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Uniformly ^{13}C-Enriched Substrates as N.M.R. Probes for Metabolic Events *in vivo*. Application of Double Quantum Coherence to a Biochemical Problem

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Glycolysis in *Escherichia coli* has been monitored *in vivo* by ^{13}C n.m.r. spectroscopy and the biochemical pathways involved in succinate biosynthesis have been evaluated by analysis of the complex resonance signals, using double quantum coherence.

The use of ^{13}C n.m.r. spectroscopy in following *in vivo* metabolism has typically involved the observation of chemical shift changes resulting from biochemical transformations of singly ^{13}C-enriched substrates.[1,2] While the resultant simplification of the spectrum is a welcome feature, the *relative* enrichments of carbon atoms of derived molecules cannot be retrieved directly and information on cycling and pathway convergence may be obscured.

In order to evaluate both the complexity and the advantages of spectra accruing from metabolism of a uniformly enriched substrate, we have re-examined glycolysis in *Escherichia coli* using D-[U-^{13}C]glucose, and now show that the analysis of such spectra provides not only useful information concerning recycling, but also adds considerable rigour to the assignment of molecular constitution. In particular, the application of a program[3] for the selective observation of ^{13}C–^{13}C couplings

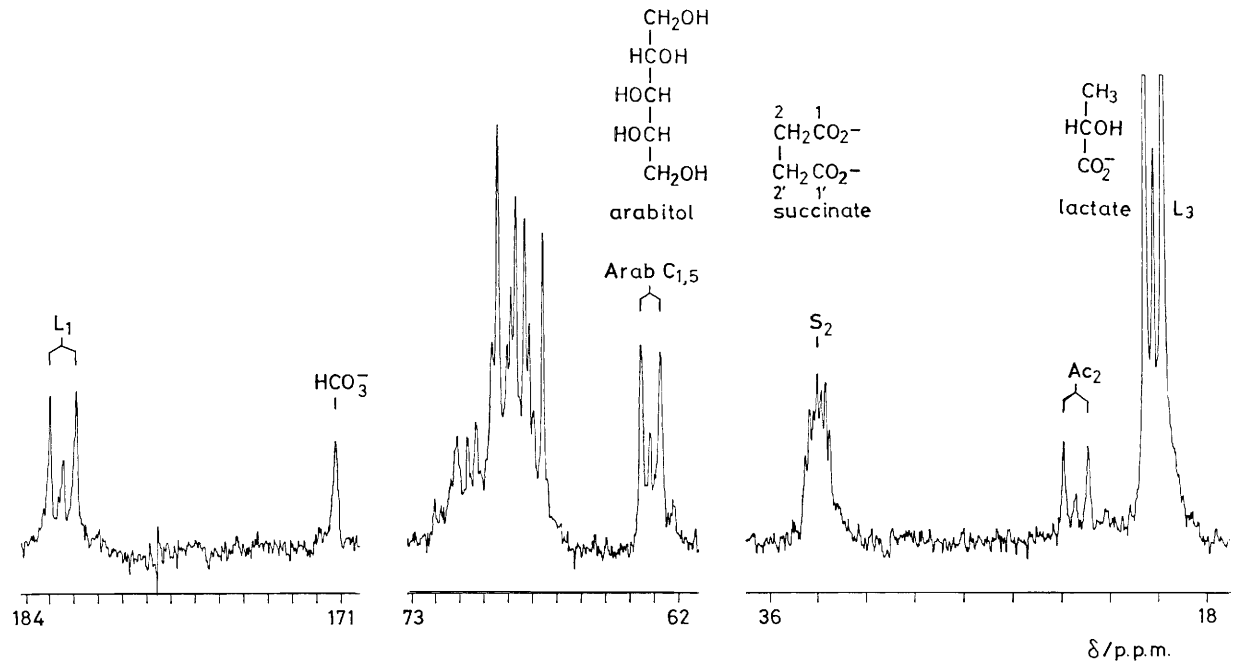


Figure 1. 50.3 MHz ^{13}C n.m.r. spectrum of the cell lysate after 45 min incubation. The spectrum represents 1500 (90°) pulses accumulated over 12.5 min. The lines were broadened *ca.* 0.7 Hz by exponential multiplication of the F.I.D. $L_{1,3}$ = C-1, 3 of lactate; Arab $C_{1,5}$ = C-1 and C-5 of arabitol; S_2 = C-2 of succinate, Ac_2 = C-2 of acetate. The stereochemistry shown is that of L-arabitol; however no assignment of stereochemistry was made in this case.

at natural abundance in symmetrical molecules provides a method of some generality in the analysis of complex signal patterns in time-course experiments derived from enriched carbon nuclei.

