KINETIC AND SPECTRAL RESOLUTION OF TWO COMPONENTS OF DELAYED EMISSION FROM CHLORELLA PYRENOIDOSA

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ABSTRACT

1. Decay kinetics of delayed emission from Chlorella pyrenoidosa have been determined with a higher degree of precision than has previously been possible. The decay in the msec-to-sec interval after excitation can be represented accurately by the sum of two exponential decays--a "fast component" whose intensity-dependent lifetime ranges between 3 msec and 10 msec and a "slow component" whose lifetime ranges between 170 msec and 215 msec.

2. The slow component can be isolated by monitoring the emission at 685 nm for long times (> 30 msec) after high or low intensity excitation, or at any time in the msec to sec interval following low intensity (< 0.9 mW/cm^2) excitation. Saturation of the slow component occurs in the low intensity region where oxygen evolution is linear with light intensity. The temperature sensitivity of the slow component indicates involvement of an enzymatic and/or diffusion-limited process.

3. The emission spectrum of the fast component is identical to the fluorescence emission spectrum of the cells, with a peak at 685 nm and a shoulder between 710 and 730 nm. The intensity of the fast component parallels the reduction of System 2 electron acceptors and reaches a maximum level when photochemistry is light saturated. This fact was established by simultaneous measurements of oxygen evolution and delayed emission.

4. The fast component can be selectively inhibited by 3(3, 4-dichlorophenyl)-1, 1-dimethylurea, heat or ultraviolet

irradiation. The slow component can be selectively inhibited by hydroxylamine or low temperatures.

5. Possible mechanisms resulting in fast and slow component delayed emission are discussed in view of the experimental results.

INTRODUCTION

The absorbtion of light by chlorophyll initiates a series of reactions which result in the oxidation of water to form oxygen and the reduction of NADP to NADPH and ultimately the reduction of CO_2 to sugars via the reactions of the Calvin cycle.

Clearly the excitation of chlorophyll must lead to the formation of physically separated oxidizing and reducing agents if "short circuiting" is to be avoided. Indeed, enhancement effects observed when red (< 700 nm) and far red (< 730 nm) exciting light tend to indicate that the formation of the reducing and oxidizing agents are two separate processes occurring in series(1, 2). The physical separation of the two processes by use of detergents confirms the existence of separate systems. This has been incorporated into the generally accepted Z scheme model of Duysens(3). It should be noted that other models exist, notably that of Arnon(4).

Quantum efficiency(5) experiments have shown that 99% or more of the chlorophylls act as antennae - absorbing photons and transferring the energy throughout the system within a matter of picoseconds(6). At specialized reaction sites, very rapid chemistry (on the order of nanoseconds)(6) can take place. These sites are the primary interface between the energy absorbing system and the metabolic systems of the cell and, as such, are vitally important to all life. The pigment molecules of both systems in green algae and higher plants are primarily chlorophyll a. Solvent and aggregation effects rather than differences in pigment type are

responsible for the absorption differences of the two systems. Chlorophyll also occurs in System 2 but is not essential to the photosynthetic process.

Fluorescence from the bulk chlorophylls can occur in competition with radiationless decay and the quenching of the chemistry at the reaction site. No evidence for formation of triplets in intact systems has been found. If the rate of radiationless decay is constant or negligible, the fluorescence will reflect the state of the reaction sites. If the primary acceptor is in the oxidized state, it will act as a quencher of fluorescence while if it is in a reduced state it will not and fluorescence yield will be expected to increase.

The spectrum of the fluorescence consists of a peak at 685 nm and a shoulder at about 720 nm. It is well established that emission at higher wavelengths is due to System 1 at liquid nitrogen temperatures(7-10). However, it is not likely that this is true at room temperature. At higher temperature the chemistry at the reaction site is much more able to compete with fluorescence and it has been argued that the fluorescence at long wavelengths is due not to System 1 but to decay into the first vibrational level of the ground state. However, indications that there may be emission from another pigment or chlorophyll in an unknown form came from Lavorel's studies of variations in the peak (685 nm)-to-shoulder (710-730 nm) ratio during the induction period(11) and studies by Vredenberg and Duysens(12) on the differential effect of light 1 and light 2 on the fluorescence spectra. In addition, it has been found(13) that the emission at 720 nm is polarized while

that at 685 nm is not, indicating heterogeneous sources of emission.

In addition to normal prompt emission or fluorescence, there is emission which lasts up to several minutes after the existing light has been cut off. Since its discovery in 1951(14), delayed emission has been one of the most intriguing and potentially rewarding subjects of investigation associated with the photosynthetic process. It is known from the work of Strehler and Arnold(14) that the action spectra for the production of delayed emission is identical to that of fluorescence.

Arnold and Davidson(15) and Clayton(16) have suggested that the variable chlorophyll fluorescence in vivo might be caused by the presence of a fast component of the delayed emission. However, the phase fluorometric measurements of Müller and Lumry (17) indicate that the intensity of the delayed emission is weaker than that of the fluorescence by at least a factor of ten.

Arnold and Azzi(18) have suggested that the array of chlorophylls within each system behaves like a semiconductor and that electrons and holes are formed which can migrate to the active sites. Their model for the delayed emission is that these electrons and holes can recombine after the exciting light has been cut off to repopulate the singlet state. Lavorel(19) and Clayton(20) in a variant of this theory, have proposed that the repopulation is due to an oxidation-reduction reaction involving the electron donors and acceptors of System 2.

Many workers have suggested that the release of stored

energy in the form of delayed light is the result of certain back reactions of the electron transport chain. Others have suggested triplet-triplet annihilation as the mechanism of repopulation of the singlet state.

In the time range of .001 to 1.0 seconds, the decay of the delayed light is not a simple exponential but has been described in past work as a superposition of as many as three different exponentials(21). This implies several sources of delayed emission.

At the present time, the apparent complexity of the delayed light and the experimental difficulties associated with measuring its extremely low intensity over the required wide time spans have prevented realization of the potential of this tool for the study of photosynthesis.

The purpose of the present paper is to investigate more quantitatively in live cells of Chlorella pyrenoidosa the above phenomena--the precise nature of the time course for delayed emission, the temperature dependence, the effect of various co-factors and inhibitors, and the precise nature of the spectrum of the delayed emission. In addition, the dependence of the delayed light on exciting light intensity from very low light levels up to levels that saturate the photochemical apparatus of photosynthesis was of interest to us because of the known relationships between light saturation and the properties of the photosynthetic unit.

MATERIALS AND METHODS

Cells of Chlorella pyrenoidosa were grown in test tube culture in Knop's medium(21) at a specific growth rate of 1.8. Temperature during growth of the samples was maintained at 26.5°C. Aeration by 5% CO_2 in air kept the cultures suspended. Samples for measurement were taken during the logarithmic growth phase and diluted with Knop's medium or centrifuged to the desired concentration.

Measurements of delayed emission and fluorescence were made using the phosphoroscope system shown schematically in Fig. 2. Excitation was provided by a 6500 W Xenon arc combined with a broad-band interference filter (Baird Atomic) with a maximum transmission at 488 nm and Corning glass filters #4-96 and #4-97. The spectral output of this excitation source is centered at 480 nm and has a half-width of approximately 40 nm. The exciting light completely illuminated a 1-ml cell suspension of between 20 and 30% transmission contained in a quartz sample holder. A belt-driven rotating cylinder with a choice of speeds and with unappreciable frequency drift served as a phosphoroscope. The light emitted from the sample passed through Corning glass filters #2-62, #2-63, and #2-73 into a $\frac{1}{4}$-m Bausch & Lomb high-intensity grating monochromator with a bandpass of 19.2 nm. The monochromator output was detected by an Amperex 56 TVP photomultiplier cooled to dry-ice temperature. The output from the photomultiplier was amplified by a Keithley MultiRange electrometer,

Model 610B, and displayed by a chart recorder, or amplified by a Tektronix oscilloscope No. 585 with a Type 1A7 plug-in unit.

Oscilloscope output was fed into a Nuclear Data memory unit to separate signal from noise. The memory unit consists of two parts: integrator and time base (ND 180 ITB), and 512-channel memory unit (ND 180 M). The triggering of the ITB unit is taken from the output of a photodiode, which is synchronized with the rotating cylinder. After averaging for 10- to 100-k counts, the output of the memory unit is printed in digital form by a Hewlett-Packard digital recorder, Model 5050B, or plotted by a Hewlett-Packard X-Y recorder. The data in digital form were subjected to kinetic analysis by an IBM 360-75 computer.

