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A Study of the Interactions of Folic Acid Analogues and 6-(Substituted)purines as Growth Inhibitors

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Introduction

A previously reported method of detection of kinetin and structurally related compounds, i.e. 6-(substituted)purines, was based upon their augmentation of growth inhibition of *Lactobacillus arabinosus* by 2,4-diamino-6,7-diphenylpteridine.[1] In the present investigation, kinetin and related compounds were studied to determine whether inhibitions by analogues structurally closely related to folic acid are augmented by these compounds, in the same organism and in other bacteria. The results indicate that many of these purine derivatives in combination with any of several inhibitory analogues of folic acid do exert synergistic growth-inhibiting effects in certain lactic acid bacteria, and, further, that certain 6-dialkylaminopurines prevent the appearance of cells resistant to the inhibitory combination of agents. Metabolic studies implicate thymidine, purines and serine as limiting products whose biosynthesis is blocked by the combination of a 6-(substituted)purine and an analogue of folic acid.

Materials and Methods

The procedure for agar diffusion assays, by means of application of small paper discs bearing the 6-(substituted)purine samples to the surface of semi-solid medium containing the pteridine analogue, has been described previously.[1] In the present study an analogous procedure was used, with the exception that amethopterin or aminopterin ($0 \cdot 01\gamma$ per ml of final volume) or methylfolic acid (80γ/ml) replaced the pteridine inhibitor. Substitution of

these analogues of folic acid for the pteridine compound also allows detection of 6-(substituted)purines in terms of augmented growth inhibition zones concentric with paper discs bearing the sample solutions, as listed in Table I. These assay plates require heavy inocula (see footnote, Table I) and incubation for about 48 h to allow background growth sufficient for convenient observation of relatively clear inhibition zones.

The procedure for tube assays, using liquid cultures, has been described elsewhere.[2]

The organisms *Lactobacillus arabinosus* 17–5 (A.T.C.C. 8014) and *Leuconostoc mesenteroides* (A.T.C.C. 8293) were maintained by monthly transfers in stab cultures containing 2 per cent agar, 1 per cent Difco yeast extract, and 0·5 per cent glucose.

Samples of various synthetic 6-(substituted)purines were generously furnished by Dr. C. G. Skinner of the Clayton Foundation Biochemical Institute.

Results and Discussion

Studies with Lactobacillus arabinosus.

With amethopterin or aminopterin in the medium, the spectrum of inhibitory potency of the group of 6-(substituted)purines (Table I) is very similar to that in the presence of diaminodiphenylpteridine,[1] with greatest inhibition zone size in the region of *n*-butyl- and *n*-pentylaminopurine in the monoalkylamino series, and a rather broad range of active substituents in the phenylalkylamino series. The most active dialkylamino derivatives (dibutyl and dipentyl) produce zones of more intense inhibition (i.e. zones of greater transparency) than the other classes of derivatives, although these zones are not especially large; these dialkyl derivatives are of special interest in preventing development of resistant colonies as subsequently indicated. Inhibition zones of moderate size are produced by some of the 6-(substituted)thiopurines (e.g. *n*-butylthio-, phenethylthiopurine), in contrast to their virtual inactivity with the diaminodiphenylpteridine inhibitor. Several members of a new class of derivatives, 2-amino-6-(substituted)purines, show activities similar to those of 6-(monoalkylsubstituted)aminopurines (Table I); the synthesis of these 2-amino derivatives and their activities with respect to

Table I. Augmented inhibition of growth of lactic acid bacteria in presence of combinations of folic acid analogues with 6-(substituted)purines

