The Role of Hygrine in the Biosynthesis of Cuscohygrine and Hyoscyamine

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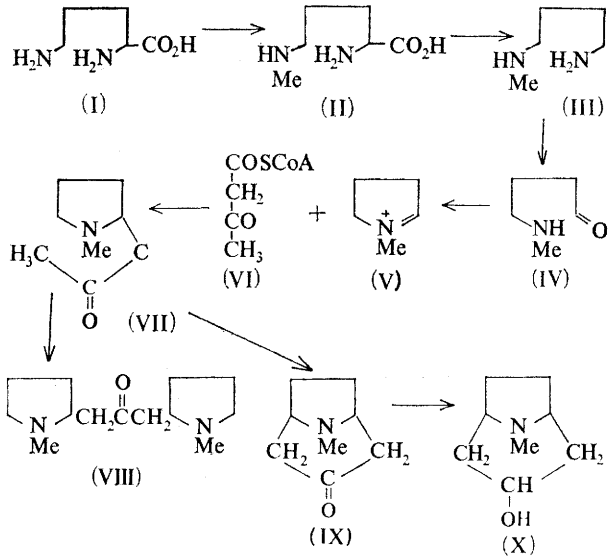
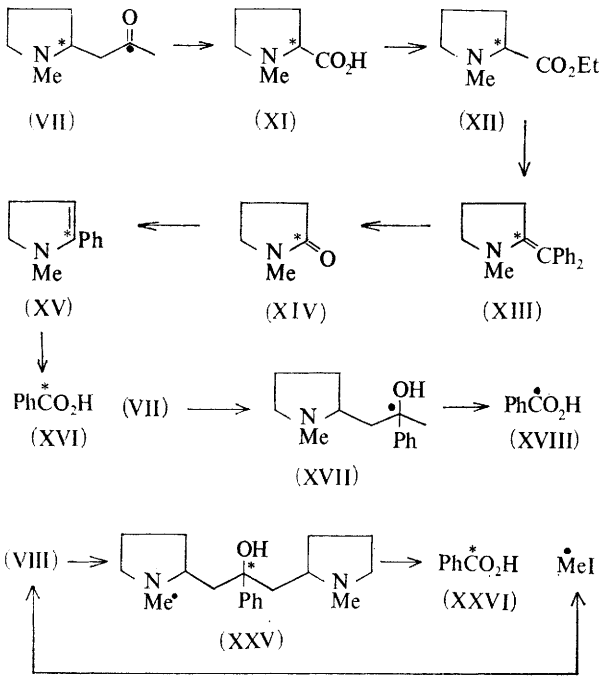
Hygrine has been shown to be derived from ornithine and acetate in accordance with accepted biogenetic theory. Hygrine has been shown to be the immediate precursor of cuscohygrine in *Scopolia lurida* and also of hyoscyamine in *Datura stramonium*.

It has been suggested [1] that the biosynthesis of hygrine and cuscohygrine is closely related to that of the tropane alkaloids and cuscohygrine has been reported in several *Datura* species [2] which produce the tropane alkaloids. A recent ontogenetic study by Verzár-Petri [3] of six *Datura* species points to a common biogenetic precursor for cuscohygrine and hyoscyamine and which we now suggest is hygrine. The non-occurrence of hygrine in the species studied may be due to a rapid turnover of this alkaloid in the plant.

Tracer studies have shown [4] that [2-^{14}C]ornithine was incorporated unsymmetrically into the tropine moiety of hyoscyamine, activity appearing only at C-1. More recent work has suggested that α-*N*-methylornithine is incorporated into hyoscyamine in the same manner as ornithine itself.[5] However Leete [6] has questioned, on the basis of double labelling experiments, similar work on the incorporation of α-*N*-methylornithine into the pyrrolidine ring of nicotine.[7] Liebisch and Schütte [8] have reported that the heterocyclic nitrogen of tropane is derived mainly from the δ-amino-group of ornithine.



The first Scheme outlines a biosynthetic pathway for hygrine, cuscohygrine, and hyoscyamine which is compatible with the studies outlined above. The ornithine (I) is methylated to δ-*N*-methylornithine (II) which on decarboxylation yields *N*-methylputrescine (III). Oxidation of the primary amino-group yields 4-methylaminobutanal (IV) which cyclises to an *N*-methyl-1-pyrrolinium salt (V). Leete [9] has shown that this intermediate does not lead to randomization of activity in the *in vitro* synthesis of hygrine and in the biosynthesis of nicotine. Condensation of the pyrrolinium salt with acetoacetyl coenzyme A (VI) yields hygrine (VII). Condensation of the hygrine (VII) with another molecule of the pyrrolidinium salt (V) yields cuscohygrine (VIII) while cyclization yields tropinone which leads by unexceptional steps through tropine (X) to hyoscyamine.



