J. Membrane Biol. 34, 277–288 (1977)

Growth Rate of Cultured Novikoff Rat Hepatoma Cells as a Function of the Rate of Thymidine and Hypoxanthine Transport

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Received 8 October 1976; revised 11 November 1976

Summary. Novikoff rat hepatoma cells were propagated in suspension culture in the presence of 1 μM methotrexate and various concentrations of hypoxanthine (or adenosine plus guanosine) and thymidine and with or without the inhibitor of nucleoside and purine transport, Persantin (dipyridamole). Methotrexate-treated cells failed to replicate and died even if the medium was supplemented with either thymidine or a purine source, but normal replication occurred when both were present. The additional presence of Persantin reduced the rate of transport of thymidine or hypoxanthine and thus their incorporation into the nucleotide pool and decreased the rate of cell replication. The growth rate of the cells was directly proportional to the rate of incorporation of thymidine (in the presence of excess hypoxanthine) or of hypoxanthine (in the presence of excess thymidine) until the normal maximum growth rate was obtained. Normal cell replication in the presence of methotrexate and Persantin occurred only when the medium was supplemented with 500 μM hypoxanthine and 30 μM thymidine. The results illustrate a dependence of the growth rate of mammalian cells on the rate of transport of essential nutrients into the cell.

Marked increases in the capacity of cells to transport nucleosides, hexoses, and phosphate have been found to be associated with the stimulation of growth of contact-inhibited normal cells in culture or of lymphocytes, and with the transformation of normal cells to tumor cells (*see* Plagemann & Richey, 1974). These findings support the view that the growth of mammalian cells may, in part at least, be regulated by the influx of certain nutrients into the cell (Pardee, 1971; Holley, 1972). Chemostatic studies have shown that the growth rate of certain yeasts (van Uden, 1967) and bacteria (von Meyenburg, 1971) can be varied by altering the substrate concentration in the medium and thus limiting the rate of uptake of essential nutrients into the cells, but similar results have not been available for mammalian cells. In the present study we

have made cultured Novikoff rat hepatoma cells dependent on thymidine (dThd) and hypoxanthine (Hyp) for growth by supplementing the medium with methotrexate. Folic acid analogs, such as methotrexate (amethopterin) or aminopterin, inhibit the synthesis of tetrahydrofolate and thus the *de novo* synthesis of purine nucleotides, the conversion of dUMP to dTMP, and the synthesis and interconversion of some amino acids (*see* Cohen, 1971). This inhibition is circumvented if appropriate substrates are available which bypass the metabolic blocks (Hakala & Taylor, 1959). Purines or their nucleosides eliminate the need for purine *de novo* synthesis and dThd is directly phosphorylated to dTMP. Cells in the body as well as cultured cells readily utilize purines or their nucleosides and pyrimidine nucleosides when supplied in the external fluid. Transport (facilitated diffusion) systems for both nucleosides and bases have been demonstrated in various types of mammalian cells, and growing cells generally possess high levels of the necessary enzymes and coenzymes for their rapid conversion to nucleotides (*see* Plagemann & Richey, 1974).

In the present study the rate of transport of dThd and purines or their nucleosides was limited by supplementing the medium also with (2,6-bis-diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*)pyrimidine (Persantin, dipyridamole). Persantin inhibits the transport of nucleosides, bases, and monosaccharides without affecting their subsequent metabolism (Plagemann & Richey, 1974). Under these conditions, the rate of replication of methotrexate-treated Novikoff cells varied as a function of the external concentration of the limiting essential nutrient in the medium, and correlated with the rate of transport of the nutrient into the cell.

Materials and Methods

Novikoff rat hepatoma cells (subline N1S1-67) and a dThd kinase deficient subline thereof (Plagemann, Marz & Erbe, 1976) were propagated in suspension culture in Swim's medium 67 as described previously (Plagemann & Swim, 1966; Ward & Plagemann, 1969). Exponential phase cultures were diluted with fresh medium to about 0.2×10^6 cells/ml. The suspensions were supplemented as indicated in the appropriate experiments and incubated at 37 °C on a gyratory shaker at 200 rpm. Duplicate samples were withdrawn and the cells enumerated with a Coulter counter (Ward & Plagemann, 1969).

