Purification, Properties, and Synthesis of Dihydrofolate Reductase from Wild Type and Methotrexate-resistant Hamster Cells*

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SUMMARY
Dihydrofolate reductase from a methotrexate-resistant hamster cell culture was purified to apparent homogeneity by affinity chromatography. A similar procedure was used to prepare enzyme from wild type cells, although the final enzyme protein concentration was too small to be measured. The purified enzymes were not significantly different with regard to substrate specificity, $K_m$ values for folate and NADPH, sedimentation coefficient, electrophoretic mobilities at pH values 8.3 and 7.0, turnover number per methotrexate-binding site, and degree of inactivation by antiserum. The rate of radioactive leucine incorporation into the reductase in wild type and resistant cells was compared by specific immunoprecipitation. By this method resistant cells showed a 20-fold increase in the rate of enzyme synthesis. These results indicate that methotrexate-resistant cells accumulate large quantities of a very similar, if not identical, reductase, due at least in part to an increased rate of enzyme synthesis.

The biochemical basis for resistance to folic acid analogs has been investigated in both microorganisms and mammalian cells (1-15). Resistance can be caused by an increased activity of dihydrofolate reductase (EC 1.5.1.3) (2-6), altered permeability (7, 8), changes in the properties of the reductase (9, 10, 14), or a combination of these mechanisms (11-13). For two kinds of methotrexate-resistant mouse cells, comparisons of the properties of the reductase in crude extracts of resistant and sensitive cells, or in partially purified preparations thereof, suggested that the increase in enzyme activity was due to an increased cellular enzyme content (3, 5, 6).

A marked increase in the specific activity of the reductase in aminopterin-resistant hamster cell sublines was described previously (15). These sublines survived in concentrations of drug up to $10^4$ times higher than that tolerated by wild type cells, and contained up to 125 times as much reductase activity. The results to be presented in this paper characterize quantitatively and qualitatively the reductase of another more highly resistant subline, and extend previous studies with mouse cells to show that the increased enzyme activity results from an increased content of enzyme protein which is similar if not identical structurally with that in wild type cells and is over-produced in the resistant subline.

EXPERIMENTAL PROCEDURE

Materials
Folic acid, NADPH, NADH, MTT-tetrazolium, sodium doxycycl sulfate, beef liver catalase (twice crystallized), ovalbumin (once crystallized), pepsin (twice crystallized), and bovine pancreatic ribonuclease (type X1-A) were purchased from Sigma Chemical Co., horse heart myoglobin (once crystallized) from Calbiochem; uniformly labeled [3H]folic acid (1,500 mCi per mmole) and [2-14C]folic acid (50 mCi per mmole) from Amersham-Searle Corp.; t-[14C]leucine (316 mCi per mmole) from Schwarz BioResearch. Aminopterin and methotrexate were kindly supplied by Lederle Laboratories, and the methotrexate-Sepharose generously donated by Dr. B. T. Kaufman, National Institutes of Health, who also advised us as to the use of this material for affinity chromatography of the reductase, following the procedure of Mell, Whiteley, and Huennekens (16). Dihydrofolate reductase was prepared by the method of Blakley (17). Concentrations of folic acid and dihydrofolate acid were determined spectrophotometrically (with molar extinction coefficients at 7,050 (at 340 nm at pH 6.1) (18) and 28,000 (at 283 nm at pH 12) (19), respectively. Methotrexate was purified on DEAE-cellulose by the method of Johns, Sperri, and Burgen (20), and a molar extinction coefficient of 8,100 (at 570 nm in 0.1 N KOH) was used to determine its concentration (21). [3H]Folic acid was purified by cellulose thin layer chromatography with 1-propanol-1%
aqueous ammonia (2:1, v/v) as a solvent (22) to remove two labeled contaminants, one of which was probably p-amino-benzoyl glutamate on the basis of its chromatographic behavior.

