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Production of Hydrocinnamic Acid by Clostridia

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Hydrocinnamic acid was found in acid extracts of spent growth medium from cultures of *Clostridium sporogenes*. The acid was identified by mass spectrometry and its identity was confirmed by gas chromatography. The acid was produced in relatively large amounts (2 to 3 μmoles/ml of medium) by *C. sporogenes*, toxigenic types A, B, D, and F of *C. botulinum*, and some strains of *C. bifermentans*. Other strains of *C. bifermentans* and strains of *C. sordellii* and *C. caproicum* produced only small amounts (0.1 to 0.4 μmoles/ml) of the acid. The acid was not detected in spent medium from toxigenic types C and E of *C. botulinum* or from 25 other strains representing eight *Clostridium* species. Resting cell suspensions exposed to *l*-phenylalanine produced hydrocinnamic and cinnamic acid; the latter compound probably functions as an intermediate in the metabolism of *l*-phenylalanine.

In earlier work in this laboratory, gas-liquid chromatography (GLC) was used to compare the fatty acid composition of various clostridia (5). It was observed that an unknown compound was present in the fatty acid extract of *Clostridium sporogenes* which distinguished this organism from 12 other *Clostridium* species. The compound, which was not identified, was acidic in nature and was readily converted to an ester by standard chemical procedures. The present report describes the identification of this compound as hydrocinnamic acid and reports its occurrence among various *Clostridium* species.

MATERIALS AND METHODS

Organisms. The cultures employed were obtained from a variety of sources and were identified by the Anaerobic Bacteriology Laboratory of the National Communicable Disease Center (3). The following 101 strains representing 13 species of the genus *Clostridium* were studied: 1 strain each of *C. histolyticum*, *C. tertium*, and *C. subterminale*; 2 strains each of *C. capitovale* and *C. difficile*; 3 strains each of *C. septicum* and *C. tetani*; 11 strains each of *C. caproicum* and *C. sordellii*; 6 strains of *C. sporogenes*; 28 strains of *C. bifermentans*; 20 strains of *C. botulinum*; and 12 strains of *C. perfringens*. Included among the *C. botulinum* and *C. perfringens* cultures were representative strains of each toxigenic type (A to F) of both species.

Growth media and culture procedures. Trypticase Soy Broth (TSB, BBL) was used as the growth medium. A growing culture was transferred from chopped-meat medium (3) to TSB and incubated anaerobically at 37 C for 15 hr. Growth from this transfer was used to inoculate a second tube of TSB (5 ml) which was incubated for 15 hr and extracted for acidic compounds.

Extraction procedure and derivative formation. The cultures were removed from incubation, acidified to approximately *p*H 2.0 with HCl, and extracted twice with 5-ml volumes of a 1:1 mixture of ethyl ether and hexane. The ether-hexane layers were combined into a small beaker and reduced to dryness under a gentle stream of nitrogen. Compounds in the dried extract were then converted to methyl ester or trimethylsilyl (TMS) derivatives for analysis by GLC. The methyl esters were prepared by transferring the dried extract to a screw-cap test tube (20 by 150 mm) by two successive rinses with 1-ml portions of Boron-Trichloride-Methanol Reagent (Applied Science Laboratories, Inc., State College, Pa.). The mixture was heated at 80 C for 5 min, cooled, and transferred to a separatory funnel containing 20 ml of the 1:1 ether-hexane mixture and 20 ml of distilled water. The container was shaken vigorously, and the ether-hexane layer was removed and evaporated to near dryness as described above. Anhydrous sodium sulfate was added, and the methyl esters were transferred to a small test tube. The sodium sulfate was washed with small portions of ether-hexane, and the ether-hexane was combined with the methyl esters to a final volume of 0.25 ml. The TMS derivative was made by adding 1 ml of Tri-Sil "Z" (Pierce Chemical Co., Rockford, Ill.) to the dried extract and transferring the mixture to a small, tightly stoppered test tube and heating at 70 C for 15 min. The heated sample was cooled, and 3 ml of chloroform was added. This mixture was washed four times by shaking with 3-ml portions of 4% HCl. After the fourth wash, the chloroform layer, which contained the TMS derivatives, was reduced to a final volume of 0.25 ml under a gentle stream of air.

