

# Structure of the Red Fluorescence Band in Chloroplasts

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**ABSTRACT.** Using Weber's method of "matrix analysis" for the estimation of the number of fluorescent species contributing to the emission of a sample, it is shown that the fluorescence<sup>1</sup> band in spinach chloroplast fragments at room temperature originates in two species of chlorophyll *a*. Emission spectra obtained upon excitation with different wavelengths of light (preferentially absorbed in chlorophyll *a* or *b*) are presented. Upon cooling to  $-196^{\circ}\text{C}$ , the fluorescence efficiency increases about twentyfold. Two additional bands, that now appear at 696 and 735  $m\mu$ , suggest the participation of four molecular species. Emission spectra observed at different concentrations of chloroplast fragments with excitation in chlorophyll *a* and *b* and excitation spectra for different concentrations of chloroplast fragments and measurements at 685 and 760  $m\mu$  are presented. Two of the four emission bands may belong to pigment system I and two to system II. The 685, 696, and 738  $m\mu$  bands respond differently to temperature changes. In the  $-196^{\circ}\text{C}$  to  $-150^{\circ}\text{C}$  range, the intensity of the 685  $m\mu$  band remains constant, and that of the 696  $m\mu$  band decreases twice as fast as that of the 738  $m\mu$  band.

## INTRODUCTION

The hypothesis (1) of two light reactions (I and II) and of two pigment systems (2), I and II, responsible for them, first derived from Robert Emerson's discovery (3) of the "enhancement effect" in photosynthesis, has been accepted by many workers in photosynthesis (4). Several types of experiments have been used to study the two light reactions (5). One of them is the measurement of chlorophyll fluorescence (6-10); if the two-pigment system hypothesis is correct, one may expect the existence of more than one fluorescent species of chlorophyll *a* in vivo. The existence of such forms of chlorophyll *a* is well known from the analysis of the absorption bands of live cells (11-13).

Since the half-band width of the fluorescence band at room temperature is not very different from that of the absorption band, the fluorescence band

<sup>1</sup> The term fluorescence has been loosely used to include all types of light emission; "emission" and "fluorescence" have been interchangeably used throughout the text.

may also be composed of several bands. Duysens (14) first noted in the blue-green alga, *Oscillatoria*, and in the red alga, *Porphyra* (15), a strong emission band in the 730  $m\mu$  region, which he attributed to an unidentified pigment. More recent experiments suggest that this complexity is not restricted to phycobilin-containing algae. Evidence has accumulated showing that during the initial induction period the emission spectrum is different from that in the steady state (Lavorel, 16; Rosenberg et al., 17). Butler (7) showed that the excitation spectrum of the fluorescence of a green leaf changes during the induction period.

A new emission band of chloroplasts at 720  $m\mu$  was discovered in *Chlorella pyrenoidosa* at  $-196^{\circ}\text{C}$  by Brody (18). Litvin, Krasnovsky, and Rikhireva (19) discovered another new band at 696  $m\mu$  (also at  $-196^{\circ}\text{C}$ ) in bean homogenates. The existence of these bands has been confirmed by Kok (20), Bergeron (21), Govindjee (5), and Goedheer (22) in a variety of organisms. That the emission spectra at  $-196^{\circ}\text{C}$  are clearly different depending on whether an accessory pigment or chlorophyll *a* is excited has also been shown (5, 20, 22).

The first part of the present work is concerned with the application of Weber's matrix analysis (23) to determine the number of fluorescing species in the emission spectrum of chlorophyll under steady-state conditions at room temperature in chloroplast fragments. The complexity of the emission band was confirmed, and the number of fluorescent species involved should be two forms of chlorophyll *a*. The second part of the paper is concerned with low temperature emission and excitation spectra of chloroplast fragments. The bands at 685  $m\mu$ , 696  $m\mu$ , and 738  $m\mu$  were shown to behave differently with change in temperature.

#### MATERIALS AND METHODS

Freshly picked young leaves of spinach, grown in a greenhouse, were treated by the usual methods of crushing and differential centrifugation (24); the resulting preparations, containing whole and broken chloroplasts, are referred to as "chloroplast fragments." Dilute suspensions (OD at 680  $m\mu$  = 0.02 for 0.2 cm path length) were employed for most of the experiments, unless otherwise specified.

Two large Bausch and Lomb grating monochromators (grating area, 100  $\times$  100 mm; dispersion: 3.3  $m\mu$  per mm of slit width) were used for excitation and for the analysis of fluorescence (25). The fluorescence was collected from the same surface which received the excitation light. The optical path of the exciting light in the sample was slightly less than 0.2 cm. The exciting light beam (band width, 2  $m\mu$ ) was filtered through a Corning 4-76 glass filter for the 400 to 550  $m\mu$  range, a Corning 3-69 filter for the 500 to 750  $m\mu$  range, and a Schott RG-10 filter for the 750 to 800  $m\mu$  range. An EMI photomultiplier (with S-20 response) was used as the light detector, and a Rubicon light beam galvanometer ( $5.8 \times 10^{-4}$   $\mu\text{a}/\text{mm}$ ) or a Keithley 150A millimicroammeter connected to a Brown recorder was used as a measuring device.

Emission spectra were corrected for spectral sensitivity of the photomultiplier and variations in the spectral efficiency of the monochromator. The excitation spectra were corrected for variations in the incident intensity as measured by a thermopile. In the intensity range used, fluorescence was proportional to incident intensity.

The temperature of the sample was measured with a thermocouple connected to the Keithley millimicrovoltmeter; the reference electrode was either in ice cold water (0°C) or in liquid nitrogen (-196°C).

For matrix analysis, the emission must be measured at several wavelengths and excited at several wavelengths, and the data are arranged in the form of a determinant. The matrix analysis is a statistical method for deciding whether emissions at different wavelengths have different emission spectra. (For a complete description and the utility of the matrix analysis, see Weber, 23). Care was taken to choose band widths, of both the exciting and the measuring beams, so that there was no overlap between two adjacent settings; this is not essential, but it is convenient. Very narrow slits (2.0 m $\mu$ , band width) were used for most of the measurements. The ratio of  $\Delta/P$  is compared with  $\delta F/\bar{F}$ , where  $\Delta$  stands for the determinant,  $P$  for the permanent ( $P = 2\bar{F}^2$  for a  $2 \times 2$  case),  $\delta F$  is the statistical error, and  $\bar{F}$  the average fluorescence. In a  $2 \times 2$  case, if  $\Delta/P$  is more than 2 to 3 times  $\delta F/\bar{F}$ , it can be assumed that more than one species are contributing to the emission spectrum. If, for the same sample,  $\Delta/P$  equals  $\delta F/\bar{F}$  in a  $3 \times 3$  case, then there are only two species contributing to the emission spectrum.

