THE FLUORESCENCE SPECTRA OF RED ALGAE AND THE TRANSFER OF ENERGY FROM PHYCOERYTHRIN TO PHYCOCYANIN AND CHLOROPHYLL

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(Received for publication, December 3, 1951)

INTRODUCTION

Since the time of Engelmann (1883, 1884) many investigators have been concerned with the possible participation of other pigments than chlorophyll in photosynthesis. The fact that photosynthesis will take place as a result of light absorption by fucoxanthin has been established by Dutton and Manning (1941). The participation of phycocyanin in photosynthesis in the blue-green alga *Chroococcus* has been shown by Emerson and Lewis (1942). The large series of action spectra of photosynthesis in the various algae used by Haxo and Blinks (1950) clearly demonstrated that light absorbed by phycoerythrin and phycocyanin is used in photosynthesis by red algae. Chlorophyll *a* is found in all plants, except bacteria, that are capable of living by photosynthesis no matter what other pigments may be present and active as light absorbers. Do these other pigments carry out photosynthesis directly themselves, or do they act solely as light absorbers and transfer their absorbed energy to chlorophyll? An answer to this question was obtained by Dutton, Manning, and Duggar (1943) and by Wassink and Kersten (1946) for the participation of fucoxanthin in the photosynthesis of the diatom *Nitzschia*. The transfer of light energy from fucoxanthin to chlorophyll was established by the finding that just as much chlorophyll fluorescence is obtained in these diatoms from light which is absorbed by fucoxanthin as from that which is absorbed directly by chlorophyll itself. Duysens (1951) has found by similar means that certain bacterial carotenoids act in photosynthesis by transferring their absorbed energy to bacteriochlorophyll. Carotenoids are quite different chemically from chlorophyll. Phycoerythrin and phycocyanin, however, are similar to chlorophyll in that they are protein complexes of bile pigments, molecules which contain the same pyrrole components as do porphyrins but which lack the ring structure and the attached metal of porphyrin compounds (Lemberg and Legge, 1949). Furthermore, Haxo and Blinks found that the chlorophyll in their red algae was so much less effective in photosynthesis than were the phycobilin pigments that the latter's direct action without the participation of chlorophyll seemed to be a real possibility.

A previous attempt has been made to investigate the possible transfer of energy from the phycobilin pigments to chlorophyll in red algae by van Norman, French, and Macdowall (1947). The overlapping of the chlorophyll fluorescence spectrum with those of phycocyanin and phycoerythrin made the results of this earlier investigation inconclusive, although a more intense red fluorescence actually was excited by light absorbed by phycoerythrin than by chlorophyll in several red algae.

We have therefore undertaken with improved equipment a further study of the fluoresence spectra of red algae to find out whether light absorption by phycoerythrin excites chlorophyll fluorescence. A detailed theoretical discussion of this approach has been presented by Wassink (1948).

The results of the present work have clearly shown that in the unicellular red alga *Porphyridium*, grown under the described conditions, light absorbed by phycoerythrin is transferred both to chlorophyll and to phycocyanin. Duysens (1951) has also observed this transfer both to phycocyanin and to chlorophyll in the same alga. It therefore appears likely that chlorophyll does have some specific function in photosynthesis in addition to its light-absorbing ability.

METHODS, APPARATUS, AND DATA

Apparatus for Measurement of Fluorescence Spectra

In order to determine the effectiveness of various incident wave lengths in exciting the fluorescence spectra of the separate algal pigments it is necessary to illuminate the cell suspension with bright monochromatic light of measured intensity. It is then necessary to collect a reasonable fraction of the emitted fluorescent light, pass this through a monochromator to separate the various wave lengths, and to measure the curve relating the fluorescence intensity to the wave length of the fluorescent light. Since the efficiency of fluorescence emission is extremely low, probably about 0.1 per cent, and only a small fraction of the fluorescent light can be collected by any practical optical system, the measurement of the fluorescence spectrum is difficult.

The equipment constructed for these measurements will be described briefly. The light from a high pressure mercury lamp[1] is dispersed by means of a 4 × 4 inch transmission-grating monochromator of aperture ratio f2.5, then focused upon the algae by a lens system. Filters are used to remove stray light. The algal suspension in a lucite cell of 0.5 mm. inside thickness is fixed on a sliding holder arranged so that a thermopile may be alternately put exactly in place of the algae for measurement of the intensity of the incident light. For calibration purposes a magnesium oxide block illuminated from a standard lamp may also be placed in this same position. The general arrangement of the equipment for illuminating the algae and collecting the fluorescent light is shown in Fig. 1. The fluorescent light, after being

[1] Obtained from Huggins Laboratories, 700 Hamilton Ave., Menlo Park, California.

chopped at 150 cycles per second, passes through a reflection-grating monochromator of the same size and aperture as the one used for illuminating the algae. The wave length setting of the analyzing monochromator is changed at a constant rate by a synchronous motor. The total band width (calculated from the slit width) of both the incident and of the fluorescent beams is 10 mμ. The light coming out of this second monochromator falls upon a photomultiplier tube the output of which is amplified by a stable alternating current amplifier. The amplified voltage is ap-

