Eur J Cancer Clin Oncol, Vol. 17, No. 12, pp. 1323–1329, 1981.
Printed in Great Britain.

0277-5379/81/121323-07$02.00/0

Apparent Methionine Auxotrophy of Some Tumour Cell Lines may be Linked to Impaired Amino Acid Transport*

MICHAEL J. TISDALE†
Department of Biochemistry, St. Thomas's Hospital Medical School, London SE1 7 EH, U.K.

Abstract—*An investigation of the* L-*methionine requirement of five cell lines* in vitro *shows an increased dependence on extracellular* L-*methionine in the order: human embryonic bladder fibroblasts (HE), human bladder carcinoma (EJ), mouse lymphoma (TLX5), mouse bladder carcinoma (MB) and Walker rat carcinoma (W.256). This order correlates with the initial velocity of transport of this amino acid and the steady state concentrations achieved. The order of* L-*methionine requirement is the same as the order of the ability of these cell lines to survive and grow in a* L-*methionine-depleted medium containing only* L-*homocysteine. The tumour cell lines all have a decreased maximal initial rate of* L-*methionine transport (v_{max}) and a lower K_m value than two normal cells investigated (hepatocytes and fibroblasts). Kinetic studies permit the identification of two transport systems for methionine in W 256 and TLX5 and only one for the other cell lines. The initial rate of transport of* L-*lysine in these cell lines follows the same order as for* L-*methionine Studies using* L-[*methyl-*^{3}H]-*methionine labelled cells show that, in addition to a defective transport system for methionine in W.256 and MB, the rate of loss of label from nucleic acids and proteins is also higher for these two cell lines. These results suggest that the apparent methionine auxotrophy of some tumour cell lines may be due to an impaired capacity to concentrate methionine and a higher turnover rate.*

INTRODUCTION

THE GROWTH and survival of a number of rodent and human tumour cell lines *in vitro* is severely reduced when L-methionine in the culture medium is replaced by L-homocysteine [1–3]. This growth dependence on L-methionine is not due to a defect in the synthesis of L-methionine from L-homocysteine by 5-methyltetrahydropteroyl-L-glutamate: L-homocysteine *S*-methyltransferase (EC2.1.1.13) [4] or in the transport of L-homocysteine into the cell [5], but may be related to a higher methionine requirement for some cell lines [6]. One possibility for a growth requirement on pre-formed methionine is a defective transport system for this amino acid. Methionine has been observed to interact with two principal transport systems for amino acids in mammalian cells, the A (alanine preferring) and L (leucine preferring) systems. System L has been thought to be the exchange system and system A has been commonly regarded as a unidirectional active transport system [7]. It has been suggested that alterations in transport activities may be of central importance in the mechanism of neoplastic transformation [8].

The present study investigates the transport systems for L-methionine and L-lysine, as well as the cellular turnover rate of methionine in five cell lines; human embryonic fibroblasts, a human bladder carcinoma, a mouse lymphoma, a mouse bladder carcinoma and the Walker rat carcinoma (W.256). These cell lines show a variable ability to proliferate in L-methionine-depleted media containing L-homocysteine only [6], the order of the ability to grow being: embryonic fibroblasts > human bladder carcinoma ≈ mouse lymphoma > mouse bladder carcinoma ≈ Walker rat carcinoma.

MATERIALS AND METHODS

L-[Methyl-^{3}H]-methionine(sp.act.78Ci/mmole) and L-[4,5-^{3}H]-lysine (sp.act.77Ci/mmole) were purchased from the Radiochemical

Accepted 27 July 1981.
*This work has been financed by grants from the Cancer Research Campaign and St. Thomas's Hospital Research (Endowment) Fund.
†Present address: Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET, U.K.

Centre, Amersham. Dulbecco's modified Eagle's medium lacking methionine and folic acid was specially prepared by Gibco, Europe Ltd., Paisley, Scotland. Methionine was removed from foetal calf serum (Gibco) by extensive dialysis against 0.9% NaCl and was sterile filtered prior to re-freezing.

