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Effects of Glucose, Glutamine, and Malate on the Metabolism of *Spodoptera frugiperda* Clone 9 (Sf9) Cells

An Initial Rate Study

NATARAJAN RAGHUNAND AND BRUCE E. DALE*

Department of Chemical Engineering, Texas A&M University, College Station, TX 77843-3122; Current Address: Dept. of Chemical Engineering, Michigan State University, East Lansing, MI 48824-1226

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ABSTRACT

Optimal design and operation of bioreactors for insect cell culture is facilitated by functional relations providing quantitative information on cellular metabolite consumption kinetics, as well as on the specific cell growth rates (μ_G). Initial specific consumption rates of glucose, malate, and oxygen, and associated changes in μ_G, were measured for *Spodoptera frugiperda* clone 9 (Sf9) cells grown in batch suspension culture in medium containing 7–35 m*M* glucose, 0–16 m*M* malate, and 4–16 m*M* glutamine. The initial specific glucose consumption rate (q_G) could be described by a modified Michaelis-Menten equation treating malate as a "competitive" inhibitor (K_I = 6.5 m*M*) and glutamine as a "noncompetitive" inhibitor (K_I = 14 m*M*) of q_G, with a K_m of 7.1 m*M* for glucose. All three carbon sources were found to increase μ_G in a saturable manner, and a modified Monod equation was employed to describe this relationship (μ_{Gmax} = 0.047 h^{-1}). The initial specific oxygen consumption rate (q_{O2}) in Sf9 cells could be related to μ_G by the maintenance energy model, and it was calculated that, under typical culture conditions, about 15–20% of the cellular energy demand comes from functions not related to growth.

*Author to whom all correspondence and reprint requests should be addressed.

Fitted parameters in mathematical expression for μ_G: K_4, Monod constant for glucose (m*M*); K_5, modified Monod constant for malate (m*M*); K_6, Monod constant for glutamine (m*M*); m_{O2}, specific consumption rate of oxygen by the cells under zero-growth conditions (nmol/cell/h); q_F, initial specific fumarate production rate (nmol/cell/h); q_G, initial specific glucose consumption rate (nmol/cell/h); q_{Gmax}, maximum initial specific glucose consumption rate (nmol/cell/h); q_M, initial specific malate consumption rate (nmol/cell/h); q_{O2}, initial specific oxygen consumption rate (nmol/cell/h); Y_{O2}, cell yield on oxygen (cells/nmol); μ, initial specific cell growth rate (h^{-1}); μ_G, initial specific cell growth rate (h^{-1}); μ_{Gmax}, maximum initial specific cell growth rate (h^{-1}).

Index Entries: *Spodoptera frugiperda* clone 9 (Sf9); insect cell culture; metabolism; maintenance energy; TCA cycle.

Nomenclature: Fitted parameters in mathematical expression for q_G: K_1, Michaelis-Menten constant for glucose (m*M*); K_2, competitive inhibition constant for malate (m*M*); K_3, noncompetitive inhibition constant for glutamine (m*M*).

INTRODUCTION

In recent years, permanent insect cell lines have acquired commercial importance as vehicles for the production of recombinant proteins. Recombinant baculoviruses have been used to express more than 200 proteins of viral, bacterial, and mammalian origin in insect cells (*1*). Insect cells may also be used for production of the wild-type baculoviruses themselves, for use as potentially viable alternatives to chemical insecticides in the control of agricultural pests (*2*). A variety of factors affect the yield of recombinant proteins or baculoviruses from an insect cell culture system, including the construction of the baculovirus vector, the choice of host cell line, the composition of the culture medium, and the construction and manner of operation of the bioreactor used.

Most published work on recombinant protein production using the Baculovirus Expression Vector System (BEVS) has involved the use of recombinant *Autographa californica* nuclear polyhedrosis virus (AcMNPV) to infect *Spodoptera frugiperda* clone 9 (Sf9) cells. AcMNPV infects more than 50 cell lines of Lepidoptera (*3*), and the choice of host cell line is important. In recent years, many cell lines besides Sf9 have been tried as hosts, and the results have generally been dependent on the recombinant product being expressed (*4–6*). Wickham et al. (*6*) have identified a *Trichoplusia ni* cell line, BTI Tn5B1-4, as being much superior to cell lines from other insects in both volumetric and specific yields. This cell line exhibits contact inhibition in monolayer culture, and early attempts to grow these cells in suspension were not successful (*7*).

The earliest media employed for in vitro cultivation of insect tissue were modeled after the blood or other bodily fluids of the parent insect. Thus, in addition to several amino acids, vitamins, and salts, early culture media contained sucrose, glucose, fructose, α-ketoglutarate, succinate, fumarate, and malate: compounds still present in most popular medium formulations today (*8–12*). These different carbon and energy sources may have different levels of importance to the cell, and various studies have been conducted to identify the nutritional requirements of insect cells. Kamen et al. (*13*) have reported specific consumption rates of glucose, glutamine, and oxygen, and specific production rates of alanine and CO_2, for Sf9 cells at specific times during a batch culture. Ferrance et al. (*14*) have estimated carbon flux through many metabolic pathways involving all major carbohydrates and most amino acids in uninfected Sf9 cells grown in IPL-41 medium. Bedard et al. (*15*) have calculated the specific consumption rates for different sugars, organic acids, and amino acids, of Sf9 and BTI-EAA insect cells grown in various media. Reuveny et al. (*16*) have utilized medium formulations with glucose, sucrose, maltose, glycerol, lactate, or pyruvate as the primary carbohydrate, and have reported the resulting specific cell growth rates and β-galactosidase yields from Sf9 cell cultures. They have provided a quantitative relationship between glucose concentration and cell growth rate, thus allowing the programmed feeding of glucose at specific times during a batch culture.

