FLUORINATED α-METHYLAMINO ACIDS AS ^{19}F NMR INDICATORS OF INTRACELLULAR pH

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ABSTRACT A series of nontoxic, fluorinated amino acids and their methyl esters have been used to measure intracellular pH in human peripheral blood lymphocytes by ^{19}F NMR. Mono-, di-, and trifluoro α-methylalanines and their methyl esters have been characterized with respect to their spectra, the pH dependence of their fluorine resonances, and the effects of temperature and cations on the NH_2 pK_a values. These pH indicators can be used singly or in combination to determine intracellular vs. extracellular pH profiles over a wide range (>2 pH units) of extracellular pH.

INTRODUCTION

Speculation on the role of intracellular and organelle pH as a triggering or regulating device in developmental processes has led to recent extensive efforts to develop better methods for measuring pH in cells and organelles. Four methods are commonly used to determine transmembrane pH gradient: (*a*) pH microelectrodes, (*b*) pH-sensitive fluorescent dyes, (*c*) equilibrium intracellular/extracellular distribution of radioactively labeled weak acids and weak bases, and (*d*) ^{31}P NMR.

Microelectrode techniques can follow rapid changes in gradient and measure the pH accurately; however, use of microelectrodes is restricted to relatively large cells immobilized on a surface. Fluorescent dyes can follow rapid changes, but, in general, probe fluorescence can respond to changes other than pH changes, such as altered partitioning between soluble and membrane phases, and changes in volume of cells and organelles. In at least one system, the lipophilic fluorescence probe catalyzes photo-oxidation reactions (Pooler, 1972; Cohen and Salzberg, 1978). Equilibrium distributions of weak acids and weak bases have been extensively used to measure intracellular pH; for a detailed summary of the advantages and disadvantages of this compared with other techniques, see the review by Roos and Boron (1981). The pH-dependent shifts in resonances of intracellular P_i and other phosphate compounds, measured by high-resolution ^{31}P NMR, have been used to determine intracellular pH (for reviews see Gillies et al., 1982 *a*; Burt et al., 1979; Radda and Seeley, 1979). ^{31}P NMR measurements require relatively large intracellular volume fractions, i.e., concentrated cell suspensions, and intracellular concentrations of probe equal to several millimoles per liter of phosphorus; also, the measurements can be made in minutes to tens of minutes. The pH-dependent chemical shift of intracellular P_i has been most useful in those cells with suitable high concentrations of intracellular P_i. However, not all cells have >1 mM levels of P_i in all phases of their growth. P_i is also an active metabolic intermediate, and chemical shifts in the P_i resonance could occur due to processes such as enzymic turnover of P_i or alterations in P_i binding to cell proteins.

These methods, while valuable in many instances, are not sufficiently sensitive or accurate in all cases, specifically in the case of human peripheral blood lymphocytes (PBL). We found that neither ^{31}P NMR nor equilibrium distribution of several radioactive weak acids and bases (Deutsch et al., 1979; and see Results below) gave a satisfactory definition of intracellular pH in human lymphocytes suspended in media of varying intracellular conditions. We therefore devoted our efforts to developing ^{19}F NMR probes of pH. Having demonstrated both the feasibility and accuracy (Taylor et al., 1981; Deutsch et al., 1982) of the ^{19}F NMR approach, we have investigated the applicability of this approach to human blood lymphocytes. We have now begun using fluorine-labeled amino acids in their ester forms as a convenient class of ^{19}F NMR pH indicators, and in this paper, we report the characteristics of such molecules and their use in biological systems.

METHODS

Preparation of Human Lymphocytes

Lymphocytes were prepared from the plateletpheresis by-product were from healthy donors. The lymphocytes were separated by gradient centrifugation, cultured, and assayed for DNA synthesis by ^3H-thymidine incorporation, as described previously (Deutsch et al., 1981).

NMR Measurements

Packed cells were diluted ~1:3 or 1:5 in a Hanks-HEPES solution that had been previously titrated to the appropriate pH and that contained 0.3–1 mM of fluorinated amino acid. This solution was used in the NMR

experiments. The samples usually contained 0.2–0.8 mM trifluoroacetate as an internal standard for chemical shift measurement, and 10–12% D_2O for the field lock.

