brief communication

New class of ^{19}F pH indicators: fluoroanilines

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ABSTRACT The pH dependence of the ^{19}F chemical shift has been characterized for a number of fluorine-substituted aniline derivatives. These compounds constitute a new class of ^{19}F nuclear magnetic resonance (NMR) pH indicators, characterized by single ^{19}F resonance lines with sensitivities ranging from 2 to 7 ppm/pH unit near the aniline pK_a; total shifts between conjugate acid and base of 5–15 ppm; and pK_as ranging from 1 to 7. One compound, *N,N*-(methyl-2-carboxyisopropyl)-4-fluoroaniline, has a pK_a of 6.8 and a sensitivity of 5 ppm/pH unit. This compound displays significant broadening of its ^{19}F resonance near the aniline pK_a (6.8), due to a decreased rate of exchange between conjugate acid and base species. Our results are consistent with slow dissociation of an intramolecular hydrogen bond in the zwitterionic species that limits the exchange rate between protonated and unprotonated forms for *N,N*-(methyl-2-carboxyisopropyl)-4-fluoroaniline.

INTRODUCTION

Nuclear magnetic resonance (NMR) techniques for measuring cellular ion concentrations offer the opportunity for spatial and temporal resolution of indicator signals. We have developed and tested a number of pH-sensitive ^{19}F NMR indicators, primarily fluorinated alanine derivatives (Deutsch et al., 1982; Taylor and Deutsch, 1983; Deutsch and Taylor, 1987*a, b*), which are generated in situ from their corresponding ester precursors. The conjugate acid and base forms of the free α-methyl amino acids are in fast exchange, giving a single line or multiplet whose chemical shift is a function of pH. The esters are easily resolved from the free amino acids over the pH range of interest, especially at fields >9T (Deutsch and Taylor, 1987*a, b*). The sensitivity of this class of indicators is 1 ppm/pH unit; their range of pKa values is 5.9–8.5.

We have synthesized and tested a similar class of ^{19}F molecules, based on a para-fluoroaniline structure (Deutsch and Taylor, 1987*a*). Fluorine substitution on the aromatic ring is highly electron-withdrawing, and consequently lowers the pKa of the aniline $-NH_2$ group. The sensitivities range from ~2–7 ppm/pH near the pK_a, with total shifts between conjugate acid and base of 5 to 15 ppm. This class of molecules has pK_as ranging from pH 1 to 6. Hence it was necessary to modify these molecules in order to move the pKa into a physiological range. Here we report the ^{19}F NMR properties of derivatives of one such compound, *N*-(2-carboxy-isopropyl)-4-fluoroaniline. Although this compound has the sensitivity, specificity, and appropriate pKa to make it useful for pH measurements, the results reported here are preliminary to development of a probe designed to minimize lipophilicity, which will incorporate the moiety represented by *N*-(2-carboxy-isopropyl)-4-fluoroaniline. In vivo tests are best deferred until such optimally designed molecules are available, because biological toxicity, binding, and compartmentation should be assessed in each individual case.

METHODS

NMR measurements

^{19}F Fourier-transform NMR spectra were obtained on a CXP 200 or AM 500 MHz spectrometer (Bruker Instruments, Inc., Billerica, MA). Titration curves, T_1 and T_2 determinations were carried out in 10-mm tubes, using an external standard insert tube containing trifluoroacetate, pH 6.8–7.0 in D_2O (uncorrected pH meter reading), with broad-band proton decoupling.

T_1s were determined from inversion recovery experiments, with pulse intervals of 0.03, 0.07, 0.1, 0.3, 0.7, 1, 3, and 10 s at pH 9, and 0.07, 0.1, 0.3, 0.7, 2, 5, 10, and 15 s at pHs 7 and 3.8. The T_1s were calculated from a 3-parameter fit to the resonance amplitudes (T_1, equilibrium magnetization value, and flip angle). T_2s were determined with the Carr-Purcell-Meiboom-Gill sequence; even echo amplitudes were used and echo times were increments of 0.1 s from 0.1 to 0.8 s, except as noted.

