^{1}H NMR detection of cerebral *myo*-inositol

Sebastián Cerdán, Roberto Parrilla, Jorge Santoro+ and Manuel Rico+*

Instituto Gregorio Marañón, CSIC Velázquez 144, and +*Instituto de Estructura de la Materia, CSIC, Serrano 119, 28006 Madrid, Spain*

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A previously unassigned group of prominent multiplets of the 360 MHz ^{1}H NMR spectrum of acid stable metabolite extracts from rat brain is shown to arise from free *myo*-inositol. This conclusion is derived from a systematic analysis of the high-resolution ^{1}H NMR spectra of brain acid extracts, in which appropriate conditions and optimal proton signals have been selected for the quantitative analysis of up to 15 metabolites. Developmental variations in the cerebral content of *myo*-inositol could be readily detected using this approach, which provides a novel alternative to study *myo*-inositol metabolism under physiological or pathological conditions.

Cerebral myo-*inositol* *^{1}H NMR quantitative analysis* *Brain development*

1. INTRODUCTION

Adequate understanding of cerebral metabolism 'in situ' demands the non-invasive study of metabolic transformations as they take place in their own intracellular environment. ^{1}H NMR provides an excellent tool for detecting in situ proton signals from a wide variety of metabolites with the important advantage over ^{13}C or ^{31}P NMR of its inherently higher sensitivity. Initial studies from different laboratories have reported 'in vivo' ^{1}H NMR spectra of rat brain [1] or high-resolution spin-echo studies in rat or rabbit brain homogenates [2,3]. However, the physiological information obtainable so far from the ^{1}H NMR spectra has been limited either by an incomplete assignment of resonances or by difficulties in signal quantitation due to partial overlapping of some relevant resonances.

Here, a systematic study of the ^{1}H NMR spectra of acid extracts from rat brain has been performed to identify the chemical nature of previously unassigned signals. Cerebral free *myo*-inositol is shown to account for a prominent group of formerly unassigned multiplets situated in the high-field vicinity of the water signal. In addition, experimental conditions leading to optimization of the ^{1}H NMR quantitation of brain acid extracts are described and applied to monitor variations in the content of *myo*-inositol and other ^{1}H NMR detectable metabolites during cerebral maturation.

2. EXPERIMENTAL

2.1. *Animals and experimental techniques*

Male Wistar rats, 200–250 g in weight, fed ad libitum and allowed free access to water, were used in these experiments. Rat brain specimens were ob-

* To whom correspondence should be addressed

Abbreviations: Ac$^-$, acetate; BHBA, β-hydroxybutyrate; Cr, creatine; CrP, creatine phosphate; 2D COSY, bidimensional correlated spectroscopy; FT NMR, Fourier transform NMR; GABA, γ-aminobutyrate; GPC, glycerolphosphorylcholine; Lac, lactate; *myo*-ino, *myo*-inositol [(1,2,3,5/4,6)cyclohexanehexol]; N-Ac-Asp, *N*-acetylaspartate; PC, phosphorylcholine; TSP, 2,2,3,3-tetradeutero-3-trimethylsilylpropionate. Abbreviations used for the amino acids are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature

