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Oxygen produced by isolated chloroplasts

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1. Introduction

The system concerned with the assimilation of carbon dioxide by the green plant has, under optimum conditions, an activity comparable with the highest rate of cellular respiration in animals. At the moment there is, in the case of animals, considerable knowledge of the subcellular* chemical mechanisms which can be connected with respiratory activity. In the green plant, on the other hand, there is still no direct indication of a single chemical mechanism connected with carbon assimilation. As the oxygen uptake of tissue preparations apart from CO_2 evolution is a guide in searching for systems connected with respiration, so might an oxygen output, apart from CO_2 absorption, indicate mechanisms characteristic of photosynthetic activity in the plant. The subcellular evolution of oxygen under illumination has been known in the case of green plants for many years (Spoehr 1926). The effect, however, was always insignificant compared with the original photosynthetic activity of the cell. The oxygen could only be detected by using certain bacteria which show either motility or luminescence with traces of this gas. But to this method we owe the classical investigations of Engelmann (Spoehr 1926) who showed that in the living cell oxygen appeared in the neighbourhood of the illuminated chloroplast and the experiments on the isolated chloroplasts of *Funaria hygrometrica* by Haberlandt, who demonstrated the production of oxygen in light. Ewart (1896) confirmed and extended these results using other mosses and *Selaginella helvetica*; in a phanerogam, *Elodea*, no oxygen could be observed to come from the isolated chloroplasts.

The same problem was approached in a somewhat different manner by Molisch (1925). The leaves of many phanerogams were allowed to dry

* The term *subcellular* is used here to imply a degree of organization less than that of the whole cell.

slowly in air and finally over a dehydrating agent. This produced a stable preparation which, if ground up in water, would show an evolution of oxygen in light which could be detected by the bacterial methods. Molisch showed that these preparations were thermolabile, indicating an enzymic process. Recently, the matter was taken up by Inman (1935) who confirmed the experiments of Molisch and showed also that fresh green extracts of many phanerogams will evolve oxygen in light, using the bacterial method. Inman brought further evidence as to the enzymic nature of the process, and, moreover, did not consider the oxygen evolved to represent photosynthesis but suggested that it was due to a limited store of oxygen-giving material.

This subcellular evolution of oxygen is then the only property specific for green plant tissue which is at the moment open to biochemical investigation. So far the oxygen had been detected qualitatively by two methods, both using bacteria. Two questions then naturally arise: can molecular oxygen be proved by an independent method, and can the activity of preparations from green tissue be measured and compared with the activity of the living cell?

It happens that there is one reagent which can be used to detect and measure traces of oxygen with certainty in a liquid medium. This reagent is haemoglobin, and it has this rare property of combining with molecular oxygen without being oxidized by it. The absorption spectra of oxyhaemoglobin and haemoglobin are very different and oxyhaemoglobin possesses so strong an absorption of light that it can be used in a dilution corresponding to concentrations of oxygen from 10^{-5} to 10^{-4} M. But as oxyhaemoglobin is a dissociable compound, the method is limited by the affinity of the haemoglobin for oxygen. However, it has this advantage, that not only can the amount of oxygen evolved be determined but also the pressure of oxygen obtaining in the fluid. Hoppe-Seyler (1879) demonstrated the use of mammalian blood in detecting the oxygen produced by the green plant in carbon assimilation. Observations on the affinity of muscle haemoglobin (Hill 1933; Theorell 1934) for oxygen suggested that this substance could be used as a sensitive method both for detecting and measuring the oxygen given off by isolated chloroplasts (Hill 1937). This spectroscopic method is much less sensitive than the bacterial methods. However, it was shown (Hill 1937) that suspensions of chloroplasts of many angiosperms can under suitable conditions give measurable amounts of oxygen in light.

The fresh suspensions of chloroplasts obtained by crushing leaves in sucrose solution would not, on illumination, evolve measurable amounts

of oxygen even in the presence of CO_2. But in the presence of an aqueous extract of acetone-treated leaf, oxygen was evolved in the light. These two findings substantiate the observations of the earlier workers and at the same time justify the contentions of Kny (1897) that the chloroplast was not perhaps a complete photosynthetic system in itself.

The present paper is devoted to an examination of this oxygen-producing property of chloroplasts. The results obtained show that for the purpose of biochemical investigation the activity of chloroplasts removed from the cell is significant, being about one-tenth the activity of the living leaf.

General procedure. The fresh leaves of a plant are crushed with a pestle and mortar in sucrose solution. The resulting mass is strained through glass-wool. The suspension of chloroplasts is then introduced into an evacuated Thunberg tube containing haemoglobin and desired reagents. The tube is then illuminated and oxygen estimated spectroscopically as oxyhaemoglobin.

2. Preparation of suspensions of chloroplasts and of mesophyll cells

The two species of flowering plant used in the present work were *Stellaria media* and *Lamium album*. Similar results however have been obtained with a variety of angiosperms. In the case of *Stellaria media* the leafy stems were picked between 9 and 10 a.m., the leaves detached and soaked in tap water for 45 min. in a diffuse light. The chloroplasts were extracted by grinding 0·5 g. of the leaves for 1 min. with 2·5 c.c. of 10% sucrose in M/30 phosphate pH 7·9. The mass was then poured into a plug of glass-wool in a funnel; the crushed tissue was almost completely retained while the green suspension containing most of the chloroplasts was collected. The suspension, 0·2–0·5 c.c., was then at once introduced into an evacuated Thunberg tube containing 5 c.c. of a solution of the haemoglobin and other substances under investigation. All these manipulations were performed in diffuse daylight. The small quantity of oxygen introduced could either be removed by evacuation or allowed to remain in the tube and measured at the beginning of an experiment. The tube was then exposed to a strong light and any oxygen measured as oxyhaemoglobin with the spectro-colorimeter.

