

# Mechanism of 3-Methylantranilic Acid Derepression of the Tryptophan Operon in *Escherichia coli*<sup>1</sup>

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3-Methylantranilic acid (3MA) inhibits growth and causes derepression of the tryptophan biosynthetic enzymes in wild-type strains of *Escherichia coli*. Previous reports attributed this effect to an inhibition of the conversion of 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate to indole-3-glycerol phosphate and a consequent reduction in the concentration of endogenous tryptophan. Our studies have shown that 3MA-resistant mutants linked to the tryptophan operon have a feedback-resistant anthranilate synthetase; mutants with an altered indole-3-glycerol phosphate synthetase were not found. 3MA or 7-methylindole can be metabolized to 7-methyltryptophan, and 3MA, 7-methylindole, and 7-methyltryptophan lead to derepression of the tryptophan operon. Furthermore, 3MA-resistant mutants are also resistant to 7-methylindole derepression. These results strongly suggest that the primary cause of derepression by 3MA is through its conversion to 7-methyltryptophan, which can inhibit anthranilate synthetase, thereby decreasing the concentration of endogenous tryptophan. Unlike 5- or 6-methyltryptophan, 7-methyltryptophan does not appear to function as an active corepressor.

Regulation of tryptophan biosynthesis in *Escherichia coli* occurs through the combined effects of repression of enzyme synthesis and feedback inhibition of the branch-point enzyme. At least one regulator gene concerned with repression has been mapped and is situated some distance from the tryptophan operon near threonine on the *E. coli* chromosome (2). Unlike some other biosynthetic operons (15, 16), tryptophanyl-transfer ribonucleic acid (tRNA) does not appear to be involved in repression (4). Regulation of tryptophan biosynthesis also occurs through feedback inhibition of the product tryptophan, on the first enzyme in the biosynthetic sequence, anthranilate synthetase (1, 14). Derepression can occur whenever the level of endogenous tryptophan becomes limiting (21). Analogues of tryptophan may be capable of causing derepression by limiting tryptophan production through feedback inhibition of anthranilate synthetase.

3-Methylantranilic acid (3MA), inhibits growth and causes derepression of the tryptophan biosynthetic enzymes in wild-type strains

of *E. coli*. Previous reports attributed this effect to an inhibition of the conversion of 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate to indole-3-glycerol phosphate and a consequent reduction in the concentration of endogenous tryptophan (12). However, anthranilate synthetase feedback-resistant mutants are known to be resistant to the effects of this analogue (18). In this report, we present evidence that 3MA is converted to 7-methyltryptophan (7MT), which can inhibit anthranilate synthetase, thereby decreasing the concentration of endogenous tryptophan and causing derepression. Unlike 5-methyltryptophan (5MT) or 6-methyltryptophan (6MT), 7-MT does not appear to function as an active corepressor.

## MATERIALS AND METHODS

**Organisms.** Wild-type strains of *E. coli* K-12, W1485, and W3110 were used in these studies. Most of the mutants described are derived from W1485 *tna* (a mutant lacking tryptophanase isolated in this laboratory) after ultraviolet (UV) or nitrosoguanidine treatment and plating on appropriate selective media. Derepressed mutants (*trpR*) and mutants with a feedback-resistant anthranilate synthetase (*trpE<sup>FBR</sup>*) were isolated by plating on minimal agar containing  $5 \times 10^{-4}$  M 5MT. 3-Methylantranilate-resistant mutants (*MAR*) were isolated by plating on minimal agar containing  $6.7 \times 10^{-4}$  M 3MA. The

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large resistant colonies were picked, purified by single colony isolation, and further characterized by assaying whole cells for elevated levels of tryptophan synthetase (7). Of the 5MT-resistant mutants, *trpR* mutants produced elevated levels of tryptophan synthetase in the presence of repressing concentrations of tryptophan ( $2.5 \times 10^{-4}$  M). The *trpE<sup>FBR</sup>* mutants had repressed enzyme levels and were identified by preparing cell-free extracts and assaying anthranilate synthetase in the presence of L-tryptophan. *MAR* mutants did not produce elevated levels of tryptophan synthetase in the presence of 0.2  $\mu$ mole of 3MA/ml. These tentative conclusions were confirmed by preparing cell-free extracts of the mutants grown under appropriate conditions and assaying the tryptophan biosynthetic enzymes.

**Transduction procedures.** The *trpR*, *trpE<sup>FBR</sup>*, and *MAR* mutants were characterized genetically as being linked, or unlinked, to the tryptophan operon by transduction. Plk lysates of the mutants were prepared by the confluent lysis technique (11). These lysates were used to transduce a *cysB trp(A-E deletion)* or other suitable recipient, selecting for *cys<sup>+</sup> trp<sup>+</sup>* recombinants. Control lysates prepared from wild-type strains and controls for sterility were done routinely. The recombinants were tested to determine whether they had concurrently received either 5MT resistance or 3MA resistance. Resistance to 5MT was determined by picking the colonies, diluting in saline, and spotting on 5MT plates. Growth within 24 hr was used as a criterion for 5MT resistance. To determine resistance to 3MA-induced derepression, recombinants were assayed for tryptophan synthetase by the whole-cell assay (7) after growth in minimal medium containing 0.2  $\mu$ mole of 3MA/ml. Cell-free extracts were prepared from a few of the recombinants, in each case, and the results of the whole-cell assay were verified by enzyme assays.

