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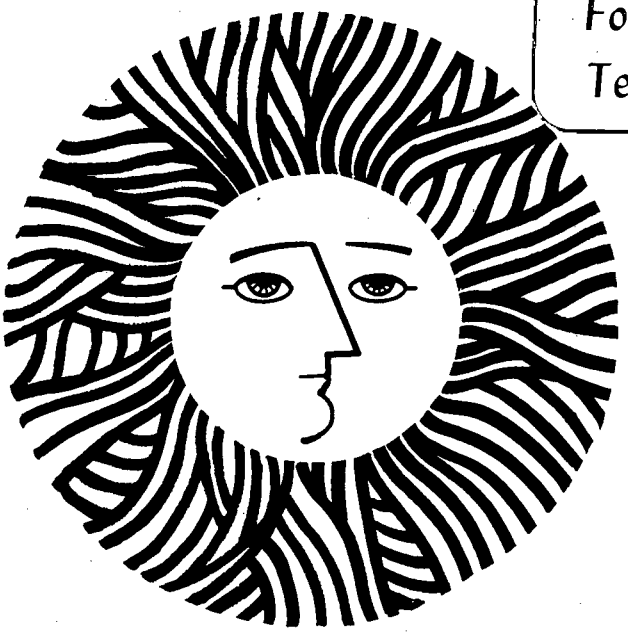

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SURFACE CHARGE CHANGES IN PURPLE MEMBRANES AND THE PHOTOREACTION CYCLE OF BACTERIORHODOPSIN

(Halobacteria, Bioenergetics, Surface Potentials)

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Abstract;

The surface potential of purple membrane fragments, determined from the distribution of the aqueous free and the membrane-bound positively charged, paramagnetic, amphiphilic probe 4-(dodecyl dimethyl ammonium)1-oxyl-2, 2,6,6-tetramethyl piperidine bromide, varied almost 60 mV as a function of ionic strength and 50 mV as a function of pH of the medium. Light induced changes in surface potential followed the changes observed in the M_{412} intermediate of the photocycle of bacteriorhodopsin as a function of pH, temperature and response to antibiotics Beauvericin and Valinomycin. The number of induced charges per M_{412} appearing at the surface of purple membranes decreases from about 0.75 to 0.45 as the surface potential becomes more negative. The stoichiometry would be twice as large if the charge changes were localized exclusively on one side of the purple membrane. Laser flash induced kinetics of the rise and decay of surface charge changes were slightly slower than the kinetics of the rise and decay of M_{412} which is associated with the reversible deprotonation of the retinal Schiff base nitrogen in the chromophore. It is suggested that the light induced charge changes monitor a dissociable amino acid residue which may be a step in the movement of protons across the purple membrane.

INTRODUCTION:

Bacteriorhodopsin, the retinal containing protein in the purple membrane of *Halobacterium halobium* functions as an electrogenic light activated proton pump (1). Upon illumination, the chromophore undergoes a photocycle in which its retinal Schiff base nitrogen is reversibly protonated (2). The detailed molecular mechanism of H^+ translocation is not understood at present, but it may involve the Schiff base alone or other charge separation events, such as tyrosine deprotonation (3,4) or charge displacement in tryptophan (5). These dissociation events could move along a sequence of amino acids, thus providing a pathway for the proton translocation across the purple membrane.

Purified purple membranes produce pH changes in their suspension medium during steady state illumination (6), which are strongly pH and temperature dependent (7). This light induced proton release (8) and its stoichiometric relation to the transient intermediates of the photoreaction cycle (9,10) were found to vary with salt concentration. The effect of ionic strength on proton release suggests the involvement of surface potential in the mechanism of the proton pump. We have, therefore, directly measured the surface potential of purple membranes and its effect on the light induced charge changes. Using continuous actinic light of moderate intensity and single turnover laser flashes, we have studied the kinetics and stoichiometry of changes in surface charge that arise on purple membranes. The relevance of these changes to the light induced intermediates of the photoreaction cycle and to proton pumping is discussed. A preliminary report of these findings has been presented (11).

MATERIALS AND METHODS:

Purple membranes were prepared from *H. halobium* by standard

methods (12), and suspended in KCl containing media at different ionic strengths and pH values, at a concentration of 0.128 mM or 0.256 mM bacteriorhodopsin, as indicated, assuming an extinction coefficient of $\varepsilon^{570} = 63{,}000\ M^{-1}cm^{-1}$ (13). The pH was adjusted with HCl or KOH and ranged from pH 2 to pH 10.

Beauvericin (Lilly Laboratories) and Valinomycin (Sigma Chemical Co.) when used to slow down the photocycle (9), were each at a 1:1 molar ratio with the protein. The positively charged paramagnetic amphiphile 4-(dodecyl dimethyl ammonium)-1-oxyl-2,6,6,6-tetramethyl piperidine bromide (CAT_{12}) which partitions between the membrane and aqueous phases, was synthesized in our laboratory by R.J. Mehlhorn and used as a probe of surface potential (14,15,16) at concentrations of 1-2 mM, depending on protein concentration. Electron paramagnetic spectra were recorded in a Varian E-109E spectrometer and the kinetics of the light induced spin-probe partitioning changes stored in a signal averager CAT 400, or through a Biomation 1010 transient recorder with a PDP 11/34 computer. Samples of 30-50 μl were held either in a 50 μl capillary, in a flat capillary 0.4 mm thick or in a flat cell 0.254 mm thick as indicated. For steady state illumination studies, a quartz iodide lamp was used through a Corning 3-67 cutoff filter (light intensity 40.5 mW/cm^2) while a Phase-R dye laser with Rhodamine 575 (0.2 joules/flash, 150 ns flash rise time) was used for the flash experiments. The photosteady state of M_{412} was measured in an Aminco DW 2 spectrophotometer. The M_{412} concentration was calculated assuming $\Delta\varepsilon^{412} = 23{,}000\ M^{-1}cm^{-1}$ (13). The photomultiplier was protected by a Beard Atomic interference filter (412 nm transmission maximum). The dye-laser was used for measurements of the kinetics of M_{412} in a flash photolysis apparatus equipped with a Biomation 1010 transient recorder interphased to the PDP 11/34 computer.

