3. An enzyme system converting anthranilate into indole has been extracted from *Esch. coli*. The reaction required ATP, inorganic phosphate, pyridoxal phosphate, Mg^{2+} or Mn^{2+} and a carbon donor. Pyruvate and α-oxoglutarate were the most effective of the donors studied.

4. The implication of these results on the problem of tryptophan biosynthesis is discussed.

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The Effect of 4-Methyltryptophan on Growth and Enzyme Systems of *Escherichia coli*

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During the study of the mechanism of tryptophan synthesis by *Escherichia coli*, the possibility of using 4-methyltryptophan (4-MT) as a blocking agent was examined. This compound has been reported to inhibit competitively the utilization of tryptophan for protein synthesis in *Bacterium typhosum* (Fildes & Rydon, 1947). However, certain discrepancies between the results of these workers and our own led to a more detailed analysis of the inhibition and, in particular, the effect of 4-MT on enzyme systems.

The results of this work are reported in this paper.

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MATERIALS AND METHODS

The strains of *Esch. coli*, general growth conditions and the methods for indole and anthranilic acid estimation have been reported in the preceding paper (Trudinger, 1956).

Cell-free tryptophan desmolase was prepared from *Esch. coli* strain ML, grown 16 hr. in aerated mineral medium (Davis & Mingioli, 1950). The bacteria (about 200 mg. dry wt.) were suspended in 10 ml. of 3% (w/v) KCl and shaken for 30 min. at 0° with 5g. of no. 12 Ballotini glass beads in the Mickle vibrator. The supernatant, after centrifuging for 15 min. at approx. 3000 *g*, was used in undialysed form. It contained no tryptophanase activity.

Cell-free tryptophanase was prepared in a similar manner, except that the bacteria were grown in a broth consisting of 1% peptone, 0·5% yeast extract (Difco) and 10^{-3} M L-tryptophan in distilled water.

RESULTS

Effect of 4-MT on growth of Esch. coli. The growth of wild-type *Esch. coli* strain W was inhibited by 4-MT at concentrations above 10^{-5} M. This inhibition was reversed in a non-competitive manner by L-tryptophan (Table 1). Maximum reversal occurred at $5–7{\cdot}5 \times 10^{-7}$ M tryptophan over a 100-fold range of 4-MT concentrations. The only evidence that 4-MT inhibited tryptophan utilization was the fact that at high levels of 4-MT (above 10^{-3} M) the maximum growth in the presence of tryptophan was 10–20% lower than that in the absence of the inhibitor.

Table 1. *Inhibition of growth by 4-MT; reversal by tryptophan*

4·5 ml. of medium (Monod, 1942) in 16 cm. × 1·8 cm. tubes and containing the appropriate amounts of tryptophan and 4-MT was inoculated with 0·5 ml. of a suspension of *Esch. coli*, strain W, prepared as described by Hirsch & Cohen (1953). The tubes were incubated for 16 hr. at 37° without shaking.

	Growth in Muenier units			
Molarity of 4-MT ...	0	10^{-3}	10^{-4}	10^{-5}
Tryptophan (molarity)				
0	95	0	0	0
10^{-5}	—	83	90	—
5×10^{-6}	—	83	94	97
10^{-6}	—	82	97	90
$7{\cdot}5 \times 10^{-7}$	—	76	90	95
5×10^{-7}	—	59	77	78
10^{-7}	—	6	21	33
5×10^{-8}	—	—	—	28

Mutants of *Esch. coli*, deficient in tryptophan (including M121–35 and M19–2 derived from W), required about 10^{-6} M tryptophan for observable growth. On the other hand, about half this concentration of tryptophan allowed full growth of strain W in the presence of 4-MT. Further, in the presence of 4-MT and trace concentrations (5×10^{-8} M–10^{-7} M) of tryptophan, growth continued for 3–4 days, although none occurred in the absence of tryptophan. No evidence for the development of strains resistant to 4-MT was obtained. Beerstecher (1954) has also reported that the inhibition of growth of *Esch. coli* and *Lactobacillus plantarum* by 5-methyltryptophan may be reversed by tryptophan concentrations too low to be the sole source of the amino acid during growth. It would seem therefore that, once growth had been initiated by small amounts of tryptophan, inhibition of synthesis of the amino acid by 4-MT was no longer complete. The fact that at low tryptophan concentrations more growth was obtained with decreasing 4-MT concentration supports this view.

Indole partially reversed 4-MT inhibition when both substances were present in low concentrations (Table 2). Some evidence was obtained which suggested that this reversal was competitive (Table 2, Expt. 2), but definite results could not be obtained, since at higher concentrations indole itself was inhibitory.

Table 2. *Inhibition of growth by 4-MT; reversal by indole*

Conditions as for Table 1.

