Effect of Medium Osmolarity on Hybridoma Growth, Metabolism, and Antibody Production

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Osmolarity is an important process variable during the cultivation of mammalian cells in vitro. Cell culture medium is designed to have osmolarity in the range of 260 and 320 milliosmoles (mOsm), basically to mimic the osmolarity of serum at 290 mOsm/kg. Fragmented information on the response of hybridoma cells to elevated osmolarity is available. A decrease in cell growth and an increase in antibody production were reported at high osmolarities.[4,7] Influence of osmolarity on antibody productivity seems to be cell line dependent.[12] Here we report the effects of osmolarity on hybridoma cell growth, metabolism, and antibody synthesis. The osmolarity of the medium was altered by the addition of both ionic and non-ionic substances.

MATERIALS AND METHODS

Cell lines, Media, and Culture Maintenance

A murine hybridoma cell line, 167.4G5.3, was used in the experiments. This cell line was provided by Dr. Latham Claflin from the Medical Center at The University of Michigan. The antibody produced by this cell line is an IgG_1, directed against phosphorylcholine.[2] Hybridoma cells were made by fusion of BALB/c spleen cells with the nonsecreting plasmacytoma fusion line P3X63-Ag8.653. Antibody was generated from mice immunized with PC-keyhole limpet henemocyanin (KLH). Cells were propagated in T-flasks (Bellco Glass, Inc., Vineland, NJ) at 37°C in a humidified incubator under 5% CO_2. The media used was Iscove's Modified Dulbecco's Medium (IMDM, Gibco Laboratories, Grand Island, NY) containing 5% Fetal Bovine Serum (FBS, Gibco). The media was supplemented by 100 Units/mL potassium penicillin G, and 100 μg/mL streptomycin sulfate (Sigma Chemical, St. Louis, MO).

Effect of Osmolarity on Cell Physiology

Cells were inoculated into IMDM medium at different osmolarities. Osmolarity in IMDM medium was increased by the addition of either an ionic (NaCl or PBS) or nonionic (sucrose) species. The IMDM medium was prepared from powder and the pH was adjusted to 7.4 by the addition of sodium bicarbonate. Medium osmolarity was measured using an Osmometer (Osmette, model 2007, Precision Systems, Inc., Natick, MA) as 290 mOsm. Media with osmolarities of about twice this value (580 mOsm) were prepared by dissolving IMDM in (1) 9 g/L NaCl, (2) in PBS, and (3) in 0.31*M* sucrose solution. The pH was also adjusted to 7.4 in these media. The osmolarities in these media preparations were measured. Then these IMDM preparations were mixed with standard IMDM media at 290 mOsm to give final osmolarities of 290, 338, 386, 435, and 580 mOsm.

Cells growing exponentially in ordinary IMDM with 5% FBS were spun down at 200*g* for 10 min. They were then washed with fresh IMDM and were inoculated with IMDM with different osmolarities. Fetal bovine serum was added to all the media preparations at 5%. Batch cultivations were carried out in 100-mL spinner flasks at an initial cell density of 4×10^4 cells/mL. The volume in spinner flasks was 60 mL and they were kept at 37°C in a humidified incubator under 5% CO_2. Agitation at 100 rpm was provided by a MultiStir magnetic stirrer (Bellco).

Cell Size Determination Under Different Osmolarities

A Coulter counter (Model ZM with Channelyzer 256) was used for size determination. First, we studied cell volume changes in different osmolarities in nongrowth medium. Concentrated PBS was diluted in distilled water to give an osmolarity range of 200–500 mOsm. Cells were suspended in these solutions and the cell size distributions were determined. Second, we studied the kinetics of cell size changes during the batch culture experiments. Cell samples (4 mL) from duplicate flasks were combined and cell size was determined without any dilution. Cell size distributions were determined in a Coulter counter with channelyzer (Coulter) as described by Adams *et al.*[1]

Analytical Methods

Usually a total of 1-mL samples was taken twice daily. Cell counts were performed using a hemacytometer. Cell viability was determined by the trypan blue exclu-

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sion method. The samples were then centrifuged at 200*g* for 10 min and the supernatants were kept frozen at −80°C for later analysis. For the cell size measurements, an additional 4 mL sample was taken. The cell suspensions from duplicate flasks were combined to give a volume of 8 mL and cell size was determined in the Coulter counter.

The glucose and lactate concentrations were measured using a model 2000 Glucose/L-Lactate analyzer (Yellow Springs Instruments, Yellow Springs, OH). Ammonia was measured with an ion selective electrode (Orion, Boston, MA). Samples (100 μL) were mixed with 500 μL pH adjusting buffer solution (Orion, Boston, MA) and the response was recorded.[9] Antibody, IgG_1, was quantified using an enzyme-linked immunosorbent assay (ELISA), as described elsewhere.[8]

Mathematical Methods

The concentration time profiles were quantified in terms of growth rates, and metabolic uptake and waste production rates were evaluated during the exponential growth, as decribed in Ozturk.[8] The standard deviation on the kinetic parameters calculated from duplicate experiments was less than 10%.

