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Alteration of Glucose Consumption Kinetics with Progression of Baculovirus Infection in *Spodoptera frugiperda* Cells

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Abstract

We have used the initial-rate approach to characterize changes in the glucose consumption kinetics of baculovirus-infected *Spodoptera frugiperda* clone 9 (Sf9) cells with the progression of the infection process. The specific glucose consumption rate (q_G) of cultured baculovirus-infected Sf9 cells was measured at 4, 8, 12, 16, and 24 h postinfection (h.p.i.) in media containing 4–35 m*M* glucose. Higher medium glucose concentrations resulted in higher final extracellular virus and recombinant β-galactosidase yields. q_G was related to the extracellular glucose concentration by means of a Michaelis-Menten relationship. The apparent Michaelis-Menten constant (K_m) for glucose consumption was found not to change significantly during the progression of the infection process, and remained between 6.2 and 7.2 m*M*. However, the maximal specific glucose consumption rate (q_{Gmax}) was found to rapidly increase after infection, peaking at 16 h.p.i. at a value four times that for uninfected Sf9 cells. The kinetic analysis of glucose consumption rates in baculovirus-infected Sf9 cells presented here will aid in the optimal design and operation of bioreactor systems for the large-scale production of recombinant products from the baculovirus/insect cell system.

Index Entries: *Spodoptera frugiperda*; insect cells; glucose consumption kinetics; baculovirus; AcMNPV; Sf9.

Introduction

In recent years, cultured insect cells have become an attractive alternative means for the production of proteins of eukaryotic origin. Insect cells

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serve as hosts for a class of viruses—the baculoviruses—which can be used as vectors for expressing recombinant proteins in insect cells *(1)*. Large-scale industrial use of the insect cell–baculovirus system necessitates the development of efficient culture strategies. But for optimal design and control of bioreactors for the culture of baculovirus-infected cells, one requires quantitative descriptions of the metabolite consumption kinetics of the cells following infection. As with mammalian cells, glucose is the most important carbon source for cultured insect cells *(2)*. Most popular growth media for insect cells are based on Grace's medium *(3)* and contain several carbon sources: sugars such as glucose, fructose, and sucrose; organic acids such as fumarate, malate, succinate and α-ketoglutarate; and amino acids such as glutamine. We have earlier quantitatively characterized uninfected *Spodoptera frugiperda* clone 9 (Sf9) cells for consumption rates of glucose at various specific growth rates, by use of initial-rate measurements *(4)*. We have used the initial-rate approach here to characterize changes in the glucose consumption kinetics of baculovirus-infected Sf9 cells, with the progression of the infection process.

The first step in the metabolism of glucose is its transport into the cell. In mammalian cells, stereospecific facilitative transport of D-glucose is accomplished by the GLUT family of homologous proteins. This class of proteins includes at least six isoforms that are encoded by different genes, and exhibit distinct tissue distributions (reviewed in ref. *5*). The GLUT-1 protein is present in many tissues and immortalized cell lines, and is thought to be responsible for basal level glucose transport into cells. It is kinetically asymmetric with respect to transport of glucose in the two directions across the plasma membrane *(6)*. GLUT-2 is a low-affinity transporter located primarily in hepatocytes and pancreatic β-cells, and is involved in the regulation of glucose-stimulated insulin release from β-cells *(7)*. GLUT-3 is a high-affinity transporter responsible for glucose transport into neurons, GLUT-4 is expressed in the insulin-sensitive adipose and muscle tissues, GLUT-5 is a fructose transporter, and GLUT-7 is thought to be localized to the endoplasmic reticulum membrane (reviewed in ref. *8*).

Glucose transporter proteins have been identified in other cell types, including yeast, which share some sequence homology with mammalian glucose transporters (reviewed in ref. *9–11*), but work on insect cells has been limited. Wang and Wang *(12)* have characterized a stereospecific glucose transport system in the Kc cells of *Drosophila melanogaster*. On the basis of inhibitor studies, they have concluded that these cells contain glucose transporters similar to mammalian transporters. The recent discovery in insect cells of an insulin-related peptide, bombyxin, suggests that mechanisms for regulation of glucose uptake similar to those seen in mammalian cells may also be present in insect cells. Bombyxin has been identified in cells from the silkmoth *Bombyx mori*, and although its functions remain unclear, it is thought to target receptors on insect ovarian cells, including the Sf9 cells studied herein *(13,14)*. In addition, bombyxin has also been demonstrated to lower the concentration of the major blood sugar trehalose in the hemolymph of *B. mori (15)*.

