Intracellular pH Recovery from Alkalination

Characterization of Chloride and Bicarbonate Transport by the Anion Exchange System of Human Neutrophils

LOUIS SIMCHOWITZ and AJUAH O. DAVIS

From the Department of Medicine, the John Cochran Veterans Administration Medical Center, and the Departments of Medicine and of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT The nature of the intracellular pH-regulatory mechanism after imposition of an alkaline load was investigated in isolated human peripheral blood neutrophils. Cells were alkalinized by removal of a DMO prepulse. The major part of the recovery could be ascribed to a Cl⁻/HCO₃⁻ counter-transport system: specifically, a one-for-one exchange of external Cl⁻ for internal HCO₃⁻. This exchange mechanism was sensitive to competitive inhibition by the cinnamate derivative UK-5099 (Kᵢ ~ 1 μM). The half-saturation constants for binding of HCO₃⁻ and Cl⁻ to the external translocation site of the carrier were ~2.5 and ~5.0 mM. In addition, other halides and lyotropic anions could substitute for external Cl⁻. These ions interacted with the exchanger in a sequence of decreasing affinities: HCO₃⁻ > Cl⁻ > NO₃⁻ > Br⁻ > I⁻ > SCN⁻ > PAH⁻. Glucuronate and SO₄²⁻ lacked any appreciable affinity. This rank order is reminiscent of the selectivity sequence for the principal anion exchanger in resting cells. Cl⁻ and HCO₃⁻ displayed competition kinetics at both the internal and external binding sites of the carrier. Finally, evidence compatible with the existence of an approximately fourfold asymmetry (Michaelis constants inside > outside) between inward- and outward-facing states is presented. These results imply that a Cl⁻/HCO₃⁻ exchange mechanism, which displays several properties in common with the classical inorganic anion exchanger of erythrocytes, is primarily responsible for restoring the pHᵢ of human neutrophils to its normal resting value after alkalinization.

INTRODUCTION

In a previous article on intracellular pH (pHᵢ) regulation in isolated human neutrophils (Simchowitz and Roos, 1985) we undertook a systematic investigation of the factors that control steady-state pHᵢ in resting cells. By identifying the ion

Address reprint requests to Dr. Louis Simchowitz (151-JC), John Cochran V. A. Medical Center, 915 North Grand Avenue, St. Louis, MO 63106.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/90/11/1037/23 $2.00 1037
Volume 96 November 1990 1037–1059
pathways responsible for maintaining pH$_i$ homeostasis, these studies serve as a basis for examining the proposed pH$_i$-dependent functions of these cells, which include phagocytosis, chemotaxis, superoxide radical generation, and degranulation (Segal et al., 1981; Klempner and Styrt, 1983; Simchowitz, 1985; Simchowitz and Cragoe, 1986). In our initial report (Simchowitz and Roos, 1985) we showed that human neutrophils possess two separate regulatory mechanisms for pH$_i$ homeostasis: one, a Na$^+$/H$^+$ exchange that restores pH$_i$ to its normal resting value (~7.25 at pH$_o$ 7.40) after imposed acidification, and the other, a Cl$^-$/HCO$_3^-$ exchange that comes into play after imposed alkalinization.

As commented upon in the past (for review, see Roos and Boron, 1981), while a Na$^+$/H$^+$ exchange mechanism has been implicated in the recovery from acidification in a variety of cell types, the recovery from alkalinization and its ionic basis have received surprisingly little attention. Passive ion fluxes (Aickin and Thomas, 1977) and metabolic acid production (Boron et al., 1979) have been proposed as possible mechanisms. However, in human neutrophils (Simchowitz and Roos, 1985), as well as in sheep cardiac Purkinje fibers (Vaughan-Jones, 1981; 1982a, b), the predominant part of the pH$_i$ recovery process from alkalinization seems to be due to a Cl$^-$/HCO$_3^-$ counter-transport (i.e., an exchange of external Cl$^-$ for internal HCO$_3^-$). In particular, it should come as no surprise that certain cells, which are likely to experience alkalinizing transients during their life histories (as, for example, neutrophils that are regularly exposed to chemotactic factors in vivo [Sha'afi et al., 1982; Grinstein and Furuya, 1984; Simchowitz, 1985]) should have evolved specific devices to facilitate the return of pH$_i$ to its normal resting value.

In our previous study (Simchowitz and Roos, 1985) we noted that pH$_i$ recovery from alkalinization could be almost completely ascribed to an exchange of external Cl$^-$ for internal HCO$_3^-$ This conclusion was based on the observations that the recovery rate was dependent on external Cl$^-$, enhanced by HCO$_3^-$, and sensitive to α-cyano-4-hydroxycinnamate (CHC), an inhibitor of anion exchange in these cells (Simchowitz and De Weer, 1986; Simchowitz et al., 1986).

The purpose of this work was to examine the properties of this intracellular alkalinization-induced Cl$^-$/HCO$_3^-$ exchange in more detail. Specifically, we sought to characterize the interaction of this transport system with its natural substrates, Cl$^-$ and HCO$_3^-$ We found that recovery is stimulated by external Cl$^-$ with $K_m$ ~ 5 mM and by internal HCO$_3^-$ with $K_m$ ~ 10 mM. In addition, pH$_i$ recovery from alkalinization is associated with an enhancement of one-way $^{86}$Cl$^-$ influxes and effluxes and with a net intracellular uptake of Cl$^-$, all of which are sensitive to competitive inhibition by UK-5099 ($K_i$ ~ 1 μM). The transport rate of the exchanger is activated by the pH$_i$. Finally, evidence compatible with a fourfold carrier asymmetry is presented. These results represent a continuing biochemical characterization of the intracellular alkalinization-induced Cl$^-$/HCO$_3^-$ exchange mechanism of human neutrophils, which plays a physiologic role in pH$_i$ regulation in these cells.

**MATERIALS AND METHODS**

**Incubation Media**

The standard medium used throughout this study had the following composition: 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM HEPES buffer, pH 7.40, and 1 mg/ml
crystalline bovine serum albumin. Experiments were performed in glucose-free media, although after isolation cells were routinely kept in the standard medium supplemented with 5.6 mM glucose for at least 1 h before any other pretreatment (see below). To test the effects of different halides or other monovalent anions, media were prepared by equimolar substitutions of Br\(^-\), I\(^-\), NO\(_2\)\(^-\), SCN\(^-\), p-aminophenylurate (PAH\(^-\)), or glucuronate for Cl\(^-\). When divalent anions were being examined, the SO\(_4\)\(^2-\) concentration was 100 mM, and that of Na\(^+\) was 194 mM, while those of K\(^+\), Ca\(^2+\), and Mg\(^2+\) were the same as for the standard medium. Unless otherwise stated, all media were exhaustively bubbled with \(\text{N}_2\) to drive off CO\(_2\) gas and thus effectively reduce the HCO\(_3\)\(^-\) concentration of the media to zero.

Bicarbonate-containing solutions were prepared as follows: A stock solution was made by equilibrating a modified standard medium (equimolar replacement of 25 mM NaCl by NaHCO\(_3\)) in a 5% CO\(_2\):95% air atmosphere. This HCO\(_3\)\(^-\) stock solution was diluted with different volumes of CO\(_2\)/HCO\(_3\)\(^-\)-free Cl\(^-\) medium containing 5 mM HEPES, which had also been brought to pH 7.40. Thus, a series of solutions was available of different HCO\(_3\)\(^-\) concentrations (0–25 mM). During the cell incubations, the tubes were overlaid with mineral oil and capped to retard diffusion of CO\(_2\).

Media of high [K\(^+\)]\(_o\) (120 mM) were obtained by equimolar substitution of KCl for NaCl.

**Neutrophils**

Human peripheral neutrophils were isolated by sequential dextran sedimentation and Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation (Boyum, 1968). The purification procedures and protocols for handling of the cells have been outlined in detail in previously published work (Simchowitz and De Weer, 1986).

