Regulation of Intracellular pH in Human Neutrophils

LOUIS SIMCHOWITZ and ALBERT ROOS

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

ABSTRACT The intracellular pH (pH_i) of isolated human peripheral blood neutrophils was measured from the fluorescence of 6-carboxyfluorescein (6-CF) and from the equilibrium distribution of [^{14}C]5,5-dimethyloxazolidine-2,4-dione (DMO). At an extracellular pH (pH_o) of 7.40 in nominally CO_2-free medium, the steady state pH_i using either indicator was ~7.25. When pH_o was suddenly raised from 7.40 to 8.40 in the nominal absence of CO_2, pH_i slowly rose by ~0.35 during the subsequent hour. A change of similar magnitude in the opposite direction occurred when pH_o was reduced to 6.40. Both changes were reversible. Intrinsic intracellular buffering power, determined by using graded pulses of CO_2 or NH_4Cl, was ~50 mM/pH over the pH_i range of 6.8–7.9. The course of pH_i 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% CO_2 or by prepulsing with 30 mM NH_4Cl, while pH_o was maintained at 7.40. In both instances, pH_i (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 α-cyano-4-hydroxycinnamate (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 NH_4Cl or by prepulsing with 18% CO_2, both at a constant pH_o 7.40. In both instances, pH_i, 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 NH_4Cl-induced alkalinization was enhanced twofold by adding 1 mM HCO_3/0.2% CO_2 to the medium (pH_o 7.40). When the membrane was depolarized from −53 to 0 mV in 115 mM K, the recovery during NH_4Cl exposure was reduced by ~30%, whereas the inward driving force on NH_4^+ was reduced by ~80%. Apparently, the entry of NH_4^+ by electrodiffusion plays only a minor role in the recovery. These results make it

Address reprint requests to Dr. Louis Simchowitz, John Cochran VA Medical Center, 151/JC, 915 North Grand Ave., St. Louis, MO 63125.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/85/03/0443/28$1.00 443
Volume 85 March 1985 443–470

likely that Cl/HCO_3 exchange is chiefly responsible for pH_i recovery from alkalinization. In conclusion, human neutrophils possess two separate regulatory mechanisms: a Na/H exchange that restores pH_i after an acid load and a Cl/HCO_3 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 (pH_i) 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, pH_i 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 pH_i. Among these functions are chemotaxis, phagocytosis, and secretion of enzymes and free radicals. However, little is known about the factors governing the pH_i of resting neutrophils. The purpose of the present work is to examine these factors.

We have measured the steady state pH_i of human neutrophils under various conditions. We also have examined their pH_i responses to intracellular acid and alkaline loads and the ionic transport mechanisms on which these responses are based. After either load, pH_i 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 alkalinization, pH_i recovery required Cl, was enhanced by HCO_3, 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/HCO_3 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 (pH_o 7.40) containing (mM): 140 NaCl, 5 KCl, 1.0 $CaCl_2$, 0.5 $MgCl_2$, 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.

pH_i Measurements

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

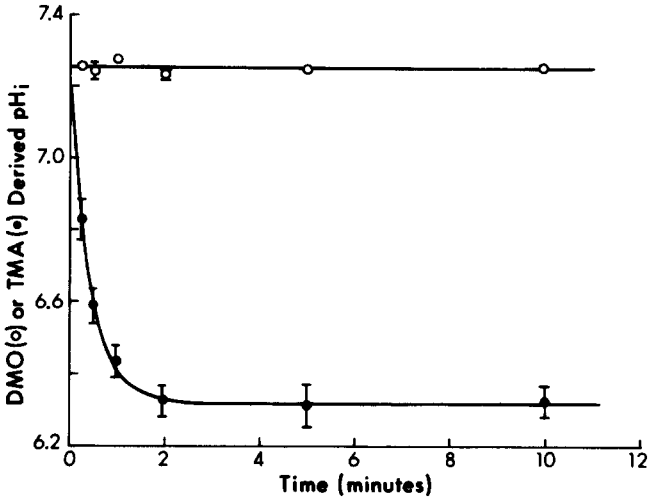

FIGURE 1. Time required for DMO and TMA to equilibrate between medium and cell water. Neutrophils were suspended in nominally CO_2-free media (5 mM HEPES buffer, pH_o 7.40). At zero time, [^{14}C]DMO or [^{14}C]TMA was added. At stated times, the cells were pelleted through oil by rapid centrifugation and counted for radioactivity. The first measurement was done at 15 s. Results represent the mean ± SEM (where larger than the symbol) of three experiments, each performed in triplicate. The DMO data were fit to a straight line, the TMA data to a single exponential. A steady DMO distribution was reached by 15 s ($\overline{pH}_{DMO}$ 7.25 ± 0.01), whereas it took ~2 min for the TMA distribution to reach a steady value ($\overline{pH}_{TMA}$ 6.32 ± 0.03).

external concentration of the neutral form: the pH_o (7.40) in these studies was more than two units lower than the pK_a' of TMA.

Samples of the neutrophil suspension (8–12 × 10^6 cells/ml), containing either [^{14}C]-DMO (1.0 μCi/ml) or [^{14}C]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_i by itself as assessed by 6-CF fluorescence. The suspension also contained [^3H]H_2O (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

~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 [^{3}H]H_2O and [^{14}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 μM 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 pH_o values (5.0–8.4) in the presence of 120 mM K and 3 μM nigericin in the external solution. Nigericin, which promotes electroneutral K/H exchange (Pressman, 1969), should lead to equality of pH_i and 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 pH_i that were in excellent agreement with the prevailing pH_o (Fig. 2*A*). The relationship between the fluorescence of 6-CF and pH_i in high-K/nigericin media of different pH (5.0–8.4) is sigmoidal (Fig. 2*B*) with an inflection at about pH_i 6.6, which probably corresponds to the apparent pK_a'. J. A. Thomas et al. (1979) measured an apparent pK_a' of 6.7 at 25°C.

(*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_i 7.0 and 8.0, namely 0.0113 ± 0.0004 min^{-1} (n = 6). At pH_i 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_a' 6.0), HEPES (pK_a'

