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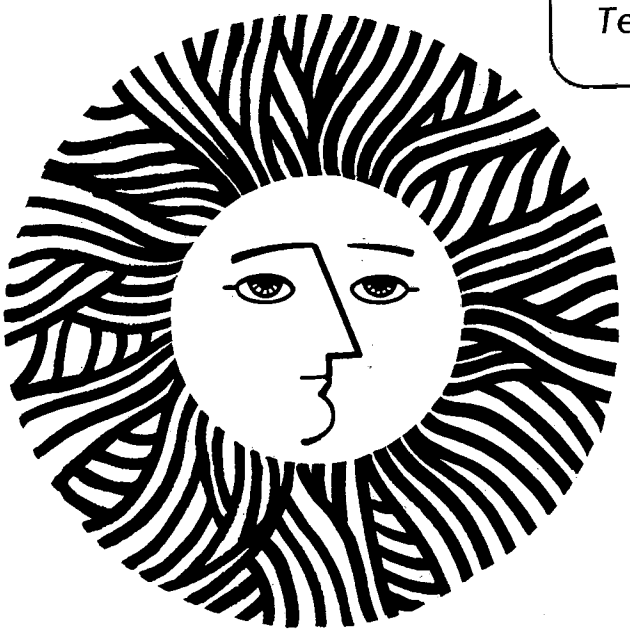

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MEASUREMENTS OF VOLUMES AND ELECTROCHEMICAL GRADIENTS WITH SPIN PROBES IN MEMBRANE VESICLES

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Volumes and electrochemical gradients in cells and vesicles can be measured by several available methods. It is important to employ at least two independent techniques for measuring these parameters to avoid misinterpretation of data due to artifacts. Spin probe techniques compliment traditional methods which focus upon extravesicular and extracellular water. By observing probe concentrations inside sealed membrane systems, these methods provide an important check on other techniques in terms of criteria of complementarity, i.e., whether inferred changes of electrochemical potentials on two sides of a membrane are opposite and equal after correcting for volume differences. Spin probes also give unique information: volume measurements are independent of assumptions of cell shape; probe binding is quantitated and corrected for; and with several probes, kinetic measurements appear to be feasible.

GENERAL CONSIDERATIONS

The technique of spin labeling [1] has recently been extended to include measurements of bioenergetics parameters like volume changes, pH gradients, and electrical potentials [2, 3]. To differentiate between the signal of spin probes on two sides of a membrane, the phenomenon of exchange broadening has been exploited. Exchange broadening of the paramagnetic signal of a spin label occurs when paramagnetic molecules collide with a high enough frequency to allow significant exchange of unpaired electrons between paramagnetic species, thus quenching the spin label signal. Quenching can be used to observe either intracellular or extracellular spin signals. The intracellular signal is elicited by quenching the extracellular signal with impermeable transition metal complexes while enhancement of the extracellular signal can be achieved indirectly be means of increased membrane binding of the probe resulting from uptake or at high spin label concentrations, where increases in intracellular probe concentrations lead to self-quenching of the nitroxides. When intracellular

signal changes are observed, these are linearly related to probe concentrations under the appropriate experimental conditions, whereas extracellular signal changes have a markedly nonlinear concentration dependence and require calibration techniques for quantitation. Therefore, accurate probe concentrations are more readily obtained by observing intracellular spin signals with impermeable quenching agents. The signal observed in the electron spin resonance spectrometer is proportional to the total number of unquenched spins in the sample, so considerable signal changes can occcur during energization of biological membrane preparations. For example, in chloroplasts it was shown the the intrathylakoid signal of a spin labeled amine increased more than a thousand-fold during illumination [4].

robe chematics

Probes useful for measuring bioenergetic parameters are shown below:

VOLUME MEASUREMENTS

Bulk ion transport can be measured directly with radioactive isotopes in flow-dialysis schemes, or by performing specific-element analysis of isolated cells or membrane vesicles. A considerably more convenient albeit indirect method for monitoring coupled ion fluxes of cations and anions (symport) is to measure volume changes associated with these bulk ion movements. The spin-probe method is ideally suited for this purpose because volumes can be measured accurately and easily.

