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False Feedback Inhibition: Inhibition of Tryptophan Biosynthesis by 5-Methyltryptophan*†

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Several normal metabolites, including isoleucine (1), valine (2), proline (3), cytidine 5′-phosphate (4), and certain of the purine ribonucleotides (5), have each been found to inhibit the action of an early enzyme functioning in its own biosynthetic pathway. Umbarger (1) has suggested that this kind of inhibition constitutes a negative feedback loop which permits a metabolite to maintain itself at a constant intracellular level. It appeared that such a feedback loop might be a site for the action of an antimetabolite, which could then prevent the biosynthesis of the corresponding normal metabolite by mimicking the latter's specific feedback inhibition (4). If the antimetabolite, in addition, were unable to mimic the positive functions of the metabolite (as a building block for macromolecules, coenzymes, etc.) inhibition of cell growth should result.

Evidence that an antimetabolite can mimic the feedback effect of the normal metabolite has been obtained by Gots and Gollub (6), who found that not only the natural purines, but also several of their analogues, can inhibit the excretion of intermediates of purine biosynthesis by purine auxotrophs. Similarly Pardee and Prestidge (7) found that azatryptophan, as well as tryptophan, inhibit the excretion of anthranilic acid by tryptophan auxotrophs. Trudinger and Cohen (8) had previously shown that such excretion was sensitive to 4-methyltryptophan. In a preliminary communication describing false feedback inhibition of enzymatic reactions Moyed and Friedman (9) reported that tryptophan and 6-fluorotryptophan are both inhibitors of the condensation of anthranilic acid with 5-phosphoribosyl 1-pyrophosphate an essential step in tryptophan biosynthesis. Similarly, both 2-thiazole alanine and histidine were found to be inhibitors of an early step in histidine biosynthesis.

In the further study of the feedback control of tryptophan biosynthesis as a possible site of antimetabolite action it became necessary to use another analogue. 5-Methyl-DL-tryptophan was examined and found to mimic the inhibitory effect of tryptophan on an earlier reaction in tryptophan biosynthesis. This paper presents evidence that such false feedback inhibition is the basis for the bacteriostatic action of 5-methyltryptophan.

MATERIALS AND METHODS

Chemicals—ATP (Pabst Laboratories), glutamine, and ribose 5-P (Schwarz Laboratories); 5-phosphoribosyl 1-pyrophosphate and 5-methyl-DL-tryptophan (Sigma Chemical Company); L-tryptophan (California Biochemical Corporation), indole and anthranilic acid (Eastman Chemical Company) were commercial preparations. The barium salt of shikimic acid 5-phosphate was a gift of Dr. Bernard D. Davis.

Growth of Bacteria—The organisms used were *Escherichia coli* strain W and its variants, strain 5-MT-R_6, which is resistant to the bacteriostatic action of DL-5-methyltryptophan, and strain M 19-2, a tryptophan auxotroph which excretes anthranilic acid. They were grown with vigorous aeration by rotary shaking. The minimal medium employed contained per liter: 18.9 g of $Na_2HPO_4 \cdot 7H_2O$, 6.3 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, and 2 g of $(NH_4)_2SO_4$. For cultivation of strain M 19-2, 10 mg of tryptophan was added per liter of medium. After this mixture was sterilized by autoclaving, 10 ml of a sterile 20% solution of glucose were added. (In some instances 2 g per liter each of Bacto Yeast Extract and of Bacto Casamino Acids were added.) Turbidity of the cultures was measured in Klett-Summerson colorimeter with a purple filter (No. 42). Growth is expressed as μg of dry weight of bacteria per ml of culture based on a previous calibration relating the turbidity of a culture to its dry weight.

Preparation of Extracts—Suspensions containing 2 g (wet weight) of cells in 12 ml of 0.03 M potassium phosphate buffer at pH 7.4 were subjected to sonic oscillation at 10 kc. for 5 minutes in a magnetostrictive oscillator (Raytheon). Intact cells and large fragments were removed by centrifugation at 25,000 $\times$ *g*.

