MIGRATION OF ELECTRONIC ENERGY FROM CHLOROPHYLL *b* TO CHLOROPHYLL *a* IN SOLUTIONS

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ABSTRACT Absorption, emission, and fluorescence excitation spectra of pure solutions of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) in diethyl ether and of equimolecular mixed solutions of the two pigments, were determined at room temperature as functions of concentration (in the range from 5×10^{-6} M to 4×10^{-3} M) and of wavelength of the exciting light (in the regions 380–465 and 550–650 nm). The efficiency of energy transfer from Chl *b* to Chl *a*, derived from these data, was found to depend on the wavelength of exciting light. Furthermore, the transfer efficiency calculated from sensitization of Chl *a* fluorescence by Chl *b* was substantially smaller than that calculated from quenching of Chl *b* fluorescence by Chl *a*. Both these effects are tentatively explained as evidence of superposition of a "fast" energy transfer (taking place *before* the Boltzmann distribution of vibrational energy had been reached) upon the "delayed" transfer, which takes place *after* vibrational equilibration. The first-named mechanism is made possible by overlapping of the absorption bands of the two pigments; the second, by overlapping of the emission band of Chl *b* and the absorption band of Chl *a*. The first mechanism can lead to repeated transfer of excitation energy between pigment molecules, the second only to a one-time transfer from the donor to the acceptor. Both mechanisms could be of the same, second-order type, with the transfer rate proportional to r^{-6}. An alternative is for the fast mechanism to be of the first order, with the transfer rate proportional to r^{-3}, but spectroscopic evidence seems to make this alternative less probable.

INTRODUCTION

Since the work of Förster (1) and Galanin (2), the transfer of electronic excitation energy from an electronically excited "donor" molecule to a neighboring, unexcited "acceptor" molecule, has been studied in solutions, crystals, thin layers, and monolayers. In photosynthesis, this transfer is supposed to permit a "reaction center" in a chloroplast to utilize quanta absorbed in several hundred pigment molecules forming a so-called "photosynthetic unit" (see summary in Rabinowitch

and Govindjee [3]). Because of the importance of this quanta collection process for the high efficiency of photosynthesis, considerable attention has been devoted to energy transfer phenomena involving chlorophyll in vitro and in vivo. A recent summary by Hoch and Knox (4), and also some papers not reviewed by them (5–10), supported the validity of Förster's theory of energy transfer with a rate proportional to the inverse sixth power of distance, r^{-6}; however, the agreement between experiment and theory was not as close, in the case of the chlorophylls, as in many other dye systems studied in vitro.

As will be shown in this paper, the efficiency of energy transfer from Chl *b* to Chl *a* in solution depends on wavelength of the exciting light. Furthermore, the efficiency of transfer appears considerably greater when derived from quenching of Chl *b* fluorescence by Chl *a* than when derived from sensitization of Chl *a* fluorescence by Chl *b*. Both effects can be tentatively attributed to superposition of a "rapid" transfer preceding vibrational equilibration upon Förster's slower transfer mechanism, in which vibrational equilibration precedes the transfer.

EXPERIMENTAL

Experimental Methods

Chl *a* and Chl *b* were prepared from fresh spinach leaves and purified chromatographically as described by Jacobs et al. (11). Chlorophyll samples in diethyl ether were considered sufficiently pure if the ratios of the blue and red absorption maxima were 1.32 in Chl *a* and 3.00 in Chl *b*, and the ratios of absorbances in the red maxima to those at 505 nm (for Chl *a*) or 520 nm (for Chl *b*) were 45–50 and 18–20, respectively. Diethyl ether was chosen because in this solvent the chlorophylls appeared most stable in the dark and most resistant to photodecomposition in light. (An exposure of approximately 1 hr was necessary to complete measurements with each sample.)

Pure Chl *a* and Chl *b* solutions, as well as their equimolecular mixtures, were prepared with concentrations of 5×10^{-6}, 5×10^{-5}, 1×10^{-4}, 2×10^{-4}, 3×10^{-4}, 5×10^{-4}, 1×10^{-3}, 2×10^{-3}, and 4×10^{-3} M. Absorption spectra of these solutions were recorded with a Cary spectrophotometer (model 11145, Cary Instruments, Monrovia, Calif.), from 380 to 700 nm (see Fig. 1). The concentrations of the solutions used were determined from the height of the red absorption peak, assuming molar decadic absorption coefficients of 8.6×10^4 for Chl *a* and 5.1×10^4 M^{-1} cm^{-1} for Chl *b*. By means of thin metal (or Teflon) sheets, inserted between Pyrex glass plates, the thickness of the solution layer was adjusted so as to keep the optical density in the red peak between 0.3 and 0.8.

Fluorescence spectra, and action spectra for the excitation of fluorescence, were recorded with an instrument described by Murty and Frąckowiak (12). The light source was a 6 v, 18 amp tungsten ribbon filament lamp; the fluorescence detector was an RCA C-7268 photomultiplier (S-20 response, RCA Electronic Components, Harrison, N.J.). The fluorescence spectra, as well as the action spectra, were determined from the front surface with excitation wavelengths from 380 to 650 nm. The wavelength at which fluorescence intensity was measured was varied from 640 to 750 nm.