The normal resting pH\(_i\) of steady-state human neutrophils bathed in nominally CO\(_2\)-free medium at pH\(_o\) 7.40 is ~7.25 (Simchowitz and Roos, 1985). The neutrophils were subjected to an intracellular alkalinization according to the following protocol. Cells were first acidified by exposing them to the weak acid DMO (70 mM) in Cl\(^-\) (78 mM)-containing medium. The time course of the pH\(_i\) transients in response to application and subsequent removal of DMO is similar to that described previously by our group in cells treated with 18% CO\(_2\) (Simchowitz and Roos, 1985): there is an immediate (<5 s) initial fall of pH\(_i\) to ~6.80, a reduction of ~0.45 pH units. Over the next 30 min of incubation, during maintained DMO exposure, the pH\(_i\) recovers by ~0.20 units to reach a value of ~7.00, and then remains unchanged. When, after 30 min, the cells are resuspended in the original DMO-free medium, the pH\(_i\) rapidly and dramatically rises to ~7.80, i.e., ~0.55 units above control. After this imposed alkalinization, the pH\(_i\) then returns to its normal resting value over the ensuing 15–20 min. The nature of this recovery process as a Cl\(^-\)/HCO\(_3\)\(^-\) exchange is the subject of this paper. It should be noted that the initial fall of pH\(_i\) is due to the entry of the neutral (i.e., uncharged or acid) form of the DMO molecule and its subsequent dissociation to form H\(^+\) and DMO\(^-\). The partial pH\(_i\) recovery during maintained DMO exposure results from the removal of H\(^+\) from the cells by an acidification-induced Na\(^+\)/H\(^+\) exchange (Grinstein and Furuya, 1984; Simchowitz and Roos, 1985). The resulting H\(^+\) deficit leads to a pH\(_i\) overshoot when the DMO is removed, similar to the pattern observed in a number of other cell types (for review, see Roos and Boron, 1981).

For some experiments, batches of Cl\(^-\)-depleted neutrophils (internal Cl\(^-\) content <2 meq/liter of cell water) were used. The intracellular Cl\(^-\) depletion was achieved by prolonged (>5 h) incubation of cells in Cl\(^-\)-free, 148 mM PAH\(^-\) medium as previously described (Simchowitz and De Weer, 1986). In this case, Cl\(^-\) levels fall via a one-for-one exchange of internal Cl\(^-\) for external PAH\(^-\). Thus, the pH\(_i\) of these Cl\(^-\)-depleted cells remains normal.
Reagents

All inorganic salts were obtained from Fisher Scientific (St. Louis, MO). The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): D-glucose, crystalline bovine serum albumin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5,5-dimethylloxazolidine-2,4-dione (DMO), 2-deoxy-D-glucose (2-DOG), D-glucuronic acid, sodium glucuronate, p-aminohippuric acid (PAH), and sodium PAH. Isotopes (H36Cl, [14C]DMO, [3H]H2O, and [14C]inulin) were purchased from New England Nuclear, Boston, MA. The specific activity of $^{36}$Cl⁻ was 13.8 mCi/g Cl⁻. The anion transport inhibitor α-cyano-β-(1-phenylindol-3-y)acrylate (UK-5099), a potent analogue of α-cyano-4-hydroxycinnamic acid (CHC; Halestrap and Denton, 1975), was a generous gift of Pfizer Central Research Laboratories (Sandwich, Kent, UK).

Intracellular pH Measurements

We derived pH from the distribution of the $^{14}$C-labeled weak acid DMO ($\text{pK}^\prime 6.13$ at 37°C). The theoretical considerations underlying the use of this method have previously been reviewed (Roos and Boron, 1981), as have details of the procedures currently used in our laboratory (Simchowitz and Roos, 1985).

Samples of the neutrophil suspensions (8-12 x 10⁶ cells/ml) containing $[^{14}$C]DMO (1.0 μCi/ml) were incubated in plastic tubes at 37°C under various experimental conditions. The indicator concentration was <0.1 mM, which does not affect pH significantly. At intervals, triplicate 0.3-0.5-ml aliquots were layered over 0.7 ml of Versilube F-50 silicone oil (General Electric, Waterford, NY) contained in 1.5-ml plastic tubes and centrifuged for 1 min at 8,000 g in a microcentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Cell separation occurred in <5 s. The neutrophil pellets were isolated, placed in scintillation vials, and counted in a liquid scintillation counter (LS 7000; Beckman Instruments, Inc.) after addition of 10 ml of Aquasol-2 (New England Nuclear).

Unidirectional $^{36}$Cl⁻ Flux Measurements

The incubations were performed at 37°C, and at stated intervals triplicate aliquots of the cell suspensions were layered on silicone oil and centrifuged as described above. The neutrophil pellets were excised and counted in a liquid scintillation counter. Influx experiments were performed in the presence of $^{36}$Cl⁻ (1.5 μCi/ml). For the efflux studies, neutrophils were first suspended at 2-3 x 10⁷/μl and incubated with $^{36}$Cl⁻ (2.5 μCi/ml) for 1-2 h at 37°C.

Intracellular Cl⁻ Determinations

Neutrophils (15-20 x 10⁶/ml) were suspended in the various experimental media. Triplicate 1-ml aliquots were layered on 0.4 ml silicone oil and spun as described above. The aqueous and oil phases were aspirated and discarded. The neutrophil pellets were lysed in 1.0% Triton X-100 in water (vol/vol) and assayed coulometrically for Cl⁻ (Cotlove et al., 1958) using a chloridometer (Buchler Instruments, Fort Lee, NJ). Results are expressed in mequiv/L of cell water after appropriate correction for total and extracellular water spaces using [3H]H2O and [14C]inulin, respectively, as markers.

Data Analysis

In all cases, the influx of $^{36}$Cl⁻, corrected for zero-time "uptake" (which represents label trapped in the extracellular space) followed equations of the form

$$C_t = C_{0}[1 - \exp(-kt)],$$

(1)
where $C_i$ is the cell label at time $t$, $C_0$ 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 $k_{C_0}$. Similar fits to the data were applied in the analysis of the $[\text{Cl}^-]$ determinations by chloridometry and of the pH transients during recovery from alcalinization. As indicated in the figure legends, the change in some of the measured variables often appeared to be linear over the period of study; in those cases, the influx rate was computed from the slope of the linear regression line.

The $^{36}\text{Cl}^-$ effluxes followed single exponentials of the form:

$$C_t = C_0 \exp(-kt), \quad (2)$$

where $C_0$ is the cell label at zero time. Curves representing the equation were fit to the various groups of data by the least-squares method.

**RESULTS**

*Intracellular pH Studies*

The ionic basis of pH$_i$ recovery after an imposed alcalinization induced by the removal of a 70-mM DMO prepulse was studied by examining the effect of a number of transport inhibitors and by different ion substitutions in the bathing medium. The time course of pH$_i$ recovery is shown in Fig. 1. The pH$_i$ immediately after DMO removal (estimated by back-extrapolation of the slow course of recovery of Cl$^-$ and HCO$_3^-$-free 148 mM glucuronate medium in the presence of 1 mM SITS [curve D]) was 7.94 ± 0.03. Thereafter, in the standard 148 mM Cl$^-$ medium containing 0.5 mM HCO$_3^-$, the pH$_i$ recovered rapidly along an exponential time course (curve A) to reach a near-normal resting value by 15–20 min of incubation. The initial recovery rate was 0.0616 ± 0.0031 pH/min. In our original study on pH$_i$ regulation in human neutrophils (Simchowitz and Roos, 1985) we reported that the recovery process from alcalinization after removal of a CO$_2$ prepulse or during exposure to NH$_4$Cl was resistant to 0.1 mM ouabain and 1 mM amiloride. In addition, the data of Fig. 1 demonstrate that this course was not significantly affected (recovery rate = 0.0744 ± 0.0055 pH/min) by 1 mM 2-DOG (curve A), a metabolic inhibitor, which also blocks active inward Cl$^-$ transport in neutrophils (Simchowitz and De Weer, 1986). The initial recovery rate for the combined data was 0.0671 ± 0.0027 pH/min.