Enzymatic Synthesis of Anthranilic Acid—The method of Srinivasan (10) was used with slight modification. The reaction mixtures contained per ml: 40 μmoles of Tris buffer at pH 8.0, 5 μmoles of $MgCl_2$, 0.5 μmole of DPN, 6.0 μmoles of glutamine, 1 μmole of shikimic acid 5-phosphate unless otherwise indicated, and a varying amount of bacterial extract. Anthranilic acid was estimated by either the Bratton and Marshal method for arylamines or by its fluorescence (activation λ = 310 mμ, fluorescent λ = 409 mμ) with an Aminco-Bowman spectrophotofluorometer.

RESULTS

L-Tryptophan and 5-Methyl-DL-tryptophan as Enzyme Inhibitors—In a previous communication (8) it was reported that tryptophan and one of its analogues, 6-fluorotryptophan, inhibited the enzymatic condensation of anthranilic acid with 5-phosphoribosyl 1-pyrophosphate, an essential reaction for the biosynthesis of tryptophan. However, in further studies of this reaction as a possible site of action for tryptophan analogues it has been found that 5-methyltryptophan, in contrast to 6-fluoro-

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† A preliminary report of this work has appeared (see reference 6).

tryptophan, has no inhibitory effect. Nevertheless, 5-methyltryptophan, as will be shown in a subsequent section, causes bacteriostasis under conditions which suggest that it too acts by inhibiting an early step in the biosynthesis of tryptophan. Such an early step is the formation of anthranilic acid. Srinivasan's (10) recent description of anthranilic acid synthesis in extracts made it possible to investigate this reaction as a possible site for the action of 5-methyltryptophan.

Extracts of cells which had been grown in the mineral salts-glucose medium supplemented with yeast extract and acid-hydrolyzed casein were used to study the effect of 5-methyltryptophan and of tryptophan on anthranilic acid formation. The rates of anthranilic acid synthesis were determined at four different levels each of tryptophan and of 5-methyltryptophan. The amount of each compound which causes 50% inhibition was estimated from a graph of the data. The results of these experiments are summarized in Table I. Both tryptophan and 5-methyltryptophan are potent inhibitors of anthranilic acid synthesis. The normal metabolite is about 4 times as effective as the analogue. The inhibition by either compound is essentially independent of the concentrations of the substrates, shikimic acid 5-phosphate and glutamine. These results might suggest that tryptophan and 5-methyltryptophan are noncompetitive inhibitors of anthranilic acid synthesis. However, such a conclusion is not yet warranted since the formation of anthranilic acid is undoubtedly a multi-step process, and the properties of the sensitive step, particularly its reactants, are still unknown.

Inhibitory Effects of 5-Methyltryptophan on Intact Cells—As might be expected from its action on cell-free preparations, 5-methyltryptophan is a potent inhibitor of anthranilic acid synthesis by intact cells. Unfortunately, it is not possible to obtain dose response curves for the partial inhibition of growth or of anthranilic acid synthesis by cells as these organisms rapidly convert 5-methyltryptophan by transamination to the corresponding keto acid which is nontoxic (11). However, the duration of the inhibitory effects of a given concentration of the analogue can be determined. The excretion of anthranilic acid by suspensions of a tryptophan auxotroph, strain M 19-2, derived from *E. coli* W, was completely inhibited for about 90 and 150 minutes by 2 and 4 μg per ml of 5-methyltryptophan (Fig. 1*B*). These amounts of the analogue caused maximum inhibition of the growth of the parent strain, W, for almost identical intervals of time (Fig. 1*A*). The average concentrations of cells during both experiments were similar. The observations that the duration of maximum inhibition in both experiments was the same are suggestive of a cause and effect relationship between the inhibitory actions of 5-methyltryptophan on anthranilic acid synthesis and on growth.

