THE ABSORPTION AND FLUORESCENCE SPECTRA OF TWO RED MARINE ALGAE

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(WITH FIVE FIGURES)

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Introduction

The problem as to whether or not there are pigments other than chlorophyll which can participate directly in photosynthesis by acting as photocatalysts has been discussed for many years. One of the earliest pieces of quantitative evidence for this possibility was the work of Warburg and Negelein which showed that the light absorbed by carotinoids was probably at least partially active in photosynthesis. Definite evidence for the participation of the carotinoid, fucoxanthin, as a light absorbing pigment for photosynthesis was provided by DUTTON and MANNING (**1**). It was later shown by DUTTON, MANNING and DUGGAR (**2**) that the light absorbed by fucoxanthin was in some way transferred to chlorophyll rather than itself acting directly upon the remainder of the photosynthesis mechanism. This was established by showing that the fluorescence of chlorophyll was excited equally well by red light which is primarily absorbed by chlorophyll and by blue-green light which is primarily absorbed by fucoxanthin. Phycocyanin was found by EMERSON and LEWIS (**4**) to be a photosynthetic pigment. It has not yet been established whether phycocyanin acts directly in the same way as does chlorophyll, or whether phycocyanin like fucoxanthin first transfers its energy to chlorophyll. Recently, HAXO and BLINKS (**8**) have reported that green light which is absorbed largely by the pigment phycoerythrin is several times as effective as is red light in photosynthesis of several red marine algae. The question arises as to whether the extra photosynthesis given by green light is due to a stronger absorption of light in this region by the algae or if this greater effectiveness of green light is due specifically to greater efficiency of phycoerythrin in photosynthesis. If the latter is the case, is phycoerythrin a pigment which acts by transferring absorbed light to chlorophyll or does it take part directly in the photosynthetic mechanism without the intermediate assistance of chlorophyll? Our measurements show only slightly greater absorption of green than of red light in two species of red algae. We have studied various aspects of the absorption and fluorescence of the pigments of red marine algae which are fundamental to the investigation of the question of the means by which phycoerythrin participates in photosynthesis.

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Results

Materials

Two batches of algae were available to us for these experiments. The first batch was collected by one of us (F.D.H.M.) near Victoria, British Columbia, and brought to the laboratory by airplane. The second batch was collected in the same place and shipped by air express in two one-gallon thermos jugs. The temperature of this shipment on arrival was 17° C. Several species of algae from each batch were tested for their capacity to perform photosynthesis. In the first batch *Gigartina radula, Iridaea sp.,* and Ulva were found to give no photosynthesis. There was no gas evolution when this Gigartina was tested for O_2 evolution by the Hill reaction while being illuminated in a solution of quinone or in a mixture of ferric oxalate and ferricyanide. From these experiments it appears unlikely that any of the algae in the first batch were photosynthetically active at the time the fluorescence experiments were performed with them. In the second batch of algae *Gigartina exasperata* and *Iridaea* showed photosynthesis rates measured manometrically in sea water of about half of those reported by Emerson and Green (**3**) for a species of Gigartina. Our lower rate may have been due to the use of a somewhat lower light intensity, which in our case was 250 f.c. The *Gigartina radula* appeared to be the least stable of all the algae since some of its red pigment diffused out into the sea water during transit. It appeared to be much more fragile than the other species with which we were dealing and did not show any photosynthesis.

The absorption spectra of several intact algae

The absorption spectra of several of the algae were measured with the Ulbricht sphere and monochromator which has been described previously (**5, 11**). The algae wet with sea water were placed in the bottom of Carrel culture flasks which were held in the center of the sphere. The correction for reflected and absorbed light in the pyrex vessel was applied as previously described (**11**). Our results are presented in figure 1 in which the light absorption in per cent. is plotted as a function of wavelength. Band widths of about 14 mμ were used and stray light was eliminated by use of the filters listed in (**11**).

