THE ACTION SPECTRUM OF PHOTOSYNTHESIS IN EUGLENA GRACILIS AT DIFFERENT STAGES OF CHLOROPLAST DEVELOPMENT

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Summary

A method for measuring the action spectra of photosynthetic oxygen evolution in unicellular algae is described. This involves embedding the cells in a thin strip of agarose gel. These gel strips can then be used for action spectrum measurements, with a bare platinum electrode of the Haxo and Blinks type in the same way as measurements are made with a piece of multicellular algal thallus.

The action spectrum of photosynthesis has been studied in etiolated cells of *E. gracilis* greening up in the light. Early in chloroplast development there is activity in red and blue light, but little or none in green. Later, activity in green light appears and increases until it reaches 50–80% of the activity obtained in red light.

Early in the greening process the relative effectiveness of red and green light is markedly affected by the concentration of cells in the gel. Green light induces little or no photosynthetic response at high cell concentrations (26·0–54·4 x 10⁶ cells/ml), but is active at low cell concentrations (6·5–13·0 x 10⁶ cells/ml); red light is effective at high or low cell concentrations. When higher cellular levels of chlorophyll are reached this dependence on cell concentration disappears.

I. INTRODUCTION

When cells of *Euglena gracilis* are grown heterotrophically in the dark they do not form chloroplasts: they contain instead small, yellow, undifferentiated proplastids (Epstein and Schiff 1961) which have no photosynthetic activity (Stern, Schiff, and Epstein 1964). When these dark-grown cells are exposed to light the proplastids begin to differentiate into chloroplasts: chlorophylls, carotenoids, photosynthetic enzymes, and the thylakoid membranes are formed, and after 1–3 days the cells contain mature chloroplasts. Photosynthetic activity develops approximately in parallel with the synthesis of these chloroplast constituents (Stern, Schiff, and Epstein 1964). In the present work the action spectrum of photosynthetic oxygen evolution has been measured at intervals during greening to study the role of various photosynthetic pigments at different stages of chloroplast development.

II. METHODS

(a) Growth of Cells

Cells of the Z strain of *E. gracilis* (strain 1224/5z, Culture Collection of Algae and Protozoa, Botany School, Downing St., Cambridge) were grown for 4–8 days at 28°C in the dark in a New Brunswick rotary shaker, in 100-ml batches in 250-ml conical flasks. The growth medium was the

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defined basal medium of Kirk and Keylock (1967) containing 0·25% (v/v) ethanol (before autoclaving) and with twice the usual concentration of NH$_4$NO$_3$.

(b) Preparation of Cell Gel

Action spectra of photosynthesis in algae are normally obtained by measuring oxygen evolution by a thin layer of cells in close contact with a bare platinum electrode, as in the pioneering studies of Haxo and Blinks (1950). In the case of multicellular algae such as Ulva, this may easily be achieved by holding a piece of thallus flat against the electrode by covering it with a piece of permeable Cellophane, close contact being maintained with elastic bands or other simple device. However, maintaining a thin layer of cells of a unicellular alga, such as Euglena, in close proximity to a platinum electrode is more difficult. Other workers have achieved this by constructing shallow, horizontal chambers with the platinum electrode at the base (Haxo and Fork 1959), or by trapping a thin film of cells over the electrode by means of Cellophane, a rubber gasket, and a suitable clamping device (French, Fork, and Brown 1961; Vidaver 1964). Since algae (such as Ulva) which take the form of thin sheets of cells are easier to handle than unicellular algae, and can be used with a simpler type of platinum electrode, it was decided in the present work to prepare the Euglena cells in the form of a thin sheet which could be handled like a piece of multicellular algal thallus. This was achieved by mixing the cells with a gelling agent and allowing the suspension to set in a shallow rectangular mould. The procedure is as follows:

Aliquots of cultures of E. gracilis containing 20–40 x 10$^6$ cells (except where otherwise stated) are taken. The cells are harvested from the growth medium and washed three times in 0·15m NaCl by centrifugation and resuspension. The pellet of cells is resuspended with circulation medium [see Section II(c)] to a final volume of 0·5 ml. The centrifuge tube is warmed for a few seconds in a water-bath at 40°C and then 0·5 ml of a 0·6% solution of agarose at 40°C is added and mixed thoroughly with the cell suspension for 15 sec with a Pasteur pipette. The mixture is now withdrawn with a pipette and used to fill the mould to make a strip of gel.