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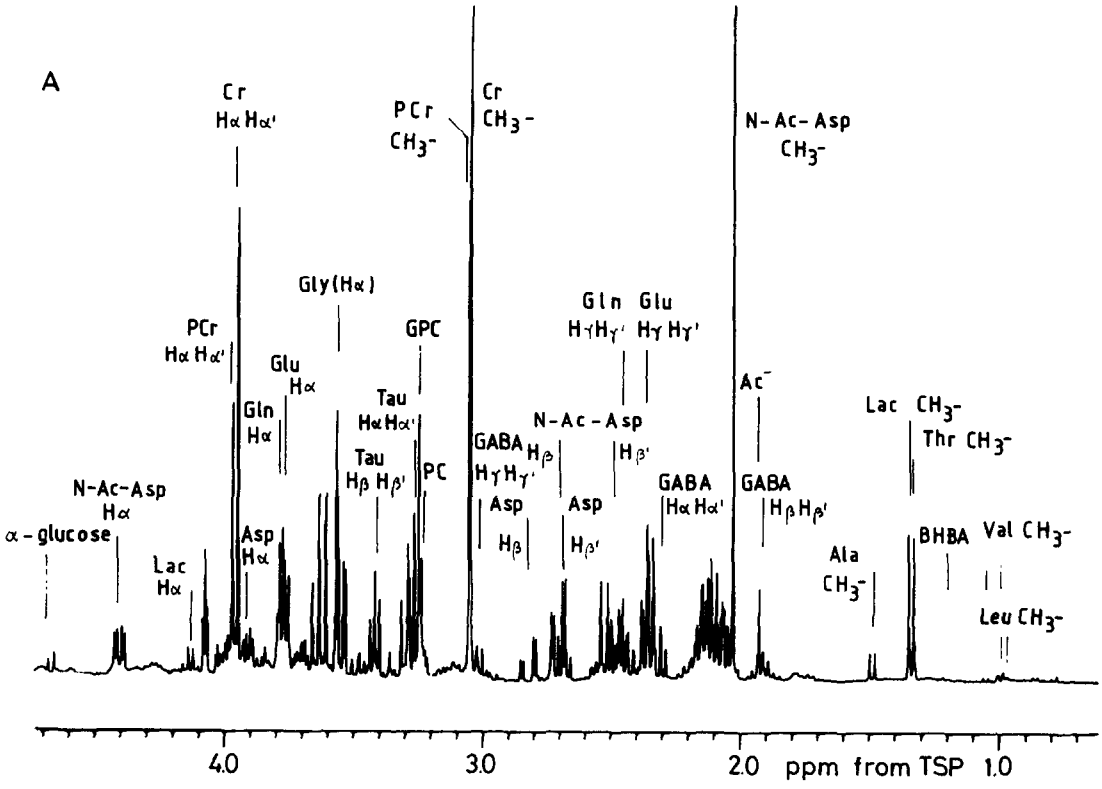
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Cr
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PCr
CH3-
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Gly(Hα)
PCr
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Glu
Hα
Gln
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Tau
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GPC
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N-Ac-Asp
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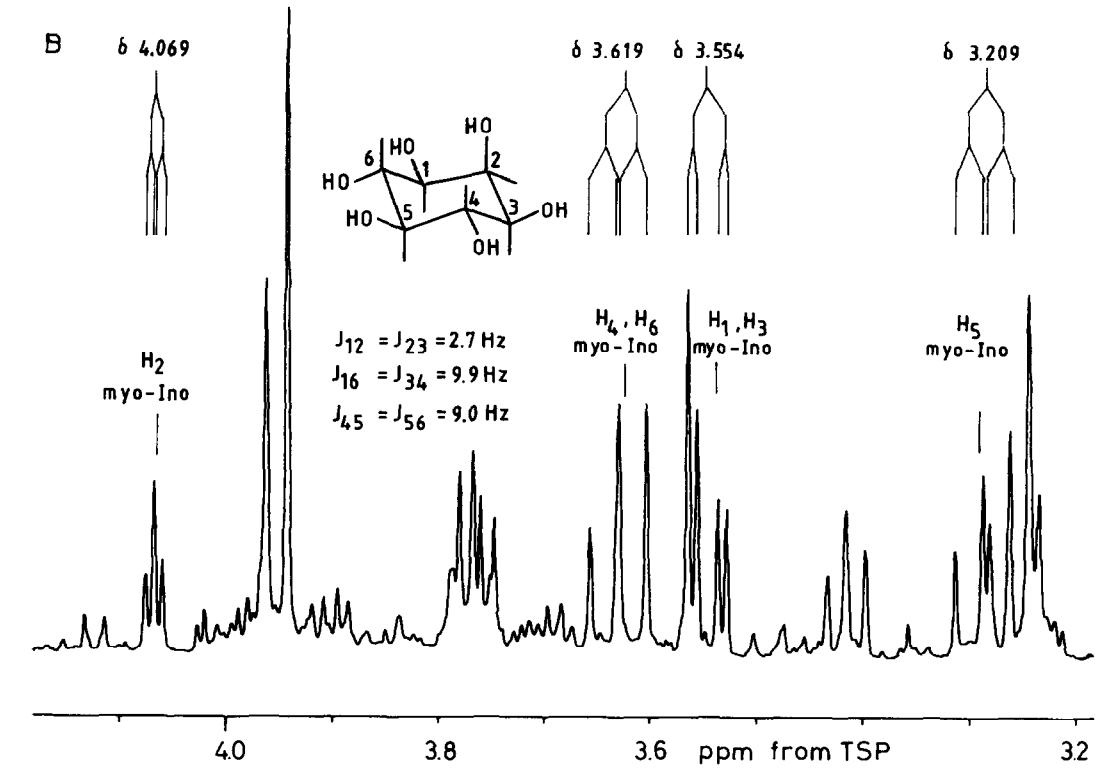
B
δ 4.069
δ 3.619
δ 3.554
δ 3.209
HO
HO
HO
HO
OH
OH
H2
myo-Ino
J12 = J23 = 2.7 Hz
J16 = J34 = 9.9 Hz
J45 = J56 = 9.0 Hz
H4, H6
myo-Ino
H1, H3
myo-Ino
H5
myo-Ino
4.0
3.8
3.6
ppm from TSP
3.2

