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SIMULTANEOUS PHOTOPRODUCTION OF HYDROGEN AND OXYGEN BY *CHLORELLA*

by

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It is known (1, 2, 3) that several species of green algae metabolize molecular hydrogen under conditions of low oxygen tension. Usually, a more or less prolonged period of incubation under very low oxygen pressures is required to activate this hydrogen metabolism. As a rule, after such a treatment hydrogen is evolved slowly in the dark and much more rapidly upon illumination. The view has long been held that *Chlorella* is devoid of this mechanism. In the course of manometric experiments on the photosynthesis of these algae at low concentrations of oxygen, we obtained indications for a production of hydrogen, especially at low light intensities (4) and confirmed this finding in various ways (5). In the latter study, it was suggested that, simultaneous with hydrogen production, some oxygen was also evolved in the light. This is in agreement with various reports (6, 7) of initial oxygen outbursts in anaerobic algae. Recently, DAMASCHKE (8) observed hydrogen production in a strain of *Chlorella*. It is evident that the simultaneous production of both gases should be of sufficient theoretical interest to warrant further study.

METHODS

In order to circumvent a problem complicating manometric experiments, viz. the rather slow distribution of gases between liquid and gas phase, we have looked for a method capable of the measurement of gases, dissolved in the suspension liquid. Moreover, the measurements should be specific for a particular gas. Rapid and specific electrochemical methods for oxygen were available (4, 13). We have used a slightly different method which has special advantages at the low oxygen concentrations encountered here and which will be described in more detail below. For hydrogen, we have not encountered a similar method, apart perhaps from mass spectrometry, which was not available to us and which also suffers from the problem of exchange between liquid and gas. The method finally adopted is a special form of polarography. After the experimental work was finished, we learned of another method, also electrochemical, but probably differing in principle from the one used here (8).

a. *Vessel.* For reasons outlined above, a gas space in contact with the algal suspension had to be avoided. This called for experiments of so short a duration that the gases produced were still completely soluble in the liquid phase.

The suspensions of algae were illuminated in a vessel of a construction, similar to the one described previously (4). Except for the stirrer and its bearing, it was made entirely from Lucite.

The stirrer axis was now placed horizontally and the various ground glass joints for the electrodes were arranged radially. There were two openings, closed by cellulose diaphragms, held between perforated Lucite discs. One of them was connected, via a short section of glass tube, filled with K_2SO_4-agar, to the reference electrode for the polarograph circuit. The other was connected in the same way to an auxilliary electrode for the generation of hydrogen or oxygen in the vessel for calibration purposes. Total volume of the vessel was 50 ml. Illumination was by a 150 Watt incandescent lamp with internal reflector, giving an intensity of white light of about 100 Kerg/cm² sec. on the front window of the vessel. The light intensity could be attenuated by means of neutral filters.

b. *Oxygen measurements.* The voltammetric method, employed previously (4) led to unexpected difficulties at the high sensitivities and low oxygen concentrations, employed here. The residual current i.e. the current flowing at zero oxygen concentration was found to be rather high at first, declining slowly over a considerable period of time. This is not so disturbing at lower sensitivities, but it was unacceptable here. Moreover, as long as this residual current had not yet reached its final (very low) value, it was found to be influenced by illumination. In the light, the current increased almost linearly with time, to return to its original value in the same way upon darkening. We found, however, that both effects were very much reduced if the electrode was not made of the usual platinum, but of gold. We have used 0.1 mm diam. Pt-wires on which a thin layer of gold had been deposited by electrolysis from a gold cyanide solution. When fresh, the residual current of such electrodes reaches a steady value in a relatively short time. During the period in which the algal suspension is flushed with nitrogen, it usually falls to a negligible value. As soon as this is the case, the sensitivity to light is also reduced to such a low level that the remaining zero shift during illumination can easily be corrected for. In some of our figures, this effect can be observed, cf. fig. 11. As soon as residual current and light sensitivity became excessive, we have removed the old gold coating and replaced it by a fresh one. In connection with the galvanometer, a KIPP en Zonen type Sc, period 1/20 sec., the sensitivity for oxygen was about 0.8 mm^3 O_2 per liter liquid for one mm deflection (i.e. a concentration of 4.10^{-8} M). BRACKETT *et al.* (7) have published curves for initial oxygen evolution, measured with a method, differing somewhat from the present one. They show a satisfactory agreement with our present measurements. We may remark here that we cannot confirm the statement of these authors that methods of convective polarography, such as those used in this study, are unreliable (see also [13]). Also, the calibration of the electrodes proved very reproducible throughout the useful life of the electrodes. As the irregularities in the diffusion current usually are small compared with the magnitude of the deflections caused by photochemical oxygen production, we have felt no need for derivative recording of oxygen concentrations (7), especially as this procedure inevitably leads to a decrease in the time resolution of the measurement. It must be admitted, however, that our method is less suited to measurements at higher oxygen concentrations, when the current becomes very irregular.

Actually, polarographic measurements at one value of the applied potential, such as the one discussed above, are not at all specific. Undoubtedly, many substances will be reduced at the cathode under these conditions. In view of previous results (9), it was not beyond doubt that all oxidising substances, formed during illumination of *Chlorella* suspensions were identical with oxygen. Complete polarograms were therefore made in the course of the illumination, as well as a number of illuminations at various fixed potentials. Such experiments have failed to reveal other reducible compounds, apart from oxygen. We therefore feel reassured that not more than insignificant amounts of oxidising substances, apart from oxygen, can have contributed to the curves, reproduced in the present paper.

