PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY

J. A. V. BUTLER
H. E. HUXLEY

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14

Editors

J. A. V. BUTLER

Professor of Physical Chemistry
University of London
Institute of Cancer Research
Royal Cancer Hospital, London

H. E. HUXLEY

Medical Research Council
Laboratory of Molecular Biology
Cambridge

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PREFACE

Volume 14 contains a representative series of articles on a variety of topics, in which we hope that all our readers will find something of value and interest. It illustrates again the wide range of knowledge which can be considered as coming within the field of Biophysics and Molecular Biology. No one volume has been able to cover more than a fraction of this subject, but it is hoped that cumulatively a picture of this branch of science is being built up. We have noted with pleasure that even the early volumes of this series are still in demand and therefore contain information of continuing value.

In the present volume Prof. L. N. M. Duysens reviews the present state of knowledge of photosynthesis, with special reference to the work of his own laboratory. Dr. J. C. Skou is concerned with the transport of sodium and potassium ions through cell membranes and discusses possible "active" mechanisms and the enzymes concerned. Dr. W. Hasselbach is concerned with ionic aspects of muscular contraction and relaxation. Dr. McKinley-McKee gives an account of some recent work on the mechanisms of enzyme actions, with special reference to the alcohol dehydrogenases. Drs. Levvy and Conchie discuss the localization of certain enzymes in the cell and Dr. D. G. Dervichian gives a comprehensive account of the physical chemistry of phospholipids, which is a necessary basis to understanding their behaviour in cell membranes and elsewhere.

Finally, it may be noted that with this volume the publishers have agreed to sell copies of the separate articles at proportionate prices. It is hoped that this will enable research workers and others to obtain articles which are of particular interest to them.

The Editors will be pleased to receive suggestions for articles in future volumes. Any such suggestions will be dealt with as expeditiously as possible.

April 1964

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PHOTOSYNTHESIS

L. N. M. DUYSENS
Biophysical Laboratory of the University of Leiden

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PHOTOSYNTHESIS

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I. INTRODUCTION

In photosynthesis by algae and higher plants, light energy is used to convert carbon dioxide, water, and relatively small amounts of inorganic salts into carbohydrates, amino acids and other organic molecules. Nearly all living beings use these molecules as building stones in the processes of growth or as sources of "energy". Energetically, photosynthesis is the most important process on earth, and the production of (Gibbs) free energy in photosynthesis exceeds the industrial utilization of energy by a factor of about hundred (Rabinowitch, 1945). This process is unique in its kind: it is the only photochemical process known to store with good efficiency the energy of visible light in the form of energy of chemical compounds.

Photosynthesis has been studied by scientists of various backgrounds. Not only plant physiologists, but also chemists, physical chemists, biochemists, physicists and biophysicists have taken part in this research, and sometimes in passionate controversy concerning the interpretation of the results. Trustworthy theories concerning the mechanism of photosynthetic reactions should be based on results of all reliable chemical and physical experiments on living cells and cell free preparations and be consistent with well established theories from physics, chemistry and biology. The fact that mastery of all these fields by one scientist is virtually impossible may perhaps make understandable that authors have sometimes proposed hypotheses based on very limited evidence, which were either inconsistent with the well established experiments of other authors, or which were much more strongly supported by unquoted evidence already obtained by others. For the interpretation of the phenomena, I have selected the simplest hypothesis consistent with practically all trustworthy experimental evidence and physical theory. The simplicity of a hypothesis is of course no guarantee for truth, but it offers in my opinion the greatest chance

Abbreviations: ADP, adenosine diphosphate;
ATP, adenosine triphosphate;
CMU, *p*-chlorophenyl-1,1-dimethylurea;
DCIP, 2,6-dichlorophenol indophenol;
DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea;
DPN(H), (reduced) diphosphopyridine nucleotide;
FMN, flavine mononucleotide;
PGA, phosphoglyceric acid;
P_i, inorganic phosphate;
PMS, phenazine methosulphate;
PN(H), (reduced) phosphopyridine nucleotide;
TPN(H), (reduced) triphosphopyridine nucleotide.

of approaching the truth and of being a fruitful starting point for further experiments.

