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ON THE PIGMENT SYSTEM OF THE RED ALGA *PORPHYRA LACINEATA*

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SUMMARY

Absorption and fluorescence around 725 mμ of suspensions of the red alga *Porphyra lacineata* were studied both with and without the phycobilins attached to the pigment system.

The *in vivo* occurrence of 2 chlorophyll *a* types was confirmed spectroscopically. Their red absorption maxima are located at 678 and 672 mμ respectively. Removal of the phycobilins did not affect the shape of the red absorption band of these types. It is therefore concluded that phycobilins are not responsible, *e.g.* by complex formation, for the occurrence of one or both of these types. It is possible that withdrawal of the phycobilins causes quenching of chlorophyll fluorescence. The "pigment X" fluorescence around 725 mμ remains unaffected by removal of the phycobilins.

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INTRODUCTION

In various respects, the pigment system of red and blue-green algae is more complicated than that of other photosynthetic species. Firstly, part of this system, the phycobilins, is readily extracted when crushing the cells in water[1]. Secondly, as soon as these phycobilins are extracted the ability to perform the HILL reaction is lost[2,3]. Thirdly, part of the chlorophyll seems to occur in a photosynthetically inactive state and does not show appreciable fluorescence *in vivo*, while it is spectroscopically different from the active chlorophyll[4–7]. In this respect, it may be briefly mentioned that KRASNOVSKY *et al.*[8] suggest the similar occurrence of such a situation in "green" plants. The chlorophyll is supposed to be present partly in an aggregated form and partly in a monomeric state. BRUGGER AND FRANCK[9], and FRANCK[10] suggested that *in vivo* chlorophyll occurs in a "protected" as well as an "exposed" state with regard to contact with liquid water and its solutes. Fourthly, the fluorescence spectrum is different from that of other plants[11], while, according to DUYSENS[4,12], its shape depends on the wave length of the exciting light. DUYSENS ascribed part of the emitted light to fluorescence of an unknown pigment, which might be chlorophyll *d*; its fluorescence maximum was found around 725 mμ. Attempts to isolate this pigment were not sufficiently successful to enable identification. The fluorescence of the unknown pigment was relatively intense at irradiation with light mainly absorbed by chlorophyll (420 mμ), but it was relatively weak when excited with light of 546 mμ which is mainly absorbed by phycoerythrin. On the other hand, BRODY[13] suggested that the fluorescence band at 720 mμ which is observed in the red alga *Porphyridium cruentum* may be due to light emission by an aggregated form of chlorophyll. In any case, both authors agree about the occurrence of chlorophyll in 2 states, one strongly and one weakly fluorescent, in the red and blue-green algae studied.

Because of the occurrence of a highly efficient energy transfer from the phycobilins to the "active" chlorophyll exclusively, both pigments seem to be in close contact with each other[4]. On the other hand, it has been suggested that the "inactive" chlorophyll is coupled with the unknown pigment mentioned and occurs spatially separated from the phycobilins[4].

Further information about both states of chlorophyll in red and blue-green algae may be gained by comparison of absorption and fluorescence phenomena in the natural pigment complex with those in complexes from which the phycobilins have been removed. In the present paper such expts. with aqueous extracts of the red alga *Porphyra lacineata*, both with and without phycobilins attached to the pigment complex, are described. With regard to fluorescence, the light emission around 725 mμ was particularly studied.

METHODS

Material

Freshly collected *Porphyra lacineata* was obtained from the Zoological Marine Station at Den Helder weekly. It was kept in seawater either in the light in a cool room or in a refrigerator at 2°. No differences in absorption or fluorescence due to differences in absorption or fluorescence due to different storage procedures were observed.

