Lifetime of the Excited State (τ) *in vivo* III. Chlorophyll during Fluorescence Induction in *Chlorella pyrenoidosa**

J.-M. BRIANTAIS**, H. MERKELO ** and GOVINDJEE****

Departments of Botany and Electrical Engineering, University of Illinois, Urbana, Illinois 61801, U.S.A.

Abstract

The lifetime of the excited state of chlorophyll (τ) was measured with the phase-delay method using a *He-Ne* mode-locked laser operated at a frequency of 102·207 MHz (100 W m^{-2}; $\lambda =$ $= 632\cdot8$ nm). Simultaneous τ and relative φ (quantum yield of fluorescence) measurements, at different points on the fluorescence transient curve, revealed a linear relationship between the two quantities. This linear relationship between τ and φ was found in (*1*) a *Chlorella* suspension flowing at different rates, (*2*) a flowing *Chlorella* suspension preirradiated with different radiant flux densities, and (*3*) in a stationary suspension treated with different concentrations of 3-(3',4'-dichlorophenyl)-1, 1'-dimethyl urea (DCMU). Within an error of $\pm0\cdot1$ ns, $\tau = \tau_0 \ \varphi$ relationship is verified *in vivo*. — We confirm the independent findings of TUMERMAN and SOROKIN (1967)[†]. These data may suggest that the statistical ("lake" or "multicentral") model of the photosynthetic units is to be preferred over the physical ("isolated puddles" or "unicentral") model. Furthermore, our preliminary measurements show that τ as a function of wavelength of observation stays constant except near 710 nm where it shows a small but significant dip.

The yield of chlorophyll fluorescence (φ) *in vivo* varies in a characteristic way during the first moments of the illumination (the Kautsky effect; see KAUTSKY and HIRSCH 1931; LAVOREL 1959; DELOSME 1967; MUNDAY and GOVINDJEE 1969; GOVINDJEE and PAPAGEORGIOU 1971). The yield increases from an initial level (0) to a maximum level (*P*); this increase in the yield is related to the reduction of a quencher of fluorescence Q (DUYSENS and SWEERS 1963). When all Q is in its oxidized state, the yield is low and when all Q is in reduced state the yield is high. LAVOREL (1963) has shown that the kinetic heterogeneity of fluorescence is accompanied by a spectroscopic heterogeneity of the chlorophyll species; the ratio of the variable (*P*-0) to the constant (0) fluorescence

---

shows a minimum at 710—720 nm. Recently, GOVINDJEE and BRIANTAIS (1972) have shown maxima at 665 and 700 nm in addition to the maxima at 685 nm observed earlier. Most of the variable fluorescence seems to be associated with the pigment system II (BUTLER and BISHOP 1963; DUYSENS and SWEERS 1963; LAVOREL 1963). BRIANTAIS (1966) and VREDENBERG and SLOOTEN (1967) demonstrated that subchloroplastic particles enriched in system II show a higher variable to constant fluorescence than the chloroplasts. On the other hand, system I enriched particles have a very small or no variable fluorescence component.

The "measured" lifetime of the excited state of chlorophyll a (τ) is proportional to the quantum yield of fluorescence (φ) as $\tau = \varphi\tau_0$, where τ_0 is the natural lifetime. Thus, during the induction of fluorescence, when φ changes, and if the quenching is of dynamic type, we expect τ to change accordingly. The "physical" and the "statistical" models of photosynthetic units predict different results (see Discussion). For example, in the "statistical" ("lake" or "multicentral") model in which all traps are accessible to any exciton in any photosynthetic unit, we expect the τ to change linearly with φ. In the "physical" ("isolated puddle" or "unicentral") model in which exciton in one photosynthetic unit can reach only the trap in that unit, we expect a non-linear relationship.

