Two Different Species of Dihydrofolate Reductase in Mammalian Cells Differentially Resistant to Amethopterin and Methasquin

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SUMMARY

The disparate cross-resistance behavior of certain amethopterin-resistant Chinese hamster cell lines to the quinazoline antifolate, methasquin, prompted an investigation of the possibility of the existence of more than one species of dihydrofolate reductase in these cell populations.

The dihydrofolate reductase of four amethopterin-resistant Chinese hamster sublines (DC-3F8/A50, 460-fold resistant; DC-3F8/A55, 4,455-fold resistant; DC-3F8/A75, 21,400-fold resistant; and DC-3F/A3, 108,400-fold resistant) and of the parental cell lines (DC-3F and DC-3F8) was partially purified and characterized. Kinetic analyses of the reduction of folate and dihydrofolate as well as inhibition analyses revealed that DC-3F8/A50 and DC-3F8/A75 synthesize the parental type of enzyme, whereas DC-3F/A3 and DC-3F8/A55 synthesize a structurally dissimilar enzyme, designated the A3 reductase. The parental enzyme interacts stoichiometrically with amethopterin and with the quinazoline as well. Inhibition of the A3 reductase is stoichiometric only with methasquin. The reversible inhibition of the A3 enzyme by amethopterin reflects a weak interaction of enzyme and this agent. The decreased sensitivity of the latter enzyme to amethopterin explains, in part, the disparate response to the quinazoline of the two most amethopterin-resistant sublines, DC-3F8/A75 (5,727-fold cross-resistant to methasquin) and DC-3F/A3 (208-fold cross-resistant). This observation in amethopterin-resistant mammalian cell populations of a structurally altered dihydrofolate reductase with decreased affinity for amethopterin, but with parental-type sensitivity to methasquin, strengthens the possibility that amethopterin may act as a mutagenic agent and suggests that methasquin may have clinical value in treating amethopterin-resistant neoplastic disease.

INTRODUCTION

A study of 9 amethopterin-resistant Chinese hamster sublines in culture, ranging in resistance from 45- to 108,400-fold, has demonstrated that an increased level of dihydrofolate reductase (5,6,7,8-tetrahydrofolate : NADP-oxidoreductase, EC 1.5.1.3) may be the primary determinant of resistance to amethopterin in several but not all of the cell lines (8). Distinctive relationships between the reductase activity levels and response to amethopterin and to the quinazoline antifolate, methasquin, differentiated the sublines into 2 groups with respect to each antifolate (6, 8). The disproportional relationships between the enzyme activity and drug resistance levels of certain lines as well as the differential response to amethopterin and methasquin suggested that the presence of more than 1 type of dihydrofolate reductase and/or differences in the kinetics of drug transport may determine the response of the sublines to each agent.

To assess the possibility of a different species of dihydrofolate reductase in those sublines for which the increased level of dihydrofolate reductase activity was insufficient to explain the degree of resistance or which responded disparately to amethopterin and methasquin, we selected 4 sublines for detailed enzymological investigation. The dihydrofolate reductase of each subline and of the 2 parental cell lines was partially purified and characterized. In a preliminary communication, 2 different forms of dihydrofolate reductase characterizing 2 sublines highly resistant to amethopterin were reported (1). The possibility of differential drug transport, however, was not investigated. The present study, describing the distinguishing properties of 2 different species of Chinese hamster cell dihydrofolate reductase, provides evidence that in this mammalian cell system another mechanism of resistance to amethopterin may be the synthesis of a dihydrofolate reductase with a structural alteration effecting a decrease in the affinity of the enzyme for amethopterin. The presence of this type of reductase in antifolate-resistant cell populations that are relatively more sensitive to methasquin than to amethopterin suggests the possible clinical value of methasquin in treating amethopterin-resistant neoplastic disease.

