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The Effect of Alternative Carbohydrates on the Growth and Antibody Production of a Murine Hybridoma

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ABSTRACT

A murine hybridoma (CC9C10) was adapted to grow in media containing alternative carbohydrates to glucose. Cell yields relative to the glucose-based culture decreased in order of the following supplements: glucose = maltose > galactose > fructose = sorbitol = xylitol, although significant yields (>50% of glucose control) were observed in all cultures. In the absence of glucose, glutamine consumption rates were enhanced significantly. Antibody production was directly related to the viable cell concentration in each culture and was independent of the phase of culture. A high specific antibody productivity (q_{Mab}) was observed in the cultures containing the polyols, sorbitol, or xylitol, even though the cell yields and growth rates were lower than the glucose-based control. The measured q_{Mab} in the xylitol culture was 5.6× that of the glucose culture and the volumetric yield of MAb was 29% higher.

Index Entries: Hybridoma; MAb; carbohydrates; sorbitol; xylitol; lactate; glutamine.

INTRODUCTION

There have been many reports that mammalian cell lines can grow in media supplemented with carbohydrates other than glucose (*1–4*). In particular, the monosaccharides, mannose, fructose, and galactose, and the

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disaccharides, maltose, trehalose, and turanose, have been shown to support the growth of a variety of cell lines.

The polyols (sugar alcohols), xylitol and sorbitol, can replace glucose and support the growth of a number of cell lines, including human diploid fibroblasts (*5–6*), although this was not substantiated in all mammalian cells (*2*).

The type of carbohydrate present in the growth medium can affect the metabolism of cultured cells. Fructose or maltose caused a decrease in production of lactate and a decreased drift in pH in MDCK cell cultures (*4*). This can be an advantage in the process control of large-scale mammalian cell cultures. Substantial increases of intracellular ribose have been reported following the presence of fructose, xylitol, D-xylulose, or tagatose in the cultures of rat hepatocytes (*7*).

MAb form part of a long list of products that are now produced routinely from mammalian cell cultures and that require process control during large-scale operations. In such operations, the role of media components in culture stability and cell productivity is important. With this in mind, we report a study of the use of alternative carbohydrates in the culture of an antibody-secreting murine hybridoma.

MATERIALS AND METHODS

Cell Line

The murine B-lymphocyte hybridoma (CC9C10), which secretes an MAb against insulin, was obtained from the American Type Culture Collection (*8*). The cells were shown to be mycoplasma-free by routine testing in an independent laboratory (Rh Pharmaceuticals).

Culture

The cells were grown in stationary suspension cultures containing 100 mL RPMI 1640 (Gibco) supplemented with 10% iron-enriched calf serum (Gibco). The basal medium was obtained glucose-free and glutamine-free. This was supplemented with glucose or an alternative carbohydrate as specified at 20 m*M* and glutamine at 2 m*M*. The glutamine was added to the media immediately before inoculation. The cultures were incubated in 150-cm^2 T-flasks (Corning) in an incubator at 37°C and under an overlay of 5% CO_2.

Cell Counting

Viable cell concentrations were determined by counting a cell suspension diluted 1:1 v/v with 0.2% trypan blue using a Neubauer hemocytometer. To ensure statistical validity, a minimum of 200 cells were counted in each sample.

Cell Adaptation

The cells were adapted for growth in each medium over 12 passages. Cultures at passage one contained 20 m*M* glucose and 20 m*M* of the substitute carbohydrate. The glucose concentration was reduced to zero by incremental changes of 5 m*M* over 10 passages. Cells were grown for two passages without glucose, but in the presence of a substitute carbohydrate before conducting the experiments.

Analysis of Culture Media

Lactate and glucose concentrations were determined using a specific analyzer (Model 27, YSI). Maltose was determined as glucose following hydrolysis by serum. The following were determined by standard spectrophotometric assays adapted for use in a multiwell plate reader (Theromax, Molecular Devices): sorbitol (*9*), xylitol (*10*), galactose (*11*), fructose (*12*), glutamine (*13*).

