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MULTIPLE INHIBITION BY 6-METHYLPURINE*

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Abstract—Inhibition of the growth of *Tetrahymena pyriformis* by 1-deaza-6-methylpurine is not readily reversed by adenine. This purine analog also causes the death and eventual lysis of the cells. While it does not have a lethal effect, 6-methylpurine (6MeP) is a more potent inhibitor of growth, and is reversed by high concentrations of adenine. Low concentrations are ineffective. Synergistic reversing effects on inhibition by 6MeP are exhibited by combinations of small amounts of adenine (10–15 μg/ml) with acetate. Metabolites such as sterols and phospholipids produced biosynthetically from acetate are capable of replacing the mixture of acetate plus adenine (10–15 μg/ml). Peptides, but not amino acids, give additional release of inhibition in the presence of lipid materials. It is suggested that 6MeP interferes specifically with the production of acyl or amino acyl compounds, probably by affecting the synthesis or utilization of ATP, as well as by interfering with adenine utilization for nucleic acid synthesis.

THE inhibition of the growth of *Tetrahymena pyriformis* by a number of analogs of adenine has already been described.[1, 2] Although adenine is not a nutritional requirement of the ciliate, since it is obtainable from guanine,[3] nevertheless specific inhibition of adenine utilization is obtained with either 2-azaadenine § or 8-azaadenine or 1-deazaadenine. Guanine has no reversing effect on these inhibitors.

The additional effect of causing death of the ciliates in cultures partially inhibited by 1-deazaadenine,[2] as distinguished from simple inhibition of growth, stimulated interest in other deazapurines. In the course of this work the compound 7-methyl-1,3,4-imidazopyridine, which could be regarded as 1-deaza-6-methylpurine, was independently synthesized by a route different from that previously reported[4] and tested. While this compound was somewhat less inhibitory than 1-deazaadenine, it possessed the property of causing death of the organisms. Unlike 1-deazaadinine, it was not readily reversed by adenine, of which it may also be regarded as an analog.

For comparative purposes 6-methylpurine (6MeP)[5] was obtained and tested.[6] This compound has proved to be the most inhibitory for *Tetrahymena* of the purine analogs so far tested (6-mercaptopurine is without activity in this system). 6-Propylpurine‖ had less than 1/100 of the activity of 6MeP. Since 6MeP did not cause the death and eventual lysis of the cells (which complicates turbidimetric measurements of growth) characteristic of the deaza-compounds, it was studied in more detail than

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1-deaza-6-methylpurine. Adenine proved to be relatively ineffective in reversal, although its effect could be greatly potentiated by the addition of acetate or certain products formed by biosynthetic pathways from acetate, as well as by addition of peptides. No inhibition was produced by 2-hydroxy- or 2-mercapto-6-methylpurine.

MATERIALS AND METHODS

Tetrahymena pyriformis W was cultivated in medium A[7] containing Tween 80 (10 mg/ml). Acetate and adenylic acid were omitted from this medium in certain experiments. Growth was measured turbidimetrically[8] after incubation for 4 days in a slanted position at 25°.

Sterols were dissolved in the Tween 80 used in preparing the basal medium. The various phospholipids were homogenized, with the aid of a small amount of Tween 80, using a Potter–Elvehjem apparatus.

Lipositol was prepared from commercial soy bean lecithin by the method of Woolley[9] and phosphatidyl ethanolamine by the method of Scholfield and Dutton.[10] Inositol phosphatides were also prepared using techniques described by Folch.[11] Crude glycolipids were extracted from beef spinal cord lipids using hot ethanol. The enzymatically hydrolyzed vitamin-free casein (VFC-EH) was obtained from Nutritional Biochemicals Corp.

By catalytic reduction of 2-amino-3-nitro-4-picoline[12] and subsequent ring closure of the resulting diamine with 98 per cent formic acid, 7-methyl-1,3,4-imidazopyridine was obtained in 65 per cent yield. Melting point 151·5–152° (corrected); literature,[4] 146–147°.

Analysis calculated for $C_7H_7N_3$: C, 63·14; H, 5·30; N, 31·6. Found: C, 63·04; H, 5·39; N, 31·6.

