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FURTHER OBSERVATIONS OF THE BINDING AND INHIBITION OF DIHYDROFOLIC REDUCTASE BY FOLIC ACID ANTAGONISTS

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Abstract—The interaction of folic acid antagonists with dihydrofolic reductase was investigated with: (a) the supernatant fraction of chicken liver homogenates; (b) a highly purified enzyme preparation from the same source; and (c) an acetone powder extract of an antifolic-resistant subline of mouse leukemia L1210 possessing high dihydrofolic reductase activity. After incubation with tritiated aminopterin and subsequent dialysis, residual radioactivity per unit of original enzyme activity was the same with the crude and the purified chicken liver dihydrofolic reductase, demonstrating that the inhibitor was not bound appreciably to other proteins. Similarly, inhibition of dihydrofolic reductase activity by graded amounts of aminopterin was the same in both the crude and the purified preparations. The amounts of inhibitor bound per unit of enzyme, as calculated from either the dialysis or inhibition experiments, were comparable. Titration of enzyme activity with aminopterin (or with amethopterin) showed that $5{\cdot}7 \times 10^{-5}$ and $6{\cdot}6 \times 10^{-5}$ μmole of inhibitor corresponded to the amount of chicken liver and tumor dihydrofolic reductase, respectively, which had an activity of 1 μmole dihydrofolate reduced per hr at pH 7·5.

The inhibition of the chicken liver and tumor enzymes was more pronounced at pH 5·2 than at pH 7·5. It was essentially stoichiometric with excess enzyme, and reversible with equivalent or excess amounts of inhibitor.

The previously reported stimulation of dihydrofolic reductase activity by K^+ and urea was confirmed for both the chicken liver and the tumor enzymes.

THE COMMONLY used folic acid antagonists (aminopterin, amethopterin, and 3′, 5′-dichloroamethopterin) are generally assumed[1] to exert their antileukemic and toxic effects at extremely low concentrations by inhibiting dihydrofolic reductase (also called "folic reductase"), an enzyme that catalyzes the reduction of both folate and dihydrofolate to tetrahydrofolate.[2, 3] As yet, no other enzyme has been found to be inhibited by the antifolates at equally low levels, although some reactions[4, 5] are inhibited at much higher concentrations. Werkheiser[6] has suggested that all the amethopterin retained in nondialyzable form by rat liver preparations corresponds exactly to that amount which is required to inhibit the reductase completely. Such measurements, however, would not necessarily detect the presence of other proteins which might also possess a strong affinity for the inhibitor. The present study was undertaken, therefore, to determine by an independent method whether dihydrofolic reductase is, in fact, the *only* protein in tissues able to bind antifolates very tightly, and to clarify further the kinetics of this inhibition.

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MATERIALS AND METHODS*

Aminopterin was labeled with tritium by the New England Nuclear Corp. and purified by stepwise elution with potassium phosphate buffer (pH 7·5) from a DEAE-cellulose column, as described previously.[7] The purified aminopterin-^{3}H, which had a specific radioactivity of 87 μc/μmole, migrated as a single component when subjected to paper chromatography.[3, 7] MTX was purified by the same method. The molarity of aminopterin and MTX solutions was calculated from the published molar absorbancies (ϵ) in 0·1 N sodium hydroxide:[8] aminopterin; 28,400 (260 mμ) and 26,200 (284 mμ); MTX, 23,000 (257 mμ) and 22,100 (302 mμ). Dihydrofolic acid was prepared by the reduction of folic acid with hydrosulfite[9] and stored frozen as a suspension in 0·001 N hydrochloric acid containing 0·01 M mercaptoethanol.[10] Hydroxylapatite was prepared according to Levin.[11]

Dihydrofolic reductase was purified 300-fold from an extract of frozen chicken liver by the method of Mathews and Huennekens.[3] After rechromatography on hydroxylapatite, the enzyme had a specific activity of 34 μmoles/hr per mg protein. The active fractions from the hydroxylapatite column were concentrated by lyophilization and were stored frozen. Crude chicken liver extracts were prepared[3] by homogenization of the tissue with potassium phosphate buffer or 0·25 M sucrose, and the supernatant fraction was obtained by centrifugation at 40,000 rev/min in the no. 40 rotor of the Spinco ultracentrifuge.

