Regulation of Intracellular pH in Human Neutrophils

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ABSTRACT The intracellular pH (pHi) of isolated human peripheral blood neutrophils was measured from the fluorescence of 6-carboxyfluorescein (6-CF) and from the equilibrium distribution of [14C]5,5-dimethyloxazolidine-2,4-dione (DMO). At an extracellular pH (pHe) of 7.40 in nominally CO2-free medium, the steady state pHi using either indicator was ~7.25. When pHe was suddenly raised from 7.40 to 8.40 in the nominal absence of CO2, pHi slowly rose by ~0.35 during the subsequent hour. A change of similar magnitude in the opposite direction occurred when pHe was reduced to 6.40. Both changes were reversible. Intrinsic intracellular buffering power, determined by using graded pulses of CO2 or NH4Cl, was ~50 mM/pH over the pHi range of 6.8-7.9. The course of pHi obtained from the distribution of DMO was followed during and after imposition of intracellular acid and alkaline loads. Intracellular acidification was brought about either by exposing cells to 18% CO2 or by prepulsing with 30 mM NH4Cl, while pHe was maintained at 7.40. In both instances, pHi (6.80 and 6.45, respectively) recovered toward the control value at rates of 0.029 and 0.134 pH/min. These rates were reduced by ~90% either by 1 mM amiloride or by replacement of extracellular Na with N-methyl-D-glucamine. Recovery was not affected by 1 mM SITS or by 40 mM a-cyano-4-hydroxy-cinnamate (CHC), which inhibits anion exchange in neutrophils. Therefore, recovery from acid loading is probably due to an exchange of internal H for external Na. Intracellular alkalinization was achieved by exposing the cells to 30 mM NH4Cl or by prepulsing with 18% CO2, both at a constant pHe 7.40. In both instances, pHi, which was 7.65 and 7.76, respectively, recovered to the control value. The recovery rates (0.033 and 0.077 pH/min, respectively) were reduced by 80-90% either by 40 mM CHC or by replacement of extracellular Cl with p-aminohippurate (PAH). SITS, amiloride, and ouabain (0.1 mM) were ineffective. The rate of recovery from NH4Cl-induced alkalinization was enhanced twofold by adding 1 mM HCO3/0.2% CO2 to the medium (pHe 7.40). When the membrane was depolarized from -53 to 0 mV in 115 mM K, the recovery during NH4Cl exposure was reduced by ~30%, whereas the inward driving force on NH4 was reduced by ~80%. Apparently, the entry of NH4 by electrodiffusion plays only a minor role in the recovery. These results make it
likely that Cl/HCO₃ exchange is chiefly responsible for pHᵢ recovery from alkalization. In conclusion, human neutrophils possess two separate regulatory mechanisms: a Na/H exchange that restores pHᵢ after an acid load and a Cl/ HCO₃ exchange that restores it after an alkaline load.

INTRODUCTION

In the past several years, a great deal has been learned about the mechanisms by which intracellular pH (pHᵢ) is regulated in a variety of cell types (Roos and Boron, 1981; Busa and Nuccitelli, 1984). The importance of such regulation for cell function is becoming increasingly apparent. Thus, pHᵢ has been found to play a role in egg fertilization (Johnson et al., 1976; Epel, 1978), mechanical properties of muscle (Fabiato and Fabiato, 1978), epithelial transport (Warnock and Rector, 1979), pancreatic β cell function (Pace et al., 1983), cell growth and division (Gerson et al., 1982; Moolenaar et al., 1983), and platelet activation (Simons et al., 1982).

It has recently been proposed (Molski et al., 1980; Segal et al., 1981; Sha’afi et al., 1982) that several functions of neutrophils, which play a prime role in host defense against microorganisms, may also be modulated by their pHᵢ. Among these functions are chemotaxis, phagocytosis, and secretion of enzymes and free radicals. However, little is known about the factors governing the pHᵢ of resting neutrophils. The purpose of the present work is to examine these factors.

We have measured the steady state pHᵢ of human neutrophils under various conditions. We also have examined their pHᵢ responses to intracellular acid and alkaline loads and the ionic transport mechanisms on which these responses are based. After either load, pHᵢ was found to return to a more normal value. The recovery after intracellular acidification required Na and could be inhibited by amiloride, which strongly suggests that the underlying mechanism is a Na/H exchange. On the other hand, after imposed alkalization, pHᵢ recovery required Cl, was enhanced by HCO₃, and could be inhibited by α-cyano-4-hydroxycinnamate (CHC), a drug that has been shown by our group to block anion transport in neutrophils (Simchowitz and De Weer, 1984). This recovery seems, therefore, to be mainly accomplished through a Cl/HCO₃ exchange.

METHODS

Cell Isolation

Neutrophils were isolated from heparinized blood of normal donors by the standard procedure of dextran sedimentation at 37°C followed by Ficoll-Hypaque gradient centrifugation at room temperature (Boyum, 1968). The cellular pellet was resuspended for 30 s in distilled water to lyse any red cells present. Isotonicity was then restored by addition of NaCl solution, after which the cells were washed twice in standard medium (pHᵢ 7.40) containing (mM): 140 NaCl, 5 KCl, 1.0 CaCl₂, 0.5 MgCl₂, 5.6 glucose, 5 HEPES, and 1 mg/ml of crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The cells were kept in this medium for 1 h at 37°C prior to experimentation. About 97% of the cell suspension consisted of neutrophils. The vital dyes eosin Y or trypan blue, when added to the suspension, were excluded by >99% of the cells, a generally accepted criterion of viability.
**pHi Measurements**

All measurements were made at 37°C. We derived pHi from the steady state distributions of the ¹⁴C-labeled weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO; pK₁ 6.13 [Boron and Roos, 1976]) or the weak base ¹⁴C-trimethylamine (TMA; pK₁ 9.62 [Everett and Wynne-Jones, 1940-41]), and from the fluorescence of the weak acid 6-carboxyfluorescein (6-CF; pK₁ 6.6; see Fig. 2).

**Measurements with DMO and TMA** These methods have recently been reviewed (Roos and Boron, 1981). They rest on the assumption that only the neutral form of the indicators is permeant. The time required for a stable DMO distribution to be reached was <15 s (Fig. 1), the shortest practical exposure time. For TMA, equilibration required ~2 min (Fig. 1). This relatively long equilibration time is probably due to the very low external concentration of the neutral form: the pHₑ (7.40) in these studies was more than two units lower than the pK₁ of TMA.

Samples of the neutrophil suspension (8–12 × 10⁶ cells/ml), containing either ¹⁴C-DMO (1.0 μCi/ml) or ¹⁴C-TMA (0.5 μCi/ml), were incubated in plastic tubes at 37°C under various experimental conditions. Unlabeled indicator was added to a total concentration of 0.1 mM, too low to affect pH; by itself as assessed by 6-CF fluorescence. The suspension also contained ²H₂O (1.0 μCi/ml), which allowed total pellet water to be measured. At intervals, triplicate 0.5-ml aliquots were layered over 0.7 ml of Versilube F50 oil (Harwick Chemical Corp., Akron, OH) in 1.5-ml plastic tubes and centrifuged for...
\[ \text{~30 s at 8,000 g in a microcentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The cells penetrate the oil and accumulate as a pellet, while the suspending medium remains above the oil. This layering method, introduced by Naccache et al. (1977), allows cell separation in <5 s. The pellet was isolated and counted in a liquid scintillation counter (Beckman LS 7000) after addition of 10 ml Aquasol-2 (New England Nuclear, Boston, MA). The extracellular water content of the pellet under a variety of conditions was assessed in preliminary studies with neutrophil suspensions containing [H]H2O and [C]inulin. Extracellular water of the pellet was 9 ± 2% (n = 7) of total water. This percentage was used to correct for extracellular DMO or TMA.} \]

**MEASUREMENTS WITH 6-CF**

The measurements are based on the pH dependence of the fluorescence of 6-CF. The diacetate of the probe, a monovalent anion, readily enters the cell where esterases remove the two acetate groups. The resulting compound is much less permeant than the esterified one (J. A. Thomas et al., 1979). We incubated a suspension of neutrophils for 15 min with 30 \( \mu \text{M} \) 6-CF diacetate. The compound had been dissolved in dimethylsulfoxide (Fisher Scientific, Pittsburgh, PA). The final concentration of the solvent was 0.1% (vol/vol). The cells were then washed three times in standard medium (required time, 10–15 min) to remove external indicator, and resuspended in the experimental solution. Fluorometric measurements (spectrofluorometer model 430, Turner Associates, Palo Alto, CA) were made at 520 nm, with excitation at 490 nm. Two aspects of the method need be examined: (a) calibration and (b) leakage of the probe from cells.

