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DIHYDROFOLATE REDUCTASE FROM MOUSE LIVER AND SPLEEN

PURIFICATION, PROPERTIES AND INHIBITION BY SUBSTITUTED 2,4-DIAMINOPYRIMIDINES AND 4,6-DIAMINOTRIAZINES*

J. L. McCULLOUGH† and J. R. BERTINO‡

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

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Abstract—Dihydrofolate reductase was purified from mouse liver and spleen, and certain physical and kinetic properties were compared. The chromatographic properties of the two enzymes on Sephadex G-75 were similar. Both enzymes exhibited two broad double pH optima, one between pH 4·5 and 5·5, and another between pH 7·5 and 8·5. The two enzymes were activated to a similar degree by KCl, urea and guanidine-HCl; methylmercuric bromide did not activate either enzyme. The presence of 5×10^{-6} M of either substrate, dihydrofolate or NADPH, protected the enzymes against thermal denaturation. The rate of reduction of dihydrofolate was 27–29 times that of folate for each of the enzymes. From the titration of the enzymes by the stoichiometric inhibitor, methotrexate, turnover numbers of 940 were calculated for both the liver and spleen enzymes.

The liver and spleen enzymes were both inactivated by an antibody prepared against the murine L1210 lymphoma enzyme. Crude liver extracts were found to contain a fraction which bound antibody and prevented inactivation of enzyme activity. The I_{50} values for a series of 2,4-diaminopyrimidine and 4,6-diaminotriazine inhibitors were determined and no significant differences were noted when the purified enzymes were tested. These studies show that when purified preparations of dihydrofolate reductase from mouse liver and spleen are compared, no differences in physical and kinetic properties are demonstrable, and stress the importance of using purified enzymes in measuring inhibition produced by analogs.

DIHYDROFOLATE reductase [5,6,7,8-tetrahydrofolate: NAD^+ ($NADP^+$) oxidoreductase, EC 1.5.1.3], which catalyzes the NADPH-dependent reduction of dihydrofolate, has been purified from a variety of sources (reviewed by Huennekens[1]). Inasmuch as this enzyme has a key role in the synthesis of tetrahydrofolate, and therefore in purine and thymidylate biosynthesis, effective selective inhibitors of this enzyme have great potential for chemotherapy of bacterial and protozoal infection in man as well as in cancer chemotherapy. Studies of Burchall and Hitchings[2] have shown that dihydrofolate reductase from several bacterial sources can be differentiated on the basis of sensitivity to certain dihydrofolate reductase inhibitors. Baker *et al.*[3] also suggested that the dihydrofolate reductase from mouse liver and spleen can be differentiated from each other as well as from the L1210 murine lymphoma enzyme by a series of "active site-directed irreversible" inhibitors. In a recent study, Perkins *et al.*[4] indicated that an

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antibody prepared against purified L1210 dihydrofolate reductase selectively inactivates the L1210 enzyme, as opposed to the liver enzyme from several mammalian sources.

Since mouse tumors are the main model for the design of chemotherapeutic agents, a detailed understanding of the properties of this enzyme in normal as well as in tumor tissue is important for the further rational development of selective inhibitors of this enzyme. This report summarizes our studies on the purification and properties of dihydrofolate reductase obtained from mouse liver and spleen; a comparison is made with selected properties of this enzyme from a methotrexate-resistant mouse lymphoma (L1210/MTX).[5]

MATERIALS AND METHODS

NADPH was purchased from Sigma Chemical Company; folic acid and *p*-chloro mercuribenzoic acid (PMB) were purchased from Nutritional Biochemicals Corp.; urea from Mann Research Laboratories; guanidine-HCl and 2-mercaptoethanol from Eastman Chemical Company; methotrexate (MTX) was a gift from Lederle Laboratories. CH_3HgBr was a gift of Dr. S. H. Chu of this department. A series of substituted 2,4-diaminopyrimidines and 4,6-diaminotriazines was kindly supplied by Dr. B. R. Baker.

Dihydrofolate was prepared from folate by the dithionite method of Futterman[6] as modified by Blakley,[7] and was stored frozen in suspension in 0·001 N HCl under nitrogen.

The antiserum, prepared by Perkins *et al.*,[4] was obtained from a rabbit injected with a highly purified dihydrofolate reductase from L1210/MTX tumor cells. The control serum was obtained from an untreated rabbit.

Dihydrofolate reductase was prepared from a methotrexate-resistant subline of the L1210 lymphoma.[5] The L1210 enzyme used in these studies was the fraction obtained from gel filtration on Sephadex G-75.