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Preparation

About 5 g of pieces of thalli of nearly the same colour and thickness were selected, quickly rinsed with tapwater, washed with a soln. of 40 g of recrystallized polyethyleneglycol "4000" or "6000" in 100 ml of glass-distilled water (briefly referred to as "40 % PEG") and minced in a mortar under the addition of 10 ml of 40 % PEG. According to McCLENDON AND BLINKS[2], this concn. of PEG prevents the release of phycobilins from the pigment system. The resulting suspension was diluted about 4 times with 40 % PEG. In order to remove cell debris, the viscous suspension was pressed through nylon tissue. It was then divided into 2 equal portions, A and B, which were centrifuged at 1200 × *g* for 15 min. The clear, colourless supernate was discarded and the pellets A and B were resuspended in 40 % PEG and distilled water respectively. Then they were centrifuged again at 1200 × *g* for 15 min. The A-supernate was clear and colourless again, while the B-supernate was clear, but coloured. Both supernates were discarded. The A-sediment was resuspended in 40 % PEG, whereas the B-sediment was taken up in distilled water.

The concn. of both suspensions was made the same with regard to absorption at 678 mμ and, with the opal glass technique of SHIBATA, BENSON AND CALVIN[14], the absorption spectra were determined in a Hilger Uvispek spectrophotometer.

Measurement

The fluorescence was measured as described earlier[15], except for the following modifications: (1) the signal was fed into a Honeywell-Brown recorder, and (2) the dimensions of the cuvette were 60·40·5 mm. Of this cuvette only a central area with a diameter of about 5 mm was illuminated.

Actinic light was obtained by passing a parallel incandescent light beam through two GAB Calflex heat-reflecting interference filters and a GAB interference filter with optimal transmission at 618, 566, or 437 mμ. Fig. 1 shows the transmission of these filters together with the absorption spectrum of the used alga in 40 % PEG.

Scattered light was eliminated by adjusting a 2 mm Schott RG 9 glass filter in front of the photomultiplier. The transmission of this filter was zero at wave lengths shorter than 695 mμ, 50 % at 725 mμ, and 77 % at 775 mμ and longer wave lengths. It was checked that illumination of blanks with water or 40 % PEG did not produce any measurable signal. As a reference, a dilute rhodamine-B soln. or a piece of black paper was used. In the latter case, the RG 9 filter was removed.

Exciting light intensities were measured with a micro vacuum thermocouple as described by MANTEN[16]. When using the 618, 566 and 437 mμ filters, the incident intensity was $8.7 \cdot 10^3$, $3.0 \cdot 10^3$ and $1.1 \cdot 10^3$ ergs $cm^{-2}sec^{-1}$ respectively.

In order to correlate fluorescence intensities in various samples at a given wave length of the exciting light absorption was expressed as the product of interference filter transmission and absorption of the suspension in question.

RESULTS

Absorption

Absorption spectra of both a thallus and an extract in distilled water are shown in Fig. 2. From the difference in absorption at 565 mμ it is evident that extraction with water resulted in an apparently complete release of phycoerythrin from the pigment system.

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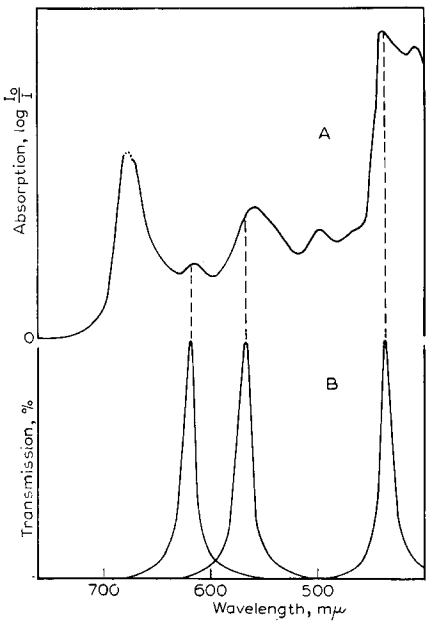

Fig. 1. A: Absorption spectrum of a suspension of *Porphyra lacineata* in 40 % PEG, B: Transmission of the used interference filters.

