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NUCLEOSIDE TRANSPORT IN NORMAL AND POLYOMA-TRANSFORMED CELLS: KINETIC DIFFERENCES FOLLOWING ADENOSINE AND SERUM OR INSULIN STIMULATION

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SUMMARY

Hamster and mouse embryo cells cultured for 24 h in serum-less medium respond to serum, insulin or adenosine with an increase in the rate of uridine uptake. Kinetic experiments indicate that serum and insulin increase the V for uridine transport without altering the K_t. In contrast, adenosine leads not only to an increased V, but also a significant alteration in K_t. Phloretin is shown to be a potent, competitive inhibitor of uridine uptake in unstimulated (S^-) cells as well as those stimulated by both serum (S^+) and adenosine (S^-:AR). The K_i for this inhibition is the same for S^- and S^+ cells but differs from the K_i for the S^-:AR cells. Furthermore, it has been found that two polyoma-transformed hamster tumor cell lines respond to serum and phloretin in the same qualitative manner as normal cells.

INTRODUCTION

Recent studies of the mechanism by which nucleoside uptake is regulated in mammalian cells has led to the view that one of several distinct processes may be rate-limiting in the uptake of individual nucleosides. For example, the rate of thymidine uptake is closely correlated with the level of the phosphorylating enzyme thymidine kinase[1,2]. In contrast, several studies have suggested that the rate of uridine uptake is limited by the rate of membrane permeation[3–5].

It is of considerable interest then that uridine transport is modulated by a variety of factors such as the addition of fresh serum to confluent cells[6], cell density[6,7], changes in the pH of culture medium[8], phytohemagglutinin stimulation of lymphocytes[3,9], and the addition of serum, fetuin, adenosine, cyclc AMP, and ATP[10] or insulin to serum-less mouse cells[5] or bone cells[11]. These fiindings suggest that permeation pathways utilized by uridine are distinct from those of thymidine and are altered in response to conditions which modify cellular function.

To investigate the mechanism by which the uridine transport system is controlled by exogenous factors, the kinetics of uptake in the presence and absence

of phloretin, a potent inhibitor of sugar transport[12–17] were studied. Previous work has shown that the apparent K_m for uridine transport in both lymphocytes stimulated by phytohemagglutinin[3] and mouse cells responding to serum[5] is not altered while the V changes significantly. Furthermore, the inhibitory effect of a variety of compounds such as heterologous nucleosides[4,18,19], colchicine[20], persantin[4,21] and phenethylalcohol[4,22] has been employed to characterize the transport system. In this report, data is presented which shows that adenosine stimulates uridine transport by a mechanism that is different than that of serum or insulin.

MATERIALS AND METHODS

Cells and medium

Hamster embryo cells and the polyoma-transformed hamster tumor cell line HTC-3049-91TC were prepared as described previously[23]. The isolation of the polyoma-transformed tumor cell line HTC-3049-3 has also been described[24].

All cells were set at $1 \cdot 10^6$ cells per 60 mm Petri dish (Falcon) in Eagle's[25] minimal essential medium (AutoPow-Flow Labs) supplemented with penicillin, streptomycin, glutamine and 10% (v/v) fetal calf serum. After the cells had been incubated for 8 h at 37 °C, most had attached to the plastic dish and were refed with serum-less minimal essential medium.

Stimulation of serum-free cells

After 24 h of incubation in serum-less medium, the cells were considered to be in the S^- (unstimulated) state. The S^- cells were then refed with fresh serum-free medium to which was added either fetal calf serum to a final volume of 10%, adenosine to a final concentration of 0.1 mM, or insulin (100 μg/ml). The cells were designated S^+, S^-: AR, or S^-: insulin, respectively.

Uridine uptake studies

Uptake of [5-^{3}H]uridine, spec. act. 26.0 Ci/mmole (New England Nuclear), was studied by applying 1.5 ml of [^{3}H]uridine dissolved in Tris-buffered saline[26] to each Petri dish. For the kinetic studies, the inhibitor and various concentrations of unlabelled uridine were combined with a constant amount of [^{3}H]uridine. Following the labeling period, the cells were put on ice, washed rapidly three times with iced Tris-buffered saline and extracted in 2.0 ml of iced 10% trichloroacetic acid. The cells were then washed twice with 10% trichloroacetic acid and dissolved in 0.5 M KOH overnight at 37 °C in order to obtain the acid-insoluble fraction. The acid-soluble and acid-insoluble fractions were counted in Bray's[27] scintillation mixture, using Omnifluor. Duplicate dishes were used in all experiments and duplicate samples were counted from each dish.

