THE IDENTITY OF THE FLUORESCENT AND DELAYED LIGHT EMISSION SPECTRA IN CHLORELLA*

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INTRODUCTION

That chlorophyll in the living plant can fluoresce has been known since the time of Stokes. The yield of this fluorescence has been determined (1) to be 0.15 to 0.30 per cent in *Chlorella*. A low fluorescence yield implies a short life-time for the excited chlorophyll. Times of 1.2 to 2.4 $\times$ 10^{-10} seconds are given by Rabinowitch (2). Two years ago a communication from this laboratory (3) described a light emission by green plants which lasted for some seconds after irradiation. In that paper it was suggested that this delayed light represented the reversal of some of the early steps in such a way that some excited chlorophyll was regenerated chemically. If this idea is valid the emission spectrum of the delayed light should be identical with or very similar to the emission spectrum of the fluorescent light. The term "very similar to" is used because the chemical formation of the excited chlorophyll and of excitation by light might give different distributions of vibrational or rotational states.

For the purpose of comparison, the present paper gives a determination of the two emission spectra, made with the same apparatus and under the same conditions. Strehler and Arnold (3) emphasized the extremely low intensity of the delayed light. It was estimated that one *Chlorella* cell was emitting only 7 quanta per second near the beginning of the decay curve. This low intensity is the first of three considerations that make it difficult to determine, in a thoroughly satisfactory manner, the emission spectrum of the delayed light. Secondly, since part of the emission spectrum falls at the same wave length as the red absorption band of chlorophyll, very thin suspensions must be used to avoid a distortion in the shape of the curve through self-absorption; thus the light intensity is still further reduced. Lastly, when this investigation was begun, it was not possible to buy a photomultiplier sensitive in the region 600 to 800 mμ.

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Materials and Methods

The cells used were *Chlorella pyrenoidosa*, Emerson's strain, grown at 20°C. in Knop's solution and suspended in the same solution for the measurements.

Optical Methods.—The cells were illuminated in a small glass bowl (150 cc.) by a neon light (40 cm. of ordinary neon sign tubing). Saturation of delayed light production was shown by the failure of extra light on the bowl to increase the signal. From the bottom of the bowl the suspension flowed through about 50 cm. of black rubber tubing, making two 180° bends, to a glass tube (5 mm. I.D., 8 mm. O.D.) mounted vertically 1.3 cm. in front of the entrance slit of the monochromator. This glass tube was held in a light-tight aluminum housing which was bolted to the monochromator. A hand-operated shutter between the tube and entrance slit allowed the light entering the instrument to be turned on and off while the suspension was illuminated continuously, thus avoiding the complicated transient effects described earlier (3). From the bottom of the glass tube the suspension flowed through black rubber tubing to a small centrifugal pump, and then back into the top of the bowl. One-half second was consumed by a given *Chlorella* cell in travelling from the illuminated bowl to the tube in front of the entrance slit.

The measurement of fluorescence was made with the cell suspension in the pumping system, the bowl being in the dark. The glass tube in front of the entrance slit was illuminated from the side. Light used to excite the fluorescence came from a small incandescent lamp whose brightness could be adjusted to make the signal of the same order as that from the delayed light. The exciting light was filtered through 14 cm. of saturated copper sulfate solution plus a Corning No. 5850 filter to remove red light.

Delayed light was measured using 1 $mm.^3$ of cells (30×10^6) per cc. A simple calculation shows that with this density, 43 per cent of the cells located on the longest ray that can send light into the monochromator will not have another cell in front of them. However, it was found that experiments made with Knop's solution and with no cells gave a small fluorescence in the region of 800 mμ. Since the most rigorous cleansing of the glass tube and the metal parts of the housing failed to stop all of it, the cell density was reduced eight times for the fluorescence spectrum to make certain that the glass tube and the metal parts were irradiated by the same intensity of blue light, whether or not cells were present. The difference between the readings with and without *Chlorella* was used as a measure of the fluorescence.

The monochromator used was the Farrand model 300VIS which has an aperture of f4. Ability to obtain any results whatsoever was due largely to the availability of an instrument of such high aperture.

