Spectrophotometric Measurements of Transmembrane Potential and pH Gradients in Chromaffin Granules

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ABSTRACT The electrical potential ($\Delta\Psi$) and proton gradient (ΔpH) across the membranes of isolated bovine chromaffin granules and ghosts were simultaneously and quantitatively measured by using the membrane-permeable dyes 3,3'dipropyl-2,2'thiadicarbocyanine (diS-C_3-(5)) to measure $\Delta\Psi$ and 9-aminoacridine or atebrin to measure ΔpH. Increases or decreases in the $\Delta\Psi$ across the granular membrane could be monitored by fluorescence or transmittance changes of diS-C_3-(5). Calibration of the $\Delta\Psi$ was achieved by utilization of the endogenous K^+ and H^+ gradients, and valinomycin or carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), respectively, with the optical response of diS-C_3-(5) varying linearly with the Nernst potential for H^+ and K^+ over the range −60 to +90 mV. The addition of chromaffin granules to a medium including 9-aminoacridine or atebrin resulted in a rapid quenching of the dye fluorescence, which could be reversed by agents known to cause collapse of pH gradients. From the magnitude of the quenching and the intragranular water space, it was possible to calculate the magnitude of the ΔpH across the chromaffin granule membrane. The time-course of the potential-dependent transmittance response of diS-C_3-(5) and the ΔpH-dependent fluorescence of the acridine dyes were studied simultaneously and quantitatively by using intact and ghost granules under a wide variety of experimental conditions. These results suggest that membrane-permeable dyes provide an accurate method for the kinetic measurement of ΔpH and $\Delta\Psi$ in an amine containing subcellular organelle.

INTRODUCTION

Changes in transmembrane potential ($\Delta\Psi$) and pH gradients (ΔpH) are essential events in cells and organelles that often control basic cellular functions. Propagation of nerve impulses, energy transformation, and metabolite or ion uptake are but a few cell processes that are directly dependent on the magnitude and time-course of $\Delta\Psi$ and/or ΔpH. In many situations where the use of microelectrodes is impossible, $\Delta\Psi$ and ΔpH have been indirectly

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/80/02/0109/32$1.00 109
Volume 75 February 1980 109–140

determined from equilibrium distributions of radiolabeled lipophilic ions (1,2) and membrane permeable acids or amines (3). In principle, the radiochemical techniques require that the labeled molecules equilibrate rapidly across the membrane and do not interact significantly with the membrane or other components of the preparation (2,4). Yet, even when these criteria are satisfied, radiochemical experiments are laborious, and, most of all, they require the separation of the cell fractions from the suspending medium, which often makes the method unsuitable for kinetic measurements. More recently, there has been considerable interest in the development and use of optical probes whose absorption and/or fluorescence are specifically sensitive to either $\Delta\Psi$ or ΔpH (5,6).

For studies of cells or vesicles in suspension, cyanine dyes, specifically diS-C_3-(5) (3,3′dipropyl-2,2′thiadicarbocyanine, have proven to be the most useful voltage-sensitive dyes because they exhibited particularly large potential-dependent fluorescence signals (7). A critical problem in the use of cyanine dyes is the calibration of the optical signals in terms of "known" values of membrane potential. In most cases, membrane potentials have been calculated from diffusion potentials induced by the addition of valinomycin in media of varying potassium concentrations. The accuracy of the calibrations depended on estimates of the free internal potassium concentration and the relative permeability of all other ionic species compared to the permeability of K^+ in the presence of valinomycin. Besides the lack of standard calibrating procedures, the spectral properties and the mechanism(s) generating the voltage-dependent response of diS-C_3-(5) also appeared to differ in some preparations (5,6). Consequently, for each new application it became necessary to examine whether or not diS-C_3-(5) acted as a membrane potential probe and to define the exact experimental conditions under which this occurred.

Acridine dyes having amine groups with high pK_a values have been used to measure pH gradients in chloroplasts, chromatophores, and liposomes (8–10). Fluorescent-like isotopically labelled amines were presumably distributed in and out of the vesicles according to the proton gradient. Dye molecules taken up by the vesicles were assumed to be completely quenched and the extent of quenching to be proportional to the dye concentration in vesicles. The fluorescence intensity was then proportional to the external dye concentration, and hence, ΔpH could be calculated. Among the dyes tested, 9-aminoacridine (monoamine molecule) and atebrin (diamine molecule) proved to be the most useful dyes for measurements of ΔpH. In some situations, the validity of ΔpH measurements with 9-aminoacridine and atebrin has been questioned on the grounds that they may bind to the preparation and fluorescence may have been quenched by factors unrelated to the proton gradients (11,12). In this report the transmembrane potential and pH differences across the membrane of chromaffin granules were simultaneously and quantitatively measured with diS-C_3-(5) and either 9-aminoacridine or atebrin, respectively.

Chromaffin granules are vesicles located in cells of the adrenal medulla which serve as the site for storage and release of endogenous catecholamines

(13,14). They were chosen for this study because their low ionic permeability and endogenous pH gradients seemed particularly suitable to evaluate and calibrate voltage and ΔpH-sensitive dyes. Isolated granules were shown to be highly impermeable to monovalent (H^+, Na^+, and K^+) and divalent (Ca^{2+}, Mg^{2+}, and Mn^{2+}) cations (15,16), and to maintain a well-buffered acidic interior with $\Delta pH \cong 1.5$ and $\Delta\Psi \cong 0$ (16–18). Consequently, membrane potential and pH gradients could be accurately manipulated by varying the experimental conditions and their magnitudes held (i.e., "clamped") at known values which could be simultaneously measured by optical and radiochemical methods. Moreover, because granules can be isolated in large quantities from bovine adrenal glands, radiochemical techniques have been extensively used to study and characterize the uptake of catecholamines during changes in electrochemical potential induced by exogenously added ATP (17,19).

In these experiments, voltage and/or pH gradients across the membrane of intact and "ghost" granules were manipulated with ionophores, added ATP, or variations of the suspending medium. The optical responses of diS-C_3-(5) and either 9-aminoacridine or atebrin were then specifically related to the predicted values of $\Delta\Psi$ and ΔpH.

The apparent mechanism(s) responsible for the potential-dependent diS-C_3-(5) response in granules are discussed in view of the different mechanism reported in red blood cell experiments, where the calibration curve of fluorescence vs. membrane potential varied with intracellular pH (20). With the cyanine and acridine dyes, the magnitude and time-course of $\Delta\Psi$ and ΔpH can be simultaneously monitored during uptake or release of catecholamines by intact granules. Moreover, the standardization of the probes in chromaffin granules would provide a basis for measurements of $\Delta\Psi$ and ΔpH in other similar secretory vesicles that are particularly difficult to obtain in large yields by available isolation procedures.

A preliminary report of this study has been previously presented (21).

MATERIALS AND METHODS

Bovine adrenal glands were obtained from a local slaughterhouse, kept on ice, and brought to the laboratory within 2 h. The chromaffin cells from the adrenal medulla were dissected out of the glands and minced in a medium at 4°C consisting of 270 mM sucrose- 10 mM Tris at pH 7.0. After homogenization and differential centrifugation, the granules were passed through a D_2O-ficoll-sucrose gradient to separate them from other subcellular organelles in order to preserve isotonicity (22). Resuspension of the purified chromaffin granules in the isolation medium was followed by two subsequent washings and final suspension in the isolation medium. The final suspension was stored at 4°C until use. Protein content was measured by the Lowry methods (23) using bovine serum albumin as a standard.

Chromaffin granule ghosts or vesicles were prepared as previously described (24). Isolated granules were lysed in a hypotonic medium of 5 mM Tris-maleate at pH 7.0 for 15 min, and the membrane fragments were resuspended in an isotonic experimental medium. The resealed vesicles were stored at 4°C and were generally used within 24 h.

Spectral Measurements

Excitation and fluorescence emission spectra of cyanine or acridine dyes in suspensions of granules and in various solutions were measured with a Hitachi MPF 2A fluorescence spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Absorption spectra were measured with a rapid scanning dual-wavelength spectrophotometer built at the Johnson Research Foundation, Philadelphia, Pa. (25). The latter instrument is equipped with microprocessors for base-line corrections to provide accurate measurements of spectral changes in a turbid suspension.

Measurements of $\Delta\Psi$ and ΔpH Optical Responses

Potential-dependent fluorescence changes of diS-C_3-(5) or pH-dependent fluorescence changes of acridine dyes were measured with a compensated fluorimeter (26). The main feature of this instrument is its trifurcated light guide; a large central light guide consisting of hundreds of randomized optical filters is split into three smaller branches. One branch is connected to a light source to conduct the excitation beam to the cuvette. The other two branches collect and conduct the scattered light from the illuminated surface of the cuvette to appropriate interference filters to detect changes in fluorescence, reflectance (scattered light at the same wavelength as the excitation), and their differences.

The compensating fluorimeter was fitted with excitation, reflectance, and emission interference filters (Omega Optical, Brattleboro, Vt.) transmitting at 670, 670, and 690 nm, respectively, for diS-C_3-(5) fluorescence, at 45, 450, and 500 nm for atebrin, and at 400, 400, and 440 nm for 9-aminoacridine. Simultaneous fluorescence measurements from pH-and potential-sensitive dyes from the same cuvette were obtained using two compensating fluorimeters with light guides oriented perpendicular to each other.

Voltage-dependent absorbance changes of diS-C_3-(5) were measured with a dual-wavelength spectrophotometer set at difference wavelength pairs of 685 minus 655 nm. Dual-wavelength techniques provide highly stable and sensitive readout of small specific absorbance changes in turbid biological material. The method is especially useful to minimize aspecific absorption changes caused by swelling or shrinking of the vesicles.

In suspensions of granules, the changes in $\Delta\Psi$ and/or ΔpH were initiated by the addition of various agents which may interact directly with the dye or alter the binding of the dye to granules. Therefore, it was important to assess the effect of these agents on the desired optical responses of the cyanine and acridine dyes. Control experiments indicated that the reagents used in these experiments did not interact with the probes, except that high concentrations of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) or valinomycin (5–10 μg/ml) produced small decreases in peak absorption, excitation, and emission of diS-C_3-(5), but no wavelength shifts. With dye bound to membrane fragments, the addition of these ionophores produced considerably smaller optical responses. Still, for quantitative measurements of membrane potential, it was important to carefully eliminate aspecific from voltage-dependent responses. This was achieved by lowering the concentrations of added ionophores (0.1–1μg/ml) and by monitoring the signals by dual-wavelength measurements.

In most experiments, the fluorimeter monitoring an acridine dye was aligned with its light guide perpendicular to the beam of the dual-wavelength spectrophotometer, which was set to measure the transmittance changes of diS-C_3-(5).

All reagents were of analytical grade. Valinomycin, 9-aminoacridine, and atebrin

were purchased from Sigma Chemical Co. (St. Louis, Mo.), and diS-C_3-(5) was the generous gift of Dr. A. Waggoner of Amherst College (Amherst, Mass). FCCP was obtained from Pierce Chemical Co. (Rockford, Ill.), and nigericin was the gift of Dr. E. Westley from Hoffman-LaRoche, Inc. (Nutley, N.J.).

RESULTS

Spectral Properties of diS-C_3-(5)

Solvent Properties In general, cyanine dyes are not very "solvatochromic," in that their absorption and fluorescence emission maxima usually shift by 20 nm or less when they are transferred from a polar solvent such as water to a nonpolar solvent such as chloroform. As a result, their extinction coefficient or fluorescence quantum yields rarely exhibit large changes unless they form aggregates (27). DiS-C_3-(5) dissolved in isotonic sucrose solution had an absorption maximum at 650 nm with a broad shoulder at 590–600 nm (Fig. 1 A). For dye concentrations ranging from 10^{-7} to 10^{-5} M, the absorption spectrum kept the same shape, which indicates that dye molecules did not form dimers or higher order aggregates. Although up to 10 μM diS-C_3-(5) could be dissolved in water, its solubility decreased with increased ionic concentration. Titrations of a diS-C_3-(5) solution with divalent (from 0 to 50 mM Ca^{2+} or Mg^{2+}) or monovalent (from 0 to 150 mM Na^+ or K^+) cations did not alter the shape of the absorption spectrum but decreased its absorbance by up to 20%.

