Effect of transmembrane ion gradients on Raman spectra of sealed, hemoglobin-free erythrocyte membrane vesicles

(cyanine dye technique/protein methyl stretching scattering/ionophores/membrane asymmetry)

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ABSTRACT Sealed hemoglobin-free erythrocyte vesicles have been isolated. Imposition of transmembrane cation gradients increases the intensity of Raman scattering in the CH3-stretching region as observed with unsealed ghosts at temperatures >35°C and pH <7.0 (Verma, S. P. & Wallach, D. F. H. (1976) Proc. Natl. Acad. Sci. USA 73, 3358-3361). Modifications in the amide I and amide III frequencies consistent with increased helicity of membrane proteins are observed upon imposition of a cation gradient. Spectrin-free vesicles also demonstrate cation gradient-sensitive intensity changes in the CH3-stretching region. However, no evidence for cation gradient-related protein conformational changes is found with these vesicles. The transmembrane potential of these vesicles has been altered by variations in anion composition and the electrogenic activity of Na+, K+-ATPase. The membrane potential was monitored by cyanine dye fluorescence. Imposeion of a membrane potential (negative inside) also increased the intensity of Raman scattering in the CH3-stretching region. These results suggest that a transmembrane potential (negative inside) and/or cation gradient can energize membranes by compression of the apolar region and transfer of protein methyl residues into polar regions.

Erythrocyte membranes undergo pH-sensitive thermotropic changes of state at near physiological temperatures (1-7): in whole cells, membrane elastic area-compressibility shifts abruptly near 45°C (1) and membrane potential breaks down near 41°C (2). In ghosts, tryptophan fluorescence can be quenched by nitrooxide analogs of stearic acid (3, 4) in a manner indicating that the protein fluorophores become abruptly more accessible about 37°C. This process is reversible below 42°C and shifts to lower temperatures upon pH reduction. Electron spin resonance studies (5) with nitroxide-labeled lipids and erythrocyte membranes also demonstrate a pH-sensitive thermal transition at 37.5-40.5°C. Proton magnetic resonance (6) shows that methyl side chains of membrane proteins become more mobile as the temperature is raised above about 35°C. Raman spectroscopy (7) reveals a sharp, thermotropic pH-sensitive discontinuity in the membrane protein CH3-stretching signal, which at pH 7.0-7.5 has a lower limit of 38°C and is irreversible above 42°C (7). Reduction of pH to 6.5 lowers the transition temperature by about 16°C (7). Raman spectra during thermally induced unfolding of ribonuclease at acid pH and evaluation of model compounds indicate that the thermotropism of the methyl-stretching signals arises from the transfer of side-chain methyl groups from apolar to more polar regions (8). We now show that transmembrane ionic gradients across hemoglobin-free, erythrocyte membrane vesicles influence membrane protein Raman signals.

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EXPERIMENTAL

All chemicals were of the highest purity grade available. Hepes, valinomycin, ATP (Na+), and ouabain were obtained from Sigma (St. Louis, MO), 1H2O from New England Nuclear (Boston, MA), egg lecithin from Lipid Products (United Kingdom), and reagents for the synthesis of 3,3'-dihexyldithiocarbocyanine iodide from Aldrich (Metuchen, NJ). Erythrocyte ghosts were isolated from freshly drawn human blood, by established procedures (9). As shown by analyses using dodecyl sulfate/polyacrylamide gel electrophoresis (10), these membranes are free of hemoglobin. Ghosts or spectrin-free vesicles, prepared as in ref. 9, were diluted with buffer of desired intravesicular ion composition and equilibrated about 16 hr at 4°C. The membrane suspensions were vesiculated by rapid expulsion from a 30-ml syringe through a 1-cm, 25-gauge needle, and this was repeated twice. The resulting vesicles were pelleted at 6 x 105 g-min and washed once at 4°C in the desired extravesicular buffer to form the transmembrane gradients. Vesiculation followed the procedure described in ref. 10, and caused no change in membrane protein composition other than elution of band 6 (9), identified as adsorbed glyceraldehyde-3-phosphate dehydrogenase (11). For Raman evaluation of amide bands, 95% 2H2O was substituted in buffers for H2O, as stated.

