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PROBLEMS
IN
PHOTOSYNTHESIS

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PROBLEMS IN PHOTOSYNTHESIS

By

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Dedicated to
the Founder of Enzyme Chemistry and Cell Physiology

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Director of the Max Planck Institute for
Cell Physiology, Berlin-Dahlem

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Preface

Civilized man, long having abandoned his worship of fire and sun, is scarcely further advanced than his ancestors, since the fundamental relation between the sun, the green plant and himself is still shrouded in mystery. Not until the time comes when he can reproduce this apparently simple process of photosynthesis in his laboratory, independent of the green plant, can Man claim to be free from the vestiges of his ancestral worship of sun and fire. When that day comes the energy awaiting his disposal is enormous.

TANG PEI-SUNG

(Green Thraldom, Allen & Unwin Ltd., London 1949)

In studying photosynthesis we have to distinguish between two main problems: energetics and chemistry. Broadly speaking, the chemical part of the process begins when the energetical part is completed. After light has exerted its photochemical action, chemical reactions set in which enable the plant to synthesize organic substance. A critical and objective review of the work done on the photochemical energetic part of photosynthesis would suggest that the work has been completed by Warburg and Burk. By contrast, the study of the chemistry of photosynthesis is still in its infancy, in spite of the many important investigations that have been carried out.

It is self-evident that in a book of this size it is not possible to refer to all the work done in the field. In making a selection I have given preference to those investigations which may be considered to-day to be classical and those which seem to make new contributions to the understanding of Nature's most important achievement. The descriptions of basic experiments are, in most instances, illustrated by numerical examples.

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W. BLADERGROEN

Basle-Binningen

Units and Symbols

γ	photosynthetic quotient
η	efficiency
λ	wave-length
ν	frequency
φ	quantum yield
$1/\varphi$	quantum requirement
$\sim$	energy-rich phosphate bond
$\text{\AA}$	Ångström unit = 10^{-8} cm
cal	= 4.186×10^7 erg
Chl	chlorophyll
Chl*	excited chlorophyll
$(\text{ChlCO}_2)^*$ or CO_2^*	photolyte of photosynthesis
G	free energy (ΔG = free energy change)
h	Planck's constant = 6.626×10^{-27} erg-sec
i	absorbed light intensity in μl quanta/min
i_0	incident light intensity in μl quanta/min
N	Avogadro's number = 6.02×10^{23}
<hr/>	
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ph	inorganic phosphate
A—S—H	coenzyme A
DPN ⁺ , DPNH	diphosphopyridine nucleotide and its reduced form
TPN ⁺ , TPNH	triphosphopyridine nucleotide and its reduced form
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
$\begin{array}{c} \text{S} \\ \diagup \quad \diagdown \\ \text{L} \quad \quad \\ \diagdown \quad \diagup \\ \text{S} \end{array}$	lipoic acid
TPP ⁺	thiamine diphosphate
TA	transaldolase
TK	transketolase

$$1 \mu\text{mole} = 22.4 \mu\text{l} \quad 1 \mu\text{l} = 0.045 \mu\text{mole}$$

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PROBLEMS
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CHAPTER 1

Introductory Remarks

§ 1 The Over-all Reaction

The Dutch physician and chemist Ingen-Housz (22) was the first to determine in 1772 that the production of oxygen ("vital air") by the green plant under the influence of light is dependent upon the green parts of the plant. Priestley (30) and van Barneveld (2) had made similar observations independently at about the same time. Other earlier investigators, whose writings were, however, less precise, were Bonnet and Hales. The name chlorophyll was given to the green substance of the leaves by Pelletier and Caventou (29) in 1818. De Saussure (33) in 1804 depicted the over-all reaction of O_2 production as follows:

He found that the ratio of the CO_2 consumed and the O_2 produced was equal to 1.

To-day this type of CO_2 -assimilation is called photosynthesis, as there are many other types of CO_2 -assimilation without the absorption of radiation energy. Sachs (31) showed in 1868 that chlorophyll is indispensable for photosynthesis. In 1881 Engelmann (11) succeeded in isolating the chlorophyll-containing particles—the chloroplasts—from various plants. He was able to show that these isolated chloroplasts develop O_2 when illuminated (see § 48).

The chemical nature of chlorophyll remained obscure until the fundamental work of Willstätter and Stoll (47) was published. Conant (6, 7) and especially H. Fischer (12) largely contributed to our present knowledge of the chlorophyll molecule.

To-day the reaction equation of photosynthesis is written as follows:

The reaction is endothermic. The product (CH_2O) indicates a non-defined primary substance of the carbohydrate group. Production of O_2 and fixation of CO_2 are characteristic of the photosynthetic reaction. For every molecule CO_2 reduced one molecule O_2 is produced. The ratio $\gamma = CO_2/O_2$ is called the assimilatory or photosynthetic quotient. Theoretically it equals 1. In reality this need not be the case, as CO_2 can be partly reduced without

the formation of carbohydrates, resulting in an assimilatory quotient greater than 1. The quotient may also be smaller than 1, e.g., growing *Chlorella* reduces the nitrate of the culture medium, resulting in a greater O_2 -production:

If the quantity of CO_2 reduced is indicated by x_{CO_2} and the quantity of O_2 produced by x_{O_2} , the general formula for the assimilatory quotient is:

$$\gamma = \frac{x_{CO_2}}{x_{O_2}} \quad (1)$$

Uptake of gas is regarded as negative and removal of gas as positive, so that x_{CO_2} and consequently γ are always negative. The quantities x_{CO_2} and x_{O_2} are expressed in μ mole or, more usually, in μ l whereby 1 μ mole = 22.4 μ l.

The reversal of the photosynthetic over-all reaction is the over-all combustion reaction of glucose (respiration):

In respiration the ratio of the CO_2 produced to the O_2 consumed is called the respiratory quotient. According to the combustion reaction given above, it equals 1. In § 54 we shall see whether or not it is correct to consider photosynthesis as the reversal of respiration, not only with respect to the over-all reactions, but also with regard to the different intermediate steps in the two processes:

Photosynthesis is composed of two distinct types of process: a photochemical process and a series of dark reactions which were originally called the Blackman reaction. The photochemical reaction or light reaction is either the photolysis of water or the photolysis of CO_2 . The dark reactions are far more complex than Blackman (5) presumed.

When green cells of *Chlorella* at constant temperature and in the presence of sufficient CO_2 are illuminated with various light intensities i , we obtain various rates v of photosynthesis. Figure 1 shows an assimilation curve obtained by plotting v against i (44, 45, 48). At low intensities the curve is nearly rectilinear and dv/di is constant. As i increases, dv/di decreases and finally becomes zero. Blackman found that the rate of photosynthesis at low intensities is independent of temperature; at higher intensities, however, it increases when the temperature is increased. It thus seems that various processes influence the rate of photosynthesis, depending on the light intensity. One of these processes is of a photochemical nature, dv/di being constant. As this process is independent of temperature, $dv/dT = 0$. The other process—the Blackman reaction—is not influenced by illumination. It is an

ordinary, chemical, temperature-dependent reaction, whereby $dv/di = 0$ and $dv/dT > 0$.

From what has been said it follows that photosynthesis comprises two great problems: energetics and chemistry. The chemical part of photosynthesis begins where the energetical part finishes. When light has exerted its photochemical action—photolysis of water or of CO_2 —chemical reactions

Fig. 1. An assimilation curve. Rates of photosynthesis v plotted against light intensities i .

set in which enable the plant to build up organic substances. As far as the energetical part of photosynthesis is concerned, a critical and impartial survey of all the work done—in which the problem of the quantum requirement occupies the most important place—shows that Warburg and Burk found the solution. The chemical part of photosynthesis, however, still remains largely obscure despite important experimental findings.

§ 2. Chlorophylls and Carotenoids

Today seven types of chlorophyll may be distinguished: the chlorophylls a, b, c, d and e, bacteriochlorophyll and bacterioviridin.* Chlorophyll a, which is blue-green, is the most abundant and occurs in all autotrophic organisms except pigment-containing bacteria. Chlorophyll b is yellow-green and is found in higher plants and certain algae. The other chlorophylls only occur, together with chlorophyll a, in algae. Bacteriochlorophyll and bacterioviridin are pigments of certain bacteria.

As can be seen from Figure 2, chlorophyll is a magnesium complex of a porphyrin. Porphyrins are composed of four pyrrole nuclei which together form a ring system. Chlorophylls a and b have, at the C_7 atom, a carboxyl group which is esterified with the high-molecular alcohol phytol. They differ with respect to the C_3 atom. This is linked with a methyl group in chlorophyll a and with an aldehyde group in chlorophyll b. In earlier work

* Literature on plant pigments: 6, 7, 12, 35, 37, 47.

Fig. 2. Constitution of chlorophyll. R $\div$ CH₃ in chlorophyll a; R $\div$ CHO in chlorophyll b.

Fig. 3. β -Carotene consisting of eight isoprene groups, four of which form two ionone rings.

Fig. 4. Structural relationship between chlorophyll and carotene. Left: chlorophyll without Mg atom, N atoms and side chains. Right: β -carotene without ionone rings (Szent-Györgyi).

Conant (6, 7) believed the isocyclic ring with the C₉ and C₁₀ atoms to be of importance in photosynthesis.

The yellow carotenoids occur in all plants together with the chlorophylls. These substances are unsaturated hydrocarbons composed of isoprene groups. Some are aliphatic molecules with open carbon chains, some are molecules containing one or two ionone rings. Of the more than 60 carotenoids known β -carotene is the most abundant. It is composed of eight isoprene groups, four of which form two ionone rings (Fig. 3).

It is surprising to see how the chlorophylls and the carotenoids differ in molecular structure. There seems to be no structural relationship whatever between the four pyrrole nuclei coordinated by a metal in the chlorophyll molecule and the isoprene chains of the carotenoids. However, Szent-Györgyi (38) pointed out that the carbon chain of β -carotene—without the ionone rings—when written as a ring bears a striking resemblance to the

porphyrin structure of the chlorophylls when the Mg and N atoms as well as the side chains are omitted (Fig. 4).

There is evidence for assuming that the carotenoids have special tasks in photosynthesis. It has even been stated that they may take an active part in the primary act of the photosynthetic process. Lynch and French (26) observed that the Hill reaction is inhibited when the carotenoids are removed from the chloroplasts by extraction. After the addition of β -carotene to such chloroplasts the Hill reaction can again be demonstrated. Fujimori and Livingston (15) found that the carotenoids are as efficient as oxygen in quenching the triplet state of chlorophyll *a*. However, for the time being there is not sufficient evidence to attribute a primary role in photosynthesis to the carotenoids. The findings of Sager and Zalokar (32) support the hypothesis that the carotenoids are not essential for photosynthesis except possibly in catalytic amounts. This is in agreement with Warburg's work on the action of blue-green light and the role of the photosynthesis enzyme to be discussed in Section B of Chapter 3. Nishimura and Takamatsu (28) found that β -carotene in various leaves—like chlorophyll—is bound to protein, an important discovery which also confirmed Warburg's views on the photosynthesis enzyme (see § 31). The group of carotenoids also includes xanthophyll and related compounds.

The light absorption of chlorophyll in the visible spectrum is relatively great. The absorption curve of a solution of chlorophyll *a* in ethyl ether shows one maximum in the red and another in the violet. In organic solvents the pigment gives a characteristic fluorescence spectrum where—as in the absorption spectrum—the position of the bands depends upon the kind of solvent used. The addition of quinone, oxygen and some other substances inhibits fluorescence to varying degrees, whereas removal of the Mg atom has practically no influence. *In vivo*, chlorophyll shows very little fluorescence (see § 11) and colloidal solutions of chlorophyll do not show fluorescence at all (23). Warburg (46) pointed out that extracts of chlorophyll in organic solvents should not be identified with the functioning chlorophyll in the chloroplasts. In the study of photosynthesis distinction has to be made between “dead” and “living” chlorophyll (see § 59).

The carotenoids absorb light in the violet, showing two or three bands, the positions of which vary according to the solvent used.

TABLE 1
Conversion factors for chlorophyll concentrations

	μ mole	mg	μ l
1 μ mole =	—	0.89	22.4
1 mg =	1.13	—	25.2
1 μ l =	0.045	0.04	—

It is customary to express chlorophyll concentrations in μmole , μl or mg . Table 1 shows the conversion factors for these units. The molecular weight of chlorophyll a ($\text{C}_{55}\text{H}_{72}\text{O}_5\text{N}_4\text{Mg}$) is 892.

§ 3 The Structure of the Chloroplast

The chloroplasts containing the chlorophylls and carotenoids vary in size and number from plant to plant.* In algae each cell may contain just one chloroplast, whereas in higher plants there may be several hundred per cell. The size of the longest axis varies from 2 to 10 μ . The chloroplasts are the site of the photochemical part of photosynthetic activity. In any case, there is no evidence to the contrary or no reason to assume that cytoplasm may also play an active part. Nevertheless, Hill and Whittingham (20), citing older work by Frenkel (13), assume that CO_2 -fixation in photosynthesis may be associated with cytoplasm. It is difficult to accept such a hypothesis since an experimentally well-established stoichiometric relationship exists between CO_2 and chlorophyll (see § 59).

The chloroplasts possess a membrane about 50 to 100 Å thick (39). The chlorophyll is localized in fairly small units—the grana—which are embedded in the stroma. Grana and stroma are composed of proteins and lipoids or lipoproteids. It has been observed that the grana have a lamellae structure probably surrounded by a membrane. Studies by Steinmann (36) with the electron microscope show that the structure of intact chloroplasts of various algae is lamellated throughout (see § 51). In such chloroplasts (e.g., *Spirogyra*) the lamellae are present in bundles between which thin layers of stroma occur (40, 41). From this it follows that distinction has to be made between two groups of chloroplasts. In the granulated chloroplasts, prevalent in higher plants, the lamellated structure is limited to the grana. Thomas *et al.* (42) propose the name chloroplast be given to this group only and that the lamellated chloroplasts be simply called grana. The monolayer state of chlorophyll in living chloroplasts has also been proposed by Trurnit and Colmano (43).

According to Frey-Wyssling (14), the chlorophyll-containing lamellae are embedded in lipid layers which are situated between protein-water layers. Hubert (21) and Wolken and Schwartz (49) have observed similar layer structures. Figure 5 shows the geometry of the chloroplast as described by Wolken and Schwartz. Baas Becking and Hanson (1) arrived at the conclusion that four chlorophyll molecules form a tetrad in such a way that the isocyclic rings are turned towards each other. It follows from Figure 5 that there is space for one carotenoid molecule per three chlorophyll molecules. This represents a weight ratio chlorophyll/carotenoids of 5 to 1. However, the spaces between the chlorophyll molecules ($15 \text{ Å} \times 15 \text{ Å}$) allow enough room for four carotenoid molecules, the diameter of which is only 6 to 7 Å. The observations, analyses and calculations of Wolken and Schwartz concern

* For more detailed information on chloroplast structure see Granick (16), Mühlethaler (27), Wolken and Schwartz (49).

Fig. 5. The structure of the chloroplast (Volken and Schwertz, *J. Gen. Physiol.*).

the algae *Euglena gracilis* and *Poterochromonas stipitata*. The figures obtained are not valid for other plants. According to Hill and Whittingham (20), the molecular ratio chlorophyll/carotenoids in higher plants may be as high as 5 to 1 (weight ratio: 8 to 1).

Stoll *et al.* (37) found that chlorophyll is bound to protein. This may be the reason for the pigment's highly specific activity. The molecular weight of the chlorophyllprotein, which has been called chloroplastin,* is about 5×10^6 . As the molecular weight of chlorophyll is about 900, the stoichiometric ratio between the protein component and the chlorophyll is 5000, if one molecule of chloroplastin contains only one molecule of chlorophyll. However, chloroplasts contain about 56% protein and 7 to 8% chlorophyll: it may therefore be assumed that one molecule of chloroplastin contains nearly 700 chlorophyll molecules. Taking the chloroplastin molecule to be a globular protein, Frey-Wyssling (14) calculated its diameter. This was found to be 100 Å, so that such a molecule has a surface area of about 32000 Å². As the surface area of a porphyrin head is 225 Å² (see Fig. 5), only 140 chlorophyll molecules can be present on the surface of one chloroplastin particle. However, the numerous side-chains of the polypeptides may also be linked with chlorophyll molecules, so that it may well be that one chloroplastin particle contains as many as 700 chlorophyll molecules. If only part of the protein in the chloroplast is used for the formation of chloroplastin, this number would, of course, be much higher. Though these considerations remain more or less speculative and exact figures are still lacking, there seems

* Lubimenko (24) was the first to prepare aqueous solutions of chlorophyll. He assumed that in these solutions as well as in the leaves chlorophyll is bound to protein. Chloroplastin contains chlorophylls a and b and the carotenoids in the same proportion as in the leaves. Volken and Schwertz (50) prepared chloroplastin by extracting chloroplasts with digitonin.

to be some evidence in favour of a morphological picture of an organized chlorophyll aggregate. We shall see in Chapter 4 that some investigators consider such aggregates of great importance from a physiological point of view.

In § 2 mention was made of the findings of Nishimura and Takamatsu (28) regarding the β -carotene-proteid. This compound has a molecular weight of 5.7×10^7 and contains about 3000 β -carotene molecules per particle.

§ 4. The Cytochrome Systems in Plants

In chlorophyll-free plant material Bhagvat (3, 4) detected the presence of the cytochromes a, b and c, doubtless performing the same functions as in yeast and animal tissues. According to Hill and Scarisbrick (19, 34), the green cells contain another hematin compound, which they called cytochrome f, instead of cytochrome c. Davenport (8) found a cytochrome b_6 in chloroplasts. As yet, a cytochrome oxidase specific to cytochrome f has not been found. It is assumed that the very small quantities of cytochrome a in chloroplasts are merely due to the surrounding tissues. The etiolated yellow plastides of barley contains the cytochromes a, b_6 and f. In Table 2 the results of these investigations are compiled. It seems that in the chloroplasts chlorophyll replaces cytochrome a in 500 to 1000 times higher quantities. There is perhaps a functional relationship between the cytochromes and photosynthesis. It is further assumed (9, 10, 25) that cytochrome f is oxidized by light, so that the cytochrome system of the chloroplasts during illumination acts like the cytochrome system of the mitochondria during respiration. No oxidation of cytochrome f can take place in the dark because there is no specific cytochrome oxidase.*

TABLE 2
Cytochrome systems

	<i>Cytochromes</i>		
	a	b	c
Mitochondria (yeast)	a	b	c
Etiolated yellow plastides (barley)	a	b_6	f
Chloroplasts	—	b_6	f

Hill (18) pointed out that mitochondria and chloroplasts show some resemblance with respect to the structure-bound cytochromes. The chlorophylls could be considered to be parts of these structures. It seems as if the chloroplasts belong to the same category as the mitochondria, the a-component of the cytochrome system being replaced by the chlorophylls.

* For literature on cytochromes in higher plants, see Hartree (17).

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CHAPTER 2

Some Photochemical Considerations

§ 5 The Einstein Law

In a photochemical reaction a molecule or an atom is brought from its normal ground state to an excited state by the absorption of radiation energy. In 1911 Einstein, encouraged by E. Warburg, extended the quantum theory to photochemistry. According to his law of photochemical equivalence, each molecule is excited (activated) by the absorption of one quantum. Thus, one mole (N molecules) is excited by the absorption of N quanta, N being the Avogadro number ($N = 6.02 \times 10^{23}$). We may consider N quanta to be one mole quanta, just as we may say that N electrons are one mole electrons. As one mole electrons is called the electrochemical equivalent (1 faraday), it is customary to say that one mole quanta (1 einstein) is a photochemical equivalent. It must be borne in mind that the law of photochemical equivalence is not an arbitrary hypothesis but, as its name indicates, a thermodynamically well-founded law.

The energy amount of a photochemical equivalent is:

$$E = N h \nu$$

Planck's constant h has the value 6.626×10^{-27} erg-sec and ν is the frequency of the radiation expressed in sec^{-1} . It follows from the theory of radiation:

$$\lambda \nu = c$$

where c is the velocity of light (2.998×10^{10} cm-sec $^{-1}$) and λ is the wave-length in cm. Hence, we find

$$E = \frac{N h c}{\lambda}$$

or

$$E = \frac{6.02 \times 10^{23} \times 6.626 \times 10^{-27} \times 2.998 \times 10^{10}}{\lambda} \text{ erg/mole}$$

or

$$E = \frac{1.197 \times 10^8}{\lambda} \text{ erg/mole}$$

or, 1 erg being 0.239×10^{-7} cal

$$E = \frac{2.86}{\lambda} \text{ cal/mole}$$

Expressing wave-lengths in Å instead of in cm, we find

$$E = \frac{2.86 \times 10^8}{\lambda} \text{ cal/mole} \quad (2)$$

The energy values of the photochemical equivalents of various radiations in the visible spectrum are given in Table 3.

TABLE 3
Photochemical equivalents of some kinds of radiation used in photosynthesis experiments

<i>Kind of Radiation</i>	<i>Wave-length in Å</i>	<i>Photochemical Equivalent in cal/mole</i>
red	6800	42100
red	6600	43300
red	6440	44400
yellow	5780	49400
green	5460	52300
blue-green	5090	56100
blue-green	4800	59500
blue-green	4680	61000
blue	4360	65500

The translational kinetic energy of a molecule can be calculated from the equation

$$u = \frac{3kT}{2}$$

The Boltzmann constant k has the value 1.381×10^{-16} erg/degree. At 20°C the energy u amounts to 6×10^{-14} erg per molecule. When illuminated with red light, one mole absorbs about 40000 cal, i.e., 280×10^{-14} erg per molecule. This simple calculation shows that the energy of the irradiated molecule is 50 times higher than before. The new energy level corresponds to a temperature of about 14000°C . This example gives an idea of the rather important difference in energy between the ground state and the excited state of a molecule.

§ 6 Examples of Photochemical Reactions

When n molecules are excited by the absorption of q quanta, the quotient n/q is called the quantum yield

$$\varphi = \frac{n}{q} \quad (3)$$

The quantum requirement is the reciprocal value of the quantum yield

$$1/\varphi = \frac{q}{n} \quad (4)$$

According to the Einstein law, each molecule is excited by the absorption of one quantum, so that

$$\varphi = 1$$

This law is applicable only to a primary photochemical reaction. Secondary dark reactions may mask the true value of the quantum yield, as the following examples show.

In the primary reaction of the photochemical dissociation of hydriodic acid

the quantum requirement $1/\varphi = 1$. This primary photochemical reaction is immediately followed by two secondary dark reactions

and

The sum of the primary photochemical and the two dark reactions is represented by

It follows from this over-all reaction that the quantum requirement $1/\varphi = 0.5$.

The photochemical decomposition of ammonia is an example of a reaction with $1/\varphi > 1$. The primary photochemical reaction

is followed by several dark reactions, e.g.,

The sum of the photochemical and the three dark reactions is

so that the quantum requirement is $1/\varphi = 5$.

These examples show that the quantum requirement of photochemical reactions may deviate considerably from the theoretical value owing to secondary dark reactions. Therefore, when it is found that a process occurring with absorption of radiation energy has a quantum requirement which is not equal to 1, it must be assumed that dark reactions are involved. The complex of reactions must then be disentangled until the correct primary photochemical reaction with $1/\varphi = 1$ is detected. In our example of the photochemical decomposition of NH_3 the quantum requirement of the over-all reaction would have other values if the experimental conditions were

changed. We shall see later that in photosynthesis also the value of the quantum requirement of the over-all reaction depends upon the experimental conditions.

§ 7 The Energy Turnover of the Photochemical Reaction

Let us consider the endothermic reaction

which proceeds to the right when one mole of the substance A absorbs the radiation energy E . The reaction thus needs E cal/mole but, according to thermochemical measurements, only U cal/mole should be necessary because the reaction would proceed to the left evolving this amount of energy. The ratio η of U and E is called the efficiency of the photochemical reaction

$$\eta = \frac{U}{E} \quad (5)$$

As an example we may take the photochemical dissociation of hydrobromic acid

As in the photochemical dissociation of hydriodic acid, the quantum yield has the value 2. E. Warburg (58), who was the first to measure photochemical yields, irradiated HBr with ultraviolet light of wave-length 2090 Å. According to equation 2, the photochemical equivalent is 137000 cal so that 2 mole HBr use this amount of radiation energy, although only 24000 cal per 2 mole HBr are necessary for the dissociation of HBr. Thus, we find for the efficiency

$$\eta = \frac{24000}{137000} = 0.18 = 18\%$$

In the same way, E. Warburg found an efficiency of only 2.1% for the photochemical dissociation of hydriodic acid.

The ozonisation reaction of oxygen has the highest efficiency. The primary photochemical reaction is the formation of an excited molecule O_2^* . This is followed by two secondary dark reactions

The over-all reaction is represented by

The quantum yield has the value 3. E. Warburg used light of wave-length 2070 Å, corresponding to the quantum energy of 137000 cal/mole. As the

endothermic formation of ozone needs 68000 cal per 3 mole O_2 , the efficiency is

$$\eta = \frac{68000}{137000} = 50\%$$

§ 8 The Nature of the Free Radicals

Many investigators believe that free radicals have important functions in photosynthesis. Free radicals are atoms or atom groups possessing unpaired electrons in their electronic structures. Using G. N. Lewis' method of writing chemical formulae, each valency electron of an element is indicated by a dot so that the usual bars between the symbols are replaced by two dots representing an electron pair. The formula for ammonia shows that the five valency electrons of the N atom are combined with the three valency electrons of the three H atoms resulting in four electron pairs

In this formula one of the electron pairs does not serve as a link between two atoms. Both electrons of this so-called lone electron pair belong to the N atom. The formula for the ammonium ion shows the same number of electron pairs

As only four and not five electrons belong to the N atom, there is an excess of one positive elementary charge characterized by a plus sign.

Coming back to the definition of the free radical, we see that the H atom and the halogen atoms with seven valency electrons must be considered to be free radicals

the unpaired electron being represented by a bold dot.

A free radical with one unpaired electron is called monoradical, whereas a free radical with two unpaired electrons is called a biradical. Thus, the H atom and the halogen atoms are free monoradicals. They are very active substances formed when halogen acids are photochemically dissociated. The primary photochemical reaction described in § 6 can be written as follows

Other examples of free monoradicals are $HO\cdot$ and the still somewhat problematic $HO_2\cdot$

The excited state of a molecule after irradiation corresponds to the state of a free radical. The irradiation of ethylene involves the formation of a free biradical

A free biradical is always produced when a double bond between two C atoms is changed into a single bond

In § 9 we shall see that in the oxidation mechanism of nicotinic amide—the active group of DPN⁺ and TPN⁺—a monoradical is formed when the double bond C=N⁺ is changed into the single bond C—N

The tendency to form electron pairs must be ascribed to the magnetic properties of the electrons. The electron possesses a spin denoted by $+1/2$ or $-1/2$. A spinning charged body must have a magnetic momentum and the magnetic momentum of the electron is called the magneton which equals $eh/4\pi mc = 9.21 \times 10^{-10}$ gauss-cm³ (e = elementary charge, m = electron mass). According to Pauli's principle, two electrons in the same orbit of an atom must have anti-parallel spin so that their magnetic momenta neutralize each other. In the case of an electron pair there can thus be no resulting spin as $+1/2 - 1/2 = 0$ and there cannot be an excess of magnetons as $+1 - 1 = 0$.

Conditions are different in the case of free radicals. The unpaired electron of the free monoradical has a magnetic momentum which is not compensated by the anti-parallel spin of a second electron. The resulting spin is then $1/2$ and the magneton excess is 1. In the free biradical we find two unpaired electrons having parallel spin so that the resulting spin is $1/2 + 1/2 = 1$ and the magneton excess has the value 2. Owing to their magneton excess, the free radicals behave as paramagnetic substances. When brought into a strong magnetic field, the axis of the magnetic momenta in excess will be orientated in the line joining the poles. As in the electron pairs the magnetic momenta neutralize each other, chemical compounds are generally diamagnetic. When brought into a magnetic field, diamagnetic substances obtain magnetic momenta the axis of which will be orientated at right angles to the line joining the poles.

In contrast to other gases, O₂ behaves like a paramagnetic substance. It is therefore assumed that the molecule contains unpaired electrons

It may be that the high reactivity of O_2 in biological systems is due to its free radical nature.

§ 9 Migration of Energy

According to Michaelis (36,37), the reversible oxidation-reduction of organic compounds proceeds in two successive, univalent steps. Instead of the simultaneous removal or uptake of two electrons, as previously assumed, only one electron at a time is removed or taken up so that free radicals are formed as intermediates (65). This is the principle of compulsory, univalent oxidation-reduction. From many examples we may select the hydrogenation of DPN^+ or TPN^+ which is nothing but the hydrogenation of nicotinic amide

The relevant part of the nicotinic amide molecule is

The change in the electronic formula shows that an intermediate free monoradical is formed

The positive charge of the N atom is neutralized by the uptake of the first electron. The double bond $\text{N}=\text{C}$ disappears and the C atom gets an unpaired electron. After the uptake of the second electron the free monoradical is changed into the reduced nicotinic amide (DPNH or TPNH).

Transport of single electrons occurs also in semiconductors. In biological systems reacting molecules are mostly bound to proteins and it is supposed that electrons must migrate through the proteins. Such a transfer of electrons shows some analogy to semiconductivity.

Semiconductors have electrical properties intermediate between those of metals which conduct electricity very well and those of insulators which conduct electricity very poorly or not at all. Crystals of germanium, silicon, copper oxide, silver sulfide, lead sulfide etc. are important semiconductors used as transistors in modern radiotechnique.* In the *n* type semiconductor

* For further details on semiconductivity the reader is referred to the excellent paper by Shockley (47).

free electrons (excess electrons) are liberated and migrate through the crystal. Conductivity is then produced by these negative carriers of current. In the *p* type semiconductor there is an electron deficit. The deficit electrons can be considered to be positively charged "holes" which act as current carriers. In the *n* type semiconductor a current of electrons proceeds from a negative to a positive pole, whereas in the *p* type semiconductor a current of "holes" proceeds from a positive to a negative pole. Figure 6 shows the principle of a type of transistor. Three zones A, B, and C can be distinguished. A and C are *n*-conductive and B is *p*-conductive. The conducting electrons produced in B migrate through A and the resulting "holes" in B are occupied by electrons coming from C.

Fig. 6. The principle of a transistor.

Szent-Györgyi (50, 51) pointed out the possibility that proteins may have an electronic structure analogous to that of semiconductors. The theoretical considerations of Evans and Gergely (19) supported this hypothesis. Eley *et al.* (18) found semiconductivity in plasma albumin, fibrinogen and edestin. In their experiments, however, the proteins used underwent such drastic treatment that the data obtained cannot be applied to native proteins. According to Vladimirov and Konev (57), there is insufficient evidence of semiconductivity in proteins.

The statement that energy can migrate through a protein molecule is not new. It is well known that light energy can be absorbed in one part of a molecule and provoke chemical change in another part of the same molecule. This has been proved by Warburg and Negelein (59, 61) for the CO-compound of the iron oxygenase (cytochrome oxidase). With the quantum requirement equal to 1, CO is photochemically dissociated from the iron of the heme part, though the light energy is absorbed by tryptophan and tyrosine in the distant protein part of the enzyme. Bücher and Kaspers (10) found a similar behavior in the photodissociation of CO-myoglobin (see also § 25).

Besides the semiconductor hypothesis, energy transfer by resonance seems very probable. According to this hypothesis, the energy of an excited oscillator is electrostatically transferred to neighboring oscillators. Such oscillators showing resonance coupling can be atom groups in the same protein molecule or two molecules of different substances oscillating at the same frequency. The energy donors must be fluorescent at frequencies which the energy acceptor is able to absorb, or in other words, the fluorescence spectrum of the energy donor must overlap the absorption spectrum of the energy acceptor. Furthermore, donor and acceptor should be sufficiently

close together (not more than 1000 Å apart). These conditions are fulfilled in protein molecules. From a theoretical point of view, Karreman and Steele (29) investigated whether such resonance energy transfer could be possible in the respiration chain. They found that the fluorescence spectra wholly or partly overlap the absorption spectra of the next steps in the chain

so that energy transfer by resonance is highly probable.

At the beginning of this paragraph it was pointed out that, as a result of theoretical considerations, free radicals are formed as intermediates in biological processes concomitant with the transfer of single electrons. However, until the participation of unpaired electrons was experimentally established, the compulsory, univalent oxidation-reduction was still unproved. It has been stated in § 8 that an unpaired electron must be influenced by an external magnetic field. Zavoisky (69) discovered that at a definite value H of an external magnetic field a resonance phenomenon—the so-called electron spin resonance—can be observed. This type of resonance is due to the interaction between the external magnetic field and the magnetic momentum of the spinning, unpaired electron. With the aid of special spectrometers, the absorption of short wave radiation energy under the influence of an appropriate external magnetic field is recorded. At a given frequency ν the value of H is changed and at a certain value of H the curve shows a deflection from the base line indicating the electron spin resonance and thus the presence of an unpaired electron.*

This technique was first applied by Commoner *et al.* (14, 15, 16) to biological systems such as enzymatic processes, chloroplasts and living cells. The oxidation-reduction reactions of the type substrate-dehydrogenase-DPN are among the simplest biochemical processes in which unpaired electrons occur. Commoner *et al.* studied the alcohol dehydrogenase system

They found that none of the participants in the reaction show electron spin resonance at any value of H . However, when the mixture of alcohol, alcohol dehydrogenase and DPN^+ is examined, the curve shows deflection at a definite value of H , indicating electron spin resonance and thus the occurrence of unpaired electrons. The same results were obtained with mixtures of acetaldehyde, alcohol dehydrogenase and DPNH . These and further experiments on other enzyme systems have established that in oxidation-reduction reactions unpaired electrons, i.e., free radicals, occur and thus prove the correctness of Michaelis' hypothesis.

§ 10 Free Radicals as Intermediates in Photosynthesis

The work done by Uri (55) seems to confirm that free radicals occur as intermediates in photosynthesis. His experiments are based on the fact

* For literature on electron spin resonance, see Wertz (66).

that free radicals give rise to polymerization of vinyl compounds. The reaction of ferrous ions with H_2O_2 (6, 7, 20) gives us an interesting example

The HO radicals formed react further either with ferrous ions

or with H_2O_2

In the presence of sufficient vinyl compounds of the general formula $\text{CH}_2=\text{CHX}$ the reactions *a* and *b* will be suppressed as the HO radical will be used for the polymerization reaction

and

The property of free radicals of inducing polymerization thus provides an indirect method of determining these substances. Uri found that the addition of methylmethacrylate* reduces the amount of O_2 developed in photosynthesis (*Chlorella*) to about one-third. The polymer produced from methylmethacrylate had a molecular weight of about 10^5 . As the molecular weight of methylmethacrylate is 100, the polymerization degree was thus 1000. No polymerization was observed in the dark under the same experimental conditions or upon illumination of killed algae. In the absence of CO_2 no polymerization could be obtained, probably because photosynthesis proceeds too slowly. The addition of methylmethacrylate had no influence upon the O_2 production in the Hill reaction (see § 49) so that apparently no polymers are formed. This negative result was independent of the hydrogen acceptors used and it may thus be concluded that no free radicals are necessary in the Hill reaction. Uri considers this highly improbable and assumes that the free radicals produced during the Hill reaction may not be able to form long chains of polymers but may act as terminal groups of such chains. This behavior has been found for the free radical $\text{SCN}\bullet$ and for the Br atom (20, 54). There is, however, insufficient evidence to assume this.

In § 9 we discussed the work of Commoner *et al.* (14, 15) dealing with electron spin resonance in enzyme systems. These authors succeeded in observing electron spin resonance in chloroplasts. Calvin and Sogo (12) confirmed these findings so that the theoretical assumption that chlorophyll is brought into its triplet state upon illumination seems to be experimentally proven *in vivo*.** However, it must be pointed out that electron spin reso-

* $\text{CH}_2=\text{C}(\text{CH}_3)-\text{COOCH}_3$.

** Fujimori and Livingston (25) succeeded in determining *in vitro* the half-life of the triplet state of chlorophyll in organic solvents.

nance in chloroplasts is also observed in the dark, though to a lesser degree. Commoner's studies on isolated chloroplasts and also on living *Chlorella* support the view that in photosynthesis there must be a univalent electron transport and that the chloroplast acts as a semiconductor (5) (see § 55). The question arises whether the electron spin resonance observed in chloroplasts is due to enzymatic processes. However, Calvin and Sogo found that cooling to -140°C did not influence the phenomenon.

§ 11 Excited States of Molecules*

When a system absorbs light, the equivalent of the energy absorbed is not lost but has to reappear either in another form or as radiation energy. It is changed into thermal energy when the temperature of the system is increased. It is changed into chemical energy when an endothermic chemical reaction is involved, as is the case in photosynthesis. The radiation energy absorbed, however, may also be emitted again as radiation energy; this is called photoluminescence. Each photoluminescence exhibits some kind of inertia, as some time passes between the removal of the incident light stimulus and the end of the luminescent phenomenon. If this time span is shorter than 10^{-4} sec, we have fluorescence. If it is longer than 10^{-4} sec, we speak of phosphorescence.**

Absorption of radiation energy means absorption of a quantum by an electron bringing the latter on to a higher energy level, i.e., bringing it into an excited state. When this process deals with electrons of the outer shells of the atoms, vibration of the nuclei occurs; this means nothing but a change of radiation energy into thermal energy. In molecules with conjugated double bonds*** we have so-called π electrons which, not being bonded to any definite nuclei, belong to the whole molecule. When radiation energy is absorbed by π electrons, emission of radiation energy of lower frequency takes place. If the molecule contains weak bonds with a dissociation energy lower than the radiation energy absorbed (excitation energy), the weak bonds may be changed, the absorbed energy being used up. This is a chemical change and no radiation will be emitted. We may illustrate this by the benzene molecule, the bonds of which require a dissociation energy of at least 126000 cal/mole. According to equation 2, the excitation energy of light of the wave-length 2700 Å amounts to 106000 cal/mole. The dissociation energy is thus higher than the excitation energy, and fluorescence, but

* For literature on the excited states of molecules, see Laidler (34), Pringsheim (39), Reid (40).

** This differentiation according to Förster (21), though arbitrary, is less complicated than the definitions given by Perrin (38) and by Lewis and Kasha (30, 35). See also page 25.

*** Conjugated double bonds are written as

Organic molecules showing photoluminescence may belong to different categories but they have such conjugated double bonds in common.

no chemical reaction, will occur. In the pyridine molecule, however, the aromatic C—N bonds are much weaker than the C—C bonds of the benzene molecule so that photochemical dissociation, but no fluorescence, has been observed (21).

Generally speaking, we have fluorescence when a molecule absorbs radiation energy without using it. As Szent-Györgyi (52) pointed out, fluorescence may not have any biological significance. The biological role of molecules is not to emit but to transmit radiation energy. As a matter of fact, biologically active molecules should exhibit very little or no fluorescence as some of the energy absorbed may be lost. Chlorophyll, which we must consider to be the most important energy transmitter, shows very little fluorescence *in vivo* while its fluorescence in organic solvents is particularly impressive (see § 2).

Fig. 7. Schematic representation of photochemical excitation. G : ground state. S_1 and S_2 : singlet states. T_1 and T_2 : triplet states. A : absorbed radiation. E : energy loss. F : fluorescence. The short arrows indicate conversions with change in spin.

Upon irradiation with ultraviolet, the red dye Rhodamin B shows orange fluorescence. Figure 7 depicts schematically what happens. The normal ground state of the molecule is indicated by the line G . The arrow GS_2 represents the excitation the molecule undergoes in absorbing the radiation energy. By a change of energy—without radiation—the molecule is brought from the state S_2 into the state S_1 . Finally the arrow S_1G shows the emission of radiation, which is called fluorescence. This type of excitation whereby an electron arrives at a higher energy level and afterwards returns to the ground state is termed singlet excitation. As has been pointed out in § 8, only two electrons with antiparallel spin can be present on the same energy level. In the case of singlet excitation one of the two electrons jumps on to a higher level and afterwards returns to its companion. This process is quite correct from a quantum mechanical point of view (Fig. 8). The possibility exists, however, that the excited electron, on its way to the higher level, obtains opposite spin so that its magnetic momentum gets the same sign as that of the electron left behind. In this case, the excited electron can no longer return to the ground state, as two electrons with parallel spins cannot coexist on the same level. The excited electron must therefore remain on the higher level indicated in Figure 7 by the lines T_1 or T_2 . The electron is then trapped in the high energy level. This is called the triplet state which, due to some loss of energy, lies somewhat lower than the corresponding singlet state. The trapped electron emitting radiation can

Fig. 8. Fluorescence. *a*: electron pair in the ground state. *b* and *c*: one electron in the singlet state S_2 or S_1 . *d*: both electrons in the ground state again. *A*: absorbed radiation. *E*: energy loss. *F*: fluorescence.

Fig. 9. Phosphorescence. *a*: electron pair in the ground state. *b* and *c*: one electron with opposite spin in the triplet state T_2 or T_1 . *d*: both electrons in the ground state again (renewed change of spin). *A*: absorbed radiation. *E*: energy loss. *Ph*: phosphorescence.

