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Regulation of intracellular pH by human peripheral blood lymphocytes as measured by ^{19}F NMR

(pH gradient/fluorine compounds)

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ABSTRACT We have measured the intracellular pH of human peripheral blood lymphocytes by means of high-resolution ^{19}F NMR spectroscopy using D,L-2-amino-3,3-difluoro-2-methylpropanoic acid (F_2MeAla) as a probe. Lymphocytes readily took up the methyl ester of F_2MeAla, and endogenous esterase hydrolyzed the ester to the free amino acid inside the cell. This α-methyl amino acid is not metabolized by the cell, and its ^{19}F NMR spectrum exhibits large pH-dependent shifts as the α-amino group is protonated. The size of the ^{19}F shifts, the high sensitivity of ^{19}F NMR, and the favorable pK_a of the α-amino group of F_2MeAla ($pK_a = 7.3$) allowed us to measure intracellular pH of lymphocytes at 25–30°C with approximately 5-min acquisition times. Measurements at various external pH values demonstrated that human peripheral blood lymphocytes regulate their internal pH, a process requiring expenditure of metabolic energy. In the pH range between 6.8 and 7.4, lymphocytes maintain a constant internal pH of 7.17 ± 0.06 pH unit. Outside this range, intracellular pH changes with extracellular pH. The accuracy of this ^{19}F pH probe has been confirmed by independent measurements of intracellular pH using equilibrium distributions of 5,5-dimethyloxazolidine-2,4-dione.

Changes in intracellular pH have been shown to be critical determinants of cell growth and development (1–10). Recent findings (11–13) indicate that there is ionic modulation of mitogen-induced proliferation of human peripheral blood lymphocytes (PBL), which could be mediated by proton exchange. It has been suggested that small changes in cytoplasmic pH control mitogen-induced proliferation of lymphocytes‡. In order to test this possibility we needed a valid means of determining cytoplasmic pH in normal, resting lymphocytes.

In initial studies of intracellular pH, we used three radioactively labeled weak acids and weak bases (14). The pH gradient across the lymphocyte cell membrane was calculated from the equilibrium distributions of trimethylacetic acid, 5,5-dimethyloxazolidine-2,4-dione (DMO), and trimethylamine. There were marked discrepancies, the magnitudes of which could not be explained by the fact that whenever there is intracellular pH inhomogeneity, weak acids always give a higher value of mean intracellular pH than did weak bases. (For a review of the mathematical considerations involved in the use of labeled weak acids and bases as pH indicators, see refs. 15, 16, and 17.) We therefore sought other methods for measuring intracellular pH in which the pH dependence of the probe molecule arises from different physical principles.

In the present work we used fluorine-labeled compounds observed by ^{19}F NMR. These fluorine-labeled compounds indicate pH not by their distribution between compartments but by the dependence of the chemical shift of their ^{19}F resonance on protonation of the amine group. Under slow-exchange conditions, separate resonance lines would be observed from the extracellular medium and from each intracellular compartment containing an observable number of indicator molecules, if significant pH differences exist between compartments.

Our first ^{19}F NMR measurements to assess the transmembrane pH gradient across human erythrocytes involved the use of trifluoroethylamine (18). This compound has a large pH-dependent chemical shift (≈1 ppm/pH unit) in the pH range 6.0–7.2, is noncytotoxic, and distributes across erythrocyte membranes. Accurate determinations of pH gradient were made for erythrocytes suspended at pH values between 7.2 and 8.0 at 4°C. However, at 25°C the exchange rate is so high that one broad ^{19}F resonance is observed. We therefore continued our search for a better probe.

In this paper we report that the ^{19}F NMR resonance of D,L-2-amino-3,3-difluoro-2-methylpropanoic acid [F_2MeAla; α-(difluoromethyl)alanine] is a useful, accurate probe of pH gradient under physiological conditions. We also report the application of these techniques to the assessment of pH gradients across human PBL. Our results confirm and extend our previous observations (14) and show that lymphocyte intracellular pH is regulated in the extracellular pH range 6.9–7.3.

METHODS

Preparation of Human PBL. PBL were prepared from the plateletpheresis by-product of healthy donors. The PBL were isolated, cultured, and assayed for DNA as described (11). PBL used in NMR experiments were maintained for short periods (5–20 min) on ice as either packed cells or a 60% suspension. The pH of such suspensions was maintained between 7.0 and 7.2 for this period.