MATERIALS AND METHODS

Materials. Most of the chemicals and the chromatographic materials were purchased from commercial sources (3, 4). Dihydrofolic acid was prepared by the Blakley modification (9) of the Futterman method (11) and was treated as described previously (3). Dr. Harriet Kiltie of the American Cyanamid Co., Pearl River, N. Y., supplied the amethopterin (methotrexate). For inhibition analyses, amethopterin was purified by ion-exchange chromatography (3). The
concentration of amethopterin was determined from the extinction coefficient of 23,000 M⁻¹ cm⁻¹ in 0.1 N NaOH at 257 nm (22). Purified aminopterin was a gift from Dr. Francis M. Sirotnak, and Dr. Harry B. Wood, Jr. (Drug Development Branch, Drug Research and Development, Chemotherapy, Bethesda, Md.) provided bioautographically pure samples of methasquin (14), the quinazoline antifolate, \(N-[\{2,4\text{-diamino-5-methyl-6-quinazolinyl}\}\text{methyl}]\text{-benzoyl}\}L\text{-aspartic acid. Standard buffer was a solution (pH 7.4) of 10 mM potassium phosphate and 1 mM EDTA.}

**Cell Lines.** The parental cell lines DC-3F and DC-3F8 (a clone of DC-3F) and the resistant sublines DC-3F/A3, DC-3F8/A50, DC-3F8/A55, and DC-3F8/A75, developed by direct exposure to amethopterin, were described previously (8).

**Preparation of Cell Extracts.** Cells of parental lines and drug-resistant sublines were grown in drug-free medium and collected for biochemically studies as previously described (8, 24). Pellets containing 5 \(\times\) 10⁸ cells were stored at \(-20^\circ\)C. On the day of enzyme purification, pellets were allowed to thaw at room temperature and were resuspended in 3.0 ml of cold standard buffer. The lysates were centrifuged (0-4°) at 15,000 rpm for 30 min. Supernatant extracts were removed after the 2nd centrifugation at 15,000 rpm and were then centrifuged at 15,000 rpm. The supernatant extracts obtained after the 2nd centrifugation at 15,000 rpm were then centrifuged at 35,000 rpm for 30 min to yield extracts suitable for molecular sieve chromatography.

**Partial Purification of Reductases.** A 2.5-ml portion (6 to 8 units of dihydrofolate reductase activity) of cell extract or an appropriate dilution of extract in standard buffer containing KCl (50 mM) was applied to a 2.5x40-cm column of (Bio-Rad P-60) polyacrylamide gel. Standard buffer containing appropriate dilution of extract in standard buffer containing 1540 CANCER RESEARCH VOL. 32

**RESULTS**

**Properties of the Cell Lines and Their Enzymes**

**Cell Lines.** The data summarized in Table 1 describe the cell populations selected for this study with respect to resistance to amethopterin and methasquin and their dihydrofolate reductase and folate reductase activity levels. The relationship between response to amethopterin and response to methasquin of the 3 highly amethopterin-resistant sublines is disproportionate. However, the disparate drug-response behavior of sublines DC-3F/A55 and DC-3F/A3 is striking.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Maintenance concentration of amethopterin (μg/ml)</th>
<th>Relative resistance levela to Amethopterin</th>
<th>Folate reductaseb</th>
<th>Dihydrofolate reductaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-3F</td>
<td>1</td>
<td>1</td>
<td>0.007</td>
<td>0.512</td>
</tr>
<tr>
<td>DC-3F8</td>
<td>1</td>
<td>1</td>
<td>0.006</td>
<td>1.171</td>
</tr>
<tr>
<td>DC-3F8/A50</td>
<td>1</td>
<td>460</td>
<td>0.080</td>
<td>1.171</td>
</tr>
<tr>
<td>DC-3F8/A55</td>
<td>5</td>
<td>4,455</td>
<td>0.050</td>
<td>1.812</td>
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<tr>
<td>DC-3F8/A75</td>
<td>25</td>
<td>21,400</td>
<td>1.171</td>
<td>1.812</td>
</tr>
<tr>
<td>DC-3F/A3</td>
<td>50</td>
<td>108,400</td>
<td>1.171</td>
<td>1.812</td>
</tr>
</tbody>
</table>

a See Ref. 8.

b Activity of cell extracts. Average values obtained from 2 to 9 determinations. These values are the results of determinations at 37° and therefore differ from those published earlier (8).

c Expressed in μmoles of tetrahydrofolate formed (folate reductase) and dihydrofolate reduced (dihydrofolate reductase)/hr/mg of protein.
Table 2