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 methionine requirement experiments test media consisted of methionine-free Eagle's medium containing the concentrations of methionine indicated in Fig. 1, 7.5 μM hydroxocobalamin, 0.1 mM folic acid and supplemented with 10% dialysed foetal calf serum. The human embryonic fibroblasts (HE), mouse bladder carcinoma (MB) and human bladder carcinoma (EJ) were kindly supplied by Dr. L. M. Franks, Imperial Cancer Research Fund, London.

Preparation of hepatocytes

Parenchymal liver cells from rats were prepared by a modification [9] of the method of Berry and Friend [10]. During ether anaesthesia the liver was perfused through the portal vein with a Ca^{2+}-free Hanks' bicarbonate buffer. The liver was excized and placed in an *in vitro* system. After 10 min of perfusion the perfusate was changed to the same buffer containing 0.05% (w/v) collagenase and 4 mM Ca^{2+}. After 10–25 min of collagenase perfusion the liver cells were released into a Hanks'/Hepes buffer and incubated for 20–30 min at 37°C. Cells were washed and purified by low speed centrifugation prior to methionine transport studies which were performed immediately.

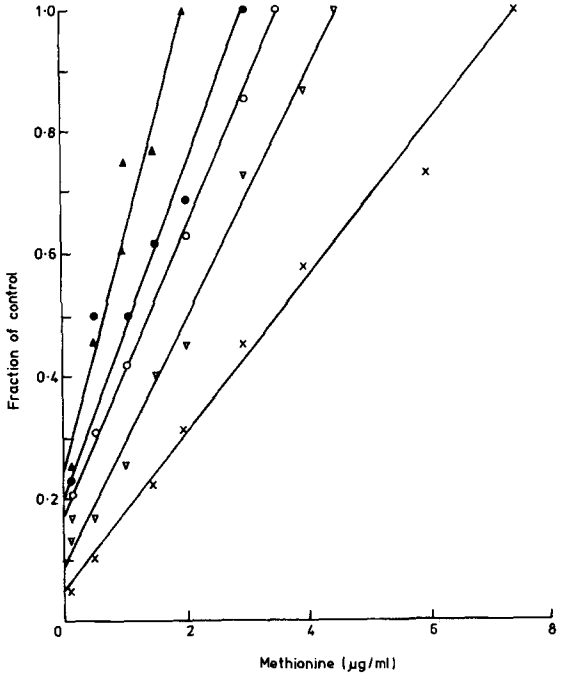

Fig. 1. Relative growth of HE (▲-▲), EJ (●-●), TLX5 (O-O), MB (▽-▽) and W.256 (×-×) with increasing concentrations of L-*methionine. Cells were incubated in Dulbecco's modified Eagle's medium with varying concentrations of* L-*methionine and the growth rate was compared with a control containing* 10 μg/ml *of* L-*methionine.*

Transport studies

Cells were washed twice in 0.9% NaCl and sedimented by low speed centrifugation prior to assay. Cells ($1–2 \times 10^6$/ml) were allowed to incubate at 37°C in a calcium-free Krebs–Ringer phosphate solution for 5 min prior to the addition of L-[methyl-^{3}H]-methionine or L-[4,5-^{3}H]-lysine at various concentrations in kinetic experiments designed to determine the Michaelis–Menten parameters. The cells were shaken to avoid clumping, aliquots (2 ml) were taken out at time intervals and transport was terminated rapidly by adding to 3 ml ice-cold Krebs–Ringer solution, followed immediately by centrifugation (300 *g* for 3 min). The supernatant was removed and the cells were re-suspended in a further 5 ml of ice-cold Krebs–Ringer solution. The suspension was centrifuged again for 3 min at 3°C, the supernatant removed and the interior of the tubes dried with paper tissue. To the washed cells was then added 2 ml 95% ethanol and a minimum period of 30 min was allowed for completion of extraction of alcohol-soluble materials. After centrifugation the supernatant was counted in a toluene/PPO scintillation fluid. The identification of the accumulated labelled compound was accomplished by radio-thin layer chromatography [11].