NMR Measurements. Packed cells were diluted approximately 1:3 or 1:5 in Hanks/Hepes solution which contained 0.3–1 mM F_2MeAla methyl ester and had been titrated previously to the appropriate pH. The samples contained an internal standard (0.3–0.8 mM trifluoroacetate) for chemical shift measurement, and 12–15% ^2H_2O for the field lock. Omission of the trifluoroacetate did not affect the measured values.

^{19}F Fourier-transform NMR spectra were obtained on a Bruker CXP 200 instrument (Middle Atlantic NMR Facility, University of Pennsylvania). Spectra were obtained by applying broad-band proton irradiation using pulse angles of approximately 40° and 10-mm sample tubes. A flow system was designed to circulate the cell suspension continuously between the 10-mm sample tube in the NMR probe and an oxygenation chamber outside the magnet in order to ensure proper oxygen-

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Abbreviations: F_2MeAla, D,L-2-amino-3,3-difluoro-2-methylpropanoic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione; PBL, peripheral blood lymphocytes.

‡ Gerson, D. (1981) Kroc Foundation Symposium, July 20–24, 1981, Santa Yvez Valley, CA.

ation of the cells during NMR measurements. All spectra shown were acquired using the flow system. The ^{19}F chemical shifts are expressed with reference to trifluoroacetate, pH 7, in 90% ^2H_2O; positive values are downfield from the reference compound.

MATERIALS

Experimental reagents were obtained from the following suppliers: Hanks balanced salt solution, Ca^{2+}- and Mg^{2+}-free, GIBCO; Ficoll, Sigma; Hypaque (sodium salt, 50% solution), Winthrop; phytohemagglutinin-M, Difco; [^3H]thymidine, New England Nuclear; trifluoroacetic acid and ^2H_2O, Aldrich; and porcine liver esterase (EC 3.1.1.1), Sigma. F_2MeAla methyl ester was made by the method of Bey *et al.* (19) and was the generous gift of Thomas E. Jackson (Stuart Pharmaceuticals Division, ICI Americas, Wilmington, DE).

RESULTS AND DISCUSSION

Characteristics of F_2MeAla and its Methyl Ester. The proton-decoupled ^{19}F NMR spectra of F_2MeAla methyl ester and F_2MeAla are shown in Fig. 1 *A* and *C*, respectively. Both compounds gave AB-type ^{19}F spectra, with $J/\Delta\nu \approx 0.3$. pK_a was 7.3 for the α-amino group on the acid and 5.1 for the methyl ester. These pK_a values were obtained from measured titration curves for the ester (not shown) and acid (Fig. 1*D*). Titrations were also carried out by using a pH meter and these gave the same pK_a values. For both ester and acid, the spacing of the two center lines of the ^{19}F quartet is $\Delta = [(\nu_A - \nu_B)^2 + J^2]^{1/2} - |J|$, in which ν_A and ν_B are the Larmor frequencies of the two nonequivalent fluorine atoms and J is the fluorine–fluorine coupling constant. This center-line spacing increases markedly as the α-amino group is protonated in these compounds and serves as a pH indicator in the pH range in which the amino group is undergoing protonation. The useful pH-indicating range is pH 6.6–7.8 for the acid and pH 4.3–5.9 for the ester; the maximum pH-dependent changes are 0.9 ppm/pH unit for the acid (Fig. 1*D*) and 1.4 ppm/pH unit for the ester. The fluorine–fluorine coupling, J, increases only 4–5 Hz upon α-amino protonation: from 277 to 283 Hz for the ester and from 275 to 278 Hz for the amino acid.

Our cell suspensions contained approximately 0.7 mM trifluoroacetate as a ^{19}F chemical shift reference. We have found no adverse effects on PBL viability or stimulated growth; trifluoroacetate has been a satisfactory reference compound in observations on several other cell types as well. However, we note that use of the center-peak spacing of the ^{19}F resonances of the probe to determine pH eliminates the need for an internal or external ^{19}F chemical shift standard. The presence of the fluorinated probe compound did not decrease viability or impair stimulated growth. PBL viability after exposure to F_2MeAla methyl ester was assessed in two ways: (*i*) by determining trypan blue exclusion and (*ii*) by determining DNA synthesis in cultures derived from PBL suspensions incubated with this probe. PBL suspensions containing 1 mM F_2MeAla methyl ester were incubated for 40 min at 37°C, washed, resuspended in minimal essential medium, and then cultured in the presence of phytohemagglutinin (25–50 mg/ml). Peak [^3H]thymidine incorporation (mean ± SD) was 62,228 ± 841 and 65,489 ± 617 cpm for control cells and cells incubated with fluorinated probe, respectively. Viability was 98.6 ± 1.1% and 97.4 ± 1.6% for these cells, respectively.