The mould was made from a microscope slide and a piece of aluminium foil, 0·16 mm thick. A rectangle of foil, the same size as the slide (2·5 by 7·5 cm), was prepared. A rectangular section, 5·5 by 0·75 cm, was cut out along the centre of the foil and discarded. The remaining foil was cemented down onto the microscope slide with Araldite epoxy resin cement thus forming a shallow chamber, 55 mm long, 7·5 mm across, and 0·175 mm deep.

To prepare a uniform strip of gel the mould is placed flat upon the bench. Another microscope slide is held so that one end is lying across one end of the mould chamber, and the slide itself is leaning back, at an angle of about 45° to the horizontal, towards the other end of the mould chamber. The tip of the pipette containing cell suspension in agarose is allowed to touch simultaneously the end of the mould chamber and the end of the microscope slide. The slide is then steadily drawn back across the top of the mould chamber and at the same time the cell suspension in agarose solution is allowed to run slowly out. The gel sets within a minute or two: it is released from the mould by cutting it around the perimeter with a razor-blade and then agitating the mould gently in a dish of circulation medium.

(c) Construction and Method of Use of Oxygen Electrode

The electrode system for polarographic measurement of photosynthetic oxygen evolution is in principle of the Haxo–Blinks type: namely a silver/silver chloride anode and a bare platinum cathode on which a piece of gel strip can be placed directly. However, the design of the apparatus differs substantially from that used by Haxo and Blinks (1950). It is shown in Figure 1. The piece of Euglena agarose gel strip (which is placed in contact with the platinum cathode) and the dialysis tubing (used to hold the gel strip in position) are not shown in the figure.

Construction and method of use of the electrode system was as follows: The end of a polypropylene centrifuge tube was cut off leaving a cylinder 9·4 cm long and 1·75 cm diameter. A rubber cap (A) was cemented on to one end of the cylinder. The silver/silver chloride anode was prepared from a silver electrode (Electronic Instruments Ltd.) consisting of a thin silver disk 1·2 cm in diameter, with its silver lead wire cemented into a glass stem (12·5 cm long, 0·6 cm
diameter). The lead wire was connected to an insulated electrical lead at the other end of the glass stem. The silver electrode was converted to a silver/silver chloride electrode by electrolysis in 1x HCl. The silver/silver chloride anode was inserted into the polypropylene cylinder in such a way that the metal disk was just inside the lower (open) end of the cylinder and the upper end of the glass stem with its attached insulated lead protruded through a hole in the rubber cap at the upper end of the cylinder. The upper part of the polypropylene cylinder was then filled with Araldite epoxy resin to fix the silver/silver chloride anode in position. The remainder of the interior cavity, i.e. the lower part, of the polypropylene tube, was filled with a 4% agar gel containing 0·15m KCl.

The platinum cathode consisted of a square piece of platinum foil (0·65 by 0·65 cm) cemented with Araldite to the outside of the polypropylene tube, its lower edge being 1·5 cm from the bottom of the tube. An electrical lead was soldered to the under surface of the platinum foil before the latter was cemented onto the polypropylene. The short length of bare copper wires of this lead were covered over with Araldite from the point where they emerged from under the platinum foil to the point where the normal plastic insulation of the lead began.

When in use this combined electrode system is immersed in a 50-ml beaker containing circulation medium (see below). Thus there is electrical connection between the cathode and anode through the liquid medium (which contains 0·15m KCl) and the KCl/agar gel inside the polypropylene tube. A polarizing voltage of 0·5 V is applied between the platinum and silver/silver chloride electrodes by a circuit utilizing a 1·5-V dry cell battery and a variable (0–2000 ohms) resistance, R1 (Fig. 1). The current through the electrode system (which is proportional to the rate of reduction of oxygen by the cathode) passes through the variable (0–2000 ohms) resistance, R2. The voltage across R2 is monitored continuously with a Honeywell (0–10 mV) Elektronik recorder. The value of R2 can be adjusted so as to ensure that all the readings in a particular experiment are within the range of the recorder.

