BBA 45055

# FLUORESCENCE BANDS AND CHLOROPHYLL *a* FORMS

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(Received February 19th, 1964)

## SUMMARY

Fluorescence spectra were determined at temperatures between 20° and −196° for a number of photosynthetic organisms. Below −90° the single fluorescence maximum around 685 mμ was replaced by a system of three bands, at 686, 696 and 717–720 mμ in algal cells. Cooling usually resulted in a decrease of the 685-mμ band. In young cultures of blue-green and red algae the three bands were of about equal intensity; in old cultures and in green algae the 717-mμ band was dominant, while in the latter the 696-mμ band was weak.

In green leaves and chloroplasts also, three bands were present at low temperatures, at 686, 696 and 735–740 mμ. Here too, the 740-mμ band was by far the major one.

During cooling of both diluted and concentrated chlorophyll *a* solutions and chlorophyll adsorbed to filter paper, the height of the 677-mμ fluorescence band and the 730-mμ vibrational level were increased by a factor of about two, provided no increased reabsorption due to increased scattering could occur. In concentrated chlorophyll *a* solutions no extra bands could be detected.

The three fluorescence bands measured *in vivo* at low temperatures are assumed to belong to three chlorophyll *a* forms: $C_a$ 670–F 686; $C_a$ 680–F 696; $C_a$ 695–F 717 in algal cells. Apart from an increase in intrinsic fluorescence yield of $C_a$ 695, the marked increase in 717-mμ fluorescence during cooling is suggested to be due to increased energy transfer from $C_a$ 670 and $C_a$ 680 to $C_a$ 695 as a result of shrinkage, when the temperature is lowered.

## INTRODUCTION

In the fluorescence spectrum of green plants and unicellular algae, when they are cooled to liquid-air temperature, an extra fluorescence band with a maximum around 730 mμ is formed. BRODY[1], who discovered this phenomenon, found also that in concentrated ($2 \cdot 10^{-2}$ M) solutions of chlorophyll *a* in organic solution an emission band is formed at 720 mμ on cooling to liquid-air temperature. He ascribes this phenomenon to emission of a chlorophyll dimer, which should be non- (or weakly) fluorescent at room temperature (BRODY AND BRODY[2]). By analogy, he ascribes the 730-mμ low-temperature emission band *in vivo* to dimer emission, while the 685-mμ

Abbreviation: DCMU, 3-(3,4 dichlorophenyl)-1,1-dimethylurea.

fluorescence band could be ascribed to emission of the chlorophyll monomer. The chlorophyll dimer *in vitro* was assumed to show absorption bands at 682 and 648 mμ (BRODY AND BRODY[3]). Fluorescence action spectra of photosynthetic organisms, measured at liquid-air temperature, showed that the fluorescence band at 730 mμ is excited by light of longer wavelengths (up to 705 mμ) than the room temperature fluorescence band (BUTLER[4]), while the action spectra were different from each other in the rest of the spectrum (BRODY AND BRODY[5]). Therefore, it was assumed that this emission originated from a 705-mμ chlorophyll component, the presence of which had been demonstrated by absorption difference spectra (KOK[6], KOK AND HOCH[7]). FRENCH[8], however, suggested that the occurrence of the 730-mμ fluorescence band at low temperatures might originate in an increase of scattering in the frozen samples, and thus may be an artifact.

BUTLER AND NORRIS[9] measured the lifetimes of the room temperature and low-temperature fluorescence. They found a lifetime of 3.1 mμsec for the low-temperature emission, against a lifetime of 0.7 for the room temperature one. The fluorescence lifetime of dilute chlorophyll *a* solutions was found to be 6.2 mμsec, but of concentrated chlorophyll *a* it was 2.5 at room temperature and 4.4 at low temperature. Thus the lifetime, and consequently the fluorescence yield, of the chlorophyll form responsible for the 730-mμ emission is of the same order of magnitude as that of chlorophyll *a* solutions.

In dilute chlorophyll *a* solutions, BRODY AND BROYDE[10] measured an extra, but weak, absorption band at about 700 mμ as a result of cooling to liquid-air temperature.

GOEDHEER[11] measured fluorescence spectra of bean leaves during different stages of greening from the etiolated state. He found that the 730-mμ low-temperature band did not occur immediately on transformation of protochlorophyll into chlorophyll *a*, but only after several hours of greening. In the first few hours the chlorophyll fluorescence spectra measured at room temperature were similar in shape to those measured at liquid-air temperature. No extra band was formed, but the fluorescence yield was increased about 75 %. The new low-temperature band (about 740 mμ in green bean leaves) and the onset of photosynthetic capacity occurred after a similar period of greening. The coincidence of these two phenomena may be due to the formation of various chlorophyll *a* forms as a result of dark reactions following the initial light-induced protochlorophyll–chlorophyll *a* transformation.

In order to investigate a possible relation between chlorophyll *a* forms and fluorescence bands, the fluorescence spectra of various divergent photosynthetic organisms were measured at temperatures between 20° and −196°. For comparison, fluorescence spectra of chlorophyll *a* solutions at various concentrations and of adsorbed chlorophyll *a* were determined in the same temperature range.

## METHODS

Fluorescence was excited by a 125-W Philips Hg lamp (HP 125). The exciting light was composed mainly of the 436, 546 and 578-mμ Hg lines without filter, and, if a Schott BG3 filter was applied, mainly of the 436-mμ line. Red light was removed by a 4-cm glass cuvette filled with a 10 % (w/v) $CuSO_4$ solution.

Fluorescence spectra were determined with a Bausch and Lomb grating mono-

chromator (500 mm, 600 lines/in, blazed at 7500 Å). The slit width used was usually 0.5 mm for both slits (total band width 3.3 mμ), but in some cases a slit width of 0.75 mm was used.

