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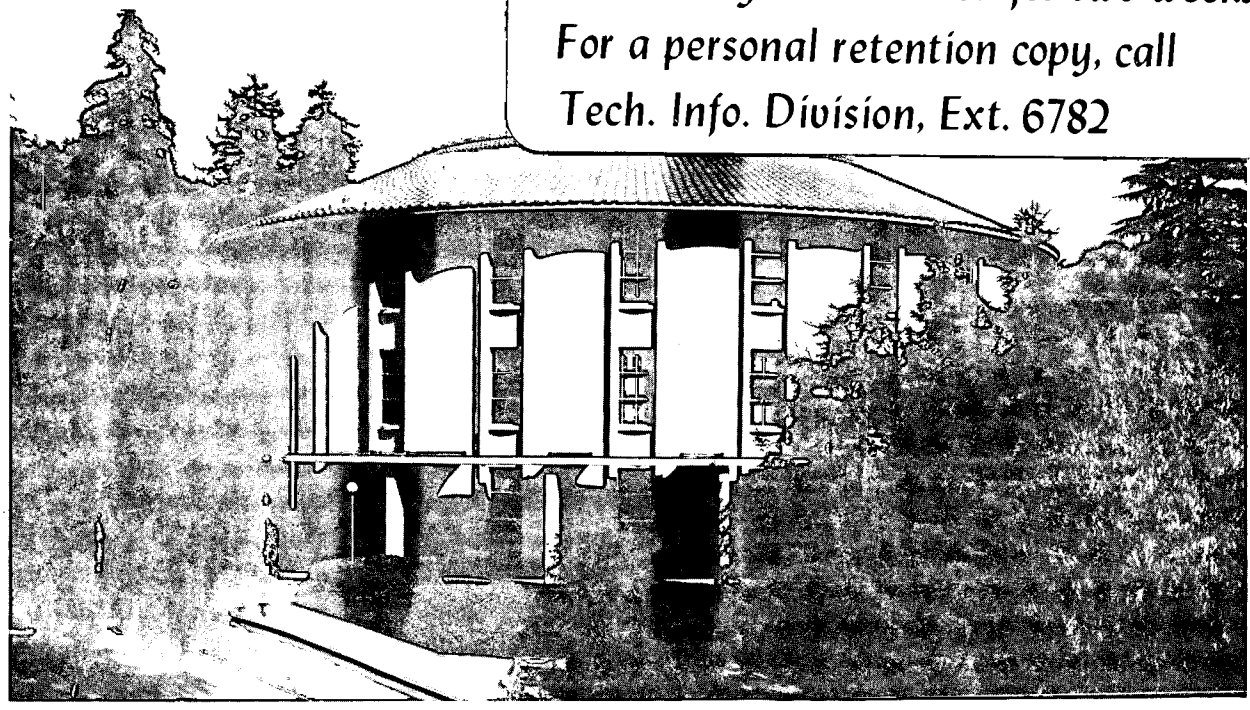

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DISCLAIMER

PICOSECOND FLUORESCENCE KINETICS AND ENERGY TRANSFER IN CHLOROPLASTS AND ALGAE

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Abbreviations: HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
DCMU: 3-(3',4'-dichlorophenyl)-1, 1-dimethyl urea

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SUMMARY

Single-photon timing with picosecond resolution is used to investigate the kinetics of the fluorescence emission of chlorophyll a in chloroplasts from spinach and pea and in the algae Chlorella pyrenoidosa and Chlamydomonas reinhardtii. The fluorescence decay is best described by three exponential components in all species. At low light intensity and with open reaction centers of photosystem II (F_0), we find lifetimes of approximately 100, 400 and 1100 ps for the three components.

Closing the reaction centers by addition of 3 - (3', 4'-dichlorophenyl)-1, 1-dimethylurea plus hydroxylamine and by increasing light intensity produces only minor changes in the almost constant fast and medium lifetime components; however, there is a dramatic increase in the yield of the slow component, by a factor of about 20, accompanied by only a modest increase in the lifetime to 2200 ps (F_{max}). In good agreement with previous fluorescence lifetime measurements we find an increase of the averaged lifetime of the three components from 0.5 to 2.0 ns, which is proportional to the four-fold increase of the total fluorescence yield. Our time-resolved results are inconsistent with models which are based on the proportionality between lifetime and yield and which involve a homogeneous origin of fluorescence that is sensitive to the state of the reaction centers. We conclude that the variable part of the fluorescence, which is dominated by the slow phase, reflects the kinetics of charge recombination in the reaction center, as proposed by Klimov, et al. [Klimov, V.V. Allakhverdiev, S.I., and Paschenko, V.Z. (1978) Dokl. Akad. Nauk. SSSR. 242, 1204-1207]. The modest increase in lifetime of the slow phase indicates the presence of some energy transfer between photosynthetic units.

INTRODUCTION

The fluorescence yield in higher plants and algae is a function of the state of the photochemical reaction centers in photosystem II. In the open state the reaction centers are photoactive, and the fluorescence yield F_0 is small. In the closed state of the reaction centers no photochemistry is possible, and the fluorescence yield F_{max} is large. Duysens and Sweers [1] concluded that the changes in the fluorescence yield are correlated with the redox state of the electron acceptor Q (originally designated as a quencher of fluorescence) of the photosystem II reaction center.

When the electron acceptor Q is reduced, the fluorescence yield increases by a factor of 3-5 [2,3]. A simple model of the photochemical apparatus [4] predicts that if photochemistry, which has a maximal quantum yield of about 0.95 when Q is oxidized [5,6], is blocked the fluorescence yield should increase about 20 fold. This apparent discrepancy could occur for two reasons. First, either photosystem I chlorophyll or chlorophyll that is not connected to any reaction center could contribute a constant fluorescence in both F_0 and F_{max} [7,8]. Second, there may be a new radiationless deactivation mechanism in the closed reaction center which does not occur in the open reaction center [9-11]. Recently, it was proposed that variable fluorescence results from recombination between the primary electron acceptor and the primary electron donor of photosystem II and that this charge recombination repopulates the excited singlet state of chlorophyll [12,13]. An analgous process leading to triplet or ground state chlorophyll molecules could be the mechanism of radiationless deactivation in closed reaction centers. Time-resolved fluorescence measurements provide a direct method for discriminating between these models.

Another aspect which has been studied by fluorescence lifetime measurements is the cooperation between photosynthetic units. Photosynthetic units in a separate package arrangement would show decay components of the fluorescence with distinct lifetimes, one for units with open and one for those with closed reaction centers. In a matrix arrangement there should be a continuous increase in the lifetime as the reaction centers are progressively closed. Most of the studies using the phase shift technique for measuring fluorescence lifetimes indicate such a proportionality between the lifetime and the fluorescence yield when the reaction centers of photosystem II are closed [14-17]. These findings are based on the assumption of a single exponential decay of the fluorescence. However, direct measurements of the kinetics after short laser pulses as well as recent measurements with the phase shift technique [18] provide evidence for two [19-22] or even three components of the decay kinetics [23].

The measurements of picosecond fluorescence kinetics with the streak camera and the photon-timing technique, both providing direct resolution of multiple exponential decays, have recently been reviewed [24]. The streak camera technique provides the fastest time resolution. However, for sufficient signal-to-noise ratios, high intensity laser pulses are needed, and these may induce deactivation of the excited state by singlet annihilation processes [24,25]. These processes do not occur at the low pulse energies used for the photon-timing technique. With this technique [26,19,21] biphasic decay kinetics were found in chloroplasts and algae with lifetimes ranging from 0.2 ns for open reaction centers [19] to 0.5 to 2.0 ns for closed reaction centers [19,21]. The disparity between the

results of different groups, even where similar techniques were used [27], makes it difficult to relate these measurements to a model of the light harvesting mechanism.

We have investigated these problems by studying the fluorescence kinetics in chloroplasts and algae with a picosecond resolution, single-photon timing instrument [27-29]. We found three different kinetic components for open as well as for closed photosystem II reaction centers. The component with the longest lifetime of 1-2 ns shows a dramatic increase in the yield as the centers become closed, but only minor changes in the lifetime. We interpret these findings in terms of charge recombination in the reaction center of photosystem II giving rise to the variable fluorescence, and we discuss the extent of energy transfer between connected photosynthetic units.

