

CELL CYCLE AND GROWTH STAGE-DEPENDENT CHANGES IN THE TRANSPORT OF NUCLEOSIDES, HYPOXANTHINE, CHOLINE, AND DEOXYGLUCOSE IN CULTURED NOVIKOFF RAT HEPATOMA CELLS

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ABSTRACT

Populations of Novikoff rat hepatoma cells (subline N1S1-67) were monitored for the rates of transport of various substrates and for their incorporation into acid-insoluble material as a function of the age of cultures of randomly growing cells in suspension as well as during traverse of the cells through the cell cycle. Populations of cells were synchronized by a double hydroxyurea block or by successive treatment with hydroxyurea and Colcemid. Kinetic analyses showed that changes in transport rates related to the age of cultures or the cell cycle stage reflected alterations in the V_{max} of the transport processes, whereas the K_m remained constant, indicating that changes in transport rates reflect alterations in the number of functional transport sites. The transport sites for uridine and 2-deoxy-D-glucose increased continuously during traverse of the cells through the cell cycle, whereas those for choline and hypoxanthine were formed early in the cell cycle. Increases in thymidine transport sites were confined to the S phase. Synchronized cells deprived of serum failed to exhibit normal increases in transport sites, although the cells divided normally at the end of the cell cycle. Arrest of the cells in mitosis by treatment with Colcemid prevented any further increases in transport rates. The formation of functional transport sites was also dependent on *de novo* synthesis of RNA and protein. Inhibition of DNA synthesis in early S phase inhibited the increase in thymidine transport rates which normally occurs during the S phase, but had no effect on the formation of the other transport systems. Transport rates also fluctuated markedly with the age of the cultures of randomly growing cells, reaching maximum levels in the mid-exponential phase of growth. The transport systems for thymidine and uridine were rapidly lost upon inhibition of protein and RNA synthesis, and thus seem to be metabolically unstable, whereas the transport systems for choline and 2-deoxy-D-glucose were stable under the same conditions.

In a previous report (1) we have demonstrated that synchronous populations of Novikoff rat hepatoma cells transport and phosphorylate thymidine (dThd) throughout the cell cycle, but that the

transport capacity of the cells increases solely during the S or late S phase (see also Fig. 1 B). dThd transport rates were estimated by determining the initial rate of incorporation of labeled dThd into total cell material (acid-soluble plus acid-insoluble [2]). At all stages of the cell cycle the transported dThd was rapidly converted to nucleotides without intracellular accumulation of free dThd, and the incorporation of dThd was competitively inhibited by specific inhibitors of the transport process. These results indicate that, throughout the cell cycle, transport was the rate-limiting step in the initial incorporation of dThd into the nucleotide pool (1).

In the present study we have extended these investigations to the systems transporting uridine (Urd), hypoxanthine (Hyp), choline, and 2-deoxy-D-glucose and have determined the effect of various metabolic inhibitors and serum deprivation on the observed transport changes during traverse of the cells through the cell cycle. In a similar manner we have attempted to assess the metabolic stability of the various transport systems and have determined the dynamics of the transport capacities of the cells as a function of the growth stage of randomly growing cells in suspension culture.

MATERIALS AND METHODS

Materials

Reagents were purchased as follows: [8-³H]Hyp, [methyl-³H]dThd, [5-³H]Urd, and [methyl-³H]choline from Schwarz/Mann, Div., Becton, Dickinson & Co., Orangeburg, N. Y.; 2-deoxy-D-[G-³H]glucose from New England Nuclear, Boston, Mass.; Colcemid from Grand Island Biological Co., Grand Island, N. Y.; cycloheximide (Actidione) from Calbiochem, San Diego, Calif.; cytosine arabinoside from Sigma Chemical Co., St. Louis, Mo.; and cytochalasin B from Aldrich Chemical Co., Milwaukee, Wis. 2,6-Bis(diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-d) pyrimidine (Persantine) and actinomycin D were gifts from Geigy Pharmaceuticals, Div. Ciba-Geigy Corporation, Ardsley, N. Y. and Merck Sharp & Dohme, Div. Merck & Co., Inc., West Point, Pa., respectively.

Cells

Novikoff rat hepatoma cells (subline N1S1-67) were propagated in suspension culture in Swim's medium 67 and enumerated by means of a Coulter counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) as described previously (3, 4). Populations of highly synchronized cells were prepared by two consecutive treatments with hydroxyurea (1). In brief, suspensions of

randomly growing cells ($5-9 \times 10^5$ cells/ml) were supplemented with 0.5 mM hydroxyurea and incubated at 37°C for 9 h. The cells were then collected by centrifugation and suspended in the original volume of warm (37°C) fresh medium 67. After another 5.5 h of incubation, the suspension was again supplemented with 0.5 mM hydroxyurea and incubated for another 5 h at 37°C. Then the cells were collected by centrifugation and suspended to $0.6-1.2 \times 10^6$ cells/ml in warm fresh medium 67 (0 time after reversal) and incubated at 37°C. All suspensions were incubated on a gyratory shaker at about 200 rpm.