For labeling experiments, cells were collected from cultures in the exponential phase of growth and suspended to about 2×10^6 or 2×10^7 cells/ml in basal medium 42 (BM42B; Plagemann & Erbe, 1974). The suspensions were supplemented with Persantin and labeled precursors as indicated in the appropriate experiments. Incorporation of radioactivity into total cell material (acid-soluble plus acid-insoluble) by wild type cells during incubation at 37 °C on a gyratory shaker was monitored as described previously (Plagemann & Erbe, 1972, 1974). dThd transport was measured with dThd kinase-deficient cells at 24 °C by the rapid kinetic technique described recently (Wohlhieter, Marz, Graff & Plagemann, 1976).

This method allows uptake measurements in 1.5-sec intervals. In brief, at sequential time points samples of cell suspension were mixed with [^{3}H]dThd and squirted into tubes containing a mixture of silicone fluid and mineral oil and mounted in an Eppendorf microcentrifuge. At a designated time the cells were collected by sedimentation through the oil mixture and analyzed for radioactivity. All values were corrected for radioactivity trapped in the extracellular space. Extracellular trapping and cell volumes were estimated by the use of [^{14}C-carboxyl] carboxylinulin and [^{3}H] H_2O as described previously (Wohlhueter *et al.*, 1976).

Unlabeled nucleosides and bases were purchased from Sigma Chemical Co., St. Louis, Mo; [^{3}H-methyl] thymidine (20 Ci/mM) and [^{14}C-8] hypoxanthine (52 mCi/mM) from Amersham/Searle, Des Plains, Ill., [^{14}C-carboxyl] carboxylinulin (2.6 mCi/g) and [^{3}H] H_2O (1 mCi/g) from New England Nuclear, Boston, Mass., and Na methotrexate from Lederle, Pearl River, N.Y. Persantin was a gift from Geigy Pharmaceuticals, Yonkers, N.Y.

Results and Discussion

Fig. 1*A* shows that Persantin, at a concentration of 8 μM, had little effect on the replication of N1S1-67 cells. Higher concentrations of Persantin caused a decrease in the growth rate and also lowered the density to which the cells grew (data not shown). Addition of 1 μM methotrexate completely inhibited the replication of the cells whether or not Persantin was also present. Minimal replication occurred when the medium was supplemented with either 40 μM guanosine plus 100 μM adenosine, or 30 μM dThd. The little replication occurring in cultures in the presence of either purine nucleosides or dThd was due to the presence of small amounts of dThd (Plagemann, 1971) and purines in a growth medium which contains 5% calf serum and 2.5% of a pancreatic autolysate (Plagemann & Swim, 1966). No replication of methotrexate-treated cells occurred in a growth medium made up with dialyzed calf serum and without pancreatic autolysate, when either purine nucleosides or dThd were added (data not shown).

Supplementation of the medium with *both* purine nucleosides and dThd, on the other hand, allowed the cells to replicate at a normal rate and to the normal maximum cell density (Fig. 1*B*). Thus at concentrations of 30 μM dThd, 40 μM guanosine and 100 μM adenosine, transport and phosphorylation of the nucleosides was rapid enough to allow cell replication at the maximum rate. At these substrate concentrations, the respective incorporation systems were saturated since the apparent K_m values for the incorporation of dThd, guanosine, and adenosine by Novikoff cells are about 0.5, 9, and 12 μM (Plagemann & Richey, 1974). Recent experiments, however, have shown that these K_m values do not reflect those of the nucleoside transport systems, but are rather a complex

