pH Homeostasis in Promyelocytic Leukemic HL60 Cells

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ABSTRACT By measuring the membrane potential using the influx of the lipophilic cation tetraphenylphosphonium and intracellular pH using 2,7-bis-carboxyethyl-5(6)-carboxyfluorescein and the distribution of the weak acid 5,5-dimethyl-2,4-oxazolidinedione, we have determined that intracellular pH is 0.9–1.1 pH units above electrochemical equilibrium in undifferentiated HL60 cells, indicating that these cells actively extrude proton equivalents. The Na/H exchanger is not the system responsible for keeping the pH above the electrochemical equilibrium, since adding inhibitors of this transport system (dimethylamiloride and ethylisopropylamiloride) or removing the extracellular sodium has no effect on intracellular pH. In contrast, the addition of the Cl/HCO₃ exchange inhibitors H₂ 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) or pentachlorophenol (PCP) causes a drop in intracellular pH, and the removal of extracellular chloride in the presence of bicarbonate leads to a large intracellular alkalinization, which indicates a role for the anion exchanger in pH homeostasis in these cells. In addition, we find that the intracellular chloride concentration is about one order of magnitude above electrochemical equilibrium. We conclude that an H₂DIDS and PCP inhibitable system, probably the Cl/HCO₃ exchanger, is at least partially responsible for keeping intracellular pH above electrochemical equilibrium in HL60 cells under resting conditions. We also find no change in intracellular pH when cells differentiate along the granulocytic pathway (having been induced by the addition of dimethylsulfoxide or of retinoic acid), which indicates that changes in intracellular pH are not causally related to cell differentiation.

INTRODUCTION

A Na⁺/H⁺ exchanger and a Cl⁻/HCO₃⁻ exchanger are important in the regulation of intracellular pH in white blood cells (Grinstein et al., 1984; Simchowitz and Roos, 1985; Grinstein and Furuya, 1986.) It is clear that intracellular pH is kept above electrochemical equilibrium in human neutrophils (Simchowitz and Roos, 1985; Grinstein and Furuya, 1986) and in rat lymphocytes (Grinstein et al, 1984). The Na⁺/H⁺ exchanger is important in the recovery from intracellular acid loads (Grinstein et al., 1984; Simchowitz and Roos, 1985; Grinstein and Furuya, 1986) and the

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Cl⁻/HCO₃⁻ exchanger mediates recovery from alkaline loads (Simchowitz and Roos, 1985). However, it is not known which transport system is responsible for maintaining intracellular pH above electrochemical equilibrium in lymphocytes and neutrophils in bicarbonate-containing media.

The human leukemic HL60 cell is a promyelocyte-like cell that can be induced to differentiate to a neutrophil-like cell by the addition of dimethylsulfoxide (DMSO) (Collins et al., 1978; Gallagher et al., 1979). This cell possesses an amiloride-inhibitable Na⁺/H⁺ exchanger (Besterman and Cuatrecasas, 1984), a Cl⁻/HCO₃⁻ exchanger (Dissing et al., 1984), and it displays Na⁺-dependent Cl⁻/HCO₃⁻ exchange activity (Ladoux et al., 1987). In this paper, we evaluate the role of these transporters in the regulation of intracellular pH in HL60 cells, and we investigate possible changes in intracellular pH homeostasis upon granulocytic differentiation of the HL60 promyelocyte.

**METHODS**

**HL60 Cell Growth and Differentiation**

HL60 cells from the American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 medium (Gibco, Grand Island, NY) at pH 7.4 with 20 mM Na⁺ bicarbonate supplemented with 15 mM HEPES and 10% fetal calf serum (HyClone Laboratories, Logan, UT) in a 5% CO₂ atmosphere. Cells were induced to differentiate by incubating them in the above medium with 1.25% DMSO for 5–6 d (neutrophil-like HL60 cells). The extent of differentiation was routinely assayed by the nitroblue tetrazolium (NBT) reduction method using the procedure of Koeffler et al. (1981). Undifferentiated cells were 5–10% NBT positive and DMSO-differentiated cells were 80–90% positive.

**Solutions**

The medium used routinely in this study was RPMI 1640 (Gibco) (without phenol red), which was supplemented with 15 mM HEPES and 20 mM sodium bicarbonate, and brought to pH 7.40 (with NaOH) at 37°C in a 5% CO₂ atmosphere (RP). The concentrations of inorganic salts in this medium (RP) were: 102.7 mM NaCl, 20 mM NaHCO₃, 5.63 mM Na₂HPO₄, 5.36 mM KCl, 0.4235 mM CaCl₂, 0.405 mM MgSO₄, 15 mM HEPES; in addition, the medium had a mixture of amino acids, glucose, and vitamins (Moore et al., 1967). The amino acids contained 0.25 mM Na⁺ and 0.75 mM Cl⁻. The Na⁺ and K⁺ concentrations measured by atomic absorption were Na⁺ = 139 ± 4 mM (mean ± SEM, n = 8), and K⁺ = 5.6 ± 0.2 mM (n = 6). The nominal Cl⁻ concentration was 109.7 mM. Ion substitutions were made by replacing all inorganic Na⁺ salts with K⁺ salts (RK), all sodium bicarbonate with choline bicarbonate, and all other inorganic Na⁺ salts with tetramethylammonium (TMA⁺) salts (RP no Na⁺), or by replacing inorganic Cl⁻ salts with gluconate salts (RP no Cl⁻).

The solutions used to assay Na⁺/H⁺ exchange activity (Fig. 3) were HTK (145 mM TMACl, 5 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 20 mM HEPES; pH 7.4 at 37°C, titrated with TMAOH) and HNK, which is the same as HTK except that the TMACl is replaced by NaCl. NHK no Cl⁻ is HNK in which Cl⁻ has been replaced by gluconate. To assay Cl⁻/HCO₃⁻ exchange (Fig. 4, B and C), we used a solution (HNG) consisting of 125 mM Na-glutamate, 20 mM NaHCO₃, 5 mM K-glutamate, 0.5 mM CaSO₄, 0.5 mM MgSO₄, 20 mM HEPES; pH 7.4 at 37°C with TMAOH. To trigger the Cl⁻-dependent acidification, a solution in which all the gluconate and sulfate had been replaced by Cl⁻ (HNC) was added.
TPP⁺ Influx

Tetraphenylphosphonium (TPP⁺) influx was monitored using [14C]TPP⁺ (Amersham Corp., Arlington Heights, IL). To start each influx, cells were diluted in media containing 0.2 μCi/ml of [14C]TPP⁺ (6.4 μM) to a final cell density of 1.5–3.0 × 10⁸ cells/ml. Influx was terminated by the dilution of 200 μl of cells into 1 ml of ice-cold Dulbecco's phosphate-buffered saline in a microfuge tube containing 0.5 ml of Versilube F50 silicone oil (General Electric Co., Wilmington, MA). The cells were immediately centrifuged through the oil in an Eppendorf microfuge (Brinkman, Westbury, NY) for 30 s. The supernatant was discarded and the tips were cut off and extracted with 0.5 ml of 0.1 M NaOH. The extract was counted in a Packard Tricarb 500C scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) after its dilution in 6 ml of Liquiscint. Results were normalized to cell densities determined through using a Coulter Counter.