The nucleosides uridine, thymidine, and adenosine and the hormone insulin (bovine pancreas, B. grade) were obtained from Calbiochem and the inhibitor, phloretin, from K and K Laboratories. A phloretin stock solution (100 mM) was prepared in absolute ethanol and diluted just prior to use in Tris-buffered saline. Ethanol controls showed no effect on the uptake of uridine by the cells at the concentrations used in treating the cultures.

RESULTS

Distinctive uridine uptake systems in hamster embryo cells stimulated by adenosine and fetal calf serum

The rate of uptake of uridine at various substrate concentrations over a 5-min period in hamster embryo cells was measured and the uptake velocity, v, plotted *versus* the substrate concentration, S, using the reciprocal Lineweaver–Burk plot[28]. Under these conditions, uridine uptake is a linear function over at least the first 5 min. Fig. 1 shows the characteristics of uridine uptake in serum-less cells (S^-) before and after 6 h stimulation with serum (S^+). It is evident that serum has only a quantitative effect on the uptake of uridine as evidenced by an increased V with the same K_t*. The results of several such experiments are summarized in Table I.

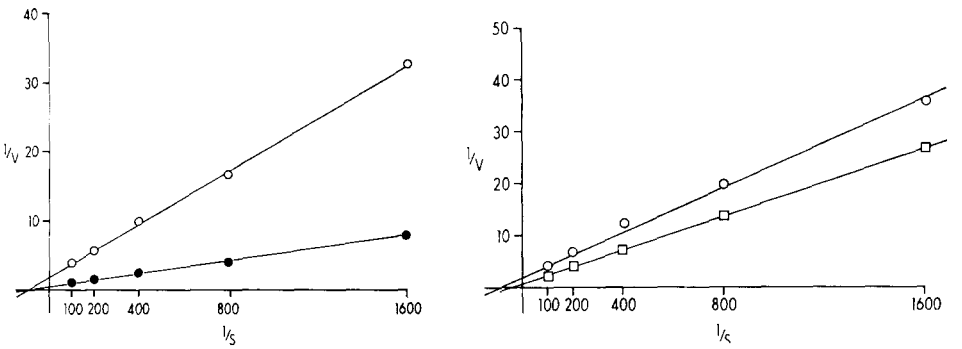


Fig. 1. Lineweaver–Burk plot of uridine uptake kinetics in hamster embryo cells cultivated in serum-less medium (○—○) or after 6 h stimulation with serum (10%) (●—●). Data were based on total uridine uptake into acid-soluble *plus* acid-insoluble fractions. See Table I for further details. V, moles/dish per 5 min $\times 10^{-10}$; S, mM.

Fig. 2. Lineweaver–Burk plot of uridine uptake kinetics in serum-less cells (○—○) and in those stimulated 6 h with 0.1 mM adenosine (□—□). See Table I for further details. V, moles/dish per 5 min $\times 10^{-10}$; S, mM.

The effect of adenosine, on the other hand, is quite different. Fig. 2 demonstrates that the uridine transport system in S^- hamster cells stimulated by 0.1 mM adenosine is characterized by a different K_t as well as an increased V. Although the effect is small, this response has been seen in repeated experiments in hamster embryo cells, summarized in Table II, as well as in mouse embryo cells. In addition, if it is assumed that a portion of the uptake function in the adenosine stimulated cells (S^-: AR) is residual serum-responsive activity, a corrected K_t can be calculated which would more nearly reflect the K_t due to the adenosine stimulated function. This corrected K_t is also shown in Table II. These findings indicate that there is a qualitative distinction between the transport systems stimulated by serum and adenosine.

* K_t is defined as the substrate concentration at which $1/2$ V is achieved. It is analogous to the K_m in enzyme kinetics.

