Tryptophan Catabolism During Sporulation in *Bacillus cereus*

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1. Two intermediates of tryptophan catabolism were isolated from a sporulating culture of *Bacillus cereus* and identified as anthranilic acid and kynurenine by their spectral properties. 2. During sporulation the rate of formation of anthranilic acid and kynurenine by whole cells increased and reached a maximum at the pre-spore stage. 3. The specific activities of tryptophan pyrrolase and formylase also increased during sporulation and exhibited a maximal activity at the pre-spore stage. 4. Kynureninase activity reached a maximum during early stages of sporulation and then started to decline. 5. There was a net increase in the activity of tryptophan pyrrolase when cells were grown in the presence of L-tryptophan or DL-kynurenine. 6. The cultures exhibited the maximal activity of kynureninase 2 h earlier in the presence of DL-kynurenine whereas L-tryptophan delayed the appearance of the maximal activity by 2 h. 7. The omission of glucose from the medium had no effect on the pattern of development of tryptophan pyrrolase during growth and sporulation. 8. On the addition of tryptophan to a chemically defined medium no significant change in the pattern of development of tryptophan pyrrolase was observed.

The differentiation of a vegetative cell into a dormant spore is a complex process during which a bacterium undergoes marked metabolic and morphological changes. The regulation of the sequence of structural and biochemical changes leading to spore formation is not clearly understood. In an attempt to correlate the biochemical and structural events, several investigations have been reported on the pattern of development of enzymes of nucleic acid, carbohydrate, acetate and phosphorus metabolism during growth and sporulation in bacilli (Hanson, Srinivasan & Halvorson, 1963; Falaschi & Kornberg, 1966; Gardner & Kornberg, 1967; Tono & Kornberg, 1967; Deutscher & Kornberg, 1968; Warren, 1968; Mandelstam, 1969). The development of enzymes follows markedly different patterns during differentiation. Studies on the metabolism of amino acids demonstrated rapid turnover, oxidation and interconversion of several amino acids during sporulation (Bernlohr, 1964). However, little is known about how the catabolism of amino acids contributes to the synthesis of spore structures.

We now report our investigations on tryptophan catabolism during spore formation in *Bacillus cereus*. Fluorescent catabolites of tryptophan were isolated from a sporulating culture of *B. cereus* and identified as kynurenine and anthranilic acid. The enzyme systems responsible for the conversion of tryptophan into anthranilic acid via kynurenine

were shown to be present. These results have been reported in a preliminary form (Prasad & Srinivasan, 1969).

MATERIALS AND METHODS

Organism. Most of the experiments were carried out with *Bacillus cereus* TR-2, a bacteriophage-resistant strain derived from *B. cereus* strain T. In some experiments *B. cereus* 49 (obtained from J. Wolf, University of Leeds, U.K.) was used. These organisms were maintained on nutrient agar (Difco) slants.

Media. *B. cereus* TR-2 was grown on either medium G (Stewart & Halvorson, 1953) or a chemically defined medium (CDGS) of Nakata (1964). The minimal medium (GGMS) used for the growth and sporulation of *B. cereus* 49 had the following composition: G minerals (Stewart & Halvorson, 1953), 0.5% glucose and 0.5% sodium glutamate. The salts, glucose and sodium glutamate solutions were sterilized separately by autoclaving and combined after cooling.

Growth and sporulation conditions. The bacteria were grown at 30°C with forced aeration in a 15-litre carboy. The morphological changes were followed by examination of the culture under a phase-contrast microscope. Growth was determined turbidimetrically at 630 nm with a Bausch and Lomb Spectronic 20 instrument. The pH was measured on a Corning model 7 pH-meter. The time at which the pH of the culture reaches a minimum is referred to as t_0 . Under our cultural conditions t_0 was also the time at which exponential growth ceased. Fig. 1 presents the correlation between the time-scale (t_0 , t_2 etc.) and the stages in the process of sporulation.

Fig. 1. Morphological and biochemical events during sporulation of *B. cereus* TR-2 (adapted from Kay & Warren, 1968). DPA, 2,6-dipicolinic acid.