**Cell Culture**

As described previously (23), a clonal subline (B1) resistant to 1 x 10^{-4} M 5-bromodeoxyuridine was isolated from an established baby hamster kidney culture (BHK 21/13), and then a subline resistant to 3 x 10^{-3} M aminopterin (Bl2A2A) isolated from B1 (15). For the present work another subline (Bl2A2A5), more resistant to aminopterin (2 x 10^{-4} M) was derived from Bl2A2A in the manner described before (15). Like BHK 21/13, B1 contains a low activity of reductase, and is regarded as "wild type" in this work; for simplicity, Bl2A2A5 and B1 are called A5 and wild type, respectively. Finally a subline called TG2 (resistant to benzoyl glutamate on the basis of its chromatographic behavior).

BlABA in the manner described before (15). Like BHK 21/13, from Bl (15). For the present work another subline (BlA2A5), more resistant to aminopterin (2 x 10^{-4} M) was derived from BHK cells in a fashion similar to that for T6A (24), except that TG2 was isolated in one step following exposure of BHK cells to N-methyl-N'-nitro-N-nitrosoguanidine.

Cells were grown as monolayers in an atmosphere of 5% CO2 in air in Eagle's minimal essential medium supplemented with non-essential amino acids, pyruvate, and 15% fetal calf serum (15). Folic acid (1 mg per liter) is present in this medium. A5 cells were carried in the same medium plus 2 x 10^{-4} M aminopterin. When our supply of this agent was exhausted, studies were continued with methotrexate, which we have found to be approximately one-fifth to one-tenth as active in our system as aminopterin.

The experimental hybridization of two sublines was carried out as described previously (24), and the hybrids were selected with methotrexate (10^{-5} M) in the presence of hypoxanthine (10^{-4} M), thymidine (4 x 10^{-4} M), and glycine (10^{-4} M). It was possible to use this selection, because none of the sublines was resistant to 5 pg per ml of 6-thioguanine was derived from BHK cells with a suitable amount of enzyme.

**Purification of Reductase**

All operations were carried out at 4°C unless otherwise stated, and NADPH, 2 x 10^{-4} M, was usually added to all fractions to stabilize the enzyme (25).

**Step 1: Preparation of Extract—**A5 cells, 35 g, washed twice with phosphate-buffered 0.9% NaCl solution and frozen as a pellet at -60°C, were thawed, mixed with 30 ml of 0.1 M potassium phosphate buffer, pH 7.0, and sonicated in a Raytheon sonicator for 2 min. Centrifugation at 6000 x g for 10 min formed with a Beckman Spinco model E ultracentrifuge, equipped with a ultracentrifugation-Analytical ultracentrifugation was performed with a Beckman Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped with a Spinco model E ultracentrifuge, equipped 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with a calibrated temperature control and schlieren optics. The MTX-Sepharose fraction from A5 cells was used after dialysis against 0.1 M potassium phosphate buffer, pH 7.0, for 12 hours and concentration by a UM-10 membrane filter (Amicon Corp.) to 7.5 mg per ml.

Polyacrylamide Gel Electrophoresis and Gel Electrophoretic Transfer— Electrophoreses were carried out in 7% polyacrylamide gels at pH 8.3 and 7.0 by the method of Ornstein and Davis (39). Enzyme was detected by incubating the gels for 15 to 30 min at 37° in 20 ml of 0.2 M potassium phosphate buffer, pH 7.0, containing 20 mg of dihydrofolate, 10 mg of NADPH, and 10 mg of MTT-tetrazolium. The Rf was calculated as the ratio of the distance traveled by the enzyme to that by a tracking dye, bromphenol blue.

Gel electrophoresis in sodium dodecyl sulfate (30) was carried out with purified commercially available proteins as standards.

Isoelectric focusing in polyacrylamide gels was carried out as described by Righetti and Drysdale (31). The sample was applied to the top of the 4% polyacrylamide gel containing 2% ampholyte (pH 3 to 10). Phosphoric acid, 0.1 M, and sodium hydroxide, 0.02 M, were used as anolyte and catholyte, respectively. After sample application, the current was maintained at a level of 1 ma per tube until the voltage had risen to 400 volts, and then the voltage was maintained at 400 volts for 4 hours.