MAb

MAb concentrations were determined by an indirect enzyme-linked immunosorbent assay (ELISA) specific for murine immunoglobulins (IgG), modified from a previously described method (*14*). Assay plates (96-well; Nunc) were coated with 100 μL goat antimouse IgG at 1/1000 dilution (Sigma, St. Louis, Mo). Plates then were washed with phosphate-buffered saline (PBS), pH 7.2, and blocked with 1% bovine serum albumin (BSA). Duplicate wells were incubated with 100 μL of culture supernatant or Ig standard for 1 h at 37°C. The wells were then washed with PBS containing 0.1% BSA (PBS-BSA). Each well then received 100 μL alkaline phosphatase-conjugated antimouse IgG (Sigma) diluted (1/2000) in PBS-BSA and incubated for 30 min at 37°C. *p*-Nitrophenyl phosphate (100 μg) in substrate buffer (100 m*M* glycine, 1 m*M* $MgCl_2 \cdot 6H_2O$, 1 m*M* $ZnCl_2$, pH 10.4) was added to each well. The plates were incubated for 30 min at 37°C in a Thermomax plate reader (Molecular Devices). The increase in absorbance was measured at 405 nm, and the kinetic data were analyzed by associated software (Softmax).

RESULTS

Cell Growth and Adaptation

The anti-insulin secreting CC9C10 murine hybridomas were adapted to cultures containing various carbohydrate sources following a period of continuous growth in glucose-based media. The adaptation process involved the addition of an alternative carbohydrate followed by 12 culture passages during which the glucose concentration was gradually reduced

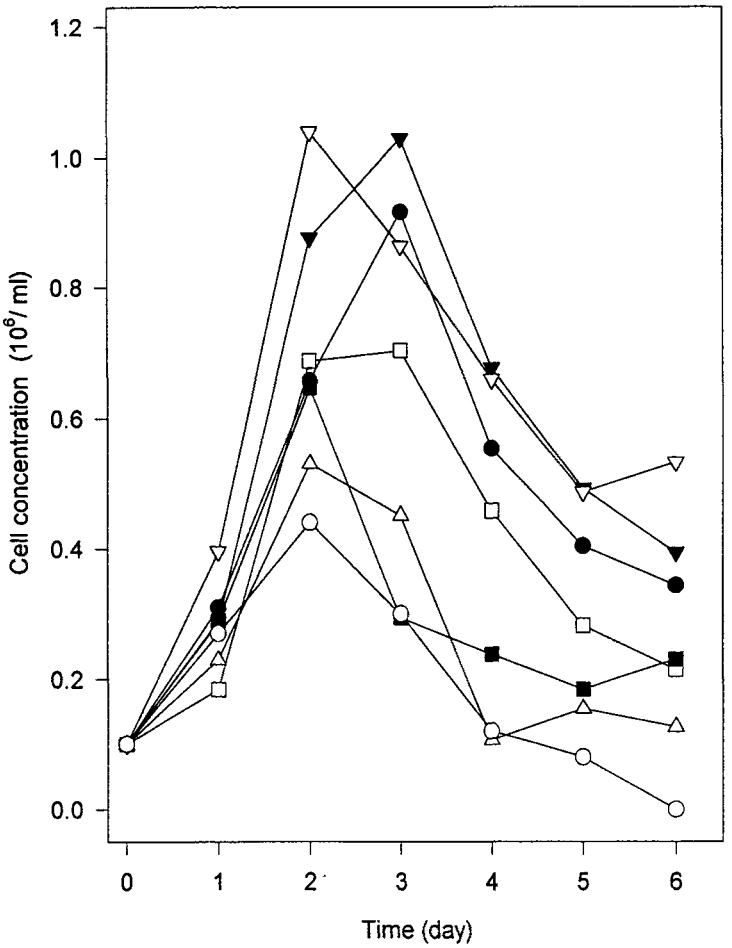

Fig. 1. Growth of hybridomas in various carbohydrates. The CC9C10 hybridomas were adapted to each media over 12 passages. The adapted cells were inoculated into 100 mL of the appropriate medium containing 20 m*M* of glucose (▽), galactose (●), maltose (▼), fructose (□), sorbitol (■), xylitol (△), or control (○) as the sole carbohydrate source. The control cultures had no added carbohydrate. The viable cell concentration was monitored over 6 d in culture. Each point represents the mean of two independent cultures. SE < 10% throughout.

to zero. Each adapted cell population was then monitored for 6 d in culture (Fig. 1). The viable cell concentration reached a maximum at day 2 or 3 of culture in all cases before a rapid decline phase, which is a typical pattern for murine hybridomas in batch culture.

