We have identified and purified to homogeneity an enzyme from rat liver that catalyzes the oxidative catabolism of 5-formyltetrahydrofolate to p-aminobenzoylglutamate and a pterin derivative. Purification of the enzyme utilized six column matrices, including a pterin-6-carboxylic acid affinity column. Treatment of crude rat liver extracts with EDTA or heat decreased the specific activity of the enzyme by up to 85%. Peptides generated from the purified protein were sequenced and found to be identical to primary sequences present within rat light chain or heavy chain ferritin. Commercial rat ferritin did not display catabolic activity, but activity could be acquired with iron loading. The purified enzyme contained 2000 atoms of iron/ferritin 24-mer and displayed similar electrophoretic properties as commercial rat liver ferritin. The ferritin-catalyzed reaction displayed burst kinetics, and the enzyme catalyzed only a single turnover in vitro. Expression of rat heavy chain ferritin cDNA resulted in increased rates of folate turnover in cultured Chinese hamster ovary cells and human mammary carcinoma cells and reduced intracellular folate concentrations in Chinese hamster ovary cells. These results indicate that ferritin catalyzes folate turnover in vitro and in vivo and may be an important factor in regulating intracellular folate concentrations.

Tetrahydrofolate (THF) is a metabolic cofactor that accepts and donates single carbon units and is a source of reducing equivalents for the thymidylate synthase reaction (1, 2). Chemically, THF consists of a quinazoline ring that is bridged to a formaldehyde group and donates single carbon units and is a source of reducing equivalents for the thymidylate synthase reaction (1, 2). Chemically, THF exists in several chemically modified forms. The quinazoline ring can be oxidized to yield dihydrofolate, the product of the thymidylate synthase reaction, and the N-5 and N-10 of the cofactor are modified with single carbon units at the oxidation state of 11. 5-Formyl-THF is the most stable derivative of THF, and its stability has been attributed in part to steric protection and other reduced thiols, which serve to protect reduced folates from oxidation (10), are proposed to protect this site from oxidation by forming transient 4a adducts. This notion is supported by studies demonstrating that lypophilized samples of pure THF contain 1 atom of sulfur/THF molecule (10). Alternatively, THF or dihydrofolate can undergo an oxidative scission reaction at the C-9–N-10 bond. Electron extraction at N-10 results in the formation of an intermediate N-10 nitrenium ion that rapidly converts to the more stable C-9–N-10 Schiff base. Upon hydrolysis of the Schiff base, the scission products 6-formyltetrahydropterin (or 6-formyldeoxypteridin) and pABG are generated (5). One carbon substitution at N-5 or N-10 can alter the reactivity of THF to oxidative degradation (11, 12). 5-Formyl-THF is the most stable derivative of THF, and its stability has been attributed in part to steric protection of the C-4a oxidation site.

Neither the role nor biochemical mechanisms of folate turnover in regulating intracellular folate concentration have been widely investigated. Humans turn over <1% of total body folate per day (13), and folate turnover has been assumed to be due to nonenzymatic degradation of labile folate cofactors (Scheme 1). Urinary pABG and its acetylated derivative, p-acetamidobenzoylglutamate, have been demonstrated to be suitable indicators of folate catabolism (14), the measurement of which reflects folate status in rats and humans (15). Once the cleavage reaction occurs, folate is no longer a viable metabolic cofactor. Increases in folate catabolic rates are associated with pregnancy and growth rate in rats (16, 17) and anticonvulsant drug therapy (18–20). These increases in folate catabolic rates result in lower intracellular folate levels (15, 17). The increase in folate catabolism associated with certain physiological states suggests that folate catabolism is a regulated, enzyme-mediated event. However, attempts to purify enzymes from mammalian tissue that catalyze folate derivatives have not been successful in vitro and readily undergoes oxidative degradation. Solutions of THF can be stabilized in vitro by the addition of reduced thiols or antioxidants, including ascorbate. Oxidation can occur by at least two distinct mechanisms that are essentially irreversible. The quinazoline ring can be sequentially oxidized to dihydrofolate and then to folic acid through a quinonoid dihydrofolate intermediate (5, 6). This mechanism is also shared by tetrahydropterin oxidation (7, 8). The site of oxidation has been proposed to occur through a 4a-carbolamine intermediate, and chemically stable deaza-tetrahydropterin 4a adducts have been synthesized (7) and shown to be analogous to intermediates associated with the nonenzymatic oxidation of tetrahydropterins. Similar intermediates are also seen for the phenylalanine hydroxylase-catalyzed oxidation of tetrahydropterins (7, 9). 2-Mercaptoethanol and other reduced thiols, which serve to protect reduced folates from oxidation (10), are proposed to protect this site from oxidation by forming transient 4a adducts. This notion is supported by studies demonstrating that lypophilized samples of pure THF contain 1 atom of sulfur/THF molecule (10). Alternatively, THF or dihydrofolate can undergo an oxidative scission reaction at the C-9–N-10 bond. Electron extraction at N-10 results in the formation of an intermediate N-10 nitrenium ion that rapidly converts to the more stable C-9–N-10 Schiff base. Upon hydrolysis of the Schiff base, the scission products 6-formyltetrahydropterin (or 6-formyldeoxypteridin) and pABG are generated (5). One carbon substitution at N-5 or N-10 can alter the reactivity of THF to oxidative degradation (11, 12). 5-Formyl-THF is the most stable derivative of THF, and its stability has been attributed in part to steric protection of the C-4a oxidation site.

