The biochemical basis for resistance to amethopterin has been investigated both in microorganisms and in mammalian cells (1, 2). Evidence has accumulated suggesting that amethopterin resistance in *Streptococcus faecalis* is associated with an increased capacity of resistant cells to form folinic acid from folic acid (3-5), altered permeability (5, 6), or changes in requirements for growth (4, 7).

In the present study, cultured Sarcoma 180 cells gradually became resistant to amethopterin and at the same time folic acid reductase, which catalyzes the reduction of folic acid to tetrahydrofolic acid, is present in the presence of triphosphopyridine nucleotide (8), increased proportionally with the degree of resistance. The kinetic characteristics of this enzyme, namely, the Michaelis constant for folic acid and the turnover number (9, 10), increased proportionally with the degree of resistance. The resistance in this case results from the production of a large excess of folic acid reductase which immobilizes amethopterin by binding it in an essentially irreversible manner (9, 10); at the same time, enough of the enzyme remains free to carry on its normal function. A preliminary report on these findings has been presented (11).

**EXPERIMENTAL PROCEDURE**

**Origin of Cells**—Sarcoma 180 cells, isolated by Foley and Droét (12), were purchased from Microbiological Associates, Inc., Bethesda, Maryland.

**Maintenance of Cultures**—Parent S-180 cells were grown on glass in Eagle’s medium (13, 14) containing 1 µg folic acid. The two amethopterin-resistant variants of S-180 cells, designated AH and AT, were grown in Eagle’s medium containing 0.2 µg folic acid and supplemented with 100 µM hypoxanthine (AH) or 30 µM thymidine (AT) together with 30 µM glycine and 0.5 µM amethopterin in each case. All the media contained 10% whole horse serum purchased from the Colorado Serum Company.

**Estimation of Growth**—Growth was measured as an increase in total protein (15). The inoculum refers to the cells attached to the glass at the time that the test medium was first applied.

**Preparation of Cells for Study of Folic Acid Reductase**—Cells grown in the presence of amethopterin were found to contain the drug in bound form. To deplete the cells of this amethopterin, all the cell lines were grown for at least 12 days² in the absence of the drug in a medium supplemented with 100 µM hypoxanthine, 30 µM thymidine, and 30 µM glycine in place of folic acid (16, 17). The cells were scraped from the glass (Roux flasks) with rubber policeman and were collected in a glass homogenizer. After centrifugation for 5 minutes at 500 r.p.m., they were washed three times with 3 volumes of cold solution of inorganic salts (13). The last centrifugation was performed at 1500 r.p.m. for 10 minutes; these cells were weighed and are referred to as “packed cells.”

**Preparation of Cell Extract**—The packed AH or AT cells were suspended in ice-cold 0.9% NaCl solution (1 ml per gram of cells) and were homogenized. All the operations were performed at 0 to 4°C. The homogenate was washed into a centrifuge tube with small amounts of 0.9% NaCl solution. The combined volume of the saline used was 2.5 ml per gram of cells. The cell debris was removed by centrifugation for 20 minutes in an International refrigerated centrifuge, model PR-2, at 18,000 r.p.m. The extract from the sensitive S-180 cells was prepared by the same procedure, but in this case the combined volume of NaCl solution used was 1.25 ml per gram of cells. Enzymatic activity and amethopterin titrations of cell extracts are expressed per unit weight of packed cells.

**Purification of Folic Acid Reductase**—Each cell extract was adjusted to pH 8.0 with NaOH and stirred for 10 minutes with Dowex 1-Cl to remove cofactors (about 1 g per 5 to 6 ml of extract). Dowex was removed by centrifugation, and the solution was adjusted to pH 4.5 by careful addition of 0.1 N HCl. The precipitated protein was removed by centrifugation, and the supernatant fluid was readjusted to pH 7.0. The folic acid reductase activity per mg of protein (18) in the supernatant at this stage of purification was twice that of the original supernatant fraction, and the preparation from AT cells was 5.5 times more active per mg of protein than our purest preparation from chicken liver (8). These preparations were used for the kinetic studies.

