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# Transport of Sugars in Chick-Embryo Fibroblasts

## EVIDENCE FOR A LOW-AFFINITY SYSTEM AND A HIGH-AFFINITY SYSTEM FOR GLUCOSE TRANSPORT

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The rate of D-glucose uptake by cells that had been deprived of sugar for 18–24h was consistently observed to be 15–20 times higher than that in control cells maintained for the same length of time in medium containing glucose. This increased rate of glucose transport by sugar-starved cells was due to a 3–5-fold increase in the $V_{max.}$ value of a low-affinity system ($K_m$ 1mM) combined with an increase in the $V_{max.}$ of a separate high-affinity system ($K_m$ 0.05–0.2mM). The high-affinity system, which was most characteristic of starved cells, was particularly sensitive to low concentrations of the thiol reagent *N*-ethylmaleimide; 50% inhibition of uptake occurred at approx. 0.01mM-*N*-ethylmaleimide. In contrast with the high-affinity system, the low-affinity system of either the fed cells or the starved cells was unaffected by *N*-ethylmaleimide. In addition to the increases in the rate of D-glucose transport, cells deprived of sugar had increased rates of transport of 3-*O*-methyl-D-glucose and 2-deoxy-D-glucose. No measurable high-affinity transport system could be demonstrated for the transport of 3-*O*-methylgucose, and *N*-ethylmaleimide did not alter the initial rate. Thus the transport of 3-*O*-methylglucose by both fed and starved cells was exclusively by the *N*-ethylmaleimide-insensitive low-affinity system. The low-affinity system also appeared to be the primary means for the transport of 2-deoxyglucose by fed and starved cells. However, some of the transport of 2-deoxyglucose by starved cells was inhibited by *N*-ethylmaleimide, suggesting that 2-deoxyglucose may also be transported by a high-affinity system. The results of experiments that measured transport kinetics strongly suggest that glucose can be transported by at least two separate systems, and 3-*O*-methylglucose and 2-deoxyglucose by one. Support for these interpretations comes from the analysis of the effects of *N*-ethylmaleimide and cycloheximide as well as from the results of competition experiments. The uptake of glucose is quite different from that of 2-deoxyglucose and 3-*O*-methylglucose. The net result of sugar starvation serves to emphasize these differences. The apparent de-repression of the transport systems studied presents an interesting basis for further studies of the regulation of transport in a variety of cells.

Chick-embryo cells progressively acquire enhanced ability to take up hexoses on exposure to culture conditions in which the sugar (D-glucose) has been omitted from the culture medium, i.e. starvation conditions (Martineau *et al.*, 1972; Shaw & Amos, 1973). Although $K_m$ and $V_{max.}$ values were not reported, the enhancement of a 5min glucose (5$\mu$M) uptake was generally 10–15 times that of cells fed on glucose (Martineau *et al.*, 1972; Shaw & Amos, 1973). Kalckar *et al.* (1973), Kalckar & Ullrey (1973) and Hatanaka (1973) have observed enhanced hexose uptake in hamster and mouse cells starved in a similar way.

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In eukaryotic cells maintained in media containing glucose and serum, it has been widely reported that there is little, if any, detectable difference between the transport of D-glucose and the transport of its analogue, 2-deoxy-D-glucose (Hatanaka, 1974). Where tested, the apparent Michaelis constants for the energy-independent transport of these sugars were approximately the same (about 1mM) and one sugar was competitive with the other. Likewise, 3-*O*-methyl-D-glucose (which is not a substrate for hexokinases and therefore is not metabolized) has been considered to enter cells through the same glucose-transport system. Interesting exceptions to these findings have been reported (Scarborough, 1970*a*,*b*; Neville *et al.*, 1971).

In the present paper, we report the results of

experiments that compare the transport of D-glucose, 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose in cultured chick-embryo cells. Kinetic evidence suggests that two systems, one apparently de-repressed by starvation, are involved in the transport of glucose. These two can be distinguished by $K_m$ values, specificity for glucose and sensitivity to metabolic poisons.

## Experimental

### *Materials*

Fertilized chicken eggs were obtained from Spafas Inc., Norwich, CO, U.S.A. Calf serum and modified Hanks' balanced salt solution (Hanks' BSS) containing glucose were obtained from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Hanks' balanced salt solution without glucose (glucose-free Hanks' BSS) was prepared from reagent-grade salts and dissolved in deionized water. Tris and Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] buffers were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-Ethylmaleimide, *p*-chloromercuribenzenesulphonic acid and iodoacetamide, also from Sigma Chemical Co., were dissolved in buffered Hanks' BSS glucose-free with 5mM-potassium phosphate, pH6.7. Cycloheximide and dithiothreitol were obtained from Calbiochem, La Jolla, CA, U.S.A. Trichloroacetic acid was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A., made 5% (w/v) in deionized water and stored at 0–4°C. Glass shell vials were obtained from Arthur H. Thomas Co., Philadelphia, PA, U.S.A., acid-washed, rinsed, covered with 25cm Bellco Ka-puts (Bellco Glass Inc., Vineland, NJ, U.S.A.) and autoclaved. Purified yeast hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1) was obtained from P-L Biochemicals Inc., Milwaukee, WI, U.S.A. Omnifluor, L-[4,5-$^3$H]isoleucine (73.5Ci/mmol), [*Me*-$^3$H]thymidine (6.7Ci/mmol), [5-$^3$H]uridine (26Ci/mmol) and radioactive sugars D-[U-$^{14}$C]glucose (196mCi/mmol), D-[2-$^3$H]glucose (540mCi/mmol), 2-deoxy-D-[G-$^3$H]glucose (7.2Ci/mmol) and 3-*O*-[$^3$H]methyl-D-glucose (6.2Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A.

### *Methods*

*Preparation of cultures.* Decapitated 10–12-day-old chick embryos were trypsin-treated (Gibco; 0.25%) and the resulting cell suspension was concentrated by centrifugation as described previously (Amos & Moore, 1963). The cells, resuspended in Eagle's basal medium supplemented with 3% (v/v) calf serum, were inoculated into 32oz prescription bottles at a concentration of $1\times10^6$–$2\times10^6$ cells/ml (final density approx. $2\times10^5$–$5\times10^5$ cells/cm$^2$). After 3 or 4 days of growth at 37°C, the cells were quickly removed from the glass surface with trypsin and protected from further action of trypsin by the addition of serum (15%, v/v). The cells were then collected by centrifugation at 1500*g* for 5min and resuspended in Eagle's basal medium containing 3% calf serum and 0.15% $NaHCO_3$ (18mM) at a density of $2\times10^5$–$5\times10^5$ cells/ml. Samples (1ml) of the cell suspension were placed in acid-washed sterile shell vials which have surface areas of 3.8cm$^2$. The loosely covered vials were placed in a humidified $CO_2$ incubator maintained at 37°C in an atmosphere of $CO_2$+air (5:95). These secondary cultures were left to grow for 3 or 4 days without further changes. Cells, near or at confluency, were twice washed with 5ml of glucose-free Hanks' BSS before exposure for 18–24h to Eagle's basal medium containing glucose (5.5mM) or Eagle's basal medium without glucose. Both media were supplemented with dialysed calf serum (0.5%).

