OXYGEN AS A FACTOR IN PHOTOSYNTHESIS

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(Received 6 December 1960)

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I. INTRODUCTION

In nitrogen, and at low concentrations of oxygen, colourless plant tissues, and green tissues in the dark, ferment sugar; as the oxygen concentration is raised, fermentation is replaced by respiration. This change in metabolism is usually accompanied by a reduction in the over-all rate of carbon loss. These phenomena have been the subject of extensive research and publication over the last 30 years. In contrast, the inhibitory effect of oxygen on the rate of photosynthesis has attracted comparatively little attention since its discovery by Warburg in 1920. The inhibition of carbon loss in respiration in the presence of oxygen has been termed the Pasteur effect (see J. S. Turner, 1960). It will be fitting and convenient to refer to the inhibition of photosynthesis (carbon gain) in high oxygen as the Warburg effect. It is by no means certain that a single explanation will serve to explain either effect, but it is at least possible that one process is associated with both, for example, the inhibition of a specific SH-containing enzyme by reversible oxidation.

The Warburg effect applies to both oxygen uptake and carbon dioxide fixation, and it is known to be of large extent when the light intensity is high and the carbon dioxide limiting. It may well reduce the efficiency of photosynthesis under optimal natural conditions. It might be envisaged as a disadvantage which has developed over the ages as the oxygen concentration of the atmosphere has increased.

A different view is that of Franck, who regards the inhibition as a form of self-narcotization. He regards the effect not as a loss of efficiency, but as a protective

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mechanism which automatically reduces the rate of both photosynthesis and photoxida-
tion: 'Plants thereby manage to prevent damage when they are irradiated in bright
sunlight in normal air whose concentration of carbon dioxide is far too low to keep
the whole photosynthetic apparatus occupied to full capacity' (Franck, 1955). The
delicacy of such control, and its reversibility, could perhaps be regarded as an evolu-
tionary adaptation to the partial pressure of oxygen in the environment.

Much of the recent work on photosynthesis has been biochemical or biophysical,
and the object has been an understanding of the complex mechanism of the process.
Our increased knowledge of this mechanism has made it possible to propose several
explanations of the Warburg effect. The testing of these has proved most difficult, and
the effect still offers a considerable challenge to the plant physiologist. This article
does not seek to offer any new theory, but supports an old one not generally favoured
today. It is hoped that the assembly of all the data and hypotheses will encourage
further experiment in a neglected field.

The Warburg effect must be clearly distinguished from an inhibitory effect on
photosynthesis of the complete absence of oxygen. So long as the green cell is pro-
ducing oxygen in the light, the steady state of production at low carbon dioxide
concentration is markedly increased by reducing the surrounding oxygen concentra-
tion to near zero. If, however, green tissue is incubated in the dark for many hours in
the complete absence of oxygen, the photosynthesis on subsequent illumination is
temporarily inhibited, and the inhibition may be prolonged indefinitely if the oxygen
evolved is continuously swept away. This inhibitory effect on induction has been
ascribed to the action of fermentation products on enzymes of the photosynthetic
mechanism. It will not be discussed further in this article. (See Rabinowitch, 1956.)

II. GENERAL FEATURES AND COMPARATIVE PHYSIOLOGY

The oxygen effect on photosynthesis was discovered by Warburg in 1920 in the
unicellular alga Chlorella. Confirmatory evidence came from various authors between
1925 and 1949, when there appeared an account by Tamiya and Huzisige of the first
thorough study of the problem. The effect was later shown to be widespread in
the plant kingdom (Turner, Todd & Brittain, 1956) and a number of the Japanese
findings were confirmed and extended (Brittain & Turner, 1962). Important contribu-
tions have come from the schools of Wassink in Wageningen, of Franck in Chicago,
and of Tamiya in Tokyo. The subject has also been reviewed by Rabinowitch (1945,
1956) and by Kessler (1960).

Most of the experiments have been done at high light intensity and high carbon
dioxide concentrations with the alga Chlorella, and photosynthesis has, in most cases,
been measured as oxygen output. For comparative purposes it is convenient to
calculate the percentage inhibition by oxygen as a percentage of the higher rate in the
low oxygen concentration, i.e.

\[
\text{percentage inhibition} = 100 \left( \frac{rP_{N_2} - rP_{O_2}}{rP_{N_2}} \right)
\]

or

\[
= 100 \left( \frac{rP_{\text{air}} - rP_{O_2}}{rP_{\text{air}}} \right)
\]

where \( rP_{N_2}, rP_{O_2} \), etc., represent the rates of real photosynthesis in nitrogen, oxygen, etc.
The inhibition by oxygen affects the carbon dioxide uptake as well as the oxygen output, as was first shown by Gaffron (1940) when he demonstrated that the assimilatory quotient (CO$_2$/O$_2$ by volume) was unchanged by the transfer of Scenedesmus cells from 0-2 to 100% oxygen. It was fully confirmed for wheat leaves by McAlister & Myers (1940), who measured Warburg effects on carbon dioxide uptake using an infra-red analyser, and by Miyachi, Izawa & Tamiya (1955) for Chlorella by the use of radiocarbon. Arnon, Allen & Whatley (1954) demonstrated a Warburg effect for radiocarbon fixation by isolated plastids capable of complete photosynthesis. It has recently been shown that the growth rate of an illuminated Chlorella culture is substantially reduced at high oxygen concentration (Brittain & Turner, 1962).

It is generally agreed that large Warburg effects cannot be due simply to an increase in the rate of normal light respiration in green tissue subjected to high oxygen concentration. Making, for the moment, the usual assumption that in such tissues the dark respiration ($R_d$) is an accurate measure of the light respiration ($R_l$), Warburg effects have been clearly established not only for the apparent photosynthesis ($aP = rP - R_d$), but for real photosynthesis ($rP$). Warburg showed that for Chlorella, whereas a change in oxygen concentration from 2 to 100% inhibited $aP$ by 41-73%, it had no effect on dark respiration. Even when there is a large percentage effect of oxygen on $R_d$, the over-all effect of this is negligible whenever the real photosynthetic rate is ten to twenty times that of dark respiration. Thus, in Cladophora (Turner et al. 1956) a gas change of 2–100% oxygen caused a 20% increase in $R_d$: nevertheless, the measured Warburg effects were as follows: (a) for $rP$, 25%; for $aP$, 28%; (b) for $rP$, 40%; for $aP$, 45%.

Most experimenters have obtained variable effects with closely comparable tissue, but there is no doubt about the significance of the inhibition, figures of the order of 30–40% being common. A summary of all published work on the green algae is given in Table 1. The percentage effects stated may be assumed to be those for true photosynthesis, although this was not always measured by the interpolation of dark periods. Such measurement is only necessary under conditions of low light intensity or when $R_d \sim aP$.

These results present one feature that must be emphasized. At high light and high carbon dioxide concentrations the effect of the change in gas concentration from 0 to 20% oxygen is very small or absent, whereas strongly marked inhibition is found for the range 20–100% oxygen. The only exception to this is a single figure given by Warburg in his pioneer paper. This has been widely quoted, but is not confirmed by later work (see Fig. 1).

There were two early records for a Warburg effect in species other than the green algae. Surbeck, Holt & Lund (1925) recorded inhibition by oxygen for the brown alga Nereocystis. McAlister & Myers (1940) were the first to show the existence of the effect in higher plants. This was obtained in wheat leaves at low carbon dioxide concentration over the range of oxygen concentrations 2–20%. Further extensive evidence for other species has been provided (Turner et al. 1956). Marked Warburg effects were obtained for six species of red algae, the inhibition of oxygen uptake ranging from 48 to 100%, even at high carbon dioxide concentrations. Similarly large
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Effects were obtained for the moss Funaria, for the fern Hymenophyllum and for eight species of higher plant, five of which were aquatics. It is concluded that sufficient evidence is now available to establish the Warburg effect as a general occurrence throughout the plant kingdom. In all, some twenty-five different species have been investigated with positive results. In most of the early work the rates were measured for short periods of from 30 to 80 min. for comparable samples each in one of the two gas mixtures. Even by these means Tamiya & Huzisige (1949) showed that the effect was reversible, as similar results for inhibition were obtained whether the plant was transferred from oxygen to nitrogen or from nitrogen to oxygen. In our own experiments the reversibility was well established for almost all the samples tested. The method adopted was to equilibrate the plant tissue successively in low, high and low oxygen partial pressures, or in high, low, high oxygen partial pressures, making allowance for any drifts in the photosynthesis over the experimental period of several hours. Figure 2 gives typical curves showing the time scale and the reversibility in such double transitions.

Table 1. Warburg effects in green algae at high carbon dioxide concentrations and high light intensity

<table>
<thead>
<tr>
<th>species</th>
<th>temp. (°C)</th>
<th>light intensity, lux</th>
<th>CO₂ mole/l.</th>
<th>% O₂</th>
<th>W.E.: % inhibition of P.S.</th>
<th>No. of expts.</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>clorella pyrenoidosa</em></td>
<td>25</td>
<td>10,000–20,000</td>
<td>91 × 10⁻⁴</td>
<td>2–100</td>
<td>57–71</td>
<td>65</td>
<td>Warburg (1920)</td>
</tr>
<tr>
<td><em>clorella pyrenoidosa</em></td>
<td>25</td>
<td>40,000</td>
<td>91 × 10⁻⁴</td>
<td>0–100</td>
<td>22–100</td>
<td>35</td>
<td>Turner et al. (1956)</td>
</tr>
<tr>
<td>Emerson’s strain</td>
<td>25</td>
<td>30,000</td>
<td>91 × 10⁻⁴</td>
<td>0–100</td>
<td>20–42</td>
<td>30</td>
<td>Wassink et al. (1938)</td>
</tr>
<tr>
<td><em>pyrenoidosa</em> vulgaris</td>
<td>25</td>
<td>30,000</td>
<td>38 × 10⁻⁴</td>
<td>0–100</td>
<td>33, 47</td>
<td>40</td>
<td>Tamiya &amp; Huzisige (1949)</td>
</tr>
<tr>
<td><em>pyrenoidosa</em> iridis</td>
<td>25</td>
<td>3,500</td>
<td>91 × 10⁻⁴</td>
<td>0–20</td>
<td>0</td>
<td>0</td>
<td>Warburg (1920)</td>
</tr>
<tr>
<td><em>pyrenoidosa</em> ellipsoidea</td>
<td>25</td>
<td>25,000</td>
<td>91 × 10⁻⁴</td>
<td>0–100</td>
<td>17–25</td>
<td>21</td>
<td>Warburg (1920)</td>
</tr>
<tr>
<td>‘adophora spp. freshwater’</td>
<td>25</td>
<td>‘High, near saturation’</td>
<td>91 × 10⁻⁴</td>
<td>2–5–20</td>
<td>5–74</td>
<td>30</td>
<td>Turner et al. (1956)</td>
</tr>
<tr>
<td>‘adophora spp. marine’</td>
<td>20</td>
<td>17,000</td>
<td>36 × 10⁻⁴</td>
<td>20–100</td>
<td>–</td>
<td>43</td>
<td>Turner et al. (1956)</td>
</tr>
<tr>
<td><em>ulerpa spp. marine</em></td>
<td>20</td>
<td>17,000</td>
<td>36 × 10⁻⁴</td>
<td>–</td>
<td>–</td>
<td>62</td>
<td>Turner et al. (1956)</td>
</tr>
<tr>
<td><em>iteromorpha spp. marine</em></td>
<td>20</td>
<td>17,000</td>
<td>36 × 10⁻⁴</td>
<td>–</td>
<td>–</td>
<td>18</td>
<td>Turner et al. (1956)</td>
</tr>
<tr>
<td><em>yopsis spp. marine</em></td>
<td>20</td>
<td>17,000</td>
<td>36 × 10⁻⁴</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>Turner et al. (1956)</td>
</tr>
<tr>
<td><em>enedesmus ‘D₅’</em></td>
<td>20:5</td>
<td>10,000</td>
<td>4 %</td>
<td>0–100</td>
<td>11, 43</td>
<td>27</td>
<td>Gaffron (1940)</td>
</tr>
</tbody>
</table>
Fig. 1. Oxygen inhibition of photosynthesis in *Chlorella* as a function of oxygen and carbon dioxide concentration. \( \Delta \Phi \), Tamiya & Huzisige (1949); \( \square \), Turner & Brittain (1956); \( \square \), Briggs & Whittingham (1952); \( \nabla \), Wassink *et al.* (1938); \( \bullet \), Warburg (1920) (note single discrepant figure at 20% oxygen).