A liquid-$N_2$-cooled photomultiplier (Dumont 6911) was used as a light detector. A correction factor for changes in multiplier sensitivity and monochromator transmission changes with wavelength was obtained by checking the experimental set up with a calibrated band lamp. The correction factor did not exceed 10 % between 650 and 750 mμ.

The samples were placed in a three-walled Dewar vessel provided with a window. Usually whole cells and chloroplasts were deposited on filter paper and pressed against the window, in this way avoiding a large increase in scattering such as occurs in suspensions during cooling. At room temperature, control spectra were determined with algal cells and chloroplasts in a cuvette. No difference was found provided the absorption did not exceed 50 % in the peaks. In the measurements the absorption was kept well below this value.

Temperatures were measured with a thermocouple pressed against the sample. The temperature was lowered by gradually filling the space between the two inner walls of the Dewar vessel with liquid $N_2$.

No fluorescence due to the filter paper could be observed either before or after, cooling in the spectral region and amplification range used in the experiments. A Schott OG2 filter was placed between sample and monochromator to remove violet Hg lines (projected on the slit by reflection) which become visible in the second-order spectrum. Chlorophyll *a* solutions and chloroplast suspensions were put into bags made of Visking transparent dialysis tubing. The bags were gently pressed against the window of the Dewar vessel. Chlorophyll *a* also was adsorbed on filter paper either as a crude solution prepared by cold extraction from the chlorophyll *b*-free alga Synechococcus with methanol, or as a solution purified by paper chromatography. A small strip of the filter paper containing chlorophyll was cut out and used in the experiments

Green leaves were placed immediately behind the window with the upper side of the leaf facing the glass.

## RESULTS

The fluorescence spectrum of the blue-green alga *Anacystis nidulans*, measured at 20° −85° and −196°, is given in Fig. 1. Instead of a single marked maximum located at 685 mμ at room temperature, three sharp maxima were present at 686, 696 and 717 mμ at liquid $N_2$ temperature. The band at 696 mμ usually appeared at a somewhat higher temperature than the band at 717 mμ. Both the band at 656 mμ from phycocyanin and the 685-mμ chlorophyll band were sharpened as a result of cooling. The 696-mμ band became clearly visible as a shoulder on the 656-mμ band at −85°.

A similar fluorescence spectrum was measured with the blue-green alga Synechococcus. The ratio of the bands in the low-temperature spectrum was not always similar to that derived from Fig. 1. Especially in aged cultures, the 717-mμ band could be much more pronounced, while the 696-mμ band exceeded the 686-mμ one. The spectral changes were nearly completed at −160°; only minor changes occurred between this temperature and liquid-$N_2$ temperature. By far the largest changes occurred between −80 and −150°.

The fluorescence spectrum of the red alga *Porphyridium cruentum* is given in Fig. 2. Here too the single 685-m$\mu$ fluorescence band changed during cooling into a system of three bands at 686, 696 and 718 m$\mu$. A marked change also occurred here in the region of phycocyanin fluorescence. The single band at 658 m$\mu$ was split into two separate bands: one at 635 and one at 660 m$\mu$. The new band overgrew the old one in intensity during cooling. This was the case with all suspensions measured,

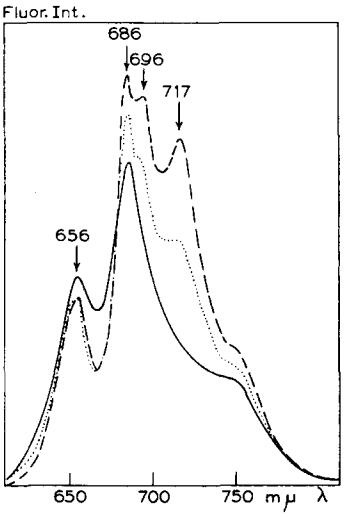


Fig. 1.

Fig. 1. Fluorescence spectrum of the blue-green alga *Anacystis nidulans* (3-days-old culture). Incident light: 437, 546 and 578 m$\mu$. ———, 20°; ·····, −85°; – – – –, −196°.

Fig. 2. Fluorescence spectrum of the red alga *Porphyridium cruentum* (14-days-old culture). Incident light: 437, 546 and 578 m$\mu$. ———, 20°; ·····, −30°; – – – –, −196°. Insert: –·–·–·, −95°.

Fig. 3. Fluorescence spectrum of the blue-green alga Synechococcus (4-days-old culture). Incident light: 437 m$\mu$. ———, steady-state spectrum after 15 min illumination; ·····, spectrum after addition of DCMU; – – – –, spectrum with DCMU reduced to equal intensity in the peak as spectrum without DCMU.

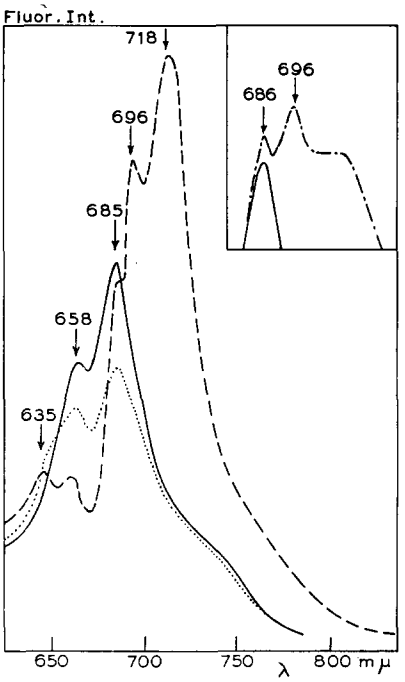


Fig. 2.