Purification of mouse liver and spleen dihydrofolate reductase

Sixty BDF_1 mice were anesthetized with ether and killed by exsanguination. The spleens from the 60 animals were pooled. An equal weight of livers, 6·0 g, was used for the enzyme purification. In all further steps, the preparations from liver and spleen were treated identically; all steps were carried out at 4°. Cold distilled water (25 ml) was added to 6·0 g tissue and homogenized for 30 sec with a Virtis No. 45 homogenizer at a setting of 70. The pH of the homogenate was adjusted to pH 6·0 by cautious addition of 0·1 N HCl. The cell homogenate was centrifuged in a refrigerated Sorvall RC-2 centrifuge at 27,000 *g* for 20 min at 4°. The supernatant fluid was decanted through two layers of cheese-cloth to remove lipid material. The supernatant solution was then fractionated with ammonium sulfate by slowly adding 27·7 g of the crystalline solid per 100 ml of homogenate. The mixture was stirred for 30 min at 4° and then centrifuged for 20 min at 27,000 *g* as before. The precipitate was discarded; to every 100 ml of the supernatant fluid was added 29·5 g ammonium sulfate. The mixture was stirred at 4° for 1 hr and centrifuged as before, except that in this case the supernatant fluid was discarded. The precipitate was dissolved in a minimum volume (3 ml) of 0·05 M Tris, pH 7·5, 0·1 M with respect to KCl. This fraction is referred to as the

45–85% ammonium sulfate fraction. This fraction was then applied to a column of Sephadex G-75 (2·5 × 80 cm), which had been equilibrated with 0·05 M tris–HCl, pH 7·5, 0·1 M with respect to KCl. Tris–KCl of the same molarity was used to elute the enzyme from the column; fractions of 5 ml each were collected subsequent to the void volume. The elution was carried out at 4°. The elution profile is shown in Fig. 1. Tubes containing the highest enzyme activity were pooled and used in the subsequent experiments.

Gel filtration of 0–45% ammonium sulfate mouse liver enzyme on Sephadex G-75

The precipitate obtained by adding 27·7 g ammonium sulfate per 100 ml of mouse liver homogenate is referred to as the 0–45% ammonium sulfate fraction. This precipitate from a mouse liver preparation was dissolved in 5·0 ml of 0·05 M tris–HCl, applied to a column of Sephadex G-75 (2·5 × 80 cm), and eluted as previously described.

Enzyme assay

Dihydrofolate reductase activity was measured by a spectrophotometric method utilizing the decrease in absorbance that occurs at 340 mμ when NADPH and dihydrofolate (FH_2) are converted to NADP and tetrahydrofolate (FH_4) respectively.[5] Assays were performed at 37°, unless otherwise indicated, using a Gilford model 2400 spectrophotometer and an expanded scale of 0–0·5 o.d. The standard spectrophotometric assay contained in a final volume of 1 ml: tris–HCl buffer, pH 7·5, 100 μmoles; (KCl, 150 μmoles; NADPH, 0.1 μmole; and dihydrofolate, 0·05 μmole, containing 1·0 μmole 2-mercaptoethanol. Purified enzyme was added last to initiate the reaction. Modifications of the standard assay procedure are indicated in the tables and figures.

Protein was determined by the biuret method in crude extracts and by absorbance at 280 mμ for the ammonium sulfate and Sephadex fractions. Specific activity is expressed as micromoles of substrate reduced per hour per milligram of protein. A unit of enzyme activity is expressed as that amount reducing 1 μmole dihydrofolate per hr under conditions of the standard assay (pH 7·5, 37°) using a molar extinction coefficient of 12,000 at 340 mμ.[5]

RESULTS

Enzyme purification

Table 1 summarizes the individual steps of the enzyme preparation. By this procedure, about 16-fold purification of liver enzyme and 37-fold purification of spleen dihydrofolate reductase were accomplished. The initial activity as well as the percentage recovery of the spleen dihydrofolate reductase was lower than that for the liver enzyme. Both enzymes eluted from Sephadex G-75 in an identical fashion (tubes 20–30; Fig. 1). In each instance the peak of enzyme activity appeared shortly after the hemoglobin peak. An average ratio of the elution volume of the enzyme to the void volume of the column (V_e/V_o) was calculated for both the liver and spleen enzymes to be 1·8. Purified preparations from both liver and spleen were stable for 2 months when sterile filtered and stored at 4°.

TABLE 1. PURIFICATION OF DIHYDROFOLATE REDUCTASE FROM MOUSE LIVER AND SPLEEN*

Purification step	Volume (ml)		Total protein (mg)		Specific activity (μmoles/hr/mg)		Total activity (μmoles/hr)		Recovery (%)	
	L	S	L	S	L	S	L	S	L	S
Crude cell extract	20·5	22·0	416	302	0·3	0·1	127	45	100	100
Ammonium sulfate (45–85%)	3·4	3·3	190	165	0·5	0·2	89	29	70	64
Sephadex G-75	·65	45	37	9	4·7†	3·7†	83	20	65	44

* Liver, L; spleen, S.
† Liver, tube 28; spleen, tube 26.

