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Physiological and Kinetic Studies with Anthranilate Synthetase of *Bacillus alvei*[1]

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Anthranilate synthetase from *Bacillus alvei* was partially purified by ammonium sulfate fractionation and was stabilized by glycerol. The reaction mechanism of the enzyme was found to be sequential with respect to substrate, and the enzyme formed a hydroxamic acid in the absence of Mg^{++}. The K_m for chorismic acid was 1.25×10^{-4} M, and the K_m for L-glutamine was 5.5×10^{-4} M. Enzyme activity was inhibited by tryptophan noncompetitively with respect to chorismic acid and uncompetitively with respect to L-glutamine. An analysis of the inhibition patterns indicated that tryptophan may act as a dead end inhibitor and bind at the catalytic site. Enzyme activity could be completely inhibited in vitro and in vivo under the appropriate conditions, and enzyme synthesis was sensitive to repression by tryptophan. A sedimentation coefficient of 5.5*S* and an estimated molecular weight of 90,000 were obtained for the enzyme.

Anthranilate synthetase catalyzes the formation of anthranilic acid from chorismic acid, the last common intermediate of the aromatic acid biosynthetic pathway (Fig. 1). This enzyme is the first enzyme unique to tryptophan biosynthesis and has been studied in a number of microorganisms (2, 3, 5, 7). Aromatic amino acid biosynthesis has recently been reviewed by Gibson and Pittard (10).

Hoch and DeMoss (12) observed that exogenously supplied tryptophan did not completely abolish the excretion of biosynthetically derived indole (early indole) in *Bacillus alvei*. They subsequently postulated that the internal pool of tryptophan was regulated by tryptophanase, a degradative enzyme synthesized constitutively by this organism, and that the internal level of tryptophan had to be closely regulated because tryptophan might inhibit a reaction which deprived the organism of an essential metabolite or vitamin (13, 14).

Because of the importance of feedback inhibition and repression in the regulation of biosynthetic pathways, we decided to undertake a study of the activity and synthesis of anthranilate synthetase in *B. alvei*, and of the involvement of this enzyme in the control of tryptophan biosynthesis.

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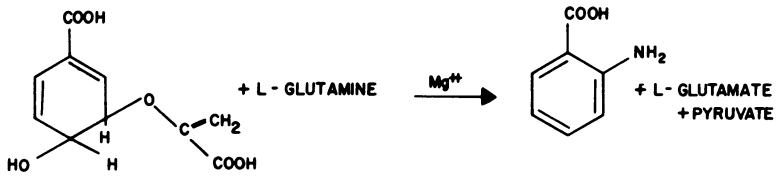
In brief, our results indicate that the reaction sequence of anthranilate synthetase is sequential with respect to substrate and that enzymatic activity can be completely inhibited by tryptophan in vitro and in vivo under the appropriate conditions. The data also suggest that tryptophan acts as a dead end inhibitor.

MATERIALS AND METHODS

Bacteria. The wild-type organism was *B. alvei* F (12). *B. alvei* TS-14, a tryptophan auxotroph which was induced by ultraviolet light, excretes anthranilic acid and requires indole or tryptophan for growth.

Media and cultivation conditions. Two of the media used have been previously described (12). The minimal salts medium with thiamine and 1% (w/v) acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) is known as STD. Glucose is usually added to a concentration of 0.5% (w/v). The medium used for the repression studies consisted of, per liter of minimal salts: 200 mg each of L-alanine, L-arginine, L-histidine·H_2O, L-isoleucine, L-lysine·HCl, L-methionine, proline, L-valine, L-serine, and glycine plus 250 mg of L-leucine, 50 mg each of L-phenylalanine and tyrosine, 10 mg of thiamine, and 5 g of glucose. The medium is known as MT. The wild-type organism was maintained by daily transfer in 2% (w/v) Trypticase. The auxotroph was maintained in Penassay Broth (Difco). Cells were grown at 37 C with vigorous shaking.

