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INHIBITION OF THE PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE OF EHRLICH ASCITES TUMOR CELLS BY GLUTAMINE ANALOGUES*

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Abstract—Azaserine and diazo-oxo-norleucine inactivated the phosphoribosyl-formylglycineamidine synthetase of Ehrlich ascites tumor cells when glutamine was substrate, but not when ammonium chloride was used instead. Inhibition was competitive with respect to glutamine, but could be made noncompetitive by prior incubation of synthetase and inhibitor. *p*-Hydroxybenzoate also inactivated this enzyme, and this effect could be reversed by glutathione and prevented by glutamine. The K_i values for azaserine and diazo-oxo-norleucine were $2{\cdot}3 \times 10^{-6}$ M and $4{\cdot}0 \times 10^{-7}$ M respectively.

O-DIAZOACETYL-L-SERINE (azaserine) and 6-diazo-5-oxo-L-norleucine (DON) are well known to inhibit the enzyme phosphoribosyl-formylglycineamidine (FGAM)‡ synthetase (5′-phosphoribosyl-formylglycineamide: L-glutamine amido-ligase (ADP); EC 6.3.5.3) in the pathway of purine biosynthesis *de novo*. Irreversible binding of these drugs to the enzyme has been demonstrated with synthetase preparations from pigeon liver,[1] chicken liver[2] and *Salmonella typhimurium*[3,4] and the kinetics of inhibition by azaserine have been studied using a pigeon liver preparation.[1] Although the irreversible nature of the binding of azaserine and DON to FGAM synthetase in animal cells has been well established,[5,6] studies of the kinetics of inhibitor binding to mammalian cell synthetases have not been reported, and inhibition constants have not been measured in such systems. In this paper, the kinetics of binding of azaserine and DON to the FGAM synthetase of Ehrlich ascites tumor cells have been investigated.

MATERIALS AND METHODS

Reduced glutathione and *p*-hydroxymercuribenzoate (PMB) were obtained from Sigma Chemical Company; 2-mercaptoethanol, from Fisher Scientific Company; and azaserine and DON, from the Cancer Chemotherapy National Service Center, National Cancer Institute. Sources of other chemicals have been reported.[7]

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‡ Abbreviations used: FGAM, phosphoribosyl-formylglycineamidine; FGAR, phosphoribosyl-formylglycineamide; DON, diazo-oxo-norleucine; PMB, *p*-hydroxymercuribenzoate.

FGAM synthetase was purified 56-fold from Ehrlich ascites tumor cells as described elsewhere.[7] Enzyme activity was measured by coupling this reaction with phosphoribosyl-aminoimidazole synthetase (EC 6.3.3.1), and measuring colorimetrically the phosphoribosyl-aminoimidazole formed; details have been reported.[7]

Inactivation studies were carried out by first incubating the enzyme preparation in 0·01 M sodium hydrogen maleate buffer, pH 6·8, with the inhibitor for 10 min at 37°. After this period, small aliquots were removed, diluted to avoid further inhibition by the inhibitor, and assayed. Controls were carried out in which the inhibitor was omitted. Substrate protection against enzyme inactivation by drugs was studied by incubating the enzyme preparation with the specific inhibitor and substrate simultaneously for 10 min; small aliquots were then removed, diluted, and assayed. Kinetic studies of drug inhibition were carried out by measuring enzyme activity at varying concentrations of glutamine while the concentrations of the other substrates were held constant. Velocity is expressed as millimicromoles of FGAM formed under the assay conditions.

RESULTS AND DISCUSSION

The irreversible inactivation of FGAM synthetase by various concentrations of azaserine and DON is demonstrated by the results in Fig. 1; 50 per cent inhibition was produced by 3×10^{-6} M azaserine or by 6×10^{-7} M DON. Inactivation by these drugs was partially prevented when glutamine was present, but Mg-ATP, NH_4Cl and phosphoribosyl-formylglycineamide (FGAR) had no effect on the rate of inactivation.

Ammonium chloride can replace glutamine as substrate for FGAM synthetase, although the maximum velocity of the reaction is reduced.[7] Under conditions where

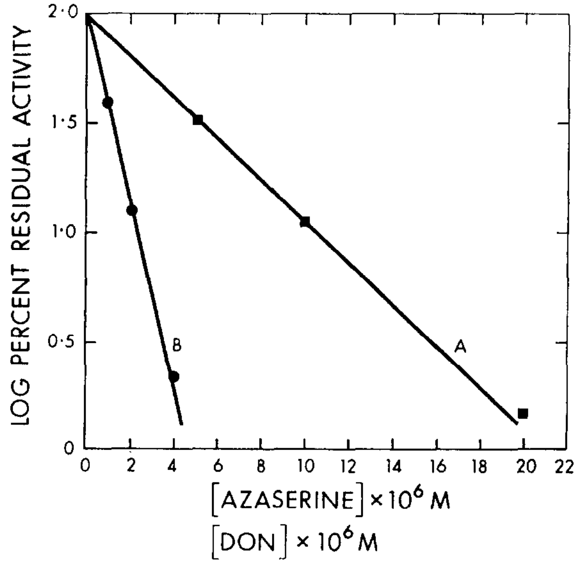

Fig. 1. Effects of azaserine and DON on FGAM synthetase activity. Enzyme (1·61 mg) was incubated with azaserine or DON in 0·15 ml of 0·01 M sodium hydrogen maleate–NaOH buffer, pH 6·8, at 37° for 10 min. Samples, 20 μl, were removed and assayed. A, azaserine; B, DON.