(a) Calibration, performed daily along with the experimental measurements, was accomplished by recording the fluorescence signal at various \( \text{pH}_c \) values (5.0–8.4) in the presence of 120 mM K and 3 \( \mu \text{M} \) nigericin in the external solution. Nigericin, which promotes electroneutral K/H exchange (Pressman, 1969), should lead to equality of \( \text{pH}_i \) and \( \text{pH}_o \), since the intra- and extracellular K concentrations are the same (Simchowitz et al., 1982). In addition, nigericin seems to abolish regional intracellular pH differences (Ohkuma and Poole, 1978). In preliminary experiments in high-K/nigericin media, using DMO and TMA, both indicators gave identical values for \( \text{pH}_i \) that were in excellent agreement with the prevailing \( \text{pH}_c \) (Fig. 2A). The relationship between the fluorescence of 6-CF and \( \text{pH}_c \) in high-K/nigericin media of different \( \text{pH} \) (5.0–8.4) is sigmoidal (Fig. 2B) with an infection at about \( \text{pH}_i \) 6.6, which probably corresponds to the apparent \( \text{pK}_a \).

(b) J. A. Thomas et al. (1979) reported significant leakage of 6-CF from Ehrlich ascites tumor cells. To test for leakage, we exposed 6-CF–loaded neutrophils to high-K/nigericin media of pH 6.0, 7.0, or 8.0. At intervals during a 60-min incubation, the fluorescence of the separated supernatants was determined at pH 10 (J. A. Thomas et al., 1979). The results (not shown) were expressed as the percent of maximal fluorescence achieved after lysing the cells with 0.2% Triton X-100. The rate coefficients of 6-CF loss were the same at pH 7.0 and 8.0, namely 0.0113 ± 0.0004 min\(^{-1}\) \( n = 6 \). At pH 6.0, the rate coefficient was somewhat less (0.0059 ± 0.0003 min\(^{-1}\) \( n = 3 \), in contrast to Thomas’ finding, in which leakage from Ehrlich tumor cells increased with decreasing pH (J. A. Thomas et al., 1979). In our studies, all fluorescence measurements were completed within 5 min of resuspension of the cells in probe-free medium. The estimated loss of 6-CF during this period was ~5%.

**Incubation Media**

All experiments were performed in the absence of glucose. In preliminary studies, we found that with the exception of the CHC series (see Results), adding glucose had no effect on the results. The media were buffered with 5 mM MES (pK\text{a} 6.0), HEPES (pK\text{a} 7.0), HEPES (pK\text{a} 7.0), HEPES (pK\text{a} 7.0).
FIGURE 2. Calibration of 6-CF. (A) Comparison between pHi and DMO- or TMA-derived pHi of neutrophils exposed to 120 mM K and 3 μM nigericin. Results, obtained 2 min after exposure of cells to high-K nigericin media, represent the means ± SEM of five separate experiments, each performed in triplicate. Over the pHi range 6.5-8.0, the pHi derived by either method closely resembled pHo. The line of identity is also shown. (B) Relationship between fluorescence of 6-CF-loaded cells and pHi. Media contained 120 mM K and 3 μM nigericin. Under these conditions, pHi and pHo are the same (see above). The measurements were made after 2 min. Results represent the means ± SEM of five separate experiments, each performed in duplicate. The data were fit to a titration curve by the least-squares method. The inflection is at pHi 6.57 ± 0.09.
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7.3), or Tricine (pK, 7.8) depending upon pH (5.0–8.4). External Na was replaced by K or N-methyl-D-glucamine, and Cl by p-aminohippurate (PAH). In NH₄Cl-containing media, 3–100 mM NH₄Cl was substituted for NaCl in equimolar amounts.

CO₂-containing solutions were prepared as follows. A stock solution was made by gassing a solution that contained 5 mM HEPES with 20% CO₂/80% O₂. Sufficient HCO₃⁻ (109 mM, replacing Cl) was added to bring the pH to 7.40. Otherwise, the ionic content was that of the standard medium. This stock was diluted without exposure to air with different volumes of CO₂-free standard medium containing 5 mM HEPES, which had also been brought to pH 7.40. Thus, a series of solutions was available of different CO₂ concentrations (1–18%) and pH 7.40. The tubes were overlaid with mineral oil and capped during the cell incubation.

Reagents
DMO, TMA, N-methyl-D-glucamine, HEPES, 2-(N-morpholino)ethanesulfonic acid (MES), N-tris(hydroxymethyl) methylglycine (Tricine), PAH, and sodium PAH were purchased from Sigma Chemical Co., St. Louis, MO. 4-Acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) was obtained from ICN Biochemicals, Cleveland, OH; α-cyano-4-hydroxycinnamic acid (CHC) from Aldrich Chemical Co., Milwaukee, WI; tritiated water ([³H]H₂O), [¹⁴C]DMO, [¹⁴C]TMA, and [¹⁴C]inulin from New England Nuclear, Boston, MA; nigericin from Calbiochem-Behring Corp., La Jolla, CA. Amiloride was a gift of Dr. Edward J. Cragoe, Jr. of the Merck, Sharp & Dohme Research Institute, West Point, PA; 6-CF diacetate was a gift of Dr. John A. Thomas, University of South Dakota, Vermillion, SD; 3,3'-dipropylthiadicarbocyanine iodide was obtained from Dr. Alan Waggoner of Carnegie-Mellon University, Pittsburgh, PA.

Membrane Voltage Determination
The membrane voltage (Vₘ) was estimated from the fluorescence of the potentiometric indicator 3,3'-dipropylthiadicarbocyanine [diS-C₃(5)]. In previous work (Simchowitz et al., 1982; Simchowitz and De Weer, 1984), we found a resting Vₘ of neutrophils in nominally CO₂-free, standard medium (5 mM K, 140 mM Na, 148 mM Cl) of approximately −53 mV, which was unaffected by complete replacement of external Cl by PAH. In the present work, we determined the effect of 30 mM NH₄Cl on Vₘ in either high-(115 mM) or low-(5 mM) K media by comparing the fluorescence levels with those observed in the absence of NH₄Cl. The extracellular K concentration varied between 5 and 120 mM, Na being adjusted reciprocally. The calibration procedure relating fluorescence to Vₘ has been reported previously (Simchowitz et al., 1982; Simchowitz and De Weer, 1984). When measured 30 s or 10 min after exposure to NH₄Cl, the fluorescence of the dye cell suspensions was the same in a solution containing 30 mM NH₄Cl and 5 mM K as that in a solution that had no NH₄Cl but contained 25 mM K. This agreement of fluorescence under the two conditions was maintained in the presence of 0.1 mM ouabain. In the absence of ouabain, Vₘ was −34 mV; in its presence, −30 mV. In 30 mM NH₄Cl and 115 mM K, Vₘ was zero. The voltage-dependent fluorescence of the cell suspensions was not affected by either pHₐ or pHₗ over the range 6.4–8.4, nor did pH changes over this range alter the fluorescence of dye solutions that contained no cells.

Intracellular Ion Concentrations, Membrane Permeabilities, and Cell Water Content
The intracellular ion concentrations and membrane permeabilities of K, Na, and Cl of neutrophils suspended in nominally CO₂-free standard medium at pHₗ 7.40 have been measured previously in this laboratory (Simchowitz et al., 1982; Simchowitz and De Weer,
The values are: [K] 120, [Na] 25, and [Cl] 80 meq/liter cell water; \( P_K \) 4.4 \( \times \) 10\(^{-8} \), \( P_{Na} \) 5.0 \( \times \) 10\(^{-8} \), and \( P_{Cl} \) 4.3 \( \times \) 10\(^{-9} \) cm/s. We measured an average neutrophil diameter of 8.8 \( \mu \)m and a fractional water content of 0.77 (Simchowitz and De Weer, 1984). Thus, the cell water volume-to-surface ratio is 1.12 \( \mu \)m.

