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The Role of Dihydrofolic Reductase in the Metabolism of One-Carbon Units*

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Dihydrofolic reductase, which catalyzes the reaction 7,8-dihydrofolate + TPNH + $H^+ \rightleftharpoons$ 5,6,7,8-tetrahydrofolate + TPN^+, and to a lesser extent the reduction of folate, has been obtained in highly purified form from chicken liver, and various properties of this enzyme have been documented. The purified enzyme has been used to prepare the *l*,L-diastereoisomer of tetrahydrofolate and to provide evidence against the possibility that alternate forms (*i.e.*, the 5,6- and 5,8-isomers) of dihydrofolate are produced in certain enzymatic and chemical reactions. The role of dihydrofolic reductase in the biosynthesis of the methyl group of thymidylate is discussed, and the identification of this enzyme as the target site for the folic acid antagonists in the leukemic leukocyte is described.

The field of one-carbon (C_1) metabolism mediated by the folic acid coenzymes had its inception in 1952, when Welch and Nichol proposed that tetrahydrofolate functions in intermediary metabolism as a "carrier" for C_1 units at the oxidation states of both formaldehyde and formate. This supposition was strengthened first when G. R. Greenberg observed that folinic acid (N^5-formyl tetrahydrofolate) was involved in the introduction of a formate unit into the C^2 of the purine ring (Greenberg, 1954), and second when two groups (Kisliuk and Sakami, 1954; Blakley, 1954) discovered independently that tetrahydrofolate is required as a co-factor in the enzymatic synthesis of serine from glycine and formaldehyde. Much information on the chemistry of folic acid compounds was also available at that time (Stokstad, 1954), and from this foundation a number of tetrahydrofolate-dependent enzymes were obtained subsequently in purified form (reviewed by Huennekens and Osborn, 1959; Rabinowitz, 1960), and mechanisms for the various reactions have been suggested (Huennekens *et al.*, 1959).

The structure of tetrahydrofolic acid, and the various C_1 units that are known to form adducts with this coenzyme during metabolic reactions, are illustrated in Figure 1. Metabolites which contain a potential C_1 unit include (*a*) at the formyl (or formimino) level: inosinic acid, histidine, N-formyl glutamate, and formate; (*b*) at the formaldehyde level: serine, glycine, and formaldehyde; and (*c*) at the methyl level: methionine and thymidylate. Diagrams illustrating the metabolic interrelationships of the various donors and acceptors of C_1 groups have been presented elsewhere (Huennekens *et al.*, 1958; Wright, 1958; Huennekens and Osborn, 1959; Rabinowitz, 1960).

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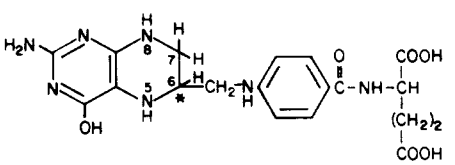
These studies on the *function* of folate coenzymes have been paralleled by other studies concerned with the *biosynthesis* of the coenzyme tetrahydrofolate from the vitamin folic acid. Two recent discoveries have given added importance to this latter area. First, the enzyme responsible for the synthesis of tetrahydrofolate (called hereafter in this paper "dihydrofolic reductase") has been shown to be the probable target site for the antileukemic agents, Aminopterin and Amethopterin, and, secondly, the enzyme has been implicated in methyl group biosynthesis.

I. ENZYMATIC CONVERSION OF FOLIC ACID TO TETRAHYDROFOLIC ACID VIA DIHYDROFOLIC REDUCTASE

Early studies (reviewed by Huennekens and Osborn, 1959) on the conversion of folic acid to folinic acid (N^5-formyl tetrahydrofolate), which were carried out with preparations from mammalian, avian, and bacterial sources, suggested the sequence of reactions shown



TETRAHYDROFOLIC ACID

Site of Attachment			C_1 Unit	Oxidation State
N^5	N^{10}	N^5, N^{10}		
H-C=O	H-C=O		formyl	formate
H-C=NH			formimino	
		CH	methenyl	
		CH_2	methylene	formaldehyde
CH_3			methyl	methanol

FIG. 1.—Structure of tetrahydrofolate and C_1 adducts.

in equation (1). Greenberg (1954) first obtained evidence that the reducing power, [H], in this sequence

$$\text{folate} \xrightarrow{[H]} \text{dihydrofolate} \xrightarrow{[H]} \text{tetrahydrofolate} \xrightarrow{C_1} \text{folinate} \quad (1)$$

was supplied by DPNH. Studies by Futterman and Silverman (1957) revealed that the "inactivation" of folic acid by pig and chicken liver extracts was due, in fact, to the enzymatic reduction of folic acid to the tetrahydro level, followed by a nonenzymatic cleavage of the latter material at the labile C^9-N^{10} linkage to yield the aromatic amine, *p*-aminobenzoylglutamate. When added to the crude systems, both DPNH and TPNH were found to enhance the "inactivation" process.

Partially purified enzyme preparations, which were obtained subsequently from chicken liver (Futterman 1957; Zakrzewski and Nichol, 1958; Obsorn and Huennekens, 1958) and sheep liver (Peters and Greenberg, 1958a; 1959), had in common the ability to catalyze the pyridine nucleotide dependent reduction of both folate and dihydrofolate to tetrahydrofolate.[1] The results of these investigations may be summarized as follows: (*a*) A single enzyme catalyzes both reductive steps; (*b*) dihydrofolate is a better substrate than folate[2]; (*c*) TPNH is the preferred reductant, although some activity is observed with DPNH; (*d*) the enzyme exhibits two *p*H optima, *viz.*, at 4.5 and 7.5; and (*e*) the enzyme is inhibited by extremely low concentrations of the folic acid antagonists Aminopterin and Amethopterin.

There were, however, some uncertainities in the above observations which resulted from the fact that the data had been obtained with relatively crude preparations. In an effort to overcome this difficulty, the chicken liver reductase has been brought to a high degree of purification by Mathews in this laboratory (Mathews, 1962). From the high-speed supernatant fraction of a chicken liver homogenate, the enzyme was purified by fractionation with protamine and ammonium sulfate, removal of unwanted proteins by treatment with chloroform-ethanol, and, finally, chromatography on hydroxylapatite. The best preparations, about 1200-fold purified over the original homogenate, have a specific activity of 2.6 μmoles of substrate converted per minute per mg of protein at *p*H 4.5 and may be compared with the preparation of dihydrofolic reductase from *S. faecalis R* (Blakley and McDougall, 1961), which has a specific activity of 4.65 when recalculated to the same units.

