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The Presence and Distribution of Reduced Folates in *Escherichia coli* Dihydrofolate Reductase Mutants*

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***Escherichia coli* DNA photolyase was overproduced and purified from each of two mutant *E. coli* strains lacking dihydrofolate reductase. The extent of overproduction in the mutants was comparable to that seen in the wild type strain. Examination of the isolated photolyase from these strains revealed that the folate cofactor, 5,10-methenyltetrahydrofolate, was present in these proteins at a level of 60–80% compared to that purified from the wild type strain. Further examination of the dihydrofolate reductase-deficient strains revealed the presence of other tetrahydrofolate derivatives. These findings demonstrate that dihydrofolate reductase is not essential for the production of tetrahydrofolates in *E. coli*.**

Folate cofactors are of critical importance in intermediary metabolism. These essential cofactors are synthesized *de novo* in bacteria and plants but are derived from dietary sources in mammals, including man. In *Escherichia coli*, folates are known to be derived from GTP as shown in Scheme 1. The reduction of 7,8-H_2folate[1] to H_4folate is an obligatory step in the synthesis of the single carbon carrier forms of the molecule (Scheme 2). This conversion is catalyzed by dihydrofolate reductase, and the reaction is considered to be the sole source of cellular H_4folates in *E. coli*.

Dihydrofolate reductase has long been the focus of mechanistic studies, particularly the targeting of drugs such as methotrexate and trimethoprim which are thought to cause cell death by inhibiting the formation of H_4folate. To facilitate structure-function studies on the enzyme by the technique of site-directed mutagenesis, construction of a dihydrofolate reductase-deficient (*fol*$^-$) strain is desirable. Because of the cellular importance of this enzyme for generating a multitude of essential compounds for the folate-dependent reactions in the cell, the survival of such a mutant would appear to be difficult. Nevertheless, the construction of two dihydrofolate reductase mutants by deletion of the *fol* gene was reported recently by two separate laboratories (2, 4). This *fol* deletion was stable only in the presence of an accompanying mutation in thymidylate synthetase.

In order to understand the details of folate metabolism in these mutants, we have analyzed the folate pool in each mutant, LH18 and PA414. Our findings are that both strains contain H_4folates. Thus, it appears that another enzyme or pathway exists in *E. coli* for the production of H_4folates in addition to the reaction or pathway involving dihydrofolate reductase.

MATERIALS AND METHODS

Chemicals and Reagents—Sodium cyanoborohydride was from Aldrich. Tetrabutylammonium hydroxide, H_4folate, 5-CHO-H_4folate, H_2folate, NADH, activated charcoal, tetracycline, kanamycin, methotrexate, thymine, adenine, glycine, L-methionine, and ammonium dihydrogen phosphate were from Sigma. NADPH was purchased from Pharmacia LKB Biotechnology Inc. Folic acid was from Nutritional Biochemicals, and HPLC grade methanol was obtained from Fisher. DL-Pantothenic acid was from Brothers Chemical Co. Isopropyl-β-D-thiogalactoside was purchased from Boehringer Mannheim Biochemicals. All other amino acids used were from Sigma.

Bacterial Strains—The *E. coli* strains used in this study are listed in Table I. LH18 and PA414 were maintained on LB plates containing 25 μg/ml kanamycin and 200 μg/ml thymine. AB1157 and NM522 were maintained on LB plates. For growth of cells for folate analysis or enzymatic assays, Fernbach flasks containing 1 liter of LB (NM522, AB1157) plus 200 μg/ml thymine and 25 μg/ml kanamycin (LH18, PA414) were inoculated with single colonies and grown for 18 h on a shaker table at 37 °C. Alternatively, Fernbach flasks were inoculated with 0.5–5 ml log-phase cultures and grown for 12 h with shaking at 37 °C.

For the determination of growth requirements, LH18 and PA414 were grown on minimal agar plates (7) containing kanamycin (50 μg/ml), kanamycin and thymine (200 μg/ml), or kanamycin, thymine, adenine (20 μg/ml), L-methionine (50 μg/ml), glycine (50 μg/ml), and pantothenic acid (1 μg/ml). Minimal plates contained additionally thiamin (1 μg/ml) and 50 μg/ml L-proline, L-arginine, L-histidine, L-leucine, and L-threonine.

MS09 was maintained on LB plates containing 20 μg/ml tetracycline. SHA10 and UNC414 were maintained on LB plates with 200 μg/ml thymine and 20 μg/ml tetracycline. Growth of these strains for photolyase induction and purification was in LB + 20 μg/ml tetracycline (MS09) with an additional 200 μg/ml thymine (SHA10, UNC414). Twenty-five-ml cultures were inoculated with single colonies and grown overnight, and 5-ml aliquots of the culture were added to 2-liter flasks containing 500 ml of the appropriate media. The flasks were shaken at 37 °C until the *A* was approximately 0.6, induced with 1 mM isopropyl-β-D-thiogalactoside, and shaken 12–16 h before harvesting.