GLC. The GLC analysis was routinely performed on a Perkin-Elmer model 900 instrument (Perkin-

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Elmer, Norwalk, Conn.) equipped with a hydrogen flame detector and a disc integrator recorder. The instrument was equipped with two 0.25 inch (6.3 mm, outside diameter) by 6 ft (1.83 meter) coiled glass columns. One column was packed with 3% SE-30 methyl silicone gum rubber coated on 80/100-mesh chromosorb P (Applied Science Laboratories, State College, Pa.); the other column contained 1% OV-17 (methyl phenyl silicone) coated on 80/100-mesh high performance Chromosorb G (Regis Chemical Co., Chicago, Ill.). Operating parameters for this instrument were: injection temperature, 285 C; detector temperature, 300 C; carrier gas, helium.

For analysis of methyl esters on the SE-30 and OV-17 columns and for analysis of the TMS derivatives on the OV-17 column, the column temperature was maintained at 130 C for 4 min and then programmed to 210 C at 6.5 C per min. For analysis of TMS derivatives on the SE-30 column, the column temperature was maintained at 100 C for 5 min and then programmed to 210 C at 6.5 C per min. Samples were also analyzed with a Barber-Colman model 5000 instrument equipped with a hydrogen flame detector. This instrument contained a U-tube glass column [8 ft (2.4 meter) by 5 mm (inside diameter)] packed with 3% tetracyanoethylated pentaerythritol (TCEPE), coated on 70–80 mesh Aeropack 30 (Varian Aerograph, Walnut Creek, Calif.). The operating parameters for this instrument were: injector, 250 C; detector, 275 C; column temperature, 165 C for methyl esters and 105 C for TMS derivatives; carrier gas, nitrogen.

RESULTS AND DISCUSSION

Isolation of unknown compound. The unknown compound from *C. sporogenes* was originally thought to be a component of the bacterial cell (5). Further study, however, showed that the compound was found in relatively large amounts in the spent growth medium and was completely removed from the cells by extensive washing with 0.85% saline solution or distilled water. The unknown compound was not completely resolved from other compounds in the spent growth medium by paper and thin-layer chromatography, but it was well separated by GLC. Therefore, isolation of the unknown compound was accomplished by preparative GLC procedures.

A preparative GLC glass column [0.5 inch (1.27 cm) by 16 ft (4.88 m)] packed with a 3% SE-30 stationary phase was placed in the Barber-Colman instrument. A splitter device (split ratio 1:9) was placed in the line leading from the column to the flame detector. One part of the effluent was passed to the detector and nine parts to a GLC fraction collector device mounted on the side of the instrument. Repeated injections (20 μliters) were made of methyl esters prepared from 250 ml of spent medium from the growth of *C. sporogenes* strain 392, and the unknown compound was collected for subsequent analysis and identification.

Identification of unknown compound. The methyl ester of the unknown compound was identified by mass spectrometry by the use of a Varian M-66 mass spectrometer (Varian Associates, Palo Alto, Calif.). A portion of the mass spectrum is shown in Fig. 1. The fragmentation pattern shows major ion peaks at *m/e* 164, 149, 133, 104, and 91. A possible pathway for the formation of these ions could result from the following fragmentation: the molecular ion of the unknown methyl ester, *m/e* 164, loses $-CH_3$ to form the *m/e* 149 ion and loses $-OCH_3$ to form the *m/e* 133 ion. The loss of -CHO from the *m/e* 133 ion produces the *m/e* 104 ion. The *m/e* 133 ion loses $-C_2H_2O$ to form the *m/e* 91 ion, which represents the benzyl ion or the fully aromatic tropylium ion (4). The presence of the tropylium ion (*m/e* 91) strongly indicated that the compound contained a benzene ring and was therefore an aromatic methyl ester. Analysis of the fragmentation pattern suggested that the molecular structure of the unknown compound was that of the methyl ester of hydrocinnamic acid (3-phenylpropionic acid).

