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The Turnover of Folate Coenzymes in Murine Lymphoma Cells*

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PETER F. NIXON,‡ GERALD SLUTSKY,§ ALY NAHAS,¶ AND JOSEPH R. BERTINO||

From the Departments of Pharmacology and Medicine, Yale University, School of Medicine, New Haven, Connecticut 06510

SUMMARY

To estimate the turnover of 5-CH_3-H_4-folate in murine lymphoma cells L1210, L1210R (a methotrexate-resistant subline), and L5178Y, suspensions of whole cells were allowed to concentrate 5-[^{14}C]CH_3-[9,3′,5′-^{3}H]H_4-folate; analysis of cell extracts showed that, for each cell line, 81 to 85% of the total cell [^{14}C]CH_3 groups were transferred to nonfolate compounds within 5 min and 82 to 91% at time intervals up to 60 min. The initial transfer of ^{14}C appeared to be into [^{14}C]methionine, but insoluble cell materials were also progressively ^{14}C labeled. Of the total cell ^{3}H, more than 87% remained identified as 5-CH_3-[$^3H_3$]H_4-folate at 60 min, showing that within this period most of the [$^3H_3$]H_4-folate derived from 5-CH_3-[$^3H_3$]H_4-folate returned to maintain the labeling of the pool of 5-CH_3-[$^3H_3$]H_4-folate.

To estimate the flux of folates through the pathway of thymidylate biosynthesis, L1210 and L1210R cells were allowed to concentrate either 5-CH_3-[9,3′,5′-^{3}H]H_4-folate in the presence of methotrexate or 5-HCO-[6-^{3}H]H_4-folate. Of total ^{3}H taken up as 5-HCO-[6-^{3}H]H_4-folate, 28% appeared to be transferred to thymidylate in 60 min by L1210 cells and 52% by L1210R cells. In methotrexate-treated L1210 cells, 23% of the total ^{3}H taken up as 5-CH_3-[$^3H_3$]H_4-folate was accumulated in 60 min as [$^3H_3$]H_2-folate, a product of thymidylate biosynthesis. However, in cells of the methotrexate-resistant L1210R line, no [$^3H_3$]H_2-folate was accumulated by the use of 2 mM methotrexate despite the demonstrated high flux of folates through the pathway of thymidylate biosynthesis. These data show the significance, for methotrexate resistance, of the 11-fold increase of dihydrofolate reductase in L1210R cells.

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‡ Merck, Sharp and Dohme International Fellow in Clinical Pharmacology. Present address, Department of Biochemistry, John Curtin School of Medical Research, Australian National University, P. O. Box 334, Canberra City, A.C.T. 2601, Australia.

§ Summer scholar. Present address, Students' Residence, Harvard Medical School, Boston, Massachusetts 02115.

¶ Present address, Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York 14620.

|| To whom requests for reprints should be addressed (at Yale University). Career Development Awardee of the National Cancer Institute (5-K3-CA-8853).

The rates of turnover of folate coenzymes through the various pathways of folate interconversions are unknown although the relative concentrations of folate coenzymes have now been measured for several tissues, including liver (1, 2, 3) and transplantable murine leukemic cells (4). An estimate of the rates of turnover of the folate coenzymes would be valuable for an understanding of the control of folate-dependent reactions in whole cells. Particularly valuable would be an estimate of the rate of flux of folates through the pathway of thymidylate biosynthesis, since it appears that the cytotoxicity of folate analogue inhibitors of dihydrofolate reductase depends almost entirely upon decreased biosynthesis of thymidylate (5).

As illustrated in Fig. 1, the folate normally available for uptake into animal cells is 5-CH_3-H_4-folate, the principal folate found in plasma (6). Suspensions of animal cells also take up 5-CH_3-H_4-folate rapidly *in vitro* (7). Within cells, 5-CH_3-H_4-folate can be converted to free H_4-folate virtually only (8) by the reaction of methionine biosynthesis, in which the CH_3 group is transferred to homocysteine. H_4-Folate can enter into several 1-carbon transfer reactions, some of which are indicated in Fig. 1.