These studies have shown that the nutrient content of the medium influences the yield of recombinant protein and cell growth rates one can obtain from a culture of insect cells. Quantitative relations linking medium composition to the behavior of both uninfected and infected insect cells are therefore needed. In this study, we have modified TNM-FH medium to contain varying amounts of glucose, glutamine, and malate. Fructose, fumarate, α-ketaglutarate, and succinate were eliminated in order to simply the analysis of the results. The medium thus had three different types of primary carbon sources available: a glycolytic substrate, glucose; an organic acid, malate; and an amino acid, glutamine. Initial metabolite consumption rates of Sf9 cells grown in medium containing different amounts of glucose, glutamine, and malate were measured, and mathematical relations linking these rates to the concentrations of glucose, malate, and glutamine have been developed. These equations, when solved in conjunction with bioreactor design equations, will permit mathematical simulations of different reactor configurations, enabling the development of better culturing strategies. This study should also aid in the development of more optimal medium formulations for Sf9 cell growth. The initial rate approach employed here suffers from some disadvantages when compared to chemostat studies, chiefly that any changes in cell behavior are owing to changes in the concentrations of several nutrients and end-products. Offsetting this disadvantage is the simplicity of these experiments, which allows us to investigate a much larger number of

medium formulations in a given amount of time than is possible with chemostat studies.

MATERIALS AND METHODS

Cells and Media

Spodoptera frugiperda clonal isolate 9 (Sf9) cells were a generous gift from M. D. Summers, Department of Entomology, Texas A&M University. Stock cultures were maintained at 27°C as a monolayer culture in TNM-FH (*9,17*) medium supplemented to a final concentration of 10% (v/v) with heat-treated fetal bovine serum (JRH Biosciences). The cells were passaged every 2–3 d to 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 an Neubauer Improved hemacytometer (Baxter, McGaw Park, IL), and viability assays were made by the trypan blue exclusion method (*18*); a final concentration of trypan blue of 0.04% was employed. All chemicals used were purchased from Sigma, 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 started from the frozen stocks.

The experimental medium formulations involved large changes in the concentrations of sucrose, glucose, malate, and glutamine (*vide infra*). It was therefore deemed necessary to acclimate the cells to these TNM-FH variations for at least 2 wk prior to beginning experiments. This was accomplished in the following manner: prior to being used in suspension culture experiments, unacclimatized Sf9 cells were passaged in 25-cm^2 tissue culture flasks in the desired medium for 2 wk, after which they were grown up in 50-mL spinner flasks in the same medium. This accomplished the dual objectives of acclimatizing the cells to suspension culture in the modified medium, and providing enough cells as inoculum for the upcoming experiment. Aliquots of the acclimated cells were frozen in their respective media and stored in liquid nitogen for future use, if needed.

Medium Composition

Before studying the carbon metabolism of Sf9 cells, the primary carbon sources present in TNM-FH were analyzed. Glucose, fructose, sucrose, and the organic acid intermediates of the tricarboxylic acid (TCA) cycle suggested themselves as possible candidates. Glutamine is also known to be an important source of energy for mammalian cells (*19*), and is possibly used by insect cells as well for energy (*15,20,21*). Sucrose is reported

to not be consumed by Sf9 cells until levels of glucose fall very low (*14*). In order to simplify the analysis, fructose was completely eliminated from all medium formulations employed in this work. In addition, malate was the only TCA cycle intermediate added to the medium. Thus, the media used in this work had three potential primary carbon sources: glucose, malate, and glutamine. The glucose levels in insect cell culture media typically range from about 4 m*M* (e.g., Grace's medium, which also contains 2.2 m*M* fructose) to about 28 m*M* (e.g., EX-CELL 400 medium). The levels of organic acids added to insect cell culture media range from none (e.g., TC-100 medium) to around 10 m*M* (e.g., Grace's medium). The glutamine levels in insect cell culture media range from about 4 m*M* (e.g., Grace's medium) to about 7 m*M* (e.g. IPL-41 medium). From preliminary studies, a minimum glucose concentration of 7 m*M* was found necessary to ensure adequate glucose levels at all times during a 4–5-d experiment. Based on this fact, and on the glucose, organic acid and glutamine concentrations typically found in insect cell culture media, experiments on the metabolism of Sf9 cells were conducted in variations of TNM-FH containing 7–35 m*M* glucose, 0–16 m*M* malate, and 4–16 m*M* glutamine.