TABLE I

THE EFFECT OF ADENOSINE, THYMIDINE AND PHLORETIN ON URIDINE TRANSPORT IN SERUM-LESS (S−) AND SERUM-STIMULATED (S+) HAMSTER EMBRYO CELLS

Cells cultivated 24 h in serum-less medium were refed with either serum-less (S−) or serum-containing (10%) medium (S+) and incubated further for 6 h. The uptake of uridine was measured for 5 min at 37 °C at different concentrations of uridine prepared by adding a constant amount of [^{3}H]uridine (26 Ci/mmole) to 2-fold dilutions of unlabeled uridine (0.01–0.00625 mM) to give a final concentration of 5 μCi/1.5 ml in Tris-buffered saline. The actual specific activity was calculated from measurements of radioactivity on each stock mixture. The uptake is the sum of acid-soluble *plus* acid-insoluble fractions. The K_t and V were calculated from the reciprocal Lineweaver–Burk plot of the uptake velocity (v) *vs* substrate concentration (S). To calculate the K_i for adenosine, thymidine and phloretin, uridine uptake in the presence of 0.1 mM, 0.2 mM and 0.05 mM concentrations, respectively, of each compound was measured and the results plotted by the Lineweaver–Burk method. The K_i was calculated from the relationship: intercept $= -1/K_t(1 + [I]/K_i)$ where: $[I]$, inhibitor concentration; intercept is that on the 1/S axis; K, calculated from the uptake kinetics in the absence of the inhibitor.

State of cell	K_t (*mM*)	$V \times 10^{-10}$ (*moles/dish per 5 min*)	*Inhibitor*	K_i (*mM*)
S−	0.010 ± 0.001	0.58	Adenosine	0.042
			Thymidine	0.200
			Phloretin	0.021
S+	0.010 ± 0.001	3.30	Adenosine	0.042
			Thymidine	0.200
			Phloretin	0.021

TABLE II

THE EFFECT OF PHLORETIN ON URIDINE TRANSPORT IN SERUM-LESS HAMSTER EMBRYO CELLS STIMULATED WITH ADENOSINE

Cells cultivated in serum-less (S−) medium or stimulated 6 h with 0.1 mM adenosine (S−:AR) were prepared and [^{3}H]uridine uptake parameters measured as described in Table I. A corrected K_t and V^* for uridine uptake following adenosine stimulation was calculated by subtracting from the total quantity of uridine transported at each concentration of extracellular uridine, that portion of transported uridine entering by way of the serum-responsive sites which remain in the S− cells kept as controls in the same experiment. The corrected V and K_t are then derived from the reciprocal Lineweaver–Burk plot.

State of cell	K_t (*mM*)	$V \times 10^{-10}$ (*moles/dish per 5 min*)	*Inhibitor*	K_i (*mM*)
S−:AR	0.024 ± 0.006	1.00	Phloretin	0.017
S−:AR	*0.045 ± 0.005	*0.50	—	—

Stimulation of uridine uptake in mouse cells by insulin

The findings presented in the previous section are of considerable interest, in view of the fact that this is the first evidence for a change in the K_t of the uridine

transport system in rodent cells. The K_t for uridine transport in mouse cells (0.01 mM) was recently found to be identical to that reported here in hamster cells[5]. It became important to measure the effect of insulin, a stimulator of uridine uptake in S^- mouse cells on the K_t of that transport function. As shown in Table III, serum-less mouse embryo cells stimulated 6 h with insulin (100 μg/ml) show a significant increase in V with no change in K_t. This finding indicates that insulin stimulates uridine transport in a way similar to serum and distinct from adenosine.

TABLE III

THE EFFECT OF INSULIN ON URIDINE TRANSPORT IN SERUM-LESS (S—) MOUSE EMBRYO CELLS

Mouse embryo cells (MEF) were prepared and studied according to the method described in Table I. [^{3}H]Uridine uptake in S— cells and those stimulated 6 h with 100 μg/ml of insulin was then measured.

State of cell	K_t (*mM*)	$V \times 10^{-10}$ (*moles/dish per 5 min*)
MEF S—	0.011	2.0
MEF S—:insulin	0.011	3.3

The effect of phloretin on uridine uptake

During studies of nucleoside uptake using transport inhibitors, it was found that phloretin was a potent inhibitor of uridine uptake. Fig. 3 illustrates that phloretin competitively inhibits uridine uptake in the S^- hamster embryo cell. Furthermore, as shown in Table I, the K_i for phloretin inhibition in the S^+ and S^- cell is identical. This finding is consistent with the data of Fig. 1, which demonstrates that the K_t of the uridine transport function in the S^+ cell is identical to that in the S^- cell.

