Submitted to Photochemistry and Photobiology

LBL-4225
Preprint

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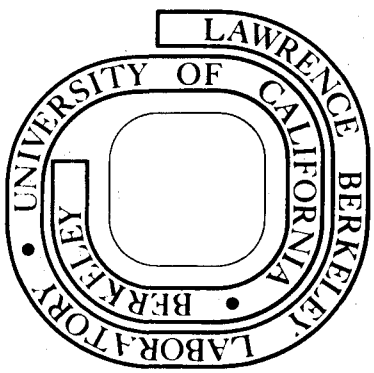
August 1975

Prepared for the U. S. Energy Research and
Development Administration under Contract W-7405-ENG-48



DISCLAIMER

THE ELECTROPHOTOLUMINESCENCE OF CHLOROPLASTS

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Abstract--Delayed light emission emanating from preilluminated chloroplasts can be perturbed with pulsed DC electric fields (200 to 4000 $v\text{-}cm^{-1}$). The perturbation produces a strong stimulation of chlorophyll luminescence. During the field perturbation the stimulated emission rises to a maximum, typically within 100 μs, and then decays. Two kinetic components, R (rapid) and S (slow), are distinguished on the basis of their rise and decay times and their field-dependence. The R component increases exponentially at high fields, decays within 100 to 300 μs during the field pulse and collapses with $t_{1/2}$ = 15 μs at the end of the field pulse. The S component occurs at low fields, exhibits near saturation at 500 $v\text{-}cm^{-1}$, decays with $t_{1/2}$ about 3 ms during the field pulse, and collapses with $t_{1/2}$ = 38 μs at the end of the field pulse. Studies using inhibitors, ionophores, electron donors and electron acceptors associate the R component with ion transport processes. The relation to electron transport associated with Photosystem II is discussed.

Abbreviations: CCCP, m-chlorocarbonylcyanide phenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; PS I and PS II, Photosystem I and Photosystem II; EPL, electrophotoluminescence.

INTRODUCTION

Delayed fluorescence from higher plant organisms is attributed to back reactions of the primary electron acceptor and donor of Photosystem II (Lavorel, 1968). The rate of the dark back reaction is stimulated by a number of perturbations, including salt (Miles and Jagendorf, 1969), acid (Miles and Jagendorf, 1969; Malkin and Hardt, 1972), acid-base (Mayne and Clayton, 1966), temperature (Mar and Govindjee, 1971), organic solvent (Hardt and Malkin, 1972) and electric field transients (Arnold and Azzi, 1971 a,b). Crofts et al. (1971) expressed the rate of delayed fluorescence in terms of electrical and chemical transmembrane potentials. They concluded that the perturbations alter one or both of these potentials, leading to increased rates of delayed fluorescence.

Fowler and Kok (1974) measured an electric field generated across chloroplasts in the light, concluding that a transfer of charges occurs in a direction perpendicular to the membrane from PS I and II electron donors located on the interior of the membrane to acceptors located on the exterior. These results are consistent with the earlier work of Witt and collaborators (Junge and Witt, 1968; Schmidt, et al., 1971) who presented evidence that the fast light-induced 518 nm absorption change is due to an electric field-induced shift in pigment spectra.

Arnold and Azzi (1971 a,b) showed that an external electric field directly applied to chloroplasts causes a stimulation of delayed light emission. Their observations were consistent with a mechanism involving a field-stimulated recombination of electrons and holes that exhibits a hyperbolic cosine dependence on field intensity.

The purpose of the present work is to explore the interaction of electric fields with preilluminated chloroplasts. We present results of experiments in which preilluminated chloroplasts were perturbed with DC electric field pulses. We have chosen to call this emission electrophotoluminescence (EPL) in deference to a similar solid state phenomenon described by Ivey (1957).

MATERIALS AND METHODS

Chloroplast preparation and treatments

Broken chloroplasts were prepared from 6 to 8 week old spinach grown in a growth chamber under a 10 hour light, 14 hour dark cycle. Typically, 10 g of deveined spinach leaves was ground for 10 s in a Waring blender. The grinding medium consisted of 75 ml of a solution consisting of .4 M sucrose, .01 M NaCl, and .05 M tricine or HEPES buffer adjusted to pH 7.6. The resulting suspension was filtered through two layers of Miracloth. Larger particles were discarded with the sediment from a 1 min, 1000 X g centrifugation, and the final pellet was collected after a 5 min, 2000 X g centrifugation. "Aqueous" chloroplasts were prepared from broken chloroplasts by resuspending the above pellet in distilled water and recentrifuging for 10 min at 2000 X g. The resulting pellet was resuspended in 1 to 5 ml of distilled water. Aliquots of that stock solution were diluted in distilled water, or distilled water plus added reagents, to chlorophyll concentrations between 10 and 50 μg/ml. "Buffered" chloroplasts were prepared similarly in 0.01 M tricine. Chlorophyll concentrations were determined according to Mackinney (1941).

Tris-washed chloroplasts were prepared by suspending broken chloroplasts for 30 to 60 min in about 20 ml of 0.8 M tris buffer,

pH 8.0, at 3°C. The chloroplasts were centrifuged from the tris buffer and resuspended twice in distilled water.

Heat treatment consisted of suspending a test tube containing chloroplasts in a water bath maintained between 55 and 60°C for about 3 min.

Chloroplasts treated with either gramicidin (Calbiochem) or valinomycin (Calbiochem) were incubated in the dark at room temperature for 5 min before making measurements.

EPL detection apparatus

The apparatus used for producing and detecting EPL (Fig. 1) consists of a 500 watt tungsten lamp (L), two stepping motor shutters (S_L and S_D), a multiplier phototube (MPT) and two platinum electrodes (E). The timing sequence of the experiment is controlled by logic circuitry (LC) whose sequential pulses directed shutter positions, voltage pulses, and recording devices. A motor drive unit (MD) activates the stepping motors (Phillips PD-22). A pulse generating circuit (PG) is employed to generate square bipolar voltage pulses. Details of the electrode and detecting assembly are found elsewhere (Ellenson, 1973).

The protocol of a typical EPL experiment is illustrated schematically in Fig. 2. Following a 3 to 5 s illumination period, t_p, the excitation is turned off and the shutter in front of the photomultiplier is opened. The delayed fluorescence is observed during all or part of an interval of 100 ms or longer (t_d), whereupon a brief electric field pulse (t_f = 0.05 to 10 ms) is applied. A dramatic rise in the chlorophyll fluorescence occurs during the field pulse. When the applied field is turned off, this EPL signal relaxes rapidly (< 100 μs) during t_r. In all of our studies the signal always returned precisely to the value characteristic

of the delayed fluorescence. Even under applied fields that produce as much as 100-fold increase in intensity during the field pulse, no evidence was ever seen of a competition that resulted in attenuation of normal delayed fluorescence. The signal detected by the MPT is stored in a Biomation 610 transient recorder (BIO-610) triggered simultaneously with the voltage pulse. The stored data is transmitted to a Nuclear Data Enhancetron or Nuclear Data ND-180M memory unit (ND-180). Both systems provide paper tape output for computer processing, or the results can be plotted in analog form on an X-Y recorder.

A modified version of this equipment was used in conjunction with a xenon flash lamp. Instead of two shutters, a single electronic shutter (Ilex) protects the MPT from direct excitation and is opened after the flash. The flash lamp is a special unit designed by ILC of Sunnyvale, Calif. (Babcock and Sauer, 1973), and has a 10 μs flash duration.

The "Bipolar energy switch", designated as PG in Fig. 1, produces either single pulses or sequential pair pulses of similar (unipolar) or opposite (bipolar) polarity. The magnitude of the field is assumed to be the magnitude of the external voltage (up to 400 v) divided by the interelectrode distance (0.25 cm generally, but 0.1 cm for some high field studies).

RESULTS

EPL kinetics

The spectrum of the emission resulting from an electric field perturbation of preilluminated chloroplasts roughly matches the spectrum of *in vivo* chlorophyll *a* fluorescence (determined by interference filters). A typical record of emission stimulated during the field

transient is shown in Fig. 3. There is a delay, or induction, of the emission at the onset of the field pulse, as shown on an expanded time scale in Fig. 4. The length of this induction decreases with increasing field strength or with increasing temperature (Arrhenius $E_a = 5 \pm 2$ kcal-m^{-1}). The time for the EPL to reach half its maximum intensity, $(t_{1/2})_f$, varies inversely with the applied field from 0 to 4000 v-cm^{-1} (Fig. 5).

The maximum intensity of the EPL response exhibits a biphasic dependence on field strength. For unbuffered chloroplasts at fields less than 1600 v-cm^{-1} the EPL intensity increases with the third power of the field (Fig. 6a), whereas at fields in the 2000-4000 v-cm^{-1} range it exhibits an exponential dependence on the field (Fig. 6b).

The EPL intensity and kinetic profile during t_f depend sensitively on the applied field strength (Fig. 7). We present evidence below showing that this field dependence arises from two distinguishable kinetic components. The first of these, component R, rises rapidly in strong fields, decays in less than a millisecond, and is unobservable at low fields (Fig. 7). The second, component S, rises slowly at low fields, persists to longer times and has a weaker dependence on field strength.

The electric field response is altered by compounds such as glycerol (30%), polyethylene glycol (0.5%) or sucrose (1-5%) that increase the viscosity and osmotic strength of the medium. Emission from such suspensions shows a retarded initial rise and an overall dominance of S-type emission. In the case of chloroplasts suspended in approximately .5% polyethylene glycol, (20,000 avg. molecular weight) the total emission was actually greater than for the control sample, owing to an increase in the S component emission.

The production of EPL depends critically on the integrity of the thylakoid membranes. Either sonication, treatment with the nonionic detergent Triton X-100 (0.01% or greater), or heating chloroplast samples to 60°C for 3 min completely abolishes EPL emission.

Extraction of carotenoids from chloroplasts using the procedure of Strichartz (1971) has little effect on EPL. This is in contrast to the field-indicating absorbance change at 515 nm, which is abolished by the carotenoid extraction.

Field dependence of the R and S components

Using single field pulses of 10 ms duration it is possible to separate the field dependence of the R and S components on the basis of their different kinetics (above, and Fig. 7). The results for one such study are shown in Fig. 8 for intermediate field strengths. Whereas the R component amplitude increases smoothly with increasing field, the S component nearly saturates at about 500 v-cm^{-1}.

From an analysis of EPL as a field-assisted detrapping process, Arnold and Azzi (1971 b) proposed that the dependence of field enhanced emission, ΔL, on field strength follows a relation of the type:

$$\frac{\Delta L}{L} = \cosh\left(\frac{\varepsilon}{kT}\right) - 1$$

or (1)

$$\frac{\Delta L}{L} = \cosh\left(AE\right) - 1$$

where L is the delayed fluorescence, $\varepsilon = cE$ is the change in trap depth produced by the external field E, and c is a constant such that $A = c/kT$. The solid line of Fig. 8 is a theoretical curve of this form. The data for the R component fit this function with a bestfit value of $A = (1.0 \pm .2) \times 10^{-3}$ cm-v^{-1}. The data for the S component cannot be fit by any

choice of the A parameter. [This analysis is consistent with the observed exponential dependence of the unseparated EPL at high fields (Fig. 6b). In the case of unbuffered chloroplasts in the range from 2000 to 4000 v-cm^{-1} the R component dominates the total signal and the function cosh (AE) - 1 approaches the limiting form exp (AE).]