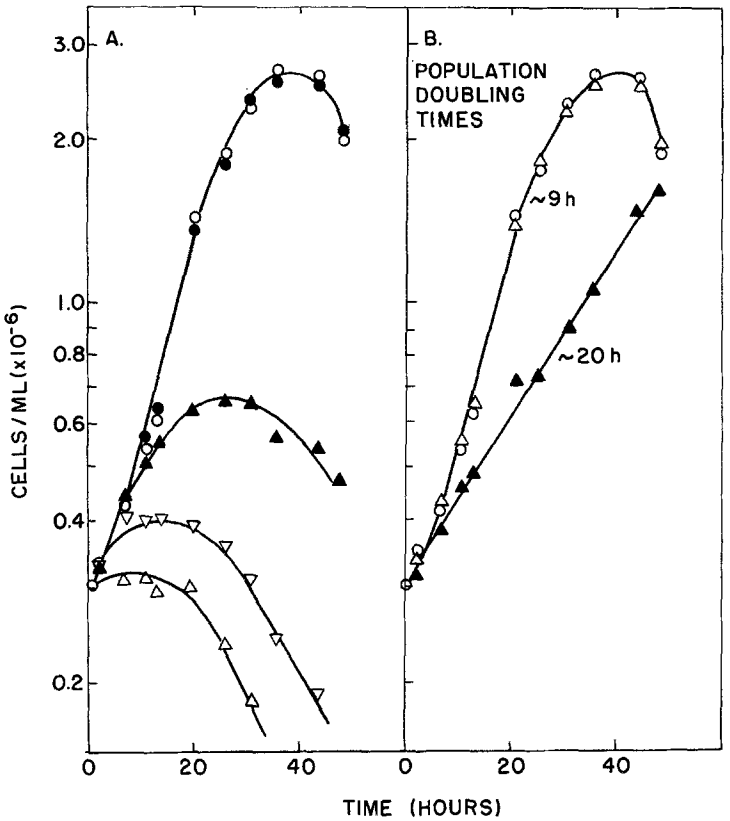

Fig. 1. The effect of methotrexate on the replication of Novikoff rat hepatoma cells, reversal of the effect by nucleosides and prevention of the reversal by Persantin. Samples of a culture of N1S1-67 cells in the early exponential phase of growth were supplemented as follows: (*A*) ○—○, no supplement; ●—●, 8 μM Persantin; △—△, 1 μM methotrexate; ▲—▲, 1 μM methotrexate and 30 μM dThd; ▽—▽, 1 μM methotrexate, 40 μM guanosine and 100 μM adenosine. (*B*) ○—○, no supplement; △—△, 1 μM methotrexate, 30 μM dThd, 100 μM adenosine, and 40 μM guanosine; ▲—▲ as △—△ and in addition 8 μM Persantin. The cultures were incubated at 37 °C and monitored for cell density. All points are averages of duplicate 1 or 2 ml of cell suspension

function of the kinetic properties of the nucleoside transport system as well as of the respective kinase activities (Wohlhueter *et al.*, 1976; Marz *et al., in preparation*). Novikoff cells have been shown to possess only a single, very rapid nucleoside transport system with wide substrate specificity and a K_m for dThd of 70–85 μM (Plagemann *et al.*, 1976; Wohlhueter *et al.*, 1976). Thus under the conditions of our experiments nucleoside transport *per se* was not saturated nor rate-limiting for nucleoside incorporation. Swim's medium 67 contains 400 μM glycine and 50 μM

cysteine, so that the supply of these amino acids was not limiting in these experiments.

When nucleoside transport was inhibited by the presence of 8 μM Persantin, however, the population doubling time of these cultures was increased from about 9 hr to about 20 hr (Fig. 1*B*). The following experiments establish that in the presence of 8 μM Persantin nucleoside incorporation became growth limiting. At the substrate concentrations used in this and subsequent experiments, 8 μM Persantin inhibited nucleoside transport 95–97% when the substrate concentration was at the transport K_m or below (Fig. 2). Fig. 2*A* illustrates the effect of various concentrations of Persantin on the time course of dThd transport into dThd kinase deficient cells in which no substrate phosphorylation occurs (Plagemann *et al.*, 1976). The plot of the dependence of the first-order rate constant (k)[1] for dThd transport on Persantin concentration (Fig. 2*B*) indicates that at the dThd concentrations employed ($<$ transport K_m) dThd transport was inhibited 95% or more by 8 μM Persantin. Other experiments have shown that transport inhibition is of a mixed type with changes in V_{max} ($K_{i,\ intercept} = 0.7$ μM) and K_m ($K_{i,\ slope} = 0.12$ μM) (Wohlhueter *et al., in preparation*). The transport of Hyp was similarly inhibited by Persantin. As measured in ATP-PRPP-depleted cells (Zylka & Plagemann, 1975) by the rapid kinetic technique, the transport of 40, 100, and 600 μM Hyp was inhibited 96, 95, and 89%, respectively, by 8 μM Persantin. Previous experiments have shown inhibition of Hyp transport by Persantin also in a line of Novikoff cells deficient in Hyp phosphoribosyltransferase activity (Zylka & Plagemann, 1975) and that Persantin up to concentrations of 50 μM has little or no effect on uridine and dThd kinase or Hyp phosphoribosyltransferase activities in cell-free preparations of Novikoff cells (Plagemann & Richey, 1974). Combined, these data indicate that the effect of Persantin on cell growth (Fig. 1) was strictly due to its inhibition of nucleoside transport and not of their subsequent metabolism. This suggests that in the presence of 8 μM Persantin nucleoside influx was too low to allow substrate phosphorylation at a rate sufficient to support maximum rates of cell replication. An inhibition of D-glucose or Pi transport did not contribute to the inhibition of replication of the methotrexate-treated cells. The apparent K_i for the inhibition of hexose incorporation in Novikoff cells by Persan-