The fluorescence emission of diS-C_3-(5) (1 μM) had a maximum at 670 nm and its maximum excitation matched its peak absorption at 650 nm. Ionic substitutions did not affect the fluorescence as long as the ionic strength remained constant. For the dye concentrations used in the present experiments (0.5–2 μM), the shape of the absorption and emission spectra were not altered by ionic substitutions or by additions of the various agents used to change the membrane potential. Thus, the spectral measurements indicate that, under these experimental conditions, dye molecules in aqueous solution remained in a monomeric state.

Spectral Characteristics of diS-C_3-(5) Bound to Chromaffin Granules DiS-C_3-(5), like other cyanine dyes, is a symmetrical molecule with a positive charge, which is delocalized over the entire structure of the chromophore. The charge delocalization is an important property of the dye, which explains its high permeability to lipid bilayers (28) and biological membranes (29).

In Fig. 1, the absorption, excitation, and emission spectra of diS-C_3-(5) were measured in an isotonic sucrose solution after the addition of chromaffin granules. The addition of granules (0.5 mg protein) decreased and displaced the peak absorption of diS-C_3-(5) from 650–662 nm (Fig. 1 A). With increased concentrations of granules (0.5–3 mg protein), the diS-C_3-(5) absorption was slightly enhanced and exhibited a maximum wavelength displacement of 20 nm, from 650 nm for dye in solution to 670 nm for dye bound to granules (Fig. 1 A).

Upon binding to granules, the excitation and emission bands of diS-C_3-(5)

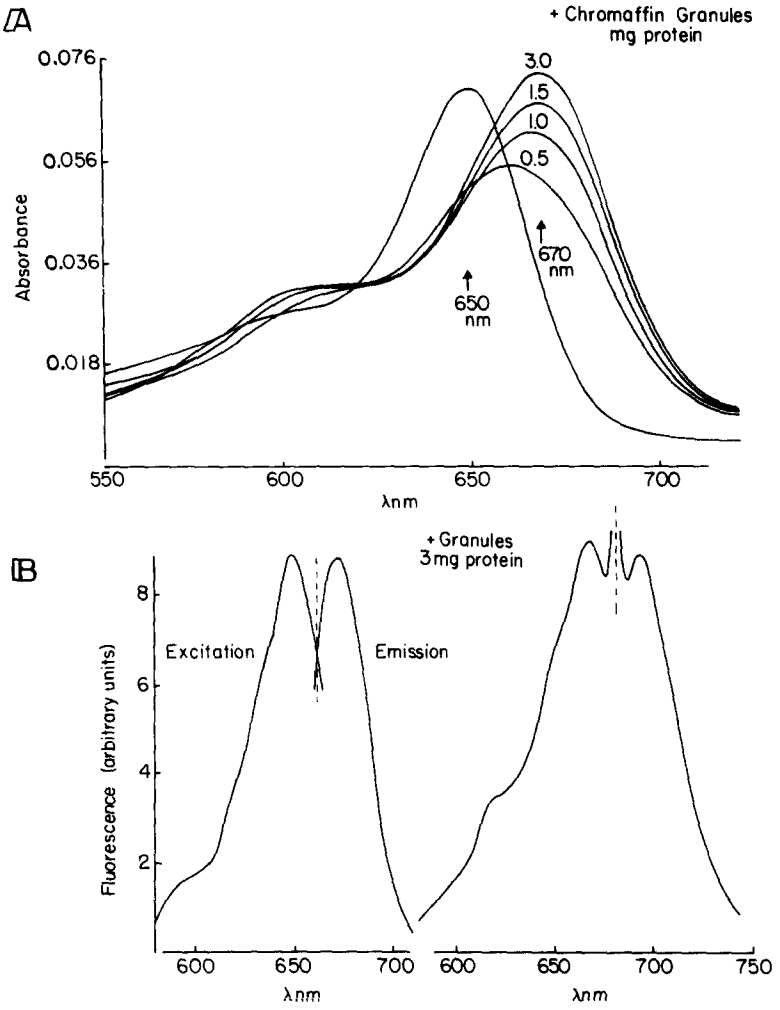

FIGURE 1. Spectral measurements of diS-C_3-(5) in the presence and absence of granules. (A) Absorption spectra of 1 μM diS-C_3-(5) solutions were normalized by subtracting the equivalent spectra from a reference cuvette free of dye, which contained the same medium: 270 mM sucrose, 10 mM Tris maleate, pH 6.9, at 24°C. The absorption spectrum of diS-C_3-(5) in solution had a maximum at 650 nm. Chromaffin granules were added to both "measure" and "reference" cuvettes, and normalized absorption spectra were measured with (*a*) 0.5, (*b*) 1.0, (*c*) 1.5, and (*d*) 3.0 mg protein per 2 ml incubation medium. The maximum absorption of dye bound to granules was displaced by ~ 20 nm from 650 to 670 nm. (B) In sucrose solutions, the excitation and fluorescence bands of diS-C_3-(5) had maxima at 650 and 670 nm, respectively (left). The addition of granules (3 mg protein/2 ml) displaced the peak wavelengths by ~ 20 nm (right). The temperature in both experiments was 24°C.

were similarly displaced by 20 nm towards longer wavelengths (compare spectra of Fig. 1 B). The spectral characteristics of diS-C_3-(5) shown in Fig. 1 were identical to measurements obtained with granule ghosts depleted of soluble proteins and with broken membrane fragments (data not shown). At low granule-to-dye concentrations, the absorption spectrum appeared to be the sum of the absorption spectra from free and bound dye molecules, whereas at high granule concentrations it suggested that the majority of the molecules are bound to granules. Although the exact binding site(s) are unknown, the absorption spectra indicate that dye molecules bound to granules did not significantly aggregate and remained in a monomer state. In principle, then, the fluorescence intensity monitored at 690 nm is proportional to the concentration of dye bound to granules.

Potential-Dependent Spectral Changes of DiS-C_3-(5) Absorption, excitation, and emission spectra of diS-C_3-(5) in a suspension of chromaffin granules were measured at steady state with membrane potential near 0 mV, then compared to spectra taken following the generation of positive and negative membrane potentials.

It has been previously documented (using [^{14}C]methylamine distribution) that the intragranular space of isolated chromaffin granules is very acidic, at pH 5.5 (16, 19). Moreover, the high internal buffering capacity and low proton conductance of the chromaffin granule allowed changing the external pH without affecting the intragranular pH (16). When ATP was added to freshly isolated granules, a potential of 50–85 mV, inside positive, was measured by [^{14}C]thiocyanate (SCN^-) distribution (19). On the other hand, the addition of FCCP, a compound which transports protons electrogenically across biological membranes in accordance with their electrochemical gradient, resulted in a reversal of the membrane potential to large negative values, the magnitude of which was proportional to the ΔpH. This observation suggested that in the presence of FCCP, a diffusion potential for protons was measured whose magnitude followed the Nernst equation, i.e., $\Delta\psi = 58\ (pH_{in} - pH_{out})$.

In Fig. 2 A, the amplitudes of excitation and emission spectra of diS-C_3-(5) bound to granules (spectrum 1) decreased when a membrane potential, positive inside, was generated by the addition of ATP (spectrum 2). The subsequent addition of FCCP established a negative membrane potential which was associated with an increase in peak excitation and emission (spectrum 3), compared to the spectra taken in the absence of any additions (spectrum 1). Thus, the excitation and emission bands of diS-C_3-(5) bound to granules varied in amplitude with altered membrane potentials but did not exhibit wavelength shifts. In Fig. 2 B, the time-course of the fluorescence changes was measured from a similar experimental sequence. Since the fluorescence at 690 nm monitors the concentration of dye bound to granules, the voltage-dependent fluorescence changes suggested that the concentration of bound diS-C_3-(5) increased for potentials more negative inside.

In Fig. 3, the absorption spectra of diS-C_3-(5) plus granules were taken in the absence of a membrane potential (spectrum 1) and following the estab-

lishment of proton diffusion potentials, first negative inside (spectra 2 and 3), then positive inside (spectrum 4). At steady state, with the membrane potential near 0 mV, as measured by [^{14}C]SCN^- distribution, the absorption spectrum had a maximum at about 665 nm (spectrum 1). As before, FCCP addition resulted in the generation of a Nernst potential for protons $\Delta\psi = 58$ ($pH_{in} - pH_{out}$). With $pH_{out} = 6.8$ (measured with a pH electrode) and $pH_{in} \simeq 5.5$ (measured by [^{14}C]methylamine distribution), FCCP induced a negative

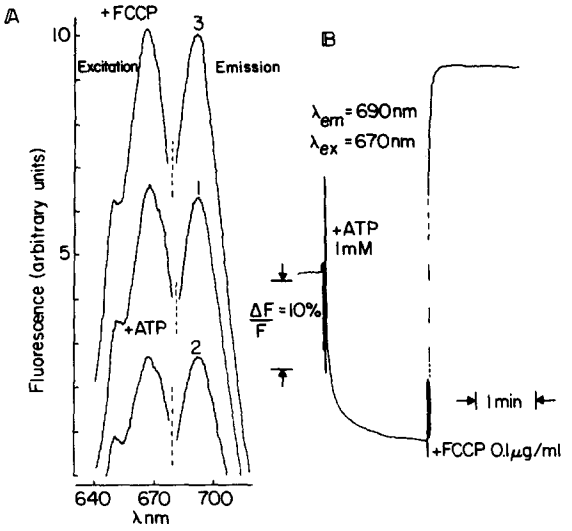

FIGURE 2. Potential-dependent changes in excitation and emission spectra of diS-C_3-(5) in the presence of chromaffin granules. (A) Excitation and emission spectra of diS-C_3-(5) were measured in the presence of granules (1 mg protein/ml), suspended in 0.27 M sucrose plus 10 mM Tris-maleate, at pH 7.0 (*a*) in the absence of additions (spectrum 1); (*b*) plus 1 mM ATP to generate a transmembrane potential, positive inside (spectrum 2); and (*c*) plus FCCP (0.1 μg/ml) to generate a Nernst potential for protons, negative inside (spectrum 3). The amplitude of the excitation and emission bands of diS-C_3-(5) decreased for membrane potentials positive inside, and increased for potentials more negative inside, but did not exhibit a wavelength shift. (B) The time-dependent fluorescence changes described in part A are shown using a wavelength excitation of 670 nm and emission of 690 nm. ATP (1 mM) and FCCP (0.1 μg/ml) were added as indicated.

membrane potential of about −75 mV, which in turn decreased the absorbance of diS-C_3-(5) and displaced its peak to 670 nm (spectrum 2).

In the presence of FCCP, the external pH could be varied without significantly altering the internal pH of chromaffin granules (19). Consequently, the magnitude and polarity of the Nernst potential for protons could be quantitatively manipulated by varying pH_{out}. The first addition of HCl lowered pH_{out} from 6.8 to 6.0, corresponding to a decrease in the $\Delta\psi$ from −75 to −30 mV, as calculated from the Nernst equation. A partial reversal of the

spectral changes of diS-C_3-(5) was observed (spectrum 3). A membrane potential, positive inside, was then obtained by the second addition of HCl, which resulted in a decrease in pH_{out} to 5.2 and a calculated $\Delta\psi$ of +18 mV (since $pH_{out} < pH_{in}$). The positive Nernst proton potential produced an increase in diS-C_3-(5) absorbance and displaced its peak absorption to lower wavelengths (spectrum 4). The potential-dependent absorption changes also confirmed that the binding of diS-C_3-(5) to granules was dependent on membrane potential, as predicted by the fluorescence measurements (Fig. 2). The generation of a negative membrane potential produced an absorption spectrum similar to that measured at high dye to granule concentrations

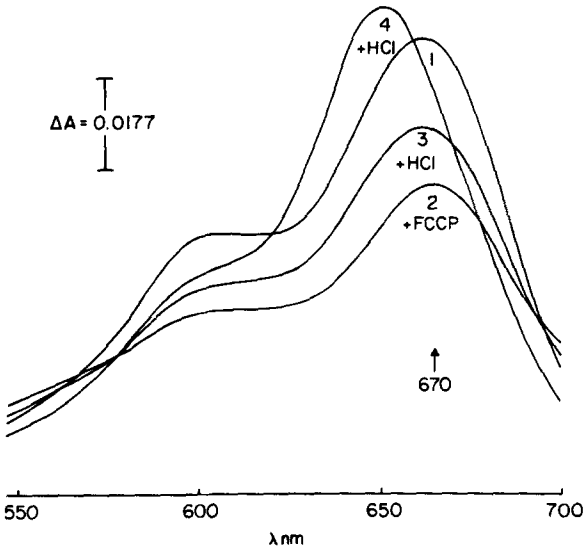

FIGURE 3. Potential-dependent absorption changes. Granules with an endogenous $\Delta pH \simeq 1.5$ (1 mg protein/ml) were suspended in 270 mM at pH 6.8. Spectrum 1: 1 μM diS-C_3-(5) plus granules (1 mg protein/ml) measured at steady state when $\Delta\Psi = 0$. Spectrum 2: 0.1 μg/ml FCCP was added to generate a proton diffusion potential, $\Delta\Psi = 58\ (pH_{in} - pH_{out}) \simeq -90$ mV, negative inside. Spectrum 3: plus 2 mM HCl, decreasing pH_{out} from 6.8 to 6.0. Spectrum 4: further addition of HCl till $pH_{out} < pH_{in}$, such that $\Delta\Psi$ became positive inside, displacing the absorption spectrum to lower wavelengths.

