INFLUENCE OF 3-METHYLANTHRANILIC AND ANTHRANILIC ACIDS ON THE FORMATION OF TRYPTOPHAN SYNTHETASE IN *ESCHERICHIA COLI*¹

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It is now well recognized that the product of a series of biosynthetic reactions can play an important controlling role in its formation, either by inhibiting the activity or the formation of the enzymes concerned with its synthesis (Vogel, 1957; Pardee, 1959). Since the extent of such control is largely dependent on the intracellular concentration of the particular metabolite involved, the levels of enzyme function or formation can be controlled by manipulations which affect the intracellular concentrations of the repressing substance (Gorini and Maas, 1957). Thus, cultures grown in the presence of high concentrations of a particular repressor show a depressed content of the enzymes concerned with its synthesis. On the other hand, the maintenance of low levels of repressor, by controlled low level feeding to strains with a requirement for the repressing metabolite, results in an elevated enzyme level in such cultures. In some strains which have a partial block in the synthesis of a repressor the level of the corresponding enzymes is also high (Yanofsky, 1960).

The latter observation suggests that specific inhibitors of reactions leading to repressor synthesis might be employed to control enzyme formation. This paper deals with an examination of this possibility with the tryptophan-synthesizing system of *Escherichia coli*, which is well suited for such a study. Tryptophan represses the formation of tryptophan synthetase in *Aerobacter aerogenes* (Monod and Cohen-Bazire, 1953) as well as other enzymes involved in tryptophan synthesis in *E. coli* (Yanofsky, 1960). Contrariwise, strains which appear to be partially blocked in tryptophan synthesis, as evidenced by the stimulation of growth by

tryptophan (Yanofsky, 1960), produce high levels of tryptophan-synthesizing enzymes. Finally, the control of tryptophan synthesis by tryptophan (or its analogues) is not limited to the formation of enzymes involved in tryptophan synthesis, but extends to the activity of these enzymes as well (Trudinger and Cohen, 1956; Pardee and Prestidge, 1958; Moyed and Friedman, 1959; Moyed, 1960a). The work to be reported here shows that tryptophan synthesis, and consequently the formation of tryptophan-synthesizing enzymes, can be regulated by inhibition of the biosynthetic sequence by a normal intermediate as well as one of its analogues.

MATERIALS AND METHODS

Organisms. The following strains used in these studies were derived from *E. coli* strain K-12. Y-mel, which is unable to utilize melibiose, was used as the wild-type reference strain. T-41R-5, which produces an elevated level of tryptophan synthetase (Eisenstein, *unpublished data*), was isolated from an ultraviolet-treated population of a tryptophan auxotroph, T-41 (Yanofsky, 1958). T-26, which is able to couple indole and serine to form tryptophan, but is unable to convert indole-3-glycerol phosphate to tryptophan, accumulates indole-3-glycerol.

Culture. The minimal medium E (Vogel and Bonner, 1956) used in these studies contained 0.12 or 0.15 per cent glucose; other additives were autoclaved separately in water solution and added aseptically to the minimal medium to give final volumes of 6 or 8 ml per test tube (18 by 150). The media were inoculated with one drop of a 1:100 dilution of an overnight culture and incubated at 37 C for 15 to 22 hr with agitation on a reciprocal shaker; deviations from these conditions will be indicated with the experiment to be described. Growth was estimated by

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turbidity measurements in the Klett-Summerson colorimeter equipped with the 660 (red) filter.

Chemicals. The compounds used in this study were obtained commercially, except the anthranilic acid analogues which were supplied by Chas. Pfizer and Co., whose gift we gratefully acknowledge.

Tryptophan synthetase assay. Indole uptake by whole cells was used as the basis for estimating tryptophan synthetase activity. Samples of cultures were centrifuged and the clear supernatants decanted. To the pellet was added 1.0 ml of 0.05 M phosphate buffer, pH 7.8, containing 0.3 or 0.4 μ mole indole, 6.3 mg DL-serine, and 10 μ g pyridoxal phosphate. After incubation at 37 C for 30 min with agitation the assay mixture was chilled in an ice bath and extracted with 4.0 ml of toluene; 1.0-ml samples of the toluene extract were assayed for indole (Yanofsky, 1955). Under these conditions the unit of tryptophan synthetase is defined as the disappearance of 0.1 μ mole of indole. The disappearance of indole was directly proportional to the amount of cells present in the assay, and, for purposes of comparison, the observed uptake of indole was converted to that expected for 1.0 ml of cell suspension giving a colorimeter reading of 300 Klett units (300 KU).

Tryptophanase. Rough estimates of tryptophanase were made using a system similar to that for tryptophan synthetase, except that the assay mixture contained tryptophan in place of indole, and no serine (in the absence of serine tryptophan synthetase activity was nil). The unit of tryptophanase is defined as the appearance of 0.1 μ mole indole. Tryptophanase activity was generally low and probably did not affect the observations of tryptophan synthetase activity, especially since the presence of serine inhibited tryptophanase activity.

