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Growth Inhibitors and their Antagonists as Mutagens and Antimutagens in *Escherichia coli*

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SUMMARY: Thirty-five substituted benzimidazoles, benzotriazoles and quinoxalines were tested as growth inhibitors on three mutants of *Escherichia coli*, strain W, having different purine requirements and on the ciliate *Tetrahymena geleii*. A number of the compounds, especially those with a nitro group in the benzene ring, were inhibitory. Both organisms were affected in a similar way by the compounds. Several of these analogues were tested for mutagenic activity at the purine and streptomycin loci in a purine-requiring mutant of *E. coli*. Only inhibitory compounds were found to be mutagenic; non-inhibitory concentrations of one analogue, 4-nitro-6-hydroxybenzimidazole (NHB), were, however, also mutagenic. No specific mutagenesis was demonstrated. NHB was found to increase the rate of mutation as determined by the papilla method. Evidence is presented which eliminates the hypothesis that selection rather than mutagenesis was involved. This analogue did not increase the frequency of mutants in non-dividing cells, and was not incorporated into bacterial DNA. The mechanism of this mutagenesis is thought to include the inhibition of enzymes concerned with DNA synthesis. DNA and RNA and their constituents, purine and pyrimidine precursors and analogues, vitamin B₁₂ and other vitamins, were found to have no effect on the inhibition or mutagenesis produced by NHB. Several amino acid combinations and one non-mutagenic analogue, 4-hydroxy-6-nitrobenzotriazole, were, however, found to interfere with mutagenesis by NHB. These compounds did not affect the frequency of spontaneous mutants. The amino acid combination was effective only when present simultaneously with the mutagen and did not act by a selection mechanism. The mechanism of antimutagenesis by amino acids may involve their ability to increase the growth rate and also to modify the inhibited nucleic acid metabolism.

The mutagenesis by 5-nitroquinoxaline, a pterin analogue, was also found to be depressed by amino acids in the same purine-requiring mutant. The annulment of mutagenic activity by amino acids was strain and mutagen specific.

Twenty-five known inhibitors of nucleic acid synthesis and antagonists to inhibition were tested for mutagenic activity in five strains of *Escherichia coli*. Several, including 6-mercaptopurine, were found to be mutagenic; in addition, NHB, 5-nitroquinoxaline and 5-aminouracil were mutagenic in most of the strains tested. The mutagenic activity of 5-aminouracil was not annulled by thymine, thymidine or other nucleic acid components, but the mutagenesis of 6-mercaptopurine was annulled by purine bases and by ribosides.

Novick & Szilard (1951) showed that purine analogues increased the rate of mutation to phage T₅ resistance in *Escherichia coli* B/1,t growing in steady-state conditions in the chemostat. On the basis of these findings and those of Fries & Kihlman (1948) who found caffeine to be mutagenic for *Ophiostoma multiannulatum*, the present investigations on chemical mutagenesis were undertaken, utilizing growth inhibitors, the majority of which are analogues

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of nucleic acid constituents or are known to interfere with nucleic acid metabolism, and growth-inhibitor antagonists. Both the inhibitors and their antagonists were tested as mutagens and antimutagens in *E. coli*. It was supposed that purine analogues, such as substituted benzimidazoles and benzotriazoles, could act by inhibiting enzymes involved in nucleic acid synthesis so that the wrong kind, arrangement, or proportion of constituents would be built into the deoxyribonucleic acid molecule, or that the analogue itself might be incorporated to form an unnatural or abnormal DNA. Further, substances able to act as antagonists to growth inhibition and nucleic acid constituents might prevent this incorporation or enzyme inhibition and thereby act as antimutagens. Novick & Szilard (1952) also reported the antimutagenic activity of purine ribosides on mutagenesis by caffeine and other purine analogues. Guanosine and adenosine depressed the induced as well as the spontaneous mutation rate. The pyrimidine ribosides were ineffective as were riboside components. The annulment of mutagenesis was effected by low concentrations of the ribosides and was competitive. The present study deals mainly with the effects of 4-nitro-6-hydroxybenzimidazole (NHB), 6-mercaptapurine and 5-aminouracil on mutation.

METHODS

The organisms used were either *Tetrahymena geleii*, strain H, or various strains of *Escherichia coli* which will be described when relevant. The medium for *T. geleii* was modified after Kidder & Dewey (1949), while a modified Gray & Tatum's medium (minimal medium) (Ryan & Schneider, 1948) was used for *E. coli*.

The compounds employed in the inhibition and mutant-screening experiments with *Escherichia coli*, strain W, and its purine-dependent mutants were benzimidazoles, benzotriazoles and quinoxalines, which are structural analogues of purines, the carcinostatic 8-azaguanine and the pterin portion of folic acid (see Fig. 1). These compounds were synthesized by Gillespie, Engelman & Graff (1954, 1956*a, b*); see Engelman, Gillespie, Greer & Graff (1952). Strain W and its purine-dependent mutants were obtained from Dr B. Davis (Davis, 1950).

Other compounds were utilized in mutant-screening experiments with *Escherichia coli* mutant strains Wp⁻, 15h⁻, 15h⁺m⁻, B/r,m⁻ and B/r,t⁻. These compounds (shown in Table 7) are inhibitors and their antagonists most of which are analogues of nucleic acid constituents; their effect on metabolism has been studied in other laboratories (Hirschberg, 1955; Woolley, 1951).

RESULTS

Inhibitory and mutagenic activity of benzimidazoles, benzotriazoles and quinoxalines

The effects of the substituted benzimidazoles, benzotriazoles and quinoxalines on the growth of *Escherichia coli*, strain W, its two purine-dependent mutants, and on *Tetrahymena geleii*, strain H, are summarized in Table 1, which also

indicates the mutagenic effect of several of the compounds on the guanine-dependent and streptomycin-sensitive loci of mutant Wp⁻. The growth inhibition analyses were performed by inoculating 500 *T. geleii* organisms into 5 ml. medium containing the test compound in test tubes and aerating in a roller at 25°. The growth of *T. geleii* was limited to one-half of maximal at a concentration of 2×10^5 organisms/ml. by adding 6 µg. guanine/ml. In studies with *E. coli*, 5×10^6 organisms of *E. coli*, strain W, or mutants Wp⁻ or Wah⁻, were inoculated into 5 ml. medium containing the test compound in test tubes and aerated on a roller at 37°. The limiting growth factor and sole purine supplement was 6 µg. guanine/ml. which supported one-half of maximal

Fig. 1. A comparison of the structure of benzimidazoles, benzotriazoles and quinoxalines with purines, 8-azaguanine and the pterin portion of folic acid, respectively. * Substitutions of OH, OCH₃, NH₂ and NO₂ groups occur at the 4- and 6-position of benzimidazoles and benzotriazoles and at the 5- and 7-position of quinoxalines. Substitutions of phenyl, ribose, OH and CH₃ groups occur in the 2-position.

growth in experiments with mutant Wp⁻. In studies with mutant Wah⁻, 18 µg. adenine/ml. served these functions. The limiting growth factor in experiments with wild-type W was 0.1 % (w/v) glucose. For both mutants, several concentrations of the test compounds were used and growth was measured by the increase in optical density as determined in the Klett-Summerson colorimeter. The optical density was calibrated against total and viable bacteria counts. To measure the inhibition of colonial growth, several concentrations of Wp⁻ bacteria (200–1000) were plated in 30 ml. of 1.5 % (w/v) agar containing minimal medium + the test compound and supplemented with 65 µg. guanine/ml. Counts of plates were made after incubation for 10 days at 37°. Determinations of this inhibitory effect of the compounds were made by comparing colony counts with appropriate controls.

