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Proton Nuclear Magnetic Resonance of Intact Friend Leukemia Cells: Phosphorylcholine Increase During Differentiation

Abstract. *Proton nuclear magnetic resonance of intact Friend leukemia cells was used to analyze their erythroid-like differentiation. The technique, which requires only 10^8 to 10^9 cells and approximately 2 minutes for acquisition of each spectrum, demonstrated the occurrence of many signal changes during differentiation. With cell extracts, 64 signals were assigned to 12 amino acids and 19 other intermediary metabolites, and a dramatic signal change was attributed to a fourfold increase in cytoplasmic phosphorylcholines.*

Noninvasive study of whole cell biochemistry has been pioneered through investigations of energy metabolism by ^{31}P nuclear magnetic resonance (NMR) spectrometry of adenosine triphosphate (*1, 2*). Addition of ^{13}C-enriched metabolites to cell suspensions has demonstrated the potential of ^{13}C NMR monitoring of metabolic processes involving these compounds (*2, 3*). Proton NMR can detect a wide range of compounds simultaneously and at higher sensitivity than ^{13}C or ^{31}P NMR (*4, 5*). It also has the advantage that isotopic substitution of ^{1}H for ^{2}H is readily observed (*6*). Moreover, this nucleus is suitable for studies of membrane transport by spin echo methods (*7*). In this report we describe the use of ^{1}H NMR in an investigation of the erythroid-like differentiation of Friend leukemia cells (FLC's) induced by dimethyl sulfoxide (DMSO). Many compounds of the cells' intermediary metabolism can be rapidly monitored by whole cell ^{1}H NMR. The number of cells required is small enough for the technique to be applicable, in general, to mammalian cells grown in culture.

Friend leukemia cells (*8*), cultured in suspension in Eagle's minimal essential medium with 10 percent calf serum, were induced into erythroid-like differentiation with concomitant hemoglobin synthesis by addition of DMSO (2 percent by volume). The cultures were monitored as previously described (*9*). Globin production was detected after 40 hours, large numbers of hemoglobin-containing cells were seen after 50 hours, and 85 to 90 percent of all cells were producing hemoglobin by 90 hours after addition of DMSO. Cells were collected at various times by centrifugation and washed twice with medium. Washed cells were resuspended in medium to a concentration of 7×10^8 cells in 0.5 ml and placed in a 5-mm NMR tube. Proton NMR was recorded on a 470-MHz instrument with an Oxford Instrument Company magnet and a Nicolet 1180 computer. Temperature was controlled at 37° ± 1°C. Signals from the medium itself were negligible except for those of glutamine and glucose, which occur in concentrations greater than 1 m*M*. Cell viability was checked after each NMR experiment by using trypan blue exclusion and found to be 90 percent or better. Other experimental details including preparation of the cytoplasmic extracts are given in the figure legends.

A spin echo (90°–τ–180°–τ) pulse sequence was used to detect, preferential-

Table 1. Changes in signal intensity during FLC differentiation. Areas of signals from spin echo spectra (τ = 60 msec) of FLC's at various stages of differentiation (0, 9, 34, 60, 88, and 105 hours after DMSO addition) were normalized to the signal at 2.12 ppm, which remained relatively constant over the entire differentiation period. Observed changes in the areas of some of the resonances numbered in Fig. 1 are shown here. Undifferentiated FLC's for 0-hour spectra were obtained from cultures of high density, 2×10^6 cells per milliliter. Thus spectra of these cells act as a control for the possible effects of increasing cell density during differentiation in which cultures undergo approximately two doublings.

Signal	Chemical shift (ppm)	Relative area during differentiation of FLC's at time (hours)					
		0	9	34	60	88	105
7	3.55	2	2	3	3	9	10
8	3.45	5	6	8	8	12	20
9, 10	3.20	24	43	46	45	73	90
16	2.35	16	12	12	14	18	22
21	1.47	1	1	2	1	2	2