**Data Analysis**

In many instances, the time course of \( pHi \) following a change in the medium could be described by a single-exponential equation of the form:

\[
pHi = pH_{in} - (pH_{in} - pHi_{initial})\exp(-kt),
\]

where \( pH_{initial} \), \( pH_{in} \), and \( pH_{initial} \) are the \( pH \) values at, respectively, zero time, time \( t \), and after steady state had been reached, and \( k \) is the rate coefficient. Curves representing the equation were fitted to the various groups of data by the least-squares method. The initial rate of \( pHi \) recovery was derived from the expression \( k(pH_{initial} - pHi_{initial}) \). In other cases, the \( pHi \) course was nearly linear over the period of study, and the slope of the linear regression represented the rate of \( pHi \) recovery.

**TABLE I**

| Cell type     | Method | \( pH_i \) |
|---------------|--------|------------|
| Neutrophils   | 6-CF   | 7.26 ± 0.02 (n = 20) |
| Neutrophils   | DMO    | 7.24 ± 0.02 (n = 25) |
| Neutrophils   | TMA    | 6.35 ± 0.03 (n = 12) |
| Erythrocytes  | DMO    | 7.30 ± 0.02 (n = 6) |
| Erythrocytes  | TMA    | 7.32 ± 0.03 (n = 5) |

Cells were isolated from heparinized blood. The standard CO\(_2\)-free medium was buffered with 5 mM HEPES. Results represent means ± SEM of 5–25 experiments, each performed in triplicate.

**RESULTS**

**Steady State \( pH_i \)**

Table I summarizes the steady state \( pH_i \) values derived from the fluorescence of 6-CF and from the distributions of DMO and TMA. The experiments were done in the nominal absence of CO\(_2\); the solutions were buffered to 7.40 with 5 mM HEPES. The DMO- and 6-CF-derived \( pH_i \)'s, 7.24 and 7.26, were nearly the same as the DMO-derived value of 7.27 reported by Molski et al. (1980) for rabbit neutrophils under similar conditions, while the TMA-derived value, 6.35, was significantly lower. In comparison, the DMO- and TMA-derived \( pH_i \)'s of erythrocytes were nearly the same, 7.30 and 7.32, in agreement with the closely similar values measured with DMO and the weak bases ammonia and nicotine (Bone et al., 1976; Brown and Garthwaite, 1979). The \( pH \) disparity between the two methods in neutrophils is probably due to \( pH_i \) inhomogeneity, which leads to a weak base-derived \( pH \) that is always lower than the \( pH \) derived from weak acid distribution (Roos and Boron, 1981). The acid lysosomal subcompartment (\( pH \sim 5 \); Ohkuma and Poole, 1978; Styrt and Klemperer, 1982), whose
relative volume of 3–7% we estimated on the basis of published electron micro-
graphs (Bainton et al., 1971; Nichols and Bainton, 1973), is probably mainly
responsible. The more alkaline mitochondria (Schulman et al., 1979) have little
effect on overall cell pH because of their small volume (Bainton et al., 1971).
The pH of the nucleus is probably similar to that of the cytoplasm because of
the high permeability of the nuclear membrane (Franke et al., 1981). In Ehrlich
ascites tumor cells, 6-CF is evenly distributed between nucleus and cytoplasm (J.
A. Thomas et al., 1982).

On the assumption of a two-compartment system, lysosomal and extralysoso-
mal, the extralysosomal pH can be obtained from:

$$\bar{pH}_{DMO} = \log \sum_{j=1}^{n} f_j 10^{pH_j},$$

where $$\bar{pH}_{DMO}$$ is the DMO-derived pH, and $$f_j$$ is the fractional volume of the jth
compartment (Roos and Boron, 1981). Assuming a lysosomal pH of 5.2 and a
fractional lysosomal volume of 0.05, the extralysosomal pH comes to 7.26, which
is only 0.02 higher than the measured $$\bar{pH}_{DMO}$$ of 7.24. Appreciable variations in
lysosomal pH or volume have a negligible effect on the calculated extralysosomal
pH. On the other hand, the TMA-derived pH, (Roos and Boron, 1981),

$$\bar{pH}_{TMA} = -\log \sum_{j=1}^{n} f_j 10^{-pH_j},$$

is very sensitive to the size and pH of the lysosomal compartment, which makes
it less useful in estimating extralysosomal pH. For instance, the measured $$\bar{pH}_{TMA}
$$ of 6.35 yields an extralysosomal pH 6.86 at an assumed fractional lysosomal
volume of 0.05, and of 7.14 at a volume of 0.06, taking the lysosomal pH as 5.2.
In agreement with the findings of Ohkuma and Poole (1978), nigericin appar-
ently abolished the regional intracellular pH differences: in its presence, both
DMO and TMA yielded similar values (Fig. 2A).

We conclude that both 6-CF and DMO yield reasonable estimates of the
average cytoplasmic pH. If, at a resting membrane voltage of -53 mV (Seligmann
and Gallin, 1980; Simchowitz and De Weer, 1984) and a pHo of 7.40, H+ were
in thermodynamic equilibrium, a pHi of 6.54 would be expected. Our measure-
ments (Table I) indicate that, in common with most other cell types (see Roos
and Boron, 1981, for review), the cytoplasm is maintained at a more alkaline
level than would be expected from passive H+ distribution.

**Effect of pHo on pHi in the Absence of CO₂**

When neutrophils, under nominally CO₂-free conditions, were suddenly trans-
ferred from medium at pHo 7.40 (5 mM HEPES) to pHo 8.40 (5 mM Tricine),
pHi rose slowly from 7.26 ± 0.01 to 7.62 ± 0.03 (n = 3) after 1 h (Fig. 3).
Sudden reduction of pHo, from 7.40 to 6.40 (5 mM MES) resulted in a slow fall
of pHi, which reached a value of 6.84 ± 0.02 (n = 3) after 1 h. The changes in
pHi under both conditions were readily reversible (Fig. 3). Amiloride, a known
inhibitor of Na/H exchange, was without effect on the intracellular acidification.
This is in contrast to the efficacy of the drug in blocking recovery from acidification induced by CO₂ or by removal of an NH₄Cl prepulse (see below). This lack of effect is due to the absence of a driving force for net Na/H exchange, since the reduction of pH₂ to 6.40 leads to an inward-directed chemical gradient for H that is nearly equal to the inward-directed chemical gradient for Na.

**Time Course of pHᵢ upon Application and Removal of CO₂: Effect of Ionic Environment**

Fig. 4 (continuous line) illustrates the time course of DMO-derived pHᵢ in response to application and subsequent removal of 18% CO₂ (pHₐ unchanged at 7.40). At the time of the earliest measurement, 30 s after start of CO₂ exposure,

\[
\begin{align*}
\text{pH}_2 & \text{, had fallen by 0.43 to 6.80. Separate measurements with 6-CF allowed pH}_i \\
& \text{to be monitored continuously, starting 5 s after CO}_2 \text{ application. At this time, pH}_2 \text{ had already fallen by ~0.4. Between 5 and 30 s, the pH}_i \text{ rose by 0.02 at the most and reached a value that was nearly the same as the 30-s DMO measurement (see below). The latter can thus be considered to nearly represent the lowest value to which pH}_i \text{ fell. Fig. 4 shows that over the next 30 min, pH}_i \text{ recovered by 0.19 units and then remained unchanged. When, after 30 min of CO}_2 \text{ exposure, the cells were resuspended in the original CO}_2 \text{-free medium, the pH}_i \text{ rapidly rose to 7.72, that is, 0.49 units above control, and then returned toward}
\end{align*}
\]

\[
\begin{align*}
&\text{(time graph)}
\end{align*}
\]
the original value over the course of the following 15 min. The initial fall in pH is due to the entry and subsequent hydration and dissociation of molecular CO₂, while the partial pH recovery during maintained CO₂ exposure can be interpreted as being due to the removal of H⁺ equivalents from the cells. The resulting intracellular H⁺ deficit leads to a pH overshoot when CO₂ is removed. A similar pH pattern has been observed in a number of cell types (see Roos and Boron, 1981, for review).