^{19}F Fourier-transform NMR spectra were obtained on a Bruker CXP 200 instrument (Bruker Instruments, Inc., Billerica, MA); ^{31}P NMR spectra were acquired on a Bruker WH 360 instrument (Middle Atlantic NMR facility, University of Pennsylvania, Philadelphia, PA). ^{19}F and ^{31}P spectra were obtained with broad-band proton irradiation. For measurements of pH in cells, 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, to ensure proper oxygenation of the cells during NMR measurements. The flow system consisted of an external lucite chamber containing a stirring magnet and accommodating a 3–5 mm deep layer of cell suspension, a peristaltic pump to circulate the suspension between the chamber and the NMR probe, and a 10-mm NMR tube with a lucite plug holding a 1.5 mm diam input tube that extended almost to the bottom of the NMR tube. For the duration of the NMR experiments, the external chamber was bubbled with O_2 at atmospheric pressure, and the cell suspension circulated continuously between the oxygen chamber and the NMR tube in the NMR probe. All spectra, with the exception of Fig. 4*A*, were acquired using the flow system. The ^{19}F chemical shifts are expressed with reference to trifluoroacetate, pH 6.8–7.0, in D_2O, and the ^{31}P chemical shifts with reference to 85% H_3PO_4; 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, from Gibco Laboratories, Grand Island, NY; Ficoll from Sigma Chemical Co., St. Louis, MO; Hypaque (sodium salt, 50% solution) from Winthrop Laboratories (New York, NY); Phytohemagglutinin-M from Difco Laboratories Inc., Detroit, MI; [^{3}H] thymidine from New England Nuclear, Boston, MA; trifluoroacetic acid and D_2O from Aldrich Chemical Co., Inc., Milwaukee, WI. α-(difluoromethyl)-alanine methyl ester and trifluoromethylalanine methyl ester and trifluoroethylalanine were obtained from Lee's Bioorganic Laboratories (Marcus Hook, PA). Hexafluorovaline was obtained from Vega Biochemicals (Tucson, AZ)and α-monofluoromethylalanine was prepared by David F. Wilson of the Department of Biochemistry and Biophysics, University of Pennsylvania, by the method of Christensen and Oxender (1963).

RESULTS

Characteristics of Fluorinated Probes

The ^{19}F NMR spectrum of 1 mM α-difluoromethylalanine is an AB-type spectrum with $J/\Delta\nu$, the ratio of the fluorine-fluorine coupling constant to the chemical-shift difference of the two nonequivalent fluorines, $\simeq$0.3. The center-peak spacing between the two fluorine resonances is pH dependent and was used to evaluate both intracellular and extracellular pH. Thus, this compound offers the advantage of containing its own internal standard. The pK_a of the acid's amino group is 7.2–7.3 at 25°C, and is dependent on the ionic composition and temperature of the medium. The methyl ester of difluoromethyl alanine has a pK_a of 5.1 at 25°C and has been described previously (Deutsch et al., 1982).

The ^{19}F NMR spectra of trifluoromethylalanine and of monofluoromethylalanine consist of a single resonance line. For trifluoromethylalanine, the $-NH_2$ pK_a is 5.9 for the acid and <4 at 25°C for the methyl ester. If the trifluoromethyl group is moved further from the amino group the maximal pH-dependent chemical shift is decreased and the pK is higher. (For example, trifluorethylalanine has a total shift of 0.72 ppm between the protonated and unprotonated amine species, and its $-NH_2$ pK_a is ~8.0 in distilled water.) The monofluoromethylalanine has a pK_a value of 8.5; the methyl ester of this compound has a pK_a value of 6.36. Table I shows the values for the pK_a and maximal shift per 0.1 pH unit (slope of titration curve) for the fluorinated amino acid derivatives studied.

The ^{19}F NMR spectrum of 1 mM hexafluorovaline is a doublet. The two resonances, from the two nonequivalent trifluoromethyl groups, are 4 ppm apart in neutral solution, with J_{F-F} = 8.5 Hz. The chemical shifts of each resonance exhibit different pH dependences. One group shows a maximal shift of 0.06 ppm upfield per 0.1 pH unit increase around the pK_a, which is 6.3 at 25°C, while the other shows a maximal shift of 0.01 ppm upfield per 0.1 pH unit increase. This compound is not useful compared with the fluoromethylalanines, since it exhibits a much smaller pH-dependent chemical shift. We therefore did not use hexafluorovaline in the cellular experiments.