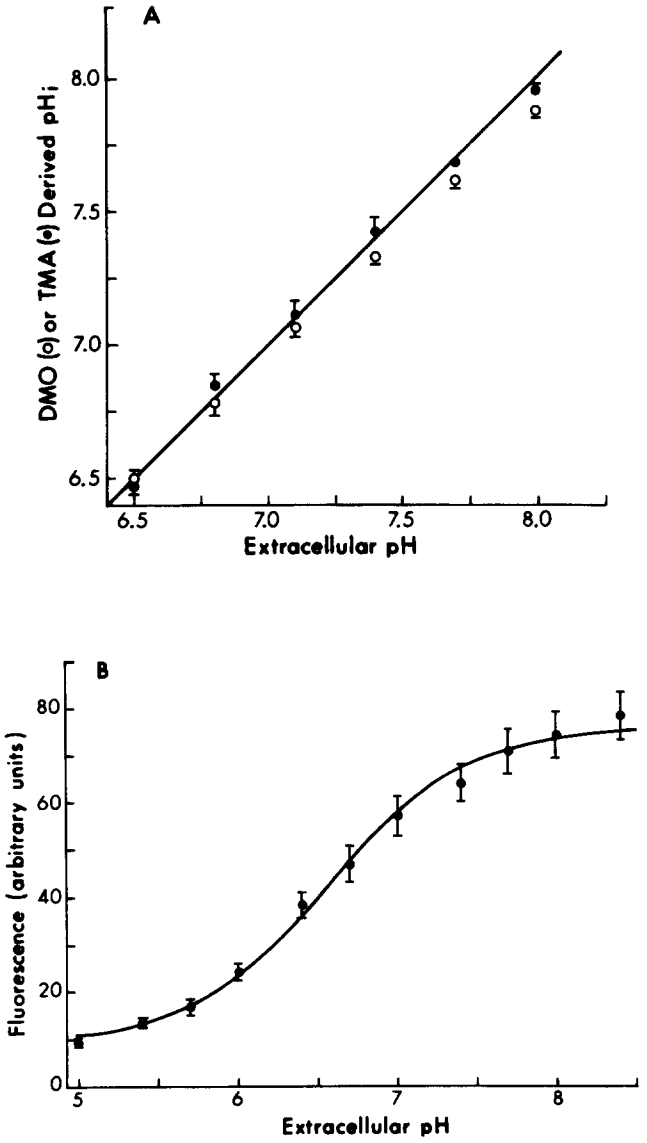

FIGURE 2. Calibration of 6-CF. (*A*) Comparison between pH_o and DMO- or TMA-derived pH_i of neutrophils exposed to 120 mM K and 3 μM nigericin. Results, obtained 2 min after exposure of cells to high-K nigericin media, represent the means ± SEM of five separate experiments, each performed in triplicate. Over the pH_o range 6.5–8.0, the pH_i derived by either method closely resembled pH_o. The line of identity is also shown. (*B*) Relationship between fluorescence of 6-CF–loaded cells and pH_o. Media contained 120 mM K and 3 μM nigericin. Under these conditions, pH_i and pH_o 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 pH_i 6.57 ± 0.09.

7.3), or Tricine (pK_a' 7.8) depending upon pH_o (5.0–8.4). External Na was replaced by K or *N*-methyl-D-glucamine, and Cl by *p*-aminohippurate (PAH). In NH_4Cl-containing media, 3–100 mM NH_4Cl was substituted for NaCl in equimolar amounts.

CO_2-containing solutions were prepared as follows. A stock solution was made by gassing a solution that contained 5 mM HEPES with 20% CO_2/80% O_2. Sufficient HCO_3 (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 CO_2-free standard medium containing 5 mM HEPES, which had also been brought to pH_o 7.40. Thus, a series of solutions was available of different CO_2 concentrations (1–18%) and pH_o 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 ([^{3}H]H_2O), [^{14}C]DMO, [^{14}C]TMA, and [^{14}C]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 (V_m) was estimated from the fluorescence of the potentiometric indicator 3,3′-dipropylthiadicarbocyanine [diS-C_3(5)]. In previous work (Simchowitz et al., 1982; Simchowitz and De Weer, 1984), we found a resting V_m of neutrophils in nominally CO_2-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 NH_4Cl on V_m in either high- (115 mM) or low- (5 mM) K media by comparing the fluorescence levels with those observed in the absence of NH_4Cl. The extracellular K concentration was varied between 5 and 120 mM, Na being adjusted reciprocally. The calibration procedure relating fluorescence to V_m has been reported previously (Simchowitz et al., 1982; Simchowitz and De Weer, 1984). When measured 30 s or 10 min after exposure to NH_4Cl, the fluorescence of the dye cell suspensions was the same in a solution containing 30 mM NH_4Cl and 5 mM K as that in a solution that had no NH_4Cl 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, V_m was −34 mV; in its presence, −30 mV. In 30 mM NH_4Cl and 115 mM K, V_m was zero. The voltage-dependent fluorescence of the cell suspensions was not affected by either pH_o or pH_i 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 CO_2-free standard medium at pH_o 7.40 have been measured previously in this laboratory (Simchowitz et al., 1982; Simchowitz and De Weer,

1984). The values are: $[K]_i$ 120, $[Na]_i$ 25, and $[Cl]_i$ 80 meq/liter cell water; P_K 4.4×10^{-8}, P_{Na} 5.0×10^{-9}, and P_{Cl} 4.3×10^{-9} cm/s. We measured an average neutrophil diameter of 8.8 μm and a fractional water content of 0.77 (Simchowitz and De Weer, 1984). Thus, the cell water volume-to-surface ratio is 1.12 μm.

Data Analysis

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

$$pH_t = pH_\infty - (pH_\infty - pH_{initial})\exp(-kt), \quad (1)$$

where $pH_{initial}$, pH_t, and pH_∞ are the pH_i 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 pH_i recovery was derived from the expression $k(pH_\infty - pH_{initial})$. In other cases, the pH_i course was nearly linear over the period of study, and the slope of the linear regression represented the rate of pH_i recovery.

TABLE I

Intracellular pH of Human Neutrophils and Erythrocytes in CO_2-free Medium (pH_o 7.40)

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_i disparity between the two methods in neutrophils is probably due to pH_i inhomogeneity, which leads to a weak base–derived pH_i that is always lower than the pH_i derived from weak acid distribution (Roos and Boron, 1981). The acid lysosomal subcompartment (pH ~5; Ohkuma and Poole, 1978; Styrt and Klempner, 1982), whose

relative volume of 3–7% we estimated on the basis of published electron micrographs (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 extralysosomal, the extralysosomal pH can be obtained from:

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

where $\overline{pH}_{DMO}$ is the DMO-derived pH_i 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 $\overline{pH}_{DMO}$ 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_i (Roos and Boron, 1981),

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

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 $\overline{pH}_{TMA}$ 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 apparently abolished the regional intracellular pH differences: in its presence, both DMO and TMA yielded similar values (Fig. 2*A*).

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 pH_o of 7.40, H^+ were in thermodynamic equilibrium, a pH_i of 6.54 would be expected. Our measurements (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 pH_o on pH_i in the Absence of CO_2

When neutrophils, under nominally CO_2-free conditions, were suddenly transferred from medium at pH_o 7.40 (5 mM HEPES) to pH_o 8.40 (5 mM Tricine), pH_i rose slowly from 7.26 ± 0.01 to 7.62 ± 0.03 (n = 3) after 1 h (Fig. 3). Sudden reduction of pH_o from 7.40 to 6.40 (5 mM MES) resulted in a slow fall of pH_i, which reached a value of 6.84 ± 0.02 (n = 3) after 1 h. The changes in pH_i 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 CO_2 or by removal of an NH_4Cl 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 pH_o 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 pH_i upon Application and Removal of CO_2: Effect of Ionic Environment

Fig. 4 (continuous line) illustrates the time course of DMO-derived pH_i in response to application and subsequent removal of 18% CO_2 (pH_o unchanged at 7.40). At the time of the earliest measurement, 30 s after start of CO_2 exposure,

