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Biosynthesis of psilocybin in submerged culture of Psilocybe cubensis

Part I. Incorporation of labelled tryptophan and tryptamine

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SUMMARY Methods have been investigated for studying the biosynthesis of psilocybin in submerged culture. DL-tryptophan-^{14}C and tryptamine-^{14}C are found to be precursors of psilocybin.

Psilocybin and psilocin are two 4-substituted indole derivatives present in a number of hallucinogenic mushrooms, mainly members of the genus *Psilocybe* (1, 2, 3), but also in a representative of *Conocybe* (2). These indoles were found to be responsible for the hallucinogenic effect of the mushrooms (1).

Few natural indole derivatives are substituted in the 4-position. In addition to psilocybin and psilocin, there are the ergot alkaloids and some *Alstonia* (4) and *Mitragyna* alkaloids (12). Certain other relations exist between psilocybin-psilocin and the ergot alkaloids. Thus, they are generally fungal metabolites, some of the ergot alkaloids also have hallucinogenic properties, and — on account of their psychotomimetic properties — mushrooms and plants containing these compounds have been used as intoxicants by certain natives (1, 2, 5, 12). At present, psilocybin[1] is being tested as a psychotherapeutic agent.

The production of psilocybin in submerged culture was studied by one of us (P. C.), and it was shown that *Psilocybe cubensis* (Earle) Singer (NRRL A-9109) was able to produce psilocybin by this type of fermenta-

[1] Indocybin®, Sandoz AG. See also ref. 11.

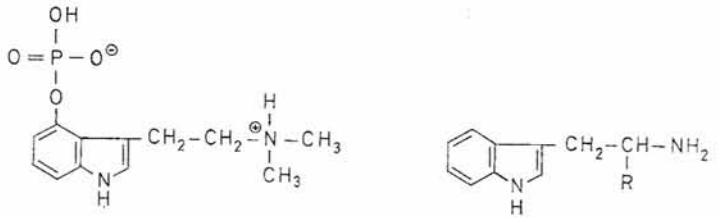

Fig. 1. Psilocybin, tryptophan R = COOH and tryptamine R = H.

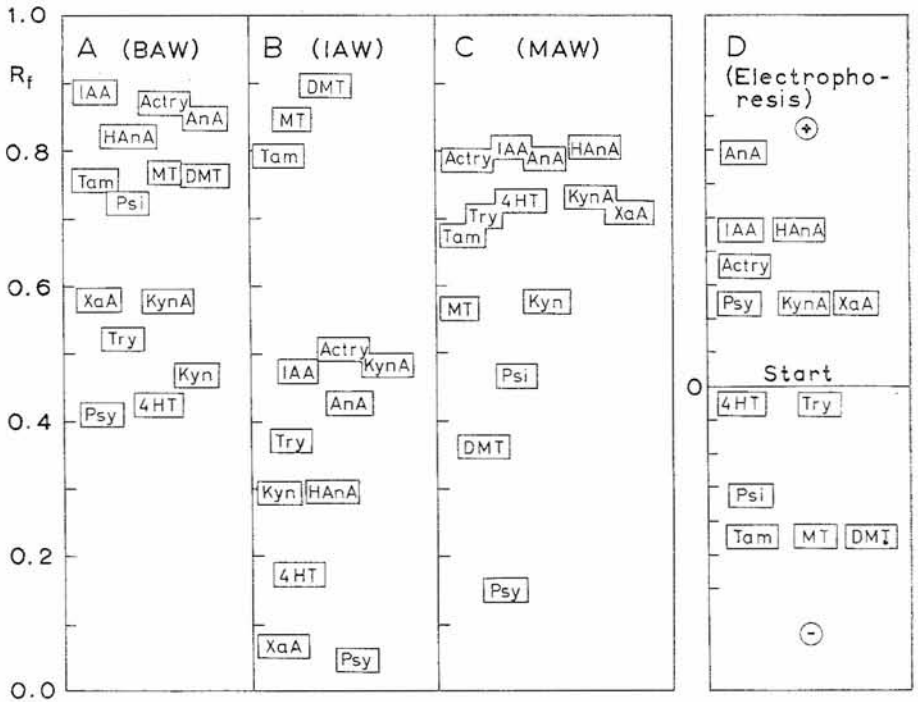

Fig. 2. Distribution of some possible tryptophan metabolites in the chromatographic systems: A. BAW; B. IAW; C. MAW; and D. the electrophoretic system.

