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Metabolic Stability of the Nucleoside Transport System of Novikoff Rat Hepatoma Cells

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Rates of transport of uridine and thymidine, estimated with a rapid sampling technique, did not change with culture age. Inhibition of cellular RNA and protein synthesis for periods up to 6 h, did not lead to a loss of nucleoside transport activity. Mild treatment of cell suspensions with trypsin or neuraminidase had no effect on the kinetics of thymidine transport. Thus we conclude, contrary to previous reports, that nucleoside transporters are metabolically stable and that the decreases in nucleoside uptake rates observed with decreased protein synthesis reflect loss of nucleoside kinase activities. These kinases (which have narrow substrate specificity) rather than the membrane-associated, transport apparatus (which has broad substrate specificity) are the most likely sites for regulation of nucleoside uptake.

Key words: hepatoma cells, nucleoside kinases, nucleoside transport, uptake into cells, transport–culture age dependence, metabolic stability of carriers

The transport* of nutrients across the cell membrane has been proposed as a key regulatory site for cellular growth processes [1]. As a consequence, uptake of various substrates has been investigated in detail in many cell culture systems. For example, cell density-dependent changes in uridine uptake rates in untransformed cells, and changes in uptake rates of uridine subsequent to transformation and as a function of the growth stage of the transformed cells, have been reported [2–8]. Thymidine uptake rates have also been observed to change with the growth stage of cells or after treatments that effect the

*"Transport" strictly denotes the transfer – mediated by a saturable, selective carrier – of a substance across the cell membrane. "Uptake" denotes the appearance of radioactivity derived from exogenous, labeled substrate within the cell regardless of metabolic conversions, and "incorporation" denotes the appearance of radioactivity derived from substrate in a specified compound or class of compounds.

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growth of the cells [7, 9, 10]. Uptake rates of isotopically labeled nucleosides have been measured typically over intervals of 1 to 10 min and have been construed generally to reflect initial rates of nucleoside transport (for review see Plagemann and Richey [11]). The results of such measurements have thus been interpreted to mean that the nucleoside transport system(s) is metabolically unstable and is rapidly lost upon cessation of cellular protein synthesis [7].

Our recent studies using a newly developed rapid-sampling technique [12, 13], however, have shown that the conclusion that nucleoside uptake rates estimated from the time points longer than 10 sec reflect initial transport rates is incorrect, at least for the five cell lines we have investigated [13–15, 35]. Instead, we find that thymidine and uridine transport are very fast processes, such that, after about 10 sec, intracellular phosphorylation determines the rate of isotope accumulation within the cell [14]. A similar finding for uridine transport in 3T3 cells has also been reported [16].

Thus, nucleoside transport per se can be assayed only if cell samples are taken at appropriately early times. Using our technique, which permits six discrete samples within the first 10 sec, we have reinvestigated in the present study the metabolic stability of the nucleoside transport system of Novikoff cells. We find, contrary to the previous conclusion, that it is metabolically stable and that the decreases in nucleoside uptake rates observed with decreasing protein synthesis reflect loss of kinase activities.

MATERIALS AND METHODS

Wild-type Novikoff rat hepatoma cells (subline N1S1-67) and a thymidine kinase-deficient subline thereof [17] were propagated in Swim's medium 67 and enumerated as described previously [18, 19]. Cells were collected by centrifugation at 400g for about 2 min and suspended in a basal medium, BM42B [20], to a concentration of $(1–2) \times 10^7$ cells per milliliter for transport measurements or about 2×10^6 cells per milliliter for uptake studies. Cells were depleted of adenosine triphosphate by incubation in glucose-free BM42B (BM42A) supplemented with 5 mM KCN and 5 mM iodoacetate for 10 min at 37° [17].

In all uptake and transport assays the influx of substrate was measured against an intracellular concentration presumed to be effectively zero; that is, a "zero-trans" protocol was used [21]. For uptake measurements over intervals of several minutes the cell suspension was supplemented with labeled substrate and incubated at 37° on a gyratory shaker at about 200 rpm. Duplicate 1.0-ml samples of suspension were analyzed for radioactivity in total cell material and acid-insoluble material as described previously [22].