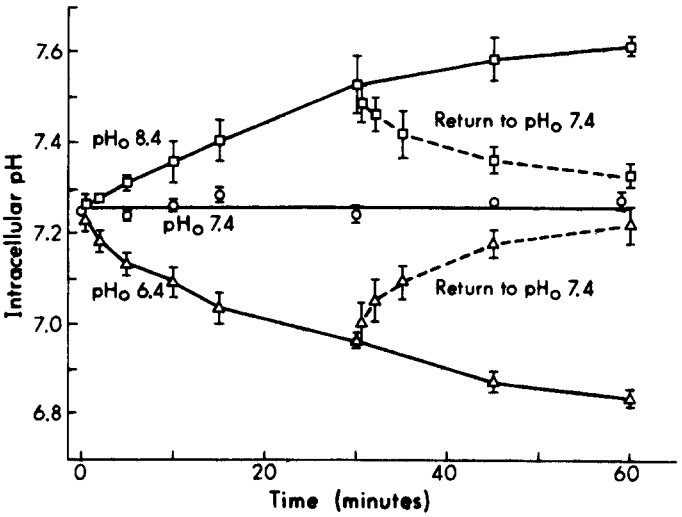

FIGURE 3. Time course of DMO-derived pH_i in response to extracellular acidification or alkalinization. A suspension of cells in standard medium (pH_o 7.40) was centrifuged and aliquots of the pellet resuspended in DMO-containing media at pH_o 6.40, 7.40, or 8.40, buffered with 5 mM MES, HEPES, or Tricine, respectively. At different times, samples were taken for pH_i measurements. In the studies at pH_o 6.40 and 8.40, part of the suspension was removed after 30 min and the pelleted cells resuspended at pH_o 7.40. Results represent the means ± SEM of three separate experiments, each performed in triplicate.

pH_i had fallen by 0.43 to 6.80. Separate measurements with 6-CF allowed pH_i to be monitored continuously, starting 5 s after CO_2 application. At this time, pH_i had already fallen by ~0.4. Between 5 and 30 s, the pH_i 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 fell. Fig. 4 shows that over the next 30 min, pH_i recovered by 0.19 units and then remained unchanged. When, after 30 min of CO_2 exposure, the cells were resuspended in the original CO_2-free medium, the pH_i rapidly rose to 7.72, that is, 0.49 units above control, and then returned toward

the original value over the course of the following 15 min. The initial fall in pH_i is due to the entry and subsequent hydration and dissociation of molecular CO_2, while the partial pH_i recovery during maintained CO_2 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_i overshoot when CO_2 is removed. A similar pH_i pattern has been observed in a number of cell types (see Roos and Boron, 1981, for review).

The ionic basis of pH_i recovery during exposure to 18% CO_2 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_i recovery could be fitted by a single exponential with a rate

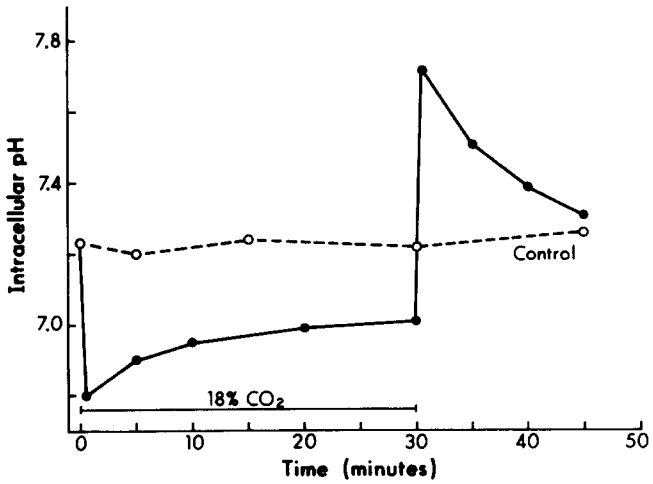

FIGURE 4. Course of DMO-derived pH_i of neutrophils upon exposure to and subsequent removal of 18% CO_2 at unchanged pH_o 7.40 (solid line). The dashed line indicates the time course of pH_i of cells kept in CO_2-free standard medium. Averages of triplicate determinations on cells from a single donor are shown.

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_i 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 (pK_a' 9.6), the initial pH_i 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 CO_2, *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_i recovery from CO_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_2-free medium, the pH_i promptly rose to exceed control ("overshoot"), after which it recovered toward

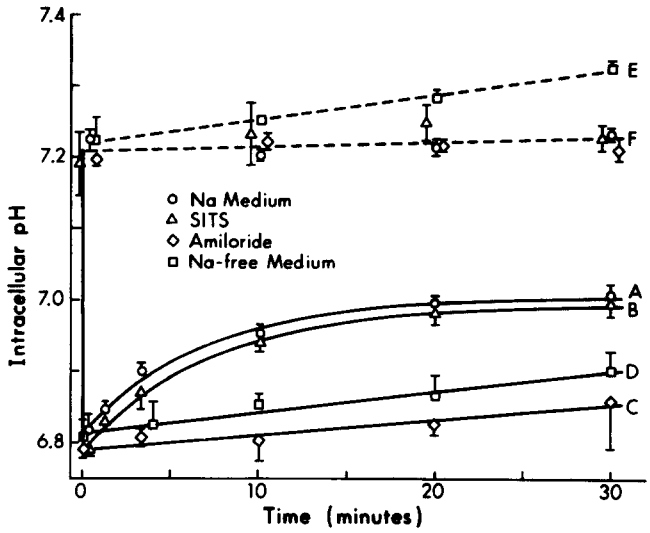

FIGURE 5. Time course of DMO-derived pH_i during exposure of neutrophils to 18% CO_2 (solid lines). Results represent the means ± SEM of three separate experiments, each performed in triplicate. The pH_o was 7.40 throughout. Exposure was started at zero time. (*A*) Standard medium without inhibitors; (*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_i under CO_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_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 (*E*), 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_3

exchange in the recovery process. (Small amounts of CO_2 and HCO_3 are undoubtedly present even in the nominal absence of CO_2.) 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/HCO_3 exchange in other cell types [Aickin and Thomas, 1977; Aickin and Brading,

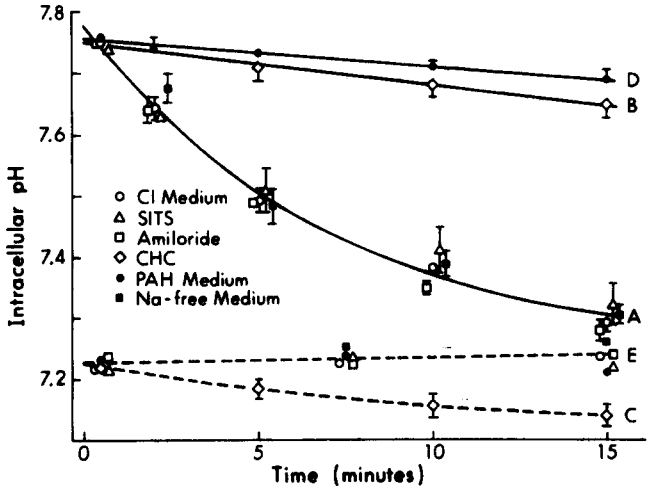

FIGURE 6. Time course of DMO-derived pH_i upon removal of 18% CO_2 (solid lines). The cells were first exposed to 18% CO_2 (pH_o 7.40) for 30 min, and then resuspended at zero time in CO_2-free solutions of the following compositions: (*A*) standard (148 mM Cl) medium with or without 1 mM amiloride or 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 pH_i after the cells, without exposure to CO_2, 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 pH_i 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 ± 0.0109 and 0.0104 ± 0.0063 pH/min, respectively). The remainder of the data were fit to straight lines with slopes (in pH/min) of 0.00671 ± 0.00204 (*B*), 0.00420 ± 0.00123 (*D*), and 0.00106 ± 0.00089 (*E*).