Under certain conditions the overall reaction of photosynthesis is:

$$nCO_2 + nH_2O \rightarrow (CH_2O)_n + nO_2$$

$(CH_2O)_n$ is the formula for carbohydrate or sugar (e.g. for glucose or another hexose, $n = 6$). In early speculations about the mechanisms of photosynthesis, it was assumed that the primary light reaction was the splitting of CO_2 into C and O_2. Subsequently the carbon reacted with water to give CH_2O. Basing himself on comparative biochemical considerations, van Niel (1941) suggested that the hydrogen is withdrawn by the light reaction from H_2O and transported to CO_2, which is reduced to carbohydrate.

Substances which yield hydrogen atoms or electrons are hydrogen donors or reductants, and substances which accept hydrogen or electrons, are hydrogen acceptors or oxidants. In photosynthesis, H_2O may function as a reductant, and CO_2 as an oxidant. The photosynthetic reactions are, however, much more complicated than earlier envisaged.

II. METHODS AND TECHNIQUES USED FOR THE STUDY OF PHOTOSYNTHESIS

The tremendous advances in the analysis of biological systems are for a great part due to the application of refined physical techniques and methods. In the field of photosynthesis, for instance, the use of radioactive carbon combined with paper chromatography enabled us to trace the main pathways of carbon in photosynthesis, and the application of sensitive and rapid fluorescence and absorption difference spectrophotometry made possible the identification and analysis of a part of the light-driven redox reactions in living cells. In this chapter I will discuss especially the new methods that were originally developed for the study of photosynthetic reactions. The biological objects should preferably be homogeneous and of low absorptance in order to ascertain that all pigment molecules are exposed to about the same intensity, and, for precise measurements of absorbed light, scattering should be low. These requirements may be fulfilled to a first approximation by the use of thin suspensions of unicellular photosynthesizing cells, of thin layers of such cells, or of a very thin leaf or of a thin thallus ("leaf") of marine algae. The nutrients are taken up from the medium. Since individual cells may have an absorptance of 50 per cent or more at the maxima of absorption (Duysens, 1952), use of suspensions having a much smaller average absorptance than this does not improve homogeneity. In order to take account of the remaining inhomogeneity and scattering, methods as described in section II, C2(*a*) can be used.

A. *Measurement of Oxygen and Carbon Dioxide*

In the Warburg apparatus, which measures gas pressures, and in many other apparatuses, the concentrations of carbon dioxide and oxygen are measured in the gas phase, which is in exchange with the solution in which the

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photosynthesizing organisms are suspended. These apparatuses in general operate rather slowly, since diffusion of gases from the water into the gas space requires a time of the order of 1 min in general. Within this time a change may occur in the rate of respiration or of other metabolic reactions, which may make uncertain the interpretation of the measurements of the rate of oxygen evolution.

None of the apparatus available is at the same time specific, rapid, precise, sensitive and absolute. I will only discuss here an apparatus which fulfills these requirements except the last one. This is the platinum polarograph for measuring oxygen, which has been used for measuring rapidly and precisely the kinetics of oxygen evolution, and for measuring action spectra of photosynthesis. A constant voltage is applied between a flat platinum electrode and a reference electrode (e.g. a large area calomel electrode). The current between the electrodes is proportional to the number of oxygen molecules diffusing towards the platinum electrode. In most applications described in this paper a thin layer of algae or a thin thallus are deposited on this electrode (Haxo and Blinks, 1950; Myers and French, 1960; Duysens and Amesz, 1962; Fork, 1962). Upon illumination of the photosynthetically active cells, the oxygen concentration in the cells rapidly rises to a steady state concentration, at which the rate of diffusion of the oxygen into the surrounding medium and to the platinum electrode is equal to the rate of evolution of oxygen by the cells. The increase of the current in the electrode circuit upon illumination was found to be proportional to the rate of oxygen evolution. This was concluded from the observation that the electrode current is proportional to light intensity under conditions in which, from measurements with other apparatuses, the rate of oxygen evolution is known to be proportional to light intensity. The response time of this apparatus is generally of the order of a second (cf. French, 1962). In another type of apparatus the algae are suspended in the liquid in which a wire-shaped electrode is immersed. This apparatus is less sensitive, and not so suitable for measurements of action spectra of photosynthesis but it can be calibrated absolutely, so that it can be used for measurements of the number of absorbed light quanta required for the evolution of one oxygen molecule (quantum requirement). By means of an apparatus in which the liquid was rapidly stirred, Joliot (1961) was able to achieve a response time of less than 0·1 sec. Since most recent measurements of action spectra of photosynthesis have been measured with an oxygen polarograph, I mention one possible error in the measurement of relative photosynthetic efficiency, when the flat platinum electrode covered with a layer of photosynthetic cells is used. For wavelengths at which appreciable absorption occurs the light will mainly be absorbed in that part of the layer which has the largest distance from the platinum, and the diffusion path for the oxygen will on the average be longer than for wavelengths which are more weakly absorbed. This effect may be small for a unicellular layer of small algae, but its possible occurrence has to be taken into account for the interpretation of action spectra of oxygen evolution measured with this type of apparatus.