Sucrose Gradient Centrifugation—Sedimentation of the reductase in sucrose density gradients was carried out as described by Martin and Ames (32). pH 5.0 supernatant fractions from wild type and A5 cells were sedimented in 8 to 20% sucrose gradients containing 0.04 M Tris-HCl, pH 7.5, with horse heart myoglobin as a standard. Centrifugation was for 24 hours at 35,000 rpm in the Spinco SW 36 rotor. The molecular weight of the reductase was estimated according to the procedure of Martin and Ames (32) based on a molecular weight of 17,200 for myoglobin (30).

Preparation of Antiserum—Two to four milligrams of the MTX-Sepharose fraction of A5 enzyme in complete Freund's adjuvant (Difco Laboratories) were injected subcutaneously into the footpads of each of six male New Zealand white rabbits (33). Four weeks later, the animals were bled, and sera separated and stored at -20°. Subsequently the sera were assayed; those from two rabbits giving the highest antibody titers were used.

Immunological Procedures—Immunoelectrophoresis of the reductase was performed on microscope slides coated with 2 ml of 1% agarose dissolved in 0.05 M barbital buffer, pH 8.6. Electrophoresis was performed at a constant current of 2.5 ma per slide for 2 hours at 4° in 0.04 M barbital buffer, pH 8.6. Immune serum, 20 μl, was placed in the center trough of the slide, and the slides were incubated at room temperature overnight. Nonprecipitated protein was washed with several changes of 0.9% NaCl; and the slides were stained for protein with Coomassie blue, destained in 5% acetic acid, dried, and photographed.

The incorporation of radioactive leucine into the reductase was measured by precipitation of the enzyme with the specific antisera as follows. Cultures of A5 or wild type cells in the exponential phase of growth in Falcon Petri dishes (100 × 20 mm) were starved for leucine for 24 hours in Eagle's medium lacking leucine, supplemented with 15% dialyzed fetal calf serum ("starvation medium"). Replicate cell cultures were then pulse labeled for 1 hour with 10 μCi of L-[3H]leucine (316 mCi per mmole) in 1.8 ml of the starvation medium or with the starvation medium only (control dish). At the end of 1 hour, media were removed and the cells washed twice with 5 ml of ice-cold phosphate-buffered 0.9% NaCl solution. Then 0.7 ml of phosphate-buffered 0.9% NaCl solution was added to each dish and the cells were scraped off with a rubber policeman. The radioactive A5 cells were mixed with nonradioactive wild type cells (wild type control dish); this mixture constitutes the A5 sample. Similarly, the mixture of the radioactive wild type cells and nonradioactive A5 cells (A5 control dish) constitutes the wild type sample. To each sample 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 5 × 10⁻⁴ M NADPH was added, and the cells were sonicated with two 30-sec bursts from a Raytheon sonicator. The cell debris was removed by centrifugation at 6000 × g for 10 min. Radioactivity incorporated into total soluble protein was determined by precipitating an aliquot (10 μl) of the crude extract with 5% trichloroacetic acid directly onto glass fiber filter papers. The precipitates were washed with 10 ml of 5% trichloroacetic acid, 5 ml of alcohol-ether (1:1, v/v), and 5 ml of ether; and the filter papers containing these protein samples were placed in 10 ml of toluene-Triton X-100 scintillation fluid for counting.

