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CONVERSION OF TRYPTOPHAN TO 2,3-DIHYDROXYBENZOIC ACID BY CLAVICEPS PASPALI

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Arcamone *et al.*[1] identified 2,3-dihydroxybenzoic acid (DHB) as a metabolite of major importance in submerged cultures of *Claviceps paspali* Stevens & Hall. The compound was also produced in replacement cultures of the organism in which tryptophan was the sole substrate. Anthranilic acid, formyl= anthranilic acid, and kynurenine were detected in these cultures, but only the latter compound was subsequently converted to DHB[2]. Neither of these studies utilized labeled precursors, and experimental details, including rates of conversion and percentage yields, were not reported.

Additional presumptive evidence linking DHB to tryptophan metabolism was obtained by Pittard *et al.*[3] in experiments utilizing a tryptophan auxotroph of *Aerobacter aerogenes*.

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These findings are in contrast to those obtained with labeled precursors in other biological systems which have shown that certain hydroxybenzoic acids derive from acetate metabolism[4] or from phenylpropanoid precursors[5]. Therefore, we administered labeled tryptophan to cultures of Claviceps paspali to determine if that amino acid could actually function as a biosynthetic precursor of DHB.

For this investigation we used Claviceps paspali strain Li 189 which had been isolated by Gröger and Tyler[6]. Its cultivation was carried out essentially as described by them, except L-tryptophan-(indole-^{3}H) was added to the fermentation medium no. 424. The tritiated L-tryptophan was prepared by exchanging the non-labeled compound with HO^{3}H/HCl [7].

Table 1

Incorporation of L-tryptophan-(indole-^{3}H) (specific activity 3.85 mC/mM) into DHB in submerged cultures of Claviceps paspali. The fermentations were carried out in 125-ml Erlen= meyer flasks containing 25 ml of nutrient medium no. 424. Incubation time was three days.

Trial no.	L-Trypto= phan-T added mg	DHB formed mg	DHB Specific activity (dpm/mM)	Specific incorpora= tion rate
1	2,5	4,9	$1,28 \times 10^9$	15 %
2	2,5	5,3	$1,00 \times 10^9$	11,8 %
3	3,0	5,3	$1,17 \times 10^9$	13,8 %
4	3,9	4,5	$9,27 \times 10^8$	10,8 %
5	5,2	6,6	$8,88 \times 10^8$	10,3 %
6	0 (control)	4,0	----	----

After extraction of the alkaloids from the culture medium, DHB was quantitatively determined by the procedure of Pittard *et al.*[3] All determinations of radioactivity were conducted by the method of Simon *et al.*[8]

A. The results of this investigation are summarized in Table 1. DHB was extracted from the acidified culture medium with ether and purified by extraction from that solvent with 8 % sodium bicarbonate solution. Isolation was effected by ascending paper chromatography on 16x36 cm sheets of S. and S. 2043 bm filter paper. After formation with *n*-butanol:acetic acid:water (4:1:5), DHB was eluted from the paper and rechromatographed with *n*-propanol:water (3:1).

DHB was eluted from the chromatograms with ethanol:1*N* hydrochloric acid (3:1). Following evaporation of the ethanol, DHB was extracted with ether, the ether evaporated, and the residue dissolved in 0,25 % succinic acid solution. Aliquot parts of this solution were utilized for radioactivity determinations.

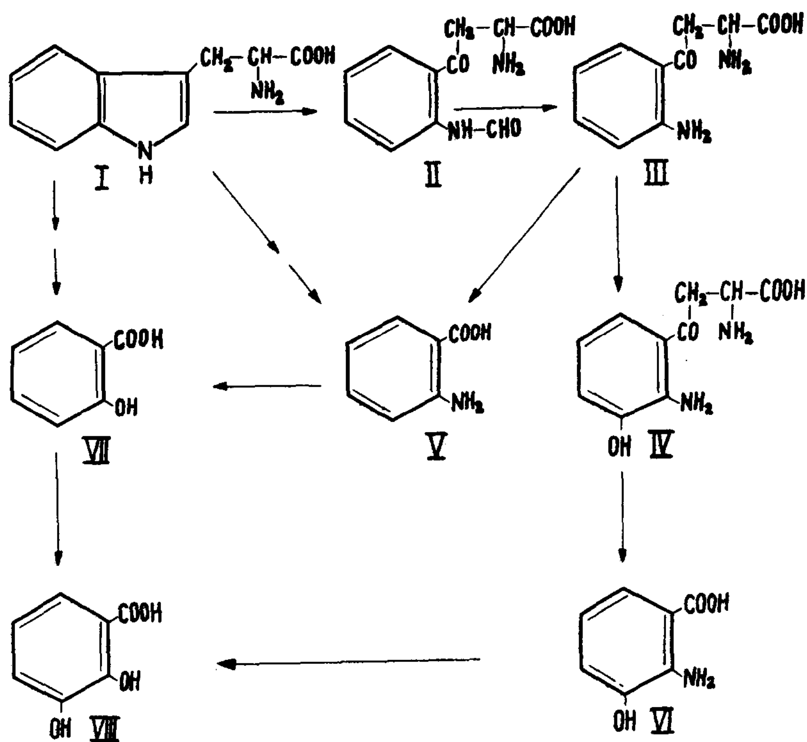
B. Crystalline DHB was obtained by the following procedure. Five 500-ml round-bottom flasks, each containing 100 ml of medium no. 424 plus 4,4 mg of *L*-tryptophan-(indole-^{3}H), were inoculated with the fungus and shaken for three days at 24°C. The filtered nutrient solutions were combined and found by colorimetric analysis to contain approximately 50 mg of DHB.

This solution was acidified (*p*H 1) with hydrochloric acid and exhaustively extracted with ether. The combined ether extract was then extracted with bicarbonate solution which was in turn acidified and re-extracted with ether. After evaporation of the ether extracts the residue was sublimed *in*

vacuo. The sublimate was found to consist of a mixture of DHB and succinic acid (the latter compound was an ingredient of the culture medium).

To separate this mixture of acids we employed an ion exchange column (0,8 x 10 cm) containing Dowex 1 x 10 resin, 200-400 mesh, in the formate form. Elution of the succinic acid was carried out with 1N formic acid, the column was washed with distilled water, and the DHB eluted with 1N hydro=chloric acid : ethanol (1:1). Following evaporation of the ethanol, the DHB was extracted with ether, the ether evaporated, and the residue sublimed *in vacuo*.

We obtained 25 mg of DHB, micro m.p.K. = 202°C. The I.R. spectrum of this compound in potassium bromide, recorded on a Unicam SP. 200 spectrophotometer, was identical with that of reference DHB. The specific activity was found to be 0,247 mC/mM; the specific incorporation rate was 6,4 %. The relatively high incorporation rate indicates that trypto=phan serves as an efficient precursor of DHB but gives no indication of the intermediates involved in the reaction. Tryptophan (I) may be converted by various pathways [9,10] to salicylic acid (VII) which is then oxidized to DHB (VIII). The latter reaction has been shown to occur in higher plants[11] but remains to be demonstrated in *Claviceps*. Alternatively, tryptophan may be degraded via formylkynurenine (II), kynurenine (III), and 3-hydroxykynurenine (IV) to 3-hydroxy-anthranilic acid (VI) which is then oxidatively deaminated to DHB (VIII). Only the last step remains to be demonstrated in *Claviceps* species[12]. This route is no doubt possible for



Claviceps and is considered to be the most likely biosynthetic pathway of DHB.

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