^{19}F-NUCLEAR MAGNETIC RESONANCE: MEASUREMENTS OF [O_2] AND pH IN BIOLOGICAL SYSTEMS

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ABSTRACT ^{19}F-nuclear magnetic resonance (NMR) has been used to determine both intracellular pH and oxygen concentrations in cell suspensions. Oxygen concentrations in *Paracoccus denitrificans* and insulinoma cells, RINm5F, in the NMR probe can be monitored directly by $1/T_1$ measurements of perfluorotripropylamine (FTPA)/lecithin emulsion added to the suspensions. With FTPA oxygen monitoring, we investigated the relative aeration capabilities of two types of NMR chambers. Both normal and transformed eucaryotic cells can be maintained in either chamber for at least 1–2 h at cytocrits of up to 20–25%, with 30% oxygen saturation and cell viabilities of 90–95%. Similar concentrations of procaryotes were maintained aerobic with high FTPA concentrations in the more efficient of the two NMR chambers. A new precursor molecule for the ^{19}F-NMR pH indicator difluoromethylalanine, the para-chlorophenyl ester, has been tested and used in RINm5F cells and *P. denitrificans*, neither of which hydrolyzes methyl esters.

INTRODUCTION

To understand the role of pH in cell function, we have had to develop sensitive, accurate methods for measuring intracellular pH in cell suspensions and intact tissues. For this purpose we chose NMR techniques since they provide spatial and temporal resolution of signals. In particular, we have exploited the advantages of the ^{19}F nucleus (Deutsch and Taylor, 1987*a*). Over the past five years we have designed, tested, and used a repertoire of pH-sensitive ^{19}F-NMR molecules, spanning a range of pKa's, sensitivities, and resonance frequencies (Deutsch and Taylor, 1987*a*,*b*). Two important problems to be addressed in the successful use of ^{19}F-NMR determination of intracellular pH are (*a*) finding appropriate indicator precursor molecules and (*b*) achieving appropriate aeration of the biological specimen in the NMR experiment. In this paper, we present our approaches and progress toward both of these goals.

Mono-, di-, and trifluoro- α-methylalanines are noncytotoxic NMR pH indicators (Deutsch and Taylor, 1987*a*,*b*; Taylor and Deutsch, 1983). With respect to the first problem, the methyl esters of α-methylfluoroalanines proved to be suitable indicator precursors in some, but not all, cells. In lymphocytes, hepatocytes, rabbit colon, and frog skin, these methyl esters diffuse rapidly into the cells and are cleaved by intracellular enzymes to the free fluoroalanines. Unfortunately, not all cells hydrolyze methyl esters. Tsien found that erythrocytes do not hydrolyze methyl or ethyl tetra-esters of a calcium chelator derived from bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) (Tsien, 1981), and we found that the methyl esters of fluorinated α-methylalanines are not hydrolyzed in human erythrocytes, 3T3 fibroblasts, RINm5F cells, *P. denitrificans*, or *E. Coli*.

Methyl esters are relatively stable to hydrolytic cleavage, compared to *p*-NO_2-phenyl, chlorophenyl, or acetoxy esters. Consequently, we selected one of the more labile esters so that intracellular pH indicator would be generated within minutes by aqueous hydrolysis, even if the requisite esterase activity were absent in the cells. The *p*-chlorophenyl ester of α-difluoromethylalanine (1.50 mM) in Hanks/Hepes buffer, pH 7.2, underwent nonenzymatic hydrolysis with a half-time of 15 min at 25°C, whereas the methyl ester, under the same conditions, showed no evidence of hydrolysis after 6 h.

With respect to our second goal, appropriate aeration of the biological specimen, it is necessary to match the rate of consumption with rate of supply so that metabolically controlled cell functions are not altered or compromised by anoxia. To achieve this we needed to measure and monitor oxygen concentrations and sample consumption rates. Our strategy was to use perfluorinated hydrocarbon derivatives as oxygen monitors; these compounds also increase the oxygen supply at high fluorocarbon concentration. The advantage of this approach is that it allows simultaneous ^{19}F-NMR measurements of other parameters, such as pH and ion concentrations (Deutsch and Taylor, 1987; Metcalfe et al., 1985; Smith et al., 1986; Levy et al., 1987).