To assay radioactivity incorporated into the reductase, 25 μl of cold 1 N acetic acid was added to the crude extract with rapid stirring to adjust the pH to 5.0. After 5 min of stirring, the mixture was centrifuged at 6000 × g for 10 min, and the precipitate discarded. The pH of the clear supernatant solution was adjusted to 7.0 by the addition of 25 μl of cold 1 N KOH again with rapid stirring. To half (150 μl) of the supernatant solution sufficient specific antisera was added to precipitate all
the enzyme activity present. The remaining supernatant solution was mixed with control rabbit serum. These mixtures were incubated at 37° for 30 min, and at 4° overnight. The resulting immunoprecipitates were collected by centrifugation, washed three times with cold 0.9% NaCl, dissolved in 0.1 ml of 0.2 M NaOH, and added to 10 ml of scintillation fluid for counting. After removal of the antigen-antibody precipitate, the supernatant solution was subjected to an identical second immunoprecipitation by adding the same amount of carrier A5 enzyme (MTX-Sepharose fraction) and of antiserum as before. The material obtained in this second precipitation (about 0.05% of the total soluble protein in both cell types) should represent nonspecific protein precipitated with the reductase-antibody complex, and its radioactivity was subtracted from that of the first precipitate. The radioactivity of the precipitate obtained with control rabbit serum was also subtracted, to give net incorporation into reductase.

Table I

<table>
<thead>
<tr>
<th>Line</th>
<th>Reductase activity</th>
<th>Methotrexate for 50% inhibition of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/µg protein</td>
<td>µM</td>
</tr>
<tr>
<td>Wild type cells</td>
<td>0.70</td>
<td>2 × 10⁻³</td>
</tr>
<tr>
<td>A5 subline</td>
<td>100</td>
<td>3 × 10⁻⁴</td>
</tr>
</tbody>
</table>

a Crude extracts were prepared and reductase assayed as described under “Experimental Procedure” with folic acid as the substrate.

b Two hundred cells were inoculated into 60-mm Petri dishes containing growth medium alone (controls) and growth medium with various concentrations of methotrexate. After incubation for 1 week, the dishes were stained and the colonies counted. Plating efficiencies of the wild type and A5 cells were 29 and 39%, respectively.

Fig. 2. Stability of reductase activity after removal of methotrexate from the medium. A5 cells grown in medium containing 2 × 10⁻⁴ M methotrexate were transferred to medium without the drug (at 0 generation), and the culture continued for 45 generations. Reductase activity was assayed in crude extracts using folic acid as the substrate as described under “Experimental Procedure.”

RESULTS

Properties of Cells

The A5 subline of BHK hamster cells survived in a concentration of methotrexate 10⁴ times greater than that tolerated by wild type cells. This resistance was associated with a 140-fold increase in the specific activity of the reductase (Table I). The doubling time of A5 cells was the same as that of wild type cells (approximately 10 hours). Chromosome analysis confirmed that wild type cells were aneuploid (23) with an average chromosome number of 40.3 (in comparison with 44 for the Syrian hamster), and that the karyotype of A5 cells was quite similar to that of wild type cells, with an average chromosome number of 39.3. The possibility that wild type cells contained an inhibitor of the reductase, or the resistant A5 cells an activator, was tested by measuring the enzyme activity with mixtures of crude extracts. When mixed in varying proportions, the activity measured was consistently the numerical average of those obtained with each crude extract alone, indicating that the observed difference in level between wild type and A5 cells was not the result of the presence of an inhibitor or activator effective in vivo.

To test the stability of the increased reductase activity, A5 cells grown in the presence of methotrexate were transferred into medium without it, and cultured for an additional 45 generations (Fig. 2). Initially just after removal of the drug, there seemed to be a small increase in reductase activity, presumably due to loss of bound methotrexate. Subsequently, the activity remained constant, indicating that high reductase activity is a stable characteristic of these cells.

