FOLIC ACID ANTAGONISTS IN CANCER CHEMOTHERAPY

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TABLE OF CONTENTS

ABBREVIATIONS

AICAR	aminoimidazole carboxamide ribotide
CF	citrovorum factor (leucovorin, 5-formyl tetrahydropteroylglutamic acid)
CNS	central nervous system
CSF	cerebrospinal fluid
DCMTX	3′,5′-dichloromethotrexate
DNA	deoxyribonucleic acid

DON	6-diazo-5-oxo-L-norleucine
DPN^+	oxidized form of DPNH
DPNH	diphosphopyridine nucleotide
FA	folic acid (pteroylglutamic acid)
FAH_2	dihydrofolic acid
FAH_4	tetrahydrofolic acid (5,6,7,8-tetrahydropteroylglutamic acid)
FGAR	formyl glycinamide ribotide
GAR	glycinamide ribotide
GTH	chorionic gonadotropin, *i.e.*, gonadotropic hormone
6-MP	6-mercaptopurine
MTX	methotrexate (amethopterin, 4-amino-10-methylpteroyl-glutamic acid)
PABA	*para*-aminobenzoic acid
TPN^+	oxidized form of TPNH
TPNH	triphosphopyridine nucleotide

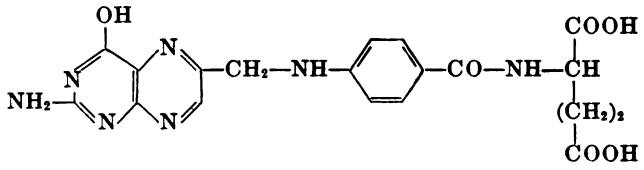
PART I. FOLIC ACID ANTAGONISTS—GENERAL DISCUSSION

I. INTRODUCTION

The chemical processes of life take place continuously in all living organisms and are brought about by special proteins, the enzymes. These react with food substances, and with small molecules arising from these substances which are termed *metabolites*. The reactions can be blocked in some instances by certain chemicals which displace metabolites from enzymatic reactions, and thus interfere with biochemical processes. Such chemicals are termed *antimetabolites* or *metabolic antagonists*. They may be recognized by the fact that their inhibitory effect is specific for certain reactions and is *reversed* either by supplying a large excess of the metabolite or by adding the normal product or products of the reaction. Woolley has discussed many aspects of antimetabolites in a book which deals exclusively with this subject (290).

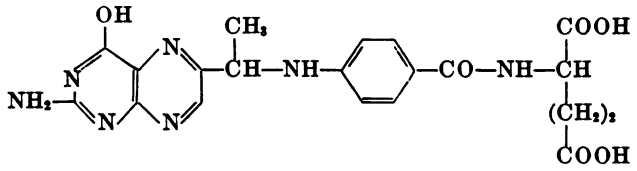
Antimetabolites are chemotherapeutic substances if they inhibit undesired growth, such as that of bacteria or cancer cells, without unduly harmful effects to the host.

Some of the coenzymes, including certain vitamins, are metabolites which are present in only very small amounts in living tissues and may be displaced by correspondingly small doses of suitable antimetabolites. These latter substances are in some cases very similar in molecular structure to the related metabolites. In other cases, although quite different in total structure, they may contain a chemical group which is similar to a corresponding group in the metabolite that attaches itself to the active region of the enzyme. For example, folic acid (FA) (I)



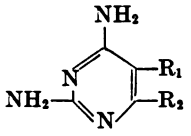
I

is blocked from its growth-promoting action in lactic acid bacteria, such as *Lactobacillus casei*, by 9-methyl folic acid (II)



II

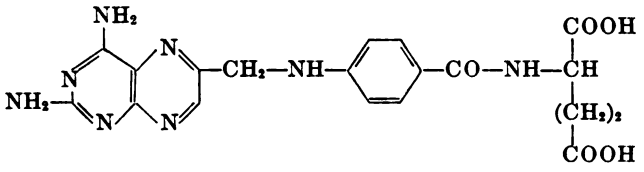
which is identical with FA except for the presence of an additional methyl group. The blocking effect is also produced by 2,4-diaminopyrimidines (III);



III

in both cases the inhibition of *Lactobacillus casei* is reversed by FA. The 2,4-diaminopyrimidines, such as pyrimethamine (R_1 = *p*-chlorophenyl; R_2 = ethyl), although much different from FA and possessing a smaller molecule, are thought to compete with folic acid by displacing its 2 - amino - 4 - hydroxypyrimidine group from a postulated attachment to certain enzymes, notably *dihydrofolic reductase* (3).

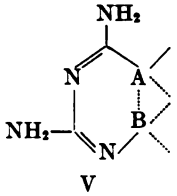
An important series of FA derivatives is represented by aminopterin (IV) and its congeners.



IV

This series may be regarded as diaminopyrimidines that closely resemble FA. They are powerful inhibitors of dihydrofolic reductase and of other FA enzyme systems. It is this series that is the most useful among the FA antagonists in cancer chemotherapy.

The FA antagonists ("antifolics") may be conveniently divided into two classes. The first of these, "class 1 antifolics," contain the group V



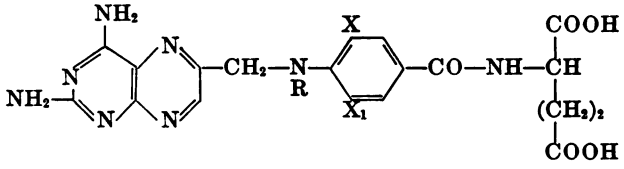
V

where A· · · ·B is C═C, N—C, or C═N. The other antifolic compounds, such as 9-methyl folic acid, may be placed in class 2; they are of little or no interest in cancer chemotherapy.

Class 2 consists of folic acid analogs which contain the usual 2-amino-4-hydroxypteridine ring. They differ from folic acid in containing one or more additional methyl groups in the side chain, or in having another amino acid, such as aspartic acid, in place of glutamic acid. They have reversible antifolic properties for some experimental animals, such as rats, and they inhibit reversibly the growth of lactic acid bacteria, but they are ineffective against neoplastic diseases.

II. CLASS 1 ANTIFOLICS

Aminopterin, methotrexate, halogenated methotrexates, and other aminopterin derivatives (VI)



VI

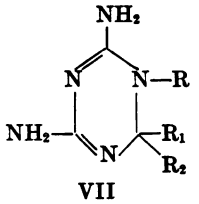
Aminopterin,	R = X = X_1 = H
Methotrexate (MTX),	R = CH_3, X = X_1 = H
Dichloromethotrexate (DCMTX),	R = CH_3, X = X_1 = Cl

It is this group of compounds that is most widely used in cancer chemotherapy, and the majority of the discussion of this subject will relate to methotrexate (MTX).

Diaminopyrimidines (III)

These are of most interest as antimalarials. Many compounds in this series have been synthesized by the Burroughs Wellcome group (67, 222). Antitumor activity is most prominent in compounds in this series that contain a 3,4-dihalophenyl group attached to the 5-position of the pyrimidine ring (122, 126).

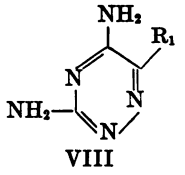
*Dihydrodiamino-*sym*-triazines (VII)*



VII

These compounds have been extensively studied and described by the Imperial Chemical Industries (I.C.I.) workers in England and by Farber, Foley, Modest, and their collaborators (32, 74, 191).

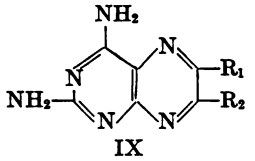
Diamino-asym-*triazines* (*VIII*)



VIII

These have been studied by Hitchings and co-workers (126) as antimalarials and as inhibitors of lactic acid bacteria

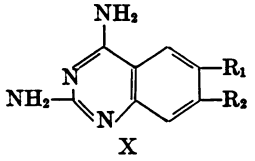
2,4-diamino pteridines without p-*aminobenzoyl in the side-chain* (*IX*) (*37, 185, 289*)



IX

R_1 and R_2 = anisyl, isopropyl, benzyl, methyl, *etc.*

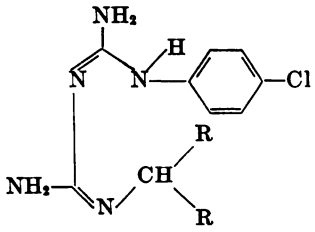
2,4-diaminoquinazolines (*X*) (*124*)



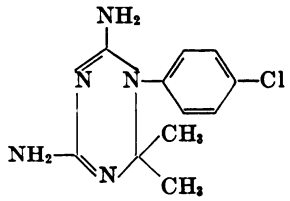
X

III. MODE OF ACTION

It is most interesting to recall that FA antagonists, like the sulfonamides, were used in clinical medicine before the metabolites with which they compete were identified. The use of FA antagonists goes back to 1945, when it was found that 5-dialkyl derivatives of 1-*p*-chlorophenylbiguanide (XI)



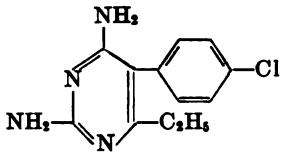
XI



XII

had antimalarial activity (47). The compound proguanil (Paludrine) (XI, R = methyl) was widely used as an antimalarial for some years before clues were found to its mode of action. It was known to be only weakly active against malarial parasites *in vitro* but to become activated *in vivo*, and in 1951 it was

shown to be transformed in the body to the dihydrotriazine (XII) which was highly active (31). The structural similarity of this compound to pyrimethamine (XIII) (Daraprim)



XIII

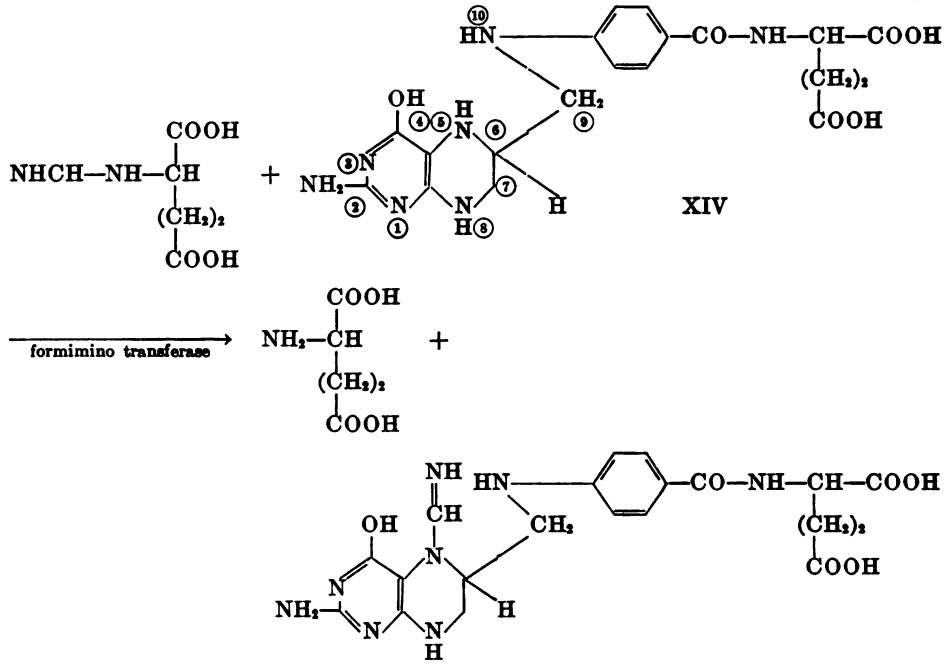
was promptly recognized, and it was found that the dihydrotriazine, like the previously studied compound pyrimethamine, was a FA antagonist for *Lactobacillus casei*, *Leuconostoc citrovorum*, and *Streptococcus faecalis* (121, 125).

The activity of these compounds against *S. faecalis* was reversed much more readily by citrovorum factor (CF, 5-formyl-5,6,7,8-tetrahydrofolic acid) than by FA, thus leading to the suggestion that the compounds prevented the conversion of FA to CF. Furthermore, the effect of pyrimethamine on experimental animals was similar to that of aminopterin (106). This has been reviewed recently by Hitchings (123).

A discussion of the mode of action of the antifolic compounds calls for a consideration of the enzyme systems involving FA and its hydrogenated derivatives. These enzymes have been discussed in detail by Rabinowitz (216).

