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Non-enzymatic synthesis of hygrine from acetoacetic acid and from acetonedicarboxylic acid

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Hygrine, an intermediate in biosynthesis of tropane alkaloids, was formed non-enzymatically by the coupling of γ-methylaminobutyraldehyde with either acetoacetic acid or acetonedicarboxylic acid. When these ketoacids were fed to a tropane alkaloid-free culture of tobacco roots, hygrine formation was observed. A possible process of this reaction is discussed.

Biosynthesis; Tropane alkaloid; Hygrine; Acetoacetic acid; Acetonedicarboxylic acid; (*Duboisia leichhardtii*)

1. INTRODUCTION

Although a biosynthetic pathway leading to pharmaceutically important tropane alkaloids, l-hyoscyamine and scopolamine, has been basically established through numerous experiments involving in vivo feeding of radioactive precursors [1–3], several steps in this pathway are still questionable.

Hygrine, an intermediate in the biosynthesis of tropane alkaloids, has been suggested to be synthesized in vivo by the coupling of γ-methylaminobutyraldehyde (MABA) with an unknown counterpart, possibly, acetoacetic acid [3], acetoacetyl CoA [4] or acetonedicarboxylic acid [5]. MABA, which is derived from the amino acids ornithine or arginine, exists in equilibrium with the *N*-methylpyrrolinium cation. Acetoacetic acid and acetonedicarboxylic acid respectively were reported to couple with MABA under physiological conditions [3,6], although the parameters and mode of action in this reaction have not been sufficiently explored. The hygrine formed is incorporated into the tropine moiety of hyoscyamine [4,7].

As a part of our investigations on tropane alkaloid biosynthesis [8–15] we have studied hygrine synthesis. Here we report the non-enzymatic formation of hygrine from either acetoacetic acid or acetonedicarboxylic acid in detail and discuss a possible in vivo process involved in hygrine synthesis.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

MABA was obtained by acid hydrolysis of γ-methylaminobutyraldehyde diethylacetal synthesized from formyl-γ-aminobutyraldehyde diethylacetal (Sigma) following [16]. MABA formed was purified through Dowex 50w × 8 as described in [17]. Acetyl CoA, acetoacetyl CoA and acetoacetic acid were purchased from Sigma.

2.2. *Cell free preparation*

Freeze dried roots of *Duboisia leichhardtii* and *Hyoscyamine albus* cultured in vitro [10,15] were ground in a chilled mortar with 2 vols of potassium phosphate buffer (pH 7.5, 100 mM), 3 mM dithiothreitol, and the same volume of polyvinylpolypyrrolidone. After the centrifugation at 10000 × *g* for 20 min, the protein was precipitated with 80% saturated $(NH_4)_2SO_4$, then collected with another centrifugation (10000 × *g*, 20 min). The pellet was dissolved in Tris-HCl buffer (pH 7.0, 50 mM) and desalted on a Pd-10 column (Pharmacia). About 1 mg protein was applied to a reaction mixture.

2.3. *Reaction condition*

In Tris-HCl buffer (pH 7.0, 50 mM), 0.1 mg acetoacetic acid and 0.5 mg MABA was incubated at 30°C for 4 h in a standard experiment.

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2.4. *Detection and identification of hygrine*

The reaction mixture was made alkaline with 50 μl of 28% NH_4OH and hygrine was extracted with 5 ml CH_2Cl_2/propanol (85:15) [18]. The solution was condensed to 1.0 ml at 30°C in reduced pressure then applied to gas chromatography. The column temperature was 140°C. Other conditions were the same as described previously [10]. Hygrine formed was identified by gas chromatography-mass spectrometry, the conditions of which were also shown in [10].

2.5. *Root culture of tobacco*

Aseptically germinated seedlings of *Nicotiana tabacum* c.v. Samsun were inoculated in liquid media containing minerals and vitamins of B5 medium [19], 3% sucrose and 10^{-5} M indolebutyric acid. The resulting root cultures have been maintained on a rotary shaker in the dark with monthly subculturings.

