URIDINE TRANSPORT PROPERTIES OF MAMMALIAN CELL MEMBRANES ARE NOT DIRECTLY INVOLVED WITH GROWTH CONTROL OR ONCOGENESIS

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1. Introduction

The transport system for cellular nutrients is the suggested target for control when cells are stimulated to grow or are transformed to a malignant state [1]. A well-studied model for growth control is the serum activation of 3T3 cells (mouse fibroblasts). Uptake of uridine into these cells is one of the earliest events to be activated by serum [2]. At low uridine concentrations intracellular metabolic trapping (e.g., phosphorylation) of uridine is activated rather than transport across the cell membrane [3]. Here we provide a kinetic analysis of the transport step in normal and in SV-40 transformed 3T3 cells. The Michaelis constant (K_m) for transport in these and several other different cell lines is shown to be some 2 orders of magnitude greater than that in [4]. Our results suggest that no changes in transport of uridine occur in growth control or transformation.

2. Experimental

2.1. *Cell culture*

3T3 cells were propagated in Dulbecco's modified Eagle's Medium (DMEM) to which 10% foetal calf serum, antibiotics and glutamine had been added. The cells were incubated in a humidified CO_2 atmosphere at 37°C.

For uptake experiments the cells were subcultured into 30 mm dishes in 5% foetal calf serum. Medium was replaced at three days. Uptake experiments were usually done after 7–10 days. In order to render the cells quiescent, the medium was changed to 0.5% serum in DMEM 16 h before an experiment. Before the uptake experiment, cells were incubated at 37°C for 45 min with DMEM containing either 10% dialysed foetal calf serum (activated cells) or 10% phosphate-buffered balanced salts medium, PBS, (quiescent cells). This treatment was followed by a further 30 min incubation at 37°C in either PBS or PBS-containing 2-deoxy glucose (2-DG). SV-40-transformed 3T3 cells were grown in 5% new born calf serum and not treated otherwise before uptake experiments.

2.2. *Uptake experiments*

Uptake of uridine (Ur) and cytosine–β-D-arabinoside (CAR) into acid-soluble pools of normal and SV-transformed 3T3 cells under various conditions was studied as a function of time. Two washes with PBS at 20°C prepared the cells for the uptake experiments. The cell cultures were then exposed to approx. 0.5 ml solutions of [5-^{3}H]uridine at 2 μCi/ml or [^{3}H]CAR at 4 μCi/ml in PBS at 20 ± 1°C, pH 7.4. At desired time intervals, the cells were washed 5 times with ice-cold PBS and extracted with 0.75 ml 5% trichloroacetic acid at 4°C for 20 min. Aliquots of 0.5 ml were taken for liquid scintillation counting.

3. Results and discussion

Meaningful transport kinetic data is difficult to obtain because intracellular phosphorylation of uridine follows the transport step. Were phosphorylation rapid enough at all accessible uridine concentrations, free uridine could never exist inside the cell, transport would always be rate limiting and hence kinetic analysis would be unequivocal. However, if phosphorylation were non-existent, classical methods of transport

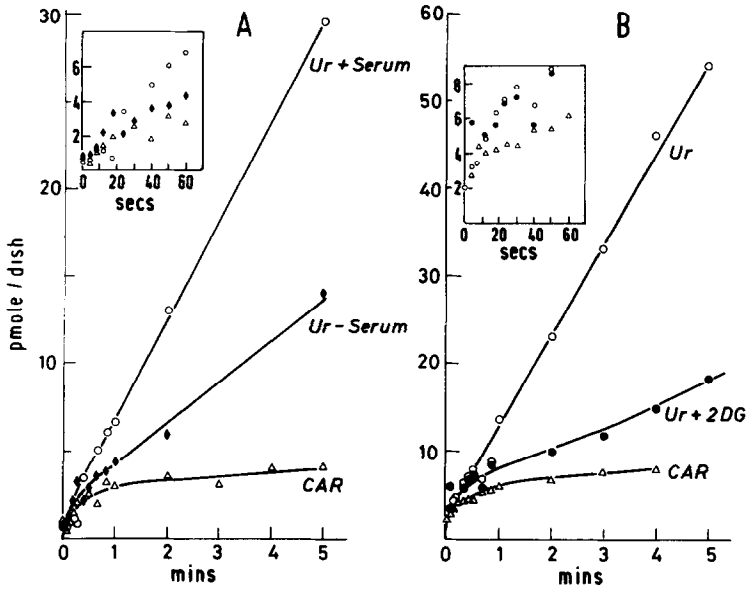

Fig.1. Uptake of uridine (Ur) and of its poorly metabolized analogue cytosine–β-D-arabinoside (CAR) into acid-soluble pools of 3T3 cells, as a function of time. (A) Uptake by activated (○) and quiescent (♦,△) cells. (B) Following the activation of the cells, they were further incubated at 37°C for 30 min with either PBS (○,△) or PBS containing 10 mM 2-deoxyglucose (2-DG, ●). Experiments A and B were performed at different times with different cell densities, as reflected by the facilitated diffusion level, reached by CAR. The different amount of uridine taken up by the cells in the 2 identical experiments (activated cells) can be accounted for by the difference in the number of cells/dish.

analysis [5] would be applicable.

