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High *in vivo* rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine*

[tumor virus/methyltransferase/homocyst(e)ine/folic acid]

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ABSTRACT **Unlike normal cells, malignant rat and two simian virus 40-transformed human cell lines can neither grow nor survive in B_{12}- and folate-supplemented media in which methionine is replaced by homocysteine. Yet three lines of evidence indicate that the malignant and transformed cells synthesize large amounts of methionine endogenously through the reaction catalyzed by 5-methyltetrahydropteroyl-L-glutamate: L-homocysteine *S*-methyltransferase (EC 2.1.1.13). (*1*) The activities of this methyltransferase were comparable in extracts of malignant and normal cells. (*2*) The uptake of radioactive label from [5-^{14}C]methyltetrahydropteroyl-L-glutamic acid (5-Me-H_4PteGlu) was at least as great in the malignant cells as in the normals and was nearly totally dependent on the addition of homocysteine, the methyl acceptor; furthermore, 59–84% of the label incorporated by cells was recovered as methionine. (*3*) The malignant and transformed cells were unable to grow in homocysteine alone, while in the presence of otherwise limiting amounts of exogenous methionine, homocysteine greatly stimulated the growth of these cells. The minimum concentration of methionine necessary to initiate growth of normal and of malignant and transformed cells was the same in all lines, and the maximal growth rates at optimal methionine concentrations did not distinguish the normal from the malignant and transformed cells. The endogenously synthesized methionine was readily incorporated into high-molecular-weight substances by the malignant and transformed cells, indicating the absence of a generalized defect in utilization. Inoculation at high density did not allow these cells to grow on homocysteine in methionine deficient media nor did the omission of the seven nonessential amino acids affect growth, and suggests that the dependence on exogenous methionine is not due to leakage of methionine alone or with other amino acids. The requirement for exogenous methionine for growth, despite high levels of endogenous synthesis, is presently unexplained but appears to distinguish at least certain oncogenically transformed, but otherwise widely varying, cell lines from normals.**

Recently it was reported that L5178Y mouse leukemia cells neither grew nor survived in folate- and vitamin-B_{12}-supplemented media in which methionine was replaced by DL-homocysteine (1). Subsequently similar results were obtained with certain malignant mammalian cell lines, e.g., Walker-256 rat breast carcinoma (W-256) and L1210 murine lymphocytic leukemia (2). In contrast, normal rat, human, and hamster fibroblasts proliferated well under these conditions (2–5). Thus, the malignant cells are methionine auxotrophs, that is, cell lines that manifest nutritional requirements not observed in the genetically-related normal cells. These authors suggested that the methionine auxotrophy of the malignant cells might result from diminished *in vivo* activity of 5-methyltetrahydropteroyl-L-glutamate:L-homocysteine S-methyltransferase (EC 2.1.1.13), the enzyme which catalyzes the terminal reaction in methionine biosynthesis, leading to deficient endogenous methionine formation and that this diminished methyltransferase activity might in turn be due to a failure to transport cyanocobalamin (CN–Cbl) necessary for cofactor formation into the malignant cells (3).

In the present studies we show that two simian virus 40 (SV40)-transformed human cell lines as well as Walker-256 carcinoma cells, which require exogenous methionine for growth, all synthesize methionine from homocysteine *in vivo* at rates at least as high as those in normal cells which grow well in homocysteine-containing, methionine-deficient medium. Further, the newly synthesized methionine is incorporated into high-molecular-weight substances in the malignant and transformed cells. Our results indicate that the methionine auxotropy of the malignant cells does not result simply from the inability to synthesize and incorporate methionine from homocysteine and 5-methyltetrahydropteroyl-L-glutamic acid (5-Me-H_4PteGlu) *in vivo*. A review of this and related folate-pathway reactions is available (6).

MATERIALS AND METHODS

The normal human skin fibroblast strains MGF316 and MGF323 were obtained and grown by standard methods (7). The SV80 (8) and W18VA2 (9) cell lines are simian virus 40-transformed human skin fibroblasts kindly provided by Drs. D. M. Livingston and P. H. Black. The Walker-256 (W-256) rat breast carcinoma line (2) was obtained from the American Type Culture Collection, Rockville, Md.