Preparation of extracts. Cells used for the partial purification of anthranilate synthetase were grown in 1-liter batches of STD + 0.5% glucose in 2.8-liter Fernbach flasks. The cells were chilled and harvested by centrifugation. The cell pellet was then suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-



CHORISMIC ACID ANTHRANILIC ACID

FIG. 1. *Reaction catalyzed by anthranilate synthetase.*

0.1 M L-glutamine, *p*H 7.5 (TG buffer). If the extract was not to be fractionated, the cells were suspended in 30% (v/v) glycerol-0.002 M ethylenediaminetetraacetic acid-0.05 M Tris, *p*H 7.5 (30% gly buffer). The suspension was then treated for 30 sec with a Branson Sonifier and was centrifuged at 120,000 × *g* for 75 min. Extracts were stored at 0 C. Because of the marked instability of the enzyme from *B. alvei*, glycerol was used to stabilize activity (Crawford, *personal communication; see also* 22).

Chemicals. Chorismic acid was purified by the method of Edwards and Jackman (6), a modification of the procedure of Gibson (9). The purification was carried through to the crystallization of the free acid. Chorismic acid was stored at −20 C with a desiccant. *Aerobacter aerogenes* 62-1, originally isolated by Gibson and Gibson (11), was kindly provided by J. A. DeMoss. All other chemicals were purchased from commercial sources.

Fractionation of extracts. Freshly harvested cells at a concentration of approximately 0.5 g/ml (wet weight) in TG buffer were sonically treated and were centrifuged at 100,000 × *g* for 1 hr. A 24-g amount of ammonium sulfate was added slowly to 100 ml of crude extract, and the solution was stirred for 30 min at 0 C before it was centrifuged at 27,000 × *g* for 15 min. The protein precipitate was dissolved in TG buffer, and this solution corresponded to the 0 to 40% protein fraction, i.e., that protein insoluble in a 40% saturated ammonium sulfate solution. Ammonium sulfate (21 g per 100 ml) was added to the 0 to 40% protein solution, and this suspension was stirred and centrifuged as described above. The protein precipitate was dissolved in a minimal amount of TG or 30% gly buffer. The specific activity of anthranilate synthetase increased approximately four-fold. This protein fraction dissolved in 30% gly buffer was used for all of the kinetic studies to be described. Activity was constant for at least 24 hr at 4 C in this buffer.

Enzyme assays. Anthranilate synthetase was assayed according to the procedure of Baker and Crawford (2). The reaction mixture, in a total volume of 2.0 ml, contained: 100 μmoles of Tris, *p*H 7.8, 25 μmoles of $MgCl_2$, 30 μmoles of L-glutamine, 0.4 μmole of chorismic acid, and enzyme. The reaction mixture was preincubated at 37 C for 2 min before the reaction was initiated with enzyme. The reaction was continuously monitored on a Westronics model LD11A strip chart recorder, with an Aminco-Bowman spectrophotofluorometer which had been fitted with a cell compartment water jacket. The temperature was maintained at 37 C. The reaction rate was determined from the first minute of increase of anthranilate fluorescence. Recrystallized anthranilic acid (Eastman Organic Chemicals, Rochester, N.Y.) was used as the standard.

Catalase was assayed according to the procedure of Martin and Ames (19). The assay mixture contained, in a final volume of 3.0 ml: 30 μmoles of potassium phosphate, *p*H 7.5, 18 μmoles of hydrogen peroxide, and the enzyme.

The hydroxamate assay procedure of Somerville and Elford (20) and Woolfolk, Shapiro, and Stadtman (23) was used to measure the hydroxamic acid formed by anthranilate synthetase. The reaction mixture contained, in a total volume of 0.9 ml: 40 μmoles of L-glutamine, 80 μmoles of Tris, *p*H 7.8, 10 μmoles of $MgCl_2$, 200 μmoles of freshly neutralized hydroxylamine, 0.4 μmole of chorismic acid, water, and enzyme to volume. Glutamic acid-γ-monohydroxamate (Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

One unit of enzyme activity is defined as the formation of 1 μmole of product in 1 min. Specific activity is units of activity per milligram of protein.

Measurement of culture turbidity. Culture turbidity was determined at 660 nm in either a Gilford 300 or a Zeiss PMQ11 spectrophotometer. An optical density (OD_{660}) of 1.0 is equivalent to a dry weight of 2.0 μg/ml. The OD_{660} was linear with respect to cell concentrations up to a value of 0.4.

