A STUDY OF OXIDATION-REDUCTION POTENTIALS IN RELATION TO THE FUNCTIONS OF LIGHT AND CARBON DIOXIDE IN CHLORELLA PHOTOSYNTHESIS

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I. INTRODUCTION

It is now generally agreed that the process of photosynthesis involves the formation by the action of light and chlorophyll of chemical agents capable of reducing certain intermediates formed in non-photochemical reactions from carbon dioxide. It has been shown, moreover, that in partial systems such as isolated chloroplasts, illumination may result in the reduction of added compounds. As such, compounds of trivalent iron (1), quinones (2, 3), dyes (4, 5), some organic compounds (6) and pyridine nucleotides (7, 8) have been employed. This reduction is accompanied by the evolution of molecular oxygen, and may give rise to the formation of hydrogen peroxide (9).

It is likely that the reactions starting with the photochemical process proper, and leading to reduction of transformation products of carbon dioxide on the one hand, and to production of oxygen on the other hand, will involve one or more steps of coupled oxido-reduction (10). Some of the intermediates in these oxido-reduction chains may react with soluble compounds able to penetrate the cell wall. It would then be possible to demonstrate the occurrence of such compounds by means of potentiometric methods. We have therefore undertaken a study of the changes of the oxidation-reduction potential upon illumination of suspensions of green cells.

Several authors (l.c.) have studied the reduction of dyes by chloroplast suspensions. As has been confirmed by the work of WESSELS *et al.* (5) this is essentially equivalent to a colorimetric determination of the oxidation-reduction potential and the same end could be achieved by direct potentiometric measurement with the platinum electrode as has been reported by Spikes *et al.* (11). Perhaps the first to study oxidation-reduction potentials in suspensions of whole cells were TANG and LIN (12). In the light of present knowledge, their findings are no longer of great importance, however. The systematic use of the platinum electrode for potential measurements in suspensions of photosynthesising cells was initiated by WASSINK (13, 14) in a study of *Chromatium* photosynthesis. For various reasons

we have considered it of interest to extend this work to the study of green non-anaerobic organisms. This has necessitated the development of some special methods, which will be described below.

II. EXPERIMENTAL METHODS

Special Warburg manometer vessels were developed to make possible the simultaneous measurement of gas exchange, oxidation-reduction potential and pH. (Fig. 1). In view of the necessity to accomodate a relatively large number of side-tubes as well as a sufficiently large inner vessel for gas absorption solutions, the total volume of the manometer vessel had to be rather large viz. about 80–100 ml. To prevent electrical leaks between the suspension in the vessel and the water in the thermostat via the joints, these were provided with glass collars protruding above the water level in the thermostat.

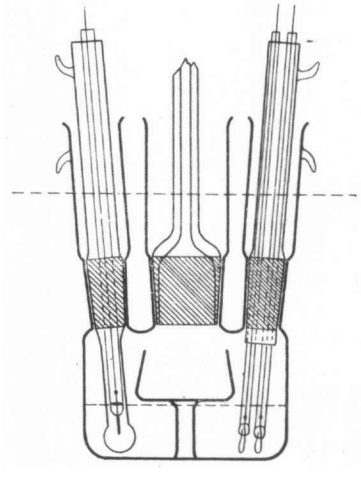


Fig. 1. Diagram of manometer vessel adapted to the measurement of pH and oxidation-reduction potential.

Great care is necessary in the use and treatment of the platinum electrodes. It is by no means easy to maintain a number of electrodes in such a condition that they indicate the same potential if submerged into the same suspension of algae, and that they keep their properties during a large number of experiments. We therefore had to devote special attention to the preparation and treatment of the electrodes. It was first found that properly prepared new electrodes, made from pure platinum wire indicated the same potential if put together in the same suspension, but that after a relatively small number of experiments deviations began to occur which eventually amounted to as much as 200 mV or even more. Originally we expected that the electrodes could be tested by putting them in a redox-mixture of predetermined potential. It was observed, however, that even badly "spoiled" electrodes (as judged from the potential reading in algal suspensions) indicated the expected potential to within 1 mV if immersed in a buffered saturated quinhydrone solution. This solution could be diluted 1000 to 10000 times before significant deviations occurred, and those were then in the same direction as found in the suspensions of algae. It is perhaps advisable to stress at this point the fact that the concentration of the redox systems excreted by living cells usually is so extremely small that minute differences in the individual properties of the electrodes become observable and behave as deviations from the ideal „inert" electrode. Earlier investigators (15) have attempted to overcome some of the difficulties inherent to this situation by adding small amounts of certain redox dyes to their solutions. After a certain period these compounds may assume the same potential as the systems excreted by the cells. They then greatly increase the poising capacity

of the solution. This in turn improves the response of the electrodes. For reasons to be outlined elsewhere in this article, such a method could not be followed in the present investigation.

Finally the following method of preparing and cleaning the electrodes was adopted and gave quite reliable results. Pieces of clean platinum wire, 2 cm long and 0.2 mm in thickness were welded to the ends of 0.6 mm copper wire in an ethanol-oxygen flame containing excess ethanol. The platinum was folded to make a loop and then cleaned in chromic acid and washed in destilled water. A bead of lead glass was molten around the platinum wire just below the weld. This was performed in an ethanol-oxygen flame containing excess oxygen. Care is required not to melt the platinum at this stage. The electrode was then sealed on to the end of a soda glass capillary with an internal diameter of about 1 mm. The soda glass should be sealed to the lead glass only and should not touch the platinum. The glass tube served as a hold and as an electrical insulation for the copper wire. The use of lead glass as a transition glass, though not recommended by some authors, was found necessary to prevent the development of troublesome cracks, which are not always easily detected but may lead to completely erroneous potential readings. There is no objection against the use of lead glass provided care is taken during sealing operations to avoid blackening of the glass. The bead should stay perfectly clear and colourless.

Such freshly prepared electrodes worked quite well, but deteriorated quickly if no special precautions were taken. The deposition of tenaciously adhering grease films on the platinum surface is probably the chief though not the only cause for this effect. The presence of greasy materials is not to be excluded completely in media of biological origin. Therefore, at the conclusion of each experiment the electrodes plus the supporting glass tubes were cleaned by immersion in warm (90° C) chromic-sulfuric acid mixture for 2 min. Before a new experiment was started this procedure was always repeated. If the electrodes are now used at this point, the potential readings usually are completely erroneous. It may be that the platinum has become saturated with oxygen and that it takes a long time before this is removed completely. In order to bring the platinum in a suitable and reproducible condition again, we have put the electrodes after the chromic acid treatment in sets of three together in a 1N KOH solution and subjected them to an electrolytic treatment. The electrodes are first connected to the positive terminal of a 6 volts battery, another Pt-wire serving as the opposite electrode. After one minute electrolysis the direction of the current is reversed and the electrodes then electrolysed for another 2 min. at the negative pole. To remove the excess hydrogen now present on the electrodes, they are immersed after careful washing in a solution of methylene blue $1 : 10^6$ containing some alkylsulfonate-type detergent. After 15 min. exposure to this solution the electrodes were very carefully washed with destilled water to remove all possible traces of methylene blue and then used immediately. We carefully checked this cleaning method and those tried, before the method was adopted definitely, by comparing such electrodes with freshly prepared non-treated electrodes in the same algal suspension. The electrodes treated in the way described above were found to indicate exactly the same potential as the fresh electrodes. The only difference is that after having been in use for some time the treated electrodes reach the final potential more quickly than fresh ones so that evidently the treatment "improves" the response. The usefull life of electrodes treated in this way varies somewhat, but may be of the order of 100–200 hours actual service. Notwithstanding the cleaning treatment this period is restricted and we have not been able to find a method keeping the electrodes in perfect condition indefinitely. In order to check the electrodes, three of them were always used together in one experiment. To this end, three electrodes were sealed into one ground joint cone with sealing wax. Very good electrodes agree to within one millivolt. As soon as the readings of the separate electrodes begin to differ by more than 30 mV we put in one new electrode. If the differences are found persistent we replace the whole lot. The agreement between the individual electrodes is least during rapid changes in the potential and there appear to be unavoidable differences in the speed of response even between otherwise very good electrodes. Bad ones often also assume the right potential if kept in the suspension for a very long time.

Between experiments the electrodes are kept immersed in destilled water.

The use of a glass electrode in the vessels is often convenient though not normally essential in buffered suspensions.

One of the joints on the manometer vessels takes a glass capillary filled with 1N KCl-agar. These capillaries are connected by means of rubber tubing to the same reservoir filled with saturated KCl solution in which the saturated calomel half cell is placed. The various electrodes can be connected separately to the electrometer (Coleman model 3D) by means of shielded polythene-insulated cables.

In most experiments, oxygen-free gas atmospheres were used. They were prepared by passing either nitrogen or nitrogen with 5 % carbon dioxide, taken from steel cylinders, over copper gauze heated electrically to about 450° C. In the case of nitrogen the gas also passes through two gas-washing bottles containing a 20 % solution of KOH and through a soda-lime tube.

