TRYPTOPHAN SYNTHETASE LEVELS IN *ESCHERICHIA COLI*, *SHIGELLA DYSENTERIAE*, AND TRANSDUCTION HYBRIDS

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Received for publication August 30, 1961

ABSTRACT

EISENSTEIN, RICHARD B. (Western Reserve University, Cleveland, Ohio) AND CHARLES YANOFSKY. Tryptophan synthetase levels in *Escherichia coli*, *Shigella dysenteriae*, and transduction hybrids. J. Bacteriol. **83**:193-204. 1962—*Shigella dysenteriae* and *Escherichia coli*, strains K-12 and B, were found to produce low levels of tryptophan synthetase, although some hybrids, formed by the introduction of the gene cluster concerned with tryptophan synthesis from *S. dysenteriae* into *E. coli*, produced high levels of this enzyme system. A revertant obtained from a tryptophan-requiring mutant also formed high levels of tryptophan synthetase. The gene or genes responsible for high enzyme production in these strains was shown to be linked to the cluster of genes concerned with tryptophan synthesis. The cause of high enzyme production was investigated. Various lines of evidence, including stimulation of growth by tryptophan precursors, sensitivity to inhibition by 5-methyltryptophan, absence of accumulation of tryptophan, and repression of enzyme formation by anthranilic acid and tryptophan, suggested that high enzyme production in the strains examined results from a partial block in the tryptophan pathway and not from resistance to repression by tryptophan. The conversion of shikimic acid-5-phosphate to anthranilic acid appears to be the partially blocked reaction in the strains studied.

It has been repeatedly demonstrated that the concentration of the end product of a biochemical pathway influences the formation of the enzymes in the pathway (Pardee, 1959). Thus, it has been shown that arginine concentration affects the formation of acetylornithinase (Vogel, 1957) and ornithine transcarbamylase (Gorini and Maas, 1958), two enzymes involved in the biosynthesis of this amino acid. Similarly, histidine influences the formation of four enzymes involved in the biosynthesis of this amino acid (Ames and Garry, 1959). Genetic effects on the level of the enzyme formed have also been observed, and, in some of these cases, it has been found that the formation of a specific enzyme or group of enzymes is no longer affected by the end product of the pathway in which the enzymes operate (Cohen and Jacob, 1959; Gorini, 1960). The present study, which grew out of an attempt to determine whether there were qualitative differences between the tryptophan synthetases of *Escherichia coli* and *Shigella dysenteriae*, is concerned with the level of this enzyme in these organisms and in various hybrids.

[1] Taken, in part, from a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine at Western Reserve University, Cleveland, Ohio.

MATERIALS AND METHODS

Bacterial stocks. The following auxotrophs of the K-12 strain of *E. coli* were employed: cys_4, td_4, A-78, T-41, T-3, T-16, and T-58. Of these, cys_4 requires cysteine for growth and the others require tryptophan. Strains B-82, B/1TN, B/1T7, and C-13T, tryptophan auxotrophs of the B strain of *E. coli*, were also used. Mutant T-3 is blocked in the synthesis of anthranilic acid and grows on minimal medium supplemented with anthranilic acid, indole, or tryptophan. Strains T-16 and T-58 are blocked in the conversion of anthranilic acid to anthranilic ribulotide (Smith and Yanofsky, 1960) and respond to indole or tryptophan. Strain A-78 also grows on indole or tryptophan but is blocked in the conversion of indoleglycerol phosphate to tryptophan. The other K-12 and the B tryptophan auxotrophs will grow only on tryptophan. Strains B/1T7, B/1TN, and C-13T are deletion mutants in which a seg-

ment of the cluster of genes controlling tryptophan formation was lost simultaneously with mutation to resistance to phage T-1 (Yanofsky and Lennox, 1959). Strain B/1T7 lacks all the enzymes involved in tryptophan synthesis; strain B/1TN lacks only those concerned with the conversion of indoleglycerol phosphate to indole; and C-13T lacks those concerned with the conversion of anthranilic acid to tryptophan. Other stocks employed included the K-12 wild-type strain and *S. dysenteriae* strain Sh/S (obtained from S. E. Luria). In addition, an unusual revertant (designated T-41R5), isolated from strain T-41, was examined. This strain forms large amounts of tryptophan synthetase when grown on an unsupplemented glucose-salts medium.

Preparation of extracts. All strains of *E. coli* were grown on single strength minimal medium (Vogel and Bonner, 1956) supplemented with 0.16 to 0.2% glucose and any required substance, e.g., tryptophan. *S. dysenteriae* was grown on the same medium with supplements of 0.05% Casamino acids, and 0.4% nutrient broth, or with 0.1% casein hydrolyzate plus low levels of anthranilic acid, indole, or tryptophan. Cells were grown 16 to 18 hr at 37 C with shaking and were harvested by centrifugation. The sedimented cells were washed once with cold 0.85% NaCl, suspended in 0.1 M potassium phosphate buffer (pH 7.8), and disrupted in a 9 kc Raytheon sonic oscillator or a Nossal disintegrator. The treated material was then centrifuged to remove cell debris, and the extracts obtained were stored at −15 C.