1984; Russell and Boron, 1976; Hoffmann, 1982; Vaughan-Jones, 1982]) had no effect. As shown by curve *B*, CHC (40 mM) greatly slowed pH_i recovery from post-CO_2 alkalinization: the initial rate was reduced by 91% to 0.00671 ± 0.00204 pH/min (n = 3). Both the small degree of remaining acidification and the slow acidification (initial rate 0.0104 ± 0.0063 pH/min; n = 3) that is induced by CHC even without prior CO_2 exposure (curve *C*) may be due to intracellular accumulation of some lactic acid resulting from the blocking effect of CHC on

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_i fall. The influx of some molecular CHC (pK_a' 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 pH_i by <0.001 pH/min, taking buffering power into account.

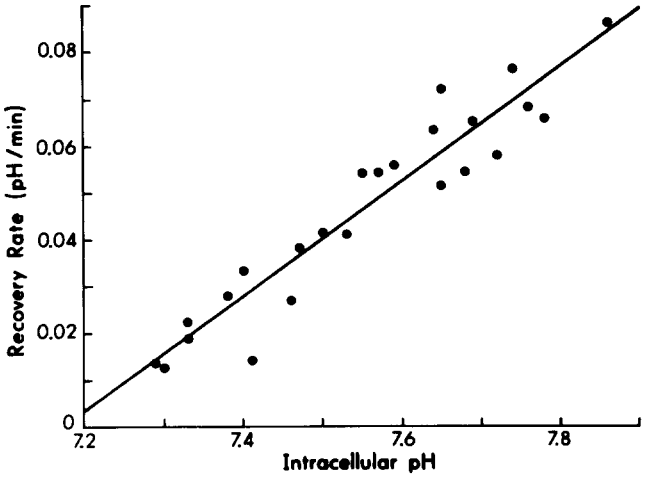

FIGURE 7. Relationship between initial pH_i recovery rate and degree of alkalinization after CO_2 removal. Separate aliquots of neutrophil suspensions from the same donor were exposed to 1, 2, 3, 5, 8, 12, 15, and 18% CO_2 at constant pH_o (7.40) for 30 min. Three different donors were used. The cells were then resuspended in CO_2-free standard media, and pH_i measurements (derived from DMO distribution) were made at 45 s, 2 min, and 5 min during the course of pH_i recovery. In order to obtain the initial recovery rates, the pH_i's at zero time (i.e., immediately after CO_2 removal) were needed. These values were separately obtained by resuspending different aliquots, previously exposed to various CO_2-containing solutions, in CO_2-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 pH_i at zero time. The data points were fit to a straight line by the least-squares method: recovery rate (in pH/min) = $-0.882 + 0.123\ pH_i$. The resting pH_i of these cells maintained in CO_2-free standard medium (pH_o 7.40) was 7.21 ± 0.02.

The effect of CHC on pH_i recovery after CO_2 removal suggests that an exchange of internal HCO_3 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 alkalinization after CO_2 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 pH_i recovery from post-CO_2 alkalinization is probably due to a Cl/HCO_3 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_i overshoot following CO_2 removal. The overshoot was varied by exposing the cells for 30 min to different CO_2 concentrations (1–18%) at pH_o 7.40. The relationship was found to be linear over the pH_i range 7.3–7.8.

Time Course of pH_i upon Application and Removal of NH_4Cl: Effect of Ionic Environment

Fig. 8 (solid line) shows a representative record of the time course of the DMO-derived pH_i of cells during application and subsequent removal of 30 mM NH_4Cl (pH_o unchanged at 7.40). At the time of the earliest measurement, 30 s after the

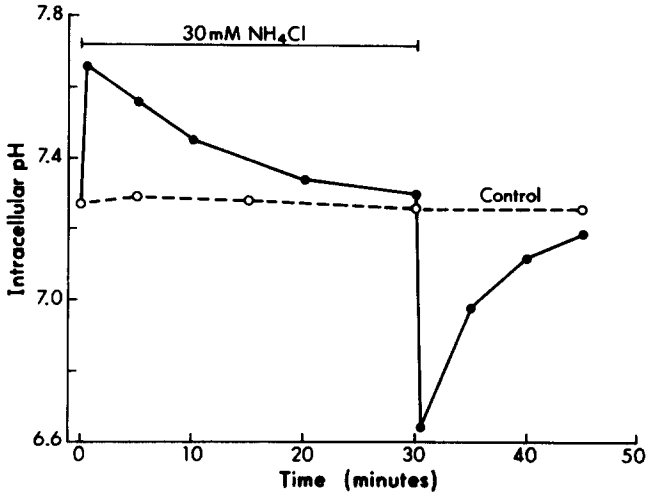

FIGURE 8. Course of DMO-derived pH_i of neutrophils upon exposure to and subsequent removal of 30 mM NH_4Cl at unchanged pH_o 7.40 (solid line). The dashed line indicates the time course of pH_i of cells kept in NH_4Cl-free standard medium. All points represent the average of triplicate determinations on cells from a single donor.

start of NH_4Cl exposure, pH_i had risen by 0.39 to 7.66. Separate measurements with 6-CF, which allowed pH_i to be monitored continuously, starting 5 s after NH_4Cl exposure, showed that pH_i had already risen by ~0.4 at this time. Between 5 and 30 s, pH_i fell by 0.02 at most. At 30 s, pH_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_i rose. Over the next 30 min, the DMO-derived pH_i fell, and almost returned to control (Fig. 8). When after 30 min, NH_4Cl was removed and the cells were resuspended in the original medium, pH_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_i pattern agrees with that observed in a variety of cell types (Roos and Boron, 1981).

We shall now examine these pH_i transients in more detail. The decline of pH_i after the initial alkalinization 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 NH_4^+ 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 NH_4^+. However, when this force was reduced by ~80% by depolarizing the membrane from −53 mV

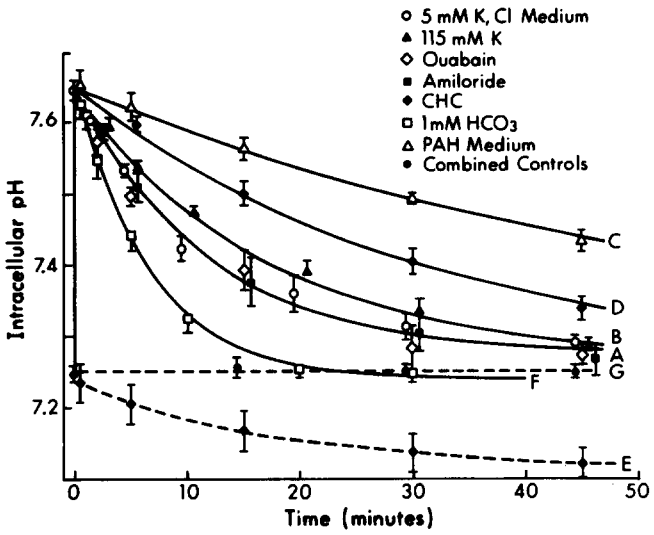

FIGURE 9. Time course of DMO-derived pH_i during exposure of neutrophils to 30 mM NH_4^+ (solid lines). Results represent the means ± SEM of three to four separate experiments, each performed in triplicate. The pH_o was 7.40 throughout. Exposure was started at zero time. (*A*) Medium containing 148 mM 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 CHC replaced 40 mM Cl; (*F*) 148 mM Cl, 1 mM HCO_3/0.2% CO_2. The two dashed lines represent the time course of pH_i in the absence of NH_4^+. (*E*) 40 mM CHC replacing 40 mM Cl; (*G*) under all other conditions. Since their pH_i'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.