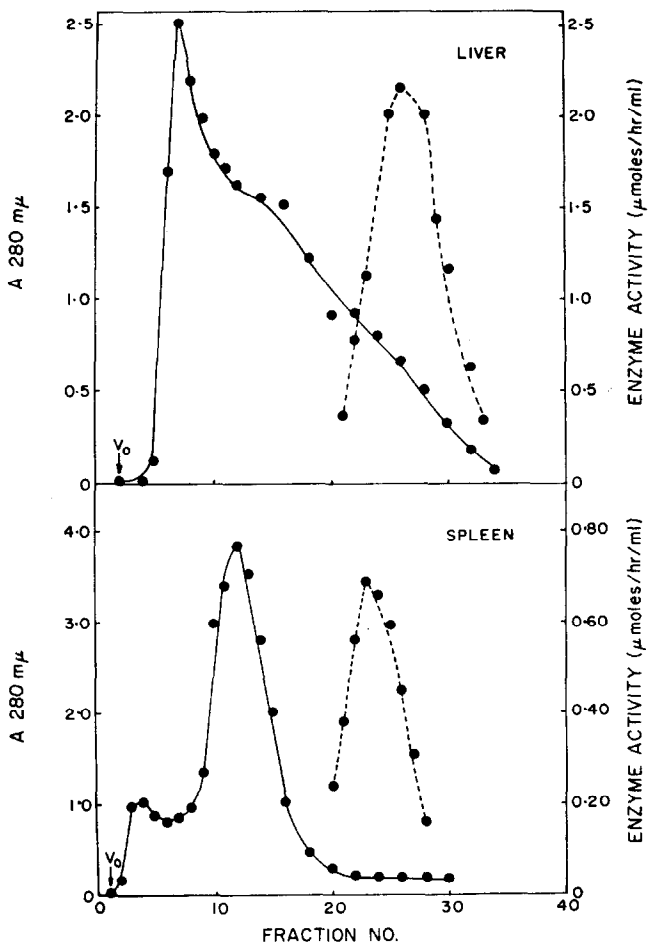


FIG. 1. Purification of dihydrofolate reductase by gel filtration on Sephadex G-75. The solid line indicates the absorbance at 280 mμ. V_0 indicates the void volume of the column. The broken line represents enzyme activity as measured in the standard spectrophotometric assay.

pH optima

The effect of pH on the activities of the two enzymes was determined. No obvious differences were observed when the liver and spleen enzymes were compared (Fig. 2). The enzymes exhibited two broad pH optima, one between pH 4·5 and 5·5, and another between pH 7·5 and 8·5. For both enzymes the reaction velocity was greater with acetate buffer than with citrate buffer.

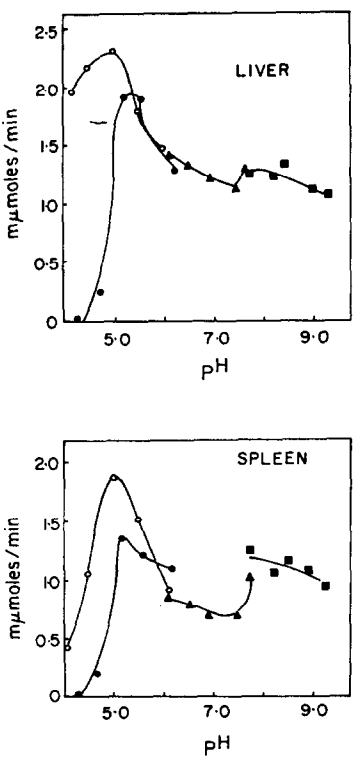


FIG. 2. The effect of pH on dihydrofolate reductase of mouse liver and spleen. Assays were performed as indicated in the text. The reaction mixture contained 100 μmoles buffer; 0·063 unit of enzyme was added to initiate the reaction. The results have been corrected for the oxidation of NADPH that occurs in the absence of enzyme. The pH values plotted are measured values obtained immediately on completion of the reaction. Sodium acetate buffer, ○——○; sodium citrate buffer, ●——●; potassium phosphate buffer, ▲——▲; tris–HCl buffer, ■——■.

Stability at 37°

The heat stability of the enzyme at 37° was determined (Fig. 3). The liver and spleen enzyme lost 28 and 18 per cent, respectively, of the initial activity after 1 hr of incubation at 37°. The presence of 5×10^{-6} M FH_2 or 5×10^{-6} M NADPH in the incubation mixture afforded significant protection of both enzymes. In each case the presence of NADPH afforded better protection than did FH_2.