Fig. 2. Reversal of oxygen inhibition of photosynthesis in *Chlorella*. A, *C. vulgaris*: \([\text{CO}_2]\) = 37.5 \( \times \) 10\(^{-4}\) mole/l., 30,000 lux, 25° C. B, *C. pyrenoidosa*: \([\text{CO}_2]\) = 91 \( \times \) 10\(^{-4}\) mole/l. 30,000 lux, 25° C. (after Turner *et al.* 1956).
III. THE INFLUENCE OF CARBON DIOXIDE CONCENTRATION

In the early work there were only two records of a Warburg effect caused by a change in oxygen concentration from 0 to 20%. The first was a single figure (20% inhibition) for *Chlorella* (Warburg, 1920). This result, obtained at high carbon dioxide concentration (91 x 10^-4 mole/l.) is anomalous. All other authors have found that, under these conditions, there is no significant difference between the rate of photosynthesis in nitrogen and that in air (Wassink, Vermeulen, Reman & Katz, 1938; Briggs & Whittingham, 1952; Tamiya & Huzisige, 1949; Turner *et al.* 1956—see Fig. 1). An inhibition of 25% caused by 21% oxygen was, however, recorded by McAlister & Myers (1940) for the wheat leaf. In this experiment the air contained the normal amount of carbon dioxide (0.03%) and light was near saturation. This result would be expected if the Warburg effect is greater when carbon dioxide is a limiting factor. This was first shown to be so by Tamiya & Huzisige (1949). At saturating light they found that the inhibition by pure oxygen varied from 5% at very high carbon dioxide concentration (1700 x 10^-6 mole/l.) to 85% at 1 x 10^-6 mole/l. In conformity with McAlister & Myers, they also obtained large Warburg effects (22%) with 20% oxygen when the carbon dioxide concentration was lowered.

In these experiments the inhibitions appear to be much less variable than those reported in other work. Their data fitted an equation of the second order:

\[
\text{degree of inhibition} = \left(1 - \frac{rP_O}{rP_N}\right) = \frac{[O_2]^2}{K^2 + [O_2]^2}
\]

where [O₂] is the partial pressure of oxygen and K is a constant representing the partial pressure of oxygen causing 50% inhibition of photosynthesis. The experimental results and the curves calculated for this equation are shown in Fig. 3A. The values of K used for CO₂ = 1.0 x 10^-6 mole/l. and CO₂ = 91 x 10^-6 mole/l. were 0.42 atmospheres and 1.71 atmospheres respectively. Incidentally, there has been, as yet, no experimental work on the effect on photosynthesis of oxygen pressures in excess of atmospheric.

An earlier attempt by the senior author to measure Warburg effects at low carbon dioxide concentration had brought to light a curious feature of the physiology of some *Chlorella* cultures and, as a result of further work on this, some doubt about the validity of the above results was expressed by Briggs & Whittingham (1952). They also found that for *C. vulgaris*, *C. pyrenoidosa* (three strains) and *Scenedesmus* the rate of photosynthesis at a low carbon dioxide concentration (Warburg buffer No. 2, 0.9 x 10^6 mole/l.), initially very low, increased by four or five times during 2 hr. of illumination. The marked rise was thought to be due to the removal of an inhibitor by a photochemical process other than photosynthesis itself, the removal taking place only at low carbon dioxide concentrations. The complications introduced by these phenomena led them to write that ‘any statement as to the depressant effect of oxygen upon photosynthesis at low carbon dioxide concentration—such as that of Tamiya & Huzisige—is of little value’.

It is now known, however, that these rising rates of photosynthesis at low carbon
dioxide concentration are found only with algal cells grown in 4% carbon dioxide; when such cells are illuminated in air the depressant is removed, and it is absent from cells grown continuously in air. In such ‘adapted’ cells the rate of photosynthesis in buffer No. 1 is remarkably high and steady, indicating that the affinity between CO₂ and the photosynthetic system is five to ten times greater than previous work has suggested. It appears, however, that the results of Tamiya & Huzisige were obtained with such adapted cells. In a personal communication Professor Tamiya has stated that the rate of photosynthesis at low carbon dioxide concentration showed no rising rates for at least 1 hr. Moreover, figures they give for photosynthetic rate in No. 2 buffer are of the same order as those given for the adapted cells of Briggs & Whittingham.

These authors also supplied a few figures showing the effects of oxygen for adapted cells, and the results are generally in agreement with those of the Japanese authors. Further confirmation is supplied by our own work with Funaria (Brittain & Turner, 1962). The leaves of this moss, taken from plants grown in air, gave a low but steady rate of photosynthesis at low carbon dioxide concentration, and the position was not complicated by upward drifting rates. Measurement of the Warburg effect was made in the usual way by manometry, and rates were compared with hydrogen, 5% oxygen and pure oxygen. Although there was a wide range of variation in the percentage inhibition figures, the mean of nine experiments in high carbon dioxide concentration (91 x 10⁻⁶ mole/l.) was 25·2%. The mean figures for the percentage inhibition at very low carbon dioxide concentration (1 x 10⁻⁶ mole/l.) was 90·9. Agreement with Tamiya’s
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results was remarkably close. We may safely conclude that the Warburg effect is greatly increased, becoming obvious even in 20 per cent oxygen, when carbon dioxide concentration limits the rate of photosynthesis.

IV. THE INFLUENCE OF TEMPERATURE

Tamiya & Huzisige (1949) have shown that the degree of inhibition caused by oxygen does not alter when the temperature is lowered from 25 to 4° C. For Chlorella, at low carbon dioxide concentration \((1 \times 10^{-6} \text{ mole/l.})\), the percentage depression of the oxygen uptake caused by pure oxygen was 87% at 25° C. and 86% at 4° C., the control gas being pure nitrogen. The depression caused by the change from air to 100% oxygen was 15% at 25° C. and 17% at 4° C. In these experiments the light was saturating and temperature affected the rate of photosynthesis in all cases. From the figures given for the range 4–25° C., and assuming an exponential relationship between the rate of photosynthesis (dark reactions) and temperature, the calculated \(Q_{10}\) was 1.5 in nitrogen, 1.5 in air and 1.4 in oxygen. Tamiya et al. used these results in support of their earlier view that the oxygen inhibited the carboxylation reaction (a dark reaction) by combining with the carboxylating enzyme.

At very high carbon dioxide concentration \((1700 \times 10^{-6})\), the \(Q_{10}\) for photosynthesis was reduced to 1.1. There was no Warburg effect at either temperature, 4° or 25° C.

We have confirmed some of these results with the moss Funaria (Brittain & Turner, 1962). For instance, at high light intensity, 30,000 lux, when the \(Q_{10}\) for photosynthesis was 1.6, we found that oxygen suppressed photosynthesis (control gas 0.05% oxygen) by 48% at 20° C. and by 45% at 30° C., the difference between these two values not being regarded as significant.

V. THE INFLUENCE OF LIGHT INTENSITY

Franck (1951a) has stated: 'Several observers have found that the inhibiting influence of oxygen is smaller if the CO₂ supply is high, and that oxygen has no influence at light intensities considerably below that necessary for saturation.' He quoted the paper of Tamiya & Huzisige, implying that in experiments in which a change of 4% could be detected they found no effect of oxygen at low light intensities. From this paper it is clear, however, that the authors worked only at saturating light intensities, and merely state in their introduction that the Warburg effect 'can be alleviated by diminution of light intensity' and that 'it is obvious that the site of attack by oxygen lies on some part of the mechanism of the dark reaction'.

Elsewhere Tamiya (1949) refers to the evidence of Wassink et al. (1938) which is discussed below, and of Warburg (1920). This author had shown that there was no oxygen effect at light intensities of 400–800 lux, conditions when the apparent assimilation rate was equal to the dark respiration rate. There appear to be no other data for such low intensities, but some figures are now available for intensities well below saturation. The evidence in this field, however, is conflicting, and can only be adequately explained by reference to Fig. 4, where all the relevant experimental evidence is set out.
Fig. 4. Summary of the available data giving the percentage inhibition of photosynthesis as a function of light intensity. Broken lines, rate of photosynthesis in low oxygen; full lines, percentage inhibition of photosynthesis by oxygen (in B, 20% O₂). A, E, Chlorella (Wassink et al. 1938); B, Triticum (McAlister & Myers, 1940); C, Callitriche; D, Chlorella; F, Funaria (Brittain & Turner, 1962).
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In each graph we plot the percentage inhibition of photosynthesis by high oxygen concentration, and on the same light axis we include a rate curve for photosynthesis at the same carbon dioxide concentration, which gives some indication as to the range of light intensities used in relation to the saturating intensity. Three results—A, B and C—by different authors are in agreement. They show that there is no change in the percentage inhibition by oxygen as the light intensity is reduced from saturation or near saturation to about one-fifth of that intensity. There is no indication of a fall in the percentage inhibition even at the lowest light intensity.

Conflicting with this evidence is that summarized in Fig. 4, D, E and F. Graph E gives the result obtained by Wassink et al. from their second experiment, which appears to be exactly comparable with that recorded in graph A. The light intensity is well below saturation over the whole range, and the rate curve is nearly linear. The percentage inhibition of photosynthesis due to oxygen increases over the light range from $0.4$ to $1.6 \times 10^4$ ergs/cm.$^2$/sec. At the low intensity it is $20\%$ and it rises at the highest intensity to $50\%$. Somewhat similar results have been obtained (Brittain & Turner, 1962) also for Chlorella (curve D). Each curve gives the degree of inhibition by oxygen for comparable samples of Chlorella at four different light intensities, the gases being changed during the course of the experiment for each sample. It was shown clearly for each of three experiments that the percentage inhibition was small or absent at $1.6 \times 10^4$ ergs/cm.$^2$/sec., and rose almost linearly to high figures varying from 20 to 50 per cent as the light intensity was raised to $7.3 \times 10^4$ ergs/cm.$^2$/sec. These high light intensities were not saturating for these samples of Chlorella, as the corresponding rate curves for photosynthesis show. Finally, there is the result given in graph F for Funaria (Brittain & Turner, 1962). At low carbon dioxide concentrations the percentage inhibition was high, as was to be expected, reaching $89\%$ at a light intensity of $6.6 \times 10^4$ ergs/cm.$^2$/sec., which is close to saturation. It fell markedly to $67\%$ at $3.9 \times 10^4$ ergs/cm.$^2$/sec., and we could perhaps expect with further data a curve approximating to that given for Chlorella (Fig. 4D). When the carbon dioxide concentration was high, however, the percentage inhibition was much lower, approximately $40\%$, and remained constant over a wide range of light intensities well below saturation, although falling sharply below $2.7$ ergs/cm.$^2$/sec.

This result indicates that we should expect to find that, at very low carbon dioxide concentration, the inhibition of photosynthesis by oxygen is strongly light-sensitive, being maximal at high light. The relative constancy of the percentage inhibition in some experiments would then be due to carbon dioxide limitation at the higher light intensities. This does not, however, explain the discrepancy noted for the experiments recorded in Fig. 4A and E (both at $91 \times 10^{-6}$ mole/l. CO$_2$), or the constant inhibition for wheat (Fig. 4B) when the carbon dioxide concentration in the gas phase was as low as $0.03\%$. It is clear that we still require a thorough experimental survey of this aspect of the problem, and the measurement of Warburg effects from compensating light intensity to saturation at different carbon dioxide concentrations is desirable. All that one can say at present is that the effects suggest that inhibition by oxygen is likely to be highest in saturating light, and especially at low carbon dioxide concentration. It is not always reduced as the light intensity falls, and may be very considerable.
at light intensities one-fifth of saturation. There is, however, some evidence that at light intensities below this the inhibition by oxygen falls to negligible proportions. It is of some interest to compare the first result of Wassink et al. (Fig. 4A) with that for urethane, a typical narcotic. The inhibition of photosynthesis caused by this substance remains constant over a wide range of light intensities, and for this reason the narcotic is supposed to act, not only on dark reactions, but also on the photochemical reaction. Were it not for the other data illustrated, one could draw similar conclusions for oxygen from the data given in Fig. 4A, B and C.

