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Enhancement of Hexose Entry into Chick Fibroblasts by Starvation: Differential Effect on Galactose and Glucose

(carrier-mediated diffusion/puromycin/cycloheximide)

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ABSTRACT **Glucose entry, as measured by 5-min uptake into the acid-soluble fraction, is enhanced 15–30 times by long-term (12–24 hr) hexose starvation of chick fibroblasts. The rate of galactose accumulation in the cells increases only 5 times under the same conditions of starvation. Several carbon and energy sources that were tested for their effect on this "derepression" can be classified as: (*i*) those resembling glucose in blocking the "stimulation," (*ii*) those permitting full "derepresssion"; and (*iii*) those partially preventing the enhanced entry. Inhibitors of protein synthesis block enhancement under conditions otherwise conducive to it. We conclude that the glucose and galactose carrier systems are not identical, based largely on the asymmetric "repression" observed when glucose and galactose are compared as "repressors."**

The mechanism of glucose entry into vertebrate cells has received considerable attention (1–5). It is now generally agreed that glucose is transported into cells (2–4) by carrier-mediated facilitated diffusion unlinked to energy-generating processes. Illiano and Cuatrecasas (4), using membrane preparations from fat cells, were able to stimulate entry by exposure of the membranes to insulin in a system uncomplicated by the problems of metabolism of the transported glucose.

In studies to determine whether D-glucose entry into chick fibroblasts was subject to modification by insulin (unpublished data), a marked enhancement of the rate of entry of D-glucose and D-galactose into the acid-soluble pool after prolonged starvation for a carbon and energy source was observed. Indeed, glucose starvation also rendered the cells responsive to insulin, whereas "fed" cells were unresponsive.

The experiments reported in this paper deal exclusively with evidence for asymmetric "derepression" of transport activity in chick fibroblasts dependent upon starvation of the cells. The results lead to the tentative proposal that glucose and galactose entry are not totally dependent on a common carrier.

MATERIALS AND METHODS

Cell Cultures. Chick embryo monolayer cultures were prepared by trypsinization of whole 10- to 12-day embryos (6). Culture vessels were inoculated to obtain initial cell densities of 10^4 to 5×10^4 cells per cm^2. After 48–72 hr the monolayers were washed with Hanks' balanced salt solution (7) and incubated in Eagle's basal medium (8) supplemented with 3% calf serum or unsupplemented as indicated for each experiment.

Glucose or Galactose Entry into Cells. Trichloroacetic acid-soluble D-glucose, 2-deoxy-D-glucose, or D-galactose contained in monolayer cells was determined after exposure to radioactive (^{14}C or ^3H) sugars for 5, 10, or 20 min. The following procedure was generally used:

(*1*) Experiments were conducted at 37°; all reagents were equilibrated to that temperature at the start of the experiment. The incubation medium was decanted from the monolayers to be used, and the last drops were removed with a Pasteur pipette.
(*2*) The monolayers were washed twice with 15 ml of balanced salt solution (without glucose).
(*3*) Radioactive hexoses were supplemented with unlabeled hexose to achieve the concentrations desired. The labeled hexose was introduced in 1.0 ml of balanced salt solution.
(*4*) The monolayers (three vessels per time interval) were incubated on a reciprocal shaker (80 oscillations per min) at 37° for 0, 5, 10, or 20 min. The "0" time background was subtracted from the timed values.
(*5*) To stop the reaction, the cells were quickly rinsed twice with 25 ml cold basic salt solution without glucose and drained.
(*6*) 1.5 ml of cold 5% trichloroacetic acid was added to each vessel. The vessel was permitted to remain cell-surface down for 3–5 hr. at 4°.
(*7*) The cells were harvested, and the soluble and insoluble fractions were separated by centrifugation.
(*8*) The acid-soluble fraction was neutralized by addition of NaOH, and duplicate or triplicate samples (0.5–1.0 ml each) were counted in a liquid scintillation counter [15 ml of Bray's scintillation fluid (11)].
(*9*) The protein content of each cell population was measured from the acid-insoluble fraction by the Lowry *et al.*, procedure (12).