It is possible to measure respiration and photosynthesis independently, at least in principle, by starting the experiment with different relative concentrations oxygen isotopes in the H_2O and in the O_2 (Brown, 1953). The changes in concentrations of the O_2 isotopes are followed by means of a mass spectrometer. In the conventional applications of this apparatus, the oxygen is passed from the gas phase to the ionization chamber of the spectrometer. In a recent application, the gases diffused directly from the water through a water-impermeable membrane into the spectrometer, which reduced the response time to less than half a minute (Hoch and Kok, 1963).

B. *Methods for Illumination and for Measuring the Light Intensity*

Until recently, the majority of the experiments with photosynthetic systems carried out by biochemists, have been done at very high intensities of unfiltered light from tungsten lamps. The intensity is usually expressed in lux. The recently discovered fact that two different photochemical reactions driven by two pigment systems with different action spectra are present in oxygen-evolving cells makes it desirable that, for qualitative biochemical work also, one or two light beams of relatively narrow wavelength regions be used, of which the intensities can be measured, preferably in einsteins/(cm^2 sec). One einstein is equal to N quanta, N being the number of molecules per gram molecule (Avogadro's number).

1. *Measurements of light intensity.* Measurements of intensity can conveniently be carried out by means of a small blackened thermopile calibrated in watts per volt by one of the national laboratories or institutes specializing in light measurements. The measurements are relatively simple, since the voltage generated by a thermopile is proportional to the energy/(cm^2 sec) incident on the light-sensitive surface of the pile. The calibration is, of course, only valid if the light beam is somewhat larger than the sensitive area and sufficiently homogeneous. The thermopile voltage can most conveniently be measured by means of a very sensitive electronic microvolt meter, but also a galvanometer of high voltage sensitivity can be used, if the voltage drop across the pile—which may have a resistance of the order of 10 ohms—is taken into account.

If the thermopile is too large, or of insufficient sensitivity, then a small 3×3 mm^2 silicon cell or other photo-electric cell can be used. The photo-electric cell is calibrated separately by means of the calibrated thermopile. In order to measure the incident intensity upon the reaction vessel, a small fraction of the incident beam is deflected onto a calibrated photo-electric cell of the vacuum emission type by means of a glass or quartz plate placed at an angle of about 45° with respect to the incident beam. The vacuum photocell with meter has the advantage of being not only stable, but also very sensitive and relatively inexpensive. The $CsCs_2O$ vacuum photocell (S_1-response) is sensitive to light in the region 300 to 1000 $m\mu$. This photo-electric cell can be calibrated by placing a calibrated thermopile at the location of the vessel with photosynthesizing cells.

2. *Light sources and monochromators.* Since the introduction of the high intensity xenon lamps and relatively inexpensive grating monochromators, it has become possible to construct from these commercially available parts relatively intense monochromatic light sources of reasonable spectral purity for wavelengths from about 240 to 1000 mμ. I will enumerate the factors which determine the energy output per sec of a monochromator and light source for a given half-width of the transmitted spectral band. In most monochromators the light source is imaged on the entrance slit and the entrance slit is imaged on the exit slit. A so-called field lens close to the entrance slit images the condenser on the dispersing element (grating or prism). A second field lens may be at the exit slit imaging the dispersing element on the object to be illuminated. For maximum output the condenser has to be so powerful that the image of the light source is slightly larger than the entrance slit. Furthermore the image of the condenser on the dispersing element should be slightly larger than this element. For optimum energy output the slits are made of equivalent width and height, i.e. that the image of the entrance slit illuminated with monochromatic light coincides with the exit slit. We assume, as is the case in most commercial monochromators, that the light coming from the entrance slit is made parallel by means of a lens or a mirror before falling upon the dispersing element. Under these conditions the energy output is equal to the product of the following factors: the surface brightness of the light source; the height of the entrance slit, divided by the focal distance of the first imaging element (or, which is the same, the height of the exit slit divided by the focal distance of the second imaging element inside the monochromator); the area of the dispersing element; the angular dispersion of this element for the transmitted wavelength; and, finally, the transmission of the monochromator for this wavelength. The transmission of the monochromator is defined as the transmitted fraction of the energy of a narrow pencil of monochromatic light which is not obstructed by the slit jaws or other diaphragms. For obtaining a high energy output, a grating is in general superior to a prism as dispersing element, since a grating is less expensive per unit area than a prism and in general may be selected to have a higher angular dispersion. To my knowledge the Bausch and Lomb monochromator with 10×10 cm^2 gratings with 1200 lines per mm is the most powerful reasonably priced monochromator available at the moment. On request, gratings are available with maximum transmission at other wavelengths than the standard one of 300 mμ. In our experiments we used as the light source an Osram (Germany) 1000 W dc xenon arc fed by a dc rectifier. The condenser system provided with the monochromator was used: with this condenser the slit was only illuminated for a little more than half of the height. A higher output may be obtained by means of the 2500 W xenon lamp, which has a larger arc, but with this lamp it will be probably necessary to cool the entrance slit and condenser of the monochromator. Another powerful light source, of somewhat higher output at some wavelengths, is the high pressure 1000 W water-cooled mercury arc (Philips SP 1000 W dc). This light source is less convenient to use than the xenon arc, because the mercury has occasionally to

