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METABOLISM OF [*METHYL*-$^{13}C_2$]HORDENINE IN HOMOGENATES FROM *HORDEUM VULGARE* ROOTS

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Key Word Index—*Hordeum vulgare*, Gramineae, barley, root homogenate, [*methyl*-$^{13}C_2$]hordenine, metabolism, ^{13}C NMR spectroscopy

Abstract—Analysis by ^{13}C NMR spectroscopy of the metabolic degradation of [*methyl*-$^{13}C_2$]hordenine by root homogenates of *Hordeum vulgare* indicated a stepwise loss of the *N*-methyl groups, hordenine being converted in *N*-methyltyramine and probably tyramine

INTRODUCTION

In the last decade, NMR spectroscopy has been developed as a useful method of studying metabolic processes *in vivo* ^{13}C NMR spectroscopy is nowadays the chosen method for viewing the metabolic flux of ^{13}C-enriched substrates in a non-invasive way [1]

In previous reports [2, 3], we proposed that hordenine was degraded by *H vulgare* plants to a C_6–C_1 unit which was incorporated into the lignin, whilst the carbon α to the nitrogen was eliminated as CO_2 As there is no conclusive evidence about the fate of the *N*-methyl groups of hordenine during its metabolism in barley [4, 5], we attempted to study this degradation by following the catabolism of ^{13}C-labelled hordenine in root homogenates by means of ^{13}C NMR spectroscopy

RESULTS AND DISCUSSION

By following a known procedure [6], we prepared [*methyl*-$^{13}C_2$]hordenine by reductive methylation of tyramine using [^{13}C]formaldehyde Mass spectrometric analysis of the labelled hordenine indicated a nearly statistic isotopic distribution in the *N*-methyl groups This compound was incubated with a buffered homogenate of 15-day-old barley roots in a NMR tube The homogenates were prepared as indicated in the Experimental, their combined enzymic activity was controlled in separate experiments by their capacity to degrade [α-^{14}C]hordenine to CO_2 (Table 1) Although concentration of the homogenate diminished the overall enzymic activity, a 20-fold concentrated homogenate had to be used in order to perform the incubation in a NMR tube As shown in Table 1, the decrease of activity produced by concentration cannot be attributed to an irreversible loss of co-factors or of enzyme activity as the regenerated homogenate maintained the original capacity to liberate CO_2 from [α-^{14}C]hordenine Thus, approximately 83% of the α-carbon of the supplied hordenine was degraded to CO_2 after 14 hr both in the original and regenerated homogenates On the other hand, in a 10-fold concentrated homogenate only 43% of this carbon was transformed to CO_2 in the same time In the 20-fold concentrated homogenate used for the NMR experiment this loss of activity was enhanced and though the degradation of hordenine took a longer time it could be readily measured (Fig 1)

Proton-decoupled ^{13}C NMR spectra were registered at different intervals detecting at the beginning of the experiment a single signal at δ43 73 which corresponded to the –N– $(^{13}CH_3)_2$ groups This signal decreased with time with the concomitant appearance and increase of a second signal at δ35 62 assigned to a –NH–$^{13}CH_3$ group Typical spectra are shown in Fig 2 After 48 hr the hordenine signal vanished, indicating the complete removal of one *N*-methyl group (Fig 1)

Table 1 Degradation of [α-^{14}C]hordenine to $^{14}CO_2$ by homogenates of *H vulgare* roots at 30°

Incubation medium	Amount and activity of [α-^{14}C]hordenine (μg/ml)	(× 10^5 dpm/ml)	Remaining act after 14 hr* (× 10^5 dpm/ml)	Amount of $^{14}CO_2$ evolved after 14 hr† (μmol/ml)
Buffer	58	4 2	4 2	0
Original homogenate	58	4 2	0 73	0 29
10 × conc homogenate	495	35 0	20	1 3
Regenerated homogenate	49	3 5	0 59	0 25

*Corresponds to all α-carbon-containing metabolites present
†Calculated values
Data are average from three experiments

