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NMR VISUALIZATION OF FREE ASPARAGINE IN POTATO TISSUE USING ADDUCT FORMATION WITH [^{13}C]FORMALDEHYDE

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; ^{13}C NMR; deuterium NMR; asparagine; formaldehyde-metabolism.

Abstract—The free asparagine in potato (*Solanum tuberosum*) tuber tissue has been observed by ^{13}C NMR, using labelled formaldehyde as a marker; formaldehyde–asparagine adduct formation is specific and leads to characteristic ^{13}C resonances. In addition, metabolism of formaldehyde to methanol and formate by potato tissue has been observed by ^{13}C and deuterium NMR. Metabolism of formaldehyde-d_2 leads to a 3:1 mixture of CD_3OH and CD_2HOH.

INTRODUCTION

Formaldehyde interacts with living tissue both as a sterilizing agent and possible carcinogen [1]. This group has previously reported the detoxifying metabolism of formaldehyde by *E. coli* as observed by ^{13}C and deuterium NMR [2, 3]. We now report investigations into the fate of exogenous formaldehyde in potato tuber tissue as observed by ^{13}C NMR spectroscopy. In particular we describe the formation of an adduct (**1**, Fig. 1), which allows the visualisation of free asparagine within potato tuber tissue.

Potato was previously known to have a high concentration of free asparagine and glutamine [4, 5]. However, the observation of these amino acids is generally difficult and unreliable: chemical degradation during the derivatisation procedures required for gas chromatography means that these amides cannot easily be differentiated from the corresponding acids [6].

Others have applied NMR to plant tissue. For example: (i) ^{31}P NMR has been used to examine the energy status of potato tubers and maize seedlings [7] and to investigate the growth characteristics of oil palm suspension cultures [8]; (ii) the fate of a labelled asparagine C–N bond has been followed in soya bean [9] and (iii) ^{13}C NMR spectroscopy has been used to observe starch in potato [10]. However, we believe this to be the first report on xenobiotic metabolism in plants observed by NMR.

RESULTS

The metabolism of [^{13}C]formaldehyde was monitored by NMR spectroscopy over a period of hours. Figure 2(a) shows the ^{13}C NMR spectrum of a sample of Maris Bard potato which had been exposed to 10 mM formaldehyde for 8 hr. The difference spectrum (Fig. 2b) between first and last spectra clearly shows that the appearance of signals at δ172.3, 55.7 and 50.2 is accompanied by a concomitant decrease in the signal corresponding to formaldehyde (δ83.2). The result was also observed in Maris Piper and Desirée cultivars. Spectroscopic changes were found to occur at a similar rate for all three varieties and were independent of incubation temperature in the range 20 to 37°.

The signals at δ172.3 and 50.2 were readily attributed to formate and methanol respectively, the chemical shifts and coupling constants corresponding to those observed previously [2]. These metabolites were also observed by deuterium NMR following the exposure of potato tissue to formaldehyde-d_2 (Fig. 3). Deuteroformate resonates as a singlet at δ8.4 and deuteromethanol as a highly asymmetric doublet at δ3.3. Similar experiments with bacteria have shown that the intense high field line of methanol is due to CD_3OH and that this obscures the high field half of a doublet due to CD_2HOH [R. P. Mason and J. K. M. Sanders, to be published]. The H–D coupling is 1.7 Hz in CD_2HOH, while the proton-induced upfield

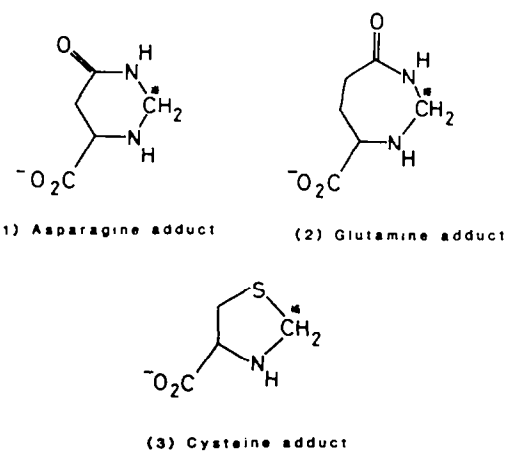

Fig. 1. Formaldehyde adducts of some amino acids.