The quartz sample holder was water cooled to maintain the temperature at 26.5°C during the measuring interval. Magnetic stirring by a quartz-jacketed stirring bar at 600 rpm kept the cells in suspension, and a flow of 5% CO_2 in air over cells prevented CO_2 limitation from occurring even at high light intensities. Exciting intensities were measured by a calibrated circular Eppley thermopile of 1-cm diameter whose photosensitive element was placed in a position corresponding to the center of the cell suspension. The scattered light contribution to measurements of fluorescence was minimized by side illumination through narrow-band interference filters (Baird Atomic) with appropriate blocking. No scattered light contribution to the delayed emission curves was detectable.

Delayed emission spectra were obtained by taking the digital output of the Hewlett-Packard 5050B recorder and averaging the values of five channels centered at the desired time after excitation and subtracting the average base line from this value. Signal averaging was done at each selected wavelength. The emission spectra were adjusted for differences in spectral sensitivity of the photomultiplier and monochromator. The correction factors were determined by using a calibrated Bureau of Standards tungsten lamp as the source and measuring the photomultiplier output with the Keithley electrometer.

The prompt emission spectrum was obtained in a similar manner except for side illumination and electrometer amplification in place of signal averaging.

The ultraviolet treatment involved using only Corning Glass Filter #7-54 between the source and the sample. This filter passed most of the ultraviolet emission from the 6500 W Xenon arc. The cells were exposed to 15.5 mW/cm^2 for 7 min. The heat treatment consisted of exposing the cells to 55°C for 85 sec.

It was possible to measure delayed emission and rates of O_2 evolution simultaneously by insertion of a Yellow Springs Instrument Co. oxygen electrode, Model 5531, into the sample compartment. Its output was measured with a Keithley picoameter, Model 417.

RESULTS

Decay Kinetics

The decay kinetics of delayed emission at three different exciting light intensities are illustrated in Fig. 2. The solid lines in the figure are generated by a least-squares fit of the data to the sum of two exponential decays. At 12 mW/cm^2 exciting light intensity, where O_2 evolution is virtually light saturated, the lifetimes of the two exponential components are 3.31 msec and 173 msec with pre-exponential coefficients of 148 and 8.62, respectively. At 0.9 mW/cm^2 exciting light intensity, in the region where O_2 evolution is linear with light intensity, the lifetimes are 5.48 msec and 215 msec with pre-exponential coefficients of 3.78 and 4.62, respectively. At 0.09 mW/cm^2 exciting light intensity, the lifetimes are 9.6 msec and 187 msec with pre-exponential coefficients of 3.13 and 4.17, respectively.

This kinetic analysis and the earlier observation of Arthur and Strehler(23) suggest the existence of two components in the time range studied here. Delayed emission in the 1 to 5 msec interval after high intensity excitation is predominantly due to the rapidly decaying "fast component", while at low exciting intensity or at times greater than 20 msec, the fast component contributes little to the emission. To understand better the underlying causes of the delayed emission and its relationship to the various photochemical events in photosynthesis, we have made a study of the physical characteristics of the delayed emission under conditions

where it will be dominated by one or the other component. Table I summarizes the results obtained.

The decay kinetics of algae exposed to 3×10^{-5} M twice-recrystallized DCMU are similar to those of the slow component of untreated algae. We observed that DCMU decreases the intensity of the fast component drastically and increases the intensity of the slow component, as previously reported by Sweetser, Todd and Hersh(25).

Strehler and Arnold(14) reported that ultraviolet light decreases the luminescence and oxygen evolution at a comparable rate. The kinetics of decay indicate that a small portion of the normal fast component, with a lifetime of 3.96 msec, is present. However, the slow component, with a lifetime of 73.0 msec, is dominant. Their respective pre-exponential coefficients are 6.02 and 15.3. The decay of the slow component is three times faster as a result of the ultraviolet treatment.

Heat treatment of cells is also effective in isolating the slow component. Both ultraviolet light and heat treatment will also eliminate the slow component upon prolonged exposure.

Hydroxylamine, at a concentration of 10^{-3} M, appears to suppress the slow component as Bertsch et al. have reported(26). Kinetic analysis of the decay of delayed light emission of hydroxylamine-treated algae gives a single exponential with a lifetime of 2.8 msec. In addition to this effect on the decay kinetics, it was found that the spectrum of delayed emission after inhibition with hydroxylamine (at 12.5 mW/cm^2, 2 msec after excitation) is similar

to that of the normal fast component. The irreversible effect of high light intensities on the delayed emission of hydroxylamine-treated cells, which will be discussed later, caused difficulty in obtaining a precise spectrum.

Lowering the temperature to 5°C also selectively suppresses the slow component. At 5°C both the decay kinetics and the emission spectrum are those of the fast component.

Intensity Dependence of Delayed Emission

We have shown that at high excitation intensities, the intensity of the fast component of delayed emission is increased relative to that of the slow component. Details of the intensity dependence of delayed emission are shown in Figs. 6 and 7.

If delayed light monitored at 690 nm is measured 2 msec after excitation, one observes the behavior of the fast component at high exciting light intensities and the slow component at low exciting light intensities. As shown in Fig. 6, the transition from slow component character to fast component character takes place when the exciting light intensity is between 0.9 mW/cm^2 and 3.0 mW/cm^2. As shown by the closed squares of Fig. 6, the intensity dependence of the fast component has a complex character, but one that can be easily explained. At excitation intensities below 12 mW/cm^2 and above 3mW/cm^2, the intensity dependence of the fast component is quite similar to the intensity dependence of fluorescence (open triangles, Fig. 6). This indicates a dependence

on the concentration of reduced products of System 2 that determine the fluorescence yield. The complementarity of the yields of the fluorescence and of oxygen evolution at low intensities (see insert, Fig. 6) is a consequence of this dependence. The rate of oxygen evolution is light saturated at an exciting intensity of 20 mW/cm^2. The intensity of the fast component, measured simultaneously, is also light saturated at this intensity.

The dependence of the slow component emission intensity upon excitation intensity is shown by the filled circles in Fig. 7 on an expanded scale. These measurements were made 30 msec after excitation where, even after high intensity excitation, the fast component is negligible. The intensity of emission rises rapidly at very low exciting intensities. Jones(29) and Clayton(28) have reported that the increase in intensity of delayed emission is proportional to the square of the exciting light intensity at very low exciting light intensities where only the slow component is present. Our data (Fig. 7) do not describe the very low intensity region in sufficient detail to show an I^2 dependence. Saturation of the slow component occurs at an exciting intensity of 0.6 mW/cm^2--somewhat below the intensity shown in Fig. 6 to be associated with increased yields of fluorescence and the fast component of delayed emission.

Inhibition of photosynthesis by 3×10^{-5} M DCMU drastically reduces the contribution of the fast component to the delayed emission. As shown by the open triangles of Fig. 7, the intensity dependence of the delayed emission after DCMU treatment is like

that of the slow component, even when measured 2 msec after excitation. Similarly, cells at 38°C exhibit little fast component emission and, as shown by the closed triangles, the resulting dependence on excitation intensity is like that of the slow component.

We observe that the delayed emission intensity of cells treated with 10^{-3} M hydroxylamine increases sharply with exciting intensity up to 8 mW/cm^2. At higher intensities there is an irreversible decrease in emission intensity. This photoinhibition is characteristically time-dependent, i.e., continued excitation by 8 mW/cm^2 results in a decrease in delayed emission intensity with time. Hence, while the slow component is eliminated by hydroxylamine addition at any exciting intensity, the fast component is also eliminated after sufficient exposure to high light intensity. We observed that photoinhibition of the fast component also occurs when cells kept at 5°C are exposed to high intensity excitation.

Temperature Dependence of Delayed Emission

Other workers have investigated the temperature dependence of delayed emission(29). In view of the many differences between the fast and slow components of delayed emission we have repeated the earlier work under conditions specific for the measurement of the fast component and the slow component. The temperature dependence of the fast component (monitored at 690 nm, at 13.5 mW/cm^2, 2 msec after excitation) and the slow component (monitored at 690 nm, at 0.9 mW/cm^2, 30 msec after excitation)

is shown in Fig. 8. The slow component is very temperature sensitive. Its intensity is almost negligible at 5°C and increases by a factor of 20 between 20°C and 40°C, while at 50°C, its intensity is irreversibly decreased by a factor of 10.