Measurement of Initial Rates of Transport and Incorporation into Nucleic Acids

Replicate 1-ml samples of cell suspensions were centrifuged and the cells were suspended in 1 ml of the following media (prewarmed to 37°C): (a) HEPES-buffered basal medium 42, BM42B (5) supplemented with either 0.1 or 0.5 μ M [methyl-³H]dThd (3,000 and 600 cpm/pmol, respectively) or 1.25 or 5 μ M [5-³H]Urd (240 and 60 cpm/pmol, respectively) or 1 μ M [8-³H]Hyp (300 cpm/pmol). (b) HEPES-buffered, D-glucose-free BM42, BM42A (6) containing 50 μ M 2-deoxy-D-[G-³H]glucose (6 cpm/pmol). (c) Choline-free BM42 (7) containing 0.5 μ M [methyl-³H]choline (600 cpm/pmol). The samples were incubated stationary at 37°C for 5 min and were then analyzed for radioactivity in total cell material (8). It has been demonstrated previously for all of these substrates that, at the substrate concentrations employed, incorporation is approximately linear with time for at least 5 min and that transport is the rate-limiting step in this incorporation, and that, therefore, the initial rates of incorporation into total cell material reflect the initial rates of transport into the cell (2, 6-8). Acid-soluble pools were extracted from labeled cells with perchloric acid as described in the same publications, and the labeled intracellular compounds were separated by ascending paper chromatography with a solvent composed of 3 vol 1 M ammonium acetate (pH 5) and 7 vol 95% ethanol. Where indicated in the appropriate experiments, samples of suspension supplemented with [³H]dThd or [³H]Urd were incubated for 30 min and then analyzed for radioactivity in acid-insoluble material as described previously (8).

Other Methods

Samples of cells were fixed and stained with quinoline blue, and the mitotic index was estimated by scoring 100 or 200 cells by phase-contrast microscopy as described previously (1). For the analysis of the karyotype, samples of cell suspension were supplemented with 0.05 μ g of Colcemid/ml and incubated for 3 h. The cells were washed in balanced salt solution (BSS [8]) and incubated at room temperature for 12 min in a solution composed of 1 part medium 67 and 4 parts of H₂O.

Then the cells were collected by centrifugation and fixed for 25 min in a mixture of 3 parts ethanol and 1 part glacial acetic acid. The cells were collected by centrifugation, suspended in 0.5 ml of the fixative, and a drop of suspension was spread over a microscope slide. The slide was air dried, stained with lactoacetic acid-orcein, and the chromosome spread was analyzed by phase-contrast microscopy as described by Brand et al. (9). Concentrations of D-glucose in samples of culture fluid were determined by the Glucostat method (Worthington Biochemical Corp., Freehold, N. J.). pH determinations were conducted immediately after removal of samples from cell suspensions with a Beckman pH meter (Beckman Instruments, Inc., Fullerton, Calif.). The microfluorometric analyses were conducted with a ICP 11 impulse cytophotometer (PHYWE Aktiengesellschaft, Göttingen, Germany) as described by Rajewsky et al. (10).

RESULTS

Synchronization of N1S1-67 Cells by Double Hydroxyurea Block

As shown previously (1), 75–85% of the N1S1-67 cells divide between 5 and 7 h after reversal of the second hydroxyurea treatment (see Fig. 1 A). The population remains sufficiently highly synchronized to allow measurements through two successive cell cycles. The second wave of cell division begins between 13 and 15 h (see Fig. 1 A). DNA synthesis resumes rapidly after removal of the hydroxyurea block and DNA synthesis in the second cell cycle begins within less than 1 h after cell division (see Fig. 1 B), indicating that these synchronized cells exhibit only a short

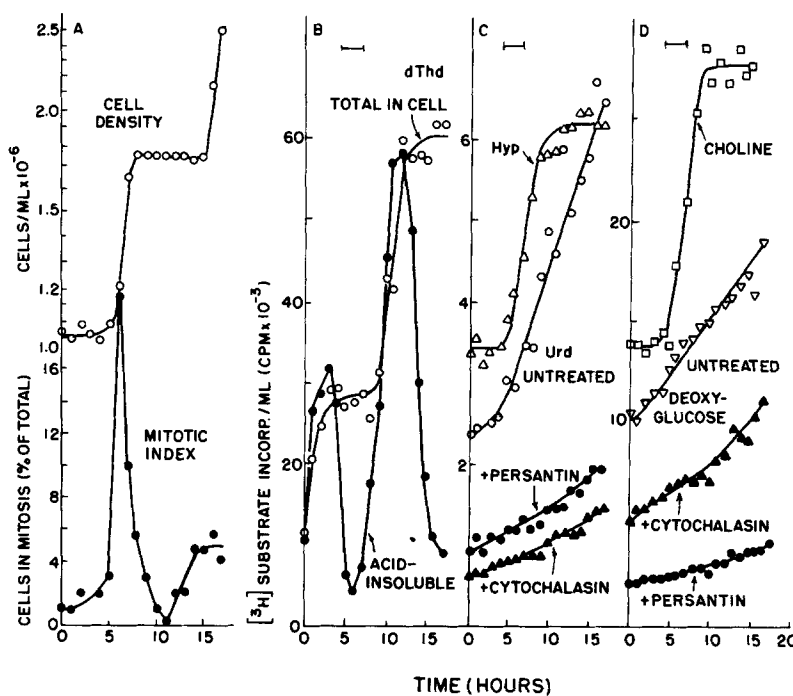


FIGURE 1 Rates of dThd, Hyp, Urd, choline, and 2-deoxy-D-glucose transport and of DNA synthesis as a function of the cell cycle stage. A population of N1S1-67 cells was synchronized by two exposures to hydroxyurea as described in Materials and Methods. At the indicated times after the reversal of the second hydroxyurea block (0 time), samples of the suspension were analyzed for cell density and mitotic index (A). From other replicate 1-ml samples the cells were collected by centrifugation and suspended in 1 ml of the appropriate basal media containing the indicated ³H-labeled substrates as described in detail in Materials and Methods. Where indicated, samples labeled with [³H]Urd (C) or [³H]deoxyglucose (D) were also supplemented with 1 and 40 μ M Persantine (C and D, respectively) or 8 and 2 μ M cytochalasin B (C and D, respectively). All samples were incubated at 37°C for 5 min and then analyzed for radioactivity in total cell material. In Fig. 1 B, additional samples were incubated with [³H]dThd for 30 min and then analyzed for radioactivity in acid-insoluble material. All points represent averages of duplicate samples. The bars in Fig. 1 B–D indicate the time period of cell division after the first cell cycle.

