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REVIEW

Controlling Mammalian Cell Metabolism in Bioreactors

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Abstract Animal cells in culture typically convert most of the glucose they consume into lactate. The accumulation of lactate, however, is commonly cited as one of the factors that inhibit cell growh and limit the maximum cell concentration that can be achieved in culture. The specific production of lactate and the amount of glucose converted to lactate can be reduced when cells are grown in a fed-batch culture in which the residual glucose concentration is maintained at low levels. Such a fed-batch culture was used to grow and adapt hybridoma cells into a low-lactate-producing state before changing into continuous culture. The cells reached and maintained a high viable cell concentration at steady state. In a similar manner, cells that were initially grown in batch culture and a glucose-rich environment reached a steady state with a cell concentration that is much lower. The feed composition and dilution rates for both cultures were similar, suggesting steady state multiplicity. From a processing perspective the desired steady state among those is the one with the least metabolite production. At such seady state nutrient concentration in the feed can be further increased to increase cell and product concentrations without causing the metabolite inhibitory effect typically seen in a cell culture. Controlling cell metabolism in a continuous culture to reduce or eliminate waste metabolite production may significantly improve the productivity of mammalian cell culture processes.

Key words: Cell culture, hybridoma, metabolism

In the past fifteen years, mammalian cell culture processing has been transformed from an exploratory protein production method to a mature manufacturing technology. Much of the transformation was propelled by the advancement in molecular genetics and the drive to meet clinical needs. Also aiding the maturation of mammalian cell-based manufacturing technology are significant advances in bioprocessing research. Notable among these are cell line and culture medium developments, a better understanding of the physical environment in bioreactors, and more insight into the kinetics and dynamics of cell growth and product formation. Serum, which was universally employed in cell culture processing in the 1980s, has been almost completely eliminated from current industrial recombinant protein production processes. In some cases, cells are cultured in an almost protein-free environment [8, 11]. The better-defined chemical environments for cell growth have allowed more consistent processes and made possible a more accurate investigation of the cell's kinetic behavior. In the past few years we have also seen major advances in analytical techniques for product characterization. One can now assess the quality of the product in great structural detail. These advanced analytical tools have allowed one to reveal the minute changes on the product caused by process fluctuations or modifications. It enables one to gain insight into means of developing a more reproducible and consistent process [9].

What has been lagging behind is the physiological studies on the centerpiece of the enterprise—the cells. Manipulation of cellular physiology to tailor the process needs has been the key contribution of applied microbiology to the commercialization of thousands of industrially important biochemicals. Classic examples include relieving the catabolite repression of antibiotic synthesis by controlled feeding of glucose [7], and the reduction of ethanol fermentation during baker's yeast production by on-line computer-aided nutrient feeding [17]. More recently the production of undesirable by-products during phenylalanine fermentaion was decreased

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through the employment of an expert system [13]. A major result of manipulating the physiological state is the redistribution of resources and redirection of metabolite fluxes away from waste product formation and toward cell mass and desired product(s).

Most of the success stories on enhanced productivity through manipulation of cellular metabolism have been on microbial fermentation systems. The potential of exploiting various metabolic states of mammalian cells has also been explored by many since the early stage of modern cell culture processing. Early work focused on the reduction of glutamine concentration [4]. Attempts were also made to alter energy metabolism by controlling glucose concentration [5, 6] or substituting glucose with a more slowly-utilized substrate [3]. The results indicate the potential of manipulating cellular metabolism via on-line monitoring and control. This paper will discuss the potential benefits of manipulating cell metabolism for the production of biopharmaceuticals.

Partially Substitutable and Partially Complementary Nature of Required Nutrients

A typical cell culture medium consists of glucose and glutamine as the main carbon and energy sources. The metabolism of glcose and glutamine in mammalian cells are related as illustrated in the simplified metabolic chart shown in Fig. 1. Both provide the carbon skeleton for cellular molecules and are essential for cell growth. They are thus complementary substrates. Depending on the cell line and other culture components, the list of essential nutrients also includes 12 essential amino acids, some viamins, nucleotides, and minerals. Most cell culture media also include many non-essential amino acids. The amino acids, in addition to being incorporated into cellular and product proteins, can also be metabolized through the TCA cycle to generate energy. The cell's energy requirements, however, are primarily