c. *Hydrogen measurements.* During a polarographic study of possible redox systems, excreted by the cells during illumination (not published), we observed the appearance of a maximum in the polarogram at a particular potential during the first minutes of the illumination. Subsequent experiments showed that this maximum was due to molecular hydrogen. We have succeeded in developing this observation into a suitable analytical method for the estimation of dissolved hydrogen. As fuller details about the effect and the procedure will be published elsewhere, we will limit ourselves to a brief description here. The effect depends upon the catalytic oxidation of hydrogen at a layer of platinum oxides, formed at the platinum electrode during previous anodic oxidation. So far, only platinum proved suitable, other platinum group metals as well as gold, silver and mercury being inactive. The platinum oxides being reduced by hydrogen, it is necessary to regenerate them continuously in an anodic cycle. By means of a slidewire potentiometer, driven by a synchronous motor and coupled to a photographic recording drum, a potential is applied to the electrode, increasing linearly with time from + 1.0 to —0.6 V, after which the cycle is repeated immediately. On the photo-

graphic paper appears a continuous series of complete polarograms. Actual recording is limited to the part where the hydrogen maximum is situated. This is done by interrupting the illumination of the galvanometer during the parts of the polarograms that are of no interest, by means of a switch coupled with the driving mechanism. In the present study, we have chosen a period of 11 seconds for one complete cycle. This proved to give sufficient time resolution and intermediate points could be obtained with sufficient accuracy by interpolation. It is possible to run through the polarogram at a higher speed, but this decreases the accuracy. There is, however, a limit to the speed, as we have found it impossible to find the hydrogen maxima in polarograms made with a 50-cycle sawtooth voltage, and observation on the oscilloscope screen. The hydrogen maximum is found at about —0.04 V at pH 7 (versus sat. cal. electr.). The height of the maxima proved proportional to the hydrogen concentration up to saturation, small deviations probably arising from losses of hydrogen by diffusion through the reference electrode connections. An example of two complete polarograms, one without, the other with added hydrogen, is given in fig. 1. The additional maximum in the curves is due to other com-

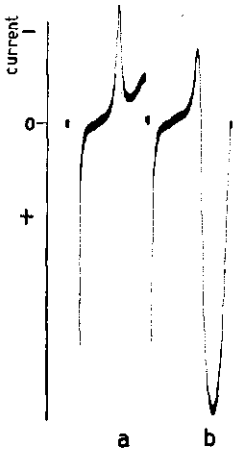

FIG. 1. Hydrogen polarograms.
a. Polarogram without added hydrogen.
b. Same with addition of about 100 mm³ hydrogen per liter.

ponents of the liquid. One important complication of the method is that the heights of the hydrogen maxima are affected by the presence of oxygen. Fortunately, this oxygen effect proved linear to oxygen concentrations, and independent of hydrogen concentrations. As oxygen concentrations in the liquid were always known from the oxygen recordings, corrections could be made easily.

The sensitivity of the hydrogen measurement was better than about 6 mm³ H_2 per liter liquid (3.10^{-7}M). The accuracy was somewhat less.

d. *Calibrations.* In order to convert the recordings to actual concentrations of gases, calibrations of the electrodes are essential. Both oxygen and hydrogen calibrations were made daily by developing known quantities of the gases in the vessel by electrolysis. To this end, an additional platinum electrode was present which could be made either cathode or anode with respect to the external auxilliary electrode mentioned under a. Amounts of oxygen and hydrogen were calculated from the measured amounts of milliampere-seconds passed through. This way of adding gases is very convenient and more accurate than the addition of aqueous solutions. The reliability of it was checked with the latter method. It proved possible to do the hydrogen calibrations in the algal suspensions at the conclusion of an experiment. Oxygen, added to the suspension in the same way, was consumed with such a speed that accurate calibration was impossible. The oxygen calibrations were, therefore, done in the pure buffer solution, before and after the actual experiments. There was very little variation in the calbration constants from one day to the other, as long as the electrodes behaved reliably. Changes in the constants usually indicated the necessity of replacement of the electrodes.

Theoretically, it should be possible to do both oxygen and hydrogen measurements simultaneously in the same suspension. Because of the complications in electrical circuitry involved, we have preferred to measure both gases in duplicate experiments with algae from the same

batch and treated identically. Control experiments confirmed that the reproducibility is sufficient to this end.

e. *Cultivation of algae, buffer solutions etc.* These methods are the same as those employed previously (4, 9). For an experiment, the washed cells were suspended in a buffer, 0.1 M in K_2SO_4 and 0.033 M in phosphate pH = 7.0. The cell density was 3 mm^3 wet cells per ml suspension medium.

RESULTS

a. Time course of H_2- and O_2- evolution

Experiments with relatively dense algal suspensions in a differential volumeter (KOK [10]), fig. 2. revealed initial "outbursts" of a gas. In some experi-

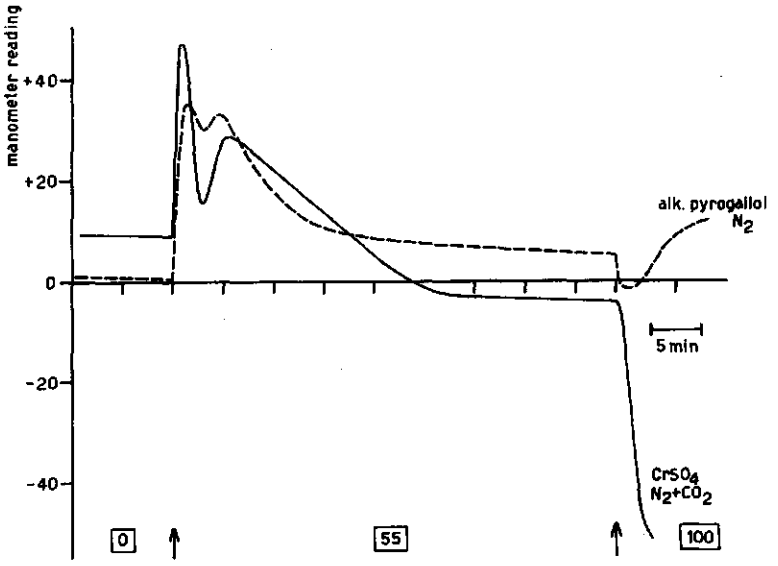

FIG. 2. Gas exchange of anaerobic algae as observed volumetrically. One vessel contained pure nitrogen and alkaline pyrogallol as an absorbent. The other had a gas atmosphere of nitrogen with 5% carbon dioxide, and chromous sulphate solution as an absorbent. The arrows indicate the moments when illumination was started or increased to a higher level. Framed figures indicate relative light intensities.

ments, negative manometer readings during the subsequent period indicated reabsorption of at least part of this gas. In the light of present knowledge, we must interpret these curves as initial production of hydrogen plus oxygen, the latter gas not being absorbed with sufficient speed by the reducing solution in the side vessel. It is evident that these transient gas exchanges are too rapid to be conveniently studied by manometric methods.