Antagonism of Bacteriostatic Action of 5-Methyltryptophan—If 5-methyltryptophan does in fact inhibit growth by blocking the synthesis of anthranilic acid, and hence of tryptophan, then the bacteriostatic action of the analogue should be antagonized by tryptophan in a noncompetitive manner. However, the nature of the relationship between tryptophan and 5-methyltryptophan cannot be demonstrated unambiguously as these two compounds gain entry into the cell by way of a common permeation mechanism (11). Thus competition between the two compounds for entry into the cell might mask the actual site of action of the analogue. For this reason indole, a compound with a presumably independent site of entry and readily converted to tryptophan within the cell, was used to antagonize the bacteriostatic action of 5-methyltryptophan. It was found that the amount of indole necessary for half-maximal growth in the presence of 5-methyltryptophan is not influenced by the level of the analogue (Table II). Thus indole, and by inference tryptophan, is a noncompetitive antagonist of 5-methyltryptophan.

TABLE I

Inhibition of conversion of shikimic acid 5-phosphate and glutamine to anthranilic acid

The assay system described under "Materials and Methods" was used for the determination of anthranilic formation except that the indicated amounts of shikimic acid 5-phosphate and glutamine were employed. In the absence of inhibitors 45 to 50 mμmoles of anthranilic acid were produced in one hour by 0.1 ml of extract from the indicated amounts of substrates.

Substrates		Inhibitors, amounts causing 50 % inhibition	
Shikimic acid 5-phosphate	Glutamine	5-Methyltryptophan	Tryptophan
M	*M*	*M*	*M*
5×10^{-4}	3×10^{-2}	1.4×10^{-4}	2.3×10^{-5}
5×10^{-4}	6×10^{-3}	1.0×10^{-4}	2.4×10^{-5}
1×10^{-4}	3×10^{-2}	1.1×10^{-4}	2.7×10^{-5}
1×10^{-4}	6×10^{-3}	0.8×10^{-4}	2.6×10^{-5}

Characteristics of 5-Methyltryptophan-resistant Mutant—If the principal action of 5-methyltryptophan is inhibition of anthranilic acid synthesis it is reasonable to expect that at least some organisms which develop resistance will do so by producing an anthranilic acid-forming system with decreased sensitivity to the analogue. To test this expectation several resistant organisms were selected by inoculating about 10^8 cells of *E. coli* W on minimal medium containing 2% agar and 2×10^{-3} M 5-methyltryptophan. By 20 hours, the interval required for colony development on noninhibitory media, several resistant colonies

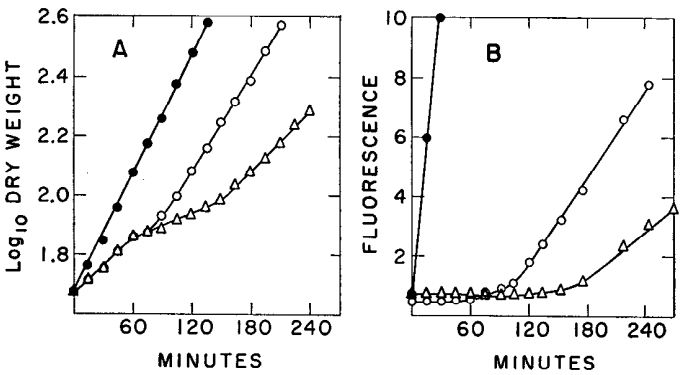

FIG. 1*A*. Inhibition of growth by 5-methyltryptophan. The inoculum was an exponentially growing culture of *Escherichia coli*, strain W. Minimal medium was employed. *B*. Inhibition of anthranilic acid excretion by 5-methyltryptophan. The organism was a tryptophan auxotroph of *Escherichia coli* W, strain M 19-2, blocked between anthranilic acid and indole. The cell suspension (55 mg dry weight of bacteria per ml of minimal medium) was aerated by shaking as in growth experiments. At intervals the fluorescence of the suspensions was measured at the activation and emission maxima of anthranilic acid, 310 mμ and 409 mμ, respectively. Fluorescence is expressed in arbitrary units. One unit is equal to 1 μg of anthranilic acid per ml.

●——●, No additions; ○——○, 2 μg of 5-methyltryptophan per ml; △——△ 4 μg of 5-methyltryptophan per ml.