Acid-Insoluble Counts. The acid-insoluble fraction (step 7) was suspended in 1.0 ml of 0.1 N NH_4OH, and 0.5 ml was used for counting in 15 ml of Bray's scintillation fluid.

Reagents. D-[*U*-^{14}C]glucose (specific activity 240 Ci/mol) was obtained from Schwartz BioResearch, Inc., Orangeburg, N.Y.; 2-deoxy-D-[^3H]glucose (specific activity 5.4 Ci/mmol); D-[^3H]galactose (6.9 and 2.04 Ci/mmol) and D-[^3H]glucose (620 Ci/mol) were obtained from New England Nuclear Corp.; D-glucose, D-ribose, and 2-deoxy-D-glucose were obtained from Nutritional Biochemical Corp.; α-glycero-phosphate, D-mannose, D-xylose, and D-glucosamine, from Sigma

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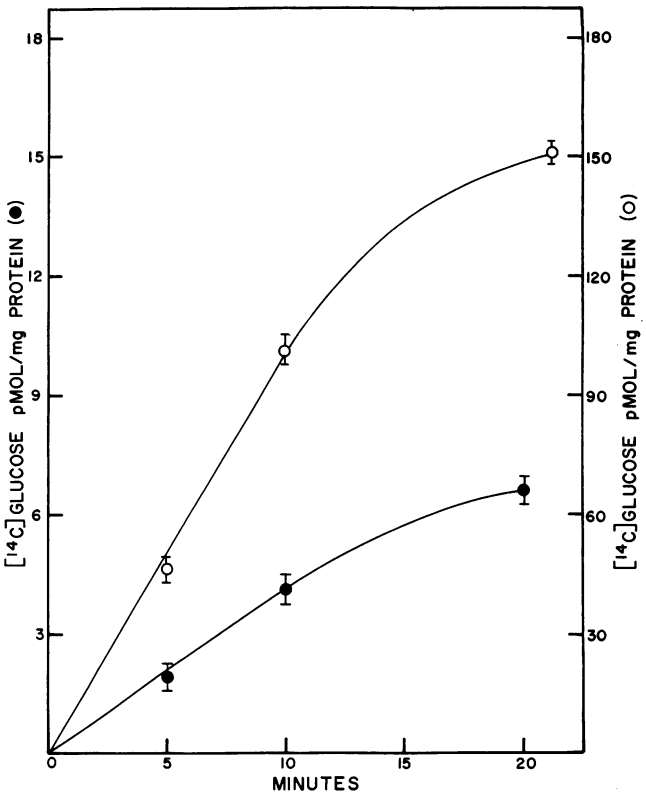

FIG. 1. Glucose uptake was plotted against time. Cells were prepared as in Table 1. After 24-hr incubation in D-glucose (1 mg/ml) or glucose-free medium, [^{14}C]glucose uptake was measured after 5, 10, and 20 min. ○, starved cells; ●, glucose-fed cells.

Chemical Co.; succinate and glycerol, from Fisher Scientific Co.; D-galactose and lactose from Calbiochem.

Glucose Determinations were made by the glucose oxidase method of the glucostat (13). Standards of 10, 50, and 100 μg were dissolved in balanced salt solution lacking glucose. Samples were diluted so as to fall in the range of 3–60 μg.

Computations of Quantities of Hexose in the acid-soluble pools were based on scintillation counting efficiency of 25% for ^{3}H-labeled hexoses and 55% for ^{14}C-labeled sugars.

RESULTS

Glucose Permeation: Effect of Prior Sugar Starvation. Chick-embryo fibroblasts grown as monolayers incorporate D-glucose into the acid-soluble pool linearly with time for at least 10 min (Fig. 1). ^{3}H- or ^{14}C-labeled glucose incorporation was measured in fresh glucose-free medium (Eagle's basal medium). The acid-soluble pool was extracted and measured as described in *Methods*. The cells in question were inoculated as monolayers in Eagle's basal medium supplemented with 3% calf serum at cell numbers designed to achieve an initial density of about 10^{4} cells per cm^{2}. After 72 or 96 hr of incubation at 37°, while the monolayers were still well below confluency, the incubation medium was replaced with glucose-containing or glucose-free Eagle's basal medium after a suitable rinse in glucose-free medium or saline.

