# Further Studies on Effect of Folic Acid Analogs on Growth of *Tetrahymena.* (20426)

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Folic acid analogs in which the hydroxyl group in position 4 is replaced by an amino group are potent inhibitors of the growth, not only of mammals, but also of bacterial and leukemic cells(1-3). However, both bacteria and leukemic cells may develop resistance to such inhibitors(4-8). *Tetrahymena* is naturally resistant to aminopterin and for that reason it was felt that other folic acid analogs capable of inhibiting the growth of *Tetrahymena* might prove useful in inhibition of aminopterin resistant cells.

A number of 3′, 5′ dichloro-substituted folic acid derivatives(9) as well as a number of pteroyl compounds containing an amino acid residue other than that of L-glutamic acid(10) have been tested for their effect on the growth of *Tetrahymena.*

*Materials and methods. Tetrahymena pyriformis (geleii)* W(11) was used. The media and other conditions of growth have been described previously(12,13).†

*Results.* Of the 20 folic acid analogs tested, it was found that 2 had the ability of replace folic acid for growth, although the activity was very low. Pteroyl-D-glutamic acid (concentrations from 0.01 to 0.1 γ/ml tested) had an activity of 3.6% and 4-dimethylaminopteroylglutamic acid, 0.43% (concentrations from 0.01 to 1.0 γ/ml tested). The analogs containing various amino acid residues in place of or in addition to glutamic acid (Table I) all showed some activity in sparing folic acid, although none would replace it when tested in concentrations up to at least 0.1 γ/ml. Such of these analogs as contained amino acids required by *Tetrahymena* were also tested for their ability to replace the amino acid. None showed any evidence of activity in this respect at concentrations up to 100 γ/ml. One compound, 3′, 5′-dichlorodiopterin exhibited both sparing and inhibitory effects.

TABLE I. Compounds Having a Sparing Effect on Folic Acid.

| Compound | Conc. range tested (γ/ml) |
|---|---|
| 4-dimethylamino-$N^{10}$-methyl PGA* | .1 –50. |
| Pteramidomalonic acid | .1 –50. |
| 4-aminopteramidomalonic acid | .1 –50. |
| Pteroyl-DL-alanine | .1 – 1.0 |
| Pteroyl-DL-seryl-L-glutamic acid | .01– .1 |
| 4-aminopteroyl-DL-seryl-L-glutamic acid | .01– .1 |
| 4-aminopteroyl-DL-valine | .01– .1 |
| 4-aminopteroyl-DL-isoleucine | .01– .1 |
| 4-aminopteroyl-DL-serine | .01– .1 |
| 3′, 5′-dichlorodiopterin* | .05– .5 |
| Pteroylglycine | .1 –10. |

* Inhibitory at higher levels.

The remaining 9 compounds were all competitive antagonists of folic acid (Table II). Only one, 3′, 5′-dichloro-$N^{10}$-methylpteroylglutamic acid had an inhibitory index small enough to give promise of usefulness.

It has recently been demonstrated(14,15) that aminopterin is grossly contaminated with folic acid. Material obtained as a major peak from the ion-exchange column was found to have no effect on the growth of *Tetrahymena,* either stimulatory or inhibitory. A minor peak showed all the characteristics of folic acid and was active in promoting growth at equivalent concentrations.

*Discussion.* The evidence now indicates that the variations in the inhibition index obtained with A-methopterin and A-denopterin(16) are probably due to the presence of folic acid or methyl folic acids in the original material rather than to deamination by the organisms as originally supposed. This may also explain the ability of these analogs to substitute for aminopterin in the growth of aminopterin-resistant bacteria. Folic acid

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TABLE II. Compounds Antagonistic to Folic Acid.

| Compound | Inhibitory index | Conc. range tested ($\gamma$/ml) |
|---|---|---|
| $N^{10}$-methylpteroylglutamic acid* | ‡ | .1 – 20. |
| 4-aminopteroylglutamic acid | i§ | .001– .1 |
| 4-dimethylaminopteroylglutamic acid | 1600 | .001– 1. |
| 3′, 5′-dichloropteroylglutamic acid | 500–600 | .1 – 5. |
| 3′, 5′-dichloro-4-aminopteroylglutamic acid | 600 | .1 – 64. |
| 3′, 5′-dichloro-$N^{10}$-methylpteroylglutamic acid | 15– 20 | .05 – 2. |
| 4-amino-$N^{10}$-methylpteroylglutamic acid* | 4 | .1 – 50. |
| 9-methylpteroylglutamic acid* | 500–750 | 1.0 – 16. |
| 4-amino-9-methylpteroylglutamic acid* | 800 | .1 –100. |
| 9, 10-dimethylpteroylglutamic acid | 200–300 | .1 –160. |
| 4-amino-9, 10-dimethylpteroylglutamic acid* | 10 | .01 –100. |
| 3′, 5′-dichloro-9, 10-dimethylpteroylglutamic acid | 125–175 | .1 – 8. |
| 3′, 5′-dichloro-4-amino-9, 10-dimethylpteroylglutamic acid | 150 | .1 –400. |
| 3′, 5′-dichlorodiopterin† | 1700 | .5 – 80. |

* See reference 13.
† 3′, 5′-dichloropteroyl-$\alpha$-glutamyl-glutamic acid.
‡ Inhibition slight and variable at high levels.
§ i = inert.

contamination is also probably the explanation of the effects of pteroyl-D-glutamic acid and 4-dimethylaminopteroylglutamic acid.