As the recordings of hydrogen evolution do not lend themselves very well to reproduction, we have redrawn most of the curves. The corrections for oxygen were applied at the same time. Figures 7, 8 and 11 are reproductions of original oxygen recordings.

In fig. 3, some examples of induction curves for O_2 and H_2 are collected. For convenience of discussion, we will divide the time course of oxygen evolution into a number of separate phases, as indicated schematically in fig. 4. The du-

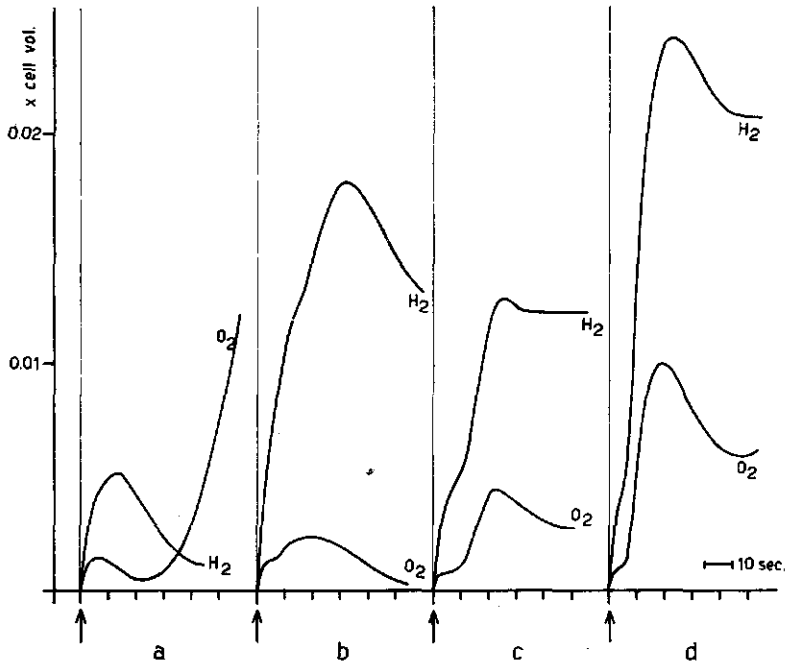

Fig. 3. Time course of hydrogen and oxygen evolution as measured electrochemically. Arrows indicate start of illumination.

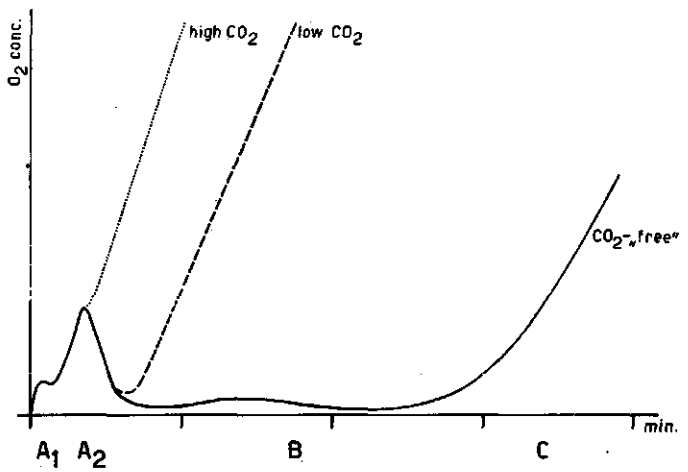

FIG. 4. Schematic representation of the time course of oxygen evolution after anaerobic incubation. Divisions on horizontal axis indicate minutes.

ration of phase B increases with increasing duration of the anaerobic dark incubation, but is also dependent upon internal factors, as it varies from one batch of algae to the other, with the same treatment. The curve, indicated as "CO_2-free" refers to suspensions that had been depleted as much as possible from this gas by vigorous bubbling with pure nitrogen. The effect of carbonic acid upon the course of oxygen evolution is chiefly in decreasing the duration of phase B. There is no observable effect upon either the position or the magnitude of the oxygen peaks A_1 and A_2. Apparently, oxygen production during these phases is not linked up with carbon dioxide supply. Addition of low concentrations of oxygen has the same effect as carbon dioxide upon phase B. This may be trivial, due to increased intracellular CO_2 as a consequence of respiration. The duration of phase B is also influenced by the intensity of the illumination: at low light intensities, it may last for very long periods.

During the whole of phases A and B there is hydrogen evolution which stops, however, as soon as phase C is well under way. Some of the hydrogen is usually reabsorbed during this phase, as in fig. 3a. Also, during the transition from phase A_2 to B, some hydrogen is often taken up, as in fig. 3b, 3d and 6c. As soon as the oxygen concentration has again reached a low value, hydrogen evolution starts anew.

Carbon dioxide has no direct effect upon the production of hydrogen. The effect is only secondary, due to oxygen evolution. It is, therefore, possible to continue hydrogen production in the presence of carbon dioxide for long periods, by keeping the light intensity at such a low value that phase C is not reached. In this case the manometric technique is preferable, as it allows the simultaneous observation at a number of different light intensities. Fig. 5 gives an example. At

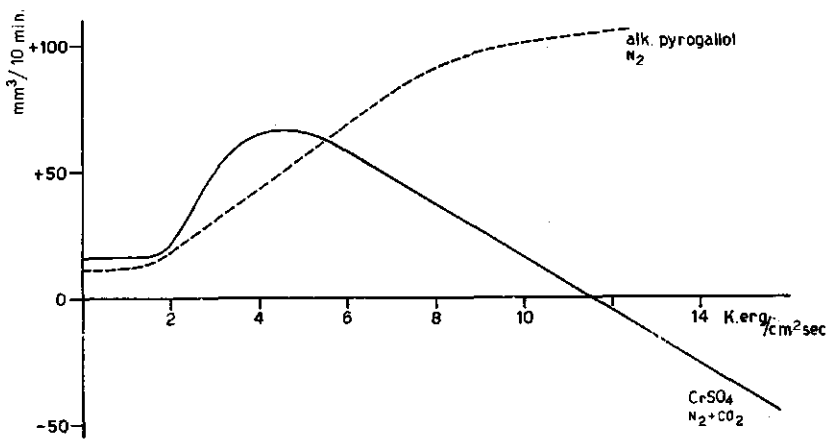

FIG. 5. Volumetric experiment, demonstrating hydrogen production in the presence of carbon dioxide (gas atmosphere nitrogen plus 5% carbon dioxide) at low light intensities, and reversal to photosynthesis at higher light intensities.

sufficiently high light intensities, normal photosynthesis starts in the presence of CO_2.