**Isolation of *MAR* mutants linked to the *trp* region (*trp MAR*).** A procedure similar to that used by Somerville and Yanofsky (18) to isolate 5MT-resistant mutants linked to the *trp* region was used to isolate *trp*-linked 3MA-resistant mutants. After UV treatment, cells were inoculated into minimal medium containing 0.2  $\mu$ mole of 3MA/ml and were grown overnight; 0.1 ml of this culture was used to inoculate a second tube containing 0.2  $\mu$ mole of 3MA/ml and again grown overnight. Since 3MA inhibits growth, this procedure enriched for mutants which were not inhibited by 3MA. A Plk lysate of the second culture was prepared and used to transduce a *cysB trp(A-E deletion)*, selecting for *cys<sup>+</sup> trp<sup>+</sup>* recombinants which were also resistant to  $6.7 \times 10^{-4}$  M 3MA.

**Culture conditions.** All strains were grown in the minimal medium of Vogel and Bonner (19) containing 0.5% glucose and other supplements as desired. The cultures were agitated on a rotary shaker at 37 C. Growth curves were obtained by growing 50-ml cultures in a 500-ml side arm flask and estimating growth at various times by turbidity at 660 nm. One hundred Klett units correspond to a plate count of  $7.2 \times 10^8$  cells/ml. The kinetics of enzyme appearance was measured in the following manner.

The prototrophic strain was grown overnight in minimal medium and harvested in the mid to late log phase of growth. The cells were washed once with ice-cold minimal medium and resuspended in pre-warmed minimal medium containing glucose and the desired concentration of inhibitor (e.g., 7-methylindole). Samples of the cell culture were removed at appropriate times and poured over crushed ice. Cell-free extracts were then prepared and the enzyme activity was determined.

**Preparation of cell-free extracts.** Cells were harvested by centrifugation, washed once in saline, and resuspended in 0.1 M  $K_2HPO_4$ -HCl, pH 7.0, containing  $10^{-3}$  M ethylenediaminetetraacetate and  $10^{-3}$  M 2-mercaptoethanol. The cells were broken by sonic oscillation, and cell debris was removed by centrifugation at  $30,000 \times g$  for 30 min.

**Estimation of 3-methylanthranilate and 7-methylindole in culture supernatant fluids.** The amount of 3MA in culture supernatant fluids was estimated by acidifying 1.0 ml of the culture supernatant with 0.1 ml of 1 N HCl and extracting in 5.0 ml of ethyl acetate. The concentration of 3MA in the ethyl acetate was determined spectrophotometrically at an absorption maximum of 336 nm. In some cases, the concentration of 3MA was determined fluorometrically with 3MA as a standard. The concentration of 7-methylindole was determined colorimetrically by use of indole reagent, after basic extraction into toluene (17). 7-Methylindole was used as a standard. Indoleglycerol was estimated spectrophotometrically after periodate oxidation to the aldehyde (17).

**Enzyme assays.** The  $\alpha$  subunit of tryptophan synthetase (A protein) was assayed according to Smith and Yanofsky (17). Indole-3-glycerol phosphate synthetase was assayed according to the method of Smith and Yanofsky (17), except that incubation was for 15 min at 37 C in  $10^{-3}$  M tris(hydroxymethyl)-aminomethane (Tris), pH 7.8. Anthranilate synthetase activity was measured by following the increase in fluorescence at 390 nm (excitation 320 nm, uncorrected) with a Farrand spectrofluorometer. The reaction mixture contained, in a total volume of 3.0 ml, Tris, pH 7.5, 50  $\mu$ moles;  $MgCl_2$ , 7.5  $\mu$ moles; L-glutamine, 15  $\mu$ moles; chorismic acid, 0.5  $\mu$ mole; enzyme extract; and distilled water. The reaction was initiated by addition of enzyme to the reaction mixture prewarmed to 37 C. Chorismic acid was prepared as the free acid according to the procedure of Gibson and Gibson (6). One enzyme unit is defined as the production or consumption of 1  $\mu$ mole of product or substrate in 1 min at 37 C. Specific activity is given as units of enzyme per milligram of protein. Protein was determined by the method of Lowry et al. (13). In most cases, the enzyme levels are expressed relative to those found in wild-type W1485 *tna*, with a value of 1.0 assigned to the level of enzymes found in W1485 *tna* grown in minimal medium.

**Chemicals.** 3-MA and 7-methylindole were obtained from Aldrich Chemical Co., Inc. Milwaukee, Wis.  $^3H$ -3MA was prepared by tritium exchange of crystallized 3MA (New England Nuclear Corp., Boston, Mass.). The material obtained from New

England Nuclear Corp. was heavily contaminated with radioactivity not associated with 3MA. Further exchange, charcoal treatment, and repeated crystallization did not remove all of the contaminating radioactivity. However, descending paper chromatography in *n*-butyl alcohol-propanol-water (1:2:1) plus 1.0 ml of 1 *N*  $\text{NH}_4\text{OH}$  per 100 ml of solvent was capable of separating the contaminating radioactivity from the radioactive 3MA. After being chromatographed twice, the resulting preparation contained no radioactivity which migrated at the solvent front, but a small amount of radioactivity migrated just behind 3MA. This latter material constituted from 5 to 10% of the total radioactivity and apparently was a breakdown product of 3MA because the amount increased with the age of the preparation. All studies with  $^3\text{H}$ -3MA were with twice chromatographed material. The specific radioactivity was  $6.9 \times 10^6$  counts per min per  $\mu\text{mole}$ .

7-MT was prepared from 7-methylindole by use of whole cells of the tryptophan auxotroph *trpC2*. The mutant was grown overnight in limiting tryptophan (so that the tryptophan enzymes were derepressed). The cells were harvested by centrifugation, washed in minimal medium, and resuspended at a density of approximately  $10^{10}$  cells/ml in an incubation mixture containing 1  $\mu\text{mole}$  of 7-methylindole/ml, 3.8  $\mu\text{moles}$  of DL-serine/ml, 0.2  $\mu\text{mole}$  of pyridoxal phosphate/ml, minimal medium, and 0.2% glucose. The cells were incubated for 2 hr at 37 C, by which time all of the 7-methylindole had been converted to 7MT. The supernatant solution was boiled for 30 min and then centrifuged at  $20,000 \times g$  for 30 min to remove

protein from lysed cells. The material was spotted along the entire length of Whatman no. 1 paper and chromatographed in a solvent system consisting of *n*-butyl alcohol-acetic acid-water (25:4:10). Strips containing the 7MT were cut out and eluted with distilled water. The resulting 7MT migrates at an  $R_F$  similar to that of 5MT or 6MT ( $R_F$ , 0.56) but different from that of L-tryptophan ( $R_F$ , 0.50). L-Tryptophan could not be detected in the 7MT preparation, although a small amount of material which migrated at the same  $R_F$  as serine was present. All other chemicals were obtained commercially.

