

# Sodium and Potassium Fluxes and Membrane Potential of Human Neutrophils

## *Evidence for an Electrogenic Sodium Pump*

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**ABSTRACT** Sodium and potassium ion contents and fluxes of isolated resting human peripheral polymorphonuclear leukocytes were measured. In cells kept at 37°C,  $[Na]_i$  was 25 mM and  $[K]_i$  was 120 mM; both ions were completely exchangeable with extracellular isotopes. One-way Na and K fluxes, measured with  $^{22}Na$  and  $^{42}K$ , were all  $\sim 0.9$  meq/liter cell water·min. Ouabain had no effect on Na influx or K efflux, but inhibited  $95 \pm 7\%$  of Na efflux and 63% of K influx. Cells kept at 0°C gained sodium in exchange for potassium ( $[Na]_i$  nearly tripled in 3 h); upon rewarming, ouabain-sensitive K influx into such cells was strongly enhanced. External K stimulated Na efflux ( $K_m \sim 1.5$  mM in 140-mM Na medium). The  $P_{Na}/P_K$  permeability ratio, estimated from ouabain-insensitive fluxes, was 0.10. Valinomycin (1  $\mu M$ ) approximately doubled  $P_K$ . Membrane potential ( $V_m$ ) was estimated using the potentiometric indicator diS-C<sub>3</sub>(5); calibration was based on the assumption of constant-field behavior. External K, but not Cl, affected  $V_m$ . Ouabain caused a depolarization whose magnitude depended on  $[Na]_i$ . Sodium-depleted cells became hyperpolarized when exposed to the neutral exchange carrier monensin; this hyperpolarization was abolished by ouabain. We conclude that the sodium pump of human peripheral neutrophils is electrogenic, and that the size of the pump-induced hyperpolarization is consistent with the membrane conductance (3.7–4.0  $\mu S/cm^2$ ) computed from the individual K and Na conductances.

### INTRODUCTION

The resting membrane potential of leukocytes and its response to stimuli have recently been studied in several laboratories. Estimates of the membrane potential of leukocytes have been made from direct measurements with microelectrodes (Gallin et al., 1975; Gallin and Gallin, 1977; Dos Reis and Oliveira-Castro, 1977; Kouri et al., 1980), from potassium flux-ratio consid-

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erations (Dunham et al., 1974), from the transmembrane distribution of  $^3\text{H}$ -labeled lipid-soluble cations (Korchak and Weissmann, 1978, 1980; Castranova et al., 1979; Seligmann and Gallin, 1980), and by means of voltage-sensitive fluorescent dyes (Utsumi et al., 1977; Castranova et al., 1979; Rink et al., 1980; Seligmann et al., 1980; Tatham et al., 1980; Jones et al., 1980).

This work was undertaken as part of a study of the physiologic mechanisms underlying neutrophil activation by chemotactic factors and other agents. To characterize the resting membrane properties upon which such agents exert their effects, we have determined the passive and active fluxes of sodium and potassium, and estimated the resting membrane potential of human peripheral neutrophils. We find that nearly all of the sodium efflux and most of the potassium influx are active, that internal sodium stimulates potassium influx, and that external potassium stimulates sodium efflux. Membrane potential was monitored with the fluorescent cationic dye 3,3'-dipropylthiadicarbocyanine (diS-C<sub>3</sub>(5) in the shorthand of Sims et al. [1974]). For calibration purposes, fluorescence readings were compared with calculated membrane potential values based on the constant-field assumption and on permeabilities estimated from passive ion movements. Our finding of a pronounced ouabain effect on the membrane potential of sodium-loaded cells is consistent with a low membrane conductance and the presence of an electrogenic sodium pump in human peripheral neutrophils.

#### METHODS

##### *Incubation Media*

The standard medium used throughout this study had the following composition: 140 mM NaCl; 5 mM KCl; 1 mM CaCl<sub>2</sub>; 0.5 mM MgCl<sub>2</sub>; 5.6 mM glucose; 5 mM Tris HCl buffer, pH 7.4; 1 mg/ml bovine serum albumin. To test the effects of varying external K<sup>+</sup>, media of appropriate [K<sup>+</sup>] were prepared by equimolar substitution for Na<sup>+</sup>. In some experiments a low-chloride medium was used, obtained by substituting Na glutamate (Sigma Chemical Co., St. Louis, Mo.) for NaCl.

##### *Neutrophils*

Human peripheral neutrophils were isolated by sequential dextran sedimentation and Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gradient centrifugation (Böyum, 1968). Contaminating erythrocytes were lysed by exposure to NH<sub>4</sub>Cl (8.5 g/liter) for 10 min at 37°C. The neutrophils were washed thrice and then counted. Purity of neutrophil suspensions averaged 97% as judged by Wrights' stain. Viability averaged 97% as assessed by eosin Y exclusion, and was not affected by any of the agents tested in this study.

Unless stated otherwise, cells were manipulated at 37°C, and spun in unrefrigerated centrifuges. In addition, the cells were kept at 37°C for 30–60 min before and during all assays. (Keeping these cells at that temperature for up to 3 h in 5-mM K medium had no effect on intracellular ion content or on the subsequent behavior of these cells.) For some specific experiments, cells were isolated in a similar manner except that the centrifuges were cooled to 4°C and the cells were washed in ice-cold, 1 mM phosphate-buffered, K-free saline, pH 7.4, during isolation. After being counted, these cells were kept in ice-cold, K-free medium for 2.5–3 h before fluorescence measurements at 37°C.

### Reagents

The fluorescent dye diS-C<sub>3</sub>(5) was a generous gift of Dr. Alan Waggoner of Amherst College, Amherst, Mass. Ouabain, valinomycin, and monensin were purchased from Sigma Chemical Co. Valinomycin was dissolved at 10<sup>-3</sup> M in dimethyl sulfoxide (DMSO), and monensin was dissolved at 10<sup>-2</sup> M in ethanol. The resultant concentrations of DMSO or ethanol in the medium (0.1%) had no effect on any of the assays performed. Isotopes (<sup>3</sup>H]-H<sub>2</sub>O, [<sup>14</sup>C]-inulin, <sup>22</sup>NaCl, <sup>42</sup>KCl) were purchased from New England Nuclear, Boston, Mass. <sup>22</sup>Na was carrier free; the specific activity of <sup>42</sup>K was 2–2.5 Ci/g K.

### Intracellular Ion Concentrations

Intracellular K (K<sub>i</sub>) and Na (Na<sub>i</sub>) concentrations were measured by flame photometry, as follows. Neutrophils (10<sup>7</sup>) were suspended in the medium described above. Triplicate 1-ml aliquots were layered on 0.4 ml silicone oil (Versilube F-50; Harwick Chemical Corp., Akron, Ohio) contained in 1.5-ml microcentrifuge tubes (Beckman Instruments, Inc., Fullerton, Calif.), and centrifuged for 1 min at 8,000 g. Cell separation occurred in <5 s. The aqueous and oil phases were aspirated and discarded. The neutrophil pellets were lysed in 0.1 N HNO<sub>3</sub>, and analyzed for K and Na in a flame photometer (Model 343; Instrumentation Laboratory, Lexington, Mass.). Results are expressed in milliequivalents per liter of cell water. In separate experiments, using [<sup>3</sup>H]H<sub>2</sub>O and [<sup>14</sup>C]inulin as markers for total and extracellular water, respectively, we determined cell water content to be 203 ± 11 (SE) μm<sup>3</sup> per cell (n = 5). The average cell radius, measured through a microscope with calibrated eyepiece reticule, was 4.2 μm.

### <sup>42</sup>K and <sup>22</sup>Na Fluxes

The technique described by Naccache et al. (1977) was used. The incubations were performed at 37°C in capped, 12- × 75-mm plastic tubes (Falcon Plastics, Oxnard, Calif.) in a total volume of 1.8 ml containing 12–18 × 10<sup>6</sup> cells. At stated intervals, triplicate 0.5-ml aliquots were layered on 0.7 ml silicone oil and centrifuged as described above. The neutrophil pellets were excised and counted in a gamma counter. Influx experiments were performed in the presence of <sup>22</sup>NaCl or <sup>42</sup>KCl (1.5 μCi/ml). For the efflux studies, neutrophils were first suspended at 2–4 × 10<sup>7</sup>/ml and incubated with <sup>42</sup>KCl or <sup>22</sup>NaCl (4.5 μCi/ml) for 30 min at 37°C. The cells were then washed twice and resuspended in unlabeled medium. Triplicate samples were then taken at stated intervals and handled as described above.

Cation effluxes followed single exponentials, and the rate coefficient was determined by least-squares fitting a straight line to a semilogarithmic plot of radioactivity remaining in the cells. Influxes, corrected for zero-time “uptake,” followed equations of the form:

$$C_t = C_\infty[1 - \exp(-kt)] \quad (1)$$

where  $C_t$  is the cell label at time  $t$ ,  $C_\infty$  is the cell label at steady state, and  $k$  is the rate coefficient. Eq. 1 was fit to the data by a nonlinear least-squares program, and the initial influx rate computed from the product  $kC_\infty$ . As indicated in the figure legends, influx was sometimes so slow as to appear linear over the period of study; in those cases the influx rate was computed from the slope of the linear regression line.

### Fluorescence Measurements

A 10<sup>-3</sup> M stock solution of diS-C<sub>3</sub>(5) in ethanol was kept at 4°C, protected from light. Experiments were performed in a total volume of 3 ml containing 4 × 10<sup>6</sup> neutrophils

and 1  $\mu\text{M}$  dye (and 0.1% ethanol). After equilibration for 5–10 min at 37°C, fluorescence of the suspension was measured in a spectrofluorometer (Model 430; Turner Associates, Palo Alto, Calif.) whose cuvette holder was maintained at 37°C. Excitation was at 622 nm; emission was at 670 nm. Fluorescence intensity is given in arbitrary units. At the concentrations used and under our conditions, neither ethanol nor the dye affected neutrophil viability as assessed by eosin Y exclusion, or functional integrity as measured (Simchowitz and Spilberg, 1979) by the superoxide generation response elicited by 5 min exposure to the synthetic chemotactic factor formyl-methionyl-leucyl-phenylalanine,  $10^{-7}$  M, or active and passive sodium and potassium fluxes, as measured by the methods described above. Intracellular concentrations of  $\text{K}^+$  and  $\text{Na}^+$  were similarly unaffected (Table I). In the absence of cells, neither ouabain or valinomycin nor changes in the medium's potassium concentration affected dye fluorescence.