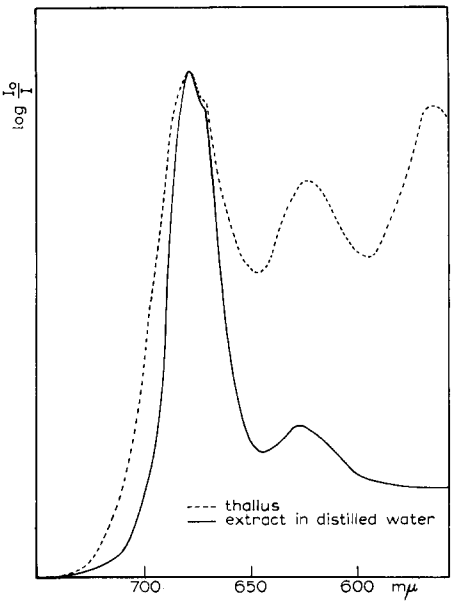

Fig. 2. Absorption spectra of a thallus and an extract in distilled water of *Porphyra lacineata*.

Extraction with water caused only a reduction of the 625 mμ maximum where phycocyanin absorption occurs. At this wave length, however, a minor absorption band of chlorophyll *a* is present. It can be concluded, in any case, that at least the major part of the phycocyanin, and probably all of it, was also released by water treatment.

The red absorption band with its maximum at 678 mμ shows a shoulder at 672 mμ. This shoulder cannot be ascribed to chlorophyll *b* absorption, for this pigment is known to be lacking in red and blue-green algae. Extraction with distilled water did not remove the shoulder. Because of very different scattering and concn. conditions, the red absorption bands of thallus and suspension do not match closely. In Fig. 3, absorption spectra of an extract in 40 % PEG as well as in water are given. The latter suspension was obtained by water treatment of a sample of the former as described under METHODS. In both suspensions scattering and concn. conditions were nearly the same. It is evident that both location and mutual height of maximum and shoulder are unaffected by extraction with water. This phenomenen was observed in all 5 expts. done.

Fluorescence

After appliance of the corrections discussed under METHODS, the intensity of the 725-mμ steady state fluorescence of the *Porphyra* suspension in 40 % PEG was considered 100 %. The fluorescence intensity of the suspension in distilled water was expressed as a percentage of that of the 40 % PEG preparation. This percentage is indicated as "measured" fluorescence.

It is possible to calculate such a percentage from the quantities of actinic light

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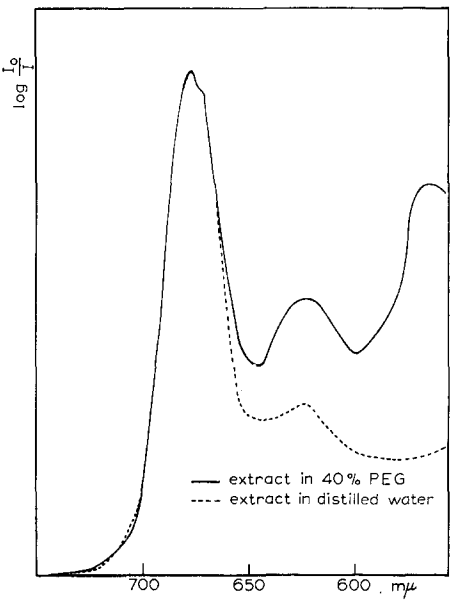

Fig. 3. Absorption spectra of extracts of *Porphyra lacineata* in 40 % PEG and in distilled water.

absorbed by the suspensions in 40 % PEG and in water. This percentage is denominated the "computed" fluorescence. If the quantum yield of the *Porphyra* fluorescence were independent of the wavelength of the exciting light, the percentage in question should be numerically the same as the "measured" one. However, it was found that the two percentages were approx. equal only at irradiation with 437 mμ light. This is quantitatively expressed by presenting the "measured" values as a percentage of those "computed", as shown in Table I. It may be emphasized that only the intensities of fluorescence excited by light of one and the same wavelength can be mutually compared.

The mean errors shown in the right-hand column of Table I vary between 12 and 15 %. Though great care was taken to prepare the suspensions under the same conditions, the errors are relatively high. The variations of the properties of the fronds with respect to the quantitative composition of the pigment system, toughness, age, different growing conditions, etc. may account for this phenomenon. In any case, it can be concluded that the yield of the 725-mμ fluorescence of suspensions deprived of phycobilins about equals that of suspensions with the phycobilins attached to the pigment system. The former fluorescence was found to be 113 ± 13 % of the latter one. However, on irradiation with light of 566 mμ, this yield of the phycobilin-free sample was about 40 % (36 ± 5 %) of that of the intact system, while this figure was about 60 % (56 ± 8 %) with light of 618 mμ.