to 0 in 115 mM K, the initial rate of pH_i 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 NH_4^+ influx, depolarization should have reduced influx, and thus the recovery rate, by a factor of 5, assuming constant-field behavior and voltage-independent NH_4^+ permeability. A large part of recovery from NH_4Cl-induced alkalinization seems, therefore, to be due to mechanisms other than electrodiffusion. These mechanisms might include: (*a*) NH_4^+ ions gaining entry

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 NH_4^+ through the Na/H exchanger; both are unlikely because recovery was not affected by 1 mM amiloride; (*c*) exchange of internal HCO_3 for external Cl. We demonstrated above that such a mechanism is probably responsible for the pH_i recovery from alkalinization following CO_2 removal. Three lines of evidence indicate that, also during recovery from NH_4Cl-induced alkalinization, Cl/HCO_3 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 NH_4^+ 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 HCO_3 (0.2% CO_2, constant pH_o) was added to the medium, the initial rate of recovery from NH_4Cl-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 CO_2 (HCO_3) are apparently sufficient for the functioning of the Cl/HCO_3 exchanger even in the nominal absence of CO_2.

When, after 30 min of exposure to NH_4Cl, the cells were returned to the original NH_4Cl-free solution, the earliest pH_i measured, 30 s after the solution change, was 6.52 ± 0.03 (n = 3), which is considerably lower than the resting pH_i (7.18) (Fig. 10, curve *A*). The pH_i then recovered rapidly along an exponential course. The pH_i immediately after NH_4Cl removal must have been ~6.45, and the initial recovery rate, 0.134 ± 0.023 pH/min. After ~15 min, pH_i 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 pH_i 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 NH_4Cl prepulse.

By varying the NH_4Cl concentration between 3 and 40 mM (exposure period constant at 30 min), the pH_i upon NH_4Cl removal could be varied. The lower this pH_i, the greater was the initial rate of recovery. The relationship was found to be linear over the pH_i range 6.3–7.2 (Fig. 11). The recovery rate extrapolates to zero at pH_i 7.25, the normal resting pH_i.

Effect of CO_2 and HCO_3 Concentrations on Steady State pH_i

The effect of various CO_2 concentrations on pH_i was examined under two conditions: (*a*) at constant pH_o, i.e., by varying $[HCO_3]_o$ and percent CO_2 proportionally, and (*b*) at constant $[HCO_3]_o$ (Table II).

(*a*) Cells were exposed to media containing 5, 10, or 15% CO_2 and 27, 54, or 81 mM $[HCO_3]_o$, respectively, at a constant pH_o of 7.40. When the cells were exposed to the CO_2-containing media, the pH_i 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 $[HCO_3]_o$ = 27 mM (Table II, results indicated by asterisks). The corresponding

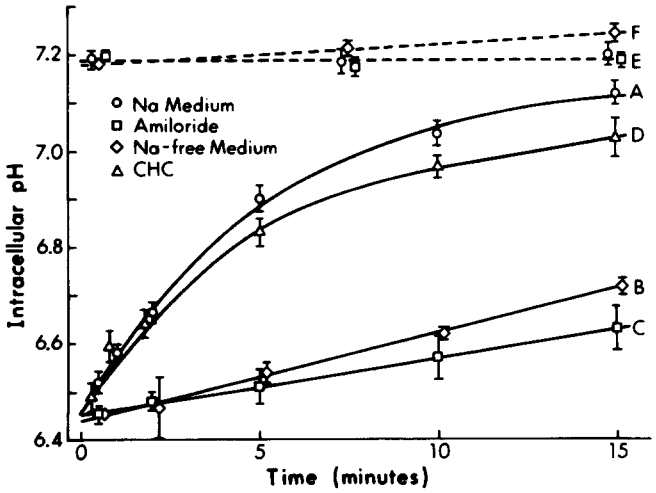

FIGURE 10. Time course of DMO-derived pH_i upon removal of 30 mM NH_4Cl (solid lines). Results represent the means ± SEM of three separate experiments, each performed in triplicate. The pH_o was 7.40 throughout the experiment. The cells were first exposed for 30 min to 30 mM NH_4Cl and then resuspended at zero time in NH_4Cl-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_4Cl, 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 ± 0.0031 (*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_4Cl, but were suspended in 40 mM CHC. The data showed a slow acidification with initial rate of 0.0103 ± 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

and 6.92 (Table II, *A*). As in the case of these early readings, the steady state pH_i's (after 1 h exposure) were also lower with CO_2 than without (Table II, *B*). In the presence of 10% CO_2, pH_i was 6.97 at $[HCO_3]_o$ = 27 mM (pH_o 7.10) and 7.06 at $[HCO_3]_o$ = 54 mM (pH_o 7.40). A similar relationship was observed with 15% CO_2 (see Table II, *B*).

Both Ahmed and Baron (1971) and Levin et al. (1976) examined the steady state pH_i levels of mixed human leukocytes (~65% neutrophils, 30% lympho-

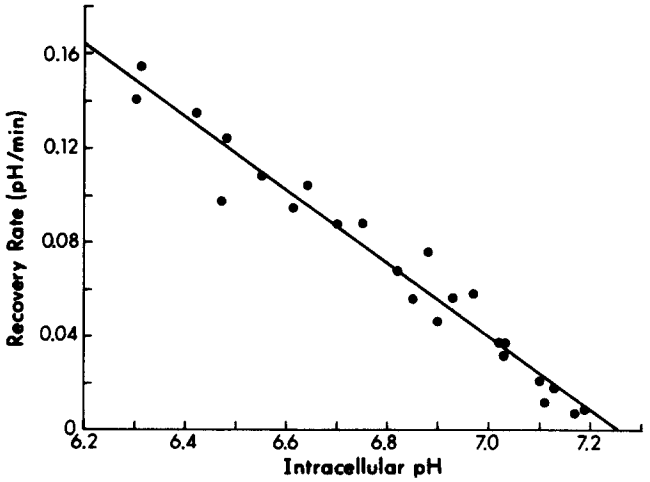

FIGURE 11. Relationship between initial pH_i recovery rate and degree of acidification after NH_4Cl removal. Separate aliquots of neutrophil suspensions from the same donor were exposed to 3, 6, 10, 15, 20, 25, 30, or 40 mM NH_4Cl at constant pH_o (7.40) for 30 min. Three different donors were used. The cells were then resuspended in NH_4Cl-free standard medium (pH_o 7.40) and pH_i measurements (derived from DMO distribution) were made at 45 s, 2 min, and 5 min during the course of pH_i recovery. In order to obtain the initial recovery rates, the pH_i's at zero time (i.e., immediately after NH_4Cl removal) were needed. These values were separately obtained by resuspending different aliquots, previously exposed to various NH_4Cl-containing solutions, in NH_4Cl-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 pH_i 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 pH_i. The resting pH_i of these cells maintained in NH_4Cl-free standard medium (pH_o 7.40) was 7.24 ± 0.03.

cytes, 5% monocytes) in response to changes in P_{CO_2} and $[HCO_3]_o$. At 6% CO_2 (pH_o 7.40), steady state pH_i measured with DMO was 7.10, nearly the same as our value (7.11). At a constant pH_o of 7.39, pH_i fell from ~7.31 to ~7.07 when CO_2 was raised from 2 to 10%.

