Biochemical Pharmacology, Vol. 18, pp. 2737–2745. Pergamon Press 1969, Printed in Great Britain

INHIBITION OF INOSINIC ACID DEHYDROGENASE BY 6-CHLOROPURINE NUCLEOTIDE*

JULIUS H. ANDERSON† and ALAN C. SARTORELLI

Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. 06510, U.S.A

(*Received* 12 *February* 1969; *accepted* 16 *May* 1969)

Abstract—The inactivation of IMP dehydrogenase of sarcoma 180 ascites tumor cells by 6-chloroinosine 5′-phosphate (ClIMP) has been examined. In the absence of substrates the inhibition of enzymatic activity by this purine nucleotide analog is progressive with time and is not reversed by dialysis. IMP, XMP and GMP protect the enzyme from inactivation by ClIMP, while NAD^+, NADH and glutathione do not protect. The data are quantitatively consistent with the hypothesis that ClIMP forms a reversible complex with the active site of the enzyme, with an apparent inhibition constant of 2×10^{-4} M (compared to a K_m for IMP of 2×10^{-5} M); this reversible EI complex then slowly decays to form an irreversibly inactivated enzyme.

INOSINIC acid dehydrogenase (IMP dehydrogenase; IMP : NAD^+ oxidoreductase, EC 1.2.1.14) is the first enzyme unique to the biosynthetic pathway leading from inosine 5′-phosphate (IMP) to guanosine 5′-phosphate (GMP). This enzyme is inhibited by the nucleotides of 6-mercaptopurine (6-MP), 6-thioguanine (TG) and 6-chloropurine (ClP), growth inhibitory compounds with clinical utility in the treatment of acute leukemia of childhood. Such blockade, which would be expected to interfere with the availability of guanine nucleotides for both coenzyme function and nucleic acid synthesis, has been suggested to be at least part of the mechanism by which these purine analogs function to inhibit the growth of neoplastic cells.[1–3]

Few studies are available on the mechanism of inhibition of IMP dehydrogenase from mammalian sources by analogs of purine nucleotides. Using the IMP dehydrogenase of Ehrlich ascites tumor cells Atkinson *et al.*[4] have shown that the inhibition produced by 6-thioinosine 5′-phosphate (6-thioIMP) is kinetically competitive with IMP. Studies from this laboratory,[3] using an enzyme isolated from sarcoma 180, have shown that preincubation of IMP dehydrogenase with 6-thioguanosine 5′-phosphate (6-thioGMP) in the absence of substrates produces progressive inhibition that can be reversed partially by glutathione (GSH) and completely by treatment with dithiothreitol. More extensive studies *in vitro* of inhibition of IMP dehydrogenase by purine nucleotide analogs have been conducted in enzymatic systems from non-mammalian sources; thus, Hampton,[5] Hampton and Nomura,[6] and Brox and Hampton[7] demonstrated progressive inhibition of IMP dehydrogenase of *Aerobacter*

*This work was presented to the Graduate School of Yale University by J. H. Anderson in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Support was provided by United States Public Health Service Grant CA-02817 and Training Grant 5-T1-GM59, and an American Cancer Society Postdoctoral Research Scholarship to J.H.A.

†Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104.

aerogenes by 6-thioIMP, 6-thioGMP and 6-chloroinosine 5'-phosphate (ClIMP). The inhibition of this enzyme by 6-thioIMP and 6-thioGMP was relieved by GSH, whereas the inactivation of IMP dehydrogenase by ClIMP was accelerated by GSH. It was suggested[5-7] that the 6-chloro and 6-thio analogs of purine nucleotides inactivate IMP dehydrogenase by exerting their effects at the active site of the enzyme by apparently forming thioether and disulfide bonds, respectively, with an enzymic sulfhydryl group.