MATERIALS AND METHODS

Broken chloroplasts were isolated as described previously [30] from 25 g of freshly harvested spinach leaves, grown either in a growth chamber or in a greenhouse, by grinding for 10 s in 100 ml 0.4 sucrose, 50mM HEPES-NaOH buffer, pH7.5 and 10 mM NaCl followed by centrifugation for 2 min at 2000 xg. After one wash with fresh grinding medium, the chloroplasts were kept for 20 min at 0°C in 0.1 M sucrose, 10 mM HEPES-NaOH buffer, pH 7.5, and 10 mM NaCl. The pellet of the subsequent centrifugation for 5 min at 2000 xg was resuspended in a small volume of 0.1 M sucrose, 10 mM HEPES-NaOH buffer, pH 7.5, 5 mM NaCl and 5 mM $MgCl_2$ to give approximately 1 mg chlorophyll ml^{-1}. In a few experiments $MgCl_2$ was omitted from the resuspending medium. For the fluorescence measurements the chloroplast suspension was diluted with the resuspending medium to a

concentration of 18 µg chlorophyll ml^{-1}. Further additions for measurements corresponding to open reaction centers (F_o) were 1.25 mM ferricyanide as electron acceptor, 1.25 mM ferrocyanide to control the redox potential and 2.5 µg . ml^{-1} gramicidin D as uncoupler. The latter was added to prevent the formation of a pH gradient across the thylakoid membrane, which is known to diminish the fluorescence intensity [31]. The reaction mixture was vigorously stirred in a 1 x 1 cm cuvette during the measurements. When prolonged data accumulation was necessary, the content of the sample cuvette was exchanged after 10 min. To produce closed reaction centers (F_{max}) 12.5 µM DCMU and 2 mM hydroxylamine hydrochloride, adjusted to pH 7 immediately before use, were present in addition to the components in the resuspending medium. The reaction mixture was illuminated with about 10 flashes of saturating intensity immediately before the fluorescence measurement. Chlorella pyrenoidosa, strain UTEX 1230, and Chlamydomonas reinhardtii, strain UTEX 89, was grown as described [32,33] and diluted with growth medium to give a chlorophyll concentration of 18-20 µg ml^{-1}. The measurements were carried out in a 0.7 x 1 cm cuvette with the suspension flowing continuously at a rate of 6 ml . min^{-1}. For measurements of samples with closed reaction centers, the algae were not flowing. They were instead incubated for 10 min with 20 µM DCMU and 10 mM hydroxylamine (pH 7) and then preilluminated with several flashes of saturating intensity. All measurements were carried out at room temperature (20-22°C). The cuvettes were painted black except for a window for the exciting beam and another window pointing in the direction of the photomultiplier. This masking minimized the broadening of the observed excitation profiles owing to reflections at the cuvette walls.

The single-photon timing apparatus employed in these studies is diagrammed in Fig. 1. The excitation pulse is provided by a Spectra Physics synchronously-pumped mode-locked dye laser which is composed of an SP 171 argon laser, an SP 362 mode locker, and modified SP 375 dye laser. The output pulses of this laser have a full-width-half-maximum duration of about 15 ps (as determined by zero background second harmonic generation [34]). All experiments were performed using the dye rhodamine 6G, and laser excitation pulses were at 620 nm.

Conventional single-photon timing systems start a voltage ramp in the TAC (Canberra 2043 Time to Amplitude Converter) upon each excitation pulse and stop the voltage ramp when a fluorescence photon is detected. The resultant height of the voltage ramp is proportional to the time between the start and the stop pulse. The recording of many such events results in a histogram which is equivalent to the fluorescence intensity versus time [36]. Because the repetition rate of our laser (82 MHz) is incompatible with the TAC input, we have adopted a reverse single-photon timing scheme. In our system, we start the TAC with a fluorescence photon and stop the TAC with the next laser flash. The jitter of the time between laser pulses is sufficiently low (< 5 ps) that we do not sacrifice resolution in this mode of operation.

Fluorescence photons are detected by a RCA 31034A photomultiplier. The output of the photomultiplier is amplified and input to a constant fraction discriminator [27,28]. One output of the constant fraction discriminator provides a pulse to the TAC resulting in the start of the voltage ramp. Two other outputs trigger the gate box (see below) and feed a count ratemeter; the count ratemeter was used to monitor the fluorescence intensity during an experiment. Laser pulses

are detected by a Texas Instruments TIED 55 photodiode mounted in a transmission line housing similar to the one described by Steinmetz [36]. The output of the photodiode triggers a level discriminator whose output provides a pulse to the TAC which stops the voltage ramp. Before reaching the TAC, the output of the level discriminator passes through the gate box. The function of the gate box is to prevent pulses from the level discriminator from reaching the TAC except when a fluorescence photon has been detected. This arrangement was required to eliminate oscillatory artifacts present in the data when a pulse from the level discriminator reached the TAC on every pulse. See ref. [29] for more details.

The output of the TAC is converted into a channel number by a Northern 1024 analog to digital converter and stored locally in a 1024 channel Northern NS636 multichannel analyzer. We collect data until the peak channel has at least 10000 counts. The contents of the Northern NS636 is transferred to a VAX 11/780 computing system for data analysis.

A fluorescence lifetime determination involves measurement of both E(t) and F(t). E(t) is the response of our system and it is measured with a scattering solution in the sample cell. F(t) is the fluorescence decay curve, and it is measured with a fluorescent sample in the sample cell (see Fig. 2). F(t) is related to E(t) by the convolution integral

$$F(t) = \int_0^t E(u)I(t-u)du \qquad (1)$$

where I(t) is the actual fluorescence decay law. We have deconvoluted Eq. (1) under the assumption that I(t) is given by a sum of N exponentials

$$I(t) = \sum_{i=1}^{N} \alpha_i \exp(-t/\tau_i) \qquad (2)$$

where α_i is the amplitude and τ_i is the lifetime of the ith component. Deconvolution was done using the method of least squares [37,38]. When the lifetime becomes greater than about 2ns, it is necessary to correct the F(t) for fluorescence due to previous pulses. This correction is necessary because the laser pulses are separated by only 12ns. For more details on the data analysis and long lifetime corrections, see ref. [39].

Based on deconvolutions of simulated data using real E(t) response functions from our system, we estimate that we can resolve fluorescence lifetimes as short as 25ps. The limiting factor is a broadening of E(t) due to time jitter in the electronics. The most probable source of jitter is the photomultiplier and constant fraction discriminator combination which is used to detect the fluorescence photon.

RESULTS

Separation of three kinetic components of the fluorescence decay

The fluorescence decay $F_0'(t)$ in spinach chloroplasts at low intensity of the exciting laser pulses is illustrated in Fig. 2. Most of the photosystem II reaction centers were open under these conditions, as indicated by the simultaneously recorded fluorescence yield (count rate). The yield did not increase during the course of a measurement. Fig. 2 includes also the response of our system to the excitation pulse, E(t). Plots of the deviation of the measured fluorescence decay from the results of the deconvolutions indicate whether the presumed decay law is matching the data. Fig. 2 shows in the middle and at the bottom these difference plots on a linear scale for the best fits of a two- and a three-exponential decay, respectively. This type of difference plot exposes deviations more clearly in the region of largest intensity than does a direct comparison

between the fluorescence decay and the calculated best fit plotted on a logarithmic scale [19,21]. As seen in Fig. 1 (middle) we found systematic deviations of the two-exponential fit well above the noise level. This pattern is typical for a two-exponential fit of most of our fluorescence kinetic data. The best fit using a three-exponential decay shows deviations due only to statistical noise. It is this fit which is the smooth line following F(t). The fluorescence decay $F_{max}(t)$ in spinach chloroplasts with photosystem II reaction centers closed by preillumination with flashes of saturating intensity in the presence of DCMU and hydroxylamine (Fig. 3) also requires a three-exponential fit to describe the fluorescence decay. The deviations of the two-exponential fit are smaller than those in Fig. 2, because the fastest component is a smaller fraction of the total decay and was close to the limit of our ability to resolve three components. When the deviations in a two-exponential fit are sufficiently small and a three-exponential fit is attempted, the three components will collapse into two. This did not happen for any of the experiments reported here.