Buffering Power

The intrinsic buffering power, defined as $-\Delta[HCO_3]_i/\Delta pH_i$ or $\Delta[NH_4]_i/\Delta pH_i$, should be derived from the decrease or increase of pH_i immediately upon

exposure of the cells to CO_2 or NH_4Cl, that is, before any recovery has taken place. Since such early pH_i values could not be obtained experimentally, they were computed by back-extrapolation of the time course of pH_i measured after 30 s exposure to 1–18% CO_2 or 3–100 mM NH_4Cl, 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 pH_i could be described by a straight line for which the least-squares regression is given by: $\beta_{intrinsic} = 137.9 - 11.9\ pH_i$, where $\beta_{intrinsic}$ is expressed in millimolar per pH unit. Thus, the buffering power under standard CO_2-free conditions (pH_o 7.40, pH_i 7.25) is ~50

TABLE II

Effect of Percent CO_2 and pH_o on Early and Steady State pH_i

(*A*) pH_i values at 30 s

	Percent CO_2			
pH_o	0	5	10	15
7.40	7.20±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	7.14±0.03			6.75±0.03*

(*B*) Steady state pH_i values (1 h)

	Percent CO_2			
pH_o	0	5	10	15
7.40	7.19±0.03	7.11±0.03*	7.06±0.02	7.01±0.02
7.10	7.10±0.02		6.97±0.03*	
6.92	7.05±0.02			6.89±0.03*

Neutrophils were exposed to 5, 10, and 15% CO_2 concentrations under two incubation conditions: (*a*) at constant pH_o 7.40, $[HCO_3]_o$ being varied proportionally with the percent CO_2 and (*b*) at constant $[HCO_3]_o$ = 27 mM (asterisks), pH_o being allowed to fall (7.40, 7.10, and 6.92, respectively) as percent CO_2 was increased. Controls were suspended in CO_2-free standard media, prepared at corresponding pH_o's using 5 mM HEPES buffer. At 30 s and at 1 h, by which time the pH_i of all cells exposed to CO_2 had reached a steady state, pH_i was measured by DMO distribution. Results represent the means ± SEM of three separate experiments, each performed in triplicate.

mM/pH, 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), $\beta_{intrinsic}$ seems to be independent of pH_i, whereas in cat skeletal muscle (Furusawa and Kerridge, 1927) and barnacle muscle (Boron, 1977; Boron et al., 1979), it increases as pH_i is reduced, as is the case in human neutrophils.

The buffering powers during and after CO_2 exposure were used to predict the post-CO_2 overshoot from the extent of recovery during the CO_2 pulse. The calculated pH_i values agreed well with those experimentally obtained. For instance, the pH_i rise of 0.19 during the 30-min exposure to 18% CO_2 (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 CO_2, $2.3[HCO_3]_i$ = 76.4 mM/pH). This should result in an overshoot of ~25.0/50.1

TABLE III

Intrinsic Buffering Power and pH_i's Measured 30 s after Exposure to 1–18% CO_2 (pH_o 7.40)

Percent CO_2	pH_i		$\beta_{intrinsic}$	
	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	7.03±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±6.3	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 CO_2 concentration and each donor, the intrinsic buffering power ($-\Delta[HCO_3]_i/\Delta pH_i$) was obtained from the difference between pH_i under CO_2-free conditions and the initial pH_i immediately upon CO_2 exposure. The latter was derived by back extrapolation of the measured pH_i data. In the case of 18% CO_2, in which the course was followed between 30 s and 30 min, the initial pH_i 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% CO_2, in which only a single 30-s measurement was made, the initial pH_i was assumed to be lower than this measurement by an amount proportional to the pH_i 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% CO_2. The initial $[HCO_3]_i$ was then derived using a CO_2 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 CO_2, $[HCO_3]_i$ was assumed to be zero.

TABLE IV

Intrinsic Buffering Power and pH_i's Measured 30 s after Exposure to 3–100 mM NH_4Cl (pH_o 7.40)

NH_4Cl	pH_i		$\beta_{intrinsic}$	
	6-CF	DMO	6-CF	DMO
mM			*mM/pH*	
0	7.26±0.03 (3)*	7.25±0.03 (13)	—	—
3	7.33±0.02 (3)	7.28±0.04 (3)	48.9±6.6	52.6±3.0
10	7.43±0.03 (3)	7.39±0.03 (3)	51.6±7.1	50.5±1.7
30	7.62±0.04 (3)	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 (3)	7.88±0.04 (3)	46.1±4.5	43.5±6.5

* Means ± SEM (number of donors).

For each NH_4Cl concentration and each donor, the intrinsic buffering power ($\Delta[NH_4]_i/\Delta pH_i$) was obtained from the difference between pH_i under NH_4Cl-free conditions and the initial pH_i immediately upon NH_4Cl exposure. The latter was derived by back extrapolation of the pH_i course. In the case of 30 mM NH_4Cl, in which the time course was followed between 30 s and 45 min, the initial pH_i 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 NH_4Cl, in which only a single pH_i measurement was made at 30 s, the initial pH_i was assumed to be higher than this measurement by an amount proportional to the pH_i 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 NH_4Cl. The initial $[NH_4]_i$ was then derived using an apparent pK_a of 8.92 for NH_4^+ (Everett and Wynne-Jones, 1938).

= 0.50 pH unit (where 50.1 is the average intrinsic buffering power during the pH_i transient after CO_2 removal), in agreement with the observed value of 0.54 (Fig. 6). In a comparable way, the pH_i fall of 0.36 during the 30-min exposure to 30 mM NH_4Cl (Fig. 9) should produce a pH_i undershoot of 0.74 upon its removal; the observed fall was 0.73 (Fig. 10).

At the same pH_i (for example, 6.80), the rate of pH_i recovery (alkalinization) after NH_4Cl withdrawal (Fig. 11) was found to be about twice (0.071 pH/min) that during CO_2 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 CO_2 exposure, and only 57.0 mM/pH after NH_4Cl withdrawal. Similarly, at a selected pH_i of 7.65, the recovery (acidification) after the overshoot following CO_2 removal was almost twice as fast (0.059 pH/min) as that during NH_4Cl application (0.033 pH/min) (Figs. 7 and 9), even though both are due mainly to Cl/HCO_3 exchange. Again, buffering power is responsible for this difference: 46.9 mM/pH after CO_2 removal and 46.9 + 37.7 = 84.6 mM/pH during NH_4Cl 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_i of human neutrophils under resting conditions. It should provide a background for the study of pH_i-dependent functions of stimulated cells.

We found that at pH_o 7.40, the pH_i of neutrophils was ~7.25 (Table I). Since the membrane voltage of these cells is about −53 mV (Simchowitz and De Weer, 1984), pH_i 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_i, 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_i recovery, after the initial rise, was mainly accomplished through a Cl/HCO_3 exchange.