Indole-3-glycerol. The assay of this compound was based on periodate oxidation to indole-3-aldehyde, which was measured spectrophotometrically at 290 m μ (Yanofsky, 1956) using a similarly treated sample of indole-3-aldehyde as a standard. The identity of indole-3-aldehyde was qualitatively confirmed by its absorption spectrum. Neither 3-methylanthranilic nor anthranilic acids interfered in this assay.

Anthranilic ribonucleoside.² The qualitative

identification of this compound was based on procedures described by Doy and Gibson (1959). Aliquots of culture supernatants were brought to pH 4 with HCl and extracted with an equal volume of ethylacetate. The ethylacetate extracts were reduced to a small volume by evaporation under a stream of nitrogen, and applied to Whatman no. 1 paper. Using an ascending system, the chromatograms were developed in methanol:*n*-butanol:benzene:water, 2:1:1:1, for about 4.5 hr at 20 C, giving a solvent front about 20 cm from the starting line. Presumptive identification of anthranilic ribonucleoside was based on (i) the appearance of a spot which fluoresced brightly under ultraviolet light, but separate from (and slower than) 3-methylanthranilic or anthranilic acids, and (ii) coinciding with this spot, a green color with a phloroglucinol reagent (Borenfreund and Dische, 1957), and a red color which appeared almost immediately (without heating) upon spraying with alkaline triphenyltetrazolium chloride (Trevelyan, Procter, and Harrison, 1950). Further identification of anthranilic ribonucleoside was based on the chromatographic identification of anthranilic acid after alkaline hydrolysis of eluates of fluorescent areas similar to those giving positive presumptive tests. Ethylacetate eluates of such areas were extracted with 0.2 M phosphate buffer, pH 7.8 to 8; half of the buffer extract was made strongly alkaline (0.5 ml of 0.5 N K₂CO₃ per 2.5 ml of extract) and heated for 3 to 4 min in a boiling water bath; the other portion of buffer extract was untreated. Both portions were adjusted to pH 4.5 with HCl and extracted with an equal volume of ethylacetate. The ethylacetate extracts were reduced in volume under nitrogen and chromatographed, as described above, to determine whether alkaline hydrolysis of the presumptively identified anthranilic ribonucleoside yielded anthranilic acid as expected.

RESULTS

Effect of various analogues of anthranilic acid and tryptophan on tryptophan synthetase formation. The pathway of tryptophan synthesis in *E. coli* has been well demarcated biochemically

designated as 1-(*O*-carboxyphenylamino)-1-deoxyribulose, but for the sake of relative brevity it will be termed here "anthranilic ribonucleoside"; the phosphorylated derivative, 1-(*O*-carboxyphenylamino)-1-deoxyribulose-5-phosphate, will be termed "anthranilic ribonucleotide."

² This compound would be more specifically

TABLE 1
Effects of analogues of anthranilic acid and tryptophan on tryptophan synthetase formation and growth

Additions	Strain Y-mel			Strain T-41R-5		
	Concn	Growth	Tryptophan synthetase	Concn	Growth	Tryptophan synthetase
	$\mu\text{g/ml}$	KU*	units/300 KU	$\mu\text{g/ml}$	KU	units/300 KU
None		128	0.51		129	4.2
L-Tryptophan	1	136	0.41	1	130	4.1
	5	136	0.24	5	128	2.8
5-Methyltryptophan	0.1	128	0.49	0.005	135	4.8
	0.5	0		0.05	8	
6-Methyltryptophan	5	120	0.22	2	4	
	20	118	0.24	10	2	
3-Methylantranilic acid	10	113	3.5	1	102	8.4
	40	90	5.2	5	25	5.4
	100	94	5.8			
4-Methylantranilic acid	10	116	0.66	0.2	98	6.1
	40	113	0.98	0.5	12	
5-Methylantranilic acid	0.2	116	0.43	0.005	130	4.3
	1.0	127	0.36	0.02	110	4.6
	5.0	2		0.1	2	
5-Fluoroanthranilic acid	0.005	125	0.40	0.005	130	4.4
	0.02	9		0.02	5	
3-Aminobenzoic acid	10	127	0.48	10	134	4.5
	100	131	0.47	100	140	4.9
3-Aminophthallic acid	10	123	0.42	10	130	4.3
	100	133	0.43	100	138	4.1
N-Acetylantranilic acid	10	123	0.42	10	129	4.9
	100	124	0.51	100	134	4.9

* KU = Klett units.