In some experiments the center well in the manometer vessel contained solutions for the removal of specific constituents from the gas atmosphere. For removing carbon dioxide, one half ml. of a 20 % solution of KOH was used. If we wanted to remove oxygen as well we added to this liquid one half ml. of a 10 % pyrogallol solution. For removing oxygen in the presence of carbon dioxide we used 1 ml of a chromous sulfate solution. This was prepared by reducing a saturated solution of potassium chrome alum acidified with sulfuric acid by means of amalgamated zinc turnings until the colour of the solution was clear blue. The oxygen absorbing capacity of this solution is not very large, however, and 1 ml usually suffices for a short time only to absorb the oxygen evolved during normal photosynthesis with the amounts of algae used in our experiments. All the oxygen absorbing solutions were added to the center well after flushing the manometer vessels with the oxygen-free gasses.

In the pH interval 6–8 use was made of M/15 phosphate buffers acc. to SÖRENSEN. Between pH 5 and 6 we used M/15 phthalate buffers or mixtures being M/15 in phthalate and M/30 in phosphate.

In some experiments we wanted to add substances to the suspensions during the course of the experiment. Some of our vessels were provided with the usual type of side bulb for this purpose. As the addition of liquid from this side arm to the main compartment involves tilting of the manometer with concomitant temporary interruption of shaking and decrease in illuminating intensity, we have found it advisable as a rule to follow a different method. Thin-walled glass bulbs were blown at the end of capillary tubes. The bulbs were filled with the solutions to be added and the capillaries were sealed. The liquids were usually freed from oxygen and care was taken to fill the bulbs completely. They were inserted into the manometer vessels with a glass rod passing through a rubber stopper which was placed in one of the side arms of the vessel. The bulbs could easily be broken at any desired time during an experiment by simply pushing down the supporting rod until the bulb shattered against the bottom of the vessel. The amount of liquid could be determined very accurately by weighing.

The manometer vessels could be illuminated in the usual way from the bottom by means of two Philips' SO 1000 sodium vapour lamps. The light was filtered through 1 cm of a 5 % copper sulphate solution to remove the infra red radiation. The maximum incident intensity obtained in this way was about 4×10^4 erg/cm^2 sec. Lower light intensities were obtained with the aid of glass filters. The light intensities were measured at the place of each vessel with a calibrated Moll thermopile immersed in the thermostat bath and connected to a galvanometer.

The algae used in this investigation were cultivated from a strain of *Chlorella vulgaris* usually employed in this laboratory. They were grown for 3–4 days in Warburg's medium (16) with fluorescent tubes as the light source. In a few experiments we used a strain of *Scenedesmus quadricauda* isolated by the author. This organism was cultivated in the same way as the strain of *Chlorella*. At the start of the experiments the algae were centrifuged and resuspended in the desired buffer to a density of 4 mm^3 wet cells per ml. It is not advisable to use much more dilute suspensions as the potential readings then tend to become erroneous. From this point of view a more concentrated suspension would have been preferable but this would have made the average light intensity in the suspension rather low.

Potential values reported in this paper are quoted as measured against the saturated calomel half cell.

III. RESULTS

a. Role of oxygen and hydrogen peroxide

At an early stage of our experiments it was realised that in green organisms the potential behaviour is complicated by the fact that in air respiratory processes are possible which may also contribute to the potential formation. As a matter of fact, if the experiments are conducted in air a stable potential is set up in the dark at a rather high level and there is hardly any change upon illumination. Even in nitrogen containing only 2 % oxygen there is practically no reaction upon illumination (fig. 2 upper curve). On the other hand, if a

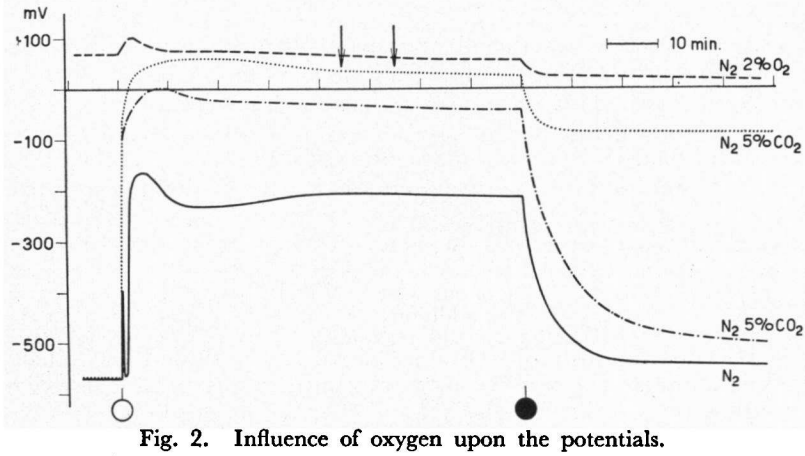


Fig. 2. Influence of oxygen upon the potentials.

———— Gas phase pure nitrogen. Center vessel with alkaline pyrogallol.
– – – – – – – Gas phase nitrogen plus 2 % oxygen.
—.—.—. Gas phase nitrogen plus 5 % carbon dioxide. Center vessel with chromous solution.
..... Gas phase nitrogen plus 5 % carbon dioxide. Center vessel with chromous solution which became saturated with oxygen during the time interval, indicated by the arrows.
In this and the subsequent figure s, ○ indicates the moment the light was turned on, ● the moment the light was turned off.

suspension of algae at pH 7 is illuminated in complete absence of carbon dioxide and oxygen, illumination produces a marked change in the oxidation-reduction potential (fig. 2, lower curve). Similar experiments can be done in gas mixtures containing carbon dioxide. In this case we have observed that the production of oxygen during the illumination does not influence the potential values but that the oxygen formed influences the dark potential set up after the suspension is put in the dark again. In fig. 2 two such experiments are given also (indicated with "N_2 + 5 % CO_2"). In one of these a sufficient amount of chromous solution was added to the center well to absorb all the oxygen produced during illumination, in the other the chromous

solution became saturated during the time interval indicated by the arrows. This could be followed accurately by observing the manometer readings which change sign as soon as the oxygen is no longer absorbed. The difference in dark level is very pronounced though there is little difference in potential in the light. It appears therefore, and more experiments of the same type have convinced us, that the potential level in the dark (DP) is strongly dependent upon the presence of oxygen. This is doubtlessly due to respiratory processes. At a sufficiently high concentration of oxygen this effect completely supersedes the effects of the illumination. For this reason we have considered it necessary to conduct most of our further experiments in the absolute absence of oxygen. More detailed examples of the potential changes upon illumination of anaerobic suspensions are given in the following figures (e.g. fig. 7) and will be discussed in a later section.

Two questions now arise. The first is: what mechanism is responsible for the establishment of the anaerobic dark potential? We will discuss this point later. The second is of fundamental importance for the interpretation of the experiments, described in this paper. It appears that, at least at pH 7, the ultimate effect of illumination in the absence of air is a considerable rise in potential (fig. 2). Is this rise due to the production of oxygen? It is known that even in the absence of carbon dioxide small amounts of oxygen are formed from intracellular hydrogen donors.

The argumentation will be divided in two sections viz. one applying to the presence of carbon dioxide, the other to the absence of it. In the experiments illustrated in fig 2, we have studied the effect of complete and partial removal of photosynthetically produced oxygen. It will be clear, that from the moment on indicated by the arrows a quite considerable amount of oxygen collected in the gas phase and this was observable both from the manometer readings and from the subsequent potential in the dark. Notwithstanding this the potential in the light (LP) does not show any influence of this oxygen-accumulation as evidenced by a comparison with a similar experiment, where the oxygen was removed rigorously throughout the illumination period. These results do not give support to the theory that oxygen is directly responsible for the rise in potential during illumination in the presence of carbon dioxide.

We will now consider the experiments in the absence of carbon dioxide (fig. 3). After the establishment of a stationary DP, in one experiment nitrogen with 0.2 % oxygen was passed through a vessel at moment *a*. The amount of oxygen present in that vessel thereby increased from practically zero to about 150 mm^3. This was accompanied by a rise in the potential of 65 mV. During illumination in the absence of carbon dioxide the potential may rise as much as 450 mV and if oxygen production was responsible for it, much more than 150 mm^3 should therefore be constantly present in the manometer vessel during the final part of the illumination period. The limit of accuracy of the manometer readings corresponds to about

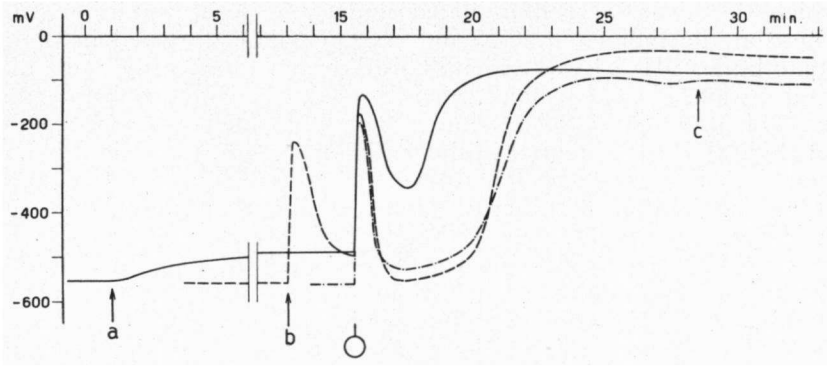


Fig. 3. Influence of oxygen. Three different vessels. At moment *a*, nitrogen plus 0.2 % oxygen was passed through the suspension. At *b*, a solution of 6 mm^3 oxygen in water was added. At *c*, a solution of 0.25 mg catalase was added.