Abbreviations: Psy = psilocybin, 4HT = 4-hydroxytryptophan, Kyn = kynurenine, Try = tryptophan, KynA = kynurenic acid, XaA = xanthurenic acid, Psi = psilocine, Tam = tryptamine, MT = methyltryptamine, DMT = dimethyltryptamine, HAnA = 3-hydroxyanthranilic acid, AnA = anthranilic acid, Actry = N-acetyltryptophan, IAA = indoleacetic acid.

tion (6). Recently, *P. baeocystis* was found to synthesize this compound in submerged culture (7). The experimental evidence on the biosynthesis of psilocybin is limited to the finding by Brack *et al.* (8) that labelled tryptophan was incorporated into psilocybin by surface cultures of *P. semperviva.*

The purpose of the present investigation was to establish conditions for the study of psilocybin biosynthesis in submerged culture, and to test the incorporation of radioactive tryptophan and tryptamine.

Experimental

Chromatographic systems

BAW. *n*-Butanol-acetic acid-water (4:1:5), Whatman 3 MM paper (6). Fig. 2 A.

IAW. *Iso*-propanol-conc. NH_4OH-water (8:1:1), Whatman 3 MM paper. Fig. 2 B.

MAW. TLC on Silica Gel G plates with methanol-acetic acid-water (75:10:15) as solvent. Fig. 2 C.

Electrophoresis

Electrophoresis (9) was carried out on 23×40 cm Whatman 3 MM paper in a collidine buffer, pH 8.0 (25.0 ml of a solution of 2.64 ml of 2,4,6-collidine in 97.36 ml of distilled water; 10.0 ml of 0.1 N HCl; 65.0 ml of distilled water) at 2000 V for 1 1/4 hr (Fig. 2 D). Compounds were located with UV light and Ehrlich's reagent (6).

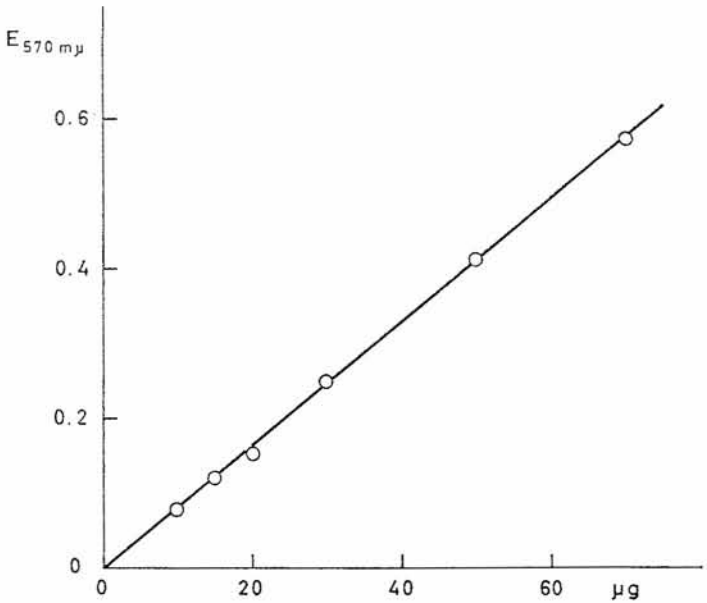

Fig 3
Calibration curve of psilocybin.

Assay procedure

Reagent: 2.00 g *p*-dimethylaminobenzaldehyde in 65 ml of conc. sulphuric acid, 35 ml of distilled water and 0.1 ml of 5 % aqueous $FeCl_3 \cdot 6H_2O$. A solution of psilocybin in 1.00 ml of distilled water and 2.00 ml of reagent was irradiated for 15 min under an UV lamp (Hanau), after which the optical density was determined at 570 mμ (Fig. 3).