Expt. no.	Indole (molarity)	4-MT (molarity) 0	10^{-4}	10^{-5}
1	0	93	—	0
	10^{-3}	65	—	13
	5×10^{-4}	84	—	32
	5×10^{-5}	—	—	61
	10^{-5}	—	—	56
2	0	90	0	0
	10^{-4}	—	10	—
	5×10^{-5}	—	24	—
	10^{-5}	—	0	44
	5×10^{-6}	—	—	20
	10^{-6}	—	—	2
	5×10^{-7}	—	—	0

Table 3. *Effect of 4-MT on indole metabolism by washed suspensions*

The reactions were carried out in 5 ml. of 0·02 M phosphate, pH 7·2, for 1 hr. at 37°, with strain M 121–35.

Expt. 1. (1·6 mg. of cell N, 2·5 μmoles of indole)

Changes in conditions	Indole removed (μmoles)
None	0·16
+50 μmoles of glucose	1·46
In vacuo	0·02
In vacuo + 50 μmoles of glucose	0·04

Expt. 2. (3·5 mg. of cell N, 100 μmoles of glucose, 5 μmoles of indole, aeration)

4-MT	Indole removed (μmoles)
—	3·0
10^{-4} M	0·7
10^{-5} M	1·9

Anthranilate did not reverse inhibition by 4-MT but enhanced the inhibition at concentrations at which it itself was not inhibitory.

Essentially the same results were obtained with wild-type *Esch. coli* strain ML. The growth of tryptophan mutants, which required 10^{-6} M or more of the amino acid, was inhibited by 4-MT only at concentrations above 10^{-3} M.

Effect of 4-MT on indole metabolism. Indole was metabolized aerobically by washed suspensions of *Esch. coli* wild types and M121–35 in the presence of glucose (Table 3, Expt. 1). The nature of the

reaction is not known. While serine did not stimulate indole removal nor was tryptophan formation demonstrated, it is possible that the reaction involved a condensation of indole with endogenous serine, the tryptophan being further metabolized during the experiment. Indole is degraded by a rod-shaped, Gram-negative bacterium by a pathway involving anthranilic acid, salicylic acid and catechol (Sakamoto, Uchida & Ichihara, 1953; Uchida, Sakamoto & Ichihara, 1953). Whether such a breakdown occurred in the present experiments has not been investigated. The metabolism of indole by washed suspensions was inhibited by 4-MT (Table 3, Expt. 2). 4-MT was a powerful inhibitor of cell-free tryptophan desmolase, the enzyme condensing indole and serine to tryptophan. The system exhibited the kinetics of competitive inhibition (Fig. 1). 50% inhibition occurred when the ratio of indole to 4-MT was about 3:1.

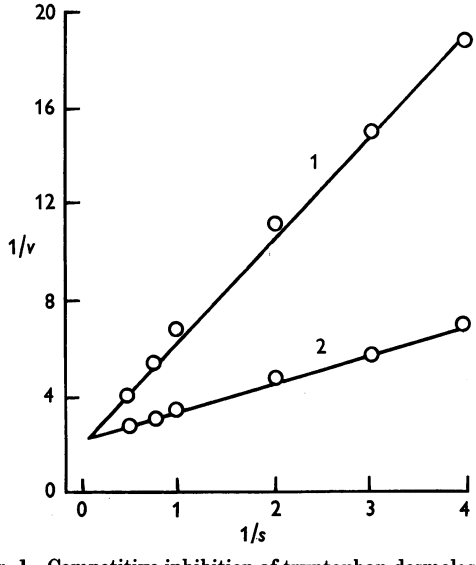

Fig. 1. Competitive inhibition of tryptophan desmolase by 4-MT. Reaction mixture contained 0·02M phosphate, pH 7·5, 25 μmole of L-serine, 20 μg. of pyridoxal phosphate and 1·5 mg. of enzyme N in a volume of 5 ml. Incubation 10 min. at 38°. Curve 1, $3{\cdot}3 \times 10^{-4}$M 4-MT added; curve 2, no 4-MT. s = μmoles of indole/ml. at zero time. v = μmoles of indole/ml. removed in 10 min.

4-MT and tryptophanase. 4-MT was itself decomposed by extracts containing tryptophanase, the enzyme system producing indole from tryptophan. The end product of 4-MT decomposition gave a colour in the Ehrlich test similar to that given by 4-methylindole. A similar reaction on 5-methyltryptophan has been described in whole cells of *Esch. coli* (Beerstecher & Edmonds, 1951). The decomposition of 4-MT and tryptophan appeared to be brought about by the same enzyme system since the amounts of indole or 4-methylindole formed from mixtures of the amino acids depended on the ratio of 4-MT to tryptophan. With a 40:1 ratio the product was almost entirely 4-methylindole, while with a 1:2 ratio indole was the main product. At intermediate ratios, mixtures of indole and methylindole were formed. 4-MT, therefore, cannot be considered an inhibitor of tryptophanase but at high concentrations will prevent tryptophan breakdown by competing for the enzyme as a substrate.