The maximum cell density of the glucose-based culture was 1.04×10^6 cells/mL after 2 d of growth. Cell yields in cultures supplemented with different carbohydrates decreased in the following rank order: glucose = maltose > galactose > fructose = sorbitol = xylitol > control (no carbohydrate), as determined by statistical analysis ($P < 0.05$) of the growth curves (Fig. 1). Significant growth was observed in all cultures, including the control culture with no added carbohydrate source.

Table 1
The Effect of Adaptation
on Cellular Growth in Alternative Carbohydrate Sources (n = 2)

Carbohydrate supplement	Max. cell concentration, 10^6/mL		
	No adaptation	After adaptation	% Increase on adaptation
Glucose	1.04	1.04	0
Galactose	0.75	0.92	22.7
Maltose	0.88	1.03	17.0
Fructose	0.62	0.70	6.1
Sorbitol	0.36	0.65	80.6
Xylitol	0.31	0.53	71.0
Control[a]	0.40	0.45	12.5

[a]Control cultures were not supplemented with a carbohydrate source SE < 10%.

Table 2
Growth and Productivity Characteristics of CC9C10
Cells Cultured on Different Carbohydrate Sources (n = 2)

Carbohydrate supplement	Specific growth rate, h^{-1}	Max. cell density as % of control	MAb productivity q_{Mab}, $\mu g/d/10^6$	Max. MAb conc. as % of control
Glucose	0.0483	231	3.29	109
Galactose	0.0388	204	6.04	105
Maltose	0.0448	229	6.43	109
Fructose	0.0397	156	10.3	113
Sorbitol	0.0385	144	10.1	125
Xylitol	0.0344	118	18.4	141
Control[a]	0.0347	100	10.9	100

[a]Control cultures were not supplemented with a carbohydrate source SE < 15%.

To test whether the adaptation process had any effect on improving the growth of cells, cultures were also monitored following cell passage directly into media containing 20 m*M* of an alternative carbohydrate. The results showed that the adaptation process led to a significant increase in the cell yield in all cultures, except for the glucose-based control (Table 1). The greatest effect occurred in the presence of the polyols. Cell yields of cultures containing sorbitol or xylitol increased by 80.6 and 71.0%, respectively, following adaptation. For adapted cells, the specific growth rates varied from a high value of 0.0483 h^{-1} in the glucose-based culture to a low value of 0.0344 h^{-1} in the xylitol culture (Table 2).

