MEMBRANE POTENTIAL CAN BE DETERMINED IN INDIVIDUAL CELLS FROM THE NERNSTIAN DISTRIBUTION OF CATIONIC DYES

BENJAMIN EHRENBERG, VALERIE MONTANA, MEI-DE WEI, JOSEPH P. WUSKELL, AND LESLIE M. LOEW
Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06032

ABSTRACT The distribution of a selection of cationic fluorescent dyes can be used to measure the membrane potential of individual cells with a microfluorometer. The essential attributes of these dyes include membrane permeability, low membrane binding, spectral properties which are insensitive to environment, and, of course, strong fluorescence. A series of dyes were screened on HeLa cells for their ability to meet these criteria and several commercially available dyes were found to be satisfactory. In addition, two new dyes were synthesized for this work by esterification of tetramethyl rhodamine. The analysis of the measured fluorescent intensities requires correction for fluorescence collected from outside the plane of focus of the cell and for nonpotentiometric binding of the dye. The measurements and analysis were performed on three different cell types for which there exists a body of literature on membrane potential; the potentials determined in this work were always within the range of literature values. The rhodamine esters are nontoxic, highly fluorescent dyes which do not form aggregates or display binding-dependent changes in fluorescence efficiency. Thus, their reversible accumulation is quantitatively related to the contrast between intracellular and extracellular fluorescence and allows membrane potentials in individual cells to be continuously monitored.

INTRODUCTION

Over the past 15 years, the utility of fluorescent dyes as indicators of membrane potential has been firmly established (Waggoner, 1979; Loew, 1988). The most sensitive dyes, and therefore the most widely used, are in the class of "slow" dyes which undergo potential dependent redistributions across the membrane (Freedman and Laris, 1981; 1988). A hydrophobic ionic dye will have some equilibrium distribution between the external aqueous medium, the cell plasma membrane, the cytosol, and the membranes and aqueous compartments of intracellular organelles. The membrane potential plays a direct role in governing the distribution across and within the plasma membrane. The more negative the potential the greater the accumulation of positively charged dye. All these are coupled equilibria, so that the amount of dye that can associate with the organelles is also indirectly controlled. Thus, the membrane potential controls the averaged environment of the dye; since the fluorescence of a dye is typically very sensitive to its molecular environment, a change in membrane potential can be reflected in a change in fluorescence. The size of this change will depend on the sensitivity of the dye fluorescence to its environment and on the sensitivity of the dye distribution to membrane potential.

Very often dye binding to membranes causes an increase in fluorescence quantum yield and a shift in the wavelength of maximum excitation and emission. If the equilibrium constant for dye binding is such that approximately half the dye is in the membrane and half is in the aqueous medium, a small change in membrane potential can cause a large change in the observed fluorescence. This situation can be obtained by manipulating the concentrations of dye and membrane and by choosing the combination of excitation and emission wavelengths which give the largest relative change in fluorescence. There are several positive cyanine (Sims et al., 1974) and negative oxonol (Smith et al., 1976) probes which appear to have adopted this variant of the redistribution mechanism.

Even more dramatic changes in fluorescence are obtained when the dye has a tendency to form nonfluorescent aggregates at high concentration. This phenomenon is commonly observed with positively charged cyanine dyes which accumulate under the influence of negative membrane potentials (Sims et al., 1974; Waggoner, 1979). The effect is especially pronounced with the more highly conjugated cyanines at higher concentrations. Aggregates can

Benjamin Ehrenberg's permanent address is Department of Physics, Bar Ilan University, Ramat Gan 51-100, Israel.

Address correspondence to Leslie M. Loew.

form in either the plasma membrane, the cytosol, or within intracellular organelles, and, by the law of mass action, their formation drives even more monomer into association with the cell. In a lipid vesicles system, a -170 mV potential caused a 1 million–fold concentration of a cyanine dye with a resultant 98% quench of the fluorescence from the total suspension (Loew et al., 1985).

If the ion is not too hydrophobic, has little tendency to aggregate, and if there are no specific binding interactions with protein or nucleic acid molecules in the cell, the distribution will be primarily governed by the Nernst equation:

$$V = -\frac{RT}{ZF} ln \frac{C_i}{C_o}, \quad (1)$$

where V is the membrane potential, Z is the charge on the ion, R is the ideal gas law constant, T is the absolute temperature, and C_i and C_o are the concentrations inside and outside the cell, respectively, of the permeant ion. This idea is the basis of the radiolabeled tetraphenyl phosphonium (TPP) assay developed by Kaback and his coworkers for measuring membrane potential (Lichtstein et al., 1979). In this assay, cells are separated from the medium and the radioactivity from each counted; the results are corrected for passive binding of the hydrophobic ions to the cell and are adjusted for the relative internal cell volume to provide the concentration ratio in Eq. 1. This procedure has the disadvantage, compared with the fluorescence methods, of a separation step which precludes the continuous monitoring of potential changes. On the other hand, the fluorescence methods are best suited to kinetic applications rather than the measurement of absolute values of the membrane potential; the latter is possible, but generally requires a cumbersome calibration protocol (Freedman and Laris, 1981).

In this paper we report an approach using both existing and custom-synthesized fluorescent cationic dyes which are designed to distribute according to the Nernst equation. The compact structures of these dyes, mainly rhodamine analogues, are similar to that of the TPP cation in that there are no long hydrophobic groups to intercalate into the lipid bilayer and there is very little tendency for them to aggregate. Thus, these probes can be used to monitor the potential on individual cells with a microfluorometer; as opposed to the other redistribution-based mechanisms, the total fluorescence from a suspension of cells containing one of these "Nernstian dyes" is not sensitive to the potential. In fact, after correction for some unavoidable background staining, the ratio of fluorescence determined from the inside and outside of a single cell is proportional to the ratio of concentrations in Eq. 1 and can therefore be used to determine the absolute value of V. The assay also contrasts with the TPP method in that an individual cell may be monitored continuously. Preliminary results of this investigation have been reported (Ehrenberg et al., 1987; Loew et al., 1986).

MATERIALS AND METHODS

HeLa Cells

The cells were grown on 22 × 30 mm microscope coverslips in Dulbecco's modified Eagle's minimal essential medium (Gibco, Grand Island, NY). They were washed three times with Earle's Balanced Salt Solution (Gibco) (EBSS) and were then incubated for 10 min in 2 ml of one of the following solutions containing the dye under investigation: EBSS only, for measuring the resting potential; EBSS, which contained valinomycin to impose a K^+ diffusion potential on the cells; potassium-rich, modified EBSS buffer (no NaCl, 170 mM KCl, and $NaHCO_3$ at 13 mM rather than 26 mM concentration), also including valinomycin, to collapse the membrane potential. The bottoms of the coverslips were wiped dry and they were then inverted onto microscope slides. The edges of the coverslips were sealed with paraffin or silicone grease and the slide transferred to the stage of the microscope fluorometer.