ig. 1

A schematic diagram representing volume measurements is shown below. The probe of choice for measuring volumes should satisfy the following criteria: efficient quenching by paramagnetic agents to minimize osmotic effects of the quencher, rapid membrane permeability and inertness towards electrical or ionic gradients. Efficient quenching is realized with probes having narrow intrinsic line widths since a given increment of line broadening causes relatively more signal decrease than is observed with broad intrinsic line widths. This

requirement is met by ring nitroxides, having effectively planar structures over the lifetimes of the excited spin states. Rapid permeability requires that the probe have weak polarity, i.e., just sufficient for adequate water solubility. Inertness towards electrochemical gradients excludes probes with charge and titratable groups. All of these criteria are met by 2,2,6,6-tetramethyl-4-oxo-piperidinooxy (TEMPONE). The ketone group on the ring confers enough polarity to give adequate water solubility and also maintains the ring in a time-averaged planar configuration which gives narrow lines. Permeability of this nitroxide is rapid (half-time for equilibration is less than 50 msec in human red cells). The procedure for absolute volume measurements consists of obtaining a TEMPONE spectrum in the presence of vesicles and a second spectrum with added quenching agent. The final aqueous signal is expressed as a fraction of the initial signal and generally converted to membrane concentration in terms of mg of protein. Relative changes can be measured in a single sample with quenching agent present during volume changes. Control experiments should be conducted without quenching agent present during the volume changes and by quantitating volumes with subsequent quenching agent additions.

MEASUREMENTS OF pH GRADIENTS

ig. 2 Procedures for measuring pH gradients are shown schematically below. Spin labeled amine and carboxylic acids have proven useful for following proton movements across membranes.

Amines with different substituents can serve several purposes: Dimethyl TEMPAMINE and other tertiary amines do not react with activated carboxyl groups and are useful for carbodiimide treated samples where the potential for such reactions exists. With various substitutions on the nitrogen, partitioning differences of spin labeled amines into lipids can be used to demonstrate whether the permeability of a probe is rate limiting.

Quaternary amines serve to determine whether a given membrane is leaky to the charged amines. Spin labeled phosphates and sulfates serve a similar purpose for the carboxyl probe.

By determining pH gradients at different probe concentrations, thresholds for inhibition can be ascertained. Decreases of pH gradients become apparent at about 20 μM of a spin labeled carboxylic acid in envelope vesicles of Halobacterium halobium [2]. These probes are accumulated inside the vesicles during illumination On the other hand, the spin labeled amine, which is extruded in envelope vesicles of Halobacterium halobium, does not inhibit pH gradient formation until its concentration exceeds 1 mM.

The high sensitivity of spin probes and availability of both weak acid and amine labels permits measurements of steady state pH gradients of small magnitude ($\Delta pH<0.1$). The procedure consists of an "effective volume" determination of cells or vesicles with both acid and amine probes. In the absence of a pH gradient these should be equal to the true volume determined with TEMPONE after correcting for binding. A small difference in the measured apparent volumes is easily converted to a pH gradient, i.e.,

$$pH = 1/2 \log\left(\frac{h_{TC}}{h_{TA}}\right)$$

Where h_{TC} and h_{TA} are the fractional intracellular signals of the acid and amine probes, respectively. As an additional control, it can be demonstrated that the difference in "effective volume" is abolished upon treating the cells with ion exchanging agents like tributyl tin or nigericin.

3 MEASUREMENTS OF ELECTRICAL GRADIENTS

Spin labeled phosphonium derivatives first introduced by Cafiso and Hubbell [3] are useful for measuring equilibrium transmembrane electrical potentials although

their permeability is insufficient to allow them to be exploited for kinetic measurements. Charge effects dictate that anionic quenching agents be used with these probes in media of low ionic strength. There is appreciable binding of these nitroxides to membranes. Due to this binding, membrane perturbation effects are a concern and careful control experiments to demonstrate that probe uptake is directly proportonal to the bulk probe concentration are required to assess inhibitory effects.

Addition of the permeable spin-labeled ion to vesicles will generate a transmembrane electrical potential which will oppose equilibration of the probe. Generally, the rate limiting process in achieving equilibrium will be the movement of some other ion, e.g. an electrogenic proton counterflow through "leaks" in the membrane. Acccordingly, equilibration may proceed slowly and the rate constant for uptake of the probe under non-energized conditions should be established for any given membrane preparation prior to attempting measurements of energized electrical gradients.

Quantitation of probe concentrations under non-energized and energized conditions must take into account the appreciable and variable fraction of probe molecules bound to the membrane. The magnitude of the total bound signal at both membrane interfaces in the energized state relative to that in the non-energized state is readily determined by quantitation of aqueous signal intensities before and after energization. With a knowledge of the energy-dependent behavior of the bound population of the probe, the net change of probe concentration outside the vesicles can be determined by quantitating its intra-vesicular concentration with quenching agent present. The electrical potential in millivolts will be related to the concentration gradient of aqueous probes directly, i.e.

$$= 60 \cdot \log\left(\frac{[T\phi E3]\ in}{[T\phi E3]\ out}\right)$$

where [TØE3] refers to the aqueous concentration of the phosphonium probe.