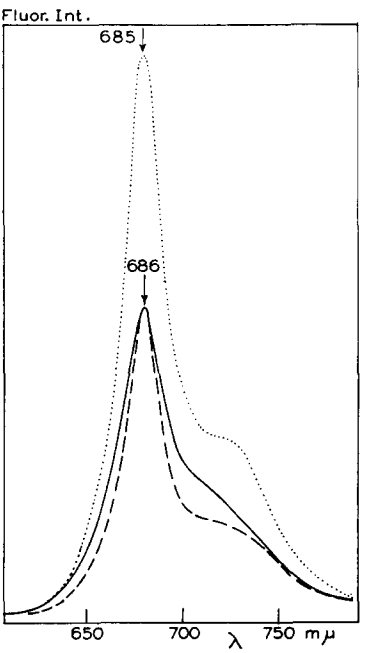


Fig. 3.

while, like the chlorophyll fluorescence changes, the phycocyanin changes were reversible during several cycles of freezing and warming. With blue-green algae such a doubling of the phycocyanin band did not occur; only a weak shoulder appeared on the short-wavelength side of the 656-m$\mu$ band. It should be remarked that the fluorescence maximum of extracted phycocyanin is located at 652 m$\mu$ at room temperature.

In Fig. 3 the effect of addition of DCMU, which reagent effectively inhibits the Hill reaction, is shown. The height of the 685-m$\mu$ band was increased by a factor of two. The percentage increase depended on the intensity of the incident light and condition of the cells. In the dashed curve of Fig. 3 the height of the 685-m$\mu$ band is adjusted to that of the main band before addition of DCMU. A marked difference occurs in the region around 700 m$\mu$.

Fig. 4 shows the fluorescence spectrum of *Chlorella vulgaris* at three different temperatures. Here also a new band arose at 718–721 m$\mu$ (depending on the sample used), which band was much more pronounced in the green than in the red or blue-green algae. The original band at 685 m$\mu$ was lowered by a factor of about two in the first stages of cooling (between −5 and −30°). A weak separate band at 696 m$\mu$ was visible, sometimes only as a shoulder on the flank of the 718-m$\mu$ band. A similar spectrum was shown by the chlorophyll *b*-deficient alga *Tribonema equale* (*cf.* ALLEN *et al.*[12]). As shown in Fig. 5, the room temperature fluorescence maximum was located at 687 m$\mu$. In the spectra of the frozen samples no separate bands at 686 and 696 m$\mu$ were observed, but a shoulder appeared on the steep slope (at about 689 m$\mu$) leading to the plateau around 695 m$\mu$.

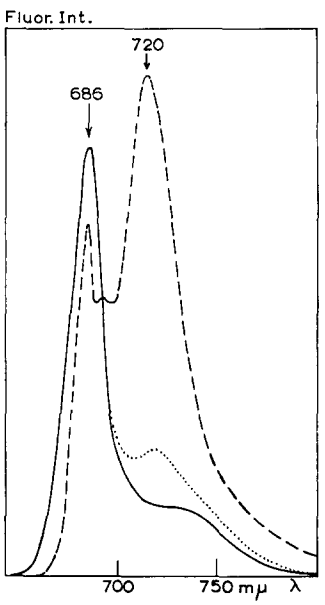


Fig. 4. Fluorescence spectrum of *Chlorella vulgaris* (4-days-old culture). Incident light: 437, 546 and 578 m$\mu$. ———, 20°; ·····, −55°; – – – –, −196°.

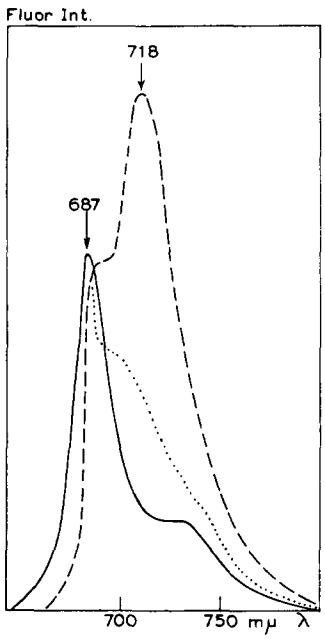


Fig. 5. Fluorescence spectrum of *Tribonema equale* (9-days-old culture). Incident light: 437, 546 and 578 m$\mu$. ———, 20°; ·····, −80°; – – – –, −196°.

It seemed interesting to compare absorption and fluorescence spectra of objects in which the red-absorption band is composed only of chlorophyll *a*. To this purpose aqueous suspensions—obtained by grinding—of Tribonema-containing chloroplasts and chloroplast fragments were compared with those of Synechococcus from which phycocyanin was eliminated by centrifugation and washing with buffer (pH 7.2).

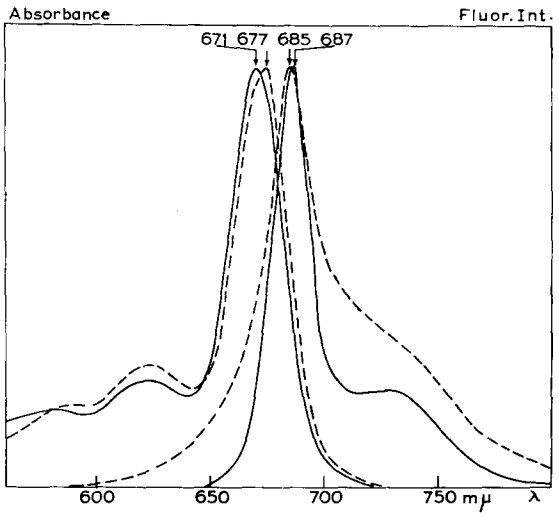


Fig. 6. Absorption spectrum of aqueous extracts of Tribonema and of Synechococcus. In Synechococcus chromatophores phycocyanin has been eliminated. The red-absorption bands of both suspensions contain only chlorophyll *a*. Fluorescence spectra of Tribonema and Synechococcus cells, excited with 437-mμ light. ———, Tribonema; – – – –, Synechococcus.