G₁ period (1). The traverse of the cells through the cell cycle has also been followed by microfluorometric analysis which scores populations of cells on the basis of their DNA content (Fig. 2), and chromosome spreads were examined for the number of chromosomes in these cells. Most of the cells were found to possess 39 (36–39) chromosomes which is three chromosomes fewer than the normal diploid complement of the rat ($2n = 42$) and similar to the chromosome number described by Hsu in 1959 (11) for the stem cells (1s) of the Novikoff rat hepatoma maintained by transplantation in rats. The karyotype of the cultured N1S1-67 (Fig. 2 C) was also still similar to that described by Hsu in 1959 (11) for the stem cells. A small and somewhat variable proportion of the N1S1-67 cell populations, however, possessed 70–80 chromosomes (2s). The presence of the 1s and 2s populations is apparent from the microfluorometer profiles of a sample of cells shortly after reversal of the hydroxyurea block (1-h sample, Fig. 2 A). These cells are expected to have G₁-DNA content. The peak at channel no. 20 represents the 1s cells, whereas the small proportion of 2s cells is indicated by the shoulder with a mean at approximately channel no. 40. The consecutive profiles (4, 6, and 8 h, Fig. 2 A and B) show that both populations of cells exhibited a synchronous increase in DNA content and cell division.

Cell Cycle-Dependent Changes in Transport Rates

As reported previously (1), the rate of dThd transport per milliliter of culture doubles during the S or late S phase (between 0 and 3 h in the first cycle and between 9 and 13 h in the second cycle, see Fig. 1 B). The summary results in Fig. 1 C and D demonstrated that the transport rates for other substrates increased in quite a different manner. Urd and deoxyglucose transport rates increased continuously during traverse of the cells through two cell cycles. The maximum increases during 14–17 h of incubation varied somewhat in repeated experiments (between two- and threefold, see Figs. 1 and 3–5), but in all instances the rates seemed to increase continuously. The transport rates for choline and Hyp, on the other hand, approximately doubled during the early part of the cell cycle (Fig. 1 C and D). Chromatographic analyses of acid extracts prepared from 5-min labeled cells at 2, 4, and 5 h after reversal of the hydroxyurea block indicated that over 95% of the radioactivity

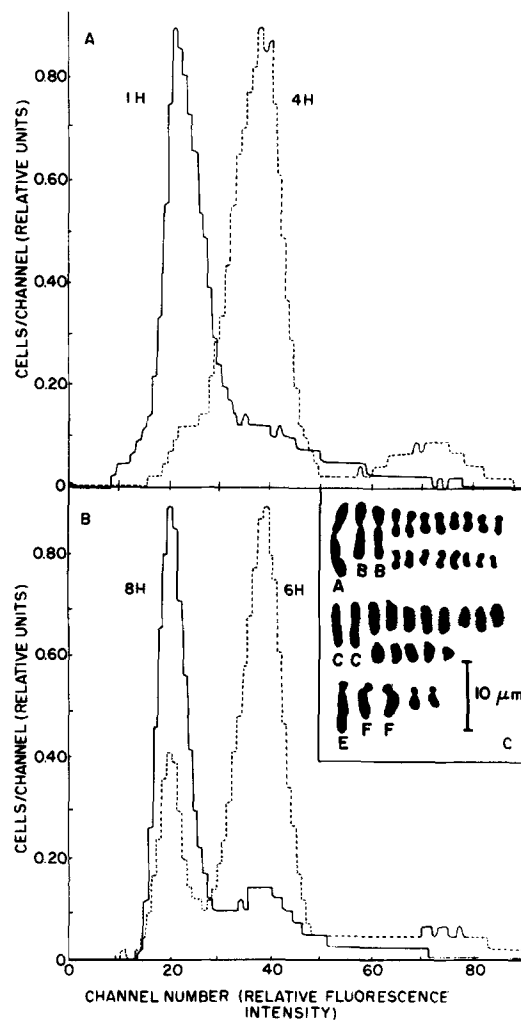


FIGURE 2 Relative DNA content of N1S1-67 cells as a function of cell cycle stage (A and B) and karyotype of the cells (C). The details of the experiment are described in the legend of Fig. 1. At the indicated times after reversal of the second hydroxyurea block, samples of 2×10^7 cells were subjected to impulse-cytophotometric analysis (A and B).

from Urd, Hyp, and choline-labeled cells and over 85% of the radioactivity from deoxyglucose-labeled cells was associated with phosphorylated intermediates (not shown). Furthermore, the initial rates of incorporation of Urd, deoxyglucose (Fig. 1 C and D), and of Hyp (not shown) were reduced to about the same extent at all stages of the cell cycle by the presence of cytochalasin B and Persantine, two substances which specifically inhibit the transport of these substrates without affecting the

activities of Urd kinase (12, 13), hexokinase (14), or Hyp-Gua phosphoribosyltransferase (15). Combined, these results indicate that, at all stages of the cell cycle, transport was the rate-limiting step in the initial incorporation of these substrates into total cell material and that these rates therefore reflect the transport rates.

Kinetic analyses showed that the increases in deoxyglucose transport during the cell cycle were due to increases in the V_{max} of the transport process, whereas the K_m remained approximately constant (Table I). In contrast, and in agreement with the data in Fig. 1 D, little if any change in V_{max} and K_m for choline transport was observed during the first 5 h after reversal of the hydroxyurea block.