For a given field the decay halftimes of the R and S components measured from the time of maximum emission vary somewhat from one preparation to another. Halftimes for the R component decay during the field pulse range from 90 μs under hypotonic buffered (pH 7.6) conditions to 300 μs for unbuffered hypotonic conditions. The halftime for decay of the S component is 2 to 4 ms, the shorter times again associated with hypotonic buffered (pH 7.6) conditions.

Two decay components have been observed in the collapse of the EPL when the electric field is removed. If the field is removed after short pulse lengths, when the R component dominates EPL emission, an exponential decay is observed [$t_{1/2}$ = (15 ± 2) μs]. However, if the field is removed after the majority of R component emission has decayed, a slower field-off exponential decay is observed [$t_{1/2}$ = (38 ± 3) μs]. Intermediate times result in a decay that can be decomposed into 15 and 38 μs components. We therefore associate the 15 μs decay with the R component and the 38 μs decay with the S component.

Bipolar pulse results

The nature of EPL emission derived from a second field pulse following the first depends both on the polarity of the second pulse relative to the first and on the length of time between the two pulses. If an electric field pulse is followed by another pulse of the same polarity (a unipolar sequence) within 50 to 500 μsec, the intense

transient of the R component emission is missing (Fig. 9 a, b and c). If the polarity of the second pulse is reversed with respect to the first (a bipolar sequence) and applied immediately after the first, the R component is missing and a dip in the emission occurs (Fig. 9 a and d) followed by a slower rise to maximum EPL emission.

By increasing the time between the two pulses of a unipolar sequence we find that the R component requires more than 20 ms to recover. This is in contrast to sequential bipolar pulse emissions where R component emission is fully recovered in less than 10 ms. Examples of this behavior are shown in Fig. 10. EPL emission resulting from a single 3.5 ms 1400 $v\text{-}cm^{-1}$ pulse occurring 9 ms after an actinic light flash is shown in Fig. 10 a. When a 300 μs field pulse of the same polarity is applied 5 ms previously, there is a decrease in the R component emission intensity of EPL during the second pulse (Fig. 10 b). However, if the polarity of the 300 μs pulse is reversed with respect to the 3.5 ms pulse, no such change in intensity is observed, and the emission is identical to the case where no previous pulse was given (compare Fig. 10 a and c).

Actinic intensity dependence

The EPL emission at 200 ms following illumination (unbuffered chloroplasts, fixed t_d and field strength) increases with the square root of actinic intensity before saturating at higher intensities (Fig. 11). A similar light dependence has been reported for the 1 ms (Clayton, 1969) and 5 ms (Ruby, 1971) delayed fluorescence, and for HCl-induced emission (Hardt and Malkin, 1973). The time profile of EPL is entirely independent of actinic light intensity, however. Curves of the response during t_f over nearly 100-fold range in actinic intensity were superimposable after normalization of their amplitudes.

10 μsec flash sequences

EPL emission yields from isotonic buffered or hypotonic buffered chloroplasts (pH 7.6) were observed at either 5 or 300 ms after each of a series of 10 μs saturating flashes spaced 1 s apart. Only the 300 ms emission from isotonic chloroplasts exhibited a reproducible flash yield dependence. The yield for the first flash was about 30% less than the yield for succeeding flashes, all of which appeared to be of the same magnitude. No oscillations of period 4 or of period 2 were observed.

Dark decay of EPL capacity

During an interval of several seconds following illumination chloroplasts lose their capacity for generating EPL in response to an electric field. This decay follows second-order kinetics (Fig. 12), as has been reported for delayed fluorescence (Mayne, 1968; Ruby, 1971; Malkin and Hardt, 1972) and for salt stimulated delayed fluorescence (Mayne, 1968; Barber and Kraan, 1970; however, see Miles and Jagendorf, 1969; Malkin and Hardt, 1972).

The relative contribution of the R component to the total EPL emission signal decreases during the interval (t_d, 5 to 300 ms) following an actinic flash (Fig. 13). The ratio of the magnitude of the R component to the S component measured at 5 ms increases with pH in the range pH 6.5 ($R/S \sim 1$) to pH 8.5 ($R/S \sim 4$).

Action of inhibitors

When the electron transport inhibitor DCMU is added (10^{-5}M) to aqueous unbuffered or buffered (pH 7.6) chloroplasts there is a small decrease in EPL yield but no discernible effect on the decay of EPL yield with time (data not shown).

The procedure of tris-washing (see MATERIALS AND METHODS), which serves to inhibit oxygen evolution by chloroplasts (Yamashita and Butler, 1968), produces a profound alteration in the profile of EPL during t_f (Fig. 14 a). The rise time for tris-treated chloroplasts is accelerated and the large initial response decays within 100 μs to a low level which then decays more slowly during t_f. Addition of o-phenanthroline (10^{-5}M) to tris-washed chloroplasts causes a further decrease in onset time, whereas addition of DCMU does not. In both cases (tris and tris plus o-phenanthroline) reciprocal plots of $(t_{1/2})_f$ were linear (Figs. 5b and c) over the range observed. o-Phenanthroline also caused, though inconsistently, an increase in the magnitude of the R component, an effect which we observed also upon chloroplast aging. DCMU did not show these anomalies.

The uncoupler CCCP suppresses the emission intensity but does not alter EPL decay kinetics during t_f. A 50% inhibition of emission occurs at about 8×10^{-7} M CCCP for unbuffered chloroplasts ([Chl] ~ 75 μg/ml).

Gramicidin D in the 10^{-7} to 10^{-6} M range with buffered or unbuffered aqueous chloroplasts decreases the R component emission more strongly than the S component emission. By contrast, valinomycin added alone (10^{-7} to 10^{-5}M) or with KCl up to 10 mM produced no noticeable effect on EPL. When gramicidin D (1×10^{-7}M) is added to tris-washed chloroplasts (Fig. 14 b), the fast decaying initial component of EPL is abolished.

Observations of the effects of hydroxylamine, DBMIB, and ferricyanide have also been made. Hydroxylamine (10^{-4}M) serves to accelerate the decay of R component emission during t_f. Addition of DCMU (10^{-5}M) with the hydroxylamine produced no further effects. DBMIB (10^{-4}M and higher) reduced EPL emission intensity overall by greater than 50%. Ferricyanide

(10^{-4}M) also lowered the overall intensity of EPL emission and resulted in a slightly faster R component decay.

Other systems tested

Chloroplasts isolated from barley, both wild-type and a mutant devoid of chlorophyll b, exhibit EPL. We did not observe any measurable EPL from suspensions of whole Chlorella cells, in agreement with the findings of Arnold and Azzi (1971 a).

DISCUSSION

The EPL of chloroplasts exhibits similarities to other stimulated delayed light emissions associated with acid-base transitions (Mayne and Clayton, 1966; Mayne, 1968), with salt, acid (Miles and Jagendorf, 1969) or organic solvent (Hardt and Malkin, 1972 a) gradients, or with a temperature jump (Mar and Govindjee, 1971). These similarities include the requirement for preillumination, dependence on the square-root of light intensity (Malkin and Hardt, 1973), association with Photosystem II, emission spectrum (Hardt and Malkin, 1974) and second-order decay of the photo-produced metastable species (Mayne, 1968). In several respects these stimulated emissions differ from EPL, however. In particular, the other stimulated emissions exhibit a sensitivity to DCMU (Mayne and Clayton, 1966; Miles and Jagendorf, 1969), a response of period four to a train of flashes (Hardt and Malkin, 1973) and a simple first-order relaxation following the stimulus (Miles and Jagendorf, 1969; Hardt and Malkin, 1971).

EPL and 100 ms delayed fluorescence

The relation between EPL and ordinary delayed fluorescence is more difficult to characterize because of the many kinetic components of

delayed fluorescence. [See Lavorel (1975) for a comprehensive review.] In our experiments the electric field pulse is applied 100 ms or longer after the illumination period, after the shorter components of delayed fluorescence have disappeared. The response of the relatively slow (100 ms to 1 s) delayed fluorescence of isolated broken chloroplasts to factors that affect system II photochemistry is similar to that of EPL in the following respects: little sensitivity to DCMU (Bertsch, et al., 1963; Mohanty, et al., 1972); the absence of period 4 phenomena at room temperature (Joliot, et al., 1971; Velthuys and Amesz, 1975); a decrease in intensity in the presence of electron acceptors like ferricyanide (Goedheer, 1962; Wells, et al., 1972) or electron donors like hydroxylamine (Arthur and Strehler, 1957). In each of these cases the fast (ms) delayed fluorescence behaves quite differently in response to the treatment.

Factors which affect membrane permeability or integrity also result in similar responses. The moderately slow delayed fluorescence from broken chloroplasts is decreased by the uncoupler CCCP (Clayton, 1969), decreased by gramicidin but unaffected by valinomycin with or without KCl (Kraan, et al., 1970), and strongly inhibited by Triton treatment (Kraan, et al., 1970). These extensive similarities suggest that the metastable intermediates of EPL are closely related or identical to those giving rise to delayed fluorescence in the 100 to 1000 ms region.

There is extensive evidence to support the idea that delayed fluorescence results from the regeneration of the chlorophyll excited singlet state from oxidized and reduced intermediates lying somewhat below it in energy (Arthur and Strehler, 1957; Lavorel, 1968). A current formulation of components associated with PS II is $(D)ZP_{680}Q(A)$, where P_{680} is the reaction center pigment, Z and D are electron donors and Q and A are electron acceptors. The parentheses indicate that the secondary

donors and acceptors are multiple species. The shortest components (ms) of delayed fluorescence arise from intermediates like $P_{680}^{+}Q^{-}$, where the separated charges are close to the reaction center. The longer-lived delayed fluorescence and the EPL that we observe arise from intermediates like $(D^{+})ZP_{680}Q^{-}(A)$ where the separated charges are initially more remotely situated. The insensitivity to DCMU and the absence of period 4 phenomena suggest that the S states on the water side (Kok, et al., 1970) and components beyond Q on the acceptor side are not involved directly in EPL.

At the end of the electric field pulse the field-generated intermediates decay via first-order processes with half-decay times of 38 μs (S component) and 15 μs (R component). The former is similar to the decay time reported for P_{680} absorption changes (Gläser, et al., 1974; Floyd, et al., 1971) and for a fast component of delayed luminescence (Zankel, 1971). This decay has been attributed to the process

$$Z + P_{680}^{+} \xrightarrow{35\ \mu s} Z^{+} + P_{680}$$

In the case of EPL, the corresponding process

$$(D)ZP_{680}^{+}Q^{-}(A) \longrightarrow (D)Z^{+}P_{680}Q^{-}(A)$$

would result in termination of the stimulated luminescence. In this view the S component of EPL would originate from the transfer of holes from the oxidized donor system to reaction centers that already contain Q^{-}.

The other obvious way in which the state $(D)ZP_{680}^{+}Q^{-}(A)$ can be deactivated is by a process in which the electron on Q^{-} is lost; for example

$$(D)ZP_{680}^{+}Q^{-}(A) \longrightarrow (D)ZP_{680}^{+}Q(A^{-})$$

The absence of a DCMU effect on EPL argues against the participation of the Q to A electron transfer step. Furthermore, the time required is reported to be much slower (Forbush and Kok, 1968) than the 15 μs relaxation time for the R component. It is possible that the R component involves species that are not in the main chain of electron transport between PS II and PS I.

