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Lack of Intracellular Conversion of Dihydrohomofolate to Tetrahydrohomofolate in Leukemia Cells with High Levels of Dihydrofolate Reductase

ALY NAHAS[1] AND MORRIS FRIEDKIN[2]

Department of Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111

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SUMMARY

The uptake of tritium-labeled dihydrohomofolate was investigated with intact leukemia cells, known to be either sensitive or resistant to amethopterin. Comparable experiments were carried out with H_2-folate. Chromatography on DEAE-cellulose was used to determine possible intracellular conversion of H_2-homofolate to H_4-homofolate, whereas microbiological assays were utilized for uptake studies of H_2-folate and conversion to H_4-folate. The amethopterin-resistant cells showed a higher uptake of H_2-folate and a higher intracellular conversion of H_2-folate to H_4-folate than the sensitive cells, consistent with high H_2-folate reductase activity in lysates of resistant cells. The situation with H_2-homofolate was quite different. Uptake of H_2-homofolate by sensitive and resistant cells was almost the same, with no detectable intracellular conversion of H_2-homofolate to H_4-homofolate, despite the fact that H_2-homofolate is an excellent substrate of H_2-folate reductase.

INTRODUCTION

Tetrahydrohomofolate inhibits the thymidylate synthetase activity of *Escherichia coli* as well as the growth of streptococci (1). Since H_2-homofolate (Fig. 1) is rapidly reduced by the action of dihydrofolate reductase to H_4-homofolate (2), we have been interested in its potential as a chemotherapeutic agent, especially in amethopterin-resistant mouse leukemia associated with a several-fold increase in dihydrofolate reductase activity (3, 4).

This investigation was carried out as part of a general study of the mechanism of H_4-homofolate action in leukemic mice (5). The major aim was to determine whether the high H_2-folate reductase activity of an amethopterin-resistant subline of mouse leukemia L1210 is actually expressed in intact cells, by measuring the possible intracellular accumulation of H_4-folate and of H_4-homofolate.

MATERIALS AND METHODS

1. *Preparation of tritium-labeled homofolic acid.* Homofolic acid (NSC 79729) was labeled with tritium and purified as described previously (6). The absorption spectra of the labeled material were identical with those reported by DeGraw *et al.* (7) (Fig. 2). Tritium was attached to C-7 of

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[1] Present address, Department of Biochemistry and Division of Oncology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642.

[2] Present address, Department of Biology, University of California at San Diego, La Jolla, California 92037.

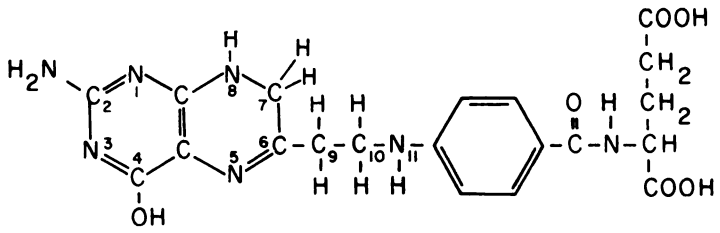

FIG. 1. *Structure of H_2-homofolic acid*

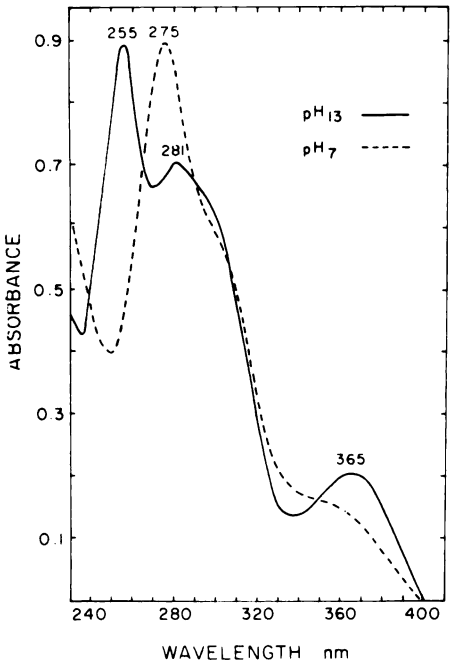

FIG. 2. *Absorption spectra of homofolate*

the pteridine ring and C-9 and C-10 of the bridge between the pteridine and the *p*-aminobenzoic acid moieties of the molecule (6).