As is seen in Fig. 6, the absorption maximum of Tribonema was found to be located at 671 mμ, while that of Synechococcus occurred at 677 mμ, with a noticeable shoulder at 671 mμ. The fluorescence maximum—measured with intact cells—of Tribonema was at 687 mμ and slightly beyond that of Synechococcus (at 685 mμ). As about the same location of absorption maxima was found in intact cells, it follows that the distance between absorption and fluorescence maxima is appreciably smaller for Synechococcus than for Tribonema. The figure also shows that Synechococcus exhibits much more fluorescence in the 700-mμ region than Tribonema.

Additionally it should be remarked, that the half width of the fluorescence bands of aqueous extracts of Tribonema (210 mμ) and Synechococcus (185 mμ) were appreciably smaller than the half width of the absorption bands (about 290 mμ) of aqueous extracts of these species containing only chlorophyll *a*.

The room temperature fluorescence spectrum of the green alga *Euglena gracilis* (Fig. 7) is quite different from that of most other green algae. Especially in aged cultures, an extra band with a maximum around 705 mμ was visible. BROWN AND FRENCH[13], who also measured fluorescence spectra of these algae at room temperature, found the appearence of this band to coincide with the appearance of a marked shoulder around 695 mμ in the absorption spectrum. They located the maximum of this special fluorescence band at 705–708 mμ. As Fig. 7 shows, cooling resulted in a shift towards 717 mμ and a pronounced increase in intensity. The shoulder at about

690 m$\mu$, which at room temperature is an indication of the 685-m$\mu$ band (this band is more pronounced in young cultures) was much decreased in intensity but was still visible at liquid-$N_2$ temperature. Also, a shoulder at about 700 m$\mu$ was visible on the 717-m$\mu$ band (Fig. 7, insert).

Neither the 705-m$\mu$ room temperature band nor the 717-m$\mu$ low-temperature band showed the marked vibrational level which is so characteristic of chlorophyll fluorescence spectra. Also their half widths exceeded that of other chlorophyll bands at equivalent temperatures.

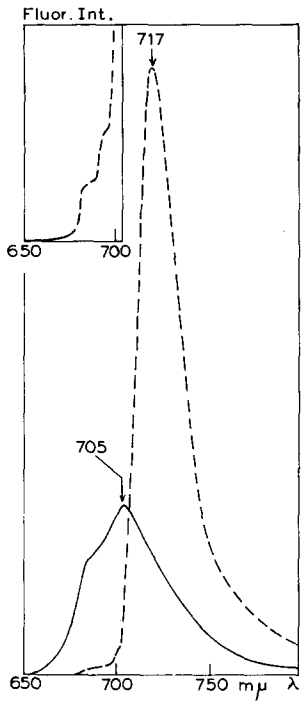


Fig. 7. Fluorescence spectrum of *Euglena gracilis* (15-days-old culture). Incident light: 437, 546 and 578 m$\mu$. ———, 20°; - - - - -, −196°. Insert: – – – –, −196°, 8 times enlarged. The room temperature fluorescence maximum at 705 m$\mu$ is enlarged and shifted to 717 m$\mu$ during cooling.

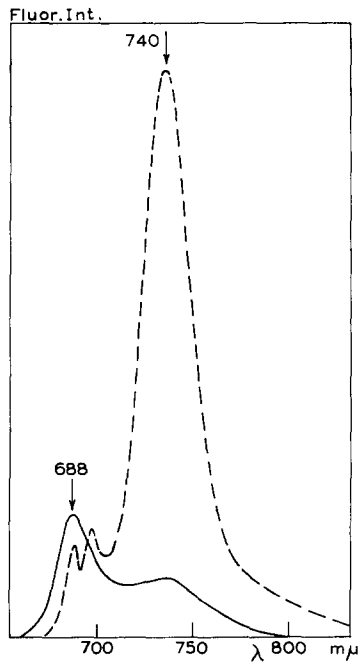


Fig. 8. Fluorescence spectrum of a thin leaf of *Adianthum caudatum*. ———, 20°; – – – –, −196°.

At room temperature, addition of DCMU resulted in an increase of the 685-m$\mu$ band, but the 705-m$\mu$ band was unchanged.

Thus, in all unicellular organisms studied, red, blue-green and green algae, an extra band at 717–721 m$\mu$ was present at liquid-$N_2$ temperature. While in the red and blue-green algae a marked band at 696 m$\mu$ was measured at low temperatures, this maximum was much less pronounced in the other species. The original single band at about 685 m$\mu$ was sharpened, often lowered in intensity but not shifted more than a few m$\mu$. The amount of 717–721-m$\mu$ fluorescence increased in aged cells.

