^{15}N relaxation and quantification of ^{15}N-labelled metabolites in cell extracts

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Abstract

^{15}N is useful for studying nitrogen metabolism by isotope labelling. However, the ^{15}N NMR analysis is hampered by negative nOe factors and sometimes long T_1 values. Moreover, these can have a marked dependency on their physico-chemical environment (pH, ionic charge, viscosity, etc.) making quantitative analysis of cell extracts from labelling experiments difficult. We have, therefore, analysed some of these dependencies in order to find conditions in which quantitative analysis of some major metabolites (amino acids, ammonium, nitrate, urea) can be performed reliably and with sufficient signal. *To cite this article: N. Houyou et al., C.R. Chimie 9 (2006).*
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Résumé

Le ^{15}N est bien adapté pour étudier le métabolisme azoté par marquage isotopique. Cependant, l'analyse par RMN ^{15}N est gênée par des facteurs nOe négatifs et certains T_1 longs. De plus, les deux peuvent dépendre de manière prononcée de l'environnement physicochimique (pH, charge ionique, viscosité, etc.) ce qui rend difficile l'analyse quantitative d'extraits cellulaires. Nous avons donc analysé quelques-unes de ces dépendances pour trouver des conditions dans lesquelles une analyse quantitative de quelques métabolites majeurs (acides aminé, ammonium, nitrate, urée) peut être réalisée d'une manière fiable et avec suffisamment de signal. *Pour citer cet article : N. Houyou et al., C.R. Chimie 9 (2006).*
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1. Introduction

Nitrogen is the fourth most abundant element in living organisms, and its metabolism plays a fundamental role in every cell. Most nitrogen taken up by man and higher animals through their food is in organic forms that have originally been formed in plants. Plants have the ability to use part of their photosynthetic energy to reduce soil inorganic nitrogen, mostly nitrate or ammonium, to organic forms, especially amino acids. So, primary nitrogen metabolism in plants from nitrate to different amino acids is subject of considerable interest.

Many studies on nitrogen metabolism are carried out by stable isotope feeding experiments with ^{15}N, especially since no widely usable radioactive isotope exists. The resulting samples, mostly cell extracts, are often analysed and quantified by mass spectroscopy techniques, but NMR has an important role to play, too [1]. ^{15}N NMR spectroscopy [2], however, is hampered by a relatively low sensitivity due a gyromagnetic factor $\gamma(^{15}\text{N}) = -2.7112 \times 10^{-7}$ Hz T^{-1} compared to $\gamma(^{1}\text{H}) = 26.752 \times 10^{-7}$ Hz T^{-1}. Sensitivity can be improved by the resulting large maximal nuclear Overhauser enhancement (nOe) factor $\eta_{\max} = -4.93$, but its negative value can also lead to signal cancellation if η is not maximal. In many situations, longitudinal relaxation times T_1 are long, too, and the signal-to-noise ratio S/N can then be improved from rapid pulsing not waiting for complete relaxation. However, for quantification, T_1 and η have to be known or their effect must be avoided, often at the expense of a lower S/N. For many metabolites of interest, this task is complicated by the presence of exchangeable protons on the nitrogen which make the relaxation properties highly pH dependent either because of exchange-modulated scalar ^{1}H–^{15}N couplings, as proposed by [3] and dismissed by [4], or because of changes in ^{1}H–^{15}N dipolar interaction [4]. A subsequent study suggested that these pH-dependent effects were mainly due to interaction with paramagnetic ions [5]. However, paramagnetic ions cannot be removed to the required level when preparing cell extracts, and therefore, independently of their origin, pH dependent relaxation effects have to be taken into account when analysing cell extracts by ^{15}N NMR. This study, therefore, examines the effects of physicochemical conditions (pH, ionic charge, viscosity) on relaxation and spectral properties of some commonly encountered ^{15}N signals in cell extracts, namely nitrate, ammonium, urea, glutamate, alanine and glycine.

