Specific Protection of Folate Reductase Against Chemical and Proteolytic Inactivation

MAIRE T. HAKALA AND ELSE-MAJ SUOLINNA

Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14203

(Received June 17, 1966)

SUMMARY

Folate reductase of amethopterin resistant S-180 cells (AT/3000), grown *in vitro*, is inactivated by *o*-phenanthroline, EDTA, *p*-chloromercuribenzoate, iodoacetamide, trypsin, subtilisin, elastase, and carboxypeptidase B, but not by carboxypeptidase-A or leucine-aminopeptidase. The enzymatic activity and the number of amethopterin binding sites were always lost in parallel fashion. When substrates or coenzymes were present, complete protection of the enzymic activity and of the amethopterin binding sites against all these agents was observed. Competitive inhibitors, 2,4-diaminopurine and -pyrimidine, which mimic just a small portion of the substrate molecule, also protected completely against *o*-phenanthroline and against the proteolytic enzymes but poorly against SH-reagents. It is suggested that folate (probably the pyrimidine portion of it) and TPNH are bound to the enzyme in the proximity of a metal and SH-groups. The protection against the proteolytic digestions is probably provided through conformational changes in the enzyme. No major proteolytic breakdown of the enzyme molecule, as a whole, was observed when folate reductase was complexed with amethopterin.

It is suggested that the intracellular presence of substrate, coenzyme or competitive inhibitor of folate reductase may alter the turnover of this enzyme by preventing the proteolytic breakdown. In certain circumstances this could lead to an increase in the folate reductase content of cells and tissues. Also, it is concluded that folate reductase can form binary complexes with both the substrate and the coenzyme. Thus, the prior presence of coenzyme on the enzyme surface is not necessary for the attachment of the substrate and vice versa. Since the concentrations of folate, TPNH, and TPN which afforded 50% protection were of the same order of magnitude as the respective K_m values, it seems that the presence of one on the enzyme surface does not affect the affinity to the other.

INTRODUCTION

The various ways in which a substrate or a coenzyme can influence the stability of enzymes in general has been thoroughly discussed in a review by Grisolia (1). These effects have been shown to vary from inactivation to stimulation or to protection against inactivation.

Folate reductase (also named dihydrofolate reductase or tetrahydrofolate dehydrogenase) is a dehydrogenase which preferably utilizes TPNH as the cofactor. It has a molecular weight of about 21,000 (2–5) and catalyzes the formation of tetrahydrofolate from both folate and dihydrofolate (6). These reductions are competitively inhibited by diamino derivatives of folate, pteridines, purines, and pyrimidines (7–9). Dihydrofolate reductases of sheep and chicken liver and of *Lactobacillus leichmannii* have been reported to be insensitive to SH-inhibitors

(10–12). In contrast, this enzyme from *Streptococcus faecalis* and from calf thymus was inhibited by *p*-chloromercuribenzoate (13, 14). Stimulation of dihydrofolate reductase activity by organic mercurials has been reported for enzyme preparations from chicken liver (2, 3), Ehrlich ascites carcinoma (15), and L-1210 leukemic cells of mouse (16). The dihydrofolate reductase of chicken liver has been reported to be insensitive to metal-binding agents (11).

In studies of folate reductase an unusual advantage is provided by the fact that it binds amethopterin, a competitive inhibitor, very tightly (8). Therefore, two parameters of folate reductase can be examined simultaneously, the enzymatic activity and the amethopterin binding capacity. The folate reductase of a subline of Sarcoma 180 cells (AT/3000) grown *in vitro* was shown to be inactivated, with a parallel loss of amethopterin binding capacity, by several chemical agents and proteolytic enzymes. Whether chemical or proteolytic, the inactivation could be prevented and the amethopterin binding capacity retained by the presence of either a substrate, coenzyme, or a competitive inhibitor. Preliminary reports on part of these findings have been presented (17, 18).

MATERIALS AND METHODS

Origin and maintenance of cells. The origin and development of the amethopterin resistant subline of S-180 cells, AT/3000, and its maintenance medium for *in vitro* culture have been previously described (19).

Preparation of the cell extract. To eliminate the folate reductase-bound amethopterin, the AT/3000 cells were grown for 10–15 days in amethopterin-free HTG-medium as has been previously described (20). The cells were then collected, washed and homogenized in saline as described. The high-speed supernatant (1 hr at 105,000 *g*) of this homogenate contained 2–3 mμmoles of folate reductase per milligram of protein as determined by titration with amethopterin. Since the molecular weight of this folate reductase is 21,000 (4), the amount of folate reductase was calculated to be 4–6% of the supernatant protein. These extracts were used as such in the present studies and were stored at −75°.

Assay of folate reductase activity. The assay of folate reductase activity is based on the enzymic conversion of folic acid at pH 6.0 in the presence of TPNH to tetrahydrofolic acid. A total volume of 0.5 ml contained 20–40 mμM enzyme, 0.1 M citrate buffer, 40 μM folate, and 400 μM TPNH. The reaction was initiated with TPNH, incubated at 37° for 20 or 30 min and the reaction was stopped by adding 0.1 ml of 5 N HCl. The blank differed from the samples by containing a supplement of 400 mμM amethopterin. Tetrahydrofolic acid, after being decomposed to *p*-aminobenzoylglutamic acid, was determined by Bratton-Marshall reaction at 560 mμ (21).

Determination of amethopterin binding capacity. This was done by titrating the folate reductase activity to zero with amethopterin as has been described (22). The basic reaction mixture was identical with that used for the assay of folate reductase activity above, except that a series of five test tubes were supplemented with 0, 8, 16, 24, and 32 mμM concentrations of amethopterin and the blank with 400 mμM, as above. This permitted a direct estimation of the concentration of amethopterin necessary for complete inhibition and thus the calculation of molar equivalents of amethopterin binding sites per milligram of protein. No change in activity per amethopterin binding site was caused by any of the various chemical or proteolytic inactivations to be described in this paper. Even a small change of this type would have been revealed by a change in the slope of the amethopterin titration curve as schematically illustrated by Fig. 1. The "per cent protection" used in several figures therefore indicates the protection of both the activity and the amethopterin binding sites.

Determination of bound and free amethopterin. The free and bound amethopterin were determined as described (23) by titrating vith folate reductase (24).

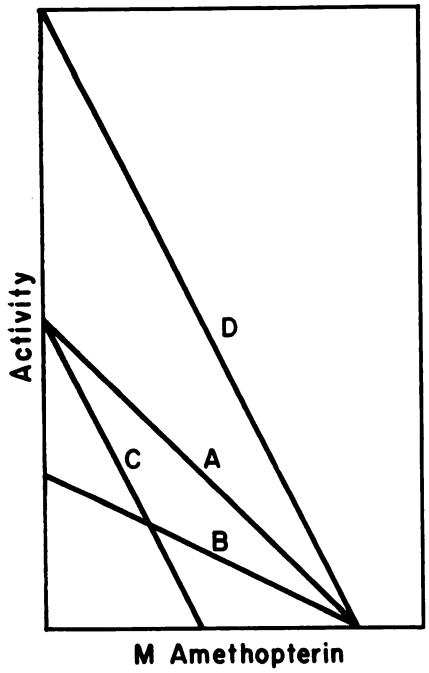

FIG. 1. *Theoretical titration curves of folate reductase with amethopterin, assuming that the enzymatic activity and amethopterin binding capacity were differentially affected*

A, Original titration curve; *B*, 50% inactivation without loss in amethopterin binding capacity; *C*, 50% loss in amethopterin binding capacity without loss in enzymic activity; *D*, 2-fold increase in the activity without change in amethopterin binding capacity.