Recovery from Acid Loads

When acidification was accomplished by exposing the cells to 5–18% CO_2 (pH_o unchanged at 7.40), recovery was not complete: the new steady state pH_i value was always lower than that before CO_2 exposure (Fig. 5 and Table II). On the other hand, when the cells were acidified by prepulsing with 30 mM NH_4Cl (pH_o again unchanged at 7.40), pH_i completely recovered (Fig. 10). This difference in pH_i behavior may be attributed, in part, to the additional acid load imposed upon the cells during CO_2 exposure caused by the outward leak of HCO_3^- down its electrochemical gradient. We also examined the rate of pH_i 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_i (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 CO_2 or by removal of NH_4Cl. 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/HCO_3 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/HCO_3 exchange, as well as Na/H exchange, is involved in pH_i 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_i recovery from alkalinization achieved either during exposure to NH_4Cl or after removal of a CO_2 pulse. In both instances, the elevated pH_i returned toward near-normal values in 15–30 min. Amiloride had no effect on recovery from alkalinization 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 NH_4Cl-induced alkalinization doubled when 1 mM HCO_3 at constant pH_o (0.2% CO_2) was added to the medium (Fig. 9). These observations suggest that recovery from alkalinization is mainly due to an exchange of internal HCO_3 for external Cl.

It thus appears that human neutrophils possess two separate regulatory mechanisms for pH_i homeostasis. One, a Na/H exchange, returns pH_i to a more normal value after imposed acidification. A second, a Cl/HCO_3 exchange, comes into play after imposed alkalinization. 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/HCO_3 exchange, whereas this compound is ineffective in neutrophils.

The question arises as to why the pH_i of resting neutrophils in nominally CO_2-free medium (pH_o 7.40) is ~7.25. Considering the presence both of Na/H and Cl/HCO_3 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:

$$RT\ln\left(\frac{[Na]_o\,[H]_i}{[Na]_i\,[H]_o}\right) - RT\ln\left(\frac{[Cl]_o\,[H]_o}{[Cl]_i\,[H]_i}\right)$$

or

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

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 2.29×10^{-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 this 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):

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

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_3(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 4.4×10^{-8} cm/s (Simchowitz et al., 1982; Simchowitz and De Weer, 1984). The equation yields a value for NH_4^+ permeability of 3.8×10^{-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 NH_4Cl was due entirely to reduction of NH_4^+ entry by electrodiffusion. The difference in NH_4^+ entry per unit surface area, ΔJ_{NH_4}, between the two conditions is given by:

$$\Delta J_{NH_4} = (\Delta[d\mathrm{pH_i}/dt])\cdot(V/A)\cdot\beta,$$

where $\Delta[d\mathrm{pH_i}/dt]$ 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 NH_3 remains equilibrated throughout recovery. The value for ΔJ_{NH_4} thus obtained was set equal to the difference in net NH_4^+ influx at −34 and 0 mV, each expressed by the constant-field equation (Goldman, 1943; Hodgkin and Horowicz, 1959):

$$J_{NH_4} = P_{NH_4}\cdot\frac{V_mF}{RT}\cdot\frac{[NH_4]_o - [NH_4]_i\exp(V_mF/RT)}{1 - \exp(V_mF/RT)}.$$

It was assumed that P_{NH_4} is voltage independent. The calculation yielded a value for P_{NH_4} 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 pH_i 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.

We acknowledge the expert technical assistance of William H. Miller and Arabella R. Kizzart and the secretarial skills of Linda Larkin and Gerlean Smith. We wish to thank Dr. John A. Thomas of the University of South Dakota for his gift of 6-CF, Dr. Alan Waggoner of Carnegie-Mellon University for his gift of 3,3′-dipropylthiadicarbocyanine iodide, and Dr. Edward J. Cragoe, Jr., of Merck, Sharp & Dohme for his gift of amiloride. We are especially grateful to Dr. Paul De Weer for providing the least-squares programs for computer fitting, to Mr. Ronald Ratzlaff for the graphic displays, and to Drs. Robert W. Putman and Luis Reuss for their comments.

This work was supported by the Veterans Administration and by a grant from the Arthritis Foundation Clinical Research Center. A. R. is recipient of National Institutes of Health Research Career Award HR-19608.

Original version received 17 August 1984 and accepted version received 22 October 1984.

REFERENCES

Abercrombie, R. F., R. W. Putnam, and A. Roos. 1983. The intracellular pH of frog skeletal muscle: its regulation in isotonic solutions. *J. Physiol. (Lond.).* 345:175–187.

Ahmed, S. A., and D. N. Baron. 1971. Intracellular pH measured in isolated normal human leukocytes, by using 5,5-dimethyl-2,4-oxazolidinedione under conditions of varying P_{CO_2} and bicarbonate. *Clin. Sci.* 40:487–495.

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. *J. Physiol. (Lond.).* 349:587–606.

Aickin, C. C., and R. C. Thomas. 1975. Micro-electrode measurement of the internal pH of crab muscle fibres. *J. Physiol. (Lond.).* 252:803–815.

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

Aronson, P. S., J. Nee, and M. A. Suhm. 1982. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature (Lond.).* 299:161–163.

Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. *J. Exp. Med.* 134:907–934.

Benos, D. J. 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* 242:C131–C145.

Bone, J. M., A. Verth, and A. T. Lambie. 1976. Intracellular acid-base heterogeneity in nucleated avian erythrocytes. *Clin. Sci. Mol. Med.* 51:189–196.

Boron, W. F. 1977. Intracellular pH transients in giant barnacle muscle fibers. *Am. J. Physiol.* 233:C61–C73.

Boron, W. F., and E. L. Boulpaep. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander. *J. Gen. Physiol.* 81:29–52.

Boron, W. F., and P. De Weer. 1976. Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *J. Gen. Physiol.* 67:91–112.

Boron, W. F., W. C. McCormick, and A. Roos. 1979. pH regulation in barnacle muscle fibers: dependence on intracellular and extracellular pH. *Am. J. Physiol.* 237:C185–C193.

Boron, W. F., and A. Roos. 1976. Comparison of microelectrode, DMO, and methylamine methods for measuring intracellular pH. *Am. J. Physiol.* 231:799–809.

Borregaard, N., and T. Herlin. 1982. Energy metabolism of human neutrophils during phagocytosis. *J. Clin. Invest.* 70:550–557.

Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77–89.

Brown, D. A., and J. Garthwaite. 1979. Intracellular pH and the distribution of weak acids and bases in isolated rat superior cervical ganglia. *J. Physiol. (Lond.).* 297:597–620.

Busa, W. B., and R. Nuccitelli. 1984. Metabolic regulation via intracellular pH. *Am. J. Physiol.* 246:R409–R438.

Cabantchik, Z. I., P. A. Knauf, and A. Rothstein. 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of "probes." *Biochim. Biophys. Acta.* 515:239–302.

Deitmer, J. W., and D. Ellis. 1980. Interactions between the regulation of the intracellular pH and sodium activity of sheep cardiac Purkinje fibres. *J. Physiol. (Lond.).* 304:471–488.

Epel, D. 1978. Mechanisms of activation of sperm and egg during fertilization of sea urchin gametes. *Curr. Top. Dev. Biol.* 12:185–245.

Everett, D. H., and W. F. K. Wynne-Jones. 1938. The dissociation of the ammonium ion and the basic strength of ammonia in water. *Proc. R. Soc. Lond. A.* 169:190–204.

Everett, D. H., and W. F. K. Wynne-Jones. 1940–41. The dissociation constants of the methyl ammonium ions and the basic strengths of the methylamines in water. *Proc. R. Soc. Lond. A.* 177:499–516.