*Short-term preincubations.* At the end of the 18–24h period of sugar-starvation or sugar-re-feeding, the cells were washed with $2\times5$ml of glucose-free Hank's BSS and chilled on ice. The washed and chilled cells were treated with *N*-ethylmaleimide, iodoacetamide or *p*-chlorobenzene sulphonic acid dissolved in glucose-free buffered 10mM-potassium phosphate, Hanks' BSS, pH6.7. Cells were transferred to 37°C for 5 or 15min, then chilled on ice before replacement of the preincubation media with 1ml of ice-cold 5mM-dithiothreitol in glucose-free Hanks' BSS. Finally, the cells were washed with 5ml of glucose-free Hanks' BSS and assayed for sugar uptake.

*Uptake assay (5min).* Before each assay, cells were washed with $2\times5$ml of glucose-free Hanks' BSS, and chilled on ice. The final wash was removed and replaced with 0.5ml of ice-cold radioactive sugar solution. The sugars for uptake (in glucose-free Hanks' BSS, pH7.0–7.5) were D-[$^3$H]glucose (2$\mu$Ci/ml, 4$\mu$M), 2-deoxy-D-[$^3$H]glucose (2$\mu$Ci/ml, 4$\mu$M) or D-[$^{14}$C]glucose (0.5$\mu$Ci/ml, 4$\mu$M). The vials were transferred to 37°C for 5min and then returned to the ice/water bath. A total of 25ml (5ml portions) of ice-cold 10mM-potassium phosphate buffer containing 1.55M-NaCl, pH7.0, was used to rinse each monolayer after aspiration of the radioactive uptake medium. Total washing time was 15–20s for each vial. Immediately after the final wash, 0.25ml of ice-cold 5% (w/v) trichloroacetic acid was placed on the cells and left at 0–4°C for a few hours (usually overnight). The trichloroacetic acid was then removed and dried on 2cm × 6.5cm Reeve–Angel glass-fibre strips. The radioactivity on the strips was counted in 10ml of a non-aqueous toluene-based scintillation fluid (15g of Omnifluor/3.8 litres of toluene) on a Beckman LS-230 counter.

*Uptake assay (10s).* The uptake procedure described above was modified somewhat to accommodate the shorter period of exposure. The temperature of the monolayer, bathed in 1.0ml of glucose-free Hanks' BSS, was raised to 37°C by a 1min incubation

just after washing and just before the addition of the radioactive sugar. The concentration of the labelled sugars was increased from 4 to 50$\mu$M. For kinetic experiments the temperature of incubation during uptake was lowered from 37°C to 25°C. At the end of each assay time, the radioactive mixture was aspirated and the monolayer washed with 5×5ml of ice-cold buffered NaCl as described above. The washed cells were immediately covered with 0.25ml of ice-cold 5% (w/v) trichloroacetic acid and processed for radioactivity counting as described above.

*Hexokinase assay.* Cells were grown in 100mm Petri dishes and fed or starved for 18–24h. The cells were scraped into 5mM-Tes/NaOH buffer (pH6.7) and homogenized in the presence of 0.1% deoxycholate. The homogenates were then cleared by centrifugation at 27000*g* for 15min and the supernatants assayed for hexokinase activities. Hexokinase activity from cells homogenized in 5mM-Tes was distributed approximately equally between the supernatant and the pellet from the 27000*g* centrifugation. Homogenization in deoxycholate (0.1%) released into the supernatant hexokinase activity equal to that of the combined pellet and supernatant. The assay mixture (1ml) contained 5mM-Tes buffer (pH6.7), 5mM-ATP, 10mM-magnesium acetate, 1mM radioactive sugar (D-glucose or 2-deoxy-D-glucose) and enzyme or homogenate protein. Assays were conducted at 37°C, and at appropriate times 100$\mu$l samples were removed and diluted in 1ml of hot 5mM-Tris/HCl buffer (pH7.6) containing 100mM-glucose (Tris/glucose buffer). After 5min at 90–100°C, the mixture was cooled and filtered through a Whatman DE81 filter pre-washed with 5ml of the above Tris/glucose buffer. The adsorbed anionic metabolic products were further washed with 10ml of Tris/glucose buffer, air-dried, and counted for radioactivity in the toluene/Omnifluor scintillation fluid.

*Paper chromatography.* Equivalent amounts of radioactive materials from hexokinase reactions *in vitro* or soluble pools extracted from monolayers were spotted on Whatman no. 1 chromatography paper. Separation (descending) was accomplished in 8–10h (solvent front travelled 25–30cm) in the Paladini & Leloir (1952) ethanol/acetate system [7.5 vol. of 95% (v/v) ethanol/3 vol. of 1M-ammonium acetate buffer, pH3.5). In this system, glucose, 2-deoxy-D-glucose, glucose 6-phosphate and 2-deoxyglucose 6-phosphate were found to have $R_F$ values of 0.6, 0.71, 0.4 and 0.52 respectively. In the hexokinase assay incubation described above, purified yeast hexokinase was used to generate glucose 6-phosphate and 2-deoxyglucose 6-phosphate; these were used, along with glucose and 2-deoxyglucose, as standard markers.

*Determination of protein.* The trichloroacetic acid-insoluble material of the monolayers was dissolved in 0.2M-NaOH. The protein content of each monolayer and protein of cell-free extracts were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

## Results

### *Culture conditions*

When secondary subcultures of chick-embryo cells were cultivated in Eagle's basal medium supplemented with 3% calf serum, they attained a well-oriented confluent monolayer in 3–4 days. At that time, the density had increased from $2\times10^5$ cells to approx. $5\times10^5$ cells per culture vessel. Similarly to the results described by Kletzien & Perdue (1973), we have found that chick cells in culture decrease their abilities to take up sugars as a function of time after subculture. The lowest uptake occurred between day 3 and day 5, corresponding to the time when little or no increase in total cell protein occurred and the cells had reached confluency (C. W. Christopher, unpublished work). Since there was no change of medium during that period, it is reasonable to assume that nutrient depletion or product accumulation was responsible for the steady loss of uptake capacity by the cells. However, when fresh medium was added to the cells, there was no detectable difference in the 5min uptake assay (see under 'Methods') between the 're-fed' cells and those that remained in the original medium. Further, experiments in which the sugar was omitted from the fresh medium showed that the depletion of glucose was not the cause of the decline in uptake capacity. In fact, as previously shown (Martineau *et al.*, 1972; Kletzien & Perdue, 1975), the glucose-uptake capacity in these 'sugar-starved' cells was increased and not decreased. It was further found that, the longer the cells remained in the original culture medium before the changes of the medium, the greater was the subsequent increase in glucose uptake by the starved cells. Attempts to use cells maintained in unchanged medium for 6–7 days were not successful because of the tendency of such cells to be washed off the surface.