Macrophage Cells

Mouse macrophage cells of the J774.2 line were grown on microscope coverslips in Dulbecco's modified Eagle's minimal essential medium, as described previously (Walter et al., 1980). The cells were washed with buffer (118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 9 mM Na_2CO_3, 5.5 mM glucose, 20 mM Hepes, pH 7.4) containing the cationic dye, and then incubated at room temperature for 10 min in the same buffer, for a measurement of the resting potential. Incubation in the same buffer which also contained 0.5 μM valinomycin, allowed measurement of a K^+-diffusion potential; incubation in a high-K^+ buffer (same as the low-K^+ buffer, in which 118 mM KCl replaced the NaCl) containing 0.5 μM valinomycin, produced depolarized cells. The coverslips were inverted onto microscope slides for microfluorimetric measurements, as for the HeLa cells.

In some cases the cells were grown in suspension by gently stirring and shaking the petri dish. They were then pelleted, washed a few times, and treated as above with the three different buffers. Finally the cells were placed on a microscope slide which had been coated with a water repellent silicon film (Sigmacote, Sigma Chemical Co., St. Louis, MO). The slide was then covered with a coverslip.

Neutrophils

Rabbit neutrophils were obtained from Dr. R. I. Sha'afi (White et al., 1984). The cells were pelleted and washed three times and then incubated in one of the three different buffers as for the macrophage cells. Microfluorometric measurements were carried out within 2 h after separation.

Microfluorimetric Measurements

A Zeiss Universal fluorescence microscope, equipped with a thermoelectrically cooled photomultiplier (RCA type PF-1011) and photon counting electronics, was used. The cells were incubated for 10 min with the appropriate buffer containing the dye to permit complete equilibration (some cell types displayed much faster equilibration times). Complete equilibration was taken as the time at which fluorescence measured from within the cell had leveled to a steady intensity. A pinhole with a projected diameter of 1 μm was placed in the image plane, allowing light collection from object areas smaller than the cells which were used in this work. Some staining by the dyes of the cytoplasmic membranes and of intracellular organelles was observed, and care was taken not to measure the fluorescence emanating from these cell areas. These measurements from inside the cells were obtained in pairs with measurements through the same pinhole from a field of view immediately adjacent to the respective cells. Approximately 20 such pairs were measured for each coverslip. Data for a given set of conditions was obtained from at least four coverslips.

To obtain the intensity of the fluorescence emanating from inside the

cells, a correction was needed for the optical depth-of-field. For this correction, all cells were bathed in media containing 1 mg/ml fluorescein isothiocyanate (FITC)-labeled dextran, 40,500 mol wt (Sigma Chemical Co.), which had been purified by passage through a Sephadex (G-50) column. The correction, based on the FITC fluorescence measured alternately from outside and inside the cells, was determined from one coverslip for each cell type, and will be discussed in the Results section.

ATP Assay

HeLa cells were grown to 90% confluency on 100-mm tissue culture dishes, washed with PBS, and suspended in fresh medium by gently detaching the cells with a stream applied from a pipet. Aliquots of 10^7 cells in 1 ml of medium were placed in 15-ml centrifuge tubes and incubated at 37°C with varying concentrations of ethyl ester of tetramethylrhodamine percholate (TMRE). After a given incubation period, the cells were pelleted, and the medium removed. The pellet was treated with 1 ml of trichloroacetic acid at 0°C for 5–10 min. The pH was then adjusted to 7.8 with ~50 mg Tris. The supernatant was removed after centrifugation, placed in a 0.5-mm path length quartz cuvette, and 200 μl of the luciferin/luciferase assay mixture (Sigma Chemical Co.) was added. The luminescence is determined with a fluorescence spectrophotometer (Perkin Elmer Model MPF 44B) and calibrated against ATP standards (Sigma Chemical Co.).

Total Fluorescence from a Cell Suspension

HeLa cells were harvested as above and aliquots added to spectrofluorometer cuvettes containing dye in EBSS. The suspension in the cuvette was stirred gently in the spectrofluorometer as fluorescence was excited at 546 nm and emission monitored at 610 nm.

Chemicals

Rhodamine-3B, rhodamine-6G, rhodamine-123, safranin 0 (Eastman Kodak Co. "Laser Grade"), *N*-methyl acridinium iodide (Molecular Probes Inc., Junction City, OR), *bis*(*N*-methyl acridinium) nitrate (Aldrich Chemical Co., Milwaukee, WI), amethyst violet (Chemetrics, Calverton, VA), di-*I*-C_1(3),di-0-C_2(5), and di-0-C_1(3) (Molecular Probes Inc.) were used without further purification and added from fresh ethanol stock solutions.

Methyl Ester of Tetramethyl Rhodamine Perchlorate (TMRM)

A mixture of 50 mg (0.102 mmol) tetramethyl rhodamine perchlorate (Eastman Kodak Laser Grade), 5.0 g (47.1 mmol) trimethylorthoformate, 10 ml methanol, and a trace of *p*-toluene sulfonic acid was heated at reflux with magnetic stirring in an oil bath (95–105°C) for a period of 45 d. Progress of the esterification was followed by thin layer chromatography (silica gel, $CHCl_3$/MeOH, 4:1). The reaction mixture was concentrated to dryness under reduced pressure to leave 40 mg of violet solids which were flash chromatographed on silica gel ($CHCl_3/CH_3OH$, 9:1). A second flash chromatography ($CHCl_3/C_2H_5OH$, 9:1) of the main fraction afforded 30 mg of dark green crystals, m.p. 274–276°C, decomposition. The analysis on silica gel ($CHCl_3/CH_3OH$, 4:1) showed one pink spot with orange fluorescence, R_f 0.275. ^{1}H NMR (DMSO-d_6, 200 MHz) 3.26 (s, 12H), 3.55 (s, 3H), 7.06 (m, 6H), 7.52 (d, 7H), 7.88 (m, 2H), 8.27 (d, 1H).