RESULTS

Reversal by adenine of the inhibition produced by 6MeP was not strictly competitive: inhibition indices varying from 0·023 to 1·46 were obtained, depending upon the adenine concentration in the medium. However, a sufficiently high concentration of adenine reversed the inhibition (Fig. 1).

The above results suggested that some compound other than adenine might be more effective in reversal. Adenosine proved to be less active than adenine, but the activity of adenosine-3′-phosphate (or adenosine-5′-phosphate) was approximately equal to that of adenine. None of the amino acids, vitamins or nucleic acid derivatives in the medium, when tested in increased concentrations, had any effect. In addition, natural materials were also investigated. "Distillers' solubles" was found to have the greatest reversing effect. Since the purine content of this material is low, non-purine compounds were implicated as having a reversing effect on the inhibition.

The fact that "distillers' solubles" was used as a starting material for the isolation of mevalonic acid,[13] suggested the possibility that the latter might be active. However, the addition of mevalonic acid* to media containing 6MeP enhanced the inhibitory effect of the purine analog to a slight extent. In turn, these results suggested that the acetate in the medium might have an effect on the inhibition, and as may be seen from Table 1, acetate was synergistic with adenine. Acetate alone, even at high concentrations, was only slightly effective, but when combined with amounts of adenine

* Obtained through the courtesy of Karl Folkers.

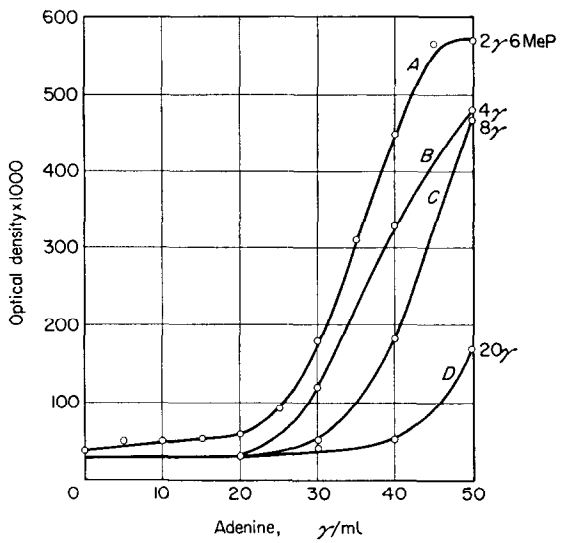

FIG. 1. Response of *Tetrahymena pyriformis* to adenine in presence of 6-methylpurine. Medium A minus acetate and adenylic acid. Curve A, 6-MeP 2 μg/ml; curve B, 6-MeP 4 μg/ml; curve C, 6-MeP 8 μg/ml; curve D, 6-MeP 20 μg/ml.

(e.g., 10–15 μg/ml) which were ineffective in themselves, a decided reversal was obtained.

The effect of acetate was limited in that relatively large amounts were required and beyond a certain point (1 mg/ml) further additions of acetate produced little further reversal of inhibition (Table 1, col. 1). The fact that "distillers' solubles" produced reversal without addition of adenine suggested that compounds other than acetate might be concerned. For these reasons substances known to be produced biosynthetically from acetate were tested.

The most active of these substances proved to be sterols (Table 2). Only those sterols (cholesterol, stigmasterol, β-sitosterol, ergosterol) having the intact side chain were active. Alterations in the side chain, as seen in the genins, or degradation to the

TABLE 1. SYNERGISTIC EFFECT OF ACETATE AND ADENINE ON INHIBITION BY 6-METHYLPURINE

Acetate (μg/ml)	Adenine (μg/ml)								
	0	5	10	15	20	25	30	40	50
	Optical Density								
0	0·04	0·04	0·04	0·06	0·10	0·16	0·27	0·43	0·48
100	0·05	0·05	0·09	0·12	0·14	0·17	0·26	0·48	0·55
250	0·07	0·09	0·13	0·17	0·20	0·24	0·30	0·46	0·52
500	0·09	0·12	0·17	0·22	0·24	0·26	0·31	0·46	0·52
1000	0·10	0·16	0·20	0·25	0·26	0·28	0·32	0·47	0·56
2000	0·13	0·17	0·20	0·24	0·26	0·29	0·34	0·53	0·61

Medium A minus acetate, adenylic acid: 6MeP 2 μg/ml.

level of the bile acids, adrenal hormones or sex hormones produced inactive or actually inhibitory compounds.[15] Acetylation of the 3-position in cholesterol reduced activity slightly, while opening the B ring (as in calciferol) completely inactivated the compound. Squalene*, a proposed precursor of sterols, had little if any activity.