Effect of KCl, urea, guanidine-HCl, PMB and CH_3HgBr on enzyme activity

Agents such as urea and guanidine-HCl, which are able to affect the conformation of proteins, have been reported to stimulate dihydrofolate reductase from chicken

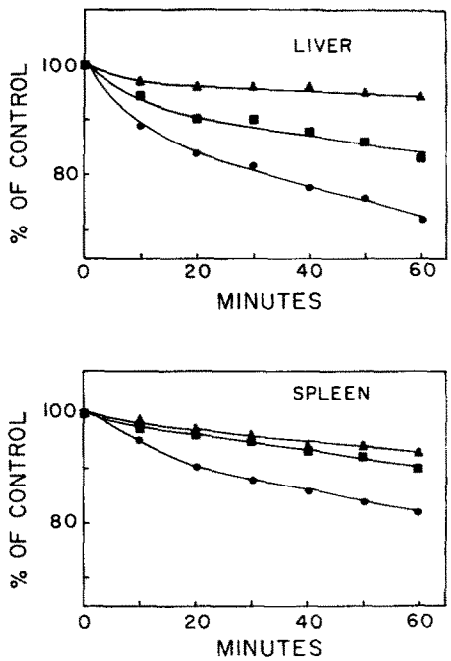


FIG. 3. Stability of dihydrofolate reductase from mouse liver and spleen and protection by substrates. A mixture containing tris–HCl, pH 7·5, KCl, and enzyme, in the presence or absence of 5×10^{-6} M FH_2 or NADPH, was incubated at 37°. At zero time and at subsequent 10-min intervals, an 0·85-ml aliquot containing 100 μmoles tris–HCl, 150 μmoles KCl, and 0·073 unit of enzyme was removed and enzyme activity was assayed by adding 0·15 ml containing 0·10 μmole NADPH, 0·05 μmole FH_2, and 1 μmole 2-mercaptoethanol. The data are plotted as the per cent of the rate obtained at zero time. In the absence of protecting substrates, ●——●; in the presence of 5×10^{-6} M FH_2, ■——■; in the presence of 5×10^{-6} M NADPH, ▲——▲.

liver,[8] Ehrlich ascites[9] and L1210 murine lymphoma.[5] It has also been observed that organic mercurials stimulate dihydrofolate reductase from these sources, but not from *Escherichia coli*.[10] It was of interest, therefore, to compare the effects of these substances on the mouse liver and spleen enzymes (Fig. 4). KCl, PMB, urea and guanidine-HCl were all found to stimulate both the liver and spleen enzyme. The degree of stimulation for the individual compounds was comparable for both the liver and spleen enzyme. CH_3HgBr did not stimulate either the liver or spleen enzyme.

Comparison of dihydrofolate reductase and folate reductase activities

The relative rates of reduction of dihydrofolate and folate by mouse liver, spleen and L1210/MTX enzymes at pH 5·5 were compared (Table 2). In each case the rate of reduction of dihydrofolate was 27–29 times that of folate.

Titration by methotrexate

The titration of mouse liver, spleen and L1210 lymphoma enzyme activities by the inhibitor, methotrexate, were compared (Fig. 5). It is clear from these data that the inhibitor binds tightly to each of these enzymes.[11] The initial linear plot of the data was extrapolated to the abscissa to give the micromoles of methotrexate bound.