tained from anesthetized animals (25 mg/kg sodium pentobarbital) using the funnel freezing technique [4]. Six-day-old rats were processed similarly.

Acid-soluble metabolites were extracted from single frozen pulverized brains using the methanol/HCl/perchloric acid procedure described by Lowry and Passoneau [5] as modified by Veech et al. [6]. The extraction protocol was repeated twice, the neutralized supernatants being combined and freeze-dried. The resulting lyophilized powder was dissolved in 99.8% D_2O to approx. 0.2 g dry wt/ml, passed through a Chelex column and its final pH adjusted to 8 just prior to ^1H or ^{13}C NMR spectroscopy.

2.2. *NMR spectroscopy*

High-resolution ^1H NMR spectra at 360 MHz were obtained on a Bruker WM-360 spectrometer at a probe temperature of 20°C. Conditions were: 8 μs pulse width (90° flip angle), 4000 Hz spectral width, 16K data points, 3 s recycle time and 256 scans. Intensity of the water signal was reduced when necessary with the help of a 0.5 s presaturation pulse. Natural abundance proton-decoupled ^{13}C NMR spectra were obtained from the same samples used in the ^1H NMR analysis but appropriately combined and concentrated after lyophilization and resuspension in D_2O. Conditions were: 20 μs pulse width (90° flip angle), 20 kHz spectral width, 16K data points and 2.4 s recycle time. ^1H and ^{13}C chemical shifts are reported vs internal TSP.

The two-dimensional homonuclear correlated spectrum, 2D COSY [7], was collected using a 512 × 2048 time domain matrix, averaging the signal over a 16 step phase cycle for each t_1 value. As in conventional ^1H NMR spectra, the solvent line was suppressed with continuous irradiation along the cycle except during the t_1 and t_2 periods. Sine-bell window functions were used along the t_1 and t_2 directions prior to 2D Fourier transformation, the final 2D frequency domain spectrum (1K × 1K) being further improved by triangular symmetrization.

^1H NMR spectral variations within the pH interval 1.5–11.0 were analyzed to assist in the assignments of resonances and select the most appropriate conditions for signal quantitation. The dissociation equilibrium of the NH_3^+ group in taurine and glutamate in the extracts happens to be a slow process on the NMR time scale and independent signals for the zwitterionic and anionic forms are observed [$\delta_{Glu\pm}$ ($H\gamma\gamma'$) 2.350, δ_{Glu-}($H\gamma\gamma'$) 2.230; $\delta_{Tau\pm}$($H\alpha\alpha'$) 3.295, δ_{Tau-}($H\alpha\alpha'$) 3.015; $\delta_{Tau\pm}$($H\beta\beta'$) 3.440, δ_{Tau-}($H\beta\beta'$) 3.035]. This inconvenience, which complicates both spectral assignments and resonance quantitation, was found to be adequately eliminated by obtaining the spectra at pH 8.0.

