SPECTRAL STUDIES OF A CHLOROPHYLL PIGMENT WITH FLUORESCENCE MAXIMUM AT 698 mμ

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ABSTRACT A chlorophyll type pigment (F698) fluorescing maximally at 698 mμ at 77°K has been observed in preparations of chlorophyll. This fluorescence is quenched by small amounts of naturally occurring materials, including plastoquinone and the ubiquinones, and by nitrobenzene, probably by formation of a nonfluorescent complex. Fluorescence quenching does not occur in the presence of carotenes, xanthophylls, or reduced plastoquinone and ubiquinone. The fluorescence is sharply temperature dependent, with a steep rise in intensity occurring at 165°K. At 77°K the fluorescence yield is between 0.8 and 1.0. The red absorption maximum of the pigment is at 675 mμ at room temperature and at 688 mμ at 77°K. In vivo, a low temperature emission is also observed at 698 mμ, and this fluorescence is quenched by nitrobenzene. It is proposed that the pigment found in vitro is also the one responsible for emission at 698 mμ in vivo. A reaction of F698 with plastoquinone is suggested as the primary photochemical step in system II of photosynthesis.

INTRODUCTION

There have been several recent observations of a fluorescence band with maximum in the vicinity of 698 mμ in photosynthetic organisms, at 77°K (1–5) and at room temperature (6). It has been speculated that this emission emanates from a form of chlorophyll *a* (1, 3, 5). Furthermore, several workers (2, 4) have proposed that this pigment participates in the primary photochemistry of photosynthesis. The preparation in pigment extracts of a material with similar spectral properties is therefore of particular interest. In this paper is reported the occurrence of a species with fluorescence maximum at 698 mμ, as well as the spectral properties of this species. The 698 mμ pigment was detected by fluorescence and absorption spectroscopy (7, 8) as a minor constituent in solutions of chlorophyll. We will refer to this material, both in vivo and in vitro, as F698.

EXPERIMENTAL

Materials. Chlorophyll was prepared from spinach. Most of the yellow and orange pigments were removed from the crude acetone extracts by chromatography on polyethylene, according to the method of Anderson and Calvin (9). The chlorophylls were then separated by sugar chromatography, using the method of Jacobs, Vatter, and Holt (10). To remove quinone impurities prior to sugar chromatography, aliquots of 0.5 g of chlorophyll were adsorbed on diatomaceous earth and washed with 5 gal of petroleum ether. Finally chlorophyll *a* was again adsorbed on sugar and washed with 2 l of isooctane. The pigment was then crystallized over water. F698 can sometimes be found in the chlorophyll *a* fraction; more often, it occurs in the chlorophyll *b* band. A sample of chlorophyll *a* unusually rich in F698 (about 6% by weight) was obtained in one preparation, and this sample was employed for most of the quantitative studies reported here.

Chlorella were grown autotropically. Young, actively growing *Chlorella* (1 to 2 days in the light) were required for the 698 mμ fluorescence to be evident.

Coenzyme Q_6, Q_{10} and alpha tocopherol were obtained from Sigma Chemical Co., St. Louis, Missouri. Plastoquinone was the generous gift of Dr. Norman I. Bishop of Oregon State University.

All other materials were obtained reagent grade from Fisher Scientific Company, New York, New York.

Procedures. All absorption spectra were obtained on a Cary model 14 recording spectrophotometer. Low temperature absorption spectra were determined in a specially designed Dewar flask (Scientific Glass Company, Bloomfield, New Jersey) which had optical windows and was of appropriate size to fit into the cell compartment of the Cary. A cuvette with a long stem was inserted into the Dewar, which was filled with dry ice–acetone mixture or liquid nitrogen. Clouding of the Dewar windows due to condensation of water vapor was not of any consequence in the short time required to run a spectrum.

The instrument used to record fluorescence spectra has been described previously (11). Briefly, a 2000 watt Xenon source is used in conjunction with a monochromator to provide monochromatic illumination of the sample. The emission spectrum of the sample is analyzed with a second monochromator and detected by a red-sensitive multiplier phototube (Dumont No. 6911). The output of the phototube is recorded on a Varian type G-11 recorder. The sample is contained in Kimax melting point tubes (1 mm diameter) and placed at the bottom of a Dewar which has an optically clear flat bottom. Liquid nitrogen is introduced into the Dewar when low temperature spectra are desired. Distortions of the spectra due to self-absorption are minimized by virture of the short optical path length of the capillary. Chlorophyll fluorescence was excited with blue light of wavelength 436 mμ. Fluorescence spectra given in this paper were determined with acetone solutions. Since the fluorescence intensity of F698 in acetone was found to decrease on standing, spectra were examined immediately after preparation.