In contrast, as previously reported, the addition of either 40 mM CHC (not shown), an inhibitor of anion exchange in human neutrophils (Simchowitz and De Weer, 1986; Simchowitz et al., 1986), or of 400 μM UK-5099 (curve B), a more potent analogue of CHC (Halestrap and Denton, 1975; Simchowitz, 1988b), moderately slowed recovery. In the latter case, the initial recovery rate was reduced from 0.0671 to 0.0375 ± 0.0029 pH/min, an inhibition of 44%. The actual extent of inhibition is much greater since at least some of the apparent recovery acidification is due to another, separate effect of the UK-5099 compound. This other, secondary consequence of UK-5099 is best seen in the moderate degree of intracellular acidification that occurs over time in control cells (curve H). As discussed previously in connection with CHC (Simchowitz and Roos, 1985), the greater part of this acidification is most likely due to the blocking effect of the drugs on lactic acid secretion: the rates and amounts of intracellular acidification and of lactic acid efflux...
FIGURE 1. Time course of pH, upon removal of a 70-mM DMO prepulse (solid lines). The pH, was 7.40 throughout. The cells were first exposed to 70 mM DMO for 30 min and then resuspended at zero time in DMO-free (except for the trace [14C]DMO label [<0.1 mM]) solutions of the following compositions: A, standard medium containing 5 mM K+, 148 mM Cl−, and 0.5 mM HCO3− (the curve also utilizes results obtained in the presence of 1 mM 2-DOG or 120 mM K+, each in the presence of 148 mM Cl− and 0.5 mM HCO3−); B, 400 μM UK-5099 and 1 mM 2-DOG in 148 mM Cl−, 0.5 mM HCO3−; C, 148 mM glucuronate, with or without 0.5 mM HCO3− (combined data); D, 148 mM glucuronate and 1 mM SITS; E, 148 mM Cl−, 1 mM HCO3−; and F, 148 mM Cl−, 2 mM HCO3−. Curves B–F were all performed in media containing 5 mM K+. The dashed line (G) represents the time course of pH, of cells not exposed to 70 mM DMO, but kept in the standard 5 mM K+, 148 mM Cl− (0.5 mM HCO3−) medium throughout. They were then resuspended in solutions identical to those described above. Since the pH, values under all these conditions (with the exception of UK-5099) were indistinguishable, each of the points is the average of the combined pH, values. The dashed curve (H) signifies the pH, of control cells resuspended at zero time in 400 μM UK-5099. Curves A–F and H represent single exponential fits to the individual sets of data. The initial recovery rates (in pH/minute) were 0.0671 ± 0.0027 (A), 0.0375 ± 0.0029 (B), 0.0153 ± 0.0019 (C), 0.0112 ± 0.0015 (D), 0.132 ± 0.021 (E), 0.248 ± 0.046 (F), and 0.0274 ± 0.0035 (H). A horizontal line (dashed, G) was drawn at 7.33, the average of all of the control data points. Results represent the means ± SEM of three to five separate experiments.
are reduced in parallel by lowering the glucose content of the media and suppressed even further in the presence of metabolic inhibition by 2-DOG. The experiments shown in Fig. 1 for 400 µM UK-5099 (curves B and H) were in fact performed in glucose-free media using cells treated with 1 mM 2-DOG, a maneuver that reduces lactic acid production by ~90%. The initial rate of acidification in resting cells (curve H) was 0.0274 ± 0.0055 pH/min. Subtracting this value from the total rate of "recovery" in the presence of UK-5099 (0.0575 pH/min, curve B leaves 0.0101 pH/min, or only ~15% that of the normal rate (0.0671 pH/min, curve A).

In previous studies (Simchowitz and Roos, 1985) we also noted recovery to be dependent on the presence of extracellular Cl\(^-\) in the bathing solutions: in its absence (equimolar replacement by PAH) only a very slow recovery (~10% of normal) was observed. A similar dependence on external Cl\(^-\) is also shown in Fig. 1: when all extracellular Cl\(^-\) was replaced by glucuronate (a nominally inert anion), the recovery rate from alkalinization fell to 0.0153 ± 0.0019 pH/min (curve C), a rate ~20% of normal.

While 1 mM SITS has little or no effect on anion exchange in neutrophils in the standard 148 mM Cl\(^-\) medium (Simchowitz and De Weer, 1986), in later studies (Simchowitz, 1988b) conducted in the presence of low external Cl\(^-\) we found that the drug behaves as a weak competitive inhibitor of Cl\(^-\) with a "true" \(K_i\) of ~100 µM in 148 mM glucuronate medium. The addition of 1 mM SITS to this nominally substrate (Cl\(^-\) and HCO\(_3\))-free glucuronate solution led to a slight further reduction in the initial rate of pH\(_i\) recovery from alkalinization to 0.0112 ± 0.0015 pH/min (curve D). The finding of the small degree of SITS sensitivity suggests the possibility that glucuronate may not be truly inert and might actually be transported inward in exchange for HCO\(_3\).

Recovery is also strikingly dependent on the amount of HCO\(_3\) in the bathing medium (and/or, through equilibration of CO\(_2\), on HCO\(_3\) in the intracellular fluid). When precautions were taken to exclude HCO\(_3\) (use of HCO\(_3\)-free NaOH and KOH, prolonged gassing of solutions with N\(_2\), overlaying of media with mineral oil to prevent inward diffusion of CO\(_2\)), the recovery rate in nominally HCO\(_3\)-free 148 mM Cl\(^-\) (0.0204 ± 0.0037 pH/min [not shown]) was significantly reduced relative to that in Cl\(^-\) medium containing 0.5 mM HCO\(_3\) (0.0671 pH/min, curve A). The rate in the nominal absence of HCO\(_3\) is slightly greater than that occurring in Cl\(^-\)-free glucuronate medium (0.0153 pH/min, curve C) and also greater than the adjusted rate of recovery derived from studies in the presence of the anion exchange inhibitor UK-5099 (0.0101 pH/min), the difference probably reflecting a small amount of residual internal HCO\(_3\). In contrast, raising the external HCO\(_3\) concentration to 1 or 2 mM (0.2 or 0.4% CO\(_2\), curves E and F) dramatically enhanced the rate of recovery (initial rates 0.132 ± 0.021 and 0.248 ± 0.046 pH/min, respectively) as compared with that under HCO\(_3\)-free conditions.

The dependence on external Cl\(^-\), enhancement by HCO\(_3\) and sensitivity to CHC and UK-5099, which block anion exchange in human neutrophils (Simchowitz and De Weer, 1986; Simchowitz, 1988b), strongly suggest that the principal part of the pH\(_i\) recovery after imposed alkalinization is mediated through an exchange of external Cl\(^-\) for internal HCO\(_3\). In other cell types (Knauf, 1979; Vaughan-Jones, 1982a, b; Reuss and Costantin, 1984) Cl\(^-\)/HCO\(_3\) exchange is electroneutral, a
finding consistent with the observed 1:1 stoichiometry of the counter-transport reaction. This also appears to be the case in neutrophils, where anion exchange has been shown to exhibit a 1:1 stoichiometry (Simchowitz et al., 1986). Moreover, recovery is independent of large changes in membrane potential: in 148 mM Cl⁻, 0.5 mM HCO₃⁻ medium the kinetics of recovery were indistinguishable at 5 mM K⁺ as compared with 120 mM K⁺ (initial rates 0.0680 ± 0.0041 vs. 0.0722 ± 0.0066 pH/min, respectively, curve A), where membrane voltage is about -60 and 0 mV, respectively (Simchowitz et al., 1982).