Cells were routinely grown in Eagle's minimal essential medium (MEM, Gibco) supplemented with 1.5 μM hydroxocobalamin (OH-Cbl) (Schwarz/Mann) and 15% fetal calf serum (Microbiological Assoc.). For growth experiments, test media consisted of MEM lacking methionine but containing 1.5 μM OH-Cbl, 0.1 mM folic acid (Sigma), and supplemented with 10% dialyzed fetal calf serum. Where indicated, various amounts of homocysteine in the form of DL-homocysteine-thiolactone-HCl (Sigma) were also added. The concentrations indicated for homocysteine are those for L-homocysteine only. Cells were grown in duplicate 100 mm petri dishes containing 10 ml of media and refed on day 5 and every 2 days thereafter or daily where indicated. Cells were enumerated in a Coulter counter.

For uptake experiments, 5 to 8 $\times$ 10^5 cells were inoculated into 60 mm petri dishes with 5 ml of MEM containing 1.5 μM OH-Cbl and supplemented with 15% fetal calf serum. Two days later the dishes were washed three times with 2 ml of warm medium which consisted of MEM but lacked serum, folic acid, homocysteine, and methionine. The final wash media were replaced with 2 ml of a solution consisting of: 1.6 ml of MEM

Abbreviations: 5-Me-H_4PteGlu, 5-methyltetrahydropteroyl-L-glutamic acid; CN-Cbl, cyanocobalamin; OH-Cbl, hydroxocobalamin; MEM, Eagle's minimal essential medium.

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containing 1.5 μM OH-Cbl and 0.90 μCi of [5-^{14}C]Me-H_4-PteGlu (57.7 Ci/mol, Amersham) but lacking methionine and folic acid; 0.2 ml of dialyzed fetal calf serum; and 0.2 ml of Dulbecco's phosphate-buffered saline containing the amount of homocysteine necessary to give the final concentration indicated. The cells were incubated at 37° for 26 hr after which the dishes were washed quickly four times with 2 ml aliquots of ice-cold Hanks' balanced salt solution (Gibco). The cells were dissolved in 1.0 ml of 0.1 M NaOH in 2% Na_2CO_3, and a 0.5 ml portion was added to 10 ml of Aquasol (New England Nuclear) which contained 5% acetic acid, 8% water and 0.2 ml of 1 M KH_2PO_4. The radioactivity was measured in a Beckman liquid scintillation spectrometer. An additional aliquot of the dissolved cells was used for protein determination (10).

For gel filtration and amino acid analyses, 10^6 cells were inoculated into 100 mm petri dishes and initial procedures followed were the same as in the uptake experiment except that all volumes were scaled up 2.5-fold. After the 26 hr incubation in the [5-^{14}C]Me-H_4PteGlu-containing medium, the cells were washed with ice-cold Hanks' solution and were processed differently for the two types of analyses. For amino acid analysis, the cells were removed from the surface of the dish by scraping into 1 ml of 6 M HCl. The contents of each dish were then transferred to a glass ampule which was evacuated, flushed with N_2, and sealed. The cells were hydrolyzed by heating at 100° overnight and the amino acid compositions were measured with a Beckman amino acid analyzer. The fractions eluted from the analyzer were counted in the above scintillation solution.

For gel filtration analysis, the cells were scraped from the surface of the dish into 1 ml of 25 mM Na phosphate buffer at pH 7, containing 11 mM of ascorbate, and frozen at −20°. The cells were thawed, disrupted by sonication (Raytheon model DF101), and, after centrifugation at 100,000 × g for 1 hr, 0.6 ml of the supernatant was applied to a 0.9 × 30 cm Sephadex G-25 column equilibrated with the above Na phosphate-ascorbate buffer. Ten drop fractions of the eluate were collected and counted in the above scintillation solution.