In order to study the temperature dependence of fluorescence intensity, a copper-constantan thermocouple was immersed into the chlorophyll solution being exam-

ined. The thermocouple was calibrated at room temperature and at 77°K (with liquid nitrogen). The sample, contained in a melting point tube or a 5ml beaker, was placed in the Dewar usually employed for fluorescence studies. The fluorescence monochromator was set at 698 mμ and intensity was recorded as a function of temperature. The temperature was varied by cooling the sample down stepwise, adding small aliquots of liquid nitrogen; after the sample reached 77°K, the fluorescence was again monitored as the sample warmed up to room temperature.

To prepare choloroplasts, *Chlorella* cells were ruptured by sonication (M.S.E. Sonerator 60 watt) at 20 kc/sec, for 10 min, while immersed in an ice bath. Unbroken material and large particles were then spun down at 2000 RPM for 2 min, in a clinical centrifuge. Then a pellet was prepared by centrifugation in a refrigerated ultracentrifuge (Spinco Model L-2) at an average centrifugal force of 13,000 *g* for 10 min.

A phosphoroscope was devised in order to examine the lifetime of the 698 mμ emission. A high pressure 1000 watt mercury lamp (Capillary type GE A-H6) operated on dc served as the light source. The lamp output was passed through a condensing lens and a blue filter (Corning 5-58). A Kerr cell filled with nitrobenzene and fitted with crossed polaroids at either end served as a high speed shutter. Samples, contained in a 5 ml Pyrex beaker with an optically clear bottom, were placed in a Dewar similar to the one described above for fluorescence. Liquid nitrogen served as the refrigerant. The emitted light passed through a deep red filter (Schott RG-9-1) and an interference filter with maximum transmission at 698 mμ, onto a red-sensitive multiplier phototube (Dumont No. 6911) the output of which was displayed on an oscilloscope. The latter was triggered by the air gap discharge of the Kerr cell, so that only the time course of the fluorescence delay was traced. A polaroid camera was employed to photograph the oscilloscope tracings. Since the over-all response time of the instrument was 10^{-7} sec, lifetimes longer than these could be determined on this instrument.

For the fluorescence quenching studies, relative emission intensities were obtained from fluorescence spectra. The intensity at 698 mμ was corrected for the contribution by chlorophyll itself at that wavelength, from the following equation:

$$[F698, \text{corrected}] = [F698, \text{measured}] - 0.16\,[F673, \text{measured}],$$

where the intensity observed at 698 mμ is [F698, measured] and [F673, measured] is the fluorescence intensity observed at 673 mμ. The fraction that chlorophyll *a* emits at 698 mμ relative to its main fluorescence band is 0.16 in acetone. The contribution of F698 to the fluorescence intensity at 673 mμ is neglected.

RESULTS

In Solution.

Fluorescence spectra. Low temperature fluorescence spectra and quantitative studies were carried out in acetone solutions, since it was found that the emis-

sion intensity of F698 was highest in this solvent. Other solvents wherein F698 fluorescence was observed are pyridine, ethyl ether, and ethanol.

The fluorescence spectrum, at room temperature, of a 2×10^{-5} M solution of chlorophyll *a* containing F698, in acetone, is shown in Fig. 1, curve I. The maxima are at 667 and 720 mμ, as usual. On cooling to 77°K, the 698 mμ band appears (curve II) and the other two peaks shift to 673 and 725 mμ. Curve III shows the fluorescence spectrum at 77°K of an acetone (2×10^{-6} M) solution of chlorophyll

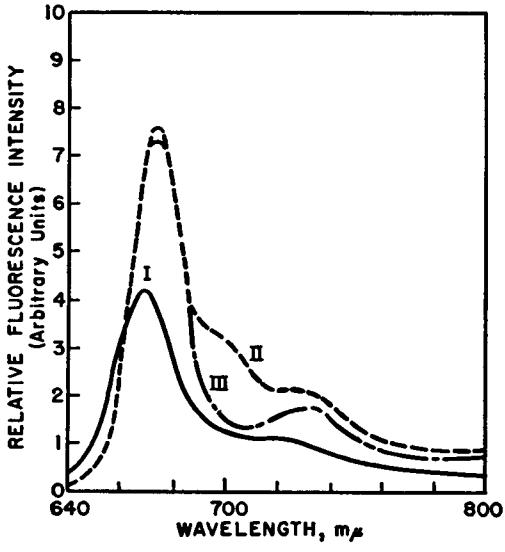

FIGURE 1 Curve I: Fluorescence spectrum of chlorophyll *a* (2×10^{-5} moles/l in acetone) containing F698, at room temperature. Curve II: Same sample as curve I, at 77°K. Curve III: Fluorescence spectrum of chlorophyll *a* (2×10^{-6} moles/l in acetone) free of F698, at 77°K.