Hybridization of one cell line to another can provide information on control mechanisms in animal cells (15). To test for possible altered regulation of reductase synthesis in A5 cells, those cells were hybridized to TG2 cells, which contain low reductase activity like wild type cells. The frequency of spontaneous hybrid cells after 24 hours of mixed growth was 5 × 10⁻⁴; in control dishes comparable numbers of A5 and TG2 cells failed to survive in the selective medium. Extracts of six hybrid clones contained reductase activity intermediate between the two parental lines, as was true with hybrids involving other BHK sublines (15). Karyotype analyses showed that about 65% of the TG2 cells had a chromosome number close to double that of wild type cells. Five of the six hybrid clones had a chromosome number close to triple that of wild type cells, and contained an average of 17% of the reductase activity of A5 cells. Probably these hybrids had derived from fusions between an A5 cell with about 39.3 chromosomes and a TG2 cell with about twice that number. The TG2 cells contained about 2.8 times as much extractable protein as A5 cells and presumably contributed this to the hybrids, which may in part explain the low enzyme activities of the latter. The sixth hybrid clone, the only one with about twice the wild type chromosome number, may be a rare revertant of TG2, a contaminated cross-feeding culture, or perhaps a more interesting clone with partial interaction between the A5 and the TG2 genomes, as discussed previously (15).

Purification and Properties of A5 Enzyme

Representative results of the purification procedure are summarized in Table II. In this case approximately 15 mg of purified enzyme were obtained from 35 g of A5 cells. MTX-Sepharose chromatography was the most effective single step, yielding
TABLE II

Purification of reductase

See text for the details of the isolation procedure.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Activity</th>
<th>Yield</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg/mg</td>
<td>units/μg</td>
<td>units X</td>
<td>%</td>
</tr>
<tr>
<td>From A5 cells*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crude extract</td>
<td>70.5</td>
<td>24.0</td>
<td>76</td>
<td>1290</td>
<td>100</td>
</tr>
<tr>
<td>pH 5.0 supernatant</td>
<td>70.0</td>
<td>7.0</td>
<td>232</td>
<td>1140</td>
<td>88</td>
</tr>
<tr>
<td>MTX-Sepharose</td>
<td>9.4</td>
<td>1.56</td>
<td>6345</td>
<td>930</td>
<td>72</td>
</tr>
<tr>
<td>From wild type cells*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>63.0</td>
<td>28.0</td>
<td>0.75</td>
<td>13.2</td>
<td>100</td>
</tr>
<tr>
<td>pH 5.0 supernatant</td>
<td>57.0</td>
<td>6.0</td>
<td>2.88</td>
<td>9.7</td>
<td>73</td>
</tr>
<tr>
<td>MTX-Sepharose</td>
<td>10.4</td>
<td>&lt;0.02</td>
<td>4.0</td>
<td>4.0</td>
<td>30</td>
</tr>
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</table>

* Enzymes were purified from 35.0 g of A5 cells and 33.5 g of wild type cells, respectively.

The concentration of protein in the MTX-Sepharose fraction from wild type cells was too low to detect.

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A 28-fold purification. The enzyme was purified three times from A5 cells in this manner with an over-all purification of 106-, 105-, and 84-fold, and 59, 69, and 72% yields, respectively. Purification of the enzyme from wild type cells was carried out twice with over-all recoveries of 30 and 36% of enzyme activity but no measurable protein. However, when the column bed volume used to purify the enzyme from A5 cells was doubled, the recovery of A5 enzyme also decreased to approximately 30%. Therefore, the poor recovery of wild type enzyme might well have been due to the greater dilution of the enzyme. Further, the reductase activity of the MTX-Sepharose fractions of A5 cells was stable when stored at -60°C in the presence of 2 x 10^{-5} M NADPH, with no loss of activity over a 3-month period despite frequent freezing and thawing. The activity of the MTX-Sepharose fractions from wild type cells was less stable. However, when the MTX-Sepharose fraction from A5 cells was diluted to a protein concentration comparable to that of the wild type preparations, similar loss of activity occurred.

The reductase obtained from A5 cells by the purification procedure outlined above was essentially homogeneous according to several criteria. Ultracentrifugation revealed a single symmetrical peak during velocity sedimentation (Fig. 3). No contaminating protein which was larger or smaller than the main peak could be observed during the run. On polyacrylamide gel electrophoresis at pH 8.3, the purified enzyme moved toward the anode as a single major protein band (Fig. 4). On isoelectric focusing the purified A5 enzyme appeared at about pH 5.8.