Fig. 10. Multiplicity and free radicals. *S*: singlet state, resulting spin = 0, magneton excess = 0. *D*: doublet state, resulting spin = $1/2$, magneton excess = 1. *T*: triplet state, resulting spin = 1, magneton excess = 2. The two unpaired electrons are characteristic of a free biradical. The one unpaired electron is characteristic of a free monoradical. The horizontal lines on the left represent energy levels.

return via the singlet state to the ground state only by changing its spin again. Such a triplet-singlet change, corresponding to a photoluminescence of longer duration, is called phosphorescence (Fig. 9) in accordance with the differentiation proposed by Lewis and Kasha (see footnote page 23). The various states including the doublet state are represented in Figure 10. The scheme shows the presence of a monoradical in the doublet state and of a biradical in the triplet state.* Such molecules are paramagnetic owing to the excess of magnetic momenta (see § 8).

* Reid (40) points out that there is some tendency to identify the triplet state with the biradical. This may often be justified but a biradical is not necessarily a triplet and triplet states may not be biradicals.

Excited molecules have a longer life in the triplet state than in the singlet state, as the electron is quasi-trapped. The probability of the singlet-triplet change can be considerably increased by the addition of oxygen or iodides. Kasha (31) found that the singlet spectrum of dichloronaphthalene can be changed into the triplet spectrum by the addition of ethyl iodide. Decrease of temperature to far below 0°C destroys the fluorescence of dyes and of some biological substances. Phosphorescence occurs instead of fluorescence because the molecules are brought into the triplet state.

Fig. 11. The photochemical behavior of excited chlorophyll.

§ 12 The Excited State of the Chlorophyll Molecule

There are two different ways in which chlorophyll can use light energy: fluorescence and the photochemical process of photosynthesis. The excited chlorophyll molecule may, depending on the conditions prevailing, either show fluorescence or change into long-lived active forms (triplet state), in which absorbed light energy can be stored for photochemical action. The scheme in Figure 11 shows both possibilities; the excited chlorophyll molecule is represented by Chl^* . The greater the fluorescence, the smaller the photosynthetic activity (32). Wassink (62) tried to establish a correlation between fluorescence and photosynthesis. Franck (22, 23, 24, 46) considered fluorescence can be ignored so that—as has also been pointed out by Uri (54)—there is no evidence to support Wassink's views.

Quite a number of hypotheses on the change of Chl^* into long-lived forms have been established. The older assumption that Chl^* may give off H atoms

Chl_{ox} representing an oxidized or a dehydrogenated chlorophyll molecule, is not accepted today (8). It is indeed difficult to imagine how a C—H bond having a bond energy of 87000 cal/mole can be split in the excited molecule by the uptake of only 40000 cal/mole radiation energy (red part of the spectrum). This might be possible in the blue or the violet (photochemical equivalent: 70000 cal/mole) together with thermal energy, but

evidence is lacking. Weigl and Livingston (63) established that all the hydrogen-carbon bonds of the chlorophyll molecule are sufficiently stable to prevent hydrogen exchange in neutral organic solvents in the dark. However, when cells of *Chlorella* or *Scenedesmus* are illuminated in water containing $^3\text{H}_2\text{O}$, it seems that tritium is incorporated at the C_{10} atom of chlorophyll *a*. These experiments were carried out by Vishniac and Rose (56), who suggested that this light-dependent labeling of chlorophyll by tritium originating from water may be the first step in the hydrogen transfer reaction chain in photosynthesis; the adherents of the water photolysis hypothesis assume this takes place. There is insufficient evidence to believe that this labeling of chlorophyll is related to photosynthesis and it must be very difficult to ascertain that tritium is really incorporated into the chlorophyll molecule and not into impurities. It is interesting to note that a distinct transfer of tritium from chlorophyll *a* to TPN^+ should occur in the light. However, why should tritium not appear in TPNH , according to the reaction equations discussed in § 50 and § 52?

Another hypothesis is based upon tautomerism

Chl_t being a tautomeric form. According to a further hypothesis, Chl^* reacts with the solvent, i.e.

or

Instead of the solvent *A*, a normal chlorophyll molecule may react with Chl^* and give rise to a kind of photodismutation

It may also be that tautomerism and photodismutation are linked together

Many experiments have been made to prove that the excited chlorophyll molecules are changed into long-lived forms to provide the starting point of photosynthesis. Though some of the results obtained are of interest, there is insufficient evidence to support this point of view. On the one hand, a direct, photochemical action of chlorophyll with regeneration of the pigment is assumed. On the other hand, the function of chlorophyll is thought to consist in a transfer of excitation energy to appropriate acceptors, making these ready to react chemically.

Strehler and Arnold (49) found that after withdrawal of illumination green plants emit for a few minutes—and even for as long as 200 minutes (48)—a

delayed light of very low intensity. According to Arnold and Davidson (2), the emission spectrum of this chemiluminescence—observed with *Chlorella*—is identical with the fluorescence spectrum of chlorophyll a. The discoverers of this phenomenon attribute it to an enzymatically catalyzed recombination of substances produced early in photosynthesis. The production of this delayed light would thus be partly a reversal of photosynthesis, in which at least one—still unknown—reaction would be reversible. If, however, the spectrum of the delayed light is identical with the fluorescence spectrum, it must be concluded that the chemiluminescence is due to the excited chlorophyll molecule. Though Brugger and Franck (9) built up a series of hypotheses, based partly upon the delayed light phenomenon, to try to explain the photochemical part of photosynthesis, the question still arises whether the phenomenon really has anything to do with true photosynthesis.

Chlorophyll is *in vivo* a stable substance with respect to air and to light. However, after strong illumination or when photosynthesis is inhibited by poisons, the leaves become yellow or colourless due to photochemical decomposition of the pigment. This bleaching process is accelerated by excess of O_2 . According to Gaffron (26), the excited chlorophyll gives its excitation energy to an acceptor A

and the excited acceptor produced reacts with O_2

Fig. 12. Kautsky's photooxidation cycle.

Kautsky (33) proposes a cyclic process. Substance A undergoes a cyclic transformation represented in Figure 12. The excited chlorophyll activates A to A^* . A metabolite X reacts with A^* to produce a reduced form A' and an oxidized form X' . The change of A via A^* to A' means nothing but the photooxidation of X . The substance A is regenerated in the dark by oxidation of A' with O_2 . This back oxidation has some similarity with the induced respiration discovered by Burk and Warburg (see § 35).

Another hypothesis based upon the formation of the HO_2 radical, has been put forward by Weiss (64).

According to Schenck (41), a photosensitizing reaction produces a biradical Chl_R of chlorophyll

The biradical Chl_R has a pronounced affinity to O_2

This compound reacts with an acceptor A

It would lead too far to discuss the arguments put forward by Schenck to support his hypothesis. They are based upon interesting investigations on the photosensitized autoxidation of α -terpinene, found in chenopodium oil, and which, in the presence of chlorophyll, results in the production by photosynthesis of ascaridol. This is an example of a particular photochemical action of chlorophyll characteristic of the chenopodium plants (43, 44, 45).

The question arises whether Schenck's findings can be linked with normal photosynthesis. He assumes that O_2 is involved in the photolysis of water, a hydroperoxide base being formed

The hydroperoxide base is dehydrogenated by a hydrogen-transferring enzyme or a cytochrome to produce the free radical $\text{HO---Chl}_R\text{---O---O}\bullet$. This compound splits off O_2 , forming a new free radical $\text{HO---Chl}_R\bullet$, which is then split into normal chlorophyll and an HO radical

Finally, four HO radicals yield water and O_2 , the intermediately formed H_2O_2 being decomposed by catalase

For the sake of clarity the reactions involved are depicted again below

The sum of these eight reactions is

Due to the action of four quanta, one molecule O_2 and four enzyme-bound reduction equivalents are produced from two molecules of water. According to the hypothesis, four quanta are required for the production of one molecule O_2 . The result shows agreement with Warburg's findings. There is, of course, no relationship whatever between the concept of Schenck and that of Warburg, the former being pure hypothesis and the latter based upon experimental work of the highest precision.

Just as Warburg compares photosynthesis and vision with respect to the carotenoids (§ 31), Schenck (42) considers the excitation of the optical nerve as a process similar to the photochemical part of photosynthesis. He assumes that the reaction of a carotenoid biradical Car_R with O_2 , i.e., the formation of the compound $\text{Car}_R\text{-O}_2$, must be the first dark reaction in the process of vision.

In spite of the many hypotheses, there is no definite evidence that the chlorophyll molecule undergoes *chemical* changes during photosynthesis. Using suspensions of isolated spinach chloroplasts, Witt *et al.* (68) found that red light flashes of 10^{-4} sec induce changes in the absorption bands of chlorophyll; this may be considered to be the result of chemical changes in the molecule.

§ 13 Interactions Between Chlorophyll and Other Pigments

Schenck (42) considers chlorophyll has no specific action in photosynthesis, the whole process being based upon the combination of chlorophyll with specific enzyme systems. Accordingly, the carotenoids should also be able to combine with such specific enzyme systems and produce similar photolytic arrangements. The carotenoids forming free radicals would thus replace chlorophyll if necessary. Stanier *et al.* (27) assume that the important role of the carotenoids consists in protecting the cells against photodynamic damage caused by photooxidation of chlorophyll. Anderson and Fuller (1) also found carotenoids in chromatophores of the purple sulfur bacteria to be essential for the protection of chlorophyll against photooxidation. Green carotenoid-less plants may indeed produce enough O_2 to cause photodynamic death. Claes (13) described carotenoid-deficient mutants of *Chlorella* which grew only in the dark and were killed by illumination. If carotenoids, in competition with chlorophyll, interfere in the transfer and change of energy, it can be imagined that, in the absence of carotenoids, the formation of peroxide and the consecutive production of O_2 will be much more active and may even be too active. Calvin (11) supports this opinion.

Kandler and Schötz (28) observed photooxidation of chlorophyll also in higher plants (*Oenothera*) containing great amounts of carotenoids. It is of importance to note that in *Chlorella* blue light is much more active than red

light. This seems incomprehensible insofar as the light absorbed by chlorophyll should have damaging properties. In certain *Chlorella* mutants the chlorophyll content—by comparison with the content of carotenoids—is so low that only a small fraction of the blue light could have been absorbed by the chlorophyll. These mutants are, nevertheless, extremely sensitive to illumination. It must therefore be concluded that in this case the photo-oxidation is catalyzed by a substance having an absorption maximum in blue. Thus, the carotenoids and not chlorophyll must be photodynamically active. Kandler assumes that an equilibrium normally exists between both pigments guaranteeing a physiologically optimal use of absorbed radiation energy. Under abnormal conditions the photodynamic action depends either upon chlorophyll or upon the carotenoids. In extreme cases—when one of the pigments is lacking—the remaining pigment undergoes photo-oxidation. This has been shown by Claes on carotenoid-less mutants of *Chlorella* and by Stanier on mutants of purple bacteria. From these considerations it follows that photosensitization is not necessarily connected with lack of carotenoids. The strong action of blue light shows that carotenoids can also be photooxidized.

It seems that energy absorption by *various* pigments may be of importance in photosynthesis. They all transfer the energy absorbed to chlorophyll a which is the pigment responsible for photosynthesis (4). The other pigments serve to render light of various wave-lengths useful for chlorophyll a (67). Teale (53) succeeded in showing in artificial pigment systems that transfer of energy may equally take place between the molecules of chlorophyll a themselves. Duysens (17), as well as Arnold and Meek (3), reached the same conclusion.

Warburg *et al.* (60) discovered in *Chlorella* a labile oxygenase system which in the dark transfers molecular O_2 to the carotenoids. This reaction is inhibited upon illumination and it disappears after short heating at $65^\circ C$ (see § 66). It is not inhibited by $10^{-3} N$ HCN and by $10^{-3} N$ phenanthroline but it is highly dependent upon the O_2 pressures. During this enzymatic oxidation of the carotenoids no oxidation of chlorophyll can be observed.

§ 14 Some Final Remarks

In this chapter the principles of photochemistry and some modern views on electron transport and excited molecules have been discussed. It is self-evident that the scope of this book permits only a few observations and hypotheses dealing with the excitation of chlorophyll molecules to be treated. It may well be that some work of interest has been passed over in silence.

The question arises whether the many hypotheses discussed—often of rather a speculative nature notwithstanding some experimental evidence—have seriously contributed to the understanding of the photosynthetic process. The main problems of photosynthesis have always been and still are energetics and chemistry. The great amount of work done on the excita-

tion of chlorophyll does not seem to give any noteworthy contribution to the elucidation of either the energetics or the chemistry of photosynthesis. Broadly speaking, this type of work—however interesting it may be from other points of view—seems to end in a blind alley when we have photosynthesis in mind. Research done with chlorophyll *in vitro*, in particular, leads to conclusions which may by no means be of any importance to the process of photosynthesis in the living plant.

In the following chapter we shall deal with quite different trains of thought and research. In contrast to what has been discussed so far, we shall meet with classical experiments based upon classical chemistry and physics worked out with an astonishingly high degree of precision. No hypotheses will be encountered, but only facts forming the basis of a real theory. By means of this theory, the energetical aspects of photosynthesis have been satisfactorily explained and clarified.

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CHAPTER 3

The Energetics of Photosynthesis

A. ENERGY TURNOVER

§ 15 *Chlorella* and Its Cultivation

To study photosynthesis use is made of a unicellular alga *Chlorella pyrenoidosa* which can be easily cultured in a nutrient solution (Fig. 13). The algal cells are 3 to 5 μ in diameter and are resistant to mechanical and chemical influences. They can be centrifuged off from the nutrient solution and suspended in other solutions. They maintain their power of assimilation in solutions of the most varied composition and with widely differing pH values, and even in distilled water. In carbonate-bicarbonate solution, at pH 9, *Chlorella* cells remain intact for days, exhibiting constant photosynthetic activity. Organisms such as *Chlorella* offer the simplest conditions for the diffusion of metabolic gases.

Ever since *Chlorella* has been used in the study of photosynthesis it has been known that there are cells which use light efficiently and cells which do not. One of the most important conditions is the intensity of light at which the cells are cultured. If use is made of artificial light burning constantly at the same degree of brightness, the conditions are too far removed from the natural conditions under which *Chlorella* has thriven for over 500 million years. Such methods of culture are highly unphysiological, and it is astonishing that some botanists who have cultivated *Chlorella* for about 40 years still do not take this into consideration. As Warburg (24, 35, 36) pointed out, under these conditions the cells are forced to produce organic substance uninterruptedly and even to produce much more than is actually needed for normal anabolic processes. It is clear that the natural defence reaction of the cells is a very inefficient transformation of light energy into chemical energy.

When the light intensity is varied so that day and night, dusk and dawn are reproduced, the culture gives rise to cells—named A-cells by Warburg—which use light most efficiently. Figure 14 depicts the equipment used for the cultivation of A-cells in a dark room. Light is proved by a 300 watt, 220 volt metal filament lamp. The resistance is changed automatically so that the voltage increases from 50 to 220 and then decreases to 50 within a 24-hour period. Not only is the light intensity changed but also, as under natural conditions, the spectral composition of the light produced. Although it is, of course, possible to produce any variation in light intensity that may be required, Warburg prefers the method depicted in Figure 15.

Fig. 13. Cells of *Chlorella pyrenoidosa*.
Diameter: 3–5 μ .

Fig. 14. Cultivation equipment for *Chlorella* in Prof. Warburg's institute.

The distance between the lamp and the culture flasks is 25 cm. The culture solution* contains the essential trace elements (see §32) and is aerated

* The culture solution K contains, per liter of quartz-distilled water, 5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ + 2.5 g KH_2PO_4 + 2 g NaCl + 2 g KNO_3 + 0.5 g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. The pH is approximately 4.2 but rises during the culture period (30, 31). Another culture solution is the salt solution S in which the nitrate is replaced by 0.2 g NH_4Cl .

Fig. 15. Cultivation of *Chlorella* with fluctuating light intensity (Warburg *et al.*, *Zschr. Naturf.*).

with a gas mixture consisting of 5 vol % CO_2 , 30 vol % O_2 and 65 vol % argon. Each culture flask contains 250 ml *Chlorella* suspension. Young cells should be used in photosynthetic experiments as older cells, although richer in chlorophyll, contain some photochemically inactive chlorophyll. Warburg (28) distinguishes various cell types in *Chlorella*, according to the culture method used:

A-cells. These are obtained when fluctuating light is used during culture. The growth increases from 60 to 160 μl in 24 hours and to 350 μl in 48 hours. The one-day cells contain 4% chlorophyll and two-day cells contain 6% chlorophyll, calculated on a dry weight basis. A-cells contain 1.5 mole glutamic acid for each mole chlorophyll. A-cells are used for the determination of quantum requirements.

South cells. These cells are cultured by exposure to south light plus a 300 watt metal filament lamp during the day and a 200 watt metal filament lamp during the night. The growth increase is from 60 to 350 μl in 24 hours and up to 650 μl in 48 hours. The two-day cells contain 7 to 8% chlorophyll calculated on a dry weight basis. South cells contain almost exactly 1 mole glutamic acid for each mole chlorophyll.

North cells. These cells are cultured in a dark room (no daylight) by exposure to two 200 watt lamps. In 48 hours the growth increases from 60 to 1500 μl . The two-day cells contain 5 to 6% chlorophyll calculated on a dry weight basis. North cells contain 1 to 2 mole glutamic acid for each mole chlorophyll.

X-cells. These cells are obtained by strong illumination from four 200 watt lamps situated close to the culture on two sides. Growth increases in 24 hours from 60 to 200 μl . On a dry weight basis, the cells contain 1.2% chlorophyll and 3 to 5 mole glutamic acid for each mole chlorophyll. The X-cells show a much higher degree of lactic acid fermentation than the other cells (see § 62).

The optimal temperature for common *Chlorella* is 25° C. Sorokin and Myers (17) succeeded in cultivating a pure strain of *Chlorella* at an optimal temperature of 39° C. Preliminary manometric studies showed that these thermophilic *Chlorella* produced, photosynthetically, about 100 μl O_2 /hour per μl cells. Burk *et al.* (8, 9) showed that the energetic behavior of this

thermophilic *Chlorella* is identical with that of common *Chlorella*, except that the rate of photosynthesis is 10 times higher and the cell growth is 100 times more rapid (6). Each cell produces 1000 cells within 24 hours. For these reasons, thermophilic *Chlorella* may be of exceptional value in studying the technical applications of photosynthesis, e.g., air purification in submarines and space ships (6).

§ 16 Principles of Manometry

The manometric methods carefully developed by Warburg (21, 22) are the methods of choice in measuring metabolic processes involving gases (respiration, fermentations and photosynthesis). Measurements are made either with a simple manometer (Fig. 16) or with the differential manometer

Fig. 16. Warburg's simple open-arm, fixed-volume manometer.

Fig. 17. The differential manometer.

(Fig. 17). The first differential manometer was constructed in 1900 by E. Warburg (18) who used it for the measurement of ozonisation. It was improved for physiological experiments by Barcroft (2) in 1908. Figure 17 shows the differential manometer used by Warburg and Negelein in their experiments on photosynthesis. The simple manometer constructed by Haldane and Barcroft (13) in 1902 for the study of blood gases is the most suitable for physiological experiments. It has been improved by Warburg. However, manometry itself was not new, as de Saussure had already made use of it. The simple manometer is connected to the manometer vessel (Fig. 18)

holding the cell suspension at one arm only, whereas the other arm is left open to the atmosphere. In the differential manometer the assimilation vessel contains the cell suspension and the compensation vessel contains the same salt solution but without any cells.

Fig. 18. Manometer vessels.

Fig. 19. Scheme of typical Warburg manometer (Burk and Hobby, *Science*).

As shown in Figure 18, the manometer vessel has two phases: the cell suspension with the volume V_F and the gas phase with the volume V_G . The vessel connected with the manometer is suspended in a thermostat and is shaken continuously during the experiment. Figure 19, after Burk (7), shows the typical, open-arm, fixed-volume manometer made classic and universal by Warburg. In the manometer vessel O_2 is consumed and CO_2 evolved and absorbed by KOH. The change of the amount of gas in the vessel causes an experimentally observed vertical displacement of the fluid in the manometer arm open to the atmosphere when the meniscus in the closed arm is maintained at a fixed level by means of the adjustable screw and the fluid-filled sac at the base of the manometer. The narrow, even-bore capillary of the manometer arms has an area of 0.5 to 3 mm². Obviously, exact calibration of the manometer and sensitivity magnification are of the utmost importance (7, 10). The theory of the simple manometer will be discussed in § 18.

The manometer fluid may be either *iso*-capronic acid or Brodie's solution. The latter contains in each 500 ml water 23 g NaCl and 5 g sodium choleinate plus a few drops of thymol solution. As the relative specific gravities are 0.926 and 1.034, respectively, we find that

$$760 \text{ mm Hg} = \frac{760 \times 13.6}{0.926} = 11160 \text{ mm } iso\text{-capronic acid}$$

and

$$760 \text{ mm Hg} = \frac{760 \times 13.6}{1.034} = 10000 \text{ mm Brodie}$$

or

$$1 \text{ mm } iso\text{-capronic acid} = 0.068 \text{ mm Hg}$$

and

$$1 \text{ mm Brodie} = 0.076 \text{ mm Hg}$$

§ 17 Light Absorption and Its Measurement

Figure 20 depicts schematically early, experimental equipment devised by Warburg and Figure 21 shows equipment as used today. The principle is the same in both, the light passing from the source through various filters to give the desired wave-length and entering the thermostat in the horizontal plane. It is then reflected by a mirror inclined at 45° and enters the manometer vessel from below. The light is then directed on to a bolometer so that its intensity can be measured. This is depicted schematically in Figure 22. It used to be the practice to measure the intensity of the light before it entered the thermostat, as can be seen from Figure 20. However, corrections always had to be made for the difference in permeability the light encountered in its passage through the thermostat. Today, the intensity is measured after the light has passed through the thermostat. The mirror M_1 in the thermostat used to be fixed so that the light was reflected vertically upwards. Now, it

Fig. 20. Early experimental equipment devised by Warburg. *Hg*: mercury lamp. *b*, *J*₁, *J*₂: diaphragms. *L*₁, *L*₂: lenses. *F*: cuvettes filled with colored solutions. *S*₂: mirror (*S*₁: position of the mirror for light intensity measurement). *B*: bolometer with Wheatstone bridge. *T*: manometer vessel. *M*: manometer. *K*: cathetometer.

Fig. 21. Newer experimental equipment in Prof. Warburg's institute. For the sake of clarity the thermostat is not shown.

is connected to the manometer and moves synchronously with it in the horizontal plane (see Fig. 22). The cross-section of the beam of light is no longer round but has the same shape and surface area as the bottom of the manometer vessel. This provides almost complete illumination of the cell suspension. After removal of the manometer vessels, the beam of light is reflected by the mirror *M*₂ into the horizontal plane and, with the aid of a

Fig. 22. Measurement of the energy of the incident light beam. *T*: thermostat. *V*: manometer vessel. *M*₁: movable mirror. *M*₂: mirror with known reflection power. *LB*: light beam. *B*: bolometer (Warburg).

lens, concentrated on the bolometer. The permeability of this mirror-lens system is a constant, dependent on the wave-length. For example, it is 0.78 for red light, which has a wave-length of 6440 Å. If i' is the intensity measured bolometrically, then the incident intensity i_o of the cell suspension is $i'/0.78$ or $1.28 i'$.

The determination of the light intensity by means of a bolometer (Fig. 23) is based on the absorption of radiation by thin blackened platinum strips. The absorption increases the temperature and gives rise to a change in the electrical resistance which is then measured in a Wheatstone bridge. Calibration of the bolometer is effected by standard carbon filament lamps supplied by the U.S. Bureau of Standards.*

The incident intensity i_o can be measured with the bolometer, taking into consideration the corrections made for permeability. However, the cell suspension absorbs only part of the incident intensity. In early experiments complete absorption was realized by using dense cell suspensions of 10 μ l cells or more per ml. This has become unnecessary to-day. With the aid of Ulbricht spheres, it is possible to measure the fraction α of the incident intensity (i_o) absorbed by the cell suspension. For this reason, the density of the cell suspension can be considerably decreased to about 0.5 to 1 μ l cells/ml.

When dense cell suspensions are used, the cells at the bottom of the manometer vessel absorb more light than those at higher levels. As the manometer vessels have to be shaken rapidly, cells in such suspensions change continuously from the light to the dark regions, resulting in photochemical inductions and consequent loss of energy. According to Warburg (35), the passage of light through the vessels remains optimal only if the intensity is kept nearly constant. This condition can be realized only with thin suspensions which absorb only 3 to 4% of the incident light in the manometer vessels.

Ulbricht spheres are hollow vessels impermeable to light, with a diameter

* Bolometry, introduced by the American physicist S. P. Langley, was developed and adapted to photochemistry by Lummer and Kurlbaum. It was Lummer's bolometer that played a decisive role in the discovery of light quanta. The bolometer of Lummer and Kurlbaum was used to measure light energy in the experiments of E. Warburg that laid the foundations of quantitative photochemistry. It is the same bolometer that has now solved the problem of the energetics of photosynthesis (24, reference 9).

Fig. 23. The bolometer (photograph taken in Prof. Warburg's institute).

of 50 cm. The inside wall of the sphere is painted in a light colour and radiation is measured with a selenium cell connected to a multiplex galvanometer. More recently, an electron multiplier has been used instead of a selenium cell. This permits the measurement of light absorption accurate to within about 3% of the absorption value. The apparatus used by Warburg (27) is depicted in Figure 24 and the interior of the sphere is shown in Figure 25. Light is provided by a 500 watt metal filament lamp. The spectral area required is isolated with the aid of an interference disc. The manometer vessel contain-

ing the cell suspension and the control vessel containing white *Chlorella* cells are placed together in the sphere and are brought alternately into the path of the light.* As in photosynthesis experiments, both vessels are shaken, the light beam moving synchronously. Determination of the degree of absorption depends on three galvanometer recordings made when: 1) the white cells are in the beam of light; 2) the green cells are in the beam of light, and 3) the white cells are again in the beam of light. If, for example, the recordings are 100 scale units, 90 scale units and 100 scale units, the permeability

Fig. 24. The measurement of light absorption with the Ulbricht sphere shown schematically. *K*: light source. *B*: diaphragm. *W*: cuvette with water. *I*: interference disc. *L*: lens. *S*: movable mirror. *a* and *b*: manometer vessels, one containing green cells and the other white cells. *Sch*: handle for moving vessel *a* or *b* into the light beam (Warburg *et al.*, *Zschr. Naturf.*).

of the cell suspension is 90%, i.e., its absorption is 10%. The problem of light absorption by thin cell suspensions is therefore satisfactorily solved by the use of the Ulbricht sphere.

If i_o is the intensity of the incident radiation—measured bolometrically—on the surface area F of the manometer vessel over the time period t sec, the incident radiation energy E_o is

$$E_o = i_o F t$$

If the fraction α of the incident intensity i_o —measured with the Ulbricht sphere—is absorbed by the cell suspension, then we have for the absorbed radiation energy E

$$E = \alpha i_o F t$$

* So-called white *Chlorella* cells are obtained when all the pigment of normal *Chlorella* cells is extracted with methanol. The concentration of the white cells in the manometer vessel must be identical with that of the green cells. The use of the white cells results in a more uniform distribution of light within the sphere than when the control vessel contains water only.

Fig. 25. The interior of the Ulbricht sphere (photograph taken in Prof. Warburg's institute).

If we write $\alpha i_o = i$, we find†

$$E = iFt \quad (6)$$

The value of E can be expressed in cal or in μl quanta. If red light with a wave-length of 6600 Å is used, then, according to Table 1,

$$1 \text{ mole quanta} = 43300 \text{ cal}$$

† Henceforth the intensity absorbed will be denoted by i

or

$$1 \mu\text{l quanta} = \frac{43300}{22400 \times 10^3} = 0.0019 \text{ cal}$$

According to equation 2, the general equation is

$$1 \mu\text{l quanta} = \frac{12.8}{\lambda} \text{ cal}$$

§ 18 Theory of Manometry

In respiration almost as much CO_2 is produced as O_2 disappears, while in photosynthesis nearly as much CO_2 disappears as O_2 is produced. Warburg succeeded in overcoming this difficulty by adopting a modified technique (19). As already pointed out, there are two phases in the manometer vessel: the liquid cell suspension and the overlying gas phase. O_2 and CO_2 are soluble to different extents in the liquid phase, much more CO_2 being absorbed than O_2 . For this reason, it is possible to measure the resulting pressure changes with a high degree of precision.

Imagine a manometer vessel partly filled with a liquid which produces a gas. As the volume is kept constant, the pressure increases and the problem now is to calculate the amount of gas produced from the change in pressure. The temperature in the vessel is kept constant. The magnitudes are:

P = pressure before the experiment

P' = pressure after the experiment

h = pressure change

V_F = volume of the liquid phase in μl

V_G = volume of the gas phase in μl

T = absolute temperature

α = absorption coefficient of the gas produced in the liquid phase

Before the experiment the amount of gas at 0°C and 760 mm Hg in the gas phase is

$$\frac{P}{760} \frac{273}{T} V_G \mu\text{l}$$

and in the liquid phase is

$$\frac{P}{760} V_F \alpha \mu\text{l}$$

In both phases the amount of gas is

$$\frac{P}{760} \left(\frac{273}{T} V_G + V_F \alpha \right) \mu\text{l}$$

and after the experiment

$$\frac{P'}{760} \left(\frac{273}{T} V_G + V_F \alpha \right) \mu\text{l}$$

The increase in the amount of gas is therefore

$$x = \frac{P' - P}{760} \left(\frac{273}{T} V_g + V_f \alpha \right) \mu l$$

Furthermore

$$\frac{P' - P}{760} = \frac{h}{760}$$

As the manometer does not contain mercury but another fluid with a normal pressure of P_o we find that

$$x = h \left(\frac{V_g \frac{273}{T} + V_f \alpha}{P_o} \right) \mu l \quad (7)$$

It follows from equation 7 that the amount of gas produced is proportional to the change of pressure and is independent of the initial pressure P . The expression between parentheses is a constant, called vessel constant K , so that

$$x = hK \quad (8)$$

Depending on the nature of the gas (O_2 or CO_2) produced, we distinguish between vessel constants K_{O_2} and K_{CO_2} ; the absorption coefficients of these gases are $\alpha_{O_2} = 0.038$ and $\alpha_{CO_2} = 1.182$ at $10^\circ C$. We therefore obtain the equations

$$x_{O_2} = hK_{O_2}$$

$$x_{CO_2} = hK_{CO_2}$$

The vessel constants are always positive and are expressed in mm^2 . The sign of h is positive when gas is produced and negative when the gas is absorbed. The values for x can thus be either positive or negative.

In measuring either respiration or photosynthesis we have to consider two gases— O_2 and CO_2 —instead of only one. The pressure change h is equal to the sum of the changes in the partial pressures of both gases, so that

$$h = h_{O_2} + h_{CO_2} \quad (9)$$

The x values of both gases are

$$x_{O_2} = h_{O_2} K_{O_2} \quad (10)$$

$$x_{CO_2} = h_{CO_2} K_{CO_2} \quad (11)$$

Further, we know that in photosynthesis

$$\gamma = \frac{x_{CO_2}}{x_{O_2}} \quad (1)$$

From the equations 1, 9, 10 and 11 we can derive

$$x_{O_2} = h \frac{K_{O_2} K_{CO_2}}{K_{CO_2} + \gamma K_{O_2}} \quad (12)$$

and

$$x_{CO_2} = h \frac{K_{O_2} K_{CO_2}}{\frac{K_{CO_2}}{\gamma} + K_{O_2}} \quad (13)$$

The fundamental equations 12 and 13 are also valid for the differential manometer.

Numerical example:

The manometer vessel contains 270 μ l cells.

$$\begin{aligned} V_F &= 7.000 \text{ ml} \\ V_G &= 6.913 \text{ ml} \\ P_o &= 10000 \text{ mm Brodie} \\ \alpha_{O_2} &= 0.03 \\ \alpha_{CO_2} &= 0.87 \\ T &= 20^\circ \text{ C} \end{aligned}$$

Hence, according to equation 7

$$K_{O_2} = \frac{V_G \frac{273}{T} + V_F \alpha_{O_2}}{P_o} \text{ mm}^2$$

and

$$K_{CO_2} = \frac{V_G \frac{273}{T} + V_F \alpha_{CO_2}}{P_o} \text{ mm}^2$$

so that

$$K_{O_2} = \frac{6913 \times 273/293 + 7000 \times 0.03}{10000} = 0.665 \text{ mm}^2$$

and

$$K_{CO_2} = \frac{6913 \times 273/293 + 7000 \times 0.87}{10000} = 1.253 \text{ mm}^2$$

The values for x_{O_2} and x_{CO_2} are calculated from equations 12 and 13. For $\gamma = -0.8$ and $h = 60$ mm Brodie after 60 min illumination, we find

$$x_{O_2} = 60 \times \frac{0.665 \times 1.253}{1.253 - 0.8 \times 0.665} = +69.4 \mu\text{l}$$

and

$$x_{CO_2} = 60 \times \frac{0.665 \times 1.253}{-1.253/0.8 + 0.665} = -55.6 \mu\text{l}$$

§ 19 The Two-vessel Method

When the value of γ is unknown, equations 12 and 13 do not suffice for calculating x_{O_2} and x_{CO_2} , because they contain three unknowns. Measurements with two vessels, however, permit the calculation of both gas amounts (25, 26). The vessels must be different in size (Fig. 18) and therefore have different vessel constants. However, in each vessel the same amount of cells is suspended in the same amount of liquid. Over equal time periods the values of x_{O_2} and x_{CO_2} should be the same for both vessels. But since the vessel

constants are different the pressure changes are also different. For vessel I we have

$$x_{O_2} = h_{O_2}K_{O_2} \quad x_{CO_2} = h_{CO_2}K_{CO_2} \quad h = h_{O_2} + h_{CO_2}$$

and for vessel II

$$x_{O_2} = h'_{O_2}K'_{O_2} \quad x_{CO_2} = h'_{CO_2}K'_{CO_2} \quad h' = h'_{O_2} + h'_{CO_2}$$

Since h and h' are measured, we can derive the following equations from these 6 equations with their 6 unknowns

$$x_{O_2} = \frac{h'K'_{CO_2} - hK_{CO_2}}{\frac{K'_{CO_2}}{K'_{O_2}} - \frac{K_{CO_2}}{K_{O_2}}} \quad (14)$$

and

$$x_{CO_2} = \frac{h'K'_{O_2} - hK_{O_2}}{\frac{K'_{O_2}}{K'_{CO_2}} - \frac{K_{O_2}}{K_{CO_2}}} \quad (15)$$

From equations 14 and 15 the photosynthetic quotient can easily be calculated

$$\gamma = \frac{x_{CO_2}}{x_{O_2}} = \frac{K'_{CO_2}K_{CO_2}}{K'_{O_2}K_{O_2}} \frac{h'K'_{O_2} - hK_{O_2}}{hK_{CO_2} - h'K'_{CO_2}} \quad (16)$$

By writing

$$\frac{K'_{CO_2}}{\frac{K'_{CO_2}}{K'_{O_2}} - \frac{K_{CO_2}}{K_{O_2}}} = A$$

$$\frac{K_{CO_2}}{\frac{K'_{CO_2}}{K'_{O_2}} - \frac{K_{CO_2}}{K_{O_2}}} = B$$

$$\frac{K'_{O_2}}{\frac{K'_{O_2}}{K'_{CO_2}} - \frac{K_{O_2}}{K_{CO_2}}} = C$$

$$\frac{K_{O_2}}{\frac{K'_{O_2}}{K'_{CO_2}} - \frac{K_{O_2}}{K_{CO_2}}} = D$$

we can simplify equations 14 and 15 to the general equations of the two-vessel method

$$x_{O_2} = h'A - hB \quad (17)$$

$$x_{CO_2} = h'C - hD \quad (18)$$

Figure 26 shows the arrangement of the vessels in the two-vessel method. Both vessels are illuminated and each contains 7 ml *Chlorella* suspension. Each non-illuminated vessel contains 7 ml of salt solution and serves as a thermobarometer for the irradiated vessels. The four vessels are connected to manometers, the open arms of which are connected with a two liter flask placed in the same thermostat as the vessels. The four vessels are shaken

Fig. 26. The two-vessel method (photograph taken in Prof. Warburg's institute).

in the horizontal plane in the thermostat at 20°C with a frequency of 210 per min and an excursion of 1 cm. The irradiation of the vessels—with which we shall deal in § 30—must be identical for both vessels because the conditions for the two-vessel method are only met if the chemical processes in both vessels are the same. It is therefore also imperative that the bottoms of the two illuminated vessels are exactly identical in size and form, because only then can light absorption be equal. This can be tested easily by means of the Ulbricht sphere since the absorption values in both vessels should be the same.

Numerical example:

Large vessel

$$V_G = 13.75 \text{ ml} \quad V_F = 7.00 \text{ ml}$$

It follows from equation 7 that at 25°C

$$K_{\text{O}_2} = 1.286 \text{ mm}^2 \quad K_{\text{CO}_2} = 2.087 \text{ mm}^2$$

Small vessel

$$\begin{aligned} V_G &= 9.50 \text{ ml} & V_F &= 7.00 \text{ ml} \\ K'_{O_2} &= 0.897 \text{ mm}^2 & K'_{CO_2} &= 1.698 \text{ mm}^2 \end{aligned}$$

From the four vessel constants it follows that

$$\begin{aligned} A &= 6.29 \text{ mm}^2 & C &= -10.31 \text{ mm}^2 \\ B &= 7.73 \text{ mm}^2 & D &= -14.78 \text{ mm}^2 \end{aligned}$$

According to equations 17 and 18 we find

$$\begin{aligned} x_{O_2} &= 6.29 h' - 7.73 h \\ x_{CO_2} &= -10.31 h' + 14.78 h \end{aligned}$$

In an experiment the pressure changes after 60 min illumination were

$$\begin{aligned} \text{for the small vessel: } h' &= +28 \text{ mm} \\ \text{for the large vessel: } h &= +12.5 \text{ mm} \end{aligned}$$

so that

$$\begin{aligned} x_{O_2} - 6.29 \times 28 &= 7.73 \times 12.5 = 80 \mu\text{l} \\ x_{CO_2} - 10.31 \times 28 + 14.78 \times 12.5 &= -104 \mu\text{l} \end{aligned}$$

Hence the photosynthetic quotient is

$$\gamma = x_{CO_2}/x_{O_2} = -104/80 = -1.30$$

§ 20 The Use of Carbonate Buffer Solutions

Instead of aerating with air containing CO_2 it is also possible to suspend *Chlorella* in mixtures of carbonate and bicarbonate which furnish the CO_2 necessary for photosynthesis. The amount of CO_2 in such solutions can easily be calculated. The two dissociation reactions of carbonic acid are

the equilibrium constants being

$$\begin{aligned} k_I &= \frac{(H^+)(HCO_3^-)}{(H_2CO_3)} \\ k_{II} &= \frac{(H^+)(CO_3^{2-})}{(HCO_3^-)} \end{aligned}$$

or

$$K = \frac{k_I}{k_{II}} = \frac{(HCO_3^-)^2}{(CO_3^{2-})(H_2CO_3)}$$

For (H_2CO_3) we may write (CO_2) , so that

$$K = \frac{(HCO_3^-)^2}{(CO_3^{2-})(CO_2)} \quad (19)$$

When

$$(\text{HCO}_3^-) = \alpha(\text{NaHCO}_3)$$

and

$$(\text{CO}_3^{2-}) = \beta(\text{Na}_2\text{CO}_3)$$

we find from equation 19

$$K = \frac{\alpha^2(\text{NaHCO}_3)^2}{\beta(\text{Na}_2\text{CO}_3)(\text{CO}_2)}$$

or

$$\frac{(\text{NaHCO}_3)^2}{(\text{Na}_2\text{CO}_3)(\text{CO}_2)} = K \frac{\beta}{\alpha^2} = K' \quad (20)$$

Equation 20 has been verified for dilute solutions. The value of K' remains constant if the total Na concentration is kept constant. At 25° C the following empirical equation has been found

$$K' = 8739 - 1671 \log C_{\text{Na}} \quad (21)$$

The equation is valid for values between 100 and 1000 millimole of the total Na concentration C_{Na} . The constant K' is dependent upon the temperature, but may be calculated approximately from the reaction heat of bicarbonate dissociation.

In this type of experiment a mixture of the following composition is used: 85 ml 0.1 molar NaHCO_3 + 15 ml 0.1 molar Na_2CO_3 .^{*} In this solution $C_{\text{Na}} = 85 + 2 \times 15 = 115$ millimole per liter, so that, according to equation 21, the value of K' at 25° C is 5300. When $(\text{NaHCO}_3) = 0.085$ mole/liter and $(\text{Na}_2\text{CO}_3) = 0.015$ mole/liter, we obtain, according to equation 20,

$$(\text{CO}_2) = 91 \times 10^{-6} \text{ mole/liter}$$

During the experiment the equilibrium between the salts is disturbed by CO_2 assimilation. However, when the CO_2 uptake is small by comparison with the salt concentration, the changes in the concentration may be ignored because the carbonate-bicarbonate mixture acts as a buffer. This is shown by the following example. The volume of the cell suspension is 10 ml. We remove 200 μl CO_2 , i.e., $0.2/22400 = 9 \times 10^{-6}$ mole/liter. The CO_2 concentration, which was 91×10^{-6} mole/liter, will thus be decreased by 10%. The CO_2 partial pressure of the carbonate buffer used is 0.20×10^{-2} atm = 20 mm manometer fluid. The removal of 200 μl CO_2 therefore means a change of only about 2 mm manometer fluid in the CO_2 partial pressure.