Other experiments were performed to determine the effect of phloretin on the uptake function stimulated by adenosine. As shown in Table II, the K_i for phloretin in S^- cells stimulated by adenosine (0.017 mM) is somewhat lower than that for S^+ or S^- cells (0.021 mM) shown in Table I. It should be noted, however,

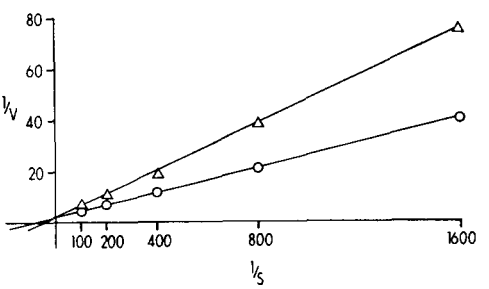


Fig. 3. The effect of phloretin (0.05 mM) on the kinetics of uridine uptake into hamster embryo cells cultivated in serum-less medium as described in Table I and plotted by the Lineweaver–Burk method. See Table I for further details. ○—○, S— control; △—△, S— *plus* phloretin.

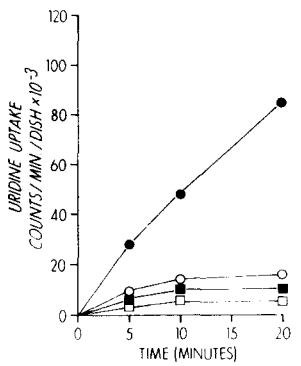
that the K_i for phloretin acting on the adenosine-stimulated uridine uptake process is a complex function due to the presence of residual activity of the serum-responsive uptake system in S^- cells, as indicated above. The evidence that the K_t and phloretin K_i in adenosine stimulated cells are distinct from those in serum or insulin stimulated cells supports the postulate that adenosine alters the uridine transport system by a mechanism distinct from serum or insulin.

Time course of phloretin inhibition

Fig. 4 demonstrates the onset and time course of the effect of 0.1 mM phloretin on simultaneous uridine uptake in S^- and S^+ hamster cells. The onset of inhibition in both cell types is rapid and increases with time. However, there is a quantitative difference in the degree of inhibition in the S^- and S^+ cells. These results demonstrate that phloretin inhibits uridine transport that is stimulated by serum, but fails to eliminate a small residual uptake.

Fig. 5 shows the results of an experiment to determine whether the residual uptake after phloretin treatment could be further reduced by pretreating the cells with phloretin to allow penetration of this hydrophobic compound to deeper sites in the membrane.

The uptake of uridine in the presence of phloretin was measured in S^- and S^+ cells, part of which had been pretreated for 20 min with phloretin (Group B). Uptake of uridine in the presence (Group B and C) and absence (Group A) of phloretin was then measured. The uptake of uridine was inhibited to the same residual level, irrespective of the uptake rate in the S^+ and S^- cells or the length of exposure to the inhibitor. This experiment further substantiates the existence of a residual permeation pathway for uridine that is not sensitive to phloretin.



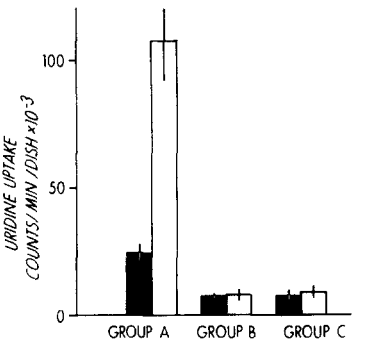


Fig. 4. Comparison of uridine uptake into serum-less (S^-) and serum-stimulated (S^+) hamster embryo cells in the presence or absence of 0.1 mM phloretin during the uptake process using [^{3}H]uridine (3 μCi/ml, 26.0 Ci/mmole) at 37 °C. ●—●, S^+ control; ○—○, S^- control; ■—■, S^+ *plus* phloretin; □—□, S^- *plus* phloretin.