Adsorption of diS-C<sub>3</sub>(5) onto glass and polypropylene complicates the use of this dye (Sims et al., 1974). However, we found that the presence of bovine serum albumin, which binds the dye (Tsien and Hladky, 1978), minimizes this problem. For example,

TABLE I  
EFFECT OF ISOLATION AND INCUBATION CONDITIONS ON  
INTRACELLULAR ION CONCENTRATIONS OF HUMAN  
NEUTROPHILS

| Conditions                                    | Intracellular ion concentrations |               |          |
|---|----------------------------------|---------------|----------|
|   | $\text{K}_i$                     | $\text{Na}_i$ | <i>n</i> |
|   | <i>meq/liter cell water</i>      |               |          |
| 37°C, 1–1.5 h                                 | 120.4±4.6                        | 24.7±3.8      | 5        |
| 37°C, 1–1.5 h; diS-C <sub>3</sub> (5), 15 min | 124.1±5.7                        | 26.2±4.1      | 5        |
| 37°C, 2.5–3 h                                 | 120.9±5.0                        | 20.9±2.8      | 3        |
| Ice-cold (K-free), 1–1.5 h                    | 98.3±5.6                         | 43.6±5.2      | 5        |
| Ice-cold (K-free), 2.5–3 h                    | 78.4±5.5                         | 68.0±5.1      | 3        |
| Ice-cold (K-free), 4 h                        | 55.7±4.6                         | 87.3±3.9      | 3        |

Results are mean  $\pm$  SE of three or five separate experiments, each performed in triplicate.

five successive transfers of a 1- $\mu\text{M}$  dye solution in protein-free medium at 15-min intervals, to a fresh glass test tube caused the loss of >90% of the dye onto the glass. In the presence of 1 mg/ml bovine serum albumin, <10% of the dye was lost.

## RESULTS

### *Intracellular Ion Concentrations*

Table I shows that the addition of dye (1  $\mu\text{M}$ ) had no significant effect on  $[\text{K}]_i$  or  $[\text{Na}]_i$  and that further incubation of the cells at 37°C for up to 3 h had little effect on intracellular ion levels. In contrast, when the cells were isolated and stored in K-free, ice-cold medium before assay, their  $[\text{K}]_i$  declined with time, and their  $[\text{Na}]_i$  rose by similar amounts. For convenience, cells prepared at 37°C will henceforth be termed “normal-sodium” cells, and those kept in the cold for 2.5–3 h, “sodium-loaded” cells.

*Potassium Fluxes*

The time course of  $^{42}\text{K}$  influx into normal-sodium cells, from a 5-mM K medium, and the effects of ouabain and valinomycin, are shown in Fig. 1. Computed initial influx rates (in meq/liter cell water·min) were 0.82 for control cells, and 0.30 (37% of control) for those exposed to 50  $\mu\text{M}$  ouabain. Valinomycin (1  $\mu\text{M}$ ), a potassium ionophore (Pressman, 1969), produced a flux increment of 0.45 meq/liter cell water·min in normal cells, and of 0.29

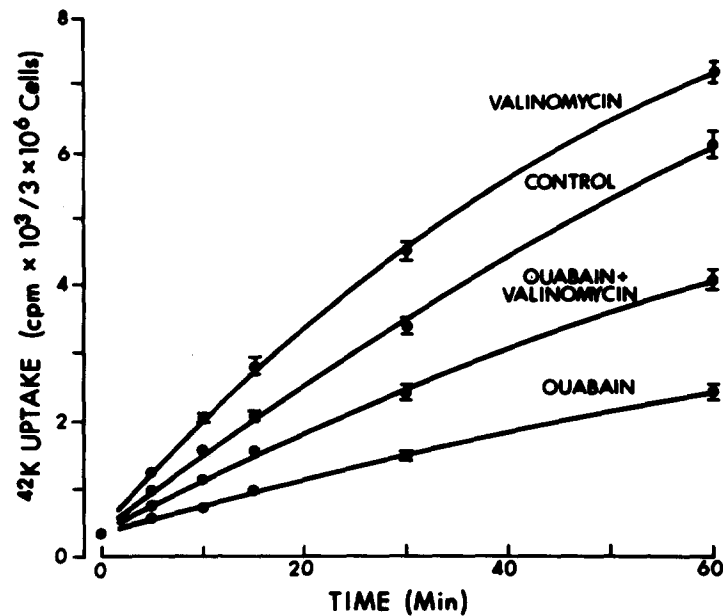


FIGURE 1. Effect of ouabain and valinomycin on potassium influx. Neutrophils were preincubated at 37°C with and without ouabain (50  $\mu\text{M}$ ) for 10 min. At zero time, the cells were delivered to tubes containing 5 mM  $\text{K}_o$  labeled with  $^{42}\text{K}$  (1.5  $\mu\text{Ci}/\text{ml}$ ) in the presence or absence of valinomycin (1  $\mu\text{M}$ ). The reaction mixtures were further incubated and the cells were assayed for radioactivity at stated times. Results represent the mean  $\pm$  SE (where larger than the symbol) of four separate experiments each performed in triplicate. Specific activity was 0.23 cpm/pmol. The zero-time value represents label present in the extracellular space. The curves are least-squares fits of single exponentials (Eq. 1) to the data.

meq/liter·min in ouabain-treated cells. Exposure of ouabain-treated cells to 10  $\mu\text{M}$  valinomycin in the presence of 0.1% bovine serum albumin, or to 1  $\mu\text{M}$  valinomycin in the absence of albumin, yielded results similar to those just reported, which suggests that a maximal valinomycin effect had been achieved. The flux increments produced by valinomycin will not be identical in control and ouabain-treated cells since both ouabain and valinomycin affect the membrane potential (see Discussion). (Our estimates for the membrane potential of control and ouabain-treated cells in 5-mM K solutions are

-59 and -50 mV, respectively [Fig. 7]; valinomycin hyperpolarizes these cells by 11 mV [not shown] and 12 mV [Fig. 7], respectively. These estimates are based [see Discussion] on a calibration method which relies, in part, on the use of valinomycin. Fortunately, errors in the assumed factor by which valinomycin increases potassium permeability have relatively little weight in the calibration procedure.) Assuming that all ouabain-resistant influx took place through passive permeability pathways, and applying constant-field (Goldman, 1943) corrections for the effect of membrane potential, we calculate that valinomycin enhances potassium permeability by a factor of 1.7 or 2.1 in the presence or absence of ouabain, respectively. Although influx was not followed for long enough periods to give very accurate estimates of exchangeable potassium, the extrapolated maximal  $^{42}\text{K}$  uptake by control cells was  $116 \pm 30$  meq/liter cell water, compatible with our assay for total potassium content.

The time course of  $^{42}\text{K}$  efflux from normal-sodium cells, both control and ouabain-treated, is shown in Fig. 2. As ouabain had no detectable effect on the rate of  $^{42}\text{K}$  release, it can be assumed that essentially all  $\text{K}^+$  efflux is passive. The efflux rate coefficient for the combined data was  $0.0078 \text{ min}^{-1}$ . Assuming a value of 122 meq/liter cell water for cell potassium (Table I), this signifies an efflux of 0.95 meq/liter cell water·min. In the presence of  $1 \mu\text{M}$  valinomycin, the efflux rate coefficient was 0.0146. Assuming that all potassium efflux occurs by simple diffusion, and correcting again for membrane potential, we calculate a 2.5-fold enhancement of potassium permeability after exposure to valinomycin.

Potassium uptake into cells that had been kept in the cold was higher than into normal-sodium cells. For example,  $^{42}\text{K}$  uptake by sodium-loaded cells ( $[\text{Na}]_i \approx 68 \text{ mM}$ ; Table I) was 2.07 meq/liter cell water·min in the absence of ouabain, and 0.32 meq/liter·min in its presence. An extreme example of the effect of sodium loading on  $^{42}\text{K}$  uptake is shown in Fig. 3. These cells had been stored in the cold for  $\sim 4$  h, and we estimate their sodium content to be 87 mM (see Table I). High intracellular sodium levels should activate the Na/K pump, and hence the potassium influx. As shown in the figure, the fraction of K influx that is ouabain sensitive is now much larger (90%) than in the normal-sodium cells of Fig. 1. Ouabain-insensitive ("passive") potassium influx was 0.26 meq/liter cell water·min (slightly less than into normal-sodium cells, as expected for these K-depleted, depolarized cells) but the active (ouabain-sensitive) flux was 2.3 meq/liter cell water·min, compared with 0.52 meq/liter·min for normal-sodium cells (Fig. 1) and 1.75 meq/liter·min for the "sodium-loaded" cells already mentioned. Theoretically, as sodium-loaded cells lose sodium during incubation at  $37^\circ\text{C}$ , the rate coefficient for active potassium uptake is expected to fall somewhat during the course of the experiment. However, since the data for unpoisoned cells in Fig. 3 are still evidently well fit by a single exponential (Eq. 1), it appears that the resolution of our influx method is insufficient to detect such time-dependent changes in rate coefficient.