A highly active dihydrofolic reductase preparation was also obtained from an antifolic-resistant subline (FR-8)[10] of mouse leukemia L1210. Acetone powders, prepared from the local subcutaneous tumors[10] and stored at $-20°$, were extracted with the appropriate buffer at 3° and centrifuged at 35,000 *g*. Extracts prepared at pH 7·5 had a reductase activity of 3–4 μmoles/hr per mg protein.

Dihydrofolic reductase activity was determined according to Osborn and Huennekens.[12, 13] The following components were added to a cuvette: enzyme, 0·24 μmole NADPH, and 0·08 μmole dihydrofolate. Each of these components was prepared in a standard buffer mixture [0·05 M potassium phosphate (pH 7·5) : 0·01 M mercaptoethanol : 0·001 M EDTA], and the final volume was adjusted to 3·0 ml with the same buffer. Absorbancy readings at 340 mμ were made at 1-min intervals against a reference cuvette from which dihydrofolate was omitted. Protein was determined from absorbancy readings at 260 and 280 mμ[14] with the chicken liver enzyme, and by the method of Lowry *et al.*[15] with the tumor extracts. Specific activity, expressed as micromoles of dihydrofolate reduced per hour per milligram protein at pH 7·5, was based on the combined decrease in absorbancy[3] for NADPH (ϵ = 6,200) and dihydrofolate (ϵ = 5,800) at 340 mμ. Any modifications of the standard assay procedure are indicated in the table and figures. In the inhibition

* Chemicals were obtained from the following sources: folic acid, aminopterin, and enzymatically reduced nicotinamide adenine dinucleotide phosphate (NADP) from the California Corp. for Biochemical Research; amethopterin (MTX) from the Lederle Division, American Cyanamid Co., through the courtesy of the Cancer Chemotherapy National Service Center, National Cancer Institute; Tris from Sigma Chemical Co.; 2-mercaptoethanol from Eastman Organic Chemicals; disodium ethylenediamine tetraacetate (EDTA) from Matheson, Coleman and Bell; diethylaminoethyl (DEAE)-cellulose (type 20) from Schleicher and Schuell; 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP) from Packard Instrument Co.; tritiated toluene for standardization from New England Nuclear Corp.; and bovine plasma albumin (fraction V) from Armour Laboratories. Cellulose tubing for dialysis was obtained from the Visking Co.

studies, the enzyme, antimetabolite, and buffer were preincubated at 37° for 5 min before the addition of NADPH and dihydrofolate.

For the dialysis experiments, enzyme and aminopterin-^{3}H were diluted appropriately to make them isotonic with the dialysis medium, and were preincubated at 37° for 5 min. One-ml aliquots were placed in bags of size 8 cellulose tubing (about 6 cm in length) and suspended in 500 ml of the dialysis medium at 3° which was stirred magnetically and changed every 8 to 10 hr.

Radioactive samples were counted with a Packard liquid scintillation spectrometer, model 314 EX, in 25-ml polypropylene vials containing 0·25–0·5 ml sample in 10 ml counting medium. The solvent system, similar to Bray's mixture,[16] consisted of naphthalene (60 g), PPO (5 g), dimethyl-POPOP (0·3 g), anhydrous methanol (100 ml), ethylene glycol (20 ml), and 1,4-dioxane (to make 1,000 ml). Counting efficiency was 10–11%, as determined by the addition of a standard amount of toluene-^{3}H after counting the aminopterin-^{3}H samples.

RESULTS

Dialysis of the aminopterin–dihydrofolic reductase complex

Previous studies have demonstrated that the stability toward dialysis of the E–I complex between dihydrofolic reductase (E) and folic acid antagonists (I) depended both on the source of the enzyme and on experimental conditions. Thus, when dihydrofolic reductase from human leukemic leukocytes was treated with aminopterin-^{3}H and the complex was dialyzed against Tris buffer, pH 7·5, dissociation and restoration of the original enzyme activity was complete within 20 hr.[7] With a purified chicken liver enzyme, conversely, there was no restoration of activity under these conditions, and only the excess aminopterin was removed by dialysis. Werkheiser[6] observed that the complex of aminopterin-2-^{14}C and the dihydrofolic reductase present in a supernatant fraction from rat liver was dissociated by dialysis against 0·25 M sucrose at a rate corresponding to a half-time of 120–360 hr. Recovery of enzyme activity was less than that calculated from disappearance of radioactivity. Bertino *et al.*[17] have shown that amethopterin was bound much more tightly at pH 5·9 than at 7·6 to a dihydrofolic reductase purified from Ehrlich ascites carcinoma, as indicated by the rate of dissociation of the E–I complex during dialysis.

