

## $\Delta$ Alkalinity: a simple method to measure cellular net acid-base fluxes

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BURBEA, ZVI-HAI, STEVEN R. GULLANS, AND SAM BEN-YAAKOV.  $\Delta$ Alkalinity: a simple method to measure cellular net acid-base fluxes. Am. J. Physiol. 253 (Cell Physiol. 22): C525-C534, 1987.—Acid-base transport across cell plasma membranes is important for cell homeostasis and growth. Current techniques for quantitatively measuring net acid-base fluxes are generally limited by either assumptions concerning properties of intracellular compartments or use of poorly buffered, nonphysiological solutions. We adapted an approach from marine chemistry to quantitate net acid-base changes in standard physiological media that obviates these problems. This method is based on conservation of charge and involves a simple acid titration of the extracellular medium to an end point, the equivalence point,  $\text{pH}_e$ . For standard physiological solutions containing buffers such as bicarbonate and phosphate,  $\text{pH}_e$  exists in the range of pH 4.0–4.7 and is identified as the pH where  $\text{dpH}/\text{dH}^+$  is maximal in an HCl titration. By determining the quantity of  $\text{H}^+$  required to reach  $\text{pH}_e$ , one can determine precisely total quantity of proton acceptors (alkalinity) present in physiological pH range. Alkalinity (in meq) is a relative measure of the charge capable of interacting with protons. We show that, unlike pH, changes in alkalinity ( $\Delta$ alkalinity) result only from net acid-base changes in medium. Therefore, by monitoring extracellular  $\Delta$ alkalinity associated with cell function, it is possible to quantitate precisely net acid-base fluxes. Moreover, through a second titration procedure,  $\Delta$ alkalinity can be divided into bicarbonate and nonbicarbonate fractions. As an example, we performed the first direct measurement of net  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchange in intact human erythrocytes and observed a  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchange ratio of  $1.01 \pm 0.03$ . Overall,  $\Delta$ alkalinity measurements are applicable to numerous cell systems, can be performed with solutions containing a mixture of buffers at normal physiological concentrations (e.g., 25 mM  $\text{HCO}_3^-$  and 2 mM  $\text{HPO}_4^{2-}$ ), do not require corrections for  $\text{CO}_2$  diffusion or loss of  $\text{CO}_2$  from the solution, and avoid assumptions about intracellular or extracellular buffer properties.

acid titration; hydrogen ion transport; bicarbonate transport; phosphate transport; intracellular pH; pH-stat; chloride-bicarbonate exchange; red blood cell

ACID-BASE REGULATION is a fundamental function of cells, and the mechanisms responsible for cellular pH regulation have been the subject of intensive investigation (19). In addition, external stimuli such as hormones and tumor promoters are known to cause changes in intracellular pH that may act as a signal for cell growth and other physiological processes (4, 11).

A variety of techniques exists to measure intracellular

or extracellular acid-base changes including fluorescence spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, pH electrodes, and weak acid (DMO) distribution (17, 18). Despite the widespread acceptance of these techniques in monitoring intracellular pH, questions concerning compartmentation, calibration, or intracellular buffering have generally thwarted accurate quantitation of acid or base fluxes. In contrast, when  $\text{CO}_2$ -mediated pH changes can be neglected, the pH-stat provides a very sensitive measurement of  $\text{H}^+$  fluxes (24). This technique involves bathing the tissue in a medium of very low buffer capacity (i.e.,  $\leq 1$  mM) and measuring the quantity of  $\text{OH}^-$  required to maintain a constant pH in the medium. As a result, the pH-stat is generally limited in its ability to study cell function at normal physiological buffer concentrations.

Conceptually, acid-base transport studies generally treat the initial pH of the intracellular or extracellular medium as the reference point and increases or decreases in pH are appropriately ascribed to net acid or base gain or loss. A problem with this approach, however, is that some cell processes produce pH changes in the absence of net acid or base fluxes. For example, changes in cell volume or relative buffer contents can alter pH without a net change in acid content. In addition, cells metabolizing glucose to  $\text{CO}_2$  will acidify their environment. However, unlike production of lactic acid from glucose, this  $\text{CO}_2$ -related decrease in pH is not associated with a net change in acid content relative to the base content because equivalent quantities of  $\text{H}^+$  and  $\text{HCO}_3^-$  are generated.

To overcome these problems, we have applied an acid titration technique to measure directly net changes in acid-base content in physiological media. This method, termed alkalinity titration, has been used successfully in marine chemistry for >30 yr to evaluate acid-base changes in lakes and oceans (6, 9, 10, 20). The titration method defines a reference pH value, the equivalence point ( $\text{pH}_e$ ) for determining the relative quantity of proton acceptors, termed alkalinity, in a solution. Alkalinity is a relative measure of the charge capable of combining with protons. Our results indicate that a change in alkalinity, termed  $\Delta$ alkalinity, is a direct quantitative measure of net acid-base changes in physiological solutions. Therefore, by sampling the extracellular solution at various time points and performing a simple acid titration, it is possible to measure with great sensitivity

the acid or base uptake or efflux rates associated with cell function. In addition, the theoretical framework for this method provides a potentially new approach for evaluation the significance of pH and acid-base in physiological systems.

## METHODS

The pH titration procedures were performed with an automated titrator system as described previously by this laboratory (2, 3). Either 50- or 1-ml samples were placed in an appropriate thermoregulated (22 or 37°C) chamber and stirred on a magnetic stir plate. pH was measured with a glass electrode (Metrohm E-124) and, when necessary, PCO<sub>2</sub> was monitored with a PCO<sub>2</sub> electrode (Orion, Cambridge, MA). The electrodes were connected via homemade electrometers to either a CBM 3032 Commodore or a Leading Edge model D computer. Titrations were performed by discrete HCl (0.1 or 1.0 N) additions (0.001 or 0.1 ml) with the use of an autotitrator (Metrohm, Brinkman Instrument, Westbury, NY) that was controlled by the computer as previously described (2, 3). Because physiological buffer solutions have a significantly greater buffer content than water from lakes and oceans, the protocol traditionally used by marine chemists was modified such that the titration was performed in two stages. Above pH 5.6, HCl additions were made only when the change in pH resulting from the preceding HCl addition was <0.01 units in 5 s, whereas below pH 5.6, HCl was added only when the pH change was <0.002 units in 5 s. For each titration, an algebraic polynomial was fit to the data, the alkalinity was calculated, and figures were plotted with an X-Y plotter (Textronix no. 4662, Beaverton, OR). In some cases, titrations were performed manually on a stirred, open 50-ml sample by adding 100 μl of 0.5 N HCl every 30 s until the pH reached 5.8 and then adding progressively smaller HCl aliquots about every minute. These manual titrations were done slowly so that each addition was made only after the pH had equilibrated with the previous addition. These manual titrations were monitored with a Markson pH electrode connected through an Orion pH meter (model 701 A) to a chart recorder (Kipp-Zonen, Holland).