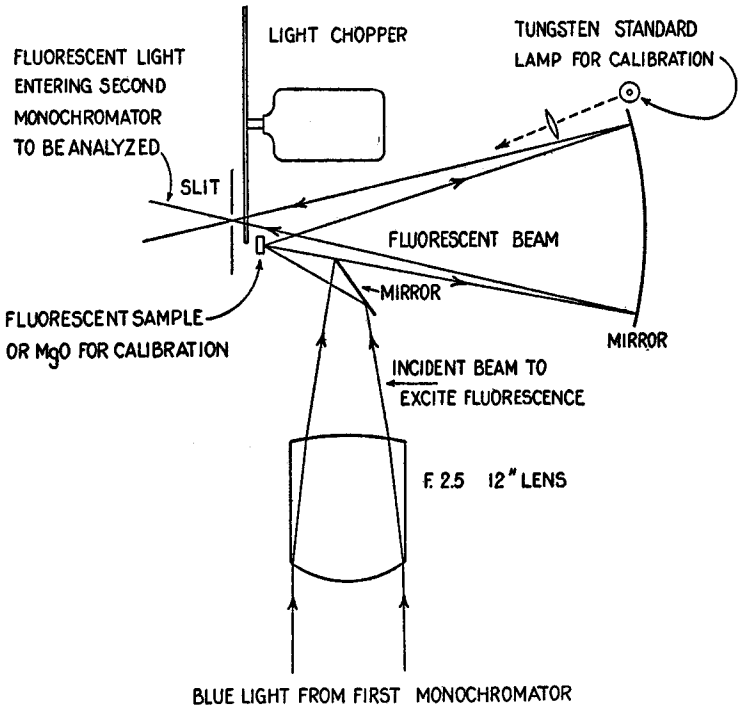

FIG. 1. The optical system for the illumination of the algae and the collection of the fluorescent beam. The arrangement was later modified by the insertion of another photographic lens in the converging incident beam, thus giving a smaller spot of light upon the sample.

plied to a correcting potentiometer which is positioned by a cam attached to the wave length drive mechanism. This provides a means of approximately adjusting for the difference in sensitivity of the photomultiplier tube, and for the difference in monochromator transmission, at various wave lengths. In order to make this correction more accurate the partially adjusted voltage is applied to another correcting mechanism the setting of which, at each wave length, is controlled through a photoelectric curve follower by a pencil line drawn upon a frosted lucite drum. This line may be easily changed for precise adjustment. The calibration of the instrument is carried out by plotting the correction curve by hand in such a way that

the deflection of the recorder at various wave lengths is proportional to the energy distribution of a standard lamp run at a known temperature. The amplified and corrected voltage passes through a calibrated attenuator so that the recorder deflection may be multiplied by known factors. An amplifier tuned to 150 cycles per second then increases the signal to noise ratio of the system. The alternating current voltage is rectified and fed to a Brown recording potentiometer. A paper speed of 2 inches per minute is used in this recorder and the drive of the monochromator changes its wave length 1 millimicron per second. ⅓ of an inch on the paper therefore represents 10 mμ in the spectrum. In order to remove the reflected incident light

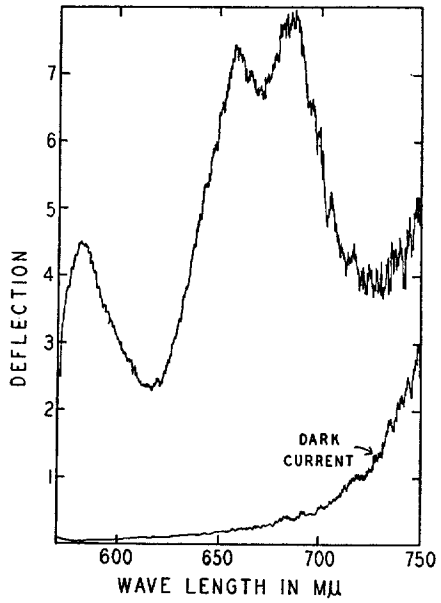

FIG. 2. The recorder trace of the fluorescence spectrum of a *Porphyridium* suspension illuminated with a wave length of 490 mμ and the corresponding dark current trace. The random variations decrease as the wave length is decreased.

from the beam of fluorescent light, a filter absorbing the incident light is placed before the photomultiplier. In the present experiments a Corning filter No. 3482 and a solution of rose bengal were used.

Apparatus for the Manipulation and Analysis of Curves

For the study and intercomparison of many plotted curves the necessary point-by-point calculation and replotting involve a very large amount of labor. In order greatly to simplify the process of replotting curves with a change of scale and for the addition and subtraction of curves from each other, an electromechanical device has been constructed. Briefly, it functions by photoelectric following of heavily inked curves placed upon movable tables. The curves are aligned on the tables with

the wave length axis parallel to the motion of the table. A cross-arm at right angles to this line of motion carries a photoelectric following head the motion of which rotates a potentiometer so that its output voltage, at any value of wave length, is proportional to the height of the curve. By the amplification or attenuation of this voltage before it is applied to a recorder, the ordinate scale may be changed at will. The recorder is similar to the follower units except for the addition of a pen. Similarly, changing the rate of motion of the follower table in relation to the speed of the recorder table makes it possible to change the wave length scale of a graph. By having two followers functioning simultaneously, adding or subtracting the output voltages, and applying the result to a recorder the sum or difference of two curves may be drawn. The analysis of the curves in this paper required the plotting of approximately four hundred individual curves most of which were for the trial and error fitting of the data with the component curves.