The fast component temperature dependence is complex. Between 20°C and 30°C the intensity is temperature independent. As the temperature is decreased from 20°C to 10°C there is a sharp increase in emission, most pronounced when the exciting light is not of sufficiently high intensity to saturate the photochemical reactions at room temperature. A similar increase, though at a somewhat lower temperature, was observed by Tollin et al(29) who measured the integrated delayed emission intensity from 1 to 10 msec. Between 10°C and 5°C there is a 25% decline in intensity. The decrease observed between 10°C and 5°C may be ascribed to the onset of photoinhibition, which we have observed to occur when cells are exposed to high intensities at 5°C. Above 30°C, there is a sharp increase in intensity of emission but then at 50°C the fast component is completely eliminated.

As one might expect after examination of Fig. 8, only the fast component is observed at 5°C. The spectrum at this temperature is the same as that obtained for the fast component of algae at room temperature, and kinetic analysis indicates a single exponential decay with a lifetime of 2.6 msec.

Further Comparisons of Prompt and Delayed Emission

As previously reported(14, 16, 30), when dark-adapted cells are exposed to light both fluorescence and delayed emission exhibit a transitory period of several minutes duration characterized by an elevated emission intensity. The decrease in emission intensity with time is complementary to an increase in the rate of photosynthesis. The relative changes during this induction period are much more pronounced for delayed emission than for fluorescence. At an exciting intensity of 10 mW/cm^2, we observe a peak-to-steady-state ratio of 10:1 for delayed emission (measured 1 to 16 msec after excitation) and of 2.5:1 for prompt emission. The difference in peak-to-steady-state ratios indicates a greater sensitivity of the fast component of delayed emission to the state of the electron transport intermediates. Clayton(28) has discussed this phenomena in terms of "live" and "dead" components of the prompt emission.

We have observed that DCMU inhibition increases the intensity of the prompt emission and of the slow component of delayed emission. At an exciting intensity of 1 mW/cm^2, the fluorescence yield due to a weak measuring beam ($\ll$ 1 mW/cm^2) is maximized, indicating that the primary electron acceptor of System 2 is largely reduced and unable to act as a quencher of fluorescence. The slow component of delayed emission from inhibited cells is also maximized at this exciting intensity.

Delayed emission from the inhibited cells can be represented by a single exponential with a lifetime of 120 msec. The signal observed after 1 mW/cm^2 excitation with the weak measuring beam turned on was a composite of the delayed emission resulting from the 1 mW/cm^2 excitation and the prompt emission stimulated by the weak measuring beam. The delayed emission signal was subtracted from the total, and the resulting time-varying component of the prompt emission was subjected to kinetic analysis. The lifetime of the decay of the fluorescence yield obtained in this way is 139 msec. The fluorescence yield decrease after excitation is attributable to reoxidation of the System 2 electron acceptor by a slow back reaction of System 2(31, 32). The similarity of the decay of delayed emission and the decay of the fluorescence yield indicates a mutual dependence on the rate of this back reaction.

A further indication that prompt and delayed emission are both sensitive to the concentration of reduced electron transport intermediates comes from observations of emission changes due to CO_2 limitation. Removal of the ultimate electron acceptor CO_2 results in an increase in concentration of reduced intermediates. Both fluorescence and delayed emission increase due to CO_2 limitation, except at saturating light intensities where CO_2 limitation would be expected to have little effect.

DISCUSSION

Since two different mechanisms appear to be involved in production of the fast and slow components of delayed emission, we will discuss the characteristics of the two components separately. The experiments here do not allow a precise delineation of the underlying reactions but do impose restrictions on possible descriptive models. It must be noted that this report deals only with delayed emission in the msec-sec interval after excitation and that the properties of much slower emission may differ from those we observe for our slow component.

Nature of the Fast Component

The prompt emission spectrum results from direct excitation of the chlorophylls in System 1 and System 2 by an external light source. The chlorophylls responsible for the fast component of delayed emission could conceivably be excited at the expense of energy stored in a System 2 photoproduct, or independently excited at the expense of a System 2 photoproduct and System 1 photoproduct at the same rate. The latter is unlikely since (1) Scenedesmus mutant No. 11 with functional System 1 and nonfunctional System 2 provides very little delayed emission(38), (2) cells inhibited by DCMU show very little fast component delayed emission(25). Thus, it appears that the metastable state whose decay results in fast component delayed emission is the result of System 2 activity.

Figure 6 shows the similarity between the dependence of prompt emission intensity and fast component emission intensity on exciting intensity.

The complementary change in the yields of oxygen evolution and the prompt emission shown in the insert of Fig. 6 is to be expected since the electron flow down the electron transport chain (which is reflected by the rate of oxygen evolution) is a process which competes with fluorescence. The fluorescence yield of the singlets which are formed as a result of the fast component mechanism will depend on both the state of the traps when the singlet state is repopulated and the concentration of the metastable state which results in System 2 emission. The latter, according to the kinetic model of Joliot(34), would be roughly proportional to light intensity before the active sites begin to become saturated, and thus would not affect the shape of a yield curve provided the concentration of the metastable state paralleled that of the primary acceptor. This would be true until the metastable state reached saturation at which point further excitation could not increase the intensity of delayed emission. Hence, if the primary acceptors remain at the same level of oxidation attained while chlorophyll was being excited for several milliseconds after the exciting light is cut off, as appears likely from the decay of fluorescence yield after excitation, the yield of delayed emission would be expected to parallel that of prompt emission. When the primary acceptor (and hence, the metastable state) is saturated, as measured by the rate of oxygen evolution, the intensity of delayed emission will

remain constant and the yield will decrease as more intense exciting light is used. This is shown to be true in Fig. 6.

The DCMU sensitivity indicates that the site of energy storage is beyond the DCMU block, generally assumed to occur between the primary and secondary acceptors of System 2. Thus, it appears likely that some reduced intermediate on the electron transport chain may act as the energy source. The relative temperature independence of the fast component indicates that the energy source is not very far removed from the System 2 chlorophylls. The pool of secondary electron acceptors denoted as A by Forbush and Kok(31) is a possible candidate for the site of energy storage responsible for the fast component of delayed emission. According to the model of Witt(34), this could be the plastoquinone pool. If this hypothesis is correct, then the fact that the fast component of delayed emission exhibits more pronounced inducation effects than does prompt emission yield simply reflects the fact that some prompt emission occurs from the bulk chlorophyll molecules even when most of the System 2 electron acceptors are in their open (oxidized) state, while the delayed emission is totally dependent upon the oxidation state of the intermediates.

Finally, it has been reported by Mantai et al(36) that ultraviolet irradiation results in a disruption of lamellar structure and of System 2 activity. Similarly, membrane structures have been shown to be disrupted over a small temperature range between 45°C and 50°C(37). Witt has shown that such disruptions eliminate the transport of electrons from the reaction site to the quinines or

the unidentified X-335 which preceeds the quinones on the electron transport chain(38). Such disruptions of the lamellar structure would account for the fact that under these conditions no energy is transferred to the metastable state responsible for the occurrence of the fast component. The dependence of this energy transfer on lamellar structure may be relevant to the geometrical arrangement of the chlorophylls within the lamellar system of the chloroplasts.

The light saturation behavior, the effect of DCMU, and the elimination of fast component delayed emission by disruption of the lamellar structure (the only time when triplets are observed)(39) eliminate the possibility of triplet-triplet annihilation as a possible source of the fast component delayed emission.

Nature of the Slow Component

As in the case of the fast component of delayed emission, the occurrence of the slow component seems dependent upon the products of System 2 activity. The temperature dependence shown in Fig. 8 of the slow component intensity is characteristic of an enzymatic and/or diffusion-limited process. Extreme heat or ultraviolet irradiation could alter either membrane permeability of the efficiency of an enzyme reaction.

It has been demonstrated(40, 41, 42) that an electrical field is set up across the thylacoid membrane upon illumination of chloroplasts. This electrical field results in the formation of ATP in the dark(39, 43, 44). The decay of the field was monitored by a

chlorophyll b absorption change. Involvement of chloroplast membranes in delayed light production is indicated by observation of emission from preilluminated chloroplasts stimulated by salt addition(40) or acid-base shifts(41) that establish a potential gradient across the chloroplast membranes. Support for this interpretation comes from the studies of Neumann and Jagendorf(43) on the variation of the pH increase outside the chloroplast as a function of light intensity. The saturation level of the pH increase was identical to the saturation level of the delayed emission. However, it must be remembered that the kinetics of decay of the pH gradient in chloroplasts has a lifetime approximately five times as long as that of the slow component delayed emission in Chlorella, while the lifetime of the decay of the electric field reflected by the decay of the chlorophyll 6 signal is one half(45) to one tenth(46) that of the slow component decay.