If whole cells were allowed to take up 5-[^{14}C]CH_3-[9,3′,5′-^{3}H]-H_4-folate, any transfer of ^{14}C to methionine would measure the conversion of 5-CH_3-H_4-folate to H_4-folate (Fig. 1), while the ^{3}H would be distributed among the various folate coenzymes and would indicate the size of the pool of cell folates. If at equilibrium it were found that the only significant fraction of cell ^{3}H remained identifiable as 5-CH_3-[^{3}H]H_4-folate then the rate of transfer of ^{14}C from 5-[^{14}C]CH_3-[$^3H_3$]H_4-folate to methionine would also provide an estimate of the turnover of the total pool of folate coenzymes.

Cells may also take up 5-HCO-H_4-folate, which then rapidly enters the metabolic pool of folate coenzymes (9). If cells were allowed to take up 5-HCO-[6-^{3}H]H_4-folate, the tritium would necessarily remain associated with H_4-folates throughout all folate-dependent enzymic reactions except for that of thymidylate biosynthesis, in which the ^{3}H would be transferred from 5,10-CH_2-[6-^{3}H]H_4-folate to thymidylate (10, 11). The transfer of ^{3}H from 5-HCO-[6-^{3}H]H_4-folate into thymidylate would then measure the flux of folates through the pathway of thymidylate biosynthesis (Fig. 1). Another approach to the estimation of the flux of folates through this pathway would be to measure the rate of accumulation of H_2-folate (Fig. 1) under conditions in which its reduction to H_4-folate was inhibited, for example by the presence of methotrexate, a tight binding inhibitor of dihydrofolate reductase (12).

This paper describes the use of each of the above approaches for the estimation of the turnover of the pool of folate coenzymes

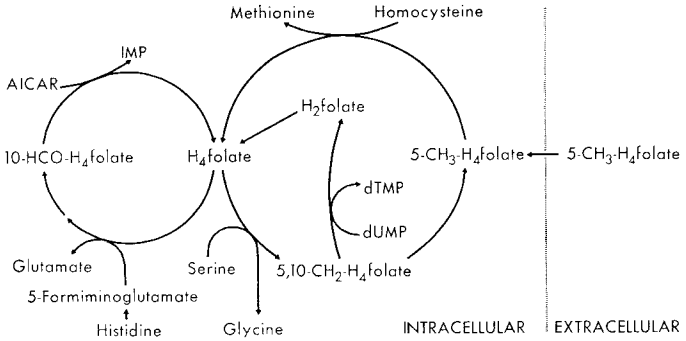

FIG. 1. The reactions of 5-CH_3-H_4-folate and H_2-folate and principal reactions of other folate coenzymes in animal cells. *AICAR*, 5-aminoimidazole-4-carboxamide ribotide.

and their flux through the pathway of thymidylate biosynthesis in whole murine lymphoma cells (L1210, L1210R, and L5178Y cells). These cell lines have a high folate requirement for growth in cell culture and are useful as models of the response of human leukemias to therapy by folate analogue inhibitors of dihydrofolate reductase. The L1210R cells, a methotrexate-resistant subline of L1210 cells, synthesize an 11-fold greater amount of dihydrofolate reductase than do L1210 cells (13). The dihydrofolate reductases of L1210R and L1210 cells are apparently identical (12, 14), and the two cell lines do not differ in methotrexate uptake (15).

MATERIALS AND METHODS

Radiolabeled Folate Coenzymes—The enzymically active diastereoisomers of the following compounds were prepared and purified as previously described (16): 5-CH_3-[9,3′,5′-^{3}H]H_4-folate (250 Ci per mole, 96% radiochemical purity) and 5-[^{14}C]-CH_3-H_4-folate (15 Ci per mole, 95% radiochemical purity). Where appropriate these materials were combined for experiments which utilized 5-[^{14}C]CH_3-[^{3}H$_3$]H_4-folate. Dr. E. J. Pastore, now of the University of California, San Diego, kindly provided 5-HCO-[6-^{3}H]H_4-folate (300 Ci per mole), which was purified (16) to 97% radiochemical purity.