Osmolality Compensation

Such wide variations in glucose, malate, and glutamine concentrations require adjustment of medium osmolality, in order to eliminate an additional variable in our analysis. Taking 1 L of medium as a basis, approximate compensation for osmolality changes was achieved by removing (adding) 1 mmol of sucrose for every millimole of "dissolved species" added to (removed from) normal TNM-FH. One millimole of glucose was taken to result in 1 mmol of dissolved species, since glucose is a nonionic solute. Ionic compounds glutamine and malate were taken to form two and three species, respectively, upon solvation in medium. In arriving at these numbers, the medium pH (6.2) was compared to the relevant pK_a values of glutamine and malate.

Medium Preparation

Sterile concentrated solutions of malate (850 m*M*, adjusted to pH 6.2 with KOH), glucose (2.33*M*), and sucrose (2.5*M*) were prepared in order to assist in medium preparation. Medium was usually prepared in 5-L batches; a weight of glutamine corresponding to the desired final concentration, and amounts of all other amino acids, vitamins, salts, yeastolate, and lactalbumin hydrolysate, corresponding to their normal concentrations in 4.5 L of TNM-FH, were added as per the procedure of Summers and Smith (*17*) to 4 L of deionized, distilled water. Malate, glucose, and sucrose were left out at this stage of medium preparation. After complete dissolution of all components, the pH of the medium was adjusted to 5.9 ± 0.05. The volume of the medium was adjusted with water to 4.2 L and the pH was adjusted to 5.9 again, if necessary. The desired final pH is

6.2, but the filtration process and the addition of serum both tend to raise the pH of the medium. It was discovered that adjusting the pH to 5.9 at this stage of medium preparation yielded a final pH close to the desired value. After sterile filtration, the medium was supplemented with 500 mL of FBS and stored in sterile bottles at 4°C. In preparing medium for a given experiment, the required volume of this incomplete medium was taken, and to it were added the stock solutions of malate, glucose, and sucrose, as well as the amount of sterile water which would yield the desired final concentrations of these three compounds in a volume of 5 L. A small sample of the complete medium was always set aside to confirm the final medium pH.

Suspension Culture

Cells in exponential growth phase which had been acclimatized to suspension culture in the appropriate medium were suspended in 100 mL of fresh medium to a density of 1×10^5 to 3×10^5 cells/mL, and divided equally into two identical 50-mL spinner flasks (Bellco) stirred at 100 rpm and incubated at 27°C. Oxygen transfer has been reported to be inadequate even in small flasks (*22*). In order to assure adequate oxygenation, the spinner flasks were modified as follows. Two hollow glass tubes (inner diameter ≈ 3 mm) holding disposable sterile 0.22 μm air filters (25 mm diameter Nalgene) at their outer ends, were passed through two holes drilled through the rubber stopper. One of these tubes extended into the flask to just above the surface of the medium. Humidified air was pumped through this tube by an air pump at a volumetric flowrate of about 50 cm^3/min to provide surface aeration (without bubbling). The air exited the flask through the other tube passing through the stopper. The connections between the tubes and the rubber stopper were airtight and there were no problems with culture contamination. The connectors were of an easily removable type so as to permit periodic transfer of the flasks to a sterile environment for sampling. For the purposes of our initial rate experiments, it was crucial that the cells be adequately oxygenated during at least the early stages of the experiment. Samples taken during the first 48 h of every experiment showed that the dissolved oxygen levels remained above 35% air-saturation during this period. Each experiment lasted 4–5 d, and all experiments were repeated twice.

Cell Counts and Specific Growth Rates

Cell counts and viability assays were done in triplicate, and the resulting cell growth plot was fitted to an exponential equation which was used to determine the initial specific cell growth rate (μ_G).

Glucose Consumption Rates

Aliquots of 1 mL were taken every 8–12 h during the incubations. The aliquots were centrifuged at 1000*g* for 1 min to remove the cells, and the

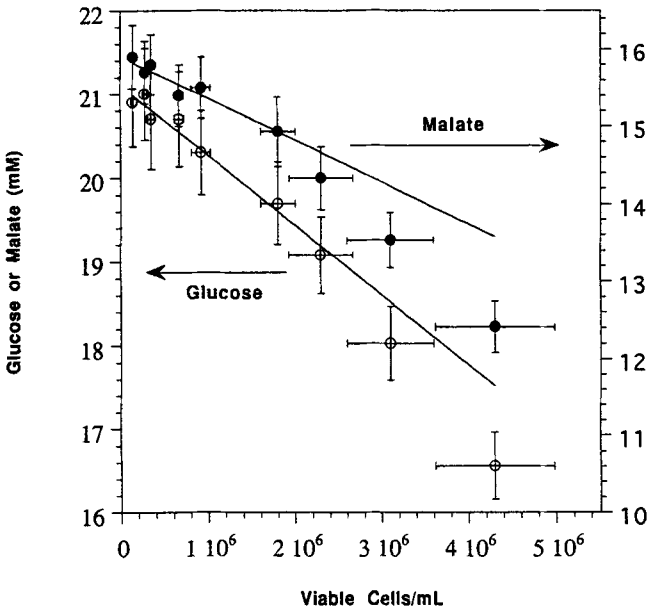

Fig. 1. Variation of glucose and malate concentrations with increase in viable cell density in medium with 10 m*M* glutamine. Y-error bars represent the range of duplicate measurements, X-error bars represent 2 SD from three viable cell counts.

