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Methionine Metabolism in Walker Carcinosarcoma *In Vitro**

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Abstract—*When homocysteine* (0.1–0.3 mM) *replaced methionine in media supplemented with folic acid* (0.01–0.1 mM) *and hydroxocobalamin* (0.1–7.5 μM) *there was no growth of Walker carcinoma in tissue culture. These cells were also unable to grow when supplemented with* 5-*methyl* (10–50 μM) *or* 5-*formyltetrahydrofolic acid* (10–40 μg/ml), *S-adenosyl-*L-*methionine* (200 μM), *S-methylcysteine* (0.05–0.5 mM), 5-*deoxy*-5′-*methylthioadenosine* (0.05–0.5 mM), *S*-(*methanethiol*)-L-*cysteine* (0.1–1.0 mM), L-*cystine* (0.1–1.0 mM) *or with added spermine* (0.2–1.0 μg/ml) *plus spermidine* (0.15–0.75 μg/ml). *The inability of Walker carcinoma to grow in methionine deficient media was not due to a loss of* 5-*methyltetrahydrofolate:* L-*homocysteine S-methyl transferase since not only was the activity of this enzyme comparable with that found in TLX5 lymphoma, which proliferated in methionine-deficient media supplemented with homocysteine, but there was also a six-fold induction of enzyme activity during* 48 hr *incubation in such deficient media. Furthermore, the activity of the methyltransferase was proportional to the methionine concentration in the medium. There was no appreciable alteration in the number of surface sulphydryl groups in homocysteine supplemented media. This suggests that the inability of Walker carcinoma to proliferate in methionine-depleted, homocysteine-supplemented media is not due to any inate biochemical defect and may be due to a higher methionine requirement of this cell line.*

INTRODUCTION

RECENT studies have shown a difference in the growth requirements between some normal and neoplastic cell lines growing in tissue culture. Normal cells were able to grow in a medium containing either methionine or homocysteine, folic acid and vitamin B_{12} (cyano-B_{12}), but malignant cells required the presence of pre-formed methionine in the medium for growth. The transformed and tumour lines whose growth is impaired by the substitution of homocysteine for methionine include L5178Y murine leukaemia cells [1], Walker 256 rat mammary carcinoma [2], L1210 murine lymphocytic leukaemia [2], SV80 and W18VA2 simian virus 40-transformed human skin fibroblasts [3], SV40-transformed BHK-21 cells [4] and transformed rat liver epithelial cells [5]. In contrast fibroblasts from human skin [3] prostate [2], breast [2] and normal rat [2] and hamster fibroblasts [4] proliferated normally in methionine-deficient, homocysteine-supplemented media.

Although the reason for this methionine auxotrophy of malignant cells is not clear the inability of SV40-transformed BHK-21 cells to proliferate in methionine deficient media appears to be due to a lack of 5-methyltetrahydrofolate ($5CH_3FH_4$) due to a low level of 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68)[4]. Also *S*-adenosylmethionine: homocysteine methyltransferase, which catalyses synthesis of methionine from homocysteine, with the use of *S*-adenosylmethionine (SAM) as methyl donor, is absent in hepatoma cells [6]. In Walker-256 mammary carcinoma the inability to utilize homocysteine in lieu of methionine was suggested to arise from a diminished activity of 5-methyltetrahydropteroyl-L-glutamate: L-homocysteine *S*-methyltransferase (EC 2.1.1.13) the enzyme which catalyses the terminal reaction in methionine biosynthesis [7] (Fig. 4). Hoffman and Erbe [3], however, showed that Walker 256 carcinoma cells synthesized

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methionine from homocysteine at rates as high as those in normal cells.

In the present study the effect of various factors on survival and growth rate in homocysteine-supplemented, methionine deprived cultures of Walker 256 carcinoma and TLX5 lymphoma has been investigated, as has the effect of such culture conditions on the ability to synthesize methionine.