be distributed evenly, the water cooling requires care and the lamp has a life time of 50 hours as compared to a life time of more than 1000 hours of the xenon lamp. We find the output of these xenon and mercury lamps to be constant within about 1 to 2 per cent. If lower energy outputs are sufficient, tungsten ribbon filaments lamps or the more intense tungsten–sodium filled quartz lamps made by e.g. Silvania in the U.S.A. and by Atlas in England are more convenient to use, because tungsten lamps require only an easily regulated power supply. When using the arc lamps, the output energy can be adjusted by changing the width of the entrance slit. For cutting off light of the second order spectra, which is always present in a grating monochromator, and also for cutting off "false light", coloured glass and interference filters are placed in the exit beam of the monochromator. By means of glass filters (e.g. Schott or Corning) it is often possible to reduce the undesired light of a certain wavelength to less than one millionth of the original value. The selection of suitable filters is especially important for fluorescence experiments. A catalogue is for sale from Schott, Frankfurt am Main, W. Germany, that gives absorption data of Schott glass filters in regions of low transmission.

If there is no objection to wavelength bands wider than 10 mμ, and continuous variation of the wavelength is not necessary, then tungsten lamps provided with interference filters of various band widths are convenient and inexpensive sources of monochromatic light. We use 500 W 2×2 in. light projectors without objective (Aldis 500 W). The slide is replaced by a diaphragm of suitable shape, which is imaged on the object by means of a simple condenser. If necessary glass filters are added to the interference filters to suppress false light.

For studying two photochemical systems, two or more strong monochromatic light beams are often desirable for illuminating the object simultaneously. These beams may fall on the cuvette from different angles, but if this is impossible because of lack of space, the two beams may be "mixed" by means of a semi-transparent mirror.

3. *Variation and modulation of light intensity.* The simplest method of changing the rate of the photosynthetic reactions is by shutting on and off light of constant intensity. By means of commercially available photographic shutters, this can be done with rise and decay times of about one thousandth of a second. Periodic flashes of variable duration and intensity can be obtained by means of rotating discs with slits. By imaging a dc high pressure mercury arc by means of a strong lens on a small slit in a revolving disk and by concentrating the light coming from the slit on a small vessel, Kok (1957, 1959) was able to obtain flash times of high intensity of the order of 10^{-4} sec and longer. By discharging a condenser through a xenon flash lamp either single or repeated flashes can be obtained of life times of 10^{-6} sec and higher. By means of a specially constructed hydrogen discharge lamp and circuit, Brody (1957) obtained flashes of 10^{-9} sec duration, but of a very low energy per flash.

As already mentioned, important methods for following photosynthetic

reactions are based on absorption and fluorescence (or luminescence) measurements.