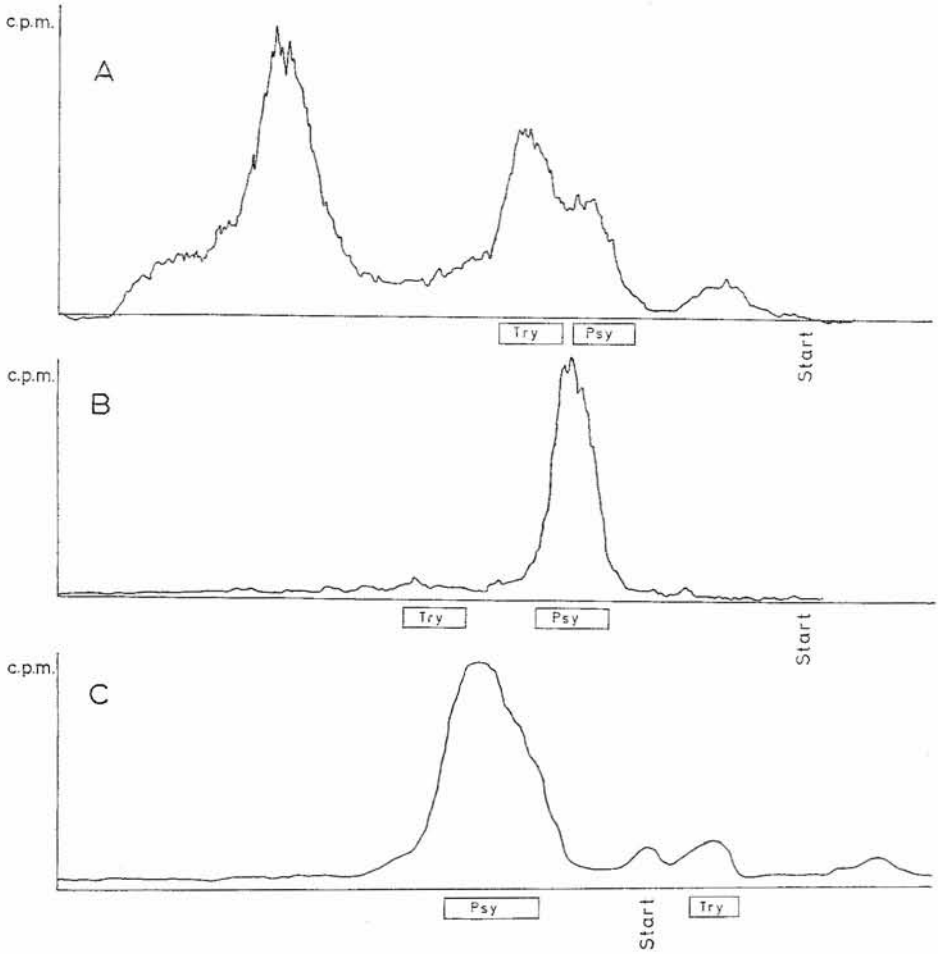

Fig. 4. A. Chromatogram scan of Dowex eluate separated in system BAW. Precursor: DL-*tryptophan-G-*^3H *(exp. no. A). B. Chromatogram scan of psilocybin-*^3H *purified by paper chromatography, separation in system BAW (exp. no. A). C. Chromatogram scan of psilocybin-*^{14}C *purified by paper chromatography (exp. no. II,* DL-*tryptophan-*^{14}C*). Electrophoretic separation. Abbreviations as in Fig. 2.*

Determination of radioactivity

The methods have been described earlier (10). Eluted, radioactive psilocybin was recrystallized with carrier (7—8 mg) from methanol to constant spec. act. Psilocybin (dried *in vacuo*) was dissolved by the addition of 0.5 ml of Hyamine® hydroxide (Packard Instrument Co.) to the XDC liquid scintillator. DL-tryptophan-3-^{14}C (side-chain label) and DL-tryptophan-G-^{3}H were purchased from the Radiochemical Centre, Amersham, and tryptamine-2-^{14}C bisuccinate from New England Nuclear Corp., Boston.

In the experiments, the association of radioactivity with psilocybin was determined in several ways, *i. e.*, by recrystallization with carrier to constant spec. act. (± 4 %), by TLC in MAW (Eastman Chromatogram sheet), by chromatography in IAW and BAW, and by electrophoresis followed by scanning (Figs. 4—5). In one experiment with each precursor, part of the labelled psilocybin was methylated with diazomethane to dimethylpsilocybin (1), which was found to be active by scanning after chromatography in BAW. In one case (DL-tryptophan, exp. II), some tryptophan-^{14}C present in the chromatographic eluate was eliminated by electrophoresis (Fig. 4 C).

Culture technique

Psilocybe cubensis was grown as described previously on medium no. 1 (6) with a glucose concentration of 1 % instead of 0.5 %. Radioactive precursors, as sterile-filtered solutions, were introduced on the 3rd day and exposed for 5—6 days.