Chemical determinations. Anthranilic acid was determined by the procedure of Gibson and Gibson (11). A 5.0-ml amount of medium was acidified with 3 drops of concentrated HCl and extracted with 3 ml of ethyl acetate. The tubes were centrifuged briefly, and the fluorescence of the ethyl acetate layer was measured at activation and emission wavelengths of 318 and 400 nm, respectively. Quench controls were also done. Anthranilic acid was identified by thin-layer chromatography in $CHCl_3$-96% acetic acid, 95:5, v/v (21).

Indole was extracted into 2 ml of toluene, and 1 ml of the toluene layer was assayed for indole content by the method of Yanofsky (24).

Protein was measured by the method of Lowry et al. (18). Crystalline bovine serum albumin was used as the standard.

Tryptophan was determined by the technique of Frank and DeMoss (8) with the use of partially purified *Escherichia coli* tryptophanase.

Column chromatography. Bio Gel P200 (Bio-Rad

Laboratories, Richmond, Calif.) was prepared by swelling the resin in distilled water while mixing for a period of 3 days. The fines were removed by decantation. A column (2.5 × 33 cm) was packed with resin and was washed overnight with the eluting buffer, which consisted of 0.1 M L-glutamine-0.02 M Tris, *p*H 7.2. The column was run in the ascending mode.

Sucrose density gradients. The technique of Martin and Ames (19) was used. Solutions of 5 and 20% sucrose were made in 0.1 M L-glutamine-0.05 M Tris, *p*H 7.5. Amounts of 2.5 ml of each solution were used to make the density gradients, and 0.2 ml of fractionated extract in TG buffer was loaded on each gradient. Catalase (Sigma Chemical Co.) was used as a marker. The gradients were centrifuged at 34,000 rev/min for 24 hr at 4 C. The tubes were then punctured, and fractions were collected.

Measurement of anthranilate excretion. A culture of *B. alvei* TS-14 in Penassay Broth was centrifuged, and the cell pellet was suspended in 400 ml of STD plus 0.5% glucose to an initial OD_{660} of between 0.05 and 0.15. The culture was incubated in a 1-liter baffled flask. Samples were removed periodically for the measurement of turbidity and anthranilic acid excretion. When the excretion of anthranilic acid commenced, the culture was divided in half. To one half, L-tryptophan was added to a final concentration of 28 μg/ml; the other half was used as a control. Samples were than taken periodically for the measurement of turbidity and of anthranilic acid, tryptophan, and indole concentrations.

Measurement of repression. One liter of an overnight culture of *B. alvei* F was inoculated into a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) which contained 10 liters of MT medium. The contents were stirred at 200 rev/min and were aerated at a rate of 8 liters of air per min. Tryptophan was added at the indicated time. Samples (250 ml) were removed periodically and were chilled in ice; turbidity readings were done immediately. The culture was centrifuged, and part of the supernatant fluid was retained. The cell pellet was washed once in 40 ml of 0.05 M Tris, *p*H 7.5, and was then suspended in 2 ml of 30% gly buffer. The crude extract was prepared as previously described.

Determination of kinetic parameters. K_m and V_{max} were calculated by iterative fitting of the data to a hyperbola by means of a computer. In all cases, duplicate assays were done.

RESULTS

Mechanism of catalysis. In crude extracts, the activity of anthranilate synthetase is not linear with increasing enzyme concentration. Attempts to remove a possible inhibitor by dialysis or ultrafiltration were unsuccessful, and, thus, the estimated specific activity of the enzyme in crude extracts is only approximate. Activity is proportional to enzyme concentration after an ammonium sulfate fractionation, and fractionated extracts in 30% gly buffer were used for all of the kinetic studies presented.

By examining reciprocal plots of activity versus substrate concentration at fixed concentrations of a second substrate, it is possible to distinguish between ping pong and sequential reaction mechanisms (4). Analyses were done with the use of fixed amounts of chorismic acid at various levels of L-glutamine (Fig. 2) and with the use of fixed amounts of L-glutamine and various chorismic acid concentrations (Fig. 3). The K_m for chorismic acid is 1.25×10^{-4} M, and the K_m for L-glutamine is 5.5×10^{-4} M. The presence of intersecting points indicates that the mechanism is sequential with respect to substrate, as is the case for the enzyme complex from *Salmonella typhimurium* (25) and *E. coli* (2, 15).