In order to test this hypothesis we initially investigated the biosynthesis of hygrine. We administered DL-[2-^{14}C]ornithine (total activity $2{\cdot}2 \times 10^8$ counts/min.) and [1-^{14}C]sodium acetate (total activity $2{\cdot}2 \times 10^8$ counts/min.) in separate experiments to six *Nicandra physaloides* plants by a wick arrangement. The plants from the ornithine feed were harvested after 16 days, and after addition of inactive hygrine as carrier (100 mg.) the active hygrine was isolated by established methods.[10] The hygrine was purified as picrate to

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[2] P. van Haga Reinouts, *Nature*, 1953, **174**, 833; M. Wellendorf, *Pharm. Tid.*, 1960, **70**, 433.
[3] G. Verzar-Petri, *Acta Biol. Hung.*, 1965, **16**, 141.
[4] E. Leete, *J. Amer. Chem. Soc.*, 1962, **84**, 55; A. A. Bothner-By, R. S. Schutz, R. F. Dawson, and M. L. Solt, *ibid.*, p. 52.
[5] H. B. Schroter and D. Neumann, *Tetrahedron Letters*, 1966 1279.
[6] T. J. Gilbertson and E. Leete, *J. Amer. Chem. Soc.*, 1967, **89**, 7085.
[7] D. Neumann and H. B. Schroter, *Tetrahedron Letters*, 1966, 1273.
[8] H. W. Liebisch and H. R. Schütte, *Z. Pflanzenphysiol.*, 1967, **57**, 434.
[9] E. Leete, *J. Amer. Chem. Soc.*, 1967, **89**, 7081.
[10] A. Romeike, *Pharmazie*, 1965, **20**, 738.

constant activity and had a specific activity of $3{\cdot}14 \times 10^5$ counts/min./mmole, percentage incorporation 0·098%.

The hygrine was regenerated from its picrate by the method of Bobbitt.[11] On oxidation of the hygrine (VII) with chromium trioxide–sulphuric acid [12] mixture, hygrinic acid (XI) was obtained. The ethyl ester (XII) of hygrinic acid was treated with phenylmagnesium bromide [12] yielding a carbinol which was dehydrated to the ethylenic compound (XIII). Treatment of (XIII) with osmium tetroxide and potassium metaperiodate yielded *N*-methylpyrrolidone (XIV), which reacted with an excess of phenylmagnesium bromide to give *N*-methyl-2-phenyl-2-pyrroline (XV).[12] Oxidation of (XV) with aqueous permanganate yielded benzoic acid (XVI). The concordance between the specific activities of hygrine and its degradation products indicates that the hygrine was labelled only on the starred carbon in (VII).

TABLE 1

Activity of hygrine and its degradation products (counts/min./mmole) × 10^{-5}

(a) Ornithine feed	
Hygrine	3·14
Hygrinic acid	3·12
N-Methyl-2-phenylpyrroline	3·13
Benzoic acid	3·10
(b) Acetate feed	
Hygrine	3·56
2′-Phenyldihydrohygrine	3·54
Benzoic acid	3·48

TABLE 2

Activity of the labelled hygrines (counts/min./mmole) × 10^{-5}

DL-[*N*-methyl-^{14}C]Hygrine	5·5
DL-[2′-^{14}C]Hygrine	3·18

Activity at *N*-methyl/activity at 2′-C = 1·77.

TABLE 3

Activity of hyoscyamine and its degradation products (counts/min./mmole) × 10^{-5}

Hyoscyamine	3·71
Tropine	3·70
3-Phenyltropine	3·69
Benzoic acid	1·33
Triethylmethylammonium iodide	2·34

Activity at *N*-methyl/activity at C-2′ = 1·75.