For transport studies at a time scale of seconds the rapid-sampling technique, which has been described in detail elsewhere [13], was employed. Briefly, fixed aliquots of a suspension of cells were rapidly mixed with a solution of radioactive thymidine (dThd) at short time intervals by means of a hand-operated, dual syringe apparatus. Samples emerging from the mixing chamber were dispensed into 12 tubes mounted in an Eppendorf microcentrifuge which contained an oil mixture (density = 1.034 g/ml). Dispensing of samples into sequential centrifuge tubes was paced with a metronome and could be accomplished, comfortably, at 1.5-sec intervals. After the last sample had been mixed, the centrifuge was started, and within an estimated 2 sec the cells had entered the oil phase, thus terminating transport. All transport assays were performed at 23–25°.

After centrifugation the supernatant medium was aspirated. The upper part of the tube was washed once with 1 ml of water, which was subsequently removed together with most of the oil. Then 0.2 ml of 0.5 N trichloroacetic acid (TCA) was added to the tube

and immediately vortexed to disperse the pellet. After 30 min of incubation at 70°C the entire tube and its contents were transferred to a vial containing 8 ml of a modified Bray's solution [18] and were analyzed for radioactivity in a liquid scintillation spectrometer.

Total water space and extracellular space in cell pellets obtained by centrifugation through oil were determined in parallel runs in which the substrate was replaced by [^{14}C] carboxylinulin in tritiated water [13]. All uptake values were corrected for substrate radioactivity in the extracellular space (generally about 12% of the total water space).

Thymidine transport was assayed in cells incapable of metabolizing this substrate; either because they were lacking thymidine kinase or because of ATP depletion. Initial transport velocities were computed as zero-time slopes of an integrated first-order rate equation to which data were fit by the methods of least squares [13]. The integrated equation employed is an approximation which somewhat underestimates influx – progressively so as exogenous concentration of substrate increases [35]. At a given substrate concentration this is a fairly constant error and has little effect on relative rates. Furthermore, at concentrations below the K_m for zero-trans influx, ie, below about 85 μM [12] the underestimation is negligible.

Initial rates of uridine transport were estimated graphically from the linear portion (5–10 sec) of the uptake curves. Use of the integrated rate equation was not possible in the case of uridine, since the intracellular breakdown of uridine to uracil could not be prevented [15]. Michaelis-Menten parameters were computed according to Wilkinson [23]. Actinomycin D, at 1.25 μg/ml, and cycloheximide, at 25 μg/ml, were used to inhibit RNA and protein synthesis, respectively. In Novikoff cells, these concentrations result in more than 95% inhibition [24].

For uridine kinase and thymidine kinase assays the cells were resuspended in B4 buffer [25], and sonicated ("Sonifier" 185, Branson Sonic Power Company, Danbury, Connecticut) using 3 bursts at maximum output for 10 sec. A supernatant fraction was used for the assays, prepared by centrifuging the lysate at 10^5g for 30 min at 4°. Uridine kinase assays [25] and thymidine kinase assays [26] were performed at 37° as described previously, except that for thymidine kinase determinations the reactions were terminated by pipetting aliquots onto polyethylene imine-cellulose plates, followed by washing to remove nonphosphorylated isotope [27]. These assays were done at saturating substrate concentrations.

Chemicals

Radiochemicals were obtained from the following sources: [methyl-^{3}H] thymidine (10 Ci/mmole) from ICN (Irvine, California); [5-^{3}H] uridine (25 Ci/mmole) from Amersham/Searle (Arlington Heights, Illinois); [carboxyl-^{14}C] carboxylinulin (2.6 Ci/g), and tritiated water (1 mCi/g) from New England Nuclear (Boston). Thymidine, uridine, and neuraminidase were obtained from Sigma Chemical Co (St Louis); trypsin from Difco (Detroit). Polyethylene imine-impregnated cellulose thin-layer plates were purchased from Brinkman (Westbury, New York). Other chemicals were reagent grade from standard suppliers.