Partial purification of Chinese hamster cell reductases

Data are from a single representative experiment for each cell line.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fraction</th>
<th>Reductase activity</th>
<th>Ratio of reductase activities, dihydrofolate/folate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-3F</td>
<td>Extract</td>
<td>0.006</td>
<td>0.38</td>
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<td>Peak filtrate</td>
<td>0.172</td>
<td>9.72</td>
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<td>DC-3F8</td>
<td>Extract</td>
<td>0.007</td>
<td>0.46</td>
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<td></td>
<td>Peak filtrate</td>
<td>0.184</td>
<td>9.78</td>
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<td>DC-3F8/A50</td>
<td>Extract</td>
<td>0.081</td>
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<td>Peak filtrate</td>
<td>1.065</td>
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<td>DC-3F8/A55</td>
<td>Extract</td>
<td>0.054</td>
<td>2.76</td>
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<tr>
<td></td>
<td>Peak filtrate</td>
<td>0.903</td>
<td>49.62</td>
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<tr>
<td>DC-3F8/A75</td>
<td>Extract</td>
<td>1.169</td>
<td>89.40</td>
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<tr>
<td></td>
<td>Peak filtrate</td>
<td>8.100</td>
<td>807.50</td>
</tr>
<tr>
<td>DC-3F/A3b</td>
<td>Extract</td>
<td>1.620</td>
<td>109.80</td>
</tr>
<tr>
<td></td>
<td>Peak filtrate</td>
<td>14.220</td>
<td>1278.30</td>
</tr>
</tbody>
</table>

*Specific activity, expressed in umoles of tetrahydrofolate formed (folate reductase) and dihydrofolate reduced (dihydrofolate reductase)/hr/mg of protein.
*Values presented are based upon 2 purification experiments; fractions of one were assayed for folate reductase activity; fractions of the other were assayed for dihydrofolate reductase activity.

Thus, only the levels of resistance to methasquin and of enzyme activity of DC-3F8/A50, DC-3F8/A55, and DC-3F8/A75 reflect a parallel relationship.

Partial Purification of Reductase. The 2-step procedure resulted in a 9- to 25-fold purification of dihydrofolate reductase activity (Table 2). Similar elution profiles with concurrent elution of folate and dihydrofolate reductase activities were obtained after filtration of the cell extracts of the 6 cell lines (Chart 1). Peak fractions were used in the enzyme characterization and inhibition studies.

Enzyme Stability. Without KCl in the eluting buffer, the activity of the DC-3F/A3 reductase was unstable during polyacrylamide filtration. The presence of KCl stabilized the activity during filtration and decreased the rate of activity loss during storage at 4°C. After 2 weeks at 4°C, however, activity decreased to 75% of the original activity. Because of the marked instability of the DC-3F/A3 reductase in solution without KCl, KCl was included routinely in the eluant for the gel filtration of all the reductases. In the standard buffer-KCl eluant, the reductases of the other cell lines, except that of DC-3F8/A55 which resembled the A3 reductase, were relatively stable. Activity of the A75 reductase, for example, was retained without loss during storage for 3 months at 4°C.

Temperature Sensitivity. Studies with cell extracts demonstrated the differential temperature lability of the DC-3F/A3 and DC-3F8/A75 reductases. After incubation at 40°C for 10 min, activity decreased by 15% (A75) and 40% (A3); after 20 min of such incubation, activity decreased by 35% and 56%, respectively. The presence of NADPH during incubation at 37°C stabilized the reductase of DC-3F8/A75 completely. With NADPH, approximately 10% of the A3 reductase activity was lost within 10 min.

Molecular Weight. A molecular weight of 22,500 was estimated for the reductases of both DC-3F/A3 and DC-3F8/A75.

Reduction of Dihydrofolate

pH Optima. The reductases of the 2 parental cell lines and those of DC-3F8/A50 and DC-3F8/A75 exhibited maximal activity between pH 5.2 and 5.5 and exhibited activity similar to the maximum over the pH range of 6.5 to 8.5 (Chart 2). The activity of the DC-3F/A3 and DC-3F8/A55 reductases displayed a sharp optimum around pH 5.5, a shoulder of lower activity between pH 6.0 and 7.0, and then a gradual decrease to pH 8.5.

Effect of Concentration of Substrates on the Initial Reaction Velocity. In responding to varied concentrations of NADPH, between 3 to 100 μM, the reductases differed slightly, with K_m values ranging from 7.7 to 17.7 μM (Table 3). The K_m values for dihydrofolate, at least an order of magnitude lower than those for NADPH, could not be determined. The initial velocity of the reduction of dihydrofolate was maximal at 1.7 μM and at higher
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Chart 2. The pH optima for the reduction of dihydrofolate by the partially purified dihydrofolate reductases of Chinese hamster cell lines. The following buffers (50 mM) were used in the otherwise standard reaction mixture: o, sodium citrate, pH 4.7 to 6.7; ü, sodium citrate-potassium phosphate, pH 5.2 to 7.0; α, potassium phosphate, pH 6.7 to 8.8.

concentrations of dihydrofolate. The standard spectrophotometric procedure was not practicable with less than 1.7 μM dihydrofolate.

