Biochemical Pharmacology, Vol. 21, pp. 1907–1919. Pergamon Press, 1972. Printed in Great Britain.

FOLATE REDUCTASE FROM MOUSE LIVER. PURIFICATION, ACTIVATION BY METALS AND INHIBITION BY SOME 6-(1-ADAMANTYL) DERIVATIVES

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(*Received* 29 *November* 1971; *accepted* 1 *February* 1972)

Abstract—Some properties of semipurified and highly purified enzyme reducing folate and dihydrofolate isolated from mouse liver have been studied. The reduction of folate at pH 5·5 was strongly activated in nonpurified and semipurified enzyme fractions by some bivalent cations. Mn^{2+} at the concentration 2×10^{-2} M increased the enzyme activity over 300 per cent. The purified enzyme was not significantly activated by Mn^{2+}. In contrast, all fractions of enzyme reducing dihydrofolate were activated by KCl to the same extent (about 180 per cent).

All halogen substituted γ-(1-adamantyl)γ-crotonlactonols tested caused less than 50 per cent inhibition of folate reductase at the concentration 2×10^{-4} M, while some of the 2,4-disubstituted 6-(1-adamantyl) pyrimidines exerted 65–93 per cent inhibition. The most potent inhibitor among these drugs appeared to be 2-hydroxy-4-amino-6-(1-adamantyl) pyrimidine ($K_i = 9{\cdot}3 \times 10^{-7}$ M), which produced greater inhibition of folate reductase than that caused by 2,4-diamino-6-(1-adamantyl) pyrimidine or 2,4-diaminopteridine. Corresponding pyrimidines without the adamantane residue did not exert any inhibition at concentrations up to 4×10^{-4} M.

The inhibition of enzyme activity was strictly pH dependent. The maximum inhibition appeared at about pH 6, whereas at neutral or alkaline pH no inhibition was found. The reduction of dihydrofolate was inhibited by 6-(1-adamantyl) pyrimidines less than the reduction of folate at pH 5·5. No difference was found in the inhibition of the partially purified and highly purified enzyme preparation.

THE INHIBITION of folate reductase has been studied by a number of authors.[1–4] Among the folic acid analogues aminopterin and its derivatives proved to be strong and "stoichiometric" inhibitors of this enzyme.[5] While 2,4-diaminopyrimidines, purines, pteridines, quinazolines, and triazines, without p-aminobenzoic acid residue in their molecules,[6–8] and also compounds related to folic acid or aminopterin with prolonged methylene bridge between the pteridine and p-aminobenzoic acid rings,[9–10] proved to be weaker and not so tightly bound inhibitors of folate reductase. However, some of these drugs showed large differences in the inhibition of bacterial dihydrofolate reductases.[11,12]

Recently the cytostatic effects of several 5-(1-adamantyl) pyrimidines were observed,[13,14] but folate reductase inhibition was not reported. However, 6-(1-adamantyl) pyrimidines proved inhibitory effect on mouse liver folate reductase.[15] The dependence of this inhibition on both the structure of 6-(1-adamantyl) pyrimidine analogues and pH are the main subject of this report. Some characteristics of the mouse liver enzyme reducing folate at pH 5·5 and dihydrofolate at pH 7·5 are also described.

MATERIALS AND METHODS

Folic acid and amethopterin were purchased from Light and Co., England and Lederle Lab., respectively. NADPH was obtained from CalBiochem. Dihydrofolic acid was prepared from purified folic acid by sodium dithionite in the presence of sodium ascorbate according to Futterman.[16]. Amethopterin, folate and dihydrofolate were dissolved in 0·01 M sodium bicarbonate, NADPH in 0·05 M sodium bicarbonate. If necessary these solutions were frozen for a few days. No decrease in their activity was observed under these conditions.

The preparation of all the 6-(1-adamantyl) pyrimidines and γ-(1-adamantyl)-γ-crotonolactonols tested was reported previously.[17–19]. α-brom-γ-(1-adamantyl)-γ-crotonlactonol (X) was dissolved in 0·05 M sodium hydroxide. All the other compounds were dissolved using 20% ethanol. These solutions were diluted further when necessary with distilled water.

Enzyme assays. The reduction of folate was assayed by determination of diazotizable amine split from tetrahydrofolate after deproteinization with 10% trichloracetic acid. The color reaction was measured after 10 min at 550 nm. The incubation mixture contained; folic acid ($1{\cdot}25 \times 10^{-4}$ M), reduced nicotinamide–adenine dinucleotide phosphate (NADPH) ($1{\cdot}56 \times 10^{-4}$ M), $MnCl_2$ ($1{\cdot}25 \times 10^{-2}$ M), sodium citrate (0·125 M) and 0·1 ml of the enzyme preparation in a total volume of 0·8 ml at pH 5·5.

