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Relationship of Cellular Folate Cofactor Pools to the Activity of 5-Fluorouracil

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SUMMARY

Human carcinoma cells (Hep-2) require 50 times more 5-fluorouracil (FUra) for growth inhibition than mouse Sarcoma 180 cells (S-180). In normal medium, dTMP synthetase inhibition by FUra is growth-limiting only in S-180. A 1000-fold excess of folates in medium increases FUra activity and makes dTMP-synthetase inhibition growth-limiting also in Hep-2. The purpose of this study was to compare the uptake of folate and the size and composition of folate cofactor pools in these two cell types. The uptake of 1–300 μM [3′,5′,7,9-^{3}H]folate by folate-depleted cells was linear with time for at least 24 hr. The maximal velocity of uptake was the same in both cells, but the K_t value was lower for S-180 (14 μM) than for Hep-2 (25 μM). S-180 cells required larger cellular pools of folates for growth than Hep-2 cells. Folates which accumulated in 48–96 hr at 1–300 μM folate were retained to the same degree, at least for 4 days, when these cells were grown in folate-free medium. To compare folate cofactor pools, folate-depleted cells were incubated for 24 hr at 1 and 10 μM [^{3}H]folate in 1640 medium containing 1% horse serum. Under these conditions Hep-2 cells took up 51% (1 μM) and 73% (10 μM) of the amount of folate taken up by S-180. The folate cofactors were extracted with hot 1% ascorbate. Gel filtration of the undigested extracts on Sephadex G-25 revealed that in S-180 cells over 90%, and in Hep-2 cells only 32%, of folates were in the form of higher polyglutamates. Moreover, in Hep-2 cells 26%, and in S-180 only 2%, of the folates coincided with markers for folate and N^5-methyltetrahydropteroylglutamate (5-CH_3H_4PteGlu). After digestion with porcine kidney γ-glutamylcarboxypeptidase, the extracts were analyzed by DEAE-cellulose chromatography. The major component in S-180 was tetrahydropteroylglutamate (H_4PteGlu), reaching 1.1 μM in cell water at 1 μM folate and 7.3 μM at 10 μM folate. In Hep-2 the major component was 5-CH_3-H_4PteGlu, reaching 0.85 μM in cell water at 1 μM folate and 5.3 μM at 10 μM folate. S-180 cells contained 5–6 times more N^{10}-formyltetrahydropteroylglutamate (10-CHO-H_4PteGlu), 3–4 times more N^5-formyltetrahydropteroylglutamate (5-CHO-H_4PteGlu), 3 times more dihydropteroylglutamate, and 2 times more H_4PteGlu than Hep-2. In contrast, Hep-2 cells contained 2–4 times more 5-CH_3-H_4PteGlu than S-180, and pteroylglutamate was present only in Hep-2. Under the conditions of these analyses, the dTMP synthetase cofactor, N^5,N^{10}-methylenetetrahydropteroylglutamate, is spontaneously converted to H_4PteGlu and N^5,N^{10}-methenyltetrahydropteroylglutamate to 10-CHO-H_4PteGlu and 5-CHO-H_4PteGlu, while 5-CH_3-H_4PteGlu is stable. It appears that the cofactors required for the biosynthesis of both dTMP and of purines were more abundant in S-180 than in Hep-2, while 5-CH_3-H_4PteGlu, the cofactor for methionine synthesis, was predominant in Hep-2. Thus, it appears that greater sensitivity to FUra and dTMP-synthetase as the site of action of FUra is associated with larger total pools of folates, greater proportion in higher polyglutamate forms, and higher content of folate cofactors for dTMP and purine biosynthesis.

INTRODUCTION

Earlier studies from this laboratory have shown that most mouse cells are more sensitive to FUra[2] than most human cells (1). dTMP synthetase activity, when determined immediately after removal of FUra, was inhibited

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[2] The abbreviations used are: FUra, 5-fluorouracil; S-180, mouse Sarcoma 180; Hep-2, human epithelial carcinoma; PteGlu, pteroylglutamic acid, or folic acid; H_2PteGlu, dihydropteroylglutamate; H_4PteGlu, tetrahydropteroylglutamate; 10-CHO-H_4PteGlu, N^{10}-formyltetrahy-

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in both types of cells at similar drug concentrations to the same degree (2). However, only in some cells, such as mouse S-180, dTMP synthetase inhibition was the growth-limiting event, while in others, such as human carcinoma Hep-2, it was not (2).

The binding of FdUMP to dTMP synthetase is greatly affected by the presence of 5,10-CH_2-H_4PteGlu (3). Waxman *et al.* (4), using Friend mouse leukemia cells, showed that in the presence of 10 μM 5-CHO-H_4PteGlu these cells became more sensitive to FUra than when grown at normal levels of folate. These kinds of observation have also been made with mouse L1210 cells (5). Our laboratory has demonstrated that a 1000-fold excess of 5-CHO-H_4PteGlu, folate, or 5-CH_3-H_4PteGlu not only potentiated FUra, but had the capacity to alter the site of action in Hep-2 cells so that dTMP synthetase inhibition became growth-limiting (6). Therefore, it seems likely that either the size or the composition of cellular folate pools, or both, may determine the significance of dTMP synthetase inhibition in growth inhibition by FUra.

The purpose of the present study was to compare S-180 and Hep-2 cells with regard to (a) uptake of [^{3}H] folate, (b) gel filtration characteristics of intact folate pools, and (c) composition of the H_4PteGlu cofactor pools after digestion with γ-glutamylcarboxypeptidase. The studies revealed significant differences, some of which have been briefly reported (7).