The thickness of the cuvette, *d*, was variable and chosen so as to keep the optical density in the red absorption peak below 0.1 (resulting in $d \leq 4 \times 10^{-4}$ cm for a 4×10^{-3} M solution), and thus limit reabsorption and secondary fluorescence to less than 1% of the primary

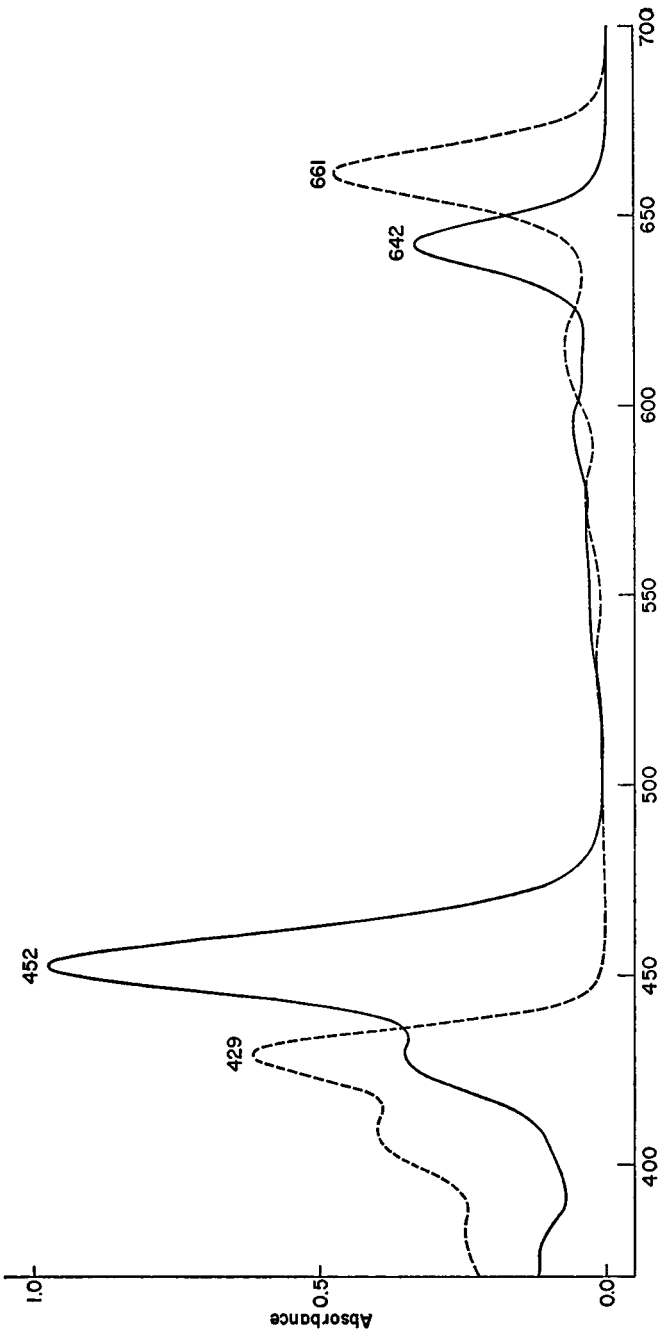

FIGURE 1 Absorption spectra of Chl *a* (dashed line) and Chl *b* (solid line) in diethyl ether. Concentration, 5×10^{-6} M. Wavelength in nanometers.

fluorescence (13, 14). For the exciting light actually used, the optical density of the solution was even less (except in the case of Chl *b* at 452 nm, in the maximum of the Soret band). One could thus assume, with an error of not more than a few per cent, that absorption was a linear function of depth.

The specially designed cuvette was made of brass with a quartz window (flat and parallel within a fraction of a micron). A second quartz plate could be made to approach the front window by means of a precise, parallel, fine (0.1 mm) thread in its holder.

Fluorescence spectra were, when necessary, corrected for the spectral response of the detection system. This system (monochromator set at a given slit width plus photomultiplier) was calibrated with a tungsten strip projection lamp of known color temperature. For correction of excitation spectra, and for quantum efficiency measurements, the spectral energy distribution of the illumination system (tungsten ribbon lamp plus monochromator at a given slit width) was measured by means of a proportional photon counter as described by Teale and Weber (15). Rhodamine B, fluorescein, and 5-aminoacridine were used for calibration of this counter. Low intensity monochromatic excitation (slit width 3.3 mμ) minimized exposure of pigment to light; to further reduce unnecessary illumination, the samples were screened from the exciting light when the latter was not needed. All spectra were found to be reproducible after the whole sequence of measurements. All measurements were carried out at room temperature.

The commonly used measure of excitation energy transfer is the quenching of donor fluorescence by the acceptor, i.e., the ratio η/η_0 (η being the yield of fluorescence of the donor in the presence of the acceptor, and η_0, that in its absence). The efficiency of transfer f_D is $1 - (\eta/\eta_0)$.

Several experimental methods for measuring f_D have been used. To check Förster's theory, systems were chosen in which the absorption and the emission spectra of the donor could be measured without substantial interference by those of the acceptor. One method, used in these experiments and described by Elkana et al. (16), permitted measurement of the fluorescence of pure donor, pure acceptor, and of their mixtures under constant geometric conditions. In Elkana's study, high optical densities were used; therefore, radiative transfer (emission and reabsorption of fluorescence) had to be taken into account. If, however, low optical densities are used, making radiative transfer negligible, this method can be used to determine the efficiency of energy transfer also between dyes with overlapping absorption and emission bands, such as the two chlorophylls; we used it in our study.

