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Physiological Studies of Biosynthetic Indole Excretion in *Bacillus alvei*

C. W. ROTH,[1] J. A. HOCH,[2] AND R. D. DEMOSS
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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Bacillus alvei excretes indole during early exponential growth in acid-hydrolyzed casein medium. L-Threonine is the amino acid responsible for "early" indole excretion, and the amount of indole excreted is directly related to the amount of L-threonine in the medium. "Early-indole" excretion can be prevented by the continuous addition of serine (3.1 μmoles per ml per hr) or by substituting a mutant with an impaired ability to degrade serine. The addition of serine to a culture during the period of indole excretion halts the excretion and stimulates indole utilization. Threonine is a competitive inhibitor of serine (K_i = 0.6 M) in the tryptophan synthetase B reaction. The internal tryptophan concentration increases during the period of indole excretion, suggesting that threonine acts by increasing the activity of the tryptophan pathway. This view is supported by experiments demonstrating that anthranilic acid and indoleacrylic acid also stimulate indole excretion. A metabolic explanation is offered and discussed.

The biosynthetic pathways of bacteria are generally considered to be efficiently regulated by one or more systems of control. Biosynthetic intermediates presumably are present intracellularly in concentrations adequate, but not excessive, for operation of a pathway at a rate consonant with the demands of the cell growth rate. Biosynthetic intermediates and biosynthetic end products are rarely excreted, except by auxotrophic mutants under certain nutrient-limiting conditions, unless the usual metabolic controls are deranged by an exogenous agent, often an inhibitor of growth. The overproduction and excretion of a pathway intermediate under apparently normal growth conditions would therefore seem to indicate either an inefficient metabolic regulatory system, or an unsuspected regulatory derangement by an apparently normal growth condition.

Indole is a biosynthetic intermediate in the pathway leading to L-tryptophan formation and is also an end product of L-tryptophan catabolism. In culture media containing L-tryptophan, *Bacillus alvei* excretes indole, as a consequence of tryptophan catabolism by tryptophanase, at the end of exponential growth (4). In media containing an acid hydrolysate of casein and no L-tryptophan, indole is excreted during the early stages of exponential growth, but not as a consequence of tryptophanase action. The excreted indole is assimilated by the bacteria before completion of exponential growth. Because of the times of indole excretion relative to the interval of exponential growth, the two phenomena are termed "late indole" (tryptophanase action) and "early indole," respectively.

Early indole excretion is completely prevented by the addition of 5μg or more of L-tryptophan per ml of medium (4), but is accentuated by the addition of L-threonine to the medium. This report offers a metabolic explanation for and extends the observations concerning early indole excretion.

MATERIALS AND METHODS

Bacteria. A nonmucoid variant of *B. alvei* ATCC 6348 isolated in this laboratory and designated strain F was used as the wild-type organism. The selection procedure was described previously (4).

Culture media. The minimal salts medium of Vogel and Bonner (11) supplemented with 1% glucose and 10 μg of thiamine-hydrochloride/ml was used as the basal medium in all experiments. Basal medium supplemented with 1% "salt-free" acid-hydrolyzed casein (Nutritional Biochemicals Corp.) instead of glucose will be referred to as standard medium (STD). A synthetic medium approximating the amino acid composition of STD but lacking L-serine, glycine, and L-threonine is called amino acid medium (AA medium). AA medium contains, in grams per liter of basal medium (*above*), L-alanine, 0.31; L-arginine, 0.25; L-aspartic acid, 0.67; L-glutamic acid, 2.00; L-histidine-hydrochloride monohydrate, 0.35; L-isoleucine, 0.25; L-leucine, 0.65; L-lysine-

[1] Present address: Department of Microbiology, University of Washington, Seattle, Wash. 98105.

[2] Present address: Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.

hydrochloride, 0.55; L-methionine, 0.20; L-phenylalanine, 0.20; L-proline, 0.86; L-tyrosine, 0.22; and L-valine, 0.40. The pH was adjusted to 7 with 10 N KOH and 10 mg of thiamine-hydrochloride was added per liter before the medium was sterilized.

TC medium contains 2% (w/v) Trypticase (BBL), adjusted to *p*H 7 and supplemented with 10 μg of thiamine-hydrochloride per liter and was used to maintain growing cultures of *B. alvei*.

Growth. For a typical experiment, *B. alvei* was grown overnight in the medium to be used in the experiment or in STD if AA medium was to be used. The cells were harvested, from an exponentially growing culture, at 4,000 × *g* for 15 min in an International clinical centrifuge at room temperature. The cells were washed twice with either minimal salts solution or the medium into which they were to be transferred.

The experimental medium was inoculated with cells to give an initial turbidity measured as 0.3 to 0.4 optical density units. The optical density was measured at 660 nm with either a Zeiss PMQ II or a Gilford 300 spectrophotometer. If the optical density was greater than 0.8 the sample was diluted to give the true turbidity. A calculated turbidity reading of 1.0 is equivalent to a dry weight of 160 μg of cells/ml. Cultures in Erlenmeyer flasks containing medium equal to no more than 20% of the volume of the flask were shaken at about 250 rev/min (high speed) with a New Brunswick gyrotory shaker bath at 37 C.

For the experiments in which either serine or threonine was continuously added to the medium, the following procedure was used. A concentrated (0.5 M) solution of amino acid was slowly added with a Sigmamotor peristaltic pump, model AL-2-E, to 200 ml of medium in a shaking 1-liter Erlenmeyer flask. Samples (5 ml) were removed every 30 min, the optical density was measured, the cells were removed by centrifugation, and 3 ml of the supernatant was used to determine the indole concentration in the medium. A control flask inoculated with the same culture as the experimental flask was grown under identical conditions except that no amino acid was added.