MATERIALS AND METHODS

Chemicals. PteGlu, H_2PteGlu, H_4PteGlu, 5-CH_3-H_4PteGlu, 5-CHO-H_4PteGlu (calcium leucovorin), PABGA, and procine kidney acetone powder were purchased from Sigma Chemical Company (St. Louis, Mo.). 10-CHO-H_4PteGlu was generated immediately before use by allowing 5,10-CH=H_4PteGlu to stand in 0.01 M ammonium acetate in the presence of mercaptoethanol. 5,10-CH=H_4PteGlu was synthesized as follows: 100 mg of calcium leucovorin were dissolved in 3 ml of 1 M mercaptoethanol and brought to pH 1.5 by a slow addition of 1 N HCl and allowed to stand at 4° for 2 days for crystallization. The crystals were washed with ice-cold 1 N HCl and absolute ether, dried overnight over dessicant, and stored in a dark bottle (8).

[^{3}H]PteGlu, [3′,5′,7,9-^{3}H]folic acid, potassium salt (28.4–43 Ci/mmole), was obtained from Amersham Corporation (Arlington Heights, Ill.). It was routinely purified on a column of DEAE-cellulose eluted with a linear gradient of ammonium acetate (0.05 M–2 M) (9). DEAE-cellulose (Cellex D) was purchased from Bio-Rad Laboratories (Richmond, Calif.).

Cells and culture conditions. The origin and maintenance of S-180 and Hep-2 cells have been described previously (1). To deplete the cells of folates they were grown for at least 1 week in folate-free RPMI 1640 medium supplemented with 5% HS, 100 μM hypoxanthine, 30 μM thymidine, and 30 μM glycine (HTG medium).

Different monolayer culture systems were used in studies of folate uptake and for chromatographic studies, i.e., 2 ml of culture (T-15 flask) for the uptake studies and 75 ml of culture (T-150 flask) for chromatographic studies.

Uptake studies. T-15 flasks were inoculated with folate-depleted cells, (200,000 S-180 or 300,000 Hep-2) in HTG medium. After 2 days the medium was changed. On the 3rd day the medium was removed and replaced with 1 ml of folate-free RPMI 1640 medium supplemented with [^{3}H]PteGlu (1 μCi/ml), varied concentrations of unlabeled PteGlu, and 1% of HS. The medium was changed daily. After incubation of duplicate cultures, the cell layers were rinsed three times with ice-cold folate- and serum-free medium and then dissolved in 3 ml of 0.2 N NaOH at 37°. Aliquots of 0.5 ml were taken for determination of radioactivity, and the amount of cells was quantitated by protein assay.

Preparation of cell pellets. T-150 flasks were inoculated with 20 million folate-deplated S-180 or 22.5 million Hep-2 cells in HTG medium. After 2 days the medium was aspirated off and replaced with 10 ml of folate-free RPMI 1640 medium supplemented with [^{3}H]PteGlu (3 μCi/ml) and 1% HS. The final concentration of PteGlu was adjusted to 1 or 10 μM with unlabeled PteGlu. At the end of a 24-hr incubation at 37°, the medium was removed and the cell layer was rinsed three times with ice-cold folate- and serum-free medium. The cells were scraped from the flask with a rubber policeman into 5 ml of rinse medium and centrifuged at 1000 rpm at 4° for 10 min. The cell pellets were weighed and stored at −70°.

Preparation of cell extract. A cell pellet of about 400–500 mg was suspended in 4 ml of hot 1% sodium ascorbate solution (pH 7.0) and the suspension was immersed in boiling water, where it was kept for 10 min. The suspension was cooled in ice and centrifuged for 10 min at 4° and 2500 rpm. The supernatant was collected and supplemented with 1% mercaptoethanol. The residual pellet was reextracted with 2 ml of hot ascorbate as above, and the two extracts were combined. Ten-microliter aliquots of the extract were analyzed for radioactivity, and the bulk of the extract was immediately digested with γ-glutamylcarboxypeptidase (below). The remaining cell pellet was dissolved in 6 ml of 0.2 N NaOH to determine the content of residual radioactivity, if any.

Digestion with γ-Glutamylcarboxypeptidase. The enzyme preparation (conjugase) was made according to the method of Bird *et al.* (10) from 550 mg of porcine kidney acetone powder which was homogenized with 10 ml of 0.32% cysteine HCl solution (pH 5.4) in a glass homogenizer. The suspension was incubated for 30 min at 37° and then centrifuged at 20,000 rpm at 25° for 30 min. The supernatant was divided into 2-ml aliquots and stored at −20°.

The digestion mixture (pH 4.7) contained (v/v): 47% cell extract, 31% γ-glutamylcarboxypeptidase, 5% of 9% sodium ascorbate solution, and 17% of 0.5 M sodium acetate buffer (pH 4.7). The mixture was incubated for 3 hr at 37° and thereafter centrifuged for 10 min at 2500

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dropteroylglutamate; 5-CHO-H_4PteGlu, N^5-formyltetrahydropteroylglutamate; 5-CH_3H_4PteGlu, N^5-methyltetrahydropteroylglutamate; 5,10-CH=H_4PteGlu, N^5,N^{10}-methenyltetrahydropteroylglutamate; PABGA, *p*-aminobenzoylglutamate; 5,10-CH_2-H_4PteGlu, N^5,N^{10}-methylenetetrahydropteroylglutamate; HS, horse serum; FdUMP, 5-fluorodeoxyuridine-5′-monophosphate conjugase, hog kidney γ-glutamylcarboxypeptidase.

rpm. Aliquots of 10 μl were taken for counting, and the supernatant was used for chromatography.