Compound	Amount, γ per paper disc	Organism: *Lactobacillus arabinosus* — Amethopterin* Inhibition zone§	*Lactobacillus arabinosus* — Aminopterin* Inhibition zone§	*Lactobacillus arabinosus* — No inhibitor† Inhibition zone§	*Leuc. mesenteroides* — Methylfolic acid‡ Inhibition zone§
Adenine sulphate	40	24	26	0	0
6-(Substituted)-purines:					
Methylamino-	20	23	23	0	
	40	27r,t,f	27r,t,f	0	23
Ethylamino-	20	28	28		
	40	32r,t,f	32r,t	0	18
n-Propylamino-	20	30	29		
	40	36r,t,f	35r,t	0	21
n-Butylamino-	10	24	25		
	20	31	32		
	40	36r,t,f	37r,t,f	0	31
n-Pentylamino-	10	26	28		
	20	33	34		
	40	37r,t,f	38r,t,f	0	31
n-Hexylamino-	10	24	24		
	20	32	30		
	40	37r,t,f	34r,t,f	0	30
n-Heptylamino-	20	28	26		
	40	33r,t,f	32r,t,f	0	28
n-Decylamino-	40	19r,t,f	25r,t,f	0	22
Dimethylamino-	40	0	0	0	0
Diethylamino-	40	18r	0	0	0
Di-*n*-propylamino-	40	17r-	20r-	20	0
Di-*n*-butylamino-	20	20	20		
	40	24r-,t-,f	23r-,t-,f	24	24
Di-*n*-pentylamino-	20	21	19		
	40	23r-,t-,f	23r-,t-,f	23	26
Di-*n*-hexylamino-	20	21	16		
	40	23r-,t-,f	21r-,t-,f	22	24
Di-*n*-heptylamino-	40	0	18	0	0
N-Benzyl-N-*n*-butylamino-	40	20r-,t-,f	17r-,t-,f	19	23
Phenylamino-	20	28	28		
	40	33r,t,f	32r,t	0	30
Phenylmethylamino-	20	29	31		
	40	34r,t,f	35r,t	0	28

Table I—*cont.*

Compound	Amount, γ per paper disc	Organism: *Lactobacillus arabinosus* — Amethopterin* Inhibition zones§	*Lactobacillus arabinosus* — Aminopterin* Inhibition zones§	*Lactobacillus arabinosus* — No inhibitor† Inhibition zones§	*Leuc. mesenteroides* — Methylfolic acid‡ Inhibition zones§
6-(Substituted)-purines:					
Phenethylamino-	20	29	29		
	40	34[r,t,f]	34[r,t,f]	0	33
(3-Phenylpropyl)-amino-	20	31	30		
	40	36[r,t]	34[r,t]	0	37
(4-Phenylbutyl)-amino-	20	28	29		
	40	34[r,t,f]	33[r,t,f]	0	34
(5-Phenylpentyl)-amino-	20	26			
	40	31[r,t,f]	30	0	30
(7-Phenylheptyl)-amino-	40	0	24	0	19
(11-Phenylundecyl)-amino-	40	0	0	0	0
(2-Phenoxyethyl)-amino-	40	30[r,t,f]	29[r]	0	
(2-Phenoxypropyl)-amino-	40	31[r,t,f]	33[r]	0	
(4-Phenoxybutyl)-amino-	40	34[r,t,f]	36[r,t,f]	0	32
(3-Methoxypropyl)-amino-	40	30[r]	29[r]	0	26
Dimethylamino-*n*-propylamino-	40	0	0	0	0
Diethylamino-*n*-propylamino-	40	0	0	0	0
*cyclo*Hexylmethyl-amino-	40	26[r,t,f]	31[r,t,f]	0	
(2-*cyclo*Hexyl-ethyl)amino-	40	32[r,t,f]	33[r,t,f]	0	
(3-*cyclo*Hexyl-propyl)amino-	40	26[r,t,f]	27[r,t,f]	0	
(4-*cyclo*Hexyl-butyl)amino-	40	21[r]	23[r]	0	32
(5-*cyclo*Hexyl-pentyl)amino-	40	0	18[r]	0	21

Table I—*cont.*

Compound	Amount, γ per paper disc	Organism: *Lactobacillus arabinosus* — Amethopterin* Inhibition zone§	*Lactobacillus arabinosus* — Aminopterin* Inhibition zone§	*Lactobacillus arabinosus* — No inhibitor† Inhibition zone§	*Leuc. mesenteroides* — Methylfolic acid‡ Inhibition zone§
6-(Substituted)-purines:					
(6-*cyclo*Hexylhexyl)amino-	40	0	0	0	23
(α-Naphthylmethyl)amino-	40	31[r,t]	31[r,t]	0	33
(2-α-Naphthylethyl)amino-	40	22[r]	24[r]	0	24
(5-α-Naphthylpentyl)amino-	40	0	0	0	30
(2-Pyridylmethyl)amino-	40	30[r,t,f]	31[r,t,f]	0	19
(3-Pyridylmethyl)amino-	40	27[r]	28[r]	0	19
(4-Pyridylmethyl)amino-	40	25[r]	25[r]	0	
Histamino-	40	0	28[r]	0	0
(2-Furfuryl)amino-(Kinetin)	10	24	24		
	20	27	30		
	40	33[r,t,f]	34[r,t,f]	0	29
(2-Thenyl)amino-	20	30	32		
	40	34[r,t,f]	36[r,t]	0	23
Adeninosuccinic acid	40	0	0	0	0
Mercaptopurine	40	0	0	19	0
Methylthio-	40	21[r,t,f]	21[r,t,f]	0	0
Ethylthio-	40	19[r]	21[r]	0	0
n-Propylthio-	40	19[r]	22[r]	0	0
n-Butylthio-	40	23[r]	22[r]	0	21
n-Pentylthio-	40	23[r,t,f]	21[r,t,f]	0	23
n-Hexylthio-	40	20[r,t,f]	20[r,t,f]	0	25
n-Heptylthio-	40	0	0	0	23
n-Octylthio-	40	0	0	0	17
n-Decylthio-	40	0	0	0	0
n-Cetylthio-	40	0	0	0	0
(2-Methylpropyl)thio-	40	0	0	0	17