The metabolic flux of D-[U-^{13}C]glucose (90% ^{13}C, 50 mM) in suspended cells of *E. coli* (MRE 600) was monitored by ^{13}C n.m.r. spectroscopy. In contrast with a previous experiment,[1] fructose-1,6-diphosphate was not detected, although signals corresponding to C-3 (CH_3; 20.2 p.p.m.), C-2 (68.5 p.p.m.), and the carbonyl (182.0 p.p.m.) of lactate appear with the expected multiplicity [^1J(C-1–C-2) 55 Hz, ^1J(C-2–C-3) 38 Hz]. The evolution of $^{13}CO_2$ derived from decarboxylation of 6-phosphogluconate and of [U-^{13}C]pyruvate can be followed *via* the HCO_3^- signal at 172.2 p.p.m. Spectra recorded after 25 min show accumulation of the pentol, arabitol (C-1 and C-5 at 62.8 p.p.m., d; ^1J 40 Hz) and ethanol (C-2 at 58.0 p.p.m., d; ^1J 38 Hz). Comparison with spectra obtained with D-[6-^{13}C]- and D-[1-^{13}C]-glucose as substrates (which label C-1 of arabitol and HCO_3^-, respectively) indicates that *ca.* 3% of the utilised glucose is diverted from the pentose phosphate pathway into arabitol.

While the succinate C-2 signal (34.0 p.p.m.) was not well resolved in the spectra of metabolising cells, the spectrum of a cell free extract (Figure 1) reveals a complex multiplet, the analysis of which was facilitated by suppression of the signals derived from uncoupled ^{13}C nuclei using the double-quantum coherence pulse sequence.[3] Spectra recorded under these conditions exhibited only three coupling constants ^1J(C-2–C-1), ^1J(C-2–C-2′), and ^2J(C-1–C-2′) (53, 36, and 2 Hz respectively). Simulated spectra of the possible labelling patterns (Figure 2) reveal that the major enriched species present are [1,2,2′-^{13}C]-, [U-^{13}C]-, and [1,2-^{13}C]-succinate. The addition of the three predicted coupling patterns, and of a central uncoupled resonance, adjusted proportionally to simulate the observed multiplet is shown in Figure 2. Quantitative analysis,[4] based on the premise of statistically independent enrichment of adjacent ^{13}C nuclei, shows that the [1,2,2′-^{13}C]-, [U-^{13}C]-, [1,2-^{13}C]-, and [2-^{13}C]-species are present in the ratio 8:3:2:1.

The high concentration of [1,2,2′-^{13}C]succinate most probably results from carboxylation of [U-^{13}C]phosphoenolpyruvate (**1**) to [1,2,3-^{13}C]oxaloacetate (**2**) and reversal of the citric acid cycle *via* [1,2,3-^{13}C]malate (**3**) under the anaerobic conditions of the experiment as indicated in Scheme 1.[5]

Alternatively, [U-^{13}C]succinate would appear to be derived *via* the glyoxalate pathway. Condensation of [U-^{13}C]acetyl

