The Mechanism of Action of the Folate Antagonists in Man*

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SUMMARY

The folate antagonists, in particular amethopterin (methotrexate), continue to be useful drugs for the treatment of certain neoplastic diseases in man. The effectiveness of amethopterin in preventing DNA synthesis and cellular replication appears to be a consequence of inhibition of the enzyme dihydrofolate reductase. This premise is supported by in vivo studies which show that in patients treated with amethopterin, the conversion of folate to reduced forms is blocked, as well as in vitro studies which demonstrate that amethopterin inhibits this enzyme activity at remarkably low concentrations. The administration of amethopterin to subjects with leukemia as well as to subjects without hematologic disease results in increased dihydrofolate reductase activity in leukocytes and erythrocytes. This “induction” of dihydrofolate reductase activity parallels the amethopterin concentration in blood cells. The binding of the folate antagonist to the reductase enzyme is tightest at pH 5.9. Because of the in vitro assay conditions (dilution of the enzyme and inhibitor and assay at pH 8.3), this increase in enzyme activity observed in vitro may not represent a true increase of enzyme activity, since most of the increased enzyme activity may be bound by amethopterin within the cell.

A knowledge of the ability of leukemic cells to transport and retain amethopterin may be of value in the prediction of responsiveness to therapy with this agent. However, other factors that deserve further investigation are cited that may also influence responsiveness.

Although aminopterin\(^1\) was first used in the treatment of neoplastic disease over 14 years ago (39), indications for the use of amethopterin or methotrexate, the derivative now used clinically, are still evolving. This drug is of temporary benefit in the treatment of acute leukemia of childhood, in which a remission rate of 47 per cent (including both partial and complete remissions) may be expected (108). An important consideration in this regard is that, in many instances, remissions can be obtained without serious host toxicity (21, 38).

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1 The terms “folate antagonists” or “antifolates” will be restricted in use to mean the 4-amino derivatives of 4-deoxyfolic acid. The three compounds of this type that will be discussed are: aminopterin (4-amino-4-deoxy]-pteroylglutamic acid), amethopterin or methotrexate (4-amino-4-deoxy]-10-methylpteroylglutamic acid), and dichloroamethopterin or dichloromethotrexate (3',5'-dichloroamethopterin).

Complete remissions in almost half the patients treated with amethopterin also may be expected in choriocarcinoma and related trophoblastic tumors in females (66, 70, 78, 79). The apparent cure of some of these patients (i.e., 5-year survival) has recently been reported (67). These striking results have not been obtained in the chemotherapy of other solid tumors, but methotrexate has caused significant objective regressions in some cases of inoperable breast carcinoma (57, 105, 135, 137) and mycosis fungoides (136), as well as epidermoid carcinoma of the head and neck (51, 63, 73). Fair results also may be produced in lymphosarcoma (23), and good to excellent results have been reported in the treatment of reticuloendotheliosis (23). Despite this impressive list of tumors that may be responsive to treatment with amethopterin, most other solid tumors usually do not respond to this drug (23, 105).

The use of amethopterin in high concentration

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intra-arterially has been explored during recent years, with (114) and without (129) protection of the systemic circulation with the \( \text{N}^4\)-formyl derivative of tetrahydrofolic acid (FH\(_4\)),\(^1\) i.e., folic acid, citrovorum factor, or leucovorin. Impressive regressions of carcinomas of the head and neck (37, 115, 116) and of the cervix (117) have been demonstrated with this method of drug administration. Large infrequent intravenous doses of amethopterin have been used by Condit (30, 32) in an attempt to increase the therapeutic index.

It is not the purpose of this paper to discuss in detail the clinical indications for and the treatment of neoplastic disease with the folate antagonists\(^1\) but rather to review what is known about the pharmacology and biochemistry of these agents in man. In those conditions in which objective benefits may be obtained when amethopterin is used, we cannot predict whether or not a particular patient will benefit from such treatment, nor can tolerance to the drug be predicted, nor even in those patients who are treated successfully (except for some women with choriocarcinoma) can the eventual emergence of drug-resistant populations of neoplastic cells be predicted or avoided. The situation at the present time is somewhat analogous to the use of penicillin without knowing what the offending organism is; in general, we can predict empirically what infections would be benefited, but there would be a significant number of nonpredictable failures.

During the last 2 decades the understanding of the functions of folate, as well as the mechanism of action of the folate antagonists, has progressed rapidly. It may not be premature, therefore, to discuss not only these studies with special reference to neoplastic disease in man, but also to indicate how an understanding of the mode of action of the folate antagonists in tumor tissue and in normal tissue may lead to the development of predictive tests of responsiveness to these drugs, as well as possible new approaches to more effective chemotherapy.

**I. THE MODE OF ACTION OF THE FOLATE ANTAGONISTS**

The elucidation of the structure of the vitamin folic acid, in 1946 (2), was soon followed by the synthesis and subsequent investigation of the biological activity of a number of potent antagonists. The compound now used clinically in place of aminopterin, the first potent folate antagonist tested, is amethopterin. On a weight basis amethopterin is actually less potent than aminopterin, but studies in mice bearing the L1210 leukemia indicated that it possessed a better therapeutic index (55). Another 4-amino analog of folate, the \( \text{N}^3,\text{N}^5\)-dichloro derivative of amethopterin—i.e., dichloromethoteprin—is of much interest because of its increased effectiveness as compared with amethopterin in mice with L1210 leukemia (54), but in those clinical studies thus far reported no advantages of this drug over amethopterin have been noted (49, 107).

The early finding of Nichol and Welch (90), who showed that in rat liver slices amethopterin blocked the conversion of folate to reduced derivatives measured as \( \text{N}^4\)-formyl-FH\(_4\), has been followed in recent years by the identification of the sensitive enzyme system—i.e., dihydrofolate reductase (also called folic acid reductase) (52, 98, 96, 139).\(^4\) This enzyme converts dihydrofolate (FH\(_2\)) or folate to FH\(_4\), a metabolically active coenzyme form of folate, which functions as a carrier of “one carbon” units in the biosynthesis of the purine ring, thymidylate, methionine, serine, and histidine (reviewed in [26, 54, 71, 72, 81, 91, 99]). Consequently, a lack of this coenzyme caused by either dietary deficiency or the administration of a folate antagonist could result in decreased synthesis of purines, thymidylate, and proteins. Although there are reports indicating that in certain tissues a defect in purine synthesis is responsible for the effects produced by the antifolates (56, 104, 106, 110), a number of studies, including investigations with rabbit bone marrow cells (120) and human chronic myelogenous leukemia cells (133), suggest that an inhibition of thymidylate synthesis is the primary event leading to cell death after treatment with the folate antagonists. In this regard, recent studies have indicated that this enzyme functions not only in the conversion of dietary folates to FH\(_4\) but also in the reduction of FH\(_2\) that is produced as the product of thymidylate synthase (181). The dual role of this enzyme is shown in Chart 1. The cyclic nature of this latter reaction may make it particularly vulnerable to interruption by inhibition of dihydrofolate reductase.

\(^1\) The following abbreviations will be used: FH\(_2\), dihydrofolic acid; FH\(_4\), tetrahydrofolic acid; TPN and TPNH, the oxidized and reduced forms of triphosphopyridine nucleotide, respectively; DPN and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide, respectively; ADP and ATP, adenosine di- and tri-phosphate; RNA and DNA, ribonucleic and deoxyribonucleic acid; dUMP, deoxyuridylate; TMP, thymidylate, DEAE, diethylaminoethyl.