Similar changes were measured in the fluorescence spectrum of leaves of higher plants and chloroplast preparations prepared from these leaves. Fig. 8 gives the

fluorescence spectrum of a thin leaf of the fern *Adianthum caudatum*. Reabsorption of fluorescence in leaves (*cf.* VIRGIN[14]) caused a decrease in the main fluorescence band and a slight shift towards longer wavelengths, but in this thin leaf the effect was not as marked as it is in thicker leaves. Cooling to −196° resulted in the appearance

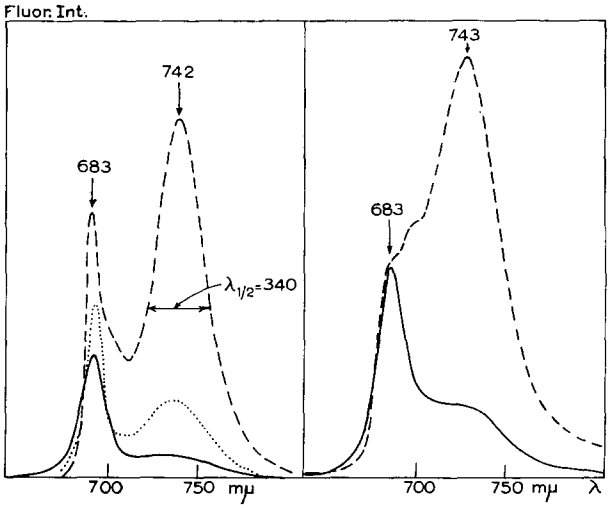


Fig. 9. Fluorescence spectra of two chloroplast preparations made of different leaves of *Aspidistra elatior*. Incident light: 437 mμ. ———, 20°; ·····, −50°; – – – –, −196°.

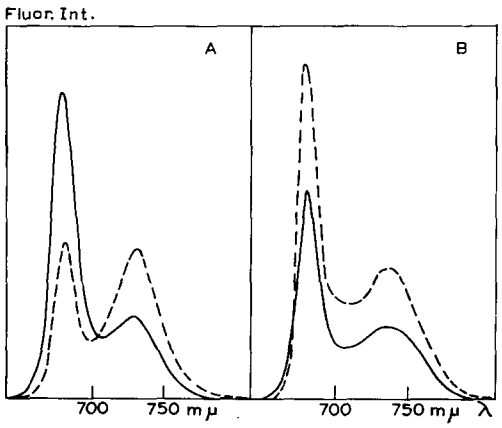


Fig. 10. Fluorescence spectrum of concentrated (3 mM) chlorophyll *a* in methanol, excited by 437-mμ light. ———, 20°; – – – –, −196°. A, thickness of layer 2 mm; B, thickness of layer 0.3 mm.

of two new bands, a weak one at 696 mμ and a strong one at 735 mμ. The original 688-mμ band again did not shift markedly, but was sharpened and decreased in intensity. Similar spectra were made of leaves of *Elodea canadensis, Spinacia oleata, Hibiscus rosa sinensis, Pelargonium zonale, Sepecio mikanoides, Marchantia polymorpha, Phaseolus vulgaris*, Liberia, Ligustrum. Some of them showed a greater amount of reabsorption of the main room temperature band. All had bands located at about 687, 696 and 735–743 mμ. With Marchantia the longest wavelength band

was located at 729 mμ. Chloroplast preparations of spinach and ligustrum leaves, made in such a concentration that no measurable reabsorption occurred, showed the same bands at liquid-$N_2$ temperature, provided freshly prepared suspensions were used.

In Fig. 9 the fluorescence spectra of chloroplast preparations made of different leaves of *Aspidistra elatior* are given. In both preparations an extra band occurred at low temperature at 742 mμ. Only one of them showed the 696-mμ fluorescence. As a result of the great separation of the remaining bands in the other, the decreased band width at low temperature of the 685-mμ band (located in Aspidistra at 683 mμ) as compared to the room temperature one, was demonstrated.

Thus fluorescence spectra of all leaves and chloroplast preparations investigated contained a predominant extra band at low temperatures at wavelengths appreciably longer than that of the unicellular algae, and all except one gave a clear, though sometimes weak, band at 696 mμ.

Fluorescence spectra were also determined of solutions of chlorophyll *a* in different concentrations. In Fig. 10B the fluorescence spectrum of a 0.3-mm layer (in bags of dialysis tubing) of concentrated purified chlorophyll *a* (3 mM) dissolved in methanol, is presented, while Fig. 10A shows the spectrum of a 2-mm layer of the same solution. It is seen that in the thin layer the intensity of both the main band and the vibrational level was increased by a factor of 1.6 as a result of cooling. In the thick layer only the 730-mμ vibrational level was increased by this factor, while the intensity of the main

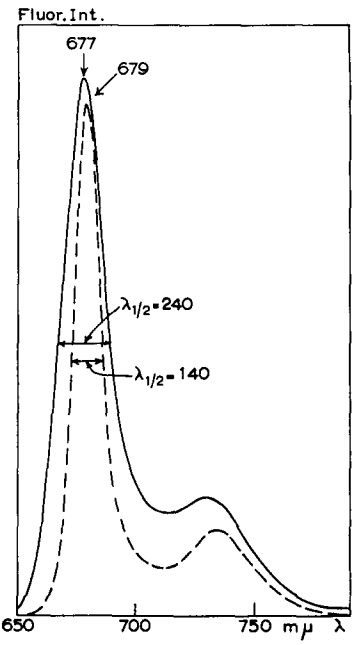


Fig. 11. Fluorescence spectrum of diluted chlorophyll *a* (2 μM) in methanol, chickness of layer 2 mm, excitation light 437 mμ; ———, 20°; – – – –, —196°. The spectrum of the cooled samples has been reduced by a factor of 1.6.