**Measurement of Enzymatic Activity**—The rate of reduction of folic acid in the presence of TPNH as the hydrogen donor was determined by the amount of diazotizable amine formed upon decomposition of tetrahydrofolic acid, as described elsewhere (8). Further details are given for each experiment separately.

**Titration of Folic Acid Reductase by Amethopterin**—Based on the observation of the essentially irreversible binding of amethopterin by folic acid reductase (9, 10), the relative quantities of this enzyme in various preparations can be estimated in terms of...

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*This investigation was supported in part by research grants (CY-2906 and CY-4175) from the National Cancer Institute of the United States Public Health Service.

¹ The following terminology is used: folic acid, pteroylglutamic acid; folinic acid, 5-formyl-tetrahydro-pteroylglutamic acid; aminopterin, 4-amino-pteroylglutamic acid; amethopterin, 4-amino,10-methyl-pteroylglutamic acid; S-180, Sarcoma 180.

² S. F. Zakrzewski and M. T. Hakala, unpublished data.
drug-binding sites. This was done by adding graded amounts of the inhibitor to a series of reaction vessels containing equal amounts of the enzyme preparation. Thus the number of moles of amethopterin required for the complete inhibition of various folic acid reductase preparations could be estimated. The precision of the results was improved by keeping the concentration of TPNH constant throughout the incubation. This was achieved by including in the incubation mixture a TPNH-regenerating system which consisted of glucose 6-phosphate and glucose 6-phosphate dehydrogenase (8).

**Chemicals**—Folic acid which was used for growth studies was Folvite supplied by American Cyanamid Company, Calco Chemical Division. A crystalline preparation purchased from Nutritional Biochemicals Corporation was used for the enzyme studies.

Folinic acid was a preparation of calcium leucovorin kindly supplied by the American Cyanamid Company, Lederle Division. The concentrations of folic acid specified in the text are one-half of the actual concentration of leucovorin used, to account for the inactive optical isomer (19, 20). Amethopterin was a product of American Cyanamid Company, Calco Chemical Division. TPNH was prepared by reduction of TPN (Pabst Laboratories) with glucose 6-phosphate dehydrogenase (8) purchased from Sigma Chemical Company.

**RESULTS**

*Development of Amethopterin-resistant Sarcoma 180 Cells*—In earlier studies (16, 17), it was demonstrated that in a medium supplemented with hypoxanthine, thymidine, and glycine, mammalian cells are insensitive to amethopterin, since the function of folic acid reductase under these conditions is not essential; omission of any one of these metabolites makes the cells susceptible to the drug. Thus, it was possible to develop different amethopterin-resistant variants by including in the medium, in addition to 0.2 μM folic acid and 30 μM glycine, 100 μM hypoxanthine (AH) or 30 μM thymidine (AT). The concentration of amethopterin was gradually increased over a period of several months from 0.02 μM to 0.5 μM.

Earlier studies with Streptococcus faecalis 8043 (21, 22) had demonstrated that thymidine (or thymine) reduced by one-half the lag period preceding the appearance of a single step aminopterin-resistant variant. The development of amethopterin-resistant S-180 was very slow in the two different media. The subline developed in the presence of thymidine (AT), however, was 2 to 3 times more resistant to amethopterin than that developed in the presence of hypoxanthine (AH). A resistant subline was also developed in a medium without either hypoxanthine or thymidine. The rate of development was also slow and the level of resistance was similar to that of subline AH. The degree of resistance to amethopterin was determined in Eagle's medium containing 1 μM folic acid by estimating the concentration of the drug necessary for 50% inhibition of growth in 7 days (Table I).