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successful, most likely due to high rates of nonenzymatic catabolism (19). The following study describes the identification and purification of an enzyme from rat liver that catabolizes folate and influences intracellular folate concentrations in cultured cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

(6S)-[^3H]Folic acid (5-formyl-THF; 40 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (6R,6S)-5-Formyl-THF was from SAPEC, and (6R)-5-formyl-THF and (6S)-5-formyl-THF were the generous gift of Eprova AG. MES, HEPES, ATP, and rat liver ferritin were purchased from Sigma. Other chemicals were reagent grade. Fetal bovine serum, minimal essential medium (MEM), and (6

**Purification of 5-Formyl-THF-catabolizing Enzyme**

All procedures were performed at room temperature, except centrifugation and dialysis steps, which were performed at 4 °C. Throughout the purification, the catalytic activity was monitored in all fractions by incubating the protein solutions (50–500 μg) with [3H]Folinic acid (5-formyl-THF; 40 Ci/mmol) at 37 °C. The amount of ρABG generated was determined by the Bratton-Marshall assay (21) described below. Throughout the purification, >90% of the 5-formyl-THF catabolic activity was associated with a single fraction, with the exception of hydroxylapatite chromatography. Folic acid, 5-formyl-THF, and pterin-6-carboxylic acid affinity columns were synthesized as described previously (22). Isolation and purification of intact mitochondria from fresh rat livers were performed as described previously (22).

**Step 1: Homogenization**—100 fresh-frozen rat livers were thawed and homogenized in 1 liter of 50 mM potassium phosphate (pH 7.2) containing 5% (w/v) polyethylene glycol 8000. The homogenate was centrifuged, and the pellet was discarded. Polyethylene glycol 8000 was added to the supernatant to a final concentration of 17% (w/v), and the solution was centrifuged. The supernatant did not contain any 5-formyl-THF catabolic activity and was discarded. The pellet was dissolved in 500 ml of 50 mM potassium phosphate (pH 7.5), and ammonium sulfate was added to a final concentration of 30%. After centrifugation, the supernatant was discarded. The pellet was resuspended in 500 mM potassium phosphate (pH 7.5). Only this fraction contained 5-formyl-THF cleavage activity.

**Step 2: Phenyl-Sepharose Chromatography**—The protein solution from Step 1 was directly applied to a 10 × 10-cm phenyl-Sepharose matrix equilibrated with 500 mM potassium phosphate (pH 7.2). The column was washed with 500 mM potassium phosphate (pH 7.5) until the A_{280} of the eluant was <0.1. The flow-through and wash fractions did not contain detectable 5-formyl-THF catabolic activity. The enzyme activity was eluted from the matrix with 5 mM potassium phosphate (pH 7.2). The activity eluted as a sharp red band. Active fractions were pooled and precipitated with ammonium sulfate (50% saturation). Following centrifugation, the protein pellet was resuspended in 500 mM potassium phosphate (pH 7.2).

**Step 3: Butyl-Sepharose**—The protein solution from Step 2 was applied to a 5.5 × 8-cm Butyl-Sepharose column. The column was washed with 500 mM potassium phosphate (pH 7.5) until the A_{280} of the eluant was <0.2. The enzyme activity was eluted with a linear gradient; buffer A (200 ml) was 500 mM potassium phosphate (pH 7.2), and buffer B (200 ml) was 5 mM potassium phosphate (pH 7.2). The activity eluted midway through the gradient as a red band. Active fractions were pooled (100 ml), precipitated with ammonium sulfate (50% saturation), and dialyzed overnight against 5 mM Tris-Cl (pH 7.5). The dialyzed protein solution from Step 3 was applied to a 5 × 10-cm hydroxylapatite column equilibrated with 5 mM Tris-Cl (pH 7.5). The enzyme activity was eluted with a linear gradient; buffer A (100 ml) was 5 mM Tris-Cl (pH 7.5), and buffer B (100 ml) was 200 mM potassium phosphate (pH 7.2). The most active fraction (see Fig. 2) eluted midway through the gradient as a red band. This fraction was diluted with water to give a final potassium phosphate concentration of 10 mM.

**Step 5: DEAE-Sephaloc**—The active protein fraction from Step 4 was applied to a 2.5 × 5-cm DEAE-Sephaloc column equilibrated with 10 mM potassium phosphate (pH 7.2). The protein was eluted with a linear gradient; buffer A (200 ml) was 5 mM potassium phosphate (pH 7.2), and buffer B (200 ml) was 200 mM potassium phosphate (pH 7.2). The activity eluted as a red band midway through the gradient. Active fractions were pooled (~100 ml), precipitated with ammonium sulfate (50% saturation), and dialyzed overnight against 5 mM potassium phosphate (pH 7.2).

**Step 6: Pterin Affinity Chromatography**—The dialyzed protein from Step 5 was applied to a 1.5 × 4-cm pterin affinity column pre-equilibrated with 5 mM potassium phosphate (pH 7.2) (buffer A). The column was washed with buffer A until the A_{280} was <0.1. The enzyme was eluted with a linear gradient; buffer A was the equilibration buffer, and the generation of laplinate and a pterin aldehyde. The generation of degradation. The scission of tetrahydrofolate by O2 results in p-aminobenzoylglutamate and a pterin aldehyde.
buffer B (100 ml) was 100 mM potassium phosphate (pH 7.2). The activity eluted near the end of the gradient as a red band. Active fractions were pooled, precipitated with ammonium sulfate (50% saturation), and dissolved in 500 μl of 20 mM sodium bicarbonate (pH 7.2).

**Step 7: Size Exclusion Chromatography**
The dialyzed protein from Step 6 was immediately applied to a 1.5 × 30 cm Superdex 200 column equilibrated with 20 mM sodium bicarbonate (pH 7.5). 1-ml fractions were collected, and active fractions were pooled.

**Peptide Sequencing**
Amino acid sequencing was performed at the Harvard Microchemistry Facility. Purified protein was applied to a 10% SDS-polyacrylamide gel containing 2% (v/v) 2-mercaptoethanol prior to loading. The upper chamber and wells were filled with the anode solution (25 mM H₃PO₄). The lower chamber was filled with the anode solution (25 mM H₂PO₄). The gel was stained with Coomassie Brilliant Blue R-250 Blue R-250 (0.25%, w/v), 10% methanol, and 10% glacial acetic acid. The protein band(s) of interest were then removed and soaked in 50% acetonitrile for 5 min. The acetonitrile was removed, and the gel slices were sent to the Harvard Microchemistry Facility for Lys-C digestion, peptide purification, and sequencing. Additionally, tryptic peptides were generated and sequenced at the Cornell Biotechnology Facility.