*Sodium Fluxes*

Fig. 4 shows the time course of  $^{22}\text{Na}$  accumulation in the presence and absence of  $50\ \mu\text{M}$  ouabain. Initial influx rates were indistinguishable (average  $0.84\ \text{meq/liter cell water}\cdot\text{min}$ ), which implies that no ouabain-sensitive sodium influx (e.g., pump-mediated  $\text{Na}:\text{Na}$  exchange) took place. However,  $\text{Na}$  uptake into ouabain-treated cells proceeded almost linearly over the entire

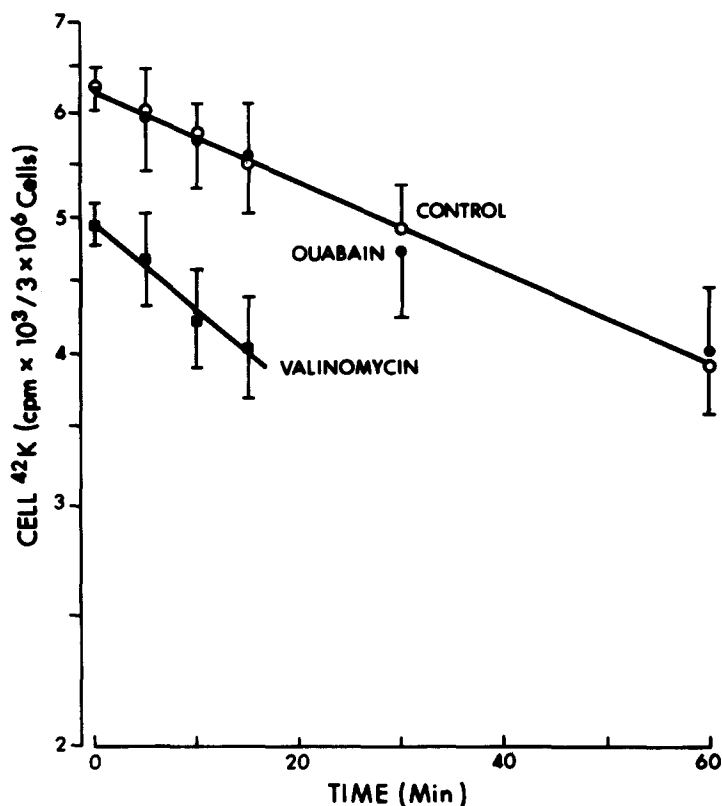


FIGURE 2. Effect of ouabain on potassium efflux. Neutrophils were incubated with  $^{42}\text{K}$  ( $4.5\ \mu\text{Ci/ml}$ ) for 30 min, after which the cells were rapidly washed with unlabeled medium. The cells were then further incubated for various periods at  $37^\circ\text{C}$  in medium with or without ouabain or valinomycin, and assayed for radioactivity. The semilog plot represents the mean ( $\pm$  SE) radioactivity left in the cell for three separate experiments, each performed in triplicate. Upper curve: efflux into medium with (open circles) or without (filled circles) ouabain ( $50\ \mu\text{M}$ ); the rate coefficient for the combined data, obtained by linear least-squares, was  $0.0078\ \text{min}^{-1}$ . Lower curve, square symbols: efflux into medium containing  $1\ \mu\text{M}$  valinomycin; the rate coefficient, obtained by linear least-squares, was  $0.015\ \text{min}^{-1}$ . To improve clarity, the lower data points and curve, which had a zero-time intercept identical to that of the upper curve, were shifted downward by 20%.

duration of the experiment, whereas uptake into control cells clearly approached an extrapolated value of  $26.0 \pm 4.5$  meq/liter cell water, comparable to the steady-state sodium concentration determined by flame photometry (see Table I). This similarity implies that essentially all cell sodium is exchangeable with extracellular  $^{22}\text{Na}$ . Valinomycin  $10^{-6}$  M (not shown) had no detectable effect on sodium influx.

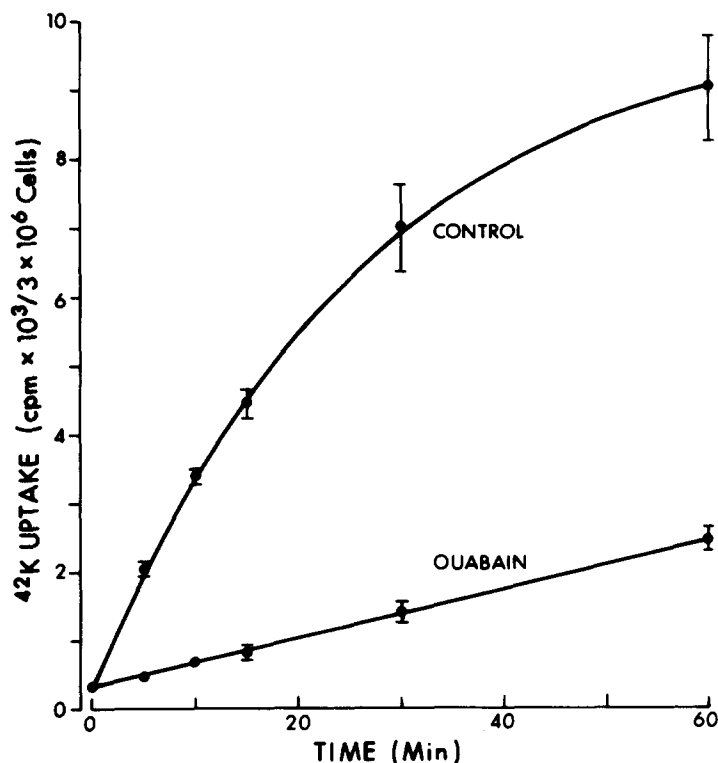


FIGURE 3. Potassium uptake by sodium-loaded cells. Procedures were as in Fig. 1 except that sodium-loaded cells were used instead of cells isolated and maintained at  $37^{\circ}\text{C}$ . Results represent the mean  $\pm$  SE (where larger than the symbol) of three experiments each performed in triplicate. Specific activity was  $0.24$  cpm/pmol. The upper curve is a least-squares fit of a single exponential (Eq. 1) to the data. The data from ouabain-treated cells were fit by a straight line.

Efflux kinetics for  $^{22}\text{Na}$  are shown in Fig. 5. The ouabain sensitivity of sodium efflux is striking: loss of  $^{22}\text{Na}$  from ouabain-treated cells over a 1-h period was negligible. Therefore, essentially all of sodium efflux can be ascribed to active pumping, and none of sodium influx to  $\text{Na}:\text{Na}$  exchange. From the rate coefficient for  $^{22}\text{Na}$  loss from untreated cells ( $0.036 \text{ min}^{-1}$ ), and an average intracellular  $\text{Na}$  concentration of  $\sim 24.4$  meq/liter cell water (Table I), we calculate a total sodium efflux of  $0.88$  meq/liter cell water  $\cdot$  min, very



near the value of 0.84 meq found for initial  $^{22}\text{Na}$  influx. The similarity of the values proves that these cells were indeed near steady state during our experiments. The effect of external K concentration on the rate coefficient of  $^{22}\text{Na}$  efflux is shown in Fig. 6. Apparent  $K_m$  for potassium activation of Na/K pump activity was  $\sim 1.5$  mM. Sodium efflux was abolished by removing  $K_o$ , and  $95 \pm 7\%$  was inhibited by  $50 \mu\text{M}$  ouabain.

*Fluorescence of diS-C<sub>3</sub>(5) in Suspensions of Normal-Sodium Neutrophils*

The fluorescent dye diS-C<sub>3</sub>(5) was used at  $1 \mu\text{M}$ , i.e., just below the concentration at which nonfluorescent dimers and polymers begin to form (Sims et

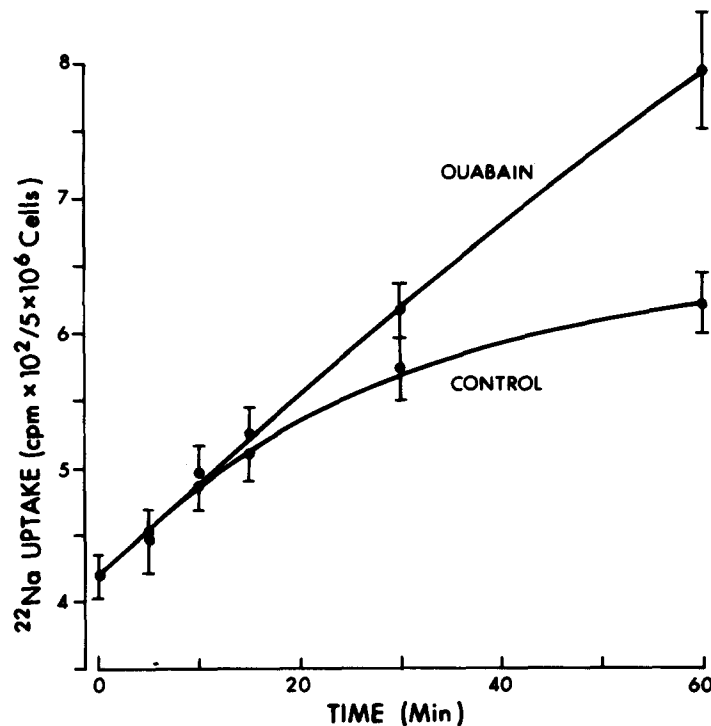


FIGURE 4. Effect of ouabain on  $^{22}\text{Na}$  influx. Experiments were performed as in Fig. 1 in the presence of  $^{22}\text{Na}$  ( $1.5 \mu\text{Ci/ml}$ ). Data points are the mean  $\pm$  SE of four separate experiments each performed in triplicate. Specific activity was  $9.1$  cpm/nmol. Zero-time value represents label present in the extracellular space. The curves are least-squares fits of single exponentials (Eq. 1) to the data.

al., 1974). In the absence of cells, fluorescence was unaffected by the Na:K concentration ratio, by substituting glutamate for chloride, or by the presence of  $50 \mu\text{M}$  ouabain and/or  $1 \mu\text{M}$  valinomycin.