Determination of pH Gradient in Human PBL. When human PBL were incubated with either the methyl ester or the free amino acid of F_2MeAla, F_2MeAla accumulated inside the cell. The extent and kinetics of uptake were dependent on the form of the compound transported. At neutral pH and temperatures from 25°C to 37°C, human PBL took up F_2MeAla methyl ester, and esterase activity gave rise to intracellular concentrations of the amino acid as high as 4–5 mM (determined from relative peak areas).

Fig. 1*A* shows the spectrum of F_2MeAla methyl ester immediately after its addition to a suspension of PBL. Fig. 1*B* shows the same sample at a later time, when some of the ester has been hydrolyzed to the acid. The down-field doublet of F_2MeAla is visible in Fig. 1*B*, bracketing the lowest-field line of the ester's quartet; the acid upfield doublet is not resolved because it is only 0.05 ppm upfield from that of the ester.

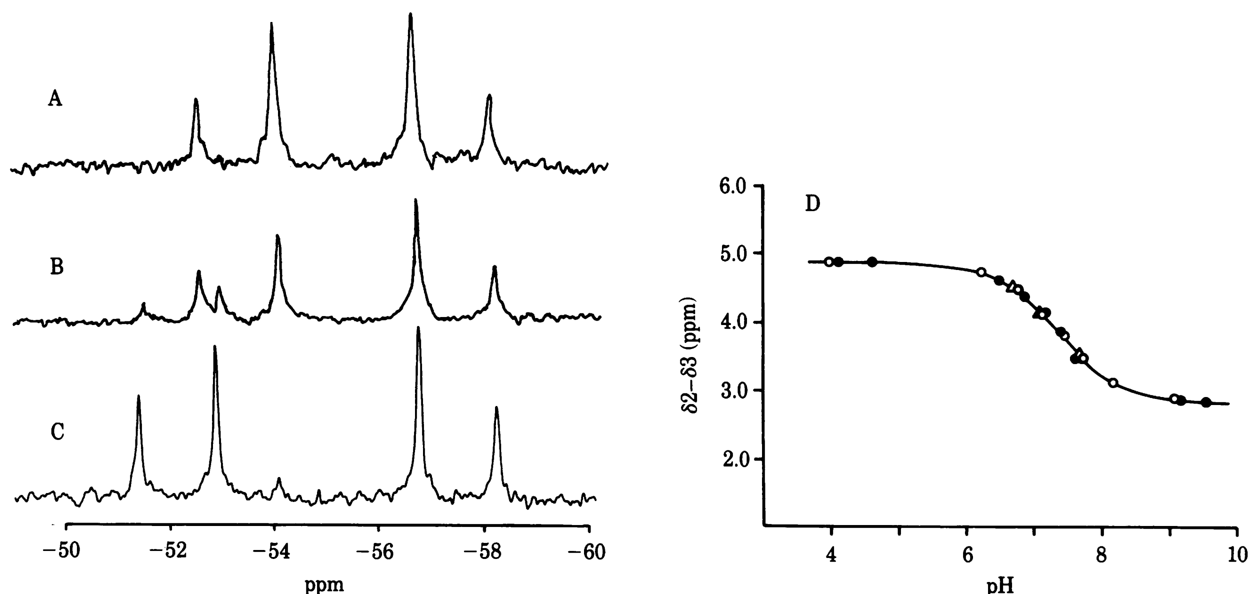

FIG. 1. ^{19}F NMR spectra (188.2 MHz) of F_2MeAla methyl ester hydrolysis to free acid. (*A*) Sample contained 2 ml of packed PBL in a total of 5.3 ml of Hanks/Hepes at 27°C (pH 7.3) plus 0.75 mM trifluoroacetate, 0.47 mM F_2MeAla methyl ester, and ^2H_2O to 12%. (*B*) Same sample after 30-min incubation. (*C*) Only 1 mM F_2MeAla and 1 mM trifluoroacetate in Hanks/Hepes containing 12% ^2H_2O at 25°C (pH 7.2). (*D*) pH dependence of the center peak spacing, δ_2–δ_3, of the ^{19}F NMR spectrum of F_2MeAla in 130 mM KCl/20 mM NaCl, containing 1 mM $MgSO_4$ (●), 3 mM $MgSO_4$ (○), or 0.5 mM $MgSO_4$ (△). For all spectra: 1,000 scans; acquisition time = 0.35 s.

For the data recorded in Fig. 2, the sample was prepared by adding 1 mM ester to 2.5 ml of packed PBL resuspended in a total volume of 10 ml of Hanks medium, incubating 30 min, centrifuging, and resuspending the cells to a total volume of 6.5 ml in medium without additional ester. ^{19}F NMR spectra were taken every 5.8 min. In Fig. 2, *A* through *C* are three sequential spectra spanning the time period 12–30 min after resuspension. These spectra show that: (*i*) after resuspension, only the amino acid remains; (*ii*) the spectra are the superposition of two quartets, of which one (marked i) has slightly broader lines and a narrower center-peak spacing than the other; (*iii*) the species i decreases with time whereas species o increases. Fig. 2*D* is the spectrum of the supernatant solution from the same sample. The spectrum was identical to that of species o at the same pH. Furthermore, addition of 0.1 mM F_2MeAla to the sample giving curve *C* resulted in a significant increase in the species o spectrum, which is therefore that of F_2MeAla in solution outside the cells.