In order to investigate to what extent the observed changes in transport rates may have been influenced by the method of synchronization, we also monitored transport rates in populations of cells synchronized by consecutive treatments with hydroxyurea and Colcemid. This treatment allowed the synchronization of the cells during the course of a single cell cycle, and about 70% of the cells were arrested in metaphase before reversal. Most of the cells divided within 2 h after resuspension of the cells in fresh medium (Fig. 3 A) and

progressed rapidly into the S phase (Fig. 3 B). The rates of dThd, choline, and deoxyglucose transport increased during traverse of these cells through the cell cycle (Fig. 3 B-D) in about the same manner observed with populations synchronized by the double hydroxyurea treatment.

Effect of Serum Deprivation on Transport Changes during Cell Cycle

Synchronized cells were suspended in either growth medium 67 or a serum-free basal medium (BM42B) and monitored for cell number, mitotic index, rates of DNA synthesis, and transport rates as in the previous experiments. The results in Fig. 4 demonstrate the following. The increases in rates of dThd transport and DNA synthesis occurred normally during the first cell cycle in the absence of serum. The serum-deprived cells also divided normally (Fig. 4 A), but few if any cells initiated DNA synthesis after mitosis (Fig. 4B). Furthermore, after the initial increase in rate of dThd transport the latter began to decrease in the serum-deprived cell population concomitantly with the end of S phase, i.e., at least 1 h before the cells began to divide (Fig. 4 B). This decrease in transport rate probably reflected a relative instability of the dThd transport system (see below). The absence of serum also prevented any of the increases in transport rates for Urd (Fig. 4 C), choline (Fig. 4 D), and deoxyglucose (Fig. 4 E), which normally accompany the traverse of the cells through the cell cycle. The transport rates for these substrates, however, remained relatively constant at the initial level.

The rate of incorporation of Urd into acid-insoluble material also failed to increase in the absence of serum (Fig. 4 B), but it seems likely that this rate reflects mainly the rate of Urd transport rather than the rate of RNA synthesis. Previous results have shown that the rate of Urd transport into the cell limits the rate of Urd incorporation into RNA by randomly growing N1S1-67 cells (8, 13) and similar results have been reported for synchronized populations of a hamster lung cell line (16).

The results indicate that serum deprivation of N1S1-67 cells which had been synchronized at the G₁-S border resulted in the arrest of the cells in the G₁ period of the next cell cycle, but that this arrest was preceded by a failure to exhibit normal increases in transport rates in the previous cell cycle.

TABLE I

Kinetic Constants for the Transport of Choline and 2-Deoxy-D-Glucose as a Function of the Cell Cycle Stage

Substrate	Time after reversal	K_m	V_{max}
	h		
Choline	0.5	7	0.048
	2.6	7	0.048
	5	7	0.048
2-Deoxy-D-glucose	0.5	2,000	7.1
	2.5	2,100	9.1
	5	2,500	12.5

Synchronized populations of cells were prepared by two successive treatments with hydroxyurea as described in Materials and Methods (see Fig. 1). At the indicated times after reversal of the second hydroxyurea block, samples of cells were collected by centrifugation and suspended in the appropriate basal media (see Materials and Methods). Initial rates of choline and 2-deoxy-D-glucose transport as a function of substrate concentration were estimated as described previously (6, 7), and the kinetic values were estimated from Lineweaver-Burk plots of the initial rates.

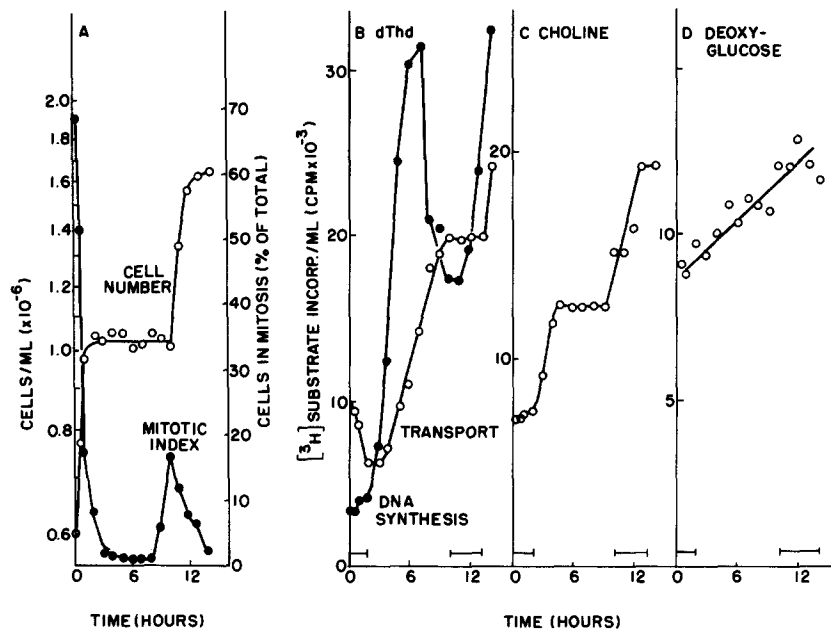


FIGURE 3 Rates of dThd, choline, and 2-deoxy-D-glucose transport and of DNA synthesis in a population of cells synchronized by successive treatment with hydroxyurea and Colcemid. The experiment was conducted as described in the legend to Fig. 1, except that the population was synchronized as follows: A culture of exponentially growing cells was supplemented with 0.5 mM hydroxyurea. After 10 h of incubation at 37°C, the cells were collected by centrifugation, suspended in fresh medium 67, and incubated for 5 h. Then the suspension was supplemented with 0.25 μ M Colcemid. After another 3.5 h of incubation the cells were collected by centrifugation and suspended in fresh medium 67 (0 time). The bars in Fig. 3 B-D indicate the periods of cell division.