At about 710 mμ the absorption of the two red algae is somewhat above that of the brown alga Alaria, although Alaria absorbs much more strongly at the chlorophyll-a maximum. The slightly greater absorption at 710 mμ may perhaps be due to the presence of chlorophyll-d. The chlorophyll-a maximum appears at about 675 mμ. The maximum obtained at 620 mμ we attribute to the presence of phycoerythrin. It is also likely that the minor absorption band of chlorophyll-a contributes to the increase in absorption at 620 mμ. The minimum absorption occurs at 600 mμ in sharp contrast to the behavior of green leaves which all show a minimum at 550 mμ. From this point on, the curve rises to a broad maximum at

about 550 mμ. In Gigartina, there appears to be a slight depression and another rise at 530 mμ. This rise has not been identified with any particular pigment. In Iridaea there is but one very broad band with the maximum at about 550 mμ. The maximum found at 495 mμ is a band also present in green leaves and may be presumed to be due either to a chlorophyll or to a carotinoid. However, we see in figure 2 below that the water extract of Iridaea containing primarily phycoerythrin also shows a sharp maximum at this point. It may be that the absorption spectrum of the intact algae rises in region of 495 mμ because of the combined effects of several pigments including phycoerythrin. From 470 mμ on toward the shorter wavelengths

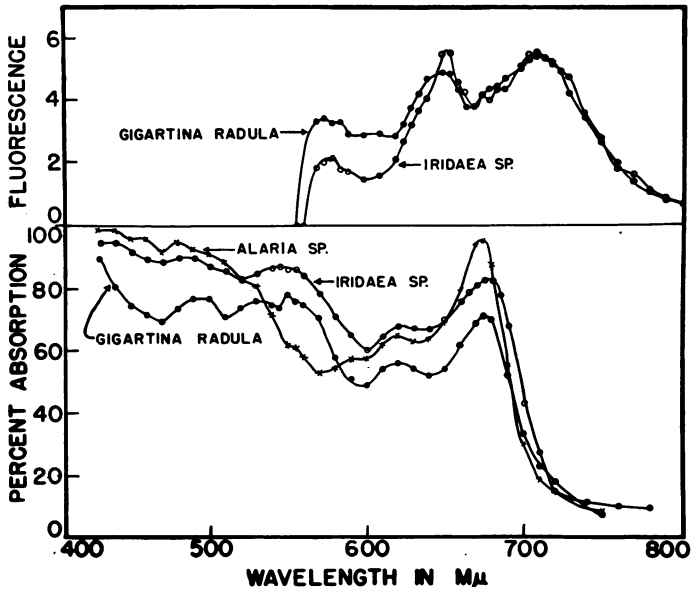

FIG. 1. The absorption spectra of two red and one brown marine algae, with the fluorescence spectra of the two red algae plotted above.

the curves rise rather sharply. This also is noted in both the methanol-soluble and the water-soluble pigments which will be discussed later. In the curve for Iridaea the bands are much less pronounced, but the maxima and minima are found in the same positions as in the curve for *Gigartina radula*. A point worthy of note is that the absorption of *Gigartina* at 550 mμ is 78% while the absorption at the red maximum, in this case 675 mμ is 71%. If the algae used by Haxo and Blinks are even approximately similar, their several times higher yield of photosynthesis in green light can not have been due to increased absorption and therefore must be due to participation of phycoerythrin in the process of photosynthesis. In comparing the height of the absorption band at 550 mμ in Gigartina with that of Lactuca (**11**), we conclude that the absorption due to phycoerythrin in *Gigartina radula* is about three times that of the absorption due to chlorophyll at this wavelength.

The curve for Alaria, a brown alga, is much more like that of a typical green leaf. The red maximum appears to be at 675 mμ with a minimum at 570 mμ. From this point on toward the blue the rise is presumed to be primarily due to absorption by fucoxanthin.

A PARTIAL SEPARATION OF THE PIGMENTS OF IRIDAEA IN EXTRACTS

In order to study both the absorption and the fluorescence of phycoerythrin and of chlorophyll for the most part separated from each other, extracts were made from a fresh piece of Iridaea. This was done by grinding with sand and fine quartz powder with added water. This macerated material was centrifuged at low speed to remove the larger particles and then placed in a high speed centrifuge at 12,000 × g for 20 minutes. Two