The experiment of fig. 6 shows the effects of increasing light intensities on hydrogen and oxygen evolution separately. It is always found that the oxygen peak A_1 is already saturated at relatively low light intensities, whereas both peak

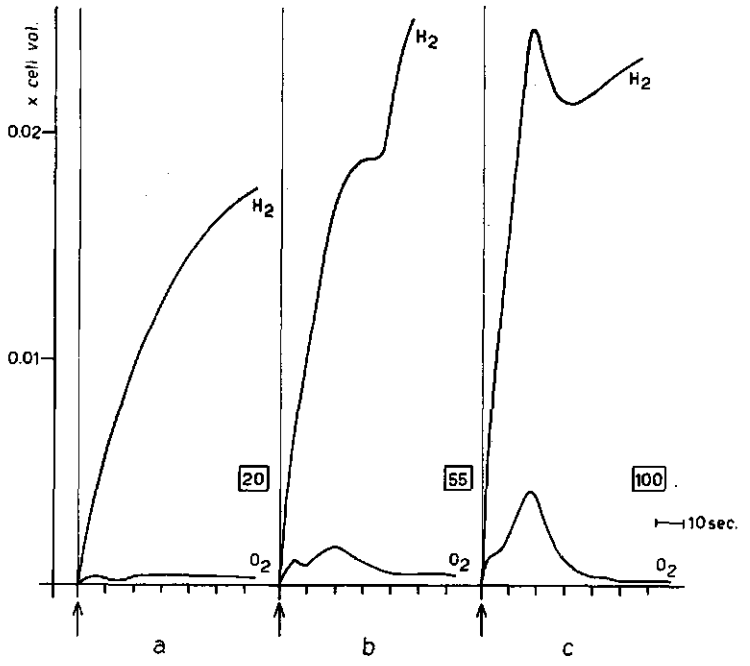

FIG. 6. Time course of hydrogen and oxygen evolution at increasing light intensities. Framed figures indicate relative light intensities. The three experiments were made with aliquots from the same batch of algae.

A_2 and the slope of the hydrogen curve do not yet saturate at the highest intensities used. It may be difficult to relate the hydrogen evolution to light intensity, as its speed is also influenced by the oxygen concentration (see section b). The apparent saturation in the total amount of hydrogen formed (fig. 6) may, therefore, not be really due to saturation of the primary steps of the hydrogen producing system.

b. Interrelation of the hydrogen and oxygen metabolism in the light

The observations mentioned above already point to some connection between the two processes. Obviously, the presence of oxygen retards hydrogen evolution, and, above a certain concentration, stops it altogether. On the other hand, hydrogen is not without influence upon the course of oxygen evolution. Fig. 7 shows an experiment, in which hydrogen was introduced into the medium to a concentration corresponding to an equilibrium pressure of 0.08 atm. Incubation was short enough to obtain a short duration of phase B. Evidently, the presence of hydrogen greatly suppresses initial oxygen production (b). Even after the hydrogen had been washed out with pure nitrogen, its influence is still apparent from the long duration of phase B (c).

The latter effect may be explained by assuming that pre-incubation with high concentrations of hydrogen aids in the formation of readily-available hydrogen donors for respiratory removal of oxygen during subsequent illumination (see Discussion). We have found that small amounts of hydrogen, added to the algal suspension shortly after an illumination period, when the

photochemically formed oxygen was already completely consumed, resulted in an uptake of considerable quantities of hydrogen by the cells.

The suppressing influence of hydrogen upon the various phases of oxygen evolution apparently is in the order $A_1 < A_2 < C$. Actually, A_1 is hardly inhibited at all. The presence of hydrogen has the same effect as lowering the light intensity. We may also express it as follows: hydrogen protects the hydrogen evolving mechanism by suppressing oxygen formation. And we may add here, that the same holds for the effect of oxygen upon oxygen evolution.

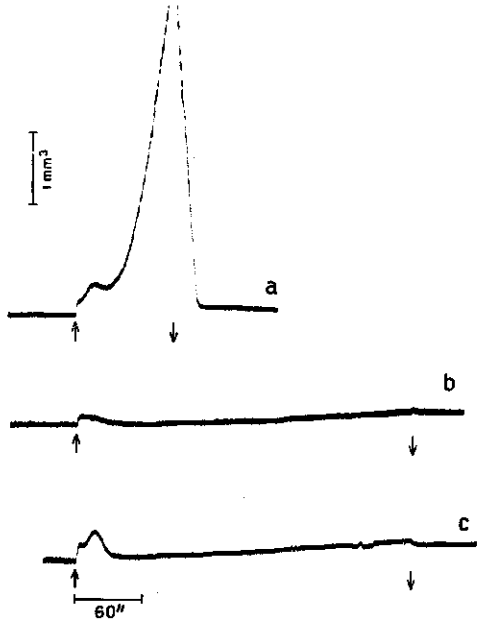

FIG. 7. Effect of hydrogen upon photochemical production of oxygen.
a. Algae flushed with pure nitrogen.
b. Aliquot, after flushing with nitrogen plus 8% hydrogen. Oxygen production suppressed, except during phase A_1.
c. As *b.*, after displacement of hydrogen atmosphere by pure nitrogen. Initial hydrogen production recovers, but duration of phase B is greatly increased.
Arrows indicate beginning and end of illumination periods.

c. Gas production by cell free extracts and damaged cells

The nature of the analytical methods used in this study almost precludes the use of poisons, often found extremely useful in the study of the metabolism of intact cells. Compounds like KCN and hydroxylamine interfere with the hydrogen measurement by poisoning the electrode. Dinitrophenol, which is of particular interest in view of Gaffrons experiments (2), is reduced at the platinum cathode and thereby interferes with oxygen measurement. It is, however, without influence upon hydrogen measurement, and we have been able to confirm the observation that it stimulates hydrogen evolution somewhat. Moreover, as a consequence of the oxygen effect upon the hydrogen measurement, in this case it is possible to conclude from the hydrogen measurements, that oxygen evolution during phases A_1 and A_2 is suppressed (conc. 10^{-3}M). Hydroxylamine,

while interfering with the hydrogen measurement, is without influence upon the oxygen polarograph. Its effects are complicated. At low concentrations (about 2.10^{-4}M) there is stimulation of initial oxygen production. At higher concentrations (10^{-3}M), oxygen production is quickly inhibited.