In the present study, crude supernatant fractions from chicken liver homogenates and a highly purified dihydrofolic reductase preparation from the same source were treated with excess aminopterin-^{3}H and dialyzed against 0·05 M phosphate buffer, pH 6·55 (Fig. 1A) or against 0·125 M sucrose containing 0·002 M phosphate buffer, pH 6·55, and 0·001 M EDTA (Fig. 1B). Aminopterin controls in the absence of enzyme were dialyzed simultaneously. Residual radioactivity was expressed in terms of micromoles aminopterin per unit of original enzyme activity (1 μmole dihydrofolate reduced/hr at pH 7·5). With both the crude and the purified enzyme preparation, about 4·5 to 5 $\times$ 10^{-5} μmole aminopterin per enzyme unit remained when essentially all the aminopterin had been removed in the control This experiment, therefore, provides good evidence that, during the extensive purification of dihydrofolic reductase from the soluble fraction of a chicken liver homogenate, no proteins were removed which possessed an avidity for aminopterin comparable to that of dihydrofolic reductase.

Continued dialysis led to a further slow decrease in the amount of residual inhibitor, suggesting that the E–I comples itself was not completely stable to dialysis. This slow dissociation of the E–I complex made it difficult to determine the exact amount of aminopterin bound to the enzyme.

In the experiment reported in Fig. 1A, the observed enzyme activity after dialysis of the crude preparation for 20 hr was 14% of the original activity without inhibitor; under the same conditions, the value was 17% with the purified enzyme. Residual

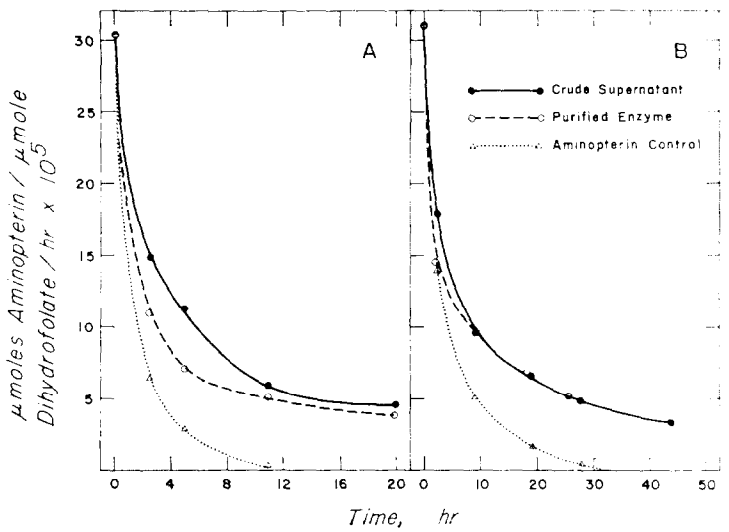

FIG. 1. Dialysis of aminopterin–dihydrofolic reductase preparations against different solutions. In experiment A, 1·15 mg of chicken liver dihydrofolic reductase purified on hydroxylapatite (activity by standard assay at pH 7·5 = 28·6 μmoles dihydrofolate reduced/hr per mg protein) and 0·010 μmole of aminopterin-^{3}H (87 μc/μmole) were diluted to 8·0 ml with the appropriate solution to render the mixture isotonic with the dialysis medium, preincubated for 5 min at 37°, and dialyzed against 0·05 M potassium phosphate buffer, pH 6·55. Samples (0·5 ml) were removed at indicated intervals for radioactivity determinations. Values (open circles) are expressed as micromoles of aminopterin remaining/unit of original enzyme activity. Similarly, 235 mg of the supernatant fraction of a chicken liver homogenate (activity = 0·14 μmole/hr per mg protein) was preincubated with aminopterin and buffer, and dialyzed (closed circles). Open triangles are data from a control containing aminopterin and buffer in the absence of enzyme. Experiment B was performed under essentially the same conditions, except that the dialysis medium was 0·125 M sucrose containing 0·002 M potassium phosphate buffer, pH 6·55, and 0·001 M EDTA.

radioactivity after the same time interval corresponded to $4{\cdot}5 \times 10^{-5}$ and $3{\cdot}8 \times 10^{-5}$ μmole aminopterin, respectively, per enzyme unit. These residual enzyme activities were somewhat lower than those measured by direct titration of the enzyme with aminopterin, as described below.