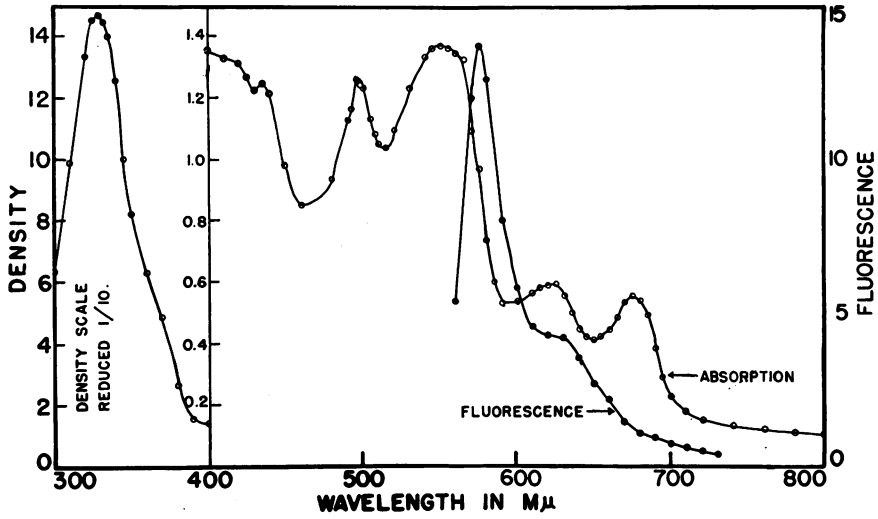

FIG. 2. The absorption and fluorescence spectra of a water extract of *Iridaea sp.*, containing primarily phycoerythrin.

successive water extracts were combined for the high speed centrifugation. The supernatant material will be referred to hereafter as the water extract of Iridaea. To the residue of the water extraction a volume of methyl alcohol was added which was equal to the total volume of cell material and quartz. The first methyl alcohol extract was slightly pinkish but contained no appreciable amount of pigment and was rejected. The second extraction with an equal volume of methyl alcohol was green in color. This, combined with a third methyl alcohol extract which was deep green was saved and will be referred to later as the methanol extract of Iridaea. A fourth extraction with methanol, made by heating the mixture to boiling, was brownish presumably due to the formation of some phaeophytin. This fraction was rejected.

The water extract was pink and highly fluorescent. It was nearly clear but did show some cloudiness which was hard to distinguish due to the

intense orange fluorescence. Its absorption curve is shown in figure 2. The absorption maximum at 675 mμ indicated the presence of some chloroplastin. The absorption due to chlorophyll present compared to that of phycoerythrin appeared to be quite small. Maximum of phycoerythrin absorption appeared at a wavelength of 550 mμ. It is a rather broad band extending from 540 to 570 mμ. We did not find a minimum at 550 mμ between two peaks as described by KYLIN (**10**). This band is followed by a minimum at 515 mμ and another maximum at 495 mμ. A broad minimum at 460 mμ leads to a steep rise at the blue end of the spectrum. In the near ultraviolet a very dense band was found with a maximum at 329 mμ. This absorption was ten times that of any of the visible part of the spectrum. Since this was a crude extract of the algae and not a

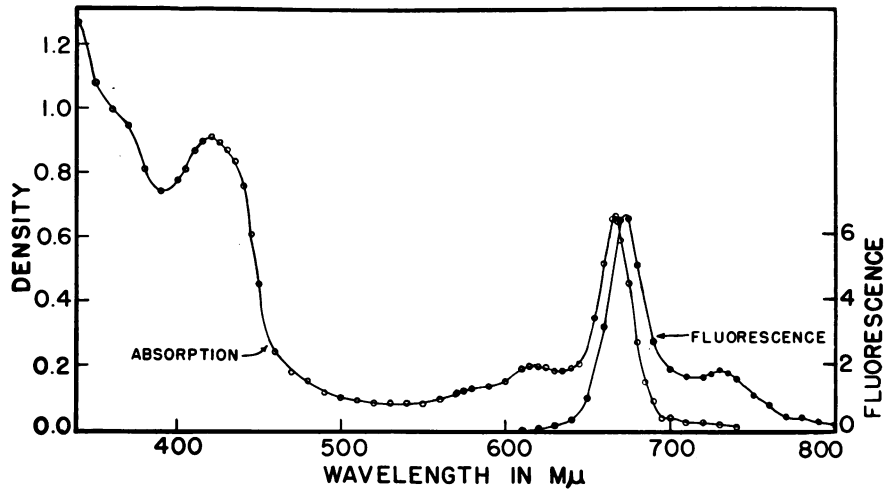

FIG. 3. The absorption and fluorescence spectra of a methanol extract of *Iridaea sp.*, containing primarily chlorophylls and carotinoids.

purified preparation it remains to be determined if this band is due to phycoerythrin.