RESULTS

When homocysteine replaced methionine in folate- and OH-Cbl-supplemented media, the normal human fibroblasts MGF323 and MGF316 grew throughout the 2–3 week test period, while growth of W-256 cells and of the two virus-transformed lines ceased after the initial 0.2–1.5 divisions (Fig. 1). The methionine auxotrophy of W-256 cells under these conditions was first reported by Halpern *et al.* (2, 3), but the corresponding methionine auxotrophy in cells known to be transformed by tumor virus has not previously been described. In additional experiments (data not shown), the malignant and transformed cells were unable to grow when the homocysteine medium was supplemented with 0.1 mM hypoxanthine and 20 μM thymidine, two other important metabolites which, like methionine, require folates for their biosynthesis; nor did these cells grow when supplemented with 10 μM 5-Me-H_4-PteGlu (with 50 μg/ml of Na ascorbate as a reducing agent), a high concentration which should overcome any deficiency of cellular 5-Me-H_4-PteGlu which might produce a methionine dependence.

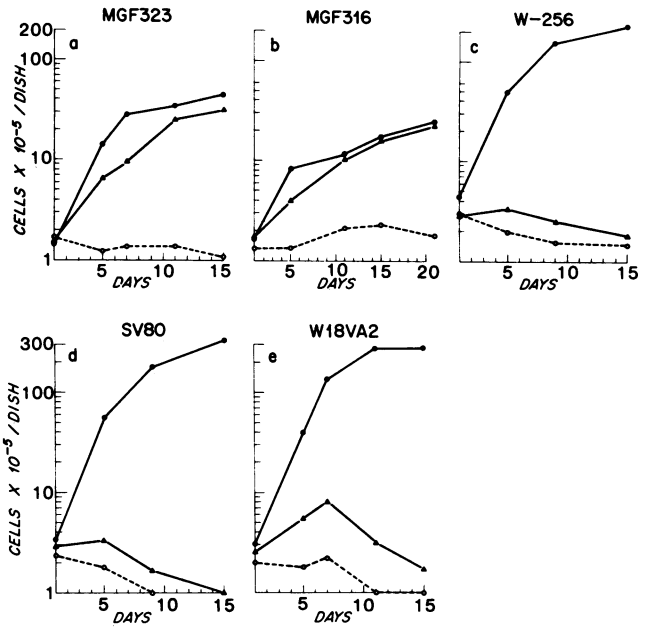

FIG. 1. Growth of normal, malignant and transformed cells in OH-Cbl-supplemented media with or without methionine. Cells were inoculated and grown in 100 mm petri dishes containing 10 ml of MEM lacking methionine and supplemented with 10% dialyzed fetal calf serum and either 0.1 mM L-methionine plus 7.5 μM OH-Cbl (●) or 0.1 mM homocysteine plus 7.5 μM OH-Cbl (▲) or 7.5 μM OH-Cbl alone (○). Cells in duplicate dishes were counted on the days indicated as described in *Materials and Methods*.

5-Methyltetrahydropteroyl-L-glutamate:L-homocysteine S-methyltransferase activity was measured in extracts of the different cells grown in cobalamin-deficient or OH-Cbl-supplemented media and with or without CN-Cbl added to the assay reaction mixture (Table 1). Under none of these conditions do the methyltransferase activities distinguish the malignant and transformed cells as a group from the normal lines. The proportion of the methyltransferase activity present as holoenzyme in extracts of cells grown in OH-Cbl-supplemented MEM was higher in the normal cells than in the malignant and

Table I. Effects of cobalamin *in vivo* and *in vitro* on 5-methyltetrahydropteroyl-L-glutamate:L-homocysteine *S*-methyltransferase activity in extracts of normal, malignant, and transformed cells

Growth media*	Assay conditions CN-Cbl	Methyltransferase activity in cell extract† (nmol of methionine formed per mg of protein) MGF323	MGF316	W-256	SV80	W18VA2
MEM + OH-Cbl	+	4.93	12.3	4.75	9.14	6.92
	−	3.72	10.4	1.96	4.12	3.27
MEM	+	0.78	2.29	0.48	2.53	1.75
	−	0.13	1.31	0.12	0.33	0.03

* Cells were grown to confluence in roller bottles with 15% fetal calf serum in MEM that either contained 1.5 μM OH-Cbl or lacked cobalamin.
† 5-Methyltetrahydropteroyl-L-glutamate:L-homocysteine *S*-methyltransferase activity in the extracts was measured as described previously (4) except that the 50 μM CN-Cbl present in the standard reaction mixture was omitted where indicated.