The molecular weight of the polypeptide chain of purified folate reductase from A5 cells was estimated on sodium dodecyl sulfate-gel electrophoresis. Standards including beef liver catalase, ovalbumin, pepsin, horse heart myoglobin, and bovine pancreatic ribonuclease, were used to calibrate the migrations of polypeptide chains (30). As shown in Fig. 5, the molecular weight of the A5 folate reductase polypeptide chain was approximately 23,000 by this method.

Comparison of Wild Type and A5 Enzymes

While the purification of reductase from A5 cells shows the capability of the method, a measurable amount of purified enzyme could not be obtained from wild type cells. Therefore it was not possible to determine the specific activity of the purified wild type enzyme, nor to study this protein by the variety of physical techniques employed above with the A5 enzyme. However, certain studies were done with enzyme activity to compare and contrast the reductases of the two cell lines.

Substrate Specificity—The purified enzymes from both wild...
A faint second activity band moving faster than the main band was often observed at pH 7.0 (RF 0.32), perhaps indicating some form of aggregate or NADPH-depended activity. It was not possible to study wild type enzyme at the same concentration.

Inhibition by Methotrexate—Increasing amounts of enzyme were added to tubes containing a constant amount of methotrexate, and after preliminary incubation for 2 min the reductase activity was assayed (Fig. 7). The activity was completely inhibited at lower amounts of enzyme, but with greater amounts of enzyme a stoichiometric relationship was noted. When the linear portions of plots were extrapolated to the abscissa, it could be estimated that 1 μmole of methotrexate was bound to about 500 units of enzyme activity for both A5 and wild type enzymes. Therefore, the enzyme activity per methotrexate-binding site was the same for both the resistant and wild type lines.

Titration Experiments —The aim of these experiments was to test for enzymically inactive, immunologically cross-reactive material in extracts of wild type cells. The antiserum obtained from immunized rabbits formed a single line of precipitation when tested against both crude extract and MTX-Sepharose fractions from A5 cells (Fig. 8). Since it was not possible to purify large amount of reductase from wild type cells, nor therefore to show with an Ouchterlony plate antigenic cross-reactivity of the enzymes from wild type and A5 cells, titration experiments were performed to see whether wild type enzyme could be as easily inactivated by the antiserum as A5 enzyme. Antibody specific for the A5 reductase was titrated against the enzyme activity in the pH 5.0 supernatant fractions from A5 and wild type cells as described under “Experimental Procedure.” A constant amount of antiserum inhibited the same amount of added enzyme activity from A5 and wild type cells (Fig. 9).

In aged preparations, two precipitation lines with the same electrophoretic mobility were often observed; both showed dihydrofolate reductase activity when stained with MTT-tetrazolium, so that the second line may be an aggregated form of the enzyme.

### Table III

<table>
<thead>
<tr>
<th>Substrate specificity</th>
<th>Enzyme source</th>
<th>Dihydrofolate reductase activity (A)</th>
<th>Folate reductase activity (B)</th>
<th>(A): (B)</th>
<th>NADPH/NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5 subline........</td>
<td>11.0 × 10⁶</td>
<td>1.52 × 10⁷</td>
<td>7.2</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>Wild type cells......</td>
<td>11.8 × 10⁶</td>
<td>1.67 × 10⁶</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MTX-Sepharose fractions from both cells were used.

* Ratio of dihydrofolate reductase activity to folate reductase activity.

* Ratio of folate reductase activity with NADPH to that with NADH. The folate reductase activity with NADH in wild type cells was too low to be determined accurately.

**Fig. 6.** pH activity profile of the reductase, MTX fractions, from A5 (●) and wild type cells (○). Assays were as described under “Experimental Procedure” except that sodium acetate buffer was replaced by potassium acetate (○) or potassium phosphate (●).