1 Theoretically, carrier-mediated transport follows first order only in the first-order range of substrate concentration ($So < K_m$), but empirically we have found that the first-order rate equation also describes adequately transport when So is above the K_m, in which case the value k is a function of So (*see* Wohlhueter *et al.*, 1976).

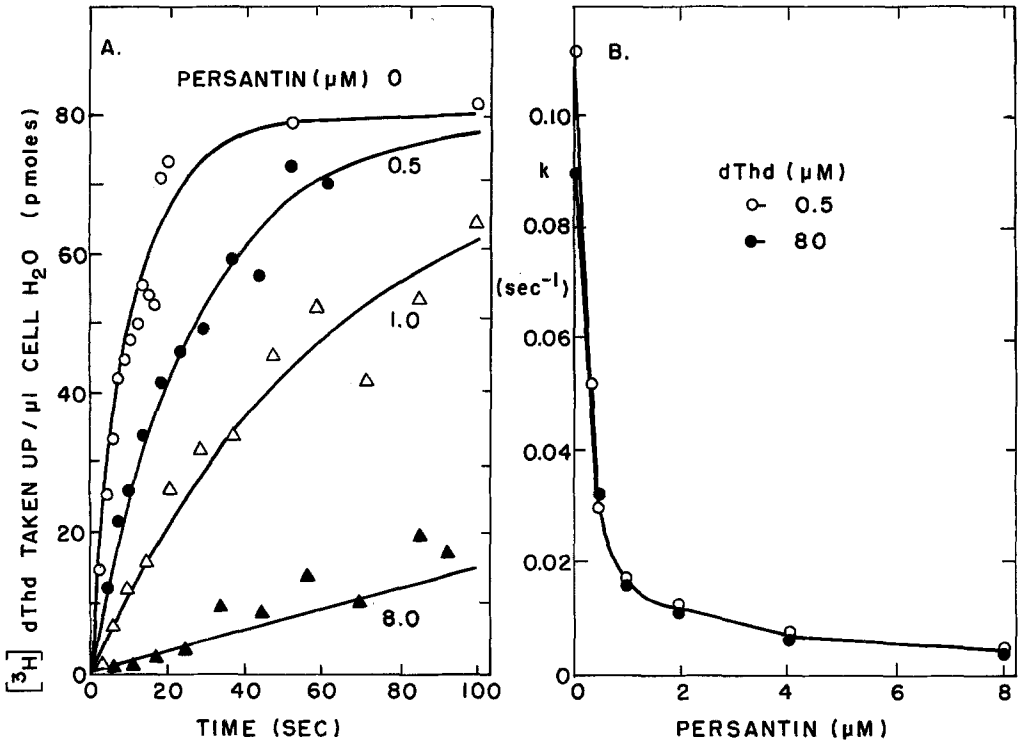

Fig. 2. Effect of Persantin on dThd transport by dThd kinase-deficient Novikoff cells. dThd transport was measured by the rapid kinetic technique described by Wohlhueter *et al.* (1976). Samples of a suspension of about 2×10^7 cells/ml of BM42B were supplemented with 0.5 or 80 μM [^{3}H] dThd (340 cpm and 14.5 cpm/pmole, respectively) and the indicated concentrations of Persantin. First-order rate constants (k) were calculated from time courses of uptake such as those in *A* for 80 μM dThd. The H_2O volume of the cells was estimated to be 13 μl/10^7 cells

tin is relatively high (25–100 μM; Plagemann & Richey, 1974) and the growth medium contained excess concentrations of D-glucose (15 mM) and Pi (1 mM). Furthermore, 8 μM Persantin in the absence of methotrexate had no effect on cell replication (Fig. 1 *A*).

