F698: A PIGMENT FOUND IN PREPARATIONS OF CHLOROPHYLL

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Introduction

We wish to report the finding of a chlorophyll-type pigment, different from chlorophyll a or b, in the extracts from green plants. This pigment, which we shall refer to as F698, has a fluorescence maximum at 698 mμ in acetone at 77°K. We find F698 to have spectral properties similar to those of a pigment with fluorescence maximum at 698 mμ in vivo, at 77°K, in Chlorella, Anacystis and Synechococcus (Kok, 1963; Bergeron, 1963; Brody and Brody, 1963; Govindjee, 1963; Goedheer, 1964). It has been suggested (Bergeron, 1963; Govindjee, 1963) that the pigment responsible for emission at 698 mμ participates in the photochemistry of System II in photosynthesis. In view of this possibility, the observation of F698 in vitro is significant, particularly since the various forms of chlorophyll observed by spectroscopy in vivo have not heretofore been detected in extracts.

Experiment

Chlorophylls were prepared by a combination of the methods of Jacobs, Vatter and Holt (1954), and Anderson and Calvin (1962). Prior to crystallization, the chlorophylls were adsorbed on diatomaceous earth and washed with five gallons of petroleum ether. Fluorescence spectra were obtained by exciting with blue light

(436 mμ) having a half band width of 6 mμ; the instrument used for these measurements has been described elsewhere (Brody and Brody, 1965). Alpha tocopherol and coenzymes Q_{10} and Q_6 were obtained from Sigma Biochemicals. Plastoquinone was the generous gift of Professor Norman I. Bishop of Oregon State University. All other materials were obtained reagent grade from Fisher Scientific Company.

Results

Absorption in Solution: F698 is present in the chlorophyll b fraction, and in some samples of chlorophyll a, which were prepared from young (Spring) spinach. Rechromatographing the chlorophylls several times results in preparations which are free of F698, demonstrating that F698 is a separate constituent, and not a material in equilibrium with the other chlorophylls.

The presence of F698 in preparations of chlorophyll is readily revealed at room temperature by difference spectroscopy

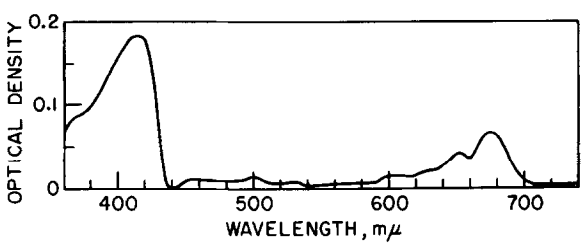

Figure I. Absorption spectrum of F698 in acetone at room temperature, obtained by difference technique using chlorophyll a with and without F698 as sample and reference, respectively. Chlorophyll a concentration was 2.2×10^{-5} moles/liter.

(in all our difference spectra the reference sample is a solution of chlorophyll a free of F698). In Fig. I isshown the spectrum of F698 obtained by this technique in acetone. At room temperature the absorption maxima of F698 are at about 415 and 675 mμ. At -80°C the red absorption band of F698 can be resolved in optically dense solutions of chlorophyll b without resorting to difference spectroscopy, the maximum being at 688 mμ. In solutions of chlorophyll a, F698 is observed as a shoulder at -80°C. Thus, cooling shifts the red absorption band of F698 by about 13 mμ toward larger wavelengths.

F698 was present to the extent of 6% in one preparation of chlorophyll a. This value was determined by comparing the total pigment concentration obtained by weighing, with the concentration of chlorophyll a, determined from its optical density. This estimate of the concentration of F698 together with its optical density at 675 mμ, obtained from a difference spectrum, permits an estimate of the extinction coefficient at 675 mμ - the value being about 5×10^4. F698 is not a chlorophyll colloid, as we find that colloids of chlorophyll a in acetone exhibit a shift in their absorption maxima of only about 3 mμ on cooling to -80°C.

That F698 is not pheophytin is evident from the dissimilarity in their absorption and fluorescence spectra; pheophytin absorbs maximally at 667 and 409 mμ (Smith and Benitez, 1955) and fluoresces at 77°K at 691 mμ in acetone. Furthermore, at room temperature pheophytin is strongly fluorescent where F698 is apparently non-fluorescent. The possibility that F698 is a material produced during the purification process has not yet been

ruled out.

Fluorescence in Solution: At room temperature, identical fluorescence spectra are obtained whether or not the chlorophyll preparations contain F698. However, upon cooling acetone solutions of chlorophyll a containing F698 to 77°K, a band with maximun at 698 mμ is observed in addition to those characteristic of chlorophyll a at 673 mμ and 735 mμ (see figure, Brody and Broyde, 1963). Similar results are obtained with preparations of chlorophyll b containing F698, i.e., at 77°K a band with maximum at 698 mμ is observed in addition to the major band at about 652 mμ. In the absence of F698, the second fluorescence band of chlorophyll b is observed at 705 mμ at 77°K.