5 mm^3. In experiments of this type we have never observed a gas production surpassing this quantity. Therefore, the large rise in potential during illumination cannot be explained by oxygen production. This conclusion is strongly supported by the results of other experiments where the oxygen pressure in the vessels was also kept constant, namely zero, by passing pure nitrogen through the vessels during the illumination period. This should effectively remove the oxygen or at least prevent a build-up in the gas phase. Nevertheless the potential course in this case is exactly the same as in a parallel experiment where no nitrogen is passed through the liquid. It appears superfluous, therefore, to give an illustration of such an experiment. Combining the evidence reported above it appears highly probable that the large rise in potential upon illumination both in the presence and absence of carbon dioxide is due to other causes than the production of oxygen. As will be shown in a later section, under certain conditions of pH the potential in the light is lower than in the dark and this is a confirmation of the conclusion arrived at above. The reasoning given here applies to the stationary LP only and the experiment *a* of fig. 3 shows that unlike in the stationary phase, oxygen has influence upon the induction phase of the potential. We will return to this point later on.

During the course of our investigations we became aware of a publication by MEHLER (9) who has shown that in the absence of hydrogen acceptors, chloroplasts may use oxygen as a hydrogen acceptor during the Hill reaction, thereby forming hydrogen peroxide. On the basis of equivalent amount of oxygen, hydrogen peroxide is a much more powerful oxidising agent than molecular oxygen and it was therefore not to be excluded that in our case the observed potential rise was due to the production of small amounts of hydrogen peroxide. To test this possibility we have added to our algal suspensions an amount of catalase very much greater than the amount naturally occurring in the algae. In fig. 3. is illustrated an experiment in

which the algae were illuminated under the usual conditions but where at the moment *c*, 0.25 mg cryst. catalase dissolved in phosphate buffer pH 7.0 was added. This did not influence the potential in any way. The same holds when the catalase is added before the start of illumination (experiment not illustrated). We therefore conclude that the potential in the stationary phase in the light is not related to the presence of hydrogen peroxide either.

An examination of the curves representing the potential changes in nitrogen at pH 7.0 reveals that there is a sudden "outburst" of an oxidising reaction during the first seconds of the illumination. A maximum is usually reached about 20 sec. after the start of the illumination. This is followed by a quick fall in potential to about the dark level after which a slower rise to the stationary level sets in. Usually there is another indistinct maximum before this final level is reached (e.g. in the lower curve of fig. 2) but this is not always very pronounced though seldom completely absent. The significance of the initial peak is not clear and the phenomenon does not occur at lower pH (section *b*). The following observation may have bearing upon the phenomenon. In experiment *b* of fig. 3 we have added at moment *b* to the suspension of algae in the dark about 6 mm^3 oxygen dissolved in water. There is a quick mixing of the oxygen with the solution and the potential rises immediately. At the same time the oxygen starts to equilibrate with the gas atmosphere so that the potential soon falls again. The general shape of this "oxygen peak" is very similar to the initial peak during illumination and it is not to be excluded that the last mentioned phenomenon is due to the sudden release of a small amount of oxygen. A similar peak can be found in the induction curve for chlorophyll fluorescence in *Chlorella* and this was supposed earlier by WASSINK *et al.* to be connected with oxygen liberation through the Hill reaction (33). More recently VAN DER VEEN (17) demonstrated such an "oxygen gush" in higher plants immediately after the start of illumination by a gas analytical method. As follows from the experiments to be discussed under section *e*, such a temporary oxygen production cannot be due to previous accumulation of carbon dioxide.

b. Influence of pH upon the potentials in the light and in the dark

Between pH 5 and 8 the hydrogen ion concentration of the suspension mainly affects the potentials in the dark and to a much smaller extent the potentials in the light. This applies equally to experiments with and without carbon dioxide. In fig. 4, the results of a number of observations are collected. Notwithstanding the fact that the behaviour of different batches of algae towards illumination is only qualitatively and never quantitatively the same, a circumstance which has been a serious limitation during our experiments, the DP's are remarkably reproducible. Between pH 6 and 8 (phosphate buffers) they follow a straight line with a slope of 60 mV per pH unit as is usual. There is a sudden sharp transition

between pH 5.75 and 5.25. In this interval the slope is much too steep to be connected with phenomena of electrolytic dissociation (18) and we must conclude that in this region there is a change in the activity of some enzyme system connected with the metabolic reactions responsible for the establishment of the dark potential.

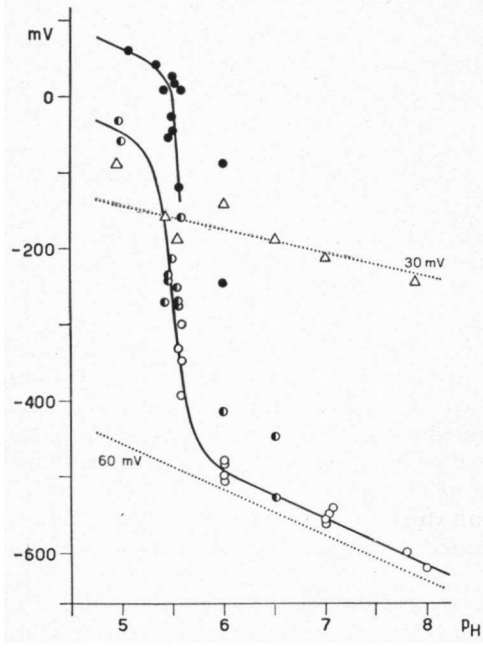


Fig. 4. The potentials as a function of pH. Dark potentials:
○ phosphate buffers.
◐ phosphate-phthalate buffers.
● phthalate buffers.
Potentials in the light (without carbon dioxide):
△ phosphate and phosphate-phthalate buffers.

In the region below pH 5.5 we have been using either phthalate buffer or a mixture of phthalate and phosphate buffers. As far as our observations go at present it appears that all buffers containing phosphate lead to DP's falling reasonably well on the same line. On the other hand the results with phthalate buffers are consistently higher by about 100 mV. As the observations with mixed buffers show, this is not due to a harmfull effect of the phthalate but must be ascribed to the absence of a sufficient concentration of phosphate. The phosphate effect is an interesting phenomenon in itself and we hope to be able to return to this matter in a later publication. Also in fig.4 have been given the points representing LP's in experiments without carbon dioxide. As will be treated in more detail under section *c*, the stationary potentials in the light are not very reproducible, depending upon a number of factors one of which is the previous anaerobic dark period. As much as possible we have collected in fig. 4 the results of experiments where the anaerobic dark period had been about 90 min. The potentials are those observed after 20–25 min. illumination and the values belonging to the same pH have been averaged. One thing is clear namely that the transition zone of the

DP's is not reflected in the LP's. The observed pH-dependency fits a 30 mV/pH line somewhat better than a 60 mV/pH line but the accuracy is insufficient to settle this question definitely.

It is evident that the mechanisms responsible for the establishment of the potential in the light, and in the dark are quite different and we must conclude that in first approximation they are entirely independent. Under anaerobic conditions in the dark there appears to be a certain metabolism leading to the production of reducing substances. It may be remarked here that our observations have confirmed the opinion expressed by other authors (17), that this anaerobic metabolism is also characterised by the production of a small amount of carbon dioxide (see section *e*).

The conclusion arrived at above is an important one as it emphasises the fact that no value is to be attached to a comparison of the potentials in the light with those in the dark. This also implies that during the first moments of the illumination there must be a transition from the dark metabolism to a metabolism in the light and though this is reflected in the potential changes, these are still influenced for a certain period by the preceding dark metabolism. If we now compare a number of induction curves at different pH such as are given in figs 5 and 6, we may conclude that after the first 20–30 seconds of the illumination the potential behaviour in the light is very similar even at widely different pH values. The experiments of figs 5 and 6 also show that the differences in DP's at the various pH values are not due to slowness in the attainment of a stationary potential level as the various potential curves return to their original level after the light is turned off again.