The following experiments were conducted in order to demonstrate more clearly the dependence of the rate of cell replication on the rate of incorporation of a single nutrient. They were based on the finding that supplementation of the medium with 30 μM dThd plus 500 μM Hyp (instead of adenosine plus guanosine) also almost completely overcame the growth inhibition by 1 μM methotrexate even in the presence of 8 μM Persantin (*see* Fig. 3 *C* and 4 *C*). Two sets of experiments were conducted. An excess of either dThd (30 μM) or Hyp (500 μM) was added

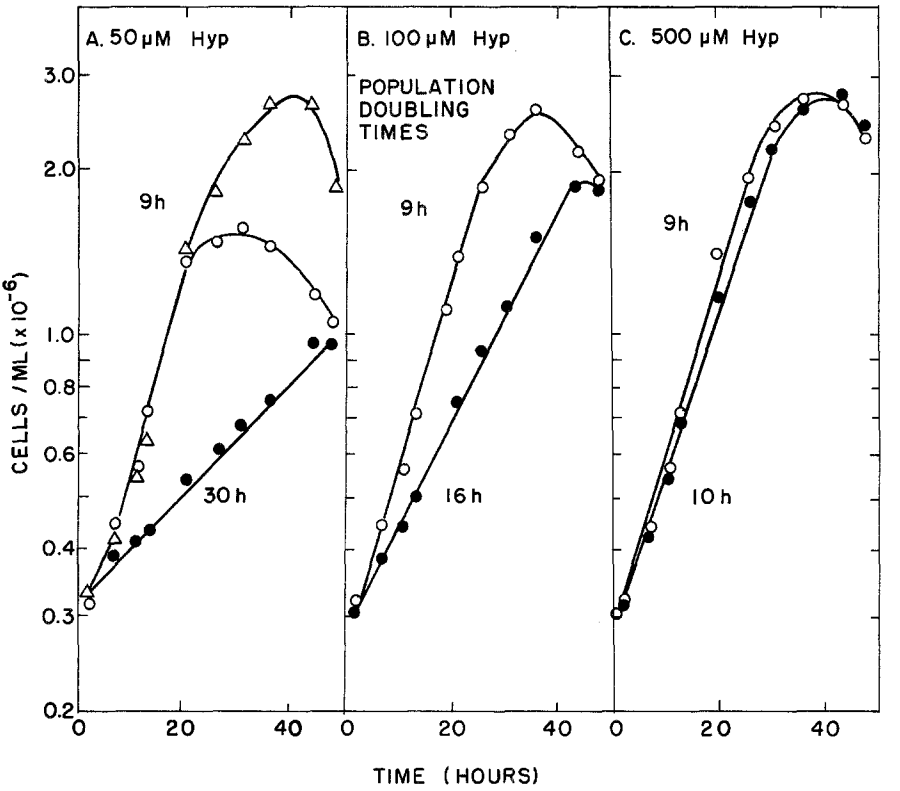

Fig. 3. Effect of various concentrations of Hyp on the reversal of the methotrexate block in the presence and absence of Persantin. Samples of an early exponential phase culture of N1S1-67 cells were supplemented with 1 μM methotrexate and 30 μM dThd, and where indicated with 50, 100, or 500 μM Hyp. One set of cultures was also supplemented with 8 μM Persantin (●—●), whereas a second set did not receive this inhibitor (○—○). △—△ indicates a control without any supplement. The suspensions were incubated at 37 °C and monitored for cell density. All points are averages of duplicate 1- or 2-ml samples of suspension

to the methotrexate-containing medium. In the first case Hyp was added at varying concentrations (50, 100, 500 μM; Fig. 3), in the second case dThd (6, 12, 30 μM; Fig. 4) was added. In each case duplicate sets of cultures were initiated, one of which was also supplemented with 8 μM Persantin. In the absence of Persantin, cells replicated at a normal rate, although the final cell density obtained was depressed if a substrate was present in a limiting amount (6 μM dThd; Fig. 4*A* or 50 μM Hyp; Fig. 3*A*). On the basis of an average DNA and RNA content of N1S1-67 cells of about 10 and 30 μg/10^6 cells, respectively (Ward & Plagemann, 1969), we estimate that between 40 and 60 nmoles of Hyp are required