Table I—*cont.*

Compound	Amount, γ per paper disc	Organism: *Lactobacillus arabinosus* — Amethopterin* Inhibition zones§	*Lactobacillus arabinosus* — Aminopterin* Inhibition zones§	*Lactobacillus arabinosus* — No inhibitor† Inhibition zones§	*Leuc. mesenteroides* — Methylfolic acid‡ Inhibition zones§
6-(Substituted)-purines:					
(3-Methylbutyl)-thio-	40	20[r,t,f]	0	0	21
Phenylmethylthio-	40	17[r,t,f]	21[r,t,f]	0	21
Phenethylthio-	40	24[r,t,f]	23[r,t,f]	0	23
(3-Phenylpropyl)-thio-	40	22[r,t]	0	0	21
(5-Phenylpentyl)-thio-	40	0	0	0	20
Purinylthio-succinic acid	40	0	0	0	0
2-Amino-6-(substituted)-purines:					
Amino-	20	28	25		
	40	33[r,t,f]	31[r]	0	0
(2-Furfuryl)amino-	20	28	30		
	40	35[r,t,f]	35[r]	0	
Phenylmethylamino-	20	28	29		
	40	33[r,t]	34[r]	0	
Phenethylamino-	20	29	32		
	40	36[r,t,f]	37[r]	0	
(3-Pyridylmethyl)-amino-	40	28[r,t]	30[r]	0	
(4-Pyridylmethyl)-amino-	40	25[r,t]	26[r]	0	
Mercapto- (Thioguanine)	40	23[r-,t-f]	22[r-]	20	0
Methylthio-	40	30[r,t,f]	28[r]	0	
Phenylmethylthio-	40	25[r]	28[r]	0	

* Medium: Same as previously described for thymidine assay[2] with diaminodiphenylpteridine, except that diaminodiphenylpteridine was omitted and amethopterin or aminopterin (0·01γ per ml) was included. Inoculation: 1·0 ml of the initial heavy saline suspension of cells per 150 ml plate. Incubation: 48 h at 30°.

† Medium: Same as described in * except that no folic acid antagonist was included. Inoculation: 0·1 ml of the initial heavy saline suspension of cells per 150 ml plate. Incubation: 24 h at 30°.

‡ Medium: Same as described previously for thymidine assay[2] with methylfolic acid, except that the amount of methylfolic acid was increased to 12 mg, and a supplement of pantethine (6γ)

augmentation of diaminodiphenylpteridine inhibition have been reported elsewhere.[3]

The 6-(substituted)purines tested fall into two classes with respect to the appearance of resistant cell strains. In plates containing amethopterin or aminopterin, after about 48 h incubation, scattered colonies of resistant organisms appear submerged in the agar within the zones of inhibition produced by one class, consisting mainly of certain 6-(monosubstituted)amino- and 6-(substituted)thiopurines. For example, an inhibition zone 34 mm in diameter surrounding a sample of 40γ of kinetin may contain from ten to several hundred such resistant colonies, in different assay plates. In contrast, the second class, consisting of 6-(dialkylamino)purines, produces zones of intense inhibition (although of relatively small size) in which the development of such resistant colonies has not been observed, as indicated in Table I.*

was included (per 150 ml plate). Inoculation: 0·5 ml of the initial heavy suspension of cells per 150 ml plate. Incubation: 24–48 h at 30°.