Ethyl Ester of Tetramethylrhodamine Perchlorate (TMRE)

A mixture of 50 mg (0.102 mmol) tetramethylrhodamine perchlorate (Eastman Kodak Co.), 7.41 g (50 mmol) triethylorthoformate, 9 ml absolute ethanol, 5 ml dimethyl formamide, and 3 mg *p*-toluene sulfonic acid was heated at reflux with magnetic stirring in an oil bath (95–105°C) for a period of 4 wk. The reaction mixture was concentrated to dryness under reduced pressure (water aspirator, then high-vacuum pump) to leave a dark violet crystalline residue which was flash chromatographed on silica gel ($CHCl_3$/ethanol, 9:1) to give 48 mg of dark green crystals, m.p. 264–266°C with decomposition and previous scintering at 240°C. TLC analysis on silica gel ($CHCl_3/CH_3OH$, 4:1) showed one pink spot with R_f 0.375. ^{1}H NMR (DMSO-d_6, 200 MHz), 0.84 (t, 3H), 3.26 (s, 12H), 3.94 (q, 2H), 7.03 (m, 6H), 7.49 (d, 7H), 7.86 (m, 2H), 8.25 (d, 7H).

RESULTS

Treatment of the Data

An ideal ionic dye for use as a Nernstian fluorescent indicator of membrane potential would have to meet several criteria. It should permeate through the cell's membrane, yet it should have a low membrane partition coefficient, i.e., low membrane staining. It also should not stain intracellular organelles or macromolecules as a result of specific or nonspecific binding. The ratio of the fluorescence intensities measured from inside and outside the cell should be related to the membrane potential through the Nernst equation. This requires that there be no self quenching or other spectral perturbations of the dye taken up by a cell, so that the ratio of fluorescence intensities can be equated with the ratio of concentrations. Other more general concerns for any vital fluorescent stains include high photostability and low toxicity.

We employed HeLa cells to screen a number of highly fluorescent cationic dyes for their ability to meet these criteria. The 170 mM K^+ concentration in the high-K^+ modified EBSS buffer, described in Materials and Methods, is very close to the reported values of the HeLa intracellular K^+ concentration (Boardman et al., 1974; Pollack et al., 1981). Thus, with valinomycin present in the solution, the cells should be depolarized, and the fluorescence of the dye should be homogeneous (i.e., the fluorescence field of view should be of uniform brightness, rendering cells invisible). All the dyes we checked, however, exhibited some membrane and intracellular binding. Therefore, a correction for the non-Nernstian binding was required.

Under conditions of $V = 0$ (i.e., high external K^+ with valinomycin) the intensities inside (F_i°) and outside (F_o°) the cell were determined. Their difference, $F_i^\circ - F_o^\circ$, reflects the inside bound dye, and an effective constant for this binding K_b, may be obtained.

$$K_b = [\text{dye}]_{\text{bound}}/[\text{dye}]_{\text{free}} = (F_i^\circ - F_o^\circ)/F_o^\circ. \quad (2)$$

Assuming that this binding constant is not significantly sensitive to potential, K_b can then be used to evaluate the Nernstian component of the fluorescence within the cell (i.e., that due to unbound dye) at $V \neq 0$. Thus,

$$F_i = F_i(\text{free}) + F_i(\text{bound}) = F_i(\text{free}) * (1 + K_b) \quad (3)$$

Given that dye fluorescence is proportional to dye

concentration, it follows from Eq. 1 that

$$V = -\frac{RT}{ZF} ln \frac{F_i(\text{free})}{F_o} = -\frac{RT}{ZF} ln \frac{F_i}{F_o(1 + K_b)}. \quad (4)$$

The fluorescence intensities, which are determined experimentally by overlaying the microscope's measuring aperture on the nuclear region and focusing on the middle of the cell, have contributions from outside the cell volume. When a focused laser beam is used for excitation, it was shown by Koppel et al. (1976) that the fluorescence collection efficiency has a Lorentzian dependence on the vertical displacement of the image plane pinhole from the in-focus object plane. Using their equations, the full-width-at-half-maximum would be 3 μm with our microscope optics. This calculation assumes laser excitation so that the thickness of the region from which light is collected would be somewhat greater with the less focused illumination which is employed here. Therefore, the integrated intensity of fluorescence that is collected from the dye present in the solution above or below the cells may not be negligible as compared with the fluorescence from inside the cell. A correction for this phenomenon is therefore necessary. FITC-dextran is not membrane permeable and is actively incorporated into cells very slowly at ambient temperatures (Steinman et al., 1976; Melmed et al., 1981). Therefore, it can be used as a marker for the external aqueous space to derive a correction factor for each cell type. This factor R_{DEX}, is the ratio of the FITC-dextran fluorescence intensity measured after focusing on the cell interior, to the intensity measured after moving the stage away from the cell without changing focus.

The observed deviation of R_{DEX} from zero reflects the efficiency of light collection from extracellular FITC-dextran when the cell's image is brought to optical focus. We obtained the following average R_{DEX} values with a 100× NA 1.30 objective: 0.61 for HeLa cells, 0.63 for the J774 macrophages grown on coverslips, 0.87 for the macrophages grown in suspension, and 0.93 for the neutrophil cells. The fluorescence intensity of the Nernstian probes emanating from inside the cells, as well as the outside fluorescence that is collected from an equal sized volume, can now be deduced from the corresponding raw intensities, I.

$$F_o = (I_o - I_s)(1 - R_{DEX}) \quad (5)$$

$$F_i = (I_i - I_s) - (I_o - I_s)(R_{DEX}). \quad (6)$$

I_s is the intensity of light that is detected in the absence of any dye in the solution, and includes the photomultiplier dark counts. I_s was at least a factor of 10, and usually 100 times lower than any of the measured intensities with the probe present, and was the same whether or not a cell was in the field of view.

Combining Eqs. 2, 3, 5, and 6, we can now express the argument of the logarithm in Eq. 4 in terms of the experimentally determined intensities.

$$\frac{F_i}{F_o(1 + K_b)} = \frac{(I_i - I_s) - (I_o - I_s)(R_{DEX})}{(I_o - I_s)(1 - R_{DEX})(1 + K_b)}. \quad (7)$$

It should be noted that the depth of field corrections need to be applied during determination of K_b, as well (i.e., in Eq. 2). If it is possible to assume that the intracellular volume is much less than the volume of the external bathing medium, then varying accumulations of dye within the cells will have little effect on the concentration of dye outside. Thus, $F_o \simeq F_o^o$, and Eq. 7 can be rewritten:

$$\frac{F_i}{F_o(1 + K_b)} = \frac{F_i}{F_o(F_i^o/F_o^o)} \simeq \frac{(I_i - I_s) - (I_o - I_s) R_{DEX}}{(I_i^o - I_s) - (I_o - I_s) R_{DEX}}. \quad (8)$$

In the following sections, we describe the results we obtained with a few cell lines, to which we applied different dye indicators. The purpose of these measurements was to demonstrate the feasibility of performing a microfluorimetric assay of the membrane potential of single cells, and to define the necessary experimental conditions. In practice we use Eq. 7 instead of Eq. 8 to calculate membrane potentials from the measured fluorescence intensities. This is because Eq. 7 references the internal fluorescence to the external fluorescence on the same slide, thus effectively controlling for many variables inherent in the comparison of fluorescence from different slides. Consideration of Eq. 8 will be useful in our discussion of some of the errors which may affect the accuracy of the method.