The results shown in Figs. 2 and 3 were measured in the presence of 5 mM $MgCl_2$. Low concentrations of monovalent cations, such as 5 mM NaCl, cause a low yield of fluorescence and unstacking of the grana membranes if divalent ions are absent [40,41]. As in the presence of $MgCl_2$, a three-exponential decay was necessary to describe the fluorescence kinetics. A detailed investigation of the complex effect of $MgCl_2$ will be presented in a subsequent paper [42]. Table I summarizes the values of the lifetimes τ and the relative amplitudes α for the best two- and three-exponential fits of these measurements and those shown in Figs. 2 and 3. Table I includes published lifetimes and amplitudes found under

experimental conditions which are not identical but are comparable to ours. In general there is only fair agreement among the values in the literature. The mean lifetime τ_{mean} calculated from equation

$$\tau_{mean} = \sum_{i=1}^{3} \alpha_i \tau_i^2 / \sum_{i=1}^{3} \alpha_i \tau_i \qquad (3)$$

is also given for comparison with published measurements of fluorescence lifetimes which presumed a single exponential decay. Our values of τ_{mean} agree closely with those found using phase fluorimetric methods [2,17]. A major new consequence of our three-exponential analysis, compared to a two-exponential one, is the resolution of a fast component with a lifetime close to 100 ps. The lifetime of the middle component is approximately 400 - 700 ps and the lifetime of the slow component is 1.2 - 2.1 ns, each depending on the experimental conditions.

Fluorescence kinetics in green algae

The kinetic components in broken spinach chloroplasts may differ from those in intact plants. This was investigated using green algae. Figs. 4 and 5 show the fluorescence decay in Chlamydomonas reinhardtii and Chlorella pyrenoidosa, respectively, when the photosystem II reaction centers are either open or closed. As with the chloroplasts, it required a three-exponential analysis to match the fluorescence kinetics. Table II shows the data for the two algae. The lifetimes and yields of the three components in the algae are almost the same as those in spinach chloroplasts except for a larger relative yield and a slightly shorter lifetime of the slow component from open reaction centers (F_o). To test for self-absorption effects we measured the fluorescence kinetics also at a twofold lower concentration of the algae, but we could not detect any

changes in the kinetics.

Comparison of the fluorescence kinetics at open and closed reaction centers

Table II summarizes the lifetimes and the fluorescence yields of the kinetic components. The (relative) total fluorescence yield ϕ_{tot} was obtained for F_o and F_{max} conditions by numerical integration of the deconvoluted fluorescence decay I(t)

$$\phi_{tot} = \int_o^\infty I(t)dt \qquad (4)$$

The values of the yield for each preparation are normalized to the total yield for open reaction centers (=100). The most obvious result upon closing the reaction centers in all of the species studied is a dramatic increase in the yield, ϕ_3, of the slowest component.

To discriminate between models involving either connected or separate photosynthetic units, it is essential to measure the fluorescence decay for partially closed reaction centers. We have used increasing light intensities to close the photosystem II reaction centers progressively. Figures 6 and 7 show the lifetimes and the yields, respectively, of the kinetic components under these conditions. While the yields of both the fast and middle components remain constant, it is the yield of the slow component which is affected by the state of the reaction centers and which is responsible for the variable part of the total fluorescence. We found the same dependence in exploratory experiments with spinach chloroplasts at lower light intensity, where the reduction potential (E_h) was decreased from +200 to -300 mV. We found a range of 20-30 for the increase in the yield of the slow component, ϕ_3, with several different batches of chloroplasts isolated either from spinach or from peas, and a range of 6-8

in the algae (cf. Table II).

In contrast to the behavior of yield, the lifetime of the slow phase at the F_o level in the range 0.7 to 1.3 ns shows only a modest increase to 2.0 - 2.4 ns at the F_{max} level for all species. The middle component with a lifetime of 350-400 ps contributes the largest portion of the F_o fluorescence. Closing the reaction centers increases this lifetime by a factor of two, but the yield is nearly unaffected. The fast component shows lifetimes between 50 and 100 ps in the different preparations. Although the yield decreases when the reaction centers become closed, this effect is less definite because of the relatively large uncertainty in our kinetic resolution of the fast component.

Intermittent light chloroplasts

Changes in the composition of the light-harvesting chlorophyll antenna might influence the lifetime of the excited state and the fluorescence kinetics. To investigate this effect, we have measured the fluorescence decay in chloroplasts from intermittent light peas. These chloroplasts have intact photosystem I and II, but are agranal and do not contain the light harvesting chlorophyll a/b protein [45,46]. This protein normally contains more than 50 percent of the antenna chlorophylls found in chloroplasts [47]. The values of the best fits are shown in Table II. The major changes compared to normal chloroplasts are a smaller increase of the lifetime of the middle and the slow component when the reaction centers become closed and an increase in the yield of the slow component by a factor not greater than 3. The low ratio $F_{max}/F_o = 2.2$ is in good agreement with previous measurements [45].

DISCUSSION

Comparison with other fluorescence lifetime measurements

The deconvolutions of our fluorescence decay kinetics indicate that three-exponential decays fit the experimental data within the statistical noise. The three components are found for Chlorella, Chlamydomonas and chloroplasts from spinach and peas, under all conditions investigated. A two-exponential decay is not sufficient to describe the fluorescence kinetics, as demonstrated by the deviations plots in Figs. 2 and 3. The deviations near the maximum fluorescence may have been missed before, because the experimental and the calculated decay were compared directly on a logarithimic scale [19,21]. We also are not able to fit our data with nonexponential decays such as those used by Barber et al. [48].

A comparison of our findings with those published in the recent literature (Table I) shows that, apart from the number of resolved components, they can be reconciled reasonably well. Compared to the previous measurements from this laboratory [19,44] the time resolution as well as the deconvolution technique have been improved, which accounts in part for the different values presented in this paper. The best two-exponential fits of our fluorescence decays give lifetimes and initial intensities comparable to those derived by Beddard, et al. [21], who applied a technique similar to ours.

Searle, et al. [22] used streak camera detection and a high laser pulse energy of about 2×10^{14} photons.cm^{-2}. Those authors resolved two components which were affected by $MgCl_2$ and closed reaction centers in a similar way to the components of our two exponential approach (Table I). Their lifetimes, however, are considerably shorter than ours. This effect was probably caused by singlet-singlet annihilation, which is induced by

picosecond pulses having energies greater than 10^{13} photons cm^{-2} [24,25]. We emphasize that the energy of our pulses (2-4 x 10^6 photons.cm^{-2}) was more than six orders of magnitude lower. In addition, the time-averaged light intensities during our measurements were similar to those used in conventional studies of the fluorescence yield.

Most of the fluorescence lifetime studies in the literature have been carried out by phase fluorimetry. The lifetimes were then calculated from the results by assuming a single-exponential decay. We find good agreement of these lifetimes with mean lifetimes calculated for our results using the best three-exponential fit of the F_o and the F_{max} kinetics (Table I). Müller et al. [15] tried to separate two decay components with phase fluorimetry by changing the modulation frequency. However, they were limited by the frequencies available to components both longer than 2 ns; therefore, they did not resolve the different components that we see. They found a steady increase of the lifetime from 0.35 ns to 1.9 ns with increasing light intensity. To compare these data with our measurements we have plotted in Fig. 8 the mean lifetime, calculated from the values in Fig. 6, versus the total fluorescence yield (Fig. 7). The almost linear relation is in quantitative agreement with the results derived from several different phase fluorimetric measurements when the photosystem II reaction centers were progressively closed [2,16,17,49]. It was in particular this result which has influenced the conclusions of many authors about the origin of the fluorescence.

A model for the origin of the fluorescence

A simple model for the fluorescence lifetime and yield of an array of light harvesting chlorophylls connected to photosystem II reaction centers P680 Q (cf. ref. [3]) leads to the relations

$$\tau = 1/(k_F + k_d + k_{ic} + k_T[P680.Q]) \quad (5)$$

and

$$\phi_F = k_F \ \tau \quad (6)$$

where k_F is the intrinsic rate constant for fluorescence, k_d is the sum of the rate constants for radiationless deactivation by internal conversion and spillover to photosystem I, k_{ic} is the rate constant for intersystem crossing, k_T is the second order rate constant for energy transfer to photosystem II reaction centers, and [P680.Q] is the fraction of open photosystem II reaction centers. If all of the reaction centers are open, the fluorescence yield is at a minimum (F_o); if all reaction centers are closed, the fluorescence yield is at a maximum (F_{max}). It is easy to show [50] that based on this model, the maximum yield of photochemistry is given by

$$\phi_{Pmax} = \frac{F_{max} - F_o}{F_{max}} \quad (7)$$

A value of $\phi_{Pmax} \geq 0.95$ has been estimated from the quantum yield of the electron transport through photosystem II [4,5]. A slightly lower value of $\phi_{Pmax} = 0.935$ can be estimated from the minimum quantum yield of the excitation losses by fluorescence of 2% [51] and by intersystem crossing of 4.5% [52]. These values give a predicted ratio F_{max}/F_o of 20 and 15, respectively, which is not consistent with the ratio of 3-5 found for chloroplasts and algae (cf. refs. 2,3, and Table II).