Effects of Metabolic Inhibitors on Transport Changes during Cell Cycle

A synchronized population of N1S1-67 cells was allowed to proceed through the cell cycle in to the G_2 period (5 h) and then samples thereof were supplemented with cytosine arabinoside, cycloheximide, or Colcemid. Both cycloheximide and cytosine arabinoside caused a rapid inhibition of DNA synthesis (Fig. 5 A), although the cells divided normally after treatment with cycloheximide or only slightly less well than the control cells after addition of cytosine arabinoside (Fig. 5 B and C). Colcemid caused an arrest of the cells in mitosis (Fig. 5 B and C), and little DNA synthesis or increase in dThd transport rates occurred subsequently (Fig. 5 A and D). The inhibition of DNA synthesis by addition of cytosine arabinoside also prevented the increase of dThd transport during the second cycle (Fig. 5 E and F). The results indicate that the increase in dThd transport rate, but not the increases in

other transport processes, is linked in some manner to the initiation of DNA synthesis in the S phase.

The inhibition of DNA synthesis by cycloheximide may result from its primary effect on protein synthesis. The inhibition of protein synthesis by cycloheximide in the G_2 period also prevented the increases in the rates of all transport processes investigated (Fig. 5 D-F). The results are similar to those observed with serum-deprived cells. Addition of cycloheximide resulted in a rapid loss in dThd transport capacity (Fig. 5 D), whereas the rates of choline and deoxyglucose transport remained relatively constant at the initial level in the G_2 period (Fig. 5 E and F). These results suggest that the dThd transport system is relatively unstable, whereas the choline and deoxyglucose transport systems are stable. Experimental evidence presented later supports this conclusion. Arrest of the cells in mitosis by addition of Colcemid also prevented any further increases in transport rates for choline and deoxyglucose (Fig. 5 E and F).

These latter results are consistent with the view that cell division is a prerequisite for the initiation of increases in transport capacity and that protein synthesis is required for these increases.

Transport Changes Related to Growth Stage of Cells

Results from a previous study (17) indicated that the rate of Urd incorporation into total cell material fluctuates markedly in suspension cultures of randomly growing N1S1-67 cells, reaching a maximum in the mid-exponential phase of growth and then decreasing to a minimum in the stationary phase. Urd kinase activity of cells was found to fluctuate in a similar manner, but subsequent work has shown that the rate-limiting step in the incorporation of Urd into the acid-soluble pool is not phosphorylation, but the transport (by facilitated diffusion) of Urd into the cells, and that the initial rate of incorporation of Urd into total cell material therefore reflects the transport rate (8, 13, 18). Similar decreases in incorpora-

tion rates for various substrates have been observed to be associated with density-dependent inhibition of growth of normal mouse and chicken cells (19-22), but the causal mechanism(s) involved in these decreases in incorporation rates in normal and transformed cells remain to be determined. In the present study with N1S1-67 cells, we have extended our investigation to other substrates (dThd, choline, and deoxyglucose) using shorter labeling periods to ascertain that transport rates were estimated.

The data in Fig. 6 illustrate that upon dilution of a late exponential phase culture with fresh growth medium the transport rates (expressed per 10^6 cells) for all four substrates increased rapidly, attaining maxima in the early or mid-exponential phase (for growth curve see Fig. 6 A). Thereafter, the transport rates decreased rapidly, reaching a minimum in the stationary phase. The rates of incorporation of Urd and dThd into acid-insoluble material changed in a similar pattern. The time-courses of transport rate changes varied somewhat from experiment to experiment, but we observed

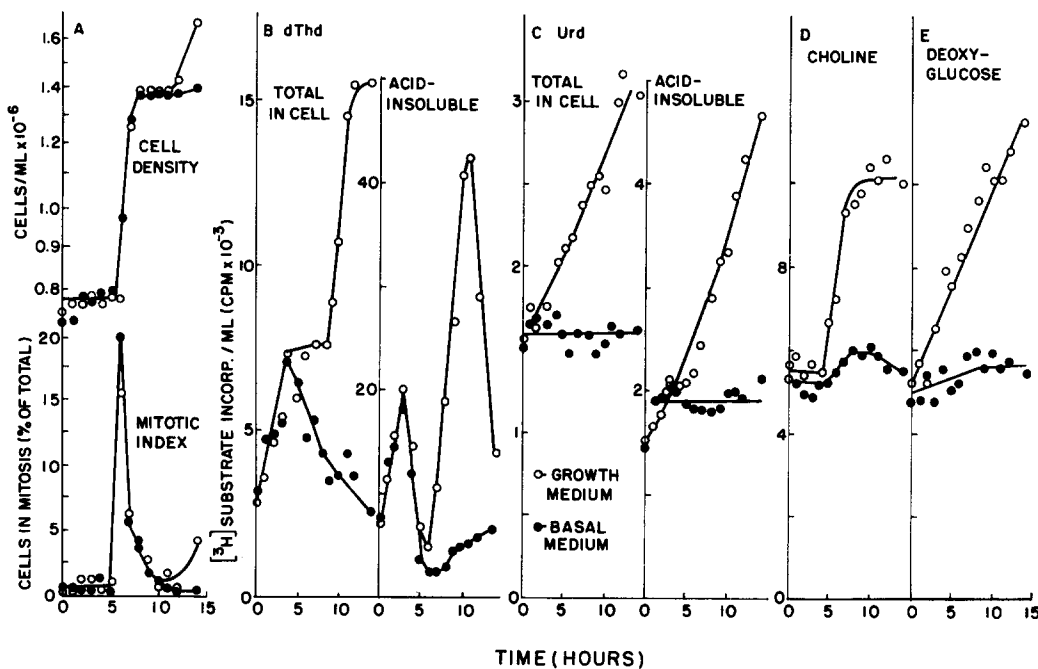


FIGURE 4 Effect of serum deprivation on the rates of transport of dThd, Urd, choline, and 2-deoxy-D-glucose during traverse through the cell cycle. A population of cells was synchronized by the double hydroxyurea treatment. The experiment was conducted as described in the legend to Fig. 1, except that samples of synchronized cells were suspended in both medium 67 (O—O) and BM42B (●—●). In addition in Fig. 4 C, samples of cells labeled with [3 H]Urd were incubated at 37°C for 30 min and analyzed for radioactivity in acid-insoluble material.