MATERIALS AND METHODS

[Methyl-^{3}H] thymidine (sp. act. 5.0 Ci/mmole), 5-[^{14}C] methyltetrahydrofolic acid, barium salt (sp. act. 58 mCi/mmole) and L-[methyl-^{3}H] methionine (sp. act. 17.0 Ci/mmole) were purchased from the Radiochemical Centre, Amersham. Folic acid (pteroylglutamic acid), 2′-deoxyuridine, cyanocobalamin (cyano-B_{12}) and L-homocysteine thiolactone hydrochloride were obtained from Sigma Chemical Co., London. Dulbecco's modified Eagle's medium lacking methionine and folic acid was from GIBCO Ltd., London. *S*-Adenosyl-L-methionine was purchased from the Boehringer Corp., London and hydroxocobalamin (aquocobalamin OH-B_{12}) from B.D.H., Poole, Dorset.

Methionine was removed from foetal calf serum (Gibco) by dialysis. *S*-(Methanethiol-L-cysteine) was prepared according to the method of Smith *et al.* [8].

Cell culture

Cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and gassed with 10% CO_2 in air. For growth experiments, test media consisted of modified Eagle's medium containing 7.5 μM hydroxocobalamin (OH-B_{12}) 0.1 mM folic acid and supplemented with 10% dialysed foetal calf serum. Cells were grown in duplicate wells (3.5 ml) of a 24-well plastic plate (Flow Laboratories, Scotland). Cell number was enumerated every 24 hr with a Coulter Counter model F_B. Cell viability was assessed by the inability to take up 0.4% trypan blue and cell numbers were determined with a haemocytometer.

Determination of 5-methyltetrahydrofolate: L-homocysteine methyltransferase

Methyltransferase activity was determined by measurement of the formation of [^{14}C] methionine from [5-^{14}C] methyltetrahydrofolate [9]. The reaction mixture contained in a total volume of 0.2 ml: 100 mM phosphate buffer (pH 7.4), 125 mM 2-mercaptoethanol, 250 μM SAM, 50 μM cyano-B_{12}, 250 μM L-homocysteine (prepared immediately before use from the thiolactone derivative by adding 1 NKOH and neutralising with 1 NHCl), 250 μM [5-^{14}C] CH_3FH_4 (3 μCi/assay) and cell extract (c.a.1 mg protein). The reaction mixtures were incubated for 1 hr at 37°C under a N_2 atmosphere and the reaction was terminated by addition of 0.8 ml of ice-cold water. The reaction mixtures were passed through a Dowex 1-Cl column, (0.5 × 3.0 cm; 100–200 mesh) which retained the [5-^{14}C] CH_3FH_4, followed by 1.8 ml of water and the [^{14}C] methionine in the pooled effluent was measured in 20 ml of PCS scintillation fluid (Hopkin and Williams). Activity was expressed per mg of cell protein, determined by the method of Lowry *et al.* [10], using bovine serum albumin as a standard.

In vivo levels of methyltransferase were determined by measuring the reversal of the methotrexate effect on deoxyuridine (dU) suppression of [^{3}H] thymidine (^{3}H-TdR) incorporation into DNA [11] (Fig. 4). Effective *de novo* synthesis of thymidylate (dTMP) from dU in intact Walker cells was measured by the ability of co-incubation with dU (10 or 1000 μM) to suppress incorporation of ^{3}HTdR (5 μCi/ml) into DNA during a 1 hr pulse at 37°C [11]. Cells were washed onto glass fibre filters (GF/C Whatman) with 0.9% NaCl solution, and the filters were washed with ice-cold 5% trichloroacetic acid, 0.9% NaCl and ethanol. The filters were dried at 70°C for 2 hr and the radioactivity on the discs was determined in a toluene/PPO scintillation fluid. Methotrexate, when present, was at a concentration of 1 μg/ml.

Determination of surface sulphydryl groups

Washed cells (5×10^7) were incubated at 25°C for 5 min with 6,6′-dithiodinicotinic acid (CPDS 3 μmole) in a total volume of 3 ml of Krebs–Ringer phosphate buffer, pH 7.2 [12]. The gas phase was air. After the incubation the cells were separated by centrifugation (800 *g* for 5 min) and the supernatant fluid was filtered through a Whatman GF/C glass fibre disc. The absorbance of the filtrate at 344 nm was determined against a blank containing the initial concentration of CPDS in an equal volume of buffer, from which the number of surface sulphydryl groups was calculated using ε_{344} 1.00×10^4 for 6-mercaptonicotinic acid [13].