For the construction of UNC414, PA414 was mated with CSR603 (5) and transformed with pMS1310. SHA10 was constructed by transforming LH18 with pMS1310.

Plasma γ-Glutamyl Hydrolase—Rat plasma γ-glutamyl hydrolase was prepared by a method based on that of Wilson and Horne (8). About 20 Zivic-Muller rats were anesthetized and decapitated. Blood was collected into a beaker containing 1 ml of sodium heparin (Upjohn Chemical Co., 1000 units/ml). The blood was centrifuged at 15,000

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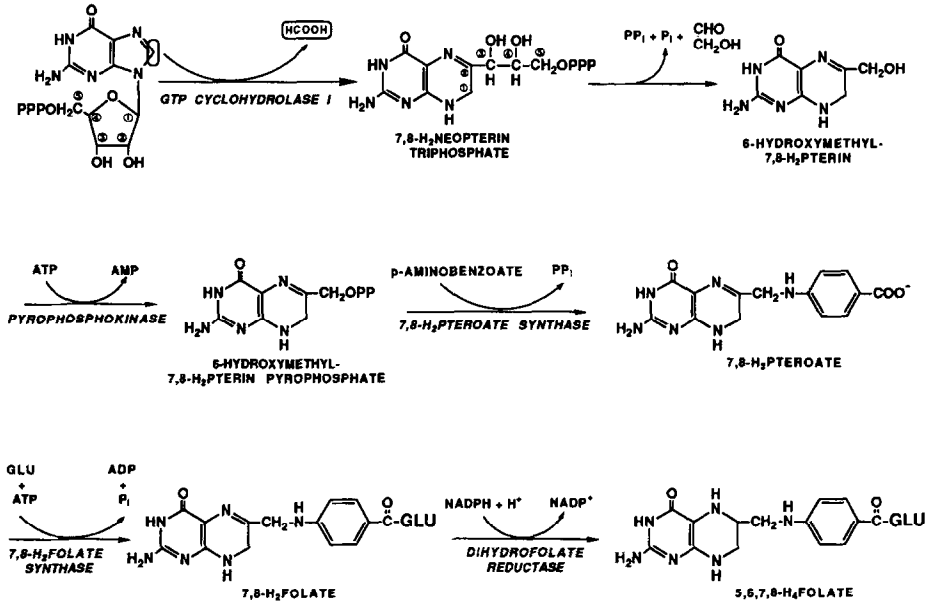
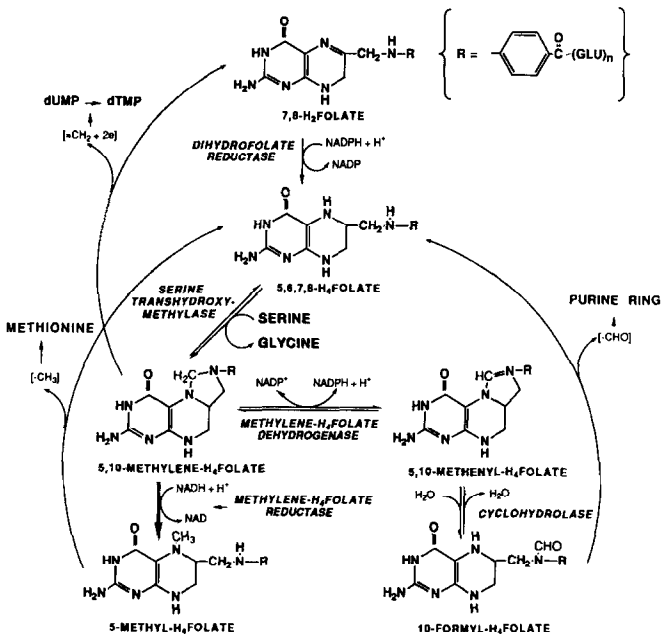
[1] The abbreviations used are: H_2folate, dihydrofolate; HPLC, high performance liquid chromatography; H_4folate, tetrahydrofolate; CH^+-H_4folate, 5,10-methenyltetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; 5-CHO-H_4folate, 5-formyltetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; 10-CHO-H_4folate, 10-formyltetrahydrofolate.

SCHEME 1





SCHEME 2

rpm for 20 min at 4 °C in the SS34 rotor of a Sorvall refrigerated centrifuge. The serum supernatant was collected and dialyzed 16–20 h against 1 liter of 50 mM potassium phosphate, pH 7.4, containing 2 g of activated charcoal. The serum was concentrated 2-fold to 3-fold by ultrafiltration using a PM10 membrane (Amicon) and frozen at −20 °C until needed.

Protein Purification—The procedure for purification of *E. coli* DNA photolyase was modified from that described previously (9). Purification was completed after elution from Blue Sepharose and passage over a Sephadex G-25 column (PD-10, Pharmacia LKB Biotechnology Inc., 9.1-ml bed volume) in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA. Photolyase concentration was determined after treatment with potassium ferricyanide to convert all residual $FADH_2$ to the stable blue flavin radical[2] followed by measurement of the absorbance at 580 nm and using an extinction coefficient of 5000 M^{-1} cm^{-1} as previously described (9).