(Fig. 1, spectrum for 1 μM dye and 0.5 mg protein), implying an increase in dye binding. On the other hand, at positive membrane potentials, the absorption spectrum resembled the spectrum of free dye in solution, suggesting a decrease in dye bound to granules.

Time-Course of Potential-Dependent Fluorescence Signals

In Fig. 4, the fluorescence of diS-C_3-(5) bound to chromaffin granules was continuously monitored at 690 nm during changes of membrane potential. The addition of ATP resulted in the establishment of a large time-dependent

decrease in the fluorescence, reaching a steady state in 1 min and corresponding to a substantial membrane potential, inside positive. In the absence of membrane-permeable ions, added FCCP would be expected to increase the membrane permeability for protons and generate a diffusion potential opposite in direction to that generated by the ATPase and at a rate which varies with the ratio of FCCP to granule concentration. In this experiment (Fig. 4) a relatively high concentration of ionophore (0.2 μg FCCP) was added to 0.5 mg protein of granules. As evidenced, the diS-C_3-(5) fluorescence in fact reversed from a large decrease in intensity to a large increase in intensity over a very short time interval. Since the internal pH of isolated granules was 5.5 and the outside pH 6.8, the Nernst potential for protons was calculated to be

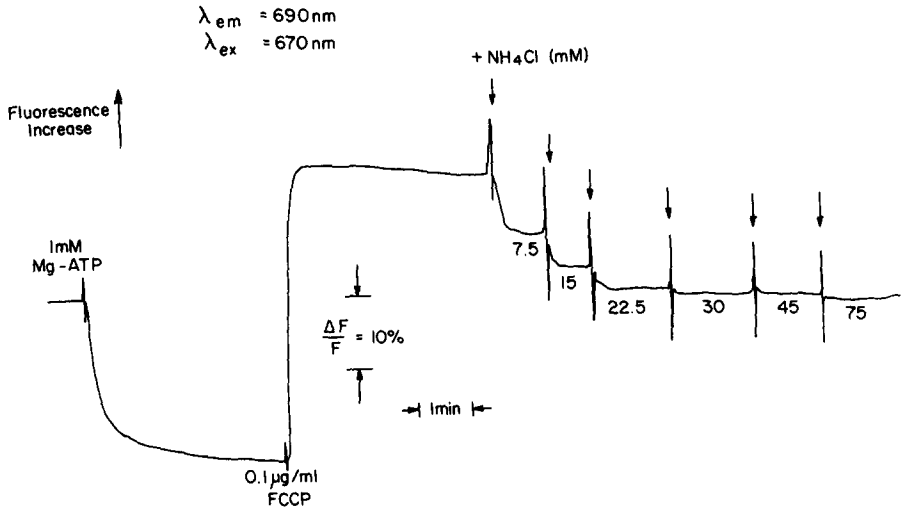

FIGURE 4. Time-course of diS-C_3-(5) potential-dependent fluorescence response. Added ATP generated a $\Delta\Psi$, positive inside, and a decrease in fluorescence. FCCP induced a proton diffusion potential $\Delta\Psi = 58\ \Delta pH \simeq -80$ mV (negative inside), and increased the fluorescence by 40%. Collapsing the pH gradient with NH_4Cl abolished the potential and the fluorescence recovered to its initial base line, where $\Delta\Psi = 0$. Granules (0.5 mg protein/ml) were suspended in 270 mM sucrose, 10 mM $MgCl_2$, and 30 mM Tris maleate, pH 6.8 and at 24°C.

−75 mV. In order to test rigorously whether in fact the diffusion potential for protons was being measured, the ΔpH was systematically varied by raising the internal pH while maintaining the external pH constant. It has previously been shown that the addition of ammonia to isolated chromaffin granules has resulted in a dose-dependent alkalinization of the intragranular space (16), because ammonia permeates the membrane in the uncharged form and reprotonates intragranularly. The addition of ammonia produced a dose-dependent decrease in the fluorescence of diS-C_3-(5) (Fig. 4). After addition of 30 mM NH_4Cl, the fluorescence reached a level identical to the initial level prior to the addition of ATP, and corresponds to a value where the pH_{in} =

pH_{out}. Since the $\Delta\psi$ is equal to zero at this point, it suggests that the value of the $\Delta\psi$ before ATP addition is also zero. Further additions of NH_4Cl did not lower the diS-C_3-(5) fluorescence below its initial level, as required, because the passive diffusion of ammonia could collapse the ΔpH (and therefore the $\Delta\psi$) but could not possibly result in a ΔpH basic inside (and therefore a positive potential).

Calibration of diS-C_3-(5) Transmittance with $\Delta\psi$

The absorption spectra of dye plus granules shown in Figs. 1 and 3 suggested that absorption changes could also be used to measure potential changes across the membrane of chromaffin granules. Changes in transmitted light (ΔT) from diS-C_3-(5) in a suspension of granules were monitored with a dual wavelength spectrophotometer at 685–655 nm to measure the relative concentration of dye bound vs. dye free in solution as a function of membrane potential.

In Fig. 5, the transmittance signal of diS-C_3-(5) at 685–655 nm was calibrated with $\Delta\psi$ by quantitatively varying the Nernst potential for protons. At steady state, the granules would be expected to maintain an endogenous ΔpH and a membrane potential equal to zero. The addition of FCCP established a calculated Nernst potential for protons, $\Delta\psi = 58\ (pH_{in} - pH_{out})$, negative inside, which was associated with a decrease in diS-C_3-(5) transmittance signal with $pH_{out} = 6.8$. $\Delta\psi$ was then systematically increased by decreasing pH_{out} with added HCl, which gradually increased the diS-C_3-(5) signal. At $pH_{out} = 5.7$ the potential-dependent signal became equal to its initial value (i.e., $\Delta\psi = 0$ before the addition of FCCP), which meant that $\Delta\psi = 58\ (pH_{in} - pH_{out}) = 0$, and that $pH_{in} = pH_{out}$ 5.7. The external medium was weakly buffered, such that addition of HCl decreased pH_{out} without significantly altering pH_{in}, since the internal volume of granules is highly buffered (17). pH_{out} was measured with a standard electrode, and pH_{in} was taken to be constant during the experiment, as shown through measurements of pH_{in} using either [^{14}C] methylamine distributions (19) or fluorescent amines (see Figs. 8 A and 10 A) under similar conditions. Thus, the Nernst potential for protons was easily calculated from ΔpH and related to the optical signals of diS-C_3-(5). A plot of the transmittance changes vs. $\Delta\psi$ yielded a linear relationship for positive and negative membrane potentials from +41 to −64 mV, with a slope of 0.1% transmittance change per millivolt (Fig. 5, *inset*). Larger positive membrane potentials could not be obtained by decreasing pH_{out} below 5.0, because at lower pH values, granules formed aggregates which tended to precipitate (30).

In Fig. 6, the diS-C_3-(5) signal was also calibrated with membrane potential determined from the Nernst potential for potassium. The internal concentration of free potassium was estimated from the potential-dependent responses of diS-C_3-(5) following additions of valinomycin. In the absence of added K^+ or other membrane permeable ions in the medium, the addition of valinomycin makes the membrane specifically permeable to potassium and consequently causes the release of K^+ from granules. Because valinomycin transports K^+ electrogenically across the membrane, the movement of the charged ion

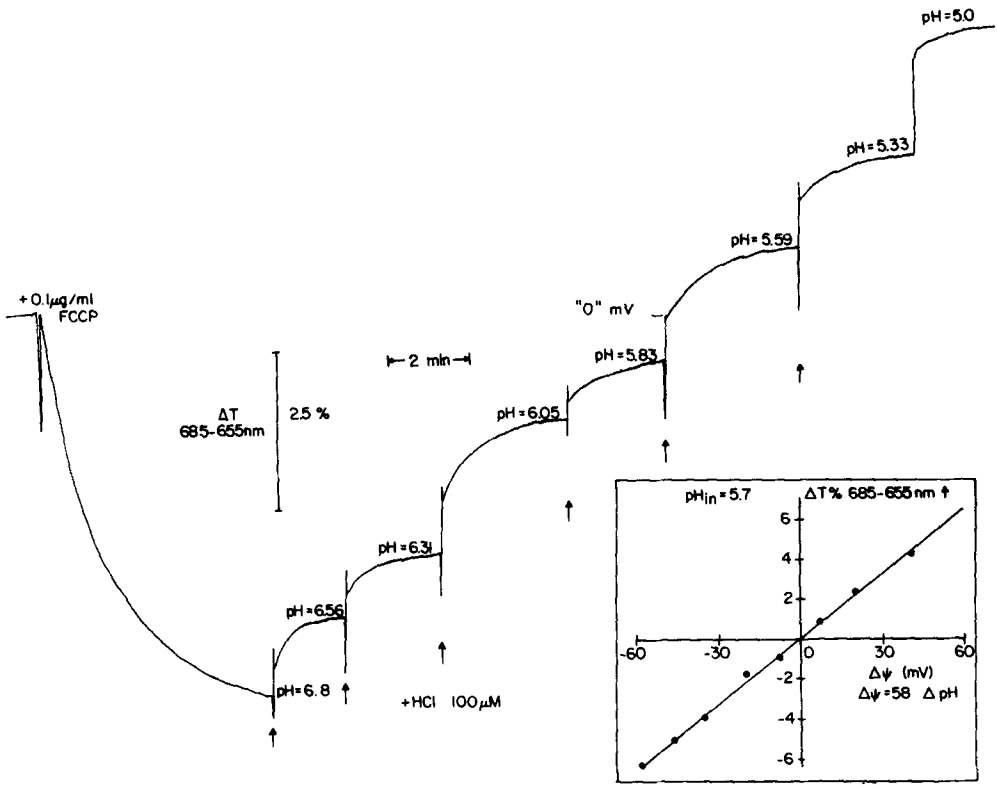

FIGURE 5. Calibration of diS-C_3-(5) with the Nernst potential for protons. Transmittance changes of diS-C_3-(5) were monitored at 685-655 nm with a dual-wavelength spectrophotometer. The output of the instrument (T) was equal to the transmittance of the experimental sample at 685 (T_{685}) minus its transmittance at 655 (T_{655}), $T = T_{685} - T_{655}$. Initially, the transmittances of the cuvette (i.e., 100% transmittance levels) at 685 and 655 nm were adjusted so that $T_{685} = T_{655}$ and $T = 0$. Changes in transmittance ($\Delta T = \Delta T_{685} - \Delta T_{655}$) were normalized as percent changes of the initial 100% transmittance of the samples. Granules (1 mg protein/ml) suspended in 270 mM sucrose, 10 mM morpholinopropane sulfonic acid, and 1 μM diS-C_3-(5) at pH 6.8 had an endogenous ΔpH and no membrane potential. Added FCCP evoked a proton diffusion potential $\Delta\Psi = 58\ (pH_{in} - pH_{out})$ and decreased the diS-C_3-(5) signal by 6.3%. pH_{out} was monitored with an electrode and was titrated with additions of HCl. Decreasing pH_{out} from 6.8 to 5.7 increased the optical signal to its initial level, $\Delta T = 0$; i.e., $\Delta\Psi = 0$ when $pH_{out} = pH_{in} \simeq 5.7$. pH_{in} was taken to be constant at 5.7, and ΔT was plotted vs. membrane potential determined from $\Delta\Psi = 58\ (5.7 - pH_{out})$. The transmittance signal of diS-C_3-(5) was linear with $\Delta\Psi$, with a slope of 0.1% ΔT/mV (*inset*).

establishes a diffusion potential (negative inside) which, in principle, limits further efflux of K^+. In this situation, the negative membrane potential induced by valinomycin produced a decrease in diS-C_3-(5) transmittance (see Fig. 16 and Discussion). When $[K^+]_{out} = 2$ mM, valinomycin had little or no

effect on the transmittance (Fig 6, bottom trace), suggesting that the Nernst potential for potassium is zero and $[K^+]_{in}$ = 2mM. For $[K^+]_{out} > 2$ mM, valinomycin generated a Nernst potential, positive inside, and accordingly, diS-C_3-(5) exhibited an increase in transmittance.