**Sparing Action by Hypoxanthine and Thymidine**—The requirement for folic or folinic acids for growth of the amethopterin-resistant cells was unchanged from that of the parent, as seen in Table II. A similar observation has been made by Fischer regarding amethopterin-resistant L-5178-Y cells (23). Folinic acid is about 100 times more effective than folic acid in promoting the growth of all the variants of S-180 cells. Maximal growth was comparable in the presence of optimal amounts of either compound. However, either hypoxanthine or thymidine caused a sparing action for folic and folinic acids which varied in the different cell lines (Table III). Thus, hypoxanthine, but not thymidine, reduced by one-half the requirement of the sensitive S-180 cells for folic acid. This effect of hypoxanthine was even more pronounced in AH cells. On the other hand, thymidine spared the folic acid requirement for the growth of AT cells to one-half of that in its absence.

### Table I

**Degree of resistance to amethopterin**

<table>
<thead>
<tr>
<th>S-180 cell line</th>
<th>In folic acid medium</th>
<th>In folinic acid medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amethopterin for 50% inhibition</td>
<td>Degree of resistance</td>
<td>Amethopterin/folic acid</td>
</tr>
<tr>
<td>Sensitive</td>
<td>0.054 ± 0.0016</td>
<td>1</td>
</tr>
<tr>
<td>Resistant</td>
<td>3.6 ± 0.41</td>
<td>67</td>
</tr>
<tr>
<td>AT</td>
<td>9.4 ± 1.1</td>
<td>174</td>
</tr>
</tbody>
</table>

* a Estimated in Eagle's medium containing 1 μM folic acid.
* b The molar ratio of amethopterin to folic acid resulting in 50% inhibition of growth was determined over a 100-fold range of concentrations of folic acid (0.003 to 0.3 μM).
* c Standard error of the mean from 12 to 16 determinations.
* d Standard error of the mean from 7 or 8 determinations.

### Table II

**Requirement of amethopterin-sensitive and -resistant Sarcoma 180 cells for folic and folinic acids**

<table>
<thead>
<tr>
<th>S-180 cell line</th>
<th>Requirement for 50% maximal growth</th>
<th>Requirement for 50% inhibition of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>Folinic acid</td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>130</td>
<td>1.4</td>
</tr>
<tr>
<td>Resistant</td>
<td>130</td>
<td>1.3</td>
</tr>
<tr>
<td>AH</td>
<td>120</td>
<td>1.7</td>
</tr>
<tr>
<td>AT</td>
<td>120</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* a To deplete the cells from folic acid and amethopterin before testing, the cells were grown for a minimum of 12 days in a medium which in place of folic acid was supplemented with 100 μM hypoxanthine, 30 μM thymidine, and 30 μM glycine.

### Table III

**Effect of hypoxanthine and thymidine on the requirement for folic acid and on amethopterin inhibition**

<table>
<thead>
<tr>
<th>S-180 cell line</th>
<th>Supplement</th>
<th>Folic acid required for 50% maximal growth</th>
<th>Amethopterin required for 50% inhibition of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Hypoxan- thine (100 μM)</td>
<td>Thymidine (50 μM)</td>
<td>None</td>
</tr>
<tr>
<td>Sensitive</td>
<td>0.15</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>Resistant</td>
<td>0.13</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>AH</td>
<td>0.12</td>
<td>0.14</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* a Estimated after 7 days of growth in Eagle's medium containing 1 μM folic acid.
SENSITIVE S-180

DAYS IN AMETHOPTERIN-FREE MEDIUM

FIG. 1. Decrease of amethopterin resistance in AT cells when maintained in absence of the drug in the following media: ○, folic acid 1 μM; △, folinic acid 0.01 μM; □, hypoxanthine 100 μM, thymidine 30 μM, and glycine 30 μM. The ordinate indicates the concentration of amethopterin required for 50% inhibition of growth when tested in a medium containing 1 μM folic acid.

These metabolites also reduced the inhibitory activity of amethopterin by a similar sparing action. Thus, in the presence of thymidine and folic acid, inhibition of AT cells required approximately 4 times more amethopterin, whereas inhibition of the parent and AH cells in the presence of hypoxanthine required twice as much amethopterin as in its absence (Table III).