TABLE 2
Effect of concentration of 3-methylantranilic acid on the formation of tryptophan synthetase

Strain Y-mel			Strain T-41R-5		
3-Methyl-anthra-nilic acid	Growth	Trypto-phan syn-thetase	3-Methyl-anthra-nilic acid	Growth	Trypto-phan syn-thetase
$\mu\text{g/ml}$	KU*	units/300 KU	$\mu\text{g/ml}$	KU	units/300 KU
0	108	0.55	0	121	3.1
2.5	112	0.72	0.1	122	3.6
5	111	1.0	0.2	119	3.7
10	108	2.7	0.4	114	4.6
20	89	5.1	0.6	108	5.6
30	85	5.2	0.8	105	5.7
40	85	4.8	1.0	99	6.2
50	86	5.0	2.5	55	5.8
75	89	4.6	5	26	3.4
100	80	4.6	10	10	3.8

* KU = Klett units.

and genetically in the sequence anthranilic acid → anthranilic ribonucleotide → indole-3-glycerol phosphate → tryptophan (Yanofsky, 1960). Hypothetically, it should be possible to limit tryptophan synthesis by the use of appropriate inhibitors of any of these steps, but practical considerations, namely availability, restricted an examination of potential inhibitors to analogues of anthranilic acid. In addition, tryptophan and a few of its derivatives were also examined for their action as repressors of tryptophan synthetase formation or as inhibitors of growth. The effects of these compounds on tryptophan synthetase formation and growth of Y-mel and T-41R-5 are shown in table 1. With both strains L-tryptophan represses tryptophan synthetase formation, and a similar effect is observed with 6-methyltryptophan in the case of Y-mel. On the other hand, 3-methylantranilic acid causes an 8- to 10-fold increase in the tryptophan syn-

Figure 1A. Growth and tryptophan synthetase formation in the absence (O) and presence (X) of 3-methylanthranilic acid. The solid lines represent growth, the broken lines the specific activity of tryptophan synthetase. KU = Klett units.

Figure 1B. Formation of tryptophan synthetase as a function of growth, in the absence (O) and presence (X) of 3-methylanthranilic acid. KU = Klett units.

thetase activity of Y-mel, and a 2-fold increase in the already elevated level of tryptophan synthetase activity of T-41R-5. None of the other analogues of anthranilic acid showed a comparable effect, although 4-methylanthranilic acid produced a modest increase in tryptophan synthetase activity in both strains.

Effect of concentration of 3-methylanthranilic acid on tryptophan synthetase formation and growth. Table 2 shows the optimal concentration of 3-methylanthranilic acid for tryptophan synthetase formation by Y-mel to be in the range 20 to 50 μ g 3-methylanthranilic acid per ml. However, a significant stimulation of trypto-

phan synthetase formation can be obtained with 5 to 10 μ g 3-methylanthranilic acid per ml. At concentrations above 50 μ g 3-methylanthranilic acid per ml, and especially above 100 μ g per ml, the growth rate is markedly decreased and lower tryptophan synthetase activities are obtained. With T-41R-5 optimal tryptophan synthetase formation was obtained with 0.6 to 2.5 μ g 3-methylanthranilic acid per ml; higher concentrations inhibit growth and also result in a decreased tryptophan synthetase activity.

Effect of 3-methylanthranilic acid on tryptophan synthetase formation during growth. The relationship of tryptophan formation to growth was examined to determine whether or not the effect of 3-methylanthranilic acid was constant during growth, or exerted its effect at a particular phase of growth. These studies were carried out using 100 ml of medium E plus 0.12 per cent glucose, plus or minus 30 μ g 3-methylanthranilic acid per ml. The medium was inoculated with a log phase culture of Y-mel to give a reading of 10 Klett units, and incubated at 37 C on a rotary shaker. Samples were taken at various intervals for turbidity and tryptophan synthetase determinations. Figure 1A shows that after about 1.5 hr incubation a slower growth rate is observed in the presence of 3-methylanthranilic acid, as well as a reduction in the level of growth finally achieved. In the absence of 3-methylanthranilic acid the specific activity of tryptophan synthetase decreased slowly and steadily. In the presence of 3-methylanthranilic acid, after a lag, the specific activity increased rapidly; the onset of increased tryptophan synthetase formation appeared to coincide with a decrease in growth rate. Figure 1B describes the formation of tryptophan synthetase as a function of growth; for the most part, the accrual of tryptophan synthetase activity is proportional to growth. It may be noted that in the presence of 3-methylanthranilic acid there appears to be a discontinuity in the rate of synthesis, with a slow rate followed by a faster rate beginning at a point coinciding with the decrease in growth rate shown in figure 1A.