**SDS-PAGE of the Purified 5-Formyl-THF Catabolic Activity**
Purified ferritin (3.6 μg) containing 5-formyl-THF catabolic activity and commercial rat liver ferritin (3.1 μg) were analyzed by 12% SDS-PAGE. Protein samples were boiled in a final solution of 2% SDS, 100 mM dithiothreitol, 10% glycerol, and 60 mM Tris (pH 6.8) prior to PAGE. Protein samples were determined using the Bratton-Marshall assay (21).

**Isoelectric Focusing**
An isoelectric focusing gel analysis of the purified protein was performed. A 0.75-mm denaturing acrylamide gel (30%, w/v) gel containing Bio-Rad ampholyte mixtures (pH 4–6) was prepared as described previously (23). Protein samples were mixed with an equal volume of lysis buffer (8% urea, 2% (v/v) amylase, 2% (v/v) Triton X-100, and 1% 2-mercaptoethanol) prior to loading. The upper chamber and wells were filled with the anode solution (25 mM H₂PO₄). The lower chamber was filled with the cathode solution (50 mM NaOH), and electrophoresis was reversed to match the polarity consistent with the electrode solutions. When electrophoresis was complete, gels were rinsed and then fixed in 10% trichloracetic acid. Gels were stained in a solution of 0.25% (w/v) Coomassie Brilliant Blue R-250 in 45% (v/v) methanol and 10% (v/v) glacial acetic acid.

**Assay for Folate Cleavage Reaction**
Purified ferritin or cell extracts (0.1–1 ml) were incubated at 37 °C with varying concentrations of 5-formyl-THF for various time intervals. The generation of pABG, a primary aromatic amine, was quantified in the clarified solutions using the Bratton-Marshall assay (21). Following the reaction, the samples were incubated at 100 °C for 2 min to precipitate protein present in the samples. This step was omitted when assaying the purified protein. 15 μl of 1.0 N HCl and 5 μl of 1% (w/v) sodium nitrite (Sigma) were added to a 50-μl sample. Samples were vortexed for 5 min. 5 μl of 3% (w/v) ammonium sulfamate (Sigma) was added, and samples were vortexed for an additional 10 min. The samples were clarified by centrifugation. The supernatant was removed, and 10 μl of 1% (w/v) N-1-naphthyl-phtaleinimide was added to 50 μl of 5% ethanol (Sigma) was added. The sample was vortexed briefly, and the absorbance at 550 nm was determined. The pABG was generated and quantified relative to a standard curve. All absorbance values were corrected with control reactions that lacked 5-formyl-THF and others that lacked cell extract or purified protein.

**Metal Analysis**
The purified enzyme was analyzed for the presence of protein-bound metals. The protein solution (0.5 mg of protein in 500 μl of 10 mM potassium phosphate (pH 7.2)) was dialyzed for 24 h against 1 liter of 10 mM Tris-Cl buffer (pH 7.2) with several buffer changes. The metal content of the protein sample was determined relative to metal standards and corrected for any metal content present in the dialysis buffer.

Iron was quantified in crude and partially purified protein fractions by modifying the colorimetric method developed by Liu et al. (24). All solutions were made with Milli-Q water. Standards were 0–50 μM ferric citrate in 0.1 N HCl. A 5-μl protein sample was diluted with 10 μl of 10 N HCl and incubated overnight at 37 °C. The reaction was diluted to a final volume of 1 ml with Milli-Q water. 51 μl of sample or standard was treated with 9 μl of 800 mM nitrotriacetic acid for 30 min at room temperature. Following incubation, 15 μl of 120 mM thiglycolic acid and 15 μl of 60 mM bathophenanthrolinedisulfonic acid disodium salt were added. After incubation for 30 min, the absorbance was determined at 537 nm.

**Removal of Catalytic Iron by EDTA**
Exterior iron bound to purified ferritin and free iron in the protein preparation were removed by incubation with 10 mM EDTA for 1 h on ice. The EDTA was removed from the protein solution by dialysis in 1 liter of Tris-buffered saline (pH 7.4) containing 150 mM NaCl, 2 μM EDTA. Control samples of purified ferritin were incubated with a volume of water equivalent to the EDTA and treated in a parallel manner. Following dialysis, the activity of the EDTA-treated and control protein samples was determined using the Bratton-Marshall assay (21).

**Reloading of Catalytic Iron**
EDTA-treated purified or commercial ferritin solutions were incubated with 500 μg ferrous ammonium sulfate for 1 h at 22 °C. Control samples were treated in a similar manner, but without ferrous ammonium sulfate. Excess iron was removed by dialyzing the samples against 0.5 liters of 0.1 M HEPES (pH 7.2) for 12 h with five buffer changes. Following dialysis, the activity of the iron-loaded and control protein samples was determined using the Bratton-Marshall assay (21).

**Vector Construction and Transfection**
Total RNA isolated from rat liver (CLONTECH) was converted to cDNA using Tth polymerase (Promega). The open reading frame of heavy chain ferritin (HCF) was amplified by polymerase chain reaction. The forward primer was 5'-CAGTTGGGTACAGACACGGCTCTCCTCTCC-3' and contained a KpnI restriction enzyme site (underlined). The reverse primer was 5'-CAGGATCTTGCCATGCTGC-3' and contained an EcoRI restriction enzyme site (underlined). The polymerase chain reaction product was subcloned into the KpnI/EcoRI sites of pcDNA3 and verified by nucleotide sequencing.

The pcDNA3 vector (Invitrogen) utilizes the cytomegalovirus major intermediate-early promoter/enhancer and the bovine growth hormone polyadenylation signal. A G418 resistance gene in pcDNA3 allows for selection of stable transfectants. The HCF construct (2.5 μg) was transfected into CHO-WTT2 or MCF-7 cells by electroporation (0.22 kV and 950 microfarads; Bio-Rad Gene Pulser II). Cells were cultured in MEM for 24–48 h prior to the addition of 400 μg/ml G418 sulfate. Over 20 colonies from each cell line exhibited resistance to G418 sulfate; eight were isolated and passaged until a stable line was generated, and two colonies were chosen at random for subsequent study.