\(^2\) A number of excellent reviews that cover these aspects have been written in the past few years (21, 22, 26, 28, 34, 40, 68, 80).

\(^3\) This enzyme has also been called tetrahydrofolate dehydrogenase (6); for reasons discussed elsewhere (71), we prefer the name dihydrofolate reductase.
Inhibition of dihydrofolate reductase activity, therefore, is almost certainly responsible for the therapeutic as well as the toxic effects that are seen after the administration of amethopterin in man. This premise is supported by the capacity of N⁷-formyl-FH₄, if given concomitantly or within a few hours after amethopterin, to reverse the effects of the antagonist, whereas folate will counteract the effects of the antagonist only if given prior to amethopterin (53). Attempts to reverse the toxicity of amethopterin with the products of biosynthesis in which the folate coenzymes participate have been only partially successful in vivo. Although adenine protected mice against relatively small doses of amethopterin, the addition of thymidine, together with histidine, methionine, and serine, gave no better results than did adenine alone (24). In tissue culture, however, the effects of amethopterin on neoplastic cells can be overcome by a combination of glycine, hypoxanthine, and thymidine (41, 50). Recently, Mead et al. (83) have reported that, in mice, FH₂, as well as leucovorin, can reverse the toxic manifestations of amethopterin. The significance and possible explanation of this finding will be discussed in a later section in studies of enzyme inhibition by the antagonists of folate.

Studies in man in vivo.—The concentration of the 4-amino-pteroylglutamate analogs of folate in body fluids and tissues was first measured by means of a microbial assay, with Streptococcus faecalis used as the test organism (25). A fluorometric procedure has also been utilized in the study of absorption, disappearance from plasma, and catabolism of amethopterin (45–47). Recently, more satisfactory assays based on the specificity and sensitivity of dihydrofolate reductase to inhibition by the antagonists of folate have been devised (13, 128). The availability of radioactive antagonists, although not used thus far in man, should also provide a sensitive method to follow the disposition of these compounds (42, 92).

In summary, these studies have shown that amethopterin is rapidly absorbed on an empty stomach (food delays absorption and also results in a lower peak level of drug in the serum), the drug is rapidly cleared from the plasma, and almost all the drug is apparently excreted unchanged in the urine, usually within the first few hours. Studies on dogs, carried out in this laboratory, indicate that when small doses of methotrexate (less than 15 µg/kg of body weight) are given intravenously the majority of the drug is held by the tissues, since very little of the material is found in urine during the next few days. These findings provide an explanation for the experimental observation that tremendous single doses can be tolerated by animals and man, whereas small repeated doses cause significant toxicity. It would appear, therefore, that only a finite amount of drug can be “bound” at one time, and administration of larger amounts of drug is probably slightly more effective only because the higher blood level produced is maintained for a longer period of time. To illustrate this last point, the blood level of amethopterin measured in a patient given a large intravenous dose of amethopterin is shown in Table 1. As compared with smaller doses (10 mg. or less) given to patients orally or parenterally, a situation in which the blood level is negligible after 6–7 hours (46), this dose produced a relatively high blood level for over 24 hours. That toxicity is related more to duration than to dose.

### Table 1

**Serum Levels of Amethopterin after the Intravenous Administration of Amethopterin**

<table>
<thead>
<tr>
<th>Time after infusion (hours)</th>
<th>Concentration of amethopterin (mole/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>2×10⁻⁴</td>
</tr>
<tr>
<td>0.50</td>
<td>1.8×10⁻⁴</td>
</tr>
<tr>
<td>1</td>
<td>7.8×10⁻⁴</td>
</tr>
<tr>
<td>2</td>
<td>4.4×10⁻⁴</td>
</tr>
<tr>
<td>6</td>
<td>2.0×10⁻⁴</td>
</tr>
<tr>
<td>24</td>
<td>2.8×10⁻⁷</td>
</tr>
<tr>
<td>48</td>
<td>1.0×10⁻⁴</td>
</tr>
</tbody>
</table>


6 Since the inhibitor constant \( K_i \) for amethopterin is less than 10⁻⁷ M, it follows that adequate blood levels to produce marked enzyme inhibition are present for greater than 24 hours.
of the contact of methotrexate with tissues than with the amount of drug present in the blood is also indicated by the studies of Miller et al. (85) and Freeman-Narrod (48).

Dichloroamethopterin, unlike amethopterin and aminopterin, is incompletely absorbed from the gastrointestinal tract and is rapidly catabolized in man and rodents, but not in the dog (80, 92); consequently, it has been necessary to use larger doses of this compound to maintain adequate blood levels in man (127). The metabolic product of this oxidative deamination (tentatively identified as 4-deamino-4,7-dihydroxy-dichloromethotrexate) is not an inhibitor of dihydrofolate reductase.  

In rodents, the tissues that bind amethopterin in the highest concentration are the liver and kidney (15, 44), organs with the highest content of the folate coenzymes (113), as well as with the highest levels of dihydrofolate reductase (15). These tissues also bind labeled folate in highest concentration (122). Although dihydrofolate reductase activity has not yet been studied in human liver and kidney, amethopterin has been detected in these tissues (obtained at post mortem) for as long as 116 days after the administration of methotrexate (29).

As might be predicted from the knowledge of the enzymic site of action of the folate antagonists, administration of amethopterin to patients rapidly and markedly decreases the urinary excretion of reduced folates as measured by microbial assay, after folic acid is administered (32, 36, 124). Of interest is the prolonged depression of the excretion of these compounds and the inability of dietary folic acid to overcome this block (32). Recently, we have measured the levels of serum folate in patients with carcinoma of the head and neck treated with methotrexate (69). In nine of ten patients treated with five daily doses of from 5 to 20 mg. of amethopterin, the folate level in the serum fell rapidly to barely detectable values and remained depressed for at least 2–3 weeks. Since the largest part of the serum activity in the serum, as measured by this microbial assay (Lactobacillus casei), is probably 5-methyl-FH4 (65), and since this compound represents the major form of folate in liver (109), it would appear reasonable to suggest that the liver and possibly the kidneys are responsible for the maintenance of this compound in the blood. Inasmuch as a deficiency in FH4 would result in a decrease in the formation of 5-methyl-FH4, the rapid fall in serum folates after amethopterin and the significant binding of this drug in the liver would imply that the liver enzyme is rapidly inactivated.  

A comparable reduction in serum folate activity would take over a month to achieve by dietary means (64). The early studies of Swendsen et al. (119) indicated that the administration of the folic acid antagonists to patients (with leukemia) resulted in the excretion of a greater amount of subsequently administered folic acid, as compared with the results obtained prior to treatment. In agreement with these findings, more recently Johns et al. (74, 76) have demonstrated that the rate of disappearance of folate-H3 in the plasma was slower in patients treated with amethopterin than the rate observed in subjects prior to treatment. These results suggest that amethopterin retards the uptake of folate into body cells and therefore results in a greater excretion of folate. This is probably not a displacement phenomenon, inasmuch as amethopterin was only moderately successful in displacing folate-H3 when given 6 hours after the radioactive folate; under these experimental conditions, “cold” folate displaced a significant amount of radioactivity (75).