On the other hand, the methods are well suited to a study of cell-free suspensions and other partial systems that do not carry out normal photosynthetic carbon dioxide reduction. Fig. 8 shows curves for the oxygen production by

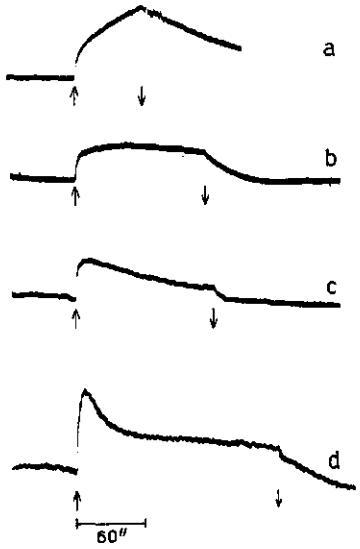

FIG. 8. Oxygen evolution by damaged cells. Experiments a through *c* were made with frozen cells, experiment *d* (sensitivity of oxygen measurement increased 3-fold) was made with a cell-free extract.

such preparations, obtained either by grinding *Chlorella* cells with fine carborundum powder or freezing them to —20°C for 30 min. followed by thawing. Experiments of this type are of interest in view of the difficulties of obtaining cell free preparations from algae that are capable of the Hill-reaction. In all these preparations, phase C is never reached, not even in the presence of carbon dioxide. A comparison with the curves for intact cells suggests that it is actually phase A_1 that remains in these preparations. Obviously, there is a sort of Hill-reaction without added hydrogen acceptors. The small amount of oxygen is reabsorbed in the dark. We have not been able to give a completely convincing demonstration of hydrogen production in cell-free *Chlorella* extracts. Frozen preparations, on the other hand, very clearly evolve this gas upon illumination. On the basis of other evidence (11), we are inclined to believe that the behaviour of cell-free extracts is in many respects very similar to that of frozen cells. We therefore are inclined to believe that the cell-free preparations also are capable of hydrogen production, but that the hydrogenase system is somewhat more sensitive than the other systems present. A great advantage of the frozen cells is, that the whole procedure, including introduction into the illumination vessel can be easily carried out in a rigorously oxygen free atmosphere, which is considerably more difficult with cell free extracts. The usual procedure for the pre-

paration of frozen cells was as follows. The required volume of *Chlorella* cells was suspended in a little buffer solution, pre-incubated in the dark under nitrogen for one hour and then frozen in the same vessel under nitrogen for 30 min. at –20°. After thawing, still under nitrogen, the suspension could be transferred without exposure to oxygen to the illumination vessel which had been previously filled with buffer solution and deaerated.

d. Ratio of hydrogen to oxygen

The experiments, reported under section b. point to an interrelation of hydrogen and oxygen metabolism. This raises the question of a possible common origin of these gases. The idea that photochemically formed hydrogen originates from water was already suggested by Gaffron in his 1942 papers ([2], p. 239) but was never put beyond doubt. Indeed, the hydrogen fermentation of algae in the dark demonstrates that non-photochemical production from internal hydrogen donors is at least one of the mechanisms for hydrogen formation. In order to answer the question if the two photochemically formed gases arise from the same source or not, a study of the ratio of oxygen and hydrogen formed upon illumination appears indicated. Some values for the ratio H_2/O_2 during the first moments of the illumination, are collected in fig. 9. Obviously, this ratio

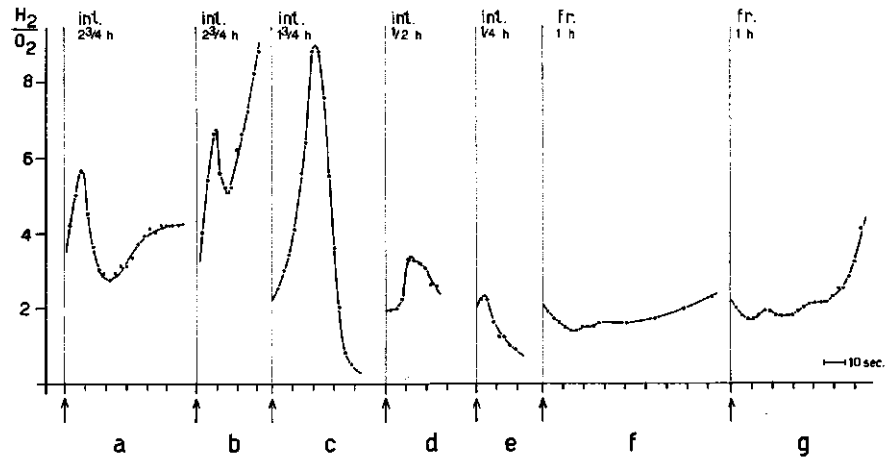

FIG. 9. Ratio of hydrogen over oxygen during the induction phase. Figures at the top indicate the duration of previous anaerobic incubation. Int.: intact cells. Fr.: frozen cells.

greatly changes in the course of time. The period of previous anaerobic dark incubation proved very important. As a rule, the longer the incubation period, the higher were the intial values for H_2/O_2. The general form of the curves is not difficult to interpret. Obviously, the first rise is related to the "dip" in the oxygen curve between A_1 and A_2. The subsequent fall coincides with the large oxygen formation during A_2. As oxygen is taken up again during phase B, there is a tendency for the hydrogen-oxygen ratio to rise again during this phase, provided it is of sufficient duration. Evidently, the curves ultimately should approach zero, as hydrogen evolution stops as soon as steady oxygen evolution in phase C starts. Now, the ratios as measured directly cannot be expected to represent the real values as part of the gases escapes measurement. Part of the oxygen evolved

