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# Paradoxical effects of cycloheximide and cytochalasin B on hamster cell hexose uptake

(glucose starvation/transport regulation/turnover)

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**ABSTRACT** **Cellular regulation of hexose uptake was studied in cultures of NIL hamster cells. Enhancements of galactose uptake were elicited most strikingly by maintaining confluent NIL cultures in culture media devoid of glucose. These glucose-starved cultures showed up to 8- or 9-fold enhancements in the galactose uptake test. When these cultures were treated for extended periods with cycloheximide, the enhanced uptake was left unimpaired, whereas the uptake by glucose-fed cells, similarly treated with cycloheximide, was inhibited greater than 90%. Addition of glucose to these starved cultures resulted in a gradual decline of uptake rates to the unenhanced level ($t_{1/2}$ approximately 3 hr). In surprising contrast, when both glucose and cycloheximide were added simultaneously, the decline was arrested for at least 12 hr. If cytochalasin B (the specific inhibitor of hexose transport) was present, the uptake of galactose by both starved and fed cells was close to completely inhibited. By several criteria, cells maintained for 24 hr in medium containing both glucose and cytochalasin B were glucose-fed. Yet, when the cytochalasin B was removed, the cells were found to have enhanced rates of galactose uptake. The regulation of the hexose uptake system may therefore not be guided by the levels of glucose catabolites. Alternative mechanisms that may control hexose uptake are considered.**

Animal cells, deprived of glucose for prolonged culture periods, were first shown to manifest striking enhancements of hexose uptake by Martineau *et al.* (1) and later by others (2–5). It was also discovered that the presence of inhibitors of protein synthesis prevented the large increases in uptake when the cells were being starved (1, 2, 4, 5). Because of these findings, it has been suggested that starvation-induced increases in hexose uptake rates may result from "derepression" (1, 2). By using the nonmetabolizable analog of glucose, 3-*O*-methylglucose, this derepression could be largely attributed to the initial step in hexose utilization, transport (2, 4). Since feeding cells 2-deoxyglucose resulted in repression and 3-*O*-methylglucose feeding did not (2), it was reasoned that the 6-ester of 2-deoxyglucose was directly interfering with derepression (1, 2). This, in turn, implied that the physiological "repressor or corepressor" may be glucose-6-phosphate. In this article we present evidence that catabolic repression does not fully explain regulation of hexose uptake in hamster cell cultures. Effects of cytochalasin B and cycloheximide on the regulation of hexose uptake have led us to suggest that turnover of hexose carriers is highly responsive to the presence or absence of glucose (or its metabolites), and this could explain increased rates of uptake after glucose starvation.

In hamster cells the regulatory changes brought about by the starvation-mediated derepression are more pronounced for galactose uptake than for 2-deoxyglucose uptake (3). For this and other reasons, galactose became the routine substrate in the uptake tests.

Abbreviations: CB, cytochalasin B; $Me_2SO$, dimethylsulfoxide.

## MATERIALS AND METHODS

**Reagents.** D-[*U*-$^{14}$C]Galactose (264–298 mCi/mmol), 2-deoxy-D-[*G*-$^{3}$H]glucose (10 Ci/mmol), [5-$^{3}$H]uridine (26 Ci/mmol), L-[4,5-$^{3}$H]leucine (30–50 Ci/mmol) and [*carboxyl*-$^{14}$C]cycloleucine (1-aminocyclopentane-1-carboxylic acid) (30–33 mCi/mmol) were obtained from New England Nuclear. Cycloheximide and puromycin-HCl were purchased from the Sigma Chemical Co., and cytochalasin B (CB) was purchased from Aldrich Chemical Co., Inc. The CB was dissolved in dimethylsulfoxide ($Me_2SO$) at a concentration of 21 mM (10 mg/ml) and then diluted in Eagle's minimal essential medium (culture medium) or phosphate-buffered saline (uptake medium) to a final concentration of 21 $\mu$M. Control culture and uptake media for the CB experiments contained 0.1% $Me_2SO$ (vol/vol) without CB. Culture media and sera (fetal calf and dialyzed fetal calf) were obtained from the Grand Island Biological Co. and from Microbiological Associates. Glucose-free culture medium contained all components of Eagle's medium except glucose.