Protein and Enzyme Assays—Protein assays were performed with BCA Protein Assay Reagent (Pierce) with bovine serum albumin as a standard. Assay of *E. coli* dihydrofolate reductase was based on that of Stone and Morrison (10, 11) and was performed as follows: 12 to 50 μl of crude *E. coli* extract was added to 2.5 ml of 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, and 100 μM NADPH. One-ml aliquots of the mixture were added to each of two cuvettes. Dihydrofolate was added to 20 μM in the sample cuvette, and an equal volume of buffer was added to the reference cuvette. The change in absorbance at 340 nm was followed using a Shimadzu UV-260 spectrophotometer. Methotrexate concentration ranged from 0.25 μM to 1 μM when present.

Synthesis of Folates—CH^+-H_4folate was synthesized from 5-CHO-H_4folate as described previously (12). CH_3-H_4folate was synthesized from CH^+-H_4folate by cyanoborohydride reduction using a modification of the procedure described previously (13). 10-CHO-H_4folate was produced by diluting CH^+-H_4folate into anaerobic 50 mM potassium phosphate, pH 7.4, containing 5 mM sodium ascorbate and 5 mM 2-mercaptoethanol (12). The product formation was complete after several hours, and the sample was stored under N_2 at −20 °C.

HPLC Analysis of Reduced Folates—HPLC analysis of reduced folates was based on the methods described by Duch *et al.* (14) and Mullin *et al.* (15). All buffers were purged with helium before use. Procedures were performed under minimal light. *E. coli* cell paste (fresh or thawed from −70 °C) was suspended in 3 volumes of 1% sodium ascorbate and 1% 2-mercaptoethanol per g of cell paste. The suspension was passed twice through a French pressure cell and then heated for 5 min at 95 °C. The mixture was centrifuged at 15,000 rpm for 20 min at 4 °C in an SS34 rotor. The supernatant was then incubated with the rat plasma γ-glutamyl hydrolase preparation for 3 h at 37 °C in the dark (1 volume of hydrolase added per 4 to 6 volumes of *E. coli* extract). After 3 h, the mixture was again heated at 95 °C for 5 min and centrifuged at 15,000 rpm for 10 min at 4 °C in an SS34 rotor. The supernatant was adjusted to pH 4.5 with 1.75 M acetic acid, and the centrifugation was repeated. Samples were then diluted by adding 1 volume of water per volume of extract and quickly processed on columns (1 × 0.625 cm) of Dowex 50X-4, 100–200 mesh (Bio-Rad Laboratories) as described (14). Processing through Sep-Pak Plus C-18 cartridges (Waters) was also as described (15, 16). The methanol eluate from the Sep-Pak cartridge was evaporated in a Speed Vac, and the remaining material was resuspended in 5 mM 2-mercaptoethanol containing 5 mM sodium ascorbate. These samples were made anaerobic and stored at −20 °C under N_2 overnight.

HPLC was performed on a 10-μ reverse phase C-18 column from Alltech Associates (Los Altos, CA). Samples were prepared for injection by centrifugation through Spin-X columns (Costar) in an Eppendorf model 5415 microcentrifuge. The HPLC solvent delivery system was a Hewlett-Packard 1090 liquid chromatograph. Spectra of the HPLC eluate were monitored continuously using a Hewlett-Packard 1040A diode array detector. Chromatography conditions were similar to those described by Duch *et al.* (14): the system utilized 98–100% of a solvent consisting of 5 mM tetrabutylammonium phosphate, 10 mM ammonium dihydrogen phosphate containing 22–25%

[2] S. Hamm-Alvarez, unpublished results.

TABLE I

E. coli strains and plasmids

Strain	Relevant genotype	Source
NM522	Δ*lac-pro supE hsd*R5 F′ *lac*(I^Q-ZΔM15)$^+$	M. Stump[a] (1)
LH18	NM522 *thy*A Δ*fol::kan*	E. Howell[b] (2)
AB1157	F^- *his*4 *thr*1 *leu*6 *thi*1 *lac*Y1 *gal*K2 *ara*1 *xyl*5 *mtl*1 *pro*A2 *arg*E3 *rps* 31 *tsx*38 supE44 λ^-	J. Imlay[a] (3)
PA414	AB1157 *thy*A Δ*fol::kan*	P. Ahrweiler[c] (4)
MS09	CSR603 F′ *lac*I^Q pMS969	Ref. 5
SHA10	LH18 pMS1310	This paper
UNC414	PA414 F′ *lac*I^Q pMS1310	This paper
MJ7	MJ1 F^- *rpsL*	Ref. 6
Plasmids		
pMS1310	*phr*$^+$*tet*$^+$	Ref. 5 A derivative of pMS969

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[c] Department of Biochemistry, Washington University Medical School, St. Louis, MO.