A 6-mm length of the agarose gel strip is placed squarely on the platinum cathode: it is held in position by a short length of dialysis tubing which is wrapped completely round the electrode assembly and held tight by two elastic bands, one below and one above the electrode. The electrode assembly is held with a clamp so that it is immersed vertically at one side of a 50-ml beaker containing circulation medium (0·15m KCl, 0·04m KH2PO4–Na2HPO4, pH 7·0, 1·0 mm MgSO4, 2·0 mm NaHCO3). The electrode system is turned so that the platinum cathode is nearest the wall of the beaker. Fresh circulation medium is continuously pumped from a reservoir (B) into the beaker by an LKB micropump at the rate of 50 ml/hr. The polythene tubing (1·5 mm bore) carrying the inflow from the pump is held (by means of an elastic band around it and the electrode assembly) pointing vertically downwards just above the platinum cathode: this ensures that there is a continual stream of fresh medium flowing past the piece of gel strip. The level of medium in the beaker is prevented from rising above a certain point by means of a siphon (C). The medium discharged by the siphon drips into a funnel (D) and then passes through tubing down to the same reservoir from which the pump is continually drawing fresh medium. The reservoir consists of a 250-ml beaker containing 150 ml of circulation medium which is stirred continuously with a magnetic stirrer to ensure that the medium is maintained in equilibrium with atmospheric oxygen and carbon dioxide.
(d) Action Spectrum Determinations

The light source for the action spectrum determinations is a Bausch and Lomb high intensity monochromator with a quartz iodine lamp and with slit widths such as to give a spectral band-pass of 9.6 nm. The monochromator is positioned just outside the 50-ml beaker (Fig. 1) and the actinic light beam is shone directly onto the piece of \( E. \) gracilis gel strip. Since the cross-sectional area of the light beam at this point is larger than that of the piece of gel, the whole of the gel is irradiated. In experiments in which activity in red and green light alone is compared, the light source is an Aldis QI 24 slide projector with Balzer interference filters (wavelengths of maximum transmission 681 and 547 nm respectively).

Before measuring the action spectrum the piece of \( E. \) gracilis gel is irradiated with red (680 nm) light. When the maximum rate of photosynthesis is reached the variable resistance \( R_2 \) (Fig. 1) is adjusted so that the recorder reading is high but on scale. The light is then switched off and the oxygen concentration falls in a few minutes to a steady state in which the consumption of oxygen by respiration and by the electrode itself is just balanced by the diffusion of fresh oxygen from the stream of medium flowing past. The monochromator is set to give the longest wavelength required in that particular spectrum, and the light is switched on. If the light is photosynthetically active, there is oxygen evolution, the recorder reading rises steadily and within 3-5 min reaches a new steady state (Fig. 2). At this point photosynthetic oxygen evolution is taking place at a steady, maximum rate. Within the gel there is a high concentration of oxygen.

![Fig. 2.—Typical time course of photosynthetic oxygen evolution by partially greened cells of \( E. \) gracilis.](image)

On either side of the gel there is a region of lower oxygen concentration: on one side there is the surface of the platinum cathode where the concentration is kept very low by the electrolytic reduction of the oxygen molecules; on the other side, separated by the dialysis membrane barrier, there is the stream of flowing medium in equilibrium with the oxygen of the atmosphere. The rate of diffusion of oxygen to the cathode should be much faster than to the stream of flowing medium, partly because there is a steeper concentration gradient to the cathode, and partly because diffusion of oxygen to the flowing medium is impeded by two layers of dialysis membrane. This will result (as previously pointed out by Haxo and Blinks 1950) in most of the photosynthetically produced oxygen being consumed by the cathode. Consequently the increase in steady-state current through the external circuit and hence the increase in voltage \( (V_L-V_D) \) across \( R_2 \), will be approximately proportional to the rate of photosynthesis. The proportionality should not, in fact, be greatly affected by the loss of oxygen through the membrane, since the rate of this loss, being proportional to the concentration of oxygen within the gel, will itself be approximately proportional to the rate of photosynthesis. In short, following Haxo and Blinks (1950) we are assuming that this electrode system gives a response which is approximately proportional to the rate of photosynthesis, but does not give absolute rates of photosynthesis.