The pH of the carbonate buffer can be calculated from the Henderson-Hasselbalch equation

$$\text{pH} = \text{pK}_1 + \log \frac{(\text{NaHCO}_3)}{(\text{CO}_2)} = 6.14 + \log \frac{0.085}{91 \times 10^{-6}} = 9.2$$

^{*} The mixture of 95 ml 0.2 moles NaHCO_3 + 5 ml 0.2 molar Na_2CO_3 is preferred today. Its pH is 8.8 and the CO_2 partial pressure at 20°C about 2 atm % (see § 29).

From equation 10 we find the amount of O_2 produced to be

$$x_{O_2} = h_{O_2} K_{O_2}$$

Owing to the small CO_2 partial pressure of the carbonate buffer, the gas phase contains very little CO_2 and the manometrically observed pressure change is equal to h_{O_2} , so that

$$x_{O_2} = h K_{O_2}$$

Since $V_G = 5000 \mu l$, $V_F = 10000 \mu l$ and $T = 298^\circ$, the value of K_{O_2} is 0.5 mm^2 .

When $200 \mu l$ CO_2 are reduced, $x_{O_2} = 200 \mu l$, i.e. for $K_{O_2} = 0.5$ the O_2 partial pressure has been changed by 400 mm . The change of the CO_2 partial pressure after removal of $200 \mu l$ CO_2 was only 2 mm , which is thus only 0.5% of the O_2 partial pressure. It follows from this that making $h = h_{O_2}$ is permissible.

§ 21 Energy Turnover

Warburg and Negelein (20, 30) were the first to study the question concerning which fraction of absorbed radiation energy in photosynthesis could be transformed to chemical energy. If the light energy absorbed is E and the

Fig. 27. Relationship between the absorbed radiation energy E and the accomplished work U .

chemical work performed simultaneously—the increase in total energy—is U , the quotient

$$\eta = \frac{U}{E} \quad (5)$$

becomes of special interest. In Figure 27 the abscissa shows the light energy absorbed per sec and the ordinate the chemical work performed per sec. We can see from the curve that η changes with the intensity of the radiation absorbed. The more intensive the radiation, the smaller η becomes, thus making that portion transformed into chemical energy smaller. With decreasing

intensity dU/dE , i.e., $\tan \alpha$, becomes larger and approaches a limit which can be mathematically expressed as

$$\eta_0 = \lim_{E \rightarrow 0} \frac{dU}{dE} = \text{constant} \quad (22)$$

Warburg set out to determine the highest value of η at low intensities. Figure 27 shows that U is generally not proportional to E , but is actually dependent upon E in a complicated manner. Mathematically, the relation may be expressed as

$$U = a + bE + cE^2$$

a , b and c being constants. Differentiation gives

$$\frac{dU}{dE} = b + 2cE$$

and for $E = 0$

$$\eta_0 = \lim_{E \rightarrow 0} \frac{dU}{dE} = b \quad (23)$$

The constant b is therefore the limiting value sought. It is shown graphically in Figure 27 by the tangent of the angle α_0 at which the curve rises from the zero point of the system of coordinates.

If $E = 0$, then $U = 0$, so that $a = 0$. Both b and c may be determined by two measurements made at two different intensities such as

$$\begin{aligned} U_1 &= bE_1 + cE_1^2 \\ U_2 &= bE_2 + cE_2^2 \end{aligned}$$

By eliminating c we find

$$b = \frac{U_2 E_1^2 - U_1 E_2^2}{E_2 E_1^2 - E_1 E_2^2} \quad (24)$$

The production of 1 mole O_2 corresponds to an increase of total energy of 112000 cal. Therefore, when $x_{O_2} \mu l$ O_2 develops, then

$$U = x_{O_2} \frac{112000}{22400 \times 1000} = 0.005 x_{O_2} \quad (25)$$

one mole being $22400 \times 1000 \mu l$. The value of x_{O_2} is determined manometrically. Warburg and Negelein used a differential manometer in their fundamental studies.

It is possible to calculate η and η_0 from the values of x_{O_2} and i obtained experimentally. If i is the intensity absorbed per cm^2 per sec, the radiation energy E absorbed in the time t of illumination is iFt . Since x_{O_2} is determined manometrically, the chemical energy U may be calculated from equation 25 and η determined. By two measurements of U and E at different intensities b and consequently η_0 may be calculated from equations 23 and 24.

Numerical example:

$$\begin{aligned} \text{I. } i &= 0.162 \times 10^{-4} \text{ cal-cm}^2\text{-sec}^{-1} \\ F &= 14 \text{ cm}^2 \\ t &= 600 \text{ sec} \end{aligned}$$

$$E_1 = iFt = 0.136 \text{ cal}$$

$$x_{\text{O}_2} = 15.4 \mu\text{l}$$

$$\begin{aligned} U_1 &= 0.005 \times 15.4 = 0.077 \text{ cal} \\ \eta &= U_1/E_1 = 0.57 \text{ cal/cal} \end{aligned}$$

$$\begin{aligned} \text{II. } i &= 0.327 \times 10^{-4} \text{ cal-cm}^2\text{-sec}^{-1} \\ F &= 14 \text{ cm}^2 \\ t &= 600 \text{ sec} \end{aligned}$$

$$E_2 = iFt = 0.275 \text{ cal}$$

$$x_{\text{O}_2} = 23.0 \mu\text{l}$$

$$\begin{aligned} U_2 &= 0.005 \times 23.0 = 0.115 \text{ cal} \\ \eta &= U_2/E_2 = 0.42 \text{ cal/cal} \end{aligned}$$

From these two experiments we find the values of E_1 , E_2 , U_1 and U_2 . Equation 24 can then be used to calculate b

$$b = \eta_0 = \lim_{E \rightarrow 0} \frac{dU}{dE} = 0.71 \text{ cal/cal}$$

Thus, Warburg and Negelein (30) found in their very first experiments in photosynthesis that an average of 70% of the radiation energy absorbed could be converted into chemical energy.

§ 22 Application of the Quantum Theory

In § 21 η was expressed in cal/cal. If we express U in $\mu\text{l O}_2$, then η and η_0 can be expressed in $\mu\text{l/cal}$; then according to equation 25

$$1 \mu\text{l O}_2 = 0.005 \text{ cal}$$

$$1 \mu\text{l/cal} = 0.005 \text{ cal/cal}$$

If U is expressed in mole O_2 , then η and η_0 would be expressed in mole/cal whereby

$$1 \mu\text{l/cal} = 4.5 \times 10^{-8} \text{ mole/cal}$$

and

$$1 \text{ mole/cal} = 1.1 \times 10^5 \text{ cal/cal}$$

All these relations may be easily calculated from equation 25.

In photosynthesis the value of η is not constant for any particular wavelength. Its value decreases as the wave-length decreases, as can be shown from experiments made in red, yellow and blue light.

Numerical example:

a) Red light (6600 Å)

$$E = 0.208 \text{ cal}$$

$$x_{\text{O}_2} = 24.2 \mu\text{l}$$

$$U = 24.2 \times 4.5 \times 10^{-8} = 108.9 \times 10^{-8} \text{ mole}$$

$$\eta = U/E = 108.9 \times 10^{-8}/0.208 = 5.23 \times 10^{-6} \text{ mole/cal}$$

b) Yellow light (5780 Å)

$$E = 0.245 \text{ cal}$$

$$x_{\text{O}_2} = 25.1 \text{ } \mu\text{l}$$

$$U = 25.1 \times 4.5 \times 10^{-8} = 113.0 \times 10^{-8} \text{ mole}$$

$$\eta = U/E = 113.0 \times 10^{-8} / 0.245 = 4.62 \times 10^{-6} \text{ mole/cal}$$

c) Blue light (4360 Å)

$$E = 0.280 \text{ cal}$$

$$x_{\text{O}_2} = 17.8 \text{ } \mu\text{l}$$

$$U = 17.8 \times 4.5 \times 10^{-8} = 80.1 \times 10^{-8} \text{ mole}$$

$$\eta = U/E = 80.1 \times 10^{-8} / 0.280 = 2.86 \times 10^{-6} \text{ mole/cal}$$

The dependence of η upon the wave-length is easily explained by the quantum theory. In § 6 we saw that the quantum yield is

$$\varphi = \frac{n}{q} \quad (3)$$

If the radiation energy absorbed is equal to E , then

$$E = qh\nu$$

i.e., the energy E consists of q quanta of $h\nu$. Since

$$\eta = U/E = n/E$$

we have

$$\eta = \frac{n}{qh\nu}$$

and therefore

$$\varphi = \eta h\nu$$

If η is expressed in mole/cal, the photochemical equivalent $Nh\nu$ must be used instead of quanta, N being Avogadro's number. Thus

$$\varphi = \eta Nh\nu \quad (26)$$

The values of η in the three examples mentioned, together with the values of φ and $1/\varphi$, are indicated in Table 4. From these experiments we can see

TABLE 4
Calculation of quantum yields and requirements

Wave-length in Å	η in mole/cal	$Nh\nu$ in cal	Quantum Yield φ	Quantum Requirement $1/\varphi$
6600	5.23×10^{-6}	43300	0.226	4.4
5780	4.62×10^{-6}	49400	0.228	4.4
4360	2.86×10^{-6}	65500	0.187	5.3

that to reduce a CO_2 molecule about 4 quanta are needed in red and yellow light and about 5 quanta in blue light. Numerous comparative experiments

have shown that in photosynthesis efficiency decreases as the wave-length decreases. The average values found in such experiments (31) are shown in Table 5.

TABLE 5
Relation between wave-length and efficiency

<i>Wave-length in Å</i>	<i>η in mole/cal</i>
6600 (red)	5.31×10^{-6}
5780 (yellow)	4.82×10^{-6}
4360 (blue)	3.04×10^{-6}

§ 23 Photooxidation of Chlorophyll

When a solution of chlorophyll in acetone is exposed to light, the pigment will be decomposed by oxidation (37). By adding certain acceptors the uptake of O_2 may be considerably increased and the oxidative decomposition of chlorophyll inhibited in compensation. The pigment still continues to absorb light but the added acceptor now becomes oxidized. Gaffron (12) in Warburg's institute measured the amount of O_2 per cal which would be transferred in such reactions after complete absorption of the radiation energy. Thus, the problem is actually the determination of the efficiency η by means of the equation

$$\eta = \frac{U}{E} \quad (5)$$

TABLE 6
Efficiency at various wave-lengths

<i>Wave-length in Å</i>	<i>Average Value of η in μl/cal</i>
6550	502
5780	461
5460	419
4360	339

TABLE 7
Efficiencies at various intensities

<i>Wave-length in Å</i>	<i>E in cal</i>	<i>η in μl/cal</i>
5780	0.0574	444
5780	0.121	450
4360	0.0487	302
4360	0.0964	303

The pigment used is crystalline ethyl chlorophyllide in 0.1% acetone solution prepared by the method of Willstätter and Stoll (37). The concentration of pigment is thus $C_p = 1.54 \times 10^{-3}$ mole/liter. The acceptor is allylthiourea, which is not oxidized in the dark in either the absence or presence of pigment.* The acceptor concentration is $C_a = 75 \times 10^{-3}$ mole/liter. The amount of radiation E absorbed in 300 sec is determined and the chemical action U calculated from x_{O_2} . The average values obtained from experiments at various wave-lengths are depicted in Table 6, η being expressed in $\mu\text{l}/\text{cal}$. Table 7 clearly shows that η is independent of the radiation intensity.

Numerical example:

Yellow light (5780 Å)

$E = 0.0574$ cal

$x_{O_2} = 25.5 \mu\text{l}$

$\eta = U/E = 25.5/0.0574 = 444 \mu\text{l}/\text{cal}$

According to Einstein's law, one molecule O_2 must be taken up for each quantum absorbed. This is in fact so, as may be seen from Table 8. The theoretical value of the quantum yield is

$$\varphi = \frac{1 \text{ mole } O_2}{1 \text{ mole quanta}}$$

or

$$\varphi = \frac{22400 \times 10^{-3}}{\text{photochemical equivalent}} \mu\text{l}/\text{cal}$$

TABLE 8
The validity of Einstein's law

Wave-length in Å	Energy per mole quanta in cal	η in $\mu\text{l}/\text{cal}$	φ in $\mu\text{l}/\text{cal}$	η/φ
6550	43300	502	517	0.97
5780	49400	461	456	1.01
5460	52300	419	431	0.97
4360	65500	339	344	0.99

The calculated value φ is identical with the experimentally obtained value of η , so that Einstein's law is valid for a relatively large spectral zone in the photooxidation of ethyl chlorophyllide solutions.

The concentration of the acceptor exerts a great influence on the yield. As can be seen from Table 9, the law holds good only for high values of C_a .

The results of similar experiments with hematoporphyrin and allylthiourea in acetone also obeyed Einstein's law. The most important fact arising from

* The importance of sulfur compounds, especially of allylthiourea, in photochemical processes used in photography was recognized by Sheppard (16).

TABLE 9
Dependence of yields upon acceptor concentrations
($\lambda = 5780 \text{ \AA}$, $C_p = 1.54 \times 10^{-3} \text{ mole/liter}$, 18°C)

C_a in mole/liter	η in $\mu\text{l/cal}$	η/φ
0.75×10^{-3}	48	0.10
1.88×10^{-3}	109	0.24
7.50×10^{-3}	199	0.44
25.0×10^{-3}	322	0.71
75.0×10^{-3}	392	0.86

these investigations is that, under suitable experimental conditions, a one-quantum reaction takes place in the photooxidation of chlorophyll solutions. Burk and Warburg (11) found that the photochemical yield is smaller than 1 if higher light intensities are used. When pure pyridine is used, the yield is less than 1, even when smaller intensities of light are used. The yields increase when certain bases, such as piperidine, are added. Ethyl chlorophyllide can be effectively replaced by pheophorbide in which case the quantum yield averages 0.70 independently of the light intensity.

§ 24 A Chemical Actinometer

Based on the experiments discussed in § 23, Warburg and Schocken (34) constructed a chemical actinometer. Instead of allylthiourea in acetone, they used thiourea in pyridine, the pigment being either acetyl chlorophyllide or protoporphyrin. 200 mg thiourea are dissolved in 5 ml pyridine in a 14 ml vessel. For measurements with red, yellow or blue light 2 mg crystalline ethyl chlorophyllide are added. For measurements with green or blue light 10 mg crystalline protoporphyrin are added. The gas phase contains pure O_2 . The vessel is attached to the usual manometer and is shaken in the dark at 20°C until there is constant pressure. The illumination sets in and the O_2 pressure decreases, as O_2 is now used for the oxidation of the thiourea. The pigment concentration must be high enough to ensure complete absorption of the radiation energy.

The vessel constant K_{O_2} is determined from equation 7. If Brodie's solution is used, $P_0 = 10000$. If $V_G = 9100 \mu\text{l}$, $V_F = 5000 \mu\text{l}$ and $\alpha_{\text{O}_2} = 0.092$, then, at 20°C

$$K_{\text{O}_2} = 0.893 \text{ mm}^2$$

In one investigation the decrease in pressure $h = 39 \text{ mm}$, so that

$$x_{\text{O}_2} = 39 \times 0.893 = 34.8 \mu\text{l}$$

or, calculated in mole,

$$x_{\text{O}_2} = 1.55 \mu\text{mole}$$

The light energy absorbed during the experiment was 1.64 mole quanta, so that

$$\varphi = \frac{1.55}{1.64} = 0.95$$

The average value of φ at various wave-lengths was 0.98.

The quantum yield in photosynthesis can be easily determined with this equipment when the intensity of the radiation is unknown. The uptake of O_2 is measured by means of this chemical actinometer. After replacing it by a manometer vessel of the same dimensions, the O_2 produced is measured in the

Fig. 28. Principle of the chemical actinometer.

same time period. The amount of O_2 absorbed is found at the quantum yield 1, whereas the photosynthetic O_2 is produced at the quantum yield φ . Thus

$$O_2 \text{ produced} : O_2 \text{ absorbed} = \varphi : 1$$

or

$$\varphi = \frac{O_2 \text{ produced}}{O_2 \text{ absorbed}}$$

The light absorption may be measured even more simply in a combined experiment, using the actinometer solution. Burk and Warburg (11) use two vessels: a 19 ml vessel A which can be fixed within a 120 ml vessel B (Fig. 28).^{*} Vessel A contains the cell suspension and vessel B contains 80 ml of actinometer fluid. Vessel B is attached to the manometer at C. The light is directed upwards into vessel A. The fraction absorbed in A is α , whereas the non-absorbed part $1 - \alpha$ is absorbed by the actinometer fluid, producing absorption of O_2 . The vessels are shaken as usual. Vessel A is filled with water and the amount of O_2 used in vessel B is read off. The pressure change is h mm, vessel B having absorbed the total radiation energy. If vessel A contains the cell suspension and the pressure change over the same

^{*} Later Warburg *et al.* (29) enlarged the actinometer to a total volume of 184 ml and a solution volume of 120 ml. The substances used were pure pyridine with 0.40 mg/ml pheophorbide a and b and 30 mg/ml thiourea.

time span is h' mm, vessel B has only absorbed the fraction $1 - \alpha$ of the radiation energy. Thus, we have

$$\frac{1}{1 - \alpha} = \frac{h}{h'}$$

Hence, the fraction of light absorbed by the cell suspension is

$$\alpha = 1 - \frac{h'}{h}$$

With this method neither the photochemical yield of the actinometer reaction nor the vessel constant need be known, as only changes in pressure are measured. For this reason, absorption in the actinometer vessel need not even be complete.

Numerical example (11):

Blue light (4360 Å)

In vessel A: water

Pressure change in vessel B in 10 min: $h = -20$ mm

In vessel A: 4 μ l cells in 7 ml

Pressure change in vessel B in 10 min: $h' = -13$ mm

$$\alpha = 1 - \frac{13}{20} = 0.35$$

35% of the light is absorbed by the cells.

In vessel A: 8 μ l cells in 7 ml

Pressure change in vessel B in 10 min: $h' = -8$ mm

$$\alpha = 1 - \frac{8}{20} = 0.60$$

60% of the light is absorbed by the cells.

In vessel A: 16 μ l cells in 7 ml

Pressure change in vessel B in 20 min: $h' = -7$ mm

$$\alpha = 1 - \frac{3.5}{20} = 0.825$$

82.5% of the light is absorbed by the cells.

For measurements of the intensity absorption of cell suspensions, the actinometer has been superseded by the Ulbricht sphere, as the latter works more rapidly. On the other hand, the actinometer is still of value in measuring quantum intensities because a single reading of the manometer at the end of an experiment immediately indicates the number of incident quanta during the time of illumination. In contrast to the bolometer, the spectral composition of the light need not be known. The bolometer measures i_o and the actinometer integrates the light intensities over time and number of quanta (29, 34).

§ 25 Photodissociation of Iron Carbonyl Compounds

In 1896 Haldane and Smith (14) discovered the photochemical dissociation of iron carbonyl compounds. They found that the affinity of CO to hemo-

globin could be significantly reduced by the action of light. Warburg and Negelein (23, 32) studied the photodissociation of various important iron carbonyl compounds with the help of manometry and calculated the quantum yields of these reactions. They studied the following substances:

Carbon monoxide-pyridine-hemochromogen, a compound of heme obtained by cleaving off globin from hemoglobin, with pyridine and CO. The bound iron is bivalent, as in hemoglobin. If heme iron is designated as [Fe], the photochemical cleavage can be represented as

Carbon monoxide ferrocysteine, a CO compound with ferrocysteine (a colourless complex of bivalent iron and cysteine*). The photodissociation of this substance can be depicted as

Iron pentacarbonyl, a compound produced by passing CO over iron filings at about 80° C. Light causes the following breakdown

Even though the chemical and optical characteristics of these three iron carbonyl compounds vary greatly, the photochemical cleavage reactions all have the quantum yield $\varphi = 1$.

By contrast, Warburg (23) found that in cleaving *carbon monoxide hemoglobin* the quantum yield was 0.25 (quantum requirement 4)

the quantum number 4 is related to the degree of polymerization of hemoglobin. According to Anderson (1), salts and acidity cleave 4-fold polymerized hemoglobin to bi-polymerized hemoglobin. Bücher and Negelein (5) found that increasing the salt concentration and decreasing the pH lowered the quantum requirement from 4 to 2. Myoglobin is a non-polymerized heme compound and, by cleaving its CO compound, Bücher and Negelein (5) and Bücher and Kaspers (4) found that $\varphi = 1$

Warburg (23, 32, 33) also studied the photochemistry of the CO compound of iron oxygenase (cytochrome oxidase). This could not be done with the pure enzyme because it has not yet been isolated and its constitution is still unknown. Instead of direct measurements being made, the inhibition by CO of cell respiration was investigated. Torula yeast and vinegar bacteria were used as test objects. The result of these experiments was $\varphi = 1$, so that in all probability cytochrome oxidase is not a polymerized heme compound.

* The addition of a ferric salt to a neutral, pure cysteine solution produces a deep-blue ferric cysteine complex which, after some time, becomes colorless. The cysteine reduces the ferric iron to ferrous iron and is thereby oxidized to cystine. If the solution is shaken in air, it turns blue again, producing trivalent iron. This process can be repeated until the iron oxidizes all the cysteine to cystine.

In contrast to photosynthesis, the dissociation of iron carbonyl compounds is reversible

In darkness the reaction may proceed from left to right. In light it proceeds only from left to right. Consequently, in illuminated solutions of carbon monoxide hemochromogen and carbon monoxide ferrocysteine there are three parallel reactions—two dark reactions and one photochemical reaction. In the case of carbon monoxide hemoglobin four dark reactions and one photochemical reaction can be differentiated, as the two following dark reactions also occur:

The experiments are carried out with the differential manometer, both vessels containing the solution of the iron carbonyl compound. The gas phases have a certain CO partial pressure. After equilibrium has been reached in the dark, one of the vessels is illuminated with light of known wave-length and intensity. Upon illumination, CO is produced and the pressure rises to constant value. When the light is removed, the pressure of the photochemically liberated CO falls to zero, as, in the dark, the equilibrium at the beginning of the experiment must be restored. From the speed of this return to zero can be calculated how many mole CO per min re-react. These are n mole. Since the number of mole quanta q which are absorbed per min is also known, the quantum yield can be calculated from equation 3.

Lautsch *et al.* (3, 15) made compounds of certain peptides with active groups from the hemin series. These served as oxidase models in their studies. Photochemical cleavage of the CO compounds of these synthetic substances also gave a value of 1 for the quantum requirement.

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B. THE QUANTUM REQUIREMENT

§ 26 Quantum Requirement and Efficiency

The experiments with red and yellow light discussed in § 22 show that the quantum requirement in photosynthesis is not dependent on the wave-length of the light used. Thus, it is the number of quanta and not the energy content which is of importance. This follows from equation 26

$$\varphi = \eta \times N h \nu = \frac{\text{mole O}_2}{\text{energy}} \times \frac{\text{energy}}{\text{mole quanta}} = \frac{\text{mole O}_2}{\text{mole quanta}}$$

However, the quantum requirement has been found to be higher in blue than in red or yellow. This discrepancy can be explained by the fact that red and yellow radiation is absorbed only by chlorophyll, whereas blue light is absorbed by chlorophyll and by yellow pigments. Many years ago Warburg and Negelein (61) found that chlorophyll absorbs only 70% of the total ab-

sorbed energy in blue (4360 Å). The energy absorbed by the other pigments is also photosynthetically active, although the efficiency is much less. This explains why the quantum requirement is higher in blue than in red and yellow.

The quantum requirement 4.4 (see Table 4) corresponds to an efficiency value lower than that obtained by Warburg and Negelein in their first experiments (see § 21). We find that

$$\eta = \frac{\text{chemical energy}}{\text{quantum energy}} = \frac{112000}{4.4 \times 43300} = 59\%$$

In later experiments utilizing an improved technique (see § 29), Warburg *et al.* found considerably lower values for the quantum requirement, i.e., higher values for the efficiency. In experiments lasting several hours (6440 Å) they obtained a value as low as 2.91. Such a low quantum requirement corresponds to the efficiency

$$\eta = \frac{112000}{2.91 \times 44400} = 87\%$$

As the quantum requirement in photosynthesis is not dependent on the wave-length, it is possible to obtain at a wave-length 6800 Å (highest value in red) an efficiency of

$$\eta = \frac{112000}{2.91 \times 41400} = 92\%$$

If the efficiency η were 100%, the theoretical quantum requirement would be

$$100 = \frac{112000}{1/\varphi \times 42100}$$

$$1/\varphi = 2.7$$

From equation 2 the following general equation for the efficiency can be obtained

$$\eta = \frac{112000}{1/\varphi \times \frac{2.86 \times 10^8}{\lambda}}$$

$$\eta = \frac{0.04\lambda}{1/\varphi} \quad (27)$$

The efficiency of photosynthesis, at the quantum requirement 3, is very high indeed by comparison with other biological processes. We shall see later that other investigators found values between 8 and 12 for the quantum requirement. According to these figures, the correctness of which we shall discuss later, the efficiency would be about 25%. This is a very low figure. Warburg's assertion (50) that the genius of living Nature cannot be satisfied with such a low photosynthetic efficiency has been proved in his more recent work (see § 29 and § 30). According to Burk (9), the fact must be envisaged that in a perfect Nature photosynthesis is perfect too (see Addendum).

The general formula for the quantum requirement is

$$1/\varphi = \frac{\text{mole quanta}}{\text{mole O}_2}$$

It is customary to use the μl as unit, so that

$$1/\varphi = \frac{\mu\text{l quanta}}{\mu\text{l O}_2}$$

or

$$1/\varphi = \frac{\text{intensity absorbed in } \mu\text{l quanta in } t \text{ min}}{\text{O}_2 \text{ produced in } \mu\text{l in } t \text{ min}}$$

The value of the numerator is determined by means of the bolometer and the Ulbricht sphere. The value of the denominator is calculated from the product of the manometrically measured pressure change and the vessel constant or, using the two-vessel method, from equation 17.

§ 27 The Significance of Compensation

The intensity of illumination can be chosen in such a way that the photosynthetic production of O_2 is equal to the consumption of O_2 in respiration. This intensity is called the compensating intensity. If the photosynthetic quotient in this compensation point is equal to the respiratory quotient, we also have compensation with respect to CO_2 . However, the two quotients do not have exactly the same value, so that the compensating intensity for O_2 is not necessarily the same as that for CO_2 . Here the term is used with respect to O_2 only. It is further assumed that the compensating intensity exists throughout the whole cell suspension so that in every part of the suspension uptake of O_2 will be equal to removal of O_2 . It looks as if neither uptake nor removal of O_2 takes place. At the compensation point respiration seems to be inhibited because apparently no O_2 is used. In reality, respiration goes on but the O_2 used is quantitatively replaced by O_2 developed photosynthetically. However, many years ago the question was raised whether illumination itself inhibited respiration. It had been imagined that illumination may cause inactivation of respiratory enzymes or that certain intermediary products formed during respiration were reduced. Warburg and Burk (44, 45, 46) pointed out that such hypotheses are incorrect when light intensities giving maximum quantum requirements are used. It is not the light itself but the O_2 production due to photosynthesis which is at the origin of the mechanism of compensation by light. Compensation does not eliminate respiration chemically but only manometrically (44).

We can express the process of O_2 production during illumination and the gain in O_2 obtained by the following equation

$$\text{gain} = \text{photosynthesis} - \text{light respiration}$$

It is only possible to determine the strength of respiration in the dark, so that

$$\text{gain} = \text{photosynthesis} - \text{dark respiration}$$

The assumption that dark respiration is equal to light respiration is not quite correct. This is only permissible when illumination is so strong that respiration will be small in comparison with photosynthesis. Then

$$\text{gain} = \text{photosynthesis}$$

It is possible to select such a low intensity that photosynthesis just compensates respiration. If the quantum yield of the gain is φ' and that of photosynthesis is φ , a relation between both magnitudes and the degree of compensation α can be expressed as follows

$$\alpha = \frac{\text{O}_2 \text{ production in light}}{\text{O}_2 \text{ removal in dark}}$$

or

$$\alpha = \frac{\text{photosynthesis}}{\text{respiration}}$$

However

$$\text{respiration} = \text{photosynthesis} - \text{gain}$$

so that

$$\alpha = \frac{\text{photosynthesis}}{\text{photosynthesis} - \text{gain}} \quad (28)$$

If we introduce φ and φ' , we find that

$$\alpha = \frac{\varphi}{\varphi - \varphi'}$$

or

$$1/\varphi' = 1/\varphi \frac{\alpha}{\alpha - 1} \quad (29)$$

At complete compensation of respiration (sufficiently weak illumination) the degree of compensation is 1. It follows from equation 28 that when $\alpha = 1$ the gain is nil, and from equation 29 that the quantum requirement of the gain $1/\varphi'$ is infinite.

If the quantum requirement of photosynthesis is kept constant at $1/\varphi = 3$, it is easy to calculate the quantum requirement of the gain for various values of α . The degree of compensation increases with the intensity. Table 10 shows the quantum requirement of the gain at various degrees of compensation. At 10-fold compensation, $1/\varphi' = 3.3$, i.e. the quantum requirement of the gain is 90% of the quantum requirement of photosynthesis. At 40-fold

compensation, $1/\varphi'$ is 98% of $1/\varphi$. By increasing the intensity, we arrive at $1/\varphi' = 1/\varphi$, which clearly proves that it is really permissible to write

$$\text{gain} = \text{photosynthesis}$$

In § 26 we found equation 27 for thermodynamic efficiency to be

$$\eta = \frac{0.04 \lambda}{1/\varphi} \quad (27)$$

At the quantum requirement 3, this equation gives for red light (6600 Å)

$$\eta = \frac{0.04 \times 6600}{3} = 88\%$$

If we introduce the degree of compensation α , we find that, according to equation 29,

$$\eta = \frac{0.04 \lambda}{1/\varphi' \frac{\alpha - 1}{\alpha}}$$

or

$$\frac{\alpha - 1}{\alpha} \eta = \frac{0.04 \lambda}{1/\varphi'} = \eta'$$

TABLE 10

Degree of compensation and quantum requirement of the gain at increasing intensities ($1/\varphi = 3$)

Degree of Compensation α	Quantum Requirement of the Gain $1/\varphi' = 3 \frac{\alpha}{\alpha - 1}$
1	∞
2	6.0
3	4.5
5	3.8
10	3.3
40	3.07

Warburg and Burk called η' the economic efficiency. At the compensation point $\alpha = 1$ we find $\eta' = 0$. At 6-fold compensation we have

$$\eta' = 88 \frac{6 - 1}{6} = 73\%$$

At 40-fold compensation $\eta' = 86\%$. With increasing compensation the economic efficiency increases and approaches the value of thermodynamic efficiency.

§ 28 Some Instructive Experiments

In analyzing a few experiments carried out by Warburg and Burk (44) in the National Cancer Institute at Bethesda and by Warburg *et al.* (47,48) in the Max Planck Institute for Cell Physiology, Berlin-Dahlem, we shall examine the influence of some factors upon the quantum requirement. The intensity (μl quanta/min) of the absorbed compensating light will be indicated by i and that of the absorbed measured light by i' . For experiments in acid culture media the two-vessel method is used. From the manometrically measured pressure changes h and h' the values of x_{O_2} and x_{CO_2} are calculated according to equations 17 and 18. The quantum requirement is found by using the equation

$$1/\varphi = \frac{i't}{x_{\text{O}_2}}$$

t being the time (in min) of illumination with the measured red light.

1. Influence of compensation

The following three experiments show that the quantum requirement is practically the same below and above the compensation point, when the same cell suspension is used. *Chlorella* is suspended in an acid culture medium and $i' = 5.4 \mu\text{l}$ quanta/min.

a. Without compensation

	<i>Small Vessel</i> Pressure Changes	<i>Large Vessel</i> Pressure Changes
10 min dark	−10.5 mm	− 2.0 mm
10 min i'	+ 0.5 mm	+ 3.0 mm
10 min dark	−10.0 mm	− 3.5 mm
10 min i'	0 mm	+ 2.0 mm
10 min dark	− 9.0 mm	− 2.5 mm
10 min i'	+ 1.5 mm	+ 3.5 mm
10 min dark	− 8.5 mm	− 2.5 mm
10 min i'	0 mm	+ 3.5 mm
10 min dark	− 9.0 mm	0 mm
10 min i'	+ 1.0 mm	+ 3.0 mm
10 min dark	− 8.0 mm	− 1.0 mm
10 min i'	+ 1.0 mm	+ 5.0 mm
60 min dark	−55.0 mm	−11.5 mm
60 min i'	+ 4.0 mm	+20.0 mm
	$h' = +59.0 \text{ mm}$	$h = +31.5 \text{ mm}$

Using the equations of § 19, we find

$$x_{\text{O}_2} = +70.4 \mu\text{l} \quad x_{\text{CO}_2} = -56.0 \mu\text{l} \quad \gamma = -0.8 \quad 1/\varphi = 4.6$$

b. Over-compensated

	<i>Small Vessel Pressure Changes</i>		<i>Large Vessel Pressure Changes</i>
5 min <i>i</i>	+ 5.0 mm		+ 6.5 mm
5 min <i>i</i> + <i>i'</i>	+11.5 mm		+ 9.0 mm
5 min <i>i</i>	+ 6.5 mm		+ 6.5 mm
5 min <i>i</i> + <i>i'</i>	+ 9.5 mm		+10.0 mm
5 min <i>i</i>	+ 6.5 mm		+ 7.0 mm
5 min <i>i</i> + <i>i'</i>	+ 9.5 mm		+ 8.5 mm
5 min <i>i</i>	+ 5.5 mm		
5 min <i>i</i> + <i>i'</i>	+13.0 mm		
5 min <i>i</i>	+ 7.0 mm		
5 min <i>i</i> + <i>i'</i>	+15.0 mm		
<hr/>			
25 min <i>i</i>	+30.5 mm	15 min <i>i</i>	+20.0 mm
25 min <i>i</i> + <i>i'</i>	+58.5 mm	15 min <i>i</i> + <i>i'</i>	+27.5 mm
<hr/>			
calculated for 25 min			
<hr/>		<hr/>	
$h' = +28.0 \text{ mm}$		$h = +12.5 \text{ mm}$	
$x_{O_2} = +46.3 \text{ }\mu\text{l}$	$x_{CO_2} = -47.6 \text{ }\mu\text{l}$	$\gamma = -1.03$	$1/\varphi = 2.9$

c. More Over-compensated

	<i>Small Vessel Pressure Changes</i>		<i>Large Vessel Pressure Changes</i>
5 min <i>i</i>	+ 18.5 mm		+14.0 mm
5 min <i>i</i> + <i>i'</i>	+ 22.0 mm		+15.0 mm
5 min <i>i</i>	+ 18.0 mm		+14.0 mm
5 min <i>i</i> + <i>i'</i>	+ 22.5 mm		+16.5 mm
5 min <i>i</i>	+ 16.5 mm		+12.5 mm
5 min <i>i</i> + <i>i'</i>	+ 22.0 mm		+16.5 mm
5 min <i>i</i>	+ 17.5 mm		+14.0 mm
5 min <i>i</i> + <i>i'</i>	+ 20.5 mm		+14.0 mm
5 min <i>i</i>	+ 17.0 mm		+11.5 mm
5 min <i>i</i> + <i>i'</i>	+ 23.0 mm		+15.0 mm
5 min <i>i</i>			+12.0 mm
5 min <i>i</i> + <i>i'</i>			+14.5 mm
<hr/>			
25 min <i>i</i>	+ 87.5 mm	30 min <i>i</i>	+78.0 mm
25 min <i>i</i> + <i>i'</i>	+110.0 mm	30 min <i>i</i> + <i>i'</i>	+91.5 mm
<hr/>			
calculated for 30 min			
<hr/>		<hr/>	
$h' = + 27.0 \text{ mm}$		$h = +13.5 \text{ mm}$	
$x_{O_2} = +36.3 \text{ }\mu\text{l}$	$x_{CO_2} = -32.6 \text{ }\mu\text{l}$	$\gamma = -0.9$	$1/\varphi = 4.5$

2. Difference between acid and alkaline culture media

Four experiments show that for the same cell suspension (200 μl cells per vessel) the quantum requirement in acid medium (pH 5) averages 3.75, whereas in alkaline medium (pH 9.2) the average value obtained, 10.15, is three times as great. For both media the quantum requirement has practically the same value below and above the compensation point. The

experiments in alkaline medium have been carried out with the one-vessel method.

a. pH 5, not compensated

	<i>Small Vessel Pressure Changes</i>	<i>Large Vessel Pressure Changes</i>
10 min dark	-12.0 mm	- 4.0 mm
10 min i'	- 5.0 mm	- 1.5 mm
10 min dark	-13.0 mm	- 5.0 mm
10 min i'	- 4.5 mm	- 1.5 mm
10 min dark	-12.5 mm	- 5.0 mm
10 min i'	- 4.0 mm	- 1.5 mm
<hr/>		
30 min dark	-37.5 mm	-14.0 mm
30 min i'	-13.5 mm	- 4.5 mm
<hr/>		
	$h' = +24.0$ mm	$h = + 9.5$ mm
$x_{O_2} = +45.6$ μ l	$x_{CO_2} = -53.0$ μ l	$\gamma = -1.18$ $1/\varphi = 3.6$

b. pH 5, compensated

	<i>Small Vessel Pressure Changes</i>	<i>Large Vessel Pressure Changes</i>
15 min i	+15.5 mm	+11.0 mm
15 min $i + i'$	+29.5 mm	+17.0 mm
15 min i	+17.5 mm	+10.5 mm
15 min $i + i'$	+29.0 mm	+16.0 mm
<hr/>		
30 min i	+33.0 mm	+21.5 mm
30 min $i + i'$	+58.5 mm	+33.0 mm
<hr/>		
	$h' = +25.5$ mm	$h = +11.5$ mm
$x_{O_2} = +41.3$ μ l	$x_{CO_2} = -43.0$ μ l	$\gamma = -1.04$ $1/\varphi = 3.9$

c. pH 9.2 (carbonate buffer), not compensated

$$K_{O_2} = 0.657 \text{ mm}^2$$

$$i' = 5.4 \text{ } \mu\text{l quanta/min}$$

	<i>Pressure Changes</i>
10 min dark	-33.5 mm
10 min i'	-23.5 mm
10 min dark	-30.5 mm
10 min i'	-22.5 mm
10 min dark	-30.0 mm
<hr/>	
20 min dark (calculated)	-62.7 mm
20 min i'	-46.0 mm
<hr/>	
	$h = +16.7$ mm

$$x_{O_2} = hK_{O_2} = 16.7 \times 0.657 = 11.0 \text{ } \mu\text{l}$$

$$1/\varphi = i't/x_{O_2} = 5.4 \times 20/11.0 = 9.8$$

d. *H* 9.2 (carbonate buffer), compensated

$$K_{O_2} = 0.657 \text{ mm}^2$$

$$i' = 5.4 \text{ } \mu\text{l quanta/min}$$

	Pressure Changes
5 min <i>i</i>	0 mm
15 min <i>i</i> + <i>i'</i>	+11.5 mm
15 min <i>i</i>	0 mm
15 min <i>i</i> + <i>i'</i>	+11.5 mm
15 min <i>i</i>	— 0.5 mm
<hr/>	
30 min <i>i</i>	— 0.5 mm
30 min <i>i</i> + <i>i'</i>	+23.0 mm
<hr/>	
	$h = +23.5 \text{ mm}$

$$x_{O_2} = hK_{O_2} = 23.5 \times 0.657 = 15.4 \text{ } \mu\text{l}$$

$$1/\varphi = i't/x_{O_2} = 5.4 \times 30/15.4 = 10.5$$

Though these experiments were published in 1950, Bassham, Shibata and Calvin (4) subsequently found that the quantum requirement of photosynthesis increases parallel with compensation. Franck (24), on theoretical grounds only, arrived at the same conclusion. Five years after Warburg and Burk had carried out their conclusive experiments, Emerson and Chalmers (21) had to refute once again these erroneous views (see, however, the remarks at the end of § 29).

§ 29 The Significance of Carbon Dioxide Pressure

The experiments described in the preceding paragraph clearly show that the quantum requirement of photosynthesis in acid medium is 3 to 4, values Warburg obtained nearly 40 years ago. It is of importance to note that in alkaline medium (carbonate buffer)—the medium preferred by many other investigators—much higher values are obtained, the quantum requirement in alkaline medium being three times greater than in acid medium. In order to explain this discrepancy, Warburg *et al.* (48) studied the influence of the CO₂ pressure in both media. They determined, in acid medium (pH 5) using the two-vessel method, the quantum yield at CO₂ pressures varying from 0.39 to 50 atm %. The maximum yield (0.342) was found at 5 atm % CO₂. At lower and higher pressures the quantum yield decreased (Fig. 29). Similar behavior has been observed in alkaline medium (pH 8.8–9.5). With the one-vessel method the quantum yields were measured at CO₂ pressures varying from 0.22 to 2 atm %. The highest quantum yield (0.304) was obtained at 2.0 atm %. If the φ values calculated are inserted in the curve of Figure 29, we see that—within the limits of experimental error—the same curve is obtained as in acid medium with the two-vessel method.

It is remarkable that the measurements in acid medium with the two-vessel method and those in alkaline medium with the one-vessel method give the same curve representing the dependency of the quantum yield on the CO₂ pressure. This result is excellent evidence for the correctness of the two-vessel method Warburg and Burk worked out several years ago.