Assay of tryptophan synthetase components. The tryptophan synthetase of *E. coli* (and of *S. dysenteriae*) is composed of two separate protein components, termed A and B (Crawford and Yanofsky, 1958). Together these two proteins catalyze the following three reactions: (I) indole + L-serine → L-tryptophan, (II) indoleglycerol phosphate ⇌ indole + triose phosphate, and (III) indoleglycerol PO_4 + L-serine → L-tryptophan + triose phosphate. Each protein component may be quantitatively assayed in the presence of a saturating amount of the second component, usually a threefold excess. Under the assay conditions employed, neither component has appreciable activity in any of the three reactions in the absence of the other. The studies reported here are concerned with reaction I, in which pyridoxal phosphate is required as a coenzyme. The unit of enzyme activity employed in this study is defined as the amount of enzyme (or of component A or B) that will convert 0.1 μmole of indole to tryptophan in 30 min at 37 C. Assays were carried out both in 0.1 M potassium phosphate buffer and in tris(hydroxymethyl)-aminomethane (tris) buffer, supplemented with 0.03 ml of a saturated solution of NaCl per ml of reaction mixture, since the activity sometimes differed under these two conditions. Tryptophan synthetase (or component A or B) specific activity is defined as the units of activity per milligram of extract protein. The protein content was determined by the method of Lowry et al. (1951).

Whole cell assay of tryptophan synthetase. Cells were grown overnight in 5 ml of single-strength minimal medium with 0.12% glucose plus supplements as indicated. Turbidity was determined with a Klett colorimeter (660-mμ filter), portions were centrifuged, and the cells were resuspended in a mixture containing potassium phosphate buffer (pH 7.8), 50 μmoles; DL-serine, 40 μmoles; pyridoxal phosphate, 0.3 μmole; and indole, 0.3 μmole; in a total volume of 1 ml. Assay tubes were incubated with shaking at 37 C for 30 min.

TABLE 1. *Tryptophan synthetase content of various hybrids*

Transduction		Isolate tested	Extract specific activity	
Donor	Recipient		Minimal medium	Nutrient medium
S. dysenteriae	B/1T7 S^{R}*	T^{+}-(1)	26	15
		T^{+}-(2)	164	
		T^{+}-(3)	80	
		T^{+}-(4)	37	11
		T^{+}-(5)	41	11
		T^{+}-(6)	203	73
		T^{+}-(7)	101	
		T^{+}-(8)	30	
S. dysenteriae	B/1T7	T^{+}-(7)	78	
		T^{+}-(8)	88	
Controls				
S. dysenteriae			—	3.2, 3.5
E. coli B			4.7	5.6
E. coli K-12			1.9	2.2, 2.8

* S^{R} (Streptomycin resistant); T^{+} (Tryptophan independent).

The unreacted indole was extracted with toluene and determined quantitatively as described elsewhere (Yanofsky, 1955). Three arbitrary classes were distinguished: low enzyme producers, less than 0.1 μmole of indole taken up per 3×10^9 cells; intermediate producers, 0.1 to 0.2 μmole of indole taken up per 3×10^9 cells; and high producers, greater than 0.2 μmole of indole taken up per 3×10^9 cells.

Assay of the conversion of shikimic acid-5-phosphate to anthranilic acid. The synthesis of anthranilic acid by cell-free extracts was carried out as described by Srinivasan (1959) and Moyed (1960). The reaction mixture contained tris buffer (pH 7.8), 20 μmoles; $MgCl_2$, 2.5 μmoles; L-glutamine, 3 μmoles; diphosphopyridine nucleotide (DPN), 0.25 μmole; shikimic acid-5-phosphate, 0.3 μmole; and extract; in a final volume of 0.5 ml. Incubation was at 37 C and 0.05-ml samples were removed at 10-min intervals and pipetted into 2 ml of 0.1 M potassium phosphate buffer (pH 6.0). The anthranilic acid present in these samples was determined fluorometrically using an Aminco-Bowman spectrophotofluorometer. The barium salt of shikimic acid-5-phosphate was kindly supplied by Bernard Davis of Harvard University.

Transduction. Phage Plkc was used in all the transduction experiments. The procedural details are described elsewhere (Lennox, 1955). Transduction hybrids bear the designations of both the donor and recipient species, with the donor always written first. For example, Sh:K-12 indicates a strain derived from a transduction from *S. dysenteriae* into a K-12 auxotroph.

RESULTS

Initial observations with high enzyme producers. The initial observations which prompted this study are shown in Table 1. Whereas *E. coli* strain B, *E. coli* K-12, and *S. dysenteriae* form relatively little tryptophan synthetase when grown on a glucose-salts medium or a rich medium, hybrids, produced by the introduction of the gene cluster concerned with tryptophan synthesis from *S. dysenteriae* into the deletion mutant, B/1T7, form large amounts of this enzyme system. Previous studies had shown that strain B/1T7 lacked all the *tryp* genes and enzymes concerned with the formation of anthranilic acid and its conversion to tryptophan (Yanofsky and Lennox, 1959; Yanofsky, 1957). It appeared that the introduction of these genes from *S. dysenteriae* into *E. coli* strain B/1T7 was responsible for the production of high levels of tryptophan synthetase. It was also shown in immunological tests that the strains with high enzyme activity formed correspondingly high levels of the A and B proteins as antigens. Thus, the increased activity detected in these strains is not due to alterations in the turnover number of the enzyme system but represents an increase in the amount of specific enzyme proteins.

