Journal of Supramolecular Structure 7:61–77 (1977)
Molecular Aspects of Membrane Transport 425–441

Nutrient Uptake and Control of Animal Cell Proliferation

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The division of fibroblast-like cells in culture can be regulated by cell density, serum, and various growth factors. This system has been widely utilized as a model to study the regulation of cell proliferation. There are many physiological and metabolic changes that correlate with the proliferative state of the cell. These include changes in morphology, cyclic nucleotide levels, enzyme activities, and certain cell surface properties such as nutrient uptake and chemical composition of the plasma membrane. Of primary concern is determination of which changes might be critical links in the control of cell proliferation and which ones are simply correlated but not causally involved with cell growth. We have discussed evidence which has strongly suggested a fundamental role for uptake of certain nutrients in the regulation of cell growth. In addition, we have presented several methods allowing a critical analysis of a putative cause and effect relationship between nutrient uptake and growth control. One method involves a dose-response study of the effect of a mitogen on uptake and DNA synthesis, while a second method involves search for a particular mitogen that may, under the appropriate conditions, stimulate cell division without stimulating uptake. These two methods are limited, however, since they are not always applicable to any given nutrient or mitogen. A third method which is not limited in its applications involves varying the concentration of a particular nutrient in the medium to control its uptake. In the case of orthophosphate (P_i) or glucose, we have used this "nutrient concentration" method to demonstrate that under normal culture conditions, uptake of these nutrients is not a causal event in the regulation of cell division.

We considered the possibility that intracellular nutrient availability might control cell growth, even under conditions where uptake did not. For P_i and glucose, we assumed intracellular pool size to be an accurate indicator of intracellular nutrient availability and measured these pools under a variety of proliferative conditions. These studies revealed, however, no correlation between pool size and proliferative state of the cells. This clearly demonstrates that for P_i and glucose, intracellular pool sizes are not causally involved in the control of growth. The possibility remains, however, that if these nutrients are compartmentalized within the cell, intracellular pool sizes may not be an accurate indicator of nutrient availability.

For P_i and glucose there are many interesting questions that remain to be answered about the transport mechanisms for these nutrients. For some other nutrients, particularly K^+ and amino acids, in addition to questions dealing with the nature of transport mechanisms, the question of uptake involvement in the control of proliferation remains entirely open. As with P_i and glucose, many observations strongly suggest a fundamental relationship between amino acid or K^+ uptake and control of cell growth. We suggest that the "nutrient concentration" technique used

Received April 20, 1977; accepted June 6, 1977.

in our studies to analyze P_i and glucose uptake is applicable to any nutrient and should, therefore, prove extremely useful for studying the involvement of any uptake change in the regulation of cell proliferation.

Key words: cell culture, growth control, glucose uptake, phosphate uptake

Numerous observations made over the past several years with a variety of different cell types and nutrients have suggested the possibility of a causal relationship between membrane transport[1] and control of cell division. The purpose of the present review is to delineate some of these observations and to point out methods allowing a careful dissection of this relationship.

There is an extensive literature dealing with the general relationship of cell surface properties to neoplastic transformation and regulation of proliferation in so-called normal cells. This subject is treated comprehensively in several recent reviews and conference proceedings (1–9). In this review, we will focus on the putative role of nutrient uptake in the growth of "normal" fibroblast-like cells.

The first part of this article will deal primarily with changes in the uptake of P_i and glucose. The relationship between cell division and changes in uptake of these nutrients is particularly well documented, and experiments have been carried out allowing a careful analysis of this relationship. The second part of this article will summarize the correlations observed between cell division and changes in uptake of nutrients other than P_i or glucose.

BACKGROUND

One line of reasoning which has led investigators to consider nutrient uptake as a key regulator of cell division is based upon the temporal and spatial considerations inherent in models of growth regulation. Stimulation of division of stationary phase cells[2] requires at least several hours from the time of serum addition until a significant increase is detected in the rate of DNA synthesis as measured by thymidine incorporation (10, 11). In addition, it seems likely that serum factors might act at the cell surface. Thus, a primary signal in the stimulation of DNA synthesis might be expected to 1) occur rapidly after the addition of serum, and 2) provide a means of transferring the signal from the cell surface to the cytoplasm and nucleus.

It was suggested as early as 1964 that changes in permeability of the cell membrane might be related to the control of cell division (12). Experimental confirmation of this hypothesis was shown for the uptake of P_i and uridine by 3T3 cells (13). We observed that uptake of these nutrients was roughly proportional to the rate of cell division. Further-

[1]In using the word "transport," we denote specifically and exclusively the rate of passage of a molecule across the plasma membrane. We will use the word "uptake" to denote experimental measurements of the initial rate of isotope accumulation into the acid-soluble fraction of the cell. Differences between transport and uptake will be further discussed in a later section.

[2]We will use the terms "stationary phase," "quiescent," and "arrested" interchangeably. In doing so, we refer to the reduction in rate of cell division and concurrent entrance into G_1 (or G_0) phase of the cell cycle observed in most nontransformed fibroblast-like cells.

