The Production of Tryptamine from Tryptophan by *Bacillus cereus* (KVT)

By J. E. PERLEY* AND B. B. STOWE

Department of Biology, Yale University, New Haven, Conn. 06520, U.S.A.

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1. A strain of *Bacillus cereus* has been isolated that can produce tryptamine when grown in a broth containing tryptophan. 2. The conditions of culture under which this conversion is optimum, as well as the general pathways of tryptophan metabolism by this micro-organism, have been examined, and the information obtained has been used to obtain the first demonstration of cell-free tryptophan-carboxy-lyase activity. 3. The significance of these findings both to the current attempts to elucidate the pathways of metabolism of tryptophan in higher plants and to the published generalizations about the previously studied amino acid carboxy-lyases is discussed.

There are several reports of the isolation of microbial strains or microbial mixtures capable of carrying out the decarboxylation of tryptophan when grown in broth containing the amino acid. In each but one, it can be demonstrated that the report is based on insufficient evidence, or that it is invalid by virtue of errors in the extraction procedures employed.

Ewins & Laidlaw (1910) isolated a physiologically active base from such a broth in which unknown micro-organisms had been allowed to grow and claimed its picrate to be identical with that produced synthetically (Ewins, 1911) in the form of long colourless needles and having m.p. 145–146°. The picrate of authentic tryptamine (Manske, 1931) is in the form of orange-red polyhedral plates and melts at 116–118°. The identification of tryptamine in the culture medium of *Bacillus mesentericus vulgatus* by Grimmer & Wiemann (1921) is based solely on the percentage platinum content of the chloroplatinate of a base isolated from the broth, and neither comparison with authentic tryptamine nor a melting point is cited.

The report of tryptamine production by the fungal plant pathogens *Taphrina deformans* and *Dibotryon morbosum* (Crady & Wolf, 1959) must be discounted, for the compound 'identified' was, by the design of the extraction procedure, either an acidic or a neutral indole. A recent examination of *Taphrina* metabolism confirms this conclusion (Perley & Stowe, 1966).

Though the report of tryptamine production by a mixed whole-cell population of intestinal bacteria

(Weissbach, King, Sjoerdsma & Udenfriend, 1959) is convincing, attempts to culture such a heterogeneous population repeatedly with the intention of obtaining a starting material for the purification of the tryptophan carboxy-lyase would be difficult.

The present paper is concerned with the production of tryptamine from tryptophan by a single strain of *Bacillus cereus* and with the demonstration of cell-free preparations from this organism that show tryptophan-carboxy-lyase activity.

METHODS AND MATERIALS

Organisms. The strain of *Bacillus cereus* used in this study is an isolate from the Laboratory of Professor K. V. Thimann of Harvard University and differs from the type description of *Bacillus cereus* in Bergey's Manual only in its inability to ferment glycerol and sucrose. This is within the range of variation considered normal for this species by Smith, Gordon & Clark (1952). The organism is referred to below as *B. cereus* (KVT) to differentiate it from the American Type Culture strains (nos. 9620, 9818, 9139, 7004, 4342 and 2), which do not show the same ability to produce tryptamine under similar culture conditions.

The organism was grown with shaking at 25° in Wickerham's (1946) medium supplemented by glucose and acid-hydrolysed casein (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) at 20 g./l. and DL-tryptophan at 1 g./l. Growth was followed by using a 250 ml. Erlenmeyer flask fitted with a Klett side-arm. This medium was chosen because it contained no tryptamine after being autoclaved, in contrast with commercial nutrient broth, which contains tryptamine in concentrations of 1 µg./250 ml. of final medium. Inoculation was achieved by transfer of cells from stock nutrient-agar slants by use of a platinum loop and turbidity was measured by using uninoculated broth as a blank in a Klett-Summerson colorimeter fitted with a red filter.

* Present address: Department of Biology, Wayne State University, Detroit, Mich. 48202, U.S.A.