MÜLLER, LUMRY and WALKER (1969) observed that for the stationary state τ increases in *Chlorella* from 0·6 ns to 1·5 ns with increasing irradiance. MÜLLER and LUMRY (1965) observed a doubling of τ when *Chlorella* cells were poisoned with DCMU. Using mode-locked *He-Ne* laser, MERKELO et al. (1969) confirmed these results. We present here a direct measurement of the relationship between τ and (relative) φ during the induction of fluorescence of *Chlorella*. Our results are in agreement with the recent independent findings of TUMERMAN and SOROKIN (1967). We will also present the differences between their experiments and ours.

Chloroplasts contain two pigment systems (see RABINOWITCH and GOVINDJEE 1969). System II, as mentioned above, is responsible for most of the fluorescence at room temperature. System I, however, is weakly fluorescent; it has a band on the long-wave side of the main system II band at 685 nm (see GOVINDJEE, PAPAGEORGIOU and RABINOWITCH 1967; BROWN 1969; MOHANTY et al. 1972). MÜLLER, LUMRY and WALKER (1969) measured τ as a function of wavelength with broad band interference filters and concluded that τ was independent of wavelength. Preliminary measurements, using a monochromator with slits having half-band widths of 7 nm, also suggest that τ as a function of λ is nearly constant (within 10—15%); however in these experiments, a small but a significant dip at 710 nm was observed.

MATERIALS AND METHODS

Chlorella pyrenoidosa (EMERSON's strain *3*) was grown in an inorganic medium under low illuminance with white light as described elsewhere (GOVINDJEE and RABINOWITCH 1960). The absorbance of *Chlorella* suspensions was measured by a *Bausch* and *Lomb* spectrophotometer equipped with an integrating sphere attachment. The absorbance of suspensions used in this work ranged from 0·10 to 0·50 at 675 nm for 1 cm path, but the path length of the fluorescence measurements was only 2 mm. In contrast, TUMERMAN and SOROKIN (1967) used a 3 mm layer and the suspension absorbed 90% light. However, they found that dilution by 3 and 5 fold did not cause changes in the results. Our suspensions were much more dilute than theirs.

To obtain different points on the fluorescence induction, we used variable flow based on the flow method of LAVOREL (1965) (Fig. 1). In this method, when the rate of flow of the suspension is fast enough (1·2 ms per cm through a capillary tubing of 2 mm diameter), the total number of quanta (intensity $\times$ time) absorbed by the sample is too small to induce any significant reduction of Q to QH, *i.e.*, we can measure the fluorescence at the "0" level. If the rate of flow is increased further, the yield of fluorescence does not change. Also, under these conditions of high rate of flow, the fluorescence intensity stays linearly proportional to the radiant flux density, and should not change with the addition of DCMU. This was the case in our experiments. In

contrast to TUMERMAN and SOROKIN (1967) we have explored the complete induction curve from   135 "0" to "*P*".

In order to establish different pseudo-stationary states of the yield, corresponding to different points of the induction curve, two methods were employed. First, τ and φ were varied by changing the rate of flow between the maximum flow and "no flow"*. Second, τ and φ were varied by pretreating the flowing-suspension with different radiant flux densities ("white light"). With this method, different levels of oxidation reduction state of Q are reached. Third, different values of quantum yield of fluorescence were maintained by adding different concentrations of DCMU; in these experiments, the suspension does not flow and receives a high irradiance of 632·8 nm from the laser.
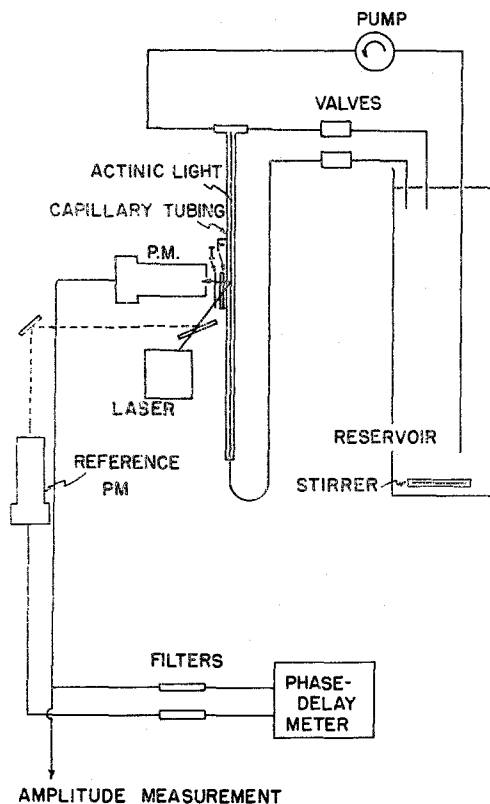