RESULTS:

In the presence of purple membranes, the spinprobe CAT_{12} partitions between the membranous and aqueous phases showing both membrane (broad) and aqueous (narrow) components to its EPR signal (Figure 1). The ratio of size of the EPR line heights corresponding to the free and bound populations of the probe, designated as the partition (P), has been shown to monitor the surface potential of membranes (15,16). A change in surface potential will result in a change in the partition of CAT_{12}. Changes in surface potential ($\Delta\Psi_s$) were calculated from the Boltzman relation (see reference 15)

$$\Delta\Psi_s = \frac{RT}{zF} \ln \frac{P_1}{P_2} \qquad \text{(I)}$$

where P_1 and P_2 stand for the partitioning of CAT_{12} at two different states; z, F, R, and T are respectively the charge on the spin-probe, the Faraday constant, the universal gas constant and the absolute temperature. One way to calculate the amount of bound CAT_{12} involves measurement of the decrease in the high field free EPR signal on titration of purple membrane into an aqueous solution of CAT_{12}. Using this method, ratios of <7:1 bound spin probes per bacteriorhodopsin were calculated under the experimental conditions used in this work. The partition of CAT_{12} was a function of the ionic strength of the medium as expected from the Gouy-Chapman theory. According to this theory, the surface charge density σ (in electronic charges/$\mathring{A}^2$), the surface potential Ψ_s (in mV), and the ionic concentration c (in moles/liter), are related by the following equation (see reference 17):

$$\sinh \frac{zF\Psi_s}{2RT} = 136 \frac{\sigma}{\sqrt{c}} \qquad \text{(II)}$$

at 22° C.

As seen from Figure 2, a theoretical curve calculated according to Equation II (assuming σ = 0.00174 negative charges/$\mathring{A}^2$) fits well with the experimental data calculated from Equation I. A Ψ_s decrease of up to 60 mV was obtained when the ionic strength was raised from 2 mM - 300 mM (monovalent).

Under constant ionic strength, the partitioning of the probe responded to variation in the pH of the medium (Figure 3). Since the positive charge of the tertiary amine in the spin probe is not affected by the change of pH within the range used in this experiment, it is likely that the changes in surface potential were due to ionization of dissociable groups in the purple membranes. Two groups of pKs around pH 3.5 and pH 10 are seen in the titration curve; since this membrane contains no phosphatidyl ethanolamine or phosphatidyl serine, the main contribution to the increase in negative surface charge as the pH is raised was probably from carboxyl groups and the free amino and hydroxyl groups of the amino acid residues in the protein.

Light Induced $\Delta\Psi_s$: At room temperature, light of moderate intensity converts only a very small fraction of bacteriorhodopsin into its M_{412} intermediate, making it difficult to measure light induced phenomena. Hence it is convenient, when studying the correlation between the photocycle and surface charge changes, to increase the steady state concentration of M_{412}; this may be done by using higher light intensities or by slowing down the rate of its decay. Continuous illumination at high light intensities may damage the system. We have used the antibiotics Valinomycin and Beauvericin at a 1:1 molar ratio with the protein, to slow down the decay of M_{412}. In the presence of the antibiotics, two distinct phases in the decay of the M_{412} were observed as in (18). The kinetics of the

flash induced rise of the M_{412} (not shown) did not change. The mode of action of these antibiotics in causing the changes in the kinetics is not understood, however, a pH titration of the surface potential changes in the dark (Figure 3) shows that the antibiotics caused a decrease in the overall negative surface charge without changing the apparent pK of the dissociable groups in the protein. Under these conditions, the spectral changes in the high field aqueous EPR line of CAT_{12} during continuous illumination, are shown in Figure 1. A reversible decrease in the free aqueous population of CAT_{12} during illumination could mean one of two things: (a) the surface potential of the purple membrane becomes more negative when H^+ are released or (b) the purple membrane becomes more hydrophobic during that process. Control studies with the uncharged spin probe 2N9 (2,2-dimethyl-5,5-methyl heptyl-N-oxazolidinyloxyl) suggested that there was very little change in the hydrophobicity of the purple membrane during illumination and that these changes were ionic strength independent up to 2M KCl. As a result of the slow down in M_{412} decay, there was an increase in the steady state level of M_{412} and a corresponding increase in the light-induced steady state change of the surface potential (Table I).

STOICHIOMETRY: The light-induced change in surface potential is independent of pH between 5.5 and 8, decreasing at lower pH's and increasing at higher pH values with pK values at about pH 4 and pH 9 (Figure 3). The change in surface potential seems to be independent of ionic strength up to about 100 mM (monovalents) decreasing only at higher ionic strengths. It is clear from Equation II that the same change in Ψ_s at two different ionic strengths (pH values) will correspond to a higher change in surface charge density at the higher ionic strength (lower pH). The stoichiometric molar

ratios between light induced charge changes and M_{412} were calculated for various ionic strengths. As seen from Figure 2, the charge changes per M_{412} decreased as the ionic strength of the medium was lowered. A similar response was observed (9) for the ratio between light induced proton release and the M_{412} level.