The rate at which glucose is accumulated depends heavily upon the treatment to which the cells have been subjected. Cells starved of glucose for 24 hr (Fig. 1) incorporate radioactive glucose 15–30 times more rapidly than cells provided with glucose during the 20 hr before the test. Though the rate of entry is sharply modified by starvation, linearity is maintained for at least 10 min.

For the determination of optimum conditions for starvation and the kinetics of the phenotypic change produced by starvation, dialyzed and undialyzed serum were provided during deprivation, and the relative rates of glucose entry were determined by a 5-min accumulation into the acid-soluble pool (Table 1). Measurement of incorporation of labeled glucose into the acid-insoluble fraction revealed that not more than 1% of the total counts were contained in that fraction (Table 1). Undialyzed calf serum in the starvation medium resulted in more modest stimulation of entry, probably due to a low amount of glucose or other hexoses in the serum. Dialyzed calf serum added to the starvation medium, on the other hand, generally resulted in even more active uptake after glucose starvation.

As might be expected, the initial concentration of glucose in the starvation medium had a predictable influence on the subsequent behavior of the cells, the rate of glucose uptake by cells after a uniform period of pretreatment being inversely related to the initial glucose concentration (Table 2). 2-Deoxy-

TABLE 1. *Effect of starvation on uptake of* D-*glucose and 2-deoxy-*D-*glucose*

Preincubation (20 hr)	D-[^{14}C]Glucose uptake* (pmol/mg protein per 5 min)		2-deoxy-D-[^{3}H]Glucose uptake* (pmol/mg protein per 5 min)	
	Acid-soluble	Acid-insoluble	Exp. 1	Exp. 2
BME − D-glucose	99 ± 12	0.8 ± 0.2	62 ± 6	129 ± 15
BME + D-glucose	3.9 ± 0.6	0.04 ± 0.01	9 ± 2.1	9 ± 3
BME + 2-deoxy-D-glucose			7.5 ± 1.5	10 ± 3
BME − D-glucose + 3% CS	33 ± 6	0.3 ± 0.1		
BME + D-glucose + 3% CS	7.8 ± 1.8	0.08 ± 0.03		
BME − D-glucose + 3% DCS	192 ± 15	1.7 ± 0.4	123 ± 9	141 ± 12
BME + D-glucose + 3% DCS	7.2 ± 1.5	0.05 ± 0.02		

Cells grown for 72 hr in Eagle's basal medium (BME) supplemented with 3% calf serum were washed and provided with new BME, some with and others without glucose or 2-deoxy-D-glucose. After an additional 20 hr at 37°, cells were incubated in 1.0 ml of balanced salt solution containing 4 μM D-[*U*-^{14}C]glucose (1.0 μCi/ml) or 2-deoxy-D-[^{3}H]glucose (2 μCi/ml) for 5 min. Samples were processed for counting. Both unlabeled D-glucose and 2-deoxy-D-glucose in the preincubation medium were at a concentration of 1 mg/ml. CS = calf serum; DCS-dialyzed calf serum.

* Each value is the average of three monolayer cultures. The deviations are average deviations from the mean of the three separate cell cultures.

D-glucose was as effective as D-glucose when used in the "starvation" medium.

Cells transferred from glucose-containing to glucose-free medium became progressively more active in glucose uptake with time of starvation (Fig. 2). Though the extent of stimulation in the early hours was not uniform from one experiment to another, there was always an effective stimulation after 24 hr of starvation. The initial values are somewhat higher than those observed in earlier experiments (Table 1) comparing fed cells with starved cells. This is probably attributable to the low amount of glucose in the medium after 3 or 4 days without change of medium. In the earlier experiments fresh glucose (1.0 mg/ml) was provided the fed cells at the start of the pretreatment period. In all cases, the effects of starvation became progressively more evident between the 24th and 48th hr.