It is generally known that cell suspensions vary greatly in their oxygen consumption, with procaryotic cells capable of consuming oxygen at rates hundred of times faster than eucaryotic cells. Table I shows the oxygen consumption rates of *Paracoccus denitrificans* and several eucaryotic

TABLE I
OXYGEN CONSUMPTION RATES FOR CELL SUSPENSIONS, 28°C

	O_2 consumption		
Cell suspension	*nmol O_2 $min^{-1}ml^{-1}$*		*μmol O_2min^{-1} mg $protein^{-1}$*
*P. denitrificans**	Basal	5.6×10^2	1×10^2
	Uncoupled	2.04×10^3	4×10^2
	nmol $O_2min^{-1}(10^6$ $cells)^{-1}$		
RINm5F	1.0 (±1.5%)		5.9
PBL	0.14 (±30%)		3.6
CEM	0.53 (±5%)		5.8
VLB	0.60 (±7%)		5.4
HPB	1.2 (±25%)		—

RINm5F are transformed cells derived from a rat insulinoma. PBL are human peripheral blood lymphocytes. CEM, VLB, and HPB are transformed human T-cell lines. All eucaryotic cells were suspended in Hanks-Hepes pH 7.4.

*Suspended in 100 mM NaCl, 10 mM Hepes. Oxygen consumption rates were measured with a Clark-type oxygen electrode in a stirred, thermostatted chamber.

cell lines, including transformed cells. Oxygen consumption rates, when expressed as μmol O_2/min per 10^6 cells, span almost one order of magnitude. Suspensions of *P. denitrificans*, prepared at 25% cytocrit in 100 mM NaCl, 20 mM Hepes, pH 7.4, respired about 20 times faster in the resting state than the eucaryotic cells. The oxygen consumption rate of the bacteria increased another fourfold in the presence of glucose plus the carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which uncouples oxidative phosphorylation.

In ^{31}P-NMR experiments, aerobiosis can often be monitored by quantifying the amounts of ATP, phosphocreatine, and inorganic P_i. In ^{19}F-NMR studies, however, where there are no endogenous metabolic indicators of aerobiosis, or when the investigators need to be assured of adequate aeration before committing an irreplaceable or valuable sample, a suitable oxygenation indicator is necessary. Emulsions of perfluorinated compounds can serve as both an indicator of [O_2] in the NMR sample and, in some cases, an O_2 reservoir. These compounds may, therefore, aid measurements of intracellular pH in selected procaryotes as a function of extracellular pH, under conditions that alter ion transport and the electrochemical gradient for H^+. The validity of such data depends on maintaining an aerobic state.

Emulsions of perfluorinated compounds and their derivatives have been used to replace blood in whole animals. Gases are ten times more soluble in the perfluorocarbon phase than in aqueous solutions or plasma (Sargent and Seffl, 1970). However, to our knowledge, no data exist for aeration of cell suspensions. ^{19}F- and ^{13}C-NMR resonances of fluorocarbons have spin-lattice relaxation rates ($1/T_1$) that are dominated by the dipolar interaction between the paramagnetic O_2 molecule and the fluorine, or carbon-13, nucleus (Hamza et al., 1981); this is also the case for perfluorotributylamine (FTBA) and perfluorotripropylamine (FTPA) emulsions exposed to O_2. Relaxation rates ($1/T_1$) for these resonances increase linearly with [O_2] in pure perfluoro compounds (Hamza et al., 1981) and in aqueous emulsions of these compounds (Parhami and Fung, 1983; this paper). We required a fluorochemical emulsion that was stable in the presence of high concentrations of actively metabolizing procaryotic cells and had no ^{19}F resonances that overlapped those of our ^{19}F pH probes. We chose FTPA-lecithin emulsion, a well-characterized blood substitute with a simple ^{19}F-NMR spectrum. We have added FTPA emulsified with egg lecithin to cell suspensions to monitor O_2 concentration directly during the course of NMR measurements and evaluate the adequacy of oxygen supply to cell suspensions in two types of NMR chambers. We have also determined the viability of a number of cell lines after exposure to FTPA and aeration in an air-lift NMR sample chamber.