Those workers who find the two-vessel method too complicated, are now in a position to correctly determine the maximum quantum yield in an alkaline medium. To begin with, the quantum yield in 0.2 molar carbonate buffer at 2 atm % CO₂ pressure is measured. Then, the same cell suspension is brought into an acid medium and the increase in the manometrically observed light action is noted when the CO₂ pressure is increased from 2 atm % to 5 atm %. This experiment can be carried on with the one-vessel method, as the photosynthetic quotient does not change between 2 atm % and 5 atm % CO₂. We see from the curve of Figure 29 that at 2 atm % CO₂ the quantum yield is 0.290 and at 5 atm % CO₂ it is 0.345. Thus, when the CO₂ pressure is increased from 2 atm % to 5 atm %, the quantum yield

Fig. 29. The quantum yield as a function of the CO₂ pressure (Warburg *et al.*, *Zschr. Naturf.*).

increases by the factor 1.19. If the maximum quantum yield in alkaline medium is 0.304, then we have to multiply this value by 1.19 to obtain the quantum yield in acid medium at 5 atm % CO₂

$$\begin{aligned} \varphi &= 0.304 \times 1.19 = 0.362 \\ 1/\varphi &= 2.76 \end{aligned}$$

This value for the quantum requirement is nearly the theoretical value (see § 26). According to equation 27, we find for $\lambda = 6440 \text{ \AA}$ the very high efficiency of

$$\eta = \frac{0.04 \times 6440}{2.76} = 93\%$$

To summarize, it has to be borne in mind that under the right experimental conditions the low, nearly theoretical value for the quantum requirement of photosynthesis can be determined in acid medium with the two-vessel method as well as in alkaline medium with the one-vessel method. A most important factor is the maintenance of the right optimal CO₂ pressure: this is 5 atm % in acid medium and 2 atm % in alkaline medium. It must be noted that many investigators preferred the alkaline medium and used a CO₂ pressure of only 0.2 atm % in their experiments. The values they obtained for the

quantum requirement were therefore three times higher. The values in Tables 11 and 12 derived from a few experiments (48) demonstrate once again the dependency of the quantum requirement on the CO_2 pressure.

TABLE 11

Influence of CO_2 partial pressure on the quantum requirement in acid medium. Temperature 20°C . Small vessel: $K'_{\text{O}_2} = 0.915 \text{ mm}^2$, $K'_{\text{CO}_2} = 1.503 \text{ mm}^2$. Large vessel: $K_{\text{O}_2} = 1.321 \text{ mm}^2$, $K_{\text{CO}_2} = 1.910 \text{ mm}^2$. Cell respiration nearly compensated with white light. Intermittent measured light 6440 Å (1 min white, 1 min white + red, etc.).

<i>CO₂ Partial Pressure in atm %</i>	<i>Absorbed Intensity in μl quanta/min</i>	<i>t min</i>	<i>h' mm</i>	<i>h mm</i>	<i>1/φ</i>	<i>φ</i>
0.33	3.42	60	+ 5.0	+ 2.5	7.3	0.137
0.66	3.50	240	+23.0	+11.5	5.6	0.179
0.74	3.36	120	+14.5	+ 7.0	4.8	0.210
1.75	3.46	90	+14.0	+ 6.5	3.6	0.278
5.0	3.65	60	+12.5	+ 6.0	2.9	0.342
26.0	3.30	120	+15.0	+ 7.0	4.2	0.240

TABLE 12

Influence of CO_2 partial pressure on the quantum requirement in alkaline medium. Temperature 20°C . Vessel constant $K_{\text{O}_2} = 0.915 \text{ mm}^2$ and 0.911 mm^2 . Cell respiration nearly compensated with white light. Intermittent measured light 6440 Å (1 min white, 1 min white + red, etc.).

<i>0.2 Molar Mixtures</i>	<i>Absorbed Intensity in μl quanta/min</i>	<i>t min</i>	<i>h mm</i>	<i>1/φ</i>	<i>φ</i>
75 NaHCO_3 25 Na_2CO_3 0.22 atm % CO_2	3.42	90	+17.0	10.0	0.10
75 NaHCO_3 25 Na_2CO_3 0.22 atm % CO_2	3.28	90	+18.0	9.0	0.11
90 NaHCO_3 10 Na_2CO_3 0.85 atm % CO_2	3.10	60	+22.0	4.6	0.216
95 NaHCO_3 5 Na_2CO_3 1.99 atm % CO_2	3.15	60	+31.0	3.33	0.30
95 NaHCO_3 5 Na_2CO_3 1.80 atm % CO_2	3.15	60	+30.5	3.4	0.294

In more recent experiments Emerson and Chalmers (20, 21) do not make any use of Warburg's results. They still obtain quantum requirements of about 10 in alkaline medium. In their experiments the CO_2 pressure is as low as 0.5 atm %. Moreover, they insist on using very dense cell suspensions (300 to 400 μl cells per 8 ml liquid) and total absorption (see § 17).

§ 30 The Action of Blue-green Light

When respiration is first compensated with white light, illumination with red or green light gives good values for the quantum yield. Monochromatic light itself at sufficient intensity would—without compensating white light—also give manifold compensation. Warburg *et al.* (56, 60) found, however, that without white light the yields become poorer and that they can be improved by the addition of white light. In particular, it was established that the blue-green part of the white light, i.e., that part of the spectrum which is absorbed by yellow pigments, plays the active role. The addition of small intensities of the light of the blue-green cadmium lines (4680, 4800 and 5090 Å) proved to be sufficient. As the action of the blue-green light sets in gradually and also decreases gradually after removal, it was assumed that this light does not directly influence photosynthesis. The required quantity is so small that, by comparison with red or green measured light, from an energetic point of view it can be ignored.

Fig. 30. The action of blue-green light. Continuous line: illumination with red measured light only ($i = 16.7 \mu\text{l}$ quanta/min). Dotted line: additional illumination with blue-green light ($i = 0.3 \mu\text{l}$ quanta/min) (Warburg *et al.*, *Angew. Chem.*).

The curve of Figure 30 shows the result of an experiment in which blue-green cadmium light is alternately added to and removed from the red measured light (6440 Å). In analyzing the curve of Figure 30 we see that after the addition of blue-green light (dotted line) the quantum requirement is 4.4 after 2 hours, corresponding to an efficiency of 59%. Removal of the blue-green light increases the quantum requirement to infinity (efficiency = 0). Addition of blue-green light again decreases the quantum requirement to 3.6 (efficiency 72%). Removal of blue-green light increases the quantum

requirement to 17 (efficiency 15%). Further addition of blue-green light brings the quantum requirement down to 3.2 (efficiency 81%). Removal again increases the quantum requirement to 12.2 (efficiency 21%). This experiment clearly shows that in the absence of blue-green light efficiency is very low. After its addition, very good efficiencies are immediately obtained. The intensity of the measured red light was $16.7 \mu\text{l}$ quanta/min, while the intensity of the added blue-green light amounted to $0.3 \mu\text{l}$ quanta/min only. The values of η were calculated with the aid of equation 27. The results of two experiments are shown in Tables 13 and 14. The figures clearly show that without blue-green light quantum requirement values increase and, in consequence, the efficiency values decrease.

TABLE 13

The effect of blue-green light on quantum requirement and efficiency (measured red light)

Incident Intensity in μl quanta/min		Time in min	Quantum Requirement $1/\phi$	Efficiency η in %
6.410 A	blue-green			
16.7	0.3	60	9.4	27
16.7	0.3	65	4.4	59
16.7	—	60	~	0
16.7	0.3	40	5.8	45
16.7	0.3	30	3.6	72
16.7	—	30	17	15
16.7	0.3	30	4.1	63
16.7	0.3	30	3.2	81
16.7	—	30	7.8	33
16.7	—	30	12.2	21

TABLE 14

The effect of blue-green light on quantum requirement and efficiency (measured green light)

Incident Intensity in μl quanta/min		Time in min	Quantum Requirement $1/\phi$	Efficiency η in %
5.460 A	blue-green			
93.5	0.3	30	4.66	47
93.5	0.3	30	4.20	52
93.5	—	30	6.6	33
93.5	—	30	8.8	25
93.5	—	30	10.6	21
93.5	0.3	30	5.6	39
93.5	0.3	60	4.7	47
49.8	0.3	30	4.0	55
49.8	0.3	30	4.0	55
171	0.3	15	5.5	40
171	0.3	15	5.5	40
171	—	15	10.3	22
171	—	15	25	9

As very small intensities of blue-green light provoke great photochemical activity, Warburg assumes an enzyme mechanism; a proenzyme is reversibly changed into an enzyme

The importance of cultivating *Chlorella* with fluctuating light has been pointed out in § 15. In § 29 we found that the maintenance of an optimal CO₂ pressure is indispensable. The investigations on the effect of blue-green light show a further factor of importance for low quantum requirements. We have seen that good or poor yields can be obtained as desired by adding or removing blue-green light. This may also explain why greatly differing values for the quantum requirement have been obtained in various institutes. Even when manometry and intensity measurements were correctly carried out, neglect of further important experimental conditions (cultivation, CO₂ pressure, blue-green light) must have led to discrepancies. However, when all the conditions are met, constant low quantum requirements will be obtained at constant illumination in experiments lasting several hours (43). Figure 31 shows the results of an experiment lasting 5 hours where the quantum requirement was always about 3. In an experiment lasting 6 hours (Fig. 32) the quantum requirement was about 4. Warburg *et al.* (43, 63, 64) carried out 23 experiments lasting 6 hours with green + blue-green. They found an average quantum requirement of 4.07. One of these experiments gave the high value of 7.51 (see Addendum, page 186).

The effect of blue-green light is determined by means of the two-vessel method (57). Each vessel is illuminated with red or green measured light and blue-green light. Thus, a total of four light beams are needed each of which is bolometrically measured. Figure 33 depicts schematically the optical equipment with the two ordinary mirrors *M* and a semi-transparent mirror *M'*. The incident intensity of each of the red (or green) beams and of each of the blue-green beams must be exactly identical for both vessels. To begin with, both red (or green) beams are bolometrically adjusted to the same intensity by means of the diaphragms *D*, the cuvette *C* being filled with pure water. Afterwards, this cuvette is filled with a solution of H₂PtCl₆ which does not absorb red (or green) so that the intensity of the blue-green beams can be adjusted (63).

Numerical example:

Determination of the quantum requirement after 2 and 5 hours at constant illumination, using the two-vessel method. Cell suspension: 0.5 μl cells/ml.

$$A = 8.595 \quad B = 11.05 \quad C = -12.46 \quad D = -18.00$$

Measured light: green. Incident intensity 24.8 μl quanta/min of which 3.3% is absorbed.

Blue-green light: 4700 Å. Incident intensity 0.95 μl quanta/min of which 20% is absorbed.

Fig. 31. Oxygen evolution under constant illumination for 5 hours with green measured light and additional blue-green light (Warburg *et al.*, *Angew. Chem.*).

Fig. 32. Oxygen evolution under constant illumination for 6 hours with green measured light and additional blue-green light (Warburg *et al.*, *Angew. Chem.*).

Fig. 33. Layout of the optical equipment for the production of two red and two blue-green light beams which are measured separately by means of the bolometer. Continuous line: red light. Dotted line: blue-green light. L_1 : 500 watt projection lamp. L_2 : 300 watt xenon high pressure lamp. M : mirrors. M' : semi-transparent mirror. D : diaphragm. C : cuvette (Warburg *et al.*, *Zschr. Naturf.*).

	<i>Small Vessel</i> <i>Pressure Changes</i>	<i>Large Vessel</i> <i>Pressure Changes</i>
120 min light	+ 9 mm	+4 mm
180 min light	+12 mm	+5.5 mm
<hr/> 300 min light	<hr/> +21 mm	<hr/> +9.5 mm

For the first two hours:

$$x_{O_2} = +33.2 \mu\text{l} \quad x_{CO_2} = -40.0 \mu\text{l}$$

For five hours:

$$x_{O_2} = +75.5 \mu\text{l} \quad x_{CO_2} = -90.5 \mu\text{l}$$

To determine the quantum requirement, the number of quanta of blue-green light absorbed must be multiplied by 0.5, as, according to Warburg and Negelein (61), chlorophyll absorbs only 50% of light with a wave-length of 4700 Å (see also § 26). It must also be considered that the O_2 consumption due to respiration of the cells at rest ($100 \mu\text{l } O_2$ per $100 \mu\text{l}$ cells per hour) must be added to the calculated value of x_{O_2} . As each vessel contains $3.5 \mu\text{l}$ cells, the respiration at rest is $3.5 \mu\text{l } O_2$ per hour. Thus, we find for the first two hours

$$1/\varphi = \frac{120 \times 24.8 \times 0.033 + 0.5 \times 120 \times 0.95 \times 0.2}{33.2 + 2 \times 3.5} = 2.72$$

and for five hours

$$1/\varphi = \frac{300 \times 24.8 \times 0.033 + 0.5 \times 300 \times 0.95 \times 0.2}{75.5 + 5 \times 3.5} = 2.95$$

If we had put in all instead of half the absorption of blue-green light, the quantum requirement would have been 3.00 and 3.25, respectively. Cell respiration is in reality greater than respiration at rest, so that in any case the quantum requirement is even lower than the figures obtained.

§ 31 The Nature of the Photosynthesis Enzyme

Warburg (50, 56, 57) determined the catalytic action of eight blue-green wave-lengths. The measured light had the wave-length 6450 Å. Figure 34 shows the action spectrum of the photosynthesis enzyme with its sharp maximum at 4600 Å. The absorption spectrum of a *Chlorella* suspension obtained under identical conditions, with the aid of the Ulbricht sphere (Fig. 35), is the absorption spectrum of all the pigments of living *Chlorella* and has a shape quite different to the action spectrum of the enzyme. The carotenoids and the flavins of *Chlorella*, like the enzyme, absorb mainly blue-green light, but the spectra of the carotenoids, xanthophylls and flavines extracted from *Chlorella* do not show the same picture as the action spectrum of the enzyme, especially with respect to the blue part. The unknown pigment acting as an enzyme must be a protein compound. It must be able to undergo a rapid chemical change upon illumination and to return rapidly to its initial state in the dark. Of the known pigments, only visual purple (rhodopsin) may

come into consideration. According to Wald (36) and Morton *et al.* (3), rhodopsin is a compound of vitamin-A-aldehyde (retinene) with a protein, i.e., a carotenoidoprotein. It fulfils the condition of a required rapid and reversible change upon illumination; this is its physiological function. The reaction consists in hydrogen transfer of the type alcohol $\rightleftharpoons$ aldehyde

Retinene reductase is probably identical with alcohol dehydrogenase. Another carotenoidoprotein which may be of interest in this connection is

Fig. 34. Action spectrum of the photosynthesis enzyme (Warburg *et al.*, *Zschr. Naturf.*).

Fig. 35. Absorption spectrum of living *Chlorella* (Warburg *et al.*, *Zschr. Naturf.*).

overdin which has been studied by Kuhn *et al.* (32). Its prosthetic group is astaxanthine which has hydrogen-transferring properties. Astaxanthine occurs not only in lobster eggs but also in the green alga *Hematococcus pluvialis*. There is, however, no evidence that this protein, like visual purple, reacts rapidly and reversibly upon illumination.

The action of blue-green light is completely inhibited by traces of HCN (1/350000 *N*). Thus, it may be assumed that the reaction caused by blue-green light is a heavy metal catalysis. It is well known that HCN reacts with complex-bound heavy metals (e.g., hemes) and with free heavy metal ions as well. In α - α' -phenanthroline we possess a substance which reacts with free heavy metal ions and loosely bound complexes, but not with com-

plex-bound heavy metals. Phenanthroline (1/35000 *N*) at pH 5 inhibits photosynthesis of *Chlorella* to about 33%. The remaining 67% photosynthesis reacts to blue-green light, as in the absence of phenanthroline. It must therefore be concluded that a heavy metal which is loosely bound or is in the state of free ions must be present, in addition to a complex-bound heavy metal. The former reacts with phenanthroline and the latter with HCN. The identity of the second metal has not yet been established, but there is some evidence that it may be vanadium.

Warburg (40) observed that *m*-phenanthroline practically does not react with heavy metal complexes. It is about 200 times less active than *o*-phenanthroline. Thus, phenanthroline does not act as a narcotic but undoubtedly influences heavy metal catalysis.

§ 32 The Significance of Vanadium

The investigations of Bertrand (5) have shown that vanadium is found throughout the whole vegetable kingdom. In the animal kingdom it is rarely found; the blood cells of the tunicates contain vanadium instead of iron. Arnon (2) established that vanadium is indispensable to the growth of certain algae. It seems that the element has a specific action and that it cannot be replaced by other heavy metals. Arnon divides the trace elements into two groups. Group A contains iron, boron, manganese, zinc, copper and molybdenum. Group B comprises cobalt, nickel, chromium, tungsten and vanadium. The stock solution of group A has the following composition: 500 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ + 1000 mg $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ + 300 mg HBO_3 + 200 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ + 22 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ + 8 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 2 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ + 1000 ml 0.005 *N* H_2SO_4 . Two ml of this stock solution are added to 250 ml of the culture salt solution (see § 15). It appears that of group B only vanadium is necessary for *Chlorella* cultures. Warburg *et al.* (54, 57) pointed out that the valency of vanadium is of importance. The blue vanadylsulfate ($\text{VOSO}_4 \cdot 2\text{H}_2\text{O}$) with tetravalent vanadium is inactive, whereas pentavalent vanadium of the yellow sodium metavanadate ($\text{NaVO}_3 \cdot 4\text{H}_2\text{O}$) is very active.

Growth with and without vanadium is identical during cultivation at high light intensities. At low light intensities, as used in measuring the quantum requirement (e.g., 50 μl quanta/min, wave-length 5460 Å), considerable differences have been found. Table 15 shows the results of two experiments.

TABLE 15

The influence of vanadium. $i = 54.3 \mu\text{l}$ quanta/min; 5460 Å. Blue-green light: $i = 0.6 \mu\text{l}$ quanta/min. Time: 30–60 min. Addition of vanadium: 100 $\mu\text{g/l}$.

	γ	$1/\varphi$
Without vanadium	−0.17	14
+ pentavalent vanadium	−1.16	3.2
+ tetravalent vanadium	0	15
+ pentavalent vanadium	−1.0	4.0

The addition of pentavalent vanadium has an immediate influence on the photosynthetic quotient. It seems that the reduction of CO_2 is primarily affected. The differences found in the quantum requirement are impressive.

§ 33 The Hyposulfite Method

Warburg and Krippahl (51) worked out a manometric method for the determination of O_2 in the presence of CO_2 . They used sodium hyposulfite, $\text{Na}_2\text{S}_2\text{O}_4$, which, in aqueous solution, absorbs O_2

The side-arm of the manometer vessel contains dry sodium hyposulfite and dry calcium chloride to protect against water vapor. When a liquid, i.e., H_2O_2 and catalase, or a *Chlorella* suspension, produces O_2 in the vessel, the contents of the side-arm will absorb all the O_2 developed when tipped into the vessel. The application of this method in photosynthesis is, according to Warburg and Schröder (63), as follows: two manometer vessels of the same size each contain $3.5 \mu\text{l}$ cells suspended in 7 ml of the usual culture medium (pH 3.8). The gas phase contains 10 vol % CO_2 , 2.5 vol % O_2 and argon. The temperature is 20°C . The side-arms each contain 20 mg $\text{Na}_2\text{S}_2\text{O}_4$ and 20 mg CaCl_2 . After tipping the salts into one vessel at time 0 and into the other vessel after 60 min illumination, two negative pressures h_0 and h are recorded, h being greater than h_0 , owing to the production of O_2 during illumination. The vessels are shaken rapidly so that after 10 min constant values of h_0 and h can be noted. We then have

$$x_{\text{O}_2} = (h_0 - h)K_{\text{O}_2}$$

To check the equipment, the determination of x_{O_2} without cells has to give the value 0. If the absorbed light intensity is known, the quantum requirement can be calculated.

The hyposulfite method has some disadvantages. When the salts are tipped into the vessel some CO_2 is developed, due to the presence of traces of bicarbonate in the salts or to the production of sodium sulfite driving out CO_2 from the cells. Corrections are therefore necessary when dense cell suspensions are used. Thus, the hyposulfite method is not of the same high scientific standing as the two-vessel method, which, because of its simplicity, reliability and precision, must be considered to be the standard method of biochemical manometry.

Numerical example:

We have for both vessels

$$V_F = 7.0 \text{ ml} \quad V_G = 26.212 \text{ ml} \quad T = 293^\circ \quad K_{\text{O}_2} = 2.47 \text{ mm}^2$$

Cell suspension: $0.5 \mu\text{l}$ cells/ml

Measured light: green. Incident intensity $24.8 \mu\text{l}$ quanta/min of which 3.3% is absorbed.

Blue-green light: 4700 Å. Incident intensity $1.0 \mu\text{l}$ quanta/min of which 19% is absorbed

Time of illumination: 60 min.

$$h_0 = -215.5 \text{ mm}$$

$$h = -220.0 \text{ mm}$$

$$h_0 - h = 4.5 \text{ mm}$$

$$x_{O_2} = (h_0 - h)K_{O_2} = 11.0 \text{ } \mu\text{l}$$

$$1/\varphi = \frac{60 \times 24.8 \times 0.033 + 0.5 \times 60 \times 1.0 \times 0.19}{11.0 + 1 \times 3.5} = 3.80$$

As discussed in § 30, the number of quanta absorbed in blue-green has to be multiplied by 0.5 and the respiration at rest taken into consideration.

§ 34 The Carbon Dioxide Outburst

Emerson *et al.* (22, 23) criticized manometry stating that in the first minutes of illumination *Chlorella* not only absorbs but also removes CO₂. Accordingly $\frac{3}{4}$ of Warburg's manometric effect would be due not to CO₂ reduction but to CO₂ development. Thus, $\frac{1}{4} \times \mu\text{l}$ O₂ would be measured instead of $x \mu\text{l}$ O₂, and the remaining $\frac{3}{4} \times \mu\text{l}$ would be not O₂ but CO₂. This would explain why Warburg and Burk found such low quantum requirements. The real quantum requirement should therefore be three times higher. It should be 9 instead of 3. There is, of course, no evidence whatsoever to support such a hypothesis. Although this photochemically and physiologically incomprehensible CO₂ outburst is still mentioned by some authors, it has never been demonstrated in quantum yield experiments (44). As it is now possible to measure the quantum requirement in experiments lasting several hours, the mysterious CO₂ outburst—if it really does occur in the first minutes of illumination—must be attributed to faulty manometric technique.

If there were any outburst of CO₂, the pressure changes would be greater at the onset of illumination than later. The photosynthetic quotient at the beginning of the experiment would also differ from that noted later. The quotient would initially be strongly positive and even attain values as high as +4. Warburg and Kubowitz (38, 39) established that neither abnormal high initial pressures nor abnormal photosynthetic quotient values are obtained. If the pressure changes are recorded at short intervals, it makes no difference whether the quantum requirement is calculated for the total duration of the experiment or for the duration of illumination only. However, at high light intensity a kind of CO₂ outburst has been observed by Warburg too. At such intensities, a sharp reversal of negative to positive pressure changes can occur and lead to the formation of foam bubbles. When using the modern experimental equipment and when all the experimental conditions are fulfilled, Warburg *et al.* have never observed any outburst of CO₂.

Emerson observed the strange effect when using the two-vessel method. However, Warburg (43, 64) and Brown and Whittingham (8) pointed out that the main prerequisite of the method was not fulfilled. Instead of both vessels being illuminated simultaneously, one was illuminated 8 to 24 hours after the other. It is essential to the two-vessel method that exactly the same

chemical process must take place in the two vessels which are of different size. When the O_2 production is measured simultaneously in both vessels with a divided light beam, there is no CO_2 outburst from living cells. In accordance with true photosynthesis, there can only be a O_2 outburst.

§ 35 The One-Quantum Reaction of Photosynthesis

From what has already been said, it follows that the quantum requirement of photosynthesis is not 8, 10 or 12; under the proper experimental conditions, it is 3. For the reduction of one molecule CO_2 , the action of 3 quanta is necessary, but according to Einstein's law one molecule should be activated by one quantum only. If more quanta seem to be necessary, the existence of dark reactions must be envisaged (see § 6). The question therefore arises whether dark reactions are also involved in photosynthesis. As a result of considerable experimental work, Burk and Warburg (13, 14, 42) succeeded in solving this problem. They found that on intermittent illumination with high intensities, when one-minute periods of light and dark alternated, the calculated quantum requirement in each light minute was 1. In each light minute one absorbed quantum therefore caused the reduction of only one molecule CO_2 and the production of nearly one molecule O_2 , the photosynthetic quotient varying between -1.0 and -1.2 . By contrast, in each dark minute the production of CO_2 and the removal of O_2 (respiration) was noted. Burk and Warburg see the solution of the quantum problem in the coupling of light and dark reactions. The light reaction in which O_2 is produced and CO_2 is fixed is a one-quantum reaction. However, it can only occur when coupled with an exergonic oxidation reaction, the so-called back reaction or induced respiration. Thus, we have a cyclic process. When 112000 cal are needed for the reduction of one mole CO_2 , the light reaction contributes only 43000 cal/mole (in red). The remaining energy, about 70000 cal/mole, is supplied by the induced respiration. The reactions can be represented as follows

Reaction 1 (light):

Reaction 2 (dark):

Reaction 3 (dark):

The over-all reaction is

In the light reaction one molecule O_2 is produced per molecule chlorophyll with the quantum requirement 1. In the dark, $\frac{2}{3}$ of the O_2 produced in the light are used for the induced respiration (reaction 2). The over-all reaction

shows that apparently 3 quanta are necessary for the reduction of one molecule CO_2 . In reality, however, only one quantum is involved, in accordance with Einstein's law. The substance obtained during illumination—indicated by C—is partly oxidized in the dark ($\frac{2}{3}$ according to reaction 2). Thus, the remaining gain ($\frac{1}{3}$) is not identical with the gain in the light reaction, but is equal to the difference between the gain during illumination and the consumption in the dark.

The remaining gain cannot be more than $\frac{1}{3}$ of the light gain, as the energy of one mole quanta in red is about 43000 cal, i.e., $\frac{1}{3}$ of the energy requirement of photosynthesis (112000 cal/mole CO_2). A higher gain is thermodynamically impossible. When $\frac{2}{3}$ of the O_2 produced are used for the induced respiration—as shown in reaction 2—the quantum requirement of the over-all reaction is 3. When $\frac{3}{4}$ of the O_2 react back the quantum requirement is 4. When $\frac{11}{12}$ of the O_2 are used for the induced respiration, the quantum requirement will have the high value of 12, a value Ehrmantraut and Rabinowitch (18) found in their experiments in alkaline medium. When all the O_2 is used, the quantum requirement will be infinite. Daniels (33) in 1938, obtained values of nearly 500.

These considerations show that the over-all reaction of photosynthesis depends upon relatively small changes in the back-reacting amounts of O_2 . The photochemical process and the back reaction mask one another. The slower the back reaction, the more apparent the light reaction.

In the dark reaction the oxygen of the CO_2 molecule is loosened in such a way that the action of one quantum is sufficient to break the bonds and to produce one molecule O_2 . The CO_2 derivative with the loosened oxygen is probably a peroxide, indicated by $(\text{ChlCO}_2)^*$. Warburg calls it the photolyte of photosynthesis (43, 64). The O_2 is developed from the photolyte, inactive CO_2 combining with the chlorophyllprotein (reaction 1). Reaction 3 shows how the inactive CO_2 is changed into the photolyte with the aid of the energy of the induced respiration. The formula of the photolyte is written as if it were a simple CO_2 derivative of chlorophyll. This is certainly not the case, as the CO_2 is probably bound to the protein part of the chlorophyllprotein. However, the formula correctly shows that the O_2 developed from the photolyte under the influence of light is equivalent to the chlorophyll content of the cells. This stoichiometric relationship will be discussed in § 39. To avoid misunderstanding, it may be better to write the formula of the photolyte as CO_2^* no indication being given of the site of the CO_2 in the chlorophyllprotein. The process is represented as follows (see also § 60)

Reaction 1 (light):

Reaction 2 (dark):

Reaction 3 (dark):

Over-all reaction:

The light energy can be transferred without notable loss from the chlorophyll molecule to the photolyte molecule. The light acts within the same molecule that absorbs it. The light reaction of photosynthesis is thus nothing but the photodissociation of a pigment. It is comparable to the photodissociation of iron carbonyl compounds (§ 25)

and the quantum requirement of 1 is almost self-evident.

Numerical example (14):

The one-quantum requirement of photosynthesis is studied by means of the two-vessel method. Each vessel contains 49 μl cells which absorb 48% of the incident green light (5460 Å). The incident intensity is 44 μl quanta/min. Photosynthesis is measured with alternate light and dark periods of 1.5 min.

	<i>Small Vessel</i>	<i>Large Vessel</i>
	$K'_{\text{O}_2} = 1.321 \text{ mm}^2$	$K_{\text{O}_2} = 2.281 \text{ mm}^2$
	$K'_{\text{CO}_2} = 1.910 \text{ mm}^2$	$K_{\text{CO}_2} = 2.870 \text{ mm}^2$
	<i>Pressure Changes</i>	<i>Pressure Changes</i>
1.5 min light	+ 1.5 mm	+0.5 mm
1.5 min dark	− 2.0 mm	−0.5 mm
1.5 min light	+ 2.0 mm	0 mm
1.5 min dark	− 2.0 mm	−0.5 mm
1.5 min light	+ 1.5 mm	+0.5 mm
1.5 min dark	− 2.5 mm	−0.5 mm
1.5 min light	+ 2.0 mm	+0.5 mm
1.5 min dark	− 2.0 mm	−0.5 mm
1.5 min light	+ 1.5 mm	+0.5 mm
1.5 min dark	− 2.0 mm	−0.5 mm
1.5 min light	+ 2.0 mm	+0.5 mm
1.5 min dark	− 2.0 mm	−0.5 mm
9 min light	+10.5 mm	+2.5 mm
9 min dark	−12.5 mm	−3.0 mm
	$h' = +23.0 \text{ mm}$	$h = +5.5 \text{ mm}$

From the values of the vessel constants, it follows that

$$A = 10.10 \text{ mm}^2 \quad B = 15.10 \text{ mm}^2 \quad C = -13.21 \text{ mm}^2 \quad D = -22.81 \text{ mm}^2$$

According to equations 17 and 18, we find

$$x_{\text{O}_2} = h'A - hB = 23 \times 10.10 - 5.5 \times 15.10 = +149 \mu\text{l}$$

and

$$x_{\text{CO}_2} = h'C - hD = -23 \times 13.21 + 5.5 \times 22.81 = -178 \mu\text{l}$$

The photosynthetic quotient is

$$\gamma = \frac{-178}{+149} = -1.19$$

In 9 min the number of quanta absorbed is

$$9 \times 0.48 \times 44 = 190 \mu\text{l}$$

so that the quantum requirement is

$$1/\varphi = \frac{190}{149} = 1.27$$

As can be seen in Table 16, the quantum requirement increases as the frequency of intermittency decreases. With increasing frequency, it decreases to a value of about 1.

TABLE 16
Time of intermittency and quantum requirement

<i>Incident Intensity</i> μl quanta/min	<i>Time of Intermittency</i> in min	<i>Duration of Experiment</i> in min	<i>Small Vessel</i> h' mm	<i>Large Vessel</i> h mm	γ	$1/\varphi$
44	1.5	18	23.0	5.5	-1.19	1.27
44	2.5	25	25.0	4.5	-1.19	1.4
44	5.0	30	16.5	6.0	-1.03	4.1
44	7.5	45	18.5	7.5	-0.97	5.3
24	5.0	30	17.5	6.5	-1.02	2.2
24	10.0	60	21.5	9.5	-0.90	4.6

§ 36 Quantum Requirement and Photosynthetic Quotient

Burk (9, 12) pointed out that the quantum requirement is low when the photosynthetic quotient is high. This fact has been confirmed by Warburg (44, 57). The quantum yield is good when γ is -1.3 but poor when γ is -0.8. It may be assumed that CO_2 is reduced in two steps: firstly, reductive carboxylation to the stage of formic acid

and, secondly, reduction of the carboxylic group to the stage of a carbohydrate

The photosynthetic quotients are

for the first stage reduction $\gamma = -2.0$

for the second stage reduction $\gamma = 0$

for the two reactions together $\gamma = -1.0$

For all further reductions, e.g., from carbohydrate to protein, $\gamma = 0$, so that γ decreases when photosynthesis is accompanied by protein formation.

It may well be imagined that some of the energy of the induced respiration is used for secondary metabolic processes. More O_2 will react back when carbohydrate acts as a hydrogen acceptor (in the case of protein formation) than when CO_2 is the hydrogen acceptor. The photosynthetically produced O_2 will then be decreased and $1/\varphi$ will increase. However, this explanation is not established. In any case, the quantum requirement measured is the correct quantum requirement of photosynthesis only when γ lies between -1.0 and -2.0 , i.e., when growth need not be taken into consideration. For reliable photosynthetic measurements use must be made of cells which produce as little protein as possible under the experimental conditions. Tolbert and Zill (34, 35) and Calvin *et al.* (15) showed in $^{14}CO_2$ experiments that *Chlorella* forms glycolic acid in photosynthesis (see § 57)

According to this reaction, $\gamma = -1.33$. It may be concluded that in glycollic acid formation, i.e. when CO_2 serves as the only hydrogen acceptor, the quantum requirement would have the best value. In the photosynthetic formation of phosphoglyceric acid

the photosynthetic quotient is only -1.2 .

§ 37 Splitting of Photosynthesis Into Light and Back Reactions

Respiration and back reaction are closely connected processes. It is possible to separate them if respiration is compensated before the beginning of the experiment. Compensating light must be of low intensity, so that manometric pressure changes will be zero. On additional illumination with green light, the positive pressure changes observed are due to the one-quantum reaction (light reaction) as well as to the back reaction. Negative pressures found after removal of the measured light must be ascribed to the back reaction only. If the quantum requirement for the complete cycle (light reaction + back reaction) is to be determined, the positive pressure changes on intermittent illumination at intervals of one minute are read every hour. However, if the quantum requirement of the light reaction alone is to be determined, the measured light is added at intermittent intervals of three minutes. The light reaction occurs almost without back reaction in the first minute of the cycle, but at the end of the third minute the back reaction is nearly complete. The true back reaction is observed after removal of the measured light and has nearly expired after a further period of three minutes. Thus, in the cycle of 6 minutes (3 minutes with and 3 minutes without measured light) it is possible to split photosynthesis into its components. Figure 36 shows the average values of five experiments (49). The quantum yield of the light reaction is 1 and that of the whole cycle 0.268.

For vessel 2

$$x_{O_2} = 8 \times 3.51 = 28.1 \mu\text{l}$$

As illumination occurs only for 15 min, during the five cycles of 6 min we have

$$\varphi = x_{O_2}/it = 28.1/7.36 \times 15 = 0.254$$

This quantum yield corresponds to that calculated under a.

- c. The same arrangement as under b. Only the average values of the pressure changes during illumination are noted. From the values observed, part I₁ of the curve in Figure 36 is obtained

after 1 min	1.9 mm
after 2 min	3.0 mm
after 3 min	3.7 mm

The tangent for $t = 0$ on addition of the measured light indicates the pressure change per minute. In Figure 36 $\tan \alpha = 2.35$. To determine the quantum yield in the light reaction alone we have

$$x_{O_2} = 2.35 \times 3.51 = 8.24 \mu\text{l}$$

and

$$\varphi = 8.24/7.36 = 1.1$$

- d. If there were no back reaction, the gain in pressure in the first three minutes of the cycle could be expressed as a straight line. After removal of the measured light, no pressure change would occur as respiration would be compensated. This behavior is illustrated by the dotted line in Figure 36. As the average value of the pressure after the third minute without measured light is 1.6 mm, the pressure difference between the two lines for one cycle is 2.1 mm. Warburg calls this value the splitting effect. For the dotted line ($\tan \beta$) we find the quantum yield

$$\varphi = x_{O_2}/it = 1.6 \times 3.51/7.36 \times 3 = 0.26$$

§ 38 Some Mathematical Considerations

The mathematical treatment of the splitting of the photosynthetic process worked out by Warburg (49) and Burk (10) is of particular interest.

Let the measured light provoke in the time t the production of $x \mu\text{l O}_2$ per unit of volume, the fraction used for the back reaction be f , and the concentration of the substance responsible for this back reaction be c (expressed in $\mu\text{l O}_2$ per unit of volume). When i is the absorbed light intensity in $\mu\text{l quanta/min}$ per unit of volume, the quantum yield is

$$\varphi = \frac{\mu\text{l O}_2/\text{min}}{\mu\text{l quanta}/\text{min}}$$

As the quantum yield in the light reaction is 1,

$$\mu\text{l O}_2/\text{min} = \mu\text{l quanta}/\text{min}$$

Hence, without the back reaction

$$\frac{dx}{dt} = i$$

If the back reaction is regarded as a monomolecular reaction

$$\frac{dx}{dt} = i - kc \quad (30)$$

k being the velocity constant. In considering the concentration c , we find

$$\frac{dc}{dt} = fi - kc$$

or after integration

$$c = \frac{fi}{k} (1 - e^{-kt}) \quad (31)$$

Substituting c in equation 30

$$\frac{dx}{dt} = i - fi(1 - e^{-kt}) \quad (32)$$

or after integration

$$x = i(1 - f)t + \frac{fi}{k} (1 - e^{-kt}) \quad (33)$$

If $t = 0$, it follows from equations 31, 32 and 33 that

$$c = 0$$

$$\frac{dx}{dt} = i$$

and

$$x = 0$$

For high values of k we find

$$c = \frac{fi}{k} = c_s \quad (34)$$

$$\frac{dx}{dt} = (1 - f)i$$

$$x = (1 - f)it + \frac{fi}{k}$$

Thus, equation 34 is valid for the steady state. The value of c , i.e. the amount of O_2 reacting back at given values of f and i , is determined by k when the measured light is removed. According to equations 31 and 34, the time t in which the steady value c_s is attained is determined by the factor $(1 - e^{-kt})$. It follows from these equations that

$$c = c_s(1 - e^{-kt})$$

At 20° C, k has been found to be 0.8. After 3 min illumination with the measured light, we have

$$c = c_s(1 - e^{-2.4})$$

or

$$c = 0.9 \ c_s$$

i.e., 90% of the steady value has been obtained. If k were 5.0, the same percentage would have been found after only 0.48 min. According to equation 34, the value of c_s would then be 6.25 times smaller than when $k = 0.8$. Needless to say, the splitting effect would no longer be measurable.

In these equations the quantum yield in the light reaction has been assumed to be 1, the value of $(1 - f)$ representing the quantum yield for the whole cycle. When $f = 0.624$, the theoretical value of the quantum yield of the cycle is 0.376. In the back reaction $0.624 \times 112000 = 70000$ cal/mole are obtained, so that the quantum energy of 42000 cal/mole just makes up the amount of energy required (112000 cal/mole). This is what happens under physiological conditions.

When $f = 1$, the total O_2 produced in the light reaction reacts back and the quantum yield of the cycle is 0. When $f > 1$, the value for x is negative, according to equation 33. Then, substance would not be gained in the light reaction, but lost by photooxidation. It follows from this mathematical analysis that when $f > 0.624$ energy is spoiled. Either more energy-rich substance than necessary will be decomposed in the light reaction or too much energy will be lost as heat when energy-rich substance is built up in the back reaction.

§ 39 Stoichiometric Oxygen Production

Chlorella brought from dark into light immediately develops O_2 at constant velocity. However, when the alga is brought from its normal culture medium into a modified, more acid medium containing only $MgSO_4$, KH_2PO_4 and $NaCl$ without the usual trace elements, the production of O_2 is much higher in the first minutes of illumination than later in the steady state. This initial O_2 production is stoichiometrically related to the quantity of illuminated cells. Thus, the cells initially do not act as catalysers but merely as reaction partners. Warburg (59) calls the stoichiometrically removed O_2 the O_2 capacity of *Chlorella*. Depending on the chlorophyll content of the cells, 150 to 250 μl O_2 can be removed from 1 ml *Chlorella* cells.

The O_2 capacity of *Chlorella* is of the same magnitude as that of erythrocytes, i.e., about 300 μl /ml. The difference between *Chlorella* and erythrocytes is that O_2 is firmly bound in *Chlorella* (42000 cal are required per mole O_2) but easily removed from oxyhemoglobin at low O_2 partial pressures.

To measure the O_2 capacity, the cells are suspended in the culture medium mentioned above. Initially, they are illuminated for 30 min with blue-green light at low intensity so that the O_2 capacity can be built up by means of the

energy of respiration. They are then illuminated with 35 to 60 μl quanta/min red measured light for 10 min. Within 5 min the O_2 capacity appears as a positive pressure. In the next 5 min the steady state of photosynthesis is attained. The much smaller pressure changes have, of course, to be deducted from the pressure changes observed during the first 5 min. Thus, the difference between the initial O_2 production and the steady O_2 production is the O_2 capacity. The measurements are carried out with the two-vessel method.

Numerical example (59):

Small vessel: $V_F = 7.0 \text{ ml}$ $V_G = 9.88 \text{ ml}$

Large vessel: $V_F = 7.0 \text{ ml}$ $V_G = 14.35 \text{ ml}$

$A = 8.05 \text{ mm}^2$ $B = 10.24 \text{ mm}^2$ $C = -11.49 \text{ mm}^2$ $D = -16.57 \text{ mm}^2$

- a. 200 μl cells per vessel. Chlorophyll content: $3.2 \text{ mg} = 3.56 \mu\text{mole} = 79.5 \mu\text{l}$.
 Blue-green: $i' = 1.2 \mu\text{l quanta/min}$.
 Measured light (6440 Å): $i = 59.8 \mu\text{l quanta/min}$.

