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# THE OCCURRENCE OF THE 'NIH-SHIFT' DURING THE FORMATION OF *N*-METHYLTYRAMINE FROM PHENYLALANINE IN BARLEY

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**Abstract**—The administration of DL-phenylalanine-4-$^{3}H$,$\beta$-$^{14}C$ to germinating barley (*Hordeum distichum*) led to the formation of *N*-methyltyramine which had retained 88% of the tritium relative to the carbon-14. A systematic degradation established that all the tritium was located *ortho* to the hydroxyl group, indicating that the 'NIH-shift' operates during the formation of this alkaloid. The barley seeds were sterilized with ethylene oxide and it was found that the major alkaloid obtained from the roots of the germinated barley was *N*-methyltyramine, in contrast to normal plants of this variety of barley which produce hordenine and *N*-methyltyramine in a ratio of 3:1.

THE HYDROXYLATION of aromatic compounds has been extensively studied by scientists at the National Institutes of Health, Bethesda, U.S.A.,[1] and they have shown that the hydroxylation frequently involves the intramolecular migration of the group or atom being displaced to an adjacent *ortho* position on the aromatic ring. This phenomenon has been given the name 'NIH-shift'. One of the earliest examples to be recognized was the conversion of phenylalanine to tyrosine in bacteria and mammalian systems.[2]

This hydroxylation of phenylalanine to tyrosine does not seem to be the major route by which tyrosine is formed in higher plants. Tracer experiments have indicated that there is little, if any, conversion of phenylalanine to tyrosine in *Narcissus incomparabilis*,[3] *Colchicum*,[4,5] or *Erythrina berterona*.[6] In other species (*Salvia splendens*,[7] *Triticum vulgare*,[8] and *Fagopyrum tartaricum*[8]) L-phenylalanine-$\beta$-$^{14}C$ was converted to radioactive tyrosine; however, the results were consistent with the formation of tyrosine via phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid. More recently, a hydroxylase has been isolated from spinach leaves (*Spinacea oleracea*) which catalyses the direct conversion of phenylalanine to tyrosine.[9] Studies on the biosynthesis of certain alkaloids using labelled phenylalanine as a precursor also have indicated that hydroxylation of phenylalanine is possible in peyote (*Lophophora williamsii*)[10] and barley (*Hordeum distichum*).[11] In the latter species it was

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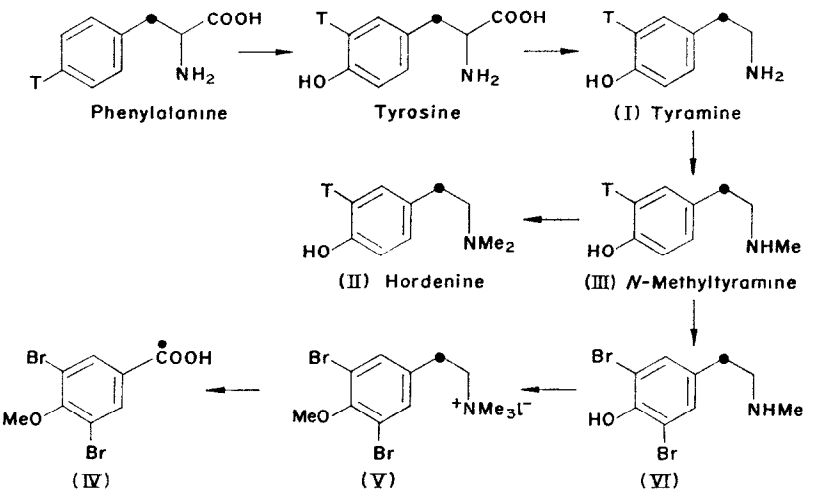


FIG. 1. BIOSYNTHESIS AND DEGRADATION OF THE LABELLED *N*-METHYLTYRAMINE DERIVED FROM PHENYLALANINE-4-$^3$H,$\beta$-$^{14}$C.

established that phenylalanine was a precursor of hordenine (2). It had been previously shown that tyrosine[12] and tyramine (1)[13] were precursors of this alkaloid, *N*-methyltyramine (3) being the immediate precursor, as illustrated in Fig. 1. Thus this species seemed to be a suitable one in which to investigate whether the 'NIH-shift' operates in higher plants. Whilst this work was in progress there have been several reports that the 'NIH-shift' does indeed function as a biosynthetic process in higher plants. It occurs during the formation of *p*-coumaric acid from cinnamic acid in *Catalpa hybrida*.[14] The same transformation is also involved in the biosynthesis of flavonoids and the retention of tritium *ortho* to its initial position in cinnamic acid-4-$^3$H was established in several compounds.[15,16] The alkaloids capsaicin and norpluviine, derived in part from cinnamic acid were also formed by hydroxylations involving an NIH-shift.[17]