The IMP dehydrogenase of sarcoma 180 ascites tumor cells has been studied in detail; the basic kinetic properties of this enzyme (i.e. apparent K_m values, substrate interaction, and activation specificity) differ significantly from those of the IMP dehydrogenase of *A. aerogenes*.[6,8,9] Furthermore, although the feedback inhibition of IMP dehydrogenase by GMP is similar for enzymes prepared from sarcoma 180 and *A. aerogenes*,[6,10] major differences exist between these enzymes and the IMP dehydrogenase of other bacterial strains with regard to the kinetics of inhibition by GMP.[6,10-12] Since 6-MP, TG and ClP have considerable clinical utility, it was of importance to study the inhibition of mammalian IMP dehydrogenase by nucleotides of these purine analogs to determine if significant differences exist between the bacterial and the mammalian enzymes. The present investigation reports the results of studies of inhibition of IMP dehydrogenase of sarcoma 180 cells by ClIMP. The results indicate that despite significant differences in the basic reaction kinetics of the IMP dehydrogenase enzymes of sarcoma 180 and *A. aerogenes*, these enzymes respond to ClIMP in a similar manner. Some of these data have appeared previously in abstract form.[13,14]

MATERIALS AND METHODS

Enzyme preparation. IMP dehydrogenase was isolated from 6-day growths of sarcoma 180 ascites cells as described earlier.[9] The procedure consisted of disruption of neoplastic cells by sonication, centrifugation at 105,000 *g* for 2 hr, and fractionation of the supernatant solution with ammonium sulfate. The precipitate which formed between 20 and 40% saturation with ammonium sulfate was collected, dialyzed and stored frozen at − 17° for 1 week prior to use. The preparation contained about 4% of the total cellular protein and did not significantly destroy IMP, NAD^+, or NADH.

Enzyme assay. The activity of IMP dehydrogenase was assayed by measurement of the net production of NADH by determination of the increase in absorbancy at 340 mμ, as indicated by Magasanik *et al.*[8] The final assay mixture usually consisted of 0·1 M Tris-Cl (pH 8), 0·1 M KCl, 0·28 mM NAD^+ (K^+ salt), 2·3 mM IMP (Na^+ salt) and 0·1 ml of enzyme preparation (generally 1–3 mg of protein); inhibitor was added where indicated. All measurements of activity were made after equilibration to 37° and involved only the initial velocity during the first 10 min after initiation of the reaction.

Chemicals. IMP, XMP, NAD^+ and NADH were obtained from P-L Biochemicals, Inc. 6-Chloropurine ribonucleoside was purchased from Sigma Chemical Co.; it was converted to the ribonucleotide (ClIMP) by the method of Hampton and Maguire.[15] The barium salt of ClIMP was converted to the potassium salt by stirring with Dowex 50-K^+ in water just prior to use.

RESULTS

ClIMP did not inhibit the activity of IMP dehydrogenase when the nucleotide analog was added directly to a complete assay mixture; at the concentrations employed,

it was necessary to preincubate the purine nucleotide analog with the enzyme in the absence of IMP to demonstrate an inhibitory effect. Figure 1 shows the results of such a preincubation experiment in which KCl and buffer were present during the preincubation of the enzyme with the analog nucleotide in the absence of substrates. The addition of substrates appeared to terminate the progression of the inhibition, since the subsequent rate of enzymatic reduction of NAD^+ was constant for at least the next 20 min. Dialysis of the enzyme–ClIMP mixture against Tris-Cl (pH 8) for 9 hr at

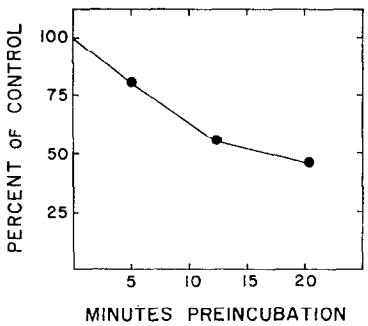

FIG. 1. Inhibition of IMP dehydrogenase by ClIMP. ClIMP ($7{\cdot}6 \times 10^{-5}$ M) was preincubated with IMP dehydrogenase (2·16 mg of protein), 0·1 M Tris-Cl (pH 8) and 0·11 M KCl at 37° in a volume of 0·9 ml. At the indicated times, 0·1 ml of a substrate mixture was added to bring the concentration of IMP to $2{\cdot}3 \times 10^{-3}$ M and NAD^+ to $2{\cdot}8 \times 10^{-4}$ M.