When an experiment was done in a 14-liter fermentor (New Brunswick Scientific, model F-14), the inoculum was grown in 1 liter of experimental medium by using a Fernbach flask (2.8 liter) and vigorous shaking. The liter of culture was added to 10 liters of experimental medium in a fermentor which was warmed in a water bath to 37 C, aerated with 10 liters of air/min, and agitated at 200 rev/min.

Isolation of strains with low L-serine dehydratase activity. Two mutants (SD-3 and SD-11) with low dehydratase activity were isolated by treating cultures with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, growing the cells overnight in TC broth and then plating on agar containing 1% acid-hydrolyzed casein for a carbon and energy source. Small colonies were picked and screened for dehydratase activity after growth in TC broth. A third mutant (SD-12) was a naturally occurring mutant which was detected because it failed to excrete early indole in STD medium containing 1% glucose.

Determination of indole. Indole was determined by a modification of the procedure described by Yanofsky (12). Samples were extracted with 2 ml of toluene, and 1 ml of the toluene layer was mixed with either 9 ml of flask assay color reagent or 3 ml of tube assay color reagent (*see below*). After incubating at room temperature for 20 min, the optical density was measured with either an Evelyn photoelectric colorimeter by using a 540 nm filter or a Gilford model 300 spectrophotometer at 568 nm. Flask assay color reagent contains 5.4 g of *p*-dimethylaminobenzaldehyde, 908 ml of 95% ethanol, and 64 ml of concentrated sulfuric acid. Tube assay color reagent contains 14.7 g of *p*-dimethylaminobenzaldehyde, 948 ml of 95% ethanol, and 52 ml of concentrated sulfuric acid.

Tryptophan synthetase assay. Tryptophan synthetase was measured in an assay based on the method of Smith and Yanofsky (10). Activity was measured in the B reaction (indole + L-serine → tryptophan) by measuring the amount of indole utilized. *B. alvei* extracts were assayed in a reaction mixture containing 12.5 μmoles of *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine) buffer (*p*H 8.0), 125 nmoles of indole, 18 nmoles of pyridoxal-5'-phosphate, 250 nmoles of reduced glutathione, 2 μmoles of L-serine, 7.5 μliters of saturated KCl, and enzyme plus water to 0.25 ml. The reaction was usually initiated by the addition of serine. The tubes were incubated at 37 C, and the reaction was stopped after 20 min by adding 5 ml of tube assay color reagent. After 20 min at room temperature, the optical density of the samples was determined at 568 nm in a Gilford 300 spectrophotometer.

One unit of activity is defined as the utilization of 1 μmole of substrate per minute. Specific activity is expressed as units of activity per milligram of protein.

L-Serine dehydratase assay. L-Serine dehydratase activity was assayed by measuring the amount of pyruvate formed from L-serine. The reaction mixture contained 64 μmoles of potassium phosphate buffer (*p*H 8.0), 3.4 μmoles of ethylenediaminetetraacetic acid (EDTA), 16 μmoles of sodium formate, 8 μmoles of serine, and extract in 2.0 ml volume. Samples were incubated for 20 min at 37 C. The reaction was stopped by adding 1.0 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl, and the pyruvate concentration was determined by the method of Friedemann and Haugen (3). Crystalline sodium pyruvate was used as a standard.

One unit of activity is defined as 1 μmole of pyruvate formed per minute; specific activity is expressed as units of activity per milligram of protein.

Determination of protein. Protein was measured by the method of Lowry et al. (7). Crystalline bovine serum albumin was used as the standard.

Preparation of extracts. Chilled *B. alvei* cells were washed once with cold 0.1 M potassium phosphate buffer (pH 7.0), containing 0.1 mM pyridoxal-5'-phosphate (PLP), and 0.1 mM mercaptoethanol (PPM buffer). Washed cells suspended in PPM buffer containing 10% (v/v) glycerol were treated with a Branson Sonifier four times, 30 sec each with 1-min intervals for cooling. The debris was sedimented by centrifugation at 93,000 × *g* for 2 hr. The supernatant was immediately brought up to 30% (v/v) glycerol and used as crude extract. Extracts prepared in this manner were used for tryptophan synthetase assays and for concentration of tryptophan synthetase activity. When serine dehydratase was to be assayed, extracts were prepared in 0.04 M potassium phosphate buffer (*p*H 8.0) containing 0.1 M sodium formate (9) and 1.4 mM EDTA

(PFV buffer). Cell suspensions were treated with a Branson Sonifier, and the debris was removed by centrifugation at 30,000 × *g* for 20 min. The supernatant was then used as crude extract. All operations were done while keeping the cells or extracts as close to 5 C as possible.

Determination of tryptophan pool. The intracellular tryptophan pool was measured by rapidly cooling a sample in an ice-water bath, harvesting the cells in a refrigerated Sorvall centrifuge at 9,000 × *g* for 15 min and resuspending the unwashed cells in a small volume of 0.1 M potassium phosphate buffer (*p*H 8.0). The cell suspension was placed in a boiling-water bath for 20 min after a sample was removed for protein determination. After centrifugation of the boiled cell suspension at 27,000 × *g* for 20 min, the supernatant was assayed for tryptophan by the method of Frank and DeMoss (2) by using partially purified *Escherichia coli* tryptophanase. Controls were run to correct for indole carried over from the medium by taking an equal amount of sample through the tryptophan assay with the exception that tryptophanase was not added. Cell protein was determined after treating a diluted cell suspension with an equal volume of 1 N NaOH.

Determination of ammonia. The ammonia concentration was estimated by a modification of the method of Johnson (6) as described by DeMoss and Moser (1). Crystalline $(NH_4)_2SO_4$ was used as a standard.

Determination of kinetic parameters. K_m and V_{max} values were calculated by fitting the data to a hyperbola by means of an iterative computer program.

Amino acid analysis. Amino acids were analyzed in *p*H 2.2 buffer (8) on a Beckman-Spinco amino acid analyzer.