Cell cultures

Human peripheral blood lymphocytes (PBL) were obtained by venipuncture from healthy donors. Cells were purified and cultured as described previously (Taylor and Deutsch, 1983). PBL were cultured at 0.5×10^6 cells/cc in 1 ml of minimum essential medium (MEM) in the absence and presence of phytohemagglutinin (10 μg/ml), pulsed for 8 h with 50 μl ^{3}[H]-thymidine (10 μC$_i$/ml stock) at 24-h intervals for a 96-h period, and then harvested onto glass-fibre filters (M12 cell harvester; Brandel, Gaithersburg, MD), in order to determine thymidine incorporation into DNA.

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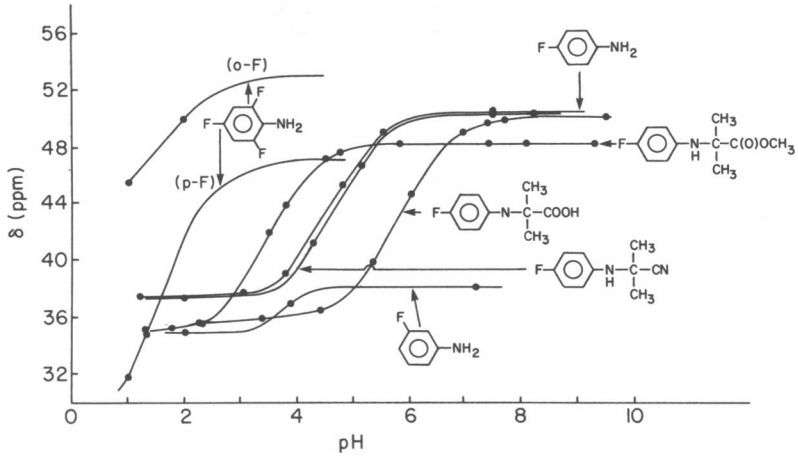


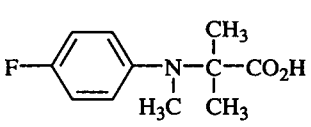
FIGURE 1 pH dependence of ^{19}F chemical shifts of fluorinated anilines and p-fluoroaniline derivatives. Chemical shifts were determined at 188.2 MHz, 25°C in Hanks-20 mM Hepes media for aniline concentrations between 1–5 mM (Chemical shifts are referenced to trifluoroacetate).

Materials

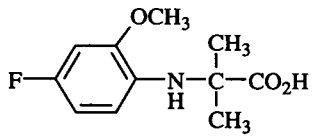
Experimental reagents were obtained from the following suppliers: Hanks balanced salt solution, Ca^{2+}- and Mg^{2+}-free, from Gibco Laboratories, Grand Island, NY; Ficoll from Sigma Chemical Company, St. Louis, MO; Hypaque (sodium salt, 50% solution) from Winthrop Laboratories (New York, NY); phytohemagglutinin-*M* from Difco Laboratories Inc., Detroit, MI; trifluoroacetic acid and D_2O from Aldrich Chemical Co., Inc., Milwaukee, WI; α-difluoromethylalanine, α-trifluoromethylalanine, O-methoxy-*N*-(2-carboxyisopropyl)-4-fluoroaniline, *N*,*N*-(methyl-2-carboxyisopropyl)-4-fluoroaniline, and the methyl esters of these four compounds were obtained from Lee's Bioorganic Laboratories (Marcus Hook, PA).

RESULTS

As shown in Fig. 1, the ^{19}F resonance line of ortho- and/or parafluorine substituted anilines shifts between 5 and 7 ppm/pH unit in the pH range near the amine pKa (pHs 3–5). The total change in parts per million between conjugate acid and conjugate base is >10 ppm for these compounds. Meta-fluoroaniline has a lower sensitivity (2.3 ppm/pH unit) and a total shift of 3.2 ppm. Monofluoro-substituted aromatic amines therefore represent viable candidates for pH indicators. However, the pK_as are in the acid range. Two strategies were used to shift the pKa of these compounds to higher pH: *N*-alkylation (compound A), and addition of an electron donating group to the aromatic ring (compound B).



Compound A



Compound B

As shown in Fig. 2, addition of an ortho-methoxy group did not have any appreciable effect on the pKa; the aniline pKa remained at a value of 5.8 for o-methoxy-*N*-(2-carboxyisopropyl)-4-fluoroaniline (compound B). However, *N*-methylation produced *N*,*N*-(methyl-2-carboxyisopropyl)-4-fluoroaniline (compound A), a compound with a pKa of 6.8. These compounds have total shifts of 12 ppm (compound A) and 17 ppm (compound B), and sensitivities of 5 and 7.5 ppm/pH unit, respectively.