As shown in Fig. 1, the pK_a of both difluoro- and trifluoromethylalanine show a marked dependence on temperature. As the temperature was increased from 17° to 37°C, the pK_a of trifluoromethylalanine shifted 0.34 pH units more acid while that of difluoromethylalanine shifted 0.80 units more acid.

The pK_a values for both difluoro and trifluoromethylalanine are similarly affected by $[K^+]$. As shown in Fig. 2, an alkaline shift of the pK_a occurs when K^+ is added to a solution of either amino acid in deionized water; the K^+ effect is saturated at 130 mM K^+. There is no similar effect observed for salt solutions of 50, 150, and 300 mM NaCl. Titration curves were also obtained for these probes suspended in Hanks-HEPES media in the presence of both

TABLE I
pK_a AND MAXIMAL pH SHIFTS FOR FLUORINATED α-METHYLALANINE DERIVATIVES

	pK_a	Δ/0.1 pH unit*
α-trifluoromethylalanine methyl ester	<4	
α-trifluoromethylalanine	5.9	0.12
hexafluorovaline	6.3	0.06
α-difluoromethylalanine methyl ester	5.1	0.14‡
α-difluoromethylalanine	7.3	0.09‡
α-monofluoromethylalanine methyl ester	6.4	0.08
α-monofluoromethylalanine	8.5	0.11

*Δ = change in chemical shift, in ppm.
‡For the difluoromethylalanines, Δ = change in spacing of the two center peaks of the ^{19}F AB spectrum, in ppm.
pK_a and Δ values were determined from titration curves done at 25°C, 1 mM indicator; chemical shifts were referenced to trifluoroacetate.

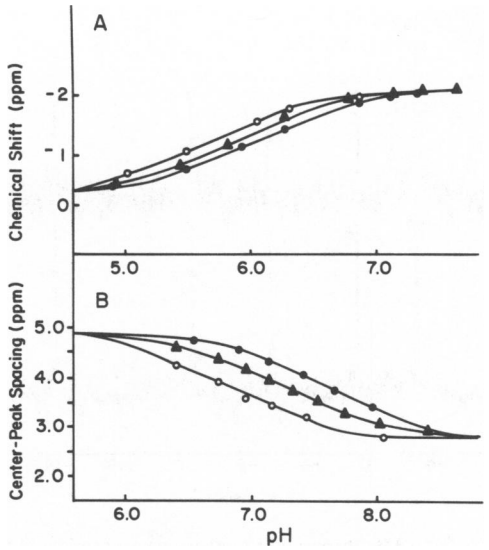

FIGURE 1 Temperature dependence of pH-dependent chemical shifts of difluoro- and trifluoromethylalanine. (*A*) pH dependence of the chemical shift (ppm from trifluoroacetate) of the 188.2 MHz ^{19}F NMR spectrum of α-(trifluoromethyl)-alanine in a Hanks-HEPES (20 mM HEPES, Ca-Mg free) medium. (*B*) pH dependence of the center peak spacing of the ^{19}F NMR spectrum of α-(difluoromethyl)-alanine in a Hanks-HEPES medium. Temperatures were 17°C (•), 25°C (▲), and 37°C (○) (200 scans, 4.2 μs pulse, 0.35 s acquisition time).

magnesium ion (0–3 mM; Fig. 3 *B* for difluoromethylalanine) and calcium ion (0–3 mM; Fig. 3 *A* for trifluoromethylalanine). In the presence of normal intracellular (130 mM KCl) or extracellular (150 mM NaCl) ionic media, neither divalent ion had any effect on the magnitude, chemical shift, or width of the fluorine resonances. A small alkaline shift of pK_a was observed for both di- and trifluoromethylalanines + 0.5 mM $MgCl_2$, compared with the amino acids in deionized water. Accurate measurement of pH with these probes thus requires knowledge of the K^+ concentrations in the cell and external medium, but is not sensitive to variations in Mg^{2+} or Ca^{2+} in physiological media. In our experiments, we used a standard curve (chemical shift or cps vs. pH) for probes in Hanks-HEPES to read extracellular pH, and a second curve for probes in 130 mM KCl, 20 mM NaCl, 10 mM HEPES, which approximates the intracellular medium of PBL, to read intracellular pH.