a free of F698. The difference between curves II and III, the fluorescence spectrum of F698, is shown in Fig. 2. F698 may fluoresce weakly at room temperature; however, such fluorescence was not detected in our samples, which contain only a few per cent of this pigment.

In the absence of F698, the fluorescence spectrum of chlorophyll *b* at 77°K has a major band at about 652 mμ and a minor band at 705 mμ, the latter being about ⅓ as intense as the main band. When F698 is present, the 705 mμ band is not resolved. Instead, a fluorescence peak, which is frequently almost as intense as the main band, is seen at 698 mμ. In addition, there is some evidence of chlorophyll *a* fluorescence, since chlorophyll *b* which has been chromatogaphed only once is not free of the *a* component. The 698 mμ emission is eliminated when the sample is chromatographed three or four additional times, demonstrating that the 698 mμ fluorescence emanates from a separate constituent.

Absorption spectra. At room temperature, the absorption spectrum of chlorophyll *a* or *b* solutions which fluoresce at 698 mμ seem identical with those whose fluorescence spectrum does not show this emission. However, by employing

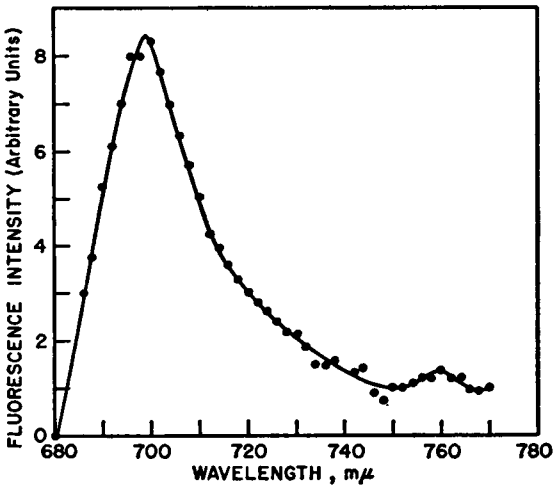

FIGURE 2 Fluorescence spectrum of F698 in acetone at 77°K. Obtained by taking the difference between curve III and curve II, in Fig. 1.

the sensitive method of difference spectroscopy, the presence of another pigment at room temperature is revealed. For this purpose, an acetone solution of chlorophyll *a* containing F698 is employed as the sample and a cholorophyll *a* solution with 2-peaked emission spectrum is placed in the reference beam of the spectrophotometer, both solutions being adjusted to the same optical densities at the red absorption maximum. Fig. 3 shows the difference spectrum obtained. The major

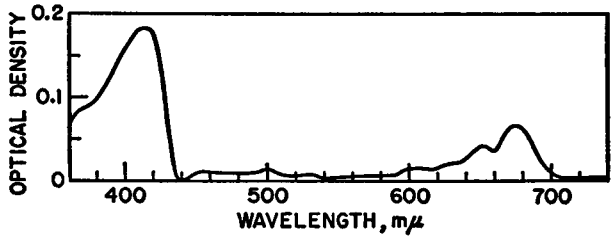

FIGURE 3 Absorption spectrum of F698 at room temperature in acetone, obtained by difference spectroscopy, using as the sample chlorophyll *a* containing F698 and as the reference chlorophyll *a* free of F698. The chlorophyll *a* concentration was 2.2×10^{-5} moles/l.

absorption maxima in the difference spectrum for F698 occur at 675 and 415 mμ. For chlorophyll *a* in acetone, the maxima are at 661 and 431 mμ.

The red absorption band of F698 can also be observed directly in solutions of chlorophyll *a* or *b* at low temperature, where the chlorophyll absorption bands are sharpened. At the temperature of dry ice–acetone mixture (−77°C) a band at

about 688 mμ is resolved in solutions of chlorophyll *b* containing F698 (Fig. 4). Similarly, a shoulder occurs at 688 mμ in cholorophyll *a* solutions containing F698 at −77°C. Thus, on cooling the red absorption band of F698 is shifted about 13 mμ toward longer wavelengths, while the red band of chlorophyll *a* only shifts 4 mμ.