The fact that DCMU does not prevent occurrence of the slow component of delayed emission indicates that the underlying mechanism does not involve an intermediate on the electron transport chain past the primary acceptor of System 2. An increase in the concentration of reduced System 2 photoproduct induced by blocking the electron transport chain by DCMU addition or CO_2 limitation increases the intensity of the slow emission, implying an increase in formation of the precursor of the slow component.

We have found that delayed emission from DCMU-treated cells shows the same rate of decay as the fluorescence yield (monitored by a weak measuring beam). This indicates that the

intensity of the slow component of delayed emission depends in part on the concentration of the primary acceptor of System 2. That the intensity is also influenced by the concentration of System 2 electron donors is shown by inhibition of the slow component by hydroxylamine. Hydroxylamine is known to interfere with electron transport on the oxidizing side of System 2(47), probably by (1) extraction of Mn from the System 2 complex(48) and (2) by reduction of an intermediate between chlorophyll and H_2O. If the slow component delayed emission is the result of a recombination involving this oxidant, then its reduction could be the cause of the selective inhibition. The first mechanism of inhibition would block electron flow and, hence, could be the cause of the slow inhibition of the fast component delayed emission.

The concept of delayed emission suggested by Lavorel(19) and extended by Clayton(20) is consistent with our observations and is in accord with reports of I^2 dependence of delayed emission at low exciting intensities(27, 28). The mechanism they suggested for production of delayed emission involves a recombination of charge across a membrane, essentially the reversal of System 2 photoactivation.

During preparation of this manuscript for publication, a report by Bennoun(32) appeared that lends support to the proposal that the slow component results from a back reaction between the reduced product of System 2 and the primary oxidant of System 2. Bennoun reported that hydroxylamine blocks one pathway for reoxidation of the reduced product of System 2 and permits reoxidation

by another pathway to proceed with unchanged velocity. In the context of the results presented here, the two pathways of reoxidation are linked to production of the slow and fast components of delayed emission.

Of the other models proposed to account for delayed emission, we find that the electron-hole recombination proposed by Arnold and Azzi(18) can account for our observations if a temperature-sensitive step is built into it. The physical model of triplet-triplet annihilation can be eliminated as a possibility due to the temperature sensitivity and the light saturation behavior of the slow emission.

Concluding Remarks

Failure to recognize the existence of two delayed emission components whose relative intensities depend upon excitation intensity, CO_2 supply, temperature, culture conditions, and presence of inhibitors has resulted in many contradictory reports concerning delayed emission. Unless these conditions are rigorously controlled during the course of measurement, scatter in the data may obscure the intensity dependence of the kinetics and necessitate use of complex kinetic schemes to fit the data. This is a possible explanation of the fact that Ruby(21) did not observe a change in decay kinetics when the exciting intensity was increased tenfold, and he had to use three exponential components to describe his decay curves.

Contradictory reports concerning light saturation of delayed emission(14, 49) and the effect of DCMU on delayed emission(28) may be resolved by realizing that the first few milliseconds after excitation will be dominated by the fast component, which is suppressed by DCMU and saturates only in the intensity range where O_2 evolution saturates, while longer times (100 msec) are dominated by the slow component, which is stimulated by DCMU and saturates in the intensity range where O_2 evolution is linear.

We find that the behavior of intact cells of Chlorella differs significantly from that of isolated chloroplasts. The intact cells produce a delayed emission whose intensity increases when photochemistry is limited by CO_2 limitation, except under high intensity excitation where the electron transport intermediates are already in a reduced state and CO_2 limitation has no effect. There have been reports(50, 51) that the absence of electron acceptors results in low levels of delayed emission in isolated chloroplasts under high intensity excitation. We suggest that the difference between chloroplast and whole cell behavior may lie in the increased interaction of the electron transport intermediates with O_2 and electron carriers in the suspension medium following isolation of chloroplasts.

FOOTNOTES

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TABLE I. Comparison of Fast and Slow Components of Delayed Emission.

	Fast Component	Slow Component
Lifetimes (msec)		
0.09 mW/cm^2 *	9.6	187
0.9 mW/cm^2 *	5.7	210
12.0 mW/cm^2 *	3.2	178
Relative Initial Intensity at 690 nm		
0.09 mW/cm^2 *	0.67	1
0.9 mW/cm^2 *	0.75	1
12.0 mW/cm^2 *	17	1
Saturation Intensity (mW/cm^2)*	20.0	0.6
Effect of Treatment		
DCMU	Strongly Suppressed	Stimulated
Ultraviolet	Strongly Suppressed	Suppressed
55°C	Strongly Suppressed	Suppressed
5°C	Stimulated	Strongly Suppressed
Hydroxylamine	Suppressed	Strongly Suppressed

* Intensity of 488 nm excitation.

FIGURE CAPTIONS

Fig. 1 The "Z scheme" proposed by Duysens (1962).

Fig. 2 Schematic representation of sample holder, phosphoroscope, and data collection components. S_1 and S_2, positions of a 6500 W Osram Xenon arc for stimulation of delayed emission (S_1) and fluorescence (S_2); F_1, F_2 and F_3, filters; L_1, L_2 and L_3, quartz lenses; M, Bausch and Lomb monochromator; PM, Amperex 56TVP photomultiplier. The multichannel analyzer was used for measurements of fluorescence.

Fig. 3 Decay kinetics of delayed emission from Chlorella pyrenoidosa excited by (A) 12 mW/cm^2, (B) 0.9 mW/cm^2, and (C) 0.1 mW/cm^2 of 488 nm light. The solid lines are plots of the indicated functions that were fit to the data by the method of least squares. The shaded region is an envelope of the data points recorded by the 5050B recorder.

Fig. 4 The emission spectrum of fluorescence (Δ) and of the fast component of delayed emission (■). The delayed emission was measured 2 msec after 12 mW/cm^2 of 488 nm excitation. Fluorescence was measured during illumination by 10 mW/cm^2 of 488 nm light.

Fig. 5 The emission spectrum of fast component or delayed emission measured 2 msec after 12 mW/cm^2 of 488 nm excitation (■). The same emission spectrum with

the delayed emission intensity decreased by a factor of 20 by a neutral density filter.

Fig. 6 Steady-state rates of O_2 evolution (●), fluorescence (△), and delayed emission (□, ■) vs. intensity of 488 nm excitation. Delayed emission 2 msec after excitation was monitored at 690 nm (□). The insert shows the intensity dependence of the yields of O_2 evolution (▲) and fluorescence (△).

Fig. 7 Intensity dependence of delayed emission at 690 nm: slow component, measured 30 msec after excitation (●); cells with 3×10^{-5} M DCMU, measured 2 msec after excitation (△); cells at 38°C, measured 30 msec after excitation (▲).

Fig. 8 Temperature dependence of 730 nm delayed emission, measured 2 msec after 12.7 mW/cm² of 488 nm excitation (■) and of 690 nm delayed emission, measured 30 msec after 0.9 mW/cm² of 488 nm excitation (●). Arrows indicate temperatures at which the spectra of Fig. 9 were obtained.

