Disposition of Endogenous Homocysteine by Mouse Fibroblast C3H/10T1/2 Cl 8 and the Chemically Transformed C3H/10T1/2 MCA Cl 16 Cells Following Methotrexate Exposure [1,2]

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ABSTRACT—The tumorigenic cell line termed "MCA Cl 16" was derived from C3H/10T1/2 clone (Cl) 8 cells by chemical transformation in the presence of 3-methylcholanthrene [(MCA) CAS: 56-49-5]. Transformed (Cl 16) cells were more sensitive toward the cytotoxic effect of methotrexate (MTX) than their normal counterpart Cl 8 cells. The disposition of endogenous L-homocysteine (Hcy) was investigated in these two cell lines after MTX exposure. Both nonmalignant and transformed cells exported Hcy into the extracellular medium, and only small amounts were retained within the cells. The Hcy efflux from the malignant cells was markedly increased after MTX exposure (0.5–10 μM), and this effect was almost completely prevented by 5-formyl-tetrahydrofolate (THF), whereas treatment with thymidine plus hypoxanthine did not inhibit the MTX-dependent Hcy efflux. Cytotoxic concentration of MCA reduced rather than increased the Hcy efflux from these cells. High concentrations of MTX (>10 μM) were required to increase the release of Hcy from nonmalignant cells. The enhancement of Hcy export from the malignant cells in the presence of MTX was not associated with cellular build-up of *S*-adenosyl-L-homocysteine (AdoHcy), indicating that the amount of intracellular Hcy was kept below the level required for inhibition or reversion of the AdoHcy hydrolase reaction. MTX-dependent Hcy efflux probably reflects cellular deficiency of 5-methyl-THF required for the salvage of Hcy to methionine and may therefore be a measure of lack of this reduced folate relative to the metabolic demand.—JNCI 1986; 77:283–289.

The antifolate drug MTX has been found useful in the treatment of acute lymphoblastic leukemia and several solid tumors (*1*). The ubiquitous enzyme DHFR seems to be the primary molecular target of MTX. This enzyme is responsible for the regeneration of THF from DHF. Inhibition of DHFR leads to cellular depletion of THF and related reduced folates, including 5-methyl-THF, and thereby blocks the various THF coenzymes-dependent synthesis of proteins, DNA, and RNA (*2, 3*). However, several questions on the action mode of MTX, as for example, the biochemical basis of several side effects of MTX (*4*) and the selective kill of sensitive tumor cells (*5*), remain to be answered. This has stimulated research on the effects of MTX on numerous biological processes, including transport of glucose (*6, 7*), folates (*8, 9*), and amino acids (*10*), and the interaction of the drug with enzymes other than DHFR (*2, 11–13*).

There has been a growing interest in the amino acid Hcy because altered cellular disposition of this compound has been suggested to play an important role in the pathogenesis of various processes, including homocystinuria (*14*), atherosclerosis (*15*), methionine-dependent cancer (*16*), and the hepatic injury induced by MTX (*3*).

Hcy is not supplied by food but is formed from the endogenous transmethylase inhibitor AdoHcy through the action of the enzyme AdoHcy hydrolase (*17*). Once formed, Hcy is either metabolized to cystathionine via the so-called transsulfuration pathway, or it is salvaged to methionine. The conversion to methionine is in most tissues catalyzed by an enzyme that requires 5-methyl-THF as methyl donor (*14*). This reaction links Hcy metabolism to the biological effect of the antifolate drug MTX, which might be expected to block methionine synthesis from Hcy. These metabolic relations are depicted in text-figure 1.

In the present paper we investigate the disposition of endogenous Hcy in a nonmalignant (C3H/10T1/2 Cl 8) and malignant (C3H/10T1/2 MCA Cl 16) cell line during MTX exposure. The malignant cells (MCA Cl 16) are derived from the nonmalignant cells (Cl 8) by chemical transformation in the presence of MCA and are tumorigenic upon implantation in immunosuppressed syngeneic mice (*18*).