For the calculation of surface charge changes using Equation II, two assumptions were made: (1) that the surface charge distributions are homogeneous on the two sides of the purple membrane, and (2) that the spin probe CAT_{12} only monitors what happens in the lipid phase, where it is almost certainly located. The first assumption has not been tested; as regards the second, we know that the lipid occupies ~37% (1) of the total area of the purple membrane and that the hyperfine splitting of the EPR signal of the bound component of the probe is ~60 gauss at room temperature (Figure 1); this suggests indeed that the probe may be in the constrained lipid environment of the purple membrane.

The charge density changes under steady state illumination were related to the level of M_{412} photointermediate under similar conditions. Table II shows the steady state levels of M_{412} at pH 7.2 for different light intensities as well as the number of charge changes per M_{412} obtained when purple membranes were suspended in 100 mM KCl and 5 mM Tris buffer. About one negative charge per M_{412} was induced by light under these conditions.

The correlation between the steady state light induced surface potential change and the level of M_{412}, its response to antibiotics and to pH, and the similarity in the stoichiometry of charges and protons per M_{412} merited a more precise evaluation of the kinetic relation between charge changes and the photointermediates.

KINETIC STUDIES: Since the generation of the $\Delta\Psi_s$ is much faster at room

temperature than the limit of sensitivity of our EPR instrumentation (0.2 ms), we studied the laser-flash induced decay of M_{412} and the corresponding decay of the surface potential change monitored with CAT_{12}. A typical decay curve for the change in the high field aqueous line of CAT_{12} is shown in Figure 4. The decay could be resolved into a fast and a slow component. The M_{412} decay was studied in a flash photolysis apparatus in the presence of CAT_{12}; since it was impossible to do these measurements at the same bacteriorhodopsin concentration as those used in the EPR experiments, the concentration of CAT_{12} was adjusted to obtain the same ratio of bound spin probe to the membrane fragments in the two experiments. The addition of CAT_{12} did not change the kinetic constants of the decay but increased by approximately 30% the contribution of the fast phase (not shown) to the overall decay of M_{412}.

A comparison of the decay of $\Delta\Psi_s$ and M_{412} over a wide range of pH values (Figure 5) indicates that the two follow similar patterns. However, the kinetics of both the fast and the slow components of surface potential changes were slower than the kinetics of M_{412} decay. This cannot be due to a limitation of the response time of the spin-probe, since changes in the EPR signal can be generated with half lives as small as 7 ms, indicating that the response time of the probe is at least in the ms range.

LOW TEMPERATURE STUDIES: Although the antibiotics used in these experiments seemed to indirectly affect the light induced charge changes through their effect on the kinetics of the photocycle, it was necessary to provide independent experimental proof that the surface potential changes are not induced only in their presence. Instrumental limitations required that the reaction be slowed down. Since low temperature decreases the rate of conversion of the intermediates in the cycle (1), it was expected

that the kinetics of the light induced surface charge changes would also be slowed down. Indeed, at -10° C, and pH 7.2, in the presence of 25% ethylene glycol, the light induced steady state $\Delta\Psi_s$ was -1.69 mV compared to -0.02 mV at room temperature. The flash induced decay of $\Delta\Psi_s$ (Figure 6B) showed biphasic kinetics. The fast and the slow components had $\tau_{1/2}$ = 200 ms and 750 ms respectively. The $\tau_{1/2}$ of the decay of M_{412} at -10° is 70 ms (19). Because of the relatively high noise contribution from the instrumentation, the rise time for $\Delta\Psi_s$ could not be accurately measured. Figure 6A shows a typical curve for the rise of $\Delta\Psi_s$ at -10° C. Linear regression of the log of these data gave a correlation coefficient of 0.769 for a $\tau_{1/2}$ = 9.3 ms. The $\tau_{1/2}$ for the rise of M_{412} at -10° is approximately 1.5 ms (20). It seems that both the rise and the decay of the $\Delta\Psi_s$ lag slightly behind the kinetics of M_{412}.

DISCUSSION:

The purple membrane functions as an electrogenic proton pump. Since the rate of uptake of H^+ is different from the rate of H^+ release, the membrane is likely to be polarized. The data presented here indicate that the changes in surface charge of purple membranes during steady state illumination can be described as the appearance (disappearance) of approximately one negative (positive) charge on the surface of the protein per M_{412} photointermediate. Under steady state illumination and conditions in which Equation II is valid (ie. $c > 0$), we estimate that the number of negative charges per M_{412} appearing at the surface of purple membranes increases from ~0.45 at 2 mM KCl to ~0.75 at 100 mM KCl, assuming that M_{412} levels are unchanged under these conditions (9). Previous studies (9) have shown that under similar conditions, the number of protons released per M_{412} formed approaches

0.9 in the presence of salts, and that this number is a factor of 2-3 times greater than that observed in the absence of salts.

The stoichiometry of proton to M_{412} has also been measured under different conditions by light induced volume changes (10). One to two protons per M_{412} were released depending on the ionic strength of the medium. Similar dependence of proton release on salt concentration of the medium (8) was interpreted to indicate a change in proton release which is due to a surface potential effect on the protein. Using the spin probe technique, it was possible to measure directly the changes in the bound and free probe and to evaluate the effect of surface potential on charge changes. If light polarizes the membrane, this could mean that the stoichiometric values given are underestimated, as the charge distribution changes were assumed to take place homogeneously on both sides of the purple membrane.