The (relative) fluorescence yield was obtained by dividing the area under the emission bands (corrected for the response curve of the photomultiplier and the efficiency of the monochromator) by the number of absorbed quanta (incident quanta  $\times$  fractional absorption). The fractional absorption, in turn, was estimated from absorbance measurements [ $A = f(\lambda)$ ] by a Bausch and Lomb Spectronic 505 spectrophotometer (equipped with integrating sphere attachment) for the same sample and for the same path length as that traversed by exciting light in fluorescent measurements. These relative yields were converted into absolute yields by assuming that the fluorescence efficiency of pure chlorophyll *a* in ether is 30% (cf. Weber and Teale, 26; and Latimer et al., 27). The geometry of the instrument when chloroplast fragments and chlorophyll solutions are used cannot be identical, due to the scattering properties of chloroplasts, and therefore we have some reservations concerning the absolute values presented here. In our earlier measurements, pure chlorophyll *a* was prepared from spinach by the method of Jacobs et al. (28). Recently, however, we have used *Anacystis* instead of spinach and the method of Strain (29) was employed (we thank Dr. Rajni Govindjee for the former preparation and Miss Barbara Acker for the latter). Both preparations gave about the same fluorescence yield.

## RESULTS

### *A. Fluorescence Spectrum at Room Temperature (22°C)*

The shapes of the emission spectra in green cells, obtained by excitation with light of different wavelengths, have been usually assumed to be identical, indicating that only one species—chlorophyll *a*—is fluorescent. A close

examination of emission spectra obtained by Duysens (14) in *Chlorella* shows that there are small differences, especially in the long wave region upon excitation with either 420 or 480  $m\mu$  light. We have confirmed this in very thin (1% absorption at 680  $m\mu$ ) suspensions of chloroplasts (see Fig. 1).

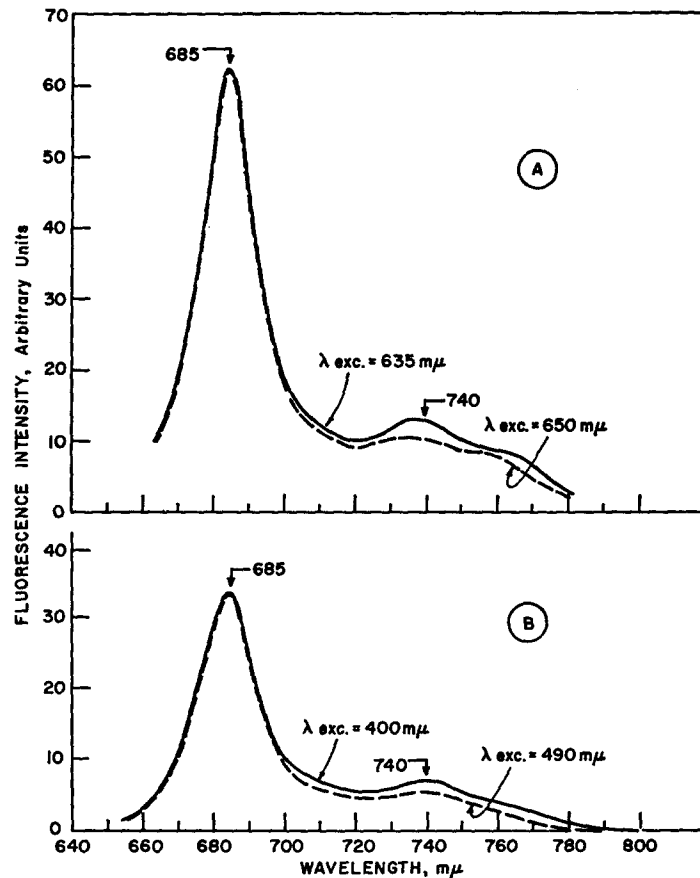


FIGURE 1. Emission spectra of chloroplast fragments from spinach upon excitation by different wavelengths of light. Measurements at 22°C.

Fluorescence was excited by 400  $m\mu$  (exciting more of chlorophyll *a* and therefore supposedly more of system I), 490  $m\mu$  (exciting more of chlorophyll *b* and thus more of system II), 635  $m\mu$  (exciting more of chlorophyll *a*), and 650  $m\mu$  (exciting more of chlorophyll *b*). The 400 and the 490, the 635 and the 650 emission curves, were normalized at 685  $m\mu$ . One may note that with 400 and 635  $m\mu$  excitation, there was about 35% more fluorescence at 740  $m\mu$  than with 490 and 650  $m\mu$  excitation. These differences are not due to reabsorption of fluorescence of the main band because the per cent absorptions at 400 and 490  $m\mu$  are about the same, and the greater absorption at

650  $m\mu$  as compared to that at 635  $m\mu$ , ought to have the opposite effect. Furthermore, identical shapes of the emission curves below 685  $m\mu$  could not have been obtained if reabsorption of fluorescence were significant. Very thin suspensions were used for these experiments; changing the chloroplast concentration so as to increase the absorption in the peak from 1% to 5% did not change the emission spectrum. These data suggest that there must be more than one fluorescent molecular species. The following "matrix analysis" was made to determine their number.

TABLE I  
 $\Delta/P$  VALUES FROM FLUORESCENCE DATA (SPINACH  
 CHLOROPLASTS) ( $2 \times 2$  ANALYSIS)  
*Excitation Pairs,  $m\mu$*

Observation pairs	400-430		430-460		460-490	
	$\Delta/P$	$\delta F/\bar{F}$	$\Delta/P$	$\delta F/\bar{F}$	$\Delta/P$	$\delta F/\bar{F}$
<i><math>m\mu</math></i>						
680-690	-0.029	0.006	+0.052	0.005	+0.017	0.004
690-700	-0.054	0.010	+0.025	0.008	+0.009	0.005
700-710	-0.028	0.017	-0.028	0.012	+0.017	0.009
	<u>680-695</u>					
730-740	-0.057	0.010				
740-750	-0.052	0.011				
750-760	+0.096	0.016				
	<u>635-650</u>		<u>650-665</u>			
680-690	-0.035	0.002	-0.052	0.001		
690-700	-0.033	0.002	-0.033	0.003		

First, we excited fluorescence at 400, 430, 460, and 490  $m\mu$ , and observed it at 680, 690, 700, and 710  $m\mu$ . The  $\Delta/P$  values for the three excitation pairs (400 to 430, 430 to 460, and 460 to 490) and the three observation pairs (680 to 690, 690 to 700, and 700 to 710) are given in the upper portion of Table I. The  $\delta F/\bar{F}$  values are also listed. Clearly,  $\Delta/P$  is many times greater than  $\delta F/\bar{F}$ . This shows that a minimum of two fluorescent species, absorbing in the 400 to 490  $m\mu$  region, produce the fluorescence in the 680 to 710  $m\mu$  region.