The present work has been carried out within studies trying to elucidate regulatory aspects of nitrate reductase in rapeseed (*Brassica napus* L.) seedlings [6]. The method mostly involves in vivo ^{15}N NMR to follow the assimilation of either K[15]NO$_3$ or [15]NH$_4$Cl, but the relative timecourses obtained from in vivo spectroscopy have to be quantified from extracts, and supplementary feeding experiments without the constraints of in vivo NMR (e.g., absence of light, densely packed excised tissue, etc.) are also performed creating a need for reliable quantitative analysis of ^{15}N in cell extracts. Though the context of this work is on primary nitrogen metabolism in plants, most of its conclusions are valid for the study of any cell extract independently of the biological source material.

2. Material and methods

To avoid the long acquisition times, often 10–20 h, necessary for getting a sufficient signal-to-noise ratio from extracts of ^{15}N-labelled plant tissues, we rather used an unlabelled perchloric acid extract prepared from 0.79 g of excised rapeseed seedling shoots, and spiked it with K[15]NO$_3$, [15]NH$_4$Cl, [^{15}N$_2$]-urea, [^{15}N]-glutamate, [^{15}N]-alanine and [^{15}N]-glycine at 200 mM each in a final volume of 1.5 ml. For comparison, a standard solution was made from the same labelled compounds in distilled water. The pH of the samples was adjusted with 12.5 M NaOH or 10 M HCl to minimise the resulting dilution. For investigating the effects of ionic charge, increasing amounts of NaCl were added to the standard solution.

^{15}N NMR spectra were recorded at 30.42 MHz on a Bruker Avance 300 WB spectrometer using an 8-mm direct detection broad-band (BBO) probehead. Reference spectra were acquired with a 90° pulse, a relatively short interpulse delay of 4.0 s, power-gated WALTZ16 ^{1}H broadband decoupling and 2048 scans. For determining the nOe factor η, a power-gated (pg) and an inverse-gated (ig) spectrum were recorded with a 90° excitation pulse, an interpulse delay of 16.0 s and 1024 scans each. From the integrals, $\eta = I_{\text{pg}}/I_{\text{ig}} - 1$ was calculated. T_1 measurements were carried out with a fast inversion–recovery sequence [7] using a 10-s interpulse delay D between the 90° detection pulse and the next scan's $90°_x 240°_y 90°_x$ composite inversion pulse [8], and 13 recovery delays τ (0.01, 0.2, 0.4, 0.7, 1.1,

1.6, 2.4, 3.6, 5.6, 9.0, 14.0, 22.0 and 35.0 s). Low-power ^1H decoupling was maintained throughout, and 80 scans were acquired per increment. T_1 was determined by curve-fitting the peak integrals I obtained to $I(\tau) = I_0 (1 - (1 + \alpha (1 - \exp(-D/T_1))) \exp(-\tau/T_1))$. The parameter α takes into account imperfect inversion due to offset and inhomogeneity effects.