Evaluation of Transfer Efficiency

Let the emission be measured from the illuminated front face of the solution cell, at an angle of 90° to the incident beam. We designate the photon flux of the incident light as n (sec^{-1}). The fluorescence intensity, observed in the maximum of the fluorescence spectrum of a solution containing a single dye (as indicated by superscript 1), is given for the donor (D) and the acceptor (A) respectively by:

$$^1F_D = anq_D\epsilon_D cd, \tag{1}$$

$$^1F_A = anq_A\epsilon_A cd, \tag{2}$$

where ϵ is the molar extinction coefficient, c is the molar concentration of the dye, d is the thickness of the solution layer (the product ϵcd is the optical density), q is the

fluorescence quantum yield, and a is a geometrical factor. The fluorescence of the donor in the presence of the acceptor (and vice versa), indicated by the superscript 2, should obey equations 3 and 4 respectively:

$$^2F_D = {}^1F_D\,(1 - f_D), \tag{3}$$

$$^2F_A = {}^1F_A + (q_A/q_D)\,{}^1F_D f_A\,, \tag{4}$$

where f_A is the efficiency of energy transfer from D to A as determined from the sensitization of acceptor fluorescence, and f_D, the same efficiency as derived from the quenching of donor fluorescence. (Usually, it is assumed that $f_A = f_D$, but we will leave the validity of this equality open.) Dividing equation 3 by ${}^1F_D$ one gets

$$1 - f_D = {}^2F_D/{}^1F_D = \eta/\eta_0\,, \tag{5}$$

(since, under our experimental conditions, the fluorescence intensities F are proportional to fluorescence yields η). Dividing equation 4 by ${}^1F_A$, and taking into account equations 1 and 2, we get:

$$f_A = (\epsilon_A/\epsilon_D)\,[({}^2F_A/{}^1F_A) - 1]. \tag{6}$$

Equations 5 and 6 are simplified forms of those given by Watson and Livingston (17), Bowen and Livingston (18), Elkana et al. (16), or Wilkinson (19) (Appendix); they can be used only if the emission spectra of the donor and acceptor solutions do not overlap. Otherwise, the fluorescence spectrum of a mixture of donor and acceptor must be analyzed in terms of the known emission bands of pure donor and pure acceptor, to calculate ${}^2F_D$ and ${}^2F_A$ in equations 5 and 6. This analysis is best done by means of a computer. From the so-calculated intensities ${}^2F_D$ and ${}^2F_A$ we can calculate f_D and f_A in equations 5 and 6. The two calculations are independent, and no assumption is made that the same values of f are involved in equations 5 and 6.

If, however, each excitation energy transfer from Chl *b* to Chl *a* leads to emission of Chl *a* fluorescence with the same quantum yield q_A as direct excitation, f_D and f_A must be identical and the efficiency of transfer can be calculated directly from the intensities of overlapping emission spectra, as follows. The observed total fluorescence intensity of the mixture (indicated by the superscript M, for "mixture") is in the maximum of the donor fluorescence,

$$^MF_D = {}^2F_D + b\,{}^2F_A\,, \tag{7}$$

where b is the ratio of fluorescence intensity of the acceptor in the maximum of donor fluorescence to its intensity ${}^1F_A$ in its own maximum. In the maximum of the acceptor fluorescence, it is similarly:

$$^MF_A = {}^2F_A + \beta\,{}^2F_D\,. \tag{8}$$

Dividing equations 7 and 8 by ${}^1F_D$ and ${}^1F_A$, respectively, one gets:

$$({}^MF_D/{}^1F_D) = ({}^2F_D/{}^1F_D) + b({}^2F_A/{}^1F_D), \tag{9}$$

and

$$({}^MF_A/{}^1F_A) = ({}^2F_A/{}^1F_A) + \beta({}^2F_D/{}^1F_A). \tag{10}$$

Taking into account that $b = {}^1F_{AD}/{}^1F_A$ (where ${}^1F_{AD}$ is the fluorescence intensity of the acceptor observed at the wavelength of the fluorescence peak of the donor), and similarly $\beta = {}^1F_{DA}/{}^1F_D$, the transfer efficiency can be calculated from equations 9 and 10, using equations 3 and 4 to eliminate the intensities ${}^2F_D$ and ${}^2F_A$, as:

$$f_D = \frac{{}^MF_D - {}^1F_D - {}^1F_{AD}}{{}^1F_{AD}(\epsilon_D/\epsilon_A) - {}^1F_D}, \tag{11}$$

$$f_A = \frac{{}^MF_A - {}^1F_A - {}^1F_{DA}}{{}^1F_A(\epsilon_D/\epsilon_A) - {}^1F_{DA}}. \tag{12}$$

In order to use the above equations, the geometrical factor a, the intensity and the wavelength of the exciting light, all concentrations, as well as the sample thickness, must remain constant during each set of measurements.

If the f values obtained from equations 11 and 12 turn out to be different (as we will find to be the case in Chl a + Chl b solutions), their meaning becomes unclear and one has to go back to the more elaborate method of analysis, using equations 5 and 6.

Corrections

At the higher concentrations (in our experiments, concentrations up to 4×10^{-3} M were used), formation of dimers could introduce a complication. To check this point, the absorption, emission, and excitation spectra of Chl a and Chl b were determined at two concentrations, 5×10^{-5} and 4×10^{-3} M (Fig. 2). In accordance with a previous report (20), these spectra showed only small effects of concentration (marked somewhat more strongly in the Soret band than in the red band, particularly in Chl b). In agreement with Watson and Livingston (17), the concentration quenching of the fluorescence of chlorophyll in ether also was found to be negligible up to 4×10^{-3} M.

The coincidence of absorption spectra of both chlorophylls with their fluorescence excitation spectra suggested constancy of the quantum yield of fluorescence over the region used (which covered the red and the Soret band), up to 10^{-3} M.

The emission spectra of Chl a and Chl b solutions, at concentrations of 5×10^{-5} and 4×10^{-3} M, differ slightly, especially in the case of Chl b, where broadening and enhancement of the long wave peak is observed at the higher concentrations.[1] These,

[1] Because comparison of the spectra was made at constant optical density this effect could not be attributed to preferential reabsorption of the first fluorescence band.