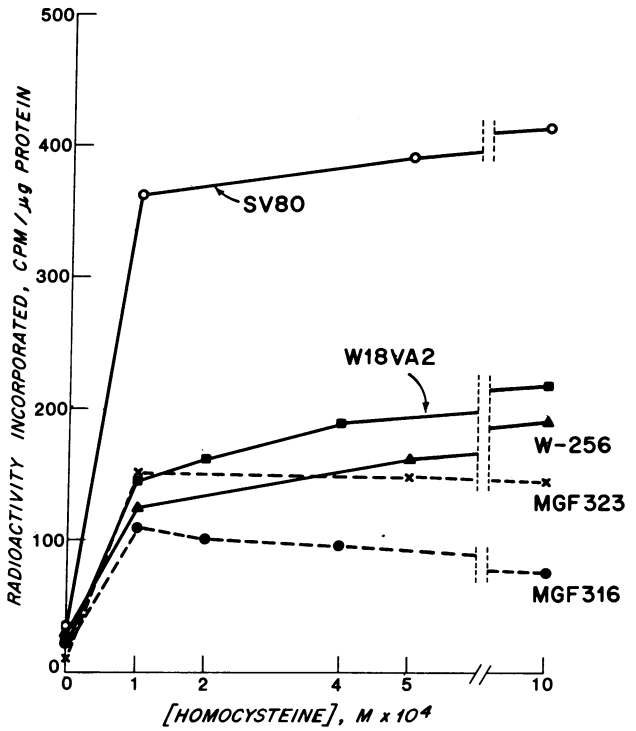

FIG. 2. Effect of homocysteine on the cellular uptake of radioactive label from [5-^{14}C]Me-H_4PteGlu. The medium in which the cells were incubated contained homocysteine at the concentration indicated on the abscissa but lacked methionine. After 26 hr, intracellular radioactivity was measured as described in *Materials and Methods*. Each point is the average of two determinations. Dashed lines, normal fibroblast strains. Solid lines, malignant and transformed cell lines. (○) SV80; (■) W18VA2; (▲) W-256; (×) MGF323; (●) MFG316.

transformed cells as was reported previously (3). Methyltransferase activities in extracts of the two normal strains assayed in the absence of added cobalamin were 75% and 85% of the respective activities when CN-Cbl was added *in vitro*, while W-256 had 41% holoenzyme and SV80 45% and W18VA2 47%, respectively. However, the methyltransferase activity levels of all cell types were substantially greater when the cells were grown in OH-Cbl-supplemented medium than in cobalamin-deficient medium, the increment ranging from 3.6- to 10-fold, indicating that relatively large amounts of OH-Cbl were entering the cells.

When incubated in OH-Cbl-supplemented media lacking both methionine and folic acid, all cell types took up radioactive label added to the medium as [5-^{14}C]Me-H_4-PteGlu, and this uptake was markedly stimulated by homocysteine, the methyl group acceptor in methionine biosynthesis (Fig. 2). Surprisingly, the rate of uptake in the malignant and the two transformed lines was high and in the case of SV80 cells was substantially higher than in either normal cell line. When the growth media contained 0.1 mM homocysteine, the uptake of label from [5-^{14}C]Me-H_4-PteGlu by four of the cell types increased to between 4.2- and 6.5-fold greater levels than the corresponding uptake seen when homocysteine was omitted from the growth medium; an increase of 10.6-fold was seen for SV80 cells. Increasing the concentration of homocysteine in the culture medium from 0.1 to 1 mM resulted in a small progressive increase in the uptake of label from [5-^{14}C]Me-H_4-PteGlu by the malignant and transformed lines, while the uptake by the two normal cell lines decreased slightly.

Amino acid analysis of whole cell hydrolyzates indicated that much of the label taken up by both normal and malignant cells was incorporated into methionine (Table 2). While the fraction of label recovered as cellular methionine was somewhat lower in W-256, the fraction recovered as methionine was higher in the two transformed lines than in either of the normal lines. Thus, the cellular uptake of radioactive label from [5-^{14}C]-Me-H_4PteGlu reflects *in vivo* methionine synthesis. The malignant and virus-transformed cells which manifest a stringent requirement for exogenous methionine for growth nonetheless synthesize at least as much methionine *in vivo* as do normal cells which grow well when methionine is replaced by homocysteine.