In many applications the "measuring" light is modulated, which is often done by means of a rotating disk mounted on a motor, or by means of a vibrating diaphragm, so that the incident light is interrupted with a fixed frequency. For the measurement of absorption, the light passes through the suspension of photosynthetic cells and falls on a vacuum photocell or on a photomultiplier. For the measurement of fluorescence, part of the emitted light, which of course is modulated with the same frequency as the incident light, falls on the photo-electric device. The alternating component of the pulsating current of the photo-electric device is amplified and the output of the amplifier is rectified by means of a phase sensitive rectifier, which only transmits the same frequency as that of the interruptor. One advantage of the modulation is that a slow drift due to changes in dark current of the photocell or to changes in the electronic tubes, which is difficult to minimize in a dc apparatus, is virtually absent. Another advantage is that simultaneous illumination of the organisms with non-interrupted light or with light modulated with a different frequency, except for transients, will not cause a deflection of the recorder. This is important in applications in which interrupted light is used for measuring certain effects which are brought about by strong non-interrupted light (continuous actinic light). Actinic light falling on the light detector will then not cause a deflection, although the noise may increase and transient deflections may occur upon the admitting or interrupting of the actinic light.

If photosynthetic effects of the actinic light are relatively long-lived, these effects may be measured a short time after the actinic illumination has been switched off. Transients can then be eliminated as follows.

In the first type of apparatus the strong actinic and the weak measuring light are admitted alternately to the suspension of photosynthesizing cells. A periodically operating shutter in front of the photomultiplier is only open during the admission of the measuring light. The modulation of the three beams may be effected by disks with diaphragms driven by the same motor. In the second type the cells may be actinically illuminated in a flow system shortly before flowing past the photomultiplier.

C. *Physical Methods for the Investigation of Intermediates and of Pigments*

1. *Introduction*

In this section methods will be discussed for the investigation of components or intermediates present in photosynthetic cells or in cell-free photosynthetic systems. Other physical methods, which require chemical purification of the substances to be measured, will be discussed in the next section. The most important methods at present are those based on the use of visible, near-ultraviolet and near-infrared light. In section II, C2 distortions of the absorption and fluorescence spectra will be discussed, which are caused by the concentration of the pigment in small particles, and by scattering of the light. Methods for correcting these distortions and for utilizing them in order to obtain

information concerning cell structures will be given. In the last section diverse techniques, such as electron magnetic resonance, and techniques for studying structure will be shortly discussed.

2. *Techniques employing light absorption*

(*a*) *Absorption spectra.* Conventional absorption spectrophotometers are in general constructed for measuring absorption spectra of non-scattering solutions or suspensions. Absorption (or extinction, or absorbancy) spectra of strongly scattering suspensions of photosynthetic cells show the following distortions, when compared with the extinction spectrum of the same molecules dispersed molecularly in a non-scattering solution. Distortions of difference spectra are discussed in section (*b*).

(1) The absorption spectrum of a scattering suspension is shifted upward. Even at wavelengths at which the pigments do not have an intrinsic absorption, this shift occurs. It is due to the fact that part of the light is scattered away from the photodetector. Since the scattering predominantly occurs in the same direction as the incident beam, the scattering can be greatly diminished by catching a larger angle of the light leaving the suspension. This can be done by using a larger photodetector, by placing the photodetector closer to the absorption cuvette, or by placing a strongly scattering layer (e.g. a piece of opal glass) close behind the absorption cuvette and the blank. The use of a scattering layer yields, except for energy losses, the same absorption spectra as a photodetector of the same area as the scattering layer in the place of this layer. The use of opal glass is due to Shibata (1958; Shibata *et al.*, 1954), and proved very convenient for adapting existing spectrophotometers for the measurement of scattering suspensions (see Amesz *et al.* (1961) for a critical discussion of the use of opal glass).

(2) Another distortion occurs in a very strongly scattering suspension. It is the flattening of the whole spectrum caused by repeated back scattering: by repeated scattering on several particles the light is scattered back towards the photodetector. If for a given wavelength and geometry the absorption is plotted as a function of the concentration of the suspension, the graph obtained will be a straight line for low concentrations (Beer's law). At higher concentrations and low absorbance the graph will bend away (downward), since, due to back scattering, more light will fall on the photo-electric detector. At high absorbance, the multiple scattered light will be strongly absorbed. Scattering plus reabsorption will result in a very low transmission, giving rise to a higher apparent absorbance (see e.g. Butler, 1962a) than in the absence of back scattering. Since it is very difficult to correct for these effects, the concentrations should be taken sufficiently low so that the deviation from Beer's law does not occur. For the small unicellular photosynthetic cells usually employed, these effects are small in dilute suspensions.

(3) A second type of flattening occurs, even in a non-scattering suspension, if the suspension contains relatively strongly absorbing particles, such as unicellular photosynthesizing cells (Duysens, 1952, 1956a). This type of flattening

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