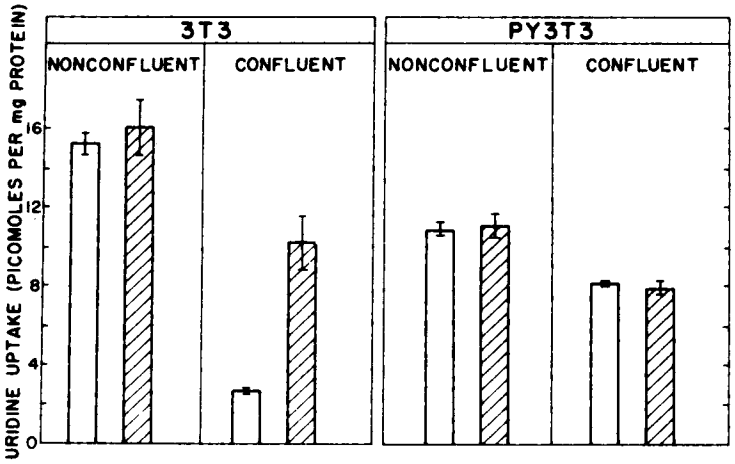

Fig. 1. Uridine uptake by 3T3 and polyoma virus-transformed 3T3 (Py3T3) cells. [^{3}H] uridine uptake was measured during a 15-min incubation period. Uptake was linear during this time interval. Plain bars) control cultures. Lined bars) uridine uptake durint the 15-min interval immediately after addition of fresh serum to a final concentration of 10%. [Reprinted from Ref. 13, with permission]

more, addition of fresh serum to arrested cells resulted in an early (within 15 min) increase in uptake. These results for uridine are presented in Fig. 1.

Changes in uptake of these nutrients are selective since uptake of some amino acids does not vary with the rate of cell division in 3T3 cells (14), and since addition of fresh serum to quiescent 3T3 cells has no effect on adenosine uptake (13).

These results were extended by the observations that addition of serum or trypsin [which can act as a mitogen (15)] to chick embryo (CE) cells stimulated an early increase in the uptake of 2-deoxyglucose (16). Furthermore, the growth rate of these cells correlated with both uridine (17, 18) and 2-deoxyglucose (16, 18) uptake. An additional observation suggesting a close relationship between chick cell division and 2-deoxyglucose uptake concerned the effect of pH on these parameters (19). In arrested cultures, raising the pH for 24 h resulted in large and parallel increases in the rate of DNA synthesis and 2-deoxyglucose uptake. However, in rapidly dividing, low density cultures, changes in pH had little effect on either parameter (19). Another finding which suggests a close relationship between initiation of cell division and early increases in nutrient uptake is the fact that both responses can be elicited by the addition of highly purified mitogenic agents (20, 21).

These and similar observations suggest a general mechanism for growth regulation. It was hypothesized that a decline in nutrient uptake as rapidly growing cells began to approach quiescence would decrease the availability of nutrients inside the cell. This, in turn, would lead to specific arrest of cell growth in G_1 (or G_0). Addition of serum or other mitogens to these stationary phase cells would cause an immediate increase in nutrient uptake, followed by increased nutrient availability inside the cell, eventually leading to initiation of DNA synthesis and cell division (22).

The idea that intracellular nutrient availability might act as a direct signal to initiate or arrest cell proliferation was lent further credence by the observation that Chinese hamster ovary cells could be arrested in G_1 phase by lowering the concentration of isoleucine or

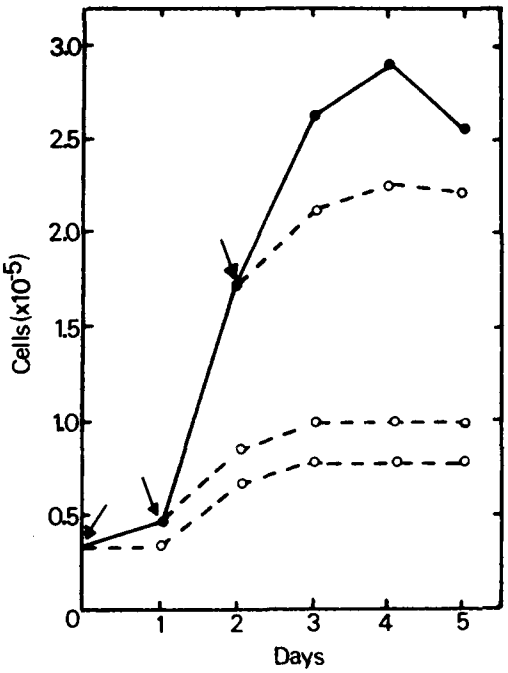

Fig. 2. Effect of reduction of P_i on 3T3 cell number. 3T3 cells were plated at a density of 5.5×10^3 per cm^2 in medium containing 10% serum. Shortly after the cells had attached, the medium was aspirated and replaced by medium containing 10% dialyzed serum and either 1.0 mM P_i or 0.005 mM P_i. On the following 2 days, some of the cultures in 1.0 mM P_i were switched to conditioned medium containing 0.005 M P_i (taken from parallel cultures) at the times indicated by the arrows. •—•) change to 1.0 mM P_i; o - - - o) changed to 0.005 mM P_i. [Reprinted from Ref. 26, with permission.]

glutamine in the medium (23). Addition of isoleucine or glutamine could then synchronously reinitiate cell division (24). Similar observations were reported for P_i and glucose in the control of 3T3 cell proliferation (25). Results from our laboratory (26) confirmed these reports and further demonstrated that arrest of 3T3 cells by lowering P_i in the medium was quantitatively dependent on initial cell density (Fig. 2).

At this point, then, we are left with the following observations: 1) Uptake of P_i, 2-deoxyglucose, and uridine is roughly proportional to the rate of cell division in a variety of cell types. Early stimulation of uptake by serum or highly purified mitogenic agents in arrested cells may fulfill a temporal expectation for a signal involved in the initiation of DNA synthesis. 2) Varying the extracellular concentrations of a variety of nutrients can specifically arrest and subsequently initiate cell division. This may be indicative of a spatial link between changes in transport at the cell surface, and direct control of DNA synthesis from within the cell.