Although hexose starvation beyond 24h resulted in further increases in the rate of uptake, beyond 48h the monolayers became fragile and susceptible to losses during procedures such as washes. An 18–24h starvation time was chosen as standard for subsequent experiments because after 24h there was no difference in cell number or total protein in monolayers that had been changed to fresh media regardless of the presence or absence of glucose.

For monolayers being re-fed with or starved of glucose, the presence of low concentrations of dialysed serum in the fresh media maintained the cells on the surfaces far better than if the media contained no serum at all. Therefore, to maintain the cells on the surface and optimize the assay system, monolayers

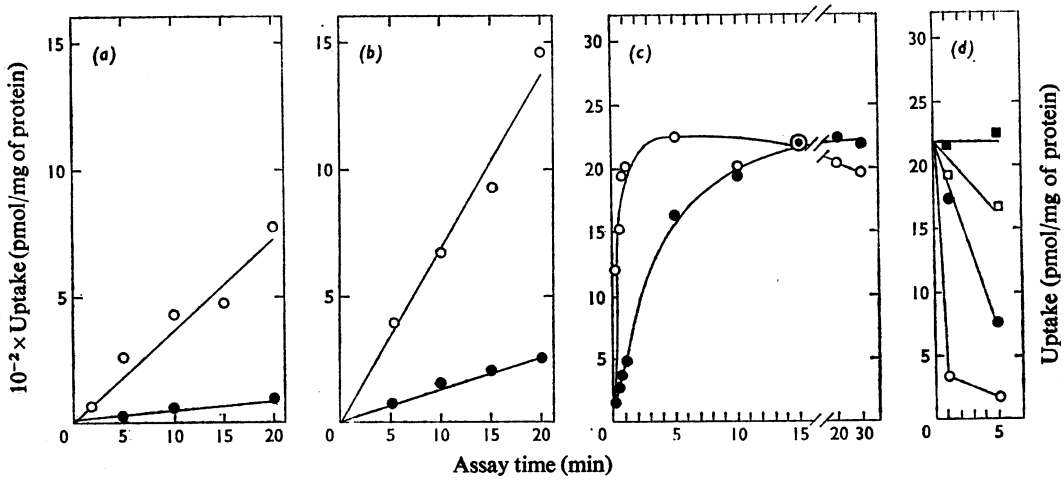


Fig. 1. *Effect of sugar deprivation on the uptake of glucose and 2-deoxyglucose and the transport of 3-O-methylglucose*

Chick-embryo cells were maintained in fresh medium containing 0.5% dialysed serum and either 1 mg of glucose/ml (●, ■) or no sugar (○, □) for 18–24h. (*a*) Uptake of D-glucose; (*b*) uptake of 2-deoxy-D-glucose; (*c*) transport of 3-*O*-methyl-D-glucose; (*d*) efflux of 3-*O*-methylglucose. In (*d*) cells were allowed to take up 3-*O*-methylglucose for 30 min, washed with ice-cold glucose-free Hanks' BSS and then placed at either 37°C (○, ●) or 0–4°C (□, ■) in 1 ml of phosphate-buffered NaCl. At the appropriate times indicated, the cells were washed and the acid-soluble material was counted for radioactivity as described under 'Methods'.

were provided with media containing 0.5% dialysed serum during the 18–24h glucose-re-feeding or -starvation period.

*Assay conditions for sugar uptake*

Fig. 1 shows that the uptake of both D-glucose and 2-deoxy-D-glucose in starved and fed cells was linear with time for at least 20 min at an assay temperature of 38°C. Further, most (85–90%) of the radioactivity originally in glucose was retained in an acid-soluble form within the cells for 30 min of additional incubation in the absence of sugar (Fig. 2). When the uptake medium collected at the end of 30 min was re-used for freshly washed cells, it proved to be unaltered; i.e. so little of the glucose had been removed that the second monolayer demonstrated the same linearity of uptake as the first.

The transport of 3-*O*-methyl-D-glucose by sugar-fed and sugar-starved cells is shown in Fig. 1(*c*). The intracellular concentration approached equilibrium with the extracellular concentration in less than 5 min in the sugar-starved cells. However, transport was linear with time only for times much less than 1 min. By comparison, the transport of 3-*O*-methyl-D-glucose by sugar-fed cells was linear for nearly 5 min, and between 10 and 15 min was required before the intracellular concentration approached the extracellular concentration. Rates of transport were 30–50% lower at 25°C than they were at 37°C. Concomitantly, at the lower temperature, linearity of transport was extended. Little or no transport of 3-*O*-methyl-D-glucose was observed at 0–4°C.

To test the rate of efflux of free sugars, cells that had transported 3-*O*-methyl-D-glucose for 30 min were washed with ice-cold glucose-free Hanks' BSS and then either warmed to 37°C or kept at 0–4°C for 5 min. Fig. 1(*d*) shows that the rates of efflux of 3-*O*-methyl-D-glucose from sugar-starved and sugar-fed cells were equal to their respective influx rates at 37°C. Efflux, however, was markedly slower at 0–4°C. Even after 5 min in sugar-free buffer at 0–4°C there was no detectable loss of the sugar from the fed cells and approximately a 20% loss of 3-*O*-methyl-D-glucose from the sugar-starved cells. These data show that at 37°C the transport of free sugar out of the cells is as rapid as the transport in to the cells. Equally important, from a procedural point of view, no detectable loss of free sugar resulted from the routine 15–20s wash procedure at 0–4°C.

To approximate initial rates of transport, assays for the transport of 3-*O*-methylglucose (as well as for 2-deoxyglucose and glucose) were conducted at 25°C for 10 or 15s. In addition, each monolayer was washed immediately after the assay with ice-cold buffered NaCl as a precaution against excessive loss of the free sugars. Under these conditions, starved cells took up 2–10 times more 3-*O*-methylglucose, 2–10 times more 2-deoxyglucose

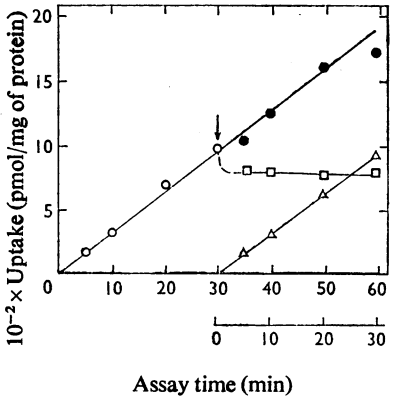


Fig. 2. *Glucose uptake and retention of radioactivity after washing*

Sugar-starved cells were washed and assayed as described under 'Methods'. After 30min (arrow) radioactive assay medium (used medium) was removed from cells, combined and placed over fresh cells (△) that had been washed preparative to assay but had not previously received the assay mixture. Some cells that had been taking up D-[$^{14}$C]glucose (0.5$\mu$Ci/ml, 4$\mu$M) were given a fresh portion of the above radioactive glucose solution and allowed to take up glucose for an additional 30min (●). Other cells that had taken up glucose for 30min were washed with 5×5ml of glucose-free Hanks' BSS and then left in 1ml of glucose-free Hanks' BSS, at 37°C for as long as 30min (□). Uptake was terminated by washing and extraction with acid as described under 'Methods'. The ordinate indicates only the acid-soluble fraction extracted from within the cells.

and 10–20 times more glucose than did fed cells. The short assay times were necessary in experiments designed to measure initial rates of uptake of 3-*O*-methylglucose and for the analysis of transport kinetics.

