CONVERSION OF DIHYDROLYSERGIC ACID TO DIHYDROERGOTAMINE IN AN ERGOTAMINE-PRODUCING STRAIN OF *CLAVICEPS PURPUREA*

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ABSTRACT.—Dihydrolysergic acid is converted to dihydroergotamine in cultures of an ergotamine-producing strain of *Claviceps purpurea*. Dihydrolysergyl coenzyme A was synthesized. Dihydrolysergyl coenzyme A was converted in a cell-free system to an unidentified product.

Lysergic acid is incorporated into the lysergic acid alkaloids (1, 2) by *Claviceps* species, and dihydrolysergic acid is incorporated into dihydroergosine in *Sphacelia sorghi* (3). The specificity of these enzyme systems for the lysergyl moiety has not been investigated. We report that an ergotamine-producing strain of *C. purpurea* utilizes dihydrolysergic acid to produce dihydroergotamine.

MATERIALS AND METHODS

CULTURE TECHNIQUES.—*Claviceps purpurea* PCCE1 was a selection from the ergotamine-producing strain Farmitalia FI 32/17 (ATCC 20102). Slants were grown on 100 g sucrose, 10 g L-asparagine, 1 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.1 g yeast extract, 0.12 g KCl, 20 mg $FeSO_4 \cdot 7H_2O$, 15 mg $ZnSO_4 \cdot 7H_2O$, and 20 g agar adjusted to pH 5.2 with sodium hydroxide and diluted to 1 liter with tap water. The preculture contained 100 g glucose, 10 g citric acid, 0.5 g KH_2PO_4, 0.3 $MgSO \cdot 7H_2O$, 7 mg $FeSO_4 \cdot 7H_2O$, 6 mg $ZnSO_4 \cdot 7H_2O$, 0.1 g yeast extract adjusted to pH 5.2 with conc. NH_4OH and diluted to 1 liter with water. The production medium contained 300 g sucrose, 15 g citric acid, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.125 g KCl, 7 mg $FeSO_4 \cdot 7H_2O$, 6 mg $ZnSO_4 \cdot 7H_2O$, and 0.1 g yeast extract adjusted to pH 5.2 with conc. NH_4OH and diluted to 1 liter. Precultures were grown for 5 to 7 days. Then 2.5 ml of preculture medium was added to 25 ml production medium or production medium containing 25 mg dihydrolysergic acid which had been steam-sterilized with the medium. Cultures were harvested 8 to 9 days after inoculation.

DETERMINATION OF DIHYDROERGOTAMINE.—The culture was filtered with suction, and the mother liquor was passed through a Dowex 50 x 4 [H^+, 25–50 mesh] cation exchange resin column. The alkaloids were eluted with 5% NH_4OH, and the eluate was concentrated to dryness on the rotary evaporator. Tlc with reference dihydroergotamine (D.H.E. 45, Sandoz) was carried out on Silica gel G in chloform-methanol, 4:1, (R_f dihydrolysergic acid 0.03, dihydroergotamine 0.66, ergotamine 0.73), ethyl acetate-methanol, 19:1, (R_f dihydrolysergic acid 0.00, dihydroergotamine 0.07, ergotamine 0.11), and ethyl acetate-dimethylformamide-ethanol, 13:1.9:0.1, R_f dihydrolysergic acid 0.00, dihydroergotamine 0.36, ergotamine 0.41). Dihydroergotamine and ergotamine were separated on Silica gel G with chloroform-methanol, 4:1, eluted with methanol-acetic acid-water, 2:1:2(4), and Van Urk's reagent was added. The absorbance at 540 nm was determined, and the amount of each alkaloid was calculated with reference to an ergotamine standard.

SYNTHESIS OF DIHYDROLYSERGYL COENZYME A.—Dihydrolysergyl coenzyme A was synthesized by the following procedure: Dihydrolysergic acid (30 mg, 90 μmole) plus 23 μl of triethylamine (165 μmole) plus 30 ml of freshly distilled THF were cooled to 0° in an ice bath under nitrogen. Ethyl chloroformate (10 μl, 105 μmole) was added dropwise. After the ice bath was removed, stirring was continued and the reaction maintained for 30 min. The solution was evaporated to dryness at 30° on a rotary evaporator. The residue was shaken with 4.5 ml of THF and the resulting solution transferred to a 15 ml flask and cooled in an ice bath under nitrogen. Nitrogen was bubbled through a solution of 23 mg (30 μmole) of coenzyme A in 7.0 ml 0.2 M $KHCO_3$ pH 8.0. The cold coenzyme A solution was added to the solution of the dihydrolysergic acid mixed anhydride. The pH was adjusted to pH 8.0 with 10% HCl. The ice bath was removed, and the pH was maintained at pH 8.0–8.5 under nitrogen for two hr. The pH was then adjusted to pH 5.0, and the solution was extracted twice with an equal volume