supernatant was frozen at −80°C for subsequent analysis. The frozen samples were later thawed and asssayed using a YSI Model 27 Glucose Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH) employing 25-μL samples. Each measurement was repeated twice. The initial specific glucose consumption rate (q_G) of the cells was first evaluated by normalizing the initial slope of a glucose concentration vs time plot to the initial viable cell density. However, this technique was very sensitive to perturbations in the values of the early glucose concentration data points. As a result, q_G was instead calculated by multiplying the initial slope of a glucose concentration vs viable cell density plot, by μ_G (*23*). Figure 1 represents such a graph for glucose (and malate); the initial slope was calculated by fitting the data-points within the exponential growth regime to a straight line. The values of q_G, which are plotted in Figs. 2–4, have all been calculated in this fashion.

Sucrose Consumption Rates

It has been reported that insect cells do not consume sucrose unless the glucose concentration falls very low (*14,24,25*). However, large changes were made to the sucrose concentration present in the different medium formulations, and it was therefore necessary to monitor changes in medium sucrose concentration. A YSI 27 Sucrose Analyzer (Yellow Springs) was used for this purpose. This assay responds to both glucose and sucrose, with a sensitivity three times as high for glucose as for sucrose. The

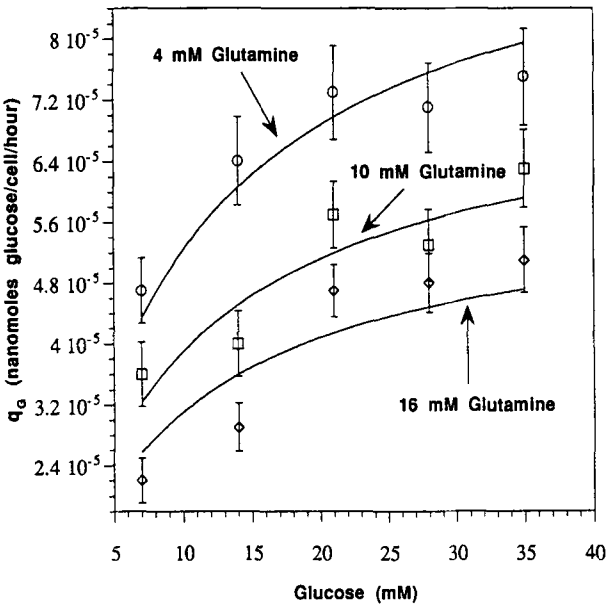

Fig. 2. Variation of initial specific glucose consumption rate of uninfected Sf9 cells with medium glucose and glutamine content; malate = 0 m*M*. Error bars represent the range of values from duplicate experiments.

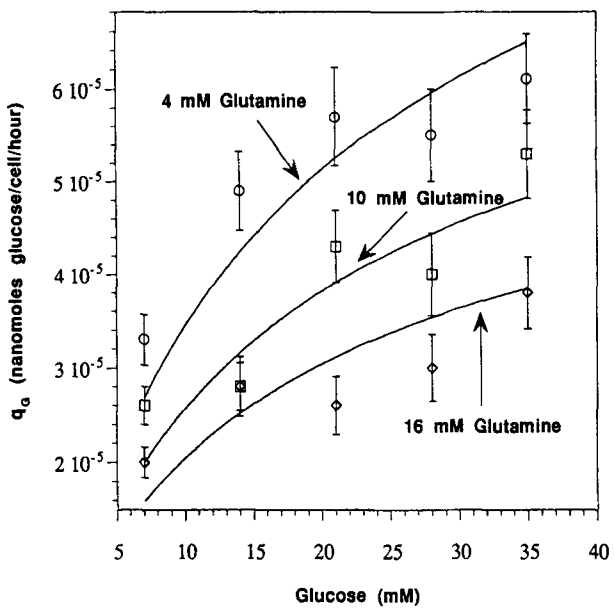

Fig. 3. Variation of initial specific glucose consumption rate of uninfected Sf9 cells with medium glucose and glutamine content; malate = 8 m*M*. Error bars represent the range of values from duplicate experiments.

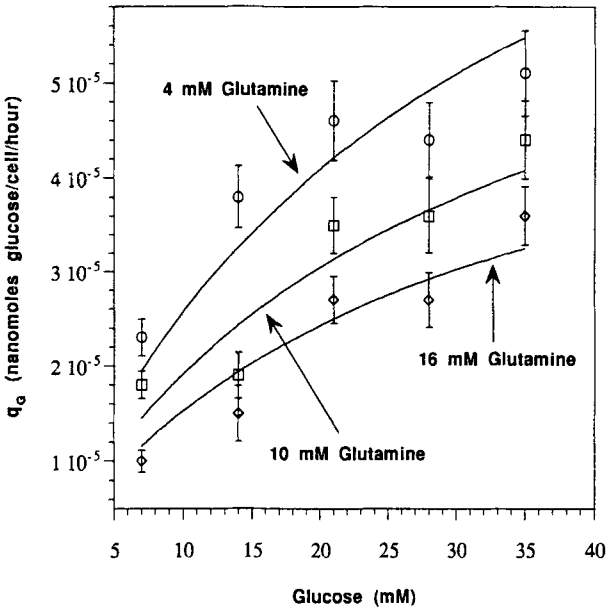

Fig. 4. Variation of initial specific glucose consumption rate of uninfected Sf9 cells with medium glucose and glutamine content; malate = 16 m*M*. Error bars represent the range of values from duplicate experiments.

sucrose concentration therefore had to be calculated by subtracting three times the glucose concentration (measured independently) from the combined reading.