Since chlorophylls have two absorption bands in the visible (the Soret band in the blue and a red band) we hoped to confirm these results also by exciting with red light. To avoid overlap of the excitation and emission wavelengths we had to choose longer wavelengths for observation than those used with blue light excitation. The results are shown in the middle part of Table I. Here again, we note that the  $\Delta/P$  values are more than three times the values

for  $\delta F/\bar{F}$ , which shows that at least two fluorescent species contribute to the emission spectrum.

Chlorophyll *b* is usually assumed to transfer its energy with 100% efficiency to chlorophyll *a*; but the efficiency may be slightly under 100%, permitting the emission of some chlorophyll *b* fluorescence. That this is so, can be inferred from the lower part of Table I. This conclusion is supported by results with blue (460 to 490) excitation (see upper part of Table I). Examination of  $3 \times 3$  determinants shows that there are, in fact, three fluorescent species—perhaps, two species of chlorophyll *a* and one of chlorophyll *b*. Table II shows a comparison of  $\Delta/P$  with  $\delta F/\bar{F}$  for groups of three excitation wavelengths: 400 to 430 to 460  $m\mu$ , 635 to 650 to 665  $m\mu$ , and 670 to 680 to 700

TABLE II  
 $\Delta/P$  VALUES FROM FLUORESCENCE DATA (SPINACH  
 CHLOROPLASTS) ( $3 \times 3$  ANALYSIS)

Excitation wave lengths	Observation wave lengths	$\Delta/P$	$\delta F/\bar{F}$
<i>mμ</i>	<i>mμ</i>		
400-430-460	680-690-700	-0.06	0.008
635-650-665	680-690-700	+0.02	0.004
670-680-700	720-730-740	+0.005	0.004

$m\mu$ ; the groups of three observation wavelengths were 680 to 690 to 700  $m\mu$ , 680 to 690 to 700  $m\mu$ , and 720 to 730 to 740  $m\mu$ , respectively. Clearly, in the region where chlorophyll *b* absorption is significant,  $\Delta/P$  is five to seven times larger than  $\delta F/\bar{F}$ , while with excitation at 670 to 680 to 700  $m\mu$ ,  $\Delta/P$  equaled  $\delta F/\bar{F}$ , suggesting that not more than two species fluoresce when chlorophyll *a* is the absorbing species. Thus, the  $2 \times 2$  analysis shows that more than one fluorescent species of chlorophyll *a* exists, while the  $3 \times 3$  analysis shows that the number of fluorescent chlorophyll *a* species is not larger than two.

The earlier finding of Olson et al. (30) that the emission band of polarized fluorescence lies in the neighborhood of 720  $m\mu$  supports the existence of at least two fluorescent species. This conclusion is confirmed by our observations (Govindjee and Weber, data to be published): if *Porphyridium cruentum* is excited by polarized light, the degree of polarization of chlorophyll fluorescence is 6% when chlorophyll *a* is excited and only 1% when phycoerythrin is excited; this also indicates the existence of two fluorescence bands—one polarized and the other nonpolarized. The chlorophyll fluorescence band of *Porphyridium cruentum* shows upon excitation by light of high intensity absorbed in phycoerythrin, a shoulder at 693  $m\mu$ , in addition to the main band at 685  $m\mu$  (31).

B. Measurements at Liquid Nitrogen Temperature ( $-196^{\circ}\text{C}$ )

At  $-196^{\circ}\text{C}$ , three major emission peaks are observed in spinach chloroplasts, in contrast to the single peak at room temperature. These peaks lie at  $685\text{ m}\mu$  (F685),  $696\text{ m}\mu$  (F696), and  $738\text{ m}\mu$  (F720).<sup>2</sup>

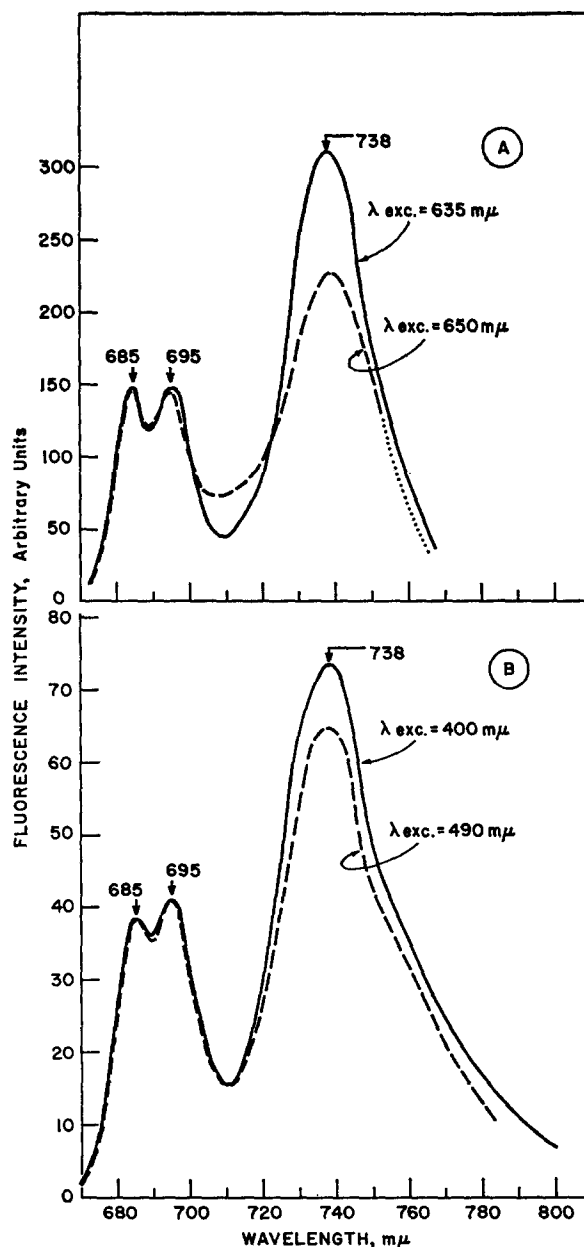


FIGURE 2. Emission spectra of chloroplast fragments of spinach upon excitation by different wavelengths of light. Measurements at  $-196^{\circ}\text{C}$ .

<sup>2</sup> The long wave band, which is a complex band, appears at different locations in different organisms; at  $712\text{ m}\mu$  in *Porphyridium*,  $718\text{ m}\mu$  in *Anacystis*,  $720\text{ m}\mu$  in *Chlorella*,  $738\text{ m}\mu$  with a shoulder at  $727\text{ m}\mu$  in spinach, and at  $712\text{ m}\mu$  in pine needles. We refer to it as F720.