2. *Preparation of reduced compounds.* H_2-folate and H_2-homofolate were prepared by dithionite reduction (8, 2). H_4-folate and H_4-homofolate were obtained by catalytic hydrogenation in 0.05 M potassium phosphate buffer, pH 6.8. After 4 atoms of hydrogen were taken up per mole of folate or homofolate, the tetrahydro compounds were protected by the addition of 6 mg of potassium ascorbate per milliliter. The yield was close to 100%, based on molar extinction coefficients of 28,000 for H_4-folate and 21,000 for H_4-homofolate at their single absorption maxima of 298 and 295 nm, respectively, at pH 7.

The specific activities of H_2-homofolate and H_4-homofolate were determined and found to be the same as the parent compound, indicating that no labilization of tritium had occurred on reduction.

3. *Assay for H_2-folate reductase and dihydro compounds.* The decrease in absorbance at 340 nm, which occurs when H_2-folate and NADPH are converted to H_4-folate and NADP, was used as a measure of H_2-folate reductase activity and also to detect dihydro compounds in column fractions (4). Initial rates were determined from absorbance readings made at 10-sec intervals with a Gilford attachment to the Beckman DU spectrophotometer. Specific activity of the enzyme was expressed as micromoles of H_2-folate reduced per minute per milligram of protein. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the reduction of 1 μmole of H_2-folate per hour under the same conditions (pH 7.4, 23°).

In a typical assay, the appropriate amount of enzyme was added to H_2-folate or H_2-homofolate (0.06 mM) and NADPH (0.1 mM) in 0.05 M potassium buffer, pH 7.5, containing 0.01 M mercaptoethanol. The blank contained the same ingredients, with H_2-folate or H_2-homofolate omitted.

4. *Microbiological assays. Pediococcus cerevisiae* (ATCC 8081) was used to assay for folates in the tetrahydro form, with calcium *dl*L-leucovorin (Lederle) as a reference standard (10). Only half of this racemic mixture is microbiologically active. *Streptococcus faecium* var. *durans* (formerly referred to as *Streptococcus faecalis* R.) (11) was used to assay for folate, H_2-folate, and H_4-homofolate (12, 1, 6). H_2-folate preparations were

also assayed for *P. cerevisiae* activity to determine possible contamination with H_4-folate (H_2-folate is about 0.2% as active as H_4-folate in this assay).

5. *Buffers commonly used in this study.* Buffer A was 0.04 M Tris-HCl, pH 7.2, and 0.56 M NaCl containing 4 mg of EDTA per milliliter. Buffer B was 1 part of buffer A plus 3 parts of water. Buffer C was 5 mM Tris-HCl, pH 7.2, and 0.2 M mercaptoethanol containing 1 mg of EDTA per milliliter. Buffer D was 60 mM Tris-HCl (pH 7.2), 65 mM NaCl, 15 mM KCl, and 8 mM $CaCl_2$ containing 3 mM ascorbate, which, according to Yunis *et al.* (13), stimulates active influx of cations. Buffer E was 5 mM Tris-HCl, pH 7.2, and 0.2 M mercaptoethanol.

6. *Chromatographic separation of H_2-homofolate and H_4-homofolate.* A mixture of 1.8 μmoles of tritium-labeled H_2-homofolate and 2.4 μmoles of unlabeled H_4-homofolate in 200 ml of buffer E was poured through an 8 × 1.2 cm DEAE-cellulose column previously washed with 1 liter of the same buffer. The adsorbed material was then subjected to linear gradient elution. The mixing chamber contained 600 ml of buffer E, and the reservoir, an equal volume of 1 M NaCl in buffer E. All operations were performed at 4°. Three-milliliter fractions were collected. Fractions from the column were initially monitored with a recording absorption meter equipped with a 280 nm filter.