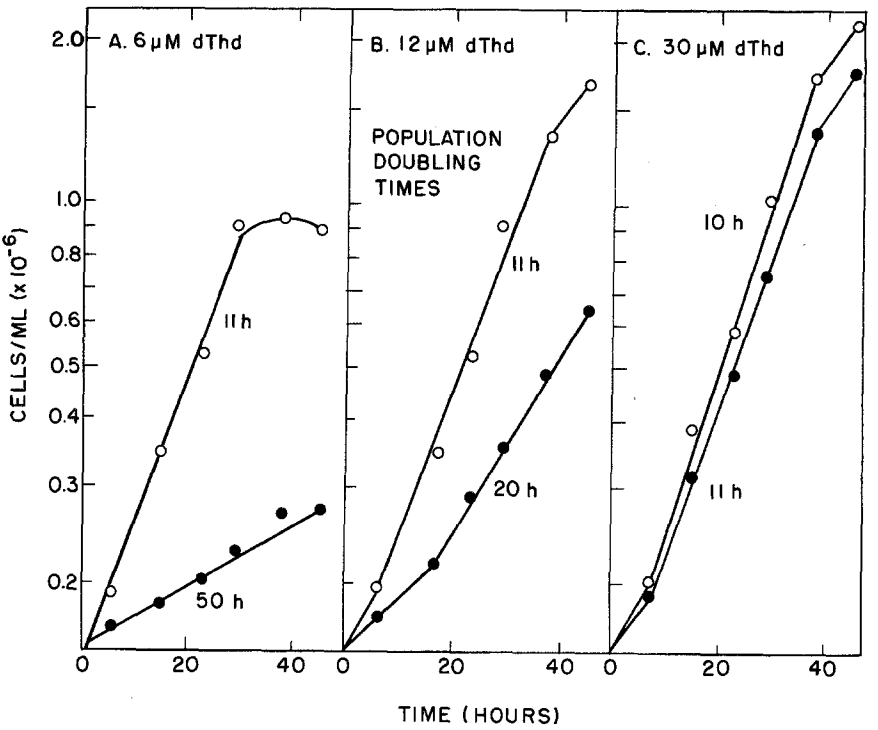

Fig. 4. Effect of various concentrations of dThd on the reversal of the methotrexate block in the presence and absence of Persantin. Samples of an early exponential phase culture of N1S1-67 cells were supplemented with 1 μM methotrexate and 500 μM Hyp and where indicated with 6, 12, or 30 μM dThd. One set of cultures were also supplemented with 8 μM Persantin (●—●), whereas a second set did not receive this inhibitor (○—○). The suspensions were incubated at 37 °C and monitored for cell density. All points are averages of duplicate 1- or 2-ml samples of suspension

to satisfy the purine requirements for pool nucleotide and net RNA and DNA synthesis for 10^6 cells. Similarly, 10^6 cells require approximately 10 nmoles dThd to satisfy the dTTP requirements for DNA synthesis. Thus the cessation of replication of cells in medium supplemented with 50 μM Hyp (Fig. 3*A*) or 6 μM dThd (Fig. 4*A*) was probably due to exhaustion of the required precursor in the medium.

The presence of 8 μM Persantin caused a marked reduction in the growth rate of the cells as a function of the concentration of either of the substrates. Fig. 3 depicts the effect of increasing the Hyp concentration in the medium; at 50 and 100 μM Hyp the population doubling time was increased by Persantin from a normal of about 9 hr to about 30 hr and 16 hr, respectively. In the reverse experiment (Fig. 4) the population doubling time was increased by Persantin to 50 and 20 hr in

the presence of 6 and 12 μM dThd, respectively. Only when 30 μM dThd was present did the replication time (11 hr) approach the control value. The results in both these experiments are consistent with the view that in the presence of Persantin substrate transport was inhibited to such an extent that the rate of cell replication became dependent on the rate of substrate transport. This conclusion is further supported by the following experiments. Samples of a cell suspension were supplemented with increasing concentrations of [^{14}C] Hyp (from 2 to 500 μM) and, where indicated, with 8 μM Persantin. The suspensions were incubated at 37 °C and monitored for the incorporation of radioactivity into total cell material (acid-soluble plus acid-insoluble). Chromatographic analysis of acid-extracts prepared from these cells showed that over 95% of the radioactivity in the acid-soluble pool was in each case in adenine and guanine nucleotides (mainly ATP, data not shown).