Fig. 2 shows relative emission spectra of chloroplast fragments normalized at 685  $m\mu$ , as obtained upon excitation at 400  $m\mu$ , 490  $m\mu$ , 635  $m\mu$ , and 650  $m\mu$ . The ratio of F720 to F685 is about 2.0 when excited with 400 and 635  $m\mu$  but it is only 1.6 when excited with 490 and 650  $m\mu$ .

The 696  $m\mu$  band is almost absent at  $-130^\circ\text{C}$ . This is shown in Fig. 3, obtained with a thin suspension of chloroplasts, spread in a 15 to 20  $\mu$  thick film between a microscope cover slip and the flat window of the Dewar flask. The emission spectrum at  $-196^\circ\text{C}$  is shown for comparison. Using different concentrations of chloroplasts excited with 430  $m\mu$  light, we observed that

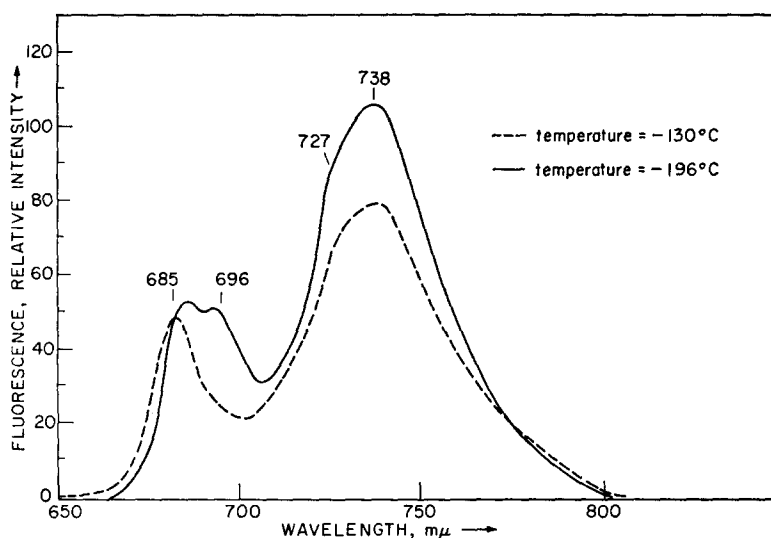


FIGURE 3. Emission spectra from 20  $\mu$  thick chloroplast film measured at  $-130^\circ\text{C}$  and at  $-196^\circ\text{C}$ ; exciting wavelength of light, 400  $m\mu$ .

the ratios of emissions at 738, 696, and 685  $m\mu$  change only at higher concentrations. When a chloroplast suspension absorbs 5% in the chlorophyll *a* absorption peak, the ratio of fluorescence at 738  $m\mu$  and 696  $m\mu$  is about 1.4; it stays so at the lower concentrations.

Fig. 4 shows emission spectra (upon excitation at 430  $m\mu$ ) as a function of varying concentrations of chloroplast fragments. The concentration is expressed on the graphs in per cent absorption at 680  $m\mu$ , ranging from about 100% to 2%. These experiments show that reabsorption of the 696  $m\mu$  and 685  $m\mu$  emission bands is insignificant at less than 5% absorption at 680  $m\mu$ , but becomes significant at the higher concentrations. Thus, the 738  $m\mu$  band must be a "real" band (i.e. not due to reabsorption of the main band). (Further quantitative investigation is, however, required because our present data have not been corrected for reabsorption within a single chloroplast fragment.)



The above experiments show that only a part of the 738  $m\mu$  band can be due to the first vibrational subband of the major 685  $m\mu$  band; and a good part must belong to a different fluorescent species. Detailed analysis of this band, in fact shows it to be complex, with a slight shoulder at 727  $m\mu$ . Also,

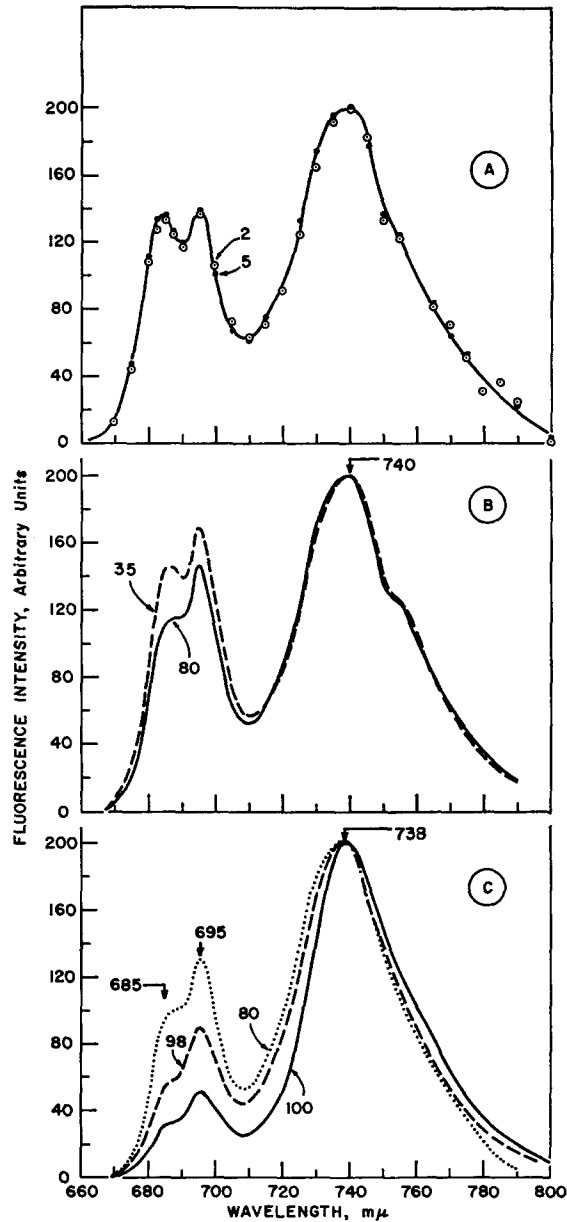


FIGURE 4. Emission spectra at  $-196^{\circ}\text{C}$  of chloroplast fragments of spinach showing the effect of using different concentrations of chloroplasts. Exciting wavelength of light, 430  $m\mu$ .

the excitation spectra of F685 and F720 are not identical. The F685 excitation spectrum shows greater participation of chlorophyll *b* (relative to chlorophyll *a*) than the F720 excitation spectrum. The excitation curves (see Fig. 5)