Measurement of Intracellular K⁺, Na⁺, and Cl⁻ Concentrations

Intracellular K⁺, Na⁺, Cl⁻, and cell volume were measured in the same sample using a triple label technique. HL60 cells were resuspended in RPMI medium at 1.5–3.0 × 10⁶ cells/ml. The cells were placed in an incubator at 37°C with a 5% CO₂ atmosphere, and 1 μCi/ml of ³⁶Cl⁻ (NaCl from ICN Radiochemicals, Inc., Irvine, CA) was added. The cells were incubated in this solution for 30 min. The time for isotopic equilibration of Cl⁻ was found to be <10 min. After 25 min, 1 μCi/ml of [14C]sucrose (Amersham) for extracellular space and 7 μCi/ml of ³H₂O (ICN) for water volume were added and 5 min later 1-ml aliquots of the cell suspension were centrifuged through Versilube F50 oil. The pellet was extracted with 1 ml of 5% TCA-10 mM CsCl. 0.4 ml of this extract was counted in a Packard Tricarb 500C counter and 0.4 ml was further diluted in 10 mM CsCl for the determination of Na⁺ and K⁺ by atomic absorption.

³⁶Cl⁻, ¹⁴C, and ³H were counted with three different energy windows. Spill of ³⁶Cl⁻ counts into the ¹⁴C channel and the ³H channel was 22.8% and 3.9% respectively (% of the total ³⁶Cl⁻ counts in the three channels). Spill of ¹⁴C counts into the tritium channel was 36.9%. Quench was the same in all samples as monitored by using an external standard. Na⁺ and K⁺ concentrations were measured with an IL251 atomic absorption spectrophotomer at 589 (Na⁺) and 766.5 (K⁺) nm. 10 mM CsCl was added to all samples to suppress ionization of the Na⁺ and K⁺ in the samples. Extracellular fluid was typically 5–20% of the total pellet water.

³⁶Cl⁻ Efflux

Cells (3 × 10⁶ cells/ml) were loaded by incubation for 30 min in RPMI containing 0.6 μCi/ml ³⁶Cl⁻. To start an efflux, 4 ml of ³⁶Cl⁻-loaded cells were centrifuged in a plastic syringe connected to a 0.4 ml tapered-end microfuge tube (Freedman and Hoffman, 1979). The supernatant was discarded and the cells (about 10μl of packed cells with 10% extracellular fluid) were diluted in 9 ml of nonradioactive media to start the efflux (t = 0). 1-ml aliquots were centrifuged through oil in an Eppendorf microfuge at specified times.

Measurement of Intracellular pH Using BCECF

The fluorescent dye 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to monitor intracellular pH. Cells were loaded at a density of 0.8–1.4 × 10⁶ cells/ml by incubating them with 2 μM of the acetoxymethyl ester of BCECF (Molecular Probes Inc., Junction City, OR). After 1 h of incubation, the cells were centrifuged, washed once, and resuspended in a stirred fluorimeter cuvette at 37°C. Fluorescence was read with an SLM 8000 fluorometer (SLM Instruments, Inc., Urbana, IL) at 530 nm with excitation at 500 nm with 4-nm slits. At the end of each run, 50 μg/ml of the detergent digitonin were added to release the BCECF.
from the cells, and the fluorescence signal was calibrated by monitoring it at different pH values attained by the addition of HCl or NaOH).

In previous work (Restrepo et al., 1987), we reported intracellular pH values that were derived by using the digitonin calibration directly. Since in this paper it is important to know the absolute value of intracellular pH, we have calibrated the dye in situ by using nigericin (Thomas et al., 1979). Cells were loaded with BCECF and resuspended in media containing 145 mM K⁺ (RK medium, see under solutions in this section). Since intracellular K⁺, measured by the double-label atomic absorption technique, is 148 ± 7 mM, the K⁺/H⁺ exchange

**Figure 1.** (A) BCECF fluorescence for cells bathed in high extracellular K⁺ (145 mM K⁺, 148 mM Kᵢ) at various extracellular pH values in the presence of 2 µg/ml nigericin (open circles) or 50 µg/ml digitonin (closed circles). Results were normalized to the value of the fluorescence at high pH (Fₘₖₙ) in the presence of digitonin after fitting data with Eq. 1. (B) Intracellular pH correction. The ordinate is the value that must be added to pH values found by using a direct calibration of the fluorescence signal versus pH in the presence of digitonin (pHₐ) to obtain the corrected value for intracellular pH. The solid line takes into account the correction for quenching of the intracellular dye only (in this case, the correction factor is pHₐ - pHᵢ where pHᵢ is found directly using Eq 1). The dashed line takes into account the correction for quenching and a correction for the fluorescence of 9% extracellular dye. This line is calculated by adding up the contributions to total fluorescence of the intracellular (91%) and extracellular (9%) dye at different intracellular pH values and an extracellular pH of 7.4, and calculating the apparent intracellular pH that would be determined if this fluorescence were used to calculate intracellular pH directly from a calibration of fluorescence versus pH after releasing the dye by addition of digitonin (pHᵢ).

Inonophore nigericin will make the intracellular and extracellular proton concentrations nearly equal. The fluorescence signal, calibrated in situ in the presence of 2 µg/ml nigericin, was compared with a calibration of the signal from the same batch of cells after the release of the dye by the addition of digitonin (Fig. 1 A).

The fluorescence was not linear with pH, but both the digitonin and nigericin calibration data were well fit by the equation:

\[ F = \frac{(F_{\text{min}} H^+ + F_{\text{max}} K^+) / (K_d + H^+)}{K_d} \]  (1)
which gives the total fluorescence ($F$) in terms of the proton concentration ($H^+$) for a fluorescent probe with dissociation constant $K_a$ and with $F_{\text{max}}$ – fluorescence when compound is in the protonated form, and $F_{\text{min}}$ = fluorescence when compound is in the unprotonated form.

The fluorescence values from each individual experiment were normalized to the $F_{\text{max}}$ with digitonin ($F_{\text{max}}^{\text{dm}}$). The data points for the in situ determinations lie below the points for the released dye. An unpaired t test indicates that this is not due to a change in $pK_a$. The average $pK_a$ is $6.97 \pm 0.02$, $n = 14$. Most of the difference between the two sets of data is due to quenching of the in situ $F_{\text{max}}$ as shown in Table I. Studies of excitation spectra in situ and after release with digitonin reveal that although there is a small red shift for the intracellular dye (~5 nm), the peak fluorescence is lower for the intracellular dye, which indicates that the effect due to intracellular quenching. A filtering effect is unlikely because the low and high pH forms of the dye are quenched to different extents.