TABLE 4

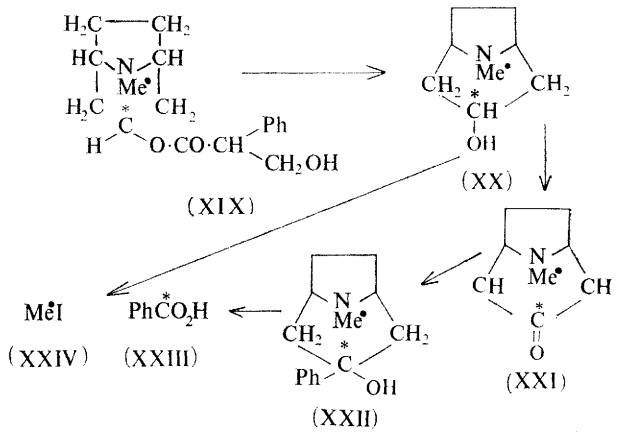
Activity of cuscohygrine and its degradation products (counts/min./mmole) × 10^{-5}

Cuscohygrine	2·73
Benzoic acid	1·45
Triethylmethylammonium iodide	1·27

Activity at *N*-methyl/activity at C-2′ = 0·87.

The hygrine (VII) from the acetate feed was treated with phenylmagnesium bromide yielding the carbinol (XVII) which on aqueous permanganate oxidation yields benzoic acid (XVIII). The concordance between the specific activities of hygrine and benzoic acid show that the hygrine was labelled only at C-2 .

To substantiate our hypothesis on the precursor role of hygrine, we synthesised DL-[*N*-methyl-^{14}C][2′-^{14}C]-hygrine. DL-[*N*-methyl-^{14}C]hygrine was synthesised from norhygrine [13] by methylation with [^{14}C]formaldehyde. DL-[2-^{14}C]hygrine was synthesised by condensation of *N*-methyl-1-pyrroline with [3-^{14}C]ethyl acetoacetate.[14] The hygrines (total activity $1{\cdot}22 \times 10^7$ counts/min.; activity at *N*-methyl/activity at C-2′ = 1·77) were fed to thirty, three-week old, *D. stromonium* plants growing hydroponically.[15] The plants were harvested after 3 days and inactive hyoscyamine (200 mg.) was added as carrier. The hyoscyamine was isolated by established methods.[16] The hyoscyamine as its picrate was purified to constant activity (specific activity $3{\cdot}71 \times 10^5$ counts/min./mmole; percentage incorporation 2·1).



The active hyoscyamine (XIX) was regenerated from its picrate [11] and hydrolysed to tropine (XX). The tropine was oxidised to tropinone (XXI) which on reaction with phenyl-lithium yielded the carbinol (XXII). Aqueous permanganate oxidation of the carbinol yielded benzoic acid (XXIII). Hertzig–Meyer reaction on tropine (XX) yielded methyl iodide (XXIV) isolated as tetramethylammonium iodide. As a result of this degradation the hyoscyamine was shown to have the same ratio, activity at *N*-methyl/activity at C-3, as the administered hygrines. This establishes that hygrine is incorporated into hyoscyamine without degradation.

In a separate experiment doubly labelled hygrine (total activity $1{\cdot}22 \times 10^7$ counts/min.; activity at *N*-methyl/activity at C-2′ = 1·77) as synthesised above, was fed to eight, three-month-old, *Scopolia lurida* plants by a wick arrangement. The plants were harvested after 7 days and active cuscohygrine isolated by established methods [17] after the addition of inactive

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[15] E. Leete, *J. Amer. Chem. Soc.*, 1956, **78**, 3520.
[16] J. Kaczkowski, H. R. Schütte, and K. Mothes, *Biochim. Biophys. Acta*, 1961, **46**, 588.
[17] A. E. Schwarting, J. M. Bobbitt, A. Rother, C. K. Atal, K. L. Khanna, J. D. Leary, and W. G. Walter, *Lloydia*, 1963, **26**, 258.

cuscohygrine (120 mg.) as carrier. The cuscohygrine, as its picrate was purified to constant activity (specific activity $2{\cdot}73 \times 10^5$ counts/min./mmole; percentage incorporation 1·2).

The active cuscohygrine (VIII) was regenerated from its picrate[11] and treated with phenylmagnesium bromide yielding the carbinol (XXV). The carbinol was oxidised with aqueous permanganate yielding benzoic acid (XXVI). A Hertzig–Meyer reaction on the cuscohygrine yielded methyl iodide, collected as tetramethylammonium iodide. The ratio of the activities at the labelled positions in the cuscohygrine was one half that in the administered hygrines showing that the hygrine was incorporated, without degradation, into cuscohygrine.