The fluorescence of dye-cell suspensions was strongly dependent on the medium's potassium concentration (Fig. 7). Fluorescence of suspensions of normal-sodium cells was maximal at  $[\text{K}]_o = 120$  mM, and fell when  $[\text{K}]_o$  was lowered, reaching a minimum at about  $2.5$  mM  $K_o$ . Fluorescence in media

containing  $[K]_o \leq 2.5$  mM was slightly higher than that at 5 mM  $K_o$ . When cells were allowed to equilibrate with dye in nearly chloride-free medium (where all NaCl was replaced with Na glutamate), a fluorescence level not statistically different from that seen in chloride medium was attained in 5–10 min. Furthermore, when cells that had previously been equilibrated with dye in chloride medium were spun down and immediately resuspended in glutamate medium, fluorescence intensity remained unchanged.

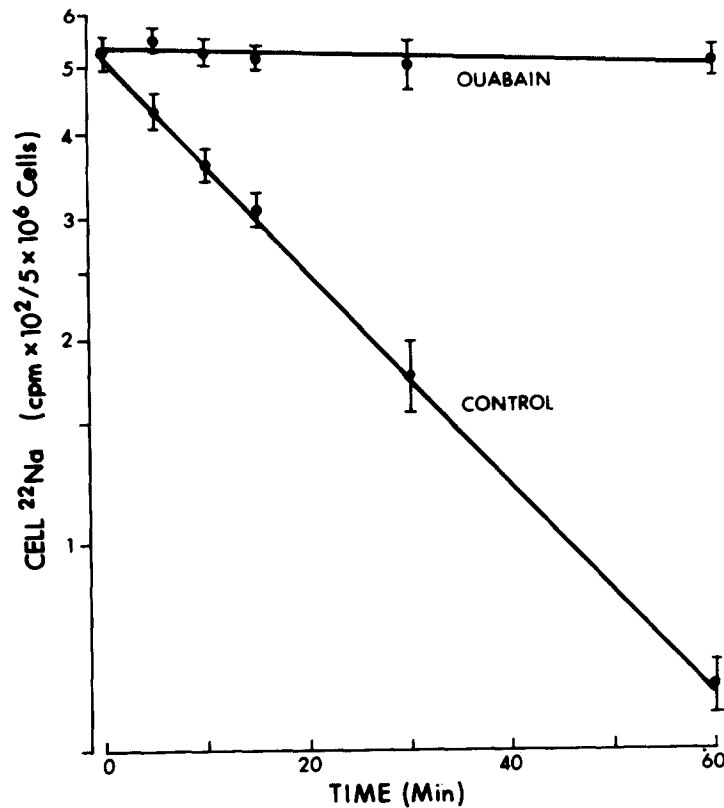


FIGURE 5. Effect of ouabain on  $^{22}\text{Na}$  efflux. Experiments were performed as in Fig. 2 except that the cells were pre-incubated with  $^{22}\text{Na}$  ( $4.5 \mu\text{Ci/ml}$ ) for 30 min. Plotted on a semilog scale is the mean ( $\pm$  SE) radioactivity left in the cells for four separate experiments, each performed in triplicate. Rate coefficient for  $^{22}\text{Na}$  loss was  $0.036 \text{ min}^{-1}$  for control cells, and  $0.001 \text{ min}^{-1}$  for ouabain-treated cells.

Inclusion of  $50 \mu\text{M}$  ouabain in the medium led to an increase in fluorescence at all  $K$  concentrations (Fig. 7). The increase was largest (almost 30%) at 2.5 mM  $K_o$ , and progressively diminished with increasing  $[K]_o$ . With  $1 \mu\text{M}$  valinomycin present as well, however, fluorescence intensity decreased again (at least for  $[K]_o \leq 10$  mM), falling even below control fluorescence when  $[K]_o \leq 5$  mM (see Fig. 7).

*Partition of diS-C<sub>3</sub>(5) between Medium and Cells*

The mechanism by which fluorescence of diS-C<sub>3</sub>(5) solutions containing suspended cells reflects the membrane potential of those cells is believed to involve a redistribution of the cationic dye between the bathing medium, where it fluoresces, and the cell interior, where fluorescence is quenched, presumably due to polymerization (Sims et al., 1974), as well as binding to cell constituents (Hladky and Rink, 1976; Tsien and Hladky, 1978). Thus, cell hyperpolarization will lead to an accumulation of dye in the cell and

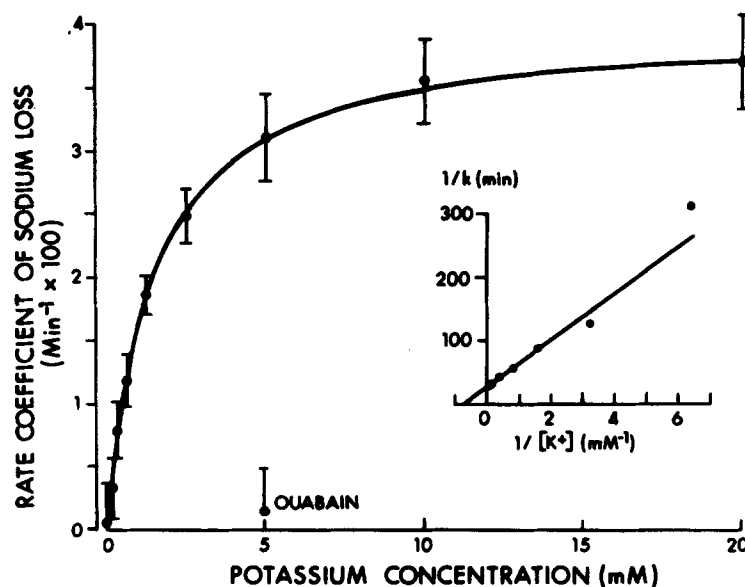


FIGURE 6. Effect of extracellular potassium on the rate coefficient for sodium efflux. Cells were pre-incubated with  $^{22}\text{Na}$  ( $4.5 \mu\text{Ci/ml}$ ) for 30 min. The loss of  $^{22}\text{Na}$  over a 15-min period into unlabeled solutions containing various potassium concentrations was then determined, and the rate coefficient of sodium loss was computed. Shown are average ( $\pm$  SE) rate coefficients for four experiments, each performed in triplicate. A Michaelis-Menten equation ( $K_m = 1.47 \text{ mM}$ ; maximal rate coefficient =  $0.040 \text{ min}^{-1}$ ) was fit to the data by a nonlinear least-squares program. The inset shows the same data, without the error bars, in double-reciprocal fashion.

consequent loss of fluorescence. Our data of Fig. 7 follow this prediction between 2.5 and 120 mM  $K_o$ , where the cells presumably depolarize with increasing  $[K]_o$ . Valinomycin, which increases potassium permeability in these cells (see Fig. 1), would normally be expected to *hyperpolarize* them, and indeed the only significant effect observed was a fluorescence decrease. Further evidence that extracellular  $[K]$  governs the distribution of the dye between cells and medium is shown in Fig. 8, where fluorescence of the cell suspension, equilibrated with dye, is shown, as well as the fluorescence of the supernatant after removal of the cells. The presence of cells always caused a reduction in

fluorescence. For this test we used cells exposed to ouabain plus valinomycin (Fig. 7) because the range of fluorescence covered, as  $[K]_o$  was varied, was widest. Even at 120 mM  $K_o$ , where the membrane potential is expected to be near zero, the presence of cells ( $1.3 \times 10^6/ml$ ) caused a fluorescence drop of ~30% (from 56 to 39 units). Removal of the cells after dye equilibration at 120 mM  $K_o$  shows that the cell-associated dye represents 37% of the quantity

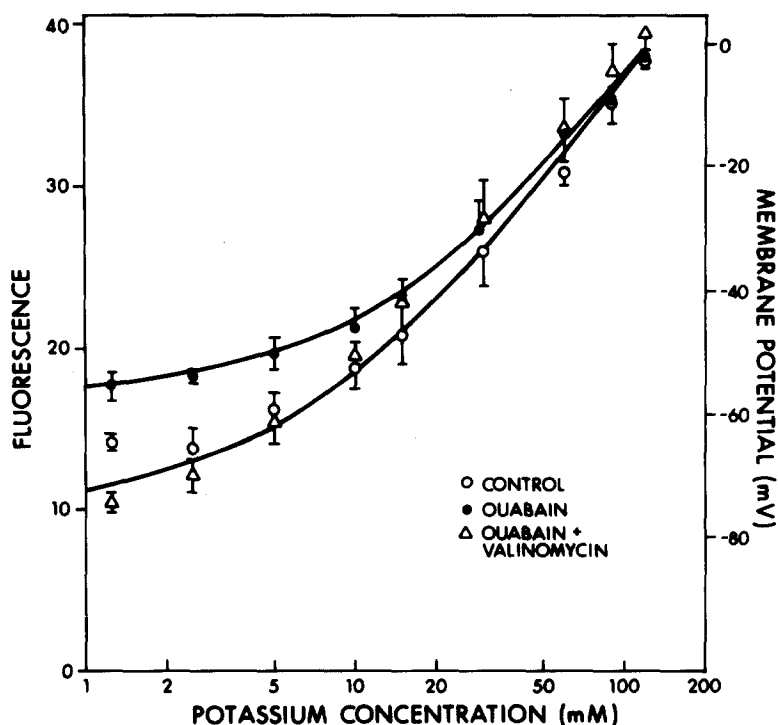


FIGURE 7. Effect of extracellular potassium on the fluorescence of normal-sodium neutrophil suspensions in diS-C<sub>3</sub>(5). Neutrophils were equilibrated with 1  $\mu$ M diS-C<sub>3</sub>(5) in media with various K concentrations, and the fluorescence was recorded. Cells from the same batch were suspended in three media: control (○); or with addition of 50  $\mu$ M ouabain (●), or 50  $\mu$ M ouabain plus 1  $\mu$ M valinomycin (△). Results represent the mean  $\pm$  SE of three separate experiments each performed in duplicate. See Discussion and Fig. 11 for calibration of righthand ordinate (membrane potential). The solid lines are drawn according to Eq. 2, with  $P_{Na}/P_K$  permeability ratio ( $\alpha$ ) set equal to 0.1 (upper curve) or 0.05 (lower curve).

originally present, yet accounts for <10% of total fluorescence. (Light scatter, measured in the absence of dye, was negligible in these experiments and was unaffected by  $[K]_o$ .) As extracellular  $[K]$  is lowered and the cells presumably become polarized, progressively less dye remains in the supernatant, but the fluorescence that can be ascribed to the cells (i.e., the difference between suspension and supernatant fluorescence) remains constant within experimental error. It appears, therefore, that the fluorescence of any dye that becomes

associated with the cells, *over and beyond* the amount already associated with unpolarized cells in equilibrium with  $\sim 0.6 \mu\text{M}$  dye, is effectively quenched; all changes in total fluorescence are accounted for solely by changes in the supernatant.