The ionic basis of pH recovery during exposure to 18% CO₂ was studied by examining the effects of SITS and amiloride, and the replacement of Na by N-methyl-D-glucamine (Fig. 5). In the presence of Na and no drugs (curve A), the time course of pH recovery could be fitted by a single exponential with a rate coefficient of 0.148 ± 0.044 min⁻¹ (n = 3); the initial recovery rate was 0.0290 ± 0.0063 pH/min. This course was not significantly affected (rate coefficient 0.141 ± 0.045 min⁻¹; n = 3) by the disulfonic stilbene SITS (1 mM; curve B), an inhibitor of anion exchange in red blood cells (Sachs et al., 1975; Cabantchik et al., 1978) and of pH recovery in a number of other cell types (R. C. Thomas, 1976; Russell and Boron, 1976; Boron, 1977; Aickin and Thomas, 1977).

In contrast, amiloride (1 mM), an inhibitor of Na/H exchange (Benos, 1982), markedly slowed recovery to a rate of 0.00213 ± 0.00151 pH/min (n = 3; curve C). Thus, the initial recovery rate was reduced by 93%. When all extracellular Na was replaced by N-methyl-D-glucamine (pKₐ 9.6), the initial pH fall was unaffected, but the recovery rate was greatly reduced to 0.00303 ± 0.00120 pH/min, (n = 3; curve D). The true rate of recovery is, in fact, still slower: in the absence of CO₂, N-methyl-D-glucamine alkalinized the cells at a comparable rate, 0.00357 ± 0.00089 pH/min (curve E). This is probably the result of entry
of N-methyl-D-glucamine as the free base. It might be added that even greater rates of alkalinization were observed when choline or methylamine was used as a replacement ion for Na. These observations strongly suggest that pH\textsubscript{i} recovery from CO\textsubscript{2}-induced acidification is due almost entirely to an exchange of external Na for internal H.

When the cells were returned to the original CO\textsubscript{2}-free medium, the pH\textsubscript{i} promptly rose to exceed control ("overshoot"), after which it recovered toward

![Graph showing time course of DMO-derived pH\textsubscript{i}](image.png)

**Figure 5.** Time course of DMO-derived pH\textsubscript{i} during exposure of neutrophils to 18% CO\textsubscript{2} (solid lines). Results represent the means ± SEM of three separate experiments, each performed in triplicate. The pH\textsubscript{i} was 7.40 throughout. Exposure was started at zero time. (A) Standard medium without inhibitors; (B) with 1 mM SITS; (C) with 1 mM amiloride. In A, B, and C, the extracellular Na concentration was 140 mM. (D) Na-free medium (substitution by N-methyl-D-glucamine). The two dashed lines represent the time course of pH\textsubscript{i} under CO\textsubscript{2}-free conditions. (E) Na-free medium; (F) standard medium (140 mM Na) with or without 1 mM amiloride or 1 mM SITS. The pH\textsubscript{i} values under the latter three conditions were indistinguishable. Curves A and B are single exponential fits to the data (initial rates 0.0290 ± 0.0063 and 0.0296 ± 0.0072 pH/min, respectively). The remainder of the data were fit to straight lines with slopes (in pH/min) of 0.00213 ± 0.00151 (C), 0.00303 ± 0.00120 (D), 0.00357 ± 0.00089 (F), and 0.00079 ± 0.00063 (F).

The control value (see Fig. 4). We have examined the nature of this recovery from alkalinization in some detail (Fig. 6). In Cl-containing standard medium, the rate of recovery was exponential, with an initial rate of 0.0770 ± 0.0169 pH/min. This time course was not significantly affected by either amiloride (1 mM), SITS (1 mM), or complete replacement of external Na with N-methyl-D-glucamine. Curve A represents the exponential fit through the combined four sets of data. The initial rate of recovery was 0.0761 ± 0.0109 pH/min (n = 12).

The inefficacy of SITS does not necessarily rule out a role of Cl/HCO\textsubscript{3}^{-}.
exchange in the recovery process. (Small amounts of CO₂ and HCO₃ are undoubtedly present even in the nominal absence of CO₂.) We have previously reported (Simchowitz and De Weer, 1984) that high concentrations of CHC, another inhibitor of anion exchange (Halestrap, 1975), competitively inhibit Cl/Cl exchange in neutrophils, while SITS (which inhibits Cl/Cl as well as Cl/HCO₃ exchange in other cell types [Aickin and Thomas, 1977; Aickin and Brading, 1984; Russell and Boron, 1976; Hoffmann, 1982; Vaughan-Jones, 1982]) had no effect. As shown by curve B, CHC (40 mM) greatly slowed pHi recovery from post-CO₂ alkalinization: the initial rate was reduced by 91% to \(0.00671 \pm 0.00204\) pH/min (\(n = 3\)). Both the small degree of remaining acidification and the slow acidification (initial rate \(0.0104 \pm 0.0063\) pH/min; \(n = 3\)) that is induced by CHC even without prior CO₂ exposure (curve C) may be due to intracellular accumulation of some lactic acid resulting from the blocking effect of CHC on

**Figure 6.** Time course of DMO-derived pHi upon removal of 18% CO₂ (solid lines). The cells were first exposed to 18% CO₂ (pH₇.40) for 30 min, and then resuspended at zero time in CO₂-free solutions of the following compositions: (A) standard (148 mM Cl) medium with or without 1 mM amiloride or 1 mM SITS; the curve also uses results obtained in Na-free medium (substitution by N-methyl-D-glucamine); (B) standard medium with 40 mM CHC replacing 40 mM Cl; (D) Cl-free medium (substitution by PAH). The two dashed lines represent the time course of pHi after the cells, without exposure to CO₂, had been kept in standard medium for 30 min. They were then resuspended in solutions of the following compositions: (C) standard medium in which 40 mM Cl was replaced by 40 mM CHC; (E) standard medium with or without amiloride or SITS, and Cl-free medium. The pHᵢ values under these four conditions were indistinguishable. For clarity, error bars have been omitted. Results represent the mean ± SEM of three separate experiments, each performed in triplicate. Curves A and C are single-exponential fits to the data (initial rates \(0.0761 \pm 0.0109\) and \(0.0104 \pm 0.0063\) pH/min, respectively). The remainder of the data were fit to straight lines with slopes (in pH/min) of \(0.00671 \pm 0.00204\) (B), \(0.00420 \pm 0.00123\) (D), and \(0.00106 \pm 0.00089\) (E).
this acid's efflux (Halestrap, 1975; Spencer and Lehninger, 1976). This would also explain that the addition of glucose (5.6 mM), which increases lactic acid formation (Borregaard and Herlin, 1982), accentuated the pH fall. The influx of some molecular CHC (pKₐ 3.8; Halestrap, 1975) might also have contributed to the residual acidification; however, at 40 mM CHC, we measured spectrophotometrically (Halestrap and Denton, 1975) an intracellular CHC accumulation that would have reduced pHᵢ by <0.001 pH/min, taking buffering power into account.

**Figure 7.** Relationship between initial pHᵢ recovery rate and degree of alkalization after CO₂ removal. Separate aliquots of neutrophil suspensions from the same donor were exposed to 1, 2, 3, 5, 8, 12, 15, and 18% CO₂ at constant pHᵢ (7.40) for 30 min. Three different donors were used. The cells were then resuspended in CO₂-free standard media, and pHᵢ measurements (derived from DMO distribution) were made at 45 s, 2 min, and 5 min during the course of pHᵢ recovery. In order to obtain the initial recovery rates, the pHᵢ's at zero time (i.e., immediately after CO₂ removal) were needed. These values were separately obtained by resuspending different aliquots, previously exposed to various CO₂-containing solutions, in CO₂-free, Cl-free (PAH substitution) medium, which blocks recovery (see Fig. 6). The initial recovery rates were computed from Eq. 1 and plotted as a function of the pHᵢ at zero time. The data points were fit to a straight line by the least-squares method: recovery rate (in pH/min) = -0.882 + 0.123 pHᵢ. The resting pHᵢ of these cells maintained in CO₂-free standard medium (pHᵢ 7.40) was 7.21 ± 0.02.

The effect of CHC on pHᵢ recovery after CO₂ removal suggests that an exchange of internal HCO₃⁻ for external Cl⁻ may be responsible. Our observations on the effect of removing extracellular Cl lend credence to this concept. When PAH was substituted for Cl, the initial alkalization after CO₂ removal was not affected, but the recovery was dramatically slowed: its initial rate was reduced by 94% to 0.00420 ± 0.00123 pH/min (n = 3; curve D). We conclude that pHᵢ recovery from post-CO₂ alkalization is probably due to a Cl⁻/HCO₃⁻ exchange that does not require Na⁺ (see above).