**Fig. 7.** Inhibition of the reductase by methotrexate. Increasing amounts of enzyme (pH 5.0 supernatant fraction) were previously incubated in the absence or presence of 1 μmole of methotrexate at 37° for 2 min. Then reductase was assayed as described under “Experimental Procedure,” except that potassium acetate was used instead of sodium acetate. Difficulty was experienced with the stability of diluted A5 enzyme on prior incubation, so the enzyme was stabilized by the use of 0.1 M potassium phosphate buffer, pH 7.0, containing bovine serum albumin (6 mg per ml). A5 enzyme (●); wild type enzyme (○).
(although approximately 140 times more of the wild type cell extract was required for an amount of enzyme activity equivalent to that of A5 cells). Also no difference was observed in the amount of antigen-antibody precipitates formed by the same amount of enzyme activity from A5 and wild type cells (Fig. 9). The wild type and A5 enzymes were no longer precipitated when the same number of enzyme units had been added to a constant amount of antiserum, this would argue for their identity. Unfortunately too little wild type enzyme was available to determine this point (Fig. 9). However the data are sufficient to suggest that the specific activity of the A5 enzyme could be no more than 50% greater than that of wild type enzyme, and certainly not 140 times greater. Thus this and the previous data suggest that A5 cells contain a marked increase in the concentration of a reductase protein closely similar, if not identical, to that in wild type cells.

Rate of Synthesis of Reductase

The concentration of an enzyme can be altered by changes in its rate of synthesis or degradation, or a combination of the two (35). The initial rate of incorporation of radioactive amino acid into reductase is independent of the size of the previously existing pool of enzyme, and can therefore be used as a measure of its rate of synthesis. If the accumulation of reductase in A5 cells results from stimulation of its synthesis, this initial rate should be faster in A5 cells than in wild type cells, whereas if it results from inhibition of degradation, the initial rate of synthesis should be the same in the two lines. Therefore the rate of reductase synthesis was measured by pulse labeling of the enzyme for 1 hour with [3H]leucine, as described under "Experimental Procedure." Prior to immunoprecipitation, the extract of the cell type was mixed with the nonradioactive culture of the other cell type, so that the composition of the mixed extract and total enzyme units were the same in the two samples, although in each the content of wild type enzyme was less than one-hundredth that of A5 enzyme. It was possible to precipitate all the dihydrofolate reductase present in the mixed extracts by a slight excess of antibody near the equivalence point of the A5 enzyme (Fig. 9).

The data in Table IV represent one of two such experiments giving very similar results. Radioactivity precipitated by the specific antibody to A5 reductase and total radioactivity incorporated into extractable protein are shown. Thus in A5 cells 2.31% of the net counts per min were incorporated into the reductase, as compared with 0.12% in wild type cells. The percentage of total radioactivity which is present in reductase reflects the initial rate of synthesis of the enzyme. Therefore by this method the rate of reductase synthesis in A5 cells is increased about 20-fold over that in wild type cells.

**DISCUSSION**

In these studies we have characterized a subline of BHK cells stably resistant in culture to an even higher concentration of a folic acid analog than those lines previously described (15). A modification of the MTX-Sepharose affinity chromatography method originally developed by Mell et al. (16) has allowed us to purify dihydrofolate reductase from A5 cells 84-fold with 72% yield. The purified enzyme is stable and appears to be essen-
tially homogeneous by velocity sedimentation, polyacrylamide gel electrophoresis, and isoelectric focusing. From velocity sedimentation an $r_{20,w}$ value of 2.56 was calculated (36), which is close to that of 2.7 estimated from the sedimentation pattern in a sucrose density gradient. The molecular weight of active enzyme (20,000) calculated from the sedimentation coefficient in a sucrose density gradient is close to the molecular weight of 28,000 observed from sodium dodecyl sulfate gel electrophoresis. Therefore it seems likely that the A5 reductase is composed of only one polypeptide chain.

