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A LABILE, SERUM-DEPENDENT URIDINE UPTAKE FUNCTION IN MOUSE EMBRYO CELLS

J. D. HARE

Department of Microbiology, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y. 14642 (U.S.A.)

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SUMMARY

Stimulation of uridine uptake in serum-less mouse embryo cells by serum repletion has been shown to require the continual presence of serum for maintenance of maximum activity. Removal of serum terminates further stimulation and is followed by a rapid decay of uptake capacity characterized by a 2–4-fold decrease in V with no change in the apparent K_m of the reaction. Acquisition of uptake capacity is sensitive to inhibitors of both RNA (actinomycin D) and protein (cycloheximide) synthesis. Insulin is shown to substitute for serum in the stimulation of uridine uptake in serum-less cells. Levels of uridine kinase activity do not correlate with uptake capacity in response to either serum or metabolic inhibitors, suggesting that this enzyme is not responsible for the control of uptake. It is proposed that uridine uptake in mouse embryo cells is controlled by a labile system which requires input from the extra-cellular environment to maintain functional capacity. The identity of the rate-limiting process is not known at this time, but is postulated to be some part of the carrier system.

INTRODUCTION

The transport of uridine and phosphate in mammalian cells has been found to be sensitive to the physiological conditions associated with cell division control. This was first shown by Cunningham and Pardee[1] who demonstrated that dialyzed serum stimulated uridine and phosphate uptake in contact-inhibited 3T3 cells. Similar findings have since been reported[2,3]. Furthermore, pH changes[4], as well as low molecular weight substances produced by contact-inhibited 3T3 cells which inhibit incorporation of uridine into RNA[5], have also been shown to reduce transport of uridine[6]. However, the ability of serum to stimulate uridine transport could not be explained e tirely by its ability to bind the inhibitory substances[6].

A second model system is the human lymphocyte which, upon stimulation by phytohemagglutinin, responds with a rapid increase in the capacity to transport uridine[7,8] and 3-*O*-methylglucose[9]. The stimulated state is associated with an increase in transport velocity in the absence of a change in the apparent K_m[8,9]. This finding suggested that the activated transport state was the result of exposure of transport sites rather than an alteration of the structure of the transport molecules.

Uridine uptake in mouse embryo cells maintained in a serum-less medium has recently been shown to decay to baseline levels which can be recalled by the addition of serum or the adenine derivatives adenosine, ATP, or cyclic AMP[10]. This paper presents the results of further studies in this model system which demonstrate the reversible nature of the uridine transport function in mouse embryo cells.

MATERIALS AND METHODS

Cells prepared from 14–18-day-old mouse embryos were grown in Blake bottles using Eagle's[11] minimal essential medium (Auto Pow-Flow Laboratories) with 5 % fetal calf serum. Cells for study were planted in 60-mm plastic petri dishes (Falcon) at $1 \cdot 10^6$ cells per dish. Labeling was carried out in the dish in 2.0 ml of Tris-buffered saline (pH 7.4) with Ca^{2+} and Mg^{2+} (ref. 12) but without glucose. Following exposure to the labeled precursor, the labeling mixture was removed, the cell monolayer rinsed rapidly thrice in iced Tris-buffered saline and an acid-soluble fraction extracted in 2 ml of cold 10 % trichloroacetic acid. The cell monolayer was then washed twice with 10 % trichloroacetic acid and an acid-insoluble fraction was prepared by extracting the residual cell material in 2.0 ml of 0.5 M KOH overnight at 37 °C. The first trichloroacetic acid extract (acid-soluble fraction) and the KOH extract (acid-insoluble fraction) were then counted in Bray's[13] scintillation mixture using Omnifluor (New England Nuclear). Duplicate dishes were used at each experimental point and duplicate samples counted from each dish. The coefficients of variation of the mean in the experiments reported was in the range of 5–10 %.

Uridine kinase was assayed as described by Lin[14] and the phosphorylated product measured by retention on DE-81 paper discs[15]. Enzyme activity is reported as n moles of UMP synthesized per mg protein per 15 min. Chemicals used were obtained as follows: insulin (bovine pancreas, B grade), Calbiochem; cyclic adenosine 3′,5′-monophosphate (cyclic AMP), Calbiochem; actinomycin D, Calbiochem; cycloheximide (Actidione), Mann.