Confirmation of the identity of the unknown compound was made by comparison with a known sample of hydrocinnamic acid (Eastman Organic Chemicals, Rochester, N.Y.). The methyl ester prepared from authentic hydrocinnamic acid gave a mass spectrum identical to that obtained for the unknown compound isolated from the spent medium of *C. sporogenes* 392 as described above. Further confirmation was obtained from GLC data in which the methyl ester and TMS derivatives of the commercial sample of hydrocinnamic acid were compared with the compound

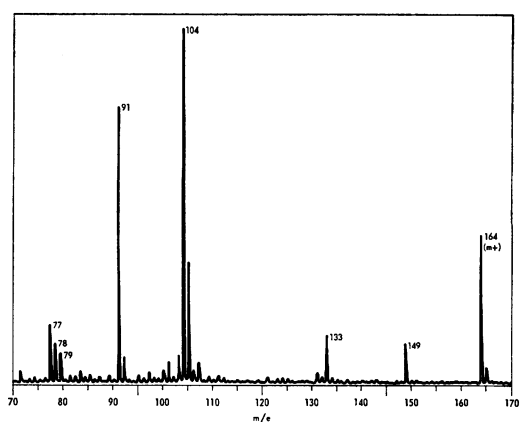

FIG. 1. *Mass spectrum of methyl ester of hydrocinnamic acid.*

TABLE 1. *Comparison of gas chromatography retention times of hydrocinnamic acid with unknown compound from C. sporogenes*

Sample	Derivative	Column packing materials[a]		
		SE-30	TCEPE	OV-17
		min	*min*	*min*
Hydrocinnamic acid	Methyl ester	2.5	5.7	4.6
Unknown	Methyl ester	2.5	5.7	4.6
Hydrocinnamic acid	TMS[b]	10.2	3.6	6.6
Unknown	TMS	10.2	3.6	6.6

[a] SE-30 = 3% SE-30 methyl silicone rubber gum; TCEPE = 3% tetracyanoethylated pentaerythritol; OV-17 = 1% phenyl methyl silicone.
[b] Trimethylsilyl.

produced by *C. sporogenes*. The derivatives were analyzed on polar (TCEPE), nonpolar (SE-30), and moderately polar columns (OV-17). Table 1 shows identical retention times for both derivatives on each of the three columns. These data together with the results of the mass spectrum firmly establish the identity of the unknown compound as hydrocinnamic acid.

Effect of growth media. In addition to TSB, four other common laboratory media were used to determine the effects of media on production of hydrocinnamic acid. Tubes containing 5 ml of chopped-meat medium (3), Heart Infusion Broth (Difco), Todd-Hewitt Broth (Difco), and Thioglycollate Medium (Fisher) were each inoculated with three strains of *C. sporogenes* and incubated anaerobically for 15 hr at 37 C. The cultures were extracted and tested for hydrocinnamic acid as described above. Relatively large amounts (2 to 3 μmoles per ml of medium) of hydrocinnamic acid were produced by each strain in each of the four media, and the amounts were comparable to those produced in TSB. In no case was there evidence of hydrocinnamic acid in uninoculated control media.

Resting cell studies. The production of relatively large amounts of hydrocinnamic acid in several media suggested that the precursor of this acid may be a simple compound common to each of the media. Phenylalanine, which is structurally related to hydrocinnamic acid and which was found in each medium, was thought to be a logical precursor. Therefore, resting cell studies were made to determine the enzymatic production of hydrocinnamic acid from *l*-phenylalanine. Cells from overnight growth of *C. sporogenes* in 250 ml of TSB were harvested by centrifugation and washed once with 0.85% NaCl solution, and then with phosphate buffer solution (*p*H 6.8) supplemented with 0.0001% pyridoxal phosphate (PBP buffer). A thick cell suspension was made in PBP buffer, and 1.0 ml of the suspension was added to 4 ml of PBP buffer containing 200 μmoles of *l*-phenylalanine. The reaction mixture was incubated anaerobically at 37 C for various periods of time up to 3 days. Control samples consisted of 4 ml of *l*-phenylalanine (i) without added cells and (ii) with the addition of heat-treated (100 C for 5 min) cells. For analysis, the supernatant fluid was tested for hydrocinnamic acid in the same manner as it was for growing cultures.