TABLE II

Antagonism of bacteriostatic effect of 5-methyltryptophan by indole

The inoculum was an exponentially growing culture of *Escherichia coli* W. The initial bacterial densities were equivalent to 6 μg per ml (Experiment 1), and 4 μg per ml (Experiment 2). Minimal medium was used for each determination. The duration of Experiments 1 and 2 were 4 and 5 hours, respectively.

5-Methyltryptophan	Indole (μg/ml)					
	0	0.1	0.2	0.3	0.4	0.6
	Growth (dry wt.)					
μg/ml	*μg/ml*					
Experiment 1						
0	82					
0.2	50					
0.4	25					
0.6	15					
0.8	8					
1.0	11	10	22	41	50	
2.0	10	14	29	34	49	
3.0	10	17	29	38	46	
4.0	10	19	27	36	44	
Experiment 2						
0	129					
1	5		38		70	87
1.5	5		40		62	80
2.0	6		40		65	82
2.5	8		42		68	80

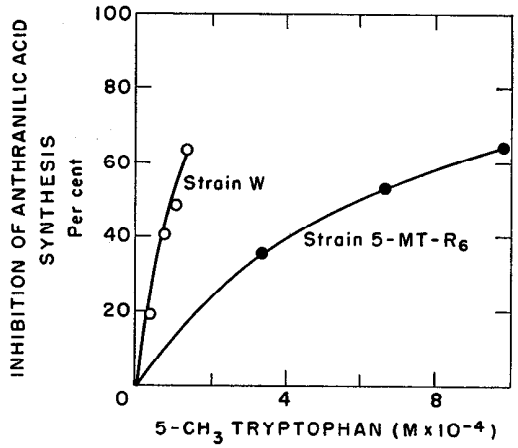

FIG. 2. Inhibition of anthranilic acid synthesis by 5-methyltryptophan in extracts of the 5-methyltryptophan resistant strain, 5-MT-R_6, and of the sensitive strain, W. Extracts were prepared from cells which had been grown in a glucose-mineral salts medium supplemented with Bacto Yeast Extract and Bacto Casamino Acids (an acid hydrolysate of casein). The assay system described under "Materials and Methods" was used for the determination of anthranilic formation. In the absence of an inhibitor 51 and 60 μmoles of anthranilic acid were produced in 1 hour by 0.1 ml of an extract of strain W and of strain 5-MT-R_6, respectively.

had appeared. Stock cultures of these were prepared. Colonies which developed after 20 hours were disregarded. (The choice of only those mutants which developed colonies rapidly on the first exposure to the inhibitor reduces the chance of selecting strains which have undergone more than one mutation.) Colony

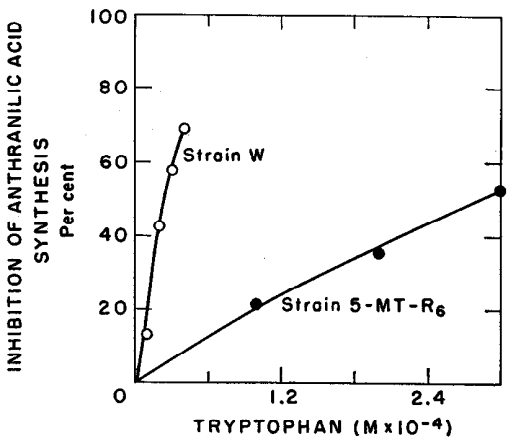

FIG. 3. Inhibition of anthranilic acid synthesis by tryptophan in extracts of the 5-methyltryptophan resistant strain, 5-MT-R_6, and of the sensitive strain, W. Experimental details are the same as for Fig. 2.

formation by each of these strains is unaffected by 2×10^{-3} M 5-methyltryptophan, whereas 2×10^{-6} M 5-methyltryptophan is sufficient to prevent colony formation by the parent strain.