RESULTS

Reversibility of serum-stimulated uridine uptake

Fig. 1 shows the results of an experiment designed to determine the length of time cells cultivated in serum-less medium must be exposed to serum to produce a persistent activation of the uridine uptake function. Cells treated with serum for up to 1.5 h show a significant stimulation of uptake in controls which declines to the baseline level by 4 h if the serum-containing medium is removed and replaced with serum-less medium. Cells stimulated for 3 h and exposed to serum-less medium for only 1 h also show a significant loss of uptake capacity. These results demonstrate that serum must be continuously present to maintain the uptake system in an activated state and that this function decays once the serum is removed.

Loss of uridine uptake capacity after serum removal

That the loss of uptake capacity following the removal of serum in the preceding experiment was not due to an unstable cell population following prolonged cultivation (48 h) in serum-less medium is shown by the results presented in Fig. 2.

Cells cultivated continuously in serum-containing medium also rapidly lose uridine uptake capacity following replacement with serum-less medium. The rate of uridine uptake declines progressively following removal of serum with a significant reduction observed within 30 min.

The basis for the marked reduction of uridine uptake following removal of serum was studied by measuring the uptake rate over a 5-min period at different substrate concentrations. The data were analyzed using the reciprocal Lineweaver–Burk plot and the results are shown in Fig. 3. Following a 4-h exposure to serum-less medium, several experiments showed a 2–4-fold decrease in V with no significant change in the apparent K_m for the association of uridine with the transport system. The apparent K_m for this reaction derived from several such experiments was approximately $1.5 \cdot 10^{-5}$ M.

Requirements for RNA and protein synthesis for maintenance of the activated uptake state

The effect of actinomycin D and cycloheximide on serum stimulation of uridine uptake activity in cells cultivated in serum-less medium is shown in Fig. 4. The drugs were used at concentrations which inhibited RNA and protein synthesis 90 % within 30 min. Both drugs added after either 2 or 4 h of serum treatment lead to a significant

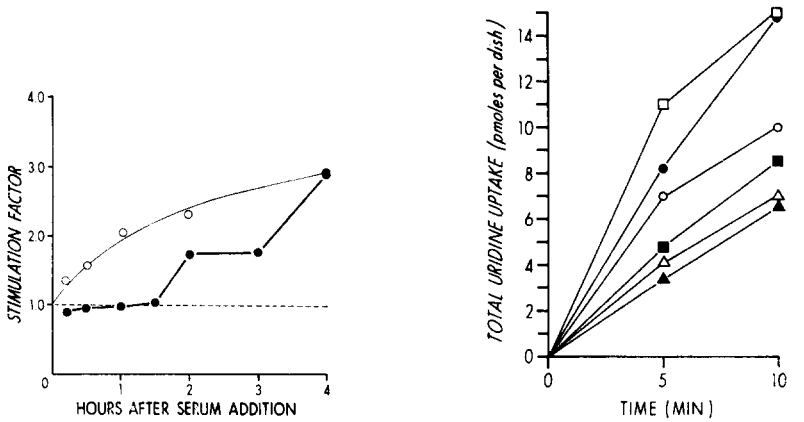

Fig. 1. Uridine uptake in serum-less mouse embryo cells following increasing periods of exposure to serum. Cells cultured 48 h in serum-less medium were exposed to 10% undialyzed fetal calf serum containing medium for increasing time periods, rinsed and refed with serum-less medium (●—●) for the balance of the 4 h experiment at which time the cells were labeled with [^{3}H]uridine (3 μCi/ml, spec. act. 25.4 Ci/mmole) for 30 min at 37 °C. Uptake at 4 h post-stimulation is plotted as a function of the time at which serum was removed. Cells maintained in serum-less medium (------) and those exposed to serum continuously (○—○) were labeled at intervals to assess the status of the uptake system. Stimulation factor is the ratio of experimental to control uptake measured at time zero.

Fig. 2. The kinetics of uridine uptake by mouse embryo cells cultivated in medium with serum compared with uptake at times after the removal of serum. The medium on all dishes was removed at time zero, replaced with fresh medium without serum and fetal calf serum (0.5 ml/5 ml medium) was added to control dishes. Uptake of uridine was measured over a 10-min period at 30 min, 1, 2, and 3 h after time zero. The uptake mixture contained 0.01 mM uridine and 5 μCi per ml of [^{3}H]uridine in Tris-buffered saline. Results are expressed in terms of pmoles of uridine incorporated into the acid-soluble *plus* acid-insoluble fractions after 5 or 10 min exposure to 2.0 ml of the labeling mixture. □—□, with serum for 30 min; ●—●, with serum for 3 h; ○—○, without serum for 30 min; ■—■, without serum for 1 h; ▲—▲, without serum for 2 h; △—△, without serum for 3 h.