TABLE 1. EFFECT OF DIALYSIS ON THE ACTIVITY OF ClIMP-TREATED IMP DEHYDROGENASE*

Inhibitor	Relative specific activity	
	Prior to dialysis	After dialysis
None	1·00	0·81
GMP	0·82	0·86
ClIMP	0·30	0·17

*IMP dehydrogenase (6·5 mg of protein with a specific activity of 1·56 mμmoles/min/mg) was exposed to either $5{\cdot}5 \times 10^{-3}$ M GMP or $1{\cdot}6 \times 10^{-3}$ M ClIMP in the presence of $7{\cdot}5 \times 10^{-2}$ M KCl in a volume of 0·43 ml. After 10 min of incubation at room temperature, 0·15 ml was removed for determination of enzymatic activity and the remainder of each mixture was dialyzed for 9 hr against approximately 2000 volumes of 0·01 M Tris-Cl (pH 8) at 4°. Portions of the dialysate were then assayed for both enzymatic activity and concentration of protein.

4° did not reverse the inhibition, whereas the inhibition of the enzyme by GMP (included as a control) was relieved by dialysis (Table 1).

Measurement of the initial rate of inactivation of IMP dehydrogenase as a function of the concentration of ClIMP is shown in Fig. 2. A half-maximal rate of inactivation occurred at a ClIMP concentration of about 2×10^{-4} M. As with the enzyme from *A. aerogenes*, the inhibition of IMP dehydrogenase of sarcoma 180 appeared to be

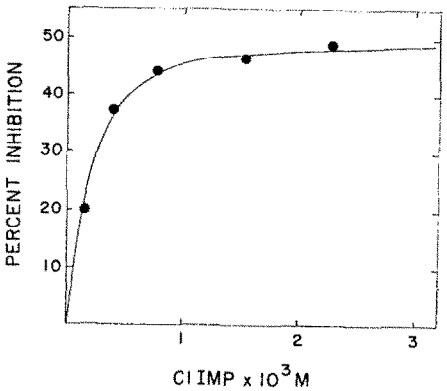

FIG. 2. Relationship between the concentration of ClIMP and the initial rate of inactivation. IMP dehydrogenase was preincubated at 37° with the indicated concentrations of ClIMP, 0·1 M Tris-Cl (pH 8) and 0·11 M KCl. After 2·5 min, a substrate mixture, which brought the concentration of IMP to $2{\cdot}3 \times 10^{-3}$ M, was added in a volume which reduced the concentration of ClIMP by 10%; the residual enzymatic activity was determined during the next 10 min.

selective for the ribonucleotide of ClP; much higher concentrations of ClP ($1{\cdot}8 \times 10^{-2}$ M) and of chloropurine ribonucleoside (7×10^{-3} M) produced only 28 and 19 per cent inhibition, respectively, after 15 min of preincubation with the enzyme in the absence of substrates; lower concentrations of these compounds were correspondingly less active as inhibitors.

The presence of $2{\cdot}6 \times 10^{-4}$ M IMP during the period of preincubation of the enzyme with ClIMP, completely protected IMP dehydrogenase from inhibition by the purine nucleotide analog; at a higher concentration of IMP ($2{\cdot}6 \times 10^{-3}$ M), a slight increase in activity was produced under these conditions (Fig. 3). The reason