The compounds shown in Table 1 have various effects on the micro-organisms. Some affect the length of the lag phase, others also depress the

Table 1. *The effects of substituted benzimidazoles, benzotriazoles and quinoxalines on the growth of Tetrahymena geleii and Escherichia coli strain W and two of its purine-dependent mutants, including the mutagenic activity of several compounds at the guanine-dependent and streptomycin-sensitive loci of E. coli Wp⁻.*

				Effect on survival and mutation of mutant Wp ⁻					
Organism				Tetrahymena geleii strain H	E. coli strain W, mutant Wp ⁻ and mutant Wah ⁻ *	Mean factor of increase over spontaneous mutant-frequency			
	Concentration of compound 200 μg./ml.					Concn. (μg./ml.)	% inhibition of visible colony formation	Purine ⁻ to purine ⁺	Strepto- mycin ^s to strepto- mycin ^r
Compound. Substitution in position				Degree of inhibition†					
Benzimidazoles									
2	4	6							
.	.	.	+	+	200	5	2	.	
.	OH	NH ₂	-	-	.	.	.	.	
.	OCH ₃	NH ₂	+	-	.	.	.	.	
.	NH ₂	OCH ₃	-	-	.	.	.	.	
.	.	NO ₂	+++++	+++++	5	20	20	4	
.	OH	NO ₂	+	++	200	98	1	2	
.	NO ₂	OH	+	+++++	85	64	33	900	
.					48	17	11	430	
.					20	0	2	35	
.					10	0	1	6	
OH	NO ₂	OH	+++++	-	200	0	1	2	
CH ₃	OH	NO ₂	-	+++	200	85	4	1	
.	OCH ₃	NO ₂	++	+	.	.	.	.	
OH	OCH ₃	NO ₂	-	-	100	0	2	1	
CH ₃	OCH ₃	NO ₂	++	-	200	0	1	1	
ribose	OCH ₃	NO ₂	+	-	.	.	.	.	
.	NO ₂	OCH ₃	-	-	75	0	1	2	
ribose	NO ₂	OCH ₃	-	-	.	.	.	.	
Benzotriazoles									
2	4	6							
.	OH	NH ₂	-	-	.	.	.	.	
.	OCH ₃	NH ₂	-	+++	.	.	.	.	
.	.	NO ₂	+	+++++	200	99.9	7	18	
.	OH	NO ₂	+++	+++++	.	.	.	.	
.	OCH ₃	NO ₂	+++++	+++	200	60	8	6	
Quinoxalines									
2	3	5	7						
.	.	OCH ₃	NH ₂	+++++	-	200	0	1	0.2
CH ₃	CH ₃	OCH ₃	NH ₂	+	-	.	.	.	.
.	.	NO ₂	.	+++++	+++++	20	99	14	14
.	.	NO ₂	OCH ₃	+++++	+	200	60	2	2
‡	‡	NO ₂	OCH ₃	-	-	.	.	.	.
.	.	OCH ₃	NO ₂	+++++	+++++	40	85	2	1
CH ₃	CH ₃	OCH ₃	NO ₂	+++++	+++++	200	85	3	3

* Wp⁻: requiring guanine, adenine, hypoxanthine, xanthine or 4-amino, 5-imidazolecarboxamide, Wah⁻: requiring adenine or hypoxanthine.

† —, no inhibition; +, time to reach stationary phase = >1 × control; ++, time to reach stationary phase = >1.5 × control; +++, time to reach stationary phase = >2 × control; + + + +, time to reach stationary phase = >2.5 × control; + + + + +, completely inhibitory to an inoculum of 10⁶ bacteria/ml. or 10⁴ ciliates/ml.

‡ Phenyl.

rate of growth. In general, the response of the micro-organisms to the compounds was similar. Furthermore, there are no differences in response to these agents by the different nutritional mutants of *Escherichia coli* W. A nitro group on the benzene ring is a highly inhibitory configuration; amino groups are less inhibitory. Adding phenyl, ribose or methyl to the 2-position tends to lessen the inhibitory activity of an analogue.

To determine the frequency of chemically-induced mutants, approximately 10^5 cells of mutant *Escherichia coli* Wp⁻ were inoculated into flasks containing 100 ml. minimal media supplemented with a superoptimal concentration of guanine (65 μ g./ml.) and the test compound. The flasks were aerated on a shaker at 37°. By restricting the glucose to 0.1% (w/v), the growth was limited to 2×10^9 bacteria/ml. after 18 generations. After the bacteria had reached the stationary phase of growth, they were washed twice in 0.85% (w/v) saline (used for all washings and dilutions) and plated. Samples containing 10^{10} bacteria/ml. were plated in Petri dishes containing minimal medium + 1.5% (w/v) agar supplemented with 65 μ g. guanine/ml. + 100 μ g. streptomycin/ml. After 4 days the number of streptomycin-resistant colonies was determined. Several dilutions of bacteria (no more than 10^9 /ml.) were plated in 1.5% minimal agar and the number of guanine-independent colonies determined at 2.5 days. The maximal number of bacteria to be plated in streptomycin agar or minimal agar for full expression of mutants was determined after reconstruction experiments wherein known numbers of mutants were added to the plates to determine the effects on mutant expression of crowding by the non-mutant background (see Ryan, 1953). After 2.5 days, guanine-independent plate mutant colonies appear; i.e. mutants not present in the growth tubes that arise on the plates from the parental guanine-dependent cells; therefore, the counts were made at this time. Several colonies were isolated from the streptomycin and minimal plates; after numerous transfers it was demonstrated that these bacteria retained their mutant characteristics. The numbers of viable organisms were measured by dilution and seeding into Difco nutrient agar pour-plates. All determinations of the frequency of mutants were made with a minimum of five replicate cultures.