Special chemicals. The A and B isomers of β-methyl-DL-tryptophan were a gift from H. R. Snyder. The isomers differ with respect to the orientation of the β-methyl group, but the actual orientation of the group on the β-carbon is not known. Indole-3-acrylate was purchased from Sigma Chemical Co.

RESULTS

Early indole excretion occurred during growth of *B. alvei* in medium containing 1% vitamin-free acid-hydrolyzed casein. To investigate whether one or more components of this complex medium might be responsible for indole excretion, the following two experiments were performed. The first experiment was designed to determine whether one or more amino acids was involved in early indole excretion. Amino acid mixtures were prepared, approximating the content of STD medium but lacking various groups of amino acids. The excretion of indole by *B. alvei* growing in these media was monitored. The result showed that the omission of L-threonine from the medium eliminated indole excretion. Figure 1 shows the results of an experiment demonstrating that the amount of indole excreted is directly related to the concentration of L-threonine in the medium. Indole excretion is apparently unaffected by the D isomer of threonine since the mixture of D and L isomers had an effect equal only to that of the approximate concentration of L isomer present. In the second experiment, amino acid utilization was monitored during indole excretion. This experiment demonstrated that the concentrations of only serine and threonine varied greatly during early indole excretion and reutilization. Figure 2 shows the results of amino acid analyses on samples taken during growth of *B. alvei* in AA medium supplemented with L-serine, glycine, and L-threonine. L-Serine was utilized rapidly and immediately, whereas L-threonine was not rapidly utilized until the serine concentration approached its minimum. Indole excretion commenced when the L-serine concentration approached its minimum. The reutilization of indole is more closely correlated with the disappearance of threonine from the medium. To examine the possibility that L-threonine influences the rate of serine utilization, an experiment was done in the same synthetic medium except that L-threonine was omitted. No indole was excreted under these conditions but the rate of serine utilization was essentially the same as when L-threonine was present. This result indicates that L-threonine does not act by inducing a threonine-serine dehydratase which is responsible for the rapid serine utilization seen in these experiments. The kinetics of serine and threonine disappearance and indole excretion were virtually identical whether *B. alvei* was grown in STD medium or STD medium containing 0.5% glucose, or in AA medium containing L-serine and L-threonine.

The rapid disappearance of serine from the medium suggested that *B. alvei* had a highly active serine-degrading enzyme. To confirm this suggestion and to investigate the type of enzyme involved, a crude extract was prepared from *B. alvei* grown on STD medium. Table 1 shows that an extract of *B. alvei* degrades L-serine to equimolar amounts of pyruvate and ammonia, indicating the presence of L-serine dehydratase activity. If L-serine dehydratase is responsible for the observed serine utilization, the presence of L-threonine should neither greatly stimulate nor repress the level of the enzyme, since it had no effect on the rate of L-serine utilization. Table 2 shows the effect of L-serine and L-threonine in the medium on L-serine dehydratase activity in *B. alvei*.. The L-serine dehydratase activity was high in all cases tested. We conclude that, in *B. alvei*, either L-serine dehydratase is a constitutive enzyme or the growth conditions employed exerted little change in the control of the synthesis of the enzyme.

The observation that indole excretion is closely correlated with the utilization of serine in the medium suggested the hypotheses that (i) indole

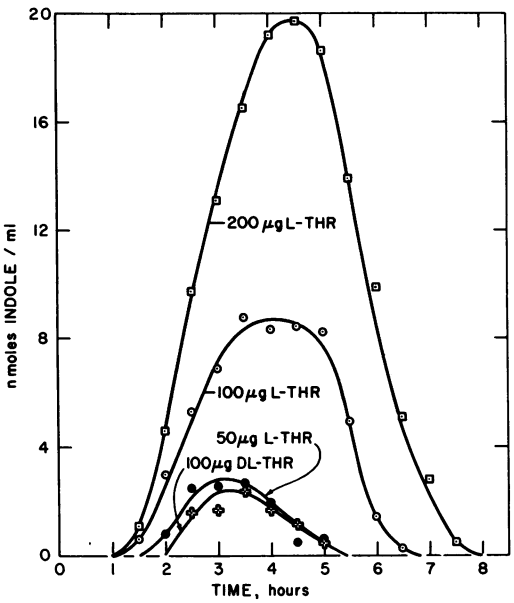

FIG. 1. *Effect of exogenous* L-*threonine on early indole excretion kinetics by B. alvei. B. alvei F was cultured at 37 C in 200 ml of amino acid medium supplemented with 4.8 μmoles of serine and 2.7 μmoles of glycine per ml. Five-milliliter samples were withdrawn at 30-min intervals, and the cells were sedimented at 8,000 × g for 10 min. The supernatant liquid was extracted with toluene, and indole was determined in the toluene layer. The initial threonine concentration of the medium was 50 μg of* L-*threonine/ml, ●; 100 μg of* L *threonine/ml, ⊙; 200 μg of* L-*threonine/ml, ⊡; 100 μg of* DL-*threonine-ml, ⊡.*

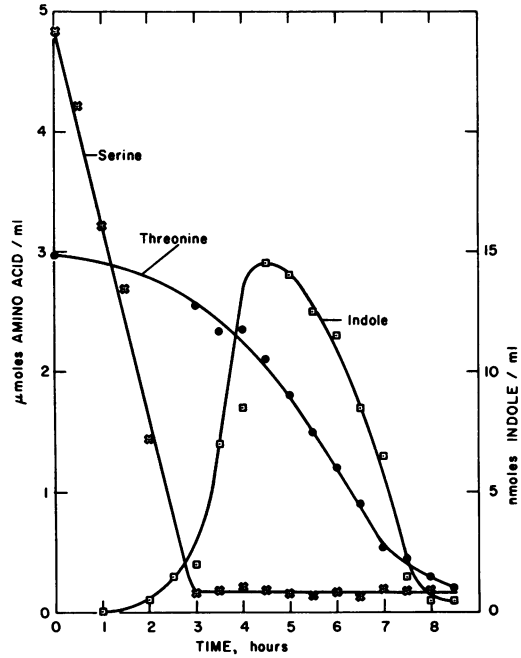

FIG. 2. *Kinetics of serine and threonine utilization during growth of B. alvei. B. alvei F was cultured at 37 C in 200 ml of amino acid medium supplemented with 4.8 μmoles of serine, 3.0 μmoles of threonine, and 2.7 μmoles of glycine per ml. Samples (5 ml) were withdrawn at 30-min intervals, and the cells were sedimented by centrifugation at 8,000 × g for 10 min. The supernatant fluid was divided. One portion was extracted with toluene and the indole concentration in the toluene layer was determined. Serine and threonine were determined by lyophilizing 0.4 ml of the supernatant, redissolving in 2.0 ml of 0.02* N *citrate buffer (pH 2.2), and applying 0.5 ml of the sample to a Beckman-Spinco amino acid analyzer.*