Mechanism of action of 3-methylanthranilic acid. There is ample evidence that the formation of tryptophan synthetase is repressed by tryptophan, and as a corollary, it may be presumed that regulation of tryptophan synthesis would also regulate tryptophan synthetase formation. As has been pointed out in the introduction such

TABLE 3

Effect of tryptophan and 3-methylantranilic acid on tryptophan synthetase and tryptophanase formation

Addition	Concn	Strain Y-mel			Strain T-41R-5		
		Growth	Tryptophan synthetase	Tryptophanase	Growth	Tryptophan synthetase	Tryptophanase
	$\mu\text{g/ml}$	<i>KU*</i>	<i>units/300 KU</i>	<i>units/300 KU</i>	<i>KU</i>	<i>units/300 KU</i>	<i>units/300 KU</i>
L-Tryptophan	0	146	0.68	0.49	157	4.0	nil
	1	151	0.72	0.45	159	3.5	nil
	2	151	0.66	0.58	161	3.5	nil
	5	152	0.63	0.64	160	3.2	0.03
	10	150	0.50	0.76	163	3.0	0.17
	20	154	0.39	1.47	160	0.53	0.71
3-Methylantranilic acid	0	137	0.74	0.54			
	50	99	4.4	0.25			

* KU = Klett units.

regulation may be achieved as the result of mutation leading to a limitation in the synthesis of tryptophan precursors. The high tryptophan synthetase content of T-41R-5 would appear to be the result of such a limitation, whereas the low tryptophan synthetase content of Y-mel could be considered to be due to a relative lack of restriction of tryptophan synthesis. This suggestion is supported by the fact that the growth of T-41R-5 is considerably more sensitive than that of Y-mel to 5- or 6-methyltryptophan, or their respective precursors 5- or 4-methylantranilic acid (table 1), whose inhibitory action can be reversed by tryptophan. Further support for a relatively low level of internal tryptophan of T-41R-5 can be adduced from estimates of tryptophanase activity, which is inducible by tryptophan. Table 3 shows that in the absence of tryptophan in the medium Y-mel produces significant amounts of tryptophanase, whereas under similar conditions the tryptophanase activity of T-41R-5 is nil; also lower levels of tryptophan are required to stimulate tryptophanase formation in Y-mel than in T-41R-5.

If it is assumed that 3-methylantranilic acid stimulates tryptophan synthetase formation by limiting tryptophan formation it would be expected to have a pronounced deleterious effect on the growth of an organism already limited in tryptophan synthesis. This can be seen to be the case in table 2 where the growth of T-41R-5 is inhibited at levels of 3-methylantranilic acid which have no effect on Y-mel; the decrease in

tryptophan synthetase formation in T-41R-5 by the highest levels of 3-methylantranilic acid used could indicate so severe a restriction of tryptophan synthesis that the formation of tryptophan synthetase is limited by an insufficient supply of this amino acid. The stimulation of tryptophan synthetase formation in T-41R-5 by lower levels of 3-methylantranilic acid than with Y-mel is more compatible with a mechanism of tryptophan limitation than with a direct action of 3-methylantranilic acid on a tryptophan synthetase-synthesizing system, since in the latter case similar concentrations of 3-methylantranilic acid might be expected to be required for a stimulation of tryptophan synthetase formation in both strains.

It is also possible that 3-methylantranilic acid might be converted to a compound which directly affects the tryptophan synthetase forming system, and that this conversion is more efficient in T-41R-5 than in Y-mel. However, the fate of 3-methylantranilic acid was examined in the experiment described by figure 1 by extracting acidified aliquots of culture with ethylacetate and determining the content of 3-methylantranilic spectrophotometrically at 335 m μ . The results given in table 4 show that the concentration of 3-methylantranilic acid did not change during the course of the experiment, nor did the ratios of optical density at 335 m μ to 255 m μ . In addition, the extracts obtained at 0, 6, and 12 hr were concentrated and chromatographed in butanol: propanol:water, 1:2:1, plus 1.0 ml 1.0 N NH₄OH

TABLE 4
Recovery of 3-methylanthranilic acid from a culture during growth of Y-mel

Incubation Time	3-Methylanthranilic Acid	Optical Density
hr	$\mu\text{g/ml}$	$335:255\text{ m}\mu$
0	28.4	2.03
3	28.0	2.17
4.5	27.8	1.96
6	27.1	1.85
12	28.2	2.0
<i>Standards:</i>		
3-Methylanthranilic acid		1.95
Anthranilic acid		0.89

per 100 ml. Only one fluorescent spot, corresponding to a 3-methylanthranilic acid standard, was observed under ultraviolet light; this chromatographic procedure was capable of separating 3-methylanthranilic acid from anthranilic acid. Thus, it appears that 3-methylanthranilic acid is not metabolized to any significant extent under these conditions (Yanofsky, 1956), nor does it cause an accumulation of anthranilic acid.

