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IN VIVO NMR ANALYSIS OF TROPANE ALKALOID METABOLISM IN TRANSFORMED ROOT AND DE-DIFFERENTIATED CULTURES OF *DATURA STRAMONIUM*

YVES-YANNICK FORD,* R. GEORGE RATCLIFFE and RICHARD J. ROBINS†‡§

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, U.K.; †Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K.

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Key Word Index—*Datura stramonium*; Solanaceae; alkaloid; biosynthesis; NMR (*in vivo*); phytohormones; root culture (transformed); tropanes.

Abstract—*In vivo* ^{15}N NMR spectroscopy has been used to study the metabolism of [^{15}N]tropinone in transformed root cultures of *Datura stramonium*. It was shown that, within only 2–4 hr of application of the labelled precursor, labelled metabolites can readily be detected. It was further demonstrated that de-differentiated cultures, derived from the roots by treatment with phytohormones, are capable of metabolising [^{15}N]tropinone to [^{15}N]tropine and simple [^{15}N]tropine esters but that these cultures cannot convert [^{15}N]tropinone to [^{15}N]hyoscyamine. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Many alkaloids are derived from amino acids by long metabolic pathways involving a series of dedicated enzymes. Although an increasing number of enzymes are being identified from these pathways, their regulation in plants remain poorly understood. In the tropane and tobacco pyrroline alkaloids, derived from arginine (Fig. 1), it has been demonstrated that putrescine *N*-methyltransferase (PMT), the enzyme which acts as the interface between primary and secondary metabolism, plays a critical role in determining the overall capacity of the biosynthetic pathways leading to these products. In transformed root cultures of *Datura stramonium* [1] and *Nicotiana tabacum* [2, 3], conditions that severely diminish – and in the long term eliminate – PMT activity lead to a rapid loss of alkaloid synthetic capacity. This occurs when cultures are treated with a combination of α-naphthaleneacetic acid (NAA) and kinetin (N^6-furfurylaminopurine). The addition of these phytohormones to a root culture leads to the breakdown of roots and the formation of a stable but, in terms of the alkaloids, biosynthetically incompetent de-differentiated suspension culture. Following restoration of phytohormone-free conditions, however, full biosynthetic capacity is restored in parallel with the restitution of the rooty phenotype [1, 4].

Other enzymes of tropane alkaloid formation are also diminished in *D. stramonium* roots treated in this way but their loss from the cultures is not complete [1, 3]. Many of the enzymes of the pathway are not defined, however, so their activities cannot be directly measured. Of particular importance in this regard are the activities responsible for forming the ester between tropine and phenyllactic acid and the rearrangement of the product, littorine, to hyoscyamine, the major component accumulated by these cultures (Fig. 1). Indirect evidence from feeding experiments [5] has suggested that the esterification/rearrangement process plays a key role in determining the rate of hyoscyamine accumulation in the root cultures. It was felt pertinent, therefore, to try to establish the extent to which enzymes of tropane alkaloid biosynthesis remained active under conditions in which the natural supply of the first substrate of the pathway, *N*-methylputrescine, is curtailed. The approach taken was to use *in vivo* ^{15}N NMR, which has proved effective as a means of measuring dynamic metabolic events in tropane alkaloid biosynthesis [6–9]. The intermediate [^{15}N]tropinone was fed to control and to de-differentiated cultures, and the subsequent metabolism of the [^{15}N]-label followed over time. [^{15}N]Tropinone was selected as it was readily synthesized [10] and would directly yield information on the residual ability to form hyoscyamine and other tropane esters.

*Present address: Horticulture Research International, East Malling, West Malling, Kent ME19 6BJ, U.K.

‡Present address: Laboratoire de RMN-RC, URA-CNRS 472, Faculté des Sciences, Université de Nantes, 2 rue de la Houssinière, F-44072 Nantes cedex 03, France.

§Author to whom correspondence should be addressed.