Mammalian cells have long been known to respond to stresses such as heat shock and viral infection by upregulating glucose uptake *(16–20)*. Our study aims to characterize changes in glucose uptake kinetics in *S. frugiperda* cells during the course of infection with a recombinant baculovirus.

Materials and Methods

Cells and Media

Sf9 cells were a gift from Dr. M. D. Summers, Department of Entomology, Texas A&M University. Stock cultures were maintained at 27°C as a monolayer culture in TNM-FH medium *(21,22)* supplemented to a final concentration of 10% (v/v) with heat-treated fetal bovine serum (JRH Biosciences, Lenexa, KS). The cells were passaged every 2 to 3 d at a seeding density of 0.5×10^6 cells/mL. Heat treatment of the serum entailed incubation of thawed serum in a water bath at 56°C for 30 min. Antibiotics were not used, either for routine subculturing or during the experiments. The cells were counted on a Neubauer hemacytometer (Baxter, McGaw Park, IL), and cell viability was assayed by trypan blue (0.04%) exclusion. All chemicals used were purchased from Sigma (St. Louis, MO), except for yeastolate and lactalbumin hydrolysate (components of TNM-FH medium), which were purchased from Difco, Detroit, MI. Sf9 cells are a clonal isolate of IPLB-Sf21-AE cells, and to prevent too great a divergence of the cell population from the characteristics of the stock Sf9 cells, cells were used for 2 mo, after which they were discarded and fresh cultures were started from the frozen stocks.

Virus and Infection

A recombinant *Autographa californica* mononuclear polyhedrosis virus (βgal-AcMNPV), constructed by homologous recombination of the wild-type virus and the pVL941 vector encoding the *Escherichia coli lacZ* gene, was obtained from Dr. Summers. This vector expresses nonfused β-galactosidase in insect cells on their infection *(23)*. In all the experiments involving infected Sf9 cells described here, cells in exponential growth phase were infected to a multiplicity of infection (MOI) of 10 infectious particles per Sf9 cell. This allowed us to achieve near-synchronous infection of all the cells *(22)*, giving a homogeneous cell population on which to carry out our studies. Virus titer was performed by the end-point dilution method *(22,24)*. To achieve high virus titers in the stock virus solution, cells in early to midexponential phase were suspended in TNM-FH medium augmented to contain 14 m*M* glucose and 10 m*M* glutamine. This medium did not contain any fructose or any of the tricarboxylic acid (TCA) cycle intermediates. The cells were then infected with βgal-AcMNPV at an MOI of 1 PFU/cell using virus of the third passage and placed in 50-mL spinner flasks (Bellco, Vineland, NJ). Virus produced from several spinner flasks was combined to form a large stock that was used in all experiments.

Experimental Medium Formulations

Primary carbon sources present in TNM-FH include glucose, fructose, sucrose, organic acid intermediates of the TCA cycle, and glutamine. Sucrose is reported not to be consumed by Sf9 cells until levels of glucose fall extremely low *(25)*. For the purposes of our glucose consumption studies, fructose and the organic acids were eliminated from the medium. Glucose was added to the medium to the desired concentration (4–35 m*M*), and the osmolality of the medium was adjusted by altering the sucrose concentration concomitantly, as described elsewhere *(4)*.

Initial-Rate Experiment

Sf9 cells in exponential growth phase were suspended in 400 mL of fresh medium at a density of 8 to 9×10^5 cells/mL, and placed in four 100-mL spinner flasks, incubated at 27°C, and stirred at 100 rpm. Virus was added to an initial MOI of 10, in order to achieve synchronous infection of all the cells. At 4, 8, 12, 16, or 24 h postinfection (h.p.i.), the cell suspension was removed from the spinner flasks, and the cells were pelletized by centrifugation at 100*g* for 10 min. The cell pellets were combined and resuspended at seeding densities of $1.4–1.7 \times 10^6$ cells/mL in 200 mL of fresh medium containing the appropriate concentration of glucose. This time point was defined as "time zero" ($t = 0$) for the subsequent initial-rate experiment. The cell suspension was divided equally into four identical 50-mL spinner flasks, incubated at 27°C, and stirred at 100 rpm. Duplicate aliquots were removed from each spinner flask at $t = 0$ and immediately used for cell count and viability assays. Adequate oxygenation in the spinner flasks was ensured by pumping humidified air through the headspace of the flasks, as described elsewhere *(4)*. Aliquots were taken every few hours for 24 h past $t = 0$, for glucose and sucrose assays.