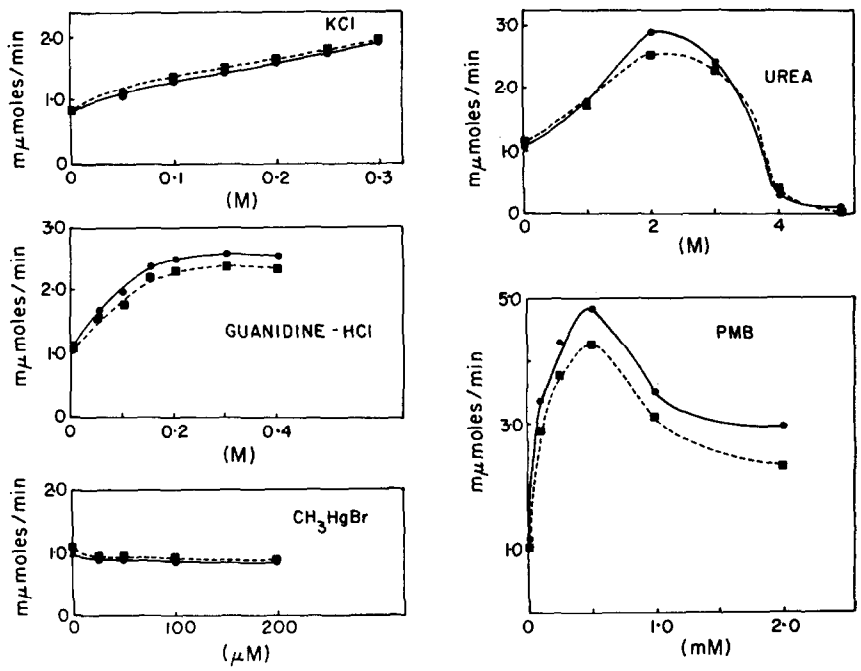


FIG. 4. The effect of KCl, urea, guanidine-HCl, PMB and CH_3HgBr on enzyme activity. KCl, guanidine-HCl, urea: the complete reaction mixture contained in a final volume of 1 ml: tris–HCl buffer, pH 7·5, 100 μmoles; KCl, 50 μmoles, except where indicated in figure; activator, as indicated in figure; 0·070 unit of enzyme; and 0·08 μmole NADPH. Dihydrofolate, 0·05 μmole, containing 1 μmole 2-mercaptoethanol was added last to initiate the reaction. CH_3HgBr, PMB: the basic reaction mixture was the same as above, except that the mixture was allowed to incubate 1 min at 37° prior to the addition of dihydrofolate. Liver, ●——●; spleen, ■——■.

TABLE 2. COMPARISON OF DIHYDROFOLATE REDUCTASE AND FOLATE REDUCTASE ACTIVITIES OF MOUSE LIVER, SPLEEN AND L1210 ENZYMES*

Enzyme source	Dihydrofolate reduction (ΔA/min)	Folate reduction (ΔA/min)	FH_2reduction/ Folate reduction
Mouse liver	2·160	0·081	27
Mouse spleen	0·850	0·031	27
L1210	0·664	0·022	29

* The complete reaction contained in a final volume of 1 ml: sodium acetate buffer, pH 5·5, 100 μmoles; KCl, 150 μmoles; NADPH, 0·10 μmole; dihydrofolate or folic acid, 0·05 μmole; and 2-mercaptoethanol, 1 μmole. The results have been corrected for the oxidation of NADPH that occurs in the absence of enzyme. Enzyme activity is expressed as ΔA/min/ml of enzyme.

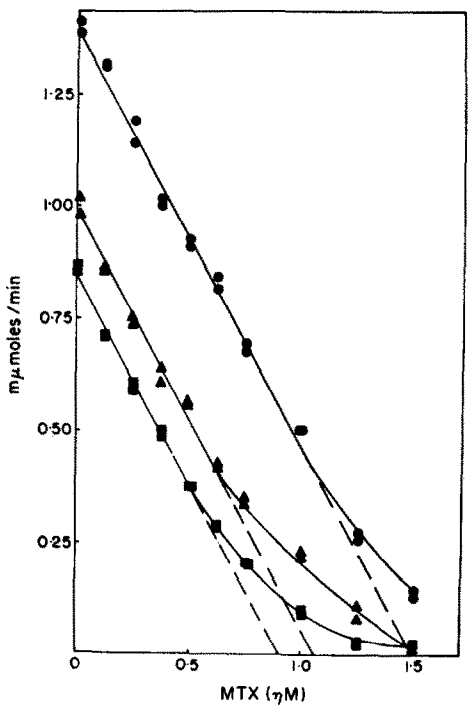


FIG. 5. Titration of mouse liver, spleen and L1210 dihydrofolate reductase by methotrexate. The complete reaction contained in a final volume of 1 ml; sodium acetate buffer, pH 6·0, 100 μmoles; KCl, 150 μmoles; NADPH, 0·10 μmole; dihydrofolate, 0·01 μmole, containing 1·0 μmole 2-mercaptoethanol; and enzyme. The reaction mixtures were allowed to incubate 2 min in the presence of methotrexate. Dihydrofolate was then added to initiate the reaction. These assays were done at 25°. Liver enzyme; ▲——▲; spleen enzyme, ■——■; L1210 enzyme, ●——●.

From these data, assuming a 1:1 stoichiometric binding of inhibitor to enzyme,[12] a turnover number of 940 moles/min/mole of enzyme (pH 6·0, 25°) was calculated for the liver, spleen and L1210 enzyme.