The data in Table I also show that the differences in intracellular/extracellular dye fluorescence are the same in DMSO-differentiated cells and in undifferentiated cells. We have used the results of these experiments to determine a correction factor for the pH calculated by the

### Table I

| Type of cell                                      | $R_{\text{ad}}$ | $R_{\text{m}}$ | $R_{\text{nd}}$ | $R_{\text{nt}}$ |
|--------------------------------------------------|-----------------|----------------|-----------------|-----------------|
| Undifferentiated                                 | 0.104 ± 0.002   | 0.1357 ± 0.005 | 0.82 ± 0.02     | 1.30 ± 0.08     |
| Neutrophil-like                                  | 0.13 ± 0.01     | 0.15 ± 0.02    | 0.81 ± 0.02     | 1.25 ± 0.26     |
| Undifferentiated and neutrophil-like             | 0.115 ± 0.008   | 0.145 ± 0.012  | 0.822 ± 0.016   | 1.28 ± 0.12     |

The constants shown were calculated by fitting the data from the nigericin and digitonin experiments described in the Methods to Eq. 1. The $R$ parameters are defined as follows:

$$R_{\text{ad}} = F^{\text{m}}_{\text{max}} / F^{\text{m}}_{\text{max}}$$

$$R_{\text{nd}} = F^{\text{m}}_{\text{max}} / F^{\text{m}}_{\text{max}}$$

$$R_{\text{nt}} = F^{\text{m}}_{\text{max}} / F^{\text{m}}_{\text{max}}$$

where the superscripts $d$ and $n$ denote fluorescence in the presence of digitonin or nigericin, respectively, and $F^{\text{m}}_{\text{max}}$ and $F^{\text{m}}_{\text{min}}$ are defined by Eq. 1 in the Methods. Values are mean ± SEM. Data are from four experiments with undifferentiated cells and three experiments with neutrophil-like HL60 cells.

digitonin technique by normalizing Eq. 1 for the digitonin data ($F^{\text{m}}_{\text{max}}$, $F^{\text{m}}_{\text{min}}$, and $H^+_a$), and Eq. 1 for the nigericin data ($F^{\text{m}}_{\text{max}}$, $F^{\text{m}}_{\text{min}}$, and $H^+_a$) by $F^{\text{m}}_{\text{max}}$, and by equating the two normalized equations assuming that $K_a$ is constant. The corrected proton concentration is given as follows:

$$H^+_a = K_a [H^+_a (R_{\text{nd}} - R_{\text{ad}}) + K_a (R_{\text{nd}} - 1)]/[H^+_a (R_{\text{ad}} - R_{\text{nt}}) + K_a (1 - R_{\text{nt}})]$$

with $R_{\text{nd}}$, $R_{\text{nt}}$, etc. defined as in Table I. To make the correction, we have used the average values of $R_{\text{nd}}$, $R_{\text{nt}}$, and $R_{\text{nt}}$ shown in Table I for undifferentiated and neutrophil-like cells combined. The solid line in Fig. 1 B shows the correction (the difference $\text{pH}_{\text{n}} - \text{pH}_{\text{d}}$) as a function of the pH found using the digitonin calibration technique alone ($\text{pH}_{\text{d}}$).

Another factor that must be taken into account in order to quantitate intracellular pH, particularly when extracellular pH is larger than intracellular pH, is the amount of BCECF in the extracellular medium (Thomas et al., 1979). Measurement of extracellular water space using $[^{14}]\text{C}$sucrose indicated that after two washes, <0.05% of the total BCECF in a typical sample would be extracellular, if there was no efflux of BCECF from the cells. However, we found that centrifugation and resuspension of BCECF-loaded cells released $5.5 \pm 0.8\%$ ($n = $
5) of the intracellular dye, presumably because of cell lysis. In addition, BCECF leaked out of
the cells with a rate constant of $0.007 \pm 0.0004$ min$^{-1}$ (n = 3). Hence, 5 min after resuspension
~9% of the dye is extracellular. Since the extracellular pH is 7.4 and the basic form of the dye
is more fluorescent, this amount of dye can contribute a large fraction of the fluorescence
when the intracellular pH is low. If this amount of extracellular dye is taken into account, the
correction factor is modified as shown by the solid line in Fig. 1 B.

For routine measurements, the fluorescence was calibrated versus pH after the addition
of digitonin. For simplicity, and since the pH range covered was usually <0.6 pH units, the
calibration data were fitted with a straight line. The pH values obtained this way were then
adjusted using the correction factor for intracellular dye quenching and extracellular dye
fluorescence and are shown as the dotted trace in Fig. 1 B. This correction modifies our
previous estimates of intracellular buffering capacity found by titrating the interior of the cell
by the addition of extracellular ammonium (Restrepo et al., 1987). Applying the correction,
the buffering capacity in the intracellular pH range 6.7–7.1 is $47.5 \pm 4.8(21)$ mM H$^+$/pH
unit (mean ± SEM, n = 21; this is independent of intracellular pH). Measurements of H$^+$
influx were obtained from continuous recordings of the fluorescent signal at times when the
changes in pH were linear with time.

**Measurement of Intracellular pH Using Radiolabeled DMO or MA**

The weak acid DMO (5,5-dimethyl-2,4-oxazolidinedione) and the weak base MA (methylam-
line) were used to estimate intracellular pH. These compounds distribute across the plasma
membrane according to the pH gradient (Roos and Boron, 1981). $^{14}$C-labeled DMO and MA
were used (Amersham; New England Nuclear, Boston, MA). HL60 cells (1.5–3.0 × 10$^6$ cells/
ml) were incubated for 1 h (in RP at 37°C with a 5% CO$_2$ atmosphere) with 1 μCi/ml of
$[^{14}$C]DMO or 0.6 μCi/ml of $[^{14}$C]MA. 5 min before the end of the incubation, 2 μCi/ml of
$[^{3}$H]sucrose were added to measure extracellular fluid. At the end of the incubation, 1-ml
aliquots were centrifuged in microfuge tubes with 0.3 ml of Versilube F50 oil and the extra-
cellular pH was determined immediately. The pellets were extracted with 1 ml of 5% TCA/10
mM CaCl$_2$. 0.4 ml of the pellet extract was counted in the scintillation counter using Liquis-
cint, and the potassium was measured by atomic absorption using the rest of the pellet
extract. Intracellular K$^+$ was used to determine the intracellular volume by using values for
intracellular K$^+$ concentration determined in a parallel incubation (see above for method).

Intracellular pH was estimated using Eqs. 3a and b. For DMO:

$$pH_i = pK_a + \log [(\text{DMO}_i/\text{DMO}_0)(1 + 10^{pH_i - pK_a}) - 1]$$  \hspace{1cm} (3a)

For MA:

$$pH_i = pK_a - \log [(\text{MA}_i/\text{MA}_0)(1 + 10^{pK_a - pH_i}) - 1]$$  \hspace{1cm} (3b)

The p$K_a$ used for DMO was 6.1 and for MA 11.8 (Roos and Boron, 1981).