**Cell Line and Medium**
CHO-WTT2 cells were obtained from Dr. Barry Shane (University of California, Berkeley, CA); MCF-7 cells were from American Type Culture Collection. Cells were maintained in MEM supplemented with 10% fetal bovine serum and incubated at 37 °C in a 5% CO₂ atmosphere. G418 sulfate was added to the medium for selection of stable cell colonies that integrated the HCF construct. For folate turnover studies, fetal bovine serum was dialyzed against 10 volumes of PBS at 4 °C for 24 h with buffer changes every 4 h to deplete serum glycylic, folate, and other small molecules. The serum was then charcoal-treated to remove any remaining folate. Defined culture medium was used (α-MEM lacking glycine, serine, and folate), which allows variation in the concentration of nutrients with relevance to folate-dependent one-carbon metabolism. For total folate analyses, defined culture medium lacking folic acid and supplemented with charcoal-treated fetal bovine serum was used.

**Microbiological Assay for Total Intracellular Folate**
Cells were cultured in MEM with 10% fetal bovine serum or in α-MEM supplemented with charcoal-treated 10% fetal bovine serum and glycylic. The medium was refreshed every 48 h as necessary. Cells were harvested at defined time points by removing the culture medium and washing cells monolayers with PBS. Cell pellets were obtained by trypsinization of cell monolayers. The pellets were washed three times and resuspended in PBS. The cell suspension was divided into two equal aliquots, centrifuged, and stored at −70 °C.

Total protein was measured in one fraction, whereas total folate was measured in the other. Protein was extracted by boiling in a buffered solution containing 2% SDS and 60 mM Tris (pH 6.8), and protein was
quantified (25). Total folates were measured by the procedure of Horne (26). Briefly, pellets were resuspended in a buffered solution containing 2% (w/v) sodium ascorbate, 0.2 M 2-mercaptoethanol, 0.05 M HEPEs, and 0.05 M CHES (pH 7.85). The solution was vortexed and boiled for 10 min. Samples were cooled and clarified by centrifugation. The supernatants were incubated at 37 °C following the addition of 0.25 volumes of rat plasma conjugase for 3 h. Following incubation, the samples were boiled to precipitate protein, and the solution was cooled and clarified by centrifugation. The experimental supernatants, as well as standards, ranging from 0 to 198 fmol of (6R,6S)-5-formyl-THF were aliquoted into black 96-well ViewPlates (Packard Instrument Co.) containing 8.1 μl of working buffer (1.6 g of sodium ascorbate, 0.5 ml of 1 M potassium phosphate (pH 6.1), and 9.5 ml of distilled water), 152 μl of folic acid Lactobacillus casei medium (Difco), and Milli-Q water for a total of 300 μl in each well. 20 μl of undiluted L. casei inoculum was added to each well, and the plates were incubated for 18 h at 37 °C in a humidified incubator. Growth of L. casei was measured at 550 nm by a MRX Microplate Reader II (Dynex Technologies, Inc., Chantilly, VA).

Western Analyses of HCF in Cultured Cells

Protein from cultured cells was extracted and quantified (25). In preparation for electrophoresis, 120 μg of protein was boiled in a final solution of 2% SDS, 100 mM sodium dodecylsulfate, 10% glycerol, 0.01% bromphenol blue, and 60 mM Tris (pH 6.8). SDS-PAGE was carried out using an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) in a Bio-Rad Transblot apparatus. The membrane was then blocked in incubating buffer containing 5.0% (w/v) nonfat dry milk and 0.5% Igepal CA-630 (Sigma) in PBS.

To detect HCF, sheep serum containing anti-heavy chain ferritin polyclonal antibodies generated against a highly conserved peptide (SQVRGQNYHSDL(D)A) (Chiron) or Y17 anti-recombinant murine HCF antiserum was used at a 1:4170 dilution in blocking buffer during incubation overnight at 4 °C. The membrane was washed with 0.1% Tween in PBS before incubation with the appropriate horseshadish peroxidase-conjugated secondary antibody (1:10,000; Pierce) for 2 h at room temperature. The membrane was then washed with 0.1% Tween in PBS and visualized using the SuperSignal West Pico chemiluminescent substrate system (Pierce).

Determination of Folate Turnover in Cultured Cells

Cell monolayers at 50% confluence were washed with buffered solution containing 10% counts/ml (6S)-5-(5-formyl)-THF (25 μCi). Cells were washed with 10 ml of PBS and then trypsinized and pelleted by centrifugation. The pellets were resuspended in α-MEM lacking folate and glycine, and cells were counted with a hemocytometer. About 0.2–1 x 10^6 cells were aliquoted into 100-mm culture plates containing 10 ml of α-MEM supplemented with 1 mM (6R,6S)-5-formyl-THF or MEM alone. Cells were harvested at defined time points. The medium was removed, and the tritium was quantified. Cell monolayers were washed with PBS and lysed with 0.2M ammonium hydroxide. Tritium remaining in the cells was quantified using a Beckman LS 8100 scintillation counter.