Titrations were performed on several solutions including a Krebs-bicarbonate buffer solution, which contained the following: 115 mM NaCl, 25 mM NaHCO<sub>3</sub>, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 2.0 mM NaH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub>, 1.2 mM CaCl<sub>2</sub>, and 10 mM glucose. This solution was prepared by sequentially combining 100 ml of KCl (stock of 50 mM), 100 ml of NaCl (stock of 1.15 M), 100 ml of MgSO<sub>4</sub>·7H<sub>2</sub>O (stock of 10 mM), 100 ml of Na<sub>2</sub>HPO<sub>4</sub> (stock of 20 mM), 100 ml of CaCl<sub>2</sub> (stock of 12 mM), and 300 ml of distilled H<sub>2</sub>O. This solution had a pH of 3.8–4.0. From this point on, the solution was bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Next, the solution was titrated to pH 5.5 with 1–1.5 ml of 1 N NaOH and 100 ml of NaHCO<sub>3</sub> was added from a 250 mM stock solution that had been bubbled with 10% CO<sub>2</sub>. The glucose was next added as a powder and the volume was completed to 1 liter, having a pH of 7.25–7.33 at room temperature. This solution was then warmed to 37°C and any slight adjustments in pH were made with NaOH or HCl to obtain a final pH

of 7.4. The concentrations of the solutes in this and other solutions prepared by us were established using an analytical balance. In addition, alkalinity determinations were performed on three solutions often used in cell culture media: Hanks' balanced salt solution (no. 310-4020), Dulbecco's modified Eagle's medium (no. 320-1965), and Ham's F12 solution (no. 320-1765). These solutions were obtained from GIBCO (Grand Island, NY).

*Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange in human red blood cells.* Fresh human blood was collected in a heparinized syringe (30 ml), transferred to four 15-ml centrifuge tubes, and centrifuged at 2,000 g for 5 min at 4°C. The plasma and buffy coat were discarded and the red blood cells were suspended in a bicarbonate buffer solution containing 100 mM KHCO<sub>3</sub>, 100 mM sucrose, 1% dialyzed, defatted albumin, and bubbled with 90% O<sub>2</sub>-10% CO<sub>2</sub> (pH 7.6). The cells were centrifuged once more, resuspended in the bicarbonate buffer to a hematocrit of 30%, and kept at 4°C. Over the next 6 h the cells were washed three more times with the bicarbonate buffer to load the cells with bicarbonate. To initiate the Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange experiment, cells were centrifuged (2,000 g) for 5 min, the supernatant was discarded, and ~1.5 ml of the packed cells were added to a 15-ml test tube containing 3.5 ml of solution composed of 100 mM KCl and 100 mM sucrose (pH 7.1). The hematocrit was 26 ± 2% (n = 7). After 10 min at room temperature, the cells were pelleted (2,000 g), and aliquots of the supernatant were taken for determination of extracellular chloride and alkalinity. During the experiment there was no detectable hemolysis. Chloride was assayed in duplicate using a Buchler chloridometer. Data are expressed as means ± SE.

## RESULTS

### *Theoretical*

It is well recognized that the apparent pK<sub>a</sub>s of buffers are sensitive to ionic strength, temperature, pressure, and numerous other physical factors (6, 22, 25). Moreover, in solutions of mixed buffers, the relative concentrations of the buffers are important determinants of pH. Considering this, the pursuit of acid-base balance in physiological fluids cannot be answered by a simple pH measurement. Rather, it must be accomplished by using an independent parameter for proton deficit or surplus, which is unaffected by ionic strength, temperature, and relative buffer quantities. The parameter "alkalinity" satisfied these criteria (6, 25). Consequently, by measuring Δalkalinity one can precisely quantitate relative changes in (free and bound) protons.

*Definition of alkalinity.* To measure changes in the proton content of a solution it is necessary to define a measurable reference point that is insensitive to non-acid-base changes which are likely to occur in physiological solutions. If this reference point is taken as zero (or constant) proton content, any net deficit or surplus of protons in a given solution can be determined by titrating the solution with a strong acid or base to this reference point. In physiology, pH 7.4 is often chosen as the reference point but this value is inherently unreliable

because non-acid-base changes can have dramatic effects. A more suitable yet convenient reference point is the equivalence point, which for a bicarbonate-buffered solution is the equivalence point for the formation of carbonic acid from bicarbonate, i.e., when  $[H^+] = [HCO_3^-]$ . For physiological fluids, this equivalence point occurs between pH 4.0 and 4.7 and it can be determined by either a Gran-type titration (9, 10) or the end-point method (6, 15). The latter is defined as the relevant deflection point in an acid titration curve or the maximum of the titration curve derivative. The simpler procedure to determine the equivalence point, titration to the end point, has been adopted in this study. Using this reference point, physiological solutions possess a relative excess of proton acceptors (i.e.,  $HCO_3^-$  and  $HPO_4^-$ ) known as alkalinity.

Total alkalinity is defined as the total charge capable of combining with protons over the physiological pH range [i.e., pH 4.9 (urine)–7.8]. This working definition divides the negative charge of a solution into two domains: protolytic (proton combining) species and non-protolytic species. Using electroneutrality and mass balance it is possible to derive an expression for alkalinity in terms of the ionic components of a physiological solution. The following expression states that the total cations equals the protolytic anions plus the nonprotolytic anions

$$H^+ + Q_i^+ = B_i^- + OH^- + A^- \quad (1)$$

where  $Q_i^+$  represents all of the cations except  $H^+$ ,  $B_i^-$  represents all of the protolytic anions except  $OH^-$ , and  $A^-$  represents the nonprotolytic anions. Rearranging this expression to separate the nonprotolytic cations and anions from the protolytic anions and protons gives the following

$$Q_i^+ - A^- = (B_i^- + OH^-) - H^+ \quad (2)$$

Based on this equation, alkalinity is defined as

$$\text{alkalinity} = (B_i^- + OH^-) - H^+ \quad (3)$$

In other words, alkalinity is the quantity of protolytic anions ( $B_i^- + OH^-$ ) minus the quantity of protons ( $H^+$ ) in a solution. Note, however, that for a given fluid volume, concentration terms are equally valid. To identify and measure alkalinity, it is necessary to define a reference value, the equivalence point.