Membrane electrical properties

Arnold and Azzi (1971 b) formulated a model for EPL in terms of a field-dependent change in the activation energy required to untrap a bound electron (reduced acceptor) and hole (oxidized donor) leading to the regeneration of excited chlorophyll. In their terms the rate of delayed fluorescence is given as

$$L = NF \exp(-E_a/kT) \qquad (2),$$

where N is the number of photosynthetic units containing an electron-hole pair, F is a frequency factor and E_a is the activation energy (trap depth). The presence of an external field produces opposite effects on the photosynthetic units on opposite surfaces of the enclosed thylakoids: on one side the activation energy is increased by an amount ε, and on the other the activation energy is decreased by the same amount. The ratio of the change in emission rate, ΔL, to the normal delayed light emission, L, becomes

$$\frac{\Delta L}{L} = \cosh\left(\frac{\varepsilon}{kT}\right) - 1 \qquad (3).$$

As ε becomes large compared with kT, the cosh function tends toward an exponential. The data of Figs. 6 and 8 suggest that at least the R component fits this hyperbolic cosine function.

The EPL induction or onset time of 100 μs or longer permits us to distinguish amongst the four different mechanisms for delayed luminescence

production presented by Arnold and Azzi (1971 a). In particular it provides a powerful argument against a mechanism involving simple recombination of electrons and holes. The rise time of our field pulse is less than 6 μs (Ellenson, 1973), and electron-hole recombination should give a luminescence response that rises as rapidly as the field. No such rapid component of the EPL was ever observed. This is the first time, to our knowledge, that such a rapid perturbation has been applied to provide stimulated luminescence from chloroplasts. The occurrence of the induction period for EPL suggests that its appearance is limited by kinetic steps involving electron transfer between specific donor and acceptor molecules. These are seen more clearly in reverse in the unimolecular decays of the R and S components at the end of the field pulse. The processes occurring upon the onset of the field are not simple and unimolecular, because the EPL rise time decreases linearly with increasing field under a wide variety of conditions (Fig. 5).

The interaction of an external electric field with thylakoid membranes requires special attention. The magnitude of the free space electric fields (600 to 4000 $v\text{-}cm^{-1}$) employed in our EPL measurements falls far below the 10^5 $v\text{-}cm^{-1}$ photoinduced transmembrane field estimated from absorption changes (Schmidt, et al., 1971; Barber, 1972) and the 1 ms component of delayed luminescence (Barber and Varley, 1972). To appreciate the relation between these different field estimates, consider that chloroplasts suspended in hypotonic media become swollen and appear somewhat like inflated balloons (Izawa and Good, 1966). Therefore, we model the swollen thylakoid as a thin dielectric shell which is placed in a uniform electric field (Fig. 15). Maxwell (1892) described a solution to this problem for the case where the shell is spherical. With the assumption that the resistivity of the membrane

is much greater than that of either the interior or the exterior medium, a term can be derived from Maxwell's general expression (Eq. 9, Chap. 10 of Maxwell, 1892) that describes the radial (or transmembrane) field as

$$E(r) = \{ 9/2[1-(a/b)^3]^{-1} E_o \cos\theta \} \cdot \underset{\sim}{r} \qquad (2)$$

where a, b and θ are defined in Fig. 16, and $\underset{\sim}{r}$ is a unit vector in the radial direction. Three conclusions are evident:

a) The induced radial field is maximum at the two horizontal poles.

b) The magnitude of the induced field is steeply dependent on the ratio of the radius to its thickness, since $[1-(a/b)^3]^{-1} = b^3/(a^3-b^3)$.

c) The induced field, although pointing in the same direction on opposite faces of the shell, is directed from the inside out on one face and from the outside in on the other.

For a "representative thylakoid" with a membrane thickness of 7 nm and a radius of 500 nm, the induced radial field at the horizontal poles is approximately 100 times the externally applied field. Free space fields of 4000 volt-cm^{-1} may thus induce local fields of 4×10^5 v-cm^{-1} across the membrane. Although thylakoid membranes probably do not form ideal spheres, they should exhibit the general features of the model. We believe that these high local fields give rise to EPL.

Any treatment which destroys the integrity of the membrane as a closed dielectric shell of high resistivity will also destroy the field enhancement mechanism. This undoubtedly accounts for our observation that detergent treatment or sonication of chloroplast membranes result in a loss of EPL activity. The decrease in chloroplast EPL with increased

osmotic strength (Arnold and Azzi, 1971 a), which should decrease the thylakoid size, is also consistent with this model. For intact cells such as Chlorella the field will be enhanced only at the outer, unpigmented cell membrane; and the intracellular space containing the chloroplast will be largely field-free. As a consequence, no EPL is observed for Chlorella.

Membrane polarization

There is good evidence that the electron donors and acceptors associated with the reaction centers are vectorially distributed across the photosynthetic membrane. [See Trebst (1974) for a recent review.] Recently, Witt and Zickler (1973) and Fowler and Kok (1974) have directly measured a photogenerated electric field across spinach chloroplast membranes. The resultant local electric moments are directed toward the exterior of the thylakoid. This is the expected result if the absorption of light at the photosynthetic reaction centers leads to the transfer of electrons from donors on the inside to acceptors on the outside of the thylakoid membranes.

The application of an external electric field immediately differentiates the reaction center complexes on opposite faces of the thylakoid. On one face (Face 1, Fig. 15) the external field is anti-parallel to the photoinduced electric moment. On the opposite face (Face 2) the field and the moment are parallel. The interaction of the anti-parallel field over Face 1 stabilizes or enhances the photoinduced charge separation $(D^+)ZP_{680}Q^-(A)$. The parallel external field over Face 2 is destabilizing and has the effect of collapsing the photo-induced charge separation by inducing the process

$$(D^+)ZP_{680}Q^-(A) \longrightarrow (D)ZP^+_{680}Q^-(A)$$

which leads to enhanced luminescence. Only when the external field is reversed will emission occur similarly from Face 1.

Our studies using paired field pulses support this model. Following a short initial field pulse, a second pulse of the same polarity (Fig. 10b) produces a significantly smaller EPL response than does a second pulse of the opposite polarity (Fig. 10c). The latter EPL signal is, in fact, indistinguishable from that resulting from a single comparable pulse (Fig. 10a). In these experiments Face 1 and Face 2 appear to respond independently. This simple picture must be modified to account for the response shown in Fig. 9 (especially Fig. 9d), however. When there is no relaxation period between the bipolar pulses, there is a pronounced decrease in EPL accompanying the transient field reversal. Furthermore, the recovery is slower than for a pair of unipolar pulses separated by a time long enough for the EPL nearly to decay (Fig. 9b). The slow recovery upon rapid field reversal may reflect the reorganization of induced gradients of ions or other mobile charged species that have formed in response to the applied field. The recovery presumably occurs in the interval between pulses (5.5 ms) used for the experiments shown in Fig. 10. There remains, however, a distinction between the behavior of the R and S components in response to paired field pulses. The R component remains intact when the second pulse after 5.5 ms has reversed polarity (Fig. 10c), but it has not yet returned after 20 ms when the second pulse has the same polarity (data not shown). This observation emphasizes the apparently quite different origins of these two kinetic components.

Witt and coworkers have shown that photoinduced absorbance changes at 515 nm in chloroplasts result from ion gradients and membrane-associated electric fields. [See Witt (1975) for a recent review.] The steady state

amplitude of this absorbance change is abolished by membrane disruptive treatments such as incubation with low concentrations of Triton X-100 (Kraan, et al., 1970) and sonication of chloroplast membranes (Strichartz and Chance, 1972). The ionophore gramicidin D greatly accelerates the decay of the 515 nm absorption change (Junge and Witt, 1968). The results of the present study suggest that common factors govern the origin of the 515 change and at least the R component of EPL.

Finally, it is important to recall that after the application of an electric field pulse up to 4000 volt-cm^{-1} the delayed fluorescence returns to precisely what it would have been in the absence of any field pulse. Similar observations were reported by Arnold and Azzi (1971 a). This suggests that the external field perturbations do not significantly deplete the reservoir of metastable charge-separated species associated with the reaction center complexes. The kinetics of the R and S components during the field pulse do not reflect changes in the concentrations of these metastable intermediates. The R and S component kinetics most probably result from changes in the ion gradients across the membrane. These gradients would respond directly to the field perturbation and undergo changes as a consequence of ion migration. The ion gradients in turn mediate the recombination of the charge-separated intermediates, which serve primarily as indicators of the ion-field interactions.

SUMMARY

The perturbation of preilluminated chloroplasts by electric field pulses provides very large (up to 100-fold) stimulation of delayed fluorescence. The method has special advantages over other stimulated

luminescences in the ability to turn the field on within a few microseconds to study onset kinetics. It is the only perturbation investigated to date that can also be turned off rapidly to study the terminal relaxation.

1. EPL is closely related to delayed fluorescence in the range 0.1 to 1 sec and probably arises from the reversal of steps in the electron transport chain close to PS II.

2. The driving force for EPL has two kinetic components that behave quite differently in the presence of ionophores. The R component is probably associated with an ion transfer process. The S component may involve the transfer of electrons to P680 from its normal donor in the electron transport chain from water.

3. The metastable species involved in EPL apparently lie between the S states associated with water oxidation and the site of interaction of DCMU.

4. The occurrence of a slow onset stage upon turning on the field rules out mechanisms involving detrapping of electrons and holes from conduction bands.

5. EPL requires intact thylakoid membranes from isolated chloroplasts. Neither disrupted membranes nor whole cells show the effect. The low dielectric constant of the membrane of swollen thylakoids provides a means of enhancing the local applied field by 100-fold or more.

6. Directional effects seen upon reversing the field occur in a time short compared with thylakoid rotational diffusion. EPL appears from charge recombination that is enhanced on one face but not the other for a given field direction. The field disturbs the ionic environment of the membrane in a way that takes several milliseconds to recover.

7. No decrease is seen in delayed fluorescence following an EPL pulse. Although the EPL may arise from the same set of metastable intermediates as does delayed fluorescence, this pool is not measurably depleted by the electric field pulse under the conditions of our experiments.

ACKNOWLEDGEMENTS

We wish to thank Dr. G. T. Babcock for his very helpful discussions of our work. The research was supported by the United States Atomic Energy Commission and the National Science Foundation (GB-36361).

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FIGURE CAPTIONS

Figure 1. Schematic diagram of experimental apparatus. See text for description of components. The electrophotoluminescence ($h\nu_{EPL}$) is monitored on the opposite side of the sample from the actinic pre-illumination ($h\nu_a$).

Figure 2. Time course of a typical EPL experiment. The sample is illuminated for time t_p (typically 3-5 sec) during which the detector is blocked. Actinic light is then blocked, and detector shutter opens to observe delayed luminescence during time t_d, typically 100 ms or longer. The electric field is applied for the time t_f. The time after the electric field pulse is designated t_r. Field pulses generally were not shorter than 50 μs nor longer than 10 ms.

Figure 3. Stimulated emission from aqueous unbuffered chloroplasts subjected to a 1600 v-cm^{-1} field pulse. t_p = 4 sec, t_d = .1 sec. The curve is an average of 10 scans.

Figure 4. The EPL onset kinetics on an expanded time scale starting with field application and showing its sigmoid nature. Aqueous chloroplasts subjected to a 1200 v-cm^{-1} field pulse and at a temperature of 3°C.