§ The tabulated figures give the diameter in mm of the relatively clear zone in the agar medium, representing augmented inhibition of bacterial growth. The symbol 0 indicates the compound was inactive. Absence of entry in the table indicates the combination was not tested. The appearance of scattered colonies of resistant organisms, after 24–48 h incubation, within the measured zone of augmented inhibition is indicated by ^{r}; the failure of such appearance is indicated by $^{r-}$. Reversal of inhibition was observed as follows: Thymidine (0·2γ) was applied to a paper disc which was placed on the agar surface close to the disc bearing the 6-(substituted)purine sample, with 1–2 mm spacing between the nearest points of the paper discs. Reversal of inhibition, i.e. continuity of the growth-stimulation zone (concentric with the thymidine disc) through the growth-inhibition zone (concentric with the purine disc) is indicated in the table by ^{t}; non-reversal of inhibition, i.e. continuity of the growth-inhibition zone through the growth-stimulation zone, by $^{t-}$. Folinic acid (2γ) was applied to an adjacent paper disc, reversal effects were observed in the same manner, and reversal indicated by ^{f}, non-reversal by $^{f-}$.

* The scattered colonies which developed in the zones of augmented inhibition produced by 6-(monosubstituted)aminopurines were shown to be resistant in the following way: From inhibition zones surrounding two such compounds [kinetin and 6-(4-phenylbutylamino)purine], in a plate containing amethopterin, several separate colonies were each transferred with an inoculating needle into medium containing amethopterin (0·01 γ/ml), to allow the growth, perferentially, of resistant organisms. These cultures were used to inoculate new plates containing amethopterin (0·01 γ/ml) which were then used for paper disc assays. These assays indicated for each culture complete insensitivity to inhibition by typical 6-(monosubstituted)aminopurines (kinetin, *n*-pentylamino- and *n*-hexylaminopurine, 40γ per paper disc), and at the same time normal sensitivity to augmented inhibition by typical 6-(dialkylamino)purines (di-*n*-pentylamino- and di-*n*-hexylaminopurine, 40γ per paper disc). To insure purity of the supposed resistant strain, one culture was streaked out on nutritionally complete agar medium and several discrete (surface) colonies were transferred separately into complete medium (containing no folic acid analogue); each such isolated strain of the organism retained through subsequent subcultures its sensitivity to augmented inhibition by 6-(dialkylamino)purines and its insensitivity to 6-(monosubstituted)purines when tested in amethopterin-containing plates as described

The inhibitory effect of those 6-(dialkylamino)purines which suppress the formation of resistant colonies appears to be independent of the inhibitory effect of those 6-(monosubstituted)-aminopurines which allow resistance development. Thus, for example, in a plate containing aminopterin, 20γ of di-*n*-pentylaminopurine produces a visually completely clear zone 23 mm in diameter, and 20γ of *n*-propylaminopurine on a separate paper disc produces a zone of inhibition 32 mm in diameter containing many scattered resistant colonies. Under the same test conditions a paper disc bearing 20γ of dipentylamino- plus 20γ of propylaminopurine produces an inhibition zone 32 mm in overall diameter, completely clear to a diameter of 23 mm but containing scattered resistant colonies in the annulus between diameters of 23 and 32 mm. On the basis of the present data it cannot be decided whether the resistant colonies observed represent spontaneous alterations in the parent line which are selectively favoured by their environment of combined inhibitory agents, to which the parent organisms are sensitive, or whether they represent mutations induced by the inhibitory agents; this distinction must depend upon further study.

In the presence of amethopterin or aminopterin, thymidine (0·2γ) applied to an adjacent paper disc reverses the augmented inhibition produced by most of the 6-(substituted)purines tested (e.g. the monoalkylsubstituted series), but does not appreciably

above. To eliminate the possibility that the original 'resistant' colonies might have consisted of the progeny of foreign organisms inadvertently present in the stock culture of *L. arabinosus*, the stock culture was streaked out on agar medium, several discrete colonies were subcultured and then used to inoculate plates for paper disc assays of typical 6-(substituted)purines as described above; all such isolated strains showed sensitivity to augmented inhibition by both 6-(monosubstituted)amino- and 6-(dialkylamino)purines, and showed development of scattered resistant colonies in the augmented inhibition zones produced by the monosubstituted 6-aminopurines but not in those produced by the 6-(dialkylamino)purines.