INCREASES IN P_i AND GLUCOSE UPTAKE CAUSED BY ADDITION OF SERUM OR OTHER MITOGENS TO STATIONARY PHASE CULTURES

There are 2 phases of cell proliferation where changes in nutrient uptake could act as a regulatory signal. The first is the decline in proliferation as cells approach a quiescent

monolayer; the second is the stimulation of quiescent cells by serum or other mitogens. The stimulation of quiescent cells is often the easier system to study since cell division can be synchronously initiated in almost the entire population.

Do Uptake Changes Reflect Changes in Membrane Transport?

One of the first questions that must be considered is whether or not changes in uptake of P_i or glucose represent actual changes in membrane transport. Serum addition causes a variety of metabolic "posttransport" effects. This raises the possibility that observed changes in P_i or glucose uptake may actually reflect changes in metabolism of these nutrients. For example, in 3T3 cells, incorporation of P_i into phospholipids (27) and total organic phosphate (28) rapidly increases two- to threefold after serum addition. In the case of glucose, the analog 2-deoxyglucose is frequently used as an indicator of transport activity since it is not significantly metabolized beyond the initial phosphorylation step (29). However, the initial phosphorylation is increased in both CE (30) and 3T3 (31) cells following addition of fresh serum. This observation is consistent with the idea that observed changes in glucose uptake may actually be a result of changes in posttransport metabolism (31, 32). This idea is supported by the observation that phosphofructokinase activity in CE cells is stimulated more than 10-fold by the addition of fresh serum (33).

One way to demonstrate that a change in uptake reflects an actual change in membrane transport is to use a nonmetabolizable analog. In the case of glucose, 3-O-methyl glucose, which is not phosphorylated at all (29, 34), has been reported to be transported by the same carrier as glucose and 2-deoxyglucose (29, 35).[3] Using this analog, several investigators have found that serum does stimulate uptake of 3-O-methyl glucose in CE (30) and 3T3 (36) cells.

Another way to demonstrate changes in membrane transport rather than "posttransport" metabolism of nutrients is to measure uptake with membrane vesicles (37). A difference in nutrient uptake by membrane vesicles prepared from cells in different proliferative states is clearly indicative of a transport change. However, the absence of an observable change does not in itself show that changes in transport might not occur in the whole cell since the vesicle isolation procedure could alter the transport properties of the membrane. In the case of P_i, vesicles derived from transformed 3T3 cells have higher transport activity than those derived from normal, quiescent 3T3 cells (38). With glucose, however, no changes in transport were observed between vesicles derived from growing, quiescent, or transformed 3T3 cells (39).

Serum Stimulation of P_i and Glucose Uptake: Quantitative Aspects

The early stimulation of both P_i and glucose uptake by serum addition to quiescent cells raises the question that these changes might be brought about by the same process. This has been termed the pleiotypic response (40, 41). One approach to answering this question has involved a quantitative characterization of uptake activation by serum. In the

[3]One disadvantage of using 3-O-methyl glucose as an indicator of glucose transport is that this analog is not "trapped" inside the cell and, therefore, is easily washed out during the rinsing procedure that follows incubation. In contrast, after 2-deoxyglucose is phosphorylated, efflux is very low. This results in an extended "initial rate" period for 2-deoxyglucose, where gross cellular accumulation of radioactivity remains linear for as long as 10–15 min. In the case of 3-O-methyl glucose, the initial rate period is usually less than 5 min. This requires extremely short incubation times to obtain an accurate indication of transport.

case of P_i, early uptake stimulation in 3T3 cells appears to be a result of a change in the observed V_{max} (42, 43). Following addition of 25% dialyzed serum to quiescent 3T3 cells, an increase in the rate of P_i uptake can be detected within 5 min (42). The uptake rate continues to increase up to about 10 min after serum addition at which time it remains constant at a level two- to fourfold over the control level (13, 42, 43). This increase appears to be energy dependent since it does not occur in glucose-free medium with oligomycin (28), or in the presence of iodoacetate and cyanide (13). The early increase does not require protein synthesis, however, since it occurs in the presence of 10 μg/ml cycloheximide (42). The early serum stimulation of glucose uptake in 3T3 cells follows a similar pattern to that of P_i, with the exception that glucose uptake appears to be mediated by facilitated diffusion in a variety of cell types and is not energy-dependent (29, 34, 44). Thus, in 3T3 cells, the rate of glucose uptake increases for 30 min to 1 h following serum addition (45, 46), and is not prevented by cycloheximide (46). In CE cells, one laboratory reported a partial inhibition by cycloheximide of early serum-stimulated glucose uptake (16), while another laboratory reported no effect of cycloheximide (30). The reasons for this discrepancy are unclear. In any case, it appears that serum does "induce" very rapid increases in P_i and glucose uptake both of which can be attributed to a change in the observed V_{max}. In some cells, this increase does not require synthesis of new protein, suggesting that the change in V_{max} is not simply a result of new carrier synthesis (30, 46).

The data cited above are consistent with the possibility that the early increase in serum activation of glucose and P_i uptake are brought about by the same process and thus are involved in the postulated pleiotypic response. This concept must be modified since hypotheses based on the pleiotypic response usually invoke one or more cyclic nucleotides as a universal regulator of transport, metabolism, and cell growth (40, 41). However, it has been demonstrated by several methods that early serum-stimulated changes in P_i (42, 43) and glucose (46, 47) uptake are not mediated by changes in cAMP.