The reduction of dihydrofolate was determined by a spectrophotometric method utilizing the decrease in absorbance that occurs at 340 nm when NADPH + dihydrofolate are converted to NADP + tetrahydrofolate. The difference in absorbances was read on Shimadzu spectrophotometer, Model QV–50, after 10 min of incubation at room temperature. The incubation mixture contained; dihydrofolic acid (1×10^{-4} M), NADPH ($1{\cdot}25 \times 10^{-4}$ M), sodium citrate (0·125 M) and enzyme (0·2 ml) in a total volume of 3 ml at pH 7·5. Each assay of enzyme activity included a reference cuvette from which substrate was omitted. In the course of all measurements the stock solutions and the enzyme preparation were kept on ice.

Protcins in the various enzyme fractions was determined by the Lowry method.[20]

Preparation and purification of the enzyme

pH 4·5 supernatant fraction. Liver harvested from adult mice (H-strain) were homogenized in ice cold 0·05 M citrate buffer pH 4·5 (50 g wet weight/150 ml buffer). The supernatant was obtained by centrifugation at 10,000 *g* for 20 min.

Ammonium sulfate fraction. The pH of the supernatant was adjusted to 7 and solid ammonium sulfate was added up to 30 per cent of saturation. The precipitate was collected by 10 min centrifugation at 6000 *g* and was discarded. The active fraction was precipitated between 30–65 per cent of saturation. The precipitate was collected by centrifugation at 6000 *g*. It was redissolved in 15 ml 0·02 M sodium citrate pH 7·5 and dialyzed against the same citrate buffer overnight. This enzyme preparation was stored frozen in small portions and used immediately after thawing.

Sephadex G–75 fraction. Ammonium sulfate fraction was concentrated by adding 2 g of Sephadex G–25 to 15 ml of the enzyme preparation. The Sephadex was allowed to swell for 10 min. Then the mixture was filtered through a sintered glass funnel (G–3). The collected filtrate, approximately one fifth of the original volume, was applied to the Sephadex G–75 column. Elution was accomplished with 0·05 M sodium

citrate solution. An automatic fraction collector, LKB 7000 UltroRac, was employed to collect 2·5 ml/5 min fractions.

All operations were performed in a refrigerated room at 4°. The specific activity of the enzyme fractions was defined as moles of the enzyme reaction product per min per milligram protein.

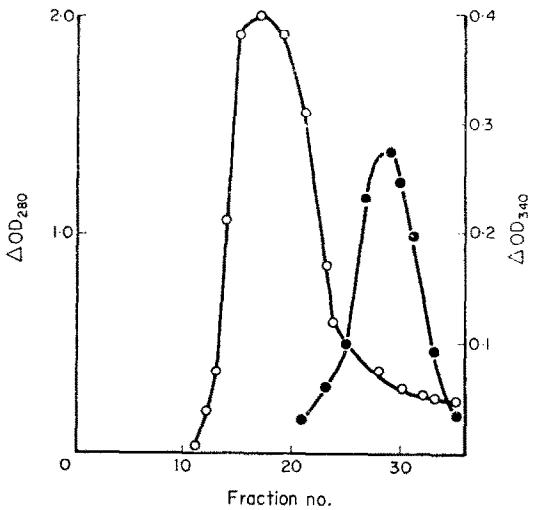


FIG. 1. Chromatography of dihydrofolate reductase on Sephadex G–75. ○—protein as determined by absorbancy at 280 nm, ●—enzyme activity as determined from decrease of absorbancy at 340 nm. Assay system was identical with that described in Table 1.

RESULTS

Purification of enzyme

A typical elution profile of Sephadex G–75 chromatography is shown in Fig. 1. The peak of enzyme activity is well separated from the main portion of the contaminating protein. The peak of the enzyme activity for folate and dihydrofolate appeared between tubes 27 and 31. Specific activity of the most active enzyme fractions was about 18 times higher than that of the 4·5 pH supernatant fraction (Table 1).

TABLE 1. PURIFICATION OF DIHYDROFOLATE REDUCTASE FROM MOUSE LIVER

Fraction	Volume (ml)	Total protein (mg)	Total activity (μmoles/min)	Specific activity (μmoles/min/mg)	Recovery (%)
pH 4·5 supernatant	15	96	360/645*	3·7/6·7*	100/100*
30–65% ammonium-sulfate saturation	3	36	162/279*	4·5/7·7*	45/43*
Sephadex G–75 tubes 29–31	7·5	0·97	66/119*	68·0/122·0*	18/18*

Complete system contained; 0·3 μmole of dihydrofolate, 0·375 μmole of NADPH, 300 μmoles of sodium citrate and variable amount of enzyme fraction in total volume of 3 ml at pH 7·5.