MATERIALS AND METHODS

Cell Lines, Culture, and Preparation

P. denitrificans was maintained on agar slants, made fresh every 90 d. Bacteria were grown and harvested in mid-log phase as described in Erecinska et al. (1981). For these experiments, the bacteria were suspended in 100 mM NaCl, 10 mM Hepes, pH 7.2 at ~ 25% cytocrit. Peripheral blood lymphocytes (PBLs) were prepared from a buffy coat preparation as described previously (Deutsch et al., 1982). The insulin-secreting RINm5F cell line was derived from a radiation-induced insulinoma from rat pancreas. RINm5F cells were maintained in culture as described by Gazdar et al. (1980) and Praz et al. (1983). RINm5F cells were harvested in log phase with trypsin/EDTA, washed twice, and resuspended in Hanks-Hepes at ~ 5% cytocrit. The CEM and VLB clones of the human T-cell lymphoblastic cell line, CCRF-CEM, were obtained from Dr. William Beck (St. Jude Children's Research Hospital, Memphis, TN). They were derived in his laboratory as described previously (Beck et al., 1979). Cells were passaged twice weekly and cultured in our laboratory in RPMI-1640 with 1% penicillin-streptomycin and 1% glutamine, and 10% fetal bovine serum. HPB-All is a human T-cell lymphoblastic cell line (Morikawa et al., 1978), which we obtained from Dr. Philip Rosoff (Tufts University, Boston, MA). HPB-All cells were cultured in RPMI-1640 with 1% penicillin-streptomycin, glutamine, and 5% fetal bovine serum. They were passaged weekly.

Oxygen Measurements

Oxygen consumption rates for cell suspensions were measured with a Clark-type oxygen electrode in a stirred, temperature controlled chamber as shown in Table I. For the standard curves ($1/T_1$ vs. [O_2]) in Fig. 2, the oxygen concentration in the air-lift NMR tube was measured in an identical air-lift tube in which the bottom of the tube was replaced by an oxygen electrode. The bubbling gas stream was split, one half going to the NMR sample tube and the other to the oxygen measuring tube.

NMR Measurements

All ^{19}F-NMR experiments were performed at 188.2 MHz on a CXP-200 (Bruker Instruments, Inc., Billerica, MA) with a 10-mm probe equipped with temperature control; temperature of the NMR sample was maintained to ±1°C. 16K data sets were collected; 90° pulse widths ranged from 7 to 12 μs. Unless otherwise noted, T_1 values were determined on the

CF_3 peak by inversion recovery (IRFT), using seven tau values, ranging from 0.03 to 10 s; a T_1 measurement required 2 min. For RINm5F suspensions, intracellular and extracellular pH were determined from the chemical shift center-peak-spacing for α-difluoromethyl alanine, as described previously (Deutsch et al., 1982; Taylor and Deutsch, 1983).

NMR Sample Chambers

We used two simple NMR chambers to maintain proper aeration of cell suspensions. One is a flow chamber, which we have previously described (Deutsch and Taylor, 1987*a*), in which the cells are circulated between the 10-mm tube in the probe and an external oxygenation chamber. The second chamber, the air lift chamber, was constructed according to Santos and Turner (1986) from a standard 10-mm NMR tube; the cell suspension is circulated constantly up the chimney and down the outer annulus of the tube.

Cytotoxicity

Cell viabilities were determined by trypan blue exclusion; cell counts were done with a hemocytometer grid or Coulter counter. The cytotoxicity of perfluorotripropylamine was tested by measuring stimulated DNA replication [^{3}H]thymidine incorporation of mitogen-stimulated PBLs in culture (Deutsch et al., 1981). Protein concentrations were determined by the Lowry method (1951) on trichloroacetic acid precipitates of samples of cell suspension.

Fluorocarbon Emulsion Preparation

The perfluoro compound emulsion was prepared as described previously (Joseph et al., 1985) in Tyrode's solution pH 7.4, 35% vol/vol of perfluoro compound and was a kind gift of H. Sloviter and B. Mukherji, Harrison Surgical Research Department, University of Pennsylvania School of Medicine. The fluorotripropylamine was obtained from SCM Chemicals, Gainesville, FL.

RESULTS

Spectrum of FTPA

Fig. 1 shows the ^{19}F-NMR spectrum of the FTPA blood substitute, with resonance lines at −8.41, −10.8, and −48.0 ppm (from trifluoroacetate as an internal standard). This ^{19}F-NMR spectrum was unchanged after periods of up to 3 h of incubation of FTPA with the eucaryotic cells listed in Table I, or up to 90 h with the bacteria *P. denitrificans*. No degradation products (F^- or other fluorine-containing species) were detected in aerated suspensions of the eucaryotic cells or bacteria. The FTPA resonances from suspensions containing 0.4% FTPA emulsion are sufficiently strong to be detected with excellent signal/noise by an H-1 decoupler coil tuned down to the F-19 frequency.