Organic Acid Assays

The samples taken for glucose analysis were also analyzed for malate, fumarate, and α-ketoglutarate by HPLC on a Carbopac PA100 anion-exchange column (25 cm, Dionex). An LDC Analytical Constametric 3500 pump served as the solvent-delivery system, and detection was by an ultraviolet detector (Spectromonitor 3100, LDC Analytical) measuring absorbance at a wavelength of 210 nm. The mobile phase consisted of 11 m*M* NaOH prepared from a 50% (w/w) solution (Fisher) and 12 m*M* sodium sulfate (Fisher) in deionized, distilled water. A constant flowrate of 1 mL/min was employed for the isocratic elutions. A Spectra Physics AS-100 auto-injector was used to inject 5 μL of sample for each run, and each sample was analyzed twice. Peak identification and quantification were achieved using fructose-free TNM-FH (with 10% FBS) containing different known concentrations of malate, fumarate, and α-ketoglutarate.

Oxygen Consumption Measurements

Cells used to seed any given culture for the measurement of q_G were also used to start a higher density culture ($\approx 7 \times 10^5$ cells/mL) in parallel.

Samples of cell suspension were placed in the temperature-controlled sample chamber of a Biological Oxygen Monitor (YSI 5300, Yellow Springs) for specific oxygen consumption rate (q_{O2}) measurements. For each medium formulation, 2–4 measurements were made about 2 h after the start of the experiment. The average of these measurements was taken as the initial time value of q_{O2} for that medium formulation. The YSI Biological Oxygen Monitor measures changes in the dissolved oxygen content of the medium (owing to consumption by the cells) in units of percent air-saturation. To convert this measurement to one involving moles of oxygen, the oxygen content of air-saturated TNM-FH at 27°C was taken to be 246 μM (*26*). The reported q_{O2} values are directly proportional to this assumed value.

RESULTS AND DISCUSSION

Specific Glucose Uptake Rate

Initial values of q_G were calculated as described in the previous section, and the averages calculated from two replicates of each experiment are plotted in Figs. 2–4; the error bars in these figures represent the range of values obtained from the replicate experiments. Figure 2 shows the variation of q_G in medium containing 0 mM malate, as the concentrations of glucose and glutamine are varied. As might be expected, q_G increases with increased glucose content between 7 mM and 35 mM glucose, but we also see that increased glutamine concentration acts to depress q_G. Figure 3 reveals a similar pattern of q_G increasing with increased glucose concentration, but decreasing with increased glutamine. The values of q_G shown in Fig. 3 were measured in medium containing 8 mM of malate. A comparison with Fig. 2 shows that, in general, q_G values at 8 mM malate are lower than q_G values at 0 mM malate. Figure 4 displays q_G values measured in medium containing 16 mM malate; once again, q_G is increased by increased medium glucose content and decreased by increased medium glutamine concentrations. From Figs. 2–4, it is apparent that both malate and glutamine act to depress cellular glucose demand. Modified Michaelis-Menten equations treating malate and glutamine as either "competitive" or "noncompetitive" inhibitors of cellular glucose uptake were employed to describe the functional relationship between q_G and the concentrations of glucose, malate, and glutamine. Power-law and polynomial form equations were found to be unsatisfactory in their ability to describe the experimental results, yielding larger serial correlations in the residuals than the Michaelis-Menten form equations. Since the concentration of sucrose in the medium was found to not be altered appreciably during the course of the experiments (data not shown), sucrose was not included in the model for q_G. Of the models tested, the data were best described by an equation which treated malate as a competitive inhibitor of glucose consumption, and glutamine as a noncompetitive inhibitor:

Table 1
Values of Fitted Parameters (±1 SD) in Mathematical Expressions Chosen to Describe the Initial Metabolic Rates of Uninfected Sf9 Cells[a]

Equation number	Fitted parameters
(1)	$q_{G\max} = (1.2 \pm 0.1) \times 10^{-4}$ nmol/cell/h; $K_1 = 7.1 \pm 1.1$ m*M*; $K_2 = 6.5 \pm 0.98$ m*M*; $K_3 = 14 \pm 1.3$ m*M*; $r^2 = 0.96$
(2)	$\mu_{G\max} = 0.047 \pm 0.001\ h^{-1}$; $K_4 = 1.3 \pm 0.2$ m*M*; $K_5 = 0.04 \pm 0.01$ m*M*; $K_6 = 1.3 \pm 0.09$ m*M*; $r^2 = 0.94$
(3)	$Y_{O2} = 124$ cells/nmol O_2; $m_{O2} = 5.6 \times 10^{-5}$ nmol O_2/cell/h; $r^2 = 0.85$

[a]Values obtained from unweighted linear regression analyses.

$$q_G = (q_{G\max}\,\text{glucose}) / (\{\text{glucose} + K_1[1 + \text{malate} / K_2]\}\{1 + \text{glutamine} / K_3\}) \quad (1)$$

The solid lines in Figs. 2–4 are described in Eq. (1) with the values for the parameters that are reported in Table 1. The parameter values were determined from an unweighted least-squares fit using the nonlinear parameter estimation program MINSQ (Micromath Inc.).