That dye partitioning reaches a true steady state was ascertained as follows.

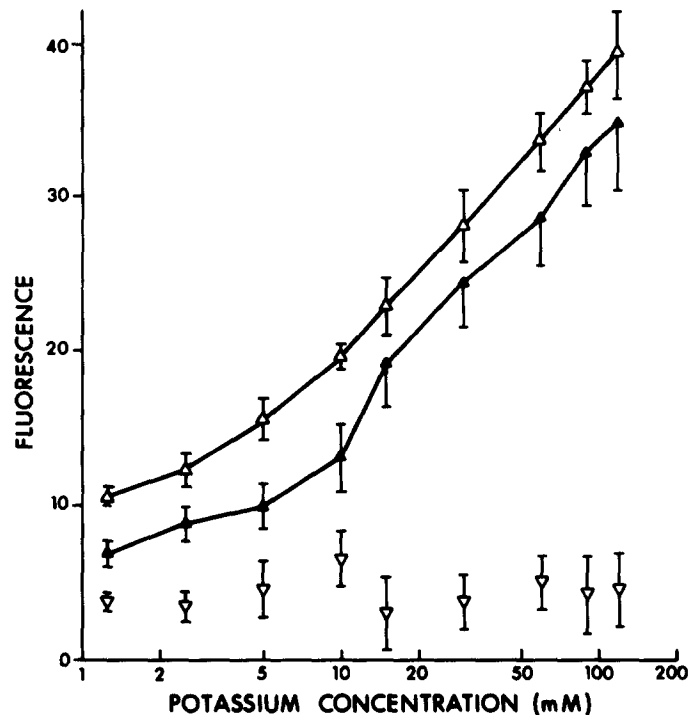


FIGURE 8. Partition of diS-C<sub>3</sub>(5) between cells and medium as a function of extracellular potassium concentration. The cells are the same ouabain- and valinomycin-treated cells as shown in Fig. 7. Total dye ( $1 \mu\text{M}$ ) fluorescence, in the absence of cells, was 56 units. Neutrophils were equilibrated with dye in the presence of various  $K_o$  concentrations and  $50 \mu\text{M}$  ouabain plus  $1 \mu\text{M}$  valinomycin, and the steady level of fluorescence was recorded ( $\Delta$ ). The cells were then removed by centrifugation and the fluorescence of the supernatant was measured ( $\blacktriangle$ ). The difference between the two is cell-associated fluorescence ( $\nabla$ ). Results represent the mean ( $\pm$  SE) of three separate experiments, each performed in duplicate.

Cells were equilibrated with  $1 \mu\text{M}$  dye in 5-mM K medium, spun down, and resuspended in various media with dye at the concentration found in the 5-mM K supernatant, but now containing 5, 30, 60, or 120 mM K. The fluorescence of the 5-mM K resuspension remained constant, whereas that of the others increased to levels indistinguishable from those shown in Fig. 7. We conclude that steady-state dye partitioning can indeed be approached from either direction, at least in media where  $[K]_o \geq 5 \text{ mM}$ .

The situation is more complicated in low-K media. The fluorescence of dye-cell suspensions prepared in  $[K]_o < 1.25$  mM (in the absence of valinomycin) was *higher* than with  $[K]_o = 5$  mM. This, we found, is indeed due to lack of dye uptake by the cells, and not to an effect of low  $K_o$  on the cells' ability to quench cell-associated dye fluorescence. On the other hand, if the cells were *first* equilibrated in 5-mM K medium and then resuspended, as described above, in low-K media containing an appropriate amount of diS-C<sub>3</sub>(5), they did not release dye: fluorescence levels remained constant or fell slightly. Thus, contrary to our observations in high-K media, it appears that identical dye partitioning cannot be reached from either direction when extracellular K levels are below 1.25 mM. It is not for lack of equilibration time that dye uptake from very low-K media is incomplete: extending incubation from 10 to 45 min produced no further fluorescence changes. Nor did 0.01–0.1  $\mu$ M tetraphenylboron, which markedly enhances the equilibration of triphenylmethylphosphonium cations across the cell membrane, affect the dye distribution in 0.3-mM K media. (Higher concentrations of tetraphenylboron interfered with dye fluorescence in the absence of cells.) Finally, incomplete dye equilibration did not result from some irreversible damage caused by low-K media: upon addition of sufficient concentrated KCl to raise  $[K]_o$  to 5 mM, cells equilibrated with dye at 0.3 mM  $K_o$  rapidly accumulated dye to the level appropriate for a 5-mM K medium.

Since the remainder of our experiments do not call for very low-K solutions, we did not further explore the origin of this apparent "rectification," but the following mechanism seems plausible. The flux and fluorescence data presented so far suggest that the cells are much more permeable to potassium than to sodium or chloride. Consequently, cells kept in very low-K media are expected to have a low membrane conductance. Sudden exposure to even small amounts of the extremely (Waggoner et al., 1977) permeant cation diS-C<sub>3</sub>(5) may produce an inward current large enough to depolarize the cells. (The exact mode of penetration of the dye strongly depends on its concentration and on ionic conditions [Krasne, 1980].) If their sodium conductance were, however slightly, voltage dependent (larger upon depolarization), these cells might be unable to recover spontaneously from depolarization in low-K media. Valinomycin, by increasing the cell's potassium conductance, would both prevent such depolarization and help the recovery. Another possibility, suggested by Rink et al. (1980), is that the dye may lower the potassium permeability by blocking calcium-induced potassium channels as it does in erythrocytes (Simons, 1976).

Whatever its mechanism, it is evident that the described anomaly must be borne in mind when voltage-sensitive dyes such as diS-C<sub>3</sub>(5) are used to estimate membrane potentials of cells bathed in very low-K media or, more generally, of cells with low intrinsic membrane conductance.

#### *Fluorescence of diS-C<sub>3</sub>(5) in Suspensions of Sodium-loaded Neutrophils*

Sodium-loaded cells were suspended in media with various K concentrations and containing 1  $\mu$ M diS-C<sub>3</sub>(5), allowed to equilibrate for 5–10 min, and

assayed for fluorescence as described above. Some cells were exposed, in addition, to 1  $\mu\text{M}$  valinomycin, and others to 50  $\mu\text{M}$  ouabain. Results are plotted in Fig. 9. The following comparisons with normal-sodium cells (Fig. 7) can be made: (a) at all K concentrations, fluorescence of sodium-loaded control cell suspensions was lower than that of normal-sodium control cell suspensions; and (b) at all but the highest K concentrations, fluorescence of ouabain-treated, sodium-loaded cell suspensions was higher than that of

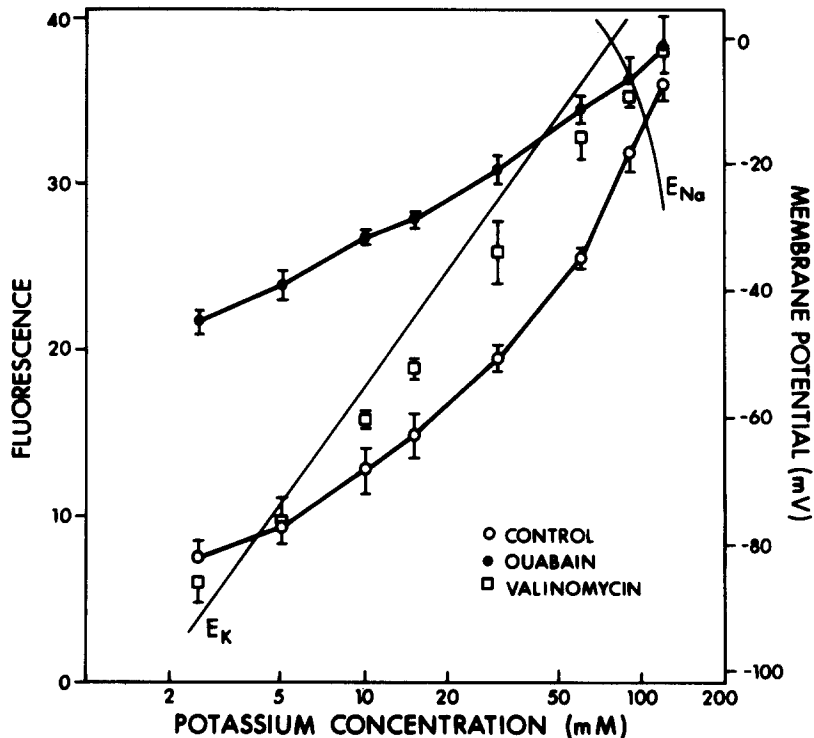


FIGURE 9. Effect of sodium loading on dye-cell fluorescence. Experiments were performed as in Fig. 7 except that sodium-loaded cells were used. Control (○); medium containing 50  $\mu\text{M}$  ouabain (●); medium containing 1  $\mu\text{M}$  valinomycin (□). Results represent the mean  $\pm$  SE of three separate experiments, each performed in duplicate. See Discussion and Fig. 11 for calibration of the righthand ordinate (membrane potential). Equilibrium potentials for potassium and sodium are also shown.

ouabain-treated normal-sodium cell suspensions. The effect of 1  $\mu\text{M}$  valinomycin (alone) depended on the potassium concentration: at  $[\text{K}]_o$  above 5 mM, valinomycin increased fluorescence (never reaching the ouabain treatment level however), whereas at  $[\text{K}]_o$  below 5 mM, fluorescence was decreased by valinomycin. To the extent that fluorescence reflects membrane potential (see Discussion), these observations suggest that sodium-loaded cells with functional sodium pumps are hyperpolarized as compared with their normal-

sodium counterparts; that nonpumping cells with high  $[Na]_i$  and low  $[K]_i$  are depolarized (as expected for cells mainly permeable to K); and that, in sodium-loaded cells with intact pumps, valinomycin depolarizes at high  $[K]_o$  and hyperpolarizes at low  $[K]_o$ , with cross-over near 5 mM  $K_o$ .