As previously reported (Simchowitz et al., 1986), glucuronate appears to be inert with respect to anion exchange and cannot be transported by the carrier due to a lack of affinity for the external translocation site. The ability of other replacement anions, each at 148 mM and in the presence of 0.5 mM HCO₃⁻, to substitute for Cl⁻ is evaluated in Fig. 2. In Br⁻ medium (initial recovery rate 0.0787 ± 0.0090 pH/min) recovery was similar to that in Cl⁻ medium (initial rate 0.0685 ± 0.0040 pH/min); those in NO₃⁻ and I⁻ were somewhat slower (initial rates 0.0531 ± 0.0033 and 0.0438 ± 0.0042 pH/min, respectively), while those in SCN⁻ and PAH⁻ media were considerably reduced (initial rates 0.0284 ± 0.0038 and 0.0209 ± 0.0031 pH/min, respectively). In contrast, recovery in 100 mM SO₄²⁻ medium (0.0164 ± 0.0017 pH/min) was not appreciably different from that in 148 mM glucuronate (0.0137 ± 0.0022 pH/min), which has been shown to be essentially inert for anion exchange. Thus, PAH⁻, SCN⁻, and especially SO₄²⁻ seem to be rather ineffective substrates for anion exchange due either to low affinity for the carrier and/or relatively slow transport rates. We shall return to this point shortly (see below). Apparently Br⁻, NO₃⁻, and, to a lesser extent, I⁻ can serve as effective substitutes for Cl⁻, since after the initial rise the pHᵢ fell with time to reach near-normal resting values (~7.35) by 15–20 min, along a time course roughly similar to that displayed in 148 mM Cl⁻ medium.

In a related series of experiments (not shown), after pretreatment with 70 mM DMO the cells were resuspended in 0.5 mM HCO₃⁻ media where the external concentrations of Cl⁻, Br⁻, I⁻, or NO₃⁻ were varied between 0 and 140 mM by equimolar replacement of glucuronate. Cl⁻-depleted cells were used so as to provide a favorable driving force for net HCO₃⁻ efflux. As [Cl⁻]₀ was gradually raised, the initial rate of pHᵢ recovery increased along a Michaelis-Menten activation curve with an apparent Kₘ(Cl⁻) of 4.2 ± 0.9 mM. Similarly, external NO₃⁻, Br⁻, and I⁻ each stimulated the rate of recovery with apparent Kₘ values of 5.6 ± 1.2, 7.5 ± 1.5, and 39.2 ± 12.8 mM, respectively.

The data of Fig. 1 indicate that recovery is very sensitive to the amount of HCO₃⁻ in the external (hence, also, the internal) solutions. The relationship of HCO₃⁻ to the rate of pHᵢ recovery is shown in a more detailed manner in Fig. 3, where the dose dependence of external HCO₃⁻ is presented in double-reciprocal fashion (Lineweaver-Burk plot). For these studies, the extracellular concentration of Cl⁻ was kept constant at 125 mM, while [HCO₃⁻]₀ was varied between 0.3 and 5 mM by equimolar replacement of glucuronate. With increasing external HCO₃⁻, the initial rate of pHᵢ recovery progressively rose. The data could be fit by Michaelis-Menten kinetics with an apparent Kₘ for external HCO₃⁻ of 8.7 ± 1.9 mM. Assuming complete equilibration of CO₂ across the cell membrane and a pHᵢ of 7.97 (average of Figs. 1 and 2) at the start of pHᵢ recovery, the internal HCO₃⁻ concentrations are
actually 3.7-fold higher than the corresponding $[\text{HCO}_3^-]_o$ values. Thus, assuming internal $\text{HCO}_3^-$ to be the relevant exchange partner for external $\text{Cl}^-$, the apparent $K_m$ for external $\text{HCO}_3^-$ of $8.7 \pm 1.9$ mM translates into an apparent $K_m$ for internal $\text{HCO}_3^-$ that is 3.7-fold higher, or $32.2 \pm 7.0$ mM.

![Figure 2](image-url)

**Figure 2.** Time course of $pH_i$ recovery from alkalinization in the presence of different anions in the extracellular fluid (see legend to Fig. 1). After a 30-min pretreatment with 70 mM DMO, the neutrophils were resuspended in 5 mM $K^+$, 0.5 mM $\text{HCO}_3^-$ medium in which the major extracellular monovalent anion (148 mM) was either $\text{Cl}^-$, $\text{Br}^-$, $\text{I}^-$, $\text{NO}_2^-$, $\text{SCN}^-$, PAH$, or glucuronate. In the case of the divalent anion $\text{SO}_4^{2-}$, its concentration in the bathing medium was 100 mM (5 mM $K^+$, 194 mM $Na^+$) in order to maintain isotonicity. The curves are all single exponential fits to the data with initial rates (in pH/minute) of $0.0685 \pm 0.0040$ ($\text{Cl}^-$), $0.0787 \pm 0.0090$ ($\text{Br}^-$), $0.0531 \pm 0.0033$ ($\text{NO}_2^-$), $0.0438 \pm 0.0042$ ($\text{I}^-$), $0.0284 \pm 0.0038$ ($\text{SCN}^-$), $0.0209 \pm 0.0031$ (PAH), and $0.0154 \pm 0.0013$ (combined glucuronate and $\text{SO}_4^{2-}$). The dashed line represents the time course of $pH_i$ of the combined controls in $\text{Cl}^-$, $\text{Br}^-$, $\text{NO}_2^-$, $\text{I}^-$, $\text{SCN}^-$, and PAH$^-\text{medium. The data sets for cells not exposed to DMO and then resuspended in 148 mM glucuronate or 100 mM $\text{SO}_4^{2-}$ medium have been omitted; they exhibited a gradual intracellular alkalinization due to an exchange of internal $\text{Cl}^-$ for the small amount of external $\text{HCO}_3^-$ present in these otherwise inert media. Results are from three to five experiments.**

As measured chemically by chloridometry, the intracellular $\text{Cl}^-$ content of neutrophils pretreated with 70 mM DMO (and 78 mM $\text{Cl}^-$) for 30 min is ~50 meq/liter of cell water (see below). On the other hand, the normal intracellular $\text{Cl}^-$ concentration of steady-state cells bathed in the standard 148 mM $\text{Cl}^-$ medium is ~80 meq/liter of
cell water (Simchowitz and De Weer, 1986). Internal Cl⁻ depletion (to a level ≤2 meq/liter of cell water) by prolonged incubation in Cl⁻-free, 148 mM PAH⁺ medium substantially altered the affinity for internal HCO₃⁻. When the experiments of Fig. 3 were repeated on cells containing little or no Cl⁻, the rate of pHᵢ recovery from alkalinization was still a direct function of the HCO₃⁻ concentration, but in this case the $K_m$ for external HCO₃⁻ was only 2.6 ± 0.8 mM, or about threefold lower

than with control Cl⁻ cells. As above, this value corresponds to a $K_m$ for internal HCO₃⁻ that is 3.7 times greater, or 9.6 ± 3.0 mM. The difference in apparent $K_m$ values for internal HCO₃⁻ in control Cl⁻ as compared with Cl⁻-depleted cells (32.2 vs. 9.6 mM) indicates that, as expected, Cl⁻ and HCO₃⁻ behave as competing substrates for the same transport sites on the Cl⁻/HCO₃⁻ exchanger.