It was noted that the amount of residual aminopterin decreased more slowly with the crude preparation than with the purified enzyme, possibly because in the former instance reversible binding of the drug to other proteins occurred or because of some hindrance to passage of the drug through the membrane. Figure 2, in which the residual radioactivity is plotted on a logarithmic scale, demonstrates that the dialysis

rate of aminopterin-^{3}H is dependent, in fact, upon the nature of the dialysis medium. The rate was reduced when dialysis was carried out against sucrose, and reduced even more markedly when bovine plasma albumin was added to the dialysis bag. Even in the latter instance, the dialysis rate was still first order with respect to aminopterin concentration.

Although the thermal inactivation of dihydrofolic reductase has been studied previously,[2] it was of interest to determine whether the presence of a bound folic acid

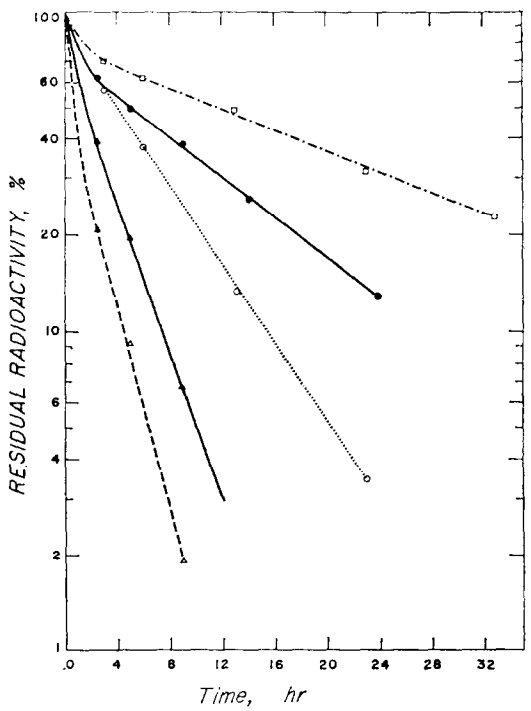

FIG. 2. Dialysis of aminopterin-^{3}H against various solutions. Initial aminopterin concentration was 11 to 14 $\times$ 10^{-7}M. Samples (0·5 ml) removed at indicated intervals. ●————●, 0·25 M Sucrose: 0·002 M potassium phosphate buffer (pH 6·55) : 0·001 M EDTA; ○·····○, 0·125 M sucrose : 0·002 M potassium phosphate buffer (pH 6·55) : 0·001 M EDTA; □— · — · —□, preceding dialysis medium, but aminopterin in 5% bovine plasma albumin; ▲————▲, 0·05 M Tris buffer, pH 7·6; △-- — —△, 0·05 M potassium phosphate buffer, pH 6·55.

antagonist would protect the enzyme from inactivation. Figure 3 shows that the purified enzyme is almost completely inactivated by heating at 55° for 5 min. Addition of aminopterin-^{3}H after such heating, followed by dialysis, indicated that the inhibitor was not bound to the denatured enzyme. On the other hand, when the enzyme was heated at 55° in the *presence* of aminopterin, the ability to retain residual radioactivity after dialysis was 90% of that observed in the unheated control. This result, however, does not necessarily mean that the enzyme activity was retained, since a catalytically inactive form of the enzyme might still be able to bind aminopterin. Indeed, when a

mixture of enzyme and aminopterin was heated at 55° for 5 min and then separated by chromatography of hydroxylapatite,[3] only 26% of the original enzyme activity was regenerated.