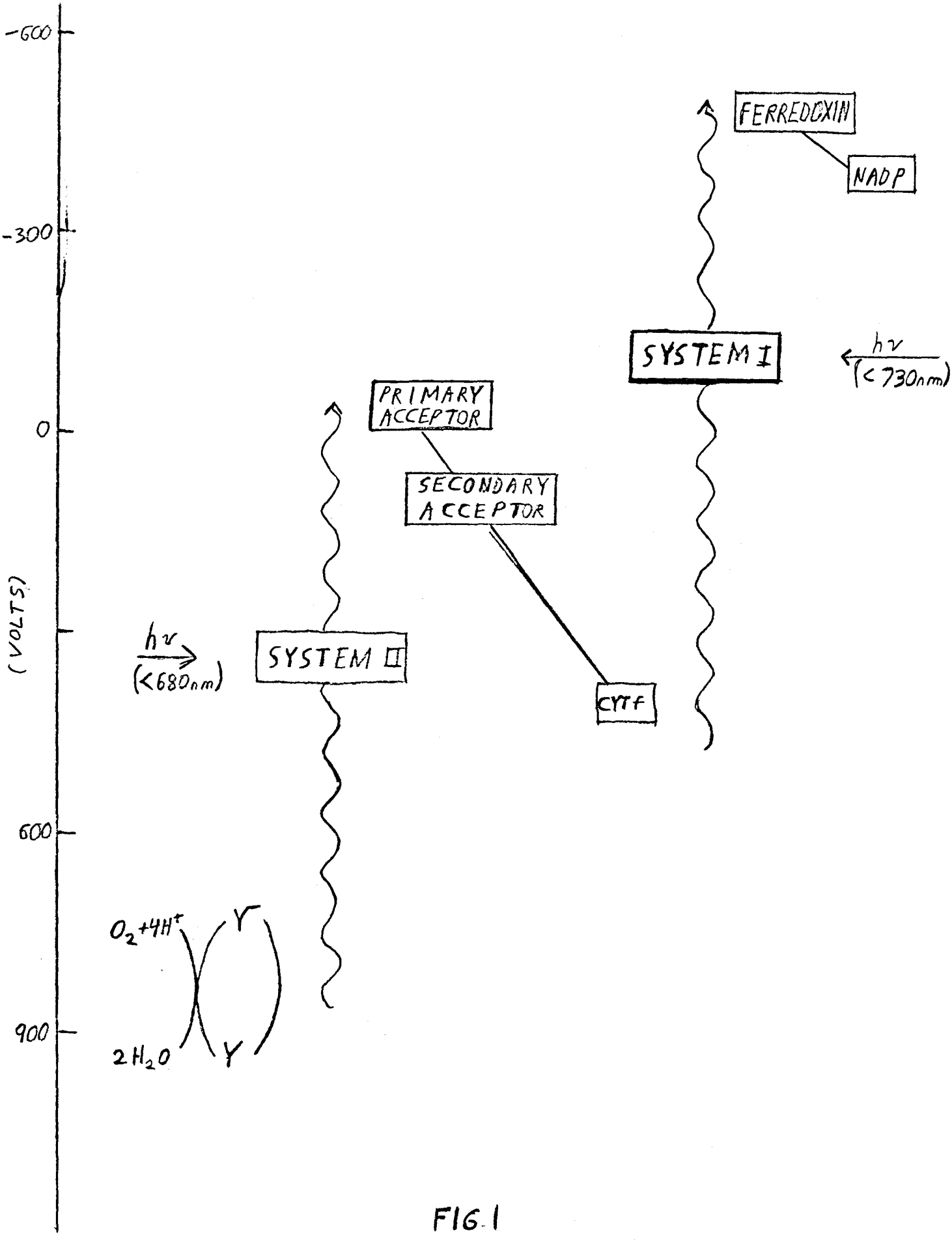
-600
-300
0
600
900
(VOLTS)
FERREDOXIN
NADP
SYSTEM I
hν
(<730nm)
PRIMARY ACCEPTOR
SECONDARY ACCEPTOR
CYTf
hν
(<680nm)
SYSTEM II
O_2+4H^+
$2H_2O$

FIG. 1

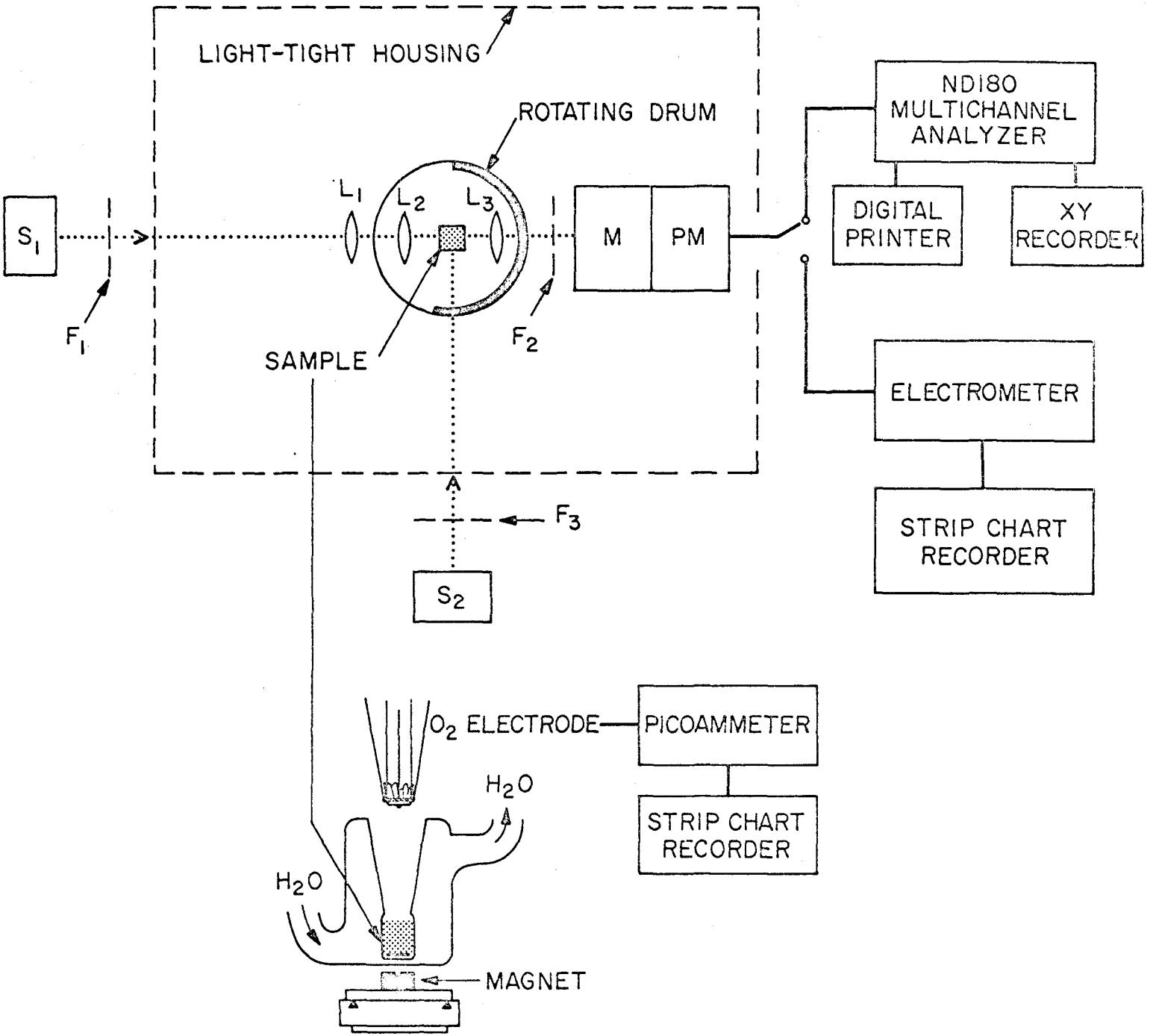
LIGHT-TIGHT HOUSING
ROTATING DRUM
ND180 MULTICHANNEL ANALYZER
DIGITAL PRINTER
XY RECORDER
S_1
L_1
L_2
L_3
M
PM
F_1
F_2
SAMPLE
ELECTROMETER
F_3
STRIP CHART RECORDER
S_2
O_2 ELECTRODE
PICOAMMETER
H_2O
STRIP CHART RECORDER
H_2O
MAGNET