RESULTS

Identification and Purification of a Folate Catabolic Activity in Rat Liver Extract—Cellular THF derivatives are chemically unstable and readily undergo oxidation in vitro, with the exception of 5-formyl-THF. 5-Formyl-THF is stable at physiological pH and therefore is an ideal substrate to identify enzymes that catalyze folate catabolism while avoiding high nonenzymatic rates of folate oxidative degradation. Soluble tissue extracts derived from rat liver homogenized in 50 mM potassium phosphate (pH 7.2) were found to catalyze the formation of pABG when incubated at 37 °C for 15 min with (6R,6S)-5-formyl-THF. The generation of pABG from 5-formyl-THF was not influenced by the addition of 1 mM NADP+, NAD+, or FAD to the rat liver extract, but all activity was lost when EDTA at 1 mM was included in the homogenization buffer. Heating the crude rat liver extract to 70 °C for 5 min decreased pABG formation by >95%, demonstrating that the catabolism of 5-formyl-THF is enzyme-catalyzed and is not due to small molecule oxidants present in liver.

| Step | Total protein | Activity* |
|------|---------------|-----------|
| 1 Homogenization | 154,000 | 0.59 |
| 2 5–17% PEG 8000 | 45,000 | 0.19 |
| 3 0–30% ammonium sulfate | 9032 | 0.30 |
| 4 Phenyl-Sepharose | 2452 | 49.0 |
| 5 Butyl-Sepharose | 428 | 3.0 |
| 6 Hydroxyapatite | 7.1 | 2630.0 |
| 7 DEAE-Sepharose | 1.6 | 2920.0 |
| 8 Pterin-Sepharose | 1.2 | |
| 9 Size exclusion | 0.9 | 12,800.0 |

*All fractions were assayed in the column elution buffer. Therefore, the specific activities derived from different purification steps are influenced by the assay buffer and are not comparable.

The 5-formyl-THF catabolic activity was purified to homogeneity as described under “Experimental Procedures” (Table I). The activity had been purified by this method >10 times starting from 100 fresh-frozen rat livers. The purification could be completed in 4 days and required the use of six column matrices. Throughout the purification, the 5-formyl-THF catabolic activity was associated with a single, red-colored fraction for all precipitation and chromatography steps, with the exception of the hydroxylapatite column. Fig. 1 displays the three major protein fractions that eluted from the hydroxylapatite column (as determined by the A280), and all three fractions contained a chromophore as demonstrated by the A310. Each fraction was found to contain high levels of elemental iron (Fig. 1, inset), which accounts for the A310. Fractions I and II both contained 5-formyl-THF catabolic activity, whereas fraction III did not. The amount of protein and catabolic activity associated with fraction I varied widely among purifications and was not seen in all purifications. Only fraction II was pooled and subjected to further purification.

The 5-formyl-THF catabolic activity bound to a pterin-6-carboxylic acid-Sepharose 4B affinity column, demonstrating that this enzyme is a pterin-binding protein. However, the enzyme did not bind to folic acid or 5-formyl-THF affinity columns. Folic acid was not effective in eluting the catalytic activity from the affinity columns. Throughout the purification, the 5-formyl-THF catabolic activity was associated with a single, red-colored fraction for all purification steps. The activity was associated with a single, red-colored fraction for all purification steps.
against 5 mM Tris-Cl (pH 7.5) and applied to a hydroxylapatite column as described under “Experimental Procedures.” The protein was eluted with a gradient of increasing concentrations of potassium phosphate. Elution of protein at A$_{280}$ and of iron at A$_{520}$ was monitored in each 5-ml fraction. The cleavage of (6R,6S)-5-formyl-THF was measured at the first peak (I; fractions 9–13), second peak (II; fractions 32–45), and third peak (III; fractions 54–59) by the Bratton-Marshall assay (21) (inset). The presence of iron was determined at fractions 10, 38, and 56 as described under “Experimental Procedures.” Values for the cleavage of 5-formyl-THF represent the mean of duplicates (peaks I and II) or triplicates (peak III). Values for iron content represent the mean of duplicate (fraction 10) or triplicate (fraction 38, 56) measures.

**Fig. 2.** SDS-PAGE of the purified 5-formyl-THF catabolic activity. Purified and commercial ferritins were analyzed by denaturing PAGE. Lane 1 contains 3.6 μg of purified ferritin, and lane 2 contains 3.1 μg of commercial rat ferritin.

**Comparison of Catalytically Active Ferritin with Commercial Ferritin**—The purified protein that catabolized 5-formyl-THF displayed several similar physical and chemical properties as commercial ferritin. The ultraviolet spectrum of the enzyme was nearly identical to that of commercial rat ferritin (data not shown). A crude mitochondrial/lysosomal fraction generated from fresh rat liver did not contain any 5-formyl-THF catabolic activity, and all activity was localized to the cytoplasmic fraction. Commercial preparations of glutamic dehydrogenase were assayed and found not to catalyze the degradation of 5-formyl-THF. Additionally, cell fractionation studies demonstrated that the 5-formyl-THF catabolic activity was localized to the cytosolic fraction (data not shown). A crude mitochondrial/lysosomal fraction generated from fresh rat liver did not contain any 5-formyl-THF catabolic activity, and all activity was localized to the cytoplasmic fraction.

**Kinetic Characterization of 5-Formyl-THF Catabolism**—Ferritin is a heteropolymer (24-mer) composed of heavy chain and light chain subunits that serve to sequester iron within cells (37, 38). Ferric iron is an effective oxidant, and the sequestration of iron by ferritin protects cells from random deleterious oxidation. The ferritin-catalyzed cleavage of 5-formyl-THF to pABG displayed burst kinetics. Fig. 5 shows that the generation of pABG following incubation of 62.5 pmol of purified ferritin (24-mer) occurred within the first 2 min of the reaction. Only 0.37 nmol of pABG was generated, and additional incubation did not result in an increase in pABG formation. The amount of product generated indicates that ~6 pABG molecules are generated per molecule of ferritin. The burst kinetics associated with 5-formyl-THF catabolism indicate that not all monomeric subunits are catalytically active and that multiple turnovers do not occur. The burst amplitude increased proportionally as a function of enzyme concentration (Fig. 6) when incubated with 2 mM (6R,6S)-5-formyl-THF.

The burst amplitude associated with pABG formation increased as a function of 5-formyl-THF concentration, and the enzyme displayed increased reactivity with the physiological versus nonphysiological isomer of 5-formyl-THF (Fig. 7). The burst amplitude did not saturate up to 4.0 mM (6S)-5-formyl-THF, the physiological isomer. Similar results were seen using (6R)-5-formyl-THF as the substrate. These data indicate that the enzyme has a very low affinity for both the physiological...
and nonphysiological isomers of 5-formyl-THF, and the possibility that the enzyme is generating \( pABG \) from a minor contaminant present in the 5-formyl-THF solutions cannot be excluded.