Any consideration of the possibility that serum activation of nutrient uptake leads to increased intracellular pool sizes and DNA synthesis must take into account levels of uptake not only immediately after serum addition, but up until the time of commitment to DNA synthesis. Several laboratories have examined these parameters, and although there are some minor discrepancies in their results, it is clear that in 3T3 cells, both P_i and glucose uptake remain elevated for at least 4 and 8 h, respectively (45, 46). In CE cells there is no stimulation of P_i uptake by serum (48), but glucose uptake remains elevated for at least 8 h (16, 30). Some laboratories have reported secondary increases in rates of uptake for P_i in 3T3 cells (46), and for glucose in 3T3 (45, 46), and CE (30) cells. These secondary increases in uptake occur 1–2 h after the first increases in uptake and without exception are dependent on protein synthesis. Thus, it appears that 1) serum activates immediate increases in uptake which are not transient, 2) the total activation may be biphasic in nature with the second phase dependent on protein synthesis, and 3) the similarity of the changes in P_i and glucose uptake is consistent with the idea that these processes may be brought about by a similar mechanism.

Other Mitogenic Agents That Stimulate Uptake

In addition to serum there are other mitogenic agents that stimulate P_i and glucose uptake in several types of cells. This may be fundamentally different from stimulation by serum alone. Trypsin (16, 49, 50) and neuraminidase (51) both stimulate 2-deoxyglucose uptake in CE cells. These enzymes are similar to serum in their effect on the time course

of 2-deoxyglucose uptake stimulation. However, levels of uptake stimulated by optimal enzyme concentrations are less than half that of uptake levels stimulated by optimal serum concentrations (50). In addition, mixing experiments suggest that neuraminidase and trypsin stimulate uptake by a common mechanism. However, low levels of trypsin and serum have a synergistic effect on 2-deoxyglucose uptake, suggesting different mechanisms of stimulation by these 2 agents (50).

In 3T3 cells, prostaglandin F2α and insulin both stimulate P_i uptake (52). Prostaglandin F2α has an effect similar to that of serum in terms of both temporal and protein synthesis dependent aspects of uptake stimulation. Insulin, however, appears to be quite different from serum in both of these aspects (52). There are other agents that stimulate uptake in different types of cells. For example, multiplication stimulating activity (MSA) (53) and fibroblast derived growth factor (FDGF) (21) stimulate 2-deoxyglucose uptake in CE and 3T3 cells, respectively. Insulin (51), Zn^{2+}, Mn^{2+}, Cd^{2+}, and the carcinogenic hydrocarbon DMBA (54), all stimulate 2-deoxyglucose uptake in CE cells. At this time, however, it is still unclear how, if at all, uptake stimulation by these agents differs from that by serum alone.

Are Increases in P_i or Glucose Uptake Causal Events in the Initiation of Cell Proliferation?

The primary question that we wish to consider is whether or not these increases in uptake caused by serum or other mitogenic agents are causal events in the initiation of DNA synthesis and cell division. There are several approaches to this problem, each of which answers a subtly different question.

One approach involves a careful analysis of the dose of serum on the response in both DNA synthesis and uptake (36). Data for 2-deoxyglucose uptake in 3T3 cells is shown in Fig. 3. Here, we have shown that 0.5% serum, which maximally stimulates 2-deoxyglucose uptake (circles), has no effect whatsoever on thymidine incorporation (triangles) or cell number (squares). Thus, a maximal increase in 2-deoxyglucose uptake is not sufficient to initiate DNA synthesis or cell division under these conditions.

A second approach to analyze the putative causality between increased uptake and initiation of DNA synthesis involves the use of agents other than serum, that when used under the appropriate conditions, stimulate DNA synthesis but not uptake (36). This directly shows that under the experimental conditions used, increased uptake is not necessary for initiation of DNA synthesis. An example of this approach is shown in Fig. 4 for the stimulation by cortisol of DNA synthesis in 3T3 cells. As shown, levels of cortisol causing a fivefold increase in DNA synthesis actually caused a decrease in 2-deoxyglucose uptake. It should be pointed out that this level of stimulation in DNA synthesis is the same as that caused by the addition of 5% fresh serum and represents a 25% increase in cell number (36). Using a similar technique with trypsin and insulin to stimulate CE cell division, we have shown that an increase in P_i uptake is not necessary for initiation of DNA synthesis (48).

A third approach to determine if a transport increase is necessary for initiation of cell division is very direct and applicable to any nutrient or mitogen. This involves controlling nutrient uptake by adjusting its concentration in the growth medium (26, 48, 55). Specifically, it is possible to lower the nutrient concentration such that its uptake, even after stimulation by serum or another mitogenic agent, remains below uptake in control quiescent cells. If, under these conditions, dialyzed serum initiates cell division to the same extent as in medium containing the "normal" nutrient concentration, we can con-

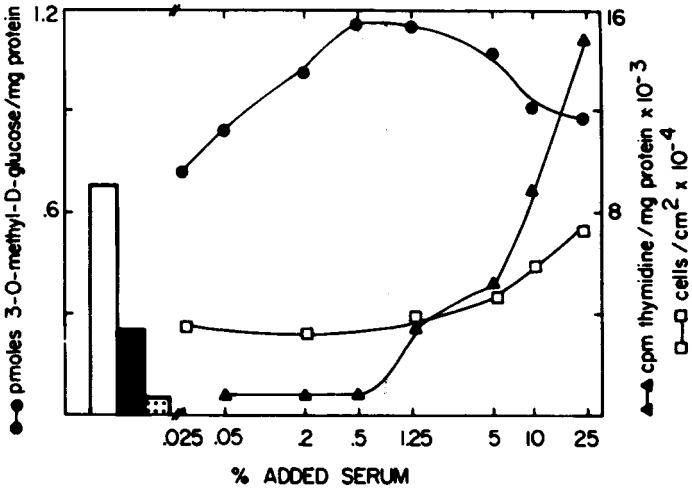

Fig. 3. Effect of concentration of added fresh serum on 3-O-methyl glucose uptake (●—●), DNA synthesis (▲—▲), and 3T3 cell number (□—□). Hexose uptake was measured during a 1-min incubation period 4 h after adding fresh serum to quiescent 3T3 cells. DNA synthesis was measured at 24 h and cell number was monitored at 72 h. Vertical bars show control values for quiescent cultures: plain bar) hexose uptake; solid bar) cell number; stippled bar) DNA synthesis. [Reprinted from Ref. 36, with permission.]