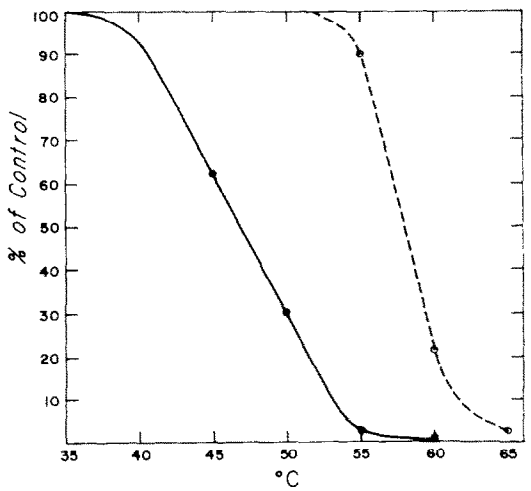

FIG. 3. Heat denaturation of purified chicken liver dihydrofolic reductase. ●——●, Enzyme samples (0·075 mg of protein, specific activity = 21 μmoles/hr per mg) in 1·0 ml of 0·05 M potassium phospate buffer, pH 7·4, in the absence of aminopterin were heated 5 min at the indicated temperatures, chilled, and assayed for residual enzyme activity (expressed as per cent of unheated control) by the standard procedure (see Materials and Methods). ○— — —○, Enzyme samples (0·14 mg of protein, specific activity = 34 μmoles/hr per mg) and 7 × 10^{-3} μmole aminopterin-^{3}H in 1·0 ml of 0·05 M Tris buffer, pH 7·5, containing 0·001 M EDTA, were heated 5 min at the indicated temperatures, chilled, and dialyzed against the same buffer. Residual aminopterin-^{3}H in each sample is expressed as per cent of that remaining under the same dialysis conditions in unheated control (3·7 × 10^{-5} μmole/μmole dihydrofolate per hr).

Inhibition of dihydrofolic reductase by folic acid antagonists

Werkheiser[6] and Zakrzewski and Nichol[2] have reported that, at pH 6 and lower, the inhibition of dihydrofolic reductase by aminopterin, MTX, and related antifolates could be described as "stoichiometric,"[18, 19] whereas Osborn *et al.*[13] and Mathews and Huennekens[3] presented evidence for reversible, noncompetitive[13] inhibition at pH 7·5. Recent studies by Bertino *et al.*,[17] as well as the present data suggest that the kinetics of inhibition depend markedly upon the experimental conditions. Figure 4 illustrates the inhibition produced when a constant amount of either purified or crude chicken liver dihydrofolic reductase was preincubated with increasing amounts of aminopterin. Enzyme activities (expressed as per cent of control) are plotted against micromoles of inhibitor per unit of enzyme activity assayed under standard conditions at pH 7·5 in the absence of inhibitor. Superimposable curves (open and closed circles) were obtained with the crude supernatant fraction and the purified enzyme, again suggesting that no other proteins in the crude preparation had an affinity toward aminopterin comparable to that of dihydrofolic reductase. Addition of bovine plasma albumin at a final concentration of 7 mg/ml to the purified enzyme (to simulate conditions in the crude system) did not change the inhibition produced by aminopterin.

It will be noted that the titration curve at pH 7·5 was a straight line until the enzyme was inhibited about 60%, but as the amount of inhibitor was increased, the curved line indicated a reversible inhibition, confirming the findings of Mathews and Huennekens.[3] As previously reported by Bertino *et al.*[17] for an enzyme preparation from Ehrlich ascites cells, inhibition was more pronounced at lower pH values (open triangles), with a concomitant extension of the straight-line portion of the curve. Extrapolation of the straight-line portion of the plot to the abscissa (dotted line, Fig. 4) indicated that, at either pH value, one unit of enzyme was titrated by $5{\cdot}7 \times 10^{-5}$ μmole of aminopterin.

Titration of a constant amount of aminopterin with increasing amounts of purified

