which demethylate the 10-position. Pteroylaspartic acid and 4-amino-pteroylaspartic acid spare, but do not replace, folic acid. Citrovorum factor is never more active, and usually less active, than folic acid.

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Growth Inhibition in Tetrahymena by Folic Acid Analogs.* (18984)

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(Introduced by C. A. Stuart.)
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Tetrahymena geleii is at present the only organism known, with the exception of *Streptococcus faecalis* 8043(1), which can utilize Aminopterin, 4-amino-pteroylglutamic acid, in place of folic acid(1). This compound has proved to be an effective inhibitor of a variety of other organisms(2-5), but complete reversal of the effects of high concentrations can be obtained in such cases only by citrovorum factor(2,6-9), by folic acid and/or desoxyribosides(2,5,10,11). Methopterin, N^{10}-methylpteroylglutamic acid, was also found(1) to have some activity for *Tetrahymena* when used in place of folic acid. However, this compound proved to be slightly inhibitory when tested at sufficiently high concentrations.

The effect on the growth of *T. geleii* of other antifolics was, therefore, of interest. The present paper deals with the inhibition obtained with 4-amino-9-methylpteroylglutamic acid (A-ninopterin), 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 4-amino-9,10-dimethylpteroylglutamic acid (A-denopterin).† These compounds have been shown to have an effect similar to that of Aminopterin in inhibiting mouse leukemias (10,11). A-methopterin reversibly inhibits the growth of *Streptococcus faecalis* R and irreversibly affects the growth of rats(12).

Material and methods. *Tetrahymena geleii* W was grown in Medium A(13) in which Tween 80 (10 mg/ml) was substituted for Tween 85. Preparation of the inoculum and all other conditions of growth have been previously described(1).

Results. Results with folic acid analogs were extremely variable quantitatively, although they were in good agreement qualitatively. Attempts to eliminate this variation by standardization of technics were made. Slight variations in the age and size of the inoculum made appreciable differences in quantitative responses. It was necessary, therefore, to repeat the experiments a large

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TABLE I. Comparison of the Inhibitory Effects of A-ninopterin, A-methopterin and A-denopterin.

Folic acid, γ/ml	A-ninopterin γ/ml*			I.I.†	A-methopterin γ/ml*			I.I.†	A-denopterin γ/ml*			I.I.†
.0005	.545 ±	.233‡	(3)§	1090	.209 ± .074‡	(2)§		418	.091 ± .008‡	(6)§		182
.001	.920 ±	.240	(3)	920	.208 ± .057	(10)		208	.093 ± .014	(16)		93
.002	2.12 ±	.52	(3)	1060	.223 ± .056	(16)		111	.138 ± .037	(20)		69
.004	3.69 ±	.29	(3)	920	.255 ± .073	(17)		64	.177 ± .057	(17)		44
.005	4.82 ±	.28	(3)	960								
.008					.298 ± .087	(19)		37	.217 ± .070	(13)		27
.01	8.65 ±	1.7	(4)	865								
.016					.324 ± .093	(14)		20	.278 ± .082	(11)		17
.02	16.6 ±	3.3	(3)	830								
.032					.393 ± .102	(12)		12	.450 ± .133	(12)		14
.05	42.2 ±	10.9	(3)	844								
.064					.554 ± .164	(8)		8.5	.648 ± .126	(4)		10
.10	75.5 ±	19.4	(3)	755								
.128					.699 ± .201	(2)		5.5	1.57 ± .017	(3)		12
.256									2.64 ± .032	(2)		10
.512									7.3 —		(1)	14
1.024									36 —		(1)	35
2.048					7.40 —		(1)	3.6	88 —		(1)	44
4.096					15.2 —		(1)	3.7				
8.192					31.2 —		(1)	3.8				

* Amount required for half max inhibition.

† Inhibition index defined as molar ratio between amount of metabolite present and amount of inhibitor required for half max inhibition at given concentration.

‡ Stand. dev.