Preparation of cell-free extract. Cells from 200 ml. of culture medium were washed three times by repetitive centrifuging at 2500g in a Spinco model centrifuge at 0° in 0.05 M-phosphate buffer, pH 5.8, containing the inorganic salts of Wickerham's (1946) medium and 1 g. of DL-tryptophan/l. These cells were resuspended in 100 ml. of the same solution and were disrupted by use of 20 kcyc./sec. ultrasonic vibration generated by a model S75 Sonifier (Branson Instrument Co., Norwalk, Conn., U.S.A.). Exposure to ultrasound was for 1 min. periods interrupted by 30 sec. rests for a total of 5 min. Any residual cells were removed by centrifuging at 20000g for 30 min. at 0°. The protein in the supernatant, after dialysis for 24 hr. against four changes of Wickerham's inorganic salts that contained no tryptophan, was precipitated by the addition of sufficient solid $(\text{NH}_4)_2\text{SO}_4$ to give a 60% saturated solution.

Determination of pH optimum of tryptophan carboxy-lyase. The precipitated protein, for studies of pH optimum, was dissolved in a minimal volume (approx. 1.5 ml.) of phosphate buffer, pH 7.0, and divided into six portions (approx. 0.2 ml.). To each portion pyridoxal phosphate (0.05 mg.) dissolved in 0.1 ml. of 0.05 M-phosphate buffer, pH 7.0, was added. To these tubes 2 mg. of L-tryptophan dissolved in either phosphate buffer (pH 5-8) or KCl-NaOH- H_3BO_3 buffer (pH 7-10) was added until the total volume in each tube was 10 ml. pH values were determined by glass electrode after the addition of these buffers and incubation was allowed for 4 hr. The borate buffer was prepared as described by Koch & Hanke (1953) and at use was 0.05 M with respect to both H_3BO_3 and KCl. Control tubes were prepared in a similar manner but placed in a boiling-water bath for 5 min. and subsequently cooled before addition of the buffers containing tryptophan. Preparations lacking pyridoxal phosphate showed no conversion of tryptophan into tryptamine.

Extraction of indolic intermediates and products. Metabolic intermediates were isolated from media from which cells had been removed at various times after inoculation by partitioning against methylene dichloride (CH_2Cl_2) purchased from the Fisher Scientific Co., New York, N.Y., U.S.A.

The volume chosen for such extraction and identification of intermediates was 25 ml. and at inoculation contained 0.5 mg. of DL-tryptophan. These samples were adjusted to pH 2.0 with H_3PO_4 . The acidified medium was extracted three times with equal volumes of CH_2Cl_2 , a procedure that left only the basic indoles in the acidified aqueous medium. The acidic indoles present in the resulting organic phase were obtained by three washings with 25 ml. volumes of double-distilled water adjusted to pH 8.0 with saturated K_2CO_3 solution. With this procedure the neutral indoles are found in the organic phase and the acidic indoles in the basic aqueous phase. The aqueous phase can then be acidified to pH 2 with H_3PO_4 and partitioned against three equal volume (25 ml.) changes of CH_2Cl_2 . The basic indoles remaining in the original acidic aqueous phase can be obtained by raising the pH to 8.0 and partitioning in a similar manner against CH_2Cl_2 .

The resulting organic phases, containing separately the acidic, basic, and neutral indoles, were evaporated to dryness over warm water and under vacuum. The resulting residues were dissolved in a minimal volume of ethanol (approx. 0.2 ml. for thin-layer chromatography and paper electrophoresis) or acetonitrile (approx. 0.2 ml. for gas-liquid

chromatography). For thin-layer chromatography and paper electrophoresis the entire sample was used, whereas for gas-liquid-chromatographic resolution 0.5-10.0 μl . samples were removed for injection. About 96-98% of samples of authentic tryptamine, tryptophol and indolylacetic acid ranging in concentration from 1 to 100 μg ./25 ml. of uninoculated medium can be extracted reproducibly by using such a procedure. Tryptophan does not partition into CH_2Cl_2 at any pH and the extracts obtained are chromatographically free of this substrate.

The cell-free incubation mixtures (10 ml.) were adjusted to pH 8.0 with a saturated solution of K_2CO_3 and partitioned against three equal volume (10 ml.) changes of CH_2Cl_2 . This procedure resulted in the isolation of a mixture of basic and neutral indoles and provided an internal check of any further metabolism of the amine, which proceeded by *N*-acetylation. The disruption of washed whole cells of *B. cereus* (KVT) resulted in the absence of the acetylating reaction from the cell-free preparations. Such preparations demonstrated only the ability to form tryptamine when incubated on tryptophan, and the initial inclusion of authentic amine (5-50 μg ./cell-free incubation mixture) resulted in the recovery of the amount of amine usually formed by such a preparation, increased by the amount that had been added. At no time were those products expected from the enzymic oxidation of tryptamine extracted from any cell incubation system.