To determine whether high enzyme production was due to some abnormality associated with B/1T7 (the recipient) or to the genic material introduced from *S. dysenteriae*, hybrids produced with other tryptophan auxotrophs were also examined. Standard transductions (Table 2) were performed with phages grown on the donors indicated, with the listed tryptophan auxotrophs as recipients. The plating medium did not contain tryptophan; thus, tryptophan independence was selected. Colonies were picked from the plates after 3 to 4 days and streaked on minimal medium for single colony isolation. A stock was prepared from one colony from each plate. The results of the examination of these stocks are summarized in Table 2. Both components, A and B, and total tryptophan synthetase activity are high in the hybrids obtained in transductions from *S. dysenteriae* into B/1T7. Furthermore, high enzyme-producing strains, although in the minority, were also isolated from transductions involving strains B-82 and td_4 as recipients. Thus, high enzyme-producing transductants are obtained with other *E. coli* auxotrophs as recipients. It is also evident from the results presented in Table 2 that genic material, permitting tryptophan synthetase formation, can be introduced from *S. dysenteriae* into K-12 and B tryptophan auxotrophs without concomitant high enzyme production. Control transductions with wild-type K-12 as the donor organism and td_4 or B/1T7 as recipient yielded strains with enzyme levels equal to those produced by the wild-type form of the recipients.

When these observations were made, it was recalled that a high tryptophan synthetase-producing strain had been isolated previously during the course of a reversion experiment with strain T-41. This strain, designated T-41R5, was included in this investigation of high tryptophan synthetase production. The tryptophan synthetase and A and B levels produced by this strain

TABLE 2. *Tryptophan synthetase and A and B specific activities of various strains*

Transduction		Stock tested	Specific activity				Ratio A/B
			Tryptophan synthetase		Component A	Component B	
Donor	Recipient		Phosphate buffer	Tris buffer	Tris buffer	Tris buffer	
		T-41R5	12.0	14.8	29.6	22.9	1.3
		E. coli K-12 wild type	1.4 (1.2-1.5*)	1.4 (0.9-1.6)	2.8 (2.2-3.3)	1.8 (0.9-2.3)	1.7 (1.1-2.5)
		S. dysenteriae	2.5	1.9	4.3	2.9	1.5
		E. coli B wild type	3.1 (2.5-3.7)	3.2 (1.8-4.5)	5.2 (3.5-6.9)	4.5 (2.3-6.6)	1.3 (1.0-1.5)
S. dysenteriae	B/1T7 S^R	T^+-(3) T^+-(6) T^+-(8)	30 150 29	18 121 27	36 306 48	29 257 45	1.2 1.2 1.1
S. dysenteriae	td_4 S^R	7 T^+ isolates T^+-(8)	1.2 (0.7-1.6) 5.3	1.1 (0.9-1.4) 4.4	3.3 (2.8-3.5) 8.1	1.4 (1.1-1.8) 7.9	2.3 (1.8-3.3) 1.0
S. dysenteriae	B-82	7 T^+ isolates T^+-(3)	2.6 (1.4-3.6) 35	2.6 (1.3-3.6) 30	6.1 (3.1-7.2) 55	4.7 (2.3-6.3) 53	1.2 (1.0-1.4) 1.0
K-12	td_4 S^R	3 T^+ isolates	1.8 (1.6-2.0)	2.0	3.4 (3.3-3.7)	3.0 (2.9-3.1)	1.1 (1.1-1.2)
K-12	B/1T7 S^R	3 T^+ isolates	3.5 (2.1-4.5)	4.3 (3.2-5.1)	7.6 (7.1-8.0)	6.6 (5.2-7.8)	1.2 (1.1-1.4)

* Values in parentheses represent range of specific activities and A/B ratios detected in separate experiments. T^+ indicates isolate which can grow in the absence of tryptophan.

are given in Table 2. Extracts of mutant T-41 lack the B component of tryptophan synthetase.

Extract preparation and analyses of the type presented in Table 2 involve many operations and as a result are time-consuming. To examine larger numbers of transductants for tryptophan synthetase activity, a whole cell assay was developed (Materials and Methods). This assay method gave good proportionality between numbers of cells added and tryptophan synthetase activity detected (Fig. 1) for both high and low tryptophan synthetase producers; therefore, it could be used to assay large numbers of individual isolates for their tryptophan synthetase activity. It can also be seen from Fig. 1 that *S. dysenteriae*, *E. coli* B, and *E. coli* K-12 show less than one unit of tryptophan synthetase activity per 3×10^9 cells. In other tests the tryptophan synthetase activity of whole cells, toluene-treated cells, and extracts was compared, and no appreciable differences in tryptophan synthetase levels were detected.

During the course of standardizing the whole cell assay, it was noticed that, although *S. dysenteriae* formed fully active tryptophan synthetase, it was impaired at some step in the formation of tryptophan, since its growth was appreciably stimulated (three- to fivefold) by tryptophan, indole, or anthranilic acid. The other amino acid requirements of the *S. dysenteriae* strain employed were not determined, but could be satisfied by tryptophan-free acid-hydrolyzed casein.