FIG. 1. **Absorption spectrum of DNA photolyase purified from SHA10.** Photolyase in 1 ml of 50 mM Tris-HCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 M KCl, 20% glycerol was incubated with 10 μl of 20 mM potassium ferricyanide for 20 min on ice. Excess ferricyanide was removed by chromatography of the mixture on a Sephadex G-25 column (PD-10) equilibrated in 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA.

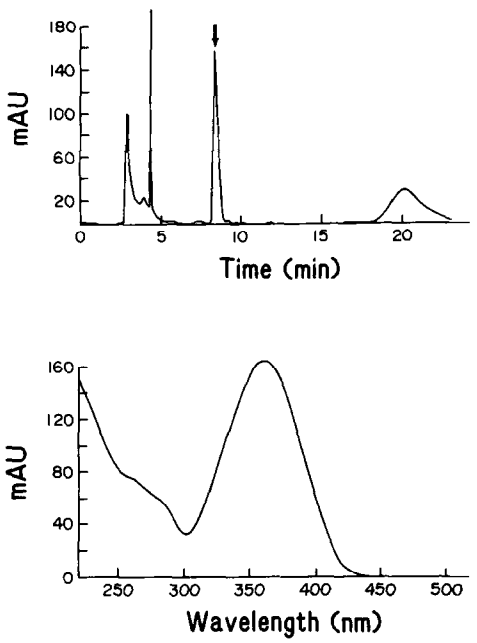

FIG. 2. *Top,* HPLC profile at 350 nm of the acid-released cofactors from photolyase purified from SHA10. 1 ml of 20 μM photolyase was acidified to pH 1 with 4 N HCl, centrifuged for 5 min in an Eppendorf microcentrifuge, and applied to a C-18 column equilibrated in 20% methanol, pH 2. The sample was chromatographed using a 20-min gradient from 20% methanol to 50% methanol at pH 2 at 1 ml/min. *Bottom,* absorption spectrum of the peak marked by the *arrow,* obtained on-line with a diode-array detector. The material in the peak is identified as CH^+-H_4folate.

methanol with a final pH of 6.1–6.2, and 0–2% of 100% methanol. The flow rate was 0.9–2.0 ml/min.

RESULTS

Overproduction of E. coli DNA Photolyase in an LH18-derived Strain—We obtained PA414 and LH18 with the hope of overproducing *E. coli* DNA photolyase in one of these strains. The *E. coli* DNA photolyase (deoxyribodipyrimidine photolyase, EC 4.1.99.3) is a monomeric protein with a molecular mass of 54,000 daltons which contains as tightly bound prosthetic groups $FADH_2$ and CH^+-H_4folylpolyglutamate (9, 17). Although the normal copy number of this enzyme is 15–20 molecules per cell, when a plasmid containing the photolyase gene, *phr,* is placed in various *E. coli* hosts and induced, the enzyme can constitute 5–10% of the total cellular protein (5). Photolyase purified from such an overproducing strain contains approximately 0.3 mol of the folate cofactor per mol of protein with some variation from preparation to preparation (12). Since the enzyme readily binds exogenously added 5,10-CH^+-H_4folate, the less than stoichiometric amount of folate in the isolated enzyme appears to be due to an insufficiency of intracellular folate to meet the demand of enzyme overproduction. By overproducing the photolyase in a *fol*$^-$ strain, we hoped to obtain enzyme completely free of folate. Although the folate cofactor can be removed by chemical treatment (12), we wished to obtain enzyme free of folate without the additional chemical treatment.

When LH18 was transformed with pMS1310 to give SHA10 and photolyase was induced and purified from this strain, the isolated enzyme displayed the spectrum shown in Fig. 1. The presence of the peak at 384 nm in this enzyme and its relative amplitude compared to the radical flavin peak at 580 nm indicated that the protein contained CH^+-H_4folate. The presence of this reduced folate was verified by HPLC; Fig. 2 shows the HPLC chromatogram of the acid-released cofactors from photolyase purified from SHA10. The spectrum shown in Fig. 2 was obtained by an on-line scan of the material in the single peak indicated by an *arrow* on the HPLC chromatogram and is identical with standard CH^+-H_4folate. From analysis of these results, it is evident that photolyase from this particular preparation was approximately 20% saturated with the re-

TABLE II

Extent of overproduction of photolyase in fol⁻ and fol⁺ strains

Values were obtained by protein assay or by gel scan.

Strain	% overproduction
SHA10	3.4
UNC414	5.8
MS09	5.5

duced folate cofactor. The photolyase purified from SHA10 was indistinguishable from that isolated from MS09 both spectrally and by its elution behavior on Blue Sepharose and Sephadex G-25.

