Identification and Biochemical Properties of 10-Formyldihydrofolate, A Novel Folate Found in Methotrexate-treated Cells*

(Received for publication, July 24, 1987)

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The folate compound 10-formyldihydrofolate (H₂folate) has not been found as a component of intracellular folates in normal tissues but has been identified in the cytosol of methotrexate (MTX)-treated MCF-7 breast cancer cells and normal human myeloid precursor cells. Its identity was verified by coelution of this compound with a synthetic marker on high pressure liquid chromatography, its reduction to 10-formyltetrahydrofolate (H₂folate) in the presence of dihydrofolate reductase, and its enzymatic deformylation to dihydrofolate in the presence of aminomimidazolecarboxamide ribonucleotide (AICAR) transformylase. Chemically synthesized monoglutamated or pentaglutamated 10-formyl-H₂folate was examined for its interaction with three folate-dependent enzymes: AICAR transformylase, glucinamide ribotide (GAR) transformylase, and thymidylate synthase. 10-Formyl-H₂folate-Glu₅ was a competitive inhibitor of thymidylate synthase (Kᵢ = 0.16 μM with 5,10-methylene-H₄folate-Glu₅ as substrate and 1.8 μM with 5,10-methylene-H₂folate-Glu₅) and inhibited GAR transformylase (Kᵢ = 2.0 μM). It acted as a substrate for AICAR transformylase (Kᵢ = 5.3 μM), and its efficiency was equal to that of the natural substrate 10-formyl-H₂folate-Glu₅. The inhibition of thymidylate synthase by 10-formyl-H₂folate was highly dependent on the inhibitor’s polyglutamation state, the -Glu₅ derivative having a 52–85-fold greater affinity as compared to the affinity of -Glu₃. Polyglutamation of 10-formyl-H₂folate did not affect its inhibition of GAR transformylase. While the actual role of 10-formyl-H₂folate contributing to the cytotoxicity of MTX has not been determined, this compound has the potential to enhance inhibition of GAR transformylase and thymidylate synthase, and at the same time provides additional substrate for AICAR transformylase. The MTX-induced intracellular accumulation of 10-formyl-H₂folate and H₂folate may play a role in the drug-related cytotoxicity through the contribution of these folates to the inhibition of thymidylate synthase and de novo purine synthesis.

MTX (2,4-diamino,10-methylpteroyl glutamic acid; MTX) is a tight-binding inhibitor of dihydrofolate reductase (DHFR). By virtue of this action, MTX produces a partial depletion of intracellular reduced folates and at the same time a marked accumulation of dihydrofolate (H₂folate). The reduced folate pool in normal and malignant tissues consists of a variety of compounds. Two of these are particularly important to DNA synthesis: 10-formyl-H₂folate, a substrate for the GAR transformylase and AICAR transformylase reactions, and thus an essential factor for de novo purine synthesis; and 5,10-methylene-H₂folate, the folate cosubstrate for thymidylate synthase.

We have previously examined the intracellular folate pool in MCF-7 breast cancer cells and in normal myeloid progenitors isolated from human marrow from normal volunteers and have found relative preservation of 10-formyl-H₂folate in MTX-treated cells despite concurrent suppression of the de novo purine pathway (2, 3) and marked cytotoxicity (3). Similarly, thymidylate synthesis was inhibited in MTX-treated MCF-7 cells without a quantitative depletion of 5,10-methylene-H₂folate (4). In the absence of depletion of the reduced folates, as has been shown in these studies, alternative mechanisms of MTX cytotoxicity related to direct inhibition of the folate-dependent enzymes of purine and thymidylate may be important. We have identified two types of compounds generated during incubation of cells with MTX that inhibit both thymidylate synthase and AICAR transformylase. One class is the series of MTX polyglutamates, found in both normal and malignant cells, which in contrast to the parent drug potently inhibit enzymes other than DHFR (5, 13). The other potential inhibitors of these enzymes are the dihydrofolate polyglutamates, which accumulate within the cells in response to DHFR inhibition by MTX and which inhibit AICAR transformylase (5) and thymidylate synthase (6, 7). During studies of the folate pools in MTX-treated MCF-7 breast cancer cells, we identified a novel folate peak by HPLC, a compound not detectable in untreated tumor cells or normal bone marrow progenitor cells but one that becomes the predominant intracellular folate in MCF-7 breast cancer cells after 12 h of drug exposure. We report here the chemical properties and biochemical action of this compound, which we believe to be 10-formyldihydrofolate. This compound is shown to inhibit both thymidylate synthase and GAR transformylase and to serve as a substrate for AICAR transformylase.