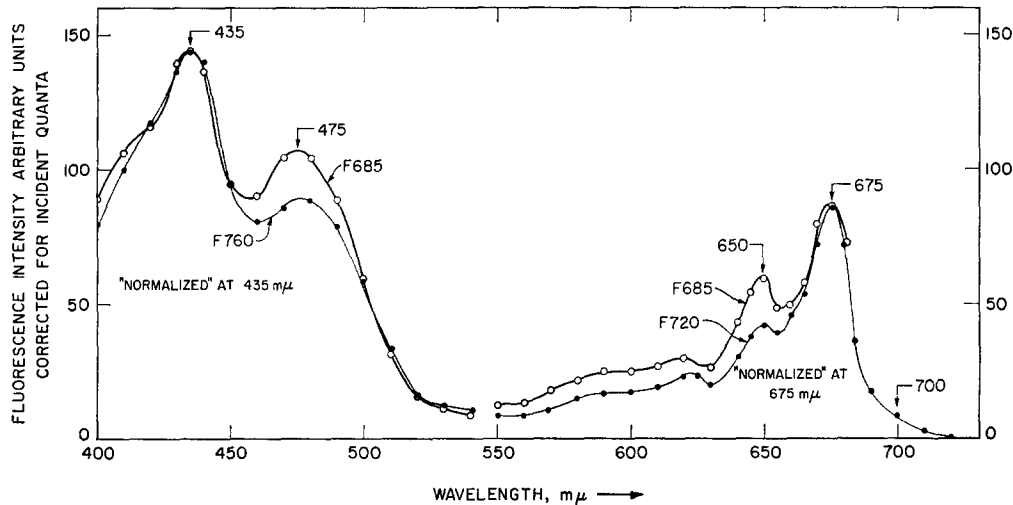


FIGURE 5. Excitation spectra of a thin suspension of chloroplast fragments for measurements at 685  $m\mu$  (F685) and at 760  $m\mu$  (F720).

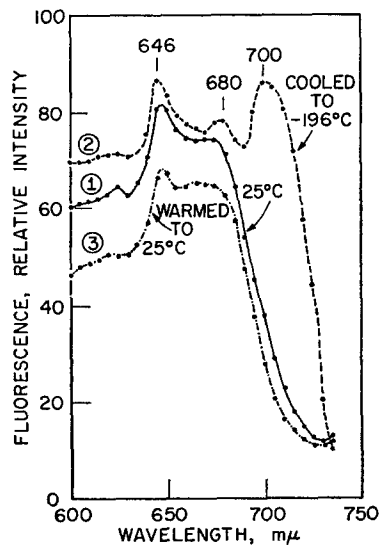


FIGURE 6. Excitation spectra of a very dense suspension of chloroplast fragments (measured at 738  $m\mu$ ), first at room temperature (solid line), cooled to  $-196^{\circ}\text{C}$  (dashed curve), and finally warmed up to room temperature (dashes and dots).

have been reduced to equal numbers of incident quanta, and normalized at 435  $m\mu$ . The ratio of fluorescence excited at 435  $m\mu$  to that excited at 475  $m\mu$  is 1.34 for F685, and 1.61 for F720; that excited at 675  $m\mu$  to that excited at 650  $m\mu$  is 1.43 for F685, and 2.04 for F720.

A large band at about  $700\text{ m}\mu$  appears in the excitation spectrum of  $738\text{ m}\mu$  fluorescence in a very dense suspension, as observed earlier by Butler (32) in bean leaves. The  $700\text{ m}\mu$  band appears (Fig. 6) upon cooling to  $-196^\circ\text{C}$ , and disappears upon warming to  $25^\circ\text{C}$ , showing clearly that it is not due to an irreversible effect of cooling to  $-196^\circ\text{C}$ .

Since the excitation spectrum should parallel the per cent absorption (or fractional absorption) spectrum of the sensitizing species, the relative heights of different peaks are expected to vary with concentration of chloroplasts. Fig. 7 shows excitation spectra reduced to equal numbers of incident quanta

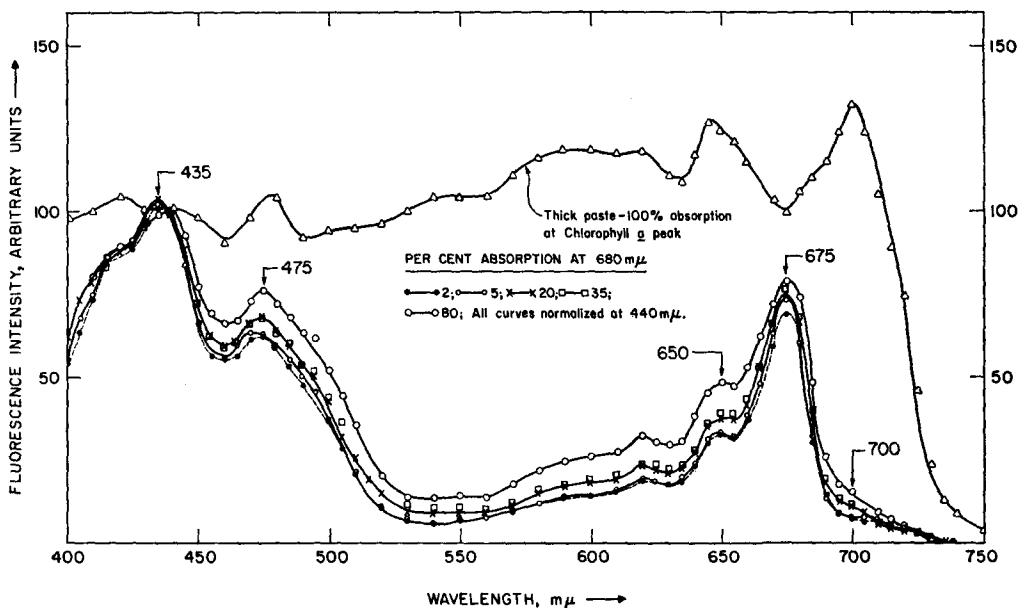


FIGURE 7. Excitation spectra at  $-196^\circ\text{C}$  of chloroplast fragments showing the effect of using different concentrations of chloroplast fragments. Measurements at  $760\text{ m}\mu$ .

and normalized at  $440\text{ m}\mu$  for different concentrations of chloroplast fragments. Measurements were made at  $760\text{ m}\mu$ . The path length was about  $0.1\text{ mm}$ . At the lower concentrations (2 to 5% absorption) the cell excitation spectra are almost identical showing participation of chlorophyll *a* and *b*; a small band indicates contribution by a pigment absorbing around  $700\text{ m}\mu$  (P700?). The  $700\text{ m}\mu$  band in the excitation spectrum is a relatively small band—about 1 to 10% or less of the main chlorophyll *a* band. At higher concentrations, the fractional absorption curve becomes flatter; as a consequence the excitation spectra change, but still retain qualitatively the same shape. The enhancement of the band at  $700\text{ m}\mu$  in very dense suspensions (as shown in Fig. 6, the uppermost curve in Fig. 7, and in Butler's studies) may be due to the "detour factor"—the path length of the  $700\text{ m}\mu$  light is

increased much more than that of the 675  $m\mu$  light. It is thus likely that the 700  $m\mu$  band observed in excitation spectra of thin suspensions of chloroplasts is due to "P700."