#### *Effect of Ouabain on Fluorescence*

Figs. 7 and 9 show that exposure of cell-dye mixtures to ouabain increases fluorescence compared to that of control cells. For sodium-loaded cells (Fig. 9), the fluorescence after treatment with ouabain in 5-mM K medium is more than twice that of untreated cells, or a difference of nearly 15 units. In the case of normal-sodium cells, the fluorescence difference is less than 5 units in 5-mM K medium (Fig. 7). We also prepared a batch of sodium-depleted cells ( $\leq 2$  mM, measured by flame photometry) by exposing cells for 2.5 h (at 37°C) to a medium in which all NaCl was replaced with *N*-methylglucamine hydrochloride. Fig. 10 shows the time course of fluorescence changes upon addition of 50  $\mu$ M ouabain to cell-dye suspensions that were previously equilibrated in 5-mM K, 140-mM Na medium, for each of the three kinds of cells mentioned above. In the case of sodium-depleted cells (trace A), fluorescence does not change perceptibly after addition of ouabain; for normal-sodium cells (trace B), there is a fluorescence increase of perhaps three units; suspensions of heavily sodium-loaded cells (trace C), however, undergo a dramatic fluorescence increase upon addition of ouabain.

Monensin is an ionophore that catalyzes the electroneutral exchange of alkali cations or protons across membranes; it has a high affinity for sodium (Pressman, 1969). Consequently, sodium-depleted cells rapidly gain sodium when bathed in a high-sodium medium containing monensin, presumably in exchange for potassium ions and/or protons. Although no net current takes place, the sodium accumulation and potassium depletion should cause gradual depolarization according to Eq. 2. Instead, sodium-depleted cells hyperpolarized when exposed to monensin (Fig. 10, trace D). That this, in all probability, results from electrogenic extrusion by the sodium pump of Na, which entered the cell via the electroneutral monensin pathway, is shown by the large fluorescence increase (i.e., depolarization) after addition of ouabain.

## DISCUSSION

### *Ion Concentrations*

Our values of  $\sim 120$  meq/liter cell water for  $[K]_i$  and  $\sim 25$  meq/liter for  $[Na]_i$  of isolated human peripheral neutrophils are similar to those of several other studies (Baron and Ahmed, 1969; Lichtman and Weed, 1969; Cividalli and Nathan, 1974). (The latter two studies used mixed peripheral blood leukocytes). It has been reported before (Hempling, 1954; Lichtman and Weed, 1969; Cividalli and Nathan, 1974) that handling and storage of white blood cells in the cold reversibly raises  $[Na]_i$  and reduces  $[K]_i$ . From the above value for  $[K]_i$ , it follows (assuming identical intra- and extracellular activity coefficients) that the potassium equilibrium potential for normal cells bathed in 5-mM K medium at 37°C is  $-85$  mV.



*Ion Fluxes*

We found a total potassium influx rate of 0.82 meq/liter cell water · min into normal cells, somewhat lower than the figures of 1.3–1.6 meq/liter cell water ·

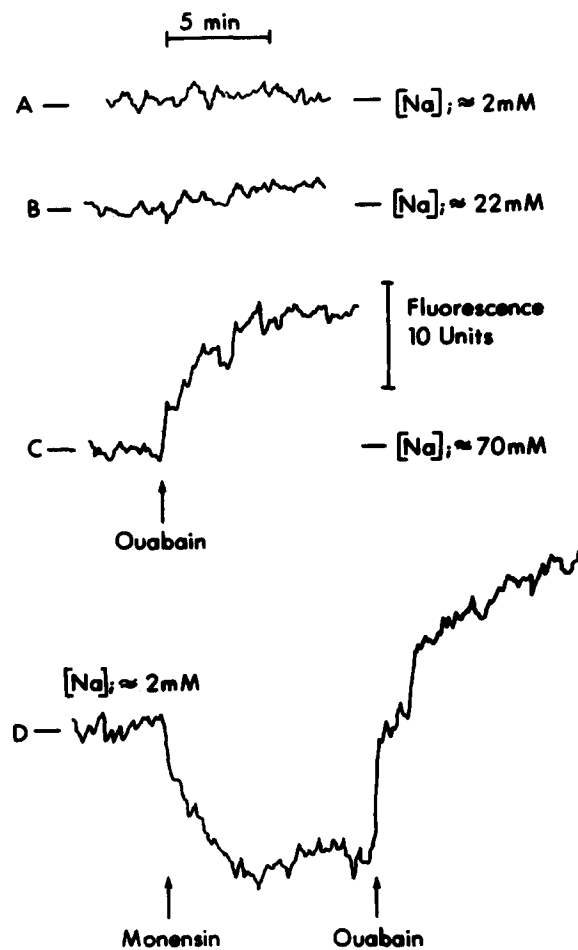


FIGURE 10. Time course of fluorescence change after addition of ouabain. Cells were suspended in 5-mM K medium with 1  $\mu$ M diS-C<sub>3</sub>(5) and equilibrated for 10 min at 37°C. Three types of cells were used: (A) exposed to 5-mM K, Na-free solution for 2.5 h at 37°C; (B) kept in 5-mM K, 140-mM Na media at 37°C throughout; (C) kept for 2.5 h in K-free, 145-mM Na solution at 4°C. At the time indicated by the arrow, 50  $\mu$ M ouabain was added to the cell-dye suspension (A–C). Trace D: sodium-depleted cells were exposed to 10  $\mu$ M monensin 10 min before addition of 50  $\mu$ M ouabain. Fluorescence increase (i.e., depolarization) is upward. Retraced from strip-chart recording.

min and 2.0 meq/liter cells · min published by others (Cividalli and Nathan, 1974; Dunham et al., 1974). Of that potassium influx, 63% was ouabain sensitive in the present study, compared with 50% in that of Dunham et al.

(1974). Our K efflux rate (0.95 meq/liter min) was also somewhat below that (1.2 meq/liter min) published by Cividalli and Nathan (1974). Similarly, our sodium fluxes were 0.84 and 0.88 meq/liter min for influx and efflux, respectively, compared with about twice these values in the work of Cividalli and Nathan (1974). The similarity of our total influx and efflux rates for both potassium and sodium, for cells kept at 37°C throughout, demonstrates that our cells were in true steady state. In addition, we found no evidence for nonexchangeable intracellular Na or K pools.

Sodium influx and potassium efflux were unaffected by ouabain. Essentially all ( $95 \pm 7\%$ ) sodium efflux from normal cells and 63% of potassium influx was ouabain sensitive. Probable changes in membrane potential upon exposure to ouabain (see below), however, preclude an exact computation of the *pumped* Na:K ratio, for the following reason: whereas all ouabain-sensitive sodium efflux can safely be assumed to have taken place through the pump (there being only a negligible passive sodium efflux), it is likely that passive K influx (which is appreciable) will decrease upon depolarization, leading to an over-estimate of true pump-mediated K influx. At any rate, pumped sodium flux appears to exceed pumped potassium flux by a ratio of 3:2 or better. We will examine further arguments in favor of the existence of an electrogenic Na/K pump in human neutrophils.

#### *Relative Permeabilities and Membrane Potential*

Ouabain-insensitive fluxes represent an upper limit to the fluxes that follow passive permeability pathways. In the case of sodium influx (all of which is ouabain-insensitive), there is very little room for Na:Na exchange in view of the small magnitude of ouabain-insensitive sodium efflux. As for the ouabain-insensitive potassium fluxes, it will be shown (see Discussion) that they appear to obey Ussing's (1949) independence criterion, which suggests that they take place through simple passive diffusion pathways. Ouabain-insensitive K influx (from a 5-mM K solution) into both normal and sodium-loaded cells amounted to  $\sim 0.30$  meq/liter cell water·min, whereas ouabain-insensitive sodium influx (from the 140-mM Na medium) was 0.84 meq/liter cell water·min. Assuming then, as a first approximation, that these fluxes take place through passive diffusion pathways exclusively, and taking into account the Na/K concentration ratio in the bathing medium, it follows that the sodium/potassium permeability ratio ( $\alpha$ ) is  $\sim 0.10$  for control cells, and, since valinomycin roughly doubles potassium permeability, the ratio is  $\sim 0.05$  for valinomycin-treated cells.

The constant-field assumption (Goldman, 1943; Hodgkin and Katz, 1949), for a cell with passive chloride distribution and/or low chloride permeability (which, considering the lack of effect of chloride substitution on diS-C<sub>3</sub>(5) fluorescence, appears to hold for human neutrophils) leads to a simplified equation (Hodgkin and Horowicz, 1959) that predicts the resting membrane potential in the absence of electrogenic pumping:

$$V_m = \frac{RT}{F} \ln \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i} \quad (2)$$

where  $R$ ,  $T$ , and  $F$  have their usual thermodynamic meaning,  $\alpha$  is the Na:K permeability ratio, and the subscripts o and i denote extra- and intracellular concentrations, respectively. Applying the above assumptions and approximations to the cells described here, we calculate, from Eq. 2, resting potentials in 5-mM K, 140-mM Na media at 37°C, of -50 mV for ouabain-treated control cells and -40 mV for sodium-loaded ( $[\text{Na}]_i \approx 70$  mM) cells. These membrane potentials should affect flux ratios (Ussing, 1949) and would easily account for our finding that passive sodium efflux is barely detectable with our methods (Fig. 5), whereas passive sodium influx is sizeable.