Paper chromatography. The following solvent systems were used for ascending paper chromatography on Whatman no. 1 paper: A, butan-1-ol-acetic acid-water (4:1:1, by vol.); B, aq. 80% (v/v) propan-1-ol; C, aq. 20% (w/v) KCl. The different intermediates on paper were detected and identified by their characteristic fluorescence under u.v. light and their R_F values.

Ion-exchange chromatography. The catabolites of tryptophan were fractionated on a column (1.3 cm diam. $\times$ 21 cm long) of Amberlite IR-120 (H^+ form; 200–500 mesh) by the method of Benassi, Veronese & De Antoni (1967) with the following modifications: the column was run at room temperature (25–26°C) and elution was accomplished with a gradient between 0.1M-pyridine-formic acid, pH 2.6 and 0.4M-pyridine-formic acid, pH 6.5. Formic acid (95%, v/v) was used to adjust the pH of the pyridine solutions. In all, 200 fractions (each 5 ml vol.) were collected at a flow rate of 20 ml/h.

U.v. and i.r. spectra. U.v. spectra were taken on a Beckman model DB spectrophotometer and i.r. spectra were measured with KBr pellets on a Perkin-Elmer model 137 spectrophotometer.

Analytical methods. Kynurenine and anthranilic acid were determined by the method of Mason & Berg (1952). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as the standard.

Preparation of cell-free extracts. Washed cells from 1 litre of culture were resuspended in 10 ml of 0.4M- KH_2PO_4 adjusted to pH 7.0 with 1M-KOH and disintegrated by sonication with a Branson ultrasonic drill. Pulses of 30 s were used with 1 min intervals between the pulses. The temperature of the samples was not allowed to rise above 10°C during sonic treatment. Cells were sonicated until 60–80% cell breakage was obtained. The samples used for the assay of tryptophan pyrrolase were

broken in the presence of 0.3 mM-L-tryptophan. The sonicated cells were centrifuged at 35000g for 30 min in a Servall RC-2 refrigerated centrifuge and the supernatant liquid was dialysed overnight against 0.04M- KH_2PO_4 adjusted to pH 7.0 with 1M-KOH; sufficient L-tryptophan was added to the buffer to bring its concentration to 0.03 mM. The non-diffusible material was used for the enzyme assay.

Enzyme assays. Tryptophan pyrrolase (L-tryptophan-oxygen oxidoreductase; EC 1.13.1.12) was assayed at 30°C in a Gilford model 2400 recording spectrophotometer by measuring the rate of increase in extinction at 321 nm ($\epsilon_{321\text{ nm}} = 3.0 \times 10^3$). The reaction mixture (2.5 ml) contained 0.02M-L-tryptophan, pH 7.0 (0.45 ml), 0.04M-dithiothreitol (0.1 ml), 0.5M-potassium phosphate buffer, pH 7.0 (0.5 ml), cell-free extract (equivalent to 10 mg of protein) and water to make 2.5 ml.

Formylase (aryl-formylamine amidohydrolase; EC 3.5.1.9) was assayed at 30°C by the rate of increase in extinction at 360 nm in a Gilford model 2400 recording spectrophotometer according to the method of Knox (1955) ($\epsilon_{360\text{ nm}} = 38.2 \times 10^5$). The reaction mixture (2.5 ml) contained 0.01M-N-formyl-DL-kynurenine, pH 7.0 (0.2 ml), 0.5M-potassium phosphate buffer, pH 7.0 (0.5 ml), cell-free extract (equivalent to 10 mg of protein) and water to make 2.5 ml.

Kynureninase (L-kynurenine hydrolase; EC 3.7.1.3) was assayed by the disappearance of kynurenine at 30°C in a Gilford model 2400 recording spectrophotometer according to the method of Dalgliesh, Knox & Neuberger (1951). The reaction mixture (2.5 ml) contained 0.75 μ mol of DL-kynurenine, 20 μ g of pyridoxal phosphate, 250 μ mol of tris-HCl buffer, pH 8.5, and water to make 2.5 ml. The disappearance of kynurenine was measured by the decrease in extinction at 360 nm ($\epsilon_{360\text{ nm}} = 38.2 \times 10^5$).

