CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF LIVING CELLS AND THEIR METABOLISM OF A SPECIFICALLY LABELED ^{13}C SUBSTRATE*

R.T. EAKIN†, L.O. MORGAN†, C.T. GREGG and N.A. MATWIYOFF‡
Los Alamos Scientific Laboratory, University of California, Los Alamos, N.M. 87544, USA

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1. Introduction

Nuclear magnetic resonance spectroscopy is an extremely valuable tool for biochemical studies in that much information can be obtained by an essentially non-destructive procedure. Extensive applications of proton NMR spectroscopy have been developed in many areas of biochemistry as a result of the high natural abundance of the ^{1}H isotope (99.98%) and its occurrence in virtually every biological molecule. Another magnetically active atomic nucleus of great potential use in biochemistry is ^{13}C. In the past the low natural abundance of this isotope (1.1%) has restricted its experimental use, but with the advent of high sensitivity signal averaging and Fourier transform techniques [1] and the development of the technology for large scale ^{13}C enrichment [2] this nucleus can become at least as useful as the proton in biochemical research. Several characteristics of the ^{13}C nucleus make it particularly suitable for biochemical studies. It exhibits a wide range of chemical shifts and these shifts are extremely sensitive to the chemical environment. Because the magnetogyric ratio of ^{13}C is small, the relaxation rates of this nucleus are relatively low even for large molecules in viscous media. This fortunate combination of large chemical shifts and favorable relaxation effects, which result in widely shifted groups of narrow ^{13}C resonance, suggests that high resolution ^{13}C NMR experiments should be possible for cell suspensions. Successful application of this technique to internal cellular components of living tissue would indicate a great potential for ^{13}C as a tracer for *in vivo* metabolic studies. Here we demonstrate that ^{13}C NMR spectroscopy is applicable to whole cells of the yeast, *Candida utilis*, and that metabolism of a specifically labeled substrate, [1-^{13}C]glucose, can be observed directly in living cells.

2. Methods

2.1. Preparation of materials

Cells of *C. utilis*, strain CU-1, were grown on an inorganic salt medium [3] with a trace element supplement [4]. Isotopically enriched cells were grown on acetic acid randomly enriched to a ^{13}C level of 20% (a gift from D. Ott and V. Kerr of this laboratory) at 35° for 42 hr. Air (CO_2-free) was used for aeration and labeled acetic acid was added automatically during growth to keep the pH at 5.7. The cells were harvested, freeze-dried, and stored at −20° until use. Unenriched *Candida* cells were grown on 2% D-glucose at 22° for 72 hr, washed twice with normal saline, resuspended in D_2O, and made anaerobic by passing a stream of N_2 through the suspension for 15 min. This cell suspension was used directly to metabolize [1-^{13}C]glucose.

2.2. Magnetic resonance methods

Proton decoupled ^{13}C NMR Fourier transform spectra were obtained at 25.2 MHz with a Varian

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† Present address: Department of Chemistry, University of Texas, Austin, Texas.
‡ Address correspondence to this author.

XL-100-15 spectrometer interfaced to a Data General Supernova computer using the deuterium resonance (15.4 MHz) of D_2O (external) or $CDCl_3$ (internal) as a lock. Free induction decays of 40 sec rf pulses were accumulated as 8192 data points in the time domain. Peak intensities were determined by digital integration. Chemical shifts were calculated in ppm upfield from external neat methanol. Bulk susceptibility corrections are estimated to be small, ± 0.2 ppm, from ^{13}C chemical shift measurements with diamagnetic materials in organic solvents and paramagnetic ions in aqueous solutions of tetramethylammonium ion.