Chemical inactivation of folate reductase. The buffers, pH, temperature, and duration of preincubations with PMB[1], IAA, EDTA, and OP are described for each experiment in the legends to the figures. The molar concentration of folate reductase in these incubation mixtures was between 5 and 15 μM. After incubation the samples were dialyzed for 23 hr at 4° against three changes of 800 ml of 0.05 M sodium citrate pH 8.0. This procedure allowed removal of practically all the unbound inactivating agents as well as protecting agents such as might have been present during the preincubation, and eliminated any complications that these substances might have caused had they been present in the folate reductase assay mixture. The only series in which dialysis was omitted was that with PMB in Fig. 3. Controls in each case were handled in an identical manner but without the agent.

Proteolytic digestion of folate reductase. Proteolytic enzymes which had pH optima in slightly alkaline pH range were used in this study. To stop the digestion (or at least to reduce it to a minimum) an equal or double volume of ice-cold 0.5 M citrate buffer pH 6.0 (the pH of folate reductase assay) was added and the whole mixture was set in ice. The concentration of folate reductase in the original incubation mixture was kept as high as practicable (~ 5 μM). In order to be able to relate the folate reductase activity to protein content, dialysis after the proteolytic digestions was avoided because this would have led to a loss of some of the digested proteins. The only complication which could have been anticipated because of this, might have occurred in samples containing a folate reductase inhibitor, such as diaminopyrimidine. Fortunately, these inhibitors completely protected folate reductase against proteolytic inactivation and, therefore, the dilution of these samples for folate reductase assay was so extensive (100- to 200-fold) that the final concentration of the inhibitor (10 to 30 μM) in the assay mixture was too low for any effect on the folate reductase reaction.

Fractionation of cell extract on Sephadex G-75. The procedure was similar to that described (25). The column, 35 mm in diameter and 40 cm long, was equilibrated with 0.1 M NaCl and eluted with same. The total amount of cell extract protein applied to this column was about 40 mg, usually in 4 ml. The size of fractions was 100 drops, and the flow rate 10–12 fractions per hour. Each fraction was analyzed for protein content using Folin-Ciocalteau reagent (750 mμ), and for folate reductase

[1] The abbreviations used are: IAA, iodoacetamide; PMB, *p*-chloromercuribenzoate; OP, *o*-phenanthroline; FR, folate reductase; S, subtilisin; T, trypsin; E, clastase; CP-B, carboxypeptidase; 2,6-DAP, 2,6-diaminopurine; DADMP, 2,4-diamino-5,6-dimethylpyrimidine; FA, folic acid; DAMP, 2,4-diamino-6-methylpyrimidine; EDTA, ethylenediamine tetraacetate (Versene).

activity. For ^{3}H-counting of the ^{3}H-amethopterin-containing fractions the Packard liquid scintillation counter was used; 0.1 ml of each fraction was measured into counting vials containing 20 ml of scintillation solution (26); this gave 15–20% counting efficiency.

Chemicals. The following proteolytic enzymes were purchased from Worthington Biochemical Corporation, Freehold, New Jersey: trypsin, 3X crystallized, sterile; α-chymotrypsin, chromatographically purified; elastase, 3X crystallized, 26 and 35 U/mg; carboxypeptidase A, 2X crystallized; carboxypeptidase B; leucine aminopeptidase, lyophilized powder from hog kidney. Pharmacia, Uppsala, Sweden, supplied Sephadex G-75, new bead form. Nutritional Biochemicals Corporation, Cleveland, Ohio, was the source of *p*-chloromercuribenzoate, sodium salt; Versene (EDTA); 2,6-diaminopurine sulfate; folic acid, and subtilisin. P-L Biochemicals, Inc., Milwaukee, Wisconsin was the source for TPN, DPN, DPNH, and TPNH; the latter two were enzymatically reduced preparations, purified by ion-exchange chromatography. Iodoacetamide was purchased from Sigma Chemical Company, St. Louis, Missouri; *o*-phenanthroline from Eastman Organic Chemicals, Rochester, New York; and amethopterin from American Cyanamid Company, Lederle Division, Pearl River, New York. We are grateful to Dr. George Hitchings from Burroughs-Wellcome & Company, Tuckahoe, New York, for supplying us with 2,4-diamino-5,6-dimethylpyrimidine and 2,4-diamino-6-methylpyrimidine. The ^{3}H-labeled amethopterin, 845 mC/mmole, >95% pure, was purchased from Nuclear-Chicago, Des Plaines, Illinois, and was stored dissolved in 0.05 M sodium citrate at −75°.

RESULTS

Effect of Iodoacetamide on Folate Reductase

The stability of folate reductase at varying pH and in different buffers in the presence and absence of iodoacetamide (IAA) is presented in Fig. 2. The enzyme by itself is quite labile below pH 5.5 and above pH 9.0 under the conditions employed. For further studies with IAA, pH 8.0 was chosen to minimize inactivation of the control samples. The time and temperature dependence of IAA effect is shown in Fig. 3 and the dependence of inactivation of folate reductase on IAA concentration at pH 8.0 and 30° is shown in Fig. 4. For inactivation of half of folate reductase in these samples in 90 min at 30° about 0.05 M IAA is required. Inactivation was always associated with a parallel loss of the number of amethopterin binding sites (see Fig. 1).

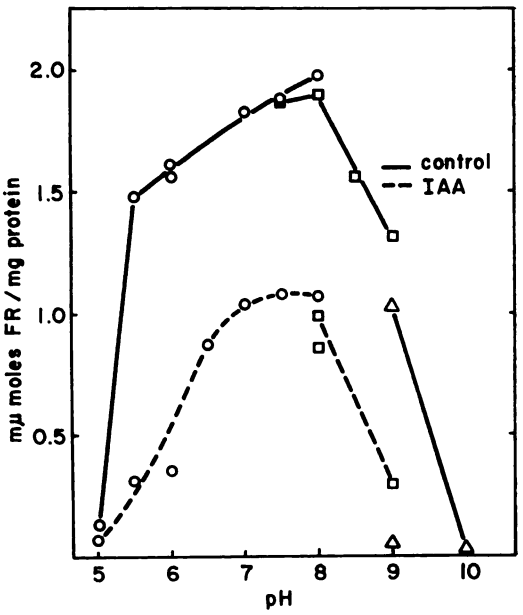

FIG. 2. *Stability of folate reductase (6 μM) at varying pH in the presence and absence of IAA (0.1 M)*

The incubation mixture (4 ml) contained 1 ml of cell extract (see Methods), 1 ml of 0.05 M sodium citrate, and 2 ml of buffer with or without IAA. ○, McIlwaine's buffer (0.1 M citric acid–0.2 M sodium phosphate); □, 0.2 M Tris buffer; △, 0.2 M sodium borate. After incubation for 60 min at 31° the samples were dialyzed and folate reductase activity and amethopterin binding sites were titrated with amethopterin as described in Methods. The control samples at pH 8.0 retained all the original activity and amethopterin binding capacity.

