New Excited State of Chlorophyll

In order to account for the high efficiency of photosynthesis, it has been suggested that chlorophyll has long-lived excited states, in which absorbed light energy can be stored for photochemical action. Until now, the only known state of this kind has been the metastable triplet state. The existence of this state has been experimentally verified only in chlorophyll *b*, where, at low temperatures, a fluorescence band presumably originating in such a state was observed at 860 mμ by Becker and Kasha (*1*).

Livingston (*2*) and Linschitz (*3*) have observed, in chlorophyll *a* and chlorophyll *b* solutions illuminated by a strong flash of light, the appearance of very broad absorption bands which they interpreted as originating in the metastable triplet state.

We now find at low temperatures (– 193°C) a new sharp fluorescence band at 720 mμ in *Chlorella* and *Porphyridium cruentum*; a similar band appeared at 715 mμ in concentrated ethanolic solutions of chlorophyll *a*. The occurrence of this band suggests the existence of an excited state of chlorophyll in the cell situated slightly below the

Fig. 1. Fluorescence spectra of *Chlorella* at room temperature (crosses) and – 193°C (open circles). The fluorescence intensities indicated are the same for both curves. The decrease in fluorescence yield at 690 mμ is probably due to the increased scattering of the exciting and fluorescent light.

well-known fluorescent singlet state. This state seems to belong to an aggregated form of chlorophyll. It could be of importance for the mechanism of photosynthesis.

The main fluorescence band of *Chlorella* lies at 685 mμ, with a relatively weak vibrational sub-band at about 740 mμ. Figure 1 shows the new fluorescence band which appears when *Chlorella* is cooled down to liquid-nitrogen temperature. The fluorescence spectra of *Chlorella* cells at room temperatures published by Vermeulen, Wassink, and Reman (*4*) and by Duysens (*5*) suggest the existence of a second fluorescence band on the long-wave side of the main one; perhaps these observations are related to ours. A very strong fluorescence peak at 720 mμ was observed at room temperature in *Porphyridium cruentum* by Duysens (*6*), who attributed this peak to an "unknown pigment" (perhaps chlorophyll *d*). However, Strain (*7*) found no chlorophyll *d* in this species of red algae. Again, this may be the same band as the one we have found at low temperatures. Identification is supported by our observation that, when *Phorphyridium* is cooled to – 193°C, the 720-mμ fluorescence band increases more than fivefold in intensity.

Light emission by chloroplast material at low temperature in the region 700 to 900 mμ was described by Tollin and Calvin (*8*), whose observations indicated a life time of several seconds. No spectral analysis of the emitted light was attempted.

Concentrated solutions (10^{-3} to $10^{-2}M$) of chlorophyll *a* (as well as those of chlorophyll *b*) also show new fluorescence bands when cooled to liquid-nitrogen temperature. This phenomenon could not be observed in dilute solutions ($10^{-6}M$); all that happened when such solutions were frozen was that the fluorescence maxima at 670 mμ (chlorophyll *a*) and 660 mμ (chlorophyll *b*) were slightly shifted toward longer wavelengths (680 and 670 mμ, respectively).

Figure 2 shows the intense fluorescence band, with a maximum at 715 mμ, appearing in concentrated ($3 \times 10^{-2}M$) ethanolic solutions of chlorophyll *a*. At this concentration, at – 193°C, the "old" band at 690 mμ shows only as a shoulder on the short-wave side of the "new" band. At somewhat lower concentrations, the two bands appear as separate peaks.

Concentrated solutions of chlorophyll *b* show, at – 193°C, a new fluorescence band at 645 mμ—that is, on the short-wave side of the "old" band; another new band may exist at 695 mμ. Lavorel (*9*) has described the absorption spectrum of a very concentrated chlorophyll *a* solution in liquid paraffin; in his curve, there is a shoulder, suggestive of a band at 700 mμ, perhaps related to the new fluorescence band.

The importance of high concentration for the appearance of the 715-mμ fluorescence band in vitro suggests that this band may be due to dimers or higher aggregated chlorophyll molecules.

That molecular aggregates do exist in highly concentrated chlorophyll solutions was shown by Rodrigo (*10*). According to his observations, at room temperature, in a $10^{-3}M$ acetone solution of chlorophyll *a*, the average molecular weight of chlorophyll is almost three times the normal one.