In Fig. 7, we have examined the initial rate of recovery in standard (Cl-
containing) medium as a function of the magnitude of the pH\textsubscript{i} overshoot following CO\textsubscript{2} removal. The overshoot was varied by exposing the cells for 30 min to different CO\textsubscript{2} concentrations (1–18\%) at pH\textsubscript{o} 7.40. The relationship was found to be linear over the pH\textsubscript{i} range 7.3–7.8.

**Time Course of pH\textsubscript{i} upon Application and Removal of NH\textsubscript{4}Cl: Effect of Ionic Environment**

Fig. 8 (solid line) shows a representative record of the time course of the DMO-derived pH\textsubscript{i} of cells during application and subsequent removal of 30 mM NH\textsubscript{4}Cl (pH\textsubscript{o} unchanged at 7.40). At the time of the earliest measurement, 30 s after the start of NH\textsubscript{4}Cl exposure, pH\textsubscript{i} had risen by 0.39 to 7.66. Separate measurements with 6-CF, which allowed pH\textsubscript{i} to be monitored continuously, starting 5 s after NH\textsubscript{4}Cl exposure, showed that pH\textsubscript{i} had already risen by \(-0.4\) at this time. Between 5 and 30 s, pH\textsubscript{i} fell by 0.02 at most. At 30 s, pH\textsubscript{i} reached a value that was nearly the same as that measured with DMO (see below): thus, the latter nearly represents the highest value to which pH\textsubscript{i} rose. Over the next 30 min, the DMO-derived pH\textsubscript{i} fell, and almost returned to control (Fig. 8). When after 30 min, NH\textsubscript{4}Cl was removed and the cells were resuspended in the original medium, pH\textsubscript{i} fell rapidly to a value 0.63 below control, and then recovered. After another 15 min, it had nearly returned to the original value. This general pH\textsubscript{i} pattern agrees with that observed in a variety of cell types (Roos and Boron, 1981).

We shall now examine these pH\textsubscript{i} transients in more detail. The decline of pH\textsubscript{i} after the initial alkalization can be described by a single exponential with a rate
coefficient of 0.0857 ± 0.0102 min⁻¹ and an initial rate of 0.0327 ± 0.0038 pH/min (Fig. 9, curve A). In other cells, this decline ("plateau phase acidification") has been ascribed to the passive entry of NH₄⁺ ions under the influence of electrical and chemical driving forces (Boron and De Weer, 1976). In neutrophils, too, there is a sizeable inward-directed driving force on NH₄⁺. However, when this force was reduced by ~80% by depolarizing the membrane from −53 mV to 0 in 115 mM K, the initial rate of pHᵢ recovery was reduced by only 32%, from 0.0327 ± 0.0038 to 0.0222 ± 0.0045 pH/min (curve B). If recovery were due solely to passive NH₄⁺ influx, depolarization should have reduced influx, and thus the recovery rate, by a factor of 5, assuming constant-field behavior and voltage-independent NH₄⁺ permeability. A large part of recovery from NH₄⁺Cl-induced alkalinization seems, therefore, to be due to mechanisms other than electrodiffusion. These mechanisms might include: (a) NH₄⁺ ions gaining entry

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**FIGURE 9.** Time course of DMO-derived pHᵢ during exposure of neutrophils to 30 mM NH₄⁺ (solid lines). Results represent the means ± SEM of three to four separate experiments, each performed in triplicate. The pHᵢ was 7.40 throughout. Exposure was started at zero time. (A) Medium containing 148 mM Cl and 5 mM K. The curve also uses results obtained in the presence of 0.1 mM ouabain and 1 mM amiloride; (B) 148 mM Cl, 115 mM K (C, D, and F were performed in 5 mM K media); (C) Cl-free medium (substitution by PAH); (D) 40 mM CHCl replaced 40 mM Cl; (E) 148 mM Cl, 1 mM HCO₃⁻/0.2% CO₂. The two dashed lines represent the time course of pHᵢ in the absence of NH₄⁺. (F) 40 mM CHCl replacing 40 mM Cl; (G) under all other conditions. Since their pHᵢ's were indistinguishable, each of the points shown is the average of various data points. Curve A–F are single-exponential fits to the data. The initial rates (in pH/min) were: 0.0327 ± 0.0046 (A), 0.0222 ± 0.0050 (B), 0.00673 ± 0.00056 (C), 0.0128 ± 0.0008 (D), 0.00694 ± 0.00351 (E), and 0.0658 ± 0.0094 (F), respectively. The data of curve G were fit to a straight line with a slope of 0.00033 ± 0.00003 pH/min.
through the Na/K pump; this can be ruled out because ouabain (0.1 mM) had no effect on recovery (Fig. 9); (b) exchange of internal Na for external H or uptake of NH₄⁺ through the Na/H exchanger; both are unlikely because recovery was not affected by 1 mM amiloride; (c) exchange of internal HCO₃⁻ for external Cl. We demonstrated above that such a mechanism is probably responsible for the pHᵢ recovery from alkalinization following CO₂ removal. Three lines of evidence indicate that, also during recovery from NH₄Cl-induced alkalinization, Cl/HCO₃ exchange plays an important role. (i) Replacing external Cl by PAH reduced the initial rate of acidification by 80%, from 0.0327 to 0.00673 ± 0.00056 pH/min (Fig. 9, curve C). (ii) Whereas SITS had no effect, CHC (40 mM) reduced the initial recovery rate by 61%, to 0.0128 ± 0.0008 pH/min (curve D). The residual recovery might be due in part to some degree of NH₄⁺ entry by electrodiffusion, and to the blocking effect of CHC on lactic acid efflux from the cells, which was discussed above (see curve E). (iii) When 1 mM HCO₃ (0.2% CO₂, constant pHᵢ) was added to the medium, the initial rate of recovery from NH₄Cl-induced alkalinization doubled to 0.0658 ± 0.0094 pH/min (curve F). Again, CHC reduced this rate by ~85% (data not shown), whereas SITS was ineffective. The small amounts of metabolically produced CO₂ (HCO₃⁻) are apparently sufficient for the functioning of the Cl/HCO₃ exchanger even in the nominal absence of CO₂.

When, after 30 min of exposure to NH₄Cl, the cells were returned to the original NH₄Cl-free solution, the earliest pHᵢ measured, 30 s after the solution change, was 6.52 ± 0.03 (n = 3), which is considerably lower than the resting pHᵢ (7.18) (Fig. 10, curve A). The pHᵢ then recovered rapidly along an exponential course. The pHᵢ immediately after NH₄Cl removal must have been ~6.45, and the initial recovery rate, 0.134 ± 0.023 pH/min. After ~15 min, pHᵢ reached a near-normal level. Removal of Na (substitution by N-methyl-D-glucamine, curve B) or addition of 1 mM amiloride (curve C) greatly slowed recovery to 0.0140 ± 0.0042 and 0.0119 ± 0.0031 pH/min, respectively, an inhibition of ~90%. CHC (40 mM), which, as stated, inhibits anion exchange, had no appreciable effect on the initial rate of pHᵢ recovery (0.115 ± 0.016 pH/min, curve D). These data strongly suggest that Na/H exchange was mainly responsible for the recovery from acidification after an NH₄Cl prepulse.

By varying the NH₄Cl concentration between 3 and 40 mM (exposure period constant at 30 min), the pHᵢ upon NH₄Cl removal could be varied. The lower this pHᵢ, the greater was the initial rate of recovery. The relationship was found to be linear over the pHᵢ range 6.3–7.2 (Fig. 11). The recovery rate extrapolates to zero at pHᵢ 7.25, the normal resting pHᵢ.

**Effect of CO₂ and HCO₃ Concentrations on Steady State pHᵢ.**

The effect of various CO₂ concentrations on pHᵢ was examined under two conditions: (a) at constant pHᵢ, i.e., by varying [HCO₃]₀ and percent CO₂ proportionally, and (b) at constant [HCO₃]₀ (Table II).