Fig. 1. Block diagram of the apparatus for measuring lifetime (τ) versus quantum yield (φ) of fluorescence.

τ was measured by an instrument described by MERKELO *et al.* (1969). Mode locked *He-Ne* laser (λ 632·8 nm; 100 W m^{-2}) provided the incident radiation. The frequency of modulation was 102·207 MHz. This high frequency (f) of modulation eliminated artifacts due to delayed fluorescence. TUMERMAN and SOROKIN (1967) had used a Hg light source (λ 436 nm, unknown radiant flux density) and the frequency of modulation was 13·4 MHz in their experiments.

The phase delay ($\Delta\varphi$) between the incident radiation and the fluorescent beam was measured and τ was calculated from the relationship $\tau = \tan \Delta\varphi/2\pi f$. The zero of the phase was adjusted by using radiation scattered by the sample; the flux density of this scattered radiation was adjusted to be the same as that of the fluorescent radiation. A *Corning C. S. 2-64* was used before the photomultiplier (*S20*) when measuring fluorescence. A 633 nm interference filter was used instead of *C. S. 2-64* when measuring scattering. In both cases, an iris of 1 mm diameter was placed in front of the photomultiplier.

---

* By varying the flow, we integrate between "0" and some intermediate state between "0" and "*P*", and thus obtain a mixed continuous range of values of φ and τ.

136   Lifetime of fluorescence (τ) as a function of the wavelength (λ) of the emission was measured by using as radiation source a powerful mode-locked *He-Ne* lase (450 W m^{-2}, λ 632·8 nm, 78 MHz). Fluorescence, emitted by algae, passed through a *C. S. 2-64 Corning* filter, and a monochromator (*Hilger-Watts*; half band width, 7 nm) before reaching the photomultiplier.

RESULTS

Lifetime as a function of the relative yield of fluorescence

A plot of τ versus φ as obtained by varying the rate of the flow of the *Chlorella* suspension shows a linear relationship (Fig. 2). This was confirmed in another experiment in which τ and φ were varied by preirradiating a flowing suspension of
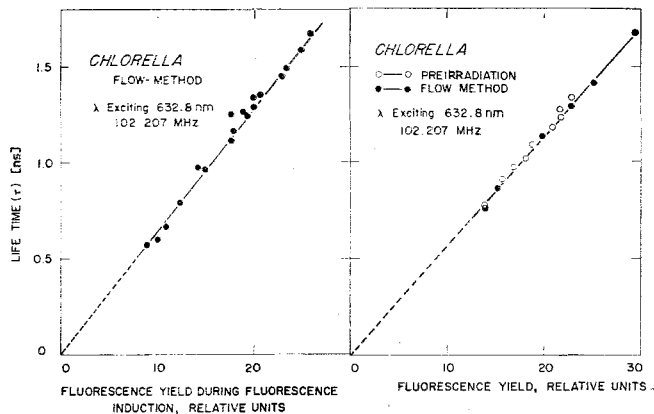

Fig. 2. Lifetime (τ) versus (relative) quantum yield of fluorescence (φ) in *Chlorella*. φ was changed by (left) varying the flow rate of algae suspension, or (right) by preirradiating a flowing suspension with different radiant flux densities ("white light"). λ exciting, 632·8 nm; frequency of modulation, 102·207 MHz; filter, *Corning C. S. 2-64*.