FIG 2

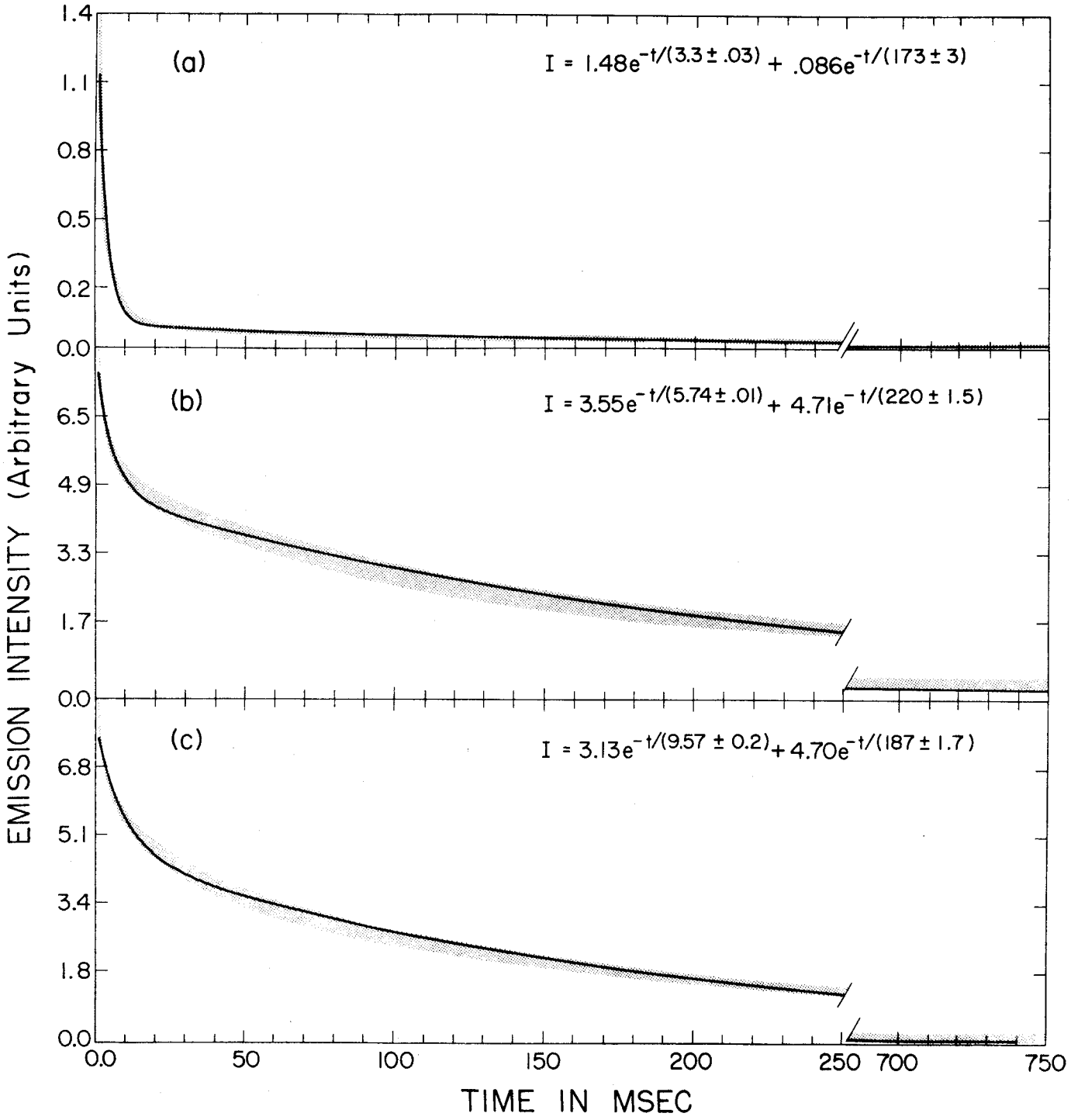
(a)
$I = 1.48e^{-t/(3.3 \pm .03)} + .086e^{-t/(173 \pm 3)}$
(b)
$I = 3.55e^{-t/(5.74 \pm .01)} + 4.71e^{-t/(220 \pm 1.5)}$
(c)
$I = 3.13e^{-t/(9.57 \pm 0.2)} + 4.70e^{-t/(187 \pm 1.7)}$
EMISSION INTENSITY (Arbitrary Units)
TIME IN MSEC
1.4
1.1
0.8
0.5
0.2
0.0
6.5
4.9
3.3
1.7
0.0
6.8
5.1
3.4
1.8
0.0
0.0
50
100
150
200
250
700
750

FIG 3

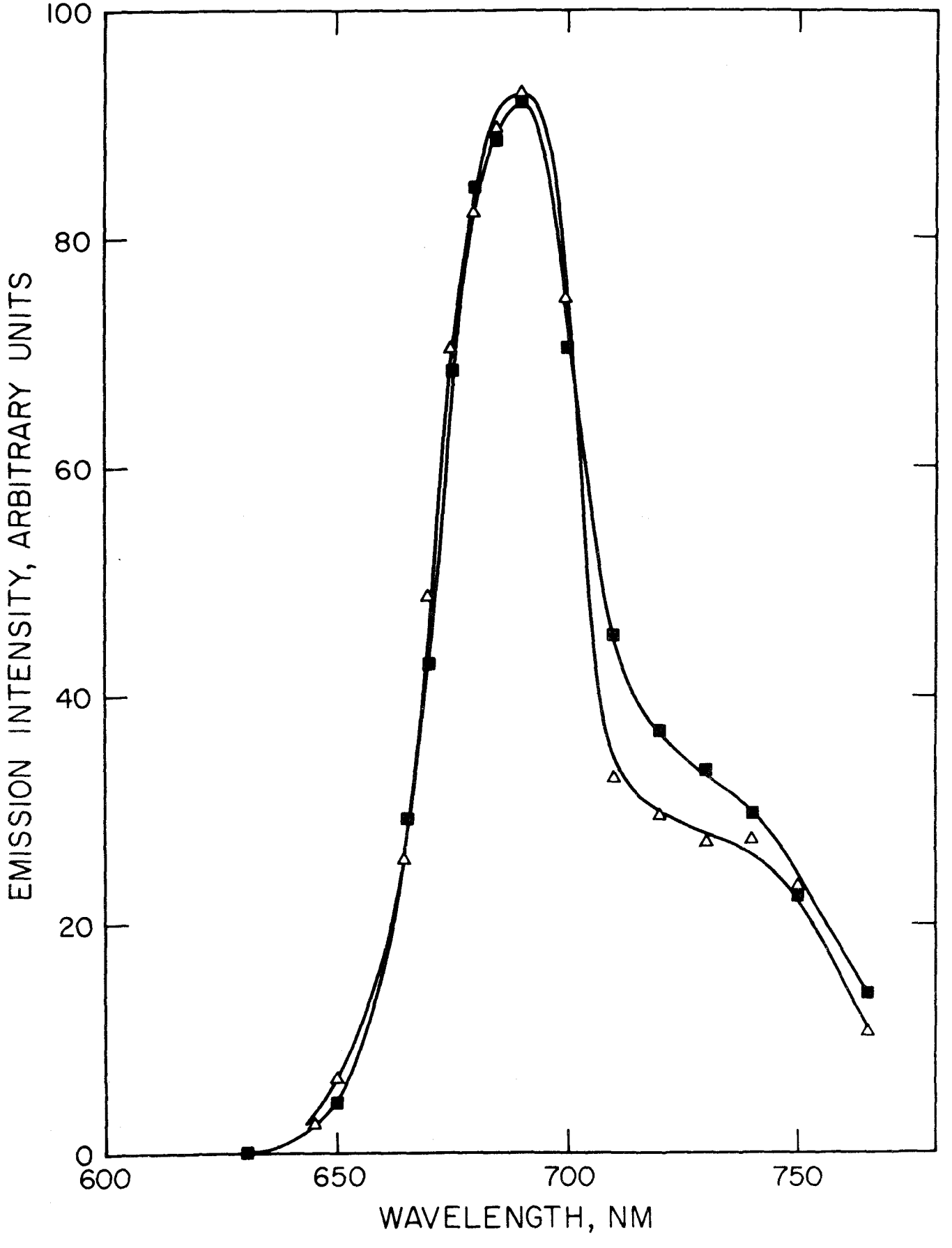
100
80
60
40
20
0
EMISSION INTENSITY, ARBITRARY UNITS
600
650
700
750
WAVELENGTH, NM