Only inhibitory compounds were found to be mutagenic; however, non-inhibitory concentrations of NHB (10 and 20 μ g./ml.) were also mutagenic. No instance of specific mutagenesis was demonstrated; that is, no case of a mutagen affecting one marker and not the other. The streptomycin locus (loci) is affected much less by 6-nitrobenzimidazole than is the guanine locus (loci); nevertheless, this low degree of mutagenesis at the streptomycin locus is reproducible in repeated experiments. Although the frequency of spontaneous guanine-independent mutants is approximately a hundred-fold greater than the frequency of spontaneous streptomycin-resistant mutants, the induced factor of increase is greater at the streptomycin locus in one case, greater at the nutritional marker in others, and equal with two of the compounds. Similar studies on specific mutagenesis were made by Ryan (1952), who found no mutagenic activity for analogues of β -galactosides at the locus involving ability to synthesize β -galactosidase.

The fact that these mutagens actually increase the number of mutations to purine independence as determined by the papilla method was more readily demonstrated with NHB than with any other active compound (Table 2). This analogue also greatly increases the frequency of streptomycin-resistant mutants (Table 1) which made it suitable for relatively simple antimutagen-screening (washing bacteria was not necessary before plating for streptomycin-resistant mutants). Therefore, extensive studies on the mutagenesis of this compound were undertaken.

Table 2. *The relationship between the concentration of 4-NO₂-6-OH benzimidazole and the mutation rate in strain Wp⁻ from purine dependence to independence as determined by the papilla method*

Concentration of NHB* (μg./ml.)	Percentage of c. 500 colonies having number of papillae equal to					Mean number of papillae/colony	Mean number of bacteria/colony (× 10 ⁶)	Mutation rate bacterium/generation (× 10 ⁻¹⁰)	Factor of increase over spontaneous rate
	0	1	2	3, 4	5-15				
0	90	7.4	2.1	0.45	0.0	0.130	19.0	47.0	—
15	84	12	3.2	0.79	0.0	0.216	16.4	91.2	1.9
35	74	22	3.5	0.20	0.0	0.305	9.00	235	5.0
48	57	38	6.2	3.9	0.30	0.600	6.45	649	14
55	34	28	15	14	9.4	1.68	6.07	1910	41

* There were too many papillae/colony to measure at a concentration of 85 μg./ml.

Mutagenic activity of 4-nitro, 6-hydroxybenzimidazole

Table 2 shows the relationship between the concentration of NHB and the mutation rate of *Escherichia coli* Wp⁻ at the purine locus (loci) as determined by the papilla method. This method has been described by Ryan, Schwartz & Fried (1955). About 200 Wp⁻ bacteria were spread with a glass rod on the surface of 30 ml. minimal agar in Petri dishes containing 1 μg. guanine/ml., 0.5% (w/v) glucose and the compounds under study in appropriate concentrations. The suboptimal concentration of guanine limits the size of the colony so that it contains only 10⁷ bacteria. After 5 days, guanine-independent mutants visibly express their selective advantage over the guanine-dependent cells and form papillae which may be readily counted under low power with a binocular microscope. Papillae counts were made at 9 days. The total number of bacteria/colony was determined by suspending a colony having no papillae in 1 ml. of saline and counting the bacteria in a Petroff-Hausser bacteria counter. This total count was done for colonies of different sizes on control and experimental plates. To investigate the possibility that as many mutant bacteria occur on plates not containing the mutagen as on experimental plates but are expressed as papillae only in the presence of the analogue, several colonies from experimental and control plates containing no papillae were resuspended in saline and plated and found to contain no mutants.

Table 3 summarizes the mutagenic effect of NHB on *Escherichia coli* Wp⁻ under conditions which permit little or no cell division; in saline, minimal medium devoid of glucose, or long exposure in the stationary phase of growth. To determine induced mutagenesis in non-growing conditions, 5 × 10⁹ bacteria

were washed twice and suspended in 5 ml. of 0.85 % (w/v) saline or media devoid of glucose and aerated in test tubes on a roller at 37°. After the organisms were exposed to the compound, they were washed twice and plated in minimal and nutrient agar for a determination of the frequency of guanine-independent mutants. A sample of washed bacteria containing 10^8 organisms

Table 3. *The mutagenic effect of 4-NO₂-6-OH benzimidazole in Escherichia coli Wp⁻ under conditions which permit little or no cell division: in saline, minimal medium devoid of glucose and long exposure in the stationary phase of growth*

Conditions of exposure to mutagen	Concn. of NHB (μg./ml.)	Mean factor of increase over spontaneous purine independent mutant frequency	Mean factor of increase over shorter exposure
A. 18 hr. of logarithmic growth	40	5.0	.
B. 5 hr. of stationary phase exposure following 18 hr of logarithmic growth	40	4.0	.
B'. Condition B + 5 generations of intermediate cultivation	40	8.2	.
C. 28 hr. of stationary phase exposure following 18 hr. of logarithmic growth	40	4.7	1.2
C'. Condition C + 5 generations of intermediate cultivation	40	5.9	0.72
D. 14 hr. of exposure in 0.85 % saline	40	0.81	.
D'. Condition D + 5 generations of intermediate cultivation	40	1.7	.
E. 40 hr. of exposure in 0.85 % saline	40	1.6	2.0
E'. Condition E + 5 generations of intermediate cultivation	40	1.8	1.1
F. 48 hr. of exposure in media devoid of glucose	80	1.5	.
F'. Condition F + 11 generations of intermediate cultivation	80	0.30	.

was then inoculated into minimal medium supplemented with 65 μg. guanine/ml. wherein the bacteria underwent intermediate cultivation as indicated in Table 3. The bacteria were then washed twice and plated. The analogue had no mutagenic activity under non-growing conditions. There was no significant mutagenesis during long exposures to these conditions, even after intermediate cultivation in supplemented minimal medium which allows for the expression or recovery of mutants induced in the stationary phase. The compound, NHB, had a lethal effect in these non-dividing conditions. Induced mutagenesis may have occurred if an energy source had been made available to the bacteria; however, this was not tested. In a few experiments there was a great increase

in the spontaneous mutant-frequency in stationary conditions. This phenomenon could not be demonstrated in later experiments. Similar increases have been found in *Streptomyces* by Wainwright (1956) and in *E. coli* by Ryan (1955*b*).

Incorporation of compound NHB into DNA

NHB was not incorporated into the DNA of *Escherichia coli* Wp⁻. In one experiment chromatographic analysis was performed on a population consisting of over 99.9% mutants, the great majority of which were grown from mutants induced by the compound NHB. The bacteria that were analyzed for incorporation of the analogue were obtained in the following two ways:

(1) 10⁶ bacteria were inoculated into each of four 4 l. flasks containing 3 l. minimal medium supplemented with 65 µg. guanine/ml. In addition, three flasks contained 50 µg. 4-nitro-6-hydroxybenzimidazole/ml. The cultures were aerated by bubbling sterile air into the flasks. By restricting the glucose to 0.1% (w/v), the bacteria reached a concentration of 2 × 10⁸ viable organisms/ml. The cells were maintained in the stationary phase of growth for a period of 24 hr., washed twice and their DNA subjected to paper chromatographic analysis.