One proposal to account for this discrepancy without modifying the simple model is that a portion of F_o and F_{max} is fluorescence from chlorophyll that is not connected to the reaction center of photosystem II. Free chlorophyll or chlorophyll separated from the light harvesting pigments could account for such a constant contribution to fluorescence. It would be expected to have a long lifetime (we estimate 2 - 5 ns), however, and we see no significant contribution from a constant long-lifetime background. Another source of constant fluorescence could be chlorophyll in photosystem I. Beddard et al [21] have measured a fluorescence lifetime of 110 ps for photosystem I particles, and this is consistent with the lifetime of 80 ps attributed to photosystem I by Paschenko et al. [23]. These results suggest that our fast phase may be due to photosystem I. The spectrum of the fast phase is very similar to those of the two other components, all peaking at 680 nm, and shows only a slightly larger relative yield around 730 nm compared to 680 nm (data not shown). We conclude that some of the fast component may be emission from chlorophyll a in the antenna pigments associated with photosystem I. These pigments have an emission spectrum similar to those of photosystem II [54]. Apart from this assignment, the yield of the fast phase, being 10% or less of F_o (Table II), is much smaller than 78-85% required to account for the discrepancy above.

Butler (11,50,53) and Duysens (9,10) account for the discrepancy between the measured and predicted ratio F_{max}/F_o by assuming a new radiationless deactivation pathway in closed reaction centers. An additional assumption that the energy transfer rate between the reaction centers and associated antenna molecules is considerably larger than the trapping rate in the reaction centers [9,10] is appropriate to describe

the apparent relation in Fig. 8 but not our data in Figs. 6 and 7. In the tripartite model of Butler et al. [11,50,53] excitation is transferred to the photosystem II reaction centers from closely connected chlorophyll a proteins which also mediate the energy transfer from remotely connected light-harvesting chlorophyll a/b proteins.

Excitation reaching a closed reaction center can undergo radiationless deactivation or be transferred back to the antennae where fluorescence is possible. If this back transfer is slower than the transfer to the reaction center, a qualitative kinetic analysis of this scheme results in the following conclusions: 1) When all reaction centers are open, the fluorescence would be dominated by two fast components whose lifetimes are close to the transfer time from the different antennae to the reaction center. 2) When reaction centers are closed, the fast components would still be present because transfer to the reaction centers is still taking place, and a new slow component would arise reflecting the kinetics of back transfer from the reaction center to the antennae. This pattern is qualitatively identical to our observations except that we see a small amount of slow fluorescence in F_o. The residual slow fluorescence could be due to a dark level of closed photosystem II reaction centers.

The results of Klimov et al. [12,13] suggest a mechanism for the processes occurring in the reaction center of photosystem II that may control the back transfer of energy to the antennae discussed by Butler and Kitajima [11,50]. In the model of Klimov et al. [12,13] a pheophytin molecule (Ph) functions as primary electron acceptor of photosystem II between P680 and Q. Charge separation is always possible at a photosystem II reaction center. When Q is oxidized a fast charge stabilization takes place $(P680^+ Ph^- Q \rightarrow P680^+ Ph Q^-)$, but when Q is reduced

charge recombination occurs ($P680^+$ Ph^- $Q^- \rightarrow P680^*$ Ph Q^-) [12,13]. The excited state may lead to fluorescence from the antennae or from the reaction center. Charge recombination to the ground state provides the new mechanism of radiationless deactivation in closed reaction centers postulated by Bulter et al. [11,50,53] and Duysens [9,10]. Our data are consistent with Butler's tripartite model for energy transfer and with the processes in the reaction center described by Klimov et al. [12,13].

We conclude that the fast phase and the middle phase represent fluorescence resulting from excitation that is on its way from the light harvesting pigments to the photosystem II reaction center and reflect the transfer times from different antenna proteins. Photosystem I antenna fluorescence may account for some of the fast phase. We propose that our component, increasing from 1 ns when all reaction centers are open to 2.2 ns when all reactions are closed, reflects the kinetics of the charge recombination. The two-fold increase indicates some connection between photosystem II units. Our slow component is shorter than the 4 ns component reported by Shuvalov et al. [55]. We did not find in either chloroplasts or algae such a slow component of the fluorescence.

In a following paper [56], we present a detailed analysis of the kinetic models for fluorescence emission, which will permit quantitative conclusions to be drawn from our data about the emission from the different antenna chlorophyll proteins and about the connectedness between photosystem II units.

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Table I

LIFETIMES AND RELATIVE AMPLITUDES OF THE FLUORESCENCE FROM SPINACH CHLOROPLASTS (cont'd)

The data in the presence of 5 mM $MgCl_2$ are derived from the kinetics shown in Figs. 2 and 3. The kinetics in the absence of $MgCl_2$ were measured under identical conditions except that $MgCl_2$ was omitted from the resuspending medium of chloroplasts. Symbols: τ, lifetime; α, relative amplitude; ϕ, relative yield. For details see text. Published values are indicated by the reference in the column at the right. In ref. [21], the measurements under low salt condition were carried out without addition of 5-10 mM monovalent cations. The techniques used were single-photon counting [19,21], streak camera with single picosecond pulse [22] and phase fluorimetry [2,17]. The chloroplasts were isolated from spinach [19], pea [17,21], lettuce [2] or barley [22].

TABLE I

LIFETIMES AND RELATIVE AMPLITUDES OF THE FLUORESCENCE FROM SPINACH CHLOROPLASTS

Experimental conditions		three-exponential fit τ(ps)	α	ϕ	two-exponential fit τ(ps)	α		τ_{mean} (ns)	
5 mM NaCl	F_o	130	0.55	26%	250	0.92		0.55	[This study]
		360	0.41	55%	1100	0.08			
		1500	0.04	19%					
					200	0.99	[19]		
					500	0.01		0.6	[2]
					410	0.96	[21]	0.4	[17]
					1460	0.04			
					160	0.77	[22]		
					600	0.23			
	F_{max}	160	0.31	8%	410	0.76			
		530	0.53	47%	1500	0.24		1.04	[This study]
		1700	0.16	45%					
					480	0.93	[19]		
					2000	0.07		1.31	[2]
					450	0.90	[21]		
					1330	0.10		0.9	[17]
					150	0.72	[22]		
					1060	0.28			
5 mM NaCl + 5 mM $MgCl_2$	F_o	100	0.34	10%	320	0.80			
		420	0.63	78%	750	0.20		0.47	[This study]
		1200	0.03	12%					
					320	0.99	[19]		
					>500	0.01		0.7	[2]
					150	0.66	[22]		
					620	0.34		0.5	[17])
	F_{max}	50	0.25	1%	790	0.37			
		750	0.26	17%	2000	0.63		1.73	[This study]
		2000	0.49	82%					
					460	0.63	[21]		
					1340	0.37		1.7	[2]
					130	0.52	[22]	1.35	[17]
					1290	0.48			

TABLE II

DECAY COMPONENTS OF THE FLUORESCENCE KINETICS IN ALGAE AND CHLOROPLASTS

species, conditions		τ_1(ps)	ϕ_1	τ_2(ps)	ϕ_2	τ_3(ps)	ϕ_3	F_{max}/F_o
Chlamydomonas reinhardtii	F_o	70	8	390	44	750	48	3.8
	F_{max}	60	2.3	850	62	2300	310	
Chlorella pyrenoidosa	F_o	60	2	390	30	840	68	3.0
	F_{max}	70	2.2	810	48	2100	250	
Spinach chloroplasts	F_o	110	10	420	78	1200	12	4.0
	F_{max}	50	4	750	68	2000	330	
ea chloroplasts	F_o	50	6	340	65	680	29	5.1
	F_{max}	70	2.5	700	68	2200	440	
ImL-pea chlorpolasts	F_o	80	14	510	35	2100	51	2.2
	F_{max}	90	9	660	50	2400	158	

The values for the algae Chlamydomonas reinhardtii and Chlorella pyrenoidosa are taken from the measurements shown in Figs. 4 and 5, respectively. The kinetics in chloroplasts were measured in the presence of 5 mM $MgCl_2$. The relative yield ϕ_i of a single component is calculated from the lifetimes τ_i and the initial intensities α_i by

$$\phi_i = \alpha_i \tau_i \Big/ \sum_{i=1}^{3} \alpha_i \tau_i$$

The entries in the table give these yields normalized to the total yield of the F_o fluorescence (=100). Pea chloroplasts were isolated by the same procedure as for spinach chloroplasts, either from 11 day old seedlings grown in a growth chamber or from peas germinated for 7 days in the dark and illuminated by 50 intermittent light periods (1700 lux) of 2 min each followed by a dark period of 118 min [45,46]. The ratio of chlorophyll a/chlorophyll b in the intermittent light (ImL) chloroplasts (two different preparations) determined by the method of Arnon [42] was >25.