As follows from fig. 4 the stationary LP is higher than the DP above pH 5.5 in carbon dioxide-free media. Below this pH the reverse holds. This is illustrated by the results of the experiments shown in fig. 5. This observation appears to form an additional argument supporting the conclusion arrived at in section *a*, viz. that the potential changes in the light are not due to the evolution of oxygen or the

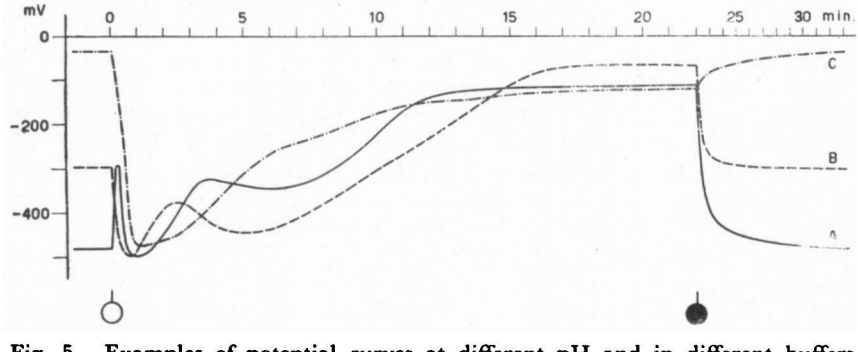


Fig. 5. Examples of potential curves at different pH and in different buffers. ———— Phosphate buffer pH 6.0. – – – – – – – Phosphate buffer pH 5.6. —.—.—. Phthalate buffer pH 5.5.

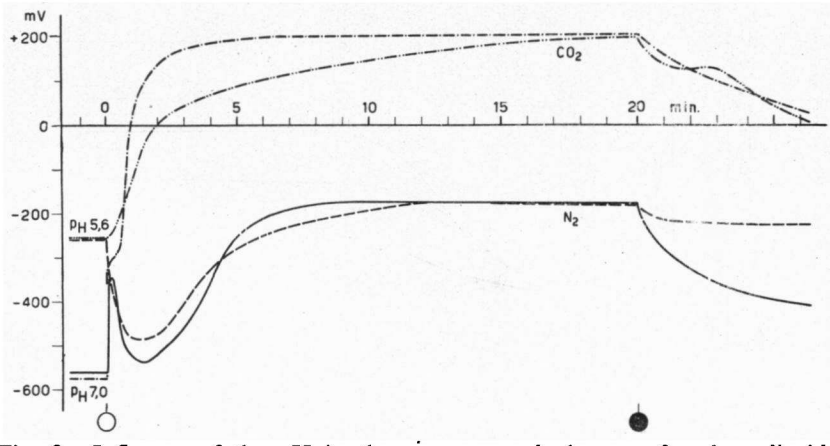


Fig. 6. Influence of the pH in the presence and absence of carbon dioxide.

production of hydrogen peroxide. Summarising the results discussed above we may say that the potential changes in carbon dioxide-free media during illumination are characterised by the attainment of a relatively low potential some moments after the start of illumination followed by a slower rise to a stationary value.

We want to discuss the influence of carbon dioxide in a later section. In the mean time it may be remarked here that carbon dioxide has no influence upon the DP as may be seen from fig. 6.

The fact that in the experiment illustrated in fig. 6 the potentials in the presence of carbon dioxide do not tend to return to the original dark level after the light is turned off, is again due to the production of oxygen during the illumination period. This gas was not removed in this particular experiment. The phenomenon was already discussed in section *a* (see fig. 2).

c. Influence of the anaerobic dark period

As has been mentioned in the previous section the length of the anaerobic dark period preceding the illumination in carbon dioxide-free media influences the potential behaviour in the light. We have been forced to make use of relatively extended periods of exposure to conditions of low oxygen pressure, as it takes a certain minimum time to expell the air from our suspensions and to allow the electrodes to attain a steady potential. An hour was usually required for this purpose, counting from the start of the introduction of pure nitrogen into the suspension in the manometer vessels. In fig. 7 we have illustrated the result of an experiment in which the anaerobic dark periods have been increased from 72 to 190 minutes. Though the general shape of the curves is similar it appears that both the initial peak and the stationary light level increase with increasing time of anaerobiosis. The reason for this effect is unknown. This makes it, however, difficult to compare different experiments if the anaerobic dark periods are not known accurately.

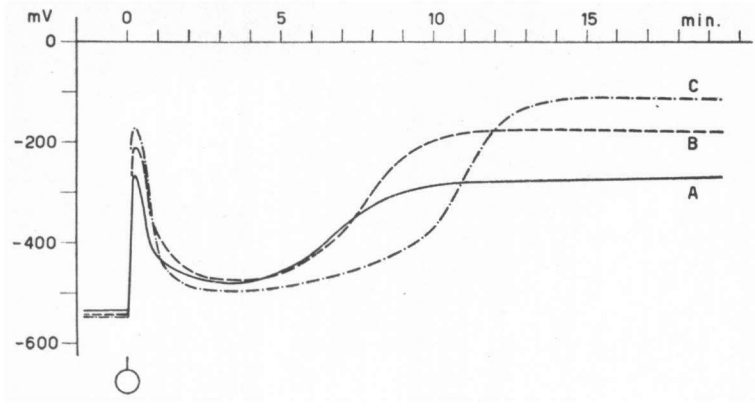


Fig. 7. Influence of the anaerobic dark period.
A: dark period 72 min. B: dark period 100 min.
C: dark period 190 min.

Even so we have found that the response to anaerobiosis varies from one batch to the next as do other details of the potential behaviour such as the depth and length of the low-potential "trough" which was for instance very long in the experiment of fig. 7 but may be quite short in other cases. In general there is a considerable divergency in the properties of different batches of algae notwithstanding the fact that we have standardised the cultivation of the algae as much as possible. It is clear that small differences in the pretreatment of the material may have important influences upon the behaviour during illumination. We have made a point of always measuring the photosynthetic activity of our experimental material simultaneous with our potential measurements in a separate vessel in carbonate-bicarbonate buffer. This has put the fact beyond doubt that minor variations in photosynthetic activity of the algae may be accompanied by important quantitative differences in potential behaviour.

d. The reducing action of intact and disintegrated Chlorella cells

From the foregoing discussions it has become clear that during a certain phase, *Chlorella* cells illuminated in the absence of carbon dioxide may develop the ability to exert a reducing action as evidenced by the oxidation-reduction potential of the liquid surrounding the cells. On the other hand the level of reduction reached during illumination never significantly surpasses the most reduced state reached anaerobically in the dark under favourable conditions. So far, evidence is therefore still lacking that during illumination the cell is developing a more powerful reducing action than in the dark. This is a consequence of the fact that a measurement of stationary oxidation-reduction potentials provides an indication of the degree of reduction of certain constituents of the cell but does not give any information as to the capacity of such systems and some sort of kinetic analysis is needed to settle this point.

The following experiments have led to a decision in this matter. If methylene blue is added to an anaerobic suspension of *Chlorella* at pH 7, the normal DP is not reached within a reasonable time.

If a concentration of methylene blue of between 10^{-5} to 10^{-6} g/L is added, a DP is usually observed of around —230 mV against sat. cal. This value is rather close to the normal redox potential of the dye (about —239 mV on our scale) i.e. in the region where the dye exerts its maximum poising capacity. After very long times of observation or sometimes with particularly active algae the potential ultimately may begin to fall again and in this case the usual DP is finally reached also. This series of events indicates that compared with the amount of dye added the reducing systems of the anaerobic dark processes are of a low capacity. If we now start the illumination before the true dark potential is reached, that is, as long as the methylene blue is still only partly reduced we may observe that illumination results in a quick fall of the potential after which the potential course is the same as in a suspension without methylene blue (fig. 8). As there are indications that illumination in the presence

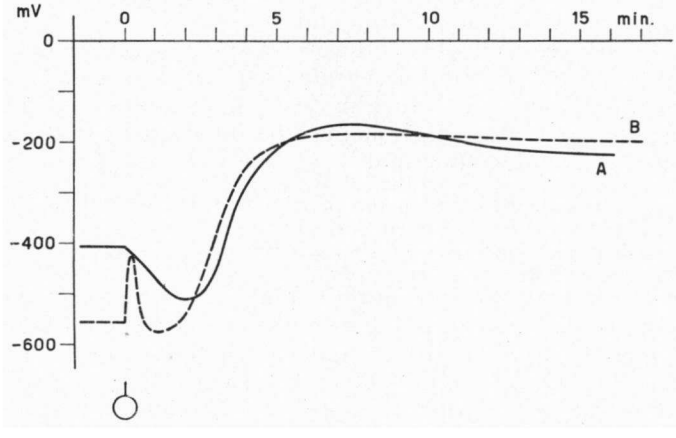


Fig. 8. Reduction of methylene blue during the induction period in the absence of carbon dioxide. A, with methylene blue, temp. 16° C. B, without methylene blue, temp. 25° C. Center vessel with alkaline pyrogallol.

of methylene blue is not a completely harmless treatment to the algae, we have conducted the experiment at a lower temperature (16° C). In fig. 8 this experiment is compared with a blank at the usual temperature of 25° C. This result indicates that at the start of the illumination the methylene blue is much more quickly reduced than in the dark and that therefore the potential determining systems operative during illumination have a larger capacity than those active in the dark. There are indications that small concentrations of oxygen may also be reduced quickly in the induction phase of illumination. We have no very satisfactory evidence at hand, however. Curve *b* of fig. 3 could be interpreted in this way if we extrapolate the potential course after the addition of oxygen in the dark to the point where the induction curve in the light has reached its minimum.

It then appears as if this minimum is lower than the extrapolated dark level. More experimental material would be required to settle this point definitely.

