Biochimica et Biophysica Acta, 1024 (1990) 233–240
Elsevier

BBAMEM 74849

Rapid and steady-state amino acid transport in perfused human fibroblasts and colon adenocarcinoma cells: effects of methotrexate

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(Received 8 January 1990)

Key words: Amino acid transport; Isotope dilution; Fibroblast; Methotrexate; (Colon adenocarcinoma)

Initial and steady-state uptakes of serine and phenylalanine by human fibroblasts and human colon tumour cells were studied applying a double isotope dilution technique to perfused populations of cultivated cells retained on microcarrier beads. This new method permits the differentiation of the unidirectional transport parameters and can also distinguish between membrane-associated processes and independently intracellular events in isolated cells. High initial L-serine uptake values in colon adenocarcinoma cells became negative under steady-state conditions. To determine if the observed negative L-serine uptake was produced by the rapid efflux of intracellular L-[^{3}H]serine, the cells were treated with methotrexate (MTX) (an inhibitor of cytosolic dihydrofolate reductase). The modified curve of L-[^{3}H]serine uptake after MTX treatment suggests that, under these experimental conditions, net serine transport is non concentrative in colon tumour cells and could be modulated by the rate of intracellular serine metabolism; it also suggests that MTX does not directly affect serine transport in perfused human colon adenocarcinoma cells. Initial and steady-state uptakes of phenylalanine were high in both fibroblasts and tumour cells and were unaffected by MTX treatment.

Introduction

The transport of amino acids and their incorporation into proteins are independent processes, except that active transport meets a demand for amino acids required for protein synthesis [1]. In addition, both transport and incorporation are interrelated, at least, through the intracellular amino acid levels and so it is difficult to differentiate between the two independent primary processes in the intact cells. Consequently, there is much contradictory evidence about the origin of the precursor amino acid molecules required for protein synthesis. Some investigators claim that protein synthesis is controlled by several types of membrane-associated ribosome that preferentially take up the amino acids from the extracellular amino acid pool [2–6]. On the other hand, if the rate of protein synthesis is limited by the rate of amino acid transport, modulation of transport might indirectly modulate the rate of protein synthesis.

Traditional methods for measuring transport in cell cultures cannot resolve the temporal parameter, and, moreover, uneven mixing and incomplete washing of the cells, may affect the accuracy of the results [7,8]. To study transport processes in tumour cells with their abnormal metabolism is even more difficult because they have considerably increased protein synthesis and uptake of amino acids [9–11].

In the present work, transport and metabolic processes were characterized independently by measuring the initial and steady-state uptakes of L-serine and L-phenylalanine of human colon adenocarcinoma cells and their modulation by methotrexate (an inhibitor of cytosolic dihydrofolate reductase) [12]. Normal fibroblasts were also studied to evaluate the proposed technique and to obtain reference data.

Different populations of isolated cells retained and perfused in columns of microcarrier beads were studied with a high-resolution, double-isotope dilution technique [13]. The proposed method gave almost instantaneous resolution of the temporal transport parameters; the cells did not have to be separated from the medium and the input and output vectors of the net transport were easily differentiated.

Abbreviation: MTX, methotrexate.

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Material and Methods

Isolation and culturing of cells

The fibroblasts were obtained from a skin biopsy by the method of Sly and Grubb [14]. The tumour cells were obtained from a resected human adenocarcinoma following the technique of Bashor [15]. All stages of the cellular isolation procedure were carried out beneath a laminar-flow hood (Gelaire Twin 30, Flow Laboratories). The two cell types were cultivated in a CO_2 incubator at 5% (Incubator 1500, Flow Lab.) in 25 ml tissue-culture flasks (Corning) with MEM-Glasgow culture medium (Gibco) supplemented with foetal calf serum 10% (Gibco), tryptose phosphate 10% (Merck) and penicillin-streptomycin 1% (Antibioticos). The fibroblast culture medium was changed twice a week until confluence and the passes were started at this time. The tumour cells were cultured in the same way as the fibroblasts and their behaviour in culture was noted, particularly their ability to grow both in suspension and on the surfaces of flask and microbeads. The medium was changed when it became acidic and the cultures were divided when they reached high cell densities (10^6 cells/ml). All the experiments were carried out during passes 5 to 12.