**Cell Cultures and Assay Conditions.** Growth of cells, uptake conditions, and other procedures have been described in detail (3, 5). Briefly mycoplasma-free cells (hamster fibroblast,, NIL strain) were cultured in Dulbecco's modified Eagle's minimal essential medium containing 4.5 mg of glucose per ml and fetal calf serum for 2 or 3 days before changes of medium were made. The confluent cells were then washed and refed with Eagle's medium or glucose-free Eagle's medium for 18–24 hr and then washed with warm phosphate-buffered saline. After the uptake period, the cells were washed with ice-cold phosphate-buffered saline and extracted with ethanol. Aliquots of the ethanol extract were monitored for radioactivity and subjected to paper chromatography for analysis of soluble metabolites. Culture time and uptake assay time are operationally different and are defined as follows: (*i*) *culture time* (usually in hours) represents the time cells were maintained in Eagle's medium (with or without sugar) in a humidified $CO_2$ incubator and in the absence of radioactive tracers; and (*ii*) *uptake assay time* (min) is the time when washed cells were incubated in phosphate-buffered saline in an air atmosphere and allowed to take up radioactive substrates. Results of uptake assays are expressed as nmol of sugar taken up/mg of cell protein per 5 (or 10) min.

## RESULTS

### Characteristics of galactose uptake and metabolism

NIL hamster cells, maintained for 18–24 hr in Eagle's medium containing 2 mg of glucose per ml and 10% dialyzed fetal calf serum (glucose-fed cells), will take up labeled galactose linearly

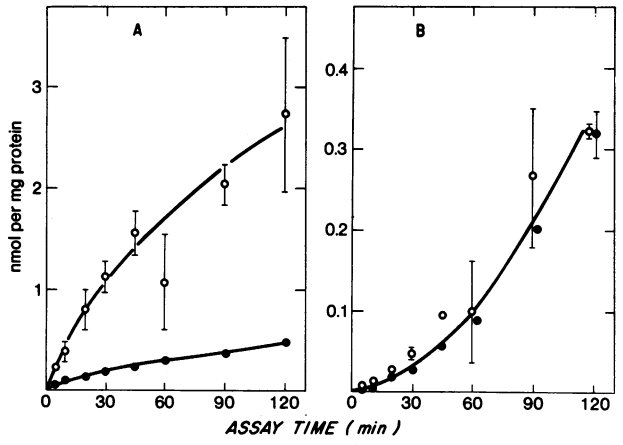


FIG. 1. Effect of 24-hr glucose deprivation on galactose uptake and incorporation. Cells were grown to confluency and changed to glucose-free medium (○) or medium containing glucose (●) for 24 hr and then allowed to take up D-galactose for the times indicated as described in *Materials and Methods*. (A) Ethanol-soluble fraction (uptake); (B) ethanol-insoluble fraction (incorporation). Points are the mean of triplicate cultures with standard error represented by the vertical bars. Where bars are not shown, error was less than the size of the symbol.

for at least 2 hr (Fig. 1A). Cells maintained for an equally long culture period in sugar-free Eagle's medium (glucose-starved cells) have higher rates of uptake and the accumulation in the ethanol-soluble pool is linear for only about 1 hr. In Fig. 1B it can also be seen that the galactose is incorporated into the ethanol-insoluble fraction equally as well by the glucose-fed cells as by the glucose-starved cells. Previous chromatographic analyses of the respective ethanol-soluble pools have shown that the glucose-fed cells accumulate the label as UDPhexoses and the glucose-starved cells accumulate greater than 90% of the label as galactose-1-phosphate (3, 5). The delay in the incorporation of label into the insoluble fraction (Fig. 1B) illustrates that the galactose label found in cells after routine uptake times of 5 or 10 min fairly well represents only the ethanol-soluble fraction.