The mode of transport into different cells may be different for each fluorinated compound. For instance, trifluoromethylalanine is transported by the anion transport system in red cells, as evidenced by its accelerated transport, compared with methylalanine and monofluoro-

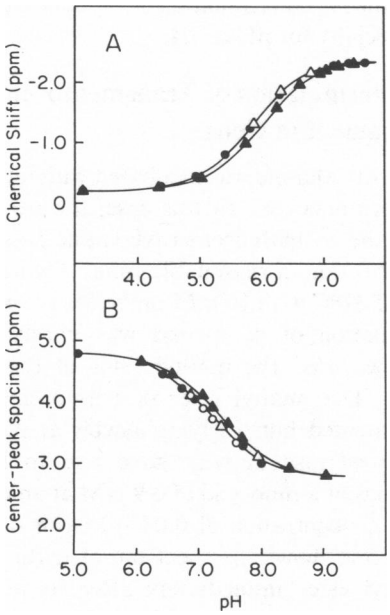

FIGURE 2 Effect of Na^+ and K^+ on the pH-dependent chemical shifts of difluoro- and trifluoromethylalanine. (*A*) pH dependence of the chemical shift (ppm from trifluoroacetate) of the ^{19}F NMR spectrum of α-(trifluoromethyl)-alanine in (▲) 0.30 M KCl, (•) 0.30 M NaCl, and (△) 0.15 M NaCl. (*B*) pH dependence of the center peak spacing of the ^{19}F NMR spectrum of α-(difluoromethyl)-alanine in (▲) 0.30 M KCl, (•) 0.30 M NaCl, (△) 0.15 M NaCl, and (○) 0.05 M NaCl. All solutions contained 10 mM HEPES.

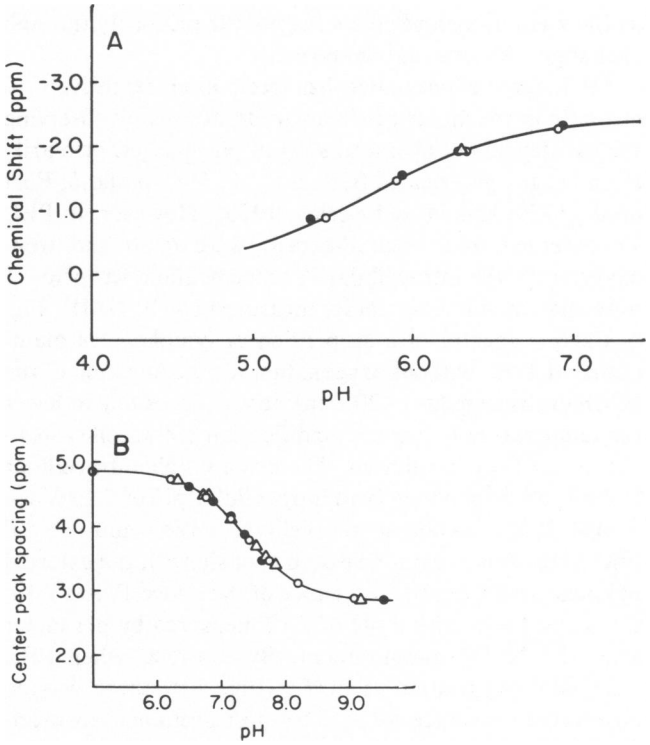

FIGURE 3 Effect of Ca^{++} and Mg^{++} on the pH-dependent chemical shifts of difluoro- and trifluoromethylalanine. (*A*) pH dependence of the chemical shift of the ^{19}F NMR spectrum of α-(trifluoromethyl)-alanine in 130 mM KCl, 20 mM NaCl, and (△) 0.5 mM $MgSO_4$, (•) 1 mM $MgSO_4$ or (○) 3 mM $MgSO_4$. (*B*) pH dependence of the center spacing of the ^{19}F NMR spectrum of α-(difluoromethyl)-alanine in a Hanks-HEPES medium containing (•) 0 mM $CaCl_2$, (○) 1.0 mM $CaCl_2$, or (△) 3 mM $CaCl_2$. Temperature of samples was 25°C.