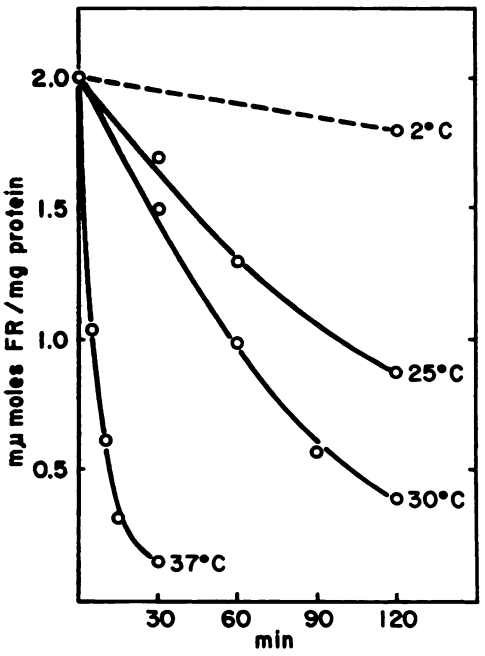

FIG. 3. *Effect of time and temperature on the inactivation of folate reductase by IAA (0.1 M) at pH 8.0 in 0.1 M Tris buffer*

Dialysis and folate reductase titration are described in Methods.

Effect of p-Chloromercuribenzoate on Folate Reductase

p-Chloromercuribenzoate (PMB) was found to be only slightly more effective as an inactivating agent of folate reductase at pH 5–6 than at pH 7–8 when McIlwain's buffer and preincubation for 30 min at 25° in 1.3 mM PMB was used. After dialysis in the usual manner, the inactivation under those conditions was found to vary from 62 to 75%. The dependence of inactivation on PMB-concentration at pH 6.0 when dialysis was omitted is seen in Fig. 4. At a concentration of folate reductase of 10 μM, about 1 mM concentration of PMB was necessary for extensive inactivation, and this was associated with an equivalent loss of amethopterin binding sites.

Effect of Metal Chelaters on Folate Reductase

As in the case of the other agents used, the inactivation of folate reductase by *o*-phenanthroline (OP) and ethylenediamine tetraacetate (EDTA) is strongly dependent on the concentration of the

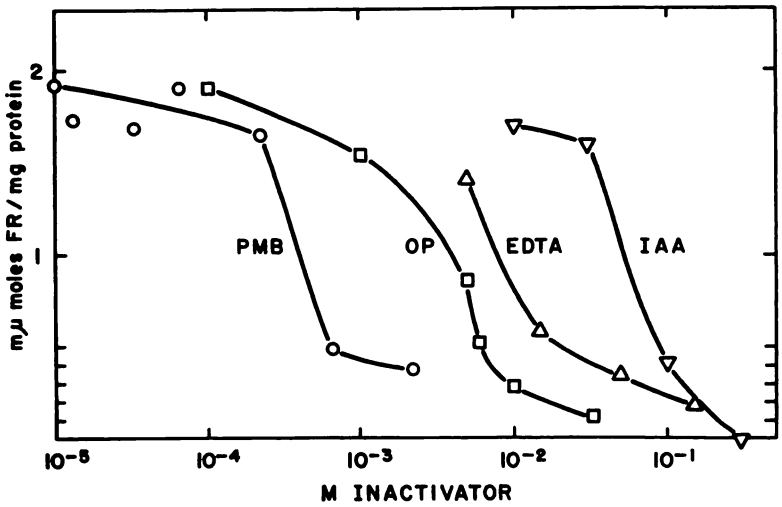

FIG. 4. *Chemical inactivation of folate reductase (FR)*

p-Chloromercuribenzoate (*PMB*): 10 μM FR, 0.05 M sodium citrate buffer pH 6.0, 90 min at 25°, no dialysis in this particular case; *o*-phenanthroline (*OP*): 5–10 μM FR, 0.07 or 0.08 M Tris-buffer pH 7.5, 60 min at 37°; ethylenediamine tetraacetate (*EDTA*): 7.5 μM FR, Tris-buffer 0.1 M, pH 7.0, 60 min at 37°; iodoacetamide (*IAA*): 6.6 μM FR, Tris-buffer 0.1 M, pH 8.0, 90 min at 30°. All the samples and controls (OP, EDTA, IAA) were dialyzed, folate reductase activity was determined, and amethopterin binding capacity was titrated as described in Methods.

inhibitor, as shown in Fig. 4. Any interference which OP or EDTA might cause by their presence in the enzyme assay mixture was avoided by thorough dialysis before assay (see Methods). This dialysis had no effect on the activity of the chelater-free controls. Thus, if metal chelation is involved, this metal cannot be removed by simple dialysis. On a molar basis EDTA was one-half as effective as OP. Whenever enzyme activity was lost, an equal loss of the number of amethopterin binding sites occurred.

Effect of Proteolytic Enzymes on Folate Reductase.

Since folate reductase in the cell extract is rather unstable at low pH (Fig. 2) and since the assays of folate reductase were carried at pH 6.0, only such proteolytic enzymes were chosen whose pH optimum is in a slightly alkaline pH range. Of the enzymes tested, five inactivated folate reductase quite effectively (Fig. 5). As in the chemical inactivations the loss of the enzymic activity of folate reductase was associated with a parallel loss of amethopterin binding sites. The controls, containing no added proteolytic enzyme, but otherwise identical in composition and handling with their respective samples, also underwent some inactivation. The inactivation of controls for elastase and subtilisin was 0–5%, for trypsin 15%, and for chymotrypsin 38%. Since the temperature (37°) and the duration of the incubations (30 min) were the same for all these controls, the variations were clearly caused by the ionic environment which was different for each proteolytic enzyme. It is possible that some of the intracellular proteolytic enzymes present in the cell extracts found certain of these ionic environments ideal and thus contributed to the inactivation. With respect to concentration, chymotrypsin was the most effective proteolytic inactivator of folate reductase. However, due to the extensive inactivation of the control samples in the chymotrypsin system, it was excluded from further investigation. Subtilisin and trypsin were about equally effective while rather large concentrations of elastase and carboxypeptidase B had to be used. Carboxypep-