**RESULTS**

**Membrane Potential of HL60 Cells**

If the transport of protons across the plasma membrane is mediated exclusively by
the diffusion of H$^+$ through the membrane, and if there is no metabolic acid pro-
duction, the intracellular pH at a given extracellular pH will be determined by the membrane potential (electrochemical equilibrium). To find out if HL60 cells actively regulate intracellular pH, we have estimated the plasma membrane potential using the TPP⁺ influx technique developed by Kimmich and co-workers (Kimmich et al., 1985; Restrepo and Kimmich, 1985). In this technique, the magnitude of the influx of the lipophilic cation TPP⁺ is used to estimate the membrane potential. Inside negative membrane potentials stimulate TPP⁺ influx into the cells. Fig.

![Graph A](image1)

**Figure 2.** (A) The influx of [¹⁴C]TPP⁺ into HL60 cells is dependent on extracellular K⁺, but not on Na⁺ or Cl⁻ concentrations. The medium used in the control situation is RP (see Methods). All other media were made up by replacing components of RP. Sodium bicarbonate was replaced by choline bicarbonate, all other Na⁺ salts were replaced by TMA⁺ and Cl⁻ was replaced by gluconate. The Na⁺ and K⁺ concentrations shown were measured by atomic absorption and the Cl⁻ concentrations are nominal. (B) Rate of TPP⁺ influx into HL60 cells (mean ± SEM, n = 4). Replacement of Na⁺ by K⁺ (RK relative to RP) results in a significant change in TPP⁺ influx. Replacement of Cl⁻ by gluconate (RP no Cl⁻ and RK no Cl⁻), of Na⁺ by TMA⁺ and choline (RP no Na⁺), or the addition of 100 μM ouabain (RP + ouabain and RK + ouabain) does not have a significant effect on TPP⁺ influx using an unpaired t test (P > 0.05). The data are normalized to make the average of the influxes in the presence of RK, RK + ouabain, and RK no Cl⁻ equal to one.

2 A shows [¹⁴C]TPP⁺ uptake into undifferentiated HL60 cells as a function of time. TPP⁺ influx is linear and its magnitude is dependent on extracellular K⁺ but it is not affected much by changes in extracellular Cl⁻ or Na⁺ (replaced by gluconate or TMA⁺, respectively). Fig. 2 B shows the average TPP⁺ influx rate for four of these ion substitution experiments. As shown, the data can be classified into two groups: those with low TPP⁺ influx have high extracellular K⁺ (solid bars), and those with high TPP⁺ influx have low extracellular K⁺ (striped bars). Differences in influx
within these two groups are not statistically significant and differences in any two influx rates with low and high K$^+$ are significant ($P < 0.05$). Since replacement of Na$^+$ with TMA$^+$ and choline, and of Cl$^-$ with gluconate, and the inhibition of the Na$^+$, K$^+$ ATPase have only small effects, the membrane potential in undifferentiated cells must be close to the K$^+$ equilibrium potential given by Eq. 4:

$$V_K = \frac{RT}{F} \ln \left( \frac{K^+}{K_e^+} \right),$$

where $RT/F = 26.73$ mV at 37°C, and $K_e^+$ and $K_i^+$ are the extracellular and intracellular K$^+$ concentrations, respectively. This method yields a membrane potential estimate of $-87 \pm 1$ mV ($K_e^+ = 148 \pm 7$ at $K_i^+ = 5.6 \pm 0.2$ mM).

Another way to estimate the membrane potential is to assume that TPP$^+$ influx follows the Goldman flux equation, that is:

$$J = -J_0 \frac{x}{1 - e^x},$$

where $x = (V_m F/RT)$, $V_m$ = plasma membrane potential and $J$ and $J_0$ are the TPP$^+$ influx rates at $V_m$ and at zero membrane potential, respectively. If the TPP$^+$ influx at zero membrane potential is known, unknown membrane potentials can be determined by solving Eq. 5 for $V_m$. For the data in Fig. 2, $J/J_0 = 3.08 \pm 0.05$, which yields a $V_m$ of $-77.8 \pm 1.2$ mv (calculated assuming that $V_m$ is zero in the presence of high K$^+$).

These values of $V_m$ should only be regarded as rough estimates because, although it is known that TPP$^+$ influx follows the Goldman flux equation in other systems (Restrepo and Kimmich, 1985), we have not shown this in HL60 cells. Also, there are probably small contributions of the Na$^+$ and Cl$^-$ gradients which make the membrane potential less negative than the K$^+$ equilibrium potential.

**Intracellular pH of HL60 Cells Is Not at Electrochemical Equilibrium**

The intracellular pH of HL60 cells was determined by three independent techniques: BCECF fluorescence, the intracellular distribution of the weak acid DMO, and the distribution of the weak base MA. Measurement of intracellular pH with weak acids and bases is subject to error because the compound accumulates in alkaline or acidic intracellular compartments, respectively. The intracellular pH measured with these compounds is given by:

$$pH_a = \log \sum_j f_j 10^{pH_j}, \quad pH_b = -\log \sum_j f_j 10^{-pH_j},$$

where $f_j$ and pH$_j$ are the fractional volume and the pH of the jth intracellular compartment (Roos and Boron, 1981).

We find that the intracellular pH estimated using the weak acid DMO is $7.12 \pm 0.02$ ($n = 4$), and using MA it is $6.46 \pm 0.01$ ($n = 3$). These results bracket the intracellular pH estimates using BCECF ($7.00 \pm 0.02$; $n = 19$). The relative volume of the acidic lysosomal compartment in HL60 cells is not known, but is probably high because the undifferentiated HL60 cell displays cytoplasmic granulation of the azurophilic type (Gallagher et al., 1979). The pH estimated with MA (6.46) would be expected if the true cytoplasmic pH is 7.00, the lysosomal pH is 5.3, and the relative lysosomal volume is 5%. These are reasonable estimates of lysosomal pH and frac-
tional volume (Bainton et al., 1971; Nichols and Bainton, 1973; Ohkuma and Poole, 1978; Styrk and Klemper, 1982). The pH estimates using DMO are expected to be better estimates of intracellular pH in HL60 cells because of the small number of mitochondria in the undifferentiated HL60 cell (Gallagher et al., 1979). Our results agree with the measurements of Simchowitz and Roos (1985) that they derived using human neutrophils, which show close agreement with the measurements of intracellular pH by DMO and 6-carboxyfluorescein, and a much lower estimate of intracellular pH by using the weak base MA.