Source of Cells—Murine lymphoma cells were grown from an inoculum of 10^6 cells in the ascitic form in hybrid strain BDF_1 mice (L1210 and L1210R lymphomas) or in strain DBA mice (L5178Y lymphoma). L1210 and L1210R cells were harvested on the 6th day (in late exponential growth phase). L5178Y cells were harvested on the 9th day (approaching stationary phase). Cells were washed with 0.9% NaCl solution and suspended in the medium described below.

Cell Suspensions—Cells at a density of 20×10^6 per ml were shaken in folate-free Eagle's basal medium with Earle's salts supplemented with 10% dialyzed horse serum and, where indicated, radiolabeled folates and 50 μM homocysteine or methotrexate. For experiments with methotrexate, cells were incubated at 37° for 30 min with the methotrexate, harvested, then suspended in the above medium containing radiolabeled folates at 37°.

Preparation of Cell Extracts—After incubation, cells were harvested and washed at 0° with 0.9% NaCl as previously described (9), suspended in 10 ml of 0.5 M 2-mercaptoethanol at 0°, disrupted by sonication for 30 s, and frozen. On thawing, the insoluble cell material was separated by centrifugation and, where appropriate, dissolved in Soluene (Packard Instrument Co.) for counting of radioactivities. Soluble cell extracts were

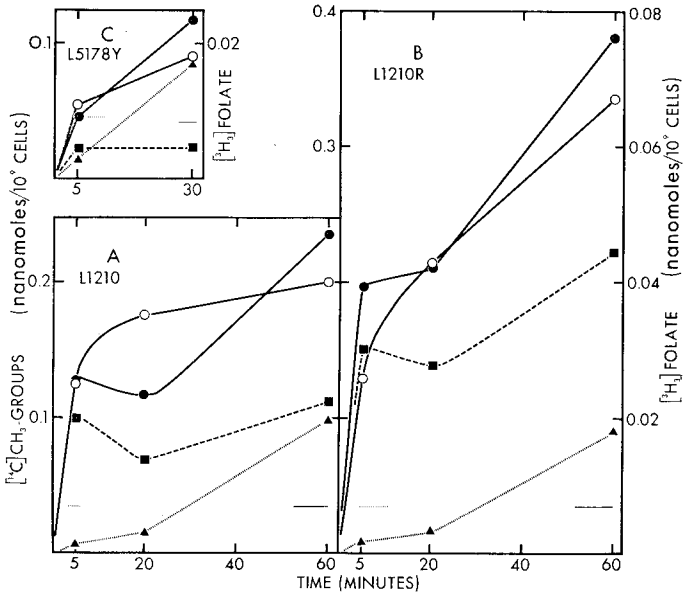

FIG. 2. Cell disposition of ^{14}C and rate of uptake of both ^{14}C and ^{3}H when cells were incubated with 5-[^{14}C]CH_3-[9,3′,5′-^{3}H]H_4-folate and homocysteine. Soluble cell extracts were passed through a column (0.5 × 2.0 cm) of the anion exchange resin AG 2IK (Cl^- form; Bio-Rad Laboratories) followed by 5 ml of 0.1 mM methionine. The unadsorbed effluent fraction contained non-folate compounds, including methionine. A fraction containing folates was then eluted by 1.0 N HCl. Each fraction was lyophilized and transferred quantitatively to a vial, and the ^{3}H and ^{14}C radioactivities were counted. Numbers of cells incubated for each time point were as follows: *A*, 10^9 L1210 cells; *B*, 0.6×10^9 L1210R cells; *C*, 0.95×10^9 L5178Y cells. The initial concentrations of radiolabels in the medium are represented by *bars*, -----, ^{14}C ——, ^{3}H; and are calculated for a volume of 0.35 ml, the approximate intracellular volume of 10^9 cells which were found to occupy a packed wet cell volume of 1.0 ml (19). The concentrations of radiolabeled compounds in the medium were as follows: 5-[^{14}C]CH_3-H_4-folate, 0.1 μM in *A* and *B*, 0.13 μM in *C*; 5-CH_3-[9,3′,5′-^{3}H]H_4-folate, 0.02 μM in *A* and *B*, 0.025 μM in *C*. ○, total cell ^{3}H; ●, total cell ^{14}C; ■, non-folate, soluble cell ^{14}C; ▲, ^{14}C of insoluble cell material.