RESULTS

The cell density of freshly diluted Novikoff rat hepatoma cells was monitored for 48 h. Aliquots from the same suspension were used to determine the initial rates of uridine transport, rates of long-term incorporation of uridine into total and acid-insoluble cell material, and uridine kinase activity (Fig 1). As had been previously reported, incorporation rates and kinase activities reached a peak in the middle log phase of cell growth, and then declined [7, 25]. Uridine transport activity, on the other hand, remained constant

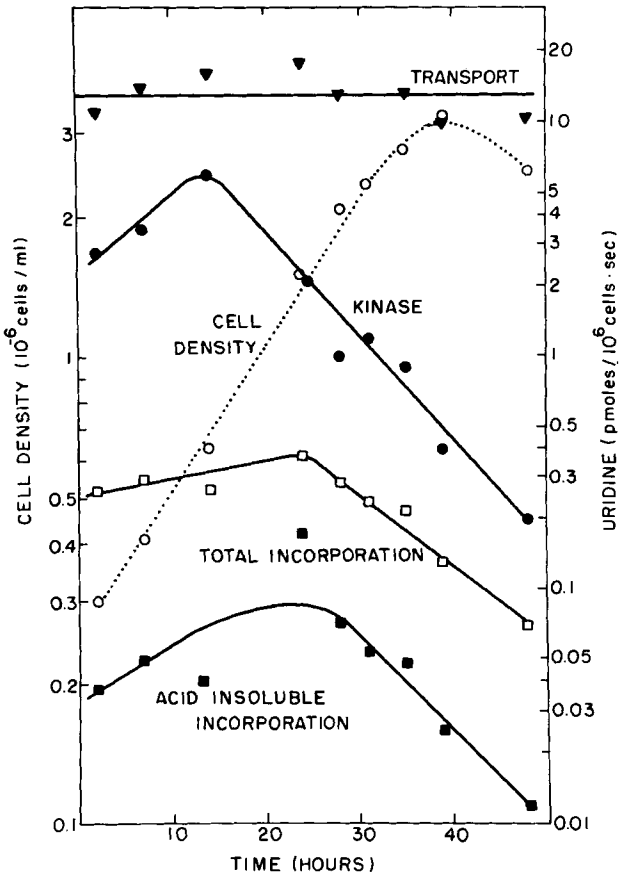

Fig 1. Effect of culture age on uridine transport, uridine incorporation into total cell material and into acid-insoluble material, and uridine kinase activity. An exponential phase culture of Novikoff cells was diluted with growth medium to about 2.5 × 10^5 cells per milliliter. The culture was incubated at 37° on a gyrotory shaker and monitored for cell density (○ · · · ○). At the indicated times aliquots were taken from the culture and analyzed as follows: 1) 1 × 10^8 cells were collected by centrifugation and depleted of ATP at a cell density of 1 × 10^7 cells per milliliter. One aliquot was analyzed for the initial rate of uridine transport. Samples of 448 μl of the cell suspension were mixed in rapid succession with 61 μl of [^{3}H] uridine (3.5 cpm/pmole; final concentration = 80 μM). The mixtures were centrifuged through oil layers and the cell pellets were analyzed for radioactivity as described in Methods. All values were corrected for trapping of substrate in the extracellular H_2O space. This space was determined using another aliquot from the same cell suspension as described in Methods. Initial velocities of uridine transport were estimated graphically from the initial linear portion (5–10 sec) of the uptake curves (▾—▾). 2) 1 × 10^7 cells were collected by centrifugation, suspended to 2 × 10^6 cells/ml of BM42B containing 5 μM [^{3}H] uridine (80 cpm/pmole). After 5 min of incubation at 37°, duplicate 1-ml samples were analyzed for radioactivity in total cell material (□ – □) and acid-insoluble material (■ – ■) as described previously [22]. 3) 1 × 10^7 cells were collected by centrifugation, sonicated in 2 ml of B4 and the 100,000g supernatant was analyzed for urdine kinase activity (● – ●) as described previously [22] with a uridine concentration of 400 μM.

regardless of the age of the cell culture. Note that both ordinates of Figure 1 are on a logarithmic scale and that the absolute velocities are not directly comparable, since except for uridine kinase activity, they do not reflect maximum velocities.

Similar results were obtained for thymidine transport (Fig 2). Cells in middle exponential growth phase (culture I) were diluted with growth medium (culture II). Initial

rates of thymidine transport and cell number (Fig 2B), as well as the cell volume (Fig 2A), were measured until the respective cultures reached maximum cell density. Thymidine transport, expressed on a per cell basis, was the same in both cultures and did not change with age. Thymidine transport, normalized on an intracellular volume basis, actually increased with age of culture, since the cell volume decreased during this time.