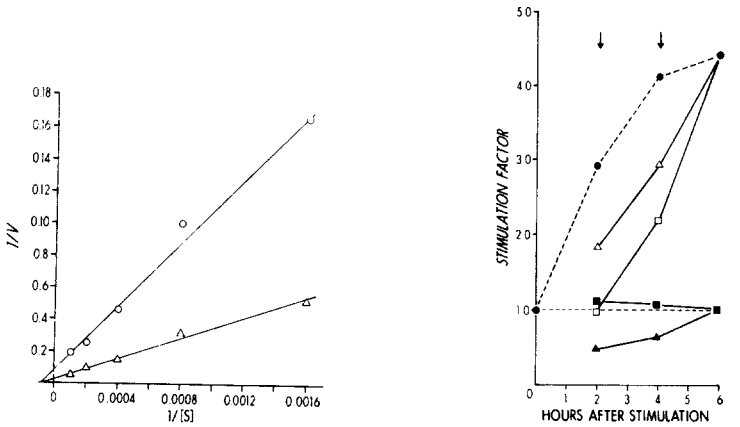

Fig. 3. Lineweaver–Burk plot of uridine uptake kinetics in mouse embryo cells cultivated 4 h in medium with and without serum. Cells cultivated continuously with serum-containing medium were refed with serum-less medium at time zero and one-half of the dishes treated with serum (5%). After 4 h incubation, the uptake of uridine for 5 min at room temperature was tested at different uridine concentrations prepared by diluting unlabeled uridine in a 2-fold series and adding a constant amount of [^{3}H]uridine (spec. act. 27.8 Ci/mmole) to give a final concentration of 5 μCi per 2 ml. The actual specific activity of each labeled uridine preparation was calculated from radioactivity measurements carried out at the time the experiment was counted. The uptake is the sum of the acid-soluble *plus* acid-insoluble fractions. Velocity of uptake (v) is expressed in pmoles per 5 min per dish and uridine concentrations ([S]) in pmoles per ml. ○—○, cells cultivated in serum-less medium; △—△, cells cultivated in medium with 5% serum.

Fig. 4. The effect of actinomycin D and cycloheximide on serum stimulation of uridine uptake by cells maintained in serum-less medium. Cells cultivated for 48 h in serum-less medium were treated by the addition of fetal calf serum (final concentration 10%) at time zero. Actinomycin D (final concentration 0.1 μg/ml) or cycloheximide (final concentration 20 μg/ml) were added (arrows) at either 2 or 4 h after serum addition. Control cells with serum (●---●) or without serum (———) were labeled 30 min with [^{3}H]uridine (3 μCi/ml) at intervals during the 6-h experiment. Cells treated with inhibitors were all labeled after 6 h and the uptake stimulation factor plotted as a function of the time of addition of the inhibitor. △—△, serum *plus* actinomycin D; ▲—▲, actinomycin D alone; □—□, serum *plus* cycloheximide; ■—■, cycloheximide alone.

decline of uptake activity when assayed after 6 h. The sensitive function was more susceptible to inhibition of protein than RNA synthesis. This is shown by the finding that uptake activity declined to lower levels after cycloheximide than after actinomycin D treatment. In contrast, uridine uptake in serum-less cells was inhibited by actinomycin D but not by cycloheximide.

Stimulation of uridine uptake by insulin in serum-less cells

The role of insulin as a stimulator of uridine uptake in the serum-less mouse cell is shown in Fig. 5. Insulin is seen to produce a significant activation of uridine uptake within 1 h followed by a partial loss of activity by 4 h, similar to that observed by Peck *et al.*[16]. Furthermore, insulin (Table I) is not capable of enhancing the stimulatory activity of serum while cyclic AMP increased the activity of both insulin and serum, as had been reported previously[10] for serum stimulation.

Uridine kinase activity following serum stimulation

The uridine kinase activity in cells cultivated in serum-less medium before

and after stimulation with serum was studied (Table II) to evaluate the possible role of this phosphorylating enzyme in the enhanced uptake of uridine. Eight experiments were performed because there was some variability between experiments. In four experiments, the uridine kinase activity was slightly greater in cells maintained in serum-less medium than those stimulated 6 h with serum, while in three other experiments the level in the serum-stimulated cells was slightly greater.