To reduce the phosphorylation capacity of the cells one can attempt to reduce the level of ATP. In our experience use of the metabolic inhibitor KCN [6] is not satisfactory, since in its presence cells fail to attach well to the dish. An alternative approach uses 2-deoxyglucose (2-DG), which enters the cells, is then phosphorylated without further metabolism [7] and thus serves as a trap for cellular ATP. Treatment of 3T3 cells with 50 mM 2-DG for 30 min at 37°C indeed reduces the level of ATP by 72% [8]. Figure 1B shows that pretreatment of 3T3 cells with 2-DG reduces the steady state level of uridine uptake by serum-activated cells to that found for the quiescent cells (fig.1A). On the other hand, as the insets to fig.1 show, uptake at short times (up to 30 s) is unaffected by 2-DG pretreatment, or by serum activation. Thus the behaviour of quiescent cells is mimicked by cells pretreated with 2-DG. Here too a pronounced curvature is seen in the uptake curve as opposed to the nearly straight line found for the serum-activated cells. After 2-DG pretreatment, phosphorylation of uridine is the rate-limiting step in the uptake process both in the activated and the quiescent cells. (Note that the differences in the absolute values of the results in panels A and B of fig.1, are due to different cell densities.)

The fact that all the uridine uptake curves are superimposable within the first 30 s, confirms the finding [3] that serum activation does not affect transport. The fact that pretreatment of serum activated cells with 2-DG generates an uptake curve which parallels that of quiescent cells (fig.1), indeed shows that serum activation is concerned with intracellular metabolic events. From about 1 min, all the uridine uptake curves are strictly linear. Thus it is not sufficient merely to demonstrate linearity with time over long times, since a subtle curvature at short times can easily be overlooked.

We have developed criteria for analysis of kinetic parameters of transport from uptake curves in which the rate-limiting transport region is well separated from the rate determining metabolic trapping region. These criteria are detailed [9]. The estimation of

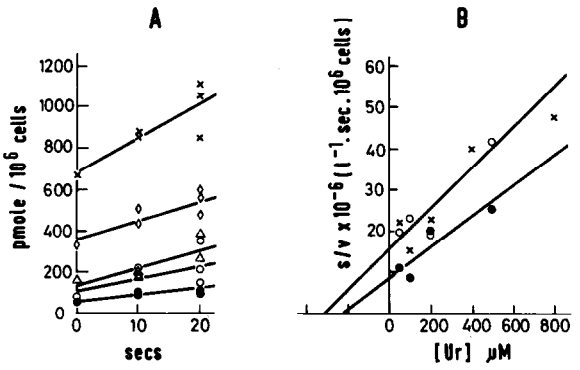

Fig.2. (A) Uptake of uridine into acid soluble pools of serum-activated, ATP-depleted 3T3 cells as a function of time and uridine concentration. The concentration of 2-DG was 50 mM and it was present during the uptake experiment. Uridine concentrations were: (×) 800 μM; (◇) 400 μM; (△) 200 μM; (○) 100 μM and (●) 50 μM. (B) Dependence of the rate of uridine uptake (v) on the uridine concentration, (S), plotted as S/v versus S. Values of v were obtained as the slopes of fig.2A (×), or in a separate experiment for ATP-depleted, serum-activated (○) or quiescent cells (●).

initial rates relies on the first portion of the uptake curve. This part is better defined after partial ATP depletion.

Using the ATP-depletion technique we studied uptake of uridine into quiescent and serum-activated 3T3 cells at short times, over a wide range of uridine concentrations. Figure 2A shows uptake curves for serum-activated cells. Figure 2B shows the kinetic analysis of these and similar data. The resulting kinetic parameters are recorded in table 1. The amount of substrate trapped outside the cells is at least 10% of its facilitated diffusion level [9]. Since initial transport rates are measured when internal substrate concentrations are well below this level, the relative contribution of the zero time amount is rather high and is proportional to the external substrate concentration. This inherent difficulty is the main reason for the noise in the data. The maximum velocities of transport and the Michaelis constants for serum activated and quiescent cells are statistically indistinguishable.

An interesting question is whether transport of uridine is affected when 3T3 cells are transformed by virus. Figure 3A depicts results of an experiment in which the time course of uptake of uridine at various concentrations into SV-40-transformed 3T3 cells was studied. Kinetic analysis of the initial rates of uptake is given in fig.3B, and the resulting kinetic parameters are also recorded in table 1. Pre-treatment with 2-DG was not necessary in this case since the bend of the uptake curve is very apparent.

