Chloride Movements in Human Neutrophils

**Diffusion, Exchange, and Active Transport**

LOUIS SIMCHOWITZ and PAUL DE WEER

From the Department of Medicine, the John Cochran Veterans Administration Medical Center, and the Departments of Medicine and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63125

**ABSTRACT** Chloride content and fluxes were measured in isolated resting human peripheral polymorphonuclear leukocytes. The intracellular Cl concentration of cells kept at 37°C in 148 mM Cl media was ~80 meq/liter cell water, fourfold higher than expected for passive distribution at the cell's estimated membrane potential (approximately −53 mV). All intracellular Cl was rapidly exchangeable with external 36Cl. Cells lost Cl exponentially into Cl-free media, and reaccumulated it when Cl was restored to the bath; this reuptake was dependent on metabolism. One-way 36Cl fluxes in steady state cells were ~1.4 meq/liter·min. The bulk (~70%) of these represented electrically silent Cl/Cl exchange mediated by a carrier insensitive to disulfonic stilbenes but blocked by the anion carrier inhibitor α-cyano-4-hydroxycinnamate (CHC). The remaining fluxes were characterized in some detail. About 20% of 36Cl influx behaved as active transport: it moved thermodynamically uphill and was absent in cells treated with 2-deoxy-D-glucose, displayed Michaelis-Menten kinetics with $K_m(\text{Cl}) = 5 \text{ mM}$, $V_{\text{max}} = 0.25 \text{ meq/liter} \cdot \text{min}$, and was inhibited by CHC ($K_i = 1.7 \text{ mM}$), ethacrynate ($K_i = 50 \mu\text{M}$), and furosemide ($K_i = 50 \mu\text{M}$). About 30% of Cl efflux and ~8% of Cl influx behaved as electrodiffusion through a low-permeability pathway ($P_{\text{Cl}} = 4 \times 10^{-6} \text{ cm/s}$; $g_{\text{Cl}} = 1 \mu\text{s/cm}^2$; $P_K/P_{\text{Na}}/P_{\text{Cl}} = 10:1:1$); these fluxes were linear with concentration and strongly voltage sensitive. The putative Cl channel does not appear to be voltage gated, and gives evidence of single filing.

**INTRODUCTION**

The mechanism of leukocyte activation is not understood, but the importance of inorganic ion movements in the process is not in doubt. Alterations of extracellular Na, K, Ca, or Cl concentrations affect chemotaxis, superoxide radical generation, and granule enzyme release, and a variety of ion movements accompanying the response of phagocytic cells to appropriate stimuli have been recorded (for reviews, see Weissmann et al., 1979; Sha‘afi and Naccache, 1981; Henson et al., 1978). Understanding these stimulus-induced responses requires characterization of the resting ion distributions and fluxes. In a previous article

Address reprint requests to Dr. Louis Simchowitz, John Cochran V.A. Medical Center (151/JC), 915 N. Grand Ave., St. Louis, MO 63125.

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(Simchowitz et al., 1982), we examined intracellular concentrations and active and passive fluxes of Na\(^+\) and K\(^+\) in human peripheral neutrophils and estimated the resting membrane potential of these cells with a potentiometric dye. We also reported a low membrane conductance and inferred a low permeability for Cl ions. We now report on Cl distribution and fluxes in unstimulated human neutrophils. Most of the steady state Cl influx and efflux is carrier-mediated, electrically silent Cl/Cl self-exchange that is insensitive to stilbene disulfonates but is inhibited by \(\alpha\)-cyano-4-hydroxycinnamate. Electrodiffusive Cl\(^-\) fluxes are small, which is consistent with a low membrane Cl conductance and a K/Cl permeability ratio of \(\sim 10\). Cl ions are not distributed at thermodynamic equilibrium, but are concentrated intracellularly to \(\sim 80\) meq/liter cell water, fourfold above the expected passive distribution. There is an active Cl uptake mechanism that requires metabolic energy and is inhibited by \(\alpha\)-cyano-4-hydroxycinnamate, furosemide, and ethacrynic acid.

**METHODS**

**Incubation Media**

The standard medium used throughout this study had the following composition (in mM): 140 NaCl, 5 KCl, 1 CaCl\(_2\), 0.5 MgCl\(_2\), 5.6 glucose, 5 HEPES buffer, pH 7.4, and 1 mg/ml bovine serum albumin. Where required, \(p\)-aminohippurate (PAH) replaced Cl and \(N\)-methyl-d-glucamine replaced Na.

**Neutrophils**

Normal human peripheral blood neutrophils were isolated by sequential dextran sedimentation and Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) gradient centrifugation (Böyum, 1968). Contaminating erythrocytes were removed by lysis in distilled water for 30 s. The neutrophils were washed twice and then counted. Purity averaged 97% as judged by Wright's stain. Viability, assessed by Eosin Y exclusion, averaged 99% and was not affected by any of the agents tested in this study. Unless stated otherwise, cells were prepared at 37°C, spun in unrefrigerated centrifuges, and kept at 37°C for at least 60 min before and during all assays. (Storage for up to 5 h at that temperature in 5 mM K medium had no effect on intracellular K, Na [Simchowitz et al., 1982, and unpublished data], or Cl content, or on the subsequent behavior of these cells.)

In some experiments, neutrophils were depleted of intracellular Cl by incubation in 148 mM PAH medium for up to 6 h at 37°C. These cells remained viable and maintained a normal intracellular pH (\(\sim 7.25\)) as measured by the equilibrium distribution of 5,5-dimethylloxazolidine-2,4-dione (DMO) (Simchowitz and Roos, 1985).

**Reagents**

We obtained crystalline bovine serum albumin, acetazolamide, PAH, \(N\)-methyl-\(d\)-glucamine, ethacrynic acid, 2-deoxy-\(d\)-glucose (2-DOG), picrotoxin, phlorizin, ouabain, and HEPES from Sigma Chemical Co., St. Louis, MO; \(\alpha\)-cyano-4-hydroxycinnamic acid (CHC) was from Aldrich Chemical Co., Milwaukee, WI; sodium 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) was from Pierce Chemical Co., Rockford, IL; 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) was from Pierce Chemical Co. and ICN Pharmaceuticals, Cleveland, OH; and \([^{3}H]\)H\(_2\)O, \([^{4}C]\)inulin, \(^{35}\)Cl (specific activity, 6.3–13.8 mCi/g Cl), and \([^{3}H]\)PAH (specific activity 2.4 Ci/mmol) were from New England Nuclear, Boston, MA. Amiloride was a generous gift of Dr. Edward J. Cragoe, Jr., of Merck, Sharp
& Dohme Research Laboratories, West Point, PA; furosemide was given by Hoechst Laboratories; and the potentiometric dye 3,3'-dipropylthiadicarbocyanine iodide [diS-C₃(5)] was a gift of Dr. Alan Waggoner of Carnegie-Mellon University, Pittsburgh, PA. When 2-DOG was used to block ATP synthesis, the neutrophils were pretreated with this inhibitor for 10–15 min at 37°C in glucose-free medium. For the other compounds tested, preincubation was found to be unnecessary.

**^36^Cl Flux Measurements**

The technique described by Naccache et al. (1977) was used. Neutrophils (7–10 × 10⁶ cells/ml) were incubated at 37°C in capped plastic tubes (Falcon Plastics, Oxnard, CA). Influx experiments were performed in the presence of ^36^Cl (1.5 μCi/ml). At stated intervals, triplicate 0.5-ml aliquots were layered on 0.7 ml of silicone oil (Versilube F-50, General Electric, Waterford, NY) and spun at 8,000 g for 1 min; cell separation occurred in <5 s. The pellets were excised, dispersed in 10 ml of Aquasol 2 (New England Nuclear), and counted for 10 min in a liquid scintillation counter (LS 7000, Beckman Instruments, Inc., Palo Alto, CA). (The right-hand ordinate of Fig. 2 shows the measured radioactivity, including zero-time "uptake" [presumably label trapped in the extracellular space], for a typical influx experiment. The trapped extracellular fluid accounted for ~10% of the total water content of the pellet.) Influxes, corrected for zero-time "uptake," followed single-exponential equations of the form:

\[
C_t = C_w[1 - \exp(-kt)],
\]

where \(C_t\) is the cell label at time \(t\), \(C_w\) 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 was computed from the product \(kC_w\). In some cases, influx was so slow as to appear linear over the period of study; the rate was then simply the slope of the straight line.

For the efflux studies, neutrophil suspensions (2–3 × 10⁶ cells/ml) were incubated with ^36^Cl (2.5 μCi/ml) for 1–2 h at 37°C. The cells were then washed twice and resuspended in unlabeled medium. Triplicate samples were taken at stated intervals and spun and counted as above. The efflux kinetics were first-order, and rate coefficients were determined by least-squares fitting of single exponentials to the time course of radioactivity remaining in the cells. (The right-hand ordinate of Fig. 3 shows the measured radioactivity for a typical efflux experiment.) Unless stated otherwise, figure symbols are averages ± SEM.