fractionated by column chromatography, as described in the legends of Figs. 2 and 3, before counting of radioactivities.

Radioactivity Assay—Radioactivities were counted in a liquid scintillation counter with channels optimized for counting of appropriately quenched samples containing ^{14}C or ^{3}H or, where appropriate, each in the presence of the other. By use of an external standard and experimentally calibrated efficiency curves the radioactivities were determined in disintegrations per min, when necessary to determine the concentrations of radiolabeled compounds.

RESULTS

Stability of Radiolabeled Folates in Incubation Media—The concentration of 5-[^{14}C]CH_3-[^{3}H$_3$]H_4-folate in media was sufficiently high so that it was not altered appreciably during incubations of cells for periods up to 60 min. After an incubation of L1210R cells for 60 min in medium containing 0.1 μM 5-CH_3-[^{3}H$_3$]H_4-folate, the ^{3}H of the medium was analyzed by the procedures outlined in the legend to Fig. 3. Of the total ^{3}H of the medium, 91% was identified with free 5-CH_3-H_4-folate, 5% appeared to be bound to proteins of the medium, 2% was identified with *p*-aminobenzoylglutamate, and 2% was identified with other compounds.

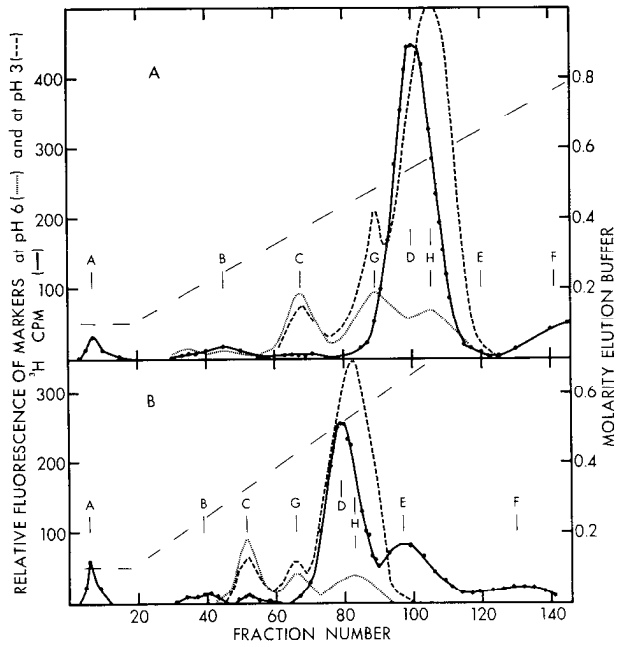

FIG. 3. Elution profiles resulting from the analysis of [^{3}H]folates of cell extracts by column chromatography on A25 DEAE-Sephadex as previously described (9, 20, 21). The positions of marker compounds were determined by their fluorescence emission at 365 nm (excitation, 305 nm). The eluted positions of peak fractions of markers and radiolabels are indicated on the figure by *letters*, which are identified (9, 20, 21) as follows: *A*, protein-bound folates not adsorbed to the column; *B*, 10-HCO-H_4-folate, 5,10-CH=H_4-folate and *p*-aminobenzoate; *C*, *p*-aminobenzoylglutamate (marker); *D*, 5-CH_3-H_4-folate; *E*, H_2-folate; *F*, late eluting compounds, perhaps folylpolyglutamates; *G*, 5-HCO-H_4-folate (marker); *H*, H_4-folate (marker). Tritium peaks (●——●) were integrated; the calculated percentage distributions of ^{3}H among principal peaks are listed in Table III. *A*, extract of 1.4×10^9 L1210 cells incubated for 60 min with 0.1 μM 5-CH_3-[9,3′,5′-^{3}H]H_4-folate. *B*, extract of 0.5×10^9 L1210 cells incubated for 30 min with 1 mM methotrexate, then incubated for 60 min with 0.4 μM 5-CH_3-[9,3′,5′-^{3}H]H_4-folate. The incubation and extraction procedures are described under "Materials and Methods."