In the presence of valinomycin, and with $[K^+]_{in} = [K^+]_{out} = 2$ mM, the diS-

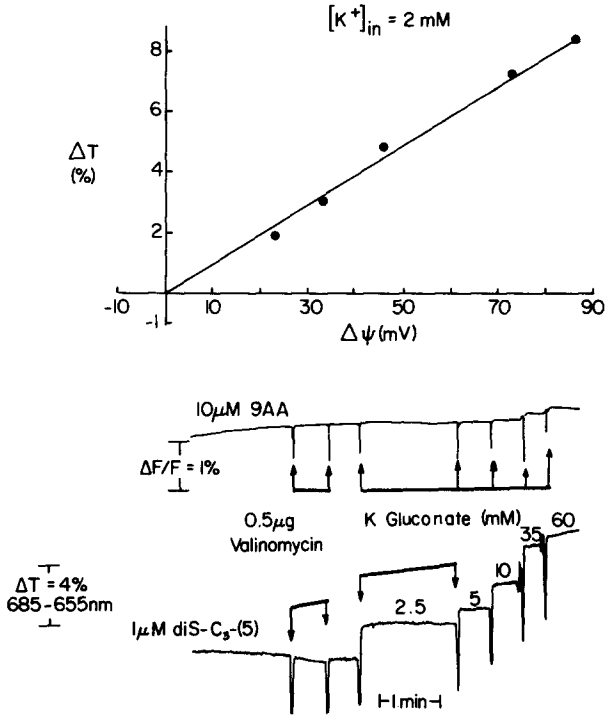

FIGURE 6. Calibration of the diS-C_3-(5) optical response with the Nernst potential for K^+. The fluorescence changes of 9-aminoacridine (top trace) and the transmittance signal of diS-C_3-(5) (bottom trace) were monitored from granules (1 mg protein/ml) in 270 mM sucrose, 20 mM Tris maleate, and 2 mM K gluconate at pH 7.0. Gluconate was used instead of the more permeable chloride anions to avoid a partial dissipation of the K^+ diffusion potential. Two additions of the potassium ionophore valinomycin did not alter the optical signals. $[K^+]_{out}$ was gradually raised to 2.5, 5, 10, 35, and 60 mM, which increased the transmittance signal of the cyanine dye (bottom trace). The membrane potential was determined from Nernst's equation, $\Delta\Psi = 58 \log ([K^+]_{out}/[K^+]_{in})$, where $[K^+]_{in} \simeq 2$ mM (see text). The plot of ΔT vs. $\Delta\Psi$ was linear with a slope of 0.095% ΔT/mV (graph).

C_3-(5) signals were monitored with increasing concentrations of $[K^+]_{out}$. A plot of ΔT vs. $\Delta\psi$ calculated from K^+ diffusion potentials was linear with a slope of 0.095% ΔT per millivolt, in excellent agreement with the previous calibration (Fig. 5). In principle, stable membrane potentials (negative inside) should be obtained in the presence of valinomycin when $[K^+]_{in} > [K^+]_{out}$. In practice,

however, membrane potentials measured optically had transient responses, such that calibrations of diS-C_3-(5) from potassium diffusion potentials were restricted to membrane potentials, positive inside (see Discussion).

The potential-dependent responses of diS-C_3-(5) were calibrated by dual-wavelength rather than fluorescence measurements because plots of ΔT vs. $\Delta\psi$ remained linear for a greater range of dye and granule concentrations.

Measurements of ΔpH with 9-Aminoacridine

The excitation and fluorescence emission spectra of 9-aminoacridine in aqueous solutions (10 μM) are shown in Fig. 7 (spectrum 1). The addition of granules having an acidic intragranular space decreased the excitation and

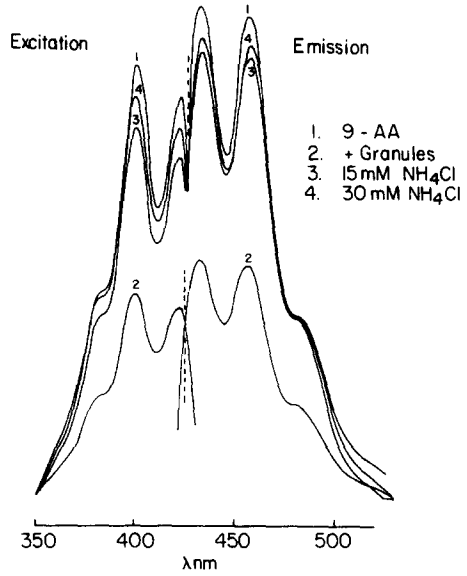

FIGURE 7. ΔpH-dependent spectral changes of 9-aminoacridine. The excitation and emission bands of 9-aminoacridine in solution (spectrum 1) were decreased in amplitude by the addition of granules (1 mg protein/ml) with an endogenous ΔpH (spectrum 2), and were enhanced by subsequent additions of NH_4Cl (spectra 3 and 4). The relative amplitudes of the excitation and emission bands were neither changed or displaced in wavelength by the presence of granules.

emission bands of the acridine dye with no wavelength displacements (spectrum 2). If the decrease in fluorescence was caused by dye accumulation in the granules according to the proton gradient, then collapsing the ΔpH should increase both the external dye concentration and the fluorescence intensity. In this experiment, ΔpH was abolished by additions of ammonia of 15 and 30 mM, which in turn enhanced the excitation and emission spectra (Fig. 7, spectra 3 and 4). The lack of wavelength shifts and the reversibility of the spectral changes as a function of ΔpH suggests that 9-aminoacridine did not

significantly bind to granules, and its fluorescence is at least partly quenched by dye accumulation in the granules.

In Fig. 8, the fluorescence of a 9-aminoacridine solution (10 μM) at pH 7.0 was quenched by the addition of chromaffin granules having an acidic intragranular space (all three traces). To demonstrate that the fluorescence quenching was caused by the proton gradient, ΔpH was collapsed by three

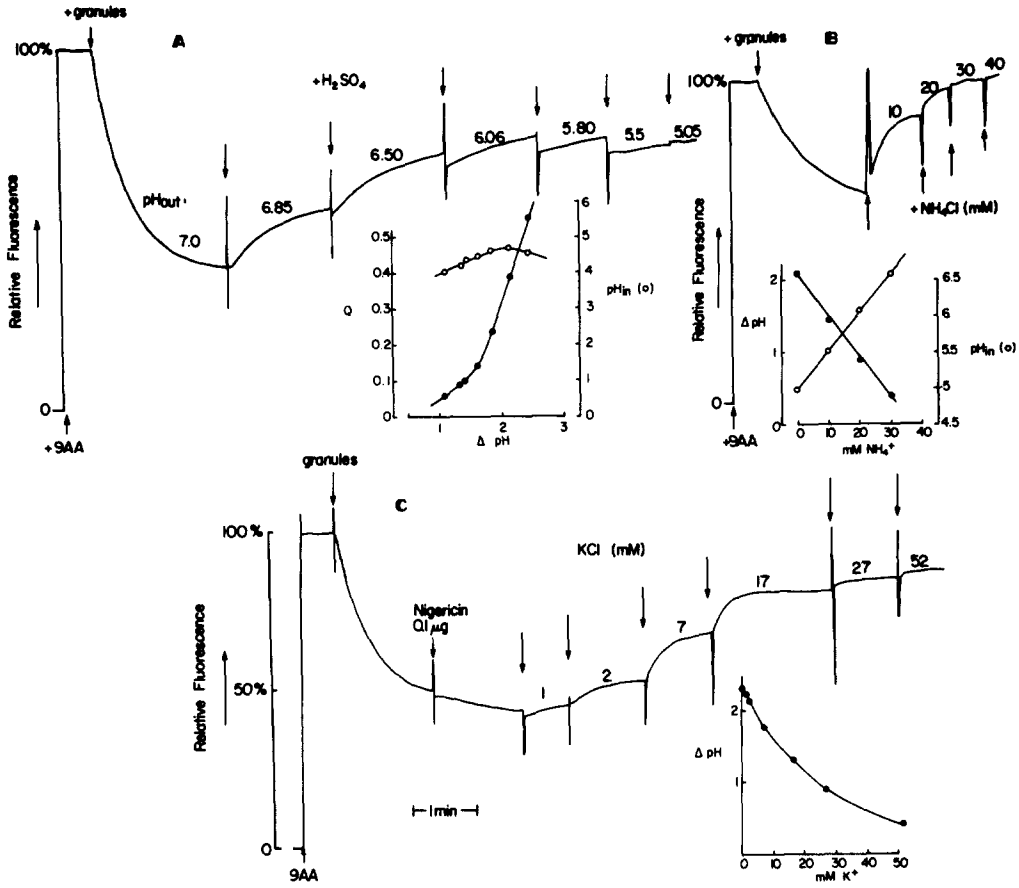

FIGURE 8. Measurements of ΔpH from the fluorescence quenching of 9-aminoacridine. The fluorescence of 9-aminoacridine was monitored from sucrose solutions buffered at pH 7 with 10 mM (A) or 30 mM (B and C) Tris maleate. The fluorescence intensity was quenched by the addition of granules and was enhanced by collapsing the ΔpH by additions of H_2SO_4 (trace A), NH_4Cl (trace B), or K^+ in the presence of nigericin (trace C). ΔpH was calculated from the fluorescence quenching (Q) and the ratio of external to internal volume (V) $\Delta pH = \log [Q/1-A] \cdot V$ (see text). In graph A, Q values from the H_2SO_4 titration were used to calculate ΔpH, then plotted vs. ΔpH. In graph B, ΔpH determined from the fluorescence quenching (trace B) was plotted as a function of $[NH_4Cl]$; in graph C, ΔpH measured with 9-aminoacridine (trace C) was plotted vs. $[K^+]_{out}$.

different methods: (*a*) by lowering pH_{out} with aliquots of H_2SO_4 until it equalled pH_{in} (trace A); (*b*) by raising pH_{in} with additions of NH_4Cl until it became equal to pH_{out} (trace B); and (*c*) by increasing pH_{in} with added potassium in the presence of nigericin (an ionophore which catalyzes the electroneutral exchange of extragranular K^+ for intragranular H^+) until it equalled pH_{out} (trace C). In all three cases, stepwise decreases in ΔpH enhanced the acridine fluorescence to higher steady-state levels until ΔpH approached zero. Then, further additions of H_2SO_4 (A), NH_4Cl (B), or $[K^+]_{out}$ (C) saturated the fluorescence at a level approximately equal to its initial intensity in the absence of granules.

For quantitative measurements of ΔpH, it was important to take into account possible fluorescence changes caused by direct interactions of 9-aminoacridine with those agents. NH_4Cl (0–80 mM), KCl (0–100 mM), and/or nigericin (10 μg/ml or less) had no effect on the fluorescence intensity. However, 9-aminoacridine in solution (10 μM) was quenched by about 25% when the pH_{out} was lowered from 8 to 5 with added HCl or H_2SO_4. As shown in trace A (Fig. 8), when aliquots of acid decreased pH_{out} from 6.85 to lower values, the fluorescence exhibited a rapid drop in signal followed by a slow time-dependent increase. Those rapid decreases in signal were attributed to quenching of dye by an increasingly acidic medium and thus were considered independent of the ΔpH-dependent response.