HeLa Cells

Previously reported measurements of the membrane potential of HeLa cells demonstrate the problems arising from the use of microelectrodes. The average values which were obtained in different laboratories were in the range −15 to −99 mV (Borle and Loveday, 1968; Okada et al., 1973; Aiton and Pitman, 1975; Husler and Lauterwasser, 1982; Roy and Sauve, 1983; Lampidis et al., 1985). Undoubtedly, this variability may be partially due to different HeLa strains that have developed over the years. Some well-known problems of the use of microelectrodes were observed in these measurements, however. These include the instability of the measured potential after penetration with the electrode, probably due to poor resealing and induced hyperpolarization.

Because molecular probes do not cause any such mechanical perturbation, they may be able to report the membrane potential reliably, albeit less directly. The following dyes were tested with HeLa cells: rhodamine-3B, -6G, and -123, safranin 0, *N*-methyl acridinium, *bis*(*N*-methylacridinium, amethyst violet, a variety of cyanine dyes, and the methyl and ethyl esters of tetramethyl rhodamine (TMRM and TMRE, respectively). These dyes all have delocalized positive charges but their hydropho-

bicities vary widely. Some of the dyes stained the cells strongly and irreversibly whereas others required long incubations to attain an equilibrium distribution. The molecular structures of the fluorescent cations which had the best properties are shown in Fig. 1. A summary of the results obtained with these dyes on HeLa cells is given in Table I.

All the dyes had significant nonpotential dependent binding to the cells, as the K_b values indicate. The fluorescence micrograph with TMRM, Fig. 2, shows that the dye stains the cytosol as well as intracellular organelles. The nuclear area appears to be stained to a lesser extent, reflecting the absence of membranous organelles. Therefore, to minimize the correction for non-Nernstian dye accumulation, the measurement of fluorescence intensity inside the cells was made at these relatively darker uniformly stained areas.

As is evident from Table I, rhodamine-6G has the highest binding constant of the five dyes, but reducing the number or size of the alkyl substituents, as in TMRM and TMRE, lowered the extent of binding by more than an order of magnitude. The higher hydrophobicity of the ethyl ester (TMRE) is reflected in its slightly higher binding constant, compared with the methyl ester molecule, TMRM. The resting potential is in the range -52 to -77 mV as reported by the different dyes. The K^+-diffusion potential induced by 0.5 μM valinomycin is in the range of

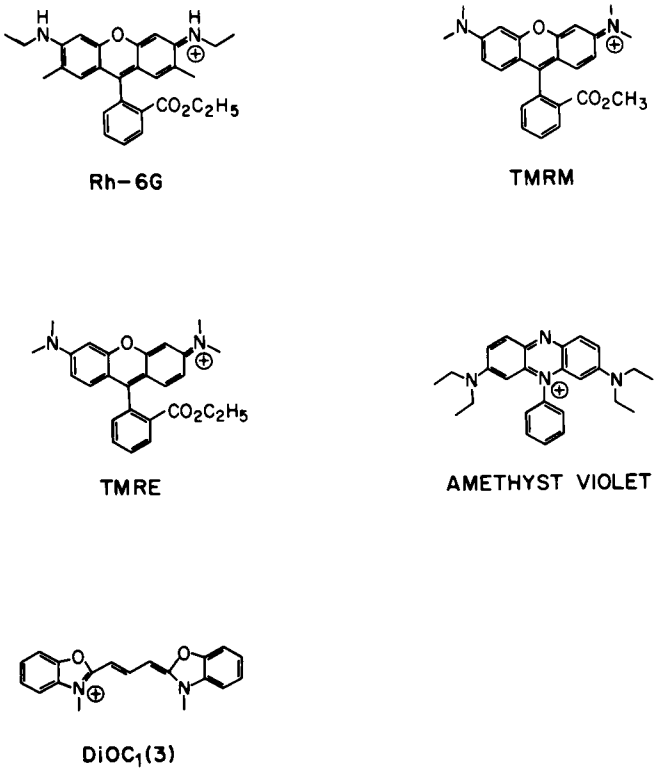

FIGURE 1 Structures of the cationic dyes with the best Nernstian behavior.

TABLE I
MEMBRANE POTENTIAL OF HeLa CELLS MEASURED BY NERNSTIAN DYE DISTRIBUTION

Dye	K_b	V^*	$V_K^\ddagger$
		mV	*mV*
Rhodamine 6G	67.7 ± 22.5	−57.5 ± 19.0	−49.2 ± 8.0
TMRM	4.8 ± 1.3	−77.3 ± 13.9	−60.9 ± 9.7
TMRE	6.4 ± 1.8	−75.9 ± 17.1	−57.4 ± 9.6
Amethyst violet	16.1 ± 7.6	−51.9 ± 14.8	−40.2 ± 14.2
Di-O-C_1(3)	58.9 ± 23.4	−60.6 ± 16.9	−59.4 ± 15.1

*Resting potential. These data are averages from ~20 cells on each of at least four cover slips. ‡Membrane potential determined in the presence of 0.5 μM valinomycin.

-50 to -60 mV for all the dyes, except for amethyst violet. We found that the results are practically independent (to <15%) of the valinomycin concentration in the range of 0.05 to 5 μM, indicating a saturation of the K^+ diffusion capacity.

We also employed the HeLa cells to see if dye accumulation led to any intrinsic change in fluorescence efficiency. This would preclude use of the corrected fluorescence intensities as a direct measure of dye concentration. To test for this, we simply measured the total fluorescence from a cell suspension in a spectrofluorometer. In Fig. 3, the level of TMRE association with the cells is modulated by successive additions of cells and subsequent introduction of valinomycin. It is apparent that dye association with HeLa cells has a very minor effect on its total fluorescence. Similar results were obtained with the other dyes in Fig. 1 except for the cyanine dye, di-O-C_1(3), which did display some fluorescence enhancement upon binding to the cells.