**Determination of Intracellular Cl**

Triplicate 1-ml aliquots of neutrophil suspensions (15–20 × 10⁶ cells/ml) were layered over 0.4 ml of silicone oil contained in 1.5-ml microcentrifuge tubes and spun for 1 min at 8,000 g. The aqueous and oil phases were discarded. The neutrophil pellets were lysed in 1% Triton X-100 and assayed coulometrically for Cl (Cotlove et al., 1958) using a Buchler Instruments (Fort Lee, NJ) chloridometer. Results are expressed in milliequivalents per liter of cell water after appropriate corrections for total and extracellular water spaces using [³H]H₂O and [¹⁴C]inulin, respectively, as markers.

**Fluorescence Measurements**

A 10⁻³ M stock solution of diS-C₃(5) in ethanol was kept in the dark at 4°C. Experiments were performed in a total volume of 3 ml containing 4 × 10⁶ neutrophils, 1 μM dye, and 0.1% ethanol. After equilibration for 5–10 min at 37°C, fluorescence of the suspension was measured at 37°C in a spectrofluorometer (model 430, Turner Associates, Palo Alto,
CA). Excitation was at 622 nm and emission measurement was at 670 nm. At the concentrations employed and for the duration and conditions of our assays, 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 formylmethionyl-leucyl-phenylalanine (10^{-7} M) or intracellular concentrations or active and passive fluxes of K^+ and Na^+ (Simchowitz et al., 1982). In the absence of cells, changes in the medium's Cl concentration did not alter the dye fluorescence.

Intracellular Water

We previously reported (Simchowitz et al., 1982) a water content of 203 μm^3/neutrophil. However, a technical error (loss of cells through clumping immediately after isolation and during 1 h incubation at 37°C) marred these measurements, and we now report a corrected estimate of 274 μm^3/cell. Assuming spherical geometry and a cell radius of 4.4 μm, a transfer rate of 1 meq/liter cell water-min is equivalent to a flux of 1.87 pmol/cm^2-s. Our previous estimates for ion conductances (Simchowitz et al., 1982) must thus be revised upward. This will be done in the Appendix, where we also present revised calculations of membrane potential.

RESULTS

Intracellular Cl Concentration; Net Cl Movements

Fig. 1A shows the effect of extracellular Cl replacement with PAH, which is very impermeant in erythrocytes (Motais, 1977), on the intracellular Cl concentration ([Cl]_i) of isolated human neutrophils. Cells isolated and kept for 1 h at 37°C in normal 148 mM Cl medium maintained a [Cl]_i of 78.9 ± 3.7 meq/liter cell water (mean ± SEM) for at least 3 more hours. (In other experiments [not shown], we ascertained that the Cl content of cells bathed in 148 mM Cl medium remained at ~80 meq/liter cell water for up to 6 h. Furthermore, the ^56Cl fluxes in such cells were similar to those seen routinely in neutrophils incubated for only 1–2 h.) In contrast, cells resuspended at 37°C in PAH medium lost Cl exponentially (rate coefficient k = 0.010 min^{-1}; initial rate, 0.81 meq/liter-min). After 3 h, [Cl]_i had fallen to 11.9 ± 1.8 meq/liter cell water. For convenience, cells kept in normal 148 mM Cl media will henceforth be called “normal Cl,” and those kept in PAH media for at least 2.5 h will be termed “Cl depleted.” The fact that 40 mM CHC, an inhibitor of carrier-mediated anion transport (Halestrap, 1976), slowed Cl loss into PAH medium by ~50% (to an initial rate of 0.39 meq/liter-min; Fig. 1A) strongly suggests that this efflux does not occur by simple electrodiffusion. It will be shown in the following article (Simchowitz et al., 1986) that much of this Cl loss occurs via carrier-mediated 1:1 exchange for external PAH. In addition, these cells maintained a normal or near-normal (within ~10%) cell water content and a normal intracellular pH of ~7.25 as measured by the DMO method (Simchowitz and Roos, 1985). Cells bathed in 108 mM Cl medium containing 40 mM CHC lost Cl nearly as rapidly as those in 108 mM PAH medium containing 40 mM CHC (initial rate, 0.31 meq/liter-min).

Since the resting potential of human neutrophils is about ~53 mV (Simchowitz et al., 1982; see Appendix), intracellular Cl appears not to be distributed at thermodynamic equilibrium, but concentrated fourfold over the expected passive
distribution level (20 mM for $V_m = -53$ mV and $[Cl]_o = 148$ mM). The presence of an active inward transport process for Cl thus seems likely. Fig. 1B shows that cells depleted of Cl to just below the expected equilibrium level reaccumulate Cl at a rate of 0.20 meq/liter cell water·min from a Cl medium, raising $[Cl]_i$, well beyond the expected equilibrium concentration. The substitution of N-methyl-D-glucamine for Na in the bathing medium had no effect on the rate of reaccumulation. Cl uptake was largely prevented, however, by the metabolic inhibitor 2-DOG, by CHC, and by the "loop" diuretic ethacrynic acid.

**Figure 1.** Time course of intracellular Cl levels in human neutrophils. (A) Normal-Cl cells. Upper curve: cells kept in 148 mM Cl medium ($n = 8$). Lower curve: cells placed, at $t = 0$, in 148 mM PAH medium; single exponential with rate coefficient $k = 0.0103 \pm 0.0007$ min$^{-1}$ ($n = 4$). Solid symbols: cells exposed to 40 mM CHC; circles: 108 mM Cl medium; rate coefficient, $0.0039 \pm 0.0006$ min$^{-1}$ ($n = 4$); squares: 108 mM PAH medium; rate coefficient, $0.0049 \pm 0.0006$ min$^{-1}$ ($n = 4$). (B) Cl-depleted cells (after 2.5 h in PAH medium). Upper curve: cells placed, at $t = 0$, in 148 mM Cl medium containing either Na ($\bullet$, $n = 8$) or N-methyl-D-glucamine ($\Delta$, $n = 4$) as the major cation; uptake for the combined data, $0.200 \pm 0.017$ meq/liter·min. Middle curve ($\square$): cells preincubated for 10 min in 148 mM PAH medium containing 1 mM 2-DOG and placed, at $t = 0$, in 148 mM Cl medium also containing 2-DOG; the curve is an empirical second-degree polynomial with an initial slope of $0.048 \pm 0.089$ meq/liter·min ($n = 3$). Lower curve: cells placed, at $t = 0$, in 148 mM Cl medium containing 40 mM CHC ($\circ$, $n = 3$) or 1 mM ethacrynic acid ($\blacksquare$, $n = 3$); initial slope, $-0.014 \pm 0.048$ meq/liter·min. The arrow at 20 meq/liter cell water indicates the level expected for passive distribution in cells with $V_m = -53$ mV.
One-Way Cl Fluxes

The time course of $^{36}\text{Cl}$ uptake by normal-Cl cells from a 148 mM Cl medium is shown in Fig. 2 (curve labeled "control"). The initial label uptake rate was $1.15 \pm 0.05$ meq/liter cell water-min, six times faster than net Cl uptake by de-

![Graph showing time course of $^{36}\text{Cl}$ uptake from 148 mM Cl medium by normal-Cl cells. The upper three curves are least-squares fits of Eq. 1 to the data. The right-hand ordinate shows the actual radioactivity measurements for the control curve; comparable activities were encountered in all similar experiments reported in this article. □, medium containing 5 mM K; initial uptake rate, $1.15 \pm 0.05$ meq/liter cell water-min ($n=5$); extrapolated maximal uptake, $81 \pm 21$ meq/liter cell water. △, medium containing 130 mM K; initial uptake rate, $1.35 \pm 0.07$ meq/liter cell water-min ($n=3$). ○, medium containing 1 mM 2-DG; initial uptake rate, $0.96 \pm 0.08$ meq/liter-min ($n=3$). ■, medium containing 40 mM CHC. The slope of the straight line is $0.22 \pm 0.02$ meq/liter-min ($n=3$). The data were obtained in 108 mM Cl medium, but pro-rated to 148 mM Cl to correct for dilution by CHC.

pleted cells (Fig. 1B). The extrapolated maximal $^{36}\text{Cl}$ level for these cells was $81 \pm 21$ meq/liter cell water, which is compatible with our value for total Cl content and with the notion that all cell Cl exchanges with extracellular $^{36}\text{Cl}$. This influx was strongly inhibited by CHC (Fig. 2, lower curve). We will show in
the following article (Simchowitz et al., 1986) that the apparent \( K_i(\text{CHC}) \) is \( \sim 9 \) mM; the inhibition by CHC shown here is therefore only \( \sim 85\% \) complete.