Since fluorescence quenching (see below) indicated the occurrence of a complex between F698 and the isoprenoid quinones, the absorption spectrum of the pigment in the presence of coenzyme Q_{10} was examined, by the difference technique, to

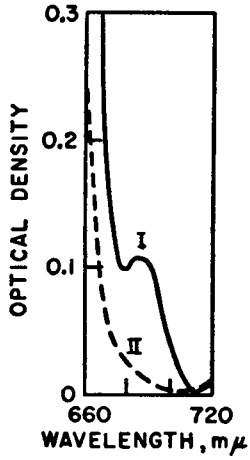

FIGURE 4 Curve I: Absorption spectrum of an acetone solution of chlorophyll *b* containing F698, at −77°C. Optical density 4.8 at 644 mμ, path length 3 cm. Curve II: Absorption spectrum of an acetone solution of chlorophyll *b* free of F698 at −77°C. Optical density 4.8 at 644 mμ, path length 3 cm.

determine if a change in absorption accompanies the formation of the complex. The spectrum was identical with that obtained in the absence of the quinone. The formation of this complex is therefore not manifested by an absorption shift.

To estimate the molar absorption coefficient of F698 the concentration of chlorophyll in solution was determined in two ways. First, 4×10^{-5} g of freshly purified and dried crystalline chlorophyll *a* containing F698 were weighed and dissolved in 5 ml of acetone. Assuming the same molecular weight for F698 and chlorophyll *a*, the concentration is 8.96×10^{-6}M. Then the absorption spectrum of this solution was determined in a 1 cm path length cell. The following simultaneous equations are solved:

$$OD_1 = C_A E_{A,1} + C_{F698} E_{F698,1}$$

$$OD_2 = C_A E_{A,2} + C_{F698} E_{F698,2} ,$$

where the E's are the molar absorption coefficients, the subscripts 1 and 2 refer to observations at 661 mμ and 675 mμ, respectively, and C_A and C_{F698} are molar concentrations of chlorophyll *a* and F698, respectively. The value of $OD_1 = 0.64$; taking $E_{A,1} = 7.66 \times 10^4$ (12) and assuming $C_{F698} E_{F698,1} = 0$, the value of C_A is calculated to be 8.41×10^{-6}M. The difference between the concentrations determined by these two methods, which is 0.55×10^{-6} M (or 6.1% by weight), is

taken as the concentration of F698 in solution. The optical density at the red absorption maximum of F698 at 675 mμ was 0.027 as obtained from difference spectra (i.e. $C_{F698}E_{F698,2} = 0.027 = OD_2 - (OD_1E_{A,2}/E_{A,1})$), and the molar absorption coefficient is estimated to be $5 \pm 1.5 \times 10^4$ 1/M.

Fluorescence yield. A combination of absorption and fluorescence data enables us to estimate the quantum yield of fluorescence at 698 mμ. From the relation $I = \varepsilon C\Phi$, (13) where I represents the fluorescence intensity at the emission maximum, εC the optical density at the exciting wavelengths (436 mμ) in a unit path length cell, and Φ the quantum yield of fluorescence, we obtain for a two pigment system:

$$\Phi_2 = \frac{I_2\epsilon_1C_1\Phi_1}{I_1\epsilon_2C_2} = \Phi_1 \frac{I_2}{I_1} \frac{[OD_1(436)]}{[OD_2(436)]}$$

assuming that the intensity at the fluorescence maximum is proportional to the integral under the emission band, and that the integrals for chlorophyll *a* and F698 (pigments 1 and 2, respectively), are the same. The fluorescence spectrum of a 1.4×10^{-6} M acetone solution of chlorophyll *a* containing F698 was obtained at 77°K. The ratio of the fluorescence intensities at the emission maxima, I_{698}/I_{673}, was 0.087. (This value was obtained for a very dilute sample -1.4×10^{-6} moles/l of chlorophyll *a* in a 1 mm capillary cell. At higher concentrations, self-absorption of the chlorophyll *a* fluorescence distorts the spectrum, making the 698 mμ band more prominent.) The ratio of the optical densities of chlorophyll *a* to F698 at 436 mμ, the exciting wavelength, was estimated from absorption difference data to be at least 16. (Since the absorption of F698 is minimal at 436 mμ, only a limit could be obtained for this ratio; furthermore the ratio could not be greater than 23, otherwise $\Phi_2 > 1$.) At 77°K, the fluorescence quantum yield of chlorophyll *a* in acetone is 1.7 times greater than the room temperature value of 0.32 (14), which is equal to 0.54. With this information, the fluorescence yield Φ_2 of F698 is estimated to be between 0.8 – 1.0.