![Graphical representation of the data from Figure 3.](image-url)
In this section we complement the work on pH\textsubscript{i} transients with direct measurements of unidirectional 36Cl\textsuperscript{-} influxes and effluxes and net Cl\textsuperscript{-} movements using a chloridometer. The time courses of one-way 36Cl\textsuperscript{-} influx and net Cl\textsuperscript{-} influx from a 0.5 mM HCO\textsubscript{3}\textsuperscript{-}, 148 mM Cl\textsuperscript{-} medium are presented in Fig. 4, A and B. In both instances the kinetics of uptake could be adequately described by a single exponential equation: the internal 36Cl\textsuperscript{-} and total Cl\textsuperscript{-} contents rose at initial rates of 19.4 ±
3.8 and 3.13 ± 0.63 meq/liter·min, respectively, to reach final extrapolated levels of 75.3 ± 4.6 and 80.2 ± 7.2 meq/liter of cell water. As shown in Fig. 4 B, the internal Cl⁻ content of alkalinized cells rose from 49.2 ± 4.5 meq/liter of cell water to reach a value of 80.2 ± 7.2 meq/liter of cell water at full pHᵢ recovery. The ~30-mM gain in intracellular Cl⁻ represents a maximal estimate for the influx of Cl⁻ through Cl⁻/HCO₃⁻ exchange during that period. Similarly, the final steady level of ³⁶Cl⁻ uptake (75.3 ± 4.6 meq/liter of cell water) signifies a net rise in intracellular Cl⁻ of 75.3 - 49.2, or 26.1 meq/liter of cell water during the full recovery period. (The reduction in internal Cl⁻ in cells pretreated with 70 mM DMO as compared with normal cells bathed in 148 mM Cl⁻ medium [~50 vs. ~80 meq/liter of cell water] is probably due to replacement of Cl⁻ by DMO⁻ after its entry and dissociation within the cytoplasm. Assuming equilibration of the uncharged form of DMO (pK' 6.13) across the membrane and a measured pHᵢ of ~7.10 at the end of the 30-min preincubation period, the cells should have contained ~33 mM of DMO in the anionic form. This amount is consistent with the extent of the observed reduction in the level of intracellular Cl⁻ in these cells.)

Assuming an average intracellular buffering power of 50 mM/pH over the entire course of pHᵢ recovery (Simchowitz and Roos, 1985) and a 1:1 exchange of internal HCO₃⁻ for external Cl⁻, total recoveries of 0.62 and 0.52 pH units (Δ[Cl⁻]/β) may be predicted. These values are in reasonably good agreement with the 0.61 and 0.64 pH unit recoveries actually observed (Figs. 1 and 2). Likewise, the initial rate of net Cl⁻ uptake (3.13 ± 0.63 meq/liter·min), if completely coupled to Cl⁻/HCO₃⁻ exchange, signifies a pHᵢ recovery rate of 3.13/β, or 0.063 pH/min, which agrees well with those (0.067 and 0.069 pH/min) estimated from the time course of pHᵢ recovery (Figs. 1 and 2).

The one-way ³⁶Cl⁻ and net Cl⁻ influxes were markedly inhibited by 400 μM UK-5099: in its presence the initial influx rates were reduced from 19.4 to 0.93 ± 0.08 and from 3.13 to 0.32 ± 0.19 meq/liter·min, respectively (i.e., inhibitions of 95 and 90%). This is as expected if these fluxes were mediated principally by transport through a UK-5099-sensitive Cl⁻/HCO₃⁻ exchange mechanism that is activated by intracellular alkalinization.

Substrate saturation is shown in Fig. 5, where the external Cl⁻ dependence of SrCl⁻ influx is depicted. The relationship obeyed saturation kinetics: as [Cl⁻], was varied between 0.3 and 140 mM (replacing glucuronate), the initial rate of ³⁶Cl⁻ influx rose along a Michaelis-Menten activation curve with an apparent Kₘ(Cl⁻) of 5.8 ± 1.5 mM, similar to the value of 4.2 ± 0.9 mM derived from the external Cl⁻ dependence of pHᵢ recovery.

Substrate interactions between Cl⁻ and several other anions are documented in Fig. 6, where the ability of extracellular Br⁻, I⁻, NO₃⁻, HCO₃⁻, PAH⁻, SCN⁻, and SO₄²⁻ to compete with ³⁶Cl⁻ at the external transport site of the exchanger was tested. In these experiments ³⁶Cl⁻ influx from a 5-mM Cl⁻ medium was measured in the presence of 0–140 mM (0–20 mM for HCO₃⁻) of the test anion. For Br⁻ and I⁻, inhibition of alkalinization-induced ³⁶Cl⁻ influx followed Michaelis-Menten kinetics with apparent Kᵢ values of 9.8 ± 2.1 and 93.4 ± 36.0 mM, respectively. In the presence of two competing substrates, S₁ and S₂, the apparent Kᵢ is related to the
true kinetic constant as given by the expression

$$\text{apparent } K_m(S_1) = \text{true } K_m(S_1) \left[ 1 + \frac{[S_2]}{K_m(S_2)} \right].$$

(3)

Since, for the experiments presented in Fig. 6, external Cl⁻ was present at a concentration near its true $K_m$, the apparent inhibitory constants for Br⁻ and I⁻ should be about twice the true $K_m$ values (7.5 ± 1.5 and 39.2 ± 12.8 mM, respectively, for [Br⁻]₀ and [I⁻]₀ activation of pHᵢ recovery). This was indeed the case.

The ability of nonhalide anions to compete with $^{36}$Cl⁻ for binding to the exchanger was also investigated. Bicarbonate, NO₃⁻, SCN⁻, and PAH⁻ all inhibited the initial rate of $^{36}$Cl⁻ influx from a 5-mM Cl⁻ medium with apparent $K_i$ values of 5.3 ± 1.4, 8.4 ± 2.0, 111 ± 28, and 154 ± 65 mM (or from Eq. 3, true $K_m$ values of ~2.6, 4.2, 56, and 77 mM), respectively. As shown in Fig. 2, we also found the initial rates of pHᵢ recovery in 148 mM Br⁻, NO₃⁻, and I⁻ media to be of the same order as that in 148 mM Cl⁻. These results imply, therefore, that Br⁻, NO₃⁻, and I⁻ are also transported inward via the carrier in exchange for HCO₃⁻. On the contrary, the divalent anion SO₄²⁻, at external concentrations of 0–95 mM, had no effect at all on the rate of $^{36}$Cl⁻ influx. These findings suggest that the anion exchange system of human neutrophils possesses little, if any, affinity for SO₄²⁻, unlike the situation in human erythrocytes where Cl⁻ and SO₄²⁻ bind to the inorganic anion exchanger with closely similar $K_m$ values (Barzilay and Cabantchik, 1977; Schnell et al., 1977; Milanick and Gunn, 1982).
Data on the time course of $^{36}\text{Cl}^-$ efflux after DMO withdrawal are presented in Fig. 7. Intracellular alkalinization caused an increase in the rate of $^{36}\text{Cl}^-$ efflux from the cells: resuspension of the neutrophils in 0.5 mM HCO$_3^-$, 148 mM Cl$^-$ medium led to a substantial, though transient, stimulation of $^{36}\text{Cl}^-$ efflux (curve A), whose time course (i.e., a single exponential) roughly paralleled that of pH$_i$ recovery under the same conditions (Fig. 1).

![Figure 6](image)

**Figure 6.** Substrate competition: effect of different halides and other mono- and divalent anions on the rate of $^{36}\text{Cl}^-$ influx from a 5-mM Cl$^-$ medium. See legend to Fig. 3. The external concentrations of each of the ions (Br$^-$, I$^-$, NO$_3^-$, SCN$^-$, or PAH$^+$) was varied between 0 and 140 mM (0 and 20 mM for HCO$_3^-$; 0 and 95 mM for SO$_4^{2-}$) by replacement of glucuronate. The $^{36}\text{Cl}^-$ influx rates were derived as in Fig. 4 and plotted against the external concentration of the competing anion. Except for SO$_4^{2-}$, all other curves are least-squares fits of Michaelis-Menten inhibition equations to the data. The apparent $K_i$ values were 5.3 ± 1.4, 8.4 ± 2.0, 9.8 ± 2.1, 93.4 ± 36.0, 111 ± 28, and 154 ± 65 mM for HCO$_3^-$, NO$_3^-$, Br$^-$, I$^-$, SCN$^-$, and PAH$^+$, respectively. In the case of SO$_4^{2-}$, the data were fit by a straight line with slope = −0.0063 ± 0.0102. Results are from three experiments for each anion.