would not be excreted if the level of L-serine in the medium were maintained at a high level, (ii) the kinetics of indole excretion would be modified upon addition of L-serine to a culture actively excreting early indole, and, (iii) a mutant with impaired ability to degrade L-serine would not excrete indole.

To test the first hypothesis, L-serine was continuously added, by means of a peristaltic pump, to growing cultures of *B. alvei*. Table 3 shows that the continuous addition of serine at the rate of 3.1 μmoles per ml of medium per hr completely stopped indole excretion. A reduced rate of indole excretion accompanied the lower rates of addition tested, but excretion was not completely prevented. The per cent inhibition was calculated by comparing the peak of indole excretion in the experimental flask with that in a control flask tested at the same time. This procedure normalized the differences in the amount of indole excreted by different cultures. It was also noted that the rate of growth increased slightly when L-serine was added.

In testing the second hypothesis, a preliminary

TABLE 1. *Stoichiometry of serine dehydratase activity in Bacillus alvei*[a]

Sample	Pyruvate (μmole/ml)	Ammonia (μmole/ml)	Ratio (pyruvate/ ammonia)
1	0.480	0.431	1.11
2	0.531	0.514	1.03

[a] *B. alvei* was cultured at 37 C in standard (STD) medium. Frozen cells harvested in the exponential phase of growth were used to prepare a cell-free extract. The reaction mixture contained 0.4 mmoles of potassium phosphate buffer (*p*H 8.0), 0.1 mmole of sodium formate, 4 mg of ethylenediaminetetraacetic acid, 0.4 mmole of L-serine, and 2.16 mg of crude extract protein in 10.0 ml. The mixture was incubated at 37 C for 30 min, and the reaction was terminated by adding 0.1 ml of 100% trichloroacetic acid. Pyruvate and ammonia in the supernatant were determined after removal of precipitated material.

TABLE 2. *Effect of growth medium on L-serine dehydratase activity in Bacillus alvei*[a]

Flask	L-Serine (μg/ml)	L-Threonine (μg/ml)	Specific activity (10^{-3} units/mg of protein)
1	100	100	61
2	100		57
3		100	62
4	200		85
5			77

[a] *B. alvei* was cultured overnight in 50 ml of amino acid medium supplemented with L-serine and L-threonine at the concentrations listed. Crude extracts were prepared, and L-serine dehydratase activity was measured.

TABLE 3. *Effect of continuous addition of L-serine on early indole excretion by Bacillus alvei*[a]

Rate of addition (μmoles per ml per hr)	Per cent inhibition of indole excretion	Growth rate (k)
0		48.5
0.62	75	62
1.07	78.5	62
3.10	100	57

[a] L-Serine was continuously added by means of a peristaltic pump to cultures of *B. alvei* growing in standard (STD) medium. Per cent inhibition is calculated as the ratio of the maximum concentration of indole excreted, compared to the control. The growth rate constant $k = (\ln B_2 - \ln B_1)/(t_2 - t_1)$, where B_1 and B_2 represent the micrograms (dry weight) of cells per milliliter at times t_1 and t_2; t is measured in hours.

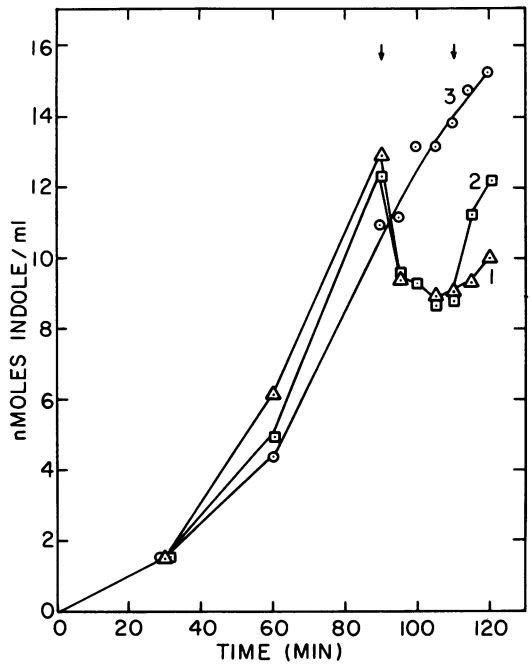

FIG. 3. *Effect of exogenous L-serine and L-threonine on the kinetics of early indole excretion by B. alvei. B. alvei F was cultured at 37 C in 200 ml of standard (STD) medium. L-Serine was added to a concentration of 1 μmole/ml to flasks 1 and 2 after 90 min growth (arrow); L-threonine was added at 4.2 μmoles/ml to flask 2 at 110 min (arrow). Four-milliliter samples were removed at consecutive 30-min intervals before L-serine addition and at consecutive 5-min intervals after L-serine addition. Cells were removed by centrifugation at 8,000 × g for 10 min. Three milliliters of the supernatant was extracted with 2 ml of toluene, and the indole concentration was determined in 1 ml of the toluene layer. Flask 3 was the control; neither L-serine nor L-threonine was added. Flask 1, △; flask 2, ⊡; flask 3, ⊙.*

experiment indicated that indole excretion was immediately halted and that the excreted indole was reutilized upon addition of L-serine to a culture during indole excretion. This result, combined with the observations in earlier experiments that L-threonine was required for indole excretion, was used to design the following experiment. L-Serine and L-threonine were added sequentially to a culture which was actively excreting indole. The concentration of indole in the medium dropped precipitously within 5 min after the addition of L-serine (Fig. 3). Upon sequential addition of L-threonine, the rate of indole excretion immediately increased. The immediate increase in indole excretion after the addition of L-threonine suggests the possibility of a competitive relationship between L-threonine and L-serine.