Effect of Folinic Acid on Inhibition by Amethopterin—In the presence of an optimal amount of folic (1 μM) or folinic acid (0.01 μM), the concentration of amethopterin necessary for inhibition of growth is about the same. However, increasing the concentration of folic acid but not of folinic acid will competitively prevent the inhibition of growth caused by amethopterin (Table I). The degree of resistance to amethopterin whether tested in folic or folinic acid media is the same.

Stability of Amethopterin Resistance—Both cell lines (AH and AT) were maintained in the absence of the drug in three types of media, containing (a) folic acid (1 μM), (b) folinic acid (0.01 μM), or (c) hypoxanthine, thymidine, and glycine. The rate at which resistance decreased was slow and independent of the type of medium used (Fig. 1). The loss of resistance closely parallels the decrease of folic acid reductase in the cells as seen in Fig. 2; half of the original resistance was lost in about 30 to 40 days, i.e. 12 to 18 generations.

Growth Patterns—The rate of growth of AH cells in the medium containing hypoxanthine, thymidine, and glycine was about 3 times as fast as that of AT cells and twice as fast as that of the sensitive ones. Microscopic examination revealed that the parent S-180 and the AH cells grow in a manner lacking organization. AT cells, on the other hand, have a tendency to adhere to each other and to grow in an oriented fashion.

Sensitivity to Other Antimetabolites—The concentrations of the drugs necessary for 50% inhibition of growth were determined. The sensitivity of S-180 cells to 6-mercaptopurine, 5-fluorodeoxyuridine, and 6-diazo-5-oxo-L-norleucine was not altered by the development of amethopterin resistance.

Folic Acid Reductase in Cell Extracts— Cultures were grown for a minimum of 12 days in the absence of the drug to deplete the cells of amethopterin which could interfere with the determination of folic acid reductase activity. However, during this period there occurs a 20 to 25% loss of resistance and presumably of folic acid reductase activity (Figs. 1 and 2). This loss will be taken into account in interpreting the results.

The activities per milligram of cells for the reduction of folic acid in the presence of an excess of TPNH were compared in extracts of sensitive and resistant cells (Fig. 3). Much more folic acid reductase activity was found in the resistant than in the sensitive cells, and a linear relationship was noted between the reaction velocity and the amount of extract.

The folic acid reductase from each extract was also titrated with amethopterin to zero activity (see "Experimental Procedure"). This revealed 33 and 85 times more folic acid re-
ductase in AH and AT cells, respectively, than was found in the sensitive S-180 cells (Table IV). By correcting for a probable loss of folic acid reductase (see above), one can estimate that AH cells (67-fold resistant) initially contained 65 times, and AT cells (174-fold resistant) 155 times more folic acid reductase than the sensitive S-180 cells. These titrations gave the same slope in a graph of initial reaction velocity against amethopterin concentration for both sensitive and resistant S-180 cell lines (Fig. 4). The same amount of amethopterin is, therefore, necessary in each case to block a given enzyme activity; i.e., the enzyme activity per drug-binding site did not change during the development of amethopterin resistance.

Titration of folic acid reductase by amethopterin in the resistant cells, taken directly from their respective maintenance media, revealed an amount of free folic acid reductase in AH cells which was equivalent to $2.1 \times 10^{-12}$ M amethopterin per mg of cells and in AT cells equivalent to $8.3 \times 10^{-12}$ M amethopterin per mg of cells. Thus the resistant cells, even when grown in the presence of amethopterin (0.5 $\mu$M), still contain much more free reductase than the parent cells.