* 450 μmoles of KCl added. Reaction was followed at 340 nm for 10 min.

Activation of enzyme

Some mono- and bivalent cations were found to activate the partially purified enzyme. The stimulating effect of cations on folate reduction at pH 5·5 and dihydrofolate reduction at pH 7·5 are summarized in Table 2. In agreement with Bertino,[21] KCl caused a considerable activation of dihydrofolate reduction at pH 7·5. Maximal effect was obtained with 0·15 M KCl. Bivalent cations did not produce any activation of dihydrofolate reduction but appeared to be very potent activators of folate reduction

TABLE 2. THE EFFECT OF SOME MONO- AND BIVALENT CATIONS ON FOLATE AND DIHYDROFOLATE REDUCTION

	Enzyme activity %	
Activator	Folate pH 5·5	Dihydrofolate pH 7·5
None	100	100
KCl	158	176
$(NH_4)_2SO_4$	97	136
$CaCl_2$	159	
$MgCl_2$	228	83
$CoCl_2$	231	
$ZnCl_2$	248	
$ZnSO_4$	252	
$CdSO_4$	250	
$MnCl_2$	318	110

The enzyme activity affected by cations are compared with the activity of the enzyme without any cation added, which is considered as 100%. The value are based on the average of three experiments. The complete system reducing folate contained; 0·1 μmole of folate, 0·125 μmole of NADPH, 100 μmoles of sodium citrate, 10 μmoles of potential activator and 0·1 ml of enzyme (ammonium sulfate fraction) in a total volume 0·8 ml, at pH 5·5. The incubation was carried out at 37° for 1 hr. The complete system reducing dihydrofolate was identical to that described in Table 1 with 0·2 ml of enzyme (ammonium sulfate fraction). The activator was added in amount 450 μmoles.

at pH 5·5. KCl also activated this enzyme reaction. The most potent activator among all salts tested was manganese chloride. Its maximal effect (over 300 per cent) occurred at 10^{-2} M concentration. The reaction rate of both Mn-activated and nonactivated enzyme was linear at least up to 90 min of incubation at 37° (Fig. 2). On the base of these results, $MnCl_2$ at the concentration $1{\cdot}25 \times 10^{-2}$ M was included in the incubation mixture when partially enzyme was used.

Whereas the stimulating effect of Mn^{2+} on nonpurified and semipurified enzyme was marked, nearly no activation was observed with highly purified enzyme (Table 3).

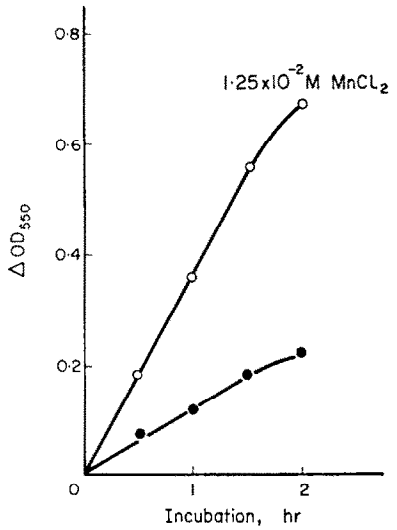


FIG. 2. Activation of folate reducing enzyme by Mn^{2+} at pH 5·5. ○—Mn-activated enzyme, ●—control without Mn^{2+}. Assay system was identical with that described in Table 2.

In contrast all enzyme fractions, regardless of their purity, were activated to the same extent by 0·15 M KCl.

Determination of K_m

The K_m values for semipurified enzyme were obtained according to the conventional Lineweaver–Burk double reciprocal plot with variable amount of the substrate. The K_m value for folate at pH 5·5 appeared to be $5{\cdot}5 \times 10^{-5}$ M, whereas a value of $5{\cdot}3 \times 10^{-6}$ M was obtained for dihydrofolate at pH 7·5. Zakrzewski[6] reported the effect of temperature on Michaelis constants and maximal reaction velocities for folate at pH 5·9. The K_m changed from 0·96 to $5{\cdot}69 \times 10^{-6}$ M in the range of temperature from 12 to 40°. In the experiments reported herein the degree of activation by Mn^{2+} influenced the K_m values too. The value for folate increased with increasing concentration of Mn^{2+}. The K_m value obtained with nonactivated enzyme was $1{\cdot}3 \times 10^{-5}$ M, compared to $5{\cdot}5 \times 10^{-5}$ M for Mn^{2+} activated.

