Volume 231, number 1, 11–15 FEB 05736 April 1988

Quantitation and identification of carbon-13 isotopomers by homonuclear polarization transfer NMR experiments

Denis Boudot*+, Daniel Canet*, Jean Brondeau* and Francis Martin+

*Laboratoire de Méthodologie RMN (UA CNRS no 406 – LESOC), Université de Nancy I, BP no 239, 54506 Vandoeuvre-les-Nancy Cedex, and +Laboratoire de Microbiologie Forestière (CRF), Institut National de la Recherche Agronomique, Champenoux 54280 Seichamps, France

Received 15 January 1988; revised version received 9 February 1988

The simplest pulse sequence $(\pi/2)_x-\tau-(\pi/2)_y$ (Acquisition) for nuclear spin polarization transfer is used in a selective fashion: transfer only flows from a nucleus A to any nucleus X J-coupled to A, provided that the carrier frequency coincides with the A resonance and that τ is slightly varied around a properly chosen value, relevant free induction decays being co-added. According to the value selected for τ ($1/2J$ or $1/4J$, J: one bond carbon-carbon coupling constant), it proves possible to sort out and compare subspectra pertaining to isotopomers involving one, two, three, and four consecutive ^{13}C. The method has been applied to an extract of mycelium grown on a medium containing ^{13}C-labeled acetate, with the aim of delineating metabolic pathways from proportions of the detected isotopomers.

^{13}C isotopomer; NMR; Polarization transfer; Metabolic pathway; Ectomyocorhizal fungus

1. INTRODUCTION

For the last decade, the capabilities of NMR spectroscopy have been greatly enhanced by the development of methods based on polarization transfer. Such methods can be applied to problems of biochemical interest for structural elucidation, for instance combined with two dimensional techniques [1]. These experiments rest on an appropriate pulse sequence and on the existence of a coupling between two nuclei which may arise from indirect (J) interaction [2,3], cross relaxation or chemical exchange [4,5]. Such transfers make possible the detection of mutual couplings, and, as a consequence, have been mainly directed towards spectral assignment or localization of nuclei in a molecule. In such instances, the quantitative aspect of signal intensity is generally disregarded. It can be altered due to instrumental and data processing reasons, or more fundamentally because of the spin system preparation. This is especially true in 2D correlated spectra involving multiple quantum coherences [6]. We address here the problem of quantitative determination of ^{13}C multilabeled metabolites, each one possibly exhibiting several distinct isotopomers. In fact, the problem is quite general since current methodologies employ ^{13}C-labeled precursor in order to delineate metabolic pathways. We shall demonstrate that quantitation can be achieved through the simplest sequence which can be devised for producing polarization transfer:

$$(\pi/2)_x - \tau - (\pi/2)_y \text{ (Acquisition)} \qquad (1)$$

(pulses are non-selective, x and y axes refer to the rotating frame), provided that one-dimensional NMR is retained with the prerequisite of some sort of selectivity (or semi selectivity) in such a way that transfer operates from one, and only one, nucleus. As recognized in a recent publication [7], 1D NMR is still advantageous in terms of spectral resolution (especially for the determination of coupling constants), spectrum phasing, measurement time, data processing, etc. We shall emphasise here the quan-

Correspondence address: D. Canet, Laboratoire de Méthodologie RMN (UA CNRS no 406 – LESOC), Université de Nancy I, B.P. no 239, 54506 Vandoeuvre-les-Nancy Cedex, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

titative properties of sequence [1]. The latter is nothing but the basic COSY sequence [2,3] with, in addition, a selective procedure which here has been devised in the following way: transfer occurs from a nucleus A, at the resonance frequency of which the carrier frequency is set, towards a X nucleus J-coupled to A; τ is slightly varied around a particular value $\tau_0 = 1/2J$ or $1/4J$ and relevant free induction decays are co-added. This experiment requires neither critical instrumental adjustments (as may be the case when resorting to the DANTE pulse train [8] for achieving semi-selectivity in polarization transfer [9,10]) nor special hardware necessary for using gaussian pulses [7,11]. The jittering of a time interval in the pulse sequence, as a means of selectively transferring coherences, has already been used by Hore et al. [12–14] and Delsuc et al. [15]. In a previous work [16], it was indeed shown that a reasonable selectivity is gained without significantly altering transfer processes by incrementing τ from $0.8\tau_0$ to $1.2\tau_0$. This imposes a minimum number of experiments but does not represent a serious drawback for ^{13}C spectroscopy since accumulations are necessary anyway.