A decrease in sensitivity to the bacteriostatic effect of 5-methyltryptophan was accompanied by decreased sensitivity to its inhibitory effect on the enzymatic synthesis of anthranilic acid. A resistant mutant, strain 5-MT-R_6, produces an anthranilic acid-forming system which is 6-fold less sensitive to the analogue than that of the parent organism, strain W (Fig. 2). There is a similar change in the sensitivity of anthranilic acid synthesis to its normal feedback inhibitor, tryptophan. Ten times as much tryptophan is necessary for 50% inhibition of anthranilic acid synthesis in extracts of strain 5-MT-R_6 as in extracts of strain W (Fig. 3).

An additional difference between the two organisms has been observed which probably contributes to the difference in their resistance. Strain 5-MT-R_6 produces 10 times more of the enzyme necessary for the synthesis of anthranilic acid than strain W when both are grown in a mineral salts-glucose medium. An extract of strain 5-MT-R_6 formed 16 mμmoles of anthranilic acid per mg of protein in an hour, whereas an extract of strain W formed only 1.5 mμmoles of anthranilic acid.[1] An increased capacity for the synthesis of anthranilic acid by 5-methyltryptophan resistant mutants has also been reported by Cohen and Jacob (12).

Strain 5-MT-R_6, unlike strain W, excretes small amounts of tryptophan into its medium. The excretion was first detected by the fact that a mutant with a specific requirement for tryptophan grows in the immediate vicinity of colonies of strain 5-MT-R_6. The fluorescent spectra of the culture fluids of the

[1] The growth of strain W in a medium supplemented with yeast extract and acid-hydrolyzed casein caused a 40-fold increase in its ability to synthesize anthranilic acid. Strain 5-MT-R_6 was stimulated 4-fold. In a typical experiment an extract of strain W prepared from cells grown in the supplemented medium formed 64 mμmoles of anthranilic acid per mg protein in an hour. Under the same conditions an extract of strain 5-MT-R_6 formed 61 mμmoles of anthranilic acid. The stimulatory action of the supplements to the growth medium does not appear to be a true cofactor effect since the addition of these materials to extracts of cells grown in the mineral salts-glucose medium did not accelerate the enzymatic synthesis of anthranilic acid.

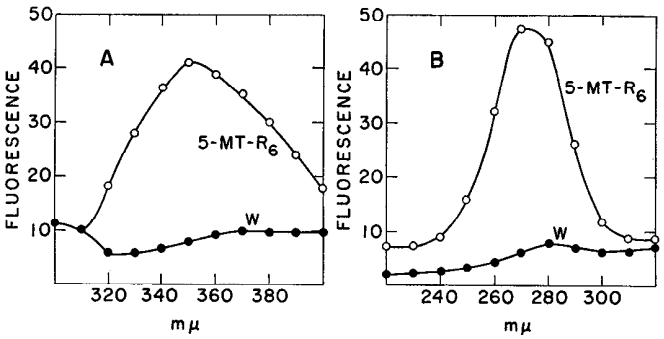

FIG. 4A. Fluorescence (emission) spectrum of culture filtrates of strains 5-MT-R_6 and W. The activating λ was 280 mμ. *B.* Activation spectrum of culture filtrates of strains 5-MT-R_6 and W. The emission λ was 350 mμ.

Filtrates were of cultures in the exponential phase of growth. The cell density had reached 350 μg dry weight of bacteria per ml.

two strains provide further evidence that the excreted factor is tryptophan (Fig. 4). The resistant mutant, strain 5-MT-R_6, excretes material with fluorescence λ_{max} = 360 mμ) and activation (λ_{max} = 280 mμ) spectra identical to those of tryptophan. On the other hand, fluorescence at these wavelengths cannot be detected in the culture fluid of the sensitive, parent strain, W. After 5 hours of growth (400 μg dry weight of cells per ml) the intensity of fluorescence indicates the presence of 0.5 μg of tryptophan per ml in the culture fluid of strain 5-MT-R_6.