LEGENDS

Fig. 1. Block diagram of the photon counting system for picosecond lifetime measurements. Abbreviations: ADC, analog to digital converter; BS, beam splitter; CFD, constant fraction discriminator; F1, neutral density filter; F2, interference filter; LEV. DISC., level discriminator; MCA, multi channel analyser; M.L., mode locker; P.D., photodiode; P.M., photomultiplier; TAC, time to pulse height converter.

Fig. 2. Fluorescence decay of spinach chloroplasts at 680 nm in the presence of 5 mM $MgCl_2$ at low excitation intensity (F_o level). The curve labelled E(t) is the excitation profile with 310 ps full width at half maximal intensity induced by the laser pulse. The curves labelled F(t) are the experimental fluorescence decay (noisy) and the calculated fit of a three-exponential decay (smooth), essentially superimposed. The lifetime τ and the relative yields ϕ of the three kinetic components are indicated. Middle and bottom, deviations of the calculated best fit of a two- and a three-exponential decay from the experimental fluorescence decay, respectively, on a linear scale. The distance between the channels was 8.2 ps.

Fig. 3. Fluorescence decay of spinach chloroplasts at 680 nm in the presence of 5 mM $MgCl_2$ at the maximum fluorescence level F_{max}. The chloroplasts were preilluminated with saturating flashes after addition of 12.5 µM DCMU and 2 mM hydroxylamine. Other details as in Fig. 2.

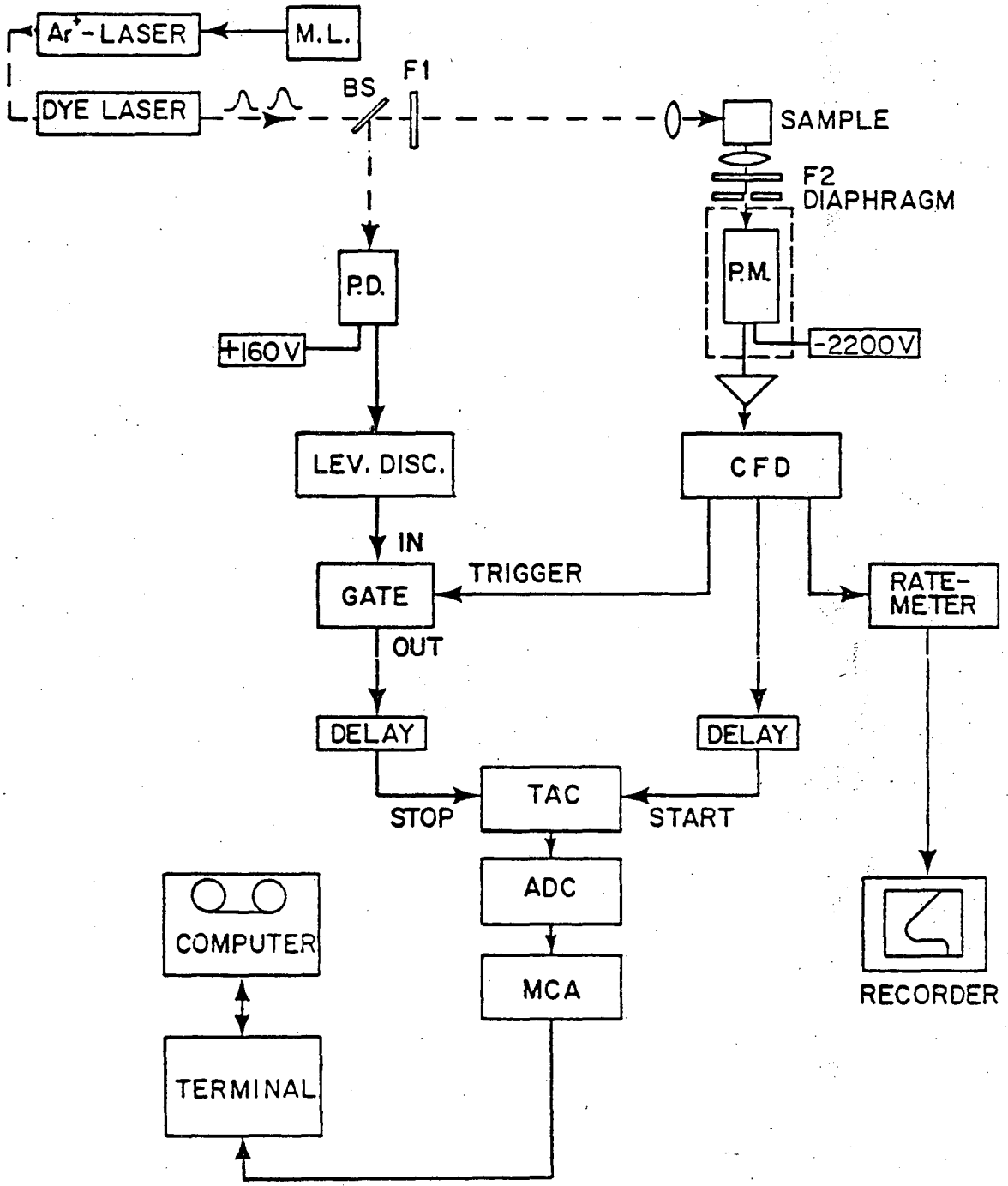
Fig. 4. Fluorescence decay of Chlamydomonas reinhardtii at 680 nm in the dark adapted state, $F_0(t)$, and in the state of maximum fluorescence, $F_{max}(t)$. The curve labeled $E(t)$ is as in Fig. 2. The noisy experimental fluorescence decays are superimposed with a smooth curve calculated from the best fit of a three-exponential decay. The differences between the decays are shown on a linear scale for F_0, middle, and for F_{max}, bottom.

Fig. 5. Fluorescence decay of Chlorella pyrenoidosa at 680 nm. Other details as in Fig. 4.

Fig. 6. Lifetimes of the components of the fluorescence kinetics in spinach chloroplasts as a function of the laser intensity. Other experimental conditions as in Fig. 2.

Fig. 7. Yields of the components of the fluorescence decay in spinach chloroplasts as a function of the laser intensity. Measurements as in Fig. 6.

Fig. 8. Average lifetime as a function of the total fluorescence yield. The average lifetime was estimated by the equation given in the text from the values of the best three-exponential fits of the fluorescence decay at different light intensities shown in Figs. 6 and 7.