The absorption curve of the methanol extract of Iridaea is shown in figure 3. It was hoped that this extract might reveal some trace of the chlorophyll-d band in the neighborhood of 690 mμ. It is, however, difficult to tell whether or not chlorophyll-d is present in traces in this extract. The chlorophyll-a maximum is found to be at 666 mμ. HARRIS and ZSCHEILE (**9**) report a major maximum for chlorophyll in methanol at 664 mμ.

We have attempted to reproduce the absorption curve of the algae by the addition of curves of the water extract and of the methyl alcohol extract. To make such an addition, an appreciable broadening of the chlorophyll band is required in addition to the shifting of its maximum. This appears to be reasonable in that the pigment combined with the protein may well have

a broader band than a pigment in true solution. However, any procedure which we were able to devise for broadening this red band resulted in curves which did not fit at the blue end of the spectrum so the attempt to reproduce the intact algae curves by some manipulation of the extract curves was abandoned. We did not, however, find any definite evidence for the presence of other pigments than those accounted for in one or another of these extracts. A possible exception to this statement may be the small maximum found at 530 mμ in the absorption of *Gigartina radula.*

THE INTENSITY OF RED FLUORESCENCE EMITTED BY INTACT ALGAE WHEN EXCITED BY EQUAL NUMBERS OF QUANTA OF VARIOUS WAVELENGTHS

We have tried to find out if the energy which is absorbed by phycoerythrin is transferred to chlorophyll. If this were the case one should be

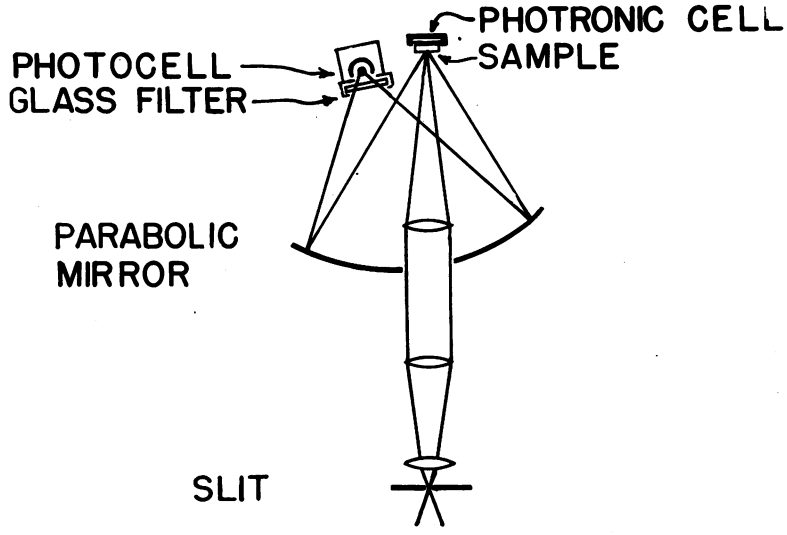

FIG. 4. The apparatus used to measure the intensity of red fluorescence emitted by intact algae when excited by equal numbers of quanta of various wavelengths.

able to get high chlorophyll fluorescence from the algae when they are absorbing light which is primarily taken up by phycoerythrin. If the red fluorescence of the algae were due only to chlorophyll the question could be settled by measuring the fluorescence passing through a red filter. This approach has been used successfully by DUTTON, MANNING and DUGGAR (2) in the study of light absorption by fucoxanthin. We were not able to answer this question satisfactorily in the case of phycoerythrin. We have, however, obtained considerable information which should help in the eventual solution of this problem.

Measurements of the relative intensity of fluorescence with different exciting wavelengths were made with the apparatus diagrammed in figure 4. A sample was held in a Carrel culture flask and illuminated by a beam of

TABLE I

A SAMPLE SET OF DATA AND CALCULATIONS OF THE RELATIVE FLUORESCENCE YIELDS IN AN INTACT ALGA EXCITED BY DIFFERENT WAVELENGTHS