Complete ultraviolet spectra were determined for each of the fractions containing most of the separated compounds [one of the very late fractions, known to be free of pteridines (tube 85), was used in the reference cell]. Each column fraction was also assayed for radioactivity.

Figure 3 shows the separation of tritium-labeled H_2-homofolate from nonlabeled H_4-homofolate.

7. *Mouse leukemia sublines.* DBA/2 male mice (Jackson Memorial Laboratories, Bar Harbor, Me.) weighing 20–25 g and carrying L1210 (amethopterin-sensitive) and L1210/MTX (amethopterin-resistant) lymphoid leukemia sublines in ascites form were used throughout this study and are henceforth designated S and R sublines. Both were generously provided by Arthur D. Little, Inc., Cambridge, Mass. The activity of H_2-folate reductase from R cells was found to be 13 times higher than that from S cells (81 and 6.25 nmoles of H_2-folate reduced per hour per 10^8 cells, respectively). Ascitic fluid was harvested from leukemic mice by

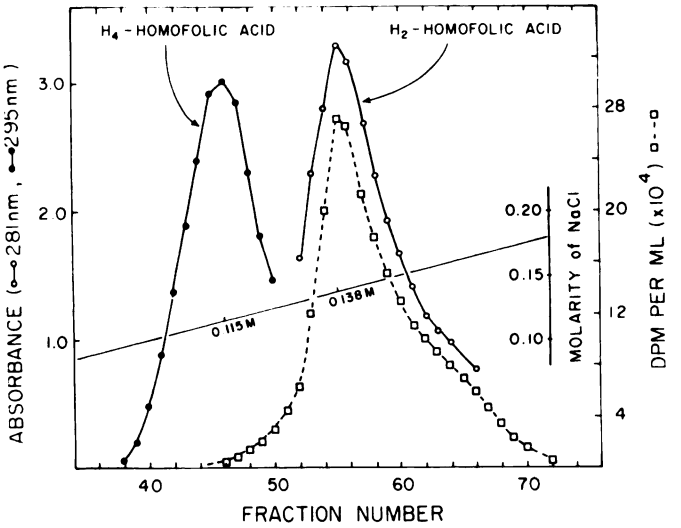

FIG. 3. *Chromatographic separation of H_2-homofolate and H_4-homofolate*

See the text (Section 6) for experimental details. It was advantageous for displaying the separation of the reduced compounds to plot peak absorbance values for H_4-homofolate at 295 nm and for H_2-homofolate at 281 nm.

the method of Sartorelli *et al.* (14) on the seventh day after transplantation.

8. *Preparation of cell lysates.* To obtain clear lysates after incubation of leukemia cells with drugs (as described later), the cells were first centrifuged and then resuspended in 5 volumes of buffer C before lysis by sonication for 2 min at maximum intensity in a Branson model LS-75 Sonifier (the temperature was not allowed to rise above 1–2°). The sonic extract was then frozen slowly overnight to denature the proteins formed as a result of an increasing concentration of mercaptoethanol as the water gradually crystallized. Centrifugation of the thawed sonic extract the following morning at 20,000 × *g* for 20 min yielded a clear solution.

RESULTS

Uptake of homofolate and its reduced forms by intact leukemic cells. Fifty million ascites cells (S or R) were resuspended in 2 ml of buffer D containing 0.9 μmole (40 μCi) of labeled homofolate, H_2-homofolate, or H_4-homofolate. The final concentration of these compounds was approximately 0.45 mM. The mixture was then equilibrated with 5% CO_2–95% air in a closed container and incubated with stirring in the dark for 45 min at 37°. The incubation period was terminated by the addition of 20 ml of ice-cold buffer D, followed immediately by centrifugation of the cells for 2 min at 1500 rpm. The cells were washed three times by resuspension in 20-ml portions of buffer D and then lysed by sonication. Aliquots of the clear lysate were assayed for radioactivity. The last wash (20 ml) just previous to lysis of the cells usually contained less than 0.02% of the original radioactivity.