The second species, i, is the spectrum of F_2MeAla inside the PBL, formed from hydrolysis of the methyl ester by intracellular esterases. As the F_2MeAla accumulated in the cell, it began to leak out (Fig. 2 *A–C*). The difference between intracellular and extracellular pH caused the spectrum of the internal F_2MeAla to be shifted relative to that in the external medium.

F_2MeAla was incubated at 1 mM with PBL suspended as 3 ml of packed cells in a total volume of 10.2 ml. A ^{19}F NMR spectrum taken shortly after sample preparation showed only one quartet, which corresponded exactly to that of F_2MeAla at the pH of the external medium. There did not appear to be significant binding of F_2MeAla to the cell membrane. The acid was taken up by PBL, but relatively long incubation times (hours) at 37°C were required to obtain observable intracellular concentrations by this method. The high internal concentrations generated by ester hydrolysis (>4–5 mM) can explain the more rapid equilibration by this route compared to uptake of the amino acid from the extracellular medium (1 mM).

Fig. 3 shows ^{19}F spectra of suspensions of PBL plus F_2MeAla methyl ester at two values of extracellular pH. Extracellular pH was calculated from a titration curve of central peak spacing vs. pH for 1 mM F_2MeAla in Ca^{2+}-, Mg^{2+}-free Hanks/Hepes buffer; the extracellular pH calculated from the standard curve agreed, within 0.02 pH unit, with the pH measured in the cell suspension with a pH meter. Intracellular pH was calculated from the titration curve for 5 mM F_2MeAla in 130 mM KCl/20 mM NaCl, corresponding to intracellular levels of these ions in PBL, containing 1 mM $MgSO_4$ (shown in Fig. 1*D*). There was a difference of 0.13 unit in the apparent pK_a between the two standard curves, the high-K^+ (intracellular) curve being shifted to higher pH. This reflected a weak specific interaction between K^+ and F_2MeAla, rather than a simple dependence of pK_a on ionic strength (unpublished data). However, there was no observable change in the titration curve of 5 mM F_2MeAla over a range of total Mg from 0.5 to 3.0 mM in the presence of 130 mM KCl/20 mM NaCl. In the experiment of Fig. 3*A*, the external pH was 7.31 and the intracellular pH was 7.15. For Fig. 3*B*, the external pH was 6.7 and the intracellular pH was 7.25, and the intracellular F_2MeAla center peaks appeared on the alkaline side, toward the center of the external difluoromethylalanine peaks.