§ No. in parentheses indicates No. of experiments.

number of times in order to obtain reliable averages. However, once these data had been obtained, the effects of the addition of other compounds could be studied with fewer repetitions. It was necessary merely to establish whether or not the results followed a similar pattern.

The 3 compounds tested fall into 2 categories. A-ninopterin behaved as a strictly competitive antagonist (Table I) over the entire range tested (0.0005-0.1 γ/ml). The inhibition index was nearly constant whether folic acid or citrovorum factor was used. On the other hand, both A-methopterin and A-denopterin have inhibition indices which decrease steadily as the concentration of folic acid is increased (Table I), until a fairly constant value is reached. Under certain circumstances it could be demonstrated that A-methopterin actually stimulates growth, when the concentration of folic acid was suboptimal and the concentration of A-methopterin was below the inhibitory level. This accounts for the fact that the amount required for half maximal inhibition at a level of 0.0005 γ/ml is the same or higher than that required at a level of 0.001 γ of folic acid /ml (Table I).

Citrovorum factor was used in place of folic acid in reversing the effects of these antifolic compounds. It was anticipated that the results would indicate a strictly competitive relationship, such as has been described in the case of Aminopterin(2). However, the data obtained were almost identical with those for folic acid. The citrovorum factor used was a synthetic product† and similar results were obtained with synthetic folinic acid.‡

The addition of various materials either known to participate or suspected of participating in folic acid metabolism has been shown to increase the effectiveness of folic acid in reversing the inhibition due to Aminopterin (2,5,9,11). It may be seen (Tables II and III) that the presence of vitamin B_{12}, thiamine, desoxyribonucleic acid (DNA), ribonucleic acid (RNA) or glycine did not have the effect of making the inhibition competitive with folic acid. In all cases the release was somewhat more effective in the presence of these compounds, although thymine, in particular, increased the inhibition at low levels

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TABLE II. Effect of Addition of Compounds Concerned in Folic Acid Metabolism Upon the Inhibition Produced by A-methopterin.

Folic acid, γ/ml	Additions: None	B_{12} (.0001 γ/ml)	B_{12} (.001 γ/ml)	Thymine (40 γ/ml)	DNA (500 γ/ml)	RNA (500 γ/ml)
.0005	.209* ± .074† (2)‡			.145* ± .035† (4)‡		
.001	.208 ± .057 (10)	.190* ± .010† (2)	.180* ± .000† (2)‡	.182 ± .053 (4)		
.002	.223 ± .056 (16)	.221 ± .004 (2)	.210 ± .012 (2)	.212 ± .055 (6)	.102* —† (1)‡	.231* ± .089† (4)‡
.004	.255 ± .073 (17)	.282 — (1)	.293 — (1)	.271 ± .068 (6)	.182 ± .044 (3)	.265 ± .099 (5)
.008	.298 ± .087 (19)	.340 — (1)	.340 — (1)	.331 ± .072 (12)	.222 ± .048 (3)	.346 ± .128 (5)
.016	.324 ± .093 (14)			.409 ± .083 (12)	.277 ± .062 (3)	.392 ± .150 (5)
.032	.393 ± .102 (12)			.495 ± .109 (5)	.326 ± .086 (3)	.502 ± .201 (5)
.064	.554 ± .164 (8)			.664 ± .169 (5)	.375 ± .005 (2)	.632 ± .316 (4)
.128	.699 ± .201 (2)			.957 ± .394 (3)		
.256				1.60 ± .73 (3)		
.512				2.20 — (1)		
1.024				4.35 — (1)		
2.048	7.40 — (1)			9.60 — (1)		
4.096	15.2 — (1)			22.6 — (1)		
8.192	31.2 — (1)			41.2 — (1)		

* Amt required for half max inhibition (γ/ml). † Stand. dev. ‡ No. in parentheses indicates No. of experiments.

TABLE III. Effect of the Addition of Compounds Concerned in Folic Acid Metabolism Upon the Inhibition Produced by A-denopterin.

