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PREVENTION OF THE INHIBITORY EFFECTS OF URETHAN, FORMAMIDE, AND N-METHYLFORMAMIDE ON THE GROWTH OF *ESCHERICHIA COLI**

G. P. WHEELER and MARGARET G. GRAMMER

Kettering-Meyer Laboratory,† Southern Research Institute, Birmingham, Alabama

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Abstract—Purines, pyrimidines, ribonucleosides, ribonucleotides, carboxylic acids, and amino acids were tested for activity in preventing the growth-inhibition of *Escherichia coli* by urethan, formamide and N-methylformamide. 2 : 6-Diaminopurine and phenylalanine were the only compounds tested that caused true reversal of the inhibition of growth of *E. coli* 9637 by these agents, but neither of these compounds re-reversed the inhibition of a purine-requiring mutant or, in experiments with urethan, the inhibition of a pyrimidine-requiring mutant. On the other hand, glutamic acid caused true reversal of the inhibition of the purine-requiring mutant and, in experiments with urethan, of the inhibition of the pyrimidine-requiring mutant, but the apparent reversal of the inhibition of *E. coli* 9637 by glutamic acid was found to be due to stimulation of the overgrowth of drug-resistant mutants. A number of other compounds also stimulated the overgrowth of drug-resistant mutants in overnight growth experiments.

Urethan, formamide and N-methylformamide, individually, inhibited the formation of diazotizable amine by resting *E. coli* B96 cells. The inhibition by urethan was partially prevented by phenylalanine; the inhibition by formamide was partially prevented by glutamic acid and by 2 : 6-diaminopurine; and the inhibition by N-methylformamide was partially prevented by 2 : 6-diaminopurine.

The data indicate that there are probably multiple sites of action for each of these inhibitors.

INTRODUCTION

THE anti-leukemic action of urethan,[1] formamide,[2] and N-methylformamide[2] has been known for a number of years. Although the mutagenic, antimitotic, and carcinogenic effects of the agents and the activities of several compounds in preventing these effects point to an influence of each agent on the biosynthesis, metabolism, or funtioning of nucleic acids,[3–11] the mechanisms of action are still unknown. However, the results of an earlier study[11] did indicate that the mechanisms of inhibition by these compounds might be closely related. Therefore, the three were studied together in the present investigation, which was undertaken to obtain information on the sites of inhibition of the growth of *Escherichia coli*. The roles of these agents in inhibiting growth of bacteria may, of course, be different from their roles in influencing the metabolism of mammalian cells; however, it was hoped that the results of studies with bacteria might serve as a guide for studies with mammalian cells.

METHODS

The basal medium for the experiments with *E. coli* 9637, the drug-resistant mutants

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of *E. coli* 9637, and the purine-requiring mutant, *E. coli* B96, was that of Davis and Mingioli.[12] For the drug-resistant mutants this basal medium was supplemented with the respective inhibitor, and for the B-96 mutant the basal medium was supplemented with hypoxanthine at a level of 20 μg/ml. The pyrimidine-requiring mutant of *E. coli*, ATCC 9723 g, was grown on the basal medium of Yates and Pardee[13] supplemented with uracil at a concentration of 22 μg/ml. The inocula were prepared by adjusting a 24 hr culture to a transmission of 26 per cent using a Spectronic "20" colorimeter at 660 mμ and diluting the resulting solution tenfold with physiological saline; 0·1 ml of this dilute suspension was used to inoculate 10 ml of medium. Incubation was at 37° C, and percentage transmission was measured by means of a Bausch and Lomb Monochromatic Colorimeter using a 660 mμ interference filter or by means of a Bausch and Lomb Spectronic "20" colorimeter with a wavelength setting of 660 mμ.