From the foregoing it is reasonable to assume that the stimulatory effect of 3-methylanthranilic acid on tryptophan synthetase formation might be due to an inhibition of tryptophan synthesis. Table 3 indicates this possibility by the reduction of tryptophanase activity in Y-mel grown in the presence of 3-methylanthranilic acid. On this basis the stimulatory effect of 3-methylanthranilic acid on tryptophan synthetase formation should be reversed by tryptophan, or other similar repressors, or by providing for an increased supply of the intermediate(s) whose metabolism is inhibited by 3-methylanthranilic acid. Table 5 shows this to be the case; tryptophan and 6-methyltryptophan, and 4-methylanthranilic acid (which can be converted to 6-methyltryptophan) reverse the effect of 3-methylanthranilic acid. In a similar fashion, the conversion of anthranilic acid to tryptophan should also lead to an inhibition of the effect of 3-methylanthranilic acid. However, although anthranilic acid does reverse the effect of 3-methylanthranilic acid on tryptophan synthetase formation, this reversal does appear to be concentration dependent as is the case with the other compounds; with increasing concentration of anthranilic acid the level of

tryptophan synthetase appears to increase. This is also true in the absence of 3-methylanthranilic acid where anthranilic acid produces a 3- to 4-fold increase in tryptophan synthetase activity.

The assumption that 3-methylanthranilic acid stimulates tryptophan synthetase formation by inhibiting the synthesis of tryptophan has been examined by studying the effect of 3-methylanthranilic acid on tryptophan synthetase formation by strain T-26, which is tryptophan- or indole-dependent. As shown in table 6, T-26 produces levels of tryptophan synthetase comparable to T-41R-5 (if tryptophan is not present in excess of its growth requirement), and that 5- and 6-methyltryptophan markedly repress tryptophan synthetase formation, without any effect on growth. However, neither anthranilic acid, 4-nor 5-methylanthranilic acid, in relatively high concentration, have any significant effect on tryptophan synthetase formation, indicating that they are not converted respectively to tryptophan, 6- or 5-methyltryptophan. Similarly, 3-methylanthranilic acid does not affect tryptophan synthetase formation, indicating that its stimulation of tryptophan synthetase formation in Y-mel requires the functioning of normal sequence leading to tryptophan synthesis. These

TABLE 5
Effect of tryptophan, anthranilic acid, and their analogues on stimulation of tryptophan synthetase by 3-methylanthranilic acid in Y-mel

Addition	Concn	3-Methylanthranilic Acid	
		0	30 $\mu\text{g/ml}$
	$\mu\text{g/ml}$	$\text{units}/300\text{ KU}^*$	$\text{units}/300\text{ KU}$
None		0.56	4.8
L-Tryptophan	1	0.64	3.9
	3	0.49	1.8
	10	0.27	0.35
6-Methyltryptophan	1	0.60	1.2
	3	0.45	0.69
	10	0.27	0.49
Anthranilic acid	10	1.5	1.5
	20	1.9	1.7
	50	2.0	2.1
4-Methylanthranilic acid	1	0.47	1.1
	3	0.53	0.75
	10	0.53	0.48

* KU = Klett units.

TABLE 6

Effects of tryptophan, anthranilic acid, and their analogues on tryptophan synthetase and indole-3-glycerol accumulation by T-26

Addition	Concn	Trypto- phan Syn- thetase	Indole-3- glycerol in Culture
	$\mu\text{g/ml}$	$\text{units}/300$ KU^*	$\mu\text{mole/ml}$
L-Tryptophan + Anthranilic acid	5	3.3	0.226
	5	3.2	0.245
	25	3.5	0.181
3-Methylantranilic acid	5	3.2	0.211
	25	3.0	0.159
4-Methylantranilic acid	5	2.7	0.214
	25	2.9	0.162
5-Methylantranilic acid	5	3.2	0.219
	25	2.8	0.186
5-Methyltryptophan	5	0.61	0.027
	25	0.25	0.038
6-Methyltryptophan	5	0.73	0.046
	25	0.40	0.047

* KU = Klett units.

results show that 3-methylantranilic acid does not have a direct action on the tryptophan synthetase forming system.

It may be noted from table 6 that anthranilic acid and its methylated analogues, especially at the higher concentration, cause a reduction of the accumulation of indole-3-glycerol. This could suggest that all are capable of inhibiting tryptophan formation, if they themselves are not converted to tryptophan or the corresponding analogue which, in turn, would repress tryptophan synthetase formation. It is of interest to note the severe depression of indole-3-glycerol accumulation by cultures of T-26 grown in the presence of low levels of 5- or 6-methyltryptophan, which could signify that these compounds repress the formation of other enzymes in the biosynthetic sequence leading to tryptophan as well as the formation of tryptophan synthetase, as does tryptophan (Yanofsky, 1960). However, this decreased accumulation could also be attributed to inhibitions of enzymatic activities required for the synthesis of indole-3-glycerol. Such inhibitions have been observed in cell extracts with 5-methyltryptophan (Moyed, 1960a) and 6-fluorotryptophan (Moyed and Friedman, 1959), and in whole cells with 4-methyltryptophan (Trudinger and Cohen, 1956)

and 7-azatryptophan (Pardee and Prestidge, 1958).