The time course of \(^{36}\text{Cl}\) efflux from normal-Cl cells into a 148 mM Cl, 5 mM K medium is shown in Fig. 3 (lower curves). The rate coefficient of \( 0.0173 \pm 0.0005 \) min\(^{-1}\) for efflux into 148 mM Cl medium (lower group of three curves): efflux into 148 mM Cl medium. The lower curve is fitted to the efflux from cells bathed in 5 mM K (O); rate coefficient, \( 0.0173 \pm 0.0005 \) min\(^{-1}\) \((n = 8)\); The middle curve is fitted to the efflux from cells exposed to 1 mM 2-DOG (\( \Delta \)); rate coefficient, \( 0.0168 \pm 0.0009 \) min\(^{-1}\) \((n = 3)\); The upper curve is fitted to the efflux from cells bathed in 130 mM K (\( \bigcirc \)); rate coefficient, \( 0.0155 \pm 0.0007 \) min\(^{-1}\) \((n = 6)\). For clarity, error bars and exponential fits have been omitted from the solid symbols, which represent cells bathed in 5 mM K media containing 1 mM of one of three inhibitors: SITS (circles): rate coefficient, \( 0.0185 \pm 0.0010 \) min\(^{-1}\) \((n = 3)\); furosemide (squares): rate coefficient, \( 0.0156 \pm 0.0008 \) min\(^{-1}\) \((n = 3)\); and ethacrynic acid (triangles): rate coefficient, \( 0.0174 \pm 0.0006 \) min\(^{-1}\) \((n = 3)\). PAH medium (intermediate curve): efflux into 148 mM PAH medium; rate coefficient, \( 0.0098 \pm 0.0002 \) min\(^{-1}\) \((n = 3)\). CHC medium (upper curve): efflux into 148 mM CHC medium; rate coefficient, \( 0.0049 \pm 0.0002 \) min\(^{-1}\) \((n = 3)\).
0.0005 min⁻¹, assuming a value of 80 meq/liter cell water for [Cl]ᵢ, signifies an efflux of 1.38 ± 0.04 meq/liter·min. Three known inhibitors of erythrocyte anion transport, SITS, furosemide, and ethacrynic acid (for reviews, see Sachs et al., 1975, and Lowe and Lambert, 1983), each at 1.0 mM, had no significant effect on ³⁶Cl efflux. DIDS (0.1 mM) was equally ineffective (data not shown). The omission of bovine serum albumin from the media did not alter these results, which suggests that the drugs' ineffectiveness was not caused by their adsorption to protein. Also, SITS from the same batch blocked >99% of ³⁶Cl uptake by human erythrocytes (data not shown).

These one-way Cl fluxes are somewhat larger than those found for K and Na (~0.9 meq/liter cell water·min) in steady state cells (Simchowitz et al., 1982). If they were mostly electrodiffusive fluxes through permeability channels, the Cl conductance would be a major fraction of total membrane conductance, and the membrane potential would be quite sensitive to changes in [Cl]ᵢ. However, the complete replacement of external Cl with PAH had no appreciable effect on the fluorescence of cell suspensions containing the potentiometric dye diS-C₆(5): using our fluorescence-potential calibration curve (Simchowitz et al., 1982, and Appendix), we found that Vₘ remained at −52 ± 2 mV. Conversely, electrodiffusive Cl fluxes should have been strongly Vₘ dependent. However, we found that depolarization to ~0 mV (with 130 mM K medium) affected only a small fraction of the one-way Cl fluxes in normal-Cl cells: influx was raised to 1.35 ± 0.07 meq/liter cell water·min from 1.15 ± 0.05 (Fig. 2, top curve), and efflux was lowered from 1.38 ± 0.04 to 1.24 ± 0.06 meq/liter·min (Fig. 3). Furthermore, we have previously reported (Simchowitz et al., 1982) that the sum of the specific conductances for K and Na approximately accounts for total membrane conductance, leaving little room for much Cl conductance. The most plausible interpretation of all these findings is that major portions of the one-way Cl fluxes do not occur via conductance pathways, but rather by voltage-insensitive pumps and/or electrically silent exchange mechanisms. The latter could be coupled, e.g., as Cl/Cl self-exchange, or in parallel pathways, e.g., via K + Cl cotransport carriers.

Active Uptake and Cl/Cl Exchange

Active inward transport of Cl must be postulated to account for the high [Cl]ᵢ (fourfold higher than predicted for thermodynamic equilibrium). We therefore tested the effect of intracellular ATP depletion on one-way ³⁶Cl fluxes, using 2-DOG, a competitive inhibitor of D-glucose. Exposure of cells to 1 mM 2-DOG in glucose-free media for 15 min at 37°C, or to 0.1 mM ouabain, depressed their ²²Na efflux rate coefficient by 97%, from 0.032 to 0.001 min⁻¹. We conclude that preincubation with 2-DOG causes sufficient intracellular ATP depletion to abolish Na/K pump activity. It is known that under these conditions, intracellular ATP levels are reduced at least 10-fold (Borregaard and Herlin, 1982). The effect of 2-DOG pretreatment on one-way ³⁶Cl fluxes in normal-Cl cells is shown in Figs. 2 and 3. Whereas 2-DOG caused a slight (17%) inhibition of the initial ³⁶Cl uptake rate, which suggests a minor ATP-dependent component of uptake, it had no effect on the rate of ³⁶Cl efflux.
The relatively modest effect of 2-DOG on one-way $^{36}$Cl fluxes in normal-Cl cells strengthens the hypothesis that the bulk of these fluxes is carrier-mediated self-exchange. We summarize here the additional evidence: (a) only small portions of one-way influx and efflux are affected by wide variations in membrane potential (Figs. 2 and 3); (b) conversely, large changes in $[\text{Cl}]_o$ have negligible effects on $V_m$; (c) label uptake by normal-Cl cells (1.15 meq/liter-min; Fig. 2) is six times more rapid than net Cl uptake by Cl-depleted cells (0.20 meq/liter-min; Fig. 1B); (d) $^{36}$Cl loss into Cl medium is nearly four times faster (1.58 vs. 0.39 meq/liter·min; Fig. 3) than into a medium where the main anion is CHC, a known competitive inhibitor of carrier-mediated anion exchange (Halestrap, 1976); (e) Cl efflux into PAH medium is still further inhibited by CHC (Fig. 1), which suggests that the putative anion exchange carrier does have affinity for PAH. An exchange carrier should exhibit 
\textit{trans} effects, saturation kinetics, and possibly competition and specific inhibition. All four were found and will be examined in detail in the following article (Simchowitz et al., 1986). The remainder of this article is devoted to the active and electrodiffusive fractions.

\textbf{Characterization of Active Cl Uptake}

To evaluate the putative ATP-dependent (operationally, 2-DOG–sensitive) influx more clearly, we minimized the contribution of Cl/Cl self-exchange (which dominates total $^{36}$Cl uptake in normal-Cl cells) by lowering $[\text{Cl}]_o$ through prolonged (~6 h) incubation of the cells in PAH media. The initial rate of $^{36}$Cl uptake by such exhaustively depleted ($[\text{Cl}]_o < 2 \text{mM}$) cells bathed in 148 mM Cl medium (Fig. 4) was 0.42 meq/liter·cell water·min, or only ~30% of the rate for normal-Cl cells. This is expected if a large fraction of $^{36}$Cl uptake by normal-Cl cells indeed consisted of Cl/Cl self-exchange.

The three curves of Fig. 4 are most straightforwardly interpreted as follows. The lower curve, $^{36}$Cl uptake in the presence of 40 mM CHC, represents (as will be argued later) electrodiffusive influx. The time course is approximately linear because the expected equilibrium $^{36}$Cl content is appreciably higher (~20 meq/liter) than the prevailing $[\text{Cl}]_o$; the influx rate is 0.10 meq/liter·min. The middle curve, $^{36}$Cl uptake in the presence of 2-DOG (which presumably abolishes active transport), should comprise both the electrodiffusive component and the carrier-mediated exchange of external $^{36}$Cl for internal Cl and PAH. (Further evidence for internal PAH/external Cl exchange will be discussed in the accompanying article [Simchowitz et al., 1986].) The data of Fig. 4 do not, of course, allow resolution of the presumed carrier-mediated $^{36}$Cl uptake, 0.11 meq/liter·min, into Cl/Cl exchange and PAH/Cl exchange portions. However, assuming that $[\text{Cl}]_o \approx 1 \text{mM}$ and taking a rate coefficient for Cl/Cl exchange in low-Cl cells of
~0.04 min⁻¹ [Simchowitz et al., 1986], we find 0.04 meq/liter-min for Cl/Cl exchange and 0.07 meq/liter-min for PAH/Cl exchange. The PAH/Cl exchange component was presumed linear in Fig. 4 because of the large intracellular PAH

![Figure 4](image-url)