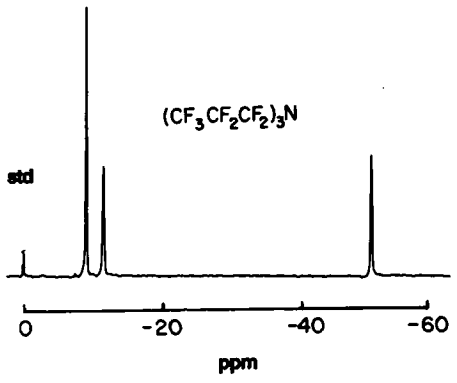

FIGURE 1 188.2-MHz F-19 spectrum of emulsified FTPA. 0.8% (vol/vol) of FTPA emulsified with egg-yolk lecithin was added to Hanks-Hepes buffer containing 10% D_2O pH 7.4. Temperature = 25°C, eight scans, 8K data points. Chemical shifts were referenced to CF_3COO^- internal standard. From left to right, the resonances shown are: CF_3COO^- standard (δ = O ppm); $-CF_3$ group of perfluorotripropylamine (δ = −8.4ppm); and the perfluoromethylene ($-CF_2-$) groups of FTPA (δ = −10.8 and −48.0 ppm).

Dependence of Relaxation Rate on $[O_2]$

Fig. 2 shows the dependence of the longitudinal relaxation rate, $1/T_1$, of the CF_3 resonance (−8.41 ppm) of FTPA on oxygen concentration in a physiological salt solution. Oxygen concentration was monitored by oxygen electrode in a parallel air-lift sample tube, simultaneously with the T_1 determination. The paramagnetic oxygen molecule causes an increase in relaxation rate, $1/T_{1P} = 1/T_1 - 1/T_{1(0)}$, where $1/T_{1P}$ is the difference between $1/T_1$ and $1/T_{1(0)}$, the relaxation rates of oxygenated and de-oxygenated solvent, respectively. This paramagnetic contribution to the relaxation rate, $1/T_{1P}$, is predicted to be proportional to oxygen concentration if the predominant T_1 relaxation mechanism for the ^{19}F nuclei is the dipolar interaction between the fluorine nuclear spins and electron spin of the paramagnetic oxygen molecules as they move in the fluorocarbon

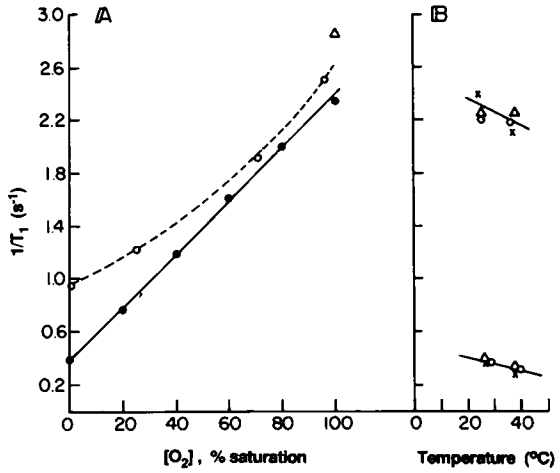

FIGURE 2 Dependence of observed spin-lattice relaxation rate, $1/T_1$, of CF_3 group of FTPA on $[O_2]$ and temperature. (*A*) ●, static (i.e., motionless) suspension of 0.4% (vol/vol) perfluoro compound in Hanks-Hepes, 10% D_2O, pH 7.7, 25°C. T_1 of the FTPA CF_3 resonance was measured by IRFT. One scan, relaxation delay = 10 s, tau values 0.03, 0.1, 0.3, 1, 3, 7, 10 s. O, 4% (vol/vol) perfluoro compound in Hanks-Hepes, 10% D_2O, pH 7.7, 25°C, in air-lift NMR chamber. Aeration was supplied by Wasthoff gas mixer; $[O_2]$ was determined simultaneously by oxygen electrode. $1/T_1$ values are mean of duplicate or triplicate measurements. The standard deviation was 6–8% measured $1/T_1$ values for 100% N_2, and 16–17% of measured $1/T_1$ for 100% O_2. (*B*) Effects of temperature and [FTPA-lecithin] on $1/T_1$ of FTPA's CF_3 group. O, 0.2%; △, 0.52%; ×, 12% (vol/vol) perfluoro compound in 100 mM NaCl, 10 mM Tris (static sample).