*Linearity extended to 5min assay*

For obvious reasons a 10s assay time for glucose uptake poses certain technical problems. High specific activities and low concentration of radioactive sugars were required as well as precise timing and washing. Whenever possible, it was desirable to decrease the amount of radioactivity and extend the assay time. In addition, the margin of intracellular glucose over the adsorbed glucose was considerably increased by extending the time from 10 or 15s to 5 or 10min. This extension of assay time is valid only if the uptake is linear for the 5 or 10min period. Figs. 1(*a*) and 1(*b*) shows this to be true for the uptake of glucose and 2-deoxyglucose in both fed and starved cells. In addition, comparison of the kinetics of glucose uptake revealed that the accumulation of acid-soluble products was linear from 5s to 20min (C. W. Christopher, unpublished work). No doubt the linearity observed in Figs. 1(*a*) and 1(*b*), is attributable, in part, to the lower substrate concentration (4$\mu$M) used as well as the immediate phosphorylation of sugar entering the cell. Since linearity is demonstrable in both fed and starved cells, there is little evidence of significant loss of glucose metabolites from the cell during the 5min period. The same might not pertain to substrate concentrations in the mM range (Kletzien & Perdue, 1975; J. Cook, personal communication). Relative values obtained with glucose and 2-deoxyglucose by the 5min assay were essentially identical with the results observed when the 10s assay was used. Starved cells again had 2–10 times higher uptake activity than did fed cells when 2-deoxyglucose was the substrate; they were at least 10–20 times more active when D-glucose was the test sugar.

*Comparison of uptake of glucose and 2-deoxyglucose in fed cells*

Direct comparisons of the uptake of D-glucose and 2-deoxy-D-glucose revealed that the absolute amounts of the intracellular sugars of starved cells were similar. However, fed cells were consistently able to take up more 2-deoxy-D-glucose than D-glucose (see Fig. 1 and controls in Table 1). This paradox cannot be explained by losses of catabolites such as $CO_2$ and lactate because, as stated above, at the low substrate concentrations used (4$\mu$M), over 85% of the radioactivity taken up in 30min was retained inside the cells for at least an additional 30min (Fig. 2). It might be argued that feedback inhibitors of key metabolic enzymes, especially hexokinase, could conceivably account for the discrepancy between 2-deoxy-D-glucose and D-glucose uptake values. However, in 5min the low substrate concentrations used in the uptake assay test could, at best, generate an internal concentration of approximately 8$\mu$M-glucose 6-phosphate (see Fig. 1*a*), which is far below the concentrations required to inhibit hexokinase *in vitro* (Kosow & Rose, 1972).

Attention should also be drawn to the fact that the cells at the time of the assay were uniformly conditioned by maintenance in 5.5mM-glucose for 18–24h. Thus the internal metabolic pools (at the time when test sugars were introduced) had to be equivalent for both the glucose and the 2-deoxyglucose assays. Hence, unless the transport systems are inherently different, feedback inhibition by product cannot account for the difference in uptake observed between the two sugars by fed cells.

*Specificity*

Radioactivity associated with the acid-insoluble fraction of the monolayers was minimal, representing less than 4, 2 and 1% of the acid-soluble material in assays with D-glucose (5min at 37°C), 2-deoxy-D-

Table 1. *Effect of cycloheximide on sugar uptake and transport*

Triplicate monolayers of chick-embryo cells were incubated for 18–24h with the amounts of cycloheximide indicated in medium containing either 1 mg of glucose/ml or no sugar. The uptake and transport assays are described under 'Methods'. The values ± S.D., are in pmol/min per mg and the values in parentheses are the ratios of starved cell to fed cell uptake rates. The D-[$^{14}$C]glucose concentration in Expts. I and II was 4 $\mu$M and the 2-deoxy-D-[$^{3}$H]glucose concentration in Expt. I was 5 $\mu$M. Expt. I involves double labelling; corrections for channel overlap were made. In Expt. III the D-[$^{14}$C]glucose and the 3-*O*-methyl-D-[$^{3}$H]glucose concentrations were 50 $\mu$M.

| Expt. no. | Additions during 24h preincubation | Assay | Uptake or transport of: D-Glucose, Starved | D-Glucose, Fed | 2-Deoxy-D-glucose, Starved | 2-Deoxy-D-glucose, Fed | Transport of 3-*O*-methyl-D-glucose, Starved | 3-*O*-methyl-D-glucose, Fed |
|---|---|---|---|---|---|---|---|---|
| I | None | 5min/37°C | 64±3 | 1.6±0.07 (40) | 53±3 | 6.1±0.35 (8.7) | — | — |
| | Cycloheximide 1 $\mu$g/ml | | 17±9.8 | 0.91±0.06 | 41±20 | 3.2±0.2 | — | — |
| | 10 $\mu$g/ml | | 3.8±0.29 | 0.52±0.08 (7.3) | 15±1 | 2.2±0.08 (6.8) | — | — |
| | 100 $\mu$g/ml | | 6.3±0.2 | 1.12±0.05 | 21±0.67 | 4.4±0.3 | — | — |
| II | None | 5min/37°C | 46±3 | 2.9±0.1 | — | — | — | — |
| | | * | 51±3 | 2.8±0.47 (16)† | — | — | — | — |
| | Cycloheximide 10 $\mu$g/ml | | 10±3.7 | 0.83±0.09 | — | — | — | — |
| | | * | 6.7±1.5 | 0.96±0.14 (12)† | — | — | — | — |
| III | None | 10s/37°C | 335±27 | 45±29 (7.5) | — | — | 271±33 | 28±7 (9.7) |
| | Cycloheximide 100 $\mu$g/ml | | 50±11 | 15±9.4 (3.3) | — | — | 50±11 | 14±5 (3.6) |

* Cycloheximide (10 $\mu$g per ml) was present in the assay mix which contained 4 $\mu$M-D-[$^{14}$C]glucose.
† Average of two experimental sets.

glucose (5min at 37°C) and 3-*O*-methyl-D-glucose (10s at 37°C) respectively. In a typical experiment where glucose uptake was 18-fold higher in starved than in fed cells, thymidine and uridine uptake in starved cells were in fact slightly decreased to approx. 80% of that of fed cells. In addition, the uptake of a representative amino acid (L-isoleucine) was only 1.8-fold higher than in the fed cells (C. W. Christopher, unpublished work).