Perfusion of cultured cells on microcarrier beads

The perfusions employed the previously described procedure of Perán and McGee [13]. Some $(8–10) \cdot 10^6$ cells were cultivated with 2 ml of a 1% suspension of microcarrier beads (Citodex 3, Pharmacia, Sweden) in the previously described medium for 24–48 h in a CO_2 incubator at 5% and 37 °C. The cells were stirred for the first hour at 40 rpm. Because tumour cells have the ability to grow either independently in suspension or as anchorage-dependent population on the surface of cultivation flasks [16], no modification of the culture medium was made to promote adhesion to the beads. Moreover, Cytodex 3 is a collagen coated microcarrier that favours cell attachment. Once covered with cells (some 40 cells per bead), the microcarrier beads were packed into a thermostatic column (C-10 Pharmacia) then perfused by a peristaltic pump (P3, Pharmacia) with Hanks' balanced salts solution (Sigma) supplemented with 10 mM D-glucose and 2% bovine serum albumin at a constant flow of 0.6 ml/min. Cell viability was determined at the end of the experiments by staining with Trypan blue (> 90%).

Uptake of tritiated L-amino acids by cultured cells

Unidirectional uptake. The rapid uptakes of L-[^{3}H]serine (37 Ci/mmol, Amersham U.K.) and L-[^{3}H]phenylalanine (43 Ci/mmol, Amersham, U.K.) were determined using a rapid single-passage, paired-tracer dilution technique [13,17]. Cells were perfused for 20 min with an amino acid-free perfusate to approach zero-trans conditions [18]. A mixture of the tritiated amino acid and an extracellular reference molecule, D-[^{14}C]mannitol (59 mCi/mmol, Amersham, U.K.), was then injected (300 μl in 30 s) into the cultured cell column inflow and a series of twenty 60 μl samples were collected from the column effluent in 120 s. In addition, a final sample was accumulated for a further 5 min to assess tracer recoveries and efflux from the perfused cells.

The fractional [^{3}H]amino acid uptake was calculated for each of the successive effluent samples according to the equation:

$$U = 1 - ([^3\text{H}]\text{amino acid}/[^{14}\text{C}]\text{mannitol}) \quad (1)$$

in which the recovered activities of the two tracer (cpm) are expressed as percentage of the dose challenged. The calculation of the efflux for each amino acid was:

$$\%\ \text{efflux} = (1 - [U_t/U_0]) \times 100 \quad (2)$$

where U_t is the net uptake (1 − total[^{3}H]/total[^{14}C]) determined from integrated tracer recoveries during a 7 min effluent collection period and U_0 is an average of the maximal uptake values, assumed to reflect unidirectional uptake [18].

Loading experiments: initial and steady-state uptakes. The design of the experimental method used to obtain the loading and washout curves is based on Boyd and Parsons model for intestinal transport studies [19], it was modified and adapted for use with perfused microcarrier cell cultures [13]. The confluent microcarrier cell cultures were packed into a small column and perfused with Hanks' solution containing a solution of the two isotopes (extracellular tracer and test). The cells were loaded with this perfusate solution for 20–25 min before being switched to an isotope-free perfusate to monitor cellular wash-out. During the wash-in, 25–40 sequential samples (50 μl each) were initially collected in 3–4 min from the column effluent. During the subsequent 20–25 min, one sample per min was collected. The perfusion medium was then changed to an isotope-free solution (wash-out period) and another series of 50–60 sequential samples were collected for 5–6 min to assess cellular wash-out (efflux) of transported amino acid. The dilution curves were constructed on the basis of the percentage activity of both tracers recovered in each sample compared with the activities of the tracers in a sample of equal volume obtained from the flask containing the perfusion medium. Initial- (U_0) and net- (U_t) uptakes of L-serine and L-phenylalanine were calculated according to Eqn. 1. Efflux of transported amino acid during loading period (3 and 23 min) were calculated according to Eqn. 2.

