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The Chloroplast as a Complete Photosynthetic Unit

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"Experimenters . . . never accept an immutable starting point; their principle is a postulate, all of whose consequences they logically deduce, but without ever considering it absolute or beyond the reach of experiment. All the theories which serve as starting points for . . . physiologists are true only until facts are discovered which they do not include or which contradict them. When these contradictory facts are shown to be firmly established . . . experimenters, far from stiffening themselves against new evidence, hasten . . . to modify their theory, because they know that this is the only way to go forward and to make progress in science. Experimenters, then, always doubt, even their starting point."—Claude Bernard, *Introduction a l'Etude de la Médicine Expérimentale* (1865).

The thesis that I am attempting to document in this article (*1*) is that "complete photosynthesis—that is, reduction of carbon dioxide to carbohydrates, and oxidation of water to oxygen, at low temperature and with no energy supply except visible light—" (*2*) has now been accomplished outside the living cell with the use of isolated chloroplasts. The conclusion drawn from the evidence presented is that chloroplasts are the cytoplasmic structures in which the complete photosynthetic process is carried out, both inside and, under suitable conditions, outside the living cell. Since the evidence in support of this conclusion is presented in detail in other publications from our laboratory (*3–7*), I am concerned here only with the principal results of our work and with a review of other experiments on extracellular photosynthesis, chiefly those in which chloroplast preparations have been used. I have not attempted to review the numerous reports (*2*) of "artificial photosynthesis" with chlorophyll solutions, dyes, or other "sensitizers."

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Photosynthesis in Chloroplasts

That chlorophyll is essential for photosynthesis was first clearly and unequivocally stated by Sachs in 1865 (*8*) almost 100 years after the discovery of the process. Of course, the association of green plants with photosynthesis had been obvious to physiologists during the preceding century, but even Theodore de Saussure, whose classical treatise (*9*) marked the beginning of modern concepts of plant nutrition, was misled by the red color of many leaves to question the indispensability of chlorophyll for assimilation of carbon dioxide in light. Sachs condemned the cautious belief of his contemporaries, that "*green plants* decompose carbonic acid," by insisting that it was chlorophyll, or more properly the "chlorophyll-body" or chloroplast, that was the organ of CO_2 assimilation in light. When it was argued that since chloroplasts carry on photosynthesis only when present in the living cell and, hence, "not the chlorophyll (chloroplasts) but the cell containing chlorophyll is the organ of assimilation," Sachs' reply was that this is "somewhat equivalent to saying that the eye is not properly the organ of sight, since, when taken out of the head, it is no longer capable of seeing" (*10*).

The basis for Sachs' firm stand that the "chlorophyll-body itself [is] the organ which decomposes carbon dioxide and consequently assimilates the organic substance" was the fact that "no cell assimilates so long as it possesses no green chlorophyll, but does so as soon as it is provided with it. The most definite proof, however, is afforded by the fact . . . that the first recognizable product of assimilation [starch] appears not in any haphazard place in the [green] cell, but in the chloroplast-body itself" (*10*). Sachs' conclusion, which was based on his classical experiments on starch synthesis in light, soon received a strikingly direct confirmation. In 1881, Engelmann (*11*) showed that the "eye" in Sachs' simile could still see after its removal from the "head." He succeeded in isolating chloroplasts from several species of green plants and demonstrated, for the first time, oxygen evolution in light by chloroplasts outside the living cell. To demonstrate oxygen evolution, he developed an extremely sensitive microscopic technique: the motility of certain bacteria in the presence of minute traces of oxygen (*11*). With the same test, he was also able to show that in the intact, illuminated *Spirogyra* cell oxygen evolution is confined to the immediate vicinity of the chloroplast (*12*).

The evolution of oxygen by isolated chloroplasts, confirmed by Haberlandt in 1888 (*13*) and by Ewart in 1896 (*14*), was then accepted as the most direct evidence yet found that the role of chloroplasts in photosynthesis was that envisaged by Sachs. In 1897 one of the most distinguished plant physiologists of the period, Pfeffer, expressed thus (*15*) what was to become a fixed tenet in plant physiology for the next 40 years: "The actual assimilation of CO_2 probably takes place entirely in the chloroplastid, for by means of the delicate bacterium-method it may be shown that isolated chloroplastids occasionally continue to evolve oxygen in the light . . . if placed in an isoosmotic sugar solution."

Extracellular Photosynthesis

The early experiments of Engelmann and his successors were not designed to test whether photosynthesis could be carried on outside the living cell. The philosophic outlook of biologists of that period on the possibility of reproducing a complex biochemical process outside a living cell was dominated by Pasteur's

views on fermentation. After the publication of his famous paper in 1857 (*16*), Pasteur maintained throughout his life that fermentation is dependent on the life and integrity of the cell and that it does not occur in the absence of the living cell. Engelmann's experience with isolated chloroplasts fitted in with this pattern of thought. His results did not suggest to him or to his contemporaries the possibility of extracellular photosynthesis. He found that isolated chloroplasts could carry on oxygen evolution, for a limited time at best, and only if their structure remained undamaged. Engelmann summed up his conclusions in what was to become an often quoted dictum: "Sobald die Struktur des Chlorophyll Körnes überall zerstört ist, hört die Möglichkeit der Sauerstoffproduktion sofort und definitiv auf." It seemed clear, in the words of Pfeffer (*15*), that "the assimilation of carbon dioxide is a *vital* function, the chloroplastids being living mechanisms specially adapted for this purpose." This conclusion also agreed with many observations that damage to whole leaves—such as that resulting from drying, freezing, or boiling—results in cessation of photosynthesis.