In some experiments, erythrocyte ghosts in 5 mM sodium phosphate (pH 8.0) were equilibrated with 1 mM ATP for 30 min at 0°C and then diluted 1:20 by addition of 65 mM NaCl/75 mM KCl/10 mM Hepes/1 mM MgCl2 (pH 7.5). After an additional 30 min at 0°C, the membranes were vesiculated and concentrated by centrifugation as described.

Total erythrocyte lipid was extracted with chloroform/methanol (2:1 vol/vol) as in ref. 12. Erythrocyte lipid and egg lecithin vesicles were prepared by suspension of N2-dried lipids into 65 mM NaCl/75 mM KCl/10 mM Hepes/0.25 mM MgCl2 (pH 7.5) followed by sonication for 30 min at 4°C. Vesicles were washed once in the same buffer or in 140 mM NaCl/10 mM Hepes/0.25 mM MgCl2 (pH 7.5).

The electrochemical potential of the vesicles was monitored with the cyanine dye technique (13, 14), using 3,3'-dihexyldithiocarbocyanine iodide, synthesized as in ref. 13. Fluorescence was measured at 25°C [Perkin Elmer MFP8 spectrophotometer; excitation at 520 nm, emission at 580 nm (slits 10 nm and 12 nm, respectively)] with 0.1 mg of membrane protein, 1 mM dye, and 1 mM valinomycin as K+ ionophore. Phase microscopy was with an Olympus instrument (Tokyo, Japan). Protein was assayed as in ref. 15.

Raman spectra were recorded at 20°C within 1 hr after establishing transmembrane gradients and concentration to ~10 mg of protein per ml by centrifugation. We then proceeded as in refs. 16 and 17, using a Ramalog 4 spectrometer (Spex Industries, Metuchen, NJ) interfaced to an Interdata computer.
and without valinomycin is incorporated into the Nernst equation as follows (13):

$$V_m = 59 \text{ mV} \cdot \log \frac{[K_e]}{[K_i]}$$

to evaluate the K⁺ equilibrium. Here $[K_e] = \text{extracellular} [K]$, $[K_i] = \text{intracellular} [K]$, and $V_m = \text{K⁺ equilibrium potential in millivolts.}$

In determining the membrane potential of spectrin-containing vesicles (Fig. 1), plots of cyanine fluorescence against external [K⁺] are nonlinear at low values of [K⁺], as with intact erythrocytes (14). However, extrapolation of the linear portions of the curves ([K⁺] > 1 mM) to the null point yields a K⁺ equilibrium potential of $-26.1 \pm 1.3 \text{ mV.}$ The K⁺ gradients that maintain this potential are stable for $\geq 4 \text{ hr.}$

The K⁺ potential of spectrin-free vesicles varied with time, with the most negative value, $-25 \text{ mV}$, determined within 1 hr after establishment of gradient. Four hours after gradient formation, the potential was $-6 \text{ mV.}$

**Raman spectroscopy**

**CH-Stretching Region.** This region of the membrane spectrum (Fig. 2A) always shows three major bands at 2850 cm⁻¹, ~2890 cm⁻¹, and ~2930 cm⁻¹ (symmetric and antisymmetric CH₂-stretching and CH₃-stretching, respectively). The intensity of the 2850 cm⁻¹ band is stable to changes of environmental conditions at temperatures above the acyl chain liquid–crystalline transition and can be used as an internal standard (7, 8). Upon imposition of a K⁺ gradient, the intensity of the 2930 cm⁻¹ feature increases relative to that at 2850 cm⁻¹; i.e., the intensity ratio $I_{2930}/I_{2850}$ becomes much larger than for unsealed ghosts (7) or vesicles without gradient (Table 1).

Amide I, III, and III' Regions. When examined in $^2\text{H}_2\text{O}$ to minimize interference at 1640 cm⁻¹ by HOH bending, the amide I region of native vesicles without a K⁺ gradient shows a diffuse feature peaking at 1665 cm⁻¹, the amide I' band (Fig. 2B). Impostion of a K⁺ gradient resolves this component into two bands at 1655 cm⁻¹ and 1640 cm⁻¹. Scattering at 1665