Antibody inactivation of enzyme activity

Perkins *et al.*[4] have prepared an antiserum from rabbits against dihydrofolate reductase from mouse L1210/MTX tumor cells. They have shown that this antibody inactivates the dihydrofolate reductase from L1210/MTX lymphoma as well as from several other mouse-borne tumors. However, they have reported that the antibody does not inactivate the dihydrofolate reductase (ammonium sulfate fraction) from *E. coli*, or from mouse, chicken, guinea pig or rabbit liver. It was of interest to compare the effect of the L1210 antibody on the activities of these enzymes (mouse liver, spleen and L1210) purified by Sephadex gel filtration. The titration of enzyme activity from these three sources by the rabbit antiserum is shown in Fig. 6. These data show that all of the enzymes were inhibited approximately 90 per cent by the addition of 0·015 ml antiserum. The possibility was considered that the inactivity of L1210 antibody reported by Perkins *et al.*[4] for the mouse liver enzyme might be attributed to a nonspecific binding of antibody to inactive protein in the enzyme preparation. There-

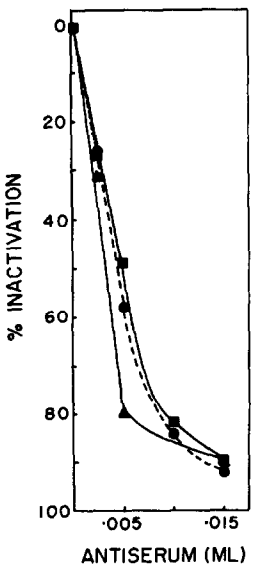


FIG. 6. Titration of mouse liver, spleen and L1210 dihydrofolate reductase by antiserum. A mixture containing tris–HCl buffer, pH 7·5, 100 μmoles; KCl, 150 μmoles; 0·09 unit of enzyme, and serum (control or antiserum), in a total volume of 0·87 ml, was incubated at 37° for 40 min. A 0·31-ml aliquot containing 0·08 μmole NADPH, 0·04 μmole dihydrofolate and 1 μmole 2-mercaptoethanol was added to initiate the reaction. The percent inactivation of control enzyme activity caused by the antiserum is plotted vs. millilitres of antiserum added. Liver enzyme, ▲——▲; spleen enzyme, ■——■; L1210 enzyme, ●——●.

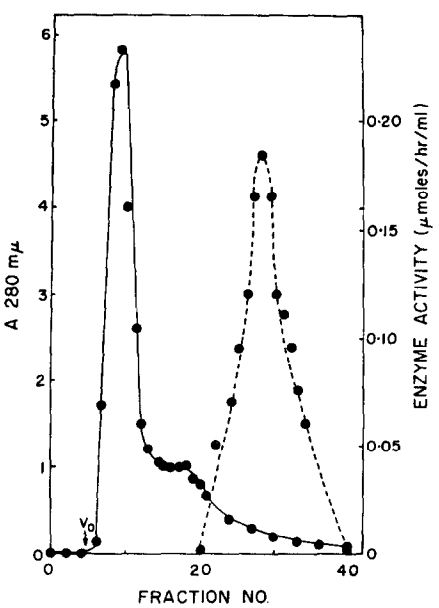


FIG. 7. Purification of the 0–45% mouse liver ammonium sulfate fraction by gel filtration on Sephadex G-75. The solid line indicates the absorbance at 280 mμ. V_0 indicates the void volume of the column. The broken line represents enzyme activity as measured in the standard spectrophotometric assay.

TABLE 3. ANTIBODY INACTIVATION OF DIHYDROFOLATE REDUCTASE FROM L1210 AND DIFFERENT FRACTIONS OF MOUSE LIVER*

Enzyme source	Antibody (ml) 0·0075 (% inactivation)	0·025
L1210	56	93
Mouse liver:		
45–85% Ammonium sulfate	63	94
Sephadex I†	60	98
0–45% Ammonium sulfate	30	51
Sephadex II‡	52	84

* Assay as in Fig. 6; approximately equal units of enzyme activity (0·087 unit) used in each assay.
† Sephadex G-75 of 45–85% ammonium sulfate fraction.
‡ Sephadex G-75 of 0–45% ammonium sulfate fraction.

fore a series of enzymes of varying degrees of purity was tested for their antigen activity against the L1210 antibody (Table 3).

Antibody equally inactivated the L1210 enzyme, the 45–85% ammonium sulfate fraction of the liver, and the fraction obtained from gel filtration of this 45–85% ammonium sulfate fraction. However, only half as much inactivation was noted when the enzyme from the 0–45% ammonium sulfate fraction was incubated with the antibody. When this fraction was filtered on Sephadex G-75, as illustrated in Fig. 7, the enzyme fraction obtained had an increased sensitivity to antibody inactivation.