In extremely thin chloroplast films, intensity changes in the 738  $m\mu$  emission band do not follow those in the 685  $m\mu$  emission band. When the cooled ( $-196^\circ\text{C}$ ) chloroplast suspension is allowed to warm up gradually, the fluorescence intensities at 685  $m\mu$ , 696  $m\mu$ , and 738  $m\mu$  decrease at different rates (Fig. 8). Up to  $-50^\circ\text{C}$ , the 685  $m\mu$  band does not change. The 696  $m\mu$  band disappears when temperature has risen to  $-130^\circ\text{C}$ ; the 738  $m\mu$  band continues to decrease in intensity up to about  $-50^\circ\text{C}$ . Around

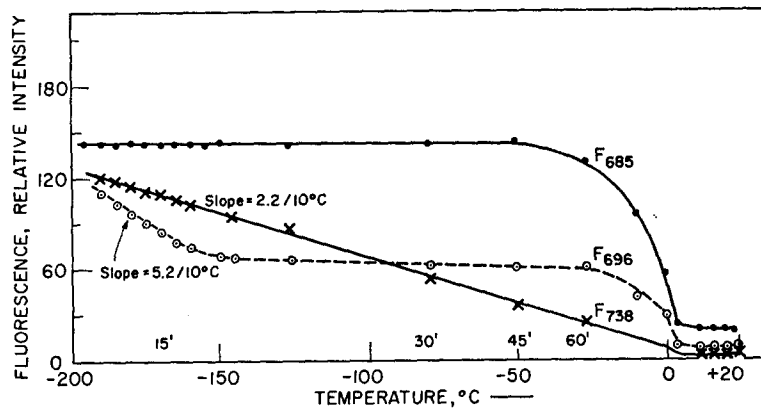


FIGURE 8. Emission at 685  $m\mu$  (F685), at 696  $m\mu$  (F696), and at 760  $m\mu$  (F738; also referred to as F720 in the text) as a function of temperature ( $-196^\circ\text{C}$  to  $20^\circ\text{C}$ ).

$-30^\circ\text{C}$  the 685  $m\mu$  and the 738  $m\mu$  bands decrease in a parallel manner, until the sample melts. There is discontinuity in the curves around  $0^\circ\text{C}$ . There are no significant changes with a further rise in temperature. These results are readily understood. In the  $0$ – $20^\circ\text{C}$  range, the maximum quantum yield of photosynthesis remains fairly constant and the fluorescence yield also remains constant. At lower temperatures, internal conversion and quenching are slowed down tremendously; the fluorescence from the bulk of the pigments and from the "traps" increases several fold.

### C. Fluorescence Yield

Assuming the fluorescence yield of our preparations (OD at  $660 m\mu = 0.05$  for the 0.2 cm optical path) of pure chlorophyll *a* in ether to be 30%, the yield of most chloroplast preparations was found to be around 1.5% when excited by 610  $m\mu$  light at  $22^\circ\text{C}$ ; the lowest yield ever measured was 0.5%, the highest 2.0%. The variation in the yield was primarily due to different age and quality of spinach leaves from which the preparations were made.

In view of this type of variation, a quantitative comparison with the fluorescence yields of whole algal cells is not very meaningful; the yields for live cells—in dim monochromatic illumination—have been estimated as 2.7% (Latimer et al., 27; and Teale, 33). A higher but variable value, depending upon the development stage, was found in greening leaves by Butler (34).

At  $-196^{\circ}\text{C}$ , the fluorescence efficiency of spinach chloroplasts was about 30%, a twentyfold increase from that at room temperature. About 5% was in the 685  $m\mu$  band, 5% in the 696  $m\mu$  band, and 20% in the 738  $m\mu$  band. No such increase was observed in chlorophyll solution.

#### DISCUSSION

Section A under Results describes experiments made with chloroplast fragments at room temperature. Observations on the different shape of the emission band upon preferential excitation of chlorophyll *a* (400, 635  $m\mu$ ) and of chlorophyll *b* (490 650  $m\mu$ ) (Fig. 1), and matrix analysis of this band presented in this paper (see also Brody and Brody, 35) indicate that there are at least two fluorescence bands at room temperature. Thus, the complexity of the fluorescence band in chloroplast fragments has been confirmed; almost all the previous work was done either with leaves or whole algal cells.

*The Nature of Emission Bands at Low Temperatures* The 685  $m\mu$  (F685), 696  $m\mu$  (F696), and 738  $m\mu$  (F720) emission bands that appear at  $-196^{\circ}\text{C}$  in chloroplast fragments may originate in the "energy traps" that become fluorescent at  $-196^{\circ}\text{C}$  due to slowing down of photochemical reaction, as suggested earlier (5, 21); in chlorophyll *a* molecules that can no longer transfer energy with high efficiency to the traps because the traps may be "bleached" (Kok, 20); or in chlorophyll *a* molecules (perhaps certain aggregates of chlorophyll *a* (Brody and Brody, 35; and Litvin et al., 19) that are non-fluorescent at room temperature, but become fluorescent at  $-196^{\circ}\text{C}$ ; or in molecular aggregates of chlorophyll *a* that are formed upon cooling, and disappear upon warming. The available data do not allow us to distinguish among these four possibilities. Perhaps the low temperature bands have multiple origin.

There are clear indications that F696 is associated with pigment "system II," and F720 with "system I." Emission at 696  $m\mu$  is likely to originate in chlorophyll *a* molecules that absorb significantly below 696  $m\mu$  (system II) rather than in P700 (system I). Using detergent-treated chloroplasts, Cederstrand (36) observed that in fractions (4000 to 7000 Å diameter) that have a lower ratio of chlorophyll *a* to chlorophyll *b*, the F696 is relatively stronger; in fractions containing more chlorophyll *a*, F738 is stronger. In experiments at  $-196^{\circ}\text{C}$  (also described in this paper), when system I is preferentially excited at 400  $m\mu$  or 635  $m\mu$ , a higher ratio of F720 to F696 results than when

system II is preferentially excited at 490  $m\mu$  or 650  $m\mu$  (see Fig. 2). This is confirmed by Fig. 5, in which excitation spectra for F685 and F720 are shown. The ratio of chlorophyll *a* to *b* peaks is lower for F685 than for F720. In addition, Krey (37) has shown that when system I is preferentially excited by 430  $m\mu$  light (in *Porphyridium cruentum* at  $-196^{\circ}\text{C}$ ), the ratio of intensity of F720 and F696 is higher than when system II is preferentially excited by 546  $m\mu$  light.