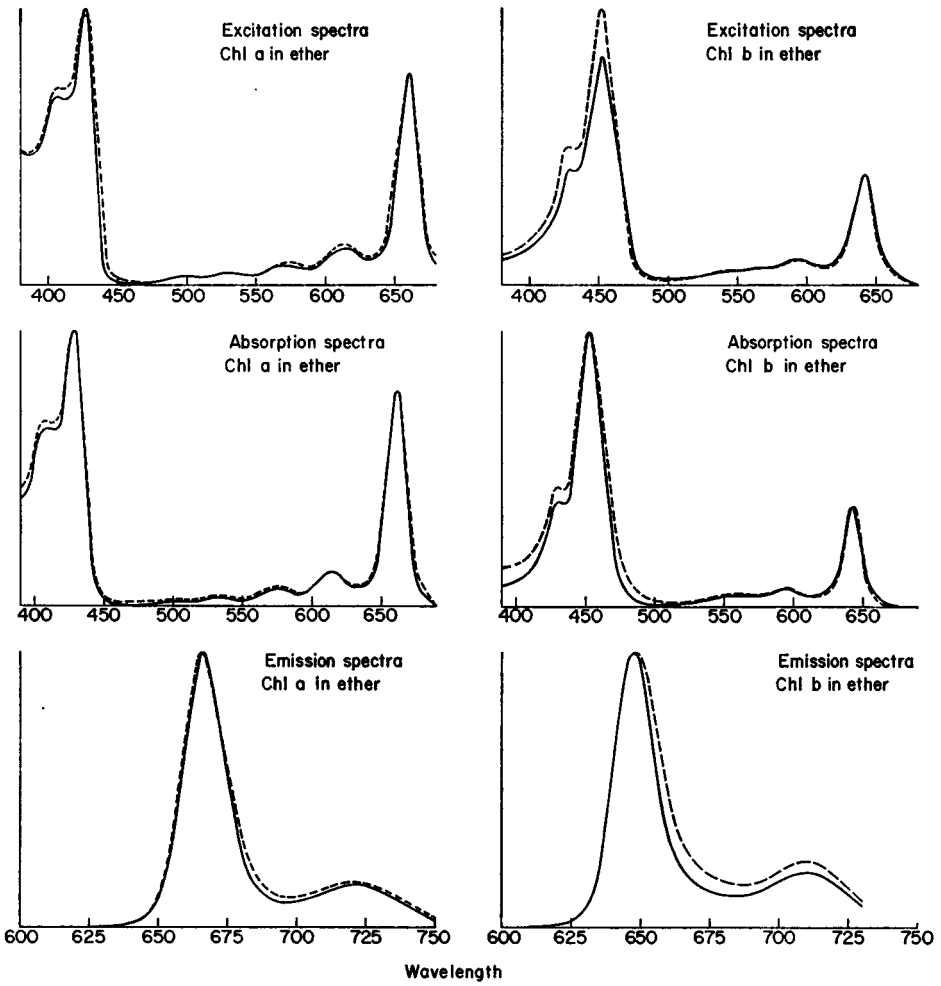

FIGURE 2 Excitation, absorption, and emission spectra of Chl *a* and Chl *b* solutions in diethyl ether for two concentrations of the pigments: 5×10^{-6} M (solid lines) and 4×10^{-8} M (dashed lines). Wavelength in nanometers; ordinate, relative scales; the two curves in each figure were arbitrarily adjusted at convenient λ's.

however, like all the other concentration effects we have observed, were minor, so that we felt entitled to consider dimer formation as of negligible importance in evaluating the efficiency of energy transfer from Chl *b* to Chl *a*.

The fluorescence intensities in equations 11 and 12 were measured at the same wavelength; therefore, no correction for the spectral sensitivity of the detector system was necessary. Since spectral bands of marked width, $\Delta\nu$, were used for excitation, in which the two absorption curves varied differently, the ratio (ϵ_D/ϵ_A) in equations 6, 11, and 12 was determined (assuming the fluorescence quantum

yield ratio of Chl *a* and Chl *b*, $q_A/q_D = 2.25$ [21], to be independent of the excitation wavelengths) from the fluorescence intensity ratios ${}^1F_D/{}^1F_A$, corrected for the spectral response of the detector (these ratios being, under our experimental conditions, equal to $\epsilon_D q_D/\epsilon_A q_A$).

RESULTS

The emission spectra of pure Chl *b*, pure Chl *a*, and their mixtures in the concentration range from 5×10^{-6} to 4×10^{-3} M, with exciting light of different wavelengths (650, 635, 625, 570, 550, 465, 452, 429, 400, or 380 nm), were measured three times using fresh chlorophyll preparation each time, and the mean values of intensities in the peak of the emission bands, ${}^1F_D$, ${}^1F_A$, ${}^1F_{DA}$, ${}^1F_{DA}$, ${}^MF_D$, and ${}^MF_A$, were determined.