DISCUSSION

The often observed inability of enzymes to discriminate completely between their substrates and related structural analogues has led to the general assumption that antimetabolites inhibit growth by competitively interfering with the incorporation of the corresponding normal compounds into essential components of the cell. However, an alternative mechanism of analogue action is suggested by the observation that 5-methyltryptophan mimics the specific inhibitory or feedback effect of tryptophan on the enzymatic synthesis of the tryptophan precursor, anthranilic acid. In this case a sensitive enzyme fails to discriminate completely between its normal feedback inhibitor, tryptophan, and 5-methyltryptophan. The cell thus responds to the analogue, as it does to externally added tryptophan, by halting further synthesis of this amino acid. As the analogue is unable to replace tryptophan for protein synthesis growth also ceases.

Several kinds of evidence support the suggestion that the bacteriostatic action of 5-methyltryptophan stems directly from its effectiveness as a false feedback inhibitor. (*a*) Anthranilic acid synthesis by cells and growth are both maximally inhibited for identical intervals of time by the same concentrations of the analogue. (*b*) Indole, which can be converted to tryptophan by a step in the synthetic pathway beyond the suspected sensitive site antagonizes the bacteriostatic action of the analogue noncompetitively. (*c*) A mutant which was selected for resistance to 5-methyltryptophan produces an increased amount of the enzyme necessary for the synthesis of anthranilic acid; furthermore, the enzyme in the mutant is less sensitive to the analogue than that in the parent strain. The changes in both the level and in the sensitivity of the enzyme could contribute to resistance if anthranilic acid synthesis is the major site of action by the analogue.

It should be pointed out that strain 5-MT-R_6 is at least a 1000-fold less sensitive than strain W to bacteriostasis by 5-methyltryptophan, but that only a sixtyfold difference in sensitivity was observed in the suspected site of action of the analogue, the anthranilic acid-forming system. This disparity probably results from the fact that 5-methyltryptophan appears to be maintained at a definite intracellular concentration by a stereospecific concentrating mechanism which is independent of the external concentration of the analogue within the limits used in these experiments (11). The concentrating mechanism may be incapable of maintaining the obviously higher concentration of the analogue which would be necessary to inhibit the growth of strain 5-MT-R_6. A small change in sensitivity at the principal intracellular site of action of the analogue could thus account for a much greater change in sensitivity to bacteriostasis by the analogue.

Anthranilic acid synthesis in the resistant strain, in addition to being less sensitive to inhibition by 5-methyltryptophan, is also less sensitive to tryptophan itself. This change and the increased level of enzymes for the synthesis of anthranilic acid are probably responsible for the slight overproduction of tryptophan by the mutant. It has been suggested that such overproduction of a normal metabolite might be a basis for resistance to an antimetabolite (13). However, this explanation would be valid only for resistance to an analogue which acts by competitively inhibiting the *utilization* of the corresponding normal metabolite. Since 5-methyltryptophan acts by inhibiting the *biosynthesis* of tryptophan, overproduction of this amino acid in the resistant organism discussed here could not readily occur in the presence of the analogue unless the susceptible enzyme had lost sensitivity to the analogue. The loss of enzymatic sensitivity to tryptophan and the consequent excretion of tryptophan, therefore, appear to be incidental changes, unnecessary for the acquisition of resistance.

SUMMARY

Tryptophan appears to be capable of regulating its intracellular level as it is a potent feedback inhibitor of an early enzymatic step necessary for its own biosynthesis, the conversion of shikimic acid 5-phosphate to anthranilic acid. An analogue, 5-methyl-DL-tryptophan, also inhibits this reaction and is, therefore, a false feedback inhibitor. Evidence is presented that this false feedback effect is the basis for the bacteriostatic action of 5-methyltryptophan.

A mutant selected for resistance to 5-methyltryptophan produces an anthranilic acid-forming system which has altered sensitivity to the analogue as well as to tryptophan itself. In addition, the mutant produces more of the enzyme system. It is because of these changes that the mutant, unlike the parent strain, overproduces tryptophan and excretes it into the culture fluid.

The basis of resistance in the mutant is altered sensitivity of the anthranilic acid-forming system to inhibition by the *analogue*. The overproduction of tryptophan is an incidental change unnecessary for resistance.

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