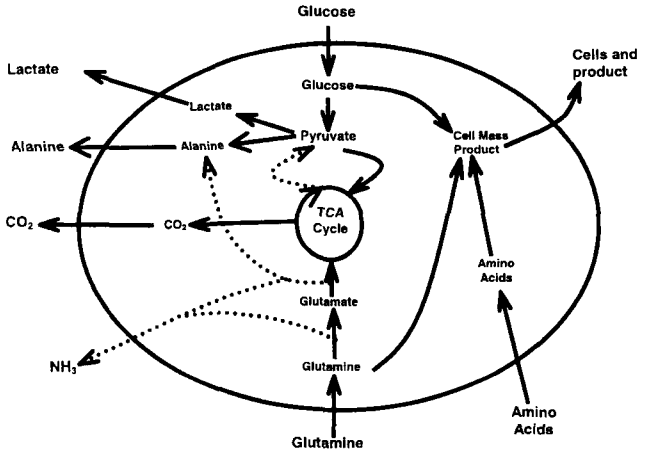

Fig. 1. Simplified chart of animal cell metabolism.

fulfilled by the metabolism of glucose and glutamine. From the perspective of energetics, cells can derive their energy needs from many combinations of these two substrates (albeit at different efficiencies and specific rates). These two substrates, with respect to supplying the cells with energy, are therefore substitutable. Since much more glucose and glutamine are consumed by cells compared to the rest of the amino acids, most physiological studies focus on the metabolism of these two nutrients.

Alteration of Cell Metabolism by Varying Glucose and Glutamine Concentrations

Mammalian cells are capable of utilizing different proportions of glucose and glutamine and metabolize them differently under different chemical environments. Early work by Zielke *et al.* [21] showed that glutamine becomes a predominant source of energy at low glucose concentrations. The metabolism of glucose is also greatly affected by its concentration [6, 22]. At high glucose levels, glucose is consumed at a faster rate on a per cell basis (or the specific consumption rate); about 70 to 95% of the glucose consumed, however, is converted to lactate. At low glucose concentrations, its specific consumption rate is lower and a larger proportion is completely oxidized to CO_2.

Glucose metabolism is linked to glutamine metabolism via the TCA cycle. Glutamine, being the favored substrate for neutral amino acid transport system A, is taken up by cells at a much faster rate than other amino acids. The uptake rate far exceeds what is needed for incorporation of glutamine into biomass (as cellular protein or nucleotides) and protein products. Glutamine enters the TCA cycle via glutamate and α-ketoglutarate where it can provide carbon skeleton for amino acids and other cellular materials or be oxidized to provide energy. Glutamine, via glutamic acid, also provides the amino group for the non-essential amino acids synthesized intracellularly. Arguably under the conditions that all-essential amino acids are supplied exogenously at sufficiently high concentrations and are transported into the cells at sufficiently high rates, glutamine will not be needed as the nitrogen source. However, it will still be required as an essential amino acid and possibly as an energy source. The excess nitrogen carried into cells with glutamine is excreted as ammonia and/or as non-essential amino acid(s), primarily alanine, and, in some cells, also asparagine, proline, and/or glutamic acid.

Glutamine has many physiological functions and is metabolized differently under different conditions. The amount of nitrogen-containing metabolite produced per mole of glutamine consumed thus varies over a wide range. Under normal growth conditions, the molar ratio of excess nitrogen excreted (NH_3 and alanine, etc.) to

glutamine consumed is less than one or in the vicinity of one. However, under the condition of low glucose concentration, glutamine also serves as an energy source and the source of carbon skeleton for other cellular constituents; under such condition, the ratio becomes greater than one.

Alteration of Metabolism in Fed-batch Cultures

Manipulation of nutrient levels in culture thus seems to be effective in altering cell metabolism for reduced metabolite accumulation. However, its realization in cell culture processing has been slow in coming. A major reason is that its implementation requires an on-line nutrient delivery system to sustain a low nutrient level. To control glucose or glutamine at low levels, they must be fed continuously to meet the metabolic demand. Too low a feed rate will cause nutrient depletion and possibly associated disastrous cell death. Too high a feeding rate, on the other hand, results in nutrient accumulation to a high level and possible failure to alter cell metabolism. In a batch process in which cell concentration and the metabolic demand change with time, a relatively precise prediction of metabolic demand is necessary in order to maintain either glucose or glutamine at a low level. Therefore, a prerequisite for applying the strategy of manipulating glucose/glutamine concentration to alter cell metabolism is to implement an on-line measurement and physiological state estimation system to implement on-demand nutrient feeding.