TABLE I

Endogenous cell folate concentrations

Endogenous cell folates were bioassayed by use of *Lactobacillus casei* (17) and their concentrations were expressed relative to folic acid standards. Prior treatment by purified chicken pancreas γ-glutamyl carboxypeptidase (18) was used to assay total folates, including folylpolyglutamates.

Cell type	Cell folates assayed by *L. casei*	
	Without treatment by carboxypeptidase	After treatment by carboxypeptidase
	nmoles/10^9 cells	
L1210	7.0	29
L1210R	9.3	27
L5178Y	7.0	42

Methyl Group Transfer from 5-CH_3-H_4-Folate—L1210, L1210R, and L5178Y cells each concentrated both radiolabels of 5-[^{14}C]-CH_3-[$^3H_3$]H_4-folate above the concentrations of the media (Fig. 2), despite many fold higher concentrations of endogenous total cell folates (Table I). In the case of L1210 cells the uptake of ^{3}H, and therefore of total 5-CH_3-H_4-folate, appeared to be approaching saturation by 60 min. By comparison, L1210R cells took up more 5-CH_3-H_4-folate and appeared to approach saturation later. At no time did the ^{14}C:^{3}H ratio of total cell radioactivities differ grossly from the ^{14}C:^{3}H ratio in the incubation medium (Fig. 2).

TABLE II

Methyl transfer from 5-CH_3-H_4-folate

The transfer of ^{14}C from 5-[^{14}C]CH_3-[$^3H_3$]H_4-folate is expressed as a percentage of the total taken up into cells at various times, and was calculated from the data shown in Fig. 2 as the sum of ^{14}C of the insoluble cell material and non-folate ^{14}C of the soluble cell extract.

Time	L1210	L1210R	L5178Y
min	%	%	%
5	84	81	85
30			91
60	89	82	

After uptake of 5-[^{14}C]CH_3-[$^3H_3$]H_4-folate into cells, more than 80% of the total cell ^{3}H remained identified with folates at all time points. In every case less than 10% of the total cell ^{3}H was found associated with either the insoluble cell material or the non-folate fraction of the soluble cell extract. Of the total cell ^{14}C only 10 to 19% remained identified with folates at the various time points. The amount of non-folate ^{14}C of the soluble cell extract increased rapidly within the first 5 min (Fig. 2) but not thereafter. The amount of ^{14}C associated with insoluble cell materials increased with time, and at the last time points constituted 25 to 70% of the total cell ^{14}C (Fig. 2). Since the amounts of ^{3}H associated with the insoluble cell material remained less than 10% of the total cell ^{3}H at all times, the ^{14}C associated with insoluble cell materials must be labeling predominantly non-folate compounds.

To further identify the nature of ^{14}C-labeled compounds separated into the non-folate fractions of the soluble extracts of L1210R cells, a portion of each of those fractions was chromatographed on cellulose thin layers with development by butanol-acetic acid-water (90:10:25). Of the total ^{14}C eluted from all areas of the thin layers, the following proportions chromatographed with methionine in this system: from cells incubated 5 min, 97%; from cells incubated 20 min, 92%; and from cells incubated 60 min, 70%.

In the experiments illustrated by Fig. 2, that proportion of the total cell ^{14}C which represented methyl group transfer from 5-[^{14}C]CH_3-H_4-folate can be estimated as the sum of ^{14}C found in the non-folate, soluble cell extract and in insoluble cell materials. As shown in Table II, this proportion was high, in the range 81 to 91%.