## RESULTS

3-MA inhibited growth and, as shown in Table 1, caused derepression in various wild-type and *trpR* (constitutive) strains of *E. coli*. At the growth-inhibiting concentrations of 3MA used, derepression was usually not coordinate, and the maximal level of enzyme formed was somewhat below the derepressed level achieved by starving tryptophan auxotrophs for tryptophan (about a 40-fold increase in enzyme level). However, feedback-resistant anthranilate synthetase mutants (*trpE<sup>FBR</sup>*), 3MA-resistant mutants (*MAR*), or double mutants, both *trpE<sup>FBR</sup>* and *trpR*, which are resistant to this analogue, were not derepressed for either enzyme.

Previous reports attributed 3MA derepression to an inhibition of indoleglycerol phosphate synthetase (12). To test this hypothesis directly,

TABLE 1. Derepression of anthranilate synthetase and tryptophan synthetase A protein by 3-methylantranilate (3MA) and 7-methylindole (7MI)

Strain	Relative specific activity <sup>a</sup>					
	Anthranilate synthetase			A protein		
	Minimal medium	3MA <sup>b</sup>	7MI <sup>b</sup>	Minimal medium	3MA	7MI <sup>b</sup>
W1485 <i>tna</i> .....	1.0	19.4	20.1	1.0	11.8	— <sup>c</sup>
W1485.....	—	13.6	—	—	8.9	—
W3110.....	—	13.1	—	—	7.0	—
<i>trpR39</i> .....	4.4	37.8	16.5	4.4	24.6	8.8
<i>trpR4</i> .....	3.4	28.6	—	3.1	18.2	—
<i>trpR trpE<sup>FBR44</sup></i> .....	3.2	3.5	—	3.7	4.3	3.3
<i>trpR trpE<sup>FBR46</sup></i> .....	2.0	4.1	—	3.8	5.7	—
<i>trpE<sup>FBR2-50</sup></i> .....	0.44	0.56	0.61	0.61	0.81	—
<i>trpE<sup>FBR1-42</sup></i> .....	—	0.87	1.62	—	0.96	—
<i>MAR 13</i> .....	0.37	0.87	2.25	0.42	0.72	2.2
<i>MAR 1-9</i> .....	—	3.4	—	—	—	—
<i>MAR 31</i> .....	—	0.50	—	—	—	—
<i>MAR 22</i> .....	—	3.2	—	—	—	—

<sup>a</sup> The enzyme levels are expressed relative to those found in W1485 *tna* grown in minimal media. A relative specific activity of 1.0 corresponds to a specific activity of 0.0027 units/mg of anthranilate synthetase and 0.0152 units/mg of the  $\alpha$  subunit of tryptophan synthetase (A protein).

<sup>b</sup> The concentration of 3MA or 7MI in the growth media was 0.2  $\mu\text{mole/ml}$ .

<sup>c</sup> Dashes indicate that the enzyme activity was not determined.

the indoleglycerol phosphate synthetase in extracts of mutants which were selected for resistance to 3MA (*MAR*), as well as those isolated as being resistant to 5MT (*trpR39* and the double mutant *trpR trpE<sup>FBR44</sup>*), were tested for inhibition by 3MA. Table 2 shows that, although *trpE<sup>FBR</sup>* and *MAR* mutants failed to derepress on 3MA, their indoleglycerol phosphate synthetase was inhibited by the analogue to the same extent as *trpR39*, which does derepress.

The genetic locus conferring feedback resistance is located in the *trpE* gene, the structural gene for anthranilate synthetase (18). That these *FBR* mutants are also located within the *trp* operon (presumably in the *trpE* region) is indicated in Table 3 by their linkage to the *cysB* gene. As has been shown previously, the *trpR* gene is unlinked to the *trp* operon. In the double mutant *trpR trpE<sup>FBR</sup>*, the recombinants were not constitutive, although they did have a feedback-resistant anthranilate synthetase, indicating that the mutant was a double mutant.

Since *trpE<sup>FBR</sup>* mutants locate within the *trp* operon, as would also be expected for mutants which become resistant to 3MA through an alteration in indoleglycerol phosphate synthetase, a preliminary test of the linkage of *MAR* mutants to *trp* was performed. Several mutants selected for 3MA resistance were tested in this manner and, as shown by the representative data in Table 4, were found not to be *trp*-linked. We conclude that *MAR 13* has a wild-type *trp* operon and have evidence that the mechanism of its resistance to 3MA is only indirectly related

TABLE 3. Linkage to *cysB* of loci governing resistance to 5-methyltryptophan (5 MT)

Donor	Recipient	No. of <i>cys</i> <sup>+</sup> recombinants resistant to 5MT <sup>a</sup>	Percentage cotransduced with <i>cysB</i>
W1485 <i>tna</i>	<i>his cysB</i>	0	
<i>trpR39</i>	<i>his cysB</i>	0	
<i>trpA trpE<sup>FBR44</sup></i>	<i>his cysB</i>	68	53.1
<i>trpE<sup>FBR1-42</sup></i>	<i>his cysB</i>	63	49.2

<sup>a</sup> The number of *cys*<sup>+</sup> recombinants tested was 128.