Fabiato, A., and F. Fabiato. 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscle. *J. Physiol. (Lond.).* 276:233–255.

Franke, W. W., V. Scheer, G. Krohne, and E. D. Jaraseh. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91:39S–50S.

Furusawa, K., and P. M. T. Kerridge. 1927. The hydrogen ion concentration of the muscles of the cat. *J. Physiol. (Lond.)*. 63:33–41.

Gerson, D. F., H. Kiefer, and W. Eufe. 1982. Intracellular pH of mitogen-stimulated lymphocytes. *Science (Wash. DC)*. 216:1009–1010.

Goldman, D. E. 1943. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* 27:37–60.

Grinstein, S., S. Cohen, and A. Rothstein. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J. Gen. Physiol.* 83:341–370.

Halestrap, A. 1975. The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J.* 148:85–96.

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 α-cyano-4-hydroxycinnamate and related compounds. *Biochem. J.* 148:97–106.

Harned, H. S., and F. T. Bonner. 1945. The first ionization of carbonic acid in aqueous solutions of sodium chloride. *J. Am. Chem. Soc.* 67:1026–1031.

Harned, H. S., and R. Davis. 1943. The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions from 0 to 50°. *J. Am. Chem. Soc.* 65:2030–2037.

Henson, P. M., M. H. Ginsberg, and D. C. Morrison. 1978. Mechanisms of mediator release by inflammatory cells. *In* Membrane Fusion. G. Poste and G. L. Nicholson, editors. Elsevier/North Holland Biomedical Publishing, Amsterdam. 407–508.

Hodgkin, A. L., and P. Horowicz. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol. (Lond.)*. 148:127–160.

Hodgkin, A. L., and B. Katz. 1949. The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol. (Lond.)*. 108:37–77.

Hoffmann, E. K. 1982. Anion exchange and anion-cation co-transport systems in mammalian cells. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:519–535.

Johnson, J. J., D. Epel, and M. Paul. 1976. Intracellular pH and activation of sea urchin eggs after fertilization. *Nature (Lond.)*. 262:661–664.

L'Allemain, G., S. Paris, and J. Pouyssegur. 1984. Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na^+/H^+ antiport. *J. Biol. Chem.* 259:5809–5815.

Levin, G. E., P. Collinson, and D. N. Baron. 1976. The intracellular pH of human leucocytes in response to acid-base changes in vitro. *Clin. Sci. Mol. Med.* 50:293–299.

Molski, T. F. P., P. H. Naccache, M. Volpi, L. M. Wolpert, and R. I. Sha'afi. 1980. Specific modulation of the intracellular pH of rabbit neutrophils by chemotactic factors. *Biochem. Biophys. Res. Commun.* 94:508–514.

Moody, W. J., Jr. 1981. The ionic mechanism of intracellular pH regulation in crayfish neurones. *J. Physiol. (Lond.)*. 316:293–308.

Moolenaar, W. H., R. Y. Tsien, P. T. van der Saag, and S. W. de Laat. 1983. Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature (Lond.)*. 304:645–648.

Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1977. Transport of sodium, potassium, and calcium across rabbit polymorphonuclear leukocyte membranes. *J. Cell Biol.* 73:428–444.

Nichols, B. A., and B. F. Bainton. 1973. Differentiation of human monocytes in bone marrow and blood. Sequential formation of two granule populations. *Lab. Invest.* 29:27–40.

Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.

Pace, C. S., J. T. Tarvin, and J. S. Smith. 1983. Stimulus-secretion coupling in β-cells: modulation by pH. *Am. J. Physiol.* 244:E3–E18.

Pressman, B. C. 1969. Mechanism of action of transport-mediating antibiotics. *Ann. NY Acad. Sci.* 147:829–841.

Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.

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. *J. Biol. Chem.* 258:12644–12653.

Russell, J. M., and W. F. Boron. 1976. Role of chloride transport in regulation of intracellular pH. *Nature (Lond.).* 264:73–74.

Sachs, J. R., P. A. Knauf, and P. B. Dunham. 1975. Transport through red cell membranes. *In* The Red Blood Cell. Second edition. D. M. Surgenor, editor. Academic Press, Inc., New York. 2:613–703.

Schulman, R. G., T. R. Brown, K. Ugurbil, S. Ogawa, S. M. Cohen, and J. A. den Hollander. 1979. Cellular applications of ^{31}P and ^{13}C nuclear magnetic resonance. *Science (Wash. DC).* 205:160–166.

Segal, A. W., M. Geisow, R. Garcia, A. Harper, and R. Miller. 1981. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature (Lond.).* 290:406–409.

Seligmann, B. E., and J. I. Gallin. 1980. Use of lipophilic probes of membrane potential to assess human neutrophil activation. *J. Clin. Invest.* 66:493–503.

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

Simchowitz, L., and P. De Weer. 1984. Chloride movements in human neutrophils. *Fed. Proc.* 43:817. (Abstr.)

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. *J. Gen. Physiol.* 79:453–479.

Simons, E. R., D. B. Schwartz, and N. E. Norman. 1982. Stimulus response coupling in human platelets: thrombin-induced changes in pH_i. *In* Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions. R. Nuccitelli and D. W. Deamer, editors. Alan R. Liss, New York. 463–482.

Spencer, T. L., and A. L. Lehninger. 1976. L-Lactate transport in Ehrlich ascites-tumor cells. *Biochem. J.* 154:403–414.

Styrt, B., and M. S. Klempner. 1982. Internal pH of human neutrophil lysosomes. *FEBS Lett.* 149:113–116.

Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 18:2210–2218.

Thomas, J. A., P. C. Kolbeck, and T. A. Langworthy. 1982. Spectrophotometric determination of cytoplasmic and mitochondrial pH transitions using trapped pH indicators. *In* Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions. R. Nuccitelli and D. W. Deamer, editors. Alan R. Liss, New York. 105–123.

Thomas, R. C. 1974. Intracellular pH of snail neurones measured with a new pH-sensitive glass microelectrode. *J. Physiol. (Lond.)*. 238:159–180.

Thomas, R. C. 1976. Ionic mechanism of the H^+ pump in a snail neurone. *Nature (Lond.)*. 262:54–55.

Vanheel, B., A. De Hemptinne, and I. Leusen. 1984. Analysis of Cl^--HCO_3^- exchange during recovery from intracellular acidosis in cardiac Purkinje strands. *Am. J. Physiol.* 246:C391–C400.

Vaughan-Jones, R. D. 1982. Chloride activity and its control in skeletal and cardiac muscle. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 229:537–548.

Vaughan-Jones, R. D. 1982. 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, New York. 239–252.

Warnock, D. G., and F. C. Rector 1979. Proton secretion by the kidney. *Annu. Rev. Physiol.* 41:197–210.

Weinman, S. A., and L. Reuss. 1984. Na^+-H^+ exchange and Na^+ entry across the apical membrane of *Necturus* gallbladder. *J. Gen. Physiol.* 83:57–74.

Weissmann, G., H. M. Korchak, H. D. Perez, J. E. Smolen, I. M. Goldstein, and S. T. Hoffstein. 1979. Leukocytes as secretory organs of inflammation. *In* Advances in Inflammation Research. G. Weissmann, B. Samuelsson, and R. Paoletti, editors. Raven Press, New York. 1:95–112.