Table 1. Effect of cytochalasin B on the uptake of sugars and amino acids by glucose-fed cells

| Uptake assay for: | Assay condition* | Uptake* | Relative uptake |
|---|---|---|---|
| D-Galactose | −CB | 0.0823 ± 0.0080 | 1.00 |
| | +CB | 0.0081 ± 0.0017 | 0.1 |
| 2-Deoxy-D-glucose | −CB | 0.3450 ± 0.0232 | 1.00 |
| | +CB | 0.0087 ± 0.0017 | 0.03 |
| L-Leucine | −CB | 15.62 ± 2.24 | 1.00 |
| | +CB | 23.18 ± 1.31 | 1.48 |
| Cycloleucine† | −CB | 0.7926 ± 0.1395 | 1.00 |
| | +CB | 0.7793 ± 0.1396 | 0.98 |

* Three-day-old cell cultures were refed with medium containing glucose 24 hr prior to the uptake assay and then washed twice with warm phosphate-buffered saline (general culture and assay conditions are described in *Materials and Methods*). In these experiments the assays were for 5 min at 37° in phosphate-buffered saline containing 0.1% $Me_2SO$ and 10 μg of cytochalasin B per ml (+CB) or in phosphate-buffered saline containing 0.1% $Me_2SO$ (−CB). Values for L-leucine are pmol/mg of protein per 5 min; all other values are nmol/mg of protein per 5 min.

† 1-Aminocyclopentane-1-carboxylic acid, a nonmetabolizable analog of L-leucine.

## Effects of cytochalasin B

Cytochalasin B (CB) inhibited the uptake of both D-galactose and 2-deoxy-D-glucose greater than 90%, but it was ineffective in altering the transport of either L-leucine or the nonmetabolizable analog, cycloleucine (Table 1). When refed Eagle's medium containing 2 mg of glucose per ml, NIL cells usually utilize about 50% of the available sugar in 24 hr (D. Ullrey, C. W. Christopher, and H. M. Kalckar, unpublished data). Cells maintained for 24 hr in complete Eagle's medium containing 10 μg of CB per ml consumed 1.2 mg of glucose per mg of cell protein, whereas control cells consumed 3.1 mg of glucose per mg of protein. Thus, in spite of a continuous severe inhibition of uptake, CB-treated cells were able to consume nearly 40% as much glucose as the control cells. Chromatographic sepa-

Table 2. Effect of cytochalasin B on galactose and leucine uptake by glucose-fed and glucose-starved cells

| Culture condition (24 hr) | Assay condition | Uptake assay (nmol/mg protein per 10 min) D-[$^{14}$C]Galactose | Relative uptake | L-[$^{3}$H]Leucine | Relative uptake |
|---|---|---|---|---|---|
| Eagle's medium (2 mg of glucose/ml) | | | | | |
| No additions | +$Me_2SO$ | 0.342 ± 0.012 | 1.00 | 0.039 ± 0.002 | 1.00 |
| | +CB | 0.012 ± 0.0001 | 0.04 | 0.035 ± 0.0006 | 0.91 |
| $Me_2SO$ | +$Me_2SO$ | 0.302 ± 0.007 | 0.88 | 0.036 ± 0.007 | 0.93 |
| | +CB | 0.014 ± 0.003 | 0.04 | 0.036 ± 0.006 | 0.93 |
| +CB | +$Me_2SO$ | 0.506 ± 0.009 | 1.48 | 0.044 ± 0.008 | 1.13 |
| | +CB | 0.007 ± 0.009 | 0.02 | 0.027 ± 0.007 | 0.70 |
| Eagle's medium (no glucose) | | | | | |
| No additions | +$Me_2SO$ | 0.754 ± 0.09 | 2.2 | 0.148 ± 0.019 | 3.8 |
| | +CB | 0.04 ± 0.012 | 0.12 | 0.141 ± 0.008 | 3.64 |
| +$Me_2SO$ | +$Me_2SO$ | 0.840 ± 0.034 | 2.49 | 0.173 ± 0.012 | 4.48 |
| | +CB | 0.04 ± 0.007 | 0.12 | 0.155 ± 0.009 | 3.99 |
| +CB | +$Me_2SO$ | 0.824 ± 0.092 | 2.41 | 0.171 ± 0.018 | 4.41 |
| | +CB | 0.042 ± 0.01 | 0.12 | 0.157 ± 0.007 | 4.06 |