For a monoamine dye such as 9-aminoacridine, ΔpH can be theoretically calculated from $\Delta pH = \log [Q/(1 - Q)] \cdot V$, where Q is the fluorescence quenching and V is the ratio of extra to intravesicular volumes (8). The intravesicular volume of granules in a 320 mosM medium was taken to be 4.3 μl/mg protein (31, 32), and ΔpH was calculated from the extent of fluorescence quenching (Q) in graphs A, B, and C (Fig. 8). In trace A, values for Q at each step of the titration were first corrected for the rapid decreases in fluorescence caused by lower values of pH_{out} and then were used to calculate ΔpH (Fig. 8, graph A—●—).

Internal pH was determined from measurements of pH_{out} with a standard electrode minus ΔpH from 9-aminoacridine fluorescence (Fig. 8, graph A—○—) and compared with measurements of pH_{in} from [^{14}C]methylamine distributions (19). In 15 experiments, pH_{in} determined from 9-aminoacridine ranged from 4.6 to 5.2, and for each preparation, equivalent measurements of pH_{in} with radiochemical techniques were higher by 0.7–0.4 pH units. Although values of pH_{in} determined from 9-aminoacridine were 20–35% lower than results from isotopically labeled amine, both methods indicated that pH_{in} remained fairly constant during titrations of the external pH.

ΔpH calculated from the Q values of traces B and C in Fig. 8 were plotted vs. $[NH_4Cl]$ (graph B) and $[K^+]_{out}$ in the presence of nigericin (graph C). Again, 9-aminoacridine predicted somewhat higher values of ΔpH compared to previous results using isotopically labeled amines (16, 19).

The excessive quenching of 9-aminoacridine fluorescence and the subsequent overestimation of ΔpH seemed to be a deviation from the ideal behavior of a fluorescent amine, possibly due to some partial but reversible binding of dye to granules.

Measurements of ΔpH with Atebrin

A diamine molecule, atebrin (also named quinacrine) was similarly used to measure ΔpH in chromaffin granules. In Fig. 9, the excitation and emission spectra of atebrin in solution (spectrum 1) were quenched by the addition of chromaffin granules (spectrum 2). This ΔpH-dependent quenching was also attributed to an accumulation of dye in the granule and was reversed by collapsing the pH gradient (spectrum 3). Note that, although the peak excitation and emission were not displaced in wavelength, the excitation band at 420 nm did not completely recover to its initial intensity. The altered shape of the excitation spectrum suggested that the dye might be interacting with

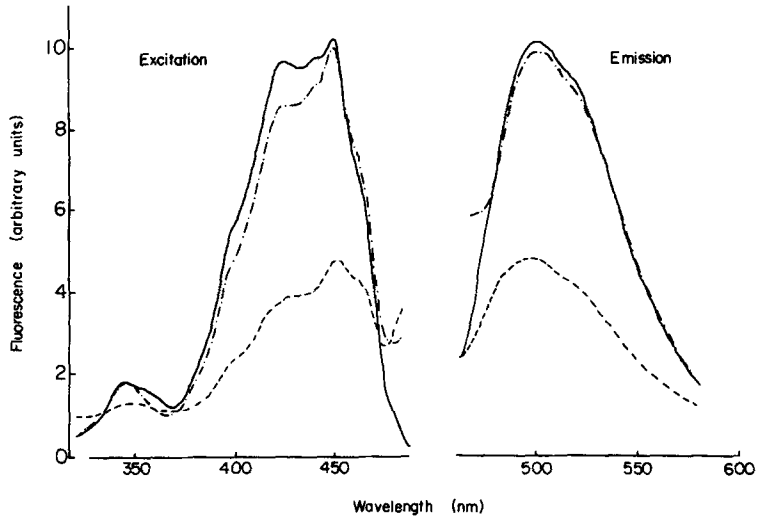

FIGURE 9. Effect of ΔpH on excitation and emission spectra of atebrin. In spectrum 1 (——), the excitation and emission spectra were measured from sucrose solutions containing 5 μM atebrin. In spectrum 2 (---), the addition of granules (0.5 mg protein) with an endogenous ΔpH of 1.5 quenched the excitation and emission bands. In spectrum 3 (····), the quenching of the excitation and emission bands by the addition of granules was reversed by 30 mM $(NH_4)_2SO_4$. However, the excitation peak at 428 did not completely recover to its original amplitude.

the granules. Yet, by using an excitation beam of 450 nm and monitoring the fluorescence at 500 nm, the quenching of atebrin was found to be accurately related to ΔpH. As shown in Fig. 10, the fluorescence of atebrin (10 μM) in a solution at pH 7.0 was quenched by the addition of granules (traces A, B, and C), and its fluorescence was enhanced by gradually decreasing the endogenous pH gradient with added HCl (trace A). For a diamine chromophore that responds to proton gradients by the same hypothetical model, the fluorescence quenching (Q) was related to ΔpH by: $\Delta pH = 1/2 \log [Q/(1 - Q)] \cdot V$ (8). In this experiment, 1 mg of protein of granules was added to a 2-ml cuvette, i.e., $V = 2{,}000\ \mu l/4.3\ \mu l = 465$, and the endogenous ΔpH calculated from Q values

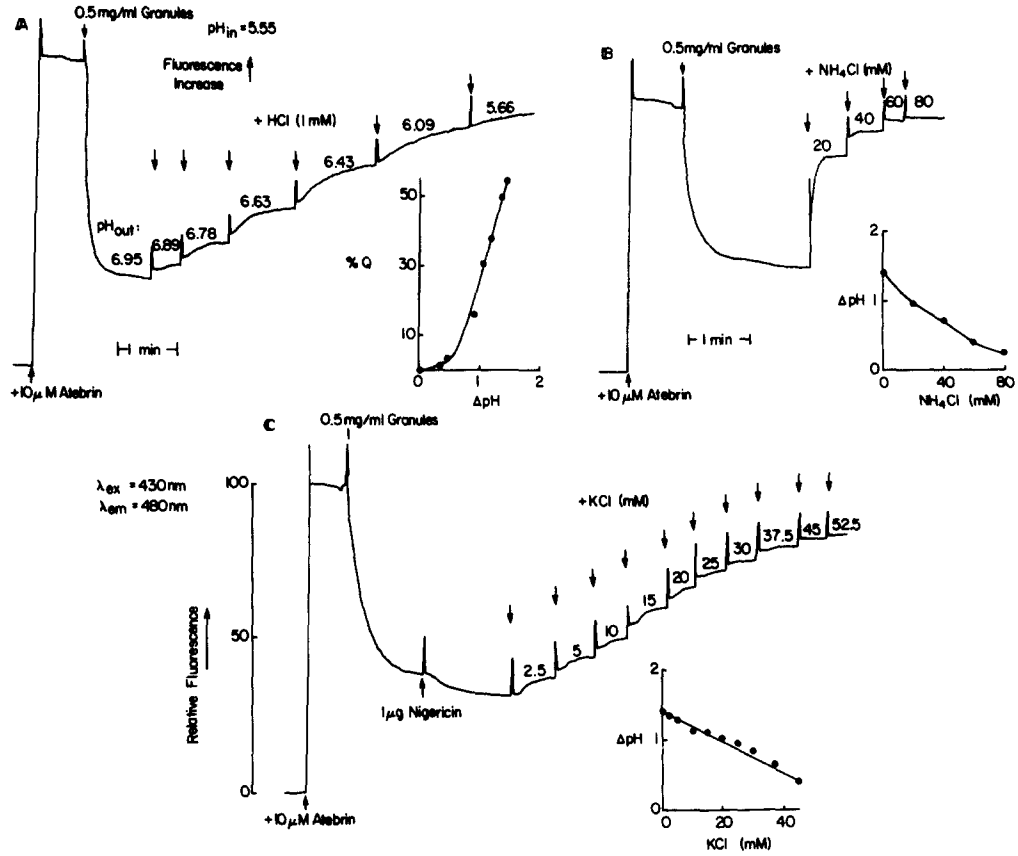

FIGURE 10. Measurements of ΔpH through the quenching of atebrin. The fluorescence of atebrin was monitored from solutions buffered with 10 mM (trace A) and 30 mM (traces B and C) Tris maleate, pH 7.0. The addition of intact granules (1 mg protein) quenched the fluorescence of atebrin. The ΔpH was gradually decreased by additions of HCl (trace A), NH_4Cl (trace B), or K^+ with nigericin, until the fluorescence gradually increased to nearly its initial intensity. ΔpH was calculated from the fluorescence quenching (Q) and the ratio of V_{out}/V_{in} (V) by $\Delta pH = 1/2 \log [Q/(1-Q)] \cdot V$. The 100% fluorescence level was taken to be the fluorescence intensity of dye plus granules at the end of each titration, where ΔpH = 0. In graph A, Q values during the HCl titration were plotted vs. calculated values of ΔpH. In graph B, ΔpH values during the ammonia titration were plotted vs. [NH_4Cl]. In graph C, ΔpH values during the KCl (plus nigericin) titration were plotted vs. $[K^+]_{out}$.

(traces A, B, and C) was equal to 1.4. For every step of the HCl titration (trace A), Q values were used to calculate pH (graph A). pH_{in} determined from pH_{out} minus ΔpH remained rather constant throughout the titration (pH 5.55–5.44). The ΔpH values measured with atebrin during titrations with NH_4Cl (graph B) and $[K^+]_{out}$ in the presence of nigericin (graph C) were in

excellent agreement with corresponding [^{14}C]methylamine experiments (16, 19).

In 12 experiments the steady-state pH_{in} calculated from the quenching of atebrin ranged from 5.3 to 5.7, and fell within 0.2 pH units of equivalent radioisotope experiments (19).

Simultaneous Measurements of $\Delta\psi$ and ΔpH

Optical responses from diS-C_3-(5) and from either 9-aminoacridine or atebrin were simultaneously monitored from suspensions of granules. At steady state, the granules had an endogenous ΔpH and zero membrane potential. The addition of 1 mM MgATP increased the diS-C_3-(5) signal by about 5% (Fig. 11, bottom trace) and did not alter the 9-aminoacridine fluorescence (top trace). The response of the cyanine dye was again associated with an increase in membrane potential, and its magnitude of +53.4 mV was determined from

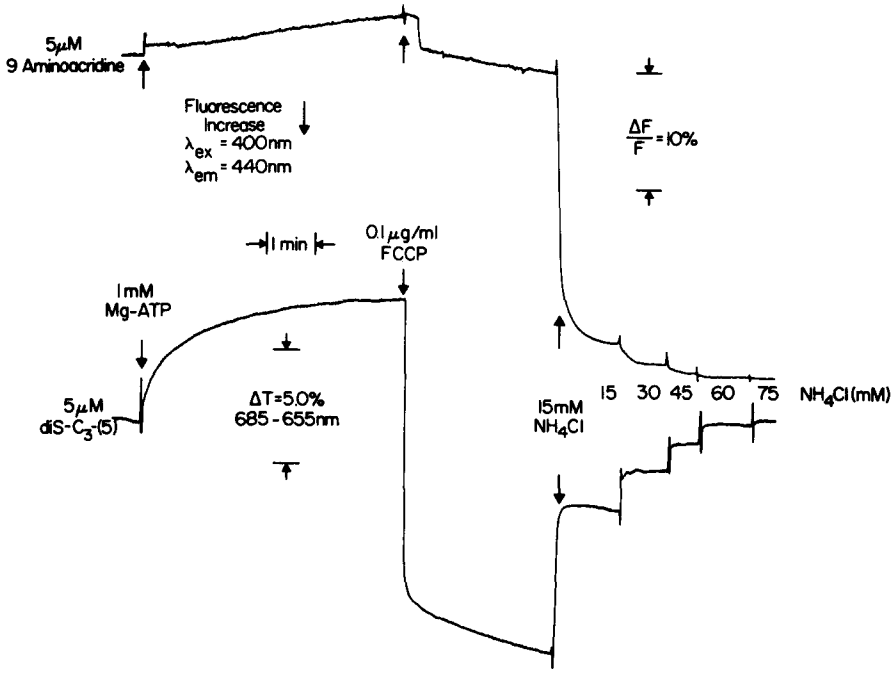

FIGURE 11. Simultaneous measurements of ΔpH and $\Delta\Psi$ with 9-aminoacridine and diS-C_3-(5). The optical responses of 9-aminoacridine (top trace) and diS-C_3-(5) (bottom trace) were monitored from the same suspension of granules in a sucrose medium at pH 7.0. ATP increased the transmittance of diS-C_3-(5) equivalent to $\Delta\Psi$ = 53.4 mV, positive inside, and did not alter the 9-aminoacridine fluorescence. Following the addition of FCCP, diS-C_3-(5) measured a proton diffusion potential $\Delta\Psi$ = −106.7 mV, negative inside, with no change in ΔpH. A titration with NH_4Cl increased both ΔpH and $\Delta\Psi$, and consequently increased the fluorescence of 9-aminoacridine (top trace) and the transmittance of diS-C_3-(5) (bottom trace).