(2) The same technique was used as in (1) with these modifications: the growth of the bacteria was limited to 2 × 10⁶ organisms/ml. by the presence of only 2 µg. guanine/ml., so that induced mutants would have a selective advantage, overgrow and comprise the majority of the population. Because these organisms utilize glucose in the guanine-limited stationary phase, glucose was added to a concentration of 0.4% to enable the mutants to overgrow the guanine-dependent population. The frequency of guanine-independent mutants was determined by differential plating in minimal and nutrient agar each day. On the fourth day, the bacterial population exhausted the glucose from the medium while the control culture growing in the absence of NHB reached this secondary stationary phase at 5 days. Each culture was maintained in the stationary phase for 24 hr. and the DNA was isolated and analysed. The isolation of DNA and the chromatographic analysis was performed by Drs M. Engelman and S. Graff (Francis Delafield Hospital, New York) by the method of Smith & Wyatt (1951). NHB was not detected in the perchloric acid-hydrolysed DNA.

Antimutagenesis

The effects of DNA and ribonucleic acid, an acid and alkaline hydrolysate of each, ribotides and ribosides (including xanthosine and inosine) singly and in combination, some deoxyribosides, purines and pyrimidines and some of their precursors (including aminoimidazolecarboxamide and orotic acid), purine and pyrimidine analogues, indole, vitamin B₁₂, riboflavin, folic acid, *p*-amino-benzoic acid and other vitamins were tested as antagonists, in various concentrations to growth inhibition and mutagenesis by compound NHB. To determine the antimutagenic activity of these compounds, 10⁵ Wp⁻ bacteria were inoculated into 6 ml. of media, supplemented with 6 µg. guanine/ml. containing the antimutagen under study, in test tubes. After 5 hr. (lag phase

+c. 2 generations) compound NHB was added aseptically to a concentration of 40 $\mu\text{g./ml.}$ Periodic determinations of the turbidity were made in a colorimeter to study the annulment of growth inhibition by the test antimutagens. After 15 generations of growth under these conditions, the unwashed stationary-phase bacteria of each tube were plated in Petri dishes to which minimal 2% (w/v) agar with 100 $\mu\text{g. streptomycin/ml.} + 65 \mu\text{g. guanine/ml.}$ was added. The number of streptomycin-resistant colonies and the number of viable bacteria was determined in the usual manner. It was determined that compound NHB had no additional effect when carried over with the undiluted, unwashed bacteria into the streptomycin-resistant mutant assay plates. None of the compounds or mixtures reversed the growth inhibition or mutagenesis of the analogue although several, including 5-aminouracil and a vitamin mixture, stimulated bacterial growth considerably.

Antimutagenesis by amino acids. Complex media which included yeast extract, Proteose-peptone, and Casamino acids were then tested as antimutagens. They were found to be active in depressing (20- to 80-fold) the frequency of mutants induced by NHB. Before examining the antimutagenesis of amino acids, studies were made to determine whether the activity of complex media was due *solely* to the great decrease in generation time that occurred in the presence of these agents. It was found that growing the bacteria at 25° instead of 37° increases both the generation time and the frequency of induced mutants; however, this temperature had no effect on the marked antimutagenesis of Casamino acids or yeast extract even though the generation time of bacteria growing in the presence of compound NHB + antimutagen is extended from 35 to 90 min. at the lower temperature. At 15° the antimutagenic effect of complex media is also unchanged in spite of the fact that the generation time of the bacteria growing in the presence of compound NHB (+ antimutagen) is extended considerably. Although the effect of changes in temperature on growing cells is not clearly understood, these experiments indicated that something other than decreased generation time was involved in the high antimutagenic activity of complex media. Experiments utilizing various concentrations of Casamino acids and the mutagen also showed the antimutagenic activity to be relatively independent of a lower generation time; for example, while there is only a slight change in the growth rate brought about by a 1000-fold decrease in concentration of casein hydrolysate (from 20 mg./ml. to 20 $\mu\text{g./ml.}$) there is a marked decrease in antimutagenic activity. These findings, as well as the finding that 5-aminouracil and a vitamin mixture have no effect on mutagenesis even though they decrease the generation time considerably, led to a series of experiments which were designed to resolve the activity of hydrolysed casein into the activity of one or more of its constituent amino acids.

A mixture of 20 amino acids was as effective as casein hydrolysate in decreasing the mutagenic activity of compound NHB. The frequency of induced streptomycin-resistant mutants was decreased 20- to 30-fold. No single amino acid was found to be effective. Many concentrations and ratios were tested. Serine was the only indispensable amino acid. Only high concentrations of the

amino acids, of the order of 0.1–1.0 mg./ml., were effective. Combinations of amino acids that result in widely different generation times are antimutagenic to the same degree and combinations that result in the same high growth rate have diverse antimutagenic properties. Glutamic acid, for example, was as effective as all the amino acids combined in increasing the rate of growth; however, it had very little effect on the mutagenesis of NHB. None of the amino acid combinations nor complex media annulled the inhibitory activity of the analogue; furthermore, some combinations were inhibitory to growth.

Table 4. *The antimutagenic activity of amino acids on the mutagenesis of 4-NO₂-6-OH benzimidazole in Escherichia coli Wp⁻ as measured by the papilla method*

Amino acids and concentration in μM		Concentration of NHB (μM)	Purine ⁺ to purine ⁻ mean mutation rate/bacterium/gen. ($\times 10^{-10}$)	Mean factor of increase over spontaneous rate	Mean factor of decrease of induced mutation rate
0	—	0	108	.	.
{ Glutamic acid 6800 Serine 8550 Methionine 3850 }		0	89.2	0.81	.
{ Glutamic acid 13600 Serine 17100 Alanine 10100 Tryptophan 2820 }		0	139	1.3	.
0	—	224 (40 $\mu\text{g./ml.}$)	1310	12	.
{ Glutamic acid 6800 Serine 8550 Methionine 3850 }		224	92.3	0.86	14
{ Glutamic acid 13600 Serine 17100 Alanine 10100 Tryptophan 2820 }		224	493	4.6	2.7

Table 4 summarizes the effect of two amino acid combinations on the NHB-induced mutation rate. The amino acid combinations did not decrease the spontaneous mutation rate as measured directly by the papilla method nor did they, as separate experiments show, affect the frequency of spontaneous guanine-independent and streptomycin-resistant mutants in liquid cultures.

The antimutagenic activity of the two amino acid combinations in the presence of the mutagen was then determined. At high concentrations of NHB (60–80 $\mu\text{g./ml.}$) there was very little reversal by the amino acids. At the lower concentrations of the mutagen the results obtained are consistent with results that would be expected in a competitive system. However, the non-linearity of the antimutagenic effect of different concentrations of the amino acids obtained at one mutagen concentration, and the non-linearity of the mutagenic effect of increasing mutagen concentrations at one amino acid concentration, indicated that this is an extremely complex system, not easily subjected to competitive reversal analysis. The degree of reversal of induced

mutagenesis was found to be independent of the molar ratios of mutagen to antimutagen at most concentrations. Although 40 $\mu\text{g./ml.}$ of the mutagen increased the nutritional mutant frequency 44-fold, and elevated the streptomycin-resistant mutant frequency 290-fold, the factor of depression of the induced mutant frequency by the amino acid combinations was almost identical for each locus. This is true for almost all concentrations.