Pasteur died in 1895. Two years later Büchner (*17*) prepared from yeast a cell-free juice that fermented sugar. Compared with the cells from which it was derived, the juice was weak and its activity short-lived. Nevertheless, Büchner's experiments electrified the scientific world at the time, for they demonstrated that—Pasteur's great authority notwithstanding—complex biochemical reactions could be carried out by enzymes in cell-free systems. It was only natural therefore to inquire whether other physiological processes, notably photosynthesis, could also proceed with "dead" cell preparations—that is, independently of the degree of cellular organization associated with a living cell.

It was in this different intellectual climate and with this new objective in view that Büchner himself suggested in 1901 to Herzog (*18*) that he investigate whether a cell-free green juice from leaves, prepared by the procedure used by Büchner with yeast, could carry out photosynthesis under the influence of light. While Herzog's work was in progress there appeared a report by Friedel (*19*) which suggested that extracellular photosynthesis had been achieved. Friedel prepared a green powder from spinach leaves dried at 100°C and, from fresh leaves, a glycerol extract containing enzymes ("diastase"). On illumination, neither preparation alone had photosynthetic activity, but after combining them Friedel reported an evolution of oxygen that he measured by gas analysis. He concluded from his experiments that "L'assimilation chlorophylienne est accomplie sans intervention de la matière vivante, par une diastase qui utilise l'energie des rayons solaires, la chlorophylle functionnant comme sensibilisateur."

All was not well, however, with Friedel's work. In the fall of the same year he tried but failed to repeat (*20*) his experiments of the spring. He attributed his failure to an assumed low photosynthetic activity of leaves in the fall. He indicated his intent to return to the problem again, but there is no record that he ever did. In fact, he himself omitted mention of his earlier successful experiments in the thesis that he submitted to the Faculté de Paris (*21*). Other investigators, who tried to repeat Friedel's work, such as Harroy (*22*), also met with failure. Herzog (*18*), upon learning of Friedel's first reported success, interrupted, on the advice of Büchner, his own experiments and proceeded to use Friedel's methods, only to obtain negative results. Herzog then returned to Büchner's methods of extracting leaf juice by high pressure, but again the results were negative (*18*).

The only reports of successful repetition of Friedel's early experiments came from Macchiati (*23*). However, the nature of Macchiati's evidence not only failed to convince others but also threw even more doubt on the original observations of Friedel. Macchiati claimed that he had obtained the formation of formaldehyde corresponding to the evolution of oxygen. Moreover, unlike Friedel, he claimed that the green powder from leaves dried at 100°C, alone and unaided by the glycerol extract of fresh leaves, possessed photosynthetic activity. The enzyme that had been supplied in Friedel's experiments by the glycerol extract was, Macchiati claimed, heat-resistant and contained in the leaf powder. But, as was properly asked by Bernard (*21*), if leaves themselves lose their photosynthetic activity when dried at 100°C, how could the dry powder possess it? Bernard carefully reinvestigated the whole problem of photosynthesis by "dead" leaf preparations and reached the conclusion that photosynthesis outside the living organism had not been attained and that all the claims to the contrary were unfounded.

Nevertheless, the unsuccessful quest for extracellular photosynthesis during this period did not end without a hopeful note. In 1901 Beijerinck published a paper (*24*) the importance of which seems to have been appreciated by only a few of his contemporaries. He showed that luminous bacteria can be used successfully to study oxygen evolution during photosynthesis. Like Engelmann's motile bacteria, Beijerinck's luminous bacteria could be used to detect minute amounts of oxygen. Moreover, the luminous bacteria had a great experimental advantage: the test for oxygen was luminescence visible to the naked eye. Instead of Engelmann's microscopic technique, which drastically limited experimental manipulation, Beijerinck's method provided an extremely sensitive oxygen "reagent" in the form of a bacterial suspension that could be mixed readily with photosynthetic tissues. In fact, because of its far greater sensitivity than the gas-analysis or bubble-counting method, it was an excellent technique to introduce into the then current debate on extracellular photosynthesis.

Beijerinck used his new technique in photosynthesis to measure oxygen evolution, not of leaf powders, but of a green extract of fresh clover leaves. He found by this method that these cell-free extracts evolved oxygen on exposure to light. Interestingly enough, Beijerinck's philosophic predilections on extracellular processes seem to have been more akin to those of Pasteur than to those of Büchner. Beijerinck interpreted his results to mean not that photosynthesis is basically separable from the integrity of a living cell but rather that "living" protoplasm is necessary for photosynthesis. He explained the observed oxygen evolution of his cell-free extracts by suggesting that they contain a water-soluble "portion" of the living protoplasm, the portion that is concerned with photosynthesis.

What was then the distinction between a living and a dead cell? Was a cell-free extract alive? It appears that desiccation was a criterion of great importance to the investigators of that period. This is particularly apparent in the work of Molisch (*25*), who embarked on the investigation of extracellular photosynthesis in 1904 with a deep conviction that the problem was of fundamental importance. Molisch appreciated the importance of Beijerinck's work and adopted his sensitive luminous-bacteria technique. He confirmed Beijerinck's results with leaves of several species and added a significant fact: when the green extracts of fresh leaves were filtered through a bacterial filter to remove all particles, including chloroplasts, the capacity for oxygen evolution was lost. Molisch then proceeded to the heart of the matter: do "dead"—that is, dry—leaves retain a capacity for photochemical oxygen evolution? He found that, contrary to the claims of Friedel and Macchiati, leaves dried at 100°C were invariably inactive. With milder drying, however, in air or at 35°C, leaves of only one species, *Lamium album,* retained activity. Other plants, including those that gave active green extracts, lost, on drying, the capacity to evolve oxygen in light. Mo-

lisch concluded from his study that the case for extracellular photosynthesis was not proved but that the exceptional results with *Lamium* offered some hope for the future.