In the case of *Lamium album*, the leaves, treated as previously described, were ground with 10% sucrose containing 0·5% of sodium potassium tartrate (Rochelle salt). The fluid was strained through glass-wool and

centrifuged at 1000 r.p.m. for 6 min. The unbroken cells and some of the chloroplasts then remained packed at the bottom of the centrifuge tube. The fluid was poured off and centrifuged for 5 min. at 1800–2000 r.p.m. This gave a deposit of chloroplasts only. The fluid, which was still green, was discarded and the sediment gently stirred into a suspension with a little fresh sucrose solution. It was also possible to remove the unbroken cells completely by filtering the original fluid through a wad of absorbent cotton-wool pressed tight to 1 cm. thickness while wet. In this case also it was necessary to centrifuge out the plastids and resuspend them in fresh fluid; the aqueous extract of *Lamium album* contains tannins which interfere with subsequent operations.

The chlorophyll content of a suspension of chloroplasts or mesophyll cells was estimated by adding 4 vol. of acetone and extracting the fluid with toluene. The toluene solution was then determined by the Nutting spectrophotometer. The value of $\log_{10} I_0/I$ at 6750 A was taken to be 4·8 for a 10^{-4} M solution of chlorophyll in toluene ($1b + 3a$) 1 cm. in thickness. The vacuum tubes used were 1·5 cm. internal diameter and the final concentration of chloroplasts, represented as a concentration of chlorophyll was between 0·2 and $0{\cdot}5 \times 10^{-4}$ g.mol./l.

3. The measurement of oxygen evolved

The chloroplasts were suspended in a liquid medium containing a known quantity of haemoglobin in a vacuum tube. The relative concentrations of haemoglobin and oxyhaemoglobin were determined in a spectro-colorimeter which has been described elsewhere (Hill 1936). This method of measurement was used previously for spectroscopic determination of oxygen dissociation curves. The presence of $0{\cdot}2$–$0{\cdot}5 \times 10^{-4}$ M chlorophyll, while showing a strong band in the red, does not interfere with the part of the spectrum used. If a glass cell containing a suspension of chloroplasts of suitable concentration is placed in the light beam illuminating the standard, two perfectly similar spectra can be obtained. This refinement was found, however, not sensibly to increase the accuracy.

The muscle haemoglobin was obtained from shin beef by a method based on that of Theorell (1932). It was important to have a preparation which did not change readily to methaemoglobin, so the purification was not carried further than the addition of basic lead acetate, removal of the lead from the filtered fluid, and dialysis.

The method was as follows: 4 lb. of shin beef after freeing from fat were cut up and rolled in about 100 g. precipitated $CaCO_3$ and minced. This gave an intimate

mixture with the $CaCO_3$. About 700 c.c. of distilled water were added and after 10 min., stirring at intervals, the mass was rapidly squeezed in portions through a cloth. The fluid was mixed with about 30 g. Kieselguhr and rapidly filtered through a layer of Kieselguhr on a large Buchner funnel. The filtrate, 700 c.c., was treated at once with 92 c.c. basic lead acetate (B. P. Fort). After removal of the precipitate by filtration the excess Pb was removed by disodium phosphate added rapidly to reach a *p*H of 7·5–8. After centrifuging, the clear fluid was dialysed against either tap or distilled water for 24–36 hr. This preparation has the property of keeping free from methaemoglobin for several days when kept at 0° C. If, however, the first process is carried out too slowly or not enough lead acetate added, the preparation rapidly formed methaemoglobin.

The oxyhaemoglobin solution usually contained about $1{\cdot}8 \times 10^{-4}$ g.atom of haemoglobin iron per litre. The strength of this stock solution was measured in the spectrophotometer. The value of $\log_{10} I_0/I$ at 5810 A was taken to be 1·6 for a solution containing 10^{-4} g.atom of haemoglobin iron per litre.

Supposing at the beginning of the experiment we have, in the vacuum tube, a suspension of chloroplasts in a fluid containing a known quantity of haemoglobin. The oxygen has been almost completely removed and the haemoglobin will be less than 5 % saturated with oxygen. If, then, minute amounts of oxygen are added to the tube (e.g. 0·5 c.c. water saturated with air), the spectrum of oxyhaemoglobin will appear, and the increase in intensity of the absorption bands is a measure of the oxygen added to the liquid in the tube.

The loss of oxygen from the liquid to the vapour phase in the vacuum tube was found to be negligible under the conditions of the experiments. If, however, the tube is shaken thoroughly and continuously, 3 min. are required to attain equilibrium with the vapour phase. Thus it is easy to remove most of the oxygen from the liquid during an experiment without opening the tap to a vacuum.

If no oxygen is added from outside, an increase in saturation of the haemoglobin is a measure of the oxygen liberated from some source in the fluid. The affinity of muscle haemoglobin for oxygen is so high that the concentration of free oxygen in the solution is negligible under the conditions used. The results of illuminating chloroplasts under different experimental conditions are given graphically as the percentage saturation of the haemoglobin with oxygen in relation to the time of illumination. The concentration and variety of the haemoglobin is stated, also the temperature and pH. From this not only can the quantity of oxygen liberated be calculated but also the pressure of oxygen in apparent equilibrium with the system at any moment. Both these quantities it is of importance to measure.

In fig. 1 are shown curves giving the percentage saturation of the haemoglobin at different pressures of oxygen. A solution of haemoglobin containing $0{\cdot}45 \times 10^{-4}$ g.atoms of haemoglobin Fe per litre represents 1 c.mm. of oxygen per c.c. when fully saturated. The pressure of oxygen for half saturation of muscle haemoglobin at 20° C, pH 7·9 is 0·6 mm. Hg. For dilute human haemoglobin from blood under the same conditions, the corresponding oxygen pressure is 1·7 mm. Hg. It can be seen from the graph that the muscle haemoglobin is suitable for detecting low pressures of oxygen.