**Kinetic Studies**—Folic acid reductase was partially purified from each cell line for these experiments as described under "Experimental Procedure." Fig. 5 shows the experimental data, and Table V lists the results of this study. The values for maximal velocities presumably reflect the purity of each enzyme preparation. Turnover numbers were determined by titrating the folic acid reductase with amethopterin and by dividing the maximal velocities by these values. No change in folic acid reductase activity per drug-binding site has occurred. Neither has there been any change in the affinity of the enzyme to the

**Table IV**

*Folic acid reductase in extracts of sensitive and resistant S-180 cells*

<table>
<thead>
<tr>
<th>S-180 cell line</th>
<th>Days in absence of drug</th>
<th>Enzyme activity/mg of cells</th>
<th>Drug-binding sites/mg of cells</th>
<th>Corrected $R/S^b$</th>
<th>Degree of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AH</td>
<td>12</td>
<td>0.742</td>
<td>0.18</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>AT</td>
<td>28</td>
<td>40.0</td>
<td>54</td>
<td>9.6</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60.2</td>
<td>81</td>
<td>15.0</td>
<td>155</td>
</tr>
</tbody>
</table>

* Medium contained in place of folic acid 100 $\mu$M hypoxanthine, 30 $\mu$M thymidine, and 30 $\mu$M glycine.

*b Test conditions described under Fig. 4. The activity was calculated from the change in optical density in the absence of the inhibitor. In addition to the enzyme, the reaction mixture contained 43.6 $\mu$M folic acid, 20 $\mu$M TPNH, 24 mM glucose 6-phosphate, 0.26 Kornberg unit (33) of glucose 6-phosphate dehydrogenase, and 0.1 M sodium citrate pH 6.0, and amethopterin as indicated, in a total volume of 0.5 ml. Incubated for 10 minutes at 37°.

*a Medium contained in place of folic acid 100 $\mu$M hypoxanthine, 30 $\mu$M thymidine, and 30 $\mu$M glycine.

*b Expressions as equivalents of amethopterin based on titration of folic acid reductase under conditions described for Fig. 4.

*c The ratio of resistant to sensitive cells was corrected on basis of days in absence of drug according to Figs. 1 and 2.

$d R/S = \text{the ratio of resistant to sensitive cells.}$
Folic Acid Reductase and Amethopterin Resistance

Vol. 236, No. 3

Degree of resistance .............................................. 1 67 174

V<sub>max</sub> (moles/liter/min/mg protein) × 10<sup>4</sup> ............ 0.05 25 42

K<sub>m</sub> for folic acid × 10<sup>4</sup> .................................. 1.1 1.25 1.1

Turnover number/ min/drug-binding site ......................... 27 25 29

* Calculated from the data presented in Fig. 5.

+ Determined by titrating each enzyme preparation with amethopterin and by dividing V<sub>max</sub> by these values.

Kinetic constants of partially purified folic acid reductase from amethopterin-sensitive and -resistant S-180 cells

Table V

Fig. 5. Reciprocal plot (34) for partially purified folic acid reductase from sensitive and resistant S-180 cells; v = moles per liter per minute per mg of protein; [S] = molar folic acid. The indicated concentrations of folic acid were incubated at room temperature in the presence of 0.16 mM TPNH and 0.1 mM sodium citrate, pH 5.2, with the following amounts of the partially purified enzyme preparations: sensitive S-180, 1.54 mg of protein; AH, 94 μg of protein; AT, 67 μg of protein; total volume 0.5 ml. AT and AH preparations were incubated for 5 minutes, and the preparation from the sensitive cells for 10 minutes.

substance, folic acid, as suggested by similar Michaelis constants.

**DISCUSSION**

According to present assumptions concerning the regulatory mechanisms of enzyme synthesis (24), it is believed that the molecular pattern (quality) is determined by genes (DNA) whereas the quantity of an enzyme is controlled also by feedback mechanisms and adaptations. The fact that amethopterin-resistant S-180 cells, after growth for long periods in absence of the drug, still contain greatly increased amounts of folic acid reductase (Figs. 1 and 2) suggests that DNA may be involved in the maintenance of this characteristic. However, the proof for this must await further studies. As an example, in **Diplococcus pneumoniae**, amethopterin resistance has been induced by DNA (25). Unfortunately, the enzymic changes associated with resistance in that case were not elucidated.