TABLE 3. ACTIVATING EFFECT OF Mn^{2+} AND K^+ ON VARIOUS ENZYME FRACTIONS

	FH_2—reduction pH 7·5			F—reduction pH 5·5		
	O.D.$_{340}$		%	O.D.$_{550}$		%
Enzyme fraction	−KCl	+ KCl	Activation	− Mn	+ Mn	Activation
pH 4·5 supernatant	0·215	0·385	178	0·135	0·385	285
30–65% ammonium sulfate	0·195	0·345	176	0·095	0·305	321
Sephadex G–75	0·130	0·245	188	0·140	0·170	121

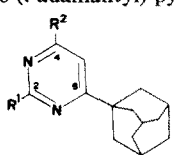
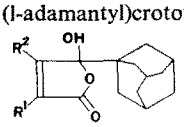
The assay systems were identical with that described in Table 1 and Table 2.

Unless otherwise noted the enzyme preparation, used in all experiments for testing the inhibitory effectiveness of the 6-(1-adamantyl) compounds, was the Mn-activated ammonium sulfate fraction.

Inhibition of folate reductase

All compounds tested on folate reductase are listed in Table 4. The upper limit of their solubility was at 4×10^{-4} M concentration. In preliminary experiments all compounds were tested at the concentration 2×10^{-4} M and compared to controls which received an equal volume of solvent. While γ-crotonlactonols did not cause 50 per cent inhibition at the concentration tested, some of the pyrimidines showed very

TABLE 4. COMPOUNDS TESTED AS INHIBITORS OF FOLATE REDUCTASE

Basic compound	Drug	Substituent		Enzyme inhibition
		R^1	R^2	%
6-(1-adamantyl)-pyrimidine 	I	OH	OH	37
	II	SH	SH	51
	III	CH_3S	OH	32
	IV	NH_2	OH	82
	V	SH	NH_2	65
	VI	OH	NH_2	93
	VII	NH_2	NH_2	67
(1-adamantyl)crotonlactonol 	VIII	H	H	39
	IX	Cl	H	27
	X	Br	H	32
	XI	H	Cl	36
	XII	H	Br	28

The assay system reducing folate at pH 5·5 was identical with that described in Table 2. Ten μmoles of $MnCl_2$ were added into the incubation mixture. The final concentration of inhibitor in the incubation mixture was 2×10^{-4} M.

good inhibitory effect. Inhibition of 93, 82 and 67 per cent was caused by 2-hydroxy-4-amino-(VI), 2-amino-4-hydroxy-(IV) and 2,4-diamino-6-(1-adamantyl)pyrimidine (VII), respectively. The analogues without the 6-(1-adamantyl) residue, (cytosine, isocytosine, 6-azacytosine, 5-azacytosine, cytidine and cytidine-5-monophosphate) when tested on folate reductase at 4×10^{-4} M concentration did not cause any inhibition. Because of the very poor inhibitory effects of the crotonolactonols and the cytosine derivatives without the adamantane ring, these drugs were omitted in further investigations. The kinetic data were determined only on the 6-(1-adamantyl)pyrimidines (VI) and (IV), (see Table 4).

The inhibitory effects of 6-(1-adamantyl) pyrimidines on folate reductase activity at pH 5·5 are shown in Table 5. The effects of two other antifolates, amethopterin and 2,4-diaminopteridine are included for comparison. With the exception of compounds I, II and III the 6-(1-adamantyl) pyrimidines exerted stronger inhibitory effect on folate reductase than the 2,4-diaminopteridine. The K_i values evaluated according to the

TABLE 5. INHIBITION OF MOUSE LIVER FOLATE REDUCTASE BY 6-(1-ADAMANTYL) PYRIMIDINES, AMETHOPTERIN AND 2,4-DIAMINOPTERIDINE

Drug	Concentration causing 50 % inhibition	K_i	Type of inhibition
	(M)	(M)	
I	4×10^{-4}		
II	$1{\cdot}8 \times 10^{-4}$		
III	$4{\cdot}0 \times 10^{-4}$		
IV	$2{\cdot}2 \times 10^{-5}$	$4{\cdot}5 \times 10^{-6}$	Competitive
V	$1{\cdot}1 \times 10^{-4}$		
VI	$7{\cdot}0 \times 10^{-6}$	$9{\cdot}3 \times 10^{-7}$	Competitive
VII	$1{\cdot}2 \times 10^{-4}$		
2,4-diaminopteridine	$1{\cdot}4 \times 10^{-4}$	$1{\cdot}2 \times 10^{-5}$	Competitive
amethopterin	$1{\cdot}7 \times 10^{-8}$		Noncompetitive

The assay system reducing folate at pH 5·5 was identical with that described in Table 2. Ten μmoles of $MnCl_2$ were added into the incubation mixture. Type of inhibition was determined by Lineweaver–Burk double reciprocal plot with varying folate concentration.