Specific Cell Growth Rates

The specific growth rates calculated from the exponential part of the cell growth curves at different environmental conditions reveal that increasing concentrations of glucose, malate, or glutamine all tend to increase μ_G. Figure 5 displays the variation of initial specific cell growth rate in uninfected Sf9 cells with changes in the concentrations of glucose and glutamine. The concentration of malate in the experiments depicted in Fig. 5 is 0 m*M*. It is apparent that an increase in extracellular glucose from 7 m*M* to 35 m*M* leads to an increase in μ_G. A similar effect is seen with an increase in the extracellular concentration of glutamine from 4 m*M* to 16 m*M*. Figures 6 and 7 show the variation of μ_G with medium glucose and glutamine content, at other malate concentrations. Once again, both glucose and glutamine act to increase μ_G in a saturable manner. Also, a comparison across Figs. 5–7 shows that the effect of malate in altering μ_G is very small. A modified Monod equation of the form of Eq. (2) was found to describe the data better than power-law form and polynomial form equations, and was adopted to describe the relationship of μ_G to the initial extracellular concentrations of glucose, malate, and glutamine. Since the cells were able to grow in the absence of malate, the term involving malate is different

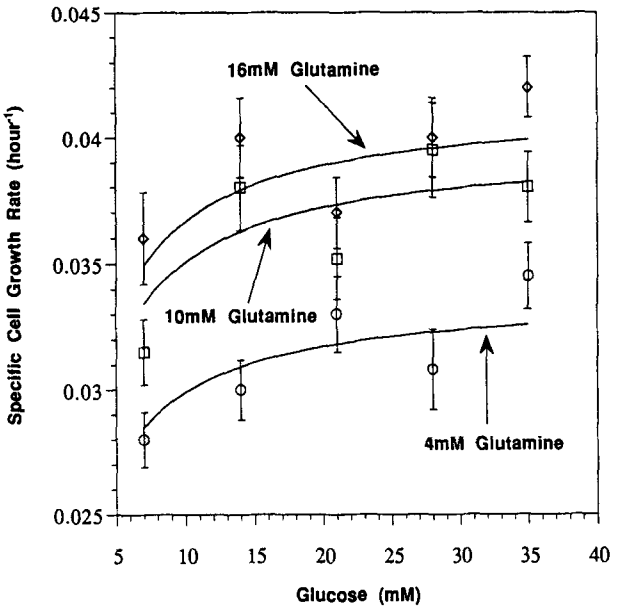

Fig. 5. Initial specific cell growth rate vs glucose and glutamine content; malate = 0 m*M*. Error bars represent the range of values from duplicate experiments.

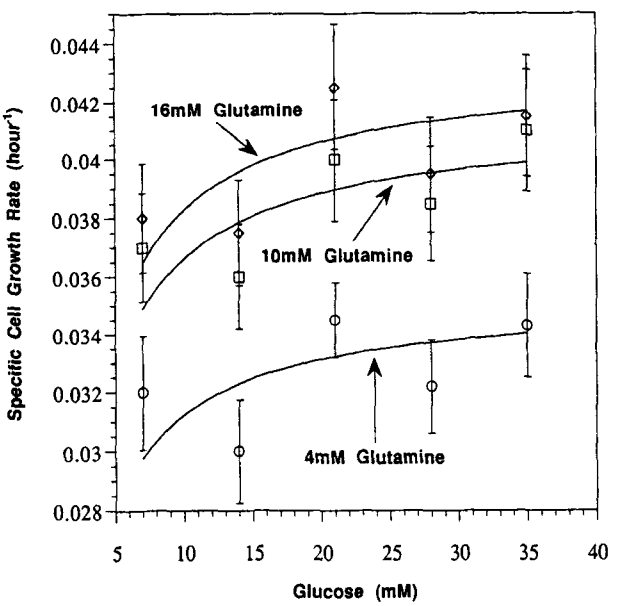

Fig. 6. Initial specific cell growth rate vs glucose and glutamine content; malate = 8 m*M*. Error bars represent the range of values from duplicate experiments.