Since it has been shown in mice (27, 45, 126), guinea pigs (15), and man (27) that amethopterin or aminopterin persists for months in liver and kidney, it was of interest to test the efficacy of some pteridines in displacing bound amethopterin-H3. Two dogs were given radioactive amethopterin, as indicated in Table 2, and a number of days later displacement of “bound” amethopterin with the substances indicated was tested by measurement of radioactivity in the urine. Control measurements of the radioactivity in 24-hour specimens of urine were made prior to the administration of the drugs. Folic acid, FH2, FH4, 5-methyl-FH4, and 5-formyl-FH4 did not displace significant amounts of radioactivity, whereas “cold” amethopterin did. These findings are in agreement with the concept that the reduced folates antagonize the effects of the folate antagonists by circumventing the induced block, rather than by competing for an enzyme site.

In conclusion, the in vivo studies reviewed here strongly support the hypothesis that dihydrofolate reductase is rapidly inactivated by amethopterin and that this inactivation persists for weeks; however, in vivo experiments such as these give only a...
general picture of what occurs in the whole body after the administration of methotrexate and cannot be used in the interpretation of events that occur in individual organs. Thus, despite the marked lowering of the level of serum folate produced by the folate antagonists, toxic effects may not necessarily result. This apparent contradiction is explained by the concept that, although the liver may be responsible for the production of the majority of circulating reduced folates, it is a non-replicating tissue, and inhibition of dihydrofolate reductase activity of this organ may not produce toxicity; on the other hand, analogous inhibition of the enzyme in bone marrow and intestinal mucosa may result in severe toxicity and death.

**TABLE 2**

**CAPACITY OF PTERIDINES TO DISPLACE "BOUND" AMETHOPTERIN-H³**

The dog in Exp. I was given 94 μc. of amethopterin-H³ subcutaneously (specific activity, 400 μc./μmole); the dog in Exp. II was given 166 μc. of amethopterin-H³ intravenously (specific activity, 15 μc./μmole). The "displacing" substances were given subcutaneously on the days indicated, after the administration of amethopterin-H³, except for FH₂, which was administered intravenously, as indicated in the legend in Table 3.

<table>
<thead>
<tr>
<th>DAYS AFTER AMETHOPTERIN-H³</th>
<th>DISPLACING SUBSTANCE</th>
<th>DOSE (μg.)</th>
<th>URINE RADIOACTIVITY (μCURIES/24 HOURS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Exp. I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>L-L-5 Methyl-FH₄</td>
<td>5</td>
<td>0.08</td>
</tr>
<tr>
<td>24</td>
<td>L-L-6 Methyl-FH₄</td>
<td>20</td>
<td>0.10</td>
</tr>
<tr>
<td>33</td>
<td>Amethopterin</td>
<td>20</td>
<td>0.14</td>
</tr>
<tr>
<td>Exp. II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>FH₂</td>
<td>25</td>
<td>0.05</td>
</tr>
<tr>
<td>12</td>
<td>N⁵-formyl-FH₄</td>
<td>20</td>
<td>0.01</td>
</tr>
<tr>
<td>24</td>
<td>Folic acid</td>
<td>20</td>
<td>0.02</td>
</tr>
<tr>
<td>29</td>
<td>Amethopterin</td>
<td>20</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Enzyme studies in vitro.*—Enzyme studies on tumor and normal tissue after drug therapy in man are limited, since, in most instances, serial sampling is not possible. Furthermore, quantitation of results may be difficult in solid tumors because of the marked variation in the composition of the cellular elements that is encountered frequently. The studies to be described below were performed on leukocytes obtained from normal and leukemic subjects. Leukocytes have the advantage of being easily obtained from whole blood as often as necessary; in addition, they can be separated easily from the other formed elements of the blood and can be accurately enumerated. Furthermore, bone marrow leukocytes may be used as a source of normal replicating cells, whereas peripheral leukocytes obtained from normal subjects represent a source of differentiated, nondividing cells. Normal and leukemic leukocytes consist of a mixture of immature cells (predominantly myeloid elements) and mature cells (predominantly polymorphonuclear cells).

Initial studies, carried out in collaboration with F. M. Huennekens, B. W. Gabnio, and R. Silber were concerned mainly with the identification and measurement of enzyme levels in the supernatant fraction of homogenates of leukocytes. A number of enzyme systems that utilize FH₄ as a coenzyme were identified and measured in normal and leukemic leukocytes: the formate-activating enzyme, serine hydroxymethylase, cyclohydrolase, N⁵,N¹⁰-methylene-FH₄ dehydrogenase (7, 8), and, more recently, folic acid isomerase. Generally, the enzyme activities are particularly well suited for use as a source of tissue in the study of folate metabolism and the mechanism of action of the folate antagonists, since leukemias both responsive and resistant (natural and drug-produced) are encountered in man.

It should be pointed out that the assumption is made in these studies that the leukemic leukocyte present in peripheral blood is representative of the leukemia cell found in the bone marrow or lymph node. "Normal" peripheral leukocytes, as isolated, consist of 80–90 percent polymorphonuclear leukocytes, the remainder of the cells being primarily lymphocytes. Leukocytes obtained from bone marrow of hematologically normal subjects are particularly well suited for use as a source of tissue in the study of folate metabolism and the mechanism of action of the folate antagonists, since leukemias both responsive and resistant (natural and drug-produced) are encountered in man.
levels of these enzyme activities were higher in acute and chronic granulocytic leukemia cells; similar results have been reported recently by Willmanns (131, 132). These enzyme systems all proved to be relatively insensitive to levels of the folate antagonists that might be expected to occur in vivo (10⁻³ moles/1 or less). After some difficulty dihydrofolate reductase activity was obtained in crude lysates from patients with acute leukemia¹¹ and chronic granulocytic leukemia, but not from leukocytes of normal subjects or subjects with chronic lymphatic leukemia (7, 14). The usual assay for this enzyme activity, involving the reaction shown in equation (1),

\[
FH_2 + TPNH + H^+ \rightleftharpoons FH_4 + TPN
\]  

(1)

based on the disappearance of \(FH_2\) and TPNH measured at 340 m\(\mu\) was not satisfactory in crude lysates because of the low enzyme activity observed (ca. one-tenth the activity of the formate-activating enzyme) and the endogenous reduction of TPN. These difficulties were overcome by measuring the \(FH_4\) formed by adding an excess of purified formate-activating enzyme formate and cofactors, as shown in equation (2). The

\[
\text{Formate} + FH_4 + ATP \xrightarrow{\text{MG}^{++} + \text{NH}_4^+} N^{10}\text{-formyl FH}_4 + ADP + P_i
\]  

(2)


N\(^{10}\)-formyl-FH\(_4\) formed was measured at 355 m\(\mu\) in acid-deproteinized extracts, since under these conditions the ring compound, \(N^4\), \(N^{10}\)-methenyl \(FH_4\), is formed (82, 94). This coupled assay system is twice as sensitive as the usual assay—\(\Delta E_{450}\) for equation (1) = 11,200 (86) and \(\Delta E_{350}\) for equation (2) = 25,900 (99). A. Cashmore in this laboratory has recently detected enzyme activity in leukocytes obtained from the bone marrow of subjects without leukemia.¹² The finding of dihydrofolate reductase in the white blood cells of patients with acute and chronic granulocytic leukemia, as well as in bone marrow leukocytes, is consistent with the capacity of these cells to synthesize DNA, as measured in studies of the incorporation of thymidine-H\(^4\) (18, 33). Other enzymes concerned with the synthesis of nucleic acids, including pyrimidine-synthesizing enzymes (112), thymidylate synthetase (108), and DNA polymerase (17), are increased in acute leukemic cells, as compared with the normal circulating leukocytes.