Loss of [methyl-^{3}H]-methionine from the acid-soluble pool, nucliec acids and proteins

The incorporation of radioactivity into nucleic acids and proteins was determined by suspending the cells (6×10^5 per ml) in the presence of 1 μCi/ml [methyl-^{3}H]-methionine for 24 hr. At the end of the incubation the cell suspension was sedimented by centrifugation at 600 *g* for 3 min, followed by re-suspension in fresh medium. At various time points 10 ml of the cell suspension was withdrawn and sedimented by centrifugation. The cell pellet was treated with 1 ml of ice-cold 0.5 M perchloric acid and the precipitate was washed four times by re-suspension and centrifugation in 1 ml of 0.5 M perchloric acid. An aliquot of the acid

supernatant, after neutralization with 5 M KOH, was counted in PCS scintillation fluid (Hopkin and Williams) to determine the acid-soluble radioactivity. A nucleic acid-soluble fraction (DNA + RNA) was prepared by heating the acid precipitate at 70°C for 20 min in 1 ml of 1.0 M perchloric acid, cooling rapidly on ice and centrifuging at 600 *g* for 10 min at 4°C. The 70°C perchlorate hydrolysis was repeated on the remaining residue and, after neutralization of a portion (1.6 ml) of the combined supernatant, the radioactivity was determined as above. The residue remaining after acid hydrolysis was dissolved in 1 M NaOH and the concentration of protein was determined by the method of Lowry *et al.* [12] using bovine serum albumin as a standard. The remaining residue was neutralized with 1 M HCl and the radioactivity determined in PCS scintillation fluid.

RESULTS

The effect of increasing concentrations of L-methionine on the growth of human embryonic bladder fibroblasts (HE/BX), human bladder carcinoma (EJ), mouse bladder carcinoma (MB), a mouse lymphoma (TLX5) and Walker rat carcinoma (W.256) is shown in Fig. 1. The growth rate is calculated from the linear part of the growth curves and is expressed as a percentage of a control growing in medium containing 10 μg/ml of L-methionine. The concentration of L-methionine required for 50% of the optimal growth rate decreases in the order W.256 (3.6 μg/ml), MB (2.1 μg/ml), TLX5 (1.4 μg/ml), EJ (1.1 μg/ml) and HE (0.7 μg/ml). This order is the reverse of the ability of these cell lines to proliferate in L-methionine-deficient media containing 0.1 mM L-homocysteine [6].

To determine whether any correlation exists between the degree of requirement for methionine and the ability of the cells to transport methionine, methionine transport was determined in the presence of 0.5 mM L-[methyl-^{3}H]-methionine. The time course of methionine uptake is shown in Fig. 2. Velocity of uptake is maximal during the first 2 min and thereafter declines and eventually reaches a plateau after about 30 min. In all of the cell lines less than 1% of the methionine taken up within 2 min was precipitable by trichloroacetic acid. The initial velocity of uptake and steady state concentration of methionine approximate to the methionine requirements of the cell lines. Thus, the steady state levels reached by EJ is 1.3 mM, by MB 0.56 mM, by TLX5 0.39 mM and by W.256 0.20 mM. Although the initial velocity of uptake by TLX5 exceeds that of MB, the accumulation reaches a maximum of 0.5 mM within 6 min and then falls off to a lower, steady state. This may be related to the incorporation of methionine into macromolecules.

Initial velocities of transport of methionine by the cell lines as a function of the external methionine concentration were determined over a 2 min period. At substrate concentrations up to 2 mM kinetic analysis revealed a transport process for methionine which conforms to the Michaelis–Menten equation (Fig. 3). The kinetic constants determined from such