2.6. *Feeding experiments*

Acetoacetic acid or acetonedicarboxylic acid (10 mM) was added to 2-week-old roots (~1 g fresh wt in 10 ml medium). The roots and media were analyzed 24 h after feeding. The roots harvested were freeze dried and the alkaloids extracted with EtOH/NH_4OH (19:1). The solution was evaporated to dryness and the residue taken up in 0.1 N HCl. Alkaloid extraction from this solution and the culture media used was the same as described above.

3. RESULTS

3.1. *Chemical coupling of acetoacetic acid and MABA*

Acetoacetic acid and MABA were incubated under the conditions described in section 2. After 24 h, 58% of the acetoacetic acid was incorporated in hygrine (fig.1). In order to determine how much substrate was left in the reaction mixture after 24 h incubation, acetoacetic acid and MABA were added separately to the mixtures. A further addition of acetoacetic acid to the reaction mixture increased hygrine yield, but an addition of MABA did not, suggesting that no acetoacetic acid was left in the reaction mixture after 24 h of incubation. Cell free preparations from *Duboisia leichhardtii* and *Hyoscyamine albus* root cultures which produce high levels of hyoscyamine and scopolamine [10,15] did not promote hygrine formation (fig.1).

3.2. *pH dependence of hygrine formation*

The maximum amount of hygrine was formed at pH 9 (fig.2). The decrease in hygrine yield at higher pH can be explained by alkaline labile nature of MABA. MABA was totally decomposed after a 4 h incubation without acetoacetic acid at pH 10 and 11.

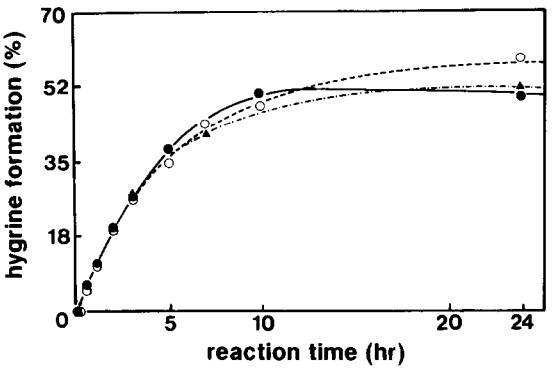


Fig.1. Time course of hygrine formation from acetoacetic acid and γ-methylaminobutylaldehyde with cell free extract from cultured roots of *Duboisia leichhardtii* (●—●); *Hyoscyamus albus* (▲···▲); or without enzyme (○---○). The conversion rates of hygrine formed to acetoacetic acid incubated are expressed.

3.3. *Hygrine formation from analogues of acetoacetic acid*

Several acetoacetic acid analogues and CoA derivatives were incubated with MABA in the presence or absence of a cell free extract of *D. leichhardtii* root culture (table 1). Acetonedicarboxylic acid (3-oxoglutaric acid) gave a considerable amount of hygrine, though no cuscohygrine was detected, in contradiction to the report by Anet et al. [6]. A trace amount of hygrine was synthesized from acetone. Acetoacetyl

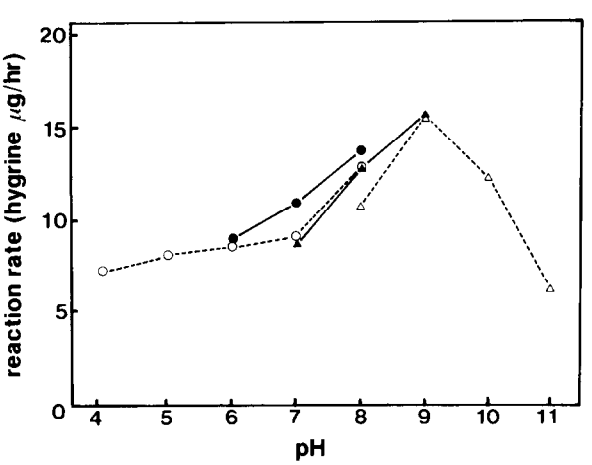


Fig.2. Effect of pH on hygrine formation. Buffers used were: 50 mM Hepes (●—●); 50 mM Tris-HCl (▲—▲); 100 mM citric acid/Na_2HPO_4 (○---○); 100 mM NH_4OH/NH_4Cl (△---△).