Enzyme unit. One unit of enzyme is defined as that amount which catalyses the appearance or disappearance of 1nmol of product or substrate/h at 30°C under the conditions of assay described in the text.

Chemicals. DL-Kynurenine was purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. L-Tryptophan was obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Anthranilic acid was a product of W. H. Curtin and Co., New Orleans, La., U.S.A. N-Formyl-DL-kynurenine was synthesized by the method of Auerbach & Knox (1957).

RESULTS

Isolation and purification of tryptophan catabolites from a sporulating culture. Medium G in a 40-litre carboy was inoculated with 2 litres of actively growing culture of *B. cereus* and was maintained at 30°C under forced aeration. At 4 h 20 g of L-tryptophan was added and the culture was allowed to reach the pre-spore stage. Then 200 ml of acetic acid was added to the culture and the mixture was kept overnight at 4°C. The contents of the carboy were filtered through Whatman no. 1 paper and the precipitate was discarded. Then 0.45 kg of activated charcoal (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) was added to the filtrate and stirred overnight at room temperature (26–27°C). The above mixture was once again filtered through Whatman no. 1 paper. The precipitate was shaken for 2 h with 2 litres of pyridine-methanol-water (1:1:2, by vol.) at room temperature 26–27°C and filtered. The precipitate was discarded and the filtrate was concentrated to dryness at 40–50°C under reduced pressure. The residue was resuspended in 200 ml of water and freeze-dried. The crude material obtained was dissolved in the minimum amount of 0.1 M-pyridine-formic acid buffer, pH 2.6, loaded on to an Amberlite IR-120 column and eluted with pyridine-formic acid buffer as described in the Materials and Methods section. The eluate from the column contained five

major fluorescent components. Two of them, termed compounds A and K, were selected for further purification and characterization. They were purified by repeated ascending chromatography on Whatman no. 1 paper with solvent systems A and C defined in the Materials and Methods section.

Characterization of compounds A and K. R_F values of the two compounds in different solvent systems are presented in Table 1; for comparison the values of kynurenine and anthranilic acid are also included. A red-purple colour with ninhydrin reagent indicated the presence of a primary amino group in compound K. A yellow reaction to Bromocresol Green suggested a carboxyl group in both the compounds. Colour reactions with Ehrlich's reagent [2% (w/v) *p*-dimethylaminobenzaldehyde in 5% (v/v) hydrochloric acid] and Pauly's reagent [0.5% (w/v) sulphanilic acid in 2% (v/v) hydrochloric acid and aq. 0.5% sodium nitrite in equal volumes, followed by 10% (w/v) sodium carbonate] indicated the possibility that compounds A and K were anthranilic acid and kynurenine respectively. The u.v. and i.r. spectra of compounds A and K were superimposable on those of anthranilic acid and kynurenine respectively.

Experiments on the formation of kynurenine and anthranilic acid by whole cells. The ability of cells, harvested at different times during the progress of sporulation, to oxidize tryptophan and kynurenine was determined in the following manner. Portions (each 1 litre) of an active culture were removed at intervals and chilled to 4°C. The cells were harvested by centrifugation at 12000g for 10 min in a Servall RC-2 refrigerated centrifuge. Cells were washed once with medium G and once with medium A (Srinivasan & Halvorson, 1963) containing chloramphenicol (250 µg/ml). Washed cells were resuspended in medium A supplemented with the same amount of chloramphenicol. The following experiment was carried out in 50 ml Erlenmeyer

Table 1. *Chromatography and colour reactions of some catabolites of tryptophan*

Ascending chromatography on Whatman no. 1 paper was used with the solvents defined in the Materials and Methods section. Chromatograms were run for 16–20 h in solvents A and B and for 3–4 h in solvent C.