2. EXPERIMENTAL

The present work is aimed at the determination of isotopomer proportions in carbon 13 spectra of enriched material and will be exemplified by the detection, identification and quantitation of glutamate isotopomers in an aqueous extract of mycelium grown on a medium containing [2-^{13}C]acetate as carbon source. Soluble compounds were extracted from the Ascomycete *Cenococcum geophilum* strain Kiffer 1973 in a methanol-water (70:30) medium. The extract was centrifuged at 20 000 × g during 20 min and the supernatant evaporated to dryness on a rotary evaporator. The residue was taken up in 2 ml of a 50 mM EDTA solution (pH 7).

The implications are obvious in metabolism studies since such information, whenever it concerns metabolites which originate from a given enriched carbon-13 precursor, provides insights into biochemical pathways. Fig.1 demonstrates the value of the method: transfers originate from a particular carbonyl in a complicated spectrum involving a lot of metabolites. Two groups of signals remain: one, at the carbonyl resonance, which is associated with the isotopomer labeled only at this position, the other with isotopomers involving an additional labeling at the neighbour position (*C and C will denote carbon-13 and carbon-12 labelings, respectively; the omission of any symbol for a given carbon will indicate that its isotopic nature is unknown). The spectrum easily lends itself to intensity measurements. Therefore, not only have the resonances been assigned, but also the ratio of these two isotopomers can be accurately determined. In practice, to ensure a good transfer and a good prevervation of the singlet on resonance (i.e. the isotopomer with a single carbon-13), pulse imperfections have to be managed and the actual sequence has the form:

$$(\pi/2)_x - \tau - (\pi/2)_{-x}\,[(\pi)_{-x}(\pi)_x(\pi/2)_{-x}(3\pi/2)_x(\pi)_{-x}(\pi/2)_x(\pi/2)_y] \quad (2)$$

The first two pulses are autocompensated as far as the singlet on resonance is concerned, and consequently cannot alter its intensity. Regarding the transfer process, it can be noticed that a double-quanta intermediate occurs after the $(\pi/2)_{-x}$ pulse following the τ interval. In fact, this scheme is comparable to that of the INADEQUATE experiment and consequently the composite pulse devised by Levitt and Ernst [18] (including the 7 pulses between brackets) can be used. Indeed, this feature has proven to be essential in order to obtain correct quantitative results, especially when off-resonance effects have to be avoided. Phase cycling is accomplished in the usual way by a global 90° rotation at each transient, on which is superposed a 180° alternation for the first two pulses.

3. RESULTS

The effect of such a polarization transfer scheme on a two-spin or a three-spin system is either well known [1,7] or easily derived using the operator product formalism [19] (or an equivalent vectorial picture [16]). Results are gathered in table 1 to make easier the analysis of experimental data. An interesting feature is the response of the three spin isotopomer M-A-X (A being selectively excited and $\tau_0 = 1/2J_{AM}$); when $J_{AM} = J_{AX}$ it is totally filtered out whilst it manifests itself only at site M if the two latter couplings differ. Labeling at dif-

Table 1

Intensities (normalized to 1) of subspectra obtained by sequence (2) as a function of the length of the evolution period for different spin systems of practical interest

System	Spins	$\tau_0 = 1/2J$	$\tau_0 = 1/4J$
A	A	1	1
A-M	A	0	$1/\sqrt{2}$
$J_{AM} = J$	M	1	$1/\sqrt{2}$
M-A-X	A	0	1/2
$J_{AM} = J_{AX} = J$	M	0	1/2
	X	0	1/2
M-A-X	A	0	$\cos(\pi J'/4J)/\sqrt{2}$
$J_{AM} = J, J_{AX} = J'$	M	$\cos(\pi J'/2J)$	$\cos(\pi J'/4J)/\sqrt{2}$
	X	0	$\sin(\pi J'/4J)/\sqrt{2}$