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§ The abbreviations used are: MTX, methotrexate; AICAR, azinomimidazolecarboxamide ribonucleotide; GAR, glucinamide ribotide; DHFR, dihydrofolate reductase; H₂folate, dihydrofolate; H₂folate, tetrahydrofolate; HPLC, high pressure liquid chromatography.
MATERIALS AND METHODS

Chemicals

Purified synthetic MTX-Glu and purified folic acid-Glu were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD) and purified by DEAE-cellulose chromatography, with elution along a linear gradient of 0.1-0.4 M NaH2PO4 (9, 9).

Folic acid, dihydrofolate (H2folate), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, aminomimidazolecarboxamide ribonucleotide (AICAR), dextran (clinical grade), dUMP, bovine albumin fraction V, and acid-washed activated charcoal were purchased from Sigma. [5-3H]dUMP (14.8 Ci/mmol) and L-[carboxy-14C]acetic acid (21 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). (6S,6S)-5-formyl-H4folate, 2.5 Ci/mmol, was purchased from Merek Biochemical (Brea, CA). 2,6-Dichloroindophenol and sodium hydroxulose were purchased from Fisher. Glucaminide ribotide (GAR) was a generous gift of Dr. S. Benkovic, Pennsylvania State University (Hershey, PA). All other chemicals were of reagent grade and were purchased from Sigma.

Pentaglutamated dihydrofate (H4folate-Glu6) was prepared by reduction of folic acid-Glu, with sodium hydroxulose (10, 11). The enzymatically active isomer of tetrahydrofolate ((S)-H4folate) was prepared by enzymatic reduction of H2folate and purified by elution from a DEAE column using linear gradient of ammonium acetate (12). The (6S)-H4folate-Glu was prepared in similar fashion from folic acid-Glu. These preparations of (S)-H4folate-Glu and -Glu did not show subtrate activity with DHFR, thus excluding folic acid and dihydrofolate impurities. Reduced folate cofactors were synthesized from (6S)-H4folate-Glu, 5,10-Methylene-H4folate-Glu, and (6R,6S)-5-formyl-H4folate-Glu. The enzymatic hydrolysis of the polyglutamated residues, the various product were measured spectrophotometrically by using the extinction of Pic Reagent A was collected at 1-min fractions and quantitated by liquid scintillation spectrometry.

Preparation of 10-Formyl-H2folate

We synthesized 10-formyl-H2folate by three different methods. Preparation of this compound by air oxidation of 10-formyl-H2folate was reported previously (20-22). In our experience, when 1 mM solution of 10-formyl-H2folate at pH 7.0 was left at 25 °C for 2-3 h, this procedure resulted in 90-95% oxidation to 10-formyl-H2folate. The product purity was verified by HPLC. The hydrolysis of 10-formyl-H2folate when the oxidation of 10-formyl-H2folate was complete. As an alternative approach, 1 mol of 2,6-dichloroindophenol (a mild oxidizing agent) was added to 1 ml of 1 mM 10-formyl-H2folate for 10 min at 4 °C, and the oxidant was extracted three times with 10-ml volumes of ether; the residual ether in the aqueous layer was removed by evaporation under nitrogen. The relative purity of 10-formyl-H2folate produced by this method (compound 1) was 97%, as the final preparation included less than 3% of 10-formylfolate. However, the product rapidly oxidized and 10-formylfolate became a major component of the preparation content within 20 min at 4 °C (see “Determination of Folate Purity by HPLC”). The UV spectrum of the freshly prepared compound 1 is shown in Fig. 2. In view of its instability when prepared by oxidation of 10-formyl-H2folate, 10-formyl-H2folate was also prepared by reduction of 10-formyl-H2folate by modification of a previously described method (23). Sodium hydroxulose (200 mg) was added to 10 ml of 0.1 M phosphate buffer, pH 7.5. The completeness of 10-formylfolate reduction was determined by disappearance of this peak on HPLC. Excess sodium hydroxulose was precipitated by cold ethanol (3 ml) and removed by centrifugation at 37 °C for 15 min. The mobile phase consisted of 78% Pic Reagent A, pH 5.2, and 24% methanol, and the compounds were eluted using isocratic conditions and a flow of 2 ml/min. The elute was collected at 1-min fractions and quantitated by liquid anilinolysis counting. The identity of radiolabeled folate peaks was verified by coelution with UV peaks of standard folate markers. We used the same HPLC system at pH 7.4 to analyze the changes in composition of radiolabeled folate extracts following their incubation with DHFR and AICAR transformylase.