Fig. 5. Comparison of uridine uptake into serum-less (solid bar) and serum-stimulated (open bar) hamster embryo cells after (A) pretreatment for 20 min at 37 °C with Tris-buffered saline and subsequent labeling with [^{3}H]uridine (3 μCi/ml, 26.0 Ci/mmole) for 20 min at 37 °C, (B) pretreatment for 20 min at 37 °C with 0.1 mM phloretin and subsequent labeling in the presence of 0.1 mM phloretin with [^{3}H]uridine for 20 min at 37 °C and (C) pretreatment for 20 min at 37 °C with Tris-buffered saline and subsequent labeling in the presence of 0.1 mM phloretin with [^{3}H]uridine for 20 min at 37 °C.

Reversibility of phloretin inhibition as seen from kinetic studies

Fig. 3 indicates that phloretin is a competitive inhibitor of uridine transport. If this is indeed the case, phloretin should interact reversibly with the inhibited transport site. To determine how readily phloretin can be removed from this site, uptake kinetics were measured in cells which had been treated with phloretin and then the inhibitor removed prior to the labeling.

Serum-stimulated cells were preincubated for 20 min at 37 °C with either 0.1 mM phloretin or Tris-buffered saline. At the end of this period, the cells were washed twice with Tris-buffered saline at 37 °C and labeled for 5 min at 37 °C. Fig. 6 shows that the uptake of uridine in cells pretreated with phloretin is indistinguishable from that in cells never exposed to the inhibitor, indicating that this compound is essentially completely removed from the transport active sites with no measurable residual inhibition. In addition, this experiment demonstrates graphically the reproducibility achieved in this study.

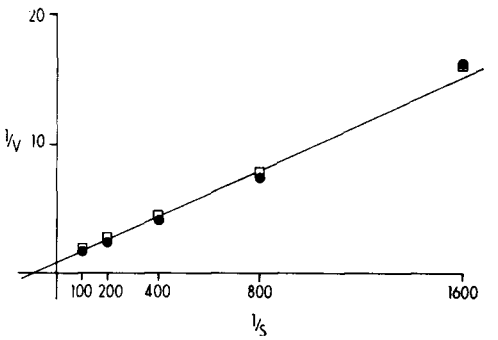


Fig. 6. Reversibility of phloretin inhibition of serum-stimulated (S^+) hamster embryo cells. S^+ cells were treated for 20 min at 37 °C with 0.1 mM phloretin. At the end of this period, the cells were washed twice with Tris-buffered saline at 37 °C and then labeled for 5 min at 37 °C with [^{3}H]uridine diluted in unlabeled uridine such that the final uridine concentrations ranged from 0.000625–0.01 mM. Control cells were pretreated for 20 min at 37 °C with Tris-buffered saline and subsequently washed and labeled as above. Results were plotted according to the method of Lineweaver–Burk. ●—●, S^+ control; □—□, S^+ *plus* phloretin treated.

Inhibition of uridine uptake by adenosine and thymidine

The nucleosides adenosine and thymidine were also found to compete with uridine for transport into S^+ and S^- hamster embryo cells when present in the uptake solution. Initially, adenosine was found to be the more potent inhibitor. Kinetic experiments were then performed to determine the K_i values for adenosine and thymidine on uridine transport and the results are shown in Table I. It is of interest that the K_i for adenosine (0.04 mM) was significantly lower than that for thymidine (0.20 mM), data which confirms the preliminary observation. Furthermore, the Lineweaver–Burk plots were characteristic of competitive inhibition by both nucleosides.

Serum stimulation of serum-less polyoma-transformed hamster tumor cells

The effect of serum on the kinetics of uridine transport in polyoma-transformed hamster tumor cells was then studied to evaluate whether transformed cells would

respond to serum in a manner different from normal cells. This possibility was suggested by the numerous studies demonstrating differences in the serum requirements for growth of normal and transformed cells[29–37]. In Table IV is recorded the K_t for the uridine transport system in two different tumor cell lines in the S^- and S^+ state as well as the K_i for phloretin. It is clear that the K_t for the transformed cells is identical to that in the untransformed, normal embryo cells (Table I). Furthermore, the K_i for phloretin inhibition in one tumor cell line is identical to that in the normal cell line.

TABLE IV

THE EFFECT OF PHLORETIN ON URIDINE TRANSPORT IN TWO SERUM-LESS (S^-) AND SERUM-STIMULATED (S^+) POLYOMA-TRANSFORMED HAMSTER TUMOR CELL LINES

The kinetics of uridine uptake in two lines of polyoma-transformed hamster cells were studied as described in Table I. Cell A is HTC-3049-91TC and cell B is HTC-3049-3.