Polarization transfer is assumed to originate only from A. The A pattern presents a difference of phase of 90° with respect to other spins which exhibit antiphase multiplets

ferent sites of a metabolite, originating from a ^{13}C enriched precursor, has no reason to be statistical and the proportions of the various isotopomers should provide decisive information concerning biochemical pathways, impossible to obtain from other techniques. It must also be recognized that the conventional NMR spectrum only provides global information [20], and that it is a unique feature of polarization transfer experiments to enable the sorting of most isotopomers. Although we have carried out systematic measurements on each carbon of glutamate, we shall focus here on spectra arising from selective excitations at C_3 because these experiments are especially rewarding. Fig.2b shows the spectrum obtained with $\tau_0 = 1/2J$ ($J = J_{23} = J_{34}$). From table 1, we know that any contribution from isotopomers possessing the arrangement $*C_2$-$*C_3$-$*C_4$ disappears, not only at the C_3 site but also at C_2 and C_4. Therefore we are left with: (i) at C_3 frequency, a singlet corresponding to isotopomer C_2-$*C_3$-C_4, (ii) at C_4 frequency, an antiphase doublet arising from the active coupling J_{34} and corresponding to isotopomer C_2-$*C_3$-$*C_4$-C_5 (it can be noticed that the contribution from isotopomer C_2-$*C_3$-$*C_4$-$*C_5$ is not visible), (iii) at C_2 frequency an intense antiphase doublet corresponding to isotopomer C_1-$*C_2$-$*C_3$-C_4, and a quartet where antiphase outer lines are clearly distinguishable which arises from the isotopomer $*C_1$-$*C_2$-$*C_3$-C_4 via the inactive coupling J_{12}. It must be emphasised that this single experiment allows in a quite unique way to quantify five different isotopomers. This information, of course, could not be extracted from a conventional spectrum. In fig.2C, all isotopomers involving the arrangement $*C_2$-$*C_3$-$*C_4$ are observed since this experiment has been carried out with $\tau_0 = 1/4J$. Isotopomers of the A-M type sorted out in the previous experiment (fig.2b) are still present but weighted by a factor of $1/\sqrt{2}$; it must be noticed that they appear as well at site A, in the inner C_3 doublet (see table 1). Of course the

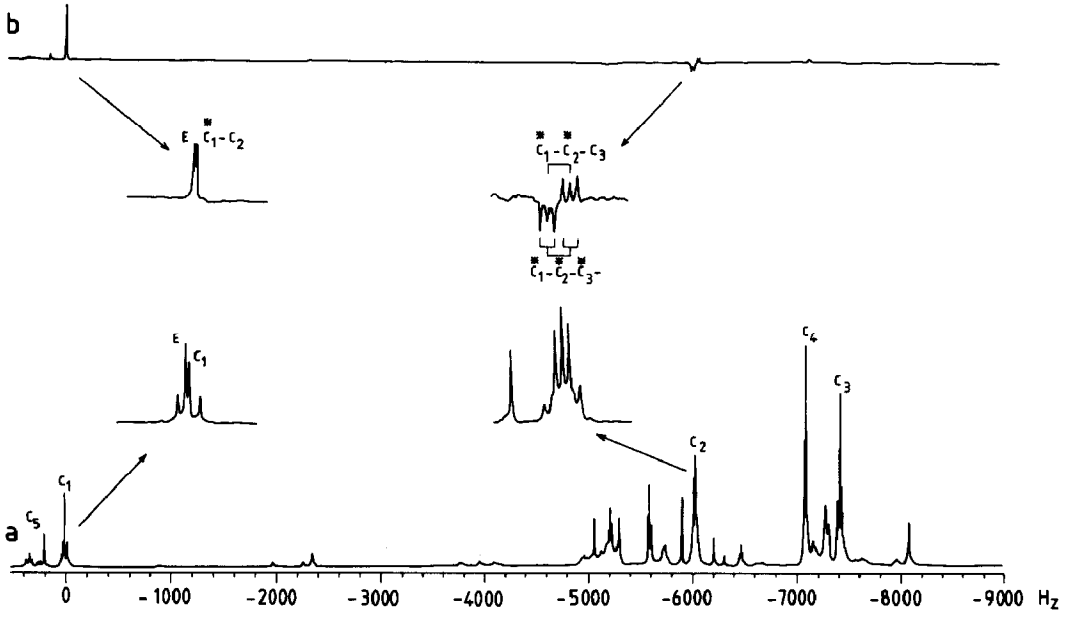

Fig.1. (a) Normal 50 MHz ^{13}C spectrum of the extract investigated here with the assignment of glutamate ($C_{(1)}OO^-$-$C_{(2)}$-HNH_2-$C_{(3)}H_2$-$C_{(4)}H_2$-$C_{(5)}OO^-$). All spectra were obtained with a modified Bruker WP-200 spectrometer interfaced to a Nicolet 1180 computer; 90° pulse: 14 μs; proton decoupling achieved according to the Waltz-16 modulation scheme[17]; 2912 scans acquired with a recycle time of 40 s. (b) Spectrum resulting from the application of sequence (2). The carrier frequency is set at the resonance of glutamate carbonyl 1 (32 transients accumulated for each τ value making a total of 2912 scans). Same recycle time as for (a). The signal pertaining to the $*C_1$-C_2- at zero frequency (E: unwanted signal from EDTA), and a response in the aliphatic region including the editing of the two isotopomers $*C_1$-$*C_2$-C_3- and $*C_1$-$*C_2$-$*C_3$- are visible. This quantitative information could not have been deduced from the reference spectrum. Inset expansions have been obtained by applying a factor of four to the frequency scale. The presentation of spectrum b results from a first order phase correction, feasible here due to the distance between the two groups of lines.