Results

Unidirectional uptake of L-serine and L-phenylalanine in colon tumour cells

Fig. 1A shows the recovery tracer dilution profiles obtained after perfusion the isolated malignant colonocytes in a thermostatic perfusion column with a supplemented Hanks' solution containing 0.06 mmol/l unlabelled phenylalanine and a mixture of L-[^{3}H]phenylalanine and D-[^{14}C]mannitol. The activities of the two tracers in each successive sample are expressed as percentages of the amounts of tracer injected and plotted against the sample number and collection time. The recovery of L-[^{3}H]phenylalanine was significantly less than that of D-[^{14}C]mannitol and this reflects both its dilution in a larger volume and (in this two compartment model) the cellular uptake of amino acid.

The uptake curve (Fig. 1B), calculated from Eqn. 1, presents two clearly differentiated phases: in the first, representing the unidirectional amino acid input, uptake increases until sample 10 (60 s approx.); in the second, the uptake declines slowly. This suggests a cellular efflux of L-[^{3}H]phenylalanine.

The net uptake (1 − [^{3}H]total/[^{14}C]total) was calculated from the integrated tracer recovery values measured in a 7 min collection period (≈ 20%); it is appreciably less than the maximum (≈ 60%) and again this suggests cellular efflux. This value is shown by the vertical bar in Fig. 1B.

As shown by Fig. 1C, the maximum uptake of L-[^{3}H]phenylalanine was inhibited by adding increasing concentrations of non-labelled phenylalanine (0.03–1 mM) to the perfusate. In addition, L-phenylalanine uptake was significantly inhibited by L-lysine, L-glutamine, L-phenylalanine and L-serine when added to the perfusate to a final concentration of 5 mM (data not shown).

Initial and steady-state amino acid uptake in loading experiments

Fig. 2 shows the dilution curves obtained after perfusing a column packed with microcarrier beads coated