Lineweaver–Burk plot for the two most effective adamantane derivatives were $9{\cdot}3 \times 10^{-7}$ M (VI) and $4{\cdot}5 \times 10^{-6}$ M (IV), and the type of inhibition appeared to be competitive.

Lineweaver–Burk plots with varying amounts of folate or NADPH are shown in Fig. 3. Compound (VI) exerted the competitive type of inhibition when plotted against folate but the uncompetitive inhibition against NADPH.

The dissociability of the complex enzyme-inhibitor was tested by titration with increasing amount of enzyme according to Ackermann–Potter (Fig. 4). The slopes of the lines indicate that (VI) was bound to the enzyme at pH 5·5 by a dissociable bond, much less than amethopterin, which exerted the typical stoichiometric binding.

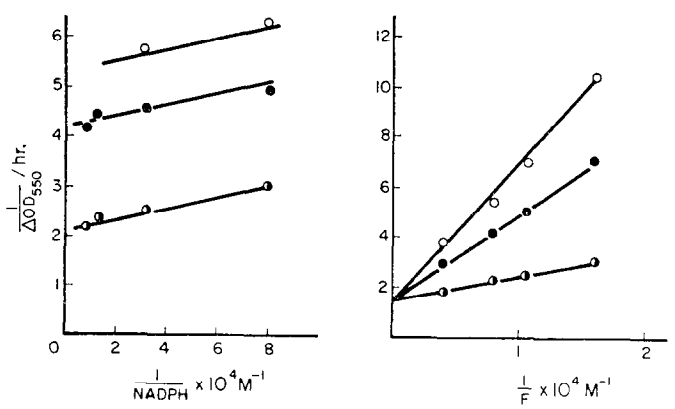


FIG. 3. Inhibition of folate reduction at pH 5·5 by 2-hydroxy-4-amino-6-(1-adamantyl) pyrimidine (VI) demonstrated by Lineweaver–Burk plots versus folic acid (F) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) respectively. ○—1×10^{-5} M (VI), ●—4×10^{-6}M (VI), ◐—no inhibitor. Assay system was identical with that described in Table 2.

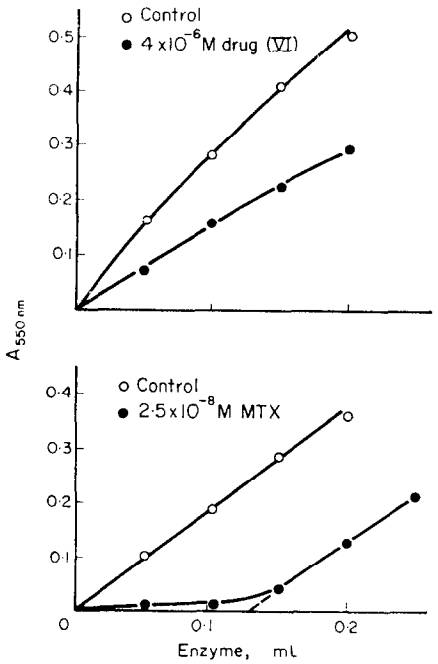


FIG. 4. Inhibition of folate reduction at pH 5·5 by 2-hydroxy-4-amino-6-(1-adamantyl) pyrimidine (VI) and amethopterin (MTX) demonstrated by Ackerman–Potter plots. Assay system was identical with that described in Table 2.

The shape of the inhibition curves and the plots described indicate that the 6-(1-adamantyl) pyrimidines represented by VI are competitive inhibitors resembling to the effect of 2,4-diaminopteridine, however effect of these drugs was greater probably because of the hydrophobic bonding of the adamantane skeleton which acted as an auxiliary interacting component.

Because all the experiments tested the inhibitory activity of the adamantane derivatives with partially purified Mn-activated enzyme, it was of interest to evaluate the inhibition of compound VI using the purified enzyme fractions. The inhibition curves in Fig. 5 indicate that there is no marked difference between the inhibitions of both purified and semipurified enzyme fractions by compound VI.

Effect of pH on the inhibition of folate and dihydrofolate reduction

It is known that folate is reduced only at acid pH reaching its maximum at about pH 5. In contrast, dihydrofolate reduction has two maxima at pH 5 and 7·5. Since the reduction of folate was inhibited very effectively by some of the 6-(1-adamantyl) pyrimidines studied, it was of interest to evaluate the inhibition of dihydrofolate reduction by these drugs. Compounds VI and IV did not cause any inhibition of dihydrofolate reduction at pH 7·5, upto the highest concentration of drug tested. When the dihydrofolate reduction was tested at pH 5·5, inhibition by these compounds was found, but it was less marked than that obtained with folate as a substrate (Fig. 6).