The Fluorescence Spectra of Porphyridium cruentum Suspensions

Complete fluorescence spectra from 570 to 750 mμ of the alga *Porphyridium cruentum* were made with eleven different incident wave lengths from 405 to 546 mμ. The exciting light thus covers the range in which chlorophyll absorbs in the blue and also the region in which phycoerythrin has its strong green absorption band. Filters[2] were used in the incident beam to reduce stray light. Control experiments with magnesium oxide in the place of the algae showed the stray light to be of negligible intensity. The sensitivity of the apparatus was adjusted so that a graph about 8 inches high at the wave length of maximum emission was produced. The amplification factor was noted and later used in the reduction of the data. After each measurement of a spectrum a Kipp and Zonen compensated thermopile was placed in the exact spot previously occupied by the suspension of algae and the relative intensity of the 1 × 8 mm. beam thus measured at the different wave lengths. Since the sensitivity of the photomultiplier changes over the region 570 to 750 mμ, being low in the red, the experimental error varied a great deal over the useful range.

[2] Filter combinations used to remove stray light:

Wave length mμ		Corning filter Nos.			
560–550		5031	4303	3486	
546		5120	4305	4303	
537		9780½	5120	5031	
530–497		"	5970	"	
495–490		5031	4303	5120	
480		"	4305	4303	
470		"	"	"	3387
	or	"	5030	4305	
460		5543	3389		
450		"	5030	3389	
450–405		5113	4305	3389	

While measurements were made up to 750 mμ, the reliable range for weak fluorescence does not extend much beyond 700 mμ. The data from 700 to 750 mμ should not be given much emphasis. Since the voltage attenuation was automatically varied as the wave length changed, it was necessary also to record for each spectrum a measurement of the dark current. This was then subtracted from the observed data. A typical record of a fluorescence curve and the dark current are shown in Fig. 2. Smooth curves were drawn through

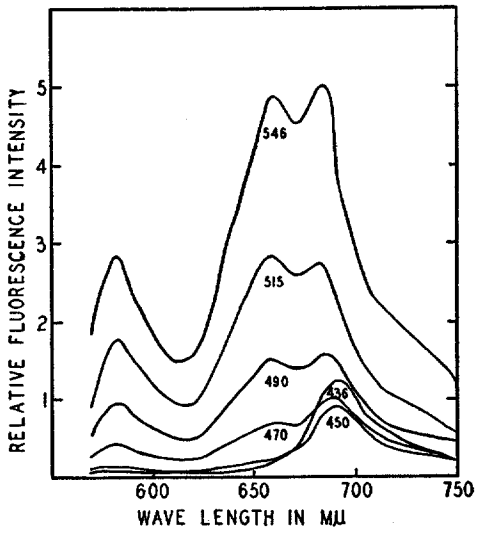

FIG. 3. The fluorescence spectra of a suspension of *Porphyridium* illuminated by various incident wave lengths (designated upon the individual curves). The curves have been adjusted in scale to the size that they would have been if it had been possible to measure them with equal numbers of incident quanta of the various wave lengths. Five of this family of curves have been omitted for the sake of clarity. The peak at 578 mμ is due to phycoerythrin, that at 655 to phycocyanin, and that at 685 to chlorophyll *a*.

these recordings and, by means of the curve analysis machine, various transformations of these curves were carried out, as will be described below. Fig. 3 shows the fluorescence curves for six of the eleven wave lengths of incident light used. They have been adjusted in height to values which would have been found if the algae had been illuminated with equal numbers of incident quanta. The fluorescence produced by λ 436 mμ is mostly due to chlorophyll as evidenced by the single maximum at 690 mμ. As the wave length of the incident light is increased two other fluorescing components appear—phycocyanin, with a maximum at 660, and phycoerythrin, at 578 mμ—their fluorescence intensity decreases at 450 mμ and then increases up to 546 mμ. In order

to determine the effect of changing the wave length of incident light upon the intensity of chlorophyll fluorescence these curves must be broken up into their component parts.

The Fluorescence Spectra of the Individual Pigments in Porphyridium

In the algae the pigments are in a very concentrated state within the chloroplasts and their spectra under these conditions are distinctly different from what they are in a dilute solution. Therefore the fluorescence spectra of purified phycoerythrin, extracted chlorophyll, and of phycocyanin in water solution

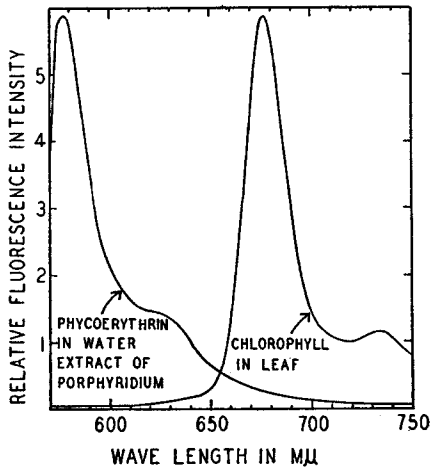

FIG. 4. The fluorescence spectra of *Porphyridium* phycoerythrin in water solution and that of chlorophyll *a* in a corn leaf. The corn leaf was grown in the dark, then exposed to light for 1 hour so that it had a very small chlorophyll content. Its spectrum therefore is that of chlorophyll *a in vivo* uncomplicated by self-absorption.

cannot be added together to reproduce the curves obtained for the fluorescence spectra of intact algae. The spectra of the pigments in solution may, however, be used as a starting point in arriving at approximate curves for the intact pigments within the cell.