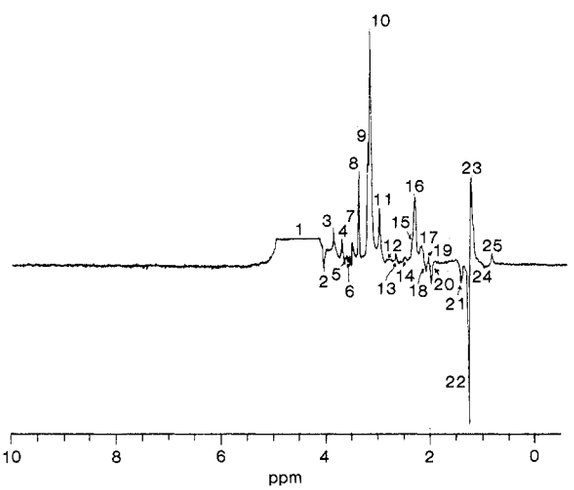

Fig. 1. Proton NMR spectrum of intact FLC's. Cells were harvested at various times after induction of erythroid differentiation. For each time period 7×10^8 cells, suspended in medium to a volume of 0.5 ml, were placed in a 5-mm NMR tube. A spin echo pulse sequence was used to collect the data at 470 MHz and 37°C. Accumulation time was 2.5 minutes (8000 points; 112 scans). The spectrum shown is of FLC's after 60 hours of differentiation and taken with τ = 60 msec. The most prominent signals are numbered; the signal from H_2O (signal 1) was truncated during plotting. The chemical shift scale is referred to external sodium 3-trimethylsilylpropionate (TSP) in a capillary. Signal assignments were made as described in the text and are as follows: 1, H_2O; 2, lactate; 3, phosphocreatine; 4, amino acid Cα; 5 and 6, glycerol and sugars; 7, glycerol; 8, unassigned; 9, phosphorylcholine (glycerophosphorylcholine); 10, choline, 11, phosphocreatine; 12, aspartate (also between signals 13 and 14); 13, citrate; 14, citrate; 15, pyruvate and succinate; 16, glutamate and proline; 17, unassigned; 18, glutamate; 19, proline; 20, isoleucine and proline; 21, alanine; 22, lactate; 23, triglycerides; 24, valine, isoleucine, and leucine; and 25, leucine and isoleucine.

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ly, small mobile metabolites in the cells. Rapidly relaxing protons of proteins and other macromolecules are effectively eliminated from the spectrum by this method (*4*, *10*). With a value for the time delay, τ, of 60 msec, a spectrum with 25 well-defined signals was obtained in 2.5 minutes, as shown in Fig. 1. Although some signals appear as shoulders on peaks in Fig. 1, changing τ from 10 to 140 msec in 10-msec steps resolved 15 additional signals through differences in their relaxation times and spin-spin coupling patterns. In whole cell suspensions, the signals observed were almost exclusively from the region of the NMR spectrum between 0 and 5 ppm (see the extract spectra shown in Fig. 2). Because of the relatively rapid relaxation time of the H_2O signal, there is no need to suppress the solvent signal in cell suspensions at high magnetic fields (*11*).

Comparison of samples that contained equal numbers of FLC's demonstrated intensity changes in many signals in the whole cell spectra during the 100-hour period of induced differentiation. Some of these changes are shown in Table 1. Many of the signal changes exhibited plateaus corresponding to the time in which differentiating FLC cultures experience a lag in growth in the first 48 hours after addition of DMSO. One of the most dramatic changes was a fourfold increase in the signal arising from incompletely resolved resonances around 3.20 ppm (Table 1).

A variety of methods was used to assign these and other cellular resonances to particular compounds. Whole cell and extract spectra were compared with standards at different τ values. Signals were further characterized by manipulating the extract solution, changing the *p*H, incubating with alkaline phosphatase to dephosphorylate some compounds, and adding standards. In most cases the last step was unnecessary. More than 70 signals are seen in the spectrum of an FLC cytoplasmic extract (Fig. 2). Some 64 resonances have been assigned to 12 amino acids and 19 other compounds of intermediary metabolism.

In whole cells the unresolved signals around 3.20 ppm (signals 9 and 10 in Fig. 1) exhibited a fourfold increase in intensity during differentiation. These signals are assigned to the methyl protons of choline, glycerophosphorylcholine or phosphorylcholine, and carnitine, which have resolved signals in extract spectra and occur at 3.20, 3.22, and 3.23 ppm, respectively (Fig. 2). The increase in cytoplasmic concentrations on the phosphorylcholines during differentiation is mainly responsible for the increase in signal intensity seen in whole cell spectra. This was demonstrated by comparison of spectra from cytoplasmic extracts of FLC's at various stages of differentiation, as shown in Fig. 2. This increase was assigned to both glycerophosphorylcholine and phosphorylcholine because a corresponding fivefold increase was observed in a signal at 3.55 ppm which is tentatively attributed to glycerol (Table 1 and signal 7 in Fig. 1) and because glycerophosphorylcholine was seen in FLC's with ^{31}P NMR (*12*).

Two signals (numbered 8 and 23) present in the whole cell spectrum of Fig. 1 were absent from the extract spectra, but reappeared in spectra of resuspended membrane and nuclear components. Signal 23 at 1.25 ppm has been tentatively assigned to the $-CH_2-$ protons of triglycerides. Increases in this signal and in the phosphorylcholine signal occurred at the same time in differentiation and were of similar magnitude. These observations could indicate synthesis of membrane components during differentiation.