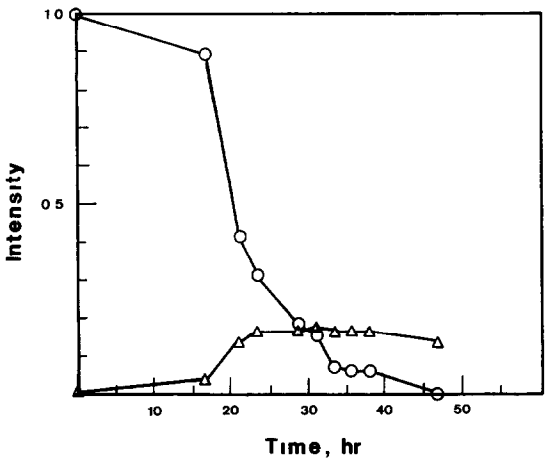

Fig 1 Intensity of N–$^{13}CH_3$ resonances of *N*-methyltyramine (△) and hordenine (○) as a function of time

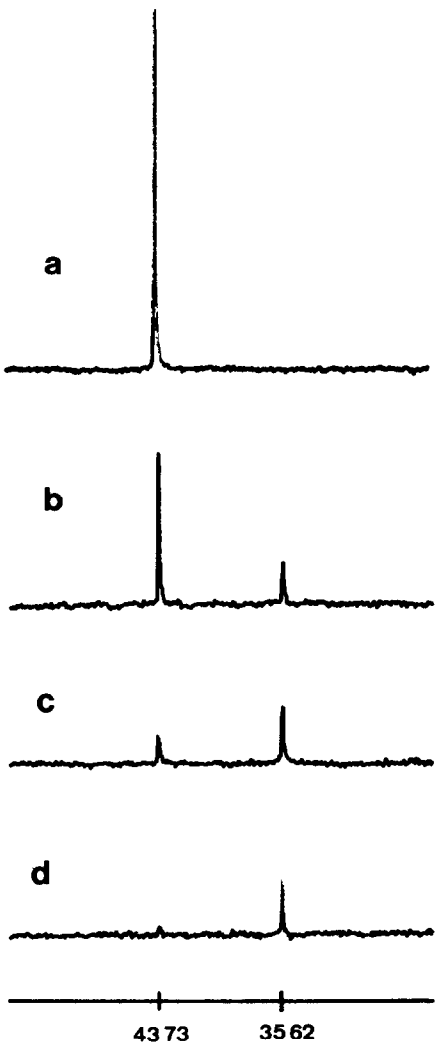

Fig 2 ^{13}C NMR spectra of incubation of [^{13}C-*methyl*]hordenine in *H vulgare* root homogenates (a) 0 hr, (b) 21 17 hr, (c) 33 46 hr, (d) 46 83 hr

These results point to a stepwise degradation of hordenine by conversion in *N*-methyltyramine and probably tyramine, as proposed previously by several authors [4, 5]

The lack of any other signal at the end of the experiment indicates either a dispersion of the labelled methyl groups among several products or its elimination as CO_2, whose resonance signal is very difficult to observe because of its long T_1 value Also, the possibility of the incorporation of the labelled methyls into high molecular weight compounds (e g proteins) cannot be dismissed, as these would not be observable under the present experimental conditions due to their extremely short T_2 values Work is in progress to elucidate the final fate of the *N*-methyl groups

EXPERIMENTAL

General ^1H NMR 100 1 MHz, ^{13}C NMR 25 2 MHz under total proton-decoupled conditions in a Varian XL-100-15 NMR spectrometer operating in the FT mode with a 620/L-100 computer interfaced to a Sykes 7000 dual disk drive unit Each ^{13}C NMR spectrum was the result of 10 000 90° pulses over a spectral width of 5120 Hz using a repetition rate of 0 8 sec D_2O (5%) was included in the incubation for internal lock Probe temp was *ca* 28° Chemical shifts are given in ppm downfield from TMS Radioactivity measurements were carried out as previously described [7] [^{13}C]Formaldehyde was purchased from Merck, Sharp & Dohme, Canada