¹¹ A patient recently studied in collaboration with S. Finch and W. Rogoway with acute monocytic leukemia did not have measurable dihydrofolate reductase; the significance of this observation is not known.

¹² A. Cashmore, unpublished observations.

Studies of dihydrofolate reductase obtained from the leukocytes of patients with chronic granulocytic and acute leukemia showed that the enzyme has a pH optimum at 8.3, with a second small optimum noted at about pH 5.5. This enzyme activity was essentially specific for TPNH rather than DPNH, and activity produced with folate as the substrate occurred only below pH 7.0 and was less than 10 per cent of the activity produced with \(FH_2\) as the substrate (7, 14). When the inhibition produced by an equimolar amount of three folate antagonists—amethopterin, aminopterin, and dichloroamethopterin—was tested at pH 8.3, each caused significant inhibition at levels of 1 \(\times 10^{-3}\) M of interest was the finding that dichloroamethopterin was more effective on an equimolar basis than was amethopterin (cf. Table 2 [14]). Similarly, Misra et al. (86) reported that dichloroamethopterin is a more potent inhibitor of dihydrofolate reductase obtained from mouse leukemia cells (L1210), with the same assay conditions, but at a slightly lower pH (7.5). Attempts to demonstrate that this inhibition is “irreversible” or stoichiometric at this pH were unsuccessful; these findings suggested that the inhibition is mainly “reversible,” i.e., that the complex of enzyme and inhibitor is dissociable under the conditions of the assay. On the other hand, Werkheiser considered that the inhibition of this enzyme activity by the 4-amino analogs of folate is essentially “irreversible” or stoichiometric and equivalent—i.e., amethopterin, aminopterin, and dichloroamethopterin produced equivalent inhibition (125). These studies were done at pH 6.0 with folate used as the substrate and a supernatant fraction of a homogenate of rat liver as the enzyme source. Under similar assay conditions but with different enzyme sources, analogous results were obtained by Zakrzewski and Nichol (139) and by Peters and Greenberg (96). Although the possibility existed that these results could be explained by the different sources of the enzymes employed, it appeared more probable that these findings could be reconciled by the different assay conditions used by the investigators. With the knowledge that this enzyme activity in most tissues is stimulated by cations (6), the effects of the concentration of substrate, pH, and salt on enzyme activity, and the inhibition produced by the folate antagonists, have been studied (9). To obtain adequate amounts of purified enzyme, a resistant subline of the Ehrlich ascites cell was used as the enzyme source (10), since this enzyme activity was 15-fold higher than that found in the sensitive subline (no qualitative differences in enzyme properties were obtained when the en-
zyme activities from these two lines were compared). After this enzyme activity was purified from 50- to 100-fold, a study was made of the influence of pH on enzyme activity and of the inhibition produced by amethopterin; the results of this study are shown in Chart 2. Double pH optima were found when FH₂ was used as the substrate. When the inhibition produced by a fixed amount of amethopterin was measured as a function of pH, a sharp inhibition maximum was produced at pH 5.9. Similar studies carried out with folate as the substrate revealed a single pH optimum at 5.0. When the tightness of binding produced by amethopterin was investigated at the two pH optima with FH₂ used as the substrate in the manner suggested by Ackerman and Potter (1), the binding at pH 5.9 was tighter than was that at pH 7.6 (Chart 3). These studies have been confirmed by experiments involving the dialysis of enzyme to which amethopterin-H₄ was bound; when dialysis was performed at pH 7.5, in the presence of 0.15 M KCl, dissociation of the enzyme-inhibitor complex was almost complete; dialysis against Tris buffer, 1 X 10⁻⁵ M; pH 7.5, or against citrate buffer, 1 X 10⁻³ M, pH 5.9, with or without 0.15 M KCl, did not result in appreciable dissociation of the enzyme-inhibitor complex. It would appear, therefore, that not only the amount of inhibition produced but also the tightness of binding of this enzyme to the 4-amino analogs of folate are influenced by pH and salt concentration. The significance of these findings, when related to events occurring in vivo, will be discussed in a later section.

**Chart 2**—Inhibition of dihydrofolate reduction as a function of pH. The reaction was followed by measuring the decrease in absorption at 340 µm (6). Readings were taken at 1-minute intervals for 4 minutes. The complete system contained buffer, 100 µmoles; KCl, 150 µmoles; TPNH, 0.06 µmole; FH₂, 0.08 µmole; purified ascites cell enzyme, 0.008 mg.; and 3-mercaptoethanol, 10 µmoles, in a final volume of 1.0 ml. O, citrate buffer; ●, Tris buffer; ■, phosphate buffer.

**Chart 3**—Inhibition by amethopterin of the reduction of FH₂ (Ackermann and Potter analysis [1]). Increasing amounts of enzyme were incubated with and without 1.1 X 10⁻⁸ M amethopterin at pH 5.9 and 7.6, respectively. The assay conditions were similar to those described in Chart 2.
II. THE "INDUCTION" OF DIHYDROFOLATE REDUCTASE

The identification of dihydrofolate reductase activity in leukemic leukocytes allowed a study to be made of the enzyme activity after the administration of amethopterin to patients. A rapid rise in enzyme activity to levels up to sixteen-fold greater than pretreatment levels was noted in patients with acute leukemia treated conventionally with amethopterin (11). These events did not occur when patients were treated with other chemotherapeutic agents (e.g., prednisone, 6-mercaptopurine, busulfan, and 5-fluorouracil).

Further investigation of this drug-induced rise in nonleukemic patients indicated that the enzyme activity appeared in normal leukocytes, and also in erythrocytes, after the administration of methotrexate (12, 16).

In order to study the kinetics of this "induced" rise in more detail, measurements of erythrocyte and leukocyte dihydrofolate reductase activity were performed in patients with and without hematologic disease after a single 20-mg. intravenous dose of methotrexate (12). Data obtained from a subject treated in this manner are shown in Chart 4. Measurable dihydrofolate reductase activity appeared in the leukocytes at about day 2, rapidly increased to a peak value at day 8, and declined rapidly during the next 2 weeks. Erythrocyte dihydrofolate reductase activities determined in a normal subject treated with amethopterin in a similar manner are shown in Chart 5. Of interest is the slow fall of enzyme activity observed. In a similarly treated patient with chronic granulocytic leukemia and an elevated leukocyte count, amethopterin was detected in the leukocyte lysate, paralleling the increase in measurable dihydrofolate reductase activity (12).

The dog has served as a useful model for the further investigation of this phenomenon. The substrate for this enzyme, folate, and FH4 did not cause a significant increase in leukocyte dihydrofolate reductase activity; nor did FH4,N5-formyl FH4, and 5-methyl-FH4. When aminopterin, amethopterin, and dichloroamethopterin were tested, each caused an increase in enzyme activity. Pyrimethamine also initiated an increase in enzyme activity. Pyrimethamine also initiated an increase in enzyme activity.