Figure 5. Variation of reciprocal of time to reach one-half maximum EPL emission, $(t_{1/2})_f^{-1}$, as a function of applied electric

field. (a), aqueous unbuffered chloroplasts, untreated. (b), tris-washed aqueous chloroplasts. (c), tris-washed aqueous chloroplasts plus 10^{-5}M o-phenanthroline.

Figure 6. Log-log, (a), and semi-log, (b), plots of maximum EPL emission measured during a 1 ms, 1600 v-cm^{-1} field pulse as a function of applied field.

Figure 7. Variation of initial EPL response with applied field. Numbers beside each curve refer to the magnitude of the externally applied field. Aqueous unbuffered chloroplasts.

Figure 8. Field dependent intensities of R and S components of EPL plotted logarithmically. The solid line is a theoretical curve for a hyperbolic cosine function. The components were educted from EPL curves resulting from 10 ms field pulses. After 10 ms the R component has completely decayed. The S component with a half decay time of 3.5 ms was extrapolated back to the peak intensity of EPL emission and subtracted from it. The difference is plotted as the magnitude of R component emission. Aqueous unbuffered chloroplasts.

Figure 9. Variation of EPL response with pulse sequence. (a) through (c), unipolar sequences. (d), dipolar sequence. Aqueous unbuffered chloroplasts.

Figure 10. (a) EPL emission from single 3.5 ms, 1600 v-cm^{-1} field pulse starting 9 ms after a single saturating 10 μs actinic flash.

(b) Same as (a), but with a 300 μs field pulse of the same polarity occurring 3.5 ms after the actinic flash.

(c) Same as (b), but with polarity of 3.5 ms pulse reversed. Aqueous unbuffered chloroplasts.

Figure 11. Plot of EPL emission maxima against the square root of preillumination intensity. Aqueous chloroplasts were preilluminated for 4 sec with broad band blue light. Maximum intensity was about 1 mw-cm^{-2}-sec^{-1}. Chlorophyll concentration was about 10 μg/ml. E = 1600 v-cm^{-1}, t_f = 2 ms.

Figure 12. Second order plot of the maximum EPL emission measured as a function of the dark time t_d after a 4 sec preillumination with broad band blue light at saturating intensity. Unbuffered aqueous chloroplasts. E = 1600 v-cm^{-1}, t_f = 2 ms.

Figure 13. Series of EPL emissions recorded at various times t_d after a 10 μs flash and normalized using intensity at t_f = 3.0 ms. Curves are labeled using t_d values. Sample: aqueous chloroplasts with a chlorophyll concentration of 20 μg/ml. pH 8.5.

Figure 14. EPL emission from (a) tris-washed aqueous chloroplasts and (b) tris-washed chloroplasts plus 10^{-7}M gramicidin. Field pulse 5 ms; chlorophyll concentration 100 μg/ml.

Figure 15. Spherical shell model discussed in text. E_o is the external applied electric field in the Z direction. Face 1 refers to the hemisphere in the -Z direction, and Face 2 is the hemisphere in the +Z direction. a is the radius of the inner spherical surface; b is the radius of the outer spherical surface. Arrows show relative magnitude and direction of electric field induced across the spherical shell.