Molisch returned 20 years later (*26*) to the still unresolved question whether "dry, dead leaves" retain the capacity for oxygen evolution in light. This time, in an extensive investigation, he was able to show, again with the sensitive luminous-bacteria technique, that water suspensions of ground dry leaves of many species retained the capacity for oxygen evolution, provided that the drying was slow, at 30° to 35°C. Quick drying at 100°C, boiling the leaves in water, or treatment with ether, destroyed activity irreversibly. Leaves killed by freezing also retained activity. The capacity to evolve oxygen in light was limited to leaves containing chlorophyll. Preparations from gently dried, etiolated leaves were inactive. On the basis of this newer evidence, Molisch concluded that photosynthesis, like fermentation, can proceed outside the living cell. He interpreted the destructive effect of drying at high temperatures as probably resulting from an inactivation of essential enzymes.

Modern Period

The evidence for Molisch's conclusions about extracellular photosynthesis, supported by the later observations of Inman (*27*), was not considered adequate by the majority of plant physiologists (*2*). There were no quantitative measurements of oxygen evolution; there was only a qualitative test by an exceptionally sensitive method. There was no evidence for a simultaneous carbon dioxide fixation. This was merely inferred from the concept, which was dominant in theories of photosynthesis for more than a century, that the source of the evolved oxygen was the photodecomposition of CO_2. From this point of view, oxygen evolution was *ipso facto* a measure of CO_2 fixation.

In the last 20 years this concept has been abandoned. The clear analysis of the process of photosynthesis by van Niel (*28*) from the standpoint of comparative biochemistry made it extremely plausible that the source of the photosynthetic oxygen is the photodecomposition of water rather than of carbon dioxide. This view soon received experimental support from the work of Hill, who demonstrated oxygen evolution without CO_2 fixation, in light, by isolated chloroplasts. It also became evident that CO_2 fixation is not peculiar to photosynthetic tissues but occurs widely in nonphotosynthetic plant and animal cells of diverse character. Both theories were firmly documented after the advent of isotopic tracer techniques. From the modern point of view, acceptable proof for complete extracellular photosynthesis would thus require evidence for *both* the evolution of oxygen and the simultaneous reduction of CO_2 to the level of carbohydrates.

There was still another basic reason why the earlier claims for extracellular photosynthesis were found inadequate in the modern period. The old philosophic controversy about duplicating a complex biochemical sequence outside a living cell ceased to be a live issue. The spectacular advances of biochemistry in elucidating the nature of fermentation and respiration by means of extracellular reactions (the important developments came about 40 years after Büchner's historic experiment), the recent reconstruction of the principal events in the citric acid and fat oxidation cycles, and many other developments left no doubt that, in principle, photosynthesis, like any other cellular process, should be susceptible of study outside the cell. What was lacking, however, was not a demonstration that a small residual photosynthetic activity remained in cell-free preparations but a quantitative biochemical technique for the separation of the photosynthetic process from other complex metabolic activities that occur simultaneously in an intact cell.

Few contemporary physiologists or biochemists doubted that only by doing away with the complexity of a whole cell could the detailed mechanism of photosynthesis be studied and interpreted with confidence in physicochemical terms. It is not surprising, therefore, that during the last 20 years the achievement of extracellular photosynthesis has remained an important target of cellular physiology and biochemistry. The objective was no longer philosophic; it was biochemical. The criteria for judging advances in this area would be whether a particular cell-free system reproduces the complete process of photosynthesis, and whether it lends itself to a systematic, quantitative study of the mechanism of the process.

The recent approaches from this modern, biochemical point of view toward extracellular photosynthesis can be divided into two main categories. One, following the first attempts of Engelmann in 1881, centered on isolated chloroplasts; the other concerned itself with preparations of whole cells without regard to any special subcellular structure. I deal with the latter first. My review is limited to those experiments in which oxygen evolution or CO_2 fixation, or both, were measured. Extracellular photosynthetic reactions have also been investigated by other techniques such as determining changes in redox potential and *p*H of whole-leaf macerates and chloroplast suspensions (*29*).

Whole-Cell Preparations

Fager (*30*) observed that cell-free macerates from spinach leaves fixed about twice as much radiocarbon in light as they did in dark. The increased fixation in light (6×10^{-9} moles of CO_2) was estimated to be 0.1 to 0.2 percent of the corresponding photosynthetic activity of intact cells. Later Fager fractionated the leaf macerate into a chloroplast and a protein fraction, which contained the CO_2-fixing enzyme (*31*). The combined fractions fixed CO_2, again in both light and dark; the increment resulting from illumination amounted to about 50 percent.

In interpreting the relationship of his findings to the over-all process of photosynthesis, Fager attached the greatest significance to his identification of phosphoglycerate as the chief product of CO_2 fixation in light. But phosphoglycerate was also the predominant compound of dark fixation. Moreover, his chloroplast preparations were almost wholly inactive photochemically when they were tested with quinone as the oxidant. The addition of photochemically active chloroplasts failed to increase CO_2 fixation in light. It is not clear how photosynthetic CO_2 fixation could be governed by photochemically inactive chloroplasts.

Another approach to extracellular photosynthesis is found in the recent experiments of Tolbert and Zill (*32*). They extruded, with a minimum of manipulation, the entire protoplasm (whole-cell contents less vacuolar sap) from a few large (1 to 2 in. long) cells of *Chara* and *Nitella* and measured CO_2 fixation in light and dark. Oxygen evolution was not determined. When illuminated, the extruded protoplasm fixed radioactive CO_2 into several compounds, including sucrose and hexose phosphates.