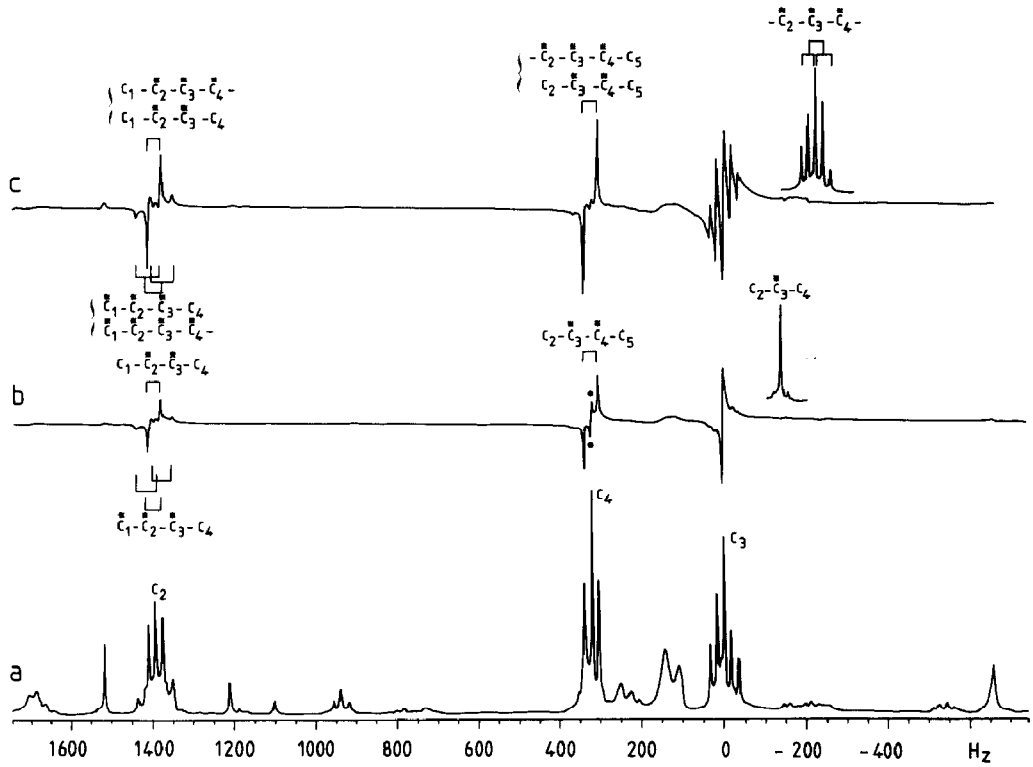

Fig.2. (a) Portion of the reference spectrum of fig.1. (b) Selective excitation of glutamate C_3 according to sequence (2) (τ_0 = 14 ms:$1/2J$). The -*C_2-*C_3-*C_4- isotopomer disappears as explained in the text. Responses are due to isotopomers involving only one active coupling with the carbon C_3. Above b: rephased C_3 singlet. o: Spurious excitation of the central C_4 line. (c) Selective excitation of glutamate C_3 according to sequence (2) (τ_0 = 7 ms: $1/4J$). The contribution of all isotopomers involved in the initial C_3 multiplet reappears with weighting factors given in table 1. Above c: rephased C_3 multiplet. Spectra b and c have been obtained under identical conditions as 1b except a recycle time of 5 s.

C_2-*C_3-C_4 remains with its normal intensity, whereas the M-A-X system (-*C_2-*C_3-*C_4) is affected by a factor 1/2 at all sites. In fact, some care must be exercised concerning the central line of the C_3 multiplet which here includes the contribution from isotopomers C_2-*C_3-C_4 and -*C_2-*C_3-*C_4-. Examination of splittings at site C_4 unambiguously indicates the negligible amount of *C_1-*C_2-*C_3-*C_4-*C_5 and C_1-*C_2-*C_3-*C_4-*C_5 isotopomers. Conversely at the C_2 site, it is possible to separate, thanks to the inactive coupling J_{12}, the contributions from isotopomers *C_1-*C_2-*C_3-*C_4-C_5 and C_1-*C_2-*C_3-*C_4-C_5. Comparison and confrontation of all line intensities in the three spectra of fig.2, which carry redundant information, show a high degree of consistency demonstrating the quantitative potentiality of the present approach.