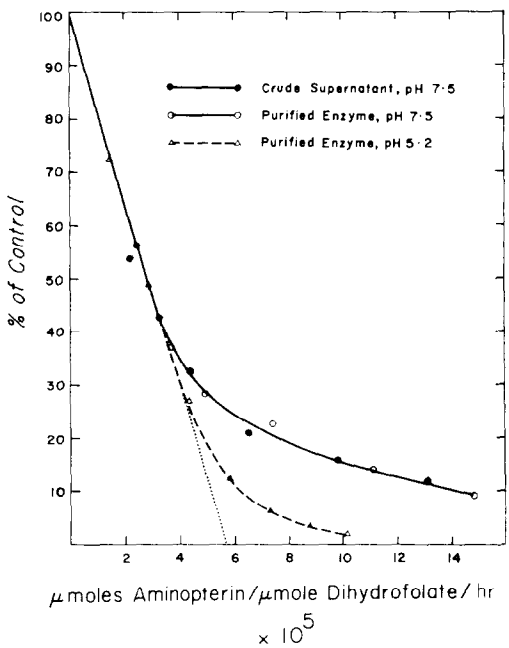

FIG. 4. Inhibition of crude and purified chicken liver dihydrofolic reductase by variable amounts of aminopterin at constant enzyme levels. ●———●, Supernatant fraction of chicken liver homogenate prepared in 0·1 M potassium phosphate buffer, pH 7·5 (specific activity = 0·145 μmole dihydrofolate/hr per mg protein). Enzyme (6·62 mg protein), aminopterin in graded amounts (range = 2·1 to $12{\cdot}6 \times 10^{-5}$ μmole), and 0·05 M potassium phosphate buffer, pH 7·5, containing 0·01 M mercaptoethanol and 0·001 M EDTA, were preincubated 5 min at 37° before addition of NADPH and dihydrofolate. Enzyme activity is expressed as per cent of control (preincubated in absence of aminopterin) and is plotted against amount of aminopterin (expressed as micromoles per unit of enzyme activity). ○———○, Enzyme purified on hydroxylapatite (specific activity = 22 μmoles/hr per mg protein). Experimental conditions as above, except 0·04 mg protein was used and aminopterin varied between 2·2 and $13{\cdot}2 \times 10^{-5}$ μmole. △— — —△, Purified enzyme (specific activity = 24 μmoles/hr per mg protein at pH 7·5 and 45 at pH 5·2). Experimental conditions as above, except that 0·03 mg protein and aminopterin (range = 1·05 to $7{\cdot}35 \times 10^{-5}$ μmole) were preincubated in 0·075 M potassium citrate buffer, pH 5·2, containing 0·01 M mercaptoethanol. In this experiment the ordinate values are based on the enzyme assays at pH 5·2, while the abscissa values are calculated for uniformity with the other data on the basis of enzyme activity at pH 7·5.

chicken liver dihydrofolic reductase at pH 7·5 is illustrated in Fig. 5. At lower enzyme concentrations the plot was concave upward, characteristic of reversible inhibition.[3] With an excess of enzyme over aminopterin, however, a straight line resulted, which was parallel to the plot of activity against enzyme concentration in the absence of inhibitor.[6, 19] Extrapolation of the straight-line portion to the abscissa again yielded a value of $5{\cdot}7 \times 10^{-5}$ μmole aminopterin per unit of enzyme activity.

Analogous results were obtained with a highly active dihydrofolic reductase preparation from an antifolic-resistant subline (FR-8)[10] of mouse leukemia L1210,

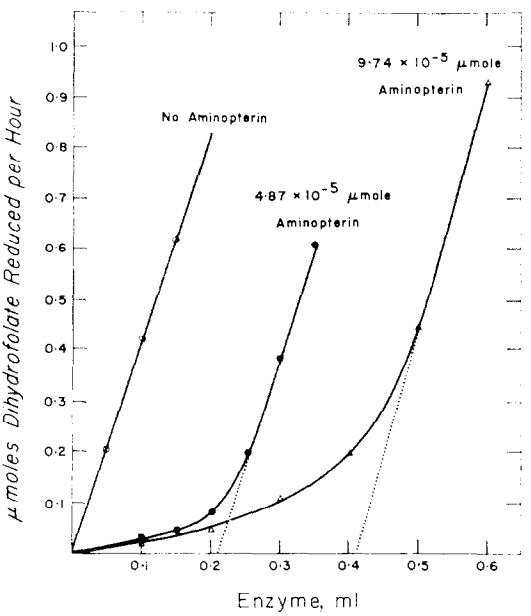

FIG. 5. Inhibition of variable amounts of purified chicken liver dihydrofolic reductase by aminopterin at constant inhibitor levels. Enzyme (specific activity = 28 μmoles/hr per mg protein), aminopterin, and 0·05 M potassium phosphate buffer, pH 7·5, containing 0·01 M mercaptoethanol and 0·001 M EDTA, preincubated 5 min at 37° before addition of NADPH and dihydrofolate (total volume 3 ml) and determination of enzyme activity. ○——○, No inhibitor; ●——●, $4{\cdot}87 \times 10^{-5}$ μmole aminopterin; △——△, $9{\cdot}74 \times 10^{-5}$ μmole aminopterin.

as shown in Fig. 6 (titration of enzyme with aminopterin)* and Fig. 7 (titration of MTX with increasing amounts of enzyme). In this instance, extrapolation of the straight-line portions in each plot (dotted lines) yielded a value of $6{\cdot}6 \times 10^{-5}$ μmole inhibitor per unit of enzyme activity. With both the chicken liver and the tumor enzyme, activity at pH 5·2 in the absence of inhibitor was about twice that obtained at pH 7·5.