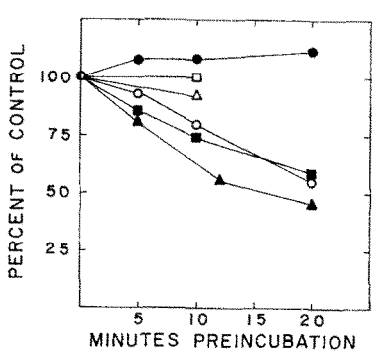

FIG. 3. Effect of several concentrations of IMP on the inhibition of IMP dehydrogenase by ClIMP. ClIMP ($7{\cdot}6 \times 10^{-5}$ M) was preincubated at 37° in a volume of 0·9 ml with IMP dehydrogenase (2·16 mg of protein), 0·1 M Tris-Cl (pH 8), 0·11 M KCl, and the following concentrations of IMP: ▲, no IMP; ■, $2{\cdot}6 \times 10^{-5}$ M IMP; ○, $5{\cdot}2 \times 10^{-5}$ M IMP; △, $1{\cdot}3 \times 10^{-4}$ M IMP; □, $2{\cdot}6 \times 10^{-4}$ M IMP; ●, $2{\cdot}6 \times 10^{-3}$ M IMP. At the indicated times, 0·1 ml of a substrate mixture, prewarmed to 37°, whose composition was $2{\cdot}3 \times 10^{-2}$ M IMP and $2{\cdot}8 \times 10^{-3}$ M NAD^+ was added, except in the cuvette containing $2{\cdot}6 \times 10^{-3}$ M IMP. The results are expressed relative to the enzymatic activity obtained in the absence of ClIMP.

for the apparent increase in enzymatic activity is unknown. Such an increase in activity also occurred when the enzyme was preincubated with IMP in the absence of ClIMP; the effect is, therefore, not dependent upon the presence of ClIMP. The findings demonstrated that concentrations of IMP below $2{\cdot}6 \times 10^{-4}$ M gave intermediate degrees of protection against ClIMP, decreasing the rate at which ClIMP produced inhibition of the activity of IMP dehydrogenase (Fig. 3). A concentration of IMP slightly greater than $5{\cdot}2 \times 10^{-4}$ M was required to reduce by one-half the rate of inactivation of enzymatic activity by $7{\cdot}6 \times 10^{-5}$ M ClIMP.

Since IMP dehydrogenase of sarcoma 180 was inhibited competitively by GMP[10, 13] (apparent $K_i = 2$ to 8×10^{-4} M) and also competitively by XMP[9] (apparent $K_i = 4 \times 10^{-4}$ M) with respect to the substrate IMP, the effects of GMP and XMP on the inhibition of the enzyme by ClIMP were determined. Relatively large concentrations of GMP and XMP added during the period of preincubation afforded complete protection of IMP dehydrogenase activity from the inhibitory effects of ClIMP (Fig. 4).

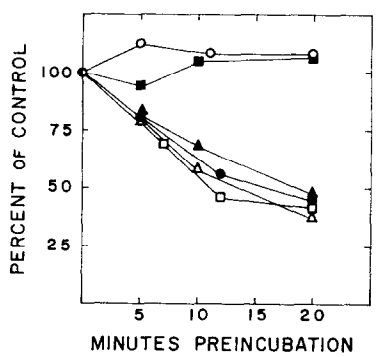

FIG. 4. Effect of several nucleotides and glutathione on the inhibition of IMP dehydrogenase by ClIMP. ClIMP ($7{\cdot}6 \times 10^{-5}$ M) was preincubated at 37° in a volume of 0·9 ml with IMP dehydrogenase (2·16 mg of protein), 0·1 M Tris-Cl (pH 8), 0·11 M KCl and the following additions: ●, none; △, $3{\cdot}1 \times 10^{-4}$ M NAD^+; ▲, $1{\cdot}6 \times 10^{-4}$ M NADH; □, $3{\cdot}8 \times 10^{-3}$ M GSH; ■, $2{\cdot}6 \times 10^{-3}$ M XMP; ○, $2{\cdot}6 \times 10^{-3}$ M GMP. At the indicated times of preincubation, 0·1 ml of a substrate mixture, prewarmed to 37°, was added to bring the concentration of IMP to $2{\cdot}3 \times 10^{-3}$ M and of NAD^+ to $2{\cdot}8 \times 10^{-4}$ M. The residual enzymatic activity was measured during the next 10 min and expressed relative to the appropriate control activities determined in the presence of NADH, GSH, XMP, or GMP.