As seen from the stoichiometry of equation (2),

$$\text{dihydrofolate} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{tetrahydrofolate} + \text{TPN}^+ \quad (2)$$

dihydrofolic reductase may be assayed conveniently by following the dihydrofolate-dependent disappearance of TPNH at 340 mμ. Because dihydrofolate, in addition to TPNH, exhibits some absorbancy at this wave length, a correction must be made to the observed decrease in absorbancy in order to obtain the absolute rate of the reaction (Misra *et al.*, 1961; Mathews, 1962). It should be noted that the spectrophotometric assay has a limitation, inasmuch as crude reductase preparations may contain TPNH-oxidizing systems or TPN-dependent dehydrogenases and their substrates. Since dihydrofolic reductase from several sources appears to be insensitive to *p*-chloromercuribenzoate and other sulhydryl reagents (Peters and Greenberg, 1958b; Blakley and McDougall, 1961; Mathews, 1962), the inclusion of such inhibitors in the assay system might be used to suppress the activity of the interfering enzymes. Another possible solution to the problem of assaying dihydrofolic reductase in crude preparations may be found in the use of fluorimetric techniques, since both dihydrofolate and TPNH, but not the reaction products, are highly fluorescent.

Other investigations (Zakrzewski and Nichol, 1958; Zakrzewski, 1961) have utilized a different type of assay for following the reduction of folate or dihydrofolate *via* dihydrofolic reductase. The method is based upon the fact that tetrahydrofolate is cleaved nonenzymatically at the C^9-N^{10} bond, with the liberation of *p*-aminobenzoylglutamate, which is detected by the Bratton-Marshall reaction for aromatic amines. The principal defect in this assay is that, while the response with a primary standard such as *p*-aminobenzoylglutamate is rapid and quantitative, the breakdown of tetrahydrofolate under the assay conditions is relatively slow and does not lead in any event to the production of more than about 30–40% of the theoretical amount of diazotizable amine (Scrimgeour, 1961). In addition, both folate and dihydrofolate give small but significant responses in this assay (Peters and Greenberg, 1958b; Scrimgeour, 1961). It has been suggested (Zakrzewski, 1961) that the yield of diazotizable amine is low because only one of the diastereoisomers of tetrahydrofolate (see Section II) is reactive under these conditions. This hypothesis is invalidated, however, by the observation that, when equal amounts of *dl*,L-tetrahydrofolate and the *l*,L-diastereoisomer are subjected to the Bratton-Marshall assay, both yield the same amount of diazotizable amine (Scrimgeour, 1961). Moreover, when a neutral solution of the *l*,L-diastereoisomer stabilized by the addition of mercaptoethanol is allowed to stand for several days, no change in the optical rotation is observed, indicating that no racemization occurs at the C^6-position (Scrimgeour and Mathews, unpublished observations).

The purified dihydrofolic reductase from chicken liver is stable to lyophilization, to freezing and thawing, and to prolonged dialysis. The enzyme is colorless and has a single absorption maximum at 280 mμ; there is no flavin or heme in the enzyme. Although the enzyme has not yet been examined spectrographically for the presence of bound metal ions, the fact that reaction (2) is not inhibited by the number of metal-binding agents (*e.g.*, EDTA, cyanide, diethyldithiocarbamate, hydroxylamine, 8-hydroxyquinoline, and 1,10-phenan-

[1] A folic acid-reducing system, discovered in *Clostridium sticklandii* by Wright and Anderson (1957), does not conform to this pattern in several respects: (*a*) The reduction of folate to dihydrofolate is linked to the oxidation of pyruvate; (*b*) Aminopterin and Amethopterin are substrates for this enzyme, rather than inhibitors; and (*c*) dihydrofolate is not reduced to tetrahydrofolate, either by the pyruvate-linked system or by the addition of DPNH or TPNH. In another exception to this generalization, Blakley and McDougall (1961) have recently obtained from *Streptococcus faecalis* a 300-fold purified dihydrofolic reductase which is apparently unreactive toward folate.

[2] We believe that the term "dihydrofolic reductase" is more suitable than "folic reductase" for the following reasons: (*a*) the chicken liver enzyme, as well as its counterpart in other tissues, uses dihydrofolate at a faster rate than folate; and (*b*) folate is inactive as a substrate at physiological *p*H values. The higher activity with dihydrofolate may reflect a heavier demand on the dihydrofolate-tetrahydrofolate cycle, *e.g.*, as a component of the thymidylate-synthesizing system (*cf.* Section III). Of interest also in this connection is the fact that in *E. coli* the biosynthesis of folic acid from a pteridine, *p*-aminobenzoic acid, and glutamate leads to the product at the dihydro level (Brown *et al.*, 1961).

throline) may be taken as presumptive evidence that the enzyme is not a metalloprotein.

The substrate specificity of the chicken liver enzyme with respect to folate *versus* dihydrofolate and TPNH *versus* DPNH is illustrated by the *p*H optimum curves in Figure 2. The enzyme displays two *p*H optima, *viz.*, at 4.5 and 7.5, with the activity being two-fold greater at the acid value. Dihydrofolate is utilized about three times faster than folate[3] at the acid *p*H optimum, and DPNH is effective as a reductant only at the acid *p*H value. A constant ratio of dihydrofolate-folate activity, measured at *p*H 4.5 with TPNH as the reductant, is observed over the course of a 600-fold purification of the enzyme; this observation provides support for the contention that a single enzyme is involved in both reductive steps.

The curious double optima seen in the *p*H-activity curve (Fig. 2) raises the question whether the enzyme catalyzes the same reaction (equation 2) in each instance. That tetrahydrofolate is, in fact, the reaction product at both *p*H values was demonstrated by means of its absorption spectrum (λ_{max} at 298 mμ) and by its conversion to N^{10}-formyl tetrahydrofolate (reaction 3) *via* the purified formate-activating enzyme from *Micrococcus aerogenes* (Whiteley *et al.*, 1959). Further-

$$\text{tetrahydrofolate} + \text{formate} + \text{ATP} \rightleftharpoons N^{10}\text{-formyl tetrahydrofolate} + \text{ADP} + P_i \quad (3)$$

more, in the presence of Aminopterin the same percentage inhibition of dihydrofolic reductase was observed at both *p*H values. These experiments establish the validity of equation (2) for the reaction at either *p*H optimum.