The results in Fig. 5*A* show that, consistent with the apparent K_m for Hyp incorporation of about 5–8 μM (Zylka & Plagemann, 1975), the initial rate of Hyp incorporation increased little with an increase in Hyp concentration above 12 μM. It can be calculated that at the normal population doubling time of 9–10 hr, the cells need to incorporate about 4–5 nmoles Hyp/10^6 cells per hr to satisfy their purine requirements for net DNA and RNA synthesis. The maximum rate of the incorporation system was just about sufficient to satisfy this requirement (Fig. 5*A*). Upon inhibition of the transport system by Persantin, on the other hand, the rate of hypoxanthine incorporation was reduced below this rate, the degree of reduction depending on the Hyp concentration in the medium (Fig. 5*B*). A comparison of these data with those in Fig. 3 shows that the decrease in the rate of Hyp incorporation with decrease in Hyp concentration was approximately proportional to the decrease in the replication rate (inverse of population doubling time) of the cells. For instance, at concentrations of 100 and 50 μM Hyp, the maximum rate of Hyp incorporation was reduced about 60 and 75%, respectively, by 8 μM Persantin (Fig. 5*B*) and the growth rate of the methotrexate-treated cells was reduced to about the same extent (Fig. 3). Only when Hyp was present at 500 μM was its residual rate of transport in the presence of 8 μM Persantin sufficient to satisfy the purine requirements of the cells and to support maximum cell replication.

Results for dThd incorporation in the presence and absence of Persantin (Fig. 5*C* and *D*) were similar to those with Hyp. Maximum rates of dThd incorporation were approached at concentrations above 3 μM dThd (Fig. 5*C*). When dThd was present at 30 μM, a rate of incorporation