TABLE 4. Nonlinkage to *trp* operon of *MAR 13*

Donor	Recipient	No. of wild-type <sup>a</sup> recombinants tested	No. derepressed on 3MA <sup>b</sup>
W1485	<i>MAR 13 trpA</i>	27	0
W1485	<i>trpA2</i>	7	7
<i>MAR 13</i>	<i>cysB<sup>-</sup> trp<sup>del</sup></i>	34	34
—	<i>MAR 13 trpA</i> revertants	7	0

<sup>a</sup> Wild type are tryptophan prototrophs.

<sup>b</sup> Wild-type recombinants were tested for derepression in the presence of 3MA by the whole-cell assay as described in Materials and Methods.

to tryptophan metabolism (see succeeding paper).

To determine whether it was possible to isolate 3MA-resistant mutants linked to the tryptophan operon, cells were selected for 3MA resistance and then transduced into a *trp* deletion (see Materials and Methods). Thus, the only mutants which should grow on 3MA would be those recombinants which received the *trp* genes along with a mutation conferring 3MA resistance. Of 76 such mutants isolated, all were also 5MT-resistant, and, of 11 tested, all had a feedback-resistant anthranilate synthetase, suggesting that anthranilate synthetase is more directly involved in 3MA derepression than indoleglycerol phosphate synthetase. Attempts were made to isolate 3MA-resistant mutants from revertants of the tryptophan auxotroph *trpC2*, which has an altered indoleglycerol phosphate synthetase; however, none was found. Thus, there appear to be two classes of 3MA-resistant mutants, one of which is linked by transduction to the *trp* operon. These mutants are 5MT-resistant and have a feedback-resistant anthranilate synthetase. The other class is unlinked by transduction to the *trp* operon and may con-

TABLE 2. Inhibition of indoleglycerol phosphate synthetase by 3-methylantranilate (3MA)

Strain <sup>a</sup>	3MA concn	Inhibition
	$\mu$	%
<i>trpR39</i>	$5 \times 10^{-4}$	60.0
	$1 \times 10^{-3}$	76.2
<i>trpR trpE<sup>FBR44</sup></i>	$5 \times 10^{-4}$	59.9
	$1 \times 10^{-3}$	71.6
<i>MAR 13 trpA</i>	$5 \times 10^{-4}$	68.4
	$1 \times 10^{-3}$	81.7
<i>MAR 22 trpB</i>	$5 \times 10^{-4}$	72.7
	$1 \times 10^{-3}$	84.5

<sup>a</sup> Strain *trpR39* derepresses when grown in the presence of 3MA, the other strains do not. *MAR 13 trpA* and *MAR 22 trpB* are double mutants containing mutations in the *trpA* and *trpB* cistrons of the tryptophan operon. The double mutants were used to increase the level of indoleglycerol phosphate synthetase in crude extracts.

stitute a heterogeneous class of mutants. Thus, it appears unlikely that 3MA derepression is caused solely by inhibition of indoleglycerol phosphate synthetase.

Previous hypotheses to explain the 3MA resistance of *trpE<sup>FBR</sup>* mutants postulated that the intracellular supply of tryptophan is high enough to antagonize the action of the analogue (18). However, neither *trpE<sup>FBR</sup>* nor *trpR* mutants appear to excrete significant quantities of tryptophan, although double mutants which are both *trpE<sup>FBR</sup>* and *trpR* do excrete large amounts of tryptophan (Table 5).

That the mechanism of 3MA-mediated growth inhibition and derepression of the *trp* enzymes involves tryptophan limitation is indicated by the ready reversal of these effects by exogenously supplied tryptophan. To examine whether the reversal was specific for tryptophan, a number of intermediates in tryptophan biosynthesis were tested. Anthranilic acid, at low concentrations, appears to be as effective as indole in reversing 3MA derepression (Table 6). If 3MA inhibits a reaction in the tryptophan biosynthetic sequence after anthranilic acid (i.e., indoleglycerol phosphate synthetase), one would expect anthranilic acid to be ineffective in reversing the inhibition, as it inhibits the same reaction (5).

Although it has been previously reported that 3MA is not metabolized to any extent by *E. coli* (12), in several experiments it was noticed that the fluorescence due to 3MA disappeared from the culture after growth of wild-type and *trpR* strains of *E. coli*. Table 7 shows that various wild-type and *trpR* strains, but not *trpE<sup>FBR</sup>* or *MAR* strains, appear to metabolize 3MA. Kinetic experiments with *trpR39* indicated rapid disappearance of 3MA from the culture after an initial lag period (Fig. 1). Concomitant with the loss of 3MA from the culture super-

TABLE 5. *L-Tryptophan accumulation in culture supernatant fluids*

Strain	Amt of L-tryptophan (nmoles)/ml of culture supernatant fluid <sup>a</sup>
W1485 <i>tna</i> .....	2.3
<i>trpE<sup>FBR</sup>2-50</i> .....	5.9
<i>trpR39</i> .....	5.2
<i>trpR trpE<sup>FBR</sup>44</i> .....	39.0

<sup>a</sup> The concentration of L-tryptophan was estimated fluorometrically in culture supernatant fluids, with L-tryptophan as a standard. Culture supernatant fluids were boiled for 30 min and centrifuged to remove any protein from lysed cells before assaying for tryptophan.

TABLE 6. *Effect of some aromatic compounds on 3MA derepression of anthranilate synthetase in W1485 tna*

Growth conditions	Additions	Concn of aromatic compounds (μmoles/ml)	Relative activity <sup>a</sup>
Minimal medium	None	—	1.0
	Indole	.10	0.55
	Anthranilic acid	.34	3.8
Minimal medium + 0.2 μmole of 3-MA/ml	None	—	17.0
	L-Tryptophan	.05	1.8
	Indole	.017	1.8
	Indole	.034	13.3
	Indole	.068	7.1
	Indole	.134	2.5
	Anthranilic acid	.134	0.30
	Anthranilic acid	.014	6.7
	Anthranilic acid	.029	2.3
	Anthranilic acid	.058	2.6
	Anthranilic acid	.116	3.3

<sup>a</sup> The anthranilate synthetase levels are repressed relative to that of W1485 *tna* grown in minimal medium.