Enzyme Source and Purification

A human breast cancer cell line, MCF-7, was used as the source of AICAR transformylase, GAR transformylase, and thymidylate synthase. The characteristics of this cell line have been previously described (24). Cells were grown in a monoculture culture in rolling flasks and stored at -40 °C. AICAR transformylase was purified 400-fold, largely through use of the Affi-Gel Blue affinity chromatography, as described previously (5). The specific activity of the purified enzyme was 216 nmol of H4folate formed per min/mg protein at 37 °C. GAR transformylase was partially purified (24-fold) by a 1%
streptomycin sulfate precipitation and ammonium sulfate fractionation. The 35–55% ammonium sulfate precipitate was dissolved in 10 mM phosphate buffer, pH 7.2, and dialyzed against the same buffer. GAR transformylase specific activity was 48.2 nmol of H$_4$folate formed per min/mg protein at 37 °C. Thymidylate synthase was purified 150-fold to a specific activity of 0.57 nmol TMP/min/mg protein according to the method described by Dolnick and Cheng (25). Lactobacillus casei DHFR was purchased from the New England Enzyme Center (Boston, MA) (specific activity 0.64 μmol of tetrahydrofolate formed/min/mg at 37 °C).

**Enzyme Assays**

**AICAR Transformylase Assay**—Activity was measured using the spectrophotometric assay developed by Black et al. (26). The reaction cuvettes contained 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 50 mM 2-mercaptoethanol, 50 μM AICAR, 4.4–7.4 units of enzyme (1 unit of enzyme defined as amount of enzyme required to form 1 nmol of H$_4$folate), and various amounts of 10-formyl-H$_2$folate-Glu; or -Glu. Reaction rates were monitored with a Gilford 2400-2 recording spectrophotometer equipped with a constant-temperature, water-jacketed sample compartment. Reaction velocities were linear for 10 min with respect to time. The absorbance changes were measured at 298 nm and converted to nanomoles of H$_4$folate formed per min/mg protein, using the extinction coefficient for the reaction of 19,700 M$^{-1}$ cm$^{-1}$ at 298 nm (26). In assays with 10-formyl-H$_2$folate as coenzyme, the absorbance changes were measured at 300 nm and converted to nanomoles of H$_2$folate formed per min/mg protein by using an extinction coefficient of 9,500 M$^{-1}$ cm$^{-1}$ (Fig. 1). Thus the change in extinction coefficient for the reaction represented the difference in extinction coefficient of these two compounds at 300 nm. At pH 7.0, the peak molar extinction coefficient for 10-formyl-H$_2$folate (22,000 M$^{-1}$ cm$^{-1}$) occurs at 272 nm. The maximum absorbancy shift during transformylation by AICAR transformylase, with conversion of 10-formyl-H$_2$folate to H$_2$folate, was measured at 300 nm (dotted line).

**Thymidylate Synthase Assay**—Thymidylate synthase was assayed by modification of the tritium-release assay of Roberts (28) as described in detail elsewhere (12). The reaction velocities were found to be linear for 10 min with 5,10-methylene-H$_2$folate-Glu, 0.45–4.0 μM, or for 30 min with 5,10-methylene-H$_2$folate-Glu, 15–90 μM, as substrates, with 0.018–0.03 units of thymidylate synthase (1 unit of enzyme defined as the amount of enzyme required to form 1 nmol of TMP/min at 37 °C).