Reduction of Folate

pH Optima. In 100 mM citrate-buffered reaction mixtures, all of the purified preparations catalyzed the reduction of folate optimally around pH 5.1.

Effect of Concentration of Substrates on the Initial Reaction Velocity. The reductases differed in their response to varied amounts of NADPH (4 to 200 μM) in the standard reaction mixture, as shown by the calculated $K_m$ values (Table 4). For the DC-3F/S/A50 and DC-3F/A3 reductases, the values were 2- to 3-fold lower than the value of 37 μM calculated for the reductases of DC-3F, DC-3F/A55, and DC-3F/A75. Saturation kinetics with varied quantities of folate could be measured because of the higher requirement for folate than for dihydrofolate in the dihydrofolate reduction system. Whereas most of the reductases were similar in their response to folate, a 2-fold lower $K_m$ value of 8.0 μM was calculated for the DC-3F/S/A50 reductase.

Inhibition of Enzyme Activity by Antifolates

Kinetic analyses of the inhibition by amethopterin and methasquin differentiated the reductase of DC-3F/A3 and DC-3F/A55 from the parental type of reductase. The general inhibition data reported for the studies with the A75 and A3 reductases are representative for the 2 types of reductase: the parental type, found in DC-3F, DC-3F8, DC-3F/A50, and DC-3F/A75; and the A3 type, found in DC-3F/A55 and DC-3F/A3. Detailed investigation of the inhibition phenomenon was carried out only with the A3 and A75 enzymes.

### Table 3

Effect of concentration of NADPH upon the initial velocity of dihydrofolate reduction

<table>
<thead>
<tr>
<th>Source of reductase</th>
<th>$K_m$ values (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-3F</td>
<td>11.2</td>
</tr>
<tr>
<td>DC-3F8</td>
<td>7.7</td>
</tr>
<tr>
<td>DC-3F8/A50</td>
<td>11.4</td>
</tr>
<tr>
<td>DC-3F8/A55</td>
<td>17.7</td>
</tr>
<tr>
<td>DC-3F8/A75</td>
<td>14.0</td>
</tr>
<tr>
<td>DC-3F/A3</td>
<td>11.0</td>
</tr>
</tbody>
</table>

### Table 4

Effect of concentration of folate and NADPH upon the initial velocity of folate reduction

<table>
<thead>
<tr>
<th>Source of reductase</th>
<th>Folate</th>
<th>NADPH</th>
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<tbody>
<tr>
<td>DC-3F8</td>
<td>18.5</td>
<td>37.0</td>
</tr>
<tr>
<td>DC-3F8/A50</td>
<td>8.0</td>
<td>13.0</td>
</tr>
<tr>
<td>DC-3F8/A55</td>
<td>15.0</td>
<td>37.0</td>
</tr>
<tr>
<td>DC-3F/A75</td>
<td>13.5</td>
<td>37.0</td>
</tr>
<tr>
<td>DC-3F/A3</td>
<td>14.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Chart 3. Inhibition of dihydrofolate reduction by amethopterin (•), methasquin (○), and aminopterin (△). Enzyme activity was measured after preincubation of each enzyme preparation (0.5 dihydrofolate reductase unit) with varied amounts of the inhibitors as described in "Materials and Methods." Reductase activity is expressed as activity relative to the reduction in the absence of inhibitor, taken as 100. Inhibitor quantities are described on the basis of 1 mg of protein.

Inhibition of the Reduction of Dihydrofolate. Inhibition of the A75 reductase by amethopterin and methasquin was essentially stoichiometric (Chart 3). Inhibition with low amounts of methasquin was slightly stronger than that with the same amounts of amethopterin. However, equivalent amounts of methasquin and amethopterin were required for complete inhibition. The inhibition by aminopterin, another 2,4-diaminopteridine antifolate, resembled inhibition by methasquin except that complete inhibition was achieved with an amount of aminopterin 40% lower than the amount of methasquin required for 100% inhibition. In contrast, only methasquin inhibited the A3 reductase stoichiometrically. The inhibition by amethopterin indicated that the interaction of this agent with the enzyme was not a firm binding.
Aminopterin resembled amethopterin in its weak inhibition of the A3 reductase.