Calculation of Initial Glucose Consumption Rate

Aliquots of cell suspension taken at various times after $t = 0$ during the initial rate experiments were centrifuged at 1000*g* for 1 min to remove the cells, and the supernatant was frozen at –80°C for subsequent analysis. The frozen samples were later thawed and assayed using a YSI Model 27 Glucose Analyzer (Yellow Springs Instruments, Yellow Springs, OH). The analyzer employed 25-µL samples, and each measurement was repeated three to four times. For each experiment, the initial data points in a plot of [glucose] vs time were fitted to a first- or second-order polynomial function by linear regression (e.g., *see* Fig. 1). The specific glucose consumption rate (q_G) was calculated by normalizing the slope at $t = 0$ of the fitted function to the viable cell density at $t = 0$ assayed earlier.

Sucrose Assay

A YSI Model 27 Sucrose Analyzer was used to measure the sucrose concentration in aliquots of medium taken during the experiment. This

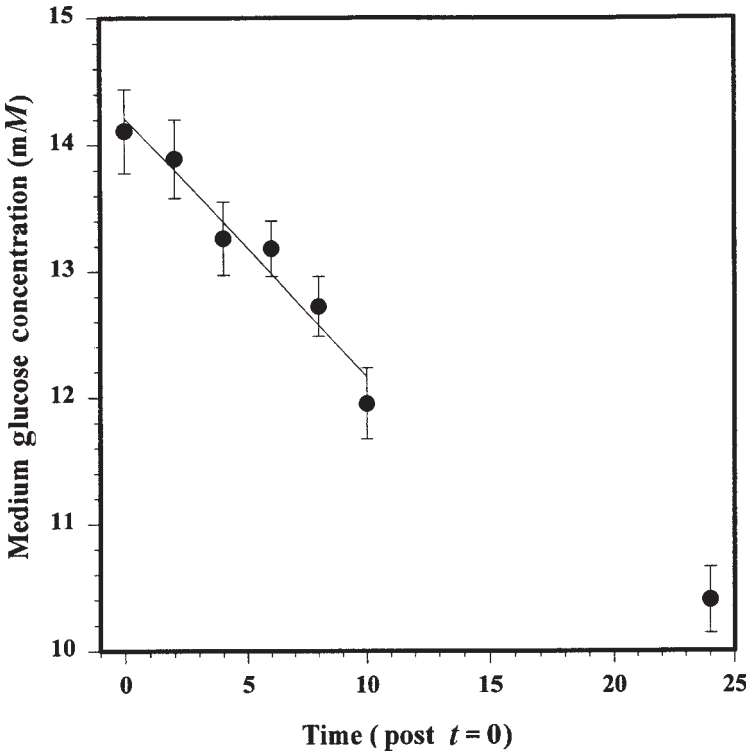

Fig. 1. Glucose disappearance from the medium during a batch incubation of βgal-AcMNPV-infected Sf9 cells in suspension culture. In this example, $t = 0$ corresponds to 4 h.p.i. Error bars represent 1 SD ($n = 3$ or 4). The slope of the fitted function was normalized to the viable cell density at $t = 0$, to obtain q_G under these conditions.

assay responds to both glucose and sucrose, with a sensitivity three times as high for glucose as for sucrose. The sucrose concentration was therefore calculated by subtracting three times the glucose concentration (measured independently) from the combined reading.