**Figure 4.** Uptake of ³⁶Cl from 148 mM Cl medium by Cl-depleted cells. Cells were depleted of Cl by incubation in PAH medium for ~6 h. The uptake of ³⁶Cl was then measured as in Fig. 2. The lower curve is a straight line. The top two curves are sums of a single exponential (Eq. 1) and a straight line. All curves represent least-squares fits. Lower curve: ³⁶Cl uptake in the presence of 40 mM CHC, corrected to [Cl]₀ = 148 mM to compensate for dilution by CHC. Slope, 0.10 ± 0.01 meq/liter-min (n = 4). Middle curve: ³⁶Cl uptake from 148 mM Cl medium in the presence of 1 mM 2-DOG (solid circles, n = 10). The curve drawn includes the assumption that a Cl/Cl exchange component exists, following Eq. 1 with compartment size = 1 meq/liter cell water and rate coefficient = 0.04 min⁻¹, in addition to electrodiffusive and PAH/Cl exchange components, both constant for the duration of the experiment. The least-squares slope for the sum of the latter two was 0.17 ± 0.01 meq/liter-min. Open squares and triangles represent experiments carried out in 148 mM Cl medium containing 1 mM ethacrynate (n = 5) and furosemide (n = 4), respectively. These data were not included in the least-squares fit. Error bars have been omitted for clarity, but were similar in size to those shown for the 2-DOG data. Upper curve: ³⁶Cl uptake from 148 mM Cl medium (n = 10). The curve includes a presumed Cl/Cl exchange component identical to the one in the middle curve, and a linear component comprising electrodiffusive, PAH/Cl exchange, and active uptake fractions. The least-squares value for this linear component was 0.38 ± 0.02 meq/liter-min.
reserve.) Finally, the top curve in Fig. 4 (no inhibitors present) reflects all three components: electrodiffusive influx and exchange carrier-mediated influx as above, and active uptake. The time course of active uptake is presumably linear, its final "destination" being ~80 meq/liter. The slope of the top curve in Fig. 4 is 0.21 ± 0.03 meq/liter·min steeper than that of the middle curve. This difference represents the active, 2-DOG-sensitive component. It is similar to the net rate of Cl gain (0.20 meq/liter·min) already seen in cells depleted to about the equilibrium level (Fig. 1B) and to the magnitude of the 2-DOG-sensitive $^{36}\text{Cl}$ influx (0.19 meq/liter·min) in normal-Cl cells (Fig. 2). The latter observation implies that intracellular Cl depletion has little effect on active uptake.

The main feature of Fig. 4 is that, unlike in normal-Cl cells, where only a small fraction (17%) of $^{36}\text{Cl}$ uptake was inhibited by 2-DOG (Fig. 2), a sizable portion of $^{36}\text{Cl}$ uptake into Cl-depleted cells is inhibited by 1 mM 2-deoxy-d-glucose, 1 mM furosemide, or 1 mM ethacrynic acid. These agents had no appreciable effect on CHC-insensitive uptake (not shown) or on the $^{36}\text{Cl}$ efflux rate from normal-Cl cells (Fig. 3). (A comparison of Figs. 2 and 4 reveals that the "baseline" uptake, resistant to 40 mM CHC, is lower in depleted cells [0.10 ± 0.01 meq/liter·min] than in normal-Cl cells [0.22 ± 0.02 meq/liter·min]. This is due to the fact that the apparent $K_r$(CHC) of the exchange carrier is ~9 mM in normal-Cl cells [Simchowitz et al., 1986], but only ~0.7 mM in Cl-depleted cells [see below]. We have not yet explored the kinetic details of this difference in apparent affinity, but its practical consequence is that 40 mM CHC inhibits the exchange carrier ~85% in normal-Cl cells, and ~98% in Cl-depleted cells. The dissimilar residual influx levels in 40 mM CHC reflect this difference in exchange inhibitor affinity.) The observations of Fig. 4 support the contention that the only detectable consequence of lowering [Cl]o is to diminish Cl/Cl exchange, and that 2-DOG, furosemide, and ethacrynic acid inhibit only the putative inward active transport process and are without effect on the self-exchange carrier or on diffusive permeability, whereas CHC inhibits both the active (0.21 meq/liter·min) and the exchange (0.11 meq/liter·min) components. Replacement of all but ~1 mM extracellular Na with N-methyl-D-glucamine, or omission of external K, had no significant effect on the rate of $^{36}\text{Cl}$ uptake. The transport inhibitors amiloride, acetazolamide, phlorizin, picrotoxin, and SITS (each at 1 mM) were ineffective. The dose-response curves for furosemide, ethacrynic acid, and CHC inhibition of active Cl uptake, defined as 2-DOG-sensitive influx, are shown in Fig. 5. Least-squares fits to the inhibition equations yielded apparent $K_r$'s of ~50 μM for both furosemide and ethacrynic acid, and ~1.7 mM for CHC.

We also determined the [Cl]o dependence of the active uptake component. In Fig. 6A, the initial rates of $^{36}\text{Cl}$ uptake by Cl-depleted cells, in the presence and absence of 1 mM 2-DOG, are plotted against the external Cl concentration (0.3–40 mM, balance PAH). The lower curve, which is the sum of a linear (presumably leak) and a saturable (presumably exchange) component, fits the 2-DOG data well at all but very low [Cl]o. The differences in $^{36}\text{Cl}$ uptake measured between control cells and the paired batch exposed to 1 mM 2-DOG are plotted in Fig. 6B. A least-squares fit yields a Michaelis-Menten curve with $K_m = 4.8 ± 1.2$ mM and $V_{max} = 0.24 ± 0.02$ meq/liter·min. This curve, when added to the 2-DOG curve of Fig. 6A, fits the original control flux data well (Fig. 6A, upper curve).
Nature of the CHC-resistant Cl Fluxes

A plausible hypothesis is that the CHC-insensitive fluxes occur by electrodiffusion through ion channels, in which case influx and efflux can be expected to display little or no evidence of saturation as a function of [Cl], to be voltage dependent, and to become equal when $V_n = E_{Cl}$.

The accurate characterization of the putative channel-mediated Cl fluxes, because of their small magnitude, requires maximal suppression of the two other components of total flux, active transport and exchange. For the experiments that follow, markedly Cl-depleted cells were used. The very low [Cl], (~2 mM) serves two purposes: (a) it lessens the already minor contribution of Cl to the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949) governing $V_m$, and (b) it reduces Cl/Cl exchange by removing the intracellular exchange partner. Active and residual exchange fluxes were suppressed with 40 mM CHC. Fig. 7 shows the concentration dependence of CHC inhibition of $^{36}$Cl efflux from these very-low-Cl cells. Since inhibition obeys Michaelis-Menten kinetics with an apparent $K_i(CHC)$ of 0.74 mM, carrier-mediated efflux from very-low-Cl cells should be 98% suppressed at [CHC] = 40 mM. From the data

**Figure 5.** Active (2-DOG-sensitive) $^{36}$Cl uptake into Cl-depleted cells: inhibition by furosemide, ethacrynic acid, and CHC. Fluxes represent the differences between the uptake into cells treated with 1 mM 2-DOG and that into cells exposed to various concentrations of furosemide, ethacrynic acid, or CHC ($n = 3$ for each compound). The data were fit with equations of the form: influx = (initial influx) · $K_i/([I] + K_i)$, where $[I]$ is the inhibitor concentration and $K_i$ is the inhibition constant. Least-squares $K_i$ values were 49.9 ± 12.6 μM for furosemide, 51.4 ± 12.4 μM for ethacrynic acid, and 1.68 ± 0.30 mM for CHC.
in Fig. 5C, we also conclude that at [CHC] = 40 mM, 96% of active influx is suppressed. This essentially complete suppression of carrier-mediated fluxes makes possible a detailed investigation of the remaining, presumably electrodiffusive, fluxes.

![Graph showing chloride uptake by low-Cl cells](image)

**Figure 6.** 

A first property generally expected of diffusive flux is illustrated in Fig. 8, where CHC-insensitive $^{36}$Cl influx into Cl-depleted cells is plotted against [Cl]$_o$. There is no evidence of saturation through the highest level tested (100 mM). The extrapolated influx for [Cl]$_o = 148$ mM is $0.083 \pm 0.004$ meq/liter·min, which is comparable to the CHC-resistant influx rate of 0.10 meq/liter·min found in Fig. 4.

Electrodiffusive fluxes should also be voltage sensitive. The resting membrane
potential of these cells is around \(-53\) mV. The \(P_{Na}/P_{K}\) ratio is 0.1, and, from indirect evidence (Simchowitz et al., 1982), the resting Cl permeability \((P_{Cl})\) appears sufficiently low to play only a minor role in determining \(V_{m}\). We manipulated the cells' membrane potential by varying \([K]_o\) between 1 and 130 mM in Na-free media (equivalent replacement with N-methyl-d-glucamine), and assumed the following intracellular ion concentrations: 120 mM \([K]_i\), 25 mM \([Na]_i\) (Simchowitz et al., 1982), and \(~2\) mM \([Cl]_i\). Given these conditions and (as

\[
V_{m} = \frac{RT}{F} \ln \frac{P_k[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_o}{P_k[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_i}
\]

will be shown later) a \(P_k/P_{Cl}\) ratio of \(~10\), the constant-field membrane potential equation (Goldman, 1943, Hodgkin and Katz, 1949),

\[
V_{m} = \frac{RT}{F} \ln \frac{[K]_o + 0.2}{133}
\]

(where the \(P's\) represent permeabilities and the other symbols have their usual meaning), reduces to (\([K]_o\) in millimolar):

Fig. 9A shows time courses of \(^3\)Cl uptake at \([K]_o = 1.3, 4, 13, 40, \) and 130 mM, or, from Eq. 3, \(V_{m} \approx -120, -92, -62, -32, \) and \(-1\) mV, respectively. As
expected for electrodiffusive fluxes and in sharp contrast to the modest effect on total uptake rate (Fig. 2), depolarization caused the CHC-insensitive \(^{36}\)Cl uptake rates to increase dramatically from 0.012 meq/liter cell water \(\cdot\) min at approximately \(-120\) mV to 0.284 meq/liter \(\cdot\) min at \(~0\) mV. At \([\text{K}]_o = 13\) mM \((V_m \approx -62\) mV, not far from the normal resting potential), an influx of 0.078 ± 0.003 meq/liter \(\cdot\) min from a 108 mM \(\text{Cl}\) medium was found. Assuming linearity, the calculated CHC-resistant \(^{36}\)Cl influx into normal cells bathed in 148 mM \(\text{Cl}\) media would be 0.11 meq/liter \(\cdot\) min, or only \(~10\)% of total \(^{36}\)Cl influx. Lowering \(V_m\) to \(~0\) mV raised this figure by 0.28 meq/liter \(\cdot\) min, which easily accounts for the modest increase in total \(^{36}\)Cl influx we observed earlier (Fig. 2) upon exposure to 130 mM \(\text{K}\) media.