MATERIALS AND METHODS

Chemicals and drugs.—DL-Homocysteine, Hcy, L-methionine, adenosine, and AdoHcy were obtained from Sigma Chemical Co., St. Louis, MO, and AdoMet was obtained from Koch-Light Laboratories, Colnbrook, England. MTX and leucovorin (5-formyl-THF) were purchased from Nyegaard & Co. (Nyco), Oslo, Norway. [^{14}C]Adenosine (0.59 Ci/mmol) and [^{35}S]methionine (1,070 Ci/mmol) were obtained from the Radiochemical

ABBREVIATIONS USED: AdoHcy=*S*-adenosyl-L-homocysteine; AdoMet=*S*-adenosylmethionine; Cl=clone; DHF=dihydrofolate; DHFR=dihydrofolate reductase; Hcy=L-homocysteine; HPLC=high-performance liquid chromatography; LD=lethal dose; MCA=3-methylcholanthrene; MTX=methotrexate; ODS=octadecylsilyl; THF=tetrahydrofolate.

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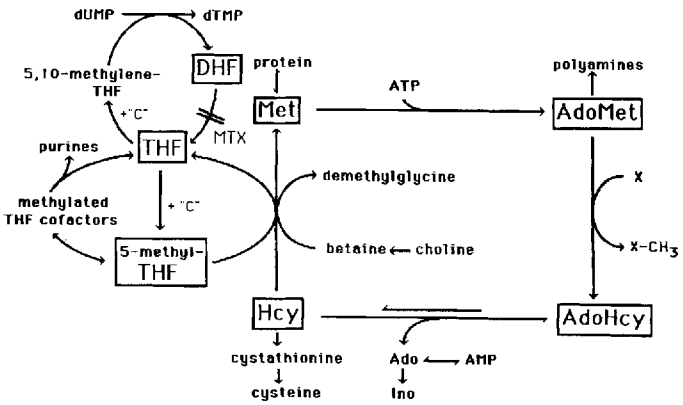


TEXT-FIGURE 1.—Metabolism of Hcy and its relation to folate coenzymes.

Centre, Amersham, Buckinghamshire, England. Cell culture medium (Eagle's basal medium) and heat-inactivated fetal calf serum were from Gibco, Paisley, Scotland, and the disposable culture flasks were from A/S Nunc, Roskilde, Denmark.

Cell lines and culture conditions.—The malignant cells, C3H/10T1/2MCA Cl 16, were obtained from mouse embryo fibroblasts, C3H/10T1/2 Cl 8, by chemical transformation in the presence of MCA (CAS: 56-49-5) (*18*). Both cell types were grown in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The Cl 8 cells were seeded at 20,000 cells per dish so that the cultured cells reached confluence on the 9th day. The MCA Cl 16 cells were seeded at 5,000 cells per dish.

MTX was dissolved in culture medium and sterile filtered immediately before addition to the culture dishes.

Determination of cytotoxicity.—The cytotoxic effect of MTX on the mouse fibroblasts was evaluated by plating efficiency and by determination of total cell count.

Cells were seeded at a density of 200 cells per 60-mm petri dish. After 24 hours, the medium was replaced with fresh medium containing increasing concentrations (0-200 μM) of MTX. The drug exposure lasted for 24 hours, and the plating efficiency was determined after 8 days of culture in a drug-free medium. Each group consisted of six dishes, and the control group corresponded to 42 colonies per dish. Only colonies comprising more than about 30 cells were counted. From parallel dishes the cells were put in suspension by trypsinization and the total number of cells per dish was determined by means of a Coulter counter.

Determination of Hcy.—Hcy was determined in both the culture medium and in the cell extract. Samples of the medium (500 μl) were mixed with concentrated perchloric acid to a final concentration of 0.6 *N*. The cells were washed with ice-cold saline and then suspended in 500 μl of 0.6 *N* perchloric acid. The cells were homogenized at sufficiently high dilution to ensure almost 100% recovery of Hcy (*19*). Proteins and other acid-insoluble material were removed by centrifugation (9,000×*g* for 5 min), and the perchloric acid was neutralized by addition of 1.44 *N* KOH-1.2 *N* $KHCO_3$. After 10 minutes at 0°C, the insoluble potassium perchlorate was removed by centrifugation.

Hcy (non-protein-bound) was routinely determined in the neutralized perchloric acid extract of the culture medium and of the cells. In some experiments, the Hcy assay was run directly in samples of the culture medium (total Hcy). The assay procedure has been described in detail elsewhere (*19*). After treatment of the samples with dithioerythritol, interfering material (adenosine and AdoHcy) was removed by adsorption to dextran-coated charcoal, leaving Hcy in solution. Hcy was then condensed with radioactive adenosine in the presence of AdoHcy hydrolase, and the radioactive AdoHcy formed was isolated by HPLC on a 3-μm ODS Hypersil column eluted with a methanol gradient. In some experiments, when the cells were grown in the presence of [^{35}S]methionine, radioactive adenosine in the assay mixture was replaced with unlabeled adenosine.