With the availability of highly purified dihydrofolic reductase, it was possible to measure the equilibrium constant for reaction (2). By admixing together tetrahydrofolate and TPN in the presence of enzyme, a small but measurable amount of TPNH was formed. Several determinations of the equilibrium constant (equation 4) by this procedure have led to an average

$$K = \frac{(\text{tetrahydrofolate})\ (TPN^+)}{(\text{dihydrofolate})\ (TPNH)\ (H^+)} \quad (4)$$

value of $K = 5.6 \times 10^{11}$, or 5.6×10^4 at *p*H 7. With a value of the standard reduction potential (E_0') for the TPN-TPNH couple as -0.32 volts (Rodkey and Donovan, 1959), it is possible to calculate from the above equilibrium constant a value of $E_0' = -0.19$ volts for the dihydrofolate-tetrahydrofolate couple; this potential falls within the range of values associated with substrates of pyridinoprotein dehydrogenases (White *et al.*, 1959). It should be noted that in the above measurements of the equilibrium constant corrections were made for the small absorbancy changes occurring in the "blanks" in which either tetrahydrofolate or TPN were omitted; this answers the criticism of Blakley and Mc Dougall (1961) that earlier measurements (Osborn and Huennekens, 1958) of the reversibility of reaction (2) might be invalid because of non-enzymatic, oxidative destruction of tetrahydrofolate.

II. Preparation of the *l*,L-Diastereoisomer of Tetrahydrofolate

Dihydrofolic reductase has also been used to prepare the *l*,L-diastereoisomer of tetrahydrofolate (Mathews and Huennekens, 1960). In addition to the L-configuration at the α-carbon atom of the glutamate residue,

[3] N^{10}-Formyl dihydrofolate (Mathews, 1962) is reduced at a slow rate by the enzyme, while N^{10}-formyl folate is inert as a substrate.

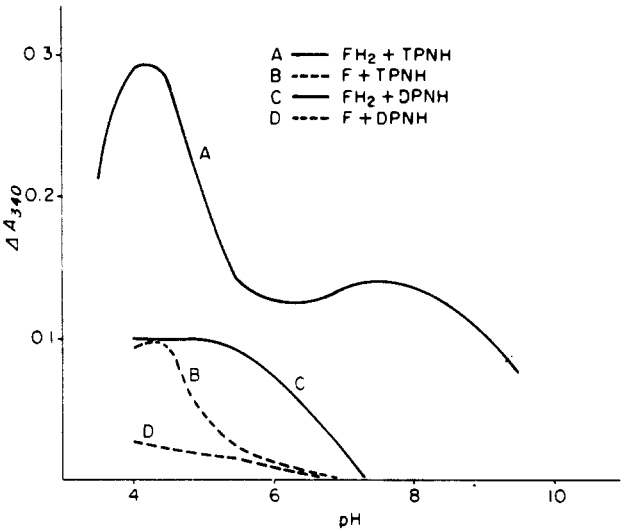


FIG. 2.—*p*H-Substrate curves for chicken liver dihydrofolic reductase (Mathews, 1962). F and FH_2 represent folate and dihydrofolate, respectively.

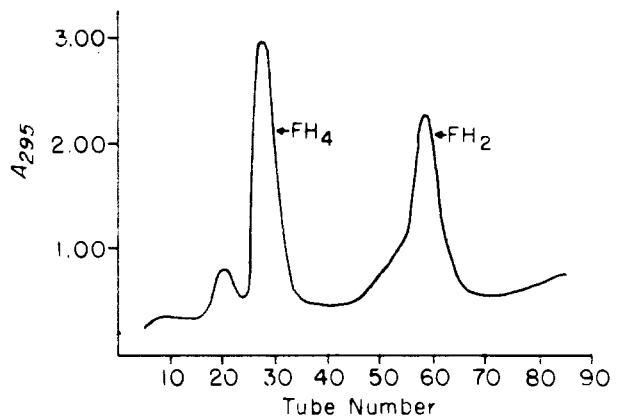


FIG. 3.—Separation of tetrahydrofolate and dihydrofolate by chromatography on DEAE-cellulose (Mathews and Huennekens, 1960). FH_2 and FH_4 represent dihydrofolate and tetrahydrofolate, respectively.

reduced forms of folic acid may have a second asymmetric center at the C^6-position (marked with an asterisk in Figure 1). It has been shown in several investigations that tetrahydrofolate prepared by the catalytic hydrogenation of folic acid is a *dl*-mixture with respect to the C^6-position, since only 50% of the material reacts in enzymatic systems (*e.g.*, equation 3). Assuming that chemically prepared dihydrofolate is the 7,8-isomer (see section IV), *enzymatic* reduction of dihydrofolate to tetrahydrofolate *via* reaction (2) should result in a single diastereoisomer, presumably the same as naturally occurring tetrahydrofolate. Dihydrofolate and TPNH were incubated together and the reaction mixture chromatographed on DEAE[4]-cellulose. Tris buffer, *p*H 7.0, was used as the eluant and the concentration was varied from 0.005 M to 0.2 M by a gradient technique. Under these conditions, tetrahydrofolate and dihydrofolate are separated readily, as shown by the elution profile in Figure 3. Enzymatically synthesized tetrahydrofolate was thus obtained in an over-all yield of about 70–80%, including the isolation step. When enzymatically prepared tetrahydrofolate was assayed with the formate-activating enzyme (equation

[4] The following abbreviations are used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

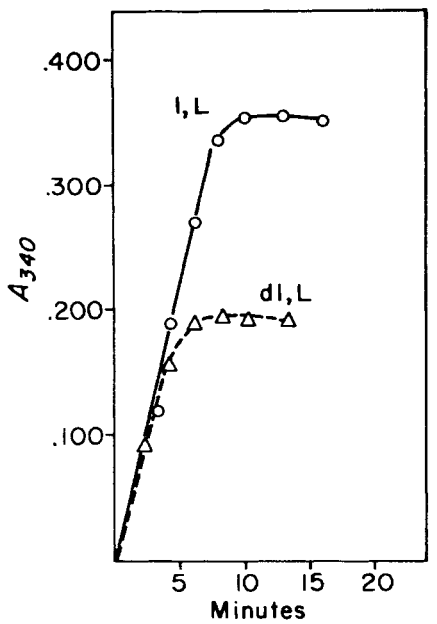


FIG. 4.—Activity of *dl*,L- and *l*,L-tetrahydrofolate samples in the coupled system, serine hydroxymethylase-N^5,N^{10}-methylene tetrahydrofolic dehydrogenase (Mathews and Huennekens, 1960).