Fig. 9B displays measurements of \(^{36}\)Cl efflux under similar conditions. The cells were \(\text{Cl}\) depleted, as before, to minimize contributions from \(\text{Cl}/\text{Cl}\) exchange. Depletion (and simultaneous labeling with \(^{36}\)Cl) was achieved by incubating the cells for at least 5 h in PAH medium containing 0.3 \(\mu\)Ci/ml \(^{36}\)Cl \((\text{[Cl]}_o = 1.3\) mM). Since the cells were exposed to \(^{36}\)Cl for 5 h, it is reasonable to assume that they had nearly reached isotopic equilibrium. From the specific activity of the loading medium, we calculate a \([\text{Cl}]_o\) of 1.9 ± 0.6 mM at the start of the efflux experiment. Altering \([\text{K}]_o\) (and hence \(V_m\)), again in contrast to the modest effect on total \(\text{Cl}\) efflux shown in Fig. 3, markedly affected the \(^{36}\)Cl efflux rates, but in
a direction opposite to that observed for uptake (Fig. 9A). The rate coefficients decreased from 0.0128 min⁻¹ at approximately −120 mV to 0.0019 min⁻¹ at ~0 mV.

The complete absence of saturation and the striking voltage dependence of the CHC-resistant fluxes described here support the notion that these fluxes are indeed channel mediated. A third finding also argues that CHC-resistant fluxes are diffusive: it will be shown that CHC-insensitive Cl influxes and effluxes become equal at the Cl equilibrium potential.

**DISCUSSION**

**Intracellular Cl Concentration**

To our knowledge, this is the first report on the intracellular Cl content of isolated human neutrophils. Our value of 80 meq/liter cell water (range, 62–93) for the [Cl]o of steady state cells bathed in normal-Cl media ([Cl]o = 148 mM), all of which appears to be exchangeable with extracellular Cl, is similar to that of

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**FIGURE 9.** Effect of membrane potential on ⁸⁶Cl fluxes in Cl-depleted ([Cl]o = 1.9 mM) cells bathed in 108 mM Cl medium containing 40 mM CHC and K at concentrations ranging from 1.3 to 130 mM. (A) ⁸⁶Cl uptake at various [K]o, following the method of Fig. 2. The rates are linear, and the values of the slopes are (in meq/liter·min): 0.012 ± 0.003 at 1.3 mM K, 0.029 ± 0.003 at 4 mM K, 0.078 ± 0.003 at 13 mM K, 0.182 ± 0.016 at 40 mM K, and 0.284 ± 0.016 at 130 mM K (n = 3). (B) Time courses, at various [K]o levels of the ⁸⁶Cl fraction remaining in cells; semilogarithmic plot, as in Fig. 3. The curves are single exponentials, and their rate coefficients (in min⁻¹) are: 0.0128 ± 0.0003 at 1.3 mM K, 0.0092 ± 0.0003 at 4 mM K, 0.0056 ± 0.0003 at 13 mM K, 0.0034 ± 0.0002 at 40 mM K, and 0.0019 ± 0.0002 at 130 mM K (n = 3).
other studies on leukocytes: ~83 meq/liter cell water for mixed horse blood leukocytes (Endres and Herget, 1929), 88–97 meq/liter cell water for rabbit peritoneal leukocytes (Wilson and Manery, 1949), 111 ± 14 meq/liter cell water for mixed human leukocytes (Baron and Ahmed, 1969), 84 ± 2 meq/liter cell water for human leukemia (HL-60 line) cells (Dissing et al., 1984), 87 ± 8 meq/liter cell water for human lymphocytes (Zieve et al., 1967), and 64 meq/liter cell water for rat alveolar macrophages (Castranova et al., 1979). Since [Cl], does not change for 3 h at 37°C (Fig. 1), our cells appear to be in true steady state. Assuming identical intra- and extracellular activity coefficients, the calculated Cl equilibriumpotential ($E_{eq}$) for normal, steady state cells bathedin 148 mM Cl medium at 37°C is -16 mV. The resting membrane potential of these cells is approximately -53 mV; it follows that Cl is not passively distributed, but must be actively transported inward.

Cl Fluxes

It has long been known that anions readily cross the leukocyte membrane. Hamburger and van der Schroeff (1902) observed reversible exchanges between (bi)carbonate and chloride (the "Hamburger shift"), nitrate, or sulfate across the membrane of horse septic leukocytes, with concomitant acidity changes. Fleischmann (1930), using horse peripheral leukocytes, demonstrated permeability to iodide, thiocyanate, and salicylate ions. Wilson and Manery (1949) showed that rabbit peritoneal leukocytes lose and gain Cl, depending on the concentration of Cl in the bathing medium. In none of the quoted studies, however, were the time courses of concentration changes established or quantitative flux measurements made.

Table I lists the magnitudes of a number of fluxes relevant to the discussion that follows. Our conclusion that the isolated neutrophils, bathed at 37°C in 148 mM Cl medium, were in true steady state is corroborated by the observation that total one-way $^{36}$Cl influx and effluxes were of equal magnitude within experimental error. Of the total steady state $^{36}$Cl efflux ($\sim$1.4 meq/liter·min), $\sim$1.0 meq/liter·min or $\sim$70% is attributable to carrier-mediated self-exchange that is inhibited by CHC, a competitive inhibitor of anion transport (Halestrap, 1976; for review, see Deuticke, 1982). The remaining $^{36}$Cl efflux, $\sim$0.4 meq/liter·min or $\sim$30% of the total (Fig. 3), probably represents electrodiffusion through channels. Steady state $^{36}$Cl uptake, on the other hand, was divisible into at least three components. The major fraction, $\sim$70% of the total, was Cl/Cl self-exchange. A much smaller CHC-resistant component, $\sim$0.1 meq/liter·min or $\sim$8% of total, behaved as diffusive influx. The third component, 0.20–0.25 meq/liter·min or $\sim$20% of the total, depended on metabolism and was inhibited by furosemide and ethacrynic acid as well as by CHC; it is probably the active process responsible for the intracellular accumulation of Cl.

The question arises whether cells transferred to 148 mM PAH medium retain their steady state volume, since such cells lose Cl with a rate coefficient $k_{net} = 0.01 \, \text{min}^{-1}$ (Fig. 1A). The total loss during the first hour is computed as

$$\text{first-hour Cl loss} = C_{in}[1 - \exp(-60k_{net})].$$
Given an initial intracellular Cl content \( (C_{in}) \) of 80 meq/liter cell water, the first-hour loss amounts to \( \sim 36 \) meq/liter. However, the net Cl loss is partly by diffusion and partly by equimolar Cl/PAH exchange, so that

\[
k_{net} = k_{diff} + k_{exch},
\]

where the rate coefficient for loss by diffusion, \( k_{diff} \), is \( \sim 0.005 \) min\(^{-1} \) (Fig. 3, Table 1).