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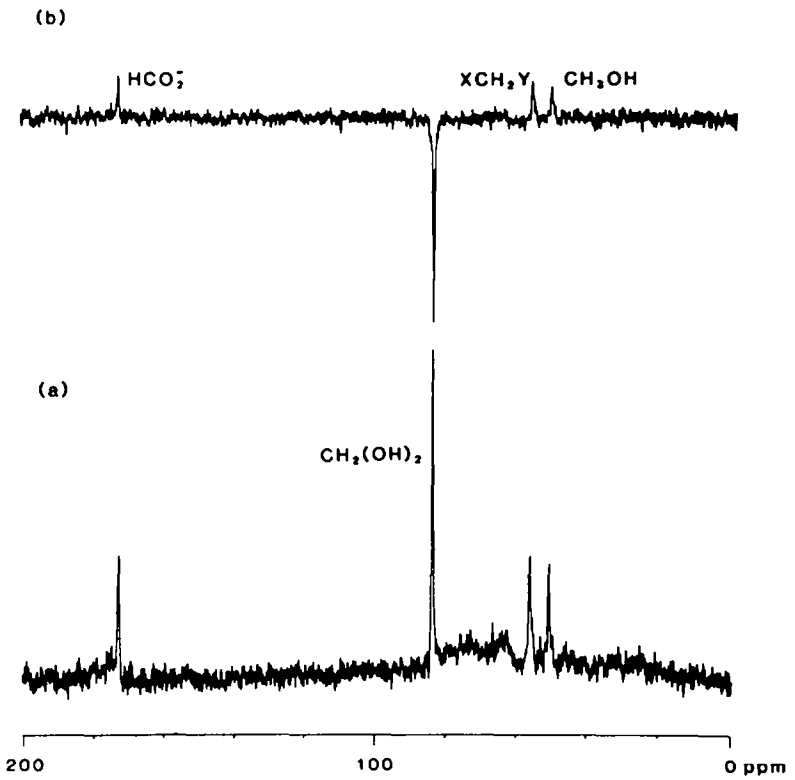

Fig. 2. (a) 100.6 MHz proton decoupled ^{13}C spectrum of a core of potato tissue (var. Maris Bard) after 8 hr exposure to 10 mM [^{13}C]formaldehyde at pH 7. 1000 transients were acquired during 35 min, and 10 Hz exponential line broadening was applied. The broad background signals arise from potato components. (b) Difference spectrum obtained by subtracting a spectrum acquired during the first hour of formaldehyde exposure from the '8 hr' spectrum. The potato background has disappeared, and it is clear that growth of signals is accompanied by loss of formaldehyde.