3), it was twice as reactive as chemically prepared tetrahydrofolate. The same effect was noted when the tetrahydrofolate samples were compared (Fig. 4) in the coupled enzyme system: serine hydroxymethylase and N^5,N^{10}-methylene tetrahydrofolic dehydrogenase (reaction 5). Final proof that the enzymatic synthesis of tetrahydrofolate yields a single diastereo-

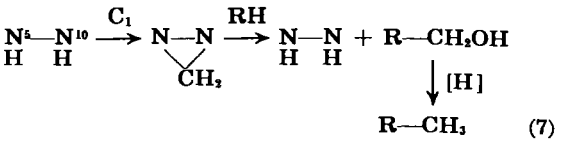
$$\text{serine} + \text{tetrahydrofolate} + \text{TPN}^+ \rightleftharpoons \text{glycine} + (N^5,N^{10}\text{-methenyl tetrahydrofolate})^+ + \text{TPNH} \quad (5)$$

isomer was provided by optical rotation measurements. Folate, dihydrofolate, and chemically prepared tetrahydrofolate each have a positive rotation ($[\alpha]_D^{27}$ = +15 to +20°) derived solely from the L-glutamate residue. Enzymatically prepared tetrahydrofolate, on the other hand, has a large *negative* rotation (*ca.* −17°), showing the overwhelming contribution from the C^6-position. Cosulich *et al.* (1952) have previously resolved chemically prepared folinic acid and shown that the *l*,L-diastereoisomer has a rotation of −15.1°. More recently, Wahba and Friedkin (1961a) have reported the electrophoretic separation of chemically prepared tetrahydrofolate into two components, possibly diastereoisomers. Kisliuk (1961) and Kaufman (1961) have also prepared, but not isolated, *l*,L-tetrahydrofolate by allowing naturally occurring *l*,L-folinic acid to react in the glutamic transformylase system:

$$N^5\text{-formyl tetrahydrofolate} + \text{glutamate} \longrightarrow \text{tetrahydrofolate} + \text{N-formyl glutamate} \quad (6)$$

III. Role of Dihydrofolic Reductase in Methyl Group Biosynthesis

Early nutritional and tracer data established that the methyl groups of methionine and thymidylate arose from a C_1 unit at the oxidation level of formaldehyde *via* a folic acid–dependent pathway (reviewed by Huennekens and Osborn, 1959). Vitamin B_{12} also appeared to be involved in these biosynthetic processes. A generalized pathway could be written for both of these methylation reactions as shown in (7), where N^5(H)—N^{10}(H)



(7)

symbolizes the N^5- and N^{10}-positions of tetrahydrofolate, N—N is "active formaldehyde," and RH is the methyl group acceptor (homocysteine in methionine synthesis, and dexoyuridylate in thymidylate synthesis). The natural occurrence of 5-hydroxymethyl cytosine gave support for the above pathway, which could be considered formally as two separate reactions: (*a*) transfer of the C_1 unit; and (*b*) reduction of the C_1 unit. The exact nature of the reducing power, [H], was unknown, although a reduced pyridine nucleotide appeared to be the likely agent.

Attractive though the above unified hypothesis might be, recent work has shown that neither thymidylate nor methionine biosynthesis follows this pathway. After the demonstration by Friedkin and Kornberg (1957) that HCHO, deoxyuridylate, and tetrahydrofolate were required for thymidylate synthesis in cell-free preparations from *E. coli*, it was observed in the laboratories of D. M. Greenberg (Humphreys and Greenberg, 1958) Blakley (McDougall and Blakley, 1960; Blakley and McDougall, 1961) and Friedkin (1959a) that tetrahydrofolate itself supplies the reducing power in reaction (6) and is thereby oxidized to dihydrofolic acid in the process.[5] With a partially purified enzyme from *E. coli*, Wahba and Friedkin (1961) have established the stoichiometry of reaction (8). Thus, a "thymidylate-synthesizing cycle" can be envisioned in which dihydrofolate, produced in reaction (9), is reconverted

$$N^5,N^{10}\text{-methylene tetrahydrofolate} + \text{deoxyuridylate} \longrightarrow \text{dihydrofolate} + \text{thymidylate} \quad (9)$$

to N^5,N^{10}-methylene tetrahydrofolate *via* dihydrofolic reductase (reaction 2) and serine hydroxymethylase (reaction 10).

$$\text{serine} + \text{tetrahydrofolate} \rightleftharpoons \text{glycine} + N^5,N^{10}\text{-methylene tetrahydrofolate} \quad (10)$$

Reaction (9), which proceeds without the formation of the 5-hydroxymethyl deoxyuridylate as an intermediate, is the most general representation for thymidylate synthesis, since it has been shown to be present also in the thymus (Nath and Greenberg, 1961), the leukocytes in acute myelogenous leukemia (Silber *et al.*, 1962), ascites tumor cells (Hartmann and Heidelberger, 1961), and *S. faecalis* (Blakley and McDougall, 1961). In phage-infected *E. coli*, however, a reaction such as that shown the first part of equation (7) occurs in the hydroxymethylation of deoxycytidylate (Flaks and Cohen, 1959; Somerville *et al.*, 1959). No requirement for a vitamin B_{12} coenzyme has yet been demonstrated for thymidylate synthesis in any of the above systems.

Reaction (9) could result from the two-step mechanism similar to that proposed by Friedkin (1959b), out-

[5] In the conversion of phenylalanine to tyrosine, Kaufman (1957) has shown that tetrahydropteridines are required in the over-all reaction, represented by equation (8), and that, during the reaction, oxidation to dihydropteridines occurs.

$$\text{phenylalanine} + \text{TPNH} + O_2 + H^+ \longrightarrow \text{tyrosine} + \text{TPN}^+ + H_2O \quad (8)$$

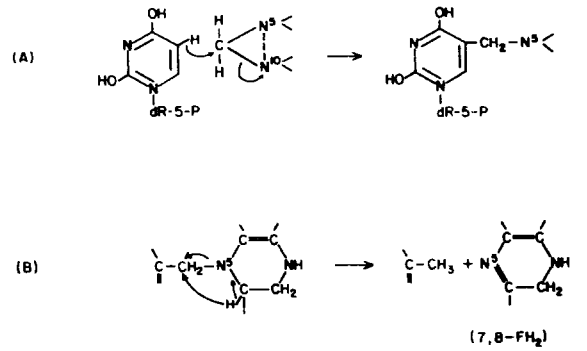


FIG. 5.—Proposed mechanism for the biosynthesis of thymidylate.