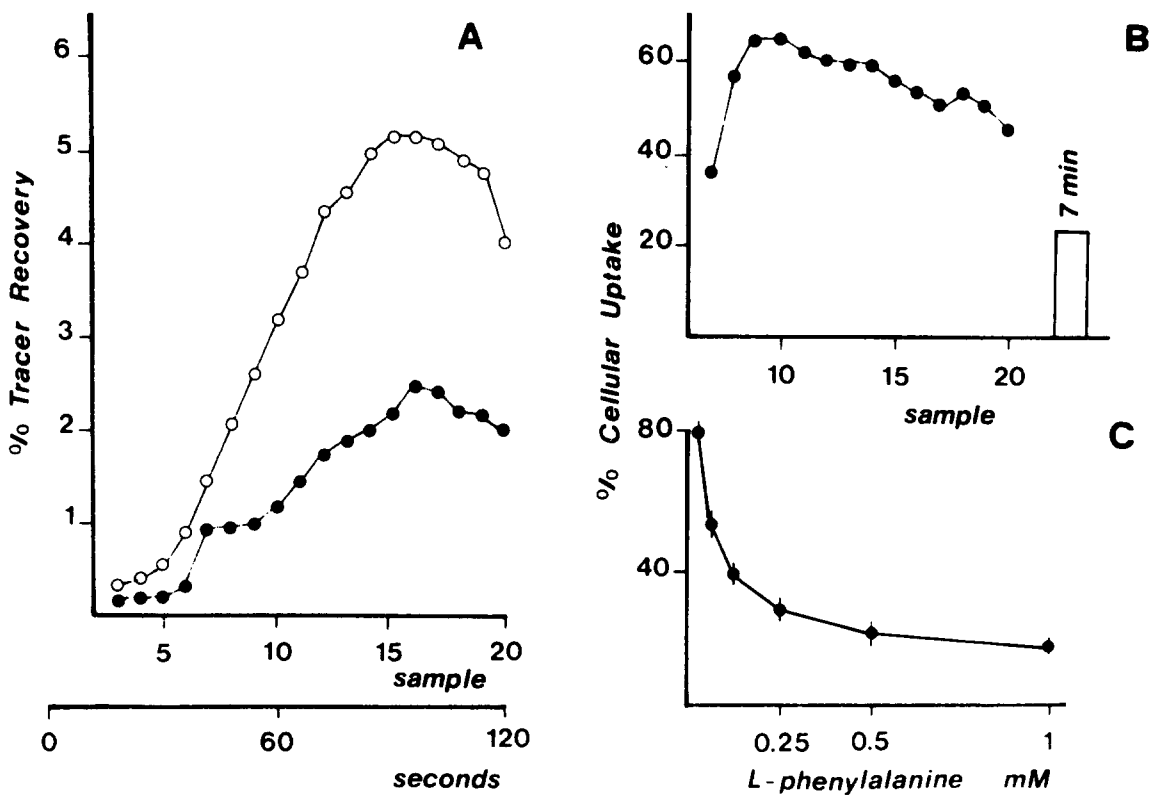

Fig. 1. Unidirectional L-phenylalanine uptake by perfused microcarrier cultures of human adenocarcinoma cells. (A) Simultaneous tracer dilution profiles for L-[^{3}H]phenylalanine (●) and D-[^{14}C]mannitol (extracellular reference, ○) following a pulse administration (300 µl/30 s) of both tracers into the inflow of a column charged with microcarriers cultures of human adenocarcinoma cells. Tracer recoveries in successive outflow samples are expressed as percentage of the respective radioactive doses supplied and plotted against sample number and collection time. (B) L-[^{3}H]phenylalanine uptake patterns: L-[^{3}H]phenylalanine uptake relative to D-[^{14}C]mannitol during the perfusion 60 µM unlabelled L-phenylalanine (dilution data shown in panel A) was calculated according to: % uptake = 1 − ([^{3}H]/[^{14}C]) × 100, and plotted against sample number. The overall uptake determined from integrated total tracer recoveries during a 7 min effluent collection period is shown in the column to the right of panel (B). (C) Self-inhibition of phenylalanine uptake: inhibition of the influx of L-[^{3}H]phenylalanine by increasing concentrations (0.03–1 mM) of unlabelled phenylalanine.