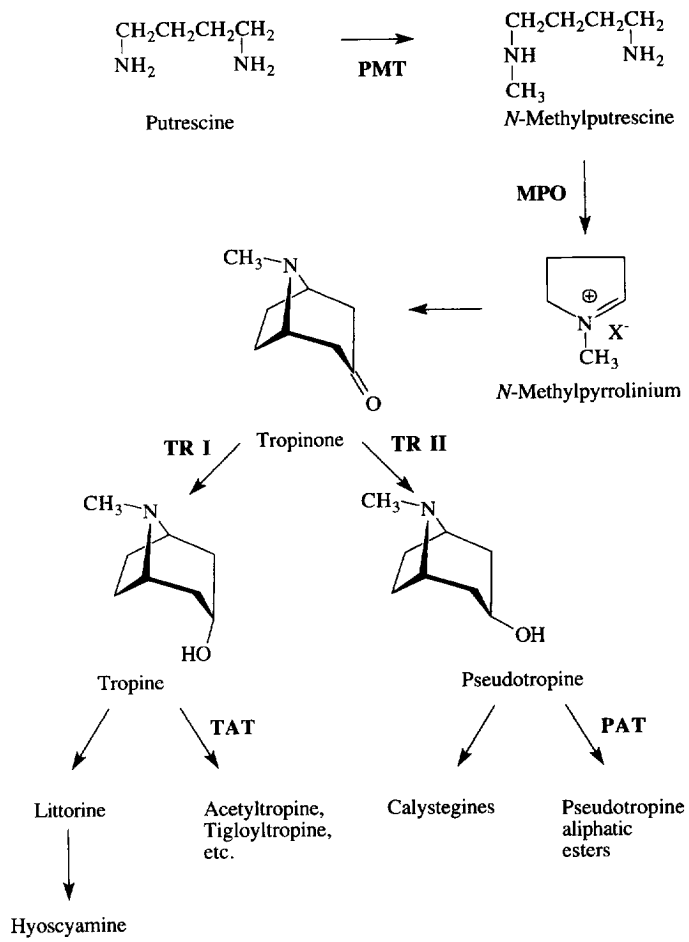

Fig. 1. Schematic pathway of tropane alkaloid biosynthesis in *D. stramonium*.

RESULTS AND DISCUSSION

Metabolism of [^{15}N]tropinone in untreated root cultures

Figure 2 shows the results obtained from a representative experiment (performed in triplicate) in which [^{15}N]tropinone (15 mM) was fed in the NMR tube to transformed root cultures of *D. stramonium* grown for 10 days in B50 medium. [^{15}N]Tropinone gave a broad resonance at about −311 ppm [6, 7] in these conditions (see legend, Figure 2). The most noticeable feature of the *in vivo* spectrum was a rapid production of [^{15}N]tropine (peak 3; −305 ppm) and, to a lesser extent, of [^{15}N]pseudotropine (peak 2; −304 ppm).

Analysis by GC and GC-mass spectrometry of the alkaloids extracted from the roots at the end of the *in vivo* NMR experiment showed tropine to have accumulated in excess of all other alkaloids. Pseudotropine was present at 20% of the level of tropine. This ratio agrees well with the accumulation levels observed *in vivo*. By GC-mass spectrometry, it was determined that the pools of tropinone, tropine and pseudotropine were all 86–90% ^{15}N-labelled. Thus, it was established by direct observation that [^{15}N]tropinone was being rapidly assimilated by the tissue and that both enzymes of tropinone reduction, TR I (tropine-forming) and TR II (pseudotropine-forming) [11] were highly active, supporting the previous *in vitro* measurements of high levels of activity for these enzymes in extracts of *D. stramonium* root cultures [12, 13].

The GC-mass spectrometric analyses also showed that the pools of tropine esters (principally acetyltropine and tigloyltropine) were 83–90% ^{15}N-labelled. Hyoscyamine and littorine were also labelled, although the extent of ^{15}N-labelling was lower (only 10–20%), presumably due to the much larger pools of these alkaloids present in the roots at the beginning of the feeding experiment diluting the labelled product. It is clear, therefore, that the fed [^{15}N]tropinone has been effectively and rapidly incorporated into simple tropine esters and that ^{15}N-labelling of littorine and hyoscyamine has also occurred. [^{15}N]Tropinone incorporation into hyoscyamine could not, however, be observed by *in vivo* ^{15}N NMR in these experiments as hyoscyamine resonates at −305.5 ppm [6, 7] and the limited incorporation is probably masked by the very large [^{15}N]tropine peak.