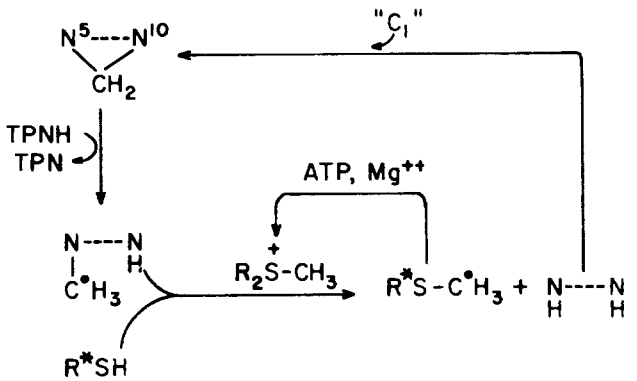


FIG. 6.—Proposed pathway for biosynthesis of methionine. $R_2\overset{+}{S}—CH_3$ represents *S*-adenosyl methionine.

lined in Figure 5. The first step involves a nucleophilic attack by the C^6 of the pyrimidine ring upon the methylene group of "active formaldehyde" to yield an intermediate in which the methylene group is bridged between the C^6 and the N^5-position of tetrahydrofolate. An intramolecular oxidoreduction follows (step B) in which reducing power from the pyrazine ring is used to cleave the bond between the methylene bridge and the N^5-nitrogen to yield 5-methyl deoxyuridylate (*i.e.*, thymidylate) and 7,8-dihydrofolate. Support for this mechanism, and especially step B, is provided by Friedkin's data (1959a) in which tritium-labeled tetrahydrofolate (presumably 5,6,7,8-H^3) was shown to label the methyl group of thymidylate.

Methionine synthesis is accomplished by a pathway quite different from that shown in reaction (9). In bacterial and mammalian systems "active formaldehyde" is first reduced by a pyridine nucleotide-dependent system to yield N^5-methyl tetrahydrofolate ("prefolic A" of Keresztesy and Donaldson, 1961). The latter compound reacts with homocysteine, perhaps *via* an intermediate, to produce methionine and tetrahydrofolate (Wilmanns *et al.*, 1960; Buchanan *et al.*, 1961; Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962) as outlined in Figure 6. It should be noted that the pyrazine ring of tetrahydrofolate is not oxidized at any point in this cycle, and hence dihydrofolic reductase is not required. Buchanan's definitive studies on methionine biosynthesis, carried out with a series of purified bacterial enzymes, have uncovered requirements for FAD and a vitamin B_{12}-containing protein (Buchanan *et al.*, 1961) in the process, although the function of these factors has not yet been elucidated. S-Adenosyl methionine, in catalytic amounts, replaces ATP and Mg^{++} in the sequence shown in Figure 6 (Mangum and Scrimgeour, 1962).

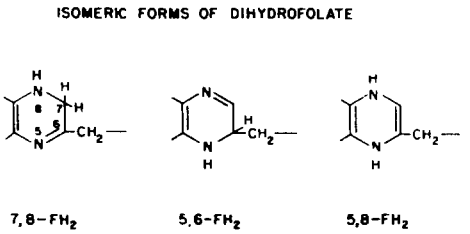


FIG. 7.—Tautomeric forms of dihydrofolate.

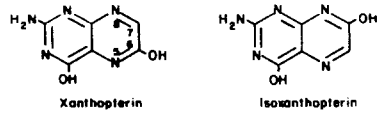


FIG. 8.—Xanthopterin and isoxanthopterin.

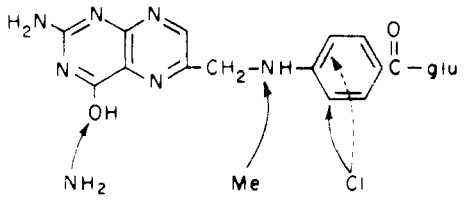
IV. Alternate Forms of Dihydrofolate

As shown in Figure 7, dihydrofolate can assume three tautomeric forms, differing only in the site of reduction. In order to provide a reference point for the present discussion, we shall define "standard" dihydrofolate as that material prepared by the reduction of folate with hydrosulfite (Futterman, 1957) or by the catalytic hydrogenation of folate in an alkaline medium (O'Dell *et al.*, 1947). Dihydrofolate prepared in this manner has been employed as the substrate for most previous studies on dihydrofolic reductase. The assignment of the 7,8-dihydro structure to "standard" dihydrofolate was based upon the following indirect lines of evidence: (*a*) xanthopterin (Fig. 8), which has a double bond at the 7,8-position (the OH group at C^6 is largely in the keto form), is readily reduced to the dihydro stage, while isoxanthopterin (Fig. 8), which has a 5,6-double bond, is refractory to reduction; and (*b*) tetrahydrofolate is quite susceptible to air-oxidation, but shielding of the N^5-position, as in folinic acid, confers upon the reduced pterin ring a resistance to oxidation. Thus, oxidation of tetrahydrofolate occurs initially at the 5,6-position to yield 7,8-dihydrofolate.

More direct evidence on this point was obtained from our earlier studies (Osborn and Huennekens, 1958) on dihydrofolic reductase in which it was shown that "standard" dihydrofolate reacted to greater than 90% in reaction (2). This observation ruled out the 5,6-isomer, which would have reacted only to the extent of 50% because the chemical route used in the synthesis of the material would have resulted in *dl*-isomerism at the C^6 position. It should be noted that this last argument would not apply if "standard" dihydrofolate were the 5,8-isomer.[6]

It has been suggested recently (Greenberg, 1961) that the dihydrofolate which appears as a reaction product in thymidylate synthesis *via* equation (9) is the 5,8-isomer. Conversion of this material to the conventional 7,8-isomer was believed to be catalyzed by an isomerase present in the thymidylate synthetase preparation from calf thymus. In

[6] Authentic 5,8-dihydropterins have been synthesized, however, by Pfleiderer and Taylor (1960). These compounds, which are characterized by resonance stabilization of the 6,7-double bond, an alkyl substituent at the 8-position, an electron-donating substituent at the 2-position, and a hydrogen atom or alkyl group at the 4-position, exhibit anomalously high absorption maxima at 405–410 mμ. On the basis of quantum mechanical calculations on the structures of various dihydrofolate isomers, Perault and Pullman (1960) believe that the 5,8-isomer would be resistant to further reduction.