The Michaelis constant for this system is indistinguishable from that for the non-transformed 3T3 cells, (whether serum activated or not). The maximum velocity of transport is statistically greater for trans-

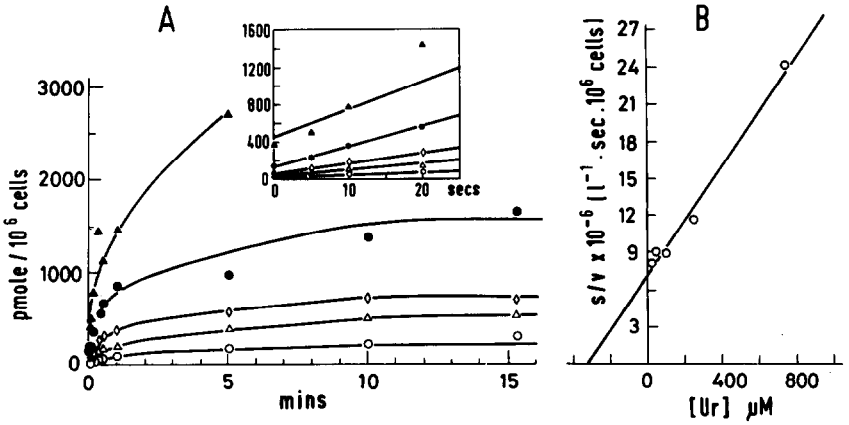

Fig.3. (A) Uptake of uridine into acid-soluble pools of SV-40-transformed 3T3 cells as a function of time and concentration of uridine. Concentrations of uridine were as follows: 20 μM (○), 50 μM (△), 100 μM (◇), 250 μM (●), 750 μM (▲). The inset shows the data at short times. The straight lines were drawn by linear regression. (B) Dependence of the rate of uridine uptake (v) on the uridine concentration (S) plotted as S/v versus S. Values of v were obtained as the slopes of fig.3A. The straight line was obtained by linear regression.

Table 1
Kinetic parameters of transport of uridine and CAR in mammalian cells in various states of growth and transformation

Type of cell	Uridine		CAR		Ref.
	K_m (μM)	V_{max} (pmol/10^6 cells.s)	K_m (μM)	V_{max} (pmol/10^6 cells.s)	
3T3 activated	220 ± 86[i]	27.0 ± 8.4	–	–	a
mouse fibroblasts quiescent	311 ± 82[i]	20.3 ± 6.2			
3T3-SV 40 virus-transformed mouse fibroblasts	323 ± 24[i]	45.5 ± 3.1	–	–	b
NIL-8 hamster fibroblasts	530 ± 160[i]	54 ± 13	–	–	[9]
NIL-8 SV virus-transformed hamster fibroblasts	400 ± 64[i]	25 ± 3	118 ± 25[i]	5.2 ± 0.3	[9]
MCT chemically-transformed hamster cells	–	–	342 ± 135[i] 503 ± 146[iii]	26 ± 4.2 41 ± 11.3	c
Rat B77 virus-transformed rat fibroblasts	–	–	414 ± 116[i]	2.7 ± 0.5	d
RBC human red blood cells	73 ± 69[i] 400 ± 120[ii] 1290 ± 110[iii]	1.3 ± 0.1 4.95 ± 0.78 18.8 ± 1.1	–	–	[12]

[i] zero trans influx
[ii] zero trans efflux
[iii] equilibrium exchange

[a] fig.2B
[b] fig.3B
[c] 20-methyl-cholanthrene-transformed hamster cells – data from [11]
[d] rat fibroblasts cell line transformed by Rous sarcoma virus (unpublished observations of Koren R. et al.). Data obtained as 1 min uptakes where time course is still linear; analysed as in fig.2,3

formed cells. This may possibly represent a difference between these cells, arising as a feature of their transformation. It may, however, represent an accidental difference which has developed between the two cultures during the time in which they have been cultured separately.

The Michaelis constant that we find for uridine transport in 3T3 cells is up to 2 orders of magnitude higher than that reported for uridine uptake in these cells [4]. Since uridine kinase, the enzyme that phosphorylates uridine, displays a low K_m for uridine of 10^{-5} M [9,10], it would appear that previous investigations of uptake were concerned with the phosphorylation step. A similar conclusion for thymidine uptake into Novikoff rat hepatoma and mouse L cells was reached by other investigations [13,14].

We have obtained also kinetic data for transport of uridine and its analogue, cytosine–β-D-arabinoside, in a number of different cells. Full details of these studies will be reported elsewhere. The results (together with results from the literature) are collected in table 1. It is clear from table 1 that uridine transport

in these mammalian cell systems displays Michaelis constants of the order of 10^{-4} M.

There is indeed a remarkable similarity between the transport kinetic parameters for these cells in spite of the fact that they are of different origins and are in different states of growth or transformation by virus or chemical mutagen. It would appear that the uridine transport system in different cells is essentially of a similar nature. Furthermore, it is clear that it is not the transport of uridine which is responsible for those changes in uridine uptake which might occur when cells are stimulated to growth or are transformed.

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