The following is an example of a membrane potential calculation carried out for envelope vesicles of the S9 strain of Halobacterium halobium: Membranes at pH 6.7 were suspended at 7mg/ml of protein and the cell volume was determined with TEMPONE as 3.1% of the total volume. An aliquot of the vesicles was spin-labeled with 30μM TØE3 and treated with 2mM sodium ferricyanide to ensure full oxididation of the probe without suffering any reduction in nitroxide line heights due to exchange broadening. The aqueous line heights in the presence of membranes after equilibration were 40% of the line heights observed in 4M NaCl, implying that 17.7μM of the probe was membrane-bound under these conditions. Under illumination there was a substantial decrease of the total aqueous signal. Careful analysis of the signal intesity of the bound component revealed that the aqueous line height reduction was due entirely to increased membrane binding of the probe and not to concentration-dependent self-quenching or chemical reduction. The final aqueous line height was equivalent to a 4.0μM aqueous solution of TØE3. Another aliquot of vesicles labeled with TØE3 was treated with 100mM sodium ferricyanide to completely quench the spin signal outside of the vesicles. Upon illumination the unquenched aqueous signal increased, reaching a final volume equivalent to a 1.5μM aqueous solution of TØE3. Subtracting this value from the total aqueous line height observed under illumination with 2mM ferricyanide, the external concentration of probe is inferred to be 2.5μM. The calculation of internal probe concentration requires that the vesicle volume be taken into account, i.e., 1.5μM divided by 0.031 yields an intravesicular probe concentration of 49μM. The resulting membrane potential is

$$= 60 \log\left(\frac{49}{2.5}\right) = 78\text{mV}$$

It is instructive to use the same data to perform a calculation which has been used in calculating membrane potentials with phosphonium ion electrode

measurements corrected for the initial binding of the phosphonium ions (5). One obtains a potential of 126mV, i.e., a 48mV overestimate. The overestimate results from neglecting the increased membrane binding of the probe during the energization process.

Structures of currently available phosphonium derivatives of nitroxide include the ester TØE3 and corresponding amides. The latter probes are resistant to hydrolysis but suffer from the disadvantage that their greater polarity renders them less permeable than ester labels. Work is in progress to synthesize other phosphonium nitroxides which may be more permeable and stable than TØE3.

COMPUTER METHODS

Increased accuracy of volume and potential determinations can be achieved with computer manipulations of the spin resonance spectra. The most useful aspect of computer analysis is correction for spectral features due to incomplete quenching of the probes. Reference spectra of partially quenched signals are obtained by performing experiments with probes and quencher either in the absence of membranes or by incorporating quenchers inside cells by sonication. Membrane bound spectra are obtained directly by subtracting a spectrum of probe and quencher in buffer from a spectrum of membranes sonicated in the presence of the probes and quenching agent. Double integration of the derived spectrum yields quantitative data on how much of the probe is membrane bound [2].

DUAL PROBE METHODS

The availability of nitroxides highly enriched in the ^{15}N isotope makes it possible to conduct simultaneous measurements of two parameters since spectra of the two nitrogen isotopes in water are clearly resolved. By having dual probe stock solutions one can avoid the difficulties associated with variability

of EPR signals among different samples. These dual probe methods have proven most useful for volume studies because volume changes asociated with lytic events occur frequently when cells or vesicles are mixed with solutions of different osmotic activities.

PRODUCTION OF SUSTAINED pH AND ELECTRICAL GRADIENTS ACROSS VESICLE MEMBRANES IN THE DARK

Prior to attempting to understand light-induced ion movements across membranes, passive as well as coupled ion movements should be characterized in the dark. In halobacterial vesicles suspended in concentrated salt solutions, substantial and stable pH gradients can be induced easily and rapidly by adding either ammonium chloride or sodium acetate solutions to the vesicles. Only the uncharged ammonia or acetic acid species are membrane permeable, hence the interior vesicle compartments will be rendered alkaline or acidic, respectively. Spin labeled acids or amines can be used to quantitate the resulting pH gradients, as well as the kinetics of their collapse, the latter providing an index of proton leakage.