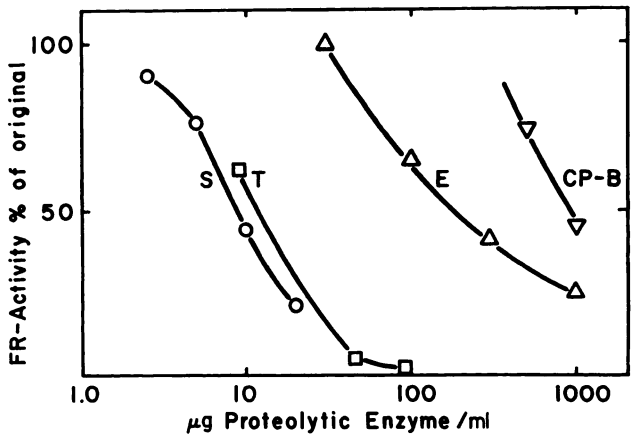

FIG. 5. *Inactivation of folate reductase in cell extracts by proteolytic enzymes at 37° in 30 min*

Subtilisin: 0.5 ml cell extract, 0.5 ml 0.2 M KCl, and 1 ml subtilisin in 0.2 M Tris buffer pH 8.0. Trypsin: 0.5 ml cell extract, 0.5 ml 0.092 M Tris buffer containing 0.023 M $CaCl_2$ pH 8.1 and 0.1 ml trypsin in 0.3 mM HCl. Elastase: 0.5 ml cell extract and 0.5 ml elastase in 0.2 M Tris buffer pH 8.8. Carboxypeptidase B: 0.5 ml cell extract and 1.0 or 1.5 ml of CP-B in 0.025 *M* Tris buffer containing 0.1 M NaCl pH 7.65. The proteolysis was stopped, folate reductase activity was determined, and amethopterin binding sites were titrated as described in Methods.

tidase A at 700 μg/ml and leucine aminopeptidase at 440 μg/ml had no effect on folate reductase activity or amethopterin binding capacity of the cell extracts when incubated for 30 min at 37°. It has been reported that a 50% pure dihydrofolate reductase preparation of chicken liver became digestible by trypsin only after the cysteine residues had been alkylated (27).

Protection of Folate Reductase against IAA

Whenever protection was observed, the activity and the number of amethopterin binding sites were equally preserved. Folic acid and TPNH offered complete protection of the enzyme against IAA (Figs. 6 and 7). Dihydrofolic acid was tested only at 0.2 mM concentration; it provided 88% protection while the unprotected control had only 13% of activity and amethopterin binding sites left. The 2,6-diaminopurine and both the 2,4-diaminopyrimidines, competitive inhibitors of folate reductase with rather large dissociation constants (7), offered only poor protection (Fig. 6 and Table 4). *p*-Aminobenzoylglutamic acid

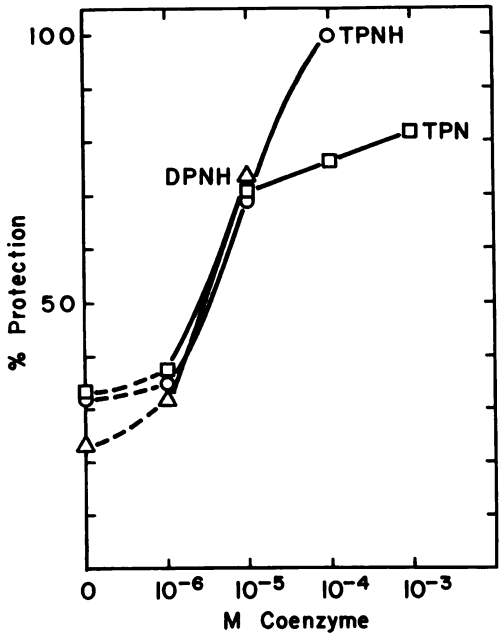

FIG. 7. *Protection of folate reductase against 0.08 M IAA by pyridine nucleotide coenzymes at 30°, in 90 min, in 0.1 M Tris buffer pH 8.0*

Dialyzed, folate reductase activity was determined and amethopterin binding capacity was titrated as described in Methods.

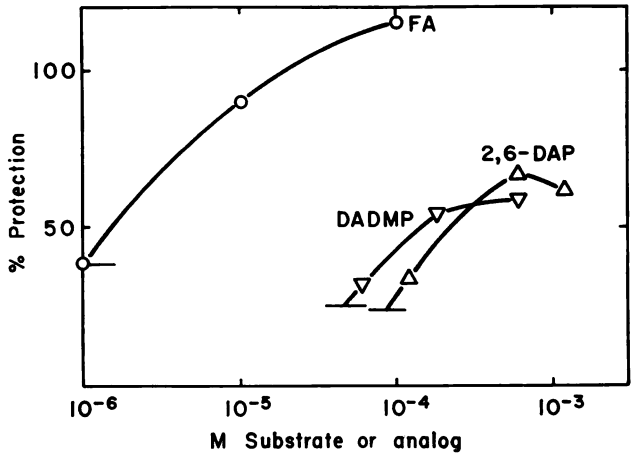

FIG. 6. *Protection of folate reductase against IAA by folate and its analogs at 30° in 0.1 M Tris buffer, pH 8.0*

Folate samples were incubated for 60 min with 0.08 M IAA, and those with folate analog for 90 min with 0.1 M IAA. Dialyzed, folate reductase activity was determined, and amethopterin binding capacity was titrated as described in Methods.

(2.5 mM) alone offered no protection and when combined with 2,4-diamino-6-methylpyrimidine (2.5 mM) did not increase the poor protection provided by the pyrimidine alone (Table 4). Amethopterin stayed bound to the enzyme through IAA treatment (Table 1).

TABLE 1
IAA-treatment of folate reductase–amethopterin complex

Incubation for 60 min at 30° in 0.1 M Tris buffer pH 8.0 was performed in the presence of amethopterin, 50% in excess over that required for saturation of the folate reductase in these samples. The samples were dialyzed, and bound amethopterin as well as free folate reductase were titrated as described in Methods.

	Folate reductase (moles/mg protein)	
IAA (M)	Bound by amethopterin	Free enzyme
0	1.09×10^{-9}	3.4×10^{-12}
0.1	1.1×10^{-9}	2.7×10^{-12}

Protection of Folate Reductase against PMB

As above, protection always involved equally the activity and the number of amethopterin binding sites. Folic acid and TPNH protected the enzyme completely, TPN and DPNH partially, and DPN

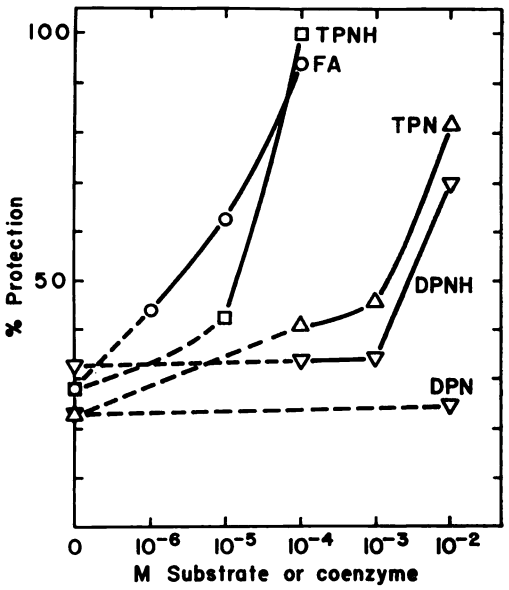

FIG. 8. *Protection of folate reductase against 1.5 mM PMB at 25°, in 60 min, in 0.067 M citrate buffer pH 6.0*

Dialyzed, folate reductase activity was determined and amethopterin binding capacity was titrated as described in Methods.