Pre- and post-treatment experiments with amino acids. The pre- and post-treatment effects of amino acids on the mutagenesis by the analogue are indicated in Table 5. The concentration of NHB used in all pre- and post-treatment experiments was 35 $\mu\text{g./ml.}$; the amino acid concentrations were L-glutamic acid, 1000 $\mu\text{g./ml.}$, DL-serine, 900 $\mu\text{g./ml.}$, and DL-methionine, 575 $\mu\text{g./ml.}$ Minimal media was supplemented with 65 $\mu\text{g.}$ guanine/ml.

Table 5. *The effect of amino acids* on the frequency of chemically-induced mutants before and after treatment with 4-NO₂-6-OH benzimidazole in Escherichia coli Wp⁻*

I. Pre-treatment experiment

E. coli Wp⁻ was grown in supplemented minimal medium with and without amino acids (pre-treatment) and then grown in media described below (subsequent treatment):

Subsequent treatment	Mean mutant frequency $\times 10^{-8}$ /bacterium purine ⁻ to purine ⁺	
	Pre-treatment	
	Minimal medium	MM. + amino acids
Minimal medium (MM)	2.3	3.0
MM + amino acids	4.0	3.9
MM + 35 $\mu\text{g.}$ NHB/ml.	200	190
MM + amino acids + 35 $\mu\text{g.}$ NHB/ml.	67	67
Cells washed; then MM	2.5	2.5
Cells washed; then MM + 35 $\mu\text{g.}$ NHB/ml.	330	230

II. Post-treatment experiment

E. coli Wp⁻ was grown in MM with and without NHB (treatment) and then grown in MM with and without amino acids (post-treatment):

Treatment (5 generations)	Post-treatment	
	Minimal medium	MM + amino acids
	(a) in agar (unsupplemented)	
Minimal medium	7.5	7.0
MM + 35 $\mu\text{g.}$ NHB/ml.	680	660
	(b) in liquid (5 gen.)	
Minimal medium	10	9.3
MM + 35 $\mu\text{g.}$ NHB/ml.	180	260

* Amino acids = glutamic acid 1000 $\mu\text{g./ml.}$; serine, 900 $\mu\text{g./ml.}$; methionine, 575 $\mu\text{g./ml.}$

In pre-treatment experiments 5×10^4 bacteria were introduced into 5 ml. of minimal medium and of minimal medium plus amino acids and grown for 15 generations. When the bacteria reached a population size of 10^8 bacteria/ml. (3 generations before saturation) they were subsequently treated as indicated in Table 5. The inoculum introduced into tubes containing 5 ml. of treatment media was 5×10^6 bacteria. When these bacteria reached the stationary phase of growth, mutant frequencies were determined. In post-treatment

experiments an inoculum of 10^5 guanine-dependent cells was introduced into 5 ml. of treatment media (see Table 5) and grown for 10 generations under standard conditions. Three generations before saturation some of the logarithmic-phase bacteria were plated in minimal agar and minimal agar containing amino acids after the cells were washed. Approximately 8×10^7 bacteria of this washed suspension were inoculated into 8 ml. of supplemented minimal medium and supplemented minimal containing amino acids, wherein the bacteria grew for 5 generations to stationary phase. The stationary-phase cells were then washed once and plated to determine the frequency of guanine-independent mutants. Growing the bacteria in amino acids had no effect on the subsequent mutagenesis of the analogue or on the subsequent antimutagenesis of amino acids, nor did the presence of amino acids in liquid or agar after treatment with the mutagen have any effect on the mutagenic activity of the analogue. Under the conditions tested, the antimutagenic effect of the amino acid combinations occurred only when they were present simultaneously with the mutagen.

If the effect of pre-treatment is solely on the very early generations in the treatment media containing the analogue, it would not be easily detected in the present experiments because the majority of the mutants are induced in the terminal generations of growth. In view of the findings of Witkin (1956), Ryan (1954, 1955*a*) and Ryan, Fried & Schwartz, (1954) the number of generations in post-treatment media may not be as critical as the rate of growth in the first generation after treatment. If post-treatment effects can only be expressed when they are applied before a bacterial division occurs as is the case with photo-reactivation and some chemical post-treatment effects on ultra-violet light, their detection in the present system would be improbable since the reversal would affect only one generation (the last) of induced mutants. However, it should be noted that in treatment medium as many divisions (and consequently as many mutations) occur in the last division as in the sum of all the preceding generations.

The amino acids do not appear to act by forming a complex with the analogue without the intervention of a biological reaction as in the instance of nucleic acid-binding acriflavine, and thus interfering with the latter's mutagenic activity in *Escherichia coli* (Witkin, 1950). The antimutagenic effect of amino acids is no greater if the cells are inoculated into tubes in which the analogue and the amino acids have been incubated together at 37° for 25 days or if the cells are inoculated into tubes containing the analogue and the amino acids are added after 5 hr. of growth. Further evidence against simple chemical binding is the fact that the antimutagenic effect of amino acids is strain specific and does not occur in B/r,t, where in some experiments the amino acids actually enhance mutagenesis.

Antimutagenesis by analogues of compound NHB. The antimutagenic effect of 4-hydroxy-6-nitrobenzimidazole and 4-hydroxy-6-nitrobenzotriazole on the mutagenesis of NHB in liquid cultures is summarized in Table 6. Neither of these compounds is mutagenic and both inhibit the growth of the bacteria. The spontaneous mutant-frequency is not affected by 4-hydroxy-6-nitro-

benzotriazole, yet the compound depresses the induced mutant frequency significantly ($P=0.001$ for each marker both before and after intermediate cultivation for 6 generations) in minimal medium supplemented with guanine. Other analogues of NHB in addition to 4-hydroxy-6-nitrobenzimidazole, which had no effect on the NHB-induced mutant frequency (not indicated in tabular form) are: 2:4-dihydroxy-6-nitrobenzimidazole, 2-methyl-4-hydroxy-6-nitrobenzimidazole, 4-nitro-6-methoxybenzimidazole, 2-ribotyl-4-nitro-6-methoxybenzimidazole, 4-nitro-6-ethoxybenzimidazole and 5-nitro-7-methoxyquinoxaline.