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**RESULTS**

**Evaluation of cation gradients**

As shown with intact cells (13, 14), addition of cyanine dye to erythrocyte vesicles causes an increase in fluorescence over that observed with dye in aqueous solution alone. This fluorescence increment depends on the transmembrane potential. This dependency forms the basis for the null method of potential measurement (13), where intracellular [K⁺], as well as intracellular and extracellular ionic strengths are kept constant, while the extracellular [Na⁺]:[K⁺] ratio is varied. Cyanine fluorescence is measured before and after establishment of a new transmembrane K⁺ equilibrium by addition of valinomycin. The external [K⁺] that yields identical cyanine fluorescence with

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**Fig. 1.** Measurement of the K⁺ equilibrium potential of erythrocyte membrane vesicles. Intravesicular ionic composition: 10 mM Hepes/65 mM NaCl/75 mM KCl/0.25 mM MgCl₂ (pH 7.5). The [Na⁺]:[K⁺] ratio was varied in the extravesicular space at constant ionic strength. (•) Without valinomycin; (O) plus valinomycin.

(model 70) and the 488 nm Ar⁺ laser line for excitation at 400-µm slit width.

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**Fig. 2.** Raman spectra of erythrocyte membrane vesicles without and with transmembrane [K⁺] gradients. Intravesicular ionic composition was as described in the legend of Fig. 1. With cation gradients, the extravesicular ionic composition is 140 mM NaCl/10 mM Hepes/0.25 mM MgCl₂ (pH 7.5). (A) 2800–3100 cm⁻¹ in H₂O; (B) 1400–1700 cm⁻¹ in $^2\text{H}_2\text{O}$; (C) 1170–1360 cm⁻¹ in H₂O; (D) 750–1000 cm⁻¹ in $^2\text{H}_2\text{O}$.
Table 1. Variation of membrane Raman spectra with transmembrane cation gradients

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<th>cm⁻¹</th>
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<td>2930/2850</td>
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<tr>
<td>CH stretch</td>
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¹ Means of three experiments with maximum standard error of 4%.

The amide III region (Fig. 2C) yields concordant results: Without a K⁺ gradient, the principal feature is at 1367 cm⁻¹. This can arise from both α-helical and unordered polypeptide (18, 19). A transmembrane K⁺ gradient reduces the scattering intensity at 1267 cm⁻¹ and causes emergence of a prominent 1310 cm⁻¹ band. The 1310 cm⁻¹ location is characteristic of α helix (18).

Without a K⁺ gradient, the amide III' region (Fig. 2D) exhibits a principal band at 960 cm⁻¹, characteristic for unordered polypeptide in H₂O, and a minor peak at 935 cm⁻¹. Upon establishing the gradient, the 935 cm⁻¹ feature becomes predominant. Since a signal at ~930 cm⁻¹ is characteristic of α helix in H₂O, the gradient-induced amide III' changes also imply enhanced α helicity (19).

Aromatic Ring Signals. Imposition of a K⁺ gradient produces minor shifts in the tryptophan signals at ~1615 cm⁻¹ and 1560 cm⁻¹ (Fig. 2B and Table 1), emergence of a tyrosine band at 1210 cm⁻¹ (Fig. 2C), and a broadening, as well as relative intensification, of the 830 cm⁻¹ tyrosine feature. These modifications are the opposite of what is observed upon denaturation of some globular proteins (19) and support the other evidence for a gradient-induced modification of protein structure.

* Frequencies due to double bonds in acyl chains of membrane phospholipid also appear in this region, but do not shift their position upon deuteration.

Other Regions of the Spectrum. The 1527 cm⁻¹ band, a resonance-enhanced 1(-C==C-) of β-carotene, is light sensitive (20). Thus, no significance can be attached to intensity changes such as observed in the samples of Fig. 2B, where light exposure was not strictly controlled. Wavelength shifts and intensity changes in the 1450-1470 cm⁻¹ range have also been observed, but correlations with the presence or absence of K⁺ gradients are not apparent (Fig. 2B).

Spectrin-free vesicles

Imposition of a transmembrane K⁺ gradient increases scattering at 2930 cm⁻¹ with spectrin-free vesicles (Table 1). This enhancement is less stable than with native vesicles and dissipates parallel to the decay of the measured K⁺ equilibrium potential (see above). Within 2 hr of establishing the gradient, I₂S900/I₂S500 is 1.37; at 5 hr I₂S900/I₂S500 equals 1.26. No consistent changes in the amide regions were noted in spectrin-free vesicles charged with K⁺ gradients. Moreover, the enhanced scattering at 1640 cm⁻¹, 1310 cm⁻¹, and 935 cm⁻¹, typical of native vesicles, is not seen with spectrin-free vesicles prepared with a K⁺ gradient (Table 1).