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of ether. The aqueous layer was evaporated partially on a rotary evaporator for 30 min at 30°. The solution was placed on a Sephadex LH-20 column (1.5 x 40 cm) and eluted with 40% ethanol. The flow rate was 2.1 ml/hr. The absorbance at 260 nm was followed. Three peaks in order of elution were obtained: coenzyme A plus coenzyme A disulfide, dihydrolysergyl coenzyme A, and dihydrolysergic acid. Dihydrolysergyl coenzyme A came off the column at an elution volume of 28 ml. The fractions which contained dihydrolysergyl coenzyme A were combined and concentrated to dryness on a rotary evaporator. Paper chromatography with water as solvent was used to monitor the synthesis and stability of dihydrolysergyl coenzyme A. The ultraviolet spectra were measured on the Cary 118 Spectrophotometer.

CELL-FREE INCUBATIONS.—Cultures of *C. purpurea* PCCE1 were harvested five days after inoculation. A mixture which contained protoplasts (5), 0.07 M sodium phosphate pH 7.5, 15 mM ATP, 5 mM $MgCl_2$, 1 mM each of alanine, proline, phenylalanine, 0.8 mM NADP, 0.8 mM glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase, 1 mM dihydrolysergyl coenzyme A, and 1 μ Ci L-[U-^{14}C]alanine (Amersham) in a final volume of 2.3 ml was homogenized with a glass homogenizer and centrifuged at 3000 g for 10 min. The supernatant was incubated at 30° for four hr and then mixed with an equal volume of ethanol. After centrifugation, the supernatant was evaporated to dryness, dissolved in 70% ethanol and separated by plc on Silica gel G with chloroform-methanol, 4:1, ethyl acetate-limethylformanate-ethanol, 86.5:12.5:1, and isopropanol-water, 70:30, as solvent systems. The plates were scanned for radioactivity in a Packard 7221 Radiochromatogram Scanner and then sprayed with Van Urk's reagent.

RESULTS

When *Claviceps purpurea* PCCE1 was incubated with dihydrolysergic acid and the alkaloid extract separated by tlc, a new Van Urk's-positive spot was observed. The compound co-migrated with reference dihydroergotamine in three solvent systems. With 25 mg dihydrolysergic acid added per 25 ml culture medium, the yield in 9-day cultures was 1.2 mg of dihydroergotamine and 10 mg of ergotamine. A control with no dihydrolysergic acid added contained 13 mg of ergotamine.

The synthesis of lysergyl coenzyme A was attempted. However, the main product was nonfluorescent, indicating loss of the 9, 10 double bond of lysergic acid. Dihydrolysergyl coenzyme A was synthesized by the mixed anhydride method in good yield. The purified product had an absorbance maximum at 260 nm (adenine) and shoulders at 288 nm (indole) and 225 nm (thioether, ref. 6). After 5 min in 0.1 N NaOH, the shoulder at 225 nm disappeared and dihydrolysergic acid was identified as the hydrolysis product by tlc. Dihydrolysergyl coenzyme A was stable in 0.1 M sodium phosphate pH 7.5 and 0.15 M Tris-HCl pH 6.7 at 25° for at least four hr. It was completely hydrolyzed after 20 min. at 25° in 0.5% β-mercaptoethanol. Dihydrolysergyl coenzyme A was incubated with a cell-free system from a five-day culture of *C. purpurea* PCCE1 in the presence of L-proline, L-phenylalanine and L-[U-^{14}C]alanine, as well as ATP, Mg^{2+} and NADPH regenerating system. The dihydrolysergyl coenzyme A was completely converted to an unidentified Van Urk's-positive nonradioactive product. No dihydroergotamine was formed.

DISCUSSION

Dihydrolysergic acid could be utilized by *C. purpurea* PCCE1, an ergotamine-producing strain, with formation of dihydroergotamine. This ability to utilize derivatives of lysergic acid may be general for organisms which synthesize lysergic acid alkaloids. It would be of interest to determine what other structural modifications could be tolerated and whether pharmacologically interesting derivatives could be synthesized by this procedure.

Lysergyl coenzyme A has been proposed as an intermediate in the biosynthesis of the lysergyl alkaloids (7). Since the chemical synthesis of lysergyl coenzyme A was not successful because of the reactive 9,10 double bond, dihydrolysergyl coenzyme A was synthesized and tested for *in vitro* incorporation into dihydro-

ergotamine. When dihydrolysergyl coenzyme A was incubated in a cell-free system from *C. purpurea* PCCE1, the dihydrolysergyl coenzyme A was rapidly converted to an unknown product. Since the product did not contain alanine, a role for the unknown product in (dihydro) ergotamine biosynthesis is questionable. Since failure to observe dihydroergotamine biosynthesis could have resulted from the rapid loss of dihydrolysergyl coenzyme A in the cell-free system, the role of (dihydro) lysergyl CoA in (dihydro) ergotamine biosynthesis remains to be established.

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