Experiments related to the accumulation of 4-amino-5-imidazolecarboxamide by non-proliferating *E. coli* B96 cells were performed by a procedure essentially the same as that of Gots and Love.[14] Following growth in the presence of hypoxanthine, the cells were collected by centrifugation, washed three times with 0·067M phosphate buffer, pH7·2, and suspended in 0·067M phosphate buffer at pH7·2 to which glucose, 360 μg/ml, and the test compounds had been added. After incubation of the suspension for 16 hr, the metabolic reactions of the non-proliferating cells were terminated by the addition of trichloroacetic acid, and the concentration of diazotizable amine was determined by a modification of the method of Bratton and Marshall.[15]

In some experiments, the test compounds were added when the bacteria were in the exponential phase of growth, and the subsequent rate of multiplication was followed turbidimetrically. For the inoculum in these experiments, the cells from 20 ml of a 24 hr culture of the bacteria were collected by centrifugation, washed once with saline, and suspended in 3 ml of saline. A sufficient volume of this suspension was added to 10 ml of medium in a 300 ml Erlenmeyer flask to give a transmission of 65–70 per cent. Determination of transmission at various periods was facilitated by fitting the flask with a standard culture tube by means of a standard taper ground glass joint; the contents of the flask were transferred to the tube by inverting the flask. The flask and contents were incubated, and the turbidity was determined at intervals of 15 or 30 min. Soon after the rate of growth had entered the exponential phase, the inhibitor and candidate reversal agents were added to the flasks, and periodic determinations of turbidity were continued. The growing cultures were not agitated or continually aerated while they were in the incubator, but the relatively large surface area of the medium in the flask and the necessary inversion of the flask for determination of turbidity caused considerable aeration.

In some of the experiments it was desirable to determine if the cells that eventually grew in the inhibited culture and the cells that grew in the presence of the inhibitor plus the test compound were less sensitive to the inhibitor than the initial culture. For these determinations, the cells were washed with saline and used to inoculate tubes of medium containing various concentrations of inhibitor. The extent of growth was measured after overnight incubation.

RESULTS

The data of Table 1 show the degree of inhibition of the growth of *E. coli* 9637 by rather large quantities of formamide, N-methylformamide or urethan and the extent

TABL 1. GROWTH OF *E. coli* 9637 IN THE PRESENCE OF INHIBITORS AND GLUTAMIC ACID, PHENYLALANINE, OR 2 : 6-DIAMINOPURINE

Test compound	Conc. (μmoles/ml)	Growth, % of control			
		Formamide*	N-Methyl-formamide†	Urethan‡	
				Expt. 1	Expt. 2
None	—	6	8	2	2
Glutamic acid	5	106 (A)§	54 (D)	118	—
	15	—	—	—	110 (G)
Phenylalanine	5	94 (B)	62 (E)	20	54 (H)
2:6-Diaminopurine	0·033	79 (C)	51 (F)	24	—
	0·1	77	38	43	—
	0·2	62	33	48	—

* Formamide was present at a concentration of 17·5 mg/ml.
† N-Methylformamide was present at a concentration of 20 mg/ml.
‡ Urethan was present at a concentration of 10 mg/ml.
§ The letters in parentheses are used to indicate which cultures were used for inocula in the tests of resistance reported in Table 2.

of growth that occurred when glutamic acid, phenylalanine, or 2 : 6-diaminopurine was present in addition to the inhibitor. The data of Table 2, however, show that the cells which grew in the presence of inhibitor and glutamic acid were somewhat resistant to the inhibitor, while those which grew in the presence of inhibitor and

TABLE 2. TESTING OF CULTURES OF TABLE 1 FOR DRUG RESISTANCE

Inhibitor	Concentration (mg/ml)	Growth, % of controls			
		Fresh culture	A*	B	C
Formamide	0	100	100	100	100
	12·5	13	87	11	12
	15·0	3	87	2	3
	17·5	0	76	0	1
	20·0	0	66	0	0
		Fresh culture	D	E	F
N-Methylformamide	0	100	100	100	100
	17·5	4	45	2	4
	20·0	1	36	0	1
	22·5	0	26	0	0
	25·0	0	19	0	0
		Fresh culture	G	H	
Urethan	0	100	100	100	
	7·5	1	91	1	
	10·0	0	64	0	
	12·5	0	34	0	
	15·0	0	12	0	
	17·5	0	3	0	
	20·0	0	0	0	