The spectra for the three fluorescing pigments *in vivo* were obtained by a trial procedure of successive approximations. We first approximated the shape of the phycoerythrin curve starting with the curve in Fig. 4, which gives the fluorescence spectrum of a phycoerythrin solution obtained from *Porpyyridium.* If we now attempt to plot this fluorescence spectrum of phycoerythrin upon the curve for the fluorescence of *Porphyridium* when excited for instance by a wave length of 530 mμ, we see that the curve for the purified material is much sharper and that it cannot be made to fit the phycoerythrin part of the fluorescence of the intact alga. If, however, the major peak at 578 mμ is lowered and

broadened in proportion to the rest of the curve, then a satisfactory match to the spectrum of phycoerythrin in the intact alga may be obtained. Such manipulation is arbitrary, but fortunately we have a test for the accuracy of the result in the final summation of all the individual pigment curves. If the assumed fluorescence spectra of the individual pigments can be multiplied by appropriate coefficients and added together to match all the spectra of the suspensions of intact algae, then they must obviously be fairly close approximations of the actual fluorescence spectra of the individual pigments. Since the manner of constructing these curves is arbitrary, their exact shape cannot be considered to be highly precise. It is, however, evident from the close fits of the summations of the derived curves to the original data that the derived curves must be fairly close to their true shapes.

The curves of algal fluorescence excited by wave lengths from 405 to 450 mμ show very little fluorescence due to either phycocyanin or to phycoerythrin. Any one of these curves might be taken as a fairly close approximation to the fluorescence spectrum of chlorophyll in this particular alga. The small amount of fluorescence by other pigments was, however, considered by taking the curve for 420 mμ incident wave length and reshaping this so that its short wave length side corresponded to the short wave length side of the fluorescence spectrum of chlorophyll *a* in a corn leaf. Chlorophyll *a* was the only pigment present in this leaf which showed any fluorescence in the region of the spectrum studied here; *i.e.*, 570 mμ to 750 mμ. This leaf fluorescence is shown in Fig. 4. The derived curves may be seen in Fig. 5.

Since we now have approximations to the fluorescence curves of phycoerythrin and of chlorophyll in the algae, it should be possible to obtain the phycocyanin component by subtracting appropriate amounts of the phycoerythrin and chlorophyll curves from the spectra of the intact algae. To do this the phycoerythrin component was first subtracted from each of the curves using a factor which brought the peak of the phycoerythrin component to the zero line. The phycoerythrin was subtracted first because the main peak at 578 mμ does not contain any overlapping fluorescence of phycocyanin or chlorophyll. We thus obtained a family of curves containing only two main peaks—that due to phycocyanin and that due to chlorophyll. For each of these two peaked curves a family of trial curves was made subtracting varying proportions of the chlorophyll component. The most reasonable looking of these was then selected for each of the eleven intact alga curves. The set of curves remaining after the chlorophyll subtraction was then adjusted to an arbitrary height of 6 inches at the peak so that these derived curves, which should represent phycocyanin fluorescence, could all be compared with each other. When this was done it was found that five out of the eleven curves were very nearly identical. The others deviated somewhat but not enough to indicate the presence of any other fluorescent pigments. These five curves were then aligned on thin

paper and a tracing of their average was made. This curve is shown in the middle part of Fig. 5 and was taken as the fluorescence of phycocyanin in *Porphyridium*. Having approximations to the fluorescence spectra of the individual pigments in the living cell, we are now in a position to analyze the original experimental curves in terms of the summation of the contributions of the individual pigments to the total fluorescence spectrum.

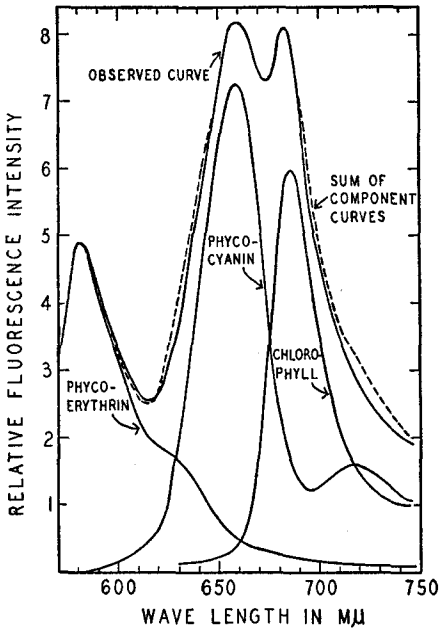

FIG. 5. The derived curves for the fluorescence spectra in *Porphyridium* of phycoerythrin, phycocyanin, and chlorophyll *a*. Their summation is compared with the fluorescence spectrum of *Porphyridium* illuminated by a wave length of 530 mμ. The sum of the individual curves is indicated by dotted lines. In all other curves of the group the fit of the sum of the component curves with the observed curves was better than in this case. The component curves were derived by a process of trial and error as described in the text.