The samples were injected into the column by means of an HPLC autosampler, which was interfaced with a programmable fraction collector equipped with a peak separator (from Isco). In this way, radioactive AdoHcy was collected during unattended analysis. Radioactive AdoHcy was determined by liquid scintillation counting.

Determination of AdoHcy and AdoMet.—Cells were extracted with perchloric acid, as described in the preceding paragraph. AdoHcy or AdoMet added to the extract was totally recovered. AdoHcy and AdoMet were assayed in the same sample on a cation-exchange column (Partisil 10 SCX, 0.46 × 25 cm), which was equilibrated at ambient temperature with 60 m*M* ammonium formate (pH 3.5). The flow rate was 1.5 ml/minute. AdoHcy eluted after 6.5 minutes. At this time, the ammonium formate concentration was increased to 330 m*M*. The detector received an auto zero signal from the autosampler at time 10 minutes, and AdoMet eluted at 11.6 minutes. The absorbance was recorded at 260 nm.

The amount of AdoHcy in the cellular extract was below the detection limit of the method based on monitoring absorbance of the effluent at 260 nm. Therefore, cellular AdoHcy was determined by incubating cells with [^{35}S]methionine (15 μCi/ml) for sufficient time to allow equilibration of radioactivity between the pools of methionine and its sulfur-containing metabolites. The radioactivity cochromatographing with AdoHcy was taken as a measure of cellular AdoHcy (text-fig. 6).

AdoHcy was almost quantitatively eluted from the cation-exchange column. AdoMet, however, was not quantitatively eluted, and the recovery was dependent on the age of the column and the mobile phase. Thus the specific area obtained by UV detection at 260 nm and integration of the chromatogram were less for AdoMet than for AdoHcy. AdoMet in tissue extract and in standard samples shows the same chromatographic behavior, and the amount of AdoMet in the tissue extract could be determined, but the specific radioactivity of AdoMet was underestimated. Therefore, the amount of AdoHcy could not be calculated with the use

of the specific radioactivity of AdoMet, but it is given as counts per minute in table 2.

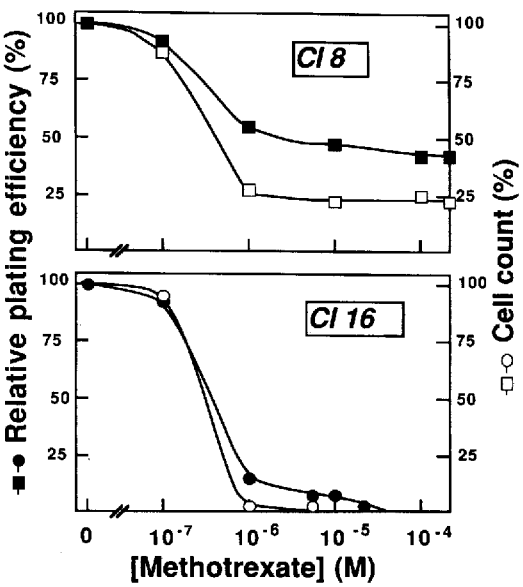
Determination of protein.—Cells were extracted with perchloric acid as described above, and the precipitated protein was collected by centrifugation. The protein pellet was dissolved in 0.1 *N* NaOH, and the protein was determined by the method of Bradford (*20*) using the Bio-Rad protein-assay kit. Gammaglobulin was used as standard.

RESULTS

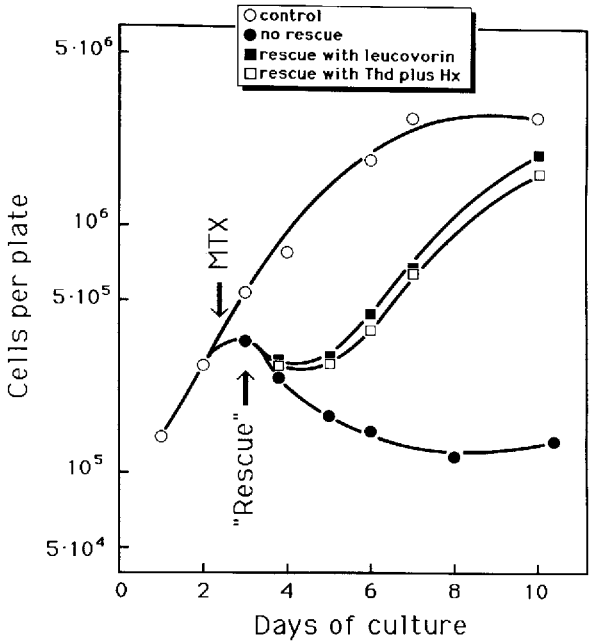
Effect of MTX on Cl 8 and MCA Cl 16 Cells