Genetic studies. The gene or genes responsible for the partial growth dependence of *S. dysen-*

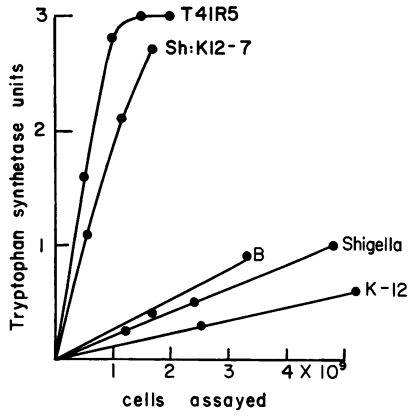

FIG. 1. *Whole cell assay: relationship between numbers of cells added and tryptophan synthetase activity. Strains T-41R5 and Sh:K-12 (7) are high enzyme producers while the others form low levels of tryptophan synthetase. A substrate concentration which gives a maximum of 3 units of activity was employed.*

teriae on tryptophan was shown to be linked to the genes controlling tryptophan synthetase formation (Table 3). Here 104 of 240 colonies tested from a transduction, with strain B-82 as recipient, responded to anthranilic acid. The test employed to detect anthranilic acid stimulation was to streak loopfuls of a bacterial suspension onto plates with or without an anthranilic acid supplement and to compare growth after 24 hr. This method is somewhat crude and it is likely that the estimate of the number of anthranilic-stimulated colonies is low. Some colonies of each type were tested for tryptophan synthetase activity, and it can be seen (Table 3) that high-producing strains appeared in both groups; however, all the anthranilic-stimulated isolates produced appreciable amounts of enzyme and were at least intermediate producers.

Strain T-41R5 was also examined in transduction tests. When T-41 was employed as a recipient, it became apparent that high enzyme production was associated with a gene distinct from the mutated region in T-41 (Table 3). Apparently strain T-41R5 differs from T-41 at two sites, one responsible for high enzyme production and the other representing a functionally effective site corresponding to the defective site in strain T-41. The high enzyme-producing determinant is obviously closely linked to the genic region controlling tryptophan synthetase formation, since 76% of the isolates which received genic material permitting tryptophan synthetase production were high producers. Thus, in both T-41R5 and *S. dysenteriae*, genic material which can cause high enzyme production is linked to the genes controlling tryptophan synthetase formation.

Attempts were made to localize further these determinants in the bacterial genome by transduction into a series of mutants lacking different segments of the *tryp* gene cluster as a result of deletion. The rationale for this approach was that if a deletion mutant lacked genic material corresponding to the region responsible for high enzyme production, then only high-producing strains could be obtained in transductions. On the other hand, if the corresponding region was not deleted, some low-producing strains would be obtained among the tryptophan-independent stocks recovered. The size of the deletions in the three stocks tested, in relation to the biosynthetic pathway of tryptophan formation, is shown in Fig. 2. The results of these transduction tests, summarized in Table 3, indicate that, with strain T-41R5 as donor, low producers are recovered with B/1TN and C-13T as recipient but not with B/1T7. These findings indicate that the genic region responsible for high enzyme production in T-41R5 is probably close to, but not in, the region deleted in C-13T and B/1TN.

Close linkage to the *tryp* region is also shown by the recovery of many high producers in the transduction into strain td_4 and into a strain bearing a closely linked marker, cys_4. The locations of these markers relative to the deletions studied are indicated in Fig. 2. Similar experiments carried out with *S. dysenteriae* or a high enzyme-producing *Shigella*:K-12 hybrid, stock Sh:K-12 (7), as donors, were complicated by the fact that many of the recombinants obtained were stimulated by anthranilic acid. Such stocks, when grown on unsupplemented minimal medium, generally fell into at least the intermediate producer class, and often produced just enough enzyme to be placed in the high enzyme producer class. With various auxotrophs as recipients and *S. dysenteriae* as donor (Table 3), both high- and low-producing strains were recovered. Furthermore, no low producers were recovered with the Sh:K-12 hybrid as donor and the deletion mutants C-13T and B/1T7 as recipients. These findings indicate that genic material closely linked to the tryptophan synthetase genic region

TABLE 3. *Whole cell assay results with isolates from various transductions*

Transduction		T^+ isolates examined	Stimulated by anthranilic acid	Isolates assayed	Number of isolates producing enzyme at following levels		
Donor	Recipient				HP*	IP*	LP*
T-41R5	T-41	50			38	0	12
T-41R5	td_4	50			40	7	3
T-41R5	cys_4	46			19	2	25
T-41R5	B-82	47			23	6	18
T-41R5	B/1T7	82			82	0	0
T-41R5	C-13T	45			42	0	3
T-41R5	B/1TN	15			9	0	6
S. dysenteriae	B-82	240	104	112 NS*	7	15	90
				19 S*	18	1	0
S. dysenteriae	B/1T7	89	89	27 S	16	11	0
S. dysenteriae	td_4	94	19	14 NS	4	0	10
				5 S	4	1	0
Sh:K-12(7)†	B/1T7	20	0	20 NS	20	0	0
Sh:K-12(7)	C-13T	18	0	18 NS	17	1	0
K-12	T-41	50	0	50 NS	0	4	46
K-12	td_4	25	0	25 NS	0	0	25
K-12	B/1T7	9	0	9 NS	0	0	9

* Abbreviations used: NS (not stimulated by anthranilic acid); S (stimulated by anthranilic acid); HP = high producing; IP = intermediate producing; LP = low producing.

† A high enzyme-producing hybrid.

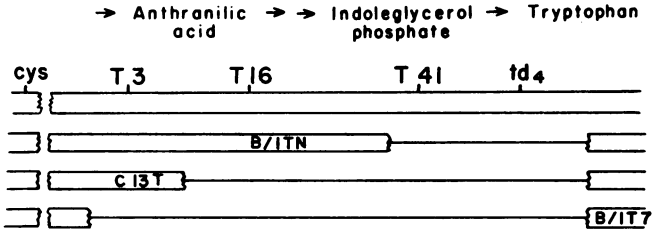

FIG. 2. *Relative location of mutated sites in the strains examined. The thin lines represent the areas deleted in mutants B/1TN, C-13T, and B/1T7.*

is responsible for high enzyme production. Transductions with K-12 as donor, also listed in Table 3, demonstrate that high producers are not recovered with this low-producing donor strain. The few intermediate producers recovered were barely out of the low producer class (1.2 units).