TABLE I

INTERRELATIONSHIPS BETWEEN SERUM, CYCLIC AMP AND INSULIN IN URIDINE UPTAKE STIMULATION IN SERUM-LESS CELLS

Serum-less mouse embryo cells were treated for 4 h with the different combinations of serum (4%), cyclic AMP (0.4 mM) and insulin (1 or 10 μg/ml), labeled for 30 min at 37 °C with [^{3}H]uridine, 4 μCi per dish, rinsed and the acid-soluble and -insoluble fractions extracted. The stimulation factor is the ratio of each experimental uptake to the serum-less, untreated control uptake, based on total incorporation.

	Stimulation factor	
	−Serum	*+Serum*
Control	1.0	3.2
Insulin (10 μg/ml)	2.0	3.2
Insulin + cyclic AMP (0.4 mM)	2.1	4.0
Insulin (1 μg/ml)	1.5	3.3
Insulin + cyclic AMP (0.4 mM)	1.9	4.2

TABLE II

URIDINE KINASE ACTIVITY IN CELLS CULTIVATED IN THE PRESENCE AND ABSENCE OF SERUM OR TREATED WITH ACTINOMYCIN D AND CYCLOHEXIMIDE

Experiment A: Mouse embryo cells cultivated 3 days on serum-less medium were treated with medium with and without 5% serum for 6 h after which they were scraped into Tris–KCl buffer (0.1 M Tris, pH 8.0, 0.15 M KCl) washed once, frozen and thawed thrice, made 0.35 M in sucrose, sonicated for 5 sec and spun at 800 × *g* for 15 min. The first pellet (1) was resuspended in Tris–KCl buffer. The supernatant was then spun at 70000 × *g* for 30 min, the second pellet (2) resuspended in Tris–KCl with a 5-s sonication. Experiment B: Mouse embryo cells cultivated 3 days on serum-less medium were refed with either medium with 10% serum or Tris-buffered saline (− serum) for 2 h in the presence of 1.0 μg/ml actinomycin D or 20 μg/ml cycloheximide. The cells were scraped into Tris–KCl buffer, washed once and sonicated for 5 s thrice with a Branson Sonifier, and the supernatant assayed following centrifugation at 70000 × *g* for 30 min. Uridine kinase activity reported in terms of nmoles of UMP produced per mg protein per 15 min ± S.D.

	Uridine kinase activity	
	− Serum	*+ Serum*
Experiment A		
Supernatant (70000 × *g*)	3.0 ± 0.4	4.2 ± 0.5
Pellet 1 (800 × *g*)	4.7 ± 0.6	6.8 ± 0.6
Pellet 2 (70000 × *g*)	3.8 ± 0.5	4.3 ± 0.5
Experiment B		
Control	5.6 ± 0.7	3.5 ± 0.4
Actinomycin D (1.0 μg/ml)	N.D.*	5.0 ± 0.6
Cycloheximide (20 μg/ml)	N.D.*	4.1 ± 0.4

* Not determined.

The results of a typical experiment are shown in Table II in which the relative specific activity of uridine kinase in a high-speed supernate and in a crude, washed, sonicated cell pellet are reported. Enzyme activity is associated with both the pellet and supernate, and serum stimulation does not change its level or distribution.

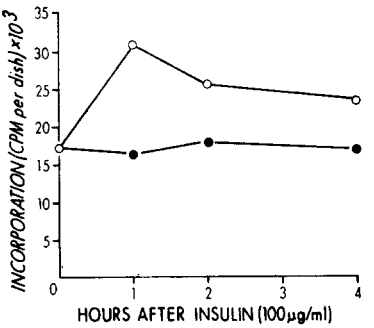

Fig. 5. Stimulation of uridine uptake in serum-less mouse embryo cells by insulin (100 μg/ml). Serum-less, untreated (●—●) and insulin-treated (○—○) cells were labeled 10 min at 37 °C with 5 μCi/ml [^{3}H]uridine beginning at the times indicated and the total uptake into acid-soluble *plus* -insoluble fractions plotted.

Furthermore, as shown in Table II, actinomycin D and cycloheximide do not significantly alter uridine kinase activity, lending additional support to the concept that the enzyme is not involved in the activation of uridine uptake by serum.