Whatever molecules produce fluorescence at 696  $m\mu$  and between 712 and 738  $m\mu$ , they are different from the ones that produce F685. This is shown by observation at rising temperatures: the 696  $m\mu$  band is observed only at  $-196^{\circ}\text{C}$  to  $-130^{\circ}\text{C}$ ; its intensity decreases in this range twice as fast as that of F738.

*A Working Hypothesis* The concept of "photosynthetic unit" is well established now (see review by Clayton, 38). There are several hundred bulk pigments associated with each energy trap. Photosynthesis is an extremely efficient photochemical process and in order for it to be so, the energy traps must efficiently use energy for photochemical reactions. In all likelihood, fluorescence from such molecules must be completely quenched at low light intensities when the quantum yield of photosynthesis is very high. The two fluorescence bands of chlorophyll *a* inferred from the matrix analysis and observed by others under such conditions may then be assumed to arise from bulk chlorophyll *a* of system I (Chl  $a_1$ ) and of system II (Chl  $a_2$ ). The two additional bands (F696 and F720) that arise at  $-196^{\circ}\text{C}$  may originate in the energy traps (trap I and trap II) because at low temperatures, all thermochemical reactions are absent and the energy arriving at the trap may be used, in part, to emit fluorescence (see review by Robinson, 39). One would expect the same behavior at room temperature at sufficiently high light intensities, but under the latter conditions, P700 is known to be bleached (40); therefore it cannot itself contribute much to fluorescence. One may assume that the hypothetical energy trap II remains colored at light saturation. It then becomes a potential candidate for the production of excess fluorescence observed at high light intensities. This trap keeps receiving energy from the bulk pigments in system II and is unable to react as the enzymes are saturated at high light intensities; hence, it releases its energy as fluorescence.

Fig. 9 shows a working hypothesis for the energy flow in photosynthetic systems based on one of the existing pictures of photosynthesis—the "separate package" model of the two systems, as used by Duysens (see Bannister and Vrooman, 41; and Myers and Graham, 42). The alternative spill-over mechanism is not included in Fig. 9, although the data presented here do not exclude that model.

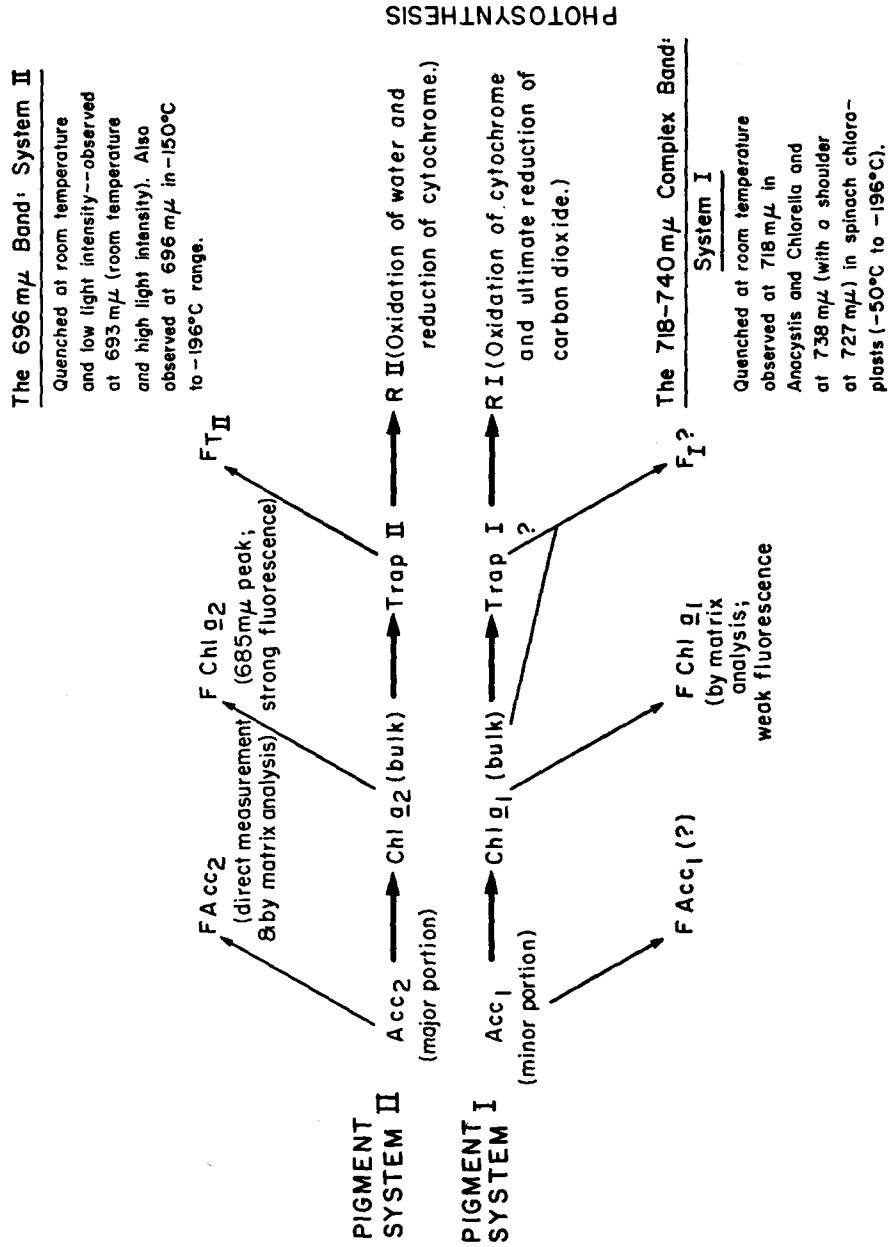


FIGURE 9. Energy flow diagram: two pigment systems—a working model (see text).

The top part of the diagram represents pigment system II, which is composed of accessory pigments (represented by  $Acc_2$  in this diagram), a fraction of chlorophyll  $a$  (Chl  $a_2$ ), and the postulated trap II. There is fluorescence from accessory pigments (F  $Acc_2$ ; observed directly in the case of the phycobilins (12, 14), or inferred from matrix analysis in the case of chlorophyll  $b$ ), from chlorophyll  $a_2$  (F Chl  $a_2$ ; with a peak at 685  $m\mu$  under all conditions), and from trap II (FT<sub>II</sub>). The latter is observed only in strong light, or at  $-196^\circ\text{C}$ , when photochemical reactions are saturated or slowed down.