3. Results

3.1. Spectroscopy of whole cells and various fractions

Candida utilis cells randomly enriched to a ^{13}C level of 20% and suspended in D_2O give the ^{13}C NMR spectrum shown in fig. 1a. The major resonances extend over a chemical shift range of 33 to −131 ppm and some insight into the chemical nature of the carbons corresponding to them is provided by the ^{13}C NMR spectra of various extracts. Osmotic shock with D_2O releases material whose ^{13}C NMR spectrum is shown in fig. 1c. The spectrum illustrated in fig. 1b is that of a suspension of cellular material left after exhaustive extraction with D_2O. The residue from the aqueous extraction was further fractionated by extraction with 2:1 (v/v) chloroform–methanol (fig. 2). Cellular components soluble in this solvent give the spectrum shown in fig. 2c, and the components insoluble in both the aqueous and organic solvents give the spectrum shown in fig. 2b when suspended in D_2O.

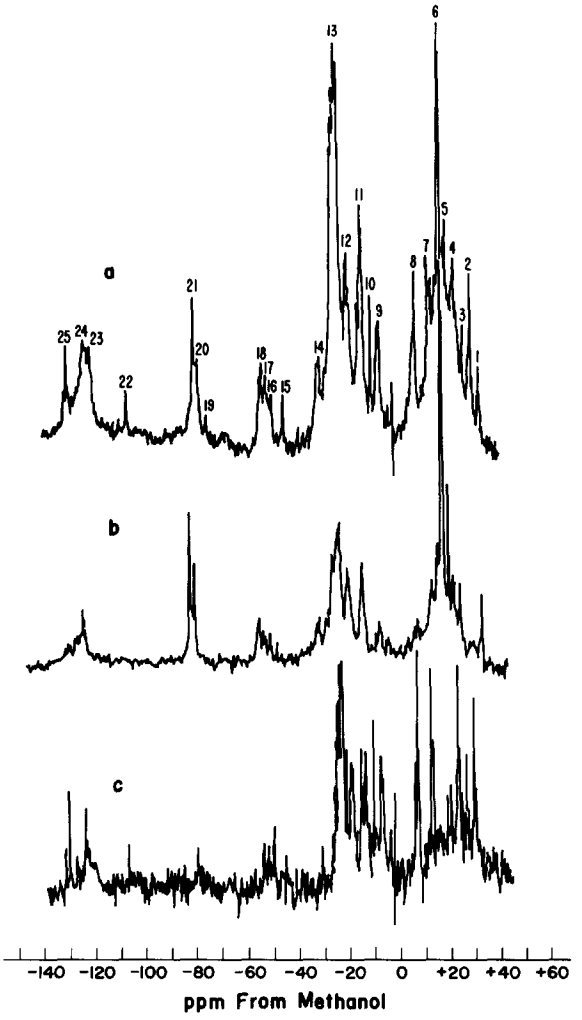

Fig. 1. ^{13}C Nuclear magnetic resonance spectra of *Candida utilis* cells. a) Whole cells suspended in D_2O, 995 pulses; b) cellular components remaining after exhaustive extraction with D_2O, 1000 pulses; c) water soluble components released by osmotic shock, 1082 pulses.

3.2. Spectroscopy of standard solutions

The metabolic substrate, an anomeric equilibrium mixture of α and β D-glucose enriched to a level of 10% ^{13}C in a C-1 (a gift from T. Whaley of this laboratory) showed ^{13}C resonances at −43.9 ppm (C-1 α) and at −47.7 ppm (C-1 β). A standard solution of glucose 6-phosphate (Calbiochem) prepared in D_2O for ^{13}C NMR analysis showed C-1 resonances at −44.0 ppm (α anomer) and at −47.8 ppm (β anomer). A solution of glycogen (Calbiochem) in D_2O showed the resonance at −51.5 ppm. Absolute ethanol (USICC) with a D_2O capillary gave a resonance corresponding to C-2 at 31.4 ppm.

3.3. Metabolism of [1-^{13}C]D-glucose

Metabolism of the labeled glucose was initiated by mixing equal volumes of 1.0 M [1-^{13}C] D-glucose and an anaerobic suspension of *Candida* cells. A sequential series of pulses during metabolism produced time-de-

pendent ^{13}C NMR spectra, some of which are reproduced in fig. 3. As can be seen from this figure, the ^{13}C label from C-1 of glucose was accumulated in C-2 ethanol and subsequently in a carbon atom chemically similar to that of C-1 in α D-glucose. After combining peak integrals for the α and β forms of [1-^{13}C]glucose, and accounting for computer scaling factors, the relative peak integrals corresponding to glucose C-1, ethanol C-2, and the second accumulated metabolite, show the time dependence illustrated in fig. 3.