DISCUSSION

It was shown previously[10] that purified fetuin, as well as the purine ribonucleoside adenosine and several of its phosphorylated derivatives (ATP and cyclic AMP), could substitute for serum as a stimulator of uridine uptake in cells cultivated for several days in the absence of serum. Now, insulin has also been shown here and in another system[17] to replace serum in this activity.

This current study has also shown that the uridine uptake function is quite sensitive to the absence of some factor in serum and that following its removal this activity is rapidly dissipated. The loss of activity is shown to be associated with a decrease in the V of the reaction with no change in the apparent K_m, suggesting that transport sites are either inhibited[6] non-competitively, or are physically lost from the membrane. Furthermore, the rapid stimulation and decay of uridine uptake activity upon addition or removal of serum, respectively, indicate that the alteration responsible for this fluctuation is fully reversible. Additionally, since no major change in uridine kinase could be demonstrated under conditions known to affect uridine uptake (Table II), the role of uridine kinase in modulation of this function is doubted, in agreement with a previous study on uridine transport in phytohemagglutinin-stimulated lymphocytes[7].

Kinetic analysis (Fig. 3) suggests several possible alternative mechanisms. First is the production of a non-competitive inhibitor of uridine transport similar to that described previously[5,6]. Second is the actual loss of transport-active molecules from the membrane. To evaluate the first possibility, culture medium which had been

in contact with mouse embryo cells for varying periods of time, has been assayed for the presence of a uridine transport inhibitor. Inhibitory activity has been found following 24–72 h exposure to cells, with and without serum, but no activity has been found after only several hours incubation (Hare, unpublished results). This would suggest that the inhibitory substances found in the medium may be the result of the spillover of active molecules once the cell sites have been saturated. Further work must be done to isolate and characterize this inhibitor before further evidence for its role in this phenomenon can be obtained.

Evidence for the presence of a labile, transport-active protein is provided by the inhibitor studies. The ability of cycloheximide to halt further acquisition of uridine transport capacity, followed by the rapid decay of the activity which remains, suggests that the continual synthesis of a labile protein is required to maintain the transport function. The ability of actinomycin D to mimic cycloheximide lends support to this hypothesis. However, due to the generalized, nonspecific effect of cycloheximide on protein synthesis, it can not be concluded that the essential protein whose synthesis is inhibited is a part of the transport active site. Further work is required to decide whether one or both of the above postulated control mechanisms is more important in the regulation of uridine transport in this cell system.

Similar studies have been carried out in other cell types. Uridine transport in rat hepatoma cells[18] cultivated in serum and, hence, in a stimulated state is not sensitive to actinomycin D. However, on the basis of data presented by Peters and Hausen[8], stimulation of uridine uptake in lymphocytes by phytohemagglutinin is partially inhibited by actinomycin D and to a lesser extent by cycloheximide and is also associated with a shift in V with no change in K_m. In contrast to the effects observed here, cycloheximide was found to stimulate rather than inhibit uridine uptake in another mouse cell system (Balb c/3T3) described recently[17]. This finding, as well as other observations, suggested that this effect on uridine transport was part of an overall cellular phenomenon similar to the stringent response to amino acid starvation in bacteria.

An alternate hypothesis involving the role of cyclic AMP as an internal signal generated by the interaction of a variety of mediator molecules with the cell membrane[19] must be considered in view of recent studies implicating cyclic AMP in contact control of cell division[20–24]. Both serum[25] and insulin[26] stimulate contact inhibited cells to undergo cell division. Since insulin leads to a reduction in cyclic AMP[27] and active cell division is associated with reduced cellular cyclic AMP[24], this suggests that stimulation of uridine uptake may be coupled with decreased intracellular cyclic AMP. The stimulating activity of exogenous cyclic AMP on uridine uptake[10] contradicts this mechanism, but would be explained if the compound were degraded to adenosine which alone is capable of stimulating uridine uptake.

At this point it is not possible to conclude which of the many functions necessary to transport uridine are under the controls described here. The nucleoside carrier complex has been suggested by Plagemann and Roth[28] and Peters and Hausen[8] as the rate-limiting site. Another potential candidate is the high energy, phosphorylated intermediate proposed on the basis of the sensitivity of uridine uptake to oligomycin[10]. Further studies will be needed to define this complex, regulated membrane function and its relationship to cell growth and division.

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