Thin-layer chromatography. The methods of Obreiter & Stowe (1964) were employed to achieve the thin-layer resolution of the extracted compounds. In each case where identifications have been made, chromatographic agreement with authentic compounds was obtained in a minimum of three solvents. Those most useful in the resolution of acidic indoles were: (1) propan-2-ol-aq. 28% (w/v) NH_3 -water (8:1:1, by vol.); (2) butan-1-ol-chloroform (3:2, v/v) saturated with 0.5 N-formic acid; (3) 3-methylbutan-1-ol saturated with aq. 2 N- NH_3 -butan-2-one (1:1, v/v). For basic indoles the solvents of choice were: (1) butan-1-ol-chloroform-water (4:1:5, by vol.); (2) propan-2-ol-aq. 28% (w/v) NH_3 -water (8:1:1, by vol.); (3) benzene-acetic acid (4:1, v/v) saturated with water. Neutral indoles were resolved most satisfactorily by: (1) butan-2-one-*n*-hexane (1:1, v/v); (2) butan-1-ol-chloroform (3:2, v/v) saturated with 0.5 N-formic acid; (3) light petroleum (b.p. 30-60°)-2-methylbutan-1-ol (3:2, v/v) saturated with 0.5 N-formic acid.

Gas-liquid chromatography. Gas-liquid chromatography was carried out by the methods of Stowe & Schilke (1963) by using a gas chromatograph equipped with an argon-ionization detector (^{90}Sr). U-shaped glass columns (6 ft. long 6 mm. inner diam.) were filled with 70-80-mesh Chromosorb W. This substrate was coated with an ethylenediamine linoleic polyamide (Versamid 900, final percentage of 11.7% on the column) prepared by General Mills Inc. (Minneapolis, Minn., U.S.A.). A second similar column was prepared with a fluorinated silicone (10% QF-1) manufactured by Dow-Corning (Dow-Corning QF-1-0065). The Versamid column was run at 240° at an argon flow rate of 100 ml./min. and the fluorinated silicone column at 212° and a gas flow rate of 120 ml./min. Indole samples were dissolved in 0.5-10.0 μl . of chloroform or acetonitrile and injected into the vaporizing block, which was maintained at a temperature 70-80° greater than that at which the column was held. Tryptophol and *N*-acetyltryptamine were

well separated at different retention volumes by both substrates.

Paper electrophoresis. Paper electrophoresis was carried out in a Durrum-type electrophoresis cell (Spinco, model-R) manufactured by Specialized Instruments Corp., Belmont, Calif., U.S.A. Two such cells, each containing eight narrow paper strips, could be run simultaneously. The 30 cm. strips were saturated with 0.05M-phosphate buffer, pH 5.8, and runs lasted for 5.5 hr. at a constant voltage of 110 v.

Synthesis of *N*-acetyltryptamine. Tryptamine hydrochloride (20 mg.) was dissolved in 10.0 ml. of distilled water and the free base was liberated by raising the pH of the solution to 8.5 with a saturated solution of K_2CO_3 . The amine was extracted into organic solvent by shaking with equal volumes of CH_2Cl_2 . The solvent was then removed under vacuum and 20.0 ml. of reagent-grade acetic anhydride added. The solution was left at room temperature overnight. The acetylated amine, being a neutral compound, could then be extracted into the CH_2Cl_2 , leaving any unchanged amine in the aqueous layer as the pH of the reaction mixture was less than 2.0. From the crude yellow oil resulting, crystals were obtained that were recrystallized from light petroleum in the form of long colourless needles having m.p. 143–145° (Spath & Lederer, 1930).

Colorimetric assays. After extraction and subsequent electrophoresis, tryptamine was qualitatively estimated by spraying with Ehrlich's reagent [ethanol–10N-HCl (4:1, v/v) containing 2% (w/v) of *p*-dimethylaminobenzaldehyde (Ehrlich & Jacobsen, 1911; Stowe & Thimann, 1954)] and then comparing the band width and the intensity of the blue-green colour formed with those of samples of authentic amine run in an identical manner.