Cause of high tryptophan synthetase production. The previous findings indicated that the introduction of certain genic material from strains T-41R5 and *S. dysenteriae* into the proper stocks resulted in the production of high levels of tryptophan synthetase. Several explanations for this observation were considered. First, it appeared possible that the high-producing strains, in contrast to normal strains, were not subject to repression by tryptophan, and thus enzyme production proceeded unregulated. According to this interpretation both T-41R5 and *S. dysenteriae* would carry the gene or genes concerned with regulating tryptophan synthetase formation in a mutated nonfunctional form, or the *S. dysenteriae* repressor gene or genes could not function in *E. coli*. Mutations relieving repression in the tryptophan pathway have been described in *E. coli* (Cohen and Jacob, 1959). A second possibility was that all the high tryptophan synthetase-producing strains

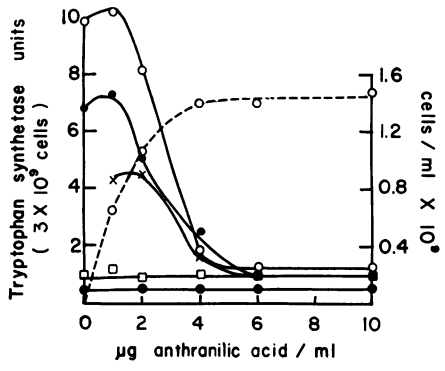

FIG. 3. *The effect of anthranilic acid concentration on tryptophan synthetase production. The dashed line represents the growth response of mutant T-3 to the levels of anthranilic acid indicated. The solid lines represent tryptophan synthetase levels,* × *(T-3);* ○ *(T-41R5);* ● *(Sh:K-12 (7), upper curve);* □ *(S. dysenteriae);* ■ *(K-12, lower curve)*

had a partial block at some step in tryptophan synthesis, and this partial block limited the supply of tryptophan. The resulting low endogenous concentration of this amino acid would thereby relieve repression. A situation analogous to this has been described by Gorini and Maas (1958) in studies of the ornithine transcarbamylase of *E. coli*. To be consistent with the data presented here it would have to be further assumed that other limitations, perhaps also in tryptophan formation, mask the effect of the partial block in *S. dysenteriae*.

To distinguish between the possibilities mentioned, two general approaches were employed; the first was intended to determine whether tryptophan synthetase production by high producers is subject to repression by tryptophan, and the second, whether high-producing strains are, in fact, partially blocked in tryptophan formation.

To examine whether the high-producing strains were resistant to repression, they were grown in the presence or absence of high concentrations of tryptophan or its precursor, anthranilic acid. More extensive data were obtained with anthranilic acid, and typical results with this compound are presented in Fig. 3. The high enzyme-producing strains examined were T-41R5 and the hybrid Sh:K-12 (7). For comparison, a mutant blocked prior to anthranilic acid, strain T-3, was also examined, as well as the parental organisms, *E. coli* K-12 and *S. dysenteriae*. It can be seen that at low anthranilate concentrations T-3 produced higher than normal tryptophan synthetase levels, but the level dropped to the wild-type control level as the anthranilic acid supplement approached a concentration which supported maximal growth. Similarly, the tryptophan synthetase level in T-41R5 and Sh:K-12 (7) dropped at approximately the same anthranilate concentration. Similar inhibitions were observed with high tryptophan concentrations. Thus, tryptophan synthetase formation in high-producing strains is clearly subject to repression by tryptophan.

Several approaches were employed to determine whether the high producers are partially blocked at some step in tryptophan synthesis. High-producing strains which were stimulated by anthranilic acid in the streaking test obviously have a partial block and were not examined further. In addition to this type, some hybrid strains

TABLE 4. *Anthranilic acid stimulation of growth**

Strain	Sample	Inoculum dilution					
		1-10		1-100		1-10³	
		Min†	AA†	Min	AA	Min	AA
Sh:K-12(7)	A‡	0	4	0	0	0	0
(H.P.)	B	0	5	0	5	0	5
Sh:K-12(10)	A	1	5	0	0	0	0
(H.P.)	B	5	5	5	5	5	5
Sh:B(7)	A	0	0	0	0	0	0
(H.P.)	B	2	5	0	5	0	5
Sh:K-12(11)	A	4	4	0	0	0	0
(L.P.)	B	5	5	5	5	5	5
T-41R5 (H.P.)	A	5	5	0	0	0	0
	B	5	5	5	5	5	5
K-12 (L.P.)	A	5	5	0	0	0	0
	B	5	5	5	5	5	5

* Cultures were grown on minimal medium to a population of 2 to 5 × 10^8 cells/ml and diluted as indicated. An inoculum of 0.1 ml of each dilution was used for 5 ml of minimal medium with and without a 10 µg/ml anthranilic acid supplement. Growth was checked at different times and recorded on a 0 to 5 scale. Turbidity measurements in the Klett Colorimeter on K-12 and T-41R5 cultures indicated that growth was identical on minimal medium and anthranilic acid-supplemented medium.