THE RESOLUTION OF THE SPECTRA OF THE INTACT ALGAE INTO THE FLUORESCENCE SPECTRA OF THE INDIVIDUAL PIGMENTS

The family of curves remaining after subtraction of phycoerythrin were one by one placed upon the recording table of the curve analyzer. Tracings of the chlorophyll curve and the phycocyanin curve were placed upon the curve follower tables of this apparatus in such a way that the sum of the two curves was indicated by the recorder on a piece of transparent paper overlying the curve to be matched. The multiplication factors of these curves were adjusted by

trial until the sum of the chlorophyll curve and the phycocyanin curve matched the experimental data. In Fig. 5 are shown the individual pigment fluorescence curves which when added together match the experimental data for the incident wave length of 530 mμ. The sum of the component curves is indicated by the dotted lines, while the curve which is to be fitted is shown as a heavy solid line. The match appeared to be within the experimental error of the original measurements. This figure shows, then, the contribution of each pigment to the total fluorescence of the algae. Each of the eleven fluorescence spectra were

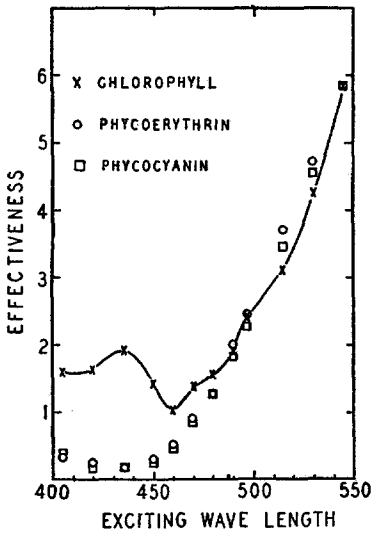

FIG. 6. The action spectrum for the excitation of the fluorescence of three separate pigments in a suspension of *Porphyridium*. Each point represents the maximum peak height of curves similar to the derived curves of Fig. 5. Above a wave length of 490 mμ these three spectra are identical with each other within the limits of experimental error. All curves in this figure have been adjusted to the same height at a wave length of 546 mμ in order to compare their shapes.

treated in the same way so that the contribution of each pigment to the complete spectrum could be determined for each wave length used to excite the fluorescence. The match between the original algal curve and the sum-of-the-components curve was even closer to the other ten cases than for wave length 530 mμ.

THE EXCITATION SPECTRA OF PHYCOERYTHRIN, PHYCOCYANIN, AND CHLOROPHYLL FLUORESCENCE IN PORPHYRIDIUM

The eleven different curves, after resolution into the fluorescence spectra of the component pigments, as illustrated by Fig. 5, provide the data for calcu-

lating the action spectra for the excitation of the fluorescence of each of the component pigments. This was done by taking the height, corrected for the amplification factor used in its measurement, of the main peak in each resolved spectrum and dividing it by the incident intensity. Action spectra so obtained

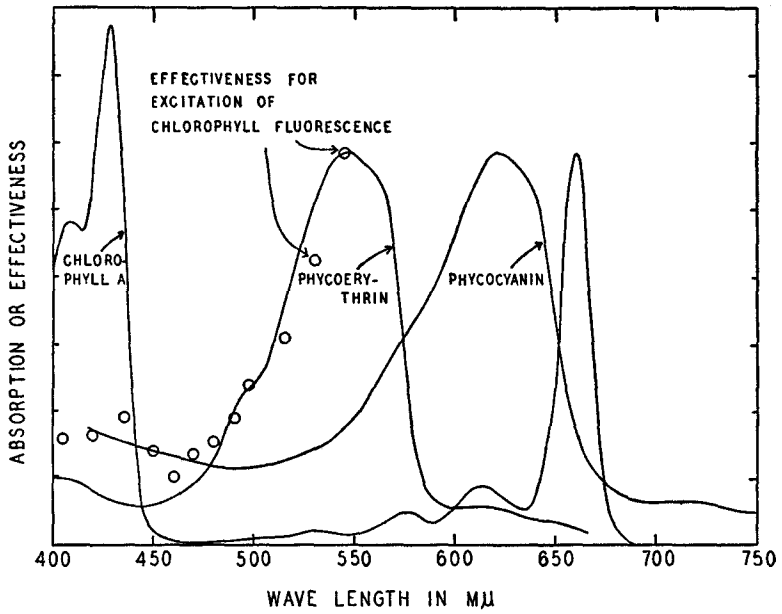

FIG. 7. The comparison of the action spectrum for the excitation of chlorophyll fluorescence (open circles) with the absorption spectrum of phycoerythrin from *Porphyridium*, with chlorophyll *a* (in methanol) and with a preparation of phycocyanin. We are indebted to Mr. L. N. M. Duysens for the absorption curve of phycocyanin which he prepared from *Oscillatoria*. The absorption curve of the phycocyanin in *Porphyridium* may not be identical with this, but it most likely is not very different. It is obvious from this figure that the chlorophyll fluorescence excitation spectrum follows the absorption spectrum of phycoerythrin as well as showing a small amount of fluorescence directly from chlorophyll absorption in the blue region. The rise in the fluorescence of chlorophyll in green corresponding to phycoerythrin absorption constitutes the evidence for a transfer of energy from phycoerythrin to chlorophyll.

for the excitation of the *in vivo* fluorescence of phycoerythrin, phycocyanin, and chlorophyll are shown in Fig. 6. In this figure the action spectra have been brought to the same height at 546 mμ. It is evident that except for the blue region, in which chlorophyll absorbed strongly, these three curves are very closely similar. This means that from 490 to 546 mμ the energy which causes the fluorescence of phycoerythrin, of phycocyanin, and of chlorophyll comes

mostly from the absorption of light by only a single substance. A comparison of these action spectra with the absorption spectra of the three pigments in Fig. 7 shows that this absorbing substance is phycoerythrin and not chlorophyll or phycocyanin. Thus it appears that light absorbed by phycoerythrin is transferred both to phycocyanin and to chlorophyll.