Fig. 3 shows the titration of a solution containing both compound A and its methyl ester. (The aniline pK_a = 3.8 for this ester.) The acid form shows significant broadening in the region of the aniline pK_a, broadening which is dependent on the strength of the external magnetic field. At 188.2 MHz the ratio of the linewidths at half height of acid:ester is 2:1, while at 470.5 MHz the linewidth ratio is 6.7:1. Compound A's methyl ester shows no such marked broadening either at pH 7 or in the vicinity of the ester pKa (pH 3.8), ruling out viscosity effects and indicating that the effect is associated with the ionized carboxylate group.

A study of the concentration dependence of linewidth of compound A at pH 7 showed no change in linewidth from 0.1 to 1 mM (data not shown). These results suggest that there is no significant degree of self association at these concentrations, which are approximately the range used in the NMR experiments. However, increasing the temperature gave a significant decrease in linewidth at pH 7 and indicated that the broadening could be due to chemical exchange (Fig. 4). Figs. 5 and 6 show the effects of temperature, Na^+, K^+, and the sulfonic acid buffer Hepes on the resonances of compound A. There is no dependence on the nature of the cation; the same exchange rate is observed in the presence of 150 mM K^+ or Na^+. Fig. 5 shows that compound A is in slow exchange

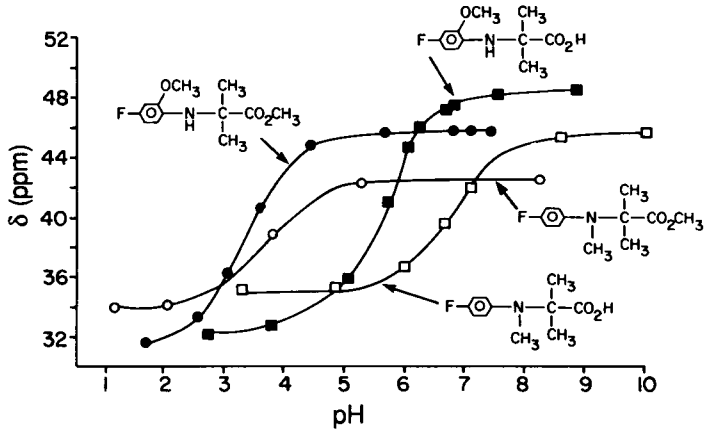


FIGURE 2 pH dependence of ^{19}F chemical shifts of *N,N*-(methyl-2-carboxyisopropyl)-4-fluoroaniline (Compound A) and o-methoxy-*N*-(2-carboxyisopropyl)-4-fluoroaniline (Compound B) and their corresponding methyl esters. Chemical shifts are referenced to trifluoroacetate. Chemical shifts were determined at 188.2 MHz, 25°C in Hanks-20 mM Hepes media for 1 mM of compound.

at 12°C in the absence of Hepes (middle row—separate peaks for protonated and unprotonated species). Increasing temperature or [Hepes] results in an increased exchange rate between conjugate acid and base, and collapse of the separate resonances. Fig. 6 shows increasing rates of exchange with increasing [Hepes]. The average exchange rate, $1/\tau$, increases from $5 \times 10^3\ s^{-1}$ at 12°C, to $1.0 \times 10^4\ s^{-1}$ at 37°C ([Hepes] = 0), or to $1.2 \times 10^4\ s^{-1}$ at 12°C ([Hepes] = 10 mM). The chemical exchange rate was estimated from the ratio of the inten-