As with uridine, the invariance of thymidine transport with cell culture age is in striking contrast to the behavior of thymidine kinase activity and long-term thymidine incorporation into total cell material by phosphorylating cells. Both of the latter activities reach a peak in the log phase of cell growth, and decline steeply as the culture age increases [7, 17, 28–30].

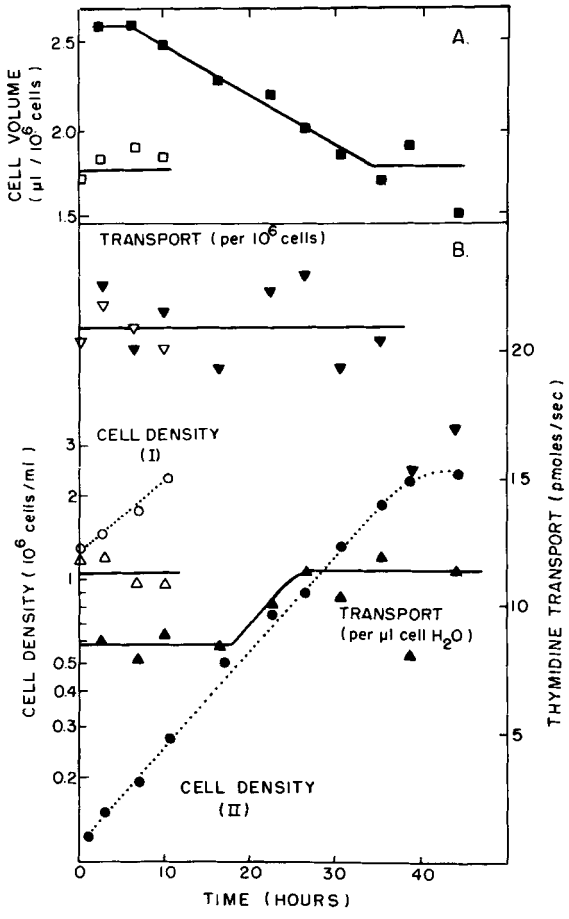

Fig 2. Effect of culture age on cell volume and thymidine transport. An exponential phase culture of Novikoff cells (culture I, open symbols) was diluted with growth medium to 1.2×10^5 cells per milliliter (culture II, closed symbols). The cultures were incubated at 37° on a gyrotory shaker and monitored for cell density (○· · ·○, ●· · ·●). At the indicated times 10^8 cells were taken from the cultures and initial thymidine transport rates were determined at a final concentration of 320 µM (2.4 cpm/pmole). Methods used were those described in the legend to Figure 1. A first-order rate equation was fitted to the uptake data, and the initial velocity of transport was calculated as the tangent at time zero [13]. Cell volumes (□ – □, ■ – ■) were determined on the same suspensions used for the transport assays as described in Methods. The transport rates were calculated per 10^6 cells (▽ – ▽, ▼ – ▼) or per µl intracellular water (△ – △, ▲ – ▲).

Constant nucleoside transport activity might result from either of two mechanisms. The transporters could be metabolically stable. Alternatively, the transporters could be subject to metabolic breakdown and resynthesis, even after the cells have stopped dividing.

To distinguish these possibilities the following experiments were undertaken. An exponential phase culture of Novikoff cells was split; protein synthesis in one flask was inhibited with cycloheximide, while the other served as control. After 2, 4, and 6 h, 150-ml aliquots were removed from each flask and the cells were collected by centrifugation, resuspended, and assayed for uridine transport. The results (Fig 3) thus are normalized to the volume of original culture rather than to cell number.

In the control, the rate of uridine transport per volume of culture increased about 70% during 6 h of incubation. This correlates well with an increase in cell density from 1×10^6 to 1.7×10^6 cells per milliliter over this time. The presence of 25 μg cycloheximide per milliliter of culture fluid inhibited the increase in uridine transport capacity just as it inhibited cell growth. It is noteworthy, however, that there was no loss in transport activity.