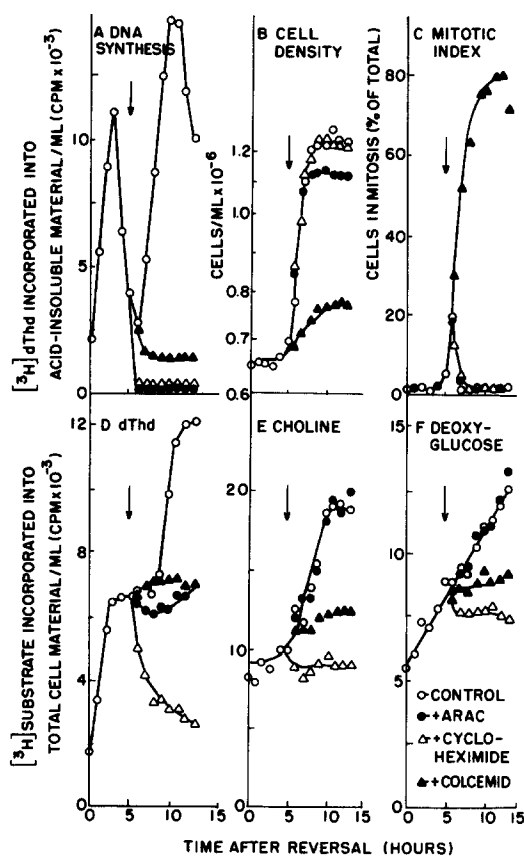


FIGURE 5 Effects of cytosine arabinoside, cycloheximide, and Colcemid on rates of transport of dThd, choline, and 2-deoxy-D-glucose in a synchronized population of cells. The population of cells was synchronized by the double hydroxyurea treatment and samples of cells were subsequently monitored for cell density (B), mitotic index (C), for rates of DNA synthesis (A), and for rates of the transport of dThd (D), choline (E), and 2-deoxy-D-glucose (F) as described in the legend to Fig. 1. Where indicated, samples of cell suspension were supplemented at 5 h after reversal of the second hydroxyurea block with 50 μ M cytosine arabinoside (ARAC), 25 μ g cycloheximide/ml, or 0.75 μ M Colcemid (see arrows).

consistently that the transport rates began to decrease at a time when the cells were still growing exponentially and when the pH of the medium had not decreased below 6.9 (Fig. 6 C). Furthermore, the fluctuations in transport rates were more pronounced for Urd and dThd than for choline and deoxyglucose. We also observed in the experiment illustrated in Fig. 6 C that the rate of deoxyglucose transport increased transiently at about the time the culture passed into stationary phase. This

observation, however, was not reproducibly made; it was observed in only three out of six experiments of the type illustrated in Fig. 6. This transient increase probably did not reflect some kind of derepression of the D-glucose transport system due to substrate exhaustion, since the medium at this growth stage still contained 2-3 mM D-glucose (Fig. 6 D), which is almost sufficient to saturate the D-glucose transport system.

Additional, more selective studies showed that the incorporation of the four substrates was approximately linear with time for at least 5 min whether exponential or stationary phase cells were investigated and that these substrates were equally phosphorylated by the cells in either growth stage. Chromatographic analysis of acid extracts from 5-min labeled cells showed that over 95% of radioactivity from Urd, dThd, and choline and over 80% of that from deoxyglucose were associated with phosphorylated intermediates. These results support the view that the decreased incorporation rates reflected decreases in transport rates and were not due to lack of phosphorylation of the substrates.

Fig. 7 compares the kinetics of dThd and deoxyglucose between exponential phase and stationary phase cells. The results show that the decreases in transport capacity were solely due to decreases in the V_{max} values for the two transport systems, whereas the K_m s remained constant.

Effects of Metabolic Inhibitors on Transport Capacity of Exponential Phase Cells

Samples of a mid-exponential phase culture were supplemented with actinomycin D, cycloheximide, or hydroxyurea and subsequently monitored for the transport rates of the cells for dThd, Urd, choline, and deoxyglucose (Fig. 8). The results show that the inhibition of DNA synthesis by hydroxyurea had little effect on the capacity of these cells to transport these substrates. The inhibition of protein synthesis by cycloheximide or of RNA synthesis by actinomycin D, on the other hand, had a marked effect. Both inhibitors caused a rapid decrease in the rates of dThd and Urd transport (Fig. 8 A and B), although it should be noted that cycloheximide caused an immediate decrease, whereas the decrease resulting from the addition of actinomycin D commenced only after a delay of about 1 h. On the other hand, neither inhibitor caused a significant loss in the capacity of the cells to transport choline or deoxyglucose for 8

h, although both inhibited the increases in transport rates which were observed in the untreated culture (Fig. 8 C and D).