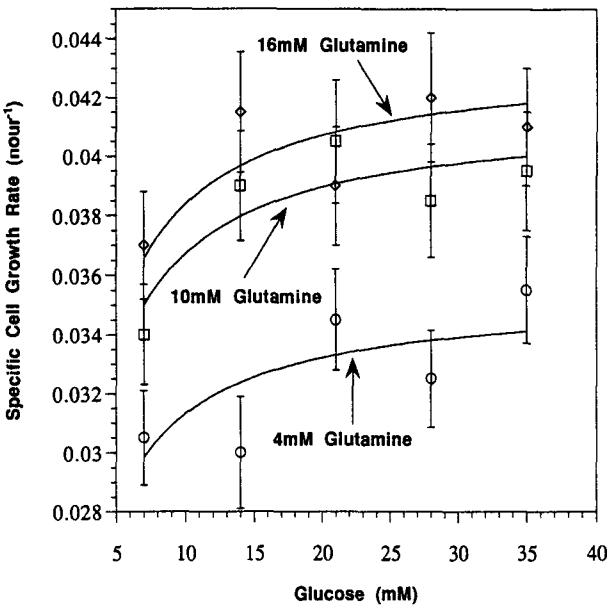

Fig. 7. Initial specific cell growth rate vs glucose and glutamine content; malate = 16 m*M*. Error bars represent the range of values from duplicate experiments.

from the terms involving glucose or glutamine. The parameter values used to fit this model are listed in Table 1.

$$\mu_G = \mu_{Gmax}\,(\text{glucose} / \{K_4 + \text{glucose}\})$$
$$(\{1 + \text{malate}\} / \{1 + \text{malate} + K_5\})\,(\text{glutamine} / \{K_6 + \text{glutamine}\}) \quad (2)$$

The solid lines in Figs. 5–7 are described in Eq. (2).

Specific Oxygen Consumption Rate

According to the maintenance energy model (27), the energy needs of cells may be divided into energy needed for growth, and a certain amount of "maintenance energy" which is required for nongrowth related processes. Thus, applying the model to the initial specific oxygen consumption rates of Sf9 cells:

$$q_{O2} = (\mu_G / Y_{O2}) + m_{O2} \quad (3)$$

Table 1 lists the values of Y_{O2} and m_{O2} obtained from a linear regression of the data shown in Fig. 8. The dashed line in Fig. 8 is represented by Eq. (3), with a specific yield on oxygen (Y_{O2}) of about 124 cells/nmol of oxygen, while m_{O2} is 5.6×10^{-5} nmol O_2/cell/h. This value of m_{O2} indicates that, in medium formulations normally employed for Sf9 cell culture ($\mu_G \approx 0.031 - 0.036$), about 15–20% of the energy generated by the cell goes toward maintenance functions, and the rest is spent on growth.

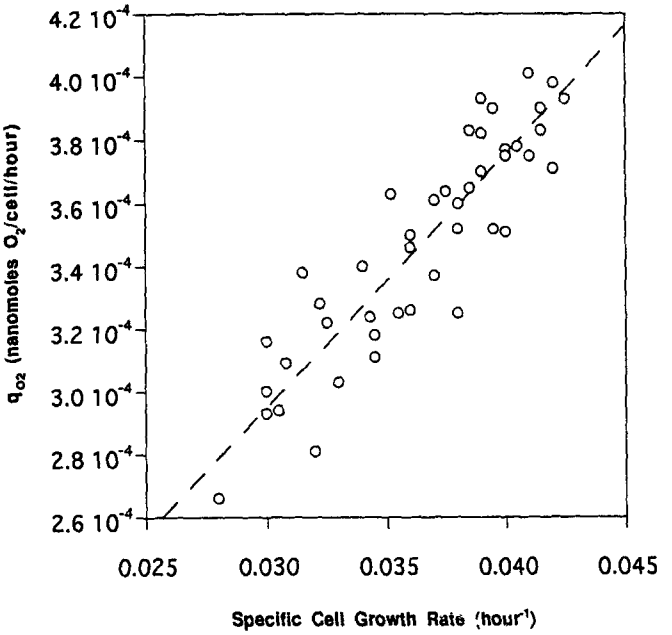

Fig. 8. The maintenance energy model applied to uninfected Sf9 cells.

Fates of Malate

The initial specific consumption rate (q_M) of malate ranged from zero to about 4 × 10^{-5} nmol/cell/h, depending on the concentrations of malate, glucose, and glutamine in the medium. Increases in the extracellular concentration of malate led to significant increases in q_M, whereas increases in the concentrations of both glucose and glutamine led to sharp reductions in the malate consumption rate of the Sf9 cells. These results will be presented in detail in a subsequent manuscript (in preparation). Malate and other organic acids were originally added to insect cell culture media because these compounds were detected in insect hemolymph. In this context, it is possible that some organic acids are produced and excreted by insect cells in culture. Ferrance et al. (*14*) have reported that Sf9 cells grown in IPL-41 medium (which contains only low concentrations of TCA cycle organic acids) produce fumarate and α-ketoglutarate.

In our experiments, fumarate was produced in significant amounts, with the initial specific production rate (q_F) being dependent on the extracellular concentrations of malate and glutamine. Preliminary data indicate that the glucose content of the medium has little effect on q_F, but increases in the malate concentration in the medium led to sharp increases in q_F. Increases in the glutamine content of the medium had a similar, but smaller, effect on q_F (manuscript in preparation). It thus appears that one of the fates of malate in Sf9 cell metabolism is conversion to fumarate. We have not observed the production of α-ketoglutarate under the conditions studied.

Other possible fates of malate include the conversion to pyruvate, and from pyruvate, to acetyl CoA (AcCoA). The conversion of malate to pyruvate requires the presence of malic enzyme activity in the Sf9 cell cytosol. The AcCoA produced from the resulting pyruvate may enter the TCA cycle (Fig. 9), leading to the production of CO_2, NADH, and $FADH_2$. *Spodoptera frugiperda* cells are reported to synthesize and excrete lipids (*28*). Thus, any AcCoA resulting from malate may also be used by the Sf9 cell to manufacture lipids.