Table 1

Hygrine synthesis from acetoacetic acid analogues

Compound	Relative hygrine yield (%)[a]
Acetoacetic acid	100
Acetonedicarboxylic acid	35
Methyl acetoacetic acid	not detected
Acetone	trace
Acetoacetyl CoA	8
Acetyl CoA	not detected

[a] Values are expressed relative to the yield obtained from acetoacetic acid

CoA also gave a small amount of hygrine, which might be due to the spontaneous breakdown of the compound to acetoacetic acid and CoA-SH. This decomposition could be followed by the decrease in UV absorbance at 303 nm due to thioester-specific enolate anion [20]. In any case, the crude extract from *Duboisia* root culture did not enhance hygrine yield.

3.4. *Effects of metal cations on hygrine synthesis*

The effect of metal cations on hygrine synthesis was examined when these were added to the reaction mixture (table 2). Some divalent cations stimulated hygrine formation. Copper ion doubled

Table 2

Effect of metal ions on hygrine formation

Compound	mM	Acetoacetic acid (%)[a]	Acetone-dicarboxylic acid (%)[a]
None	–	100	100
NaCl	10	101	113
K_2SO_4	10	142	92
$(NH_4)_2SO_4$	10	111	95
$MgCl_2$	10	172	76
$CaCl_2$	10	133	99
$MnSO_4$	2	153	122
$FcSO_4$	0.5	154	133
$FeCl_3$	0.5	118	272
$CoCl_2$	0.5	129	125
$NiCl_2$	0.5	167	144
$CuSO_4$	0.5	200	234
$Zn(NO_3)_2$	0.5	164	140
$HgCl_2$	0.5	85	123
$Pb(NO_3)_2$	0.5	111	159

[a] Values are expressed relative to the yield without any metal ion

Table 3

Feeding of ketoacids to cultured tobacco root

	Hygrine formed (μmol/g dry wt)	
	Roots	Media
Control	N.D.	N.D.
Acetoacetic acid	4.3	N.D.
Acetonedicarboxylic acid	0.35	N.D.

N.D., not detected. Roots were incubated for 24 h with 10 mM acetoacetic acid or acetonedicarboxylic acid

hygrine synthesis from either acetoacetic acid or acetonedicarboxylic acid. Ferric ion markedly enhanced hygrine yield from acetonedicarboxylic acid, but did not change the yield from acetoacetic acid.

3.5. *Feeding of acetoacetic acid and acetone-dicarboxylic acid to cultured root of tobacco*

Tobacco alkaloids such as nicotine share a common precursor, MABA, with tropane alkaloids.

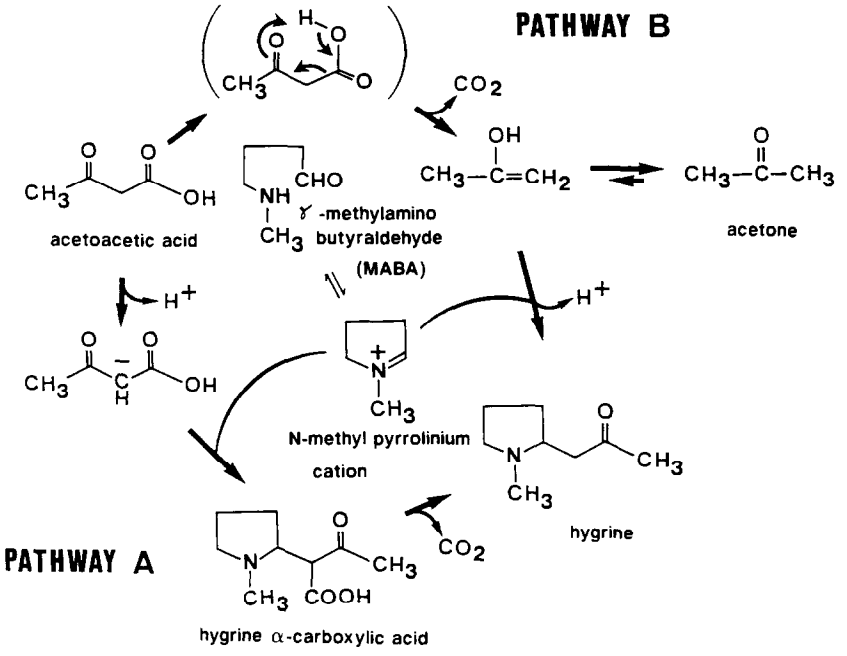
Acetoacetic acid and acetone dicarboxylic acid separately were fed to a root culture of tobacco, which produced a considerable amount of nicotine but not tropane alkaloids, including hygrine. Hygrine was detected when either keto acid had been fed to the tobacco roots (table 3).