Resting cell studies were performed with three strains of *C. sporogenes* each of which produced hydrocinnamic acid from *l*-phenylalanine. Two strains produced relatively large amounts (2 to 3 μmoles/ml) of the acid, whereas the third strain produced only moderate amounts (0.5 to 1.5 μmoles/ml). No explanation can be given for this difference since each strain produced approximately equal amounts (2 to 3 μmoles/ml) of the acid in growing cultures. The heat-treated cell mixtures and uninoculated *l*-phenylalanine controls contained no detectable hydrocinnamic acid or other acidic components. In addition to hydrocinnamic acid, another compound subsequently identified as cinnamic acid was found in the acid extract from resting cells exposed to phenylalanine. Preliminary experiments showed relatively large amounts of cinnamic acid and small amounts of hydrocinnamic acid during early stages (5 hr) of incubation. As the incubation time was extended, the relative amount of cin-

TABLE 2. *Survey of hydrocinnamic acid (HCA)[c] production by various clostridia[a]*

Organism	No. of strains tested	No. of strains producing HCA	HCA/TSB[b]
Clostridium sporogenes	6	6	2–3[c]
C. botulinum (types A-F)	20	13	2–3
C. bifermentans	28	16	0.1–2
C. sordellii	11	2	0.2–0.3
C. caproicum	11	6	0.1–0.4

[a] Cultures were incubated anaerobically for 15 hr at 37 C in 5 ml of Trypticase Soy Broth.
[b] TSB, Trypticase Soy Broth. Ratio of HCA to TSB is expressed in micromoles of HCA to milliliters of TSB as a range.
[c] HCA determined by gas chromatography using a standard curve of known amounts of authentic HCA (determined as the methyl ester).

namic acid present decreased with a concomitant increase in hydrocinnamic acid. These data suggest that cinnamic acid may function as an intermediate in the formation of hydrocinnamic acid from *l*-phenylalanine.

Production of hydrocinnamic acid by growing cultures of various clostridia. The results of a survey of hydrocinnamic acid production in TSB by 101 strains representing 13 *Clostridium* species are shown in Table 2. Each of the six strains of *C. sporogenes* and 13 of 20 strains of *C. botulinum* produced relatively large amounts (2 to 3 μmoles/ml) of hydrocinnamic acid. Each strain of *C. botulinum* of toxigenic types A (three strains), B (five strains), D (two strains), and F (three strains) produced hydrocinnamic acid; none was produced by the two type C and the five type E strains. Of the *C. bifermentans* strains, fifty-seven per cent (16/28) produced the acid in amounts ranging from 0.1 to 2 μmoles/ml. The two *C. sordellii* and the six *C. caproicum* cultures produced only small amounts (0.1 to 0.4 μmoles/ml) of hydrocinnamic acid. No hydrocinnamic acid (<0.01 μmoles/ml) was detected in cultures of *C. perfringens* (types A–F), of 12 strains tested; *C. tetani*, of 3 strains tested; *C. capitovale*, of 2 strains tested; *C. septicum*, of 3 strains tested; *C. difficile*, of 2 strains tested; *C. histolyticum*, of 1 strain tested; *C. tertium*, of 1 strain tested; and *C. subterminale*, of 1 strain tested.

Little information is available on the production and metabolism of hydrocinnamic acid. The acid has been isolated from sheep rumen fluid (6), and is metabolized by *Achromobacter* (2) and *Pseudomonas* (1) to various phenyl hydroxy acids. To our knowledge, this is the first report which describes the production of hydrocinnamic acid by clostridia. The observation that only certain *Clostridium* species, and certain toxigenic types within a species, produce the acid may be useful in the identification and classification of these organisms. A rapid test for hydrocinnamic acid can be performed by GLC techniques as described in this report or by paper chromatography (1).

The studies with resting cell suspensions show that hydrocinnamic acid is derived from *l*-phenylalanine; they also indicate that cinnamic acid is a possible intermediate compound. The presence of a deaminase with a reaction mechanism similar to that of aspartase (7) could account for the production of cinnamic acid and ammonia; subsequent reduction of cinnamic acid could lead to the formation of hydrocinnamic acid.

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