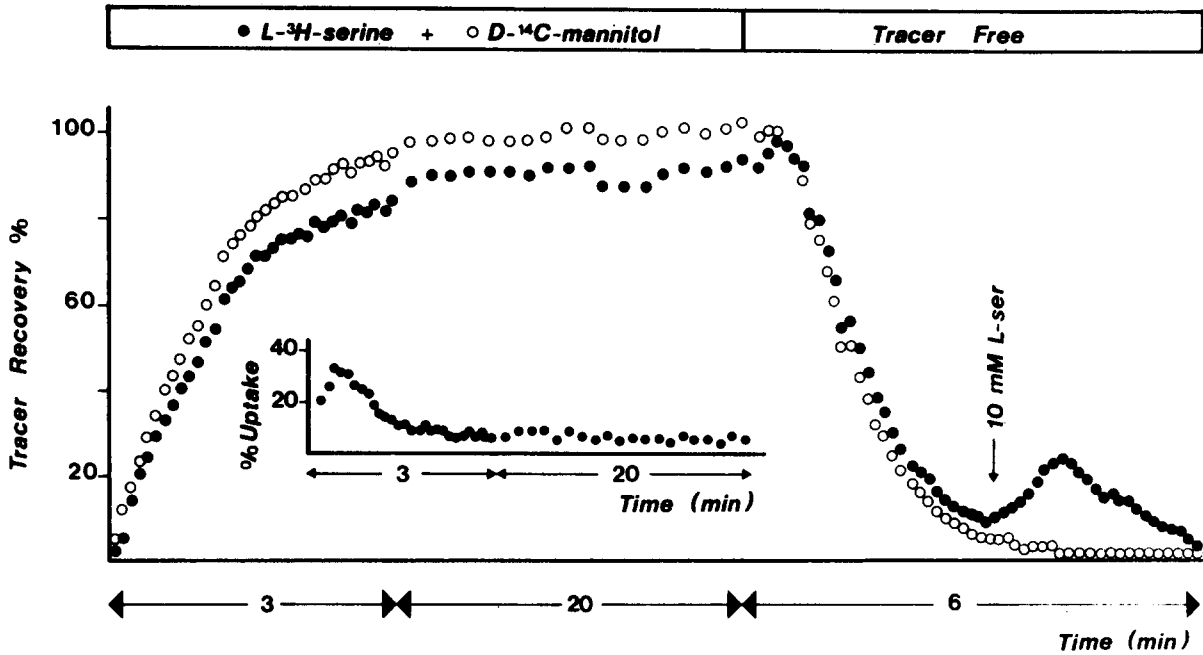

Fig. 2. Initial and steady-state amino acid uptake by perfused cultures of human fibroblasts in loading experiments. A column of confluent microcarrier cultures of human fibroblasts was loaded with a mixture of a labelled amino acid L-[^{3}H]serine, D-[^{14}C]mannitol and 30 μM unlabelled L-serine. The column effluent was collected sequentially; thirty samples in the first 3 min, then once a minute for the remaining loading time. Upper lines express the tracer dilution profiles expressed as percentage of activity of sample of similar volume taken from the inflow reservoir. After a 23 min cell-loading period, the perfusate was rapidly switched to an isotope-free solution and sequential samples were collected from the effluent column for 6 min. When 10 mM of unlabelled L-serine was injected into the extracellular compartment (arrow), a trans-stimulation of L-[^{3}H]serine was detected. The inset shows the initial and steady-state L-[^{3}H]serine uptakes relative to D-[^{14}C]mannitol, calculated from successive effluent samples. The abscissae of both illustrations represent the total elapsed time divided into three periods (separated by the two-headed arrows).

with human fibroblasts with a supplemented Hanks' solution containing L-[^{3}H]serine, D-[^{14}C]mannitol and 0.03 mmol/l of unlabelled L-serine. The change to the isotope-free perfusate (the washout phase) is reflected by the decrease in the radioactivity of both tracers recovered from the column effluent.

TABLE I

L-[^{3}H]serine and L-[^{3}H]phenylalanine uptake and efflux in perfused human fibroblasts

Human fibroblasts cells were grown to confluence on Cytodex 3 microcarrier beads in MEM-Glasgow medium containing 10% foetal calf serum. Confluent microcarrier cultures were packed into a thermostatically controlled Pharmacia C-10 column to a final volume of 0.5 ml and were perfused with a balanced Hanks' salt solution supplemented with 2% bovine serum albumin and 10 mM D-glucose at constant flow rate of 0.6 ml/min. After a 20 min pre-equilibration period, cells were loaded with L-[^{3}H]serine or L-[^{3}H]phenylalanine, D-[^{14}C]mannitol (extracellular reference) for 23 min in the presence of 30 μM of the appropriate unlabelled amino acid. Initial 30 or 60 s uptake and net 3 and 23 min uptake and [efflux] were calculated under different experimental conditions, as described in Materials and Methods. Data represent the mean value in 3–4 independent detrminations ± S.E. (Student's *t*-test, * a-b, $P < 0.01$).

Amino acid	Experimental condition	30 μM uptake [efflux] %, mean ± S.E. (*n*)		
		30 s	3 min	23 min
L-[^{3}H]Serine	control[a]	33 ± 4 (3)	13 ± 2 (3)	9 ± 0.5 (3)
			[59 ± 9 (3)]	[72 ± 5 (3)]
	+ methotrexate[b]	43 ± 2 (4)	14 ± 1 (4)	6 ± 0.7 (4) *
	0.5 mg/ml, 20 min		[67 ± 2 (4)]	[85 ± 3 (2)]
		60 s	3 min	23 min
L-[^{3}H]Phenylalanine	control	70 ± 3 (4)	68 ± 3 (4)	49 ± 4 (4)
			[4 ± 1 (4)]	[31 ± 4 (4)]
	+ methotrexate	76 ± 0.5 (3)	71 ± 1 (3)	51 ± 3 (3)
	0.5 mg/ml, 20 min		[7 ± 2 (3)]	[34 ± 4 (3)]