When the extracellular pH of PBL suspensions was varied

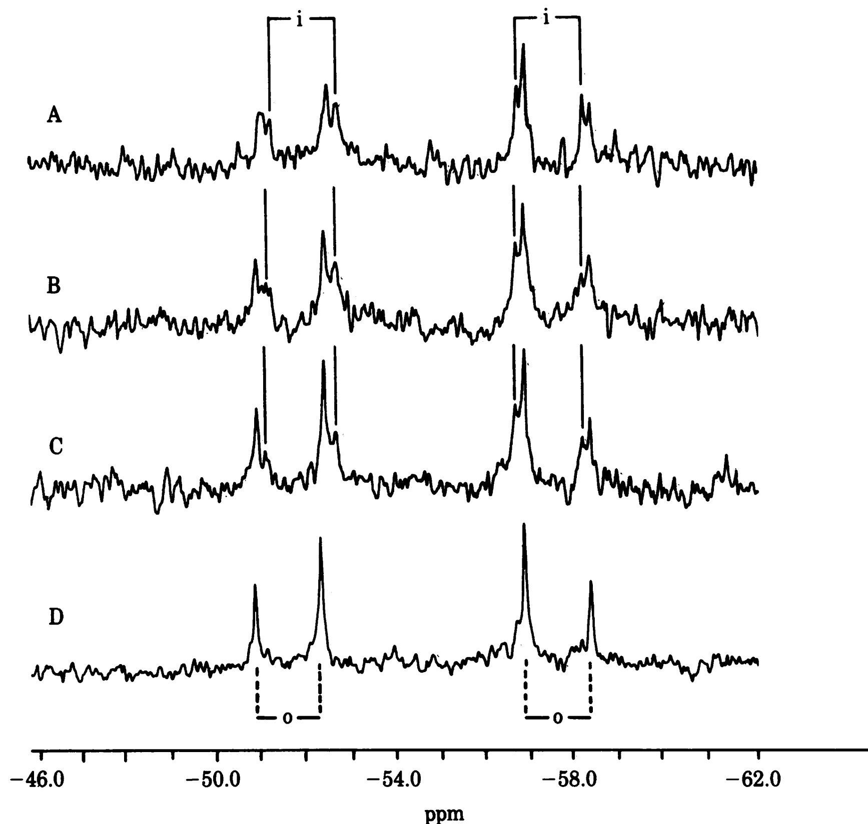

FIG. 2. Time sequence of ^{19}F NMR spectra of F_2MeAla in PBL suspensions. Packed PBL (2.5 ml) were incubated with 1 mM F_2MeAla methyl ester for 30 min at 37°C in a total volume of 10 ml of Hanks/Hepes medium, centrifuged, and resuspended in a total volume of 6.5 ml of Hanks medium containing 0.62 mM trifluoroacetate and 15% ^2H_2O; the initial pH was 6.88. Spectra were taken every 5.8 min (1,000 scans; acquisition time = 0.35 s). *A*, *B*, and *C* show the spectra obtained beginning at 5.8, 11.6, and 17.4 min, respectively. The sample was removed and centrifuged, and the supernatant solution was returned to the spectrometer for spectrum in *D*.