suspension (Swift and Connick, 1962; Luz and Meiboom, 1964). $1/T_1$ for a static sample (fluorocarbon suspension gassed with O_2/N_2 mixture at desired O_2 saturation, and tightly capped in 10-mm NMR tube) showed the expected linear dependence on $[O_2]$ (Fig. 2 *A, solid circles*). However, several processes can decrease the apparent T_1 obtained for aerated samples. The first is sample flow. To aerate cell suspensions, it is generally necessary to circulate the sample. For any sample that flows through the sensitive volume of the detector coil in a time $\leq 3T_1$, a significant fraction of the spins inverted by the initial (180°) pulse of the IRFT sequence exit from the detected volume and are replaced by relaxed spins from outside the coil, before M_z is sampled by the 90° pulse. In this case, the IRFT sequence will still, in general, show an exponential recovery of M_z, but the apparent T_1 calculated can be very much shorter than the true T_1. The second process is liquid-vapor exchange. The vapor pressure of FTPA is sufficiently high that it is possible that FTPA liquid-vapor interchange can be significant in vigorously aerated solutions. Gas-phase spin rotation relaxation is very fast compared with liquid relaxation rates, so that even a small amount of vapor in equilibrium with the FTPA-lecithin phase can shorten T_1 at very fast gas flow rates (see Farrar et al., 1972).

For example, the air-lift NMR tube described by Santos and Turner (1986) provides excellent aeration without significant degradation of magnetic field homogeneity; the sample constantly circulates in and out of the sensitive volume of the detector coil. However, as expected from the preceding considerations, the apparent T_1 was a function of the gas flow rate. As the solid line in Fig. 2 *A* shows, T_1 ranged from 0.4 s for O_2-saturated solutions to 2.4 s for static solutions with $[O_2]$ equal to zero. For the air-lift cell there was substantial movement of sample out of the detector coil volume in times of 1–8 s; we therefore expected to see a shorter apparent T_1 for flowing samples. The dashed line in Fig. 2 *A* shows that the apparent T_1 is indeed shorter ($1/T_1$ is increased) for flowing samples in the air-lift cell. The increment in $1/T_1$ increases markedly with increasing gas flow rates (data not shown).

In Fig. 2 *B*, the upper line represents 100% O_2 saturation and the lower line represents solutions without O_2. The FTPA relaxation rate was slightly sensitive to temperature, particularly at 100% saturation with O_2 because O_2 solubility changes with temperature. There was no dependence of the relaxation rate as the percent volume replacement blood substitute ranged from 0.2 to 12% (Fig. 2 *B*). There was no difference in the observed $1/T_1$ for FTPA when the aqueous phase was changed from Hanks-Hepes to 100 mM NaCl, 10 mM Hepes, pH 7.2 (data not shown), which is consistent with the observed relaxation rate being determined by $[O_2]$ within the FTPA-lecithin phase.

Aeration of Cell Suspensions

Even though only an apparent T_1 value can be obtained for aerated suspensions, the relaxation rate of FTPA can easily be used qualitatively to monitor the oxygenation of suspensions of respiring cells in the NMR probe. Fig. 3 shows the changes in FTPA $1/T_1$ for a concentrated suspension of *P. denitrificans* (20% cytocrit in 100 mM NaCl, 20 mM Hepes, pH 7.0). The solution was high in O_2 but not saturated, when the cells were initially respiring at a resting level. On addition of glucose, the respiration of the bacteria increased and the $[O_2]$ approached a lower steady-state value. Addition of the uncoupler, FCCP, which typically stimulated respiration threefold, caused a rapid depletion of O_2. The steady-state value of $1/T_1$ in the presence of FCCP (compare with curves of Fig. 2) indicated that the rapidly respiring bacteria reduced $[O_2]$ to <1% of its saturating level. Addition of CN^-, which inhibits 80% of the O_2 consumption of these organisms, was followed by a rapid rise to saturating $[O_2]$ levels. Quantitative $[O_2]$ measurements from FTPA $1/T_1$s would require very reproducible flow rates and a standard curve for each flow rate and sample tube geometry. However, the apparent $1/T_1$ values corresponding to 0 and 100% O_2 saturation were easily obtained: at the end of the NMR experiment, adding CN^- and continuing to aerate with O_2 yielded the 100% value; the zero-O_2 value was obtained by subsequent bubbling with N_2 until the apparent relaxation rate reached a minimum.