For example, addition of a sodium acetate solution to vesicles equilibrated at pH 7 produces a pH gradient as freely permeable acetic acid carries protons into the vesicle interior with a concomitant alkalinization of the vesicle exterior. The magnitude of the resulting pH gradient is difficult to estimate mathematically due to the unknown concentrations of buffering substances at membrane interfaces and in the aqueous phases. However, these gradients can be determined experimentally. Thus with spin labeled amines it was observed that addition of 400 mM sodium acetate at pH 7 to envelope vesicles of S9, a wild type strain of *Halobacterium halobium* suspended in 4 M NaCl, generated a pH gradient of 0.8. The subsequent collapse of this pH gradient was slow - the time required for the gradient to diminish to half its initial maximum value was about one hour.

RAPID DETERMINATION OF MEMBRANE SURFACE AREAS

Amphiphilic nitroxides partition between aqueous and membrane domains giving rise to easily resolved narrow aqueous and broad membrane-bound spectral components, respectively. Membrane translocation of quaternary amine probes is slow (several hours for 50% uptake in envelope vesicles of halobacteria) so their bound signal can be ascribed to the membrane surface facing the extravesicular aqueous phase. To determine the aqueous and membrane fractions of probes, quantitatively, the aqueous signal is determined by direct measurement of the high field aqueous line in the presence of membranes. The membrane fraction is deduced by subtraction of this component from the measured line height of the probe in the absence of membranes. In concentrated salt solutions surface potential effects are negligible and partitioning is a direct function of the membrane concentration. This was confirmed for several envelope vesicles preparations derived from wild-type and mutant strains of *Halobacterium halobium* where serial dilutions of several sets of membranes were spin labeled with the cationic nitroxide CAT10 [6].

This is reflected in the table below which summarizes partitioning data obtained as a function of binary volume dilutions of different cell envelope vesicle preparations of several strains of halobacteria. The ratio of bound-to-free signal fractions was plotted against protein concentrations of the samples diluted serially from the initial concentrations shown in the table.

Cell Strain	L-33	L-33	L-33*	R_1mW	S9
Protein mg/ml	48	7.7	4	20	28.3
Slope	0.21	0.54	0.16	0.20	0.34
Coefficient of determination	0.999	0.996	0.998	0.988	0.997

L-33* vesicles were prepared in 3.8M choline chloride, 0.2M NaCl; all others in 4M NaCl.

An excellent linear fit to the data was obtained by least squares as reflected in the coefficients of determination in the table and all lines passed through the origin within the experimental error.

Assuming that membrane surface areas are proportional to membrane protein concentrations, the slopes in the table should be equal (except for the choline chloride solution which may solubilize CATIO differently than sodium chloride solutions). However, slopes shown in the table vary significantly, perhaps due to differing amounts of cytoplasmic proteins entrapped in the vesicle during the membrane isolation prodecures, or, less likely, due to variability in membrane composition.

QUENCHING AGENTS

Line broadening of nitroxides by transition metal ions and chelates is proportional to the concentration of the transition metal (7). The observed linewidth, w, is the sum of the intrinsic linewidth, w_o, and the linebroadening, Δw, caused by the broadening agent. The lineheight of a broadened spectrum h, is related approximately to the intrinsic line height, h_o, by the expression:

$$h = \left(\frac{w_o}{w}\right)^2 h_o$$

Therefore, at sufficiently high concentrations of a quenching agent, quenching

effectiveness is proportional to the square of its concentration. Linebroadening data for several nitroxides and two useful quenching agents are presented in Fig. 4. Intrinsic linewidths for the probes shown are: TEMPONE, w_o=0.4G; TEMPACID, w_o=1.6G; TEMPAMINE, w_o=1.8G; TØE3, w_o=1.5G.

Generally, 100 mM of quenching agent is required for efficient eradication of the extracellular concentration of nitroxides in the absence of charge effects. However, suboptimal quencher concentrations can be used in conjunction with careful mathematical analysis, of quenched and unquenched spectral components preferably with a computer, to ascertain that the quenching agent does not exhibit inhibitory effects on activity at high concentrations.

The most important feature of useful quenching agents is impermeability in the membrane system being studied. Uptake of the quenching agent into cells should cause negligible broadening effects inside the cells on the time scale of a given experiment. This constraint is most difficult to meet for volume change studies because such phenomena generally occur over long time spans. Other important requirements of a quenching agent include: that no interfering resonances be produced by the agent, that quenching occur at the lowest possible concentrations so as to minimize osmotic effects, and for work with pH sensitive probes, that the quenching agent have negligible buffering capacity in the pH range of interest. Alternatively, extracellular buffering can be made large so that observed pH changes can be ascribed to the intracellular compartment. Finally, the quenching agent should not inhibit or promote the reactions being assayed, e.g. by altering electron transport. In practice, not all of these criteria are met by a single quenching agent so a combination of agents or other control experiments must be employed for accurate work.