Quantitation of the ^1H NMR resonances in terms of concentration was achieved through comparison of the intensity per contributing proton of the appropriate resonance with that of the alanine methyl doublet, after determining enzymatically the concentration of this amino acid [8]. To determine the intensity, the integral value was used for isolated signals. For partially overlapping signals, the sum of heights of the multiplet component lines measured on an expanded scale was found to be more reliable. Optimal proton signals used in quantitation (table 1) were selected among those arising from the same metabolite on the basis of either complete resolution or minimal coalescence with neighboring resonances. Correction factors were introduced in the calculations to account for small differences in signal amplitude due to different relaxation times or non-resolvable long-range couplings.

2.3. *Amino acid analysis*

Amino acid content in the same samples used in the ^1H NMR quantitative analysis was also determined by quantitative ionic exchange chromatography on an automatic amino acid analyzer (Biotronik Corp., FRG).

←

Fig.1. High-resolution (360 MHz) ^1H NMR spectrum of acid soluble metabolites from adult rat normoxic brain. (A) High-field portion; proton signal positions for different metabolites are indicated. (B) Expanded region (δ 4.2–3.2 ppm) of the spectrum depicted above showing *myo*-inositol multiplets; assignments, couplings and chemical shifts are also presented. NMR conditions were as described in section 2.

2.4. *Materials*

Creatine phosphokinase, lactate and alanine dehydrogenases were obtained from Boehringer Mannheim (FRG). Creatine, creatine phosphate, amino acids and nucleotides were purchased from Sigma (St. Louis, MO). Deuterated solvents and TSP were obtained from Stohler (Waltham, MA). The rest of the reagents were of the highest purity available commercially.

3. RESULTS AND DISCUSSION

Fig.1A depicts the high-field portion of a representative high-resolution ^{1}H NMR spectrum of an extract of acid-soluble metabolites from rat brain. Well resolved resonances of various amino acids, creatine, creatine phosphate, glycerol phosphorylcholine, phosphorylcholine and lactate, which have been previously identified [1–3], have been pointed out. We have further checked their correct assignment on the basis of their pH titration behavior, correlations found in the 2D COSY ^{1}H NMR spectrum and internal standard additions. Expected proton signal connectivities were found in the 2D COSY spectrum (not shown) for all metabolites detailed in tables 1 and 2.

The group of signals indicated in fig.1B remained unassigned. These resonances were also correlated in the 2D COSY ^{1}H spectrum and displayed the proton coupling values presented in the inset of fig.1B. Couplings, shifts, relative intensities and the fact of being non titratable signals lead us to identify this relatively major spectral component as *myo*-inositol. This assignment was further checked through the addition to the sample of a *myo*-inositol internal standard which coresonated with the formerly unidentified signals. Further evidence was obtained from the natural abundance ^{13}C NMR spectrum of the original samples in which *myo*-inositol gave rise to 4 prominent signals in a clean zone of the spectrum at δ 77.00, 75.06, 74.09 and 73.77 ppm (relative intensities 1:2:1:2) which corresponded to carbons C5, C1–C3, C2 and C4–C6, respectively (not shown). It should be noted that no *myo*-inositol signals have been observed in reported ^{1}H NMR spectra of normal muscle [9], liver [10], serum [11] or tumor cells [12]. In addition, preliminary experiments indicated that cerebral free *myo*-inositol content might show important interspecies variations, since analysis of the ^{1}H NMR spectra of acid extracts from adult dog brain revealed significantly higher concentrations than those found in the rat (M. Rico and S. Cerdán, unpublished).