**Role of Iron in 5-Formyl-THF Catabolic Activity**—The oxidative cleavage of 5-formyl-THF to \( pABG \) by ferritin likely involves molecular iron as a cofactor. This suggestion is supported by the observation that the addition of EDTA to the homogenization buffer eliminated 5-formyl-THF catabolic activity in rat liver extracts. Ferric iron is an oxidant, and some studies have demonstrated that non-ferritin-bound iron catalyzes the oxidation of folate derivatives \( \text{in vitro} \) (6, 39). Passage of purified ferritin through a 0.5\( \times \)2-cm Chelex 100 cation affinity column to remove non-ferritin-bound iron did not diminish the catalytic activity, indicating that if iron is a catalyst, it functions only when bound to ferritin. Accordingly, incubation of purified ferritin with 10 mM EDTA for 60 min and subsequent removal of the chelator by dialysis reduced catalytic activity by up to 85%. Although EDTA is effective in chelating metals in solution, it is not effective in removing iron from the mineral core of ferritin under the dialysis conditions (40). These results demonstrate that ferritin contains a bound metal cofactor that participates in the catabolism of 5-formyl-THF that is accessible to EDTA. The activity can be restored by the addition of ferrous ammonium sulfate to the enzyme followed by removal of unbound Fe(II) by dialysis, confirming the necessity of iron for catalysis. Additionally, commercial ferritin can acquire 5-formyl-THF catabolic activity with the same

### Table II

| Sequence | Fragment type | Sequence identity | Identity% |
|----------|---------------|------------------|-----------|
| SEGAERLLK<sup>a</sup> | Lys-C | LCF | 100 rat |
| GNHLTLRRVAGPQPAQ<sup>b</sup> | Lys-C | LCF | 100 rat |
| LQNERRGRALFQDVQKPSDQEXYG<sup>b</sup> | Lys-C | LCF | 100 rat |
| NLNQALLDHALGSARTDPHL<sup>b</sup> | Lys-C | LCF | 100 rat |
| TLEAEMEAALAR<sup>c</sup> | Trypsin | LCF | 100 rat |
| QIYSTEVAEAVN<sup>c</sup> | Trypsin | LCF | 100 rat |
| ELGDIVTNLR<sup>d</sup> | Trypsin | HCF | 100 rat |
| DDVALEGVCHFR<sup>d</sup> | Trypsin | LCF | 100 rat |
| MGNHITNL<sup>d</sup> | Trypsin | LCF | 100 rat |

<sup>a</sup> HCF sequence was compared with NCBI Accession number U58829. LCF sequence was compared with NCBI accession number 120527. The underlined amino acid differs from the sequence reported.

<sup>b</sup> Sequenced by Cornell Sequencing Facility.

<sup>c</sup> Sequence GNHLTLRRVAGPQPAQ matched 100% to the amino acid sequence from Denis (80) (GenBank™/EBI accession number L01122).

<sup>d</sup> Sequenced by Harvard Microchemistry Facility.

**FIG. 3.** Isoelectric focusing of purified and commercial ferritins. Purified and commercial ferritins were analyzed by denaturing isoelectric focusing at pH 4–6 and stained for protein with Coomassie Blue. Lane 1 contains 4.7 \( \mu \)g of rat liver ferritin; and lane 2 contains 3 \( \mu \)g of purified ferritin.

**FIG. 4.** Western analysis of the purified 5-formyl-THF catabolic activity. Heavy chain ferritin immunoreactivity was detected with antiserum containing Y17 antibodies generated against recombinant mouse heavy chain ferritin. 3.1 \( \mu \)g of commercial rat ferritin (lane 1), 2.5 \( \mu \)g of recombinant mouse heavy chain ferritin (lane 2), 2.5 \( \mu \)g of purified active ferritin (lane 3), and 5.0 \( \mu \)g of purified active ferritin (lane 4) were analyzed by Western blot analysis.

**FIG. 5.** 5-Formyl-THF catabolism displays burst kinetics. 62.5 pmol of purified ferritin protein (24-mer) was added to a solution containing 2 mM (6\( R \),6\( S \))-5-formyl-THF in 18 mM NaHCO\(_3\) (pH 7.2) and incubated at 37 °C. At various time points, the reaction was terminated by placing the sample in a dry ice/ethanol bath, and the amount of \( pABG \) generated was determined using the Bratton-Marshall assay (21) as described under "Experimental Procedures."

**FIG. 6.** 5-Formyl-THF catabolism increases with increasing enzyme concentration. The cleavage of 2 mM (6\( R \),6\( S \))-5-formyl-THF in 20 mM NaHCO\(_3\) (pH 7.2) by 0, 25, 50, and 75 pmol of purified ferritin (24-mer) was measured at 37 °C for 10 min. The appearance of \( pABG \) was determined by the Bratton-Marshall assay (21). All values represent duplicate measures, and error bars represent S.D.
specific activity as purified ferritin when treated in a similar manner with ferrous ammonium sulfate.

Expression of Rat HCF in Mammalian Cells—The effect of rat HCF expression on folate turnover was investigated in human MCF-7 and CHO cells by pulse-chase analysis. Cells were transfected with a G418-resistant plasmid containing the rat HCF cDNA driven by the pCMV promoter, and stable clonal cell lines were established. Studies have indicated that high levels of HCF expression in mammalian cells are difficult to achieve in stable cell lines, which may be due to the deleterious effect this has on intracellular labile iron concentrations and proliferation rates (41-43). Fig. 8 shows a Western blot of the control and rat HCF-expressing cell lines probed with sheep anti-HCF antibodies. These antibodies were generated from a peptide that is 100% conserved in mouse, rat, and human HCF proteins. This antibody would be expected to bind rat, human, and most likely Chinese hamster HCF proteins with similar affinity and thereby yield an estimate of rat HCF expression. The results in Fig. 8 suggest that HCF levels were increased 2–3-fold in CHO cells and 5–10-fold in human MCF-7 cells. Fig. 9 displays rates of folate turnover in control and rat HCF-expressing cells. Rates of folate turnover in MCF-7 cells were biphasic, with a rapid phase occurring within the initial 4 h following the chase with unlabeled folic acid and a slower phase that was nearly linear for the subsequent 22 h. Rat HCF expression increased both the rate and amplitude of the rapid phase that was nearly linear for the subsequent 22 h. Rat HCF-expressing cells. The results clearly indicate that rat HCF expression increases rates of folate turnover, presumably by increasing rates of folate catabolism. Since increased rates of folate turnover were most pronounced during the early stages of the chase, this indicates that folate derivatives become less susceptible to ferritin-mediated turnover with increased residency time within the cell.