J774 Macrophage Cells

Numerous measurements of the transmembrane potential of mouse macrophage cells, performed by both microelectrodes and molecular probes, have been reported in the literature. Values in the range -25 to -90 mV were observed for macrophage cells of varying origins (Dos Reis and Oliveira-Castro, 1977; Castranova et al., 1979; Kouri et al., 1980). Generally, values obtained by microelectrode impalements and by uptake of TPMP, did not coincide. J774 cells, the macrophage-derived tumor line which we employed in this work, exhibited a resting membrane potential of -14 or -35 mV, when assessed by the uptake of the lipophilic cations tetraphenyl phosphonium (TPP) (Young et al., 1982), and triphenyl methyl phosphonium (TPMP) (McCaig and Berlin, 1983), respectively. By impaling with microelectrodes, a resting potential of -25 mV was observed (McCaig and Berlin, 1983). It was demonstrated, however, that plating the cells from suspension onto a substrate caused an increase of the membrane potential from -15 to -70 mV over a period of 6–8 h (Sung et al., 1985). We therefore decided to test our

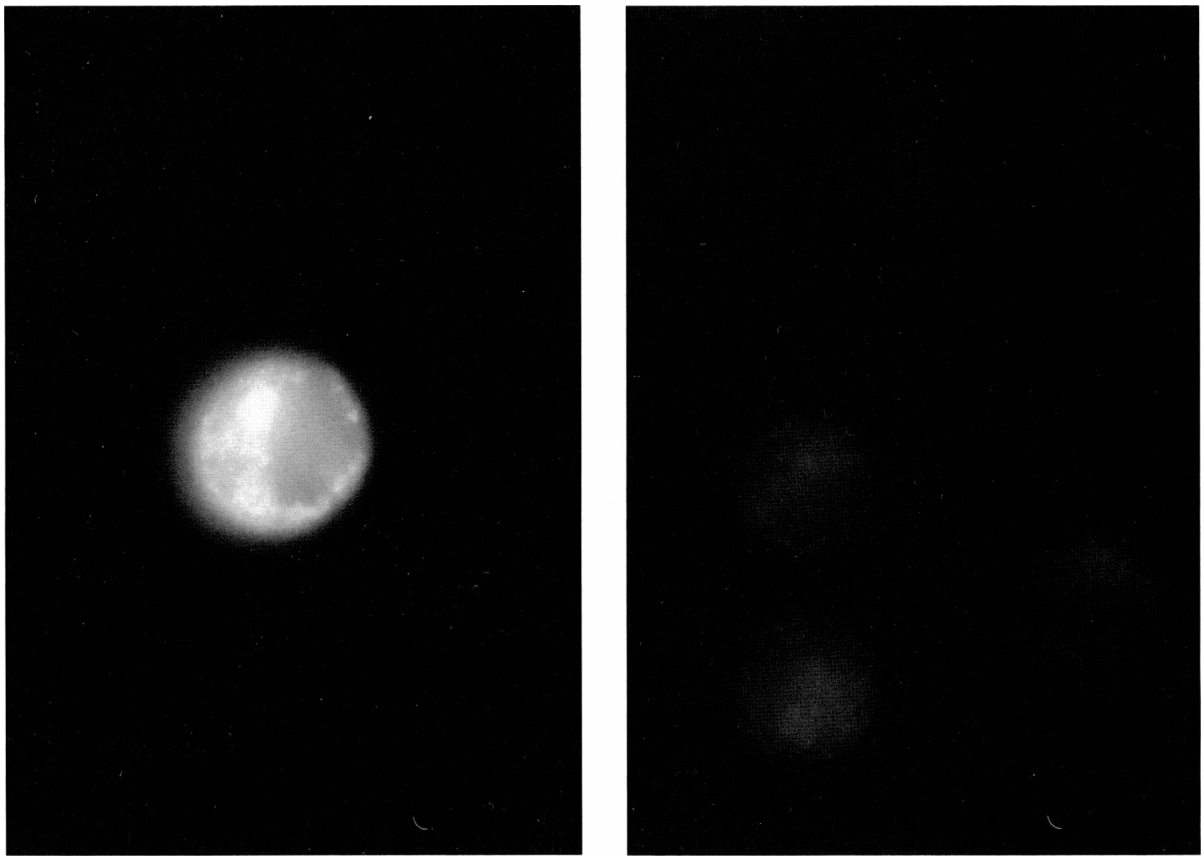

FIGURE 2 Fluorescence of TMRM in HeLa cells. The cell on the left is bathed in normal EBSS containing 0.5 μM dye. The three cells on the right were depolarized by adding 0.5 μM valinomycin to a high K^+ bathing medium containing the same amount of dyes. Both images were obtained with a Zeiss 100× n.a. 1.3 neofluor objective and a 2-s exposure of ASA 400 film. Fluorescence was excited through a 546-nm interference filter and emission collected above a 610-nm highpass filter.

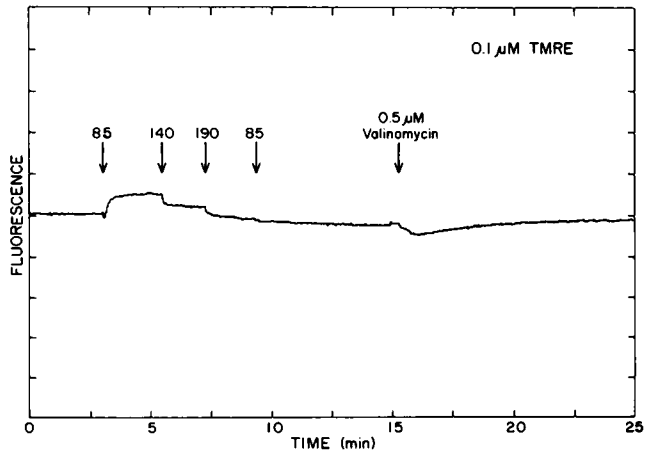

FIGURE 3 Total fluorescence of TMRE in EBSS. Successive additions of HeLa cells are indicated by arrows, with numbers representing the size of each aliquot in units of 1,000 cells/ml. Fluorescence from the continuously stirred suspension was determined in a spectrofluorometer as described in Methods.

Nernstian dyes on the J774 macrophages, both in suspension and plated on coverslips.