	<i>Small Vessel</i> Pressure Changes h'	<i>Large Vessel</i> Pressure Changes h
30 min i' only	-22.0 mm	-11.5 mm
2.5 min $i' + i$	+14.5 mm	+ 7.5 mm
2.5 min $i' + i$	+10.0 mm	+ 6.5 mm
5.0 min $i' + i$	+ 3.0 mm	+ 2.0 mm
5.0 min $i' + i$	+ 3.0 mm	+ 2.0 mm

$$h' = 14.5 + 10.0 - 3.0 = +21.5 \text{ mm}$$

$$h = 7.5 + 6.5 - 2.0 = +12 \text{ mm}$$

According to equations 17 and 18,

$$x_{\text{O}_2} = +48 \mu\text{l (O}_2 \text{ capacity)}$$

$$x_{\text{CO}_2} = -46 \mu\text{l}$$

$$\gamma = -0.96$$

$$\frac{\text{O}_2 \text{ capacity}}{\text{chlorophyll content}} = \frac{48}{79.5} = 0.6$$

- b. 200 μl cells per vessel. Chlorophyll content: $1.17 \text{ mg} = 1.305 \mu\text{mole} = 29.2 \mu\text{l}$
 Blue-green: $i' = 1.2 \mu\text{l quanta/min}$.
 Measured light (6440 Å): $i_1 = 37.5$ and $i_2 = 59.5 \mu\text{l quanta/min}$.

	<i>Small Vessel</i> Pressure Changes h'	<i>Large Vessel</i> Pressure Changes h
30 min i' only	-10.0 mm	- 6.0 mm
2.5 min $i' + i_1$	+ 7.0 mm	+ 4.0 mm
2.5 min $i' + i_1$	+ 1.0 mm	+ 0.5 mm
5.0 min $i' + i_1$	- 3.5 mm	- 1.5 mm
30 min i' only	-56.0 mm	-30.0 mm
2.5 min $i' + i_2$	+10.0 mm	+ 5.5 mm
2.5 min $i' + i_2$	+ 3.5 mm	+ 1.0 mm
5.0 min $i' + i_2$	+ 1.5 mm	+ 0.5 mm

It follows from the first three values that

$$\begin{aligned} h' &= 7.0 + 1.0 + 3.5 = 11.5 \text{ mm} \\ h &= 4.0 + 0.5 + 1.5 = 6.0 \text{ mm} \end{aligned}$$

and hence

$$x_{\text{O}_2} = +29.6 \mu\text{l} \quad x_{\text{CO}_2} = -30.0 \mu\text{l} \quad \gamma = -1.0$$

From the last three values it follows that

$$\begin{aligned} h' &= 10.0 + 3.5 - 1.5 = 12.0 \text{ mm} \\ h &= 5.5 + 1.0 - 0.5 = 6.0 \text{ mm} \end{aligned}$$

and hence

$$x_{\text{O}_2} = +33.4 \mu\text{l} \quad x_{\text{CO}_2} = -36.0 \mu\text{l} \quad \gamma = -1.08$$

Thus, the average value for the O_2 capacity is $31.5 \mu\text{l}$ so that

$$\frac{\text{O}_2 \text{ capacity}}{\text{chlorophyll content}} = \frac{31.5}{29.2} = 1.08$$

In Example a the cells used contained 6.4% chlorophyll ($200 \mu\text{l}$ cells = 50 mg dry weight); in Example b, where the ratio of O_2 capacity and chlorophyll content was 1, *Chlorella* contained only 2.34% chlorophyll. It has been established that the ratio 1 is valid for a chlorophyll content up to 5% of

Fig. 37. Stoichiometric oxygen production (oxygen capacity) from *Chlorella*. Constant light intensity. End value: $23.4 \mu\text{l} = 1.045 \mu\text{mole O}_2$. Chlorophyll content: $22.8 \mu\text{l} = 1.1018 \mu\text{mole}$ (Warburg *et al.*, *Naturw.*).

the cell dry weight. Figure 37 depicts the stoichiometric O_2 production in the form of a graph. O_2 production is initially rapid but becomes slower and slower and reaches its end value after 5 min. In this experiment the end value is $23.4 \mu\text{l O}_2$. Thus, under the influence of light, $23.4 \mu\text{l} = 1.045 \mu\text{mole O}_2$ have been produced by $100 \mu\text{l}$ cells. The chlorophyll content of the cells used is $1.018 \mu\text{mole}$.

When the chlorophyll content of the cells is decreased to $1/2$ or $1/7$ —by increasing the light intensity during cultivation— O_2 capacities decrease in the same proportion. Warburg (58) concludes that the O_2 must originate from

a carrier substance, one molecule of which is stoichiometrically linked to one chlorophyll molecule. This chlorophyll-carrier-substance can be denoted by $(\text{ChlCO}_2)^*$ or CO_2^* (see § 35).

§ 40 Oxygen Capacity and Quantum Requirement

The investigations on O_2 capacity have resulted in chlorophyll being included in the reaction equation of photosynthesis. Thus, the action of chlorophyll is not only physical (absorption of radiation energy) but also chemical. This new concept of the chemical participation of chlorophyll partly rehabilitates the earlier views of Willstätter and Stoll (69).

Warburg distinguishes between two kinds of chlorophyll molecule: those which are linked to the carrier substance (bound chlorophyll) and those which are not (free chlorophyll). Only light absorbed by the bound chlorophyll can be photochemically active. Light absorbed by the free chlorophyll is lost to any photochemical activity (62).

Let the O_2 capacity be K and equal to the chlorophyll content of the cells. After t min the removed capacity x corresponds to the free chlorophyll. The remaining capacity present after t min is then $K - x$, which is equal to the bound chlorophyll. The incident intensity is i_o and the fraction absorbed α .

Thus, the free chlorophyll molecules absorb the fraction $\alpha \frac{x}{K}$ and the bound

chlorophyll molecules the fraction $\alpha \frac{K - x}{K}$.

In the time dt the total absorption is

$$\alpha i_o dt \text{ } \mu\text{l quanta}$$

In the same time dt the O_2 produced by the bound chlorophyll is

$$\alpha i_o \frac{K - x}{K} dt \text{ } \mu\text{l O}_2$$

For the quantum requirement we have

$$1/\varphi = \frac{\text{absorbed energy}}{\text{produced O}_2} = \frac{\alpha i_o dt}{\alpha i_o \frac{K - x}{K} dt}$$

or

$$1/\varphi = \frac{K}{K - x} \quad (35)$$

As can be seen from Table 17, this new equation for the quantum requirement is particularly instructive. The value of x determines the quantum requirement.

The quantum yield can be calculated by drawing the tangents of the O_2 capacity curves. When the total concentration of chlorophyll is C_o and that of the free chlorophyll is $C_o - C$, equation 35 can be written as follows

$$\varphi = \frac{K - x}{K} = \frac{C}{C_o} \tag{36}$$

as K is equal to the total chlorophyll and $K - x$ corresponds to the bound chlorophyll.

TABLE 17
Removed O₂ capacity and quantum requirement

x	$1/\varphi$
0	1
$\frac{1}{2} K$	2
$\frac{7}{8} K$	8
$\frac{11}{12} K$	12
K	$\sim$

It is now possible to calculate the chlorophyll content C_o of the cells from two determinations of φ during stoichiometric O₂ production. When y μ l O₂ are produced after t min,

$$C = C_o - y \tag{37}$$

and hence, according to equation 36

$$\varphi = \frac{C_o - y}{C_o} \tag{38}$$

or

$$C_o = \frac{y}{1 - \varphi} \tag{39}$$

However, the renewed formation of the chlorophyll-carrier-substance, occurring at the velocity constant k , has not been taken into consideration. Therefore, equation 37 has to be corrected to

$$C = C_o - y + kt$$

Hence, instead of equation 38 we have

$$\varphi = \frac{C_o - (y - kt)}{C_o} \tag{40}$$

or

$$C_o = \frac{y - kt}{1 - \varphi} \tag{41}$$

If φ_1 and φ_2 are determined at the times t_1 and t_2 , two equations 41 are obtained. After elimination of k we find

$$C_o = \frac{\frac{y_1}{t_1} - \frac{y_2}{t_2}}{\frac{1 - \varphi_1}{t_1} - \frac{1 - \varphi_2}{t_2}} \tag{42}$$

Numerical example (52):

After 1 min: $y_1 = 16.8 \mu\text{l}$ and $\varphi_1 = 0.380$
 After 3 min: $y_2 = 27.2 \mu\text{l}$ and $\varphi_2 = 0.091$

According to equation 42

$$C_o = \frac{16.8 - 9.06}{0.620 - 0.303} = 24 \mu\text{l}$$

Chemical determination of chlorophyll: $22 \mu\text{l}$. Hence, two determinations during stoichiometric O_2 production enable the chlorophyll content of the cells to be correctly calculated.

The numerical examples of § 39 show that on the addition of measured light the negative pressure changes are followed by positive pressure changes. In Example a the negative pressure change of 22 mm in the small vessel during illumination with blue-green light only is due to the stream of O_2 passing from the gas phase through the liquid into the cells. During 2.5 min illumination with the measured light, this negative pressure change is followed by the positive pressure change of 14.5 mm, due to the reversal of the O_2 stream. The reversal in pressure change takes some time and though vigorous shaking (190 excursions per min) may decrease this time lag, it cannot be completely avoided. The following experiment by Warburg (59) shows that positive pressure change is still noted in the first dark minute after one minute illumination.

	<i>Small Vessel</i> <i>Pressure Changes</i> <i>mm</i>	<i>Large Vessel</i> <i>Pressure Changes</i> <i>mm</i>
10 min dark	-5.0	-2.5
1 min light	+4.5	+2.5
1 min dark	+1.5	+0.5
	<hr/>	<hr/>
5 min dark	-5.0	-3.0

The values for γ and φ are somewhat lower if the time lag is not taken into consideration. To avoid these complications in experiments with short illumination times, the manometric readings should be made one minute after the change from light to dark and one minute before the change from dark to light, so that it is not necessary to interrupt the shaking at the onset of illumination (59).

§ 41 Oxygen Capacity and Induced Respiration

According to the reaction equation of photosynthesis discussed in § 35, the O_2 is produced by a chlorophyll-carrier-substance. When the end value of stoichiometric O_2 production is attained in light, a dark period is necessary if further O_2 is to be developed. Needless to say, O_2 must be present in this recovery period (e.g., 20 min) in order that the induced respiration, which, according to Warburg (59, 62), makes possible O_2 production in the light, can proceed. After the dark recovery period, complete O_2 capacity can be

restored. In Figure 38 the dark intervals between the illumination periods are indicated on the abscissa. After a dark interval of 30 min the maximum end value is again attained following 1-minute illumination. In Figure 38 this value corresponds to a pressure change of 5.5 mm. When the recovery period lasts only 20 min, the pressure increase after 1-minute illumination amounts to 4.7 mm, representing 85% of the maximum end value.

Fig. 38. Regeneration of oxygen capacity in the dark. The values of the dark intervals between the periods of illumination are plotted against the positive pressure changes during the periods of illumination (1 min) (Warburg *et al.*, *Zschr. Naturf.*).

Fig. 39. Influence of oxygen on the regeneration of the oxygen capacity. Continuous line: aerobic. Dotted line: anaerobic. "light" indicates that green light ($i = 25.7 \mu\text{l quanta/min}$) has been absorbed (Warburg *et al.*, *Zschr. Naturf.*).

Hence, the induced respiration restores the O_2 capacity. When O_2 is removed, this recovery is no longer possible. After saturation with 10 vol % CO_2 and argon the capacity cannot be restored, even after very long recovery periods in the dark. At normal O_2 pressures small quantities of HCN ($10^{-4} N$) have the same effect as O_2 deficit. From this analogous action it follows that it is not the O_2 pressure but a chemical action of O_2 —induced respiration—which restores the O_2 capacity. HCN thus acts on the induced

Fig. 40. The action of phenanthroline on the oxygen capacity. Continuous line: without phenanthroline. Dotted line: with 0.01 molar phenanthroline. "Light" indicates that green light ($i = 15.3 \mu\text{l quanta/min}$) has been absorbed (Warburg *et al.*, *Zschr. Naturf.*).

TABLE 18

Influence of HCN and phenanthroline. ++: complete inhibition. +: partial inhibition. -: no inhibition

	HCN	Phenanthroline
Normal respiration	-	-
Induced respiration	++	-
Photosynthesis	+	+
Action of blue-green light	++	+
O ₂ capacity	++	++
Fluoride reaction	++	-
Carotenoid oxygenase reaction (55)	-	-

respiration. This is not self-evident as HCN does not inhibit the normal respiration of *Chlorella* (37, 59). Phenanthroline inhibits neither normal respiration nor induced respiration (62). However, the addition of phenanthroline after the dark recovery period inhibits the restoration of O₂ capacity. As discussed in § 31, this substance inhibits photosynthesis to a great extent.

Figure 39 illustrates the pressure changes under aerobic and anaerobic conditions. The influence of O₂ deficit upon the restoration of O₂ capacity is clearly indicated. Figure 40 shows the action of phenanthroline.

These experiments confirm early observations that O₂ must be present in photosynthesis in order that O₂ can be produced. We shall see in the next paragraph that there is no photosynthesis without respiration.

Certain investigators rejected the existence of O_2 capacity and induced respiration by stating that the observed manometric effects could be due to thermic influences. Figure 39 and Figure 40 clearly show the irrelevance of such statements. In spite of illumination or darkness no pressure changes occur when the chemical reactions in *Chlorella* are inhibited (62).

Table 18 shows the influence of HCN and phenanthroline on some functions and reactions of *Chlorella*.

§ 42 Photosynthesis and Respiration

Since the studies of Boussingault (6), it has been assumed—but not proved—that O_2 is indispensable to photosynthesis. Willstätter and Stoll (69) drew attention to the fact that plants do not exhibit assimilation after long anaerobiosis. According to the one-quantum theory of photosynthesis, O_2 is used in the induced respiration to liberate the energy necessary for the change of CO_2 into the photolyte CO_2^* . For this reason, photosynthesis cannot proceed without O_2 . The use of manometry and *Chlorella* has made it possible to establish conclusive proof. Hill and Whittingham (27) were the first to employ the new methods to solve this problem. Hemoglobin was added to a *Chlorella* suspension, and the O_2 produced upon illumination was determined by optical measurement of the oxyhemoglobin formed. They found that at a O_2 pressure of 2 mm Hg photosynthesis already began to decrease. Later, Whittingham (68) corrected this statement, indicating a pressure of less than 10^{-3} mm Hg.

The following experiments carried out by Warburg *et al.* (43, 53, 64) provide definite proof. In the manometer vessel 100 μ l *Chlorella* suspended in carbonate buffer (pH 8.8) are in equilibrium with 2 vol % CO_2 and argon in the gas phase. According to § 20, pressure changes registered by the manometer can only be changes in O_2 pressure. The gas phase contains very little O_2 . The cells themselves are used to produce the desired low O_2 pressures in the vessel. If the O_2 pressure has to be decreased, the cells are allowed to use O_2 in the dark. If the O_2 pressure has to be increased, the cells are illuminated to produce O_2 . Initially, the cells are allowed to respire in the dark until all the O_2 is used up and respiration is zero. Afterwards, the cells are illuminated: O_2 pressure increases until photosynthesis attains a constant end value. The cells are then saturated with O_2 so that photosynthesis reaches its saturation value. After removal of the light, the O_2 pressure due to respiration decreases to about zero. Figure 41 shows two curves obtained with vessels of different size. Tangents can be drawn at different O_2 pressures p , indicating photosynthesis $+ dp/dt$ on the ascending branches and respiration $-dp/dt$ on the descending branches for the various O_2 pressures. The experiment—utilizing intermittent periods of light and dark—can be repeated at will without opening the manometer vessels.

The increase of photosynthesis with time is dependent only upon the O_2 pressure. This can be proved if equal cell amounts are illuminated under

Fig. 41. Oxygen evolution in the light and oxygen uptake in the dark as a function of oxygen pressure. In both experiments 100 μ l cells are suspended in 7 ml. 0.2 molar carbonate buffer, pH 8.8 (95% bicarbonate + 5% carbonate). Gas phase: 2 vol % CO_2 + argon. Curve I: $V_G = 9.759$ ml, $K_{O_2} = 0.931$ mm 2 . Curve II: $V_G = 14.544$ ml, $K_{O_2} = 1.377$ mm 2 . Incident light intensity (green): 30 μ l quanta/min (Warburg *et al.*, *Zschr. Naturf.*).

Fig. 42. Respiration and photosynthesis at low oxygen pressures (Warburg *et al.*, *Zschr. Naturf.*).

equal conditions in two vessels of different volume. The curves in Figure 41 were obtained in this way. By drawing the tangents at points of equal O_2 pressure, it can be established, for instance, that photosynthesis at point A on curve I for the small vessel is the same as at point B of the curve II for the larger vessel.

$$dp^I/dt \times K_{O_2}^I = dp^{II}/dt \times K_{O_2}^{II} \quad (43)$$

The points of equal pressure and equal photosynthesis on the two curves are, however, not attained at the same time. At equal chemical turnover the O_2 pressure in the small vessel (curve I) will of course increase more rapidly than the O_2 pressure in the larger vessel (curve II).

In Figure 42 the changes in photosynthesis and respiration are plotted against the O_2 pressures. We see that respiration as well as photosynthesis show identical behavior when the O_2 pressure changes. For both processes the half saturation value is reached at 3 mm H_2O and the whole saturation value at 20 mm H_2O . Below an O_2 pressure of 1 mm H_2O , respiration and photosynthesis are both very small.

The light intensity is of particular importance in these experiments. With weak illumination, i.e., when respiration is just compensated after saturation with O_2 , the O_2 pressure, and therefore photosynthesis, cannot increase. Thus, when photosynthesis is zero at very low O_2 pressures, it remains zero under these conditions no matter how long illumination lasts. However, with strong illumination the saturation value of photosynthesis is so rapidly reached that the effect of low O_2 pressure can no longer be recorded. For this reason, the intensity should be chosen in such a way that respiration is 1-fold overcompensated, as is the case in Figure 41. The auto catalytic increase will then last about 30 min and it will be possible to measure the increase of photosynthesis with increasing O_2 pressure exactly. As photosynthesis is the sum of O_2 production in the light and O_2 consumption due to respiration, we must write, at 1-fold over-compensation,

$$2dp/dt \times K_{O_2}$$

In equation 43 factor 2 has been omitted.

Various experiments have been performed to prove that O_2 is not indispensable to photosynthesis. Allen (1) kept *Chlorella* under strong anaerobic conditions. His method is based upon the fact that traces of O_2 decrease the phosphorescence of many dyes. A stream of CO_2 -containing nitrogen is conducted over glowing copper, from there over water, then over a *Chlorella* suspension, over liquid nitrogen, and finally over the dye acriflavin adsorbed on silica gel. Photosynthesis was still detectable at an O_2 pressure of 10^{-6} mm Hg. As described above, manometry in closed vessels showed, according to Warburg, that below 10^{-1} mm Hg photosynthesis in *Chlorella* is very small. Allen's method necessitates empirical calibration, i.e., the O_2 pressures inducing a certain degree of phosphorescence have to be analytically determined. This means that in a rapid stream of gas O_2 pressures of the order of magnitude of 10^{-6} mm Hg have to be produced, maintained and measured. Needless to say, this seems to be a problem which is rather difficult to solve and Allen gives no information on the analytical determination of these traces of O_2 .

This kind of experiment, however, does not give any information on the O_2 pressure in the cells. As a matter of fact, it is not the O_2 pressure in the gas phase but the O_2 pressure in the cells which is of primary importance. If the O_2 pressure outside the cells were zero, a sufficiently high O_2 pressure must, of necessity, be present in the cells. A rough calculation, with the aid of the diffusion constant of O_2 in the cells, makes it possible to estimate a value of

the order of magnitude of 10 mm H₂O for the O₂ pressure in the cells, when the O₂ pressure outside the cells is zero.

Brown (7) tried to prove that no additional respiration (induced respiration) occurs in photosynthesis. In his experiments *Chlorella* respire O₂ enriched with ¹⁸O. As the alga is suspended in normal water, normal O₂ should be developed, according to the concept of water photolysis. However, under normal conditions of photosynthesis when the light reaction and the back reaction overlap, ²/₃ of the O₂ produced in light is reabsorbed so rapidly that the O₂ can be imagined to be oscillating between the free state and the C-bound state. If the molecular O₂ at the disposal of the cells is labeled and the CO₂ provided is not labeled, we cannot expect that more labeled O₂ will be used during illumination than in the dark. As a matter of fact, on spatial grounds, the normal O₂ produced by the cells upon illumination will be used much more rapidly than the labeled O₂ introduced from outside that must diffuse into the cells. Brown found, in fact, that labeled respiration was not increased but was often decreased during illumination. This result shows that not only induced respiration but also dark respiration favors the unlabeled O₂ produced in the cells from the unlabeled photolyte. Warburg (43, 64) regards this as a beautiful example of isotopic discrimination. Further weighty arguments against the logic of Brown's method have been put forward by Decker (17).

The necessity of O₂ in photosynthesis is also demonstrated by experiments carried out by Krall and Burris (31) who found that CO₂-fixation by barley leaves is inhibited to about 97.5% by keeping the leaves anaerobic in an atmosphere of nitrogen in the light or in the dark for 30 min or more before exposure to ¹⁴CO₂. Due to O₂ evolution, this inhibition decreases with increasing time of exposure to CO₂ in the light. Though these experiments are quite convincing, Krall tries to explain his results in a different way. Because of his further research on the influence of CO on photosynthesis of barley leaves, he considers cytochrome oxidase participates in the photosynthetic fixation of CO₂ (29, 30).

Calvin *et al.* (66) tried to measure induced respiration by illuminating the cells in the presence of labeled CO₂, expecting that more unlabeled CO₂ would be given off in the light. However, they found no increase in unlabeled CO₂. This finding is quite in agreement with Warburg's equations of photosynthesis, from which it follows that the induced respiration must be labeled when the CO₂ is labeled. The induced respiration can, of course, only be measured as it was discovered by Burk and Warburg: when it is separated in time from O₂ development (43).

The close connection existing between photosynthesis and aerobic resynthesis of glutamic acid, to be discussed in Part D of Chapter 4, gives further irrefutable evidence of the necessity of O₂ in photosynthesis.

In § 68 and § 70 the necessity of respiration in photosynthesis will be further discussed.

§ 43 Some Final Remarks

The quantum requirement 3 determined by Warburg and Burk could not be confirmed by various authors* who obtained values between 8 and 12. For this reason, Warburg repeated his experiments of 1922 and 1923 in the Max Planck Institute for Cell Physiology in Berlin-Dahlem and at the National Cancer Institute, National Institutes of Health, Bethesda, and the Marine Biological Laboratory, Woods Hole. He worked together with Burk and others and used both the old and the improved methods. His earlier findings were confirmed (11, 45, 46). The rather bitter controversy which ensued seems quite out of date today, especially as recent investigations by Warburg and Burk have completely changed views on photosynthesis energetics. It is regrettable that nevertheless Emerson continued to criticize in a rather polemical manner the fine results Warburg and Burk obtained. However, his review (19) published in 1958 on the quantum yield of photosynthesis is completely out of date. It intentionally ignores Warburg's work done since 1954, as, according to a footnote, the survey of literature was concluded in September 1957.

Although the opponents of Warburg mostly used manometry in their experiments, this method was not considered to be accurate enough. Those who have seen Warburg's highly experimental technique must refute this objection. As long as some investigators utilizing manometry found quantum requirements of 8 to 12, manometry was not criticized. In reality, Warburg's manometric methods—an indispensable part of experimental biochemistry today—and radiation measurement with such instruments as the bolometer and the Ulbricht sphere, have reached a very high degree of perfection. These methods are based upon the classical laws of gases and radiation. A rejection of these methods would be tantamount to a rejection of these laws. It is therefore a regrettable error to assume that other methods which are more complicated and which are less well known to many investigators, such as mass spectrography, infrared recording, paramagneto recording, etc., are superior. If the findings obtained with such modern equipment do not confirm the findings obtained with methods based upon well-established classical principles, it is logical to conclude that they must be unsuitable for experimental photosynthesis (42).

It is of course essential that all the conditions are fulfilled so that no objection can be sustained. To begin with, *Chlorella* must be cultivated in appropriate culture media and under fluctuating light. The cell suspensions used must be thin: light absorption must not be more than a few percent of the incident light. Dense cell suspensions may not be used. Total absorption of the incident light is not permissible. The cells must be young, as old cells contain inactive chlorophyll. Small intensities of blue-green light have to be added to the measured light. Traces of vanadium must be added to the

* Daniels (16), Emerson and Lewis (22), Franck and Gaffron (25), Gabrielsen (26), Kok (28), Wassink (65), Went (67), Yuan (70).

culture medium. The maintenance of a suitable CO_2 pressure is of the highest importance. In addition to these conditions, Warburg's arrangements for the measurement of intensities and their absorption by the cells have to be carefully observed. It does not matter whether the quantum requirement is determined in acid medium with the two-vessel method or in alkaline medium with the one-vessel method (see also compensation method on page 186).

The discovery of the one-quantum reaction of photosynthesis showed that every possible value for the quantum requirement could be obtained depending on the experimental conditions, as induced respiration depends upon these

Fig. 43. Comparison of photosynthetic efficiencies in the field and in the laboratory (Burk. *Fed. Proc.*).

conditions. This means that, after all, the magnitude of the quantum requirement of the over-all reaction is not so important as was believed. Of primary importance is the fact that under optimal conditions it is 3. If higher values are found, it may be concluded that experimental conditions are not optimal. Since more attention has been paid to exact measurement with the large area bolometer, Daniels (71) found, in 1955, quantum requirements of 8.9 in green and 6 in blue, whereas he obtained a value of 157 in 1938 (33). Calvin (4) succeeded in obtaining a value of 4.9 in red in 1955. In accordance with errors shown by Warburg (41), these values should be reduced by 25 to 50%.

Figure 43 shows, according to Burk (9), that in Nature the photosynthetic efficiency for the conversion of light energy into chemical energy attains 1% as an upper limit. However, in the laboratory it is possible to obtain an efficiency of at least 90% under optimal experimental conditions. Photosynthesis indeed is a thermodynamic perfection of Nature (see addendum).

As already stated in § 35, the light reaction of photosynthesis is nothing but the photodissociation of a pigment comparable with the photodissociation of iron carbonyl compounds. It is therefore self-evident that the quantum requirement in the light reaction must necessarily be 1. The problem of the energetics of photosynthesis can be considered to be definitively solved.

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CHAPTER 4

The Chemistry of Photosynthesis

A. INTRODUCTORY NOTES

§ 44 Essentials of Glycolysis

Originally Lépine (19) called glycolysis the breakdown of hexoses in the tissues. Later, Warburg (34) restricted this term to the anaerobic breakdown to the stage of lactic acid. It has long been known that inorganic phosphate is indispensable to carbohydrate breakdown. During glycolysis, phosphorylations take place giving rise to the formation of hexose phosphates and triose phosphates.

The first step of glycolysis concerns the conversion of C_6 -molecules characterized by the following reactions

The second step deals with the formation of C_3 -molecules. It begins with the splitting of fructose-1,6-diphosphate into 3-phosphoglyceraldehyde and phosphodihydroxyacetone

An equilibrium exists between the two triose phosphates

Due to the triosephosphate isomerase, about 96% of phosphodihydroxyacetone is present at the equilibrium state so that the reaction practically proceeds from right to left. However, another enzyme, phosphoglyceraldehyde dehydrogenase, drives the 3-phosphoglyceraldehyde so rapidly away that the reaction proceeds from left to right. Warburg and Christian (36), who discovered phosphoglyceraldehyde dehydrogenase, called this enzyme the "oxidizing fermentation enzyme." With DPN^+ and in the presence of inorganic phosphate it catalyses the formation of 1,3-diphosphoglyceric acid, which is afterwards dephosphorylated to produce 3-phosphoglyceric acid.

In the first of these reactions an energy-rich phosphate bond is produced which, according to the second reaction, is accumulated in ATP. The active group of phosphoglyceraldehyde dehydrogenase has been considered by Racker (24) to be the sulfhydryl group of its glutathion part. However, recently, Warburg (38) confirmed the stoichiometric character of the aldehyde oxidation in the presence of phosphate, which he and Christian (36) first reported in 1939.

The further oxidation of 3-phosphoglyceric acid is preceded by an intramolecular transfer of the phosphate group due to the action of phosphoglyceromutase

In the presence of Mg ions, the enzyme enolase catalyses the conversion of 2-phosphoglyceric acid into phospho-*enol*-pyruvic acid

The energy-rich phosphate bond produced is transferred by pyruvic phosphokinase to ADP and pyruvic acid is formed. Mg ions and K ions are indispensable to this reaction.

In glycolysis there are two main reactions producing energy-rich phosphate bonds:

1. the conversion of 3-phosphoglyceraldehyde into 3-phosphoglyceric acid.
2. the conversion of 2-phosphoglyceric acid into pyruvic acid.

All the reactions discussed are reversible. Under appropriate conditions both pyruvic acid and the intermediate products can be converted back to glucose. The only irreversible reaction is the formation of glucose-6-phosphate from glucose. However, this reaction can be reversed by a special phosphatase.

Pyruvic acid is reduced to lactic acid by lactic acid dehydrogenase (Warburg's "reducing fermentation enzyme").

It is to be noted that DPNH produced during the oxidation of 3-phosphoglyceraldehyde is oxidized during the reduction of pyruvic acid. Thus, there exists an oxidoreduction reaction between 3-phosphoglyceraldehyde and pyruvic acid. Glycolysis is therefore neither an oxidation nor a reduction reaction. This is shown by the over-all reaction of glycolysis

The whole reaction mechanism can be represented as follows:

§ 45 Oxidative Decarboxylation of Pyruvic Acid

The opinion used to be held that the further oxidation of carbohydrates was continued immediately after glycolysis. The lactic acid produced was considered to be the primary substance for further breakdown. This is certainly not the case. In muscle cells of some animals some of the lactic acid produced can be used for resynthesizing glycogen as all steps in glycolysis are reversible. However, further oxidation of lactic acid is out of the question. The primary substance for the continued breakdown of the carbohydrates is not lactic acid but pyruvic acid. When lactic acid is produced in cell metabolism, it must be dehydrogenated to pyruvic acid again before any further oxidation is possible. This dehydrogenation reaction is the reversal of the lactic acid dehydrogenase reaction discussed in the preceding paragraph

It follows from this that lactic acid production is a "blind alley" in the reaction series of carbohydrate breakdown. It only serves for the back oxidation of DPNH formed during the oxidation of 3-phosphoglyceraldehyde (see the reaction equation at the end of § 44). Under aerobic conditions, this back oxidation of DPNH is effected in quite a different manner, namely in the normal way via the respiratory chain.

Pyruvic acid dehydrogenase catalyses the oxidative decarboxylation of pyruvic acid

However, free acetic acid does not occur and, as a matter of fact, this reaction is far more complicated than would be supposed from the above equation. Recent investigations have shown that at least five cofactors are indispensable. They are Mg ions, thiamine diphosphate (TPP^+), DPN^+ , coenzyme A ($\text{A}-\text{S}-\text{H}$) and lipoic acid.* Gunsalus (9) proposed the following four reactions

1. Formation of a hypothetical intermediate product with TPP^+

* Lipoic or thioctic acid has the formula

For literature on lipoic acid, see Reed (28).

2. Formation of an acetyl-lipoic acid-complex

3. Transfer of the acetyl group to coenzyme A

4. Oxidation of the reduced lipoic acid by DPN^+

Hence, the over-all reaction is

According to Reed and De Busk (29), the true coenzyme of decarboxylation is lipothiamide, a compound of lipoic acid with TPP^+ . The reaction scheme of these investigators corresponds basically with that of Gunsalus. The possible importance of lipoic acid in photosynthesis is discussed in § 55.

Hence, the product obtained by oxidative decarboxylation of pyruvic acid is not free acetic acid but acetylcoenzyme A. It is the primary product which enters the tricarboxylic acid cycle where it reacts with oxaloacetic acid. It may be stressed here that Wood, Werkman, Hemingway and Nier (39) found direct carboxylation of pyruvic acid to oxaloacetic acid in bacteria and animal tissues

It is possible that this reaction may help to supply the oxaloacetic acid necessary for the first step in the tricarboxylic acid cycle.

§ 46 The Tricarboxylic Acid Cycle

The first step in the tricarboxylic acid cycle is the condensation of acetylcoenzyme A with oxaloacetic acid to form citric acid (21). Many investigators contributed to our present knowledge of this most important cycle according to which the acetyl group of acetylcoenzyme A is oxidized to CO_2 and H_2O . Due to the work of Krebs (18), the cycle depicted in Figure 44 is well established and universally recognized. More recently, a dicarboxylic acid cycle has been discovered by Kornberg *et al.* (15, 16, 17), in different microorganisms and mammalian tissues effecting the oxidation of glycolate or glycine via glyoxylate and formate to CO_2 and H_2O .

The tricarboxylic acid cycle proceeds with the aid of enzymes localized in

Fig. 44. The tricarboxylic acid cycle.

the mitochondria and their various cofactors. There are four oxidation steps linked with the action of particular dehydrogenases. The substrates *iso*-citric acid, α -ketoglutaric acid, succinic acid and malic acid are dehydrogenated, each giving 2 H atoms to appropriate hydrogen acceptors. The H atoms are finally oxidized to H ions.

As depicted in Figure 44, one acetyl group of acetylcoenzyme A is oxidized in one cycle, 3 molecules H_2O being used and 2 molecules CO_2 and 8 H atoms being produced. This corresponds to the over-all reaction

The molecule of coenzyme A which is produced reacts with another molecule of pyruvic acid to form a further molecule of acetylcoenzyme A.

The first oxidation step is the conversion of *iso*-citric acid to oxalosuccinic acid catalysed by the *iso*-citric dehydrogenase. This reaction is followed by the decarboxylation to α -ketoglutaric acid. Thus, the over-all reaction represents an oxidative decarboxylation.

In the second oxidation step, α -ketoglutaric acid is converted to succinic acid by oxidative decarboxylation. According to Green (7, 8), Kaufman (14) and Sanadi (30) the following scheme with its three main reactions may be accepted:

1. Formation of succinylcoenzyme A

This reaction corresponds to the oxidative decarboxylation of pyruvic acid (§ 45). The same 5 cofactors are indispensable.

2. Formation of succinic acid

Inorganic phosphate is used. The enzyme succinylcoenzyme A-ADP-phosphorylase discovered by Hift *et al.* (10) needs guanosine diphosphate (GDP) as an additional cofactor (30).

3. Formation of ATP

Finally, a GTP-ADP-transphosphorylase transfers the phosphate group from GTP to ADP

The third oxidation step is the dehydrogenation of succinic acid catalysed by the succinic dehydrogenase. The H atoms are not accepted by either DPN^+ or TPN^+ ; they are probably oxidized to H ions by cytochrome b. The enzyme fumarase catalyses the hydration of fumaric acid to malic acid.

In the fourth and last oxidation step, malic acid is dehydrogenated to oxaloacetic acid by malic dehydrogenase and DPN^+ . This reaction terminates the tricarboxylic acid cycle. Starting from one molecule acetylcoenzyme A, one molecule of oxaloacetic acid is regenerated and can condense with a further molecule of acetylcoenzyme A.

The so-called malic enzyme which is closely related to malic dehydrogenase can convert malic acid into pyruvic acid with DPN^+ or TPN^+ .

Badin and Calvin (2) consider the reversal of this reaction, i.e., the reductive carboxylation of pyruvic acid, may play a role in photosynthesis (see § 58). The reaction discovered by Ochoa (20, 22, 23) is reversible; the presence of Mn ions is essential. When it proceeds from right to left and the malic acid produced is converted by the malic dehydrogenase to oxaloacetic acid, the over-all reaction is identical with the Wood-Werkman reaction discussed in § 45.

In various cells fixation of CO_2 is a most important process.* It is mostly due to reductive carboxylation, i.e., the reversal of oxidative decarboxylation. Other reactions of CO_2 fixation are the Wood-Werkman reaction and the carboxylation of phospho-*enol*-pyruvic acid with inosine diphosphate (IDP).

* For literature on CO_2 fixation, see Utter and Wood (32) and Vishniac *et al.* (33).

The latter reaction discovered by Utter *et al.* (31) produces oxaloacetic acid

It is possible that ADP and not IDP may be the phosphate acceptor.

During the breakdown of pyruvic acid 10 H atoms are transferred to the respiratory chain. As the free energy decrease in oxidation of pyruvic acid is about 275000 cal/mole, about 55000 cal/mole are liberated per 2 H atoms. This amount of energy is accumulated in energy-rich phosphate bonds and it is assumed that 3 molecules ATP are produced per 2 H atoms. This oxidative phosphorylation is different to anaerobic phosphorylation in glycolysis, the latter being substrate phosphorylation, the chemistry of which has been clearly defined (see § 44). In § 51 a third form of phosphorylation, due to the action of light, is discussed.

§ 47 The Pentose Phosphate Pathway

In addition to glucose, various carbohydrates—tetroses, pentoses, hexoses, heptoses—have been found to occur in living matter. These may also be sources of energy or fulfil important biological functions; thus, pentoses are parts of nucleotides. The existence of ways of carbohydrate breakdown other than glycolysis and the tricarboxylic acid cycle might therefore be suspected. In fact, in the most varied types of cell it has been found that glucose-6-phosphate can be oxidized in quite a different way. It had been noted that glucose breakdown continues in various cell extracts even when potent inhibitors of glycolysis such as iodoacetate or NaF are added. Furthermore, enzymes had been discovered which are TPN-specific whereas the enzymes of glycolysis are mostly DPN-specific.

Warburg *et al.* (37) isolated in 1931 the enzyme glucose-6-phosphate dehydrogenase from yeast. This enzyme, which they called the “Zwischenferment,” catalyses, with TPN, the oxidation of glucose-6-phosphate to 6-phosphogluconate. An intermediate product formed is the δ -lactone of 6-phosphogluconic acid, the lactone ring of which is split by a lactonase (5).

By means of 6-phosphogluconic acid dehydrogenase, TPN^+ and TPP^+ , 6-phosphogluconic acid is converted by dehydrogenation and decarboxylation to ribulose-5-phosphate or xylulose-5-phosphate. This is changed into ribose-5-phosphate by phosphoriboisomerase. These reactions were also discovered by Warburg and Christian (35). According to Horecker (11), the reaction scheme can be written as follows (see also Dickens (6), Racker (25) and Cohen (4)):

From the pentose phosphate produced two C atoms are oxidized to CO_2 , leaving triose phosphate. As pentose phosphate is continuously regenerated, there is a cyclic process using hexose phosphate and producing triose phosphate and CO_2 . These reactions do not involve the glycolytic pathway, so that the pentose phosphate pathway is often denoted as the hexose phosphate shunt. Two main reactions are responsible: transketolation and transaldolation.

The enzyme transketolase (TK) transfers the ketol group ($-\text{CO}\cdot\text{CH}_2\text{OH}$) to aldehydes:

The enzyme thus splits xylulose-5-phosphate into a C_3 fraction and a C_2 fraction. However, the C_2 fraction is not produced as free glycolaldehyde but reacts with ribose-5-phosphate to give the phosphate of a C_7 sugar, sedoheptulose-7-phosphate. Transketolase is also involved in the following conversion

where the ketol group is transferred from ribulose-5-phosphate to erythrose-4-phosphate to produce fructose-6-phosphate and phosphoglyceraldehyde:

The transaldolase (TA) transfers the C_3 fraction $-\text{CHOH} \cdot \text{CO} \cdot \text{CH}_2\text{OH}$ and therefore catalyses the formation of tetrose and hexose from heptose and triose (13, 26). The following reaction shows how this fraction of sedoheptulose-7-phosphate is transferred to phosphoglyceraldehyde to produce fructose-6-phosphate:

Transketolase and transaldolase need TPP^+ as a cofactor. It has now been found that transketolation and subsequent transaldolation result in an over-all reaction representing the above-mentioned oxidation of one molecule pentose phosphate to one molecule triose phosphate and two molecules CO_2 . Starting from one molecule glucose-6-phosphate as the primary substance, this oxidation reaction produces one molecule triose phosphate and three molecules CO_2 . Figure 45 shows the reaction scheme as indicated by Racker (25). The ketol donor in the formation of sedoheptulose-7-phosphate is xylulose-5-phosphate and not ribulose-5-phosphate as was formerly assumed. An equilibrium state exists between both pentose phosphates; this is maintained by the enzyme epimerase (12, 27).

If the fate of one molecule glucose-6-phosphate in the presence of one molecule ribose-5-phosphate is studied, it is seen that the first three C atoms of glucose-6-phosphate are oxidized to CO_2 , whereas the other three C atoms re-

Fig. 45. The pentose phosphate pathway (Racker).

main together in a molecule triose phosphate. In Figure 46 I represents the oxidative decarboxylation of glucose-6-phosphate to xylulose-5-phosphate as well as the condensation of the latter with ribose-5-phosphate. This is the transketolase reaction producing sedoheptulose-7-phosphate and triose phosphate. In II we have the transaldolase reaction in which erythrose-4-phosphate and fructose-6-phosphate are produced. The latter gives off CO_2 by oxidative decarboxylation, leaving pentose phosphate. Finally, III shows

Fig. 46. The fate of the C atoms of glucose-6-phosphate (Racker).

the other transketolase reaction condensing pentose phosphate with erythrose-4-phosphate and producing fructose-6-phosphate and triose phosphate. Oxidative decarboxylation of the fructose-6-phosphate produced again gives ribose-5-phosphate which can condense with a further molecule of xylulose-5-phosphate derived from glucose-6-phosphate. Thus, we have a cyclic process in which ribose-5-phosphate acts as a catalyst, like oxaloacetic acid in the tricarboxylic acid cycle.