* The letters indicate the cultures of Table 1 that were used as inocula.

phenylalanine or 2 : 6-diaminopurine were as sensitive to the inhibitor as cells that had never been exposed to the inhibitor. The possibility that the growth was due to the presence of glutamic acid that was carried along with the cells of the inoculum seems unlikely because of the small size of the inoculum. Also, the cells that grew in the presence of inhibitor and glutamic acid were about equally as resistant to the inhibitor as cells that were exposed to the inhibitor alone and which proliferated during a long period of incubation. These results are interpreted to indicate that glutamic acid stimulated the overgrowth of resistant cells, while phenylalanine and 2 : 6-diaminopurine caused true prevention of the inhibition. In Table 3 data are given which show

TABLE 3. GROWTH OF *E. coli* 9637 IN THE PRESENCE OF INHIBITORS AND COMBINATIONS OF PHENYLALANINE AND GLUTAMIC ACID

Inhibitor	Concentration (mg/ml)	Growth,* % of controls reversal agent			
		No reversal agent	Phenylalanine†	Glutamic acid‡	Phenylalanine† plus glutamic acid‡
Formamide	0	100	98	94	94
	15·0	24	94	53	77
	17·5	12	91	48	75
	20·0	4	54	37	82
N-Methylformamide	0	100	98	90	90
	17·5	17	64	17	43
	20·0	8	59	9	31
	22·5	5	45	11	18
Urethan	0	100	95	90	—
	10·0	2	4	79	59
	12·5	2	1	29	14
	15·0	1	0	13	1

* Growth was measured turbidimetrically after incubation at 37 °C for 16 hr.
† Phenylalanine was used at a concentration of 7·6 μmoles/ml.
‡ Glutamic acid was used at a concentration of 10 μmoles/ml.

the reversal of growth inhibition by phenylalanine and glutamic acid, as added to the medium singly and in combination, with various concentrations of the inhibitors. Cells that grew in the presence of both of these compounds retained sensitivity to the inhibitors.

A number of other compounds in quantities that were equimolar to glutamic acid were tested for prevention of inhibition by each of the three inhibitors, and, when growth occurred, the cells were tested for resistance. The results are indicated in Table 4. Glutamic acid stimulated the overgrowth of resistant cells much more than any other compound, and none of these other compounds caused true prevention of inhibition.

Fig. 1 shows that *E. coli* 9637 is inhibited by urethan when the inhibitor is added to the culture during the exponential phase of growth and that the inhibition is partially prevented by phenylalanine. This figure also shows that the inhibition is partially overcome even if the addition of the phenylalanine is delayed until 1 hr or longer after the addition of the inhibitor. Under the same conditions glutamic acid did not prevent or overcome the inhibition, even though it was added simultaneously

TABLE 4. EFFECTS OF VARIOUS COMPOUNDS UPON THE GROWTH OF *E. coli* 9637 IN THE PRESENCE OF FORMAMIDE, N-METHYLFORMAMIDE, OR URETHAN

No effect		Stimulation of overgrowth of drug-resistant cells	True reversal
Adenosine	Glycine	Ureidosuccinic acid	Phenylalanine
Guanosine	Alanine	Orotic acid	2:6-Diaminopurine
Uridine	Serine	Uracil	
Cytidine	Threonine	Cytosine	
Adenylic acid (2′ + 3′)	Leucine	Thymine	
	Lysine	Thymidine	
Uridylic acid (2′ + 3′)	Ornithine	Cytidylic acid (2′ + 3′)	
	Citrulline		
	Arginine	Glutamic acid	
Citric acid	Histidine	Aspartic acid	
*iso*Citric acid	Proline	Asparagine	
*cis*Aconitic acid	Hydroxyproline	Methionine	
Succinic acid	Cystine	Valine	
Fumaric acid	Tyrosine	*iso*Leucine	
Malic acid	Tryptophan		
Oxalacetic acid			
α-Ketoglutaric acid			