DEAE-cellulose chromatography. DEAE-cellulose columns in acetate or phosphate form were used. The acetate column was prepared according to the procedure of Zakrzewski and Himberg (11) with the following modifications: 8.5 g of Bio-Rad Cellex D (OH form) were suspended in 0.25 M NaOH, stirred for a few min, and allowed to stand for 30 min. Portions of the suspension were transferred to a Buchner funnel and washed with water. The material was then suspended in 1 M NH_4-acetate buffer (pH 6.0), and washed again with water. These slurries were suspended in 0.01 M NH_4-acetate (pH 7.0) containing 1% mercaptoethanol and packed in a glass column (0.8 cm × 75 cm). The acetate column was eluted by a convex gradient of 0.01–1.5 M NH_4-acetate with the mixer volume maintained at 600 ml.

The sample applied to the column consisted of cell extract and marker compounds as follows: 0.5 mg each (about 1 μmole) of H_4PteGlu, H_2PteGlu, and PABGA, and 0.25 mg each (about 0.5 μmole) of PteGlu, 5-CH_3-H_4PteGlu, 10-CHO-H_4PteGlu, and 5-CHO-H_4PteGlu. The flow rate of the column was controlled by a Buchler peristaltic pump and was maintained at 0.3–0.4 ml/min. About 4-ml fractions were collected. The absorbance of the eluate was scanned at 280 nm with a Gilson UV monitor, and 0.5-ml aliquots of each fraction were taken for counting.

The fractions from the acetate column which contained Peaks 4–5 (Fig. 4) were pooled and rechromatographed on a DEAE-phosphate column. This was prepared by suspending 9 g of Bio-Rad Cellex D in 0.25 M KH_2PO_4 (pH 4.4), and then packing such slurries in a glass column (0.8 cm × 100 cm). The column was equilibrated and eluted with the same buffer containing 1% mercaptoethanol. The flow rate was maintained at 0.8–1.0 ml/min with a pump. Absorbance of the eluate at 280 nm was monitored with a Gilson UV monitor. Fractions of about 3.5 ml were collected and analyzed for radioactivity.

Gel filtration on Sephadex G-25. The procedure was similar to that of Shin *et al.* (12) Sephadex G-25 (fine, Pharmacia Fine Chemicals, Piscataway, N. J.) was swollen for 1 hr in a boiling water bath with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.001% sodium azide. A glass column (0.8 cm) was packed by gravity to a height of 185 cm and washed with 0.1 M potassium phosphate buffer (pH 7.0), which was also used as the eluant. All buffers contained 1% 2-mercaptoethanol. Two milliliters of the undigested cell extract or the extract after digestion with γ-glutamylcarboxypeptidase were applied to the column together with blue dextran (2000, Pharmacia Fine Chemicals) for determination of the void volume and markers for 5-CH_3-H_4PteGlu (0.5 μmole) and PteGlu (0.57 μmole). Fractions of 2.5 ml were collected at a flow rate of 12.5 ml/hr. The fractions were analyzed for radioactivity and for optical density at 280 nm.

Measurement of radioactivity. Aliquots (0.5 ml) of the column fractions were diluted with an equal volume of water and counted in 10 ml of Liquiscint-2 (National Diagnostics, Somerville, N. J.) using a Packard Tri-Carb Model 2450 liquid scintillation spectrometer. In uptake studies, 0.5-ml aliquots of cell solutions in 0.2 N NaOH were acidified with 0.2 ml of 1 N HCl and counted as above.

Molarity in cell water. The amount of intracellular water per milligram of total cell protein is 4.9 mg for S-180 and 5.5 mg for Hep-2 (2). The number of moles of folate metabolites per milligram of protein was calculated on the basis of the specific radioactivity of the labeled folate in the initial incubation medium and the radioactivity per milligram of total cell protein. The intracellular water values then allowed an easy means of converting these quantities to molarities in cell water.

Protein determination. Protein was determined by the method of Lowry *et al.* (13), using crystalline bovine serum albumin as the standard.

Cellular retention of [^{3}H]folate. T-15 flasks were inoculated with folate-depleted cells, 200,000 S-180 and 300,000 Hep-2 cells, respectively, in HTG medium; 24 hr later the medium was removed and the cell layers were incubated for 48–96 hr with 1 ml of folate-free RPMI 1640 medium containing 1–300 μM [^{3}H]folate and 1% HS. The medium was thereafter replaced with 2 ml of folate-free RPMI 1640 medium (1% HS), which was changed daily. Duplicate T-15 flasks were analyzed at time zero and at 1, 2, and 4 days for radioactivity and protein. This was done after rinsing each flask three times with cold serum- and folate-free medium and then dissolving the cell layer into 3 ml of 0.2 N NaOH.

RESULTS

Cellular uptake of folate. The cells were first depleted of folates as described under Materials and Methods. At 1 μM folate the uptake in both cell lines was linear for 48 hr (Fig. 1, left); at that time S-180 and Hep-2 cells had accumulated in cell water 8.2 μM and 4.5 μM, respectively. Even at higher concentrations (up to 300 μM) the uptake was linear for 24 hr and, therefore, a 24-hr period of incubation was chosen for the study of kinetics of uptake (Fig. 1, right). The maximal velocity was about equal in both cells, 2.3 and 2.6 μM folate in cell water per hour for S-180 and Hep-2, respectively. The half-saturation values for folate uptake were 25 μM for Hep-2 and 14 μM for S-180.