The uptakes of homofolate and its reduced derivatives by amethopterin-sensitive and amethopterin-resistant leukemia sublines were almost identical (Table 1). In both lines the uptake of homofolate was about 4 times higher than that of H_2-homofolate, and 40 times higher than H_4-homofolate. When cells were incubated with H_2-homofolate at 0° the uptakes were only 12% and 16% of that at 37° for sensitive and resistant sublines, respectively.

TABLE 1

Comparison of uptake of homofolate and its reduced forms by ascites leukemia cells

Fifty million sensitive and resistant cells were incubated with [^{3}H]homofolate or one of its reduced derivatives as described in the text.

Folate analogue	Radioactivity taken up	
	Sensitive line	Resistant line
	%	%
Homofolate	1.02	1.11
H_2-homofolate	0.25	0.20
H_4-homofolate	0.02	0.03

Enzymatic synthesis of H_4-homofolate by cell lysates. H_4-homofolate is formed from H_2-homofolate by the action of dihydrofolate reductase in lysates of disrupted leukemia cells. An incubation mixture was prepared containing NADPH (30 μM), tritium-labeled H_2-homofolate (60 μM; specific activity, 10 μCi/mg; added last to start the reaction), a cell lysate of amethopterin-resistant ascites cells (50 times the amount that gave a decrease of 0.01 absorbance unit/min at 340 nm, in 1 ml of a similar reaction mixture), and 0.05 M Tris-HCl (pH 7.4)–0.01 M mercaptoethanol to a final volume of 50 ml. The reaction was followed by measuring the decrease in absorbance at 340 nm every 2 min. Although the reaction was essentially complete in 20 min, the incubation was allowed to continue for a total of 40 min. Since a limiting amount of NADPH was added, the final incubation mixture should have contained equal quantities of tritium-labeled H_2-homofolate and H_4-homofolate. That this was essentially so was shown by subjecting the mixture to chromatography as described in Section 6. The elution profile is displayed in Fig. 4.

Lack of conversion of H_2-homofolate to H_4-homofolate by intact cells. To 8 ml of ascitic fluid containing 1.75 × 10^9 cells were added 10 μmoles of sodium isocitrate, 10 μmoles of glucose, and 3 μmoles of neutralized potassium ascorbate in 1 ml of 0.9% NaCl. The mixture was incubated under 5% CO_2–95% air at 37° for 15 min. Then 10 μmoles of tritium-labeled H_2-homofolate (5

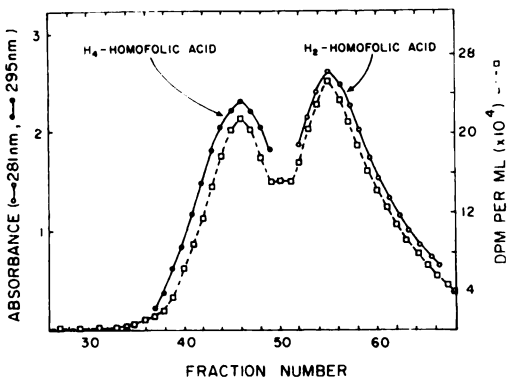

FIG. 4. *Chromatographic separation of H_2-homofolate and H_4-homofolate from an enzymatic reaction mixture*

H_2-folate reductase from a lysate of L1210/MTX mouse ascites cells was incubated with tritium-labeled H_2-homofolate in the presence of limiting amounts of NADPH. See the text for experimental details.

μCi/μmole) in 1 ml of 0.9% NaCl were added, and the incubation was continued for another 45 min under the same conditions. The pH before and after incubation was 7.18. At the end of incubation, the cells were centrifuged and washed and a lysate was prepared as described in Section 8. Nonlabeled carrier H_4-homofolate was usually added to the washed cells before lysis. The clear lysate was chromatographed and assayed for radioactivity and optical density as described in Section 6.