Purple membranes contain approximately 25% lipid by weight (1). Monomers of bacteriorhodopsin have been prepared from the purple membrane which retain proton pump activity (21). It would appear that the lipid in the purple membrane has no direct effect on bacteriorhodopsin activity. Hence, the high amount of CAT_{12} employed, <7/mole of bacteriorhodopsin, is probably not a serious factor in evaluating surface potential changes as this amount of spin probe does not modify the kinetic constants of M_{412} rise and decay. In this context, it is interesting to note that the large change in surface charge introduced by the probe hardly affects the photocycle.

In the presence of the antibiotics at room temperature, the rate of decay of the M_{412} is at least three orders of magnitude slower than its formation (9). Under similar conditions, the unresolved rise time of $\Delta\Psi_s$ is less than 7 ms, while the decay is at least two orders of magnitude

slower. At low temperatures (-10° C), the rate of decay of the M_{412} is almost two orders of magnitude slower than its formation (19,20). Although we do not have a precise measurement (correlation coefficient 0.76) of the kinetics of the rise of light induced negative surface charge changes, the data suggests that at low temperature the rise is approximately two orders of magnitude faster than the decay. Under steady state conditions, the spin probe measures the overall charge change arising from the amount of M_{412} in the photostationary state.

The decay of the surface charge changes closely followed the decay of the M_{412} intermediate dependence on pH, temperature and response to antibiotics. Biphasic decay kinetics were characteristic for both processes. A biphasic decay of M_{412} has also been reported (22) for flash induced changes under different experimental conditions. However, both the rise and the decay of the surface potential changes were slightly slower than the M_{412} changes, indicating that the dissociation of a proton from the retinal Schiff base preceeds the appearance of changes on the surface of the membrane fragments. It can be argued that the charge changes were due to changes in pK of some amino acid residue of the protein which is not directly related to the pump activity. However, measurements of flash induced proton absorbance changes in pH indicators in purple membrane suspensions which were interpreted to measure proton release, were slower than the formation of M_{412} (23). Flash induced volume changes interpreted to measure proton changes in the medium also showed that the release and uptake of protons is slower than the formation and the decay of the M_{412} (24).

It is suggested that the charge changes measured on the surface of

the purple membrane may reflect dissociation of amino acid residues which are on the path of transfer of protons across the membrane. The observed pK at pH 3.5 indicates that carboxyl groups are involved. Indeed, chemical modification of carboxyl residues with carbodiimides inhibit the photoreaction cycle, increase M_{412} in the photostationary state and the light induced surface potential changes measured with the CAT_{12} probe (25).

ACKNOWLEDGMENTS

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Legends to Figures:

Figure 1

EPR spectrum and light induced amplitude changes of the high field aqueous CAT_{12} signal in a purple membrane suspension. A 50 µl sample contained 0.256 mM bacteriorhodopsin, 0.256 mM Valinomycin, 0.256 mM Beauvericin, 100 mM KCl, 2 mM CAT_{12}, pH 7.2 at room temperature. Time course of the light (intensity 40.5 mW/cm^2) induced changes in h_o were measured in a flat cell (0.254 mm light path).

Figure 2

Purple membrane surface potential as a function of the ionic strength of the medium. Purple membranes (0.128 mM bacteriorhodopsin) in the presence of 1 mM CAT_{12}, 0.128 mM Valinomycin and 0.128 mM Beauvericin, pH 6.1, at room temperature, were titrated with KCl to give the indicated final concentrations. The line represents a theoretical curve calculated according to Equation II, while the points (•) were calculated from Equation I. Ratio of light induced charge changes to M_{412} (o) were measured and calculated as described under Table II.

Figure 3

The effect of pH on surface potential of purple membranes. Conditions as in Figure 2, but in the presence of 100 mM KCl. The surface potential in the dark is shown in the lower graph. The steady state light induced changes in surface potential ($\Delta\Psi_s$) are shown in the upper graph. Values for the surface potential in the absence of Beauvericin and Valinomycin are shown as (-o-).

Legends to Figures (cont):

Figure 4

Kinetics of the laser flash induced decay of CAT_{12} binding to purple membranes. The inset is a trace average of 50 flashes. The EPR time constant was 8 ms. To analyze the kinetics, the decay of the EPR signal (-o-) was plotted on a similog plot and the "curve peeling" technique was used to distinguish the slow ($\tau_{1/2}$ = 574 ms) from the fast (-•-) ($\tau_{1/2}$ = 103 ms) first order kinetic components. Other conditions as in Figure 1.

Figure 5

The pH dependence of the half life of the decay of laser flash induced formation of M_{412} and of the changes in CAT_{12} partitioning to purple membranes. The half life of the decay of the flash induced partitioning changes of CAT_{12} were obtained as described in Figure 4. The decay of the flash induced M_{412} phototransient was measured using a suspension of purple membranes (3.85 μM bacteriorhodopsin) in a medium containing 100 mM KCl, 3.85 μM Beauvericin, 3.85 μM Valinomycin, 36.6 μM CAT_{12}.

Figure 6

Laser flash induced binding and release of CAT_{12} to purple membranes at -10° C. Conditions as in Figure 1, but with 25% ethylene glycol. A flat capillary (0.4 mm light path) was used. (A) For measurement of the rise time of the partition changes of CAT_{12}, 600 flashes were averaged using an EPR time constant of 0.2 ms. (B) For the decay time of partition changes of CAT_{12}, 100 flashes were averaged using an EPR time constant of 4 ms.

Table I

Effect of Beauvericin and Valinomycin on Light Induced Steady State Changes in M_{412} and $\Delta\Psi_s$

Additions	M_{412} (μM)	Potential $\Delta\Psi_s$, (mV)
None	4.1	-0.046
Beauvericin & Valinomycin	21.8	-0.235

Conditions as in Figure 1, calculations of CAT_{12} distribution as in Equation I and of M_{412} absorbance as in "Methods." For the measurement of the M_{412}, the same EPR flat cell was used in the Aminco DW-2 spectrophotometer at 45° to the light source and at 45° to the measuring beam.