The initial $^{36}\text{Cl}^-$ efflux rate was 15.6 ± 2.3 meq/liter·min, sevenfold greater than that of controls (2.11 ± 0.18 meq/liter·min) where resting pH$_i$ was ~7.35. Therefore, the net $^{36}\text{Cl}^-$ influx rate, computed from the difference between one-way $^{36}\text{Cl}^-$ influx and efflux was 18.1 (average of Figs. 4 A and 5) minus 15.6 = 2.5 meq/liter·min, a value similar to that measured chemically by chloridometry (3.13 ± 0.63 meq/liter·min, Fig. 4 B). The augmentation of $^{36}\text{Cl}^-$ efflux that was
FIGURE 7. Time course of $^{36}\text{Cl}^-$ efflux into 148 mM Cl$^{-}$, 0.5 mM HCO$_3^-$ medium during pH$_i$ recovery from alkalinization. The neutrophils had been preincubated with 2.5 $\mu$Ci/ml $^{36}\text{Cl}^-$ in 148 mM Cl$^{-}$ medium for ~2 h. Aliquots of the cell suspensions were then exposed to 70 mM DMO for 30 min (solid lines). Controls (dashed lines) were kept in 148 mM Cl$^{-}$ medium. After being pelleted, the cells were resuspended in 148 mM Cl$^{-}$, 0.5 mM HCO$_3^-$ in the presence or absence of 400 $\mu$M UK-5099. Results are from four experiments. The curves represent declining single exponential fits (Eq. 2) to the individual sets of data. In the special case of cells alkalinized by pretreatment with DMO (curve A), the data points were fit to a program in which the efflux was divisible into two components: (a) a falling single exponential representing the alkalinization-induced transient, and (b) upon complete pH$_i$ recovery, a residual rate coefficient equal to that of control cells. The efflux rate coefficients for curves A–D were (in minutes$^{-1}$) as follows: 0.474 ± 0.069 (the exponential terminating at a relative Cl$^{-}$ content of 0.341 ± 0.021), 0.0180 ± 0.0010, 0.0264 ± 0.0023, and 0.0064 ± 0.0012, respectively. Assuming a zero-time internal Cl$^{-}$ content of 50 meq/liter of cell water (from Fig. 4 B) for cells pretreated with 70 mM DMO (curves A and B) and a zero-time [Cl$^{-}$], value of 80 meq/liter of cell water (the normal resting value [Simchowitz and De Weer, 1986]) for control cells (curves C and D), these rate coefficients signify initial $^{36}\text{Cl}^-$ efflux rates of 15.6 ± 2.3, 0.90 ± 0.05, 2.11 ± 0.18, and 0.52 ± 0.09 meq/liter-min for curves A–D, respectively.
associated with pH$_i$ recovery from alkalinization was markedly sensitive to 400 µM UK-5099: efflux in the presence of the drug was reduced from 15.6 to 0.90 ± 0.05 meq/liter-min, an inhibition of 94%.

Data on the rate of $^{36}$Cl$^-$ efflux as a function of external Cl$^-$ (0.6-140 mM, replacing glucuronate) are given in Fig. 8. Raising [Cl$^-$]$_o$ from 0.6 toward 140 mM led to a gradual enhancement of the rate of $^{36}$Cl$^-$ efflux from the cells. External Cl$^-$ stimulated its own efflux along a Michaelis-Menten activation curve with an apparent $K_m$ of 7.2 ± 1.4 mM. Since the carrier appears to be devoid of affinity for glucuronate, the Michaelis constant for Cl$^-$ in glucuronate medium is a reasonable estimate of its true $K_m$. This value agrees well with the $K_m$ values of 4.2 ± 0.9 and 5.8 ± 1.5 mM for the [Cl$^-$]$_o$ dependence of pH$_i$ recovery and $^{36}$Cl$^-$ influx (Fig. 5).

![Figure 8](image_url)

**Figure 8.** Activation of intracellular alkalinization-induced $^{36}$Cl$^-$ efflux by external Cl$^-$ (replacing glucuronate). See legend to Fig. 5. Cells were labeled with $^{36}$Cl$^-$ and pre-treated with 70 mM DMO as described in Fig. 7. The neutrophils were then resuspended in media in which the concentration of Cl$^-$ was varied between 0.6 and 140 mM by equimolar replacement of glucuronate. The efflux of $^{36}$Cl$^-$ was measured at 2 and 4 min and the initial efflux rates calculated by fitting the data points to a declining exponential (Eq. 2). A UK-5099-insensitive background of ~0.5 meq/liter-min, representing a leak flux, was subtracted from all values. The starting (zero time) internal Cl$^-$ content was taken as 50 meq/liter of cell water. The initial $^{36}$Cl$^-$ efflux rates were plotted against [Cl$^-$]$_o$. The curve represents a Michaelis-Menten activation equation which yielded a $K_m$ for Cl$^-$ of 7.2 ± 1.4 mM and a $V_{max}$ of 14.1 ± 0.7 meq/liter-min. The results are from three experiments.

Fig. 9 displays the effect of HCO$_3^-$ on $^{36}$Cl$^-$ efflux. The data have been graphed in the form of a Dixon plot (1/v vs. I). For these experiments, the medium contained Cl$^-$ at 125 mM, a saturating concentration, while [HCO$_3^-$]$_o$ was varied between 0 and 5 mM by equimolar replacement of glucuronate. At first sight, the potential interactions between HCO$_3^-$ and Cl$^-$ at both the internal and external translocation sites of the carrier appear to be quite complex. For instance, HCO$_3^-$ might be expected to have two opposite effects on the efflux of $^{36}$Cl$^-$ from the intracellular compartment: (a) inhibition, by competing with Cl$^-$ on the cis (internal) side, and (b) activation, by stimulating exchange as an additional exchange partner on the trans side. However, under the conditions of the experiment, namely saturating external Cl$^-$ (i.e., [Cl$^-$]$_o$ ) ~25 times its $K_m$, this latter effect becomes negligible. This is
especially true since the maximal transport rates for Cl\(^-\) and HCO\(_3^-\) are the same. Thus, under the conditions outlined above, the principal effect of added HCO\(_3^-\) is at the carrier's internal binding site. As shown in Fig. 9, increasing [HCO\(_3^-\)]\(_o\) between 0 and 5 mM caused a progressive reduction in the \(^{36}\)Cl\(^-\) efflux rate. Inhibition followed Michaelis-Menten kinetics with an "apparent \(K_m\)" for external HCO\(_3^-\) of 11.1 ± 2.8 mM. This value is in reasonable agreement with the determination of 8.7 ± 1.9 mM for the "apparent \(K_m\)" for external HCO\(_3^-\) activation of pH\(_i\) recovery (Fig. 3). As noted above, at a pH\(_i\) of ~7.97 at the start of recovery and a pH\(_o\) of 7.40, [HCO\(_3^-\)]\(_i\) exceeds [HCO\(_3^-\)]\(_o\) by a factor of 3.7. Thus, the data of Fig. 9 imply an apparent \(K_m\) for internal HCO\(_3^-\) that is 3.7-fold higher, or 41.1 ± 10.4 mM.