TABLE 7. *3MA disappearance from culture supernatant fluids*

Strain	3MA remaining after 18 hr of growth <sup>a</sup>
	μmoles/ml
None.....	.33
W1485 <i>tna</i> .....	.11
W1485.....	.073
Y mel.....	.21
<i>trpR39</i> .....	<.007
<i>trpR trpE<sup>FBR</sup>44</i> .....	.30
<i>trpR4</i> .....	.007
<i>trpE<sup>FBR</sup>2-50</i> .....	.33
<i>trpE<sup>FBR</sup>1-2</i> .....	.34
<i>MAR 13</i> .....	.35
<i>MAR 22</i> .....	.22

<sup>a</sup> The 3MA concentration was estimated fluorometrically as described in Materials and Methods.

natant fluid, the cells began to accumulate indoleglycerol. Therefore, it appeared that 3MA can be metabolized by *E. coli* and was metabolized by the tryptophan biosynthetic enzymes. It should be noted, in regard to Fig. 1, that the amount of 3MA being metabolized was larger than the amount of indoleglycerol formed.

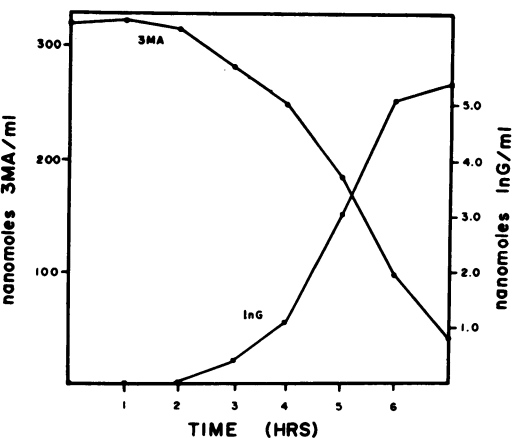


FIG. 1. Kinetics of 3MA disappearance by *trpR39*. Strain *trpR39* was grown overnight in minimal medium. The cells were resuspended at a density of  $2 \times 10^8$  cells/ml in minimal medium containing  $0.325 \mu\text{mole}$  of 3MA/ml and 0.5% glucose. 3MA concentration in the culture supernatant was estimated spectrophotometrically as described in Materials and Methods. InG = indoleglycerol.

TABLE 8. Conversion of 3MA to 7-methylindole (7MTIn) by *trpR39* in the presence of hydroxylamine<sup>a</sup>

Time	3MA disappeared	7MTIn formed	Indole formed (no 3MA)
min	$\mu\text{moles/ml}$	$\mu\text{moles/ml}$	$\mu\text{moles/ml}$
0	—	—	—
30	.034	.035	.000
60	.053	.055	—
90	.063	.069	.013
120	.072	.076	—
180	.085	.088	.013

<sup>a</sup> The incubation mixture consisted of a minimal medium containing  $0.3 \mu\text{mole}$  of 3MA/ml, 0.2% glucose, and  $8.7 \mu\text{moles}$  of hydroxylamine/ml. At 30 and 90 min, an additional  $8.7 \mu\text{moles}$  of hydroxylamine/ml was added.

To establish whether 3MA was indeed metabolized, possibly all the way to the corresponding tryptophan derivative, 7-MT, several additional experiments were performed. A *trpR* strain was grown overnight in the presence of 3MA so that the tryptophan enzymes were derepressed. The cells were washed and incubated with 3MA in the presence of hydroxylamine [hydroxylamine inhibits tryptophan synthetase and causes accumulation of indole (20)]. If 3MA was being metabolized via the tryptophan pathway, it should accumulate as the indole derivative, 7-methylindole. Table 8 shows that the 3MA was quantitatively converted to the correspond-

ing indole derivative, whereas cells incubated without 3MA accumulated very little indole. The indole derivative was extracted from the culture supernatant fluid by basic extraction into ether, crystallized, and shown to have the same melting range (79 to 81 C) as commercially available 7-methylindole.

With the demonstration that 3MA was converted to 7-methylindole, it became necessary to determine whether 3MA is converted to 7MT by whole cells of *E. coli*. W1485 *tna* was grown overnight on  $0.267 \mu\text{mole}$  of 3MA/ml so that the tryptophan enzymes were derepressed. After the cells were harvested and washed, they were suspended in minimal medium containing  $0.2 \mu\text{mole}$  of  $^3\text{H}$ -3MA/ml (see Materials and Methods),  $0.95 \mu\text{mole}$  of DL-serine/ml, and 0.4% glucose, and were incubated for 2 hr at 37 C. The culture supernatant fluid was co-chromatographed with partially purified 7MT made from methylindole (see Materials and Methods) in *n*-butylalcohol-acetic acid-water (25:4:10). The paper was sprayed with ninhydrin, and then was cut into 1.5-cm squares and counted. Figure 2 shows that  $^3\text{H}$ -3MA is converted to  $^3\text{H}$ -7MT. Chromatography in *n*-butyl alcohol-propanol-water (1:2:1) plus 1.0 ml of 1 N  $\text{NH}_4\text{OH}$  per 100 ml of solvent gave essentially the same results, although the peaks were not as well separated.