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14 Daraprim (2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine). This compound is a potent inhibitor of dihydrofolate reductase activity at concentrations of $10^{-4}$ to $10^{-2}$ M, with guinea pig liver or bone marrow used as the enzyme source (unpublished observations).
folate reductase activity, while 4-amino-pteroylglutamic acid did not. These results are summarized in Table 3. The presence of amethopterin in dog leukocytes, proportional to the level of "induced" dihydrofolate reductase, was also demonstrated by use of amethopterin-H^4 (Chart 6). These results confirm the findings obtained in the patient with chronic myelocytic leukemia and demonstrate that amethopterin-H^4 may be useful as a label in the study of the life span and kinetics of formation and disposition of leukocytes.

Detailed observations on two patients with acute leukemia treated with methotrexate are presented in Charts 7 and 8. Patient 1 (Chart 7) has a rapid response to the drug, given orally; within 5 days after initiation of treatment the leukocyte count decreased to below 5000 and was kept between 2000 and 3000 cells per cu. mm. by maintenance therapy with amethopterin. This child subsequently had a complete remission that lasted 6 months. Leukocyte dihydrofolate reductase activity rose rapidly after the first dose of amethopterin, paralleled by an equally rapid increase of intracellular amethopterin. These measurements were not continued after the 4th day, since the percentage of lymphoblasts, as well as the leukocyte count, had markedly decreased. In contrast to this dramatic response to amethopterin, patient 2, an adult male with acute leukemia, showed only a slight, transitory decrease in the leukocyte count after administration of amethopterin (Chart 8). Although therapy with methotrexate resulted in a rise in enzyme activity in the leukocytes of this subject, the increase began more slowly and was associated with the entrance of less amethopterin into the cells than occurred in the other patient. The drug was stopped after 5 days because of gastrointestinal toxicity; nevertheless, both enzyme activity and intracellular methotrexate increased during the next 4 days. Reinstitution of therapy resulted in a slight further decrease in the leukocyte count, but the percentage of myeloblasts in the peripheral blood remained unchanged (80-95 per cent). Therapy was discontinued again because of toxicity, but enzyme activity, as well as intracellular amethopterin, continued to rise, as measured 7 days later, after which both enzyme activity and intracellular methotrexate declined slowly.

The observation that the administration of

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**TABLE 3**

"INDUCTION" OF DIHYDROFOLATE REDUCTASE ACTIVITY IN LEUKOCYTES OBTAINED FROM DOGS TREATED WITH VARIOUS PTERIDINES

Leukocytes were isolated, lysed, and assayed for dihydrofolate reductase activity, as previously described (14). Pyrimethamine was dissolved in a water-ethanol mixture and administered intravenously; FH^4 also was administered intravenously in a 4-ml. volume containing 5 mg. of sodium ascorbate per ml. The other substances were given subcutaneously.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Does</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg.)</td>
</tr>
<tr>
<td>Folic acid</td>
<td>20</td>
</tr>
<tr>
<td>FH^4</td>
<td>20</td>
</tr>
<tr>
<td>dL-N'formyl-FH^4</td>
<td>20</td>
</tr>
<tr>
<td>L-N'methyl-FH^4</td>
<td>20</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>5</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>5</td>
</tr>
<tr>
<td>Dichloroamethopterin</td>
<td>10</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>6 Days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid 20</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>FH^4 20</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>dL-N'formyl-FH^4 20</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>L-N'methyl-FH^4 20</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Amethopterin 5</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>Aminopterin 5</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>Dichloroamethopterin</td>
<td>0.00</td>
<td>0.18</td>
</tr>
<tr>
<td>Pyrimethamine 100</td>
<td>0.01</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Dog leukocytes usually have measurable levels of dihydrofolate reductase activity (range, 0.01-0.04 μmole/hr/mg), in contrast to leukocytes from normal humans (14).
methotrexate causes an appearance of dihydrofolate reductase activity in normal leukocytes and erythrocytes has been interpreted in the following manner (12). Circulating amethopterin is taken up by immature bone marrow leukocytes, and the since they would not be affected by this antagonist.

These considerations do not apply to the cells found in acute leukemia, because these neoplastic cells apparently lack the ability to differentiate.

**Chart 6.**—Leukocyte dihydrofolate reductase activity and radioactivity in a dog administered 166 μc. of amethopterin-H³ (15 μc/μmole).

Drug is retained within these cells; as a consequence of this binding, enzyme activity is "induced" in those cells that survive and continue to differentiate. When these cells emerge into the peripheral blood a measurable enzyme activity is found, whereas ordinarily this enzyme activity would be essentially undetectable. These events allow the suggestion that certain immature bone marrow cells are affected by this inhibitor in such a manner that enzyme activity remains throughout the life of the cells. In essence, a "biochemical" label is produced, comparable to tagging leukocytes with thymidine-H³ or erythrocytes with Fe⁵⁹ in vivo. These events are apparently unique to blood cells; a recent study of dihydrofolate reductase activity in guinea pig liver, kidney, and intestinal mucosa, after the administration of amethopterin, did not demonstrate a significant rise in enzyme activity (15). Normal bone marrow probably does not become refractory to repeated courses of amethopterin, because the cells that incorporate a nonlethal amount of amethopterin and, as a result, develop increased levels of dihydrofolate reductase continue to differentiate and after a normal life-span subsequently disappear.

The increased level of enzyme confers no added advantage to bone marrow cells, except possibly for 1 or 2 days while they are in the bone marrow and continue to replicate; once they reach a stage at which they are not capable of further division, an increased enzyme level would be of no value,
The rapid fall in the leukocyte count observed in patient 1 (Chart 7) after therapy with methotrexate suggests that this type of cell replicated rapidly—i.e., the cell has a short life span (1–3 days). A decrease in the synthesis of DNA caused by the administration of methotrexate consequently could have caused cell death. The cells that did not take up a lethal amount of methotrexate and remained in the peripheral blood developed an increase in enzyme activity in proportion to the amount of bound drug. As amethopterin therapy was continued these cells incorporated a lethal amount of drug. This hypothesis assumes that these cells had no difficulty in transporting amethopterin; that this is true was suggested by the rapid appearance of high levels of methotrexate in these cells after the administration of the drug and also by experiments performed in vitro by G. A. Fischer, discussed in a later section.

The studies performed on the patient with acute myeloblastic leukemia shown in Chart 8 suggest that, after the first dose of amethopterin, the leukemia cells of this patient contained less drug (per liter of leukocytes) than did those of the previous patient. The cells from this patient probably had a longer life span—i.e., they replicated less often than did the cells of the patient described in Chart 7. This conclusion is based on the continued increase in the concentration of intracellular amethopterin, as well as the continued rise in dihydrofolate reductase activity after administration of the drug was stopped. Some additional support for this contention is afforded by the relatively slow rise in leukocyte count prior to therapy, and the gradual decrease in leukocyte count produced as a result of therapy. The increase in enzyme activity and amethopterin content of the cell for a period of time after the drug was stopped probably means that in this subject the bone marrow was the source of some of the circulating myeloblasts and that these cells were formed in the bone marrow subsequent to release.