The intracellular pH at electrochemical equilibrium \( \text{pH}_{eq} \) is determined by the membrane potential \( (V_m) \) and the extracellular pH \( (\text{pH}_o) \) as follows:

\[
\text{pH}_{eq} = \text{pH}_o + \frac{V_m}{(2.303RT/F)}.
\]

Using the two \( V_m \) values from above, we find that \( \text{pH}_{eq} \) is 6.0–6.1 for an extracellular pH of 7.4. Our estimates of intracellular pH using BCECF or DMO are 0.9–1.1 pH units higher than \( \text{pH}_{eq} \), and even the artifactually low intracellular pH found by using MA is 0.4–0.5 pH units higher than electrochemical equilibrium. This indicates that the cell must be actively extruding protons.

**Involvement of Na/H Exchange in pH Regulation of HL60 Cells**

Fig. 3 shows that, when the cytoplasm of HL60 cells is made acidic by the addition of nigericin in media without Na\(^+\), and then Na\(^+\) is added to the medium, a Na\(^+\)-dependent alkalization can be observed. This Na\(^+\)-dependent alkalization is inhibited by 20 \( \mu \)M of the Na\(^+\)/H\(^+\) exchange inhibitors DMA or EIPA (5-[N-ethyl-N-isopropyl]-amiloride). These data are in agreement with work of Besterman and Cuatrecasas (1984) who first showed that HL60 cells possess a Na\(^+\)/H\(^+\) exchanger.
If the Na\(^+\)/H\(^+\) exchanger is involved in keeping pH above electrochemical equilibrium, three criteria must be fulfilled: (a) the Na\(^+\) gradient must be away from electrochemical equilibrium in the proper magnitude and direction. (b) If the Na\(^+\) gradient is reversed, the intracellular pH must change. (c) If the exchanger is inhibited by addition of DMA or EIPA, the intracellular pH must change. As expected, the Na\(^+\) gradient is away from electrochemical equilibrium when measured by atomic absorption (Na\(^+\) \(=\) 15.65 ± 2.1, \(n = 3\); Na\(^+\) is ~140 mM, see Methods). With this driving force, an electroneutral Na\(^+\)/H\(^+\) exchanger could theoretically set intracellular pH to 8.3 at an extracellular pH of 7.4. However, when the Na\(^+\) gradient is reversed (by replacing extracellular Na\(^+\) with TMA\(^+\) and choline, or by replacing Na\(^+\) with K\(^+\)) or when the exchanger is inhibited by the addition of DMA or EIPA, the intracellular pH does not change significantly (Table II and Fig. 6). This indicates that, although thermodynamically the Na\(^+\)/H\(^+\) exchanger could actively par-

Table II

| Solution | Inhibitor | Change in pH | Net initial H\(^+\) influx |
|----------|-----------|--------------|---------------------------|
| RP       | DMA       | -0.009 ± 0.004(5) | 0.05 ± 0.14(5) |
| RP       | EIPA      | 0.00 ± 0.03(2) | NA |
| RP no Na\(^+\) | —        | 0.05 ± 0.05(4) | NA |
| RK       | —         | 0.20 ± 0.07(5) | NA |

Solutions are detailed in Methods and values are shown as mean ± SEM. Briefly, RP is our standard solution with ~140 mM Na\(^+\). In RP no Na\(^+\), Na\(^+\) bicarbonate is replaced with choline bicarbonate and other Na\(^+\) salts are replaced with TMA\(^+\) salts. In RK all Na\(^+\) salts are replaced by K\(^+\) salts. pH change is reported with respect to RP alone and was measured at steady state (when the intracellular pH stabilized 4–6 min after ion replacement). Control intracellular pH is 7.0. A t test indicates that the changes in pH are not significant at the 0.05 level. The change with RK is marginally significant (\(P = 0.07\)), but in the opposite direction from that expected if the Na\(^+\)/H\(^+\) exchanger were regulating the steady state pH. NA indicates that initial rates could not be measured in these cases.

Involvement of Cl\(^-\)/HCO\(_3^-\) Exchange in pH Regulation in HL60 Cells

Fig. 4 A shows that isotopic Cl\(^-\) exchange in undifferentiated HL60 cells is inhibited by the Cl\(^-\)/HCO\(_3^-\) exchange inhibitor dihydro-4'4'-dinitrostilbene-2,2'-disulfonic acid (H\(_2\)DIDS). Dissing and co-workers (1984) have shown that this isotopic Cl\(^-\) flux in HL60 cells has the properties of electroneutral exchange. Fig. 4 B shows that, as expected if this transport system exchanges Cl\(^-\) for HCO\(_3^-\), a Cl\(^-\)-dependent acidification can be measured at high intracellular pH, which is inhibited by the addition of H\(_2\)DIDS.

To test whether or not the Cl\(^-\)/HCO\(_3^-\) exchanger is setting intracellular pH above
electrochemical equilibrium, we monitored the effect of changes in the Cl\(^-\) gradient on intracellular pH. Fig. 5A shows that if the chloride chemical gradient is reversed by replacing extracellular Cl\(^-\) with gluconate, there is a dramatic increase in intracellular pH, which is as expected if intracellular Cl\(^-\) is exchanged for extracellular HCO\(_3^-\) (see also Table III). This alkalinization is inhibited by H\(_2\)DIDS (Fig. 5A and Table III) and is absent in media without bicarbonate (Fig. 5B). On the other hand, the steady state intracellular pH decreases upon the addition of 1 mM H\(_2\)DIDS (Fig. 6A and Table III). These observations indicate that the Cl\(^-\)/HCO\(_3^-\) exchanger is actively involved in setting intracellular pH above electrochemical equilibrium. Fig. 6A and Table III also show that intracellular pH decreases even more if both H\(_2\)DIDS and DMA are added simultaneously even though the initial rates of proton influx are the same for the addition of H\(_2\)DIDS in the presence or absence of DMA. This is expected if, in the absence of DMA, the Na\(^+\)/H\(^+\) exchanger is activated as intracellular pH decreases (Restrepo et al., 1987), which would thereby limit the extent of a decrease in pH, but not in its initial rate.

**Figure 4.** Anion exchange in undifferentiated HL60 cells. (A) \(^{86}\)Cl\(^-\) efflux in undifferentiated HL60 cells is inhibited by H\(_2\)DIDS (\(^{86}\)Cl\(^-\) exchange was assayed as explained in Methods). The solution used in this experiment is RP (which contains 109.65 mM Cl\(^-\) and 20 mM HCO\(_3^-\)). Open circles, control (rate constant = 0.5 min\(^{-1}\)); closed circles, 10 μM H\(_2\)DIDS (0.49 min\(^{-1}\)); open triangles, 100 μM H\(_2\)DIDS (0.314 min\(^{-1}\)); closed triangles, 1 mM H\(_2\)DIDS (0.07 min\(^{-1}\)). (B) When intracellular pH is increased by replacing external Cl\(^-\) with gluconate and by adding of 15 μM monensin (10\(^6\) cells/ml in 1.2 ml of HNG medium), addition of 50.8 mM Cl\(^-\) (open circles) triggers cytoplasmic acidification (Cl\(^-\) was added by diluting cells with HNC). The Cl\(^-\)-dependent acidification is inhibited by adding 1 mM H\(_2\)DIDS simultaneously with Cl\(^-\) (closed circles). The time span between points is 0.5 min.
Even with both DMA and H$_2$DIDS present, the pH does not decrease to the level expected for electrochemical equilibrium. If we consider, for simplicity's sake, a system with a proton conductance (which follows the Goldman flux equation) and the Cl$^-$/HCO$_3$ exchanger, then the pH should have fallen to 6.1–6.2 upon the addition of 1 mM H$_2$DIDS (90% inhibition according to Fig. 4 A). However, one should take into consideration that by lowering intracellular pH from 7.0 to 6.8, the intracellular HCO$_3$ concentration is halved. This reduction in the bicarbonate gradient could stimulate the anion exchanger by an amount determined by the kinetic characteris-