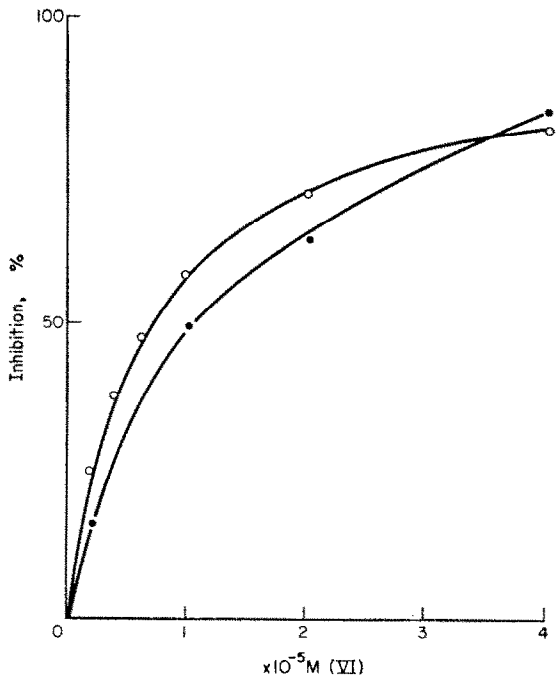


FIG. 5. The inhibitory effect of 2-hydroxy-4-amino-6-(1-adamantyl) pyrimidine (VI) on folate reducing system isolated by; ammonium sulfate fraction–○, Sephadex G–75 chromatography–●. Assay system was identical with that described in Table 2.

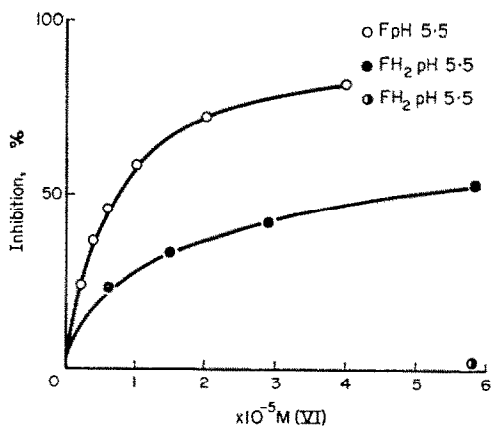


FIG. 6. The inhibition of folate and dihydrofolate reducing system by 2-hydroxy-4-amino-6-(1-adamantyl) pyrimidine (VI) at pH 5·5 and 7·5 respectively. The assay system was identical with those described in Tables 1 and 2.

The K_i values obtained with dihydrofolate at pH 5·5 were $3{\cdot}4 \times 10^{-6}$ M and $7{\cdot}5 \times 10^{-6}$ M for compounds VI and IV respectively, in contrast to the values of $9{\cdot}3 \times 10^{-7}$ M and $4{\cdot}5 \times 10^{-6}$ M found with folate. These K_i values show that the inhibition of enzyme activity was about 2–3 fold greater with folate than with dihydrofolate as the substrate.

Consequently, the dependence of the inhibition of folate and dihydrofolate reduction on pH was studied (Fig. 7). The inhibition of dihydrofolate reduction caused by compound VI appeared to be strictly pH dependent, with inhibitory maximum at

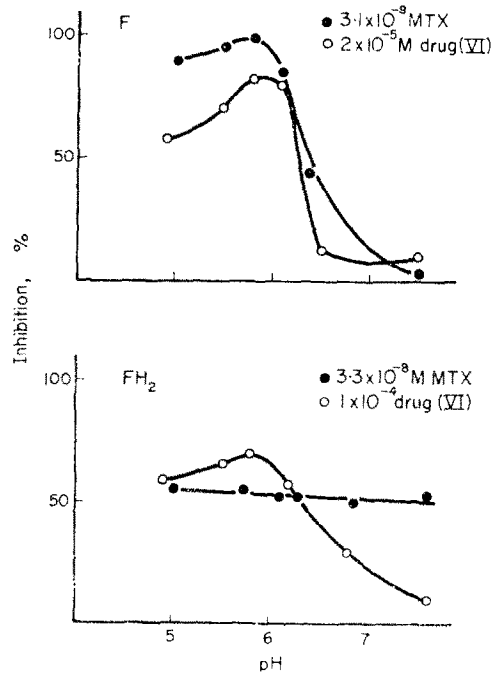


FIG. 7. The effect of pH on the inhibition of folate and dihydrofolate reduction caused by 2-hydroxy-4-amino-6-(1-adamantyl) pyrimidine (VI) and amethopterin (MTX). The assay systems were identical with those described in Tables 1 and 2.

pH 5·8. In contrast, the inhibition caused by amethopterin was pH independent. The inhibition of folate reductase activated by Mn^{2+} was pH dependent in the presence of both drugs with optimum inhibition at about pH 6. These results lead to the assumption that the inhibition of folate reductase by 6-(1-adamantyl)pyrimidines is both pH and substrate dependent.