If only a small number of leukemia cells from this patient were undergoing cell division at any one time, and since amethopterin produces cell death by virtue of this capacity to prevent the synthesis of DNA, it is reasonable to suggest that in this patient only a small proportion of the leukemia cells were affected by short-term therapy with amethopterin; thus, this situation would result in a slow decrease in the leukocyte count as was observed. Whether or not the lower concentration of amethopterin present in the cells from this patient 24 hours after therapy, as compared with

---

**Chart 8.**—The "induction" of dihydrofolate reductase activity in the leukocytes of a patient with acute myeloblastic leukemia treated with amethopterin.
that in the previous patient, was a reflection of the small number of cells in the DNA-synthesizing phase, probably can be solved by autoradiography with amethopterin-\textsuperscript{3}.\textsuperscript{18}

III. "INDUCED" ENZYME ACTIVITY

AS A FUNCTION OF pH

Studies of the inhibition and binding by amethopterin become significant when considered in light of the "induction" phenomenon. It should be emphasized that the measurements of enzyme activity on crude leukocyte lysate preparations, as shown in Charts 4, 5, 6, 7, and 8, were made by the coupled dihydrofolate reductase-formate activating enzyme system (equations 1 and 2) at the pH optimum for leukocyte dihydrofolate reductase—i.e., 8.3. Since this may not be the pH of the cell studied or, more correctly, the pH of the enzyme within the cell, the measurements of enzyme activity performed \textit{in vitro} may not reflect the actual enzyme activity within the cell. As an approach to this problem, studies of enzyme activity as a function of pH in leukocytes from patients treated with amethopterin have been carried out. The separation of enzyme activity from amethopterin in a leukocyte homogenate obtained from a patient with chronic myelocytic leukemia given amethopterin has been reported (12). In a recent experiment, the leukocytes from a patient with acute lymphatic leukemia in relapse after 6 months of continuous treatment with methotrexate were separated from the other formed elements of the blood, lysed, and dialyzed overnight against distilled water; amethopterin bound to the enzyme was not removed by this procedure. The dialysate was then chromatographed on DEAE-cellulose (Table 4), in a manner previously described (12). This procedure permitted the separation of enzyme activity from inhibitor\textsuperscript{18} and resulted in an increase in total enzyme activity (Table 5), a situation that would be expected if some of the enzyme activity were inhibited by amethopterin. Activity as a function of pH of the bound enzyme (prior to DEAE cellulose chromatography) was compared with the amethopterin-free enzyme (Tubes \#3 and 4 after DEAE cellulose chromatography). DEAE cellulose chromatography resulted in a tenfold concentration of the enzyme, as measured at pH 8.3; however, when activity also was measured at pH 5.9, 6.6, and 7.6, before and after chromatography (Table 5), a 90-fold concentration of the activity was attained at pH 5.9, whereas at pH 6.6 only a 26-fold concentration of the enzyme activity resulted. These results indicate that 9 times as much inhibition occurred, as measured at pH 5.9, than that measured at pH 7.6 or 8.3, whereas assay of the enzyme at pH 6.6 indicated that only about 2\frac{1}{2} times as much inhibition occurred as that measured at pH 7.6. The implications of these findings will be obvious: if dihydrofolate reductase is func-

### TABLE 4

<table>
<thead>
<tr>
<th>Step</th>
<th>Total amethopterin ((\mu\text{moles}))</th>
<th>Total enzyme activity ((\mu\text{moles/hr}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dialyzed enzyme</td>
<td>1.8(\times)10(^{-4})</td>
<td>2.9</td>
</tr>
<tr>
<td>2. DEAE chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005 M KCl eluate</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.15 M KCl eluate (tubes 3 &amp; 4)</td>
<td>0.0</td>
<td>7.8</td>
</tr>
<tr>
<td>0.5 M KCl eluate (tubes 3 &amp; 4)</td>
<td>1.5(\times)10(^{-4})</td>
<td>0.0</td>
</tr>
</tbody>
</table>

### TABLE 5

<table>
<thead>
<tr>
<th>pH</th>
<th>Amethopterin-bound enzyme (dialyze)</th>
<th>Amethopterin-free enzyme (0.15 M KCl eluate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>specific activity—(\mu\text{moles/hr/mg})</td>
<td></td>
</tr>
<tr>
<td>5.9</td>
<td>0.04</td>
<td>3.6</td>
</tr>
<tr>
<td>6.6</td>
<td>0.16</td>
<td>3.6</td>
</tr>
<tr>
<td>7.6</td>
<td>0.40</td>
<td>5.1</td>
</tr>
<tr>
<td>8.3</td>
<td>0.73</td>
<td>7.6</td>
</tr>
</tbody>
</table>

\textsuperscript{18} It is conceivable that amethopterin is transported by a mechanism ordinarily used for the transport of folates; it is also reasonable to speculate that this transport mechanism may operate only during the period of time when the cell is synthesizing DNA.

\textsuperscript{16} The separation of inhibitor from chicken liver dihydrofolate reductase also has been reported by Matthews (in [71]), who employed chromatography on hydroxylapatite.
tioning in a cell at pH 5.9, the activity may be almost completely inhibited by a finite amount of amethopterin, whereas, if the pH at the enzyme site is higher—e.g., 8.9—the enzyme activity will require more amethopterin to cause equivalent inhibition. Since the binding is less tight at the higher pH, this may mean that less drug would be retained and higher intracellular concentrations of amethopterin would be difficult to attain unless the drug were given continuously. These experiments also provide an explanation for the capacity of dihydrofolate to reverse the toxic effects of amethopterin (88). If one assumes that the pH of the cell (bone marrow or intestinal mucosa cell) is approximately 6.9, then some reversal of the inhibition by large doses of dihydrofolate could be obtained, since this inhibition is partially competitive in vitro (9). Although knowledge of the manner of transport of this drug into the cell and the occurrence of nonenzyme binding could modify these considerations, studies of the capacity of amethopterin to cross the semipermeable membranes and to be retained within the cell should take into account the characteristics of enzyme binding within the cell.

IV. STUDIES CONCERNED WITH THE PREDICTION OF RESPONSIVENESS TO AMETHOPTERIN OF PATIENTS WITH LEUKEMIA

Although the factors that determine whether or not a patient with neoplastic disease will respond to a drug obviously are complex, some factors can be listed, in the light of the previous discussion, that may be concerned with the failure of amethopterin to affect neoplastic cells: (a) either poor transport of the drug into the neoplastic cell or inability of the cell to retain the drug, or both; (b) a high level of dihydrofolate reductase activity per cell; (c) an enzyme that is not "irreversibly" inhibited by amethopterin; (d) an alkaline pH of intracellular dihydrofolate reductase (in essence a cause for [e]); (e) the capacity of the neoplastic tissue, as compared with normal tissue, to utilize either available reduced folates or performed purines, or thymidine or DNA, or any combination of these; and (f) the capacity of the cell to inactivate the drug.

It is realized that this is only a partial list of the possible causes for natural resistance to the folate antagonists; however, almost all these possibilities can be subjected to experimental test. It will be apparent that, if one of these mechanisms becomes operative neoplastic cells previously sensitive to methotrexate, refractoriness to the drug may result. Thus, the mechanisms of natural resistance may be similar to those that develop in sensitive cells, and this may be responsible for acquired resistance.

It should be emphasized that responsiveness to a chemotherapeutic agent is a relative term and may depend in part on the method of administration, dosage used, and ability of the patient to tolerate the drug. For example, subclinical folate deficiency may predispose a patient to excessive toxicity from the folate antagonists (5, 63, 118), thus limiting the amount of drug that can be given. Such a deficiency may be dietary or may result from excessive demand for folate because of rapid proliferation of tissue (67, 69, 118). Oral administration of amethopterin may be associated with delayed absorption in some patients, with resultant increased toxicity, probably as a result of sustained blood and tissue levels (48). Other proposed causes of unexpected toxicity have been poor renal excretion of amethopterin (even with a normal level of urea nitrogen in the blood) and prior administration of testosterone (32).