Further support for the applicability of Eq. 2 is the following. As stated above, valinomycin reduces  $\alpha$  by half (or even more if one allows that not all ouabain-insensitive influx be by passive diffusion), but Fig. 7 shows that in the presence of ouabain the ionophore has a negligible effect on dye distribution and hence, presumably, on membrane potential, for bathing medium K concentrations above 10 mM. This is precisely what would be expected if membrane potential obeyed an equation like Eq. 2 with small  $\alpha$ , rather than an equation where the major permeability is to an ion other than potassium. For example, Hoffman and Laris (1974), working with red blood cells, whose membrane potential is governed mainly by the chloride distribution ratio, showed that valinomycin induces a strong depolarization in high-K media and a hyperpolarization in low-K media, as expected for cells that initially have a low potassium permeability. We found significant valinomycin-induced fluorescence reductions (presumably, hyperpolarizations) only in solutions where  $[\text{K}]_o$  is not much larger than the product  $\alpha[\text{Na}]_o$  in Eq. 2.

#### *Relation between Membrane Potential and Fluorescence*

Our experiments leave little doubt that changes in fluorescence of diS-C<sub>3</sub>(5) in suspensions of human neutrophils reflect changes in the membrane potential of these cells. On theoretical grounds, the fluorescence of a dye-cell suspension cannot be expected to bear a simple, let alone linear, relationship to the membrane potential of the suspended cells: the quantity of dye is finite, and the intracellular/extracellular partition of the cationic dye monomer is exponentially related to membrane potential. Further complications arise with multiply charged dye polymers (Hladky and Rink, 1976) and binding to intra- and extracellular proteins (Tsien and Hladky, 1978). Precise calibration cannot be achieved without an independent measurement of membrane potential. However, if it be assumed here, for the reasons mentioned above, that Eq. 2 is a reasonable predictor of membrane potential (at least for  $[\text{K}]_o \geq 1.25$  mM and in the absence of electrogenic pumping), then a convenient fluorescence-voltage calibration curve can be constructed that is sufficient for the semi-quantitative arguments that follow.

In Fig. 11 we have gathered the fluorescence readings from the three sets of data for which Eq. 2 may be valid: normal-sodium cells treated with ouabain or ouabain plus valinomycin (Fig. 7), and sodium-loaded cells treated with ouabain (Fig. 9). These fluorescence readings were plotted against the theoretical membrane potential computed from Eq. 2. Two points from Fig. 9 (at

$K_o = 90$  or  $120$  mM), for which Eq. 2 predicts a positive membrane potential, were left out. Considering the differences among the three kinds of cells—(a) normal  $[Na]_i$ , normal  $P_{Na}/P_K$  ratio; (b) normal  $[Na]_i$ , low  $P_{Na}/P_K$  ratio; and (c) high  $[Na]_i$ , normal  $P_{Na}/P_K$  ratio—the constancy of the relationship between fluorescence and calculated membrane potential is quite satisfactory and supports the applicability of the constant-field assumption. Moreover, the relationship between fluorescence intensity and computed membrane poten-

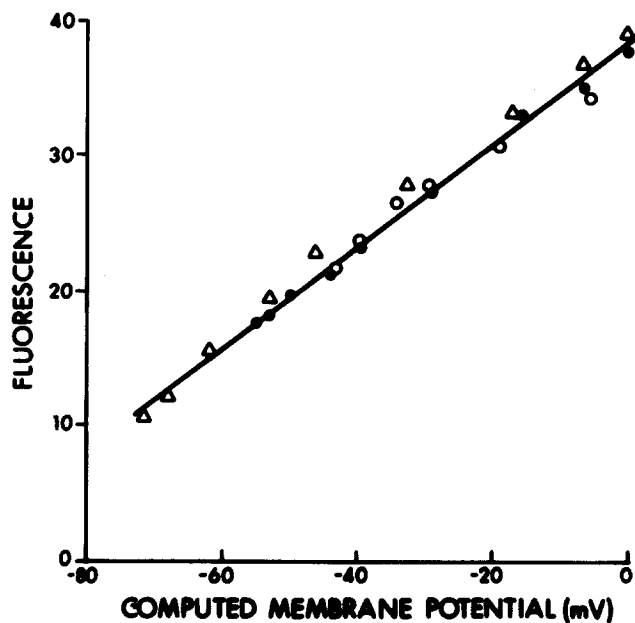


FIGURE 11. Fluorescence: potential calibration. Fluorescence readings from three experiments were plotted against the corresponding theoretical membrane potential calculated from Eq. 2. The  $P_{Na}/P_K$  permeability ratio ( $\alpha$ ) used for cells treated with ouabain was 0.10, and 0.05 for cells treated with ouabain plus valinomycin. Intracellular  $[K]$  was 120 mM for normal-sodium cells and 78 mM for sodium-loaded cells; intracellular  $[Na]$  was 25 mM for normal cells and 68 mM for sodium-loaded cells. Normal-sodium cells treated with  $50 \mu\text{M}$  ouabain (from Fig. 7) (●); normal-sodium cells treated with  $50 \mu\text{M}$  ouabain plus  $1 \mu\text{M}$  valinomycin (from Fig. 7) ( $\Delta$ ); sodium-loaded cells exposed to  $50 \mu\text{M}$  ouabain (from Fig. 9) (○). The equation for the least-squares regression is: fluorescence (in arbitrary units) =  $38.8 + 0.38 V_m$  (in millivolts).

tial is very nearly linear over the range covered. Whether this extended range of dye linearity (compare, for example, Freedman and Hoffman, 1979) is only apparent and due to compensating errors in the constant-field computation of membrane potential, or real and due perhaps to our use of bovine serum albumin, which serves as an extracellular buffer for the dye, remains to be determined. The least-squares line through the data of Fig. 11 represents a calibration slope of  $2.63$  mV/fluorescence unit. This conversion factor was

then used in the construction of the righthand (membrane potential) ordinates of Figs. 7 and 9.

#### *Membrane Potentials*

With the above calibration curve, we can estimate the actual membrane potentials of cells treated in various ways. In Fig. 7, the resting potential of normal-sodium cells bathed in 5-mM K, 140-mM Na medium is estimated at  $-59$  mV, or 9 mV more negative than the potential calculated from Eq. 2. This hyperpolarization relative to the constant field prediction is abolished by ouabain. Such ouabain-sensitive hyperpolarization with respect to the predicted constant-field potential was observed for all  $K_o$  concentrations between 1.25 and 20 mM. The hyperpolarization upon exposure to valinomycin (in the lower ranges of  $[K]_o$ ) predictably results from the reduction of  $\alpha$  in Eq. 2 by a factor of two.

In sodium-loaded cells (Fig. 9), the ouabain-sensitive hyperpolarization is more pronounced. At 5 mM  $K_o$ , the estimated membrane potential is  $-78$  mV, or hyperpolarized 38 mV with respect to the constant-field prediction, and also hyperpolarized 19 mV *with respect to normal-sodium cells* (even though the latter have higher  $[K]_i$ ). This hyperpolarization extends throughout the range 2.5–120 mM  $K_o$ . At all but the lowest  $[K]_o$ , the membrane is hyperpolarized beyond the nominal potassium equilibrium potential. Even valinomycin-treated cells, when sodium-loaded, are hyperpolarized beyond the potential predicted by Eq. 2.

#### *Potassium Flux Ratio, Permeabilities, and Conductances*

The computed membrane potential for ouabain-treated, normal-sodium cells ( $V_m = -50$  mV) is sufficiently different from the corresponding potassium equilibrium potential ( $E_K = -85$  mV) to allow a reasonable test of Ussing's (1949) equation for independent fluxes, as modified by Hodgkin and Keynes (1955):

$$\frac{M_{out}^K}{M_{in}^K} = \exp[(V_m - E_K) Fn'/RT]$$

where  $M_{out}^K$  and  $M_{in}^K$  represent passive potassium efflux and influx, respectively,  $n'$  is an empirical term whose deviation from unity is a measure of non-independent behavior, and the other symbols have their usual meaning. Taking the ouabain-insensitive flux ratio  $0.95/0.30 = 3.17$  for  $M_{out}^K/M_{in}^K$ , we find  $n' = 1.15$ , not appreciably different from unity. Put another way, the observed ratio of ouabain-insensitive potassium fluxes requires, if these fluxes are independent, that the cells have a membrane potential of  $-54$  mV (we find  $-50$  mV). This suggests that potassium ions indeed move independently of one another and are not subject to, for example, K-K exchange or single-filing (Hodgkin and Keynes, 1955) constraints. Measurements of the small passive sodium efflux are too inaccurate to allow reliable calculation of the sodium flux ratio.