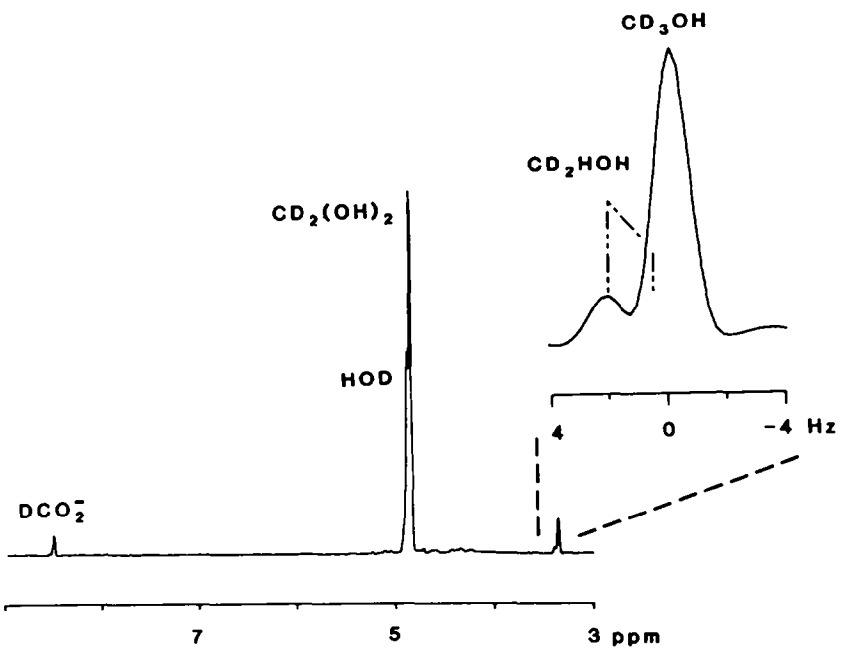

Fig. 3. 61.4 MHz resolution enhanced deuterium NMR spectrum of a core of potato tissue after 24 hr exposure to 20 mM formaldehyde-d_2. Total acquisition time was 4 min.

isotope effect on the deuterium resonance is 0.020 ppm (1.2 Hz). The CD_3OH:CD_2HOH ratio is approximately 3:1. The assignments have been proved by separate addition of authentic deuterated methanols, and by proton decoupling.

Most organisms that we have studied to date simultaneously produce formate and methanol from formaldehyde. However the signal at δ55.7 in the ^{13}C NMR spectrum (Fig. 2) had not been observed previously in our work; in proton coupled spectra it appears as a triplet, $J_{CH} = 158$ Hz. The combination of shift and coupling constant implies a structure of the form $X^{13}CH_2Y$ where X and Y are each O, N or S. In such a structure, the formaldehyde-derived carbon is at an unchanged oxidation level and the product is generally the result of adduct formation rather than metabolism. Some obvious possibilities on the basis of chemical shift [11] are shown (Figs 1 and 4). These were synthesised from [^{13}C]formaldehyde, and their ^{13}C NMR properties are shown in Table 1.

The cysteine adduct, 3, was rejected as a possibility because of its large coupling constant. Synge [5] and Davis [4] had shown that potatoes contain large amounts of free asparagine and glutamine, which suggested 1 or 2 as likely structures. The one bond ^{13}C–^{1}H coupling constant was 158 Hz in each case and chemical shifts were found to be very similar and pH dependent. A shift from 53.5 to 55.7 for both adducts was observed between pH 3.7 and pH 7, preventing a distinction from being made on purely chemical shift grounds. Therefore, solutions of the adducts were added to potato samples (Fig. 5). Peaks due to the major asparagine adduct were coincident with the potato product whereas the glutamine adduct was shifted 11 Hz from the potato-derived signal in resolution enhanced spectra.

The appearance of these spectra is pH dependent. Figure 2 shows a sample in buffer at pH 7, whereas Fig. 5 shows the sample in H_2O–D_2O at pH 4.6. Under these acid conditions there are extra peaks which we attribute to ring-opened adducts. Peaks due to these ring-opened forms also correspond to those observed in potato for the asparagine adduct but not the glutamine adduct. The amino methylol (NCH_2OH) resonances are assigned (Table 1) on the basis of previous work on formaldehyde

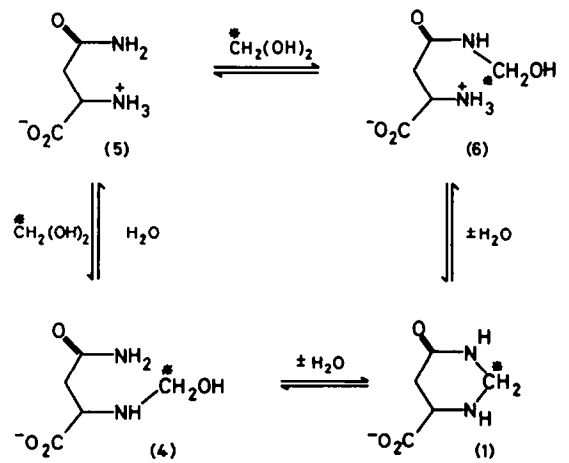

Fig. 4. The formaldehyde–asparagine equilibrium.