The metabolic stability of thymidine transport, uptake, and kinase activities were investigated in the experiment shown in Figure 4. A culture of exponentially growing cells was split in three (zero time). Flask 1 served as control; in flask 2 RNA synthesis was inhibited with actinomycin D; and flask 3 received cycloheximide to inhibit protein synthesis. Thymidine transport and long-term uptake, as well as thymidine kinase, were assayed at time zero and every hour thereafter. The experiment was terminated after 6 h, since the actinomycin D-treated cells became appreciably permeable to trypan blue after this time.

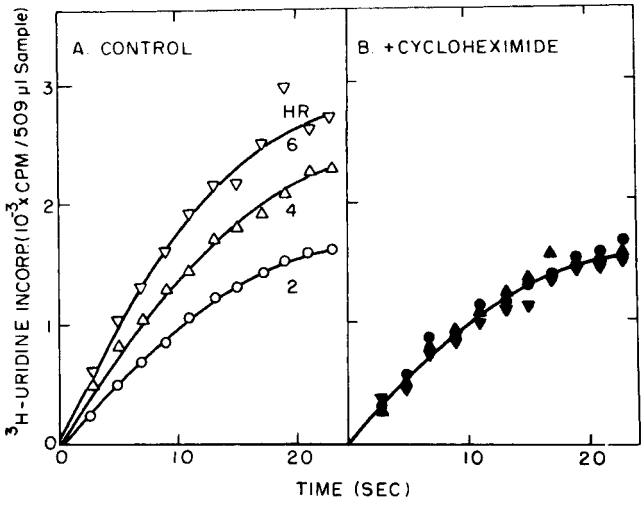

Fig 3. Effect of cycloheximide on uridine transport. Part of an exponential phase culture of Novikoff cells (at 1×10^6 cells/ml) was supplemented with 25 μg cycloheximide/ml. This suspension (B) and the other part without inhibitor (A) were incubated at 37°. At 2, 4, and 6 h cells from 150 ml of each suspension were collected by centrifugation and suspended in 12 ml of BM42B. The suspensions were analyzed for uridine transport rates by the rapid-kinetic technique with 80 μM [^{3}H] uridine (3.4 cpm/pmole), as described in the legend to Figure 1. The cell densities of the control and cycloheximide-treated culture samples increased to 1.7 and 1.3×10^6 cells per milliter during the 6-h incubation period, respectively.