To determine whether loss of the ability to degrade serine affects the excretion of early indole, three mutants with less than 20% of the wild-type level of L-serine dehydratase were isolated and tested. None of these mutants excreted indole when grown in AA medium supplemented with 4.2 μmoles of L-threonine per ml but all did excrete late indole when grown in Trypticase (TC) medium. One mutant, SD-3, was grown in AA medium with L-threonine continuously added at the rate of 1.7 μmoles per ml per hr. Indole was not excreted under these growth conditions. These results suggest that indole excretion is not solely a consequence of the presence of L-threonine, but in addition requires that the availability of L-serine be low.

On the basis of the experiments described, it was tentatively concluded that the function of L-threonine in early indole excretion might be one of competition with L-serine. One possible site of such competition is the tryptophan synthetase reaction. L-Threonine conceivably could compete

with L-serine in the reaction catalyzed by tryptophan synthetase. If L-threonine interferes with tryptophan synthesis, it is plausible to expect the accumulation of indoleglycerolphosphate. The latter could be degraded in the tryptophan synthetase A reaction to yield indole. The hypothesis was tested by studying in vitro the effects of L-threonine on the tryptophan synthetase B reaction.

The tryptophan synthetase activity was enriched twofold by slowly adding an equal volume of a neutralized aqueous solution of ammonium sulfate saturated at 0 C to a cell-free extract prepared as previously described. After stirring for 20 min in an ice bath, the mixture was centrifuged for 20 min at 30,000 × *g*, and the resulting pellet was redissolved in a minimal volume of PPM buffer containing 30% (v/v) glycerol.

The effect of L-threonine on the rate of tryptophan synthesis in the B reaction was investigated by using an extract of *B. alvei* enriched as described above. Figure 4 shows a Lineweaver-Burk plot for the tryptophan synthetase B reaction with various concentrations of L-serine in the presence and absence of 0.1 M L-threonine. The apparent K_m and V_{max} values calculated from these data and those from a similar experiment are shown in Table 4. These data show that the K_m for L-serine in the absence of L-threonine is approximately 10 mM and that this value is increased in the presence of 0.1 M L-threonine. The V_{max} remains essentially the same in either the presence or absence of L-threonine. The simplest interpretation consistent with these data is that L-threonine acts as a competitive inhibitor of L-serine in the tryptophan synthetase reaction of *B. alvei*. The apparent K_i for L-threonine calculated from these data is approximately 0.6 M. It is concluded from these results that L-threonine inhibits the B reaction of tryptophan synthetase and thus may also inhibit the overall conversion of indoleglycerol phosphate (InGP) to tryptophan. The observed inhibition is also consistent with the proposed role of L-threonine in the excretion of early indole.

If the primary mode of action of L-threonine is in blocking the tryptophan synthetase reaction, it could be predicted that L-threonine would cause a decrease in the tryptophan pool during the period of indole excretion. To test this prediction, the indole concentration in the medium and the apparent intracellular tryptophan concentration was measured in samples of *B. alvei* growing in STD medium. The results are shown in Fig. 5. The tryptophan pool increased during the period of indole excretion and decreased during the period of indole reutilization. This result is not consistent with the hypothesis that L-threonine acts

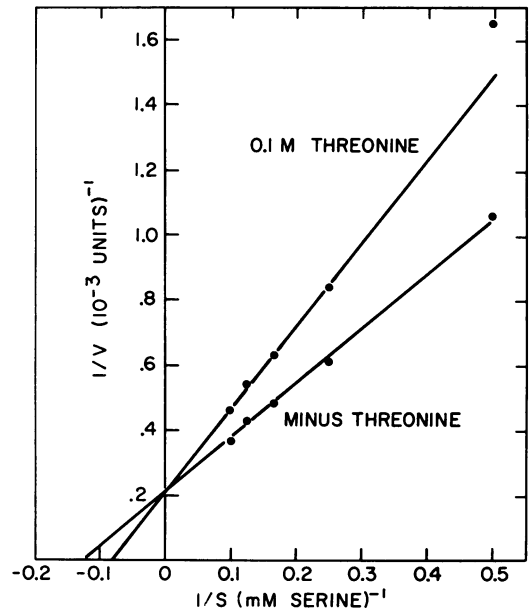

FIG. 4. *Lineweaver-Burk plot of tryptophan synthetase B activity (V) as a function of L-serine concentrations (S) in the presence of L-threonine. Each reaction mixture contained 12.5 μmoles of Bicine buffer (pH 8.0), 125 nmoles of indole, 18 nmoles of pyridoxal-5'-phosphate, 250 nmoles of glutathione (reduced form), 7.5 μliters of saturated KCl, B. alvei F extract, and various concentrations of L-serine in a 0.25-ml volume. L-Threonine was present at 0.1 M. After 20 min of incubation at 37 C, the reaction was terminated, and the amount of indole utilized was measured by adding 5 ml of tube assay color reagent to the mixture.*

by blocking the synthesis of tryptophan and indeed suggests that L-threonine stimulates tryptophan synthesis. To explain the observed increase in the tryptophan pool, it was tentatively hypothesized that threonine might act by altering the regulation of tryptophan synthesis. The increase in tryptophan synthesis could result from either an increase in the levels of the tryptophan biosynthetic enzymes or an increase in the activity of

TABLE 4. *Michaelis-Menten constants for L-serine in the Bacillus alvei tryptophan synthetase B reaction*[a]

Constant[b]	Expt 1		Expt 2	
	Minus threonine	Plus threonine	Minus threonine	Plus threonine
K_m (M)	(8.8 ± 0.3) × 10^{-3}	(14.4 ± 0.1) × 10^{-3}	(10.8 ± 0.3) × 10^{-3}	(20.9 ± 0.5) × 10^{-3}
V_{max}	5.13 ± 0.11	5.3 ± 0.2	3.54 ± 0.24	3.85 ± 0.41

[a] The reaction conditions are described in Fig. 4.