PHOTON TIMING SYSTEM

XBL811-4422

FIG 1

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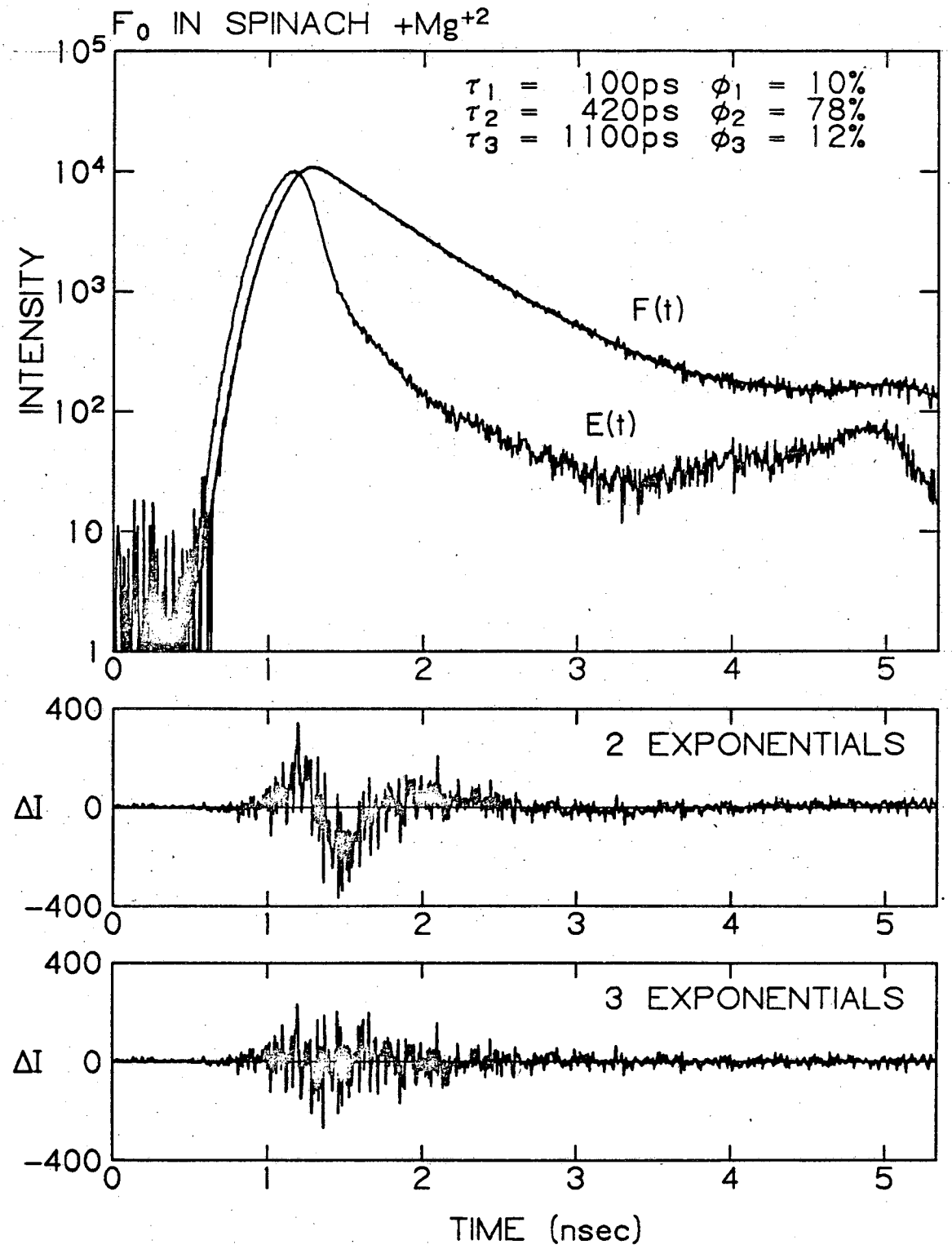

XBL 811-4430

FIG 2.

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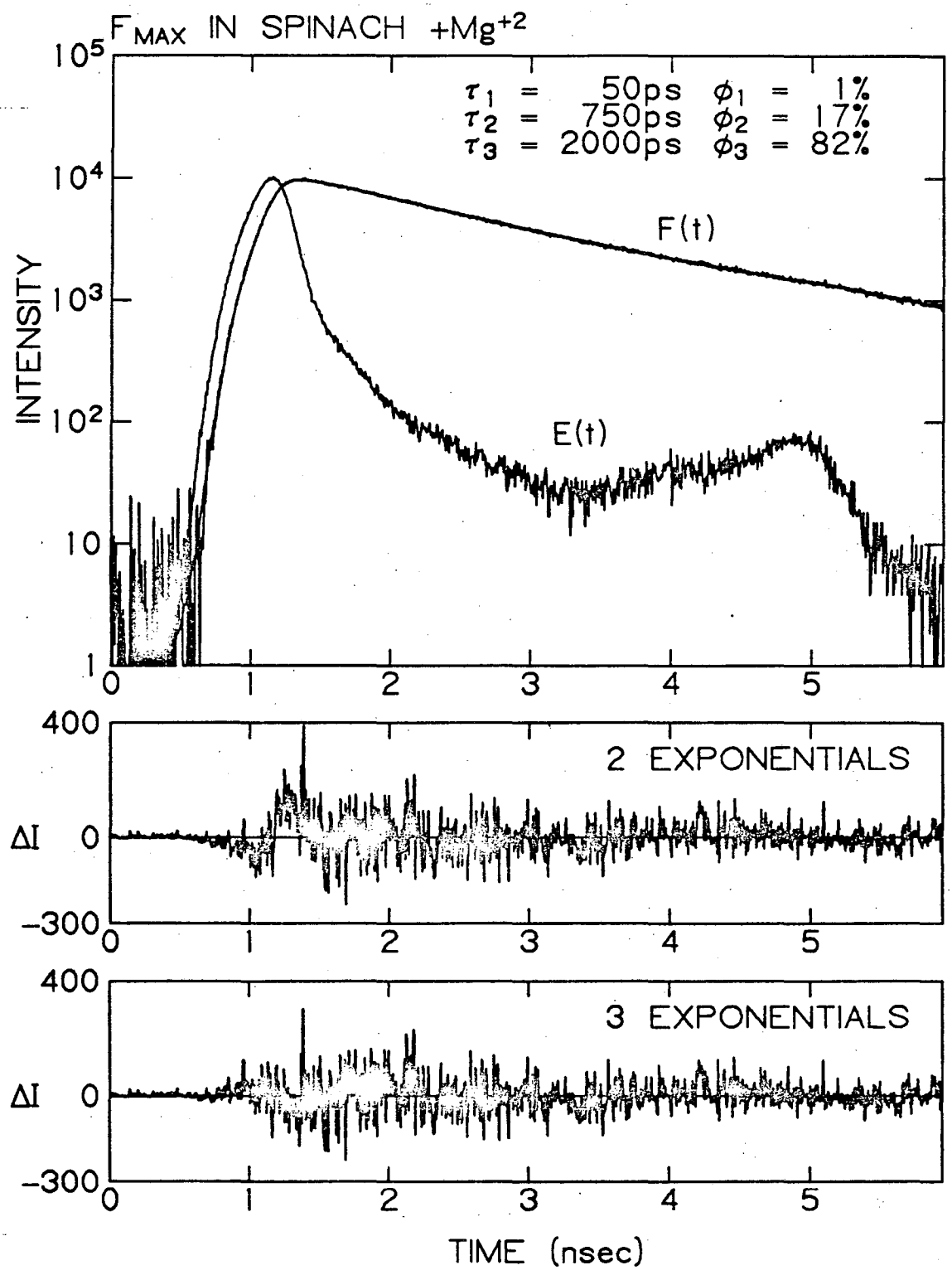

XBL 811-4431

FIG 3

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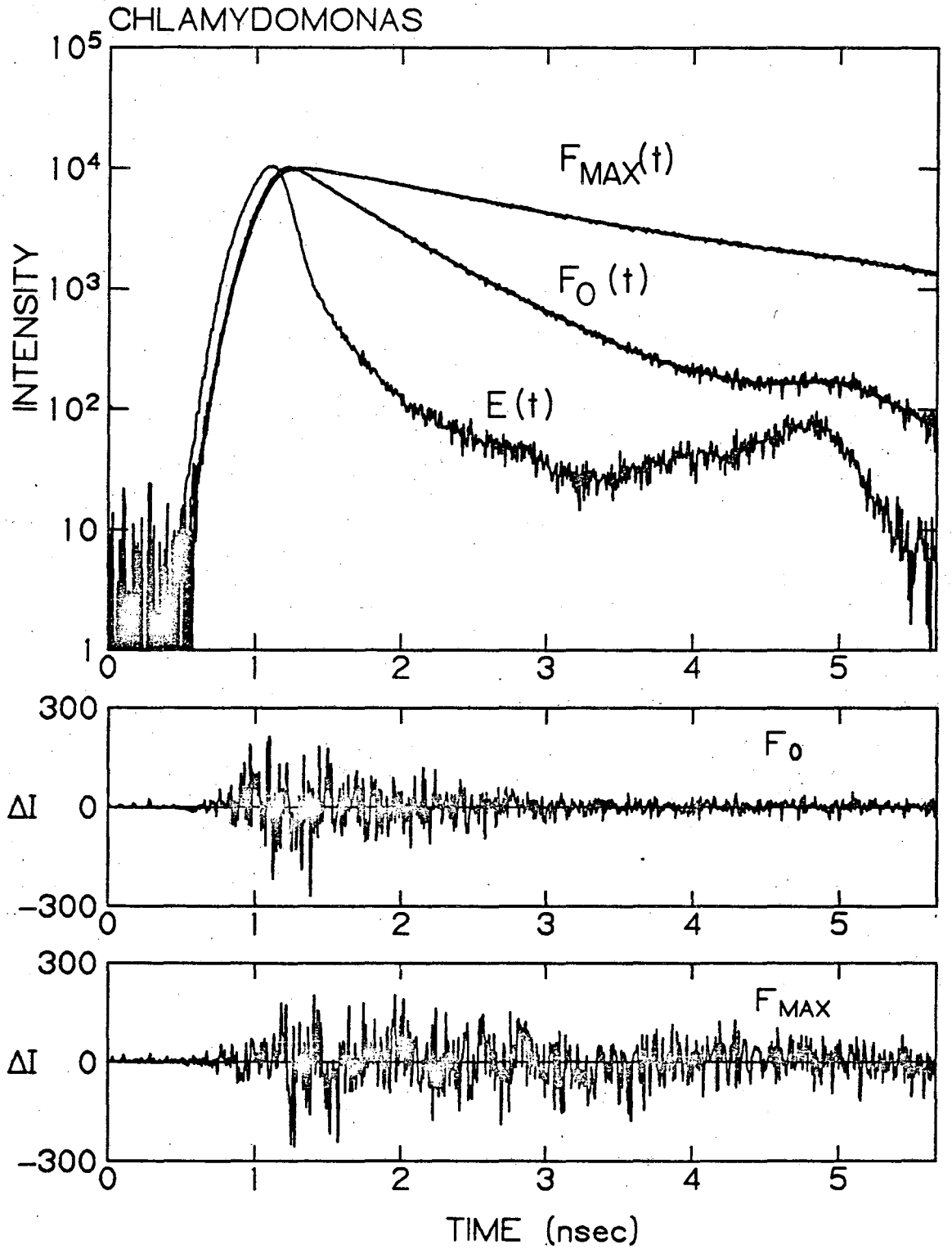