Both base addition (in response to lactic acid accumulation) and oxygen uptake rate (OUR) measurements were explored as possible means of estimating metabolic demand [18]. Base addition offers the advantage of simplicity. However, because of the buffer capacity of the culture media, it is not a very sensitive method. Futhermore, if the metabolic alteration is completely successful and no lactic acid is produced, then no base will be added and renders the method completely inapplicable. It thus appears that oxygen uptake rate measurement, coupled with base addition and turbidity measurement, offers the best possibility of success.

On-line measurements of oxygen uptake were employed to estimate the metabolic demand of nutrients and the feeding rate of concentrated medium (without bulk salts) in fed-batch culure. The fed-batch culture was started with rather low initial glucose and glutamine concentrations (1.39 mM and 0.3 mM, respectively). The amount of glucose consumed was estimated on-line using cumulative oxygen consumption and the stoichiometric relation between glucose and oxygen consumption. The feeding of a salt-free concentrated medium was initiated automatically after the estimated glucose concentration dropped to 0.56 mM. The feeding nutrient solution was a 15.5-fold concentrated medium without inorganic components and with glucose and glutamine concentrations (258.3 mM and 93 mM, respectively). By using the estimated stoichiometric relation (α) between glucose and oxygen consumption, glucose consumption were determine on-line. Subsequently feeding nutrient solution was added to replenish glucose and other nutrients and to maintain glucose concentration around the set point. The feeding rate (F_{t_c}) of the nutrient feeding solution for the period between time points t_i and t_{i+1} was determined as

$$F_{t_c} = \frac{\int_{t_i}^{t_{i+1}} OUR \cdot V \, dt}{\alpha_i C_f (t_{i+1} - t_i)}$$

where V is the culture volume and C_f is the concentration of glucose in the feed solution, t_i and t_{i+1} are the current and next time points of the OUR measurements. The initial value of α was 0.08 g glucose/mmol oxygen consumed as determined from previous experiments. During the fed-batch stage α was estimated off-line from OC and cumulative glucose consumption (GC):

$$\alpha_i = \frac{\int_{t_{i+1}}^{t_i} OUR \cdot V \, dt}{GC_{t_i} \cdot V_{t_i} - GC_{t_i-1} \cdot V_{t_i-1}}$$

Using this feeding strategy, the glucose concentration was maintained at a low level throughout the exponential growth period [19] (Fig. 2). Lactate production was reduced to near zero towards the end of the cultivation. At the beginning of the cultivation, 85% of the glucose carbon was excreted as lactate; each mole of glucose consumed was accompanied by one mole of oxygen consumption. Towards the end of cultivation almost no glucose carbon was excreted as lactate and the stoichiometric ratio between oxygen to glucose increased to six. The results indicate that a complete change of metabolic state from a highly glycolytic mode (with high lactate production) to a highly oxidative mode (with high

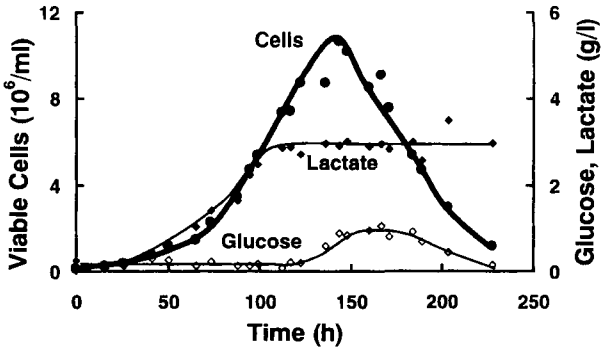

Fig. 2. Fed-batch culture with controlled glucose concentration and metabolic shift.
(●), Viable cell concentration; (◇) glucose concentration; (◆), lactate concentration.

oxygen consumption) was gradual. With the lower degree of accumulation of lactate, a high cell concentration of 10^7 cells/ml and a high viability (> 95%) was achieved.

In a subsequent experiment, both glucose and glutamine were maintained at low concentrations of 0.5 mM and 0.2 mM, respectively, during the growth stage [20]. The cell metablism was altered from that observed in batch culture resulting in significant reduction of lactate, alanine and ammonia production. The final product concentration in batch culture for this particular clone in the defined medium was only 8 μg/ml. In the first fed-batch culture the IgG titer was 65 μg/ml while in the second one it was 250 μg/ml. The higher viable cell concentration and product titer were attributed to the reduced accumulation of metabolites. The alteration of metabolism was also observed in a myeloma cell line that was used for industrial production when the same strategy was applied in a fed-batch culture.