(a) Cells were exposed to media containing 5, 10, or 15% CO₂ and 27, 54, or 81 mM [HCO₃]₀, respectively, at a constant pHᵢ of 7.40. When the cells were exposed to the CO₂-containing media, the pHᵢ fell within 30 s from 7.20 to 7.03, 6.90, and 6.81, respectively (Table II, A), and then slowly recovered. After 1 h
(Table II, B), the pH$_i$ had reached new steady values of 7.11, 7.06, and 7.01. These values represent the balance between acid extrusion (Na/H exchange; see Fig. 5) and intracellular acidification due, in part, to passive HCO$_3$ efflux.

(b) In a second series, cells were exposed to 5, 10, and 15% CO$_2$ at constant [HCO$_3$]$_e$ = 27 mM (Table II, results indicated by asterisks). The corresponding

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{Time course of DMO-derived pH$_i$ upon removal of 30 mM NH$_4$Cl (solid lines). Results represent the means ± SEM of three separate experiments, each performed in triplicate. The pH$_o$ was 7.40 throughout the experiment. The cells were first exposed for 30 min to 30 mM NH$_4$Cl and then resuspended at zero time in NH$_4$Cl-free solutions of the following compositions: (A) standard (140 mM Na) medium; (B) Na-free medium (substitution by N-methyl-D-glucamine); (C) standard medium with 1 mM amiloride; (D) 40 mM CHC replacing 40 mM Cl. The two dashed lines represent the time course of pH$_i$ of cells not exposed to NH$_4$Cl, but kept in standard medium for 30 min. They were then resuspended in solutions of the following compositions: (E) standard medium with or without amiloride; (F) Na-free medium. Curve A is a single-exponential fit to the data (initial rate 0.134 ± 0.023 pH/min. Curve D was empirically fit to a third-degree polynomial; the initial rate was 0.115 ± 0.016 pH/min. The remainder of the data were fit to straight lines with slopes (in pH/min) of 0.0184 ± 0.0029 (B), 0.0119 ± 0.0051 (C), 0.00014 ± 0.00140 (E), and 0.00436 ± 0.00131 (F). For clarity, we have omitted the curve for cells that had not been exposed to NH$_4$Cl, but were suspended in 40 mM CHC. The data showed a slow acidification with initial rate of 0.0105 ± 0.0039 pH/min, similar to curves C and E of Figs. 6 and 9, respectively. This slight acidifying effect of CHC (discussed in the text) probably explains the small difference between curves A and D.

pH$_o$'s were 7.40, 7.10, and 6.92. We compared the earliest (30 s) pH$_i$ values in these studies (Table II, A) with those obtained with CO$_2$-free solutions of the same three pH$_o$'s (buffered with 5 mM HEPES). As expected, in the presence of CO$_2$, the early falls in pH$_i$ were more striking and largely independent of pH$_o$: 10% CO$_2$ reduced pH$_i$ by ~0.30 both at pH$_o$ 7.40 (from 7.20 to 6.90) and at pH$_o$ 7.10 (from 7.17 to 6.86); 15% CO$_2$ reduced pH$_i$ by 0.39 both at pH$_o$ 7.40
As in the case of these early readings, the steady state pH's (after 1 h exposure) were also lower with CO₂ than without (Table II, B). In the presence of 10% CO₂, pH was 6.97 at [HCO₃]₀ = 27 mM (pH₀ 7.10) and 7.06 at [HCO₃]₀ = 54 mM (pH₀ 7.40). A similar relationship was observed with 15% CO₂ (see Table II, B).

Both Ahmed and Baron (1971) and Levin et al. (1976) examined the steady state pH, levels of mixed human leukocytes (~65% neutrophils, 30% lymphocytes, 5% monocytes) in response to changes in P_CO₂ and [HCO₃]₀. At 6% CO₂ (pH₀ 7.40), steady state pH measured with DMO was 7.10, nearly the same as our value (7.11). At a constant pH₀ of 7.39, pH fell from ~7.31 to ~7.07 when CO₂ was raised from 2 to 10%.

**Buffering Power**

The intrinsic buffering power, defined as \(-\Delta[HCO₃]/\Delta pH_i\) or \(\Delta[NH₄]/\Delta pH_i\), should be derived from the decrease or increase of pH immediately upon

![Figure 11. Relationship between initial pH, recovery rate and degree of acidification after NH₄Cl removal. Separate aliquots of neutrophil suspensions from the same donor were exposed to 3, 6, 10, 15, 20, 25, 30, or 40 mM NH₄Cl at constant pH₀ (7.40) for 30 min. Three different donors were used. The cells were then resuspended in NH₄Cl-free standard medium (pH₀ 7.40) and pH measurements (derived from DMO distribution) were made at 45 s, 2 min, and 5 min during the course of pHᵢ recovery. In order to obtain the initial recovery rates, the pHᵢ's at zero time (i.e., immediately after NH₄Cl removal) were needed. These values were separately obtained by resuspending different aliquots, previously exposed to various NH₄Cl-containing solutions, in NH₄Cl-free standard medium with 1 mM amiloride, which blocks recovery (see Fig. 10). The initial recovery rates were computed from Eq. 1 and plotted as a function of the pHᵢ at zero time. The data points were fit to a straight line by the least-squares method: recovery rate (in pH/min) = 1.125 - 0.155 pHᵢ. The resting pHᵢ of these cells maintained in NH₄Cl-free standard medium (pH₀ 7.40) was 7.24 ± 0.03.
exposure of the cells to CO₂ or NH₄Cl, that is, before any recovery has taken place. Since such early pHᵢ values could not be obtained experimentally, they were computed by back-extrapolation of the time course of pHᵢ measured after 30 s exposure to 1–18% CO₂ or 3–100 mM NH₄Cl, as described in the legends to Tables III and IV. The values for intrinsic buffering power derived from 6-CF fluorescence and from DMO distribution are shown to be in good agreement. The relationship between buffering power and pHᵢ could be described by a straight line for which the least-squares regression is given by: $β_{\text{intrinsic}} = 137.9 - 11.9$ pHᵢ, where $β_{\text{intrinsic}}$ is expressed in millimolar per pH unit. Thus, the buffering power under standard CO₂-free conditions (pHᵢ 7.40, pH 7.25) is ~50

| pHᵢ | 5 | 10 | 15 |
|-----|---|----|----|
| 7.40 | 7.29±0.03 | 7.03±0.01* | 6.90±0.02 | 6.81±0.02 |
| 7.10 | 7.17±0.02 | 6.86±0.02* | 6.92±0.03 |
| 6.92 | 7.19±0.03 |

| pHᵢ | 0 | 5 | 10 | 15 |
|-----|---|---|----|----|
| 7.40 | 7.19±0.03 | 7.11±0.05* | 7.06±0.02 | 7.01±0.02 |
| 7.10 | 7.10±0.02 | 6.97±0.05* |
| 6.92 | 7.05±0.02 | 6.89±0.05* |

Neutrophils were exposed to 5, 10, and 15% CO₂ concentrations under two incubation conditions: (a) at constant pHᵢ, 7.40, [HCO₃]ᵢ being varied proportionally with the percent CO₂ and (b) at constant [HCO₃]ᵢ = 27 mM (asterisks), pHᵢ being allowed to fall (7.40, 7.10, and 6.92, respectively) as percent CO₂ was increased. Controls were suspended in CO₂-free standard media, prepared at corresponding pHᵢ's using 5 mM HEPES buffer. At 30 s and at 1 h, by which time the pHᵢ of all cells exposed to CO₂ had reached a steady state, pHᵢ was measured by DMO distribution. Results represent the means ± SEM of three separate experiments, each performed in triplicate.

mM/pHᵢ in reasonable agreement with previously measured values in mammalian cells (see Table 13 of Roos and Boron, 1981). In crab muscle (Aickin and Thomas, 1975) and in rat thymic lymphocytes (Grinstein et al., 1984), $β_{\text{intrinsic}}$ seems to be independent of pHᵢ, whereas in cat skeletal muscle (Furusawa and Kerridge, 1927) and barnacle muscle (Boron, 1977; Boron et al., 1979), it increases as pHᵢ is reduced, as is the case in human neutrophils.