In a comparison of the possible pathways of carbohydrate degradation the question arises as to which factors induce the cells to follow a particular pathway. It is probable that the quantitative distribution of the enzymes is of utmost importance. Glycolysis is nearly non-existent in various micro-organisms, but is predominant in muscle cells. Although the enzymes of the pentose phosphate pathway are present in yeast, they barely participate in carbohydrate breakdown, as the enzymes of glycolysis are present in much higher concentrations. In respiration of higher plants the pentose phosphate pathway may play a role since its enzymes have been found to be present. However, at present, no exact information on the importance of the pentose phosphate pathway is available (1, 3).

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B. THE PROBLEM OF WATER PHOTOLYSIS

§ 48 Introductory Remarks

Even the early investigations with isolated chloroplasts (15, 21, 39, 47, 74) showed O₂ production upon illumination. The first experiments *in vitro* were of a qualitative nature only. Engelmann (21) demonstrated O₂ production under the microscope utilizing certain bacteria which move to spots where traces of O₂ occur. These bacteria accumulate around the isolated chloroplasts as long as these are illuminated. Beijerinck (15) and Molisch

(39) used bacteria which show luminescence in the presence of O_2 traces, a technique that allows the observation of O_2 production with the naked eye. At that time, there was no evidence of simultaneous CO_2 fixation. It was assumed—without any proof—that O_2 was produced by the photolysis of CO_2 . Later, views were modified. It was admitted that O_2 was produced by the photolysis of water and not of CO_2 . The comparative biochemical considerations of Kluyver (34) and van Niel (57), as well as Hill's investigations (24, 25) on O_2 production of isolated chloroplasts in the absence of CO_2 , provided considerable support for the new hypothesis. In 1946 Nicol (42) wrote that CO_2 photolysis had been accepted as a well-established fact though it was not proven. Stressing this as a typical example of unscientific thinking, he proposed to accept the concept of water photolysis, though at that time the latter was, as it still is to-day, not proven either. We know now that CO_2 photolysis *is* a fact (see § 49 and § 67).

In the presence of H_2S , certain green sulfur bacteria reduce, upon illumination, CO_2 to carbohydrate to produce sulfur.

Other pigment-containing bacteria, the purple bacteria, produce sulfuric acid

If the over-all reaction of photosynthesis of green plants is written as follows

a striking resemblance between these reactions is evident. Van Niel therefore proposed to represent photosynthesis by the following general equation

The compound H_2A is a hydrogen donor; it is H_2O in the green plant and H_2S in the sulfur bacteria. Organic compounds may also act as hydrogen donors in bacterial photosynthesis, as has been shown by Foster (22) for *iso*-propanol

Comparative biochemistry was found by Kluyver and van Niel to be very useful in microbiology. Its application to photosynthesis *does not prove* that the O_2 produced originates from H_2O , as the sulfur originates from H_2S , but does provide support for this view.

Experiments with labeled O_2 and CO_2 are often quoted as evidence for these purely theoretical considerations.* When a plant has at its disposal

* See Ruben *et al.* (48, 49, 50), Kamen and Barker (30), Dole and Jenks (20), Yoshida *et al.* (77), Winogradow and Teis (75).

water with ^{18}O and normal CO_2 , the O_2 produced contains ^{18}O . By contrast, when normal water and CO_2 labeled with ^{18}O are available to a plant normal O_2 should be produced. Thus,

However, the experimental results show that neither reaction proceeds in this theoretical way. Neither 100% $^{18}\text{O}_2$ nor 100% O_2 has ever been found. A comparison of the work of various investigators revealed marked discrepancies. It may be said that 15 to 40% of the O_2 produced seems to originate from CO_2 . This result is not surprising, if one bears in mind that it is not known whether CO_2 or H_2CO_3 is the reaction partner in photosynthesis. It is very probable that H_2CO_3 is intermediately formed so that the following equilibria have to be taken into consideration

According to Warburg (60, 61, 67), Brown and Frenkel (17) and to van Niel himself (57), the experiments with ^{18}O do not prove that the O_2 produced in photosynthesis originates from H_2O . Similarly, they do not prove the contrary view, so that they must be considered to be useless.

Fig. 47. Van Niel's scheme.

In Figure 47 van Niel's views are depicted schematically. The symbols E' and E'' represent unknown factors which prevent the recombination of H and OH produced by the photolysis of water. The factor E' combines with H and transfers it to CO_2 which is reduced to (CH_2O) . This is the process occurring in green plants and in photosynthetic bacteria. The factor E'' combines with OH and transfers it to the hydrogen donor H_2A in bacterial photosynthesis. In green plants OH is transferred to special mechanisms which convert it to H_2O and O_2 . However, the intermediate for-

mation of H_2O_2 , though accepted by other investigators, is still very doubtful.

According to this scheme, light should decompose H_2O to H and OH . Other investigators write water photolysis as follows

where $[\text{H}]$ and $[\text{O}]$ do not represent molecular hydrogen and oxygen, but reduced and oxidized hypothetical products.

§ 49 Hill Reactions

Hill (24) was the first to observe that isolated chloroplasts are able to reduce compounds other than CO_2 upon illumination. He proposed the following general reaction equation

where reducible substances serving as hydrogen acceptors are denoted by A . Thus, the chloroplasts possess a mechanism by which O_2 is developed upon illumination and a hydrogen acceptor—but not CO_2 —is reduced. Hill replaced the qualitative bacteria method by a quantitative method measuring the O_2 production spectroscopically by means of the conversion of myoglobin to oxymyoglobin.

In his first experiments Hill (24, 25, 27) used ferric oxalate as a hydrogen acceptor

However, the ferrous oxalate produced is autoxidizable and as ferric oxalate in light—without chloroplasts—is easily reduced and CO_2 is produced, photochemical experiments with this salt lead to complications which Hill was able to avoid by adding potassium ferricyanide. Warburg and Krip-pahl (62) used potassium ferricyanide in their experiments. This salt is much more stable than ferric oxalate and its reduction product is not autoxidizable. The correctness of the iron reduction equation has also been confirmed by Holt and French (28).

The Hill reaction can also be realized with quinone as a hydrogen acceptor. Warburg and Lüttgens (59, 64) studied the action of quinone on cell-free leaf extracts, intact and disintegrated chloroplasts and on living *Chlorella*

As no CO_2 occurs in the Hill reaction, Hill concluded that the primary photochemical process must be the decomposition of water. However, this conclusion was over-hasty as nothing was definitely known about the mechanism of this type of reaction.

When *Chlorella* is suspended in an appropriate nitrate medium, and is illuminated in the absence of CO₂ it produces O₂ for several hours, according to the following equation

This reaction, discovered by Warburg and Negelein (66) in 1920, must be considered to be the over-all reaction of a light and a dark reaction

In a dark reaction the NO₃ ions oxidize C to CO₂. After conversion to the loosely bound CO₂ in the photolyte CO₂*, this is split in the light.

It was tentative to explain the reaction with iron salts in the same way. In 1955 Warburg and Krippahl (62) succeeded in distinguishing the following reactions with living *Chlorella* and ferric ions

Thus, the Hill reaction can be considered to be an over-all reaction composed of dark reactions and a light reaction. The latter is identical with normal photosynthesis. Schwartz (52) and Good (23) came to similar conclusions. It is to be noted that the iron reduction with living *Chlorella* is a relatively powerful reaction. In an experiment lasting 45 hours with 20 μl cells (5 mg dry weight) to which 61 μmole = 20 mg K₃Fe(CN)₆ have been added, 61:4 = 15 μmole = 0.66 mg CO₂, i.e., 13% of the dry weight of the cells are produced. The addition of the iron salt does practically no damage to the cells, and their normal reproduction is not impaired.

As already mentioned, Hill used as a reagent ferric oxalate which, after reduction to ferrous oxalate, produces CO₂ in the light. Thus, the presence of CO₂ was evident so that Hill's original experiments concerned nothing but true photosynthesis.

In replacing ferric ions by quinone, we would expect the following reactions

A difficulty arises here insofar as quinone seems to inhibit reactions b and c. The addition of quinone and CO₂ stops the production of O₂ as soon as the quinone—not the CO₂—is used up. Thus, until now it was impossible to

assume reactions b and c to be intermediate in the quinone reduction by *Chlorella*. Two kinds of reactions had to be accepted, one with the light reaction c and one unknown light reaction of another type. Such a conclusion is of course far from satisfactory and very improbable (61, 67). Warburg and Krippahl (63) found that CO₂ is indispensable to the quinone reaction in such small amounts that its necessity had been overlooked in hundreds of papers since 1944, but, nevertheless, in amounts still great enough to enable the CO₂ pressures to be measured. However, in his study of the Hill reaction with quinone in 1948, Boyle (16) observed that the presence of very small amounts of CO₂ was indispensable. Extracts of young spinach leaves were used. The gas phase contained commercial nitrogen only. In the presence of some KOH in the side-arm of the manometer vessel, traces of CO₂ were removed with the result that O₂ production ceased. Without KOH, the traces of CO₂ in the commercial nitrogen were sufficient to obtain considerable gas pressures due to O₂ production. These findings could not be confirmed by either Clendenning and Gorham (19) or Hill (26) so that the necessity for intermediary CO₂ in the quinone reaction was rejected. Today, we know why Boyle's experiments could not be reproduced (63). Spinach grana are very rich in oxalic acid; on illumination this is oxidized to CO₂ by quinone

In spite of KOH, illuminated spinach grana normally develop so much CO₂ that sufficient CO₂ still remains in the gas phase. Thus, spinach grana are certainly not an appropriate test object for proving the necessity of CO₂. It is highly probable that Boyle's extracts had, by chance, a low oxalic acid content. It is obvious that if extracts with a high oxalic acid content are employed, no difference can be expected, irrespective of whether KOH is used or not (63).

The necessity of CO₂ means that CO₂ is an intermediate product in the quinone reaction as described in the above equations. It is not paradoxical that with the simultaneous addition of quinone and CO₂ only quinone and not CO₂ is used up in the over-all reaction. Quinone inhibits O₂ respiration but only because it is replaced by "quinone respiration" which energetically permits reaction b. The above equations show that "quinone respiration" produces as much CO₂ as disappears in light, so that the total CO₂ turnover does not appear in the over-all reaction. Thus, reaction c ceases when all the quinone is completely reduced, as no more energy is available for reaction b.

These conclusions are in good accord with the ferric cyanide experiments. In contrast to quinone, the iron salt does not inhibit respiration of *Chlorella*. When ferric cyanide and CO₂ are added to *Chlorella*, the end value of O₂ produced in light is equivalent to the sum of ferric cyanide and CO₂, as, under

these circumstances O_2 respiration + "iron respiration" produce the necessary energy for reaction b.

With isolated chloroplasts which do not respire, the end value of the O_2 produced is equivalent only to the amount of ferric cyanide, no matter how much CO_2 the gas phase may contain. It is not the Hill reagent but the O_2 respiration which decides whether CO_2 disappears or not in the light reaction, i.e., whether sufficient energy is available for converting CO_2 to CO_2^* in reaction b.

Of primary importance for the understanding of the Hill reactions is respiration—not normal respiration, but induced respiration. The Hill reagents do not replace CO_2 , but they do replace molecular O_2 . The Hill reactions must therefore be considered to be O_2 -free respiration processes; this type of respiration, like O_2 respiration, produces the necessary energy for the splitting of CO_2 in the light (reactions b and c). These considerations lead to the firm conclusion that the hypothesis of water photolysis has to be abandoned. The explanation of the Hill reactions must be found in respiration processes which are directly and inseparably connected with the photolysis of CO_2 . In photosynthesis and in the Hill reactions the light reaction is the splitting of CO_2 . In photosynthesis CO_2 decreases in the over-all reaction, whereas in the Hill reactions CO_2 remains constant and the Hill reagents decrease.

In Section D Warburg's conclusive experiments dealing with the action of quinone on living *Chlorella*, isolated chloroplasts and lyophilized cells will be further discussed. These experiments provide definite evidence that the hypothesis of water photolysis can no longer be maintained (63). This is embarrassing insofar as many important contributions to the chemistry of photosynthesis are based upon this hypothesis.

Phenylurethane and ethylurethane inhibit true photosynthesis (65) as well as the Hill reactions (27). This was considered by Hill and Scarisbrick (26, 27) to be a very valuable argument in favor of the mechanism of O_2 production *in vitro* being identical with that in true photosynthesis. According to Hill (24), the chloroplast reaction is not influenced by HCN.

Phenanthroline inhibits the Hill reactions by about 60% (62). The addition of Zn ions, which are bound by phenanthroline, cancels this inhibitory action (65). Schwartz (52) arrived at similar results with lyophilized cells. According to Arnon and Whatley (9), other metal ions exert this effect. Thus, phenanthroline (0.002 molar solution) specifically and reversibly inhibits photosynthesis, O_2 production of isolated chloroplasts with quinone, the ferric cyanide reaction with living *Chlorella* and the O_2 capacity. Respiration is not inhibited by phenanthroline (see Table 18).

§ 50 Phosphorylations

Ochoa and Vishniac (43, 44) found that, under appropriate conditions, illuminated isolated chloroplasts can reduce DPN^+ and TPN^+ . These re-

actions were confirmed by Arnon (3), Tolmach (55) and San Pietro and Lang (51), although Jagendorf (29) using highly purified chloroplasts observed the Hill reaction with TPN^+ only. The reactions proceed as follows

With the $\text{DPNH}(\text{TPNH})$ produced, reductive carboxylations may occur. In § 46 some examples of reductive carboxylations were discussed. As these are reactions of CO_2 fixation, it was reasonable to examine whether such reactions were responsible for CO_2 fixation in photosynthesis. Ochoa showed that illuminated chloroplasts are able in the presence of malic enzyme, TPN^+ , Mn ions and pyruvic acid, to produce appreciable amounts of malic acid. When α -ketoglutaric acid and *iso*-citric dehydrogenase were used, much *iso*-citric acid was obtained. Malic enzyme occurs in the cytoplasm of plant cells but not in the chloroplasts; this may explain why isolated chloroplasts, which cannot catalyze reductive carboxylations, are not capable of exhibiting true photosynthesis. However, the question arises whether reductive carboxylations are really the main reactions of CO_2 fixation in photosynthesis. In sections C and D of this chapter other important reactions of CO_2 fixation will be discussed.

It is doubtful if the reduction of $\text{DPN}^+(\text{TPN}^+)$ must be considered to be a primary reaction. Although this has been postulated by Arnon in the case of TPN^+ , other authors, for reasons to be discussed later, assume that a primarily acting hydrogen acceptor has to be inserted. Calvin (18) considers this primary hydrogen acceptor to be lipoic acid but Wessels (70) supposes it to be vitamin K. If the primary hydrogen acceptor is indicated by X, the reduction of TPN^+ is

The reduction of phosphopyridine nucleotides in the Hill reaction links the latter with enzymatic processes and with the reaction of oxidative phosphorylation found by Lehninger (37)

The enzymes responsible for oxidative phosphorylation are localized in the mitochondria so that a relationship between chloroplasts and mitochondria should exist. Vishniac and Ochoa (58) proved in their experiments with chloroplast-mitochondria systems that formation of ATP takes place with simultaneous oxidation of reduced phosphopyridine nucleotides by molecular O_2 . Investigations by Arnon (4, 6, 7, 10, 73), to be discussed in § 51, showed, however, that both intact chloroplasts and chloroplast fragments without any external enzyme systems—i.e., without mitochondria—are able to produce ATP without being able to fix CO_2 . It is also to be noted that cells

rich in chloroplasts are generally poor in mitochondria. Wassink (68, 69), Wintermans (76) and Kandler (31, 32) also observed the uptake of inorganic phosphate by illuminated chloroplasts. According to Wassink, it is possible that not ATP but another polyphosphate with a sulfhydryl group is produced. The existence of such a still unidentified organic phosphate ester is also claimed by Krall (35).

Allen *et al.* (1) showed that the rate of ATP production by chloroplasts, anaerobically, upon illumination is far higher than observed with mitochondria in oxidative phosphorylation. In § 57 it will be shown that, according to Kandler (33), the rate of phosphorylation in photosynthesis of whole cells is of the same magnitude as that of oxidative phosphorylation.

It must be pointed out however that Ohmura (45) observed oxidative phosphorylation with chloroplast fragments in the dark: these fragments showed the Hill reaction in the light when intermediate products of the tri-carboxylic acid cycle were added. Thus, it is possible to prepare chloroplast fragments which show oxidative phosphorylation and photosynthetic phosphorylation (see § 51). Ohmura concludes that the latter is nothing but a combination of water photolysis and oxidative phosphorylation. This assumption would accord with the views of Ochoa and Vishniac.

§ 51 Light Phosphorylation

Arnon (4–14, 56, 71, 72, 73) called ATP formation by illuminated intact chloroplasts *photosynthetic phosphorylation*. With the aid of light energy and under appropriate conditions, chloroplasts can synthesize ATP

According to Anderson and Fuller (2) and Newton and Kamen (41), isolated chromatophores of purple sulfur bacteria are also capable of light-dependent ATP formation.

The most important difference between photosynthetic phosphorylation and oxidative phosphorylation is that the former does not need molecular O_2 . In earlier work, Arnon (4) was not able to establish the role of DPN^+ or TPN^+ in light phosphorylation. Later, he found that TPN^+ —but not DPN^+ —acts as a catalyst, a further fact demonstrating the difference between the two types of phosphorylation, DPN^+ being indispensable to oxidative phosphorylation. Arnon's photoreduction of TPN^+ is expressed as follows

This reaction needs a TPN^+ -reducing factor which occurs in chloroplast extracts. It was found that when 1 mole O_2 is produced 2 mole TPN^+ are reduced and 2 mole inorganic phosphate are esterified, as the following equation shows

This reaction, which is the over-all reaction of both the light reactions indicated above, shows that only a part of the light energy would be used for ATP production (conversion into phosphate bond energy) and the remaining part would be used for the reduction of TPN^+ .

It is to be noted that in Lehninger's reaction of oxidative phosphorylation (§ 50) ATP formation is accompanied by oxidation of reduced phosphopyridine nucleotide. In Arnon's reaction ATP formation is accompanied by reduction of oxidized phosphopyridine nucleotide. However, Marrè and Forti (38) found that reduction of TPN^+ in illuminated chloroplasts is not dependent on the production or presence of ATP.

Thus, light energy provides ATP and TPNH. Both substances form what Arnon calls the *assimilatory power*. He considers them to be the true "first products of photosynthesis," a term usually applied to products formed during CO_2 fixation (see §53).

More recently, Arnon (5) succeeded in combining his various findings into an interesting hypothesis of light phosphorylation. Photosynthetic phosphorylation as shown in reaction a, in which ATP is the sole product of the light action, is designated as *cyclic photophosphorylation*. The process shown in the over-all reaction of a and b, in which light energy is used for the production of ATP and TPNH is termed *non-cyclic photophosphorylation*. This process, which provides O_2 and assimilatory power for the reduction of CO_2 , must be considered to be the light phase of photosynthesis in green plants. In cyclic photophosphorylation light energy is also converted into chemical energy, but it does not seem to be related to true photosynthesis in green plants as neither O_2 production nor CO_2 fixation is possible. This type of photophosphorylation is peculiar to the photosynthetic bacteria. They follow the vitamin K pathway of cyclic photophosphorylation (see Fig. 48). After absorption

Fig. 48. Arnon's scheme for photosynthesis

of light quanta, chlorophyll molecules are brought into the excited state and expel electrons which are transported in a cyclic system as follows:

A modification of the vitamin K pathway is the FMN pathway occurring in green plants. In this pathway the electrons also return to chlorophyll in a cyclic system. However, this cycle includes TPN^+ and a second cytochrome cyt_{11} . Both pathways of cyclic electron transport as well as the non-cyclic pathway for the generation of assimilatory power are shown schematically in Figure 48.

Arnon's general hypothesis of photosynthesis embracing green plants as well as photosynthetic bacteria does not necessitate water photolysis. In green plants the O_2 production results from an "open" non-cyclic electron transport. The electrons expelled from the excited chlorophyll molecules do not return to chlorophyll, as in the cyclic pathways, but are removed by TPN^+ , together with H ions originating from water in the presence of a light-dependent reductase.

The electrons removed must be continuously replenished. This is made possible by the interaction of the remaining OH ions from water and a cytochrome (cyt_{11}) peculiar to the green plant and not found in photosynthetic bacteria. Thus, the role of water is to produce H ions for TPNH formation and OH ions to yield molecular O_2 and donate electrons to a cytochrome chain. According to this hypothesis water is still the source of O_2 though the process of photolysis is not involved:

In bacterial photosynthesis external hydrogen donors are still needed. However, their function is not the reduction of a precursor of O_2 but merely the production of H to reduce phosphopyridine nucleotides (PN), i.e. produce one of the components of assimilatory power. The other component of assimilatory power is ATP which photosynthetic bacteria are able to produce with light energy by cyclic photophosphorylation. Figure 49 shows, according to Arnon, the reaction schemes of photosynthesis in green plants and photosynthetic bacteria.

Warburg (59) discovered that Cl ions are essential for photosynthesis in green plants. Arnon's experiments confirm this finding, Cl ions being required for reaction c as well as for the FMN pathway. They are not re-

quired for the vitamin K pathway which is the only type of bacterial photosynthesis. Thus, in the absence of Cl ions chloroplasts behave like chromatophores in being able only to follow the vitamin K pathway.

	GREEN PLANTS	PHOTOSYNTHETIC BACTERIA
LIGHT	CYCLIC PHOSPHORYLATION: $ADP + ph \longrightarrow ATP$ NON-CYCLIC PHOSPHORYLATION: $2 TPN^+ + 2 H_2O + 2 ADP + 2 ph \longrightarrow$ $2 TPNH + 2 H^+ + O_2 + 2 ATP$	CYCLIC PHOSPHORYLATION: $ADP + ph \longrightarrow ATP$
DARK	ASSIMILATION OF CO_2 : $CO_2 + 2 TPNH + 2 H^+ + nATP \longrightarrow$ $(CH_2O) + H_2O + 2 TPN^+ + nADP + nph$	REDUCTION OF PN: $2 PN^+ + 2 H_2 \longrightarrow 2 PNH + 2 H^+$ ASSIMILATION OF CO_2 : $CO_2 + 2 PNH + 2 H^+ + nATP \longrightarrow$ $(CH_2O) + H_2O + 2 PN^+ + nADP + nph$
OVER-ALL	$CO_2 + 2 H_2O \xrightarrow{LIGHT} (CH_2O) + O_2 + H_2O$	$CO_2 + 2 H_2 \xrightarrow{LIGHT} (CH_2O) + H_2O$

Fig. 49. Similarities and differences in photosynthesis of green plants and bacteria (Arnon)

Arnon (5, 13) considers that the Hill reactions are non-physiological variants of the over-all reaction, e.g.:

TPN^+ does not participate. It is replaced by the Hill reagents, e.g. ferric ions. This non-physiological non-cyclic photophosphorylation also requires Cl ions. According to Arnon's recent investigations, the reduction of TPN^+ does not result from water photolysis. In his earlier work it was the mysterious product [H] which elicited the reduction of TPN^+ . Today he claims that H ions from water, together with electrons expelled from excited chlorophyll molecules are sufficient for this reduction in the presence of a light-dependent reductase. Thus, water is split into H ions and OH ions, the latter being converted into O_2 . In photosynthetic bacteria the hydrogen donor which is still needed does not reduce CO_2 , as had been claimed by van Niel, but gives off hydrogen to reduce phosphopyridine nucleotides. In terms of comparative biochemistry, the common denominator of photosynthesis in green plants and in bacteria is, according to Arnon (5), the conversion of light energy absorbed by chlorophyll into phosphate bond energy. It is not the photochemical formation of a CO_2 reductant.

Thomas *et al.* (54) succeeded in preparing chloroplast fragments of *Spirogyra* which show evolution of O_2 as well as uptake of CO_2 without the addi-

tion of enzymes or cofactors to the suspension. The rates of both reactions were about the same as those observed in intact cells. These findings are in contrast to those of Arnon, who stated that the addition of certain enzymes and cofactors is indispensable for obtaining photosynthetic phosphorylation and CO_2 uptake in isolated chloroplasts and chloroplast fragments. Thomas *et al.* explain the discrepancy as follows. Arnon used spinach chloroplasts which are of the granulated type, whereas *Spirogyra* chloroplasts are lamellated, spiral-shaped chloroplasts that are grana-free. In the latter thin layers of stroma occur between bundles of lamellae (see § 3). This layered structure may be capable of protecting the stroma against damage far more effectively than may be possible in granulated chloroplasts. The lamellae are highly resistant to experimental conditions (crushing of the cells), whereas the granulated chloroplasts are easily damaged. The presence of intact stroma seems to be necessary if complete photosynthesis is to be obtained. Either the stroma contains important enzymes and cofactors or it protects enzymes located on the surface of the lamellae.

According to Arnon, the light reaction in photosynthesis only serves for the production of ATP and TPNH, these substances being necessary for the dark CO_2 fixation reaction:

Arnon (5, 8, 56) found that the assimilatory power, i.e., ATP and TPNH, generated in the light in the absence of CO_2 in the green water-insoluble parts of the chloroplasts (grana) is able to carry out CO_2 fixation in the dark in chlorophyll-free extracts (stroma) obtained by suitable centrifugation of the chloroplasts. These extracts contain the assimilatory power produced in the previous light reaction together with soluble enzymes. Small amounts of glucose-1-phosphate and $^{14}\text{CO}_2$ were added. The dark fixation of $^{14}\text{CO}_2$ by the chlorophyll-free extract to which assimilatory power was added was of the same order of magnitude as CO_2 fixation in the light in the presence of $^{14}\text{CO}_2$ since the commencement of illumination. The products of CO_2 fixation were identical in both cases, as was shown by paper chromatography and autoradiography. Instead of using ATP and TPNH generated by a light reaction, both substances were supplied from external, chemical or enzymatical sources. They were as effective in producing CO_2 fixation as the assimilatory power generated by illuminated chloroplasts.

Thus, Arnon found a physical separation of the light and the dark phase through the identification of ATP and TPNH produced in the light and the presence of CO_2 fixing enzymes in aqueous extracts of chloroplasts. The light phase is localized in the grana and the dark phase in the stroma (5, 8, 56). However, the necessity of the TPN^+ -reducing factor shows that, in the light, some stroma factors must be involved. It follows from these important investigations that CO_2 fixation of isolated chloroplasts must be considered to be a process independent of the photosynthetic pigment system.

It does not matter whether assimilatory power is produced by the action of light or is supplied to the system from outside sources. *In vivo*, however, photochemical generation of assimilatory power remains closely connected with CO_2 fixation.

More recently, Krogmann (36) provided evidence of a third type of light-dependent ATP synthesis—a type associated with the oxidation of the dye trichlorophenol indophenol. Incubation of spinach chloroplasts with this dye in its oxidized form, phosphate and ADP results in ATP synthesis only in the presence of light and O_2 . The oxidized dye is reduced by the action of light and reoxidized by O_2 . When the photoreduction of the dye is inhibited by certain chemicals (e.g. dichlorophenyl dimethylurea), no reoxidation by O_2 is possible and ATP synthesis stops. Upon addition of glutathione, cysteine or ascorbic acid, the dye can be reduced. This chemical reduction cannot be inhibited by dichlorophenyl dimethylurea. Light-dependent reoxidation does occur but, under these circumstances, light must have another site of action, as it cannot participate in a photoreduction process. Nakamoto *et al.* (40) described the effect of O_2 on other chloroplastic photophosphorylation systems. All these experiments clearly show how complex phosphorylation must be in photosynthesis and support the finding that photosynthetic phosphorylation may also be of the oxidative type.

In § 69 we shall further examine the problem of light induced phosphorylation in connection with Warburg's findings concerning quinone catalysis. According to Warburg's experiments light induced phosphorylation does not exist. It is nothing but oxidative phosphorylation in the dark.

§ 52 Some Final Remarks

In this section as well as in Section C of Chapter 4 the hypothesis of water photolysis forms the basis of many tentative approaches to the chemistry of photosynthesis. Recent investigations of Warburg have shown that the mechanism of the Hill reaction can be brought into accord with his findings in living *Chlorella*. The action of ferric ions and of quinone must be considered to be the same in isolated chloroplasts as in living *Chlorella*. The photodecomposition of water can thus be replaced by the photodecomposition of the photolyte CO_2^* . As a matter of fact, there is no evidence whatsoever that light quanta of low energy can decompose water (46), though efforts have been made to render this acceptable with the aid of excited states of chlorophyll, free radicals, etc. It seems extremely abnormal that water, which plays a most important but nevertheless more or less passive role in living Nature, should exert such a highly "explosive" action in photosynthesis. It must be admitted that nothing is impossible in Nature; nevertheless, it is hardly permissible to accept this adage as the only evidence of reactions we would like to see proceeding because they fit in well with our hypotheses.

Part of the O_2 of the photolyte certainly originates from water, due to the intermediary formation of H_2CO_3 . The origin of the photosynthetically pro-

duced O_2 is of no importance to those in favor of CO_2 photolysis. However, for those who accept water photolysis, all the O_2 produced *must* of necessity originate from water.

Comparative biochemistry contributed greatly to the general acceptance of water photolysis. However, the question arises whether it would not be possible to apply CO_2 photolysis to the photosynthetic sulfur bacteria.

In the green plant a part of O_2 is visibly produced. The remaining part is used in the induced respiration providing additional energy. In the anaerobic sulfur bacteria it is, of course, impossible that the O_2 produced is freely developed. It must be used entirely for a reaction, specific and indispensable to this organism, i.e., the oxidation of H_2S . A comparison of this reaction with that of induced respiration in plant photosynthesis shows some similarity insofar as both reactions provide additional energy for the conversion of CO_2 to CO_2^* . It is evident that these considerations are pure speculation. There is no proof whatever that the reactions indicated above would proceed in photosynthetic sulfur bacteria. The question is whether these reactions are less probable than those discussed in § 48 and whether experimental evidence instead of theoretical considerations will one day bring enlightenment.

It is doubtful whether comparative biochemistry, i.e. the thesis of unity in biochemistry, can serve as a reliable guide in all the fields of biochemical research. In any case, Cohen (19a), Stanier (53) and others warn against exaggerations and, thus, question the general validity of this thesis. Nevertheless, Bassham and Calvin (14) still take it for granted that Ruben's isotope studies and van Niel's suggestion clearly indicate that the photosynthetic O_2 has its ultimate origin in the O atoms of H_2O .

In Section D of this chapter the important role which must be imputed to CO_2 in the chlorophyll-polypeptide is discussed. It is becoming increasingly apparent that the photosynthetic process centers around CO_2 . CO_2 is not merely a reaction partner but must be considered to be the primary substance. In the green plant, CO_2 is loosened in the photolyte so that it can be split by the relatively weak light energy. The energy needed for the formation of the photolyte is provided by the induced respiration. It is very probable that a peroxide may be intermediately produced and that phosphorylations take place (§ 69).

It must be borne in mind that phosphorylation always occurs in respiration or, in other words, no phosphorylation, no respiration. The converse, no respiration, no phosphorylation, seems incorrect as Arnon found phosphorylation upon illumination independent of O_2 . Arnon does not take into consideration the induced respiration which, being respiration, must be con-

nected with phosphorylation. He considers back reactions unlikely since chloroplasts would not be able to carry out oxidative phosphorylation when supplied with O_2 in the dark with appropriate substrates and TPNH. It has already been pointed out that there is no photosynthesis without respiration. As there is no respiration without phosphorylation, we logically arrive at the conclusion that there is no photosynthesis without phosphorylation, i.e., oxidative phosphorylation.

As a matter of fact, the phosphate turnover in photosynthesis is still a subject of many contradictory investigations. As yet, anaerobic phosphorylation is the only kind of phosphorylation the chemistry of which is known (see the reaction with the oxidizing fermentation enzyme discovered by Warburg and Christian discussed in § 44). This is still not the case with oxidative phosphorylation and it is far less the case with phosphorylations proceeding upon illumination. The hypothesis of ATP formation by means of light energy may be an interesting outlet, but, for the time being, it belongs to the same category as water photolysis which was at the time an equally interesting outlet for which no evidence was available.

On the other hand there is evidence that reduction of TPN^+ takes place under the influence of light. However, we may readily admit here the same mechanism as found in the Hill reaction with ferric ions.

Traces of reactive C may be sufficient to drive the reaction. In the over-all reaction only TPN^+ determines the amount of O_2 produced. Until now this mechanism has not been proved.

Parts of Arnon's work are of importance, though the reduction of TPN^+ with simultaneous formation of ATP is rather unconventional. It does not seem impossible to bring Arnon's findings into accord with Warburg's theory. The light reaction $CO_2^* \rightarrow C + O_2$ could be accompanied by phosphorylation (See § 69).

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C. INTERMEDIATE PRODUCTS OF PHOTOSYNTHESIS

§ 53 The Carbon Cycle of Photosynthesis

To establish the intermediate products of photosynthesis, Calvin *et al.* (8, 9, 10, 19, 22) carried out a series of basic experiments with labeled CO_2 , opening up new interesting ways of research. The results of similar experiments have been published by Fager *et al.* (24). The test object *Chlorella* is illuminated during 10 to 60 sec in an atmosphere of $^{14}\text{CO}_2$. Then, the alga is killed in hot 80% alcohol and the alcoholic extract examined by means of paper chromatography and autoradiography. Even the first experiments showed that the first products to appear were carbohydrates; and after longer illumination the labeled carbon was also found in fats, amino-acids and proteins. As will be discussed later, other investigators found different sequences, e.g., amino-acids before sugars. By steadily decreasing the time of illumination, Calvin tried to trap the first CO_2 fixation product to appear; this was considered to be 3-phosphoglyceric acid. The further reduction of this substance to sugars was represented as follows:

The condensation of two molecules triose phosphate is the reversal of the anaerobic degradation of fructose-1,6-diphosphate (§ 44). The distribution of the radioactive isotope ^{14}C among the C atoms of 3-phosphoglyceric acid and of fructose-1,6-diphosphate shows that the CHO group of 3-phospho-

glyceraldehyde joins with the CH_2OH group of phosphodihydroxyacetone. Therefore, the labeled C atoms in fructose-1,6-diphosphate should be symmetrically distributed (see § 57).

Further research (18) showed, however, that ribulose-1,5-diphosphate was a precursor of 3-phosphoglyceric acid. This pentose diphosphate is carboxylated, producing 3-phosphoglyceric acid.

A labile ketoacid is intermediately produced (40). With this carboxylation reaction proceeding much faster than the carboxylation reactions discussed in § 46, the cycle represented in Figure 50 is started. The ribulose-1,5-diphosphate is regenerated, according to the Horecker cycle, thus enabling renewed uptake of CO_2 .

Ribulose-1,5-diphosphate is produced from ribulose-5-phosphate. Calvin assumed that one molecule 3-phosphoglyceraldehyde and one molecule sedoheptulose-7-phosphate are converted to two molecules ribose-5-phosphate. However, more recent investigations (7) have shown that in this conversion only one molecule ribose-5-phosphate and one molecule xylulose-5-phosphate are produced instead of two molecules ribose-5-phosphate. By the action of phosphoketopentose epimerase, xylulose-5-phosphate is changed into ribulose-5-phosphate. In the conversion of fructose-1,6-diphosphate + 3-phosphoglyceraldehyde to erythrose-4-phosphate + ribulose-5-phosphate, xylulose-5-phosphate occurs as an intermediate (see Fig. 50). The enzymes indicated in Figure 50 are needed for the various reactions of the carbon cycle. Their presence in cell-free extracts of green plants has been determined by Racker (47). When DPN^+ , ATP, labeled bicarbonate and small amounts of ribose-5-phosphate are added to such extracts, paper chromatography shows, after some time, the presence of labeled sugars and sugar phosphates.

The carbon balance of Calvin's cycle can be written as follows:

Fig. 50. The photosynthetic carbon cycle (Calvin).

1, Carboxydismutase. 2, Triose phosphate dehydrogenase. 3, Triose phosphate isomerase. 4, Aldolase. 5, TK. 6, Phosphatase. 7, Phosphopentoisomerase. 8, Phosphopentokinase. 9, Phosphoketopentose epimerases

According to Figure 50, the following reactions must also be considered

§ 54 Is Photosynthesis the Reversal of Respiration?

The main pathway of respiration is constituted by the sequence glycolysis, tricarboxylic acid cycle and biological oxidation. Ochoa (43) assumed that in photosynthesis the tricarboxylic acid cycle may proceed in the opposite direction. The reductive carboxylation of α -ketoglutaric acid to *iso*-citric acid, which is assumed to occur in photosynthesis, is indeed the reversal of the oxidative decarboxylation of *iso*-citric acid to α -ketoglutaric acid occurring in respiration. The same could be said for the reductive carboxylation of pyruvic acid and the oxidative decarboxylation of malic acid (44, 45), all the reactions of the tricarboxylic acid cycle being reversible. With the exception of the conversion of glucose to glucose-6-phosphate, all the reactions of glycolysis are also reversible.

Ochoa and Vishniac (46) reduced 3-phosphoglyceric acid to fructose-1,6-diphosphate upon illumination *in vivo*. This process goes as follows:

1. $2 \text{ DPN}^+ + 2 \text{ H}_2\text{O} \xrightarrow{\text{light}} 2 \text{ DPNH} + 2 \text{ H}^+ + \text{O}_2$
2. $2 \text{ 3-phosphoglyceric acid} + 2 \text{ ATP} \xrightleftharpoons{\text{Mg ions}} 2 \text{ 1,3-diphosphoglyceric acid} + 2 \text{ ADP}$
3. $2 \text{ 1,3-diphosphoglyceric acid} + 2 \text{ DPNH} + 2 \text{ H}^+ \rightleftharpoons 2 \text{ 3-phosphoglyceraldehyde} + 2 \text{ DPN}^+ + 2 \text{ ph}$
4. $2 \text{ 3-phosphoglyceraldehyde} \rightleftharpoons \text{fructose-1,6-diphosphate}$

In these experiments the chloroplasts were suspended in a solution of 3-phosphoglyceric acid containing ATP, DPN^+ , Mg ions and the necessary enzymes. Only reaction 1 is photochemical. No fructose-1,6-diphosphate was obtained without illumination. The over-all reaction

is the reversal of the degradation reaction of hexose phosphate to triose phosphate, in which reaction ATP is produced and DPN^+ reduced (see § 44). It was assumed that in the photochemical reaction light energy produces water photolysis. ATP serves as an additional energy source.

The investigations with labeled CO_2 carried out by Calvin (§ 53), Kandler

(§ 57) and Warburg (§ 61) show, however, that citric acid and other intermediary products of the tricarboxylic acid cycle do not belong to the primary products of CO_2 fixation. Moyse and Jolchine (29, 41, 42) observed that in certain leaves the tricarboxylic acid cycle proceeds in the light as freely as in the dark. Besides the carboxylation of ribulose-1,5-diphosphate proposed by Calvin, they found irreversible carboxylation of phospho-enol-pyruvic acid to produce oxaloacetic acid which is then reduced to malic acid (6, 55) (see § 46).

With the use of $^{14}\text{CO}_2$, the production of labeled malic acid was established. Moreover, labeled citric acid, *iso*-citric acid and fumaric acid as well as amino acids were found. These findings are in contrast to Calvin's views regarding the blocking of the tricarboxylic acid cycle by the photoreduction of lipoic acid (see § 55).

Warburg, also, does not admit photosynthesis to be the simple reversal of respiration. There is no definite evidence that the occurrence of the products of the carbon cycle cannot be due to the induced respiration. In Figure 51 some relationships between the carbon cycle and respiration are depicted schematically.

§ 55 The Significance of Lipoic Acid

When lipoic acid is added to a system of illuminated algae and quinone, the rate of O_2 production is considerably increased (15). It seems as if lipoic acid is intercalated between the light activation reaction and the quinone reduction. Calvin (20) originally assumed that lipoic acid acts as both a hydrogen acceptor and an oxygen acceptor

Fig. 51. Relationship between photosynthetic carbon cycle (bold lines), respiration and pentose phosphate pathway (thin lines) (Calvin).

Later, Calvin (19) regarded lipoic acid as a hydrogen acceptor only

The electron transfer of the process is best represented by the following reactions

Calvin and Barltrop (20) assume that lipoic acid reacts with chlorophyll as follows

The biradical produced accepts, according to Bickel and Kooymans (12), the H atoms of a hydrogen donor HD

It is evident that another compound must serve as the oxygen acceptor, i.e., the acceptor of electrons from O_2 . According to these views, illumination creates an excited state of chlorophyll (oxidation) and the energy of this state (electron energy) must be converted to chemical energy by means of the acceptor. Levitt (36) postulates the formation of the oxidized chlorophyll as follows:

Thus, the acceptor molecule removes an electron from the chlorophyll so that the remaining positive chlorophyll molecule can accept another electron from another source. This is the process represented in Figure 52. Hérisset (28), comparing chlorophyll and Mg-free pheophytin, considers the Mg atom should play an important role in photosynthesis.