Cellular retention of [^{3}H]folate. The retention of [^{3}H]folate was examined in order to get an indication whether the cellular folates were in forms, such as polyglutamates, that are not able to penetrate through the plasma membrane. At the end of 4 days of incubation in folate-free medium, the T-15 flask cultures had lost only 25%–35% of the initial radioactivity regardless of the initial content of folate (data not shown). Because of the uniformity of this loss at all levels of folate, it appears that the loss of radioactivity was due to a loss of cells, which detached from the monolayer and were removed when the medium was changed daily. During 4 days in folate-free medium the cell mass increased 3.8- to 4.4-fold. This resulted in a corresponding dilution of folate per unit amount of cells. Thus, it appears that the amount of intracellular folates escaping from the cells was insignificant.

Recovery of [^{3}H]folates from cells by extraction. As can be seen in Table 1, the higher the extracellular folate concentration, the greater was the fraction of radioactiv-

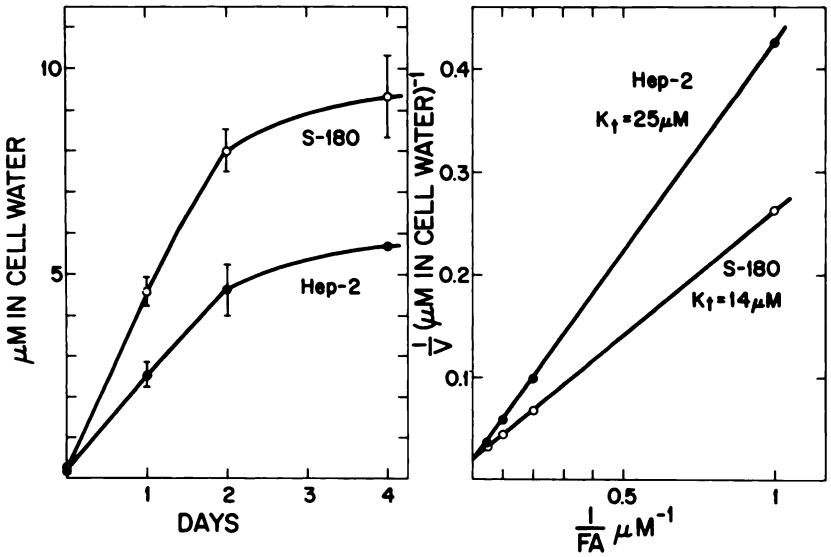

FIG. 1. *Kinetics of uptake of folate by S-180 and Hep-2 cells*

Monolayers of folate-depleted cells in T-15 flasks were incubated with 1 μM [^{3}H]folate (1 μCi/ml) (left) in a medium supplemented with 1% HS. The medium was changed daily. Right, the double, reciprocal plot of 24-hr data obtained at varied concentrations of folate (*FA*).

ity that was not released by the routine extraction with hot 1% sodium ascorbate. Thus, almost all folate was extracted after 1 μM, about 90% after 10 μM, but only 60%–70% after 300 μM folate.

An attempt was made to examine the unextracted radioactivity remaining after 300 μM folate. After routine extraction with four washes of 2 ml of hot 1% sodium ascorbate, S-180 cell pellets contained 30% of original radioactivity. When the extraction was repeated four more times with 2 ml of ascorbate solution, the remaining cell pellet still retained 15% of the original radioactivity. Incubation of such pellets for 3 hr with hog kidney conjugase failed to release more radioactivity than the enzyme-free controls. In contrast, a 1-hr incubation with elastase (1 mg/ml) released 43% as compared with 2.4% in the controls.

It appears that the unextracted radioactivity does not represent polyglutamylfolates, but is more likely a breakdown product or the result of redistribution of tritium.

Folate pools versus growth. To determine the size of cellular folate pools required to support growth, folate-depleted cells were exposed to varied concentrations of folate for 24 hr, rinsed, and thereafter transferred to folate-free medium. Figure 2 reveals that S-180 cells required larger pools (8 μM) than Hep-2 cells to support one-half of maximal growth in the absence of folate. These pools were achieved in 24 hr at 2 μM folate for S-180 and at 0.35 μM for Hep-2. Thus, the slower uptake of folate by Hep-2 (Fig. 1) is associated with lesser requirement for intra- and extracellular folate for growth. For maximal growth the required pools as well as the extracellular folate were about equal for both cell lines, namely 30 μM folate in the medium (for 24 hr) and 35 μM folate compounds in cell water.

Gel filtration of [^{3}H]folates. Figure 3 shows the frac-

TABLE 1

Extractability of cellular folates

After a 24-hr incubation of folate-depleted cells at the indicated levels of [^{3}H]folate, the cells were rinsed and extracted with hot 1% sodium ascorbate as described under Materials and Methods. The numbers in parentheses indicate the number of separate experiments.