Many of the above criteria are met by ferricyanide and nickel chelates. These agents exhibit no ESR signals to interfere with the nitroxides. Ferricyanide is quite impermeable while a number of nickel chelates are only slightly permeable. However, most effective nickel complexes, unlike ferricyanide, suffer from the

disadvantage that they have appreciable buffering capacity at physiological pH values.

FIGURE LEGENDS

Fig. 1: Impermeable ferricyanide broadens the external spin probe signal and the remaining unbroadened spin signal intensity is directly proportional to the cell or vesicle volume.

Fig. 2: Unprotonated amines and protonated carboxylate spin probes are freely membrane permeable whereas the charge species are not; hence, as protons are pumped across membranes a redistribution of the amine or acid probes occurs and the equilibrium concentration gradient of the probes provides a direct measure of the pH gradient.

Fig. 3: The large phosphonium ion, whose charge center is surrounded by hydrophobic groups, is membrane permeable and thus responds to electrical transmembrane potentials by being accumulated within the more negative aqueous compartment.

Fig. 4: Linebroadening, Δw, of several nitroxides by paramagnetic quenching agents in 4 M NaCl. □ TEMPACID, △ TØE3, ○ TEMPONE, ◇ TEMPAMINE.

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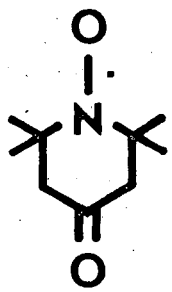

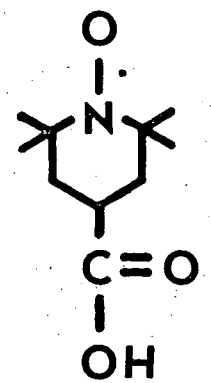

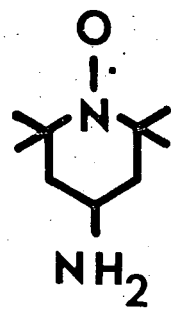

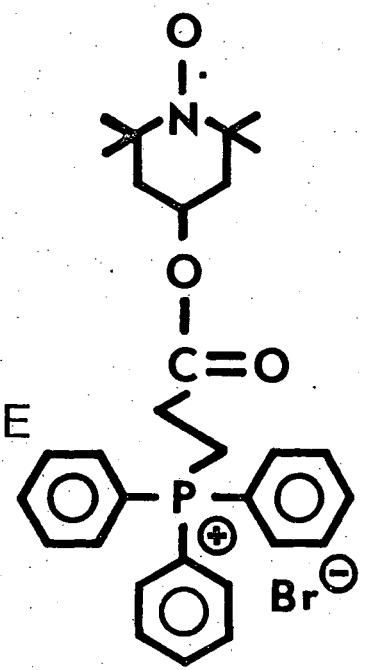

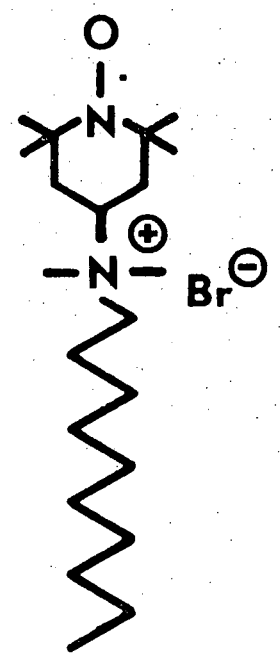

Probe Schematics (See p.2)

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Volume Measurements with Freely Permeable Nitroxide