In contrast, NAD^+ at a concentration optimal for enzymatic activity and NADH at a concentration approximating its K_i value, did not significantly affect the rate at which ClIMP produced inactivation of the activity of IMP dehydrogenase. Furthermore, in contrast to results obtained with the enzyme from *A. aerogenes*,[5,6] GSH did not influence the rate of inactivation by the purine nucleotide analog of IMP dehydrogenase of sarcoma 180.

The effect of the cofactor K^+ on the inhibition of enzymatic activity by ClIMP was determined (Fig. 5). The absence of K^+ in the preincubation mixture decreased the rate at which ClIMP produced inhibition of IMP dehydrogenase; thus, only 30 per cent inhibition of enzymatic activity was produced by $7{\cdot}6 \times 10^{-5}$ M ClIMP after

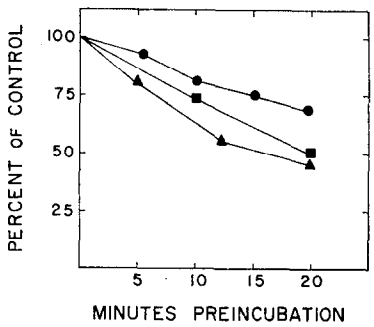

FIG. 5. Effect of inorganic cations on the inhibition of IMP dehydrogenase by ClIMP. ●, ClIMP ($7{\cdot}6 \times 10^{-5}$ M) was preincubated at 37° with IMP dehydrogenase (2·16 mg of protein) and 0·1 M Tris-Cl (pH 8) in a total volume of 0·9 ml. At the indicated times, 0·1 ml of a substrate mixture, prewarmed to 37°, was added to bring the concentration of KCl to 0·10 M, IMP to $2{\cdot}3 \times 10^{-3}$M, and NAD^+ to $2{\cdot}8 \times 10^{-4}$ M. The residual enzymatic activity was determined during the next 10 min and was plotted relative to the enzymatic activity obtained in the absence of inhibitor. The value at 0 min was determined by addition of IMP, 20 sec prior to the addition of ClIMP, to enzyme which was preincubated for 5 min with NAD^+ and KCl. ▲, KCl (0·11 M) was present during the preincubation period described above, and KCl was omitted from the substrate mixture in order that the concentration of KCl during the assay of enzymic activity would be 0·10 M. ■, NaCl (0·14 M) was present during the preincubation period and KCl was omitted from the substrate mixture in order that the concentration of salt during the assay of enzymic activity would be 0·126 M NaCl. These results are expressed relative to the activity of IMP dehydrogenase in the presence of 0·126 M NaCl rather than 0·10 M KCl.

incubation for 20 min in the absence of K^+, as compared with 55 % inhibition of the activity of IMP dehydrogenase by the purine nucleotide analog in the presence of this cation. Substitution of K^+ by Na^+ gave an intermediate rate of inactivation. These results are of interest, since Na^+ can partially replace K^+ as an activator of the IMP dehydrogenase of sarcoma 180.[9]

To assess the kinetics of the inactivation of IMP dehydrogenase by ClIMP, the following model, which is basically that proposed by Hampton,[5] was employed. The free enzyme was assumed to rapidly form the following reversible complexes which are mutually exclusive:

$$E_f + P \overset{\text{fast}}{\rightleftharpoons} EP$$

$$E_f + I \overset{\text{fast}}{\rightleftharpoons} EI$$

where P is a protector molecule, I is an inhibitor, and E_f is the free enzyme.
The EI complex is envisioned to slowly decay to an irreversible complex with loss of enzymatic activity.

$$EI \overset{\text{slow}}{\longrightarrow} E'$$

Initially the enzyme would be expected to be distributed between three forms:

$$E = E_f + EI + EP.$$

The following kinetic constants can be calculated:

$$K_p = (E_f)\,(P)/(EP).$$

$$K_I = (E_f)\,(I)/(EI).$$

If r_o is the initial rate of inactivation in the absence of a protecting agent and r_p is the initial rate of inactivation of enzymatic activity in the presence of an agent capable of imparting protection against the inhibitor, it can be shown algebraically that:

$$K_p = \frac{P}{(I/K_I + 1)\,(r_o/r_p - 1)}.$$

To estimate protection constants for IMP, GMP and XMP using the above equation, an experiment was performed with a relatively high concentration of ClIMP using a relatively short period of preincubation to measure the initial rate of inactivation of enzymatic activity. The results and the calculated values of K_p are shown in Table 2.