VI. THE ASSIMILATION QUOTIENT AT HIGH AND LOW OXYGEN CONCENTRATION

Oxygen at high partial pressure causes a large reduction in the rate of both the oxygen output and carbon dioxide uptake of green cells. Miyachi, Izawa & Tamiya (1955) have measured photosynthesis at four oxygen concentrations by supplying Chlorella with labelled bicarbonate and measuring the $^{14}$C fixed in the light over a period of fifteen minutes. The degree of inhibition ($h$) obtained fitted the equation

$$\text{degree of inhibition} = \frac{[O_2]^2}{\phi^2 + [O_2]^2},$$

where $\phi$ is a constant corresponding to the oxygen partial pressure causing 50% suppression. The inhibition was of 'second order', the equation being the same as that obtained by Tamiya & Huzisige (1949) for oxygen inhibition of oxygen output (Fig. 3A, B). No direct comparison, however, may be made between the two experiments and the results give us no information about the assimilatory quotient. A few actual measurements of the quotient in high and low oxygen have been made. Gaffron (1940) reported no change or a slight increase in the volume quotient for CO$_2$/O$_2$ for Scenedesmus at high oxygen partial pressure. A considerable decrease has been found for Chlorella—0.93 to 0.63 (Brittain, 1957). Further measurements over short and long time intervals are necessary before firm conclusions can be drawn. Meanwhile, we can be certain that the Warburg effect applies both to the oxygen-liberating and to the carbon-fixing processes of photosynthesis.

VII. THE WARBURG EFFECT: EXPLANATORY HYPOTHESES

We have now set out most of what is known about the direct effect of oxygen on the rate of photosynthesis under various conditions. Further observations bearing on the problem will be presented in the discussion of the various hypotheses which have been put forward in explanation of the Warburg effect; these may be classified as follows.

(1) Back reactions involving molecular oxygen

In his original paper, Warburg (1920) stated that the explanation of the oxygen effect that he had established must first take account of two possibilities. 'At high light intensity the assimilate forms at higher concentration and may, before it can turn into a stable form, be reoxidized by the oxygen back to CO$_2$' (photorespiration).
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'This is the less interesting possibility. It might also be imagined that oxygen reacts with the primary photochemical product, so that it acts as acceptor, like the carbon dioxide derivative' (Mehler reaction). A third possibility of this kind is a direct photooxidation.

(a) Photorespiration

This is an acceleration of normal respiration (R.Q. 1.0) to a rate of the order of photosynthesis itself, the substrate being a carbon compound, an intermediate of both respiration and photosynthesis. Photorespiration is supposed to cease in the dark as instantaneously as does photosynthesis.

It has been distinguished from photooxidation as being enzymatically controlled and probably saturated at low oxygen concentration; we shall also assume that it is not sensitized by chlorophyll. Rabinowitch (1945) has referred to its possible existence as 'a nightmare oppressing all who are concerned with the exact measurement of photosynthesis'.

The methods first used to investigate photorespiration included the measurement of respiration immediately following the darkening of tissues, the effect of temperature on gas exchange at various light intensities and the use of poisons, such as hydroxylamine and cyanide, which were by some believed to differentiate between respiration proper and photosynthesis. In 1945 Rabinowitch, after reviewing the available evidence, concluded that there was 'no final proof of the non-existence of "photorespiration", and that no evidence of such a phenomenon has as yet been found, all definitely established cases of light-stimulated respiration were of the persistent type and could be attributed either to an accumulation of sugars or to an indirect photochemical effect of blue-violet light absorbed by the carotenoids'.

New methods, however, became available at about this time, and work with isotopically labelled carbon dioxide indicated that photosynthesis and respiration were processes that involved certain common intermediate carbon compounds, for example, phosphoglyceric acid. It was suggested that some of these might provide cross links between the two processes. This appeared the more likely because at that time it was thought that both respiration and photosynthesis occurred simultaneously in the plastid, and that cytoplasmic enzymes were also concerned in both processes. Today it appears to be more likely that the plastid is the sole site of photosynthesis, while the major oxidative cycle of respiration is confined to the mitochondrion (Arnon, 1959). This spatial separation of the catabolic and anabolic processes makes the existence of a true photorespiration rather less likely, and it is significant that Calvin & Benson (1947) showed that the C₅ and C₆ acids of the Krebs cycle were not present after short-term photosynthesis, but appeared within 90 sec. of the extinction of the light. This could mean that Krebs cycle respiration, in the mitochondrion, is inhibited rather than stimulated in the light, or that the cycle is maintained in the light at the expense of glutamic acid coming from proteins, instead of from pyruvic acid derived from the photosynthetic products (Steward & Thompson, 1950). We know, however, that if we supply green leaves with labelled carbon dioxide, translocation of labelled sucrose takes place in the phloem while the leaves are still being illuminated.
Therefore, photosynthetic products must leave the plastids in the light, and one might expect some of it to be metabolized in the mitochondria.

Calvin’s hypothesis, light-inhibition of respiration, was at first supported by a direct study of gas exchange for the barley leaf with \(^{14}\text{C}\) present (Weigl & Calvin, 1949; Weigl, Warrington & Calvin, 1951). The conclusion drawn was that the respiration rate was halved in the light, but the analysis of the results was greatly complicated by the existence of large isotopic exchange reactions and a surprisingly strong isotopic discrimination, \(^{14}\text{C}\) uptake being \(17\%\) less active than \(^{12}\text{C}\) uptake. In a repetition of experiments of this type by van Norman & Brown (1952) the mass-spectroscope was used. They made the assumption that light- and dark-respiration were identical in rate, and were then able to confirm the existence of marked isotopic discrimination. From the point of view of the present discussion it is more important that they confirmed their own hypothesis—for both barley leaves and *Chlorella* they showed, by using the oxygen isotope \(^{18}\text{O}\), that there was no difference between the oxygen uptake in light and darkness. This important experiment and those confirming it by Brown (1953) will be discussed in more detail after we have dealt with other back reactions which could lead to oxygen uptake in the light.

(b) Photoxidation

This is the oxidation of a carbon compound, presumably a carbohydrate; it is a non-enzymatic process sensitized by chlorophyll.

Photosynthesis itself is primarily the reduction of a carbon compound, following the photolysis of water in the presence of the chlorophyll pigments. *In vitro*, however, the most frequent of the reactions sensitized by chlorophyll in solution or adsorbed on to a protein is oxidation; Rabinowitch (1945) lists substances that can be photoxidized in this way. Such photoxidations take place *in vivo* as well as in freshly killed green cells. For instance, Noak (1925) showed that benzidine, when added to living or boiled green tissue, was oxidized in the light to a brown pigment. An added substrate is not essential. Photoxidations of metabolites or cellular reserve materials have been clearly demonstrated (a) when photosynthesis is inhibited by poisons such as urethane, nitrous and sulphurous oxides (Noak, 1925; Wehner, 1928), (b) when no external carbon dioxide is supplied, and (c) when the light intensity is abnormally high. As the rate of photoxidation under condition (b) is increased by oxygen concentration over the whole range from 0 to 100\% (Franck & French, 1941) it has been suggested that at high oxygen concentration a substantial photoxidative back reaction

\[
\text{CO}_2 + \text{H}_2\text{O} \xrightarrow{\text{photosynthesis}} \text{C(H}_2\text{O)} + \text{O}_2 \xrightarrow{\text{photoxidation}}
\]

masks photosynthesis and is responsible for the Warburg effect. This hypothesis is supported by the observation that the Warburg effect is increased in extent at low carbon dioxide concentration. Van der Pauw (1932) and Franck & French (1941) illuminated green tissue above strong alkali so that the only carbon dioxide available for photosynthesis was that liberated by respiration (and photoxidation) and assimilated
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before it was trapped by the potash. At high light intensities (near that saturating the normal photosynthesis) there was an oxygen uptake which amounted to two or four times the dark respiration rate. This photooxidation was shown to vary in rate with oxygen partial pressure in the same way as does the Warburg effect when carbon dioxide is in low concentration. Franck & French (1941) pointed out, however, that the loss in oxygen production due to the Warburg effect was ten to twenty times the oxygen consumption by photooxidation in the absence of carbon dioxide. They therefore concluded that the Warburg effect was associated with a real inhibition of photosynthesis and could not be due simply to a masking of part of photosynthesis by a massive photooxidation.

The comparison of the magnitude of the Warburg effect and photooxidation was based on Warburg's figure of a 35% oxygen effect in Chlorella at a high carbon dioxide concentration. The discrepancy is even greater if the Warburg effect (like the photooxidation) is measured at low CO₂ concentration (see § III).

This is not to imply that Franck & French did not consider photooxidation important. On the contrary, they held the view—since discussed many times by Franck—that a small amount of photooxidation was indirectly responsible for inhibiting photosynthesis by producing a narcotic substance. This view was first put forward by Myers & Burr (1940). Since the early work of Blackman (1905) the phenomenon of 'light saturation' in photosynthesis has attracted so much attention that there has been a tendency to neglect the existence of 'solarization'—i.e. an apparent or real inhibition of photosynthesis in very strong light. In very high light intensity oxygen output may even be replaced by oxygen uptake, ascribed to photooxidation in the presence of carbon dioxide (Fockler, 1938; Myers & Burr, 1940). In short-term experiments the effects are reversible, even when the rate of oxygen uptake has reached two to four times the dark respiration rate. If insolation is prolonged (e.g. for 2 hr.) the photosynthetic mechanism is irreversibly damaged and eventually (perhaps as other substrates are used up) the chlorophyll is bleached. Myers & Burr found it difficult to believe that photooxidation went on at such a high rate as to mask the photosynthesis completely (i.e. rate photooxidation (max.) = rate photosynthesis (max.) + 4Rₐ). For this reason, and because its full extent was established rather slowly in solarizing light, they concluded that photooxidation actually inhibited photosynthesis.* Evidence in favour of this view has been provided by Fockler (1938), Myers & Burr (1940), Stählfelt (1939) and Steeman-Nielsen (1952), all of whom showed that there was an induction period for the photosynthesis at low light after solarization. Thus, Steeman-Nielsen found that light at 100,000 lux inhibited maximal photosynthesis by 50%. If the light intensity was then reduced to 3000 lux (well below saturation) the photosynthesis did not regain its full value at this intensity for several hours.

Photooxidation then is a real phenomenon, demonstrable under specified conditions in living green tissue. It is apparently sensitized by chlorophyll as it occurs in both red and blue light. It differs from respiration and probably, therefore, from photorespiration in not being saturated by low partial pressures of oxygen (c. 1-5%), and

* Photooxidation in the complete absence of carbon dioxide and at saturating light intensity is rapidly established (Franck & French, 1941).
also in its survival after the enzymes of the leaf have been destroyed by heat or inhibited by cyanide (Myers & Burr, 1940); it presumably occurs in the plastid itself. The endogenous substrate is unknown, but is presumed to be a carbohydrate. The process is believed to involve not only the uptake of molecular oxygen but the evolution of carbon dioxide, but the photoxidation quotient (+ CO₂/O₂ vol.) is not known. Rabinowitch (1945) has proposed as a working hypothesis, that photosynthesis and photoxidation share the same primary photochemical process—the photolysis of water—and differ only in the nature of the secondary dark reactions.

The available evidence (Franck & French, 1941) suggests that a photoxidative back-reaction is unlikely to account for the Warburg effect directly, that is, by masking photosynthesis. The only way to discover whether such a reaction is of appreciable extent at (or below) saturating light and with carbon dioxide present, is by the use of isotopes. Recent work along these lines will be discussed at the end of the next section. It does, however, appear probable that a small-scale photoxidation could accompany photosynthesis at high light, low carbon dioxide concentration and high oxygen pressure and that, as Franck suggests, the indirect effect of this could be of great significance (see § VII, 2, 3).