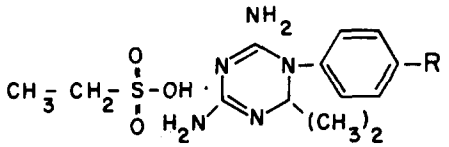
This experiment suggested that a nonspecific binder of the antibody was present in the 0–45% ammonium sulfate fraction which was removed by Sephadex gel filtration. That this was so was demonstrated in the following way. When increasing amounts of a fraction (tube 9, Fig. 7), containing no dihydrofolate reductase activity, were

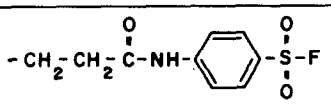
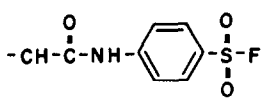
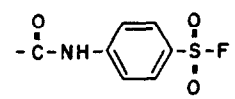
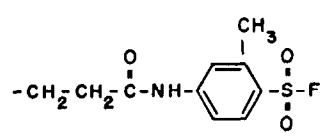
TABLE 4. EFFECT OF ENZYMATICALLY INACTIVE SEPHADEX II PROTEIN ON ANTIBODY ACTIVITY*

Fraction† (ml)	% Inactivation
0	60
0·10	56
0·20	46
0·30	41
0·40	35

* An 0·87 ml mixture containing tris–HCl, pH 7·5, 100 μmoles; KCl, 150 μmoles; inactive protein; 0·1 unit of 45–85% ammonium sulfate mouse liver enzyme, and 0·0075 ml serum (control or antiserum) was incubated at 37° for 40 min; a 0·15-ml aliquot containing NADPH, 0·08 μmole; dihydrofolate, 0·04 μmole; and 2-mercaptoethanol, 10 μmoles, was added to initiate the reaction.
† Peak tube 9, Fig. 7.

TABLE 5. INHIBITION BY SUBSTITUTED 4,6-DIAMINOTRIAZINES



Compound	R	I_{50}* Liver	Spleen	L 1210
113220		2.8×10^{-9}	3.2×10^{-9}	7.5×10^{-9}
113221		5.5×10^{-8}	4.6×10^{-8}	2.0×10^{-8}
113226		3.5×10^{-5}	4.2×10^{-5}	1.0×10^{-4}
113423		2.2×10^{-9}	3.3×10^{-9}	2.5×10^{-9}

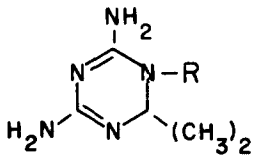
* The complete system contained in a final volume of 1 ml: tris–HCl buffer, pH 7·5, 100 μmoles; KCl, 150 μmoles; 0·075 unit of enzyme; NADPH, 0·05 μmole; and inhibitor. After mixing and allowing the mixture to incubate at 37° for 2 min, dihydrofolate (0·01 μmole containing 1 μmole 2-mercaptoethanol) was added to initiate the reaction. The reaction rate was measured for 5 min. Several inhibitor concentrations were studied for each compound in order to determine the concentration necessary to obtain 50 per cent inhibition (I_{50}).

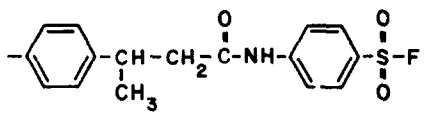
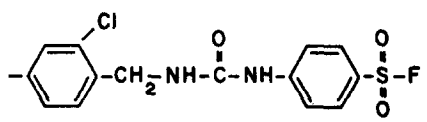
added to the standard assay, the per cent inactivation decreased from 60 per cent in the control to 35 per cent in the presence of 0·40 ml of this fraction (Table 4). The possibility that this fraction, which was present in mouse liver and which bound antibody, was denatured dihydrofolate reductase enzyme was tested by adding an equivalent amount of heat-inactivated enzyme to the assay; however, no effect on the per cent of enzyme inactivation caused by the antibody was observed.

*Inhibition of mouse liver, spleen and L*1210 *dihydrofolate reductase by substituted 2,4-diaminopyrimidines and 4,6-diaminotriazines*

The studies of Burchall and Hitchings[2] have shown that dihydrofolate reductase from several bacterial sources can be differentiated on the basis of sensitivity to certain inhibitors. It was of interest therefore to see if the dihydrofolate reductase from mouse

TABLE 6. INHIBITION BY SUBSTITUTED 4,6-DIAMINOTRIAZINES



Compound	R	I_{50}^{*} Liver	Spleen	L1210
6		$2{\cdot}6 \times 10^{-9}$	$4{\cdot}6 \times 10^{-9}$	$2{\cdot}4 \times 10^{-9}$
8		$1{\cdot}7 \times 10^{-9}$	$2{\cdot}7 \times 10^{-9}$	$1{\cdot}8 \times 10^{-9}$

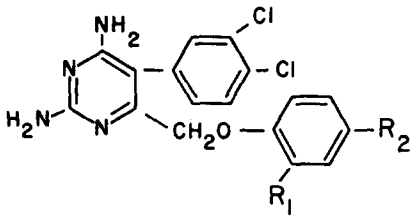
* Assay conditions were the same as in Table 5.