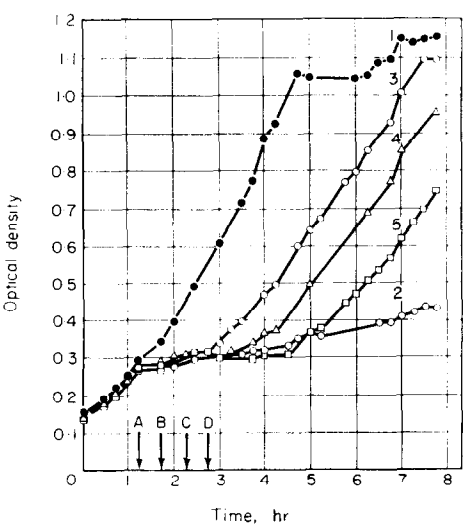

FIG. 1. Reversal of urethan inhibition of *E. coli* 9637 by phenylalanine. Culture (1) is the control. To cultures (2), (3), (4) and (5) urethan was added at time *A* to give a concentration of 10 mg/ml, and to cultures (3) (4) and (5) phenylalanine was added at times *B*, *C* and *D*, respectively, to give a concentration of 0·81 mg/ml.

with the inhibitor. Similar results were obtained with formamide and N methylformamide. Evidently, no significant overgrowth of resistant cells occurred in the presence of glutamic acid under these conditions and during this relatively short period of growth.

In order to obtain information regarding the correlation of molecular structure and activity in preventing inhibition, several purines and related compounds were

tested with exponentially growing cultures, and some of the results are shown in Fig. 2. For comparison, phenylalanine was included in this experiment. 2 : 6-Diaminopurine was the most active of the purines, and 2-aminopurine had considerable activity, but 2-hydroxypurine was inactive. In other experiments not shown here, 2 : 6-diamino-8-azapurine possibly had slight activity, but 4-amino-5-imidazolecarboxamide, hypoxanthine, inosine, *iso*guanine, 2-hydroxypurine, guanine, xanthine, adenine and 8-azaguanine had little or no activity. It was also found that 2 : 6-diaminopurine could overcome the effects of urethan when the reversal agent was added to cultures that were already inhibited by urethan.

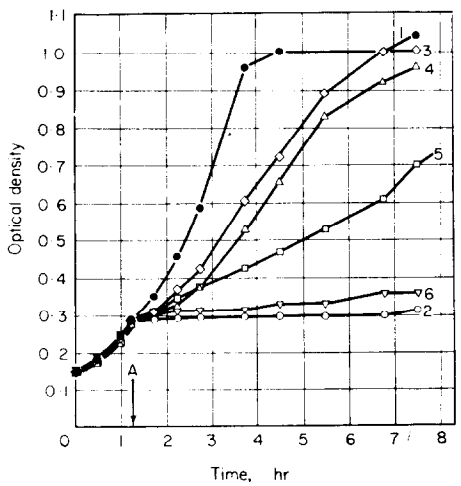

FIG. 2 Prevention of urethan inhibition of *E. coli* 9637 by phenylalanine and by purines. Culture (1) is the control. To cultures (2), (3), (4), (5) and (6) urethan was added at time *A* to give a concentration of 10 mg/ml. To cultures (3), (4), (5) and (6) the test compounds were added at time *A* to give the indicated concentrations: culture (3)—phenylalanine, 0·81 mg/ml; culture (4)—2:6-diaminopurine, 0·1 mg/ml; culture (5)—2-aminopurine, 0·09 mg/ml; culture (6)—2-hydroxypurine, 0·09 mg/ml.