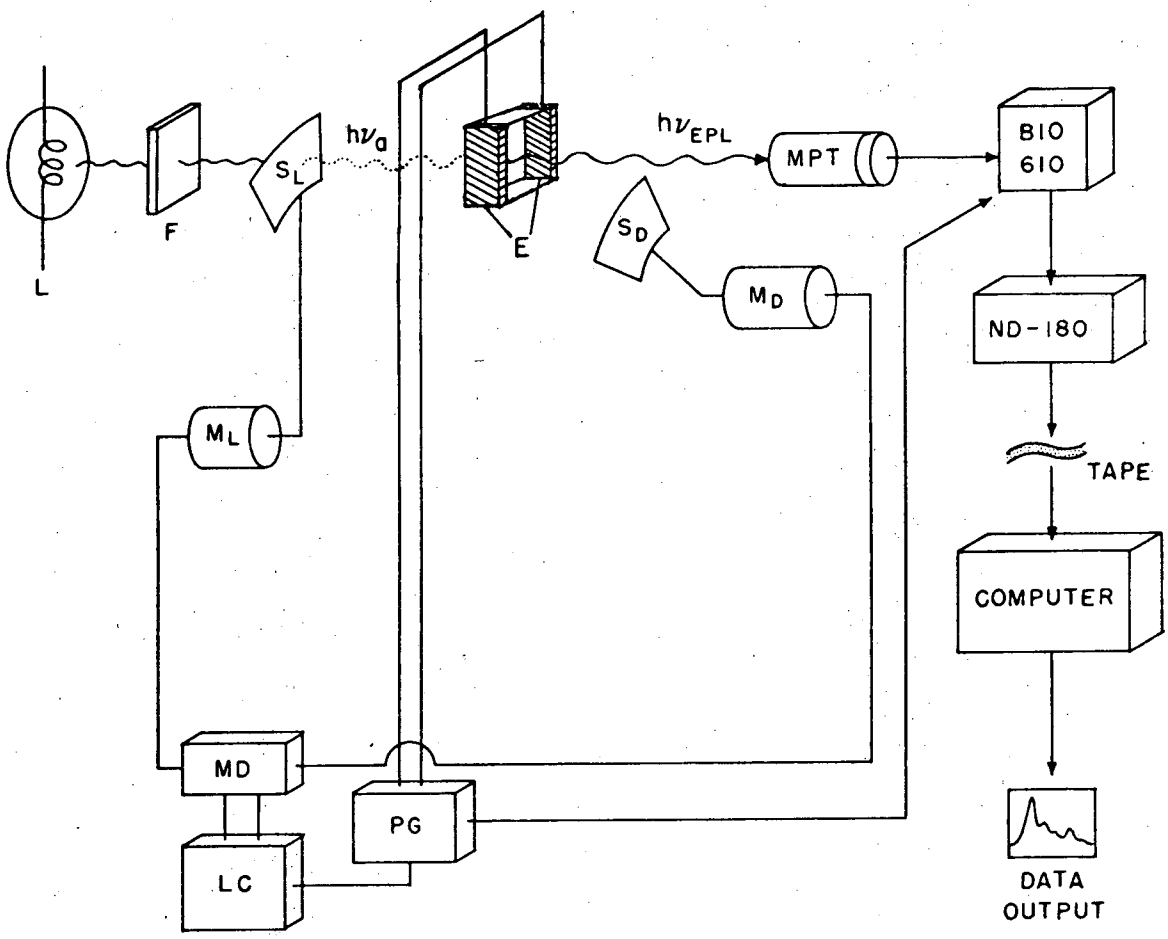

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Fig. 1

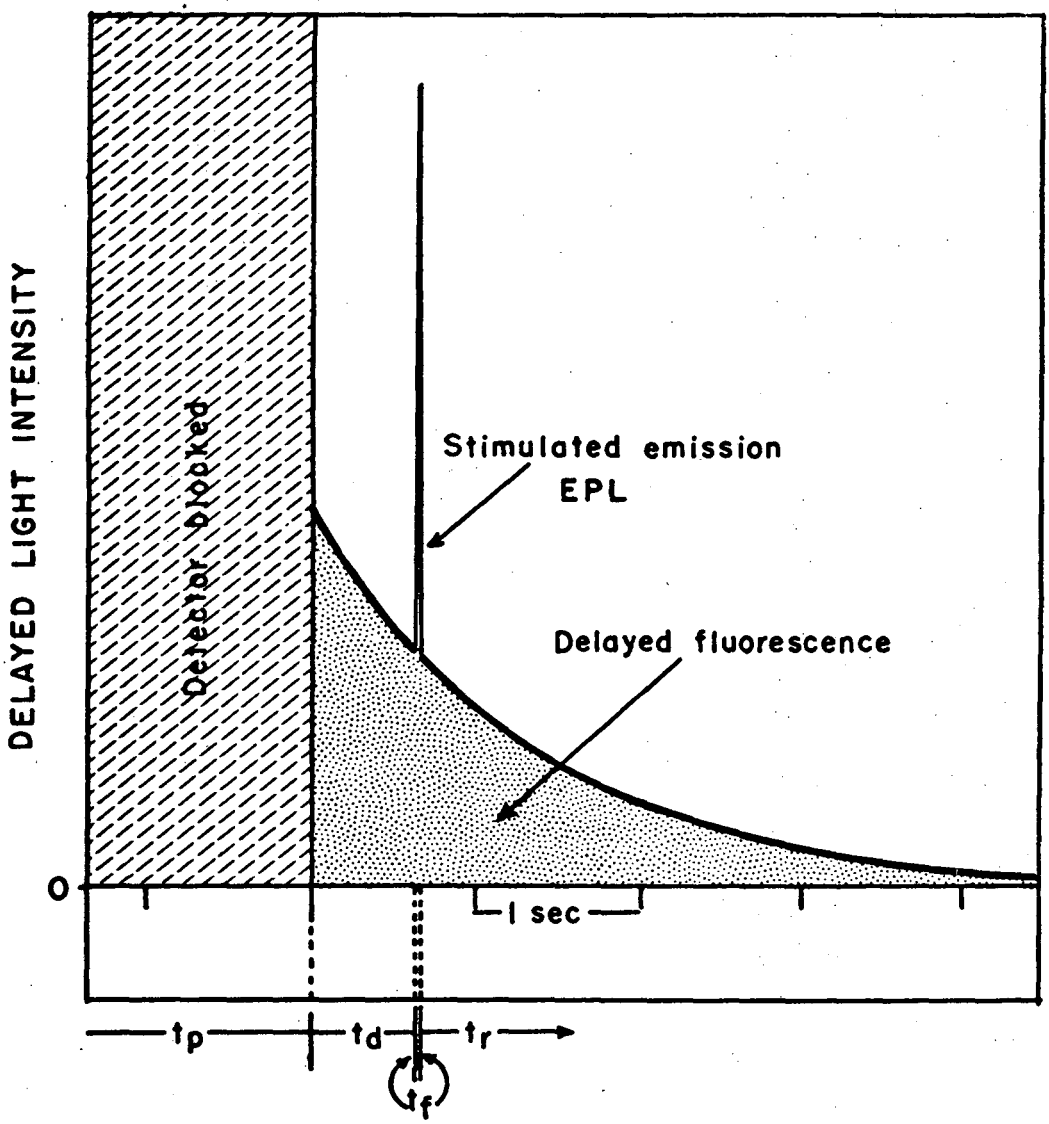

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Fig. 2

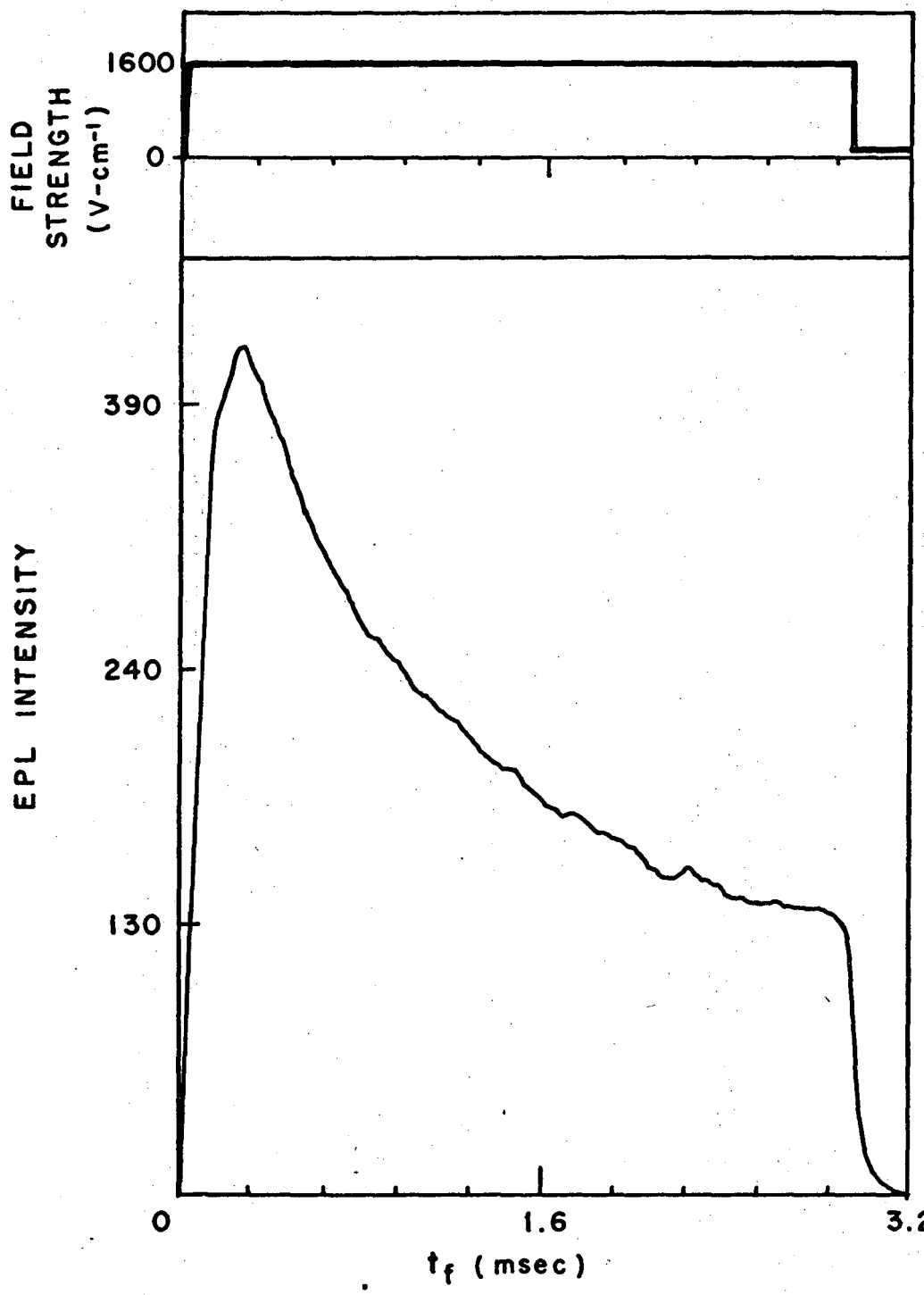

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Fig. 3

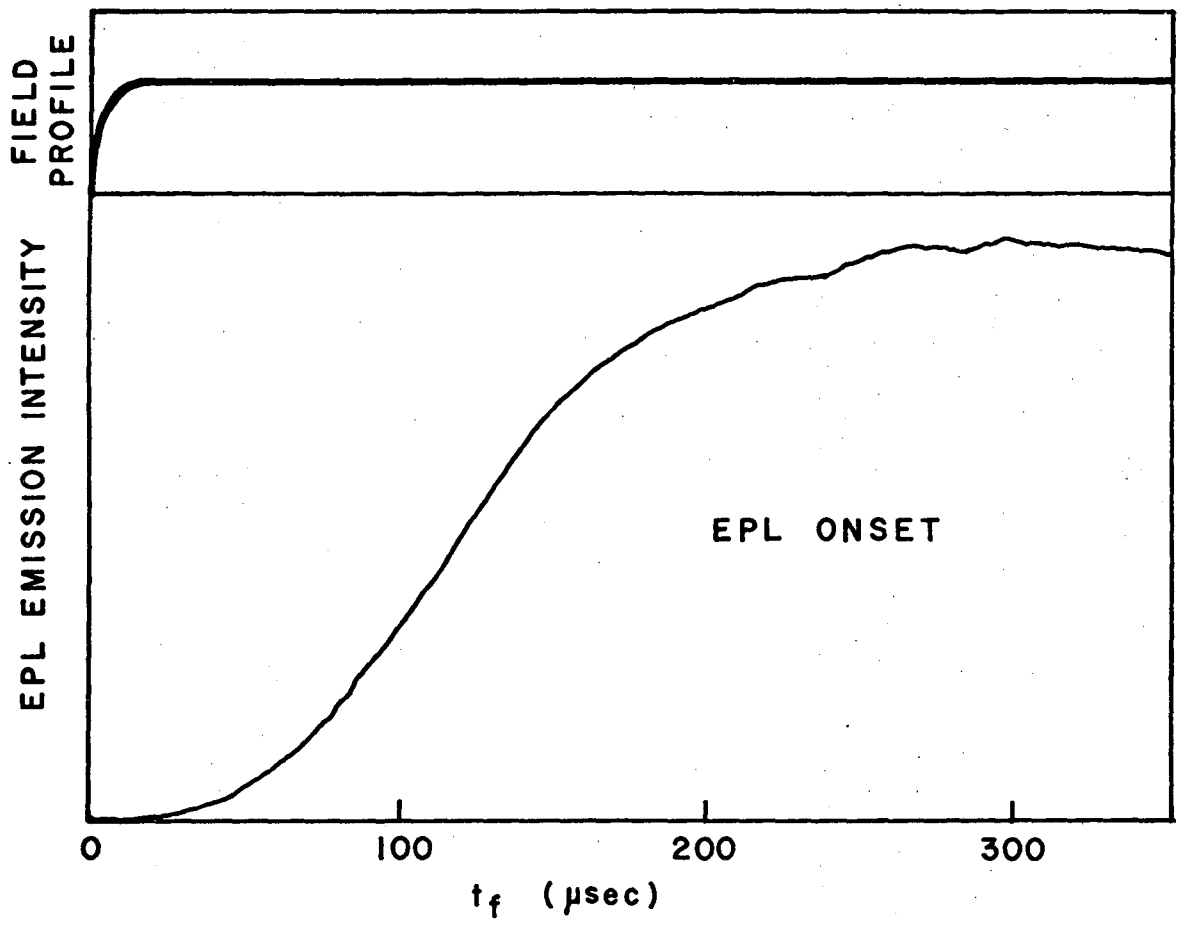