The quantitative determination of tryptamine was achieved by use of a modification of the Salkowski reagent (1885), the most frequently used version of which is found in the work of Gordon & Weber (1951). As used in the present study, the reagent contained $HClO_4$ (3.5N) and Fe^{3+} as $Fe(NO_3)_3$ (5mM) in the final reaction mixture.

As the coloured products formed by both tryptamine and tryptophan show virtually identical absorption spectra with maxima at about 450 m μ as measured in a Bausch and Lomb Spectronic 20 spectrophotometer, preliminary extraction of the amine was necessary for quantitative determination.

After the tryptamine present had been extracted and the extract submitted to electrophoresis, those sections of the air-dried electrophoresis strips 8–10.5 cm. from the origin and towards the cathode were removed and extracted with CH_2Cl_2 . After removal of the solvent under vacuum, 2.0 ml. of distilled water and 2.0 ml. of the reagent were added such that the final concentrations of acid and Fe^{3+} were as given above.

Colours formed by use of the Salkowski reagent as given above were read in a Klett–Summerson colorimeter with a blue Corning glass filter (no. 5031), which has a transmission range 400–510 m μ . Colours were read against a reagent-water blank and were compared with those formed when the Salkowski reagent was added to samples containing known concentrations of amine ranging from 1 to 120 μ g./ml. Maximum colour is obtained in 60 min. when development is allowed in white fluorescent light (approx. 750 ft.-candles). Within this range the yellow colour produced is proportional to amine concentration and 10 μ g. of authentic tryptamine gives a Klett value of 40 units.

Protein determinations were made by the methods of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as the standard.

RESULTS

The presence of tryptamine after 5 days' growth of *B. cereus* (KVT) in growth medium containing tryptophan was demonstrated by the presence in the basic extracts of this medium of a compound whose thin-layer-chromatographic properties were identical with those of authentic tryptamine in the three solvents cited above, whose electrophoretic behaviour was identical with that of the authentic compound and whose colour reactions with both the Ehrlich and Salkowski reagents were likewise identical with those of the authentic amine. No such tryptamine production could be observed resulting from the incubation of A.T.C.C. strains of *B. cereus* (nos. 9620, 9818, 9139, 7004, 4342 and 2). The amounts of tryptamine accumulating in the medium represent a 0.4% conversion of tryptophan.

It could be shown by standard manometric techniques that washed whole cells, removed from medium that contained tryptamine and incubated in buffer containing lysine at pH 5.8, exhibited a pronounced production of carbon dioxide when a comparison was made with control respiration levels, indicating the presence of a carboxy-lyase for lysine in this organism. Though cell-free preparations obtained from the same weight of cells were about 2½ times as effective in carrying out the same reaction, similar indications of the ability of these cells under the same conditions to decarboxylate tryptophan were lacking.

To obtain washed whole cells capable of decarboxylating tryptophan it became necessary to define the general pathway(s) of tryptophan metabolism by this organism, and to discover the conditions of culture under which the observed amine production was optimum.

Fig. 1 represents the growth and pH changes in the growth medium of this organism as a function of time.

Point 1 represents the point in time at which tryptamine first appears in the medium, and point 2 the first appearance of a neutral indole that has been shown to have chromatographic properties identical with those of synthetic *N*-acetyltryptamine (Fig. 2). The low- R_f neutral 'compound' seen can be shown to be a compound present in autoclaved non-inoculated medium and to yield 11 ninhydrin-positive spots after alkaline hydrolysis, one of which chromatographs identically with tryptophan. Growth of *B. cereus* (KVT) on medium containing this peptide as the sole nitrogen source did not result in tryptamine production.