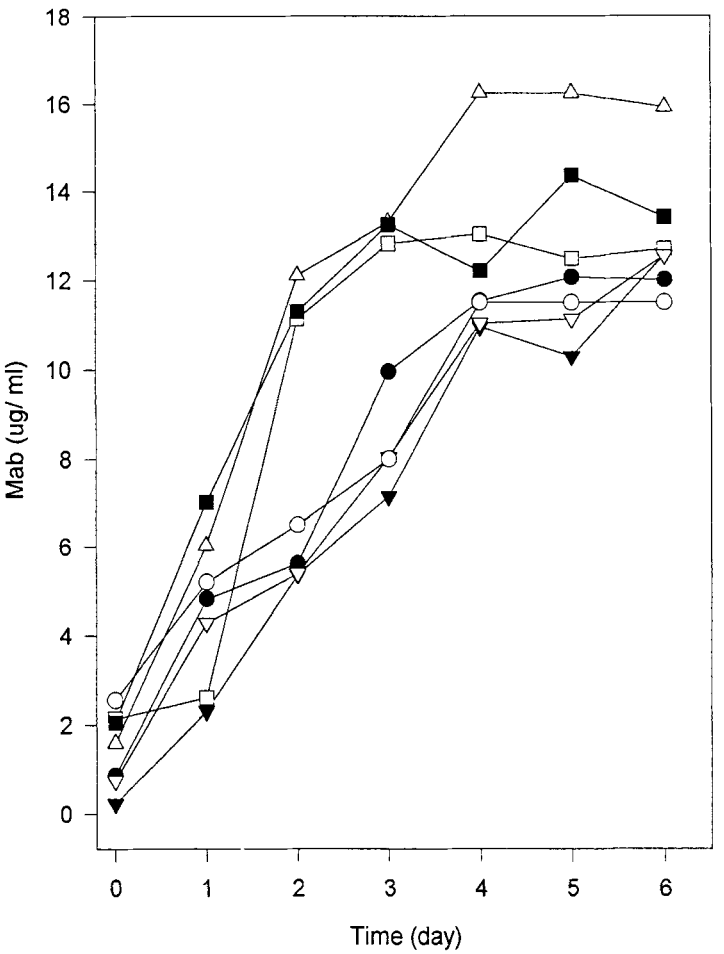

Fig. 2. MAb concentration in cultures of CC9C10 hybridomas grown in various carbohydrates. The MAb concentration was measured in the media of cultures of the CC9C10 hybridomas grown in medium containing 20 m*M* of glucose (▽), galactose (●), maltose (▼), fructose (□), sorbitol (■), xylitol (△), or control (○) as the sole carbohydrate source. The control cultures had no added carbohydrate. Each point represents the mean of two independent cultures. SE < 15% throughout.

MAb Production

The MAb concentrations were analyzed in all cultures on a daily basis by ELISA (Fig. 2). The highest concentration (MAb_{max}) was measured in the xylitol-based culture at 16.24 μg/mL, which was 29% higher than the concentration found in the presence of glucose and 41% higher than the control. The specific antibody productivity (q_{Mab}) was determined by plotting the MAb concentration against the integral of viable cell density vs time (*18*). In all cultures, this resulted in a straight line, indicating a correlation between MAb production and the concentration of viable cells.

The q_{Mab} was determined from the gradient of each plot. The value varied from 18.4 $\mu g/d/10^6$ cells for the xylitol culture and decreased significantly in the other cultures in the following rank order: control = sorbitol = fructose > maltose = galactose > glucose with respect to the carbohydrate source (Table 2). The MAb productivity in the xylitol culture was a factor of 1.7× higher than in the control and 5.6× higher than the glucose-based culture.

The pattern of cell growth is typical for hybridoma cultures with a rapid decline in viable cell concentration following the maximum cell yield at day 2 or 3. The MAb concentration accumulates even after the maximum cell density is attained owing to continued synthesis by viable cells or by release from lysed cells.

Cellular Metabolism

The carbohydrate concentrations of all cultures were measured on a daily basis by spectrophotometric asays (Fig. 3). The assays for four of the carbohydrates (galactose, fructose, sorbitol, and xylitol) depended on a 100× dilution of media samples to obtain absorbance values within the standard scale and may account for the variability in initial carbohydrate concentrations. The data show that 37% of the added glucose and 16% of maltose were utilized by cells over 2 d in culture. However, the cellular utilization of the other carbohydrates was minimal. In the case of xylitol, any uptake was below the level of detection of the assay (< 0.1 m*M*).

The concentration of lactate was measured in all cultures on a daily basis (Fig. 4). Data showed that the production of this byproduct of glycolysis was greatest in the maltose-based culture and reached a level of 12 m*M* during the growth phase. This corresponded to a metabolic coefficient, $Y_{lac/malt}$ of 2.9. A substantial increase in lactate concentration was also observed in the glucose-based culture, but with a lower coefficient ($Y_{lac/glc}$ = 0.97). However, minimal levels of lactate production (< 4 m*M*) were observed in all other cultures.

Daily measurements of glutamine concentration indicated that this substrate was completely utilized in all cultures after 3 d. Determination of the specific consumption rate (q_{gln}) over the first day indicated a significant variation between cultures with the highest q_{gln} for control cultures followed by fructose > xylitol = sorbitol = maltose = galactose > glucose-based cultures (Table 3). Control cultures that did not contain a carbohydrate showed a q_{gln} 3.5× greater than the glucose-based cultures.