We first attempted to evaluate the results (obtained at $\lambda_{exc} = 650, 625, 452, 429$ nm) by the simplified procedure, assuming $f = f_D = f_A$, i.e., using equations 11 and 12. The resulting ratios, $(\eta/\eta_0) = 1 - f$, are plotted in Fig. 3 *a* vs. the logarithm of concentration. Clearly, the ratios (η/η_0), calculated from the quenching of donor fluorescence, according to equation 11, are, in Fig. 3 *a*, at all the excitation wavelengths used, substantially lower than those calculated from the sensitization of the acceptor fluorescence according to equation 12. In other words, the quenching of Chl *b* fluorescence by Chl *a*, attributable to energy transfer, is not fully compensated by sensitization of the fluorescence of Chl *a*, as presumed in equation 4. We note in Fig. 3 *a*, however, that the closest pair of curves, that marked by crosses, corresponds to $\lambda_{exc} = 452$ nm, a spectral region where absorption by Chl *a* is very weak, so that energy transfer from Chl *b* and Chl *a* can be expected to occur predominantly by the Förster mechanism (at the other wavelengths used, absorption by Chl *a* is almost equal to that by Chl *b*).

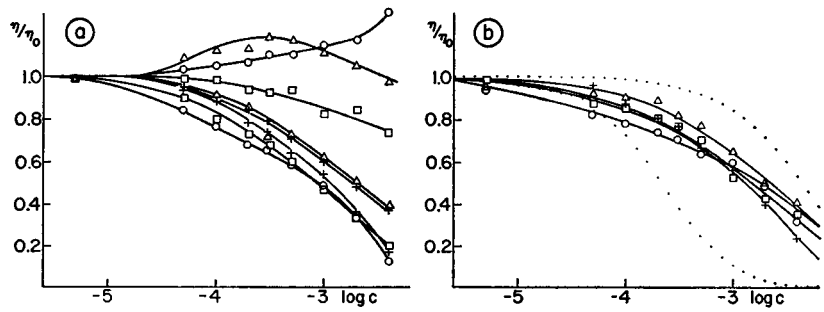

FIGURE 3 The concentration dependence (for different excitation wavelengths: △, −650 nm; □, −625 nm; +, −452 nm; ○, −429 nm) of the ratios η/η_0. (*a*) The four lower curves (△, □, +, ○), calculated by means of equation 11; the four higher ones (△, □, +, ○), by means of equation 12. (*b*) Solid curves, calculated from equation 5 ($\eta/\eta_0 = {}^2F_D/{}^1F_D$); dotted curves calculated from Förster's equation (equation 17), with $c_0 = 5 \times 10^{-4}$ M (upper) and $c_0 = 10^{-2}$ M (lower), respectively.

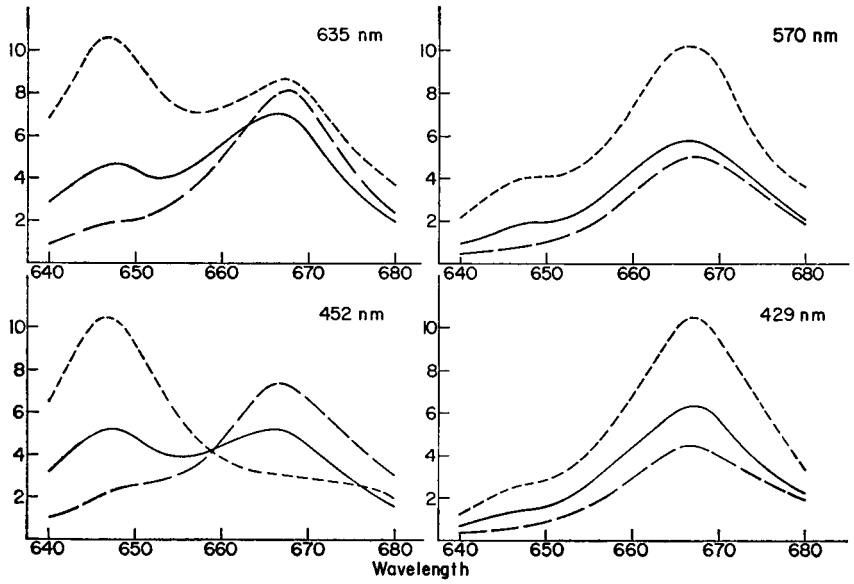

FIGURE 4 Emission spectra of mixed (equimolecular) Chl *b* and Chl *a* solutions in diethyl ether for different excitation wavelengths (635, 570, 452, 429 nm) and concentrations (——— ——— ———, $c = 4 \times 10^{-3}$ M; ———, $c = 5 \times 10^{-4}$ M; - - - - - - - - -, $c = 5 \times 10^{-5}$ M). Abscissa, wavelength in nanometers; ordinate fluorescence intensity, relative scale.

Because of the failure of equations 11 and 12 to give the same f values, we must return to equations 5 and 6 and calculate f_D (the meaning of f_A is becoming unclear in this case) by first analyzing the emission bands and calculating ${}^2F_D$ and ${}^2F_A$, as suggested previously.

The emission spectra of mixed (Chl *b* + Chl *a*) solutions are shown in Fig. 4 for three concentrations (5×10^{-5}, 5×10^{-4}, and 4×10^{-3} M) and four excitation wavelengths (635, 570, 452, and 429 nm). This figure shows strong overlapping of the two emission bands, and the predominance of Chl *a* fluorescence at all but the lowest concentration used (5×10^{-5} M). Striking is the (already mentioned) relatively small increase of Chl *a* fluorescence with rising concentration, compared with the strong decrease of Chl *b* fluorescence.

The emission spectra in Fig. 4 were analyzed into their components, and the fluorescence intensities ${}^2F_D$ and ${}^2F_A$ calculated. The results are given in Table I as ratios of fluorescence intensities of each component in mixed and in pure solutions ($\phi_D = {}^2F_D/{}^1F_D = 1 - f_D$ for Chl *b*, and $\phi_A = {}^2F_A/{}^1F_A$ for Chl *a*); they are plotted for several wavelengths in Fig. 3 *b*. Because of the similarity of results obtained within certain wavelength regions, Table I contains, in addition to values obtained at $\lambda_{exc} = 650$ nm, only mean values of those found in the regions 550–635, 452–465, 429–436, and 380–400 nm.