The effect of rat HCF expression on intracellular folate concentration was determined (Fig. 10). CHO cells expressing rat HCF showed 16% decreased levels of intracellular folate when cultured in MEM containing 2 μM folic acid. This indicates that HCF-catalyzed cleavage of folate can influence intracellular folate concentrations. CHO cells expressing HCF displayed 25% (CHO-HCF28) and 40% (CHO-HCF29) decreases in intracellular folate when cultured in medium lacking folic acid or other forms of folate for 48 h relative to wild-type CHO cells. This indicates that HCF expression influences intracellular folate concentrations and that the effect of HCF levels on intracellular folate concentrations is more pronounced under conditions of folate deprivation.
Folate Catabolism

In this study, we have demonstrated that ferritin purified from rat liver can catabolize 5-formyl-THF in vitro and that stable expression of rat ferritin in mammalian cell culture lines results in increased rates of folate turnover and reduced intracellular folate concentrations. The ferritin-catalyzed reaction is dependent upon an EDTA-accessible iron molecule. Ferritin is a 24-subunit heteropolymer containing light chain and heavy chain subunits. The relative distribution of light and heavy subunits displays tissue-specific variations (44). The structure of recombinant HCF reveals a novel dinuclear ferroxidase center that is not present in recombinant LCF (45). The metal ligand residues Glu$_{27}$, Glu$_{63}$, Glu$_{319}$, Glu$_{377}$, and His$_{605}$ constitute the ferroxidase center embedded in the centers of helical bundles (46), which lies 8 Å from the inside surface of the molecule and 10–12 Å from the outside surface (45). Channels formed by the 432 symmetry of the ferritin 24-mer permit the sequestration and release of iron (37). The x-ray structure indicates that the ferritin channels are narrow (3–5 Å) (47), although small molecules including dihydroflavins have been demonstrated to diffuse through the ferritin shell and to reduce Fe(III) at the mineral core, presumably through channels (37, 48, 49). During the in vitro catabolism of 4 mM (6S)- or (6S)-5-formyl-THF, less than stoichiometric equivalents of pABG are generated per ferritin subunit. The ferroxidase sites may be the sites containing the EDTA-chelatable iron molecules that catalyze the oxidation of 5-formyl-THF. Alternatively, Fe(III) produced by the ferroxidase sites may be located in ferritin channels that are not associated with ferroxidase activity (37).

The mechanism and kinetics associated with the horse liver and spleen ferritin-catalyzed oxidation of dihydrofavin analogs have been previously investigated (48). These studies demonstrated that dihydro flavins pass through the channels of ferritin and reduce Fe(III) at the mineral core. This oxidation reaction does not exhibit burst kinetics, but rather an accumulation of Fe(II) with time that is linear. The ferritin-catalyzed reaction occurs with a high $K_m$ (250 μM) and displays saturation kinetics with respect to substrate concentration. The oxidation rate is limited by dihydrofavin diffusion through the channels, and dihydrofavin bound to Sepharose beads is resistant to ferritin-catalyzed oxidation. This mechanism cannot account for 5-formyl-THF oxidation by ferritin. The oxidation of 5-formyl-THF monoglomerate by purified ferritin displays burst (not linear) kinetics and exhibits less than a single turnover per ferritin subunit at 4 mM 5-formyl-THF. If 5-formyl-THF were oxidized by the iron core, many turnovers per subunit would be expected.

In this study, 5-formyl-THF was utilized as a convenience substrate due to its relative stability compared with other forms of THF, especially in crude tissue extracts. Although other efforts have been made to purify a protein with folate catabolic activity from mammalian tissue, these efforts did not lead to the isolation of a catalytic protein (19). Some of these studies utilized other reduced forms of folate and therefore suffered from high rates of nonenzymatic catabolism, whereas other studies used fully oxidized folic acid as a substrate. Our purified ferritin did not react with oxidized folic acid. These catalytic properties exhibited by ferritin, including the low affinity for 5-formyl-THF and less than a single turnover per subunit, make detailed kinetic study of the purified enzyme difficult. Attempts to measure the reactivity of ferritin with other reduced forms of folate and therefore suffered from high rates of nonenzymatic folate catabolism during the reaction as well as during the Bratton-Marshall assay (21). Additionally, analytical methods for pABG detection were confounded by the necessity to remove ferritin prior to chromatography without releasing ferric iron. Previous studies have demonstrated that iron can catabolize the THF derivatives in vitro (6, 39, 50). However, it is unlikely that 5-formyl-THF is the only, or even the primary, substrate for folate catabolism catalyzed by ferritin. Since 5-formyl-THF is the most stable form of oxidized THF and one of the least abundant intracellular forms of THF, it would be predicted to be a poor substrate for catabolism. Both the ability of ferritin to bind to a pterin affinity column and the lack of reaction specificity with respect to the physiological and nonphysiological isomers of 5-formyl-THF indicate that ferritin does not display strict substrate specificity for THF derivatives.