Assuming spherical geometry for resting neutrophils ( $4.2 \mu\text{m}$  radius) and a cell water content of  $203 \mu\text{m}^3/\text{cell}$ , each meq/liter cell water  $\cdot$  min of flux is

equivalent to  $1.52 \text{ pmol/cm}^2 \cdot \text{s}$ . Thus, in normal-sodium, ouabain-treated neutrophils, net passive potassium efflux amounted to  $(0.95-0.30) \times 1.52 = 0.99 \text{ pmol/cm}^2 \cdot \text{s}$ . From this figure, potassium permeability can be estimated using the equation of Goldman (1943):

$$P_K = M_{\text{net}}^K \cdot \frac{RT}{V_m F} \cdot \frac{1 - \exp(-V_m F/RT)}{[K]_o \exp(-V_m F/RT) - [K]_i}$$

Entering the appropriate values for ouabain-treated, normal-sodium cells, we find  $P_K = 3.3 \times 10^{-8} \text{ cm/s}$ . Such relatively low permeability to potassium, presumably the most permeant ion, may explain why, at extremely low  $[K]_o$ , the very permeant cationic dye diS-C<sub>3</sub>(5) can apparently induce depolarizations (see Results) even at  $1 \mu\text{M}$  concentration. A similar calculation for  $P_{\text{Na}}$  yields a figure one order of magnitude smaller ( $4.0 \times 10^{-9} \text{ cm/s}$ ), in agreement with the value of  $\alpha$  obtained earlier.

From the electrochemical driving force responsible for the passive net movement of potassium,  $E_K - V_m$ , the potassium chord conductance can be computed:

$$g_K = \frac{M_{\text{net}}^K \cdot F}{E_K - V_m}$$

Our values,  $0.99 \text{ pmol/cm}^2 \cdot \text{s}$  of net K efflux under a 35-mV driving force, yield a specific potassium chord conductance ( $g_K$ ) of  $2.7 \mu\text{S/cm}^2$ . (Constant-field potassium *slope* conductances computed for  $-85$  and  $-50 \text{ mV}$  are  $2.0 \mu\text{S/cm}^2$  and  $3.6 \mu\text{S/cm}^2$ , respectively.) Similarly, net Na influx of  $\sim 1.3 \text{ pmol/cm}^2 \cdot \text{s}$  is driven by  $E_{\text{Na}} - V_m = 46 \text{ mV} - (-50 \text{ mV}) = 96 \text{ mV}$ , which yields a value for specific sodium chord conductance ( $g_{\text{Na}}$ ) of  $1.3 \mu\text{S/cm}^2$ . A second estimate of  $g_{\text{Na}}$  uses the relationship

$$g_{\text{Na}}(V_m - E_{\text{Na}}) = g_K(E_K - V_m),$$

which holds at rest in nonpumping cells where  $\text{Na}^+$  and  $\text{K}^+$  are the only conducting species. Entering the proper values we obtain  $1.0 \mu\text{S/cm}^2$  for  $g_{\text{Na}}$ .

#### *Electrogenic Na/K Pump and Membrane Conductance*

Conceivably, the observed ouabain-sensitive hyperpolarization beyond the diffusion potential predicted by Eq. 2 or even beyond the nominal potassium equilibrium potential (Fig. 9) could result from potassium depletion, in an unstirred layer surrounding the cell, brought about by the operation of an electroneutral Na/K pump. The required layer thickness,  $\delta$ , would be:

$$\delta = \frac{D_K}{M_K} \Delta C_K$$

where  $D_K$  is the diffusion coefficient for potassium in water,  $M_K$  is the pumped potassium flux ( $2.7 \text{ pmol/cm}^2 \cdot \text{s}$  for sodium-loaded cells at  $5 \text{ mM } K_o$ ), and  $\Delta C_K$  is the putative potassium concentration drop between bulk solution and cell surface. With  $D_K = 1.9 \times 10^{-5} \text{ cm}^2/\text{s}$  (Friedman and Kennedy, 1955), and  $\Delta C_K \approx 2 \text{ mM}$  (enough to produce a large hyperpolarization at  $5 \text{ mM } K_o$ ), we find  $\delta > 10 \text{ cm}$ , an absurd requirement indeed.

The most likely cause for the observed hyperpolarization, then, is the operation of an electrogenic Na/K pump. It was noted above that the ouabain-sensitive sodium flux exceeds the sensitive potassium flux. Electrogenic (i.e., ouabain-sensitive) hyperpolarization should be maximal in sodium-loaded cells, and less pronounced in normal-sodium cells; high  $[K]_o$ , although it stimulates the Na/K pump, should shunt the electrogenic hyperpolarization through an increase in membrane conductance; ouabain should cause a strong (and rapid) depolarization in sodium-loaded cells, a weak one in normal-sodium cells, and none in sodium-depleted cells. All these expectations were verified (Figs. 7, 9, and 10), which supports the hypothesis that human neutrophils, like other animal cells (Thomas, 1972; De Weer, 1975), including erythrocytes (Hoffman et al., 1979), possess an electrogenic Na/K pump.

The membrane potential of true steady-state cells possessing an electrogenic sodium pump is predicted by the equation of Mullins and Noda (1963):

$$V_m = \frac{RT}{F} \ln \frac{r[K]_o + \alpha[Na]_o}{r[K]_i + \alpha[Na]_i} \quad (3)$$

where  $r$  is the Na:K pumping ratio, and the meaning of the other symbols is as in Eq. 2. If the latter correctly predicts the membrane potential in the absence of electrogenic pumping, then the difference between Eqs. 3 and 2 represents the size of the ouabain-induced depolarization. With  $r = 1.5$  and  $\alpha = 0.1$ , a 7.5-mV depolarization is predicted for steady-state, normal-sodium neutrophils bathed in 5-mM K medium. It can also be shown (see Thomas, 1973; De Weer, 1975) that the contribution of a 3:2 electrogenic Na/K pump to the membrane potential of a steady-state cell at 37°C cannot exceed 11 mV. Our average ouabain-induced depolarization in steady-state neutrophils (Fig. 7) was  $9.2 \pm 3.8$  mV. In an additional experiment we exposed normal-sodium cells to 5 mM  $Ba^{2+}$ , which reduces their potassium permeability by more than half (unpublished results). This treatment enhanced the ouabain-induced depolarization of such cells by a factor of 2–3, as expected.

The magnitude of the electrogenic hyperpolarization in sodium-loaded cells is striking: ~35 mV in trace C of Fig. 10. The active potassium uptake rate of these cells was 1.75 meq/liter cell water·min, or 2.7 pmol/cm<sup>2</sup>·s. Net charge transport by a 3:2 Na/K pump would be half this amount, or a current density of 0.13  $\mu$ A/cm<sup>2</sup>. For the pump to generate a 35-mV hyperpolarization with this current density, membrane conductance must be

$$g_m = \frac{I_{\text{pump}}}{\Delta V_{\text{pump}} \cdot \text{cm}^2} = \frac{0.13 \times 10^{-6} \text{ A}}{0.035 \text{ V} \cdot \text{cm}^2} \approx 3.7 \mu\text{S}/\text{cm}^2.$$

This magnitude (membrane resistance = 270  $k\Omega \cdot \text{cm}^2$ ) is entirely compatible with the values already computed for the individual potassium and sodium conductances. The fact that the combined potassium (2.7  $\mu$ S/cm<sup>2</sup>) and sodium (1–1.3  $\mu$ S/cm<sup>2</sup>) conductances account for the estimated total membrane conductance is also compatible with our finding that the membrane potential of human neutrophils is unaffected by external chloride.

A relevant question is how much conductance is contributed by the

permeant cation diS-C<sub>3</sub>(5) itself. The rate coefficient for passive potassium loss from the cell is  $\sim 0.008 \text{ min}^{-1}$  (Fig. 2), whereas from Fig. 10 it appears that the dye can be lost with time constants as short as 2–1 min (rate constants  $0.5\text{--}1 \text{ min}^{-1}$ ), which suggests a dye permeability two orders of magnitude larger than that for potassium. Clearly, since the prevailing dye concentration (well below  $1 \mu\text{M}$ ) is about four orders of magnitude lower than that of potassium ( $K_o = 5 \text{ mM}$ ), no substantial contribution of the dye to total membrane conductance is to be expected. However, at extremely low  $K_o$  ( $\leq 0.5 \text{ mM}$ ), cells exposed to  $1 \mu\text{M}$  dye may acquire an extra conductance comparable in magnitude to their potassium conductance.

#### *Valinomycin Null Point in Sodium-loaded Cells*

In the presence of electrogenic pumping, valinomycin has two distinct and opposing effects on membrane potential. By lowering the  $P_{Na}/P_K$  ratio ( $\alpha$  in Eq. 2) it hyperpolarizes the cell; this effect is most pronounced at low  $[K]_o$ . By increasing the membrane's potassium conductance, on the other hand, valinomycin blunts the hyperpolarizing effect of the electrogenic sodium pump, and thus depolarizes the cell. Since the return current of the electrogenic circuit is carried mainly by external potassium, this second effect is most pronounced at high  $[K]_o$ . The relative contributions of these two actions to the net valinomycin-induced potential change vary with  $[K]_o$  and with the intensity of electrogenic pumping. The "null point" where valinomycin has no effect (near  $5 \text{ mM } K_o$  in Fig. 9) therefore represents not simply the potential at which the prevailing potassium concentrations are at equilibrium across the membrane, as was the case for erythrocytes (Hoffman and Laris, 1974; Freedman and Hoffman, 1979), but rather a complex situation in which the hyperpolarization resulting from the reduction of  $\alpha$  in Eq. 2 is exactly offset by the depolarization resulting from the drop in resistance over which the electrogenic pump current flows. The following illustrates this point for sodium-loaded cells. At  $5 \text{ mM } K_o$ , doubling the potassium permeability (i.e., reducing  $\alpha$  from 0.1 to 0.05) is expected to bring  $V_m$  (in the absence of pumping) from  $-40$  to  $-51 \text{ mV}$ , a hyperpolarization of  $11 \text{ mV}$ . At the same time, since  $g_K$  constitutes 0.7 of total membrane conductance, doubling  $g_K$  will reduce electrogenic hyperpolarization from  $35 \text{ mV}$  to  $35/1.7 = 21 \text{ mV}$ , a depolarization of  $14 \text{ mV}$ . Thus, little or no depolarization should result from valinomycin treatment at  $[K]_o = 5 \text{ mM}$  in sodium-loaded cells, a prediction verified by our data in Fig. 9.

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