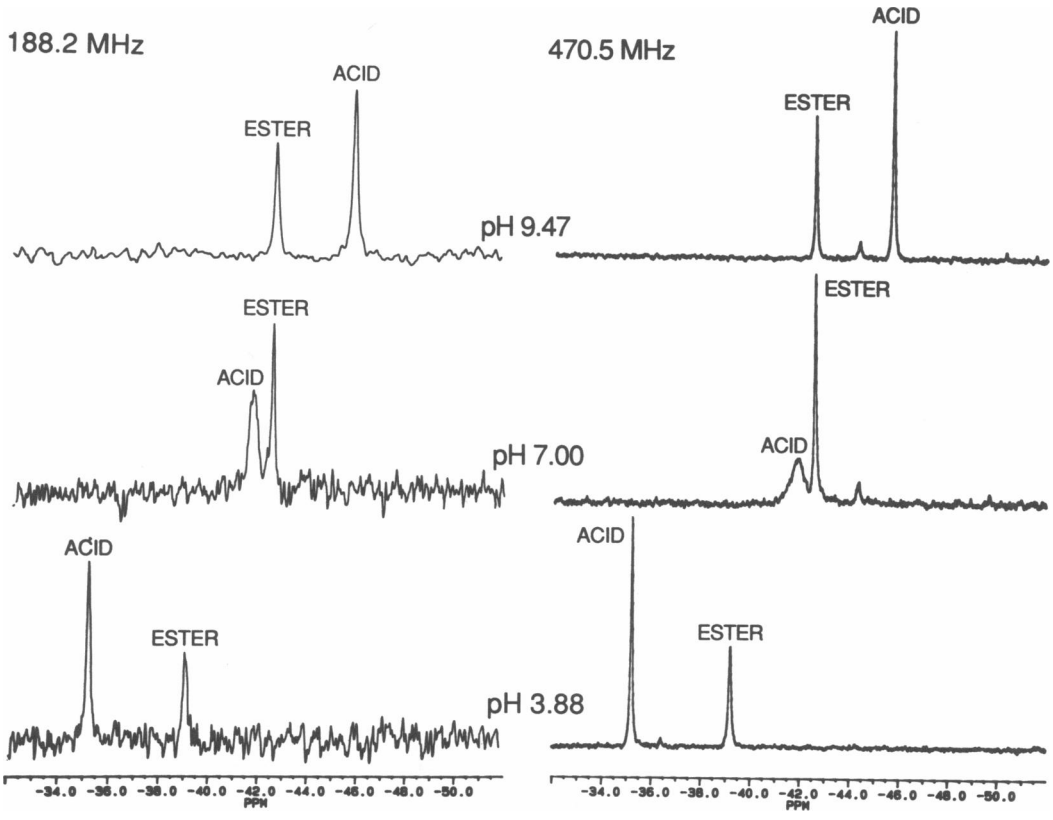


FIGURE 3 ^{19}F NMR spectra of Compound A and its methyl ester, 5 mM of each in Hanks, 20 mM Hepes, at pHs 4, 7, and 9. Conditions for spectra at 188.2 and 470.5 MHz are given in legend of Table 1.

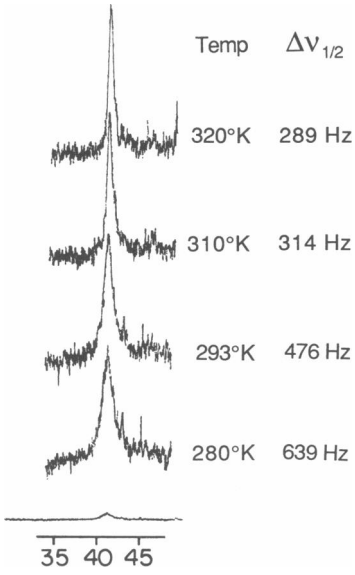


FIGURE 4 Temperature dependence of ^{19}F resonance of Compound A at 470.5 MHz. Solution contained 1 mM compound A in Hanks – 25 mM Hepes. Temperatures as indicated. PW = 35 μs, SW = 37,000 Hz, 16 K data points, scans = 300. Full width at half-maximum ($\Delta\nu_{1/2}$) is given in hertz. pH = 7.0.