XBL811-4432

FIG. 4

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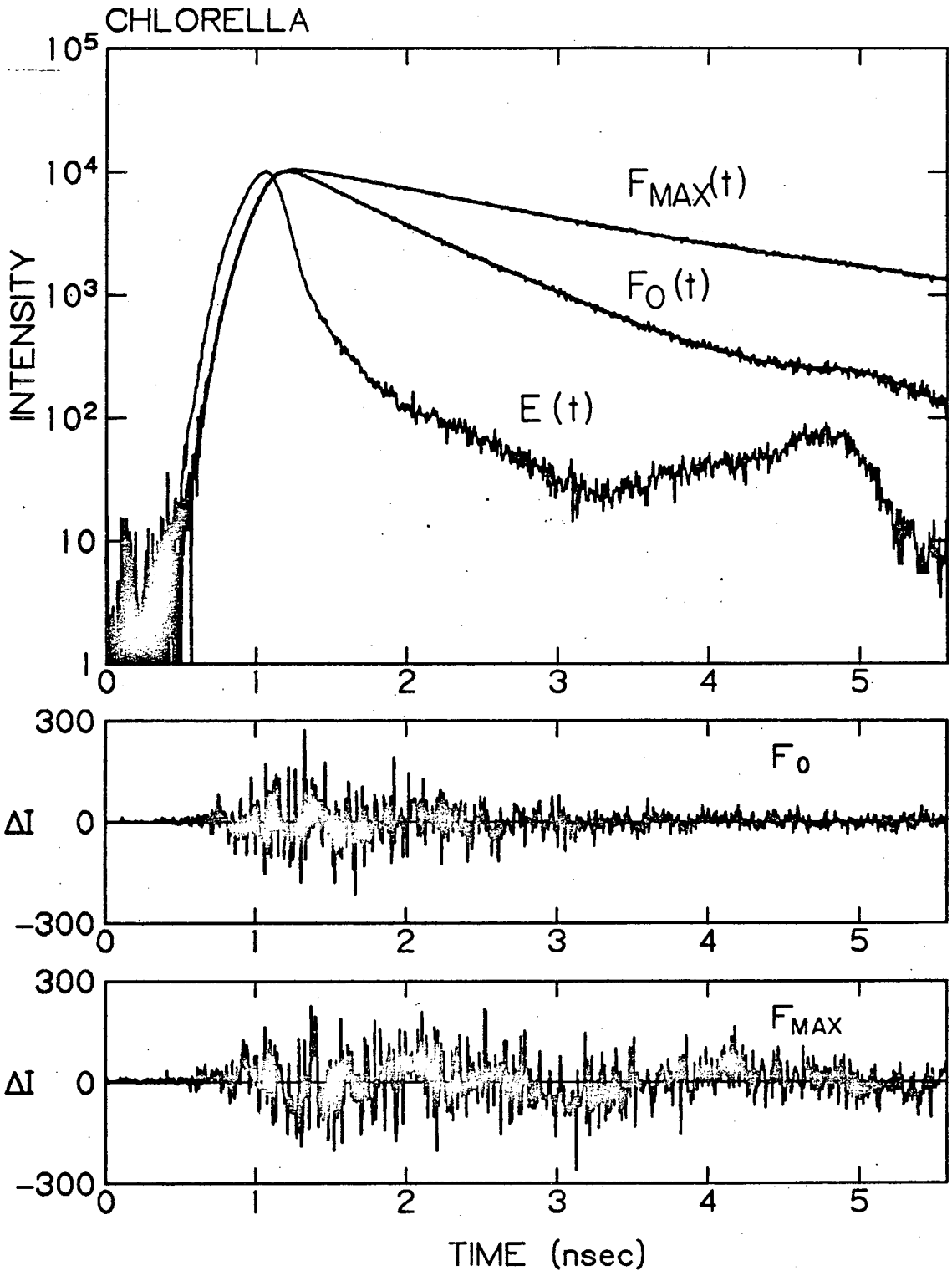