DISCUSSION

With regard to spectra, red and blue-green algae differ from other plants in that they show phycobilin absorption as well as a distinct shoulder at the short-wave side of the red chlorophyll *a* absorption band, while chlorophyll *b* is lacking. Thus,

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TABLE I

FLUORESCENCE AROUND 725 mμ OF SUSPENSIONS OF *Porphyra lacineata* IN 40% POLY ETHYLENE GLYCOL (PEG) AND IN DISTILLED WATER

Wavelength of exciting light mμ	*Expt.*	*Fluorescence in scale divisions. Suspension in*		*Absorption* Suspension in*		*Fluorescence of suspension in water in % of that in PEG*		*Fluorescence "measured" in % of fluorescence "computed"*
		PEG	*water*	*PEG*	*water*	*"Measured"*	*"Computed"*	
437	a	6.6	3.6	617	360	54	58	93
	b	4.6	3.3	397	240	72	59	118
	c	3.3	2.7	437	213	82	49	167
	d	4.6	2.0	763	347	44	46	96
	e	4.7	2.7	607	273	57	45	127
	f	5.6	2.3	420	213	41	51	80
								mean: *113 ± 13*
566	a	68.5	11.4	186	60	17	32	53
	b	33.1	5.1	206	107	15	52	29
	c	30.8	1.5	253	84	5	33	15
	d	56.6	4.4	413	91	8	22	36
	e	47.3	2.7	343	47	6	14	43
	f	42.9	4.3	257	58	10	23	43
								mean: *36 ± 5*
618	a	11.0	3.2	184	62	29	34	85
	b	8.0	2.4	170	99	30	59	52
	c	7.1	0.9	259	114	13	44	30
	d	12.9	1.9	434	125	13	29	45
	e	10.9	1.8	315	79	16	25	64
	f	9.2	1.7	254	76	18	30	60
								mean: 56 ± *8*

* When irradiating with light of 437 mμ, absorption is expressed in log $I_0/I \cdot 10^3$ at 680 mμ. When using actinic light of 566 or 618 mμ, because of difference in composition of the spectra of both related samples, it is expressed as the area of the graph obtained by plotting the product of absorption of the sample and percentual transmission of the interference filter used *vs.* wave length. Figures represent scale divisions of the plane geometer.

some relation may exist between the presence of this shoulder and the occurrence of phycobilins.

The present expts., however, demonstrate that the shape of the red absorption band of chlorophyll *a* is the same in suspensions with or without phycobilins attached to the pigment complex. Consequently, neither the maximum at 678 mμ nor the shoulder at 672 mμ are due to any influence, *e.g.* complex formation, exerted by phycobilins on chlorophyll *a*. The present results support the suggestion of HALLDAL[7] that in red and blue-green algae this chlorophyll occurs in 2 different "forms". Furthermore, HALLDAL suggested that, since only one "form" of chlorophyll *a* is observed in solution, the different *in vivo* "forms" may be due to the occurrence of different complexes of this pigment with, presumably, proteins. On the other hand, it is also possible that the 2 types represent a monomeric and an aggregated form of chlorophyll as suggested by others[8,13]. If the latter hypothesis were true, the aggregated form is likely to consist of more than three associated molecules (*cf.* RODRIGO[17]). It may be added that both forms are supposed to be attached to protein carrier molecules.

The presence of more than one long-wave absorption maxima of chlorophyll *a*

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in vivo with only a single one occurring in solution recalls the situation with bacteriochlorophyll. Still there seems to be a considerable difference. In the living cell three bacteriochlorophyll types, denominated according to the location of their infrared absorption maxima: B890, B850, and B800, may be observed. Energy absorbed by B850 or B800 is transferred to B890 with an efficiency approaching 100 % and enters the chain of photosynthetic dark processes via this type exclusively[12]. Thus, the absorption maximum of the photosynthetically most important bacteriochlorophyll type is situated at the low-energy side of the *in vivo* spectrum. However, according to FRENCH AND YOUNG[6], the chlorophyll *a* type with a maximum at the long-wave side of the *in vivo* absorption band (around 680 mμ) is photosynthetically inactive, while that with a maximum at 670 mμ represents the active chlorophyll type. It would be interesting to study this matter in more detail.