Uptake of methionine

Cells (2×10^7/ml) were allowed to incubate at 37°C in a calcium-free Krebs–Ringer phosphate solution for 5 min prior to the addition of [^{3}H] methionine (0.2 μCi/μmole; final concentration of methionine 2 mM). The cells were shaken to avoid clumping and aliquots (2 ml) were taken out at time intervals. The samples were added to 3 ml ice-cold Krebs–Ringer solution, centrifuged; the supernatant was removed and the cells were resuspended in a further 5 ml of ice-cold Krebs–Ringer solution. The suspension was centrifuged again, the supernatant removed, and the interior of the tubes was dried with paper tissue. To the washed cells was than added 2 ml of 95% ethanol and a minimum period of 30 min was allowed for completion of extraction of the alcohol-soluble materials. After centrifugation 3 ml of the supernatant was counted in a toluene/PPO scintillation mixture.

RESULTS

The following growth regimens were unable to support proliferation of Walker carcinoma in Dulbecco's modified Eagle's medium, lacking methionine, but supplemented with homocysteine (0.1–0.33 mM), folic acid (0.01–0.1 mM) and OH.B_{12} (0.1–7.5 μM).

(a) Addition of 5-CH_3FH_4 (10–50 μM, with sodium ascorbate as a reducing agent) which should overcome any cellular deficiency which might produce a methionine dependence, or with L-serine (0.1 mM) which also contributes methyl groups for 5-CH_3FH_4 biosynthesis (Fig. 4).

(b) Supplementation with 5-formyltetrahydrofolic acid (folinic acid) (10–40 μg/ml), a source of reduced folate.

(c) In the presence of a high extracellular concentration of SAM (200 μM), a stimulator of 5-CH_3FH_4: L-homocysteine *S*-methyltransferase [14].

(d) Addition of spermine (0.2–1.0 μg/ml) plus spermidine (0.15–0.75 μg/ml) which act as growth factors for both micro-organisms and mammalian cells [15].

(e) In the presence of L-cystine (0.1–1.0 mM) which exerts a methionine sparing effect [16] or with the methylthio compounds *S*-methylcysteine (0.05–0.5 mM), 5-deoxy-5′-methylthioadenosine (0.05–0.5 mM) or *S*-(methanethiol)-L-cysteine (0.1–1.0 mM).

No growth was observed when homocysteine thiofactone substituted for homocysteine, or by daily supplementation with extra homocysteine since the latter has a short half life (0.375 hr at pH 7.4). The stability of homocysteine is much greater in media of low pH (the half life is increased from 0.65 hr at pH 7.0 to 1.5 hr at pH 6.5), although altering the pH of the media had no effect on the proliferative capacity of Walker cells in methionine-deficient medium. Cells in such methionine-deficient media not only loose the ability to proliferate, but also have a decreased viability compared with cells in normal media, which was evident within 3 hr of the medium change and increased steadily with time (Table 1). There was no significant effect on the number of surface sulphydryl groups in homocysteine supplemented medium as measured by reaction with 6,6′-dithiodinicotinic acid (Table 1).

Table 1. Effect of methionine deprivation on the viability and number of surface sulphydryl groups in Walker carcinoma

Conditions	Viability (% of control)	Formation of 6-mercaptonicotinic acid (thione) molecules/cell × 10^8
Normal medium + 0.1 mM homocysteine	100	7.04
Methionine deprived medium + 0.1 mM homocysteine		
t 1.5 hr		7.42
t 2.5 hr	91	9.82
t 2.5 hr		9.17
t 24 hr	66	8.3
t 48 hr	55	6.64
t 72 hr	49	7.06

*Supplemented with 0.5 mM homocysteine. Methionine deprived cultures were also supplemented with 0.1 mM folic acid and 7.5 μM OH-B_{12}.