The level of staining at zero membrane potential with all the dyes was lower than that of the HeLa cells. The results obtained with the cells which were grown either on glass coverslips or in suspension, are shown in Table II. With cells on glass, the resting potential as reported by the various rhodamine dyes and by amethyst violet is between −60 and −72 mV, and the K^+ diffusion potential is −43 to −70 mV. Cells that were grown in suspension and

TABLE II
MEMBRANE POTENTIAL OF J774 CELLS

Dye	K_b	V	V_K
		mV	*mV*
Rhodamine 6G	34.5 ± 11.8	−72.5 ± 9.7	−66.7 ± 11.3
TMRM	3.1 ± 0.7	−69.1 ± 8.8	−51.2 ± 6.4
TMRE	4.4 ± 1.0	−60.2 ± 9.5	−54.6 ± 7.1
Amethyst violet	11.1 ± 8.9	−66.2 ± 21.3	−53.7 ± 20.6
Di-O-C_1(3)	71.3 ± 26.0	−50.1 ± 15.8	−43.7 ± 12.9
Rhodamine 6G*	7.6 ± 7.0	−25.4 ± 22.9	−63.8 ± 22.7

*Cells grown in suspension and examined immediately after deposition on a silanated glass slide.

deposited on silicon-coated microscope slides, had the same K^+-diffusion potential, but their resting potential, as measured with rhodamine-6G, was only −26 mV.

Neutrophil Cells

The plasma membrane potential of neutrophil cells from different sources has been determined previously by a variety of methods (Simchowitz et al., 1982). Because of the small size of these cells, molecular probes have been the primary tools in these experiments. Values from −45 to −86 mV (Seligmann and Gallin, 1980; Seligmann et al., 1980; Tatham et al., 1980; Bashford and Pasternak, 1985) have been reported. The potential of the rabbit neutrophils was estimated to be in the range of −66 to −86 mV (Tatham et al., 1980).

The resting and K^+-diffusion potentials of the rabbit neutrophil cells, as obtained with rhodamine-6G, TMRM, and TMRE are shown in Table III. Again, the resting potentials we obtained are within the range of the literature values. Amethyst violet and di-O-C_1(3) could not be used because they did not equilibrate into the cells within a reasonable period of time.

Cell Viability in the Presence of the Dyes

Possible toxic effects of the dyes used in this work on the various cell lines were tested by the exclusion of trypan blue (Paul, 1970). After washing the cells with the appropriate low-K^+ buffers, which also contained 0.4% trypan blue and the dye, we counted stained cells on a hemocytometer. We found that with all the combinations of cells and dyes used in this work, the fraction of viable cells (i.e., those not stained by trypan blue) was within 3% of the value obtained for the same cells without the Nernstian dye present. All the viability values were 85% and higher. We can thus conclude that the dyes are not significantly toxic to the cells.

In addition, we probed for any effects of TMRE on the metabolic activity in HeLa cells by measuring ATP levels with the luciferin–luciferase assay (Leach and Webster, 1986). Cells were incubated with varying concentrations of dye for either 75 or 200 min (in membrane potential determinations, the total exposure time of the cells to the dyes seldom exceeded 60 min). After 75 min, there was no significant reduction in ATP content (3.6 ± 0.2 pg/cell) compared with control for any dye concentration in the range tested (0.1–1.0 μM). After 200 min, ATP levels were reduced by 3, 10, and 13% for 0.1, 0.5, and 1.0 μM TMRE, respectively.

It should be stressed that these dyes may influence some cell-specific physiological function (e.g., secretion, phagocytosis, etc.) and appropriate controls should be devised in all applications.

TABLE III
MEMBRANE POTENTIAL OF NEUTROPHILS

Dye	K_b	V	V_K
		mV	*mV*
Rhodamine 6G	76.8 ± 20.0	−49.7 ± 11.6	−44.5 ± 13.7
TMRM	4.0 ± 2.9	−60.1 ± 20.3	—
TMRE	4.8 ± 2.3	−74.1 ± 21.6	−28.1 ± 17.5

DISCUSSION

We have demonstrated in this work that certain positively charged dye molecules are taken up by cells in a potential dependent fashion. We have shown that this accumulation exists in addition to a nonpotential dependent staining of the cells which can vary significantly from dye to dye. By correcting for this binding and for fluorescence emission collected from outside the cells, we can use the measured intensities to determine the membrane potential. The very wide range of membrane potential values that are reported for the same cell type by different laboratories makes it difficult to assess the accuracy of the technique. All of the potentials measured by us for the three cell types do, however, fall within the range of values reported for these cells in the literature. In this respect, the thorough investigation of the J774 mouse macrophage cells by Kaback and co-workers (Young et al., 1983; Sung et al., 1985), using the uptake of TPP^+, might be used as a point of comparison. Their reported values for the membrane potentials of cells in suspension and on a solid surface were −15 and −70 mV, respectively. With the same cells on coverslips, we obtained membrane potentials in the range of −51 to −70 mV. We obtained −26 mV with cells which were grown in suspension and were layered on a microscope slide which had been coated with a silanizing agent. Thus, our method is able to confirm the adhesion dependence of the membrane potential on these cells. Interestingly, we find that the K^+ diffusion potential generated in the presence of valinomycin is not sensitive to cell–substrate interactions. This suggests that either K^+ channels open or other ion channels close when the cells bind to the glass.

The standard deviations associated with the membrane potential measurements in Tables I, II, and III are quite large. This is not necessarily a reflection of poor precision in the measurements, however. It is likely that there is much more variation in physiological parameters such as membrane potential than is commonly appreciated from measurements of large populations of cells. Clearly, the degree of substrate adhesion is one parameter which strongly influences membrane potential. It is also clear that the membrane potential can fluctuate with the metabolic activity of the cell which, in turn, is likely to be a sensitive function of factors such as the position of the cell in the cell cycle, the degree of differentiation, cell viability, etc.

In a preliminary report (Loew et al., 1986; Ehrenberg et al., 1987), we employed diffusion potentials on cell-sized liposomes to test the functional relationship of dye accumulation on membrane potential. This system is not com-

plicated with ion channels and pumps which preclude the a priori prediction of the potential. Indeed, the agreement was excellent, especially in view of the lability of the diffusion potential in this system.

Ideally, the simplest microfluorimetric measurement of membrane potential with a Nernstian dye would consist of the measurement of the fluorescence intensity of the dye inside and outside the cell, and the potential would be directly derived from Eq. 1. However, we have shown that two corrections are necessary, and as a result, additional measurements are needed to obtain the accurate value.

First, a microfluorimetric measurement of a membrane-impermeable dye such as FITC-labeled dextran is needed to evaluate the contribution of fluorescence originating from outside the cell to the measured intensity apparently collected from inside the cell. Second, the nonpotential dependent uptake of dye has to be separated from the Nernstian accumulation. This latter problem is also relevant to the existing redistributing lipophilic probes, such as TPP or TPMP (Altenbach and Seelig, 1985; Gibrat et al., 1985; Demura et al., 1985). We therefore performed our measurements under conditions where the membrane potential is set to zero, in which case the contrast in the fluorescence between the inside and outside of a cell defines the non-Nernstian binding.