In our opinion the conclusion arrived at above is an important one in relation to an interpretation of the causes of the potential changes during illumination of algal suspensions. It strongly supports the opinion that these potential changes form a reflection of the formation and transformation of reducing substances formed by the photosynthetic system. In this light, the absolute potential values observed may also have some significance. It is perhaps not a mere coincidence that the lowest potential reached during illumination, namely say —560 mV at pH 7.0, is close to the normal potential of the pyridine nucleotides reported recently by BURTON (19). This author has estimated the E'_0 at pH 7.0 of diphosphopyridine nucleotide to be —320 mV (hydrogen scale) that is equivalent to about —570 mV on our scale (sat. cal.). We might attempt to interprete a curve like *C* of fig. 5 in such a way that about one minute after the start of illumination the pyridine nucleotides in the cells became about half-way reduced. This interpretation gains additional significance in the light of recent experiments whereby it was shown that isolated chloroplasts may reduce pyridine nucleotides (7) and it has been suggested that this reaction may be involved in the photosynthetic carbon dioxide reduction.

In a few experiments with *Scenedesmus quadricauda* at pH 7 a DP of —540 mV was observed. The potential changes during illumination, both in the presence and absence of carbon dioxide, were found very similar to those of *Chlorella*.

Above pH 6 the DP is about the same as the lowest potential reached in the light. One is inclined to assume that in both cases the same reduced compounds are involved. This leads to the following generalised picture. The reductive processes occurring anaerobically in the dark and those induced by the action of the light both lead to the reduction of the pyridine nucleotides. As the two kinds of reductive processes have a limited though different capacity and as dehydrogenative processes may be going on simultaneously, the comparatively large reservoir of pyridine nucleotides in the cell may be considered an effective buffer keeping the oxidation-reduction potential relatively constant notwithstanding important variations in the total amounts of reducing substances present under the different conditions. This region of "buffered" potentials should of course be in an interval of a few tens of millivolts above and below the normal potential of the buffering compound involved.

It is difficult to resist the temptation to speculate a bit further. During photosynthesis the reduced pyridine nucleotides could be used up by two processes. On the one hand they might serve as the sources of reducing power for the reduction of carbon dioxide. On the other hand their removal by simultaneously occurring oxidative processes might furnish the energy for the completion of this carbon dioxide reduction cycle (20). Ultimately the energy is then derived from the photochemical process through the reduction of the pyridine nucleotides. The occurrence of a high concentration of reduced pyridine nucleotides during anaerobiosis in the

dark is an indication of their formation also under normal conditions of respiration. They are then continuously removed through oxidative processes so that they cannot reach the same steady-state concentration as in the absence of respiratory processes. Nevertheless their continuous oxidative removal likewise produces a continuous source of the same sort of chemical energy as formed in photosynthesis and they may also be active in similar, though not necessarily identical processes of carbon dioxide reduction. As in this case the energy required for carbon dioxide reduction ultimately is derived from the respiratory consumption of cellular constituents there is no nett uptake of carbon dioxide as is quite obvious.

We have supplemented the experiments reported in this section, with some observations with disintegrated *Chlorella* cells. Though as far as we are aware no method has as yet been described for the isolation of chloroplasts from algae, the method of preparing colloidal extracts from photosynthetic organisms by grinding with carborundum powder (KATZ *et al.* 21) should lead to preparations comparable in many respects to disintegrated chloroplast suspensions of higher plants.

Such disintegrated *Chlorella* preparations, prepared at 2° C and suspended in phosphate buffer, show a pronounced change in oxidation-reduction potential upon illumination (fig. 9). The potential

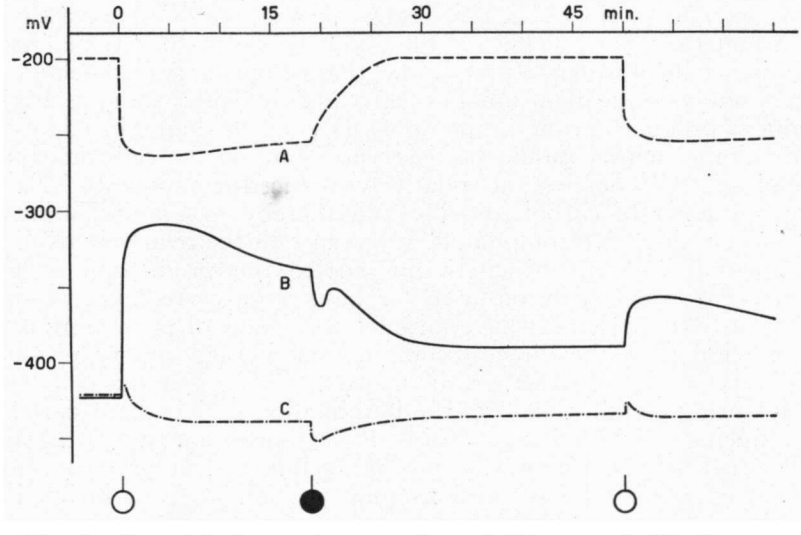
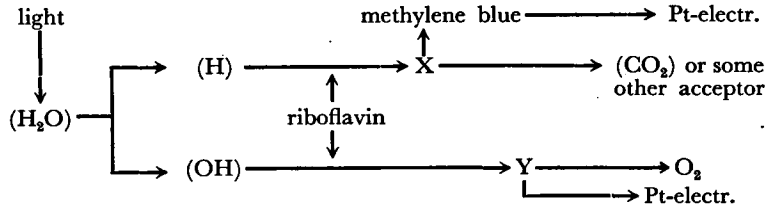


Fig. 9. Potential changes in suspensions of disintegrated *Chlorella* cells. A: with addition of 1: 10^6 methylene blue. B: no addition. C: with addition of 1: 10^6 riboflavin.

reached anaerobically in the dark is not quite as low as with whole cells but may in favourable cases approach this value. Unlike in suspensions of intact cells the illumination provokes a potential change in the positive direction (curve *B*). As the light is turned off the potential falls again and this series of events can be repeated

a number of times though the changes tend to become gradually smaller. This is probably due to an inactivation of the material. That these preparations nevertheless may unfold reductive powers during illumination becomes evident if a small amount of methylene blue (10^{-6} g/ml) is added (curve *A*). This effectively keeps the potential high in the dark, and a comparison with the experiments with whole *Chlorella* cells demonstrates that disintegration has considerably reduced the reductive capacity of the material. However, illumination in this case leads to a quick fall in potential to a level indicating a considerable degree of reduction of the methylene blue. These changes too are reversible upon interruption of the illumination. The addition of a small amount of riboflavin (10^{-6} g/ml) has no influence upon the DP. This is a consequence of the fact that the normal reduction potential of this compound (—220 mV at pH 7.0, i.e. about —470 mV on our scale) is somewhat below the DP. However, upon illumination the presence of this compound effectively suppresses any potential change except for some slight changes at the start and at the end of illumination (curve *C*). It should be added here that addition of riboflavin has no measurable influence upon the potential behaviour of intact *Chlorella* cells.

We would like to offer the following tentative explanation for the reported effects. If we base our discussion upon the simplified assumption that the primary effect of the absorption of light is the splitting of a molecule of water (10), it follows that this primary photochemical act produces equivalent amounts of relatively oxidised and relatively reduced products. From this point of view, no nett change in oxidation-reduction potential should be observable outside the cell under any condition. Ultimately, the relatively reduced compounds lead to the reduction of carbon dioxide and thereby to the formation of non-electrode active compounds. In green cells the relatively oxidised compounds formed, ultimately give rise to the production of molecular oxygen which on a molar basis is only weakly oxidising. Obviously the cell must have an effective means of preventing or at least regulating, the direct recombination of the primary products of the photolysis of water. At any rate, it is more or less a coindence that during certain phases of the illumination period the occurrence of reducing compounds can be directly demonstrated. In disintegrated cell preparations where the normal cellular structure has been destroyed quite other conditions may prevail. We are inclined to conclude that in such disintegrated preparations there is a mixture of various redox-compounds which may be able to react with different speed with the intermediates of the reactions of the photolysis products of water. Depending upon the relative speed with which certain compounds are either hydrogenated or dehydrogenated, an oxidising or a reducing effect may be "transmitted" to the platinum electrode.



Under normal conditions obviously a link is established in a disintegrated preparation between the electrode and the "oxidised line" (see above diagram). Methylene blue, however, appears to react much more quickly with some intermediate (X) of the "reduced line" and this is reflected in a fall in potential upon illumination. It would be in line with the above explanation to assume that whereas riboflavin quickly reacts with the "reduced line" its dihydro form produced in this way will be able to react very quickly with some intermediate of the "oxidised line" as indicated schematically by the arrows. Riboflavin therefore acts so to speak as a short-cirquit between the "reduced" and the "oxidised line". We believe that this picture is not in disagreement either with the observations or with the theoretical deductions made from the experiments with intact cells.