Light Measurement.—The low intensity of the delayed light makes its measurement difficult. The obvious method is the use of photographic plates. Plates were used in the earlier experiments but the long exposures required (approximately 10 days), made it so difficult to maintain the cell suspension in a constant condition that electrical methods were adopted. Modulating the light at 1080 cycles per second by means of a shutter and synchronous motor was tried but neither a Cs-O photocell nor a Pb-S cell feeding into a tuned amplifier enabled detection of a signal from the delayed light coming from the monochromator. A combination of image tube and photomultiplier was tried next. The CV-148, which is being sold as war surplus by a

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number of firms, was mounted directly in front of a 5819 photomultiplier. At a distance of about 5 mm. the transparent photocathode of the image tube is parallel to a fluorescent screen that emits green light when hit by electrons. The screen is held 3000 to 4000 volts positive with respect to the photocathode. Thus an image formed on the photocathode by red or near infrared light is copied on the screen in green light. The combination image tube–photomultiplier was mounted inside a metal tube which ran horizontally through a metal tank. The whole device was arranged in such a way that the metal tube was connected to the monochromator by a light-tight joint which permitted the light leaving the exit slit to fall on the photocathode of the image tube. The metal tank was insulated on the outside and packed with dry

TABLE I

Relative Signals for the Same Energy at Different Wave Lengths

Wave length	CV-148-5819 combination	Special photomultiplier (RCA)
$m\mu$		
600	0.99	1.19
625	0.82	1.33
650	0.75	1.49
675	0.70	1.64
700	0.66	1.71
725	0.54	1.84
750	0.49	2.04
775	0.45	2.18
800	0.32	2.35
825	0.27	2.16
850	0.19	1.90
875	0.16	1.51
900	0.12	1.10
925	0.099	0.71
950	0.061	0.37

ice. In order to find an image tube that did not give off any green light in the dark when the high voltage was applied, eight tubes were examined; only two could be used. The 5819 photomultipliers had to be selected for high sensitivity and low dark current. On cooling from room temperature to dry ice temperature, the dark current fell about a thousand times; despite this, the signal was never as large as the reduced dark current. The sensitivity of the combination CV-148-5819 to various wave lengths of light is shown in Table I.

A special red-sensitive photomultiplier, made by the Radio Corporation of America on the advice of their Dr. G. A. Morton who had intimate knowledge of our problems, was used in another trial. This is an end-window type tube and has an S1 response. Again, cooling the tube with dry ice was essential to lower the dark current. The sensitivity of this tube, as shown in Table I, is several times greater than that of the CV-148-5819 combination over the wave length region involved in these experiments.

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In both devices just discussed it was found that the ratio of signal to fluctuation in dark current could be improved by lowering the voltage on the photomultiplier. Best results were obtained with about 60 volts per stage. Current from the photomultiplier in both cases was measured with a vibrating-reed electrometer. The collecting anode was connected to the input of the reed by a 10^{10} ohm resistor, and was connected to ground through a 0.0005 microfarad condenser which had a very high resistance. The reed input was connected to the feedback lead through a 10^{11} ohm resistor. When used in this way the vibrating reed measures the voltage drop over the 10^{11} ohms, while the time constant of the circuit is largely determined by the external resistance and capacity. Our final time constant was 14 seconds. A long time constant was used to average out the fluctuations of the dark current. The output of the reed was continuously recorded by an Esterline-Angus graphic ammeter.

Experiments were customarily made with the shutter alternately open for 1 minute and then closed for 1 minute. During the time that the shutter was closed the wave length drum was changed to the next setting. From the curve made by the recorder the average was taken of the last half minute of each light period and of each dark period. The difference between the values in the light and the average of the preceding and following dark values was used as the measure of light intensity.

In order to absorb any blue light that might be scattered through the monochromator during the experiments on fluorescence, two sheets of yellow cellophane were placed between the exit slit and the light detector. These sheets, which transmit about 80 per cent in the red above 600 mμ and about 1 per cent in the blue below 480 mμ, were kept in place for all measurements except during the search for delayed light in the blue region.

If the entrance and exit slits of the monochromator are identical, the signal increases as the square of the slit width, and as the slits are widened the intensity can be determined with a corresponding increase in accuracy. But as the slit width increases, the detailed structure of the spectra is "smeared out," thus making detection of a difference between the delayed light and fluorescence more unlikely. A slit width of 0.35 mm. seemed to be the best compromise. At the peak of the delayed light emission spectrum this gave a signal of 20 mv. on the 10^{11} ohm resistor. Very roughly, this signal stands in relation to the fluctuations in the dark current in about the same ratio as the width of the emission band stands to the slit width. At 700 mμ a slit width of 0.35 mm. corresponds to 14 mμ.

