Inhibition of Dihydrofolic Reductase by Aminopterin and Amethopterin.*† (23764)

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(Introduced by C. A. Finch)
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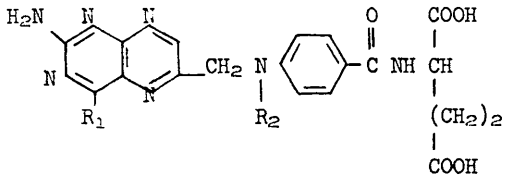
Since the folic acid antagonists, Aminopterin (I, $R_1 = NH_2$, $R_2 = H$) and Amethopterin (I, $R_1 = NH_2$, $R_2 = CH_3$) have proved to be of considerable value in the treatment



of certain human and experimental leukemias (see(1) for a general review of this subject), it would be of interest to ascertain the specific site of action of these antagonists at the enzymatic level. Extensive studies by Nichols (2) have shown that the inhibition occurs at some point in the conversion of the vitamin, folic acid. (I. $R_1 = OH$, $R_2 = H$) to folinic acid (N^5-formyl, 5,6,7,8-tetrahydrofolic acid). From recent findings, reviewed elsewhere(3,4, 5), it is now known that 5,6,7,8-tetrahydrofolic acid is the coenzyme form of folic acid (*i.e.* the carrier for one-carbon fragments at the oxidation level of formate and formaldehyde), and that folinic acid is probably a stable storage form for tetrahydrofolic acid. These relationships are outlined in equation (1):

$$\text{Folic} \longrightarrow \text{Dihydrofolic} \longrightarrow \text{Tetrahydrofolic} \longrightarrow \text{Folinic} \qquad (1)$$

As a secondary effect, understandable from the above considerations, inhibition by folic acid antagonists has been observed with certain of the metabolic reactions which involve a folic acid coenzyme, *e.g.* incorporation of formate into purines(6) or thymine(7), or interconversion of serine and glycine(8). The second step in the conversion of folic acid to the coenzyme form (*i.e.* reduction of FH_2 to FH_4)§ is mediated by a TPN-linked dehydrogenase, which has recently been obtained in purified form from chicken liver(9).†† The stoichiometry of the reaction is shown in equation (2):

$$FH_2 + TPNH + H^+ \longrightarrow FH_4 + TPN^+ \qquad (2)$$

In this communication evidence is presented that the dihydrofolic reductase is inhibited non-competitively by extremely low concentrations of Aminopterin and Amethopterin.

Materials and methods. TPNH was a product of the Sigma Chemical Co. Folic Acid was obtained from the California Foundation for Biochemical Research, 9, 10-dimethyl folic acid from the Lederle Laboratories, Aminopterin from the Mann Research Laboratories, and Amethopterin from the Bios Laboratories. The absorption spectra of Aminopterin and Amethopterin in 0.1 N NaOH or 0.1 N HCl agreed well with the published spectra(10) of these compounds. Paper chromatographic examination of the antagonists in 3 solvent systems ((a) 1 M sodium formate containing 2% formic acid; (b) 0.1 M glycine, pH 9.5, containing 2% sodium versenate; and (c) ethanol:water (70:30 v/v)) revealed in each case a principal component with traces of other fluorescent materials; such inhomoge-

* This work was supported by grants from U.S. P.H.S., Life Insurance Medical Research Fund, and Initiative 171. State of Washington.

† Paper VI in *Folic Acid Coenzymes and Active One-Carbon Units;* for Paper V see M. J. Osborn, E. N. Vercamer, P. T. Talbert, and F. M. Huennekens, *J. Am. Chem. Soc.*, 1957, v79, 6565.

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§ The following abbreviations are used: F, folic acid; FH_2, 7,8-dihydrofolic acid; FH_4, 5,6,7,8-tetrahydrofolic acid; f^5FH_4, $f^{10}FH_4$, N^5-formyl and N^{10}-formyl FH_4; ATP, ADP, adenosine tri- and diphosphate; P_i, inorganic phosphate; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide.