Previous studies of folate turnover in humans, animals, and cell cultures have indicated that not all intracellular folate is equally susceptible to degradation and that several kinetically distinct turnover pools exist in cells (51, 52). Our studies demonstrate that rates of folate turnover are markedly different in MCF-7 and CHO cells. Additionally, folate turnover in MCF-7 cells was clearly biphasic over the initial 24 h of chase with unlabeled folate. In contrast, folate turnover in CHO cells did not proceed in a clearly biphasic manner over the initial 130 h of chase. However, it was apparent in both cell lines that expression of rat HCF stimulated folate turnover and that its effect was greatest during the initial periods of the chase. This suggests that intracellular folate becomes less susceptible to degradation by ferritin with increased residency time. One possible mechanism to account for these observations involves...
the protection of folate from catabolism by polyglutamylation. The addition of the polyglutamate chain to folate would be expected to make the cofactor less available for catabolism due to the higher affinity of folate polyglutamates for folate-binding proteins (53). In general, folate-dependent enzymes display 1–2 orders of magnitude increased affinity for the polyglutamate forms of folate relative to the monoglutamate forms. Therefore, folate polyglutamates are likely to be tightly bound to folate-binding proteins and not as accessible to ferritin, whereas the monoglutamate and diglutamate derivatives of THF are less likely to be enzyme-bound and therefore would be available for catabolism. This mechanism suggests that ferritin serves to “scavenge” unbound folate. Interestingly, several studies have demonstrated that cells do not contain excess folate relative to the concentration of intracellular folate-binding proteins (54).

Our studies also demonstrate that HCF can regulate intracellular folate concentrations. The level of increased HCF expression in the CHO cells is very modest relative to changes in HCF expression that occur under physiological conditions. HCF mRNA levels can be elevated 8–10-fold in uterine stromal cells, leading to significantly high expression of the protein (55). Ferritin protein levels have been shown to be elevated 6-fold in carcinomas, but not in normal tissue (56), whereas HCF mRNA levels have been demonstrated to increase 10-fold in chemically induced carcinomas (57). However, even the modest level of increased HCF expression in CHO cells has fairly dramatic effects on intracellular folate concentrations. CHO cells expressing HCF have a 16% reduction in intracellular folate when cultured in the presence of pharmacological levels of folic acid in the culture medium. It is possible that all of the decreased folate represents loss of cytoplasmic folate. Mitochondrial ferritin combines 50% of the intracellular folate, and the catalytic ferritin was found exclusively in the cytoplasm. Therefore, the cytoplasmic folate levels may have been depressed by as much as 32% resulting from rat HCF expression. The effects of HCF expression are even more pronounced during folate deprivation. These data suggest that folate catabolism is an important variable in determining intracellular folate concentrations and suggest a complex relationship between folate and iron status in cells.

There is increasing evidence that HCF, independent of its role in protective iron storage, is essential for regulating many diverse cellular processes. HCF acts as a modulator of cell proliferation and differentiation and as an immunosuppressive role in protective iron storage, is essential for regulating many physiological states may be relevant to folate homeostasis. Recently, we have demonstrated that iron chelators influence the expression of the cytoplasmic serum hydroxymethyltransferase gene and disrupt folate metabolism in cultured cells (79). This study provides more evidence that iron status and metabolism can have profound effects on folate metabolism and provides biochemical evidence for an association that has long been inferred from clinical studies (79).

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is mediated by progesterone (55). In addition, the serum level of human placental isoforms, which consists of LCF and a super heavy chain ferritin (p43) usually present in low levels, rises sharply from the start of pregnancy until the end of pregnancy (67) and is present in placental and fetal tissues (68, 69). The increased expression of p43 and HCF and the increased catabolism observed during pregnancy in the rat model suggest a means by which catabolism may be modulated in vivo.

There is also evidence that folate catabolism is increased in malignancy. Cancer patients with active, untreated, or metastatic malignancies have folate deficiency without evidence of malnourishment, malabsorption, or increased folate excretion (70–72). Ascitic tumors in mice increase the rate of folate catabolism after administration of [14C]folate, as measured by urinary [14C]pABG and acetylated [14C]pABG (73, 74). Correspondingly, increased levels of HCF mRNA and/or protein have been observed in cancer tissues and in the sera of patients (57, 74). Normally, HCF mRNA is not detected in normal mammary tissues, but is present in breast cancer tissue (75). The p43 protein has also been observed in malignant breast tissue, but not in normal mammary tissue (76–78). The increases in folate turnover and incidence of folate deficiency as well as the increases in ferritin during cancer, especially of HCF and p43, suggest a possible relationship between folate turnover and the presence of ferritin with respect to the data that we have presented on the 5-formyl-THF catabolic activity of ferritin.

Our demonstration of folate catabolism by ferritin and the influence of rat HCF expression on intracellular levels of folate indicates that HCF has a novel role in maintaining intracellular folate levels and that its increased synthesis in certain physiological states may be relevant to folate homeostasis. There is increasing evidence that HCF, independent of its role in protective iron storage, is essential for regulating many diverse cellular processes. HCF acts as a modulator of cell proliferation and differentiation and as an immunosuppressive agent (37). Although the regulation of both HCF and LCF synthesis is known to be modulated by iron status (58), factors independent of iron, such as tumor necrosis factor, cAMP, thyroid-stimulating hormone, and glucose, have been shown to up-regulate HCF mRNA levels, but either not to affect or to down-regulate LCF levels (59–64). Our studies demonstrate a new catalytic function for this protein, namely the regulation of intracellular folate concentrations.

Increased rates of ferritin synthesis can be detected in various physiological states where increased rates of folate catabolism or perturbations in folate levels have been observed. Increased folate catabolism in pregnancy is thought to contribute to the high incidence of folate deficiency seen in pregnancy (16). In rats, folate catabolism, as demonstrated by increases in urinary acetylated pABG, correlates with increasing needs of hyperplastic growth of placental and fetal tissues (16). In humans, these increases in catabolism have also been detected in some (65), but not all (66), studies. Consistent with the studies in rodents, HCF (but not LCF) protein levels are increased significantly during pregnancy in the endometrial stromal cells of rats, with the highest levels of HCF mRNA expression corresponding to the proliferation of stromal cells (55). This effect...