Effect of 4-MT on anthranilate synthesis. In a preceding paper (Trudinger, 1956) it was shown that various mutant strains of *Esch. coli* used in this laboratory produced indole, anthranilate or an unidentified, diazotizable compound (BDC) in their growth media. The addition of 10^{-4}M 4-MT to the growth media prevented these accumulations. Further, wild-type *Esch. coli*, grown in the presence of 4-MT and tryptophan, did not produce indole as might be expected if 4-MT inhibited only indole utilization. Fildes (1938, 1945) demonstrated that indole accumulated when *Esch. coli* and *Bact. typhosum* were grown in the presence of indole acrylate, an inhibitor of the conversion of indole into tryptophan (Fildes, 1941). This suggested that 4-MT might inhibit further back in the biosynthetic chain.

Table 4. *Effect of 4-MT on anthranilate synthesis*

The reactions were run in 5 ml. of 0·02M phosphate, pH 7·5, for 1 hr. at 37° with aeration. Cell N: Expt. 1, 1·8 mg.; Expt. 2, 1·5 mg.

Expt. no.	Additions	Anthranilate formed (mμmoles)
1	None	5
	100 μmoles of glucose	70
	Glucose + 5 μmoles of L-methionine	1020
	Glucose + 5 μmoles of NH_4Cl	1040
	Glucose + L-methionine + NH_4Cl	1840
2	(All tubes contained 100 μmoles of glucose, 5 μmoles of L-methionine and 5 μmoles of NH_4Cl)	
	—	1300
	10^{-3}M 4-MT	0
	10^{-4}M 4-MT	35
	10^{-5}M 4-MT	155
	5×10^{-6}M 4-MT	600

Washed cells of strains B 37 and M 19–2, blocked between anthranilate and indole, synthesized anthranilate aerobically from glucose, methionine and NH_4Cl (Table 4, Expt. 1). The end product supported the growth of the *Esch. coli* mutant M 121–35, which grows on anthranilate, had the same absorption spectrum as authentic anthranilate and moved as anthranilate on Whatman no. 1

paper irrigated with *iso*pentanol or butanol, both saturated with water (Tabone & Robert, 1948).

The synthesis of anthranilate was inhibited strongly by 4-MT above 5×10^{-6} M (Table 4, Expt. 2).

Effect of 4-MT on the metabolism of anthranilate. The synthesis of indole and BDC from anthranilate by washed suspensions of ML 328e and ML 304g has been described in the preceding paper (Trudinger, 1956). This reaction was unaffected by the addition of 2×10^{-4} M 4-MT. Anthranilate removal and indole formation by extracts of ML 328e were also unaffected by this concentration of 4-MT.

DISCUSSION

From the foregoing results it may be seen that 4-MT inhibits at least two enzymes involved in the biosynthesis of tryptophan, namely tryptophan desmolase and an enzyme concerned with the synthesis of anthranilate. In addition, 4-MT inhibits the production of tryptophan desmolase during growth (Monod & Cohen-Bazire, 1953). The fact that concentrations of 4-MT below 10^{-3} M do not inhibit completely the enzymes involved in tryptophan biosynthesis may explain the slow growth which occurred in the presence of the inhibitor and subgrowth-factor concentrations of tryptophan. However, the reason for the complete inhibition of growth in the absence of tryptophan is not clear.

Although the growth inhibition studies reported in this paper suggest that 4-MT primarily inhibits tryptophan synthesis, too much reliance should not be placed on this type of approach. The contrary results of Fildes & Rydon (1947) have already been quoted. Also Marshall & Woods (1952) have reported that inhibition by 4-MT of the growth of *Esch. coli* is reversed competitively by tryptophan and non-competitively by some tryptophan dipeptides. Both these observations would suggest that 4-MT primarily inhibits tryptophan utilization. On the other hand, Beerstecher (1954) has reported that tryptophan reversed the inhibition of *Esch. coli* and *Lb. plantarum* by 5-methyltryptophan in a non-competitive manner. In these cases indole also reversed non-competitively.

Since 4-MT can, in certain cases, be degraded to 4-methylindole, it was necessary to exclude the latter as the active inhibitor in these experiments. The breakdown of 4-MT occurred only in preparations containing active tryptophanase. In the growth experiments and enzyme experiments involving cells grown on growth-factor levels of tryptophan, no production of 4-methylindole was detected.

SUMMARY

1. The growth of *Esch. coli* was inhibited by 4-methyltryptophan (4-MT), and the inhibition was reversed by tryptophan in a non-competitive manner.

2. 4-MT inhibited tryptophan desmolase in cell extracts in competition with indole.

3. Extracts containing tryptophanase degraded 4-MT to a compound reacting as 4-methylindole.

4. 4-MT inhibited strongly the synthesis of anthranilate and indole removal by washed cells of *Esch. coli*.

5. The conversion of anthranilate into indole was not affected by 4-MT.

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