DISCUSSION

Evidence from kinetic analyses, substrate specificities, and inhibitor effects have previously indicated that Urd and dThd are transported by different transport systems of N1S1-67 cells (2) and that deoxyglucose (6) and choline (7, 8) are transported by still other systems. These data and the present results support the view that the plasma membrane is a mosaic of a large number of different transport systems and that these transport systems also differ with respect to their metabolic stability and their pattern of synthesis during the cell cycle. The affinities of the various systems for their respective substrates appear to be quite constant and alterations in transport rates either during traverse of the cells through the cell cycle or as a function of the growth stage of the culture seem to reflect changes in the number of functional transport sites in the membrane (see reference 23). This conclusion is

indicated by the fact that it is the V_{max} that fluctuates with the growth stage or cell cycle stage, whereas the K_m remains constant (see also reference 24). The number of functional transport sites for dThd increases only in the S or late S phase, whereas those for Hyp and choline are formed early in the cell cycle and those for deoxyglucose and Urd increase continuously throughout the cell cycle. Similarly, in Chinese hamster cells an increase in dThd transport capacity is confined to the S phase (25), whereas Urd transport rates increase continuously during the cell cycle (16). However, in these cells the Urd transport rate increased seven- to 10-fold during the period from G_1 to G_2 , the only periods investigated. This increase seems unphysiologically high, except if one assumes that most of these newly formed transport sites are again lost or inactivated between G_2 and G_1 . The large increase is probably not an artifact related to the Colcemid treatment used in the synchronization, since in N1S1-67 cells synchronized by consecutive treatments with hydroxyurea and Colcemid the transport rates for

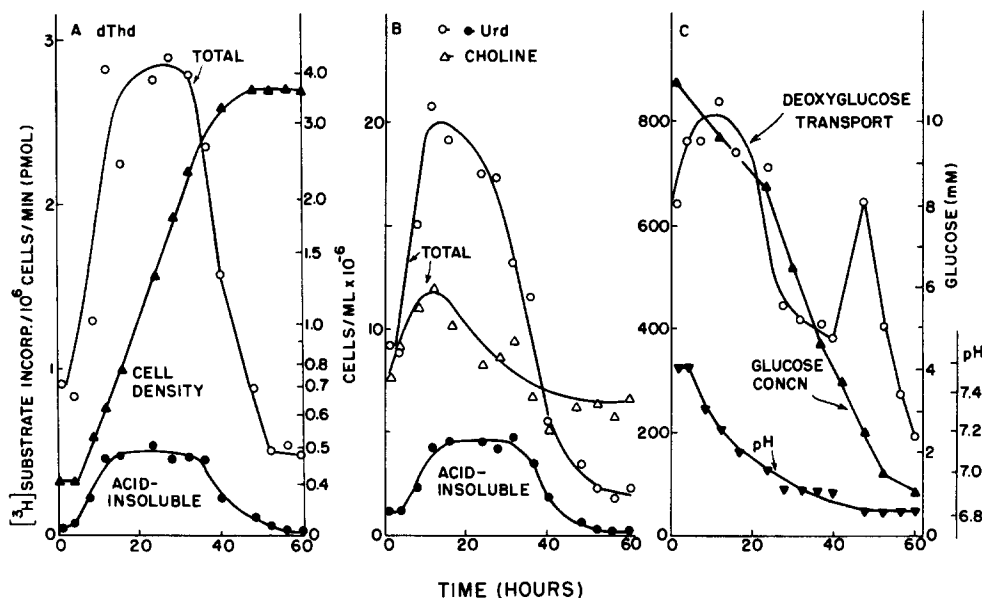


FIGURE 6 Rates of transport of dThd, Urd, choline, and 2-deoxy-D-glucose and of the incorporation of dThd and Urd into acid-insoluble material as a function of age of a culture of randomly growing N1S1-67 cells. A culture in the late exponential phase of growth (at about 2.5×10^6 cells/ml) was diluted approximately sixfold with fresh medium 67 and incubated at 37°C . Samples of suspension were analyzed for cell density (A) and D-glucose concentration and pH (C). From other 1-ml samples the cells were collected by centrifugation and analyzed for their rate of incorporation of ^3H -labeled precursors in total cell material (transport) or acid-insoluble material as described in Materials and Methods. All points represent averages of duplicate samples.

dThd, Urd, and deoxyglucose did not increase more than twofold during the cell cycle (Fig. 3). Thus, upon cell division the transport rates (on a per cell basis) returned to the base level. Similar observations have been made for the transport of aminoisobutyric acid in Chinese hamster ovary cell cultures (24). It was also reported in the later study that the V_{max} for Urd and dThd transport by these cells had approximately doubled 3 h after mitosis (i.e., in G_1), but it was not determined whether increases in transport rates occurred in other stages of the cell cycle (24). These results are contrary to our data with N1S1-67 cells and

to those with hamster cells reported by other investigators (16, 25) and discussed already.

Various lines of evidence (Fig. 1 [16]) indicate that the increases in incorporation rates are due to changes in transport rates, and do not reflect increases in the respective kinase activities. Nevertheless, the dThd (26, 27) and the Urd kinases (16) increase during the cell cycle in patterns similar to those of the changes in transport rates for the respective substrates. Whether this observation is more than coincidental is not known.

Serum deprivation has been found to cause a decrease in the transport of a number of substrates in various types of cells (see reference 23). The present study indicates that serum deprivation also markedly affects the formation of transport systems in synchronous populations of N1S1-67 cells. The finding that the dThd transport capacity of the cells is rapidly lost during serum deprivation (Fig. 4) or upon inhibition of protein synthesis in either synchronous populations in the G_2 period (Fig. 5 D) or in randomly growing cultures (Fig. 8 A) indicates that the dThd transport system is metabolically unstable and must therefore be continuously synthesized to be retained at a certain level. Thus the doubling of the transport sites during the S phase would require the *de novo* synthesis of transport sites in addition to those produced as a result of normal turnover. The Urd transport system also exhibits a certain degree of instability (Fig. 8 B), but the decrease in transport rates in the absence of protein synthesis occurs more slowly than the decrease in dThd transport rates. The finding that treatment of the cells with actinomycin D also results in a loss of transport capacity for both substrates, although only after a short delay (Fig. 8), is consistent with the view that the messenger RNA (mRNA) for the synthesis of one or more proteins involved in the transport of these substrates turns over and that the cells possess only a limited amount of this mRNA. Another interpretation of the data is that both cycloheximide and actinomycin D exert a general toxic effect on the cells which indirectly causes the loss of the transport capacities for dThd and Urd. This interpretation seems unlikely, however, since the transport systems for choline and deoxyglucose seem to be completely stable under the same experimental conditions (Figs. 5 and 8). The increases in transport rates for the latter two substrates during the cell cycle, however, are also