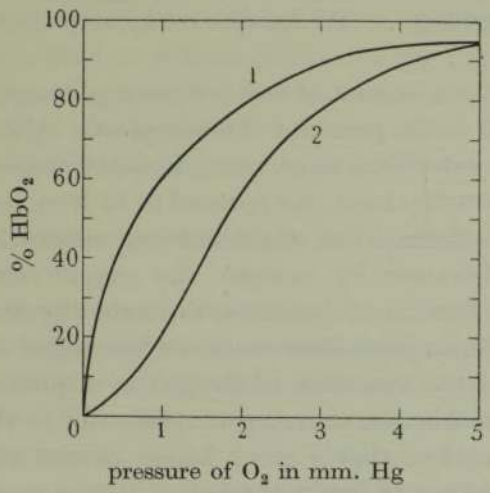

FIG. 1. Oxygen dissociation curves of haemoglobins at 19° C and pH 8. Curve 1, muscle haemoglobin; curve 2, haemoglobin from human blood.

In order to obtain the evolution of measurable amounts of oxygen a strong source of light was used. A concentrated beam from a projection lens was used and the illumination was approximately equal to that obtainable from a 1200 c.p. lamp 6 in. from the vessel. This light source was kept constant for all the experiments described in the present paper.

4. PREPARATION OF EXTRACTS FROM LEAVES

The chloroplasts of *Lamium album* or *Stellaria media*, when suspended in solutions of sucrose, would not evolve oxygen in the light even in the presence of CO_2. If, however, they were suspended in a fluid prepared from an acetone powder of the leaves, oxygen was evolved in the light and rapidly taken up in the dark. The extracts were made as follows:

40 g. fresh leaves of *Lamium album* were ground in a mortar with 160 c.c. of acetone until the chlorophyll was dissolved. The powder was

filtered off and washed first with 80% acetone and finally with pure acetone. Then the powder was allowed to dry. 2 g. of the powder were stirred up with 20 c.c. of water and the fluid filtered off at once by suction through Kieselguhr. This extract was best kept *in vacuo* to avoid browning due to oxidation. The addition of a fresh extract of the leaf prepared in this way did not form methaemoglobin when mixed with muscle haemoglobin. In these extracts from leaves, the property of producing oxygen with chloroplasts was thermolabile. In many cases only traces of iron were initially present and ferric salts (as shown by the reaction with $\alpha\alpha'$-dipyridyl and a reducer) could be removed completely without loss of activity.

It was found that an extract of acetone yeast prepared in a similar way would yield oxygen in the presence of chloroplasts. Also an extract made by boiling yeast in water for a short time. In an attempt to fractionate the active part of the yeast extract, it appeared to be ferric iron compounds of organic acids. Moreover, after a preliminary adsorption on tricalcium phosphate, and liberation by oxalate, the oxygen output was nearly proportional to the ferric iron content as estimated by $\alpha\alpha'$-dipyridyl. Then it was found that ferric potassium oxalate when added to a suspension of chloroplasts caused the evolution of oxygen in a quite startling manner on illumination. In this case the oxygen uptake due to the ferrous oxalate formed was less rapid so that a much higher partial pressure of oxygen could be reached than with leaf extracts.

5. Chloroplasts and ferric salts

When ferric potassium oxalate was added to muscle oxyhaemoglobin at pH 8, no reaction took place. If now the mixture was evacuated, as the oxygen was removed, some methaemoglobin was formed along with the reduced haemoglobin. If all the oxygen was removed and the fluid exposed to white light no further change took place.

On the other hand, if a large excess of ferric potassium oxalate was added to methaemoglobin in the presence of oxygen and the mixture exposed to white light, the spectrum of oxyhaemoglobin gradually appeared. As the ferric iron became reduced photochemically at the expense of some of the organic substances, the ferrous iron formed would then reduce the methaemoglobin to haemoglobin which then became oxygenated.

The presence of oxygen therefore shifts the equilibrium of the iron oxalate-haemoglobin system towards more reduction of the haemoglobin, owing to the oxygenation of the latter. This was shown clearly by adding a

trace of ferrous potassium oxalate to methaemoglobin in the presence of air; oxyhaemoglobin was produced at once. If, however, the experiment was performed *in vacuo*, very little reduction to haemoglobin occurred. It also follows that ferrous potassium oxalate is oxidized more quickly by methaemoglobin than by oxygen. Hence the formation of methaemoglobin when ferric oxalate is present *in vacuo* does not interfere with measurement of molecular oxygen subsequently present. With haemoglobin of blood, however, there is no formation of methaemoglobin.

If a mixture of ferric potassium oxalate and muscle haemoglobin was completely deprived of oxygen in the presence of chloroplasts, exposure to light rapidly produced oxyhaemoglobin. In the dark the oxygen was slowly absorbed, the system tending to approach the state before illumination. If $\alpha\alpha'$-dipyridyl was added after illumination, the red colour was immediately produced indicating reduction of the iron to the ferrous state.

The percentage of the saturation of the haemoglobin was measured and plotted against time.

The fluid in the vacuum tube contained: 1 c.c. of M/2 potassium oxalate, 0·7 c.c. of water containing the necessary amount of ferric potassium oxalate, 1 c.c. of M/15 phosphate buffer pH 7·9 and 2 c.c. of a stock solution of oxyhaemoglobin. The oxygen was first removed completely and then 0·3 c.c. of a chloroplast suspension added through the side tube.

Oxygen evolutions with different amounts of iron are shown in fig. 2*a*. In Table I some figures are given showing the total amounts of oxygen evolved under various conditions. The evolution of oxygen is seen to be proportional to the ferric iron added and corresponds almost to 1 mol. of oxygen for 4 ferric iron atoms. If the amount of chloroplasts was varied the total evolution is not greatly affected, but the rate was altered (fig. 2*b* and Table II). The amount of ferric iron added does not greatly alter the initial rate above $0{\cdot}5 \times 10^{-4}$. Below this the rate fell off (Table III). The initial rate with ferric oxalate is very nearly the same as with the chloroplasts in presence of leaf extracts which contain no oxalate and very little ferric iron; this is shown in fig. 3. In Table II the initial rate of oxygen production is expressed in terms of molecules of oxygen produced per hour per molecule of chlorophyll present. This again is converted by the factor 44/900 to the equivalent value of the photosynthetic number of Willstätter, represented here as *Pc* (Spoehr 1926).