### Table 1

**Summary of (Initial) Flux Magnitudes**

| Type of flux                              | Magnitude         | Figure |
|------------------------------------------|-------------------|--------|
| Net Cl fluxes                            |                   |        |
| Loss into PAH medium                     | 0.81±0.06         | 1A     |
| Loss into PAH medium + 40 mM CHC         | 0.39±0.05         | 1A     |
| Loss into Cl medium + 40 mM CHC          | 0.31±0.05         | 1A     |
| Gain \( ([Cl] \sim 16 \text{ mM}) \) from Cl medium | 0.20±0.02         | 1B     |
| 2-DOG resistant                          | 0.043±0.089       | 1B     |
| Inhibitor resistant\*                   | -0.014±0.048      | 1B     |

One-way \(^{36} \text{Cl} \) uptake \( ([Cl]_0 = 148 \text{ mM}) \)

| Normal-[Cl] \_ cells                     |                   |        |
| From control medium                      | 1.15±0.05         | 2      |
| From 130 mM K medium                     | 1.35±0.07         | 2      |
| 2-DOG sensitive                          | 0.19±0.08         | 2      |

| Low-[Cl] \_ cells                        |                   |        |
| Total influx                             | 0.54±0.06         | 4      |
| 2-DOG sensitive                          | 0.50±0.01         | 6\( ^{A4} \) |
| CHC resistant\*                           | 0.21±0.04         | 4      |
| CHC resistant\*                           | 0.27±0.02         | 5\( A, B \) |
| CHC resistant\*                           | 0.23±0.02         | 5\( C \) |
| CHC resistant\*                           | 0.22±0.04         | 6\( A4 \) |
| CHC resistant\*                           | 0.10±0.01         | 4      |
| CHC resistant\*                           | 0.083±0.003       | 8      |
| CHC resistant\*                           | 0.078±0.003       | 9\( A4 \) |

One-way \(^{36} \text{Cl} \) efflux

| Normal-[Cl] \_ cells                     |                   |        |
| Into control (Cl) medium\*               | 1.38±0.04         | 3      |
| Into 130 mM K medium                     | 1.24±0.06         | 3      |
| In the presence of 1 mM 2-DOG            | 1.34±0.07         | 3      |
| Into PAH medium                          | 0.78±0.02         | 3      |

| CHC resistant\*                           | 0.39±0.02         | 3      |
| Low-Cl \_ cells normalized to \([Cl] = 80 \text{ mM}\) | 0.42±0.10         | 7      |
| CHC resistant\*                           | 0.45±0.03         | 9\( B4 \) |

\* Inhibitors: 1 mM ethacrynic acid, 40 mM CHC.
\( ^{\dagger} \) Influxes in the presence of 40 mM CHC have been normalized to \([Cl]_0 = 148 \text{ mM}\) to compensate for dilution by CHC.
\( ^{\dagger} \) \([Cl]_0 = 40 \text{ mM}\).
\( ^{\dagger} \) Na-free, 13 mM K medium; calculated \( V_m = -60 \text{ mV} \).
\( ^{\dagger} \) Unaffected by 1 mM SITS, furosemide, and ethacrynic acid.
CHC medium) and that for loss by exchange, \( k_{\text{exch}} \), is also \( \sim 0.005 \text{ min}^{-1} \) (Fig. 3, difference between PAH and CHC media). Only the diffusive loss has osmotic consequences, and its magnitude is given by

\[
\text{first-hour diffusive Cl loss} = C_{\text{init}} \frac{k_{\text{diff}}}{k_{\text{diff}} + k_{\text{exch}}} \{1 - \exp[-60(k_{\text{diff}} + k_{\text{exch}})]\}.
\]

Using the above parameters, we find a net anion loss of 18 meq/liter cell water in the first hour. (Our analysis assumes that PAH has negligible diffusive permeability.) If net anion loss is accompanied by an equimolar loss of, e.g., K, we project a first-hour net osmotic loss of 36 mosmol/liter cell water or \( \sim 12\% \) of the cell's osmotic content. Theoretically, then, our cells might be expected to shrink \( \sim 12\% \) in the first hour of exposure to 148 mM PAH medium. In practice (unpublished measurements), we did not observe any significant shrinking over 3 h, although it would have been difficult to detect volume changes of \( < 10\% \). Since the cell volume remains approximately constant over several hours in PAH media, it appears that some unknown regulatory volume increase mechanism compensates for the modest volume loss expected on theoretical grounds.

\textbf{Active Cl Uptake}

Cells whose \([\text{Cl}]_{i}\) had been lowered to \( \sim 16 \text{ mM} \), close to the expected thermodynamic equilibrium level (20 mM) for \( V_m = \) approximately \(-53 \text{ mV} \), neither gained nor lost Cl for some time when placed in 148 mM Cl medium containing 2-DOG, CHC, or ethacrynic acid (Fig. 1B). Without inhibitors present, these cells gained Cl at a rate of \( \sim 0.2 \text{ meq/liter} \cdot \text{min} \) (Fig. 1B), which can be regarded as an estimate for the rate of active inward transport. Several models regarding the nature of this active transport were considered: (a) an ATP-driven Cl transport mechanism, or "Cl pump," (b) an Na gradient-driven transport in which the electrochemical gradient of Na energizes uphill movement of Cl, and (c) an exchange carrier mechanism where the downhill efflux of an unidentified anion drives the uphill influx of Cl.

Regardless of the exact nature of this putative Cl uptake process, one would expect a differential effect of any active transport inhibitor on one-way fluxes: a sizable inhibition of \(^{36}\text{Cl}\) uptake, and little or no effect on \(^{36}\text{Cl}\) efflux. This minimum requirement appears to be met by 2-DOG, by furosemide, and by ethacrynic acid. The former blocks anaerobic glycolysis and lowers intracellular [ATP] enough to abolish Na transport. Treatment with 1 mM 2-DOG in glucose-free media caused a reduction of the \(^{36}\text{Cl}\) influx rate (Figs. 2, 4, and 6A), most obviously in low-Cl cells (Figs. 4 and 6A). Influx from 148 mM Cl medium dropped by 0.21 meq/liter \cdot min (Fig. 4), in agreement with the observation made on net Cl uptake (Fig. 1B). On the other hand, no significant effect of 2-DOG was seen on \(^{36}\text{Cl}\) efflux (Fig. 3), which suggests that ATP depletion does not affect Cl self-exchange or diffusive permeability. Similarly, furosemide and ethacrynic acid reduced \(^{36}\text{Cl}\) uptake to the same extent that 2-DOG did (Figs. 4 and 5), but had no effect on uptake by 2-DOG–treated cells or on Cl efflux (Fig. 3). All these observations are consistent with the hypothesis that active Cl uptake
occurs via an ATP-requiring "Cl pump," which is inhibited by furosemide, ethacrynic acid, and CHC.

In the case of Na electrochemical gradient-driven transport, removal of extracellular Na should abolish active Cl uptake, whether the mechanism is direct Na + Cl or Na + K + Cl cotransport or indirect linkage, e.g., through parallel Na/H and Cl/HCO₃ exchanges, all of which have been described in a variety of cells (Kregenow, 1981; Spring and Ericson, 1982). If the linkage were indirect via Na/H exchange, one might expect the phenomenon to be inhibitable by amiloride. It turns out, however, that neither amiloride nor removal of extracellular Na or K had any effect on Cl uptake. It is unlikely, therefore, that Na + Cl cotransport or Na/H exchange plays a significant role in Cl accumulation. It could be argued that Na replacement with N-methyl-D-glucamine was incomplete, and that trace amounts of Na were sufficient to saturate an Na + Cl cotransport carrier with high affinity for Na. (Our assays indeed detected residual Na levels of 0.5–1 mM in nominally Na-free media.) Fig. 1B shows, however, that thermodynamically uphill net Cl accumulation continues unabated in nominally Na-free media. This observation demonstrates conclusively that the Na electrochemical gradient cannot serve as the energy source for Cl accumulation via Na + Cl cotransport unless an absurd Na/Cl stoichiometry is invoked. In addition, we found (unpublished experiments) no effect of external Cl on ²²Na influx into these cells.

The third model, Cl uptake driven by efflux of an unidentified anion, appears plausible on thermodynamic grounds. For example, since the intracellular pH (~7.25) in these cells is very near pH₀ (Simchowitz and Roos, 1985), OH/Cl exchange could conceivably raise [Cl]₀ to near [Cl]₀; obviously, the same would apply to HCO₃/Cl exchange since [HCO₃][OH] = [HCO₃][OH] through the Henderson-Hasselbalch equation and the equality of pCO₂ inside and outside the cell. (These two models are thermodynamically indistinguishable from one another and from a Cl + H cotransport model.) Other intracellular anions such as lactate or pyruvate, generated inside the cell but not present outside, would have the thermodynamic potential to drive Cl inward, and their generation could conceivably be blocked by 2-DOG. If such an exchange carrier were responsible for the accumulation of Cl, it would by necessity be distinct from the exchange carrier responsible for the bulk of the Cl traffic across the membrane, since the two processes (a) exhibit different Michaelis constants for inhibition by CHC, and (b) are differentially affected by furosemide and ethacrynae. However, the kinetic constraints on such a carrier, required to fit our observations, are quite formidable. Although a carrier should possess, by definition, bilateral (not necessarily equal) affinity for its substrate(s), we found no effect of a 40-fold [Cl]₀ reduction on ³⁶Cl influx through this pathway, and no ³⁶Cl efflux that was sensitive to 2-DOG, furosemide, or ethacrynae. This requires an unusually asymmetrical substrate affinity and/or an extremely high affinity toward the other (unknown) substrate. Furthermore, if the action of 2-DOG were to limit the availability of this putative substrate, then the competition for the internal site would vanish pari passu with the disappearance of the unknown substrate, and during exposure to 2-DOG the carrier would become available for ethacry-
nate- and furosemide-sensitive Cl/Cl exchange fluxes (without net accumulation). We saw no such fluxes. The rather high demands placed on a model of this third kind make it appear, for the moment, less economical and less likely than a model in which ATP hydrolysis drives primary active Cl influx.