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Fig. 4

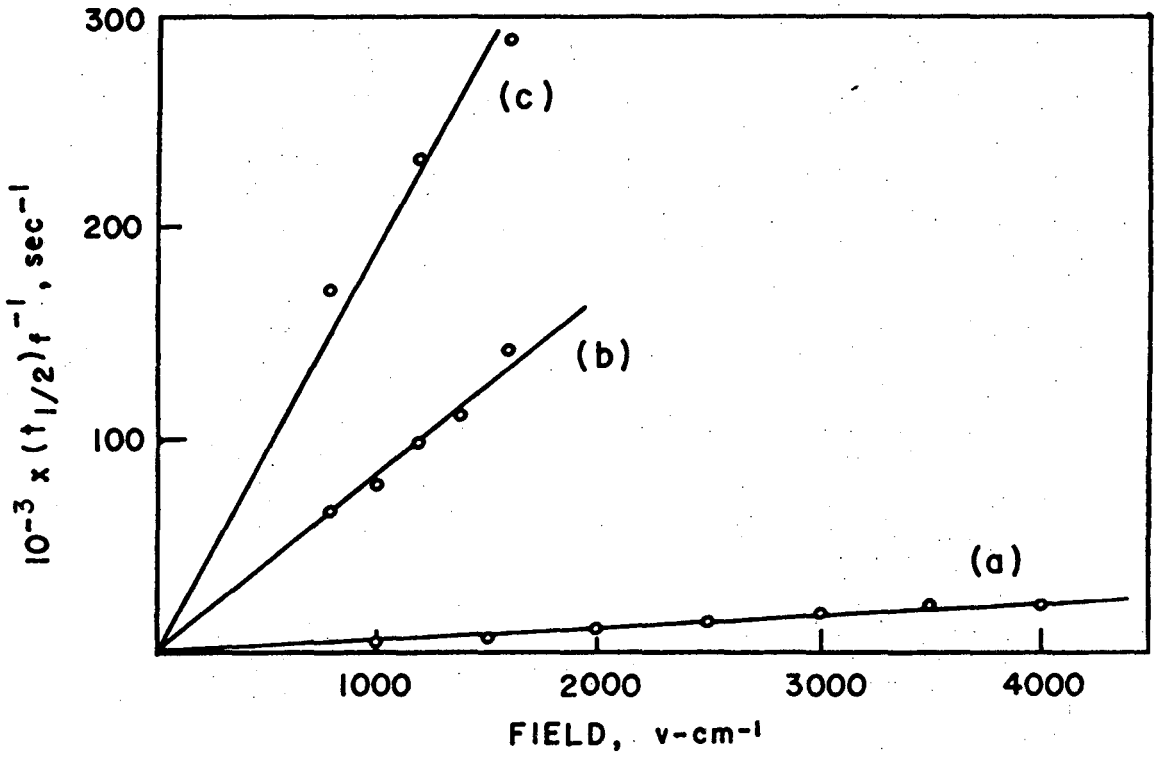

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Fig. 5

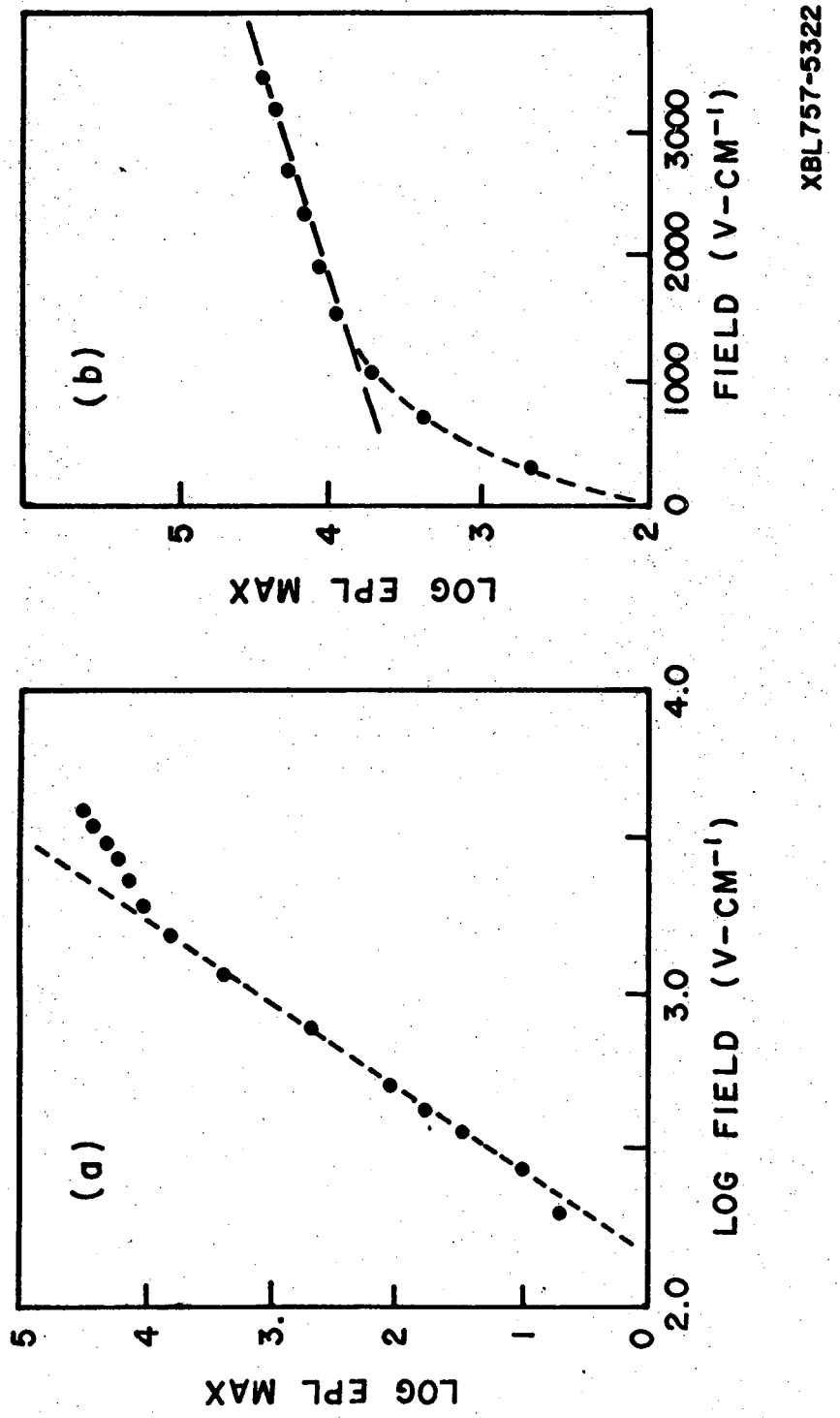

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Fig. 6

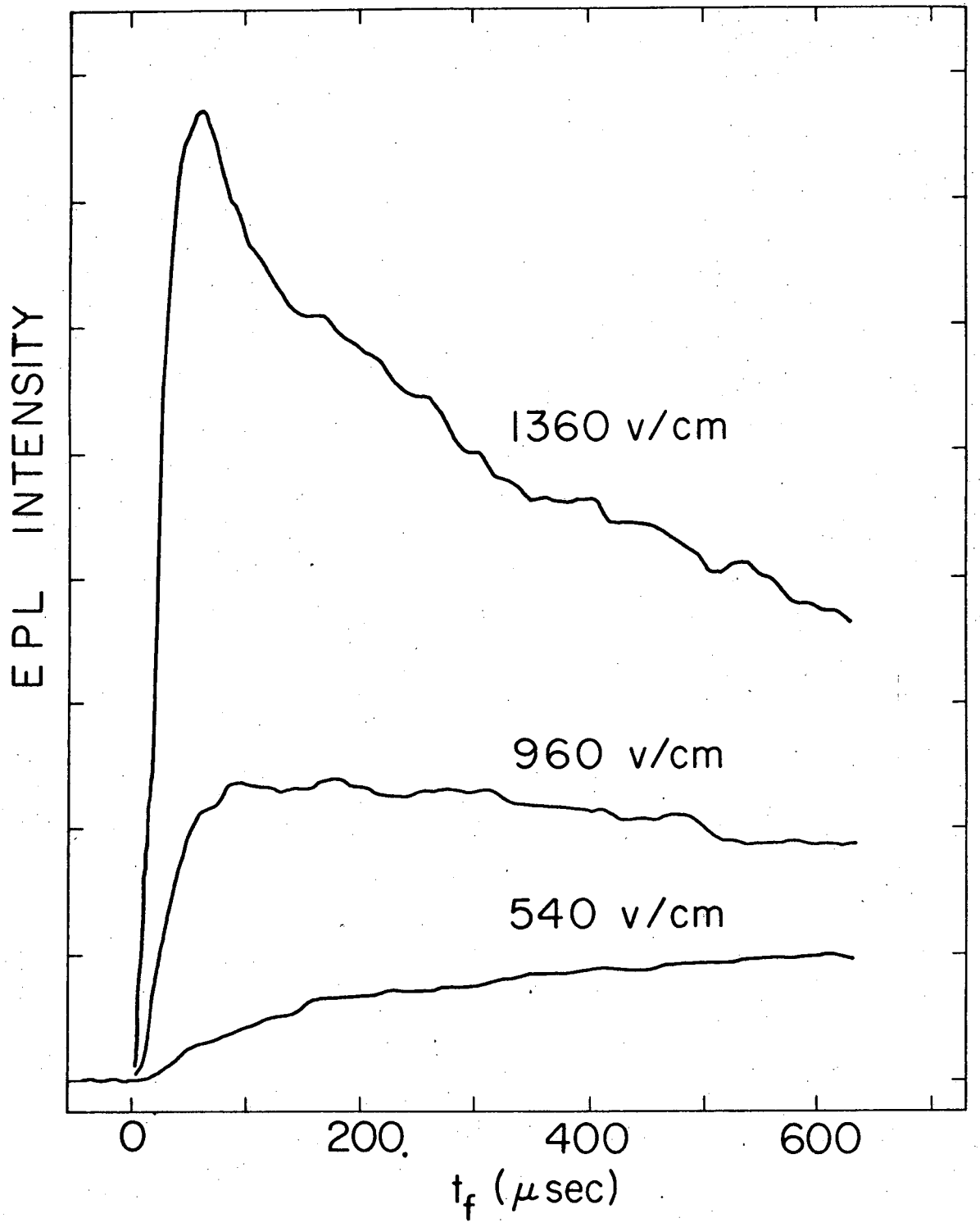

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Fig. 7