In the case of fluorescence very much narrower slits could have been used since the brightness of the fluorescence is proportional to the intensity of the exciting light. Inasmuch as the prime purpose was a comparison of the two spectra, it was felt that they should be determined under as nearly the same conditions as possible.

After determining the emission spectrum for the delayed, or fluorescent, light by the method just given, the emission spectrum of a standard lamp was measured in the same manner. The United States Bureau of Standards had determined the energy emitted per unit wave length as a function of the wave length for this lamp. For the purposes of this experiment, the intensity had to be reduced by a large factor in order to be measured. The standard lamp was placed about 50 cm. from a wall, on the other side of which was the monochromator. A hole in this wall was covered by aluminum foil in which there was a small pinhole. The light from this pinhole, after

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traveling a meter or so, was scattered by a block of magnesium carbonate and the scattered light reflected by clear glass into the entrance slit of the monochromator. Readings made on the standard lamp were used to correct the data for the dispersion of the monochromator and for the sensitivity of the photomultiplier. If, at each wave length, the signal of our delayed light emission spectrum is divided by the signal determined for the standard lamp, and then multiplied by the energy emitted by the standard lamp, a number proportional to the energy emitted at that wave length by the *Chlorella* is obtained.

TABLE II

Relative Energy Emitted per Unit Wave Length at Different Wave Lengths

Wave length	Delayed light		Fluorescent
$m\mu$			
610	0.02		
620	−0.16		
630	5.2	1.8	0.1
640	5.2	−1.8	1.3
650	4.8	4.7	3.5
660	10.2	9.1	11.1
670	24.2	23.1	22.6
680	31.0	31.5	26.6
690	26.7	29.4	29.1
700	{17.5	14.4	18.4}
	{19.3	18.2	17.4}
710	11.2	13.0	10.9
720	9.4	13.2	10.0
730	11.5	8.2	8.8
740	7.7	7.1	8.1
750	5.8	5.8	5.7
760	3.0	4.3	4.9
770	3.4	2.3	3.7
780	1.3	0.7	2.9
790	0.2	0.1	2.6
800	−2.1	0.2	2.0

RESULTS

Table II gives two separate determinations of the emission spectrum of the delayed light and one of the spectrum for fluorescence. The numbers are proportional to the energy emitted at the indicated wave length in each case. Two of the sets of numbers have been multiplied by arbitrary constants to bring the three sets to the same scale. Each entry is an individual determination of the light intensity at that wave length; the negative values are due to the dark current fluctuations. Collection of this data was started at a wave length of 700 mμ, taking the even multiples of ten up to 800, down on odd

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multiples to 610, and then back to 700 on the even ones. This was done to minimize any effects of changes in the suspension that might have taken place in the 30 to 40 minutes of the run. Curves showing the data (Fig. 1) can be compared with each other and with those published for the fluorescence of *Chlorella* (1).

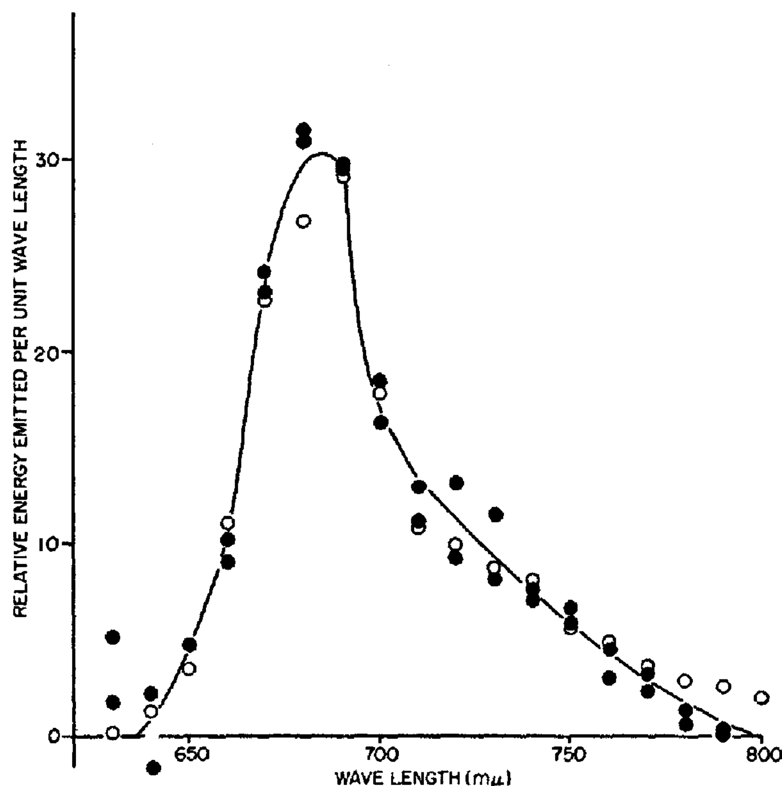

Fig. 1. Relative energy emitted per unit wave length as a function of the wave length. Filled circles, delayed light; open circles, fluorescent light.