*Effects of cycloheximide*

The dramatic increases in the transport of sugars in starved cells were prevented by the inclusion of cycloheximide in the sugar-free medium during the long incubation time (Table 1). Table 1 shows that cells fed with glucose for the same time-periods were also affected by cycloheximide. In the same experiment (double labelling) the 2-deoxyglucose uptake was also decreased by cycloheximide. The transport of 3-*O*-methylglucose in Expt. III (Table 1) was similarly decreased by long exposure to the inhibitor of protein synthesis. In addition to these observations, Table 1 shows that: (1) in Expt. I, in a double-labelling experiment, fed cells transported more 2-deoxyglucose (6.1 pmol/min per mg) than glucose (1.6 pmol/min per mg); (2) starved cells always transport sugars more rapidly than fed cells but the absolute values vary from experiment to experiment; (3) cycloheximide, when present during the assay (Expt. II), does not alter the effect of uptake regardless of the long preincubation condition (i.e. starved or fed with or without cycloheximide); and (4) paradoxically, there appears to be a concentration of cycloheximide (10 $\mu$g/ml) above which the inhibitor is less effective (Expt. I).

*Competition for sugar transport by glucose and its analogue*

Simple tests for the inhibition of the uptake or transport of 5 or 50 $\mu$M radioactive sugars in starved cells were done at two concentrations (1 and 10mM) of potentially competitive sugars. The results (Table 2) indicated that all the sugars tested were at least partially competitive with each other. However,

Table 2. *Competition for sugar transport and uptake in starved cells*

Chick-embryo cells were deprived of sugar for 18–24h as described under 'Methods'. The uptake of D-[$^{14}$C]glucose (4$\mu$M) or 2-deoxy-D-[$^{3}$H]glucose (5$\mu$M) was for 5min at 37°C and the transport of 3-*O*-methyl-D-[$^{3}$H]glucose (50$\mu$M) was for 15s at 37°C in the presence of unlabelled sugars as indicated. The average value from triplicate samples are shown±S.D. in parentheses.

| | | Sugar uptake | | | | Transport | |
|---|---|---|---|---|---|---|---|
| | | D-Glucose | | 2-Deoxy-D-glucose | | 3-*O*-Methyl-D-glucose | |
| Competing sugar | Concn. (mM) | (pmol/min per mg) | Inhibition (%) | (pmol/min per mg) | Inhibition (%) | (pmol/min per mg) | Inhibition (%) |
| None | — | 84.7 (±9) | 0 | 152 (±5.8) | 0 | 54.5 (±4.5) | 0 |
| D-Glucose | 1 | — | — | 7.65 (±0.17) | 95 | 25.2 (±3.8) | 54 |
| | 10 | — | — | 0 | 100 | 15.1 (±0.95) | 72 |
| 2-Deoxy-D-glucose | 1 | 51.5 (±5.5) | 39 | — | — | 22.9 (±3.53) | 58 |
| | 10 | 10.2 (±2.7) | 88 | — | — | 13.6 (±3.1) | 75 |
| 3-*O*-Methyl-D-glucose | 1 | 67.5 (±9.6) | 20 | 119 (±16.2) | 21 | — | — |
| | 10 | 25.7 (±1.6) | 70 | 69.6 (±5.4) | 54 | — | — |

only when glucose (1 and 10mM) competed with 2-deoxyglucose for uptake was inhibition greater than 90%. By comparison, both glucose and 2-deoxyglucose were, at best, only 75% effective in preventing 3-*O*-methylglucose transport. In these competition experiments, the concentration range of the radioactive sugars (5–10$\mu$M) was well below that where passive diffusion could be considered a significant factor in the apparent transport (Renner *et al.*, 1972; Kletzien & Perdue, 1974).

*Effects of thiol reagents*

Iodoacetamide and *p*-chloromercuribenzenesulphonic acid inhibited uptake of D-glucose and 2-deoxy-D-glucose in sugar-starved cells to various degrees. *N*-Ethylmaleimide was the most consistently effective reagent used to block the uptake of these two sugars (Fig. 3). These reagents did not decrease the uptake activities of starved cells to zero. Instead, 5–10% of the control activity (which corresponds roughly to the uptake in fed cells) appeared to be unaffected by the reagents. When this residual activity was subtracted and the results were presented as a semi-logarithmic plot (as shown in Fig. 3, inset), the apparent first-order inactivation gave nearly 100% on extrapolation suggesting a single-hit phenomenon (Lea, 1955). All of the inhibitions that did occur were prevented by the addition of a 10-fold molar excess of dithiothreitol to the thiol reagents before they were added to the cells. There was also no effect on the subsequent uptake of the sugars when the radioactive sugars were first preincubated with the thiol reagents for 5min at 37°C and then neutralized with dithiothreitol. Dithiothreitol alone had no effect on the sugar-uptake activities of sugar-starved and sugar-fed cells.

Occasionally, *N*-ethylmaleimide caused a decrease in the already low uptake by sugar-fed cells, but the resultant value was identical with that of the sugar-starved cells similarly treated with the reagent. The inhibition of the uptake capacity in the starved cells by *N*-ethylmaleimide (0.5mM) could not be altered by preincubation of the cells and reagent with high concentrations of glucose or 2-deoxyglucose (5mM). Hence no substrate protection of the sensitive site was observed.

*Chromatography and hexokinase activity*

Fig. 4 shows that both sugar-fed and sugar-starved cells converted glucose into at least four different metabolites resolved by descending paper chromatography. These were designated I, II, III and IV. Peaks of material occasionally appeared at the origin (Fig. 4*c*), but these varied from experiment to experiment and no attempt was made to characterize the metabolite(s). Markers, glucose and glucose 6-phosphate, had $R_F$ values of 0.58–0.61 and 0.38–0.41 respectively. Excluding the variable peak of material at the origin, the remaining peaks from fed cells had $R_F$ values 0.22 (I), 0.47 (II), 0.6 (III) and

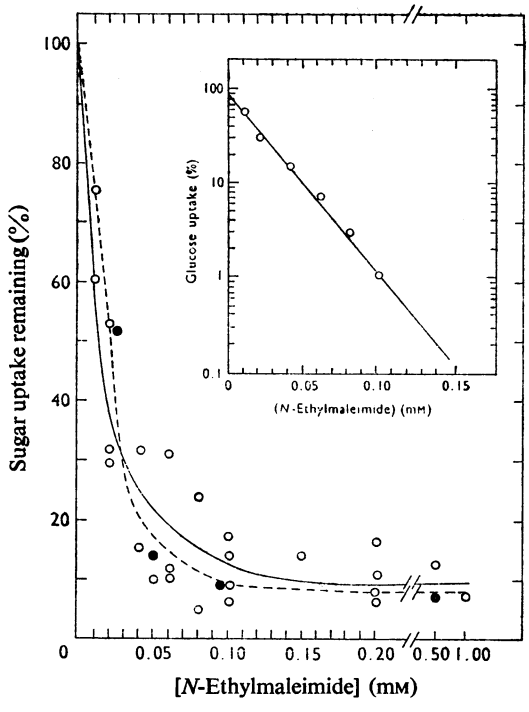


Fig. 3. *Effect of N-ethylmaleimide on the uptake of glucose and 2-deoxyglucose in starved cells*