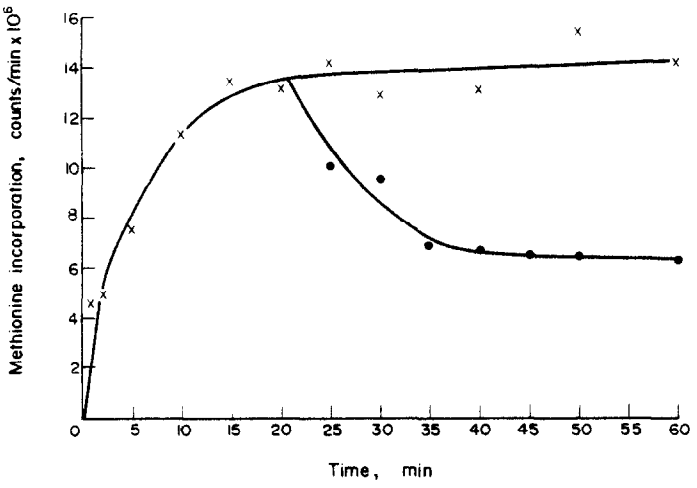

Fig. 1. The effect of homocysteine on the steady state level of [^{3}H]methionine in Walker carcinoma. Cells were incubated with 2 mM methionine (× —— ×) in Krebs–Ringer phosphate solution and after 20 min homocysteine (1 mM) (● —— ●) was added directly to the incubation medium. Samples were removed at intervals throughout and the alcohol-soluble radioactivity was assayed as described in methods.

The inability to grow in methionine-deficient medium was not due to the inability of Walker cells to take up homocysteine. The results presented in Fig. 1 show that when homocysteine was added to cells which were in equilibrium with a methionine-containing medium there was a rapid efflux of methionine and the steady state level fell by almost 60%. Since two amino acids which are transported by the same system should be capable of exchange diffusion [17] this shows that homocysteine is transported by the methionine carrier system in Walker cells.

The inability to grow in methionine-deficient medium does not appear to be due to loss of methyltransferase, since activity is present using either cyano-B_{12} or methylcobalamin as the source of vitamin B_{12} (Table 2). Furthermore, the activity of the methyltransferase in both normal and methionine-depleted media is similar to that found in TLX5 lymphoma which is able to proliferate in methionine-depleted media. Addition of 0.1 mM homocysteine to medium containing folic acid, vitamin B_{12} and methionine did not alter methyltransferase activity. In contrast increasing the methionine concentration of the medium, containing homocysteine, folic acid and cyano-B_{12}, resulted in a progressive decrease in methyltransferase activity of Walker carcinoma (Fig. 2). Such a decrease of methyltransferase activity with increasing methionine concentration is also observed with BHK cells [9] which show no methionine requirement [4]. As the methionine concentration was increased from the normal level of 2×10^{-7} M to 10^{-4} M the sp. act. of both the holoenzyme (assayed in the absence of

*Table 2. Activity of 5-methyltetrahydropteroyl-L-glutamate: L-homocysteine S-methyltransferase in extracts of Walker carcinoma and TLX5 lymphoma**

Growth conditions	Assay conditions (Cyano-B_{12})	Methyltransferase activity (nmole of methionine formed/mg protein/hr)	
		Walker	TLX5
Normal media	−	0.99	0.52
	+	1.84	1.05
Methionine deprived media	−		3.09
(+0.1 mM folate)	+		4.12
Methionine deprived media (+0.1 mM folate + 7.5 μM OH-B_{12} +0.3 mM homocysteine)	−	6.1	7.89
	+	7.1	9.11

*Cells were incubated in the respective media for 24 hr and enzyme activity was determined as described in methods.