Folate for 24 hr	% Cellular folates extracted	
	S-180	Hep-2
μM		
1	97.9 ± 0.92 (4)	98.0 ± 0.66 (3)
10	88.8 (2)	88.4 ± 1.92 (3)
300	70.1 ± 4.2 (3)	60.0 ± 1.0 (3)

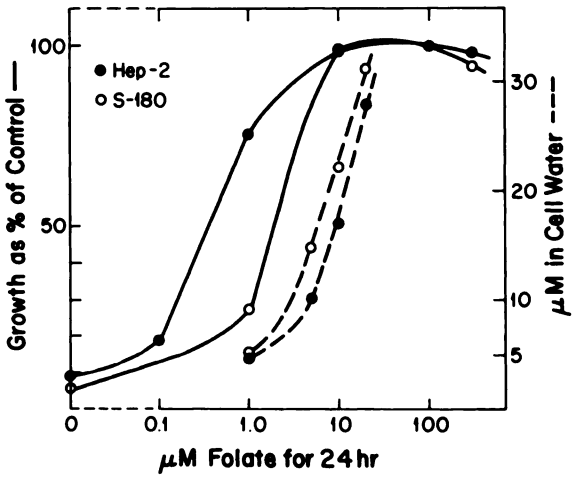

FIG. 2. *Folate pools versus capacity for growth*

Monolayers of folate-depleted cells were preincubated for 24 hr at varied concentrations of folate in a medium supplemented with 1% HS. The cell layers were rinsed. Triplicate sets at each concentration were dissolved in 0.2 N NaOH and analyzed for protein and radioactivity to determine micromolar [^{3}H]folate in cell water (- - -). Another triplicate set was grown in folate-free RPMI 1640 medium with 5% HS. The medium was changed daily. After 5 days (S-180) and 6 days (Hep-2) the amount of cells was determined by protein assay (——). Control growth at 10–100 μM folate was 15- to 20-fold.

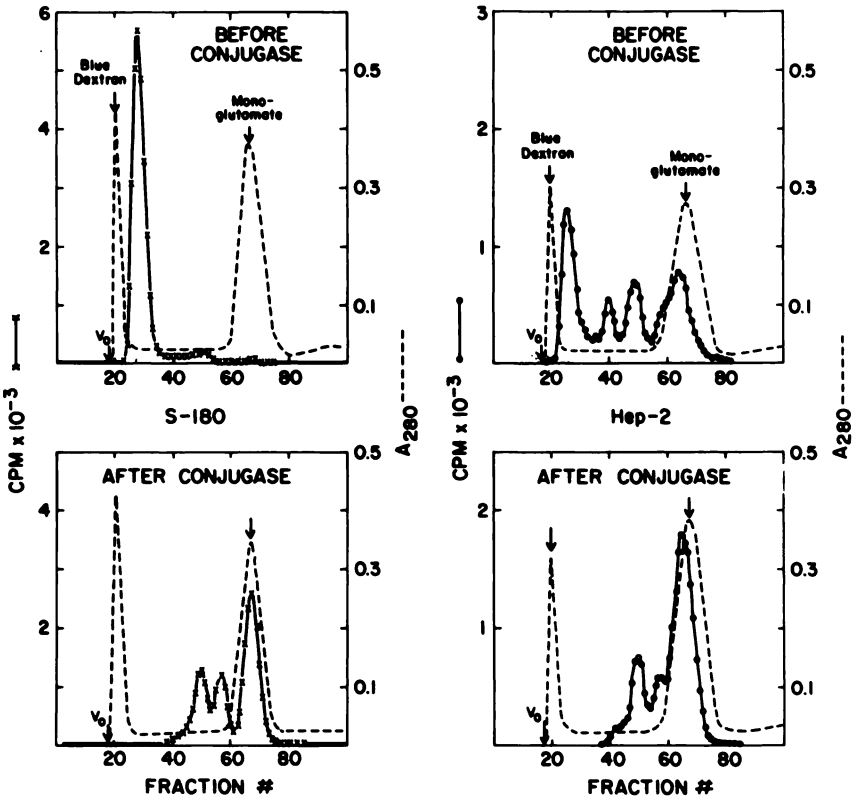

FIG. 3. *Fractionation of cellular [^{3}H]folates on Sephadex G-25 before and after digestion with conjugase*

Monolayers of folate-depleted cells in T-150 flasks were incubated for 24 hr with 10 ml of folate-free RPMI 1640 medium supplemented with 1 μM [^{3}H]folate (3 μCi/ml) and 1% HS. The cell layer was rinsed, and the cell pellet was collected and extracted twice with hot 1% sodium ascorbate (pH 7.0). An aliquot of 2 ml plus markers for folate, 5-CH_3-H_4PteGlu, and blue dextran were applied to a Sephadex G-25 (fine) column (185 cm × 0.80 cm), equilibrated, and eluted with 0.1 M potassium phosphate buffer (pH 7.0) containing 1% mercaptoethanol. Another aliquot was digested with hog kidney conjugase as described under Materials and Methods and applied to a column with marker compounds as above. Fractions of 2.5 ml were collected with a flow rate of 12.5 ml/hr and analyzed for radioactivity and light absorption at 280 nm. These studies were repeated twice with separate cell pellets, and the results were identical.

tionation patterns of cellular folates (after 24 hr with 1 μM [^{3}H]folate) on a Sephadex G-25 column before and after digestion with conjugase. Two separate cell samples were analyzed and gave identical results. One can see that before the digestion there was a striking difference between the folates in S-180 and Hep-2 cells. Whereas in S-180 cells 91% of the folates were present as high molecular weight polyglutamates, the Hep-2 cells had only 32%. Also, whereas in Hep-2 cells 26% of the total coincided with monoglutamylfolates (markers used: folate and 5-CH_3-H_4PteGlu), in S-180 only 2% did. The remainder in Hep-2 (42%) appeared to behave as intermediate forms, possibly oligoglutamates, while this was observed in S-180 for only 7%. After conjugase digestion the patterns of distribution on Sephadex G-25 were similar for both cells.