Table 6. *The antimutagenic effect of two analogues of 4-NO₂-6-OH benzimidazole in Escherichia coli Wp⁻; 4-OH-6-NO₂ benzimidazole and 4-OH-6-NO₂ benzotriazole*

Test antagonist*		None			4-OH-6-NO ₂ benzimidazole			4-OH-6-NO ₂ benzotriazole		
		196 μ M (35 μ g./ml.)			559 μ M			695 μ M		
Marker	Time of plating	Spont.	NHB induced	Mean factor of increase	NHB	NHB	Mean factor of decrease	NHB	NHB	Mean factor of decrease
		m.f.† (a)	m.f.† (b)	(b/a)	m.f.†	m.f.† (c)	(b/c)	m.f.†	m.f.† (d)	(b/d)
Purine p ⁻ to p ⁺	Zero	9.3	953	103	46	442	2.2	25	173	5.5
	After intermediate cultivation	18	4190	320	14	3430	1.2	7.0	975	4.8
Streptomycin S ^s to S ^r	Zero	0.25	137	550	1.6	28.4	4.8	0.25	14.8	9.2
	After intermediate cultivation	1.0	205	205	0.80	254	0.80	4.0	32.4	6.3

* Antagonist was added to growing bacteria in liquid cultures 3 hr. before addition of NHB.

† m.f. = mean mutant frequency $\times 10^{-9}$ /bacterium.

Mutagenesis and antimutagenesis by some compounds related to nucleic acid metabolism

The mutagenic activity of several inhibitors and inhibitor antagonists on 5 loci in 5 mutants of *Escherichia coli* are summarized in Table 7. The bacteria grew in the presence of these compounds for 15 generations; the exposure to the chemicals was continued for 24 hr. in stationary phase conditions; this was followed by a period of intermediate cultivation for 5 generations, washing in saline, and plating for the determination of the mutant frequency. NHB, 5-nitroquinoxaline, and 5-aminouracil are general in their effect. *E. coli* 15h⁻ appears to be the most mutagen-stable, while B/r, t is the most mutagen-sensitive. There is no correlation between the spontaneous mutant frequency and the factor of increase of a single compound from one strain to another; for example, NHB has no effect on *E. coli* 15m⁻, although the spontaneous mutant frequency is approximately 10^{-5} mutants/bacterium. There are no major differences in response of these strains to the inhibitory action of these compounds. Bacterial growth is not inhibited by 5-aminouracil but it is a potent mutagen.

Table 7. *The mutagenic activity of several inhibitors and antagonists to inhibition in 5 mutants of Escherichia coli**

Strain:	...	...	...	Wp ⁻	15h ⁻	15h ⁺ m ⁻	B/r,m ⁻	B/r
Marker tested:	...	...	...	S ^s to S ^r	h ⁻ to h ⁺	m ⁻ to m ⁺	m ⁻ to m ⁺	t ⁻ to t ⁺
Spontaneous mutant frequency × 10 ⁻¹⁰ /bact.:	...	...	...	3	8400	90,000	10	20
Compound	(μg./ml.)	Effect on growth†	Induced factor of increase over spontaneous mutant-frequency‡					
2,6-Diaminopurine	250	++	4	0.76	1.6	79	3	
	325	+++	.	.	2.7	.	.	
6-Chloropurine	400	-	0.8	0.50	1.3	2	1	
	700	-	.	.	2.2	.	.	
2-Thioadenine	250	-	3	0.89	1.2	1	1	
6-Mercaptopurine	100	+++	.	.	7.8	.	1	
	150	++++	.	0.5	.	.	.	
Dithio-6-oxypurine	1000	-	.	.	1.7	.	2	
4-Amino, 5-imidazole- carboxamide	500	-	4	0.80	1.1	1	.	
8-Azaguanine	500	+	1	.	2.1	1	1	
Caffeine	250	-	.	.	1.6	.	1	
	500	-	.	.	.	.	6	
Theophylline	500	-	.	.	2.1	1	1	
Benzimidazole	300	+++	1	1.1	.	.	14	
	800	++++	.	.	0.50	1	31	
4-NO ₂ -6-OH—	40	+	370	0.60	.	.	26	
Benzimidazole	80	++	.	4.7	1.6	10	37	
5-Aminouracil	300	-	7	.	.	.	6	
	500	-	.	1.6	7.0	.	.	
	700	-	.	.	14	2	48	
2-Amino-4-methyl- pyrimidine	1000	-	.	.	1.5	.	1	
Barbituric acid	1000	-	.	.	1.1	.	.	
Uric acid	1000	-	.	.	1.1	.	.	
Thiouracil	1000	+	.	.	1.4	.	1	
Aminopterin	100	++++	.	0.43	0.82	1	1	
Ca leucovorin	1000	-	1	.	1.3	.	.	
5-Nitroquinoxaline	15	++	.	.	4.6	.	.	
	20	++	7	0.41	41	.	.	
	25	+++	.	.	59	47	19	
Sulphanilamide	2000	++++	.	0.45	1.5	.	.	
Sulphathiazole	500	++++	1	.	.	.	.	
2-Chloro-4-aminobenzoic acid	500	+	.	.	.	.	2	
p-Aminobenzoic acid	1000	-	1	0.60	0.91	.	1	

Figures in black show greater than 4-fold mutagenic activity.

* Abbreviations: p, purine; S, streptomycin; h, histidine; m, methionine, t, tryptophan; -, dependence; +, independence; s, sensitivity; r, resistance.

† See Table 1 for definition of degree of inhibition.

‡ Mutant-frequencies were determined after: (a) growth of bacteria in liquid cultures containing test mutagen; (b) continued long exposure of bacteria in stationary phase of growth to test mutagen; (c) intermediate cultivation in appropriately supplemented minimal medium.

The effects of several compounds on the mutagenesis on NHB, benzimidazole, 5-nitroquinoxaline, 5-aminouracil, and 6-mercaptopurine are summarized in Table 8. For example, in the presence of 300 μg. 5-aminouracil/ml. the induced frequency of purine-independent mutants in *E. coli* Wp⁻ is 173×10^{-9} /bacterium. This is a 17-fold increase over the spontaneous mutant

frequency of 10.2×10^{-9} /bacterium. In the presence of an amino acid mixture and 5-aminouracil the mean mutant-frequency is 127×10^{-7} /bacterium—only 1.4-fold lower than the induced mutant frequency in the absence of amino acids. The antimutagenic activity of the combination of glutamic acid, serine, and methionine is strain specific; they do not depress the mutagenic activity of NHB and 5-nitroquinoxaline in any strain other than Wp⁻. Furthermore, the amino acids have no effect on the mutagenic action of 5-aminouracil in *Escherichia coli* Wp⁻. In all cases, the amino acids stimulated growth considerably. The mutagenesis of 5-aminouracil cannot be reversed by any of the compounds or mixtures employed in these studies. Purines and purine ribosides reverse the growth inhibition and the mutagenic activity of 6-mercaptopurine; furthermore, the depression in the frequency of mutants is retained through 5 generations of intermediate cultivation in liquid medium. Folic acid does not affect the inhibition or mutagenesis of 6-mercaptopurine. 6-Mercaptopurine was found to be extremely inhibitory in the presence of low concentrations of amino acids. Although the inhibitory activity of benzimidazole is reversed by guanosine and adenosine in *E. coli* B/r, t there is no reversal of the mutagenesis of this compound. None of the test antagonists have any effect on the spontaneous mutant-frequency. This is also true for all the compounds tested as antagonists to the mutagenesis of NHB.