The effect of intensity of illumination was not studied in detail because the experimental conditions were not suitable. The thickness of the vessel being 1·5 cm., a concentration greater than $0{\cdot}5 \times 10^{-4}$ M chlorophyll could

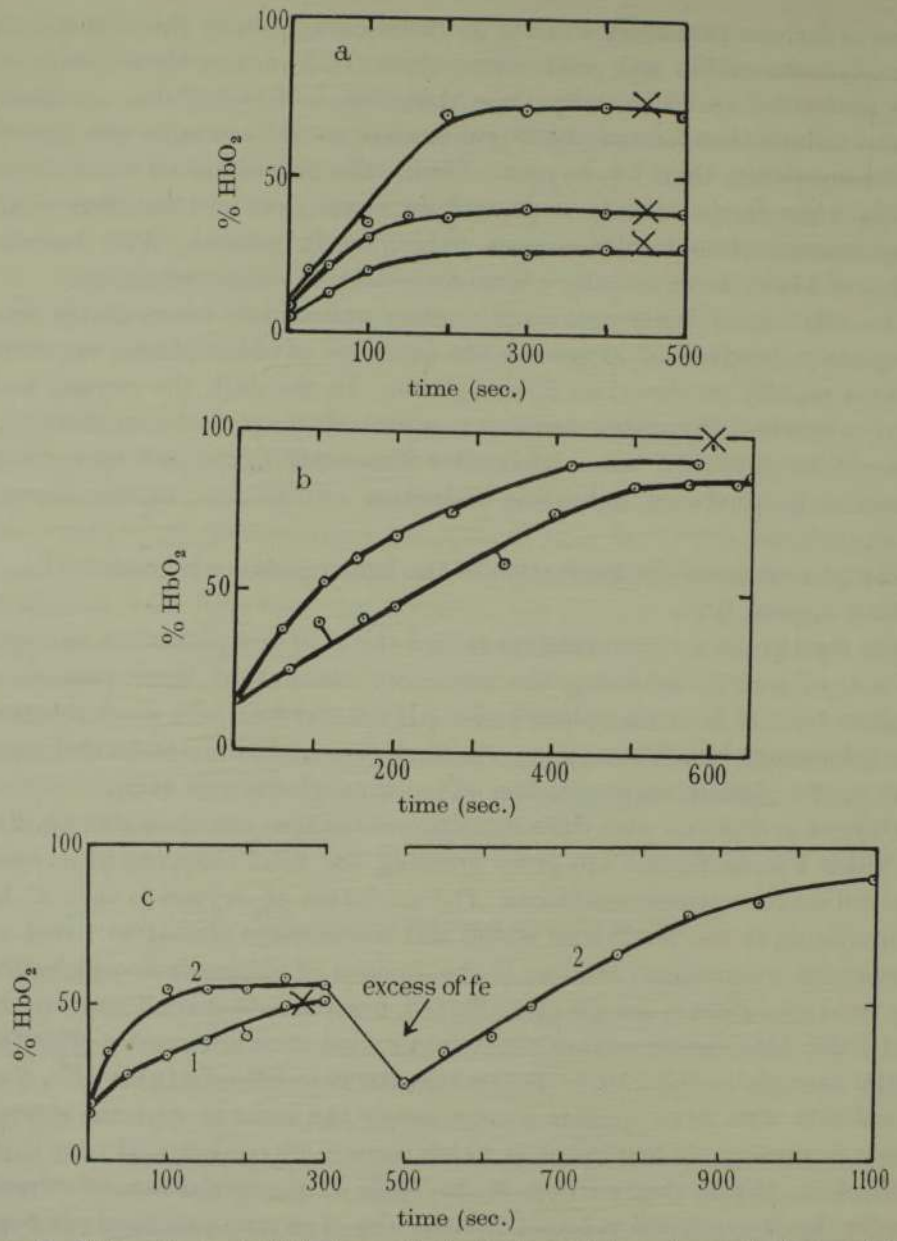

FIG. 2. Chloroplasts of *Stellaria media* in ferric oxalate and muscle haemoglobin. 20° C, pH 8.

The crosses represent the theoretical saturation of the haemoglobin for equation (2) in text. (*a*) Three curves corresponding to ferric iron concentrations of $0{\cdot}76 \times 10^{-4}$, $1{\cdot}0 \times 10^{-4}$ and $2{\cdot}0 \times 10^{-4}$ g.atoms/l.; haemoglobin $0{\cdot}6 \times 10^{-4}$. (*b*) Two curves corresponding respectively to two chloroplast concentrations $0{\cdot}17 \times 10^{-4}$ and $0{\cdot}35 \times 10^{-4}$ M chlorophyll; ferric iron 4×10^{-4}, haemoglobin 1×10^{-4}. (*c*) Two curves corresponding respectively to two chloroplast concentrations $0{\cdot}15 \times 10^{-4}$, $0{\cdot}5 \times 10^{-4}$ M chlorophyll; ferric iron 1×10^{-4}, haemoglobin $0{\cdot}7 \times 10^{-4}$. Curve 2 shows the effect of removing oxygen by evacuation and then adding an excess of ferric oxalate.