FIG 4

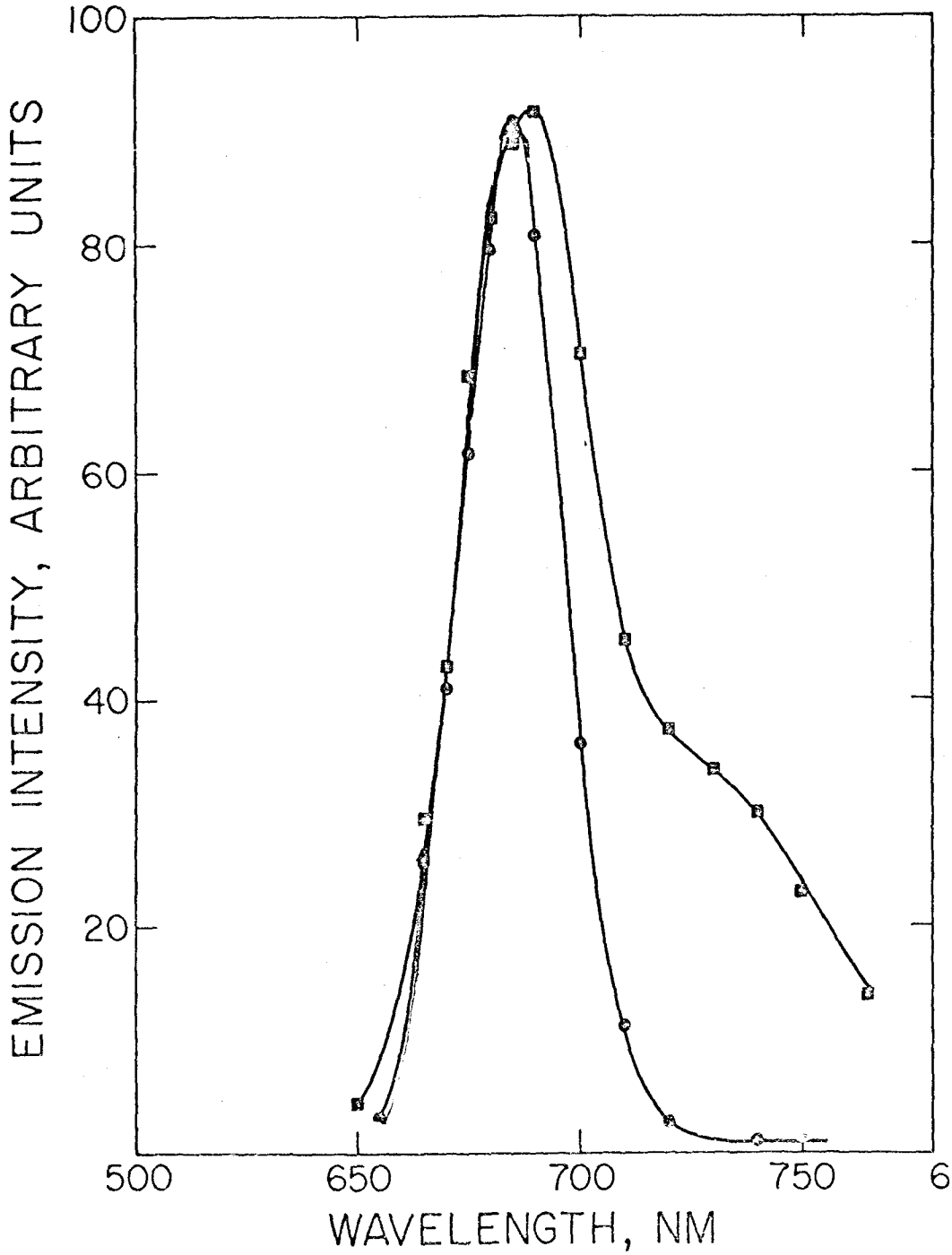
100
80
60
40
20
EMISSION INTENSITY, ARBITRARY UNITS
500
650
700
750
6
WAVELENGTH, NM

FIG 5

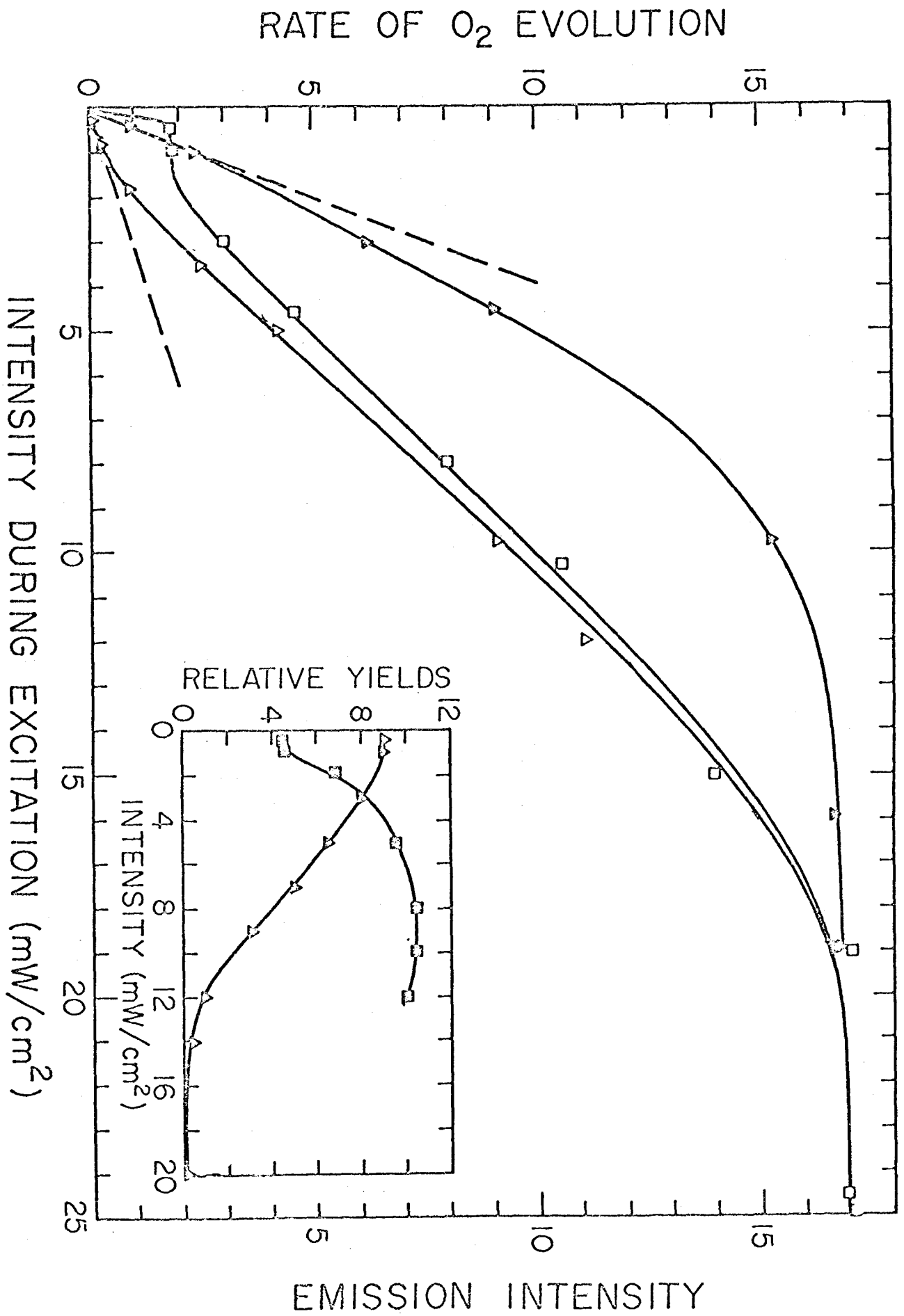
RATE OF O_2 EVOLUTION
0
5
10
15
INTENSITY DURING EXCITATION (mW/cm^2)
5
10
15
20
25
EMISSION INTENSITY
RELATIVE YIELDS
0
4
8
12
INTENSITY (mW/cm^2)
0
4
8
12
16
20