3. Results

Fig. 1 shows a series of spectra from the spiked extract with increasing pH taken with power-gated broadband decoupling to get the nOe and with a relatively short interpulse delay that does not allow for complete relaxation thus highlighting differences in T_1. The
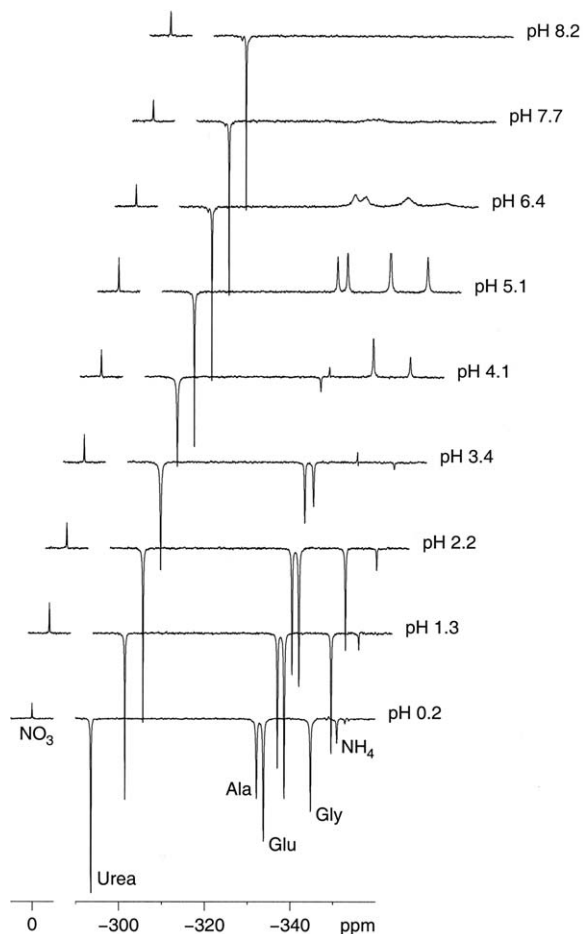
first point to note is that the spectra at acid pH contain all six expected peaks, whereas the amino and ammonium resonances broaden at more alkaline pH leading to their disappearance. This can be attributed to base catalysed proton exchange and suggests that any useful NMR analysis has to be done at acid pH values. Strong variations in signal intensity and even peak inversions can also be seen. These are the combined effect of variations in the nOe factor η and the relaxation time T_1.

A back-titration from pH 8 down (data not shown) showed that the recovery of narrow signals occurred at lower pH than the broadening did. The main difference between the forward and back-titration is the amount of NaCl created by the subsequent addition of NaOH and HCl. We, therefore, recorded a series of spectra with the standard solution at pH 7 and increasing NaCl concentration (Fig. 2). Without NaCl, the peaks were still reasonably narrow but on addition of NaCl the peaks rapidly broadened in much the same fashion as when increasing the pH.

Fig. 3 shows the changes in the nOe factor η versus pH that were qualitatively apparent from Fig. 1. Values for amino acids and ammonium at pH 7 and 8 could not reasonably be extracted given the peak broadening.
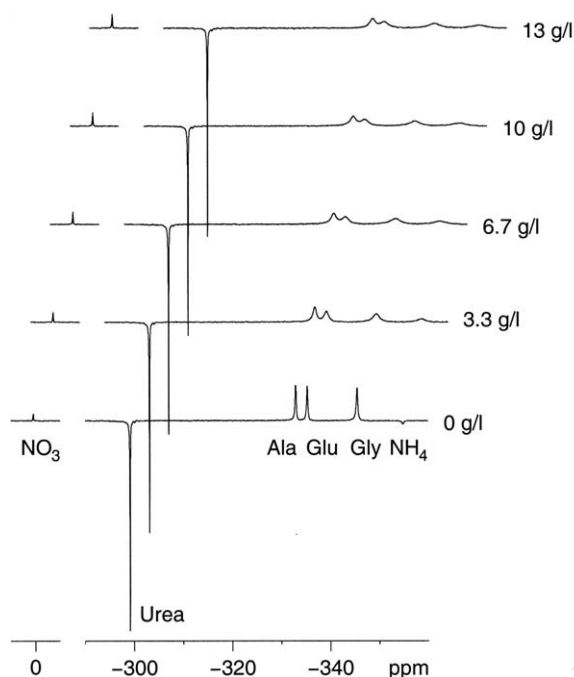

Fig. 1. ^{15}N NMR spectra of a spiked rapeseed seedling shoot extract at different pH values. NMR conditions: $\nu = 30.42$ MHz, power-gated ^1H decoupling, 90° pulse, 4.0 s interpulse delay, 2048 scans.

