ADVANCES IN ENZYMOLOGY

AND RELATED SUBJECTS OF BIOCHEMISTRY

Volume XII

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OXIDOREDUCTION IN CHLOROPLASTS

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I. Introduction

The present article will be concerned with the biochemical knowledge we have at the moment of the nature of the green plastids of plants. We shall attempt to show how much of the behavior of the chloroplasts and of their constituents can usefully be applied to the more general conception of assimilation in the green plant. Many of the most important aspects of the physiological problem of photosynthesis have formed the subjects in recent reviews (13, 16, 37, 54, 61). Our knowledge from a strictly biochemical standpoint is still of a most elementary kind, but it may well have reached the stage at which some further critical appreciation is desirable.

The study of the photosynthetic anaerobes has established the occurrence of definite types of chemical transformation; these gave the basis for the important generalization by van Niel (52, 53) which has had so profound an effect on views concerning photosynthesis during the last two decades. This was the suggestion that the light energy is converted into a splitting of water and that the reduction of CO_2 is a secondary process. By his definition of principles derived from comparative biochemistry van Niel was able to generalize from a wide range of chemical activities shown by microorganisms. Important from the comparative point of view is the work of Gaffron

(54) with certain algae which he found would become adapted to photosynthesize anaerobically with hydrogen. These and other recent developments now show clearly that photosynthesis is a many-sided problem which indeed had been foreshadowed in classical physiological investigations. The effect of external factors and the use of inhibitors on the living green cell had established the existence of separate light and dark reactions. The use of intermittent illumination made it possible to separate these two types of reaction in a physiological sense. The analysis of the kinetic data has suggested that an interrelated series of reactions, analogous to those occurring in fermentation and respiration, take place in photosynthesis. This aspect is now clearly brought out by the tracer method for following the path of carbon in a green plant. But the rapid utilization of the carbon compounds in cellular metabolism has made it difficult to identify the initial stages. It is just here that advances in the biochemical method with isolated systems would be of fundamental importance. At this moment, however, experiment has not covered the necessary region.

II. Production of Oxygen by Cell-Free Preparations

The classical investigations of Engelmann (59) proved that in the living green cell oxygen appeared in the neighborhood of the illuminated region of a chloroplast. This was observed by mounting a filament of a green alga in fluid containing a motile aerobic bacterium; after a period of dark the bacteria were at rest when all the oxygen was used up; on illumination the motility was restored starting in the closest proximity to the chloroplast. Haberlandt, by cutting the green cells of the moss *Funaria* in a small volume of fluid, obtained the free chloroplasts and showed that they behaved as they did in the living cell, using bacteria to indicate oxygen. Thus it could be concluded the chloroplasts isolated from the cell and placed under anaerobic conditions would evolve either oxygen or something very like it on exposure to light. Ewart (14) extended this to certain other plants; the leaves were cut in strong sucrose (20–30%) solution, which prevents the chloroplasts from breaking up. With *Selaginella helvetica* the activity could be maintained for several hours. Ewart considered that he had observed the formation of starch in the free chloroplasts in one case during a long exposure to light, and therefore concluded that the oxygen production represented

photosynthesis outside the living cell. From what we know now both from the point of view of starch formation and the chloroplasts of other plants it is difficult to accept this conclusion, but it must be remembered that chloroplasts of certain *Selaginella* spp. have a rather peculiar structure and that the method of isolation used by Ewart was essentially different from the larger scale methods, involving blending or grinding, used in subsequent work.

Molisch (51) brought the problem more within the scope of biochemical methods. He showed that aqueous suspensions prepared by grinding from both fresh and dried leaves of a number of plants produced oxygen in light as indicated by the use of luminescent bacteria. He further showed that the activity was markedly thermolabile; this proved that the activity was due to an enzyme system stable enough to be examined *in vitro*, especially since the activity could be preserved for long periods in the dried leaves of phanerogams. At this time no substance other than CO_2 was known to give oxygen with the green cells in light—with the exception of H_2O_2 in both light and dark. The tendency was therefore to regard these preparations from leaves *in vitro* as showing a very feeble, but otherwise normal, photosynthesis.