Acid indoles identified, by the methods listed

Fig. 1. pH changes (Δ) and growth ($\circ$) of *B. cereus* (KVT) as a function of time at 25°. Inoculation was by platinum-loop transfer of cells from stock agar slants into 500 ml. Erlenmeyer flasks containing 125 ml. of broth as described in the text.

above, as tryptophan metabolites of this organism include indol-3-ylpyruvic acid, indol-3-ylacetic acid, indol-3-yl-lactic acid, anthranilic acid and *N*-acetyltryptophan. Neutral indoles found included tryptophol, indole-3-carboxaldehyde and *N*-acetyltryptamine. The indol-3-ylpyruvic acid was identified by virtue of its characteristic pattern of decomposition products (Kaper & Veldstra, 1958; Schwarz & Bitancourt, 1960; Winter, 1964).

If washed whole cells are incubated separately with the indicated intermediates of tryptophan metabolism, it is possible by identification of the resulting products to obtain an idea of the overall metabolic pathway for tryptophan in this organism. Table 1 indicates the results of such experiments.

Tryptamine is observed only after the pH of the medium begins to rise on day 5 of culture, and artificial raising of the pH of the growth medium to pH 8.0 with 0.1*N*-sodium hydroxide or 0.2*M*-potassium carbonate after the culture has reached its maximum density (12 hr.) causes the amine to accumulate in the medium within 5 hr. of the alteration of pH. In addition, under such conditions, two new neutral compounds are produced. Incubation of authentic tryptophol with acetic anhydride overnight at room temperature results in the production of two compounds whose chromatographic behaviour is identical with those of the new compounds in the medium, suggesting that the new

Fig. 2. Thin-layer-chromatographic separation of indoles produced by *B. cereus* (KVT). The solvent used in separating the neutral indoles was butan-2-one-*n*-hexane (1:1, v/v), that for bases was butan-1-ol-acetic acid-water (4:1:5, by vol.), and that for acids was butan-1-ol-chloroform (3:2, v/v) saturated with 0.5*N*-formic acid. Neutral indoles: (1) 20 μ g. of authentic tryptophol; (2) 20 μ g. of authentic indole-3-carboxaldehyde; (3) 20 μ g. of synthetic *N*-acetyltryptamine; (4) the neutral indoles extracted from *B. cereus* (KVT) growth medium after 8 days of growth at 25°. Basic indoles: (5) 20 μ g. of authentic tryptamine; (6) the basic indoles of 8-day-old *B. cereus* (KVT) growth medium. Acidic indoles: (7) 20 μ g. of authentic indol-3-ylacetic acid; (8) 30 μ g. of aged indol-3-ylpyruvic acid; (9) the acidic indoles extracted from the same *B. cereus* (KVT) medium.

Table 1. *Metabolism of tryptophan derivatives by washed whole cells of B. cereus* (KVT)

In each case washed whole cells were removed from 50.0 ml. of growth medium 8 days after inoculation and, after repeated washing in 0.05*M*-phosphate buffer, pH 8.0, resuspended in 25 ml. of the same buffer containing 10.0 mg. of the indicated indoles. Products were extracted after incubation for 2 hr. with shaking at 30° and identified as described in the text.

Substrate for incubation	Observed products
Tryptophan	Tryptamine <i>N</i> -Acetyltryptophan Indolylpyruvic acid decomposition products Tryptophol Indolylacetic acid Anthranilic acid
<i>N</i> -Acetyltryptophan	Same as for tryptophan
Tryptamine	<i>N</i> -Acetyltryptamine
Indolyl-lactic acid	Tryptophol Indolylacetic acid

compounds may be acetylated derivatives of tryptophol.

Cells of *B. cereus* (KVT) harvested from 25 ml. of Wickerham's medium 24 hr. after inoculation, and 12 hr. after the pH has been artificially raised, can be shown to produce tryptamine on incubation in 1 g./l. concentrations of tryptophan dissolved in a solution of Wickerham's salts containing 0.1 mg. of pyridoxal phosphate. Incubation of such a weight of cells (usually 25 mg. fresh wt.) yields an incubation mixture containing 25–30 μ g. of tryptamine/ml. within 2 hr. Though this concentration does not change with time, the amount of extractable *N*-acetyltryptamine does increase with time, being present at concentrations of 5 μ g./ml. after 2 hr. and reaching 40–45 μ g./ml. after incubation for 4 hr. The presence of pyridoxal phosphate is essential to the demonstration of this conversion in both whole-cell and broken-cell preparations.