The buffering powers during and after CO₂ exposure were used to predict the post-CO₂ overshoot from the extent of recovery during the CO₂ pulse. The calculated pHᵢ values agreed well with those experimentally obtained. For instance, the pHᵢ rise of 0.19 during the 30-min exposure to 18% CO₂ (see Fig. 5) corresponds to removal of 0.19 (55.4 + 76.4) = 25.0 mmol of H⁺/liter cell water (average intrinsic buffering power 55.4 mM/pH; buffering due to CO₂, 2.3[HCO₃]ᵢ = 76.4 mM/pH). This should result in an overshoot of $25.0/50.1$
TABLE III
Intrinsic Buffering Power and pH's Measured 30 s after Exposure to 1–18% CO₂ (pH, 7.40)

| Percent CO₂ | pHᵢ | βᵢntrinsic |
|------------|-----|------------|
|            | 6-CF | DMO        | 6-CF | DMO    |
|            | mM/pH |           |       |        |
| 0          | 7.24±0.03 (4)* | 7.21±0.02 (14) | —     | —      |
| 1          | 7.19±0.02 (4)  | 7.14±0.03 (3)  | 55.9±6.9 | 49.8±5.7 |
| 2          | 7.13±0.04 (4)  | 7.09±0.02 (3)  | 53.4±7.8 | 48.5±3.0 |
| 5          | 7.05±0.04 (4)  | 7.00±0.01 (3)  | 55.2±5.5 | 54.1±7.5 |
| 10         | 6.95±0.04 (4)  | 6.90±0.02 (3)  | 66.5±5.2 | 54.2±7.6 |
| 15         | 6.86±0.02 (4)  | 6.81±0.01 (3)  | 59.1±6.5 | 51.7±1.2 |
| 18         | 6.82±0.01 (4)  | 6.80±0.02 (11) | 58.9±4.9 | 56.7±5.5 |

* Means ± SEM (number of donors).

For each CO₂ concentration and each donor, the intrinsic buffering power (Δ[HCO₃⁻]/ΔpH) was obtained from the difference between pHᵢ under CO₂-free conditions and the initial pHᵢ immediately upon CO₂ exposure. The latter was derived by back extrapolation of the measured pHᵢ data. In the case of 18% CO₂, in which the course was followed between 30 s and 30 min, the initial pHᵢ was derived from the exponential curve fitted to this course (see Fig. 5 and Eq. 1), and was 0.014 less than the 30-s value. With 1, 2, 5, 10, and 15% CO₂, in which only a single 30-s measurement was made, the initial pHᵢ was assumed to be lower than this measurement by an amount proportional to the pHᵢ fall at 30 s (0.002, 0.004, 0.007, 0.010, and 0.013, respectively), the proportionality factor being derived from the measurements at 18% CO₂. The initial [HCO₃⁻] was then derived using a CO₂ solubility coefficient of 0.0326 mol/liter-mm Hg (Harned and Davis, 1943) and an apparent first pK of carbonic acid of 6.03 (Harned and Bonner, 1945). In the nominal absence of CO₂, [HCO₃⁻] was assumed to be zero.

TABLE IV
Intrinsic Buffering Power and pH's Measured 30 s after Exposure to 3–100 mM NH₄Cl (pH, 7.40)

| NH₄Cl (mM) | pHᵢ | βᵢntrinsic |
|------------|-----|------------|
|            | 6-CF | DMO        | 6-CF | DMO    |
|            | mM/pH |           |       |        |
| 0          | 7.26±0.03 (3)* | 7.25±0.03 (13) | —     | —      |
| 3          | 7.53±0.02 (3)  | 7.28±0.04 (5)  | 48.9±6.6 | 52.6±3.0 |
| 10         | 7.49±0.03 (3)  | 7.39±0.03 (5)  | 51.6±7.1 | 50.5±1.7 |
| 30         | 7.62±0.04 (5)  | 7.62±0.01 (13) | 44.8±5.7 | 45.0±1.7 |
| 60         | —     | 7.78±0.02 (3) | —     | 42.6±3.5 |
| 100        | 7.89±0.02 (5)  | 7.88±0.04 (3)  | 46.1±4.5 | 45.5±6.5 |

* Means ± SEM (number of donors).

For each NH₄Cl concentration and each donor, the intrinsic buffering power (Δ[NH₄⁺]/ΔpH) was obtained from the difference between pHᵢ under NH₄Cl-free conditions and the initial pHᵢ immediately upon NH₄Cl exposure. The latter was derived by back extrapolation of the pHᵢ course. In the case of 30 mM NH₄Cl, in which the time course was followed between 30 s and 45 min, the initial pHᵢ was derived from the exponential fit (see Fig. 9 and Eq. 1), and was 0.016 higher than the 30-s value. With 3, 10, 60, and 100 mM NH₄Cl, in which only a single pHᵢ measurement was made at 30 s, the initial pHᵢ was assumed to be higher than this measurement by an amount proportional to the pHᵢ rise at 30 s (0.003, 0.007, 0.023, and 0.026, respectively), the proportionality factor being derived from the measurements at 30 mM NH₄Cl. The initial [NH₄⁺] was then derived using an apparent pKᵢ of 8.92 for NH₂ (Everett and Wynne-Jones, 1958).
= 0.50 pH unit (where 50.1 is the average intrinsic buffering power during the pH transient after CO₂ removal), in agreement with the observed value of 0.54 (Fig. 6). In a comparable way, the pH fall of 0.36 during the 30-min exposure to 30 mM NH₄Cl (Fig. 9) should produce a pH undershoot of 0.74 upon its removal; the observed fall was 0.73 (Fig. 10).

At the same pH, (for example, 6.80), the rate of pH recovery (alkalinization) after NH₄Cl withdrawal (Fig. 11) was found to be about twice (0.071 pH/min) that during CO₂ exposure (0.029 pH/min, Fig. 5), even though presumably both are due to Na/H exchange. This difference can be ascribed to the difference in buffering power, which was 57.0 + 56.6 = 113.6 mM/pH during CO₂ exposure, and only 57.0 mM/pH after NH₄Cl withdrawal. Similarly, at a selected pH of 7.65, the recovery (acidification) after the overshoot following CO₂ removal was almost twice as fast (0.059 pH/min) as that during NH₄Cl application (0.033 pH/min) (Figs. 7 and 9), even though both are due mainly to Cl/HCO₃ exchange. Again, buffering power is responsible for this difference: 46.9 mM/pH after CO₂ removal and 46.9 + 37.7 = 84.6 mM/pH during NH₄Cl exposure.

**DISCUSSION**

Neutrophils play a vital role in the normal host defense mechanisms against microorganisms. Through the secretion of degradative enzymes, free radicals, and other mediators of inflammation, they contribute to tissue damage in a variety of diseases (Henson et al., 1978; Weissmann et al., 1979). Several neutrophil functions, such as chemotaxis, phagocytosis, and mediator release, have been proposed to be regulated by intracellular pH. The present work represents a systematic study of the factors that determine pH of human neutrophils under resting conditions. It should provide a background for the study of pH-dependent functions of stimulated cells.

We found that at pH 7.40, the pH of neutrophils was ~7.25 (Table 1). Since the membrane voltage of these cells is about -53 mV (Simchowitz and De Weer, 1984), pH is considerably higher than would be expected from electrochemical equilibrium, as with most other cells (Roos and Boron, 1981). Upon exposing the neutrophils to an acid load, pH, after the initial fall, returned toward its resting value. At least 90% of this recovery could be ascribed to a Na/H exchange. On the other hand, when an alkaline load was imposed on the cells, pH recovery, after the initial rise, was mainly accomplished through a Cl/HCO₃ exchange.

**Recovery from Acid Loads**

When acidification was accomplished by exposing the cells to 5–18% CO₂ (pH₀ unchanged at 7.40), recovery was not complete: the new steady state pH value was always lower than that before CO₂ exposure (Fig. 5 and Table II). On the other hand, when the cells were acidified by prepulsing with 30 mM NH₄Cl (pH₀ again unchanged at 7.40), pH completely recovered (Fig. 10). This difference in pH behavior may be attributed, in part, to the additional acid load imposed upon the cells during CO₂ exposure caused by the outward leak of HCO₃⁻ down its electrochemical gradient. We also examined the rate of pH recovery as a function of the degree of acidification. As in other systems (Boron et al., 1979; Aronson et al., 1982; Moolenaar et al., 1983; Grinstein et al., 1984), the rate
rose linearly with decreasing pH
(6.30–7.25; Fig. 11). Amiloride (1 mM) or external Na removal reduced the recovery rate by ~90%, irrespective of whether the acidification had been accomplished by exposure to CO₂ or by removal of NH₄Cl. It is, therefore, highly likely that recovery from an acid load is predominantly achieved through an exchange of external Na for internal H. CHC (40 mM), an inhibitor of anion exchange in neutrophils (Simchowitz and De Weer, 1984), had little effect on recovery rate, which rules out a significant role of Cl/HCO₃ exchange. SITS (1 mM), which has no effect on Cl fluxes in these cells (Simchowitz and De Weer, 1984), did not affect recovery. It is of interest that in mouse soleus muscle (Aickin and Thomas, 1977), Cl/HCO₃ exchange, as well as Na/H exchange, is involved in pH recovery from acidification, whereas in neutrophils, anion exchange seems to play a significant role only in recovery from an alkaline load, as will be discussed below.