TABLE I. EFFECT OF FERRIC OXALATE AND CHLOROPLAST CONCENTRATION ON TOTAL OXYGEN PRODUCED

Exp.	Concentration in g.atoms of iron per litre: Muscle haemoglobin	Concentration in g.atoms of iron per litre: Ferric oxalate	Chloroplasts corresponding to molecular concentration of chlorophyll	Total increase in % saturation of the haemoglobin during exposure to light	Equivalents O / Equivalents Fe
p. 257	$1{\cdot}0 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$	$0{\cdot}2 \times 10^{-4}$†	46	0·92
	$1{\cdot}0 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$0{\cdot}2 \times 10^{-4}$†	75	0·75
p. 255	$0{\cdot}6 \times 10^{-4}$	$0{\cdot}76 \times 10^{-4}$	$0{\cdot}3 \times 10^{-4}$	22	0·69
	$0{\cdot}6 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	$0{\cdot}3 \times 10^{-4}$	32	0·76
	$0{\cdot}6 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$	$0{\cdot}3 \times 10^{-4}$	65	0·78
p. 256	$1{\cdot}0 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$0{\cdot}17 \times 10^{-4}$	75	0·75
	$1{\cdot}0 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$0{\cdot}35 \times 10^{-4}$	76	0·76
p. 276	$0{\cdot}7 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	$0{\cdot}3 \times 10^{-4}$	45	1·2
	$0{\cdot}7 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$	$0{\cdot}3 \times 10^{-4}$	80	1·1
p. 271	$1{\cdot}0$* $\times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$0{\cdot}3 \times 10^{-4}$	82	0·82

* Blood haemoglobin. † *L. album.*

TABLE II. EFFECT OF CHLOROPLAST CONCENTRATION ON INITIAL RATE OF OXYGEN EVOLUTION

Exp.	Concentration in g.atoms of iron per litre: Hb	Concentration in g.atoms of iron per litre: Ferric oxalate	Concentration of chloroplasts as chlorophyll in g.mol. per litre	Time in sec. for saturation of Hb; initial rate	Mol. O_2 per hr. / Mol. chlorophyll	P.c.
p. 256	$1{\cdot}0 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$0{\cdot}17 \times 10^{-4}$	610	35	1·5
	$1{\cdot}0 \times 10^{-4}$	$4{\cdot}0 \times 10^{-4}$	$0{\cdot}35 \times 10^{-4}$	290	35	1·5
p. 278	$0{\cdot}7 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	$0{\cdot}15 \times 10^{-4}$	430	40	1·8
	$0{\cdot}7 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	310	16	0·7

TABLE III. EFFECT OF FERRIC OXALATE CONCENTRATION ON INITIAL RATE OF O_2 EVOLUTION WITH CHLOROPLASTS OF *S. MEDIA*

Exp.	Concentration of Fe in g.atoms per litre: Hb	Concentration of Fe in g.atoms per litre: Ferric oxalate	Initial rate. Time in sec. for saturation of Hb
p. 276	$0{\cdot}7 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	290
	$0{\cdot}7 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$	200
p. 275	$0{\cdot}7 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	740
	$0{\cdot}7 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$	250
	$0{\cdot}7 \times 10^{-4}$	$6{\cdot}0 \times 10^{-4}$	320

not be used. At the lower concentration, $0{\cdot}3 \times 10^{-4}$, it was found that cutting off all wave-lengths shorter than 6000 A with red glass filter, produced no appreciable effect on the reaction. Under the conditions of these experiments the reaction goes as fast in red as in white light. This shows that chlorophyll is acting as part of the light-sensitive system and not the ferric oxalate.

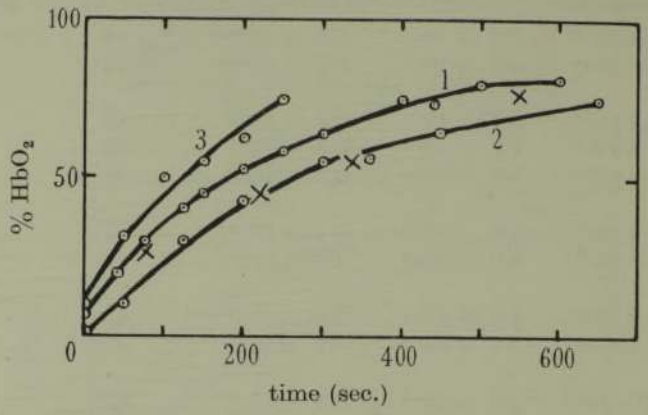

FIG. 3. Chloroplasts in leaf extracts and muscle haemoglobin. (1) *Stellaria media* plastids in extract from *Lamium album* Hb, $0{\cdot}9 \times 10^{-4}$. (2) *Lamium album* plastids in extract from *Anthriscus sylvestris* Hb, $0{\cdot}9 \times 10^{-4}$. The crosses represent the same plastids in ferric oxalate 4×10^{-4}. (3) *Stellaria media* plastids in extract from *Alliaria officinalis* Hb, $0{\cdot}44 \times 10^{-4}$.

The production of oxygen from CO_2 in the living green plant is inhibited by moderate concentrations of cyanide and hydroxylamine. It had been suggested that catalase (present in almost all plant cells) was concerned in the liberation of oxygen, although more recently indirect evidence against catalase has accumulated (Emerson 1936). Hence the effect of substances like azide, cyanide and hydroxylamine must be determined. Here, however, was a difficulty; the ferric oxalate, as has been mentioned earlier, reacts with muscle haemoglobin to form methaemoglobin. The methaemoglobin combines with azide, etc., to give compounds which are not easily reduced; thus the whole method of detecting and measuring oxygen would break down. It was found, however, that fresh diluted human blood about 1/50, could be mixed with dilute hydrocyanic acid and ferric oxalate at pH 8 in a vacuum without any immediate reaction taking place. While the affinity for oxygen of human blood haemoglobin is much less than that of muscle haemoglobin, the rate of reoxidation of the ferrous oxalate did not make it impossible to measure the oxygen evolved in light. Fig. 4 shows the effect of cyanide and azide and hydroxyl-

amine on the evolution of oxygen. In the case of hydroxylamine a certain amount of combination with methaemoglobin occurred—if this is allowed for, the oxygen output does not appear to be influenced. The same effect occurred with cyanide, but the experiments show a relatively small oxygen deficiency with two widely differing cyanide concentrations.