methylalanine, and the fact that its transport is not competitively inhibited by other amino acids (Christensen and Oxender, 1963). However, trifluoromethylalanine was not rapidly transported into lymphocytes, which do not have a comparable anion transport system. Incubation times of 1–2 h at 37° in 1 mM difluoromethylalanine were required to obtain observable intracellular concentrations of difluoromethylalanine. In contrast, the methyl esters of this compound and of the mono- and trifluoromethylalanines were taken up by lymphocytes rapidly, probably via free diffusion, and all three esters were hydrolyzed by endogenous esterases (see Figs. 5 and 6) to yield substantial concentrations (>1 mM) of the free acids within 3–10 min after addition of methyl ester to the cell suspension. The fluxes of difluoromethylalanine and trifluoromethylalanine are probably mediated by amino acid transport systems in lymphocytes, but not necessarily by the same transport system in each case, since the exported species differ in charge, due to a difference in the pK_a's of the fluorinated amino acids. Experimentally, at an extracellular suspension pH of ~6.6, the efflux of trifluoromethylalanine is at least 10 times slower than that of difluoromethylalanine. This may be because trifluoromethylalanine exists at this intracellular pH (6.8) primarily as an anion, whereas the difluoromethylalanine exists primarily (>6%) as the zwitterion, and efflux may occur primarily through exchange with external amino acids.

^{31}P magnetic resonance has been used extensively to estimate intracellular pH in a variety of cells by observing the pH-dependent chemical shift of phosphates, primarily P_i and sugar phosphates (Gillies et al., 1982 *a* and *b*; Burt et al., 1979; Radda and Seeley, 1979). However, in PBL we observed that when the cells were fresh and well-oxygenated, the intracellular P_i concentration was below 1 mM and could not be readily measured by ^{31}P NMR. Fig. 4 *A* shows spectra of a suspension of lymphocytes maintained at 12°C without oxygenation for the duration of the NMR measurement (~20 min); it was necessary to lower the temperature to prevent acidification of the cell suspension under these conditions. There is a visible intracellular P_i peak, corresponding to an intracellular pH of 7.2. When 5 mM P_i was added to the cell suspension and the ^{31}P NMR spectrum again measured (not shown), the external pH measured from the resonance of the added P_i was 7.14; this agreed well with a pH of 7.12 measured by pH meter after the NMR measurement. By contrast, when PBL were kept oxygenated at 20°C, (Fig. 4 *B*) there was no observable resonance for P_i. The most prominent intracellular phosphate resonance under these conditions is at +4.3 ppm, which is probably due to one or more sugar phosphates. Gillies et al. (1982) have noted the virtual absence of an intracellular P_i peak separate from the extracellular P_i peak in suspensions of respiring Ehrlich ascites cells at 37°C. Under optimum conditions for the cells, therefore, the ^{19}F NMR probes allowed more accurate simultaneous determination of intracellular and external pH, especially for pH ≥ 7.4.

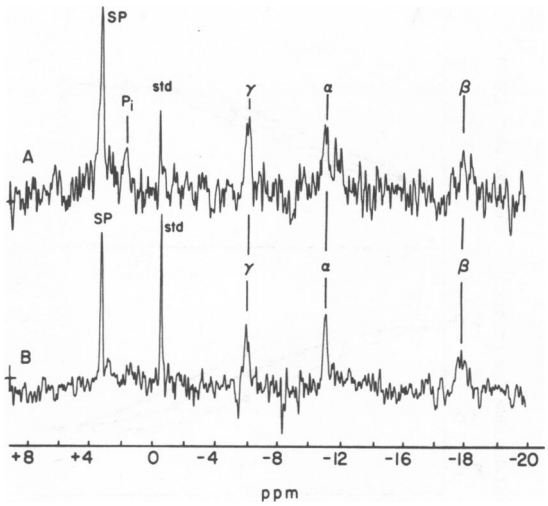

FIGURE 4 ^{31}P NMR spectrum of lymphocyte suspensions. 145.7 MHz ^{31}P NMR spectra of lymphocyte suspensions (under poorly oxygenated and well-oxygenated conditions). (*A*) 0.51 ml packed PBL resuspended in a total volume of 2 ml Hanks-HEPES medium plus 15% D_2O. The sample was cooled to 12°C, transferred to a normal 10 mm sample tube, and the NMR spectrum taken at 12°C (3,000 scans, acquisition time = 0.34 s). (*B*) 0.64 ml packed PBL resuspended in a total volume 1.5 ml phosphate-free Hanks-HEPES medium plus 15% D_2O and 6.6 mM L-glycero-phosphoryl choline (internal standard). The spectrum was taken at 20°C, using the flow system to keep the suspension oxygenated (3,000 scans, acquisition time = 0.34 s). *SP*, sugar phosphate; P_i, inorganic phosphate; α, β, γ, the α, β, γ-phosphorus resonances of ATP; peaks marked *STD* are glycerophosphorylcholine.