The bottom part of the diagram represents energy flow in system I; the latter consists of a minor fraction of accessory pigments ( $Acc_1$ ), of chlorophyll  $a_1$  (Chl  $a_1$ ), and of trap I (P700). The loss of energy by fluorescence may be from accessory pigments (F  $Acc_1$ ; but for this there is as yet no evidence), from chlorophyll  $a_1$  (F Chl  $a_1$ ; inferred from matrix analysis and from polarization at room temperature; observed directly at  $-196^\circ\text{C}$ ), and from trap I itself (FTI?) when photochemical energy utilization is slowed down. At room temperature and low light intensities, both traps are engaged in efficient photosynthesis—in oxidation of water and reduction of cytochrome (RII) and in oxidation of cytochrome and reduction of carbon dioxide (RI).

The above is only a working model; it is presented here to stimulate further discussion. Obviously, more experiments are needed to confirm this, especially in view of the Franck-Rosenberg hypothesis (43), which explains the existing data with only one "energy trap."

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#### REFERENCES

1. HILL, R., and BENDALL, F., *Nature*, 1960, **186**, 136.
2. DUYSSENS, L. N. M., AMESZ, J., and KAMP, B. M., *Nature*, 1961, **190**, 510.
3. EMERSON, R., *Science*, 1957, **125**, 746; 1958, **127**, 1059; *Ann. Rev. Plant Physiol.*, 1958, **9**, 1.
4. Photosynthetic Mechanisms of Green Plants, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences-National Research Council Publication No. 1145, 1963.
5. GOVINDJEE, in Photosynthetic Mechanisms of Green Plants, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences-National Research Council Publication No. 1145, 1963, 318.
6. GOVINDJEE, ICHIMURA, S., CEDERSTRAND, C., and RABINOWITCH, E., *Arch. Biochem. and Biophysics*, 1960, **89**, 322.



7. BUTLER, W. L., *Biochim. et Biophysica Acta*, 1962, **64**, 306.
8. TEALE, F. W. J., *Biochem. J.*, 1962, **85**, 14P.
9. DUYSSENS, L. N. M., and SWEERS, H. E., in *Studies on Microalgae and Photosynthetic Bacteria*, (Japanese Society of Plant Physiologists, editors), Tokyo, The University of Tokyo, 1963, 353.
10. ROSENBERG, J. L., and BIGAT, T., in *Photosynthetic Mechanisms of Green Plants*, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences-National Research Council Publication No. 1145, 1963, 122.
11. KRASNOVSKY, A. A., and KOSOBUTSKAYA, L. M., *Dokl. Akad. Nauk SSSR*, 1952, **85**, 177; 1953, **91**, 343.
12. FRENCH, C. S., and YOUNG, V. M. K., in *Radiation Biology* (A. Hollaender, editor), New York, McGraw-Hill Book Co., 1956, **3**, 343.
13. THOMAS, J. B., and MARSMAN, J. W., *Biochim. et Biophysica Acta*, 1959, **35**, 316.
14. DUYSSENS, L. N. M., Doctoral Thesis, University of Utrecht, 1952.
15. DUYSSENS, L. N. M., *Nature*, 1951, **168**, 548.
16. LAVOREL, J., *Biochim. et Biophysica Acta*, 1962, **60**, 510.
17. ROSENBERG, J. L., BIGAT, T., and DEJAEGERE, S., *Biochim. et Biophysica Acta*, 1964, **79**, 9.
18. BRODY, S. S., *Science*, 1958, **128**, 838.
19. LITVIN, F. F., KRASNOVSKY, A. A., and RIKHIREVA, G. T., *Dokl. Akad. Nauk SSSR*, 1960, **135**, 1528.
20. KOK, B., in *Photosynthetic Mechanisms of Green Plants*, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences-National Research Council Publication No. 1145, 1963, 45.
21. BERGERON, J. A., in *Photosynthetic Mechanisms of Green Plants*, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences-National Research Council Publication No. 1145, 1963, 527.
22. GOEDHEER, J. H. C., *Biochim. et Biophysica Acta*, 1965, **88**, 304.
23. WEBER, G., *Nature*, 1961, **190**, 27.
24. GOVINDJEE, R., GOVINDJEE, and HOCH, G., *Plant Physiol.*, 1964, **39**, 10.
25. GOVINDJEE, and SPENCER, J., Paper presented at the 8th Annual Biophysics Congress, Chicago, February 1964; unpublished manuscript.
26. WEBER, G., and TEALE, F. W. J., *Tr. Faraday Soc.*, 1958, **54**, 640.
27. LATIMER, P., BANNISTER, T. T., and RABINOWITCH, E., in *Research in Photosynthesis*, (H. Gaffron, A. H. Brown, C. S. French, R. Livingston, E. I. Rabinowitch, B. L. Strehler, and N. E. Tolbert, editors), New York, Interscience Publishers, Inc., 1957, 107.
28. JACOBS, E. E., VATTER, A. E., and HOLT, A. S., *Arch. Biochem. and Biophysics*, 1954, **53**, 228.
29. STRAIN, H. H., 32nd Annual Priestley Lectures, The Pennsylvania State University Phi Lambda Upsilon and Associated Departments, University Park, Pennsylvania, 1958, 26-31.
30. OLSON, R. A., JENNINGS, W. H., and BUTLER, W. L., *Biochim. et Biophysica Acta*, 1964, **88**, 331.
31. KREY, A., and GOVINDJEE, *Proc. Nat. Acad. Sc.*, 1964, **52**, 1568.
32. BUTLER, W. L., *Arch. Biochem. and Biophysics*, 1961, **93**, 413.

33. TEALE, F. W. J., *Biochim. et Biophysica Acta*, 1960, **42**, 69.
34. BUTLER, W. L., *Arch. Biochem. and Biophysics*, 1961, **92**, 287.
35. BRODY, S. S., and BRODY, M., in *Photosynthetic Mechanisms of Green Plants*, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences—National Research Council Publication No. 1145, 1963, 455.
36. CEDERSTRAND, C., Ph.D. thesis, University of Illinois, 1965.
37. KREY, A., M.S. thesis, University of Illinois, 1965.
38. CLAYTON, R., *Science*, 1965, **149**, 1346.
39. ROBINSON, W., *Ann. Rev. Physic. Chem.*, 1964, **15**, 311.
40. KOK, B., and GOTT, W., *Plant Physiol.*, 1960, **35**, 802.
41. BANNISTER, T. T., and VROOMAN, M. J., *Plant Physiol.*, 1963, **39**, 105.
42. MYERS, J., and GRAHAM, J., *Plant Physiol.*, 1963, **38**, 105.
43. FRANCK, J., and ROSENBERG, J. L., in *Photosynthetic Mechanisms of Green Plants*, (B. Kok and A. T. Jagendorf, editors), Washington, D. C., National Academy of Sciences—National Research Council Publication No. 1145, 1963, 101.