**Data Analysis**

All kinetic data were first analyzed by Lineweaver-Burk double-reciprocal plots, and the graphic estimates of parameters derived from these plots were used as initial estimates for computerized curve-fitting using a weighted nonlinear least squares method. Curve-fitting was performed by a program designated "enzyme" that was developed by Drs. D. Rodbard and R. Lutz (National Institute of Child Health and Human Development, Bethesda, MD).

**RESULTS**

**Identification of 10-Formyl-H$_2$folate in Cellular Extracts**

The major intracellular folates were identified in extracts of MCF-7 cells after incubation with [3H]5-formyl-H$_2$folate. In the extracts of untreated MCF-7 cells, we identified 10-formyl-H$_2$folate, H$_2$folate, 5-formyl-H$_2$folate, and 5-methyl-H$_2$folate (Fig. 2, panel A). Analysis of intracellular folates from MCF-7 cells, pretreated with 1 μM MTX for 4 h before the extraction, revealed characteristic changes, i.e. a marked decrease in the 5-methyltetrahydrofolate pool (peak 5), 20–40% decrease in 10-formyl-H$_2$folate (peak 1), and accumulation of H$_2$folate (peak 4), as reported previously (Fig. 2, panel B) (2, 3). An unanticipated finding was the preservation of and later increase in the peak eluting at 9 min retention time.

**Fig. 1.** UV spectra of 10-formyl-H$_2$folate and H$_2$folate. Curve 1 (solid line) represents the spectrum of 3.2 × 10$^{-4}$ M 10-formyl-H$_2$folate (product 1) and an equimolar H$_2$folate concentration at pH 7. The maximum absorbancy shift during transformylation by AICAR transformylase, with conversion of 10-formyl-H$_2$folate to H$_2$folate, was measured at 300 nm (dotted line).

**Fig. 2.** The effect of formaldehyde on the extracts of intracellular folates. MCF-7 cells were incubated with [3H]5-formyl-H$_2$folate 0.1 μM for 4 days and then were either left untreated or were exposed to 1 μM MTX for 4 h. Following MTX exposure, folates were extracted with 2% ascorbate and 2% mercaptoethanol (see "Materials and Methods") and separated by HPLC at pH 5.2 before or after an additional incubation with 0.1 mM formaldehyde for 1 h at 31 °C. Panels A and C, extracts of control cells before (panel A) and after (panel C) incubation with formaldehyde. Panels B and D, extracts of MTX-treated cells before (panel B) and after (panel D) incubation with formaldehyde. Peak 6, p-aminobenzoic acid; peak 1, 10-formyl-H$_2$folate; peak 2 H$_2$folate or 10-formyl-H$_2$folate; peak 3, 5-formyl-H$_2$folate; peak 4, H$_2$folate; peak 5, 5-methyl-H$_2$folate; peak 6, 5,10-methylene-H$_2$folate.
which corresponded to the H$_4$folate peak in untreated cells (peak 2). To determine the identity of this peak at 1 min in treated cells, extracts from MTX-treated and normal untreated cells were subjected to incubation with 0.1 mM formaldehyde for 1 h at room temperature, a procedure that converts H$_4$folate to 5,10-methylene-H$_4$folate (Fig. 2, panels C and D). While peak 2 in the MTX-untreated cells underwent quantitative conversion to 5,10-methylene-H$_4$folate (peak 6) during incubation with formaldehyde (panel C), little effect was seen on the 9-min peak from MTX-treated cells (panel D). The migration of this radioactive peak on HPLC was compared to that of authentic unlabeled 10-formyl-H$_4$folate, which by UV analysis eluted 1.5 min before the labeled peak at 9 min (Fig. 3, peak A). However, when authentic 10-formyl-H$_4$folate was treated using an extraction procedure identical to the intracellular folates, its relative elution time was prolonged and corresponded to peak 2 (9.6 min) (Fig. 3, peak B). Thus, the retention time of the material in peak 2 and its failure to react with formaldehyde were consistent with its identification as 10-formyl-H$_4$folate. Additional studies showed that ascorbate was responsible for the alteration in HPLC retention time of 10-formyl-H$_4$folate. The deletion of ascorbate from the extraction procedure resulted in a retention time (7.5 min) identical to that of standard 10-formyl-H$_4$folate, while the deletion of either 2-mercaptoethanol or the conjugase enzyme preparation had no effect on the prolongation of retention time associated with the extraction procedure. While ascorbate increased the retention time of 10-formyl-H$_4$folate, [14C]ascorbate did not co-migrate with the 10-formyl-H$_4$folate peak.