Inhibition by tryptophan. In contrast to the enzyme from *E. coli*, anthranilate synthetase from *B. alvei* was completely inhibited by tryptophan (Fig. 4). The inhibition was noncompetitive with respect to chorismic acid (Fig. 5) and un-

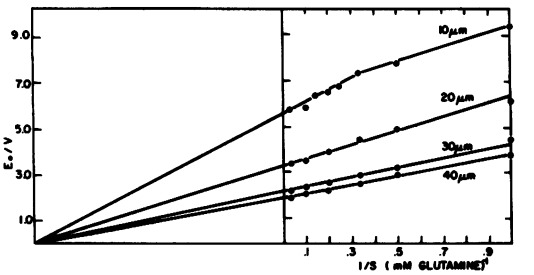


FIG. 2. *Reciprocal plots of anthranilate synthetase activity at fixed levels of chorismic acid. E_0 is defined as the activity of the preparation under saturating substrate conditions. The concentrations of chorismic acid used were 10, 20, 30, and 40 μM.*

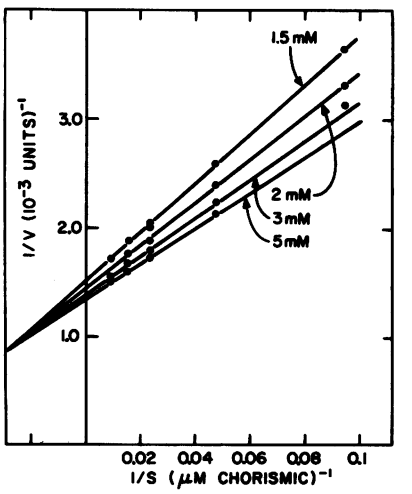


FIG. 3. *Reciprocal plots of anthranilate synthetase activity at fixed levels of L-glutamine. The concentrations of L-glutamine used were 1.5, 2.0, 3.0, and 5.0 mM.*

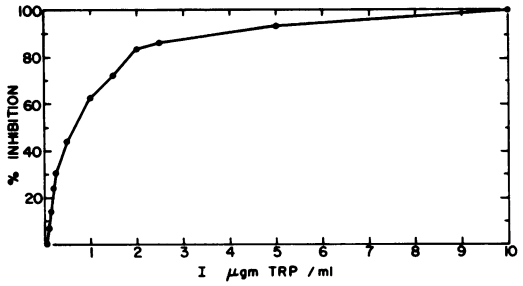


FIG. 4. *Inhibition of anthranilate synthetase activity by tryptophan. The substrate and Mg^{++} concentrations used were those listed in Materials and Methods.*

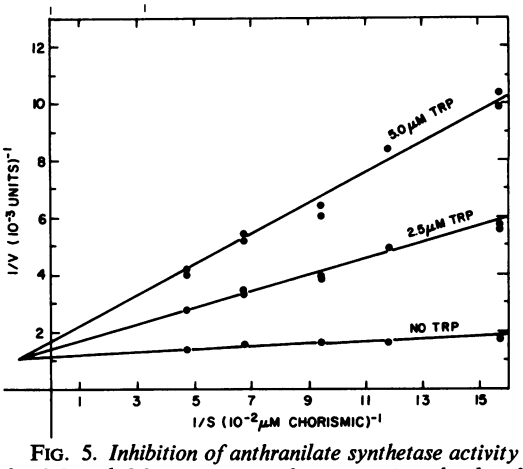


FIG. 5. *Inhibition of anthranilate synthetase activity by 2.5 and 5.0 μM L-tryptophan at various levels of chorismic acid.*

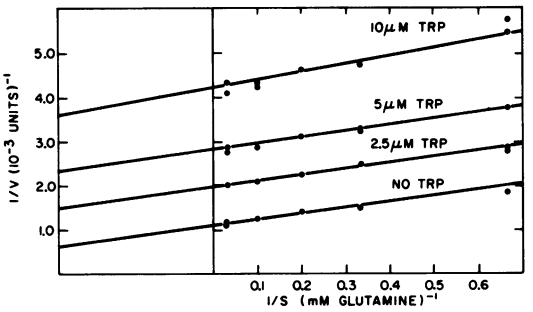


FIG. 6. *Inhibition of anthranilate synthetase activity by 2.5, 5, and 10 μM L-tryptophan at various levels of L-glutamine.*