Determination of Transmembrane pH Gradient in Cells

Trifluoromethylalanine was incubated with human peripheral blood lymphocytes. In this case, the permeability of the membrane to trifluoromethylalanine was too low to allow equilibration in reasonable times. Under incubation conditions of 37°C with 10 mM probe for 1–3 h, intracellular accumulation of compound was barely observable. Therefore, we used the methyl ester of the fluorinated amino acid. The methyl ester of trifluoromethylalanine rapidly permeated human lymphocytes at 25–37°C, and endogenous esterase activity gave rise to intracellular concentrations of amino acid of 0.9 mM at an extracellular amino acid concentration of 0.04 mM (an ~20-fold gradient). Fig. 5 *A* shows the spectrum of trifluoromethylalanine methyl ester immediately after its addition to a suspension of lymphocytes, and Fig. 5 *B* shows the sample at a later time, when some of the ester has been hydrolyzed to the acid. The largest peak, which is at lowest field (labeled 0 ppm) is the trifluoroacetate reference. The doublet is the spectrum of extracellular and intracellular trifluoromethylalanine, the peak closest to the standard being the extracellular amino acid. The peak at highest

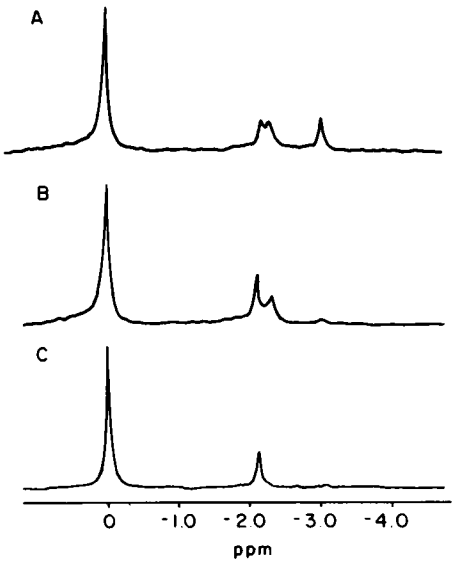

FIGURE 5 ^{19}F NMR spectra of human lymphocytes plus trifluoromethylalanine methyl ester, 25°C. Human PBL were suspended in Hanks-HEPES buffer, initially at pH 6.69, at 25% by volume cells, 0.8 mM α-(trifluoromethyl)-alanine methyl ester, 2 mM trifluoroacetate, and 11% D_2O; spectra were taken at 6 min intervals (1,000 scans, 4.2 μs pulse, acquisition time = 0.35 s). (*A*) 14–19 min after mixing. (*B*) 70–75 min after mixing. (*C*) Cell suspension was centrifuged after the spectrum *B* was completed; the supernatant was returned to the flow cell.

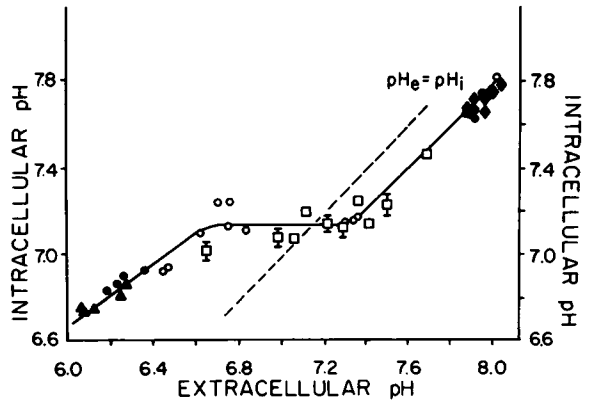

FIGURE 7 Intracellular pH vs. extracellular pH in resting human peripheral blood lymphocytes. Data points are taken from individual ^{19}F NMR experiments using monofluoromethylalanine methyl ester (♦, 1 mM), difluoromethylalanine methyl ester (○, ●, 1 mM) or trifluoromethylalanine methyl ester (▲, 0.5 mM). The filled symbols represent NMR determinations made with two probes simultaneously present in the same sample. DMO (7–14 μM, □) measurements were carried out as described previously (Deutsch et al., 1979). Error bars represent mean value ± SD; these DMO experiments were carried out on suspensions at 2 × 10^6 cells/cm^3 maintained under culture conditions. Those squares without error bars represent DMO experiments carried out on suspensions at 30 × 10^6 cells/cm^3. Cell water and trapped extracellular water were assayed simultaneously. Temperature ranged from 24–25°C.

field is trifluoromethylalanine methyl ester. In Fig. 5 *C*, the spectrum of the supernatant solution from the same sample as in Fig. 5 *A* and 5 *B* is shown; only the extracellular free amino acid is present. The trifluoro compound has the advantage of high sensitivity due to the 3 equivalent F atoms. This probe gave accurate intracellular pH values for lymphocytes when intracellular pH was ≤ 6.9; the results agreed with those previously obtained using difluoromethylalanine (Deutsch et al., 1982).