State of cell	K_t (*mM*)	$V \times 10^{-10}$ (*moles/dish per 5 min*)	*Inhibitor*	K_i (*mM*)
Cell A S^-	0.010±0.001	2.50	Phloretin	0.021
Cell A S^+	0.010±0.001	6.70	Phloretin	0.021
Cell B S^-	0.010±0.001	14.30	—	—
Cell B S^+	0.010±0.001	50.00	—	—

DISCUSSION

Several kinetic parameters of the uridine transport system in serum-less (S^-) normal and polyoma-transformed hamster and normal mouse cells following stimulation with serum, insulin and adenosine are reported. Normal and neoplastic hamster cells respond to serum by increasing the V with no change in K_t, a finding similar to that shown previously for phytohemagglutinin-stimulated lymphocytes[3] and serum-stimulated mouse cells[5]. This study also demonstrates that insulin increases the V in S^- mouse cells without affecting the K_t. Of added interest is the fact that the K_t for uridine transport in both mouse and hamster cells is the same (0.01 mM).

These findings suggest that the mechanism by which serum and insulin stimulate uridine transport in S^- cells is through an increase in the number of functionally active transport sites identical to those that already pre-exist in that cell. This hypothesis is supported by the fact that the K_i for phloretin (0.02 mM), adenosine (0.04 mM) and thymidine (0.20 mM) inhibition of uridine transport in the S^+ state is identical to that in the S^- state. Thus, three distinctly different compounds which competitively inhibit uridine transport (Table I), each with a different K_i, cannot distinguish the transport active sites in the S^- and S^+ state.

Phloretin has been extensively studied as an inhibitor of sugar transport in hamster[12–14,38] and guinea pig[38] small intestine, cat kidney[15] and the red blood cell[16,17]. Both competitive[16,17,38] and non-competitive[12] inhibition of sugar transport

has been observed. In this study, phloretin has been shown to be a competitive inhibitor of uridine transport in both S^- and S^+ normal embryo as well as tumor cells. Furthermore, both adenosine and thymidine act as competivite inhibitors of uridine transport in a manner similar to that shown in other cell systems[18,19].

In view of these findings, the demonstration that, following adenosine treatment of S^- cells, the K_t for uridine transport is significantly altered suggests that the fundamental mechanism by which adenosine enhances uridine transport is different from that of serum and insulin. In an attempt to calculate the 'real' K_t for the adenosine sites, a corrected K_t (Table II) was derived from the observed uptake data following adenosine stimulation by subtracting from it that portion of the transported uridine entering by way of the serum-responsive sites which remain in the S^- cell. This calculation gives a K_t of 0.045 mM which is significantly higher than the K_t of the serum-responsive site (0.01 mM). However, due to the complexity of the overall uridine transport process, it is not possible to conclude from these findings the mechanism by which uridine transport is modulated by the compounds studied here.

Other studies have indicated, however, that nucleosides utilize the same transport carrier in rabbit polymorphonuclear leucocytes. Taube and Berlin[18] demonstrated that several purine and pyrimidine nucleosides (thymidine, adenosine and uridine) share a common transport system. This conclusion was based on the similarity of both the transport K_m and the K_i for transport inhibition of each nucleoside by other nucleosides and their derivatives. It was proposed on the basis of this and other data that the transport active carrier molecule for nucleosides is flexible and susceptible to conformational changes by the substrate. At this point, it is difficult to reconcile this concept of the rather nonspecific nature of the nucleoside carrier system with the evidence for distinctive permeation pathways under independent control as evidenced by the fact that uridine transport can be altered by a variety of conditions[3,5–11], some of which do not affect thymidine transport[10].

The mechanism by which control over uridine transport is exerted is unclear. The rate of permeation through the membrane has been suggested to be the rate-limiting process[3–5] although phosphorylation, as in the case of thymidine[1,2], cannot be excluded. Several possibilities for the effect of serum, insulin or adenosine on S^- cells may be suggested. These potentiators may act by induction of synthesis of additional transport sites, by counteraction of an inhibitor(s)[39] or by modification of pre-existing transport sites[18]. No experimental evidence is available at present to distinguish among these possibilities. Studies are currently in progress to elucidate the mechanism by which selective alteration of uridine transport is affected.

ACKNOWLEDGEMENTS

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