7-MT does not appear to be incorporated into

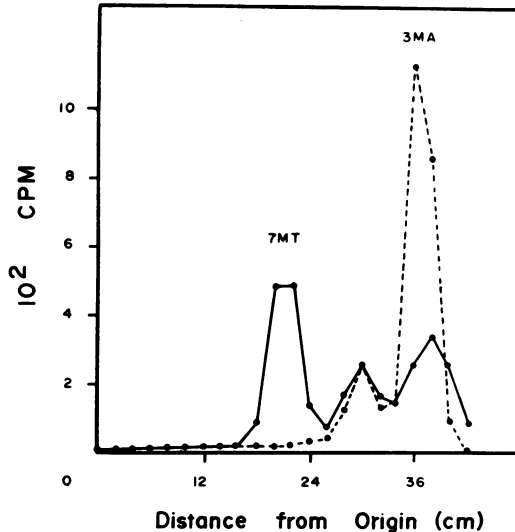


FIG. 2. Conversion of  $^3\text{H}$ -3MA to  $^3\text{H}$ -7MT by W1485 *tna*. Dashed line indicates radioactivity of  $^3\text{H}$ -3MA, sample taken before 2-hr incubation. Solid line indicates radioactivity after 2-hr incubation. The position of 3MA was determined by its blue fluorescence; 7MT, by ninhydrin staining.

protein. Table 9 indicates that most of the radioactivity after 12 hr of growth of *trpR39* on  $^3\text{H}$ -3MA remained in the supernatant fluid. Less than 0.018% of the original counts were precipitated with trichloroacetic acid, and more than 85% of the  $^3\text{H}$ -3MA had been metabolized.

Since 3MA can be converted to 7MT by *E. coli*, the question whether 3MA or 7MT causes derepression needed to be resolved. If 7MT causes derepression, then 7-methylindole, which can be converted to 7MT, should also cause derepression in wild-type strains. Table 1 shows that 7-methylindole does effectively cause derepression in wild-type and *trpR* strains of *E. coli*. Also, it is evident that *trpE*<sup>FBR</sup> and *MAR* mutants are relatively unaffected by 7-methylindole. The kinetics of enzyme appearance when low concentrations of 7-methylindole (0.02  $\mu\text{mole/ml}$ ) were used indicate that derepression continued for a period of time and then the culture began to be repressed (Fig. 3). In this experiment, samples of the culture were tested at various times for the presence of 7-methylindole. After 1.5 hr, 7-methylindole was not detectable in the culture supernatant fluid, although derepression continued for another 1.5 hr, indicating that 7-methylindole probably does not directly cause derepression.

Since both 3MA and 7-methylindole are converted to 7MT, it appeared probable that 3MA and 7-methylindole cause derepression through their conversion to 7MT. This could be tested directly by using 7MT prepared from 7-methylindole (see Materials and Methods). The following experiment was designed to determine the relative effectiveness of 3MA, 7-methylindole, and 7MT in causing derepression and growth inhibition in a wild-type strain. W1485 *tna* was grown overnight in minimal medium; the cells were harvested, washed in cold minimal medium, and resuspended in each of three flasks containing equal molar concentrations of 3MA, 7-methylindole, and 7MT. The initial concentration of inhibitor was 0.01  $\mu\text{mole/ml}$ . At 1-hr intervals, growth was determined and an additional 0.01  $\mu\text{mole}$  of inhibitor/ml was added to maintain a source of inhibitor. 3MA only slightly inhibited growth at this low concentration of inhibitor, although it did cause some derepression (Fig. 4). Both 7-methylindole and 7MT inhibited growth very strongly and caused derepression to a similar extent.

As shown in Table 10, 7MT inhibited anthranilate synthetase and this inhibition appears to be competitive with chorismic acid. Previous reports have shown that L-tryptophan inhibition of anthranilate synthetase is competitive with chorismic acid (1). Although 7MT is not as

TABLE 9. Recovery of radioactivity from a culture of *trpR39* grown in the presence of  $^3\text{H}$ -3MA<sup>a</sup>

Sample	3MA ( $\mu\text{moles/ml}$ )	Total counts/min
Supernatant fluid (0 hr)	.224	$6.68 \times 10^6$
Supernatant fluid (12 hr)	.033	$5.52 \times 10^6$
Crude extract (cold, trichloroacetic acid)	—	$1.18 \times 10^3$
Crude extract (hot trichloroacetic acid)	—	$9.21 \times 10^3$

<sup>a</sup> Strain *trpR* was grown for 12 hr in 50 ml of culture medium containing 0.224  $\mu\text{mole}$  of  $^3\text{H}$ -3MA/ml.

<sup>b</sup> The total counts per minute represents the total radioactivity in the supernatant fluid (50 ml) or crude extract (3 ml).

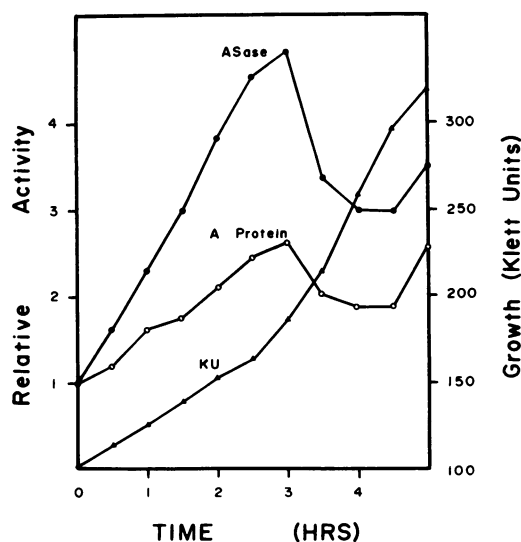


FIG. 3. Kinetics of derepression of W1485 *tna* on 0.02  $\mu\text{mole}$  of 7-methylindole/ml. Enzyme activity is expressed relative to the level of enzyme in W1485 *tna* grown in minimal medium. ASase = anthranilate synthetase.

effective a feedback inhibitor of anthranilate synthetase as L-tryptophan, it is more effective than 5MT or 6MT.