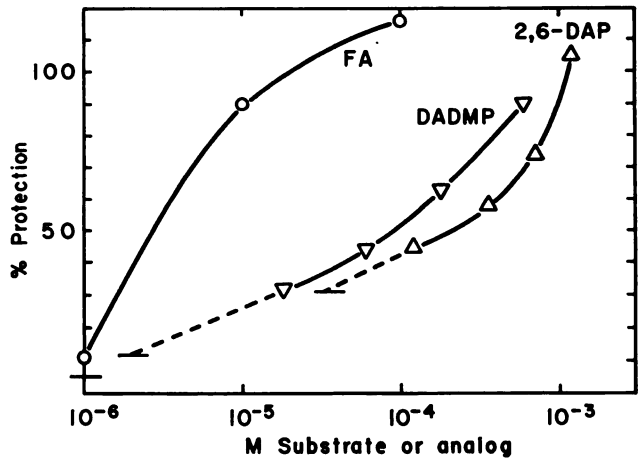

FIG. 9. *Protection of folate reductase against o-phenanthroline by folate and its analogs at 37° in 60 min in 0.1 M Tris buffer pH 7.5*

With folate and 2,6-diaminopurine, the concentration of *o*-phenanthroline was 6 mM with DADMP 10 mM. Dialyzed, folate reductase activity was determined and amethopterin binding capacity was titrated as described in Methods.

not at all even at 10^{-2} M, against PMB (Fig. 8). As with IAA, 2,4-diamino-6-methylpyrimidine (8 mM) provided poor protection of folate reductase against PMB (Table 4).

Protection of Folate Reductase against OP

As before, activity and amethopterin binding sites were equally affected. Folic acid, TPNH, TPN, 2,6-diaminopurine, and 2,4-diamino-5,6-dimethylpyrimidine completely protected the enzyme against OP-inactivation (Figs. 9 and 10). Also dihydrofolic acid, 9×10^{-5} M, offered complete protection. As seen in Table 2, AMP-5′, PO_4, *p*-aminobenzoylglutamic acid, and pyrimidines, other than 2,4-diamino-, offered only insignificant or no protection even at concentrations 1–3 mM.

Protection of Folate Reductase against Proteolytic Enzymes

Folic acid, TPNH, and 2,4-diamino-pyrimidines were the only compounds tested in this respect and only at one single concentration each. These compounds offered complete protection of folate reductase activity as well as amethopterin binding capacity (Fig. 11), i.e., the presence of these compounds in the active site prevented the proteolytic destruction of this site. In respect to protection against trypsin digestion it is to be noted that the unprotected, trypsin-free control also lost some folate reductase activity, as was discussed earlier. The loss of activity and of the amethopterin binding sites in the control samples was also prevented by the presence of the protecting agents; this is shown in Fig. 11. Results in agreement with ours have been reported by Burchall (28). He demonstrated protection of *Escherichia coli* dihydrofolate reductase by substrates, coenzymes, and competitive inhibitors against inactivation by pronase.

Incubation of folate reductase–amethopterin complex with elastase failed to release free amethopterin, whereas the free folate reductase was inactivated by 93% (Table 3). This indicates that elastase was unable to digest the amethopterin-bound enzyme to such a degree as would be necessary for dissociation of amethopterin.

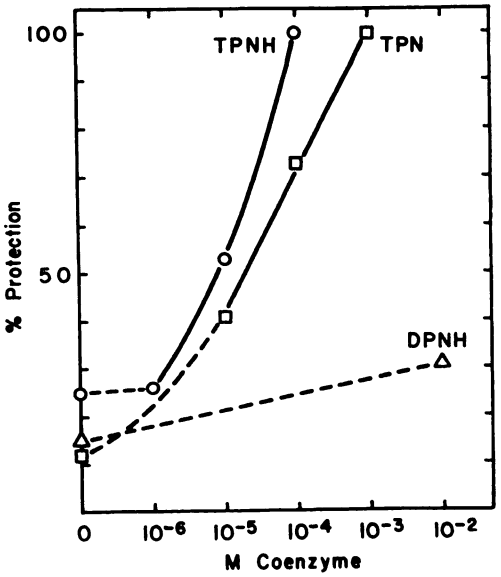

FIG. 10. *Protection of folate reductase against 6 mM o-phenanthroline by pyridine nucleotide coenzymes at 37°, in 60 min, in 0.1 M Tris buffer pH 7.5*

Dialyzed, folate reductase activity was determined and amethopterin binding capacity was titrated as described in Methods.

It is possible that proteolysis of folate reductase in the presence of a protector still occurs even though it has no effect on the enzymic activity, or integrity of the active site. To detect whether major digestion of folate reductase in the presence of a protector had occurred, the folate reductase–^{3}H-labeled amethopterin complex was incubated with large quantities of each enzyme, elastase twice, trypsin 10 times and subtilisin 20 times the highest concentration used in Fig. 5. After incubation the digestion mixture was combined with the native folate reductase (*T*) or the undigested labeled complex (E and S) before application to Sephadex G-75 column (Fig. 12). In respect to Fig. 12 it must be realized that the absolute positions of the peaks in the four different fractionations are not comparable, since the columns were not identical. The profile of

TABLE 2

Compounds that do not protect folate reductase against o-phenanthroline

Incubations were at 37° for 60 min in 0.1 M Tris buffer pH 7.5. Samples were dialyzed and analyzed as described in Methods.

Compound (mM)	OP (mM)	Folate reductase remaining	
		Moles/mg protein	Per cent of control
2-Amino-4,6-dihydroxypyrimidine			
0	0	1.9×10^{-9}	100
0	6.0	4.8×10^{-10}	25.2
3.0	6.0	5.0×10^{-10}	26.3
2,5-Diamino-4,6-pyrimidine dione			
0	0	1.6×10^{-9}	100
0	6.0	5.8×10^{-10}	36.2
3.0	6.0	7.1×10^{-10}	44.3
2,4,5-Triamino-6-hydroxypyrimidine			
0	0	1.5×10^{-9}	100
3.0	0	1.9×10^{-9}	127
0	10.0	6.4×10^{-10}	42.7
3.0	10.0	4.2×10^{-10}	28.0
AMP-5′			
0	0	1.9×10^{-9}	100
0	6.0	2.4×10^{-10}	12.6
1.0	6.0	3.2×10^{-10}	16.9
PO_4 1.0	6.0	2.8×10^{-10}	14.7
p-ABGA[a] 1.0	6.0	2.7×10^{-10}	14.2

[a] *p*-Aminobenzoylglutamic acid.