Aminopterin = 4-amino PGA

Amethopterin = 4-amino, 10-methyl PGA

Dichloroamethopterin = 4-amino, 10-methyl, 3,5-dicnloro PGA

FIG. 9.—Structures of folic acid antagonists. PGA represents pteroyl glutamic acid.

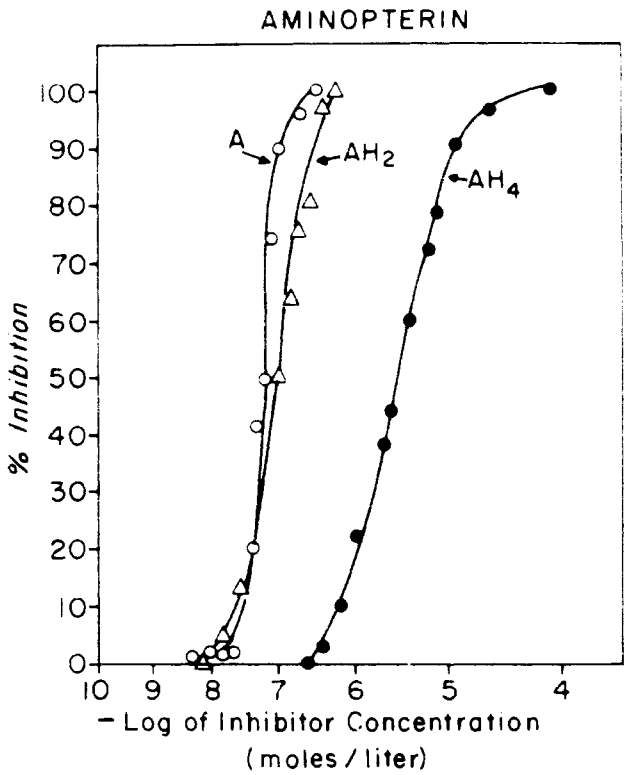


FIG. 10.—Inhibition of chicken liver dihydrofolic reductase by aminopterin and its reduced derivatives (Ozols, 1962). A, AH_2, and AH_4 refer to aminopterin, dihydroaminopterin and tetrahydroaminopterin, respectively.

contrast, however, Wahba and Friedkin (1961a) found the 7,8-isomer of dihydrofolate as the co-product of thymidylate in the purified thymidylate synthetase system from *E. coli.* Nath and Greenberg (1961) also suggested that the dihydrofolate produced by the pterin reductase from *C. stricklandii* (Wright and Anderson, 1957) is the 5,8-isomer. In still another instance, Kaufman (1961) has reported that, in the tetrahydropterin-dependent hydroxylation of phenylalanine to tyrosine [*cf.* equation (8), footnote 5], a labile dihydropterin produced in the primary reaction is isomerized rapidly to the more stable 7,8-dihydro form. The same labile isomer was also reported to be produced by the oxidation of tetrahydrofolate with dyes such as 2,6-dichlorophenolindophenol. In most of the examples cited above, however, the dihydrofolate in question was not actually isolated from the enzymatic or chemical reaction mixture in which it was synthesized.

In order to investigate this important question, we have repeated the preparation of dihydrofolic acid by four of the above routes: (1) reduction of folic acid with hydrosulfite; (2) reduction of folic acid with the pterin reductase from *C. sticklandii;* (3) oxidation of tetrahydrofolic acid during the reductive synthesis of thymidylate; and (4) oxidation of tetrahydrofolic acid with indophenol. Each preparation of dihydrofolate was isolated from its reaction mixture by the chromatographic method using DEAE cellulose, as described in Figure 3. When examined after purification, all four dihydrofolate samples were identical with respect to the following physical and chemical properties: (*a*) absorption maximum at 282 mμ (*p*H 7) with a shoulder at 300 mμ; (*b*) fluorescence maximum at 425 mμ; (*c*) R_F values in three different solvent systems; (*d*) conversion to a yellow degradation product (absorption maximum at 420 mμ) when treated with trichloracetic acid (Wright *et al.*, 1958); (*e*) facile reduction to tetrahydrofolate with borohydride; and (*f*) optical rotation, $[\alpha]_D^{25}$, of *ca.* + 16°. Finally, when assayed enzymatically with dihydrofolic reductase preparations from chicken liver (Mathews, 1962), sheep liver (Peters and Greenberg, 1958b), and calf thymus (Greenberg, 1961), each sample of dihydrofolate was reduced at the same initial rate and to the same extent (95–100%).

From these data it would appear that only one isomer of dihydrofolate is produced by the above four routes. The possibility that a labile form, produced in the primary reaction, isomerizes to the more stable 7,8-form during the chromatographic step is inconsistent with the fact that the *l*,L-diastereoisomer of tetrahydrofolate does not racemize during column chromatography, *i.e.*, at least the carbon-bound hydrogen at C^6 is not labilized during this process.

V. Inhibition of Dihydrofolic Reductase by Folic Acid Antagonists

As a corollary to studies on the nutritional aspects of folic and folinic acids, it was found that the utilization of the former compound, but not the latter, was markedly inhibited by the structural analog (Fig. 9) of the vitamin, Aminopterin and Amethopterin (reviewed by Nichol and Welch, 1950). The site of action of these antagonists was thus localized at some point in the enzymatic conversion of folic to folinic acid (*cf.* equation 1). As the details of this sequence became understood, it was shown by Futterman and Silverman (1957) and by Osborn *et al.* (1958) that the antagonists were potent inhibitors of dihydrofolic reductase. The latter investigators reported K_I values of *ca.* 10^{-9} M for the noncompetitive inhibition of dihydrofolic reductase by these analogs.[7] Of interest is the fact that reduced forms of aminopterin, *i.e.*, dihydroaminopterin and tetrahydroaminopterin, also inhibit dihydrofolic reductase (Ozols, 1962). As shown in Figure 10, the dihydro compound is about equal to aminopterin in inhibitory power, but the tetrahydro compound displays a considerably weaker affinity for the enzyme.