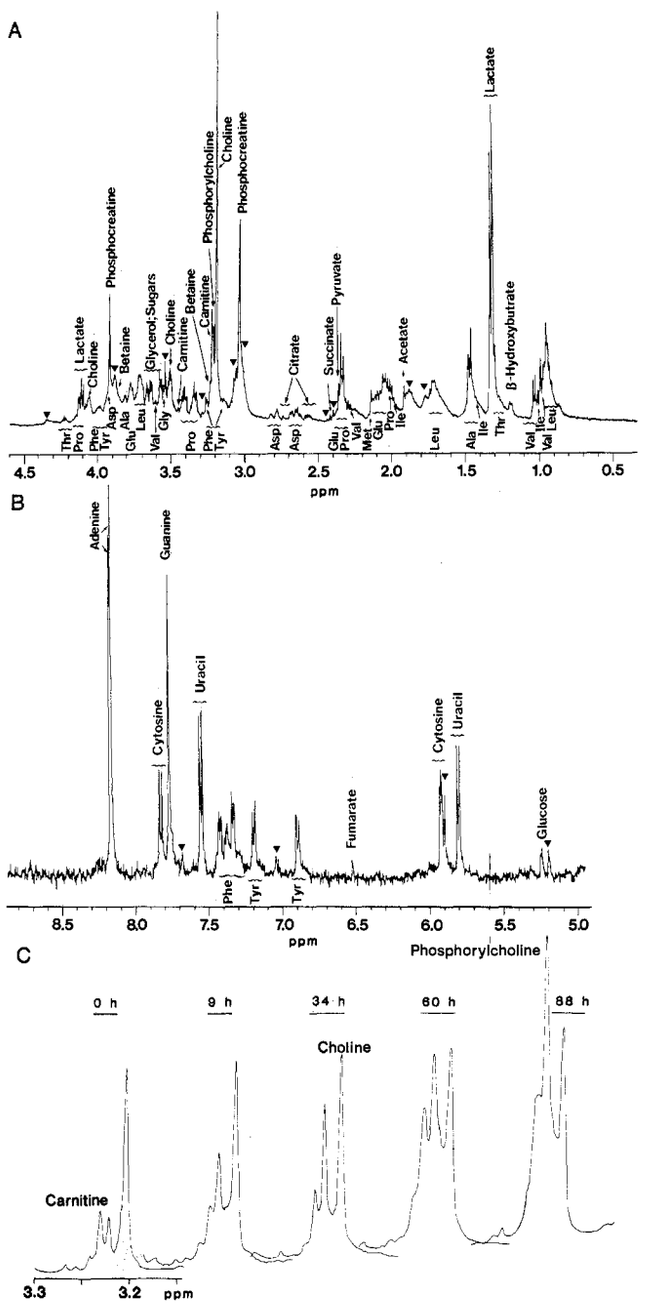

Fig. 2. One-pulse spectra of cytoplasmic extracts from undifferentiated and differentiating FLC's. Some signal assignments are shown in the full spectrum composed of (A) and (B); arrows denote unidentified signals. Small regions of the spectra of cytoplasmic extracts from cells at various stages of differentiation are shown in (C) to illustrate the increase in signal intensity of the resonance from glycerophosphorylcholine and phosphorylcholine methyl protons relative to that of carnitine and choline. After addition of alkaline phosphatase to these solutions the peak for phosphorylcholines was converted to that of the cholines. Extracts were prepared by sonication of each FLC sample (7×10^8 cells); removal of membrane, nuclei, and other high molecular weight components by centrifugation; treatment of the cytoplasm with an equal volume of 8 percent perchloric acid; and neutralization of *p*H with potassium carbonate. Neutralized extract was centrifuged to remove salts and the supernatant fluid evaporated to dryness under vacuum. Cytoplasmic components were dissolved in phosphate-buffered saline (0.5 ml, *p*H 7.2) that was approximately 95 percent D_2O and contained 5 m*M* TSP. The spectrum was obtained with a 45° pulse, 0.5-second delay, and 1000 scans. The aromatic region of the spectrum (B) has been expanded eightfold in height. We believe the appearance of the nucleic acid bases in the cytoplasmic extract is due to action of endogenous ribonuclease on RNA during preparation of the cytoplasm, followed by acid hydrolysis of the nucleosides during perchloric acid treatment.

Changes in membrane phosphorylated proteins (*13*) and cell-surface antigens (*14*) during FLC differentiation have been reported. Pools of phosphorylcholine have been observed in Chinese hamster ovary cells (*15*).

Thus, ^1H spin echo NMR can be used to observe specific changes in metabolite levels in both intact cells and extracts during a controlled experiment such as the induced FLC differentiation. Methods described here have been easily adapted to the study of antimycin-resistant and -sensitive strains of cells grown in monolayer cultures (*16*). Whole cell ^1H NMR allows simultaneous monitoring of many cytoplasmic compounds at low concentrations in H_2O. The methods are applicable to lower field (300 MHz) instruments when cells are washed in D_2O medium or solvent suppression is used. Cytoplasmic extracts can be useful as tools for the assignment of signals as well as sources of substrates, competitors, and inhibitors of enzymes, whose activities can be assayed by ^1H NMR.