Monoglutamylfolates on DEAE-cellulose. Figures 4 and 5 show the profiles of distribution of radioactivity and their coincidence with the marker compounds. Peaks 4 and 5, 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu, respectively, were overlapping on the DEAE-acetate column (Fig. 4) and were separated by rechromatography on DEAE-phosphate (Fig. 5).

Table 2 lists the percentage distribution of the various forms in cells exposed for 24 hr to 1 and 10 μM folate. Significant differences are apparent between the amounts of different forms of folates in S-180 and Hep-2 cells. Thus, in S-180 cells H_4PteGlu was the major species, constituting 24% and 32% of the total radioactivity after 1 and 10 μM folate, respectively. 5-CHO-H_4PteGlu plus 10-CHO-H_4PteGlu constituted 36% and 28% of the total, while 5-CH_3-H_4PteGlu comprised 9% and 6% and H_2PteGlu only 4% and 3% of total.

In Hep-2 cells the major single species was 5-CH_3-H_4PteGlu, which made up 31% of the total, whereas H_4PteGlu made up 20% and 21%. 5-CHO-H_4PteGlu plus 10-CHO-H_4PteGlu comprised 17% and 8% of total after 1 and 10 μM folate, respectively, while H_2PteGlu made up 2%. One clear difference between the two cell lines concerns folate itself, which was undetectable in S-180 after 1 and 10 μM folate, but was found at 7% and 2% levels in Hep-2.

When one compares the molar concentrations in cell water (Fig. 6), one finds that S-180 cells contained 2 times more H_4PteGlu, 3 times more H_2PteGlu, and 4 and 5 times more 5-CHO-H_4PteGlu plus 10-CHO-H_4PteGlu than Hep-2. On the other hand, Hep-2 cells contained 2 times and 4 times more 5-CH_3-H_4PteGlu than S-180 after 1 and 10 μM folate, respectively.

300 μM Folate. FUra action had been shown to be potentiated by 300 μM folate (6). Unfortunately, at this high level of [^{3}H]folate the extractability of the cellular

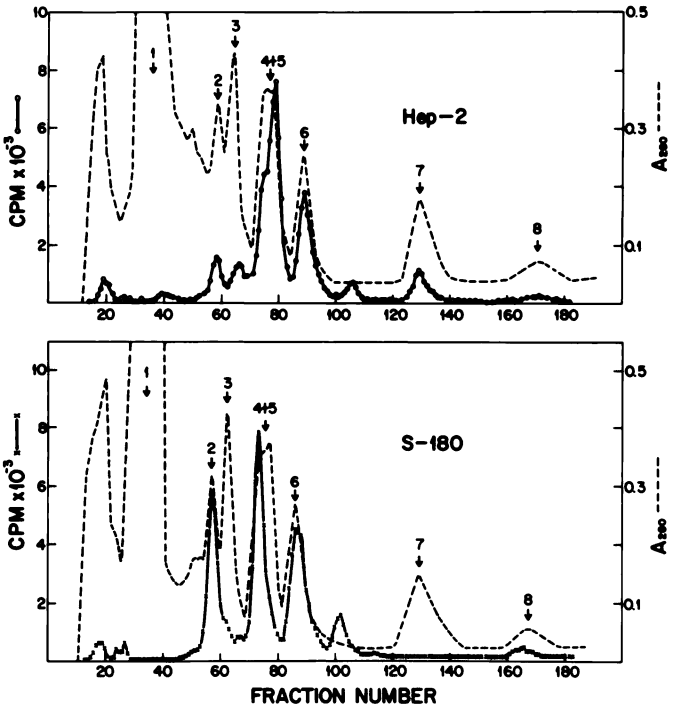

FIG. 4. *DEAE-cellulose fractionation of cell extracts after digestion with porcine kidney γ-glutamylcarboxypeptidase*

Monolayers of folate-depleted cells were incubated with 1 μM [^{3}H] folate for 24 hr, and cell extracts were prepared and digested as described in the legend to Fig. 3. The digested extract was applied together with marker compounds to a column (0.8 cm × 75 cm) of Cellex D (Bio-Rad Laboratories). It was eluted with a convex gradient of 0.01 M–1.5 M ammonium acetate containing 1% mercaptoethanol with a mixer volume maintained at 600 ml. Peak *1* indicates ascorbate; Peak *2*, 10-CHO-H_4PteGlu; Peak *3*, PABGA; Peak *4*, 5-CHO-H_4PteGlu; Peak *5*, 5-CH_3-H_4PteGlu; Peak *6*, H_4PteGlu; Peak *7*, PteGlu; and Peak *8*, H_2PteGlu.

radioactivity was limited (Table 1). Also, the increase in "unidentified" radioactive compounds, which were mainly breakdown products (Table 2), made a reliable quantitation difficult. Thus, the data obtained at 300 μM folate must be treated with reservation. However, the relative amounts remained largely similar in S-180 so that the content of various forms decreased in the sequence H_4PteGlu > 10-CHO-H_4PteGlu > 5-CHO-H_4PteGlu > 5-CH_3-H_4PteGlu. In Hep-2 cells, on the other hand, 5-CH_3-H_4PteGlu became overwhelmingly predominant.