(c) The Mehler reaction

The third possible back-reaction (considered by Warburg as more significant than photorespiration) is the reaction of molecular oxygen with the photochemical primary product, competing for it with the carbon dioxide or its derivative. In modern terms this would mean that oxygen could act as a Hill reagent, becoming reduced to hydrogen peroxide and eventually to water by the hydrogen or reduced substance formed during the photolysis of water. Part of the photosynthetic process would be running in a circle (equations 1–4).

\begin{align*}
4H_2O & \xrightarrow{\text{light}} 4(H) + 4(\text{OH}) \quad (1) \\
4(\text{OH}) & \rightarrow 2H_2O + O_2 \quad (2) \\
4(H) + 2O_2 & \rightarrow 2H_2O_2 \quad (3) \\
2H_2O_2 & \rightarrow 2H_2O + O_2 \quad (4)
\end{align*}

This hypothesis has taken a central place in recent discussions of the Warburg effect and must be considered in some detail.

This back-reaction, unlike photorespiration and photoxidation, may be studied in the isolated plastid in the absence of carbon dioxide and of endogenous substrate. Hill's discovery that such plastids, when illuminated in the presence of a suitable added oxidant, evolve oxygen, is now well known; possible Hill reagents and reactions are set out in the scheme of Fig. 5, due to Brown & Good (1955). It will be seen that oxygen is included as a Hill reagent in reactions k or c. Mehler in 1951 showed that reaction c (the Mehler reaction) could occur in isolated plastids in the absence of oxidants other than molecular oxygen.

This discovery arose from the attempt to show whether or not hydrogen peroxide was formed during normal photosynthesis. Mehler (1951 a), working with plastids,
attempted to trap any hydrogen peroxide so formed, by the Keilin & Hartree reaction. He added an excess of catalase, together with ethanol, to the plastid suspension in the presence of a Hill reagent (e.g. quinone). If hydrogen peroxide were formed it would be expected to react according to equation (5), the catalase acting as a peroxidase:

$$H_2O_2 + CH_3 \cdot CH_2 \cdot OH \xrightarrow{excess catalase} \xrightarrow{catalase} 2H_2O + CH_3CHO.$$ (5)

He could detect no acetaldehyde after illumination in air and concluded that no hydrogen peroxide is normally formed during oxygen production in the Hill reaction (i.e. as an intermediate in equation (2) above). When, however, this experiment was repeated in the presence of oxygen and the absence of another oxidant or Hill reagent, oxygen output was replaced by oxygen absorption and acetaldehyde was formed according to the overall equation (6):

$$O_2 + 2CH_2 \cdot CH_2OH \rightarrow 2CH_3CHO + 2H_2O.$$ (6)

This equation is the sum of the partial equations (1), (2), (3) and (5). The trapping reaction for hydrogen peroxide did not work in the presence of adequate quantities of other known oxidants, although later Mehler (1951) found that it was stimulated by a prior Hill reaction with quinone. He concluded that oxygen is an efficient Hill reagent, but is excluded from reactions with chloroplasts by other oxidants. He went on to suggest that, although in normal photosynthesis an oxidant other than oxygen is always present, nevertheless, reactions (1) to (4) could be of significance, not only when carbon dioxide is absent and light in excess, but also when the oxygen concentration is abnormally high. He, therefore, postulated that the Mehler reaction is the explanation of the Warburg effect.

The existence of a Mehler reaction in plastids was confirmed in an elegant way by
Mehler & Brown (1952) with the use of oxygen isotopes. They obtained the concomitant production and consumption of oxygen only after the chloroplast preparation had photochemically reduced quinone. Later Brown (1953) clearly demonstrated the reliability of such experiments with oxygen isotopes. He showed that 'neither isotope discrimination nor isotope exchange is of a magnitude or velocity which could vitiate the assumption that the utilization of tracer oxygen (\(^{18}O\)) is a true measure of the respiratory rate'. It is, therefore, possible to study the output of oxygen (\(^{18}O\)) by photosynthesis simultaneously with the uptake of oxygen (\(^{18}O\)) due to respiration or to a Mehler reaction. It has also been established that the oxygen of hydrogen peroxide does not exchange with that of water (Dole, Hawkins & Baker, 1947; Cahill & Traube, 1952). Hence, in further isotope experiments with plastids, Brown & Good (1955) were able to show that, in the presence of oxygen, illuminated plastids, without other added oxidants, evolved oxygen at the same rate as they absorbed it, the catalase under these conditions being presumably endogenous in the plastid. It was assumed, therefore, that the catalase under these conditions dismuted the hydrogen peroxide with the formation of water and oxygen, the net gas exchange being zero (equations (1), (2), (3) and (4)). Such balanced oxygen exchange can only be detected by the use of isotopes. It could, of course, be argued that in this experiment the reduction of the oxygen took place with the formation of water rather than hydrogen peroxide, and the authors presented evidence derived from the addition of cytochrome C or polyphenol oxidase that such a reaction could occur. When, however, they repeated their experiment and added the hydrogen peroxide trap, the system began to take up oxygen and was presumed to return to that set out in reactions (1), (2), (3) and (5).

Good & Hill (1955) subsequently found that isolated plastids, active with normal Hill reagents, do not always reduce molecular oxygen. They can be made to do so, however, by adding substances (some of which can be obtained from leaves) whose reduced forms can be oxidized by molecular oxygen. They argued, therefore, that such intermediate hydrogen transport systems might couple the chloroplast reaction with oxygen reduction. (This could be the explanation of Mehler’s discovery that the Mehler reaction in plastids is accelerated by prior reduction of quinone.) It might, therefore, be thought that Good & Hill would argue that the Mehler reaction can go on in the cell and that it survives in the plastid (as shown by Mehler) only when the intermediate H-transport systems are retained during plastid isolation. On the contrary, they say:

‘Therefore we might suppose that the frequently observed inactivity of isolated chloroplasts towards molecular oxygen represents the photochemical situation as it exists in the cell. But if, during the process of isolation the chloroplasts lose a component which normally prevents the short-circuiting of the reducing system by oxygen, the resulting system might reduce oxygen directly.’

Evidence that a Mehler reaction can occur in living cells, as well as in plastids, has, however, been provided by the very active school of Tamiya in Japan. This evidence is derived from studies of carbon fixation in the dark following the pre-illumination of Chlorella cells. Calvin & Benson (1947) showed that if green algae are strongly illuminated in the absence of oxygen and carbon dioxide, they will then fix tracer carbon dioxide supplied immediately after the light is turned out. Gaffron & Fager (1951),
after further experiments in this field, supported the view that this dark fixation after pre-illumination represents the normal carboxylation step in photosynthesis. With *Scenedesmus* the sole product of fixation is phosphoglyceric acid. With *Chlorella* the tracer is more widely distributed. Experiments on this type of dark fixation should provide useful information on photosynthesis itself. Tayima and his school have adopted this viewpoint, and some of their results are summarized in Fig. 6.

*Chlorella* cells were illuminated for various periods in the absence of oxygen and of carbon dioxide (other than that provided by respiration), and then placed in darkness in contact with labelled bicarbonate. At the concentration of NaH$_{14}$CO$_3$ saturating for the dark fixation ($1\cdot0 \sim 1\cdot7 \times 10^{-3}$M) the fixation was virtually complete in 30 sec.

![Fig. 6. The effect of oxygen on the level of *R*, the reducing substance formed in *Chlorella* by pre-illumination in the absence of carbon dioxide. (After Tamiya *et al.* 1957.)](image)

Its extent was estimated by counting after the cells had been killed by acid. It was shown that the reducing substance *R* (measured by a standard 30 sec. fixation) reached a steady level of about $5\cdot7 \times 10^{-6}$ molar CO$_2$-equivalent after 30 min. of pre-illumination. It is now assumed (Tamiya, Miyachi & Hirokawa, 1957) that the steady state of *R* in nitrogen gas is the resultant of the photochemical formation of *R* and a Mehler reaction with the oxygen formed during the 30 min. pre-illumination. The decay of *R* in darkness and without exogenous oxygen was normally complete within 15 min., and was also presumed to be due to the reaction of *R* with endogenous oxygen.

It will be seen from Fig. 6 that molecular oxygen present during the pre-illumination depresses the *R*-level (this depression being reversible and a function of the partial pressure of oxygen), and that oxygen also accelerates the decay of *R* in the dark. Miyachi, Izawa & Tamiya (1955) argued that these results were due, not to an oxygen effect on the rate of production of *R*, but to the Mehler reaction, that is, to the
interaction of $R$ with molecular oxygen to form hydrogen peroxide and the subsequent rapid dismutation of this under the influence of endogenous catalase.

That the reducing substance $R$ is similar if not identical with that formed in the Hill reaction with plastids was shown by Katoh, Hirokawa & Miyachi (1958). The dark reduction of the dye 2-6-dichlorophenol indophenol (a Hill reagent) was greatly enhanced by pre-illumination. It was further shown that quinone will replace carbon dioxide as the oxidant and that, so long as azide is added to inhibit catalase, hydrogen peroxide will also decrease the steady $R$-level in the light and accelerate its decay in the dark (Hirokawa, Miyachi & Tamiya, 1958). At the concentrations of the oxidants used in all these experiments it was found that the reactivity of the various Hill reagents was in the order $O_2 < CO_2 < H_2O_2 <$ quinone.

As a result of all this new work Tayima and his colleagues have abandoned their earlier hypothesis concerning the cause of the Warburg effect (see pp. 137-56) and now propose that it is due to the coexistence with photosynthesis of a Mehler back-reaction. 'In the photosynthesis occurring under natural conditions, the concentration of carbon dioxide available is usually suboptimal, and, moreover, there is a large amount of oxygen in the circumambient medium. Under such a condition, the competition between CO$_2$ and O$_2$ for $R$ will be more or less in favour of oxygen' (Tamiya et al. 1957). They apparently no longer invoke an enzyme inhibition by oxygen, nor do they believe that the photosynthetic machinery is damaged by the hydrogen peroxide first formed in the Mehler reaction—'according to our notion, it is to protect the photosynthetic machinery from such contingent accumulation of hydrogen peroxide that catalase is playing a role in the mechanism of photosynthesis'. It is important to point out that Tamiya regards the back-reaction with oxygen (Mehler reaction) as a normal accompaniment of the whole photosynthetic process, so much so that it has been a factor involved in the evolution of the siting of catalase in the green plastid.

In supporting their earlier hypothesis, that oxygen affects carboxylation, Tamiya & Huzisige adduced as evidence the facts that the Warburg effect is greatest at high light and low carbon dioxide concentration and independent of temperature. These same facts do not also support the new hypothesis. If oxygen inhibits photosynthesis in saturating light, when $R$ is in excess, by destroying $R$ (which is unlikely), then low temperature should raise the photosynthetic rate by decreasing the rate of this back-reaction.

\textit{(d) Direct tests for back-reactions}

While the results of the Japanese work on pre-illumination remind one of the similar effects of oxygen on photosynthesis proper, they provide no direct evidence for the existence of a massive Mehler reaction under the normal conditions of photosynthesis. It has been presumed up to now by the Japanese workers that the extent of carbon fixation after pre-illumination is a measure of $R$, the reducing power. It has been shown, however (Arnon, 1960), that fixation depends upon the concentration of two components of assimilatory power—$R$ and adenosine triphosphate, both of which are formed during pre-illumination. Even if we accept Tamiya's premise it must be remembered that in all these pre-illumination experiments the reaction with oxygen
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is taking place in the virtual absence of carbon dioxide and when the level of assimilatory power has been substantially and artificially raised. The destruction of $R$ by oxygen follows the first order equation (Miyachi et al. 1955):

$$h = \frac{[O_2]}{\phi + [O_2]}$$

whereas the corresponding equation for the Warburg effect is of second order see pp. 135, 140).

Again, although we may take it for granted that a Mehler reaction has been established in the isolated plastid, it has been demonstrated to occur only when other oxidants (including carbon dioxide) are absent. Mehler himself (1951a) could detect no hydrogen peroxide by the trapping method while another Hill reagent was present with the oxygen. Mehler & Brown (1952) stated, ‘Until the quinone (Hill reagent) is nearly exhausted there is no significant change in tracer oxygen partial pressure... after 20 minutes the quinone has been almost quantitatively reduced and molecular oxygen begins to be absorbed’. We could, therefore, deduce that oxygen should have no effect on the rate of the Hill reaction. Direct confirmation of this is not a simple matter. Care must be taken that oxygen does not merely reoxidize the reduced Hill reagent—as in Hill’s first experiment—or cause destruction of the pigments in the isolated chloroplasts. In our experiments (Brittain & Turner, 1962), we have been unable to detect any oxygen effect on the rate of the Hill reaction with ferricyanide or dichloro-phenol-indophenol as oxidants. Confirmation of these results would be of great importance. Still more direct evidence for or against back-reactions in vivo may one day be obtained by the use of oxygen isotopes.