The 4-amino analogues of folic acid have been shown to be bound to folic acid reductase in a manner that is practically irreversible (9, 10). The greatly increased amount of folic acid reductase in amethopterin-resistant S-180 cells acts as a unique physiological inactivator by binding the antagonist. Yet, in media containing ordinarily lethal concentrations of amethopterin, enough of the enzyme remains free in the resistant cells to carry on its normal functions. The possibility that an increase in the amount of other enzymes occurs in other cases of resistance to irreversibly bound inhibitors merits investigation.

Folic acid reductase has been found to be extremely sensitive to aminopterin (26); dihydrofolic acid reductase, most probably identical with folic acid reductase (27), is also sensitive to aminopterin and amethopterin (28). Other recent investigations of amethopterin resistance in mammalian cells have also revealed an increase in the activity of folic acid or dihydrofolic acid reductase in the resistant cells (23, 20). Several explanations are possible for this phenomenon; (a) an increase in the quantity of the enzyme, (b) an increase in the number of the active sites per enzyme molecule, (c) an increase in turnover number per active site, (d) a decrease in K<sub>m</sub> value for the substrate (if the the sensitive line had been tested at suboptimal substrate concentration), or (e) some combination of these changes.

In the present case, we have demonstrated that both the turnover number per amethopterin binding site and K<sub>m</sub> value for folic acid remained constant despite the development of resistance. At the same time the total enzyme activity, together with the drug-binding capacity, had increased in the cells proportionally with the degree of resistance. Assuming that the drug-binding site is the active site, then either the quantity of the unchanged enzyme or the number of active sites per enzyme molecule had increased. The first alternative is the more probable one, although final proof must await the study of purified enzyme preparations from the sensitive and resistant cells.

Assuming that the molecular weight of folic acid reductase is 10<sup>6</sup> and that there is only one drug-binding site per enzyme molecule, one can calculate that in AT cells (which contain 155 times more reductase than the sensitive ones) the folic acid reductase makes up about 5% of the cellular protein. One can speculate that an increase of this enzyme cannot be the only cause of resistance. Although the studies of the other enzymes in AT cells have been reported, it is believed that the molecular pattern (quality) is determined by genes (DNA). Worthy of mention is the possibility that the regulatory mechanisms of enzyme synthesis of amethopterin-resistant S-180 cells act as a unique physiological inactivator by binding the antagonist. Yet, in media containing ordinarily lethal concentrations of amethopterin, enough of the enzyme remains free in the resistant cells to carry on its normal functions. The possibility that an increase in the amount of other enzymes occurs in other cases of resistance to irreversibly bound inhibitors merits investigation.

Folic acid reductase prevents competitively the growth inhibition caused by amethopterin at concentrations of folic acid which are only a fraction of that of the inhibitor. One might assume that folic acid or some product of it competitively prevents inhibition of folic acid reductase by amethopterin. There are two lines of evidence which indicate otherwise. (a) In an enzymatic assay with folic acid reductase from chicken liver, 43 μM folic acid failed to interfere with the inhibition caused by 0.01 to 0.06 μM amethopterin, indicating that folic acid itself
is unable to protect folic acid reductase against amethopterin. (b) Studies on amethopterin binding in growing cultures of S-180 cells, the other hand, have demonstrated that cells grown in the presence of 1 μM folic acid and 0.1 μM amethopterin bound and concentrated amethopterin from the medium to the same extent as in two other media lacking folic acid and that the amount bound was proportional only to the amount of folic acid reductase in these cells. Thus, folic acid or its product also failed to prevent the binding of amethopterin to folic acid reductase. The latter observation, in addition, excludes the involvement of competition between amethopterin and folic acid at the site of entry into the cell.