Folic acid, γ/ml	Additions: None	B_{12} (.0001 γ/ml)	B_{12} (.001 γ/ml)	Thymine (40 γ/ml)	DNA (500 γ/ml)	RNA (500 γ/ml)	Glycine (100 γ/ml)
.0005	.091* ± .008† (6)‡			.076* ± .027† (2)‡	.064* ± .007† (2)‡	.215* —† (1)‡	
.001	.093 ± .014 (16)	.092* ± .017† (2)‡	.085* ± .021† (3)‡	.096 ± .034 (7)	.089 ± .006 (2)	.258 — (1)	.152* —† (1)‡
.002	.138 ± .037 (20)	.128 ± .033 (2)	.121 ± .031 (3)	.176 ± .070 (8)	.145 ± .040 (2)	.420 — (1)	.204 ± .057 (4)
.004	.177 ± .057 (17)	.168 ± .053 (2)	.153 ± .058 (3)	.217 ± .105 (6)	.238 ± .068 (2)		.292 ± .086 (3)
.008	.217 ± .070 (13)	.216 ± .079 (2)	.204 ± .073 (3)	.338 ± .154 (4)	.374 ± .106 (2)		
.016	.278 ± .082 (11)	.401 ± .119 (2)	.397 ± .077 (3)	.985 ± .325 (2)	.740 — (1)		
.032	.450 ± .133 (12)	.570 ± .120 (2)	.557 ± .100 (3)	1.66 ± .44 (2)			
.064	.648 ± .126 (4)						
.128	1.57 ± .017 (3)						

* Amt required for half max inhibition (γ/ml). † Stand dev. ‡ No. in parentheses indicates No. of experiments.

of folic acid.

It is of interest that fermentation folic acid and folic acid conjugate had exactly the same degree of effectiveness as folic acid on a molar basis.

Since Aminopterin can replace folic acid in the nutrition of *T. geleii*, it was used in an attempt to reverse the inhibition produced by these compounds. It was found to have the same efficiency in reversal as it has in growth promotion (18%).

Discussion. At the present time only conjectures can be offered in explanation of the results obtained with the antifolic compounds. It must be assumed that Aminopterin is either utilized as such or is deaminated to folic acid by *Tetrahymena*(1). There is no means of deciding at the moment which process occurs. However, it is more logical to assume that folic acid (or possibly the citrovorum factor) is the only growth-promoting compound in the group under consideration.

If it is true that *T. geleii* is capable of deaminating the 4-position of the pteridine ring, then it is possible that all of the analogs used in the present study are also deaminated. If that were the case, then A-methopterin would be converted to Methopterin, a compound which has growth-promoting activity in the concentrations used in these experiments. Thus, although only 0.0005 γ of folic acid/ml was added to the medium, the presence of a relatively large amount of deaminated A-methopterin would considerably increase the "folic acid activity". As the amount of folic acid in the medium increases, the contribution of the methyl folic acid would become relatively less important and would eventually become negligible. At this point one would expect the inhibition index to become constant or nearly so, as it does. The fact that A-methopterin is capable of sparing suboptimal amounts of folic acid is an indication that it can be converted to an active compound.

From the data at hand it is possible to calculate the amount of A-methopterin deaminated. In the expression $y = kx$, if y is the concentration of the metabolite and x is the concentration of the inhibitor required for half maximum inhibition, then k is the reciprocal of the inhibition index. In the case of A-methopterin, this index becomes constant at a value of about 4. It has previously been reported(1) that Methopterin can replace folic acid with an activity of about 2%. If, then, one lets z equal the amount of A-methopterin deaminated the following relationship would hold: $y + 0.02\ z = k\ (x–z)$. Substituting the values from Table I into this equation and solving for z, one obtains the values shown in Table IV. It may be seen that the values for z are reasonably constant. They might be expected to be somewhat lower at the lower concentrations of folic acid where growth is not quite optimal.

TABLE IV. Deamination of Folic Acid Analogs by Tetrahymena.