TABLE 3

Elastase digestion of free and amethopterin-bound folate reductase

Cell extract (0.5 ml) was supplemented with 0.5 ml of amethopterin solution, twice the amount required for saturation of the folate reductase in the cell extract. To this, 0.5 ml of elastase 1 mg/ml in 0.2 M Tris buffer pH 8.8 was added, and the mixture was incubated for 30 min at 37°. The control samples contained 0.5 ml of H_2O in place of amethopterin solution. Digestion was stopped and bound amethopterin as well as free folate reductase were titrated as described in Methods.

Elastase (μg/ml)	Amethopterin	Folate reductase (moles/mg protein)	
		Bound by amethopterin	Free enzyme
0	−	0	1.6×10^{-9}
333	−	0	1.1×10^{-10}
0	+	1.6×10^{-9}	0
333	+	1.6×10^{-9}	0

the undigested labeled cell extract was included in the diagram to demonstrate the protein pattern (solid line) of the native extract and the relative position of the free amethopterin. While the other proteins in the cell extracts were digested extensively by T, E, and S, the FR–^{3}H-amethopterin complex always formed a single, symmetrical peak when mixed with an equal amount of undigested complex (E and S) or with an equal amount of native reductase (T). Furthermore, just as was found for elastase in another experiment (Table 3), no free ^{3}H-amethopterin was released by any of these digestions with E, T, or S.

Reactivation of PMB-Treated Folate Reductase

Folate reductase after being inactivated by PMB, could not be reactivated by immediate dialysis against high concentra-

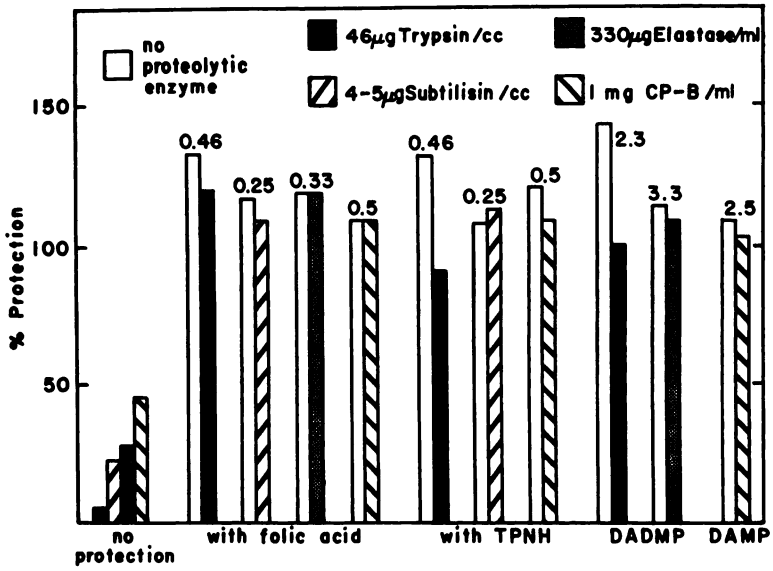

FIG. 11. *Protection of folate reductase against proteolytic enzymes*

Trypsin: 0.5 ml cell extract, 0.5 ml 0.092 M Tris buffer, pH 8.1, containing 0.023 M $CaCl_2$ and the protecting agent, and 0.1 ml 0.3 mM HCl containing trypsin. Subtilisin: 0.5 ml cell extract, 0.5 ml 0.2 M KCl, and 1 ml 0.2 M Tris buffer, pH 8.0, containing subtilisin and the protecting agent. Elastase: 0.5 ml extract and 1 ml 0.2 M Tris buffer, pH 8.8, containing elastase and the protecting agent. Carboxypeptidase-B: 0.5 ml cell extract and 1 ml 0.025 M Tris buffer, pH 7.65, containing 0.1 M NaCl, CP-B, and the protecting agent. All others, except subtilisin samples, were incubated at 37° for 30 min, the latter at 30° for 2 hr. The proteolysis was stopped, folate reductase activity was determined and amethopterin binding capacity was titrated as described in Methods. The numbers at the top of the bars indicate the millimolar concentration of the protecting agent. Per cent protection describes the activity and amethopterin binding capacity compared with the appropriate control, free of protector, but treated otherwise identically with the corresponding samples. These controls also lost activity; this is discussed in the text.

tions (10 mM) of either cystine, glutathione, or β-mercaptoethanol in 0.05 M sodium citrate.

Reactivation of OP-Treated Folate Reductase

Folate reductase after being inactivated by OP could not be reactivated by immediate dialysis against a variety of metal chlorides (10 mM) dissolved in 0.05 M sodium citrate pH 8.0. The metal chorides tested included Ca^{++}, Mg^{++}, Mn^{++}, Fe^{++}, Fe^{3+}, Zn^{++}, Ni^{++}, Co^{++}, Cu^{++}, Mo^{++}, and Mo^{5+}. Further studies with folate reductase preparation (30% pure) purified by Sephadex G-75 fractionation have indicated that OP causes a precipitation of the enzyme indicating denaturation.

DISCUSSION

Although the studies on folate reductase described here have been done with cell extracts, we have had the unusual advantage of being able to titrate the enzyme content in terms of the number of amethopterin binding sites. Moreover, the content of folate reductase in these extracts made from the amethopterin resistant subline, AT/3000, is high: 2–3 mμmoles/mg protein, or 4–6%. Thus, this extract is as pure in respect to folate reductase as enzyme preparations purified 400- to 500-fold from chicken liver (3). However, using a cell extract one must realize that all the inactivating agents, whether chemical or proteolytic, react not only with folate

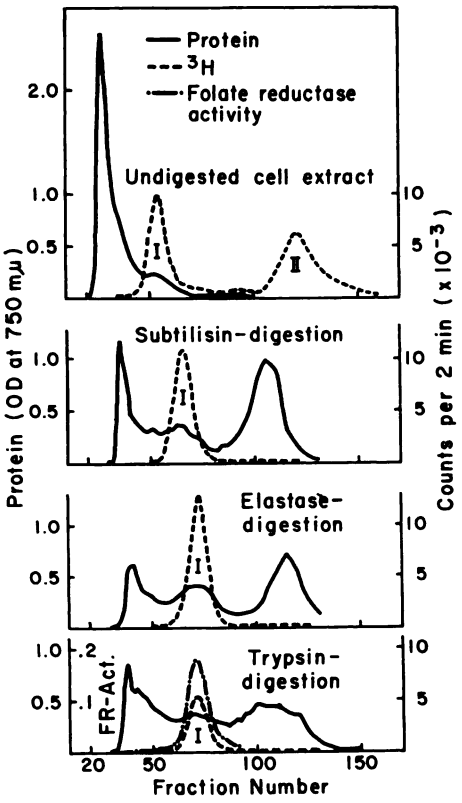

FIG. 12. *The effect of proteolytic digestion on the Sephadex G-75 fractionation-profile of cell extracts containing folate reductase ³H-labeled-amethopterin complex*