EXPERIMENTAL

Melting points are corrected. Radioactive assays were carried out in a Nuclear-Chicago model D-47 Q gas-flow counter with a Micromil window on samples of finite thickness with appropriate corrections for efficiency and self-absorption. The [3-^{14}C]ethyl acetoacetate was purchased from the Radiochemical Centre, Amersham.

Administration of Tracers to N. physaloides *and Isolation of Hygrine.*—DL-[2-^{14}C]Ornithine (total activity 0·1 mc) dissolved in water (12 ml.) was fed by a wick arrangement to six, three-month-old, plants of *Nicandra physaloides* growing in soil in a greenhouse. The solution was absorbed by the plants after 24 hr. Water was fed to the plants through the wick for 3 days. The plants were then watered normally and grown on for 16 days after which time they were harvested. The plants were mascerated in a Waring Blendor with 95% ethanol (300 ml.) and concentrated hydrochloric acid (3 ml.). After storing for 48 hr. the mixture was filtered and the plant residue washed with 95% ethanol. The combined washings and filtrate were concentrated to about 6 ml. and dilute hydrochloric acid was added. The aqueous solution was washed with chloroform–ether (1 : 1) to remove non-basic material. The acidic solution was basified with 10% sodium hydroxide and extracted with chloroform. After drying (Na_2SO_4) the chloroform was evaporated under reduced pressure to yield a colourless oil. This oil was dissolved in ether and treated with a solution of picric acid in ether. Recrystallisation of the resulting precipitate from ether yielded hygrine picrate (264 mg.), m.p. 156—154.

In a second experiment [1-^{14}C]acetate (0·1 mc) was fed to 6 more plants and the hygrine isolated in the above manner.

Degradation of Hygrine.—(a) *Acetate feed.* The hygrine picrate was decomposed by the method of Bobbitt[11] to give an almost quantitative yield of hygrine.

2′-Phenyl-dihydrohygrine. The active hygrine (95 mg.) was dissolved in absolute ether (5 ml.) and treated slowly with an excess of phenylmagnesium bromide. After stirring under nitrogen for 48 hr. the magnesium complex was hydrolysed by the addition of 5% hydrochloric acid (15 ml.) and the ether layer separated. The acid layer, after washing with ether was basified with 10% sodium hydroxide and extracted with chloroform. After drying (Na_2SO_4) the chloroform extract was evaporated to yield a viscous oil which solidified on standing. Sublimation of this solid yielded 2′-phenyl-dihydrohygrine (110 mg.), m.p. 88—90° (Found: C, 76·2; H, 9·3; N, 6·3. Calc. for $C_{14}H_{21}NO$: C, 76·7; H, 9·6; N, 6·45%).

Benzoic acid. The carbinol (100 mg.) was refluxed with a solution of potassium permanganate (600 mg.) in water (60 ml.) for 4 hr. The reaction mixture was cooled and some ethanol added to decompose excess permanganate. After filtering, the solution was acidified with 10% hydrochloric acid and extracted with ether (6 × 20 ml.), after drying (Na_2SO_4) the ether was evaporated to yield benzoic acid, which was purified by sublimation (30 mg.), m.p. 120—121°.

(b) *Ornithine feed.* The active hygrine picrate was diluted with inactive picrate to give a total weight 1060 mg. and the hygrine regenerated as above.

Hygrinic acid. Chromium trioxide (560 mg.) in water (8 ml.) was added to a solution of the active hygrine (400 mg.) in sulphuric acid (8 ml.; 20% v/v) and the reaction mixture kept at 90° for 4 hr. The solution was treated with water (20 ml.) and extracted with ether. This extract was discarded.

Sulphur dioxide was passed through the aqueous layer which was then heated to remove the excess of sulphur dioxide. Sulphate ion was removed by dropwise addition of barium hydroxide solution and then filtered. The filtrate was neutralised with a little ammonium hydroxide and concentrated to a small volume (3 ml.). The solution was applied to a column (30 × 1 cm.) of Amberlite 1R-120 (H^+ form), washed with water (100 ml.), and then eluted with 1% ammonium hydroxide (100 ml.). The eluate was evaporated under reduced pressure to yield a white solid. Crystallisation of this compound from methanol–ether followed by sublimation yielded hygrinic acid (210 mg.), m.p. 165—170°. The hygrinic acid was shown to be identical (i.r., m.p.) with an authentic specimen prepared by methylation of DL-proline with formic acid–formaldehyde.