To observe the formation of tropine esters *in vivo* by ^{15}N NMR spectroscopy, [^{15}N]tropinone was fed at a

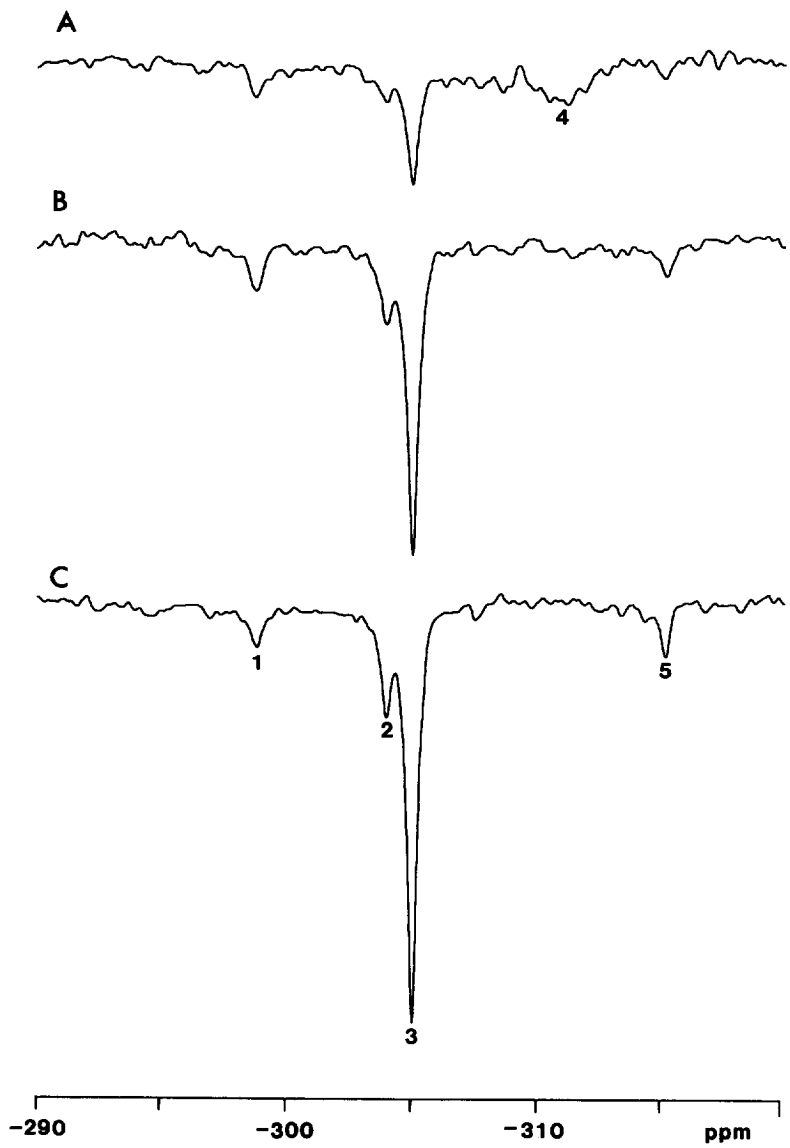

Fig. 2. *In vivo* ^{15}N NMR spectra of transformed *D. stramonium* roots showing the metabolism of [^{15}N]tropinone. The roots were grown for 10 days in B50 medium and were then transferred to a 20-mm NMR tube containing 10 mM MES–0.1 mM $CaSO_4$, pH 6.0. The roots were allowed to stabilize for 3 hr and then a [^{15}N]tropinone solution was added to give a concentration of 15 mM. ^1H-decoupled ^{15}N NMR spectra were recorded over (A) 2–4 hr, (B) 8–10 hr and (C) 14–16 hr exposure to the [^{15}N]tropinone solution. Peak assignments: 1, urea (included in a capillary to generate a chemical shift reference and intensity reference signal); 2, pseudotropine; 3, tropine; 4, tropinone; 5, unassigned. [^{15}N]Tropinone gives a broad signal with a pH-dependent shift under physiological conditions and is difficult to observe. The pH-dependence can be attributed to the low pK_a of tropinone [11] and the peak width presumably arises from exchange broadening. The fresh weight of the roots at the end of the experiment was 2.3 g. The experiment was performed three times.

much lower concentration (2.4 mM) to root cultures grown for 10 days in ^{15}N-enriched B50N medium prior to observation by ^{15}N NMR. In such roots, the major resonance observed in the *in vivo* ^{15}N NMR spectrum (Fig. 3A) is primarily hyoscyamine (peak 3a) with a shoulder due to tropine [6]. The other tropine esters present, notably acetyltropine and tigloyltropine, are not resolved from hyoscyamine. Figures 3B–3D show difference spectra obtained by subtracting the spectrum recorded from the ^{15}N-labelled roots in the absence of added [^{15}N]tropinone (i.e. Fig. 3A) from the spectra obtained during [^{15}N]tropinone metabolism. During the first 8 hr following [^{15}N]tropinone addition, increases in [^{15}N]tropine (peak 3; −305 ppm) and [^{15}N]pseudotropine (peak 2; −304 ppm) are again visible. An accumulation of tropine esters is also observed (peak 3a; −305.3 ppm). After 24 hr, the [^{15}N]tropine esters (peak 3a) are seen to have accumulated to a greater level than [^{15}N]tropine. Thus it is apparent that tropine and a number of simple tropine esters are formed