The fixation was appreciable (12 to 15 percent of that of whole cells) and left no doubt that whole protoplasm retains outside the cell walls the capacity to reduce CO_2 photosynthetically to the level of sugars. Photosynthesis, however, was not separated from other cellular processes. Thus in the dark, the extruded protoplasm fixed CO_2 into compounds that were characteristic of dark respiration. The extrusion technique, although it dispenses with the structural integrity, seems to retain the biochemical complexity of a whole cell, without conferring any obvious advantage in isolation of enzyme systems peculiar

to photosynthesis. The material was extremely sensitive to manipulation and could be used only for short-duration experiments. Whether the further improvement of this technique will lead to the attainment of the biochemical objectives of research in photosynthesis remains to be seen.

Photolysis of Water by Chloroplasts

The first fruitful step toward the realization of the modern biochemical objective of reconstructing photosynthetic events outside the living cell was taken by Hill in 1937 (*33*). In his 1937 paper and subsequent papers (*34, 35*), Hill demonstrated that the evolution of oxygen by isolated chloroplasts under the influence of light—first observed by Engelmann in 1881—could be measured quantitatively by reliable biochemical techniques in accordance with the general equation:

$$A + H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} H_2A + 1/2O_2 \quad (1)$$

in which A represents a hydrogen acceptor other than CO_2. When a suitable hydrogen acceptor was supplied, a stoichiometric amount of molecular oxygen was evolved.

This reaction fell short of being complete photosynthesis because CO_2 could not serve as the hydrogen acceptor A. Hill concluded that chloroplasts contain "a mechanism, the activity of which can be measured apart from the living cell, which under illumination simultaneously evolves oxygen and reduces some unknown substance that is not carbon dioxide" (*34*).

The most useful hydrogen acceptors were substances usually considered foreign to the metabolism of the cell. Among these, benzoquinone (*36*) and ferricyanide (*37*) have been found particularly useful for what Hill designated as "the chloroplast reaction" (*35*), but others have appropriately called it the *Hill reaction.*

Hill's conclusion that isolated chloroplasts, unaided by other enzyme systems, were incapable of reducing CO_2 was confirmed later with the sensitive $C^{14}O_2$ tracer technique by Brown and Franck (*38*) and by Aronoff in Calvin's laboratory (*39*). Later experiments by Vishniac and Ochoa (*40–42*), Tolmach (*43*), and Arnon and Heimbürger (*44, 45*) have also substantiated this conclusion. It thus became a currently established concept that the Hill reaction is "photosynthesis with a substitute oxidant"—that is, photosynthesis without CO_2 fixation (*35, 46*). The chloroplast, in this view, was a "system much simpler than that required for photosynthesis" and was the site of only "the light-absorbing and water-splitting reactions of the over-all photosynthetic process" (*47*).

Reports of CO_2 fixation by isolated chloroplasts came from the laboratory of Boichenko (*48*), but they lacked sufficient experimental details to permit a critical evaluation. The earlier work from that laboratory on photosynthetic CO_2 fixation by cell-free preparations and other Russian work was reviewed by Rabinowitch (*46, 49*).

Pyridine Nucleotide Bridge

The abandonment of the classical concept that chloroplasts are the sites of the complete process of photosynthesis intensified the search for a link between the photolysis of water, now shown to be localized in the chloroplasts, and the CO_2 fixation reactions. A step forward was the finding that, under proper conditions, illuminated, isolated chloroplasts can reduce the well-known physiological electron carriers triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) (*40, 43, 44*). This permitted the linking of the Hill reaction to many enzymatic reactions, including carboxylations, that are dependent on reduced pyridine nucleotides. Indeed, the expectation that any enzyme system that uses reduced tri- or diphosphopyridine nucleotide can do so whether the reduced coenzymes are produced by respiration or by illuminated chloroplasts was amply documented, particularly by the work of Vishniac and Ochoa (*41*).

The energetically difficult reaction in carbohydrate formation during photosynthesis is the reduction of a carboxyl to a carbonyl group (*46*). The reducing potential of pyridine nucleotides is insufficient to accomplish this step without a lowering of the potential barrier, as by phosphorylation of the carboxyl group by adenosine triphosphate (ATP) (*50*). A workable scheme for photosynthesis should therefore provide for the conversion of light into high-energy chemical bonds, for example, the pyrophosphate bonds of ATP. There are two approaches to this problem. One envisages photosynthesis as a special process involving a direct conversion of light into the chemical-bond energy of ATP or some other energy-rich compound by mechanisms peculiar to green cells. The other regards photosynthesis as a process that, except for the initial photochemical reactions localized in chloroplasts, is in essence a reversal of respiration (*42, 51*). In the latter view, both phosphorylation and carbohydrate formation during photosynthesis would proceed by the same mechanisms and with the aid of the same enzymes that govern cellular metabolism in the dark.

Until now there has been scant experimental evidence for a *sui generis* photosynthetic mechanism of phosphorylation. It is not surprising, therefore, that the mechanisms invoked to explain phosphorylations in the light were modeled on those known to operate in nongreen cells. The high-energy pyrophosphate bonds of ATP are generated in nongreen cells by esterification of inorganic phosphate coupled with oxidation of a variety of substrates. Two pathways are recognized in this process: (i) substrate phosphorylation, essentially independent of oxygen, exemplified by the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate; and (ii) oxidative phosphorylation in which molecular oxygen is the ultimate electron acceptor. The former is characteristic of fermentations, whereas the latter typifies the principal aerobic pathway in respiration, which is linked with the tricarboxylic acid cycle, and accounts for the conversion of 65 to 80 percent of the total energy content of the substrate into the pyrophosphate bonds of ATP (*52*). In aerobic phosphorylation, the electron transport chain is the same, regardless of the substrate that is being oxidized (*52, 53*). The substrate reduces DPN or TPN, and the reduced pyridine nucleotides (PN_{red}) are reoxidized by flavoproteins, cytochromes, and molecular oxygen. This is represented by the following scheme (the arrows indicate the direction of the flow of electrons):

$$PN_{red} \longrightarrow \text{flavoproteins} \longrightarrow \text{cytochromes} \longrightarrow O_2$$