When considering the fluorescence data, it may be recalled that the fluorescence yield around 725 mμ of pigment systems deprived of phycobilins is about 100, 40, and 60 % of that of complete systems when irradiated with light of 437, 566, and 618 mμ respectively.

The fluorescence spectrum of *Porphyra lacineata* was extensively studied by DUYSENS[12]. According to him, the spectrum varies with variation in the wavelength of the exciting light. He concluded from analysis of these spectra that they are composed of contributions by chlorophyll *a*, phycobilins, and an unknown pigment with a fluorescence maximum around 725 mμ. As mentioned already, DUYSENS did not succeed in determining this pigment with certainty, whereas BRODY[13] suggested that the 720-mμ fluorescence may be ascribed to an aggregated form of chlorophyll. This question will not be considered further here. Whatever the nature of the pigment may be, for brevity's sake it will be termed "pigment X" in the following discussion.

By using DUYSENS' fluorescence analyses, the composition of the fluorescence excited by 437 and 566 mμ light and entering the photomultiplier after passing a Schott RG 9 glass filter was determined approx. This was done by plane geometry of the area of graphs obtained by plotting the product of the intensity of the fluorescence emitted by the mentioned components of the pigment system separately and the transmission of the RG 9 filter *vs.* wavelength. Approx., it was found that on excitation with 437 mμ light the measured fluorescence around 725 mμ was composed of light emission by chlorophyll *a*, phycobilins and pigment X in the proportions 6, 2 and 92 % respectively. On irradiation with 566 mμ light, these percentages became about 12, 43, and 45.

From these figures it is clear that the contribution of the phycobilin fluorescence at irradiation with 437-mμ light is considerably below the mean error of our measurements. Consequently, if no additional effects occur, removal of the phycobilins should not change the fluorescence yield measurably. Actually, with the phycobilin-deprived system this yield was found to be about 100 % of that of the intact pigment complex. This indicates that extraction of phycobilins does not affect the pigment X fluorescence.

If, however, exciting light of 566 mμ is used, the phycobilins provide about 43 % of the total 725-mμ fluorescence. Thus, apart from decreasing the absorption around 566 mμ, removal of the phycobilins results in the loss of one of the major contributors to the studied fluorescence. Instead of the same fluorescence yield as observed with the intact system, a yield of about 57 % could be envisaged with

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phycobilin-deprived complexes. Actually, this percentage was measured to be about 40; the difference, however, is slightly above the accuracy of the used method. Therefore, it can only be concluded that the possibility of the simultaneous disappearance of a minor contributor to fluorescence exists. The single "minor contributor" is chlorophyll *a*, which accounts for about 12 % of the measured light emission. Since no chlorophyll is extracted, the fluorescent "active" portion of this pigment is possibly brought into a non-fluorescent state by loss of the phycobilins. This possibility fits in an explanation of the fact that the ability to perform the HILL reaction is lost after extraction of phycocyanin from a blue-green alga[3]. It may be added that if chlorophyll *a* "inactivation" actually occurred, a reduction of the fluorescence yield of about 6 % would be expected on irradiation with 437-mμ light after removal of the phycobilins. The scattering of the results, however, prevents observations of such a relatively small reduction.

Around 618 mμ, the ratio of chlorophyll absorption to phycobilin absorption is higher than that around 566 mμ, as demonstrated in Figs. 2 and 3. Therefore, removal of the phycobilins should cause a smaller decrease of fluorescence on irradiation with 618-mμ light than with 566-mμ light. Actually, it is found that the fluorescence excited by the former light is decreased only to approx. 60 % instead of the 40 % in the latter case. Because of the mentioned variations this result cannot be evaluated quantitatively.

In conclusion it can be stated that the fluorescence data are easily understood in terms of annihilation of phycobilin fluorescence, and possibly of chlorophyll *a* fluorescence on withdrawal of the phycobilins. The results indicate that the pigment X fluorescence is unaffected by removal of the phycobilins. This means that, whatever the nature of pigment X may be, its state is probably independent of the presence of phycobilins.

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