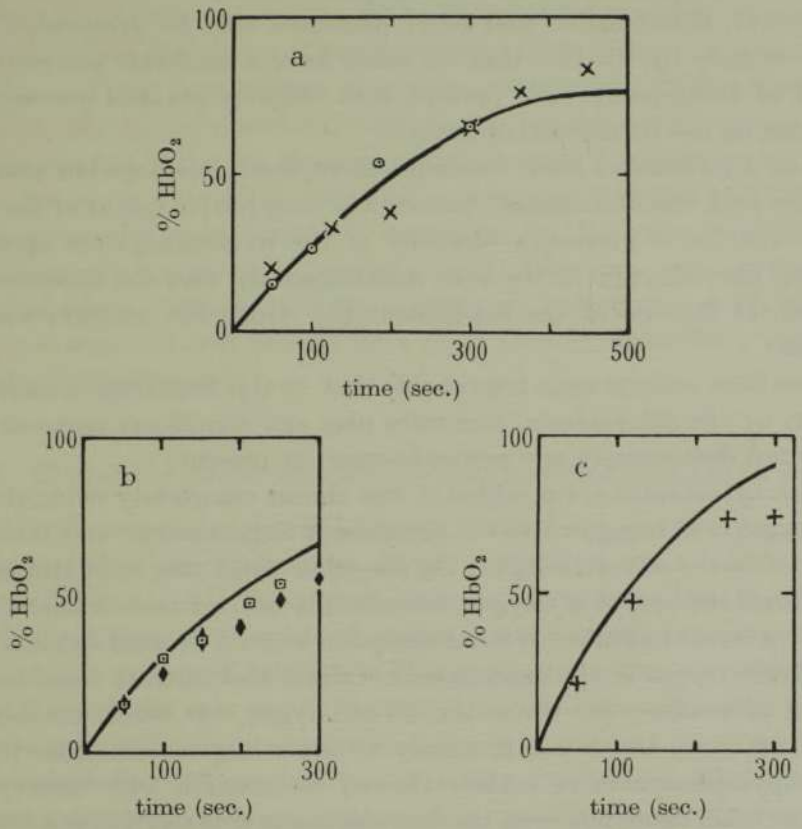

FIG. 4. Effect of certain inhibitors. Chloroplasts of *Stellaria media* in ferric oxalate, 4×10^{-4}, and haemoglobin from human blood, 1×10^{-4}. 20° C, pH 8. *a*. Curve, no inhibitor. Crosses, sodium azide, 4×10^{-4} M. Circles, sodium fluoride, 4×10^{-4} M. *b*. Curve, no inhibitor. Squares, HCN, 3×10^{-4} M. Diamonds, HCN, 60×10^{-4} M. *c*. Curve, no inhibitor. Crosses, hydroxylamine, $0{\cdot}2 \times 10^{-4}$ M.

Thus the oxygen output does not seem to be influenced by substances which have an effect on many oxidations and which also combine with catalase and with methaemoglobin. The combination with the last mentioned we use as evidence, that when the oxygen output is measured from illuminated chloroplasts, the effect is not due to some property of the haemoglobin.

That hydrogen peroxide is not directly involved in the reaction producing molecular oxygen it seems safe to conclude from the above facts. An additional point of evidence is that a relatively high concentration (about 10^{-5} M) of peroxidase with the chloroplasts was without influence on the evolution of oxygen. (I am grateful to Professor Keilin and Dr Mann for a preparation of horseradish peroxidase.)

However, it is possible that other peroxides may be involved. This is made unlikely by the fact that we could have a moderate concentration (M/20) of thioglycollic acid present with chloroplasts and yet have no influence on the production of oxygen.

When a mixture of ferric oxalate, chloroplasts, haemoglobin and thioglycollic acid was illuminated, the original deep purple colour of the ferric —SH complex is gradually bleached as the oxyhaemoglobin appeared, showing the reduction of the iron, simultaneously with the appearance of oxygen; at the end of the experiment the strong SH reaction was still obtained.

These facts make it seem improbable that, in this formation of molecular oxygen by the chloroplasts, peroxides play any significant part—at least we cannot demonstrate any peroxide stage, at present.

When cytochrome *c* was added it was almost completely reduced while the oxygen was being evolved in presence of ferric oxalate, and there was no effect on the oxygen output. On the other hand, free haematin greatly diminished the output of oxygen, because it is autoxidizable when reduced.

Only a limited number of ferric compounds could be used as a source of photolytic oxygen in the experiments. Citrate and tartrate could be used instead of oxalate, but the evolution of oxygen was much less rapid in these two cases, and it was necessary to add a large excess of ferric iron. Ferric pyrophosphate or malate showed no reaction with chloroplasts. In many other cases, however, the iron was not in true solution as a complex ion and in these cases there was no reaction with the chloroplasts. Thus it seems probable that the reagent, such as ferric oxalate, used for producing an oxygen output, must have free access to the surface of the chloroplasts. This assumption is supported by the fact that a great dilution of the fluid used when the chloroplasts are removed from the cells produces no influence on the rate of reaction with ferric oxalate. If, however, ferric potassium oxalate is used in the absence of excess of oxalate no reaction occurs with chloroplasts. There must always be an excess of the oxalate ion as compared with the iron present.