In discussing the oxidative decarboxylation of pyruvic acid (§ 45) we learnt that lipoic acid acts as an indispensable cofactor. Lipoic acid is considered to be a part of the pyruvic acid dehydrogenase, so that it is supposed that this enzyme also plays an important role in photosynthesis. Calvin (18) observed that upon illumination practically no labeled carbon is found in intermediate products of the tricarboxylic acid cycle, in contrast to what happens

Fig. 52. Electron transfer of excited chlorophyll.

Fig. 53. The status of lipoic acid.

in the dark. Thus, it seems that the action of light blocks the link between the photosynthetic carbon cycle and the tricarboxylic acid cycle. The photo-reduction of lipoic acid would strongly inhibit the conversion of pyruvic acid to acetylcoenzyme A. Figure 53 shows the position of lipoic acid, schematically. This means that respiration proceeding in the dark through the tricarboxylic acid cycle—a process producing CO_2 —would be diverted in light to proceed through another cycle—the carbon cycle—showing uptake of CO_2 . According to Steward and Thompson (49), it is physiologically improbable that the tricarboxylic acid cycle is suppressed in the light. First of all, there is no proof that respiration proceeds only through this cycle. As there is no photosynthesis without respiration (see § 42), it is impossible to admit that green cells in the light do not respire. Secondly, Steward and Thompson assume that the tricarboxylic acid cycle may well proceed in the light with substrates other than those used in the dark, and utilize carbon sources drawn from amino-acids (e.g., glutamic acid) and synthesize proteins.

Fig. 54. The chlorophyll aggregate as a semiconductor.

According to a further hypothesis, chlorophyll acts photochemically not as a single molecule but as an organized, orientated aggregate. The lamellae structure of the grana discussed in § 3 support the view that the chlorophyll aggregate can be considered to be a kind of photoelectric battery and the absorbed light energy its driving force. On one side of the aggregate a fat or lipoproteid layer and on the other side a hydrophilic layer act as “electrodes.” The former would be composed of the lipid soluble lipoic acid and the latter would be water (Fig. 54).

Commoner's experiments discussed in § 9 lead to the assumption that the chloroplast acts as a semiconductor. Katz (35) and Calvin (9) compare the chlorophyll aggregate to a semiconductor. As in the transistor (Fig. 6), the electrons set free in the chlorophyll layer migrate to the lipoic acid layer where they are accepted by lipoic acid. The “holes” produced are occupied by electrons originating from water (Fig. 54). As sufficient water is present, no back reaction can take place. The lipoic acid molecules are continuously regenerated so that the electrons produced are continuously accepted.

According to Levitt (36), the chlorophyll excited by *one* quantum would give only *one* electron to lipoic acid and, after absorption of a second quantum, a second electron. Bearing this in mind, we can imagine what would happen at the “electrodes” of the chlorophyll aggregate. By the absorption of one quantum about 42000 cal/mole would be taken up. Assuming a loss of 5000 cal/mole, only 37000 cal/mole of the light energy would be available. Considering the relationship between potential and change of free energy

$$\Delta G = -23074 nE \quad (44)$$

we have for $n = 1$ a potential difference between the aforementioned layers of the chlorophyll aggregate (“electrodes”) of 1.6 volt. It is now possible to determine the potential of each of the “electrodes.” According to reaction b, the increase of free energy during the transfer of one electron to lipoic acid is about 7000 cal/mole, corresponding to a potential of 0.3 volt. The remaining energy is $37000 - 7000 = 30000$ cal/mole, corresponding to a potential of 1.3 volt. Thus, the potential difference of 1.6 volt is distributed over the photoelectric battery in such a way that the potential is 0.3 volt on the negative side (lipoic acid side) and 1.3 volt on the positive side (water side).

Fig. 55. Relationship between photosynthetic carbon cycle (bold lines) and respiration (dotted lines).

The following reaction is supposed to proceed on the positive side

In fact, this reaction corresponds to a potential of 1.2 volt. The H₂O₂ produced may react with cytochrome f

The Calvin scheme and its connections with respiration are shown in Figure 55. Calvin's views are intriguing but they are based upon quite a number of pure hypotheses needing further experimental evidence.

The significance of lipoic acid as the primary hydrogen acceptor still remains doubtful. Lipoic acid occurs in chloroplasts in low concentrations only. The chlorophyll concentration is about 1000 times that of lipoic acid, so that the chlorophyll aggregate should be composed of at least 1000 chlorophyll molecules. Arnon *et al.* (2, 4) and Geller (25) point out that water photolysis with isolated chloroplasts is not influenced by inhibitors of sulfhydryl groups (e.g., arsenite), findings which are certainly not in favor of the lipoic acid hypothesis. Biswas and Sen (13) incubated cells of *Scenedesmus* and *Chlorella* with labeled NaHCO_3 and various concentrations of lipoic acid. They observed practically no stimulation of CO_2 uptake upon illumination. By contrast, with concentrations higher than 0.25 mg/ml, a marked inhibitory effect of lipoic acid upon CO_2 uptake was noted. Similar experiments with simultaneous addition of quinone showed a slight increase of CO_2 uptake with low concentrations of lipoic acid. Paper chromatography revealed that, with low concentrations of lipoic acid, intermediate products of the tricarboxylic acid cycle and related amino-acids were more radioactive, and sugars and sugar phosphates less radioactive than without lipoic acid. According to Calvin, if lipoic acid were reduced to its thiol form, a greater incorporation of ^{14}C in sugars and sugar phosphates would be expected. It follows from these investigations that it is very doubtful whether lipoic acid is of significance in photosynthesis. Reed (48) arrives at the same conclusion.

§ 56 The Significance of Vitamin K

Wessels and Havinga (59, 60, 61) examined the Hill reaction with various quinones and dyes as hydrogen acceptors. They found that the reduction power of these substances is strongly dependent upon the redox potential. This is not astonishing, as the redox potential of a system is a measure of its reducing or oxidizing power. It was found that reduction does not occur with quinone derivatives and dyes having a redox potential lower than +0.040 volt. It would therefore be questionable whether DPN^+ and TPN^+ , which have a redox potential of -0.300 volt, could serve as a Hill reagent.

Wessels and Havinga suggest vitamin K_1 instead of lipoic acid as the primary hydrogen acceptor, i.e., reduced vitamin K_1 as the primary hydrogen donor to TPN^+ . The redox potential of vitamin K_1 is about -0.030 volt, whereas that of lipoic acid is about -0.300 volt. For a better understanding of the energetic situation, the various reactions are indicated below, the equations a and b of § 55 being repeated

or in the over-all reaction

In the case of vitamin K₁

or in the over-all reaction

For reaction b (lipoic acid) the redox potential is, according to equation 44,

$$E = - \frac{13800}{46148} = -0.300 \text{ volt}$$

and for reaction d (vitamin K₁) we find

$$E = - \frac{1500}{46148} = -0.032 \text{ volt}$$

It follows from these figures, which are not standard values, that, according to the findings of Wessels and Havinga, vitamin K₁ should have the preference over lipoic acid. The latter has the same low potential as TPN⁺ and could therefore not function as the primary hydrogen acceptor.

Accepting the requirement of one quantum per two electrons in reactions c and e, we find for the efficiencies in red

$$\text{lipoic acid: } \frac{43000}{51300} = 84\%$$

$$\text{vitamin K}_1: \frac{43000}{39000} = 110\%$$

Of course, an efficiency of more than 100% is impossible as has been pointed out by Bassham and Calvin (9). However, Wessels thinks that the reduction of TPN⁺ by reduced vitamin K₁ proceeds with the aid of energy-rich phosphate bonds produced in a partially back oxidation of reduced vitamin K₁. The denominator will therefore be higher, and give more reasonable figures. Figure 57 shows the various conversions in the vitamin K₁ cycle.

Generally speaking, there are some arguments in favor of the importance of this vitamin, as can be seen from its role in oxidative phosphorylation and its possible function in the respiratory chain. It is a striking fact that vitamin K₁ occurs in chloroplasts only and is not present in the cytoplasm. Anti-vitamin-K substances inhibit Hill reactions. The light-sensitive vitamin K₁ is protected by Cl ions. As Warburg and Lüttgens (56) and Arnon and What-

Fig. 56. Vitamin K₁ (2-methyl-3-phytyl-1,4-naphthaquinone).

Fig. 57. Vitamin K₁ cycle (Wessels).

ley (3) have shown, Cl ions exert a protective action upon chloroplasts *in vitro*. The fact that the molecules of vitamin K₁ and chlorophyll possess a phytol chain (Fig. 56) may also be of interest. Both substances probably occur in the monomolecular layers between the lipid and the protein-water lamellae of the grana. In spinach leaves the vitamin K₁ content amounts to about 0.04 mg per g dry weight, so that the ratio chlorophyll/vitamin K₁ is of the magnitude 200. The ratio chlorophyll/lipoic acid is about 1000.

According to Marrè and Servettaz (38), the reduction of TPN⁺ does not follow that of a compound with a more positive redox potential, such as vitamin K or FMN ($E = -0.06$). They found a distinct effect of TPN⁺ on the reduction of cytochrome c (or f) in illuminated chloroplasts and on the rate of photosynthetic phosphorylation, indicating that reduction of TPN⁺ precedes and conditions the production of ATP. This is in agreement with the findings of Marrè and Forti (37) mentioned in § 51. Thus, it seems that the role of TPNH in photosynthesis would be not only that of a reducing agent of some product of CO₂ fixation, but also that of an electron carrier in photosynthetic phosphorylation, as Arnon (4) postulated.

§ 57 Some Other Investigations

According to Calvin's hypothesis, the essential reduction process in photosynthesis is the reduction of 3-phosphoglyceric acid

This reaction is the reversal of the oxidation of triose phosphate discussed in § 44. According to Bassham *et al.* (11), the concentration of 3-phosphoglyceric acid in the light is about 30% lower and that of triose phosphate about 70% higher than in the dark. Nevertheless, it is assumed that in the light the reaction distinctly proceeds from left to right. Kandler (30) points out that this can only be possible when the concentrations of the necessary cofactors are changed accordingly and when ATP is present in particularly high concentrations. We learnt in § 53 that 3 mole ATP would be used per mole CO₂ in photosynthesis. Thus, Calvin postulates a stoichiometric relationship between CO₂ reduction and ATP turnover. In respiration, 6 mole ATP are produced per mole CO₂ (3 mol ATP per 1/2 mole O₂ used). When respiration of *Chlorella* is overcompensated 40-fold, 40 mole CO₂ are reduced in photosynthesis against one mole CO₂ produced in respiration. This means that the ATP turnover is increased 20-fold. However, the investigations of Kandler (30), Strehler (50) and Bradley (14) clearly show that the ATP content of the cells does not increase upon illumination. A stoichiometric relationship between CO₂ reduction and ATP turnover is therefore improbable. These results make the conversion of 3-phosphoglyceric acid to triose phosphate questionable.

The symmetric distribution of radioactivity in fructose-1,6-diphosphate is due to the condensation of two similar trioses. Investigations by Kandler and Gibbs (27, 34) show a distinct asymmetrical distribution as can be seen from the following reaction scheme:

The C₄ atom of fructose-1,6-diphosphate shows the highest radioactivity. The labile intermediate compound—a carboxylation product of ribulose-1,5-diphosphate (cf. Calvin's intermediate ketoacid)—is reduced in the light, and a polyhydroxyacid, probably hamamelonic acid, is produced as an intermediate (31, 32). It may be that, instead of the carboxyl group of the labile compound, a peroxide group is formed being able to split off O₂ exothermically. This splitting would be associated with its reduction to aldehyde.

It is to be noted that according to these views O₂ is produced from CO₂. Kandler suggested that Warburg's labile CO₂ would thus more or less find its place in this reaction scheme, a somewhat bold assumption indeed (see § 59).

Furthermore, Kandler assumes that the labile intermediate compound may partially produce 3-phosphoglyceric acid, providing the cells with other important constituents such as amino-acids. This is represented in Figure 58. By means of transaldolation and transketolation, a part of the fructose-1,6-diphosphate produced is converted to ribulose-1,5-diphosphate again, so that Kandler's cycle may be written as follows

According to the over-all reaction, the minimal requirement of ATP is only 1 mole per 6 mole CO₂. The mole ATP used in the cycle compensates

Fig. 58. Kandler's scheme. Separation of photosynthesis and respiration.

for the loss of inorganic phosphate due to the conversion of fructose-1,6-diphosphate to saccharides. The ATP turnover in the light at 40-fold overcompensation is thus not increased 20-fold but remains practically constant.

In the light, the labile intermediate compound is reduced, via hamamelonic acid, to fructose-1,6-diphosphate when sufficient reducing power is available. If not, the labile compound is slowly split to 3-phosphoglyceric acid (respiration). Kandler compares this clear separation of photosynthesis and respiration with Warburg's induced respiration. It is difficult however to find anything common to the mechanisms. Thus, according to Kandler, the production of 3-phosphoglyceric acid from ribulose-1,5-diphosphate is a step in the direction not of photosynthesis, but of respiration. The decisive, photochemical reduction mechanism in the direction of photosynthesis starts with the conversion of the labile compound to hamamelonic acid.

Bascham *et al.* (8) found, after extrapolation to very small times, the activity of 3-phosphoglyceric acid to be only 75% of the theoretical value of 100%. The sugar phosphates showed an activity not of 0% but of about 17%. These figures justify the assumption that 3-phosphoglyceric acid and sugar phosphates are simultaneously produced from a common, not yet identified precursor substance. There are two possible explanations for this deviation from the theoretical values of 100% 3-phosphoglyceric acid and 0% sugar phosphates. Either the alcohol added to the extracts stops carboxylation more rapidly than reduction, or the enzymatic processes occurring after the production of 3-phosphoglyceric acid are much faster than the formation of 3-phosphoglyceric acid itself. The latter explanation would imply the dependence of the activity distribution upon the interactions of substrates and enzymes, so that it would be independent of the sequences in the carbon cycle.

For this reason, only the first explanation is worthy of consideration. Kandler (33) found that alcohol poisoning blocks the reduction reactions which lead to the formation of sugar phosphates somewhat more rapidly than the carboxylation to 3-phosphoglyceric acid. Thus, the statement made by Bassham *et al.* that the reduction of 3-phosphoglyceric acid may continue to a certain extent in the alcoholic extract seems unjustified. By contrast, the alcoholic extraction may give rise to some splitting of labile intermediate products produced in the photosynthetic process to 3-phosphoglyceric acid and cause further deviations from the real activity values.

Though Kandler's investigations may upset the correctness of Calvin's carbon cycle, many questions remain unanswered. The hypothetical labile intermediate compound is difficult to visualize chemically.

Tolbert and Zill (51, 52, 53) observed that a part of the fixed ^{14}C is excreted in the culture medium as glycolate. Calvin *et al.* (21) also found that glycolate appears among the first labeled products. Warburg (58) obtained, in experiments with the addition of blue-green light, γ -values approaching 1.3 which corresponds to the formation of glycolic acid ($\text{CH}_2\text{OH}\cdot\text{COOH}$) from CO_2 and H_2O (see § 36). As has been discussed in § 54, Moyse and Jolchine obtained labeled malic acid and other acids of the tricarboxylic acid cycle from certain leaves. In § 61 we shall discuss important investigations by Warburg from which it follows that ^{14}C -labeled amino-acids appear prior to 3-phosphoglyceric acid.

Gibbs (26) postulated that during photosynthesis CO_2 is reduced first to formate and then to the formaldehyde level and transferred to either a C_5 or a C_2 acceptor to form a hexose or a triose respectively. ^{14}C -labeled formate was added to *Chlorella* and after 4 min of photosynthesis the tracer in the glucose was located in the C_4 atom. This primary reduction to formate is reminiscent of the early hypothesis of Willstätter and Stoll (62) according to which formate bound to the Mg atom of chlorophyll should be intermediately produced.

§ 58 Some Final Remarks

The use of heavy and radioactive isotopes as tracers has considerably enriched our knowledge of metabolic processes. However, it must be kept in mind that the tracer methods may give rise to erroneous conclusions if the interpretations of experimental results are incorrect or when certain necessary experimental conditions are not fulfilled. We have met examples of this in § 42 and § 48. The various investigations with labeled carbon for determining substances produced in photosynthesis show relatively little agreement. In principle, it is possible that labeled carbon appears in all the products of intermediate metabolism when cells are exposed to an atmosphere containing $^{14}\text{CO}_2$. There is no irrefutable evidence that the labeled products found concern photosynthesis only. Furthermore, there is still considerable ignorance of and disagreement on the fate of $^{14}\text{CO}_2$ in the dark. It is ex-

tremely difficult to separate CO_2 fixation upon illumination from that which normally takes place in the dark. Investigations by Miyachi *et al.* (39) clearly show how slight our knowledge is with respect to CO_2 fixation in the dark aerobically and anaerobically and how careful one has to be in concluding whether CO_2 fixation observed in a given experiment is really related to photosynthesis or to other metabolic conversions.

Wood (63) and Utter and Wood (54) point out that it must be far from easy to determine the first product of photosynthesis in which labeled carbon is incorporated. The sequence of the products which occur in the carbon cycle after short illumination is deduced from the measured activities of these products. When, for instance, CO_2 is reduced to (CH_2O) via the compounds A_1, A_2 etc. A_1 must attain the same specific activity as CO_2 before A_2 does, A_2 must reach it before A_3 , etc. This has been correctly observed by Calvin (21). Figure 59 shows a theoretical example by Utter and Wood. CO_2 can be reduced to (CH_2O) in three ways, according to the sequences A_1, A_2 etc.,

Fig. 59. Different ways of ^{14}C incorporation in photosynthesis (Wood).

B_1, B_2 etc. and C_1, C_2 etc. The reaction times are represented by the lengths of the arrows. It is supposed that by decreasing the time of illumination the sequences B and C will become more and more improbable so that finally only sequence A will come into consideration. This, however, does not mean that sequence A is the most important. It may be possible that in sequence A the reaction $A_3 \rightarrow A_4$ proceeds so slowly that the over-all speed of the reaction $\text{CO}_2 \rightarrow (\text{CH}_2\text{O})$ in sequence A might be of the same magnitude as that of the sequences B and C . If A_1 represents 3-phosphoglyceric acid, labeled carbon certainly appears very rapidly in the carboxyl group of this acid, but this provides no evidence that 3-phosphoglyceric acid is the primary product on the way to (CH_2O) and that all other ways leading to this end product are excluded. As a matter of fact, Badin and Calvin (5) observed, at low light intensities, carboxylation to malic acid as the first reaction (see § 46). Thus, in any case CO_2 fixation can occur in two ways: at high intensities via 3-phosphoglyceric acid and at low intensities via carboxylation to a dicarboxylic acid. There is no reason to believe that other reactions may not be involved if the experimental conditions are changed.

In investigations with radioactive isotopes measurement is made not of the specific activities but of the counts per minute (cpm). However, the relationship between the specific activity of a substance produced and that of $^{14}\text{CO}_2$ is of much more importance than the cpm values (63). When, for instance, 1 $\mu\text{mole } A_1$ gives 2000 cpm and 0.1 $\mu\text{mole } A_2$ 1000 cpm, the latter

substance has a far higher concentration of labeled carbon than the former. Thus, A_2 should have its place in the sequence before A_1 and not after A_1 , even though A_2 has a lower cpm value.

For analysis, the labeled products are extracted from the whole cell, though the reactions involved occur in certain areas of the cell (grana, mitochondria). Only the specific activity at the site of photosynthesis is of importance. The shorter the exposures to light, the less the likelihood that the decisive equilibria have been reached throughout the whole cell. The extracted compounds therefore do not give a true picture of what happens at the site of photosynthesis.

As will be discussed in § 61, Warburg in similar experiments found glutamic acid to be one of the primary products in photosynthesis. Although Calvin observed that *Chlorella* extracts are very rich in glutamic acid, he did not find any noteworthy incorporation of labeled carbon in this amino-acid. It could be concluded from this finding that the tricarboxylic acid cycle does not participate in photosynthesis, as one of its intermediate products, α -ketoglutaric acid, is in a state of equilibrium with glutamic acid. It is highly probable that the tricarboxylic acid cycle is not involved in photosynthesis, but the exclusion of glutamic acid is certainly not justified. We shall see later that glutamic acid, which is part of the chlorophyllprotein, is of the greatest importance in photosynthesis (see § 64).

There is some morphological evidence that chlorophyll occurs in the chloroplast in units each containing several hundreds of molecules. In some hypotheses a physiological significance has been attributed to such units. According to Calvin and others, the chlorophyll aggregate having semiconductive properties would be composed of about 1000 molecules. Arnold and Meek (1) believe that one quantum absorbed deals with several hundreds of chlorophyll molecules. For the time being, the assumption of chlorophyll units with physiological functions is still speculation, though such units may perhaps exist from a morphological point of view. Emerson and Arnold (23) determined with very short-lasting light flashes (10^{-5} sec) of high intensities and long dark intervals the maximal O_2 production during one light flash. They compared the amount of O_2 observed with the chlorophyll content of the cells, and calculated that 2500 chlorophyll molecules are involved in the production of one molecule O_2 . Warburg *et al.* (57) directly measured the light reaction without intermittent light and found, per one molecule chlorophyll, the production of one molecule O_2 (see § 39). Thus, a comparison of the ratio chlorophyll/ O_2 with intermittent and with continuous illumination reveals a discrepancy of three powers of 10. Burk (16, 17) pointed out that Emerson's calculations are based upon the assumption that enough light is absorbed per flash to permit complete photosynthesis. In reality, however, it is impossible to imagine light saturation during such short illumination times. The light intensity of the flashes is some 1000 times too low, i.e., absolutely insufficient to decompose the photolyte in 10^{-5} sec. Burk regrets with good

reason that investigations of this kind, which, lacking physical significance, contributed so much to the stoichiometric relationship between chlorophyll and O_2 not having been discovered much earlier.

The photosynthetic carbon cycle as proposed by Calvin and his group is based upon a series of hypotheses. Like many other contributions to the chemistry of photosynthesis, it is based upon the photolysis of water, a hypothesis which seems to be improbable today. The participation of primary hydrogen acceptors such as lipoic acid or vitamin K_1 lacks support. Further, there is not sufficient evidence to assume a chlorophyll aggregate and its semi-conductive properties. There is no doubt that the work done with $^{14}CO_2$ has opened new and interesting perspectives in the field of photosynthesis. However, too hasty conclusions and too much combination of experimental results with unproven theoretical considerations are extremely dangerous. They may certainly lead to intriguing schemes which suggest the whole problem is solved, but as long as no definite evidence is provided, there is no reason whatever to accept them as true and irrefutable.

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D. CARBON DIOXIDE

§ 59 The Fluoride Reaction

If stoichiometric O_2 production is to take place after a dark period, the presence not only of O_2 but also of CO_2 is necessary. Without CO_2 , re-illumination would not yield any O_2 (14, 22). Warburg *et al.* (4, 16) found that *Chlorella* contains chemically bound CO_2 which is stoichiometrically connected with chlorophyll. Warburg calls this CO_2 fraction the labile CO_2 of photosynthesis.

Fig. 60. Manometer vessel for the determination of labile carbon dioxide. S: *Chlorella* suspension. F: NaF solution.

Fig. 61. Anaerobic removal of labile carbon dioxide in the dark by NaF from 200 μ l *Chlorella* (pH 3.8, 20° C). Chlorophyll content: 3.83 μ mole. Curve I: without NaF. Curve II with $1/80$ N NaF. Pressure difference: 49.5 mm. $K_{CO_2} = 1.73$ mm². CO_2 removed = $49.5 \times 1.73 = 85.7$ μ l = 3.86 μ mole (Warburg *et al.*, *Zschr. Naturf.*).

The addition of NaF to *Chlorella* removes all the labile CO_2 . A conical manometer vessel (Fig. 60) contains a *Chlorella* suspension (e.g., 200 μ l cells in 3 ml salt solution at pH 3.8). The gas phase contains pure, CO_2 -free argon and the side-arm a solution of NaF (e.g., 0.2 ml 0.2 N NaF at pH 3.8). The fluoride is given to the cells in the dark and CO_2 is developed. A clear-cut end value is reached after about 20 min. As Figure 61 shows, the amount of CO_2 removed in the dark from 200 μ l cells in 25 min is 85.7 μ l = 3.83 μ mole, i.e., about 43% of the cell volume (more than the amount of O_2 erythrocytes can bind). In the experiment shown in Figure 61, the chlorophyll content is 3.83 μ mole* so that the stoichiometric relationship between chlorophyll and labile CO_2 is obvious. It is possible to vary the chlorophyll content by cultivating at various light intensities. The end value of the CO_2 removed will, however, vary accordingly. These experiments prove that the

* This high value corresponds, at 22.5% dry weight, to a chlorophyll content of 7.7%.

CO₂ removed by NaF must be linked in some way or other with chlorophyll.

If the NaF concentrations are varied, the end values remain the same but the lower the NaF concentration, the slower the rate of CO₂ removal. The CO₂ removal by NaF is not a simple reaction. It is inhibited by small amounts of HCN and by heating for 5 min at 65° C. Thus, it seems that NaF does not have a direct action. Its addition starts an enzyme reaction responsible for the CO₂ removal by means of a heavy metal compound. The addition of phenanthroline does not inhibit the action of NaF, so that the enzyme does not seem to be a dissociating heavy metal compound (see § 31). As phenylurethane does not influence the CO₂ removal, it may be concluded that the enzyme is not structure bound.

The fluoride reaction is reversible if the concentration of NaF is not too high. When the NaF is washed out after CO₂ removal, the cells are again able to take up CO₂. This renewed uptake is only possible in the presence of O₂. Therefore, the binding of labile CO₂ in the cells is not a reaction by itself but needs respiration, i.e., energy.

Warburg and Krippahl (15) established that the addition of a drop of octanol to *Chlorella* removes 1 mole CO₂ from 1 mole chlorophyll. This CO₂ removal is irreversible because of the cytolytic properties of octanol. It follows from this that chlorophyll isolated by means of organic solvents does not contain labile CO₂. As a matter of fact, there is no place for CO₂ in the chlorophyll formula. We must therefore distinguish between isolated and functional chlorophyll, i.e., between "dead" and "living" chlorophyll (see § 2).

Numerical example (15):

Vessel I: $V_F = 3.2$ ml $V_G = 15.48$ ml $K_{CO_2} = 1.725$ mm²

Vessel II: $V_F = 3.2$ ml $V_G = 15.53$ ml $K_{CO_2} = 1.732$ mm²

Per vessel 200 μl cells in 3.0 ml salt solution, pH 3.8. Chlorophyll content 7.7%, i.e., at 22.5% dry weight: 3.83 μmole. In the side-arm of vessel I: 0.2 ml H₂O. In the side-arm of vessel II: 0.2 ml 0.2 N NaF (pH 3.8). Gas phase: argon. Experiment carried out in the dark. Temperature: 20° C.

	<i>Vessel I</i> Pressure Changes After Tipping	<i>Vessel II</i> Pressure Changes After Tipping
2.5 min	+0.5 mm	+32.0 mm
2.5 min	0 mm	+11.5 mm
5 min	+1.0 mm	+ 7.0 mm
5 min	+0.5 mm	+ 1.0 mm
10 min	+1.0 mm	+ 1.5 mm
20 min	+0.5 mm	0 mm
45 min	+3.5 mm	+53.0 mm

Vessel I: $x_{CO_2} = 3.5 \times 1.725 = 6.0$ μl. Vessel II: $x_{CO_2} = 53.0 \times 1.732 = 91.8$ μl.
Labile CO₂: $91.8 - 6.0 = 85.8$ μl = 3.86 μmole

$$\frac{\text{labile CO}_2 \text{ in } \mu\text{mole}}{\text{chlorophyll in } \mu\text{mole}} = \frac{3.86}{3.83} = 1.0$$

§ 60 The Reaction Equations of Photosynthesis

The stoichiometric relationships between chlorophyll and O_2 and between chlorophyll and CO_2 show that the O_2 produced in the light originates from the CO_2 bound to chlorophyll. Warburg (22) shows this clearly in the following experiment: a cell suspension is divided into three samples. All CO_2 is removed from the first sample by NaF, i.e., 1 mole CO_2 per 1 mole chlorophyll. From the second sample only 0.5 mole CO_2 per mole chlorophyll is removed and from the third sample no CO_2 is removed. Afterwards the three samples are saturated with 10 vol % CO_2 . Illumination shows that no O_2 can be produced in the first sample, only 0.5 mole O_2 is produced in the second sample and 1 mole O_2 per mole chlorophyll in the third sample. When NaF is washed out of the first sample, so that CO_2 can combine again with chlorophyll, 1 mole O_2 is produced per mole chlorophyll upon illumination. The conclusion of this very instructive experiment is that upon illumination the O_2 produced originates only from the labile CO_2 , i.e., from the CO_2 which can be removed from chlorophyll by the addition of NaF. All other forms of CO_2 present in the cells (dissolved CO_2 , bicarbonates) are not influenced by the action of light. As already shown in § 35, O_2 capacity and labile CO_2 and their connections with chlorophyll can be represented by the following reaction equations:

Reaction 1 (light):

Reaction 2 (dark):

Reaction 3 (dark):

Over-all reaction:

The production of the photolyte (reaction 3) is not a simple reaction in itself. It needs about 70000 cal/mole, an amount of energy produced by induced respiration (reaction 2).

Of the 110000 cal necessary to split one mole CO_2 , 70000 cal are supplied by a respiratory process and the remaining 40000 cal are provided by one mole quanta. All difficulties with respect to the quanta are eliminated by this scheme. As Warburg (6) pointed out, all has been found experimentally and measured in living *Chlorella*. Reaction 1 is measured by O_2 production and CO_2 consumption in the light. Reaction 2 is measured by O_2 consumption and CO_2 production in the dark. Reaction 3 is measured by the time of recovery that elapses before the light is again able to produce as much O_2 as in reaction 1. This recovery period lasts about 20 min in Warburg's experiments (see § 41).

§ 61 Amino-acids in *Chlorella*

Warburg *et al.* (10, 11) found that *Chlorella* contains a very active glutamic acid decarboxylase which anaerobically splits glutamic acid into γ -aminobutyric acid and CO_2 . Under aerobic conditions the reaction proceeds in the opposite direction.

With respect to HCN, this reaction behaves like the fluoride reaction, so that it was supposed that the latter is nothing but decarboxylation of glutamic acid. It has indeed been established that the CO_2 removed by NaF originates from glutamic acid. It has been possible to show by means of paper chromatography that during the fluoride reaction glutamic acid disappears and γ -aminobutyric acid is produced. *Chlorella* cultivated under special conditions is able to develop CO_2 anaerobically without the addition of NaF (see § 62). Paper chromatography with aspartic acid, glutamic acid, alanine and γ -aminobutyric acid as test substances showed, according to Warburg (6), that *Chlorella* under its normal living conditions contains little aspartic acid, much glutamic acid, very much alanine but no γ -aminobutyric acid.

Warburg *et al.* (6, 12) used labeled CO_2 to study the behavior of glutamic acid in photosynthesis. A manometer vessel contains a *Chlorella* suspension. The side-arm of the vessel is divided into two compartments, one containing ^{14}C -carbonate and the other an excess of lactic acid. When the lactic acid is given to the carbonate, a pressure of radioactive CO_2 develops in the vessel. The vessels prepared in this way are illuminated for 0.5, 1 and 5 min. Afterwards, they are, together with control vessels kept in the dark, immersed for 5 min in water at 75°C to stop enzymatic reactions and, at the same time, to extract the soluble substances from the cells. The extracts obtained are analyzed by two-dimensional paper chromatography and the activities of the spots measured with a Geiger counter. It follows from these experiments that amino-acids become radioactive much more rapidly than 3-phosphoglyceric acid, which was generally thought to have priority in similar investigations. Furthermore, it was found that aspartic acid and alanine became radioactive more quickly than glutamic acid. From this it may be concluded that a chain of amino-acids acts as a catalyst in *Chlorella*. In this peptide chain glutamic acid does not seem to occupy the first place.

In his investigations on chloroplastin, Kaufmann (1) observed that the grana proteins of various higher plants contain a series of loosely bound amino-acids, glutamic acid being quantitatively predominant. The grana were washed by the method of Warburg and Lüttgens (3) to remove the greater part of the glutamic acid. Thus, living chlorophyll must be considered to be

a chlorophyll peptide composed of aspartic acid, alanine and glutamic acid the last-mentioned being of particular importance in photosynthesis.

Chlorella contains glutamic acid in concentrations varying from 0.5 to 1.3% of the cells' dry weight, depending on the method of cultivation employed. In chlorophyll-rich cells (5 to 8%) 1 to 2 mole glutamic acid is present per mole chlorophyll. It is possible that in such cells all the glutamic acid is bound to chlorophyll. In chlorophyll-poor cells only a part of the glutamic acid is bound to chlorophyll and the remaining part exists in a free state.

It is an important fact that O_2 inhibits the breakdown of glutamic acid. The addition of NaF increases respiration and the energy derived from the increased respiration resynthesizes the decomposed glutamic acid until an equilibrium state is reached between decomposition due to NaF and resynthesis due to respiration. Anaerobically, 0.001 *N* NaF completely decomposes glutamic acid. In the presence of O_2 the decomposition is incomplete because of the above-mentioned resynthesis.

The action of light decreases as the glutamic acid decomposes, and increases as it is resynthesized. Thus, glutamic acid must be closely connected with the light action. Two different NaF concentrations are added, in the presence of O_2 , to a *Chlorella* suspension. The decomposition of glutamic acid and the decrease in the light action are measured when the steady state is reached. Table 19 clearly shows the close relationship mentioned (6, 13).

TABLE 19
Glutamic acid and light action

NaF Concentration	Remaining Glutamic Acid in %	Remaining Light Action in %
$1/640$ <i>N</i>	79	82
$1/320$ <i>N</i>	36	36

The objection could be raised that NaF inhibits the light action not because of the decomposition of glutamic acid, but because of some other, unknown reasons. A comparison of the inhibition of the light action by 0.001 *N* NaF under anaerobic and aerobic conditions shows—as already mentioned—that all the glutamic acid is decomposed under anaerobic conditions and that little glutamic acid is decomposed under aerobic conditions. With the same NaF concentration, the inhibition of the light action is very pronounced under anaerobic conditions and very weak under aerobic conditions. Thus, under all conditions the decomposition of glutamic acid runs parallel to the decrease in the light action; it must be concluded that the former is the cause of the latter. The inhibitory action of long-lasting withdrawal of O_2 upon photosynthesis must be attributed to the decomposition of glutamic acid.

The addition of O_2 results in glutamic acid being resynthesized and photosynthesis starting again. This is further evidence of the absolute necessity of O_2 in photosynthesis (see § 42).

Under appropriate cultivation conditions, the ratio between the chlorophyll content, the O_2 capacity and the CO_2 removed in the dark after the addition of NaF is 1:1:1. This very important observation has been confirmed by Vishniac and Fuller (2) with *Chlorella*, *Scenedesmus* and *Euglena*, using continuous and fluctuating illumination and cultivation at pH 6.8 instead of 3.8. After removing the CO_2 with NaF, the algae were washed and allowed to recover $^{14}CO_2$ in the presence of O_2 . After further addition of NaF the CO_2 produced was radioactive: it was concluded that the CO_2 fixed during the recovery is located at the same site as the CO_2 removed by NaF. Paper chromatography showed 90% of the radioactivity in the α -carboxyl group of glutamic acid. Chlorophylls a and b did not exhibit any radioactivity.

§ 62 Lactic Acid Fermentation in *Chlorella*

As has been stated in § 15 and § 61, the glutamic acid content of the cells depends upon the kind of cultivation. The behavior of the X-cells is particularly atypical (§ 15). They are produced in very strongly illuminated cultures—without day light—with four 200 watt metal filament lamps. The action of light in the X-cells is at least 10 times smaller than in other types of cells, probably to counteract over-illumination during cultivation. Anaerobically, the X-cells produce lactic acid in far greater amounts and at a higher rate than ordinary cells (13). Indeed, it has been found that anaerobic lactic acid fermentation depends upon cultivation conditions (8). The immediate inhibition of photosynthesis upon withdrawal of O_2 has, however, no connection whatever with this lactic acid formation. This inhibition is solely the result of the fact that induced respiration cannot occur in the absence of O_2 .

In 1936 Warburg and Christian (7) demonstrated that lactic acid fermentation proceeds as follows (see also § 44)

It should be noted that the active enzyme of *Chlorella*, lactic acid dehydrogenase, is a D-enzyme and the lactic acid produced is D-(−)-lactic acid. As yet, no other D-enzyme has been observed in *Chlorella*. Glutamic acid decarboxylase and malic acid dehydrogenase, which also occurs in *Chlorella*, are L-enzymes. The alanine present in large amounts in the alga is L-alanine.

The significance of lactic acid in photosynthesis probably lies in its regulation of the pH in *Chlorella* to assure the optimal value for the activation of glutamic acid decarboxylase. Figure 62 shows that D-lactic acid—but not L-lactic acid—exerts an influence upon light absorption. Both isomers of

lactic acid are added to a solution containing *Chlorella* extract and DPN^+ . The light absorption at 3400 Å increases only when D-lactic acid is added, as only this isomer can be dehydrogenated to pyruvic acid by D-lactic acid dehydrogenase.

Fig. 62. The lactic acid dehydrogenase of *Chlorella* and D- and L-lactic acid. Curve I: L-(+)-lactic acid. Curve II: D-(-)-lactic acid. Only curve II shows light absorption at 3400 Å indicating that only D-lactic acid reduces DPN^+ (Warburg *et al.*, *Zschr. Naturf.*).

The X-cells exhibiting high lactic acid production are particularly useful when the glutamic acid of *Chlorella* is to be decomposed without adding NaF. Owing to lactic acid formation in these cells, glutamic acid is decomposed anaerobically without NaF and aerobically resynthesized.

§ 63 Breakdown and Resynthesis of Glutamic Acid

Lyophilized cells of *Chlorella* cannot divide and cannot reduce CO_2 upon illumination. When suspended in water or in salt solutions, they are able to decarboxylate L-glutamic acid but not other amino-acids or pyruvic acid. They are thus particularly useful for determining glutamic acid. The side-arm of a conical manometer vessel contains 20 mg lyophilized cells. The vessel itself contains 3 ml of the glutamic acid solution to be analysed (pH 3.8). After tipping the contents of the side-arm into the vessel, the pH increases to about 5. The temperature is 20°C and the gas phase contains argon only. Figure 63 shows that after 280 min 4.49 μmole CO_2 are produced by decarboxylation of the 4.8 μmole glutamic acid added (curve II). Curve I represents a control experiment without the addition of glutamic acid. The CO_2 of curve I originates from the glutamic acid present in the lyophilized cells where it is so tightly bound that extraction is very difficult even at 100°C .

The breakdown and resynthesis of glutamic acid can also be demonstrated by means of paper chromatography. Various concentrations of NaF are added under anaerobic and aerobic conditions to a suspension of living *Chlorella* (100 μl cells in 3 ml salt solution, pH 3.8, temperature 20°C). When the

Fig. 63. Decarboxylation of L-glutamic acid under anaerobic conditions. 20 mg *Chlorella* powder, pH 3.8, 20° C. Curve I: 3 ml salt solution in the manometer vessel. Curve II: 3 ml salt solution + 4.8 μ mole L-glutamic acid in the manometer vessel. $a = 4.49 \mu$ mole CO_2 ; $b = 0.96 \mu$ mole CO_2 (Warburg *et al.*, *Zschr. Naturf.*).

Fig. 64. Influence of NaF on photosynthesis under aerobic conditions. 100 μ l cells suspended in 7 ml salt solution in each manometer vessel. pH 3.8, 20° C. Gas phase: 10 vol % CO_2 , 30 vol % O_2 , 60 vol % argon. Curve I: without NaF; $V_G = 10.808$ ml, $K_{\text{O}_2} = 1.031$ mm², $K_{\text{CO}_2} = 1.624$ mm². Curve II: with 0.001 N NaF; $V_G = 10.624$ ml, $K_{\text{O}_2} = 1.016$ mm², $K_{\text{CO}_2} = 1.609$ mm². Incident light intensity: 200 μ l quanta/min. Result: practically no inhibition of photosynthesis (Warburg *et al.*, *Zschr. Naturf.*).

steady state is reached, the suspension is centrifuged, the cells are again suspended in 3 ml water and heated to 90° C for 5 min. The liquid is centrifuged again, dried *in vacuo* and examined. The chromatograms show the presence of aspartic acid, glutamic acid, alanine and γ -aminobutyric acid. There are no variations in the concentrations of aspartic acid and alanine. Table 20 shows the expected changes, which have already been discussed, with regard to glutamic acid and γ -aminobutyric acid.

The experiments with lyophilized cells and those using paper chromatography clearly show that the labile CO₂ of *Chlorella* developed when NaF is added or when other measures are applied originates from decarboxylation of glutamic acid. By adding NaF to living cells we are able to elicit the breakdown and resynthesis of glutamic acid in life and to study the function of this amino-acid in living *Chlorella*.

TABLE 20
Results of chromatography studies

	Glutamic Acid Breakdown	γ -Aminobutyric Acid Formation
anaerobic $1/1600$ N NaF	+++	+++
anaerobic $1/80$ N NaF	++++	++++
anaerobic $1/80$ N NaF + 10^{-3} N HCN	0	0
aerobic $1/1600$ N NaF	+	+
aerobic $1/80$ N NaF	++	++
aerobic $1/80$ N NaF + 10^{-3} N HCN	0	0

It follows from Table 20 that noteworthy differences exist between high and low NaF concentrations. The concentration $1/80$ N is very high and decomposes glutamic acid irreversibly, under anaerobic and aerobic conditions. With low concentrations ($1/1000$ to $1/300$ N NaF) glutamic acid is reversibly decomposed and respiration is increased. Thus, it is not possible to determine the presence of glutamic acid by means of manometric pressure changes when low NaF concentrations are used under aerobic conditions. To measure aerobic glutamic acid breakdown manometrically, Warburg (13) uses initially a low concentration of NaF and afterwards a high concentration. If, for instance, 40 μ l CO₂ are directly produced aerobically with $1/80$ N NaF and only 30 μ l CO₂ when previously $1/1000$ N NaF had been given, it may be concluded that the low NaF concentration decomposed 25% of the glutamic acid.