Table II

Stoichiometry of Steady State Charge Changes in

Purple Membranes to Amount of M_{412} in the Photostationary State

Light Intensity (mW/cm^2)	M_{412} (μM)	$\frac{M_{412}}{BR}$	Potential $\Delta\Psi_s$, (mV)	$\frac{Charge}{BR}$	$\frac{Charge}{M_{412}}$
40.5	21.8	0.0854	-0.235	0.0643	0.753
32.0	18.3	0.0715	-0.205	0.0559	0.782
16.5	10.7	0.0417	-0.074	0.0270	0.647

Conditions as in Table I, calculation of charge distribution as in Equation II. BR - Light Adapted Bacteriorhodopsin.

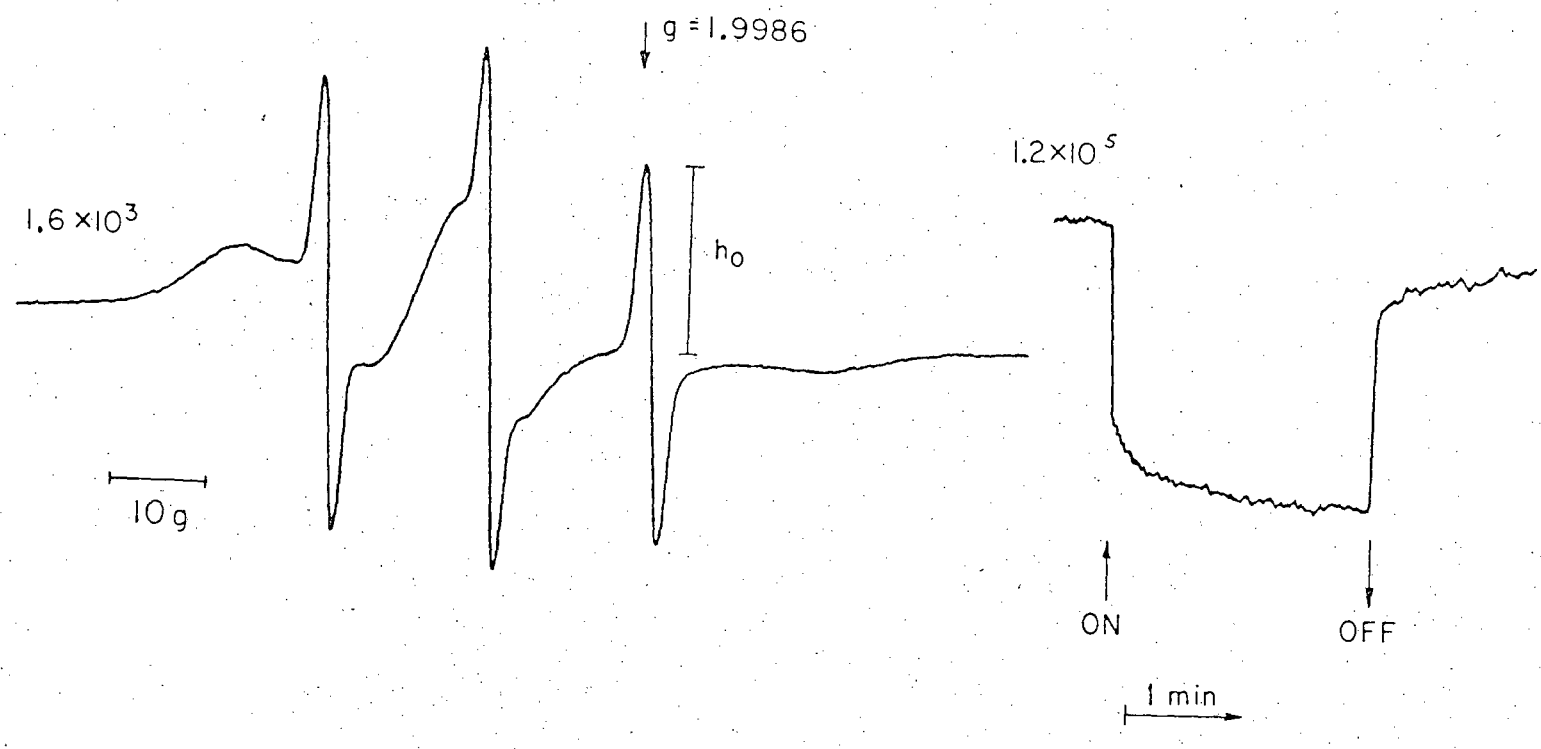

Fig. 1

XBL 797-10700

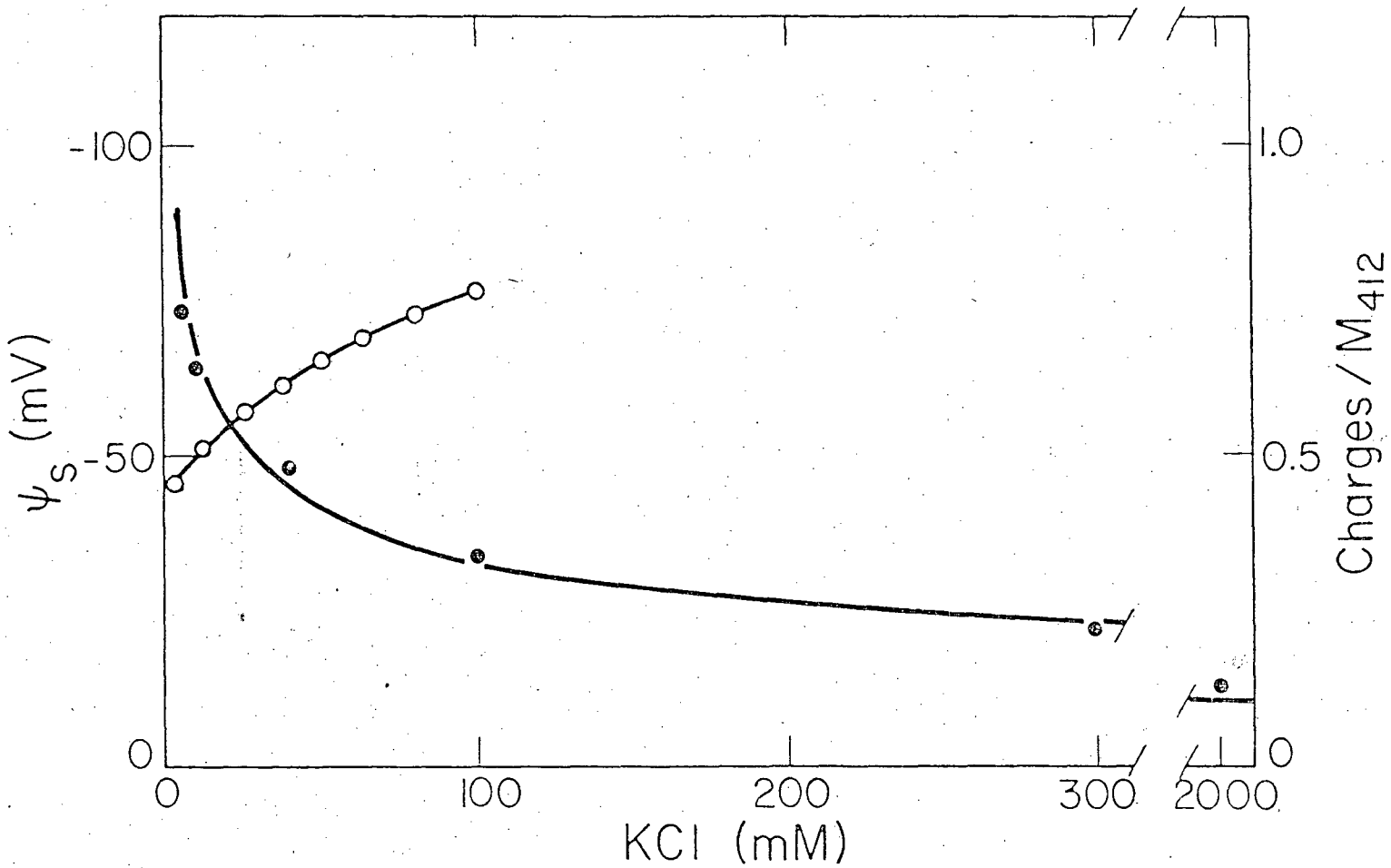

Fig. 2

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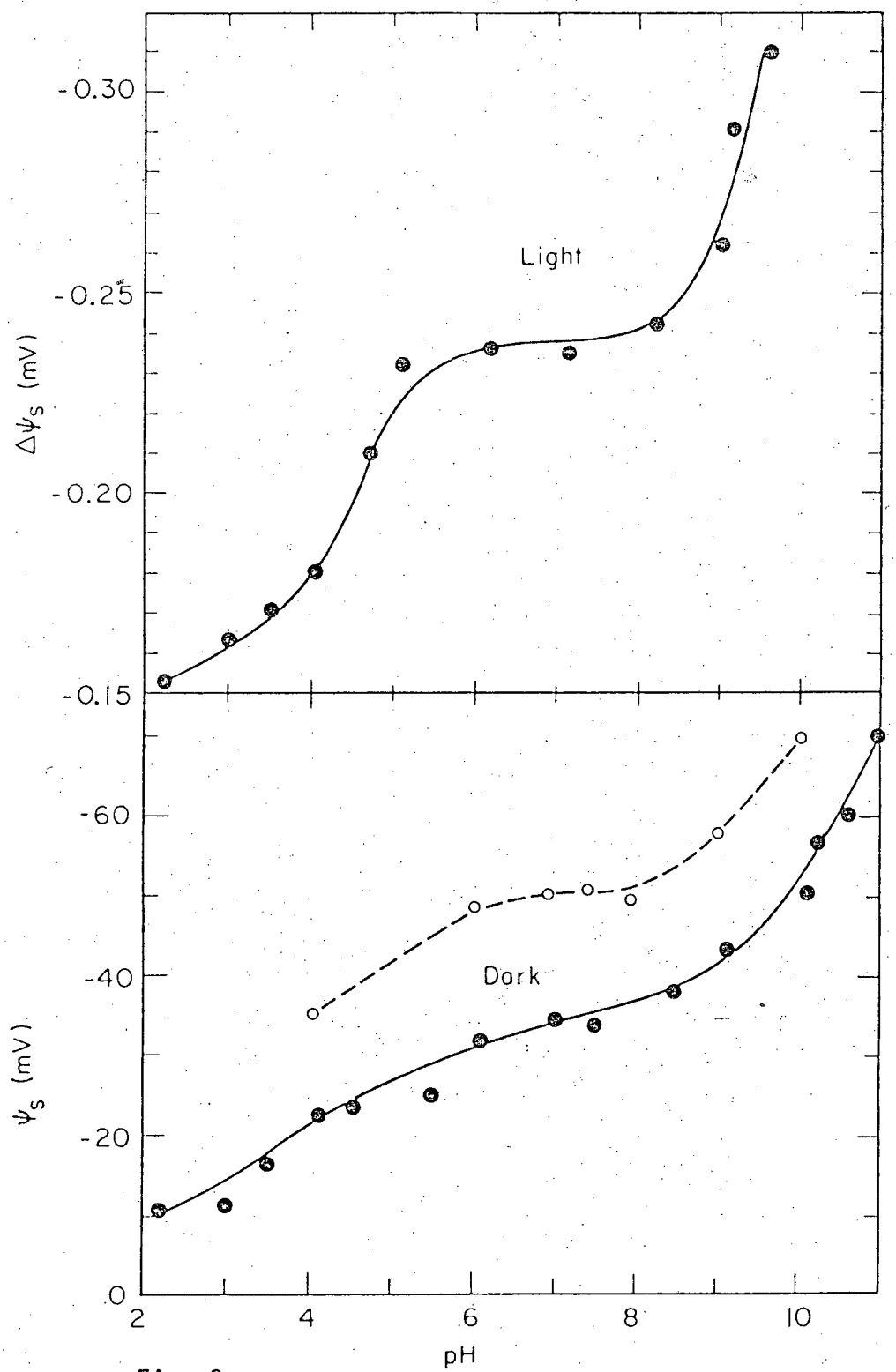