It was desirable to determine whether a culture that had been inhibited by formamide, N-methylformamide, or urethan after it had entered the exponential phase of growth would resume exponential growth if the inhibitor was removed. Accordingly, the following experiments were performed. After the cultures were well into the exponential phase of growth, the inhibitors were added, and the cultures were exposed to the inhibitors for 2 hr, during which time essentially no growth occurred. At the end of the 2 hr, the control cultures and the inhibited cultures were centrifuged, and the cells were washed once with saline and resuspended in fresh medium that contained no inhibitor. The process of collecting cells, washing them, and resuspending them required about 60 min, and since some cells were lost by decantation, the optical densities of the reconstituted cell suspensions were less than those of the original cultures prior to centrifugation. Upon incubation of the suspensions, the control cultures immediately resumed exponential growth, and after an initial lag phase the inhibited cultures also grew exponentially.

Table 5 contains data obtained in tests of cross-resistance with various mutant lines of *E. coli*. The azaserine-resistant mutant was somewhat resistant to formamide, N-methylformamide, and urethan. The DON-resistant mutant was also somewhat

TABLE 5. TESTS OF CROSS-RESISTANCE

Lines of *E. coli*	Concentration (mg/ml) of inhibitor required to cause 50% inhibition of growth				
	Azaserine	DON*	Formamide	N-Methyl-formamide	Urethan
9637	0·0045	0·000053	7·9	8·3	10·5
Azaserine-resistant	>0·2†	0·083	27·7	16·3	18·0
DON-resistant	0·00057	0·073	26·6	20·2	NT‡
Formamide-resistant	0·000053	0·00005	24·3	18·3	NT
N-Methylformamide-resistant	0·000052	0·00005	27·9	25·0	NT
Urethan-resistant	0·000051	0·00005	NT	NT	22·9

* 6-Diazo-5-oxo-L-norleucine.
† Azaserine at a concentration of 0·2 mg per ml caused only 6% inhibition of growth.
‡ NT indicates that no test was performed.

resistant to formamide and N-methylformamide; this mutant was not tested for resistance to urethan. However, it is of interest that the formamide-resistant mutant, the N-methylformamide-resistant mutant, and the urethan-resistant mutant showed mutual cross-resistance to formamide, N-methylformamide, and urethan as previously reported,[11] yet retained their sensitivity to azaserine and DON.

Table 6 contains data that show the extent of growth of *E. coli* B96 cells in the

TABLE 6. GROWTH OF *E. coli* B96 IN THE PRESENCE OF INHIBITORS AND GLUTAMIC ACID, GLUTAMINE, PHENYLALANINE, OR 2 : 6-DIAMINOPURINE

Test compound	Concentration (μmoles/ml)	Growth, % of control		
		Formamide*	N-Methylformamide*	Urethan†
None	—	2	8	2
Glutamic acid	5	64	20	78
	10	66	15	76
Glutamine	5	2	6	8
	10	1	5	10
Phenylalanine	5	2	5	0
	10	2	5	0
2:6-Diaminopurine	0·33	1	5	7
	0·66	—	—	8
	1	2	4	—
	2·64	—	—	0

* The inhibitor was present at a concentration of 30 mg/ml, and the turbidity was determined after incubation at 37 °C for 16 hr.
† Urethan was present at a concentration of 10 mg/ml, and the turbidity was determined after incubation at 37 °C for 16 hr.

presence of the inhibitors and glutamic acid, glutamine, phenylalanine, or 2 : 6-diaminopurine. Extensive growth occurred in the presence of the inhibitors only when glutamic acid was present, and the results of tests for resistance showed that this growth was not due to the overgrowth of resistant cells. Formamide slowed the growth of exponentially growing B96 cells, and N-methylformamide stopped the growth of such cells. The quantities of inhibitor required to inhibit the growth of cells under these conditions were considerably greater than those required to inhibit the growth of the 9637 cells under similar conditions and also greater than the quantities required to inhibit the growth of B96 cells in overnight growth studies. Glutamic acid partially prevented the inhibition by each of these agents. As shown in Fig. 3, the inhibition of B96 cells by N-methylformamide was partially overcome by glutamic acid even if the inhibitor was added two hours before the addition of the glutamic acid.