Ethyl hygrinate. The ester was prepared by refluxing the hygrinic acid (200 mg.) in absolute ethanol saturated with dry hydrogen chloride. After 4 hr. the solution was cooled and concentrated to a small volume. Water (10 ml.) was then added and the solution neutralised with sodium carbonate. Extraction of the neutralised solution with ether yielded ethyl hygrinate (200 mg.) which was shown to be identical (i.r.) with an authentic specimen.

N-Methylpyrrolidone.[12] Ethyl hygrinate (180 mg.) was dissolved in ether and added to a solution of phenylmagnesium bromide cooled to −30°. The reaction mixture was stirred under nitrogen for 24 hr. and then acidified with 2% hydrochloric acid. The aqueous layer was separated, basified with sodium hydroxide, and extracted with ether. After drying (Na_2SO_4) the ether was evaporated to yield the carbinol which was then dehydrated by refluxing with a mixture of acetic acid (10 ml.) and acetic anhydride (10 ml.). Evaporation of the solvent yielded the ethylenic compound (XIII). This oil was dissolved in ether (16 ml.) containing pyridine (0·3 mg.) and cooled to −40°. Osmium tetroxide (100 mg.) in ether (6 ml.) was added. The mixture was stirred for 20 min. and a yellow precipitate formed. The solution was allowed to come to room temperature and stand for 2 hr. The osmate ester–pyridine complex was filtered off and washed with ether.

The complex was air dried and added to a solution of sodium sulphate (100 mg.) and potassium carbonate (40 mg.) in 50% ethanol (6 ml.) and stirred at room temperature for 1 hr. The solution was extracted with ether several

times and the ether extract evaporated *in vacuo*. The residue was dissolved in a little water and added to potassium metaperiodate (90 mg.) in water (18 ml.). After standing for 1 hr. the solution was extracted with chloroform. Evaporation of the chloroform yielded slightly impure *N*-methylpyrrolidone.

N-*Methyl-2-phenylpyrroline*.[12] The pyrrolidone was treated with an excess of phenylmagnesium bromide and *N*-methyl-2-phenylpyrroline (picrate m.p. 140—141°) was isolated by the method of Craig.[18]

Benzoic acid. *N*-Methyl-2-phenylpyrroline was refluxed for 2 hr. with 1% aqueous potassium permanganate, alcohol was added to decompose the excess of permanganate, and the filtered solution was acidified with dilute hydrochloric acid. Extraction with ether yielded benzoic acid which was purified by sublimation, m.p. 119—121°.

Administration of Labelled Hygrine to D. stramonium *and Isolation of Hyoscyamine*.—Thirty three-week-old plants of *D. stramonium* were grown hydroponically with their roots immersed in an aerated inorganic nutrient solution.[15] After 2 weeks [*N*-methyl-^{14}C][2′-^{14}C]hygrine (4 mg., $4{\cdot}3 \times 10^8$ counts/min./mmole) was added to the solution. After 3 days essentially all the activity had been absorbed by the the plants. The plants were then harvested and mascerated in a Waring Blendor with chloroform (200 ml.) + concentrated ammonium hydroxide solution (3 ml.). Inactive hyoscyamine (300 mg.) was added as carrier for the active material and the solution allowed to stand for 48 hr. The mixture was then filtered through cloth and the filtrate extracted with 5% hydrochloric acid (4 × 40 ml.). After washing with ether the acidic solution was basified with 2% sodium hydroxide solution and extracted with chloroform. The extract was dried (Na_2SO_4) and evaporated to yield crude hyoscyamine. The alkaloid was converted into its picrate and recrystallised from ethanol to constant activity. (452 mg.), m.p. 164—165°.

Degradation of Hyoscyamine.—The free base was regenerated from its picrate as in the case of hygrine above.

Tropine.[19] The active hyoscyamine (250 mg.) was refluxed in 10% sodium hydroxide (10 ml.) for 30 min. The mixture was cooled and extracted with chloroform to yield tropine (110 mg.); picrate m.p. 291—292°. The aqueous layer was acidified with 1% hydrochloric acid and extracted with ether to yield tropic acid (200 mg.), m.p. 116—117°.