liver and spleen could be differentiated by using a series of closely related 2,4-diaminopyrimidines and 4,6-diaminotriazines. Three 2,4-diaminopyrimidines, as well as several substituted 4,6-diaminotriazines, were tested; the results are shown in Tables 5–7. Only slight differences could be demonstrated in the I_{50} values for the liver and spleen enzymes. Data obtained with the L1210 dihydrofolate reductase are included for comparison. Some slight differences, but less than 3-fold, were noted in the I_{50} values obtained for the L1210/MTX enzyme as compared to the liver and spleen enzymes. A decrease in the length of the hydrophobic chain, R, in Table 5, resulted in an increase in the I_{50} value for all of the enzymes. The attachment of a methyl group to the benzene ring of compound 113220 to form compound 113423 (Table 5) resulted in a slight decrease in the I_{50} values for the L1210 enzyme. The addition of an orthochlorine atom to the R group (compound 8) in Table 6 resulted in a slight decrease in the I_{50} value for all enzymes. The attachment of a chlorine atom (R_1) to the benzene ring (compound 5) also resulted in a decrease in I_{50} for all enzymes (Table 7).

DISCUSSION

In these studies we have been unable to detect any significant differences between dihydrofolate reductase from mouse liver and spleen when the following physical and kinetic properties were compared: behavior during purification, stability, substrate specificity, inhibitor specificity, pH optima, inhibition by antiserum and activation by organic mercurials, urea, guanidine-HCl, and KCl. The properties of the

TABLE 7. INHIBITION BY SUBSTITUTED 2,4-DIAMINOPYRIDINES



Compound	R_1	R_2	I^*_{50} Liver	Spleen	L1210
5	H	$-CH_2-NH-C(=O)-C_6H_4-SO_2F$	$1{\cdot}5\times10^{-7}$	$1{\cdot}1\times10^{-7}$	$1{\cdot}6\times10^{-7}$
12	H	$-NH-C(=O)-C_6H_4-SO_2F$	$1{\cdot}4\times10^{-7}$	$1{\cdot}7\times10^{-7}$	$0{\cdot}8\times10^{-7}$
13	Cl	$-NH-C(=O)-C_6H_4-SO_2F$	$7{\cdot}0\times10^{-8}$	$9{\cdot}5\times10^{-8}$	$5{\cdot}2\times10^{-8}$

* Assay conditions were the same as in Table 5.

mouse liver and spleen dihydrofolate reductase here examined are comparable with those reported for the L1210 enzyme.[5] The elution characteristics of the mouse liver, spleen and L1210 enzymes on Sephadex G-75 are similar, indicating a molecular weight close to 20,000 for the liver and spleen enzymes.[5] The properties of the mouse liver dihydrofolate reductase are similar to those reported for the guinea pig liver enzyme, indicating no apparent species differences in the enzymes. The dihydrofolate reductase from mouse liver and spleen can be differentiated from that of chicken liver by virtue of the lack of stimulation of these murine enzymes by CH_3HgBr.[13] The mouse liver and spleen enzymes can also be easily differentiated from the *E. coli* enzyme in several ways, including the lack of stimulation of the bacterial enzyme by either mercurials or guanidine.[10]

The antiserum prepared against dihydrofolate reductase from mouse L1210/MTX tumor cells was found to inactivate both the liver and spleen enzymes, if they were purified more than 15-fold by Sephadex gel filtration, as well as the L1210 enzyme. These data show that crude liver extracts contain a fraction which does not have dihydrofolate reductase activity, but which can bind antibody and thereby prevent inactivation of enzyme activity. The presence of this nonenzyme active fraction in the crude ammonium sulfate enzyme from mouse liver used by Perkins *et al.*[4] may

explain the previously observed lack of inactivation of the mouse liver enzyme by the L1210 antiserum.

The I_{50} values obtained for the inhibition of mouse liver and spleen reductase enzyme were similar, although slight differences in the inhibition of these enzymes and the L1210 enzyme were noted with three compounds (Table 5). Since some of these inhibitors have extremely low I_{50} values, identical amounts of enzyme activity were used to compare the enzymes. Assuming similar turnover numbers for the enzymes, K_m values for the substrate, FH_2, and that the compounds are all competitive inhibitors with respect to FH_2, this method of comparing enzymes is valid. Baker *et al.*,[3] using different assay conditions, have reported differential "irreversible" inhibition of dihydrofolate reductase from L1210, mouse liver and spleen with some of these compounds. Fölsch and Bertino[14] have shown that, although certain substituted 4,6-diaminotriazines are potent inhibitors of L1210 dihydrofolate reductase *in vitro*, they are ineffective inhibitors in the mouse *in vivo*. These inhibitors appear to be inactivated by a protein present in mouse serum. In view of these findings, it is apparent that, unless reasonably pure enzymes are used, alteration of inhibitors could take place, thus yielding ambiguous results concerning species and even organ specificity. Further studies of the "irreversible" inhibition[3] of these purified enzymes by these and other closely related inhibitors are in progress.

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