Some other previously unassigned signals in the high-field region of the spectrum corresponding to minor components are also marked in fig.1A: α-glucose (H1′, δ 4.672) and β-hydroxybutyrate (methyl, δ 1.210). The low-field zone of the spectrum (not shown) presents resonances assignable to AMP (H8, δ 8.596; H1′, δ 6.155), ADP (H8, δ 8.514; H1′, δ 6.160), ATP (H8, δ 8.522; H1′, δ 6.160), NAD (H2, δ 9.330; H6, δ 9.130; H4, δ 8.830; H8, δ 8.432), GTP (H8, δ 8.166), Phe (H_{ortho}, δ 7.342; H_{meta} and H_{para}, δ 7.442), Tyr ($H_{\delta\delta}$, δ 6.912 and $H_{\epsilon\epsilon}$, δ 7.206) and His (H2, δ 7.740; H5, δ 7.050). The relative intensity of this portion of the spectrum is considerably smaller than that of the high-field zone.

An important aspect derived from the identification of *myo*-inositol ^{1}H NMR signals is the possibility of obtaining by NMR quantitative information on the *myo*-inositol content of the brain and its eventual changes during physiological or pathological situations. Simultaneously, information on the concentration of a fairly large number of cerebral metabolites can be obtained quickly and reliably. However, before a correct interpretation of the quantitative NMR results can be made, validation of the NMR data by comparison with values obtained by other techniques is needed. Table 1 shows an illustrative comparison between the cerebral content of a number of metabolites as determined by ^{1}H NMR and values obtained for the same samples using more conventional techniques such as enzymatic analysis or ion-exchange chromatography. Specific proton signals used in the quantitation along with their chemical shifts, multiplicities, assignments and the range of previously reported values for the corresponding metabolites [13,15,16] are also included. A high degree of agreement is found between the values determined by ^{1}H NMR, those obtained by chemical analysis and the range of concentrations previously reported in the literature, thus supporting the results obtained in our NMR quantitative analysis.

As a first application of this methodology, table 2 summarizes the variations observed in ^{1}H NMR detectable metabolites during brain maturation.

Table 1

Characterization of ^{1}H NMR signals (360 MHz, 20°C, pH 8.0) used in the quantitation of rat brain metabolites and comparison of metabolite content (μmol/g dry wt) as determined by ^{1}H NMR with other analytical techniques

Metabolite	Signal identification			Content (^{1}H NMR)	Content[b] (other techniques)	Range[c] of reported values
	δ (ppm)	Mult[a]	Assignment			
Thr	1.324	d	Me	1.6 ± 0.2	2.5 ± 0.2	0.4– 1.3
Lac	1.336	d	Me	7.4 ± 1.2	4.7 ± 0.9	4 – 6
Ala	1.483	d	$H_\gamma H_\gamma$,	1.8 ± 0.2	1.9 ± 0.2	0.6– 4.3
N-Ac-Asp	2.027	s	Me	22.9 ± 2.4		21.7–44.5
GABA	2.306	t	$H_\alpha H_\alpha$,	5.5 ± 0.5		1.1– 3.8
Glu	2.348	m	$H_\gamma H_\gamma$,	32.2 ± 2.5	31.2 ± 5.4	36.0–57.6
Gln	2.453	m	$H_\gamma H_\gamma$,	16.3 ± 2.4	14.1 ± 2.1	9.9–25.8
Asp	2.800	dd	H_β	8.9 ± 0.5	7.1 ± 1.4	7.1–12.5
PC	3.234	s	N-CH_2-	1.4 ± 0.2		1.2
GPC	3.244	s	N-CH_2-	3.0 ± 0.2		2.2
Tau	3.420	t	$H_\beta H_\beta$,	11.8 ± 2.2	10.1 ± 1.9	5.8–24.7
Gly	3.562	s	$H_\alpha H_\alpha$,	3.4 ± 0.3	3.5 ± 0.5	2.5– 6.7
myo-Ino	3.619	dd	H4,H6	18.8 ± 2.5		14.1–28.2
Cr	3.942	s	$H_\alpha H_\alpha$,	20.3 ± 0.5	19.5 ± 2.1	24.8–35.4
PCr	3.962	s	$H_\alpha H_\alpha$,	12.7 ± 1.5	10.8 ± 1.6	15.0–22.7