The equivalence point,  $pH_e$ , is the pH at which alkalinity is zero, hence

$$[H^+] = [B_i^-] + [OH^-] \quad (4)$$

The equivalence point can be recognized from an acid titration as the deflection point, i.e., where  $dpH/dH^+$  is maximal. (For a more detailed discussion of the equivalence point,  $pH_e$ , see the APPENDIX.) For physiological solutions, an equivalence point can be identified in the pH range of 4.0–4.7. Consequently, relative to this reference point, the protolytic species ( $B_i^-$ ) of a physiological solution can be identified and substituted into Eq. 3. These chemical species include the inorganic carbon buffers,  $HCO_3^-$  and  $CO_3^{2-}$ , and the phosphate buffers,  $HPO_4^{2-}$  and  $PO_4^{3-}$  (see Ref. 6). Therefore, maintaining

charge balance and solving for alkalinity

$$\begin{aligned} \text{alkalinity} = & [HCO_3^-] + 2 \cdot [CO_3^{2-}] + [HPO_4^{2-}] \\ & + 2 \cdot [PO_4^{3-}] + [OH^-] - [H^+] \quad (5) \end{aligned}$$

In addition to these components, a portion of some organic anions can represent alkalinity and when appropriate each of these must be added to Eq. 5 as  $n \cdot [O^-]$  where  $n$  is the protolytic anionic charge of the protolytic organic species ( $O^-$ ). The inclusion of a particular organic anion ( $n \cdot [O^-]$ ) in this expression is based on its  $pK_a$ , and this situation will be addressed in the DISCUSSION. It should be emphasized that the protolytic species, and hence charge, is determined relative to the equivalence point.

Using this definition, a change in the relative acid-base content of a solution can be easily measured by determining the change in alkalinity where alkalinity is determined by titration to  $pH_e$ . As expressed above and as demonstrated in experiments below, a change in the alkalinity of a solution is produced only by a net acid or base addition. Changes in alkalinity, termed  $\Delta$ alkalinity, are defined as

$$\Delta \text{alkalinity} = \text{alkalinity}_t - \text{alkalinity}_0 \quad (6)$$

where  $\text{alkalinity}_0$  is the initial alkalinity of a solution and  $\text{alkalinity}_t$  is the alkalinity of the same solution after some period of time. For physiology, the measurement of  $\Delta$ alkalinity is the most powerful application of this new method to resolve quantitative changes in acid-base balance. The following experiments will outline how alkalinity is measured, show its insensitivity to various physical parameters, and finally demonstrate how alkalinity changes in a solution are a direct measure of acid-base changes.

### Experimental

*Measurement of alkalinity.* Figure 1 is a typical acid titration curve of a standard Krebs-bicarbonate buffer solution. As HCl is added, the pH of the solution falls

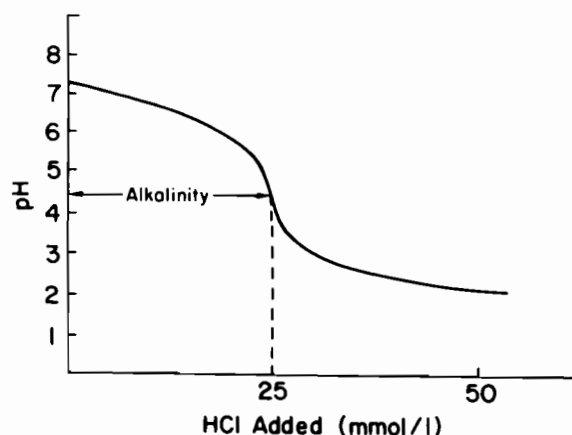


FIG. 1. Titration of 50 ml of Krebs-bicarbonate buffer solution carried out by incremental additions of 1 N HCl. pH was sampled every 5 s after each addition and points were recorded only when the pH change was  $<0.005$  units between consecutive additions. Continuous line was drawn through points. Total alkalinity was indicated by quantity of HCl required to titrate solution to equivalence point (pH 4.2), which was 1.255 meq or 25.2 meq/l in this case.

slowly until there is a precipitous decline between pH 5 and 4. Alkalinity is the quantity of  $H^+$  required to titrate the pH to the  $pH_e$ , which was pH 4.25 in this case. The equivalence point is recognized by the point of maximal  $dpH/dH^+$  in the titration curve and is calculated according to the formula

$$\text{alkalinity} = [V_a \cdot N_a / (V_0 + V_a)] \cdot 1,000 \quad (7)$$

where  $V_0$  is the initial volume (ml) of the sample,  $V_a$  is the volume (ml) of the acid added to titrate to  $pH_e$ , and  $N_a$  is the normality of the acid. Alkalinity is expressed as a charge concentration (meq/l) that is easily converted to content by considering the  $V_0$  of the sample. The solution titrated in Fig. 1 contained 25.1 meq/l alkalinity. The shape of this titration curve is typical for a solution containing bicarbonate and phosphate such that it displays a relatively flat slope until pH 5.8, since the  $HCO_3^-$  and  $HPO_4^{2-}$  are protonated to  $CO_2$  and  $H_2PO_4^-$ . After the steep decline in pH at  $\sim$ pH 4.2, a final stage in the titration, below pH 3.5, again displays a relatively flat contour that relates primarily to the high proton concentration so that a relatively large addition of HCl is required to produce a significant decrease in pH.

**Alkalinity of physiological buffers.** The predominant inorganic physiological buffers are bicarbonate and phosphate. To demonstrate the ability of an alkalinity measurement to distinguish the quantity of these species in solution, titrations of simple buffer solutions were performed. As shown in Table 1, a 50 mM  $NaHCO_3$  solution, having a pH of 8.3, exhibited a  $pH_e$  of 4.2 and a total alkalinity of 50.1 meq/l. Dibasic phosphate ( $Na_2HPO_4$ ) at a concentration of 50 mM had a pH of 8.6, a  $pH_e$  of 4.45, and a total alkalinity of 50.1 meq/l. In contrast, the monobasic sodium phosphate salt ( $NaH_2PO_4$ ) at a concentration of 50 mM had a pH of 4.67 and displayed virtually no alkalinity (<0.1 meq/l).

If alkalinity is a conserved parameter (see *Theoretical*) then combinations of  $NaHCO_3$  and  $Na_2HPO_4$  should produce a measured total alkalinity equal to the sum of the alkalinities of each species. The results of such combination experiments are shown in Table 2. As expected for a conserved parameter, varying the proportion of  $NaHCO_3$  and  $Na_2HPO_4$  in a solution did not affect the total alkalinity (50 meq/l) of the solution, despite wide variation in pH and a modest shift in  $pH_e$ . In other words, the total alkalinity was equal to the sum of the added  $HCO_3^-$  and  $HPO_4^{2-}$  despite the fact that  $CO_2$ ,  $CO_3^{2-}$ , and  $PO_4^{3-}$  were also generated by mixing them.

**Bicarbonate and nonbicarbonate alkalinity.** Because physiological processes depend on the types of buffers as well as their quantities, it is important to distinguish

TABLE 2. Alkalinity and  $pH_e$  of buffer mixtures

Buffer Concentration, mM		pH	$pH_e$	Alkalinity, meq/l		
$NaHCO_3$	$Na_2HPO_4$			Total	Bicarbonate	Nonbicarbonate
50	0	8.30	4.2	50.1	50.1	0
30	20	8.17	4.3	50.1	29.0	21.1
25	25	8.15	4.4	50.0	24.2	25.8
20	30	8.14	4.4	50.1	19.0	31.1
0	50	8.60	4.45	50.1	0	50.1
KBB + 5% $CO_2$		7.39	4.2	25.1	23.7	1.4

$pH_e$ , equivalence point; KBB, Krebs-bicarbonate buffer.