Glucose may be used by the cells for energy production via the TCA cycle, or for production of precursors for nucleic acid synthesis and reducing equivalents in the form of NADPH, through the pentose-phosphate pathway. Since uptake of glucose is competitively inhibited by malate, it is possible that Sf9 cells utilize malate for one or more of the same purposes as glucose, such as energy production via the TCA cycle. The relatively small effect of malate on μ_G indicates that malate is probably not useful to the cell for biosynthesis purposes.

Fates of Glutamine

Glutamine consumption rates in Sf9 cells were not monitored. However, the non-competitive type inhibition of glucose uptake by glutamine indicates that Sf9 cells have needs for glutamine which are not fulfilled by glucose. These needs may involve the supply of nitrogen for nucleic acid and protein synthesis. This is consistent with the observed positive effect of glutamine on the specific cell growth rate. Glutamine may also enter the TCA cycle (Fig. 9), from where it may undergo fates similar to those discussed for malate earlier.

CONCLUSIONS

In conclusion, metabolic quotients in Sf9 cells have been measured as a function of the concentrations of three carbon sources: glucose, malate, and glutamine. Mathematical relations have been developed which describe cell behavior at different concentrations of these nutrients in the medium. These relations will help determine optimal schedules for programmed feeding of nutrients for Sf9 cell culture when used in conjunction with design equations for fed-batch or repeated-batch bioreactor configurations. A modified Monod model was used to describe cell growth at different concentrations of these three carbon sources. Values of the "Monod constants" for glucose and glutamine (both 1.3 m*M*) were found to be lower than, but of the same order of magnitude as, the concentrations of these two compounds in common insect cell culture media. The value of the malate-dependent constant in Eq. (2) (0.04 m*M*) is very low compared to the concentration of malate in TNM-FH medium. Even taking into account the difference between the malate-dependent term in

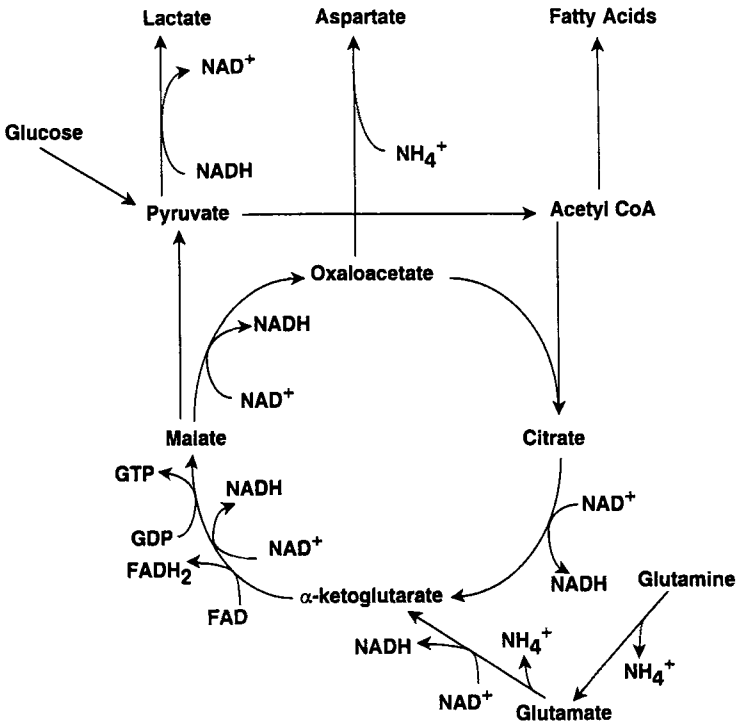

Fig. 9. Carbohydrate metabolism via the tricarboxylic acid cycle.

Eq. (2), and the terms for glucose and glutamine, this indicates that malate does not contribute significantly to cell growth. Increasing the glutamine and glucose concentrations, at the expense of some of the sucrose, would thus be a more efficient way to enhance cell yields on the medium. The cells appear to follow the maintenance energy model when q_{O2} is plotted against μ_G, and this may permit the estimation of cell growth rates from on-line measurements of oxygen uptake rates when such measurements become routinely possible.

From the results presented here, it is recommended that higher glucose concentrations (in the range of 35 m*M*) and higher glutamine concentrations (in the range of 16 m*M*) than commonly employed in commercial insect cell culture media, be used for growing up Sf9 cells. Glutamine metabolism results in the extrusion of ammonium by insect cells (*21*), and one might expect this to cause some problems in high-density cultures when using media with high concentrations of glutamine. However, Sf9 cells have been reported to exhibit only slight decreases in cell growth in the presence of ammonium ion concentrations up to 10 m*M* (*15*). Increased malate also improves cell growth, but only very slightly, and it may be better to leave malate out of Sf9 cell culture media. Since fumarate is actually excreted by the cells, it is not likely that fumarate is an essential nutrient for cultured Sf9 cells. It is not known at this time how the results obtained with malate and fumarate apply to the use of succinate and α-ketoglutarate in insect cell culture media

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