**TABLE III**

*Effect of Na$^+$/H$^+$ and Cl$^-$/HCO$_3$ Exchange Inhibitors and of Replacement of Extracellular Cl$^-$ on Intracellular pH in Undifferentiated HL60 Cells*

| Solution     | Addition                  | Change in pH       | Net initial H$^+$ influx | mM H$^+$/min |
|--------------|---------------------------|--------------------|--------------------------|--------------|
| RP no Cl$^-$ |                           |                    |                          |              |
| RP no Cl$^-$ | H$_2$DIDS                 | 0.86 ± 0.16(5)     | NA                       |              |
| RP           | H$_2$DIDS                 | 0.02 ± 0.01(2)     | NA                       |              |
| RP           | H$_2$DIDS + DMA           | -0.11 ± 0.01(7)    | 3.13 ± 0.39(9)           |              |
| RP           | PCP                       | -0.22 ± 0.03(7)    | 2.83 ± 0.34(5)           |              |
| RP           | PCP + EIPA                | -0.40 ± 0.05(2)    | NA                       |              |
| RP           | PCP + EIPA                | -0.79 ± 0.06(2)    | NA                       |              |

Compositions of solutions are shown in Methods and values are shown as mean ± SEM (n). RP has 109.7 mM Cl$^-$ and RP no Cl$^-$ has 0.75 mM Cl$^-$. Both solutions have 20 mM HCO$_3$. Control intracellular pH is 7.00. The change in intracellular pH in RP no Cl$^-$ plus H$_2$DIDS is not significantly different from zero. All other values are significantly different from zero (t test at the 0.05 level). The concentration of H$_2$DIDS is 1 mM, and of DMA and EIPA it is 20 μM. NA denotes cases in which initial rates were not measured.
tics of the exchanger. On the other hand, the rate of Na\(^+\)/H\(^+\) exchange in HL60 cells is stimulated by intracellular acidification (Restrepo et al., 1987), and the proton conductance may be dependent on intracellular pH. Thus, it may be possible that after the addition of H\(_2\)DIDS and DMA, the small proton efflux rates mediated by the inhibited Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)exchangers are able to balance the sum of the influx of proton equivalents from outside and the production of acid equivalents by metabolism. If this is so, the addition of more potent inhibitors of Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\) exchange should lead to larger changes in intracellular pH. In agreement with this premise, Fig. 6 B and Table III show that when EIPA, which is six times more potent than DMA as an inhibitor of Na\(^+\)/H\(^+\) exchange (Vigne et al., 1983), and pentachlorophenol (PCP), an inhibitor of the red blood cell anion exchanger (Motaïs et al., 1978), which is four times more potent than H\(_2\)DIDS as an inhibitor of the Cl\(^-\)/HCO\(_3\) exchanger in HL60 cells (our own unpublished data), are added to HL60 cells, there is a 0.8 pH unit drop in intracellular pH.

These data suggest that the Cl\(^-\)/HCO\(_3\) exchanger plays an important role in keeping intracellular pH away from electrochemical equilibrium, but they do not rule out the existence of other pH regulatory mechanisms acting in parallel with the Cl\(^-\)/HCO\(_3\) exchanger to keep intracellular pH above electrochemical equilibrium (these other mechanisms must be Na\(^+\)-independent because of the failure of Na\(^+\) replacement to elicit a pH change, see Table II).
Transmembrane Cl⁻ Gradient in HL60 Cells

The Cl⁻/HCO₃⁻ exchanger can increase intracellular pH above electrochemical equilibrium only if intracellular Cl⁻ itself is above electrochemical equilibrium. To explore the energetic status of the Cl⁻ gradient, we have measured total exchangeable Cl⁻ in HL60 cells with a triple label technique using ³⁶Cl⁻ to measure exchangeable Cl⁻, H₂O to measure total water and [¹⁴C]sucrose to measure extracellular fluid (see Methods). The results (Table V) indicate that intracellular Cl⁻ is 35.7 ± 1.8 (mean ± SEM, n = 3, for a nominal Cl⁻ = 109.65 mM). This value is about one order of magnitude larger than the Cl⁻ concentration at electrochemical equilibrium (3–5 mM using the membrane potential values estimated above).

The Cl⁻ gradient calculated with an intracellular Cl⁻ of 35.7 mM is roughly equal to the transmembrane proton gradient (see Discussion). From the point of view of thermodynamics, two mutually exclusive situations are possible: the proton gradient could be set by the Cl⁻/HCO₃⁻ exchanger at the expense of energy supplied by the Cl⁻ gradient, or vice versa. Fig. 7 A shows that, when extracellular Cl⁻ is lowered in a HCO₃⁻-free medium, intracellular Cl⁻ decreases, but the chloride gradient ([Clᵢ⁻]/[Clₒ⁻]) increases. This contrasts with intracellular pH (Fig. 7 B), which does not change when extracellular Cl⁻ is lowered under the same HCO₃⁻-free conditions. Thus, at low extracellular Cl⁻ the transmembrane Cl⁻ gradient is three to four times larger than the transmembrane HCO₃⁻ (or OH⁻) gradient. This experiment shows that intracellular Cl⁻ is brought above electrochemical equilibrium by mechanisms independent of the pH gradient and suggests that the Cl⁻ gradient is not set above electrochemical equilibrium by the Cl⁻/HCO₃⁻ exchanger.

We have not identified the transport system responsible for keeping Cl⁻ above electrochemical equilibrium, but we can estimate the magnitude of this transport component as follows: if in the steady state Cl⁻ loss through Cl⁻/HCO₃⁻ exchange is balanced by Cl⁻ uptake through some other transport pathway, then this uptake should be equal to the H⁺ influx measured after addition of H₂DIDS, which is ~3 mmol/liter cell water per min as shown in Table III. This transport system cannot be an H₂DIDS-inhibitable Cl⁻/HCO₃⁻ exchanger, or a bumetanide- or furosemide-sensitive Na⁺/K⁺/2Cl⁻ cotransport system (Haas and McManus, 1983) because, as shown in Table IV, the net Cl⁻ loss from HL60 cells after the addition of these inhibitors is much smaller than the magnitude of the H⁺ influx calculated above.