Werkheiser (1961) has criticized the results of Osborn *et al.* (1958) on the grounds that conventional Michaelis-Menten kinetics are not applicable since the binding of enzyme (E) and inhibitior (I) is sufficiently tight to preclude any possibility of a reversible E-I interaction. In support of this argument, he found in a rat liver extract that when reductase activity is plotted against enzyme concentration in the presence of a fixed level of inhibitor a biphasic curve (*A* in Fig. 11) is obtained; this type of inhibition has been termed "stoichiometric."

[7] A value of 5×10^{-7} M has been reported (Osborn and Huennekens, 1958) for the Michaelis constant (K_m) of the chicken liver reductase with dihydrofolate.

Using the highly purified reductase from chicken liver, Mathews (1962) repeated this interesting experiment but found that the data conformed to a hyperbola (curve *B*, Fig. 11) rather than a sharp biphasic curve. This behavior is consistent with the existence of a small but measureable reversibility of the E-I complex.[8] It should be noted, however, that less purified preparations of dihydrofolic reductase from human leukemic leukocytes (Bertino *et al.*, 1962b) and from intestinal epithelial cells (Nutter and Huennekens, unpublished results) show plots which are intermediate between curves *A* and *B*. Thus, the type of kinetic behavior may depend upon the source and purity of the enzyme.

Additional evidence against the possibility that the antagonists inhibit the reductase irreversibly is provided by the finding that identical concentrations of Aminopterin, Amethopterin, and Dichloramethopterin produce different degrees of inhibition with the reductase from leukocytes in acute leukemia (Bertino *et al.*, 1960b) and from the spleens of mice with L-1210 leukemia (Misra *et al.*, 1961). Pertaining to the same point, the Aminopterin-inhibited reductase from rat liver can be dissociated by dialysis against a relatively high concentration of folic acid (Werkheiser, 1961), and the inhibited leukocyte enzyme is reactivated even by dialysis against phosphate buffer. On the other hand, the inhibited chicken liver reductase is not dissociated by prolonged dialysis against various buffers. The Aminopterin-inhibited forms of both the chicken liver and the leukocyte reductase can be dissociated, however, by passing the material through hydroxylapatite or DEAE-cellulose columns (*cf.* Fig. 12). This somewhat unexpected lability of the E-I complex toward chromatography unfortunately obviates the possibility of tagging the "active center" of the purified chicken liver reductase with H^3-Aminopterin and subsequently isolating a small labeled peptide.

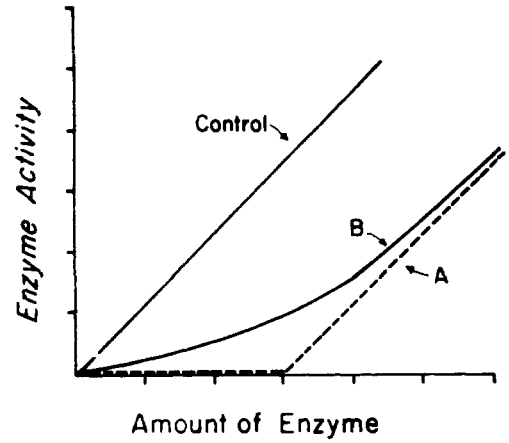


FIG. 11.—Titration of dihydrofolic reductase with aminopterin.

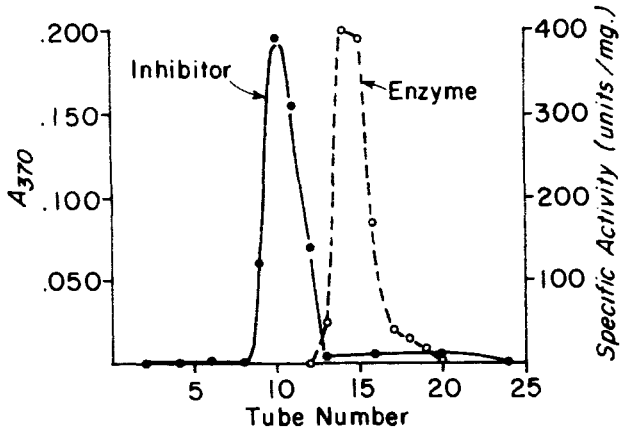


FIG. 12.—Dissociation of reductase-aminopterin complex by chromatography on hydroxylapatite (Mathews, 1962).

VI. Dihydrofolic Reductase in Normal and Leukemic Leukocytes

For many years biochemists and clinicians have searched for biochemical differences between normal and leukemic leukocytes, especially those which might be exploited as targets for chemotherapeutic agents. It is evident that the most profitable metabolic areas for exploration are those involved directly in cell replication, namely, RNA, DNA, and protein synthesis. Folic acid coenzymes are known to participate in the biosynthesis of key precursors in each of these metabolic areas (reviewed by Huennekens and Osborn, 1959), and it is perhaps significant that leukemic leukocytes contain an elevated level of both folic and folinic acids (reviewed by Ellison and Hutchison, 1957). Finally, the folic acid antagonists are used extensively, since it is now well established that certain patients with acute leukemia respond satisfactorily, although transiently, to this regimen. The inhibition of dihydrofolic reductase by extremely low levels of the folic acid antagonists suggested that in the leukocyte this enzyme might represent the target site for these chemotherapeutic agents. The interrelationship of dihydrofolic reductase with thymidylate synthesis (*cf.* Section III), and hence with DNA synthesis, would provide an explanation for the manner in which folic acid antagonists could eventually block cell replication.

In collaboration with Drs. Beverly Gabrio, Joseph Bertino, and Robert Silber, a general study was undertaken in normal and leukemic leukocytes of the enzymes concerned with one-carbon metabolism, especially dihydrofolic reductase. Leukocytes were isolated from whole blood and lysates were prepared by one of three methods: (*a*) fragmentation in a high-speed Virtis homogenizer; (*b*) repeated freezing and thawing; and (*c*) treatment with acetone at −20°. Initially, it was found that the levels of formate-activating enzyme, serine hydroxymethylase, and N^5N^{10}-methylene tetrahydrofolic dehydrogenase are elevated about 2-fold in cells of acute leukemia as compared to the normal cells (Bertino *et al.*, 1960b, 1962b).