To illustrate the relation between decrease of donor and increase of acceptor

TABLE I

λ_{exc}	650 nm			550–635 nm			452–465 nm			429–436 nm			380–400 nm		
c	ϕD	ϕA	S	ϕD	ϕA	S	ϕD	ϕA	S	ϕD	ϕA	S	ϕD	ϕA	D
M															
5×10^{-6}	0.95	0.98	0.95	0.96	1.04	0.96	0.99	1.32	0.85	0.95	0.97	0.93	0.98	1.00	0.99
5×10^{-5}	0.92	0.98	0.94	0.89	1.07	0.84	0.95	2.42	0.60	0.86	0.93	0.82	0.94	0.98	0.96
10^{-4}	0.90	0.96	0.91	0.85	1.09	0.81	0.88	4.38	0.51	0.81	0.92	0.78	0.91	0.96	0.92
2×10^{-4}	0.88	0.96	0.90	0.79	1.12	0.76	0.81	6.20	0.53	0.76	0.91	0.73	0.89	0.93	0.90
3×10^{-4}	0.82	0.95	0.87	0.75	1.15	0.72	0.76	9.68	0.59	0.72	0.90	0.70	0.87	0.91	0.87
5×10^{-4}	0.77	0.94	0.84	0.67	1.25	0.70	0.67	13.70	0.62	0.64	0.93	0.68	0.82	0.90	0.84
10^{-3}	0.65	0.94	0.79	0.53	1.41	0.66	0.56	19.44	0.67	0.57	0.95	0.66	0.74	0.86	0.78
2×10^{-3}	0.50	0.93	0.74	0.41	1.57	0.65	0.40	26.27	0.68	0.46	0.97	0.62	0.63	0.80	0.70
4×10^{-3}	0.41	0.89	0.68	0.32	1.68	0.64	0.24	33.50	0.68	0.30	0.98	0.55	0.50	0.75	0.62

excitation, caused by energy transfer, we can use the ratio $S = \phi_A/\phi_A\ (D)$, where $\phi_A\ (D)$ is the ratio of intensity of acceptor fluorescence in the presence of the donor to that in its absence, as derived from equation 6, and ϕ_A is the actually observed value of the same ratio.

$$\phi_A(D) = 1 + (\epsilon_D/\epsilon_A) f_D\,, \tag{13}$$

$$S = \phi_A/\phi_A(D). \tag{14}$$

Table I shows the mean values of S for the five above-mentioned wavelength regions as function of concentration. All values of S in Table I are less than 1, i.e., the fluorescence of Chl *a* is always less than expected from quenching of the fluorescence of Chl *b*. In fact, in some cases, ${}^2F_A < {}^1F_A$, i.e. the Chl *a* fluorescence is quenched together with that of Chl *b*, so that ϕ_A itself plunges below 1, particularly at 380–400 nm.

To compare the results with the predictions of Förster's theory, we recall that, according to Förster, the efficiency of energy transfer from a given donor molecule to a given acceptor molecule is generally

$$f = \frac{(R_0/R)^j}{1 + (R_0/R)^j} = \frac{(c/c_0)^{j/3}}{1 + (c/c_0)^{j/3}}, \tag{15}$$

where j is the exponent in the expression $U = \text{const}\ R^{-j}$ for the interaction energy. A similar equation is given by Duysens (22) for two- and three-dimensional lattices. Latt et al. (23) used equation 14 to confirm the validity of the r^{-6} distance law for energy transfer between two different dye molecules separated by several "bisteroid" molecular layers, and Stryer and Haugland (24) did the same for energy transfer between two different chromophoric groups separated by carbohydrate chains of varying length.

Equation 15 may be rewritten as

$$\log\,[(1/f) - 1] = j/3 \log c_0 - j/3 \log c. \tag{16}$$

The exponent in equation 15 can be found from the slope of the plot of log $[(1/f) - 1]$ vs. c. For random distribution of molecules in solutions, the so-calculated exponent is not identical with the exponent characterizing the dependence of energy transfer on distance between two molecules. Equation 16, however, may be used to compare the experimental results with predictions derived from Förster's equation:

$$f = \sqrt{\pi}\, c/c_0 \exp (c/c_0)^2 [1 - \text{erf}\,(c/c_0)], \qquad (17)$$

where erf (c/c_0) is the gaussian error function. The results of such a comparison are shown in Fig. 5, where experimental results obtained in the wavelength regions 380–400, 452–465, and 550–650 nm, are plotted together with a "theoretical" curve (calculated from equation 17 for $c_0 = 10^{-3}$ M). The latter curve is obtained by calculating numerical values of f from equation 17 and plotting them as log $[(1/f) - 1]$ vs. concentration; as a result one gets a fairly straight line with a slope that decreases in the concentration region 3×10^{-3}–10^{-5} M from 1.3 to 1.1. For concentrations from 2×10^{-4} to 4×10^{-3} M, the four experimental curves are practically straight lines, with the slopes 0.76, 0.9, 0.75, and 0.87, respectively, while the slope of the theoretical curve in the same region is 1.2 (variation in c_0 will cause only a small change in the slope; for $c_0 = 10^{-2}$ M, for instance, the slope decreases from 1.2 to 1.0 when c is varied in the above-mentioned concentration region). This may mean either that the transfer dependence on distance follows, in the case of the two chlorophylls, a law