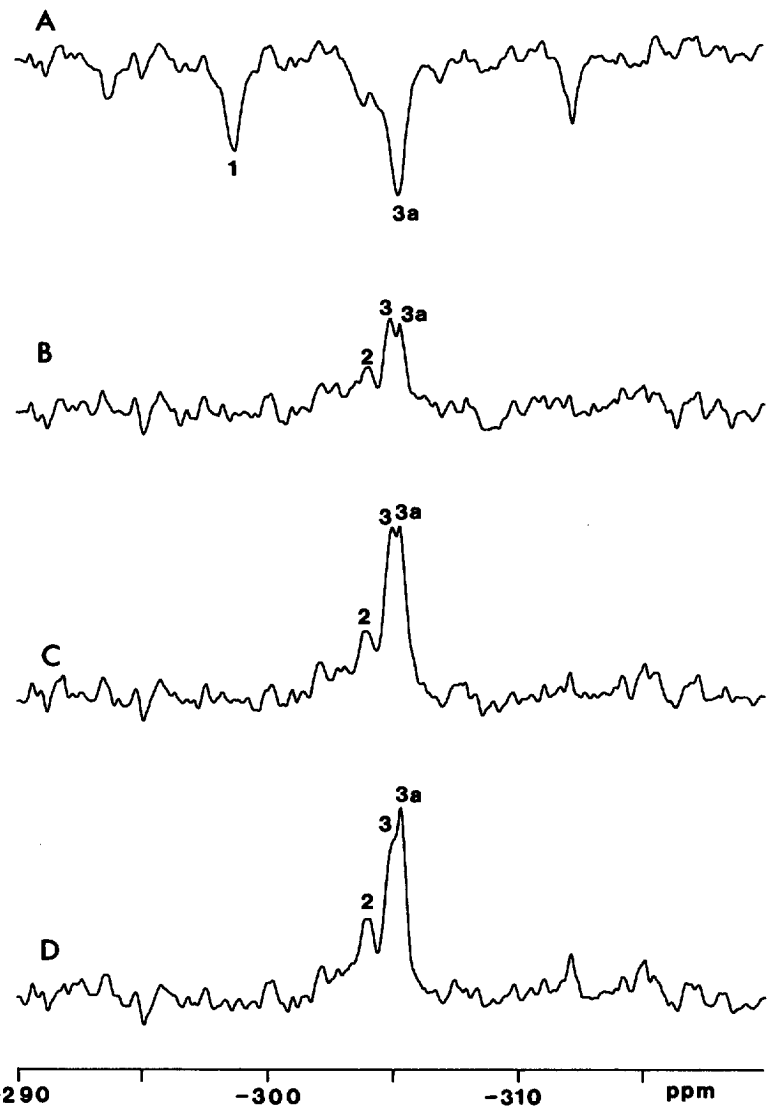

Fig. 3. *In vivo* ^{15}N NMR difference spectra of transformed *D. stramonium* roots showing the metabolism of [^{15}N]tropinone. The roots were grown for 10 days in B50N medium and were then transferred to a 20-mm NMR tube containing 10 mM MES–0.1 mM $CaSO_4$, pH 6.0. A ^1H-decoupled ^{15}N NMR spectrum (A) was recorded for 2 hr and then a [^{15}N]tropinone solution was added to give a concentration of 2.4 mM. Further 2 hr spectra were recorded over the following 24 hr and the spectroscopic changes are shown in the form of difference spectra corresponding to (B) 0–8 hr, (C) 8–16 hr and (D) 16–24 hr exposure to the [^{15}N]tropinone solution. Peak assignments: 1, the urea capillary; 2, pseudotropine; 3, tropine; 3a, tropine esters, including hyoscyamine. Positive signals in the difference spectra correspond to increases in intensity relative to the signals observed in (A). The fresh weight of the roots at the end of the experiment was 2.2 g.

rapidly from [^{15}N]tropinone. The increase in peak 2 (Figs 3B–3D) demonstrates that pseudotropine is also being labelled at a measurable rate.