Chromatography of lysates of cells previously incubated with H_2-homofolate was carried out to ascertain whether intracellular conversion of H_2-homofolate to H_4-homofolate had occurred. No radioactivity could be detected in column fractions containing carrier H_4-homofolate. This was true for both S and R sublines. *In other words, H_4-homofolate formed by H_2-folate reductase in cell-free lysates could be detected easily, whereas none could be recovered from intact cells previously incubated with H_2-homofolate.*

Intracellular conversion of H_2-folate to tetrahydro derivatives. H_2-folate, 10 μmoles, was incubated with cell suspensions exactly as described for experiments with H_2-homofolate (see previous section). The uptake of H_2-folate by ascites leukemia cells was 9.8% in the resistant subline, as compared to 1.5% in the sensitive one (Table 2). These values were based on microbiological activity with *S. faecium*.

Since the possible folate derivatives would include H_2-folate, H_4-folate, or any of their derivatives except 5-methyl-H_4-folate and polyglutamate forms of folate (15), it became imperative to ascertain what forms of folates were actually present. Lysates were prepared as described in Section 6. A portion of the lysate from cells previously incubated with H_2-folate was formylated in order to convert any H_4-folate possibly formed to the more stable 5-formyl derivative (citrovorum factor). This was accomplished by the procedure of Futterman and Silverman (16). To 1 ml of the lysate were added 1 ml of 240 mg of potassium ascorbate per milliliter (pH 6) and 2 ml of 88% formic acid (23 M). The mixture was heated for 1 hr at 80° and then cooled. Two milliliters of the same ascorbate solution were added, as well as enough 12 M KOH to adjust the pH of the mixture to 6–7. The neutralized mixture was autoclaved for 25 min under 20 pounds of steam pressure and then diluted for microbiological assays. Autoclaving isomerizes 10-formyl-H_4-folate to the much more stable 5-formyl-H_4-folate.

TABLE 2

Uptake of H_2-folate and H_2-homofolate by L1210 ascites cells

Lysates from cells incubated with H_2-folate were assayed microbiologically for *S. faecium* and *P. cerevisiae* activities. Lysates from cells incubated with tritium-labeled H_2-homofolate were chromatographed and assayed for radioactivity as described in Section 6.

Compound	Subline[a]	Uptake of radioactive intracellular folate	Intracellular folate as	
			Dihydroform	Tetrahydroform
		%	%	%
H_2-folate	S	1.5	21	79
	R	9.8	0	100
H_2-homofolate	S	1.2	75	<3
	R	1.3	72	<3

[a] S and R stand for L1210 and L1210/MTX leukemia sublines, respectively.

TABLE 3

Uptake of H_2-folate by intact leukemia cells and intracellular conversion to tetrahydro derivatives

The experimental details for incubation, preparation, and assay of lysates are described in the text.

Cell line	Expt.	Conditions of experiment	Formylation	Autoclaving	*S. faecium* activity	*P. cerevisiae* activity
					nmoles	*nmoles*
L1210	1	Complete system, 37°	−	−	180	120
	2	Complete system, 37°	+	+	160	136
	3	Complete system, 37°	−	+		71
	4	Complete system, 0°	+	+	20	38
	5	Complete system, 37°, with dihydrofolate omitted	+	+	30	18
L1210/MTX	6	Complete system, 37°	−	−	1000	900
	7	Complete system, 37°	+	+	1060	1100
	8	Complete system, 37°	−	+		850
	9	Complete system, 0°	+	+	25	25
	10	Complete system, 37°, with dihydrofolate omitted	+	+	20	14
H_2-folate	11		−	−		0
H_4-folate	12		−	+		0

As a control, a known amount of H_4-folate (prepared by catalytic hydrogenation) was autoclaved without previous formylation in the presence of ascorbate. Under these conditions H_4-folate was completely destroyed, as indicated by microbiological assay with *P. cerevisiae*.

When *P. cerevisiae* was used to determine the total tetrahydro forms, all the H_2-folate that entered the resistant cells was accounted for as tetrahydro derivatives whereas 79% was the value obtained with sensitive cells (Table 2). Although not actually identified, the tetrahydro compounds could include H_4-folate or its N^5-formyl, N^{10}-formyl, N^5-formimino, and N^5,N^{10}-methylene-H_4-folate derivatives. The H_2-folate preparation used for various incubations was tested for *P. cerevisiae* activity and was shown to have no contamination with H_4-folate.