After 18–24h in fresh medium containing no sugar, cells were washed with glucose-free Hanks' BSS and then incubated for 5min at 37°C in either 10mM-potassium phosphate-buffered glucose-free Hanks' BSS, or the same medium also containing 0.5mM-*N*-ethylmaleimide. The cells were twice washed with unbuffered glucose-free Hanks' BSS and then assayed for glucose (○) or 2-deoxyglucose (●) uptake. The details of the uptake assay are presented under 'Methods'. For the effect of *N*-ethylmaleimide on glucose uptake, a composite of four experiments is shown, one of which was plotted as a semi-logarithmic function and is shown in the inset. The inset shows the shape of the plot of the logarithm of the percentage activity versus concentration of *N*-ethylmaleimide after subtracting the activity that was refractory to *N*-ethylmaleimide.

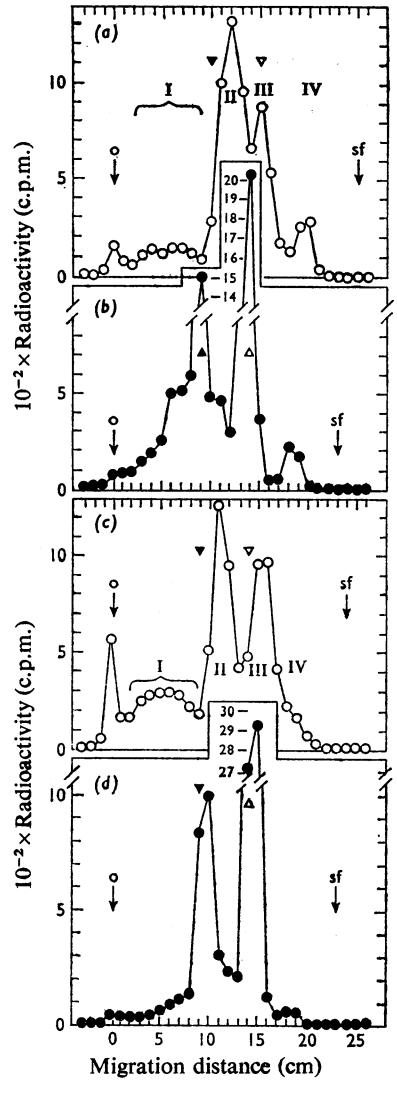


Fig. 4. *Chromatographic patterns of glucose metabolism*

After 18–24h in media containing 1mg of glucose/ml (*a* and *b*) or no sugar (*c* and *d*), cells were preincubated in 10mM-potassium phosphate-buffered glucose-free Hanks' BSS, (○) or the same medium also containing 0.5mM-*N*-ethylmaleimide (●). Uptake of 50μM-D-[$^{14}$C]glucose was for 15min at 37°C after the cells had been twice washed with unbuffered glucose-free Hanks' BSS. Equal amounts of the radioactivity in the acid-soluble extracts were spotted on the chromatographic paper. Markers, glucose (▽) and glucose 6-phosphate (▼) were spotted separately. Origin (o) and solvent front (sf) are indicated.

0.78 (IV) (Fig. 4*a*), and those from starved cells had $R_F$ values 0.21 (I), 0.47 (II), 0.65 (III) and 0.77 (IV) (Fig. 4*c*). Except for the amount of glucose converted (10 times more for starved cells), the only obvious difference between the chromatograms of fed and starved cells was in the migration of peak III. In addition, little, if any, glucose 6-phosphate accumulated within either starved or fed cells, suggesting rapid movement of glucose through that metabolic step and that hexokinase was not limiting or inhibited in either starved or fed cells.

When sugar-fed or sugar-starved cells were pre-treated with 0.5mM-*N*-ethylmaleimide and then allowed to take up glucose, they had nearly identical uptake rates and their soluble pools yielded fewer peaks (Fig. 4*b* and 4*d*). The majority of the radio-

activity migrated to the position of D-glucose ($R_F$ 0.6) and glucose 6-phosphate ($R_F$ 0.4) indicating that the sugar not only entered the cells but that the hexokinase remained active after the treatment with *N*-ethylmaleimide. However, the amount of glucose 6-phosphate relative to that of glucose varied from experiment to experiment. Thus comparative estimates of hexokinase activities *in vivo* with and without treatment with *N*-ethylmaleimide were complicated by the lack of measurable glucose 6-phosphate in the untreated cells and the variation in the ratio of glucose 6-phosphate/free glucose in the treated cells. In an effort to resolve the questions of whether or not hexokinase activities differed between fed and starved cells, the ATP-dependent hexokinase activities in homogenates of fed and starved cells were assayed. For conversions of either the D-glucose or 2-deoxyglucose, there were no detectable differences with time between the specific activities of the homogenates from fed or starved cells (Fig. 5).

*Kinetics of transport*

Kinetic analyses of the transport of the three sugars over concentration ranges from 5$\mu$M to 1.25mM by the double-reciprocal method of Lineweaver & Burk (1934) are shown in Fig. 6. The insets in Fig. 6 show the shapes of the plots at high sugar concentrations. These inset plots are best-fit extensions of the lines shown at the lower concentrations. They are included because they provide clear evidence for saturation kinetics through the highest substrate concentration used (i.e., within the 5$\mu$M–1.25mM concentration range, simple diffusion does not contribute significantly to the kinetic analysis). In addition, the inset of Fig. 6(*b*) clearly demonstrates the break in the curve, which is less evident on the full-range plot.

A comparison of open symbols in the plots of Fig. 6 show that differences in the transport of D-glucose, 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose exist. There appears to be a low-affinity transport system for all three sugars that have a $K_m$ of 1–3mM and this value is unaffected by sugar starvation. Starvation, however, causes an increase in the $V_{max.}$ for all three sugars: 3-*O*-methylglucose, 5–8-fold (Figs. 6*e* and 6*f*), 2-deoxyglucose, 2–3-fold (Figs. 6*c* and 6*d*), and glucose, 3–5-fold (Figs. 6*a* and 6*b*). In addition to the low-affinity system there is a second high-affinity system for D-glucose in sugar-fed and sugar-starved cells (Figs. 6*a* and 6*b*). Less obvious is a break in the plot for the transport of 2-deoxyglucose by the starved cells (Fig. 6*d*). The break occurs between 0.05 and 0.1mM (approx. 10–20mM$^{-1}$ on the plot). There is, however, no break in the plot for the transport of 2-deoxyglucose by fed cells (Fig. 6*c*). There are also no breaks in the plots for the transport of 3-*O*-methylglucose by either fed (Fig. 6*e*) or starved (Fig. 6*f*) cells.