We have been able to obtain results essentially similar to those reported above, with chloroplast preparations of higher plants but we do not intend to go into details in this paper. In the mean time our observations speak against the opinion, expressed by some authors (9, 5) that only redox-compounds with relatively high oxidation-reduction potentials are suitable as Hill reagents, and that in particular, methylene blue is not or only weakly active. We must emphasise that, unlike the majority of previous authors, we have conducted all our experiments in oxygen-free gasses. Undoubtedly, the failure to recognise the effects of oxygen and the presence of oxidising enzyme systems in their preparations, has been responsible for unwarranted conclusions by several investigators as regards the ability of various compounds to serve as Hill reagents. In particular, the failure to demonstrate directly by means of spectro-photometric techniques the formation of dihydropyridine nucleotides in illuminated chloroplast preparations may be due, according to our observations, by the lack of appreciation for the part played by oxygen.

e. The influence of carbon dioxide upon the potential behaviour

As was explained already, the presence of carbon dioxide (as a gas atmosphere of nitrogen containing about 5 % carbon dioxide) has no measurable effect upon the DP's. Upon illumination its presence manifests itself by a very quick rise of the potential to a level which may be as high as 700–800 mV above the dark potential (fig. 6). Again this potential in the presence of carbon dioxide is only weakly dependent upon the pH although we have insufficient

material to give a more accurate estimation of the influence of the pH. It appeared that the concentration of carbon dioxide present under these conditions is much too high to study its effect upon the early stages of the potential curve. Actually, very low concentrations of carbon dioxide already produce marked effects upon the course of the potential in the first minutes of illumination. As it was not so easy to add such small doses of carbon dioxide in an accurate way, we have made use of the production by the algae of some carbon dioxide during the anaerobic dark period. In the first place it was shown that anaerobiosis in the dark in a vessel containing no KOH in the center well, led to differences in the potential behaviour during the following illumination. This could be prevented either by adding KOH to the center well or by constantly flushing the vessels with pure nitrogen during the dark period. This led to curves, exactly identical in both cases and we have taken the fact that a volatile compound is obviously formed which can be absorbed by KOH, as sufficient evidence that the compound was carbon dioxide. Actually the effects can be duplicated by the intentional addition of carbon dioxide. By allowing the vessels to accumulate carbon dioxide as the result of varying periods of anaerobiosis, by closing off from the central nitrogen supply at different times, a series of experiments with increasing amounts of carbon dioxide could easily be performed. In fig. 10 the result of such an experiment is illustrated, the numbers in brackets indicating the time in minutes during which carbon dioxide was allowed to accumulate in the dark. In the same figure the result of an experiment in which the gas phase consisted of nitrogen with 5 % carbon dioxide, that is, with a large excess of carbon dioxide is also given. It is of importance to note that the amount of carbon dioxide, formed during anaerobiosis in the dark is so small

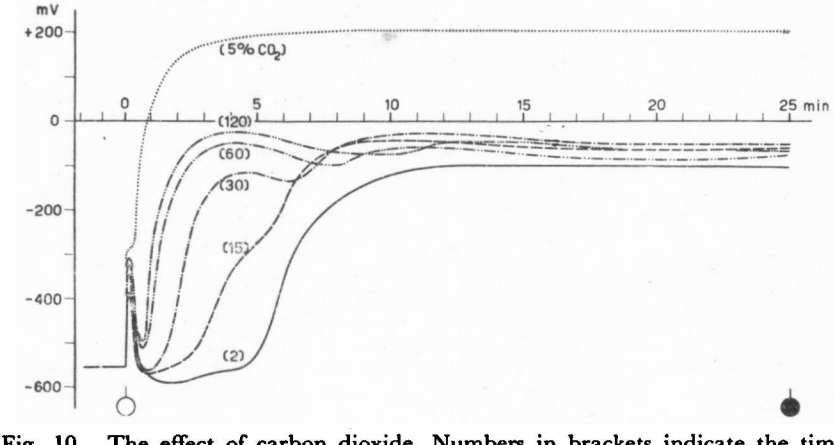


Fig. 10. The effect of carbon dioxide. Numbers in brackets indicate the times in minutes of anaerobic carbon dioxide accumulation and are a relative measure of the amount of carbon dioxide present. Dotted curve: gas phase with 5 % carbon dioxide.

that it is hardly observable manometrically even after two hours. This amount of carbon dioxide is therefore used up quickly during illumination and the righthand parts of the curves in fig. 10 therefore apply to essentially carbon dioxide-free conditions (except of course the upper curve).

From experiments such as the one illustrated in fig. 10 it becomes clear how carbon dioxide influences the potential course during illumination. If we first look at the curve marked (2), i.e. the one applying to an experiment in which carbon dioxide was accumulated during 2 minutes only and which therefore contained the smallest quantity of the lot, we see a slight inflection about 3–4 minutes after the start of the illumination. As the amount of carbon dioxide increases, this inflection grows more and more. The fall, occurring at higher concentrations of carbon dioxide after about 5 min. illumination undoubtedly is associated with the exhaustion of the carbon dioxide. Taking into account this fact, we see that there is a gradual transition to the curve for excess carbon dioxide (top).

The different heights of the peaks immediately after the start of illumination, are due to the fact that in experiments of this kind also the total anaerobic dark period was varied. This in itself leads to an increase in peak height as has been discussed in section *c*. This difference in peak height therefore has nothing to do with the presence of carbon dioxide. We may therefore conclude that the influence of carbon dioxide upon the potential changes in the light is not present immediately after the start of illumination but only sets in gradually and reaches its maximum after about 4 minutes (in this particular experiment!). It is important to note that in several cases we have observed a slight inflection in the potential curve for nitrogen plus 5 % carbon dioxide, about 20–30 sec. after the start of illumination, as illustrated also in fig. 10. This should correspond to the transition from the initial peak to the "carbon dioxide peak" just described. Though it is difficult to measure this inflection accurately owing to its short duration, its demonstration nevertheless is of significance. The consequences of the delayed action of carbon dioxide are important and will be discussed in the next section. We may confine ourselves here to the statement that the effect of carbon dioxide is not immediately observed during illumination, notwithstanding the fact that the carbon dioxide was already present for a considerable time before the start of illumination.

f. The problem of the so-called "persistence of reducing power"

In our opinion, the observations reported in the preceding section form a strong support for the theory that the reducing action of the illumination such as was evidenced already by other experiments (section *d*) is also involved in the mechanisms of the photochemical reduction of carbon dioxide in so far as carbon dioxide removes this reducing material.

Our experiments, therefore, may also throw some light upon the problem of the so-called "persistence of reducing power" formed

during preillumination in the absence of carbon dioxide. There has been an important controversy on this question between CALVIN and his collaborators on the one hand and GAFFRON and his co-workers on the other hand (22, 23, 24, 25). From their experiments with labelled carbon dioxide, CALVIN and BENSON (22) concluded, that illumination in the absence of carbon dioxide leads to the formation of a capacity to fix carbon dioxide during a subsequent dark period. They found that this ability to fix carbon dioxide persisted in the dark for some time, the amount of carbon dioxide taken up declining to about one half during a dark period of 5 minutes. In a later paper (23) they demonstrated that the products formed from carbon dioxide during this type of dark fixation are more similar in nature to the products formed during normal photosynthesis than to those formed from labelled carbon dioxide during respiration. It was therefore concluded by these authors that the illumination in the absence of carbon dioxide leads to the formation of compounds, able to reduce carbon dioxide during subsequent exposure in the dark, in the same way as during photosynthesis.

This view was strongly criticised by GAFFRON *et al.* (24, 25), who maintain that the effect of preillumination is only in removing carbon dioxide from intracellular "carbon dioxide reservoirs" and that subsequent exposure to carbon dioxide in the dark leads to a replenishment of such reservoirs. This point of view received support from the experiments of MEHLER (9) who observed no persistence of the ability to reduce Hill reagents after preillumination of chloroplast preparations.

It would appear that our experiments in principle are able to give a decision between these controversial viewpoints. We have therefore performed the following experiments. *Chlorella* cells were preilluminated for a certain period without carbon dioxide and the potential was observed. At a suitable moment, the light was turned off, and carbonic acid was added to the suspension as quickly as possible.

The method employed was as follows. As preliminary experiments had indicated, introduction of carbon dioxide gas did not guarantee a sufficiently quick addition. We therefore have put into our suspensions small thin-walled glas bulbs completely filled with an oxygen-free solution of sodium bicarbonate. The bulbs could be broken without opening the vessels or interrupting the shaking (see experimental part). In this way it was possible to add the carbonic acid within half a second after the light was turned off and the first potential reading could be taken about ten seconds later.