†† S. Futterman (*J. Biol. Chem.*, 1957, v228, 1031) has also studied the enzymatic reduction of folic acid in chicken liver preparations and has observed a similar inhibition by the folic acid antagonist, Aminopterin.

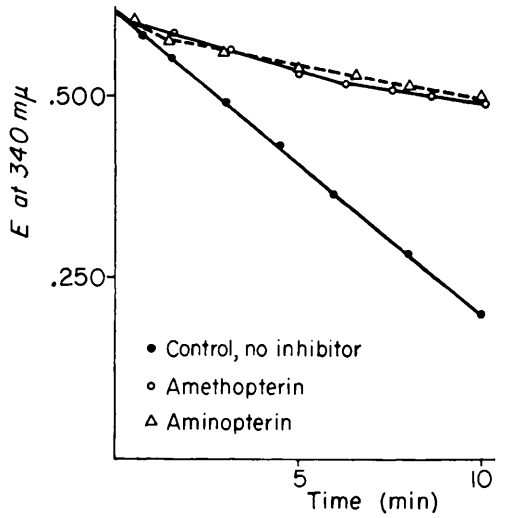

FIG. 1. Standard spectrophotometric assay system contained (in 1 cm Corex cuvette) .03 ml of purified FH_2 reductase, .07 μmoles of FH_2, .10 μmoles of TPNH, 50 μmoles of phosphate buffer, pH 7.5, 10 μmoles of 2-mercaptoethanol, and 1.4×10^{-4} μmoles of Aminopterin or Amethopterin, where indicated, in volume 1.25 ml. Decrease in light absorption (E) at 340 mμ was measured against time, and values obtained were corrected for small changes in separate, blank cuvettes omitting FH_2 or TPNH.

neity is routinely observed during paper chromatography of folic acid compounds, and is reminiscent of the known lability of the flavin nucleotides(11) under similar conditions. FH_2 was prepared by catalytic hydrogenation of folic acid in 0.1 N NaOH, essentially according to the method of O'Dell *et al.*(12). The purification of the FH_2 reductase, and the spectrophotometric assay for reaction(2) are described elsewhere(9).

Results. In the presence of purified reductase, FH_2 is rapidly reduced by TPNH, as shown in the control curve of Fig. 1. The addition of Aminopterin or Amethopterin, each at a final concentration of 1.1×10^{-7} M, to the assay system, results in an inhibition of approximately 70%. Higher concentrations (*ca.* 5×10^{-7} M) of the inhibitors produce complete inhibition. It should be noted that the level of substrate, FH_2, used in the experiment (5.3×10^{-4} M) is far above that necessary to saturate the enzyme ($K_m = 5.0 \times 10^{-7}$ M, see below).

A more detailed kinetic study of inhibition by Aminopterin and Amethopterin is presented as the conventional Lineweaver-Burk double reciprocal plots in Fig. 2. The affinity of the enzyme for both inhibitors is extraordinarily high. Calculation of the inhibitor constants yields K_I values of 1.0×10^{-9} M and 2.3×10^{-9} M for Aminopterin and Amethopterin, respectively. It is of interest that the Michaelis constant for FH_2, $K_m = 5.0 \times 10^{-7}$ M,¶ is also unusually low for a substrate of a pyridino-protein enzyme. The fact that the inhibitor curves do not pass through a common intercept on the ordinate axis suggests that both inhibitors act *non-competitively,* rather than competitively. Although folic acid and 9,10-dimethyl folic acid have been shown also to inhibit the dihydrofolic reductase(9), they are effective only at concentrations greater than 10^{-6} M. Thus, the