The effect of MTX on Cl 8 and Cl 16 cells during exponential growth was evaluated by determining plating efficiency and total cell count in the presence of various concentrations of MTX. The dose-response curves based on total cell count gave a steeper slope and lower LD_{90} values than the curves obtained from determination of plating efficiency. The LD_{90} for the Cl 16 cells was about $3 \cdot 10^{-7}$ *M* (cell count), whereas a fraction (20-30%) of the Cl 8 cells were not killed even in the presence of high-concentration (200 μM) MTX (text-fig. 2).

Addition of 5 μM MTX to Cl 8 and MCA Cl 16 cells during exponential growth caused an immediate growth arrest of both cell types (data not shown). The Cl 16 cells treated with MTX showed a marked drop in cell count after MTX exposure. These cells were "rescued" by addition of leucovorin or thymidine plus hypoxanthine (text-fig. 3). Similar results were obtained with Cl 8 cells (data not shown).



TEXT-FIGURE 2.—Cytotoxicity of MTX against C3H/10T1/2 Cl8 and MCA Cl 16 cells. Cells were seeded at a density of 200 cells/60-mm petri dish. After 24 hr, the medium was replaced with fresh medium containing MTX at concentrations given on the *abscissa*. The drug exposure lasted for 24 hr, and plating efficiency (■,●) and cell count (□,○) were determined after 8 days of culture in a drug-free medium. Each group consisted of 6 dishes, and the control group was set at 100%.



TEXT-FIGURE 3.—Growth curves of C3H/10T1/2 Cl 16 cells during MTX exposure and "rescue" with leucovorin or thymidine plus hypoxanthine. MCA Cl 16 cells were seeded in 60-mm petri dishes (5×10^4 cells/dish). When the cell count reached 3×10^5 cells, the medium was replaced with fresh medium, which either contained 5 μM MTX (●) or no drug (○, control cells). The MTX exposure lasted for 16 hr. At this time, the medium was again changed, and one-half of the cells exposed to MTX received a medium containing 10 μM leucovorin (■) or 100 μM thymidine plus 100 μM hypoxanthine (□). The cell growth was determined by trypsinization of two dishes from each group, followed by cell counting by means of a Coulter counter. Thd=thymidine; Hx=hypoxanthine.

Disposition of Endogenous Hcy

Both nonmalignant (Cl 8) and malignant (MCA Cl 16) cells exported Hcy into the extracellular medium, and only a small fraction was retained within the cells (table 1). Most extracellular Hcy (≈90%) was recovered in a medium deproteinized with perchloric acid and therefore exists as free Hcy not associated with proteins in the culture medium. Total Hcy was assayed directly in the medium (*see* "Materials and Methods" for details). The amount of Hcy in the culture medium was proportional to the cell density (data not shown).

Nonmalignant (Cl 8) and the chemically transformed (MCA Cl 16) cells were grown to confluence and then exposed to MTX. Export of Hcy from Cl 16 cells was greatly enhanced in a dose-dependent manner by MTX (text-fig. 4). The amount of extracellular Hcy increased progressively for at least 96 hours. Notably, addition of leucovorin (5-formyl-THF) to Cl 16 cells exposed to MTX for 24 hours markedly reduced the MTX-dependent efflux of Hcy from these cells, whereas addition of thymidine plus hypoxanthine did not reduce Hcy efflux (text-fig. 5).