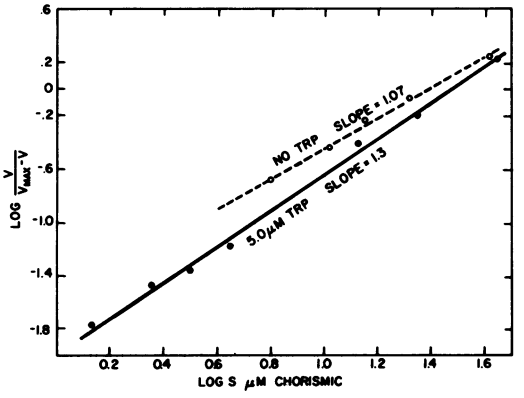


FIG. 7. *Anthranilate synthetase activity as a function of chorismic acid concentration in the presence and absence of L-tryptophan plotted according to the Hill equation. Symbols: ○, no tryptophan; ●, 5.0 μM tryptophan.*

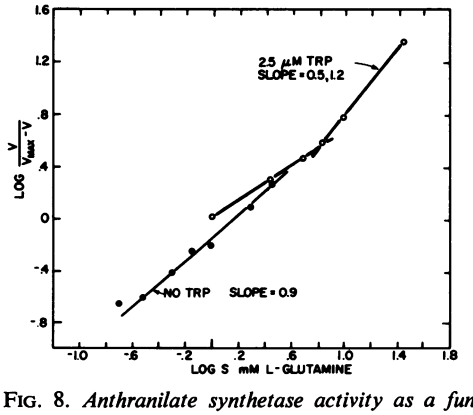


FIG. 8. *Anthranilate synthetase activity as a function of L-glutamine concentration in the presence and absence of L-tryptophan, plotted according to the Hill equation. Symbols: ●, no tryptophan; ○, 2.5 μM tryptophan.*

competitive with respect to L-glutamine (Fig. 6). According to Cleland, the pattern of intersecting curves indicates that the inhibitor binds to the catalytic site at a step in the reaction sequence which is separated from the binding of the variable substrate (chorismic acid, in this case) by reversible steps only (4). Parallel curves indicate that irreversible steps occur between the binding of the inhibitor and the binding of the variable substrate (L-glutamine). Irreversible steps could be either the binding of a substrate at infinite concentration or the release of a product at zero concentration.

Curves plotted according to the Hill equation (1) in the absence of tryptophan indicated that there is little cooperativeness between substrate binding sites, but in the presence of tryptophan binding site interactions appear (Fig. 7 and 8).

Tryptophan also inhibited the formation of a hydroxamic acid catalyzed by anthranilate synthetase, as has been found by Somerville and Elford (20) for the enzyme from *E. coli* (Table 1). The concentration of tryptophan used inhibited

the complete reaction approximately 60%, whereas this inhibitor concentration decreased hydroxamate formation by about 50%. The hydroxamate has not been identified.

Gel filtration and sucrose gradient centrifugation. The elution profile of anthranilate synthetase on Bio Gel P200 showed one peak of activity (Fig. 9). The elution volume used suggests a molecular weight of approximately 90,000. The sucrose density centrifugation profile also indicated one peak of activity (Fig. 10), and an uncorrected sedimentation coefficient of 5.5*S* was obtained. These size and weight estimates are less than those obtained for the anthranilate synthetase complexes of *S. typhimurium* and *E. coli*, but they are somewhat higher than those obtained for component I from these complexes (16, 25). We have no information concerning the subunit structure of anthranilate synthetase from *B. alvei*, and attempts to assay phosphoribosyl transferase, the second enzyme of the tryptophan pathway, have been unsuccessful.