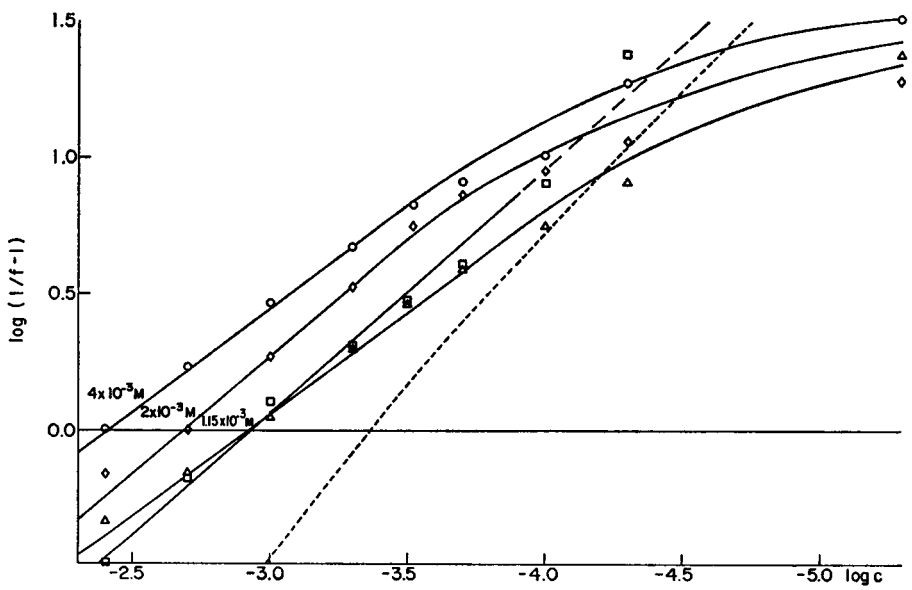

FIGURE 5 The log of $[(1/f] - 1]$ values plotted vs. concentration for different wavelengths: ○, 380–400 nm; ◇, 650 nm; □, 452–465 nm; △, 550–635 nm. The dashed curve is obtained from Förster's equation (equation 17), with $c_0 = 10^{-3}$ M.

containing r^{-x} with $x < 6$, for example, $x = 3$; or that because of repeated back and forth transfer the final, net transfer follows not a simple r^{-6}, but a more complicated function.

Under these conditions, Förster's "critical concentration" c_0 cannot be determined from the experimental curves. The "half-transfer" concentration $c_{1/2}$ is 4×10^{-3} M in the 380–400 nm excitation region; at 650 nm, it is 2×10^{-3} M; in all other excitation regions studied, 1.1×10^{-3} M.

DISCUSSION

The results presented in the preceding section suggest that excitation energy transfer in the system Chl *b* + Chl *a* is not adequately described by Förster's mechanism alone. On the other hand, Förster's r^{-6} law has been confirmed experimentally for many dye couples used in measurements of sensitized fluorescence. There is also a case in vivo which apparently follows Förster's law: energy transfer from phycoerythrin to Chl *a* in red algae (25). Here, as in the studies of sensitized fluorescence in vitro, little overlapping of the absorption bands exists. (Phycoerythrin absorbs most strongly in green, midway between the two main absorption bands of chlorophyll.) This is not true of the two chlorophylls, where both types of overlapping are strong (cf. Fig. 1). The situation is still more complex in chloroplasts, which also contain, in addition to two chemically different chlorophylls, Chl *a* and Chl *b*, several spectroscopically different forms of Chl *a* (3).

It will be further noted that the system Chl *a* + Chl *b* has also a second spectroscopic property not found in other dyestuff pairs used in the study of energy transfer. In addition to the overlapping of the absorption bands of the two components, each of them has (at least) *two* excited electronic levels contributing to the visible absorption spectrum, corresponding to the "red" and the "violet" (or Soret) bands; the relative location of the bands of the two components is, in the case of the "violet" band, the reverse of that in the "red" one (cf. Fig. 4). This second complication could be eliminated by extending the study to pigment pairs with overlapping absorption bands, but with only one excited electronic level, such as thionine and methylene blue; this we plan to do in the future.

In the present discussion, we consider only the possible role in the observed quenching and sensitization phenomena, of "fast" transfer, completed before vibrational relaxation, in addition to Förster's "slow" transfer from vibrationally equilibrated states of the donor (cf. references 25 and 26). This could be either a "first-order" r^{-3} proportional transfer, or a second kind of r^{-6} proportional transfer, which becomes possible when the rate of vibrational equilibration is not fast compared with that of energy transfer (27). Because of the lack of strong concentration effect on the absorption band (which seems to exclude a stronger than "very weak" interaction between excited and nonexcited molecules) the second explanation seems more plausible.

In agreement with this suggestion, experimental curves in Fig. 3 *b* follow approximately Förster's curve with $c_0 = 5 \times 10^{-4}$ M at the lower concentrations (10^{-4}–10^{-5} M) and approach Förster's curve with $c_0 = 10^{-2}$ at the higher concentrations. A better illustration is provided by Fig. 5. It shows that the difference between the experimental and "theoretical" slopes is smallest in the region 452–465 nm (□ curve), where overlapping of the two absorption bands is minimal, and Förster's simple equation is therefore most likely to apply. The discrepancy increases at the shorter waves, where this overlapping is stronger.