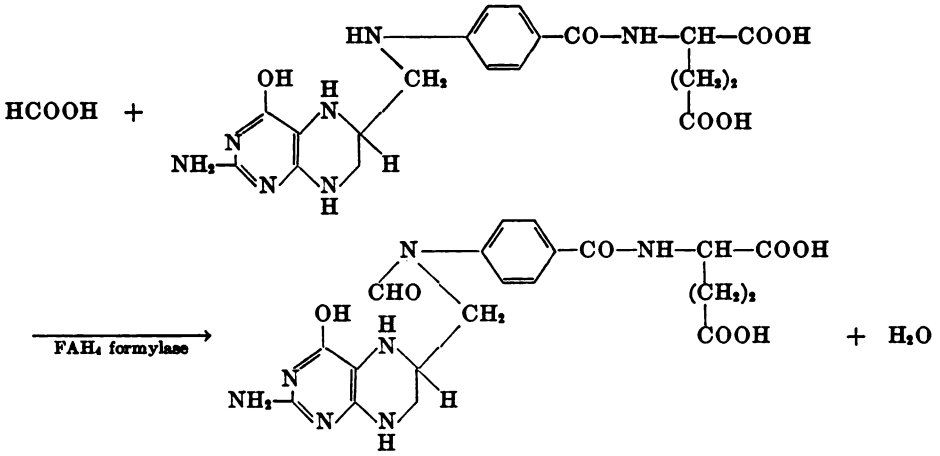
Tetrahydrofolic acid (FAH_4) (XIV) can accept a "single-carbon unit" as follows:

(1) Formiminoglutamic acid or formiminoglycine can transfer $-CHNH$ to the 5-position of FAH_4.

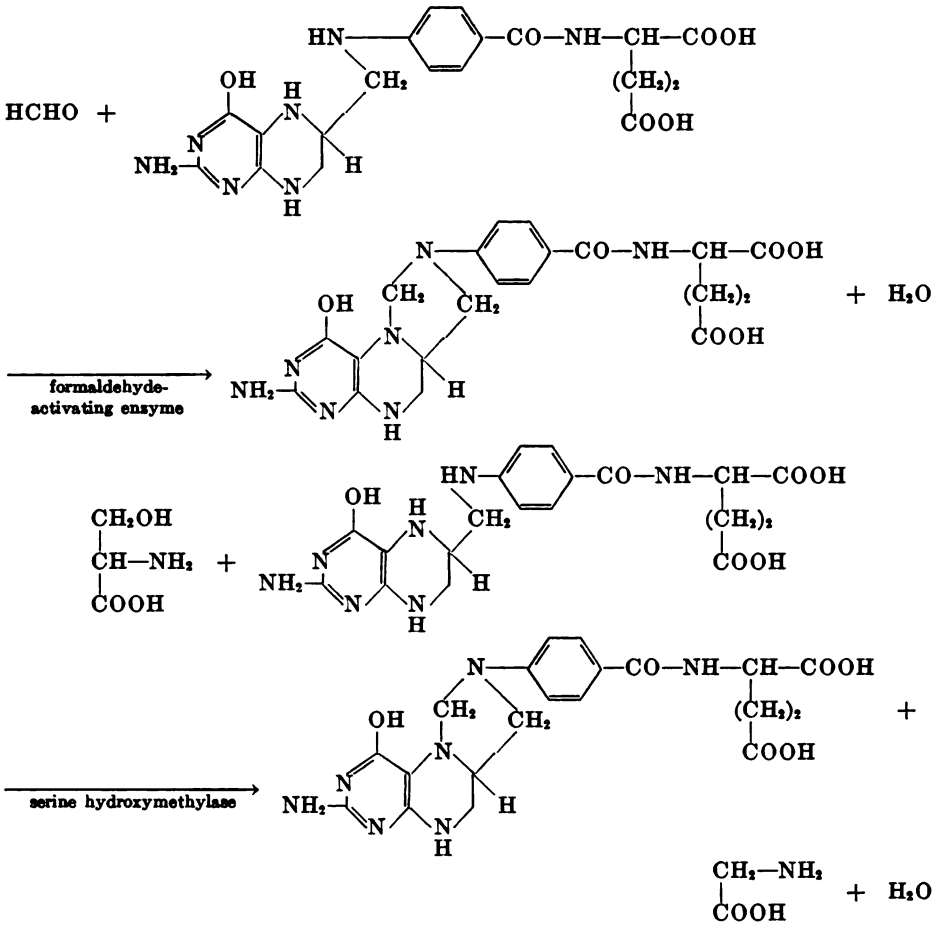


XIV

(2) Formate can transfer $-CHO$ to the 10-position of FAH_4.

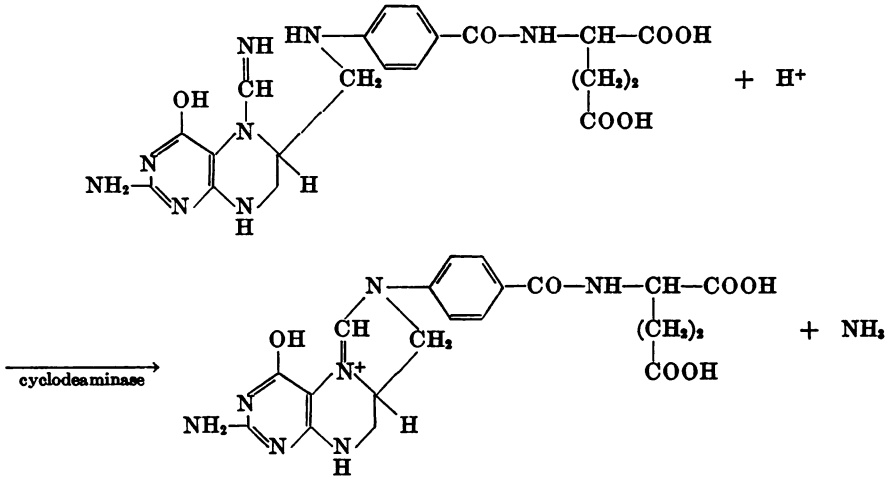


(3) Formaldehyde or serine can transfer $-CH_2OH$ to the 10-position of FAH_4, followed by cyclization and loss of water to form 5,10-methylene FAH_4.

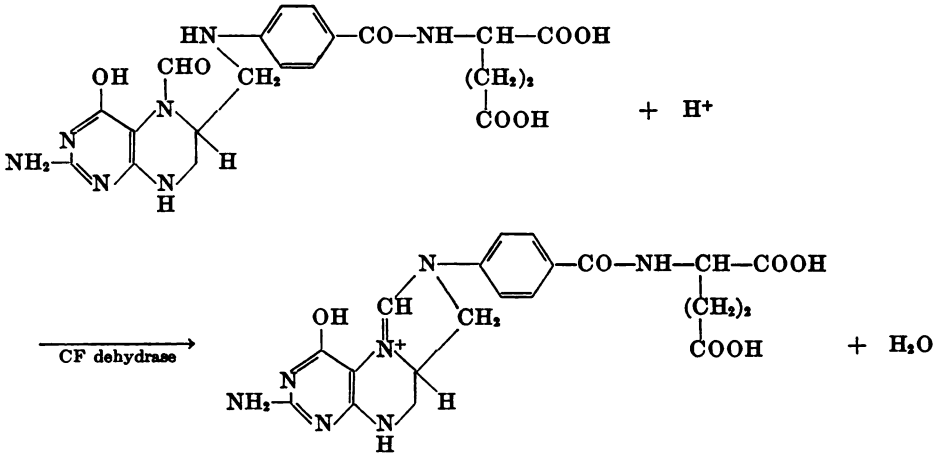


The three FAH_4 compounds produced in these reactions can undergo the following transformations.

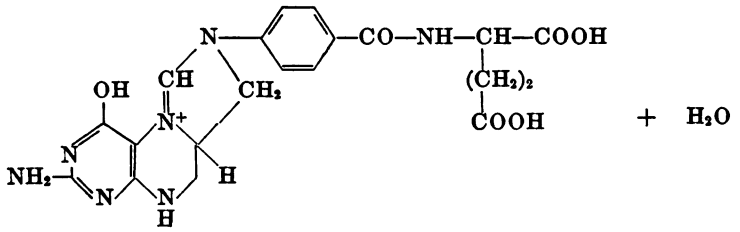
(4) 5-Formimino-FAH_4 loses ammonia and cyclizes to 5,10-methenyl-FAH_4.

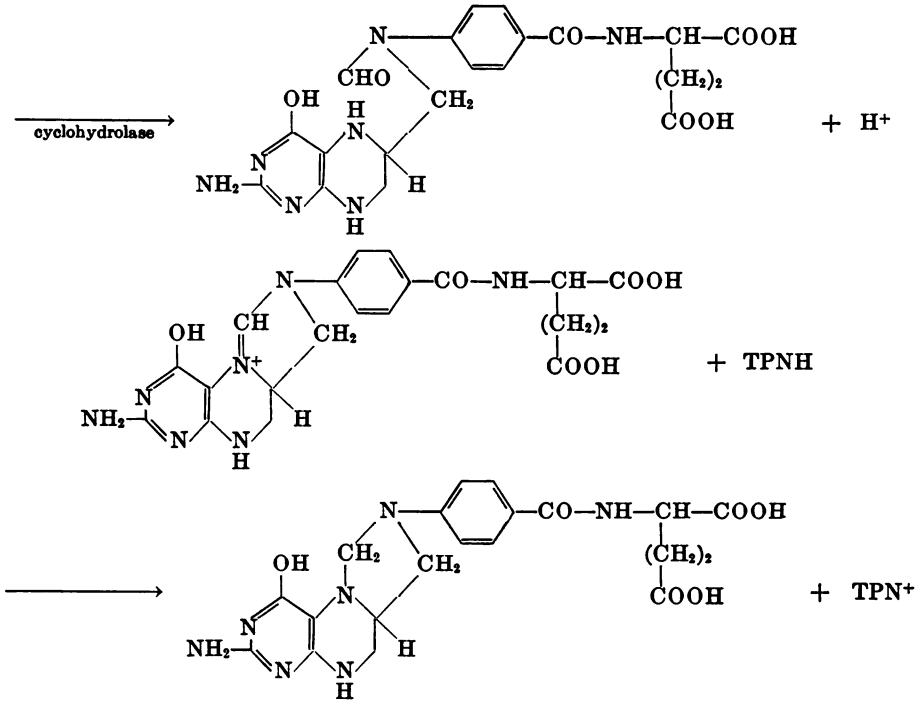


This compound can also be produced from 5-CHO-FAH_4 (leucovorin, citrovorum factor) which is made synthetically and also occurs in nature.

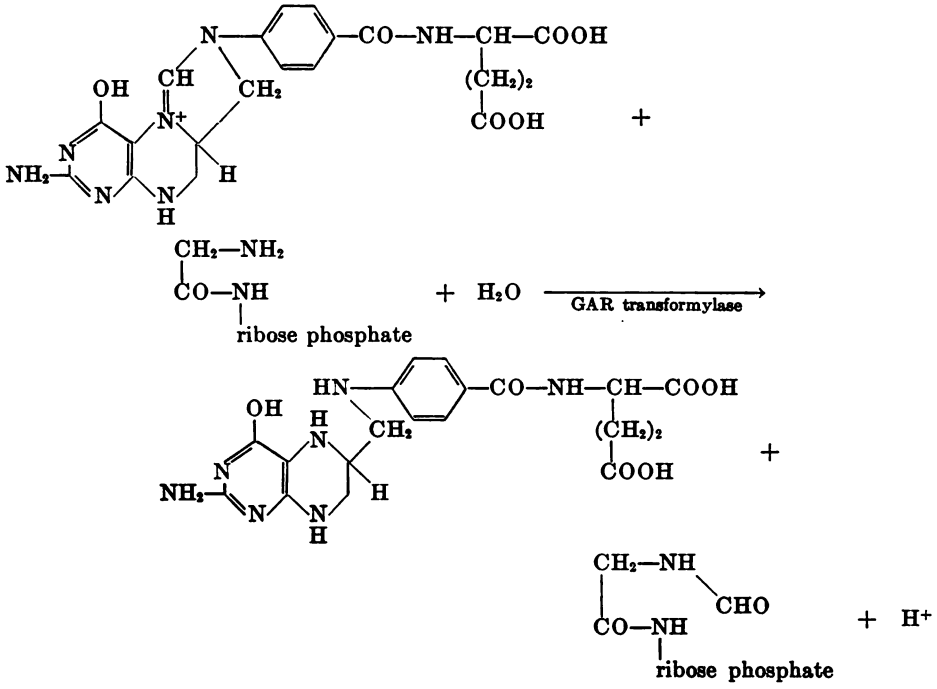


(5) 5,10-Methenyl-FAH_4 can be hydrated to form 10-formyl-FAH_4 or hydrogenated to form 5,10-methylene-FAH_4.