Since stigmasterol has been used to supply the sterol requirement of another ciliate, *Paramecium aurelia*,[16] it was used in most of the present experiments. More detailed

TABLE 2. EFFECT OF STEROLS ON 6-METHYLPURINE INHIBITION

Steroid	μg/ml					
	0	5	10	25	50	100
			Optical Density			
Sitosterol	0·19	0·26	0·29	0·36	0·44	0·45
Ergosterol		0·24	0·29	0·32	0·37	
Stigmasterol		0·23	0·25	0·30	0·36	
Cholesterol		0·22	0·26	0·30	0·40	0·42
Calciferol		0·20	0·19	0·19	0·20	
Digitonin		0·17	0·17	0·14	0·17	
Smilagenin		0·16	0·17	0·17	0·18	
Squalene	0·14		0·13	0·13	0·11	0·08
Hydroxybisnorcholenic acid	0·10	0·11	0·07	0·10	0·13	
Cholesteryl acetate	0·08	0·08	0·12	0·13	0·30	0·32
Dehydroepiandrosterone	0·10			0·06	0·04	0·03
Cortisone	0·14		0·08	0·06	0·04	0·03

6-MeP 2 μg/ml, acetate present

TABLE 3. COMPARISON OF EFFECTS OF β-SITOSTEROL AND OF STIGMASTEROL IN REVERSAL OF INHIBITION BY 6-METHYLPURINE

	β-Sitosterol (μg/ml)							
	0	5	10	15	20	30	40	50
				Optical Density				
	0·04	0·13	0·19	0·20	0·22	0·24	0·25	0·25
+ Acetate	0·08	0·24	0·33	0·36	0·36	0·41	0·43	0 44
				Stigmasterol				
	0·04	0·10	0·11	0·12	0·12	0·12	0·13	0·14
+ Acetate	0·07	0·21	0·23	0·26	0·28	0·31	0·32	0·33

Medium A minus acetate, adenylic acid; 6MeP 2 μg/ml.

comparisons in which various sterols were tested at low concentrations in the same experiment showed that β-sitosterol was the most active sterol (Table 3). Stigmasterol, cholesterol and ergosterol were less active, but about equal to one another.

Like acetate, the sterols also were of limited effectiveness. While smaller amounts were required and activity was present even without the addition of adenine, there was a point beyond which added sterol was without effect. Since the presence of acetate improved growth over that obtained with sterols, it was apparent that still other substances are active. It was considered possible that the unknown materials might be

* Obtained through the courtesy of Karl Folkers.

products peculiar to the metabolism of the ciliate. Therefore, a lipid extract of *Tetrahymena* was chromatographed on silicic acid by the method of Fillerup and Mead.[17] The most active fraction obtained was the one containing the phospholipids.

Various commercial preparations of animal and plant phospholipids (lecithins) were then tested for activity. Considerable variation was found between preparations of supposedly similar material. For this reason, one of the more active preparations was chromatographed on silicic acid by the method of Marinetti *et al.*,[18] but none of the phospholipid fractions showed any significant activity alone, or when all were combined; on the other hand, when stigmasterol was present in the medium, all the

TABLE 4. EFFECT OF LIPIDS ON INHIBITION BY 6-METHYLPURINE

Additions	μg/ml							
	0		10		50		100	
	a	b	a	b	a	b	a	b
	Optical Density							
Lipositol	0·03	0·11	0·3	0·21	0·04	0·26	0·07	0·25
Phosphatidyl Ethanolamine	0·03	0·10	0·02	0·20	0·03	0·27	0·03	0·15
Inositol Phosphatides	0·03	0·11	0·03	0·18	0·04	0·23	0·03	0·21
Glycolipids	0·04	0·11	0·04	0·17	0·05	0·24	0·05	0·28
Fatty Acids								
soy bean	0·03	0·13	0·03	0·16	0·05	0·24	0·07	0·25
C_{20}unsat.	0·03	0·12	0·03	0·19	0·04	0·22	0·03	0·23

6-methylpurine 2 μg/ml. a = medium A minus acetate and adenylic acid. b = same as a plus stigmasterol, 50 μg/ml.