## DISCUSSION

The results of these experiments indicate: (i) that 3MA is metabolized to 7MT in wild-type strains of *E. coli*; (ii) that 3MA-resistant mutants linked to the tryptophan operon have a feedback-resistant anthranilate synthetase and that mutants with an altered indoleglycerol phosphate synthetase do not occur; and (iii) that 3MA, 7-methylindole, and 7MT lead to derepression

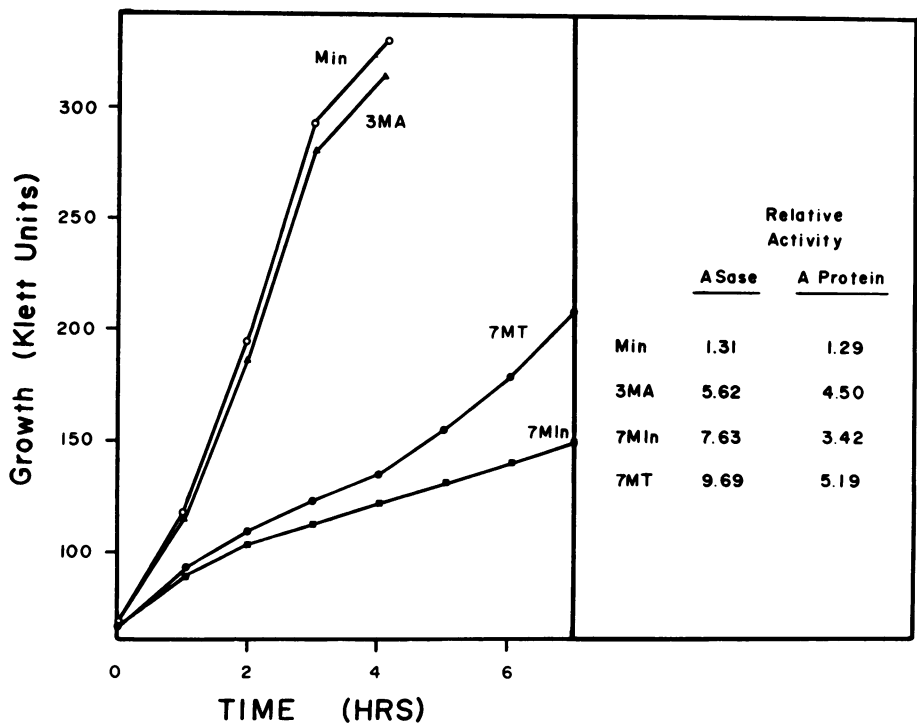


FIG. 4. Derepression and growth inhibition by 3-methylantranilic acid (3MA), 7-methylindole (7Min), and 7-methyltryptophan (7MT) in W1485 tna. Cultures were harvested at the end of the growth period and cell-free extracts were prepared. Enzyme activities are relative to the level of enzymes in W1485 tna grown in minimal medium.

TABLE 10. Inhibition of anthranilate synthetase<sup>a</sup> by tryptophan analogues

Inhibitor	Inhibitor concn	Chorismate concn	Inhibition
	M	M	%
L-Tryptophan	1.7 × 10 <sup>-5</sup>	1.7 × 10 <sup>-5</sup>	50.0
	3.4 × 10 <sup>-5</sup>	1.7 × 10 <sup>-5</sup>	68.0
	3.4 × 10 <sup>-5</sup>	1.7 × 10 <sup>-4</sup>	16.9
	1.7 × 10 <sup>-4</sup>	3.4 × 10 <sup>-5</sup>	87.0
5-Methyl-tryptophan	3.4 × 10 <sup>-5</sup>	1.7 × 10 <sup>-5</sup>	26.2
	1.7 × 10 <sup>-4</sup>	3.4 × 10 <sup>-5</sup>	25.0
6-Methyl-tryptophan	1.7 × 10 <sup>-4</sup>	3.4 × 10 <sup>-5</sup>	10.0
7-Methyl-tryptophan	1.7 × 10 <sup>-5</sup>	1.7 × 10 <sup>-5</sup>	35.4
	3.4 × 10 <sup>-5</sup>	1.7 × 10 <sup>-5</sup>	46.2
	3.4 × 10 <sup>-5</sup>	1.7 × 10 <sup>-4</sup>	26.2
	1.7 × 10 <sup>-4</sup>	3.4 × 10 <sup>-5</sup>	40.0

<sup>a</sup> Partially purified anthranilate synthetase from *trpA2/F'A2* was used as a source of the enzyme.

of the *trp* operon. These results suggest strongly that the primary cause of derepression by 3MA is through its conversion to 7MT. Formation of relatively small amounts of 7MT inhibits the

initially low concentration of anthranilate synthetase present, reducing the concentration of endogenous L-tryptophan. This leads to derepression of the tryptophan operon, the formation of more 7MT, and further inhibition of anthranilate synthetase, etc. 7MT, unlike 5MT or 6MT, apparently does not function as an active corepressor so that inhibition of anthranilate synthetase can lead to derepression. Mutants with a feedback-resistant anthranilate synthetase are not inhibited by 7MT, do not derepress, and, therefore, convert very little of the 3MA or 7-methylindole to 7MT.

3MA inhibition of indoleglycerol phosphate synthetase may contribute somewhat to 3MA derepression but is not the primary cause. Anthranilic acid causes a low-level derepression in wild-type strains, and 3MA would be expected to cause a similar low-level derepression except for its conversion to 7MT. Recent reports have also indicated that anthranilic acid causes product inhibition of anthranilate synthetase from *Salmonella typhimurium* (3). However, since anthranilic acid reverses 3MA derepression, product inhibition of anthranilate synthetase by 3MA is probably not relevant to 3MA derepression.