4. Discussion

The spectra presented in figs. 1 and 2 clearly indicate that biological systems at the cellular level are indeed suitable for investigation by ^{13}C NMR methods. Whole cells represent a multiphase, non-homogeneous system consisting of thousands of components, yet the resonances of many groups of the carbon atoms in similar chemical environments can be resolved. Thus the ^{13}C NMR spectra of suspensions of *C. utilis* consist of a series of broad peaks each representing a set of chemically similar carbon atoms. Comparison of the spectrum of whole cells with previously reported spectra of carbohydrates [5, 6], polypeptides [7, 8], protein [9], and lipids [10, 11], along with the observations on chemical fractionation permits association of many of the resonances with particular types of carbon atoms.

The cellular components released by osmotic shock (fig. 1c) include low molecular weight compounds and probably a significant amount of protein since in bacterial systems many specific proteins are known to be released by such treatment [12–15]. A comparison of fig. 1a and 1c shows that the soluble constituents also contribute significantly to all portions of the ^{13}C spectrum except in the methine carbon region (−78 to −82 ppm). The chemical shifts of the resonances in fig. 1c are not inconsistent with their assignments primarily to the ^{13}C atoms of amino acids. In particular the resonance at −6.8 ppm (no. 9) is in the location expected for the α carbon of polypeptides, resonances in the region 25 to 15 ppm probably correspond to the β and γ methylene carbons of alkyl side chains, and the resonance at −124.1 ppm (no. 24) can be attributed to peptide carbonyl carbons [7, 8].

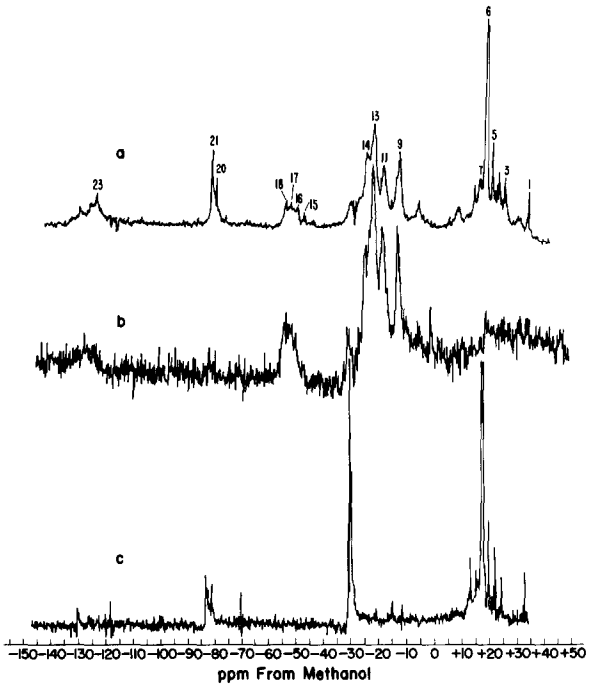

Fig. 2. ^{13}C Nuclear magnetic resonance spectra of the water insoluble fraction of *Candida* cells. a) Complete water insoluble fraction, 3023 pulses; b) residue from subsequent 2:1 chloroform–methanol extraction, 2399 pulses; c) material soluble in chloroform–methanol, evaporated to dryness and dissolved in $CDCl_3$, 4236 pulses.