The column and the analytical procedures are described in Methods. The absolute elution volumes for the four fractionations are not comparable. Peak I: folate reductase activity and folate reductase-³H-amethopterin complex. Peak II: Free ³H-amethopterin. Undigested cell extract: 4 ml of cell extract (37.5 mg protein, 78 mμmoles folate reductase) supplemented with 155 mμmoles of ³H-amethopterin equal to 12.5 μc. The proteolytic digestions were made at 37° for 30 min, and the samples were then set immediately into ice. The labeled cell extract used for these contained per milliliter: 11.9 mg protein, 19.7 mμmoles folate reductase and 19.3 mμmoles ³H-labeled amethopterin equal to 1.56 μc. Subtilisin digestion: 2 ml labeled cell extract, 1 ml 0.2 M KCl in 0.2 M Tris buffer, pH 8.0, and 1 ml subtilisin in Tris (400 μg/ml). Elastase digestion: 2 ml of labeled cell extract and 2 ml of elastase in 0.2 M Tris buffer, pH 8.8 (2 mg/ml). Trypsin digestion: 2 ml of labeled cell extract, 2 ml of 0.092 M Tris buffer containing 0.023 M $CaCl_2$, pH 8.1 and 0.4 ml of trypsin in 0.3 mM HCl (1 mg/ml). After digestion and prior to application to the column, 2 ml of undigested ³H-labeled cell extract were added to the subtilisin and elastase digested extracts. To the trypsin-digested ³H-labeled cell extracts were added, instead, 2 ml of undigested, amethopterin-free native cell extract prior to application to the column.

reductase, but also with other proteins present in these extracts. It should also be noted that these extracts apparently contain intracellular proteolytic enzymes which most likely were the cause of the extensive inactivation of the control samples under the conditions used for chymotryptic digestion.

In all the inactivation experiments, whether chemical or proteolytic, particular care was taken to assure that the inactivation was not complete. The residual activity permitted a rough estimation of the rate of inactivation. It also made the quantitative determination of amethopterin binding sites through the titration method convenient. It was considered quite conceivable that the enzymic activity and the amethopterin binding sites might be destroyed to a different degree by the various agents which were used. Even a small change of this type would have been revealed by a change in the slope of the amethopterin titration curve as schematically illustrated in Fig. 1. This type of change should be quite pronounced when 75–95% loss of activity occurred, but it was never observed. Instead, whenever activity was lost, either chemically or through proteolysis, a parallel loss in the number of amethopterin binding sites occurred. This must mean that the intactness of the binding site for amethopterin is a prerequisite for folate reductase activity and vice versa.

In relation to all the protection studies described in this paper and summarized in Table 4, one should consider the small size of folate reductase (mol. wt. 21,000). The substrate and coenzyme of this enzyme have molecular weights of 440 and 800, respectively, and it is well known that the

glutamate end of folate molecule can be extended by γ-carboxyl-linkages to polyglutamates (di-, tri-, and hepta-) with no change in reactivity (29). The presence of folic acid at the active site does not prevent the entry of TPNH to this site, nor does the presence of TPNH prevent the entry of folic acid. Thus, the approach of these rather large molecules is spatially unhindered. It seems unlikely that either the substrate or the coenzyme would spatially prevent the approach of even smaller molecules such as IAA (mol. wt. 185), PMB (mol. wt. 340) or OP (mol. wt. 198) to this general area. Yet substrate and coenzyme prevent the destruction of

TABLE 4
Summary of protection of folate reductase against inactivation[a]

Protector and highest concentration used (mM)	Chemical inactivation				Proteolytic inactivation[b]			
	IAA pH 8.0	PMB pH 6.0	OP pH 7.5	50% Protection (μM)	T	S	E	CP-B
Substrates								
Folic acid	+++	+++	+++	1–10	+++	+++	+++	+++
Dihydrofolic acid, 0.2	+++		+++					
Coenzymes								
TPNH	+++	+++	+++	10–20	+++	+++		+++
TPN	++	++	+++	10–30				
DPNH, 10.0	++	++	+					
DPN, 10.0	+	−						
Competitive inhibitors								
Amethopterin[b]	+++				+++	+++	+++	
2,6-Diaminopurine	++		+++	300				
2,4-Diamino-5,6-dimethylpyrimidine	++		+++	100	+++		+++	
2,4-Diamino-6-methylpyrimidine, 8.0	++	++						+++
Other derivatives								
2,4,5-Triamino-6-hydroxypyrimidine, 3.0			−					
2,5-Diamino-4,6-pyrimidinedione, 3.0			−					
2-Amino-4,6-dihydroxypyrimidine, 3.0			−					
p-Aminobenzoylglutamic acid, 2.5	−		−		−			
AMP-5′, 1.0			−					
PO_4, 1.0			−					
Combination								
2,4-Diamino-6-methylpyrimidine, 2.5 p-Aminobenzoylglutamic acid, 2.5	++							

[a] +++, complete protection; ++, partial; +, slight; −, no protection. The protection always involved equally the activity and the amethopterin binding sites.

[b] IAA and elastase were unable to release free amethopterin from folate reductase–amethopterin complex (Tables 1 and 3). T-, S- or E-treated complex moved on Sephadex G-75 column together with the undigested enzyme (Fig. 12).

amethopterin binding site (active site) by these small molecules, and therefore it seems more likely that the reactive groups are directly shielded by the substrate and the coenzyme. It is interesting to note, however, that while the diaminopyrimidines protect the active site against metal chelaters, they protect very poorly against SH-inhibitors.

Digestion by leucineaminopeptidase or carboxypeptidase A had no effect on folate reductase activity or the amethopterin binding sites whereas carboxypeptidase B destroyed both of these. Thus, it seems that the N-terminal is less critical for the active site than the C-terminal. Moreover, the critical C-terminal is more likely either a basic amino acid or cysteine than aromatic or branched-chain aliphatic amino acid. The N-terminal of chicken liver dihydrofolate reductase has been shown to be leucine, and the C-terminal is glutamic acid.[2]

The protection of folate reductase by folate, TPNH, and diaminopyrimidines against proteolysis involved not only the activity, but also the binding sites, for amethopterin. Thus, the presence of these compounds on the enzyme prevented the digestion of those peptide bonds which are essential for the maintenance of the binding site and activity. It is possible that proteolysis of other bonds less critical for folate reductase activity or binding sites might have occurred even in the presence of the protector. However, the study of Markus (30) suggests that even those peptide bonds not concerned with the binding site would be digested more slowly. His evidence is based on the general slowing down of proteolytic digestion of serum albumin when it is attached to dye molecules. The absence of major proteolytic breakdown of folate reductase molecule, when it was bound to amethopterin (Fig. 12), seems to confirm the observations of Markus.