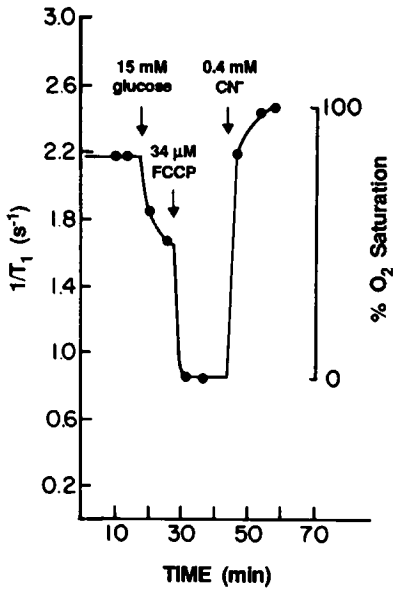

FIGURE 3 A suspension of *Paracoccus denitrificans* (3.1 mg protein/ml, 0.4% [vol/vol] FTPA) in 100 mM NaCl, 20 mM Hepes, 10% D_2O, was aerated with O_2 in air-lift NMR chamber at 25°C. $1/T_1$ measurements of the CF_3 resonance of FTPA were made at the times indicated. Additions: 15 mM glucose, 34 μM FCCP, 0.4 mM NaCN. T_1 determined as in Fig. 2.

Oxygen gas flow rates of >50 ml/min per 5 ml suspension are required to keep 20–25% cytocrit suspensions of *P. denitrificans* aerated; satisfactory aeration is manifested by a significant increase of the relaxation rate, $1/T_1$ app, above the zero-O_2 value. This agrees well with the gas flow rates cited by Ugurbil et al. (1979) for similar

suspensions of *E. coli*. Thus, the air-lift cell can maintain very concentrated suspensions of procaryotic cells in an aerobic state. This aeration cell has the additional advantage that the NMR sample temperature was easily regulated by the spectrometer's temperature control, because the entire sample remains in or near the NMR probe.

We have not observed cytotoxic effects on the eucaryotic cell lines tested from either the FTPA-lecithin emulsion or from aeration in the air-lift chamber. Lymphocytes incubated for 72 h in the 38% (vol/vol) perfluoro compound in culture (MEM) were 97% viable. Perfluoro compound concentrations up to 50% were noncytotoxic.

Cell viabilities, as determined by trypan blue exclusion, were measured for samples of suspensions aerated in Hanks-Hepes buffer for 1 h in the NMR air-lift chamber. Viabilities ($n = 4$) for CEM, VLB, and HPB were 91.6 ± 1.1 (92.7 ± 1.5), 91.4 ± 2.7 (95.3 ± 0.5), and 95.0 ± 1.4 (96.3 ± 1.3)%, respectively. The percentages given in parentheses are the corresponding control viabilities for each cell type, for suspensions maintained in a shaker bath.

In the NMR experiment, RINm5F cells were well-oxygenated as shown by the values for $1/T_1$ obtained from suspensions in both the flow and air-lift chambers (Fig. 4). While the air-lift chamber permits extremely fast gas-flow rates, it can be difficult to control frothing with dense and viscous cell suspensions. This is the primary cause of the larger variability of the measured T_1s for the air-lift chamber, seen in Fig. 4. Antifoam can alleviate this problem with bacterial suspensions, but it is not standard practice for eucaryotic cell suspensions. It is clear from Fig. 4 that the air-lift chamber was more effective in aerating the suspension, with a $1/T_1$ value of >2.1, compared with a flow cell $1/T_1$ of 1.6. However, Fig. 4 also shows that the flow chamber provides adequate aeration for dense suspensions of the RINm5F cells. Hence, we used the flow cell for the pH measurements of RINm5F cells described below; according to the curves of Fig. 2 *A*, during these pH measurements O_2 saturation was >50%.