† Min (single strength *E. coli* minimal medium); AA (the same medium supplemented with anthranilic acid).

‡ A and B represent different sampling times, with B the later time.

were recovered which were not stimulated by anthranilic acid but which were high producers (Table 3). Furthermore, T-41R5 and all high-producing stocks derived from it were not stimulated by anthranilic acid.

A fairly sensitive method of detecting differences in growth rate was employed in examining high enzyme-producing strains. This involved growing a log-phase culture in minimal medium, diluting the cells, and using equal inocula in fresh minimal medium and in minimal medium supplemented with anthranilic acid or tryptophan. The cultures were then observed for visible turbidity and scored, using an arbitrary scale. The results obtained in one series of experiments are presented in Table 4. The data obtained indicate that three high-producing strains, Sh:K-12 (7), Sh:K-12 (10), and Sh:B (7), none of which was detectably stimulated by anthranilic acid in the streaking test, reached visible turbidity first in the anthranilic-supplemented medium. The controls and T-41R5 grew equally rapidly in both media. Similar results were obtained with several of these strains when tryptophan, rather than anthranilic acid, was employed as supplement. As an independent test of the conclusions reached in the experiments in liquid medium, small numbers of cells, ca. 5 to 20, were plated on minimal agar plates and on minimal agar plates supplemented with anthranilic acid; and after 2 to 3 days, colony size (diameter) was measured. The results were entirely consistent with those obtained in liquid medium. For example, colonies of strain Sh:K-12 (7) on minimal medium averaged 2.3 ± 0.3 mm and on minimal medium plus anthranilic acid averaged 3.2 ± 0.3 mm.

Only strain T-41R5, of the high-producing types, failed to respond to tryptophan or tryptophan precursors. This behavior need not exclude the existence of a partial block in tryptophan synthesis in this strain, since it is possible that K-12 normally overproduces tryptophan and that this slight overproduction is responsible for repression of the enzymes involved in tryptophan biosynthesis. T-41R5 could have a partial block which reduces the rate of tryptophan synthesis to a level at which tryptophan is never present at a concentration sufficient to repress. It still might be capable of synthesizing tryptophan at a rate which permits normal growth.

To examine this possibility, a test was sought which would effectively measure the endogenous levels of tryptophan and tryptophan precursors present during growth rather than growth dependence on tryptophan. The test ultimately selected was to examine sensitivity to an analogue of tryptophan, 5-methyltryptophan. It was reasoned that, since inhibition by this analogue is specifically reversed by tryptophan and tryptophan precursors, the level of growth obtained in the presence of the analogue would be an indication of the internal concentrations of tryptophan and tryptophan-specific precursors. Sensitivity of various high-producing and low-producing strains to 5-methyltryptophan is shown in Table 5, where it can be seen that all the high producers, but none of the low producers, were inhibited by the level of 5-methyltryptophan employed. It is also evident that strain T-41R5 was more sensitive to the analogue than the wild type or a low-producing revertant, T41-R8, which was also isolated from T-41. However, T-41R5 was the only high producer to form visible colonies after 3 days. These results, although they do not give any information on the mechanism of inhibition by 5-methyltryptophan, indicate that the concentration of tryptophan and tryptophan-specific precursors is probably lower in the high-producing strains, including T-41R5.

As a verification of the method and the interpretation, several presumed partial revertants

TABLE 5. *5-Methyltryptophan sensitivity of high and low producers*

Strain	Colony size in mm (average of 5 or more colonies)			
	Minimal medium		Minimal medium plus 0.05 μg 5-MT*/ml	
	2 days	3 days	2 days	3 days
K-12, wild type	2.8	4.2	2.9	4.1
T-41R5 (H.P.)	3.2	4.6	0	2.5
T-41R8 (L.P.)	2.7	—	3.1	—
Sh:K-12(7) (H.P.)	2.0	3.4	0	0
Sh:K-12(18) (H.P.)	2.0	3.3	0	0
Sh:K-12(11) (L.P.)	2.8	3.9	2.0	3.4
Sh:K-12(15) (L.P.)	2.9	4.0	2.5	3.8
Sh:B(7) (H.P.)	3.1	—	0	0
Sh:B(20) (L.P.)	4.2	—	4.5	—

* 5-MT (5-methyltryptophan).

TABLE 6. *Conversion of shikimic acid-5-phosphate to anthranilic acid*

Extract	Tryptophan synthetase components, specific activity		S-5-P → Anth. A. / μMoles anth. A. formed/hr/mg extract protein	Tryptophan* inhibition of S-5-P → Anth. A
	A	B		
				%
T-58†	31	17	9.2	45 to 60
A-78	99	76	3.2	—
T-41R5	22	19	0.6	43
Sh:B (6)	151	147	0.06‡	33
Sh:K-12 (7)	71	72	0.12	50

* 4×10^{-5} M L-tryptophan. S-5-P (shikimic acid-5-phosphate).

† Grown in the presence of low levels of indole (tryptophan auxotrophs, T-58 and A-78). Grown on unsupplemented minimal medium (high enzyme producers, T-41R5, Sh:B(6), and Sh:K-12(7)).

‡ Barely detectable.

were isolated from strain T-16, a mutant blocked between anthranilic acid and anthranilic ribulotide. (Genetic tests were not performed to distinguish between partial reversion and suppression.) These were obtained by plating T-16 cells on minimal medium and picking slow-growing tryptophan-independent colonies. These cells invariably accumulated anthranilic acid. The results of whole cell assays on a number of these partial revertants showed that they were all high producers. These revertants were then examined for sensitivity to 5-methyltryptophan, and all were found to be inhibited by 5-methyltryptophan concentrations which do not inhibit the wild type.