**Electrodiffusive Fluxes: Conductance and Permeability**

CHC-resistant fluxes were larger in the outward than in the inward direction: efflux from normal-Cl cells into a 148 mM CHC medium was \(-0.40\) meq/liter-min (Fig. 3), whereas CHC-resistant influx into Cl-depleted cells was \(0.08-0.10\) meq/liter-min (Figs. 4 and 8). Three lines of evidence suggest that these fluxes represent electrodiffusion through channels. First, influx was proportional to \([\text{Cl}]_o\) (Fig. 8). Second, these fluxes meet, to a first approximation, Ussing's (1949) flux-ratio criterion for independence, as modified by Hodgkin and Keynes (1955):

\[
\frac{M_{\text{Cl}}^{\text{out}}}{M_{\text{Cl}}^{\text{in}}} = \exp[(E_{\text{Cl}} - V_m)n'/RT],
\]

where \(M_{\text{Cl}}^{\text{out}}\) and \(M_{\text{Cl}}^{\text{in}}\) represent passive \(^{36}\text{Cl}\) efflux and influx, respectively, \(n'\) is an empirical term whose deviation from unity is a measure of nonindependent behavior, and the other symbols have their usual meanings. The test can reasonably be applied here because the resting membrane potential of these cells bathed in normal-Cl media (approximately \(-53\) mV; Simchowitz et al., 1982; but see Appendix) is sufficiently removed from the Cl equilibrium potential \((E_{\text{Cl}} = -16\) mV). Taking the ratio \(M_{\text{Cl}}^{\text{out}}/M_{\text{Cl}}^{\text{in}}\) of CHC-resistant fluxes listed above, we find, from Eq. 4, \(n' = 0.91-1.14\). Put another way, the observed CHC-resistant flux ratio requires, for these fluxes to be independent, that the membrane potential be between \(-50\) and \(-58\) mV. This suggests that CHC-insensitive Cl fluxes are indeed independent, or nearly so, and not carrier mediated (which would lower \(n'\)) or subject to strong single-filing constraints (which would raise \(n'\); Hodgkin and Keynes, 1955). We will give evidence below that there is, in fact, a slight single-filing effect.

A third observation compatible with CHC-resistant fluxes being electrodiffusive is that altering \(V_m\), which caused little change in the total one-way \(^{36}\text{Cl}\) fluxes (Figs. 2 and 3), displayed the dramatic and opposite effects on one-way CHC-insensitive \(^{36}\text{Cl}\) movements predicted for electrodiffusive fluxes (Fig. 9).

It was previously concluded from indirect evidence (Simchowitz et al., 1982) that the Cl conductance of human neutrophils must be quite low. The data of Fig. 1A afford a direct estimate of its magnitude. The initial net Cl loss, \(M_{\text{Cl}}^{\text{out}}\), into CHC-containing 108 mM Cl medium (Fig. 1A) was \(0.31\) meq/liter-min or (see Methods) \(0.58\) pmol/cm².s. Under these conditions, \(E_{\text{Cl}} \approx -8\) mV and \(V_m \approx -53\) mV; hence, the Cl chord conductance \((F\) is the Faraday constant),

\[
g_{\text{Cl}} = \frac{M_{\text{Cl}}^{\text{out}}F}{E_{\text{Cl}} - V_m},
\]

is \(1.24\) \(\mu\)S/cm². This conductance is comparable to \(g_{\text{Na}}\) (\(1.3-1.6\) \(\mu\)S/cm²) but smaller than the \(g_K\) (\(2.8\) \(\mu\)S/cm²) calculated previously (Simchowitz et al., 1982;
but see Appendix). Added together, these three conductances satisfactorily account for the total membrane conductance, 5.3 \mu S/cm², estimated from the hyperpolarization induced by the electrogenic Na pump (Simchowitz et al., 1982; but see Appendix).

Assuming constant-field behavior (Goldman, 1943), the Cl permeability coefficient can be computed from either net or one-way fluxes (inward flux is positive):

\[ P_{Cl} = \frac{RT}{V_m F} \frac{1 - \exp(-V_m F/RT)}{[Cl]_o - [Cl]_i \exp(-V_m F/RT)}. \]

Taking \( V_m = -53 \) mV, the CHC-resistant net efflux into PAH medium (Fig. 1A) and \(^{36}\text{Cl}\) efflux into CHC medium (Fig. 3) both yield \( P_{Cl} = 3.9 \times 10^{-9} \) cm/s, and net efflux into CHC-containing Cl medium (Fig. 1A) yields \( P_{Cl} = 3.7 \times 10^{-9} \) cm/s. Our best estimate from \(^{36}\text{Cl}\) influx measurements (Fig. 8) is \( P_{Cl} = 3.5 \times 10^{-9} \) cm/s. The above estimates are based on flux measurements at a single, not necessarily very accurately known, membrane potential. (In the next section, a value of \( 5.1 \times 10^{-9} \) cm/s will be derived from a least-squares fit to fluxes obtained at a variety of membrane potentials.) This very low permeability, \( 3.5-5.1 \times 10^{-9} \) cm/s, is only \( \sim 1/10 \) of that estimated previously for \( P_{K} \) in these cells and is similar to our estimate for \( P_{Na} \) (see Appendix). It easily accounts for our observation that variations in \([Cl]_o\) had little effect on \( V_m \).

Voltage Dependence of Electrodiffusive Fluxes

Independent electrodiffusive fluxes should satisfy Ussing's (1949) criterion, i.e., \( n' \) in Eq. 4 should be unity. Using the more convenient transformation,

\[ \log_{10} \frac{M_{Cl}}{M_{Cl}} = \frac{n'}{61.5} (E_{Cl} - V_m), \] (5)

a plot of \( \log_{10} \) (flux ratio) against \( (E_{Cl} - V_m) \), where the latter are in millivolts, should have a slope of \( 1/61.5 \).

To check this, we plotted in Fig. 10A the flux ratios taken from Fig. 9; the resulting slope gives \( n' = 1.15 \) in Eq. 5, which suggests that the CHC-resistant fluxes, if purely electrodiffusive, are subject to a slight single-filing constraint. The question arises whether the measured fluxes still comprise nonelectrodiffusive components (e.g., residual exchange or active uptake) that might bias the Ussing criterion. Given \( K_i(CHC) = 0.74 \) mM for the inhibition of exchange in low-Cl cells (Fig. 7), 1.8% of residual exchange is expected in the presence of 40 mM CHC. In a 1.9 mM Cl cell bathed in 148 mM Cl medium, this would leave \( \sim 0.0014 \) meq/liter·min of carrier-mediated \(^{36}\text{Cl}\) efflux and \( \sim 0.0026 \) meq/liter·min of \(^{36}\text{Cl}\) influx (the latter including some exchange of external Cl for internal PAH). Similarly, given \( K_i(CHC) = 1.7 \) mM for inhibition of active influx (Fig. 5C), a residual active influx of 4.1% (of \( \sim 0.21 \) meq/liter·min; Fig. 4) or 0.0086 meq/liter·min might be expected. If these corrections are applied (arrows in Fig. 10A), the flux ratio exponent \( n' \) becomes, if anything, larger rather than smaller. We are reluctant to apply these corrections, however, for the following reason. The inhibitor CHC blocks not only Cl/Cl exchange, but other anion
(lactate, pyruvate, bicarbonate) movements as well, so that vigorously metabolizing cells tend to acidify in the presence of CHC. We have unpublished evidence that intracellular acidification inhibits the exchange carrier. Consequently, the exact extent of exchange carrier inhibition by CHC in our experiments (between

**FIGURE 10.** (A) Decimal logarithm of the ratio of Cl fluxes taken from Fig. 9, A and B, plotted against $E_{Cl} - V_m$, where $E_{Cl}$ is $-108$ mV. The symbols represent ratios as calculated from the data of Fig. 9. The arrows indicate the maximal change these ratios might undergo if corrections for residual exchange and active transport were applied as discussed in the text. The solid line is a fit of Eq. 5 to the uncorrected data; the least-squares value for $n' = 1.15 \pm 0.04$. The dashed line is a plot of Eq. 5 with $n' = 1$ exactly. (B) Replot of the slopes from Fig. 9, A and B, against the corresponding membrane potentials computed from Eq. 2. Top curve (solid circles): slopes (least-squares value $\pm$ SE) from Fig. 9A, representing rates of $^{36}$Cl influx from $108$ mM Cl medium into $1.9$ mM Cl cells kept at the indicated membrane potential. The upper solid line is a least-squares fit of constant-field Eq. 6 to the variance-weighted data, with $P_{Cl}$ as the sole adjustable parameter. The best value was $P_{Cl} = 5.1 \pm 0.1 \times 10^{-6}$ cm/s. Lower solid curve (open circles): slopes (least-squares value $\pm$ SE) from Fig. 9B, representing rates of $^{36}$Cl efflux from $1.9$ mM Cl cells, kept at the indicated membrane potential, into $108$ mM Cl medium. The solid line is a least-squares fit of Eq. 4 to the variance-weighted data and to the upper curve, with the flux-ratio exponent $n'$ as the sole adjustable parameter. The best value was $n' = 1.07 \pm 0.01$. The dashed curve is the prediction of Eq. 4 for $n' = 1.0$, signifying the absence of single-filing.