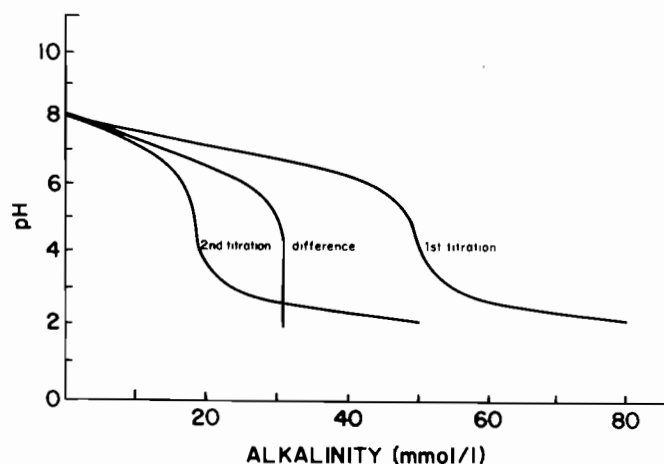


FIG. 2. Double acid titration of solution containing 30 mM  $NaHCO_3$  and 20 mM  $Na_2HPO_4$ . First titration measured total alkalinity. After 1st titration, solution was bubbled with nitrogen at pH 2.5 to remove all  $CO_2$ . pH was then restored to original value with 1 N NaOH and a 2nd acid titration was performed to measure nonbicarbonate alkalinity. By fitting a polynomial to each titration and performing a mathematical subtraction of 2nd titration from 1st, a titration curve (difference) of bicarbonate alkalinity was obtained. Total alkalinity was 50.1 meq/l, nonbicarbonate alkalinity was 20.9 meq/l, and bicarbonate alkalinity was 29.0 meq/l in this case.

between the various buffer species in a solution. We have recently described a simple method to distinguish bicarbonate alkalinity (i.e.,  $[HCO_3^-] + 2 \cdot [CO_3^{2-}]$ ) from nonbicarbonate alkalinity in a solution containing a mixture of buffers (2). This experimental protocol is illustrated in Fig. 2, which shows a double titration of a buffer solution containing  $NaHCO_3$  and  $Na_2HPO_4$ . The first HCl titration measured the total alkalinity (50.1 meq/l) of the solution. After this titration, while the pH was 2.0, the solution was bubbled with  $CO_2$ -free nitrogen to remove all the dissolved  $CO_2$ . Because  $HCO_3^-$  and  $CO_3^{2-}$  had been fully converted to  $CO_2$  at this low pH, this bubbling procedure effectively removed all the  $CO_2$  from the solution. Removal of the  $CO_2$  took  $\sim$ 10 min and was monitored with a  $PCO_2$  electrode. Subsequently, the pH was restored to the original value of eight by the rapid addition of NaOH, and a second HCl titration was performed. Because this second titration measured only the nonbicarbonate alkalinity (21.1 meq/l), mathematical subtraction of the second titration from the first titration generated a third titration curve for the bicarbonate buffers only and a value of 29.0 meq/l for the bicarbonate alkalinity. Therefore, this double titration procedure

TABLE 1. Total alkalinity and  $pH_e$  of simple buffer solutions

Solution	pH	$pH_e$	Total Alkalinity, meq/l
50 mM $NaHCO_3$	8.3	4.2	50.1
50 mM $Na_2HPO_4$	8.60	4.45	50.1
50 mM $NaH_2PO_4$	4.67	4.5	<0.1

$pH_e$ , equivalence point.

measures the total alkalinity, bicarbonate alkalinity, and nonbicarbonate alkalinity. As a result, it is possible to distinguish whether acid-base changes in a solution are the result of  $\text{HCO}_3^-$  changes. Furthermore, when the non- $\text{CO}_2$  buffers are known precisely (e.g., phosphate) it is possible to recognize the nature of nonbicarbonate acid-base changes.

To illustrate the utility of this double titration procedure, we have distinguished the alkalinity properties of several mixed buffer solutions containing both bicarbonate and inorganic phosphate (Table 2). A 50 mM  $\text{NaHCO}_3$  solution had a total alkalinity of 50.1 meq/l, which was entirely bicarbonate alkalinity. Conversely, a 50 mM  $\text{Na}_2\text{HPO}_4$  solution possessing a total alkalinity of 50.1 meq/l was entirely nonbicarbonate alkalinity. Various mixtures of these two solutions altered the pH but did not change the total alkalinity of 50 meq/l. Note that the relative quantities of bicarbonate and nonbicarbonate (phosphate) alkalinity reflected the relative quantity of each added to the solution. In each instance, however, the bicarbonate alkalinity was ~1 meq/l less than the total  $\text{HCO}_3^-$  added, whereas the nonbicarbonate alkalinity was ~1 meq/l more than the added  $\text{HPO}_4^{2-}$ . This experimental deviation reflects the chemical interaction of these buffer salts in solution where maintenance of an isohydric state requires a redistribution of the protons and hence a transfer of protons from the  $\text{HPO}_4^{2-}$  to the  $\text{HCO}_3^-$ . As a result, a fraction of the  $\text{HCO}_3^-$  is converted to  $\text{CO}_2$  and the level of  $\text{PO}_4^{3-}$  is concomitantly elevated at the expense of  $\text{HPO}_4^{2-}$ . Because alkalinity measures the total anionic charge capable of combining protons and not the total  $\text{CO}_2$  or total phosphate, it is understandable that mixing these buffers having different  $\text{pK}_a$ s results in a redistribution of protons and thus a rearrangement of the alkalinity charge among all the alkalinity species, although the total alkalinity is unaffected. Finally, the total alkalinity of the Krebs-bicarbonate buffer was 25.1 meq/l with 23.7 meq/l present as bicarbonate and 1.4 meq/l present as nonbicarbonate alkalinity.

*The influence of  $\text{CO}_2$ , ionic strength, and temperature.* Carbonic acid forms spontaneously in water whenever  $\text{CO}_2$  is present. As a result, pH can vary considerably depending on the quantity of dissolved  $\text{CO}_2$  in a solution. The dissociation of carbonic acid reduces pH by generating  $\text{H}^+$  but it also produces an equivalent amount of  $\text{HCO}_3^-$ . Consequently, the decrease in pH associated with elevating  $\text{CO}_2$  is not accompanied by a net increase in acid relative to the quantity of base. For cell physiology this distinction is lost when acid fluxes are based on pH, since cell-induced extracellular  $\text{CO}_2$  changes will appear as  $\text{H}^+$  secretion when pH is monitored, but this fails to recognize the concomitant secretion of  $\text{HCO}_3^-$ . Table 3 shows the effects of bubbling with  $\text{CO}_2$  or air on pH and alkalinity of a Krebs-bicarbonate buffer solution. At 5%  $\text{CO}_2$ , the pH of the solution was 7.39 and the alkalinity was 25.1 meq/l. An increase in  $\text{Pco}_2$  by bubbling with 10%  $\text{CO}_2$  decreased the pH to 7.20 but it did not affect the total alkalinity. Likewise, there was no significant change in alkalinity when the solution was equilibrated with air. These data highlight a fundamental property of

TABLE 3. *Effects of  $\text{CO}_2$  and air on pH,  $\text{pH}_e$ , and total alkalinity of a Krebs-bicarbonate buffer solution at 37°C*

Gas	pH	$\text{pH}_e$	Total Alkalinity, meq/l
5% $\text{CO}_2$	7.39	4.2	25.1
10% $\text{CO}_2$	7.20	4.2	25.1
Air	8.25	4.25	25.1

$\text{pH}_e$ , equivalence point.