Interestingly, a resistant cell strain, isolated as just described from a zone of augmented inhibition produced by phenylbutylaminopurine and shown to be resistant to combinations of amethopterin with 6-(monosubstituted)aminopurines, when used for inoculation of a paper disc assay plate containing the diaminodiphenylpteridine inhibitor,[1] showed normal sensitivity to inhibition by combinations of diaminodiphenylpteridine with 6-(monosubstituted)aminopurines. In other words, e.g., a strain resistant to the amethopterin-kinetin combination shows no cross resistance to the diaminodiphenylpteridine-kinetin combination.

reverse that exerted by the dialkylamino series (Table I). The inability of thymidine to reduce appreciably the size of the augmented inhibition zones produced by 6-dialkylaminopurines is the result of the activity of these purine derivatives in inhibiting growth alone (Table I) at a concentration not substantially higher than that necessary for all of the 6-(substituted)purines to augment the effects of the folic acid antagonists. While most of the 6-(substituted)purines exert at high concentrations this independent inhibitory effect, the 6-dialkylaminopurines do so at concentrations low enough to interfere with reversal studies in the paper disc assay. That single carbon unit metabolism is not involved in this secondary inhibited system is indicated by the inability of thymidine to reverse inhibition in the paper disc assay, and also by tube type, liquid culture tests in which the amount of di-*n*-pentylaminopurine just necessary to prevent growth of the organism (e.g. 50γ per 5 ml), in the absence of any folic acid analogue, is essentially independent of *p*-aminobenzoic acid, folic acid, folinic acid, or thymidine, over the range of concentrations tested (0·1–10, 0·01–1, 0·01–1, 0·1–10 γ/5 ml, respectively). High concentrations of folinic acid (2γ per paper disc) exert a reversing effect on the dialkylaminopurine inhibition in the paper disc assay (Table I), but this reversal by folinic acid has been variable in liquid culture experiments, and a number of reducing agents (e.g. glutathione, 100γ/5 ml) exert a similar effect, suggesting that the reversal may be a non-specific effect of reducing agents.

Although the paper disc test results do not show reversal of dialkylaminopurine inhibition by thymidine, in experiments carried out with liquid cultures evidence is available that relatively low concentrations of dialkylaminopurines do interfere with one-carbon unit transfer. Thus, with thymidine (and thymine) in the medium, *purines* appear to be the limiting products whose biosynthesis is blocked by amethopterin or aminopterin, or by a combination of one of these agents with the 6-(substituted) purine. As the tube type assay data of Table II indicate, the inhibition of growth produced by amethopterin is augmented by the presence of 10γ of dipentylaminopurine per 5 ml. Growth inhibition by the folic acid analogue alone or in combination with the substituted purine is reversed very effectively by a

Table II. Reversal by purines of inhibition exerted by amethopterin alone and in combination with dipentylaminopurine

Amethopterin γ per 5 ml	Supplements					
	Without 6-(substituted)-purine			With 6-Di-*n*-pentylamino-purine, 10 γ per 5 ml		
	None	Adenine and guanine 50γ each per 5 ml	AICA* 100γ per 5 ml	None	Adenine and guanine 50γ each per 5 ml	AICA* 100γ per 5 ml
	Galvanometer readings†					
0	98	98	98	98	98	98
0·01	65		72	29		44
0·02	38		47	9		17
0·05	11		22	3		2
0·1	3	74	4		76	
0·2		66			68	
0·5		57			60	
1		55			53	
2		50			53	

* AICA = 5-amino-4-inidazolecarboxamide.
† Distilled water reads 0, an opaque object 100.

Organism: *Lactobacillus arabinosus.*
Incubation: 26 h at 30°.
Medium: Same as that used for the experiments of Table IV, except that (a) adenine and guanine were omitted from the medium, (b) the concentration of thymine was 50γ per 5 ml, (c) that of *p*-aminobenzoic acid was 0·1γ per 5 ml, and (d) thymidine (2·5γ per 5 ml) was included.

combination of adenine and guanine, but not appreciably by the purine precursor lacking a single carbon unit, 5-amino-4-imidazole-carboxamide, which is known to be assimilated by *L. arabinosus* to form purines under normal growth conditions.[4] These data suggest that the augmentation of inhibition exerted by the 6-(substituted)purine at the concentration used represents prevention of purine biosynthesis at the stage of the one-carbon unit incorporation into the 2 position and possibly also into the 8 position of the purine ring, under these conditions. An analogous experiment with thymidine omitted from the medium and purines included, shows reversal by added thymidine of the augmented inhibition effect.

Table III. Augmentation of diaminodiphenylpteridine inhibition by some 6-(substituted)purines

2,4-Diamino-6,7-diphenylpteridine γ per 5 ml	Supplements												
	No addition	6-*n*-Pentylaminopurine γ per 5 ml			6-Phenylaminopurine γ per 5 ml			6-(4-Phenylbutyl)-aminopurine γ per 5 ml			6-Di-*n*-pentylaminopurine γ per 5 ml		
		3	10	30	3	10	30	3	10	30	3	10	30
	Galvanometer readings												
0	97	95	95	94	96	96	94	96	95	95	93	76	30
3	90			65			80			84			18
5	82		39	30		61	50		48	28		70	6
7	56	32	23	9	54	48	14	29	17	8	42	28	
10	25	8	3	2	16	4	5	10	6	2	9	5	
15	9	3			1			3			3		
20	1												

Organism: *L. arabinosus.*
Incubation: 22 h at 30°.
Medium: Same as described previously for thymidine assay,[2] except that the diaminodiphenylpteridine was omitted from the medium and added in graded amounts as tabulated.