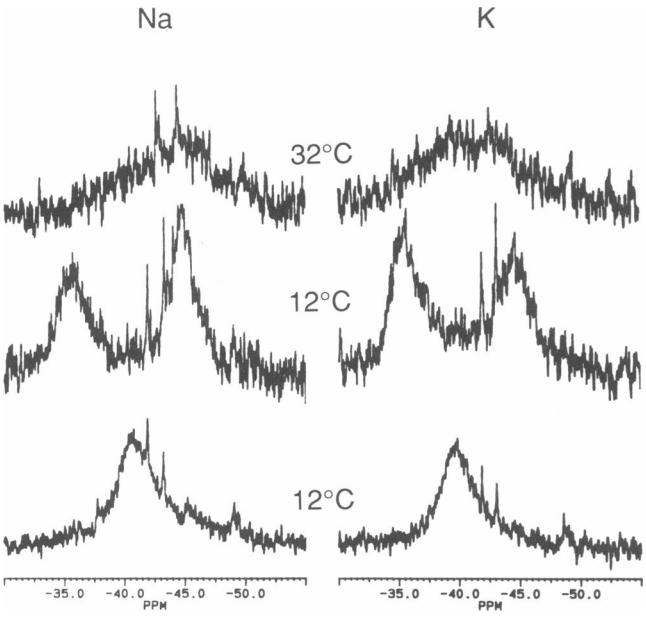


FIGURE 5 Exchange-broadening of ^{19}F resonance of Compound A as a function of cations (Na^+, K^+), temperature and (Hepes) at 470.5 MHz. All solutions contained 1.0 mM Compound A. For all spectra shown in the left column, solutions contained 150 mM NaCl; those on the right contained 150 mM KCl. Temperatures are as shown. (*Bottom row*): solutions contained, in addition to Compound A and alkali chloride, 10 mM Na-Hepes. Conditions for NMR spectra are as described in the legend to Table 1. pH = 7.0.

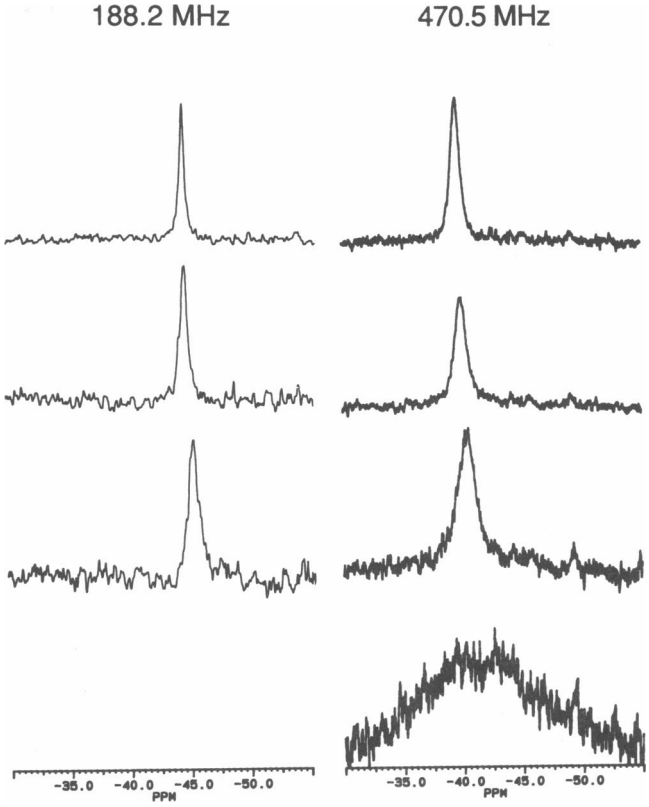


FIGURE 6 Effects of (Hepes) on exchange-broadening of ^{19}F spectra of Compound A at 188.2 and 470 MHz. Solution contained 1.0 mM Compound A, 150 mM KCl and Hepes concentration of 40 mM (*top*), 20 mM, 10 mM, and zero (*bottom*). T = 26°C for 188.2 MHz measurements and 32°C for 470.5 MHz. Conditions for NMR spectra as in legend to Table 1. pH = 7.0.

sity at the frequency of the conjugate acid, ν_1, to the intensity at the center of the coalescing peaks, $(\nu_1 + \nu_2)/2$ at 470.5 MHz, (Dimitrov, 1976; Martin et al., 1980). This calculation assumes that there are two equally populated exchange sites, no strong coupling between the two sites, and T_2 (or T_2^*) of site 1 equals that of site 2. The two exchanging species are the conjugate acid and base forms of compound A. The assumption of equal population of both sites holds at pH equal to pKa (7.0), and the assumption that the T_2^*s are equal is reasonable (Fig. 3). However, we have not verified that the simple two-site model is valid. Therefore, the exchange rates above must be considered estimates.