Table 1. ^{13}C NMR properties of formaldehyde-derived carbons in amino acid adducts (pH 4.6)

Compound	δ (ppm)	J (Hz)
(1) Cyclic asparagine adduct	54.7	158
(2) Cyclic glutamine adduct*	54.6	158
(3) Cysteine adduct	53.6	162
(4) N_α-Asparagine adduct	69.1	159
(6) N_γ-Asparagine adduct	64.1	159
N_α-Glutamine adduct*	66.3	158

*In addition, glutamine–formaldehyde solns show signals from as yet unidentified compounds at δ47.6 (J = 142 Hz) and 64.0 (J = 144 Hz).

adducts of amino acids and their *N*-acetyl derivatives [12].

Synge [5] suggests that potatoes have similar concentrations of glutamine and asparagine. The fact that we have specifically observed the asparagine adduct is an inevitable consequence of the relative stability of 6- and 7-membered rings. Adduct formation is 16 times more favourable for asparagine, as determined by integration. The apparent equilibrium constant for adduct formation between asparagine and formaldehyde at pH 4.6 is 42 ($\pm$ 12) mol^{-1}. Using this value, the concentration of free asparagine in the various potato samples examined in this work is estimated to be in the range 0.25–0.75% dry weight, in good agreement with the range of values quoted by Davies [4], 0.4–3‰, for a variety of cultivars.

DISCUSSION

The observation of formaldehyde oxidation and reduction by potato tissue is perhaps not too surprising, but the fact that the major reduction product of formaldehyde-d_2 is methanol-d_3 is unexpected. Reduction by NADH should give methanol-d_2, but this in fact constitutes only a small proportion of the methanol produced. There are two possible explanations for the formation of methanol-d_3: either there is a formaldehyde dismutase operating [13, 14] on *two* molecules of formaldehyde to give one formate and one methanol (eqn 1) *or* the pool of NADH is very small, and NADD formed by formaldehyde oxidation (eqn 2) then reduces formaldehyde-d_2 (eqn 3).

$$2CD_2(OH)_2 \rightarrow DCO_2H + CD_3OH + H_2O \quad \text{(eqn 1)}$$

$$CD_2(OH)_2 + NAD^+ \rightarrow DCO_2H + NADD + H^+ \quad \text{(eqn 2)}$$

$$CD_2(OH)_2 + NADD + H^+ \rightarrow CD_3OH + H_2O + NAD^+ \quad \text{(eqn 3)}$$

The present results do not distinguish between these two possibilities.

The selective detection of free asparagine using adduct formation with [^{13}C]formaldehyde is interesting for two reasons. Firstly, it is possible that it will provide a more convenient assay for asparagine in plant tissues than conventional methods, although it remains to be demonstrated what precision will be achieved. Given the apparent equilibrium constant of *ca* 40 for adduct formation, it should be possible to assay free asparagine up to about 25 mM in any plant tissue that is permeable to formaldehyde. Glutamine, although present in