Measured fluorescence lifetime. To determine if the 698 mμ emission originated from a singlet or a metastable state, the emission, at 77°K, was examined using a Kerr cell phosphoroscope. A 10^{-5} M acetone solution of chlorophyll *a* which manifested the 698 mμ band was studied. The lifetime of the excited state of F698 proved to be less than 10^{-7} sec, as no emission of longer duration than this was detected by our instrument.

Fluorescence quenching, qualitative results. The fluorescence at 698 mμ in chlorophyll solutions is selectively quenched by certain natural substances present in the crude pigment extract, as evidenced by the fact that the fluorescence of F698 is not observed before washing with petroleum ether. Adding back the washings quenches the fluorescence. The fluorescence is also quenched by a material present in the orange xanthophyll fraction of the polyethylene column. In order to identify the natural quencher, the petroleum ether washings were chromatographed on sugar to

free them of small amounts of chlorophyll. The chlorophyll remained adsorbed on the sugar and a pale yellow eluate passed through the column. This yellow substance had an intense ultraviolet absorption band with maximum at 260 mμ in petroleum ether. A class of compounds possessing similar spectral properties and found in plants are the isoprenoid quinones. The isoprenoid quinones are also similar to the natural quencher in that they are extractable with petroleum ether and chromatograph on sugar in the same manner (16). Furthermore the addition of small amounts of such quinones (coenzyme Q6 and Q10, plastoquinone) to chlorophyll solutions fluorescing at 698 mμ quenches the 698 mμ band (see Fig. 5). Alpha tocopherol, another

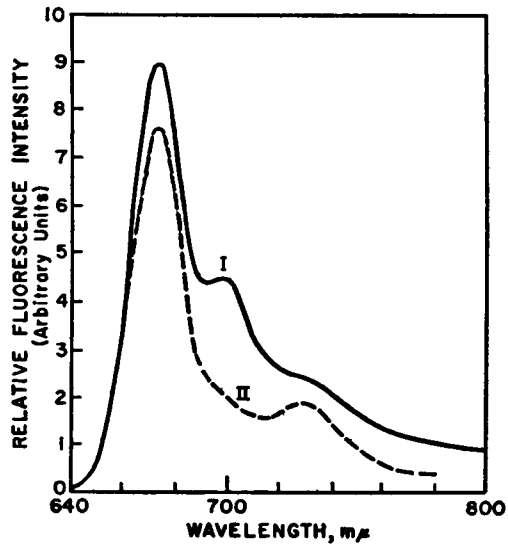

FIGURE 5 Curve I: Fluorescence spectrum of chlorophyll *a* (2.4×10^{-5} moles/l in acetone) containing F698, at 77°K. Curve II: Same sample as curve I, after the addition of 1.6×10^{-5} moles/l of coenzyme Q_6.

natural isoprenoid material, also quenches the fluorescence at 698 mμ. Synthetic substance which are efficient quenchers include nitrobenzene and p-phenylenediamine hydrochloride.

Of particular interest is our observation that reduced plastoquinone does not quench fluorescence of F698. In addition, commercial preparation of vitamin K, beta carotene and alpha carotene, also did not act as quenchers, and xanthophylls which we extracted from spinach were similarly inactive.

Quenching quantitative results. The intensity of the 698 mμ fluorescence was studied quantitatively at 77°K, as a function of concentration of nitrobenzene, coenzyme Q_6, and plastoquinone. Acetone solutions of chlorophyll *a* containing F698 were used for this purpose. Quenching follows the Stern-Vollmer relationship in the concentration ranges investigated: up to 10^{-4} moles/l for nitrobenzene, 1.5×10^{-6} moles/l for coenzyme Q_6 (see Fig. 6), and 5.6×10^{-6} moles/l for plastoquinone. (The F698 concentration was estimated to be 1.5×10^{-6} moles/l,