[b] K_m and V_{max} were calculated by fitting the data to a hyperbola by means of an iterative computer program. Apparent K_i for experiment 1, 6.7 × 10^{-1} M; for experiment 2, 5.7 × 10^{-1} M.

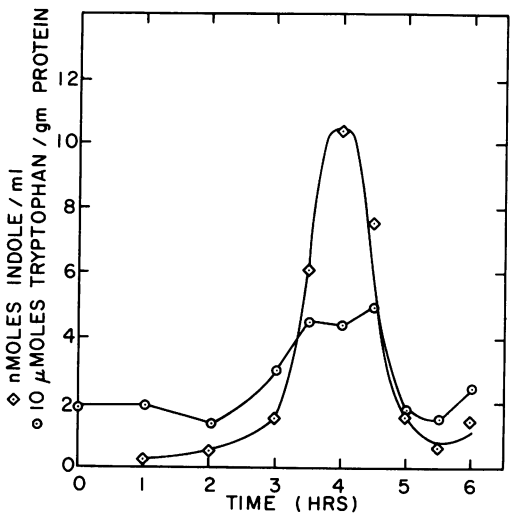

FIG. 5. *Kinetics of intracellular tryptophan pool expansion during early indole excretion. B. alvei was cultured at 37 C in a fermentor containing 11 liters of standard (STD) medium. Samples (500-ml) were removed at 30-min intervals, and the cells were removed by centrifugation at 8,000 × g for 15 min. Four-milliliter samples of the supernatant were extracted with 2 ml of toluene, and the indole in 1 ml of the toluene layer was determined. The cells were analyzed for free tryptophan content. One unit on the ordinate represents: 1 nmole of indole per ml, ◇; 10 μmoles of tryptophan/g of protein, ○.*

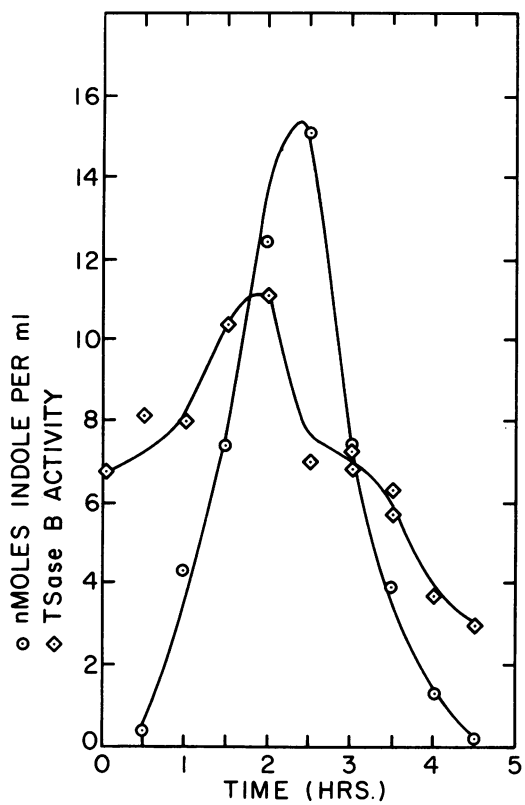

FIG. 6. *Kinetics of tryptophan synthetase formation during the excretion of early indole. B. alvei was cultured at 37 C in a fermentor containing 12 liters of standard (STD) medium supplemented with 5 nmoles of L-serine/ml. Samples were withdrawn at 30-min intervals, and the cells were removed by centrifugation at 8,000 × g for 15 min. Samples of the supernatant were extracted with toluene, and the indole in the toluene layer was measured. The tryptophan synthetase (TSase) was measured in cell-free extracts and is expressed in the following dimension: 10^{-3} units per milligram of protein. Concentration of indole in the medium, ○; tryptophan synthetase specific activity, ◇.*

the biosynthetic enzymes. To differentiate between these possibilities, the concentration of tryptophan synthetase in cells during growth in STD medium was examined (Fig. 6). The tryptophan synthetase activity increased during the period of indole excretion and decreased during the period of indole reutilization. The increase in tryptophan biosynthetic capacity explains the results of the previous experiment in which the internal tryptophan pool increased during the period of indole excretion. In this experiment anthranilate synthetase activity also increased during the period of indole excretion (A. Catena, *unpublished data*), indicating that the activity of the entire pathway probably increased.

The in vivo experiments demonstrating the necessity for exogenous L-threonine in stimulation of indole excretion, and the experiments establishing the correlation between intracellular tryptophan and tryptophan synthetase activity are consistent with the hypothesis that L-threonine directly affects early indole excretion and indirectly deranges the normal regulation of tryptophan biosynthesis.

To test the hypothesis that L-threonine is responsible for the increased specific activity of tryptophan synthetase during early indole excretion, the effect of L-threonine on the level of tryptophan synthetase was examined in an experiment of different design. *B. alvei* was grown to a density of 176 μg (dry weight)/ml in AA medium containing 0.5% glucose. The culture was divided into three portions. L-Threonine was added to a concentration of 4.17 μmoles/ml to one portion and indoleacrylate (*see below*) was added to a concentration of 1 μmole/ml to a second portion. The three cultures were incubated for an additional 60 min at 37 C, at which time each culture was harvested and used for the preparation of crude extracts. The activity of tryptophan synthetase in the extracts was determined. The results, presented in Table 5, show that neither L-threo-

nine nor indoleacrylate effected an increase in the specific activity of the tryptophan synthetase B reaction compared to the activity from cells in the untreated sample. These results appear to be inconsistent with the hypothesis that L-threonine is responsible for the increased specific activity of tryptophan synthetase observed during growth of *B. alvei* in STD medium if we assume that there is either no loss of enzyme activity or a proportional loss of activity in all samples. The reason for the inconsistency is not understood.