DISCUSSION

The data presented provide information on the stimulating effect of Mn^{2+} on folate reductase activity and on the nature of the binding of 6-(1-adamantyl) pyrimidines to the enzyme.

The activation of dihydrofolate of different origin by various compounds has been the subject of numerous studies.[21–23] Bertino[21] showed clearly the activation of the

enzyme from liver of guinea-pigs by different monovalent and bivalent ions at pH 7·5. Dihydrofolate reductase from different sources may be activated by different cations in a different way. The activation of folate reductase at pH 5·5 by metals has not yet been reported. As described in this paper the reduction of folate at pH 5·5 was markedly activated by Mn^{2+} ions, and this activation was very pronounced with both non purified and ammonium sulfate semipurified enzyme. The activation can be explained partially by the presence of the concomitant enzyme participating in the formation of NADPH from the oxidized form (probably isocitrico dehydrogenase). In this system NADP can be used instead of NADPH without any marked change in enzyme activity. The folate reductase highly purified by Sephadex G–75 chromatography is activated by Mn^{2+} ions only very slightly and NADP is inactive in this system, because the NADPH regenerating enzyme system is not present. In contrast, the activating effect of K^+ is constant at all purification steps and seems to be due to a direct effect on the enzyme activity.

The effect of cations on the catalytic function of folate reductase has not been completely explained as yet. Nevertheless, the presence of cations influences strongly the enzyme activity and its basic kinetic parameters. Therefore it should be noted that the K_m and V_{max} values reported in this paper with semipurified enzyme were obtained with enzyme maximally activated by manganese ions. Thus the different K_m and V_{max} values for folate reductase reported by others[1,4,6] may be due to the possibility that these authors did not use maximally activated enzymes.

The competitive character of the inhibition by compound VI vs. folate and the non-competitive one vs. NADPH suggests that 6-(1-adamantyl) pyrimidines are bound to the binding site of folate or dihydrofolate respectively. Two parts of the inhibitor molecule seem to participate in the reversible binding, i.e. the 2,4-disubstituted pyrimidine and the adamantane skeleton. Zakrzewski[6] reported that 2,4-diaminopyrimidines are bound competitively to one part of the enzyme molecule via hydrogen bonding. This bond seems to be strong enough to cause the reversible binding of the 2,4-diaminopyrimidine ring itself without auxiliary bonds. However, 2-amino-4-hydroxy- and 2-hydroxy-4-aminopyrimidine are bound in a different way and the binding forces do not seem to be strong enough to cause the binding of these compounds to the enzyme in such a way that the substances could behave as inhibitors of dihydrofolate reductase. In this case an auxiliary, probably hydrophobic bond, is necessary to achieve a binding resulting in an inhibitory effect. Such a hydrophobic bond could exist between the enzyme and the adamantane molecule in the position C_6 which increased strongly the inhibitory effect of 2,4-diaminopyrimidine. The fact that 2-hydroxy-4-amino- and 2-amino-4-hydroxy-6-(1-adamantyl) pyrimidines exerted stronger inhibition of folate reductase than 2,4-diaminoderivative may be explained by postulating that the adamanatane moiety is the most important factor for the binding of these compounds to the enzyme, and that the pyrimidine part of the molecule acts only as the "pilot group" introducing the inhibitor to the binding site of the enzyme. According to Gerzon[24,25] the extraordinary properties of the adamantane molecule are determined not only by its lipofilic character but also by its spheric conformation. The spheric shape of the adamantane molecule would enable its maximal interaction with the lipoid area of the protein molecule formed by the aggregation of the side carbon chains of amino acids leading to conformation with minimum energy.[26]

The importance of the hydrophobic bond for the attachment of the pyrimidine ring

has been shown in a series of folate reductase-inhibiting alkylating agents prepared by Baker *et al.*[8,27,28] According to Baker the inhibition of the enzyme is dependent on the protonation of the enzyme and the inhibitor respectively.[29] The tightness of the enzyme–inhibitor complex formed is determinated by whether or not the proton is firmly associated with the enzymic acid group or with the strongly basic pyrimidine. On the basis of this assumption the degree of inhibition is dependent on the pK value of the inhibitor and/or on the pH value of the incubation mixture. The higher pK value of compound VI than that of IV is in agreement with this theory.