TABLE 1.—*Amount of free Hcy associated with the cells and in the culture medium*[a]

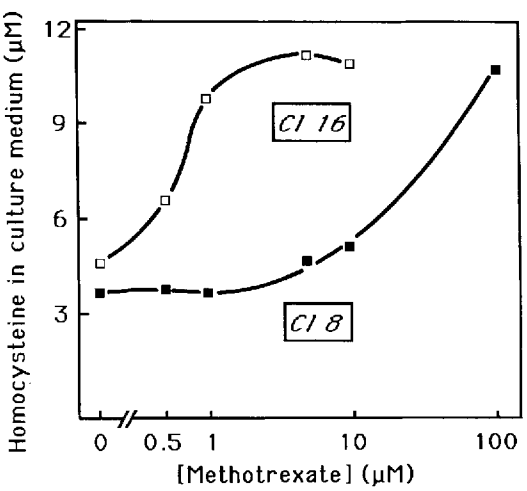
Cell line	Time after medium shift, hr	Hcy associated with cells[b]	Hcy in culture medium	
		pmol/10^6 cells	μM	pmol/10^6 cells
Cl 8	24	6.5	0.9	900
	48	15.1	1.6	1,600
	72	21.5	2.5	2,500
Cl 16	24	11.7	1.6	1,600
	48	20.2	2.3	2,300
	72	25.4	3.2	3,200

[a] C3H/10T1/2 Cl 8 and MCA Cl 16 cells were grown to confluence, and the culture medium was replaced with fresh medium. The concentrations of Hcy in the medium before the medium was changed were 1.5 μM for the Cl 8 cells and 2.1 μM for the Cl 16 cells and undetectable immediately after medium replacement. The amount of Hcy in the medium and in cellular extract at various times after the medium is changed is given in the table.

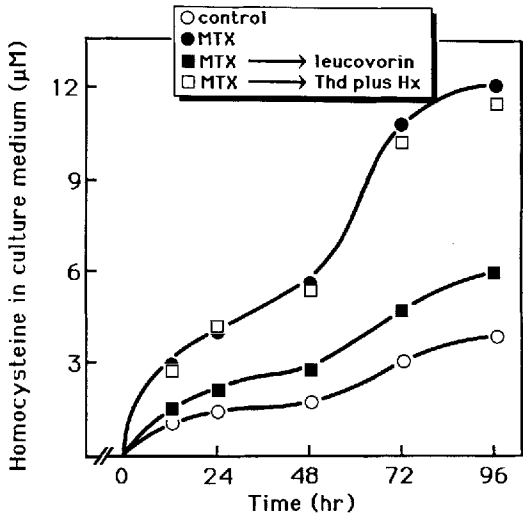
[b] Includes Hcy in adherent extracellular water.

A high concentration (>10 μM) of MTX was required to increase the Hcy release from Cl 8 cells (text-fig. 4).

To investigate whether the MTX-dependent Hcy efflux (text-figs. 4 and 5) is specific to MTX or is a phenomenon induced by cytotoxic agents in general, the release of Hcy was determined from Cl 16 cells exposed to MCA. This compound is metabolized to an electrophilic alkylating agent reacting with and causing damage of numerous cellular components (*21*). The Hcy efflux from confluent Cl 16 cells treated with 3.7 μM MCA was reduced by about 40% (data not shown). This concentration reduced the viability of Cl 16 cells in exponential growth by about 40% (data not shown).



TEXT-FIGURE 4.—Hcy efflux from C3H/10T1/2 Cl 8 and MCA Cl 16 cells exposed to various concentrations of MTX. C3H/10T 1/2 Cl 8 and MCA Cl 16 cells were seeded at 2×10^4 and 6×10^4 cells/dish, respectively, and were grown until confluence, which corresponded to 1×10^6 cells per dish for Cl 8 cells and $\approx3\times10^6$ cells per dish for MCA Cl 16 cells. The culture medium was then replaced with a fresh medium containing MTX at the concentrations indicated on the *abscissa*, and after 24 hr the extracellular drug was removed by changing the medium. The amount of Hcy accumulating in the medium the following 72 hr was plotted versus the MTX concentration.