The enzymes responsible for aerobic phosphorylation are not distributed at random in the cell but are localized in mitochondria. The coupling of phosphorylation with the oxidation of reduced pyridine nucleotides by molecular oxygen was demonstrated experimentally by Lehninger (*54*). The over-all reaction, in which he used mitochondria from animal sources, is represented by Eq. 2. (The analogous reaction with TPNH has not, as yet, been experimentally demonstrated.)

$$DPNH + H^+ + 1/2O_2 + 3ADP + 3Pi \xrightarrow[\text{mitochondria}]{} DPN^+ + H_2O + 3ATP \quad (2)$$

where Pi represents inorganic phosphate.

Since illuminated chloroplasts had already been found to be capable of reducing pyridine nucleotides coupled

with carboxylating enzymes, it was only a step further to attempt a linkage between chloroplasts and the mitochondria in the Lehninger reaction. This was accomplished by Vishniac and Ochoa (*55*), who have shown that in a chloroplast-mitochondria system DPN, reduced by the chloroplasts in light, can be reoxidized by molecular oxygen with the aid of enzymes contained in the mitochondria. Vishniac and Ochoa (*55*) concluded from their experiments with the chloroplast-mitochondria system "that the generation of phosphate bonds in photosynthesis may occur through the oxidation by molecular oxygen of photochemically reduced nucleotides." The role of chloroplasts was limited, in this view, to the photochemical transfer of hydrogen from water to coenzymes, most probably DPN and TPN (*42, 55*). Once this was accomplished, all other reactions in photosynthesis would proceed by the same mechanisms as were operative in nonphotosynthesizing cells.

Photosynthetic Phosphorylation

It soon became evident that this new scheme of photosynthesis fell short of explaining certain aspects of the process *in vivo*. I can perhaps best illustrate this part of the story by following the sequence of experiments in our laboratory. When we started our work in photosynthesis about 6 years ago, we selected as our objective the reconstruction of a complete extracellular photosynthetic system using only enzyme components from green tissues. This limitation was self-imposed in the belief that it offered at least a partial safeguard against the construction of a model system which, although functional, did not mirror the photosynthetic events in the intact cell.

We began by examining several conflicting observations which at that time cast doubt on the identity of the oxygen-liberating mechanism in isolated chloroplast fragments with the mechanism in intact green cells (*56*). Our results supported the conclusion that the two were the same. Next, in attempting to link oxygen evolution by chloroplasts to CO_2 fixation, we found the TPN-dependent "malic enzyme" in the cytoplasmic fluid of the same leaves from which we isolated the chloroplasts (*44, 45*). This added plausibility to the view that photosynthesis involves an interaction between initial photochemical events, localized in chloroplasts, and subsequent dark reactions catalyzed by enzyme systems outside the chloroplasts. However, we soon had to abandon this hypothesis because of discordant conclusions reached from parallel experiments on the dark reactions of chloroplasts.

We set out to measure the respiration of isolated chloroplast fragments in the dark. We observed an oxygen uptake and CO_2 evolution which we thought at first (*57*) to be endogenous respiration. Subsequent experiments, however, have shown that this gas exchange was an oxidative decarboxylation of oxalate (*58*) by an enzyme contained not in whole chloroplasts but in smaller, nonchloroplast particles that are associated with the "chloroplast fragments" fraction (*59*). The endogenous respiration of chloroplasts was found to be very low. Our results have also suggested that it was unlikely (*59*) that green leaves contained enough of other cytoplasmic particles, such as mitochondria, for the generation of ATP in *photosynthesis,* by the oxidative phosphorylation reaction (Eq. 2) of the chloroplast-mitochondria system (*55*).

We then proceeded to reexamine the question of ATP synthesis by illuminated chloroplasts. Here we encountered a number of surprises. First, we learned that the method of isolating chloroplasts was of far greater importance than we had suspected. Although we had used whole chloroplasts on occasion (*59*), we preferred to work, as a rule, with chloroplast fragments (*56*). Since both preparations were equally active photochemically (*3*), as measured by the quinone reduction test, it was an obvious advantage from a biochemical point of view to use the structurally less complex chloroplast fragments.

In light, isolated whole chloroplasts, unlike chloroplast fragments, vigorously synthesized ATP (*4*) from inorganic phosphate and adenosine mono- or diphosphate (AMP or ADP) (Fig. 1). Whereas, without added mitochondria, the chloroplast-mitochondria system was inactive (*55, 60*), the addition to our chloroplast system of all the other cytoplasmic particles from the leaf gave no increase in phosphorylation (*4*). ATP synthesis in light by whole chloroplasts (the chloroplast system) was not enhanced by the addition of DPN or TPN (*3, 4*); in the chloroplast-mitochondria system the omission of the pyridine nucleotide halved the phosphorylation (*55*).

Phosphorylation by the chloroplast system may thus be distinguished from that by the chloroplast-mitochondria system by its coenzyme requirements and its independence from external enzymes. The most unexpected finding, however, was that phosphorylation by whole chloroplasts was an anaerobic process (*6*). A salient feature of oxidative phosphorylation by mitochondria, which is the basis of aerobic respiration, is molecular oxygen consumption (*52*). "The strict dependence of phosphate exchange on the presence of oxygen" (*55*) was also observed by Vishniac and Ochoa in their model system of illuminated chloroplasts linked to mitochondria of plant or animal origin.