In some experiments the voltage begins to decrease again shortly after the steady state is reached: in these cases \( V_L \) is taken to be the highest value reached during the early steady state. When the value of \( V_L \) has been determined, the light is switched off again to allow the dark steady state to be re-established. The monochromator is set to the next wavelength required and then the light is switched on again. In this way a set of values for \( (V_L-V_D) \) at a series of wavelengths
proceeding from the red end of the spectrum down to the blue is obtained. When particularly accurate values of \((V_L-V_D)\) are required, \(V_L\) and \(V_D\) are measured with a Solartron digital voltmeter, model LM 1420.2.

The spectral energy distribution of the monochromator over the range 400–750 nm was determined with an ISCO spectroradiometer. The monochromator emits its highest energy in the red (660–700 nm) region. The energy falls off progressively at shorter wavelengths: the energies at 550 and 450 nm are about 77 and 38%, respectively, of the energy at 680 nm. The energy at the \(E.\ gracilis\) gel surface is 5000 ergs/cm²/sec with light of wavelength 680 nm. To convert the rates of photosynthesis at the different wavelengths to equal incident energies, the value of \((V_L-V_D)\) at each wavelength is divided by the energy \((E_\lambda)\) incident on the gel at that wavelength. The values of \((V_L-V_D)/E_\lambda\) are plotted against \(\lambda\) to give the action spectrum. Ideally in an action spectrum determination the incident energy should be the same at all wavelengths. However, since the purpose of the present work was to look for changes in the action spectrum at different stages of chloroplast development, rather than to establish an absolute action spectrum for this alga, the experimentally simpler procedure outlined above was used. When comparisons of activity in red and green light alone were carried out, using the interference filters, the energy was adjusted to be 5000 ergs/cm²/sec in each case.

(e) Chlorophyll Determination and Cell Counts

When the cells were allowed to green up within the gel, the pieces of gel were removed from the electrode after determination of the action spectrum and extracted with 1 ml of 80% acetone. The debris was removed by centrifugation and the extinctions of the supernatant at 645 and 663 nm were measured in microcells of path length 2 cm in a Unicam SP 500 spectrophotometer. When the cells were greened up in liquid medium aliquots of the culture were taken, the chlorophyll extracted from the cells with 80% acetone, and extinctions measured at 645 and 663 nm. Chlorophyll concentration was calculated with the help of a nomogram (Kirk 1968) based on the published (MacKinney 1941) extinction coefficients of chlorophyll \(a\) and chlorophyll \(b\).

Cell counts on aliquots of the cultures were performed with a Coulter counter.

(f) Absorption Spectra of Gel Strips

A 3-cm length of moist gel strip is placed along the inner face of one of the optically clear sides of a spectrophotometer cuvette. The cuvette is put into the "second sample position" of a Unicam SP 500 recording spectrophotometer, with the gel strip on the side nearest to the photomultiplier. An absorption spectrum is taken with air as the blank. The gel strip is then immersed in 80% acetone for 2 hr followed by 100% acetone for 2 min, to extract the pigments. The extracted strip (now white in colour) is immersed in circulation medium for 15 min to rehydrate the cells. An absorption spectrum is then taken as before. This latter spectrum gives an approximate indication of what proportion of the first spectrum is actually due to light scattering rather than absorption.