The residue from aqueous extraction (figs. 1b and 2a) consists primarily of membrane bound species and cell wall material. The further fractionation of this residue by chloroform–methanol extraction should have solubilized lipid components (fig. 2c) while leaving cell wall carbohydrate and membrane protein insoluble (fig. 2b). In the lipid fraction the intense triplet at −30 ppm is the signal from the solvent $CDCl_3$. The other prominent peaks can be associated with fatty acids. In *C. utilis* palmitic and stearic acids constitute the major saturated fatty acids, and oleic and palmitoleic acids are the predominant unsaturated fatty acids [16, 17]. The peak at 33 ppm (no. 1) represents the terminal methyl function of long chain fatty acids and the resonances in the region 30 to 10 ppm, in which peak nos. 3, 5, 6 and 7 are prominent, are those expected for internal methylene carbons in fatty acids [10, 11]. Peaks

at −78.5 ppm (no. 20) and −80.5 ppm (no. 21) represent the methine carbons of unsaturated fatty acids [11]. Perhaps the small signals between −10 and −15 ppm in fig. 1c are generated by glycerol carbons.

The residue from lipid extraction (fig. 2b) includes membrane protein and cell wall carbohydrate, which in the case of *C. utilis* consists primarily of glucose and mannose units [18]. The protein α carbon peak expected at −6.8 ppm (no. 9) is prominent in the spectrum of fig. 2b and careful examinations reveals that there are extremely broad signals in the region −110 to −140 ppm where peptide carbonyl resonance is expected and in the region 50 to 10 ppm where the resonances of alkyl side chain carbons of protein amino acids are expected to occur. The peak at −13.6 ppm (no. 11) most likely represents the hydroxymethyl function of carbohydrates; the peaks in the region −20 to −35 ppm, including no. 13 and no. 14, represent the internal hydroxymethylene carbons of carbohydrates; and the peaks in the region −45 to −55 ppm, including no. 16, no. 17, and no. 18, represent the aldehyde and acetal functions of cell wall carbohydrate. The ^{13}C NMR spectra of the various fractions of *C. utilis* indicate that the major components of the cell are sufficiently mobile to yield high resolution spectra. A significant exception to this is the tightly bound protein associated with the cell membrane [19] which remains in the residue following chloroform–methanol extraction. These materials show extremely broad resonances in two regions in the spectrum of fig. 2c: ~250 Hz (−125 to −135 ppm) for protein carbonyl carbon atoms, and ~750 Hz (45 to 15 ppm) for the alkyl side chain carbons of protein amino acids. The breadth of the resonances presumably reflect an unfavorable correlation time for molecular motion of the membrane bound protein. We were not able to distinguish a ^{13}C resonance for the $N(CH_3)_3^+$ group of choline containing phospholipids in the spectra of methanol/chloroform extracts but a weak signal at the expected chemical shift (−5 ppm) is apparent in the spectrum (fig. 2b) of the residue following extraction. As suggested by a referee, it is possible that only the neutral fats, and not the phospholipids, are extracted from these cells by the chloroform/methanol treatment.

In this context it should be emphasized that the ability to observe high resolution spectra for the cell suspensions does appear to depend strongly on the

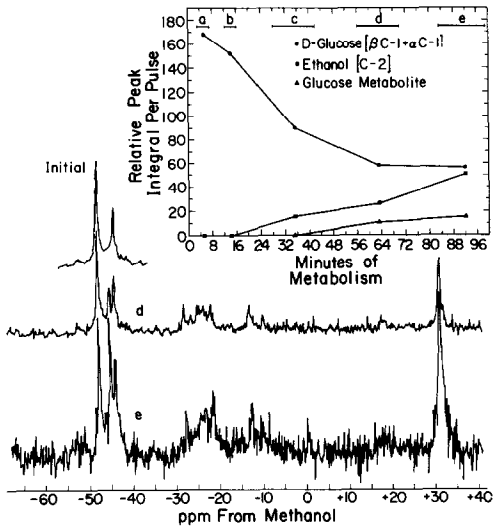

Fig. 3. Metabolism of [1-^{13}C] glucose. a) 500 pulses, 3–7 m after initiation of metabolism; b) 500 pulses, 12–16 min; c) 1500 pulses, 28–42 min; d) 1500 pulses, 56–70 min; e) 15(pulses, 83–99 min. The spectra obtained during the initial time periods show only the signals corresponding to the sub strate, [1-^{13}C] glucose. The C-1 region is illustrated. Spectra generated during the time periods (d) and (e) are illustrated for the region between 40 ppm and −65 ppm. Signals in the region between −12 ppm and −28 ppm are generated from the natural abundance ^{13}C in glucose carbon atoms other thai C-1.