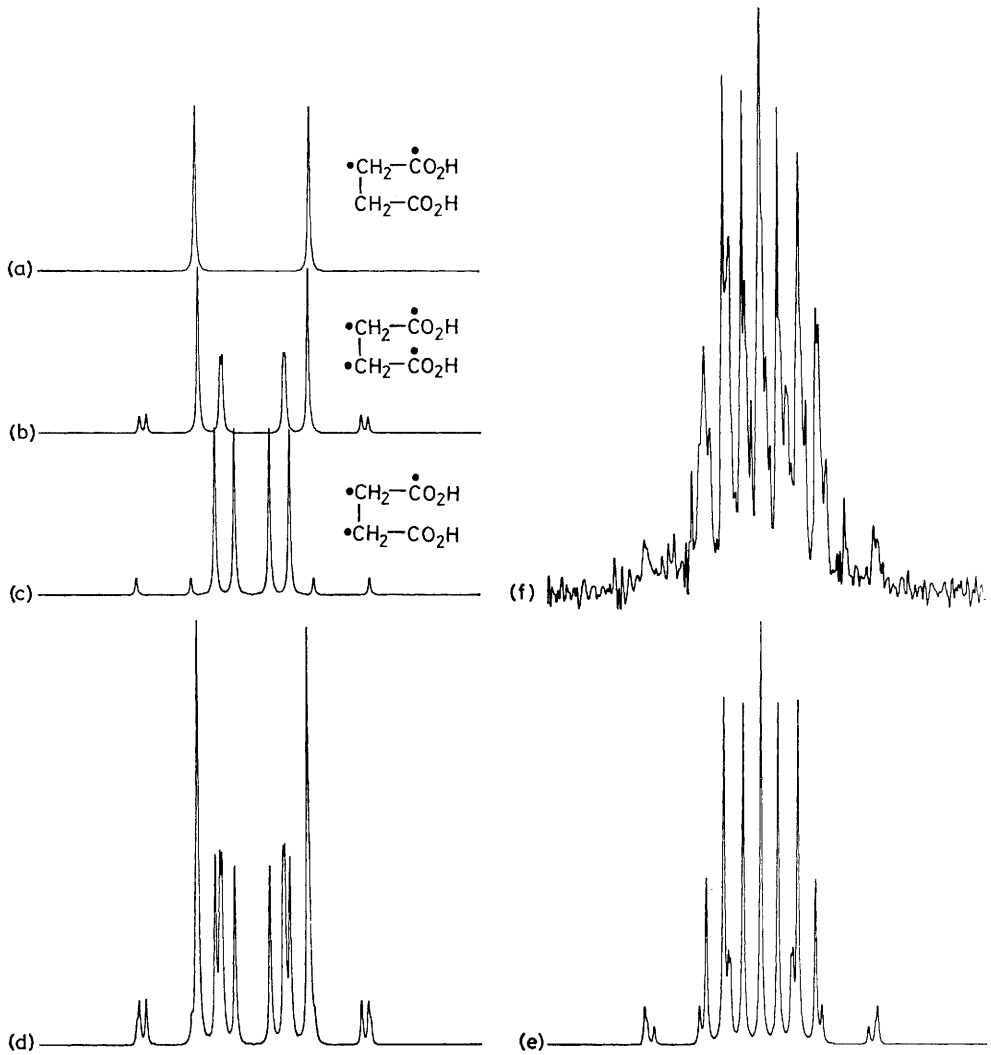
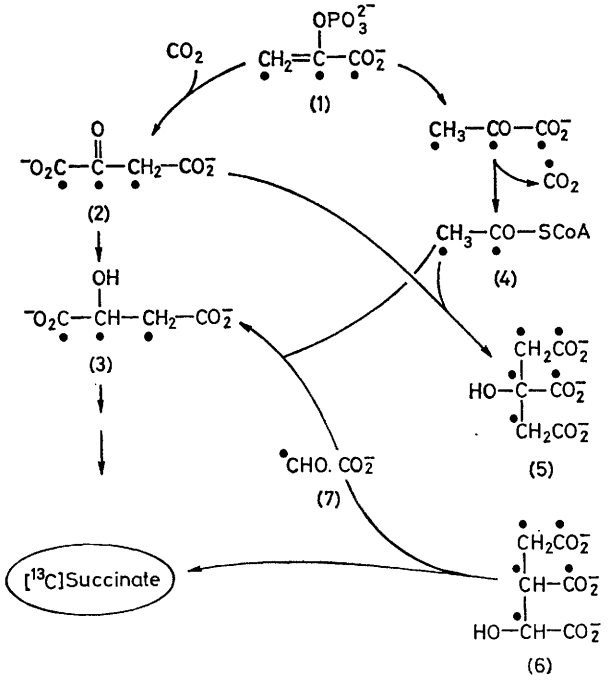


Figure 2. Simulated coupling patterns for the C-2 resonance of enriched succinate. (a) [1,2-^{13}C]; (b) [U-^{13}C]; (c) [1,2,2′-^{13}C]; (d) 1:1:1 addition of (a), (b), and (c); (e) 1:5:25 addition of (a), (b), and (c), respectively with addition of an uncoupled centre line; (f) observed multiplet. For spectra (b) and (c) ^2J(C-1–C-2), and ^3J(C-1–C-1′) were each assigned a value of —2.0 Hz. No adjustment was made for ^{13}C–^{13}C shift effects. Simulated spectra were obtained using the SIMEQ2 programme on a Varian XL-200 Spectrometer.



Scheme 1. The formation of ^{13}C-enriched succinate from [U-^{13}C]-phosphoenolpyruvate (derived from D-[U-^{13}C]glucose).

CoA (**4**) with [1,2,3-^{13}C]oxaloacetate gives rise to labelled citrate (**5**) and hence isocitrate (**6**). Under the action of isocitrate lyase [U-^{13}C]succinate and [2-^{13}C]glyoxalate (**7**) are formed, the latter being condensed with a further molecule of [U-^{13}C]acetyl CoA affording [1,2,3-^{13}C]malate which further augments the contribution of [1,2,2′-^{13}C]succinate to the enriched succinate 'pool' (Scheme 1). Formation of [1,2-^{13}C]- and [2-^{13}C]-succinate can be rationalised by similar sequences initiated by condensation of [U-^{13}C]acetyl CoA with unlabelled oxaloacetate and by condensation of unlabelled acetyl CoA with [1,2,3-^{13}C]oxaloacetate, affording [1,2-^{13}C]- and [2,3,3′-^{13}C]-citrate, respectively.

Hence analysis of the ^{13}C-labelled succinate formed provides not only information on the pathways being utilised but also quantitative data on the flux of metabolism *via* these pathways. By employing uniformly enriched precursors, complex spin–spin couplings due to the unsymmetrical labelling of symmetrical molecules may be observed in many biochemical processes. We suggest that the present treatment of such systems holds promise for a wide range of applications involving both the unravelling of recycling problems and the rigorous assignment of chemical shifts of labile intermediates.

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