The properties of the reductase from these hamster cell cultures are similar to those of the enzyme from other animal sources. Thus substrate specificity, molecular weight of 20,000 to 23,000, activation by potassium ion, and stoichiometric inhibition by aminopterin or methotrexate are properties of the reductases from chicken liver (25), mouse sarcoma (5), and murine lymphoma cells (6). The apparently stoichiometric nature of methotrexate inhibition permits a calculation of the turnover number of the enzyme (37). Thus from the data of Fig. 7 it can be calculated that both A5 and wild type enzymes have a turnover number of approximately 33 molecules of folate reduced per min per methotrexate-binding site. This value is slightly higher than that of 25 reported for the enzyme from mouse sarcoma cells (38). The turnover number of A5 enzyme can also be calculated from the specific activity in the presence of potassium acetate. If the molecular weight of the active enzyme is assumed to be 23,000, this yields a value of 39, close to that calculated above by an independent method, and confirms the likelihood that each molecule of enzyme contains one methotrexate-binding site.

In these studies we have also sought to compare the nature and amount of the reductase in wild type and A5 cells. The two enzymes are similar in regard to substrate specificity, $K_m$ values for folate and NADPH, pH optimum, sedimentation coefficient, electrophoretic mobility, and turnover number per methotrexate-binding site. Since it was not possible to purify a large amount of reductase from wild type cells, antisera was made against A5 enzyme, and the degree of its inactivation and precipitation was the same as that of A5 enzyme, to the extent they could be compared. From all these results the most plausible explanation for the high reductase activity in A5 cells is an increase in the quantity of an enzyme similar if not identical with that in wild type cells.

To determine whether the increase of reductase protein in A5 cells results wholly or in part from an increased rate of synthesis, both A5 and wild-type cells were pulsed with $\left[14C\right]$leucine for 1 hour, a short period of labeling relative to the turnover time of reductase when protein synthesis is inhibited by puromycin (15). Under these conditions there was an approximately 20-fold increase in the rate of synthesis of reductase in A5 cells over wild type cells.

Such a 20-fold increase in the rate of reductase synthesis is not enough to account for the 140-fold increase in reductase specific activity in A5 cells. It may be that for some reason these experiments underestimate the rate of synthesis in A5 cells or overestimate that in wild type cells. For example, if the antibody precipitates cell proteins other than the reductase, this would cause a falsely high impression of the rate of reductase synthesis in wild type cells. On the other hand, the 140-fold increase in reductase activity in A5 cells may represent the summation of two or more different mechanisms, as suggested by the several steps of drug resistance through which the resistant lines were selected (15). Thus in addition to increased synthesis, the rate of degradation of reductase might be slower in A5 cells, from either a change in primary structure undetectable in the present studies but conferring added stability or a decrease in an extraneous degradative mechanism. It has not yet been possible to compare the turnover of the reductase in A5 and wild type cells because of the small amount of enzyme in the latter.

Nonetheless it would be most interesting to understand the nature of the stable, heritable change causing the 20-fold increase in the rate of reductase synthesis in A5 cells. As previously (15), the hybridization experiments do not particularly suggest that the resistant and wild type genomes interact when placed together in a single cell, as would be expected if the resistant cells were similar to regulator-constitutive mutants of bacteria. An operator-constitutive mutation (as in bacteria or perhaps here due to translocation of the structural reductase gene to a different location in the genome) or extensive duplication of the structural gene remain possibilities (15). Other possibilities include increased mRNA stability, increased efficiency of mRNA transcription or translation, altered mRNA transport from nucleus to cytoplasm, etc. Finally A5 cells might contain a structural gene mutation undetectable in the present studies but similar to those recently described in Diplococcus pneumoniae by Shrotnak (37), which cause 2- to 120-fold increase in reductase activity. Methotrexate-binding and immunological data indicate increased amounts of the altered protein to be present, and indirect evidence implicates increased rates of its synthesis (37).

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Purification, Properties, and Synthesis of Dihydrofolate Reductase from Wild Type and Methotrexate-resistant Hamster Cells
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