4. DISCUSSION

It must be emphasized that the understanding of biochemical processes which occur at advanced stages of the precursor assimilation rests on accurate data concerning doubly, triply or (more) labeled metabolites. They can only be obtained by polarization transfer experiments such as those presented in this paper. A complete analysis based on the present data and some others obtained at different stages of the precursor assimilation will be published elsewhere [21]. For now, we have demonstrated the existence of the glutamate isotopomer *C_1-*C_2-*C_3-*C_4-C_5, which until now was suspected without any physical evidence [22–24]. The proportion of this isotopomer is indicative, in a quantitative fashion, of recycling within the TCA cycle.

Acknowledgements: Financial support from CNRS and INRA is gratefully acknowledged.

REFERENCES

[1] Bax, A. (1982) Two Dimensional Nuclear Magnetic Resonance in Liquids, Reidel, Dordrecht.
[2] Aue, W.P., Bartholdi, E. and Ernst, R.R. (1976) J. Chem. Phys. 64, 2229–2247.
[3] Bax, A. and Freeman, R. (1981) J. Magn. Reson. 44, 542–561.
[4] Jeener, J., Meier, B.H., Bachmann, P. and Ernst, R.R. (1979) J. Chem. Phys. 71, 4546–4553.
[5] Macura, S. and Ernst, R.R. (1980) Mol. Phys. 41, 95–117.
[6] Piantini, U., Sørensen, O.W. and Ernst, R.R. (1982) J. Am. Chem. Soc. 104, 6800–6801.
[7] Kessler, H., Oschkinat, H., Griesinger, C. and Bermel, W. (1986) J. Magn. Reson. 70, 106–133.
[8] Morris, G.A. and Freeman, R. (1978) J. Magn. Reson. 29, 433–462.
[9] Brondeau, J., Millot, C. and Canet, D. (1984) J. Magn. Reson. 57, 319–323.
[10] Millot, C., Brondeau, J. and Canet, D. (1984) J. Magn. Reson. 58, 143–148.
[11] Bauer, C.J., Freeman, R., Frenkiel, T., Keeler, J. and Shaka, A.J. (1984) J. Magn. Reson. 58, 442–457.
[12] Hore, P.J., Zuiderweg, E.R.P., Dykstra, K. and Kaptein, R. (1982) J. Am. Chem. Soc. 104, 4286–4288.
[13] Hore, P.J., Scheek, R.M., Volbeda, A. and Kaptein, R. (1982) J. Magn. Reson. 50, 328–334.
[14] Hore, P.J., Scheek, R.M. and Kaptein, R. (1983) J. Magn. Reson. 52, 339–342.
[15] Delsuc, M.A., Guittet, E. and Lallemand, J.Y. (1985) J. Magn. Reson. Chem. 23, 213–215.
[16] Canet, D., Brondeau, J., Boubel, J.C. and Retournard, A. (1987) Magn. Reson. Chem. 25, 798–803.
[17] Shaka, A.J., Keeler, J. and Freeman, R. (1983) J. Magn. Reson. 53, 313–340.
[18] Levitt, M.H. and Ernst R.R. (1983) Mol. Phys. 50, 1109–1124.
[19] Sørensen, O.W., Eich, G.W., Levitt, M.H., Bodenhausen, G. and Ernst, R.R. (1983) Prog. Nucl. Magn. Reson. Spectrosc. 16, 163–192.
[20] London, R.E., Kollman, V.H. and Matwiyoff, N.A. (1975) J. Am. Chem. Soc. 97, 3565–3573.
[21] Martin, F., Boudot, D. and Canet, D. Plant. Physiol, submitted.
[22] Chance, E.M., Sechalzer, S.H., Kobayashi, K. and Williamson, J.R. (1985) J. Biol. Chem. 258, 13785–13794.
[23] Sherry, A.D., Nunnally, R.L. and Peshock, R.M. (1985) J. Biol. Chem. 260, 9272–9279.
[24] Dickinson, J.R., Dawes, J.W., Boyd, A.S.F. and Baxter, R.L. (1983) Proc. Natl. Acad. Sci. USA 80, 5847–5851.