In our early experiments on ATP synthesis by chloroplasts (*3, 4*), oxygen was neither consumed nor evolved. These observations led us to a tentative formulation of the over-all mechanism of phosphorylation by chloroplasts as involving a recombination of an oxidized and a reduced product of water photolysis without the evolution of molecular oxygen (*3, 4*). There remained, however, the discordant observation that a sustained rate of phosphorylation was obtained only under aerobic conditions (*3, 4*). Further investigation then led to the identification of some of the cofactors of chloroplast phosphorylation (*6*). When these were added and conditions were so arranged that traces of oxygen originally present or possibly formed during the reaction were eliminated (shaking in a nitrogen atmosphere in the presence of chromous chloride), phosphorylation proceeded at rates substantially greater than those observed aerobically (*61*). We have concluded, therefore, that ATP synthesis by chloroplasts represents a direct anaerobic synthesis of pyrophosphate bonds at the expense of light energy by a mechanism peculiar to photosynthesis. We have

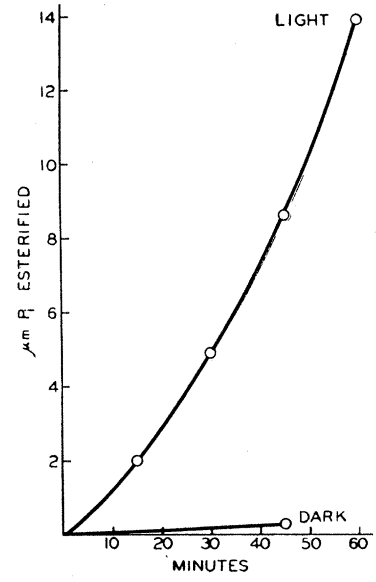

Fig. 1. Photosynthetic phosphorylation, the light-dependent esterification of inorganic phosphate by spinach chloroplasts under strictly anaerobic conditions (*6*). No evolution even of traces of molecular oxygen during the progress of the reaction was detected by the sensitive luminous bacteria method (*24*).

named this light-dependent ATP synthesis by chloroplasts *photosynthetic phosphorylation* to distinguish it from the oxidative phosphorylation by mitochondria in the dark. The over-all reactions in photosynthetic phosphorylation are represented by Eqs. 3, 4, and 5.

$$h\nu + H_2O \xrightarrow{\text{chloroplasts}} 2[H] + [O] \quad (3)$$

$$AMP + 2Pi + 2[H] + [O] \xrightarrow{\text{chloroplasts}} H_2O + ATP \quad (4)$$

Sum:

$$h\nu + AMP + 2Pi \xrightarrow{\text{chloroplasts}} ATP \quad (5)$$

The oxygen and hydrogen atoms in brackets represent an oxidized and a reduced product of the photodecomposition of water (*not* molecular oxygen or hydrogen).

Equation 4 differs from the parallel Eq. 2 of oxidative phosphorylation as carried out by the chloroplast-mitochondria system in that DPN does not appear as a hydrogen carrier and no molecular oxygen is involved. Although certain individual steps in the electron transfer during photosynthetic phosphorylation may be similar to those in oxidative phosphorylation, the two systems seem to represent distinctive structural and functional adaptations to two types of metabolism in green plants: (i) the chloroplast system for the direct conversion of light into chemical energy during photosynthesis, and (ii) the mitochondrial system (*62*) for the interconversions of chemical energy during respiration.

Some of our recent experiments (*7*) support the conclusion that photosynthetic phosphorylation is different from oxidative phosphorylation. Whole chloroplasts that carried out vigorous photosynthetic phosphorylation were unable to oxidize tricarboxylic acid cycle substrates either in light or in dark. On the other hand, smaller cytoplasmic particles (mitochondria?) that were isolated from the same leaves as the whole chloroplasts exhibited oxidative phosphorylation, but not photosynthetic phosphorylation, in a citrate medium.

As is shown later, whole chloroplasts fix CO_2 in light. It became important, therefore, to determine whether photosynthetic phosphorylation proceeded at the expense of energy released by the reoxidation of partly or wholly reduced products of photosynthetic CO_2 fixation (*63*). We have found that, under both aerobic (*4*) and anaerobic conditions (*7*), photosynthetic phosphorylation is independent of CO_2 fixation. Photosynthetic phosphorylation proceeded unimpaired when CO_2 was excluded from the reaction vessels.

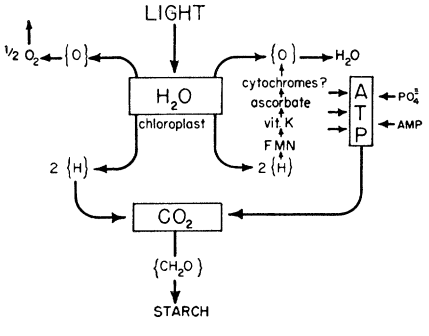

Fig. 2. Scheme for photosynthesis by isolated chloroplasts. Photolysis of water (center) leading either to ATP synthesis and the reconstitution of water (right) or to CO_2 reduction (below) linked to oxygen evolution (left).

A 1-Quantum Process

Since the energy content of 1 quantum of red light is about 44 kcal (per Einstein) and that of a pyrophosphate bond of ATP 12 kcal (*52*), it is evident that photosynthetic phosphorylation, to be highly efficient, must convert the energy of 1 quantum of light into the energy of two or more pyrophosphate bonds. This could be accomplished if the recombination of the products of water photolysis, [H] and [O], occurred not in one but in several successive steps, each step transforming a portion of the electron energy into phosphate bond energy, in a manner analogous to that envisaged for respiration by Lipmann (*64*).