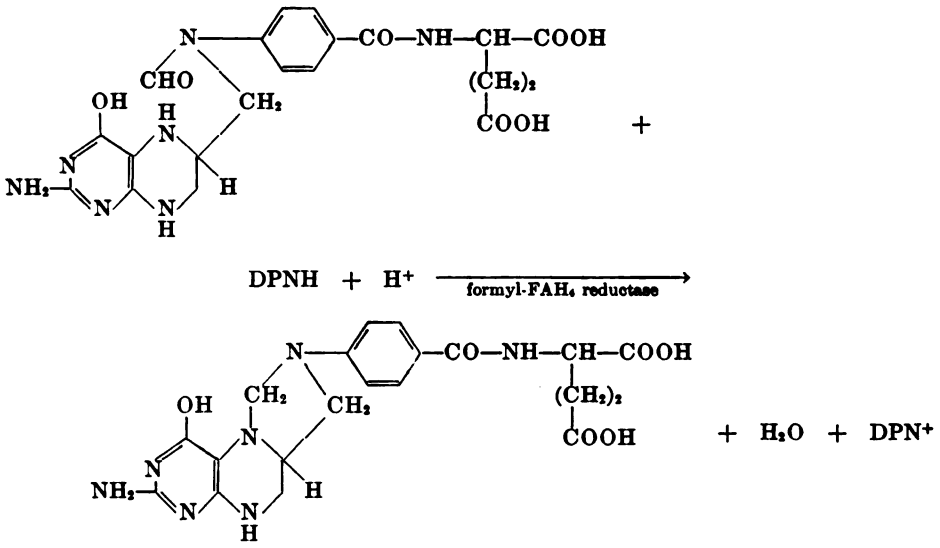




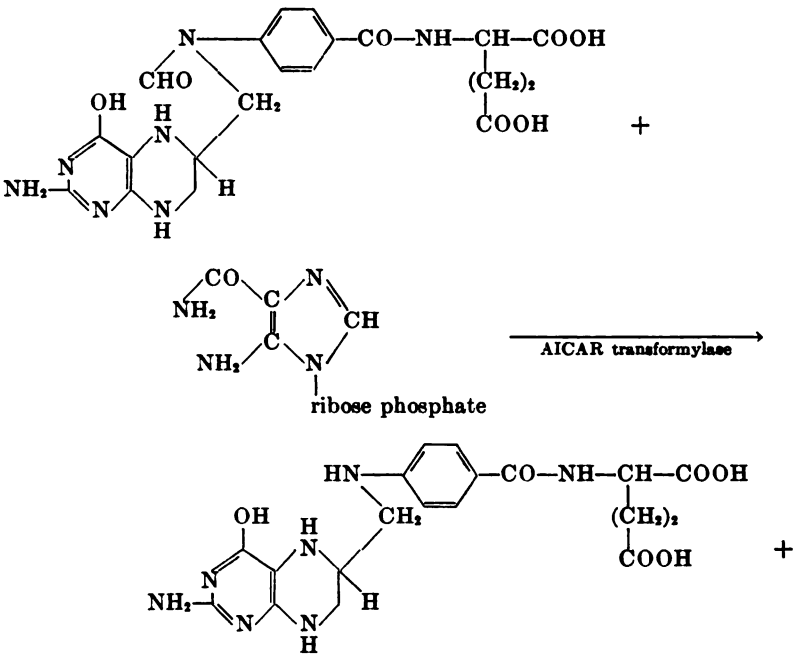
It can also give up its bridge carbon atom to glycinamide ribotide (GAR) to produce formyl glycinamide ribotide (FGAR).

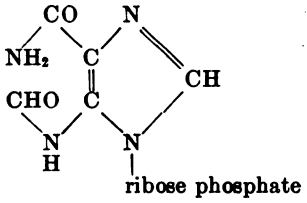


(6) 10-Formyl-FAH_4 can be reduced and dehydrated to form 5,10-methylene-FAH_4.

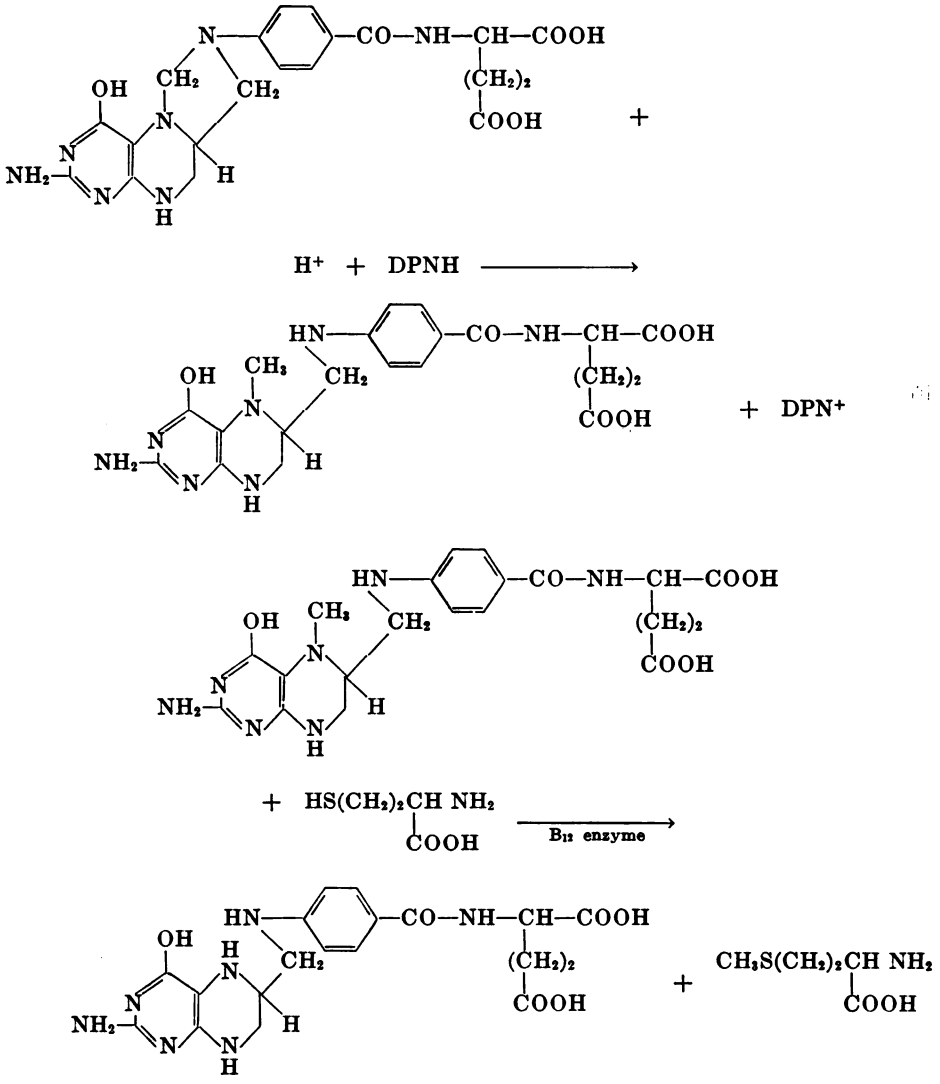


It can also give up its 10-carbon atom to aminoimidazolecarboxamide ribotide (AICAR) to form formyl-AICAR, the immediate precursor of purines and an intermediate compound in histidine biosynthesis.

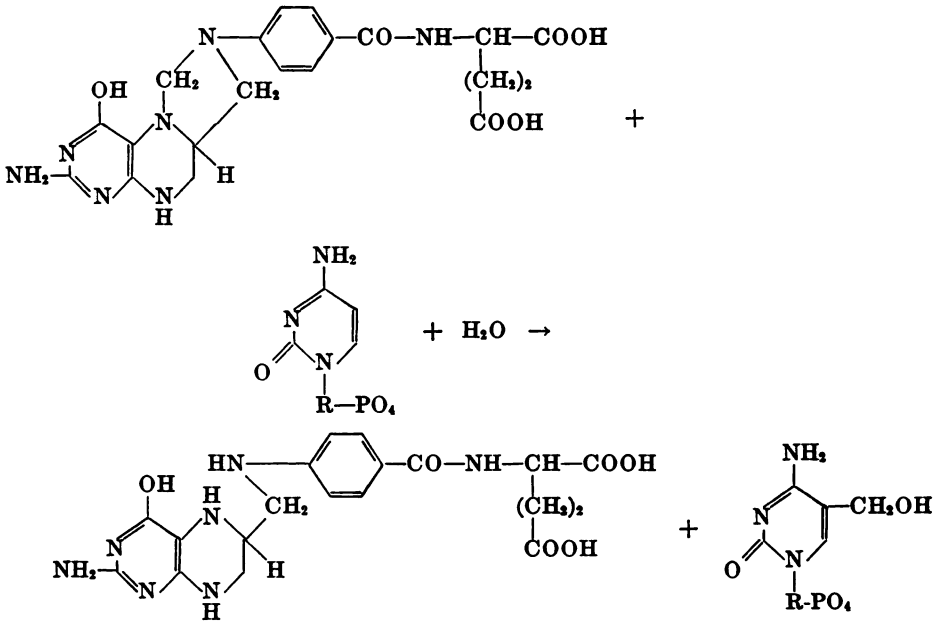




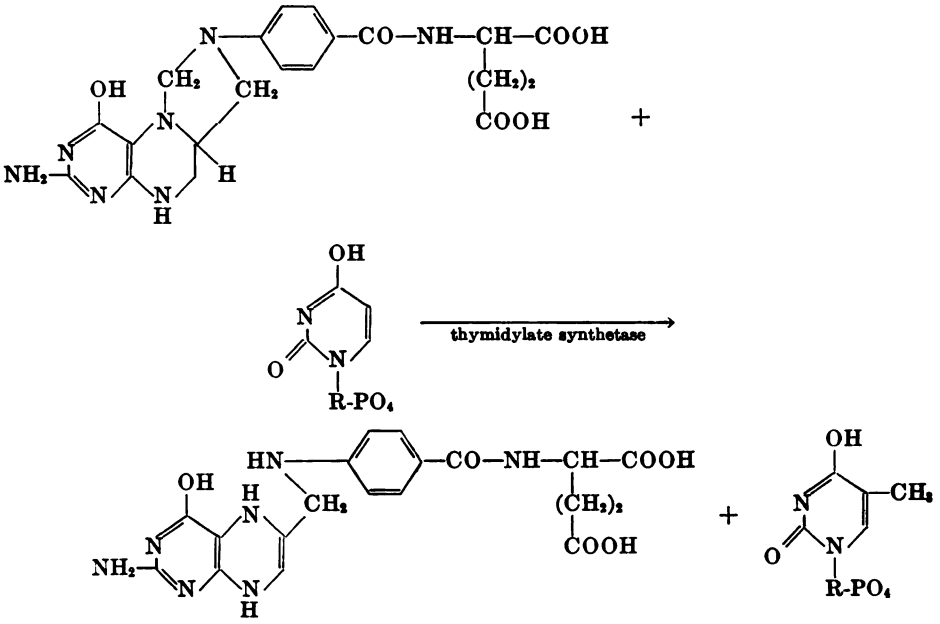
(7) 5,10-Methylene-FAH_4 can be reduced to 5-CH_3-FAH_4 which then transfers its methyl group to homocysteine to form methionine. Vitamin B_{12} is involved in the transmethylation step.



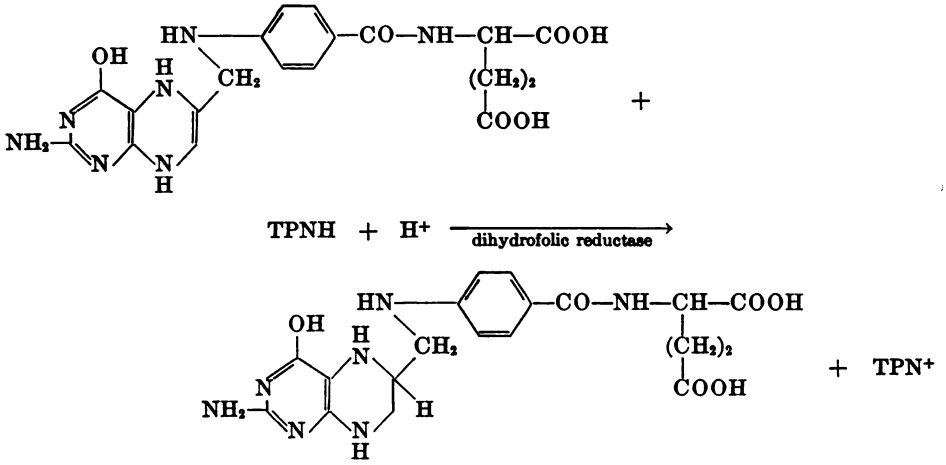
The bridge carbon can also be transferred to deoxycytidine-5′-phosphate to form 5-hydroxymethyldeoxycytidine-5′-phosphate.



Finally, the bridge carbon, together with two hydrogen atoms from the reduced pteridine ring, may be transferred to deoxyuridylic acid to form thymidylic acid and dihydrofolic acid (FAH_2).



(8) Dihydrofolic acid obtained from the last reaction, or from *de novo* biosynthesis in autotrophic organisms, or possibly from reduction of dietary FA, is reduced to FAH_4, the essential starting point of the network of reactions described in (1) to (7).