Isolation of psilocybin

The mycelial pellets were dried, ground and extracted with methanol (6) and the extract evaporated to about 5 ml (»methanol extract»: Table 1). After addition of 20 ml of 50 % aqueous methanol, the filtered extract was slowly passed through a 1×12 cm column of ion exchanger Dowex 50 W×8 (H^+). The column was washed in sequence with 50 ml each of: 50 % aqueous methanol, methanol, chloroform-methanol (1:1),

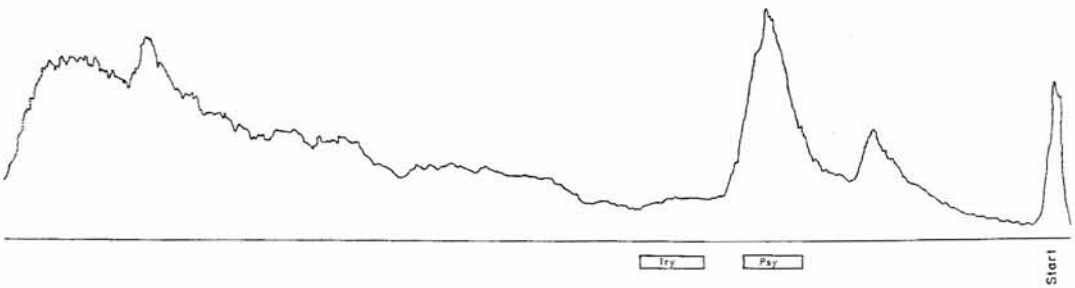

Fig. 5. Chromatogram scan of Dowex eluate separated in system BAW. Precursor: tryptamine-^{14}C.

methanol, and 50 % aqueous methanol. The column was then eluted with 100 ml of 5 % conc. NH_3 in 50 % aqueous methanol (»Dowex eluate»: Table 1). The evaporated extract was dissolved in 2 ml of 50 % methanol, and streaked on washed (10 % acetic acid followed by methanol) Whatman 3 MM paper, and chromatographed in system BAW overnight. The band containing psilocybin was cut out and eluted with water (»psilocybin eluted from BAW»: Table 1.).

Identification of psilocybin in P. cubensis

Psilocybin (2.6 mg by colorimetric estimation), purified twice by paper chromatography from 26.2 g of dried mycelial pellets, was found to have an IR spectrum (Perkin-Elmer Infracord 237, microfame) agreeing with that of similarly chromatographed authentic psilocybin. Furthermore UV (Beckman DB), chromatographic and electrophoretic data agreed with those of a reference sample.

Results and discussion

In a number of trial runs with and without added tritiated tryptophan (*e. g.* exp. A, Table 1), suitable procedures were investigated for the biosynthetic labelling and subsequent isolation of radioactive psilocybin. The following method was devised for isolation of radioactive psilocybin from incorporated labelled precursors (*cf.* Experimental). Psilocybin is extracted with methanol from the dried mycelium, followed by adsorption on an ion-exchange resin, (Dowex 50W$\times$8 H^+). After elution from the resin, psilocybin was isolated by preparative paper chromatography in a butanol-acetic acid-water system. The ion-exchange step eliminates a certain amount of activity (Table 1), and the following separation in BAW is also greatly improved. The yield in the ion-exchange step was found to be 95—100 % with pure psilocybin, whereas the recovery in the paper-chromatographic step, after elution from the paper, was about 60 %. Purification of the Dowex eluate by TLC in MAW gave more impurities and lower yield.

The method described was also used to isolate psilocybin from *P. cubensis* for identification purposes. The amount of psilocybin in cultures ranged from 100—1100 μg/g mycelium (dry wt.), as determined from the chromatographic eluate. Since the usual van Urk's reagent (10) could not be used for the colorimetric determination of psilocybin, a modified reagent was developed.

Biosynthesis of psilocybin

The investigation of Brack *et al.* (8) showed that tryptophan was the biogenetic precursor of psilocybin. The tryptophan molecule would require the following modifications for conversion to psilocybin (Fig. 1):

1) decarboxylation
2) methylation of the amino group
3) hydroxylation of the 4-position of the indole moiety, followed by phosphorylation.