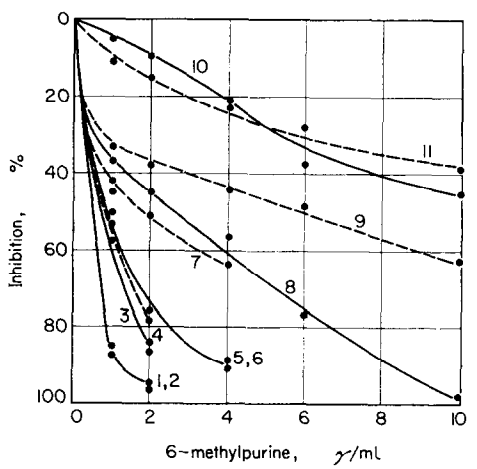

FIG. 2. Effect of combinations of active compounds in reversal of inhibition by 6-methylpurine; Curve 1, no addition; Curve 2, adenine (10 μg/ml); Curve 3, acetate (1 mg/ml); Curve 4, enzymatically hydrolyzed vitamin-free casein (1 mg/ml); Curve 5, lecithin (100 μg/ml); Curve 6, stigmasterol (50 μg/ml); Curve 7, casein hydrolyzate (Curve 4) + stigmasterol (Curve 6); Curve 8, lecithin (5) + stigmasterol (6); Curve 9, lecithin + stigmasterol + casein hydrolyzate; Curve 10, lecithin + stigmasterol + casein hydrolyzate + adenine; Curve 11, lecithin + stigmasterol + casein hydrolyzate + adenine + acetate.

fractions were active. It would appear that the more active of the commercial samples were those which contained a certain proportion of sterols.

In view of the fact that all fractions showed some activity, in the presence of sterols, no attempt was made to characterize the material present in each. Instead, standard isolation procedures were employed to prepare compounds such as lipositol,[9] phosphatidyl ethanolamine[10] Folch inositides[11] or glycolipids. Table 4 shows the ineffectiveness of these compounds in the absence of sterol and their effectiveness in its presence. There was little difference between the various lipids and, in fact, a mixture of unsaturated fatty acids (C_{20} unsaturated) gave essentially the same effect as the conjugated lipids. Inositol, on the other hand, had no effect on the inhibition, either alone or in combination with other compounds. Single long chain unsaturated fatty acids showed little activity, but combinations, either from natural sources or prepared from the isolated fatty acids, were effective.

These results suggest that 6MeP interferes with the activation of acetate in the synthesis of compounds of higher molecular weight. Since amino acids are activated by

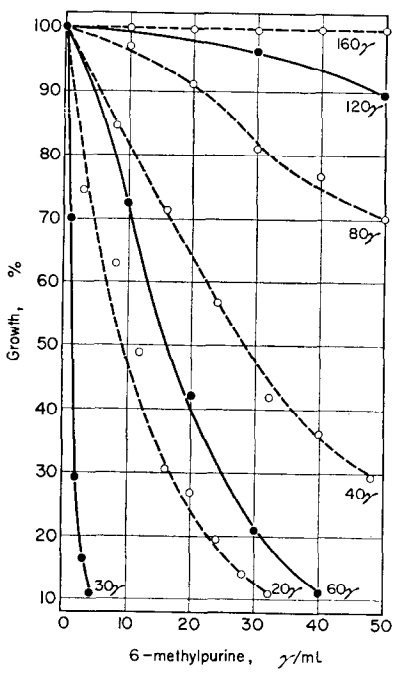

FIG. 3. Response of *Tetrahymena pyriformis* to 6-methylpurine. Open circles, medium A minus acetate and adenylic acid. Solid circles, medium A minus acetate and adenylic acid, plus stigmasterol 50 μg/ml, lecithin 100 μg/ml, and enzymatic hydrolysate of vitamin-free casein 1 mg/ml. Numbers beside curves indicate adenine concentration/ml.