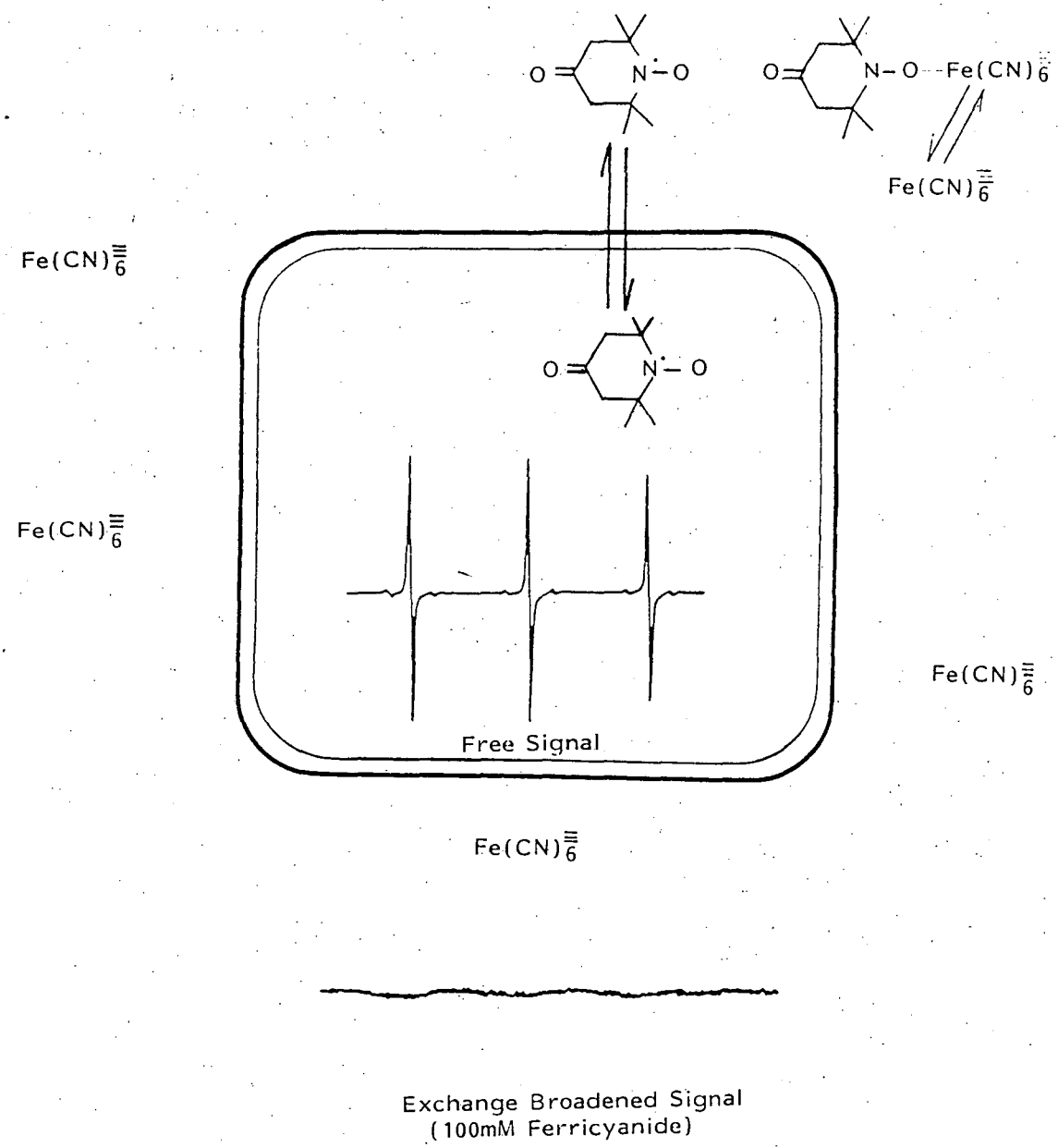

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Fig. 1

Measurement of pH Gradient

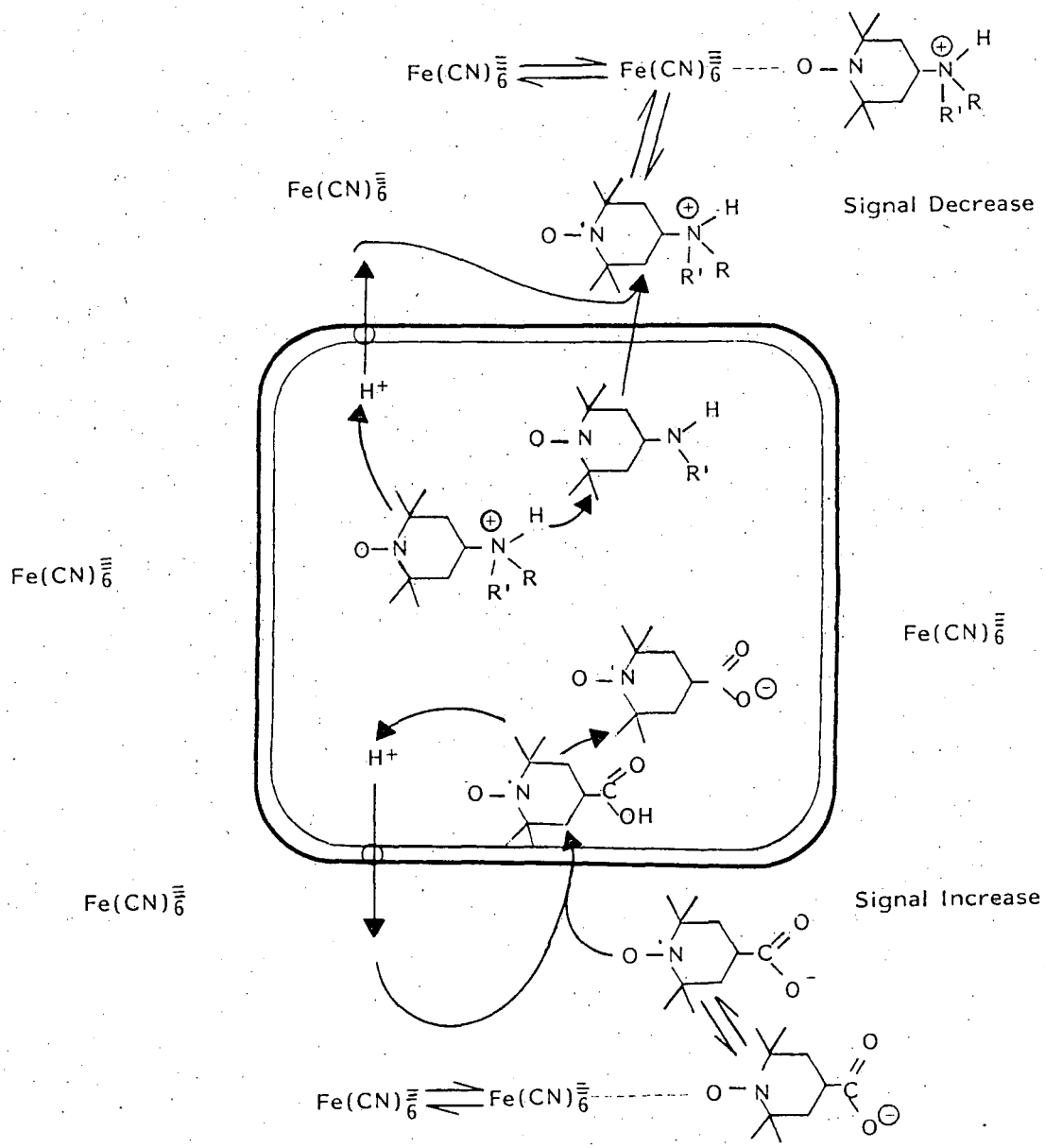