Fig. 2. ^{15}N NMR spectra of a model solution with increasing amounts of NaCl. NMR conditions as in Fig. 1.
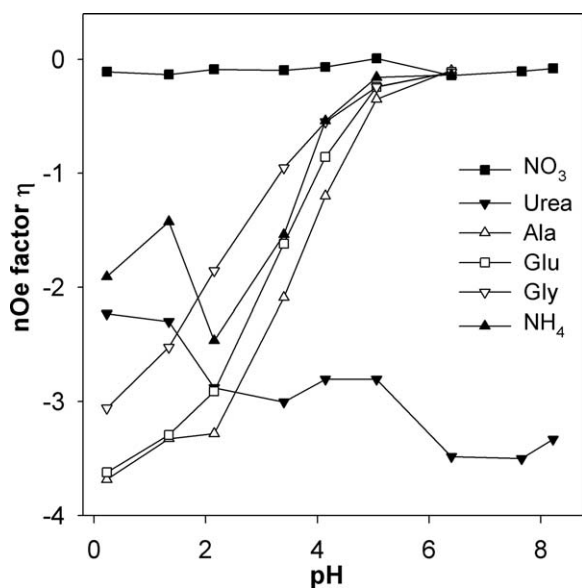
Fig. 3. The pH dependency of the nOe factor $\eta = I_{pg}/I_{ig} - 1$.

These compounds change peak sign between pH 3 and 5, corresponding to η comprised between –1 and 0. An increase in absolute intensity, i.e. $\eta < -2$, is only obtained at pH < 2. Nitrate has nearly no nOe, which was expected given that the molecule contains no hydrogen, and urea is the only compound tested with a gain in absolute signal throughout. Its η decreases with pH though it does not reach the theoretical maximum (numerically a minimum) $\eta_{max} = -4.93$.

T_1 values for ammonium (Fig. 4A) and the amino acids (Fig. 4B) decrease with increasing pH attaining surprisingly low values when the line broadening sets in. The concomitant loss in nOe suggests that the relaxation mechanism that becomes efficient with increasing pH is another than dipolar relaxation by the attached ^{1}H. The relaxation mechanism in question could either be exchange modulation of the scalar coupling [3] as base catalysed proton exchange increases with pH or for the amino acids enhanced interaction of paramagnetic ions (e.g., Cu^{2+}, Mn^{2+}) [5] with the prevalent zwitterionic form at pH above the pKs which are around 2.3, 2.2 and 2.3 for Ala, Glu and Gly, respectively. The pH dependence of the T_1 of urea (Fig. 4A) is the contrary of the amino groups, like its nOe, meaning that a non-dipolar mechanism becomes less efficient with increasing pH. It should be noted that urea does not change its protonation state in the pH range considered. The T_1 of nitrate (Fig. 4A) is as expected the long-
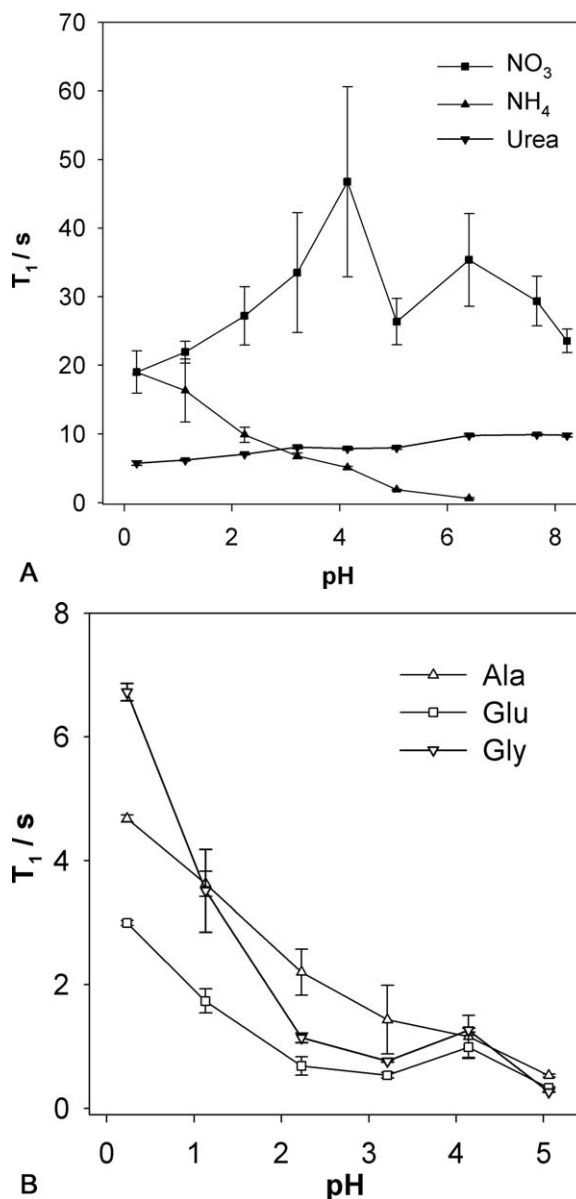