Table 1

Incorporation of radioactive precursors into psilocybin by P. c u b e n s i s

Exp. no.	Precursor introduced (spec. act.)	No. of cultures	Dry wt. mycelium g	Radioactivity d. p. m. introduced	Radioactivity d. p. m. in methanol extract[1]	Radioactivity d. p. m. in Dowex eluate[1]	Radioactivity d. p. m. of psilocybin eluted from BAW[1] (µg isolated)	Precursor incorp. into psilocybin[2] %	Spec. act. of psilocybin µC/mM[3]	Dilution[4]
A	DL-Tryptophan-G-^{3}H (159 mC/mM)	3	0.92	$444 \cdot 10^6$	$36.0 \cdot 10^6$	$5.15 \cdot 10^6$	$1.56 \cdot 10^6$ (1000 µg)	0.35	185	—
Ia	DL-Tryptophan-3-^{14}C (3.37 mC/mM)	4	0.93	$33.3 \cdot 10^6$	$0.84 \cdot 10^6$	$0.72 \cdot 10^6$	$0.029 \cdot 10^6$ (45 µg)	0.086	92.0	37
IIa	DL-Tryptophan-3-^{14}C (3.37 mC/mM)	3	0.92	$33.3 \cdot 10^6$	$2.08 \cdot 10^6$	$0.68 \cdot 10^6$	$0.144 \cdot 10^6$ (325 µg)	0.43	57.7	58
Ib	Tryptamine-2-^{14}C (2.73 mC/mM)	4	1.10	$33.3 \cdot 10^6$	$4.03 \cdot 10^6$	$3.08 \cdot 10^6$	$0.560 \cdot 10^6$ (220 µg)	1.68	275	9.9
IIb	Tryptamine-2-^{14}C (2.73 mC/mM)	3	0.98	$33.3 \cdot 10^6$	$6.60 \cdot 10^6$	$2.55 \cdot 10^6$	$1.72 \cdot 10^6$ (320 µg)	5.17	612	4.5

[1] *cf.* Experimental: »Isolation of psilocybin».

[2] Determined from figure in previous column.

[3] Determined after recrystallization with carrier psilocybin.

[4] Defined as spec. act. of precursor/spec. act. of product.

The sequence of events may be strict, although not necessarily in the above order, or a number of reaction steps in varying order may lead to the final product, psilocybin. This is to be investigated.

The experiments with labelled DL-tryptophan (Table 1) showed that the biosynthesis of psilocybin could be studied in submerged culture of *P. cubensis*. Although the percentage of precursor incorporated and the amount of psilocybin formed was small, the isolated psilocybin was of high spec. act., and the dilution (10) (defined as spec. act. of precursor/ spec. act. of product), was fairly low, which strongly indicates that tryptophan was not incorporated via breakdown products, as also indicated by the investigation of Brack *et al.* (8). Degradation procedures for the psilocybin molecule are being investigated.

A comparison between the incorporation rates from tryptophan and tryptamine under similar conditions (*e. g.* exp. I a and exp. I b were started with the same inoculum) showed that tryptamine functioned as a better precursor than tryptophan, also if it is assumed that only the L-form of tryptophan was utilized. In exp. II with tryptamine, the high spec. act. of the labelled psilocybin showed that not less than 22.4 % of the psilocybin formed was derived from the labelled precursor. Thus, it seems possible that a decarboxylation of tryptophan to tryptamine is the first step in the biosynthesis of psilocybin.

Acknowledgements

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References

1. A. Hofmann, R. Heim, A. Brack, H. Kobel, A. Frey, H. Ott, Th. Petrzilka and F. Troxler. *Helv. Chim. Acta* **42**, 1557 (1959)
2. R. G. Benedict, L. R. Brady, A. H. Smith and V. E. Tyler Jr. *Lloydia* **25**, 156 (1962)
3. A. Hofmann, R. Heim and H. Tscherter. *Compt. rend.* **251**, 10 (1963)
4. T. R. Govindachari, N. Viswanathan, B. R. Pai and T. S. Savitri. *Tetrahedron Letters* 901 (1964)
5. R. Voigt. *Pharmazie* **17**, 317 (1962)
6. P. Catalfomo and V. E. Tyler Jr. *Lloydia* **27**, 53 (1964)
7. A. Y. Leung, A. H. Smith and A. G. Paul. *J. Pharm. Sci.* 54, 1576 (1965)
8. A. Brack, A. Hofmann, F. Kalberer, H. Kobel and J. Rutschmann. *Arch. Pharm.* **294**, 230 (1961)
9. G. Samuelsson. *Svensk Farm. Tidskr.* **66**, 201 (1962)
10. S. Agurell. *Acta Pharm. Suecica* in press
11. E. Jacobsen. *Clin. Pharmacol. Therapeut.* 4, 480 (1963)
12. A. Hofmann. *Planta Medica.* **12**, 341 (1964)

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