In Fig. 7 we noted that 400 \(\mu\)M UK-5099 inhibited the alkalinization-induced rate of \(^{36}\)Cl\(^-\) efflux by >90%. Fig. 10 presents data on the efflux of \(^{36}\)Cl\(^-\) into 148 mM Cl\(^-\) or 2.5 mM HCO\(_3^-\) (balance glucuronate) medium as a function of the concentration of UK-5099 (0–400 \(\mu\)M). Raising the amount of added drug caused the \(^{36}\)Cl\(^-\) efflux rate to fall along Michaelis-Menten inhibition curves which yielded apparent \(K_i\) values for UK-5099 of 43.9 ± 10.5 and 2.3 ± 0.5 \(\mu\)M in 148 mM Cl\(^-\) and 2.5 mM HCO\(_3^-\), respectively. The difference in apparent \(K_i\) values may be satisfactorily explained by considering the effects of a competitive inhibitor (UK-5099) in the presence of widely different concentrations of two high affinity substrates (i.e., Cl\(^-\) and HCO\(_3^-\) are present at roughly 30 and 1 times their respective \(K_m\) values at the external site of the exchange carrier). On this basis, and from Eq. 3, the apparent \(K_i\) for UK-5099 in 148 mM Cl\(^-\) should exceed that in 2.5 mM HCO\(_3^-\) by a factor of
~15, which it does (43.9 vs. 2.3 \( \mu M \)). Substituting the appropriate values (\( K_m(Cl^-) = 5.0 \text{ mM} \) and \([Cl^-]_o = 148 \text{ mM} \) or \( K_m(HCO_3^-) = 2.6 \text{ mM} \) and \([HCO_3^-]_o = 2.5 \text{ mM} \)) into Eq. 3, one may derive the two closely similar estimates of 1.4 and 1.2 \( \mu M \) for the true \( K_i \) value of UK-5099 in Cl\(^-\) and HCO\(_3^-\) media, respectively.

**DISCUSSION**

*Bicarbonate-dependent Transport Systems in Other Cells*

The existence of HCO\(_3^-\)-transporting pH\(_i\) regulatory mechanisms has been reported in a wide variety of animal cells. For example, a Na\(^+\)/HCO\(_3^-\)-Cl\(^-\)/H\(^+\) exchanger is present in several invertebrate species (Thomas, 1977; Boron et al., 1981; Boron and Russell, 1988). It is likely that a similar mechanism occurs in human epidermoid carcinoma cells (Rothenberg et al., 1983), hamster fibroblasts (L’Allemain et al., 1985), and monkey kidney cells (Tonnessen et al., 1987), where a Na\(^+\)-dependent Cl\(^-\)/HCO\(_3^-\) exchange has been described. On the other hand, in human erythrocytes (Knauf, 1979), mouse skeletal muscle (Aickin and Thomas, 1977), mouse Ehrlich ascites tumor cells (Hoffmann, 1982, 1986), and guinea pig smooth muscle (Aickin and Brading, 1984, 1985), to name a few, a Na\(^+\)-independent Cl\(^-\)/HCO\(_3^-\) exchange system has been implicated in the regulation of intracellular pH.

Compared with the abundance of information in diverse cell types concerning mechanisms of acid extrusion, the processes underlying recovery from intracellular alkalization have received relatively little attention. Using a sheep cardiac Purkinje
Simchowitz and Davis  

Cl⁻/HCO₃⁻ Exchange in Human Neutrophils

...
to the internal translocation site with a $K_m \sim 10$ mM and to the external site of the
exchanger with a $K_m \sim 2.5$ mM strongly suggests the possibility of a fourfold
asymmetry between inward- and outward-facing conformations. Since thermody-
namic considerations dictate the same relative rank order of affinities for all ions on
both sides of the membrane (Frohlich and Gunn, 1986), these findings imply that the
true $K_m$ for internal $\text{Cl}^-$ is also fourfold greater than its $K_m$ for external $\text{Cl}^-$ ($\sim 5$ mM)
or $\sim 20$ mM. If the true $K_m$ (internal $\text{HCO}_3^-$) and the true $K_m$ (internal $\text{Cl}^-$) were $\sim 10$
and $\sim 20$ mM, then, substituting the appropriate values in Eq. 3, the apparent $K_m$
for internal $\text{HCO}_3^-$ measured in $\text{Cl}^-$-containing cells ($[\text{Cl}^-]_i \sim 50$ meq/liter of cell water)
ought to come to $\sim 35$ mM. Indeed, this is similar to the value of $\sim 32$ mM actually
observed.

Other evidence consistent with an asymmetrical carrier in neutrophils was first
published in our original report on anion exchange in resting cells (Simchowitz et al.,
1986), although it was not pursued at the time. In that study we noted that $\text{Cl}^-$ binds
to the carrier's external site with a true $K_m$ of 5.0 mM and with an apparent $K_m$ of
$\sim 35$ mM when intracellular $\text{Cl}^-$ was varied reciprocally with $\text{PAH}^-$ (i.e.,
$[\text{Cl}^-]_i + [\text{PAH}^-]_i = 80$ meq/liter of cell water). Under these conditions, activation
of $\text{Cl}^-$ efflux by internal $\text{Cl}^-$ follows a Michaelis-Menten equation where

$$\text{apparent } K_m(\text{Cl}^-) = K_m(\text{Cl}^-) \cdot \frac{K_m(\text{PAH}^-) + 80}{K_m(\text{PAH}^-) - K_m(\text{Cl}^-)}. \quad (4)$$

Since, for reasons given above, $K_m(\text{PAH}^-)$ must always exceed $K_m(\text{Cl}^-)$ by a factor of
10, substituting 10 $K_m(\text{Cl}^-)$ for $K_m(\text{PAH}^-)$ and $35$ mM for apparent $K_m(\text{PAH}^-)$ in Eq. 4,
one may derive an estimate of the true $K_m$ value for internal $\text{Cl}^-$ of 24 mM. Compared with a measured $K_m$ for external $\text{Cl}^-$ of only 5 mM, this result also
suggests the existence of an approximately fourfold asymmetry between inside and
outside carrier states. Presently we cannot definitely exclude the presence of an
unidentified competing ligand inside the cell, rather than a genuine carrier asymme-
try.

$\text{Cl}^-$-independent $\text{pH}_i$ Recovery

As stated above, the exchange carrier appears to be devoid of affinity for gluc-
uronate and $\text{SO}_4^{2-}$. Nonetheless, from the data of Figs. 1 and 2, it is evident that there
is an appreciable $\text{pH}_i$ recovery in these media ($\sim 20\%$ of that in 148 mM $\text{Cl}^-$
medium), which is also comparable to the UK-5099-resistant component. Moreover,
resuspension of alkalinized cells in substrate-free glucuronate or $\text{SO}_4^{2-}$ media
would be expected to lead to a further rise in $\text{pH}_i$ due to reversed $\text{Cl}^-/\text{HCO}_3^-$ exchange,
the latter being driven by the large outward gradient for $\text{Cl}^-$. Such a secondary
alkalinization could not be detected, however, further implying the presence of a
separate $\text{pH}_i$ recovery process or processes independent of anion exchange.

This as yet unidentified process could conceivably represent metabolic acid
production or perhaps passive leak fluxes of $\text{HCO}_3^-$, $\text{H}^+$, and/or $\text{OH}^-$. A significant
contribution of metabolic acid production seems unlikely, since 2-DOG, which
blocks anaerobic glycolysis and leads to intracellular ATP depletion and reduced
lactic acid production, did not appreciably alter the slow recovery in 148 mM
glucuronate medium. On the other hand, this minor component of recovery could in
theory be due to passive electrodiffusive fluxes of $\text{HCO}_3^-$, $\text{H}^+$, and/or $\text{OH}^-$ occurring under very favorable electrical and chemical gradients (membrane voltage $\sim -60 \text{ mV}$, $\text{pH}_o 7.40$, $\text{pH}_i \sim 7.95$). When in fact the electrical driving force was abolished by completely depolarizing the cells in 120 mM K+, we were still unable to measure any significant change in the $\text{pH}_i$ recovery rate in 0.5 mM $\text{HCO}_3^-$, 148 mM glucuronate medium. Therefore, it would appear that the vast proportion of this apparent recovery remains largely unexplained.

We acknowledge the expert technical assistance of William H. Miller and Jacquelyn T. Engle, and the secretarial skills of Annette Irving and Teri L. Becker. We are especially grateful to Dr. Paul De Weer for providing the computer programs and least-squares fits for data analysis and to Ronald W. Ratlaff and Milton F. Pfeiffer for their efforts in connection with the computer files, graphic displays, and illustrations. We also wish to thank Dr. Paul De Weer and Dr. Albert Roos for their helpful discussions while the work was in progress and for their comments on the manuscript.

This work was supported by the Veterans Administration and by NIH grant GM-38094.