Such a strategy of altering cell metabolism by controlling glucose concentration was applied to a myeloma cell line which has been genetically engineered to produce IgG. Interestingly, controlling glucose concentration at 0.05~0.1 g/l failed to change glucose metabolism from a glycolytic state to an oxidative state completely. Subsequently, two consecutive fed-batch cultures were used; cells from the first fed-batch culture were used to inoculate the second culture, thus extending the duration for which cells were exposed to a low glucose concentration while at the exponential growth phase. In the second fed-batch culture, the stoichiometric ratio of lactate production to glucose consumption was drastically reduced, indicating a shift of cell metabolism [2]. Furthermore, both viable cell and antibody concentration increased by nearly threefold from those of a batch culture. The results indicate that such a method of altering cell metabolism is probably generally applicable to different cell lines.

The results shown above demonstrated that cellular metabolism can be manipulated in a fed-batch culture to maximize the efficiency of biopharmaceutical productions. It is possible that cells in a continuous culture can also be grown with different uptake patterns of glucose and glutamine. For example, in one range of concentrations of glucose and glutamine, the cells may preferentially utilize glucose and convert it to lactate; in another range of concentrations, the cells may consume a considerably larger proportion of glutamine and convert the bulk of glucose and glutamine to CO_2. This variability in the metabolic fates of glucose and glutamine is accompanied by a variation in the efficiency of nutrient utilization (i.e., the yield of biomass). Thus, for a given nutrient feed under the same dilution rate (imposing a constant specific growth rate) in a continuous culture, one may envisage multiple steady states characterized by cells of radically different metabolic states with a corresponding change in the efficiency of conversion to biomass.

Multiple Steady States

Although the physiological reasoning and the results from fed-batch cultures suggests that multiple steady states may exist. Experimental observation has been elusive. Under normal operations, namely starting up a culture in batch mode and switching to a continuous mode after certain cell concentration is reached, the steady states realized always correspond to those associated with the high lactate production state. This elusiveness of putative steady state associated with low lactate production is possibly due to the instability of the state. Alternatively it may be caused by the fact that only certain reaction paths will lead the particular steady state.

Recently we employed fed-batch cultures to alter the cell's metabolic state to a low lactate producing state before switching the culture to continuous operation. Using

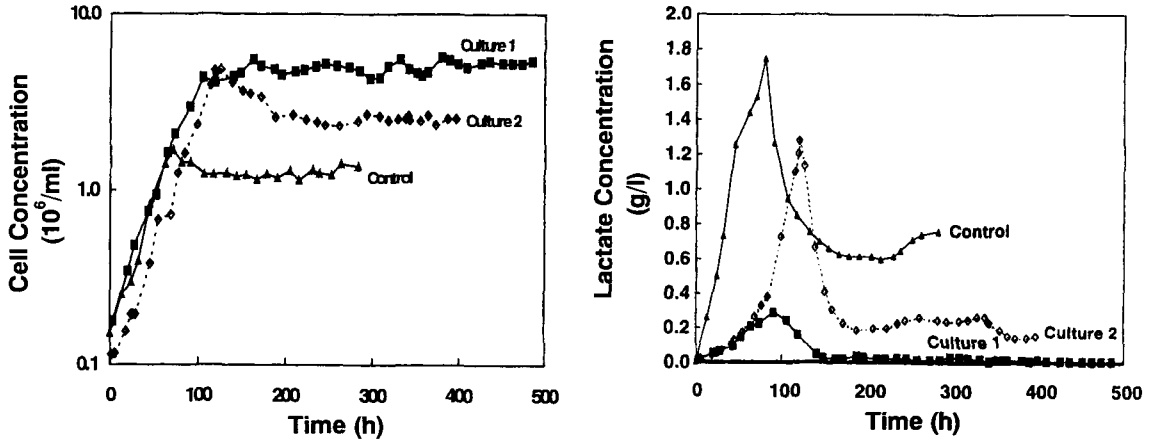

Fig. 3. Multiple steady states achieved with different initial conditions.
(a), viable cell concentration; (b), lactate concentration.

the same strategy of controlling glucose concentration at a low level as described above, lactate production was suppressed (at 90 h, only 0.3 g/l), indicating a successful manipulation of metabolic state to a low-lactate-producing one. The culture was then switched to a continuous mode of operation using the same feed as that used in the conventional method. A viable cell concentration of about 5×10^6/ml was achieved (Fig. 3, culture 1).