Table 2. *In vivo* incorporation of label from [5-^{14}C]Me-H_4PheGlu into cellular methionine

Cell line*	Percentage of label from [5-^{14}C]Me-H_4PheGlu recovered as [^{14}C]methionine†
MGF323	76
MGF316	68
W-256	59
SV80	84
W18VA2	83

* MGF323 and MGF316 were grown in medium containing 0.1 mM homocysteine. W-256, SV80, and W18VA2 were grown in medium containing 1 mM homocysteine.

† Results are expressed as the percentage of total radioactivity in the acid hydrolyzates of cells applied to the amino acid analyzer column recovered as [^{14}C]methionine in the peak which cochromatographed with the L-[*methyl*-^{3}H]methionine standard.

While the malignant cells were unable to grow on homocysteine alone, they grew rapidly on homocysteine in the presence of otherwise limiting amounts of methionine. In normal cells, homocysteine and small amounts of methionine had an additive effect (Fig. 3a). MGF323 grew slowly at a methionine concentration of 1 μM, with a doubling time of about 5 days. In medium containing 100 μM homocysteine, the cells grew more rapidly while, in medium containing both, the growth was approximately the sum of the rates in either com-

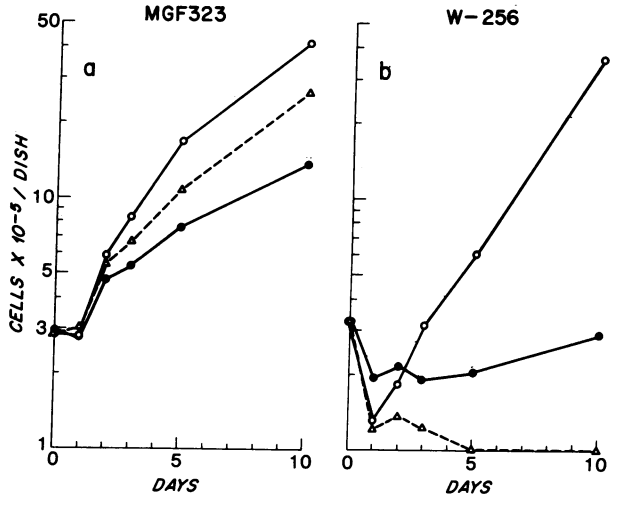

FIG. 3. Effect of a limiting concentration of methionine on the ability of normal and malignant cells to grow on homocysteine. Normal human skin fibroblasts (a) and W-256 rat breast carcinoma cells (b) were grown and counted as described in Fig. 1 with the following differences: Cells were refed daily with media as indicated supplemented with penicillin-streptomycin (Microbiological Assoc.). ○—○, 1 μM L-methionine + 100 μM L-homocysteine; △- - -△, 100 μM L-homocysteine; ●—●, 1 μM L-methionine.

ponent alone. In contrast, the presence of a small amount of methionine resulted in disproportionately greater growth of the malignant cells (Fig. 3b). W-256 cells were, as before, unable to grow on homocysteine and grew very slowly in the presence of 1 μM methionine. In medium containing both, however, W-256 cells grew rapidly, with a doubling time of about 2 days. A similar permissive effect, but of smaller magnitude, was observed in W18VA2 and SV80 cells.

Even at methionine concentrations above a limiting amount, homocysteine exerted a growth-stimulating effect. At methionine concentrations ranging from 1 μM, which is distinctly limiting, to as high as 50 μM, the addition of 100 μM homocysteine substantially increased the extent of W-256 cell growth in the 10-day period of observation.

Folate repletion studies yielded further evidence of high *in vivo* methyltransferase activity. Cells were depleted of folates by growth in folate-deficient medium containing 0.1 mM methionine until division ceased. When present as the sole folate source, 1–100 μM 5-Me-H_4-PteGlu was as effective as folic acid in restoring normal growth rates in normal fibroblasts and W-256 carcinoma cells. In catalyzing the reaction from homocysteine to methionine, the methyltransferase also converts 5-Me-H_4PteGlu into H_4PteGlu, the latter being necessary for many other essential folate-dependent reactions. The fact that 5-Me-H_4PteGlu and folic acid are interchangeable indicates that the activity of this enzyme is sufficiently high that it is not rate limiting for growth when the cells must depend on its 5-Me-H_4PteGlu substrate as the sole source of folates.