TABLE 4. *Effects of temperature and ionic strength on pH,  $\text{pH}_e$ , and total alkalinity of a Krebs-bicarbonate buffer*

Temperature, °C	Ionic Strength, M	pH	$\text{pH}_e$	Total Alkalinity, meq/l
37	0.280	7.39	4.2	25.1
25	0.280	7.25	4.2	25.1
37	0.305	7.31	4.2	25.1

Ionic strength was increased by addition of 60 mM NaCl.

TABLE 5. *Effects of acid or base on pH, total alkalinity content, and Δalkalinity of 1 liter of Krebs-bicarbonate buffer solution bubbled with 5%  $\text{CO}_2$  at 25°C.*

Acid-Base Addition, mmol	pH	Total Alkalinity Content, meq	ΔAlkalinity, meq
None	7.25	25.1	0
+2 mmol HCl	6.97	23.1	-2.0
+2 mmol NaOH	8.20	27.1	+2.0
+2 mmol $\text{NaHCO}_3$	8.10	27.1	+2.0

In this case, total alkalinity is calculated as content to correct for volume changes associated with additions of HCl and NaOH.

alkalinity, namely, that alkalinity measures the net quantity of proton combining species and not the activity of free protons in a solution. Furthermore, this experiment demonstrates that experimental error related to the gas content of a solution is not a problem when measuring total alkalinity.

Temperature and ionic strength are well-established modulators of pH and thus can complicate analysis of acid-base balance using a traditional approach. In contrast, alkalinity is virtually unaffected by temperature or ionic strength (6, 22, 25). As an example, Table 4 shows the effects of changing temperature on pH and alkalinity of a Krebs-bicarbonate buffer solution. The solution bubbled with 5%  $\text{CO}_2$  had a pH of 7.39 and an alkalinity of 25.1 meq/l at 37°C. Reduction of the temperature to 25°C decreased the pH to 7.25, but alkalinity was unaffected. Similarly, addition of 60 mmol/l of NaCl crystals, a 20% osmolality change comparable to that used in studies of volume regulation, decreased the pH but the alkalinity remained 25.1 meq/l. The pH changes caused by temperature changes and ionic strength changes represent well-known effects on  $\text{pK}_a$ s and dissolved  $\text{CO}_2$ , but since alkalinity is measured by titrating to  $\text{pH}_e$ , these changes don't significantly affect this alkalinity end-point determination.

*Net acid-base changes: effects on alkalinity.* Table 5 shows how changes in the acid or base content of a physiological solution affect alkalinity. An initial Krebs-

bicarbonate buffer solution with a volume of 1 l had a pH of 7.25 and an alkalinity of 25.1 meq at 25°C. Addition of 2 mmol of HCl decreased the pH to 6.97 and decreased the total alkalinity content of the sample from 25.1 to 23.1 meq. ΔAlkalinity was therefore -2 meq with the negative value, indicating the net gain of acid. When 2 mmol of NaOH was added to the initial solution, the pH rose to 8.20 and Δalkalinity was +2 meq. Similarly, addition of 2 mmol of NaHCO<sub>3</sub> increased the pH to 8.10 and also increased alkalinity by 2 meq.

In a related series of experiments, we measured the alkalinity of solutions containing HEPES instead of HCO<sub>3</sub><sup>-</sup> as the predominant buffer. Though not shown, additions of HCl or NaOH produced equi-equivalent effects on Δalkalinity, analogous to those seen with the Krebs-bicarbonate buffer (Table 5). In addition, the alkalinities of three solutions used to culture cells were measured. Dulbecco's modified Eagle's medium, Ham's F12 solution, and Hank's balanced salt solution displayed total alkalinities of 36.6, 16.8, and 4.3 meq/l, respectively, and bicarbonate alkalinities of 34.8, 15.3, and 2.8 meq/l, respectively. Moreover, changes in their relative acid-base contents caused equivalent changes in alkalinity.

*Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange in human red blood cells.* Although extensive information exists concerning the Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange process of the red blood cell, there has never been a direct measurement of the net Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange ratio in intact red blood cells. To examine this question, we preincubated red blood cells in a medium containing 100 mM bicarbonate to bicarbonate load the cells. Cells were then exposed to a medium containing 100 mM Cl<sup>-</sup> to initiate Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange. In seven experiments, there was a decrease in extracellular Cl<sup>-</sup> of 22.3 ± 0.9 meq/l and a corresponding increase in extracellular alkalinity of 22.1 ± 0.9 meq/l. On a paired basis, the observed chloride-alkalinity exchange ratio was 1.01 ± 0.03, which is not significantly different from 1.00. In addition, distinction of bicarbonate alkalinity with a second titration showed that all of the alkalinity flux was bicarbonate.

## DISCUSSION

Alkalinity is a direct measurement of the total charge capable of combining with protons over the physiological pH range. In accord with observations made by others in seawater (6), we have found that the equivalence point, pH<sub>e</sub>, is a suitable reference point for measuring the alkalinity of physiological solutions. Titration of simple buffer solutions of NaHCO<sub>3</sub> and Na<sub>2</sub>HPO<sub>4</sub> (Tables 1 and 2) showed that the quantity of acid required to titrate these solutions to pH<sub>e</sub>, hence total alkalinity, was equal to the amount of HCO<sub>3</sub><sup>-</sup> and HPO<sub>4</sub><sup>-2</sup> added. Furthermore, using a variety of physiological buffer solutions including a Krebs-bicarbonate solution (Table 5), we showed that additions of acid or base produced equivalent effects on Δalkalinity. Therefore, pH<sub>e</sub> is a suitable end point for quantitating changes in the relative acid-base content of standard physiological solutions.