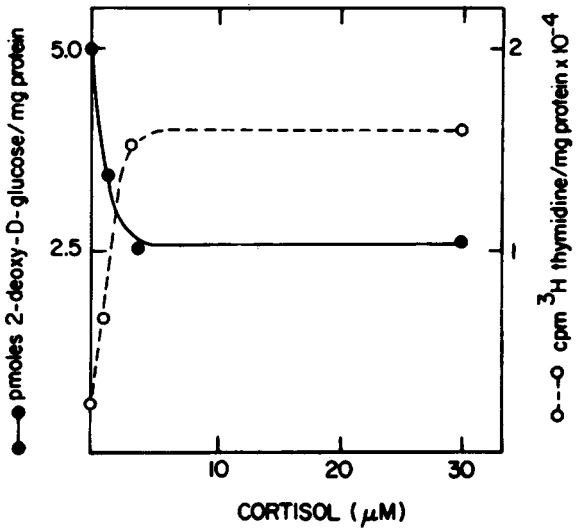

Fig. 4. Effect of cortisol concentration on 2-deoxyglucose uptake and DNA synthesis by quiescent 3T3 cells. Uptake of 2-deoxyglucose (●—●) was measured 4 h after cortisol addition; DNA synthesis (o- - -o) was measured 24 h after cortisol addition. [Reprinted from Ref. 36, with permission.]

clude that increased uptake is not necessary for initiation of cell division. The data in Fig. 5 show the use of this approach to analyze the serum stimulated increase in P_i uptake (48). Quiescent 3T3 cells were stimulated by switching either to medium containing fresh serum and the "normal" amount of P_i (circles), or to medium containing fresh serum and 5% of the "normal" amount of P_i (triangles). The upper panel of Fig. 5 shows that this low level of P_i kept uptake below the level of unstimulated control cultures. When we examined the subsequent effects on cell proliferation (middle panel), we found that this low concentration of P_i did not inhibit the initiation of cell division. Thus, the increase in P_i uptake that occurs under normal culture conditions is not necessary for initiation of cell division. Similar studies on CE and mouse embryo cells have led to the same conclusion (48). We have also used this approach to demonstrate that under normal culture conditions, an increase in glucose uptake is not necessary for initiation of division in 3T3, CE, or human foreskin (HF) cells (55).

Although increased uptake of P_i or glucose was not required for initiation of DNA synthesis, the possibility remained that intracellular nutrient availability (as measured by intracellular pools of P_i or glucose) might still increase under our experimental conditions even though extracellular nutrient concentration and nutrient uptake were below the control or unstimulated level. Increased intracellular availability of P_i or glucose could result from alterations of certain metabolic events. It could also result from decreased rates of P_i or glucose efflux, since serum stimulates influx of P_i more than efflux (13). Thus, it was possible that even though increased uptake of P_i or glucose was not necessary for initiation of proliferation, increased nutrient availability might still be required. When we made direct chemical measurements of intracellular inorganic P_i, however (Fig. 5, lower panel), we found that P_i pool size in the cells stimulated by fresh serum in low P_i media (triangles) was not significantly different from unstimulated control cultures (squares) (48). Moreover, intracellular P_i pool size in the cells stimulated by fresh serum in normal media remained virtually constant. We have also shown that intracellular pool size of glucose did not significantly change after quiescent CE cells in medium containing the usual amount of glucose are stimulated with fresh serum (55). Taken together, these results demonstrate that under normal culture conditions, neither an increase in the uptake of P_i or glucose, nor an increase in the intracellular pools of these nutrients is required for the initiation of cell proliferation.

CHANGES IN P_i AND GLUCOSE UPTAKE CORRELATED WITH GROWTH TO QUIESCENCE

Although changes in nutrient uptake and intracellular pool size were not causal events in the initiation of DNA synthesis in nonproliferating cultures, the possibility remained that the observed decrease in P_i or glucose uptake as proliferating cells began to approach a quiescent monolayer might limit cell division. As mentioned earlier, there is less information available on this aspect of growth regulation since an unsynchronized population of low density cells is difficult to study. Most reports dealing with the involvement of uptake in the control of division in proliferating cells have only dealt with differences in uptake between exponentially growing and stationary phase cultures. Generally, most investigators have found that exponentially growing cells take up P_i and/or glucose at rates three- to fourfold higher than their stationary phase counterparts (13, 16, 35, 56–58). In addition, the change in uptake, as with the serum stimulation of