The longitudinal and transverse relaxation rates, T_1 and T_2, were measured at pH 9.4, 7.0, and 3.9 for the acid and ester forms of compound A at both 188.2 and 470.5 MHz. As Table 1 shows, the ester and acid have very similar T_1s at all pHs. The T_2 estimates are much less accurate (although the precision of the fits, shown by the standard deviations in Table 1, is good). However, it can

TABLE 1 T_1 **and** T_2 **values of compound A (acid and its methyl ester)**

470.5 *MHz*	Compound A	188.2 *MHz*
$T_1 = 0.73 \pm .13$ s $T_2 = 0.29 \pm .08$ s	Ester pH 9.4	$T_1 = 1.0 \pm 0.2$ s $T_2 = 0.21 \pm 0.04$ s
$T_1 = 0.79 \pm .15$ s $T_2 = 0.14 \pm .05$ s	Acid pH 9.4	$T_1 = 1.0 \pm 0.2$ s $T_2 = 0.30 \pm 0.04$ s
$T_1 = 2.7 \pm 0.04$ s $T_2 = 0.26 \pm 0.10$ s	Ester pH 7.0	T_1 = ND $T_2 = 0.67 \pm .06$ s
$T_1 = 2.5 \pm 0.1$ s T_2 = ND	Acid pH 7.0	T_1 = ND T_2 = ND
$T_1 = 2.4 \pm 0.05$ s T_2 = ND	Ester pH 3.9	$T_1 = 4.2 \pm 0.6$ s $T_2 = 0.08 \pm 0.03$ s
$T_1 = 2.2 \pm 0.4$ s $T_2 = 0.46 \pm 0.16$ s*	Acid pH 3.9	$T_1 = 3.8 \pm 0.2$ s $T_2 = 1.6 \pm 0.4$ s*

T_1 and T_2 pulse sequences are described in the Methods. At 188.2 MHz, 90° PW = 15 μs, sweepwidth 2,800 Hz, 8 K zero-filled to 16 K data points, 16 scans, T = 26°C; at 470.5 MHz, 90° PW = 35 μs, sweepwidth 16,000 Hz, 16 K data points, 8 scans, T = 32°C. Values for T_1s were calculated using a 3-pm fit to the resonance amplitudes (T_1, equilibrium magnetization value, and flip angle). Errors are ± standard deviation of the fit. ND = not determined. *These values should be regarded as estimates due to drift in the RF pulse angle for these measurements.

be seen that in all cases, except for the acid at pH 7, the natural linewidths, given by T_2, are of the order of 1–16 Hz at 470.5 MHz, and 0.5–4.0 Hz at 188.2 MHz. Therefore the dominant contribution to the observed linewidths (Fig. 3) is the field inhomogeneity for all resonances except the acid at pH 7.

The ester of compound A can be hydrolyzed to yield the acid, compound A, by purified porcine carboxylase (Sigma Chemical Co.). It is also cleaved by human peripheral blood lymphocytes. Human peripheral blood lymphocytes were suspended at 5×10^6 cells/cc in MEM medium, in the presence of 3 mM ester of (compound A) for 4 h in a CO_2-incubator at 37°C. The supernatant was diluted to 30% with Hanks-20 mM Hepes and titrated to pH 9 for an accurate determination of the relative peak areas of methyl ester and compound A. Twenty-six percent of the ester was converted to compound A under these conditions. Trypan blue is excluded by these cells to 95% after 5 h of incubation with 1 mM ester at 37°C. To evaluate cytotoxicity, lymphocytes were stimulated with phytohemagglutinin (PHA) after short term (2 h or 4 h, 37°C) and long term (72 h, in culture) incubation under culture conditions with 1 mM compound A methyl ester or 1 mM difluoro-α-methyl alanine methyl ester. (The latter was included as a cytotoxicity reference; Taylor and Deutsch, 1983.) Thymidine incorporation (cpm ± SD, n = 3) at 72 h was identical for 2 h incubations with both probes, while incorporation was 30,980 ± 1,058 and 56,269 ± 2,480 for cultures incubated for 4 h with compound A methyl ester and difluoro-α-methyl alanine methyl ester, respectively.