Chlorella with different radiant flux densities. As mentioned under Materials and Methods, data on τ versus φ in Fig. 2 cover the complete range of the induction curve from "0" to "*P*". There is an optimal rate of the flow corresponding to the *P* level; indeed the values of τ and φ obtained when 10^{-5} M DCMU was added to the sample that was not flowing were the same as that with the optimum flow; thus, all Q was in the reduced state. The level of fluorescence, obtained with the maximum rate of flow, was indeed the minimum (0) because DCMU did not affect it and it was linearly dependent on the intensity of excitation.

Extrapolation of τ versus φ curve below the φ and the "0" level shows that as $\varphi \to 0$, τ also approaches zero. (However, more experiments should be done to explore this area further.)

The linear relationship between τ and φ was also confirmed by measuring the stationary value of τ and φ in the presence of different concentrations of DCMU. Fig. 3 shows that the ratio τ/φ versus φ remains constant when the DCMU concentration is varied from 10^{-8} to 5×10^{-6} M. This confirms the more extensive data of TUMERMAN and SOROKIN (1967) on the effect of DCMU on τ and φ. Thus, within an error of 0·1 ns, $\tau = \tau_0\,\varphi$ is verified *in vivo*.

Fig. 3. Ratio of lifetime (τ) and (relative) quantum yield of fluorescence (φ) versus φ. φ was changed by adding different concentrations of DCMU to a stationary sample of *Chlorella*; see legend of Fig. 2.
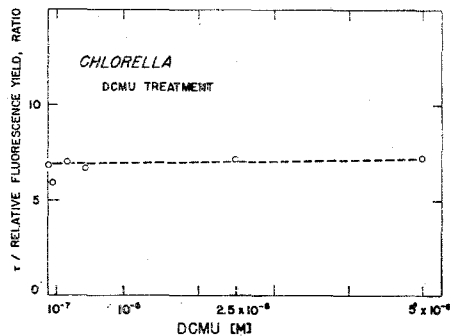
We interpret the (almost) linear dependence of τ on φ to mean that the "lake" (or the statistical or the multicentral) model of the photosynthetic units II is to be preferred over the "isolated puddles" (or the physical or the unicentral model; also see JOLIOT, JOLIOT and KOK 1968, and LAVOREL and JOLIOT 1972). Since τ versus φ curve intercepts the τ axis very close to zero, we suggest that there may not be a large difference between the τ values of the major chlorophyll molecules responsible for the variable and the constant fluorescence. However, we note that no data are available for τ as a function of φ below the "0" level, and thus, this interpretation must be made with caution. The absence of a significant heterogeneity of fluorescence (from the τ versus φ curve) when all traps are closed and when all traps are open suggests that most, but not all, of the variable fluorescence arise from the same bulk chlorophyll molecules that produce the "constant" fluorescence. Minor differences cannot and should not be ruled out because we know that the emission spectrum at "P" is different from that at "0" (LAVOREL 1963; GOVINDJEE and BRIANTAIS 1972), the excitation spectrum at high irradiances is different from that at low irradiances (BUTLER and BISHOP 1963) and the emission spectrum at high irradiances is different from that at low irradiances (KREY and GOVINDJEE 1966). If indeed more than one species were present, τ as a function of the wavelength of emission would show it.

Lifetime as a function of wavelength

Preliminary measurements of τ as a function of λ of emission both in normal and DCMU poisoned *Chlorella*, at the steady state of fluorescence, suggest that fluorescence is dominated by a single species as τ is nearly constant (within 10—15%) at different wavelengths; a small but significant dip at 710 nm is, however, present.

138   The position of this dip corresponds to the minimum observed by LAVOREL (1963) when he plotted the ratio of the variable to constant fluorescence as a function of wavelength of observation.

DISCUSSION

Among several possible models for photosystem II of photosynthesis, the two extreme ones are (*1*) the isolated puddles model in which the units are independent of each other with no connection between them, and (*2*) the lake model in which the reaction centers (or energy traps) are embedded in a "lake" of "bulk" (sensitizer or antenna) pigments (ROBINSON 1967, also see CLAYTON 1966).