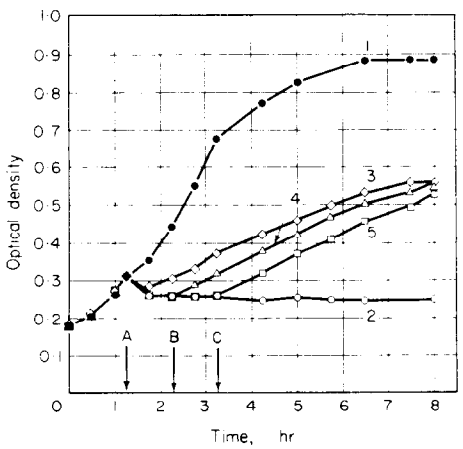

FIG. 3. Reversal of N-methylformamide inhibition of *E. coli* B96 by glutamic acid. Culture (1) is the control. To cultures (2), (3), (4) and (5) N-methylformamide was added at time *A* to give a concentration of 50 mg/ml, and to cultures (3), (4) and (5) glutamic acid was added at times *A*, *B* and *C*, respectively, to give a concentration of 2·19 mg/ml.

The data of Table 7 show that formamide, N-methylformamide, and urethan partially inhibit the formation of diazotizable amine (4-amino-5-imidazolecarboxamide) by resting B96 cells. The inhibition by formamide was prevented to a greater extent by glutamic acid than by 2 : 6-diaminopurine or phenylalanine, although these compounds also were somewhat effective. The inhibition by N-methylformamide was partially prevented by 2 : 6-diaminopurine but not by glutamic acid or phenylalanine. The inhibition by urethan was partially prevented by phenylalanine but not by glutamic acid or 2 : 6-diaminopurine. These results indicate a certain degree of specificity of the inhibitors.

A number of compounds including phenylalanine and glutamic acid were tested for the prevention of the inhibition of growth of the pyrimidine-requiring mutant 9723 g by urethan. Glutamic acid was the only compound that was found to give true prevention of inhibition (Table 8); 2 : 6-diaminopurine was not tested. No similar tests were performed with formamide or N-methylformamide.

TABLE 7. EVALUATION OF COMPOUNDS FOR PREVENTING THE INHIBITION OF THE PRODUCTION OF DIAZOTIZABLE AMINE BY *E. coli* B96

Test compound	Concentration (μmoles/ml)	Production of diazotizable amine (% of control)		
		Formamide* 35 mg/ml	N-Methylformamide† 20 mg/ml	Urethan† 30 mg/ml
None	0	49	33	47
Glutamic acid	5	64	31	50
Phenylalanine	10	55	32	80
2:6-Diaminopurine	0·05	59	53	47

* The recorded values are the average values for nine experiments. Although the order of the activities was not consistent for all of the experiments, glutamic acid was the most active compound in six of the experiments.

† The recorded values are the average values for four experiments, and the order of the activities of the compounds was the same for all of the experiments.

TABLE 8. GROWTH OF *E. coli* 9723 g IN THE PRESENCE OF URETHAN PLUS GLUTAMIC ACID AND TESTING OF THE RESULTING CULTURE FOR RESISTANCE TO URETHAN

A. Growth

Tube	Urethan (mg/ml)	Glutamic acid (μmoles/ml)	% growth, after 16½ hr.
1	0	0	100
2	15	0	2
3	15	5	78

B. Test for Resistance

Urethan (mg/ml)	% growth	
	Culture from tube 1*	Culture from tube 3*
0	100	100
10·0	36	22
12·5	2	1
15·0	1	0
17·5	0	0
20·0	0	0

* This number indicates the tube of *A* that was used as inoculum in the test for resistance.