Pure lipid vesicles

Vesicles from erythrocyte lipid extracts or egg lecithin were prepared with and without K⁺ gradients. The ~1.26 1.22 to 1.37 at 2S900/I₂S500 ratio was not altered by establishment of a K⁺ gradient (Table 1). These gradients were stable for several hours, as verified by valinomycin-induced K⁺ diffusion potentials (data not shown).

Membrane potential and ~1.26 1.22 to 1.37

Attempts to measure ~1.26 1.22 to 1.37 with valinomycin present to increase the membrane potential (negative inside) have not been consistently successful. Both ~1.26 1.22 to 1.37 and the hyperpolarization induced by valinomycin dissipate rapidly at high membrane protein concentrations (>0.1 mg/ml). The range of values for ~1.26 1.22 to 1.37 in four such experiments is 1.4-2.4. Higher concentrations of valinomycin could not be used since the ionophore solvent, ethanol, contributes to this region of the Raman spectrum. To circumvent this problem, we have manipulated the extravesicular anion composition and have used the endogenous membrane Na⁺,K⁺-ATPase. Table 2 compiles experimental results correlating membrane potential with ~1.26 1.22 to 1.37.

In experiments 1 and 2 of Table 2, membrane potential was calculated from Fig. 1, assuming a linear relationship between potential and cyanine fluorescence. Thus, the fluorescence yield at the null point was equated to 0 mV and the intersection point of the + valinomycin curve at [K⁺⁺] = 0 was assigned a value of ~−26 mV.

In experiment 3, extravesicular Cl⁻ was replaced by the impermeant anion, Hepes (21, 22) to positively increase the membrane potential to +10 mV. This anion substitution did not alter the K⁺⁺ equilibrium potential as measured by cyanine fluorescence.

In experiment 4, the membrane potential was made more negative by addition of NaSCN to the extravesicular volume. SCN⁻ is a lipophilic anion with a greater diffusion rate across biomembranes than Cl⁻. Unfortunately, SCN⁻ as well as another lipophilic anion, NO₂⁻, effectively quench cyanine fluorescence whether membranes are present or not. The diffusion potential for the SCN⁻ gradient is the value obtained with vesicles isolated from 3T3 cells (23).

In experiments 5 and 6, 1 mM ATP plus 1 mM Mg²⁺ were incorporated into vesicles with Na⁺⁺ and [K⁺⁺] identical on each side of the membrane. After 30 min at 20°C, ~1.26 1.22 to 1.37 was measured in the presence or absence of 1 mM ouabain. Al-
though the membrane potential was not measured, a ouabain-sensitive decrease in cyanine fluorescence was observed.

To summarize Table 2, conditions that increase the membrane potential (negative inside) are reflected by an intensification of $\sim I_{2930}/I_{3850}$.

**DISCUSSION**

The Donnan contribution of hemoglobin to the membrane potential of intact erythrocytes is $\sim -8 \text{ to } -10 \text{ mV}$ (24). The K$^+$ equilibrium potential of intact erythrocytes, calculated from cyanine dye fluorescence measurements, is about $-40 \text{ mV}$ (13). The membrane contribution to the potential of intact cells, $\sim -30 \text{ to } -32 \text{ mV}$, thus closely approaches the value of $-26 \text{ mV}$ we obtain with hemoglobin-free vesicles. These results bear on the controversy (25) about the relative significance of membrane transport versus water-structure/ion-binding in cellular cation selectivity. Indeed, our demonstration that the electrochemical gradient across the membranes of vesicles free of cytoplasmic proteins closely corresponds to the gradient across the membranes of intact erythrocytes, weighs against ion-exchange or resin-cytoplasmic-protein models (see, e.g., refs. 26 and 27) for cellular ion gradients.