It is this reaction which in many species is, figuratively, the bottleneck or "Achilles' heel" of the biochemical systems in which folic acid is involved. Folic acid antagonists of class 1 can combine firmly with dihydrofolic reductase and thus block the "folic acid cycle," cutting off the supply of FAH_4 that is needed for reactions leading to the formation of purines and thymine, essential to the production of DNA. The effect of FA antagonists on the other reactions has not been completely explored; however, it appears that none of these antagonists that have been tested so far will inhibit thymidylate synthetase from *E. coli* (274) with the exception of the tetrahydro derivatives of methotrexate and, to a lesser extent, of aminopterin.

The organism *Leuconostoc citrovorum* responds very weakly to FA but readily to the FAH_4 compounds, the formation of which is blocked by class 1 folic acid antagonists. Consequently, these antagonists are often described, particularly in the earlier literature, as blocking the conversion of FA to CF (123, 198), this being a loose term for biological activity of compounds in the FAH_4 series. However, it was shown that the toxic effects of aminopterin were reversed by FAH_4 alone (15), which indicated that aminopterin blocked the reduction of FA to FAH_4. Furthermore, an increase in the resistance of leukemic cells to MTX is paralleled by a proportionate increase in their content of dihydrofolic reductase (76, 105).

These observations strongly support the inference that the biological effects, including the anticancer effect, of aminopterin and of the related compounds MTX and its halogenated derivatives are predominantly due to their blocking of the enzyme dihydrofolic reductase. It seems evident, however, that these antagonists can block other enzymatic reactions in which FA coenzymes are involved (147, 155).

IV. EFFECTS IN BIOLOGY

A. Antibacterial effects

Inhibition of the growth of bacteria, especially those in the lactic acid group, is produced by both classes of antifolics and has been extensively studied. Interesting differences occur between the responses observed with various species of bacteria, presumably reflecting differences in the amounts and combining power of the enzyme systems that are subject to inhibition, or differences in the accessibility of the enzyme systems to the antagonists.

1. Lactic acid bacteria. a. Lactobacillus casei. This organism is inhibited by both classes of antifolics, including "x-methyl folic acid" (82), MTX (124), and various 2,4-diaminopteridines and 2,4-diaminopyrimidines (125). It is remarkable that the inhibitory effect of class 1 antifolics for *L. casei* is reversed equally well by FA and CF. This seems to indicate that the folic reductase system of this organism is not blocked by these antagonists and that they block one or more of its single-carbon-transfer enzyme systems.

b. Streptococcus faecalis. The response of this organism to antifolics in many respects resembles the pattern seen in animals. The inhibition by class 2 antifolics was competitively reversible by either FA or CF. In contrast, class 1 antifolics were reversed only at high concentrations by FA, and even this slight effect of FA diminished or vanished at high levels of the antagonists (80).

Competitive reversal of the antagonists is produced by CF or FAH_4. These observations fit the concept that class 1 antifolics block the reductase system of *S. faecalis* and that both classes of antagonist are able to block some of the single-carbon-transfer enzyme systems. It is of additional interest that *S. faecalis* resembles leukemic cells in developing resistance to MTX and other class 1 antifolics (16). The resistant strain was found to produce CF from FA more than 100 times as rapidly as the parent strain, perhaps indicating an increased content of folic reductase in the resistant strain.

c. Leuconostoc citrovorum (*Pediococcus cerevisiae*). The unique requirement of this organism for FAH_4 or for derivatives of FAH_4, such as CF, indicates that it is unable to reduce FA to FAH_4. Aminopterin inhibited its growth and was reversed competitively by CF and noncompetitively by thymidine (17). Hydrogenation of aminopterin increased its inhibitory effect (155). It is of interest that hydrogenation of aminopterin and MTX makes these compounds active as inhibitors of thymidylate synthetase (274). Class 2 antifolics have only slight inhibitory activity for *Leuconostoc citrovorum.*

2. E. coli. Growth of this organism was inhibited by very high concentrations of aminopterin. No reversal was produced by FA or CF, but thymidine restored growth (83). Evidently the mechanism that produces thymidine was blocked but the point at which the block occurred is unknown.

B. Antiprotozoal effects

Certain class 1 antifolics are effective inhibitors of a number of protozoal parasites, including *Plasmodia, Toxoplasma,* and *Eimeria.* The compound,

chlorguanide (Proguanil, Paludrine), was introduced in 1945 for the treatment of malaria as discussed above. The dihydrotriazine arising from chlorguanide, together with other 2,4-diaminodihydrotriazines and 2,4-diaminopyrimidines are active against toxoplasmosis and coccidiosis, and are synergistic with the sulfonamides. This synergism is due presumably to the blocking of two sequential steps in the formation of FAH_4 from PABA (123). The antiprotozoal effect of these antifolics is not reversed by CF, although CF protects against the toxic effects of the drugs in animals (123). Presumably these protozoans are not permeable to CF. However, the effect of sulfonamides against *Plasmodia* is abolished by adding PABA to the diet of animals under treatment (109).

The inhibitory effect of class 1 antifolics on *Protozoa* varies. For example, *Tetrahymena* was found by Kidder (153) to be inhibited by MTX; the effect was competitively reversed by FA over a wide range, but aminopterin was inert. The protozoan *Crithidia fasciculata* is readily inhibited by aminopterin and MTX (140). The susceptibility of protozoan parasites has been investigated with chlorguanide and pyrimethamine far more carefully than with MTX and aminopterin. As indicated above, typical representatives of these organisms apparently utilize PABA for the formation of FAH_4 and two of the steps in the synthesis are blocked, respectively, by sulfonamides and certain class 1 antifolics.

C. Effects on normal animals

The effects of antifolics on leukemia were foreshadowed by experiments with normal rats (82). These animals, when treated with "x-methyl folic acid," a class 2 antifolic, became anemic and agranulocytic. The blood dyscrasia was prevented or reversed by raising the FA content of the diet to overcome the action of the antagonist. Other changes in the treated animals, preventable by FA, included aplasia of the bone marrow, severe diarrhea, necrotic, and ulcerative changes in the mouth, and small and atrophic uteri. Comparable effects were produced in mice, chickens, and dogs treated with "x-methyl folic acid" (148), but no effects were produced in a human subject with chronic myeloid leukemia (111), and interest soon changed to the use of class 1 antifolics. The first of these to be tested was aminopterin which produced death in about six days in mice when they were given a diet containing one part per million of the drug (84). The addition of folic acid did not reverse its effects. The LD50 of aminopterin was found to be 1.9 ± 1.4 mg for rats (208). The intestinal mucosa was severely affected and the feces became watery and bloody. Changes took place rapidly in the bone marrow of rats, including liquefaction, with disappearance of the blood-forming elements in less than three days. The changes in the intestinal mucosa in rats are so rapid that differences in histology have been reported to be produced within three hours (273).

The severity of these changes is all the more remarkable for the fact that they may be completely prevented by treatment with CF. Changes in rats and mice similar to those caused by aminopterin are produced by MTX in somewhat higher dosage (80). Human beings are similarly subject to the toxic effects of these class 1 antifolics. However, there is considerable variation among other species,

and some animals, including guinea pigs and hamsters, are resistant to these antagonists (299). Dogs and monkeys are susceptible, and changes in the bone marrow and intestinal mucosa of these species have been described (208).

The effect of antifolics on pregnancy in rats and mice is conspicuous and the substances are also highly toxic for pregnant women; aminopterin was found to terminate pregnancy when given in the first trimester (257).

The effects of x-methyl folic acid on reproduction in female rats were extensively studied by Nelson and her collaborators (195). Female rats were given a diet deficient in folic acid and containing a sulfonamide and x-methyl folic acid. Control animals that received the same diet with high levels of added FA had a normal reproductive performance. Deficient rats showed various degrees of resorption and fetal abnormalities, depending on the conditions of dosage with respect to level and timing. Edema and anemia were produced in the groups fed the deficient diet from the 12th to 21st days. Abnormalities of a striking nature occurred even when the period on the deficient diet lasted only two or three days for the mothers. The abnormalities included malformation of the face and mouth, deformed and misshapen bones, kidney defects, and abnormalities of the cardiovascular and genito-urinary systems. Indeed it was commented that "no system or organ yet studied has escaped some damage" (195).

Other effects on normal animals include the urinary excretion of formiminoglutamic acid and formate. These substances accumulate due to the absence of the folic enzyme systems that are necessary for their normal metabolism (216).

The normal response of the oviduct of young chickens to estrogens was blocked by adding x-methyl folic acid to the diet (81, 114).

Methotrexate and aminopterin are not known to be broken down in animals, and the intact substances appear in the blood, tissues and urine following their administration (85). In contrast, dichloromethotrexate (3′,5′-dichloro-4-amino-10-methyl-pteroylglutamic acid, DCMTX) was found to be rapidly changed by rats into a compound that was excreted in the urine and bile. The compound was tentatively identified as 4,7-dihydroxy-4-deamino DCMTX, which is identical with the parent compound except for the change of the 4-amino group to a 4-hydroxy group and the addition of an hydroxyl group to the 7-position of the pteridine ring (201). This compound was found not to inhibit dihydrofolic reductase. No analogous 4,7-dihydroxy compound was produced from MTX. Dogs did not form the compound from DCMTX, and a human subject converted from one-half to one-third of a dose of DCMTX into the metabolite. The conversion of DCMTX into an inert compound undoubtedly explains the fact that DCMTX is less toxic than MTX for rats.

D. Effects on tumors in animals

Early studies by Leuchtenberger and co-workers with pteroyltriglutamic acid drew attention to the use of FA derivatives in tumors of animals (162, 166).

Even before this, FA had been shown to be involved in the synthesis of purines and thymidine, so that its relation to cellular proliferation was indicated. Great interest was aroused by the report by Farber's group (73) of the effect of aminop-

terin in producing temporary remission in leukemic children, and investigators turned to mice and rats for further explorations of the effect of antifolics on experimental leukemia (24). Aminopterin and MTX prolonged the survival time of mice with AK4 and C1398 strains of leukemia. Further studies were made with AK4 and a large number of antifolics together with other heterocyclic compounds (25). Only those containing a 2,4-diaminopyrimidine group were active.

The voluminous literature of experimental cancer chemotherapy in the past decade has contained many articles describing the effectiveness of methotrexate and other class 1 antifolics in the treatment of leukemia and lymphosarcoma in mice and other animals. A few examples are cited for reference (22, 97, 239, 240).

V. RESISTANCE TO FOLIC ACID ANTAGONISTS

The usefulness of chemotherapeutic substances is limited by the extent to which resistance develops to their selectively toxic effects. For example, strains of pathogenic staphylococci that are resistant to penicillin have become an increasingly frequent problem in hospitals, and it is known that such strains emerge when staphylococci are repeatedly cultured in media containing penicillin. This illustrates the development of resistance by the elimination of susceptible organisms accompanied by the survival and multiplication of inherently resistant individuals that were originally present in the same group.

The use of antifolics in the treatment of leukemia is restricted by what appears to be a similar train of events. It was shown by Burchenal and co-workers (29) and by Law and Boyle (158) that Leukemia AK4 and L1210 became resistant when exposed to MTX during successive transplants in mice. In the investigation by Law and Boyle, three separate sublines of strain L1210-Y lymphoid leukemia became resistant in mice receiving 3 mg of MTX per kg. Resistance persisted through seven successive transplants into mice which were not given the antagonists. Similar results have been reported by various investigators (135).

Certain strains of leukemic mice were found to survive actually for a shorter time when MTX was given than when untreated. It was stated that they were infected with a contaminant, presumably lymphocytic choriomeningitis virus. This virus retarded the rate of tumor growth and MTX was thought to inhibit the protective effect of the virus (104).

Information on the possible mechanism of resistance of leukemic cells has come from two studies carried out in tissue culture. It was found by Hakala and co-workers (105) that the folic reductase content per cell in cultures of sarcoma 180 increased in direct relation to methotrexate resistance. Similar findings were reported by Fischer (76) with L5178-Y mouse leukemic cells in tissue culture. These results indicate that in untreated leukemia in mice the majority of the leukemic cells are very low in dihydrofolic reductase. A few cells contain much larger amounts of this enzyme; these survive treatment with the antagonists, and eventually multiply to the point where it is no longer possible for the host to tolerate treatment with a dose of MTX that is adequate to inhibit the leukemic cells.