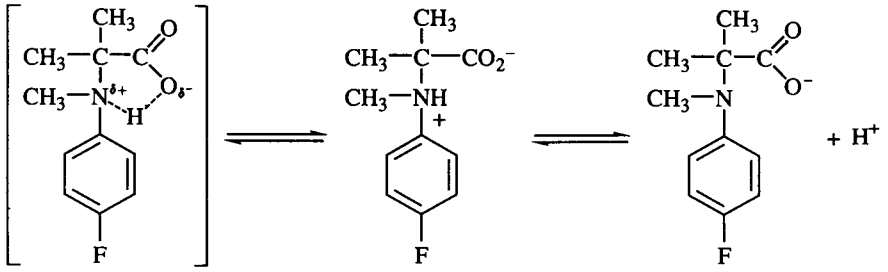
The corresponding incorporation in 72-h cultures was 147 ± 90 and 56,822 ± 3,238; incorporation in stimulated and unstimulated controls were 62,119 ± 642 and 246 ± 70, respectively. These data suggest that (*a*) neither the aliphatic nor the aromatic fluorinated probe is toxic after 2-h incubation with cells, (*b*) 4-h incubation causes 50% inhibition of stimulated proliferation in the case of compound A methyl ester, and 10% inhibition in the case of difluoro-α-methyl alanine methyl ester, and (*c*) 72-h incubation with Compound A ester leads to total inhibition, and <3% inhibition with difluoromethylalanine ester. Long term incubation (72 h) is an extremely stringent condition; during such extended times a number of processes can contribute to observed cytotoxicity, which are not significant in shorter times.

DISCUSSION

To evaluate pH in situ, we need sensitive pH NMR indicators. Toward this end we have developed exogenous fluorinated pH probes. Thus far we have developed and tested a series of aliphatic amines, which have a sensitivity of 1 ppm/pH near the pK_a. Further increases in sensitivity would allow us to measure the pH of small-volume compartments (such as intracellular organelles) and small pH gradients. We therefore decided to exploit the resonance interaction between amine and ortho or para fluorine substituents on an aromatic ring. We were encouraged by three reports in the literature: Smith et al. (1983) showed that 5-fluoro-BAPTA, a fluorinated calcium indicator, had a large sensitivity to pH of the fluorine chemical shift; Chang and Graves (1985) showed large pH-dependent shifts for fluorinated pyridoxals; Metcalfe et al. (1985) showed that the fluorinated analog of quene-1, a *trans*-analog of the calcium indicator quin 2, could be used to measure pH by fluorescence or by NMR spectroscopy and also displayed a large total shift of the ^{19}F resonance with pH. In seeking the simplest, least hydrophobic fluorinated aromatic amine that would have maximum sensitivity, we found that a simple system with significant electronic resonance, fluoroaniline, possessed the same large chemical shift with pH observed for the larger aromatic systems cited above.

N,*N*-(methyl-2-carboxyisopropyl)-4-fluoroaniline (compound A) is a promising ^{19}F NMR pH indicator with a single resonance line, a pKa of 6.8 and chemical shift/pH unit = 5 ppm around its pKa. Its methyl ester, like those of the α-methyl fluoroalanines we have previously investigated, can be cleaved by human peripheral blood lymphocytes. Initial tests showed no cytotoxicity when lymphocytes were incubated with the ester for times

approximating NMR experiments (2 h). Compound A is in fast exchange at lower fields and higher (~30) temperature. Chemical exchange broadening is marked at temperatures below 25° at 188.2 MHz and 37° at 470.5 MHz. The decreased exchange rate (~10^4 s^{-1}) between protonated and unprotonated forms is much slower than proton exchange rates for amines in aqueous solution (Loewenstein and Meiboom, 1957). One possibility is that intramolecular hydrogen bonding occurs in the zwitterion of compound A between the carboxylate and protonated amine groups:



The rate of dissociation of the H-bond then will limit the chemical exchange rate for the conjugate acid and base forms of compound A. The same exchange broadening was observed for compound B at its pKa, but not for the methyl esters of either compound at their pKas (~4–5). The T_1s for conjugate acid and base forms of compound A (free acid) and the conjugate base of compound A methyl ester, are 1-4s. The ester of compound A shows no broadening at its pKa (3.8). This is consistent with chemical exchange as the mechanism for line broadening of compound A at pH 7. Due to the exchange broadening of the ^{19}F resonance, the optimal conditions for the use of compound A, then, are at 188.2 MHz and 32–37°C, where the effect of exchange is minimized. Alternatively, the molecule can be modified to inhibit intramolecular hydrogen bonding in the zwitterionic species, thereby restoring fast exchange between conjugate acid and base species.

Clearly, compound A has the sensitivity and specificity to qualify as a useful indicator. However, for use in biological systems its lipophilicity should be altered to obviate any partitioning into hydrophobic portions of cells, e.g., introduction of polar side-groups. We are optimistic that further chemical modifications of compound A and/or incorporation of the *p*-fluoroaniline moiety into other compounds can provide useful probes for in situ determinations of pH in biological systems.

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