mobility of the cell consituents. For example the ^{13}C spectra of freeze-dried cells consist of featureless broad resonances in the carbonyl, carbohydrate, and alkyl carbon regions. Freeze-dried cells suspended in water, however, exhibit the high resolution spectrum illustrated in fig. 1a. We have also observed that if an aqueous suspension of cells is frozen, a high resolution spectrum is still obtainable with only the carbohydrate and carbonyl carbon atom resonances showing pronounced line broadening.

The knowledge that intracellular components can be characterized readily by ^{13}C NMR suggest the possibility of investigating *in vivo* metabolism of a specifically labeled substrate. The anaerobic metabolism of [1-^{13}C] glucose which produces the time dependence of the ^{13}C NMR spectra illustrated in fig. 3 is an example of the technique, admittedly under adverse physiological conditions. The use of [1-^{13}C] glucose labeled at a 90% level would permit, from the viewpoint of NMR analysis, administration of glucose to

Table 1
^{13}C Chemical shifts of [1-^{13}C] glucose and its metabolic products.

Substance	Peak position*	Peak assignment
[1-^{13}C] Glucose	−12.6	C-6
	−21.5	C-4
	−23.3	C-2, 5
	−24.6	C-3
	−26.0	C-2
	−27.7	C-3, 5
	−43.9	C-1 α
	−47.7	C-1 β
Glucose 6-phosphate	−14.9	C-6
	−21.1	C-4
	−22.8	C-2, 5
	−23.2	
	−24.1	C-3
	−26.0	C-2
	−27.0	C-3, 5
	−44.0	C-1 α
	−47.8	C-1 β
Glycogen	−12.6	C-6
	−23.4	C-2, 3, 5
	−28.7	C-4
	−51.5	C-1
Ethanol	+31.4	C-2
	+ 8.4	C-1

* ppm with respect to external methanol, a positive sign denotes an upfield shift.

the extracellular medium at more nearly normal physiological concentrations (~ 5.5 mM).

The data presented in table 1 and fig. 3 show that isotopic carbon from C-1 of glucose accumulates in C-2 of ethanol as expected [20]. However, there is also a subsequent accumulation of the isotopic label in another molecule, the resonance of which does not correspond to C-1 in standard solutions of glucose 6-phosphate or glycogen. The chemical shift of the peak corresponding to the unknown species is in a range which does not include many carbons other than aldopyranose C-1 atoms. Furthermore the chemical shift (−45.0 ppm) is very close though not identical to that for C-1 of the α anomers of glucose (−43.9 ppm) and glucose 6-phosphate (−44 ppm). It is thus likely that this second accumulated compound is an α aldopyranose, but the only such species present in the metabolic pathways of glucose are α glucose itself and α glucose 6-phosphate. This leads to the intriguing speculation that the second accumulated species represents intracellular glucose or glucose 6-phosphate. Certainly the difference in intracellular pH and ionic strength are potential sources of small differences in chemical shift and differences such as observed between glucose and the second accumulated product (> 1 ppm) have in fact been observed between extracellular and intracellular cyanide [21].

This successful demonstration of the use of ^{13}C as a label both for cellular constituents and for monitoring the *in vivo* metabolism of a substrate illustrates the potential usefulness of this isotope in biochemistry. The labeling of cellular components can have applications analogous to those now being used with *N*-oxyl-oxazolidine paramagnetic electron spin labels in membrane biochemistry [22, 23] but with the advantage that the use of the internal ^{13}C nuclear spin does not require external modification of biological material. The specific labeling of substrates can have numerous applications in *in vivo* metabolic studies.

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