The significance of these two corrections can be appreciated by considering Eq. 8. If the dye has a high binding affinity, even at zero membrane potential, i.e., $K_b \gg 1$, then $I_i \gg I_o > (I_o)(R_{DEX})$ for both the case of zero potential as well as for any inside-negative potential, and thus all the terms containing R_{DEX} in Eq. 8 can be ignored. Therefore, for a dye with a high binding affinity such as rhodamine 6G, the depth of field correction is not as important as the correction for this binding. On the other hand, when K_b approaches 1, I_i^o approaches I_o, and R_{DEX} assumes primary importance. The fact that the sensitivity of the measurements to these corrections depends in opposite ways on the hydrophobicity of the probes is significant for several reasons. It is worthy of note, particularly, that the membrane potentials measured with different dyes are essentially identical for a given cell type; this suggests that both the binding and depth of field corrections are being correctly applied. It also suggests that our assumption that K_b is not potential dependent is probably valid. The choice of dye can be guided by the need to minimize these corrections. For example, when experimental constraints force the use of low numerical aperture objectives on relatively thin cells so that R_{DEX} becomes intolerably close to 1, the choice of a high-K_b dye, such as rhodamine 6G, would be appropriate.

The problem of how to account for lipophilic cation accumulation within mitochondria has plagued membrane potential measurements with TPP, TPMP, and the cyanine dyes. A study by Korchak et al. (1982), for example, pointed out the possibility that the dye di-O-C_6(3), when applied to neutrophil cells, might act as a mitochondrial, and not a plasma membrane, potential probe. Rhodamines have, in fact, been successfully employed as specific mitochondrial stains that can provide a qualitative indication of mitochondrial membrane voltage (Johnson et al., 1981). We were therefore very careful to measure the intracellular fluorescence at points which were free of stained and fluorescing organelles. The ability to visualize the dye distribution is, therefore, a major advantage of this method. Also, the rhodamine probes have fluorescence quantum yields which are insensitive to membrane binding (Fig. 3), making the contribution to total fluorescence from dye bound to mitochondrial or other organelle membranes much less than with the cyanine dyes.

Microelectrode and patch techniques are available to measure the electrical properties of cell membranes. They are capable of defining the properties of individual channels and pumps in much greater detail than the method described in this paper. The Nernstian dyes should complement direct electrical methods in several important ways, however: (*a*) Many cells can be processed quickly. Typically, 20 cells are examined on a single slide in 45 min. This avoids any bias associated with choosing cells that are easily impaled (i.e., large cells) which may be built into the electrical measurements. (*b*) Not all cell types are amenable to patch or microelectrode methods for technical reasons; the cells may be too small or their membranes too resistant for efficient impalement or whole cell patching. (*c*) Heterogeneity in the response of a population of cells to a stimulus can be followed by monitoring fluorescence from a group of cells simultaneously using a multisite detector (e.g., photodiode array or low–light level video). Simultaneous electrical measurements on several cells are physically much more cumbersome and impractical. (*d*) The Nernstian dyes offer the opportunity to monitor, albeit qualitatively, the membrane potential of mitochondria in situ. (*e*) Uncertainties associated with membrane potential measurements with electrodes include the possibility of mechanical injury to the membrane and, for whole cell clamps, the need to match the ionic composition of the electrolyte in the pipet with the cytosol. The uncertainties associated with dye accumulation are quite different, so that the two types of measurements can serve to reinforce each other.

Dyes with a low K_b, such as TMRM and TMRE, would be expected to display readily reversible potential dependent uptake, which might be especially useful in kinetic studies. The probes should be especially suitable for applications involving fluorescence-activated cell sorting. These dyes could also be combined with the emerging digital video microfluorescence technology to permit experiments in which the membrane potential of individual cells may be correlated with a variety of inter- and intracellular events. The ability to distinguish cytoplasmic from mitochondrial fluorescence should be particularly useful in this connection. It is the intention of this work, by demonstrating that the dye accumulation can be quantitatively interpreted, to

provide assurance that the method is reliable in more qualitative applications where a monitor of membrane potential in individual cells is desirable.

We are pleased to acknowledge several valuable discussions with Richard D. Berlin, Carlos Gitler, and Rose Johnstone. We are also grateful to Daniel Farkas for help with the ATP assay and to Joan Jannace for typing the manuscript.

This work was supported by the United States Public Health Service through National Institutes of Health grants GM35063 and AI22106.

Received for publication 14 August 1987 and in final form 8 December 1987.

REFERENCES

Aiton, J. F., and R. M. Pitman. 1975. Measurement of membrane potential in HeLa cells. *J. Physiol. (Lond.).* 251:9P–10P.

Altenbach, C., and J. Seelig. 1985. Binding of the lipophilic cation tetraphenylphosphonium to phosphatidylcholine membranes. *Biochim. Biophys. Acta.* 818:410–415.

Bashford, C. L., and C. A. Pasternak. 1985. Plasma membrane potential of neutrophils generated by the Na^+ pump. *Biochim. Biophys. Acta.* 817:174–180.

Boardman, L., M. Huett, J. F. Lamb, J. P. Newton, and J. M. Polson. 1974. Evidence for the genetic control of the sodium pump density in HeLa cells. *J. Physiol. (Lond.).* 241:771–794.

Borle, A. B., and J. Loveday. 1968. Effects of temperature, potassium and calcium on the electrical potential difference in HeLa cells. *Cancer Res.* 28:2401–2405.

Castranova, V., L. Bowman, and P. R. Miles. 1979. Transmembrane potential and ionic content of rat alveolar macrophages. *J. Cell. Physiol.* 101:471–480.

Demura, M., N. Kamo, and Y. Kobatake. 1985. Determination of membrane potential with lipophilic cations: correction of probe binding. *Biochim. Biophys. Acta.* 820:207–215.

Dos Reis, G. A., and G. M. Oliveira-Castro. 1977. Electrophysiology of phagocytic membranes. I. Potassium-dependent slow membrane hyperpolarization in mice macrophages. *Biochim. Biophys. Acta.* 469:257–263.

Ehrenberg, B., M. D. Wei, and L. M. Loew. 1987. Nernstian dye distribution reports membrane potential in individual cell. *In* Membrane Proteins. S. C. Goheen, editor. Bio-Rad Laboratories Publishers, Richmond, CA. 279–294.

Freedman, J. C., and P. C. Laris. 1981. Electrophysiology of cells and organelles: studies with optical potentiometric indicators. *Intern. Rev. Cytol.* 12(Suppl.):177–246.

Freedman, J. C., and P. C. Laris. 1988. Optical potentiometric indicators for nonexcitable cells. *In* Spectroscopic Membrane Probes. L. M. Loew, editor. CRC Press, Boca Raton, FL, Ch. 16.