DISCUSSION

Glucose is metabolized by hybridoma cells in culture mainly by glycolysis (*15*). This leads to production of a substantial concentration of lactate, which is released into the medium in an acidic form causing a decrease in

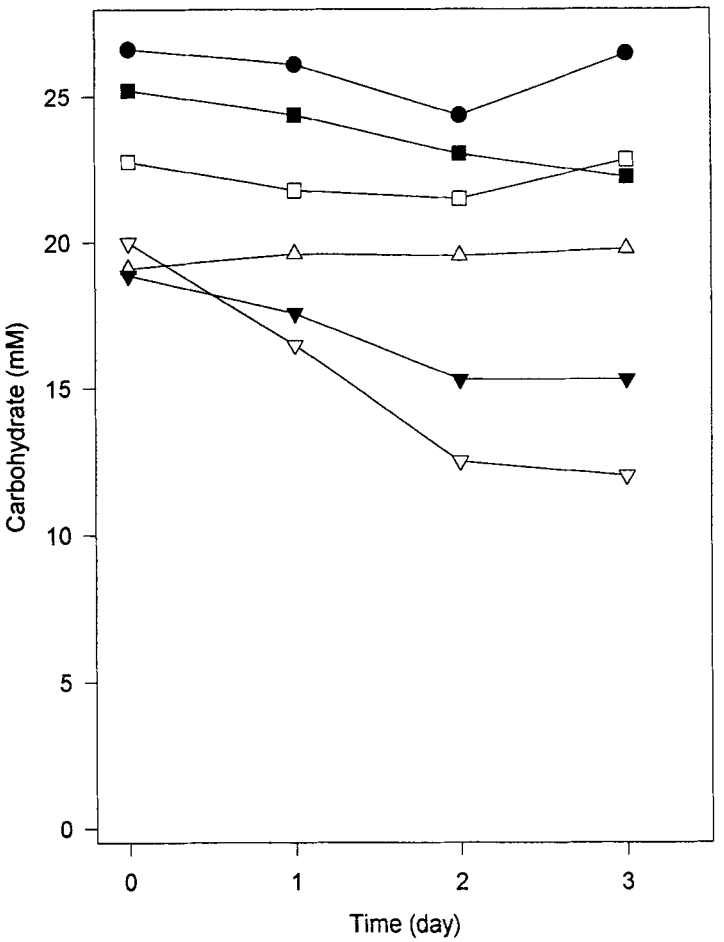

Fig. 3. Utilization of carbohydrates by CC9C10 hybridomas. Cultures contained glucose (▽), galactose (●), maltose (▼), fructose (□), sorbitol (■), xylitol (△) as the sole carbohydrate source. Each point represents the mean of two independent cultures. SE < 10% throughout.

culture pH. This necessitates a system of pH control in bioreactor cultures of mammalian cells in order to maintain a consistent growth rate. Alternative carbohydrates, such as fructose or galactose, can reduce the lactate production without having a substantial effect on growth (*4*). For the CC9C10 cells used in the experiments reported here, only glucose- and maltose-based cultures led to significant lactate production in the medium. Maltose can be hydrolyzed into glucose, so its utilization is likely to be similar to that of glucose.

Galactose, fructose, sorbitol, or xylitol substituted for glucose to produce significant growth and lower cell yields, but with a reduced production of lactate.

Although glucose metabolism provides a substantial proportion of the energy requirement of the cell via glycolysis, an important role of glu-