GC and GC-mass spectrometric analysis of the alkaloids at the end of the experiment indicated that tropine, pseudotropine and tropine esters had accumulated in the roots. The pool of acetyltropine was 2.8 times greater than the pool of un-esterified tropine, confirming the observations made by *in vivo* ^{15}N NMR. Tropine, pseudotropine and the tropine esters were all highly ^{15}N-enriched (87–92% incorporation). The hyoscyamine pool, in contrast, was only *ca.* 79% ^{15}N-enriched. Growth of the roots in ^{15}N-enriched medium typically leads to an enrichment of *ca.* 70% for the alkaloids [6, 7]. Thus, the GC-mass spectrometric data indicate that the rate of incorporation of ^{15}N label into hyoscyamine was lower than into the other esters during the 24-hr period of the experiment. From this, it may be inferred that the majority of the label observed to accumulate in peak 3a of the ^{15}N NMR difference spectra (Figs 3B–3D) represents the formation of acetyltropine, as found previously in tropinone-fed cultures [5, 14], rather than of hyoscyamine. Nevertheless, the level of ^{15}N-enrichment in hyoscyamine has increased, indicating ^{15}N-labelling of hyoscyamine from the fed [^{15}N]tropinone. As no net accumulation of

hyoscyamine is apparent in tropinone-fed roots [5, 14], this might suggest that hyoscyamine is steadily turning over. This suggestion is supported by the kinetics of ^{15}N-labelling previously reported [9].

Metabolism of [^{15}N]tropinone in de-differentiated cultures

The influence of phytohormones on the extent to which these activities remained active during the associated de-differentiation was investigated by feeding [^{15}N]tropinone to plant growth regulator-treated *D. stramonium* root cultures, grown for 10 days in NK5 medium. Figure 4 shows the results obtained from a representative experiment (performed in duplicate). [^{15}N]Tropinone (10 mM) was added as a solution at pH 6.0, and gave a broad peak at *ca.* −306 pm. As with the untreated transformed *D. stramonium* roots, metabolism of [^{15}N]tropinone into [^{15}N]tropine (peak 3; −305 ppm) and [^{15}N]pseudotropine (peak 2; −304 ppm) was observed. Interestingly, there was a greater initial accumulation of [^{15}N]pseudotropine than in the control, untreated *D. stramonium* roots. This observation is compatible with the previous finding that more pseudotropine is accumulated in conditions in which the formation or utilization of tropine is inhibited [14]. GC and GC-mass spectrometric analyses indicated an accumulation of tropine, pseudotropine and acetyltropine, with these alkaloids all being between 89 and 95% ^{15}N-labelled. There was very little hyoscyamine present, as expected for plant growth regulator-treated roots [1], with no observable incorporation of the ^{15}N-label into this alkaloid.

These results show that a residual capacity to metabolize intermediates in the tropane alkaloid biosynthetic pathway (Fig. 1) is retained in de-differentiated cultures of *D. stramonium* transformed roots. Although PMT is, essentially lost from the de-differentiated cultures and the activity of MPO is severely diminished [1], TR I, TR II, acyl-CoA: tropine (TAT) and acyl-CoA: pseudotropine (PAT) acyltransferases are demonstrated to be still active. This finding adds further support to the hypothesis [9] that one of the primary effects of the plant growth factors involves the re-channelling of putrescine away from PMT-catalysed methylation, with the consequent repression of alkaloid production. Whether these activities will persist at a low level in long-term de-differentiated cultures – as does MPO – remains to be established.

The apparent rapid loss of an ability to convert [^{15}N]tropinone to hyoscyamine also merits further attention.