In Figs. 6 and 7 the small peak at 436 mμ of the chlorophyll fluorescence excitation spectrum indicates the excitation of chlorophyll fluorescence from light absorbed directly by chlorophyll itself. Why is the fluorescence of chlorophyll lower here than that at 546 mμ where the chlorophyll receives its energy by transfer from the phycobilins? This effect may be partially due to internal filtering action of light by carotenoids which are present and absorb in the blue region of the spectrum. This would reduce the fraction of incident light that can be absorbed by chlorophyll. It is also conceivable in view of the Haxo and Blinks' experiments that the active and the inactive chlorophylls differ in their ability to fluoresce or to receive energy by transfer from the phycobilins. This raises the question as to the origin of chlorophyll fluorescence. Does it come from the chlorophyll molecules that are active in photosynthesis or does it come from the inactive chlorophyll fraction? This question cannot be answered with the data available at present.

POSSIBLE SOURCES OF ERROR IN BIOLOGICAL FLUORESCENCE SPECTROSCOPY

Instrumental Errors

The accuracy of the wave length scale of the monochromator is within half of 1 millimicron, so that this factor does not enter into a consideration of the possible errors. However, lack of synchronization of the paper with the wave length drive mechanism and changes in the length of the paper may put in errors up to 1 or 2 millimicrons in the position of the peaks of the recorded spectra. The linearity of response of the photocell and amplifier system to light has been held to about 0.1 per cent. The calibration by means of a standard lamp for response at different wave lengths should be correct to within 2 per cent, as nearly as can be estimated.

The extremely low yield of fluorescence makes stray light a very important problem. The filters used to remove stray light are given in footnote 2. The necessity for complete removal of stray light is due to the fact that reflected as well as fluorescent light is collected by the optical system. The incident beam must therefore be free of stray light of the wave lengths in the fluorescence region. With all the filters used this has been held to within a few per cent of the fluorescence intensity.

The greatest instrumental source of error lies in the fact that we are measuring very weak light and in the region in which the 1P22 photomultiplier is not particularly sensitive. With the sensitivities normally used in recording the fluorescence spectra of algae the random variation is appreciable, due to the noise of the multiplier tube. To reduce the fluctuation from this cause the rectified voltage was fed to a condenser, the value of which may be selected by a switch to give a time constant of about 1 to 8 seconds. Under the conditions of use, the noise level as a per

cent of the signal is at 750 mμ, about 25 per cent, at 700 mμ 10 per cent, at 650 mμ 5 per cent, and at 600 mμ about 2 per cent. These estimates are based on measurements of the random fluctuations of the recordings similar to that in Fig. 2.

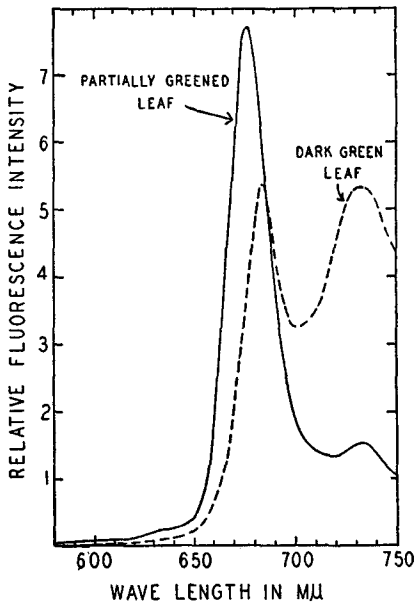

FIG. 8. The fluorescence spectrum of a leaf containing very little chlorophyll compared with that of a leaf containing a large amount of chlorophyll. The curve for the partially greened leaf is taken from Fig. 4. The curve for the dark green leaf (*Dendromecon rigida*) was measured at the same sensitivity of the apparatus and with the same intensity of incident light of wave length 436 mμ. The difference in shape of these two curves is due to reabsorption of the fluorescent light by the red absorption peak of chlorophyll which lies just to the left of the main fluorescence band. This effect shifts the position of the main peak and greatly decreases its height in proportion to that of the 730 mμ band which does not show as much reabsorption. This effect of reabsorption of fluorescent light by the pigment itself appears to be one of the most serious errors likely to be encountered in fluorescence spectroscopy of biological material.