Calculation of Glucose Consumption Kinetics

At each time postinfection, q_G was seen to increase with increasing medium glucose concentration. Michaelis-Menten type saturation kinetics are often seen for carrier-mediated glucose transport in mammalian cells. Such kinetics might also be expected if metabolism, and not transport, is limiting for the glucose consumption rate in these cells. We attempted to relate q_G to the glucose concentration by means of saturation Michaelis-Menten type kinetics:

$$q_G = q_{Gmax} \cdot [\text{glucose}]/(K_m + [\text{glucose}]) \quad (1)$$

where q_{Gmax} is the maximum specific glucose consumption rate, [glucose] is the glucose concentration in the medium at $t = 0$, and K_m is the apparent Michaelis-Menten constant. In addition, the data were also fitted to an

equation of the following form, which includes a term for unsaturable passive diffusion:

$$q_G = q_{Gmax} \cdot [\text{glucose}]/(K_m + [\text{glucose}]) + P_D \cdot [\text{glucose}] \quad (2)$$

where P_D is the specific plasmalemmal permeability of glucose (a diffusion constant that includes information on the diffusive distance and surface area). The intracellular concentration of glucose is very low in most cells *(26)*, and the reverse flux of glucose out of the cell was ignored in Eq. 2. Values of q_G, calculated from the initial slopes of the [glucose] vs time plots for each time postinfection, were fitted to Eqs. 1 and 2 by nonlinear least-squares regression. Goodness-of-fit was estimated from the correlation coefficient, as well as by analysis of residuals, using the nonlinear parameter estimation program MINSQ (Micromath Scientific Software, Salt Lake City, UT).

Monolayer Experiments

For measurement of extracellular virus (ECV) yield per cell and total β-galactosidase yield per cell, Sf9 cells were incubated in 24-well plates at low cell densities ($\approx 3 \times 10^5$ cells/well) in media containing different concentrations of glucose. Cells in suspension in 50-mL spinner flasks were inoculated with virus at an MOI of 10 PFU/cell. After 5 min of stirring to allow mixing of the virus inoculum with the cell suspension, enough cell-virus suspension was transferred from the spinner flask to wells in a 24-well plate to give about 3×10^5 cells/well. The plate was then kept in the incubator for 3 h, to permit the viruses to attach to the cells, and the cells to attach to the wells. After the 3-h attachment period, the medium in each well of that plate was aspirated off and immediately replaced with 1 mL of the appropriate medium, and the plate was returned to the incubator. Four wells were used for each medium condition. At about 96 h.p.i. any attached cells in each well of each plate were dislodged by use of sterile Pasteur pipets, and the medium and cells from each well were collected in sterile microcentrifuge tubes. After vortexing the tubes in order to disrupt any intact cells, two tubes for each medium condition were stored at –80°C for subsequent analysis of β-galactosidase activity, and the other two tubes were stored at 4°C for assay of virus titer by end-point dilution.

β-*Galactosidase Assay*

β-Galactosidase activity was determined by a colorimetric assay based on cleaving *o*-nitrophenyl-galactopyranoside (ONPG) by β-galactosidase to form D-galactoside and *o*-nitrophenol. *O*-nitrophenol formation produces a characteristic yellow color, which was monitored spectrophotometrically as the change in absorbance of the solution at a wavelength of 420 nm. One unit of β-galactosidase was defined as the amount capable of cleaving 1 nmol of ONPG per minute at pH 7.0 and 27°C.

Results

A series of experiments was carried out to measure the initial specific rates of glucose consumption (q_G) of βgal-AcMNPV-infected Sf9 cells during batch incubations in media containing varying initial concentrations of glucose. The TNM-FH medium was modified to eliminate fructose and the organic acid intermediates of the TCA cycle, leaving only glucose and glutamine as potential primary carbon sources in the medium. Sucrose assays of aliquots taken at various points during the incubations showed that sucrose is not consumed by the infected Sf9 cells under these incubation conditions (data not shown). The range of glucose concentrations used, 4–35 m*M*, was chosen on the basis of previous experiments with uninfected Sf9 cells *(4)*, and included concentrations in the neighborhood of, as well as much higher than, the expected apparent K_m. Five sets of experiments were carried out, on cells at 4, 8, 12, 16, and 24 h.p.i., in order to investigate changes in glucose consumption kinetics during the infection process.