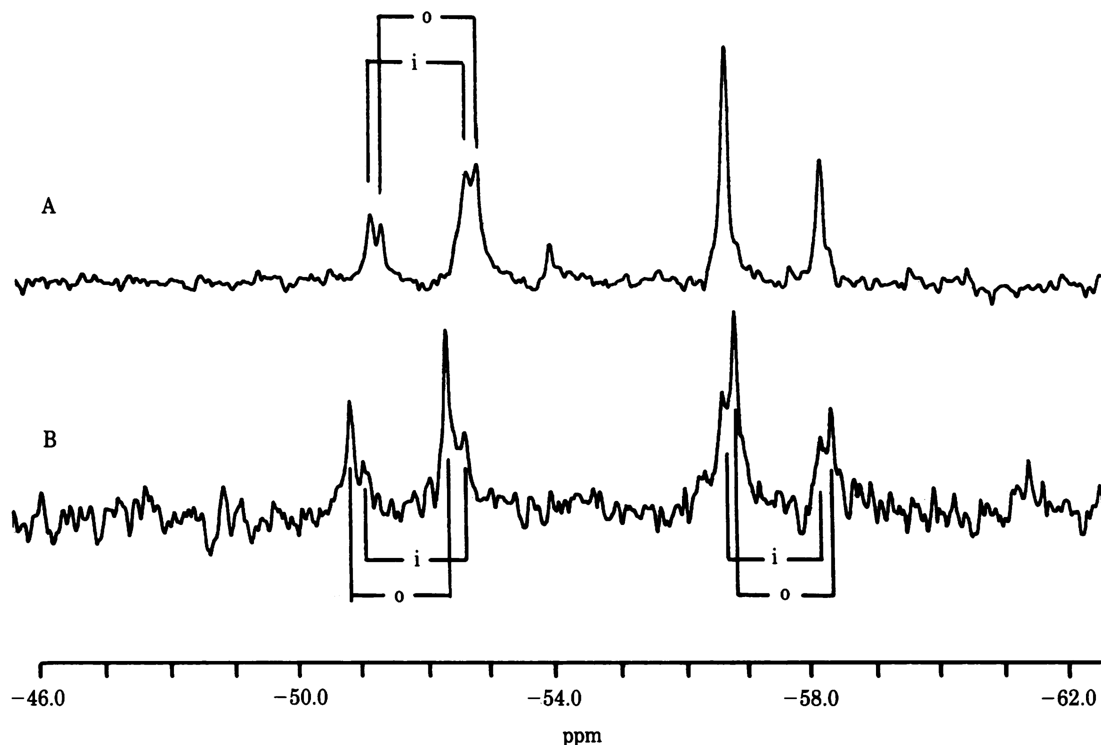

FIG. 3. ^{19}F NMR spectrum of intracellular and extracellular F_2MeAla in PBL suspensions at different external pH values. (*A*) Packed PBL (0.80 ml) were suspended in a total volume of 5.8 ml of Hanks/Hepes containing 0.70 mM trifluoroacetate and 12% ^2H_2O. F_2MeAla methyl ester (0.43 mM) was added and a NMR spectrum taken within 6 min (1,000 scans; acquisition time = 0.30 s). The external pH was 7.3. (*B*) As in Fig. 2C. The external pH was 6.7.

from 6.4 to 8.02, the ^{19}F NMR spectra of the intracellular fluorinated amino acid gave the intracellular pH values shown in Fig. 4. Intracellular pH was a polyphasic function of extracellular pH and was insensitive to extracellular pH between pH values 6.8 and 7.4. In this range, the mean (± SD) intracellular pH was 7.17 ± 0.06. This SD was calculated for the intracellular pH values on the plateau of Fig. 4, corresponding to extracellular pH 6.8–7.4. The intracellular pH as calculated from the equilibrium distribution of DMO (open circles) agrees well with that determined from ^{19}F NMR measurements.

We have characterized several ^{19}F NMR pH probes (unpublished data) of which F_2MeAla was the most suitable for measurements in the physiologically important range of pH for most animal cells. The F_2MeAla methyl ester will complement and extend the range of techniques presently available for measurement of intracellular pH. The ester is expected to permeate most cell membranes, and esterase activity has been demonstrated in a number of animal cells (20). Neither the ester nor the amino acid is cytotoxic, and the inherent high sensitivity of ^{19}F NMR makes accurate determinations of pH possible in short measurement times (1–5 min).

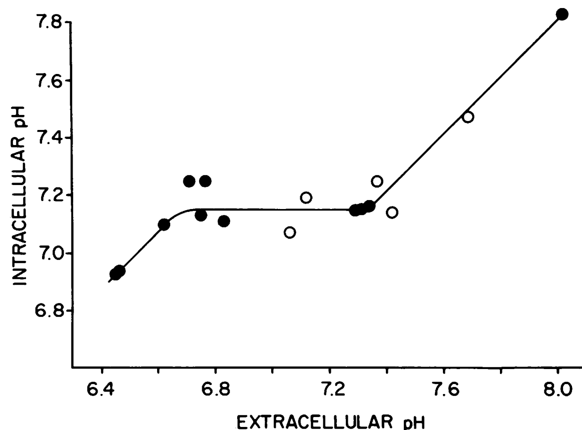

FIG. 4. Intracellular pH as a function of extracellular pH in resting human PBL. ●, Data from experiments described in Figs. 2 and 3; ○, DMO determinations. In these experiments cells were suspended to a concentration of 25–30 × 10^6/ml in Hanks/Hepes medium, incubated for 5 min with DMO (14 μM), and then centrifuged. Cell water and trapped extracellular water were assayed simultaneously (15).