Regulation of Intracellular pH in DMSO-differentiated HL60 Cells

HL60 cells kept in medium with 1.25% DMSO for 5–6 d differentiate into neutrophil-like cells. Table V shows that the intracellular pH, K⁺, and Cl⁻ concentrations of these cells are not significantly different from those of undifferentiated HL60 cells, even though there is a large decrease in cell volume. The intracellular pH of HL60 cells differentiated along the granulocytic pathway with 1 μM retinoic acid for 5–6 d (7.02 ± 0.04, n = 12) was also not significantly different from the intracellular pH of undifferentiated or DMSO-differentiated cells.

Table VI shows that, as in undifferentiated cells, the intracellular pH of neutrophil-like cells increases when extracellular Cl⁻ is replaced by gluconate, and that this increase is inhibited by H₂DIDS. In contrast, if Na⁺ is replaced by TMA⁺ and chol-
[Text from the page]

**DISCUSSION**

Since the membrane potential of HL60 cells is ~-80 mV, and since intracellular pH is 7.0 at an extracellular pH of 7.4, cells must extrude protons to counteract a net increase in intracellular acid equivalents mediated by inflow of H⁺ (or by out-

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**TABLE IV**

**Net Intracellular Cl⁻ Changes after the Addition of Bumetanide, Furosemide, or H₂DIDS**

| Compound        | Concentration | Net Cl⁻ loss  | n  |
|-----------------|---------------|---------------|----|
|                 | µM            | mM/min        |    |
| No addition     | -0.05 ± 0.1   | 5             |
| Bumetanide      | 100           | -0.03 ± 0.14  | 2  |
| Furosemide      | 1,000         | 0.20 ± 0.07   | 2  |
| H₂DIDS          | 1,000         | 0.13 ± 0.09   | 4  |

Intracellular Cl⁻ was measured every 5 min for a total of 25 min after the addition of the drugs to cells incubated in RP.
TABLE V

Intracellular K⁺, Cl⁻, pH, and Volume of DMSO-differentiated and
-undifferentiated HL60 Cells

| Quantity measured | Undifferentiated cells | DMSO-differentiated cells |
|-------------------|------------------------|--------------------------|
| K⁺ (mM)           | 148 ± 7(6)             | 150 ± 20(3)              |
| Cl⁻ (mM)          | 35.7 ± 1.8(6)          | 33.5 ± 3.4(2)            |
| pH                | 7.00 ± 0.02(19)        | 6.98 ± 0.05(19)          |
| Volume (fl/ceil)  | 773 ± 27(7)            | 340 ± 28(4)              |

All values are mean ± SEM (n). pH values were measured using the BCECF technique, and K⁺, Cl⁻, and cell volume was measured using isotopic techniques explained in Methods.

Flow of OH⁻ or HCO₃⁻ through conductance pathways and by metabolic acid production. The Na⁺/H⁺ exchanger is not active at steady state intracellular pH as indicated by the lack of dependence of intracellular pH on extracellular Na⁺ and by the absence of changes in intracellular pH upon the addition of Na⁺/H⁺ exchange inhibitors. Hence, the Na⁺/H⁺ exchanger in HL60 cells is not involved in keeping intracellular pH at its steady state value, but rather may serve to protect the cell from severe cytoplasmic acidification, as is suggested by its activation by acidic intracellular pH, which is shown in Fig. 3 and in previous work (Restrepo et al., 1987). The exchanger can also act to change intracellular pH when cells are stimulated by osmotic shock, activation of kinase C, or an increase in intracellular free Ca (Restrepo et al., 1987, and unpublished observations). Similar quiescence of the Na⁺/H⁺ exchanger at steady state intracellular pH has been observed in human neutrophils (Simchowitz and Roos, 1985; Grinstein and Furuya, 1986) and in rat lymphocytes (Grinstein et al., 1984).

Replacement of extracellular Cl⁻ by gluconate in media containing bicarbonate triggers an increase in intracellular pH. This indicates that a Cl⁻ and HCO₃⁻-dependent transport system can transport H⁺ equivalents at a rate greater than or comparable to that of other H⁺ transport systems that are active at steady state intracellular pH.

TABLE VI

Effect of H₂DIDS, H₂DIDS Plus DMA, and of Replacement of Extracellular Cl⁻ and Na⁺ on Intracellular pH in DMSO-differentiated HL60 Cells

| Solution | Addition | Change in pH | n   |
|----------|----------|--------------|-----|
| RP no Na⁺|          | 0.05 ± 0.02* | 4   |
| RK       |          | 0.13 ± 0.02* | 3   |
| RP no Cl⁻|          | 1.00 ± 0.14† | 4   |
| RP no Cl⁻| H₂DIDS   | 0.08         | 1   |
| RP       | DMA      | −0.05 ± 0.01†| 5   |
| RP       | H₂DIDS   | −0.12 ± 0.02†| 6   |
| RP       | H₂DIDS + DMA | −0.32 ± 0.04† | 8 |

Ion contents of different solutions are given in Methods. DMA was 20 μM and H₂DIDS 1 mM.

*Marginal significance (P between 0.051 and 0.15).
†Significantly different from zero (P < 0.05).
lar pH. This transport system is probably not a Na+-dependent Cl-/HCO₃⁻ exchanger (L'Allemain et al., 1985) since replacement of extracellular Na⁺ produces no change in intracellular pH. The other likely candidate is a stilbene-inhibitable anion exchanger, which was shown to exist in HL60 cells by Dissing et al. (1984), that could exchange Cl⁻ for HCO₃⁻.

If this Cl⁻/HCO₃⁻ exchanger is active at steady state intracellular pH, intracellular pH should change if the system is inhibited. As shown in Table III, we find that intracellular pH decreases after the addition of 1 mM H₂DIDS or of 200 μM PCP, both inhibitors of the anion exchanger in HL60 cells, which indicates that a stilbene- and PCP-sensitive transport system is actively extruding H⁺ equivalents. This is consistent with an involvement of the Cl⁻/HCO₃⁻ exchanger in setting intracellular pH above electrochemical equilibrium, but does not preclude the possibility that some other stilbene- and PCP-inhibitable transport system is also participating in setting the steady state intracellular pH.

Another requisite for participation of Cl⁻/HCO₃⁻ exchange in setting intracellular pH to 7.00 is that the transmembrane Cl⁻ gradient must be away from electrochemical equilibrium. We find that the Cl⁻ gradient is indeed away from electrochemical equilibrium (intracellular Cl⁻ = 35.7 mM and extracellular Cl⁻ = 109.65 mM). The equilibrium intracellular pH that could be set by this Cl⁻ gradient for a 1:1, Cl⁻/HCO₃⁻ exchanger is:

$$pH_i = pH_o + \log \left( \frac{aCl^-}{aCl_o} \right), \quad (8)$$

where $aCl_i$ and $aCl_o$ are the intracellular and extracellular Cl⁻ activities, respectively. For an extracellular pH of 7.4, and assuming that the activity coefficient for Cl⁻ is the same in the cytoplasm as in the extracellular medium, Eq. 8 gives an equilibrium pH of 6.91, which is 0.09 pH units lower than the steady state intracellular pH of 7.00. This difference is significant at the 0.05 level using a t test.