In cells previously incubated with H_2-folate, a major proportion of the intracellular H_4-folate is stable to autoclaving: sensitive, 71 vs. 136 nmoles; resistant, 850 vs. 1100 nmoles (Table 3).

Since unmodified H_4-folate loses all its *P. cerevisiae* activity on autoclaving, most of the intracellular folate is probably in a formylated form. This form is convertible to a thermostable form by autoclaving for 20 min. Thus, after incubation with H_2-folate in sensitive cells, about 52% of the total H_4-folate is in a stable form, and in resistant cells, about 77%.

The data in Table 3 also demonstrate the temperature sensitivity of the folate uptake system in both sensitive and resistant cells.

DISCUSSION

A comparison of the uptakes of H_2-folate and H_2-homofolate by leukemia cells revealed important dissimilarities. Resistant cells not only took up much more H_2-folate than sensitive cells, but also more effectively converted H_2-folate to tetrahydro derivatives (Table 2). With H_2-homofolate the situation was quite different. Sensitive and resistant cells took up the same amount of H_2-homofolate. Furthermore, no conversion of H_2-homofolate could be demonstrated in intact cells, despite the fact that intracellular H_2-folate reductase activity is expressed in experiments with H_2-folate (Table 2) and

even though H_2-homofolate is a substrate of H_2-folate reductase when tested *in vitro* (Fig. 4).

The lack of formation of H_4-homofolate from H_2-homofolate by leukemic cells remains a puzzling finding. In an attempt to explain our negative results five possibilities were considered: (a) H_4-homofolate was formed but immediately broke down; (b) one or both of the reduced forms were unstable under the conditions used to isolate them; (c) H_2-homofolate was metabolized to a compound that inhibited intracellular dihydrofolate reductase; (d) as soon as H_4-homofolate was formed it escaped into the medium by rapid efflux from the cells; and (e) the incubation conditions *in vitro* were unfavorable for conversion of H_2-homofolate to H_4-homofolate.

The first and second possibilities were excluded by the following experiments. Incubation of ascitic cells with tritium-labeled H_4-homofolate led to recovery of 96% of the intracellular radioactivity as unchanged H_4-homofolate. Obviously, H_4-homofolate did not undergo any appreciable breakdown either intracellularly or during lysis of the cells. When labeled H_2-homofolate was diluted with buffer C, allowed to stand for 45 min at 37°, subjected to sonication, and then chromatographed, the same distribution of radioactivity was obtained as with the untreated preparation, meaning that labeled H_2-homofolate did not break down with this technique.

The third possibility, i.e., metabolic conversion of H_2-homofolate to an inhibitor of dihydrofolate reductase, was interesting in view of the conversion of H_2-homofolate to nonadsorbable material when incubated with cells (Table 4). About 10% of the radioactivity in H_2-homofolate was not adsorbed on DEAE-cellulose. After H_2-homofolate was incubated with cells, nonadsorbable radioactivity increased markedly and radioactivity in the eluted H_2-homofolate decreased proportionately (Table 4). In none of these experiments were we able to notice any difference between sensitive and resistant cells.

In separate experiments the nonadsorbable metabolite(s) was eluted from DEAE-cellulose with 5% NH_4OH, freeze-dried, and tested for its possible effect on H_2-folate reductase (Section 3). *No inhibition could be demonstrated.* This would seem to indicate that this breakdown product could not account for the lack of intracellular synthesis of H_4-homofolate. The nature of the breakdown product remains unknown.

The fourth possibility, i.e., rapid efflux of H_4-homofolate from cells, was approached as follows. In separate experiments S and R cells were incubated with tritium-labeled H_2-homofolate, the cells were centrifuged, and to the clear supernatant medium, in which the cells had been previously suspended, was added nonlabeled carrier H_4-homofolate. After chromatography (see Section 6) no radioactivity could be detected in column fractions containing carrier H_4-homofolate. Thus rapid efflux of H_4-homofolate from cells incubated with H_2-homofolate apparently had not occurred.