In the isolated puddles model, the exciton in one unit can only reach the trap of that unit. Here, we assume that there is fluorescence from the units with open traps, as well as from those with closed traps. We must, therefore, consider two kinds of units with two independent values of τ and φ as discussed earlier by TUMERMAN and SOROKIN (1967):

$$\varphi = \alpha \, \varphi_1 + (1 - \alpha) \, \varphi_2, \quad \text{and} \quad \tau = \frac{\alpha \varphi_1 \tau_1 + (1 - \alpha) \, \varphi_2 \tau_2}{\alpha \varphi_1 + (1 - \alpha) \, \varphi_2} ,$$

where α is the fraction of units that have open traps, and $(1 - \alpha)$ the fraction of units that have closed traps. The τ/φ versus φ would then be affected by the change in α. It can be easily shown by selecting values for τ_1, φ_1, *etc.* that τ/φ would first rise and then decrease as φ is increased. The magnitude of this change would depend on the values of $\tau_1, \tau_2, \varphi_1$ and φ_2. This model predicts a non-linear relationship between τ and φ. Data in Figs. 2 and 3 do not support a non-linear relationship between τ and φ within the error of measurement ($\pm 0 \cdot 1$ ns).

If, in the "isolated puddles" model, both exciton migration and trapping (quenching) are faster than deactivation by fluorescence, the units with "open traps" may not fluoresce*. When the traps close, the photosynthetic units become highly fluorescent. In this case τ would be independent of φ. This is also the scheme of static quenching. The units with open traps may be considered equivalent to the complex in the ground state. Our results do not support such a relationship. We also note that in the above model we have not given any role to the fluorescence at "0". This indeed exists, and may be assumed to come, at least partly, from system I, or unconnected Chl having a fixed τ and φ.

The lake model is a simple model: there are only statistical system II units; there is a two dimensional bed of bulk pigments of system II with randomly located traps. In this lake model, if there is "fast" energy transfer, the trapping — not the migra-

---

* LAVOREL (personal communication) has, however, pointed out to us that even in a fast transfer case this situation may not occur as there is always some probability of fluorescence when we deal with a situation where a large number of transfers are involved.

tion — is the limiting process; the efficiency of trapping per encounter need not be very high, but the overall efficiency is still very high because of many encounters in a short time. Furthermore, all traps are accessible to any excitation in any photosynthetic unit. In this case, the scheme of dynamic quenching can be applied as the rate of trapping can be expressed as a product of the concentrations of excitons and open traps. A linear relationship between τ and φ is predicted. Figs. 2 and 3 show such a linear relationship between τ and φ within the error of our measurements.

In the lake model, and the slow transfer case, the exciton migration — not trapping — is diffusion limited, and the exciton must be trapped very efficiently at each and every encounter with the quencher (or trap) molecule. In this case, one could predict (LAVOREL, personal communication) that when the excitons are close to the trap, they disappear faster than if they are farther from the trap. This would predict a non-exponential fluorescence decay. Such a non-exponential decay has not yet been observed (SINGHAL and RABINOWITCH 1969). There is a significant probability of fluorescence from the bulk pigments, and this would increase as more and more traps are closed. There will be a rise in both τ and φ. Whether this increase will be linear or not, under our experimental conditions, is difficult to predict. Perhaps, the actual relation of τ with φ will depend on the method with which τ is measured. Thus, it may be worthwhile to obtain data on τ versus φ with different methods.