	INCIDENT ENERGY MEASUREMENTS	
Wavelength:	560 mμ	650 mμ
Band width: Stray light filters:	14 mμ (2nd order; 2-mm. slits) 2.6 cm. $FeSO_4$ sat. in 25% H_2SO_4 2.6 cm. $CuSO_4$ dilute soln. Corning Nos. 3484, 4305, 512	14 mμ (1st order; 1-mm. slits) 2.6 cm. $FeSO_4$ sat. in 25% H_2SO_4 2.6 cm. $CuSO_4$ dilute soln. Corning No. 241
Total of thermopile deflections at 1-mm. intervals across the beam × 1/width of receivers:	6.75 cm.	3.56 cm.
Defln. of thermopile exposed to standard lamp giving 2.35×10^{-3} cals./min. at 1 M.:	15.5 cm.	
Energy incident on algae:	$\frac{6.75}{15.5} \times 3.35 \times 10^{-3} =$ 1.46×10^{-3} cals./min.	$\frac{3.56}{15.5} \times 3.35 \times 10^{-3} =$ 7.7×10^{-4} cals./min.
Energy of one quantum:	8.39×10^{-20} cals./min.	7.22×10^{-20} cals./min.
Correction for Pyrex cell transmission:	0.91	
Energy incident on algae:	$0.91 \times \frac{1.46 \times 10^{-3}}{8.39 \times 10^{-20}} =$ 1.55×10^{16} quanta/min.	$0.91 \times \frac{7.7 \times 10^{-4}}{7.22 \times 10^{-20}} =$ 0.97×10^{16} quanta/min.
	FLUORESCENCE MEASUREMENTS	
Photronic cell galv. defl. with no sample:	51.0 cm.	13.3 cm.
Photronic cell galv. defl. with algae:	7.0 cm.	4.3 cm.
Fraction absorbed:	0.86	0.68
Energy absorbed:	1.33×10^{16} quanta/min.	6.59×10^{15} quanta/min.
Photocell response with MgO: Filter before photocell: (RG8) (88) (5850 + 241)	 0 0 0	 7.2 4.0 4.0
Correction for reflection: (RG8) (88) (5850 + 241)	 0 0 0	 $0.2 \times 7.2 = 1.4$ $0.2 \times 4.0 = 0.8$ $0.2 \times 4.0 = 0.8$
Deflection from algae: (RG8) (88) (5850 + 241)	 43.0 32.0 31.5	 18.0 13.8 11.5
Deflection corrected for reflection: (RG8) (88) (5850 + 241)	 43.0 32.0 31.5	 16.6 13.0 10.7
Fluorescence per 10^{15} absorbed quanta: (RG8) (88) (5850 + 241)	 $\frac{43.0}{13.3} = 3.23$ $\frac{32.0}{13.3} = 2.40$ $\frac{31.5}{13.3} = 2.36$	 $\frac{16.6}{6.59} = 2.52$ $\frac{13.0}{6.59} = 1.97$ $\frac{10.7}{6.59} = 1.62$

light of about two sq. cm. area which came from the monochromator. Filters (**11**) were used in front of the monochromator to reduce stray light of other wavelengths. The intensity of the incident light was measured by a thermopile calibrated in absolute units. The fraction of light which was absorbed by the algae was measured by a photronic cell placed directly behind the vessel. Its diffuse reflection was estimated as a fraction of incident light, by coating half the vessel with magnesium oxide and weakening that portion of the beam of light which fell on the magnesium oxide with a set of filters of known transmission until the brightness of the magnesium oxide and of the algae appeared equal. This value was used in making a correction for scattered light as noted below. The fluorescent light was collected by means of a curved mirror which concentrated a constant fraction thereof on a photocell located behind red glass filters. The photocell was connected to a "Photovolt" amplifier which permitted reading

TABLE II

THE RATIOS OF FLUORESCENCE YIELD FROM RED AND FROM GREEN EXCITING LIGHT ON THE BASIS OF EQUAL ABSORBED QUANTA. THIS SHOWS THAT MORE RED FLUORESCENCE IS OBTAINED FROM LIGHT ABSORBED PRIMARILY BY PHYCOERYTHRIN THAN FROM LIGHT ABSORBED BY CHLOROPHYLL

SPECIES	FILTER			
	RG8	88	5850 + 241	AVERAGE
	Fl. from 560 mμ / Fl. from 650 mμ			
Gigartina radula	.....	.....	1.9	1.9
" "	2.1	2.1	1.8	2.0
" "	1.7	4.3	1.3	2.6
Iridaea sp.	.....	.....	1.2	1.2
"	1.3	1.2	1.5	1.3
Bean leaf	0.66	0.79	0.70	0.72
Magnolia acuminata	0.67	0.80	0.61	0.69

of the relative intensity on the photocell as a galvanometer deflection. When magnesium oxide was substituted for the sample there was a measureable galvanometer deflection. The scattered light was corrected for by multiplying the reflection of a sample by the galvanometer deflection introduced by the magnesium oxide, and subtracting this number from the apparent fluorescence of the sample.