In tube assays with liquid medium, the relative potencies of the various 6-(substituted)purines in augmenting growth inhibition by diaminodiphenylpteridine do not completely parallel the values obtained by the paper disc–agar diffusion assay as previously reported;[1] the effects of four typical 6-(substituted) purines in liquid medium are shown in Table III. The variation in apparent order of potency between the two assay methods may involve diffusion rate factors, or the relative abilities of the derivatives to prevent resistance development. The amount of pentylamino-, phenylamino-, or phenylbutylaminopurine required to inhibit growth completely in the absence of a folic acid analogue is greater than 1 mg per 5 ml, and the corresponding amount of dipentylaminopurine is about 100γ per 5 ml. These purine derivatives in concentrations which do not alone exert any effect diminish by one-third to one-half the amount of the pteridine required to inhibit growth.

Demonstration of augmentation of amethopterin or aminopterin inhibition in liquid culture requires heavy inocula (one drop of a 1 : 30 to 1 : 10 dilution of the initial saline suspension of cells, per assay tube). A synergistic relationship between amethopterin and three representative 6-(substituted)purines is shown in liquid culture medium, as indicated in Table IV. In these experiments the amount of phenylamino-, phenylbutylamino-, or dipentylaminopurine required in the absence of a folic acid analogue for complete growth inhibition is greater than 2 mg, approximately 1 mg, or 100γ per 5 ml, respectively. Thus concentrations of these purines which alone exert no effect decrease by factors of from 2 to 100 the amount of amethopterin necessary for inhibition of growth.

In a medium completely lacking exogenous purines, *L. arabinosus* is capable of moderate growth provided *p*-aminobenzoic acid is present. The 6-(substituted)purines were tested under these conditions for possible stimulation or depression of purine biosynthesis. None of the derivatives tested stimulated growth (i.e. replaced purines). Under these conditions 6-mercaptopurine is strongly inhibitory, and a few of the 6-(substituted)thiopurines are weakly inhibitory (about 2–5 per cent as potent as 6-mercaptopurine); among the amino derivatives, only 6-methylamino-, 6-di-*n*-butylamino- and 6-di-*n*-pentylaminopurine are markedly

inhibitory. Growth inhibition by *N*-methyladenine (6-methylaminopurine) is reversed effectively and non-competitively by adenine or hypoxanthine, or less actively by inosine, adenosine,

Table IV. Augmentation of amethopterin inhibition by some 6-(substituted)purines

Amethopterin γ per 5 ml	Supplements: 6-Phenylaminopurine* γ per 5 ml			6-(4-Phenylbutyl)-aminopurine* γ per 5 ml				6-Di-*n*-pentylaminopurine* γ per 5 ml			
	0	30	100	0	10	100	300	0	20	30	50
	Galvanometer readings										
0	94	93	93	96	96	96	95	97	96	93	36
0·01		83						85		36	
0·02		72				70		78	72	5	
0·05		51				46		75	15	2	
0·1	58	26		52	40	30		60	1		
0·2	39			33	17	12		62			
0·5	28			16	10			42			
1	27			11	7			32			
2	13			6				9			

* Separate assays.

Organism: *L. arabinosus*.
Incubation: The separate assays were incubated 23, 29, and 34 h, respectively, at 30°.
Medium: Same as described previously for thymidine assay with diaminodiphenylpteridine inhibitor,[2] except that the diaminodiphenylpteridine was omitted and amethopterin was included in amounts as tabulated.

or 5-amino-4-imidazolecarboxamide; inosinic acid is less than 10 per cent as effective as inosine in reversing this inhibition.

In a medium including adenine and guanine, with no folic acid analogue, and thus essentially complete for optimal growth, only the dialkylaminopurines inhibit growth when tested by the paper disc method, as indicated in Table I. Under these conditions 6-methylaminopurine is not inhibitory since adenine in the medium reverses its toxicity.

Studies with Leuconostoc mesenteroides.