Finally, there are several possibilities of "mixed" models. In one such model, system II units are isolated but not completely isolated as they have the possibility to exchange some energy between them when the traps are closed (JOLIOT, JOLIOT and KOK 1968). If the transfer is "fast", and if the traps are open, these units may not fluoresce. However, if the trap in a unit is closed, there is the probability of emission of some fluorescence in competition with the energy transfer to neighboring units. This situation also resembles a scheme of dynamic quenching, but in details, it is a different picture. We can either expect a transfer from the "bulk", or from "near the trap" to the neighboring unit(s). If the units have the pigments arranged in an order such that the transfer is directional, *i.e.*, towards the trap, we cannot expect a high rate of transfer from near the trap to the other Chl molecules of its own unit, otherwise the probability of transfer to another unit would be very low. Thus, it seems equally difficult to visualize a transfer to the bulk Chl of another unit. We are left with the choice of excitation energy transfer from one closed trap directly to another open trap. This may also appear unreasonable because of very low concentration of traps (1/300 Chl). However, this difficulty can be overcome if several photosynthetic units (say, 4—10) are arranged in a super unit (FRANCK and ROSENBERG 1964) such that the traps are close neighbors. However, the possibility of excitation energy transfer from the bulk Chl of the unit with closed trap to the bulk Chl of the unit with open trap is the other alternative.

In the above pictures, the "0" level must either be due to some "inactive" or "unconnected" Chl molecules in system II or due to the fact that the trap is not 100% efficient (see MAR and GOVINDJEE 1972, and MAR *et al.* 1972). In the latter case, the overall picture becomes more complicated as the exciton at the trap has the

140 possibility to fluoresce, to be trapped, or to be transferred to other molecules. The prediction of the exact relationship between τ and φ becomes difficult. Thus, we cannot yet rule out these possibilities! From the point-of-view of similicity, however, the lake model with a "fast" transfer case appears to be the favored one as it clearly predicts the linear relationship between τ and φ observed in our study, and in those of TUMERMAN and SOROKIN (1967).

REFERENCES

BRIANTAIS, J. M.: Isolement et activité de particules chloroplastiques correspondant aux deux systems photochemiques. — Photochem. Photobiol. 5: 135—142, 1966.

BROWN, J. S.: Absorption and fluorescence of chlorophyll *a* in particle fractions from different plants. — Biophys. J. 9: 1542—1552, 1969.

BUTLER, W. L., BISHOP, N. I.: Action of two-pigment system on fluorescence yield of chlorophyll *a*. — In: Photosynthetic Mechanisms in Green Plants. Nat. Acad. Sci. — Nat. Res. Council, Misc. Publ. 1145: 91—100, 1963.

CLAYTON, R. K.: Physical processes involving chlorophylls *in vivo*. — In: VERNON, L. P., SEELY, G. R. (ed.): The Chlorophylls. Pp. 609—641. Academic Press, New York—London 1966.

DELOSME, R.: Étude de l'induction de fluorescence des algues vertes et des chloroplastes au début d'une illumination intense. — Biochim. biophys. Acta 143: 108—128, 1967.

DUYSENS, L. N. M., SWEERS, H. E.: Mechanism of two photochemical reactions in algae as studied by means of fluorescence. — In: ASHIDA, J. (ed.): Studies on Microalgae and Photosynthetic Bacteria. Pp. 353—372. Univ. Tokyo Press, Tokyo 1963.

FRANCK, J., ROSENBERG, J. L.: A theory of light utilization in plant photosynthesis. — J. theor. Biol. 7: 276—301, 1964.

GOVINDJEE, BRIANTAIS, J.-M.: Chlorophyll *b* fluorescence and an emission band at 700 nm at room temperature in green algae. — FEBS Letters 19: 278—280, 1972.

GOVINDJEE, PAPAGEORGIOU, G.: Chlorophyll fluorescence and photosynthesis: Fluorescence transients. — In: GIESE, A. C. (ed.): Photophysiology. Vol. 6. Pp. 1—46. Academic Press, New York—London 1971.

GOVINDJEE, RABINOWITCH, E.: Action spectrum of the "second Emerson effect". — Biophys. J. 1: 73—89, 1960.

GOVINDJEE, PAPAGEORGIOU, G., RABINOWITCH, E.: Chlorophyll fluorescence and photosynthesis. — In: GUILBAULT, G. G. (ed.): Fluorescence Theory, Instrumentation and Practice. Pp. 511—564. M. Dekker, Inc., New York 1967.