Original version received 25 November 1988 and accepted version received 8 May 1990.

REFERENCES

Aickin, C. C., and A. F. Brading. 1984. The role of chloride-bicarbonate exchange in the regulation of intracellular chloride in guinea-pig vas deferens. *Journal of Physiology.* 349:587–606.

Aickin, C. C., and A. F. Brading. 1985. The effects of bicarbonate and foreign anions on chloride transport in smooth muscle of the guinea-pig vas deferens. *Journal of Physiology.* 366:267–280.

Aickin, C. C., and R. C. Thomas. 1977. An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *Journal of Physiology.* 273:295–316.

Barzilay, M., and Z. I. Cabantchik. 1979. Anion transport in red blood cells. II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. *Membrane Biochemistry.* 2:255–281.

Boron, W. F., W. C. McCormick, and A. Roos. 1979. pH regulation in barnacle muscle fibers: dependence on intracellular and extracellular pH. *American Journal of Physiology.* 237:C185–C193.

Boron, W. F., W. C. McCormick, and A. Roos. 1981. pH regulation in barnacle muscle fibers: dependence on extracellular sodium and bicarbonate. *American Journal of Physiology.* 240:C80–C89.

Boron, W. F., and J. M. Russell. 1983. Stoichiometry and ion dependencies of the intracellular pH-regulating mechanism in squid giant axons. *Journal of General Physiology.* 81:373–399.

Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation.* 21(Suppl 97): 77–89.

Cotlove, E., H. V. Trantham, and R. L. Bowman. 1958. An instrument and method for automatic, rapid, accurate, and sensitive titration of chloride in biological samples. *Journal of Laboratory and Clinical Medicine.* 51:461–468.

Frohlich, O., and R. B. Gunn. 1986. Erythrocyte anion transport: the kinetics of a single-site obligatory exchange system. *Biochimica et Biophysica Acta.* 864:169–194.

Grinstein, S., and W. Furuya. 1984. Amiloride-sensitive Na+/H+ exchange in human neutrophils: mechanism of activation by chemotactic factors. *Biochemical and Biophysical Research Communications.* 122:755–762.

Halestrap, A. P., and R. M. Denton. 1975. The specificity and metabolic implications of the inhibition of pyruvate transport in isolated mitochondria and intact tissue preparations by $\alpha$-cyano-4-hydroxycinnamate and related compounds. *Biochemical Journal.* 148:97–106.
Hoffmann, E. K. 1982. Anion exchange and anion-cation co-transport systems in mammalian cells. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences.* 299:519–555.

Hoffmann, E. K. 1986. Anion transport systems in the plasma membrane of vertebrate cells. *Biochimica et Biophysica Acta.* 864:1–31.

Klempner, M. S., and B. Styr. 1983. Alkalinizing the intralysosomal pH inhibits degranulation of human neutrophils. *Journal of Clinical Investigation.* 72:1799–1800.

Knauf, P. A. 1979. Erythrocyte anion exchange and the band 3 protein: transport kinetics and molecular structure. *Current Topics in Membrane Transport.* 12:249–363.

L’Allemain, G., S. Paris, and J. Pouyssegur. 1985. Role of the Na⁺-dependent Cl⁻/HCO₃⁻ exchange in regulation of intracellular pH in fibroblasts. *Journal of Biological Chemistry.* 260:4877–4883.

Lowe, A. G., and A. Lambert. 1983. Chloride-bicarbonate exchange and related transport processes. *Biochimica et Biophysica Acta.* 694:353–374.

Madhus, I. H., and S. Ohlens. 1987. Selective inhibition of sodium-linked and sodium-independent bicarbonate/chloride antiport in vero cells. *Journal of Biological Chemistry.* 262:7486–7491.

Milanik, M. A., and R. B. Gunn. 1982. Proton-sulfate cotransport. H⁺ and sulfate addition to the chloride transporter of human red blood cells. *Journal of General Physiology.* 79:87–113.

Olsnes, S., T. I. Tonnessen, and K. Sandvig. 1986. pH-regulated anion transport in nucleated mammalian cells. *Journal of Cell Biology.* 102:967–971.

Parsons, D. S. 1982. Role of anions and carbonic anhydrase in epithelia. *Philosophical Transactions of the Royal Society of London, Series B.* 299:369–381.

Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiological Reviews.* 61:406–409.

Rothenberg, P., L. Glaser, P. Schlesinger, and D. Cassel. 1983. Activation of Na⁺/H⁺ exchange by epidermal growth factor elevates intracellular pH in A431 cells. *Journal of Biological Chemistry.* 258:12644–12653.

Schnell, K. F., S. Gerhardt, and A. Schoppe-Fredenburg. 1977. Kinetic characteristics of the sulfate self-exchange in human red blood cells and red blood cell ghosts. *Journal of Membrane Biology.* 30:319–350.

Sha’afi, R. I., P. H. Naccache, T. F. P. Molski, and M. Volpi. 1982. Chemotactic stimuli-induced changes in the pH of rabbit neutrophils. *In Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions. R. Nuccitelli and D. W. Deamer, Editors. Alan R. Liss, Inc., New York.* 513–525.

Simchowitz, L. 1985. Intracellular pH modulates the generation of superoxide radicals by human neutrophils. *Journal of Clinical Investigation.* 76:1079–1089.

Simchowitz, L. 1988a. Interactions of bromide, iodide, and fluoride with the pathways of chloride transport and diffusion in human neutrophils. *Journal of General Physiology.* 91:835–860.

Simchowitz, L. 1988b. Properties of the principal anion exchange mechanism in human neutrophils. *In Society of General Physiologists Series, Vol. 43 (Cell Physiology of Blood). R. B. Gunn and J. C. Parker, editors. The Rockefeller University Press, New York.* 193–208.

Simchowitz, L., and E. J. Cragoe, Jr. 1986. Regulation of human neutrophil chemotaxis by intracellular pH. *Journal of Biological Chemistry.* 261:6492–6500.

Simchowitz, L., and P. De Weer. 1986. Chloride movements in human neutrophils. Diffusion, exchange, and active transport. *Journal of General Physiology.* 88:167–194.

Simchowitz, L., R. Ratzlaff, and P. De Weer. 1986. Anion/anion exchange in human neutrophils. *Journal of General Physiology.* 88:195–217.
Simchowitz, L., and A. Roos. 1985. Regulation of intracellular pH in human neutrophils. *Journal of General Physiology.* 85:443-470.

Simchowitz, L., I. Spilberg, and P. De Weer. 1982. Sodium and potassium fluxes and membrane potential of human neutrophils. Evidence for an electrogenic sodium pump. *Journal of General Physiology.* 79:455-479.

Thomas, R. C. 1977. The role of bicarbonate, chloride, and sodium ions in the regulation of intracellular pH in snail neurons. *Journal of Physiology.* 273:317-338.

Tonnessen, T. I., J. Ludt, K. Sandvig, and S. Olsnes. 1987. Bicarbonate/chloride antiport in vero cells. I. Evidence for both sodium-linked and sodium-independent exchange. *Journal of Cellular Physiology.* 132:183–191.

Vaughan-Jones, R. D. 1979. Regulation of chloride in quiescent sheep-heart Purkinje fibres studied using intracellular chloride and pH-sensitive micro-electrodes. *Journal of Physiology.* 295:111–137.

Vaughan-Jones, R. D. 1981. Ammonia stimulates chloride-bicarbonate exchange in sheep cardiac Purkinje fibres. *Journal of Physiology.* 320:33P.

Vaughan-Jones, R. D. 1982a. Chloride-bicarbonate exchange in the sheep cardiac Purkinje fibre. In Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions. R. Nuccitelli and D. W. Deamer, editors. Alan R. Liss, Inc., New York. 239–252.

Vaughan-Jones, R. D. 1982b. Chloride activity and its control in skeletal and cardiac muscle. *Philosophical Transactions of the Royal Society of London, Series B. Biological Sciences.* 299:537–548.