Culture and assay conditions are described in *Materials and Methods* with modification regarding $Me_2SO$ and CB as indicated in the legend for Table 1. The assay was for 10 min at 37°. Samples were doubly labeled with D-[$^{14}$C]galactose, 0.5 μCi/ml ($1.55 \times 10^{-5}$ M), and L-[$^{3}$H]leucine, 2 μCi/ml ($4 \times 10^{-7}$ M). Corrections for channel overlap were made, and results are expressed as nmol/mg of protein per 10 min. Eagle's medium is minimal essential medium fortified with 10% dialyzed fetal calf serum.

ration of the ethanol-soluble pools extracted after a 10-min uptake of [$^{14}$C]galactose revealed that the level of glucose consumption over the 24-hr period (even in the presence of CB) was sufficient to maintain the cells in a glucose-fed condition (data not shown, see refs. 3 and 5). Moreover, Dolberg *et al.* (6) have shown that, although CB virtually eliminates facilitated uptake of sugars, simple diffusion is not affected by CB, and thus glucose catabolism cannot be prevented as a result of treatment of cells with this reagent. Only those cells intentionally deprived of glucose for 24 hr (with or without CB) had typical glucose-starved chromatographic patterns (data not shown, see refs. 3 and 5). As was shown in studies of hexose uptake by chick cells (7), the rate of uptake by glucose-fed cells released from 24-hr treatment with CB was higher than control, glucose-fed cultures (Table 2). This enhanced level in glucose-fed cells approached the level of the glucose-starved cells (Table 2). Table 2 also shows that increases in uptake by fed cells treated with CB is not additive to the starvation effect. Although in this experiment the CB effect is relatively small (1.5-fold), in other experiments the relative increases in uptake by fed cells released from CB treatment were as much as 5-fold, which was close to the increases caused by glucose starvation alone. Again, as in Table 1, amino acid transport is not significantly inhibited by CB, and long exposure of the cells to CB does not affect the level of leucine transport by either glucose-fed or glucose-starved cells (Table 2). In Table 2 it can also be seen that the glucose-starved cells show greatly enhanced active transport, much like the previous demonstration of cycloleucine transport enhancement (3).

## Effects of cycloheximide

The lack of coupling between the control of hexose uptake and hexose catabolism gains further support from a series of experiments in which cycloheximide was used to modify uptake rates and galactose metabolism. Although puromycin treatment of cells caused similar results, cycloheximide was an effective inhibitor of protein synthesis (see below and Fig. 3), and was found to be less likely to cause the cells to detach from the surface of culture vessels. Cycloheximide has no effect on the uptake of sugars when it is present in the assay (data not shown, see refs. 4 and 5). However, over prolonged culture times cycloheximide does partially inhibit the development of enhanced galactose uptake rates by glucose-starved cells (Fig. 2). Even in the presence of concentrations of cycloheximide capable of sustaining a greater than 90% inhibition of protein synthesis (data not shown), the glucose-starved cells were repeatedly found to sustain a 1.5- to 2-fold increase in galactose uptake rates. These cells retain the ability to metabolize trace amounts of galactose as if they had been fed glucose instead of being starved (5).

Interesting comparisons of the effect that cycloheximide has on uptake rates of sugar-starved cells were provided through studies of galactose uptake by glucose-fed cells. Cells that have been refed medium containing glucose and 10 μg of cycloheximide per ml steadily lose the ability to take up galactose (Fig. 2). As can be seen in Fig. 3, this loss can be appreciable, since the uptake capacity of these fed cells after 24 hr of cycloheximide treatment often amounted to only 5% of the initial capacity. Fig. 3 also demonstrates the paradoxical but critical range of cycloheximide required for the expression of the maximal loss of uptake ability and the parallel inhibition of protein synthesis. High concentrations of cycloheximide (and puromycin, data not shown) contained in the culture medium promoted a modest uptake enhancement in sugar-fed cells while protein synthesis continued to be inhibited by greater