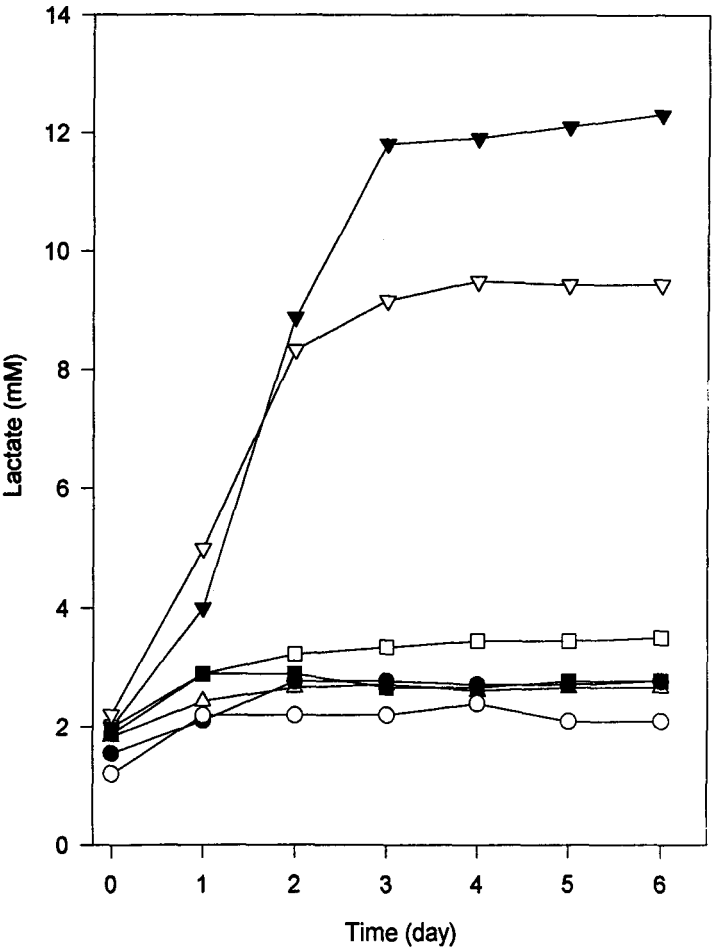

Fig. 4. Lactate concentration in cultures of CC9C10 hybridomas grown in various carbohydrates. The lactate concentration was measured in the media of cultures of the CC9C10 hybridomas grown in medium containing 20 m*M* of glucose (▽), galactose (●), maltose (▼), fructose (□), sorbitol (■), xylitol (△), or control (○) as the sole carbohydrate source. The control cultures had no added carbohydrate. Each point represents the mean of two independent cultures. SE < 10% throughout.

cose is to act as a substrate for the pentose phosphate pathway, which provides ribose for nucleic acid synthesis. However, Zielke et al. (*16*) showed that this anabolic function of glucose could be provided by supplements of purine and pyrimidines. In the absence of glucose, the cellular energy requirement could be provided by glutamine, which is metabolized via glutaminolysis. The anabolic role of glucose could also be provided by galactose or fructose, which are converted into glycolytic intermediates by isomerization and phosphorylation. Their slower rate of utilization is the most likely explanation for the decreased lactate formation as shown in the cultures of the CC9C10 cells.

Table 3
Specific Glutamine Consumption Rates of Cells Cultured in Alternative Carbohydrate Sources (n = 2)

Carbohydrate supplement	Q_{gln}, nmol/min/10^6 cells	Gln consumption as a % of glucose-based culture
Glucose	3.19	100
Galactose	4.18	131
Maltose	4.48	140
Fructose	8.30	260
Sorbitol	5.05	158
Xylitol	5.62	176
Control[a]	11.3	354

[a]Control cultures were not supplemented with a carbohydrate source SE < 10%.

The metabolism of sorbitol and xylitol has received considerable attention because of their use as food sweeteners (*17*). Their use in cell culture was investigated by Demetrakopoulos and Amos (*5*), who reported the rapid growth of mammalian fibroblasts in D-xylose and xylitol, although poor growth in sorbitol. The utilization of the polyols depends on the induction of dehydrogenases for their conversion to xylulose or fructose, which would be suitable substrates for the pentose phosphate pathway. In our experiments, we gradually reduced the glucose available to the cells over a period of 12 passages to allow adaptation. This had a significant effect on the lag phase of the cultures and also the cell yields attainable. After adaptation, cell yields in the xylitol or sorbitol cultures increased significantly, with maximum densities reaching 18% and 44% higher than the control.

Glutamine can provide a significant proportion of the energy requirement of the CC9C10 hybridomas (*15*). The consumption rates of glutamine would appear to be related to the availability of a carbohydrate as energy source. Thus, q_{gln} was high in carbohydrate-free control cultures, but low in glucose-based cultures. The fact that minimal growth did occur in control cultures may indicate the availability of trace quantities of substrates in serum (possibly nucleotides) to satisfy the minimal anabolic requirement of a carbohydrate source. It is unlikely that glucose was present in the serum because of the low levels of lactate produced in the control cultures.

The data with respect to MAb productivity were unexpected. The xylitol-grown cells showed a specific productivity of 5.6× greater than the glucose control and a 29% higher volumetric yield. In all cultures, a correlation was found between released MAb and the integral of cell concentration and time (viability index). This relationship has been shown previously for hybridomas (*18*) and indicates that the specific productiv-

ity is related to the number of viable cells in culture, and not to whether the cells are in the growth or stationary phase.