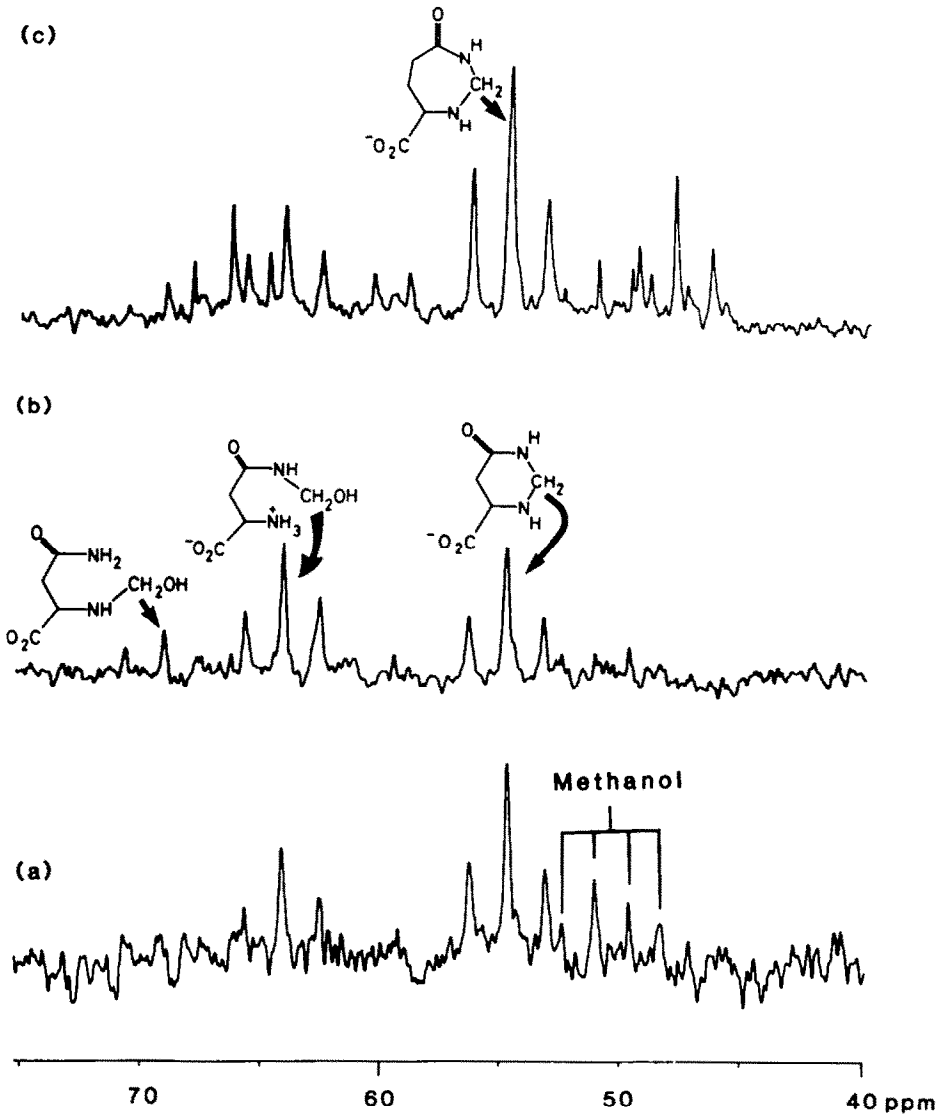

Fig. 5. Partial 100.6 MHz proton coupled ^{13}C spectra. (a) Potato tissue (var. Maris Piper) after 3 days incubation with 10 mM [^{13}C]formaldehyde, pH 4.5. (b) A portion of the same sample as (a) after addition of 3 mM asparagine–[^{13}C]formaldehyde adduct. (c) As (a) after addition of 3 mM glutamine–[^{13}C]formaldehyde adduct.

comparable concentrations, will not interfere because the stability of its adduct with formaldehyde is much lower. Note that deuterium NMR is not suitable for assaying adduct formation, even though it provides such detailed information on the isotopic composition of product methanol. This is because deuterium is a quadrupolar nucleus, so signals from even moderately large molecules are severely broadened. In Fig. 3, the adduct signals are barely visible above the noise in the 4–5 ppm region.