Three different sets of filters were used between the fluorescent algae and the photocell: Corning ♯5850 with ♯241, which cuts off 50% of the light at about 720 mμ; Wratten ♯88, which has a 50% transmission at about 710 mμ; and Jena RG8, which transmits 50% of the light at about 703 mμ. A list of sample calculations for a complete set of measurements is presented in table I. The results of all such measurements are presented as ratios in table II. No attempts were made to measure the percentage yield of the incident light which was turned into fluorescent light.

It was observed (table II) that in the red algae the fluorescence excited by green light having a wavelength of 560 mμ is definitely larger than that excited by red light having a wavelength of 650 mμ. The green light is largely absorbed by phycoerythrin and the red by chlorophyll. In comparison with this behavior of red algae it is seen that in green leaves the relative fluorescence yields are reversed; that is, more red fluorescence is excited by red light than by green light. These results could be due either to a red fluorescence from other pigments than chlorophyll or to a more efficient excitation of chlorophyll fluorescence by phycoerythrin than by chlorophyll itself. A study of the fluorescence spectra of the live algae and of the extracts was therefore undertaken.

THE FLUORESCENCE SPECTRA OF INTACT ALGAE AND OF THEIR EXTRACTS

The algae in a glass vessel containing sea water were exposed to the green and blue lines of the mercury arc obtained from an H-4 mercury lamp filtered through a double Corning ♯9780 filter and also a Corning #512 thus transmitting the 365, 405, 436 and 546 mμ lines. An image of the mercury lamp fell on the surface of the algae at an angle of incidence of about 70°. The fluorescent light was collected by a short-focus lens and focused on the slit of the monochromator. Behind the entrance slit was placed an orange Corning ♯3842 filter to absorb the blue light completely and a very large fraction of the green light. Measurements were made at every 10 mμ over the range from 560 to 800 mμ of the intensity of fluorescence with a photocell which was placed behind the exit-slit of the monochromator. At each wavelength the deflection of the photocell was compared with the deflection produced when light from a standard lamp of known energy distribution was allowed to go through the same optical system. Its intensity was reduced by means of wire screens so that it was of a magnitude comparable with the intensity of the fluorescent light from the algae. In this way the relative energy distribution throughout the spectrum of the fluorescent light could be determined and the photocell sensitivity and monochromator transmission were thus cancelled out. The curves which were obtained using the apparatus shown in figure 5 have been plotted in figure 1 to show their position in relation to the absorption of the algae. It will be noted that the fluorescence of phycoerythrin which had its maximum at about 575 mμ is small in proportion to the height of the fluorescence bands in the red region. There are two red maxima in the fluorescence spectra of both algae investigated and we have so far been unable to attribute these bands definitely to any particular pigment. It is striking, however, that the minimum between these two bands comes at exactly the same place as the maximum of the chlorophyll absorption band in the red region.

It does appear that the fluorescence is probably due more to chlorophyll than to phycoerythrin, or that the fluorescence spectrum of phycoerythrin in intact algae is very different indeed from the fluorescence spectrum of

the extracted material. We are indebted to Professor Robert Livingston for the suggestion that phycoerythrin and chlorophyll may in the intact algae be in combination with each other thus influencing the fluorescence spectrum of the complex and facilitating the transfer of energy from one to the other. Professor L. R. Blinks has suggested that the 655 mμ maximum is due to the presence of phycocyanin in the algae; an explanation which we have accepted as the most likely after seeing some of his unpublished absorption curves of similar algae. The maximum at 710 mμ would then presumably be due to chlorophyll fluorescence. Its position is, however, quite different from the 675 mμ found in chlorella fluorescence (12).