The aerobic resynthesis of glutamic acid should not be considered to be a simple reversal of the anaerobic decarboxylation reaction. As the resynthesis occurs only under aerobic conditions, O_2 , i.e., respiration, must be indispensable. In fact, when small amounts of NaF are added to *Chlorella* under aerobic conditions, respiration increases.

§ 64 The Necessity for Glutamic Acid in Photosynthesis

The foregoing paragraphs have shown that, upon addition of NaF, the glutamic acid of *Chlorella* can be decomposed and resynthesized by alternating anaerobic conditions and aerobic conditions. It has been found that 0.001 *N* NaF inhibits photosynthesis anaerobically but not aerobically. The decomposition of glutamic acid and the inhibition of photosynthesis on the

Fig. 65. Influence of NaF on photosynthesis under anaerobic conditions changing into aerobic conditions. This experiment is the continuation of the experiment in Fig. 64, at the end of which the composition of the gas phase was changed to 10 vol % CO_2 and 90 vol % argon. After 50 min anaerobiosis in the dark glutamic acid is decarboxylated. At $t = 0$ illumination with 200 μl quanta/min. pH, temperature and vessel constants as in Fig. 64. Result: initial marked inhibition of photosynthesis steadily decreasing with increasing O_2 pressure and resynthesis of glutamic acid (curve II) (Warburg *et al.*, *Zschr. Naturf.*).

one hand, and the resynthesis of glutamic acid and the reincrease of photosynthesis, on the other, are simultaneous processes. Figure 64 shows an aerobic experiment without NaF and with 0.001 *N* NaF described by Warburg (17). The gas phase contains, initially, 10 vol % CO_2 , 30 vol % O_2 and 60 vol % argon. Under these aerobic conditions, photosynthesis is not inhibited by 0.001 *N* NaF. After changing the content of the gas phase to 10 vol % CO_2 and 90 vol % argon, anaerobic conditions set in. The vessels are shaken for one hour in the dark so that all the glutamic acid is decomposed. Illumination with the same intensity as in the experiment depicted

in Figure 64, results in the curves shown in Figure 65. It follows from curve II that photosynthesis with 0.001 *N* NaF is initially inhibited, but reincreases when O₂ production changes the initial anaerobic conditions to aerobic conditions. Respiration of the cells sets in and this energy resynthesizes glutamic acid, so that photosynthesis can increase again. By ascertaining the glutamic acid content at the beginning and at the end of the anaerobic experiment in NaF-free controls, the constant value of 1.7 $\mu\text{mole}/100\ \mu\text{l}$ cells is found. In the presence of 0.001 *N* NaF the glutamic acid content at the beginning of the experiment is zero. At the end it is 1.47 $\mu\text{mole}/100\ \mu\text{l}$ cells. Therefore, because of the autocatalytic O₂ production the glutamic acid content increases from 0 to 86% of the normal value (1.7 $\mu\text{mole}/100\ \mu\text{l}$ cells). During this time photosynthesis increases from a very low value to 82% of its normal value, as follows from the difference of the slopes of curves I and II in Figure 65. This quantitative parallelism between the resynthesis of glutamic acid and the increase of photosynthesis (see also Table 19) proves most convincingly the close connection between photosynthesis and glutamic acid.

For these experiments illumination with high intensity is necessary so that the O₂ saturation pressure is obtained within about 10 min in the NaF-free controls. If the intensity is too low, the saturation pressure would not be obtained for one hour, and six hours would be required for the resynthesis of glutamic acid in 0.001 *N* NaF.

In this type of experiment it is sufficient to know the ratio of the pressure changes in NaF and in the control. However, if the exact amount of the O₂ produced is to be ascertained, equation 12 can be used. Owing to the high light intensity, it is permissible to call $\gamma = -1$, so that

$$x_{\text{O}_2} = h \frac{K_{\text{CO}_2} K_{\text{O}_2}}{K_{\text{CO}_2} - K_{\text{O}_2}} = 2.8\ h\ \mu\text{l}$$

With regard to the ratio of the pressure changes, it must be borne in mind that in the experiment depicted in Figure 65 CO₂ is bound when glutamic acid is resynthesized. To this amount of CO₂ we have to add the so-called aerobically dissociating CO₂ which will be discussed in § 65. With 10 vol % CO₂ in the gas phase, this amounts to about half of the other CO₂. As 1.47 $\mu\text{mole} = 1.47 \times 22.4\ \mu\text{l}$ glutamic acid is resynthesized,

$$x_{\text{CO}_2} = 1.47 \times 22.4 \times 1.5 = 49\ \mu\text{l}$$

Hence, the binding of CO₂ in NaF elicits the negative pressure change

$$h_{\text{CO}_2} = \frac{-49}{k_{\text{CO}_2}} = \frac{-49}{1.61} = -30.5\ \text{mm}$$

It follows from Figure 65 that the difference Δ in the pressure changes of both curves is 157 mm. The CO₂ binding is thus 20% of Δ . The remaining 80% must be attributed to the decreased O₂ production, i.e., indicate the inhibition of photosynthesis.

It must be emphasized that the reaction

does not represent CO_2 fixation in photosynthesis. The more γ -aminobutyric acid produced, the greater the inhibition of photosynthesis. § 65 shows that the aerobically dissociating CO_2 —a great amount of CO_2 fixed in the presence of O_2 and removed in the absence of O_2 —must be considered to be of utmost importance. This fraction of CO_2 is also quantitatively connected with glutamic acid. If half of the glutamic acid is decomposed by the addition of NaF, photosynthesis and also aerobically dissociating CO_2 are halved.

§ 65 Dissociating Carbon Dioxide

When *Chlorella* is saturated at various CO_2 pressures and NaF is added, not only glutamic acid but also bicarbonates develop CO_2 , e.g.,

Furthermore, it may be possible that CO_2 originates from carbamino-acids produced in the cells by the action of CO_2 upon amino-acids (e.g. glutamic acid).

The CO_2 derived from such carbamino-acids is dependent on CO_2 pressure, whereas the CO_2 derived from glutamic acid is not dependent on the CO_2 pressure.

Warburg (13) found that more CO_2 is bound under aerobic than under anaerobic conditions. When, for instance, 100 μl cells containing 1.84 μmole chlorophyll and suspended in 3 ml salt solution at pH 3.8 are saturated with CO_2 pressures varying from 5 to 50 vol %, the amount of CO_2 removed by $1/80$ *N* NaF in 20 min at 20 °C is as shown in Table 21.

TABLE 21
Dissociating CO_2 , anaerobically and aerobically

<i>CO</i> ₂ Pressure in Vol %	0	5	10	20	50
anaerobically (<i>CO</i> ₂ + argon) μl <i>CO</i> ₂	38.8	44.7	48.2	51.8	56.3
aerobically (25 vol % <i>CO</i> ₂) μl <i>CO</i> ₂	38.0	51.5	63.0	79.5	97.0

If the CO_2 removed from glutamic acid at zero CO_2 pressure is subtracted from the anaerobic values, the *anaerobically dissociating CO*₂ is obtained. If the anaerobic values are subtracted from the aerobic values, we find the

aerobically dissociating CO₂, i.e., the CO₂ which is bound under the influence of O₂ only. Table 22 and Figure 66 show the results of these subtractions.

TABLE 22
Dissociating CO₂, anaerobically and aerobically

CO ₂ Pressure in Vol %	5	10	20	50
anaerobically dissociating CO ₂ in μl	5.9	9.4	13.0	17.5
aerobically dissociating CO ₂ in μl	6.8	14.8	27.7	40.7

It follows from Figure 66 that, both anaerobically and aerobically, the degree of saturation is reached at a CO₂ pressure of 50 vol %. The degree of saturation is about 2.5 times greater for aerobically dissociating CO₂ than for anaerobically dissociating CO₂.

Fig. 66. Evolution of dissociating CO₂ from *Chlorella* with 1/80 N NaF. pH 3.8, 20° C. Curve I: anaerobically dissociating CO₂. Curve II: only aerobically dissociating CO₂ (Warburg *et al.*, *Zschr. Naturf.*).

Curve II for aerobically dissociating CO₂ is strongly dependent upon the method of *Chlorella* cultivation. Cells cultivated with fluctuating light (A-cells) give the best quantum yields and bind CO₂ aerobically much more strongly than south cells which give poor quantum yields (see § 15). It is also of interest to note that the saturation value for aerobically dissociating CO₂ is equal to the glutamic acid content of the cells. In the experiment shown in Figure 66, the latter was 38.4 μl and the saturation value for aerobically dissociating CO₂ was 41 μl. As the chlorophyll content was 41.2 μl, it follows that, within the limits of experimental error, chlorophyll content, glutamic acid content and aerobically dissociating CO₂ are identical.

In X-cells containing 5 times as much glutamic acid as chlorophyll Warburg found that 100 μl cells contained 1.9 μmole glutamic acid and 0.418 μmole chlorophyll and that the saturation value for aerobically dissociating CO_2 was 1.9 μmole . Thus, in these cells, too, the saturation value for aerobically dissociating CO_2 is equal to the glutamic acid content, but not to that of the chlorophyll content.

To summarize these most interesting findings, *Chlorella* contains three types of CO_2 :

1. CO_2^{I} of glutamic acid which is not dependent on CO_2 pressure.
2. CO_2^{II} , originating from bicarbonates, which is dependent on CO_2 pressure.
3. CO_2^{III} , i.e., the dissociating CO_2 , originating in the presence of O_2 , which is dependent on the CO_2 pressure and occurs in a concentration of the same magnitude as that of glutamic acid.

It is possible to establish the three CO_2 values by means of the fluoride method (5):

saturation of the cells with argon:	$x \mu\text{l CO}_2$
saturation of the cells with argon + 10 vol % CO_2 :	$y \mu\text{l CO}_2$
saturation of the cells with argon + 10 vol % CO_2 + O_2 :	$z \mu\text{l CO}_2$

We then have

$$\begin{aligned}\text{CO}_2^{\text{I}} &= x \mu\text{l} \\ \text{CO}_2^{\text{II}} &= (y - x) \mu\text{l} \\ \text{CO}_2^{\text{III}} &= (z - y) \mu\text{l}\end{aligned}$$

In the light glutamic acid does not decrease, but aerobically dissociating CO_2 decreases markedly. After 5 min illumination at high intensity (800 μl quanta/min) the aerobically dissociating CO_2 decreased in one experiment (100 μl A-cells) from 77 μl to 49 μl . When the cells were put in the dark afterwards $1/80 \text{ N NaF}$ developed 75 μl CO_2 after 20 min. Thus, the light value (49 μl) increased within 20 min in the dark to its initial value. On illumination the O_2 capacity of cells which were previously in the dark sets in, and the dissociating CO_2 decreases markedly during the stoichiometric O_2 production. However, in the dark, when induced respiration restores O_2 capacity, the dissociating CO_2 increases to its initial value (12).

§ 66 The Carotenoid Oxygenase of *Chlorella*

When a small amount of octanol or quinone is added to *Chlorella* (south cells suspended in salt solution K or S) in the dark, respiration ceases. Nevertheless, there is a very pronounced O_2 uptake without production of CO_2 . The O_2 consumption of 200 μl cells at 20°C and at pH 6.5 is about 500 μl per hour, i.e., 5 times greater than the O_2 uptake during respiration. Warburg *et al.* (20), who discovered this reaction, showed that lyophilized cells of *Chlorella*—without addition of quinone—behave in the same way. Brief heating to 65°C stops the reaction, which is therefore assumed to be of an enzymatic

nature. The reaction is not inhibited by either 10^{-3} *N* phenanthroline or 10^{-3} *N* HCN. It was found that the carotenoid concentration decreases during the reaction so that, according to Warburg, the reaction is an enzymatic oxidation of carotenoids by molecular O_2 . The enzyme responsible, carotenoid oxygenase, is activated by the addition of small amounts of quinone. In lyophilized cells the reaction proceeds spontaneously. From experiments with fresh cells it follows that the rate of O_2 uptake depends upon the amount of quinone and upon the pH (optimal 6.5). The rate rises with increasing O_2 pressure.

Fig. 67. Oxygenase reaction and carotenoid concentration. 200 μ l *Chlorella* in the nanometer vessel suspended in salt solution S. pH 3.8. In the side-arms no quinone or 2 mg quinone. Air in the gas phase. Curve I: control without quinone, normal respiration. Curve II: 2 mg quinone, incident intensity of white light 730 μ l quanta/min. After 225 min 60 μ l O_2 used. Curve III: 2 mg quinone in the dark. After 225 min 370 μ l O_2 used (Warburg *et al.*, *Zschr. Naturf.*).

The action of the oxygenase is light-sensitive. Less O_2 is consumed in the light than in the dark and the carotenoid concentration is not decreased as much in the light as in the dark. The simplest explanation is that the oxygenase transfers O_2 in the dark and removes it in the light. Figure 67 shows the behavior of the carotenoids. A *Chlorella* suspension of 200 μ l cells in salt solution S is divided into three samples. Sample I, which serves as a control, is shaken with air in the dark for 225 min and no quinone is added. Sample II is shaken with air and strongly illuminated for the same time with

2 mg quinone added. Sample III is shaken with air in the dark for the same time with 2 mg quinone added. The cells are afterwards washed with water, centrifuged and extracted with methanol. Using Willstätter's method, the chlorophylls are removed by saponification and the remaining carotenoid ether solution filled up with ethanol to 100 ml. For 1 cm layer thickness $\ln i_0/i$ is plotted against the wave-length. It follows from Figure 67 that the activation of the oxygenase by means of quinone reduces the carotenoid concentration in the dark to about 50% of the initial value, but reduces it in the light to about 25% of the initial value.

Fig. 68. Oxygenase reaction with lyophilized cells of *Chlorella*. In the manometer vessel 3 ml $1/50$ M phosphate, 0.1% KCl (pH 6.5). In the side-arms 25 mg lyophilized cells. Air in the gas phase. KOH in the center well. At time $t = 0$, the lyophilized cells are tipped into the vessel. Curve I: dark. Curve II: incident intensity of white light 200 μ l quanta/min. Curve III: incident intensity of white light 750 μ l quanta/min (Warburg *et al.*, *Zschr. Naturf.*).

Figure 68 illustrates the behavior of lyophilized cells in the dark and upon illumination with two light intensities. A suspension of 25 mg lyophilized cells corresponding to 100 μ l fresh *Chlorella* consumes 160 μ l O_2 in the dark after 190 min (curve I). On illumination the uptake decreases more markedly at high light intensities than at lower light intensities (curves II and III). This experiment shows some similarity to the compensation of respiration by photosynthesis. However, it should be kept in mind that lyophilized cells produce neither CO_2 due to respiration nor O_2 from CO_2 (see § 63).

In the manometric experiments discussed certain precautions have to be observed. Lyophilized cells must be brought into the side-arm of the manometer vessel and only at time $t = 0$ are they tipped into the salt solution of the main compartment, where O_2 uptake immediately sets in. If the lyophilized cells were suspended in the salt solution before the manometric reading, the greater part of the oxidation process would pass unobserved. Precautions are also necessary for the activation with quinone. The center

well of the manometer vessel should contain KOH, as is shown, for instance, in Figure 19. The addition of quinone produces CO_2 from glutamic acid and this CO_2 must, of course, be absorbed by KOH. However, the quinone added may distil into the KOH where it may be oxidized to tetrahydroxyquinone. This reaction also consumes O_2 . For this reason, it is necessary to carry out control studies without cells and to subtract the pressure changes obtained in the controls from those obtained in the experiment. There is a further complication: on illumination quinone produces O_2 (Hill reaction). To avoid this, quinone should be completely reduced: this can be done by means of brief illumination with very high light intensity. Otherwise, the O_2 production due to quinone would prevent the correct measurement of O_2 uptake due to the carotenoid oxygenase. Thus, there are so many sources of error in the activation with quinone that it is preferable to use lyophilized cells to measure oxygenase activity.

§ 67 Experiments with Quinone

When quinone is added to *Chlorella* in the dark and under anaerobic conditions, a considerable amount of CO_2 is developed in a short time, and quinone is reduced to hydroquinone. The amount of quinone reduced is not equivalent to the amount of CO_2 produced. The major part of the CO_2 produced originates from decarboxylation of glutamic acid, a reaction which is not an oxidation process. Warburg and Krippahl (18) found that addition of 0.1 mg quinone gives the greatest CO_2 production, whereas addition of 2.0 mg quinone elicits the smallest production of CO_2 . Whereas 0.1 mg quinone added to 100 μl cells in 3 ml salt solution K has no poisoning effect, 0.2 mg quinone inhibits O_2 respiration and, therefore, photosynthesis completely and irreversibly. It has been found that, for instance, after addition of 0.1 mg quinone the end value of 57 μl CO_2 developed from 100 μl cells was attained after 30 min. Of this amount, 40 μl CO_2 were due to glutamic acid breakdown; the origin of the remaining 17 μl CO_2 was unknown.

Similar experiments in the dark under aerobic conditions also show that 0.1 mg quinone does not inhibit O_2 respiration but that 0.2 mg quinone does. However, under these conditions O_2 is used to a great extent *without CO_2 production*, due to the action of the carotenoid oxygenase.

The light reaction of *Chlorella* with quinone has been discovered by manometry with KOH in the center well of the vessels (3), so that the influence of CO_2 could only have been observed with very low CO_2 pressures. In these experiments the pH should be low and constant (3.8) to decrease the bicarbonate content of the medium as much as possible. The suspension of 100 μl cells in 3 ml salt solution K is previously treated with 0.1 mg quinone in the dark to drive out the major part of the labile CO_2 . The suspension is first aerated with argon and a little CO_2 for 20 min. After closing the stop cocks, the vessels are shaken—also in the dark—for 40 min. Afterwards, 2 mg quinone are tipped from the side-arm and strong illumination (725 μl quanta/

min) sets in. It was found that at CO_2 pressures between 0.125 vol % and 1.49 vol % the end values for the O_2 produced are practically identical. They are equivalent to the amounts of quinone added. However, the rates of O_2 production increase with the CO_2 pressures. Under 0.015 vol % CO_2 the rates are very low (Fig. 69). These experiments can only be explained if we admit that CO_2 is an intermediate product of the Hill reactions, as has been proved in other ways for the Hill reagents: NO_3 ions and ferric ions (see § 49).

Fig. 69. Influence of CO_2 pressure on the quinone light reaction. In the main compartment, 100 μl *Chlorella* (south cells) suspended in 3 ml salt solution K + 0.1 mg quinone. In the side-arm 2 mg quinone dissolved in 0.2 ml water. In the gas phase argon with various contents of CO_2 . Illumination; 725 μl quanta/min (Warburg *et al.* Zschr. Naturf.).

In isolated grana and in lyophilized cells of *Chlorella* the O_2 evolution in the light upon addition of quinone proceeds twice as quickly when the gas phase contains 1.4 vol % CO_2 in addition to argon. In the grana the end value for the O_2 produced is the same whether the gas phase contains, besides argon, CO_2 or not. It corresponds to about 83% of the amount of quinone added. The end values for O_2 production in lyophilized cells are also independent of the presence of CO_2 in the gas phase. They are about 50% of the values calculated from the quinone turnover (17).

The investigations discussed in this paragraph are still being carried out in Warburg's institute. The results already obtained clearly show the prime importance of CO_2 in the Hill reactions.

§ 68 Quinone as a Catalyzer

In §49 we discussed the necessity of CO_2 in the quinone reaction of grana:

The necessary half-value pressure of CO_2 should be 10 mm H_2O . At a half-value pressure of 1 mm H_2O no O_2 is produced. However, the grana do not consume CO_2 , so that the action of CO_2 must be that of a catalyzer. Intermediate reactions must therefore take place and it seems quite logical to assume the formation of a peroxide. As a matter of fact, the O_2 developed is molecular O_2 and therefore has to be formed from a peroxide. From a chemical and energetical point of view evolution of O_2 in the atomic state must be excluded. Warburg (21) proposes the following reaction scheme:

Thus, quinone reduces CO_2 to percarbonic acid, a well-known substance with the formula

It is reduced to formic acid giving off molecular O_2 . The formic acid produced *in statu nascendi* reacts with a second molecule quinone to produce CO_2 . The intermediate products do not appear in the over-all reaction. It must be assumed that they are all bound to chlorophyll. These reactions do not take place in solutions and are inhibited by 0.001 *N* phenylurethane.

It is of course possible to advance a similar scheme assuming that quinone does not oxidize CO_2 but water to produce H_2O_2 :

The over-all reaction is the same as before. However, the percarbonic acid pathway seems *a priori* to be more probable as the experiments show that CO_2 pressure is absolutely indispensable. On the other hand, a formic acid stage has never been established. An important argument against the H_2O_2 pathway lies in the fact that catalase is present. This enzyme is inhibited by HCN but the O_2 evolution in the quinone reaction is not inhibited by HCN .

An important condition for the quinone reaction is a sufficiently acid medium so that the hydroquinone produced cannot react back with O_2 . Warburg (21) pointed out how interesting it is to study conditions for obtaining

this back reaction in which quinone would act as a catalyzer. If the pH of the grana suspension is increased from 6.5 to 8 quinone reaction changes from the stoichiometric state to the catalytic state and we find an over-all reaction of zero:

The back reaction of hydroquinone may be either enzymatic by means of the copper of the phenoloxidase or directly chemical by autoxidation. The enzymatic reoxidation takes place rapidly at low O_2 pressures; it is inhibited by HCN. By contrast, the autoxidation proceeds only at higher O_2 pressures and—in contrast to the enzymatic reoxidation—results in formation of H_2O_2 :

The ratio $\text{H}_2\text{O}_2/\text{O}_2$ equals 1. The less quinone added in these catalytic quinone reactions, the closer conditions are to the physiological. According to Warburg, the optimal amount of quinone to be added is equal to the chlorophyll content of the grana, e.g., 1 μmole benzoquinone or 1 μmole β -naphthaquinone sulfonic acid for a quantity of grana containing 1 μmole chlorophyll. In stoichiometric experiments 20 μmoles quinone are added to 1 μmole chlorophyll.

As already mentioned, the enzymatic back reaction is inhibited by HCN. However, the strange thing is that the forward reaction of O_2 cannot be inhibited by HCN. This can be shown in the stoichiometric quinone reaction—i.e. under the proviso that no back reaction of O_2 can take place—when HCN is added. The O_2 evolution is then not inhibited by 0.01 N HCN and only slightly inhibited by 0.1 N HCN. Thus, as the O_2 production is not inhibited by HCN and the enzymatic back reaction of O_2 is inhibited by HCN, quinone catalysis at low O_2 pressures must always be inhibited by HCN. If the O_2 pressure is increased to such an extent that autoxidative back reaction proceeds rapidly enough, neither the forward reaction nor the back reaction of O_2 will be inhibited by HCN. Thus, in 0.01 N HCN we have complete quinone catalysis; however, H_2O_2 is produced instead of water, HCN inhibiting the catalase of the grana. In 0.01 N HCN we therefore have the following reactions:

The over-all reaction is no longer zero. O_2 is consumed and H_2O_2 produced. The addition of HCN thus gives, manometrically, an alteration in pressure changes from zero to markedly negative values. In contrast to simple autoxidation, the ratio $\text{H}_2\text{O}_2/\text{O}_2$ now equals 2 (see Table 23).

TABLE 23

O₂ consumption and H₂O₂ formation in grana (about 1 μmole chlorophyll) in air, in 0.01 *N* HCN, 0.5 to 1 μmole benzoquinone or β-naphthaquinone sulfonic acid added. pH 8.3. Incident energy about 36 μmoles quanta/min.

O ₂ μ moles	H ₂ O ₂ μ moles	H ₂ O ₂ /O ₂
-6.2	+12.5	2.04
-7.9	+17	2.16
-7.2	+13	1.80
-5.6	+11	1.96
-6.7	+13	1.94
-8.2	+15.7	1.92
average values -6.97	+13.7	1.97

Warburg (21) compared these findings obtained with grana in the presence of quinone with those obtained with *Chlorella* without quinone. When *Chlorella* in CO₂-containing air is illuminated normal photosynthesis is obtained. Addition of HCN up to 0.01 *N* elicits complete inhibition and no pressure changes are noted; no H₂O₂ is produced. However, when the O₂ pressure is increased from 0.2 to 1 atm, considerable negative pressures and H₂O₂ production are obtained upon illumination but not in the dark. Addition of CO₂ accelerates the H₂O₂ production which can be completely inhibited by 0.0001 *N* phenanthroline. In these experiments the ratio H₂O₂/O₂ is not equal to 1, as is the case in simple autoxidation, but has the value 2 (Table 24). This means that the reaction equations found for quinone catalysis in grana in 0.01 *N* HCN are the same for *Chlorella* without quinone in 0.01 *N* HCN. Warburg assumes the presence of an oxidant E, which he considers to be an enzyme, in *Chlorella*. The following reaction scheme can then be applied to *Chlorella*:

These experiments prove that *Chlorella* contains a natural catalyzer which is reduced upon illumination evolving O₂. The catalyzer is steadily re-oxidized in the dark. It behaves in the same way as quinone added to grana or *Chlorella* (see Tables 23 and 24).

TABLE 24

O₂ consumption and H₂O₂ formation in 100 μl *Chlorella* per hour, in 0.01 *N* HCN, in 20 vol % CO₂-O₂. pH 4. Incident energy about 36 μmoles quanta/min.

O ₂ μmoles	H ₂ O ₂ μmoles	H ₂ O ₂ /O ₂
-1.32	+2.40	1.82
-1.26	+2.60	2.04
-1.97	+4.95	2.50
-2.04	+4.12	2.02
-1.36	+2.72	2.00
-2.14	+4.12	1.92
average values -1.68	+3.48	2.06

It should be borne in mind that quinone reactions involve heavy metal catalysis. The forward reaction of O_2 is catalyzed by the unknown heavy metal which is inhibited by $0.0001\ N$ phenanthroline but not by $0.01\ N$ HCN. The enzymatic back reaction of O_2 is catalyzed by the copper of the phenol-oxidase. It is still doubtful whether a third heavy metal—the iron of the very active catalase of green cells and grana—plays a role in the quinone reactions.

Fig. 70. Manometer vessel for determining the CO_2 partial pressure

We come now to the question how CO_2 fixation is related to the experimental results described. To study this, the partial pressure of CO_2 must be established. For this purpose, Warburg worked out a new type of manometer vessel (Fig. 70). The main compartment contains the suspension of grana or cells. The side-arm A connected with the main compartment contains H_2SO_4 . The trough contains $KMnO_4$ and the side-arm B connected with the trough contains $K_4Fe(CN)_6$. When H_2SO_4 is tipped into the main compartment CO_2 evolves from any bicarbonate present in the suspension. When $K_4Fe(CN)_6$ is tipped from the side-arm B to the trough containing $KMnO_4$ the contents of the trough become strongly alkaline and very rapidly absorb all the CO_2 present:

In this way it is possible to determine the CO_2 partial pressures at times t_0 and t and to establish the exact change in CO_2 partial pressure in the time $t - t_0$. Any decrease in the CO_2 partial pressure means CO_2 fixation. In spite of many variations in his experiments, Warburg always found that the O_2 evolutions in the presence of quinone and HCN were never accompanied by changes in the CO_2 partial pressure. The explanation of these results lies in the fact that there is no respiration. It was, indeed, found that no CO_2 is fixed in grana as they do not respire. Similarly, *Chlorella* cells do not respire in the presence of quinone and no CO_2 fixation is observed. However, when *Chlorella* cells are treated with $0.01\ N$ HCN in the dark, they show respi-

ration as well as CO_2 fixation, but under the same conditions upon illumination they exhibit neither respiration nor CO_2 fixation. They do not respire because the H_2O_2 produced in the light destroys respiration. Thus, the rule that no CO_2 fixation is possible without respiration, has been established.

§ 69 Quinone Catalysis and Phosphorylation

In § 51 we discussed Arnon's discovery that green grana suspended in phosphate solution at pH 8 are able to synthesize ATP from ADP upon illumination. Warburg (21) found that substances, such as 0.0001 *N* phenanthroline and 0.01 *N* HCN (at low O_2 pressures), which inhibit quinone catalysis also inhibit light phosphorylation. However, at high O_2 pressures 0.01 *N* HCN does not or only very slightly inhibits both quinone catalysis and light phosphorylation. This specific behavior with respect to HCN proves that a relationship between both processes must exist. It seems therefore contradictory that nevertheless ATP production has no influence whatever upon quinone catalysis. The speed of O_2 evolution elicited by the quinone remains the same whether ADP has been added or not, provided, of course, that phosphate is present in sufficient quantities. The explanation lies in the fact that in quinone reactions phosphate is bound under all circumstances. In the absence of ADP this bound phosphate is freed again during the O_2 evolution and the amount of free phosphate remains constant in the over-all reaction. It acts as a catalyzer. In the presence of ADP, phosphate is trapped and the over-all reaction shows its decrease.

Warburg found that, in the presence of ADP, one molecule phosphate is bound when one molecule H_2O_2 is produced. In other words, one molecule phosphate is bound when one molecule quinone takes up two H atoms. This relationship of 1 ATP and 2 H is the same as that Warburg and Christian found in the oxidation reaction of fermentation (see §44), the only reaction in which the mechanism of phosphorylation has been chemically defined. Thus, the energetics of light phosphorylation can be identified with the energetics of dark phosphorylation (21).

It is certainly permissible to speculate about the substance in the quinone reactions which may be responsible for the binding of phosphate. Warburg proposes a phosphorylated peroxide of CO_2 according to the following reaction scheme:

If we accept the hypothesis of water photolysis, a similar scheme can be outlined with the intermediate production of a phosphorylated H_2O_2 :

The formation of $\text{H}_4\text{P}_2\text{O}_9$ instead of H_3PO_5 may be envisaged:

It does not seem very probable that such phosphorylated hydrogen peroxides (peroxyphosphoric acid and peroxyphosphoric acid) play a biological role.

The investigations discussed in this Section are being continued in Warburg's institute. This experimental work is difficult and painstaking but the results already obtained show the prime importance of CO_2 . The many interesting facts drawn from the experiments with fluoride and quinone have enriched our knowledge of the very complicated process of photosynthesis.

§ 70 Some Final Remarks

In this chapter some basically important results of research in the chemistry of photosynthesis have been discussed. It must be admitted that, with respect to the extreme complexity of the problem, these results are modest. Most of them are far from being certain and are not universally accepted. Generally, the work done consists of tentative approaches and there is a considerable amount of disagreement between the investigators working in the field. The main risk in all the schemes worked out in the last years to describe chemical pathways in photosynthesis lies in the fact that the student may forget that most of it is pure "paper" chemistry. It is evident that such schemes may be useful as working hypotheses but more or less definite conclusions should only be drawn when every intermediate reaction of a reaction scheme is found to accord with experimental facts. It must be emphasized again that at the present time the chemistry of photosynthesis is still in its infancy.

Three main directions of research can be distinguished to-day. They are inspired by Arnon, Calvin and Warburg. Arnon's concept of light phosphorylation and Calvin's photosynthetic carbon cycle certainly show new and important ways, though the general theories as advanced by these investigators are difficult to accept. All theories based upon the photolysis of water—a hypothesis for which not the slightest evidence can be advanced and which was put forward about 20 years ago to explain the reactions discovered by

Hill—must be regarded as more or less suspect. Phosphorylation due to the action of light still remains very problematical. Warburg's work in the field of photosynthesis chemistry deals mainly with the importance of CO_2 . His studies with fluoride and with quinone have already resulted in many important discoveries which were discussed in the last section of this chapter. They are all based upon reliable measurements by means of new manometric methods. Warburg found considerable evidence that respiration belongs to the chemical mechanism of photosynthesis. This follows from the one-quantum theory discovered in 1950. In 1958 he found that respiration and photosynthesis decrease in the same proportion at very low O_2 pressures (§ 42). The experiments discussed in § 67 showing that no CO_2 fixation without respiration can be detected provided further support. Recently Warburg and Kayser (9) measured the CO induced inhibitions of respiration and photosynthesis in cotyledons of barley of various ages (6 to 21 days) and found a significant relationship. When respiration in older leaves was less inhibited by CO, the same decrease in CO inhibition was noted in photosynthesis. Six day old leaves showed inhibition values of 56% and 59% for the two processes. In 14 day old leaves values of 24% and 20% were found, whereas in 21 day old leaves there was no CO induced inhibition in respiration and in photosynthesis. Thus, these experiments clearly show that when respiration is inhibited to a certain degree, photosynthesis is equally inhibited to the same extent. We may also recall here the inhibitory action of O_2 withdrawal upon photosynthesis attributed to the decomposition of glutamic acid (§ 61).

In § 36 and § 57 we discussed the production of glycolic acid in photosynthesis of *Chlorella*. There is evidence that this compound may be of particular importance. Zelitch (24) draws the attention to the central position of glycolic acid in the processes of respiration and photosynthesis in tobacco leaves. Warburg and Krippahl (19) show that *Chlorella* suspensions in appropriate carbonate buffer solutions provoking a low CO_2 pressure (0.05 atm %) produce upon strong illumination (800 μl quanta/min) with pure O_2 in the gas phase about one molecule glycolic acid for two molecules CO_2 consumed. In increasing the CO_2 pressure, glycolic acid formation is inhibited. At a CO_2 pressure of 1.70 atm % only traces of the acid are detectable. It was possible to separate CO_2 fixation and glycolic acid formation. When the gas phase contains 2 atm % CO_2 and O_2 , it is shown that in the beginning of the experiment CO_2 disappears without formation of glycolic acid. After a while the CO_2 pressure is decreased to such an extent that glycolic acid can be produced in more or less pure O_2 atmosphere. The chemical mechanism is not yet elucidated but there is evidence that CO_2 reduction on the one hand (e.g., to glycolaldehyde) and oxidation of the resulting reduction products (e.g., to glycolic acid) on the other hand are competitive reactions with respect to an oxidizing enzyme. This view is in full agreement with the fact that in photosynthesis CO_2 has to be oxidized (to the state of the photolyte) before it can be reduced (19).

Recently, the half-value pressures of CO_2 in the quinone reactions have been determined by Warburg (23) with the aid of a special manometric arrangement allowing the measurement of very low CO_2 pressures. The values obtained are 7 to 8 mm H_2O for *Chlorella* and only about 0.5 mm H_2O for chloroplasts and grana. These results provide definite proof that CO_2 is indispensable for the Hill reactions. The hypothesis of water photolysis, therefore, can no longer be accepted. The following original scheme of the quinone reaction in *Chlorella* as well as in grana

has to be replaced by

According to Hill, Calvin and others, quinone oxidizes water and hydroquinone reduces CO_2 in the first scheme. The second scheme shows how, according to Warburg (23), quinone oxidizes CO_2 (orthocarbonic acid: H_4CO_4) and hydroquinone reduces O_2 .

With respect to CO_2 , the difference between photosynthesis and the Hill reactions lies in the fact that in photosynthesis the reduction product of CO_2 is only partially (about two-thirds) reoxidized to CO_2 . However, in the Hill reactions the reduction product of CO_2 is completely reoxidized to CO_2 , so that CO_2 does not appear in the over-all reaction.

In the early part of this exposé a clear-cut distinction was made between the energetics and the chemistry of photosynthesis. We considered them, for the sake of simplicity, to be two distinct problems. However, the reader will have noted that, in reality, there is no such line of demarcation. The more recent findings in chemistry have been successively developed from the earlier work on energetics. Warburg's discovery of the quantum requirement 3 led to the one-quantum theory advanced by Burk and Warburg. From this fruitful concept started the work done on the oxygen capacity, the labile carbon dioxide and their relationships to chlorophyll which, in turn, led to Warburg's equations of photosynthesis and the notion of the photolyte. This further enabled Warburg to give a new explanation for the mechanism of the Hill reactions.

Warburg's important fundamental discoveries are the result of clear-sighted studies which are based not on simple theorizing but on painstaking measurements—measurements of a high standard with irreproachable techniques carried out along the lines of scientific thought and carefulness of the classical old style and which can stand up to objective criticism. The many unjustified attacks of a polemical nature mostly hide the incompetence or lack of experimental ability of those making the attacks and will not stop the ad-

vance of scientific research. However, they will undermine the authority of Science over the uninitiated and, still worse, will bring confusion to the minds of the young generation of scientists from whom Humanity expects, as in the past, the search for truth. We may cite here Louis Pasteur's exclamation at a session of the Academy of Medicine in Paris when he had to counter the attacks made by the ignoramus Colin on his experiments dealing with the etiology of anthrax: "Jeunes gens qui siégez au haut de ces gradins et qui êtes peut-être l'espoir de l'avenir médical de notre pays, ne venez pas chercher ici les excitations de la polémique; venez vous instruire des méthodes."

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Addendum

A New Method for the Determination of the Quantum Requirement

Recently Warburg and Krippahl* worked out an improved compensation method for determining the quantum requirement of *Chlorella*. In using the compensation method, dense cell suspensions are used so that the resulting great respiration has to be compensated with diffuse bluish light (20% blue and blue-green, 80% green, yellow and red). The compensation light is provided by a 100 watt metal filament lamp with a reflector fixed above the thermostat. Light absorption is nearly complete. As it is not possible to measure the absorbed intensity of such a diffuse compensation light with a bolometer, the manometer vessels are replaced by actinometer vessels containing a solution of pheophorbide and thiourea in pyridine (see § 24). The uptake of O_2 in the actinometer divided by the O_2 evolution in the cell suspension equals the quantum requirement for the compensation light. The determination of the quantum requirement for the measured light is carried out in the usual way with the two-vessel method, the light beams entering the vessels from below. Respiration is compensated; the intensities are measured with the bolometer and the degree of absorption which varies between 93 and 95% is found with the aid of the Ulbricht sphere.

It has been found that the quantum requirement decreases with decreasing light intensity. At $i = 0$, a limit of $1/\varphi$ is obtained which has the value 3.06 for the compensation light and 2.85 for the measured light (Fig. 71). The low value of 2.85 is very near to the theoretical quantum requirement 2.7 at 100% efficiency (see § 26). It is of course irrelevant that the value for the quantum requirement is not a whole number. According to the one-quantum reaction, one quantum causes the splitting of one molecule photolyte producing one molecule O_2 . As discussed in § 35, at the quantum requirement 3 of the over-all reaction, 2/3 of the O_2 evolved are used to produce the energy necessary for renewal of the photolyte. If the O_2 , reacting back were somewhat less than 2/3, the quantum requirement of the over-all reaction would of course be somewhat less than 3.

It may seem at first glance that quantum yield determinations in photosynthesis should be as easy as those in simple photochemical reactions. However, this is not the case and it must be repeated once again that the cells must be forced to utilize the energy of light at its maximum. As a matter of fact they do not do so when they are not cultivated under fluctuating illumination, when they are illuminated without blue-green light, when the neces-

* Warburg, O. and Krippahl, G. *Zschr. Naturf.*, 15b: 190, 1960.

Fig. 71. Determination of the quantum requirement. Dotted line: compensation of respiration. Continuous line: measured green light (5400–5680 Å). Abscissa: i in μl quanta/min. When $i = 0$, the values of $1/\varphi$ are 3.06 for the compensation of respiration and 2.85 for the measured green light.

sary trace elements are absent, when there is no optimal CO_2 pressure, etc. Though all factors inhibiting the action of light may be of biological significance, the physical chemist is of course only interested in obtaining the *minimum* quantum requirement. In discovering these different inhibitory factors, Warburg in his latest work arrived at the value 2.85 for the quantum requirement of the over-all reaction of photosynthesis. According to this value, we find by means of equation 27, efficiency values of the photosynthetic process of 96% in red (6800 Å), of 77% in green (5460 Å) and of 61% in blue (4360 Å).

Numerical example:

200 μl cells in 7.2 ml suspension. Two-vessel method. Gas phase: air + 10% CO_2 . Temperature 20° C. Duration of experiment 8 hours.

1. Quantum requirement in diffuse compensation light.

Incident intensities determined by means of the actinometer. 20% of the diffuse light is blue-green and about half of it is absorbed by chlorophyll. The calculated value of $1/\varphi$ must therefore be multiplied by 0.9 so that

$$1/\varphi = 0.9 \text{ it}/x_{0_2}$$

i μl quanta/min	Time of illumination in min	x_{0_2} in μl	$1/\varphi$
16	30	+72.9	5.92
8.27	30	+52.3	4.28
4.18	30	+31.3	3.60
0	limiting value (Fig. 71, dotted line)		3.06

2. *Quantum requirement in green measured light.*

Incident intensities determined by means of the bolometer. Degree of absorption 94% so that

$$1/\varphi = 0.94 \text{ it}/x_{0_2}$$

i $\mu\text{l quanta/min}$	<i>Time of illumination in min</i>	x_{v_2} in μl	$1/\varphi$
compensation light + 18.8 green	15	+43.3	6.12
compensation light + 9.6 green	30	+60.4	4.42
compensation light + 4.86 green	75	+95.2	3.59
compensation light + 0 green	<i>limiting value (Fig. 71 continuous line)</i>		2.85

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