Identity of Tritiated Compounds Found in Extracts of Cells Incubated with 5-CH_3-[9,3′,5′-^{3}H]H_4-Folate—L1210 cells took up 5-CH_3-[$^3H_3$]H_4-folate essentially unchanged. In fact, after a 60-min incubation, 87% of the ^{3}H of extracts of L1210 cells remained identifiable as 5-CH_3-[$^3H_3$]H_4-folate (Fig. 3*A* and Table III). When L1210R cells preincubated with methotrexate were then incubated for 60 min with 5-CH_3-[$^3H_3$]H_4-folate, a similar distribution of ^{3}H resulted; namely, 91% of the total ^{3}H of the cell extract was identified as 5-CH_3-[$^3H_3$]H_4-folate (Table III). The distribution of ^{3}H of the cell extracts differed, however, when L1210 cells were first incubated with methotrexate and then with 5-CH_3-[$^3H_3$]H_4-folate. In this case an additional ^{3}H-labeled compound, identified as [$^3H_3$]H_2-folate, was detected (Fig. 3*B*); only 60% of the ^{3}H of the cell extract could be identified as

TABLE III

Distribution of tritium among cell folates

Cells were extracted after a 60-min incubation with the indicated tritiated folate; the extract was analyzed as described for Fig. 2. The distribution of tritium identified with particular compounds is expressed as a percentage of the total. Principal tritium peaks are listed; residual tritium from each experiment was distributed in small amounts (1 to 11%) among *p*-aminobenzoylglutamate, *p*-aminobenzoate, 10-CHO-H_4-folate or 5,10-CH=H_4-folate, and late eluting compounds, presumably folylpolyglutamates. In no case was any tritium found identified with 5-HCO-H_4-folate. The number of cells incubated was in the range 0.5 to 2.0 × 10^9; the concentration of tritiated folate in the medium was in the range 0.1 to 0.4 μM. Where indicated, cells were preincubated for 30 min with methotrexate.

Incubation with	Cell type	Methotrexate concentration	Protein-bound folates and thymidylate	5-CH_3-H_4-Folate	H_2-Folate
		mM	% total ^3H		
5-CH_3-[9,3′,5′-^{3}H]H_4-Folate	L1210	0	1	87	0
	L1210	1	3	60	23
	L1210R	2	2	91	0
5-HCO-[6-^{3}H]H_4-Folate	L1210	0	28	48	0
	L1210R	0	52	28	0

5-CH_3-[$^3H_3$]H_4-folate and 23% was identified as [$^3H_3$]H_2-folate (Table III).

Identity of Tritiated Compounds Found in Extracts of Cells Incubated with 5-HCO-[6-^{3}H]H_4-Folate—Analysis of extracts of L1210 and L1210R cells which had been incubated with 5-HCO-[6-^{3}H]H_4-folate showed a distribution of about 80% of the total cell ^3H into two peaks (Table III), one identifiable as 5-CH_3-[^{3}H]H_4-folate and the other as ^3H which was not adsorbed to the analytical column. Small amounts of the latter ^3H may have been protein-bound folate, as appeared to be the case for 5-CH_3-[$^3H_3$]H_4-folate of cell extracts, but when extracted from whole cells thymidylate is not adsorbed to DEAE-Sephadex, and thymidylate synthesized in the presence of a [6-^{3}H]H_4-folate would be labeled by ^3H as described in the introduction. It is therefore reasonable, as well as consistent with the above result for the incubation of methotrexate-treated L1210 cells with 5-CH_3-[$^3H_3$]H_4-folate, to identify tentatively as thymidylate that ^3H which, in these experiments, was not adsorbed to DEAE-Sephadex. Thus after incubation with 5-HCO-[6-^{3}H]H_4-folate, about 28% of the ^3H of the extract of L1210 cells and 52% of that of L1210R cells was tentatively identified as thymidylate (Table III).