In broken-cell preparations, usually totalling 1.2–1.5 mg. of protein, activity can be observed in the supernatant obtained after disruption by ultrasound if the incubation mixtures contain the elements listed above. Dialysis of the original supernatant against four changes of 0.05 M-phosphate buffer, pH 7.0, does not result in a decrease in the activity subsequently observed. From incubation mixtures containing a doubled weight of protein,

approximately twice the amount of tryptamine can be extracted. The production of 15 μ g. of tryptamine was observed in 25 ml. of reaction mixture (pH 8), containing a total of 1.4 mg. of protein, after incubation for 4 hr. A similar incubation mixture containing 2.5 mg. of protein after the same time produced 27 μ g. of tryptamine. No tryptamine could be extracted from incubation mixtures of tryptophan and pyridoxal phosphate or in incubation mixtures containing an equivalent amount of boiled supernatant and tryptophan.

The concentration of amine produced by such preparations may be taken to be a measure of the activity of the tryptophan carboxy-lyase, since the amine is not further metabolized by the broken-cell preparation under the experimental conditions used.

Accordingly, the pH optimum for the tryptophan carboxy-lyase of *B. cereus* (KVT) was determined on the cell-free extract (Fig. 3).

DISCUSSION

It is suggested that Scheme 1 represents the pathways by which tryptophan is metabolized by *B. cereus* (KVT).

In his comprehensive review of the studies on bacterial amino acid decarboxylases (carboxy-lyases), Gale (1946) states that no single strain among the more than 1000 strains of microorganisms investigated in his Laboratory could decarboxylate tryptophan, or any of the common amino acids other than the six for which enzymes had been described, namely arginine, histidine, glutamic acid, lysine, ornithine and tyrosine. The highest specific activity of tryptophan-carboxy-lyase preparations encountered in the present work was 17 μ moles of substrate converted/mg. of protein/hr. This activity is such that, even at optimum pH, the enzyme would have been overlooked in the manometric methods employed by these earlier workers.

The optimum pH 8 observed in these studies sets it apart from the other amino acid carboxy-lyases,

Fig. 3. Effect of pH on the activity of tryptophan carboxy-lyase. The reaction mixtures contained the following components in a total volume of 25.0 ml.: 0.1 mg. of pyridoxal phosphate, 10.0 mg. of L-tryptophan, 0.05 M-phosphate or NaCl–NaOH–H₃BO₃ buffer at the indicated pH and 1.4 mg. of protein. Incubation was for 4 hr. with shaking at 30°. Tryptamine was estimated as described in the Materials and Methods section.

Scheme 1.

all of which have pH optima less than 6.0. This fact must be taken as the first evidence against the previously inclusive generalizations of Gale (1946) that the amino acid carboxy-lyases function to raise the pH of an excessively acidic medium and that 'in an alkaline environment, the formation of amino acid decarboxylases is completely suppressed, and that amino acids are attacked instead by deamination ...'. The ability of washed whole cells harvested late in the culture of *B. cereus* (KVT) to produce both tryptamine and indolylpyruvic acid indicates that both pathways are functional at the same time in this organism.

The acetylation of indoles such as tryptamine, tryptophol and tryptophan at high pH values by *B. cereus* (KVT) suggests that the enzyme responsible for this acetylation is most likely non-specific, and also that this strain of *B. cereus* should be of use in the synthesis of unusual compounds of similar structure, such as melatonin.

The low levels of activity observed, which necessitate extraction of the product before assay, may be due to any of a number of factors. The enzyme may be present in small quantities in the organism, or it may be unstable, as is the corresponding carboxy-lyase for ornithine, or its synthesis may be repressed under the conditions of culture employed. Although the rates observed are low, this work represents the first isolation of a single microbial species that can effect the decarboxylation of tryptophan.

Tryptamine has been supposed to be an intermediate in the biosynthesis of the plant growth hormone indolylacetic acid in the tissues of higher plants (Stowe, 1959; Mahadevan, 1963). This role has been assigned despite the lack of evidence of a functional tryptophan carboxy-lyase in these same tissues. The further study of the properties of this enzyme should provide the plant biochemist with new facts with which he may investigate the possibility that the decarboxylation of tryptophan is a limiting step in the biosynthesis of indolylacetic acid in higher plants.

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