Whereas the dihydrofolate concentration of the reaction mixture did not strongly influence the interaction of inhibitors with the A75 reductase, it did affect the interaction of inhibitors with the A3 reductase (Chart 4). Despite the fact that enzyme and inhibitors had been preincubated prior to the addition of dihydrofolate to the reaction mixture, dihydrofolate partially reversed the inhibition. Reversal was noted at high concentrations of methasquin, but the titration with lower amounts of methasquin was relatively less affected (Chart 4). Dihydrofolate was more effective in reversing the inhibition by both low and high amounts of amethopterin and aminopterin.

Folate, a substrate for which the enzyme apparently has a lower affinity, did not prevent the inhibition by amethopterin and methasquin. On the contrary, 1 and 3 μM folate decreased the reduction of dihydrofolate by 15 and 40%, respectively. Therefore, additive inhibition resulted from preincubation of the A3 reductase simultaneously with folate and the antifolates.

The absence of NADPH during the preincubation of the A3 enzyme and methasquin or amethopterin had essentially no effect upon the interaction of the enzyme with these 2 antifolates (Table 5). On the other hand, the stoichiometric interaction of the A75 enzyme with amethopterin or methasquin was strongly dependent upon the presence of NADPH during preincubation of enzyme and inhibitor in the reaction mixture that contained KCl (Table 5). The omission of KCl from the reaction mixture eliminated the requirement for NADPH in the titration of the A75 enzyme.

Inhibition of the Reduction of Folate. Inhibition of the reductases in catalyzing the reduction of folate for the most part reflected the same enzyme-inhibitor interactions expressed in the inhibition of dihydrofolate reduction (Chart 5). The reductases of the parental cell lines, of DC-3F8/A50, and of DC-3F8/A75 resembled one another in their susceptibility to stoichiometric inhibition by amethopterin and methasquin. Methasquin was a slightly better inhibitor of folate reduction (Chart 5). Similar to the inhibition of dihydrofolate reduction, inhibition of folate reduction catalyzed by the reductases of DC-3F/A3 and DC-3F8/A55 was stoichiometric only with methasquin.

Folate reversed the inhibition of the A3 reductase in catalyzing the reduction of folate just as dihydrofolate reversed the inhibition of the dihydrofolate reduction.

Calculations Based on Titration with Methasquin. On the assumption that methasquin is more generally suitable than amethopterin as a titrating agent of dihydrofolate reductase (2), the reductase in cell extracts was quantitated in terms of methasquin-binding sites/mg of total protein (25). A methasquin-binding capacity of 9 pmoles/mg of total protein was calculated for DC-3F8. For DC-3F/A3 and DC-3F8/A75,
with the A3 enzyme.


categorized the reductase of the parental cell lines and of the experiment described in Table 6, the Chromatographie was recovered. As demonstrated by the results of the least 98% of the A75 reductase and 80% of the A3 reductase were eluted later. In control experiments without inhibitors, at eluants at pH 7.5 and 8.5. Reductase activity was eluted and the A3 reductase was studied by means of filtration through short columns of Bio-Rad P-30 polyacrylamide gel DC-3F8/A50 with the A75 reductase and that of DC-3F8/A55 of the purified reductase preparations of the other cell lines a 2.3-fold difference.


The tightness of binding of amethopterin and methasquin to the parental type of dihydrofolate reductase. The tightness of the binding of amethopterin and methasquin to the parental type and the A3 reductase was studied by means of filtration through short columns of Bio-Rad P-30 polyacrylamide gel with eluants at pH 7.5 and 8.5. Reductase activity was eluted from such columns in the early fractions, and the inhibitors were eluted later. In control experiments without inhibitors, at least 98% of the A75 reductase and 80% of the A3 reductase was recovered. As demonstrated by the results of the experiment described in Table 6, the chromatographic procedure caused a rapid dissociation of the A3 reductase-amethopterin complex. The release of a smaller amount of active enzyme from the A3 reductase-methasquin complex reflected the firmer binding of the quinazoline to the enzyme. Therefore, amethopterin resistance in this mammalian cell system can be assigned, at least in part, to mutation to an unusual insensitivity to amethopterin apparently provides sublines DC-3F8/A50 and DC-3F8/A55 with the A3 enzyme.