The theory of alkalinity is based on the conservation of charge and mass (6). This was evident when total alkalinity was measured in solutions containing a mix-

ture of NaHCO<sub>3</sub> and Na<sub>2</sub>HPO<sub>4</sub> (Table 2). Upon mixing, the pH of these buffer solutions changed reflecting the redistribution of protons and the consequent generation of CO<sub>2</sub>, CO<sub>3</sub><sup>-2</sup>, and PO<sub>4</sub><sup>-3</sup>. Nonetheless, the total alkalinity remained constant, indicating that the total negative charge of the physiological buffer species in the solution was unaltered. Therefore, unlike traditional approaches to acid-base chemistry of physiological solutions, alkalinity provides an important ability to quantitate acid-base quantities in terms of electroneutrality. Using a simple double titration procedure, we also showed that the bicarbonate alkalinity (HCO<sub>3</sub><sup>-</sup> + 2·CO<sub>3</sub><sup>-2</sup>) could be separated from the nonbicarbonate alkalinity (Fig. 2 and Table 2). In fact, double acid titration of the mixed bicarbonate-phosphate buffer solutions provided direct evidence that there had been a transfer of protons from phosphate to bicarbonate, since the nonbicarbonate alkalinity was slightly greater than the added HPO<sub>4</sub><sup>-2</sup> and the bicarbonate alkalinity was correspondingly less than the added bicarbonate. This observation has important implications for studies of acid-base transport where the ion being transported (e.g., bicarbonate) may transfer its charge to another chemical species in accordance with the prevailing chemical conditions. Consequently, in circumstances where cells are transporting more than one buffer species simultaneously, such as bicarbonate and phosphate transport in the renal proximal tubule, traditional approaches can't reliably quantitate the net flux. In contrast, direct measurement of bicarbonate and nonbicarbonate alkalinity offers a simple means of tracing the charge transfer across cell membranes.

Alkalinity and Δalkalinity offer several distinct advantages over conventional approaches to measuring acid-base fluxes across cell membranes. Alkalinity is not influenced by changes in ionic strength (Table 4) that are encountered in studies of cell volume regulation or urine formation. Furthermore, the insensitivity of alkalinity to temperature (Table 4) enables experimentation at 37°C with analysis of the sample at room temperature. CO<sub>2</sub>, despite its effects on pH, did not influence alkalinity (Table 3) because of its equivalent formation of H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>. As a result, analysis of PCO<sub>2</sub> with its attendant difficulties is unnecessary when total alkalinity is used to identify acid-base changes in a solution. This insensitivity to CO<sub>2</sub> is useful to eliminate concerns of CO<sub>2</sub> diffusion when measuring HCO<sub>3</sub><sup>-</sup> fluxes. In addition, total alkalinity remains constant despite the loss of CO<sub>2</sub> from an HCO<sub>3</sub><sup>-</sup>-containing solution. In other words, the net loss from a solution of CO<sub>2</sub> that results from the conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> does not affect the total alkalinity of the solution, since this conversion requires that another alkalinity charge species be created (e.g., conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>3</sub><sup>-2</sup> or of H<sub>2</sub>O to OH<sup>-</sup>) to maintain electroneutrality. This was apparent in Table 2 where the total alkalinity of mixtures of buffers was constant (50 meq/l), although the alkalinity charge could be redistributed among the various buffer species and CO<sub>2</sub> was presumably lost to the atmosphere.

ΔAlkalinity can also accurately measure acid-base changes in standard, well-buffered physiological solutions. This was apparent when HCl, NaOH, and Na-

HCO<sub>3</sub> additions to the Krebs-bicarbonate buffer (Table 5) produced equi-equivalent changes in alkalinity. Alkalinity measurements can be used with complex solutions containing a mixture of buffers having different pK<sub>a</sub>s, a feature that is advantageous when the cells actually transport more than one buffer ion. In addition, when  $\Delta$ alkalinity is used to evaluate acid or base fluxes, there is no need to make assumptions concerning the characteristics of the intracellular compartment such as buffer properties, compartmentation, or changes in metabolic rates. Finally, alkalinity titrations can be done easily and routinely either manually or with an automated system as described in METHODS.

For the solutions evaluated in this study, pH<sub>e</sub>, where alkalinity is zero, was in the pH range of 4.2–4.5. Because pH<sub>e</sub> is determined by the types and relative quantities of buffers, it is to be expected that a bicarbonate-phosphate buffered solution would display a pH<sub>e</sub> in this range (see Table 2). From a practical perspective, however, several points can be emphasized. For solutions containing only nonvolatile buffer species (e.g., phosphate and HEPES), changes in pH<sub>e</sub> will result only from changes in the relative buffer concentrations. In contrast, for solutions containing volatile species such as CO<sub>2</sub>, pH<sub>e</sub> will also depend on the titration procedure itself. During the HCl titration, CO<sub>2</sub> is formed and, in a vessel that is open to the atmosphere like the one that we used, there will be a continual loss of CO<sub>2</sub> from the solution. Because pH<sub>e</sub> is a function of total CO<sub>2</sub> (see APPENDIX), the equivalence point will occur at a higher pH (i.e., 4.4–4.8) than would be observed in a closed or nonvolatile system (i.e., 4.0–4.3). In this vein, acid titrations can be performed in either a closed system or an open system. Our experience has shown that a closed system is difficult to work with and doesn't provide any advantages over an open system. In an open system, i.e., a vessel open to the atmosphere, we tried both continuous bubbling of the sample with air and no bubbling during the titration. In the end, we found that our best reproducibility in measuring alkalinity was obtained when we did not bubble air during the titration and the titration was performed in two stages (see METHODS). In this case, we obtained a very consistent pH<sub>e</sub> for a given solution. Titration of the Krebs-bicarbonate buffer generally took ~15–20 min and the variability in pH<sub>e</sub> was <0.1 units. In the range of pH<sub>e</sub>, 0.1 mM increases in HCl would generally decrease the pH by 0.2 units so that errors related to misidentification of the exact pH<sub>e</sub> are negligible. In fact, we found that when a particular acid titration protocol was consistently implemented, pH<sub>e</sub> was virtually constant and could be treated as a fixed value for that solution in the determination of alkalinity. Nonetheless, it is important to recognize that, provided the titration is performed slowly, loss of CO<sub>2</sub> with the accompanying change in pH<sub>e</sub> does not affect the measured alkalinity or  $\Delta$ alkalinity.

The ability to measure alkalinity and to resolve precisely small changes in alkalinity depends in large part on the titration procedure. The equipment used in this study was originally designed for relatively large volumes of fluid (50 ml) that are generally available to marine chemists. By decreasing the volume of the titration

chamber and adding smaller aliquots of HCl, we were also able to titrate 1-ml samples. In addition, a simple manual titration procedure (see METHODS) was equally effective for 50-ml samples through the use of any of these methods our resolution was 0.1 meq/l, and the alkalinity of any given solution including the Krebs-bicarbonate buffer was extremely reproducible with <0.1 meq/l error. Interestingly, however, we found that there was significant variability in the alkalinity of the Krebs-bicarbonate buffer solution prepared at different times. Our range of values was 25.0–26.6 meq/l. We attribute this to slight variations in several factors that can affect the final PCO<sub>2</sub> of the solution such as the percent of CO<sub>2</sub> in the gas tank, bubble size, stirring rate, temperature, and barometric pressure. Consequently, because PCO<sub>2</sub> can vary slightly, the amount of NaOH used to titrate the buffer solution to pH 7.40 (see METHODS) will not be consistent and variations in alkalinity will result. Fortunately, when measuring  $\Delta$ alkalinity, these day-to-day variations are unimportant, because the initial solution serves as an internal alkalinity reference (alkalinity<sub>0</sub>).