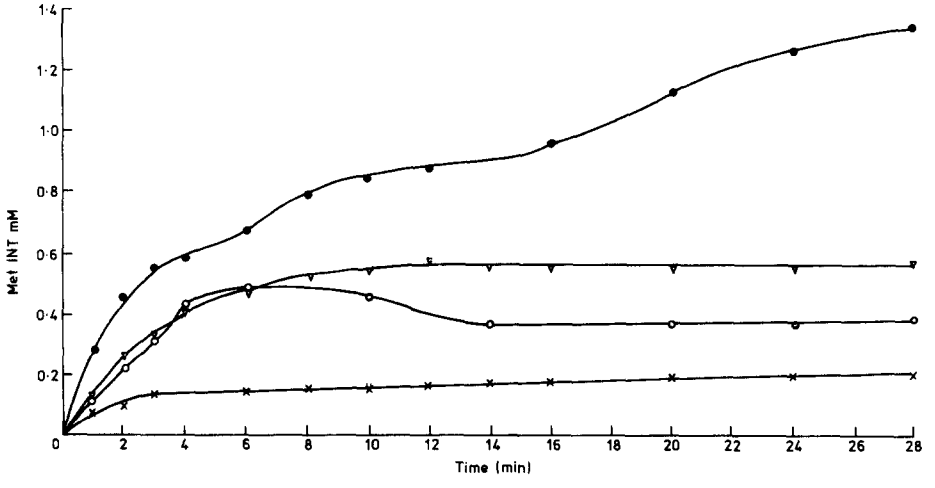

Fig. 2. Time course of L-*methionine uptake by EJ* (●-●), *MB* (▽-▽) *TLX5* (○-○) *and W.256* (×-×). *Cells were incubated with* 0.5 mM L-[*methyl-*^{3}H]-*methionine* (20 mCi/mmole) *at* 37°C *in a shaking water bath. Samples were removed in duplicate at various time intervals and washed as described in Methods. Cell volume was determined with a Coulter counter model D.*

plots for each of the cell lines and in addition freshly prepared hepatocytes are presented in Table 1. The maximal velocities of uptake of methionine (ν_{max}) for the two normal cell types HE/BX and hepatocytes exceed that of any of the neoplastic cell lines investigated, although the K_m values are higher. A similar situation has been observed in a comparison between normal bone marrow cells and those obtained from leukaemic patients (M. J. Tisdale and S. Eridani, unpublished results). In addition, the ν_{max} values for methionine uptake approximate to the methionine requirements of the cell lines, although TLX5 and MB have the same values of K_m and ν_{max}. The diffusion constants for each cell line were estimated from these data by the method of Akedo and Christensen [13]. The rate of diffusion of methionine into HE, EJ and TLX5 are all similar and much greater than for WS and MB.

The Lineweaver–Burk plots of all cell lines except W.256 and TLX5 show linear curves, suggesting that only the transport system for methionine is operative in these cells. When the kinetic constants for methionine uptake by W.256 and TLX5 are evaluated without correcting for diffusion, the Lineweaver–Burk plot breaks downward (Fig. 3), a result which would be expected if the cells had two transport systems for methionine. The kinetic constants under these conditions are given in Table 1. At methionine concentrations less than 5 mM diffusion can be ignored, since the contribution would be negligible to the total uptake.

A comparison of lysine transport by the four tumour cell lines shows the same order of ν_{max}