The studies of Perkins and Bertino (15) suggest that the organic mercurials react with reductase even when it is protected by TPNH. These authors incubated the enzyme from Ehrlich ascites cells with PMB in the presence of high concentrations of TPNH (0.2 mM), adequate in our experiments for complete protection of folate reductase activity and amethopterin binding capacity (Fig. 8). A marked stimulation of dihydrofolate reductase activity in such preparations was noted only if mercaptoethanol was present in the dihydrofolate reductase assay mixture (15). Inability of glutathione, β-mercaptoethanol or cysteine in our study to reactivate folate reductase of S-180 cells (AT/3000) after PMB treatment, might indicate irreversible denaturation of the enzyme.

Dihydrofolate reductase of guinea pig liver has been reported to be activated by mono-(K^+, NH_4^+) and divalent (Ba^{++}, Ca^{++}, Mn^{++}) cations (31). This type of activation has not been observed in our laboratory with folate reductase of S-180 cells (AT/3000). It is possible that the reported activations actually reflect a stabilization of the enzyme against nonspecific inactivations. This type of stabilization or protection has been observed with lactic dehydrogenase of chicken heart by inorganic ions (32). Our demonstration, that metal chelaters cause an inactivation not caused by a simple dialysis and prevented only by the presence of substrates, coenzymes and their analogs, suggests that a metal is actually a structural part of the enzyme molecule. Thus, it must not be confused with the "activation," "stabilization," or "protection" offered by extraneous K^+ or Ca^{++}.

These studies have also demonstrated that folate reductase is able to form binary complexes with substrates and coenzymes with stabilities such as are required for competition with all the irreversible inhibitors described here. Thus, the prior presence of the coenzyme on the site is not necessary for the binding of the substrates as it is in the cases of dehydrogenases exhibiting a compulsory order of binding.

It also appears (Table 4) that the 50% protective concentrations of folate, TPNH and TPN are of the same magnitude as

[2] Oral communication by Dr. Frank Huennekens (1966).

their K_m values (5, 12). Thus, it seems that the K_m values in this case represent true dissociation constants. This has earlier been suggested for the K_m of folate (29). It also suggests that the prior presence of TPNH on the enzyme surface does not increase the affinity of folate, and vice versa. A method, based on the alteration by a protecting agent of the rate of inactivation of an enzyme by a slow acting, irreversible inhibitor, such as PMB, has been previously used to measure dissociation constants which have been found to agree with those established by other methods (33).

It was by no means to be predicted that the substrate, coenzyme, and competitive inhibitor would provide complete protection of folate reductase against proteolytic digestions. Grisolia (1) has listed several examples of enzymes for which the situation is reversed. On the basis of the present results then, one can conclude that in this case the intracellular presence of substrates, coenzymes, and inhibitors could exert a significant influence on the turnover of folate reductase by shutting off the proteolytic breakdown. This could conceivably result in an increase in the enzyme content in the cells and might provide a partial explanation for the observed increase in folate reductase content of cells exposed to amethopterin (34). The increase in folate reductase was at that time described as induction but might, at least partially, be explained by protection of the existing enzyme by amethopterin against proteolytic breakdown while synthesis of the new enzyme would proceed with normal or possibly accelerated rate. A similar explanation has been offered by Bertino *et al.* (35) for the increase in dihydrofolate reductase in leukocytes and erythrocytes of dog and man which was observed after administration of amethopterin.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mrs. L. Kenny and Miss D. Sugg for their skillful technical assistance.

This investigation was supported in part by research grant CA-04175 from the National Cancer Institute of the U.S. Public Health Service.

REFERENCES

1. S. Grisolia, *Physiol. Rev.* **44**, 657 (1964).
2. B. T. Kaufman, *J. Biol. Chem.* **239**, 669 (1964).
3. B. T. Kaufman and R. C. Gardiner, *J. Biol. Chem.* **241**, 1319 (1966).
4. S. F. Zakrzewski, *Federation Proc.* **24**, 540 (1965).
5. J. R. Bertino, J. P. Perkins and D. G. Johns, *Biochemistry* **4**, 839 (1965).
6. S. F. Zakrzewski and C. A. Nichol, *J. Biol. Chem.* **235**, 2984 (1960).
7. S. F. Zakrzewski, *J. Biol. Chem.* **238**, 1485 (1963).
8. D.-H. Wang and W. C. Werkheiser, *Federation Proc.* **23**, 324 (1964).
9. G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.* **27**, 417 (1965).
10. J. M. Peters and D. M. Greenberg, *J. Am. Chem. Soc.* **80**, 6679 (1958).
11. C. K. Mathews and F. M. Huennekens, *J. Biol. Chem.* **238**, 3436 (1963).
12. D. Kessel and D. Roberts, *Biochemistry* **4**, 2631 (1965).
13. R. L. Blakley and B. M. McDougall, *J. Biol. Chem.* **236**, 1163 (1961).
14. R. Nath and D. M. Greenberg, *Biochemistry* **1**, 435 (1962).
15. J. P. Perkins and J. R. Bertino, *Biochemistry* **4**, 847 (1965).
16. J. P. Perkins and J. R. Bertino, *Federation Proc.* **24**, 541 (1965).
17. M. T. Hakala, *Federation Proc.* **24**, 540 (1965).
18. M. T. Hakala, *Federation Proc.* **25**, 750 (1966).
19. M. T. Hakala and T. Ishihara, *Cancer Res.* **22**, 987 (1962).
20. M. T. Hakala, *Biochim. Biophys. Acta* **102**, 198 (1965).
21. A. C. Bratton and E. K. Marshall, *J. Biol. Chem.* **128**, 537 (1939).
22. M. T. Hakala, S. F. Zakrzewski and C. A. Nichol, *J. Biol. Chem.* **236**, 952 (1961).
23. M. T. Hakala, *Biochim. Biophys. Acta* **102**, 210 (1965).
24. W. C. Werkheiser, S. F. Zakrzewski and C. A. Nichol, *J. Pharmacol. Exptl. Therap.* **137**, 162 (1962).
25. S. F. Zakrzewski, M. T. Hakala and C. A. Nichol. *Mol. Pharmacol.* 423–431.
26. G. A. Bruno and J. E. Christian, *Anal. Chem.* **33**, 1216 (1961).
27. G. P. Mell, G. H. Schroeder and F. M. Huennekens, *Federation Proc.* **25**, 277 (1966).
28. J. J. Burchall, *Federation Proc.* **25**, 277 (1966).
29. S. F. Zakrzewski, *J. Biol. Chem.* **235**, 1780 (1960).

30. G. Markus, *Proc. Natl. Acad. Sci. U.S.* **54**, 253 (1965).
31. J. R. Bertino, *Biochim. Biophys. Acta* **58**, 377 (1962).
32. G. Di Sabato and N. O. Kaplan, *J. Biol. Chem.* **240**, 1072 (1965).
33. A. S. Mildvan and R. A. Leigh, *Biochim. Biophys. Acta* **89**, 393 (1964).
34. M. T. Hakala, *Federation Proc.* **21**, 236 (1962).
35. J. R. Bertino, A. Cashmore, M. Fink, P. Calabresi and E. Lefkowitz, *Clin. Pharmacol. Therap.* **6**, 763 (1965).