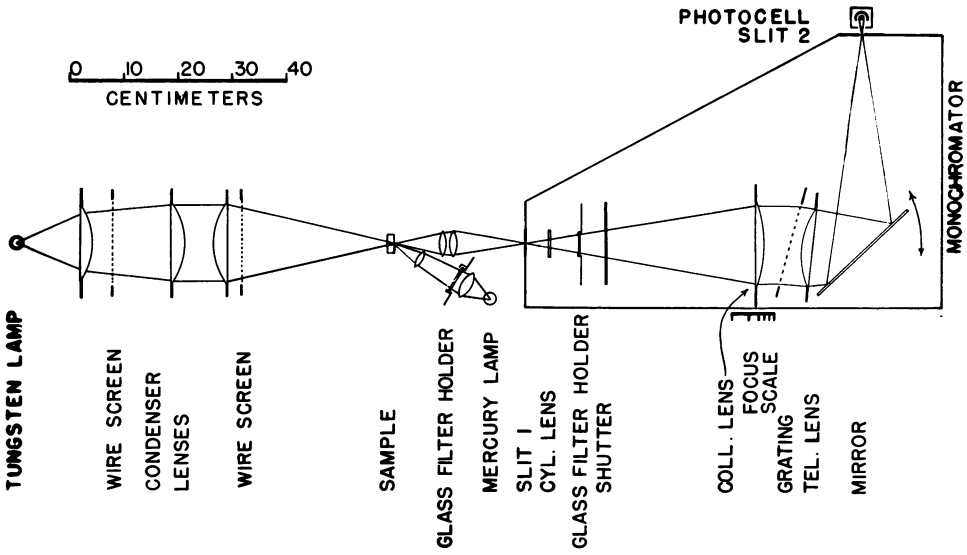

FIG. 5. The apparatus used to measure the fluorescence spectra of the intact algae by the comparison of its emission with that of a standardized tungsten lamp.

The fluorescence spectrum of the extracted material, measured with a slight modification of this apparatus, is presented in figure 2 in comparison with its absorption spectrum. In measuring the fluorescence spectra of the extracts a greater intensity was obtained by illuminating one corner of the vessel containing the extract and taking the fluorescent light out at right angles from the same corner. The cell containing the extract was placed adjacent to the entrance slit of the monochromator and illuminated from the side by the mercury lamp. Radiation from the standard lamp was arranged to pass through the same optical system as the fluorescent light, as in the original apparatus. We have determined the fluorescence curves for both the water extract containing phycoerythrin and the methyl alcohol extract containing chlorophyll. By shifting the chlorophyll fluorescence spectrum so that its maximum comes at 707 mμ and adding this curve to the fluorescence spectrum for phycoerythrin we are completely unable to

reproduce the shape of the fluorescence curves of the intact cells. It must therefore be concluded that the fluorescence spectrum of the phycoerythrin and of the chlorophyll is widely different in the intact algae from its shape in extracts, or that other pigments not present in either the water or the methyl alcohol extracts are causing some of the fluorescence of the intact material. The determination of the fluorescence curves of algae when excited by various wavelengths has not yet been attempted since it would require two monochromators and a more sensitive photocell. Until this can be done it will probably be difficult to come to a definite conclusion as to whether or not the energy absorbed by phycoerythrin is used directly for photosynthesis or is first transferred to chlorophyll.

THE BEHAVIOR OF WATER EXTRACTS OF ALGAE WHEN ILLUMINATED IN THE PRESENCE OF DYES

The dye phenol-indophenol has been shown by HOLT and FRENCH (**6, 7**) to be reduced to the leuco form when illuminated in the presence of active chloroplasts of spinach with a concomitant evolution of oxygen. This is believed to take place by means of a part of the photosynthesis system of the cell. Chloroplasts from our first shipment of *Gigartina radula* were not active in the reduction of the dye. A very slight activity was obtained from the Ulva chloroplasts. The extract of phycoerythrin from the first batch of Gigartina appeared to lose color when illuminated with added dye at pH 6.5. At this pH phenol-indophenol and phycoerythrin are both pink. Further investigation using slightly alkaline solutions of the dye which are blue or acid solutions of 2,6-dichlorophenol-indophenol showed that it was not the dye but the phycoerythrin which was bleached upon illumination in such solutions. Dye reduction experiments were later made with fresh phycoerythrin extracts of algae which had been shown to be photosynthetically active, again with negative results.

Summary

The absorption and fluorescence spectra of several marine algae are presented. The fluorescence yield in *Gigartina radula* and in *Iridaea sp.* is greater from green light than from red light. The fluorescence spectra of two red algae show a band at 575 mμ corresponding to phycoerythrin fluorescence, and also peaks at 675 mμ and 710 mμ.

It is a pleasure to thank the Graduate School of the University of Minnesota for grants which made this work possible. DRS. C. O. ROSENDAHL and JOHN H. MOORE gave invaluable assistance in the identification of the algae used. The photosynthesis experiments and the dye reduction experiments were done by DR. A. S. HOLT to whom we are also greatly indebted.

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