Effect of Glucose Concentration Upon Rate of Entry. Although the value of attempting to determine the K_m for glucose entry into the acid-soluble pool is uncertain, the effect of glucose concentration was determined as an indicator of concentrations of glucose that must be achieved for saturation. Saturation is achieved at a concentration of D-glucose above 1 mM (Table 3). The choice of 4 μM glucose as the concentration for standard test of uptake assures that the assay is linear with concentration.

Disappearance of Glucose from Medium. Determination of the glucose concentration of the medium at intervals after its addition to established monolayers, reveals a relatively rapid and exponential disappearance. By 72 hr of incubation, about 10–50% of the initial glucose (1 mg/ml) remained (unpublished results), depending upon initial cell density.

2-Deoxy-D-Glucose. When permeation of the glucose analogue, 2-deoxy-D-glucose, was examined, uptake was linear for at least 10 min, as with D-glucose (unpublished data); moreover, the effects of starvation upon its uptake were similar to those observed with D-glucose as the test sugar (Table 1). Since 2-deoxy-D-glucose is apparently not metabolized further than 2-deoxyglucose-6-PO_4 (14, 15), the kinetics of incorporation of D-glucose into the acid-soluble cell pool appear not to be unduly complicated by the rate of its disappearance by conversion to usable metabolic products. Glucose and 2-deoxyglucose were equally effective in maintaining the low entry rates observed for fed cells.

TABLE 2. *Effect of glucose concentration in "starvation" medium*

Initial concentration of D-glucose (20 hr preincubation)	D-[^{14}C]Glucose uptake (pmol/mg protein per 5 min)
Experiment 1	
0	162 ± 21
50 μg/ml	45 ± 6
1.0 mg/ml	11 ± 1.8
1.0 mg/ml 2-deoxy-D-glucose	7 ± 1.2
Experiment 2	
0	141 ± 9
50 μg/ml	51 ± 9
1.0 mg/ml	6.5 ± 2
1.0 mg/ml 2-deoxy-D-glucose	7.5 ± 0.9

Cells were prepared as described for Table 1. D-[*U*-^{14}C]-glucose 1.0 μCi/ml (4 μM) was incorporated for 5 min. Each value is the average of three monolayer cultures. The deviations are average deviations from the mean of the three determinations.

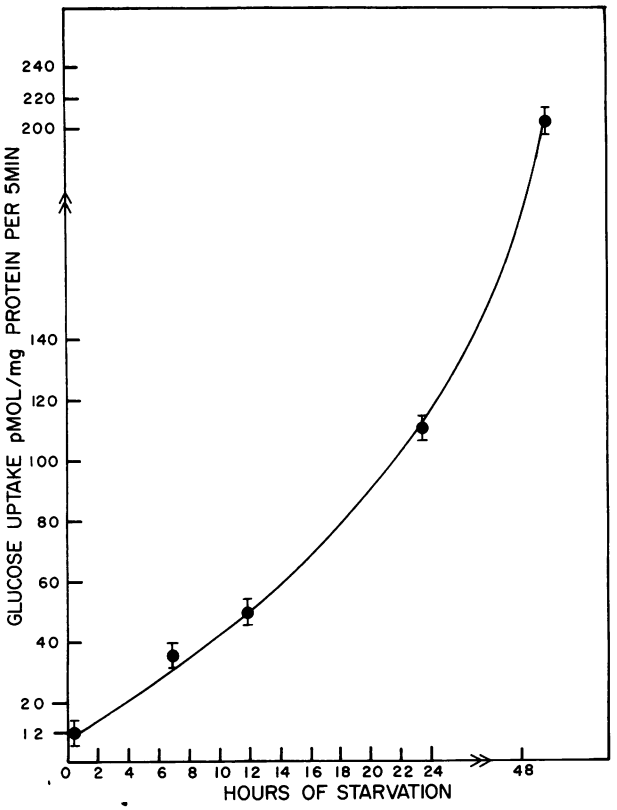

FIG. 2. Effect of starvation on glucose entry. Cells were prepared as in Table 1. 5-min entry after starvation for times indicated after removal from glucose-containing medium. Each point is the average of three monolayers.