We have already shown that Mehler & Brown (1952), and later Brown & Good (1955), used isotopic oxygen to demonstrate the reality of the Mehler effect in plastids. When the same technique was used for whole Chlorella cells, however, it was demonstrated that oxygen uptake continued unchanged in rate in light and darkness (van Norman & Brown, 1952). Subsequently, in a study of light-respiration, Brown (1953), using molecular oxygen highly enriched in mass 34, measured oxygen uptake and oxygen output simultaneously for one sample of Chlorella in light and darkness. He could obtain no evidence for a photo-stimulation of oxygen uptake even with intermittent light, for which Warburg had claimed a greatly accelerated light respiration. He repeated these experiments with similar results with several other green algae and some vascular plants. Brown’s experiment may be taken as establishing the absence, not only of photo-respiration, but of photoxidation and the Mehler reaction under the special conditions of his experiment.

Brown & Good (1955), having shown the existence of a Mehler reaction in plastids, asked themselves the question, ‘Can the photochemical reduction of oxygen be a significant reaction in intact cells?’ They stated that as yet an answer cannot be given, but their experiments were negative. There was little photochemical reduction of oxygen superimposed on respiration, even when the cells were illuminated in the absence of exogenous carbon dioxide.

These conclusions, however, do not apply to the blue-green alga Anabaena. Brown
Webster (1953) obtained variable results with this form. At very low external oxygen concentrations—0.2–1.1%—the isotope experiments indicated that light caused an inhibition of respiration, while at 0.8–1.6% oxygen there was an increased oxygen uptake in the light. These experiments served to demonstrate the value of this important technique, but are not at present relevant to the argument, in the first place because in the blue-green algae the sites of respiration and photosynthesis are not spatially separated as in other plants, and secondly because as yet there has been no demonstration of a Warburg effect in the blue-green algae—nor is there likely to be one at such low oxygen concentrations.

The crucial experiment with oxygen isotopes has not yet been made. In all the isotope experiments reported above the oxygen concentration surrounding the plastids or the living cells was of the order of 0.1% to a maximum of 9%. The carbon dioxide concentration was usually high. Under these conditions one would not expect a Warburg effect. All we can say at the present time is that the evidence for back-reactions in living cells is negative in experiments where the oxygen concentration is below 9%; there is no result available at present for cells exposed to 20 or 100% oxygen. If it proves impossible to use the oxygen isotope method at these high concentrations, a possible alternative would be to return to the method developed by Weigl & Calvin (p. 142). We could use the data obtained from experiments with oxygen isotopes to estimate the isotopic exchange and discrimination for the carbon isotopes at low external oxygen concentration; then repeat the experiment with carbon isotopes at high oxygen concentration and assume that oxygen has no effect on the isotope exchange or discrimination. In this way it would be possible to make a direct test of the hypothesis that back-reactions mask normal photosynthesis and are entirely or partially responsible for the Warburg effect.

(2) The narcotic hypothesis

This involves the production of a narcotic substance capable of inhibiting photosynthesis by covering the surface of the active part of the chlorophyll complex: such a substance could be produced by reactions VII 1 (b), 1 (c) or following a mass action effect of oxygen on the oxygen-liberating reaction of photosynthesis.

In 1941, Franck, French & Puck first postulated the photochemical production of surface-active narcotics to explain induction effects in photosynthesis. The production and destruction of natural narcotics was invoked to account for temporary rises in the fluorescent yield accompanied by depression of the photosynthetic rate. Franck (1951a, b, 1955, 1957, 1960) expanded this hypothesis to cover the Warburg effect.

The fluorescent yield of green tissue is of the order of 0.1–1% of the incident light energy. Measurements of the intensity of the fluorescence have frequently shown an inverse relationship with the rate of photosynthesis, and simultaneous measurements of both processes have been used in studying the mechanism of the photochemical reactions (Wassink, 1951; Franck, 1960). For instance, when green plant cells are kept in the dark for some hours and then illuminated, the rate of photosynthesis is at first low; as it rises in the subsequent induction period (lasting a few minutes) the fluorescent intensity falls from its initial high level to the normal low steady state.
Franck's explanation was that during the dark period the oxygen-liberating enzyme became inactivated; its substrate, a 'photo-peroxide', then accumulated and was able to oxidize sugars, presumably to an acid which acted as a narcotic and settled on the chlorophyll surface. Narcotics such as phenyl urethane are known to inhibit photosynthesis and to raise the fluorescent yield in vivo, and the naturally formed narcotic is supposed to do the same, cutting down the production of the photo-peroxides to a level 'which the available oxygen-liberating enzyme can handle'. The narcotic is then supposed to be removed by oxidation, mainly respiration, while the enzyme is being reactivated during induction.

The induction effects are accentuated if the plant is continually swept with pure nitrogen. It was also found that a dark period of strict and prolonged anaerobiosis greatly prolongs induction, and a secondary hypothesis was erected—that the products of fermentation also act as a narcotic, which can be oxidized away. The same idea was expanded to account for photosynthetic inhibition at very high light intensities (see p. 143). Photoxidation was thought to produce a narcotic which inhibits both photosynthesis and photoxidation. Finally, it was proposed (Franck, 1951a, b) that at saturating light and low carbon dioxide concentration photoxidation goes on at a rate sufficient, not to mask photosynthesis, but to depress it by the secondary production of a natural narcotic.

These hypotheses, incorporated in his general theory of photosynthesis, have been argued with great skill and knowledge by Franck, but are not entirely accepted even by some working in the field of fluorescence studies (e.g. Wassink, 1951). The evidence is derived mainly from studies of fluorescence and it is very difficult to disentangle the results obtained from the induction and from the steady-state effects, and to explain the variation in results for different species and groups of plants and photosynthetic bacteria. Moreover, the different types of inhibitor, including narcotics, may have multiple effects. For the physico-chemical aspects, therefore, the reader must be referred to recent reviews by Franck (1955), Wassink (1956) and Rabinowitch (1956). The physiologist may perhaps be excused for a degree of scepticism when he reads such statements as, 'The general shape of the fluorescent/time curves can only be understood if it is taken into account that two natural narcotics are present. One is slowly made by anaerobic metabolism and is shown by the high value of the initial fluorescence intensity... the other is made during illumination by the reaction of the peroxides with carbohydrates. The first mentioned has its maximum concentration at the start of the illumination, and this decreases under the influences of the light, since this narcotic is more quickly removed by the attack of photo-peroxides than produced by narcotic metabolism' (Schiau & Franck, 1947).

Before examining the evidence for the production of narcotics as part of the Warburg effect, we set out Franck's views as developed up to 1957. He applied the ideas of Weiss (1938) and Franck & Livingstone (1941) that photoxidation sensitized by chlorophyll in vitro is a peroxidation, the hydrogen peroxide being formed by a reaction of hydrogen (produced photochemically) with molecular oxygen. With the demonstration of the Mehler reaction he argued that, in vivo, oxygen competes with carbon dioxide complexes, acting, therefore, as a Hill reagent. The smaller the carbon
dioxide concentration the more effective the competition. Other experiments in carbon dioxide-free air showed that oxygen consumption in the light is very much smaller than the difference in the saturation rate of photosynthesis in air and nitrogen (Warburg effect). This result, together with fluorescence studies, led him to the narcotic hypothesis. This is that all kinds of photoxidation of carbohydrate produces substances that are adsorbed by chlorophyll, raise its fluorescence and, at the same time, act as a protective cover. These substances (narcotics) restrict access of oxygen to the excited chlorophyll molecule, therefore checking excessive photoxidation and reducing photosynthesis. Franck returns continually in his reviews to this idea of a self-regulating, protective mechanism, and has not, so far, discussed other suggested mechanisms of this kind (for example, that the function of the carotenoids is to prevent photoxidations—Kohl (1902), Anderson & Robertson (1960)). Franck (1951b) supposes that during photosynthesis in air at low carbon dioxide concentration the narcotic is continually produced and destroyed—'only enough chlorophyll remains active to allow photosynthesis to balance the formation and consumption of the CO₂ complexes, i.e. the photosynthetic apparatus adjusts itself to limited carbon dioxide by inactivating so much of the chlorophyll that for the part which remains active the so-called carbon dioxide limitation does not apply. The narcotic layer lowers photosynthesis and checks photoxidation very efficiently. The photoxidation would, in fact, go to zero if the narcotic substance were stable. Since it is unstable, sufficient photoxidation remains to replenish its loss.'

The first data published on the relation between fluorescence yield and the Warburg effect did not support these views of Franck. Wassink et al. (1938) found a linear increase of fluorescence intensity as the light intensity was increased from zero to...
values beginning to saturate the photosynthesis. The same straight line was given whether the plant (Chlorella) was in equilibrium with oxygen or with nitrogen gas, although the oxygen caused a typical Warburg effect (Fig. 7). Similarly, a cyanide concentration which markedly depressed photosynthesis at high light intensities had no effect on the fluorescence yield, while the narcotic substance, phenyl urethane, also a depressant of photosynthesis, increased the fluorescence intensity at all light intensities. Wassink and his colleagues accounted for these data by the hypothesis that oxygen and cyanide acted on a dark reaction in photosynthesis, while urethane, a surface-acting narcotic, inhibited the photochemical reaction also.

Franck has relied, however, on other published data in which measurements of both fluorescence intensity and photosynthesis were carried out simultaneously on the same plant material (McAlister & Myers, 1940). These workers exposed the wheat leaf to either 2 or 20% oxygen, both gases containing 0.03% carbon dioxide. The saturating light intensities (Fig. 7) were much higher than those applying to the Chlorella in Wassink's experiments, and the leaves were under conditions of carbon dioxide limitation at the higher light intensities. As might be expected from the results discussed above, there was a marked Warburg effect between 20 and 2% oxygen. The curves for photosynthesis were similar to those published by Wassink. The results for fluorescence intensity curves, however, were different. The curve for fluorescence intensity against light intensity was linear over the whole range of light intensity for the leaf in nitrogen, whereas the curve for air diverged upwards, 'the first indications of the rise being visible at those light intensities which caused the first deviations of the saturation curve from linearity' (Franck, 1951b). Franck has argued that the difference between this curve and that of Wassink et al. is due to the fact that the latter authors did not measure the curves far enough into the region of high light intensity. It should be pointed out, however (Fig. 7), that in Wassink's experiments there is no increase in the fluorescence intensity over the region in which a Warburg effect is well established. It seems, therefore, that further data are required to establish the truth of this matter. Franck himself (personal communication) has no doubt about the faster rise of fluorescence in the saturation region in the high oxygen concentrations, but published data are scanty. Brugger (1957) presents curves which show a rise in fluorescence in both nitrogen with 2% carbon dioxide and in 20% oxygen with 4% carbon dioxide, and his explanation of the anomaly is hardly convincing. The only other published curves for the steady state fluorescence intensity against light are those of Schiau & Franck (1947). These, however, are not strictly comparable with the others because extra pure nitrogen was used, traces of oxygen were continually removed and one does not know whether the photosynthesis was inhibited or accelerated during the anaerobic period.

(3) The chlorophyll radical hypothesis

This is the hypothesis that photoxidation, damaging the plastid, leads to the production of additional chlorophyll radicals which are photochemically inactive.

We have presented the narcotization hypothesis at some length because it has taken a prominent place in photosynthesis theory for nearly twenty years. We must agree
with Wassink (1951) that these narcotics have no better foundation than that they make comprehensible certain fluorescent phenomena. Direct experimental evidence for their genesis and their role in metabolism is lacking. And in his latest review Franck (1960) has abandoned the concept of a natural narcotic, while retaining his explanation of the Warburg effect as due to the indirect consequence of photoxidations. The new theory is based largely on work on the after-glow of chlorophyll, discovered by Strehler & Arnold (1951) and considered to be of similar importance to fluorescence in attempts to understand the photochemistry of photosynthesis (Brugger & Franck, 1958). It is now proposed by these authors that chlorophyll \( a \) exists mostly in the ‘protected’ state—as a crystalline layer between proteins and lipoids, protected from water and solutes but capable of receiving excitation energy, either directly or from other pigments; in turn the energy is transferred to a small number of ‘unprotected’ chlorophyll molecules. These molecules combine with water at the \( \text{C}=\text{O} \) bond of the carbon atom 10 and each is supposed to have as its neighbour (adsorbed on to the same protein) a prosthetic group of the enzyme (EN) accepting \( (-\text{OH}) \) and concerned in the liberation of oxygen. This enzyme may be a cytochrome. The unprotected hydrated chlorophyll molecules are supposed to take part cyclically in the photochemical processes as in Fig. 8.