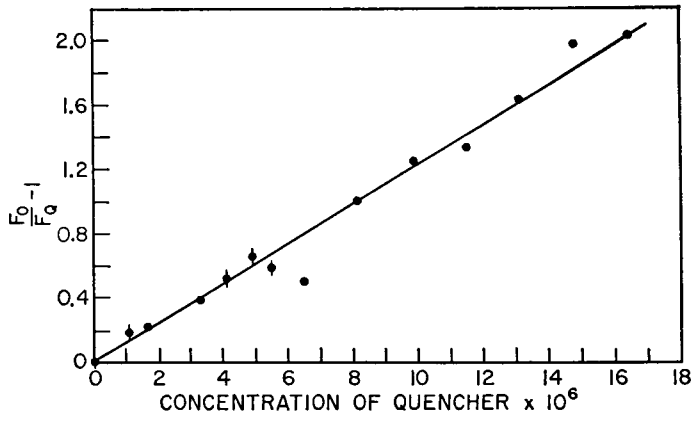

FIGURE 6 Stern-Volmer plot for fluorescence quenching of F698, at 77°K. F_0 is the fluorescence intensity at 698 mμ in the absence of quencher. F_Q is the intensity after the addition of quencher. Data for quenching by coenzyme Q_6 depicted by solid circles. Data for quenching by plastoquinone depicted by solid circles with lines.

from absorption data.) Quenching constants obtained were 8.6×10^2 l/mole for nitrobenzene and 1.2×10^5 l/mole for both coenzyme Q_6 and plastoquinone. In the range of concentrations used it was observed that nitrobenzene slightly enhances the fluorescence of chlorophyll *a* and shifts the emission maximum several mμ to shorter wavelengths, and that plastoquinone also quenches the fluorescence of chlorophyll *a*.

Temperature dependence of fluorescence of F698. The temperature dependence of F698 emission was examined in a 1.8×10^{-4} M solution of chlorophyll *a* containing F698 (concentration of F698 estimated at about 10^{-5} moles/l, by absorption difference spectroscopy). A plot of relative fluorescence intensity at 698 mμ as a function of temperature is shown in Fig. 7. The onset of fluorescence occurs at about −90°C and the intensity rises rather steeply over a temperature range of 60°. Below −150°C, the intensity increases only slightly. (A similar temperature dependence was recently described by Goedheer (5) for an emission which he detected at 696 mμ in vivo in *Synechococcus*.)

Spectral properties of other pigments. Pheophytin *a* was found to absorb maximally in acetone at 409 and 667 mμ, and to fluoresce strongly both at room temperature and at 77°K, with maxima at 673 and 691 mμ, respectively. Magnesium-purpurin 7-trimethyl ester (allomerized chlorophyll *a*, fraction 1) fluoresces only very feebly at room temperature in acetone. At 77°K it manifests an intense emission with maximum at 698 mμ, and this fluorescence is susceptible to quenching by nitrobenzene and coenzyme Q_{10}. Mixing samples of chlorophyll *a* and *b* which do not contain F698 does not produce the 698 mμ band.

In vivo fluorescence spectra. An emission band with maximum at about

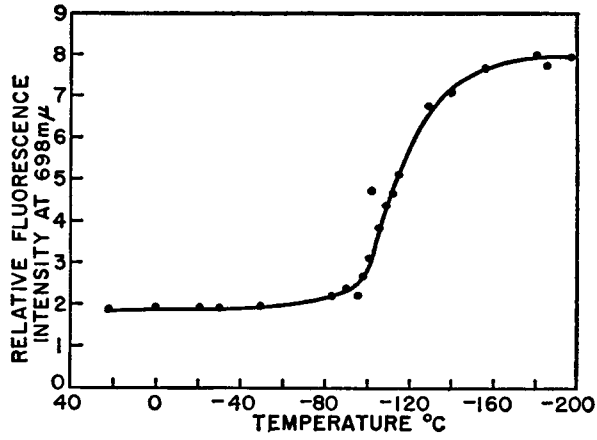

FIGURE 7 Temperature dependence of fluorescence at 698 mμ from an acetone solution of chlorophyll *a* containing F698.

698 mμ, at 77°K, is observed in young organisms, and chloroplast fragments. We have been able to partially isolate the 698 mμ fluorescent species by fractional centrifugation. The fluorescence spectrum of an aqueous suspension of a pellet prepared from sonicated *Chlorella* cells is shown in Fig. 8, curve I. The emission maximum is at 704 mμ. When the pellet was resuspended in water saturated with nitrobenzene, the fluorescence spectrum (curve II) showed a minimum at 698 mμ. Nitrobenzene apparently quenches the 698 mμ fluorescence in vivo as it does in vitro. There was no evidence that the nitrobenzene-water solution extracted any chlorophyll.