A-methopterin			A-denopterin		
x*	y†	z‡	x*	y†	z‡
.209	.0005	.192	.091	.0005	.086
.208	.001	.190	.093	.001	.083
.223	.002	.199	.138	.002	.118
.255	.004	.221	.177	.004	.137
.298	.008	.246	.217	.008	.137
.324	.016	.241	.278	.016	.118
.393	.032	.245	.450	.032	.130
.554	.064	.276			
.699	.128	.172			

* Amt (γ/ml) of inhibitor required for half max inhibition.
† Amt (γ/ml) of folic acid present.
‡ Calculated amt (γ/ml) deaminated.

The values for z in the case of A-denopterin (Table IV) were obtained in a somewhat different manner. Since nothing is known of the properties of the deamination product of A-denopterin, it is not possible to use the relationship given for A-methopterin. If one assumes that 9,10-dimethylpteroylglutamic acid neither promotes nor inhibits growth, then the equation becomes: $y = k\ (x–z)$. A value of $k = 1/10$ was used, since it gave the most constant values of z and is reasonably close to observed values. It may be seen however, that these values are not as constant as those obtained in the case of A-methopterin. This may be attributed to the fact that we do not know the properties of 9,10-dimethylpteroylglutamic acid in the metabolism of *Tetrahymena*.

The inhibition indices of A-methopterin and A-denopterin, when they reach constant values, are rather low, indicating that the compounds are very inhibitory. It is this fact that is

responsible for the peculiar behavior of the inhibition indices of these compounds at intermediate concentrations of folic acid, since A-ninopterin, which is much less inhibitory, behaves as one would expect of a strictly competitive inhibitor. Since the amounts of A-ninopterin are relatively large, it is of little consequence whether or not small amounts are deaminated.

Summary. The antifolics, 4-amino-9-methylpteroylglutamic acid (A-ninopterin), 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 4-amino-9,10-dimethylpteroylglutamic acid (A-denopterin) are all inhibitory to *Tetrahymena*. The inhibition produced by A-ninopterin is strictly competitive, being released over wide ranges of concentration of folic acid. The inhibition indices of the other two analogs decrease as the amount of folic acid in the medium increases until a certain level is reached, at which they become constant. This shifting index is tentatively explained on the basis of the ability of *Tetrahymena* to deaminate the 4-position of the analogs.

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Effect of Estrogen-Treatment and Castration on ACTH Content of Rat Adenohypophysis.* (18985)

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In a previous paper(1) we were able to show that the ACTH content of the rat's adenohypophysis is not affected by the severe depletion of acidophilic and beta-granular material which follows thyroidectomy. In the present study we sought to determine the effect of radical changes in the amount of the third described type of adenohypophysial secretory granulation, that of the delta cells, on the ACTH stores of the pituitary. For this purpose, the hypophyses of estrogen-treated and castrated male rats were assayed for their ACTH content against those of controls. Massive doses of estrogen were previously found to lead to a dramatic reduction in the number of delta cells(2), whereas castration is characterized by a progressive hypertrophy and hyperplasia of this cell type(3).

Method. The pituitaries of 46 young adult male Sprague-Dawley rats were used in this experiment. Twelve rats received daily injections of 62.5 μg α-estradiol in sesame oil over a period of 32 days, 10 rats were castrated and kept for 30 days and 24 intact animals served as controls. At the time of their sacrifice the estrogen-treated rats weighed 215-245 g (mean 226 g), the castrates 288-345 g (mean 313 g) and the controls 288-350 g (mean 328 g). All animals were killed by decapitation. The pituitaries of 4 castrates, 4 estrogen-treated and 6 intact rats were saved for quantitative histological examination. The remaining hypophyses were prepared for ACTH-assay by the method of Richards and Sayers(4). The simultaneous assay of the 3 groups of pooled donor pituitaries was carried out on hypophysectomized rats by the adrenal ascorbic acid depletion method of Sayers *et al.*(5). The recipients, the administered doses of the extracts, the procedure for the determination of the adrenal ascorbic acid concentration and the method for the statistical analysis of the assay data, as well as the

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