Starved cells preincubated with 0.5mM-*N*-ethylmaleimide (closed symbols in Fig. 6) lost virtually all of the high-affinity transport system (Figs. 6*b* and 6*d*). There was also about a 30% loss of the high-affinity system for glucose transport in the sugar-fed cells (Fig. 6*a*). *N*-Ethylmaleimide had a less-severe effect on the transport of 2-deoxy-D-glucose in fed cells (Fig. 6*c*), but again primarily affected the high-affinity system for 2-deoxy-D-glucose (Fig. 6*d*) in the sugar-starved cells. The transport of 3-*O*-methyl-D-glucose (which is apparently without a higher-affinity system in either fed or starved cells) was also unaffected by *N*-ethylmaleimide.

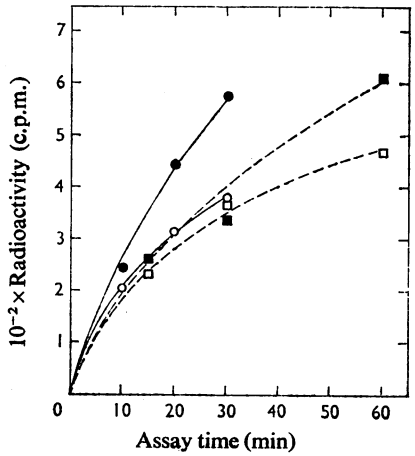


Fig. 5. *Hexokinase activity in vitro from fed and starved cells*

Cell-free, deoxycholate-treated homogenates were assayed for hexokinase activity as described under 'Methods'. ○, ●, Assay mixture containing 60$\mu$g of protein and 1mM-D-[$^{14}$C]glucose. □, ■, Assay mixture containing 180$\mu$g of protein and 1mM-2-deoxy-D-[$^{3}$H]glucose. ●, ■, Fed cells; ○, □, starved cells.

## Discussion

The starvation-dependent increase in the expression of a high-affinity glucose-transport system in chick cells appears similar in many respects to the de-repressible active-transport system demonstrated in *Neurospora crassa* (Scarborough, 1970*b*; Neville *et al.*, 1971). Both the chick and *Neurospora* high-affinity systems are inhibited by thiol reagents. They both appear gradually on the depletion of glucose from the medium and, in both cases, these changes can be blocked by cycloheximide. However, important major points of difference between the two systems are revealed in a comparison of the transport of 3-*O*-methylglucose. The non-phosphorylated sugar was concentrated as much as 100-fold over the

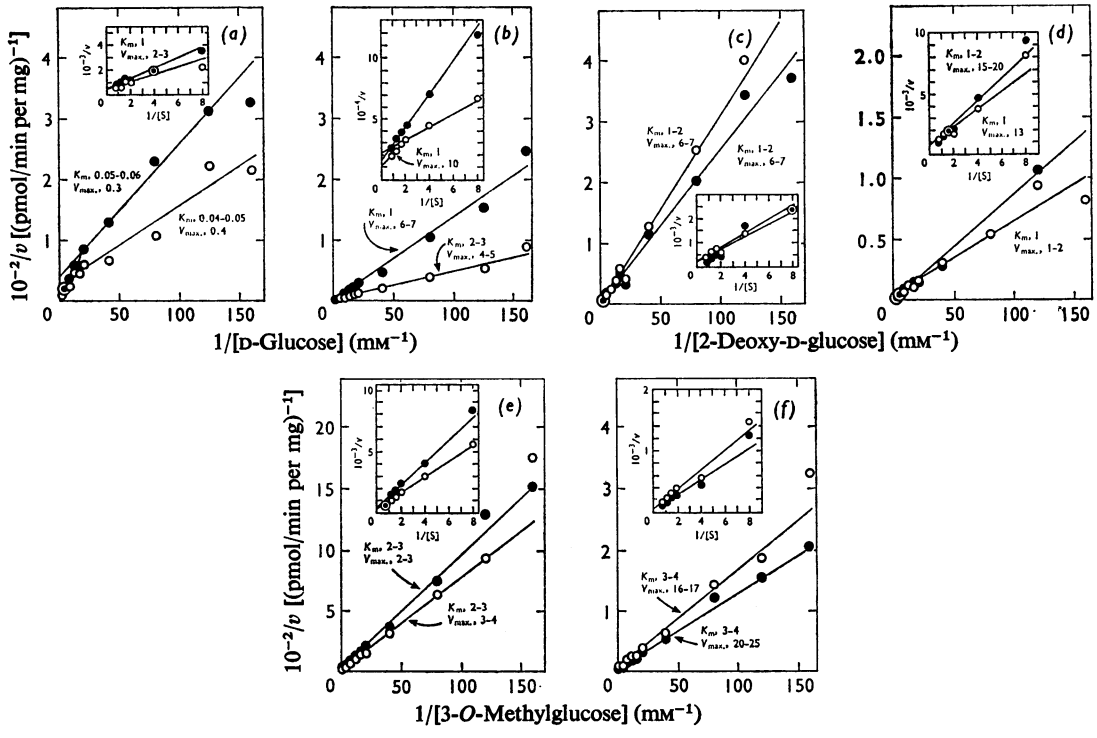


Fig. 6. *Effect of N-ethylmaleimide on the transport kinetics for* D-*glucose, 2-deoxy-*D-*glucose and 3-O-methyl-*D-*glucose*

After incubation for 18–24h in the presence of 5.5mM-glucose (*a*, *c* and *e*) or no sugar (*b*, *d* and *f*), washed cells were pre-incubated for 5min at 37°C in either 10mM-potassium phosphate-buffered glucose-free Hanks' BSS (○) or the same buffer also containing 0.5mM-*N*-ethylmaleimide (●). The preincubated cells were washed and then assayed for sugar transport (glucose, *a* and *b*; 2-deoxyglucose, *c* and *d*; 3-*O*-methylglucose, *e* and *f*) for 10s at 24°C. The concentrations of the sugars varied between 5 $\mu$M and 1.25 mM. Initiation of the transport assay was accomplished by pipetting 0.5ml of the pre-warmed assay media on to the cell monolayer which had also been warmed to 25°C. The reaction was stopped by aspiration of the assay medium immediately, followed by five washes with 5ml each of ice-cold wash solution and application of ice-cold 5% trichloroacetic acid. Radioactivity was counted as described under 'Methods'. Each point is the average of triplicate cultures. Radioactivity in c.p.m. per $\mu$g was converted into pmol/min per mg and plotted by the double-reciprocal method of Lineweaver & Burk (1934). Extensions of the plots in the concentration range 0.13–1.25 mM are shown in the insets. Michaelis constants ($K_m$) are expressed as mM; $V_{max.}$ values are expressed as nmol/min per mg. Slopes and axes intercepts were determined by a linear least-squares regression-analysis program (computerized) without weighted factors.

external medium by *Neurospora* (Neville *et al.*, 1971). Chick cells, however, did not concentrate the sugar, strongly suggesting that there is no active transport of sugars by these cells. In *Neurospora*, 3-*O*-methylglucose transport was inhibited by *N*-ethylmaleimide; the corresponding chick cell system was not inhibited by the reagent.