TABLE 2. CALCULATED PROTECTION CONSTANTS FOR IMP, GMP AND XMP*

Protecting nucleotide	Concn (M)	r_p†	K_p (M)
IMP	$2{\cdot}3 \times 10^{-4}$	11	$3{\cdot}2 \times 10^{-5}$
GMP	$2{\cdot}6 \times 10^{-4}$	20	$8{\cdot}8 \times 10^{-5}$
XMP	$2{\cdot}6 \times 10^{-4}$	16	$5{\cdot}7 \times 10^{-5}$

*IMP dehydrogenase was preincubated with $3{\cdot}8 \times 10^{-4}$ M ClIMP, 0·1 M Tris-Cl (pH 8) and 0·11 M KCl in a volume of 0·9 ml at 37° in the presence of the indicated concentrations of IMP, GMP, or XMP. After 2·5 min, 0·1 ml of a substrate mixture was added to bring the concentration of IMP to $2{\cdot}3 \times 10^{-3}$ M and of NAD^+ to $2{\cdot}8 \times 10^{-4}$ M. The residual enzymatic activity was then monitored by the change in the absorbancy at 340 mμ. The rate of inactivation in the absence of IMP, GMP, and XMP was 38 per cent in 2·5 min. The K_i for ClIMP as determined from Fig. 2 was 2×10^{-4} M. K_p was calculated using the equation given in the text.

† Initial rate of inactivation in per cent per 2·5 min.

The K_p values for IMP and GMP agree within a factor of 2 to 3 with the measured kinetic constants for these nucleotides; the apparent K_m for IMP being 2×10^{-5} M and the apparent K_i for GMP being 2 to 8×10^{-4} M. The K_p for XMP is within a factor of 5 of the dissociation constant for XMP ($1{\cdot}4 \times 10^{-5}$ M) calculated from experiments employing NAD^+ as the variable substrate using the equations of Cleland[16] for an ordered bi bi mechanism.[9] A difference of this magnitude (5-fold) cannot be considered to be outside of the realm of experimental error in these experiments. Thus, all of the data appear to be quantitatively consistent with the proposed model.

DISCUSSION

The studies on the effects of ClIMP on IMP dehydrogenase reported here extend the findings of Hampton *et al.*[5–7] with the IMP dehydrogenase of *A. aerogenes* to a mammalian enzyme (sarcoma 180). The results support the contention that ClIMP reacts to form a stable complex with the active site of IMP dehydrogenase. Thus, the interaction of the enzyme with ClIMP appears to be stable to dialysis, and IMP, GMP and XMP are capable of preventing the inactivation of IMP dehydrogenase by ClIMP. These data are compatible with a model that involves an initial reversible association of the enzyme with ClIMP with an apparent K_i of about 2×10^{-4} M, followed by the