Peak 2 HPLC eluate from MTX-treated MCF-7 cell extracts was pooled, re-analyzed by HPLC under similar conditions, and found to consist of a predominant peak coeluting with authentic 10-formyl-H$_4$folate marker (Fig. 4, panel A), with less than 10% of material eluting in a small second peak with the authentic 10-formyl-H$_4$folate peak. The product, when analyzed by HPLC, had an elution time of 8 min, corresponding to the elution profile of 10-formyl-H$_4$folate (Fig. 4, panel B). Authentic 10-formyl-H$_4$folate underwent the same conversion to 10-formyl-H$_4$folate in the presence of DHFR. We next examined the ability of peak 2 material to serve as a substrate for 400-fold purified AICAR transformylase. Eluate from peak 2, to which was added authentic unlabeled 10-formyl-H$_4$folate, was incubated with AICAR transformylase. Incubation resulted in a conversion of 80% of peak 2 radioactivity to material that eluted with H$_4$folate or its further oxidation product folic acid (Fig. 4, panel C, peak 5). An identical rate/extent of conversion was observed simultaneously for the unlabeled 10-formyl-H$_4$folate, as monitored by the elution of UV-absorbing material by HPLC (Fig. 4C).

These studies indicated that the peak 2 material, found only in MTX-treated cells, migrated on HPLC in a pattern consistent with 10-formyl-H$_4$folate and underwent the same transformations with DHFR and AICAR transformylase as observed with authentic 10-formyl-H$_4$folate.

**Biochemical Characteristics of 10-Formyl-H$_4$folate**

To understand the biochemical reactivity of 10-formyl-H$_4$folate, we studied the effects of this folate on three folate-dependent enzymes: thymidylate synthase, GAR transformylase, and AICAR transformylase.

**Effect of 10-Formyl-H$_4$folate-Glu$_6$ on Thymidylate Synthase—Synthetic 10-formyl-H$_4$folate-Glu$_6$ and 10-formyl-H$_4$folate-Glu$_4$ were examined as inhibitors of MCF-7 thymidylate synthase at variable concentrations of mono- and pentaglutamated 5,10-methylene-H$_4$folate. Inhibition of this enzyme by H$_4$folate-Glu$_6$ and H$_4$folate-Glu$_4$, which are products of the thymidylate synthase reaction, was also examined. With 5,10-methylene-H$_4$folate-Glu$_6$ as substrate, the $K_i$ of 10-formyl-H$_4$folate-Glu$_6$ was 0.16 μM. The Glu$_6$ derivative of 10-formyl-H$_4$folate was 85-fold more potent than the parent monoglutamate (Table I). Pentaglutamation of H$_4$folate resulted in a relatively greater increase in the inhibition potency of H$_4$folate (155-fold). The $K_i$ value of 10-formyl-H$_4$folate-Glu$_4$, or Glu$_4$, however, was generally 3-5-fold lower than the
corresponding H$_2$folate derivative. An approximate 10-fold increase in the $K_i$ values of 10-formyl-H$_2$folate and H$_2$folate was observed in the presence of the pentaglutamated substrate, as compared to the $K_i$ values in the presence of 5,10-methylene-H$_2$folate-Glu$_5$. 10-Formyl-H$_2$folate and H$_2$folate differed also in the pattern of inhibition. While the inhibition pattern was consistently competitive with 10-formyl-H$_2$folate-Glu$_5$ and -Glu$_6$, a noncompetitive pattern was observed for inhibition by both H$_2$folate and H$_2$folate-Glu$_5$ (Fig. 5).