Substitution of Other Sugars or Potential Carbon and Energy Sources for Glucose During the "Starvation Period." If during 23 hr of starvation in medium free of glucose but supplemented with dialyzed calf serum one of several compounds was substituted, some relieved the apparent "starvation" and others did not (Table 4). D-mannose, D-fructose, lactose, and D-ribose resembled D-glucose in their effect. Succinate, glycerol, α-glycerophosphate, and D-galactose appeared unable to substitute for D-glucose. D-Xylose and fumarate were intermediate. In general, those compounds likely to

TABLE 3. *Rate of glucose entry as function of glucose concentration*

Concentration of D-glucose (μM)	pmol/mg protein per 5 min
1	30 ± 6
4	96 ± 9
10	240 ± 9
100	1680 ± 60
1000	6204 ± 306

Cells were incubated for 20 hr in hexose-free Eagle's basal medium. Unlabeled D-glucose was added to D-[^{3}H]glucose (2–10 μCi/ml) to achieve the molar concentrations indicated. Each value is the average of three monolayer cultures. The deviations are average deviations from the mean of the three determinations.

TABLE 4. *Effect of various carbon sources on "starvation"*

Carbon source	D-[^{3}H]glucose uptake (pmol/mg protein per 5 min) Exp. 1	Exp. 2
None	192 ± 15	126 ± 2.1
D-Glucose (1 mg/ml)	16.8 ± 1.8	7.5 ± 1.5
D-Mannose (1 mg/ml)	12.9 ± 3	10 ± 3
D-Fructose (1 mg/ml)	9.6 ± 0.9	4.5 ± 1.8
Lactose (1 mg/ml)	23 ± 6	22 ± 3
D-Ribose (1 mg/ml)	25 ± 1.8	27 ± 4.2
Succinate (1 mg/ml)	222 ± 24	135 ± 12
Glycerol (0.9% volume)	186 ± 18	111 ± 15
α-Glycerol phosphate	201 ± 9	138 ± 2.4
D-Galactose (1 mg/ml)	213 ± 12	159 ± 9
D-Xylose (1mg/ml)	72 ± 6	36 ± 3
Fumarate (1 mg/ml)	48 ± 2.1	57 ± 6
D-Glucosamine (1 mg/ml)	42 ± 9	33 ± 12

Cells were prepared as described for Table 1. Starvation period was 23 hr in balanced salt solution at 37°. Each value is the average of three monolayer cultures. The deviations are average deviations from the mean of the three determinations. Samples were incubated with 4 μM D-[^{3}H]glucose (2 μCi/ml).

generate glucose by interconversion (D-mannose and D-fructose), by enzymatic hydrolysis (lactose), or by rapid biosynthetic modification (D-ribose) behaved like glucose during 24 hr. If the rate of conversion to glucose was limited [D-galactose (16)], cells reacted as to starvation. D-Glucosamine was, surprisingly, also effective as a glucose substitute. Glucose contamination would have had to be in excess of 5% to account for any of the effects observed with glucosamine, lactose, or ribose. By paper chromatography, the glucose contamination of none of the three exceeded 0.5% (unpublished data).