The observation that conditions which led to indole excretion, i.e., addition of L-threonine to cells growing in AA-glucose medium, could not be shown to cause an increase in tryptophan synthetase activity suggested that L-threonine might act by increasing the flow of metabolites in the tryptophan pathway. This effect could conceivably result if L-threonine or a derivative of L-threonine interfered with the normal feedback inhibition capacity of tryptophan. The possibility was examined in an indirect manner by adding anthranilate to *B. alvei* growing in AA-glucose medium. By analogy, it was expected that if L-threonine acted by causing an increase in the levels of tryptophan intermediates, the addition of anthranilate might also cause a similar effect, resulting in indole excretion. Figure 7 shows that the addition of anthranilate to the culture medium resulted in the excretion of indole by *B. alvei* and that indole excretion was halted upon addition of L-serine to the medium during indole excretion. These results indicate that indole excretion can be stimulated in the absence of L-threonine by adding a tryptophan precursor to the medium and is consistent with the working hypothesis that L-threonine acts by stimulating the flow of intermediates in the tryptophan pathway.

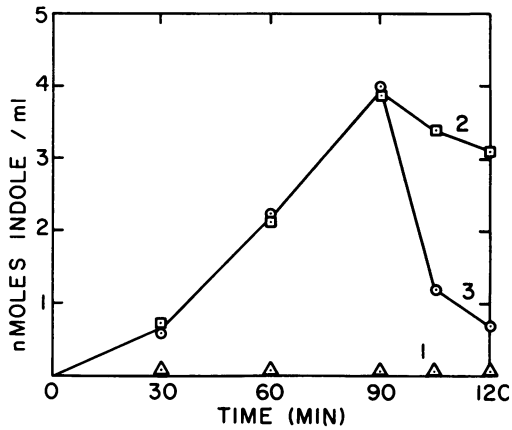

FIG. 7. *Effect of anthranilate on indole excretion by B. alvei. B. alvei F was cultured at 37 C in 50 ml of amino acid medium containing 0.5% glucose. The medium in flasks 2 and 3 was supplemented with anthranilate at an initial concentration of 0.1 μmoles/ml. After 90 min of growth, L-serine was added to flask 3 to the concentration 2 μmoles/ml. Samples (4 ml) were withdrawn at the times indicated; 1 ml was used for determination of the turbidity of the culture, 3 ml was extracted with 2 ml of toluene, and the concentration of indole in 1 ml of the toluene layer was measured. Flask 1, no anthranilate, △; flask 2, anthranilate, □; flask 3, anthranilate with serine added at 90 min, ⊙.*

TABLE 5. *Effect of L-threonine and indole-3-acrylate on the apparent specific activity of the tryptophan synthetase B reaction*[a]

Effector	Concn (μmoles/ml)	Specific activity (10^{-3} units/mg of protein)
None		8.6
L-Threonine	4.17	7.7
Indoleacrylate	0.1	8.7

[a] *B. alvei* was cultured at 37 C in amino acid medium supplemented with 0.5% glucose. When the cells were in the exponential phase of growth the culture was divided into three equal portions. L-Threonine was added to the first portion, indoleacrylate was added to the second, and no addition was made to the third. Growth was continued for 60 min at 37 C; the cultures were rapidly chilled, harvested, and used to prepare crude cell-free extracts. Crude extracts were prepared, and tryptophan synthetase B activity was measured.

The effects of some tryptophan analogues, indole-3-acrylate (W. F. Doolittle, Ph.D. Thesis, Stanford University, 1969), and β-methyltryptophan (J. A. Hirst and J. A. DeMoss, *personal communication*) were studied because of reports that they effect increases in the size of the tryptophan pool in *E. coli*. *B. alvei* was grown in the presence of either 10 or 20 μg of indoleacrylate per ml (Fig. 8). Indole excretion occurred in the presence of indoleacrylate at either concentration employed. More indole was excreted in the presence of 10 μg of indoleacrylate/ml than in the presence of 20 μg of indoleacrylate/ml; in a separate experiment in which the culture contained 50 μg of indoleacrylate/ml, only 1 nmole of indole was excreted per ml, and growth was completely inhibited. These observations are consistent with the working hypothesis, and we assume that indoleacrylate effects increases in the activities of the pathway enzymes and does not inhibit the formation of tryptophan from indole. The latter assumption was tested by the experiment described in Table 6. The results indicate that in the presence of excess indole and serine, indole-3-acrylate is only a weak inhibitor of the tryptophan synthesis reaction. The results of this experiment together with the finding that the