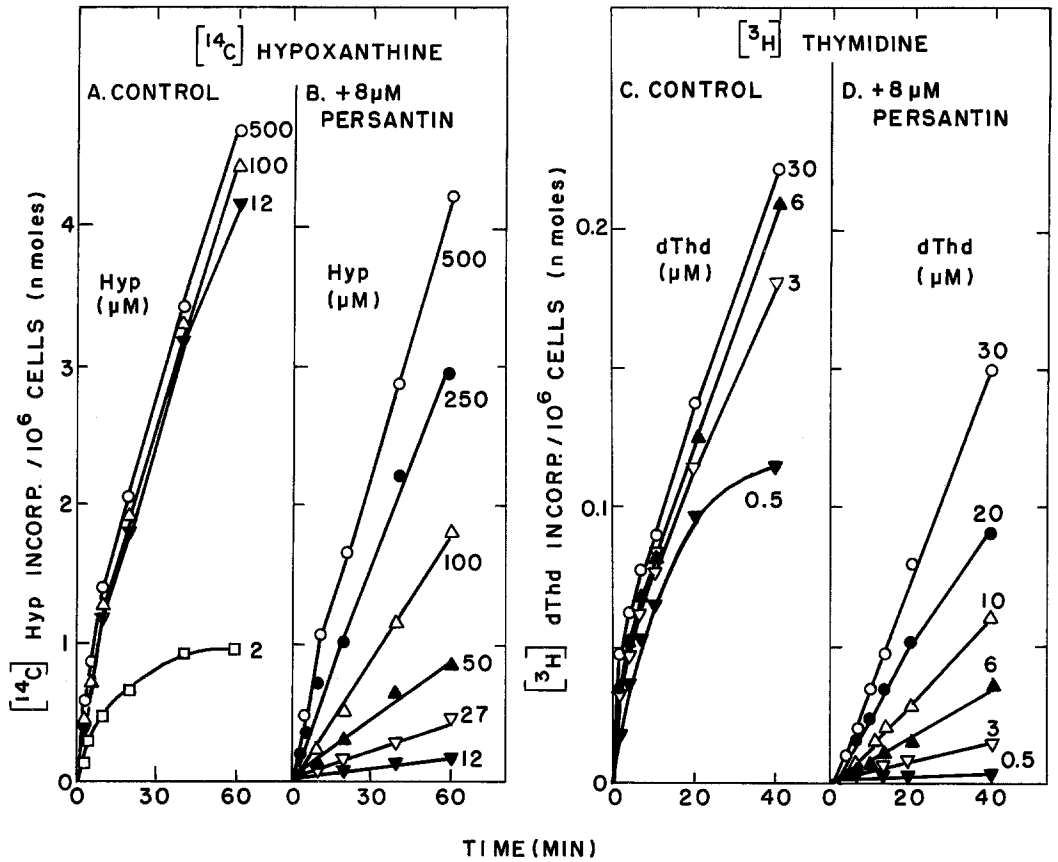

Fig. 5. Effect of Persantin on the incorporation of Hyp and dThd as a function of substrate concentration. Samples of a suspension of 2×10^6 N1S1-67 cells/ml of BM42B were supplemented (*A, B*) with 2 μM [^{14}C] Hyp (80 cpm/pmole) or (*C, D*) with 0.5 μM [^{3}H-methyl] dThd (700 cpm/pmole) and with unlabeled Hyp and dThd to indicated concentrations. All suspensions in *B* and *D* were also supplemented with 8 μM Persantin. The suspensions were incubated at 37 °C and duplicate 1-ml samples were analyzed for radioactivity in total cell material. All points represent averages of the duplicate samples

close to the maximum was maintained, even in the face of a 95% inhibition of its transport into the cell by 8 μM Persantin (compare Figs. 2, 5 *C* and *D*), and this rate was sufficient to supply enough dTTP to support a maximum rate of cell replication (Fig. 4 *C*). With decrease in dThd concentration in the medium, on the other hand, dThd transport was sufficiently inhibited by 8 μM Persantin to cause a limitation in the supply of dTTP (Fig. 5 *D*) and a proportional decrease in the growth rate of the cells (Fig. 4). Other studies showed that treatment of the cells with methotrexate in the presence of 500 μM Hyp and 30 μM dThd

did not alter the capacity of the cells to incorporate either Hyp or dThd (data not shown).

In a previous study Warnick, Muzik and Paterson (1972) have shown that 6-[(4-nitrobenzyl) thio]9-β-D-ribofuranosylpurine (NBMPR) inhibits the toxic effects of methylthioinosine and of other nucleoside analogs for L5178Y mouse lymphoma cells by inhibiting their transport into the cell. It was also reported that NBMPR inhibited the growth of methotrexate-treated cells in the presence of dThd and Hyp. This effect was attributed to an inhibition of dThd transport by NBMPR, but no detailed experimental results were presented with respect to an inhibition of dThd transport by NBMPR or to the relationship between dThd incorporation rates and the growth rates of the cells. An effect of NBMPR on Hyp transport was also not considered. Our study clearly shows that growth of methotrexate-treated cells in the presence of dThd and Hyp can be inhibited by an inhibition of either dThd or Hyp transport. Persantin is especially effective in this respect because it inhibits the transport of all nucleosides and purines. Furthermore, because the mixed type of inhibition exhibits a strong K_m component, the efficacy of Persantin in preventing cell replication of methotrexate-treated cells is particularly great when the essential nucleosides or bases are present in relatively low concentrations as, for instance, 1 μM dThd plus 2 μM Hyp. Other experiments have shown that, under these conditions, the cells failed to grow in the presence of Persantin, whereas some growth occurred in the absence of Persantin (data not shown).

Our results therefore demonstrate that under certain experimental conditions the rate of replication of mammalian cells is dependent on the rate of transport of essential nutrients into the cells. They also indicate that in the cell culture system a transport inhibitor, such as Persantin, markedly potentiates the toxicity of methotrexate in the presence of low concentrations of required nucleosides and purines, but whether this observation may also apply to the *in vivo* situation in whole animals needs further investigation.

This work was supported by Public Health Service research fellowship CA00800 to R.M., research grants CA 16228 and 16959, and Division of Cancer Treatment contract NOI-CM-43788 from the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare. The authors thank Cindy Ovshak and Marsha Behrens for competent technical assistance and Geigy Pharmaceuticals for a gift of Persantin.

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