is reabsorbed as a consequence of respiratory processes. On the other hand, there is also reabsorption of hydrogen (cf. fig. 6). We have observed that the respiratory capacity, expressed as the speed with which a given amount of added oxygen is consumed, is dependent upon the time of previous dark treatment. The longer this period, the more rapid the oxygen consumption. This observation gives an explanation for the general rise of initial hydrogen-oxygen ratios with increasing duration of anaerobic dark incubation. Further, at very low oxygen tensions, this respiration may be expected to be oxygen-limited. For these reasons, we expect the "true" hydrogen-oxygen ratios to be most closely approached 1. at the very start of an illumination period, 2. with algae that have had only a short incubation period. An examination of fig. 9 reveals that under such conditions, the ratio H_2/O_2 always is not far from 2. Unfortunately, the first points in the oxygen and hydrogen recordings give rise to rather large inaccuracies in the calculated concentrations of gases. In general, the accuracy of the method, with its inherent corrections of hydrogen measurements for presence of oxygen, is not too great, and H_2/O_2 values may be estimated to be accurate to about 30%. Even so, the low initial values of this ratio are of considerable interest. It appears rather fruitless to attempt to pin these values down more accurately in view of the many complicating reactions involved. Therefore, experiments with damaged cells assume special importance, as with such materials, side reactions may be expected to be of much smaller magnitude. As a matter of fact, the experiments with frozen cells gave hydrogen-oxygen ratios, that were close to 2 for a surprisingly long time. Such preparations evolve pure "Knallgas" for more than one minute during illumination. It is obvious that those parts of oxygen and hydrogen that escape measurement as a consequence of the hydrogen serving as respiratory substrate ("Knallgas" reaction) do not affect the ratio H_2/O_2.

e. Initial oxygen production and carbon dioxide

As mentioned under section a, the presence of carbon dioxide manifests itself only in the later stages of the oxygen induction curve. This raises the question of the carbon dioxide metabolism during the initial phases. We have not yet studied this point exhaustively, but a few observations may be worth mentioning. Simultaneous with oxygen measurement, we have followed the pH changes in an unbuffered medium. The method was described previously (4). Fig. 10 gives some results. As a consequence of the presence of CO_2, phase B is absent, but A_1 and A_2 can still be recognised clearly. In the initial phases, pH changes as a rule are small and a high sensitivity is required. It appears that significant acid consumption is not observed until the oxygen evolution is definitely in phase C. It should be pointed out that, so far, it has not been demonstrated that pH changes under these conditions are exclusively due to carbon dioxide exchange. But at any rate, results such as those of fig. 10 harmonize with the view that carbon dioxide is not consumed during the early stages of the illumination.

In a surprisingly large number of cases, we found some acidification of the medium immediately after illumination was started. This may be related to the so-called carbon dioxide gush, observed also under aerobic conditions. Examples of both acidification and delayed carbon dioxide uptake can be found in a previous publication (4). Figures 6 and the first part of fig. 9 of the latter article are especially good examples.

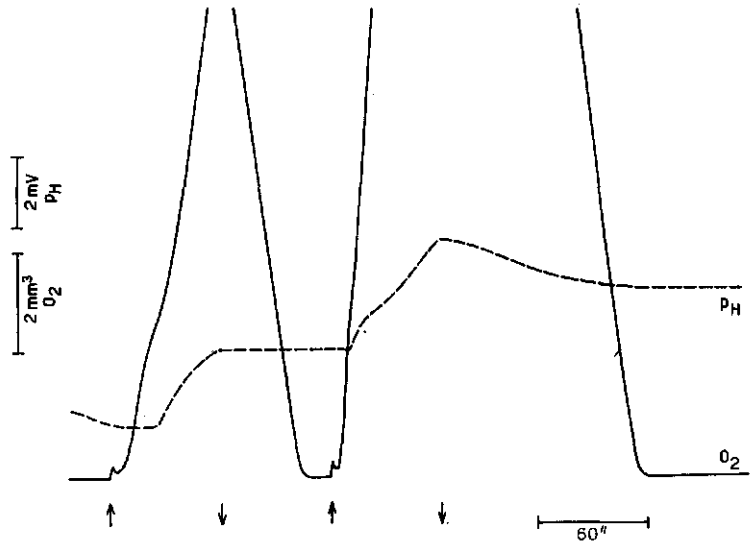

FIG. 10. Simultaneous recording of oxygen production and pH changes in an unbuffered suspension. Five minutes before the start of the illumination, 10 ml per liter of a 0.1 molar solution of sodium bicarbonate, saturated with carbon dioxide, was added to the suspension.

It should be pointed out here that most of our experiments are made with algal suspensions from which carbon dioxide had been removed as much as possible, by flushing with pure nitrogen. So, even oxygen production in phase C can hardly be regarded as "normal photosynthesis". As it behaves very similar to oxygen production at low concentrations of added carbon dioxide, it may perhaps be looked upon as photosynthesis under conditions of extreme carbon dioxide limitation. It is known, and we have confirmed (see also the first part of the pH curve of fig. 10) that anaerobic algae evolve some carbon dioxide in the dark by fermentative processes, and this production may well go on during illumination, forming a constant very small supply of this gas. The often observed individual difference of various batches of *Chlorella* with regard to the duration of phase B in pure nitrogen, may therefore find its explanation in differences in the intensity of this carbon dioxide production, which in turn may be governed by the nature and concentration of reserve substances in the cells.

Whatever may be the detailed explanation for such phenomena, it is obvious that the continuous, though small, production of oxygen in an anaerobic atmosphere devoid of carbon dioxide, must be accompanied by reductive processes. So far, the nature of the compounds, serving ultimately as hydrogen acceptors, is not known with certainty.

f. Oxygen induction and manganese

KESSLER (14) made the interesting observation that some algae (*Ankistrodesmus*), grown in a medium deficient in manganese, show a greatly reduced capacity for photosynthesis, whereas hydrogenase activity is unimpaired, or even stimulated. It is sometimes suggested that manganese has a specific role in the oxygen evolving mechanism. Accordingly, we have tested our *Chlorella*,

grown in a medium without manganese, for initial oxygen production. Oxygen maxima A_1 and A_2 were found unaffected or slightly increased. It is not very likely that the effect of manganese is directly upon the oxygen mechanism.