4. DISCUSSION

In this report, we have shown that non-enzymatic formation of hygrine takes place in physiological pH and temperature. The result of feeding acetoacetic acid to tobacco roots suggests the possibility of non-enzymatic hygrine formation in vivo.

Two hypothetical processes for the coupling of acetoacetic acid and MABA are proposed. Acetoacetic acid may lose a proton to form enolate anion (pathway A in scheme 1) [21] or it can decompose to carbon dioxide and an enole (2-hydroxypropene) which is easily turned into acetone (pathway B in scheme 1) before Mannich condensation with MABA takes place.

Formation of a trace amount of hygrine from acetone suggests the possibility of pathway B. The hygrine yield from acetone was only one thousandth the yield from acetoacetic acid (not



Scheme 1.

shown), indicating that acetone formed from the enole can turn back into the enole at a very low rate. This may explain the result of in vivo feeding [22] in which sodium [4-^{14}C]acetoacetate was incorporated into only C4 position of hygrine. If pathway B is indeed the process in vivo, a putative intermediate, hygrine α-carboxylic acid is not involved in this reaction, although further experiments are needed to establish the actual in vivo pathway. From acetonedicarboxylic acid, hygrine may be formed by a similar process. But in this case a second decarboxylation must be considered.

Although we cannot deny the possible involvement of an enzyme system such as acetoacetic acid decarboxylase in hygrine formation, no indication for the involvement of any enzyme could be obtained from this study. Acetoacetic acid and acetonedicarboxylic acid are two possible counterparts of MABA for the formation of hygrine in vivo because they both formed considerable amounts of hygrine when combined with MABA at physiological pH and temperature. Feeding experiments indicate that endogenous MABA produced by tobacco root cultures could couple with exogenously supplied acetoacetic acid or acetonedicarboxylic acid to form hygrine. Since it is plausible to assume that the tobacco root, which produces no tropane alkaloids does not have an enzyme which catalyzes hygrine formation, the coupling observed must result from a non-enzymatic process. This suggests that, if pools of either acetoacetic acid or acetonedicarboxylic acid and of MABA are present together, non-enzymatic hygrine formation takes place in vivo. The presence or absence of an available pool of these keto acids may result in the difference in alkaloids formed in tobacco and in plants which produce tropane alkaloids (*Duboisia, Hyoscyamus, Datura, Atropa,* etc.).

In animals acetoacetic acid is formed from acetoacetyl CoA with CoA transferase, deacylase, or activating enzyme [20]. Acetoacetyl CoA, which can be formed from two acetyl CoA (thiolase reaction), is involved in terpenoid synthesis and the degradation of fatty acids and some amino acids (leucine, phenylalanine, tyrosine, tryptophan and lysine). Acetonedicarboxylic acid is formed in the course of citric acid degradation by some micro-organisms [23,24].

Although the metabolism of these ketoacids has been partly revealed in animal and micro-organism systems, in higher plants it is largely unknown.

Carbons 2, 3 and 4 of the tropane ring have been demonstrated to be derived from acetic acid in *Datura metel* roots [25], while labeled citric acid was not incorporated into the tropane ring [26]. It is essential to study the metabolism of the ketoacids in plants in order to obtain a clearer understanding of tropane alkaloid biosynthesis because these compounds are key intermediates which link primary and secondary metabolism in plants which produce tropane alkaloids.

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