It is likely that this apparent discrepancy is due to small errors in the calculation of the intracellular Cl⁻ concentration and pH, or to differences between internal and external activity coefficients. Alternatively, it is possible that intracellular anions other than Cl⁻ (e.g., lactate or pyruvate) could contribute to the driving force for HCO₃⁻. If some other intracellular anion is involved, it must also be away from electrochemical equilibrium. The other possibility is that the Cl⁻/HCO₃⁻ exchanger does have a set point at intracellular pH 6.91 and that another yet unidentified stilbene- and PCP-inhibitable transport system is responsible for setting intracellular pH. This system cannot be the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger postulated to exist in HL60 cells by Ladoux and co-workers (1987) because of the lack of an effect of extracellular Na⁺ removal on intracellular pH.

In conclusion, undifferentiated HL60 cells keep their intracellular pH 0.9–1.1 pH units above electrochemical equilibrium. The DMA-sensitive Na⁺/H⁺ exchanger does not contribute to maintaining the intracellular pH above electrochemical equilibrium. In contrast, Cl⁻ replacement experiments, and the effect of anion exchange inhibitors on intracellular pH, suggest a role for the Cl⁻/HCO₃⁻ exchanger in maintaining intracellular pH above electrochemical equilibrium. Measurement of the magnitude of the Cl⁻ gradient indicates that if the Cl⁻/HCO₃⁻ exchanger contributes to maintaining intracellular pH, it does so in a unique man-
ner, since intracellular pH is kept at a value such that the transmembrane chemical gradient for protons is nearly equal to the Cl\(^-\) gradient. As a corollary, since the driving forces are near equilibrium, the net fluxes of protons and Cl\(^-\) through the exchanger are small compared with the unidirectional fluxes. The advantage of such a near-equilibrium situation from the point of view of intracellular pH homeostasis is that large fractional increases in the factors that contribute to intracellular pH acidification (proton conductance, metabolic acidification, etc.) are of small magnitude compared with the unidirectional fluxes through the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger, and hence will lead to small changes in intracellular pH. Thus, near equilibrium, the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger is very efficient at maintaining intracellular pH at a set point. This premise is consistent with our observation that a large fractional inhibition of the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger is needed to decrease intracellular pH in the undifferentiated HL60 cell.

In contrast to the studies of Ladoux et al. (1987) we find no change in intracellular pH upon DMSO- or retinoic acid–induced cell differentiation. This discrepancy could be due to differences in the intracellular pH assay conditions since, in contrast to Ladoux et al. (1987), we assayed intracellular pH in HCO\(_3\)\(^-\)-containing media at 37°C. Regardless of the reason for the discrepancy, our observations indicate that HL60 cells can differentiate along the granulocytic pathway under conditions in which there is no change in intracellular pH. Thus, changes in intracellular pH are not causally related to cell differentiation in HL60 cells.

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REFERENCES

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. Journal of Experimental Medicine. 134:907–934.

Besterman, J. M., and P. Cuatrecasas. 1984. Phorbol esters rapidly stimulate amiloride-sensitive Na/H exchange in human leukemic cell line. Journal of Cell Biology. 99:340–343.

Collins, S., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1978. Terminal differentiation of promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proceedings of the National Academy of Sciences, USA. 75:2458–2462.

Dissing, S., R. Hoffman, M. J. Murane, and J. F. Hoffman. 1984. Chloride transport properties of human leukemic cell lines K562 and HL60. American Journal of Physiology. 247(Cell Physiology. 16):C53–C60.

Freedman, J. C., and J. F. Hoffman. 1979. Ionic and osmotic equilibria of human red blood cells treated with nystatin. Journal of General Physiology. 74:157–185.

Gallagher, R., S. Collins, J. Trujillo, K. McCredie, M. Ahearn, S. Tsai, R. Metzgar, G. Aulakh, R. Ting, F. Ruscetti, and R. Gallo. 1979. Characterization of the continuous differentiating myeloid cell line (HL60) from a patient with acute promyelocytic leukemia. Blood. 54:713–733.
Grinstein, S., S. Cohen, and A. Rothstein. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na/H antiport. *Journal of General Physiology.* 83:341–369.

Grinstein, S., and W. Furuya. 1986. Characterization of the amiloride-sensitive Na-H antiport of human neutrophils. *American Journal of Physiology.* 250(Cell Physiology. 19):C283–C291.

Haas, M., and T. J. McManus. 1988. Bumetanide inhibits (Na+K+2Cl) cotransport at a chloride site. *American Journal of Physiology.* 245:C235–C240.

Kimmich, G. A., J. Randles, D. Restrepo, and M. Montrose. 1985. A new method for determination of relative ion permeabilities in isolated cells. *American Journal of Physiology.* 248(Cell Physiology. 17):C399–C405.

Koeffler, H. P., M. Bar-Eli, and M. C. Territo. 1981. Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation. *Cancer Research.* 41:919–926.

Ladoux, A., E. J. Cragoe, Jr., B. Geny, J. P. Abita, and C. Frelin. 1987. Differentiation of human promyelocytic HL60 cells by retinoic acid is accompanied by an increase in the intracellular pH. *Journal of Biological Chemistry.* 262:811–816.

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

Moore, G. E., R. E. Berner, and H. A. Franklin. 1967. Culture of normal human leukocytes. *Journal of the American Medical Association.* 199:519–524.

Motais, R., F. Sola, and J. L. Cousin. 1978. Uncouplers of oxidative phosphorylation. A structure-activity study of their inhibitory effect of passive chloride permeability. *Biochimica et Biophysica Acta.* 510:201–207.

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

Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of intralysosomal pH in living cells and the perturbation of pH by various agents. *Proceedings of the National Academy of Sciences, USA.* 75:3327–3331.

Restrepo, D., and G. A. Kimmich. 1985. The mechanistic nature of the membrane potential dependence of sodium-sugar cotransport in small intestine. *Journal of Membrane Biology.* 87:159–172.

Restrepo, D., D. J. Kozody, and P. A. KnauL 1987. Changes in Na⁺-H⁺ exchange regulation upon granulocytic differentiation of HL60 cells. *American Journal of Physiology.* 253:C619–C624.

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

Simchowitz, L., and A. Roos. 1985. Regulation of intracellular pH in human neutrophils. *Journal of General Physiology.* 85:443–470.

Styrt, B., and M. S. Kempler. 1982. Internal pH of human neutrophil lysosomes. *FEBS Letters.* 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.

Vigne, P., C. Frelin, E. J. Cragoe, and M. Lazdunski. 1983. Structure-activity relationships of amiloride and certain of its analogues in relation to the blockade of the Na⁺/H⁺ exchange system. *Molecular Pharmacology.* 25:131–136.