Since we measure equivalent K$^+$ equilibrium potentials and enhanced scattering of the $\sim 2930 \text{ cm}^{-1}$ band with both native and spectrin-free vesicles, spectrin cannot act as an essential component in the $\sim 2930 \text{ cm}^{-1}$ response to transmembrane K$^+$ gradients. However, the lack of signals, in spectrin-depleted vesicles, of gradient-induced conformational changes suggests that spectrin might be the protein changing its helicity in native vesicles. The relative instability of both the cation gradients and $I_{2930}/I_{3850}$ with spectrin-free vesicles also suggests that spectrin may be important in stabilizing membrane structure.

**Protein and Lipid Contributions to $\sim I_{2930}$**. There are two major intensity contributions to the $\sim 2930 \text{ cm}^{-1}$ Raman scattering feature of erythrocyte membranes. At $>20^\circ \text{C}$ this region is dominated by the symmetric CH-stretching of protein CH$_3$ groups which represents $>90\%$ of the erythrocyte membrane CH$_3$ mass (7, 8). Data on model compounds and RNase (8) indicate that intensification of the $\sim 2930 \text{ cm}^{-1}$ membrane band with temperature ($>38^\circ \text{C}$) and pH reduction (7) is caused by the transfer of CH$_3$ groups from a medium of low to one of higher polarity. The second contribution at $2926 \text{ cm}^{-1}$ has been assigned to asymmetric CH$_2$-stretching of lipid CH$_2$ groups (28, 29). Although the intensity of this band increases with melting of the fatty acid chain, $I_{2926}/I_{2850}$ in pure lipid liposomes does not exceed 1.0 (28, 29). Moreover, we have demonstrated that applied cation gradients do not alter this ratio in pure lipid vesicles.

**Transmembrane Ion Gradients and $I_{2930}/I_{3850}$**. The erythrocyte membrane is more permeable to anions than cations and, thus, the potential across the membrane is primarily governed by anion conductance. In this regard, it is surprising that we measure a significant potential difference between vesicles with and without transmembrane cation gradients. Although we have not further explored reasons for this potential difference, it is possible that with preparation of vesicles either anion conductance is reduced relative to that of K$^+$ or K$^+$ permeability is enhanced relative to Cl$^-$. Alternatively, the cyanine dye may enhance K$^+$ or decrease anion permeabilities. Measurements of the transmembrane potential of *Rhodospirillum rubrum* chromatophores indicate that low concentration of cyanine dyes can increase the ion permeability of membranes (30).

This problem aside, we have provided several lines of evidence indicating a relationship between the potential across the erythrocyte membrane and $I_{2930}/I_{3850}$. Changing the extravascular anion composition by adding either impermeant or lipophilic anions, a procedure that, respectively, decreases or increases the membrane potential (negative inside), results in corresponding decrease or intensification of $\sim I_{2930}/I_{3850}$.

Moreover, we have used the endogenous Na$^+$/K$^+$-ATPase activity to increase the negative inside membrane potential. Laris et al. (31), using Ehrlich ascites carcinoma cells, have measured a ouabain-sensitive decrease in cyanine fluorescence under conditions that generate cellular ATP and have suggested that this represents electrogenic activity of Na$^+$/K$^+$-ATPase. We have similarly observed ouabain-sensitive cyanine fluorescence decrease in sealed erythrocyte vesicles containing ATP. This hyperpolarization of the membrane was associated with intensification of $\sim I_{2930}/I_{3850}$.

As discussed above, intensification of the $\sim 2930 \text{ cm}^{-1}$ feature reflects transfer of protein CH$_3$ groups from apolar to more polar environments. Thus, our data suggest an intriguing molecular model for membrane energization. We hypothesize that enhancement of the methyl CH-stretching results from a potential induced compression of the apolar membrane core with concomitant partial extrusion of apolar protein residues from the core. Compression of black lipid bilayers by electric fields near physiological potentials ($\sim 10^5 \text{ V/cm}$) has been shown to squeeze the n-decane (in such films) from lipid bilayer domains into segregated visible lenses without separation of lipid molecules (32, 33). This potential, if applied across the 22-Å apolar width of the erythrocyte membrane (34), would correspond to about 1 kcal/mol, or approximating the molar energy required to transfer methyl groups from apolar solvents to water (35).
This concept of membrane energization relates, in principle, to the molecular model for elastin elasticity (36), as well as to the observations of Packer (37) that the intramembranous particles of mitochondrial and chloroplast membranes shift perpendicularly to the plane of the membrane as a function of membrane energization.