Strain UNC414 was constructed from the *fol⁻* strain PA414 in order to determine whether photolyase overproduction in this strain also yielded an enzyme preparation partly saturated with the reduced folate cofactor. Our analysis revealed that the photolyase purified from UNC414 was 25% saturated with CH^+-H_4folate (data not shown).

Previous experiments have indicated that the overproduced photolyase is only partially saturated with folate *in vivo* in wild type MS09; supplementation of the MS09 crude extract with CH^+-H_4folate resulted in isolation of photolyase containing virtually a full complement of the folate cofactor after purification (12). Therefore, it appears unlikely that the extent of overproduction in the *fol⁻* mutants would be affected by intracellular folate levels. In fact, photolyase purified from the *fol⁻* mutants is saturated with the folate cofactor to 60–80% of the level seen for photolyase purified from the wild type strain, and both of the *fol⁻* mutants overproduce photolyase to approximately the same extent as the wild type strain. As shown in Table II, the extent of overproduction of photolyase in SHA10 was 3–4%. Even though the extent of overproduction for SHA10 is slightly less than that obtained with MS09, this lower yield probably reflects the poorer growth of this particular *fol⁻* strain. The more vigorous PA414-derived strain, UNC414, overproduces photolyase as well as the wild type strain, MS09 (5.8% for UNC414 compared to 5.5% for MS09). It is evident that the amount of the CH^+-H_4folate present in both *fol⁻* mutants is considerable. There is no evidence that photolyase can produce CH^+-H_4folate from a H_2folate precursor; our findings are that it binds only 5,10-CH^+-H_4folate/10-CHO-H_4folate (12). The results of photolyase overproduction in both of these *fol⁻* strains have led to the conclusion that another source for fully reduced folates besides the dihydrofolate reductase must exist.

Analysis of Reduced Folates by HPLC—Since the *fol⁻* strains UNC414 and SHA10 were able to maintain enough CH^+-H_4folate to partly saturate the overproduced photolyase, it was of interest to determine whether other H_4folates were also present in these dihydrofolate reductase-deficient strains. Most of the analytical procedures for folate analysis that have been developed were intended for mammalian cells, but seemed adaptable to *E. coli* cells. The analytical procedure of choice involved cleavage of the polyglutamates to monoglutamates in the crude extracts using rat plasma γ-glutamyl hydrolase, passage of the treated extracts over Dowex which retains the folates, elution of the folates from Dowex, and concentration of the folates on a Sep-Pak C-18 cartridge.

Representative HPLC chromatograms of extracts from NM522 and AB1157, the two parent strains of LH18 and PA414, are shown in Fig. 3. Each chromatogram represents about 2.5 g of *E. coli* cell paste. In all cases, the presence of the individual folates in the extracts was verified by co-elution with the corresponding folate standard, as well as by comparison of the spectrum of the eluting species obtained on line by a diode-array detector to the spectrum of the standard

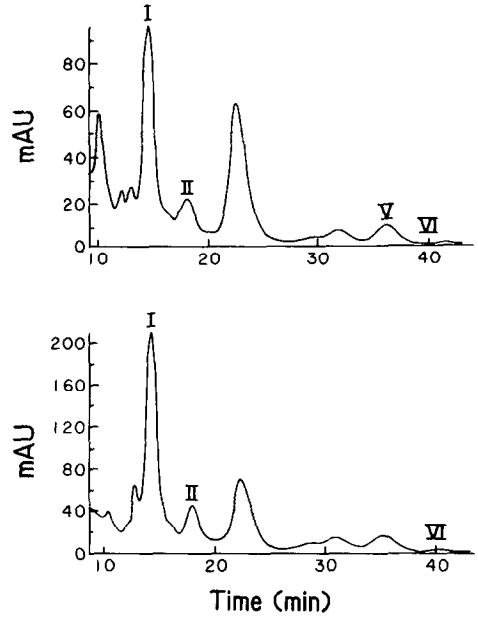

FIG. 3. **HPLC profile at 280 nm of folate cofactors from 2.5 g of NM522 cells (*top*) and from 2.5 g of AB1157 cells (*bottom*) treated as described in the text.** Before HPLC, the extracts in 5 mM sodium ascorbate, 5 mM 2-mercaptoethanol were passed through Spin-X columns by centrifugation in an Eppendorf microcentrifuge. Chromatography was in 5 mM tetrabutylammonium phosphate, 10 mM ammonium dihydrogen phosphate, 22% methanol, pH 6.1, at a flow rate of 0.9 ml/min. The *numbered peaks* are labeled as follows: *I*, 10-CHO-H_4folate; *II*, H_4folate; *III*, 5-CHO-H_4folate; *IV*, H_2folate; *V*, folate; and *VI*, CH_3-H_4folate.

folate compound. The numerals indicate the folates identified in each of the extracts. In these extracts, 10-CHO-H_4folate, H_4folate, CH_3-H_4folate, and traces of folic acid were identified.