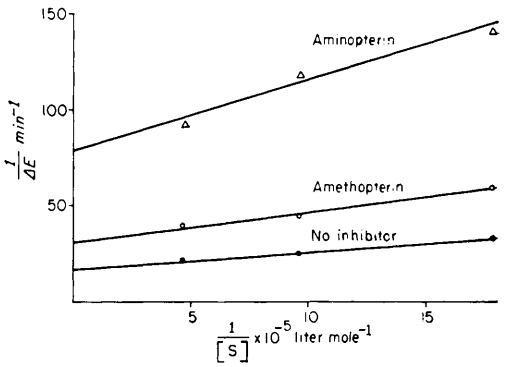

FIG. 2. Silica cells with 10 cm light path initially contained 0.1 ml of purified FH_2 reductase, 0.13 μmoles of TPNH, and 9×10^{-5} μmoles of Aminopterin or 4.4×10^{-5} μmoles of Amethopterin, where indicated, in 25 ml of 0.05 M phosphate–0.01 M 2-mercaptoethanol, pH 7.5. The reaction was started by addition of FH_2 to final conc. of 5.6×10^{-7} M, 1×10^{-6} M, or 2.2×10^{-6} M. Decrease in light absorption at 340 mμ (△E) was measured against time, and initial velocity (expressed as △E/min.) was corrected for the small blank oxidation of TPNH in the absence of FH_2. In the figure the reciprocals of velocity and substrate conc. [S] are plotted as ordinate and abscissa respectively. K_m values for FH_2, and K_I values for inhibitors were calculated by the conventional Lineweaver-Burk method. The extremely low values for K_m and K_I made it necessary to work at high dilutions of FH_2. This, in turn, produced only small changes in TPNH (*cf.* the stoichiometry of equation (2)) and necessitated the use of long optical paths (10 cm) to achieve measurable values of △E.

‖ We are indebted to Dr. P. T. Talbert and Mr. J. G. Ozols for carrying out this preparation.

¶ Separate determinations of K_m for FH_2 have yielded values of 6.0×10^{-7} M. and 3.5×10^{-7} M.

degree of inhibition observed with Aminopterin and Amethopterin is several orders of magnitude greater than could be accounted for on the basis of any possible contamination by folic or methyl folic acids. Moreover, the possibility that other trace contaminants in the antagonists are the actual causative agents for the observed inhibition is unlikely since the inhibitor constants would then be even lower, *i.e.* 10^{-10} or 10^{-11} M.

The inhibition of the dihydrofolic reductase is the first example of an appreciable effect by low levels of Aminopterin and Amethopterin on an isolated enzyme system. Although several investigators(2) have observed an inhibition by these folic acid antagonists on the conversion of folic acid to folinic acid, the exact localization of the block has required the characterization of the individual enzymes participating in the multi-step process shown in equation (1). The reduction of folic acid to FH_2 (*cf.* equation (3)) has recently been studied by Wright and Anderson(13) in extracts of *E. coli.*

$$\begin{array}{ccc} \text{Folic} & \longrightarrow & FH_2 \\ \text{pyruvate} & \curvearrowright & \text{acetate} + CO_2 \end{array} \qquad (3)$$

It was reported that this reaction, which requires some component of the pyruvic oxidase system to supply the reductive power, is not inhibited by Aminopterin. The formate-activating enzyme(14), which mediates the formation of N^{10}-formyl FH_4 according to equation (4) is also not sensitive to

$$\text{ATP} + \text{formate} + FH_4 \longrightarrow f^{10}FH_4 + \text{ADP} + P_i \qquad (4)$$

Aminopterin or Amethopterin.** The conversion of $f^{10}FH_4$ to f^5FH_4 (folinic acid) is still not well understood.

Therefore, it appears that the primary site of action of the 4-amino analogues of folic acid in blocking the conversion of folic acid to its coenzymatically active forms is probably only at the FH_2 reductase reaction. Furthermore, the finding that the inhibition is *non-competitive* with the K_I values of approximately 10^{-9} M adequately accounts for both the physiological potency of the drugs, and also for the relative ineffectiveness of folic acid, even in massive doses, in reversing the toxicity of the antagonists.

Summary. The enzyme, dihydrofolic reductase, which catalyzes the TPNH-linked reduction of dihydrofolic acid to tetrahydrofolic acid, is inhibited non-competitively by the folic acid antagonists, Aminopterin and Amethopterin. Values of 1 x 10^{-9} M and 2.3 x 10^{-9} M were obtained for the inhibitor constants (K_I) of Aminopterin and Amethopterin, respectively.

** Unpublished experiments with Dr. H. R. Whiteley.

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Received December 11, 1957. P.S.E.B.M., 1958, v97.