In vivo studies. When *B. alvei* TS-14 was transferred from a medium containing tryptophan to one containing neither tryptophan nor indole, the growth rate decreased, and anthranilic acid excretion commenced after a short lag. When tryptophan was added to a culture excreting anthranilic acid, excretion stopped within 15 min (Fig. 11). In some experiments, anthranilic acid excretion did not terminate until 30 min after tryptophan addition, but in all cases excretion stopped completely. During the course of the experiment, indole was not excreted, nor was the tryptophan concentration significantly reduced. Under these conditions, then, anthrani-

TABLE 1. *Formation of hydroxamic acid by anthranilate synthetase*[a]

Conditions	A_{500}/10 min
Complete reaction mixture	0.137
Without L-glutamine	0.0
Without chorismic acid	0.020
Without Mg^{++}, with 2.2 mM ethylenediaminetetraacetic acid	0.107
With 5 μM L-tryptophan	0.07

[a] The reaction mixture contained, in a total volume of 1.0 ml: 20 μmoles of L-glutamine, 80 μmoles of Tris, *p*H 7.8, 10 μmoles of $MgCl_2$ (when Mg^{++} is omitted, 2.22 μmoles of ethylenediaminetetraacetic acid are added in its place), 200 μmoles of NH_2OH, *p*H 7.8, 0.4 μmole of chorismic acid, water, and enzyme to volume. After 10 min of incubation at 37 C, 0.25 ml of equal volumes of 24% trichloroacetic acid, 6 N HCl, and 10% $FeCl_3$ in 0.02 N HCl were added to the reaction mixture. Protein was removed by centrifugation, and the supernatant absorbancy was read at 500 nm. Approximately 10.4×10^{-3} anthranilate synthetase unit was used for each assay. The molar extinction coefficient of glutamic acid-γ-monohydroxamate is 492 at 500 nm.

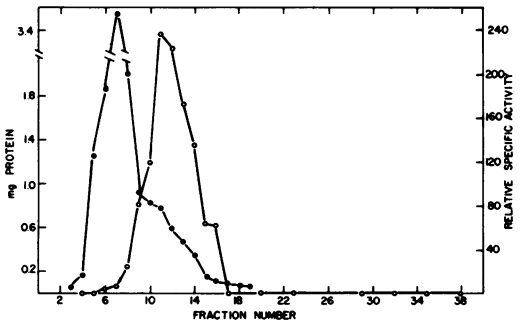


FIG. 9. *Elution profile of anthranilate synthetase from Bio Gel P200 (8.3-ml fractions were collected). Symbols: ●, protein; ○, anthranilate synthetase activity.*

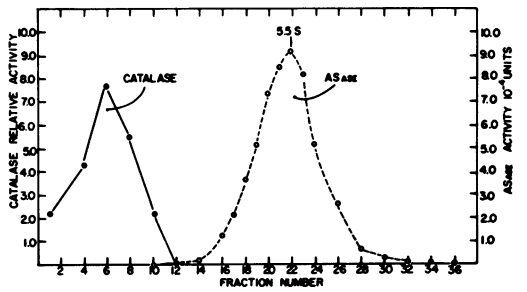


FIG. 10. *Sucrose density centrifugation of anthranilate synthetase. Fractions consisted of 10 drops each. A total of 36 fractions was collected.*

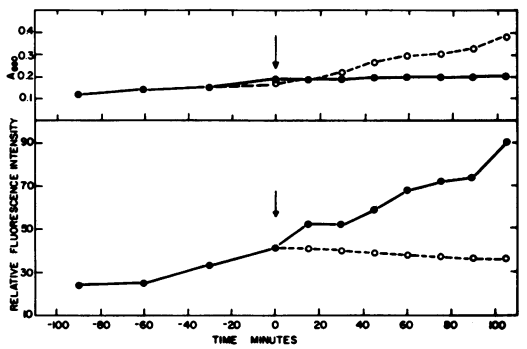


FIG. 11. *Excretion of anthranilic acid by B. alvei TS-14 in the presence and absence of tryptophan. Tryptophan was added to one half of the culture to a final concentration of 28 μg/ml at zero time (arrow). Symbols: ●, no tryptophan; ○, 28 μg of tryptophan per ml.*

late synthetase activity can be completely inhibited.

It should be noted that in this organism tryptophan also inhibits the first enzyme of the aromatic acid biosynthetic pathway, 3-deoxy-D-*arabino*-heptulosonate acid 7-phosphate synthetase (DAHP synthetase; 17). It is possible that cessation of anthranilic acid excretion is due in part to the inhibition of the DAHP synthetase. However, when tryptophan was added to a culture of *B. alvei* F growing without aromatic amino acids, there was no detectable decrease in growth rate, as measured by turbidity, over a 2.5-hr period. The primary effect of exogenous tryptophan must be on the activity of anthranilate synthetase.