FIG 6

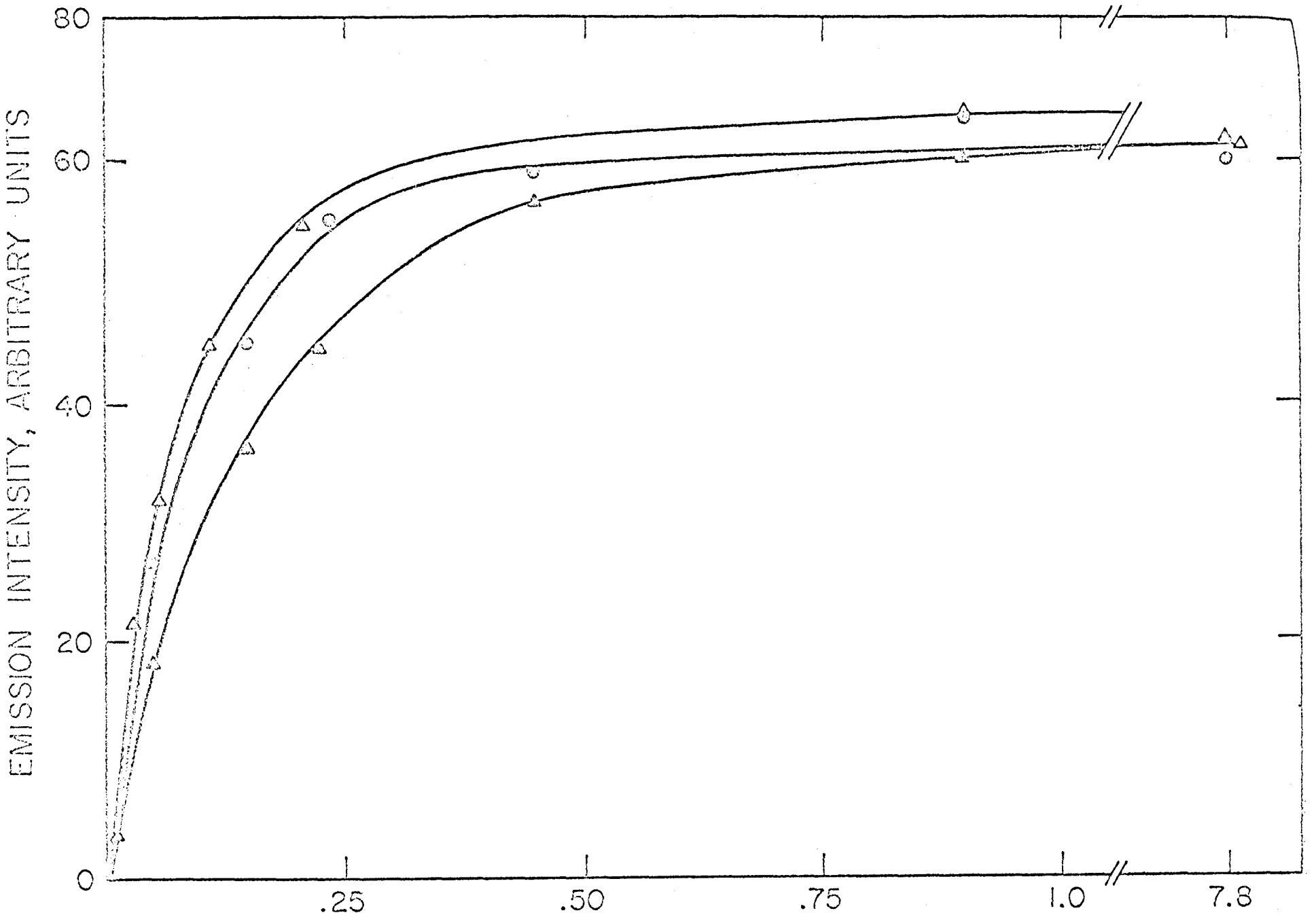
80
60
40
20
0
EMISSION INTENSITY, ARBITRARY UNITS
.25
.50
.75
1.0
7.8
EXCITING INTENSITY, mW/cm²

FIG 7

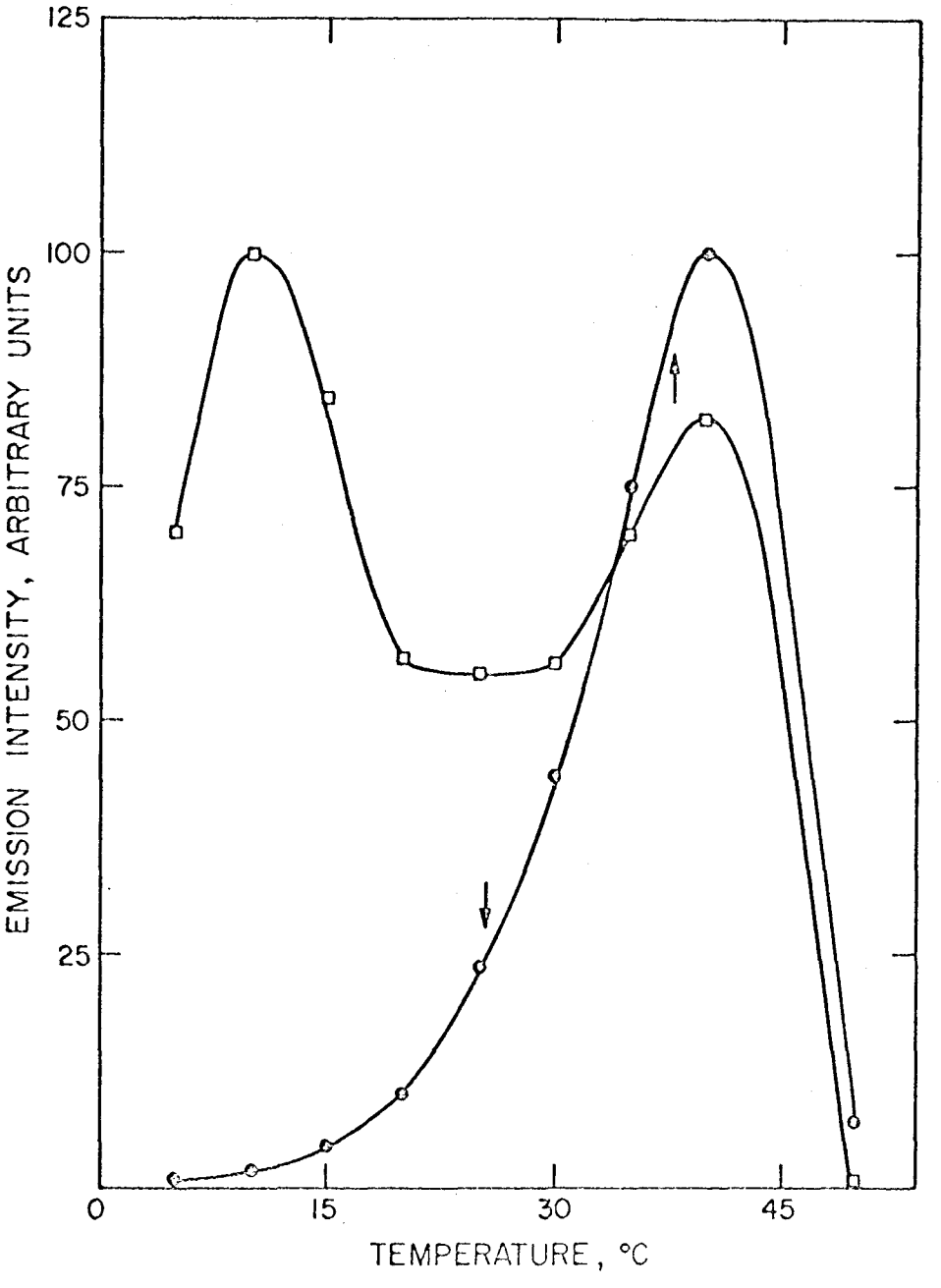
125
100
75
50
25
0
15
30
45
EMISSION INTENSITY, ARBITRARY UNITS
TEMPERATURE, °C

FIG 8

APPENDIX

The first measurements of the system of the slow component revealed no shoulder between 710 nm and 740 nm. Measurements by another research group appeared to contradict this finding and so the method outlined above was reexamined.

By placing neutral density filters between the sample and the photomultiplier it was possible to measure the spectrum of the fast component under the same conditions as previously, only with the intensity of emission cut down. The result was a spectrum that lacked the shoulder between 710 nm and 740 nm as did the original slow component spectrum as shown in Figure 5.

There are several possible causes of this distortion:

1. The averaging process could be inadequate when there is very low input from the photomultiplier,
2. The photomultiplier could be responding in a non-linear fashion to different intensity levels, or
3. The photomultiplier could have a different response vs intensity curve at different wavelengths.

By using calibrated neutral density dilters between the standard tungsten light source and the photomultiplier, it was possible to measure the response of the system as recorded on the averaging unit as a function of intensity at 690 nm and 740 nm. The response vs intensity curves were similar at both wavelengths and were nonlinear. This would eliminate the third possibility.

The experiment was repeated using the electrometer to record the response. There was still nonlinearity but not as much as when the averaging unit was used. Thus the distortion appears to be due primarily to the inadequacy of the averaging unit at low input levels, although there might also be some distortion due to the photomultiplier response as a function of intensity also.