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17. We thank I. Craig for the use of cell culture facilities and K. M. Brindle and C. G. Eliot for their advice on NMR of whole cells and extracts. P.F.A. was a Fogarty Senior International Fellow (1-FOG-TW00440) on sabbatical leave from the Division of Biological Sciences, University of Missouri, Columbia, and is now a National Research Service Award Senior Fellow (1-F33-GM07826). I.D.C. is a member of the Oxford Enzyme Group, which is supported by the Science Research Council.

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A Brain Heater in the Swordfish

Abstract. *The brain and eye of swordfish are warmer than the water. Associated with one of the eye muscles is a tissue that heats the brain. This brain heater is rich in mitochondria and cytochrome c and is supplied with blood through a vascular heat exchanger. It protects the central nervous system from rapid cooling during daily vertical excursions which may take the swordfish through a wide temperature range.*

Swordfish, *Xiphias gladius*, are large pelagic fish that range over the world's temperate and tropical oceans. The preponderance of white fibers in their swimming muscle indicates that they are stalkers and sprinters (*1*). They do not maintain the high level of continuous activity that we associate with warm fishes with elevated body temperatures such as the tunas (*2*) and, indeed, their muscle and viscera are close to water temperature (*3*). Swordfish are creatures of semidarkness, spending the night near the surface, but going as deep as 600 m during the day (*4*). In these vertical excursions water temperature may change as much as 19°C in less than 2 hours (*4*). The large and abrupt temperature changes that swordfish experience daily would chill the brain and affect central nervous system processes in most fish (*5*), but swordfish have developed a heater, which warms the brain and eye. This mass of specialized tissue and its associated vascular heat exchanger warm these organs to temperatures significantly above that of the surrounding water and reduce the extent of temperature fluctuations.

Associated with one of the eye muscles, the rectus superior, is a large swelling closely applied to the ventral side of the brain case. The tissue in this structure is brown, with the color and consistency of liver. Its blood supply is by way of a highly developed rete mirabile that arises from the carotid artery and forms a dense mass of small (80 to 100 μm in diameter), parallel arteries and veins. The rete is large for the mass of tissue it serves: in a 120-kg swordfish, the rete leading to a 50-g mass of brown tissue and muscle has a cross section area of 2 cm^2. As it merges with the brown tissue, the rete divides into strands of parallel vessels and blood is delivered to the surrounding brown tissue cells by a radiating pattern of sinusoids that have some resemblance to those of liver. The brown tissue cells are cuboidal with dense brown cytoplasm and clear distinct nuclei. They are packed with mitochondria and contain numerous small vacuoles. The distinctive brown color of the tissue is due to its high concentration of cytochromes. Cytochrome c concentration was 35 ± 3 nmole/g (*6*), similar to the 22 to 33 nmole/g reported for the brown fat of various small mammals (*7*). The ample blood supply, numerous mitochondria, and high cytochrome c content are similar to mammalian brown fat and indicate an unusually high metabolic rate in the brown tissue.

The brain of swordfish is warm (*8*). For fish caught on longline fishing gear (*9*), the brain, eye, and brown tissue are significantly warmer than the water (Table 1). Because swordfish captured on longline are usually dead or in poor condition, temperatures of undisturbed fish are probably higher than those shown in Table 1 (*10*). The highest temperatures yet recorded in swordfish were obtained from a free-swimming fish in an acoustic telemetry experiment where during a 36-hour period temperature in the cranial cavity was 10° to 14°C warmer than the water (*4, 11*).

The temperatures of fishes are tightly coupled to water temperature by circulation of the blood, which acts as a convective cooling system. Metabolic heat produced in the tissues is carried away by the blood and lost to the environment through the gills (*12*) so that fish remain close to water temperature. Warm fishes have developed countercurrent heat exchangers in their circulatory system and these retain metabolic heat and raise temperatures (*13*). The large rete mirabile serving the swordfish brown tissue is such a countercurrent heat exchanger. The venous and arterial flow in the retial vessels is in opposite directions and the alternating arrangement of the tightly

Table 1. Temperatures in billfish heads; *N* is indicated in parentheses.

Fish	Water (°C)	Temperatures (°C above water)		
		Retina	Brain	Brown tissue
Swordfish	19.8 ± 3.4 (11)	3.4 ± 1.7 (8)	4.7 ± 2.0 (9)	4.3 ± 2.0 (11)
White marlin	20.9 ± 1.8 (4)			3.4 ± 1.1 (4)
Sailfish	25.6		3.2	1.1

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