After cooling to – 193°C, concentrated chlorophyll solutions could conceivably have formed crystalline molecular aggregates. However, when crystalline chlorophyll *a* and *b* (or the corresponding chlorophyllides) prepared according to
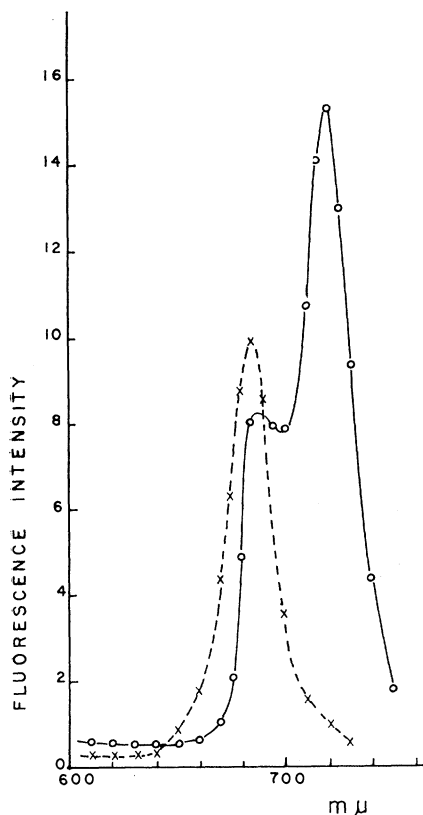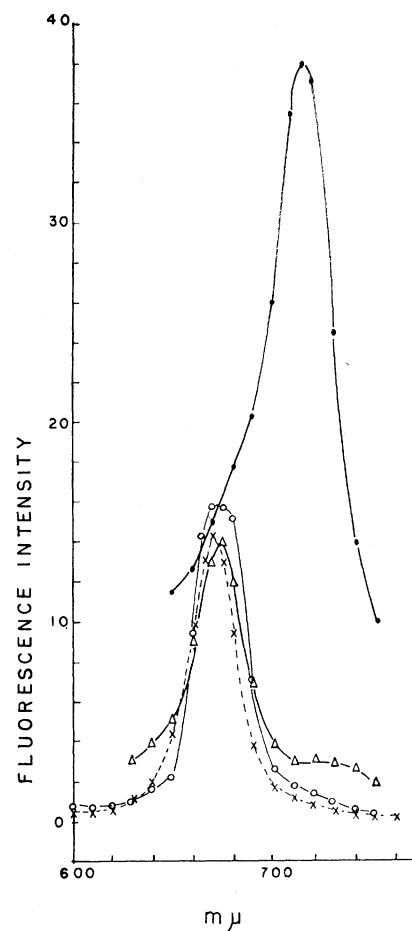

Fig. 2. Fluorescence spectra of concentrated and dilute ethanolic solutions of chlorophyll *a* at room temperature and – 193°C: (crosses) spectrum of dilute solution room temperature; (open triangles) spectrum of concentrated solution at room temperature; (open circles) spectrum of dilute solution at – 193°C; (solid circles) spectrum of concentrated solution at – 193°C. The fluorescence spectra of dilute solutions of chlorophyll *a* do not change radically at low temperature. However, concentrated solutions at – 193°C. develop a large new fluorescence band at 715 mμ. At slightly lower concentrations the two fluorescence maxima can be resolved.

Holt and Jacobs (*11*) were examined, no fluorescence bands were found, at either room temperature or at liquid-nitrogen temperature, which could be considered to correspond to the 715 mμ band found for concentrated solutions. (These crystals were described as "non-fluorescent," but we noted a weak fluorescence band at 680 mμ. It cannot belong to the crystals, whose absorption band lies at > 720 mμ; it may have been due to the presence of a small amount of colloidal material, with an absorption band at 670 mμ.)

In the cell, the fluorescence band at 720 mμ could conceivably be due to a new molecular species, such as chlorophyll *d*, having an absorption and a fluoresence band on the long-wavelength side of those of chlorophyll *a* (as suggested in the case of *Porphyridium*). However, alcoholic extracts from *Porphyridium* showed no fluorescence band at 720 mμ, at either + 20°C or − 193°C. This supports the hypothesis that the 720-mμ band is due to an aggregated form of chlorophyll *a*.

Kok (*12*) and Coleman *et al.* (*13*) have observed a "difference" band at 705 mμ in the absorption spectra of dark and illuminated cells of *Chlorella* (and some other algae). This may mean that the 720-mμ fluorescence band is correlated with an absorption band close to this position.