The following concept of the development of resistance to MTX in leukemic mice and rats may be drawn from the available experimental observations. The leukemic cells contain varying amounts of an enzyme, dihydrofolic reductase, which is essential for their multiplication. This enzyme normally catalyzes the reduction of FAH_2 to FAH_4, using a reduced pyridine nucleotide as a hydrogen donor. The enzyme as found in rat liver has a far greater affinity, in the order of 100,000-fold, for MTX than for FAH_2 which is its normal substrate. Methotrexate binds the enzyme so firmly that the drug may be retained for prolonged periods in combination with the enzyme (280) in the tissues of animals. The above discussion illustrates one mechanism by which resistance may develop to antifolics; namely, (a) *Cells sensitive to the drug are eliminated and their place is taken by cells that are inherently insensitive.*

Improved results may be obtained by using DCMTX which is considerably less toxic than MTX for rats and mice. The lower toxicity may be due to the rapid breakdown of the dihalogenated compound to an inert product; MTX does not undergo such a breakdown. The approach of using a drug which is less toxic to the host than to the unwanted cells because of the breakdown of the drug to inert products is a valuable one in the field of selective toxicity.

There are a number of other mechanisms by which resistance may be developed to drugs by unwanted cells, such as pathogenic bacteria or cancer cells. The best understanding of these mechanisms is through the study of enzyme systems. The following possibilities should be considered.

(b) *The enzyme may become less accessible to the drug.* Nichol and Welch (199) studied a MTX-resistant strain of *S. faecalis*. They found that the CF-forming enzyme system present in the contents of the ruptured cells was not resistant to MTX, even though the intact cells could, in contrast, convert FA to CF in the presence of large amounts of the drug. Furthermore, it was found in other investigations in which aminopterin was added to the cultures that unaltered aminopterin could be recovered from resistant *S. faecalis* cells in quantities larger than those obtained from susceptible cells.

The measurement of CF production from FA may be presumed to be an indication of dihydrofolic reductase activity, since the conversion of FA to FAH_4 and to other compounds that are active for *Le. citrovorum* is controlled by dihydrofolic reductase. The finding that the resistant cells contained the antagonist indicates that the resistance was not due to impermeability but that, apparently, the enzyme in the cells was not accessible to the antagonist.

It is of interest that guinea pigs are highly resistant to MTX toxicity (93), but their liver, kidney, and intestinal tissues contain about the same amounts of dihydrofolic reductase as are present in the corresponding tissues of rats and mice, which are easily susceptible to the toxic effects of MTX (280). Here perhaps is another example of variation in the accessibility of the enzyme to the drug.

(c) *The cell may develop an alternate metabolic pathway.* This may bypass the enzymatic reaction that is blocked by the antagonist, or it may result in a decreased requirement for the metabolite that is produced by the reaction.

It was reported by Woolley and Koehelik (291) that *E. coli* grown in the presence of 3-methylaspartic acid was able to convert this compound into thymine, thus bypassing the thymidylate synthetase reaction by which thymine is produced from uracil and 5,10-CH_2-FAH_4. A biological reaction involving vitamin B_{12} is known to lead to the production of 3-methylaspartic acid. However, Abramsky, Rowland and Shemin found that *E. coli w.* produced isoleucine rather than thymine when incubated with 3-methylaspartic acid (1a).

(d) *The cell may produce increased quantities of the normal metabolite.* Bacterial cells that produce large quantities of FAH_2 would be expected to be resistant to MTX, because FAH_2 and MTX compete for the same enzyme surface. This might explain the resistance of *E. coli* to any but high levels of aminopterin (83) since this organism can synthesize the "folic acid coenzyme" (18). No examples of this mechanism have been described in studies of the relation of antifolics to cancer.

(e) *The "fine structure" of the enzyme may become modified so that its combining power for the antagonist becomes diminished.* This occurs in sulfonamide-resistant pneumococci (132), but no studies in this field have been reported with antifolics.

(f) *The cell may become less permeable to the antagonist or it may develop enzyme systems that destroy the antagonist.* These effects are not known to occur in cancer cells treated with antifolics. Although normal animals, especially rats and mice, can metabolize DCMTX, there is no indication that tumor cells are more effective than the host in breaking down this substance or other antifolics.

The principal known causes of the development of resistance by cancer cells to antifolics are therefore (a) increased production of folic reductase and (b) diminished accessibility of this enzyme to the antagonists. A third possible cause is the development of alternate pathways for the formation of essential products such as thymine.

Part II. Folic Acid Antagonists—Clinical Application

I. Introduction

The first attempts to study the antineoplastic activity of FA and its derivatives were undertaken by Leuchtenberger, Lewisohn and their co-workers (162, 166) who reported that "fermentation *Lactobacillus casei* factor" (pteroyltriglutamic acid, Teropterin[1]) inhibited the growth of transplanted Sarcoma 180 and induced 30 to 40% regression of spontaneous mammary carcinoma in mice. However, Teropterin (13) showed no activity in human tumors (294). The findings reported by Lewisohn's group have not been confirmed.

A search for FA analogs with greater activity on human neoplasms led to the synthesis of a series of FA antagonists (139, 186, 232). Of these only the 4-amino congeners, *i.e.*, the "class 1 antifolics"—aminopterin, MTX, and halogenated methotrexates—are of clinical interest.

Aminopterin (4-amino-pteroylglutamic acid), synthesized by Seeger and coworkers in 1947, was reported to induce a high frequency of remissions in acute

[1] Reg. U. S. Pat. Off., American Cyanamid Co.

leukemia in children (68, 73) as well as occasional remissions in adults (51, 52). Aminopterin was soon largely replaced in clinical practice by its methylated derivative, methotrexate (MTX, 4-amino-10-methyl-pteroylglutamic acid), which has a similar therapeutic spectrum but shows greater activity and manageability at comparable host-toxicity levels. Rapid confirmation of the therapeutic activity of aminopterin (10, 49, 136, 146, 177, 190, 210, 213, 215, 237) and MTX (5, 10, 26, 49–53, 55, 179, 215, 221, 223, 243, 284, 287) within the next few years resulted in the incorporation of these drugs into the meager arsenal of useful antileukemic agents.

The antileukemic activity of subcutaneously injected DCMTX has been demonstrated to be superior to that of orally administered MTX or DCMTX in mice (229, 272) but not in man (230, 281).

II. METABOLISM

A. Absorption

Aminopterin and MTX are readily and more or less completely absorbed, while DCMTX is incompletely absorbed from the gastrointestinal tract. Peak serum levels of DCMTX can be increased ten-fold when the absorption barrier is overcome by parenteral administration (217).

Following oral administration, antifolic activity was detected in the blood and urine within thirty to sixty minutes (30, 253). A linear relationship existed between the one-hour peak of plasma MTX concentration and the dose per kg body weight (85). According to Rall and Dion (217), peak plasma DCMTX concentrations are attained only after two hours.

In fasting patients the rate of MTX absorption was doubled, demonstrable amounts of drug appearing in the blood (50 mμ/ml) and urine as rapidly as 15 to 30 minutes, and peak levels of approximately 100 mμ/ml occurring in the serum within 30 to 60 minutes after ingestion of a 5-mg dose (30).

The degree of plasma-protein binding showed great individual differences, but appeared to vary inversely with the drug concentration. As measured by the fluorimetric method, it was about 50% at therapeutic serum concentrations of MTX (85) and DCMTX (217). Comparable therapeutic efficacy was obtained by oral administration of one-tenth as much aminopterin, and three to five times as much DCMTX (281) as MTX calculated on a drug-dose per body-weight basis. A DCMTX:MTX dosage ratio of 5:1 gave a plasma ratio of only 1.2:1 following oral administration, but a thirty-minute plasma ratio of 2.5:1 following parenteral administration (281). However, since DCMTX, unlike aminopterin and MTX, is partially converted *in vivo* to its inactive 4,7-hydroxy metabolite (201, 202), the plasma ratio, after a two-hour peak (217), dropped to 1:1 by the end of four and one-half hours (281). Thus, therapeutic concentrations of DCMTX remained in the blood for much shorter periods than comparably active concentrations of MTX.

Freeman *et al.* (87) and Freeman and Narrod (253) reported that acute leukemia patients developed progressive retardation of MTX absorption as therapy continued and resistance began to build up. Toxic symptoms could be correlated

directly with, and were observed only during the time of, delayed drug absorption (87, 253).

B. Distribution

In both man and mice, the persistence of aminopterin, MTX, and DCMTX was observed in those tissues which normally have high folic acid (FA) and citrovorum factor (CF) contents—notably liver, kidneys, and spleen (35, 78, 79, 201, 279). Methotrexate and its metabolites (methopterin, aminopterin) remained longest in the liver, where they were demonstrable for periods up to several months after suspension of therapy (35, 78, 79). The duration and amount of drug persistence are independent of the dosage, route of administration, and duration of drug therapy (35, 85). Werkheiser (278) concluded that this drug persistence was due to binding of MTX by folic reductase in the tissues.

Absorption and distribution were affected by dosage schedules, route of administration, type and stage of disease, nutrition, and other factors associated with physiologic variability.

Better absorption and maintenance of drug blood levels, but also coincidental enhancement of toxicity (39, 40, 218), were obtained with repeated small doses than with single large doses of MTX.

Parenteral administration—intramuscular or intravenous for trophoblastic tumors (115, 116, 129, 167–172), intracranial for the central nervous system (CNS) involvement of leukemia (192, 226, 283), and intraarterial infusion for certain epidermoid tumors (246–249)—may facilitate the antifolic agent's penetration to neoplastic sites, thus greatly enhancing its efficacy.

Because of poor transmeningeal drug diffusion, only intrathecal administration resulted in high cerebrospinal fluid (CSF) levels which, since they were associated with prolonged (four to six days) but low serum levels, evoked no apparent adverse systemic effects (283). As little as 0.5 mg of MTX, given intrathecally, could be detected within 15 minutes in the CSF of dogs (192) and patients (283), and yielded drug levels 30 to 100 times higher than those obtained in the CSF even after a massive single oral dose. Since neurologic involvement is a frequent complication of advanced acute leukemia (226, 245, 277, 283), the drug must gain access to the CNS for adequate effect on cerebral metastases.

Methotrexate metabolism can be altered by dietary variants. Inanition, which accelerated drug absorption in man (30), potentiated the inhibitory effect of MTX on formate incorporation (4) in rats and tended to retard the fatal evolution of leukemia (134). In man, on the other hand, considerable deterioration of the clinical condition and acceleration of the neoplastic growth rate occurred (8) during negative nitrogen balance.

Improved drug diffusion across body barriers (nerve trunks, meninges, adipose and fibrous tissue) to malignant neoplastic foci may be obtained eventually by further chemical modification of the antifolics. Of the many FA analogs recently tested in laboratory animals, the greatest promise, *i.e.*, lower toxicity and higher antileukemic activity, was shown by the mono- and dichlorinated derivatives of MTX, especially DCMTX (97, 98). However, clinical investigation indicated

that DCMTX was merely comparable if not actually inferior to MTX in toxicity and therapeutic efficacy in leukemia and other lymphomatous processes (230, 281).

C. Excretion

Within thirty to sixty minutes of oral administration MTX appeared in the urine (30). About 85 to 100% of the drug was recovered in the urine by twelve hours following oral administration of MTX (85) and parenteral administration of DCMTX (201), and within less than three hours following intravenous administration of MTX (253). Only 38 to 72% of aminopterin was excreted over a 72-hour period in acute leukemia patients and even less (19 to 62%) in chronic granulocytic leukemia (252).

The plasma concentrations of MTX and DCMTX dropped to 50% of maximum levels within 2.3 (85) and three hours (217), respectively. Following oral dosage, 40% of the MTX and 7% of the DCMTX administered were excreted in the form of the unchanged drug (281). Following parenteral dosage, these values rose to 65 and 13%, respectively. Despite this relatively rapid excretion of MTX and DCMTX, detectable serum levels persisted for several days after withdrawal.

Freeman and Narrod (253) reported that in leukemic patients manifesting signs of toxicity and retardation of MTX absorption, the rate of drug excretion was reduced following oral but not intravenous treatment.

D. Effects of antifolics on metabolic patterns

Antifolic therapy interferes with existing metabolic patterns. The presence of abnormal chromosomes and mitoses as well as the phenomenon of mitotic arrest prior to anaphase results in accumulation of morphologically normal prophase and metaphase cell stages. Thus, abnormal chromosomes and mitotic figures probably reflected drug interference with intracellular nucleic acid synthesis, and provided morphologic evidence of drug damage to the cells (145, 154, 259, 260). There was no morphologic evidence of direct damage to the nucleolus and nuclear membrane prior to onset of cell dissolution. Physiologic evidence of drug interference with metabolic patterns—correlatable in part with therapeutic response in neoplastic disease—was provided by changes in urinary excretion levels of uric, formic, formiminoglutamic, and folic acids and citrovorum factor.

An increase in uric acid excretion (to over 100 mg per day) may occur during antifolic therapy, despite continued nucleic acid synthesis, in patients with sensitive leukemias (175, 225) or trophoblastic tumors (174). Breakdown of both tumor tissue and erythrocytes, which resulted in a negative nitrogen balance, was thought to be responsible in part for this phenomenon. Although the nitrogen balance returned to normal between drug courses, uric acid excretion never completely reverted to base level.