Attempts to determine the site of the partial block. Culture filtrates of the various partially blocked high-producing strains (other than those derived from T-16) were examined for anthranilic acid, anthranilic ribuloside, indoleglycerol, and indole. One of these compounds might be expected to accumulate if the block in tryptophan synthesis were at a reaction involving one of the compounds as substrate. No trace of any of these compounds was detected; therefore, it was concluded that either the partial block was not severe enough to cause accumulation or was at a step prior to anthranilic acid.

As a further test of this conclusion, extracts of representatives of the different types of high-producing classes were examined semiquantitatively for the enzymes involved in the conversion of anthranilic acid to indoleglycerol phosphate. In all cases the enzyme activities were very high, at least 5 to 10 times that of the low-producing strains. Thus, none of the enzymes between anthranilic acid and tryptophan appears to be present in a reduced amount, supporting the view that the partial block is prior to anthranilic acid. Culture filtrates were also examined for tryptophan and none was detected. Neither was this amino acid found in the supernatant solutions obtained following heating and centrifugation of crude extracts of high-producing strains.

Extracts of various high producers were also examined in the conversion of shikimic acid-5-phosphate to anthranilic acid. Two tryptophan auxotrophs, strains A-78 and T-58, were employed as controls. These strains form high levels of tryptophan synthetase and other enzymes of the tryptophan pathway when grown on growth-limiting levels of indole or tryptophan. The high tryptophan synthetase-producing strains, T-41R5 Sh:B (6), and Sh:K-12 (7), which presumably have partial blocks, are clearly distinguishable from the control stocks in that they showed little anthranilic acid synthesis (Table 6). The absence of appreciable activity could not be due to enzyme imbalance or a rapid conversion of synthesized anthranilic acid to other intermediates, since the extracts employed did not metabolize added anthranilic acid in the presence of the supplements provided. Furthermore, the extracts do not contain an inhibitor, since mixtures with T-58 extracts were as active as T-58 extracts alone. The synthesis of anthranilic acid is a complex reaction and the activity values presented should be considered only a qualitative measure of enzyme activity. In fact, with the control extracts, T-58 and A-78, there was a definite threshold effect, with little or no activity when less than 2 to 3 mg of extract protein are present per ml. When the extract concentration was increased there was a sharp nonproportional increase in enzyme activity. This lack of proportionality could be eliminated by the addition of an extract of the deletion mutant B/1T7. Extracts of this mutant were devoid of activity but increased the rate of anthranilic acid synthesis by T-58 and A-78 extracts 10-fold to 20-fold. The stimulatory effect of B/1T7 extracts is probably not due to the presence of one of the enzymes involved in the synthesis of anthranilic acid, since extracts

TABLE 7. *Tryptophan synthetase levels in tryptophan-independent isolates derived from Shigella dysenteriae*

Transduction		T^+ isolates examined	Stimulated by anthranilic acid	Isolates assayed	Number of isolates producing enzymes at following level		
Donor	Recipient				HP	IP	LP
T-41R5	*S. dysenteriae*	20	0	20	20	0	0
K-12	*S. dysenteriae*	39	11	28 NS*	0	0	28
				11 S*	11	0	0

* See Table 3 for explanation of symbols.

of B/1T7 grown on repressing levels of tryptophan were fully effective in stimulating the activity of T-58 extracts. Extracts of B/1T7 did not stimulate the synthesis of anthranilic acid by extracts of strains T-41R5, Sh:B (6), and Sh:K-12 (7), suggesting that the activity of the latter extracts is rate-limiting. In other tests it was shown that tryptophan inhibited the synthesis of anthranilic acid by extracts of the various strains to the same extent (Table 6).

Studies with S. dysenteriae as recipient. One of the as yet unexplained observations is that, although introduction of *S. dysenteriae* genic material into *E. coli* stocks may result in high enzyme production, *S. dysenteriae* itself does not form high levels of tryptophan synthetase. Only low levels of enzyme are formed by this organism despite the fact that it appears to be partially blocked in tryptophan synthesis and grows only poorly in the absence of this amino acid or one of its precursors. To obtain further information relevant to the behavior of this organism, several transduction experiments were performed with *S. dysenteriae* as recipient. In these experiments the limited ability of *S. dysenteriae* to grow without tryptophan was used as a selective character, and recombinants which grew well in the absence of tryptophan were selected.

The results obtained with wild-type K-12 and T-41R5 as donors are presented in Table 7. It can be seen that with T-41R5 as donor all the recombinants tested were high producers. Thus it would appear that the inability to produce high levels of enzyme is not due to the other nutritional requirements of *S. dysenteriae* but is associated with the genic region concerned with tryptophan synthesis, since replacement of a portion or portions of this region yields high enzyme producers. The results obtained with wild-type K-12 as donor show that replacement of a portion or portions of this region from a low enzyme producer, wild-type K-12, also gives high enzyme producers, although less frequently. It should also be noted that the recombinant high producers obtained from the transduction K-12 → *S. dysenteriae* responded to anthranilic acid in the streaking test, indicating that they were still partially blocked in tryptophan synthesis. However, they were capable of much faster growth in the absence of tryptophan than the parental strain, *S. dysenteriae*.