DISCUSSION

The activity of phenylalanine in preventing the inhibition of the wild strain of *E. coli* by formamide, N-methylformamide, and urethan is in contrast to the lack of activity of tyrosine and tryptophan and to the failure of phenylalanine to prevent the inhibition of the growth of the purine-requiring mutant and of the pyrimidine-requiring mutant. The various roles of phenylalanine in the metabolism of *E. coli* are not known at present. It is known that phenylalanine will prevent the inhibition

of the growth of *E. coli* by azaserine,[16, 17] but the mechanism of this prevention is not established. Phenylalanine is one of several compounds that prevented the azaserine inhibition of the production of diazotizable amine, an intermediate in the biosynthesis of purines, by resting B96 cells, [17–19] and, in the presently reported experiment, it partially prevented the urethan inhibition and formamide inhibition of the production of diazotizable amine. These results imply that phenylalanine may be involved in the biosynthesis of purines, but this evidently is not its only role, because it is more effective than intact purines in preventing the inhibition by azaserine, formamide, N-methylformamide, and urethan. The possibility that phenylalanine exerts its effect by preventing the entry of the inhibitor into the cell[20] has not been eliminated, but the growth of the cells can be arrested for an hour or more prior to addition of the phenylalanine, and growth will resume soon after the addition. Since phenylalanine does not prevent inhibition of the purine-requiring and pyrimidine-requiring cells, phenylalanine must not perform as important a function in these mutant lines as it does in the wild strain, or supplementation by phenylalanine alone is not sufficient to overcome the inhibition by the higher concentration of inhibitor. Phenylalanine also did not prevent inhibition of *E. coli* 9637 at higher concentrations of inhibitor. This might indicate that the inhibitor prevents the utilization of phenylalanine, rather than its synthesis, or it may indicate that there are several sites of action and that at the higher concentration the inhibitor becomes effective at additional sites.

2 : 6-Diaminopurine was the most active, on a molar basis, of the compounds that were tested as reversal agents for these inhibitors. However, the effectiveness of 2 : 6-diaminopurine is limited because this compound itself is toxic at higher concentrations. Comparison of the activities of the various substituted purines show that there is some specificity for an amino group on carbon-2. 2-Aminopurine showed some activity, but adenine was inactive. Guanine, *iso*guanine, 2-hydroxypurine, xanthine and hypoxanthine were also inactive. Therefore, it appears that an amino group on carbon-2 is essential for activity, but the activity is greater if there is also an amino group on carbon-6. A hydroxyl group on carbon-6 eliminates any activity that might be conferred by the 2-amino group. Replacement of carbon-8 of 2 : 6-diaminopurine by a nitrogen atom results in a great decrease of activity. 2 : 6-Diaminopurine has an activity qualitatively similar to that of phenylalanine, in that it prevented inhibition of *E. coli* 9637 without stimulating the overgrowth of resistant cells, and it did not prevent inhibition of the B96 mutant. Like phenylalanine, 2 : 6-diaminopurine partially prevented the inhibition of formation of diazotizable amine by formamide, and, unlike phenylalanine, it also partially prevented the inhibition by N-methylformamide.

Table 9 shows a comparison of the effects of urethan, formamide, N-methylformamide, azaserine and DON upon certain responses of bacteria and of resistant lines of the plasma-cell neoplasm 70429. This table contains data that were taken from the literature as well as those obtained in the present study. This comparison shows that the five inhibitors have certain effects in common, but each inhibitor has certain effects that are peculiar to that particular inhibitor.

The inhibitory action of formamide, N-methylformamide, and urethan may occur within the cell or at the cell wall.[20, 21] The inhibitory effect is transient, and growth resumes if the inhibited cells are transferred to fresh medium that does not contain the inhibitor. If inhibition is dependent upon the continual entrance of the inhibitor into the cell, then the reversal agent might function by competing with the inhibitor

for the transport system.[20] If inhibition is dependent simply upon the presence of the inhibitor within the cell, then the reversal agent is perhaps competing with the inhibitor for an enzyme. Another possibility is that the reversal agent effectively detoxifies the inhibitor by chemically reacting with it, but this mechanism seems unlikely in view of the known chemical properties of these compounds.