Fig. 3

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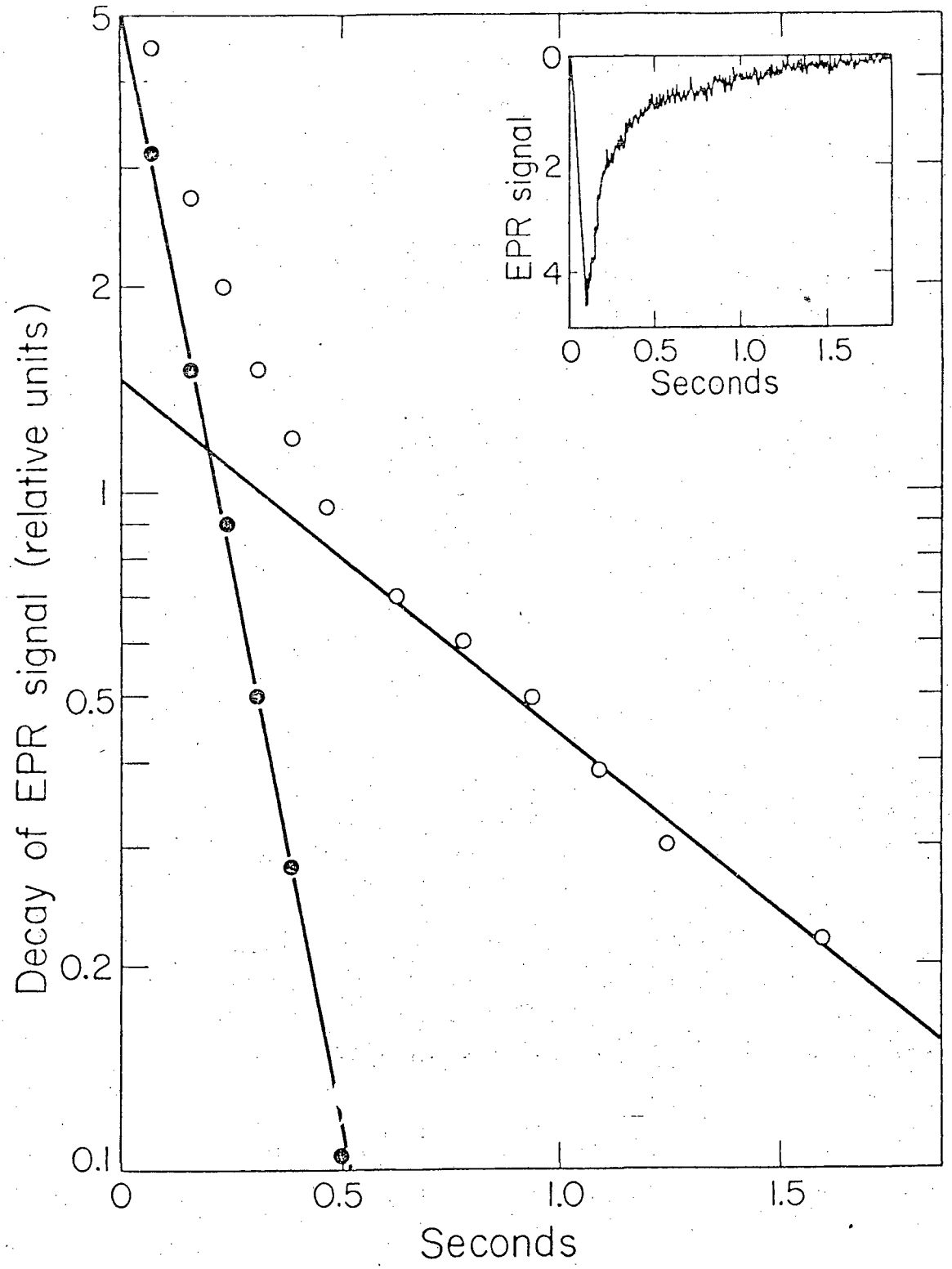