Effect of 10-Formyl-H$_2$folate-Glu$_5$ and H$_2$folate-Glu$_5$ on GAR Transformylase—We studied the effects of mono- and pentaglutamated 10-formyldihydrofolate and dihydrofolate as direct inhibitors of MCF-7 GAR transformylase. Inhibition constants for each compound were determined at a constant GAR concentration of 300 $\mu$M and at variable concentrations of the mono- or pentaglutamated 10-formyl-H$_2$folate (Table II). The inhibition pattern was competitive with the folate substrates. The $K_i$ values of 10-formyl-H$_2$folate-Glu$_5$ (3.3 $\mu$M) and Glu$_5$ (2.1 $\mu$M) were virtually identical and were 7-10-fold lower than the $K_i$ values of corresponding H$_2$folate compounds. Pentaglutamation of the substrate resulted in only a small decrease in $K_i$ (6.5 $\mu$M with monoglutamated versus 4.9 $\mu$M with pentaglutamated 10-formyl-H$_2$folate).

The effect of polyglutamation state of the inhibitor on GAR transformylase catalysis was further investigated with MTX-Glu$_5$ to Glu$_6$ (Table III). A stepwise increase in inhibition potency occurred with the addition of three or more glutamyl groups, with a 32-fold greater inhibition with MTX-Glu$_5$ than MTX-Glu$_1$. However, the $K_i$ of MTX-Glu$_5$ (2.5 $\mu$M) was approximately the same as that of 10-formyl-H$_2$folate-Glu$_5$ when these inhibitors were used in conjunction with the monoglutamated substrate, 10-formyl-H$_2$folate.

The Interaction of 10-Formyl-H$_2$folate-Glu$_5$, with AICAR Transformylase—10-Formyl-H$_2$folate-Glu$_5$ had no inhibitory effect on AICAR transformylase from MCF-7 cells. It was an efficient substrate for this enzyme and as such was comparable with the physiological substrate 10-formyl-H$_2$folate-Glu$_5$.

The relative specificity constants, $V_{max}$, were similar for the two pentaglutamated substrates (3.92 and 3.85 for 10-formyl-H$_2$folate-Glu$_5$ and 10-formyl-H$_2$folate-Glu$_6$, respectively) (Table IV).


discussion

10-Formyl-H$_2$folate has been previously described as a product of 10-formyl-H$_2$folate oxidation, but it was not considered to have physiologic significance (22, 29) and was not detected in prior studies of normal cell extracts. The recognition of this compound in cellular extracts was complicated by the proximity of its peak on HPLC to other folates, and in particular its elution with H$_4$folate under the conditions used in this study. Using the HPLC methodology described in this study, it is not possible to detect 10-formyl-H$_2$folate in measurable amounts in normal untreated cells. It is detectable after treatment with MTX, as is H$_2$folate. Two questions were addressed in the first part of this study: Is the new compound indeed 10-formyl-H$_2$folate? Secondly, if the compound is 10-formyl-H$_2$folate, is it an experimental artifact of 10-formyl-H$_2$folate oxidation or is it actually generated as a result of MTX effects on folate metabolism? The identity
TABLE III

Inhibition of GAR transformylase by MTX-Glu,

Polyglutamate derivatives of MTX (Glu, to Glu,) were tested as inhibitors of the human GAR transformylase with respect to the monoglutamated 10-formyl-H,folate as substrate.

| MTX     | K*  |
|---------|-----|
| µM     |
| Glu,   | 80 ± 7.0* |
| Glu,   | 57 ± 10.0 |
| Glu,   | 7.1 ± 0.7  |
| Glu,   | 5.1 ± 0.8  |
| Glu,   | 2.5 ± 0.6  |

* Based on competitive model.

S.E.