Although simple buffered salt solutions are often used in physiology, there is frequently a need to analyze a solution containing organic anions that can interact with protons. These anions include organic buffers, substrates, and proteins. In terms of alkalinity, these anions can be divided according to their pK<sub>a</sub>s. For all anions having pK<sub>a</sub>s greater than 5.5, such as the standard organic buffers HEPES, 2-(*N*-morpholino)propanesulfonic acid (MOPS), 1,4-piperazine-bis(ethanesulfonic acid) (PIPES), and so forth, the alkalinity titration measures their protolytic charge exactly. Conversely, all organic acids with pK<sub>a</sub>s <3.5 are not detected by the titration. Therefore, the organic anions which must be reconciled are those with pK<sub>a</sub>s >3.5 and <5.5. Generally, however, these anions do not present a significant problem when  $\Delta$ alkalinity is used to measure net acid or base fluxes because the effects of these anions will be evident in the titration curve itself. If these anions should significantly affect the acid-base flux measurements, serial titrations still allow differentiation of bicarbonate alkalinity (Table 2 and Fig. 2) changes. Furthermore, through the use of precipitation procedures (28), direct measurement of the conflicting anion (e.g., citrate), and/or mathematical analysis (8), it will also be possible to distinguish directly the phosphate alkalinity as well as the contribution of organic anions. Overall, potential problems can exist with organic anions, but we stress that when measuring  $\Delta$ alkalinity these problems will generally be inconsequential, will always be apparent in the shape of the titration curves, and can be solved.

Ammonium (NH<sub>4</sub><sup>+</sup>) serves as a mediator of H<sup>+</sup> excretion by the body. In addition, NH<sub>4</sub><sup>+</sup> is often used experimentally to acid load cells to evaluate cellular acid extrusion pathways (19). Therefore, recognizing the role of NH<sub>4</sub><sup>+</sup> and NH<sub>3</sub> in alkalinity is important. NH<sub>3</sub>, with a pK<sub>a</sub> of 9.37, is an alkalinity species, since it will be converted to NH<sub>4</sub><sup>+</sup> in an acid titration to ~pH 4.5. NH<sub>4</sub><sup>+</sup>, therefore, is not an alkalinity species. Although it is necessary to include NH<sub>3</sub> in the definition of alkalinity

(Eqs. 4 and 5), at normal levels of  $\text{NH}_3$  and over the physiological pH range, virtually all  $\text{NH}_3$  exists as  $\text{NH}_4^+$ . Still, two considerations must be recognized. First, unlike bicarbonate or phosphate alkalinity,  $\text{NH}_3$  alkalinity is electroneutral. Second, cells can consume or generate  $\text{NH}_3$  through metabolic processes such as urea synthesis or glutamine oxidation. These processes will produce changes in total alkalinity commensurate with the change in  $\text{NH}_3$ ; however, because  $\text{NH}_3$  is readily converted to  $\text{NH}_4^+$  at physiological pH,  $\Delta$ alkalinity will be evident as a change in another chemical species such as  $\text{HCO}_3^-$  or  $\text{HPO}_4^{2-}$ . Consequently,  $\Delta$ alkalinity properly assesses net acid-base changes when  $\text{NH}_3$  and  $\text{NH}_4^+$  are involved. However, under conditions in which  $\text{NH}_3$  may be involved, identification of the cellular mechanisms responsible for the acid-base changes requires direct measurement of  $\text{NH}_3/\text{NH}_4^+$ .

#### *$\Delta$ Alkalinity: Application to Cell Physiology*

Three criteria must be satisfied for  $\Delta$ alkalinity to be useful in measuring net acid-base transport across cell membranes. First, it must be possible to sample the extracellular medium so that a titration of the buffer solution can be performed at a given time point. Direct sampling of the extracellular fluid in studies of sheet epithelia is feasible, whereas centrifugation and filtration methods should be applied to studies of cells or vesicles in suspension. Second, the predominant buffer(s) in the solution must have  $\text{pK}_a$ s less than 3.7 and greater than 5.5. These buffers include bicarbonate, phosphate, HEPES, MOPS, and most of the standard buffer solutions used by cell physiologists. When it is necessary to include an organic anion with a  $\text{pK}_a$  between 3.5 and 5.5 such as a metabolic substrate, it is preferable to select an anion with a relatively low  $\text{pK}_a$  and to use it in low concentration. Finally, the resolution of the  $\Delta$ alkalinity measurements must be sufficient to distinguish cellular acid-base fluxes. In this study, our resolution was 0.1 meq/l in either a 50- or 1-ml volume, corresponding to 50  $\mu\text{eq}$  and 100 neq, respectively. Beyond this, however, sophisticated titration techniques currently available in marine chemistry can resolve alkalinity changes of 15 neq in a 1-ml sample (A. G. Dickson, personal communication). By combining this technology with microtitrator and electrometric techniques in use by physiologists (16, 23), alkalinity titrations of small volumes ( $<1 \mu\text{l}$ ) with comparable resolution should be feasible.

To demonstrate the ability of an alkalinity titration to measure net fluxes, the net  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchange ratio of the intact human red blood cell was measured. Interestingly, despite numerous studies of the  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchange mechanism in human erythrocytes (14, 27, 29), the net  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchange ratio has not been directly measured because of the uncertainty associated with correcting for the permeation of  $\text{CO}_2$ .  $\text{HCO}_3^-$ -loaded cells lost  $\text{HCO}_3^-$  to the extracellular medium in exchange for  $\text{Cl}^-$  and the observed  $\Delta\text{Cl}^-$ - $\Delta\text{HCO}_3^-$  alkalinity ratio was  $1.01 \pm 0.03$ , a value not significantly different from the expected value of 1.00.

Red blood cells represent perhaps an ideal biological system to use  $\Delta$ alkalinity, because they can be studied

at high densities and they transport large quantities of bicarbonate. However, based on known rates of acid-base transport in other cells, it is evident that adequate resolution ( $\leq 0.1 \text{ meq/l}$  or  $0.1 \mu\text{eq/ml}$ ) exists to apply  $\Delta$ alkalinity in many studies. For example, the stomach, the proximal colon, and the gallbladder exhibit transepithelial acid and bicarbonate fluxes of  $2\text{--}4 \mu\text{eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$  (7, 26, 30). In addition, the renal proximal tubule can reabsorb bicarbonate at  $155 \text{ neq}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$  (5), and along the nephron, the bicarbonate concentration normally varies between 24 and 1 mM (13). Studies of intermediary metabolism can also benefit from alkalinity determinations, because numerous substrate conversions involve utilization or production of acid and base. Although direct assays of acid utilization or production rates by tissues are generally unavailable, it is possible to estimate these rates based on changes in substrate levels. For example, lactate utilization rates in the production of  $\text{CO}_2$  and glucose are  $332 \text{ nmol}\cdot\text{h}^{-1}\cdot\text{mg protein}^{-1}$  in suspensions of proximal tubules (11). Furthermore, glycolytic rates of lactate production in hepatocytes and tumorigenic cells are  $100 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g wet wt}^{-1}$  (21) and  $180 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mg protein}^{-1}$  (1), respectively. Consequently, with appropriate adjustments in extracellular fluid volume or cell density, it will be possible to quantitate directly numerous cell-mediated acid-base events using  $\Delta$ alkalinity.