Certain inhibitors of photosynthesis, such as hydroxylamine and, less certainly \( \alpha \)-phenanthroline, give, like the narcotics, a percentage inhibition of photosynthesis and an increase of the fluorescent yield, which are not dependent on light intensity. It is now supposed that they interfere with the further reactions of the \( \text{OH–EN} \) complex (Fig. 8). This will lead to an inhibition of the formation of this complex. On receipt of excitation energy, the transfer of \( \text{H} \) from \( \text{C}_9 \) of the chlorophyll molecule to the oxidant occurs, but the rest of the molecule retains the two \( \text{OH} \) groups and the cycle is interrupted at \( (a) \). The resulting chlorophyll radical, \( \text{Chl}\{\text{OH}_\text{OH}\} \) according to Weller (1954) absorbs in longer wavelengths than chlorophyll, and will thus be a more efficient collector of excitation energy. It will not, however, normally promote chemical reactions but will dissipate the energy thermally or in fluorescence, or conserve it temporarily in the metastable state of the chlorophyll radical. Franck (1960) believes that the chlorophyll radical has, therefore, all the properties earlier ascribed to a natural narcotic; these are used to explain, for instance, the after-glow, fluorescence and induction data, the inhibition of photosynthesis after long anaerobiosis and the Warburg effect.

In applying this new concept to the Warburg effect Franck no longer assumes that oxygen replaces the normal oxidant and forms hydrogen peroxide. Relying apparently on the work of Brown & Good (1955) with oxygen isotopes (which we have seen to be inadequate), he now states categorically that ‘oxygen is not a Hill reagent’ (Brugger & Franck, 1958). The competition is now not between oxidant and oxygen for the hydrogen, but between oxidant and oxygen for the excitation energy. He assumes that impacts of oxygen with excited chlorophyll remove the excitation energy and utilize it for activating oxygen molecules; impacts of these with easily oxidizable
substances cause ‘oxidations connected with peroxide formation’. This process, called photooxidation, instead of forming a natural narcotic which blankets the chlorophyll, is supposed to damage the chloroplast, with the result that some of the protected chlorophylls come into contact with water. At high light intensities and low carbon dioxide concentration, however, when the oxidant is in low supply, the additional hydrated chlorophyll molecules formed as a result of photooxidation will lack the necessary OH-accepting enzymes. The chlorophyll radicals then produced will preferentially divert the energy from the chlorophyll which promotes chemical reactions and, in consequence, both photosynthesis and further photooxidations will be suppressed. ‘The ability of the chlorophyll radical to collect and dissipate excitation energy makes it useful in protecting the plant against photooxidation under conditions when oxidants are in low concentration.’

Fig. 8. Franck’s hypothesis of the participation of chlorophyll in photosynthesis. (After Brugger & Franck (1958), Franck (1960).)

Whether this theory will gain general acceptance and how it will be related to other concepts (e.g. see Arnon, 1959; Kandler, 1960) remains to be seen. In so far as it explains the Warburg effect, the same objections apply to it as to the narcotization hypothesis—that it is not readily tested by experiment, especially biochemically. One objection to it is the rapid and complete reversibility of the Warburg effect. On the narcotization hypothesis it was apparently assumed that for each concentration of oxygen a net amount of narcotic was present, the resultant of its formation and destruction. Nothing is known, however, of the manner in which photooxidation causes damage to the chloroplast and converts protected- into unprotected-chlorophyll molecules. The complete reversal of this process at low oxygen concentrations does not seem to be very likely.

(4) Enzyme inhibition

This involves the direct or indirect oxidation of an enzyme or enzymes of the dark reactions of photosynthesis.

Several workers have suggested that the oxygen effect might be wholly or in part due to the inactivation of an enzyme of the dark reactions, brought about by reversible oxidation or oxygenation. The first proposal of this kind came from Gaffron in 1937,
who worked with the alga *Scenedesmus*. Sufficient cyanide was used to reduce respiration and catalase activity almost to zero, while leaving the photosynthesis practically unimpaired. It was then found that the dark reactions of photosynthesis were extremely sensitive to traces of hydrogen peroxide. Gaffron equated the effect of hydrogen peroxide with that of atmospheric oxygen, and argued that an enzyme concerned in the dark reactions was put out of action by oxidation.

Subsequently Franck & French (1941) suggested that an excess of photoperoxides (produced at high light in the presence of oxygen) can oxidize certain metabolites, and that the products of this oxidation 'are capable of inhibiting photosynthesis by poisoning ''catalyst B'', the enzyme responsible for light saturation. This offers a natural explanation for the fact that the limiting influence of high oxygen vanishes at low light intensities, for at these intensities a great part of catalyst B can be inactivated without influencing the rate of photosynthesis.' Later, Franck preferred to explain the same facts in terms of the effect of a natural narcotic or additional chlorophyll radicals produced at high light intensity by photoxidation.

(a) Inhibition of the carboxylating enzyme

Tamiya & Huzisige (1949) showed that the Warburg effect was increased at low carbon dioxide concentration and argued that there was competition between oxygen and carbon dioxide for the carboxylating enzyme. They speculated that this might be a direct reversible combination of oxygen with this enzyme as with haemoglobin. As they pointed out, however, their own evidence indicates that the analogy between the carboxylation enzyme and haemoglobin breaks down, in that carbon monoxide does not combine with the carboxylating enzyme, nor does it influence the Warburg effect.

Cyanide, like oxygen, inhibits photosynthesis in *Chlorella* at high (but not at low) light intensities, and recent work shows that the cyanide inhibition is also greater the lower the carbon dioxide concentration (Whittingham, 1952; Osterlind, 1952; Gaffron, (in Rabinowitch, 1956)). It has been supposed that cyanide acts on the reactions involved in carbon dioxide fixation. Some workers (e.g. Hill & Whittingham, 1953) have suggested, however, that cyanide acts, not on the carboxylation itself, but on a reaction closely coupled with it. This was the view taken by Tamiya & Huzisige. They found that the inhibitory effects of cyanide and oxygen were not additive. The percentage inhibition caused by a given partial pressure of oxygen is diminished in the presence of cyanide (Table 2 (a)); conversely, the degree of inhibition by cyanide is decreased progressively as the oxygen concentration is raised (Table 2 (b)).

The non-additive nature of the inhibitions is perhaps seen more clearly if we compare the percentage inhibitions for each inhibitor separately with that for the inhibitors combined. Thus, HCN at $10^{-4.08}$ m/l. applied in nitrogen caused 72–76% inhibition, while 75% oxygen alone also caused 73–79% inhibition. The two inhibitors combined, at these concentrations, gave only 87–90% inhibition of the rate in nitrogen.

These results have been described (Rabinowitch, 1956) as showing that oxygen inhibition is 'competitive' with cyanide inhibition.* However, the non-additive nature

* The term 'competitive inhibition' should perhaps be reserved for competition between inhibitor and substrate for a given enzyme.
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of the inhibitions shows merely that if the inhibitors act on the same enzyme they must compete with one another for the site. Tamiya & Husige did not take this view (which seems unlikely considering the properties of oxygen and cyanide) but interpreted the results as indicating that oxygen and cyanide act upon separate reaction steps in the mechanism of dark reactions. They derived equations for the actions of the two inhibitors (which could satisfactorily reproduce the experimental results) on the hypothesis that cyanide and oxygen attacked two enzymes of successive links in a chain of dark reactions involving carboxylation. The extreme difficulty, however, of coming to a definite conclusion of this type from inhibitor experiments, is shown by the following quotation from Franck (1951b). 'We cannot accept the interpretation of the oxygen effect given by Tamiya... his observations fit his particular assumptions as well as ours (the narcotic hypothesis). The non-additivity of the two inhibitors indi-

Table 2. Cyanide and oxygen inhibition of photosynthesis
(Data of Tamiya & Huzisige, 1949)

(a) The effect of cyanide on oxygen inhibition

The percentage inhibition by oxygen at a given cyanide concentration calculated on the basis of the rate in nitrogen at the same cyanide concentration

<table>
<thead>
<tr>
<th>oxygen-atmosphere</th>
<th>concentration of HCN; mole/l.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10^{-5.66}</td>
<td>10^{-6.66}</td>
</tr>
<tr>
<td>percentage inhibition by oxygen</td>
<td>1.00</td>
<td>87 %</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>73, 79 %</td>
<td>74 %</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>62 %</td>
<td>—</td>
</tr>
</tbody>
</table>

(b) The effect of oxygen on cyanide inhibition

The percentage inhibition by cyanide at a given oxygen concentration calculated on the basis of the rate without cyanide at that oxygen concentration

<table>
<thead>
<tr>
<th>HCN mole/l</th>
<th>oxygen-atmosphere</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.50</td>
<td>0.75</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>percentage inhibition by cyanide</td>
<td>10^{-4.66}</td>
<td>72, 73, 76 %</td>
<td>60 %</td>
<td>47, 51 %</td>
<td>34 %</td>
</tr>
<tr>
<td></td>
<td>10^{-5.66}</td>
<td>26 %</td>
<td>—</td>
<td>11 %</td>
<td>—</td>
</tr>
</tbody>
</table>

cates only that oxygen and cyanide do not attack the same enzyme. If, by the addition of oxygen, the photosynthetic rate is lowered, less of the cyanide-sensitive enzyme is needed to keep up with the lower rate. Thus, a part of these enzyme molecules can be inactivated without influencing the rate.'

The enzyme now known to be concerned in carboxylation is diphosphoribulose carboxylase, which catalyses the reaction

\[
\text{Ribulose 1,5-diphosphate} + \text{CO}_2 \rightarrow 2 \text{D-glycerate-3-phosphate.}
\]

Mayaudon, Benson & Calvin (1957) have called it carboxydismutase, because its action involves an internal dismutation of the substrate. It has been isolated from green tissue and purified by three independent groups of workers. It is activated by —SH compounds, such as cysteine and glutathione, and inhibited by reagents attacking the
thiol group, such as p-chloromercuribenzoate and iodoacetamide, the inhibition being reversed by cysteine. It also requires Mg$^{++}$ or other divalent ions such as nickel or cobalt. As cyanide and oxygen resemble one another in several respects as inhibitors of photosynthesis, it is worth enquiring what is known about their effects on this enzyme.

(i) Its sensitivity to cyanide has not been directly tested in vitro. Cyanide is not, however, specific for enzymes with prosthetic groups containing heavy metals, and there is no reason why it should not inhibit this enzyme as it does some other carboxydrases (Dixon & Webb, 1958). There is some indirect evidence for an in vivo inhibition of carboxydismutase by cyanide, but it is not conclusive. Gaffron, Fager & Rosenberg (1951) showed that after pre-illumination of Scenedesmus, in the absence of oxygen and carbon dioxide, there was a rapid dark fixation of labelled carbon dioxide ($^{14}$CO$_2$), the product being largely phosphoglycerate. Cyanide strongly inhibited this fixation—a result confirmed by Tamiya et al. (1959) and again by Miyachi (1959). In 1951 Gaffron et al. thought that pre-illumination produced a carbon dioxide acceptor rather than a reducing agent, and they, therefore, argued that ‘the carboxylation of the 2-carbon acceptor is a reaction quite specifically sensitive to cyanide’. As a result of the work arising from the discoveries of Benson, Bassham & Calvin, the reactions of dark fixation following pre-illumination are now known to involve a cyclic process summarized in Fig. 9 (see Vishniac, 1955). Several enzymes are concerned in this cycle, and we cannot yet be certain that it is interrupted by cyanide at the carboxylation step (a).