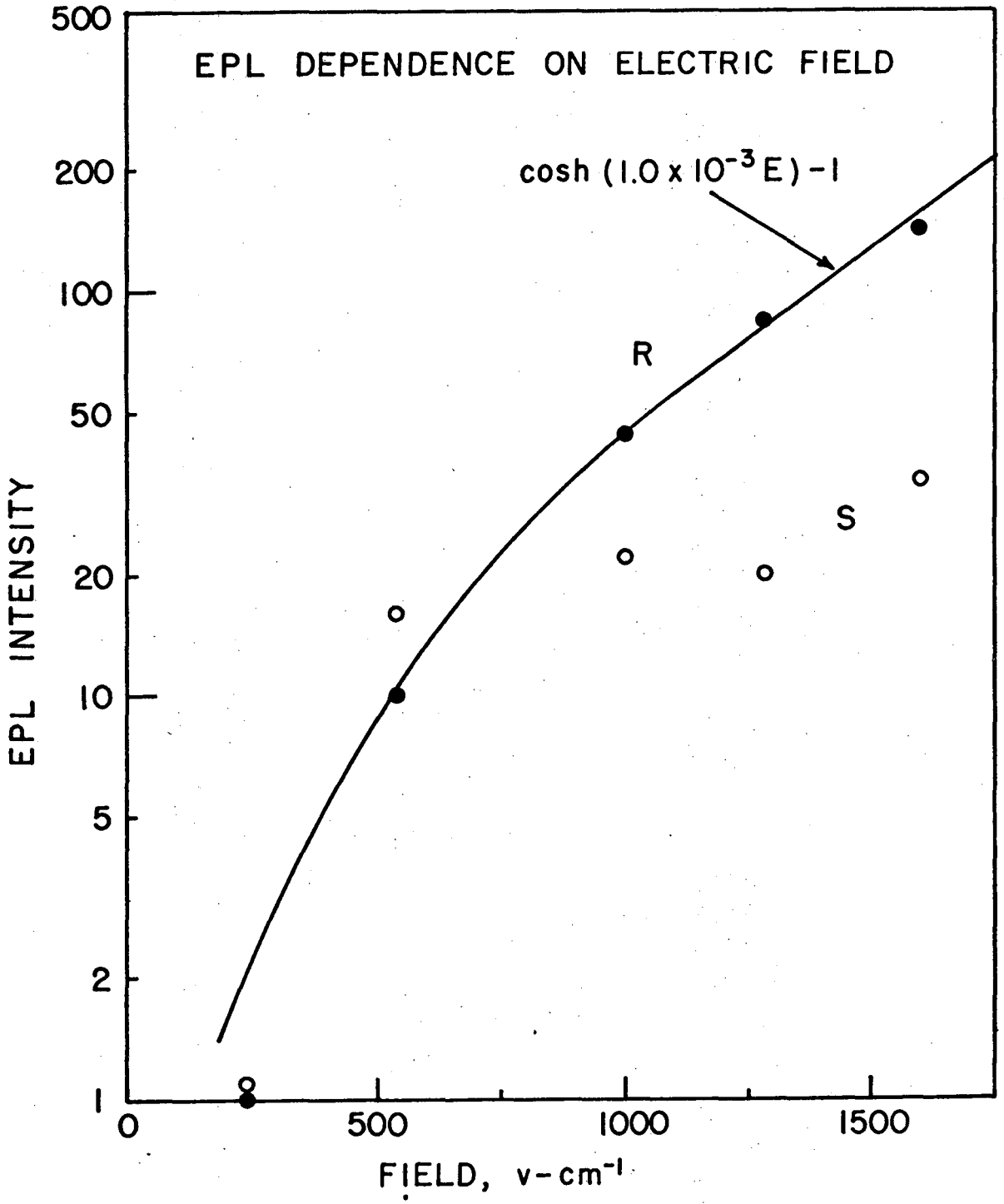

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Fig. 8

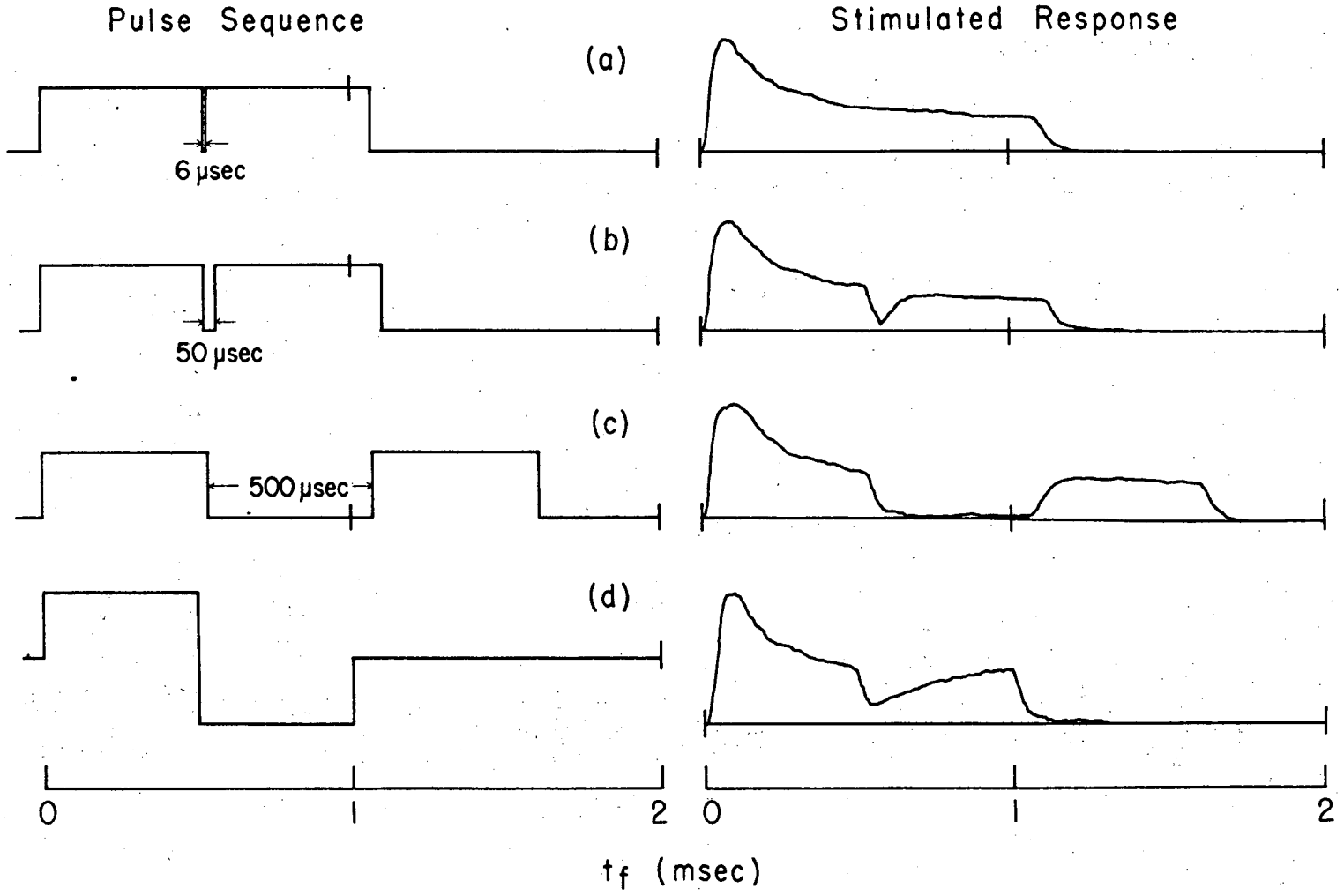

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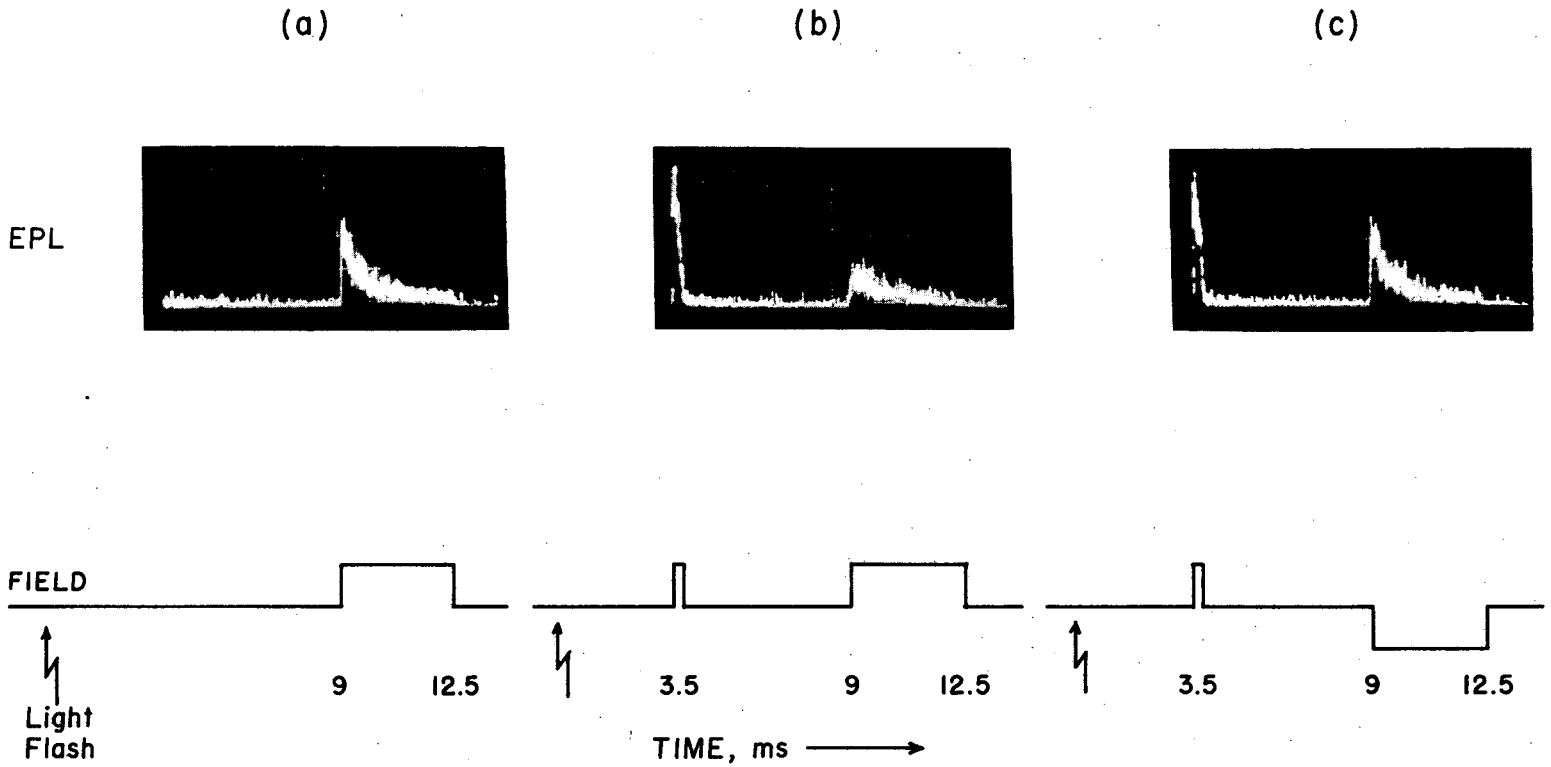
Fig. 9



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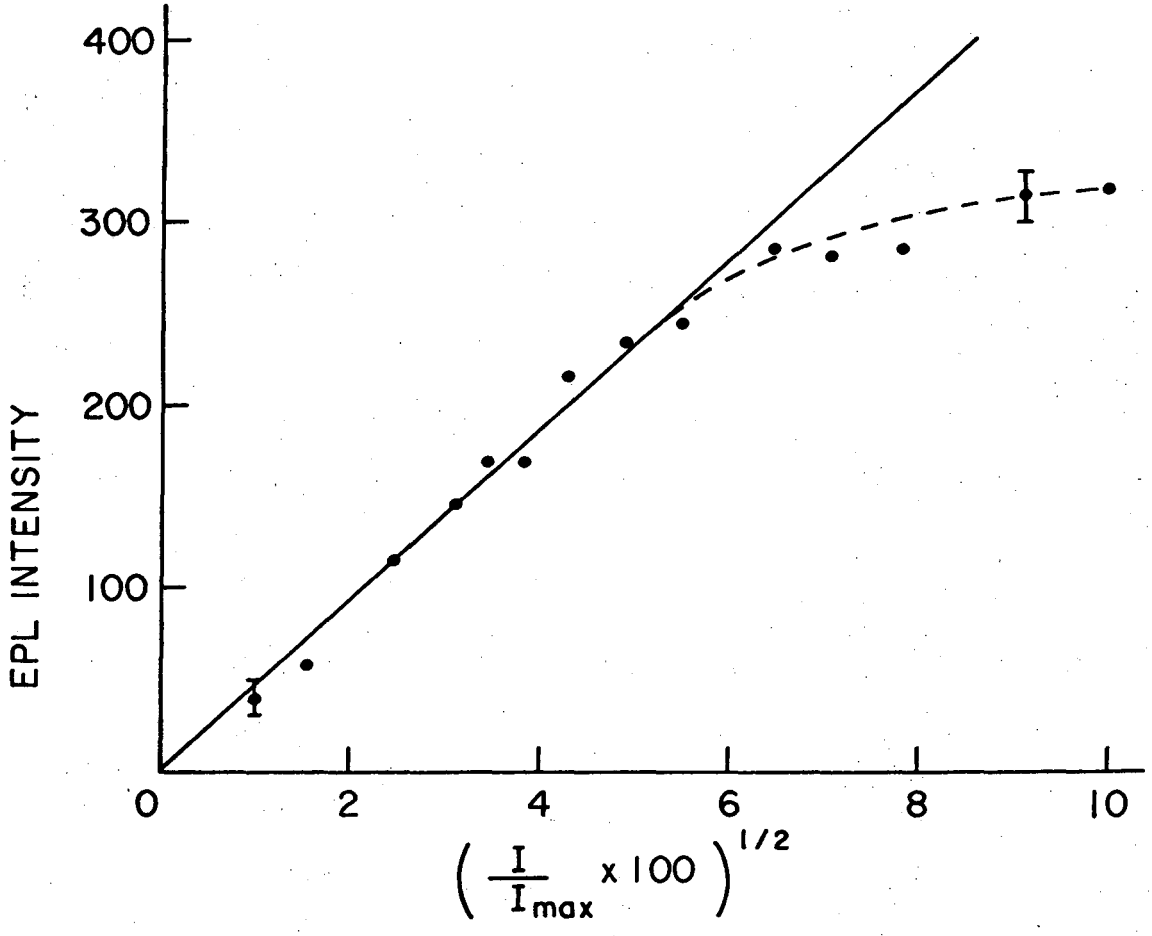