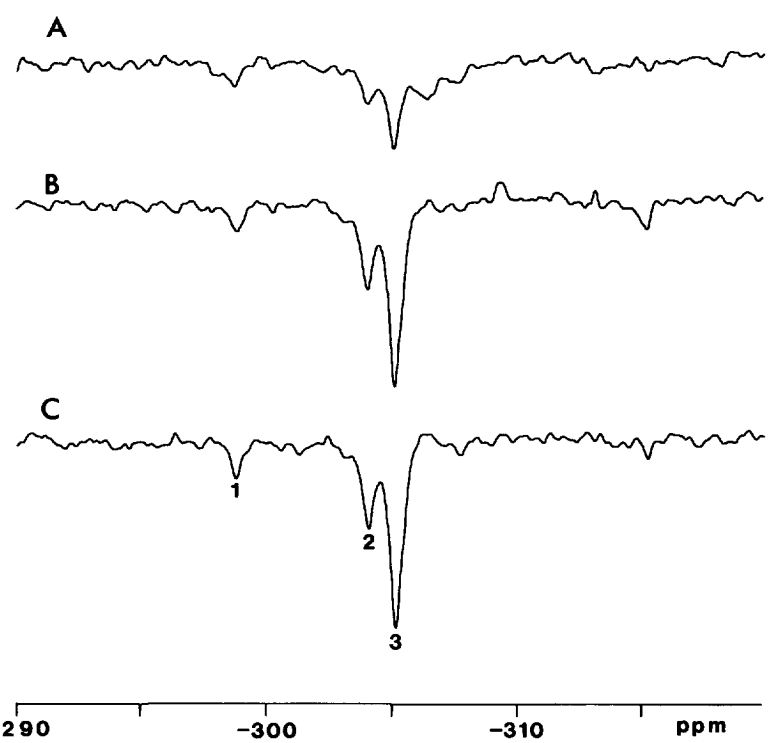

Fig. 4. *In vivo* ^{15}N NMR spectra of de-differentiating transformed *D. stramonium* roots showing the metabolism of [^{15}N]tropinone. The roots were grown for 10 days in NK5 medium and were then transferred to a 20 mm NMR tube containing 10 mM MES–0.1 mM $CaSO_4$ pH 6.0. The roots were allowed to stabilize for 3 hr and then a [^{15}N]tropinone solution was added to give a concentration of 15 mM. ^{1}H-decoupled ^{15}N NMR spectra were recorded over (A) 2–4 hr, (B) 6–8 hr and (D) 14–16 hr exposure to the [^{15}N]tropinone solution. Peak assignments: 1, the urea capillary; 2, pseudotropine; 3, tropine. The fresh weight of the roots at the end of the experiment was 3.0 g. The experiment was performed twice.

EXPERIMENTAL

Plant material and growth conditions. Transformed root cultures of *D. stramonium* line D15/5 were grown in full strength Gamborg's B5 liquid medium plus 30 g l^{-1} sucrose (B50 medium) as described in [5]. For non-specific ^{15}N-labelling of the root metabolites, roots were sub-cultured into a ^{15}N-enriched, low nitrate medium (B50N), consisting of Gamborg's B5 medium except that 1 mM $(^{15}NH_4)_2SO_4$ (98 atom% ^{15}N) and 2.5 mM $K^{15}NO_3$ (98 atom% ^{15}N) were used in place of 1 mM $(NH_4)_2SO_4$ and 25 mM KNO_3 [6, 7]. In experiments to investigate metabolism in the presence of phytohormones, roots were grown for 10 days in NK5 medium, which is B50 medium supplemented with α-NAA at 11 μM (2 mg l^{-1}) final concn and kinetin at 11 μM (0.2 mg l^{-1}) final concn.

Preparation of roots for in vivo *NMR spectroscopy.* Transformed roots were harvested from 3 or 4 flasks, suspended in 15–20 ml of a soln of 10 mM MES (2-(*N*-morpholino)ethanesulphonic acid) and 0.1 mM $CaSO_4$ at pH 6.0 in a 20 mm diameter NMR tube and *in vivo* ^{15}N NMR spectra were recorded at 30.42 MHz using an oxygenated airlift system as described previously [6, 7].

Investigation of tropinone metabolism in D. stramonium *cultures.* Roots, grown for 10 days on B50, B50N or NK5 medium were harvested and suspended in the MES–$CaSO_4$ solution. After a 3 hr equilibration period, a solution of [^{15}N]tropinone in the MES/$CaSO_4$ solution was injected into the NMR tube, the final concn (2.4–15 mM) depending on the particular experiment (see Figure legends). *In vivo* ^{15}N NMR spectra were recorded in 2 hr blocks over a period of up to 24 hr. Under these conditions, antibiotic was not required in the NMR tube.

Extraction and analysis of alkaloids. Alkaloids were extracted and analysed by GC and GC-MS as described previously [14, 15].

Chemicals. $(^{15}NH_4)_2SO_4$ (98 atom% ^{15}N) and $K^{15}NO_3$ (98 atom% ^{15}N) were purchased from the Aldrich Chemical Company (Poole, U.K.). Gamborg's B5 liquid medium salts were obtained from ICN Chemicals (Irvine, Scotland). [^{15}N]Tropinone was prepd [10] using a modified Robinson synthesis by H. D. Boswell (Department of Chemistry, University of Glasgow, Scotland). All other chemicals were obtained from commercial suppliers and were of the highest grade available.

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