There is a second, and ultimately more compelling, reason why these results are interesting. They demonstrate the possibility of visualizing and quantifying an unlabelled, naturally occurring species within tissues by formation of a specific product with an infiltrated, labelled reagent. The labelled product then has characteristic spectroscopic properties which can be used to report on the concentration of the species of interest. This concept is widely used in radioimmunoassay, and has recently been applied to assay by NMR of Ca^{2+} and other metal ions in animal cells [15] and glutathione in *E. coli* [16]. The present work demonstrates that the same idea is applicable to organic molecules in plant tissues.

EXPERIMENTAL

Labelled materials. Deuterium oxide (99%) was supplied by Aldrich, and [^{13}C]paraformaldehyde (90%) and paraformaldehyde-d_2 (98%) were obtained from Merck, Sharp and Dohme.

Preparation of samples. Cylindrical cores (8 mm diameter by 10 mm length) through the parenchyma and cortex were removed from newly peeled potato tubers (*Solanum tuberosum* var. Maris Bard, Maris Piper or Desirée). These were sliced repeatedly with a steel scalpel and immersed in 2 ml of 0.2 M NaPi buffer (pH 7, 20% D_2O) or 2 ml of a 1:1 mixture of H_2O–D_2O. 100 μl of 250 mM ^{13}C-labelled aq. formaldehyde (prepared as described

by Hunter *et al.* [2] and assayed using the Nash test [17]) were added, giving a concn of about 10 mM formaldehyde. The samples were incubated for various times, at different temps, prior to examination by ^{13}C NMR.

Samples for investigation by deuterium NMR were prepared similarly except that they were immersed in 3 ml of NaPi buffer *without* D_2O and 60 μl of 1.4 M aq. formaldehyde-d_2 (final formaldehyde concn 20 mM).

NMR. The samples were observed as described previously [2] by ^{13}C NMR at 100.6 MHz in a Bruker WH400 spectrometer using deuterium lock. Decoupled spectra were acquired from 1000 transients over about 30 min with gated proton broadband decoupling to avoid heating. Low level decoupling was applied during a relaxation delay of about 2 sec to improve sensitivity via the nuclear Overhauser enhancement; high power decoupling was applied during acquisition. 45° pulses were applied and 8192 data points were acquired with a spectral width of 200 ppm. The spectra were obtained by zero filling once, and applying exponential line broadening or Gaussian resolution enhancement prior to Fourier transformation. Coupled spectra were obtained by omitting the high powered decoupling during acquisition. Chemical shifts are quoted relative to the internal standard $CH_2(OH)_2$ at 83.2 ppm.

Deuterium spectra were obtained at 61.4 MHz on the WH 400 instrument. Potato samples and supernatant in an 8 mm o.d. tube were placed in a concentric 10 mm o.d. tube. The outer tube contained hexafluorobenzene which was used for a ^{19}F lock signal. 100 transients were acquired using 4096 data points across 16 ppm and a two second relaxation delay.

Synthesis of adducts. The cyclic adducts (Fig. 1) were prepared by a method similar to that described by Dewar *et al.* [11]. A 3:1 mixture of amino acid and ^{13}C-labelled formaldehyde was incubated in 0.2 M NaPi buffer at pH 5.5 at 37° for 3–4 days. The pH of the solns was finally adjusted to match that of the potato samples by adding HCl.

Assay of free asparagine. Potato cores were weighed, prepared and incubated with [^{13}C]formaldehyde (10, 20 or 40 mM) as above for periods in the range 2 days–1 week. The final pH was about 4.6. Similar cores were weighed and lyophilized to determine dry wt. Equilibrium solns of asparagine and [^{13}C]formaldehyde were similarly prepared in pH 4.6 NaPi buffer and allowed to stand at 37° for 1 week. Relative concns of species were determined by integrating the ^{13}C spectra obtained as above but processed with no weighting functions.

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