[a] Multiplicity: s, singlet; d, doublet; dd, doublet of doublets; t, triplet and m, multiplet

[b] All values were determined on the same samples used in the ^{1}H NMR analysis by quantitaive ion-exchange chromatography, except values of lactate, creatine and phosphocreatine, which were determined by enzymatic analysis [5]

[c] Values are taken from [13] and quotations therein, except those of creatine, upper limit of creatine phosphate, PC, GPC and *myo*-inositol which were taken from [4], [15] and [16], respectively. A wet wt/dry wt ratio of 4.6 determined experimentally was used to convert literature data expressed in wet wt units to dry wt units

Results are the mean ± SE of 6 independent experimental observations on adult rats

Well defined changes in free amino acid content have been described to occur during cerebral development. ^{1}H NMR results obtained in this study closely parallel those obtained by ion-exchange chromatography [14]. *N*-Acetylaspartate, glutamine, γ-aminobutyrate and aspartate content increased with age while alanine and taurine decreased. Interestingly enough, free *myo*-inositol content increased 2-fold during the first 8 weeks after birth and continued to rise until the sixth month.

Finally, if our rat brain methanol/HCl/perchloric acid extract is compared to those reported in [1] for (a) rat brain acid extract, (b) excised brain tissue or (c) in vivo, very similar relative concentrations of the different ^{1}H NMR observable metabolites are found, and most relevant, *myo*-inositol signals (particularly those of protons H1–H3 and H4–H6) can be followed in the spectrum of excised brain tissue or even in vivo.

Decreased *myo*-inositol content has been associated with the protective action of lithium in mania [16] and the development of diabetic neuropathy [17]. In addition, a triphosphorylated derivative of *myo*-inositol, *myo*-inositol 1,4,5-triphosphate is thought to act as a second messenger of intracellular calcium-mobilizing hormones [18]. This report opens a promising way of studying *myo*-inositol metabolism non-invasively using ^{1}H NMR.

Table 2

Effect of development on the content (μmol/g dry wt) of some relevant ^{1}H NMR (360 MHz) detectable metabolites of rat brain

Metabolite	Age (days)		
	6	60	180
Thr	3.5 ± 0.3	1.4 ± 0.2	1.9 ± 0.3
Lac	5.8 ± 0.8	7.5 ± 1.2	7.0 ± 0.9
Ala	5.8 ± 0.7	1.8 ± 0.2	1.8 ± 0.3
N-Ac-Asp	3.5 ± 0.4	25.0 ± 1.8	29.0 ± 2.1
GABA	3.3 ± 0.5	5.6 ± 0.5	6.9 ± 0.5
Glu	13.0 ± 1.1	30.5 ± 1.9	33.8 ± 2.2
Gln	11.1 ± 1.1	16.7 ± 2.4	20.2 ± 2.5
Asp	3.8 ± 0.4	9.3 ± 0.5	11.3 ± 0.5
PC	2.8 ± 0.4	1.5 ± 0.2	1.4 ± 0.2
GPC	0.9 ± 0.3	2.7 ± 0.2	3.3 ± 0.6
Tau	26.7 ± 2.0	12.9 ± 2.2	15.0 ± 2.0
Gly	6.3 ± 0.7	3.4 ± 0.3	3.6 ± 0.2
myo-Ino	9.7 ± 1.0	21.5 ± 2.5	24.4 ± 1.8
Cr	12.7 ± 1.1	20.4 ± 0.5	20.0 ± 1.0
PCr	5.8 ± 0.9	12.1 ± 1.5	14.0 ± 0.8

Results are the mean ± SE of not less than 5 independent experimental observations

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