Properties	DL-Kynurenine	Compound K	Anthranilic acid	Compound A
R_F values in:				
Solvent A	0.44	0.44	0.90	0.90
Solvent B	0.32	0.33	—	—
Solvent C	0.73	0.73	0.58	0.58
Fluorescence under u.v. light	Strong pale blue	Strong pale blue	Blue-violet	Blue-violet
Colour reactions with:				
Ninhydrin	Red-purple	Red-purple	—	—
Ehrlich's Reagent	Orange	Orange	Yellow	Yellow
Pauly's Reagent	Brown	Brown	—	—

flasks, which were incubated for 1 h on a rotary shaker maintained at 30°C. The reaction mixture consisted of 6 ml of cell suspension (20 mg of protein) and 1 ml of substrate (10 μ mol). After 1 h 1 ml of 100% (w/v) trichloroacetic acid was added to the flask and the mixture shaken for another 5 min. The contents of the flask were transferred to a centrifuge tube. The flask was washed with 2 ml of water and the washing was also transferred to the same centrifuge tube. The mixture was centrifuged at 12000g for 10 min in a Servall RC-2 centrifuge maintained at 4°C. The supernatant was used for the assay of kynurenine and anthranilic acid.

The production of kynurenine from tryptophan and of anthranilic acid from tryptophan or kynurenine by chloramphenicol-inhibited cells of *B. cereus* harvested during various stages of sporulation is presented in Fig. 2. The rate of formation of kynurenine and anthranilic acid from tryptophan

started rising at t_2 (20 nmol/h per mg of protein) and reached a maximum (50–60 nmol/h per mg of protein) at t_8 . However, if kynurenine was used as the substrate, the rate of formation of anthranilic acid increased about eightfold from that at t_2 and reached its maximum between t_8 and t_{10} .

Enzyme activities in cell-free extracts of B. cereus. The specific activities of three enzymes involved in tryptophan catabolism were determined in extracts of cells harvested at different stages of sporulation, and are shown in Table 2. Two markedly different patterns of development of the enzymes were observed. The activities of tryptophan pyrrolase and formylase started increasing at t_1 and reached a maximum at t_8 . On the other hand, kynureninase activity attained its maximum at t_4 and then gradually declined.

Effect of nutritional conditions on the development of tryptophan-catabolic enzymes during sporulation.

Fig. 2. Transformation of tryptophan and kynurenine by chloramphenicol-inhibited cells of *B. cereus* TR-2. Details of this experiment are given in the Results section. Δ , Anthranilic acid formed from kynurenine; $\blacktriangle$, kynurenine formed from tryptophan; $\circ$, anthranilic acid formed from tryptophan; $\bullet$, pH; $\times$, E_{600} .

Table 2. *Tryptophan pyrrolase, formylase and kynureninase activities during sporulation*

Assay methods are described in the Materials and Methods section.

Age	Specific activity (nmol/h per mg of protein)		
	Tryptophan pyrrolase	Formylase	Kynureninase
t_{-2}	13	0	11
t_0	14	0	13
t_2	41	7	43
t_4	41	41	167
t_6	76	66	115
t_8	156	124	38
t_{10}	117	83	52

Table 3. *Effect of the cultural conditions on the pattern of development of tryptophan pyrrolase*

A 10-litre medium G culture of *B. cereus* TR-2 was grown in a 15-litre carboy as described in the Materials and Methods section. At time t_1 the culture was divided into two 5-litre portions in separate carboys. In one carboy L-tryptophan (100 μ g/ml) or DL-kynurenine (100 μ g/ml) was added and the other was maintained as the control. At various intervals samples were taken for the assay of protein and enzymic activity in extracts as described in the Materials and Methods section.

Age	Specific activity (nmol/h per mg of protein)			
	Set A		Set B	
	Medium G	Medium G + L-tryptophan	Medium G	Medium G + DL-kynurenine
t_{-2}	11	—	10	—
t_0	15	—	18	—
t_2	37	19	32	60
t_4	37	34	60	108
t_6	68	156	89	122
t_8	137	196	73	119
t_{10}	105	198	62	113

Table 4. *Effect of the cultural conditions on the pattern of development of kynureninase*

Details of the experiment were as described in Table 3.