TABLE IV

AICAR transformylase catalysis with 10-formyl-H,folate-Glu, or 10-formyl-H,folate-Glu, as substrate

The Michaelis-Menten constants (K,) and V,, with AICAR transformylase and V,,/K, ratios of 10-formyl-H,folate-Glu, and Glu, were measured and compared to values for 10-formyl-H,folate-Glu,.

| 10-Formyl-H,folate-Glu, | 10-Formyl-H,folate |
|-------------------------|-------------------|
| µM         | mmol/min/mg      | µM         | mmol/min/mg      |
| Glu, 13.4 ± 0.5* | 13.6 ± 1.2       | 1.01 | 38.8 ± 4.7       | 16.1 ± 0.4 | 0.41 |
| Glu, 5.3 ± 0.2    | 20.8 ± 0.1       | 3.92 | 5.9 ± 2.3        | 22.7 ± 0.8 | 3.85 |

S.E.

of 10-formyl-H,folate in the cellular extracts was verified by co-elution of the radioabeled peak 2 with the unlabeled 10-formyl-H,folate marker when both the cellular folate and the standard underwent the same extraction procedure before HPLC separation. Furthermore, treatment of the putative intracellular 10-formyl-H,folate peak with dihydrofolate reductase and with AICAR transformylase revealed characteristic shifts in elution pattern that support the identity of this compound as being 10-formyl-H,folate.

Untreated MCF-7 cells did not contain 10-formyl-H,folate; the folate peak eluting at 9 min included only H,folate. In an extract from untreated cells, conversion of 10-formyl-H, to 10-formyl-H,folate during the processing of cell extracts would be possible under conditions allowing oxidation, but this process is almost completely prevented by inclusion of high concentrations of 2-mercaptoethanol and ascorbate; furthermore, the absence of 10-formyl-H,folate in untreated cells indicates that the material found in treated cells was not an experimental artifact. While both MTX-treated and untreated cells contained 10-formyl-H,folate, 10-formyl-H,folate was found only in MTX-treated cells, an observation that further supports its existence in the cells.

We examined the effect of 10-formyl-H,folate on thymidylate synthase, GAR transformylase, and AICAR transformylase, all folate-dependent enzymes that synthesize the precursors of DNA and/or RNA. When the inhibition of thymidylate synthase by 10-formyl-H,folate was compared to inhibition by H,folate, the product of the thymidylate synthase reaction, three main differences emerged: 1) a competitive pattern of inhibition with 10-formyl-H,folate versus noncompetitive with H,folate, 2) lower K values in experiments with 10-formyl-H,folate in comparison to identical experiments with H,folate, and 3) polyglutamation of 10-formyl-H,folate induced relatively less enhancement of inhibition than the polyglutamation of H,folate. These findings may be explained by the existence of separate binding sites present on each of two monomeric units of thymidylate synthase, with H,folate and 10-formyl-H,folate binding to different sites. This model was suggested previously in studies of human thymidylate synthase derived from leukemic blast cells (6). H,folate was noncompetitive, while 10-formyl-H,folate, which is closely related to 10-formyl-H,folate, was a competitive inhibitor with a relatively lower K value. The authors suggested that a combination of these two compounds may bind to different sites on the enzyme and inhibit the catalysis without interference of either inhibitor with the binding of the second inhibitor (mutually nonexclusive pattern). A similar relationship probably exists between H,folate and 10-formyl-H,folate, thus producing a stronger net effect than might result from either compound alone. Previous work (2, 3) has shown that the concentration of formyl-H,folate in MTX-exposed cells approaches the inhibition constants for thymidylate synthase and GAR transformylase (0.5–1.0 µM), suggesting that this compound may contribute to the inhibition of these enzymes during MTX treatment.

10-Formyl-H,folate was also a more potent inhibitor than H,folate when both were tested against GAR transformylase, the first folate-dependent enzyme in the de novo purine synthetic pathway. In contrast to thymidylate synthase, GAR transformylase catalysis and inhibition were minimally affected by the polyglutamation state of the folate compounds. This observation was further tested with MTX-Glu,, as inhibitions of this enzyme, surprisingly, MTX-Glu,, was 32-fold more potent than MTX (Table III). A similar enhancement of inhibition was found previously in studies of chicken liver GAR transformylase, as inhibited by folic acid and its polyglutamates (30). Although the inhibition of GAR transformylase is enhanced by polyglutamation of MTX or folic acid, this enhancement is modest compared to the 150–2500-fold enhancement of inhibition of AICAR transformylase (5) or thymidylate synthase (12) by polyglutamation of MTX and H,folate.