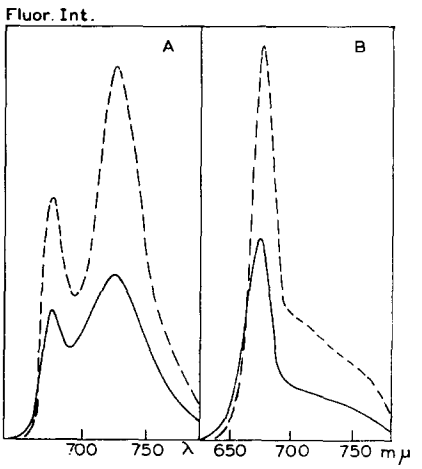


Fig. 12. Fluorescence spectrum of chlorophyll *a* adsorbed on filter paper; excitation light 437 mμ. A, high concentration; B, low concentration. ———, 20°; – – – –, —196°.

band was decreased by a factor of two. It is likely that the decrease of the main band in the thick layer of concentrated chlorophyll *a* was an artifact, caused by a strong increase in scattering as a result of freezing of the solution. As the turbidity is strongly increased, freezing results in an increase in reabsorption of scattered light, and thus of fluorescence. With diluted chlorophyll *a* (2 $\mu$M) both a 0.3-mm and a 3-mm layer yielded a fluorescence spectrum in which no new bands were found to occur. The main maximum and the vibrational level were increased by a factor of 1.6. In Fig. 11 the fluorescence spectrum of a diluted methanolic chlorophyll *a* solution before and after cooling is given.

When a "crude" solution of chlorophyll *a* was chromatographed on filter paper and the region containing chlorophyll was cut out and dried, the fluorescence spectrum of adsorbed chlorophyll gave the result shown in Fig. 12. The intensity of fluorescence was not more than a few per cent of that of dissolved chlorophyll. Possibly this fluorescence of adsorbed chlorophyll was due to traces of lipids in filter paper or solution. In Fig. 12A the spectrum shown was obtained if only two drops of concentrated chlorophyll solution were used, while the spectrum in Fig. 12B was obtained when 18 drops were used. The room temperature spectrum of Fig. 12A resembles that of a thick leaf in having a higher vibrational level than the main fluorescence band. The difference between Figs. 12A and 12B is assumed to be due to stronger reabsorption in the highly scattering filter paper of the concentrated sample. Cooling, however, did not change the conditions much and resulted in an approximately equal increase of both bands in both cases.

## DISCUSSION

DUYSENS[15], KRASNOWSKII AND BRIN[16] and others have concluded from their experiments that chlorophyll *a* in photosynthesizing cells is present in two or more forms: a form with relatively high fluorescence and one (or more) with relatively low fluorescence yield.

In the blue-green and red algae lacking chlorophyll *b* the red-absorption band was clearly asymmetric (*cf.* Fig. 6). The main maximum was located at 677 m$\mu$, while a shoulder was present at about 670 m$\mu$. Such an asymmetry seems plausibly explained by the assumption that in these algae two overlapping chlorophyll *a* forms are present with a slightly different location of absorption maximum (called here $C_a$ 670 and $C_a$ 680) and possibly a somewhat different shape of the absorption band. In these algae the amount of $C_a$ 680 exceeds that of $C_a$ 670. The asymmetric structure is not due to the presence of phycocyanin, as it is retained after removal of this pigment (*cf.* THOMAS AND MARSMAN[17]). Using derivative spectrophotometry, FRENCH[18] found that the red chlorophyll band is complex in all photosynthetic organisms studied.

If the forms $C_a$ 670 and $C_a$ 680 are chlorophyll forms with widely different fluorescence yields, the asymmetry in the fluorescence spectrum will be different from the one in the absorption spectrum and dependent on various factors. This implies that the shape of the fluorescence spectrum will depend on such factors as temperature, inhibitors, light intensity or time of measurements. As shown in Figs. 3 and 6, the fluorescence spectrum of Synechococcus measured at room temperature was asymmetric in a way different from the absorption spectrum, while its shape depended on the

addition of DCMU. Addition of this reagent, namely, resulted in an enhancement of fluorescence which was proportionally much higher in the main peak at 685 mμ than it was around 700 mμ. The room temperature fluorescence spectrum of this alga thus is, like the absorption spectrum, also composed of at least two components. The same was found to be true for Anacystis and Porphyridium. DUYSENS AND SWEERS[19] for red algae and LAVOREL[20] for Chlorella came to a similar conclusion from measurements of changes in steady-state fluorescence and induction phenomena.

The two mentioned chlorophyll components are not the only ones present in red or blue-green algae. Derivative spectrophotometry showed (*cf.* ALLEN *et al.*[12]) that in various photosynthetic organisms also a chlorophyll component with absorption maximum at about 695 mμ (sometimes at 705 mμ) is present. Usually this chlorophyll component was present in such a low concentration that it was barely or not at all visible on the curves, but in some algae, especially in aged cultures of Euglena, Porphyridium and Ochromonas, it was present in a measurable percentage of total chlorophyll concentration. Cooling the samples to liquid-$N_2$ temperature (*cf.* FREI[21]) resulted in a sharpening of the bands of the components or in the appearance of the $C_a$ 695 component in samples in which it had not been detected before; but no new components were detected in the samples studied.

As is seen in Figs. 1 and 2, cooling blue-green and red algae to liquid-$N_2$ temperature resulted in the appearance of three marked fluorescence band at 686, 696 and 717 mμ. It is tempting to attribute these fluorescence bands to each of the chlorophyll components mentioned before and to term them accordingly: $C_a$ 670–F 686, $C_a$ 680–F 696 and $C_a$ 695–F 717.

If this denomination is justified, $C_a$ 670–F 686 is a "short-wavelength chlorophyll *a*" component preferably active in the luminescence promoting "*p*" system (*cf.* GOEDHEER[22], also called System II by DUYSENS *et al.*[23], or accessory pigment system by FRENCH AND FORK[24]). This system covers about half the energy requirement of photosynthesis and performs $O_2$ production by water decomposition, thereby raising an electron from the potential of water decomposition to that required for reduction of a cytochrome. Addition of DCMU poisons this system, resulting in an increased energy flow towards fluorescence.