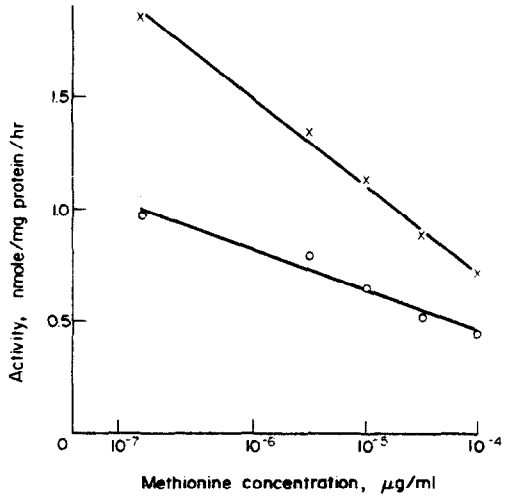

Fig. 2. Effect of methionine concentration on the activity of 5-methyltetrahydrofolate: L-homocysteine S-methyltransferase. Walker cells were taken from normal growth medium, washed with 0.9% NaCl *and resuspended in methionine-free medium containing* 0.1 mM *homocysteine,* 7.5 μM *OH-B_{12}.* 0.1 mM *folic acid and the indicated concentrations of* L-*methionine. After* 4 hr *incubation at* 37°C *the cells were washed with* 0.9% NaCl, *sonicated in assay buffer and the supernatant obtained by centrifugation at* 2000 ***g*** *for* 60 min *at* 4°C *was assayed for methyltransferase as described in methods in the absence* (○—○) *or presence* (×——×) *of cyano-B_{12}.*

cyano-B_{12}) and apoenzyme decreased by about 50%. Furthermore, in methionine-depleted medium containing homocysteine, folic acid and OH-B_{12} the activity of the methyltransferase increased about six-fold (Fig. 3). There was a very rapid increase in enzyme activity which was appreciable within 3 hr of the medium shift. Thus the activity of the methyltransferase was greatly enhanced in media in which growth inhibition and eventual cell death was observed.

Since this situation appeared paradoxical, and could possibly have arisen by activation of latent enzyme by proteases released during cell rupture, it was decided to measure the activity of the methyltransferase by an indirect method. Thymidylate (dTMP) for DNA synthesis can arise either via the salvage pathway from performed TdR or via the *de novo* pathway from deoxyuridylate (dUMP) (Fig. 4). Thus in normal medium deoxyuridine (dU) blocks the incorporation of [^{3}H] TdR into DNA due to conversion to dTMP by the *de novo* pathway [11] (Fig. 5). In the *de novo* sequence, the one-carbon unit is reduced and transferred from 5,10-methylenetetrahydrofolate (5,10-CH_2FH_4) to dUMP. This process is accompanied by the oxidation of tetrahydrofolate (FH_4) to dihydrofolate (FH_2) Methotrexate blocks *de novo* biosynthesis of dTMP by inhibiting dihydrofolate reductase [18]. Thus in the presence of methotrexate the suppression of [^{3}H] TdR incorporation into DNA by dU is much reduced. In the biosynthesis of methionine from homocysteine 5-CH_3FH_4 is converted into FH_4 which is available for *de novo* dTMP synthesis and can overcome the methotrexate block [11]. This presumably accounts for the report by Chello and Bertino [19] that the cell-killing effect of methotrexate in L5178Y cells was blocked by methionine deprivation. The results presented in Fig. 6 show a decreased ability of methotrexate to reverse the dU suppression of [^{3}H] TdR incorporation into acid-insoluble material when Walker cells were incubated in methionine-depleted media, such that after 10 hr the methotrexate effect was completely

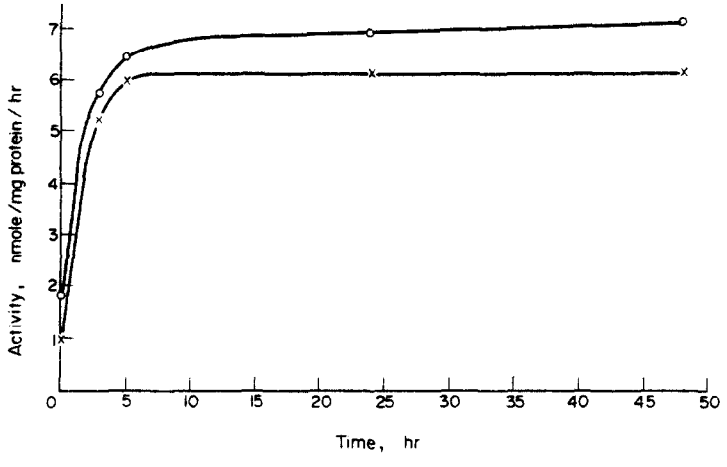

Fig. 3. Effect of methionine deprivation and homocysteine supplementation on methyltransferase activity in Walker carcinoma. After washing with 0.9% NaCl *cells were resuspended in methionine-free medium containing* 0.1 mM *homocysteine,* 7.5 μM *OH-B_{12} and* 0.1 mM *folic acid. At the indicated times cells were removed and methyltransferase activity was assayed as described in the absence* (×——×) *or presence* (○——○) *of cyano-B_{12}.*