Fig. 4

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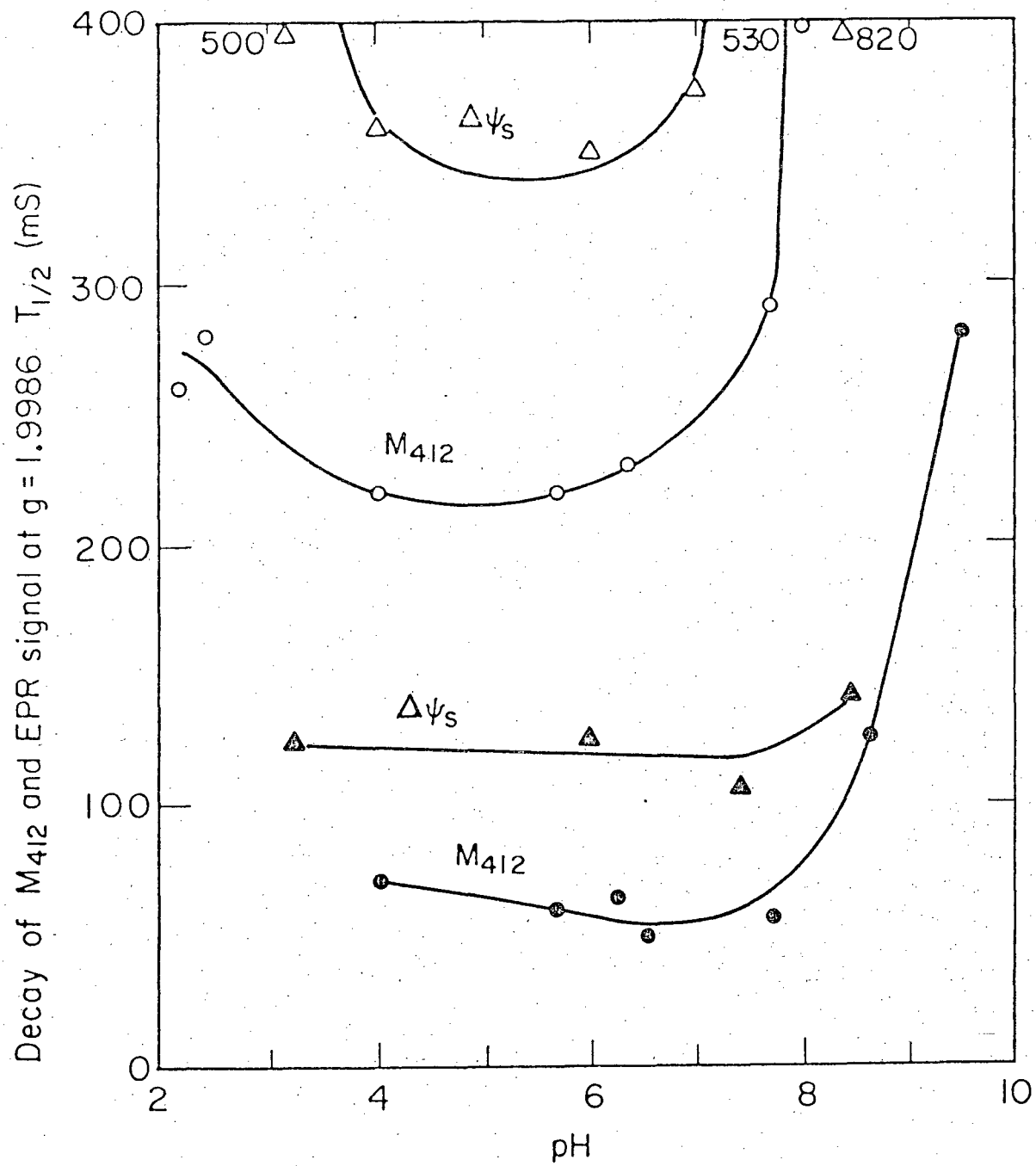

Fig. 5

XBL 7910-12237

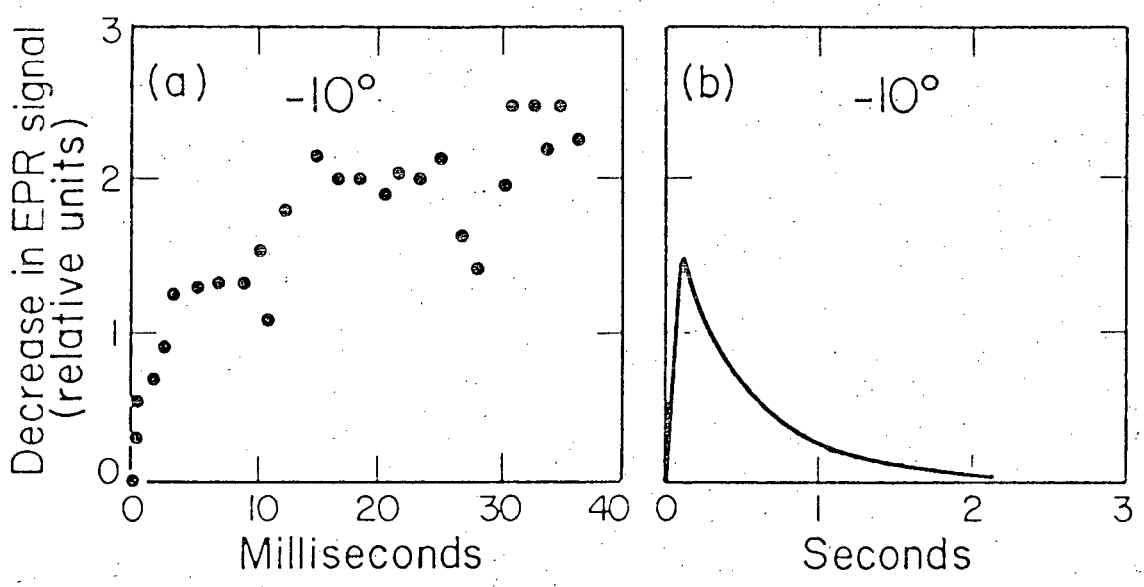

Fig. 6

XBL 799-2909

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