DISCUSSION

Our experiments leave no doubt that the two strains of *Chlorella* studied by us, contain a hydrogenase. This enzyme apparently is inactivated by oxygen, a relatively short incubation in nitrogen sufficing to bring it to full activity. The time course of hydrogen evolution during illumination shows that the enzyme is already inactivated to a considerable degree by oxygen pressures as low as 10^{-4} atm. After removal of this amount of O_2, the activity is regained rapidly. At this point it should be remarked that many authors have been unable to demonstrate hydrogenase activity in *Chlorella* and other organisms. This may be due to a difference in the sensitivity of the respective hydrogenases towards oxygen. As there seems to be always a little oxygen produced upon illumination, this may, in very sensitive organisms, be sufficient to inactivate the hydrogenase almost instantly, so that no hydrogenase activity in the light can be observed. On the other hand, some organisms seem to be relatively insensitive to oxygen. In those organisms where very long "adaptation periods" are required, this long duration may be essential to the formation of a sufficient concentration of respiratory substrates to keep the oxygen concentration in the light below the critical level for hydrogenase inactivation. If this is the correct explanation, the "adaptation" as defined by GAFFRON is not due to development of the system producing hydrogen in the light, but rather to activation of the secondary processes that affect the oxygen concentration during illumination. Results by DAMASCHKE (8) with a strain of *Chlorella pyrenoidosa* indicate persistence of hydrogen production under relatively high oxygen pressures, indicating relative insensitivity of the hydrogenase to oxygen. In view of the wide-spread occurrence of hydrogenases in aerobic bacteria, it should be interesting to look for algae, capable of hydrogen evolution in the presence of moderate or high oxygen concentrations.

The evolution of hydrogen in the presence of carbon dioxide is rather surprising. It is hard to see how this observation agrees with GAFFRON'S demonstration of so-called photoreduction: reduction of carbon dioxide in the light with hydrogen gas as reducing agent and without evolution of oxygen. It has been pointed out by HORWITZ and ALLEN (12) that adapted *Scenedesmus* in the presence of hydrogen and carbon dioxide can carry out photosynthesis at a rate, sufficient to account for all its photochemical activity, the oxygen produced being immediately used up by the "Knallgas" reaction. They used oxygen isotopes and a mass spectrometer to follow the fate of various gasses. Unfortunately, they have not measured their hydrogen exchange, and their conclusion that under certain conditions a true photoreduction in the sense of Gaffron's definition exists, appears to be only weakly founded. In our opinion, the demonstration that, whatever the composition of the gas phase, oxygen is always formed during illumination, along with hydrogen, points to normal operation under anaerobic conditions of the photochemical proces forming oxygen. The experiments, reported above, seem to indicate that, whereas oxygen evolution is essentially unconnected with carbon dioxide reduction, it is more closely related to hydrogen production. As soon as conditions favour full activity of

the hydrogenase, the cells apparently get rid of their reduced products rather in the form of hydrogen gas than by transfer to reaction products of carbon dioxide. It may be that hydrogen removal via the hydrogenase effectively drains the supply of reduced products to carbon dioxide. An alternative explanation could be that a minimum concentration of oxygen is essential for the normal operation of the carbon dioxide reduction, e.g. by influencing the formation of carbon dioxide acceptor. In the absence of the latter, photochemically formed reduced products are forced to escape by way of hydrogen evolution via the hydrogenase. Preliminary experiments, made in cooperation with Dr. W. LINDEMAN of this Laboratory have already shown that under the conditions of our experiments, phosphoglyceric acid is very low. These experiments are to be continued. Meanwhile, we suggest as a working hypothesis, that, in the absence of oxygen, the components of the CALVIN cycle are of such a low concentration that the carbon dioxide acceptor (ribulose diphosphate) is strongly limiting carbon dioxide consumption. Apparently, first a little oxygen has to be formed to bring the cycle into operation, before "normal" photosynthetic carbon dioxide consumption can start. In the mean time, the cell continues its photochemical functions by producing oxygen in the usual manner, and by discharging its reduced products in the form of hydrogen gas. In the presence of carbon dioxide, the steady state concentration of carbon dioxide acceptor builds up along with oxygen evolution in an autocatalytic proces (the attainment of the necessary oxygen concentration being counteracted by respiratory processes. Hence the almost unlimited duration of hydrogen evolution in the presence of carbon dioxide at low light intensities). This picture is in agreement with WARBURG's contention (15) that oxygen is essential to photosynthesis, if "photosynthesis" is defined in the original sense of oxygen liberation with the simultaneous consumption of equivalent quantities of carbon dioxide. Photosynthesis in a more restricted sense of simultaneous production of oxygen and reducing substances is a proces that, according to our present findings, requires no threshold quantities of oxygen.

According to the shape of the oxygen induction curves, the mechanism of its formation is not simple. During phase B, there is a steady formation and consumption of this gas, as evidenced by its quick disappearance as soon as the light is interrupted. Obviously, there is, during this phase, a balance between oxygen production and its removal by strongly oxygen limited respiration. As to the origin of the two oxygen peaks A_1 and A_2, we are inclined to believe, that they may not represent true peaks in the oxygen production, but that they are rather due to transients in the respiratory removal of this gas. As there is no respiration at all during the dark period, preceeding illumination, it may be that at the start of oxygen evolution, the respiratory enzymes are not yet active. Inactivation of respiratory enzymes in normally aerobic organisms by exposure to anaerobic conditions is not an unusual phenomenon. If this applies also to our *Chlorella*'s the oxygen induction curve would also reflect the induction of respiratory processes. This conclusion receives some support from experiments like the one of fig. 11. After the algae have reached "aerobic" conditions during an illumination period, the most readily oxidisable substrates may be supposed to be exhausted. After a short dark period, little can have been produced, and a new illumination finds the algae depleted of respiratory substrates, with the consequence that phase B is much shorter than during the previous period.

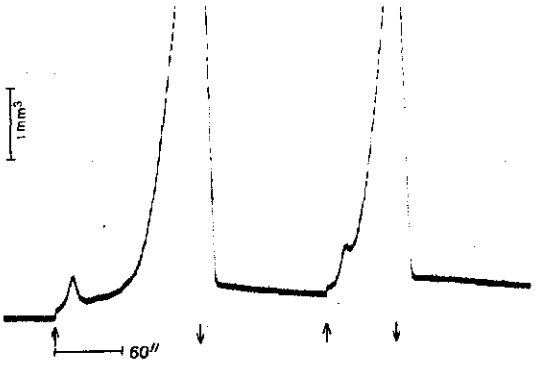

FIG. 11. Oxygen evolution in two successive illumination periods, with a short dark period interposed. During the second illumination oxygen concentrations in phase C rise much more rapidly than during the first.