Anthranilate synthetase and phospho-ribosyl (PR) transferase activities normally exist as an enzyme complex in *E. coli* (8), and both activities are inhibited by L-tryptophan. Recent investigations on the nature of this enzyme complex indicate that anthranilate synthetase and PR transferase probably have separate tryptophan binding sites, as tryptophan inhibition of PR transferase is not related to feedback sensitivity of anthranilate synthetase (9). Furthermore, tryptophan inhibition of PR transferase appears to reach a limiting value at about 60% inhibition at  $10^{-4}$  M L-tryptophan. Anthranilate synthetase, however, is more sensitive to tryptophan inhibition and is completely inhibited (at low chorismic acid concentrations) at about  $3 \times 10^{-5}$  M. This differential sensitivity presumably allows metabolism of 3MA under conditions where anthranilate synthetase may be strongly inhibited (i.e., in the presence of 7MT).

Kinetic experiments indicate that 7-methylindole derepression ceases rather abruptly, followed by a period of relative repression and then slight derepression again (see Fig. 3). Derepression may allow the cells to overcome the inhibitory effect of 7MT and to make L-tryptophan even though 7MT is present. Synthesis of the tryptophan enzymes may "over-shoot" the critical level of enzymes required to overcome the inhibition by 7MT, resulting in temporary repression until the new "equilibrium state" is established.

Although 7MT does not appear to be incorporated into protein to any extent, studies were not carried out to determine whether 7MT is charged to the tryptophanyl-tRNA. Previous investigators have shown that mutations affecting tryptophanyl-tRNA synthetase probably do not have a direct effect on regulation (4), although the analogue 5MT, long known to cause repression of the *trp* operon, has been shown to be incorporated at a low level into proteins of *E. coli*. Presumably this incorporation proceeds through a charged tRNA intermediate (10).

7-MT appears to be unique among tryptophan analogues in causing derepression. Other tryptophan analogues such as 4-, 5-, or 6-MT and 6-fluorotryptophan cause repression (4). Presumably, either 7MT does not bind or binds very weakly to the tryptophan operon repressor, or 7MT may bind to the repressor but the complex is relatively inactive in causing repression.

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#### LITERATURE CITED

1. Baker, T. I., and I. P. Crawford. 1966. Anthranilate synthetase. Partial purification and some kinetic studies on the enzyme from *Escherichia coli*. *J. Biol. Chem.* 241:5577-5584.
2. Cohen, G. N., and F. Jacob. 1959. Sur la repression de la synthesis des enzymes intervenant dans la formation du tryptophane chez *Escherichia coli*. *Compt. Rend.* 248:3490-3492.
3. Cordaro, J., H. Levy, and E. Balbinder. 1968. Product inhibition of anthranilate synthetase in *Salmonella typhimurium*. *Biochem. Biophys. Res. Commun.* 33:183-189.
4. Doolittle, W. F., and C. Yanofsky. 1968. Mutants of *Escherichia coli* with an altered tryptophanyl-transfer ribonucleic acid synthetase. *J. Bacteriol.* 95:1283-1294.
5. Gibson, F., and C. Yanofsky. 1960. The partial purification and properties of indole-3-glycerol phosphate synthetase from *Escherichia coli*. *Biochim. Biophys. Acta* 43:489-500.
6. Gibson, M. K., and F. Gibson. 1964. Preliminary studies on the isolation and metabolism of an intermediate in aromatic biosynthesis: chorismic acid. *Biochem. J.* 90:248-256.
7. Ito, J., and I. P. Crawford. 1965. Regulation of the enzymes of the tryptophan pathway in *Escherichia coli*. *Genetics* 52:1303-1316.
8. Ito, J., and C. Yanofsky. 1966. The nature of the anthranilic acid synthetase complex of *Escherichia coli*. *J. Biol. Chem.* 241:4112-4114.
9. Ito, J., and C. Yanofsky. 1969. Anthranilate synthetase, an enzyme specified by the tryptophan operon of *Escherichia coli*: comparative studies on the complex and the subunits. *J. Bacteriol.* 97:734-742.
10. Lark, K. G. 1969. Incorporation of 5-methyltryptophan into the protein of *Escherichia coli* 15T<sup>-</sup> (555-7). *J. Bacteriol.* 97:980-982.
11. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* 1:190-206.
12. Lester, G., and C. Yanofsky. 1961. Influence of 3-methylanthranilic and anthranilic acids on the formation of tryptophan synthetase in *Escherichia coli*. *J. Bacteriol.* 81:81-90.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
14. Moyed, H. S. 1960. False feedback inhibition: inhibition of tryptophan biosynthesis by 5-methyltryptophan. *J. Biol. Chem.* 235:1098-1102.
15. Neidhardt, F. C. 1966. Roles of amino acid activating enzymes in cellular physiology. *Bacteriol. Rev.* 30:701-719.
16. Roth, J., D. Silbert, G. Fink, M. Voll, D. Anton, P. Hartman, and B. Ames. 1966. Transfer-RNA and the control of the histidine operon. *Cold Spring Harbor Symp. Quant. Biol.* 31:383-392.
17. Smith, O. H., and C. Yanofsky. 1962. Enzymes involved in the biosynthesis of tryptophan, p. 748-806. In S. P. Colowick and N. O. Kaplan (ed.), *Methods in enzymology*, vol. 5. Academic Press Inc., New York.
18. Somerville, R. L., and C. Yanofsky. 1965. Studies on the regulation of tryptophan biosynthesis in *Escherichia coli*. *J. Mol. Biol.* 11:747-759.
19. Vogel, H. J., and D. M. Bonner. 1956. Acetyl-ornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* 218:97-106.
20. Yanofsky, C. 1955. On the conversion of anthranilic acid to indole. *Science (Washington)* 121:138-139.
21. Yanofsky, C. 1960. The tryptophan synthetase system. *Bacteriol. Rev.* 24:221-245.