The reason for the increased MAb productivity in the presence of the polyols is unclear. There is evidence that parameters that adversely affect cell growth can promote MAb productivity (*19*). This argues for a non-specific effect observed with the CC9C10 cells in our experiments. However, the effect of MAb productivity in the xylitol cultures is unlikely to be due merely to the lower growth rate or absence of glucose because of the significantly higher q_{Mab} and MAb_{max} compared to either carbohydrate-free controls or glucose-based cultures.

The data presented here show minimal cellular utilization of sorbitol or xylitol at a millimolar level. However, metabolic effects could be produced by an interaction with the cell membrane, which could affect antibody secretion, or by low-level (micromolar) uptake, which would be undetected by the spectrophotometric assays used. It has been shown previously that xylitol can cause major changes to the intermediary metabolism of mammalian cells by increasing the NADH/NAD ratio and the concentration of available ribose (*7*). These changes are both likely to improve the metabolic state of the cell for product synthesis. It may be that these specific metabolic effects promoted the observed higher MAb productivity in the experiments reported here.

The data presented in this article indicate that xylitol may be a suitable substitute for glucose in mammalian cell-culture processes for enhanced MAb yields and lower byproduct formation.

ACKNOWLEDGMENT

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REFERENCES

1. Eagle, H., Barban, S., Levy, M., and Schulze, H. O. (1958), *J. Biol. Chem.* **233,** 551–558.
2. Burns, R. L., Rosenberger, P. G., and Kleke, R. J. (1976), *J. Cell Physiol.* **88,** 307–316.
3. Cristafalo, V. J. and Kritchevsky, D. (1965), *Proc. Soc. Exp. Biol. Med.* **118,** 1109–1112.
4. Imamura, T., Crespi, C. L., Thilly, W. G., and Brunengraber, H. (1982), *Anal. Biochem.* **124,** 353–358.
5. Demetrakopoulos, G. E. and Amos, H. (1976), *Biochem. Biophys. Res. Commun.* **72,** 1169–1178.
6. Turner, J. L. and Bierman, E. L. (1978), *Diabetes* **27,** 583–588.
7. Vincent, M. F., van den Berghe, G., and Hers, H. G. (1989), *FASEB J.* **3,** 1862–1867.
8. Schroer, J. A., Bender, T., Feldman, R. J., and Kim K. J. (1983), *Eur. J. Immunol.* **13,** 693–700.

9. Bergmeyer, H. U., Gruber, W., and Gutmann, I. (1974), in *Methods of Enzymatic Analysis*, Bergmeyer, H. U., ed., Acedemic, New York, p. 1323.
10. Bassler, K-H. (1974), in *Methods of Enzymatic Analysis*, Bergmeyer, H. U., ed., Academic, New York, p. 1381.
11. Kurz, G. and Wallenfels, K. (1974), in *Methods of Enzymatic Analysis*, Bergmeyer, H. U., ed., Academic, New York, p. 1279.
12. Gerlach, U. (1983), in *Methods of Enzymatic Analysis*, Bergmeyer, H. U., ed., Academic, New York, p. 112.
13. Lund, P. (1985), in *Methods of Enzymatic Analysis*, Bergmeyer, H. U., ed., Academic, New York, p. 357.
14. Long, W. J., Palombo, A., Schofield, T. L., and Emini, E. A. (1988), *Hybridoma* **7,** 69–77.
15. Petch, D. and Butler, M. (1994), *J. Cell Physiol.* **161,** 71–76.
16. Zielke, H. R., Ozand, P. T., Tildon, G. T., Sevdalian, D. A., and Cornblath, M. (1976), *Proc. Natl. Acad. Sci.* **73,** 4110–4114.
17. Wang, Y.-M. and Eys, J. van (1981), *Ann. Rev. Nutr.* **1,** 437–475.
18. Renard, J. M., Spagnoli, R., Mazier, C., Salles, M. F., and Mandine, E. (1988), *Biotechnology Lett.* **10,** 91–96.
19. Oh, S. K. W., Vig, P., Chua, F., Teo, W. K., and Yap, M. G. S. (1993), *Biotech. Bioeng.* **42,** 601–610.