a calibration curve that was obtained by varying the Nernst potential for protons (see Fig. 5). Following the addition of FCCP, the decrease in diS-C_3-(5) transmittance (bottom trace) was equivalent to a shift in $\Delta\psi$ from +53.4 mV, positive inside, to −106.7 mV, negative inside, with no significant change in 9-aminoacridine fluorescence (top trace). In the presence of FCCP, $\Delta\psi$ = −58 ΔpH, such that ΔpH = 1.80, acidic inside, according to the potential measured with the cyanine dye. The potential and pH gradients were then simultaneously decreased to zero by adding large concentrations of amines which raised pH_{in} until $pH_{in} = pH_{out}$. When NH_4Cl was gradually added to the cuvette, the transmittance of diS-C_3-(5) and the 9-aminoacridine fluorescence increased simultaneously until they reached a maximum response of 10 and 25%, respectively. The change in ΔpH was calculated from the fluorescence increase of 9-aminoacridine by taking $Q = 0.25$ and $V = 310$; then ΔpH = 2.0, thus overestimating ΔpH by 0.4, compared to the independent measurement obtained with diS-C_3-(5). Again, the transmittance signal of diS-C_3-(5) at the end of the ammonia titration was exactly equal to its value at the beginning of the experiment; an important observation, which indicates that $\Delta\psi$ = 0 initially, since $\Delta\psi$ is known to be 0 mV in the presence of large concentrations of NH_4Cl (19).

In Fig. 12, a similar experiment was repeated using atebrin instead of 9-aminoacridine. Added ATP produced a positive membrane potential ($\Delta\psi$ = +26 mV) with no change in ΔpH (top trace); added FCCP generated a Nernst potential for protons of −72.5 mV (bottom trace), such that the endogenous ΔpH = 1.25. As before, the titration with ammonia increased both the atebrin fluorescences (top trace) and the transmittance signal of diS-C_3-(5) (bottom trace). In the presence of 60 mM ammonia, ΔpH was completely collapsed and the fluorescence of atebrin increased by 48%. Therefore, the endogenous ΔpH quenched atebrin by 48%, such that $Q = 0.48$, $V = 465$, and ΔpH was 1.32, in excellent agreement with a ΔpH of 1.25 estimated from the Nernst proton potential measured by diS-C_3-(5).

In the absence of the proton ionophore FCCP, ΔpH measured with an acridine dye could be altered by adding HCl or NH_4Cl with no changes in $\Delta\psi$ measured with diS-C_3-(5) (not shown). On the other hand, ATP-dependent membrane potentials, positive inside, could be decreased by large concentrations of lipophylic anions, without altering ΔpH (19). In Fig. 13, a positive membrane potential generated by ATP was detected as an increase in diS-C_3-(5) transmittance (top trace) and was gradually collapsed by 10 mM aliquots of thiocyanate (SCN^-) with no changes in the ΔpH monitored with 9-aminoacridine (bottom trace). With 10 mM SCN^- the membrane potential decreased from +65 to +58 mV, with 30 mM SCN^- $\Delta\psi$ = +15 mV, and with 40 mM SCN^- the diS-C_3-(5) signal recovered to its initial value, i.e., $\Delta\psi$ = 0 (Fig. 13 A, top trace). Moreover, further additions of SCN^- produced no other changes in either $\Delta\psi$ or ΔpH (nor in diS-C_3-(5) or aminoacridine signals). In Fig. 13 B, the stepwise addition of 30 mM SCN^- in the absence of ATP (i.e., $\Delta\psi$ = 0) did not alter the membrane potential detected by diS-C_3-(5). A subsequent addition of 1 mM MgATP generated a small membrane potential

of +15 mV, which was equal to the membrane potential measured in Fig. 13 A when the sequence of SCN^- and ATP additions was reversed. These experiments demonstrate that the two probes can be calibrated precisely and yield consistent results.

Measurements of $\Delta\psi$ and ΔpH in Granule Ghosts

In granule ghosts devoid of the endogenous soluble intragranular components, the spectral characteristics of the cyanine and acridine dyes were analogous to those described in intact granules. The ghosts were resealed in a weakly buffered medium at pH 6.9, containing 1 mM Tris maleate plus 185 mM sodium isethionate, such that at steady state, both $\Delta\psi$ and $\Delta pH = 0$. In ghosts, added ATP was shown to generate a positive membrane potential of 30–40

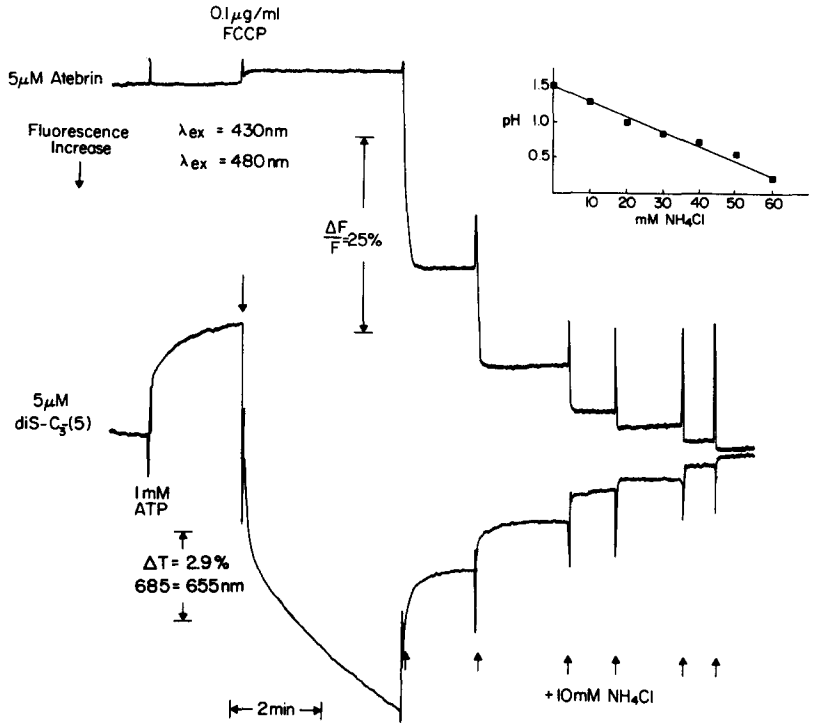

FIGURE 12. Simultaneous measurements of ΔpH and $\Delta\Psi$ with atebrin and diS-C_3-(5). The fluorescence of atebrin (top trace) and the transmittance of diS-C_3-(5) (bottom trace) are simultaneously monitored from the same suspension of granules (0.85 mg protein) in 0.27 mM sucrose plus 10 mM Tris-maleate at pH 7.0. Added ATP evoked a positive membrane potential associated with a 3% increase in ΔT (bottom trace), i.e., $\Delta\Psi = 4.30$ mV, but no change in ΔpH or atebrin fluorescence (top trace). FCCP induced a Nernst proton potential associated with an 8.9% decrease in ΔT (bottom trace), and no change in atebrin fluorescence (top trace). Titration with NH_4Cl collapsed ΔpH, which enhanced the fluorescence of atebrin (top trace), and $\Delta\Psi$, which enhanced the diS-C_3-(5) signal (bottom trace).

mV (33, 34). However, in the presence of membrane-permeable anions, such as chloride, the magnitude of the potential was greatly reduced, but instead added ATP generated a ΔpH acidic inside. Thus, by increasing the ratio of chloride to impermeant anion concentrations from 0 to 100% chloride, ATP-induced ΔpH increased from 0 to 1 while $\Delta\psi$ decreased from +40 to 0 mV (34).

In Fig. 14, the diS-C_3-(5) fluorescence was measured from vesicles (1 mg protein/ml) suspended in a Cl^--free medium. Added ATP reduced the diS-C_3-(5) fluorescence, which, as in granules (Fig. 4), indicated the development

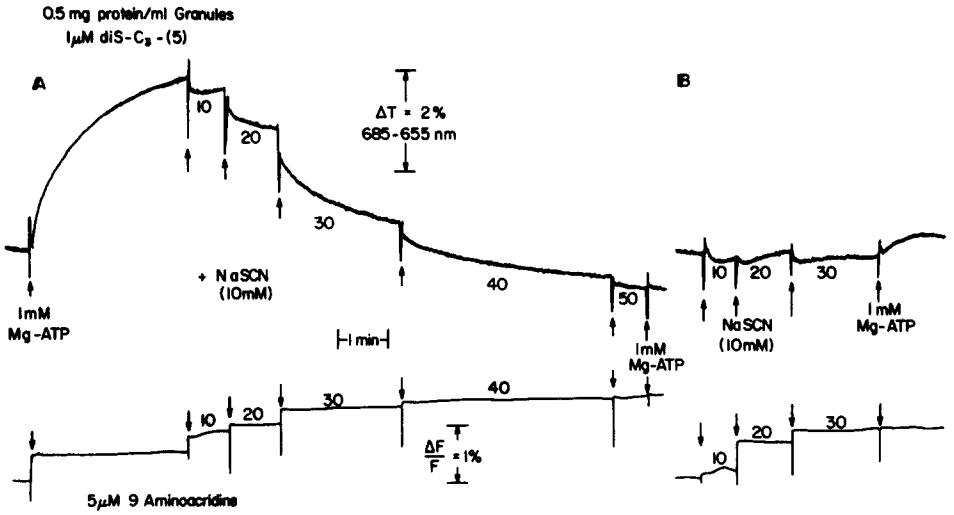

FIGURE 13. Effect of lipophylic anions on ΔΨ and ΔpH. The fluorescence of 9-aminoacridine (bottom trace) and the transmittance of diS-C_3-(5) (top trace) are monitored from granules (0.5 mg protein) suspended in a sucrose medium at pH 7.0. (A) A membrane potential, positive inside (top trace) was generated by added ATP, with no change in ΔpH measured with 9-aminoacridine (bottom trace). The membrane potential measured with diS-C_3-(5) (top trace) was decreased by additions of thiocyanate (SCN^-), with no changes in ΔpH measured with 9-aminoacridine (bottom trace). Further additions of SCN^- produced no changes in ΔΨ or ΔpH. (B) In the absence of ATP, three additions of 10 mM SCN^- did not alter ΔΨ (top trace) or ΔpH (bottom trace). A subsequent addition of ATP generated a ΔΨ equal to the potential just measured in the previous experiment, where ATP and SCN^- were added in the reverse order.

of a membrane potential, positive inside. FCCP enhanced the fluorescence above its initial intensity, indicating a slight negative potential before reaching a steady state at its initial level, i.e., $\Delta\psi = 0$, since there was no endogenous ΔpH, and a ΔpH was not generated in the absence of chloride. To calibrate the response of the cyanine dye vs. membrane potential, the vesicles were suspended in a medium where ATP could generate a substantial ΔpH, such that in the presence of FCCP the Nernst potential for protons could be systematically varied.