Figure 1 shows a sample calculation of the initial rate of glucose consumption from a plot of [glucose] vs time: the initial time points were fitted to a first-order function. The slope of the function was normalized to the viable cell density $t = 0$, to yield the value of q_G. The short-term (24-h) incubations of the βgal-AcMNPV-infected Sf9 cells were started with relatively high seeding densities. Consequently, there was a significant rate of disappearance of glucose from the medium at the early time points, permitting a reliable determination of the initial slope.

Figure 2 shows the variation in q_G with medium glucose content at 4, 8, 12, 16, and 24 h.p.i. Each data point represents the mean value, and error bars represent 1 standard deviation (SD), from four replicate experiments. The variation of q_G with medium glucose content may be expected to follow saturation Michaelis-Menten type kinetics if the rate of glucose utilization by the cells is limited either by the rate of glucose metabolism or by carrier-mediated uptake of glucose: this situation is described by Eq. 1. It is also possible that, at high extracellular glucose concentrations, glucose uptake may occur by a combination of facilitated and simple diffusion *(27–30)*. Thus, if glucose uptake into the cell is limiting for glucose consumption, and if significant passive diffusion also occurs, one may expect q_G to exhibit the behavior described by Eq. 2. The data shown in Fig. 2 were fitted to both Eqs. 1 and 2. Regression using Eq. 2 did not result in a satisfactory description of the data (not shown), but Eq. 1 was found to describe the data well. The regression results for all five sets of experiments are also shown in Fig. 2. Table 1 lists the values of the fitted parameters (mean ± SD) for each set of experiments. It can be seen from Table 1 that, as compared to uninfected Sf9 cells, the maximum specific rate of glucose consumption (q_{Gmax}) increases following infection. The increase is apparent as early as 4 h.p.i., and continues through to 16 h.p.i., after which there appears to be no further increase at 24 h.p.i. It can also be seen from Table 1 that during this period, there appears to be no significant change in the apparent Michaelis-Menten constant, K_m.

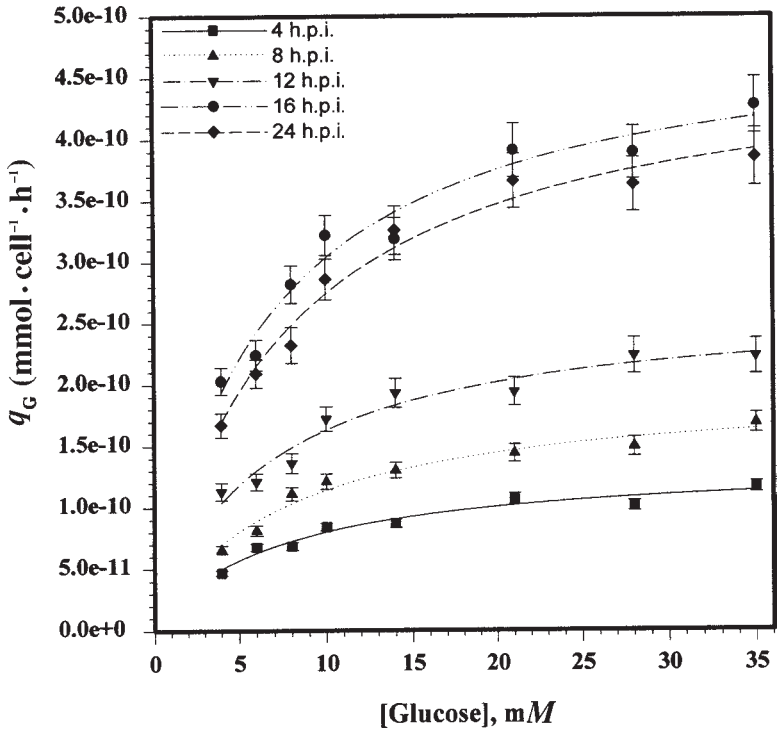

Fig. 2. q_G vs [glucose] at 4–24 h.p.i. Error bars represent 1 SD (n = 4). Fitted curves represent Eq. 1, with parameter values as in Table 1.