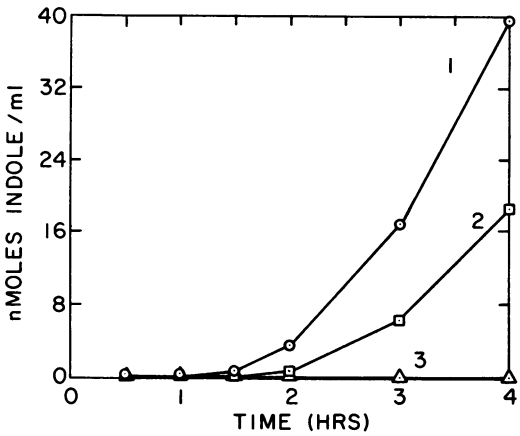

FIG. 8. *Effect of indole-3-acrylate on indole excretion by B. alvei. B. alvei F was cultured at 37 C in amino acid (AA) medium containing 0.5% glucose and supplemented with the tryptophan analogue. Samples (4ml) of the culture were withdrawn at the indicated times; 1 ml was used for measurement of turbidity. Three milliliters was extracted with toluene, and the indole concentration in the toluene layer was determined. Flask 1 contained 10 μg of indoleacrylate per ml; flask 2 contained 20 μg of indoleacrylate per ml; flask 3 represents the control in the absence of indoleacrylate. During the 4-hr course of the experiment, the density of the cultures increased, in dry weight, as follows: control, 314 μg/ml; 10 μg of indoleacrylate/ml, 179 μg/ml; 20 μg of indoleacrylate/ml, 141 μg/ml.*

amount of indole excreted is inversely related to the amount of indoleacrylate in the medium suggest that inhibition of tryptophan synthetase is not the primary mode of action of indole-3-acrylate in vivo.

The effect of indole-3-acrylate on the formation of tryptophan synthetase was tested by the experiment described in Table 5. The specific activity was not changed as a consequence of incorporating indoleacrylate into the growth medium.

Indole was not excreted when *B. alvei* was grown in AA-glucose medium containing either 20 μg of the A isomer per ml or 30 μg of the B isomer of β-methyltryptophan per ml. These analogues did not affect the rate of growth, but may not have been able to enter the cell.

DISCUSSION

The results presented in this paper show that L-threonine stimulates the excretion of early indole by *B. alvei*. L-Serine was also shown to play an important role in early indole excretion based on the following observations. (i) Serine was rapidly removed from casein hydrolysate medium before indole excretion commenced, (ii) continuous addition of serine to a growing culture prevented

TABLE 6. *Inhibition of Bacillus alvei tryptophan synthetase B activity by indole-3-acrylate*[a]

Concn (μg/ml)	Indole utilized (nmoles)	Inhibition (%)
0	170.6	
100	145.4	12.6
200	132.8	18.9

[a] *B. alvei* was cultured at 37 C in standard (STD) medium. Cells in the exponential phase of growth were harvested and used to prepare crude extracts. Tryptophan synthetase B activity was measured by a modification of the assay described in the text. The reaction mixture contained 25 μmoles of Bicine buffer (*p*H 8.0), 250 nmoles of indole, 36 nmoles of pyridoxal-5'-phosphate, 500 nmoles of reduced glutathione, 40 μmoles of L-serine, 15 μliters of saturated KCl, 50 μliters of indole-3-acrylate in 50% ethanol, and enzyme. Water was added to a total volume of 0.5 ml. The control tube contained 50 μliters of 50% ethanol. The reaction was stopped after 20 min at 37 C by adding 0.1 ml of 1 N NaOH. The reaction mixture was extracted with 2 ml of toluene, and the indole concentration was measured by mixing 1 ml of the toluene layer with 3 ml of tube assay color reagent.

indole excretion, (iii) addition of serine immediately stopped indole excretion after it had commenced, and (iv) strains with impaired ability to degrade serine did not excrete early indole. A competitive effect between L-serine and L-threonine was suspected and demonstrated. L-Threonine is a competitive inhibitor of L-serine in the tryptophan synthetase B reaction. The high K_i (0.6 M) for threonine and the observation that the internal tryptophan pool increases during the period of indole excretion suggest that the cause of early indole excretion is more complex than a simple inhibition of tryptophan synthetase. Threonine may act primarily by stimulating the activity of the tryptophan pathway since the addition of anthranilic acid or indoleacrylic acid also stimulate indole excretion in the absence of L-threonine.

One interpretation of the data presented suggests the following model. The amount of serine in the cell is assumed to be maintained at a low level adequate for normal growth conditions. The addition of L-threonine to the medium stimulates the activity of the tryptophan pathway so that the flow of tryptophan intermediates is increased. An increased flow of intermediates forms large amounts of InGP which in the absence of an adequate supply of serine and, possibly, antagonism by threonine, is partially degraded to indole which is excreted. When the level of threonine is reduced the flow of tryptophan intermediates is slowed or stopped, and the excreted indole is reutilized by the cell to form tryptophan. Two varia-

tions on the primary site of threonine action can be accommodated by this model. The first predicts that threonine or some derivative of threonine is directly responsible for the increased flow of tryptophan intermediates which build up at the tryptophan synthetase reaction due to the low relative availability of serine. There is sufficient serine to synthesize an increased amount of tryptophan, but not enough to utilize all of the accumulated InGP. The alternative possibility is that threonine acts primarily by antagonizing the tryptophan synthetase reaction which causes an accumulation of InGP. The accumulated InGP then interferes with feedback inhibition causing a further increase in InGP which can be degraded to indole by the tryptophan synthetase A reaction. The increased intracellular tryptophan concentration is assumed to result from partial relief of the threonine inhibition by the increased InGP concentration. Our data with *B. alvei* cannot distinguish between these possibilities.

This model suggests the possibility that other species might excrete biosynthetically produced indole under conditions of serine limitation. It will be shown in another communication that *E. coli* and *Salmonella typhimurium* do excrete indole under these conditions and that L-threonine stimulates indole excretion. Studies with *S. typhimurium* indicate that, at least in that organism, threonine does not act unless indole is also available. It would thus appear that an analogue of tryptophan might be the active species. For this reason the effect of β-methyltryptophan in the active species cannot be eliminated since the compound may not have been able to enter the cell from the medium.

It is not clear why the serine pool in *B. alvei* is at an apparently lower level than in either *E. coli* or *S. typhimurium*. The difference may signify variations in the role of serine in the general metabolism of these organisms. It is also of interest that the growth of a serine auxotroph of *Bacillus subtilis* is inhibited by high concentrations of L-serine, suggesting that it is important for the bacilli to maintain small pools of serine. The importance of maintaining a small pool could be envisioned if serine proved inhibitory to another cellular process.

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