The temperature dependence of the relative fluorescence yield of F698 is shown in Fig. II for a 10^{-4} molar solution of

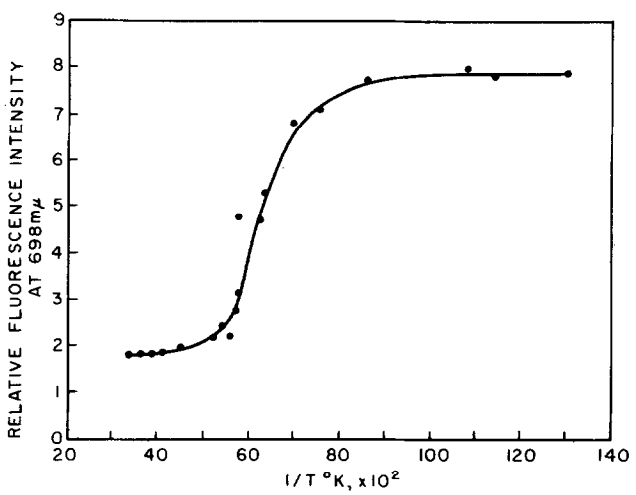

Figure II. Fluorescence intensity at 698 mμ as a function of temperature. Sample is a 10^{-4} molar solution of chlorophyll a in acetone, containing about 6% F698.

chlorophyll a in acetone. It is seen that the fluorescence intensity increases only slightly upon cooling to -80°C. Between the temperatures of -80°C and -160°C, the intensity increases sharply, close to four fold. On cooling further to -196°C the yield increases only a few percent.

In Synechococcus, Goedheer (1964) has shown that the temperature dependence of fluorescence at 698 mμ is similar to what we find in solution.

To determine if the emission from F698 was long-lived, its lifetime was examined, using a Kerr cell phosphoroscope. No emission longer than 10^{-7} seconds (the decay time of our exciting light) was detected, demonstrating that the 698 millimicron emission is a short-lived one and does not originate from a metastable state of chlorophyll a (Brody and Broyde, 1963).

Prior to washing the chromatographed chlorophyll with petroleum ether F698 is non-fluorescent because of quenching by some naturally occurring materials. (However, the presence of F698 in the samples can be established by absorption spectroscopy.) In order to obtain some information as to the identity of these naturally occurring quenchers, we examined the ultraviolet absorption spectrum of the concentrated petroleum ether washings. A band with maximum at 260 mμ was observed, which is the vicinity where the plastoquinones and other terpenoid quinones are known to absorb (Crane and Dilley, 1963; Bishop, 1956). Furthermore, it was found that fluorescence of F698 can be quenched selectively by Coenzymes Q_{10} and Q_6, plastoquinone and alpha tocopherol. Fluorescence of F698 is similarly quenched by nitro-

benzene (Brody and Broyde, 1963). Of particular interest is our finding that reduced coenzyme Q_6 does not quench fluorescence at 698 mμ. A quantitative study of fluorescence quenching at 698 mμ in acetone showed that the Stern-Volmer relationship is obeyed, in the range of quencher concentration examined - up to 10^{-3} moles/liter for nitrobenzene, 10^{-5} moles/liter for coenzyme Q_{10}, and 5×10^{-6} moles/liter for plastoquinone; the concentration of chlorophyll a used for these studies was in the 10^{-4} to 10^{-5} molar range. The observed quenching constants were 8.6×10^{2} liters/mole for nitrobenzene and 1.2×10^{5} liters/mole for both coenzyme Q_{10} and plastoquinone. The quenching mechanism probably entails the formation of a non-fluorescent complex,

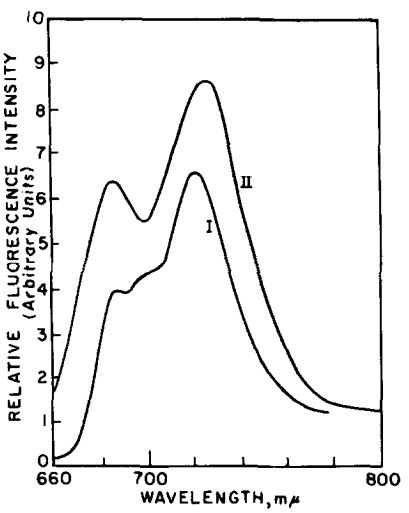

Figure III. Curve I - Fluorescence spectrum of Chlorella pyrenoidosa (2 days in the light) in aqueous suspension, 77°K.

Curve II - Same material as Curve I, in suspension of water saturated with nitrobenzene.

since diffusional encounters are ruled out at 77°K.

In vivo: We find emission at 698 mμ is quenched in intact cells and chloroplast fragments by nitrobenzene. Fig. III, Curve I, shows the low temperature fluorescence spectrum of an aqueous suspension of Chlorella pyrenoidosa. Curve II, shows the same sample suspended in water saturated with nitrobenzene, revealing a minimum at 698 mμ. It should be pointed out that water saturated with nitrobenzene does not extract chlorophyll from the cells.

Discussion

We have shown the occurrence in vitro and in vivo of a chlorophyll type pigment with fluorescence maximum at 698 mμ. The temperature dependence of fluorescence of the 698 mμ band and some of its quenching properties are similar in vivo and in vitro. Based upon these facts, it is suggested that the two substances may be identical. The possibility that F698 is the site for sensitizing the photochemistry of System II in photosynthesis is particularly interesting in this connection. Our finding that F698 probably complexes with plastoquinone is relevant since the photoreactive pigment for System II is thought to interact directly with plastoquinone (Witt, Miller and Rumberg, 1963). Kok (1963) has observed that the onset of 698 mμ fluorescence is photo-initiated in vivo. In keeping with the results of our quenching studies, Kok's observation may be interpreted in the following way: The pigment fluorescing at 698 mμ is originally complexed with plastoquinone and hence non-fluorescent; when the cells are irradiated the quinone becomes reduced, whereupon it no longer

acts as a quencher and emission at 698 mμ appears.

Acknowledgement

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