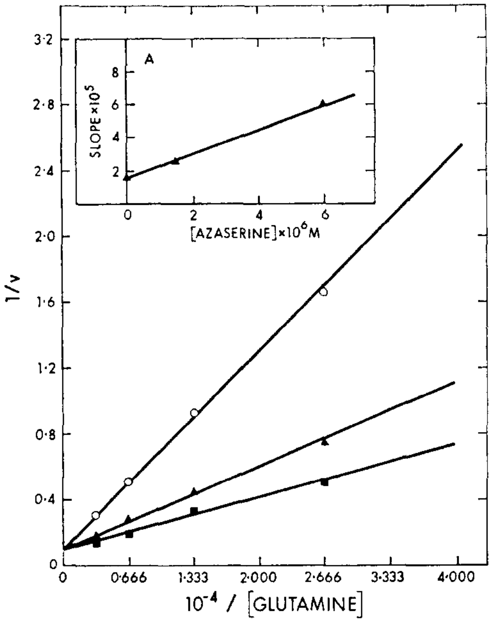

FIG. 2. Inhibition by azaserine. Double reciprocal plot of initial velocity against glutamine concentration at an FGAR concentration of $3{\cdot}75 \times 10^{-4}$ M and an ATP concentration of $3{\cdot}75 \times 10^{-3}$ M. Azaserine concentrations were: 0 (■); $1{\cdot}5 \times 10^{-6}$ M (▲); and 6×10^{-6} M (○). Insert: replot of slopes against azaserine concentration.

azaserine produced 84 per cent inactivation of enzyme activity when glutamine was substrate (8×10^{-6} M azaserine, 10 min of incubation), the maximum velocity with ammonium chloride was increased 21 per cent. Similarly, at $1{\cdot}6 \times 10^{-6}$ M DON, activity with glutamine was inhibited 86 per cent, whereas that with ammonium chloride was stimulated 14 per cent. Qualitatively similar results have been reported by others,[1,2] and it has been suggested that, although these glutamine analogues share a common binding site with glutamine, the amide nitrogen of glutamine binds to a different site after its release from this amino acid. Thus ammonium ion can bind independently of azaserine or DON.

Kinetic studies of the inhibition of FGAM synthetase by azaserine and DON are shown in Figs. 2 and 3, when glutamine was the varied substrate and the inhibitors and substrates were added essentially simultaneously. Inhibition by both glutamine analogues was competitive with respect to glutamine, as previously shown for azaserine and pigeon liver synthetase.[1] The K_i values for azaserine and DON were found to be $2{\cdot}3 \times 10^{-6}$ and $4{\cdot}0 \times 10^{-7}$ M respectively, these are 93 per cent (for azaserine) and 66 per cent (for DON) lower than those reported for pigeon liver FGAM synthetase.[1] Whether the lower K_i values are typical of mammalian FGAM synthetases in general, or are tissue specific differences, remains to be established.

Figures 4 and 5 show that when azaserine and DON are incubated with FGAM synthetase prior to addition of substrates, inhibition with respect to glutamine is noncompetitive rather than competitive, and the extrapolated lines intersect on the $1/S$ axis. This is to be expected for compounds which compete with glutamine for

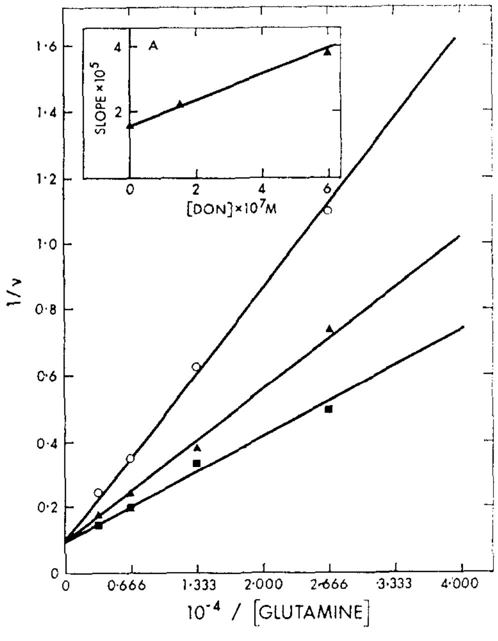

FIG. 3. Inhibition by DON. Double reciprocal plot of initial velocity against glutamine concentration at an FGAR concentration of 3·75 × 10^{-4} M and an ATP concentration of 3·75 × 10^{-3} M. DON concentrations were: 0 (■); 1·5 × 10^{-7} M (▲); and 6 × 10^{-7} M (○). Insert: replot of slopes against DON concentration.