In our work we prepared phenylalanine-4-$^3$H by the reduction of the methyl ester of 4-chlorophenylalanine with tritium in the presence of palladium on barium carbonate, followed by hydrolysis of the resultant methyl ester of phenylalanine. A systematic degradation established that essentially all the tritium was located at the 4-position (see Experimental). This tritium labelled phenylalanine was mixed with phenylalanine-$\beta$-$^{14}$C and fed to germinating barley. Since we experienced considerable difficulty in germinating the barley free of mold using previously described methods,[12] we decided to fumigate the seeds with ethylene oxide. The seeds were then grown inside a plastic bag in glass trays. In this way healthy mold-free plants were obtained. However, the composition of the alkaloids isolated from the roots of the seedlings derived from the ethylene oxide-treated seeds was significantly different from that obtained from untreated barley. The amount of *N*-methyltyramine was much higher than hordenine in the plants derived from the ethylene oxide-treated seeds,

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TABLE 1

| | Specific Activity (dis/min/m-mole) $^{3}H$ | $^{14}C$ | $^{3}H/^{14}C$ |
|---|---|---|---|
| DL-phenylalanine-4-$^{3}H$,$\beta$-$^{14}C$ | $1{\cdot}96 \times 10^{10}$ | $2{\cdot}09 \times 10^{9}$ | 9·35 |
| *N*-Methyltyramine | $1{\cdot}88 \times 10^{7}$ | $2{\cdot}28 \times 10^{6}$ | 8·25 |
| 3,5-Dibromo-*N*-methyltyramine HBr | $0{\cdot}06 \times 10^{7}$ | $2{\cdot}38 \times 10^{6}$ | 0·25 |
| *O*-Methyl-3,5-dibromohordenine methiodide | | $2{\cdot}16 \times 10^{6}$ | |
| 3,5-Dibromoanisic acid | | $2{\cdot}24 \times 10^{6}$ | |
| 3,5-Dibromo-4-methoxyaniline | | 0 | |
| Barium carbonate | | $2{\cdot}18 \times 10^{6}$ | |

Specific incorporation of $^{3}H$ into *N*-methyltyramine = 0·096%. Specific incorporation of $^{14}C$ into *N*-methyltyramine = 0·109%. Retention of $^{3}H$ = 0·096/0·109 = 88%.

whilst in the normal plants there was an excess of hordenine. The usual ratio of hordenine to *N*-methyltyramine in the variety of barley (Charlottetown No. 80) we have used in all our experiments is about 3:1.[12] It may be that the ethylene oxide treatment destroys or inhibits the enzyme responsible for the methylation of *N*-methyltyramine to hordenine. Mann *et al.*[18] have shown that there seem to be two distinct methylating enzymes involved in the methylation of tyramine to hordenine. There is considerable evidence[19,20] that the alkaloid content and composition can be affected by external conditions of growth, although there has been little systematic study of this interesting phenomenon.

In the isolation of the *N*-methyltyramine, care was taken to avoid excessively basic or acidic conditions to avoid loss of tritium from the position *ortho* to the hydroxyl group, by exchange with water. The purified *N*-methyltyramine was found to contain both tritium and $^{14}C$, and the retention of tritium relative to the $^{14}C$ was 88% (see Table 1). All the tritium was shown to be present at a position *ortho* to the hydroxyl group, and all the $^{14}C$ at the $\beta$-position of the side chain, by the degradation illustrated in Fig. 1. Bromination of the *N*-methyltyramine yielded 3,5-dibromo-*N*-methyltyramine (VI), which was methylated to afford *O*-methyl-3,5-dibromohordenine methiodide (V). Oxidation of this methiodide with permanganate yielded 3,5-dibromoanisic acid (IV) which afforded carbon dioxide and 3,5-dibromo-4-methoxyaniline on subjecting to a Schmidt reaction.

We have thus established that the 'NIH-shift' operates during the conversion of phenylalanine to *N*-methyltyramine, an alkaloid formed in germinating barley.

## EXPERIMENTAL

### *General Methods*

Radioactivity was measured in a Nuclear Chicago liquid scintillation system, Model 724, using as solvents wither dioxane or toluene, with the usual scintillators.[21] The mass spectra were determined by Adrian Swanson and his associates at the University of Minnesota, using an Hitachi–Perkin–Elmer RMU-6D mass spectrometer. Microanalyses were determined by the Clark Microanalytical laboratories, Urbana, Illinois.