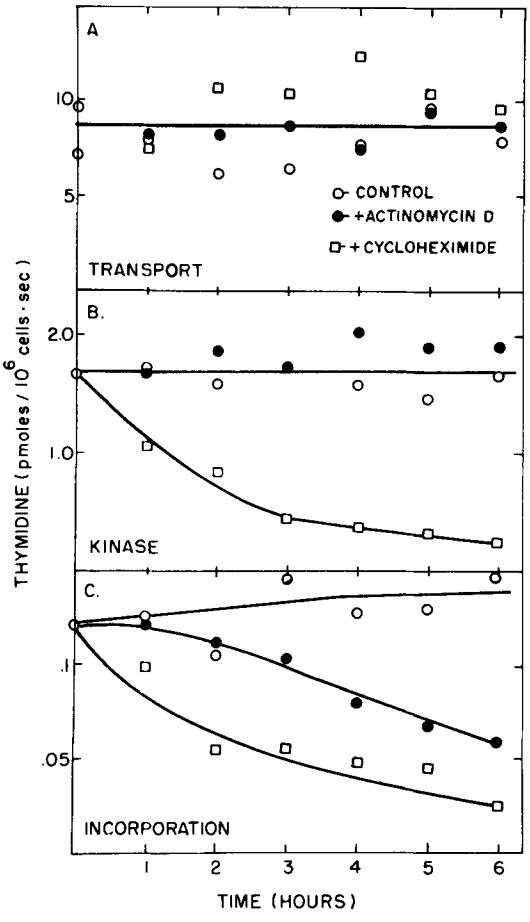

Fig 4. Effect of actinomycin D and cycloheximide on thymidine transport, thymidine incorporation into total cell material, and thymidine kinase activity. An exponential phase culture of Novikoff cells (at 1.1×10^6 cells per milliliter) was divided into three aliquots. One served as control, the second received 1.25 μg actinomycin D per milliliter, and the third was supplemented with 25 μg cycloheximide per milliliter. At time zero, and at 1-h intervals thereafter, aliquots were taken from each suspension. For thymidine transport assays 200-ml aliquots were withdrawn, resuspended in 11 ml BM42A containing 5 mM KCN and 5 mM iodoacetate, and incubated at 37° for 10 min to deplete them of ATP. The transport assay and initial rate determination were performed using 320 μM [^{3}H] thymidine as described in the legend to Figure 2. Cell extracts were prepared from 50 ml of cell suspension and assayed for thymidine kinase activity as described in Methods. For thymidine incorporation measurements, aliquots were resuspended in an equal volume of BM42B containing 0.5 μM[^{3}H] thymidine (520 cmp/pmole). After 3 min of incubation at 37°, duplicate 1-ml samples were analyzed for radioactivity in total cell material [22].

Initial rates of thymidine transport did not change over the course of the experiment, regardless of treatment (Fig 4A). In contrast, thymidine kinase activity decayed to 20% of control after protein synthesis was inhibited (Fig 4B). Inhibition of RNA synthesis, on the other hand, did not result in a decrease of thymidine kinase activity.

Rates of long-term thymidine uptake decreased as a result of inhibition either of RNA or protein synthesis (Fig 4C). The decrease of uptake in the presence of cycloheximide

paralleled the decrease of thymidine kinase. But uptake also decreased in the presence of actinomycin D, which had no effect on thymidine kinase activity.

Since we were unable to show metabolic turnover of the nucleoside transporters, it was of interest to determine if a mild digestion with a protease would cause loss of transport activity. Novikoff cells were incubated for 15 min at 37° suspended in a trypsin solution (1 g/liter of balanced salt solution). A 5-min incubation under these conditions, followed by mechanical agitation, is sufficient to disperse these cells into a single cell suspension. On the other hand, a 30-min digestion is not sufficient to render these cells stainable by trypan blue.

Figure 5 shows that the trypsin digestion had no effect on the thymidine transport characteristics of the cells. Plotted are the initial velocities of transport versus the substrate concentration for both control and trypsin-treated cells. In the same experiment another aliquot of cells was preincubated with neuraminidase and then analyzed for thymidine transport. The solid line is a Michaelis-Menten hyperbola fit to all 24 points; best-fitting parameters were: $K_m = 98 \pm 12$ μM; $V_{max} = 13.3 \pm 0.6$ pmoles/μl cell water • sec. This hyperbola also describes adequately each individual data set. In particular, the V_{max} for the individual data sets (see legend to Fig 5) – the parameters which should reflect most closely a change in number of carrier molecules – do not differ significantly.