Representative chromatograms of extracts from LH18 and PA414 as well as a chromatogram of several folate standard compounds are shown in Fig. 4. Each of the chromatograms for the *fol⁻* extracts represents approximately 4 g of *E. coli* cell paste. The chromatography conditions were slightly different for the chromatograms in Fig. 4, and all folates exhibit shorter retention times relative to the chromatograms in Fig. 3 as a result. Under these conditions, the H_4folate peak, indicated by the *arrows* in Fig. 4, is obscured by UV-absorbing material. The chromatography was repeated under conditions that better resolved the H_4folate peaks in the mutants, and a distinct H_4folate peak was observed for each of the *fol⁻* mutants with a magnitude of about 50% less than that of the H_4folate peak shown in Fig. 3 for the parent strains (data not shown). By these methods, we have verified the presence of H_4folate, CH_3-H_4folate, folic acid, and 10-CHO-H_4folate in the *fol⁻* strains.

The relative amounts and the distribution of these folates can be seen by a comparison of the different HPLC chromatograms in Fig. 3 and Fig. 4, keeping in mind that the chromatograms for the *fol⁻* strains were obtained using 1.6-fold more sample. 10-CHO-H_4folate is the major folate species in both of the *fol⁻* strains and the parent strains. However, there appears to be slightly more of this species in the parent strains, since the amounts are approximately equal in the chromatograms but more material was injected for the *fol⁻* strain analysis. The serum γ-glutamyl hydrolase controls indicated that there was not any folate material in the serum preparations (data not shown). Extracts of the strains containing internal folate standards were analyzed for folate content, and the recovery of the internal standards was com-

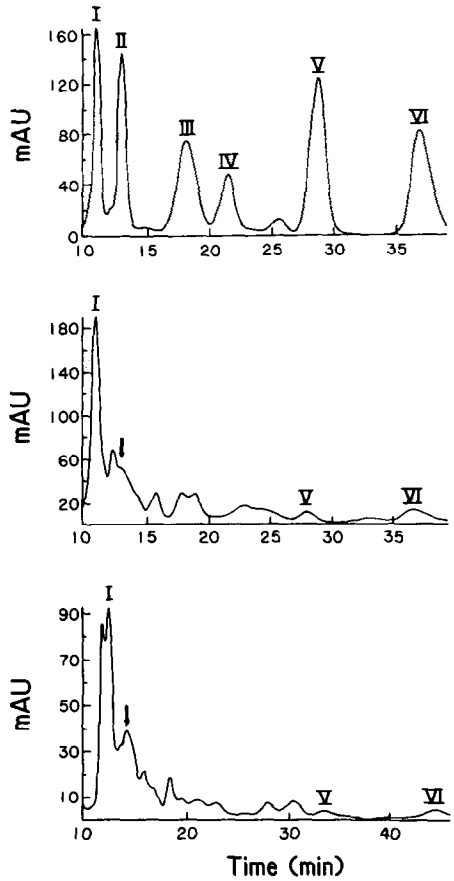

FIG. 4. **HPLC profile at 280 nm of standard folates (*top*) and folates from 4 g of LH18 cells (*middle*) or 4 g of PA414 cells (*bottom*).** Extracts in 5 mM sodium ascorbate, 5 mM 2-mercaptoethanol were centrifuged through Spin-X columns and immediately injected. Chromatography was in 98% solvent A and 2% solvent B at a flow rate of 1.8 ml/min. Solvent A consisted of 5 mM tetrabutylammonium phosphate, 10 mM ammonium dihydrogen phosphate, 24% methanol, pH 6.1, and solvent B consisted of 100% methanol. The *labeled peaks* are as follows: *I*, 10-CHO-H_4folate; *II*, H_4folate; *III*, 5-CHO-H_4folate; *IV*, H_2folate; *V*, folate; and *VI*, CH_3-H_4folate. The *arrows* indicate the position of the H_4folate peaks which were obscured by UV-absorbing material in these chromatograms.

parable between the strains (data not shown). These recoveries were also comparable to yields of the folate standard controls.

The yields of each standard when carried through the procedure with and without added *E. coli* crude extract are somewhat lower than those reported by Duch *et al.* (14) and vary between 20 and 50% for all species but H_2folate and 5-CHO-H_4folate which are on the order of 10%. The low yields of H_2folate and 5-CHO-H_4folate were expected, although the yields of the other folates have been reported to be higher than ours (14). We attribute our lower yields to the fact that the ionic strengths of our extracts were certainly higher due to the relative impurity and proportionally greater volume of γ-glutamyl hydrolase that was used. It was disappointing that we could not obtain better yields of H_2folate, since it would have been useful to see how much H_2folate was present in the *fol*$^-$ strains relative to the *fol*$^+$ strains.