As an approach to developing predictive tests for determining the sensitivity of acute leukemia in man to amethopterin, our group at New Haven (G. A. Fischer, R. Zanes, D. Clement, P. Calabresi, A. D. Welch, and S. Finch), in collaboration with M. Lyman and J. Burchenal of the Sloan-Kettering Institute in New York, has attempted to carry out a series of tests in patients treated with methotrexate. Thus, 24 hours prior to treatment, blood is obtained from the patient (in the fasting state for a determination of the level of serum folates), and leukocytes are isolated for studies of dihydrofolate reductase activity and studies of in vitro transport of amethopterin-H3. Blood is obtained again, and the studies are repeated prior to the initiation of oral therapy with methotrexate (10 mg. for adults; 5 mg. for children); then, after 2, 4, 6, and 24 hours, respectively, the levels of amethopterin in the serum and cells as well as dihydrofolate reductase enzyme activity in the leukocytes are determined. Treatment with methotrexate is continued for a minimum of 5 consecutive days. To obtain a significant number of leukocytes to carry out the above studies, patients with elevated leukocyte counts, with over 70 per cent characteristic blast forms present in the peripheral blood, have been studied. Although some studies have been done with leukocytes isolated from aspirated bone marrow, the lack of adequate numbers of cells and the difficulty in obtaining serial samples has greatly

limited the value of this approach. In approximately seven patients sufficient data have been obtained to allow analysis. These results should be interpreted with a consideration of the uncontrolled variables characteristic of clinical research e.g., the necessary administration of other drugs, the possible heterogeneity in what is called acute leukemia, and the different status of each patient's disease i.e., early or late. With these reservations in mind, some tentative conclusions can be drawn.

1. The measurement of dihydrofolate reductase activities alone may not be of value as an in vitro assay of drug sensitivity. The manner in which this measurement is expressed (i.e., per cell, per mg. of protein, etc.) is complicated by the differences in cell sizes and in the apparent ratios of size of nucleus to that of cytoplasm, which are observed in different types of malignant cells. To illustrate this point, cells obtained from children with acute lymphoblastic leukemia are small as compared with cells obtained from patients with acute myeloblastic or monocytic leukemia; consequently, the amount of soluble protein recovered per 10^9 cells would be less in the former instance. Thus, although the level of enzyme is similar in these two cells (expressed on the basis of milligrams of soluble protein), the acute lymphoblastic leukemia cells have less enzyme per cell. Table 6 shows the level of this enzyme activity before treatment and the subsequent response of patients to amethopterin.

2. The finding that an active transport process for amethopterin exists for murine L5178Y cells maintained in tissue culture and, further, that a defective transport system is associated with a 70-fold increase in the resistance to amethopterin (42) indicated that similar studies would be of interest in human leukemia. By use of technics developed in the study of transport of amethopterin in murine leukemia cells, preliminary studies performed by G. A. Fischer in this department have shown that an active (energy-dependent) transport system for amethopterin is present in human leukemic leukocytes; furthermore, study of the initial rates of uptake of the drug have varied

### TABLE 6

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Dihydrofolate Reductase</th>
<th>Cell Amethopterin</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (F)</td>
<td>65</td>
<td>AML</td>
<td>0.012</td>
<td>1.5</td>
<td>1+</td>
</tr>
<tr>
<td>2 (M)</td>
<td>45</td>
<td>AML</td>
<td>0.019</td>
<td>4.5</td>
<td>1+</td>
</tr>
<tr>
<td>3 (M)</td>
<td>25</td>
<td>ALL</td>
<td>0.070</td>
<td>5.0</td>
<td>1+</td>
</tr>
<tr>
<td>4 (M)</td>
<td>48</td>
<td>AML</td>
<td>0.026</td>
<td>7.5</td>
<td>3+</td>
</tr>
<tr>
<td>5 (M)</td>
<td>71</td>
<td>Reticular-cell leukemia</td>
<td>0.011</td>
<td>8.0</td>
<td>1+</td>
</tr>
<tr>
<td>6 (M)</td>
<td>6</td>
<td>ALL</td>
<td>0.081</td>
<td>9.5</td>
<td>4+</td>
</tr>
<tr>
<td>7 (F)</td>
<td>21</td>
<td>ALL</td>
<td>0.028</td>
<td>16.5</td>
<td>4+</td>
</tr>
</tbody>
</table>

*Response was graded as follows: 1+, decrease in WBC, but not below 10,000/cmm; 2+, decrease in WBC to below 10,000/cu. mm, but abnormal forms still present; 3+, disappearance of abnormal forms with subsequent increase in erythrocytes and platelets; 4+, complete remission.

†Patient put on steroids 7 days after start of therapy because of bleeding.
the cell to retain this compound. The results summarized in Table 6 indicate that some correlation may exist between clinical responsiveness and high levels of intracellular amethopterin.

It should be emphasized again that these findings are preliminary and are presented here to indicate the feasibility of carrying out studies of this type in patients with acute leukemia. The relationship of other factors to natural resistance may also prove to be of importance. Cells with a long intermitotic time may be resistant by virtue of limited uptake of amethopterin. Another important consideration is the distribution of the leukemic cells in the organs of the body, since the folic acid antagonists may not reach certain areas, especially portions of the central nervous system, in adequate concentrations. Thus, natural or acquired resistance in some instances might be attributable to the persistence of leukemia cells in those areas impermeable to amethopterin (123). Finally, the capacity of leukemia cells to utilize preformed nucleosides, or conceivably even intact DNA, consequent by-passing the block imposed by amethopterin, should be mentioned. These possibilities deserve further investigation.

V. ACQUIRED RESISTANCE TO THE FOLIC ACID ANTAGONISTS

The apparent cure of a significant number of women with malignant trophoblastic tumors treated with methotrexate (67), and the occasional long-term remissions obtained in children with acute leukemia treated with this drug, encourage the hope that, with more understanding of the origins of drug resistance, improvement in the duration of remissions and perhaps even cures may be possible, at least in those malignancies that can respond to amethopterin. A sobering thought, with respect to the "cure" of choriocarcinoma, is the possibility that, in addition to the large doses of amethopterin that can be tolerated by these young females and in addition to the availability of urinary choriongonadotropin assays to determine tumor activity, is the possibility that, since these tumors are of fetal origin, some immunologic response "turns the tide" and results in complete eradication of the tumor cells.

The question must be asked: why do apparently sensitive tumors return even after apparently complete regression (as far as can be determined) after therapy with amethopterin or, for that matter, other chemotherapeutic agents? The emergence of resistant cells in a previously successfully treated patient implies that (a) all the malignant cells were not reached by sufficient concentration of drug to be lethal to the cell, thereby allowing certain adaptive changes in the cell to take place that result in drug resistance; (b) some mutant cells exist, although few in number, that are naturally resistant to the drug (perhaps by a mechanism discussed in IV), which by virtue of the selective pressure of the chemotherapeutic agent continue to increase in number, finally resulting in a resistant population of cells; or (c) the malignant cells may be completely eradicated—however, since the basic cause has not been removed (e.g., virus, metabolic deficiency), therefore, the tumor recurs. In this situation resistance would be produced by another mechanism, possibly a host response.