A perfectly fresh suspension of chloroplasts which is free from other matter has a characteristic silky appearance when agitated, similar to a

suspension of red blood corpuscles. At this stage the chloroplasts appear on examination to be similar to those in the cells. On keeping the suspension at 20 or at 0° C in the dark the appearance of the suspension soon changes, it becomes darker in colour and less silky on agitation. At the same time the chloroplasts are seen to be losing their clear regular outlines, although they do not appear to be actually breaking up. Before this visible alteration is reached the suspension will be found to be incapable of producing O_2 in the light under any of the experimental conditions. But it must be emphasized that the property of evolving oxygen in light is not proved to be due only to uninjured chloroplasts for the following reasons. After drying the leaf (following Molisch) a slight oxygen evolution up to 0·6 mm. pressure of O_2 is detectable by the haemoglobin method. Owing to the large amount of opaque material necessary for this experiment no actual measurements are given at present. Again, after crushing a living leaf in distilled water, when most of the plastids are broken, this fluid, when the cells are removed, will behave for a short time (2 min.) like a suspension of intact chloroplasts.

6. Evolution of oxygen by mesophyll cells of *Lamium album*

The cell suspension obtained from the leaf by centrifuging (see § 1) contained also free chloroplasts. It was introduced into a tube containing haemoglobin and all the oxygen removed by evacuation. In order to produce a measurable output of oxygen it was necessary to add some CO_2. 0·2 c.c. of a saturated solution of CO_2 in air-free water was added to the tube through the side arm. The rate of O_2 production per unit of chlorophyll obtained by these preparations was only half that obtained from the chloroplasts in ferric oxalate under similar conditions. Allowing for the presence of inactive chloroplasts outside the cells, the active chloroplasts inside the cells did not greatly exceed the rate of O_2 production of the isolated chloroplasts in the ferric oxalate. Moreover, the CO_2 remaining in the fluid after evacuation is shown to be very small in amount. Hence the oxygen evolved by the isolated chloroplasts in ferric oxalate must all come from the change from ferric to ferrous iron and not from CO_2— as indeed is shown by the relation of the original ferric iron concentration and the oxygen evolved. To remove with certainty all the CO_2 from a suspension of chloroplasts before they have become inactive is at present impossible, but this is at the moment an essential step in elucidating the mechanism of the formation of oxygen. The probability is, however, that CO_2 does not take part in the production of oxygen from ferric salts as no

difference was found after attempting to remove all the CO_2 at pH 6·8 by evacuation, and after the addition of CO_2.

Discussion

The first question to decide is whether the isolated chloroplasts are acting in a catalytic capacity or whether they possess some store of oxygen which is only liberated in light. Inman (1935) considered that the oxygen he had detected came from a store of oxygen-producing substance. Kautsky (1938) points out that ferric oxalate causes decomposition of peroxides in light. This latter effect would be far the simplest qualitative explanation of the experiments where ferric oxalate is concerned.

The reaction would be of the type

$$2Fe^{\cdot\cdot\cdot} + R\begin{matrix}O\\|\\O\end{matrix} + 2H_2O \rightarrow 2Fe^{\cdot\cdot} + R(OH)_2 + O_2 + 2H^{\cdot}. \qquad (1)$$

In the present experiments only half the oxygen for this reaction is obtained. It is possible that some oxygen is lost by other oxidations; yet this is improbable because the oxygen/iron ratio is constant for a series of experiments with different initial concentrations of ferric iron.

The second inference from the experiments is that if there is a store of a peroxide it must be at least 1/10 M in the chloroplasts, because at least 5 mol. of O_2 can be liberated per molecule of chlorophyll. This large quantity of a peroxide would be of great interest if it could be detected by a direct method, but so far there is no evidence for it.

The third inference from the experiments is that there is something which can be extracted from leaves which contains neither $Fe^{\cdot\cdot\cdot}$ nor oxalate; this however behaves towards chloroplasts very much like ferric oxalate. We will therefore leave aside the question of the store of oxygen-giving substance and consider the chloroplast as a catalytic system.

The conclusion, then, to be drawn from the present investigation is that light energy can be utilized by a subcellular system containing chlorophyll; the work done can be measured in terms of the production of molecular oxygen and reduction of a ferric complex salt.

With potassium ferric oxalate the photochemical reaction in presence of chloroplasts gives nearly the theoretical yield of molecular oxygen for the reaction

$$4K_3Fe(C_2O_4)_3 + 2H_2O + 4K^{\cdot} = 4K_4Fe(C_2O_4)_3 + 4H^{\cdot} + O_2. \qquad (2)$$

In the dark the reaction will proceed in the reverse direction.

The oxidation-reduction potential of the iron oxalate system is E_0H = zero at pH 8 (Michaelis and Freidheim 1931), while the potential of the oxygen electrode at a pressure of 1 mm. of oxygen at pH 8 would be $EH = 0{\cdot}7$ V. The reduction of the iron goes nearly to completion, but even if 99% of the iron were reduced the change in free energy would be less than half that required for a direct reduction of carbon dioxide. However, the maximum energy has not been measured as no definite equilibrium could be shown with ferrous and ferric iron in the system.

A suspension of chloroplasts will also evolve oxygen under illumination when in presence of other ferric complex salts with organic acids, and also from extracts of leaves that contain calcium ions and do not give reactions for iron salts. Thus the chloroplasts are not specific for ferric oxalate. The ferric oxalate could then be regarded simply as a reagent to demonstrate a property of the chloroplast. This might be compared to the use of indophenol reagent by Keilin (1929) in measuring the activity of the oxidase cytochrome system in the respiration of cells. There must therefore be some primary substance which is reduced, while at the same time giving oxygen. If this primary substance is A,* and the reagent B, such as ferric oxalate, represented in terms of hydrogen transport, we have the following reactions.

$$2A + 2H_2O \rightarrow 2AH_2 + O_2, \tag{3}$$

$$AH_2 + B \rightarrow BH_2 + A. \tag{4}$$

These two reactions together will represent the type of reaction (2). It must be concluded that the substance A is not easily removed from the chloroplasts because great dilution of the suspending fluid did not diminish the rate of reaction with ferric oxalate.