Difluoromethylalanine has a pK_a of 7.2–7.3, and proved to be optimal for determining the intracellular pH in lymphocytes suspended over a wide range of extracellular pH (Deutsch, et al., 1982). When human peripheral blood lymphocytes were incubated with the methyl ester of α-difluoromethylalanine, the uncharged ester readily crossed the cellular membrane to the cytoplasm, where it was hydrolyzed to the free amino acid. The free amino acid is thus accumulated inside the cell where it serves as a pH probe. When monofluoromethylalanine methyl ester was

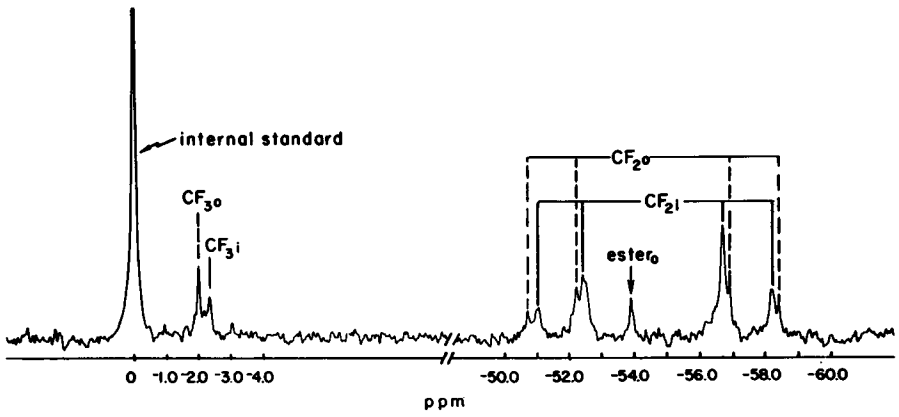

FIGURE 6 ^{19}F NMR of human lymphocytes plus difluoromethylalanine methyl ester (1 mM, labeled CF_2) and trifluoromethylalanine methyl ester (0.17 mM, labeled CF_3) at 25°C. Human lymphocytes were suspended in Hanks-HEPES buffer, initially at pH 6.60 at 25% by volume cells with 11% D_2O. Spectra were taken at 6 min intervals (1,000 scans, 4.2 μs pulse, acquisition time = 0.35 s). The internal standard (2 mM trifluoroacetate) is the downfield peak; the next pair of peaks is trifluoromethylalanine (CF_3) and the upfield peaks are difluoromethylalanine (CF_2). The resonances marked *o* arise from extracellular species while those marked *i* arise from intracellular species.

incubated with human peripheral blood lymphocytes, it too was taken up and hydrolyzed by endogenous esterases. Both the ester and the acid are maintained at high intracellular concentrations (1–2 mM), enabling the calculation of intracellular pH. These values agreed with those obtained using difluoromethylalanine.

Experiments in which difluoro- and trifluoromethylalanine methyl esters were added simultaneously to the same lymphocyte suspension (Fig. 6) showed that intracellular pH values obtained from the two probes in the same cell suspension agreed to within 0.05 pH unit. In similar experiments, mono- and difluoromethylalanines simultaneously gave intracellular pH values that agreed to within 0.06 pH units. Fig. 7 shows the intracellular pH at various extracellular pHs as determined by mono-, di-, and trifluoromethylalanine. All three probes give results consistent with those reported previously for lymphocytes. In addition, the intracellular pH calculated from equilibrium distributions of DMO is included, and this shows reasonable agreement with the ^{19}F NMR results.