Dihydrofolate Reductase Assays of PA414, LH18, AB1157, and NM522—Both PA414 and LH18 were constructed by deleting the *fol* gene so that spontaneous reversions could not occur (2, 4). The *fol* gene was in fact replaced with the gene coding for kanamycin resistance so that the *fol*$^-$ phenotype was easily verifiable. The phenotypes of both mutants have been confirmed by enzymatic assays and Southern blot experiments (2, 4). We have also confirmed that crude extracts of PA414 and LH18 contain no dihydrofolate reductase activity. Assays of crude extracts of PA414 and LH18 and the two parent strains AB1157 and NM522 as well as another *fol*$^+$ strain, MJ7, are shown in Table III. The *fol*$^-$ strains show a very small and inconsistent rate of change in absorbance at 340 nm. This rate is similar to the background rate of NADPH oxidation in *fol*$^+$ strains. If this rate is significant, then the observation that it is not eliminated completely in assays of PA414 incubated with methotrexate might indicate that it represents an "alternative" reductase activity with dihydrofolate that might be present in all strains but elevated in PA414. In separate assays, extracts of SHA10 and UNC414 were also shown to contain no dihydrofolate reductase activity (data not shown).

Growth Requirements for PA414 and LH18—The reports of the growth requirements for PA414 and LH18 (2, 4) seemed inconsistent. PA414 reportedly required only added thymine for growth, but LH18 required all of the folate-dependent end products (thymine, adenine, glycine, methionine, and pantothenic acid) for growth. Both of the strains were constructed with mutations in the gene coding for thymidylate synthetase since the *fol* mutations were otherwise lethal. Both mutants would be expected to require thymine for growth because of the thymidylate synthetase mutation. However, the report that LH18 requires all folate end products for growth is inconsistent with the presence of the reduced folates in the cell that we observed. We have found that both LH18 and PA414 demonstrate a requirement for thymine but not for the additional folate end products. Both grew on minimal plates containing kanamycin and thymine or kanamycin, thymine, adenine, glycine, methionine, and pantothenate. Neither grew on minimal plates containing only kanamycin. This is consistent with the fact that each strain contains H_4folates.

DISCUSSION

Our results show unambiguously that two *E. coli* strains that are devoid of dihydrofolate reductase activity contain significant quantities of reduced folates. The presence of these reduced folates was first inferred in LH18; photolyase, an enzyme containing a tightly bound reduced folate cofactor, was overproduced and purified from SHA10, a derivative of LH18, and shown to contain the reduced folate cofactor. This reduced folate cofactor was also isolated from photolyase purified from UNC414, a derivative of PA414. This finding, that a H_4folate was present in sufficient quantity in these *fol*$^-$ strains to partly saturate the overproduced photolyase, was

TABLE III

Activities of E. coli extracts

Assays are composite. Values were normalized to 1 mg of protein from crude extract added per assay. The concentration of methotrexate (MTX) was 1 μM.

Strain	Decrease in absorbance at 340 nm
NM522	0.043
NM522 + MTX	0.000
LH18	0.0016
LH18 + MTX	0.000
AB1157	0.036
AB1157 + MTX	0.000
PA414	0.0015
PA414 + MTX	0.002
MJ7	0.047
MJ7 + MTX	0.000

unexpected. The extent of saturation of the enzyme with the CH^+-H_4folate in both cases was 60–80% of that of enzyme obtained from the *fol*$^+$ strain. The dihydrofolate reductase lesion in LH18 and PA414 had abolished the activity presumed to be responsible for the generation of all cellular H_4folates in *E. coli*. That the dihydrofolate reductase activity was in fact absent was verified by assays of crude extracts of LH18 and PA414. Reversion to the wild type is impossible for these *fol*$^-$ mutants since the dihydrofolate reductase mutation was created by deleting the *fol* gene.

Quantitative folate analysis of both mutants revealed that in fact several different reduced folates were present; 10-CHO-H_4folate, CH_3-H_4folate, H_4folate, and folic acid. The presence of 10-CHO-H_4folate as the major species in chromatograms of all of the strains is consistent with its essential role in the cell; it is responsible for the generation of N^{10}-CHO-methionine which is required as the initial amino acid for protein synthesis. 10-CHO-H_4folate also serves a critical role as a cofactor in *de novo* purine biosynthesis. There is a small amount of H_4folate in the *fol*$^-$ strains, and about twice that in the parent strains. Most of the CH_2-H_4folate present in the cell is converted to H_4folate during the isolation procedure (14). Since the major cellular reaction requiring CH_2-H_4folate is the reaction catalyzed by thymidylate synthetase, it is not surprising that there is more H_4folate in the parent strains containing this enzyme than in the *fol*$^-$*thy*A$^-$ mutants. There appears to actually be more of the CH_3-H_4folate species in the *fol*$^-$ strains than in the parent strains. The fact that the major folate species in the cell was the 10-CHO-H_4folate explains the finding that photolyase from SHA10 and UNC414 was saturated with 5,10-CH^+-H_4folate to almost the same extent as photolyase from the *fol*$^+$ strain, MS09.