DISCUSSION

The analogues active as growth inhibitors in *Escherichia coli* are, in general, the same as those which were found to be inhibitory to developing *Rana pipiens* embryos by Liedke, Engelman & Graff (1954, 1955). An exception is NHB which does not affect the embryos. Loveless, Spoerl & Weismann (1954) have shown that 4-hydroxy-6-nitrobenzotriazole specifically inhibits cell division in yeast and *E. coli*; the proposed mechanism of inhibition is through the formation of a carbonium ion. This may be true for other related compounds employed in the present studies. Although Woolley (1944) has shown that benzimidazole inhibition can be reversed by adenine or guanine, there are several other investigations in which nucleic acid constituents failed to reverse benzimidazole inhibition (Kushner & Pascal, 1955; Pollock, 1947; Roblin *et al.* 1945). Novick & Szilard (1952), using high concentrations of ribosides, could not decrease by more than one-third the mutagenesis by 150 µg. benzimidazole/ml.

Because induced mutagenesis was found to occur only under growing conditions, a mechanism involving the selection of pre-existing mutants by the analogue seemed likely; however, the papilla method of determining the mutation rate demonstrates that the action of NHB results in a greater number of mutational *events* rather than simply a greater number of mutants. Ryan *et al.* (1955) have shown that NHB increases the mutation rate at the histidine locus fourfold as measured by the papilla method in *Escherichia coli* 15, h⁻. The fact that there is an increase in streptomycin-resistant mutants without any detectable killing or growth inhibition in liquid cultures suggests

Table 8. *The effect of various compounds on the mutagenesis by 4-NO₂-6-OH benzimidazole, benzimidazole, 5-nitroquinoxaline, 5-aminouracil, and 6-mercaptopurine in 3 strains of Escherichia coli*

(1) = Strain (2) = Marker (3) = Mean spontaneous mutant frequency × 10 ⁻⁹ /bact. (a)	Mutagen	Concn. (μg./ml.)	Mean induced mutant frequency (× 10 ⁻⁹) (b)	Mean factor of increase (b/a)	Test antimutagen*	Concn. (μg./ml.)	Mean mutant frequency × 10 ⁻⁹ /bact. mutagen + antagonist (c)	Mean factor of decrease (b/c)									
(1) Wp ⁻ (2) p ⁻ to p ⁺ (3) 10-2, 29-0	5-Aminouracil	300	173	17	aa†	.	127-0	1-4									
	5-Nitroquinoxaline	20	495	17	aa	.	68-0	7-3									
(1) 15h-m ⁻ (2) m ⁻ to m ⁺ (3) 122	5-Nitroquinoxaline	15	7240	59	aa	.	4500	1-6									
	6-Mercaptopurine	90	2410	20	Folic acid	100	5060	1-4									
250						61-0	40										
250						231	10										
250						111	22										
83						238	10										
(3) 59-0	Guanine	250	41-0	83	250	127	2660	0-90									
									83	19							
									100	2580	0-81						
									Folic acid	aa	250	780	1-0				
														Guanosine	Thymidine	Uridine	Cytidine
	250	617	1-3														
				250	498	1-6											
							250	498									

(1) B/r, t	5-Aminouracil	750	558	110	aa	.	697	0.80
(2) t ⁻ to t ⁺					{Guanosine}	500	354	1.6
(3) 4.95-7.05					Adenosine			
					Thymidine	800	428	1.3
					Uridine	800	350	1.6
					Cytidine	800	437	1.3
		300	44.0	6.3	Thymidine	1000	118	0.37
					Uridine	1000	94.1	0.47
					Cytidine	1000	94.4	0.47
	Benzimidazole	200	96.1	14	aa	.	65.2	1.5
					Vit. B ₁₂	16	184	0.52
					{Guanosine}	500	126	0.76
					Adenosine			
	4-NO ₂ -6-OH	75	261	37	aa	.	344	0.76
	Benzimidazole	75	402	80	aa	.	620	0.65
		40	215	31	aa	.	1170	0.18
		40	129	26	aa	.	119	1.1
		15	70.4	14	aa	.	33.1	2.1
		75	261	37	Vit. B ₁₂	16	242	1.1
					{Guanosine}	500	549	0.48
					Adenosine			

* Test antimutagen was added to growing cells in liquid cultures 3 hr. before addition of mutagen. When the bacteria subsequently reached the stationary phase of growth they were washed and the frequency of prototrophs was determined by plating in minimal and nutrient agar.

† aa: L-glutamic acid, 1000 μ g./ml.; DL-serine, 900 μ g./ml.; DL-methionine, 575 μ g./ml.

that the analogue is not acting by decreasing the proportion of parental cells. Even at concentrations of the compound which kill, there is an *absolute* increase in the number of mutants. In addition, mutant frequencies of six different markers have been shown to be affected by NHB. Several different mechanisms would have to be postulated for the selective action of NHB against amino acid-requiring strains, purine-requiring strains, streptomycin-sensitive strains and selection for streptomycin-resistant organisms; on the other hand, the single mechanism of increased genetic instability could account for these results. Moreover, in the presence of NHB, auxotrophs, prototrophs, streptomycin-resistant and -sensitive cells grow, independently, at the same rate. Thus the action of NHB is thought to involve a mechanism whereby the rate of mutation is increased.

The action of NHB is very different from that of the mustards and most other mutagens. Because of the elimination of many selection problems and lack of clonal variation in conditions of exposure in stationary phase, almost all mutagen-screening studies have been made with non-growing bacteria. If the same techniques were employed in the present studies, the mutagenic activity of the compounds would not have been detected. This may imply that these compounds, as well as caffeine and theophylline, do not act via a free radical mechanism or that they have a unique mode of action since formaldehyde, peroxides, the mustards, acriflavin and the Mn ion act on stationary populations. These findings are consistent with those of Lee (1953), who has shown that caffeine and theophylline are not mutagenic at very long generation times.

Analogues of nucleic acid constituents possessing conjugate bond stability, and which do not inactivate Transforming Principle (Zamenhof, Leidy, Hahn & Alexander, 1956), more likely act by interfering with DNA synthesis. Because benzimidazole is known to inhibit the activity and synthesis of several enzymes, it is possible that benzimidazole derivatives also inhibit enzymes concerned with nucleic acid synthesis. This seems likely in view of the studies of Koch & Lamont (1956) who have shown that caffeine, theophylline and theobromine (as well as some natural bases) inhibit some enzymes involved in nucleic acid metabolism.