DISCUSSION

The studies reported in this paper demonstrate that strain T-41R5 and some *E. coli-S. dysenteriae* transduction hybrids form high levels of the A and B proteins of tryptophan synthetase. Two alternative explanations were considered for the production of high levels of enzyme by these strains: a partial block in an earlier reaction, and resistance to repression by the end product of the pathway, tryptophan. The findings obtained in this study have been interpreted as favoring the partial block hypothesis. The growth of most of the high enzyme producers, with the notable exception of T-41R5, was stimulated by anthranilic acid or tryptophan. Stimulation of the growth of high producers is obviously an indication of an impairment in tryptophan formation. In addition, all the high enzyme producers, including T-41R5 and strains derived from it, were extraordinarily sensitive to inhibition by 5-methyltryptophan, an analogue which interferes with tryptophan formation (Moyed, 1960). This extreme sensitivity would be expected if the rate of tryptophan synthesis was low in the high enzyme producers. Consistent with this conclusion is the observation that tryptophan was not accumulated in the culture medium of the high enzyme producers, although most of the assayed enzymes in the tryptophan pathway were present in greater than normal amounts. Tryptophan might be expected to be accumulated by organisms which are resistant to repression and form

high levels of enzymes; and, in fact, the one repression-resistant mutant (tryptophan pathway) which has been isolated and studied (Cohen and Jacob, 1959) does accumulate this amino acid. Finally, levels of anthranilic acid and tryptophan which just barely supported maximal growth of tryptophan auxotrophs were found to repress enzyme production in the high producers. Taken together, these observations strongly suggest that partial blocks in tryptophan synthesis are responsible for the production of high levels of tryptophan synthetase in the strains examined.

Enzymatic studies did lead to the detection of a partial block in the conversion of shikimic acid-5-phosphate to anthranilic acid. The nature of the partial block is not known; all that has been established is that there is very little enzyme activity in this reaction. One possible explanation of the partial block (suggested by Bernard Davis of Harvard University), that the enzyme(s) responsible for the synthesis of anthranilic acid is particularly sensitive to feedback inhibition by tryptophan, is unlikely in view of the finding that the formation of anthranilic acid by extracts of strain T-41R5 is not unusually sensitive to tryptophan. In view, however, of the fact that feedback-resistant mutants have been obtained (Moyed, 1960), it is not unlikely that mutants with an increased feedback sensitivity will also be found. Studies with other microbial systems have shown that partial blocks in the preceding reactions may lead to the formation of high levels of specific enzymes (Gorini and Maas, 1958; Ames and Garry, 1959). In view of these observations, it might be expected that any compound which competes with one of the intermediates in the tryptophan pathway could be employed to control the internal supply of tryptophan in much the same way that a partial block would operate. Indeed, recent studies with many of the strains employed in the present investigation have shown that high levels of enzyme are produced when 3-methyl anthranilic acid is added to the culture medium (Lester and Yanofsky, 1961).

The transductions with strain T-41R5 as donor demonstrate that the locus responsible for high enzyme production is distinct from the original mutant site in strain T-41, but is closely linked to this site. A group of 50 additional revertants of strain T-41 was examined in other tests to determine whether stocks of the T-41R5 type are frequent among these revertants. High enzyme producers were not detected. Furthermore, attempts to recover high enzyme producers by transduction of T-41 to tryptophan independence with phage grown on wild-type K-12 were also unsuccessful. Thus, it would appear that the mutation responsible for high enzyme production was a rare event in strain T-41. The locus from *S. dysenteriae* which is responsible for high enzyme production by some of the hybrids was also found to be closely linked to the genes controlling tryptophan synthetase.

The results of the transductions from wild-type K-12 and T-41R5 into *S. dysenteriae*, and the fact that *S. dysenteriae* itself is a low enzyme producer, are difficult to explain. One could assume that *S. dysenteriae* has not one but two blocks in anthranilic acid synthesis and any level of tryptophan supplement supplied represses activity. The recovery of high producers from the transductions with wild-type K-12 as donor could be accounted for by assuming that the more severe block in *S. dysenteriae* was eliminated but not a second, partial block. Thus, enough tryptophan could be produced in the recovered strains to allow rapid growth in the absence of tryptophan, but not enough to effectively repress tryptophan synthetase production. When both blocks are eliminated by transduction, tryptophan would be produced in amounts which allowed regulation of tryptophan synthetase formation at the repressed wild-type level and low producers would be recovered. The T-41R5 transductions gave only high producers in the sample examined, probably by the introduction of genic material carrying the T-41R5 partial block along with the gene or genes permitting rapid growth in the absence of tryptophan.

It is also of interest to compare the levels of enzyme activity observed in the high producers with those reported for a strain which is resistant to repression by tryptophan (Cohen and Jacob, 1959). In the latter case tryptophan synthetase production was increased only three- to fourfold, although in the present study, instances in which enzyme levels were consistently increased by a factor of 50 were observed. Theoretically, a partial block could not lead to enzyme levels higher than those obtained in an organism which is completely resistant to repression. Thus, other factors must play a role in the regulation of enzyme formation, or mutations may occur which impart different degrees of repression resistance.

The explanation for the variety of tryptophan synthetase levels observed in the *S. dysenteriae-E. coli* high enzyme-producing hybrids is not known.

ACKNOWLEDGMENTS

This investigation was supported by grants from the U. S. Public Health Service and the National Science Foundation.

The authors are greatly indebted to Virginia Horn and Mary Hurd for technical assistance.

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