Förster's energy transfer theory implies that energy can not oscillate repeatedly between two molecules, but is transferred ireversibly from one to the other. (Vibrational equilibration takes place in the acceptor molecule so rapidly as to make return transfer to the donor impossible.) If the rate constant of vibrational relaxation is assumed to be of the order of 10^{12} sec^{-1}, the transfer time must be, in this case, greater than 10^{-11} sec. If the transfer rate is somewhat faster, say, of the order of 10^{12} sec^{-1}, several transfers can occur before (or during) vibrational relaxation, particularly if they involve the loss of a considerable number of vibrational quanta. Such repeated transfers can occur either between the same two molecules, or they can involve a sequence of them ("energy migration"). Explanation of the second unexpected effect observed in the Chl *b* and Chl *a* system, the difference between the transfer rates derived from the quenching of Chl *b* fluorescence and those calculated from sensitization of Chl *a* fluorescence, may be sought in such migration.

As postulated by Förster in the interpretation of concentration quenching of fluorescence, energy migration, covering a considerable number of molecules, permits an otherwise insignificant number of energy "traps" (such as dimer molecules) to exert a marked quenching effect; the same type of energy loss may occur in repeated transfer between Chl *b* and Chl *a* and cause the fluorescence of Chl *a* to be quenched, together with that of Chl *b*.

Also to be considered is the probability of a relatively high concentration of *mixed* Chl *a* and *b* dimers. We found, however, that the absorption spectra of Chl *a* + Chl *b* solutions in ether are very similar at concentrations of 5×10^{-5} and 4×10^{-3} M (Fig. 6) thus making a marked concentration of mixed dimers unlikely.

The suggested tentative interpretation of energy loss during Chl *b* → Chl *a* energy transfer process requires postulation of a more rapid energy transfer between unlike molecules Chl (*b* → *a*) and back, compared with symmetric pairs (Chl *a* → Chl *a*, Chl *b* → Chl *b*). (Otherwise, a fast energy exchange would have to be postulated also in one-pigment systems; and yet mutual quenching of the two chlorophylls was observed in our experiments down to 10^{-4} M, whereas concentration quenching in pure Chl *a* or pure Chl *b* solutions becomes noticeable only above 5×10^{-3} M.) This suggestion requires further analysis. The fact that overlapping between the fluorescence band of Chl *b* and the absorption band of Chl *a* is, because of the Stokes shift, more efficient than that between the fluorescence and the absorption band of each pigment is not a sufficient explanation, because this type of overlapping is important

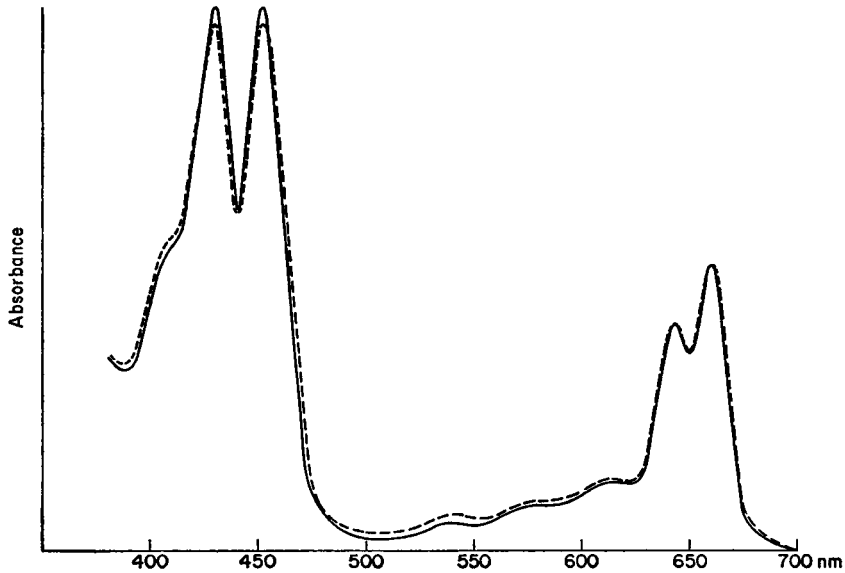

FIGURE 6 Absorption spectra of mixed Chl *a* and Chl *b* solutions in diethyl ether for two concentrations, 5×10^{-5} M (solid line) and 4×10^{-3} M (dashed line).

only for transfer occuring *after* vibrational equilibration. Obviously, additional experiments are needed for more complete explanation of our findings.

This paper is presented here to honor Professor Eugene Rabinowitch in whose laboratory this work was done.

We wish to thank the Chemistry Department of the State University of New York at Albany for its hospitality.
Dr. Bauer is grateful to Professor A. Jabonśki for discussions of the mechanism of energy transfer.
We thank Professor E. Rabinowitch upon whose suggestion this investigation was carried out, and for his continued interest in it.

We gratefully acknowledge the financial support of the National Science Foundation.

Received for publication 14 June 1971.

APPENDIX

Expressing, after Wilkinson (19), the ratio of the fluorescence intensities of the donor in the absence and in the presence of the acceptor as

$$ {}^1F_D/{}^2F_D = 1 + (k_{10}A/k_2 + k_3) = 1/(1 - f), \qquad (A\,1) $$

(where k_{10} is the rate constant of energy transfer, A is the acceptor concentration, and k_2 and k_3 are the rate constants of fluorescence and nonradiative energy dissipation, respectively) and comparing equation A 1 with equation 5, one gets:

$$ k_{10}A/k_2 + k_3 = [1/(1 - f)] - 1 = f/(1 - f). \qquad (A\,2) $$

Thus, Wilkinson's equation for the ratio of fluorescence intensities of the acceptor in the presence and in the absence of the donor,

$$^2F_A/^1F_A = 1 + (\epsilon_D/\epsilon_A)(k_{10}A/k_2 + k_3 + k_{10}A)$$
$$= 1 + f(\epsilon_D/\epsilon_A), \qquad (A\,3)$$

is equivalent to our equation 8.

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