Tropinone.[19] Tropine (80 mg.) was refluxed with the equivalent amount of chromium trioxide in 85% acetic acid (15 ml.). Basification of the reaction mixture with 10% sodium hydroxide followed by chloroform extraction yielded tropinone (52 mg.); picrate m.p. 218—220°.

3-*Phenyltropine*.[19] Tropinone (45 mg.) was dissolved in ether (5 ml.) and treated slowly with an excess of phenylmagnesium bromide. The phenyl derivative was isolated in the same manner as in the case of hygrine. Recrystallisation from ethanol gave 3-phenyltropine (50 mg.), m.p. 177—179°

Benzoic acid. The phenyltropine (40 mg.) was refluxed with an excess of aqueous potassium permanganate for 4 hr. The excess of permanganate was decomposed by addition of ethanol and the reaction mixture filtered. After acidification with 2% hydrochloric acid, the solution was extracted with ether to yield benzoic acid which was purified by sublimation (5 mg.), m.p. 120—121°.

Hertzig–Meyer Reaction on Tropine.—Tropine (20 mg.) was pyrolysed with hydriodic acid under a stream of nitrogen (3 ml./min.). Iodine and hydriodic acid were removed from the gas stream by passage through a tube of Amberlite IR-120 (Na^+ form) [20] moistened with 0·1M-sodium carbonate. After drying (silica gel) the nitrogen was passed into a solution of triethylamine in toluene. After standing at −20° for 1 hr., triethylmethylammonium iodide crystallised out (10 mg.), m.p. 196—297°.

Administration of Labelled Hygrine to S. lurida *and Isolation of Cuscohygrine*.—Eight, three-month old, plants were fed DL-[*N*-methyl-^{14}C][2′-^{14}C]hygrine (4 mg.; $4{\cdot}3 \times 10^8$ counts/min./mmole) in 0·01N-HCl (12 ml.) by a wick arrangement as in the hygrine case. After 7 days the plants were harvested and mascerated in a Waring Blendor with 95% ethanol (200 ml.) and concentrated hydrochloric acid (3 ml.). Inactive cuscohygrine (120 mg.) was added and the cuscohygrine isolated as its picrate, m.p. 221—223°, in the same manner as hygrine above.

Degradation of Cuscohygrine.—The cuscohygrine was regenerated from its picrate as in the case of hygrine.

Hertzig–Meyer reaction on cuscohygrine. This reaction was carried out in the same manner as that on tropine above, the methyl iodide being isolated as triethylmethylammonium iodide.

2′-*Phenyldihydrocuscohygrine*. Cuscohygrine (100 mg.) was dissolved in absolute ether (4 ml.) and treated with an excess of phenylmagnesium bromide and stirred under nitrogen for 24 hr. The phenyl derivative (90 mg.), m.p. 118—120°, was isolated in the same manner as 3-phenyltropine above.

Benzoic acid. The phenyl derivative was oxidised with aqueous permanganate as above to yield benzoic acid which was purified by sublimation.

DL-[*N*-methyl-^{14}C]*Hygrine*. Norhygrine (hydrochloride m.p. 114—115°) was prepared by condensation of 1-pyrroline with ethyl acetoacetate. Norhygrine (508 mg.) was added to a mixture of 35% labelled formaldehyde (120 mg.; specific activity $5{\cdot}5 \times 10^8$ counts/min./mmole) and 90% formic acid (960 mg.). The mixture was allowed to stand for 4 hr. and then heated at 90° in a sealed tube for 1 hr. Concentrated hydrochloric acid (5 ml.) was added and the formic acid and excess of formaldehyde were evaporated on a steam-bath. The residue was basified with 5% sodium hydroxide and extracted with chloroform. After drying (Na_2SO_4) the chloroform was evaporated to yield a yellow oil which was dissolved in ether, filtered, and treated with an ethereal solution of picric acid. Recrystallisation of the resulting precipitate from ethanol yielded hygrine picrate (400 mg.), m.p. 149—151 (specific activity $5{\cdot}5 \times 10^8$ counts/min.mmole).

DL-[2′-^{14}C]*Hygrine*. The labelled hygrine was prepared by the method of Galinovsky.[14] [3-^{14}C]Acetoacetic acid (specific activity $3{\cdot}1 \times 10^8$ counts/min./mmole) was condensed with *N*-methyl-2-hydroxypyrrolidine. The resulting hygrine was converted into the picrate and recrystallised to constant activity (specific activity $3{\cdot}18 \times 10^8$ counts/min./mmole).

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