It is perhaps appropriate to recall here the old observations of BEIJERINCK (16) on oxygen production by algae and chloroplasts, that were done under absolutely anaerobic conditions (luminous bacteria dimmed!). His observations of oxygen productions at all probability refer to the oxygen peaks of phase A. This is even more evidently so in the experiments by HARVEY (17), made with the same luminous bacteria method. He observed that algae that had been kept anaerobic for 45 min. evolved oxygen during the first seconds of the illumination, but not after an illumination period of 30 sec.

Although we do not claim that our present experiments give a complete answer as to the origin of the photochemically formed oxygen and hydrogen, it seems likely that they have a common origin, as mentioned already under section d. Especially experiments such as those of fig. 9f and g strongly suggest their formation from water. Unlike the hydrogen formed during the anaerobic dark fermentation, there is no evidence that the photochemical hydrogen is formed from organic donors. GAFFRON has already pointed out that the mechanisms of both processes must be different, mainly on the evidence that only the dark process is inhibited by dinitrophenol. An independent formation of both gases could not easily be reconciled with the apparently constant ratio of their volumes produced under suitable conditions. That in experiments of longer duration, no definite ratio between the two gases can be found, must be ascribed to secondary reactions such as respiration and poisoning of the hydrogenase by oxygen. It is not likely that the cessation of hydrogen production at higher oxygen concentrations is due simply to back reactions ("Knallgas" reaction), as in this case it would be hard to see why the hydrogen-oxygen ratio ever could fall below 2 in the absence of carbon dioxide reduction (fig. 9a, c, e). So, the oxygen inactivation of the hydrogenase is real. The apparent increase of the ratio after prolonged anaerobic incubation has to be explained as due to an increase in the capacity of the respiratory system. Apparently the dark fermentation produces suitable respiratory substrates. Another support for the idea that photochemically formed hydrogen arises in a major reaction, directly connected with the photolytic processes of photosynthesis, is found in the high efficiency of this hydrogen production, as pointed out previously (5). Actually, if hydrogen is produced in the primary photochemical steps from water, its volume yield per quantum of absorbed energy should be twice that of the aerobic photosynthetic oxygen production. We have observed about equal yields for

both processes under appropriate conditions. It should be remembered that an unknown part of the photochemically formed hydrogen may escape measurement because of recombination with oxygen in the oxyhydrogen reaction. Keeping this in mind, the observed quantum yield of hydrogen evolution appears too high to be accounted for by anything, except a process, closely linked up with photochemical oxygen production. Even if this conclusion is accepted, the nature of the compounds, directly acting as hydrogen donors to the hydrogenase, remains in the dark. It is evident that during normal aerobic activity of the algae, sufficient hydrogen acceptors are present, both in light and darkness. It is likely, therefore, that anaerobiosis first has to create conditions of almost complete saturation of internal hydrogen acceptors in such a way that the subsequent production of reducing agents by light must necessarily be accompanied by their removal in the form of hydrogen. We do not, therefore, want to maintain that photochemically formed hydrogen comes directly from the photolysis of water, but rather that the amount released during illumination should be equivalent to the quantity of reducing matter generated by the illumination, as suggested by the observed H_2/O_2 ratios.

Nevertheless, the assumption of a common photochemical process for both hydrogen and oxygen formation raises some difficult theoretical points. Although the maximum hydrogen pressure that can be reached during illumination is not known, it certainly can exceed 10^{-3} atm. As demonstrated by GAFFRON, at high hydrogen pressures, this gas can be consumed by the algae in the light, so that there may be an intermediate equilibrium pressure with no net hydrogen conversion. Several years ago, we have demonstrated (9) that anaerobic *Chlorella* suspensions may develop oxidation-reduction potentials in the dark as low as —330 mV (at pH = 7, hydrogen scale). This value may decrease to about —350 mV after half a minute illumination. Although the nature of the reducing substances is not yet completely clear, it is evident that there must be a connection with the hydrogen mechanisms, discussed in the present paper.* Calculated as reversible hydrogen potentials, these values correspond to hydrogen pressures of between 10^{-3} and 2.10^{-2} atm. If both oxygen and hydrogen ultimately arise from the decomposition of water, such gas pressures would correspond to free energy changes of at least 50 kcal per mol water decomposed, or 100 kcal per mol oxygen formed. Obviously, this could not be accomplished by one quantum of red light. Taken at their face value, the experiments, described in the present paper seem to indicate, that illumination is able to generate such highly reducing compounds that several light quanta are required for their formation from water. The reaction products would even contain almost sufficient energy to drive the complete photosynthetic process (112 kcal per mol oxygen).

SUMMARY

The production of oxygen and hydrogen by anaerobically incubated *Chlorella* has been studied with the aid of rapid and sensitive electrochemical as well as volumetric methods.

A relatively short anaerobic treatment suffices to activate a hydrogenase, and rapid hydrogen production follows upon illumination. Simultaneously, there is production of some oxygen. As soon as the oxygen concentration has reached a

*We will discuss oxidation-reduction potentials in the light of the present work in a subsequent publication.

certain level, the hydrogenase becomes poisoned and hydrogen evolution stops. Before the inactivation is complete, some of the hydrogen is reabsorbed.

The ratio of the volumes of hydrogen and oxygen formed during illumination in the absence of added carbon dioxide, is variable, depending upon the duration of the previous anaerobic period and of the illumination. Arguments have been put forward that in the absence of secondary reactions, the ratio of hydrogen to oxygen formed in the light is close to two. Such values were regularly observed with algae, killed by freezing.

Whereas oxygen suppresses hydrogen formation, high concentrations of hydrogen were found to inhibit oxygen production. It is concluded that hydrogen and oxygen metabolisms are closely related, and that, ultimately, both gases arise during illumination from the same source, water.

Carbon dioxide apparently has no direct influence upon either initial oxygen or hydrogen formation. Its effect is rather indirect, through accelerated oxygen production during later stages of the illumination, when the hydrogenase becomes poisoned.

The experiments indicate that minimal quantities of oxygen are essential for normal photosynthetic carbon dioxide reduction. On the other hand, no threshold oxygen concentration is required for photochemical oxygen and hydrogen production.

Illumination generates reducing compounds of a very low oxidation-reduction potential. Several quanta of red light (>2) per mol must be involved in their formation from water.

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