JOLIOT, P., JOLIOT, A., KOK, B.: Analysis of the interactions between the two photosystems in isolated chloroplasts. — Biochim. biophys. Acta 153: 635—652, 1968.

KAUTSKY, H., HIRSCH, A.: Neue Versuche zur Kohlensäureassimilation. — Naturwissenschaften 19: 964, 1931.

KREY, A., GOVINDJEE: Fluorescence studies on a red alga, *Porphyridium cruentum*. — Biochim. biophys. Acta 120: 1—18, 1966.

LAVOREL, J.: Induction of fluorescence in quinone poisoned *Chlorella* cells. — Plant Physiol. 34: 204—209, 1959.

LAVOREL, J.: Indications d'ordre spectroscopique sur l'hétérogénéité de la chlorophylle *in vivo*. — Colloq. int. C.N.R.S. No. 119: La Photosynthèse. Pp. 161—176. Gif-sur-Yvette et Saclay 1962. Paris 1963.

LAVOREL, J.: A study of chlorophyll fluorescence *in vivo* by a flow method. — Photochem. Photobiol. 4: 819—828, 1965.

LAVOREL, J., JOLIOT, P.: On a connected model of the photosynthetic unit. — Biophys. J. **12**: (in press), 1972.

MAR, T., GOVINDJEE: Kinetic models of oxygen evolution in photosynthesis. — J. theor. Biol. (in press), 1972.

MAR, T., GOVINDJEE, SINGHAL, G. S., MERKELO, H.: Lifetime of the excited state *in vivo* I. of chlorophyll *a* in algae, at room and at liquid nitrogen temperature; rate constants of radiationless deactivation and trapping. — Biophys. J. **12**: (in press), 1972.

MERKELO, H., HARTMAN, S. R., MAR, T., SINGHAL, G. S., GOVINDJEE: Mode-locked lasers: Measurements of very fast radiative decay in fluorescent systems. — Science **164**: 301—302, 1969.

MOHANTY, P., BRAUN, B. Z., GOVINDJEE, THORNBER, P.: Chlorophyll fluorescence characteristics of system I chlorophyll-protein complex and system II particles at room and liquid nitrogen temperatures. — Plant Cell Physiol. **13**: 81—91, 1972.

MÜLLER, A., LUMRY, R.: The relation between prompt and delayed emission in photosynthesis. — Proc. Nat. Acad. Sci. U.S. **54**: 1479—1485, 1965.

MÜLLER, A., LUMRY, R., WALKER, M. S.: Light-intensity dependence of the *in-vivo* fluorescence lifetime of chlorophyll. — Photochem. Photobiol. **9**: 113—126, 1969.

MUNDAY, J. C. Jr., GOVINDJEE: Light-induced changes in the fluorescence yield of chlorophyll *a in vivo*. III. The dip and the peak in the fluorescence transient of *Chlorella pyrenoidosa*. — Biophys. J. **9**: 1—21, 1969.

RABINOWITCH, E., GOVINDJEE: Photosynthesis. — J. Wiley & Sons, Inc., New York—London—Sydney—Toronto 1969.

ROBINSON, G. W.: Excitation transfer and trapping in photosynthesis. — In: Energy Conversion of the Photosynthetic Apparatus. Brookhaven Symp. Biol. **19**: 16—48, 1967.

SINGHAL, G. S., RABINOWITCH, E.: Measurement of the fluorescence lifetime of chlorophyll *a in vivo*. — Biophys. J. **9**: 586—591, 1969.

TUMERMAN, L. A., SOROKIN, E. M.: Fotosinteticheskaya edinitsa: "fizicheskaya" ili "statisticheskaya" model'? [The photosynthetic unit: a "physical" or "statistical" model?] — Mol. Biol. **1**: 628—638, 1967.

VREDENBERG, W. J., SLOOTEN, L.: Chlorophyll *a* fluorescence and photochemical activities of chloroplast fragments. — Biochim. biophys. Acta **143**: 583—594, 1967.