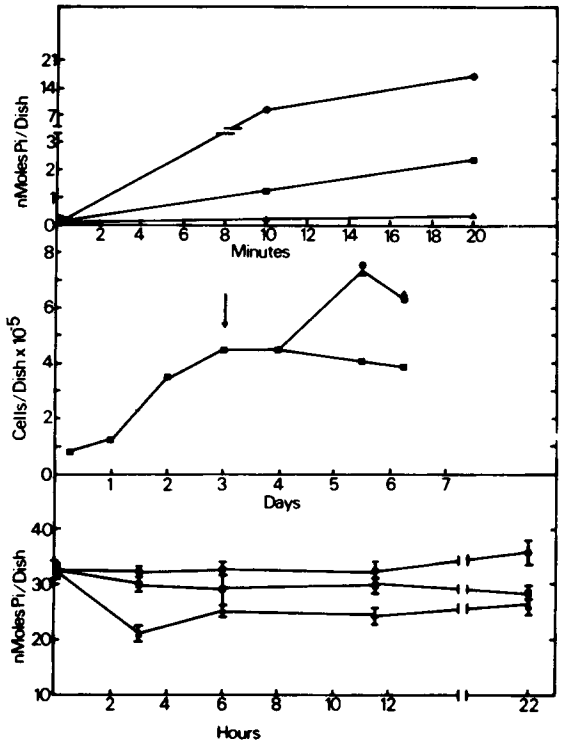

Fig. 5. Effect of reduction of P_i concentration on P_i uptake, initiation of cell division, and pool size of P_i in 3T3 cells. 3T3 cells were seeded at a density of 1.0×10^4 cells/cm^2 in medium containing 10% calf serum. After 3 days, at the time indicated by the arrow (middle panel), the medium was changed to one containing 25% dialyzed calf serum and 1.1 mM P_i (•), or 25% dialyzed calf serum and 0.05 mM P_i (▲). The medium was not changed on control cultures (■). After the medium change, P_i uptake (upper panel), cell number (middle panel), and intracellular pool sizes of P_i (lower panel) were measured at the indicated times. Error bars in pool size measurements designate one standard deviation from the mean. The absence of an error bar means that it was smaller than the symbol. [Reprinted from Ref. 48, with permission.]

quiescent cells, usually involves a change in the observed V_{max} of the transport system (26, 35). Representative data for P_i uptake by 3T3 cells is shown in Fig. 6 (26). A Lineweaver-Burk plot of this data (lower panel) shows a twofold change in the observed V_{max} with no apparent change in the observed K_m. What is particularly interesting, however, is the observation that diffusion of P_i, as indicated in the upper panel by the plain solid line (quiescent cells) and plain dashed line (proliferating cells), two- to threefold greater in the proliferating cells than in the quiescent cells. This probably reflects a greater exposed surface area per μg of protein in the proliferating cells.

In any case, what is of primary concern is whether or not this decrease in diffusion as well as carrier-mediated uptake as proliferating cells begin to approach quiescence is causally involved in the inhibition of DNA synthesis. A first requirement for a primary causal signal is a temporal one. That is, if we ask what is the earliest time that proliferating cells are committed to entering stationary phase, any putative signal controlling DNA synthesis must precede that time. Under normal culture conditions, a decline in thymidine

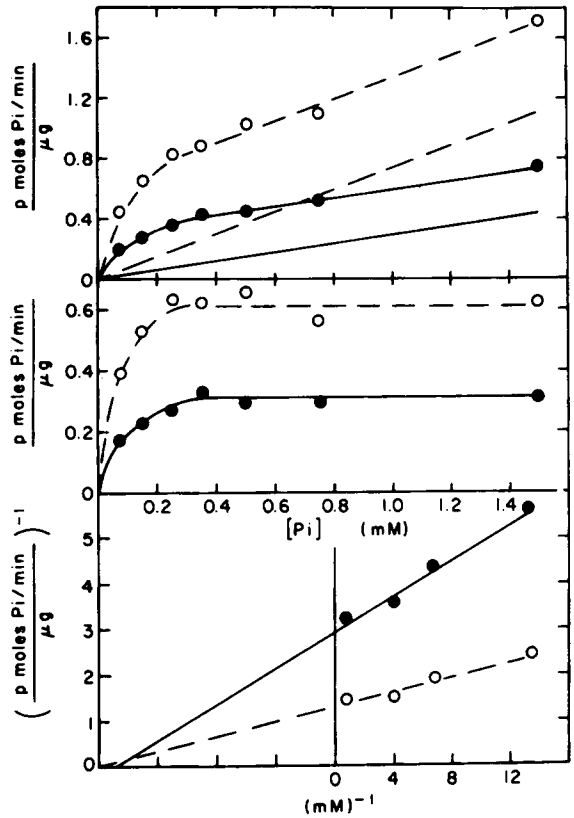

Fig. 6. Kinetics of P_i uptake in quiescent and growing 3T3 cells. 3T3 cells were plated in medium containing 10% serum at a density of either 1.1×10^3 or 1.1×10^4 per cm^2. Three days later, the cultures set up at the higher density were quiescent (•—•). To remove residual P_i, the cultures were preincubated for 2 h at 37°C in dialyzed depleted medium. After the preincubation, the cells were incubated for 15 min with $^{32}P_i$ in dialyzed depleted medium containing the indicated concentrations of P_i. Upper panel: total uptake with diffusion estimated by drawing a line through the original that was parallel to total uptake at regions where uptake appeared to be linear (0.4–1.5 mM). Middle panel: carrier-mediated uptake calculated by subtracting diffusion from total uptake. Lower panel: Lineweaver-Burk plot of carrier-mediated uptake data. [Reprinted from Ref. 26, with permission.]

incorporation in proliferating cells occurs some time before these cells actually show a decrease in their rate of division. This probably indicates the first sign of decreased DNA synthesis and must, therefore, follow any putative regulatory signal. Experiments that compared the decrease in nutrient uptake with the decline in thymidine incorporation have suggested that a decrease in P_i (26) and glucose (16, 55, 59) uptake usually slightly precedes the decline in thymidine incorporation. This is consistent with the possibility that a decreased rate of uptake signals a decline in DNA synthesis.