Age	Specific activity (nmol/h per mg of protein)			
	Set A		Set B	
	Medium G	Medium G + L-tryptophan	Medium G	Medium G + DL-kynurenine
t_{-2}	10	—	12	—
t_0	18	—	13	—
t_1	20	20	22	28
t_2	45	44	41	191
t_4	71	44	160	133
t_6	48	65	110	130
t_8	51	58	47	132
t_{10}	50	60	37	132

Tables 3 and 4 present the specific activities of tryptophan pyrrolase and kynureninase in the extracts of *B. cereus* TR-2 cells grown in medium G in the presence or absence of L-tryptophan or DL-kynurenine. There was a net increase in the activity of tryptophan pyrrolase when cells were grown in the presence of L-tryptophan or DL-kynurenine without any change in the pattern of the development of the enzyme. On the addition of DL-kynurenine, the immediate substrate of kynureninase, the culture exhibited the maximal kynureninase activity about 2 h earlier than the control. On the other hand, in the presence of tryptophan the maximal activity of kynureninase was delayed by 2 h.

The effect of glucose and tryptophan on the development of tryptophan pyrrolase activity in a *B. cereus* TR-2 culture is presented in Table 5. The

presence of 0.1% glucose in medium G had no effect on the pattern of development of tryptophan pyrrolase during growth and sporulation. The effect of tryptophan was studied in a chemically defined medium (CDGS) to determine whether it results in a change in the activities of tryptophan pyrrolase in the vegetative and sporulating cultures. On the addition of tryptophan in CDGS medium no significant change in the pattern of development of tryptophan pyrrolase was noticed.

The results from a typical experiment performed with *B. cereus* 49, an organism that has properties intermediate between those of *B. cereus* and *B. megaterium*, is presented in Table 6. This organism was chosen because of its ability to grow and sporulate well in a chemically defined medium (GGMS) composed of glucose, glutamate and G minerals.

Table 5. *Effect of media on the development of tryptophan pyrrolase activity in a B. cereus TR-2 culture*

The 10-litre cultures of *B. cereus* TR-2 were grown in 15-litre carboys as described in the Materials and Methods section. The cultures were inoculated with 1 litre of actively growing vegetative cells. Washed vegetative cells grown in medium G were used to inoculate CDGS-medium cultures. At various intervals samples were taken for the assay of protein and enzymic activity in extracts as described in the Materials and Methods section.

Expt. no.	Medium	Age of culture (h)	pH	Morphology	Specific activity (nmol/h per mg of protein)
1	Medium G	1.0 (t_{-3})	7.0 (decreasing)	Vegetative cells	5
		2.0 (t_{-2})	6.9 (decreasing)	Vegetative cells	7
		5.0 (t_1)	5.1 (increasing)	Sporulating cells	52
	Medium G minus glucose	1.0 (t_{-3})	7.2 (decreasing)	Vegetative cells	2
		2.0 (t_{-2})	7.1 (decreasing)	Vegetative cells	12
		5.0 (t_1)	7.1	Sporulating cells	86
2	CDGS medium	2.0 (t_{-2})	6.4	Vegetative cells	8
		4.0 (t_0)	6.4	Vegetative cells	14
		9.0 (t_5)	6.4	Sporulating cells	115
	CDGS medium + L-tryptophan (100 μ g/ml)	2.0 (t_{-2})	6.4	Vegetative cells	10
		4.0 (t_0)	6.4	Vegetative cells	13
		9.0 (t_5)	9.0	Sporulating cells	118

Table 6. *Effect of L-tryptophan on the development of tryptophan pyrrolase activity in B. cereus 49 culture grown in GGMS medium*

Two 10-litre cultures of *B. cereus* 49 were grown in 15-litre carboys as described in the Materials and Methods section. One of the cultures was grown in GGMS medium and the other in GGMS medium containing L-tryptophan (100 μ g/ml). At various intervals samples were taken for the assay of protein and enzymic activity in extracts as described in the Materials and Methods section.

Age of culture (h)	Cell morphology	Specific activity (nmol/h per mg of protein)	
		GGMS medium	GGMS medium + L-tryptophan
4	Vegetative cells	18	18
8	Vegetative cells	27	34
22*	Sporulating cells	80	86

* Cells at this stage correspond to stage t_{12} in a *B. cereus* TR-2 culture (see Fig. 1).