98.2 and 100%) is unknown, and the magnitude of the required correction, if any, is uncertain. What does appear certain, however, is that the flux ratio exponent $n'$ must be at least 1.15 and may be larger. Some single-filing must therefore occur.
In Fig. 10B the uncorrected one-way $^{36}\text{Cl}$ flux rates taken from Fig. 9 are plotted, after conversion to picomoles per square centimeter per second, against $V_m$ computed from Eq. 3. As required on thermodynamic grounds for passive fluxes, influx and efflux become equal at about the computed equilibrium potential—further proof that our experimental manipulations of $V_m$ are effective. If constant-field behavior (Goldman, 1943) prevails, electrodiffusive influxes and effluxes should show the following dependence on $V_m$:

$$M_{\text{in}}^{\text{Cl}} = P_{\text{Cl}} \frac{V_m F}{RT} \frac{[\text{Cl}]_o}{1 - \exp(-V_m F/RT)}$$

and

$$M_{\text{out}}^{\text{Cl}} = P_{\text{Cl}} \frac{V_m F}{RT} \frac{[\text{Cl}]_i}{\exp(V_m F/RT) - 1},$$

where $P_{\text{Cl}}$ is the Cl permeability coefficient. In fact (Fig. 10B), only the $^{36}\text{Cl}$ influx data (i.e., from 108 mM Cl medium into 1.9 mM Cl cells) fit the constant-field prediction well, with a least-squares permeability coefficient of $5.1 \pm 0.1 \times 10^{-9}$ cm/s. Since this passive $^{36}\text{Cl}$ influx closely follows the prediction of the economical constant-field model over an $\sim 100$-mV range, no additional features such as voltage-dependent permeability need be invoked to describe the putative Cl channel's behavior.

The constant-field prediction for $^{36}\text{Cl}$ efflux (from 1.9 mM Cl cells into 108 mM Cl medium), using the same permeability coefficient, is shown as the dotted line in Fig. 10B. (Different values for $P_{\text{Cl}}$ would cause only parallel vertical displacements of the curve.) The dotted line is simply the prediction of Ussing's flux-ratio equation for isotope efflux, given its influx. The discrepancy shows that, independently of the form (constant-field or other) of the equation governing influx, Ussing's flux-ratio equation is not obeyed: efflux is lower than expected, except at very negative potentials, and the deviation is relatively larger at more depolarized potentials. Errors in the assumed specific activity, intracellular [Cl], or intracellular binding cannot account for the finding; they, like errors in assumed permeability, only cause vertical parallel displacements of the curve.

The most economical way to interpret the deviation of our $^{36}\text{Cl}$ efflux data from Ussing's prediction is to invoke some single-filing (Hodgkin and Keynes, 1955) in the Cl channel. Efflux should be most visibly retarded, since it encounters up to 100-fold-higher fluxes in the opposite direction. (It should be recalled that the experiments of Fig. 9 were carried out on Cl-depleted cells.) The lower solid line of Fig. 10B was a least-squares fit to the efflux data using Eq. 4 and the assumption that the upper solid line properly describes the voltage dependence of $^{36}\text{Cl}$ influx under identical conditions. The resulting best value for the flux ratio exponent, $n'$, was $1.07 \pm 0.01$. If this interpretation is correct, then electrodiffusive $^{36}\text{Cl}$ efflux into a Cl-free medium should always exceed that into Cl-containing media, at least, if in the latter condition, $E_{\text{Cl}} < V_m$. To check this, we measured $^{36}\text{Cl}$ efflux from two batches of very-low-Cl cells, bathed in 40 mM CHC and 130 mM K (i.e., with $V_m \approx 0$ mV) in the presence and absence of
extracellular Cl, and found that 108 mM extracellular Cl inhibits the presumed electrodiffusive efflux of $^{36}$Cl by 40–60%.

**APPENDIX**

*Recalibration of the diS-C$_3$(5) Fluorescence–Membrane Potential Curve*

In a previous publication from this laboratory (Simchowitz et al., 1982), the membrane potential ($V_m$) was estimated with the potentiometric indicator diS-C$_3$(5). Calibration was based on the assumption of constant-field behavior where K and Na are the only permeant species. Because external [Cl] had no effect on $V_m$, Cl was assumed to be either passively distributed or effectively impermeant. Since we now have a reasonable estimate of Cl permeability, we can recalibrate our original fluorescence measurements.

This is done in Fig. A1, which shows fluorescence readings from three sets of data based on Eq. 2 of the present article. The $P_K/P_{Na}/P_{Cl}$ permeability ratio used was 10:1:1 for cells treated with ouabain and 20:1:1 for cells treated with ouabain plus valinomycin. Intracellular [K] was 120 mM for normal-Na cells and 78 mM for Na-loaded cells; intracellular [Na] was 25 mM for normal cells and 68 mM for Na-loaded cells. Intracellular [Cl] was 80 mM for normal cells and 55 mM for Na-loaded cells. Normal-Na cells treated with 50 µM ouabain (from Fig. 7, S/82) (□); normal-Na cells treated with 50 µM ouabain plus 1 µM valinomycin (from Fig. 7, S/82) (Δ); Na-loaded cells exposed to 50 µM ouabain (from Fig. 9, S/82) (O). The equation for the least-squares line is: fluorescence (in arbitrary units) = 39.7 + 0.44 $V_m$ (in millivolts).

**Figure A1.** Fluorescence-potential calibration. Fluorescence readings from the three experiments displayed in Fig. 11 of Simchowitz et al. (1982) ("S/82") were plotted against the corresponding theoretical membrane potential calculated from Eq. 2 of the present article. The $P_K/P_{Na}/P_{Cl}$ permeability ratio used was 10:1:1 for cells treated with ouabain and 20:1:1 for cells treated with ouabain plus valinomycin. Intracellular [K] was 120 mM for normal-Na cells and 78 mM for Na-loaded cells; intracellular [Na] was 25 mM for normal cells and 68 mM for Na-loaded cells. Intracellular [Cl] was 80 mM for normal cells and 55 mM for Na-loaded cells. Normal-Na cells treated with 50 µM ouabain (from Fig. 7, S/82) (□); normal-Na cells treated with 50 µM ouabain plus 1 µM valinomycin (from Fig. 7, S/82) (Δ); Na-loaded cells exposed to 50 µM ouabain (from Fig. 9, S/82) (O). The equation for the least-squares line is: fluorescence (in arbitrary units) = 39.7 + 0.44 $V_m$ (in millivolts).
where Eq. 2 (which now includes Cl terms) appears valid: (a) normal-Na cells treated with ouabain \((P_K/P_{Na}/P_{Cl} \text{ ratio of } 10:1:1)\), (b) normal-Na cells treated with ouabain plus valinomycin \((P_K/P_{Na}/P_{Cl} \text{ ratio of } 20:1:1)\), and (c) Na-loaded cells treated with ouabain \((P_K/P_{Na}/P_{Cl} \text{ ratio of } 10:1:1)\). Fluorescence is plotted against the theoretical membrane potentials computed from Eq. 2; the relationship appears linear over the range covered, as before (Simchowitz et al., 1982). The least-squares line through the data of Fig. 1A represents a revised calibration (slope, 2.26 mV per fluorescence unit). The new conversion factor was then used to recompute the right-hand ordinates \((V_m)\) of Figs. 7 and 9 of Simchowitz et al. (1982). The corrected \(V_m\) values are related to the previous estimates as follows:

\[
V_m^{\text{corrected}} = -2.0 \text{ mV} + 0.86 V_m^{\text{previous}}.
\]

**Revised Estimates for Resting Potential and Membrane Conductances of Human Neutrophils**

With this new calibration curve, our original estimate of the resting \(V_m\) of steady state neutrophils bathed in standard medium should be revised slightly downward from \(-59\) to \(-53\) mV. In combination with our corrected measurement of cell water content (see Methods), the new membrane potential calibration curve produces a revised estimate for total membrane conductance of \(5.3 \mu S/cm^2\). Similarly, the combined effects of the cell water content correction and the revised fluorescence-potential calibration curve lead to the following specific membrane conductance estimates: \(2.8 \mu S/cm^2\) for \(g_K\) (up from \(2.7 \mu S/cm^2\)) and \(1.3-1.6 \mu S/cm^2\) for \(g_{Na}\) (up from \(1.0-1.3 \mu S/cm^2\)). We have also recalculated our estimates of the absolute permeabilities for K and Na from unidirectional and net passive fluxes from the original Figs. 1, 2, 4, and 5 of Simchowitz et al. (1982). The derived values are \(3.3-5.5 \times 10^{-8} \text{ cm/s for } P_K\) and \(4.3-5.6 \times 10^{-9} \text{ cm/s for } P_{Na}\). It should be emphasized that our original estimate of 10 for the \(P_K/P_{Na}\) ratio is not subject to the uncertainty of the precise voltage calibration since the passive influx measurements on which this estimate is based were performed under identical conditions.

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