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Fig. 11

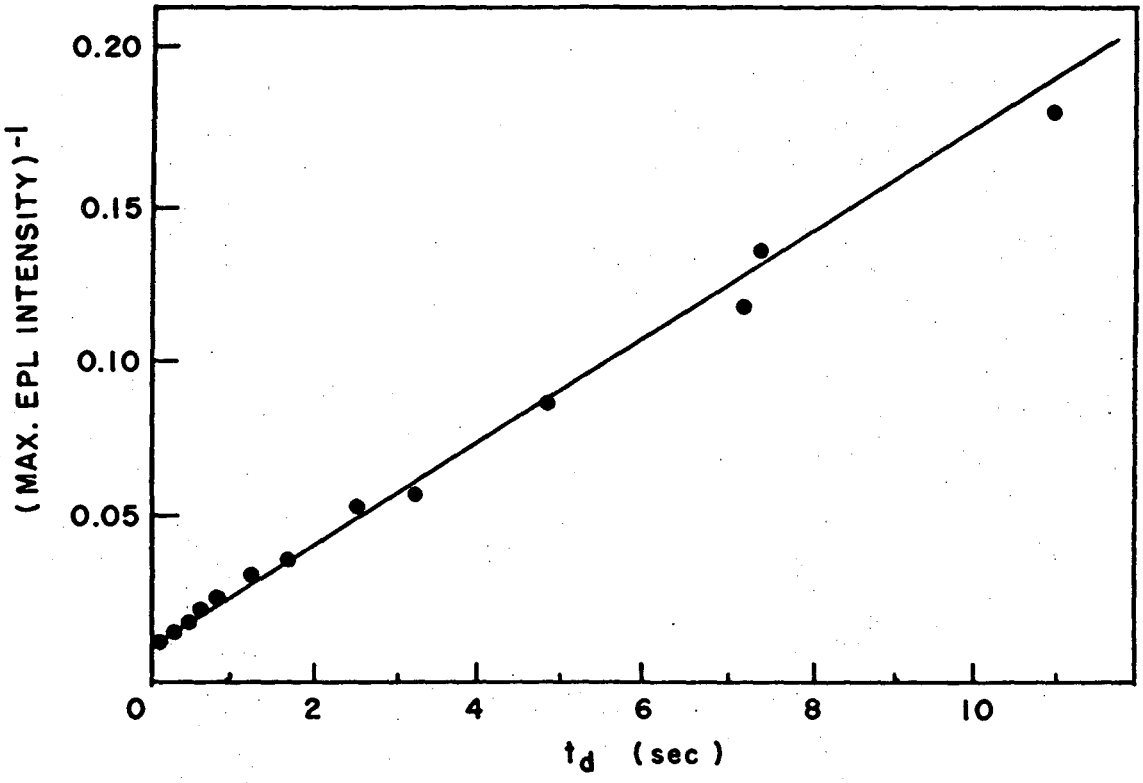

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Fig. 12

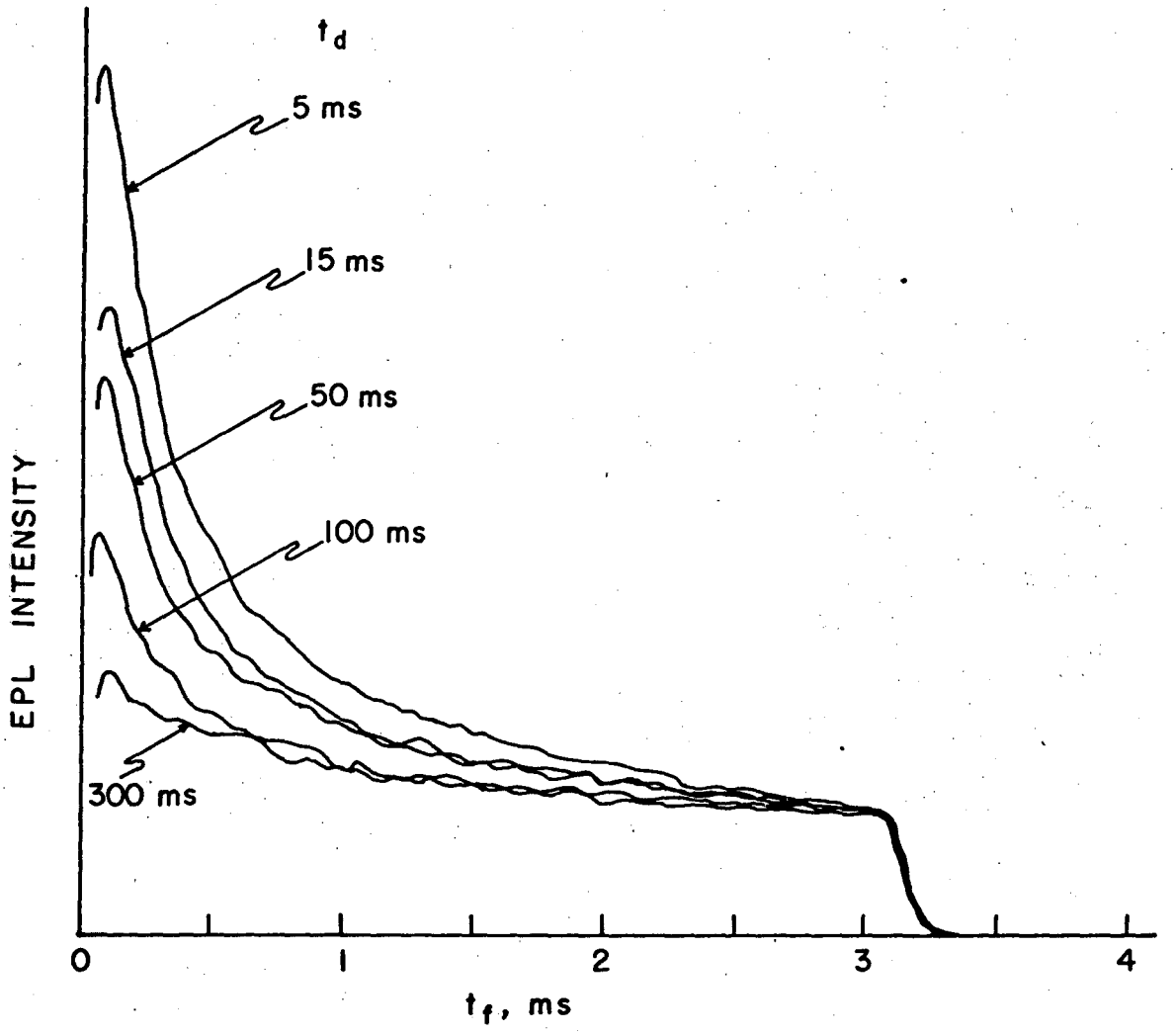

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Fig. 13

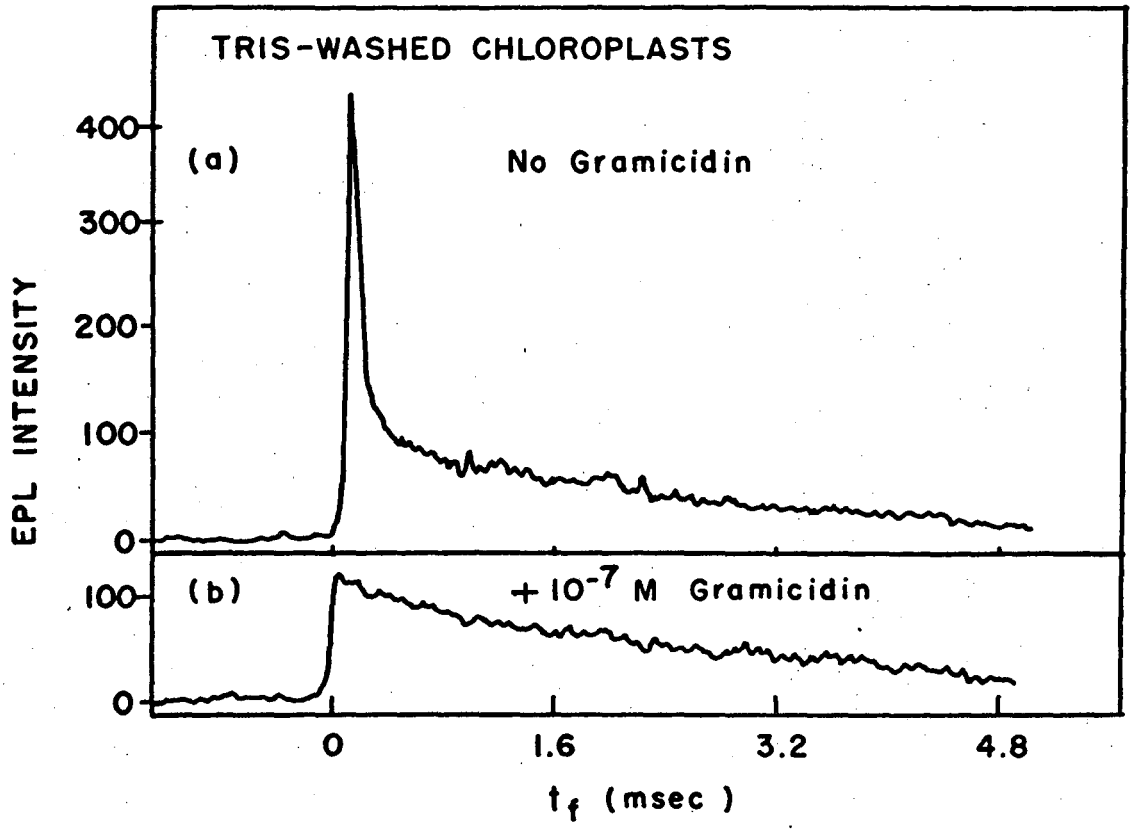

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Fig. 14

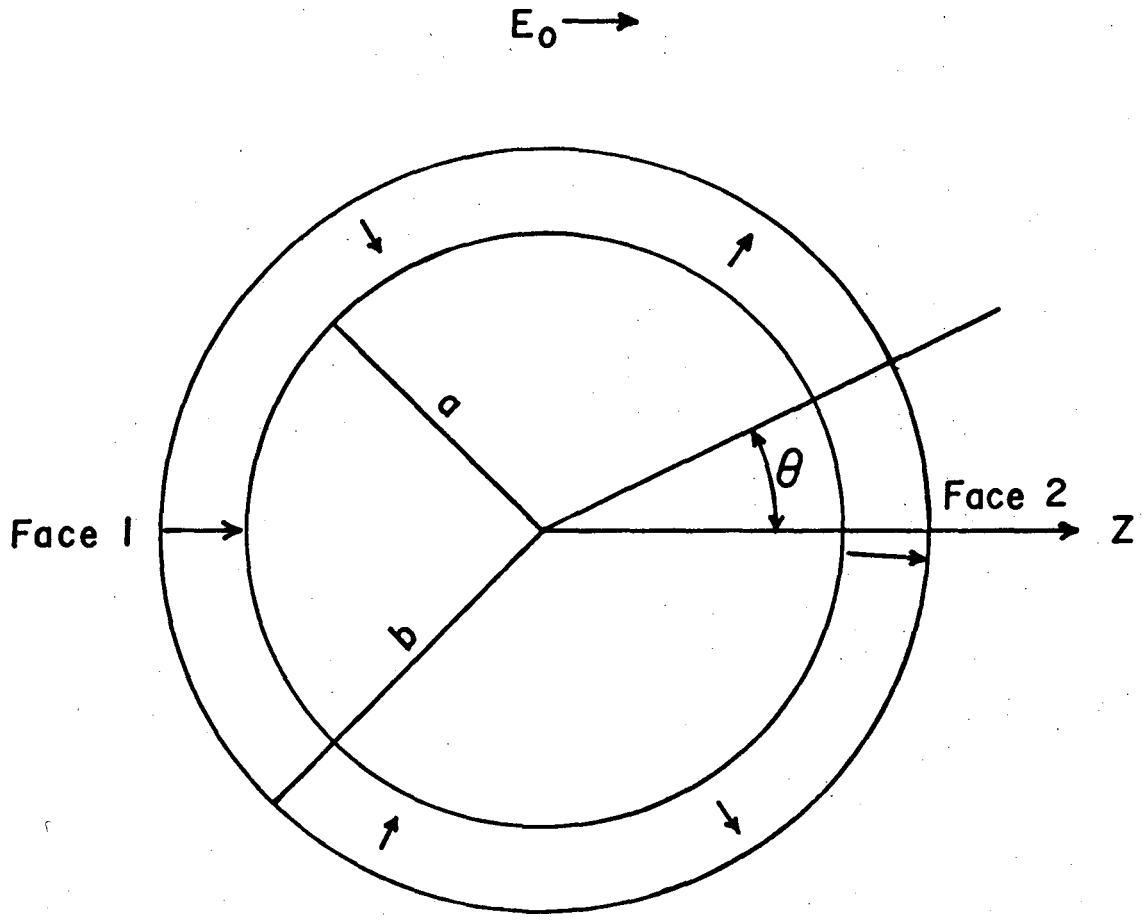

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Fig. 15

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