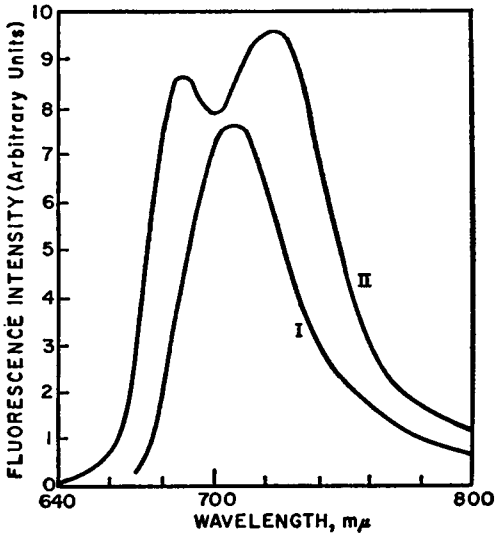

FIGURE 8 Curve I: Fluorescence spectrum of *Chlorella* fragments in culture medium, at 77°K. Pellet prepared at 13,000 *g*. Curve II: Same sample as curve I, resuspended in medium saturated with nitrobenzene (1.54×10^{-3} moles/l).

Similar attempts to quench this fluorescence in vivo by addition of coenzyme Q, plastoquinone, and alpha tocopherol have not as yet been successful. The quinones were introduced into the aqueous suspensions of the sonicated cell in small volumes of ethanol or in petroleum ether (which was subsequently removed with a stream of nitrogen).

In young, light green spinach, emission from F698 is much more intense than that from monomeric chlorophyll at 685μ (see Fig 9). As greening progresses, the

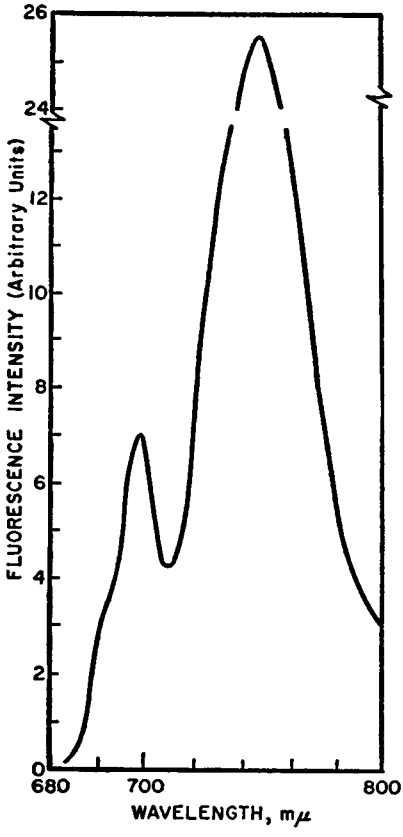

FIGURE 9 Fluorescence spectrum of early spring spinach leaf, at 77°K.

relative intensity of 698 mμ fluorescence to fluorescence at 685 mμ (monomer) and 735 mμ (aggregate) decreases, until in dark green leaves F698 is no longer detectable. A similar situation with respect to greening is observed with *Chlorella* and *Anacystis*.

DISCUSSION

We have found that solutions of chlorophyll *a* or *b*, which are apparently pure as judged from their absorption spectra, contain small amounts of another pigment

whose fluorescence is very readily quenched by naturally occurring quinones. Originally (7), the susceptibility to quenching and the temperature dependence of the 698 mμ fluorescence led us to believe that the band emanated from a metastable state of chlorophyll *a*. However, lifetime measurements ruled out this possibility. That F698 is a mixed dimer of chlorophyll *a* and *b* was also considered and ruled out, since mixing solutions of chlorophyll *a* and *b* which did not fluoresce at 698 mμ did not produce the 698 mμ band. Since the 698 mμ band is eliminated by repeated chromatography, the presence of an additional constituent, not an equilibrium product, was indicated. The pigment is poorly separated from the chlorophylls by sugar chromatography with isopropanol in petroleum ether, and we are currently developing chromatographic methods to obtain it pure. The possibility that F698 is a decomposition product produced during the extraction or purification procedure cannot be ruled out (for this or any other minor constituent). However the similarities of its properties to those of a pigment fluorescing at 698 mμ in vivo are suggestive. In particular, the steep temperature dependence of the fluorescence over a fairly short temperature range is unusual, since fluorescence intensity generally increases as a continuous inverse function of temperature. A possible interpretation of the temperature dependence of F698 fluorescence is that at room temperature intersystem crossing between the excited singlet state and the triplet level occurs very readily, excitation energy being trapped in the metastable state, thereby precluding fluorescence. At the temperature where fluorescence ensues, the vibrational level at which the cross-over point occurs may no longer be populated.