As a control, cells were cultivated in a batch mode with glucose concentration at high levels before the culture was switched to a continuous mode. In the continuous culture phase, the same feed medium was used, the cells convert most of the glucose consumed to lactate and cell concentration reached about 1.3×10^6 cells/ml (control culture).

In a follow-up experiment the glucose concentration was maintained at 0.03 g/l and a dilution rate of 0.033 h^{-1} (same as previous two experiments) was used. Much to our surprise, another steady state with a viable cell concentration of 2.5×10^6 cells/ml (viability 95%) was achieved (Fig. 3, culture 2). The residual glucose concentration was very similar to that observed in previous continuous culture. The lactate concentration was lower than the conventional continuous culture but higher than the first culture. It is possible that the small difference in the glucose concentrations between these two cultures was rather significant physiologically and accounted for the differences in the cell metabolism. The only difference between this experiment and the one that resulted into a high cell concentration in Fig. 3 was that the glucose concentration set point during fed batch was slightly higher. The results not only provided further evidence of metabolic shift and the existence of multiple steady states, but also suggest that the steady state achieved could be path dependent. The antibody concentrations in these cultures were virtually proportional to the cell concentration (data not shown).

Technological Relevance of Cellular Metabolism

In a typical continuous culture, most of the glucose consumed is converted to lactate, resulting in insignificant accumulation of lactate even at steady state. This prevents further increase in glucose concentration in the feed as it will give rise to even higher lactate concentration. To increase the cell concentration in a continuous culture, a high flow rate, instead of high nutrient concentrations, is used in the feed. However, too high of a flow rate results in cell washout. To achieve a high cell concentration at a high flow rate of medium, continuous culture with cell retention or recycle, usually referred to as perfusion culture, is often used.

Many cell retention/recycle devices have been developed in the past decade, including settling device [1, 12], centrifuge [10, 14], wire cage [16], and acoustic device [15]. In some cases a perfusion rate as high as ten culture volumes a day is employed. Such a system is used for the manufacturing of some products including Factor VIII, truncated factor VIII and some monoclonal antibodies. Most of these cultures are operated as batch cultures initially and are later switched to a continuous mode with cell retention.

The cost of goods for producing recombinant proteins used to be of little concern to pharmaceutical industry. Although this remains true for some drugs that are used in small doses, the price pressure is making the production cost increasingly a concern. A few years ago, a $2000/g cost of bulk therapeutic protein was considered economical. Today the target cost, for products like monoclonal antibodies, is often closer to $200/g. To devise an efficient process, one aims at increased cell and product concentrations and an extended production period. In many cases this is achieved through perfusion culture.

An industrial perfusion process consumes up to thousands of liters of medium a day. The reason for using a high perfusion rate in these cultures is primarily to remove the inhibitory metabolites. The results of multiple steady states presented above demonstrate that it is possible to manipulate cell metabolism to minimize metabolite accumulation and operate the metabolically shifted continuous culture at a steady state. At such a steady state both the cell and product concentrations are higher than those of a culture in which the cellular metabolism is not altered. This will allow for the same product throughout at a much reduced medium consumption rate. The full potential of altering cell metabolism for enhanced productivity of mammalian cell culture processes is yet to be realized. The cellular and molecular events leading to such metabolic shifts are yet to be unveiled. Further investigations on such metabolically shifted multiple steady states are warranted.

References

1. Batt, B. C. and D. S. Kompala. 1990. Verification of immune response optimality through cybernetic modeling. *J. Theoretical Biol.* **142**: 317–340.
2. Europa, A. F., A. Gambhir, and W.-S. Hu. (submitted). Altering mammalian cell metabolism using consecutive fed-batch culture.
3. Fleischaker, R. T. 1982 An experimental study in the use of instrumentation to analyze metabolism and product formation in cell culture. In *Nutrition and Food Science.* Massachusetts Institute of Technology, Cambridge.
4. Glacken, M. W., R. J. Fleischaker, and S. J. Sinskey. 1986. Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yeilds on serum in cultures of mammalial cells. *Biotechnol. Bioeng.* **28**: 1376–1389.