The great increase in intensity of the 715-mμ fluorescence band in vitro (and of the 720-mμ fluorescence band in the cell) at low temperature could be due either to an increase in the relative *concentration* of the aggregates or to an increase in their *yield of fluorescence*. The latter is not impossible if the new fluorescence band originates in a metastable level, which at room temperature is too rapidly used up by photochemical reactions, collision deactivation, and internal conversion, to emit fluorescence. At low temperatures, all these deactivation processes are slowed down. Lifetime determination, and other quantitative studies, should permit a check of this hypothesis.

The new energy level could correspond to a triplet or nπ level. The new level would lie closer to the singlet ππ excited state in the aggregate than in the monomer.

In vivo, the chlorophyll fluorescence yield increases (according to Franck, French, and Puck, *14*) and the absorption in the neighborhood of 705 mμ decreases (according to Kok, *12*) with incident light intensity, until the saturation of photosynthesis is reached. These changes can be related to changes in the concentration of aggregated chlorophyll in light. When the intensity of the incident light increases, some aggregates may break up into monomers. This would cause a decrease in absorption at 705 mμ

and an increase in chlorophyll fluorescence at room temperature (if the monomeric form of chlorophyll is fluorescent at all room temperatures, while the aggregated form only becomes flourescent at low temperatures).

Possibly the drop in photosynthesis on the long-wavelength side of the chlorophyll absorption band (*15*) may be due to increased absorption of light energy by the aggregated form of chlorophyll. The absorption band leading to the new level should be located at about 700 mμ. Because of the strong selective scattering in this region (*16*), it would not be easy to identify. The most careful measurements of absorption spectra of live cells do not exclude the possibility that the red absorption band contains more than one component. To utilize the energy stabilized in the new energy level (aggregated chlorophyll), light of shorter wavelengths may be required—perhaps, to produce a doubly excited state, as suggested by Franck (*17*) (*18*).

SEYMOUR STEVEN BRODY
Department of Botany,
University of Illinois, Urbana

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18. I would like to thank Professor Eugene Rabinowitch for reviewing this paper, and Mr. Krishan Kumar for his assistance in performing these experiments. This work was supported in part with aid from the Office of Naval Research.

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Immunogenetic Dissection of the T5 Bacteriophage Tail

Studies of the antigenic structure of several bacterial viruses (*1-4*) have led to the recognition of two non-cross-reacting surface antigens, which are designated the head and tail antigens, respectively, according to their apparent localization in the virus particle (*3*).

Since the ability to react with neutralizing antibody appears to be an exclusive property of the phage tail (*2, 3*), which contains also the sites of attachment to host cells (*5*), specific neutralization and host adsorption afford tools for studying the structure and function of this virus organelle. The relation between the host-attachment sites and the antigenic sites of the tail has not yet been fully settled, but in some instances the sites show a close genetic association (*6*).

Several lines of evidence (*4, 7*) have suggested that the virus tail itself may be antigenically heterogeneous; however, evidence is yet to be presented that one and the same virus particle can react through different critical sites with two dissimilar neutralizing antibodies. This report is a brief description of experiments (*8*) which supply this kind of evidence for phage T5.

The principal conclusion, that this virus contains at least two distinct antigenic determinants in that portion of the virus particle which is sensitive to neutralizing antibody, rests on the following facts, some of which have been reported previously.

1) Phage T5 stocks form plaques with equal efficiency on *Escherichia coli* strains B and F (smooth derivative of FCb) (*9*).

2) Either of these hosts can adsorb the bulk (over 99 percent) of the plaque formers for both. This fact is crucial to the conclusion.

3) All T5 stocks contain readily isolated host-range mutants, designated BF⁻, which adsorb onto and infect strain B but not F (*9*).

4) These mutants cross-react reciprocally with T5 in specific neutralization, but both of the homologous reactions are stronger than the cross reactions (*10*).

5) The cross-reacting antibody can be removed readily from a T5 antiserum (rabbit) by absorption with BF⁻, leaving behind a large fraction of antibody (about 50 percent as measured by neutralization of T5 for strain F) which has a striking behavior against phage T5. Like the original serum, it neutralizes the infectivity for strain F in an almost exponential fashion down to 1 percent survivors and beyond; but the infectivity of the same sample for B, instead of decreasing progressively, levels off and remains at about 50 percent, while the infectivity for F vanishes. The leveling off is not due to antibody depletion, since a fresh input of T5 is neutralized just as is the first input.

6) The stable survivors infective for strain B no longer adsorb to F (the adsorption rate constant is less than 0.3 percent of that for untreated T5). They adsorb to B at about half the initial rate. When allowed to grow on B, they produce progeny that are again capable of infecting F. Hence, treatment of T5