A Na/H exchange mechanism has been implicated in the recovery from acidification in several mammalian cell types: rat lymphocytes (Grinstein et al., 1984), mouse soleus muscle fibers (Aickin and Thomas, 1977), sheep cardiac Purkinje fibers (Deitmer and Ellis, 1980), hamster lung fibroblasts (L'Allemain et al., 1984), and cultured human epidermoid carcinoma cells (Rothenberg et al., 1983). A similar mechanism plays a role in other cells (Moody, 1981; Boron and Boulpaep, 1983; Weinman and Reuss, 1984; Abercrombie et al., 1983).

Recovery from Alkaline Loads

We also examined pH recovery from alkalization achieved either during exposure to NH₄Cl or after removal of a CO₂ pulse. In both instances, the elevated pH returned toward near-normal values in 15–30 min. Amiloride had no effect on recovery from alkalization by either method (Figs. 6 and 9), in marked contrast to its efficacy in blocking recovery from acidification (Figs. 5 and 10). Either total replacement of extracellular Cl or application of CHC (40 mM) greatly inhibited recovery (Figs. 6 and 9). SITS and external Na removal were ineffective. The recovery rate from NH₄Cl-induced alkalization doubled when 1 mM HCO₃ was added to the medium (Fig. 9). These observations suggest that recovery from alkalization is mainly due to an exchange of internal HCO₃ for external Cl.

It thus appears that human neutrophils possess two separate regulatory mechanisms for pH homeostasis. One, a Na/H exchange, returns pH to a more normal value after imposed acidification. A second, a Cl/HCO₃ exchange, comes into play after imposed alkalization. A similar differentiation of function of the two exchange processes has been demonstrated in sheep Purkinje fibers (Deitmer and Ellis, 1980; Vaughan-Jones, 1982; Vanheel et al., 1984). However, in this tissue, SITS effectively inhibits Cl/HCO₃ exchange, whereas this compound is ineffective in neutrophils.

The question arises as to why the pH of resting neutrophils in nominally CO₂-free medium (pH₀ 7.40) is ~7.25. Considering the presence both of Na/H and Cl/HCO₃ exchangers, and assuming that both types are electroneutral, the total transmembrane chemical potential difference of the three ions, Na⁺, H⁺, and Cl⁻, is given by:
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\[
RT \ln \frac{[\text{Na}_i]}{[\text{Na}_o]} \frac{[\text{H}_i]}{[\text{H}_o]} - RT \ln \frac{[\text{Cl}_i]}{[\text{Cl}_o]} \frac{[\text{H}_i]}{[\text{H}_o]} 
\]

or

\[
RT \ln \frac{[\text{Na}_i]}{[\text{Na}_o]} \frac{[\text{H}_i]^2}{[\text{H}_o]^2} \frac{[\text{Cl}_i]}{[\text{Cl}_o]},
\]

where the H distribution represents the inverse of the HCO_3 distribution. Given the internal and external concentrations of Na and Cl, and the extracellular pH (see Methods), the equilibrium value for [H_i] can be obtained by setting this expression to zero. The [H_i] thus obtained comes to \(2.29 \times 10^{-8}\) M, which corresponds to a pH_i of 7.64. This value is quite different from that actually observed (7.25). Therefore, other, unknown mechanisms are responsible for maintaining steady state pH_i.

In general, recovery of pH_i from alkaline loads has not been extensively studied. Passive ion fluxes (Aickin and Thomas, 1977) and metabolic acid production (Boron et al., 1979) have been proposed as possible mechanisms. The pH_i recovery that occurs during NH_4Cl-induced alkalinization in squid giant axon (Boron and De Weer, 1976), snail neuron (R. C. Thomas, 1974), and giant barnacle muscle fiber (Boron et al., 1979), has generally been ascribed to the entry of NH_4^+ down its electrochemical gradient. In neutrophils, the principal part of the recovery during exposure to 30 mM NH_4Cl seems to be due to a Cl-/HCO_3 exchange, as discussed above. However, entry of NH_4^+ still may play some role, since a definite though modest reduction in the rate of recovery did occur when the driving force on NH_4^+ was reduced by depolarizing the cells. We have estimated the NH_4^+ permeability (P_{NH_4}) of neutrophils, which may be responsible for the residual rate of recovery, by two methods.

(a) On the assumption that no electrogenic exchange is taking place, P_{NH_4} was derived from the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

\[
V_m = \frac{RT}{F} \ln \frac{P_{K}[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_i + P_{NH_4}[NH_4]_i}{P_{K}[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_o + P_{NH_4}[NH_4]_o},
\]

where R, T, and F have their usual thermodynamic meanings, and P represents the permeability coefficients. The Na/K pump was inhibited by 0.1 mM ouabain. In the presence of ouabain, the membrane voltage during the pulse of 30 mM NH_4Cl measured with diS-C_6(5) (see Methods) was \(-30\) mV. Intracellular K, Na, and Cl concentrations were taken as 120, 25, and 80 mM, respectively (Simchowitz et al., 1982; Simchowitz and De Weer, 1984), while the respective extracellular concentrations were 5, 110, and 148 mM. On the assumption of concentration equality of NH_3 across the membrane, intracellular NH_4^+ concentration was 16.4 mM (pK_a 8.92 [Everett and Wynne-Jones, 1938]). The K:Na:Cl permeability ratio was taken as 10:1:1, the P_K as \(4.4 \times 10^{-8}\) cm/s (Simchowitz et al., 1982; Simchowitz and De Weer, 1984). The equation yields a value for NH_4^+ permeability of \(3.8 \times 10^{-8}\) cm/s.

(b) It was assumed that when the cells were depolarized from \(-34\) to 0 mV (in 5 and 115 mM K media), the reduction in pH_i recovery rate during exposure to
30 mM NH₄Cl was due entirely to reduction of NH₄⁺ entry by electrodiffusion. The difference in NH₄⁺ entry per unit surface area, ΔJ_{NH₄⁺}, between the two conditions is given by:

$$\Delta J_{NH₄⁺} = (\Delta [dPH_i/dt]) - (V/A) \cdot \beta,$$

where Δ[dPH_i/dt] is the difference in acidification rates at the two voltages (0.0105 pH/min), V/A is the ratio of cell water volume over cell surface (1.12 μm), and β is the total intracellular buffering power at the start of recovery (84.6 mM/pH). The assumption was made that NH₃ remains equilibrated throughout recovery. The value for ΔJ_{NH₄⁺} thus obtained was set equal to the difference in net NH₄⁺ influx at -34 and 0 mV, each expressed by the constant-field equation (Goldman, 1943; Hodgkin and Horowicz, 1959):

$$J_{NH₄⁺} = \frac{P_{NH₄⁺} V_m F [NH₄⁺_i] - [NH₄⁺] \exp(V_m F/RT)}{RT} \cdot \frac{1}{1 - \exp(V_m F/RT)}.$$

It was assumed that P_{NH₄⁺} is voltage independent. The calculation yielded a value for P_{NH₄⁺} of 5.7 × 10⁻⁸ cm/s, which is in reasonable agreement with the value derived by the first method. It is of interest that these values are comparable to the P_K of 4.4 × 10⁻⁸ cm/s obtained by us from isotopic flux measurements (Simchowitz et al., 1982; Simchowitz and De Weer, 1984). This agreement would be expected because of the similarities of the crystal radii and mobilities of the two ions.

The results on pHᵢ regulation in resting human neutrophils given in the present work should be useful in providing the background necessary for evaluating the role of intracellular pH of activated cells.

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