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DL-*Phenylalanine-4-*$^3$H

Hydrogen and tritium (100 mCi) were admitted to a flask containing a magnetically stirred mixture of the methyl ester of *p*-chlorophenylalanine (165 mg), KOAc (100 mg) and 10% Pd on $BaCO_3$ (25 mg) in dry MeOH (5 ml) at room temp. and atm pressure. After 30 min the absorption of hydrogen ceased. The solution was then allowed to evaporate in a fume hood, the residue suspended in methanol (25 ml), and then filtered and evaporated. The residue was stirred in 2 N $Na_2CO_3$ (10 ml) at room temp. for 5 hr. This solution was added to a column of Dowex 50 (H+ form) after adjusting its pH to 2·5 by the addition of HCl. The column was washed with $H_2O$ until free of $Cl^-$, and the phenylalanine was eluted with N $NH_3$. Evaporation of the fractions containing phenylalanine afforded material (131 mg) having an activity of $6{\cdot}8 \times 10^{10}$ dis/min/mmole. Dilution with non-radioactive DL-phenylalanine and crystallization from water afforded DL-phenylalanine-4-$^3$H having an activity of $2{\cdot}59 \times 10^{10}$ dis/min/mmole.

*Degradation of the* DL-*phenylalanine-4-*$^3$H *to Establish its Radiochemical Purity*

Some of the tritium labelled phenylalanine was diluted to afford material having an activity of $1{\cdot}63 \times 10^8$ dis/min/mmole. The diluted phenylalanine (500 mg), $Na_2CO_3$ (500 mg) and $KMnO_4$ (2·0 g) were refluxed in $H_2O$ (20 ml) for 4 hr. $SO_2$ was then passed into the reaction mixture, the solution acidified with dilute HCl, and extracted with ether. Evaporation of the dried ($MgSO_4$) extract afforded a residue which was crystallized from water yielding colorless plates of benzoic acid (330 mg, 90%), m.p. 120–121° ($1{\cdot}59 \times 10^8$ dis/min/mmole). The benzoic acid (295 mg) was dissolved in a mixture of $CHCl_3$ (8 ml) and conc. $H_2SO_4$ (4 ml) and stirred with $NaN_3$ (275 mg) at 40° for 2 hr. The mixture was then diluted with ice (15 g), made basic with $Na_2CO_3$, and extracted with ether. Evaporation of the dried ($MgSO_4$) extract yielded crude aniline which was dissolved in HOAc (0·3 ml) and $Ac_2O$ (0·3 ml) and refluxed for 30 min in the presence of a trace of Zn dust. The reaction mixture was cooled, diluted with $H_2O$ and the resultant solid crystallized from water yielding colorless plates of acetanilide (268 mg), m.p 114° ($1{\cdot}66 \times 10^8$ dis/min/mmole). The acetanilide (135 mg) was dissolved in HOAc (0·5 ml) and treated with a slight excess of $Br_2$ (165 mg) in HOAc (0·25 ml). After 30 min at room temp *p*-bromoacetanilide was precipitated by the addition of $H_2O$ (containing a little $NaHSO_3$ to remove excess $Br_2$) and then crystallized from aq MeOH affording colorless prisms, (161 mg, 76%), m p 167–168° ($< 0{\cdot}02 \times 10^8$ dis/min/mmole).

*Fumigation and Germination of Barley Seeds*

Barley seeds (250 g) (*Hordeum distichum*, Charlottetown No. 80) were treated in an ethylene oxide sterilizer at 50° and 50–60% r.h. for 4 hr. The gas mixture (Linde Chemical Co.) was 11% ethylene oxide and 88% Freon 11 and 12. The concentration of ethylene oxide was 450 mg/l. The seeds were then soaked in aerated $H_2O$ for 24 hr. The swollen seeds were then transferred to three 18 × 22 × 1·5 in. Pyrex glass trays, lined with cotton gauze. Aqueous $CaCl_2$ (0·005 M, 100 ml) was added to each tray, and the trays individually enclosed in polyethylene plastic bags, closed by means of a wire twist. $CaCl_2$ solution (0·005 M, 100 ml) was added daily to each tray until day 5 after germination

*Administration of* DL-*Phenylalanine-4-*$^3$H,$\beta$-$^{14}$C *to the Germinating Barley and Isolation of the* N-*Methyltyramine*