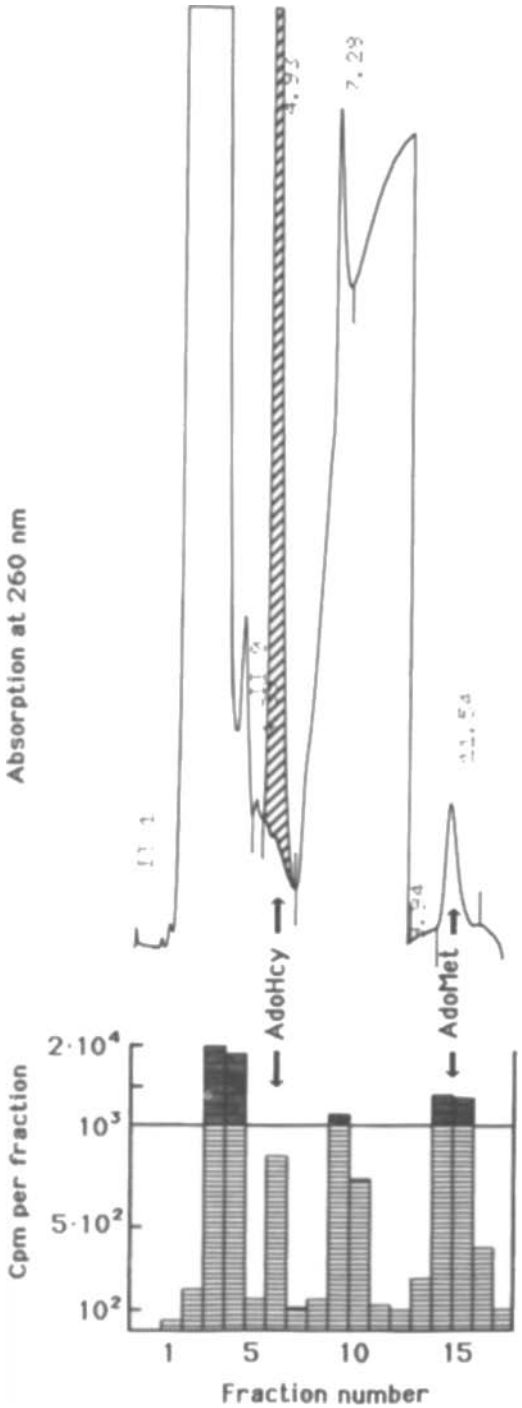
TEXT-FIGURE 5.—Time course of the Hcy efflux from C3H/10T1/2 Cl 16 during MTX exposure and following treatment with leucovorin or thymidine plus hypoxanthine. The MCA Cl 16 cells were grown to confluence, as described in the legend to text-fig. 4. A portion of the cells was exposed to 5 μM MTX for 24 hr. At this time point, the medium was replaced with fresh medium containing either no drug or supplemented with leucovorin (10 μM 5-formyl-THF) or 100 μM thymidine plus 100 μM hypoxanthine. Samples of 0.2 ml from the culture medium were withdrawn at the times indicated and frozen at −80°C until analysis. The text-fig. shows the Hcy efflux from cells not exposed to drug, i.e., control cells (○), from cells treated with MTX for 24 hr (●) and from cells treated with MTX followed by addition of leucovorin (■) or thymidine plus hypoxanthine (□). Thd=thymidine; Hx=hypoxanthine.

Cellular Content of AdoHcy and AdoMet

The amount of AdoHcy in Cl 8 cells and MCA Cl 16 cells, whether exposed to MTX or not, was below the detection limit of an HPLC assay based on UV detection. Therefore, the cellular pools of methionine and adenosyl sulfur amino acids were uniformly labeled by incubating cells for 24 hours with [^{35}S]methionine. (*See* "Materials and Methods" for details.) The chromatogram including the radioactive profile of extract from labeled MCA Cl 16 cells is shown in text-figure 6. A chromatographic system, based on gradient elution of a cation-exchange column, was developed, which allowed the simultaneous determination of AdoHcy and AdoMet in a single run. The amount of radioactivity cochromatographing with AdoHcy (text-fig. 6) was taken as a measure of cellular AdoHcy.

MTX (5 μM) had no effect on cellular AdoHcy in either Cl 8 or MCA Cl 16 cells (table 2), despite the fact that the Hcy concentration in the culture medium of the latter cell type was greatly increased by this drug (text-figs. 4 and 5).