similar mechanisms,[19] it seemed possible that peptides might also have an effect on 6MeP-inhibition. Therefore, an enzymatic hydrolysate of vitamin-free casein was tested. From Fig. 2 it is apparent that peptides were also capable of antagonizing the effect of 6MeP, particularly in the presence of sterol and lecithin. While crude lecithin was used in these experiments, entirely similar results were obtained with the more

purified preparations. Single simple dipeptides, such as leucylglycine, caused only small effects.

Included in the figure are the results obtained when adenine (10 μg/ml) was added in the presence of these various compounds. Under these conditions, in contrast to the addition of adenine alone, the effect was marked, especially at lower concentrations of 6MeP. The addition of acetate instead of adenine had no effect, but when added in the presence of adenine at 10 μg/ml a slight stimulation occurred. Therefore, it must be assumed that still other products of acetate metabolism (such as carotenoids and coenzyme Q) are capable of influencing the response to 6MeP.

As shown in Fig. 3, adenine in sufficiently high concentration completely reversed the inhibitory effect of 6MeP. Concentrations of adenine above 160 μg/ml were not tested, since adenine itself becomes inhibitory above this level. Also, it is apparent from the figure that the presence of sterols, lecithin and peptides considerably enhanced the activity of adenine.

A few experiments similar to those described above have been carried out using 6-methyl-deazapurine as the inhibitor. The pattern of reversal appears to be very similar to that of 6MeP, in that the effectiveness of adenine was enhanced by the presence of acetate, sterols and lipids. In the absence of adenine these compounds were relatively ineffective, as was adenine alone. Death of a certain proportion of the cells occurred even in the presence of the reversing compounds, so that definite quantitative results cannot be presented. Death and lysis of the cells, instead of clarifying the cultures, led to increased turbidity through the release into the medium of fat globules, glycogen granules or other formed elements.

DISCUSSION

A possible interpretation of the above experiments is that 6MeP exerts a multiple inhibitory effect, having as its source the inhibition of the activation of acetate and amino acids, and perhaps other similar compounds as well. These effects are mediated through adenosine triphosphate (ATP), so that 6MeP may prove to be a specific analog of adenine in the synthesis or utilization of ATP or other coenzymes, as well as in nucleic acid synthesis. The inactivity of 6-methylguanine or 6-methylpyrimidine points to a very definite specificity of inhibition.

While 6-mercaptopurine did not inhibit *Tetrahymena*, it is a potent inhibitor in other systems. Thus, in *Escherichia coli* cultures, Bolton and Mandel[20] reported inhibition of acetate incorporation by 6-mercaptopurine, and in cultures of cells of Sarcoma 180, Biesele[21] found that mitotic aberations produced by 6-mercaptopurine or 6-thioguanine[22] were relieved by the presence in the medium of either coenzyme A or thioctic acid (α-lipoic acid) or both. Biesele[21] has suggested that there may be interference with the metabolism of 2-carbon compounds in such inhibited cultures.

That acetate and therefore sterols may have an even more intimate connection with ATP-metabolism is demonstrated by the fact that stigmasterol overcame the inhibitory effect of dinitrophenol[23]. Here the action of the sterol appears to have been on the uncoupling of phosphorylation by dinitrophenol, rather than on ATP-synthesis, although the two inhibitors might prevent acetate activation through different mechanisms.

The results obtained with 6MeP resemble those described by Elion[24] using synergistic combinations of inhibitory analogs. In both cases, much larger concentrations

of the reversing compounds were required to release the inhibition than is the case with inhibitors assumed to have a single effect. This finding serves as another illustration of the possible multiple nature of the inhibition produced by 6MeP.

Although 6MeP is toxic in higher animals[25] and relatively ineffective against Sarcoma 180[26, 27] it may prove useful as a tool in studies of adenine metabolism in certain systems.

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