The experiments of Molisch were confirmed and extended by Inman (39); and both investigators concluded that the activity depended not only on the insoluble green part of the preparation but also on a dissolved fraction removable by washing. Inman, however, went further and concluded from his experiments that the oxygen-producing property did not depend on CO_2 but was due to a limited store of oxygen-producing substance in the active preparations. We can easily see now how justified this conclusion was, and again its importance in separating experimentally the oxygen production from the assimilation of CO_2. This made it clear that the only property, specific for the green cell, which could be investigated with the help of biochemical methods was the evolution of oxygen in light. The use of bacteria—either by motility or by means of luminescence—had the advantage of detecting minute traces of oxygen produced under otherwise anaerobic conditions. It was, however, a qualitative method, and although oxygen was the only substance known to cause the motility or the luminescence, it was not certain that these reactions were absolutely specific.

Thus it was that hemoglobin became applied by the writer (25)

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to this problem of determining oxygen, and indeed Hoppe-Seyler (38) had used it long before to demonstrate the oxygen evolved from a green plant in photosynthesis. The high affinity for oxygen possessed by muscle hemoglobin as distinct from the hemoglobin in the blood (24, 25) gave a sensitive and specific test for the production of oxygen under anaerobic conditions. The combination with molecular oxygen can be followed spectroscopically; thus from a knowledge of the dissociation curve both the amount of oxygen present and its pressure can be determined. Provided that the experiment covers only the lower region of the dissociation curve of the hemoglobin used, a progress curve of oxygen production (or consumption) can be obtained equivalent to that resulting from manometric procedure. There is actually a choice of hemoglobin ranging from a very high oxygen affinity in *Ascaris* hemoglobin (12) to that of mammalian blood; muscle hemoglobin occupies an intermediate position.

In the experiments with hemoglobin in which oxygen was being determined as oxyhemoglobin with the spectroscope, it was necessary to have a moderately transparent fluid. The preparations described by Molisch and Inman were too dense or opaque to be used in this way; nor, as was subsequently found, did they usually show a sufficient degree of activity. This was overcome by using a fresh dilute suspension of chloroplasts, prepared by grinding leaves in 12% sucrose; the soluble part of the system was supplemented by the addition of an aqueous extract of acetone-treated leaves*. When muscle oxyhemoglobin was added to this system, and all the atmospheric oxygen removed by gently shaking the mixture in a vacuum, the spectrum of the hemoglobin could be seen. But very rapidly, during the exposure to light, the spectrum of oxyhemoglobin became visible. When the hemoglobin was about half saturated with oxygen there was no further immediate change, but if the exposure to light was continued the oxygen appeared slowly to be absorbed. By placing the system in the dark after a short exposure to light, the oxyhemoglobin was gradually converted to a corresponding amount of hemoglobin, and in light oxyhemoglobin would then reappear. The effect of light and dark could be repeated several times but each new exposure to light tended to give a lower degree of saturation of the hemoglobin. The initial degree of saturation obtained in the light was not affected by changes in the hemoglobin concentration, indicating that in these

* Extract of acetone leaf.

experiments the store of oxygen-producing material itself was not limiting the amount of the oxygen being produced.

These experiments showed that a measurable amount of oxygen was evolved in the light. It could also be shown that the extract of acetone leaf was necessary for the production of oxygen in this way by the chloroplasts. The activity of both component parts was shown to be thermolabile. No difference in the course of this reaction could be observed whether or not additions of CO_2 had been made. The experiments could therefore be seen to support the conclusions of Molisch, that an enzymic system was involved, and of Inman (39, 54), that the production of oxygen arose from some store in the soluble part of the preparation and did not depend on the presence of CO_2. It could also be concluded that with this system in light there was something resembling a photostationary state with an oxygen pressure of less than 1 mm. Hg, and also that the chloroplasts might catalyze both the forward and backward reactions because the reabsorption of O_2 in the dark was relatively slow. The degree of saturation of the hemoglobin at a given light intensity was found to vary both with the particular sample of chloroplasts and independently with the sample of extract of acetone leaf; it was therefore difficult at the time to analyze the system further. But having now defined a test for active chloroplasts it was possible to extend the range of experiment.