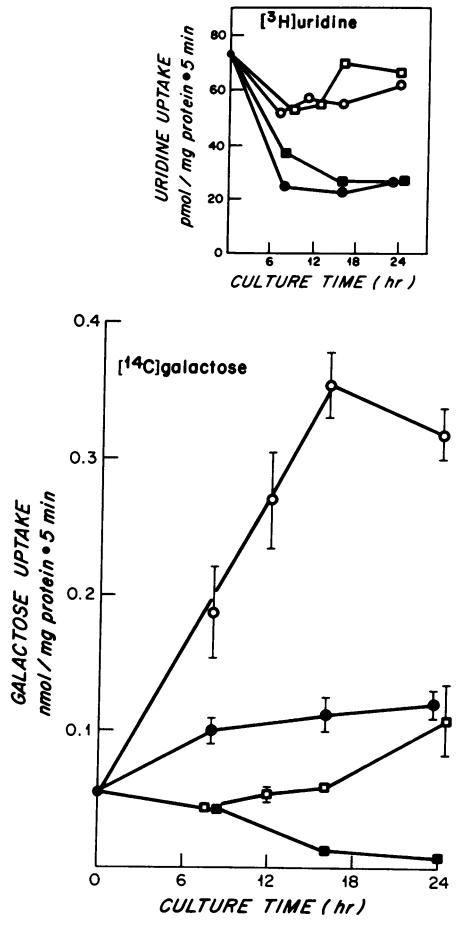


FIG. 2. Kinetics of galactose and uridine uptake changes. Cells were cultured as described in Fig. 1. The double-label assays, [$^{14}$C]-galactose and [$^3$H]uridine (inset), were for 5 min at the culture times indicated (corrections for channel overlap were made). Glucose-starved cells (○); glucose-starved cells plus 10 μg of cycloheximide per ml of culture medium (●); glucose-fed cells (□); and glucose-fed cells plus 10 μg of cycloheximide per ml of medium (■).

than 90%. The inset to Fig. 2 shows the effects of sugar starvation and cycloheximide treatment on subsequent uptake of uridine by the hamster cells. In contrast to galactose uptake in this double-label experiment, uridine uptake is unaffected by sugar starvation. Cycloheximide (10 μg/ml) caused a steady loss of uridine uptake regardless of the presence or absence of glucose.

## Reversal of high hexose uptake rates in glucose-starved cells

The characteristically high galactose (or 2-deoxyglucose) uptake rates of cells deprived of glucose for 24 hr can be lowered to the glucose-fed level of uptake (reversal) by exposure of the starved cells to medium containing glucose for 6–8 hr (Table 3, Fig. 4). The decrease was moderate for 2-deoxyglucose uptake but was usually more marked for galactose uptake (Table 3). The data shown in Fig. 4 are reversals of derepressed uptake rates in response to refeeding with 4.5 mg of glucose per ml (22 mM) and show a half-time ($t_{1/2}$) for galactose uptake reversal of between 2½ and 3 hr. Treatment of the starved cells with a combination of glucose and cycloheximide (or puromycin) did not permit any decrease in uptake rates (Table 3, Fig. 4), yet the consumption of glucose in the cells so treated was considerable (40–60% of that of cells treated with glucose alone), and