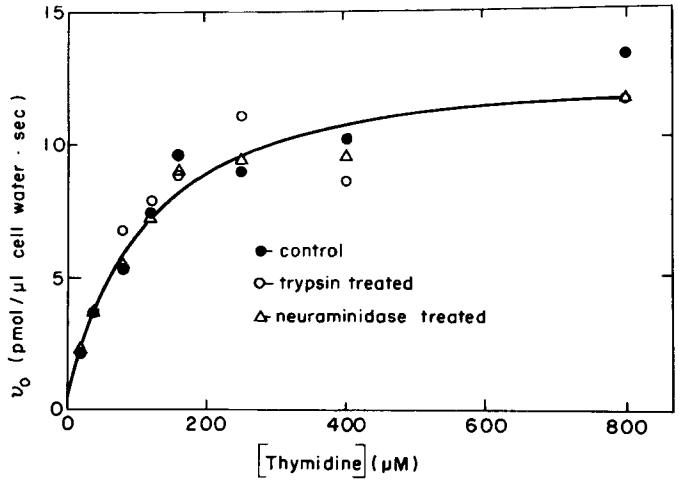

Fig 5. Lack of effect of trypsin and neuraminidase treatment on the kinetics of thymidine transport. Three equal samples of an exponential phase culture of a thymidine kinase-negative subline of Novikoff cells were resuspended in 100 ml BM42B each to give final cell densities of 12×10^6 cells per milliliter. One flask contained 1 mg trypsin per milliliter, while the second contained 3 mg neuraminidase per milliliter. The suspensions were incubated on a gyratory shaker at 37° for 15 min. The cells were collected by centrifugation and resuspended in BM42B at a density of 15×10^6 cells per milliliter. Initial rates of thymidine transport were determined as described in the legend to Figure 2. The final [^{3}H] thymidine concentrations were 20, 40, 80, 120, 160, 250, 400, and 800 μM (680 cpm/μl at all concentrations). Intracellular and extracellular spaces in the cell pellets (6.7×10^6 cells) were respectively 12.3 and 2.1, for the control 11.4 and 2.1 for the trypsin-treated cells, and 12.4 and 2.0 μl for the neuraminidase-treated cells. Michaelis-Menten parameters were computed according to Wilkinson [23]. K_m and V_{max} values were respectively 125 ± 26 and 14.7 ± 1.1 for the control, 76 ± 22 and 12.5 ± 1.1 for the trypsin-treated cells, and 101 ± 15 μM and 13.0 ± 0.6 pmoles/μl cell water•sec, for the neuraminidase-treated cells.

DISCUSSION

The results presented indicate that the transport capacity of Novikoff cells for both thymidine and uridine stays constant while the cells traverse exponential growth to cessation of cell division. This constancy of transport capacity could be achieved by breakdown and resynthesis of the nucleoside carrier, or by their metabolic stability. The results presented in Figures 3 and 4, where no decay in transport capacity over 6 h could be observed, favor the latter possibility. In the experiment shown in Figure 5, nucleoside transporters were resistant to digestion by trypsin or neuraminidase. Resistance to trypsin suggests that the presumptive transport proteins may be invulnerable to protease by virtue of their location in the membrane, and hence, metabolically stable. Trypsin, of course, was applied to the outer membrane surface; the proteases responsible for intracellular protein turnover presumably operate at the inside surface.

The similar behavior of the thymidine and uridine transport systems supports our previous suggestion that in Novikoff cells all nucleosides are transported by a single system with broad substrate specificity [15, 31]. Another important confirmatory point is the fact that under all conditions examined, nucleoside transport capacity greatly exceeded the capacity of the respective kinases and long-term uptake systems. For example, even at 80 μM (about ½ of transport K_m [15]) uridine transport was twice as rapid as the V_{max} of uridine kinase activity. Thus, as previously reported, intracellular nucleoside phosphorylation, rather than nucleoside transport, is the rate-determining step in long-term nucleoside incorporation into Novikoff cells. Such is also the case for Chinese hamster ovary cells [35] and for 3T3 cells [16], and may be the situation in cultured mammalian cells generally.

The finding of the metabolic stability of the nucleoside transport system is contrary to conclusions derived from a previous study [7] in which long-term uptake rates were incorrectly equated to transport rates and which suggested rapid turnover of the nucleoside transporter. Our present results clearly show that it is a decrease in kinase activity alone that accounts for the decrease in nucleoside uptake when cultures approach stationary phase, or after inhibition of protein synthesis.

Thus the kinases, rather than the membrane-associated transport step, are potentially important regulatory steps in nucleoside uptake. This is also suggested by the finding that the substrate specificity of long-term nucleoside uptake reflects that of the different nucleoside kinases [11] and not of a single nucleoside transport system with broad specificity [15, 31]. Thymidine kinase is of particular interest, since its activity is also rapidly lost from cells during G2 and reaches a minimum in M and G1 [10, 32]. Just how thymidine kinase activity is modulated as a function of growth stage or the cell cycle is not entirely clear [33]. Our results in Figure 4 suggest that the mRNA for thymidine kinase may be quite stable. Evidence for the importance of uridine kinase in regulating uridine uptake was recently reported by Rozengurt, Stein, and Wigglesworth [16]. Upon serum stimulation of 3T3 cells, it was the kinase activity, rather than transport, that increased.

There exists an apparent inconsistency with our interpretation that phosphorylation paces uptake in the experiment depicted in Figure 4. In the presence of actinomycin D both thymidine transport and kinase activity were unaffected for 6 h, yet thymidine incorporation declined after 1 h. A possible resolution may be as follows. Thymidine kinase is allosterically inhibited by nucleoside triphosphates [34], the concentrations of which, in the absence of RNA synthesis, are likely to increase. The resultant effective decrease in kinase activity would be reflected in decreased thymidine uptake. Another possibility

which cannot be excluded, however, is that thymidine kinase activity as measured in vitro is not an adequate measure of kinase activity in situ.

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