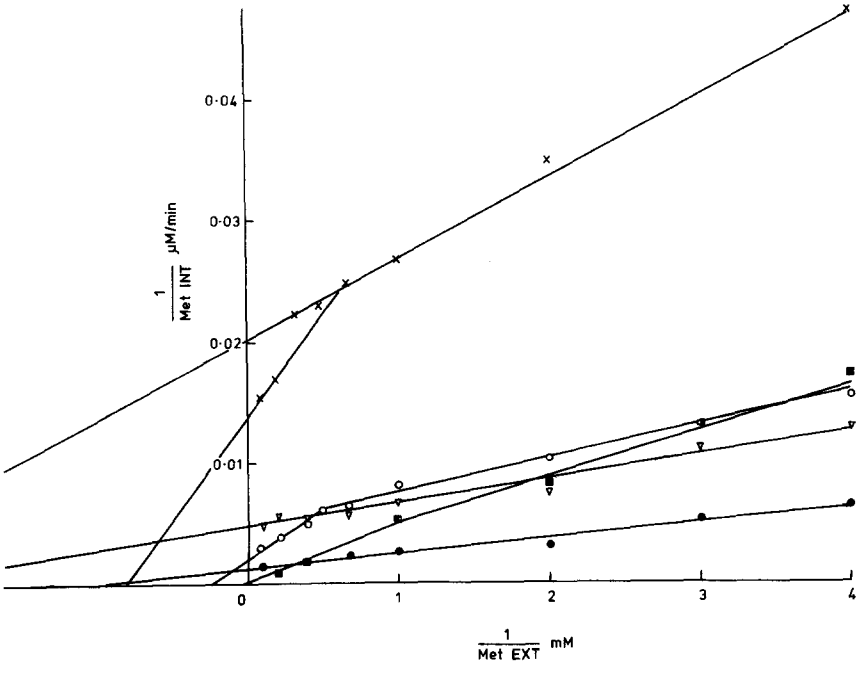

Fig. 3. Double reciprocal plot of initial rate of L-*methionine transport against substrate concentration by freshly isolated hepatocytes* (■-■), *EJ* (●-●) *TLX* 5 (○-○) *MB* (▽-▽) *and W.*256 (×-×). *Cells were exposed to varying concentrations of* L-[*methyl-*^{3}H]-*methionine* (0.25–10 mM) *for* 2 *min at* 37°C. L-[*Methyl-*^{3}H)-*methionine was present at* 5–10 µCi/ml *and at a specific activity of* 0.1–20 mCi/mmole. *All uptake determinations were made in duplicate.*

Table 1. Kinetic parameters for L-*methionine and* L-*lysine uptake*

	Methionine			Lysine	
Cell line	K_m (mM)	K_D (min^{-1})	v_{max} (µM/min)	K_m (mM)	v_{max} (µM/min)
W-256	1.25; 0.32	0.005	50; 72	2.5	66
MB	0.64	0.01	222	2.5	125
TLX5	4.1; 0.64	0.03	222; 500	2.5	286
EJ	0.93	0.03	727	2.5	286
HE	2.8	0.03	888		
Hepatocytes	7.2	—	2000		

values as for methionine uptake (Table 1 and Fig. 4). The K_m value for lysine uptake is 2.5 mM for all cell lines. These results suggest a general impairment of amino acid transport in some tumour cells.

In addition to transport of methionine, the cellular turnover rate may be important in determining sensitivities to methionine-depletion. Methionine is a constituent of some, but not all, proteins and, after conversion to *S*-adenosyl-L-methionine, is an important source of cellular methyl groups particularly in the post-transcriptional modification of nucleic acids. The rate of loss of L-[methyl-^{3}H]-methionine from the acid-soluble pool, nucleic acids and proteins for each cell line is shown in Table 2 and Fig. 5. The rate of loss has been calculated from the linear part of the decay curve, which in the case of nucleic acids extends for the whole 24 hr of measurement (Fig. 5). The rate of loss of methionine from the acid-soluble pool is approximately the same for all cell lines except MB, which shows a six-fold greater loss. The rate of loss of label from both nucleic acids and proteins is higher for the two cell lines with a higher methionine requirement (MB and W.256).

Table 2. Rate of loss of radioactivity (cpm/mg protein/hr) *from the acid-soluble pool, nucleic acid and protein*

Cell line	Acid soluble	Nucleic acid	Protein
W.256	125	1083	10232
MB	1244	2118	64232
TLX5	254	647	4419
EJ	286	786	5489
HE	277	859	31562

DISCUSSION

The present experiments suggest a correlation between the methionine requirements of various cell lines and the ability to concentrate methionine. The methionine requirement of the cell lines also correlate with their ability to survive in a L-methionine-depleted medium containing only L-homocysteine. Many reports [1, 3, 4, 6] have shown that normal fibroblasts are able to utilize homocysteine in lieu of methionine and this has been correlated with a decreased minimal concentration of L-methionine sufficient to support optimal growth [6]. This study shows the v_{max} for methionine uptake to be greater for normal fibroblasts than for the other cell lines. Changes in v_{max} reflect the number of sites involved in the carrier-mediated process, while differences in K_m reflect qualitative alterations of these sites. The two cell lines with the most stringent methionine requirement (W.256 and MB) show a decrease in the number of membrane sites involved in the uptake of methionine. Although the v_{max} values for methionine uptake by these two cell lines are reduced, the K_m values are also lower than for the other cell lines. A similar situation is shown for leukaemic marrow cells when compared