The extract of acetone leaf could be replaced by an extract of acetone yeast; but this did not usually allow as high a pressure of oxygen to be reached as with leaf extract. The addition of coenzymes I and II (DPN and TPN) to the crude chloroplast system gave evidence in both light and dark of an increased rate of disappearance of oxygen (29). Many substances connected with fermentation were tried, but none of them produced any enhancement of oxygen production. Some samples of baker's yeast were found to give a boiled extract which caused a marked production of oxygen in the light. This seemed to be of a rather different nature from the case of the leaf extract; the degree of saturation could be higher and after reaching a certain point no further evolution of oxygen took place after re-evacuation or a dark period. The attempt to concentrate an active material was more successful than had been the case with the leaf extract. The activity seemed to depend on the presence of iron salts and it was found eventually that ferric potassium oxalate was

an almost ideal substance for studying the production of oxygen by chloroplasts with the hemoglobin method. When the extract of acetone leaf was re-examined, it was found that most of the activity could be recovered from a lead precipitate if decomposed by phosphate; this active solution, however, was found to contain negligible amounts of iron.

This shows that ferric oxalate must be regarded simply as a reagent, which is assumed artificially to replace some system in the plant extract in relation to the chloroplasts. The early experiments with the leaf extracts have been mentioned in some detail because it will be necessary to refer to them later, after the oxygen-producing reaction of the chloroplasts has been examined.

III. Oxygen-Producing Reaction of the Chloroplast

On illumination in the presence of chloroplasts, a mixture of muscle hemoglobin and potassium ferric oxalate gave oxyhemoglobin which represented nearly 100% saturation with oxygen. This showed that this system in light could give a pressure of oxygen of at least 4 mm. Hg. It was thus possible to use the hemoglobin from human blood as the oxygen-determining reagent. In this case there was no oxidation of the hemoglobin to methemoglobin at the beginning of the experiment as there was with muscle hemoglobin. For the measurement of the low pressures obtaining in the experiments with extracts of acetone leaf, as can be seen from the figure in Hill's paper (26), blood hemoglobin would give practically no indication that oxygen was being produced at all. The evolution of oxygen from this ferric oxalate system was found to be proportional to the amount of ferric iron initially present; the ratio was almost one molecule of oxygen to four equivalents of ferric iron. This was not affected by varying the quantity of the chloroplasts used; while the initial rate of oxygen production in the range used was proportional to the chloroplast concentration. After a reaction had been completed with a small amount of iron, the addition of more caused a further production of oxygen. The appearance of ferrous iron was shown by the production of the red color on the addition of α,α'-dipyridyl.

Before drawing what appeared to be the simplest conclusion from these experiments, it had to be decided whether the chloroplasts were acting in a photocatalytic manner or whether they were contributing anything besides the energy derived from illumination. After the

first note on this work (25), Kautsky (40) pointed out that ferric oxalate would cause decomposition of peroxides in light. This reaction would involve only two equivalents of ferric iron for one molecule of oxygen. The experiments showed that at least four were required; but this result is not in itself sufficient to rule out Kautsky's interpretation, for it could be argued that the yields of oxygen obtained might be low owing to side reactions. When, however, we examine the effect of independently varying the concentrations of ferric iron and of the chloroplasts there is no indication of the much higher yield of oxygen demanded. Again it was found that at least five molecules of oxygen could be produced in the presence of one molecule of chlorophyll. This would represent a concentration of a peroxide of about 0.1 *M* in the chloroplast; it was not possible to show the presence of a peroxide by means of any added reagents. The reason that this question has to be considered in detail is due to the fact that *in vitro* the activity of the chloroplast system is found to decline during the photochemical reaction. But it seemed best to conclude from these experiments that the loss of activity was due to the inactivation of the catalyst rather than the depletion of a store of substance taking part in the reaction.

The photochemical reaction in the presence of chloroplasts, at 25°C., and pH 7, with an oxygen pressure of 4 mm. Hg, is represented as follows:

$$2\,K_3Fe(C_2O_4)_3 + H_2O + 2\,K^+ \longrightarrow 2\,K_4Fe(C_2O_4)_3 + 2H^+ + {}^1/_2\,O_2$$

$$\Delta F = +\,32{,}000 \text{ cal.}$$

As the ferrous oxalate is oxidized directly by molecular oxygen the reaction in the dark will proceed in the reverse direction. The rate of this reaction depends on the pressure of oxygen and becomes negligible at low pressure; this then sets an oxygen limit of about 4 mm. Hg that was reached in these experiments. Although there seemed to be no doubt whatever that oxygen was a product of the reaction it was not proved that the hemoglobin itself took no part in the reaction.