The chlorophyll *a* forms $C_a$ 680–F696 and $C_a$ 695–F 717 then should be weakly fluorescent at room temperature but become more fluorescent as a result of cooling. They can be the chlorophyll *a* forms preferably active in the luminescence quenching "*q*" system (GOEDHEER[22], System I of DUYSENS *et al.*[23] or long-wavelength chlorophyll system of FRENCH AND FORK[24]). Absorption by this system covers the second half of the energy requirement of photosynthesis. An electron is assumed to be raised from the potential of cytochrome oxidation to that required for reduction of NAD, or ultimately, reduction of $CO_2$. In this way absorption by this system may enhance photosynthesis (for the enhancement effect in Chlorella *cf.* EMERSON AND RABINOWITCH[25], MYERS AND GRAHAM[26]). It may also quench afterglow (GOEDHEER[22]) or fluorescence (BUTLER[27]) emitted by the first mentioned system containing $C_a$ 670–F 686. As the blue-green and red algae were found to contain a much larger amount of $C_a$ 680–F 696 than other cells, the low apparent activity of chlorophyll *a* in the action spectra of photosynthesis (HAXO AND BLINKS[28], BRODY AND EMERSON[29], AMESZ[30]), fluorescence (DUYSENS[15]) and afterglow (ARNOLD AND THOMPSON[31]) in these algae is understandable.

Justification of the denomination $C_a$ 695–F 717 finds support from the measurements with Euglena. As was shown, the 705-m$\mu$ room temperature band in the fluorescence spectrum was shifted towards 717 m$\mu$ and increased four-fold during cooling. Apart from a sharpening, the band kept its typical shape, resembling more the fluorescence spectrum of phycobilins than that of chlorophyll *a*.

The presence of the 717 m$\mu$ band at low temperatures in all algal cells measured indicates that, most probably, $C_a$ 695–F 717 is present in all these cells, though in such a low concentration that it often cannot be measured by absorption spectroscopy. In other species, as in Euglena, its concentration increases with the age of the cells.

About the nature of this chlorophyll form it should be mentioned that our measurements with chlorophyll *a in vitro* do not yield support for the chlorophyll dimer theory. In contrast to the extra low-temperature bands of intact cells and chloroplasts, the extra bands in concentrated chlorophyll *a* solutions or adsorbed chlorophyll were found to be only apparent and thus artifacts.

A simple alternative to the assumption that the 717-m$\mu$ band is due to emission of a chlorophyll dimer which fluoresces only at low temperature, is to assume that energy transfer from $C_a$ 670 and $C_a$ 680 is strongly increased as a result of structural shrinkage by excessive cooling. Energy transfer by inductive resonance is proportional to $1/r^6$—if all other parameters are kept constant—in which $r$ is the distance between pigment molecules. A slight decrease in mutual distance may bring about a great increase in energy transfer. If we assume that in most algae $C_a$ 695 does not account for more than 1 % of total chlorophyll (a value which is taken from various direct and derivative absorption spectra) a hypothetical energy increase from 0 to 100 % would increase the fluorescence of $C_a$ 695 by a factor of 100. From Figs. 4, 5 and 11 it may be inferred, that most of the fluorescence around 705 m$\mu$ at room temperature in Chlorella and Tribonema is due to the vibrational level of the main 685-m$\mu$ band, while not more than about one-fifth can be due to $C_a$ 695. Cooling thus results in an increase of $C_a$ 695 fluorescence by a factor of about 30. With Euglena fluorescence at 705 m$\mu$ at room temperature (Fig. 7) is nearly totally due to $C_a$ 695, and cooling results in an increase by only a factor of 4. Thus, if we assume that the 717-m$\mu$ band is in all algal species emitted by a same $C_a$ 695 form, the percentage increase $C_a$ 695 fluorescence is much less in Euglena than it is in Chlorella and Tribonema. This wide discrepancy cannot easily be explained by assuming that $C_a$ 695 is a kind of pigment which is weakly fluorescent at room temperature and highly fluorescent at low temperature. It seems more plausible to assume, that $C_a$ 695 shows a relatively high intrinsic fluorescence also at room temperature, but due to its low concentration n most types of algae and its wide fluorescent band no separate band in the fluorescence spectrum is measurable here.

It should be remembered that BUTLER AND NORRIS[9] found the life time of the low-temperature fluorescence band (F 717) to be about 4.5 times higher than that of the 685-m$\mu$ room temperature band. This implies, that the intrinsic fluorescence yield of the former also is higher than that of the latter, and will be nearly as high as that of chlorophyll *a in vitro*.

The hypothesis of increase in energy transfer as a result of cooling finds support in the behaviour of $C_a$ 670–F 686 during cooling. In bean leaves in the "post etiolated state" (GOEDHEER[11]) and chlorophyll *a in vitro* the fluorescence intensity increases

by 60–100 %, while in algal cells the intensity of this band decreases by a factor of about 2 (more in Euglena). It seems unlikely that *in vivo* in the low concentrations used self absorption can account for this effect as this should occur within one cell or chloroplast. It seems more likely that fluorescence is quenched by transfer to $C_a$ 695.

In young cultures of blue-green and red algae the 717-m$\mu$ band is not as marked as it is in aged cultures. In the young cultures probably the number of $C_a$ 695 molecules is too small to act as a very efficient energy trap even at liquid-$N_2$ temperature. Of course energy transfer from $C_a$ 670 and $C_a$ 680 to $C_a$ 695 can never be a full 100 %, otherwise the fluorescence bands at 686 and 696 m$\mu$ would not be visible. The ratio of intensities of the three bands at liquid-$N_2$ temperature suggests, that energy is transferred more easily from $C_a$ 670 to $C_a$ 695 than it is from $C_a$ 680. This is probably due to the low intrinsic fluorescence yield of the latter.