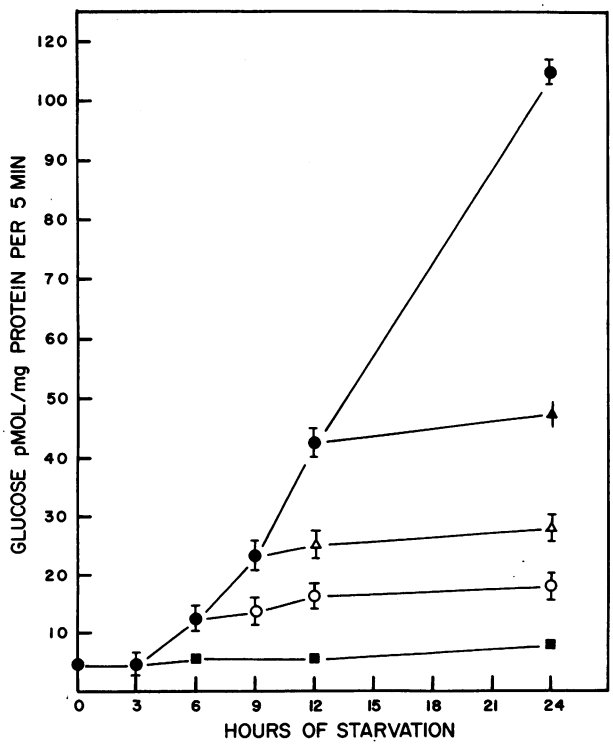

FIG. 3. Cycloheximide inhibition of enhancement. Conditions were the same as in Fig. 2. ●, No cycloheximide; ■, cycloheximide (10 μg/ml) at "0" time; ○, cycloheximide at 6 hr; △, cycloheximide at 9 hr; ▲, cycloheximide at 12 hr.

Galactose Entry. The effect of starvation upon galactose entry was similar to that observed with glucose, showing an increased rate of accumulation in cells starved of carbon source for 24 hr (Table 5). Again, uptake proved linear for at least 10 min (unpublished result). Starvation for a carbon source produces a maximum enhancement of only 5 or 6 fold for galactose entry while permitting a 15- to 25-fold increase in glucose entry.

Nonreciprocal "Starvation": Comparison of Glucose and Galactose. Glucose and galactose, when supplied through a range of concentrations, compete for uptake by the fibroblasts (unpublished results). Nonetheless, they fail to show reciprocity in their effects upon the accelerated entry resulting from long-term starvation. Whereas glucose at 1 mg/ml prevents enhancement of both glucose and galactose entry (Table 5), galactose at the same concentration permits the enhancement of glucose permeation as though the cells were starved of a carbon source.

Blockage of Enhancement by Inhibitors of Protein Synthesis. The 15- to 30-fold enhancement of glucose entry observed after prolonged starvation of carbon source is prevented by inhibitors of protein synthesis, either puromycin (100 μg/ml) or cycloheximide (5, 10, or 100 μg/ml). Indeed, if the inhibitor of protein synthesis is added at intervals during starvation, the increase in rate of entry ceases on addition of the inhibitor (Fig. 3). The altered permeation of galactose observed on prolonged starvation is also blocked by cycloheximide (unpublished results).

DISCUSSION

Transport of glucose, at least in adipose tissues, occurs by a carrier-mediated facilitated diffusion mechanism (4). The translocation occurs through a bidirectional system that has similar properties in both directions. The transport of glucose in both directions is stimulated by insulin—outside the cell membrane. The contribution of allosteric transitions and of covalent modifications, such as phosphorylation of carrier proteins, must have a place in current speculation about this problem.

TABLE 5. *Effect of starvation upon galactose entry*

Preincubation (24 hr)	Test sugar	D-[^{3}H]Galactose uptake (pmol/mg protein per 5 min) Exp. 1	Exp. 2
BME − D-glucose	D-Glucose	102 ± 6	80 ± 5
BME + D-glucose	D-Glucose	7.5 ± 1	3 ± 0.5
BME + D-galactose	D-Glucose	52 ± 4	86 ± 4
BME − D-glucose	D-Galactose	51 ± 5	27 ± 1.2
BME + D-glucose	D-Galactose	12 ± 3	6 ± 0.2
BME + D-galactose	D-Galactose	11 ± 1.5	7 ± 1

Cells were prepared as in Table 1. [^{3}H]glucose and [^{3}H]galactose were used as test sugars for entry. Each value is an average of 3 monolayer cultures. When provided during the preincubation, glucose and galactose were at a concentration of 1 mg/ml.