In conclusion, we have described an analytical technique and concept to quantitate net changes in the relative acid-base content of standard physiological buffer solutions. By monitoring the extracellular  $\Delta$ alkalinity associated with normal cell function, it is possible to quantitate precisely the net acid-base transport rates across plasma membranes. This method, though less sensitive, has several distinct advantages over traditional approaches to quantitate net acid fluxes. In particular, fluxes can be performed with solutions containing a mixture of buffers at normal physiological concentrations (e.g., 25 mM  $\text{HCO}_3^-$  and 2 mM  $\text{HPO}_4^{2-}$ ), the method is insensitive to changes in ionic strength, temperature, and  $\text{CO}_2$ , and no assumptions concerning the properties of the intracellular compartment are required. Finally, as a supplement to standard measurements of intracellular pH, alkalinity measurements will offer a direct assessment of the acid-base regulatory properties of many types of cells.

#### APPENDIX

Unlike fixed cations and anions such as  $\text{Na}^+$  and  $\text{Cl}^-$ , quantitation of net acid-base changes in a solution requires one to define an arbitrary reference point. In this study we have chosen an equivalence point,  $\text{pH}_e$ , as the zero proton level. For physiological solutions, where  $\text{HCO}_3^-$  is generally the predominant buffer species,  $\text{pH}_e$  was identified in the pH range of 4.0–4.7 so that at physiological pH (generally 7.0–7.4) these solutions contain an excess of base equivalents termed alkalinity. Although other approaches exist to determine  $\text{pH}_e$ , we have adopted the approach of MacIntyre (15) and have operationally defined  $\text{pH}_e$  as the point of maximum  $\text{dpH/dH}^+$  in an acid titration of a solution. MacIntyre showed that alkalinity determination by this method was always within 4  $\mu\text{eq/l}$  of that measured by the more rigorous Gran-type titration. Further-

more, for measurements of Δalkalinity, the error using this method to determine alkalinity would be considerably less than this value and, hence, well beyond the limits of experimental resolution.

To appreciate why the deflection point occurs at pH<sub>e</sub>, consider a simple solution containing CO<sub>2</sub> as its buffer components so that HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>-2</sup> are the only relevant protolytic anions (B<sub>1</sub><sup>-</sup>). According to Eq. 4, the alkalinity of this simple buffer solution is

$$\text{alkalinity} = [\text{HCO}_3^-] + 2 \cdot [\text{CO}_3^{2-}] + [\text{OH}^-] - [\text{H}^+] \quad (8)$$

By setting alkalinity to zero and solving for [H<sup>+</sup>] one can determine pH<sub>e</sub>, which is the unique pH (i.e., -log[H<sup>+</sup>]) that satisfies the following expression

$$[\text{H}^+] = [\text{HCO}_3^-] + 2 \cdot [\text{CO}_3^{2-}] + [\text{OH}^-] \quad (9)$$

During an alkalinity titration in which H<sup>+</sup> is added to the solution, the chemical reactions are driven toward association, and the dominant reaction when approaching the equivalence point is



Assuming that kinetic effects are negligible this reaction abides the relationship

$$K_1 = [\text{H}^+][\text{HCO}_3^-]/[\text{H}_2\text{CO}_3] \quad (11)$$

Knowing that pH<sub>e</sub> for this solution is in the range of pH 4.0–4.7, the only protolytic species (B<sub>1</sub><sup>-</sup>) that exists in significant quantity is HCO<sub>3</sub><sup>-</sup> (i.e., HCO<sub>3</sub><sup>-</sup> ≫ 2 · CO<sub>3</sub><sup>-2</sup> + OH<sup>-</sup>). Therefore, based on Eq. 9, the equivalence point occurs when [H<sup>+</sup>] = [HCO<sub>3</sub><sup>-</sup>]. Substituting this relationship into Eq. 11, we find

$$K_1 = [\text{H}^+]^2/[\text{H}_2\text{CO}_3] \quad (12)$$

or

$$\text{p}K_1 = 2 \cdot \text{pH}_e - \text{pH}_2\text{CO}_3 \quad (13)$$

where

$$\text{p}K_1 = -\log K_1 \quad (14)$$

$$\text{pH}_e = -\log[\text{H}^+] \quad (15)$$

at the equivalence point

$$\text{pH}_2\text{CO}_3 = \log[\text{H}_2\text{CO}_3] \quad (16)$$

or, solving Eq. 13 for the equivalence point

$$\text{pH}_e = \frac{1}{2}(\text{p}K_1 + \text{pH}_2\text{CO}_3) \quad (17)$$

Because pH<sub>e</sub> is in the range of pH 4.0–4.5, virtually all the CO<sub>2</sub> species have been converted to H<sub>2</sub>CO<sub>2</sub> so that [H<sub>2</sub>CO<sub>2</sub>] ≫ [HCO<sub>3</sub><sup>-</sup>] + [CO<sub>3</sub><sup>-2</sup>]. Hence, at pH<sub>e</sub>, total CO<sub>2</sub> (TCO<sub>2</sub>), defined as

$$[\text{TCO}_2] = [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}] \quad (18)$$

can be approximated by

$$[\text{TCO}_2] = [\text{H}_2\text{CO}_3] \quad (19)$$

Equation 17 can be rewritten as

$$\text{pH}_e = \frac{1}{2}(\text{p}K_1 + \text{pTCO}_2) \quad (20)$$

It is thus evident that for this example of a simple bicarbonate-buffered solution, pH<sub>e</sub> is a function of TCO<sub>2</sub>. Accordingly, within the very small error of the approximations above, it is possible to calculate the pH<sub>e</sub> for a bicarbonate-buffered solution. For example, assuming pK<sub>1</sub> is 6.1, if total CO<sub>2</sub> is 1 mM, pH<sub>e</sub> will be 4.55, whereas if total CO<sub>2</sub> is 25 mM, pH<sub>e</sub> will be 3.85. Finally, by a simple expansion of the logic herein it is possible to demonstrate that for multibuffered solutions whose

predominant buffers have pK<sub>a</sub>s above 5.5 and below 3.5, the deflection point represents the equivalence point for the determination of alkalinity (15).

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