The presence of reduced folates in *E. coli* lacking dihydrofolate reductase explains the previously reported failure of the PA414 mutant to display folate end product auxotrophy (4). Depletion of the folate pool by thymidylate synthetase in the absence of dihydrofolate reductase seems to be the most likely explanation for the deleterious effect on cells of *fol*$^-$*thy*A$^+$.

Our findings imply that there must be another enzyme or metabolic pathway responsible for the generation of H_4folates in *E. coli fol*$^-$ mutants. The recently identified dihydropteridine reductase of *E. coli* (18, 19) appears to be a likely candidate for this activity. Dihydropteridine (or dihydropterin) reductases are found primarily in mammalian cells containing the aromatic amino acid hydroxylases. These aromatic amino acid hydroxylases require H_4biopterin as a cofactor; during the course of the reaction, quinonoid-7,8-H_2biopterin is generated and requires reduction to the 5,6,7,8-H_4biopterin cofactor (20). This reduction step is catalyzed by the dihydropteridine reductase in a reaction requiring NADPH.

E. coli does not contain any known aromatic amino acid hydroxylases, yet the presence of the dihydropteridine reductase has been recently documented. The function of this enzyme in *E. coli* is unknown. Analysis of the pterin composition of *E. coli* by HPLC reveals mainly monapterin with trace amounts of pterin and biopterin (18). These three pterins have no known function in *E. coli*. Also present in *E. coli* is molybdopterin (6); this pterin forms the molybdenum cofactor for enzymes such as nitrate reductase and dimethyl sulfoxide reductase. The cofactor itself is tightly bound to enzymes, but during biosynthesis the pterin may require ring reduction by an enzyme such as dihydropteridine reductase.

Characterization of the *E. coli* dihydropteridine reductase indicates that it is a monomeric enzyme of molecular mass 27,000 daltons, contains a flavin prosthetic group, accepts NADH and NADPH as a reducing substrate (although NADH is preferred *in vitro* with the quinonoid-H_2pterin substrates), and can use a variety of quinonoid-H_2pterin substrates (18, 19). A recent report states that it has activity with H_2folate as well, at a rate 5% of that seen with the quinonoid-H_2pterin substrates (19).

The activity observed *in vitro* with H_2folate is not unprecedented. Rat liver dihydropteridine reductase acts efficiently on quinonoid-H_2folates and, less efficiently, on H_2folates (20). The rat liver enzyme is also competitively inhibited by several folate analogues including 2,4-diaminopteroate and aminopterin. Other mammalian dihydropteridine reductases are inhibited by methotrexate (20), as is the *E. coli* enzyme (18, 19). In addition, dihydropteridine reductases have been identified in mammalian tissues which do not contain amino acid hydroxylases. One explanation proposed to explain this finding is that the nonenzymatic oxidation of H_4folate produces a quinonoid-H_2folate species which might be regenerated to the H_4folate by dihydropteridine reductase (20). In this way, H_4folate pools might be protected under conditions where dihydrofolate reductase activity is minimal. This same scenario is applicable to the *fol*$^-$ mutants. It is possible that in cells lacking dihydrofolate reductase this enzyme can catalyze the reduction of 7,8-H_2folate to H_4folate at a slow but significant rate.

If the dihydropteridine reductase of *E. coli* is responsible for the generation of the cellular reduced folate pool, then the lethal nature of the *fol*$^-$*thy*A$^+$ combination is explained. In the absence of thymidylate synthetase, the dihydropteridine reductase catalyzes a slow but steady reduction of 7,8-H_2folate to H_4folate. In the presence of thymidylate synthetase, this rate is unable to keep up with the rapid production of H_2folate by thymidylate synthetase. Under these conditions, the cells cannot maintain a reduced folate pool necessary to carry out the other folate-requiring reactions essential for growth, and the cells die. It would be interesting to see if dihydropteridine reductase levels are elevated in the *E. coli fol*$^-$ mutants.

Reduction of H_2folate by either a primary or auxiliary function of dihydropteridine reductase is only one scenario for these *fol*$^-$ mutants, although it appears to be the most likely. Another possibility is that folates may be synthesized as quinonoid species and that the terminal step in folate biosynthesis in *E. coli* is not catalyzed by dihydrofolate reductase but rather by the dihydropteridine reductase. In this scheme, one of the postulated H_2-precursors shown in Scheme 1 might undergo a rearrangement to a quinonoid precursor. Dihydrofolate reductase would function in this scheme by reducing the 7,8-H_2folate produced in the thymidylate synthetase reaction. The fact that the folate levels in LH18 and PA414 are not quite as high as the levels in the parent strains makes this scenario less likely. However, in the absence of thymidylate synthetase, overall folate production may be decreased since one major folate-requiring pathway is inoperative. It is possible that another as yet uncharacterized enzyme exists in *E. coli* with the primary or secondary function of reducing H_2folate. Finally, it is also possible that another pathway for folate biosynthesis exists in *E. coli* that does not derive the folate ring from GTP; this, however, is extremely unlikely.

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