Site of action of 3-methylantranilic acid. The data thus far presented indicate that the site of action of 3-methylantranilic acid probably does not involve the reaction anthranilic acid $\rightarrow$ anthranilic ribonucleotide since (i) anthranilic acid is not accumulated by cultures grown in the presence of 3-methylantranilic acid, and (ii) anthranilic acid itself stimulates tryptophan synthetase formation. It has been determined that neither the normal nor 3-methylantranilic-stimulated tryptophan synthetase activity in Y-mel or T-41R-5 is inhibited by 3-methylantranilic acid; also, no indole-3-glycerol could be detected in cultures of Y-mel or T-41R-5 grown in the presence or absence of 3-methylantranilic acid. Correspondingly, the reduction of indole-3-glycerol accumulation by T-26 grown in the presence of 3-methylantranilic acid (table 6) suggests that 3-methylantranilic acid inhibits a reaction prior to that catalyzed by tryptophan synthetase. Thus, it appeared likely that the site of 3-methylantranilic acid action might be the reaction anthranilic ribonucleotide $\rightarrow$ indole-3-glycerol phosphate, and it might also be expected that cultures grown in the presence of 3-methylantranilic acid should accumulate anthranilic ribonucleoside.

Cultures of Y-mel and T-26 grown in the absence and presence of 3-methylantranilic acid (50 or 200 $\mu\text{g/ml}$) were qualitatively examined for anthranilic ribonucleoside by paper chromatography. Only extracts of cultures grown in the presence of 3-methylantranilic acid yielded a spot corresponding to anthranilic ribonucleoside. Ethylacetate eluates of these spots were hydrolyzed and reextracted with ethylacetate. These extracts were chromatographed and yielded a spot corresponding to anthranilic acid, as would be expected from the hydrolysis of anthranilic ribonucleoside. These results, which are summarized in table 7, indicate that 3-methylantranilic acid inhibits the conversion of anthranilic ribonucleotide to indole-3-glycerol phosphate.

Effect of anthranilic acid on tryptophan synthetase formation. The observation (table 5) that anthranilic acid stimulates the formation of tryptophan synthetase made it of particular interest to determine whether this normal intermediate acts in a similar fashion as 3-methylan-

TABLE 7
Effect of 3-methylantranilic acid on the accumulation of anthranilic ribonucleoside

Strain	3-Methyl-antranilic Acid	L-Tryp-tophan	Growth	Trypto-phan Syn-thetase	Anthranilic Ribo-nucleoside
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	KU*	units/300 KU	
Y-mel	0	0	137	0.81	—
	50	0	99	4.4	+
	200	0	8		
	0	6	137	0.64	—
	50	6	136	1.2	+
T-26	200	6	93	3.0	+
	0	6	104	4.6	—
	50	6	106	4.2	+
	200	6	99	4.5	+

* KU = Klett units.

TABLE 8
Effect of anthranilic acid on formation of tryptophan synthetase by Y-mel

Anthranilic Acid	Growth	Tryptophan Synthetase
$\mu\text{g/ml}$	KU*	units/300 KU
0	163	0.89
10	161	2.0
25	163	2.3
50	161	2.7
100	148	3.0
200	135	4.4
350	6	
500	0	

* KU = Klett units.

thranilic acid. Table 8 shows that anthranilic acid can stimulate tryptophan synthetase formation to a level similar to that attained with 3-methylantranilic acid, although the optimal concentration of anthranilic acid is much higher than that of 3-methylantranilic acid (table 2). This latter observation is explicable if it is considered that anthranilic acid serves both as a precursor as well as competitor for anthranilic ribonucleotide.

Table 8 shows that levels of anthranilic acid above 200 $\mu\text{g/ml}$ severely restrict growth. If this is due to an inhibition of tryptophan synthesis, it would be expected that the addition of tryptophan to the medium should reverse the inhibitory effect of anthranilic acid on growth.

Also, the level of tryptophan synthetase formed should decrease with increasing concentration of tryptophan. Table 9 shows that the inhibition of growth by anthranilic acid (300 $\mu\text{g/ml}$) is almost completely reversed at the lowest level of tryptophan used. (At concentrations of anthranilic acid above 500 $\mu\text{g/ml}$, the inhibition of growth is not reversed by tryptophan, and indicates a secondary inhibition by anthranilic acid.) A large stimulation of tryptophan synthetase formation is achieved at the lowest tryptophan concentration which is slightly suboptimal for the growth of tryptophan-dependent strains, under these conditions. With increasing tryptophan concentration tryptophan synthetase formation decreases to very low values. It is of interest to note, however, that a complete abolition of tryptophan synthetase formation

TABLE 9
Effect of tryptophan on growth inhibition and stimulation of tryptophan synthetase formation in Y-mel by anthranilic acid

Anthranilic Acid	L-Tryptophan	Growth	Tryptophan Synthetase
$\mu\text{g/ml}$	$\mu\text{g/ml}$	KU*	units/300 KU
0	0	168	0.70
300	0	9	1.1
300	3.4	150	3.1
300	10.2	150	1.4
300	34	158	0.26
300	102	160	0.17
300	340	160	0.09
0	340	166	0.09

* KU = Klett units.