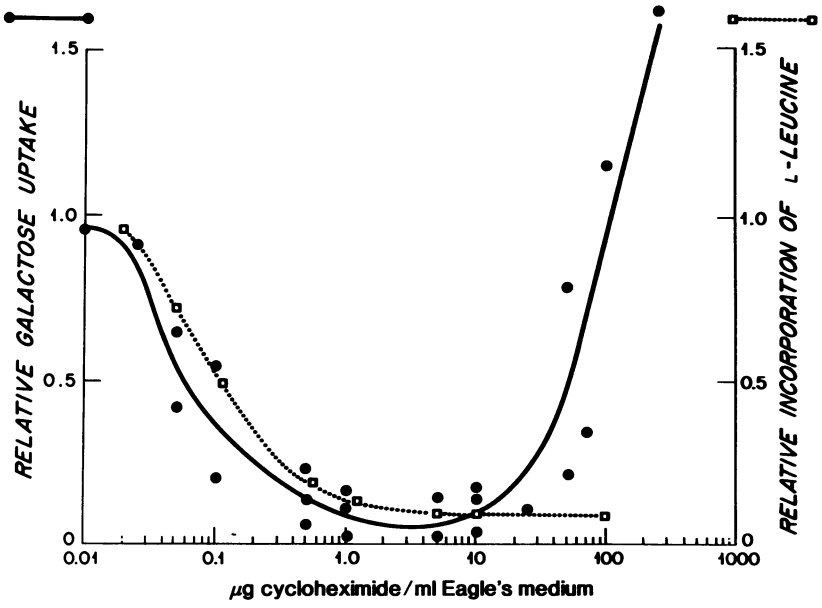


FIG. 3. Effect of varying concentrations of cycloheximide. Cells were cultured as described in the legend of Fig. 1, washed, and refed with medium containing glucose and the various amounts of cycloheximide indicated. After 18 or 24 hr the cells were washed and allowed to take up galactose for 5 or 10 min. Results are nmol of galactose taken up per mg of protein per assay time (●) and are expressed relative to uptake by cells cultured without cycloheximide. Protein synthesis (the incorporation of [$^3$H]leucine into ethanol-insoluble material, □) was for 2 hr at both the beginning and the end of the culture time while the cells were in media containing cycloheximide. This figure is compiled from several experiments; individual points are averages of duplicate or triplicate samples.

lactate production into the media showed that the glycolytic pathway was functional. Evidently glucose metabolic products (e.g., glucose-6-phosphate) did not interfere with the derepressed state of the uptake system. Consonant with this view, 2-deoxyglucose, replacing glucose in the refeeding medium for 6–8 hr, also did not interfere with the high uptake rates (Table 3, Exp. 3). These results contradict earlier speculations that the 6-ester, the main metabolic product of 2-deoxyglucose, controls hexose uptake rates. Other controls not affecting the high uptake levels are described in Table 3 and Fig. 4.

Table 3. Reversal of hexose uptake

| | Sugar uptake (nmol/mg per 10 min) | | | | |
|---|---|---|---|---|---|
| | D-Galactose | | | 2-Deoxy-D-glucose | |
| Culture conditions | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 1 | Exp. 2 |
| No additions (zero time) | 0.657 ± 0.044 (1) | 0.733 ± 0.019 (1) | 0.702 ± 0.003 (1) | 1.99 ± 0.058 (1) | 1.56 ± 0.141 (1) |
| + Glucose | 0.145 ± 0.018 (0.22) | 0.143 ± 0.01 (0.2) | 0.342 ± 0.019 (0.49) | 1.037 ± 0.12 (0.52) | 0.687 ± 0.022 (0.44) |
| + 2-Deoxyglucose | — | — | 0.713 ± 0.037 (1.02) | — | — |
| + Cycloheximide | 0.701 ± 0.129 (1.07) | — | 0.705 ± 0.105 (1.01) | — | 1.76 ± 0.011 (1.13) |
| + Puromycin | — | 0.639 ± 0.088 (0.87) | — | — | — |
| + Glucose and cycloheximide | 0.566 ± 0.136 (0.86) | — | 0.642 ± 0.025 (0.91) | 1.681 ± 0.165 (0.84) | 1.055 ± 0.012 (0.67) |
| + 2-Deoxyglucose and cycloheximide | — | — | 0.714 ± 0.038 (1.02) | — | — |
| + Glucose and puromycin | — | 0.61 ± 0.098 (0.83) | — | — | — |

Cells were deprived of sugar for 16–18 hr, washed, and assayed for the uptake of 15 μM D-galactose or 2-deoxy-D-glucose at 37° for 10 min (zero time). Glucose (or 2-deoxyglucose) (1 mg/ml) and cycloheximide (10 μg/ml) or puromycin (100 μg/ml) were added to various cultures as indicated and the cultures were placed in a $CO_2$ incubator. After 6–8 hr, the cultures were washed and assayed for galactose or 2-deoxyglucose uptake. Results are the average nmol of sugar taken up per mg of cell protein per 10 min ± standard error of the mean for duplicate or quadruplicate samples. Values in parentheses are the uptake activities at the end of 6–8 hr under the various conditions relative to the zero time control (no additions).