III. RESULTS

(a) Action Spectra of Cells after Different Periods of Greening within the Agarose Gel

Thin strips of agarose gel, containing 20–40 \(\times\) 10⁶ dark-grown cells of \(E.\ gracilis\) per millilitre of gel were prepared as described in Section II(b). The strips of gel were placed in a Petri dish containing 20 ml of circulation medium to which ammonium nitrate (0.03%) was added to enhance greening. The Petri dish was kept at room temperature. The gel was illuminated with light at an intensity of about 1500 lux from a tungsten lamp. At intervals 6-mm lengths of gel strip were removed, placed on the oxygen electrode, and tested for photosynthetic activity in red (680 nm) light. With pieces of gel that showed measurable levels of activity in the red, measurements were also carried out at a series of other wavelengths to give an action spectrum.
To begin with, the dark-grown cells gave no detectable oxygen evolution with actinic light of any wavelengths in the visible range. No activity in red light could be detected after illumination for 1, 2, or 3 hr. Oxygen evolution in red light was usually just barely detectable after illumination for 4 hr. Since there was measurable activity after 6 or 7 hr (1·0–2·0 μg chlorophyll/10^6 cells), the first action spectrum was measured at about this time: this showed activity in the red (620–700 nm) and the blue (440–460 nm) regions, but no activity in the 480–600 nm range (Fig. 3). As greening proceeded the height and width of the red and blue peaks of the action spectrum increased: for instance after 9 hr (2·0–4·0 μg chlorophyll/10^6 cells) these extended from 580–690 nm, and from 420–500 nm, respectively. However, there was still no measurable photosynthetic activity in green light. Oxygen evolution in green (550 nm) light first became detectable after illumination for c. 12 hr, and after 24 hr (7·0–14·0 μg chlorophyll/10^6 cells) the relative rate had risen to 25–30% of the rate obtained in red light (Fig. 3). In fully green cells (30·0 μg chlorophyll/10^6 cells) which had been grown in continuous light, green light was, relatively speaking, even more effective: the rate of oxygen evolution per unit of incident energy at 550 nm was as much as 70% of the rate obtained in red (680 nm) light.

![Fig. 3.—Action spectra of photosynthesis in cells of E. gracilis, after different periods of greening. Agarose gel strips containing dark-grown cells of E. gracilis were prepared and then exposed to light to permit chloroplast development. Action spectra of photosynthetic oxygen evolution were determined with gel strips exposed to light for 6 (○), 9 (△), or 24 (□) hr.](image)

(b) Dependence of Relative Effectiveness of Red and Green Light on Cell Concentration

Experiments were carried out to determine whether the relative effectiveness of red and green light at bringing about photosynthesis was affected by the concentration of cells in the gel strip. Cells were grown in the dark as usual and when they had reached the late logarithmic or early stationary phase the flask was transferred to a window-sill receiving diffuse daylight (1000–4000 lux during the day; approximately 8 hr of darkness every night), at 3·00–4·00 p.m. Cultures were sampled on the following morning (i.e. after 11–12 hr light) and again after either one or three further 24-hr periods (each 16 hr light/8 hr dark). Each sample of culture was used to make two gels, one containing 6·5–13·0×10^6 cells per millilitre, the other containing 26·0–54·4×10^6 cells per millilitre, i.e. the second gel had a concentration of cells four times as high as that in the first gel. The rate of photosynthetic oxygen evolution was measured in red (681 nm) or green (547 nm) light from a slide projector used with interference filters. The total incident light energy was the same (5000 ergs/cm^2/sec) at both wavelengths. The order of the light treatments was—dark period, red, dark period, green.
The results of three typical experiments are shown in Table 1. From these and other measurements it is clear that at relatively low chlorophyll levels (e.g. up to \( c.7\cdot0 \mu g/10^6 \) cells) green light is almost totally ineffective at promoting photosynthesis at high cell concentrations, but is quite active at low cell concentrations. Red light is effective at high or low cell concentrations. In experiment 1 it can be seen that the photosynthetic effectiveness of green light (relative to that of red light) is 26 times as high with \( 12\times10^6 \) cells/ml of gel as it is with \( 48\times10^6 \) cells/ml of gel. However, this drastic falling-off in the relative effectiveness of green light at high cell concentrations (which we shall call "the cell concentration effect") disappears at higher levels of chlorophyll (upwards of \( 11 \mu g \) chlorophyll/\( 10^6 \) cells), as may be seen in Table 1.