In Fig. 15, the diS-C_3-(5) transmittance (bottom trace) and the 9-aminoacridine fluorescence (top trace) were simultaneously monitored from vesicles (0.9 mg protein/ml) suspended in a 30% chloride medium. Initially, $\Delta\psi$ and ΔpH were both equal to zero. Added ATP generated both a positive membrane potential of 13.3 mV (see the calibration of Fig. 15, *inset*) associated with an increase in diS-C_3-(5) transmittance (bottom trace) and a ΔpH (acidic inside), which quenched the 9-aminoacridine fluorescence (top trace). In the presence of FCCP, the Nernst potential for protons, $\Delta\psi = 58$ ($pH_{in} - pH_{out}$), was detected through a decrease in the transmittance of the cyanine dye (bottom trace). When pH_{out} was reduced from 6.9 to 5.7 with aliquots of HCl, the 9-aminoacridine fluorescence (top trace) and the diS-C_3-(5) transmittance signals (bottom trace) increased. The potential-sensitive response of diS-C_3-(5) was then calibrated vs. membrane potential by the same method described for intact granules. The Nernst potential for protons was 0 mV when $pH_{out} = pH_{in}$ and the diS-C_3-(5) signal reached its initial value prior to the addition

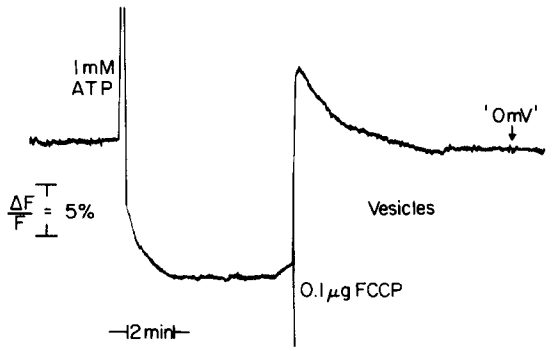

FIGURE 14. Fluorescence measurements of $\Delta\Psi$ in granule ghosts. Vesicles were suspended in a Cl^--free medium containing 185 mM K gluconate and 30 mM MOPS at pH 7.0. At steady state, $\Delta\Psi$ and ΔpH = 0. The ghosts generated a positive membrane potential with added ATP which produced a decrease in diS-C_3-(5) fluorescence, as in intact granules. The ionophore FCCP collapsed the membrane potential, which evoked a fluorescence increase above the original base line, followed by a phasic recovery to the initial fluorescence level.

of ATP; this occurred when $pH_{out} = 6.2$. For $pH_{out} > 6.2$, the Nernst potential was negative inside, and for $pH_{out} < 6.2$, it was positive inside. ΔT was plotted vs. $\Delta\psi$ in the narrow range of -45 to $+30$ mV, where pH_{out} varied by 1.2 units, such that pH_{in} remained fairly constant during the titration (Fig. 15, *inset*). The response of the cyanine dye was linear with membrane potential with a slope of 0.15% ΔT per millivolt, in good agreement with calibration curves obtained with intact granules (Figs. 5 and 6). The similarity of the calibration curves in ghosts and granules becomes more impressive when one considers that granules depleted of soluble proteins weigh ~35% less than intact granules (6.6 vs. 4.3 μl/mg protein), such that the membrane permeable diS-C_3-(5) molecules (28, 29) inside the ghost can bind to fewer aspecific sites where a transmembrane potential cannot be detected.

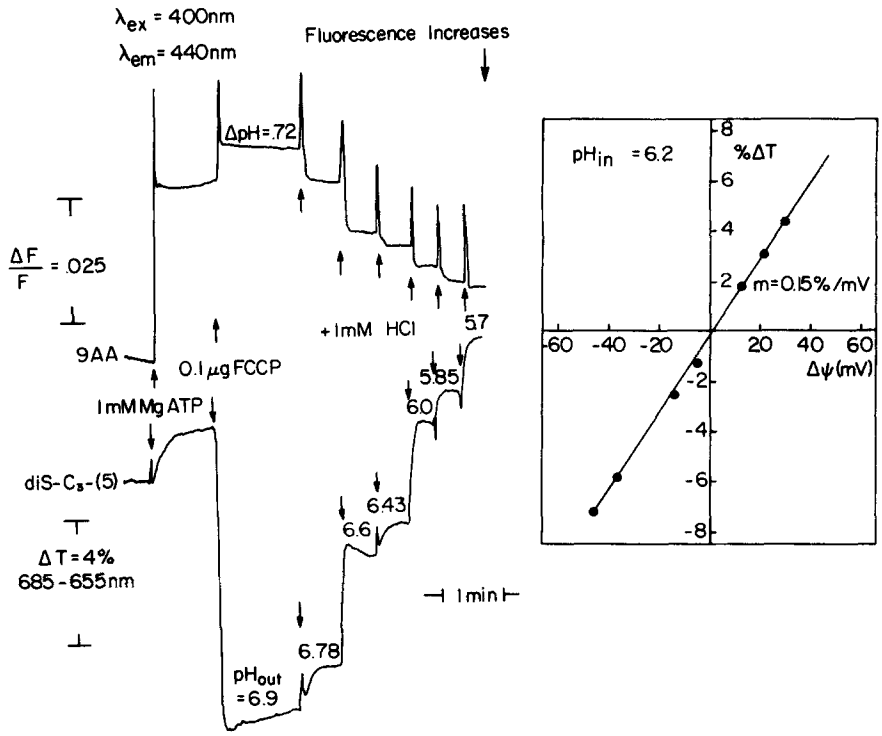

FIGURE 15. Calibration of diS-C_3-(5) transmittance with the Nernst potential for protons in granule ghosts. The fluorescence of 9-aminoacridine (top trace) and the transmittance of diS-C_3-(5) were monitored from granule ghosts (0.9 mg protein/ml) in 185 mM sodium isethionate, 10 mM Tris-maleate, 40 mM $MgCl_2$, 5 μM 9-aminoacridine, and 1 μM diS-C_3-(5) at pH 7.0. At steady state, the potential and pH gradients of the ghosts are zero. With added ATP, the vesicles generated both pH and potential gradients which were detected as a quenching of 9-aminoacridine (top trace) and an increase in diS-C_3-(5) signal (bottom trace). FCCP decreased the diS-C_3-(5) signal by establishing a Nernst proton potential $\Delta\Psi = 58\ \Delta pH$. Titrating the external pH with aliquots of acid reduced ΔpH to 0, thereby increasing the 9-aminoacridine fluorescence and the diS-C_3-(5) voltage-sensitive response. When $\Delta T = 0$, $pH_{out} = pH_{in} \simeq 6.2$. From measurements of pH_{out} and pH_{in} taken to be constant, ΔT was linear with the Nernst proton potential, with a slope of 0.15% T/mV (see *inset*).

The intravesicular volume was taken to be 6.6 μl/mg protein (35), and the ΔpH-dependent fluorescence decrease as 3.0%. Then, $V = 2{,}000\ \mu l/11.8\ \mu l \simeq 170$, $Q = 0.03$, and $\Delta pH = 0.72$. Thus, with $pH_{out} = 6.9$, added ATP generated a $\Delta pH = 0.72$, such that $pH_{in} = 6.18$. This result is in excellent agreement with estimates of 6.2 from the potential-sensitive cyanine and 6.35 from radiochemical experiments done on vesicles in a 30% chloride medium (34).

These experiments demonstrate that the cyanine dye acts as a membrane potential probe in granule ghosts as well as intact granules, and therefore that its voltage-sensitive response in intact granules was not mediated by a dye interaction with intragranular soluble proteins.

DISCUSSION

The main results of this paper are that the optical responses of diS-C_3-(5) and of either 9-aminoacridine or atebrin can be used to measure quantitatively and simultaneously $\Delta\psi$ and ΔpH in chromaffin granules. At low dye-to-granule concentration (1 μM dye/mg protein), optical responses of diS-C_3-(5) are linear with respect to positive and negative transmembrane electrical potentials. The diS-C_3-(5) signals appear to be generated by a potential-dependent dye binding to granules, such that membrane potentials more negative inside increase the concentration of bound dye molecules, and vice versa for potentials more positive inside. The presence of cyanine dye does not alter the magnitude of either pH gradients or membrane potentials, which suggests that the $\Delta\psi$ indicator is nontoxic in that it does not perturb the basic properties of the chromaffin granules. The quenching of 9-aminoacridine or atebrin can be used to measure ΔpH and appears to be produced by a distribution of dye in and out of the granules, according to the proton gradient.

Calibration of diS-C_3-(5) Response vs. $\Delta\psi$

When $\Delta\psi$ was varied according to the Nernst potential for protons (Fig. 5) or the Nernst potential for potassium (Fig. 6), the transmittance of diS-C_3-(5) measured at 685–655 nm was linear with the membrane potential, with a slope of about 0.1% ΔT per millivolt. The calibration of the dye was necessarily constrained to within the range of potential (from −90 to +100 mV) attained under conditions that do not lyse the granules or induce their aggregation (i.e., osmotic shock or low external pH).

The calibration of diS-C_3-(5) with the proton diffusion potential (Fig. 5) required two plausible assumptions that were verified experimentally: first, that $\Delta\psi = 0$ mV before the addition of FCCP; and second, that pH_{in} remained constant during the acid titration. In freshly prepared chromaffin granules, the membrane potential at the beginning of an incubation at 24°C was found to be 0 mV but then gradually decreased to −20 mV in ~20 min (19). Although the present experiments were run at room temperature, they began within 5 min of transferring the granules from a 4 to a 24°C medium. The potential-dependent diS-C_3-(5) signals also confirmed that $\Delta\psi = 0$ at the beginning of the experiment. In the presence of FCCP and large concentrations of ammonia, $\Delta\psi$ is known to be 0 mV, and under those conditions the potential-dependent signals were equal to signals measured at the beginning of the experiment (see Figs. 4, 12, and 13). Moreover, even if $\Delta\psi$ were not exactly 0 mV at the onset of the experiment, a slight error could be introduced which would merely shift the calibration curve (Fig. 5, *inset*) without affecting the linearity of ΔT vs. $\Delta\psi$. During titrations of pH_{out} (from pH 7 to 5) in the presence of FCCP, pH_{in} was shown to remain constant (17, Figs. 8 A and 10 A). This is a consequence of the high buffering capacity within the granules (15, 36). The calibration of ΔT vs. $\Delta\psi$ from the Nernst potential for potassium depended on the concentration of free potassium in the granules, $[K^+]_{in}$. To determine $[K^+]_{in}$, granules were suspended in a medium of varying $[K^+]_{out}$, such that added valinomycin induced potential-dependent diS-C_3-(5) re-

sponses as a decrease in transmittance for $[K^+]_{out} < [K^+]_{in}$, and increase in transmittance for $[K^+]_{out} > [K^+]_{in}$, and presumably no change in signal for $[K^+]_{out} = [K^+]_{in}$. Thus, when added valinomycin did not change the diS-C_3-(5) signal, $[K^+]_{in}$ was taken to be equal to $[K^+]_{out} \simeq 2$ mM, and then $[K^+]_{out}$ was further increased to obtain a calibration curve (Fig. 6).

Measurements of ΔpH with fluorescent amines also indicated that $[K^+]_{in} \simeq 2$ mM. For granules in a K^+-free medium, the electroneutral co-transport of K^+/H^+ catalyzed by nigericin increased the pH gradient (more acidic inside since the efflux of K^+ was accompanied by an influx of protons (Figs. 8 C and 10 C). In principle, this increased acidity of the intragranular volume depends on $[K^+]_{in}/[K^+]_{out}$, and since the addition of 2 mM KCl reversed this change in ΔpH, this confirms that $[K^+]_{in} \simeq 2$ mM.

It is important to note that a calibration curve could not be obtained when valinomycin was first added to granules suspended in a K^+-free medium. In this case, $\Delta\psi$ became transiently negative inside, then collapsed to 0 mV instead of maintaining a stable negative K^+ diffusion potential (Fig. 16, bottom trace). This phasic time-course of $\Delta\psi$ measured with diS-C_3-(5) could not be attributed to a dye-valinomycin interaction (see Materials and Methods), and was furthermore supported by radiochemical measurements, which detected a membrane potential of 0 mV following a 5-min incubation in a K^+-free medium containing valinomycin instead of a stable negative membrane potential.[1]

The linear relationship between diS-C_3-(5) response and $\Delta\psi$ was observed for ratios of dye-to-granule concentrations of 1 μM diS-C_3-(5) per milligram protein or lower, but deviated from linearity when this ratio was increased by a factor of 8, or with more turbid suspensions.

In previous studies, the fluorescence enhancement of ANS (1-amilinonaphthalene-8-sulfonic acid) upon the addition of Mg^{++}/ATP was interpreted as an "energized state" of the chromaffin granules (37). Through analogies with experiments on mitochondria, the changes of ANS fluorescence appeared to reflect the movement of protons into or out of the granules but could not be linked to a measurement of transmembrane potential (37).