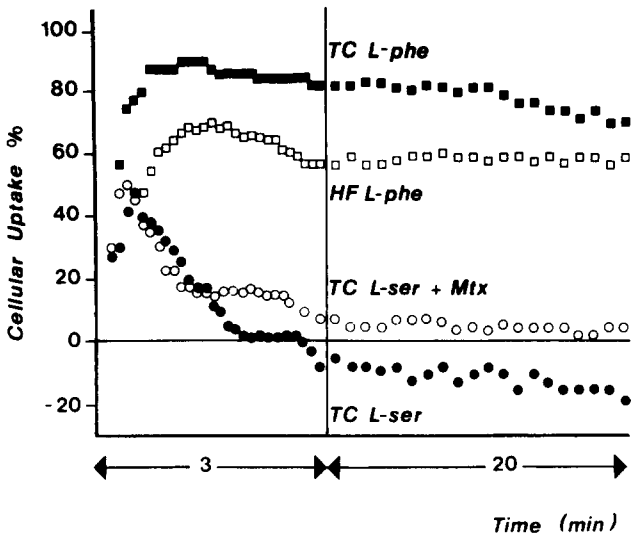

Fig. 3. Amino acid uptake time-course in loading experiments. Initial and steady-state uptakes of 30 μM L-[^{3}H]phenylalanine by perfused cultures of human fibroblasts (HF L-phe, □) or perfused cultures of human adenocarcinoma cells (TC L-phe, ■) and initial and steady-state uptakes of 30 μM L-[^{3}H]serine in perfused cultures of human adenocarcinoma cells in the absence (TC L-ser, ●) or presence (TC L-ser + Mtx, ○) of 0.5 mg/ml methotrexate. Uptake values were calculated from the isotope dilution data as in Fig. 1B (see Tables I and II).

The cellular uptake of L-[^{3}H]serine during the loading period is shown by the fact that the profile of its dilution curve falls below that of D-[^{14}C]mannitol. During the washout phase the dilution curves of both tracers quickly converge and cross, indicating the cellular excretion of L-[^{3}H]serine. Tracer efflux, following the loading of the cells with L-[^{3}H]serine, was stimulated by the injection of a pulse of 10 mM L-serine (most probably this was due to trans-stimulation). The very short transit-time observed suggests that re-uptake of tracer was insignificant.

The inset in Fig. 2 shows the corresponding sequential uptake calculated for the loading period. In the first part of the curve the uptake increases steeply to reach its maximum value (33 ± 4%, $n = 3$); thereafter, because of the efflux of cellular tracer, it decreases gradually to equilibrate close to the steady-state level (9 ± 0.5%, $n = 3$) (Table I).

In the tumour-cell experiments, the results (Fig. 3) show that the initial L-[^{3}H]serine uptake reached a maximum (43 ± 3%, $n = 3$) in the first 30 s and then decreased rapidly to reach negative values within 3 min. Uptake remained negative for the whole of the loading period (Table II). However, during the wash-phase, the profiles were very similar to those obtained for fibroblasts and again, in this case, the unlabelled L-serine trans-stimulated cellular reflux (data not shown).

In fibroblasts and colonocytes the uptake of L-[^{3}H]phenylalanine was much greater, the initial values were (70 ± 3, $n = 4$) and (86 ± 3, $n = 5$), respectively. The steady-state phase values were very similar for both normal and malignant cells: (49 ± 4, $n = 4$) and (59 ± 9, $n = 5$), respectively (Tables I and II).

Effects of methotrexate on amino acid transport in fibroblasts and malignant colonocytes

The effects of methotrexate on the previously described amino acid parameters were calculated in the same way. The experiments used a cell population previously exposed to a concentration of 0.5 mg/ml of methotrexate for one of three different periods: 20 min, 4 h and 24 h.

TABLE II

L-[^{3}H]serine and L-[^{3}H]phenylalanine uptake and efflux in perfused human colon adenocarcinoma cells

As with human fibroblasts (Table I), L-[^{3}H]serine (30 s) and L-[^{3}H]phenylalanine (60 s) initial uptake and net 3 and 23 min uptake and [efflux] by human colon adenocarcinoma cells were measured in the absence or presence of 0.5 mg/ml methotrexate. Details in legend to Table I. (Student's t-test ** a-b, a-c, a-d, $P < 0.001$).