It might be argued that the higher-affinity system reflects altered hexokinase activity. However, Kletzien & Perdue (1975), using culture and uptake-assay conditions nearly identical with those described in the present paper, produced convincing evidence that hexokinase is not rate-limiting for sugar uptake when short assay times and low substrate concentrations are used (see Fig. 1 in Kletzien & Perdue, 1975). The same considerations apply for other enzymes in the metabolic pathways for glucose (Renner *et al.*, 1972). The low substrate concentrations (5 $\mu$M) and short assay times (10s) that we have used make it extremely unlikely that enzyme activities subsequent to transport could be measured. For these reasons and the fact that there was no significant difference between hexokinase activities *in vitro*, in fed and starved cells, it is unlikely that the margin of difference in uptake rates between fed and starved cells can be due to anything other than differences in transport rates.

The interpretation of kinetic data involving

transport is often difficult and, at best, requires certain assumptions that may not apply to carrier systems. The most critical assumption is that in studying a carrier system one is dealing with a situation analogous to a simple enzyme–substrate relationship, i.e. Michaelis kinetics. From this, it follows that a kinetic study must utilize initial-rate measurements and, in a multi-step process such as glucose metabolism in whole cells, the step of interest must be rate-limiting. It also follows that $K_m$ and $V_{max}$. data assume a straightforward analysis of enzymes essentially unmodified by substrate or product concentrations. These criteria are not easily satisfied, especially in experiments using whole cells. In fact, a carrier system (in a strict sense) is not an enzyme at all since it does not modify a substrate, but presumably binds it and moves it.

Non-metabolizable substrates have been considered good molecules for the transport measurements since they do not present problems that might arise through subsequent enzymic modification of the initial substrate. To minimize such complications, 2-deoxy-D-glucose and, more ideally, 3-*O*-methyl-D-glucose have become favoured substrates for determining the parameters of D-glucose transport. We have attempted to satisfy reservations about uptake and transport of D-glucose and its analogues 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose, in chick cells by using all three potential substrates for the carrier system(s) under strict assay conditions. Because of these studies, the interesting comparisons of the kinetic data (Fig. 6), as well as competition studies (Table 2), lead us to suggest that the facilitated diffusion of D-glucose (and perhaps 2-deoxy-D-glucose) is more complex than at first anticipated and certainly more complex than 3-*O*-methyl-D-glucose transport.

We have found that depriving cells of a sugar source for extended periods sharpens the contrast between D-glucose transport and the transport of its analogues. Even though modification of culture conditions in this way leads to increases in carrier activity, it is clear that more than one model can satisfy the data accumulated At least two distinct carrier-mediated systems could be involved. We have elected to place principal weight on the distinct high- and low-affinity systems observed when glucose is the substrate. That there are at least two systems is supported by the *N*-ethylmaleimide inhibition pattern, which results in blockage of the high-affinity ($K_m$ 0.05mM) system and has little or no effect on the low-affinity ($K_m$ 1mM) system. Essentially, *N*-ethylmaleimide decreases the capacity of the sugar-starved cell to that of the sugar-fed cells.

The transport of 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose presents an interesting variation of the generally accepted interpretation that these sugars are transported on the glucose-transport system as though it were a single unit. Indeed, reservations have been expressed by Mizel (1973) with respect to identity of handling of D-glucose and 2-deoxy-D-glucose. Our results show that 2-deoxy-D-glucose is recognized principally by the low-affinity system when glucose is provided and perhaps by both the high- and low-affinity systems when the cells are sugar-starved. The high-affinity recognition is, however, much less significant for 2-deoxy-D-glucose than for D-glucose. On the other hand, 3-*O*-methyl-D-glucose is virtually unrecognized by the high-affinity system. From the kinetic data (Fig. 6), the apparent $K_m$ for 2-deoxy-D-glucose and D-glucose transport was 1–2mM, and that for the carrier system that transports 3-*O*-methyl-D-glucose was 2–3mM.

In the competition experiments, the concentration range of the radioactive sugars (5–50$\mu$M) was well below that where passive diffusion could be considered a significant factor in the apparent transport (Renner *et al.*, 1972; Kletzien & Perdue, 1974). Therefore, unless there are separate transport systems or unless the substrates are either selecting or modifying components of the transport systems, it is difficult to explain the failure of either D-glucose or 2-deoxy-D-glucose (1–10mM) to compete successfully (only 55–75% inhibition; Table 2) with the transport of 50$\mu$M-3-*O*-methyl-D-glucose.

Our data show that at least two separate systems are available in chick cells for the transport of glucose. The low-affinity system has been described by a number of laboratories (Renner *et al.*, 1972; Mizel, 1973; Weber, 1973; Hatanaka, 1974; Kletzien & Perdue, 1974, 1975), but to our knowledge only the report by Mizel (1973) has indicated high-affinity sugar-transport systems in animal cells. The descriptions are made possible by using very low substrate concentrations, for only at concentrations below 0.1mM can saturation kinetics be demonstrated for the high-affinity system. This may explain why others, using higher substrate concentrations, have not been able to detect high-affinity systems. In addition, most glucose-transport studies have been done in well-fed cultures, where the predominant system is the low-affinity one. Moreover, 2-deoxyglucose has been the favourite indicator of glucose transport, and in our hands this glucose analogue is transported by fed cells primarily by the low-affinity system.

Over long periods of maintenance in culture, some cells may so deplete the medium of glucose that they effectively approach sugar starvation. Although starvation appears to derepress the high-affinity system for glucose, the mechanism of this control is not understood. Some form of feedback inhibition or modification of the transport molecules cannot be ruled out at this time. Likewise, the apparent preference for 2-deoxyglucose over glucose by the low-affinity system cannot be explained. These data suggest that a single system must have a preference for 2-deoxyglucose, or else two separate low-affinity

systems, each specific for one of the substrates, must exist.

Further speculation that cells (under the threat or stress of sugar starvation) would find a de-repressible high-affinity transport system advantageous are obvious and have been discussed by Scarborough (1970*b*). We have not attempted to provide an explanation for the differences in substrate specificity nor for the mechanism of de-repression. We have instead elected to describe these new findings and also to point out important reservations concerning the use of analogues as faithful indicators of events involving natural substrates. These studies serve as an interesting basis from which comparisons can be made. For example, the insulin-stimulated increase in the rate of glucose transport by chick cells (Shaw & Amos, 1973; Vaheri *et al.*, 1972) was due to changes in the low-affinity system. Characterization and studies on the regulation of transport by highly glycolytic transformed cells (Hatanaka, 1974) is also of obvious interest.

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