Mechanism Controlling the diS-C_3-(5) Potential-Dependent Optical Responses

In human red blood cells, the fluorescence of diS-C_3-(5) was found to decrease during a hyperpolarization (potential more negative inside) and decrease during a depolarization (38). These signals were opposite in direction to those measured in granules. The large fluorescence changes (80%) from diS-C_3-(5) were related to expected diffusion potentials generated by varying the $[K^+]$ in the presence of valinomycin, or $[Na^+]_o$ in the presence of nystatin (39) or $[Cl^-]_o/[Cl^-]_{in}$. Through centrifugation and spectrophotometric measurements, Sims et al. (29) showed that a membrane hyperpolarization increased the dye concentration within the cells compared to the extracellular dye concentration, and that cell-associated dye molecules formed nonfluorescent aggregates. Thus, the mechanism responsible for the voltage-sensitive response of diS-C_3-

[1] Salama, G., R. G. Johnson, and A. Scarpa. Unpublished results.

(5) was explained as a displacement of dye molecules from a fluorescent monomeric state in the extracellular medium to a nonfluorescent, cell-associated dimer state (29, 38). Experiments in dye binding to cell hemolysates further indicated that in the human red blood cell the principal site for dye binding and aggregation was oxyhemoglobin (20). From such a mechanism, a calibration of the diS-C_3-(5) fluorescence (ΔF) vs. membrane potential ($\Delta\psi$) changes could not be readily standardized, but was found to depend on

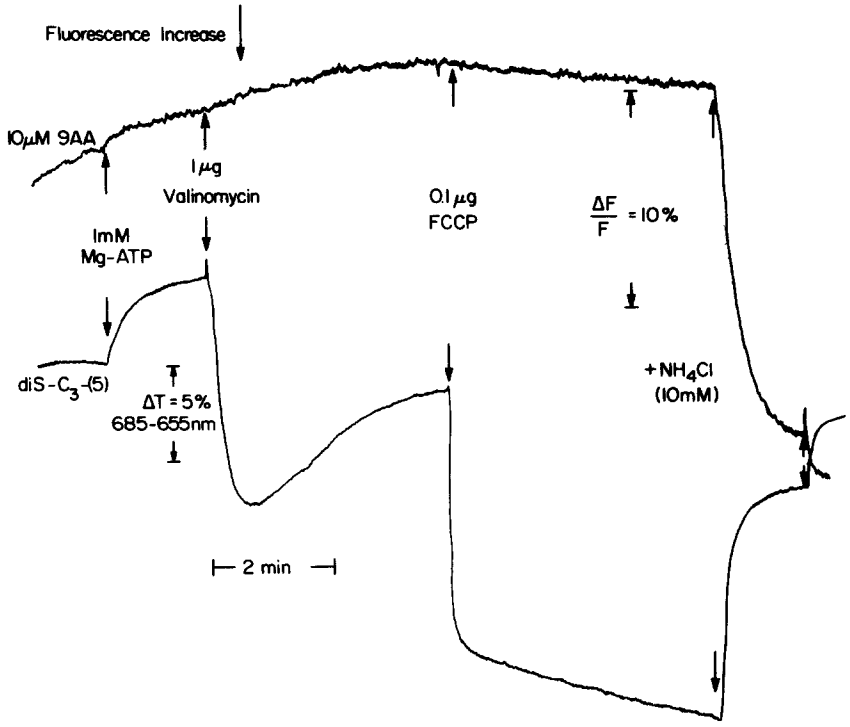

FIGURE 16. Effect of valinomycin on the membrane potential of granules in a K^+-free medium. The 9-aminoacridine fluorescence (top trace) and the voltage-sensitive response of diS-C_3-(5) were measured from intact granules in a potassium-free solution at pH 7. ATP evoked the usual positive membrane potential (bottom trace) with no change in ΔpH (top trace). Since $[K^+]_{in} > [K^+]_{out}$, valinomycin (0.5 μg/ml) induced a negative Nernst potential for potassium, but the signal was phasic, and decreased to its initial base line in 4 min. As before, FCCP established a negative proton diffusion potential with a decrease in ΔT (bottom trace) and NH_4Cl collapsed both ΔpH (top trace) and $\Delta\Psi$ (bottom trace).

the manner in which $\Delta\psi$ was varied, the internal pH, the relative concentration of dye and cells, and the choice of wavelengths (39, 40). Moreover, at low dye concentrations, the voltage-dependent signals of diS-C_3-(5) were produced by a different process, possibly through changes in fluorescence yield of membrane-bound dye rather than dye aggregation in the cells (40).

Experiments with granule ghosts (Figs. 14 and 15) and the potential-dependent spectral data (Figs. 3 and 4) ruled out the formation of dye aggregates in chromaffin granules. Instead, the spectral changes were best interpreted as a potential-dependent dye binding to the granules. Membrane potentials more positive inside reduced the concentration of bound dye (the chromophore is positively charged), thereby decreasing the fluorescence emission at 690 nm (Fig. 3 A, spectrum 2). Concurrent with a reduction of bound dye, the absorption of the suspension at positive membrane potentials has a maximum at about 650 nm (Fig. 3 B, spectrum 4), similar to the absorption spectrum of diS-C_3-(5) in solution in the absence of granules (Fig. 1 A). Membrane potentials, negative inside, increased the concentration of bound dye and accordingly generated an enhanced fluorescence at 690 nm (Fig. 3 A, spectrum 3), and shifted the peak absorption towards longer wavelengths (Fig. 3 B, spectrum 2).

The lack of an absorption peak at lower wavelengths representing dye molecules in an aggregate state (29) and the similarity in the slope of ΔT vs. $\Delta\psi$ for intact granules (Figs. 5 and 6) and vesicles (Fig. 15) strongly suggested that changes in dye binding to the membrane of chromaffin granules generated the potential-sensitive response of diS-C_3-(5). Thus, in red blood cells, diS-C_3-(5) was bound to the cell membrane and to intracellular oxyhemoglobin as a function of both $\Delta\psi$ and intracellular pH (39). In granules, however, the dye did not interact with intragranular proteins, and consequently its optical responses specifically followed the changes in $\Delta\psi$ and did not depend on internal pH.

Measurements of ΔpH with Acridine Dyes

In earlier studies, the uptake of [^{14}C] methylamine and the quenching of 9-aminoacridine by granules were found to vary with external pH, which was interpreted as a redistribution of amines according to the transmembrane potential rather than ΔpH (31). In the present experiments, the fluorescence quenching of 9-aminoacridine (Fig. 7) or atebrin (Fig. 9) was functionally related to ΔpH. A determination of ΔpH using the formulae given by Shuldiner et al. (8) for fluorescent monoamine or diamine molecules necessarily takes on all the assumptions of the model previously described for chloroplasts (8). To test the validity of the model and to demonstrate that the fluorescence responses to changes in ΔpH were not merely coincidental, ΔpH was determined from the acridine fluorescence and from [^{14}C]methylamine distributions under various conditions (Figs. 8, 10, 11, 12, and 13). The optical and radiochemical techniques were found to be in rather good agreement. It is important to note that the two methods are based on similar principles. Both methods rely on the distribution of charged amines according to the ratio of hydrogen ion concentrations in and out of the vesicles. However, the optical techniques where a chromophore is attached to the amine groups require the further assumption that dye molecules accumulated in the vesicles become nonfluorescent (8). Several factors may contribute to the fluorescence quenching: (*a*) the intrinsic fluorescence of the dye may be quenched by a more acidic medium; (*b*) dye in the intragranular compartment may be

"screened" by the surrounding pigmented membrane; (*c*) at high concentrations, the fluorescence of dye molecules accumulated within the granules may be self-quenched through energy transfer between molecules of the same species; and (*d*) dye may bind to other molecules located on the membrane or within the granules. The transfer of dye molecules from a basic to an acidic medium inside the granules would not entirely explain the large ΔpH-sensitive responses, since the fluorescence of acridine dyes in solution was not significantly quenched by decreasing the pH from 7 to 5. The pigmented membrane of chromaffin granules is not likely to severely screen the strong fluorescence of dye accumulated within the granules. On the other hand, for ΔpH = 1.5–1.8, sufficiently high concentrations of amines would build up within the granules (310–650 μM) to induce self-quenching and thus would readily account for those large and reversible fluorescence changes. It would be desirable if self-quenching prevailed as the dominant mechanism, since it would imply that the dye molecules remain freely permeable across the membrane. However, dye binding to membrane or molecules within the vesicles couldn't be conclusively excluded as contributing to the quenching of both 9-aminoacridine and atebrin. The spectral measurements suggested that some atebrin remained bound to the granules (Fig. 9), whereas a similar interaction was not apparent for 9-aminoacridine.

However, ATP (10 mM) was found to quench the fluorescence of 9-aminoacridine by ~15% without altering the shape of its excitation and emission spectra. Since granules have a high content of endogenous ATP (approximately 1000 nmol/mg of protein), 9-aminoacridine might not be distributed solely as a function of proton gradient, but excess dye may be accumulating in the granules and binding to ATP. The reversible binding of dye to ATP could explain why 9-aminoacridine gave higher values for ΔpH and why the time-course of its fluorescence changes was three times slower than with atebrin (compare time scales in Figs. 8 and 10 and Figs. 11 and 12). This hypothetical effect of high ATP concentrations on the response of 9-aminoacridine was supported by experiments done on ghost granules depleted of endogenous ATP. In ghosts, values of ΔpH from 9-aminoacridine were in much better agreement with values for ΔpH determined with diS-C_3-(5) or isotope experiments (see Fig. 15 and text), and most of all, the time-course of the fluorescence changes was considerably faster in ghosts than with intact granules.

Thus, in spite of some interactions between acridine dye and granules, the values for ΔpH determined from fluorescent and isotopically labeled amines were similar and within experimental error of both methods.

In summary, the membrane-permeable dyes have been shown to provide a highly reproducible method for the measurement of $\Delta\psi$ and ΔpH in isolated chromaffin granules and ghosts. The magnitude and polarity of $\Delta\psi$ and ΔpH obtained through the cyanine and acridine dyes were not only self-consistent, but were also in excellent agreement with values obtained by other methods. The advantage of optical measurements are fourfold when compared to existing techniques. First, minimal amounts of material are needed per incubation sample (~0.5 mg of protein/ml), compared with measurements by

radiolabeled compounds (~5 mg of protein/ml), or by ^{31}P nuclear magnetic resonance (NMR) techniques (~50 mg of protein/ml), which require one to two orders of magnitude higher concentrations of granules for each experimental data point of $\Delta\psi$ or ΔpH. Second, optical methods are particularly well suited for kinetic measurements during the generation or collapse of ΔpH and/or $\Delta\psi$, because, unlike radiochemical or NMR techniques, these measurements do not depend upon individual sampling times, centrifugation, or long acquisition times. Third, in each preparation of granules, the endogenous pH gradient allows for a rapid internal calibration of both optical responses with the proton gradient and the membrane potential by accurately controlling the Nernst proton potential in the presence of FCCP. Fourth, the absence of dye-dye interactions and the wavelength separation of the cyanine and acridine dyes' fluorescence and absorption spectra made it possible to monitor their responses simultaneously, and hence to determine both $\Delta\psi$ and ΔpH from the same sample.

In a broader sense, because of its endogenous pH gradient and the generation of a proton diffusion potential of known magnitude, the chromaffin granule may prove to be an excellent system for the rapid screening of potential sensitive dyes. In conclusion, these results indicate that the application of these indicators may provide a practical alternative for studying the properties of membranes from other amine-containing secretory granules (such as platelet granules or mast cell granules), which can only be isolated in extremely small quantities and which, up to now, could not be examined by standard techniques.

The authors are indebted to David Pfister and Sally Carty for preparing the chromaffin granules and to Dan Brannen for preparing the manuscript.
This work was supported by grants 77-675 from the American Heart Association and HL-18708 and HL-24010 from the National Institutes of Health. This research was carried out during Dr. Salama's tenure of a postdoctoral fellowship from Muscular Dystrophy Association. Mr. Johnson is the recipient of grant GM-20246 of the Medical Student Training Program of the National Institutes of Health.

Received for publication 25 June 1979.

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