DISCUSSION

Turnover of Whole Cell 5-CH_3-H_4-Folate—In whole cells which have taken up 5-[^{14}C]CH_3-H_4-folate, the transfer of [^{14}C]CH_3 groups into non-folate compounds is a measure of whole cell methyltetrahydrofolate:homocysteine methyltransferase activity. Indeed, results confirmed that the initial transfer of [^{14}C]CH_3 groups from 5-[^{14}C]CH_3-H_4-folate was into [^{14}C]methionine, the product of this reaction. Since the equilibrium constant for the reduction of 5,10-CH_2-H_4-folate to 5-CH_3-H_4-folate (Fig. 1) lies well in the forward direction (22), 5-CH_3-H_4-folate can lose its CH_3 group virtually only by that methyltetrahydrofolate:homocysteine methyltransferase reaction. Thus the rate of transfer of [^{14}C]CH_3 groups from 5-[^{14}C]CH_3-H_4-folate into non-folate compounds also provides a minimum value for the turnover of 5-CH_3-H_4-folate. It is clear from the results that the turnover of 5-CH_3-H_4-folate of whole L1210, L1210R, and L5178Y cells is very rapid.

By the use of 5-[^{14}C]CH_3-[$^3H_3$]H_4-folate for the estimation of [^{14}C]CH_3 transfer from 5-[^{14}C]CH_3-H_4-folate, it was possible to ensure that there were no gross discrepancies between the total amount of ^{14}C found in cells and the total amount of 5-[^{14}C]CH_3-[$^3H_3$]H_4-folate taken up by cells (Fig. 2). The ^3H label also provided an indication of the approach to equilibrium between newly taken up 5-CH_3-H_4-folate and the endogenous cell pool of 5-CH_3-H_4-folate, as well as an indication of the relative size of that pool.

Relative Concentrations of [^{3}H]Folate Coenzymes in Whole Cells—Despite the rapid turnover of 5-CH_3-H_4-folate in L1210 and L1210R cells, demonstrated by the rapid transfer of CH_3 groups into non-folate compounds, 80 to 90% of the ^3H of cell extracts remained identifiable as 5-CH_3-[$^3H_3$]H_4-folate after a 60-min incubation of cells with 5-CH_3-[9,3′,5′-^{3}H]H_4-folate. As illustrated in Fig. 1, H_4-folate derived from 5-CH_3-H_4-folate will participate in various folate coenzyme interconversions. Apparently the rate constants for the interconversions of the folate coenzymes are such that 5-CH_3-H_4-folate itself represents 80 to 90% of the pool of all folate coenzymes with which [$^3H_3$]H_4-folate equilibrates within a period of 60 min in the cells studied. That is, the major metabolic pathway for H_4-folate derived from 5-CH_3-H_4-folate is its return to 5-CH_3-H_4-folate as illustrated in Fig. 1. In following this circuit, H_4-folate would form adducts with unlabeled single carbon groups within the cell and these would flush [^{14}C]CH_3 groups of 5-[^{14}C]CH_3-H_4-folate out of the pool of 5-CH_3-H_4-folate.

It follows from the above observations that the 10 to 19% of ^{14}C which remained associated with folate coenzymes after 60 min of incubation of the cells with 5-[^{14}C]CH_3-H_4-folate must represent 5-CH_3-H_4-folate sequestered in a pool the turnover of which is very slow compared to the major portion of cell 5-CH_3-H_4-folate. The nature or significance of this pool is not yet known.

Flux of Cell Folates through the Pathway of Thymidylate Biosynthesis—In L1210 cells the flux of cell folates through the pathway of thymidylate biosynthesis was estimated by two different approaches reported in "Results." The first approach was to incubate L1210 cells with 5-HCO-[6-^{3}H]H_4-folate and measure transfer of ^3H to thymidylate. In 1 hour, about 28% of the cell folates had been used for thymidylate biosynthesis. This value was confirmed by the second approach, in which methotrexate-treated L1210 cells were incubated with 5-CH_3-[9,3′,5′-^{3}H]H_4-folate. In the absence of methotrexate any [$^3H_3$]H_2-folate derived from the thymidylate synthetase reaction would be quickly reduced to [$^3H_3$]H_4-folate (Fig. 1). However, methotrexate inhibits dihydrofolate reductase, and in its presence any [$^3H_3$]H_2-folate generated in whole cells would accumulate as such. In fact, of the total ^3H found in methotrexate-treated L1210 cells which were incubated for 1 hour with 5-CH_3-[$^3H_3$]H_4-folate, 23% had accumulated as [$^3H_3$]H_2-folate.