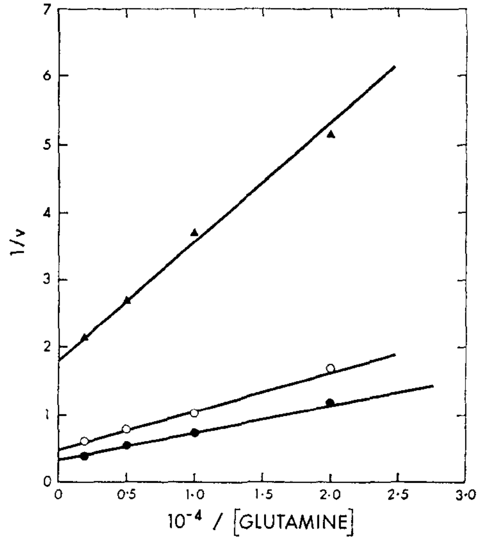

FIG. 4. Inhibition by azaserine. Double reciprocal plot of initial velocity against glutamine concentration at an ATP concentration of 4 × 10^{-3} M and an FGAR concentration of 4 × 10^{-4} M. Enzyme was preincubated with azaserine in 2 ml of 0·01 M sodium hydrogen maleate–NaOH buffer, pH 6·8, at 37° for 5 min. Samples 75 μl, were removed for initial rate studies. Concentrations of azaserine were: 0 (●); 2·4 × 10^{-6} M (○); and 1·2 × 10^{-5} M (▲).

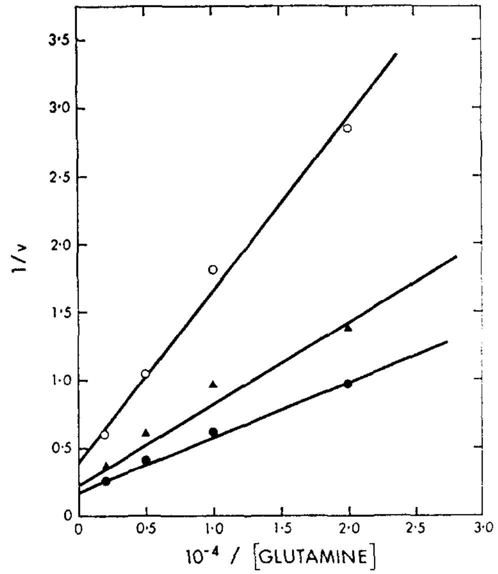

FIG. 5. Inhibition by DON. Double reciprocal plot of initial velocity against glutamine concentration at an ATP concentration of 4 × 10^{-3} M and an FGAR concentration of 4 × 10^{-4} M. Enzyme was preincubated with DON in 2 ml of 0·01 M sodium hydrogen maleate–NaOH buffer, pH 6·8, at 37° for 5 min. Samples, 75 μl, were removed for initial rate studies. Concentrations of DON were: 0 (●); 4 × 10^{-7} M (○); and 8 × 10^{-7} M (▲).

TABLE 1. FACTORS AFFECTING ENZYME INACTIVATION BY PMB

PMB (M)	Additions	Residual activity (%)
Reversal of inactivation*		
0	None	100
1·6 × 10^{-4}	None	18
1·6 × 10^{-4}	Glutathione (1·8 × 10^{-4} M)	37
1·6 × 10^{-4}	2-Mercaptoethanol (2·1 × 10^{-4} M)	42
Protection against inactivation†		
0	None	100
2 × 10^{-4}	None	11
2 × 10^{-4}	Glutamine (1 × 10^{-4} M)	82
2 × 10^{-4}	Mg-ATP (1 × 10^{-3} M)	12
2 × 10^{-4}	FGAR (1 × 10^{-4} M)	10

* Reaction mixtures (0·05 ml) contained: enzyme (0·89 mg); 10 mM sodium hydrogen maleate–NaOH buffer, pH 6·8; and the indicated amounts of PMB. The mixtures were incubated at 37° for 10 min. Glutathione, 2-mercaptoethanol or water was then added, and the mixtures were further incubated for 10 min at 37°. Samples 10 μl, were removed for enzyme assay.

† Enzyme (1·65 mg) was incubated for 10 min at 37° with PMB in 0·15 mg of 0·01 M sodium hydrogen maleate–NaOH buffer, pH 6·8, and the addition indicated. Samples, 20 μl, were removed for enzyme assays.

binding to the enzyme, but then attach irreversibly. Such a noncompetitive pattern of inhibition was previously shown for azaserine and a pigeon liver FGAM synthetase.[1]

Azaserine has been shown to react with a sulfhydryl group in the *Salmonella typhimurium* FGAM synthetase,[3,4] and the synthetases from various sources are inactivated by sulfhydryl reagents such as iodoacetate.[2,8] Table 1 shows that the tumor cell FGAM synthetase is inactivated by PMB, that this effect can be at least partially reversed by glutathione and mercaptoethanol, and that glutamine but no other substrate would at least partially protect the synthetase against the action of PMB; ammonium chloride had no effect.

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