Tryptophan not only inhibits enzymatic activity, but it also represses enzyme synthesis. It was of interest to know the kinetics of repression so that the contribution of repression to the modulation of the excretion kinetics of anthranilic acid and of early indole could be assessed (13). Accordingly, tryptophan was added to a culture of the wild-type organism growing in MT medium. Samples were withdrawn, and extracts were assayed for anthranilate synthetase activity (Fig. 12). A control culture containing no tryptophan showed a slight increase in anthranilate synthetase specific activity over the course of the experiment. It is evident that, in the presence of tryptophan, enzyme specific activity decreases rapidly; however, because of the difficulty of measuring activity in crude extracts, the rate of decrease was not estimated quantitatively. Experiments with dialyzed extracts have indicated that the decrease in activity is not due to feedback inhibition by tryptophan remaining in the extract.

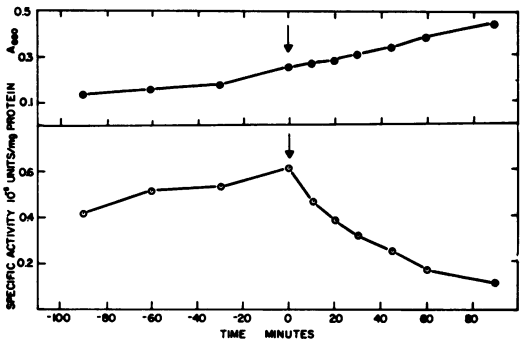


FIG. 12. *Repression of anthranilate synthetase by tryptophan. Tryptophan at a final concentration of 28 μg/ml was added to a culture of B. alvei growing in MT medium at zero time (arrow).*

DISCUSSION

The mechanism of catalysis of anthranilate synthetase appears to be similar in *E. coli* (2, 15), *S. typhimurium* (25), and *B. alvei*. Both substrates must bind to the enzyme before a product is released, but little information is available as to the order of substrate binding or product release. There are considerable differences, however, when tryptophan inhibition is considered. In both *E. coli* and *S. typhimurium*, tryptophan inhibits activity competitively with respect to chorismic acid and noncompetitively (V_{max} is decreased) with respect to L-glutamine; tryptophan does not inhibit enzymatic activity completely, but rather a maximum of approximately 80% inhibition is reached. In contrast to the enzymes from these organisms, anthranilate synthetase from *B. alvei* is inhibited noncompetitively (both the slope and intercept of reciprocal plots are altered) with respect to chorismic acid, whereas inhibition is uncompetitive with respect to L-glutamine, and the inhibition is complete. The enzyme from *A. aerogenes* can also be completely inhibited by tryptophan, but a detailed analysis of the inhibition patterns has not been done (7).

Our data are insufficient for statement of possible reaction mechanisms, but they are consistent with the conclusion that tryptophan acts as a dead end inhibitor which binds at the catalytic site. We cannot exclude the possibility that allosteric sites are present. Direct binding studies with pure protein are needed to corroborate any mechanism postulated.

Tryptophan is capable of inhibiting the excretion of anthranilic acid completely in vivo, as shown here; yet, a previous study showed that it did not inhibit indole excretion completely (12). The apparent paradox may derive from a difference in the design of the two sets of experiments. The organisms were not growing (increasing in turbidity) in the anthranilic acid experiment, whereas growth was exponential in the indole excretion study. It thus appears that tryptophan can effectively control the flow of metabolites into the tryptophan pathway and, under conditions of unbalanced growth, it can abolish anthranilate synthetase activity.

The role of tryptophan in the regulation of the aromatic acid biosynthetic pathway is of interest because tryptophan, but neither tyrosine nor phenylalanine, inhibits DAHP synthetase (17). With the addition of exogenous tryptophan, both DAHP synthetase and anthranilate synthetase would be inhibited, though the degree of inhibition would be much greater for anthranilate synthetase than for DAHP synthetase. Thus, as far as modifier effects are concerned, tryptophan appears to act as an end product of an unbranched

pathway which has at least two enzymatic steps under feedback control. It is possible that, to insure the formation of adequate levels of the other products of the aromatic acid biosynthetic pathway, the internal level of tryptophan must be closely regulated by the combined mechanisms of feedback inhibition and metabolism of the inhibitor.

ACKNOWLEDGMENTS

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