Greater interest, however, centered about the dihydrofolic reductase (Bertino *et al.*, 1960b). It was not possible to detect the presence of this enzyme in normal leukocytes whether the assays were performed *via* the direct reaction (equation 2) or indirectly by coupling reaction (2) with reaction (3) catalyzed by endogenous formate-activating enzyme. Through the use of the coupled assay, evidence was obtained, however, for the presence of the enzyme in acute leukemic cells. Dihydrofolic reductase was then purified about 30-fold from acute leukemic leukocytes by ammonium sulfate fractionation followed by adsorption and elution from calcium phosphate gel. It was possible to assay the purified enzyme by means of the direct assay, *i.e.*, the dihydrofolate-dependent disappearance of TPNH *via* reaction (2). The leukocyte enzyme has somewhat different properties than its counterpart from chicken liver. For example, the *p*H optimum occurs at 8.3, with a smaller optimum near *p*H 5, and it is completely

[8] Blakley and McDougall (1961) have also shown that the highly purified dihydrofolic reductase from *S. faecalis* is inhibited noncompetitively by aminopterin.

TABLE I

INHIBITION OF LEUKOCYTE DIHYDROFOLIC REDUCTASE BY FOLIC ACID ANTAGONISTS[a]

Inhibitor (10^{-8} M)	% Inhibition
Amethopterin	57
Aminopterin	64
Dichloroamethopterin	85

[a] From Bertino *et al.* (1960b).

TABLE II

ACTIVITY OF DIHYDROFOLIC REDUCTASE IN LEUKOCYTES[a]

Diagnosis[b]	No. of Patients	Activity (μmoles/hr./mg of protein)
Normal	15	*ca.* 0.001
CLL	8	0.002 ± 0.001
CML	10	0.042 ± 0.008
AL	22	0.034 ± 0.004

[a] From Bertino *et al.* (1962b). [b] CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; AL, acute lymphoblastic and myeloblastic leukemias.

TABLE III

LEVELS OF DIHYDROFOLIC REDUCTASE IN VARIOUS TISSUES

Tissue	Level (μM/ml./hr./mg protein)
Chicken liver	0.07
Acute leukemic leukocytes	0.05
Rat intestinal mucosa	0.03
Ascites tumor cells	0.01

specific for TPNH; dihydrofolate is about 3- to 4-fold more active than folate as the substrate. As shown in Table I, the leukocyte enzyme is inhibited by extremely low levels of the folic acid antagonists.

The level of dihydrofolic reductase in various types of leukoctyes is given in Table II, and, for comparison, levels of the enzyme in several tissues are listed in Table III. It should be noted that the level in chronic lymphocytic leukemic cells and normal cells is very low, but still detectable, especially when the 55 to 90% ammonium sulfate fraction from the lysate is assayed.

Of particular interest to the leukemia problem is the finding that patients treated with Amethopterin develop an *increased* level of dihydrofolic reductase in their leukocytes even under conditions in which the total white cell count has decreased (Bertino *et al.*, 1962a). The increased level of the enzyme (expressed as μmoles of substrate converted per hour per mg of cell lysate protein) is not due to any change in the properties of the protein, since dihydrofolic reductase, isolated from the same patient before and after treatment with Amethopterin, was shown to have identical properties with respect to its *p*H optimum and its K_m values for both TPNH and dihydrofolate. In a number of patients it has been shown that the level of dihydrofolic reductase increases from 5- to 20-fold during treatment with the folic acid antagonists, and the rise in level may be correlated with the onset of resistance to the drug. On the other hand, when patients are treated with other chemotherapeutic agents, such as 6-mercaptopurine, prednisone, or Myleran, no increase in dihydrofolic reductase is noted. Similar findings regarding the rise in reductase level upon treatment with antagonists have been reported by Misra *et al.* (1961), working with mice with L-1210 leukemia, and by Hakala *et al.* (1961) and Fischer (1961), using cells in tissue culture. While it is difficult at the present time to provide an adequate biochemical explanation for the development of resistance to the folic acid antagonists, the obvious relevance of dihydrofolic reductase to the chemotherapy of leukemia will undoubtedly stimulate further investigation in this direction.

ACKNOWLEDGMENTS

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Spermine Reversal of Microbial Growth Inhibition by Thioesters of *p*-Aminosalicylic Acid*

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Natural extracts were found to reverse partially the toxicity of the thiobenzyl ester of *p* aminosalicylic acid. Properties of the active factor(s) indicated that spermine is the major principle present in hot water extract of beef liver, which reverses this inhibition.

The incorporation of a reactive thioester group into an analog of a metabolite has resulted in derivatives which bind and interact with certain enzymes utilizing the metabolite (Ravel *et al.*, 1958; Skinner *et al.*, 1958). Thus, *S*-carbamoyl-L-cysteine inhibits certain enzymatic processes involving glutamine by inactivating the enzymes. Recently, other thioester analogs of various metabolites were prepared and shown to be inhibitory to the growth of certain lactic acid bacteria (Hayashi *et al.*, 1961; Skinner *et al.*, 1961). The corresponding metabolite as anticipated exerted very little effect upon reversing the toxicity of the antagonists. For example, the growth inhibitory effects of the thiobenzylester of *p*-aminosalicylic acid for *Streptococcus lactis* is not overcome to any appreciable extent by *p*-aminobenzoic acid.

In the present investigation it was observed that supplements of natural extracts such as hot water extract of beef liver caused a two-fold increase in the amount of thioester required for inhibition of growth. The identity of the reversing agent(s) was desired in order to determine whether or not it might be related to a biochemical role of *p*-aminobenzoic acid. In the course of this work, an improved technique for the synthesis of thioesters of *p*-aminosalicylic acid was utilized and several new analogs were prepared.

Preliminary chemical evidence concerning the properties of the naturally occurring reversing agent(s) for these thioesters suggests that the compound(s) were probably strongly basic polyamines. Thus, a number of naturally occurring amines of this type were examined, and spermine, and to a lesser extent spermidine, were found to reverse the toxicity of the thiobenzyl ester of *p*-aminosalicylic acid.

Experimental[1]

Microbiological Assays

A previously described assay medium (Lansford *et al.*, 1958) which had been utilized for an "*L. arabinosus-*

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