Biological Errors

(*a*) *Reabsorption of the Fluorescence.*—The reabsorption of fluorescence either by the fluorescent pigment itself or by other pigments probably presents the most difficult problem in fluorescence spectroscopy of biological materials. In the study of solutions it may be avoided by using thin layers of dilute solutions. In living material, however, the situation is not easily controlled. The difficulty lies in the fact that the fluorescence peaks are usually quite close to the absorption peaks. The result is the selective reabsorption of one side of the fluorescent band. This leads to

a shift of the main peak towards the red end of the spectrum in addition to a reduction of the total emitted light. These effects are shown in Fig. 8. The spectrum of the partially greened leaf was made with a corn leaf which was grown in the dark and exposed to light for 1 hour. The amount of chlorophyll formed was so small that the leaf was just barely green to the eye. This curve probably comes very close to representing the true fluorescence spectrum of chlorophyll *a* in a leaf. The peak at about 680 mμ is much higher than the secondary maximum at 730 mμ. The influence of reabsorption on the chlorophyll fluorescence spectrum is shown in the same figure by the curve representing the fluorescence from a dark green leaf, *Dendromecon rigida*, containing a large amount of chlorophyll. The position of the main peak is shifted by the reabsorption of the shorter wave length part of the main band. The secondary maximum at 730 mμ, being relatively less reabsorbed, is quite high. The total amount of fluorescence, corrected for reabsorption, could perhaps be estimated by multiplying the curve for the leaf containing rather little chlorophyll by a curve for the leaf transmission until its secondary peak came to the height of the secondary peak of the dark green leaf. Thus the areas of the corrected curves could be compared. The total fluorescence may be diminished by reabsorption to a rather small fraction of the fluorescence actually produced within the leaf. Fortunately, the shift of the peaks and the relative height of the main and of the secondary peaks give an indication as to whether serious difficulties are being encountered by reabsorption. In the fluorescence spectra of red algae described in this paper it appears unlikely that the reabsorption of chlorophyll fluorescence by chlorophyll is a serious factor, since the secondary peaks at 730 mμ do not appear to be high in proportion to the main fluorescence peak of chlorophyll.

For phycoerythrin it may be that self-absorption of the fluorescent light is partially responsible for the difference in the shape of the algal fluorescence spectrum and the spectrum of the pure solution. Since the phycoerythrin fluorescence peak is well removed from the absorption bands of other substances, it is unlikely that its fluorescence curve is distorted by the absorption of other pigments. This is not true, however, in the case of phycocyanin. It may be that the long wave length side of the phycocyanin fluorescence band has its shape altered by the presence of chlorophyll. If this effect were very great, one might expect to obtain different shapes of fluorescence curves for phycocyanin from the different incident wave lengths, which was not found. The fluorescence and the absorption bands of phycocyanin are well enough separated so that self-absorption can play only a relatively small part in determining the shape of the phycocyanin fluorescence curve.

(*b*) *Change of Fluorescence Intensity during Illumination.*—In green leaves and in green algae the change in the intensity of fluorescence after a dark period is well known, due to the work of Kautsky (1948 and many earlier papers), of McAlister and Myers (1940), and of Franck *et al.* (1941). The outburst of fluorescence which occurs immediately upon illumination may decline to approximately one-half of its peak value in the course of a minute or two. This phenomenon is known as the Kautsky effect. The shape of this curve relating fluorescence intensity to time is highly dependent upon the prevailing conditions and the immediate history of the material. Attempts to interpret the shape of this curve have led to much theoretical discussion by Franck (1949, 1951) and by Wassink (1951).

We have observed this change with time in the chlorophyll fluorescence of many marine algae, but have found that the phycoerythrin fluorescence and the phycocyanin fluorescence always remain constant with time. The Kautsky effect in red algae is limited to chlorophyll. In red algae, as also in the green algae of Shiau and Franck (1947), the effect is much greater in older specimens. It was found that in

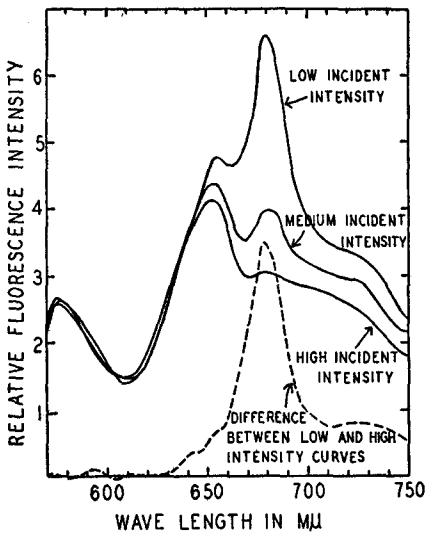

FIG. 9. The fluorescence spectra of the marine red alga *Porphyra naiadum* when illuminated by different intensities of green light, 546 mμ. The ordinates of the original curves have been divided by the relative intensities; therefore the fluorescence peak of phycoerythrin appears the same height in all. The intensities used were 0.9×10^3, 2.0×10^3, 4.4×10^3 ergs/cm.^{2}/sec. It is evident from this figure that the intensity of fluorescence of phycoerythrin and of phycocyanin is proportional to the light intensity. This is, however, far from being true for the chlorophyll fluorescence. The higher light intensity produces far less than a proportionate amount of chlorophyll fluorescence. That the change in the shape of the curves is due only to lack of chlorophyll fluorescence at the higher intensity is shown by the difference curve which itself corresponds closely to the fluorescence spectrum of chlorophyll *a*. These curves were all measured after an exposure of 6 minutes when the chlorophyll fluorescence intensity had become constant. There is no change with time in the intensity of the fluorescence of the phycoerythrin or phycocyanin peaks.

young *Porphyridium* grown in the laboratory the effect was entirely negligible. This finding is in accord with results of Shiau and Franck in young green algae and proved to be of great value in facilitating the type of measurements upon which this paper is based.