**Tightness of Binding of Amethopterin and Methasquin to the Different Forms of Reductase.** The tightness of the binding of amethopterin and methasquin to the parental type and the A3 reductase was studied by means of filtration through short columns of Bio-Rad P-30 polyacrylamide gel with eluants at pH 7.5 and 8.5. Reductase activity was eluted from such columns in the early fractions, and the inhibitors were eluted later. In control experiments without inhibitors, at least 98% of the A75 reductase and 80% of the A3 reductase was recovered. As demonstrated by the results of the experiment described in Table 6, the chromatographic procedure caused a rapid dissociation of the A3 reductase-amethopterin complex. The release of a smaller amount of active enzyme from the A3 reductase-methasquin complex reflected the firmer binding of the quinazoline to the enzyme.

The dihydrofolate reductase activity of the eluted fractions was expressed in terms of the amount added to the complex.

**DISCUSSION**

These studies provide evidence for the synthesis by amethopterin-resistant Chinese hamster cells in vitro of a dihydrofolate reductase structurally dissimilar to the parental enzyme. Therefore, amethopterin resistance in this mammalian cell system can be assigned, at least in part, to mutation to quantitative and qualitative (structural) alteration of dihydrofolate reductase. Elevated levels of the parental type of enzyme contribute to the resistance of sublines DC-3F8/A50 and DC-3F8/A75 and may represent the primary basis of resistance (8). Another type of dihydrofolate reductase with an unusual insensitivity to amethopterin apparently provides subline DC-3F/A3 with a mechanism for an extremely high degree of resistance. This type of reductase, called the A3 reductase, is found also in DC-3F/A55 at a lower level. The presence of the A3 enzyme in DC-3F/A3 and DC-3F8/A55 cells strengthens our alternative proposal (8) to classify these 2 sublines into the same group, i.e., cell lines for which increased enzyme activity levels may be inadequate to explain the degree of resistance. This type of reductase, called the A3 enzyme, is expressed in terms of the amount added to the complex.

A new molecular species of dihydrofolate reductase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pH</th>
<th>50 mM KCl</th>
<th>75%</th>
<th>50%</th>
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<tr>
<td>Amethopterin</td>
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<td>–</td>
<td>24</td>
<td>75</td>
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<tr>
<td></td>
<td>7.5</td>
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<td>26</td>
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<td></td>
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<td>54</td>
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<tr>
<td>Methasquin</td>
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differing from that of the sensitive parental cells has been found in the amethopterin-resistant murine leukemia L4946 (10). Two electrophoretically distinct species of hepatic dihydrofolate reductase have been isolated from nontumor-bearing rats that were given injections of amethopterin (18). One of these 2 species (molecular weight, 24,800) and was inhibited competitively by amethopterin. The other enzyme was larger (molecular weight, 24,800) and was inhibited noncompetitively. In most mammalian cell lines investigated, only the quantity of folate (dihydrofolate) reductase has been found to increase with development of resistance to amethopterin (12, 15, 17, 20, 21).

Like most mammalian folate reductases studied, the Chinese hamster enzymes catalyze the reduction of folate and dihydrofolate. With folate as substrate, activity is 50- to 100-fold lower than with dihydrofolate at 37°, and the range of the \( K_m \) values for folate is at least 10-fold higher. This range includes values at least 2- to 4-fold higher than those established for the folate reductase of sarcoma 180 (27). The Michaelis constants for NADPH in folate and dihydrofolate reduction are also slightly higher than those determined for certain other mammalian reductases (5, 20, 27). The variations that exist among the \( K_m \) values calculated for the reductases of the Chinese hamster cell lines perhaps reflect small structural differences which may or may not contribute to the major distinguishing biochemical properties.

The criteria for structural dissimilarity between the parental- and A3-type enzymes are the different pH optima for reduction of dihydrofolate and the differential inhibition kinetics.

Double pH optima for the reduction of dihydrofolate, a characteristic of most mammalian dihydrofolate reductases (5, 20), belong to the parental-type reductase of these Chinese hamster cell lines. The single acid pH optimum for the A3 enzyme is in sharp contrast. Apparently the structural dissimilarity affects the reduction of folate to a smaller degree, because for both types the optimum is near pH 5.