With chloroplasts alone we obtain no oxygen either because A is present in small amount or that a catalyst is needed to oxidize AH_2. But if a reagent is added which will reoxidize AH_2 sufficiently fast, O_2 can accumulate, but this is only possible if the reduction of the reagent B is more rapid than the oxidation by molecular oxygen. Thus the conditions are limited in which it is possible to produce a measurable oxygen output with the preparations of isolated chloroplasts.

In the absence of a reagent B, no oxygen evolution could be detected, that is, it would be less than 1/10 mm. of mercury pressure. CO_2 was also found to exert no influence on the evolution of oxygen in the case of substances which could be reduced. The next most important step would be to ascertain whether CO_2 is actually the primary substance A, giving

* This is not necessarily the substance A in Kautsky's (1937) scheme.

oxygen in the experiments. This cannot be decided directly from the results of the present work. If, however, CO_2 is the primary substance concerned with oxygen output by isolated chloroplasts, it follows directly from the experiments that the first product of reduction of the CO_2 must be as rapidly oxidized as it is formed, and hence it could not be normal carbohydrate. Also it must react with ferric oxalate at great speed. Again, if CO_2 is the primary substance undergoing reduction, the chloroplasts must have the greater part of the photosynthetic mechanism intact. This last assumption is rendered unlikely by the behaviour with cyanide and other poisons. Thus while it is not possible to show directly that CO_2 plays a part in this system giving oxygen, there is circumstantial evidence against this assumption.

It seems then, that the isolated chloroplasts have very definite and peculiar catalytic properties; it is therefore of use to bring the conclusions into relation with the living cell. This is done, not in order to add yet another theory of carbon assimilation to the many existing, but in order to prepare ground for future experiments, concerned with the evolution of oxygen.

The activity of the isolated chloroplasts measured as rate of oxygen output per unit of chlorophyll is only about 1/10 the activity of the leaf under optimum conditions. When, however, the oxygen production from CO_2 of isolated mesophyll cells of *Lamium album* is compared with the oxygen production from ferric oxalate or leaf extract with isolated chloroplasts of the same plant, the activity is nearly the same. We may therefore be justified in assuming that as molecular oxygen is a photochemical product in each case, so is the isolated chloroplast behaving in part as it does in the intact cell. In crushed leaf tissue when all cells are broken it can be inferred that oxygen is actually evolved by chloroplasts in light but practically as readily absorbed again. For it is a matter of common experience that crushed green tissue will not assimilate to any measurable extent, even though it may show respiration. Once an assimilating cell is destroyed and a false balance of the systems established, even if they may not be inactivated, any net effect of a complex chemical reaction is likely to be small. When oxygen production by isolated chloroplasts is measured in the presence of leaf extracts the pressure of oxygen obtainable is only 1 mm. of mercury. In the living plant the pressure of oxygen reached is several hundred mm. of mercury. In the dark it has been shown that the oxygen is taken up again when chloroplasts are suspended in leaf extract. The speed of reoxidation rapidly increases with increase in oxygen pressure; thus the balance is reached under the present experimental conditions at

1 mm. pressure. But in the living cell the final product of reduction appears as carbohydrate which is but slowly broken down in respiration, hence a high pressure of oxygen can accumulate.

This subcellular evolution of oxygen, then, does not represent normal assimilation and very probably has no direct connexion with carbon dioxide. Yet the activity of the system is relatively high and oxygen as a photochemical product is a characteristic of photosynthesis in green plants.

The most suggestive view is to regard the chloroplast as containing 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 which is not carbon dioxide. This substance is capable of rapid reoxidation, being the converse of reaction (3)

$$2AH_2 + O_2 \rightarrow 2H_2O + 2A. \qquad (5)$$

Organisms are known which can assimilate CO_2 in the dark while oxidizing inorganic compounds, that is, during the progress of a reaction of the type of (5). Thus it is proved that CO_2 can be reduced by living cells when free oxygen is present quite independently of light and chlorophyll derivatives. So that reactions (3) and (5) provide, qualitatively at least, a means of carbon assimilation; the net production of oxygen in the process will then be a function of the CO_2 reduced. This type of process will obviously have a low efficiency. The substance A in the chloroplast is, however, assumed to be of the type of a respiratory catalyst. This mode of linking assimilation with a part of respiration may give a high efficiency at low light intensities. The autotrophic anaerobes, which have no possibility of carrying out reaction (5) directly, obtain their energy by a system similar to the green plant. The oxygen, however, never appears as such because it is removed by hydrogen donators in the growth medium, and when these are fully oxidized assimilation ceases.

This hypothesis acknowledges the reduction of CO_2 as being a rather general phenomenon in organisms independently of photosynthesis, and yet it can explain the nature of a similar type of pigment system in the green plant and in autotrophic anaerobes for utilizing radiant energy.

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Summary

1. Haemoglobin has been used in a spectroscopic method of measuring both the appearance and disappearance of small quantities of oxygen.

2. Cell-free suspensions of chloroplasts have been obtained from the leaves of various angiosperms by grinding in isotonic sucrose solutions.

3. The observations of earlier workers, that chloroplasts will produce molecular oxygen apart from the living cell, have been confirmed by an independent method, and the quantities of oxygen evolved under different conditions have been measured.

4. The chloroplasts after removal from the cells only evolve oxygen in light when in the presence of extracts of leaves or certain ferric salts, and do not evolve oxygen from carbon dioxide.

5. In particular the chloroplasts will cause a photoreduction of ferric oxalate to ferrous oxalate and oxygen; the oxygen obtained corresponds to the iron reduced.

6. The illuminated chloroplasts in the presence of leaf extracts evolved oxygen up to a pressure of 1 mm. mercury; in the presence of ferric oxalate a pressure of 4 mm. of mercury can be reached.

7. It is concluded that a system can be removed from the cell of a green plant which will convert light energy into a measurable amount of chemical work. That molecular oxygen is produced in these circumstances, places the system in a category unique among other chemical systems.

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