Fig. 2

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Measurement of $\Delta\psi$

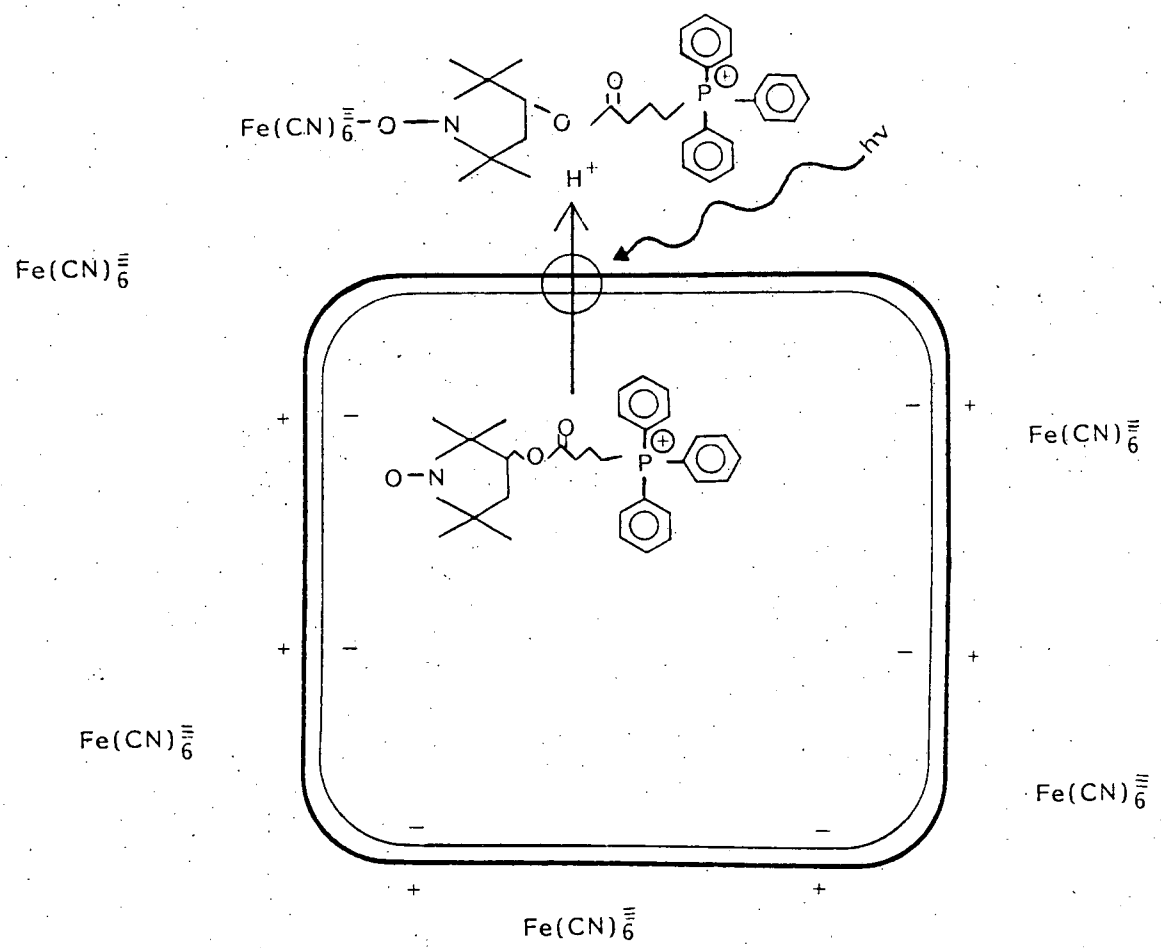

Fig. 3

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