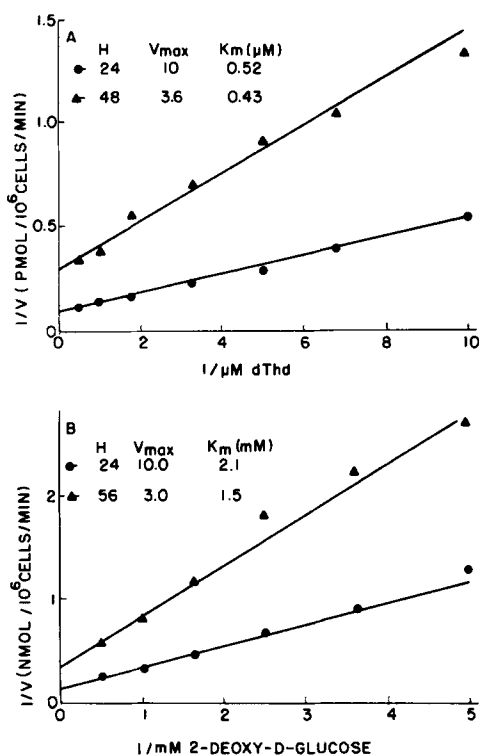


FIGURE 7 Lineweaver-Burk plots of the initial rates of transport of dThd (A) and 2-deoxy-D-glucose (B) by cells in the mid-exponential phase and in the stationary phase of growth. The details of the experiment are described in the legend to Fig. 6. At 24, and 48 or 56 h of growth, samples of cells were collected, suspended to 2×10^6 cells/ml in the appropriate basal media (see Materials and Methods), and the initial rates of dThd and 2-deoxy-D-glucose transport were determined as function of the substrate concentration as described previously (2, 6). All points represent averages of duplicate samples.

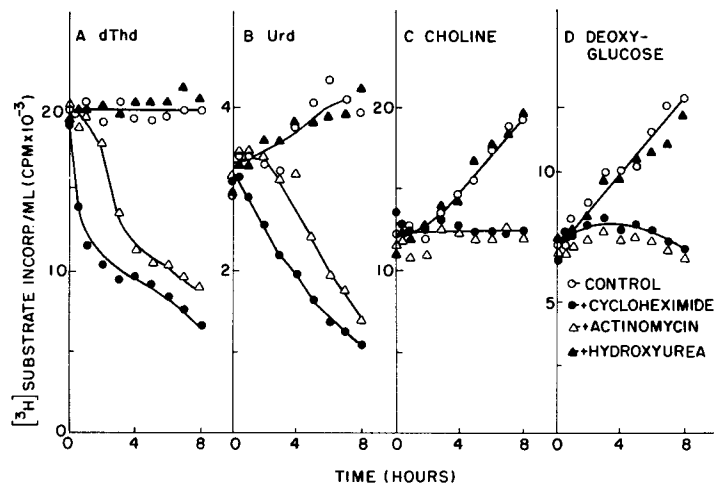


FIGURE 8 Effects of cycloheximide, actinomycin D, and hydroxyurea on the rates of transport of dThd, Urd, choline, and 2-deoxy-D-glucose in mid-exponential phase cultures of randomly growing cells. Samples of a suspension of mid-exponential phase cells at 1.6×10^6 cells/ml were supplemented where indicated with 25 μ g cycloheximide or 1.25 μ g actinomycin D/ml, or 0.5 mM hydroxyurea. The suspensions were incubated at 37°C and monitored at various times for initial rates of transport of dThd, Urd, choline, and 2-deoxy-D-glucose as described in Materials and Methods. All points represent averages of duplicate samples.

inhibited by actinomycin D and cycloheximide and thus also, require *de novo* RNA and protein synthesis. The requirement for protein synthesis in the increase in transport rates during the cell cycle has also been observed for Chinese hamster ovary cells (24). The finding that actinomycin D inhibits the increase in transport rates as rapidly as cycloheximide also suggests a very limited supply of mRNA for the transport proteins in these cells.

Arrest of N1S1-67 cells (Fig. 5) or Chinese hamster ovary cells (24) in metaphase by treatment with Colcemid inhibits any further increases in transport rates. This finding suggests that the initiation of transport rate increases requires normal cell division. Cell division, however, is not sufficient since increases in transport rates do not occur in the absence of serum, i.e., when the cells become arrested in G_1 (Fig. 4). Furthermore, when the initiation of DNA synthesis is prevented in synchronous cultures the initiation of increases in dThd transport rates are inhibited (Fig. 5 B), whereas the formation of the other transport systems and probably other proteins is not affected (Fig. 5 E and F), leading to unbalanced growth (28). These results suggest that the *de novo* synthesis of components for the dThd transport system, but not for other transport systems, is

coupled to the initiation of DNA synthesis in the cell cycle. The nature of this relationship is unknown.

The reason for the decreases in functional transport sites during normal growth of randomly growing cells in culture (Fig. 6) is not clear. The most likely explanation is that it is a consequence of a decrease in the overall rate of protein synthesis which commences midway through the exponential phase of growth (4). The finding that the metabolically more stable transport systems for choline deoxyglucose are less affected than the dThd and Urd transport systems is consistent with this conclusion. Similar decreases in transport rates for a number of substrates are also associated with density inhibition of growth of "normal" cells (see reference 23), but it is uncertain whether these decreases are also related to decreases in rates of protein synthesis. An understanding of the mechanism of regulation of transport processes requires additional work and more information on the molecular basis of facilitated diffusion systems per se.

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