If there is any incorporation into DNA of compound NHB at all, it is far less than the incorporation found for 5-bromouracil (Zamenhof & Griboff, 1954) and 6-methyladenine (Dunn & Smith, 1955) in *Escherichia coli* and for 8-azaguanine in the RNA of *Bacillus cereus* (Mathews & Smith, 1956). The studies of Koch (1956) have shown that radioactive caffeine and theophylline are not incorporated into bacterial DNA. They failed to detect (as did the present studies) incorporation of these analogues into analogue-induced mutants.

Although their ability to decrease the generation time may not be solely responsible for the antimutagenesis of amino acids, the effect on the growth rate may contribute to decreasing the efficiency of the action of the mutagen. The antimutagenic activity of amino acids may be due to the role they play in the synthesis of purines and pyrimidines whereby they could supply the

bacteria with 'correct' nucleic acid constituents. The simplest amino acid combination (serine, glutamic acid and methionine) found empirically to be the most effective antimutagen, contains amino acids which have been shown to be actively concerned with the synthesis of purines and pyrimidines (see Reichard, 1955, and Schlenk, 1955). For example, glutamic acid may contribute to the amino group on pyrimidines and to N₁ of the ring. Serine takes part in the formation of C₂, C₄, C₈ and N₇ of purines and the methyl group of thymine. Methionine can act as a methyl donor in the conversion of aminoimidazolecarboxamide to purines. NHB may interfere with vitamin B₁₂ activity (this vitamin contains 5-6-dimethylbenzimidazole and is involved in thymine synthesis). Although vitamin B₁₂ had no effect on the activity of NHB, methionine, a methyl donor which can substitute for vitamin B₁₂ in many reactions, may be involved in this system. Vitamin B₁₂ can reverse substituted benzimidazole inhibition in a B₁₂- or methionine-requiring mutant of *Escherichia coli* strain W (Scott, Rogers, Rose & Chu, 1957).

The mechanism of the reversal of the mutagenic activity of 5-nitroquin-oxaline by amino acids (see Table 7) is subject to similar speculation. Folic acid and the citrovorum factor are involved in single carbon unit metabolism and are concerned with the synthesis of formate (a purine and pyrimidine precursor) from glycine and serine; therefore, the addition of exogenous serine may circumvent the inhibited reaction. It is also possible that glutamic acid, together with *p*-aminobenzoic acid (*p*-AB), acts as a trap for the pterin analogue, since, normally, *p*-AB, glutamic acid and pterin form folic acid.

Adding 'ready made' amino acids may increase protein synthesis; this may then lead to a number of changes which could affect the mutation process, such as an increase in the synthesis of enzymes involved in DNA duplication that may be blocked due to the possible ability of NHB to interfere with RNA synthesis which, in turn, appears to be necessary for protein synthesis. If NHB were interfering with the *action* of DNA-synthesizing enzymes as caffeine does in Koch's studies, then increased enzyme synthesis could antagonize this inhibition. Witkin (1956) has found that the mutant frequency of ultraviolet-induced tryptophan mutants is a function of the concentration of amino acids in the mutant-assay plates before the first bacterial division occurs. Witkin considers one possible explanation to be that increased protein synthesis leads to genetic repair of mutant cells that would normally die in absence of enrichment. If increased protein synthesis results in reversal of genetic alteration (reversal of mutation) as well as reversal of genetic killing of potential mutant cells, the results obtained in the present work may be reconciled with Witkin's findings, in spite of the fact that a decrease rather than an increase in the mutant frequency is obtained. The repair in Witkin's instance, may, however, be repair of the physiological damage to bacteria that have received ultraviolet irradiation; these are the organisms from which the mutants arise. It should be noted that the amino acids did not decrease the frequency of NHB-induced mutants in *Escherichia coli* B/r,t, the same strain in which Witkin found an increase in ultraviolet-induced prototrophs with amino acid post-treatment; furthermore, in the present system there is little killing by the mutagenic agent.

The absence of an effect of the amino acids on the spontaneous mutant frequency or mutation rate indicates that a selection mechanism is not involved in the antimutagenesis of amino acids, and suggests that spontaneous mutations may not be caused by benzimidazole-like compounds in the cell. The amino acids actually decrease the number of mutational events induced by NHB as has been demonstrated by the papilla method. These properties of the amino acid combination, in addition to their equal effect on the growth rate of mutant and parental cells, argue against a selection mechanism. The results obtained in pre- and post-treatment experiments also support an antimutagenesis mechanism; for if amino acids selected against induced or spontaneous mutants, pre- and post-treatment with amino acids would have had an effect on the frequency of mutants. Furthermore, a single concentration of amino acids does not result in a decrease of a constant number of induced mutants. The antimutagenesis of amino acids depends, to a limited degree, on the concentration of NHB (fewer mutants are annulled at lower concentrations of NHB).

None of the non-mutagenic analogues are as effective as 4-hydroxy, 6-nitro-benzotriazole in reducing the 4-nitro-6-hydroxybenzimidazole induced-mutant frequency. The antimutagenesis does not appear to be the result of inhibition alone since several other analogues which have similar inhibitory properties have no antimutagenic effect. Although studies on competitive reversal of induced mutagenesis were not pursued, an attractive hypothesis for the action of 4-hydroxy-6-nitrobenzotriazole is that it replaces NHB at certain sites in the cell.

Novick & Szilard (1951) and Novick (1955) found none of the pyrimidine analogues they tested to be mutagenic in *Escherichia coli* B/1, t at the T5- and T6-resistant loci; nevertheless, 5-aminouracil has been shown in the present studies to be an effective mutagen at several loci including the tryptophan locus of B/r, t. This is extremely interesting in view of Dunn & Smith's (1955) finding that a new purine, 6-methyladenine, replaces thymine in the presence of 5-aminouracil in *E. coli*. The mutagenesis of 5-aminouracil in these investigations suggests that the inability of pyrimidine analogues to act as mutagens in Novick & Szilard's studies was not due to the impermeability of the cell to the compounds as has been suggested by Marshak (Novick & Szilard, 1951). On the other hand, Novick & Szilard found that theophylline affects the T5 and T6 locus of B/r, t⁻, whereas theophylline has no effect on the tryptophan locus in the present studies. This demonstrates the strain and locus specificity of mutagenic reactions. Consistent with the present findings are the results of Duncan & Woods (1953) who have shown that the effect of 5-aminouracil on chromosome breakage could not be reversed by thymine.

Although Novick & Szilard found no mutagenic activity for the components of the ribosides that were active antimutagens for caffeine and theophylline, in these studies the bases adenine and guanine, which annulled the growth inhibition of 6-mercaptapurine in these and other investigations (Elion, Hitchings & Vanderwerf, 1951), also annulled the mutagenic activity of the antimetabolite. This annulment by purine and pyrimidine bases indicates

that the antimutagenic activity of ribosides is not due to the action of their ribose component as a trap for mutagenic purine and pyrimidine bases as Kalckar (1954) has suggested. The mechanism of reversal may be more simple and direct than that proposed for the antimutagenic activity of amino acids.

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