slower production of an irreversibly inactivated enzyme; similar affinity of ClIMP for IMP dehydrogenase from *A. aerogenes* and data supporting a two-stage reaction of the inhibitor with the enzyme have been recently reported.[7] In the present studies, which employed limited concentrations of ClIMP, inhibition of IMP dehydrogenase activity could not be demonstrated unless the ClIMP was preincubated with the enzyme in the absence of IMP. The postulated mechanism of inhibition by ClIMP implies, however, that demonstrable competitive inhibition of enzymatic activity should be obtainable with the use of larger concentrations of ClIMP in the reaction mixture containing IMP. The limited availability of ClIMP precluded such experiments. For the second stage of the interaction Hampton *et al.*[5-7] have postulated that ClIMP reacts with a sulfhydryl group at the active site of IMP dehydrogenase to produce an inactive complex. The persistence of the inhibition of the sarcoma 180 enzyme after dialysis and the absence of any effect upon the inhibition by the presence of GSH suggest that the concepts of Hampton *et al.*[5-7] may apply to the mammalian enzyme as well as to the IMP dehydrogenase of *A. aerogenes*. The inhibition of enzymic activity by the purine nucleotide analog is accelerated by inorganic cations. It is not clear whether inorganic cations alter the apparent K_i for ClIMP, accelerate the irreversible phase of the enzyme–inhibitor interaction, or displace ClIMP from an impurity in the enzyme preparation, thereby raising the effective concentration of the inhibitor. However, the findings that the relative effects of Na^+ and K^+ on the rate of inactivation are similar to their relative effects as activators of IMP dehydrogenase activity in the absence of ClIMP and occur at similar concentrations suggest that the cations are functioning at the level of the enzyme itself.

It is of interest that complete inactivation of IMP dehydrogenase by ClIMP was not obtained, and that an apparent maximum initial rate of inactivation of only 50 per cent was obtained. Hampton and Nomura,[6] using an enzyme from *A. aerogenes*, observed similar results and speculated that only a fraction of the native enzyme population was capable of binding ClIMP in the absence of GSH, since GSH accelerated the rate of inactivation of this enzyme by ClIMP. This hypothesis does not appear to explain the data obtained with the IMP dehydrogenase from sarcoma 180, since GSH did not affect the rate of inactivation of this enzyme by ClIMP (Fig. 4). Conceivably, an impurity in the enzyme preparation which binds ClIMP could be responsible for the maximum inhibition of only 50%.

The protection of this enzyme by purine nucleotides (especially by GMP) presumably also occurs *in vivo*; such a phenomenon complicates the kinetics *in vivo* and may well limit the effectiveness of these purine analogs as antineoplastic agents.

The results presented in this report are of particular interest because despite differences in the basic kinetics of the IMP dehydrogenase enzymes of sarcoma 180 and *A. aerogenes*, these two enzymes appear to interact in a similar manner with ClIMP.

REFERENCES

1. N. H. CAREY and H. G. MANDEL, *J. biol. Chem.* **236**, 520 (1961).
2. A. C. SARTORELLI and B. A. BOOTH, *Biochem. Pharmac.* **12**, 847 (1963).
3. R. P. MIECH, R. E. PARKS, JR., J. H. ANDERSON, JR. and A. C. SARTORELLI, *Biochem. Pharmac.* **16**, 2222 (1967).
4. M. R. ATKINSON, R. K. MORTON and A. W. MURRAY, *Biochem. J.* **89**, 167 (1963).
5. A. HAMPTON, *J. biol. Chem.* **238**, 3068 (1963).
6. A. HAMPTON and A. NOMURA, *Biochemistry* **6**, 679 (1967).

7. L. W. Brox and A. Hampton, *Biochemistry* **7**, 2589 (1968).
8. B. Magasanik, H. S. Moyed and L. B. Gehring, *J. biol. Chem.* **226**, 339 (1957).
9. J. H. Anderson and A. C. Sartorelli, *J. biol. Chem.* **243**, 4762 (1968).
10. J. H. Anderson and A. C. Sartorelli, *Biochem. Pharmac.* **18**, 2747, (1969).
11. D. H. Buzzee and A. P. Levin, *Biochem. biophys. Res. Commun.* **30**, 673 (1968).
12. K. Ishii and I. Shiio, *J. Biochem.* **63**, 661 (1968).
13. J. H. Anderson and A. C. Sartorelli, *Fedn Proc.* **25**, 745 (1966).
14. J. H. Anderson and A. C. Sartorelli, *Fedn Proc.* **26**, 730 (1967).
15. A. Hampton and M. H. Maguire, *J. Am. chem. Soc.* **83**, 150 (1961).
16. W. W. Cleland, *Biochim. biophys. Acta* **67**, 104 (1963).