TABLE 10
Effect of anthranilic acid on tryptophan synthetase formation and accumulation of indole-3-glycerol and anthranilic ribonucleoside by strain T-26

Anthranilic Acid	Growth*	Tryptophan Synthetase	Indole-3-glycerol in Culture	Anthranilic Ribo-nucleoside
$\mu\text{g/ml}$	KU†	units/300 KU	$\mu\text{moles/ml}$	
0	175	2.9	1.64	—
100	166	3.0	0.92	+
300	138	2.9	0.25	+

* The medium contained 6 μg L-tryptophan per ml and a level of 0.33 per cent glucose.

† KU = Klett units.

was not observed, even though 50 to 60 times the optimal requirement for dependent strains was used. The low tryptophan synthetase activity observed here is probably minimal, since with high tryptophan concentrations tryptophanase activity was elevated and would tend to lower the values obtained for indole uptake.

To determine whether the site of action of anthranilic acid is the same as that of 3-methylanthranilic acid the accumulation of anthranilic ribonucleoside was determined in cultures of T-26 grown in the presence and absence of anthranilic acid. Strain T-26 was used so that the effect of anthranilic acid on the accumulation of indole-3-glycerol could again be ascertained. The results given in table 10 show that high levels of anthranilic acid have only a small effect on growth and no effect on tryptophan synthetase formation. On the other hand, the accumulation of indole-3-glycerol³ is markedly inhibited, and the accumulation of anthranilic ribonucleoside is promoted by the presence of anthranilic acid. These results indicate that anthranilic acid and 3-methylanthranilic acid have the same site of action, an inhibition of the conversion of anthranilic ribonucleotide to indole-3-glycerol phosphate. The inhibitory action of 3-methylanthranilic acid and anthranilic acid on this reaction has been recently confirmed with cell-free extracts (Gibson and Yanofsky, 1960).

DISCUSSION

The data presented here indicate that anthranilic acid and 3-methylanthranilic acid can stimulate tryptophan synthetase formation by limiting the synthesis of tryptophan through an inhibition of a reaction in the biosynthetic sequence leading to tryptophan. The action of these compounds, per se, is probably not unique since other analogues could act similarly; this has been indicated by the small stimulation of tryptophan synthetase formation by 4-methylanthranilic acid, and by the reduction of indole-3-glycerol accumulation in cultures of T-26 by 4- and 5-methylanthranilic acid and anthranilic acid. Gibson and Yanofsky (1960)

³ The high control level of indole-3-glycerol is probably due to the presence of glucose in excess of what is required to give maximal growth with the amount of tryptophan supplied, so that although growth stops, production of indole-3-glycerol continues.

have also found that these compounds inhibit the conversion of anthranilic ribonucleotide to indole-3-glycerol phosphate by the corresponding purified enzyme. A practical prerequisite for eliciting a stimulation of enzyme formation would be that the inhibitors are not converted to toxic compounds or highly effective repressors, as would be the case with 4- or 5-methyl- or 5-fluoroanthranilic acids (Yanofsky, 1956).

The delayed effect of 3-methylanthranilic acid on tryptophan synthetase formation during growth probably reflects the presence of tryptophan or its precursors in the cells, and indicates that anthranilic acid or 3-methylanthranilic will exhibit a maximal stimulation of tryptophan synthetase formation when the normal level of tryptophan is reduced. In this respect, it is noteworthy to mention that an attempt was made to identify tryptophan in culture filtrates and in heated extracts of cells grown on minimal medium, and no significant amount of tryptophan was observed. This could suggest that at its site of action the effective amount of tryptophan required for repression is low, considerably less than would be indicated by the amounts of exogenous tryptophan required to reduce tryptophan synthetase formation. It would also indicate a rather firm association between tryptophan and its site of repressive action, from which it is shifted when the supply or synthesis of tryptophan is restricted, or when the demand for tryptophan for other metabolic purposes is high.

Although the evidence presented indicates that 3-methylanthranilic acid (or anthranilic acid) does not have a direct effect on the tryptophan synthetase forming system, there is a superficial resemblance between the effect of 3-methylanthranilic acid and that of inducers of enzyme formation. In this respect, 3-methylanthranilic acid could be considered as a pseudoinducer. A similar situation has been observed (Moyed, 1960b) in the case of 2-thiazole alanine, which inhibits an early step in histidine synthesis and consequently causes an increased formation of an enzyme whose formation is repressed by histidine. This, of course, raises the question of whether some inducers of the formation of other enzymes might not also act by inhibiting the synthesis of repressors, rather than by exerting a direct effect on enzyme formation or competing with repressors for an enzyme forming site. It also raises the possibility that a pseudoconstitutiveness might be achieved by a

block which reduces the supply of repressor, and at the same time causes an accumulation of an intermediate which inhibits subsequent reactions. Drawing from the work presented here, a partial block in the reaction anthranilic $\rightarrow$ anthranilic ribonucleotide would reduce the rate of tryptophan synthesis, and an accumulation of anthranilic acid would emphasize the restriction of tryptophan synthesis by inhibiting the subsequent reaction anthranilic ribonucleotide $\rightarrow$ indole-3-glycerol phosphate.