DISCUSSION

The effect of 5,10-CH_2-H_4PteGlu in increasing the affinity of FdUMP to dTMP synthetase and the significance of this cofactor for the maintenance of the ternary complex have been proven in isolated enzyme systems (3). However, in intact cells the effect of an increase in extracellular folates on this system was by no means predictable. Yet, excess folinic acid (1000 times the normal level) not only increased the potency of FUra in all cells examined (4–6) but, in addition, it made dTMP synthetase inhibition growth-limiting even for human Hep-2 cells (6). These effects were due to the stabilization of the ternary complex, resulting in slower spontaneous recovery of the enzyme activity (6). In Hep-2 cells, at normal levels of folate, the recovery of dTMP synthetase activity was too rapid to affect growth.

The observations, briefly summarized above, suggested that mouse S-180 and human Hep-2 cells were likely to differ with regard to their normal pools of folates. These speculations led to the present study which, indeed, revealed striking differences between the two cell lines.

Most cell culture media contain 1–2 μM folate, which is sufficient for supporting optimal growth. At these "physiological" levels of folate, the velocity of its uptake was almost twice as fast in S-180 as in Hep-2 (Fig. 1). Interestingly, these uptake velocities correlate directly with the dihydrofolate reductase levels, which are about twice as high in S-180 as in Hep-2 (14). For half maximal growth, S-180 cells required 3 times higher extracellular levels of folate than Hep-2 (6). At the same time, S-180 cells also required larger intracellular pools of folate than Hep-2 cells (Fig. 2). Thus, S-180 cells, when compared with Hep-2, are characterized by faster uptake, faster reduction, and greater requirement for folate.

It is well recognized that natural forms of folate are often polyglutamates. Thus, a study of fibroblasts from patients revealed that 88%–92% of folates were polyglutamates (15). In livers of rats, guinea pigs, and hamsters, 51%–85% were in the form of pentaglutamates; in monkeys both penta- and hexaglutamates were present (16). In contrast, in human plasma, folates were entirely in the form of monoglutamates. Thus, the number of glutamyl

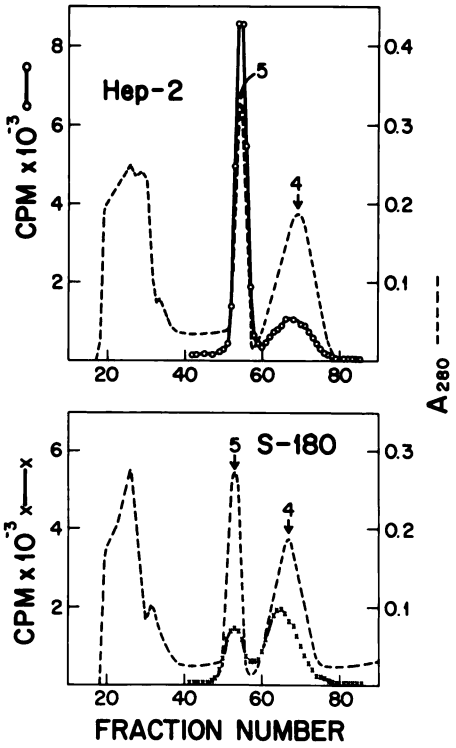

FIG. 5. *Separation of Peaks 4 and 5 on a DEAE-phosphate column*

The fractions under *4* and *5* in Fig. 4 were pooled and rechromatographed on a column (0.8 cm × 100 cm) of Cellex-D in phosphate form. It was eluted with 0.25 M KH_2PO_4 (pH 4.4) containing 1% mercaptoethanol. Fractions of 3.5 ml were collected with a flow rate of 0.8–1.0 ml/min. Peak *4* indicates 5-CHO-H_4PteGlu; Peak *5*, 5-CH_3-H_4PteGlu.

TABLE 2

Quantities of various folates in cells after 24 hr at 1, 10, and 300 μM folate

Peak[a]	Folate species	% of Total[b]					
		S-180			Hep-2		
		1 μM	10 μM	300 μM	1 μM	10 μM	300 μM
2	10-CHO-H_4PteGlu	15.8	9.9	7.6	5.3	2.2	3.1
4	5-CHO-H_4PteGlu	20.2	17.7	5.9	11.5	5.6	2.2
5	5-CH_3-H_4PteGlu	9.3	6.2	3.7	31.2	31.1	38.0
6	H_4PteGlu	24.1	31.9	12.3	19.7	20.7	2.9
7	PteGlu	ND[c]	ND	3.3	6.5	2.3	3.2
8	H_2PteGlu	3.6	3.0	ND	2.2	1.5	ND
—	Unidentified[d]	10.2	11.9	42.6	6.5	16.9	29.5

[a] Peak numbers refer to those in Figs. 4 and 5.

[b] Total refers to the sum of counts recovered in all column fractions and includes also those present between the distinct peaks.

[c] ND, nondetectable.

[d] Includes peaks in the area of Fractions 20 and 100–110 in Fig. 4, which are (at least partially) breakdown products.

units depends not only on the species but on the tissue type as well. Our studies showed that in S-180 cells over 90% of folates were in the form of higher polyglutamates as evidenced by their elution from Sephadex G-25 near blue dextran (Fig. 3). In contrast, in Hep-2 cells only 32% of the folate pools were in that fraction. Thus, human carcinoma Hep-2 cells differ not only from mouse sarcoma S-180, but also from human fibroblasts mentioned above. It may be of significance that human fibroblasts (U-cells) differed from other human cells and resembled S-180 cells not only with respect to their sensitivity to FUra, but also by having similar enzyme profiles (1).