Gibrat, R., H. Barbier-Brygoo, J. Guern, and C. Grignon. 1985. Membrane potential difference of isolated plant vacuoles: positive or negative? Evidence for membrane binding of cationic probes. *Biochim. Biophys. Acta.* 819:206–214.

Husler, D. F., and U. Lauterwasser. 1982. Membrane potential oscillations in homokaryons. *Exp. Cell Res.* 139:63–70.

Johnson, L. V., P. C. Walsh, B. J. Bockus, and L. B. Chen. 1981. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. *J. Cell Biol.* 88:526–535.

Koppel, D. E., D. Axelrod, J. Schlessinger, E. L. Elson, and W. W. Webb. 1976. Dynamics of fluorescence marker concentration as a probe of mobility. *Biophys. J.* 16:1315–1329.

Korchak, H. M., A. M. Rich, C. Wilkenfeld, L. E. Rutherford, and G. Weissmann. 1982. A carbocyanine dye, Di-O-C_6(3), acts as a mitochondrial probe in human neutrophils. *Biochem. Biophys. Res. Commun.* 108:1495–1501.

Kouri, J., M. Noa, B. Diaz, and E. Niubo. 1980. Hyperpolarisation of rat peritoneal macrophages phagocytosing latex particles. *Nature (Lond.).* 283:868–869.

Lampidis, T. J., Y. Hasin, M. J. Weiss, and L. B. Chen. 1985. Selective killing of carcinoma cells in vitro by lipophilic-cationic compounds: a cellular basis. *Biomed. & Pharmacother.* 39:220–226.

Leach, F. J., and J. J. Webster. 1986. Commercially available firefly luciferase reagents. *Methods Enzymol.* 133:51–70.

Lichtstein, D., H. R. Kaback, and A. J. Blume. 1979. Use of a lipophilic cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. *Proc. Natl. Acad. Sci. USA.* 76:650–654.

Loew, L. M. (ed.) 1988. Spectroscopic Membrane Probes. CRC Press, Boca Raton, FL, Ch. 14–21.

Loew, L. M., L. Benson, P. Lazarovici, and I. Rosenberg. 1985. Fluorometric analysis of transferable membrane pores. *Biochemistry.* 24:2101–2104.

Loew, L. M., B. Ehrenberg, E. Fluhler, M. D. Wei, and V. Burnham. 1986. A search for nernstian dyes to measure membrane potential in individual cells. *Biophys. J.* 49:308a.

McCaig, D. J., and R. D. Berlin. 1983. Transmembrane potential of J774.2 mouse macrophage cells measured by microelectrode and ion distribution methods. *Experientia (Basel).* 39:906–907.

Melmed, R. N., P. J. Karanian, and R. D. Berlin. 1981. Control of cell volume in the J774 macrophage by microtubule disassembly and cyclic AMP. *J. Cell Biol.* 90:761–768.

Okada, Y., M. Ogawa, N. Aoki, and K. Izutsu. 1973. The effect of K^+ on the membrane potential in HeLa cells. *Biochim. Biophys. Acta.* 291:116–126.

Paul, D. 1970. Cell and Tissue Culture. 4th ed. Livingstone, Edinburgh.

Pollack, L. R., E. H. Tate, and J. S. Cook. 1981. Na^+, K^+-ATPase in HeLa cells after prolonged growth in low K^+ or ouabain. *J. Cell. Physiol.* 106:85–97.

Roy, G., and R. Sauve. 1983. Stable membrane potentials and mechanical K^+ responses activated by internal Ca^{2+} in HeLa cells. *Can. J. Physiol. Pharmacol.* 61:144–148.

Seligmann, B. E., and J. I. Gallin. 1980. Use of lipophilic probes of membrane potential to assess human neutrophil activation. *J. Clin. Invest.* 66:493–503.

Seligmann, B. E., E. K. Gallin, D. L. Martin, W. Shain, and J. I. Gallin. 1980. Interaction of chemotactic factors with human polymorphonuclear leukocytes: studies using a membrane potential-sensitive cyanine dye. *J. Membr. Biol.* 52:257–272.

Simchowitz, L., I. Spilberg, and P. DeWeer. 1982. Sodium and potassium fluxes and membrane potential of human neutrophils: evidence for an electrogenic sodium pump. *J. Gen. Physiol.* 79:453–479.

Sims, P. J., A. S. Waggoner, C. H. Wang, and J. F. Hoffman. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry.* 13:3315–3330.

Smith, J. C., P. Russ, B. S. Cooperman, and B. Chance. 1976. Synthesis, structure determination, spectral properties, and energy-linked spectral responses of the extrinsic probe oxonal V in membranes. *Biochemistry.* 15:5094–5105.

Steinman, S., E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A sterologic analysis. *J. Cell Biol.* 68:665–687.

Sung, S. S. J., J. D.-E. Young, A. M. Origlio, J. M. Heiple, H. R. Kaback, and S. C. Silverstein. 1985. Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic [Ca^{2+}], and inhibits phagocytosis in mouse macrophages. *J. Biol. Chem.* 260:13442–13448.

Tatham, P. E. R., P. J. Delves, L. Shen, and I. M. Roitt. 1980. Chemotactic factor induced membrane potential changes in rabbit neutrophils monitored by the fluorescent dye 3,3′-dipropylthiodicarbocyanine iodide. *Biochim. Biophys. Acta.* 602:285–298.

Waggoner, A. S. 1979. Dye indicators of membrane potential. *Annu. Rev. Biophys. Bioeng.* 8:847–868.

Walter, R. J., R. D. Berlin, J. R. Pfeiffer, and J. M. Oliver. 1980. Polarization of endocytosis and receptor topography on cultured macrophages. *J. Cell Biol.* 86:199–211.

White, J. R., C. K. Huang, J. M. Hill, P. H. Naccache, E. L. Becker, and R. I. Sha'afi. 1984. Effect of phorbol 12,13-didecanoate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils. *J. Biol. Chem.* 259:8605–8611.

Young, J. D.-E., T. M. Young, L. P. Lu, J. C. Unkless, and Z. A. Cohn. 1982. Characterization of a membrane pore-forming protein from *Entameoba histolytica*. *J. Exp. Med.* 156:1677–1690.

Young, J. D.-E., J. C. Unkeless, H. R. Kaback, and Z. A. Cohn. 1983. Mouse macrophage F_c receptor for IgG 2b/1 in artificial and plasma membrane vesicles functions as a ligand-dependent ionophore. *Proc. Natl. Acad. Sci. USA.* 80:1636–1640.