For this organism, which requires folic acid for growth, thymidine is known to exert a limiting product effect under

appropriate conditions in the presence of the folic acid analogue, x-methylfolic acid.[2] As shown by the data of Table V, growth is inhibited to the same extent by 200γ of methylfolic acid, or by 100γ of methylfolic acid plus 100γ of phenylbutylaminopurine, or by 20γ of methylfolic acid plus 200γ of the substituted purine (per 5 ml), indicating an augmentation of the folic acid analogue action by the purine derivative. This augmented metabolic block appears to prevent thymidine synthesis, as shown by the effective reversal of inhibition when thymidine is added in the presence of either of the combinations of folic acid analogue with 6-(substituted)purine just mentioned (Table V). Phenylbutylaminopurine in the absence of a folic acid analogue inhibits growth

Table V. Reversal by thymidine of inhibition exerted by methylfolic acid alone and in combination with phenylbutylaminopurine

x-Methylfolic acid γ per 5 ml	6-(4-Phenylbutyl)aminopurine, γ per 5 ml									
	None		100		200		500		1000	
	O*	T*	O	T	O	T	O	T	O	T
	Galvanometer readings									
0	80	80	83	82	82	84	60	64	3	4
10	76		79		81					
20	75		72		16					
50	58	80	33	83	12	81				
100	47	80	16	80	6	79				
200	20	78		79		66				
500	2	64		55		39				

* The symbol O indicates no thymidine supplement; T, thymidine supplement, 5γ per 5 ml.

Organism: *L. mesenteroides* 8293.

Incubation: 20 h at 30°.

Medium: Essentially the same as previously described for thymidine assay with this organism;[2] the final concentration of folic acid, however, was 0·05γ per 5 ml, x-methylfolic acid was omitted from the medium and added as tabulated, and a supplement of pantethine (0·1γ per 5 ml) was included in the medium.

completely only at higher concentrations (about 1 mg per 5 ml) and such inhibition apparently involves interference with some metabolic process other than thymidine synthesis, since addition of thymidine has little effect.

In medium containing thymidine but lacking purines, growth inhibition of *L. mesenteroides* by methylfolic acid is reversed by purines (Table VI). The addition of dipentylaminopurine (10 or 20γ per 5 ml) augments the inhibition by the methylfolic acid; this augmented inhibition still represents a limitation of purine biosynthesis since exogenous purines extensively reverse the inhibitory effect. However, at a concentration of dipentylaminopurine sufficient to prevent growth in the absence of any folic acid analogue (30γ per 5 ml in the experiment of Table VI) some

Table VI. Reversal by purines of inhibition exerted by methylfolic acid alone and in combination with dipentylaminopurine

x-Methylfolic acid γ per 5 ml	6-Di-*n*-pentylaminopurine, γ per 5 ml							
	None		10		20		30	
	O*	P*	O	P	O	P	O	P
	Galvanometer readings							
0	65	80	60	80	58	67	4	19
2	60		60		48		4	
5	55		53		20			
10	26		13		5	68		3
20	5	78	3	78		70		
50	2	79		77		61		
100		77		68		45		

* O, without purine supplement; P, with purine supplement (adenine and guanine, 50γ each per 5 ml).

Organism: *L. mesenteroides* 8293.
Incubation: 23 h at 30°.
Medium: Same as that used for the experiment of Table V, except that adenine, guanine, and xanthine were omitted from the medium, except as tabulated, and thymidine (5γ per 5 ml) was included.

metabolic process other than purine synthesis apparently is limiting, since the addition of purines then fails to reverse the inhibition. Separate experiments show that in the absence of any inhibitory analogue of folic acid, the amount of dipentylaminopurine (30γ per 5 ml in a typical experiment) or of phenylbutylaminopurine (1 mg per 5 ml) necessary to prevent growth completely is essentially independent of the amount of folic acid

or of folinic acid present over the concentration range tested (0·01 to 1γ of either acid per 5 ml), affording further indication that folic acid coenzyme function is not the growth-limiting process at such high levels of these 6-(substituted)purines.

Since *L. mesenteroides* does not synthesize its own methionine but requires it pre-formed in the medium, it is not possible to study the effect of 6-(substituted)purines upon one-carbon incorporation into methionine by the organism. Serine is not required by the organism, although its addition is somewhat stimulatory to growth in an otherwise complete medium including thymidine, purines, methionine and folic acid. In such a medium lacking serine, growth inhibition by (a) 100γ of methylfolic acid per 5 ml is approximately duplicated by (b) 50γ of methylfolic acid plus 10γ of dipentylaminopurine per 5 ml; the addition of 200γ of DL-serine in each of these two cases raises the inhibition ratio (methylfolic acid/folic acid) about fourfold for a defined growth response. The nearly complete growth inhibition produced by higher concentrations of dipentylaminopurine (greater than 20γ per 5 ml) in the absence of any folic acid analogue is not reversed by DL-serine (200γ per 5 ml), so that as in the case of limiting purine synthesis above, this higher level of dialkylaminopurine appears to block another process in addition to serine biosynthesis.