The selective quenching of fluorescence of F698 by nitrobenzene, both in vivo and in vitro further supports the view that the F698 pigment found in solutions is the same as that occurring in vivo. The identity of F698 in solution cannot be determined until the material is obtained pure. However we can eliminate several possibilities. It is not a form of chlorophyll *a* whose absorption and fluorescence spectra are distorted by complexing with protein, as has been suggested (1, 5) for F698 in vivo, since it occurs in protein-free solutions. It can also be concluded that it is not pheophytin, since the room temperature absorption maxima of F698 do not correspond with those of pheophytin. Also, the fluorescence properties of the two pigments are dissimilar both at room temperature and at 77°K.

In our search for a chlorophyll type pigment with spectral properties similar to those of F698 we examined Mg-purpurin 7-trimethyl ester. This pigment, which is fraction 1 of allomerized chlorophyll, has visible absorption maxima in ethyl ether at 420 and 670 mμ (17). Its fluorescence and quenching properties are not unlike those of F698 both at room and liquid nitrogen temperature. Whether F698 is a pigment similar to this purpurin or whether the similarity in the spectra of the two species is coincidental remains to be determined. Michel-Wolwertz and Sironval (18) have also found a form of allomerized chlorophyll in pigment extracts. Gould, Kuntz, and Calvin (19) have reported the preparation of a chlorophyll-like pig-

ment, an oxidation product of bacteriochlorophyll, with absorption in the vicinity of 680 mμ.

The fact that F698 fluorescence is quenched by plastoquinone is of interest, since plastoquinone is involved in the electron transport chain of photosynthesis (16). An understanding of the quenching mechanism could be pertinent to the reactions of plastoquinones in vivo. Fluorescence quenching may be by kinetic encounters between the quencher and the dye, which dissipate the excitation energy as heat, or by the formation of nonfluorescent complex. In the rigid system studied here, diffusion controlled encounters are ruled out. However, dynamic quenching, resulting from repeated interactions between dye and quencher frozen together in a "cage" must be considered in its stead. In order to ascertain if this mechanism is the origin of quenching in our case, an estimate of the average distance between molecules was made. For a statistically uniform distribution of molecules in solution, the distance between molecules ($2R_0$) is obtained from the equation

$$R_0^3 = \frac{2}{\sqrt{\pi}} \cdot \frac{3}{4\pi N' C_0},$$

where N' is Avogadro's number/millimole and C is the concentration of dissolved molecules in moles/liter (reference 15, 227.) For the plastoquinone case, the concentration of F698 was estimated at 5×10^{-6} moles/l and the quencher was a maximum of 5×10^{-6} moles/l. Using 10^{-5} moles/l for C_0, $2R_0$ is calculated to equal 710 A. Therefore, if the molecules are randomly distributed in the frozen solutions they would be too far apart for dynamic quenching to occur. The remaining possibility that quenching occurs via the formation of a nonfluorescent complex, is the likely mechanism.

Recent work has revealed that two photochemical reactions, mediated by two chlorophyll type pigments, occur in photosynthesis (20, 21). It has been proposed that the 698 mμ fluorescing pigment, in vivo, is the site for one of these photoreactions, namely system II (2, 4). On excitation, the reactive pigment in system II is believed to sensitize the reduction of plastoquinone by an unknown material (21). Our proposal that the excited F968 molecules are primarily in a metastable state at room temperature fits well with the hypothesis that it is one of the photoreactive sites. The finding that in solution the fluorescence of F698 is readily quenched by plastoquinone, probably by formation of a complex, is also relevant. Kok (1) has shown that the appearance of the 698 mμ fluorescence in vivo is photo-initiated, even at 77°K. An explanation of this phenomenon, in keeping with our results, is that in vivo F698 is complexed with plastoquinone, and is therefore nonfluorescent. When illuminated, F698 sensitizes the photoreduction of the quinone, which, in its reduced state, we have found does not act as a quencher.

The apparent decrease of emission from F698, during greening, may be due to a relative increase in emission from aggregated chlorophyll, at about 720 mμ,

which obscures emission at 698 mμ. Another possible explanation is that the relative concentration of plastoquinone to F698 may increase to such an extent during aging that there is almost complete fluorescence quenching.

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