The pathways of methionine biosynthesis and thymidylate biosynthesis have a common origin in 5,10-CH_2-H_4-folate (Fig. 1). The relative flux of folate coenzymes through these competing pathways can be partly estimated from the above results. In L1210 cells the flux of folate coenzymes through the pathway of thymidylate biosynthesis in 1 hour appeared to be about 28% of the cell folates. In the same time period the flux of folate coenzymes through the pathway of methionine biosynthesis appeared to be not less than 90% of the cell folates.

In L1210R cells the flux of folate coenzymes through the pathway of thymidylate biosynthesis was estimated to be 52% of the cell folates during a 1-hour incubation with 5-HCO-[6-^{3}H]-H_4-folate. However, it proved impossible to accumulate [$^3H_3$]H_2-folate by incubating methotrexate-treated L1210R cells with 5-CH_3-[$^3H_3$]H_4-folate.

Methotrexate Resistance of L1210R Cells—The methotrexate resistance of L1210R cells is known to depend, at least in part, on their increased concentration of dihydrofolate reductase (13). The significance of this increase is apparent in the results obtained above; despite a high flux of folate coenzymes through H_2-folate and preliminary incubation of the cells in a high concentration of the dihydrofolate reductase inhibitor methotrexate, no H_2-folate was accumulated. The failure to accumulate H_2-folate might be attributed to efflux of methotrexate from the cells (9) during the course of the incubation, sufficient to allow activity of a significant portion of the dihydrofolate reductase of L1210R cells. Presumably, continuation of the methotrexate treatment throughout the incubation, perhaps at still higher methotrexate concentrations, should result in accumulation of H_2-folate in L1210R cells; but such treatment would also decrease the cell uptake of 5-CH_3-[$^3H_3$]H_4-folate (7).

Another finding pertinent to the methotrexate resistance of L1210R cells might be that their pool of 5-CH_3-H_4-folate appeared to be larger than that of the methotrexate-sensitive L1210 cells (Fig. 2), although the two cell lines did not differ significantly in their concentrations of total folates (Table I).

Relative Concentrations of [^{3}H]Folylpolyglutamates in Whole Cells—Some tritiated late eluting compounds, perhaps [^{3}H]-folylpolyglutamate coenzymes, were detected in the analyses of cell extracts reported in Fig. 3 and Table III, but quantitatively they were of little significance. These results are quite different from the recent finding (2, 23) of mostly [^{3}H]H_4-folyltetraglutamate coenzymes in liver 24 hours after the administration of [^{3}H]folic acid to intact rats. The reason for the difference might be that in 1-hour incubations the biosynthesis of folylpolyglutamates is insignificant or that in the experiments reported above any endogenous γ-glutamyl carboxypeptidase was not specifically inactivated. There is therefore the possibility that the results reported in Fig. 3 and Table III might represent the products of removal of glutamic acid residues from [^{3}H]H_4-folylpolyglutamate coenzymes by endogenous γ-glutamyl carboxypeptidase. However, the estimations of the turnover of 5-CH_3-H_4-folate and of the flux of folates through the pathway of thymidylate biosynthesis would not be affected by these considerations.

Control of Cell Folate Metabolism—The results indicate that whole cell folate coenzymes turn over rapidly although only one, 5-CH_3-H_4-folate, accumulates to a significant concentration. The metabolism of folate coenzymes must be finely controlled in a manner which could be manipulated so as to potentiate the effects of folate analogue inhibitors of dihydrofolate reductase.

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