As a rule, potentials start to return to the dark level immediately after the light is turned off. This process is rather slow however. If there was any persistence of the ability to reduce carbon dioxide, and this ability should persist for periods of minutes, we might expect to see the potential approach the level of a carbon dioxide-containing experiment during the first seconds after the light was turned off and the carbon dioxide added. The curve should then slowly fall to the dark level again. As may be seen from fig. 11, this expectation was not fulfilled under any experimental condition. Experiment 11*a* shows

what happens if carbon dioxide is added in the manner described, to a carbon dioxide-free suspension during illumination (open circles). In the same figure is given the course of the potential if the light is simply turned off at the same moment without addition of carbon dioxide (black circles). As was expected, the curve for carbon dioxide rises more quickly than does the curve for the darkened suspension

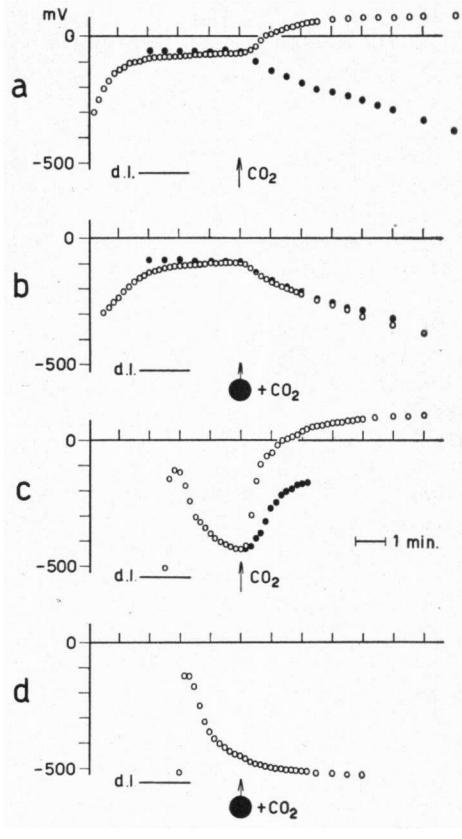


Fig. 11. Addition of carbon dioxide during illumination and after pre-illumination in the absence of carbon dioxide.

a. Stationary phase. Bicarbonate added in the light (○), or light turned off. (●).

b. Stationary phase. Bicarbonate added immediately after light was turned off (○), and no bicarbonate added in the dark (●).

c. Induction phase. Bicarbonate added in the light (○), or no bicarbonate added (●).

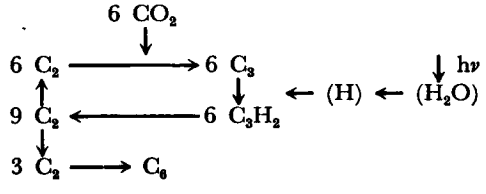
d. Induction phase. Bicarbonate added immediately after light was turned off.

fall. In experiment 11*b*, carbon dioxide was added to one vessel immediately after the light was turned off (open circles). It is obvious that the potential follows exactly the same course in the dark as in a suspension to which no carbon dioxide was added (black circles). Both experiment *a* and *b* were done in the stationary state of the potential curve (for the sake of clarity, all parts of the potential curve, not essential to the present experiments, have been omitted. The dark potentials before the illumination was started are indicated by the lines marked d.l.). One could remark, however, that the reducing tendency of the light would become apparent only during the first minutes of the illumination. The experiments described above have

been repeated, therefore, during this phase of the potential changes (expt. 11*c* and 11*d*). The results were essentially the same. In experiment 11*c*, the potentials in the light, after carbon dioxide addition (open circles) have been compared with the potentials in the light without such addition (black circles). In experiment 11*d*, the addition of carbon dioxide had been preceded by interruption of the illumination and it is seen that the potential returns to the dark level quickly.

The experiments reported in this section have therefore failed to demonstrate a survival of reducing power following illumination in the absence of carbon dioxide and from this point of view, they are in better agreement with the views expressed by GAFFRON and co-workers than with those of CALVIN and his associates. We believe, however, that our experiments do not support the conclusion that no reducing power survives in the dark but merely that in the dark, carbon dioxide does not react with such reducing compounds, if formed. We want to stress the fact, that such a situation is not at all unexpected. It is now generally accepted that it is not the carbon dioxide itself which enters into the reaction with the reducing agents but that the carbon dioxide is first incorporated into certain intermediates in non-photochemical processes. Such intermediates then may react with the reducing compounds to form products which may be looked upon as representing reduction products of carbon dioxide. Such a scheme supposes the presence in the photosynthesising cell of specific compounds able to react with carbon dioxide to form the intermediates which can be reduced by the products of the photochemical process. We may call such a compound the photosynthetic carbon dioxide acceptor. The experiments of CALVIN (26) and of GAFFRON (25) have indicated that this carbon dioxide acceptor may be a two-carbon compound forming with carbon dioxide phosphoglyceric acid which may react with the reducing compounds from the photochemical process to form phosphoglyceraldehyde, which is then transformed in a reversal of the glycolytic pathway into carbohydrate.

In the following discussion we will base ourselves upon a scheme for the photosynthetic cycle which is a modification of those proposed by other workers. It must be stressed, however, that the precise details of such a process are not important for our present purpose and that this scheme therefore merely serves as an example.



In section *e* it was shown that carbon dioxide, even if present from the start, does not consume reducing power in the light until after some moments. Nevertheless, this reducing power is formed

immediately upon illumination (e.g. fig. 5*C*, fig. 8*A*). In our opinion this indicates that at the start of the illumination only a small amount of the carbon dioxide acceptor (C_2) is present, which has to be built up before carbon dioxide reduction can assume a measurable speed. This acceptor is therefore not formed from carbon dioxide in the dark to any appreciable extent.

If carbon dioxide is added to a suspension of algae during illumination, several minutes are required before the potential reaches its maximum steady value (fig. 11*a* and *c*). As it must be supposed that in this case, the reducing systems were already operating, we may draw the following conclusion from this induction period in the effect of carbon dioxide. During illumination in the absence of carbon dioxide the reducing system is present but the concentration of the carbon dioxide acceptor system remains low. As soon as carbon dioxide is added, more acceptor begins to be formed until the final level required for steady state photosynthesis is reached. The addition of carbon dioxide after pre-illumination (fig. 11*b* and *d*) therefore is not followed by a rise in potential as the carbon dioxide is not transformed to a significant extent into a compound which can react with the reducing compounds formed in the light. This picture therefore does not exclude the possibility that reducing compounds survive in the dark. It merely indicates that they cannot be demonstrated because of the lack of suitable carbon dioxide acceptors. The conclusion we may therefore draw from our experiments is that the amount of carbon dioxide acceptor required for steady state photosynthesis cannot be formed unless by the simultaneous and combined action of light and carbon dioxide.

At first sight it might appear that the picture, presented above, does not agree with the results of isotope studies (24). From experiments of such kind, GAFFRON and co-workers have concluded that the carbon dioxide pick-up following preillumination in the absence of carbon dioxide leads mainly to the formation of phosphoglyceric acid, so that under "carbon dioxide-free" illumination the same carbon dioxide acceptor appears to have been formed as during normal photosynthesis. The discrepancy with our experiments probably is only of a quantitative nature. In order that photosynthesis can start at all, at least a small amount of carbon dioxide acceptor has to be present at the start of illumination. The amount of hydrogen acceptor formed from it may be much too small to be demonstrated by our potentiometric methods but the same does not apply to tracer methods. Moreover, as we have shown it is practically impossible to work in the complete absence of carbon dioxide even if respiration is excluded. What we believe to have demonstrated is therefore, that though some carbon dioxide acceptor may be present after a long dark period, the bulk of this substance present during normal photosynthesis is only formed as a result of the action of light and carbon dioxide, that is to say that it is itself a product of photosynthesis. This point of view agrees well with the observation that already after relatively short periods of photosynthesis with

labelled carbon dioxide the phosphoglyceric acid formed becomes uniformly labelled (27). We are of the opinion that the picture presented above may throw considerable light upon the induction phenomena associated with the photosynthetic process.

g. Influence of KCN

The influence of KCN upon photosynthesis is variable, some organisms being strongly inhibited, others hardly (28). For *Chlorella* it has been shown that concentrations of 10^{-4} M already are markedly inhibitory to the gas exchange in the presence of carbon dioxide (29). We have studied the influence upon the potential behaviour and have been very surprised to see that this poison is already active in very small concentrations indeed (fig. 12). Concentrations as low as

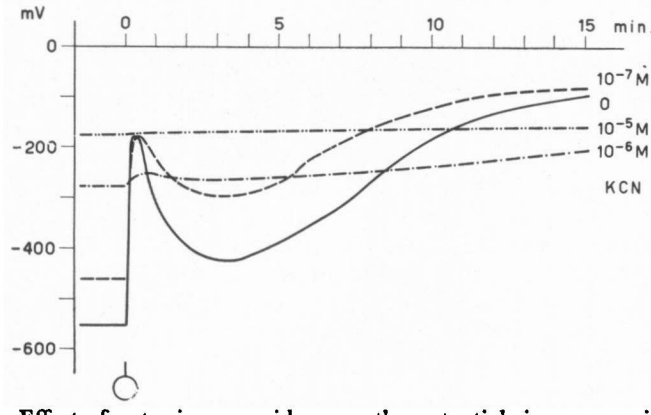


Fig. 12. Effect of potassium cyanide upon the potentials in an experiment without carbon dioxide. Cyanide concentrations in the suspension indicated behind the curves.