Most published studies on the composition of H_4PteGlu-cofactor pools in tissues have been carried out using microbial assays. Using such methods, 5-CH_3-H_4PteGlu was found to be the principal folate in plasma (17, 18). Also, in the livers of rats and monkeys, 5-CH_3-H_4PteGlu constituted 60%–80%, in human plasma 90%, and in red cells 100% of total folates (16). A study of human fibroblasts revealed that in normal individuals 5-CH_3-H_4PteGlu constituted 87% of total folates, whereas in patients afflicted with 5,10-CH_2-H_4PteGlu reductase deficiency, only 35% were in that form (15).

Using a column separation technique similar to the one employed here, mouse leukemia L1210 cells were found to have only 2%–10% of the total folate pools in the form of 5-CH_3-H_4PteGlu (9). Mouse S-180 cells resemble L1210 cells in that 3%–9% of the pools were 5-CH_3-H_4PteGlu (Table 2). In contrast, human Hep-2 cells had 31 to 38% in that form. The figures concerning 5-CH_3-H_4PteGlu content should be reliable in view of the known stability of this folate under the conditions used.

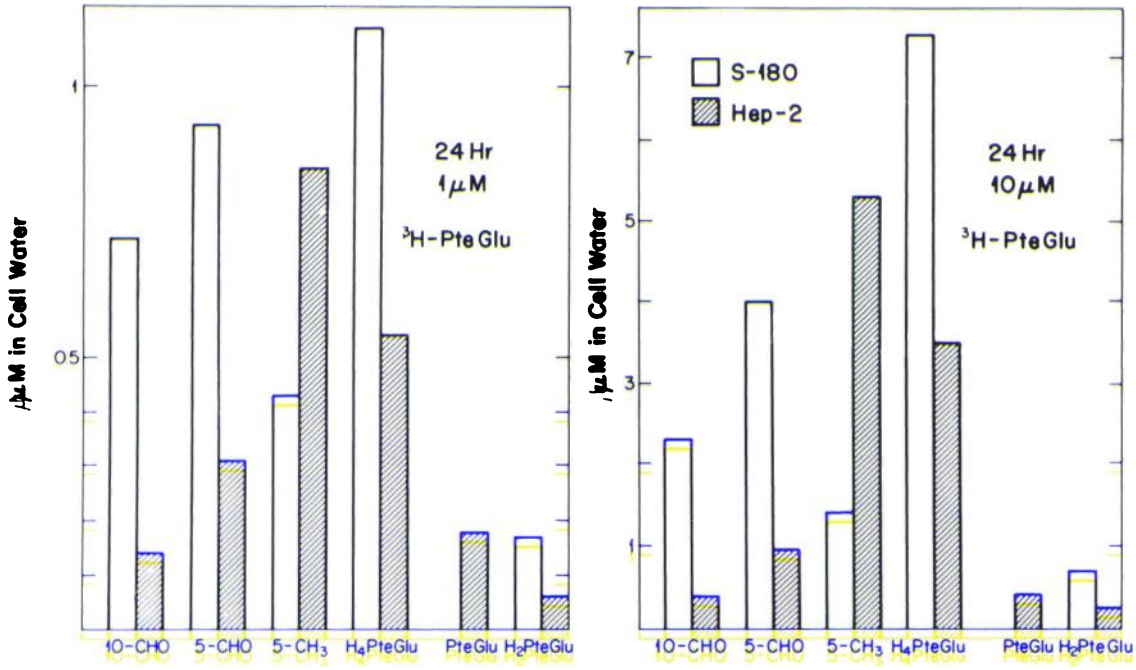

FIG. 6. *Molarity of various forms of folate in S-180 and Hep-2 cells as determined after digestion with conjugase*

Monolayers of folate-depleted cells were incubated with 1 μM (left) or 10 μM [^{3}H]folate (right) and analyzed as described in the legends to Figs. 4 and 5. The percentage distribution was determined (Table 2) and was used to calculate the molarity in cell water on the basis of total uptake.

Whether the activities of 5,10-CH_2-H_4PteGlu-reductase and/or methionine synthetase differ in the two cell types remains to be seen. It is of interest that the capacity of S-180 and Hep-2 cells to grow on homocysteine paralleled their 5-CH_3-H_4PteGlu content.[3]

The source of carbon 8 of the purine ring, 5,10-CH=H_4PteGlu, is unstable under the conditions of these experiments. It is converted to both 5-CHO-H_4PteGlu and 10-CHO-H_4PteGlu (19). The latter folates were far more abundant in S-180 than in Hep-2 cells, both in terms of percentage of total (Table 2) and in terms of molarity in cell water (Fig. 6).

The cofactor for dTMP synthetase is also unstable under the conditions used. It is spontaneously converted to H_4PteGlu (19). H_4PteGlu was the single most abundant ingredient in S-180 cells, constituting up to 32% of the total folates (Table 2). The molarity of H_4PteGlu in cell water of S-180 was twice that in Hep-2 (Fig. 6). Again, S-180 cells resembled L1210 cells, which reportedly contain 30%–32% of total pools in the form of H_4PteGlu (9). Both S-180 and L1210 cells are sensitive to FUra, and dTMP synthetase is the site of action in both (1). Thus, it is attractive to speculate that, indeed, the H_4PteGlu content reflects the 5,10-CH_2-H_4PteGlu content in the intact cells and thus parallels the response to FUra. However, a direct determination of the unaltered cofactor content in undigested cell extracts is essential before far-reaching conclusions can be drawn.

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