The reconstruction of an "electron ladder" in photosynthetic phosphorylation depends on the identification of the participating cofactors. Among these the following have been identified so far: Mg^{++}, flavin mononucleotide (FMN), vitamin K, and ascorbate (*6*). The Mg^{++} probably has a catalytic function in the transfer of phosphate groups (*65*). The other cofactors can serve as electron carriers. The quantities in which they are required indicate that these substances act as catalysts and not as substrates (*6*). The identity of the electron carriers above ascorbate is unknown, but they may very likely prove to be components of a cytochrome system (*66*).

A tentative scheme for the "electron ladder" in photosynthetic phosphorylation, based on evidence now available, is shown in Fig. 2. The relative positions assigned to FMN and vitamin K are provisional; they are based solely on published values of redox potentials (*67*). It is possible that, *in vivo*, their positions are reversed, with vitamin K as the primary electron acceptor. Wessels (*68*) has recently postulated, on theoretical grounds, such a role for vitamin K in photosynthesis.

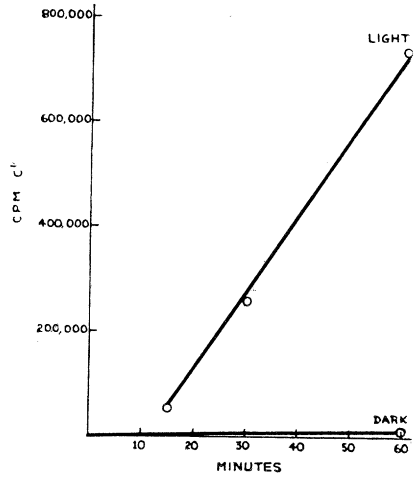

Fig. 3. Time course of CO_2 fixation by isolated spinach chloroplasts in light and in dark. The lag in CO_2 fixation during the first few minutes of illumination is attributed to a time lag in equilibration after the release of $C^{14}O_2$ gas from the sidearm of the reaction vessel (*5*).

An interesting consequence of the proposed scheme for photosynthetic phosphorylation (Fig. 2) is that with either FMN or vitamin K as the primary electron acceptor, the photolysis of water could be accomplished by 1 quantum of red light (44 kcal). In this case, photosynthetic phosphorylation would conform to the Einstein law of photochemical equivalence, which states that in a primary photochemical process each molecule is activated by the absorption of 1 quantum of radiation.

Computations based on known redox potentials (*67*) suggest that, if photosynthetic phosphorylation is a 1-quantum process, either FMN or vitamin K rather than pyridine nucleotide is the primary electron acceptor. Assuming an oxidation level of [O] equal to that of molecular oxygen at 0.2 atm, enough energy would be available in 1 quantum of red light to maintain the ratio of FMN_{red}/FMN_{ox} at approximately 5 at *p*H 7; the comparable ratio for vitamin $K_{1\ red}$/vitamin $K_{1\ ox}$ would be approximately 1000. By contrast, the comparable ratio for DPN_{red}/DPN_{ox} would be approximately 0.00001.

The study of photosynthetic phosphorylation seems to me to offer a new and promising approach to the problem of conversion of light energy into chemical energy during photosynthesis.

Carbon Dioxide Fixation

Isolated whole chloroplasts were found, on exposure to light, to fix CO_2 (Fig. 3) with a simultaneous evolution

Table 1. Carbon dioxide fixation and oxygen production by isolated chloroplasts (*5*).

Expt.	$C^{14}O_2$ fixed (μM)	O_2 produced (μM)
979	1.0	1.5
1010	0.81	0.83
1011	0.38	0.50
1050	1.04	1.0

of oxygen (*5*). The reaction was strictly light-dependent and proceeded at an almost constant rate for at least 1 hr (Fig. 3). There was approximate correspondence between the oxygen evolved and the CO_2 fixed, in agreement with the well-known photosynthetic quotient of 1 (Table 1). Both soluble and insoluble products resulted from the fixation of radiocarbon by chloroplasts. The insoluble product has been identified as starch, which appeared to be the only insoluble compound formed (*5*). The identification of the soluble products of CO_2 fixation is not complete. A typical autoradiogram of the compounds found in the soluble fraction is shown in Fig. 4. Among the compounds so far identified are (i) phosphate esters of fructose, glucose, ribulose, and dihydroxyacetone; (ii) glycolic, malic, and aspartic acids; (iii) alanine, glycine, and free dihydroxyacetone (*5*).

The light-dependent reduction of CO_2 to the level of carbohydrates with a simultaneous evolution of oxygen suggests that whole chloroplasts possess all the enzymes needed for complete photosynthesis.

Specialized Photosynthetic Unit

In the light of our present evidence, chloroplasts emerge as remarkably complete cytoplasmic structures, which contain multienzyme systems divided into three main groups, each controlling an increasingly complex phase of photosynthesis: photolysis of water, photosynthetic phosphorylation, and CO_2 fixation. The suggested interrelationships among the three are shown in Fig. 2.

In vivo, photolysis is linked either with phosphorylation, resulting in the production of ATP and the reconstitution of water, or with CO_2 fixation, resulting in the evolution of oxygen and the reduction of CO_2. Carbon dioxide reduction required the participation of all three groups of enzymes, and phosphorylation required two, whereas photolysis of water can proceed without the others, provided that an artificial hydrogen acceptor is supplied. The last process is, of course, the Hill reaction, which has already been discussed.

The experimental separation of the three phases of photosynthesis is readily accomplished with isolated chloroplasts, either by variations in preparative technique or with the use of inhibitors. In our early experiments (*3*), we reported that the intact structure of whole chloroplasts was essential for both photosynthetic phosphorylation and CO_2 fixation. This is no longer true for photosynthetic phosphorylation (*7*). Chloroplasts can now be prepared that retain a capacity for photolysis alone, or for both photolysis and phosphorylation, or for all three reactions. This interpretation requires, as is experimentally verified, that chloroplasts capable of carrying out a subsequent phase of the process should also be able to carry out the one that precedes it (*3*).