The effect of MTX on cellular AdoMet varied somewhat for unknown reasons from one experiment to another. The amount of AdoMet in Cl 16 cells was not affected or decreased slightly (<10%) following MTX exposure for up to 72 hours. In contrast, the AdoMet content in the nonmalignant cells (Cl 8) was in some



TEXT-FIGURE 6.—Chromatogram of extract from MCA Cl 16 cells treated with [^{35}S]methionine. Cl 16 cells (3×10^6 cells/dish) were incubated with [^{35}S]methionine, as described in the text. The perchloric acid extract of these cells was analyzed on a Partisil SCX column, which was eluted with 60 m*M* ammonium formate buffer for the first 6.5 min, and then with 330 m*M* ammonium formate. The increase in buffer concentration resulted in a steep increase in base-line absorption, which was compensated for by an auto zero signal (from the autosampler) at time 10 min. The effluent was fractionated (1.5 ml/fraction) during unattended analysis by interfacing the autosampler to a programmable fraction collector (from Isco). The *upper profile* shows the absorption at 260 nm. The chromatogram is spiked by adding unlabeled AdoHcy to the extract. The elution of radioactive material is shown in the *lower part* of the text-fig. The radioactivity in fractions 3 and 4 and in fractions 9 and 10 coelutes with methionine and 5-methylthioadenosine, respectively. The retention times of AdoHcy and AdoMet standards are indicated by *arrows*.

experiments drastically decreased (to 15-20% of the AdoMet content in control cells) in the presence of MTX (5 μM), whereas in other experiments essentially no effect on AdoMet content was observed (table 2). Cl 8 cells, which showed a low AdoMet content during MTX exposure, exported less Hcy into the medium than cells containing normal amounts of AdoMet (data not shown).

DISCUSSION

Inhibition of DHFR by MTX blocks the regeneration of THF from DHF and thereby interferes with the biosynthesis of thymidine as well as purines. These are compounds essential for DNA synthesis in proliferating cells. These aspects of the pharmacology of MTX have been studied in great detail during the last decades (*2, 3*).

TABLE 2.—*Cellular content of AdoHcy and AdoMet in C3H/10T1/2 Cl 8 and MCA Cl 16 cells exposed to MTX*[a]

Cell line	Time after medium shift, hr	MTX	AdoHcy	AdoMet		[AdoHcy]:[AdoMet]
			cpm/10^6 cells	cpm	pmol/10^6 cells	
Cl 8	24	−	5,793	65,763	330	0.088
	48	−	5,276	40,123	300	0.13
	72	−	6,663	45,988	285	0.14
	24	+	5,603	65,356	330	0.085
	48	+	4,635	48,414	255	0.095
	72	+	4,179	30,850	180	0.13
C16	24	−	2,886	46,359	520	0.065
	48	−	2,606	33,883	342	0.076
	72	−	2,975	28,482	330	0.10
	24	+	2,560	42,654	385	0.060
	48	+	2,611	28,750	300	0.090
	72	+	3,566	30,314	460	0.11

[a] C3H/10T1/2 Cl 8 and MCA Cl 16 cells were grown to confluence ($1.1 \cdot 10^6$ Cl 8 cells and $3.5 \cdot 10^6$ Cl 16 cells/dish), and then half of the dishes were exposed to 5 μM MTX for 24 hr. The culture medium was at this time replaced with fresh medium containing [^{35}S]methionine, and the cells were further cultured for 24, 48, and 72 hr. The number of Cl 8 cells decreased to $0.76 \cdot 10^6$ cells, and the number of Cl 16 cells, to $2.4 \cdot 10^6$ cells and $1.7 \cdot 10^6$ cells (in the presence of MTX) during the experimental period. The cellular extract was analyzed by HPLC, and the effluent from the column was fractionated as shown in text-fig. 6. The [AdoHcy]-to-[AdoMet] ratio was obtained by dividing cpm associated with AdoHcy with cpm associated with AdoMet. Each value represents the mean of duplicate determinations.

Recently, some reports have focused on the metabolic consequences of cellular depletion of 5-methyl-THF and the effect on metabolism of methionine. MTX has been shown to decrease the cellular content of 5-methyl-THF (*4*) as well as 5-methylene-THF (*22*). Furthermore, methionine transport in lymphocytes is inhibited by MTX (*10*), and methionine deprivation reduces the antineoplastic effect of MTX (*23*).

In the present paper we have studied the effect of MTX on the disposition of endogenous Hcy in a benign and malignant fibroblast line grown to confluence. These cell types differ with respect to sensitivity toward MTX during exponential growth. MTX has a moderate cytotoxic effect against the malignant Cl 16 cells, whereas the benign Cl 8 cells are less sensitive, and a fraction seems MTX resistant (text-fig. 2). When these cells reached confluence, MTX induced essentially no cell kill. This made it possible to study the time-dependent Hcy efflux during MTX exposure. Determination of Hcy efflux from cells exposed to MTX during exponential growth might have given information on its relation to cytotoxicity, but it is hampered by a drastic decrease in cell count (text-figs. 2 and 3).