Thus, one would conclude that some other reaction in addition to the reduction of folic acid is also inhibited by amethopterin. This reaction cannot be the immediate synthesis of a purine ring, as has been suggested (31), or introduction of the methyl group of thymidine, since growth inhibition by amethopterin is observed even in the presence of (a) folic acid and hypoxanthine or (b) folic acid and thymidine. If the synthesis of either purines or thymidine were inhibited by amethopterin in the presence of folic acid, no inhibition would have occurred under one of these conditions. This evidence suggests that the reaction(s) inhibited by amethopterin, yet competitively protected in living cells by folic acid, is concerned with some step in the formation of a common precursor derived from folic acid and used for one-carbon transfer reactions.

The metabolite (either thymidine or hypoxanthine) which was present during the development of the amethopterin-resistant strain, exerts a sparing action with respect to the requirement for folic or folic acid for growth of these resistant cells. Also, AT cells are more resistant to amethopterin in the presence of thymidine than in its absence, and the parent and AH cells are more resistant in the presence of hypoxanthine. If this can happen in vivo, the availability of thymidine or a purine during amethopterin treatment can influence the character of the resistant population of cells which eventually develops.

It is significant that hypoxanthine demonstrates a sparing action in the sensitive S-180 cells whereas thymidine has no such effect. Thus purines, rather than thymidine, are the limiting products with respect to the one-carbon transfer reactions reflected by growth. This is logical when one considers the known manifold functions of purines in the living cell as compared with the single role of thymidine as a constituent of DNA. Indeed, it was pointed out by Lieberman and Ove (32) that the formation of one rabbit liver cell in a culture requires 15 times more adenine than thymidine. In the present study, a sparing action of thymidine could be created only by forcing the cells to grow in the presence of amethopterin together with folic acid and thymidine.

The sparing action of purines seems to be the natural or "in-born" characteristic, not only of S-180, but also of several other mammalian cell strains. Further studies may reveal whether these amethopterin-resistant variants differ from the parent with respect to purine- and thymidine-synthesizing enzymes in addition to their increased content of folic acid reductase. The cause correlation between the content of folic acid reductase and the level of resistance to amethopterin in the cell lines studied provides a basis for questioning whether a similar relationship exists in neoplasms which are known to differ in their responsiveness to treatment with 4-amino antagonists of folic acid.

**SUMMARY**

Two lines of cultured Sarcoma 180 cells resistant to amethopterin have been studied. Resistance developed gradually in a medium containing hypoxanthine (AH cells; 67-fold resistant), and in a medium containing thymidine (AT cells; 174-fold resistant). The rate of decrease of resistance in the absence of the drug was independent of the type of medium used; after one month of cultivation, the cells were about half as resistant.

The requirement for folic or folinic acids for growth was not altered during the development of resistance. Folic acid prevented competitively the inhibition of growth caused by amethopterin; 9 times more amethopterin than folic acid was necessary for inhibition of the growth of sensitive Sarcoma 180 cells, whereas 600 times more was necessary for inhibition of AH cells and 1600 times more for inhibition of AT cells. The relationship between folic acid and amethopterin is discussed and a suggestion is made concerning the possible site of action involved. The sensitivity to the growth-inhibitory effects of 6-mercaptopurine, 6-diazo-5-oxo-L-norleucine, and 5-fluorodeoxyuridine remained unchanged despite the development of amethopterin resistance.

The resistant AH cells were found to contain 65 times more folic acid reductase than the sensitive Sarcoma 180 cells, and the AT cells contained 15 times more. Only the quantity of this enzyme has increased (or the number of active sites per enzyme molecule) whereas the kinetic characteristics, Michaelis constant, $K_m$, value for folic acid (1.1 to 1.25 x $10^{-5}$ M) and turnover number per amethopterin-binding site (25 to 29 per minute) had remained constant. Resistance to amethopterin in this case results from the large excess of folic acid reductase which acts as a specific physiological inactivator by binding the antagonist. Enough of the enzyme remains free to carry on its normal functions.

**ACKNOWLEDGMENTS**—The authors gratefully acknowledge the technical assistance of Miss Edythe Taylor, Miss Luella Pecetti, and Mrs. Jadwiga Drobiak.

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Maire T. Hakala, Sigmund F. Zakrzewski and Charles A. Nichol


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