TABLE 9. COMPARISON OF EFFECTS OF INHIBITORS

Property	Inhibitor				
	Azaserine	DON	Urethan	Formamide	N-Methyl-formamide
Resistance of azaserine-resistant mutant of *E. coli*.	−	−	−	±	...
Resistance of DON-resistant mutant of *E. coli*.	−	−	NT *	−	±
Resistance of urethan-resistant mutant of *E. coli*.	−	−	...	±[11]	...[11]
Resistance of formamide-resistant mutant of *E. coli*.	−	−	±[11]	...	...
Resistance of N-methylformamide-resistant mutant of *E. coli*.	−	−	±[11]	+	±
Resistance of azaserine-resistant line of neoplasm 70429.	+[21]	+[21]	NT	NT	−[21]
Resistance of DON-resistant line of neoplasm 70429.	+[21]	+[21]	NT	NT	±[21]
Resistance of N-methylformamide-resistant line of neoplasm 70429.	+[21]	+[21]	NT	NT	±[21]
Effectiveness of phenylalanine in preventing inhibition of growth of *E. Coli*.	+[16]	−	...	−	...
Effectiveness of 2 : 6-diaminopurine in preventing inhibition of growth of *E. coli*.	NT	NT	±	+	±
Effectiveness of purines other than 2 : 6-diaminopurine in preventing inhibition of growth of *E. coli*.	+[22]	+[23]	−	−	−
Inhibition of *de novo* biosynthesis of purines.	+[22]	+[23]	±[3, 4], −[4]	NT	±[4], +[5]
Inhibition of formation of diazotizable amine by *E. coli*. B96.	+[18]	NT	±	+	−
Reversal of inhibition of formation of diazotizable amine by phenylalanine.	+[18]	NT	+	±	−
Reversal of inhibition of formation of diazotizable amine by 2 : 6-diaminopurine.	NT	NT	−	±	±
Reversal of inhibition of formation of diazotizable amine by glutamic acid.	+[18]	NT	−	+	−
Accumulation of 1-(N-formylglycinamide) ribose by inhibited *E. coli*.	+[24]	+[25]	−[25]	−[25]	−[25]

* NT = not tested.

The data that are presented in this report suggest that in *E. coli* there are multiple sites of action for each of these inhibitors—formamide, N-methylformamide, and urethan. Possible sites might be represented schematically as follows:

(1) A → B, reversed by phenylalanine and 2 : 6-diaminopurine.

(2) C → D, reversed by glutamic acid.

Each of these steps might be inhibited by each of the agents, but process (1) is more sensitive to the agents, and hence the effects of low levels of the inhibitors can be overcome by phenylalanine or 2 : 6-diaminopurine. At these low concentrations of inhibitor, process (2) is not affected. However, at higher levels both processes are affected, and phenylalanine or 2 : 6-diaminopurine does not prevent the inhibition. If process (1) is sufficiently less critical in the purine-requiring cells and the pyrimidine-requiring cells than in the wild-type cells, then these auxotrophic cells would not be sensitive to low levels of the inhibitors, but at higher levels they would be inhibited because of interference with process (2). This would explain why larger quantities of the agents would be required to inhibit the mutant cells. Since the characteristic features of these mutants are the lack of purine synthesis and the lack of pyrimidine synthesis, it might be suspected that process (1) is related directly or indirectly to the synthesis of purines and pyrimidines. This possibility is consistent with the observed interference with the synthesis of diazotizable amine. Perhaps both process (1) and process (2) actually consist of several different biochemical transformations, and this

accounts for the observed differences with the individual inhibitors. This possibility is consistent with the fact that combinations of phenylalanine and glutamic acid do not completely prevent inhibition. Present knowledge does not permit further definition of processes (1) and (2), but it is hoped that experiments in progress with radioactive substrates will elucidate them.

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