It is interesting to note that the introduction of the 4-amino group into 3′, 5′-dichloropteroylglutamic acid has little effect on the inhibitory index, while its introduction into either of the methylated folic acids greatly increases the inhibitory effect. This increase in inhibition is also obtained by the introduction of the dichloro substitution into the 10-methyl compound. Neither alone gives much inhibition; together they give one of the most inhibitory of the compounds studied.

Of the dihalogenated compounds reported by Cosulich *et al.*(9) the most inhibitory appear to be those with the 4-amino substitution, especially when this is combined with a 9- and/or 10-methyl substitution. However, these results apply to a strain of *Streptococcus faecalis* sensitive to aminopterin. It is possible that cells with a resistance to aminopterin, either natural or acquired, would show a different pattern of response to the halogenated analogs, as does *Tetrahymena*. For this reason we suggest that 3′, 5′-dichloro-$N^{10}$-methylpteroylglutamic acid might prove to be a better inhibitor of aminopterin-resistant cells than it is of the sensitive cells.

The fact that the amino acid analogs of folic acid cannot be utilized as sources of amino acids indicates that the bond between the *p*-aminobenzoic acid and the amino acid portion of the molecule cannot be hydrolyzed. This would indicate that folic acid cannot be utilized as a source of PABA(17). How these compounds act in sparing folic acid is difficult to explain unless certain apoenzymes exhibit little or no specificity for the amino acid portion of the folic acid molecule.

*Summary.* 1. The growth of *Tetrahymena pyriformis,* which was produced when aminopterin was added to a folic-acid deficient medium, was due to the presence of folic acid in the aminopterin. 2. A similar contamination may explain the replacement effects of pteroyl-D-glutamic acid and 4-dimethylaminopteroylglutamic acid. 3. Folic acid is spared by a number of folic acid analogs in which the glutamic acid is replaced by other amino acids. 4. These compounds cannot serve as sources of amino acids. 5. Of several 3′, 5′-dichloro-substituted folic acid analogs, the most inhibitory is 3′, 5′-dichloro-$N^{10}$-methylpteroylglutamic acid. 6. It is suggested that this compound may also be effective in inhibiting aminopterin-resistant cells.

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## Influence of Dietary Factors and the Adrenal on Hyaluronidase Spreading Reaction in the Rat.* (20427)

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The influence of adrenalectomy and adrenal cortical hormones on the spreading reaction of hyaluronidase has been studied in mice and rabbits(1). Conflicting results have been reported in the rat(2,3). The present study is concerned with observations on the intradermal spread of hyaluronidase in intact, cortisone treated intact, in adrenalectomized or sham operated rats, and the influence of nutritional factors on the reaction.

*Materials and methods.* A total of 185 adult male and female rats, bred in the laboratory, were used in these experiments. Both intact and adrenalectomized rats received a 1% sodium chloride solution as drinking water. No hormonal therapy was given the adrenalectomized rats. One group of intact rats was given 5 mg of cortisone intramuscularly daily for 4 days prior to the skin test. One hour before the skin test was performed another dose of cortisone was given. The rats received one of the following *diets*: 1) a dog chow diet; 2) an experimental diet adequate in all nutritional substances for the rat; 3) the experimental diet supplemented with excessive amounts of calcium pantothenate (43.6 mg per 100 g of diet); 4) the experimental diet supplemented with large amounts of vit. $B_{12}$ (50 $\mu$g of $B_{12}$ concentrate/100 g diet); and 5) the experimental diet deficient in pantothenic acid. The composition of the experimental diet (per 100 g diet) was as follows: vitamin free casein 22 g, sucrose 62 g, primex 9 g, salt mixture (ferric citrate 2.3%, calcium phosphate dibasic 44%, potassium citrate 42%, magnesium citrate 11.6%, copper sulfate 0.8%, and potassium iodide 0.01%) 5 g, cod liver oil 2 g, thiamine 0.3 mg, pyridoxine 0.3 mg, riboflavin 0.5 mg, folic acid 0.1 mg, biotin 0.05 mg, calcium pantothenate 1.0 mg, niacine 1.0 mg, choline 30.0 mg, inositol 5 mg, mixed tocopherols 7.5 mg, and synthetic vit. K 5 mg. The animals on the dog chow diets were divided into 4 groups. *One group* was skin tested as a control group, *one* received cortisone intramuscularly for 4 days before the skin test, and the *third* group was bilaterally

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