Most studies concerned with the regulation of enzyme activities or formation have emphasized the regulatory role of the end product of a series of reactions on the activity of that sequence as a feed-back control mechanism. The demonstration that anthranilic acid can affect the activity of a subsequent reaction indicates that "feed-forward" control mechanisms might play a role in the regulation of cellular activities. In either case, whether feed-back or feed-forward, the work described here (*also see* Davis, 1952) indicates the possibility that intermediates, as well as end products, in a biosynthetic sequence might influence its operation.

SUMMARY

The formation of tryptophan synthetase, which is repressed by tryptophan, is stimulated by the presence of anthranilic or 3-methylantranilic acids in the medium. This effect could be attributed to an inhibition of the conversion of anthranilic ribonucleotide to indole-3-glycerol phosphate, presumably causing a reduction in the concentration of endogenous tryptophan.

REFERENCES

- BORENFREUND, E., AND Z. DISCHE 1957 A new spray for spotting of sugars on paper chromatograms. *Arch. Biochem. Biophys.*, **67**, 239-240.
- DAVIS, B. D. 1952 Aromatic biosynthesis. V. Antagonism between shikimic acid and its precursor, 5-dehydroshikimic acid. *J. Bacteriol.*, **64**, 749-763.
- DOY, C. H., AND F. GIBSON 1959 1-(*o*-Carboxyphenylamino)-1-deoxyribulose. A compound formed by mutant strains of *Aerobacter aerogenes* and *Escherichia coli* blocked in the biosynthesis of tryptophan. *Biochem. J.*, **72**, 586-597.
- GIBSON, F., AND C. YANOFSKY 1960 The partial purification and properties of indole-3-glycerol phosphate synthetase from *Escherichia coli*. *Biochim. et Biophys. Acta*, **43**, 489-500.
- GORINI, L., AND W. K. MAAS 1957 The potential for the formation of a biosynthetic enzyme in *Escherichia coli*. *Biochim. et Biophys. Acta*, **29**, 208-209.
- MONOD, J., AND G. COHEN-BAZIRE 1953 The effect of specific inhibitors in the biosynthesis of tryptophan desmolase in *Aerobacter aerogenes*. *Compt. rend.*, **236**, 530-532.
- MOYED, H. S. 1960a False feedback inhibition: inhibition of tryptophan biosynthesis by 5-methyltryptophan. *J. Biol. Chem.*, **235**, 1098-1102.
- MOYED, H. S. 1960b Induced phenotypic resistance to an antimetabolite. *Science*, **131**, 1449.
- MOYED, H. S., AND M. FRIEDMAN 1959 Interference with feedback control: a mechanism of antimetabolite action. *Science*, **129**, 968-969.
- PARDEE, A. B. 1959 Mechanisms for control of enzyme synthesis and enzyme activity in bacteria. In *Ciba Foundation symposium on cell metabolism*, pp. 295-304. Edited by G. E. W. Wolstenholme and C. M. O'Connor. J. & A. Churchill Ltd., London.
- PARDEE, A. B., AND L. S. PRESTIDGE 1958 Effects of azatryptophan on bacterial enzyme and bacteriophage. *Biochim. et Biophys. Acta*, **27**, 330-344.
- TREVELYAN, W. E., D. P. PROCTER, AND J. S. HARRISON 1950 Detection of sugars on paper chromatograms. *Nature*, **166**, 444-445.
- TRUDINGER, P. A., AND G. N. COHEN 1956 The effect of 4-methyltryptophan on growth and enzyme systems of *Escherichia coli*. *Biochem. J.*, **62**, 488-491.
- VOGEL, H. J. 1957 Repressed and induced enzyme formation: a unified hypothesis. *Proc. Natl. Acad. Sci. U.S.*, **43**, 491-496.
- VOGEL, H. J., AND D. M. BONNER 1956 Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.*, **218**, 97-106.
- YANOFSKY, C. 1955 Tryptophan synthetase from *Neurospora*. In *Methods of enzymology*, vol. II, pp. 233-238. Edited by S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York.
- YANOFSKY, C. 1956 The enzymatic conversion of anthranilic acid to indole. *J. Biol. Chem.*, **223**, 171-184.
- YANOFSKY, C. 1958 Restoration of tryptophan synthetase activity in *Escherichia coli* by suppressor mutations. *Science*, **128**, 843-844.
- YANOFSKY, C. 1960 The tryptophan synthetase system. *Bacteriol. Rev.*, **24**, 221-245.