XBL 811-4433

FIG 5

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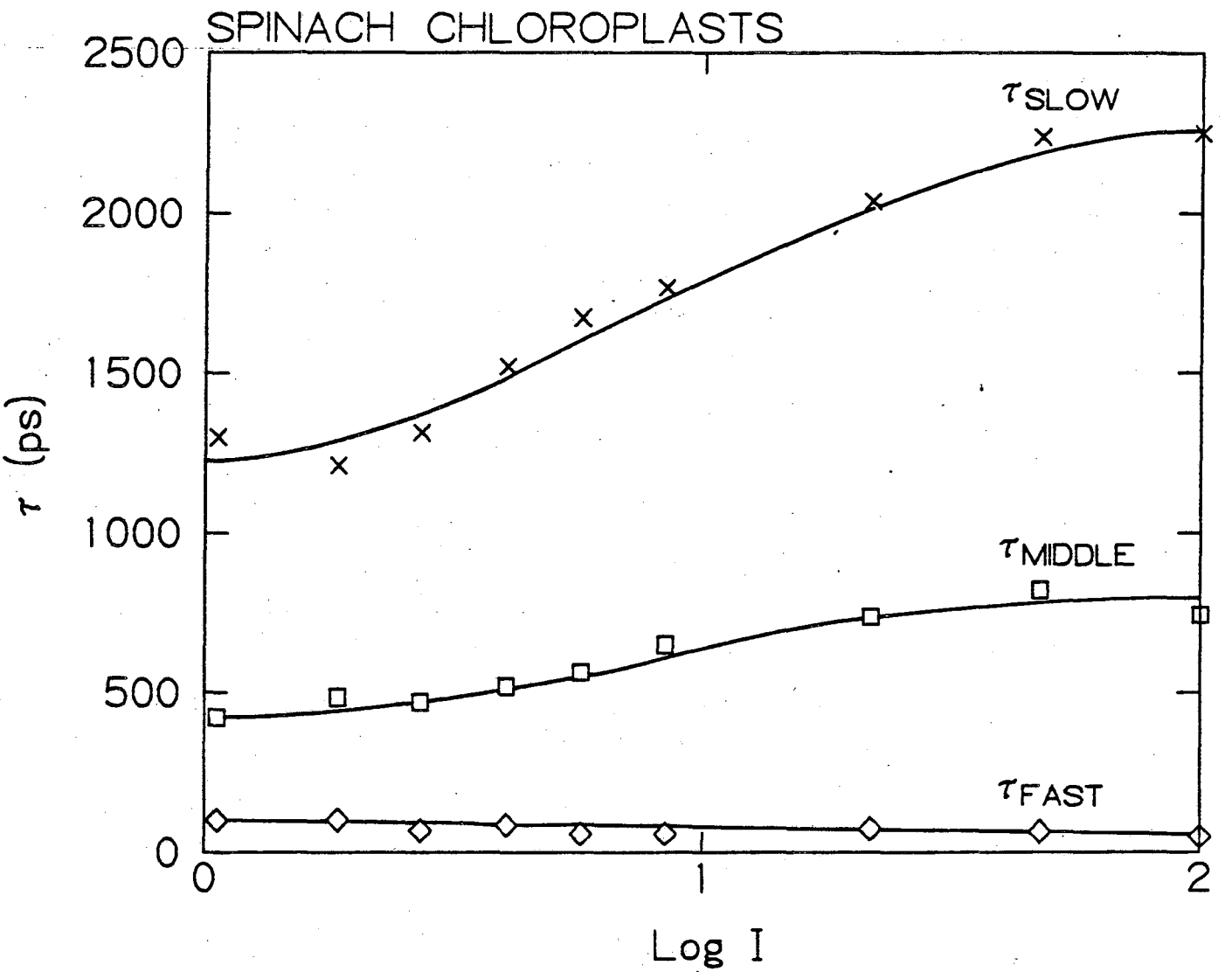
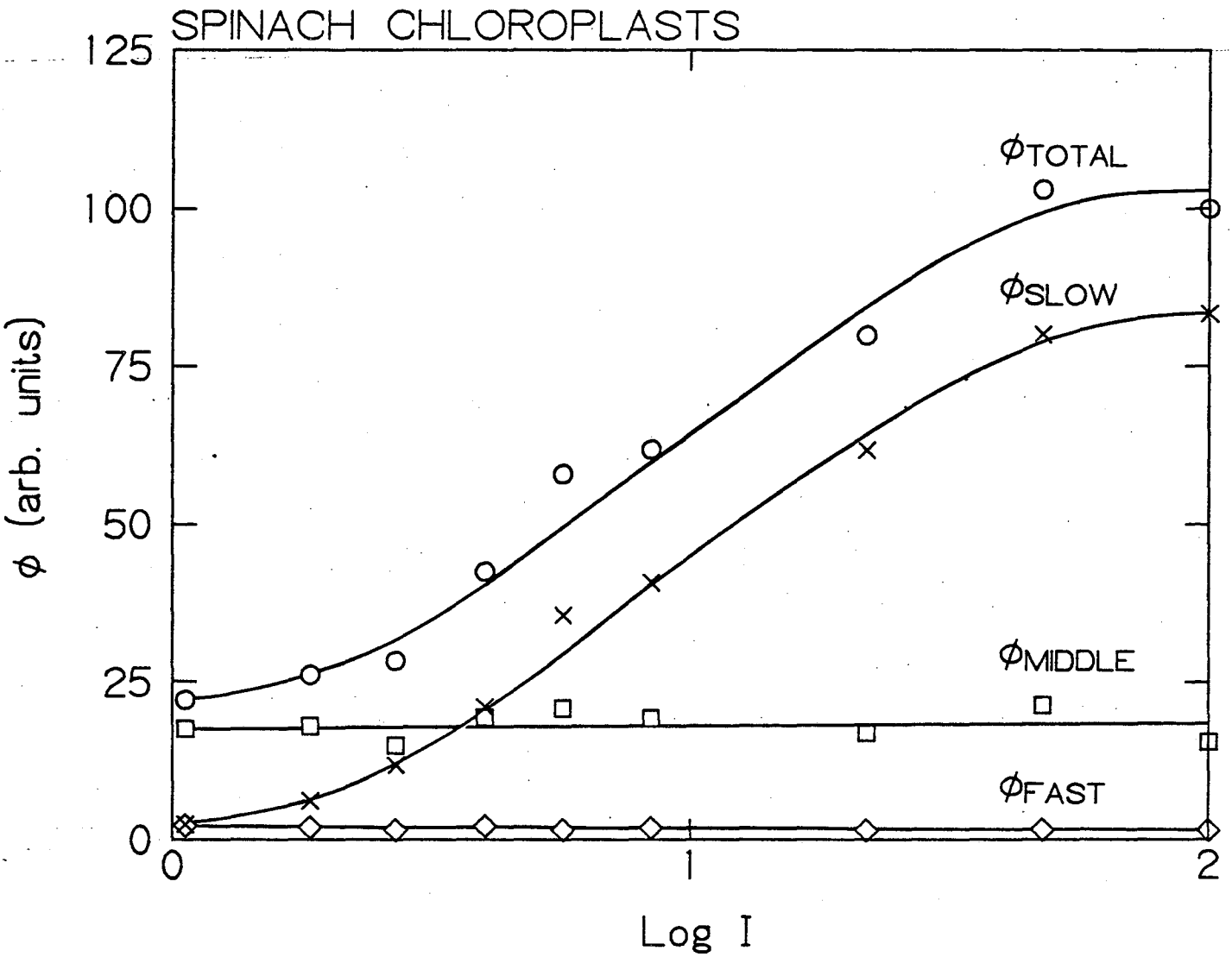


XBL 817-1025

FIG 6

Huchne



XBL 817-1026

FIG 7

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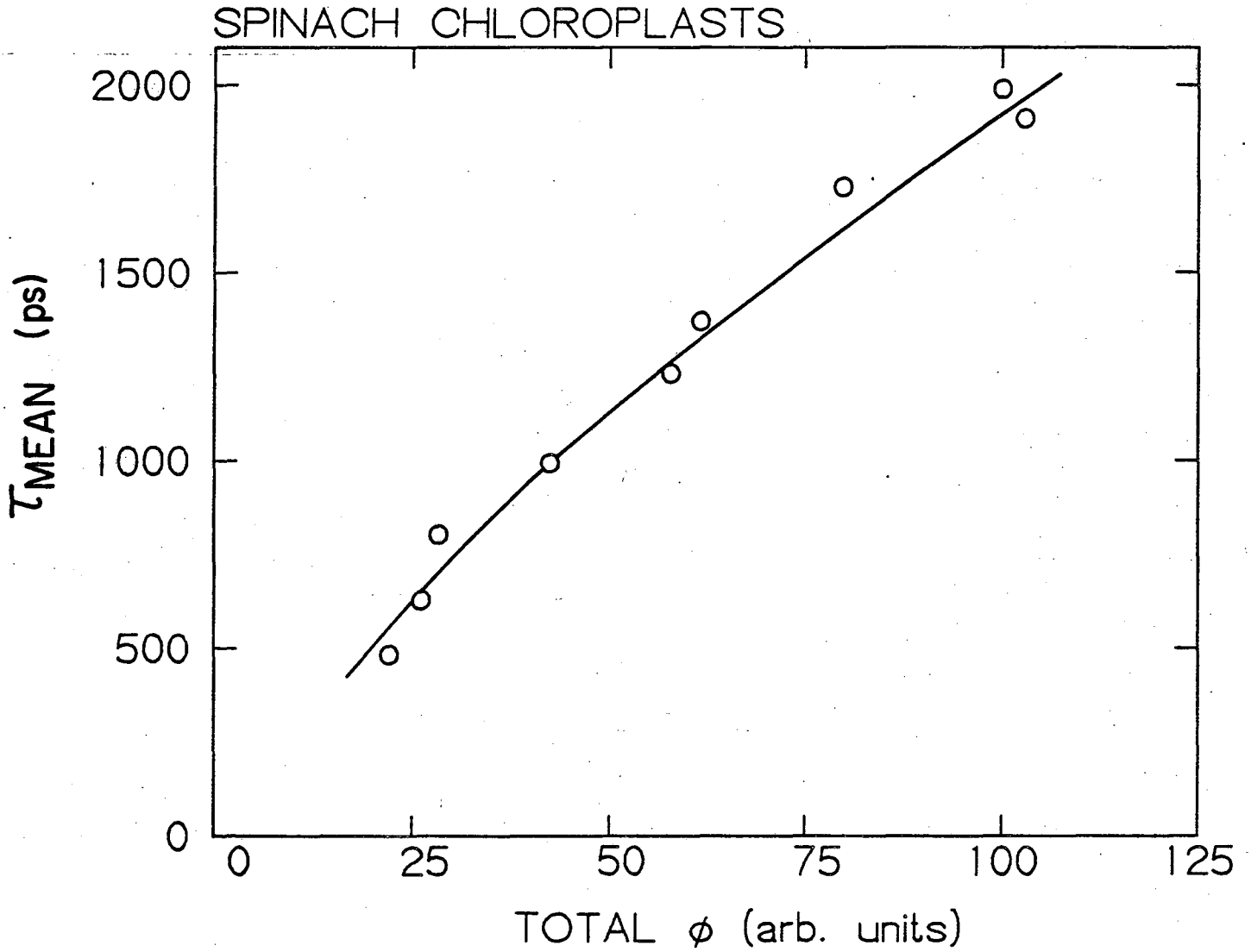

XBL 817-1024

FIG 8

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