Galactose permeation in mammalian intestines is considered to be mediated by the glucose transport system since competition can be demonstrated between the two (9, 19). A single carrier is postulated (19), and transport is active, i.e., intestinal sacs accumulate sugars against a concentration gradient (8, 10, 19).

The striking increase in the rate of glucose entry that we observe after prolonged starvation for a carbon and energy source requires protein synthesis and is prevented by either puromycin or cycloheximide. This result suggests that the increased activity is due mainly to synthesis of elements of the permeation system. Sefton and Rubin (20) have described a similar increase in glucose transport in density-inhibited cells on treatment with trypsin and serum. The enhancement they observe also requires protein synthesis. The "derepression" reported here has been effected for the most part in nonconfluent cultures. Similar "derepression" has been obtained with dense cultures (unpublished results), which in our hands also show the reduction in rate of glucose entry reported by Sefton and Rubin (20). The mechanism by which glucose and metabolically related hexoses block the "derepression" is unknown. That 2-deoxyglucose is as effective as glucose suggests that the phosphorylated product of hexokinase action could also be involved in the "repression." It is important to note that after starvation for an energy source for as long as 72 hr, cells remain attached to the surface and will multiply normally when supplied with glucose and serum (unpublished results).

Presumptive evidence for a different carrier system for D-glucose and D-galactose is presented. It consists primarily of the different degrees of increase of entry after starvation for a carbon source, that for glucose being from 15–30 times while that for galactose rarely was greater than 5 times. The fact that hexokinase activity (unpublished results) did not vary significantly in cells subjected to various conditions of starvation tends to rule out "derepression" as a reflection of the specific activity of hexokinase. This interpretation is supported by the nonreciprocal effect of glucose and galactose on "repression" of permeation activity for the two sugars. Glucose blocks the "derepression" of both its own and galactose uptake, while galactose, as effective as glucose blocking increased galactose entry, permits full "derepression" of glucose entry machinery.

Recent evidence has revealed that galactose and glucose entry into virus-transformed mammalian cells is enhanced compared to controls (17, 18). In some cases (17) the effect is a reflection of change in K_m and V_{max} of the transport system.

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1. Rodbell, M., James, A. B., Chiappe di Ciugolani, G. E. & Birmbaumer L. (1968) *Recent Progr. Hormone Res.* **24**, 215–254.
2. Crofford, O. B. (1968) Renold, A. E. (1965) *J. Biol. Chem.* **240**, 3237–3244.
3. Crofford, O. B. (1967) *Amer. J. Physiol.* **212**, 217–220.
4. Illiano, G. & Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 2472–2479.
5. Kaback, H. R. (1970) *Annu. Rev. Biochem.* **39**, 561–598.
6. Amos, H. & Moore, M. O. (1963) *Exp. Cell Res.* **32**, 1–13.
7. Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200.
8. Eagle, H. (1955) *J. Biol. Chem.* **214**, 839–852.
9. Crane, R. K. Forstiner, G. & Eicholz, A. (1965) *Biochim. Biophys. Acta* **109**, 467–477.
10. Schultz, S. G. & Curran, P. F. (1970) *Physiol. Rev.* **50**, 637–718.
11. Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285.
12. Lowry, O. H. Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
13. Huggett, A. St. G. & Nixon, D. A. (1957) *Lancet ii*, 368–370.
14. Kipnis, D. M. & Cori, C. F. (1959) *J. Biol. Chem.* **234**, 171–177.
15. Smith, D. E. & Gorski, J. (1968) *J. Biol. Chem.* **243**, 4169–4174.
16. Robinson, E. A., Kalckar, H., Troedsson, H. & Sanford, K. (1966) *J. Biol. Chem.* **241**, 2737–2745.
17. Hatanaka, M., Augl, C. & Gilden, R. V. (1970) *J. Biol. Chem.* **245**, 714–717.
18. Isselbacher, K. J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 585–589.
19. Goldner, A. M., Hajjar, J. J. & Curran, P. E. (1969) *J. Gen. Physiol.* **53**, 362–383.
20. Sefton, B. M. & Rubin, H. (1971) *Proc. Acad. Sci. USA* **68**, 3154–3157.