On day 6 after germination the seedlings were removed from the trays. A healthy root mat had developed and there was no visible sign of microbial contamination. Ungerminated seeds (less than 10%) were shaken loose and the root mats were washed by dipping them three times in distilled water. A mixture of DL-phenylalanine-4-$^3$H (18 6 mg, $2{\cdot}92 \times 10^9$ dis/min) and DL-phenylalanine-$\beta$-$^{14}$C (6·0 mg, $3{\cdot}12 \times 10^8$ dis/min, New England Nuclear Corp.) was dissolved in $H_2O$ (450 ml) and divided equally between the three trays which were then re-enclosed in plastic bags. The plants were watered daily and harvested on day 12 after germination. The leaves were cut off and discarded. The roots (fr. wt. 389 g) were mascerated with 95% EtOH in a Sorvall Omnimixer The mixture was filtered and the residual marc further extracted with MeOH in a Soxhlet extractor for 3 days. The combined alcoholic extracts were filtered and evaporated to dryness *in vacuo*. TLC was carried out on a small portion of the residue on $Al_2O_3$ G (Merck), developing with 95% EtOH. Development of the plate with Millon's reagent indicated the presence of two phenolic compounds having $R_f$s 0 30 and 0 62, corresponding to *N*-methyltyramine and hordenine respectively, the former being the major component. The crude alcoholic extract of the roots was then subjected to preparative TLC on $Al_2O_3$ PF-254, developing with 95% EtOH. The zone corresponding the *N*-methyltyramine was extracted with methanol affording *N*-methyltyramine (114 mg) purified by sublimation (105°, 2 mm) and crystallization from anisole. The *N*-methyltyramine was diluted prior to degradation, however the activities reported in Table 1 are for carrier free material.

*Degradation of the Radioactive* N-*Methyltyramine* 3,5-*Dibromo-*N-*methyltyramine*

$Br_2$ (320 mg, 2 mmole) dissolved in HOAc (10 ml) was added with stirring to a solution of *N*-methyltyramine (151 mg, 1 mmole) and NaOAc (168 mg, 2 mmole) in HOAc (5 ml). After 1 hr the precipitated solid was filtered off, washed with HOAc and ether and then dried over KOH in vacuum. The resultant 3,5-dibromo-*N*-methyltyramine hydrobromide (371 mg, 95%) had m.p 231–235° dec.

3,5-Dibromo-*N*-methyltyramine was obtained as colorless needles, m.p. 254–256°, mass spectrum *m/e* 307, on addition of ammonia to a solution of the hydrobromide salt in water. The picrate was obtained as yellow needles from EtOH, m.p. 225–226°. *Anal.* Calc. for $C_{15}H_{14}Br_2N_4O_8$: C, 33·48; H, 2·62; N, 10·41; Br, 29·70. Found: C, 33·62, H, 2·73; N, 10·43; Br, 29·26%.

*O-Methyl-3,5-dibromohordenine Methiodide*

3,5-Dibromo-*N*-methyltyramine (247 mg, 0·80 mmole) and NaOMe (92 mg, 1·7 mmole) were refluxed in MeOH (10 ml) with MeI (1 ml) for 3 hr. The reaction mixture was filtered hot and the filtrate evaporated to about 5 ml. On cooling, *O*-methyl-3,5-dibromohordenine methiodide separated as colorless needles (294 mg, 77%) m.p. 229–231° dec. *Anal.* Calc. for $C_{12}H_{18}Br_2INO$: C, 30·08; H, 3·78; N, 2·92; Br, 33·36; I, 26·50. Found: C, 30·15; H, 3·72; N, 2·83; Br, 34·25; I, 26·58%.

*3,5-Dibromoanisic Acid*

*O*-Methyl-3,5-dibromohordenine methiodide (170 mg), $KMnO_4$ (1 g) and NaOH (1·5 g) were refluxed in $H_2O$ (25 ml) for 18 hr. The reaction mixture was acidified with dilute $H_2SO_4$ and decolorized by the addition of sodium sulfite. The precipitated 3,5-dibromoanisic acid was collected and crystallized from ethanol which afforded colorless needles, (45 mg, 40%) m.p. 214–215° (lit.[22] 214°).

*Schmidt Reaction on 3,5-Dibromoanisic acid*

3,5-Dibromoanisic acid (155 mg) was dissolved in conc. $H_2SO_4$ (0·5 ml), cooled to 0°, and treated with $NaN_3$ (51 mg). A stream of $N_2$ was passed through the reaction mixture which was heated to 60–70° during 1 hr. The $N_2$ stream was washed with a 5% $KMnO_4$ in 2 N $H_2SO_4$, and then passed into a saturated solution of $Ba(OH)_2$ resulting in the formation of $BaCO_3$ (59 mg, 60%). The contents of the reaction flask were added to ice and neutralized with NaOH. The tan-colored solid was filtered off, dried in air, and crystallized from petroleum ether, affording colorless leaflets of 3,5-dibromo-4-methoxyaniline (67 mg, 48%), m.p. 62–63°, lit.[23] 64–65°).

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