Table 1
Maximum Specific Rates of Glucose Consumption (q_{Gmax}) and Apparent Michaelis-Menten Constants for Glucose Consumption (K_m) in βgal-AcMNPV-Infected Sf9 Cells, at Various Times Postinfection[a]

Hours postinfection (h.p.i.)	q_{Gmax} (mmol · cell^{-1} · h^{-1}) ± SD (n = 4)	K_m (m*M*) ± SD (n = 4)
4	$(1.35 \pm 0.07) \times 10^{-10}$	6.8 ± 1.1
8	$(1.97 \pm 0.09) \times 10^{-10}$	7.3 ± 1.0
12	$(2.65 \pm 0.13) \times 10^{-10}$	6.2 ± 0.99
16	$(4.91 \pm 0.21) \times 10^{-10}$	6.2 ± 0.84
24	$(4.72 \pm 0.18) \times 10^{-10}$	7.2 ± 0.83
Uninfected Sf9 cells[a]	1.2×10^{-10}	7.1

[a]Also shown, for comparison, are the values for uninfected Sf9 cells reported elsewhere *(4)*.

Monolayer incubations of βgal-AcMNPV-infected Sf9 cells were carried out to measure specific yields of ECV and β-galactosidase at 96 h.p.i. As can be seen from Fig. 3, there is a significant increase in ECV yield on increase of the initial medium glucose concentration from 7 to 14 m*M*. Further increases in medium glucose concentration resulted in an increas-

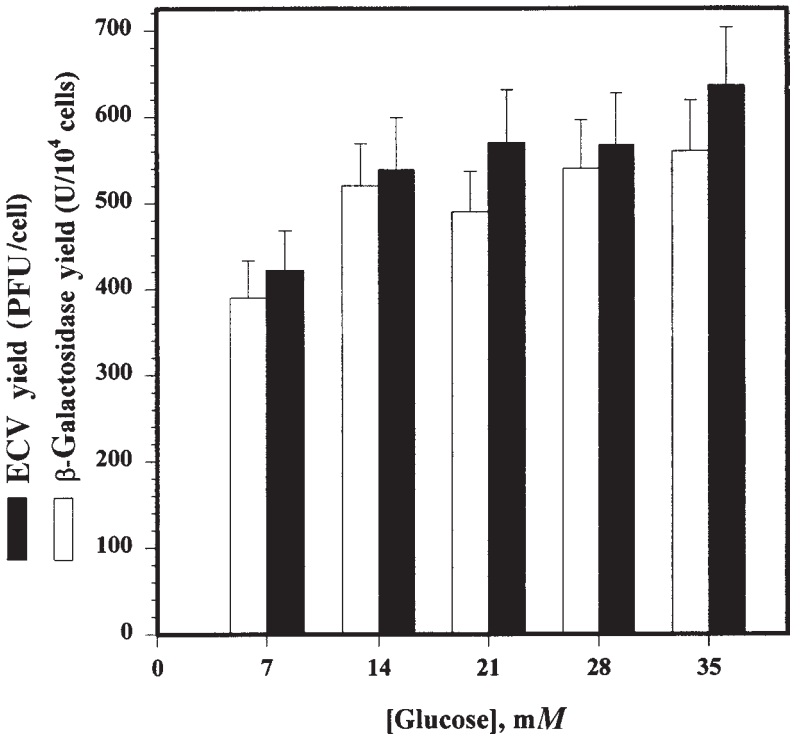

Fig. 3. Yields at 96 h.p.i. of β-galactosidase and extracellular virus particles from βgal-AcMNPV-infected Sf9 cells cultured in media containing 7, 14, 21, 28, or 35 m*M* glucose. Error bars represent the range of values from duplicate experiments.

ing trend in ECV yield, but this trend is not statistically significant. An increase in β-galactosidase yield on increase of the initial medium glucose concentration from 7 to 14 m*M* was observed, but further increases in medium glucose content did not result in greater increases in recombinant β-galactosidase yield (Fig. 3).