According to Jonak[14] the cytostatic effect of 5-(1-adamantyl) pyrimidines increased considerably if these compounds were substituted in position C_6. As shown in this study some pyrimidines became inhibitors of folate reductase when substituted with adamantane in position C_6. These data suggest that position C_6 in pyrimidine molecule is of a critical importance for both the cytostatic and inhibitory effect on folate reductase.

The enzyme molecule seems to be a flexible protein as suggested in a number of papers dealing with dihydrofolate reductase inhibition by inhibitors of structures far different from those of the natural substrate. This might be the main reason for the good inhibitory effect of this enzyme obtained with 6-(1-adamantyl) pyrimidines. The results of this study suggest that folate reductase is the target enzyme of 6-(1-adamantyl) pyrimidines, which represent a new group of compounds with cytostatic effect.*

Acknowledgements—The authors are indebted to Dr. Karel Slavík for his active interest and valuable discussions during the course of this work. They would also like to thank Dr. Enrico Mihich and Dr. Sigmund Zakrzewski for their valuable suggestions.

REFERENCES

1. S. F. ZAKRZEWSKI, *J. biol. Chem.* **235,** 1776 (1960).
2. R. L. BLAKLEY and B. M. MCDOUGALL, *J. biol. Chem.* **236,** 1163 (1961).
3. W. C. WERKHEISER, *Cancer, Res.* **23,** 1277 (1963).
4. J. R. BERTINO, B. A. BOOTH, A. L. BIEBER, A. CASHMORE and A. C. SARTORELLI, *J. biol. Chem.* **239,** 479 (1964).
5. W. C. WERKHEISER, *J. biol. Chem.* **236,** 888 (1961).
6. S. F. ZAKRZEWSKI, *J. biol. Chem.* **238,** 1485 (1963).
7. G. H. HITCHINGS and J. J. BURCHALL, *Adv. in Enzymol.* **27,** 417 (1965).
8. B. R. BAKER, in *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, p. 192. John Wiley, New York (1967).
9. K. SLAVÍK, V. SLAVÍKOVÁ, K. MOTYČKA, E. HERMANOVÁ, J. SOUČEK, Z. TOMSOVÁ, M. ŠPUNDOVÁ and E. NOVÁKOVÁ, *Molec. Pharmac.* **5,** 137 (1969).
10. J. SOUČEK and K. SLAVÍK, *5th FEBS Meeting*, Prague (1968).
11. J. J. BURCHALL and G. H. HITCHINGS, *Molec. Pharmac.* **1,** 126 (1965).
12. B. R. BAKER and HO BENG-THONG, *J. pharmaceut. Sci.* **53,** 1137 (1964).
13. J. P. JONAK, S. F. ZAKRZEWSKI, L. H. MEAD and M. T. HAKALA, *J. Med. Chem.* **13,** 1170 (1970).
14. J. P. JONAK, S. F. ZAKRZEWSKI and L. H. MEAD, *J. Med. Chem.* **14,** 408 (1971).
15. J. SOUČEK, V. SLAVÍKOVÁ and M. KUCHAŘ, *7th International Congress of Chemotherapy*, Prague (1971).
16. S. FUTTERMAN, *J. biol. Chem.* **228,** 1031 (1951).
17. M. KUCHAŘ, Thesis "Some new pharmacodynamically interesting compounds with the adamantyl residue," Prague, VUFB (1968).
18. M. KUCHAŘ, J. ŠTROF and J. VACHEK, *Colln. Czech. chem. Commun.* **34,** 2278 (1969).
19. M. KUCHAŘ and B. KAKÁČ, *Colln. Czech. chem. Commun.* **34,** 3343 (1969).
20. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
21. J. R. BERTINO, *Biochim. biophys. Acta* **52,** 377 (1962).
22. B. T. KAUFMAN, *Biochim. biophys. Res. Commun.* **10,** 449 (1963).

* E. Hermanová and H. Veselá, unpublished data.

23. B. T. KAUFMAN, *J. biol. Chem.* **239,** 669 (1964).
24. R. T. RAPALA, J. R. KRAAY and K. GERZON, *J. Med. Chem.* **8,** 580 (1965).
25. K. GERZON and D. KAU, *J. Med. Chem.* **10,** 189 (1967).
26. E. W. GILL, *Progr. Med. Chem.* **4,** 39 (1965).
27. B. R. BAKER and G. J. LOURENS, *J. heterocyclic. Chem.* **2,** 344 (1965).
28. B. R. BAKER, B. T. HO and D. B. SANTI, *J. Pharm. Sci.* **54,** 1415 (1965).
29. B. R. BAKER and J. H. JORDAAN, *J. Pharm. Sci.* **54,** 1740 (1965).