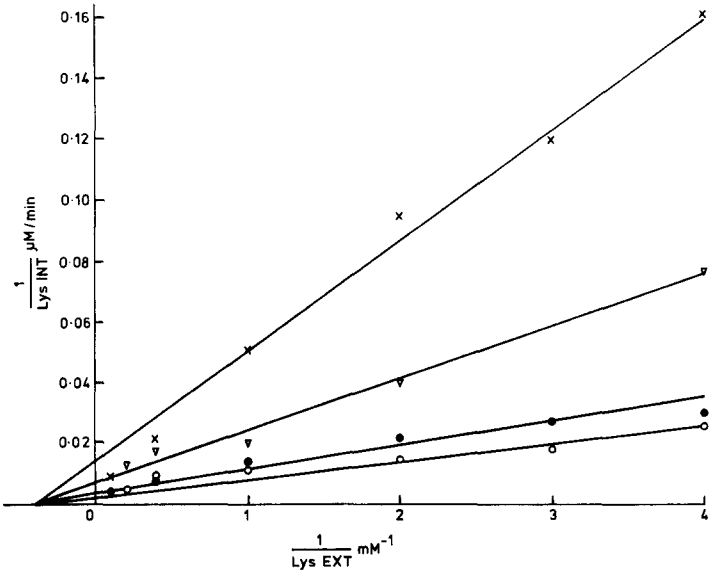

Fig. 4. Lineweaver–Burk plots of initial velocity of L-[4,5-^{3}H]-*lysine uptake by EJ* (●-●), *TLX5* (○-○), *MB* (▽-▽) *and W.256* (×-×). *Varying concentrations of* L-[4,5^{3}H]-*lysine* (0.25–10 mM) *was added to the cell suspension for* 2 *min at* 37°C. L-[4,5^3-H]-*lysine was present at* 5–10 µCi/ml *and at a specific activity of* 0.1–20 mCi/mmole. *All uptake determinations were made in duplicate.*

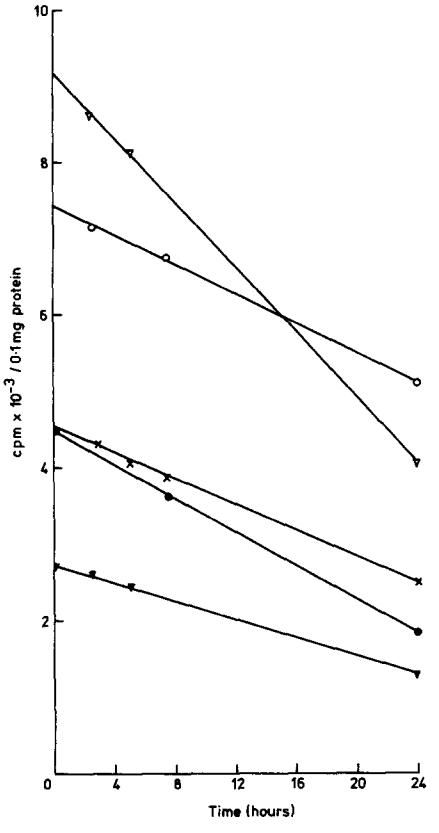

Fig. 5. Rate of loss of [^{3}H]-*methyl groups from* L-[*methyl*-^{3}H]-*methionine-labelled TLX5* (▼-▼), *W.256* (●-●), *HE* (×-×), *MB* (▽-▽) *and EJ* (○-○). *Cultures were incubated with* 1 μCi/ml *of* L-[*methyl*-^{3}H]-*methionine and the nucleic acids were isolated as described in Methods.*

with normal marrow cells (Tisdale and Eridani, unpublished results). Lineweaver–Burk plots of the kinetic data indicate that W.256 and TLX5 have two transport systems for methionine. These probably correspond to the L (leucine preferring) and the A (alanine preferring) systems [7]. A similar analysis of HE, EJ and MB yielded a single straight line suggestive of one transport system.

It has been suggested by some workers that there is an increased amino acid transport in tumour cells when compared with normal cells [14–16]. However, a decreased uptake of aspartate and glutamate has recently been reported in rapidly growing hepatomas [17]. This study shows a wide variability in the ability to transport both a neutral and a basic amino acid in epithelial tumours.