Retention of folic, formic, and formiminoglutamic acids as well as abnormally high excretion of CF were observed in patients with untreated acute and (much

less frequently) terminal chronic granulocytic and chronic lymphatic leukemia (44, 117, 118, 176, 251, 252). These phenomena were reversed in drug-sensitive patients during antifolic therapy (117, 131). However, urinary excretion of these acids was not found useful for predicting response to or tolerance of MTX. Nevertheless, urinary levels of formic acid can be considered a measure of drug-induced FA deficiency (118).

Following aminopterin therapy, the 24-hour urinary FA excretion rose to nearly normal levels in patients with acute leukemia but remained greatly depressed in those with chronic leukemia. Titers had a much wider range of dispersal in leukemic than in normal individuals.

Excessive excretion of formic and formiminoglutamic acids was observed in patients with acute leukemia and choriocarcinoma at onset of response to antifolic therapy (44, 117, 118, 169, 252).

Urinary CF excretion was depressed during antifolic therapy (66, 252) and for several weeks thereafter (42). The severity of this phenomenon was proportional to the magnitude of the dose as well as to host sensitivity to antifolic agents. During the recovery period, there was overcompensation of CF excretion (42). Depression of CF excretion was more profound and recovery slower 1) on multiple than on single drug dosage, and 2) on daily as against intermittent schedules.

Phenylalanine hydroxylation to tyrosine was impaired in women with choriocarcinoma during MTX therapy, without concomitant changes in serum levels of these amino acids (100). It is possible that this phenomenon may be attributed to interference by the drug with a cofactor necessary for the enzymatic hydroxylation of phenylalanine (150, 151).

Urinary chorionic gonadotropin (GTH) titers frequently decrease during repeated courses of antifolic therapy of chorionic tumors (115, 129, 133, 167–170). Although there appears to be some correlation between tumor destruction and GTH reduction, the latter has been known to remain elevated in some patients despite objective evidence of tumor response.

Endocrine mediation and potentiation—*via* the pituitary-adrenal axis—of the lymphopenic and other more severe toxic effects of FA antagonists have been demonstrated in rodents (59, 119, 163–165), but not in man (9, 29). In fact, Cramblett (45) found that in leukemic children MTX appears to act by direct inhibition of metastatic cell metabolism, independently of concomitant steroid therapy.

III. TOXICITY OF CLASS 1 ANTIFOLICS

The toxic effects of class 1 antifolics possibly are due to the inhibition of synthesis of nucleic acid in rapidly proliferating cells. The rate of proliferation is high in cells of the hemopoietic and leukopoietic systems, gonads, and oral and intestinal mucosa, but low in nonproliferative cells such as neurons.

The relatively linear relationship of the tolerated therapeutic dosage to the total amount of drug administered was modified by the dosage schedule and, insofar as it determined the rates of absorption, distribution and elimination of the drug, the route of administration.

Drug tolerance limits were higher in adults than in children by body surface criteria (mg/m^2) (214).

On a dose per kg body weight basis, MTX was tolerated much better in mice and slightly better in dogs than either aminopterin or DCMTX (209, 218). Clinical toxicity appeared similar for the three drugs at therapeutic dose levels where the dosage ratio of aminopterin:MTX:DCMTX was approximately 0.1:1:4. During prolonged therapy, however, aminopterin produced a higher frequency and degree of toxicity than MTX, possibly because of the latter's more rapid and complete excretion (85, 252).

Single large oral doses of antifolics, resulting in high but transitory serum levels and associated with rapid urinary drug elimination, were better tolerated by normal animals (75, 217, 218) and man (40, 41) than repeated smaller doses giving rise to moderately high, prolonged drug levels in the serum. In cancer patients, maximum though moderate toxicity, fully reversible within two weeks, was attained within four to ten days of initiation of massive dosage of MTX administered in either a 5-day course or a single dose. In the former case (1), the daily dosage levels exceeded the customary dose of 5 mg/day by a factor of five; in the latter case (41), by a factor of 80 to 200. With even higher single doses —1 to 10 mg/kg (max. 450 mg) administered parenterally at intervals of one to six weeks (40, 41)—toxicity was more severe and usually was reversed only two to three weeks following the last dose.

Impaired renal function resulted in delayed plasma clearance (85) and drug excretion (30). This increases the danger of early systemic toxicity (65) and requires great care in the adjustment of antifolic dosage in such patients.

Recently R. D. Sullivan (253) postulated that toxicity depended on the duration of drug contact with the tissues rather than on the concentration of the drug in the blood: a 5-day course of continuous 12-hour intraarterial infusions of 5 mg/day resulted in toxic manifestations considered quantitatively similar to those observed after a 5-day course of single intraarterial doses of 25 mg. His contention tended to find confirmation in Freeman and Narrod's (253) observations that, following oral and intravenous administration of MTX, toxicity can be related directly to delayed drug absorption but not to the rate of excretion; the latter is reduced only as a consequence of delayed drug absorption, unless renal damage is present.

Regardless of the size and schedule of antifolic dosage, toxic symptoms may set in as long as four to seven days following withdrawal, due to continuation of drug-initiated disruption of essential enzymatic activity within the cells as well as to persistence of drug in the body in the case of aminopterin. The above data and, indeed, the preponderance of clinical evidence suggest that, in contrast to the duration of the drug effect, development of reversible toxicity is more time- than dose-dependent.

Signs of toxicity due to antifolics may include major associated gastrointestinal and hematologic effects (26, 41, 58, 62, 68, 90, 92, 112, 127, 144, 192, 215, 223, 227, 255, 256, 262, 285) and minor and indirect ones (38, 128, 178). At the first signs of toxicity—1) oral ulceration or bleeding, 2) diarrhea, or 3) acutely

progressive leukopenia, marked bone marrow depression, or both—discontinuation or reduction of antifolic therapy was found advisable until all manifestations of toxicity had subsided. Subsequently, therapy could be resumed at lower dose levels. Preresponse malaise, weakness, weight loss, fever, pallor, and hemorrhages were considered signs of mild toxicity but also indicated responsiveness to therapy. They were reversed during remission.

In general, less toxicity was observed for a unit dose when the antifolic was administered orally instead of parenterally to animals (209, 218), the lowest degree of tolerance being observed with intravenous administration. In man, the tolerance level of MTX, a rapidly absorbed and excreted antifolic, following parenteral injection is similar to that following oral administration. However, a highly enhanced systemic tolerance existed for the intrathecally administered drug (283), due to its slow transmeningeal diffusion rate (276), and for the intra-arterially infused drug where simultaneous parenteral administration of CF conferred protection (246–249). At dose levels used to date in man, no increased toxicity was observed when DCMTX, which is poorly absorbed from the gastrointestinal tract although excreted almost as rapidly as MTX, was administered intravenously instead of orally.

A. G-I tract toxicity

Buccal lesions preceded or accompanied warning symptoms of intolerance such as anorexia, abdominal cramps, nausea, vomiting, and leukopenia. They began as small, shallow, painful, white or yellow, red-edged lesions on the lips, tongue or other buccal mucosa and developed into ulcers, if ignored. More severe toxicity was manifested by *diarrhea*. This coincided with the development of extensive areas of atrophic degeneration and denudation of the villous epithelium and inhibition of mitosis in crypt cells—particularly in areas of the small intestine—as well as of multiple hemorrhagic and morphologic mucosal ulceration along the entirety of the lower gastrointestinal tract (262).

B. Hematologic toxicity

A sudden onset of rapidly progressive leukopenia in the *peripheral circulation* reflected bone marrow depletion and required interruption of drug administration for at least several days. Neither primary leukopenia, thrombocytopenia or anemia, nor development of macrocytosis and neutrophil hypersegmentation—abnormalities which reflect the effect of the drug on the erythroid and myeloid elements of the bone marrow—required suspension of therapy.

The development of pancytopenia, gradually progressing toward *bone marrow* aplasia (256) during prolonged therapy, represented not only toxic but, possibly also, initial clinical response. A severe myeloblastic crisis generally preceded total aplasia, indicating overdosage. Acute bone marrow aplasia was reversible by temporary withdrawal of the antifolic, associated in severe cases with specific measures antagonistic to the drug. Chronic bone marrow aplasia resulting from prolonged drug administration may become irreversible unless treated. It is characterized by severe chronic leukopenia, with progressive anemia, and

thrombocytopenia. The last mentioned sign, coupled with vascular damage, may accentuate the hemorrhagic diathesis in leukemic processes or provoke primary gastrointestinal hemorrhages.

C. Other associated effects

Transitory *alopecia*, due to reversible atrophy of anagenetic hair bulbs (266, 267), frequently developed during prolonged MTX treatment. Although a correlation may exist between this phenomenon and other signs of drug toxicity (46), hair regrowth occurred within one to two months despite continuation of therapy. *Brownish skin pigmentation* may also occur, with no ulterior sequelae detrimental to the patient. Occasional, minor signs of acute toxicity were erythematous or even exfoliative *dermatitis*, the latter limited to the hands and feet. The nonallergic nature of these phenomena was indicated by their absence on readministration of adjusted doses of the drug, following temporary withdrawal. *Middle ear hemorrhages* (50), which may entail serious consequences such as deafness, have been observed in isolated cases as a rare toxic manifestation.

Weight loss may occur in untreated as well as treated patients, but this reflects dysphagia and poor physical condition rather than drug toxicity.

There was a high incidence of *hepatic* portal *fibrosis associated with leukemia* (128, 220, 221, 282) in both untreated and antimetabolite-treated patients. Clinical symptoms included diarrhea, jaundice, and ascites; objective signs were impaired liver function, biliary thrombi and stasis, disruption of liver cell cytology, and fibrous distortion of liver cord structure. Wetherley-Mein and Cottem (282) considered hepatic fibrosis to be independent of the dose, duration, and type of therapy, although they conceded that antimetabolites could be a contributing factor to the development of hepatic fibrosis. Colsky *et al.* (38) attributed hepatic fibrosis to drug injury at the sites of dissolution of leukemic infiltration in the liver and interference with the normal healing process. Murphy (192) believed that there was a definite but indirect drug-fibrosis relationship; *i.e.*, that repeated shrinkage of the liver with fibrous replacement of leukemic foci during drug-induced remission, and subsequent hepatomegaly due to leukemic infiltration during relapse, may have been responsible for the portal cicatrization of hepatic fibrosis. In only 14 leukemic patients in remission was hepatic fibrosis ever associated with prolonged MTX therapy administered alone (38, 128) or in conjunction (128, 178) with steroids and antipurines. Liver toxicity was not considered inevitable, for responsive MTX-treated patients with acute leukemia have retained (203) the ability for normal liver recovery following infectious hepatitis. Be it spontaneous or drug-induced, the development of hepatic fibrosis in leukemic patients makes the interruption of antifolic therapy advisable if not imperative.

Antifolic therapy is strongly contraindicated in *pregnancy*, at least during the first trimester (241), for toxicity may manifest itself in the form of abortion (257), severe maternal intoxication (14, 207, 258), or *teratogenic effects* such as severe congenital malformation (hydrocephalus, harelip, *etc.*) (189, 257, 275).

However, Frenkel and Meyers (91) observed that teratogenic effects are not inevitable sequelae of antifolic therapy during pregnancy. Inhibition of reproductive functions, transitory azoospermia (266) and amenorrhea, were infrequent.

Some authors (226, 277) believe that the high incidence of *cerebral metastases* observed in leukemic patients during therapy may be due, at least in part, to drug-induced acceleration of pathomorphosis. However, both treated and untreated patients surviving to the late stage of disease developed multiple CNS metastases which, according to the preponderance of clinical evidence (45, 141, 192, 276, 277, 283), appeared to be palliated rather than accelerated under the influence of adequate CSF drug levels. Welch (276) attributed the absence of toxicity during such palliation of metastases in the CNS to the nonproliferative neurons' extremely low requirement for DNA synthesis, which renders them resistant to drug concentrations adequate for destruction of neoplastic cells.

During advanced toxicity, *susceptibility to infection* increased progressively, eventually culminating in a chronic septic condition. Possible factors in the development of this phenomenon were 1) reduction in the number of circulating phagocytes, as a result of the drug's leukopenic effect, 2) inhibition of the phagocytic function of leukocytes by the antifolic agent (254), 3) facilitation of bacterial migration, due to ulceration of the intestinal wall, and 4) depression of antibody formation, similar to that elicited with cytostatic agents in rabbits (231) and guinea pigs (56). However, one must not lose sight of the fact that increased susceptibility to infection is generally associated with the advanced leukemic or otherwise malignant process itself, and that it is more frequently correlated with severe neutropenia than with an antibody deficiency syndrome (103).

A high fluid intake during the first weeks of therapy (156) may reduce the danger of *anuria*, a rare but often fatal complication of leukemia.