Our previous studies on the intracellular pH of lymphocytes (14) showed marked discrepancies (0.2–0.5 pH unit) in the intracellular pH calculated from the equilibrium distributions of radioactively labeled weak acids and bases. Such measured distributions can yield erroneous values for cytoplasmic pH due to the effects of compartmentation, ionic permeation, or complexation between these probes and ions, proteins, and other cell components and partitioning of the probe into the membrane lipid phase. In the case of lymphocytes, consideration of a 4% mitochondrial volume compartment (14) with a gradient from cytosol to mitochondria (alkaline intramitochondria) of 0.4 pH unit would explain only 0.01 pH unit of this discrepancy between weak acid and weak base calculations. (Lysosomes in lymphocytes appear to be an exceedingly small compartment.) However, the observed discrepancy between DMO and trimethylamine is more than 10 times this value (14), indicating that most of the difference must arise from other sources.

In general, ionic permeability would be expected to cause serious errors in intracellular pH determinations by equilibrium distribution of weak acids and bases only in those cases in which specialized transport systems are present in the membrane or the extracellular pH is several units away from the pK_a. It is unlikely that ionic permeation accounts for the discrepancy between the DMO, trimethylacetic acid, and trimethylamine results. We therefore suggest that complexation to intracellular

or membrane components is the most reasonable source of these discrepancies.

When more than one weak acid or weak base is available, the one giving the lower calculated pH gradient (having the lower concentration gradient) should have the least binding and give the most accurate value. It is therefore significant that the ^{19}F NMR results are in better agreement with the DMO results ($pH_i = 7.2$ at $pH_e = 7.4$) than with the trimethylacetic acid results ($pH_i = 7.5$ at $pH_e = 7.4$). For the following reasons, it is likely that the agreement of the DMO and ^{19}F NMR methods results from accurate measurements of pH_i. (*i*) DMO and F_2MeAla are quite different in structure. (*ii*) In the pH range 6.4–8.0, DMO exists as negatively charged and neutral (mostly charged) species but F_2MeAla exists as a zwitterion and a negatively charged species. (*iii*) All types of complexation and partitioning cause DMO to indicate a more alkaline internal pH. Of these factors, only some complexation reactions would cause a shift in the observed ^{19}F resonant frequencies corresponding to an alkaline pH shift for the F_2MeAla. Complex formation between F_2MeAla and another molecule or ion would cause errors in pH_i determination only if it altered either the apparent pK_a of the $-NH_2$ or the ^{19}F chemical shifts. There is no satisfactory theoretical or pragmatic basis for predicting a particular direction or magnitude of shift in ^{19}F resonant frequency from the molecular structure of such a complex.

We have examined the behavior of human PBL in media at various extracellular pH values from 6.4 to 8.0. We have shown that, in the range 6.8–7.4, PBL maintain a constant intracellular pH of 7.17 ± 0.06. Outside this range, intracellular pH changes in parallel with extracellular pH. Previous reports of measurements of intracellular pH in lymphocytes are conflicting. Zieve *et al.* (21), using the DMO method, found that intracellular pH changes in parallel with extracellular pH over the extracellular pH range 6.8–8.0. Levin *et al.* (22), using the same method, found that intracellular pH is relatively insensitive to changes in extracellular pH between 6.7 and 7.3 and that intracellular pH increases with increasing extracellular pH above pH 7.4. The discrepancy between these two groups may be due to cell preparation techniques (22).

The data of Levin *et al.* (22) are consistent with our findings. We used two independent methods, DMO and ^{19}F NMR, and found regulation of intracellular pH over a wide range of extracellular pH. These data are consistent with our earlier findings (14) that the measured pH gradients are too small, especially in the physiological range, for the H^+ and OH^- to be in electrochemical equilibrium since the measured transmembrane electrical potential is approximately -52 mV. Thus, our findings indicate that human PBL do have a mechanism for utilizing metabolic energy to regulate their intracellular pH. This regulation may be particularly significant in view of the possible role of intracellular pH in stimulated growth and our observation that mitogen-induced growth of human PBL is optimal in the external pH range throughout which the cells can maintain a constant intracellular pH.

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