Fig. 4. The pH dependency of longitudinal relaxation time T_1 determined by fast inversion–recovery. Error bars correspond to the curve-fitting error. **A**: Nitrate, ammonium, urea; **B**: alanine, glutamate, glycine.

est with the absence of a directly attached ^{1}H. More surprising is that it shows some pH dependency nearly doubling at pH 4 compared to acid and neutral pH. However, it should be noted that the measurements carry relatively large error bars since the longest recovery delay was 35 s, slightly shorter than the maximum

T_1 itself, and that a big change in T_1 does not seem to be supported by the rapid pulsing spectra in Fig. 1.

Finally we compared the T_1 values for nitrate and urea at pH 7 between the spiked cell extract ($T_1(NO_3) = 29.4 \pm 3.6$ s and T_1(urea) = 9.9 ± 0.2 s) with those obtained with the standard solution after addition of 13.3 g l^{-1} of NaCl ($T_1(NO_3) = 64 \pm 11$ s and T_1(urea) = 17.1 ± 0.4 s). The approximate doubling of relaxation times suggests another role of the sample matrix. Possibly the much higher viscosity of the cell extract leads to slower molecular movements which would favour relaxation when $\omega \tau_c < 1$ which is certainly the case for the small molecules and the low NMR frequency under consideration.

4. Discussion

Relaxation measurements of ^{15}N in small biomolecules found some interest in the early days of ^{15}N NMR [2] and have then only reappeared occasionally to help interpret in vivo NMR spectra [9–11]. Though the different physico-chemical conditions preclude a quantitative comparison of absolute values (and incidentally, η beyond η_{max} have been reported by Scharff et al. [11]), the tendency in pH dependence can be compared. For the amino groups, the stronger nOe for more acid pH values is confirmed, but for ammonium we found a similar behaviour whereas Scharff et al. [11]) found a rather stable value. The difference could be explained by earlier observations [10] that the nOe for ammonium changes drastically between a neat solution and solution containing cell debris. In the extracts, an intermediate situation is found since large cell debris is eliminated by centrifugation but all soluble cell constituents are retained. The strong dependence of nOe effects on difficult-to-control parameters in extracts like ionic charge, viscosity, cell constituents thus suggests that quantitative ^{15}N analysis should only be performed without nOe, and our results indicate that in many situations $\eta > -2$ thus rather reducing signals than enhancing them.

Still, conditions have to be chosen with respect to T_1. Here the pH zone around pH 3–4 looks the most promising with peaks that are still reasonably narrow and rather short T_1s under 2 s for the amino resonances. This would allow to obtain fully relaxed spectra in reasonable experimental time and thus be quantitative. Under such conditions, other signals like nitrate or urea would not be sufficiently relaxed, and for quantification it is necessary to record a second spectrum with a longer interpulse delay. Since ^{15}N-nitrate occurs essentially in nitrate-incubated samples, and there in rather high concentrations, this second spectrum could be recorded with less scans.

Thus the quantitative analysis of cell extracts by ^{15}N NMR seems feasible when experimental conditions are carefully chosen. Nevertheless an alternative avoiding many of these problems might be found by using ^{1}H NMR for indirect detection of ^{15}N as has been demonstrated on rice coleoptile extracts [12] though quantification is not easy as transfer efficiencies have to be evaluated and again problems due to proton exchange have to be avoided by selecting an appropriate pH where the exchange rates are much lower than the scalar couplings.

Acknowledgements

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