(*c*) *Chlorophyll Fluorescence Not Proportional to Light Intensity in Many Cases.*— This lack of proportionality of fluorescence intensity to incident intensity has been reported by Franck *et al.* (1941) in green leaves and by Wassink (1939) in algae in

which the yield increases with intensity. In the red alga *Porphyra naiadum* we found, as shown in Fig. 9, that the chlorophyll fluorescence was much less in proportion to the fluorescence of the other pigments when measured at high intensity than it was when measured at low intensity. It appears as though the products of photosynthesis were quenching the chlorophyll fluorescence. These curves were measured after a 6-minute exposure of the algae to light during which time the fluorescence at the chlorophyll peak had become constant. The lack of proportionality to incident intensity is limited to chlorophyll fluorescence. The overlapping of chlorophyll with the phycocyanin fluorescence at 655 mμ introduces small changes in the height of the peak but not in the actual phycocyanin fluorescence. In *Porphyridium* grown in the laboratory and used while young the chlorophyll as well as the phycoerythrin and phycocyanin fluorescence was proportional to intensity.

The possible sources of error in biological material discussed in (*a*), (*b*), and (*c*) may influence the precise position of the fluorescence peaks, the shape of the fluorescence curves, and even to some extent the relative intensity of fluorescent light emitted by wave lengths penetrating to different depths. None of these effects appears to be capable of introducing such large errors as to influence the main conclusions of this paper.

THE MOLECULAR MECHANISM OF RESONANCE ENERGY TRANSFER

The transfer of energy from one dye to another has been studied in some detail. For instance, the fluorescence of naphthacene is excited by light absorbed by anthracene in a mixture of the two. Similarly, acriflavine is known to transfer its energy to rhodamine *b* in concentrated but not in dilute solution. A review of the data and a theoretical discussion of this mechanism of resonance transfer of energy have been given by Franck and Livingston (1949) and its implications in photosynthesis pointed out. The conditions necessary for such a process to take place are twofold. In the first place, the molecules must be close to each other, generally less than 1 wave length apart. Transfer of this type over distances greater than 100 mμ is highly unlikely, but within 10 mμ the efficiency of such transfer may be nearly 100 per cent. A second condition which must be fulfilled for the process of resonance transfer to take place between two dye molecules is that the two structures have common resonance frequencies. The existence of such common resonance frequencies can be shown by the overlapping of the fluorescence bands of the sensitizing dye molecule with an absorption band of the receiving molecule. A discussion of these principles has been applied to the transfer of energy from phycocyanin to chlorophyll in blue-green algae by Arnold and Oppenheimer (1950). Since the fluorescence of chlorophyll is at an appreciably longer wave length than the absorption band of phycoerythrin, the amount of overlap might well be insufficient for a high yield of energy transfer from phycoerythrin to chlorophyll. On the other hand, the fluorescence band of phycoerythrin lies much closer to the absorbing bands of phycocyanin, so that according to theoretical considerations energy transfer should be easier from phycoerythrin to phycocyanin than

to chlorophyll. Correspondingly, the transfer from phycocyanin to chlorophyll should also be easier than for the resonance transfer from phycoerythrin to chlorophyll. It therefore appears that phycocyanin may function as a link between the absorbing pigment phycoerythrin and the chemically active pigment chlorophyll. This has also been suggested by Duysens (1951). Such an interpretation is in line with the finding that energy absorbed by phycoerythrin is received both by phycocyanin and by chlorophyll.

SUMMARY

1. The fluorescence spectra of the alga *Porphyridium* have been recorded as energy distribution curves for eleven different incident wave lengths of monochromatic incident light between wave lengths 405 and 546 mμ.

2. In these spectra chlorophyll fluorescence predominates when the incident light is in the blue part of the spectrum which is strongly absorbed by chlorophyll.

3. For blue-green and green light the spectrum excited in *Porphyridium* contains in addition to chlorophyll fluorescence, the fluorescence bands characteristic of phycoerythrin and of phycocyanin.

4. From these spectra the approximate curves for the fluorescence of the individual pigments phycoerythrin, phycocyanin, and chlorophyll in the living material have been derived and the relative intensity of each of them has been obtained for each of the eleven incident wave lengths.

5. The effectiveness spectrum for the excitation of the fluorescence of these three pigments *in vivo* has been plotted.

6. From comparisons of the effectiveness spectrum for the excitation of each of these pigments it appears that both phycocyanin and chlorophyll receive energy from light which is absorbed by phycoerythrin.

7. It is suggested that phycocyanin may be an intermediate in the resonance transfer of energy from phycoerythrin to chlorophyll.

8. Since phycoerythrin and phycocyanin transfer energy to chlorophyll, it appears probable that chlorophyll plays a specific chemical role in photosynthesis in addition to acting as a light absorber.

We are indebted to Professor Lawrence R. Blinks and Dr. Conrad Yocum of the Hopkins Marine Station for many stimulating discussions of photosynthesis and fluorescence in red algae. Dr. Francis Haxo kindly supplied the culture of *Porphyridium*. The electronic components of the light-measuring system and of the curve analyzer were designed, constructed, and tested by Mr. George H. Towner. The mechanical parts of the monochromators and of the curve analyzer were made by Mr. Frank Schuster.

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