To directly determine if this decrease in uptake is a causal signal in the entrance of cells into stationary phase, we used a similar technique to the one used in Fig. 5. After plating the cells at a low density, we changed them to media containing a lowered nutrient concentration and subsequently monitored cell number, DNA synthesis, and nutrient uptake (55). Representative data for glucose uptake in 3T3, HF, and CE cells are shown

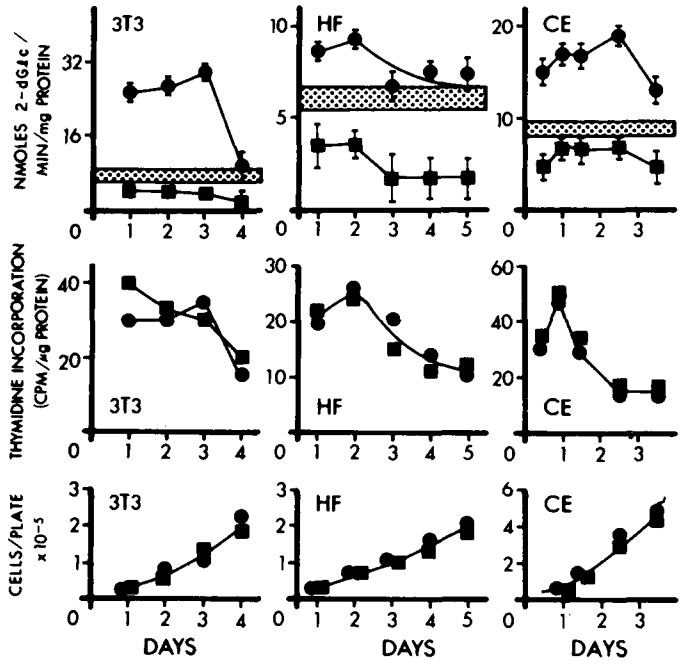

Fig. 7. Effect of reduced concentration of glucose on glucose uptake, DNA synthesis, and cell number. 3T3, HF, and secondary CE cells were plated at densities of 4.0×10^3, 5.0×10^3, and 1.0×10^4 cells, respectively per cm^2, The medium contained 10% calf serum (3T3 cells), 5.0% calf serum (HF cells), or 2.0% calf serum (CE cells). Shortly after the cells attached, the medium was changed to fresh medium containing the same concentrations of dialyzed fresh calf serum and either 22 mM glucose (•) or 2.8 mM glucose (▪). Time zero indicates the time at which the cells were plated. Glucose uptake, thymidine incorporation, cell number, and cell protein were measured at the indicated times. Each glucose uptake data point represents an initial velocity of uptake determined from a time course of uptake using 4 cultures and incubation times between 10 sec and 4 min. The shaded areas in the panels for glucose uptake designate the uptake rates characteristic of quiescent cells plus and minus one standard deviation from the mean. Error bars for the measurements of glucose uptake show one standard deviation from the mean. The absence of an error bar means that it was smaller than the symbol. [Reprinted from Ref. 55, with permission.]

in Fig. 7. After changing to medium containing 10% of the normal amount of glucose (squares), we found no differences from control cultures (circles) in either thymidine incorporation (middle panel) or cell number (lower panel). Glucose uptake (upper panel) in the low glucose cultures, however, was kept below the level characteristic of stationary phase cultures (as indicated by the shaded bars). We found similar results for P_i uptake in these cells (26). Since levels of uptake below those characteristic of quiescent cells had no effect on DNA synthesis or cell division over a several day period, we concluded that the decrease in P_i or glucose uptake that very slightly precedes the decline in DNA synthesis was not regulating cell division during growth to quiescence (26, 55). As with the case of serum stimulation of quiescent cells, however, the possibility remained that intracellular pool size might be regulating proliferation under our experimental conditions. When we measured intracellular pool sizes of both P_i (26) and glucose (55) during growth to

quiescence, however, we found either no changes or actual increases (in the case of P_i in 3T3 cells) during growth to quiescence. This directly demonstrated that changes in intracellular pool size were 1) not exclusively controlled by changes in uptake, and 2) not controlling DNA synthesis under normal culture conditions.

It should be pointed out that one group of investigators has reported changes in intracellular P_i pool sizes in 3T3 cells during growth to quiescence and after serum stimulation that are at variance with our results (60). However, these investigators measured intracellular P_i pools using an isotope equilibration technique with a 2-h incubation. If a radioisotope equilibration method is used, it is exceedingly important to make sure that a long enough incubation time is employed to allow the isotope to become completely equilibrated. In the case of P_i, uptake appears to be linear for at least 3 h (48, 56), indicating that a 2-h incubation probably reflects measurements of uptake rather than intracellular pool sizes. The results presented here are from experiments which utilized a direct chemical method to measure pool sizes (26, 48). We have also used a radioisotope equilibration method with an incubation period of 2 days and obtained essentially identical results (27).

In conclusion, it appears that under normal culture conditions neither uptake nor intracellular pool size of P_i or glucose serves as a causal signal in the regulation of proliferation.

CHANGES IN UPTAKE OF OTHER MOLECULES

A number of investigators have described changes in the uptake of other molecules that appear to be related to the control of cell proliferation. At the outset, we can conclude that for some molecules changes in uptake are not critical for the regulation of cell division since these molecules are not required for growth and are not present in serum or the usual cell culture media. On the other hand, some of the changes in uptake involve molecules that are required for cell growth. There is not enough information at the present time to decisively determine whether these latter changes participate as causal events in the regulation of DNA synthesis or cell division.

Molecules Not Required for Cell Proliferation and Not Present in Serum or Most Cell Culture Media

Although changes in uptake for this class of molecules are not causally involved in the control of cell proliferation, it is noteworthy that some of the changes are closely correlated with certain aspects of growth control. As noted in the background section, uptake of uridine decreases four- to fivefold during growth of 3T3 or CE cells to quiescence (13, 17). This same difference in uridine uptake has been observed with membrane vesicles prepared from growing and nongrowing 3T3 cells, indicative of a transport change (37). Addition of fresh serum to quiescent 3T3 or CE cells brings about a severalfold increase in uridine uptake that can be detected within several minutes (13, 42, 61). Although this increase slightly follows the increase for P_i and glucose uptake (42), it is one of the earliest events that has been detected following initiation of division in arrested cells. As mentioned earlier, serum addition to quiescent 3T3 cells has no effect on adenosine uptake (13). In view of these findings about uridine and adenosine uptake, it is curious that in 3T3 and BHK cells (62–64) added adenosine potentiates serum stimulated DNA synthesis while uridine does not (62).