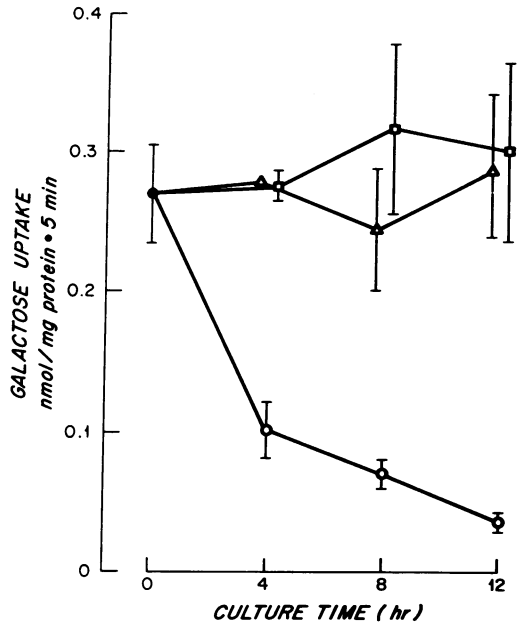


FIG. 4. Reversal of uptake. Cells were deprived of glucose for 12 hr (●) and then refed with glucose-free media containing 4 mg of glucose per ml of culture medium (○), 4 mg of glucose and 10 μg of cycloheximide per ml (△), or 10 μg of cycloheximide per ml (□). After refeeding, the cells were washed and allowed to take up galactose for 5 min at the culture times indicated. Cells maintained in medium containing glucose for the total culture period (glucose-fed cells, not shown) had an average uptake value of 0.055 ± 0.003 nmol/mg per 5 min. With some variability, the fully reversed uptake rate was the same as the rate from cells that had been fed glucose from the start, and neither reversed rates nor glucose-fed cell rates ever reaches zero uptake. Half the difference in rate between the glucose-starved level and the glucose-fed level was attained by the reversed cells in about 2½ hr ($t_{1/2}$). In this experiment, points are averages of duplicate cultures.

## DISCUSSION

Ever since it was found that inhibition of protein and RNA synthesis could block the development of glucose-starvation-mediated enhancements of hexose uptake rates (1, 2), it has been speculated that hexose transport is controlled by repression. However, in hamster cells a "repression" mechanism does not appear to be enough. Our data also support a model of control where increases in uptake rates, caused by synthesis of uptake system components, are poised against decreases in rates, caused by inactivation or physical loss (i.e., "turnover") of the same uptake components. When cells are maintained in a glucose-rich environment, a balance between synthesis and turnover is achieved. Synthesis of the uptake components can be prevented by inhibitors of protein synthesis. The turnover can be prevented in at least two ways: by the presence of cytochalasin B for prolonged culture periods and by extended glucose starvation. The factors responsible for turnover (whatever the mechanism) seem to be stable under conditions when the cells are fed, and labile under conditions when the cells are deprived of glucose (Fig. 2). Once lost, the turnover factor(s) must be resynthesized in order again to interfere with the overall uptake system (Fig. 4).

Uptake or transport systems fall roughly into two classes, those that are affected by glucose starvation (e.g., hexose uptake and leucine transport) and those that are not (e.g., uridine uptake). Since cytochalasin B treatment does not seem to modulate leucine transport, turnover of hexose uptake systems seems to have additional distinct characteristics. This implies that either the turnover factors are specific for transport systems or the CB affects hexose uptake systems in ways different from other systems.

In addition to considerations of carrier functions, the problem of regulation of hexokinase, galactokinase, and subsequent enzymes as part of the uptake system cannot be ignored. Besides the use of analogs like 3-*O*-methylglucose, which cannot be phosphorylated, there are other approaches. The isolation of plasma membrane vesicles, free of kinase activity, for the study of transport regulations of amino acids and hexoses is already being explored (8, 9). By using culture conditions similar to the ones described in this paper, it may be possible to obtain membrane vesicles from cells with uptake rates ranging from very low (cycloheximide-treated fed cells) to very high (starved cells). Cell populations with up to 50-fold differences in uptake rates can be obtained (Fig. 2). Isolation of vesicles and further attempts to isolate transport carriers are needed to get more insights into the nature of carrier function and turnover.

**Note Added in Proof.** Results of our recent 3-*O*-methylglucose transport assays (10 s at 37°) show unambiguously that carrier activity is subject to regulation in response to the above changes in culture conditions. Moreover, assays of the galactokinase activities *in vitro* did not show any significant differences as a result of changes in culture conditions (10).

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