The similarity of the two spectra leaves little doubt that it is excited chlorophyll which emits the delayed light. Since the lifetime of the excited chlorophyll is too short for the delayed light to be the "tail end" of the fluorescence, it must be that excited chlorophyll is regenerated (3).

The Absolute Intensity of the Delayed Light.—The absolute intensity of the delayed light was estimated by putting into the monochromator-photomultiplier combination light from a small incandescent lamp attenuated with a pile of neutral filters to make the signal small enough to be measured. The photomultiplier was then replaced with a thermopile, neutral filters were

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removed, and the light was measured again. The thermopile was calibrated with a standard lamp. Calculations then showed that the peak of the emission spectrum for the delayed light corresponds to 4.7×10^{-14} watts falling on the photomultiplier. This figure is for 0.35 mm. slit width on the f4 monochromator and for a cell density of 30×10^6 *Chlorella* per cc.

Using the absolute intensity given and the added information that slit length was 3 cm., the emission per cell can be estimated. A graphical integration of the spectrum is made in order to find the ratio between total light emission and that in the peak with 0.35 mm. slits. Calculations of the solid angle subtended by the collimator lens as seen through the entrance slit and of the number of cells in position to radiate into the monochromator at any instant show that each *Chlorella* cell emits 33 quanta per second near the beginning of the decay curve. This figure is several times larger than the value of 7 given by Strehler and Arnold (3), but, considering the assumptions and approximations that must be made in such a chain of calculations, the agreement is very good indeed.

Since 1 $mm.^3$ of packed *Chlorella pyrenoidosa* contains about 30×10^6 cells, the delayed light emission will be at a rate of about 10^9 quanta/second/$mm.^3$ at the beginning of the decay curve and at a temperature of 25°C.

SUMMARY

1. The delayed light emission of *Chlorella pyrenoidosa* over the wave length range 400 to 950 mμ has been investigated.

2. Emission of delayed light is confined to the range 600 to 800 mμ.

3. To the precision with which the low light intensities involved can be measured with the apparatus in these experiments, the emission spectrum of the delayed light is the same as the spectrum of the fluorescent light.

4. Thus the delayed light must come from excited chlorophyll.

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APPENDIX 1

It was mentioned in the introduction that thin suspensions had to be used in order to avoid the absorption of the light by chlorophyll on its way out of the tube. The

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diameter of the average *Chlorella* cell is about 4 μ; its area A is 1.25×10^{-7} cm². If we now imagine a beam of parallel light penetrating the suspension, then at a distance X cm., the fraction F of the area of the beam which has not hit a cell will be given by

$$F = Exp.(-ANX),$$

in which N is the number of cells per cm³.

We set as our requirement that F should fall to $1/e$ in a distance equal to the radius of the tube, 0.25 cm. N is then given by

$$N = \frac{1}{AX} = 32 \times 10^6 \text{ cells/cc.}$$

The longest ray in the tube that can send light into the monochromator will be just the diameter D. For this ray the fraction of the cells that do not have another cell in front of them will be given by

$$\frac{\int_0^D N\, Exp.(-ANX)\, dX}{\int_0^D N dX} = \frac{1}{AND}[1 - Exp.(-AND)] = 0.43$$

for the values we have chosen.

APPENDIX 2

Perhaps the simplest test for a possible difference between the spectrum for the delayed light and that for fluorescence is given by the sum of squares. Take the fifteen values from 650 to 790 mμ (shown in Table II), using the average of the two at 700 as one. Then the sum of the squares of the differences between the two sets for the delayed light, divided by 2×15 (15 is used as the number of degrees of freedom), gives 1.553. If we now take the sum of the squares of the differences between 2 times the fluorescent light and the sum of the two values for the delayed light and divide by 6×15 we have 1.967. The two are not statistically different. Thus we can say that, to the precision with which we can measure these small light intensities, the two spectra are the same.

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