If the system could be set up in some other way without hemoglobin, so that the oxygen could actually be collected as gas and identified, as we may do with a living green plant, then the chloroplast reaction *in vitro* would appear to have more significance. It was found that potassium ferricyanide readily oxidizes the iron in ferrous oxalate to ferric, and the ferrocyanide produced would not be oxidized by

molecular oxygen. The chloroplast system with ferric oxalate was set up with ferricyanide in an evacuated tube. On exposure to light liberation of gas was evident from the bubbling which was seen to occur. By filling the tube with air-free water a bubble of gas was obtained at atmospheric pressure; this, after treatment with KOH, was shown, by the way it was absorbed by alkaline pyrogallol, to be mainly oxygen. The much smaller bubble obtained in a control experiment contained but traces of oxygen. From this Hill and Scarisbrick (32) were able to conclude that the hemoglobin used for measuring the oxygen plays no part in the chloroplast reaction. The use of ferricyanide, which has a more oxidizing potential than ferric oxalate, as the final hydrogen acceptor meant that here the system was converting less of the light energy to a chemical form. But as the dark reabsorption of the oxygen was largely eliminated it was possible to study the chloroplast reaction by the more familiar manometric method. Using this method Hill and Scarisbrick obtained results which supported the conclusions they had drawn from the experiments using the spectroscopic hemoglobin method.

The manometric method was used in an important study of the chloroplast reaction by Warburg and Lüttgens (60). They showed that benzoquinone was an active hydrogen acceptor for the process of oxygen evolution in light. With this system almost complete reduction to hydroquinone took place together with nearly the theoretical production of oxygen. The reaction was represented as follows:

$$C_6H_4O_2 + H_2O \longrightarrow C_6H_4(OH)_2 + {}^1/_2\,O_2$$
$$\Delta F = +26{,}000 \text{ cal.}$$

They found that the speed of the reaction with a given amount of quinone was proportional to the quantity of the chloroplast preparation used in the range of their experiments, while the final extent of the reaction was not affected. More important still, it was found that the chloroplast preparation could transform its own weight of quinone during the photochemical reaction. This established, more certainly than had the previous work, the conclusion that the chloroplast system was essentially a photocatalytic one. The plants used in these experiments were spinach and spinach beet as the source of the chloroplasts. In addition it was found that the chloroplasts in (initially) living cells of *Chlorella* would give the same reaction when illuminated in the presence of quinone. The use of quinone in these experiments

gave a simpler system, in a practical sense, than ferric oxalate. Since quinone is rather a reactive substance toward many types of protein, the fact that the chloroplast activity for oxygen production persists in this system shows how completely it may be removed from the cell as a living unit.

Fan, Stauffer, and Umbreit (15) had shown that *Chlorella* cells, in the absence of CO_2, would produce oxygen in the presence of several compounds, including acetaldehyde and benzaldehyde. The two aldehydes do not seem to react with isolated chloroplasts (29). We have to assume that quinone, an active chloroplast reagent, enters a system in the plant at a point different from the aldehydes. The characteristic property of the chloroplast activity, as we have seen, is that oxygen production *in vitro* depends on the presence of a suitable acceptor for an exact equivalent of hydrogen. In our own preliminary experiments, although very few suitable reagents could be found, there was no indication of any specificity on the part of the chloroplasts toward the hydrogen acceptor.

The properties required seemed to be that the reduced form of the reagent should not react at all easily with molecular oxygen and that the reagent should behave as a good oxidoreduction indicator. The combination of these two properties retains, as possible reagents, mainly substances of relatively high oxidizing potential (5a). No satisfactory reagent was found having a more reducing potential than iron oxalate, $E_0' H$ of zero at *p*H 7 (50a); the iron tartrate system would react but the rapid reoxidation of the iron seemed to preclude its use.

In this preliminary discussion the object is to define as nearly as possible the properties of the chloroplast system simply with regard to the production of molecular oxygen. The conclusion that the system is purely photocatalytic is the foundation on which the subsequent discussion is to be based.

IV. Some Properties of the Photochemically Active Chloroplast Material

An inspection of the equation for the photochemical reaction

$$4\ K_3Fe(C_2O_4)_3 + 2\ H_2O + 4\ K^+ \longrightarrow 4\ K_4Fe(C_2O_4)_3 + 4\ H^+ + O_2$$

suggests three measurements by which the course of the reaction could be followed: the quantity of O_2, the amount of the reagent

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