In contrast to the inhibitory effect on the two previously mentioned enzymes, 10-formyl-H,folate was a substrate for human AICAR transformylase. The relative specificity constant (V,,/K, of the pentaglutamated 10-formyl-H,folate was identical to 10-formyl-H,folate-Glu, the natural substrate of AICAR transformylase. This is consistent with a recently reported finding using AICAR transformylase isolated from chicken liver (22).

We have previously observed that important intracellular reduced folates, 10-formyl-H,folate and 5,10-methylene-H,folate, are not significantly depleted with up to 24 h of incubation with cytotoxic concentrations of MTX, at a time when de novo purine and thymidylate synthesis were strongly inhibited (2–4). We suggest that these pathways may be inhibited directly by polyglutamated metabolites of H,folate and 10-formyl-H,folate. These compounds are formed in the presence of MTX and both induce inhibition of thymidylate synthase and GAR transformylase, the latter being the first of the two folate-related enzymes of the de novo purine pathway. The effects of these two oxidized compounds differed, however, with respect to AICAR transformylase. While H,folate inhibits AICAR transformylase, 10-formyl-H,folate acts as a substrate. This substrate activity of 10-formyl-H,folate is inconsistent with the assumption that inhibition of purine synthesis results from depletion of folate substrate for AICAR transformylase. We previously demonstrated that in MCF-7 cells treated with MTX the 10-formyl-H,folate pool undergoes little change. The present work demonstrates the formation of an additional substrate, 10-formyl-H,folate, for this enzyme, a compound that does not exist in the untreated cells. This finding adds further evidence for the importance of direct inhibition of de novo purine and thymidylate pathways by H,folate and MTX polyglutamates rather than an
indirect inhibition through depletion of the required folate cofactors.

The origin of 10-formyl-H$_2$folate remains an enigma. Its appearance only in the extracts from MTX-treated cells is not consistent with a simple oxidation of 10-formyl-H$_2$folate during the extraction procedure. This, however, does not exclude the occurrence of spontaneous oxidation of 10-formyl-H$_2$folate within the intact cells prior to the folate extraction. While the cells that were not treated by MTX retain the capacity to reduce 10-formyl-H$_2$folate by DHFR, and thus maintain the 10-formylfolates in the fully reduced tetrahydro form, MTX-treated cells may accumulate 10-formyl-H$_2$folate due to the DHFR block by MTX. This explanation, which assumes spontaneous intracellular oxidation, seems unlikely because of the stabilizing antioxidant intracellular milieu. An alternative explanation appears more likely. Preliminary evidence suggests that 10-formylhydrofolate may be produced by an enzymatic formylation of dihydrofolate. This hypothesis stems from the observation that this reaction can be measured in MCF-7 cytosol and requires the presence of ATP and sodium formate. No formylation can be measured when MCF-7 cytosol is heat-denatured (31). The dihydrofolate-formylating enzyme appears to represent a new enzyme other than that capable of formylating tetrahydrofolate (32) since its dihydrofolate-formylating activity is unaltered by the presence of excess tetrahydrofolate. The abundant H$_2$folate within MTX-treated cells provides a high concentration of substrate, which does not exist in the untreated cells. We have previously shown both in MCF-7 cells (2) and in bone marrow progenitors (3) that after 12 h or longer exposure to MTX, the H$_2$folate level declines while 10-formyl-H$_2$folate continuously increases such that the sum of these two folates remains constant. These findings are consistent with an active enzymatic mechanism for conversion of H$_2$folate to 10-formyl-H$_2$folate. The identification and characterization of this H$_2$folate formylase activity is the subject of continuing investigations.

Acknowledgments—We thank Drs. I. Wong and S. J. Benkovic (Pennsylvania State University, University Park, PA) for providing glycinamide ribonucleotide and Kathy Moore for preparation of the manuscript.

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