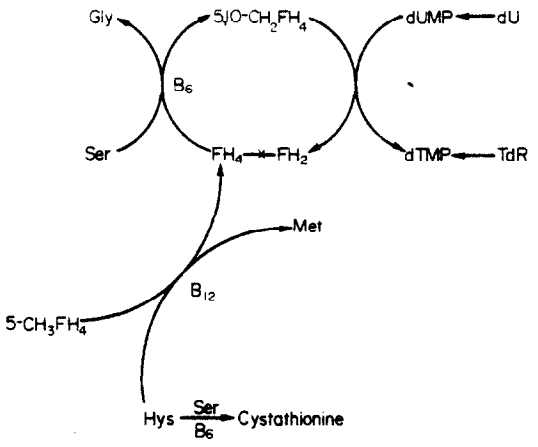

Fig. 4. Pathways of thymidylate formation (×) indicates the block by methotrexate.

abolished. This presumably reflects an increased *de novo* biosynthesis of methionine in the intact cell.

DISCUSSION

Although Jacobsen *et al.* [4] attributed the inability of SV40-transformed BHK-21 cells to proliferate in methionine-deficient medium supplemented with homocysteine and vitamin B_{12} to be due to a deficiency of 5-CH_3FH_4 we have been unable to stimulate proliferation of Walker-256 rat mammary carcinoma by addition of extracellular 5-CH_3FH_4 in confirmation of the results of Hoffman and Erbe [3]. L-Serine is required for the formation of

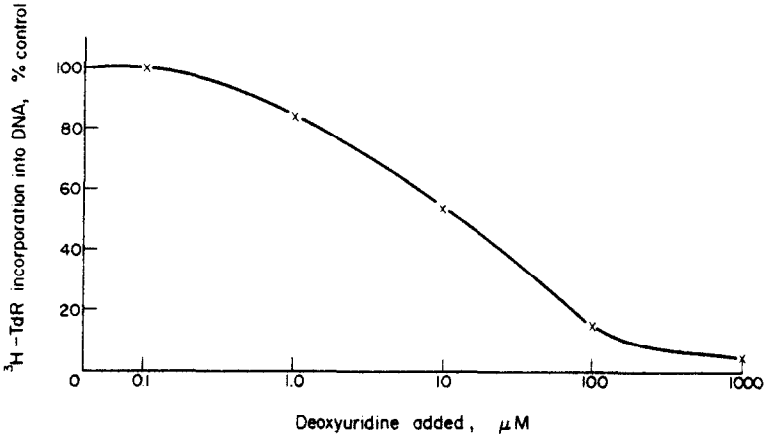

Fig. 5. Effect of preincubation of Walker cells with various concentrations of dU on [^{3}H]TdR incorporation into acid-insoluble material.

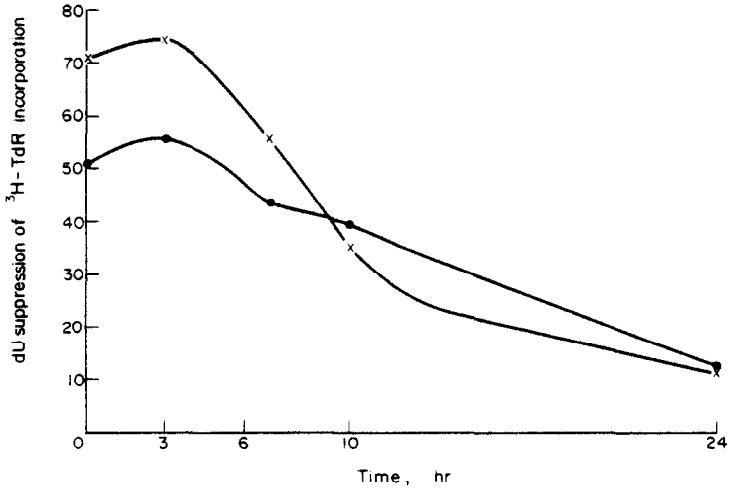

Fig. 6. Effect of methotrexate (1 μg/ml) on dU (10 μM) suppression of [^{3}H]TdR incorporation into acid-insoluble material in methionine-free medium supplemented with 0.1 mM homocysteine, 0.1 mM folic acid and 7.5 μM OH-B_{12}. The ability of dU to suppress the incorporation of [^{3}H]TdR (5-μCi/ml) in a 1 hr pulse was measured in the absence (●——●) or presence (×——×) of methotrexate.