Some evidence exists for the first of these possibilities. Amethopterin does not penetrate into tissues readily, in particular, the central nervous system (130). Accordingly, the opportunity exists, especially in the acute leukemias, in which neoplastic cells may be found in the central nervous system, for sublethal concentrations of amethopterin to exist. In humans, the single biochemical event that has been found to occur in leukemia cells after administration of amethopterin has been the rise in dihydrofolate reductase activity (11, 16). That this increase probably does not result from cell selection has been shown by the rapidity with which this change occurs and in instances not associated with selective pressure—i.e., the destruction of most sensitive cells. These findings are complicated by similar events that occur in normal leukocytes and erythrocytes after amethopterin is given to subjects, but this response, even with doses of amethopterin that do not significantly decrease leukocyte or erythrocyte production, may be used to support the argument that this phenomenon does not represent selection. The relation of these events to drug resistance is not entirely clear. As indicated in an earlier section, the pH at which the enzyme carries out its activity will affect the amount of product formed in the presence of inhibitor; therefore, although an increase in enzyme activity as assayed in vitro has been unequivocally demonstrated, the amount of uninhibited enzyme actually present in these leukocytes is not known, since this would require knowledge of conditions that obtain in vivo. If one makes the assumption that the intracellular pH of the milieu in which dihydrofolate reductase operates is above 6.5, and appropriate cofactors are available to the enzyme, the activity of the "induced" enzyme would be greater than the enzyme activity per cell assayed prior to treatment and thus could account for amethopterin resistance. Natural resistance could be explained in

18 J. Burchenal, personal communication.
terms of the pH of the enzyme within the cell; if high, the cell would not be susceptible to routine amethopterin therapy. Acquired resistance also could involve an intracellular change in pH such that the enzyme is no longer completely inhibited by amethopterin. This hypothesis would explain the ineffectiveness of subsequent courses of amethopterin even after dihydrofolate reductase activity has returned to pretreatment levels.

The second possibility listed, i.e., selection of genetically stable mutants, while not demonstrated to occur as yet in human resistance, should not be discounted. Ample experimental evidence exists in bacteria (3, 88, 89), as well as in murine neoplasms propagated in vitro (41, 42, 60), as well as in vivo (42, 86), which indicates that resistance to amethopterin may arise from the emergence of genetically stable drug-resistant mutant cells. Two mechanisms in particular have been shown to be responsible for resistance in bacteria (19, 87, 88, 97, 134), as well as in murine leukemia (10, 41, 42, 58, 60, 86): (a) defective transport of amethopterin and (b) an increase in dihydrofolate reductase activity. In contrast to the studies in humans, however, this latter event has been shown to result from genetically stable changes that are attributable to a different mechanism. Although it may be argued that the extrapolation from bacteria and murine leukemias (both in culture and as transplanted tumors) to human disease may be considerable, it is difficult, nevertheless, to imagine that these events cannot take place in human malignancies—if not in leukemia cells, at least in solid tumors that become resistant. Other mutations that might result in amethopterin resistance also should be considered (88, 123). Among these are (a) the capacity of the resistant cell to inactivate amethopterin, (b) an altered enzyme with less affinity for amethopterin or greater affinity for FPs, (c) increased concentration of a metabolite pathway by-passing the metabolite, and (d) utilization of exogenous metabolites that are products of the inhibited reactions. Although not all these possibilities have been studied in drug-resistant populations from patients with acute leukemia, the first two possibilities have not been found in a number of patients studied (11).

As pointed out in a review by Welch (123), other factors that are concerned with the metabolic transformation and physiological disposition of the drug should not be overlooked as causes for drug resistance. The apparent incapacity of normal tissues (i.e., bone marrow and intestinal mucosa) to develop resistance to the folate antagonists, however, afford evidence against a significant change in metabolic alteration or excretion pattern occurring when refractoriness develops (20). Studies of the urinary excretion of amethopterin in sensitive and resistant subjects have not revealed any change in the disposition of this drug (20). A study of patients who developed toxicity after prolonged oral administration of amethopterin indicated that this increase in host toxicity might result from slower absorption, resulting in persistent blood levels (48). Although this change might lead to relative resistance, since less drug would be tolerated, the observations that resistance can occur without a change in drug tolerance, and also in patients treated parenterally with amethopterin, suggests that other factors are usually involved when amethopterin loses its effectiveness.

VI. FUTURE CONSIDERATIONS

The purpose of this paper has been to indicate how knowledge of the mechanism of action of the folate antagonists, at present confined to leukemia cells in man, might be used to develop predictive tests for assessment of the responsiveness to methotrexate and the development of drug resistance. Amethopterin is a potent anticancer agent when the conditions are "right," i.e., (a) if the inhibitor is brought to the cell in high enough concentration, (b) if the drug can cross the cell membrane, and (c) if the conditions for inhibition and binding to dihydrofolate reductase are optimal. Furthermore, the sensitive cell should depend on synthesis of thymidylate de novo and perhaps also on that of purines and should have a short generation time. It would seem that future progress in the development of newer folate antagonists should take into account the possible reasons why this drug does not work in certain types of neoplastic disease. Thus, tumors that are refractory because of defective transport may respond to analogs that are better transported across cell membranes (138) or to induced dietary deficiency of folic acid (62, 98, 102); tumors refractory because of conditions unfavorable to irreversible binding of dihydrofolate reductase by amethopterin may respond to other analogs with different binding properties or to continuous drug administration. Attempts to develop antifolates

18 Hakala has recently suggested that increased levels of dihydrofolate reductase may arise, in amethopterin-resistant Sarcoma 180 cells grown in culture, by virtue of a selection of cells having the capacity to induce high levels of enzyme (38).

20 Some evidence for limited resistance developing in normal rats has been reported (101). Another possible example of resistance occurring in a non-neoplastic cell is the development of refractoriness of some patients with psoriasis to aminopterin (100).
with more selective properties, i.e., inhibition of specific enzymes utilizing FH4, have been initiated (4, 77, 84, 111). The pharmacologic problem of obtaining high concentrations of drug at all tumor locations should not be overlooked. It is well known that solid tumors previously treated with either x-ray or surgery, or both, respond less well to chemotherapy, probably because of poor blood supply (114). Welch (123) has called attention to the poor transport of the antagonists of folate into the central nervous system and has advocated initial intrathecal treatment of subjects with acute leukemia. Attempts to develop compounds that are better transported across lipide membranes have been initiated (35).

Clearly, a better understanding of the mechanisms of drug resistance will be of considerable importance. If resistance is shown to occur by virtue of mutant cells spontaneously arising, the use of combination chemotherapy, in particular combinations of drugs that do not give additive host toxicity, will be an important therapeutic approach. If “induction” of dihydrofolate reductase does indeed cause resistance, attempts to block “induced” dihydrofolate reductase activity, or perhaps to use this rise in enzyme activity to advantage (50), should be of much interest.

Finally, although the studies in man that have been described initially have used leukemic leukocytes, it should be possible to extend these observations to solid tumors.11 Although it may be pointed out that no new significantly improved folate antagonist has been found since the discovery of aminopterin, this does not justify pessimism, since we are just beginning to appreciate how these drugs should be used and what we are looking for in developing new drugs.

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