Uptake of putrescine by cultured human fibroblasts is also closely related to the proliferative state of these cells. Uptake decreases as the cells grow to quiescence and markedly increases within 30 min after initiating proliferation with fresh serum (65). Although these cells do not require added putrescine in the culture medium for growth, it is significant that they produce putrescine, and that putrescine can function as a growth factor under certain conditions (66).

Uptake of choline by cultured CE cells also increases soon after initiating proliferation by some but not all methods (61). Serum, elevated pH, excess Zn^{2+}, DMBA (a carcinogenic hydrocarbon), and insulin accelerate the progress of growth-inhibited CE cells into the S phase of the cell cycle. All of these treatments except Zn^{2+} addition increase choline uptake (61).

In addition to these early increases in uptake after initiation of proliferation, increased uptake of pyrimidine deoxynucleosides occurs at a time which coincides closely with initiation of DNA synthesis (67, 68). This increase is selective for uptake of thymidine and deoxycytidine; uptake of other DNA precursors such as deoxyadenosine, deoxyguanosine, and P_i does not increase at this time (68). The increase in thymidine uptake is not a result of higher rates of DNA synthesis since inhibition of DNA synthesis does not prevent the increase in uptake. In addition, it is not a result of increased phosphorylation of thymidine, since it takes place in cells which lack thymidine kinase. Thus, it is probably a direct change in membrane transport of thymidine (68). These increases in thymidine and deoxycytidine uptake at the time of DNA synthesis suggest that increased pool sizes of these compounds or their derivatives might influence the initiation of DNA synthesis. However, we could detect no significant change in either the timing or extent of DNA synthesis after adding varying levels of deoxynucleosides to quiescent cells or to quiescent cells initiated with low levels of fresh serum (69).

Nutrients Required for Growth

It is particularly important to examine the changes in uptake that occur for this class of compounds since changes that correlate with growth rate might be involved in the regulation of cell proliferation. In this section, we will examine changes in amino acid and potassium ion uptake that correlate with some aspects of cell division.

Two amino acid analogs are commonly used to measure amino acid uptake. α-Aminoisobutyric acid (AIB) is taken up by the Na^+-dependent A system, and cycloleucine is transported by the Na^+-independent L system. Transport of amino acids by these 2 systems has been extensively studied, although many important questions about the fundamental aspects of amino acid transport remain to be answered (70).

Early studies on growing and nongrowing 3T3 cells revealed that AIB and cycloleucine uptake decreased about 30–50% after growth to a quiescent state, while uptake of arginine, glutamic acid, and glutamine did not change (14). Similar density-dependent changes in AIB uptake have recently been described for membrane vesicles prepared from cells at different densities (39, 71). Studies on synchronized cell populations have revealed that uptake of AIB and cycloleucine are generally low during early G_1 and then increase about two- to threefold late in G_1 or during S, although the exact patterns are somewhat different for the 2 amino acid analogs (72, 73). Addition of fresh serum to quiescent fibroblasts brings about a rapid decrease in the uptake of leucine and lysine (74) and an increase at about 3 h in the uptake of cycloleucine (75). It is noteworthy that stimulation of human lymphocytes with phytohemagglutinin also brings about an increase in AIB uptake (76). Furthermore, injection of rats with growth hormone brings about a twofold

increase in the maximum velocities of the AIB and cycloleucine transport systems in the perfused liver under conditions where growth (protein synthesis) is stimulated (77). Taken together, these studies show that rates of uptake of some amino acids are related to the growth state of the cell and that there are cell cycle-dependent changes in the uptake of certain amino acids.

Similarly, there are now several lines of evidence showing that changes in potassium ion uptake are associated with alterations in cell growth. Early experiments showed that relatively high intracellular K^+ levels are necessary for protein synthesis and growth in cultured mammalian cells (78) and that intracellular K^+ levels vary during the cell cycle of cultured mouse lymphoblasts (79). Studies on human lymphocytes have revealed that the stimulation of DNA synthesis by phytohemagglutinin is quite sensitive to ouabain, an inhibitor of membrane Na^+, K^+-ATPase. Importantly, excess extracellular K^+ reverses the inhibitory effect of ouabain on DNA synthesis (80, 81). It has also been shown that treatment of human lymphocytes with phytohemagglutinin leads to an early stimulation of K^+ uptake (82). These studies have led to the conclusion that increased uptake of K^+ seems to be a necessary condition for the biosynthetic and morphological events of lymphocyte stimulation (82). More recent studies have shown that Na^+,K^+-ATPase fluctuates during the cell cycle of cultured hamster fibroblasts (83) and that there are cell cycle-dependent changes in K^+ transport in cultured Ehrlich ascites tumor cells (84). Also, active K^+ uptake decreases in 3T3 and SV40-transformed 3T3 cells when the growth rate of both cell types decreases (85). Stimulation of DNA synthesis in 3T3 cells brings about rapid increases in K^+ uptake (86) and Na^+,K^+-ATPase (52). All of these findings point to the importance of further investigations to determine if the changes in K^+ uptake and intracellular levels of K^+ are causal events in the regulation of DNA synthesis and cell division in these cases.

ACKNOWLEDGMENTS

This work was supported by Grant CA-12306 from the National Cancer Institute. D.D.C. is a recipient of Research Career Development Award CA-00171 from the National Cancer Institute.

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