The methionine requirement of the malignant and transformed cells, despite high levels of endogenous methionine synthesis, might be expected if these cells grew more rapidly or simply required greater amounts of methionine for growth. Growth rates of normal and malignant cells were compared over a range of methionine concentrations in media lacking homocysteine. At 1 μM methionine, the normal fibroblasts grew slowly, the two transformed lines grew more slowly and the W-256 cells survived without appreciable growth. The growth of the transformed cells and survival of the W-256 cells at this very low exogenous methionine concentration contrasts markedly with the rapid loss of viability in homocysteine from which the studies described above have demonstrated substantial amounts of endogenous methionine synthesis. Furthermore, the fact that all the cell lines initiate growth, or survive in the case of W-256, at this low methionine concentration indicates that the normal, transformed and malignant cells do not differ markedly in their minimum exogenous methionine requirements. The growth rates of the two normal lines and the two transformed lines correspond closely over the whole range of methionine concentrations tested. The maximal growth rates observed with the various lines do not distinguish the normal from the transformed and malignant cells. These results suggest that the inability of transformed and malignant cells to grow when homocysteine replaces methionine is due neither to a more rapid growth rate nor to an abnormally high threshold in the minimum amount of methionine required for growth.

In order to test for a leakage of methionine, the transformed and malignant cells were seeded at high density where cross-feeding may overcome cellular leakage (12). When 2×10^6 cells per 100 mm petri dish were inoculated into methionine-containing media, the W-256, SV80, and W18VA2 cells all grew quite rapidly and doubled three to four times. In contrast, these lines at the same initial density inoculated into methionine-deficient media which contained 0.1 mM homocysteine survived for a few days while cell numbers increased only very slightly (0.1–0.3 doubling), then ceased to divide and died.

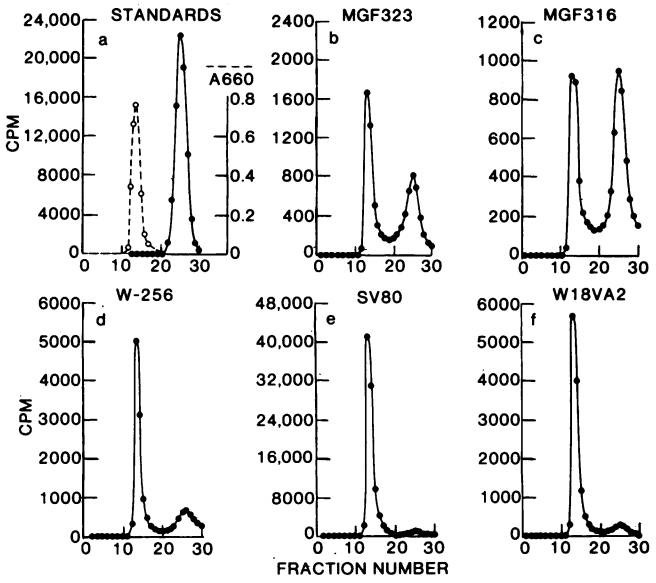

FIG. 4. Distribution of radioactive label taken up from [5-^{14}C]-MeH_4PteGlu into soluble low- and high-molecular-weight fractions. High-speed supernatants of sonicates from cells incubated in medium containing [5-^{14}C]MeH_4PteGlu and homocysteine but lacking methionine and folic acid were prepared and analyzed by gel-filtration chromatography as described in *Materials and Methods*. (a) Standards consist of blue dextran (O) to indicate the fractions of the Sephadex G-25 column eluate containing high molecular weight (>5000) substances and L-[*methyl*-^{3}H]methionine (●) to indicate the fractions of the eluate containing methionine.

Evidence against a more generalized leakage of amino acids was provided by the observation that the growth of the malignant and transformed cells in media containing both methionine and the nonessential amino acids did not differ in rate or extent from their growth in media containing methionine but lacking the seven nonessential amino acids (alanine, asparagine, aspartic acid, glutamic acid, proline, serine, and glycine). Thus, these seven amino acids are not only presumably synthesized by the cells but also are retained within the cells in amounts sufficient to support normal growth.