Bertino[20] has reported a stimulation of dihydrofolic reductase activity by cations, and Kaufman[21] has recently observed a similar effect with urea. As shown in Table 1,

* Identical data were obtained in Fig. 6 when aminopterin was replaced by MTX.

these effects were confirmed with both the purified chicken liver dihydrofolic reductase and with the enzyme preparation from the antifolate-resistant mouse leukemia. With the tumor enzyme, but not with the avian enzyme, the stimulations produced by K^+ and by urea were approximately additive. Maximal stimulation was observed

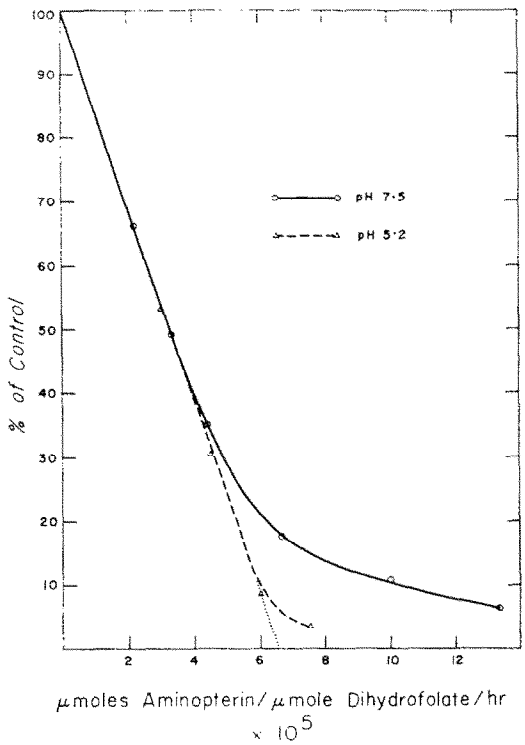

FIG. 6. Inhibition of L1210/FR-8 dihydrofolic reductase by variable amounts of aminopterin at constant enzyme levels. Acetone powder of antifolic-resistant tumor (see Materials and Methods) was extracted with appropriate buffer. Experimental conditions similar to those in Fig. 4. ○———○, Extract (0·28 mg protein, specific activity = 3·5 μmoles dihydrofolate/hr per mg protein at pH 7·5) and aminopterin in phosphate buffer (pH 7·5) mercaptoethanol : EDTA (see standard assay) preincubated 5 min at 37°. △— — —△, Extract (0·13 mg protein, specific activity = 5·1 μmoles/hr per mg at pH 7·5 and 12 μmoles/hr per mg at pH 5·2) and aminopterin preincubated in 0·075 M potassium citrate buffer (pH 5·2) : 0·01 M mercaptoethanol. Values on the abscissa are calculated on the basis of enzyme activity at pH 7·5.

at 3 M to 4 M urea. It is of interest that both K^+ and urea produced the same stimulation of activity in the resistant variant (FR-8) of leukemia L1210 as in an antifolate-sensitive strain,* suggesting that the susceptibility of the enzyme toward this type of activation had not been altered by the establishment of resistance.

* W. L. Yu and F. M. Huennekens, unpublished findings.

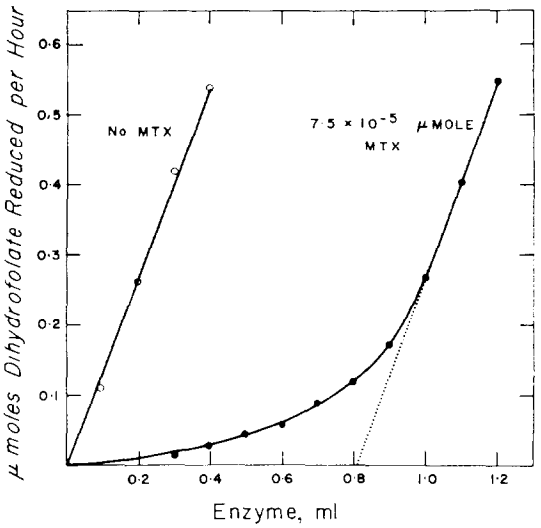

FIG. 7. Inhibition of variable amounts of L1210/FR-8 dihydrofolic reductase by amethopterin (MTX) at constant inhibitor level. Experimental conditions similar to those in Fig. 5. Acetone powder extract (specific activity = 4 μmoles/hr per mg protein), MTX, and phosphate buffer (pH 7·5) : mercaptoethanol : EDTA (see standard assay) preincubated 5 min at 37° before assay (total volume 3 ml). ○——○, No MTX; ●——●, $7{\cdot}5 \times 10^{-5}$ μmole MTX.