**Table 1**

DEPENDENCE OF RELATIVE PHOTOSYNTHETIC EFFECTIVENESS OF RED AND GREEN LIGHT ON CELL CONCENTRATION, AND CELLULAR LEVELS OF CHLOROPHYLL

Cells of *E. gracilis* were grown in the dark and then transferred to light to permit chloroplast development. After suitable intervals of time samples were taken, chlorophyll was estimated, and agarose gel strips at appropriate cell concentrations were prepared for measurement of photosynthetic oxygen evolution in red or green light.

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<th>Expt. No.</th>
<th>Period of Exposure to Light (hr)</th>
<th>Chlorophyll (( \mu g/10^6 ) cells)</th>
<th>Millions of Cells per 1 ml of Gel</th>
<th>( (V_L - V_D)_{\text{red}} ) (mV)</th>
<th>( (V_L - V_D)_{\text{green}} ) (mV)</th>
<th>( \frac{100 \times (V_L - V_D)<em>{\text{green}}}{(V_L - V_D)</em>{\text{red}}} )</th>
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As was also found in the experiments described in the previous section, there is within any one experiment, at a given cell concentration, a substantial increase in the photosynthetic effectiveness of green light (relative to that of red light) as the cellular chlorophyll concentration increases (Table 1). Because of the simultaneous diminution of the cell concentration effect as greening proceeds, this increase in the relative effectiveness of green light is much more marked at high cell concentrations than at low cell concentrations.

**IV. Discussion**

Two main findings emerge from these results. One is the very marked increase in the photosynthetic efficiency of green light relative to that of red light that takes place during greening. The other is the almost complete loss of photosynthetic
effectiveness of green light, but not of red light, when the concentration of cells in the gel is raised from about $12 \times 10^6$ cells/ml to about $48 \times 10^6$ cells/ml; this cell concentration effect being observed at low, but not high, cellular levels of chlorophyll.

In attempting to interpret the increased photosynthetic effectiveness of green light during chloroplast development we must bear in mind the fact that the effectiveness of light of a given wavelength is related to the percentage of light that is absorbed (100–T) rather than to the extinction (log $I_0/I$, or 2–log T). In the case of chlorophyll in solution the ratio of percentage absorption of green light to percentage absorption of red light increases as the concentration of chlorophyll increases. For instance, for chlorophyll $a$ dissolved in diethyl ether it can be calculated, using the published extinction coefficients (French 1960), that for a 1-cm light path the ratio of percentage absorption of green light (550 nm) to percentage absorption of red light (662 nm) rises from 0.082 at 10 $\mu$g chlorophyll/ml to 0.538 at 100 $\mu$g chlorophyll/ml. This effect may partly, or perhaps even wholly, account for the increased photosynthetic effectiveness of green light relative to that of red light, as chloroplast development proceeds.

We are as yet unable to offer any plausible explanation for the cell concentration effect. The possibility that at high cell concentrations all the green light, but not the red, is absorbed before it can penetrate to that part of the gel nearest to the platinum electrode, can immediately be rejected. Absorption spectrum measurements on the gel show that, as expected, green light is absorbed to a much lesser extent than red light. For instance, the concentrated gel strip ($48 \times 10^6$ cells/ml), prepared with cells illuminated for 11 hr in experiment 1 (Table 1), had an apparent percentage absorption of about 58% at 550 nm and 82% at 680 nm. Control measurements on the strip after extraction of pigments with acetone indicated that nearly all the apparent percentage absorption at 550 nm, and slightly over half that at 680 nm, were due to scattering. These measurements show that even at the high cell densities, green light penetrates the gel better than red light.

Whatever the explanation of the effect, its existence raises the possibility that the actual shape of an action spectrum of photosynthesis (in particular, the relative effectiveness of green light) measured on a piece of algal thallus may be affected by the concentration of cells (or of chloroplasts) in the thallus, a factor which in the case of a multicellular alga is not under the experimenter’s control. However, if the present results with E. gracilis can be generalized to other algae, then this effect should be significant only when the cellular levels of chlorophyll are low.

V. References