Amino acid	Experimental condition	30 μM uptake [efflux], mean ± S.E. (n)		
		30 s	3 min	23 min
L-[^{3}H]Serine	control [a]	43 ± 3 (3)	0.9 ± 2 (3)	−16 ± 2 (3)
			[98 ± 5 (3)]	[138 ± 6 (3)]
	+ methotrexate [b]	46 ± 2 (3)	17 ± 2 (3) **	6 ± 1 (3) **
	0.5 mg/ml, 20 min		[63 ± 5 (3)]	[86 ± 3 (3)]
	+ methotrexate [c]	43 ± 4 (3)	18 ± 2 (3) **	7 ± 1 (3) **
	0.5 mg/ml, 4 h		[59 ± 2 (3)]	[83 ± 3 (3)]
	+ methotrexate [d]	41 ± 0.5 (3)	18 ± 3 (3) **	6 ± 0.5 (3) **
	0.5 mg/ml, 24 h		[57 ± 7 (3)]	[85 ± 2 (3)]
		60 s	3 min	23 min
L-[^{3}H]Phenylalanine	control	86 ± 3 (5)	80 ± 4 (5)	59 ± 9 (5)
			[7 ± 4 (5)]	[32 ± 8 (5)]
	+ methotrexate	76 ± 6 (3)	72 ± 5 (3)	56 ± 5 (3)
	0.5 mg/ml, 20 min		[5 ± 2 (3)]	[25 ± 5 (3)]

The initial uptake of L-[^{3}H]serine in human fibroblasts increased following the perfusion with methotrexate for 20 min; however, a significant decrease was noted at steady state (Table I). In colon tumour cells, however, methotrexate had no effect on the initial uptake maximum, but it induced an increase in uptake during the steady-state period that changed the values from negative to positive so that they were similar to those of normal fibroblasts. (Compare Fig. 3, [TC L-Ser + Mtx] with the inset of Fig. 2.) The results obtained after incubating the colon tumour cells with methotrexate for periods of 4 h and 24 h were not significantly different from those obtained after 20 min.

The transport of L-[^{3}H]phenylalanine in both fibroblasts and malignant colonocytes were not affected by methotrexate (Tables I and II).

Discussion

The process of tumoral development taking place in previously normal cells is associated with the reorientation of the cellular metabolism towards increased production of protein and nucleotides. In neoplastic cells, the amino acids become particularly important because of their accelerated protein turn-over. Characteristically, tumour cells exhibit very early in their development increases in their uptakes of amino acids [10,20,21].

In normal cells, amino acids enter the cell through several different energy-dependent transport systems that can establish a concentration gradient across the membrane [7,22,23]. Once in the cytosol, the amino acids are directed into the different metabolic pathways. Although it is fundamentally important to elucidate the mechanisms that control each stage of amino acid cellular transport and metabolism, present techniques cannot differentiate sequentially the several components of the amino acid transport from the metabolic events that occur in the cytosol [7–8].

To obtain information that would permit us to differentiate between transport and metabolism, the cellular uptakes of two neutral amino acids, phenylalanine and serine, were studied in human fibroblasts and in human adenocarcinoma cells because it is known that these two amino acids are transported by different systems and metabolized by different intracellular pathways [22]. Of course, one cannot meaningfully compare the amino acid uptakes of these two dissimilar cell types like the human adenocarcinoma cells and human fibroblasts used in this work. The human fibroblasts were chosen because they are fast-growing cells, they are a convenient, easy-to-manage experimental model and they serve as a technical reference because there is a great deal of information about their amino acid transport systems in the literature [25–28].

As expected, phenylalanine uptakes by both fibroblasts and malignant colonocytes were high and were not affected by MTX. In most animal cells, L-phenylalanine transport is basically mediated by the sodium-independent L system. This system has been found in human fibroblasts and has been shown to have a large transportation capacity that can be potentiated by trans-stimulation [25,26]. The results of this present work show that the fibroblasts had high uptakes of L-phenylalanine (Table I) and that the addition of unlabelled L-phenylalanine produced auto-inhibition (Fig. 1C). However, on the basis of the cross-inhibition experiments, it seems that L-phenylalanine transport by human colon adenocarcinoma cells could be mediated by two or more transport systems with overlapping specificities. In the loading experiments, the colonocytes had higher uptakes of phenylalanine than the fibroblasts (Table II). Similar results are reported by White [29] for S1814, DSI fibroblasts and HT29 rectal-cancer cells.