Toxic effects of overdosage were reversible (23, 94, 228), provided a 3- to 12-mg dose of leucovorin (citrovorum factor, CF), the effective antidote, was administered intramuscularly immediately or at least within less than four hours of onset of toxicity. Citrovorum factor also neutralized the drug's therapeutic effects (22, 23, 27, 94, 96, 228). Other vitamins such as FA (68, 101, 228), vitamin B_{12} (68), and ascorbic acid (86) were ineffective for reversal of toxicity.

In healthy mice and rats, aminopterin and methotrexate toxicity was reported to be potentiated by feeding large amounts of ascorbic acid (86) and inhibited by administration of adenine (28) and deoxypyridoxine (58). The effect of these compounds on the antifolic agents' neoplastic activity is unknown, as is the possible clinical significance of these findings.

IV. LEUKEMIAS AND LYMPHOMATOUS PROCESSES

Leukemia, with about 11,000 new cases occurring each year, accounts for over 10,000 deaths annually in the United States (160). Whereas the total incidence of leukemia is similar for children under twenty years and for adults (65, 180, 261), the frequency of various types of leukemia, such as granulocytic,

lymphocytic, and monocytic varieties of both acute and chronic leukemia, varies according to age. In children under 15 years, acute lymphatic leukemia is most common (80%); in adults, chronic granulocytic and chronic lymphocytic leukemia are most prevalent (10, 160).

Remission, which has a "spontaneous" incidence of 10% in children (57, 69, 242, 260), has been induced with folic acid antagonists in 40 to 68% of children with acute leukemia (26, 69, 71, 89, 131, 288) but in only about 5 to 14% of similarly affected adults (26, 65, 131). Only about one-half of these were complete remissions. Methotrexate was the most effective of the clinically used antifolics (69, 230). Opinions differed on the relative roles played by the antifolics and "spontaneous" factors such as infection (57, 69, 242) and endogenous adrenal and gonadal steroids (69, 101, 261) in the induction of these remissions. In acute leukemia responsive to folic acid antagonists, duration appeared to be approximately proportional to the size of the administered dose (39), whereas survival seemed to be inversely proportional to the degree of initial leukosis (107, 108, 192) and thus independent of therapy. This was confirmed by the findings of Haut *et al.* (107, 108) suggesting that consecutive use of several chemotherapeutic agents appeared to increase survival only among groups of responsive patients.

Although analysis of results is rendered difficult by the common practice of using multiple therapy as well as by great individual variability in degree and frequency of remission thus obtained, the average survival time for acute leukemias (all cell types) from onset of disease to death has been estimated to have increased from about three to four months without therapy (261, 292) to as much as one year by the use of chemotherapy (92, 108, 264). Recently Farber (71) reported 14-month survival in 50% and 32 months in 10% of 800 children with acute leukemia who had been treated with antifolics (MTX and aminopterin) over the past 11 years. Isolated cases of patients surviving over five years on such therapy have been reported (71, 110). No statistics are available on the degree and duration of remission or the possible prolongation of life in leukemic processes, other than acute leukemia, which occasionally respond to antifolic therapy.

A. Susceptibility to antifolics

The morphologic characteristics of the leukemia (54, 286) often, although not invariably, furnish a valuable index of susceptibility to antifolic therapy. For example, the affinity of leukocytes for the drug varies greatly with the type of cell and the type of leukemia (6, 7). Responsiveness to antifolic therapy is greatest where the leukemic process is characterized by a preponderance of "blast" cells. On the other hand, leukemias frequently are of mixed morphologic type; a change in predominance from one cell type to another during the course of disease may reverse the patient's original pattern of positive or negative response to folic acid antagonists.

Best results with antifolic therapy were induced in acute stem cell leukemia

and "blast" stages of most other forms of acute leukemia of children. The latter generally were more responsive than adults in whom response to antifolics was infrequent (49, 243, 296). The non-"blastic" stages of acute monocytic and granulocytic leukemia were almost invariably resistant (52, 53, 92, 296) at all age levels.

Susceptibility of the more slowly progressing varieties of acute leukemia, the so-called subacute leukemias, to antifolic agents followed the above morphologic index.

Chronic leukemias generally responded poorly to antifolic therapy (5, 6). Occasionally remissions were induced in adults with chronic granulocytic leukemia during the radiation-resistant late and "blastic" stages but not during acute terminal exacerbation (5, 26, 190, 192). However, such remissions as did occur were of short duration and inferior to those obtained with other agents such as 6-mercaptopurine (6-MP).

The antifolics also afforded extremely transitory control of Hodgkin's disease (63, 68, 70, 197, 230, 300) and were clinically effective in lymphosarcomas (68, 90, 192, 227, 230, 300), nonlipid reticuloendotheliosis (227, 250) and mycosis fungoides (292, 294). Lymphosarcomas which have evolved to the leukemic phase can be treated like primary acute leukemia.

The findings of an apparent correlation between the efficacy of antifolic agents and the number (239) as well as the degree of maturity (6) of the susceptible leukemic cells indicated that more consideration should be given to early therapy. Several years ago, Skipper *et al.* (239) reported favorable correlation between early therapy with MTX and "curability" of certain mouse leukemias. More recently, Welch (276) advocated initial or early antifolic therapy of acute leukemia, before the stage of CNS infiltration has been reached, for a direct relationship appears to exist between the degree of response of metastases to intracranial MTX and the degree of CNS involvement.

B. Administration and dosage

Antifolic dosage levels and schedules, initially set on an empirical basis of mg drug per kg body weight, must be adjusted according to individual time-dose response and variations in drug tolerance. The average safe course of MTX in leukemias was 50 to 150 mg, generally administered on a basis of daily or biweekly doses of about 0.1 mg/kg/day—1.25 to 5 mg for infants and children, and 2.5 to 10 mg for adults. Equivalent activity but greater danger of toxicity can be obtained with about one-tenth as high a dose of aminopterin. Results of clinical trials indicated that daily subcutaneous doses of 12 mg/m^2 of DCMTX were as safe as but less effective than daily oral doses of 3 mg/m^2 of MTX. No patient who has not been treated for at least six to eight weeks—the usual time lapse between initiation of therapy and onset of response—can be considered drug-resistant. During the remission-relapse interval, therapy may be interrupted or continued on a maintenance basis. Palitzsch (203) recommended giving half the therapeutic dose of MTX thrice weekly, while Condit (40, 43) preferred infre-

quent massive dosage. Following suspension of MTX for the duration of signs of toxicity, Holland (131) recommended one-half the original dose during the first week of readministration.

Frei *et al.* (88, 89) reported that the incidence of remission and rate of survival appeared to be dependent on the total dose rather than on the schedule of administration. However, when remission did occur, it was more prolonged in patients treated once a day rather than in those treated three times per week.

Intravenous, intramuscular, or subcutaneous administration has no known advantage over the oral route with respect to dosage requirements, tolerance, or rate and degree of drug absorption and distribution—except in the case of DCMTX—but may be useful where oral administration is impossible.

Intrathecal MTX is reserved for palliation of neurologic complications due to CNS infiltration in acute leukemia (141, 142, 157, 192, 234, 283), its effects paralleling those of X-rays (142). Most investigators (63, 192, 233, 283) preferred single or multiple courses of two to three doses of 0.25 to 0.33 mg/kg administered intrathecally on alternate days or 0.5 mg/kg every four to seven days. Larger individual doses may cause convulsions due to the slowness of transmeningeal diffusion of MTX in the CSF and, hence, persistence of high localized drug concentrations, as has been demonstrated in dogs (287). Methotrexate concentrations of 20 to 60 mμg/ml persisted in human CSF for three days after an intrathecal dose of 0.30 to 0.46 mg/kg (45). Multiple small doses were well tolerated in man; up to ten such doses have been administered without causing systemic toxicity (192). Although oral MTX is generally suspended during intrathecal administration as a precaution against overdosage, Shambron's (233) results indicated that the small amounts diffusing from the CSF apparently do not bring the drug concentration in serum up to levels capable of inducing systemic toxicity. Onset of response to intrathecal MTX was rapid, generally occurring within a few to 24 hours. Clinical remission and normal CSF cell counts were seen by the third day, and lasted from a few weeks up to three months (45).

C. Therapeutic effects

Preresponse manifestations of mild toxicity may be reversed by the onset of remission, or may develop into serious signs of intolerance requiring drug withdrawal before the time necessary to obtain remission has elapsed.

Remission, with or without objective benefit, is characterized by subjective changes including disappearance or palliation of bone pain, of neurologic symptoms, and of manifestations of purpura and bleeding, as well as by improvement of appetite with weight gain, by normalization of temperature, and by return of the patient to normal performance. The accepted criteria for response to therapy in leukemia have been well summarized by Bisel (12), who described three types of remission: complete, partial, and clinical.

In the *bone marrow*, early response to antifolics and initial remission were characterized by pancytopenia and maturation arrest of both erythroid and myeloid elements (41), with gradual progression toward total aplasia. The char-

acteristic picture (62) showed 1) reduction in leukocyte numbers (especially in polymorphonuclear and metamyelocytic elements) and appearance of abnormal cell types such as hypersegmented polymorphonuclears and giant metamyelocytes, and 2) degeneration of normoblastic and mature erythroid cells, with appearance of abnormal mitoses and nuclear remnants, and an erythroid shift toward basophilic forms and altered patterns in mature cells. Following prolonged therapy, primitive erythroblasts and megaloblasts were observed.

The *peripheral blood picture* of patients treated with antifolics resembled that of nutritional or drug-induced FA deficiency anemia in swine (33) and of pernicious anemia in man. Leukocyte numbers dropped sharply to normal or even leukopenic levels, minimum counts for each cell line being attained after periods approximately corresponding to their life span *in vivo* (41). Most circulating "blast" cells disappeared rapidly from the circulation but not necessarily from the bone marrow. The appearance of mature granulocytes, characterized by relative neutrophilia and some subsequent eosinophilia, signalized the onset of remission. Slight improvement of secondary anemia was observed, despite development of macro- and megaloblastosis during prolonged therapy. Early normalization of platelet values followed therapeutic thrombocytopenia.

Partial or even complete, though temporary, drug-induced regression of *leukemic invasion foci* in the liver, spleen, CNS, and bone (61) was clinically and radiologically demonstrable. Occasionally, similar response was observed in lymphatic, renal, or ophthalmic metastases (141).

D. Combination therapy

Combination therapy was based on the principle that two or more agents which block a given metabolic pathway at different levels might delay or prevent development of drug resistance (22). The most widely used combination therapy in acute leukemia is that of MTX, 6-MP, and adrenocorticosteroids or ACTH. For initial treatment of patients in fair to good clinical condition, the majority of investigators appeared to prefer MTX where the leukocyte count was relatively low, and 6-MP where the count was high; subsequently, combination therapy schedules may be useful. Adrenocorticosteroids or ACTH are generally used to confer immediate relief of acute disease to patients in poor clinical condition, although subsequent transfer to or alternation with MTX, 6-MP, or both, may be indicated for prolonged therapy.

Goldin and Mantel (95) reported that in patients with advanced, but not in those with early stages of acute leukemia, simultaneous therapy with low doses of MTX and 6-MP resulted in longer survival time and less toxicity than treatment with optimal doses of either drug alone. In both man and mice, Venditti *et al.* (270, 271), Holland (130, 131), and Frei *et al.* (88) observed a more or less additive antileukemic effect of simultaneously administered therapeutic doses of MTX and 6-MP.

A cooperative study (in 13 institutions) of 318 patients with acute leukemia revealed (88) that the frequency of remission resulting from sequential or simultaneous administration of MTX and 6-MP was higher in children, but not in

adults, than during treatment with either drug alone. For children, remission rates were 59% with combination therapy, 47% with 6-MP, and 29% with MTX; for adults, 15% with combination therapy, 21% with 6-MP, and 7% with MTX. The incidence and duration of remission as well as the survival time were similar for all three types of combination therapy—1) MTX followed by 6-MP, 2) 6-MP followed by MTX, and 3) MTX and 6-MP given simultaneously. However, long-lasting remissions occurred more frequently during simultaneous therapy. Toxic manifestations of the bone marrow and gastrointestinal tract were quantitatively similar for all three combination schedules, even when the full (classical oral) dosage of both drugs was administered simultaneously. These findings are consistent with the hypothesis of independent action of MTX and 6-MP (88), and would appear to refute the concept (64, 95) of therapeutic synergism.