The results with green leaves and chloroplast preparations did not deviate essentially from those with algal cells. At low temperatures also three bands were present in the fluorescence spectrum. The main low-temperature band, however, was located at longer wavelengths, but it was of similar shape. The absorption band corresponding to this 740-m$\mu$ fluorescence band was probably located at a longer wavelength: we thus will write this tentitatively $C_a$ 705–F 740.

The other two bands were located within close distance of their position in algal cells. As in most algal species, $C_a$ 705 does not comprise much more than about 1 % of total chlorophyll, while its amount also is increased in aged leaves.

Summarising, our picture leads to the following scheme: $C_a$ 670–F 686, about 50 % of chlorophyll, moderate fluorescence yield; $C_a$ 680–F 696, about 50 % of chlorophyll, low fluorescence yield; $C_a$ 695–F 695, about 1 % of chlorophyll, high fluorescence yield.

The percentages, of course, are only rough approximations. They may vary from species to species and within one species with age and growth condition of the cells.

If the above picture holds and little energy transfer occurs at room temperature between $C_a$ 680 and $C_a$ 695, the value of the latter as an energy trap in the "*q*" system (System I) is doubtful. The occurrence of a band around 700 m$\mu$ in the action spectra of photosynthetic enhancement (*cf.* EMERSON AND RABINOWITCH[25], MYERS AND GRAHAM[26]) or quenching of afterglow (*cf.* GOEDHEER[22]) need not be an indication of participation of $C_a$ 695 in these processes, as such bands can as well be due to the ratio of effective absorption of $C_a$ 670 to $C_a$ 680 in the enhancing (quenching) light. In such a case the location of the maximum of the action spectrum of the quenching (enhancing) effect may depend on external factors, such as temperature. With the luminescence quenching effect this indeed was found to occur.

It should be mentioned, however, that the chlorophyll *a* system *in vivo* may be more complex and comprise more components than the ones mentioned (*cf. e.g.* THOMAS[32]). As the fluorescence spectra of the samples investigated here did not give more than three components even with very narrow slits, the most simple system was chosen.

## REFERENCES

1 S. S. BRODY, *Science*, 128 (1958) 838.
2 S. S. BRODY AND M. BRODY, *Trans. Faraday Soc.*, 58 (1962) 416.
3 S. S. BRODY AND M. BRODY, *Biochim. Biophys. Acta*, 54 (1962) 495.
4 W. L. BUTLER, *Arch. Biochem. Biophys.*, 93 (1961) 413.

[5] S. S. BRODY AND M. BRODY, *Arch. Biochem. Biophys.*, 95 (1961) 413.
[6] B. KOK, *Acta Botan. Neerl.*, 6 (1957) 316.
[7] B. KOK AND G. HOCH, in W. D. MC. ELROY AND B. GLASS, *Light and Life*, Johns Hopkins Press, Baltimore, 1961, p. 397.
[8] C. S. FRENCH, in W. RUHLAND, *Handbuch der Pflanzenphysiologie*, Vol. 5, Springer Verlag, Berlin, 1960, p. 252.
[9] W. L. BUTLER AND K. H. NORRIS, *Biochim. Biophys. Acta*, 66 (1963) 72.
[10] S. S. BRODY AND S. B. BROYDE, *Nature*, 199 (1963) 1097.
[11] J. C. GOEDHEER, *Biochim. Biophys. Acta*, 53 (1961) 420.
[12] M. B. ALLEN, C. S. FRENCH AND J. S. BROWN, in M. B. ALLEN, *Comparative Biochemistry of Photoreactive Systems*, Academic Press, New York, 1960, 33.
[13] J. S. BROWN AND C. S. FRENCH, *Biophys. J.*, 1 (1961) 539.
[14] H. I. VIRGIN, *Physiol. Plantarum*, 7 (1954) 560.
[15] L. N. M. DUYSENS, *Thesis*, University of Utrecht, 1952.
[16] K. K. KRASNOWSKII AND G. P. BRIN, *Doklady Akad. Nauk SSSR*, 63 (1948) 163.
[17] J. B. THOMAS AND J. W. MARSMAN, *Biochim. Biophys. Acta*, 35 (1959) 31.
[18] C. S. FRENCH, *Biol. Colloq. Photobiology*, Oregon State College, Corvallis, 1959, p. 52.
[19] L. N. M. DUYSENS AND H. E. SWEERS, *Microalgae and Photosynthetic bacteria*, special issue of *Plant Cell Physiol.*, (1963) 353.
[20] J. LAVOREL, *Biochim. Biophys. Acta*, 60 (1962) 510.
[21] Y. E. FREI, *Biochim. Biophys. Acta*, 57 (1962) 82.
[22] J. C. GOEDHEER, *Biochim. Biophys. Acta*, 64 (1962) 294.
[23] L. N. M. DUYSENS, J. AMESZ AND B. M. KAMP, *Nature*, 190 (1961) 510.
[24] C. S. FRENCH AND D. C. FORK, *Carnegie Inst. Wash. Yearbook*, 60 (1961) 357.
[25] R. EMERSON AND E. I. RABINOWITCH, *Plant Physiol.*, 35 (1960) 477.
[26] J. MYERS AND J. R. GRAHAM, *Plant Physiol.*, 38 (1963) 105.
[27] W. L. BUTLER, *Biochim. Biophys. Acta*, 66 (1963) 273.
[28] F. T. HAXO AND L. R. BLINKS, *J. Gen. Physiol.*, 33 (1950) 389.
[29] M. BRODY AND R. EMERSON, *J. Gen. Physiol.*, 43 (1959) 251.
[30] J. AMESZ, *Thesis*, University of Leiden, 1964.
[31] W. ARNOLD AND J. THOMPSON, *J. Gen. Physiol.*, 39 (1956) 311.
[32] J. B. THOMAS, *Biochim. Biophys. Acta*, 59 (1962) 202.