Discussion

Wang and Wang *(12)* have characterized a glucose transport system in the Kc cells of *D. melanogaster*, but other studies on glucose transport in insect cells are hard to find in the literature. Although studies on the glucose transport kinetics of baculovirus-infected insect cells are not to be found in the literature, mammalian cells have long been known to upregulate glucose uptake rates as part of a general response to stresses such as heat shock and viral infection. Gray et al. *(18)* have reported that baby hamster kidney (BHK) cells exhibit increased rates of glucose uptake in response to infection with vesicular stomatitis virus, Semliki Forest virus (SFV), and herpes simplex virus. They have also presented evidence that this increase in glucose uptake is the result of increased transport of glucose

into the cell, and not increased metabolism. Warren and Pasternak *(19)* have found that the response of BHK cells to stress (including viral infection) is mediated in a manner similar to the action of insulin, with three- to fivefold increases in the maximal rates of glucose transport (V_{max}), and little effect on the K_m. Insulin and viral infection have both been found to cause a reversible redistribution of glucose transporter molecules from intracellular stores to the plasma membrane, thus presumably increasing the V_{max}, but leaving the K_m for transport unaffected *(31–33)*. This is particularly interesting in light of the recent discovery of bombyxin in cells from the silkworm *B. mori*. Bombyxin is an insulin-related peptide that targets receptors on insect ovarian cells, including Sf9 cells *(13,14)*. Bombyxin has been shown to lower the hemolymph concentration of trehalose in *B. mori (15)*. This demonstrated function, as well as the homology between insulin and bombyxin, suggests a possible role for the peptide in the regulation of glucose transport into insect cells, such as an upregulation of glucose transport in response to stresses such as viral infections by recruitment of glucose transporter molecules from intracellular stores, in a manner similar to the action of insulin. In the current study, we have measured the kinetics of glucose consumption by Sf9 cells at various points during the infection process to look for such changes in glucose uptake kinetics.

We do not know whether the glucose consumption rate in these cells is transport limited or metabolism limited, and this needs to be addressed separately, possibly through the use of radiolabeled glucose analogs. It is possible that, as with virus-infected mammalian cells, the increase in q_G seen with the progress of infection in baculovirus-infected Sf9 cells results from an increase in the rate of glucose transport itself and not from an increased rate at some other "rate-limiting" step(s) in glucose metabolism. Such a change in transporter kinetics could potentially result from either new protein synthesis or a change in V_{max} or K_m of the putative glucose transporter. The stress-induced increase in hexose transport seen in mammalian cells is not dependent on new protein synthesis *(19,33)*. It remains to be seen whether this is also the case with infected insect cells. Increased glucose transport in transformed fibroblasts has been reported to occur as a result of a decrease in K_m for glucose of the transporter, arising out of altered *N*-glycosylation of the protein *(34)*. However, the K_m for glucose consumption in βgal-AcMNPV-infected Sf9 cells does not appear to change much during the course of the infection process (Table 1). There is, however, a significant increase in q_{Gmax} during the course of the infection (Table 1). It is possible that, as with infected mammalian cells, this increase in q_{Gmax} arises out of a translocation of glucose transporter molecules from intracellular stores to the cell membrane. From Table 1 we can see an increase in q_{Gmax} by 4 h.p.i., as compared to uninfected Sf9 cells. The value of q_{Gmax} continues to increase in the next few hours, apparently peaking at 16 h.p.i. Comparable data on other baculovirus-infected insect cell lines are unavailable in the literature, but a comparison with virus-infected mammalian cells is possible. The increase in glucose uptake rates following viral

infection have been reported to peak at between 12 and 15 h.p.i. in various mammalian cells *(16,18)*.

Batch incubations of βgal-AcMNPV-infected Sf9 cells reveal that the yield of recombinant β-galactosidase increases for an increase in medium glucose content from 7 to 14 m*M*, but further increases to 35 m*M* do not result in enhanced yields of β-galactosidase. Two-stage reactor systems have been employed for the production of recombinant products and baculovirus from insect cells, in either continuous mode *(35)* or repeated batch mode *(36)*. Our results suggest the use of media containing no more than 14 m*M* glucose for optimal yields of recombinant product from the AcMNPV/Sf9 system in batch reactor configurations. The optimal glucose concentration for use in continuous mode baculovirus/insect cell culture systems, with no buildup of metabolic waste products, remains to be determined. The kinetic analysis of glucose consumption rates in βgal-AcMNPV-infected Sf9 cells presented here will aid in the modeling and design of better bioreactors for the large-scale production of recombinant products from the baculovirus/insect cell system. Glucose is the primary substrate of insect cells, and the availability of quantitative information on glucose consumption kinetics will assist in the optimal operation of continuous bioreactor systems, as well as in the programming of optimal feeding schedules for fed-batch and repeated-batch bioreactor configurations.

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