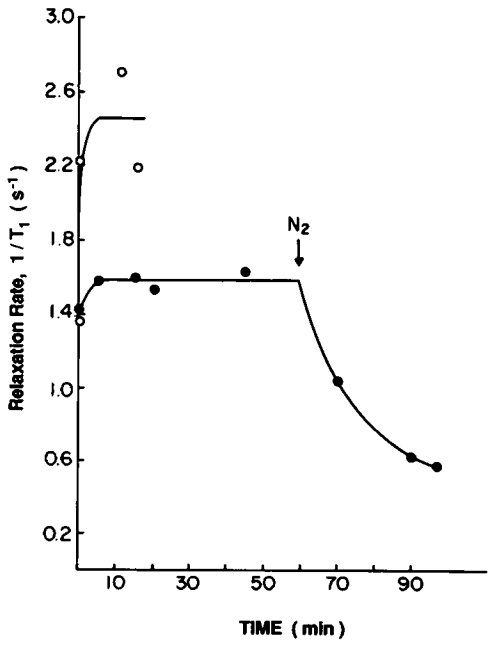

FIGURE 4 O_2 saturation estimated from $1/T_1$ of FTPA for RINm5F cells aerated in the flow chamber (●) or the air-lift chamber (○) at 25°C. Cells were suspended at 9% cytocrit in Hanks-Hepes media pH 7, containing 10% D_2O and 0.4% (vol/vol) FTPA. T_1's were measured as in Fig. 2.

^{19}F-NMR Measurement of pH in Cells Lacking Methyl Esterase

The fluorinated methylalanines, mono-, di-, and trifluoro-α-methylalanine, are a useful family of ^{19}F-NMR pH indicators which we have tested and used to measure intracellular pH in lymphocytes, hepatocytes, and several tissues (Taylor and Deutsch, 1983; Deutsch and Taylor, 1987*a,b*). The fluoromethylalanines are not, in general, taken up by cells or tissues rapidly enough, or in sufficient quantity, for rapid, accurate intracellular pH measurements. However, we have shown that methyl esters of the fluoromethylalanines are rapidly taken up by many cells. Once inside, the methyl esters are cleaved by intracellular esterases to the amino acid pH indicators. However, not all cells display methyl esterase activity.

The RINm5F cells possess no detectable methyl ester-

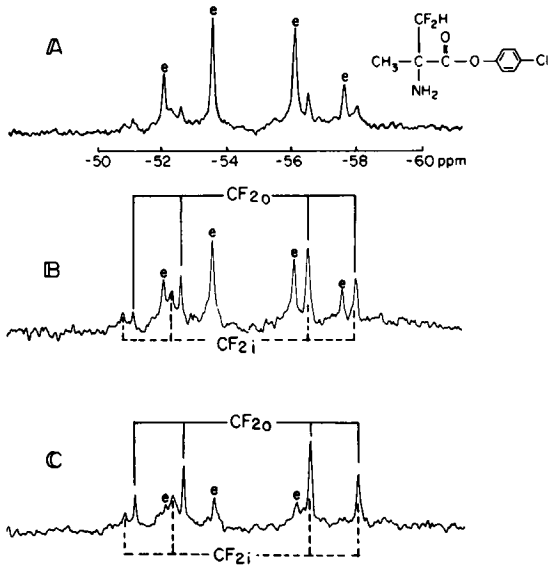

FIGURE 5 ^{19}F-NMR of RINm5F cells (~4% cytocrit) plus difluoromethylalanine para-chlorophenyl ester (1 mM) suspended in RPMI 1640/Hanks-Hepes at 25°–27°C. Spectra were taken at 4-min intervals (1,600 scans, 30° pulse, repetition rate = 5/s) with broadband proton decoupling. The resonances marked o arise from extracellular free amino acid, whereas those marked *i* arise from intracellular free amino acid. The resonances marked *e* arise from the ester form of the amino acid. Spectra *A*, *B*, and *C* represent scans taken (*A*) 1–5 min; (*B*) 5–9 min; and (*C*) 9–13 min after addition of ester to the RIN cell suspension. The ester quartet (*e*) lines decreased in intensity, while the quartets of lines from the product of ester hydrolysis, intracellular difluoromethylalanine (CF_{2i}) and extracellular difluoromethylalanine (CF_{2o}), increased with time.