The presence or absence of tryptophan in the medium did not affect the activities of tryptophan pyrrolase in the vegetative and sporulating cells.

DISCUSSION

The chemical and physical evidence indicated clearly the formation and the presence of kynurenine and anthranilic acid in a sporulating culture of *B. cereus*. The formation of these intermediates from tryptophan has been previously linked with differentiation in mammals, insects and amphibians (Dalglish, 1952; Brown & Price, 1956; Baglioni, 1959; Amer, Abdel-Daim & Abdel-Tawab, 1968; Hanks, Brown, Schiffer & Schmaeler, 1968; Paik, Christinzio & Kim, 1968; Rose, Yao & Brown, 1968).

The studies on the transformation of tryptophan and kynurenine by cells in the presence of chloramphenicol showed that the onset of tryptophan

catabolism coincided with the spore formation (Fig. 2). The maximal rate of formation of anthranilic acid from kynurenine was fourfold higher than that for the formation of anthranilic acid from tryptophan. This difference may be attributed to the reaction(s) responsible for the formation of kynurenine from tryptophan catalysed by tryptophan pyrrolase and formylase. A change in the absolute amount of these enzyme(s) or their kinetic properties may bring about this phenomenon, which could also be explained by a permeability difference in the cell for the two substrates.

It is a well-known phenomenon that inducible enzymes are repressed by glucose. Since glucose was present in medium G and it was utilized during vegetative growth, it was decided to determine whether the onset of tryptophan catabolism concomitant with sporulation was an artifact due to the release of glucose repression. Since *B. cereus*

TR-2 did not grow in chemically defined medium in the absence of glucose the organisms were grown in a complex medium without glucose. Comparison of the activities of tryptophan pyrrolase in extracts of such cells with those of the extracts from cells grown in the presence of glucose indicated that the low activity of the enzyme in the vegetative cells cannot be due to its repression by glucose. Further, the addition of tryptophan to a glucose-containing defined medium did not significantly alter the enzyme activities. Similar results were obtained with a different organism, *B. cereus* 49. Our experimental results are highly indicative that the induction of tryptophan catabolism occurs concomitantly with the differentiation of the organism after completion of the vegetative growth.

An analysis of the enzyme activities in cell-free extracts showed that tryptophan pyrrolase and formylase attained maximal activities at late sporulation phase whereas kynureninase reached its maximal activity at a very early stage of sporulation. However, in experiments with whole cells, we noticed a maximal activity of all three enzymes at later stages of sporulation. As an explanation of these apparently anomalous results, one may speculate that kynureninase may be synthesized during early stages of sporulation but remains inactive until later. The inactivation *in vivo* may be accomplished by the formation of an enzyme-inhibitor complex, which dissociates from the enzyme on sonication. Another possibility may be the existence *in vivo* of an apoenzyme that changes to holoenzyme in the presence of substrate or cofactor(s).

Our understanding of the mechanisms involved in the control of tryptophan pyrrolase is still *in statu nascenti*. The complexity of this problem has been discussed in detail in two recent reviews (Paul, 1968; Recheigl, 1968). 'Under conditions of starvation, when no net synthesis is possible, turnover (of protein and RNA) is probably the only mechanism available for achieving adaptation.' This remark (Mandelstam, 1960) very well applies in the case of sporulation. The involvement of stable mRNA for the synthesis of certain enzymes during sporulation has already been suggested (Rosas del Valle & Aronson, 1962; Aronson & Rosas del Valle, 1964; Sterlini & Mandelstam, 1969). The participation of stable mRNA in the synthesis of mammalian tryptophan pyrrolase is well documented (Garren, Howell, Tomkins & Crocco, 1964).

The present results establish the presence of an aromatic pathway of tryptophan catabolism during sporulation. However, it does not rule out the possibilities of the existence of other pathways of tryptophan catabolism. The function of tryptophan catabolism during sporulation is obscure. Our preliminary studies have indicated, however, a

possible involvement of tryptophan catabolism in heat-resistance of bacterial spores.

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