5. Hu, W. S., T. C. Dodge, K. K. Frame, and V. B. Himes. 1987. Effect of glucose on the cultivation of mammalian cells. *Develop. Biol. Standard.* **66**: 279–290.
6. Hu, W. S. and M. G. Oberg. 1990. Monitoring and control of animal cell bioreactors: biochemical engineering considerations. *Bioprocess Technol.* **10**: 451–481.
7. Hu, W.-S. and A. L. Demain. 1979. Regulation of antibiotic biosynthesis by utilizable carbon sources. *Proc. Biochem.* **14**: 2–6.
8. Inoue, Y., L. B. Lopez, S. Kawamoto, N. Arita, K. Teruya, K. Seki, M. Shoji, M. Kamei, S. Hashizume, Y. Shiozawa, H. Tachibana, H. Ohashi, K. Yasumoto, A. Nagashima, H. Nakahashi, T. Suzuki, T. Imai, K. Nomoto, M. Takenoyama, Y. Katakura, and S. Shirahata. 1996. Production of recombinant human monoclonal antibody using Ras-amplified Bhk-21 cells in a protein-free medium. *Biosci. Biotechnol. Biochem.* **60**: 811–817.
9. Jenkins, N., R. B. Parekh, and D. C. James. 1996. Getting the glycosylation right–implications for the industry. *Nature Biotechnol.* **14**: 975–981.
10. Johnson, M., S. Lanthier, B. Massie, G. Lefebvre, and A. A. Kamen. 1996. Use of the centritech lab centrifuge for perfusion culture of hybridoma cells in protein-free medium. *Biotechnol. Progress* **12**: 855–864.
11. Keen, M. J. and C. Hale. 1996. The use of serum-free medium for the production of functionally active humanised monoclonal antibody from NS0 mouse myeloma engineered using glutamine synthetase as a selectable marker. *Cytotechnology* **18**: 207–217.
12. Kitano, K., Y. Shintani, Y. Ichimori, K. Tsukamoto, S. Sasai, and M. Kida. 1986. Production of human monoclonal antibodies by heterohybridomas. *Appl. Microbiol. Biotechnol.* **24**: 282–286.
13. Konstantinov, K. B., N. Nishio, T. Seki, and T. Yoshida. 1991. Physiologically motivated strategies for control of the fed-batch cultivation of recombinant *Escherichia coli* for phenylalanine production. *J. Ferment. Bioeng.* **71**: 350–355.
14. Tokashiki, M., T. Arai, K. Hamamoto, and K. Ishimaru. 1990. High density culture of hybridoma cells using a perfusion culture vessel with an external centrifuge. *Cytotechnology* **3**: 239–244.
15. Trampler, F., S. A. Sonderhoff, P. W. Pui, D. G. Kilburen, and J. M. Piret. 1994. Acoustic cell filter for high density perfusion culture of hybridoma cells. *Bio/Technology* **12**: 281–284.
16. Varecka, R. and W. Scheirer. 1987. Use of a rotating wire cage for retention of animal cells in a perfusion fermentor. *Develop. Biol. Standard.* **66**: 269–272.
17. Wang, H., C. L. Cooney, and D. I. C. Wang. 1977. Computer-aided baker's yeast fermentation. *Biotechnol. Bioeng.* **19**: 68–86.
18. Zhou, W. and W.-S. Hu. 1994. On-line characterization of a hybridoma cell culture process. *Biotechnol. Bioeng.* **44**: 170–177.
19. Zhou, W. C., J. Rehm, and W. S. Hu. 1995. High viable cell concentration fed-batch cultures of hybridoma through on-line nutrient feeding. *Biotechnol. Bioeng.* **46**: 579–587.
20. Zhou, W.-C., J. Rehm, A. Europa, and W.-S. Hu. 1997. Alteration of mammalian cell metabolism by dynamic nutrient feeding. *Cytotechnology* (in press).
21. Zielk, H. R., P. T. Ozand, J. T. Tildon, D. A. Sevdalian, and M. Cornblath. 1976. Growth of human diploid fibroblasts in the absence of glucose utilization. *Proc. Natl. Acad. Sci. USA* **73**: 4110–4114.
22. Zielke, H. R., P. T. Ozand, J. T. Tildon, D. A. Sevdalian, and M. Cornblath. 1978. Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts. *J. Cell. Physiol.* **95**: 41–48.