The postribosomal supernatant fractions from sonicates of the [5-^{14}C]MeH_4PteGlu-labeled cells were analyzed by gel filtration column chromatography to compare the proportions of the intracellular radioactivity present in the high- and low-molecular-weight fractions (Fig. 4). The peak of radioactivity eluting in fractions 20–30 reflects the intracellular pool of free methionine, while the high-molecular-weight peak in fractions 11–18 includes newly-synthesized methionine which has been incorporated into proteins as well as radioactive nucleic acids and other substances to which the [^{14}C]methyl group of methionine was transferred through *S*-adenosyl-L-methionine. In the normal fibroblasts (Fig. 4b and c), the amounts of radioactivity in the two peaks were similar, the ratio of high- to low-molecular-weight radioactivity being 1.1 in MGF323 and 0.6 in MGF316. In contrast, W-256 (Fig. 4d) showed much greater incorporation of radioactivity into the high- than into the low-molecular-weight peak (ratio 2.7), with the preponderance of the high-molecular-weight peak being even greater in the transformed cells, namely, ratios of 17 in SV80 and 7 in W18VA2 (Fig. 4e and f). Although it is not possible to quantitate the absolute amounts of free intracellular methionine from these data, it is clear that the malignant and transformed cells, in spite of being unable to grow under these conditions, are incorporating the newly-synthesized methionine extensively into

high-molecular-weight substances either directly or via transmethylation.

DISCUSSION

The results of the present studies indicate that the malignant and transformed cells, although methionine auxotrophs, do synthesize methionine endogenously from 5-MeH$_4$PteGlu and homocysteine at rates which are at least as high as in normal cells. Three lines of evidence indicate high *in vivo* activity of the methyltransferase in these cells. First, the levels of 5-methyltetrahydropteroly-L-glutamate:homocysteine *S*-methyltransferase activity measured directly in extracts of cells grown in the presence or absence of OH-Cbl varied between the different strains and lines but did not distinguish the normal from the malignant or transformed cells in regard to total methyltransferase activity. Second, the uptake of radioactive label from [5-^{14}C]Me-H$_4$PteGlu, the methyl donor in methionine biosynthesis, was greatly stimulated in both normal and malignant or transformed cells by homocysteine (Fig. 1), the methyl group acceptor in methionine biosynthesis. The validity of this approach to measuring endogenous methionine biosynthesis was confirmed by the observation that in all the cell types a majority of the radioactive label taken up from [5-^{14}C]Me-H$_4$PteGlu was recovered as methionine as measured by amino acid analysis of hydrolyzates of whole cells.

Third, in the presence of otherwise limiting amounts of exogenous methionine, homocysteine markedly stimulated the growth of the malignant and transformed cells. The magnitude of the growth stimulation in these cells was disproportionately large when compared with the additive effect seen in the normal cells. The results suggest that in the malignant and transformed cells the small amount of exogenous methionine complements the high level of endogenous synthesis to fulfill one or more functions necessary for rapid growth.

Furthermore, the equal effectiveness of 5-Me-H$_4$PteGlu and folic acid in repleting folate-starved malignant cells is further evidence of high endogenous 5-methyltetrahydropteroyl-L-glutamate:L-homocysteine *S*-methyltransferase activity. Recent studies have indicated that 5-Me-H$_4$PteGlu can also be used to some extent to form formaldehyde through a reaction catalyzed by methylene-H$_4$PteGlu reductase (11). However, both the fact that little label was incorporated from [5-^{14}C]Me-H$_4$PteGlu in the absence of homocysteine (Fig. 2) and the recovery of the majority of the incorporated label in methionine (Table 2) suggest that cellular utilization through reactions other than that catalyzed by this methyltransferase must be quite small.

In addition to synthesizing methionine endogenously, the malignant and transformed cells incorporated a substantially greater proportion of this methionine into high-molecular-weight substances, and provides evidence that they are not prevented from utilizing this methionine for at least some cellular functions. Why, then, are the malignant and transformed cells unable to grow in the homocysteine-containing, methionine-deficient media? Our current hypothesis is that there are one or more specific functions in the malignant and transformed cells that can be fulfilled only by exogenous methionine.

It should be noted that the results of these studies differ in important respects from those reported previously by Halpern *et al.* (2, 3) and suggested as a basis for possible therapy of malignancy (13).

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