both 5,10-CH_2FH_4 and cystathionine from homocysteine (Fig. 4) and therefore might be rate limiting in cultures deprived of methionine and supplemented with excess homocysteine. However, this amino acid also had no effect on the ability of Walker cells to proliferate in methionine-depleted media. Although the intracellular level of SAM in such methionine-deprived cultures has been shown to be much reduced [2], addition of a high extracellular concentration of SAM had no effect on growth even though exogenous SAM has been shown to be taken up by isolated rat liver [21] and rabbit erythrocytes [22]. This may reflect a difference in the uptake mechanism between tumour cells and normal cells. The polyamines spermine and spermidine are growth factors which are biosynthesized from SAM by SAM decarboxylase (EC 4.1.1.50) [15]. A cellular depletion of SAM could therefore result in a lowered concentration of these polyamines, which in turn could result in the inability to proliferate in methionine-deficient media. Addition of extracellular spermine and spermidine, however, had no effect on the ability of Walker cells to grow under conditions of methionine deficiency. Although L-cystine has been reported to exert a methionine sparing effect [16], no effect of L-cystine was observed in the present experiments. Certain malignant mammalian cells require methylthio groups for division *in vitro* [23] and such methylthio-dependent cells can be maintained in culture by supplementing the medium with small disulphides of the type R-S-S-CH_3, such as *S*-(methanethiol)-L-cysteine or methylthioadenosine. Since the concentration of methylthioadenosine in Walker carcinoma cells in methionine-deprived, homocysteine-supplemented media is likely to be low, due to the low level of SAM the effect of cysteine-methyl disulphide and methylthioadenosine on cell growth was investigated. Neither compound showed any effect under the conditions employed in the present study. The failure of methylthioadenosine to support growth may be due to the absence of a nucleotidase which catalyses the phosphorolytic cleavage of methylthioadenosine to yield adenine and 5-methylthioribose-1-phosphate [24]. At the concentrations employed in the present studies homocysteine had no effect on the growth rate of Walker cells in complete medium, which suggests that the inability to proliferate in homocysteine-supplemented medium is not due to a growth inhibitory effect of homocysteine.

Short term incubation of rat lymphocytes with L-cysteine in concentrations not altering cell viability increased the number of surface sulphydryl groups and induced an immediate total loss of response to adrenaline [25]. Since the number of sulphydryl groups on the surface of Walker cells did not increase in homocysteine-supplemented media, however, the loss of proliferative ability and cell viability does not appear to be due to an alteration of the surface architecture.

Kamely *et al.* [9] showed that the activity of 5-CH_3FH_4:homocysteine methyltransferase of BHK cells which show no methionine requirement [4] was regulated by the methionine content of the media, such that there was a derepression of enzyme synthesis on substitution of the substrate homocysteine for the product methionine. A similar induction of enzyme activity in media of low methionine concentration, and in media lacking methionine, but supplemented with homocysteine, was observed in the present experiments with Walker carcinoma. Further confirmation of an increased enzyme synthesis in methionine-depleted media was provided by the reversal of the methotrexate blocking effect on dU suppression of [^{3}H] TdR incorporation into acid-insoluble material. In contrast to Walker carcinoma TXL5 lymphoma is capable of proliferating in media lacking methionine and substituted with homocysteine, albeit at a somewhat reduced rate. Although no difference is found in the activity of the methyltransferase in these two cell lines there is a large difference in the ability to proliferate in medium of low methionine concentration with TXL5 lymphoma being much less dependent on a high extracellular methionine concentration [20]. Thus the inability of Walker carcinoma to grow in media lacking methionine seems to be due more to the high methionine requirement of this cell line than in its inability to methylate homocysteine as previously suggested [7].

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