TABLE 4

Distribution of radioactivity in lysates after incubation of tritium-labeled H_2-homofolate with amethopterin-resistant leukemia cells

In a final volume of 10 ml, 1.73×10^9 cells were incubated at 37° with 10 μmoles of tritium-labeled H_2-homofolate under 5% CO_2–95% air for the time indicated. The incubation mixture also contained sodium isocitrate (1 mM), glucose (1 mM), and potassium ascorbate (0.3 mM). After disruption of the cells by sonication, the lysates were chromatographed on DEAE-cellulose and assayed for radioactivity and absorbance as described in Section 6.

Experimental conditions	Radioactivity	
	In column-fraction containing H_2-homofolate	Non-adsorbable on column
	%	%
Original H_2-homofolate substrate	85–89	9–12
Lysates from 30-min incubation	76	22
Lysates from 45-min incubation	65	34
Lysates from 60-min incubation	53	44
Lysates from 120-min incubation	39	59

The fifth possibility, i.e., that the incubation mixture *in vitro* was unfavorable for intracellular H_4-homofolate synthesis, prompted us to carry out the following experiment: 5 mg (50 μCi) of tritium-labeled H_2-homofolate in 0.5 ml of 0.9% NaCl containing 0.3 mg of potassium ascorbate (previously adjusted to pH 7.2) were injected intraperitoneally into each of four leukemic mice on the seventh day after intraperitoneal transplantation of the S or R subline. Two hours later the ascitic fluid was collected and pooled. The leukocytes were washed three times with buffer B, lysed by sonication in buffer C (see Section 8), and chromatographed by the procedure described in Section 6. No radioactivity could be detected in column fractions containing carrier H_4-homofolate from either the S or R subline.

Since none of the above explanations seem probable, we are at a loss to explain why intact leukemic cells do not convert H_2-homofolate to H_4-homofolate.

In the present study, in which some of the techniques of Silverman *et al.* (17) were applied to lysates of cells previously incubated with H_2-folate, formylated forms of H_4-folate and/or the N^5,N^{10}-methylene derivative (since this compound too has *P. cerevisiae* activity) predominated (Table 3).

H_4-folate was synthesized within cells from exogenous H_2-folate, with more reduction occurring in resistant cells than the sensitive ones because of high levels of H_2-folate reductase in the former. The H_4-folate thus synthesized was further formylated. The increase in H_2-folate reductase activity in resistant cells, according to Sartorelli *et al.* (14), is specific. Those investigators found no similar increase in N^5-formyl-H_4-folate synthetase, N^5,N^{10}-methylene-H_4-folate dehydrogenase, N^5-formyl-H_4-folate isomerase, or serine hydroxymethylase. If these conditions apply to the present study, elevated H_2-folate reductase would be the main controling factor in the increased synthesis of formylated H_4-folate observed with the resistant cells. In other words, the increase in the level of H_2-folate reductase in resistant cells would be reflected as an increase in formylated H_4-folate as an end product.

Since the completion of the present study, new and important aspects of homofolate action have been uncovered and reviewed (18). It now appears likely that the chemotherapeutic efficacy of H_4-homofolate in mice (5) can be attributed to a block in folate transport as well as inhibition of thymidylate synthetase. Kisliuk and Gaumont (19) have shown that in *S. faecium* and *Lactobacillus casei* growth is inhibited by the diastereoisomer of H_4-homofolate having the configuration at carbon 6 *opposite to that found in naturally occurring H_4-folate*. Since both diastereoisomers were present in the H_4-homofolic acid preparations used in the treatment of leukemic mice (5), a dual antagonism was possible: inhibition of folate transport into cells, and of thymidylate synthetase.

In preliminary experiments[3] H_4-homofolate was found to inhibit the uptake of N^5-methyl-H_4-folate and N^5-formyl-H_4-folate by L1210 cells.

We conclude that homofolate, despite its early promise and unusual actions, does not have the unique properties needed in a therapeutic strategy specifically aimed at taking advantage of high H_2-folate reductase activities in amethopterin-resistant leukemia cells (20, 4, 5). We still believe that a dihydrofolate derivative capable of intracellular reduction to an inhibitory form could be a useful antileukemic agent.

ACKNOWLEDGMENT

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