(ii) The sensitivity of carboxydismutase to oxygen has not yet been tested either in vitro or in vivo, although such tests would be of crucial importance to the interpretation of the Warburg effect. In 1949 Tamiya & Huzisige remarked that the validity of their assumption, that the carboxylation enzyme becomes reversibly activated by combining with oxygen, ‘would have been easily and conclusively brought to light by testing directly the effect of oxygen upon the enzyme, using radioactive carbon as a tracer’.

When radiocarbon did become available to the Japanese workers, the test proved more difficult than had been anticipated. This is because Tamiya and his colleagues found that the reducing substance $R$, produced as a result of pre-illumination, was itself reoxidized by molecular oxygen. The Tamiya group has now established that in living green tissue:

(a) during pre-illumination without carbon dioxide a reducing substance ($R$) is produced;
(b) the steady state level of $R$ is lowered by oxygen given in the light in the absence of carbon dioxide;

(c) $R$ decays in the dark in the absence of carbon dioxide. The decay is thought to be due to oxidation by oxygen produced during pre-illumination. Decay is promoted by added oxygen or by hydrogen peroxide when catalase is inhibited;

(d) the rate of production of $R$ is not affected by oxygen. (The evidence for this statement is not very strong.)

These facts were ascertained by estimating the quantity of $R$ at any moment by placing the cells in contact with an excess of labelled carbon dioxide in the absence of oxygen and in the dark. They led Tamiya to abandon his 1949 hypothesis and to regard the Warburg effect as due to a back-reaction of $R$ with molecular oxygen (see p. 148). The evidence for this did not, however, include the establishment of an absence of oxygen inhibition of carbon fixation. A test of this would at first sight appear possible by Tamiya's methods. Unfortunately the results of such a test, if positive, could not be unequivocally interpreted.

Suppose, for instance, that the amount of carbon fixation resulting from a known amount of $R$ were diminished when the dark fixation was carried out in the presence of oxygen. This could result from some destruction of $R$ by oxygen without any effect of oxygen on the carboxylation cycle (Fig. 9), from some destruction of $R$ by oxygen combined with inhibition of the carboxylation cycle, or from absence of destruction of $R$ by oxygen (because of the competition by carbon dioxide) and inhibition of the carboxylation cycle.

On the other hand, the experiment is worth doing, for a negative effect of oxygen on carbon fixation would mean that when carbon dioxide was present oxygen would neither destroy $R$ nor inhibit carboxylation. If such a result were obtained it would presumably support Franck's current hypothesis on the nature of the Warburg effect.

(b) Inhibition of glyceraldehyde phosphate dehydrogenase or of other enzymes of the carbon cycle

The enzyme glyceraldehyde phosphate dehydrogenase (GPD), which catalyses step (b) in the carboxylation cycle (Fig. 9), has also been considered as a possible site for the inhibition of photosynthesis by oxygen (J. S. Turner, J. F. Turner, Shortman and King, 1958). This enzyme reacts with the reduced pyridine nucleotides DPN and TPN, one of which may well be the reducing substance $R$ produced during photosynthesis. It has been obtained from chloroplasts and has some of the properties of carboxydismutase (Gibbs, 1952; Arnon, 1952; Turner et al. 1958); it is activated by cysteine and inhibited by low concentrations of the thiol reagents, iodoacetate and $p$-chloromercuribenzoate. The activity of GPD in the presence of added cysteine is also inhibited by oxygen at high concentration (Turner et al. 1958). This was assumed to be due to the oxidation of sulphydryl groups of the enzyme protein, although the in vitro results could, of course, have been due to a direct effect of oxygen or iodoacetate, not on the enzyme protein, but on a soluble SH compound present in vivo and corresponding to the cysteine which formed a part of the enzyme digest.
Lippmann, in 1933, proposed that the Pasteur effect in respiration could be due to the reversible oxidation of sulphydryl groups in the dehydrogenases concerned with glycolysis. J. F. Turner & Mapson (1958) and Hatch & J. F. Turner (1959) have shown that the decreased rate of glycolysis in pea seed extracts is due to the inhibition of GPD by oxygen through the oxidation of sulphydryl groups of the enzyme (see also J. S. Turner, 1960). If GPD acted as a pace-maker in photosynthesis, which it might do at high light intensity and high carbon dioxide concentration, then its inhibition by oxygen could depress both the rate of reoxidation of reduced pyridine nucleotides and the rate of carbon fixation. This could explain the relatively high Warburg effects obtained frequently at high light and high carbon dioxide concentration. The mechanism, however, is not adequate to explain all the effects, because this enzyme would not be expected to act as a pace-maker at low carbon dioxide concentration when the Warburg effect is at its maximum.

Iodoacetic acid and iodoacetamide inhibit the respiration of green cells (Arnon, 1952; Holzer, 1954), but photosynthesis is even more sensitive to these reagents than is respiration (Kohn, 1935; Holzer, 1954; Calvin et al. 1951). Arnon, Allen & Whatley (1956) have also shown that carbon dioxide fixation in green plastids is inhibited by iodoacetamide and parachloro-mercuri-benzoate. There is some evidence that the iodoacetate inhibition is greatest at high light and high carbon dioxide (Frazer, 1954).

It is not probable that the inhibitory oxygen, cyanide and iodoacetate are specific for any one enzyme, but there is now sufficient evidence to make it likely that they inhibit photosynthesis by inhibiting dark reactions of the carboxylation cycle. Many difficulties remain. Thus Tamiya et al. (1958) and Miyachi (1959) believe that cyanide, by inhibiting catalase, promotes the decay of \( R \) by hydrogen peroxide produced when oxygen acts as a Hill reagent. Miyachi (1960) has reopened the whole question of the site of action of iodoacetate on photosynthesis. His pre-illumination experiments have led him to the view that iodoacetic acid has no effect on the carboxylation cycle, but inhibits the photochemical formation of reducing substance. This is in opposition to the results obtained by Wessels (1954) Arnon et al. (1954) and Frazer (1954), who reported that iodoacetic acid had no effect on the Hill reaction in plastids or on quinone reduction by Chlorella cells. Gibbs and Calo (1960) have made a study of the effects of iodoacetamide on the enzymes of photosynthesis. They found that at \( 5 \times 10^{-15} \text{M} \) it inhibited carbon dioxide fixation by plastids, but even at \( 5 \times 10^{-18} \text{M} \) it did not affect ATP, TPNH₂ or oxygen formation. The sole site of inhibition was, therefore, ascribed to some action on the carbon cycle. The lower concentration, however, did not inhibit either the TPN-linked glycerophosphate dehydrogenase or carboxydismutase. Its effect was apparently due to an inhibition of phospho-ribulokinase, an enzyme which is concerned only with photosynthesis. Finally, Mortimer (1960) has concluded that the iodoacetate-sensitive GPN reaction could account for only part of the assimilation by sugar beet leaves and did not appear to be utilized by soybean leaves. It is perhaps not surprising that the solution of the problem of oxygen inhibition has proved so intractable, when we find that the more extensive results with other inhibitors are still in doubt.
(5) Oxygen and the electron carriers: photosynthetic phosphorylation

This is the interference of molecular oxygen with the components of the electron carrier chain, with or without effects on photosynthetic phosphorylation.

It now seems to be generally agreed that, upon illumination, a green plastic produces substances endowed with 'assimilatory power', that is, the power of reducing carbon dioxide and forming sugars. One component of the assimilatory power is adenosine triphosphate and the other is suspected to be a reduced pyridine nucleotide. Other electron carriers undoubtedly exist in the plastid, and recent research suggests that several of them take part in photosynthesis. Cytochromes, apparently specific to the plastids, have been discovered by Hill and his colleagues, and there are also present ascorbic acid, lipoic acid, flavine nucleotides and vitamin K. It is proposed (e.g. Arnon, 1959, 1960) that, while some of the electrons move 'uphill' during the operations driven by the assimilatory power, others move along electron carrier chains 'downhill', as in the respiratory process, eventually reducing not oxygen but the oxidized chlorophyll. In this passage some of the energy is transferred to adenosine triphosphate.

The capture of the electrons (with hydrogen ions) by oxygen at some step of the chain would be a back-reaction which would lower the rate of photosynthesis by lowering the concentration of one or both components of the assimilatory power. The possibility of the reduction of oxygen by the reducing substance $R$, presumably the first link in the chain, has already been discussed. The reduction of oxygen by other autoxidizable carriers is also possible, but is more likely to require a terminal oxidase. Hill (1956) could find no such oxidase specific for cytochrome $f$ and the evidence for the presence of cytochrome oxidase itself in plastids is not convincing (Rabinowitch, 1956). Inhibition of photosynthesis could also be caused by the oxidative inactivation of an enzyme of the electron-carrier chain. It seems, therefore, that an investigation of the effects of light and darkness, under aerobic and anaerobic conditions, on the components of the electron-carrying system might aid analysis of the Warburg effect.

It is fortunate that many of the biologically important electron carriers are pigments whose absorption spectrum changes on oxidation and reduction. Their importance in respiration was made clear in the classical studies of Keilin on the cytochromes. Until recently, however, the study of such absorption changes in densely pigmented green cells remained impracticable. In the hands of Duysens, Lundegårdh and Chance, improved methods of spectrophotometry have been developed with apparatus ten to one hundred times more sensitive than that used for colourless cells (Duysens, 1957; Smith & Chance, 1958). By using intermittent light or by passing an 'exciting' light beam through a cell suspension at right angles to the weaker measured light beam, it is possible to measure difference spectra showing the change in absorption in passing from darkness to light, or from oxygen to nitrogen. These new methods, combined to a limited extent with the more conventional enzymatic studies, have provided us with information about the electron carriers operating in photosynthesis, and in a few more years the extension of this technique will undoubtedly inform us of the effect of oxygen in the light upon these systems. At present the data are conflicting.
(a) Pyridine nucleotides

It might be expected that, upon illumination, the pyridine nucleotides DPN and TPN present in green cells or plastids would show an increased reduction, and such a photochemical reduction by nucleotides added to chloroplast suspensions was first reported by Vishniac & Ochoa (1952). Confirmatory work with plastids has been reviewed by Vishniac (1955). Duysens (1955) obtained, in illuminated cells, an increase in absorption in the neighbourhood of 350 m\(\mu\). He argued that this implied the reduction of pyridine nucleotide by light. Chance & Strehler (1957) have, however, questioned this result and provided more detailed data, in which the net effect of illumination was not a reduction, as expected, but an oxidation of pyridine nucleotides. These authors pointed out that these changes were measured for the intact green cells, and that the overall oxidation, possibly induced by the oxygen liberated in photosynthesis, could have masked a small reduction in the green plastid. The most recent work in this field still further complicates the situation. Miyachi, Oh-hama & Tamiya (1960) measured, by enzyme analysis, the amounts of oxidized and reduced pyridine nucleotides in Chlorella illuminated in the presence and absence of carbon dioxide. They concluded that TPN played a more prominent part than DPN in photosynthesis. In other work, however, Oh-hama & Miyachi (1959, 1960) used the pre-illumination technique and measured \(R\), the hypothetical reducing substance, by dark fixation of carbon dioxide. Their experimental results were in many respects contrary to expectation. They could detect no parallelism between the levels of \(R\) and PN as they changed during the period of illumination. Nor could changes in the level of the nucleotides be related to the apparent destruction of \(R\) in oxygen. The effect of light upon the pyridine nucleotides appeared to lie in the main in causing the transformation of DPN to TPN. Although the authors did not discuss it in relation to Arnon’s recent work, it seems likely that this change could have been associated with photosynthetic phosphorylation in the absence of carbon dioxide. Their conclusion was (Oh-hama & Miyachi, 1960): ‘\(R\) is a reducing substance more primarily generated by the photochemical process than TPNH’. The ensuing theoretical complications, together with those introduced by similar work on the enzymic poisons (Miyachi, 1960), lead us to suggest that the results of these elegant pre-illumination experiments may require re-interpretation in view of the fact that there are two components of assimilatory power—\(R\) and ATP—both of which may be produced by pre-illumination.