10^{-6} M are sufficient to suppress the potential changes during illumination almost completely. The effect of KCN is mainly upon the dark potentials, in agreement with the conclusion, arrived at in section *b* viz. that the DP's and LP's are governed by separate processes. It is, however, difficult to give an explanation for the unusually low concentrations in which the poison is active. One possibility is that hydrocyanic acid is poisonous to the platinum electrodes. A direct chemical reaction with the redox-compounds, transmitting the potentials to the electrode is also not to be excluded completely. At the moment we are unable to offer a satisfactory explanation for the effect of KCN.

We have also studied the influence of KCN in experiments with carbon dioxide. It is difficult under these conditions to keep the hydrocyanic acid in the suspension during the long-continued deaeration with the carbon dioxide containing gas mixture. This has made the estimation of the amounts of HCN present in the sus-

pension a bit uncertain, but there can be no doubt that also in this case the effect upon the potential is very strong and is in the same concentration range as for experiments without carbon dioxide. We have been able to confirm by direct measurement of gas exchange that in this concentration region (10^{-7} — 10^{-5}) there was no significant influence of the HCN upon carbon dioxide assimilation.

h. Influence of light intensity upon the potentials

We have measured the changes in oxidation-reduction potential as a function of time during illumination, for a number of intensities of the irradiating light.

In all the experiments discussed so far, the light intensity was approximately the same, viz. about 4×10^4 erg/cm² sec. We have obtained the lower intensities by placing glass filters under the manometer vessels. A set of 6 vessels was always employed simultaneously, each containing suspensions of the same batch of algae and receiving a different irradiating intensity. In this way it was possible to obtain from one experiment the potential-time curves at six different intensities. Afterwards sections could be made through the sets of curves so obtained, giving the potentials as a function of light intensity at any desired time from the start of illumination.

In fig. 13 some of the curves so obtained are given, the numbers behind the various curves indicating the times in minutes after the start of illumination. We have chosen for illustration two curves for each experiment, one for a moment during the induction period, the other in the stationary phase of the potential-time curve. The

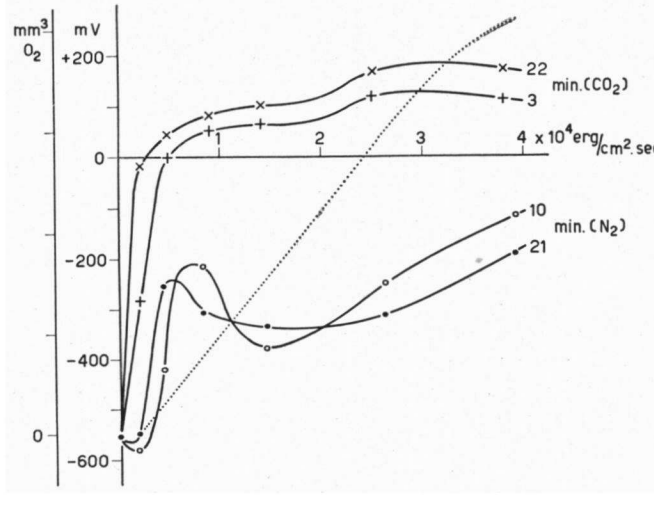


Fig. 13. Potentials as a function of light intensity. Upper two curves with addition of carbon dioxide. Lower two curves without carbon dioxide. Numbers behind curves indicate the times after start of illumination. Dotted curve: photosynthesis. This applies to the experiments with carbon dioxide.

lower two curves represent the results of an experiment with pure nitrogen as the gas phase, the others apply to an experiment with nitrogen plus 5 % carbon dioxide. For the latter experiment, the result of the simultaneous determination of gas exchange is also given in arbitrary units as the dotted line.

This assimilation curve was obtained with the one-vessel method assuming an assimilatory quotient of unity.

We have felt the necessity for carefully checking the results of this type of experiments. As has been explained in the Experimental Part, the platinum electrodes may become unreliable, and a vessel containing such electrodes would lead to wrong conclusions. We have therefore proceeded as follows. After the conclusion of a complete measurement the vessels were put in the dark again. As soon as the potentials had nearly reached the original dark potential the light filters under the vessels were replaced (in the dark) so that each vessel received a different filter than before. The complete experiment was then repeated. Though we have never been able to reproduce exactly the curves found in the first run (this was not expected either, on the basis of our previous experience with repeated illumination) we were satisfied to note that the maxima and minima in the curves fell at approximately the same light intensities and were not, therefore, due to pecularities of a particular set of platinum electrodes. We therefore feel confident that the unusual form of the curves in fig. 13 is real.

An attempt to explain the shape of the potential-intensity curves would appear impossible at the moment. We will only note that as far as we can judge from these experiments, there is no relation with light-saturation of photosynthesis. If we may speak of a light-saturation of the potentials in experiments with carbon dioxide present, this saturation occurs at an intensity at least one tenth of that required for saturation of photosynthesis. Moreover, a comparison with the curves for pure nitrogen shows that the light-intensity region of $0.5 - 1 \times 10^4$ erg is of particular importance in both conditions and that the occurrence of extrema in this region is not, therefore, connected with the presence of carbon dioxide.

In making a comparison between the light intensity functions of gas exchange and potentials, it should be kept in mind that photosynthesis represents a reaction velocity whereas the potentials, especially those measured during stationary conditions, indicate concentration ratios of oxidation-reduction systems and thereby of reducing or oxidising capacities [1].

Finally, the outcome of these experiments bears some relation to the work of other authors. STREHLER *et al.* (30 and 31) have reported experiments on the luminescence of chloroplasts and intact *Chlorella* cells, following irradiation. They have also measured potentials in such systems, assuming them to give a measure of the rate of the Hill reaction. In the first place we believe that our experiments have shown that the last-mentioned conclusion is very dangerous. Further, these authors have measured the potentials in such systems as a function of light intensity and have shown that luminescence and potentials follow almost the same course. The results discussed in this section clearly demonstrate that the light

[1] The author is indebted to Prof. Dr E. C. WASSINK for this remark.

"saturation" observed by *Strehler*, is no real saturation and is not related to light saturation of photosynthesis. It is nevertheless very interesting to compare the figure 3 on page 811 of their publication (30), which gives the luminescence of *Chlorella* as a function of the exciting light intensity, with our figure 13. They are of exactly the same nature and it is tempting to suggest that the luminescence phenomena observed by these authors are related to the oxidation-reduction reactions we have discussed in the present paper.

IV. CONCLUDING REMARKS

Many of the conclusions, arrived at in this paper have a preliminary nature. We believe to have shown beyond doubt, however, that the changes in oxidation-reduction potentials measured in suspensions of *Chlorella* are connected in some way or other with processes, particular to the mechanisms of photosynthetic carbon dioxide reduction. They are not, however, connected directly with the effects of over-all photosynthesis, an observation already reported earlier by WASSINK for experiments with purple bacteria (13, 32). In addition, our experiments strongly support the idea that, at least during certain phases of the induction period the potentials are a reflection of the formation of reducing compounds by the primary photochemical process which are suitable as hydrogen donors for carbon dioxide reduction.

Finally we want to direct attention to the fact that the induction phenomena reported here bear a great deal of resemblance to induction phenomena of other aspects of photosynthesis. This does not so much apply to the induction of total gas exchange but more to phenomena such as chlorophyll fluorescence (WASSINK, 33, 34) and phosphate metabolism (KANDLER 35). We hope to be able to study the relations between these various phenomena in some detail later.

SUMMARY

A study has been made of the oxidation-reduction potentials in suspensions of algae. The potential was found to be governed by three systems, viz. *1*, the respiratory system, *2*, a metabolic process occurring in the dark in the absence of oxygen, and *3*, a system connected with the photosynthetic processes, exclusively determining the potential changes in the light below a certain oxygen tension. The activities of these systems may be distinguished on the basis of their different sensitivities to oxygen, pH and enzyme poisons.

At the start of illumination the potential shows a pronounced induction phenomenon and a stationary potential is reached after about 15 min. illumination. Oxygen and hydrogen peroxide are not involved in the establishment of this stationary potential.

The essential feature of the potential behaviour in the light and in the absence of carbon dioxide irrespective of pH is that a relatively reduced state is reached some minutes after the start of illumination.

Afterwards the potential rises again slowly to its stationary value. During the initial phase of low potentials, methylene blue and possibly also oxygen may be reduced by the suspensions. Similar experiments with disintegrated *Chlorella* cells demonstrated that both oxidising and reducing reactions occur.

The presence of carbon dioxide during illumination results in a more positive potential. This can be explained by assuming that the reducing compounds formed by the action of light are removed by reaction with carbon dioxide. The action is exerted by extremely small concentrations of carbon dioxide. Its maximum effect occurs about 1–2 min. after the start of illumination.

No persistence of the ability to reduce carbon dioxide in the dark after preillumination in the absence of carbon dioxide was observed.

Our observations lead to the hypothesis that in the dark a very low concentration of the photosynthetic carbon dioxide acceptor is present. The establishment of steady state photosynthesis involves the formation of a sufficient amount of this carbon dioxide acceptor under the simultaneous and combined action of light and carbon dioxide.

The potentials depend upon the light intensity in an unusual way. There is no relation to the light saturation of photosynthesis.

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