In contrast to the stoichiometric inhibition by the 4-aminofolates established for most dihydrofolate reductases studied previously (5, 20, 24) and observed in this study with the parental-type enzyme, the inhibition of the A3 enzyme reflects a weak interaction with 4-aminofolates. A reversible inhibition, it resembles the inhibition by amethopterin of the dihydrofolate reductase of the amethopterin-resistant mutant strain, R6 ame4 of *Diplococcus pneumoniae* studied by Sirotnak et al. (23). Under appropriate reaction conditions, folate and dihydrofolate partially reverse the inhibition of the A3 enzyme by the 4-aminofolates. The folates also partially reversed the inhibition by methasquin without grossly influencing the stoichiometric interaction of enzyme and methasquin. These subtle differences in the reversal of inhibition may be secondary effects of the altered structure of the A3 enzyme and thus also may contribute to the relatively greater sensitivity of the DC-3/A3 cell line when challenged with methasquin.

The loss of a structural element of the enzyme required for stoichiometric interaction with the 4-aminofolates may have concomitantly effected a configuration facilitating a firmer interaction of the A3 enzyme with methasquin and thus allowing less dissociation of the A3 reductase-methasquin complex than noted for the A75 reductase-methasquin complex (Table 6).

Another consequence of the structural dissimilarity between the A3 and parental-type (A75) reductases was observed in the study of the experimental conditions of inhibition. The necessity for preincubation with NADPH in the presence of KC1 for stoichiometric titration of the A75 enzyme with amethopterin and methasquin, by contrast to the NADPH independence of the inhibition of the A3 enzyme, emphasizes not only the differential effect of experimental conditions on the interaction of inhibitors with the reductases but also the suggestion previously presented (2) that the intracellular environment influences drug action. Differential effects of the intracellular conditions on inhibitor-drug binding and dissociation could also contribute to the disparate drug response of the Chinese hamster sublines.

The lability of the A3 enzyme, recognized early in our studies, prompted a gentle purification procedure. Approximately 10- to 25-fold purification of the reductases was achieved with 20% purity values for the enzymes of the most highly amethopterin-resistant sublines, DC-3F/A3 and DC-3F8/A75. The simplicity of the preparative procedure, however, prevented the recognition of physical or chemical differences among the reductases.

Our biochemical studies indicate that the A3 and parental-type enzymes are structurally different. The kinetic data, however, offer no positive evidence that any preparation contains more than 1 form of reductase. The titration data (Charts 3 and 5) demonstrate that folate and dihydrofolate reduction are catalyzed by the same enzyme. Nonetheless, the results do not exclude the possibility of multiple forms within any or all of the cell lines (13). The presence of more than 1 form could explain the inconsistent ratios of dihydrofolate to folate reductase activity calculated for the cell extracts and purified preparations.

A large part of this study has dealt with the inhibition of 2 different Chinese hamster dihydrofolate reductases. Differential inhibition, indeed, can explain the disparate response to amethopterin and methasquin of the sublines DC-3F/A3 and DC-3F8/A75 and of certain of the other amethopterin-resistant sublines previously described (8). It is probable that the high levels of enzyme (2% of the total protein) in DC-3F/A3 and DC-3F8/A75 are in fact augmented as mechanisms of resistance because intracellular factors prevent complete inhibition. The total resistance phenomenon exhibited by the amethopterin-resistant sublines could also involve other biochemical processes not as yet investigated.

The synthesis by sublines DC-3F/A3 and DC-3F8/A55 of a dihydrofolate reductase structurally different from the parental enzyme and thereby less sensitive to amethopterin supports our earlier consideration of amethopterin as a mutagenic agent (7). Impairment of nucleic acid metabolism indirectly by amethopterin could cause a mutational event, an altered genetic code for dihydrofolate reductase. The occurrence of such mutational events during clinical utilization of amethopterin is probable and could lead to the development of cell populations having altered forms of
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dihydrofolate reductase. If the structural alteration were to modify the affinity of the target enzyme for antifolates, resistance to amethopterin could be expressed. The observation of an atypical mammalian dihydrofolate reductase with markedly decreased affinity for amethopterin but with unaltered sensitivity to methasquin suggests the potential value of this newer antifolate in the chemotherapy of certain types of clinical disease in which resistance to amethopterin is encountered.

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Two Different Species of Dihydrofolate Reductase in Mammalian Cells Differentially Resistant to Amethopterin and Methasquin

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