In parallel experiments (*3, 7*) starting with chloroplasts capable of accomplishing complete photosynthesis, it was possible to inhibit a more advanced phase of the process without affecting the simpler one that precedes it. Thus iodoacetamide (*3*) inhibited CO_2 fixation but not photosynthetic phosphorylation or the Hill reaction. Methylene blue ($10^{-5}M$) inhibited both CO_2 fixation and photosynthetic phosphorylation but not the Hill reaction (*7*). On the other hand, as would be expected, *o*-phenanthroline, which inhibits the photolysis reaction (*36, 56*), also inhibited photosynthetic phosphorylation and CO_2 fixation.

There is good reason to believe that the separation of the chloroplast as the "photosynthetic unit" will prove to be a fruitful approach to the study of the detailed mechanism of photosynthesis. I do not suggest that we are ready yet to equate without reservation photosynthesis by isolated chloroplasts with photosynthesis in intact cells. It seems best now to explore the characteristics of extracellular photosynthesis as a separate process and to test later their validity for photosynthesis in whole cells. The most interesting properties of extracellular photosynthesis found so far are (i) total independence of photosynthesis from respiration or from any other process that requires the consumption of molecular oxygen and (ii) direct conversion of light energy into phosphate bond energy by an anaerobic process. Among the unsolved problems of extracellular photosynthesis that are now under consideration are the nature of the reductant in CO_2 fixation, the path of carbon in the formation of sugars and starch, the identity of other cofactors, and the quantum efficiency in photosynthetic phosphorylation.

It is sometimes suggested that extracellular photosynthesis by isolated chloroplasts represents merely a transfer of the process from the familiar environment of the cell to the unfamiliar environment of the test tube with a loss of most of its activity. This argument, if it is not to be taken as sterile neovitalism, could also be applied to respiration. Half a century after Büchner's memorable experiments (*17*) it is still impossible to prevent the loss of 80 to 90 percent of respiration on disrupting yeast cells (*52*). Yet the impressive advances in the understanding of the mechanism of respiration made during this period

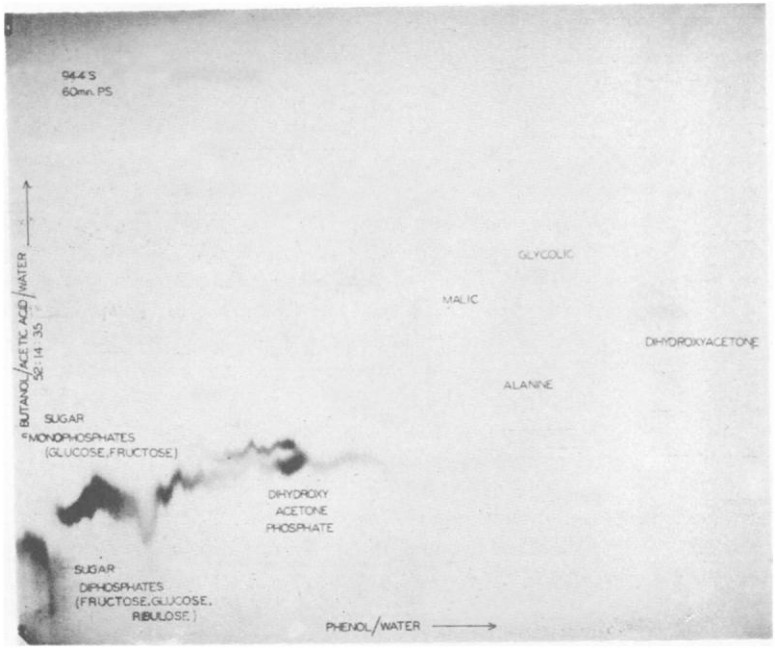

Fig. 4. Typical autoradiograph of the soluble products of CO_2 fixation by isolated chloroplasts. Further details are given elsewhere (*5*).

would have been impossible without dispensing with the complexity of the whole cell. A more optimistic philosophy for present-day approaches to the reconstruction of metabolic sequences outside the cell is aptly expressed by Green (*69*): "The disintegration of the cell is usually attended by the liberation of many destructive enzymes which degrade coenzymes and interfere with or nullify the action of those enzymes whose activity is essential for the metabolic sequence. Thus, even a residual trace of activity is encouraging because there are many ways and means eventually of muzzling the destructive agents and of restoring cofactors which are not present at the levels for maximal activity. . . . Once an *in vitro* system can be found in which a metabolic sequence can be shown to proceed, at least one can be certain that all components needed are present in that system, and by the stepwise simplification and analysis of the system, it is only a question of time, patience, and ingenuity before the entire process is fully reconstructed."

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I cannot express the amazed awe, the crushed humility, with which I sometimes watch a locomotive take its breath at a railway station, and think what work there is in its bars and wheels, and what manner of men they must be who dig brown iron-stone out of the ground and forge it into that. What assemblage of accurate and mighty faculties in them, more than fleshly power over melting crag and coiling fire, fettered and finessed at last into the precision of watchmaking; Titanian hammer-strokes beating out those glittering cylinders and timely respondent valves, and fine ribbed rods, which touch each other as a serpent writhes in noiseless gliding and omnipotence of grasp, an infinite complex anatomy of active steel. What would the men who beat this out, who touched it with its polished calm of power, who set it to its appointed task and triumphantly saw it fulfill the task to the utmost of their will, feel or think about this weak hand of mine timidly leading a little stain of water color which I cannot manage into the imperfect shadow of something else . . . what, I repeat, would these iron-dominant genii think of me, and what ought I to think of them?—JOHN RUSKIN.