(b) Cytochromes

We are not yet certain of the full cytochrome complement of the green plastids, but Hill & Scarisbrick (1951) have shown that cytochrome \(f\) and cytochrome \(b_6\) are both present in large amounts. It is unlikely that cytochrome \(c\) is also present, although Marre & Serbattaz (1958) have demonstrated the existence in plastids of a cytochrome reductase which reacted with cytochrome \(c\) in vitro. Lundegårdh (1954) was the first to show that cytochrome \(f\) is strongly oxidized in the living cell when this is illuminated and this was confirmed by Chance & Sager (1957), who state that cytochrome \(f\) responds rapidly to illumination under aerobic conditions and sluggishly under anaerobic
Oxygen in photosynthesis

conditions. There is some doubt as to the role of cytochrome $b_6$. Lundegårdh showed that it was moderately reduced in the light. Using golden leaves of higher plants Hill (1954) demonstrated that cytochrome $b_6$ is reduced in the light, while cytochrome $f$ is oxidized. Chance & Sager found that cytochrome $b$ responds most sensitively to illumination anaerobically, but very little to illumination when oxygen is present. There has up to now been no systematic study of the effect of oxygen itself on the oxidation-reduction state of these pigments in the light. Absorption spectrum changes of cytochromes similar to those seen on reaction with oxygen are apparent on illumination (Smith & Chance, 1958).

c) Other electron carriers

The data on other electron carriers are still meagre. Lundegårdh found that added ascorbic acid had no effect on the oxidation-reduction state of cytochromes $f$ and $b_6$ in Chlorella. Tamiya & Huzisige (1949) report that ascorbic acid in algal cells, both in total quantity and in the ratio of its oxidized and reduced forms, is not directly influenced by the presence of oxygen. This was so irrespective of whether the plant was illuminated or not and in what quantity the carbon dioxide was supplied. Copper sulphate (M/800) inhibited photosynthesis slightly, but brought about a remarkable shift of the redox ratio of ascorbic acid from 88% to 7% reduced. The implication is that such a change in ascorbic acid has little effect upon the photosynthetic rate.

New discoveries which may have some bearing on the Warburg effect have been reported by Dixon, Maynard & Morrow (1960), and Armstrong, Coates & Morton (1960). These workers show that, while most flavoprotein enzymes are reasonably stable in vitro, the lactic dehydrogenase of yeast and the cytochrome $c$ reductase of heart muscle are both unstable. This instability is due to a new type of autoxidation reaction. Both enzymes are rapidly inactivated in the presence of air, with or without substrate. They believe that these enzymes contain an autoxidizable protein group (probably with an SH bond) bound to a prosthetic flavine group. On the oxidation of the SH bond the protein can no longer bind the flavine and the enzyme as a whole is irreversibly inactivated, and at the same time becomes fluorescent. Such an inhibition of an enzyme system concerned with the oxidation and reduction of cytochromes could play a part in the Warburg effect, but it must be remembered that this effect is reversible and that, as yet, nothing is known about the stability of the plastid enzymes in vitro or in vivo.

d) Photosynthetic phosphorylation

Arnon (1960) has set out the evidence for the view that light causes the production in green cells of the two components of assimilatory power. The ATP is required at two steps of the carboxylation cycle—the formation of ribulose diphosphate from ribulose monophosphate and the reduction of phosphoglyceric acid to triosephosphate—this last process also requiring TPNH$_2$. He states categorically that for isolated plastids provided with all the known cofactors, there is no formation of sugars when only TPNH$_2$ (reducing power) is formed in the light: both TPNH$_2$ and ATP are required.
The formation of ATP in green cells in the light was at first described as an anaerobic process. It does not consume oxygen and hence was differentiated from the oxidative phosphorylation in mitochondria, in spite of the fact that in both processes the formation of a pyrophosphate bond is supposed to be coupled with a release of free energy which occurs when the electrons drop from higher to lower energy levels. It is, however, known that when the cofactors vitamin K and riboflavin phosphate are present in very small concentration in isolated plastids the photosynthetic phosphorylation does not occur unless oxygen is present. It is independent of oxygen concentration (at least up to 20%) when the cofactors are present at the ‘adequate catalytic concentration’—as they presumably are in the living cell.

Arnon has, therefore, postulated the existence of three types of photosynthetic phosphorylation:

(i) Anaerobic cyclic photosynthetic phosphorylation. As a working hypothesis he assumes that the electrons expelled from chlorophyll in the light themselves return to the chlorophyll along a chain of electron carriers, at some step(s) of which there is phosphorylation.

(ii) Non-cyclic photosynthetic phosphorylation. Here the electrons expelled from chlorophyll are used, with a proton from water, to form TPNH₂. The oxidized chlorophyll is now reduced by electrons passed from the (OH)¹ of water when these give rise to oxygen: here again the electron passage is at some stage(s) associated with phosphorylation.

(iii) Oxygen-catalysed photosynthetic phosphorylation.

It is now suggested that under certain conditions the electrons of the cyclic process (i) may be trapped by molecular oxygen and, with a proton, form water. Another cyclic process results (Fig. 10) and this may be the explanation of the action of molecular oxygen in sustaining the yield of phosphorylation when some of the cofactors are in short supply. The cyclic process consumes and produces no net oxygen.

Arnon provisionally concludes that process (iii) may increase the over-all rate of ATP formation when light is abundant and cofactors in short supply, but would be an energy-wasteful step when light is limiting. From our point of view this hypothesis is of interest in two respects. It suggests the possibility of oxygen modifying the concentration of adenosine triphosphate; it also means that there is a possibility of isotope exchange between oxygen and water in the illuminated plastid (Fig. 10).

According to Arnon there is now some direct evidence for this exchange. If so, the
isotope test (with $^{18}O$) for back-reactions becomes suspect. In his original work with this test Brown (1953) concluded that there was no isotope exchange for $^{16}O$ and $^{18}O$, but this view was based on his observation that in dark respiration these two isotopes were used up in proportion to their partial pressures.

VIII. DISCUSSION AND CONCLUSIONS

There is no agreement as to the cause of the inhibition of photosynthesis by oxygen. Franck’s chlorophyll radical hypothesis assumes that oxygen directly damages the photochemical mechanism. It explains many of the data on induction, fluorescence and the after-glow, and does not conflict with the kinetic data on the Warburg effect; the rapid and complete reversibility of the effect tells against it. Here a close comparison of the fluorescence yield in tissue whose photosynthesis was depressed to the same degree by oxygen, cyanide and $-SH$ reagents would be valuable. It should also be worth while to investigate the Warburg effect in diatoms and blue-green algae, for which anomalous fluorescent data have been obtained. And we still require further data on oxygen inhibition at low light intensity. The Tamiya school has recently concentrated on pre-illumination experiments and now explain the Warburg effect in terms of a back-reaction (the Mehler reaction) between oxygen and the reducing compound formed in light. There is no evidence that this reaction is of significance when natural oxidants are present. Oxygen does not appear to inhibit the Hill reaction in isolated plastids. It has its greatest effect on cells when light is in excess and carbon dioxide limiting, and under these conditions the Warburg effect is independent of temperature. A back reaction of oxygen with $R$, the first photochemical product, would be expected to have its greatest effect at low light intensity. Further, if $R$ does limit photosynthesis at high light intensity and is destroyed by a back-reaction with oxygen, low temperature should slow down the back-reaction and increase the over-all rate of photosynthesis in oxygen. On the contrary, the facts show that low temperature diminishes the rate of photosynthesis to the same extent in oxygen and nitrogen. Experiments with carbon and oxygen isotopes, which have been quoted as proving the absence of back-reactions, require repetition at higher oxygen concentration, but it is still possible that such experiments may prove invalid because of isotope exchange.

We favour the earlier hypothesis, never disproved, that oxygen acts on photosynthesis primarily by reversibly inactivating one or more of the enzymes of the carbon cycle—e.g. carboxydismutase, glyceraldehyde phosphate dehydrogenase or phosphoribulo-kinase. The available kinetic data are not at variance with this hypothesis and all three enzymes appear to possess $-SH$ groups which might be destroyed by oxygen and reactivated at low oxygen concentration by natural reductants. Further detailed study (cf. Gibbs & Calo, 1960) of the action of enzyme inhibitors (including oxygen) on such enzymes would be of value. Similar work on the enzymes and carriers of the electron chain in photosynthesis may show to what extent these (and photosynthetic phosphorylation) are implicated in the Warburg effect. Lastly, an extension of the work of the Calvin school (see Rabinowitch, 1956) on the $^{14}C$-labelling of the initial photosynthetic products is clearly desirable. If we could find whether the absolute
and relative amounts of (say) ribulose diphosphate and phosphoglyceric acid changed with increasing oxygen concentration it should help us to differentiate between the various hypotheses. Some work along these lines has been started (Whittingham & Graham, 1960). As far as can be seen at present the results support the view that oxygen does not merely mask or suppress photosynthesis, but modifies its course by operating on the carbon cycle.

**IX. SUMMARY**

An inhibition of photosynthesis by molecular oxygen (the Warburg effect) has been demonstrated for 25 species, representing most of the major groups of green plants.

The inhibition, often of the order of 30–40%, applies to both oxygen output and carbon dioxide uptake; it is not yet known whether the assimilatory quotient is modified by high oxygen concentration. The inhibition is rapidly produced and equally rapidly reversed. The inhibition is increased as the carbon dioxide concentration is lowered, and at low concentrations of carbon dioxide the Warburg effect operates over the range 2–100% oxygen. At high concentrations of carbon dioxide there is oxygen inhibition only over the range 20–100% oxygen.

The inhibition is greatest when light is saturating; it does not operate at very low light intensities but there is evidence that substantial oxygen inhibition occurs at light intensities down to one-fifth of the saturation level.

The temperature coefficient of photosynthesis at high light is unchanged by oxygen. Cyanide and oxygen both appear to inhibit dark reactions, but their effects are not additive. The effects of oxygen are not explicable in terms of an increase in the rate of normal (mitochondrial) respiration at high oxygen concentration.

The greatest part of this article consists of a discussion of the diverse hypotheses which have been put forward in explanation of the Warburg effect. So far it has not been found possible to obtain experimental results which eliminate one or more of these hypotheses. In view of the great complexity of the photosynthetic mechanism, it is considered unlikely that oxygen is a specific inhibitor of only one reaction.

There is strong evidence in favour of the view that oxygen inhibits photosynthesis by inactivating one or more enzymes of the carbon cycle; there is also a possibility of the inhibition of an enzyme of the electron-carrier chain.

Some workers favour an explanation in terms of back-reactions which effectively reverse the photosynthetic process. These include photorespiration and photoxidation in which molecular oxygen oxidizes the assimilate. Another important back-reaction is the Mehler reaction, in which oxygen acts as a Hill reagent, being reduced by the hydrogen formed by the photolysis of water. Most of the available data tell against the concepts that massive back-reactions are responsible directly for the Warburg effect, but there may be important indirect inhibition of photosynthesis resulting from small-scale photoxidation.

Suggestions for future work are put forward and, in particular, it is felt that considerable progress may be made by the further use of oxygen and carbon isotopes and a critical examination of the mode of action of known enzyme inhibitors which depress the rate of photosynthesis.
Oxygen in photosynthesis

X. REFERENCES


KOHN, H. I. (1935). Inhibition of photosynthesis in *Chlorella pyrenoidosa* by the iodoacetyl radical.

*J. gen. Physiol.*, 19, 23-34.


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XI. ADDENDUM

We are indebted to Professor Franck and Professor Whittingham for discussion of some of these problems. It has been pointed out to us that, of the many effects which Warburg has discovered, the enhancing of photosynthesis due to illumination by one wavelength superimposed on that by a second wavelength was at one time referred to as a 'Warburg effect', but current usage in the U.S.A. is to call it the 'Emerson effect'.

With reference to Arnon's work discussed on p. 164, since our manuscript was completed he writes (Arnon, 1961*): 'Under special conditions oxygen gas can replace TPN as the electron acceptor B in cell-free systems. If, at the same time ... water is used as the electron donor ... an oxygen exchange reaction would result—oxygen would act as a catalyst (of photosynthetic phosphorylation). This would explain the catalytic effect of oxygen which we observed in our first experiments on photosynthetic phosphorylation. Molecular oxygen can also serve as the electron acceptor B in an experimentally contrived 'bacterial-type' of non-cyclic photophosphorylation by chloroplasts, when the use of water is blocked by inhibitors. There is no experimental evidence, however, that under physiological conditions molecular oxygen is a reactant in photosynthetic phosphorylation by chloroplasts.'

* Reference in Section X.