Treatment with methotrexate did not modify phenylalanine transport in the two types of cell used in this work. This ineffectiveness could be due to the fact that methotrexate does not directly affect phenylalanine metabolism, except for the modulation of phenylalanine hydroxylase through inhibition of its coactor H_4-biopterin [30].

Serine plays an important role in the synthesis of nucleotides and proteins. Several authors [31–33] report that in tumour cells there are increases in the activities of those enzymes involved in serine biosynthesis and also in the activities of those which control the use of serine as a precursor of purine nucleotides. On the other hand, those enzymes involved in serine catabolism are either absent, or are only present in very small concentrations [33]. These observations indicate that in tumour cells the serine metabolism has been reorientated towards the synthesis of those nucleotides required to support the greatly accelerated growth. Consequently, serine appears to be a particularly suitable metabolic indicator to differentiate between membrane associated transport processes and those which are essentially cytosolic events.

In human fibroblasts, the ASC system shows a significant affinity for serine and it can be trans-stimulated by intracellular amino acids [26]. In the present work, measurements of L-serine uptake in normal fibroblasts during the steady-state periods were less than those of the initial phase (Fig. 2, inset); most probably due to cellular efflux. Moreover, when the isotope-containing perfusion medium was changed to one free of isotopes, the injection of a pulse of 10 mM L-serine trans-stimulated the tracer efflux by homo-exchange: this suggests that the ASC system has a bidirectional transport capacity. The treatment with MTX potentiated initial L-serine uptake by human fibroblasts (Table I), probably because inward transport via the ASC system was trans-stimulated by the intracelular accumulation of the

L-serine [26]. However, these results do not exclude a direct action of MTX on amino acid transport systems, since steady-state uptakes were affected in the opposite direction (Table I).

High initial uptakes of serine by colon tumour cells were measured in this study (Table II). Increased uptake of serine by tumour cells is also described in the literature. Thus, Sauer et al. [34] measured the differences between arterial and venous amino acid concentrations in experimental animals following transplants of hepatomas and observed that, quantitatively, tumours take-up more glutamine than serine. Thorndike et al. [28] reported elevated serine uptakes and increased incorporation of serine in leukaemic leukocytes and Rowe et al. [35] showed that in lectin-activated leukocytes serine became an essential amino acid; it could not be synthetised sufficiently to meet demand because it was the main source of carbon for nucleotide synthesis.

The most characteristic feature of serine transport by malignant colon cells in this present work was the negative uptake measured during the steady-state period (Fig. 3). To determine if this negative serine uptake was produced by the rapid efflux of intracellular L-[^{3}H]serine, the cells were treated with methotrexate to eliminate the main degradation pathway of serine [33]. MTX-treatment had no effect on initial uptakes, but it reversed the previous negative steady-state uptake values. The modified curve of net L-[^{3}H]serine uptake after methotrexate treatment suggests that the radioactivities detected in non-treated cells may be due, in part, to the metabolites of L-[^{3}H]serine and also that methotrexate does not directly affect serine transport in perfused human colonic tumour cells. Therefore, most of the differences induced in serine uptake by treatment with methotrexate could represent the serine consumed in the tumour cells to synthesize nucleotides. Should this be the case, the net transport of serine could be modulated by the rate of intracellular metabolism of serine and, in particular, by the activity of serine hydroxymethyltransferase which acts in conjunction with the tetrahydrofolate cofactor inhibited by methotrexate [12]. In this way, in colon adenocarcinoma cells, the concentrative serine transport of normal fibroblasts would become non-concentrative and this suggests a control model similar to that of monosaccharides, nucleotides and nucleobases [36].

Acknowledgements

This work was financially supported by a Project Grant FIS (Fondo de Investigaciones Sanitarias, Spain) 86/1015 and Plan Andaluz de Investigación Project Grant 1988 to Prof. S. Perán. M. Muñoz was the recipient of a Spanish Education Ministry Reincorporation Fellowship. We gratefully acknowledge the expert advice of Prof. Nuñez de Castro. We thank Dr. A. Maté for providing the tumoral tissue and Miss Marina Muñoz, Mr. G. Perán and Mr. J. Lopez for their assistance with some of these experiments and Mr. D.W. Schofield for translating the manuscript.

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