Because these probes accumulate to a level of 0.1–5 mM inside cells, we have tested these compounds for cytotoxicity. Lymphocytes incubated in the presence of 0.5–1 mM mono-, di-, and trifluoromethylalanine methyl esters, or hexafluorovaline for 1–2 h at 37°C had no effect on stimulated DNA synthesis, as measured by ^3H-thymidine incorporation (see Table II), or on viability, as measured by trypan blue exclusion. Furthermore, measured ATP/ADP ratios for suspensions of rat liver cells that had been incubated with 1 mM difluoromethylalanine methyl ester and circulated in the NMR flow cell for 25 min at 27°C were not significantly different from the ratios obtained for control cells (Kashiwagura, Deutsch, Taylor, Erecinska, and Wilson, unpublished data).

DISCUSSION

We have succeeded in developing some nontoxic, sensitive ^{19}F NMR indicators of intracellular pH. These probes were specifically designed to be nonmetabolizable and noncytotoxic. The presence of the α-methyl group in amino acids greatly suppresses their metabolism (Christensen and Oxender, 1963; Greenstein and Winitz, 1961); these fluorinated alanines bear an α-methyl group and, as our results indicate, are not cytotoxic. Intracellular pH values obtained with one of these probes agree with pH readings obtained from another probe simultaneously present in the same cell, and with pH values obtained by measuring equilibrium distributions of 5,5-dimethyloxazolidine-2,4-dione (DMO). Any two of the fluorinated probes allow one to construct a reliable profile of intracellular pH over a 2 pH unit range of extracellular pH. The precision of these pH measurements was ±0.06 pH unit, for data such as those in Fig. 6, and is limited by the precision of determining the chemical shifts. In using difluoromethylalanine, poor temperature control can lead to significant determinate error in the pH reading.

An expanded repertoire of pH probes and methods is required to cover all systems and conditions of interest: (*a*) cells and organisms (such as bacteria, plants, mammalian cells) that exist in drastically different pH environments will require probes that have pK_a's in the range of the environmental pH. (*b*) Cell membrane permeabilities may be quite different from cell type to another, making intracellular accumulation of certain probes in reasonable times impractical or impossible, thus requiring different molecules for different types of cells. (*c*) Some cells may lack methyl esterase activity and will require the use of other esters that are susceptible to hydrolysis by the esterases of that particular cell. This has already proven to be the case in the human erythrocyte (Tsien, 1981; Deutsch and Taylor, unpublished data). (*d*) To separate and evaluate subcellular compartment gradients in situ, a number of probes with high detection sensitivity and different pK_a's must be used to assess the actual magnitude of the pH gradient accurately. This will help avoid the possibility that any one particular probe (when only one is

TABLE II
STIMULATED DNA SYNTHESIS IN LYMPHOCYTES INCUBATED WITH FLUORINATED pH INDICATORS

Probe molecule*	^3H-thymidine incorporation			
	Day 1	Day 2	Day 3	Day 4
	cpm	*cpm*	*cpm*	*cpm*
Control	398 ± 4	23,111 ± 165	57,572 ± 7089	41,812 ± 1352
Monofluoromethylalanine methyl ester	432 ± 42	25,068 ± 4697	60,960 ± 4285	43,322 ± 96
Difluoromethylalanine methyl ester	492 ± 110	21,228 ± 574	58,201 ± 9612	46,257 ± 1711
Trifluoromethylalanine methyl ester	423 ± 20	21,799 ± 2010	57,017 ± 1277	45,943 ± 811
Hexafluorovaline	362 ± 63	24,175 ± 761	56,615 ± 1104	34,135 ± 4846

*The concentrations of monofluoromethylalanine and trifluoromethylalanine methyl esters were 0.5 mM while those of difluoromethylalanine methyl ester and hexafluorovaline were 1.0 mM. Cells (0.57×10^6 cells/ml) were incubated in Hanks-HEPES medium for 1–2 h at 37°C, centrifuged and resuspended in culture media containing minimal Eagle's medium, 10% serum and 50 μg/ml succinyl Concanavalin A and cultured in a 5% CO_2-incubation for the next 4 d. Cultures were pulsed with ^3H-thymidine (0.25 μCi) and harvested 8 h later.

used) is in error due to a cell-specific interaction. Finally, since all of the methods of intracellular pH determination, including the one presented here, have the possibility of giving erroneous results under some circumstances, prudence dictates that at least two independent methods should be used to determine intracellular pH whenever possible. The fluorinated α-methylalanine derivatives described here are the first members of a new class of noncytotoxic ^{19}F NMR pH indicators that should be applicable in a wide variety of biological systems.

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