Hcy is exported into the extracellular medium by both nonmalignant (Cl 8) and malignant (Cl 16) cells (text-figs. 4 and 5), and small amounts are retained within the cells (table 1). Similar results have been obtained with other cell types, including rat hepatocytes, mouse fibroblasts L-929 (unpublished data), and human lymphoid cells (*24*). These observations are consistent with the finding of low levels of Hcy in tissues (*19*) and high concentrations in extracellular media like plasma and urine (*25*). A unidirectional transport system for Hcy may exist, which ensures low intracellular content of Hcy under conditions favoring accumulation of this compound.

The fate of intracellular Hcy includes its conversion to methionine or condensation with serine to form cystathionine via the so-called transsulfuration pathway. The former pathway is catalyzed by two enzymes, one of which requires 5-methyl-THF as methyl donor. This enzyme is believed to be responsible for the salvage of Hcy to methionine in most tissues, except kidney and liver [text-fig. 1 and (*14*)]. MTX probably blocks the 5-methyl-THF-dependent salvage of Hcy and thereby increases the intracellular level of this amino acid. This may explain the enhancement of the Hcy efflux from cells by MTX (text-figs. 4 and 5). The possibility that release of Hcy induced by MTX is an unspecific process related to leakage of cellular constituents into the medium after cell damage seems unlikely because Hcy efflux is reduced rather than increased by cytotoxic levels of MCA. In addition, rescue with thymidine plus hypoxanthine (*26*) prevents the cell kill induced by MTX (text-fig. 3), but it does not interfere with the MTX-dependent Hcy efflux (text-fig. 5).

The Hcy efflux from Cl 16 cells was markedly enhanced in the presence of 0.5–10 μM MTX. Higher concentrations (>10 μM) were required to increase the Hcy release from Cl 8 cells (text-fig. 4). This should be related to the fact that Cl 16 cells are more sensitive toward the cytotoxic effect of MTX than Cl 8 cells (text-fig. 2). However, Hcy efflux is not intimately associated with cytotoxicity since MTX is not cytotoxic to confluent cells, and MTX-dependent Hcy efflux is not inhibited by addition of thymidine plus hypoxanthine, which prevents the killing of growing cells exposed to MTX (text-fig. 3). It is conceivable that Hcy efflux is a measure of the ability of MTX to reduce the amount of reduced folates, including 5-methyl-THF, relative to the metabolic demand. The decrease in Hcy efflux upon addition of 5-formyl-THF (text-fig. 5) is in accordance with this interpretation. Cellular depletion of reduced folates may only cause cell death under conditions of high thymidylate synthase activity, i.e., during DNA synthesis and cell division (*2, 3*).

The effect of MTX on cellular content of AdoMet and AdoHcy was determined (table 2) because alterations of Hcy metabolism may induce effects on the amounts of these adenosyl sulfur amino acids. It is well established that addition of Hcy to isolated cells increases the AdoHcy content (*17*) and in some cases the amount of AdoMet (*27*). In addition, Hilton et al. (*28*) have recently reported on effects on MTX of AdoMet content in liver of mice.

We found no effect of MTX on the amount of AdoHcy in Cl 16 cells (table 2), in spite of the fact that this drug increases the concentration of Hcy in the medium severalfold (text-figs. 4 and 5). This finding shows that the intracellular content of Hcy in Cl 16 cells exposed to MTX is kept below the level required to reverse or inhibit the AdoHcy hydrolase reaction. One may speculate whether a low concentration of Hcy in the intracellular compartment may be critical for some vital cellular function.

In conclusion, MTX induced Hcy export from cultured fibroblasts but no accumulation of intracellular Hcy, as judged by no build-up of AdoHcy. Therefore, Hcy efflux may be an efficient mechanism compensating for inhibition of Hcy salvage to methionine. The Hcy release into extracellular medium may be a measure of insufficient amount of 5-methyl-THF relative to the metabolic demand. Exploitation of Hcy efflux in monitoring MTX effects, role of Hcy in long-term effects of MTX (*29–31*), and altered MTX response under conditions of altered Hcy metabolism (*16, 17*) are subjects that obviously deserve further attention.

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