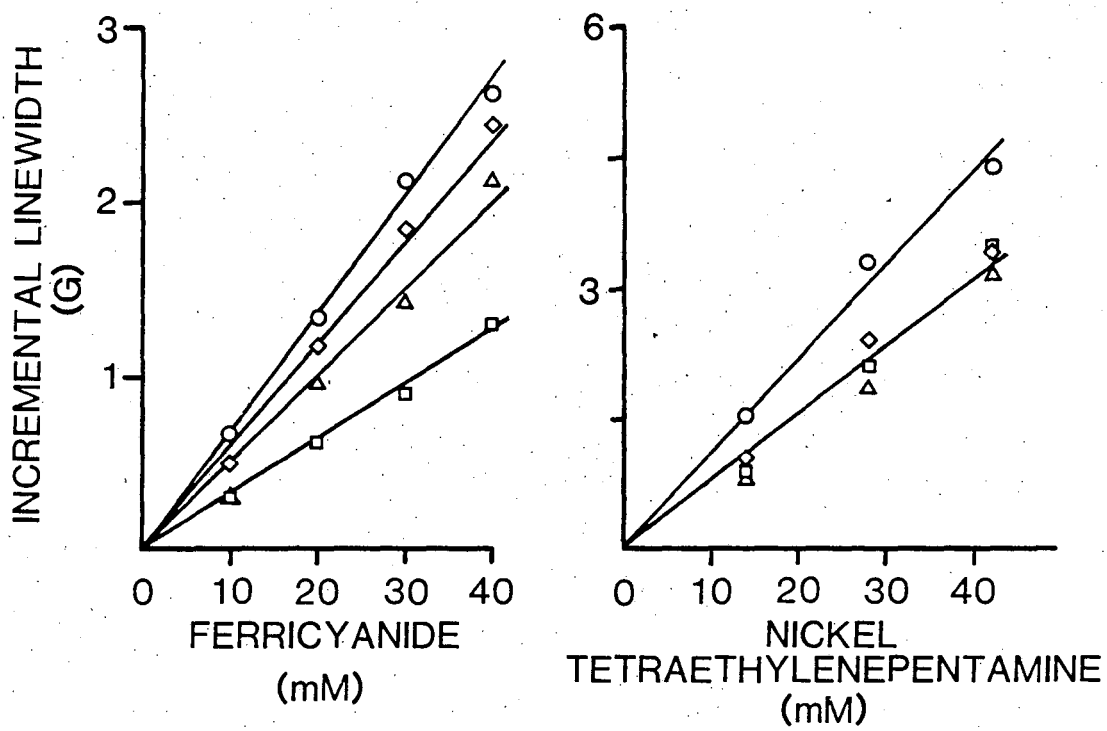

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Fig. 4

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