TABLE 1. STIMULATION OF DIHYDROFOLIC REDUCTASE ACTIVITY BY POTASSIUM CHLORIDE AND UREA

Addition*	Enzyme activity†	
	Chicken liver	FR-8 tumor
None	100	100
0·1 M KCl	135	195
3 M urea	410	405
4 M urea	425	370
2 M urea + 0·1 M KCl	350	470
2·5 M urea + 0·1 M KCl	385	530
3 M urea + 0·1 M KCl	380	550
4 M urea + 0·1 M KCl	335	305

* Molarities are expressed as final concentrations. The basic medium was 0·05 M potassium phosphate vuffer, pH 6·55, which is 0·07 M in K^+ ion. The total molarity of K^+ after addition of 0·1 M KCl was, therefore, 0·17 M.

† Expressed as per cent of activity determined in the basic medium. The enzyme preparations were, respectively, a chicken liver enzyme purified by chromatography on hydroxylapatite and an acetone powder extract from the antifolic-resistant subline (FR-8)[10] of leukemia L1210.

DISCUSSION

Both the dialysis studies and the results of titration experiments support the hypothesis that no soluble proteins in chicken liver, other than dihydrofolic reductase, possess any appreciable affinity toward aminopterin and related antifolic compounds. Specifically, it appears unlikely that a separate folic reductase is present in this tissue,

unless it is insensitive to the folic acid antagonists. This would confirm the previous findings[2, 3] that, in chicken liver, dihydrofolic and folic reductase activities are associated with only one enzyme. A recently reported[22] separation of folic and dihydrofolic reductases from sheep liver might suggest differences in this respect between avian and mammalian tissues.

The most highly purified dihydrofolic reductase from chicken liver had a specific activity of 85 μmoles/hr per mg protein.[3] From the present data it can be calculated that 5×10^{-3} μmole aminopterin would combine with 1 mg protein. Assuming one binding site per molecule, an upper limit of 200,000 can be placed on the molecular weight of the enzyme. Since the above preparation does not represent the pure enzyme, the actual molecular weight is undoubtedly lower. Blakley and McDougall[23] have described a purified dihydrofolic reductase from *Streptococcus faecalis R* with a specific activity of 280 μmoles/hr per mg protein; the turnover number of the bacterial reductase could be different, however, from that of the avian enzyme.*

The properties of the dihydrofolic reductase from the antifolic-resistant subline (FR-8) of leukemia L1210 were quite similar to those of the chicken liver enzyme, both with respect to inhibition by the antifolates and to stimulation by K^+ and urea. This stimulation, which is also of the same order of magnitude as with the sensitive parent leukemia L1210, makes it likely that the increased dihydrofolic reductase activity of the resistant subline is caused by increased synthesis of enzyme rather than by activation of inactive subunits.

As did the recent results of Bertino *et al.*,[17] the present data may provide a basis for reconciliation between the apparently contradictory reports[2, 3, 6, 13] concerning the kinetics of inhibition of dihydrofolic reductase. When the amount of enzyme is large relative to that of the inhibitor, essentially all of the latter is enzyme bound.[6,18] In this case the kinetics follow that of stoichiometric inhibition.[18, 19] However, with increased concentrations of the antifolic compound, the kinetics are characteristic of reversible inhibition.[3, 12] Analogous findings have been reported[24] for the inhibition of thymidylate synthetase by fluorodeoxyuridylic acid. At lower pH values, the inhibition of dihydrofolic reductase resembles more closely the stoichiometric type, probably because the inhibitor is more tightly bound.[17]

The similarlity of the liver and the tumor dihydrofolic reductase with respect to their inhibition by the folic acid antagonists makes it likely that the selective chemotherapeutic action against the tumor is related principally to different metabolic requirements of the cells and to differential uptake of the drugs.

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* From the amounts of inhibitor corresponding to one unit of enzyme activity and assuming one binding site per molecule, turnover numbers of 290 and 250 molecules of dihydrofolate reduced/min per molecule enzyme at pH 7·5 can be calculated for the chicken liver and the L1210/FR-8 tumor dihydrofolic reductase respectively. Higher turnover numbers are possible under conditions *in vivo* because of stimulation by cations and by a more favorable pH.

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