Thus, differences in the transport of methionine and the size of the cytosolic pool could greatly influence the ability of cells to grow in minimal concentrations of this amino acid. Tumour cells may also have an increased requirement for methionine which is utilized via *S*-adenosylmethionine both in the methylation of macromolecules and in the biosynthesis of the polyamines spermidine and spermine. The latter are growth factors produced in substantial amounts by rapidly growing cells and increased levels have been detected in the serum of cancer patients [18]. The extent of methylation of tRNA is also higher in tumour cells, as is also the specific activity of tRNA-methylating enzymes [19]. This suggests an approach to anti-tumour chemotherapy based on interference with the methionine concentration in the cell. Reduction of the plasma level of this amino acid by L-methioninase (L-methionine-α-deamino-γ-mercaptomethane-lyase EC 4.4.1.11) coupled with the reduced transport of some tumour cells could result in the selective inhibition of tumour growth. Selective inhibition of the growth of malignant, but not normal cells results after L-methionine depletion by L-methioninase followed by L-homocysteine thiolactone 'rescue' therapy [20].

REFERENCES

1. Halpern BC, Clark BR, Hardy DN, Halpern RM, Smith RA. The effect of replacement of methionine by homocysteine on survival of malignant and normal adult mammalian cells in culture. *Proc Natl Acad Sci USA* 1974; **71**: 1133–1136.
2. Wilson MJ, Poirier LA. An increased requirement for methionine by transformed rat liver epithelial cells *in vitro*. *Exp Cell Res* 1978; **111**: 397–400.
3. Kreis W, Goodenow M. Methionine requirement and replacement by homocysteine in tissue cultures of selected rodent and human malignant and normal cells. *Cancer Res* 1978; **38**: 2259–2262.
4. Hoffman RM, Erbe RW. High *in vivo* rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. *Proc Natl Acad Sci USA* 1976; **73**: 1523–1527.
5. Tisdale MJ. Methionine metabolism in Walker carcinosarcoma *in vitro*. *Eur J Cancer* 1980; **16**: 407–414.
6. Tisdale MJ. Effect of methionine replacement by homocysteine on the growth of cells. *Cell Biol Int Rep* 1980; **4**: 563–567.
7. Matthews RH, Zand R. Methionine transport in S37 cells. Substrate-dependent

function of amino acid transport system A in exchange processes. *Biochim Biophys Acta* 1979; **554**: 227–238.

8. HOLLEY RW. A unifying hypothesis concerning the nature of malignant growth. *Proc. Natl Acad Sci USA* 1972; **69**: 2840–2841.
9. CHRISTOFFERSEN T, BERG T. Glucagon control of cyclic AMP accumulation in isolated intact rat parenchymal cells *in vitro*. *Biochim Biophys Acta* 1974; **338**: 408–417.
10. BERRY MN, FRIEND DS. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J Cell Biol* 1969; **43**: 506–520.
11. HEISER W, ENGLESBERG E. Isolation and characterization of L-methionine-resistant mutants of SV40-transformed Balb 3T3 (SVT2) affecting L-methionine transport. *Somat Cell Genet* 1979; **5**: 345–361.
12. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
13. AKEDO H, CHRISTENSEN HN. Nature of insulin action on amino acid uptake by the isolated diaphragm. *J Biol Chem* 1962; **237**: 118–122.
14. FOSTER DO, PARDEE AB. Transport of amino acids by confluent and nonconfluent 3T3 and polyoma virus-transferred 3T3 cells growing on cover slips. *J Biol Chem* 1969; **244**: 2675–2681.
15. ISSELBACHER KJ. Increased uptake of amino acids and 2-deoxy-D-glucose by virus-transformed cells in culture. *Proc Natl Acad Sci USA* 1972; **69**: 585–589.
16. BHARGAVA PM, SZAFARZ D, BORNECQUE CA, ZAJDELA F. A comparison of the ability of normal liver, a premalignant liver, a solid hepatoma and the Zajdela ascites hepatoma to take up amino acids *in vitro*. *J Membr Biol* 1976; **26**: 31–41.
17. KOCH MR, KHALIL FL, LEA MA. Decreased uptake of ^{14}C labelled dicarboxylic amino acids in rapidly growing hepatomes. *Cancer Res* 1980; **40**: 4053–4058.
18. NISHIOKA K, ROMSDAHL MM. Elevation of putrescine and spermidine in the sera of patients with solid tumours. *Clin Chim Acta* 1974; **57**: 155–161.
19. NAU F. The methylation of tRNA. *Biochimie (Paris)* 1976; **58**: 629–645.
20. KREIS W. Tumour therapy by deprivation of L-methionine: rationale and results. *Cancer Treat Rep* 1979; **63**: 1069–1072.