Plant material Seeds of *H vulgare* (Magnif 102, INTA 78/79) were provided by INTA, Castelar They were sterilized by immersion in 1% $Ca(ClO)_2$ soln for 30 min and germinated in plastic trays over sand in a growth chamber as previously described [7]

Homogenates Roots from 200 15-day-old seedlings were blended in 0 1 M NaPi buffer (pH 7 2, 200 ml) for 1 hr at 5° The suspension was exposed to ultrasound (1 sec bursts) for 15 min at 5° and then centrifuged at 15 000 *g* for 2 hr The supernatant was concd to either 10 or 20 ml by ultrafiltration through a YM-10 Diaflo membrane (Ultrafiltration cell model 202 from Amicon)

Synthesis of [methyl-$^{13}C_2$]*hordenine* A soln of tyramine hydrochloride (107 mg) in MeOH (10 ml) was treated with 10% Pd/C (10 mg) and $H_2{}^{13}CO$ (90% ^{13}C, 14 6% aq soln, 0 5 ml) The mixture was hydrogenated at room temp and atmospheric pres for 16 hr The catalyst was filtered off, the filtrate was made basic by addition of NH_4OH (2 ml) and evapd to dryness The resulting solid was dissolved in MeOH–HCl (95 5, 10 ml) and left standing for 12 hr for complete hydrolysis of residual polymeric formaldehyde It was then treated with NH_4OH soln and evapd Sublimation (0 001 torr, 110°) of the residue afforded pure (IR, ^1H and ^{13}C NMR) hordenine (100 mg, 98%) MS (70 eV) *m/z* (rel int) 167 $[M+2]^+$ (5), 121 (7), 107 (15), 91 (10), 77 (18), 60 $[^{13}C_2{}^{12}CH_8N]^+$ (100), 59 $[^{13}C^{12}C_2H_8N]^+$ (20), 58 $[^{12}C_3H_8N]^+$ (2) Isotopic distribution calcd from ions at *m/z* 60, 59 and 58 gave [*methyl*-$^{13}C_2$]hordenine 82 0%, [*methyl*-$^{13}C_1$]hordenine 16 4% and unlabelled hordenine 1 6% ^1H NMR (D_2O–DSS) δ 2 70–3 50 (*m*, A_2B_2 zone from a $A_2B_2X_2$ system, $C\underline{H}_2$–$C\underline{H}_2$–$N(^{13}CH_3)_2$), 2 90 (*d*, $^3J_{C,H}$ = 4 Hz, $^{12}C\underline{H}_3$–N–$^{13}CH_3$), 2 90 (*dd*, $^1J_{C,H}$ = 143 Hz, $^{12}CH_3$–N–$^{13}C\underline{H}_3$), 2 90 (*dd*, $^1J_{C,H}$ = 143 Hz, $^3J_{C,H}$ = 4 Hz, $N(^{13}C\underline{H}_3)_2$), 6 85 and 7 18 (*dd*, J_{AB} = 8 Hz, $C_6\underline{H}_4$), ^{13}C NMR (D_2O–DSS) δ 30 23 ($PhC\underline{H}_2CH_2$), 43 73 ($N(^{13}\underline{C}H_3)_2$), 59 54 ($\underline{C}H_2CH_2N$), 116 70 (C-2 and C-6), 128 46 (C-4), 130 90 (C-3 and C-5), 155 59 (C-1) Neither $^2J_{C,C}$ nor $^3J_{C,C}$ was observed (< Hz)

Incubation assays [*Methyl*-$^{13}C_2$]Hordenine (19 3 mg) was dissolved in D_2O (1 ml) and aliquots (200 μl) of this soln were

added to a 20-fold conc. homogenate (4 ml) contained in a NMR tube. ^{13}C NMR spectra were recorded under the conditions and at the times indicated above. [α-^{14}C]Hordenine (0.99 mg, 1.2 $\times 10^9$ dpm/mmol) was added to the incubation mixtures as indicated in Table 1. After 14 hr in a shaking water bath at 30°, samples were taken for the determination of the remaining radioactivity by liquid scintillation counting.

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