ase activity: no free difluoromethylalanine occurs after ~ 1 h incubation of cells (3% cytocrit) with difluoromethylalanine methyl ester at 25°C. However, the *p*-chlorophenylester of α-difluoromethylalanine was rapidly cleaved in RIN cells to give strong intracellular α-difluoromethylalanine resonances. The $-CF_2$ group of α-difluoromethylalanine gives an AB quartet of lines centered about −54.7 ppm from CF_3COO^-. The chemical shifts of the quartet depend on the degree of protonation of the α-NH_2 group, which has a pK_a of 7.2. The δ in ppm between the two central lines of the quartet is a measure of the pH of the compartment containing the α-difluoromethylalanine. Fig. 5 shows the ^{19}F-NMR spectra of an RIN cell suspension to which we added 1.0 mM difluoromethylalanine *p*-chlorophenyl ester, at 25°C. Scans *A*, *B*, and *C* were taken at 4-min intervals after ester addition. An intracellular α-difluoromethylalanine signal persisted for at least 20 min. This is long enough to observe the effects of depolarizing extracellular K^+ concentrations, for example, on intracellular pH. The estimated integrated intensity of the intracellular α-difluoromethylalanine, compared to extracellular α-difluoromethylalanine, indicated that the fluorinated amino acid was concentrated in the cells to levels at least 30 times greater than extracellular levels. This indicated that the intracellular rate of *p*-chlorophenyl ester hydrolysis was much greater than the rate of hydrolysis in the external medium. Measurements of intracellular pH in RIN cells are easily made with this precursor. In addition, we have used the relaxation rate of FTPA emulsified with lecithin to verify that this cell suspension was properly aerated during measurements of intracellular pH by ^{19}F-NMR. Furthermore, cell counts (Coulter counter), cell volume (Channelyzer), and viability (trypan blue exclusion) of RINm5F cells incubated with the fluorinated precursor in the NMR experiment varied by <7% from the values obtained from control cells (data not shown). Having generated the appropriate pH indicator in situ, under aerobic conditions, we used the observed chemical shifts for the intra- and extracellular free amino acid pools (Fig. 5) to determine intra- and extracellular pH. For example, for the cells in Fig. 5, intracellular pH is 7.02 at an extracellular pH of 7.26.

DISCUSSION

The use of ^{19}F-NMR has many advantages compared with other approaches for determination of pH. However, this technique becomes still more useful with the addition of the ability to measure sample oxygenation and the availability of better pH indicator precursors. We have made considerable progress in both of these areas.

In this paper, we show that oxygen concentrations in cell suspensions in the NMR probe can be monitored directly by $1/T_1$ measurements of FTPA/lecithin emulsion added to the suspensions. Low concentrations of FTPA (0.4% vol/vol) suffice. With FTPA oxygen monitoring, we investigated the relative aeration capabilities of air-lift and flow NMR chambers. The air-lift vessel is much more efficient in supplying oxygen and is the system of choice for concentrated suspensions of procaryotic cells. Both normal and transformed eucaryotic cells can be maintained in either chamber for at least 1–2 h at cytocrits of up to 20–25%, with 30% oxygen saturation and cell viabilities of 90–95%. These results demonstrate the feasibility of monitoring oxygenation of cellular suspensions during ^{19}F measurements of intracellular H, or Ca^{2+} and/or Na^+ ion concentration (Deutsch and Taylor, 1987; Metcalfe et al., 1985; Smith et al., 1986; Levy et al., 1987).

We also tested a new precursor molecule for the ^{19}F-NMR pH indicator difluoromethylalanine in RINm5F cells and *P. denitrificans*. The precursor, the para-chlorophenyl ester of difluoromethylalanine, is suitable for intracellular pH measurements in cells lacking methyl esterase activity, such as RINm5F cells and *P. denitrificans*. The *p*-chlorophenyl ester was used to determine intracellular pH in a suspension of RINm5F cells under aerobic conditions at 25°C; pH_i = 7.02 for pH_e = 7.26. Cell viabilities were 98% at the end of the experiment.

P. denitrificans also took up the *p*-chlorophenyl ester of a-difluoromethylalanine and cleaved it very rapidly to generate high levels of intracellular difluoromethylalanine.

These experiments showed that the *p*-chlorophenyl ester was readily hydrolyzed inside these cells, neither of which hydrolyzes the methyl esters of our α-methylalanine probes. The *p*-chlorophenyl ester of difluoromethylalanine can be used to load the pH indicator difluoromethylalanine into cells lacking methyl esterase activity. Furthermore, since this relatively reactive ester is hydrolyzed nonenzymatically in aqueous solution it should be a cell type-independent precursor molecule, and therefore useful in a variety of biological systems.

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