The effect of phenylbutylaminopurine upon the ratio of methylfolic acid to folinic acid just necessary for prevention of growth of this organism is shown in Table VII. If the purine derivative interfered with some metabolic process at the same site as the methylfolic acid, i.e. if its effect were simply additive with that of the analogue, then it would be expected that a given amount of phenylbutylaminopurine would lower the inhibitor-substrate ratio to a greater extent at low absolute concentrations of inhibitor and substrate than at high concentrations. Actually, as the data indicate, the inhibition ratio (for a defined growth response) is lowered by a constant factor for a given amount of the 6-(substituted)purine over a tenfold range of concentrations of methylfolic and folinic acid tested. Analogous results with phenylbutylaminopurine have been obtained using folic acid in place of folinic acid, over a range of concentrations.

Leuconostoc mesenteroides inhibited by methylfolic acid, which

has been used for thymidine determination,[2] may be utilized in the form of a paper disc–agar diffusion assay[1] for the detection of the inhibitory action of certain 6-(substituted)purines. Inhibition zone diameters determined by the latter procedure are included in Table I. The spectrum of 6-(substituted)purines

Table VII. Effect of phenylbutylaminopurine upon the inhibition index for methylfolic acid inhibition of folinic acid utilization

x-Methylfolic acid γ per 5 ml	Folinic acid γ per 5 ml	Supplement	
		None	PBAP*
		Galvanometer readings	
0	0·001	66	63
10	0·001		15
20	0·001	40	5
50	0·001	10	1
100	0·001	1	
0	0·01	72	68
100	0·01		52
200	0·01	60	18
500	0·01	15	3
1000	0·01	2	
Inhibition index:		100,000	50,000

* PBAP 6-(4-Phenylbutyl)aminopurine, 20γ per 5 ml.

Organism: *L. mesenteroides* 8293.
Incubation: 44 h at 30°.
Medium: Same as that used for the experiment of Table V, except that methylfolic acid and folic acid were omitted from the medium; folinic acid and methylfolic acid were added as indicated above.

which augment the methylfolic acid inhibition is rather broad, including monoalkylamino-, dialkylamino- and 6-(substituted)-thiopurines. Separate paper disc assays, not tabulated, using the same medium except that methylfolic acid was omitted, show no inhibitory action exerted by a selection of typical 6-(substituted)purines, with the exception of small-diameter inhibition zones produced by 6-methylamino-, 6-di-*n*-butylamino-, 6-di-*n*-pentylamino- and 6-di-*n*-hexylaminopurine (40γ of each compound; zone diameters 18, 18, 20, and 18 mm, respectively).

Conclusions. These results indicate that the site of action of the 6-(substituted)purines in augmenting the inhibitory effects of folic acid analogues involves either the formation of the folic acid coenzyme or some separate function in the transfer of single-carbon units. These observations with bacteria suggest the need for further study of possible synergistic combinations of antagonists related to folic acid (a) with 6-(substituted)purines in general, for the purpose of inhibiting malignant cell growth, and (b) with certain members of the 6-(substituted)purine series, for preventing the appearance of malignant cells resistant to the inhibitory agents.

Summary. Kinetin and a number of other 6-(substituted)purines have been found to augment growth inhibitions of typical lactic acid bacteria exerted by certain folic acid analogues (amethopterin, aminopterin, and methylfolic acid). These augmented inhibition effects have been measured in agar diffusion assays in terms of growth inhibition zones concentric with paper discs bearing the 6-(substituted)purine samples, and turbidimetrically in liquid culture experiments. Metabolic studies with these bacteria indicate that under appropriate conditions thymidine, purines, or serine may be the limiting product whose biosynthesis is blocked by the combination of inhibitory agents. At higher concentrations than those which augment folic acid analogue inhibitions, the 6-(substituted)purines appear to block some (unidentified) metabolic system other than that involving the products of folic acid coenzyme. Resistant strains of *Lactobacillus arabinosus* appear in the presence of inhibitory combinations of amethopterin or aminopterin with most of the 6-(substituted)purines, but not with certain 6-(dialkylamino)purines.

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