Adrenocorticotropic hormone (ACTH), *corticosteroids*, and *other steroids* have themselves been found to be highly active and rapidly effective antileukemic agents (11, 72, 120, 205, 219, 224, 298). Ullman (265) has suggested that the clinical use of combination therapy with antifolics and corticosteroids for leukemia would offer the possibility of synergistic effects and delay the onset of drug resistance. Sampey's (224) recent review presented some evidence of a higher incidence, degree, and duration of remissions (72, 77, 173, 182, 183, 184, 187, 203, 264) as well as an increase of the average survival time (152, 161, 211, 212, 220) in leukemic patients receiving antifolics and corticosteroids simultaneously instead of either compound alone. However, the effects of antifolics and corticosteroids are less than additive. In fact, clinical results indicate independent rather than synergistic activity of the two types of compounds. For Bethell (11) has shown that the acute leukemia therapeutic index for antifolics plus steroids (1.53), though slightly superior to that of antifolics alone (1.50) and much superior to that of corticosteroids alone (1.05), is inferior to that of 6-MP given alone (1.56) or together with corticosteroids (1.78). Also, one cannot ignore the evidence that in animals the antileukemic activity of antifolics was reduced by simultaneous administration of cortisone (238) and other steroids (205), while in man concomitant administration of testosterone (236) enhanced the toxicity of classic as well as of massive doses of MTX. Thus it remains a moot point whether the leukemic patient benefits more 1) from the slightly higher therapeutic index offsetting the reduced antileukemic potency and increased toxicity of antifolics during simultaneous administration of steroids, or 2) from alternate or consecutive therapy with antifolics and steroids, where the two compounds neither potentiate nor interfere with each other's efficacy and toxicity.

Synergism of MTX with *diaminopyrimidine* has been observed in mouse leukemia L1210 at dose levels of 1.5 mg/kg and 2.5 mg/kg, respectively, which are suboptimal for the individual drugs (193). At high dose levels, combination therapy with these two drugs was less than additive. *Reserpine* potentiated the teratogenic effect of aminopterin in the fruit-fly (99).

V. SOLID TUMORS

Although a variety of malignant tumors, including trophoblastic neoplasms (34, 200, 204), occasionally evidenced spontaneous regression, their prognosis was more favorable following successful treatment. Antifolic therapy has resulted in varying degrees of response such as shrinkage of the primary or recurrent tumor due to localized destruction of neoplastic tissue (115, 167–170, 172, 174, 244), reduction in size and occasional resorption of pulmonary and hepatic metastases, osteolytic, ulcerative and other metastatic lesions (36, 113, 292), as well as retardation of tumor recurrence. In choriocarcinoma, urinary GTH titers frequently dropped to normal levels.

Solid tumors responded best to massive parenteral dosage (19, 20, 21, 39, 41, 48, 115, 129, 167, 235, 246–249). Occasionally, regression lasting a few months to one and one-half years was observed during prolonged treatment with classical oral (63, 68, 149, 192, 197, 227, 250, 292, 293) or parenteral (268) dosage of approximately 5 mg per day.

In more than one hundred cases of solid tumors treated with repeated courses of massive doses of MTX, systemic toxicity has been moderate to severe, but generally reversible. Fatalities were extremely rare and were attributed to the combined effects of drug toxicity and extreme physical debilitation.

A. Trophoblastic tumors

Remission has been elicited with repeated courses of massive parenteral doses of MTX in about 65% of female patients with malignant chorionic tumors (all histologic types) but not in those suffering from related trophoblastic diseases (19, 20, 21, 60, 115, 116, 129, 167–170, 172, 174, 181, 204, 206, 244, 269). Especially favorable therapeutic results were obtained when the drug was administered during or after surgical excision of the primary tumor. Dosage levels recommended by Li, Hertz, and their associates for female chorionic tumors ranged from 5 mg (116) to as high as 10 to 30 mg (115, 167) per day, administered in multiple intramuscular or intravenous courses of three to six days each. The maximum tolerated total dose recorded in a single patient was 2,750 mg given over a period of 15 months (115).

Response generally occurred within a few days to a few weeks. Several courses of therapy were sometimes necessary. Hertz *et al.* (116) reported that the incidence of complete remissions, lasting from six months to over five years, was 48%; that of incomplete remission, lasting from one month to over three years, 41%. Up to April 1961, three patients had survived four to five years (116). Response to intramuscular DCMTX has been reported (133) in a patient with choriocarcinoma resistant to oral MTX. Whether this response was related to the route of administration or to drug activity *per se* is debatable.

B. Miscellaneous solid tumors

Employed as a last resort in palliative chemotherapy, the classical dosage and schedule of antifolic therapy infrequently induced temporary regression of

primary or metastatic tumors in selected cases of miscellaneous solid neoplasms, including mammary carcinoma (196, 227, 253, 293, 295), testicular seminoma (227), embryonal carcinoma of the testes (297), bladder carcinoma (68, 292), basal cell carcinoma (268), rhabdomyosarcoma (197, 227), and neuroblastoma (68, 197). Wright reported more striking albeit temporary response to MTX in occasional patients with Kaposi's sarcoma (292) and in three of eight patients with mycosis fungoides (292, 294).

Condit, Shnider, and their associates (43, 235) and Zubrod (300) preferred infrequent massive doses of MTX for patients with miscellaneous solid tumors. Their treatment—2.5 to 10 mg of MTX per kg in single intravenous injections given at two- to three-week intervals—was even more vigorous than the short courses of 0.75 to 1.5 mg per kg advocated by Li *et al.* (167) and Hertz *et al.* (115). Shnider, Condit and Owens (235) recorded moderate objective and subjective response in 20% and 38% of their patients, respectively; the responses lasted two to nine weeks. However, the massive doses provoked considerable toxicity in about one-third of the cases (235).

C. Combination therapy

Using antimetabolite-metabolite therapy, Sullivan *et al.* (246–249, 253) obtained an incidence of about 70% complete or partial, though temporary, regression in epidermoid tumors of the head and neck and of the pelvic region. Treatment consisted of single or multiple five- to ten-day courses of massive continuous intraarterial infusion of MTX—as much as 40 to 80 mg per 24-hour period—plus intermittent intramuscular injection of 6 to 9 mg of leucovorin (CF) every four to six hours. Antimetabolite-metabolite infusion was used as primary therapy in a large percentage of a total of 51 patients (246–249, 253). The findings of Westbury (253) and Espiner (253) suggest that prior radiation therapy reduced the incidence and degree of tumor response to MTX-CF therapy. Therapy by antimetabolite-metabolite infusion has also been employed with a degree of objective success in 15 patients with cervical and vaginal carcinoma (263) as well as in a few patients with lymphosarcoma (248), primary and metastatic brain tumors (248, 253), and metastatic hepatic involvement (36, 113). Systemic drug tolerance generally was good, despite signs of oral toxicity. The latter were particularly prevalent during infusion of head and neck tumors. In patients with renal impairment, accumulation of toxic concentrations of the antifolic was avoided by reducing the duration of individual courses of MTX but not of leucovorin by approximately one-half (249).

Testicular seminoma and male choriocarcinoma generally failed to respond to antifolics administered alone or in combination with other antimetabolites such as antipurines (2, 167, 170). However, combination therapy of aminopterin of MTX with irradiation or radiomimetic agents—*e.g.*, following Co^{60} alone or Co^{60} plus triethylenethiophosphoramide (Thio-TEPA) (235), or simultaneously with Actinomycin D and chlorambucil (171, 172)—has produced notable, though transitory, regression of pulmonary, epigastric, and other metastases, but was associated with substantial albeit reversible hematologic, gastrointestinal and

dermatologic toxicity. Similarly, response to combination therapy with MTX and a radiomimetic agent has been observed in other tumors—MTX plus Thio-TEPA for advanced ovarian carcinoma (102) and MTX plus triethylene melamine (TEM) for cancer of the gastrointestinal tract (137, 138).

VI. RESISTANCE

A. Direct resistance

Primary resistance may reflect either very severe host intolerance of therapeutically effective drug levels or absolute refractoriness of the neoplastic cells. This holds true also for *acquired resistance* which results from the adaptation and survival of some malignant cells within an initially drug-sensitive neoplastic process. The mechanism of resistance to antifolics has been discussed in the first part of this review.

Greenspan (101) suggested that the incidence of primary resistance to antifolics is greater in adults than in children because of: 1) a lower FA requirement of leukemic cells in adults, 2) a reluctance to administer adequately high doses, in view of the augmented danger of hemorrhage during toxicity, 3) a higher incidence of acute myeloid leukemia in adults, and 4) a reduced tolerance of antifolics, due to a higher output in adults of endogenous adrenal and gonadal steroids which potentiate antifolic toxicity.

B. Cross resistance

Cells which are resistant to a given antimetabolite also are refractory, *i.e.*, cross-resistant, to those structural analogs of the antimetabolite which inhibit the enzymes affected by this given antimetabolite. Primary or acquired resistance to MTX engenders cross-resistance to aminopterin, DCMTX and other FA analogs, all of which interfere with *de novo* purine synthesis by competing with the metabolite for cellular uptake, and inhibit its conversion to FAH_4. No cross-resistance develops between the FA antagonists and glutamine antagonists [*e.g.*, azaserine and 6-diazo-5-oxo-L-norleucine (DON)], which inhibit the utilization of formate and glutamine for purine synthesis, or between the former and purine analogs (*e.g.*, 6-MP, thioguanine, 2,6-diamino-purine, 8-azaguanine, and adenine), which interfere with purine incorporation into nucleic acids. In fact, responsiveness to MTX has been frequently associated with responsiveness to or dependence on 6-MP or 8-azaguanine (159). The phenomenon of cross-resistance helps to explain why patients resistant to purine analogs may be responsive to MTX. It also elucidates the rationale of combination therapy with these two classes of antimetabolites.

Since antifolics and ACTH or corticosteroids have no biochemical relationship and thus cannot engender cross-resistance, therapy with the former may be beneficial, where indicated, in certain neoplastic processes resistant to these hormones.

VII. COMMENT

The biological functions of folic acid are blocked by various synthetic analogs of this substance, termed "folic acid antagonists." A large amount of information

is available on the enzymatic nature of these functions, which are essential to the multiplication of cells. Folic acid is reduced to tetrahydrofolic acid which forms derivatives that carry "single carbon units" in biochemical reactions in the synthesis of purines, thymine, methionine, serine, and several other metabolites. Certain antagonists, "antifolics," block some of these reactions, and the enzyme system that produces tetrahydrofolic acid is particularly susceptible to blocking by antagonists containing the 2,4-diaminopyrimidine group. The most useful compound in this class is 4-amino-10-methyl folic acid or *methotrexate* (MTX).

Methotrexate and its dihalogenated derivative, 3′,5′-dichloromethotrexate (DCMTX), are used in the treatment of leukemia, principally acute leukemia in children, and chorionic tumors. Dichloromethotrexate has a therapeutic ratio superior to that of MTX in the treatment of mouse leukemia. This superiority does not extend to man. DCMTX is converted by mice to its 4,7-dihydroxy derivative which appears to be inert.

The use of MTX in cancer chemotherapy is based on its toxic effects in cancer cells. It is similarly toxic for the cells of rapidly proliferating normal tissues of the host and there is no indication that MTX is preferentially injurious to neoplasms. Its usefulness seems primarily due to firm combination with the folic acid reductase system and this blocks the enzymatic reactions that are dependent upon folic acid. Cells that contain large amounts of the enzyme are resistant, and the emergence of a cell population containing larger amounts of this enzyme is a possible explanation for the development of resistance to MTX by leukemic patients. It has been emphasized that in the treatment of leukemia, MTX is most useful in the early stages when the cells are predominantly sensitive to the drug. Apparently MTX is not reduced by the folic reductase system and once it has combined with the enzyme the antagonist takes no further part in the biochemistry of the cell. It is possible to hydrogenate MTX chemically, and the product of this reaction is able to block other enzyme systems, perhaps indicating another use for antifolics, although at present the reduced compound does not seem to be selectively toxic against cancer cells in favor of the host (188).

There are some recent findings that indicate the need for caution in adopting a theory that the effect of MTX is solely to prevent the "regeneration" of FAH_4 from FAH_2. It was found by Nath and Greenberg (194) that the form of FAH_2 produced in the thymidylate synthetase reaction as occurring in rat thymus tissue was 5,6-FAH_2 or 5,8-FAH_2, but *not* 7,8-FAH_2, the compound that is prepared synthetically and has been used in most studies of the folic reductase enzyme system. Furthermore, the reductase from chicken pancreas did not reduce the form of FAH_2 isolated by Nath and Greenberg (194). This finding makes it necessary to re-examine the relationships of the enzymes from different sources to various substrates. It is also evident that MTX can inhibit several of the folic acid enzyme systems (253a).

Perhaps the most encouraging information obtained from studies in the folic acid field is the knowledge that the formation of DNA is dependent upon enzymes which are susceptible to blocking by suitable antagonists. The synthesis of new antagonists in this field with the appropriate selectively toxic action against

unwanted cells is a task for the future. Studies of the individual enzymes involved in single-carbon transfer reactions are necessary to further progress in this approach to cancer chemotherapy. However, the reactions of the individual enzymes are no more than a clue to the behavior of the cell, and such studies must extend to investigations with intact cells, where a delicate balance is maintained among the folic acid enzyme systems.

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