RESULTS

Cell growth, substrate, product, and antibody concentration during batch cultivation at different osmolarities are presented in Figure 1. At 580 mOsm, cells did not grow. Different osmolarities in Figure 1 were obtained by the addition of PBS components into IMDM, as described in the Materials and Methods section. Using PBS, the physiological Na^+/K^+ concentration ratio was kept constant. Similar data was obtained for the addition of NaCl alone and for the addition of sucrose. Thus, these effects described below seem to be solely due to medium osmolarity.

Cell Growth

Cell growth was depressed at higher osmolarities, as illustrated in Figure 1. Specific growth and death rates

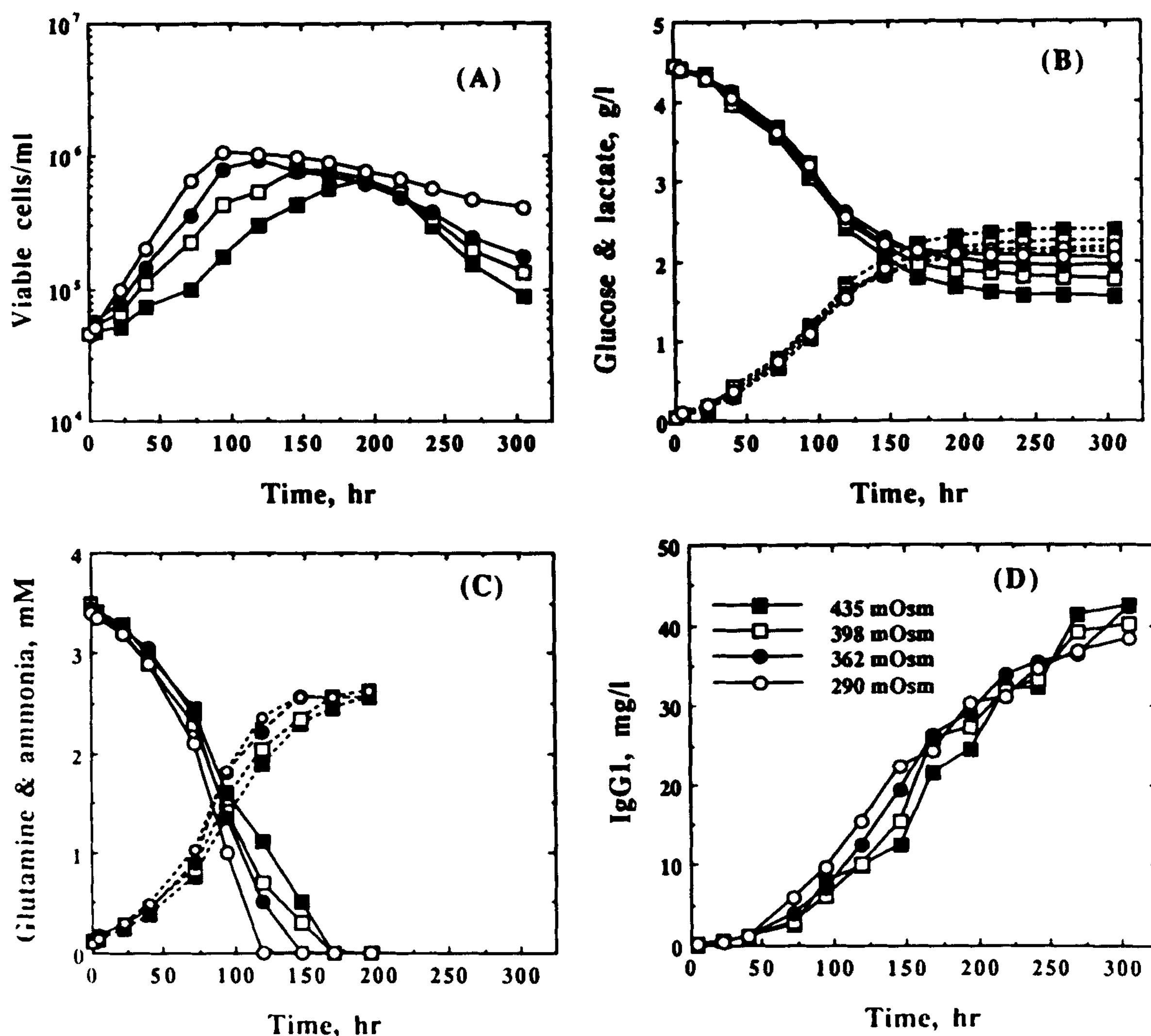

Figure 1. The time profiles of (A) viable cells, (B) glucose and lactate concentration, (C) glutamine and ammonia concentrations, and (D) monoclonal antibody concentrations for the batches of different osmolarities. Legend shows 290 mOsm (○), 326 mOsm (●), 380 mOsm (□), and 435 mOsm (■).

were calculated in the exponential phase and summarized in Table I. Increasing osmolarity from 290 to 435 mOsm decreased the specific growth rate by a factor of about 2. The maximum cell concentrations obtained in the batch mode were also dependent on media osmolarity, as can be seen from Figure 1. The specific death rates evaluated in the exponential phase were also influenced by the media osmolarity (Table I). About a threefold increase in the death rate was observed at 435 mOsm compared to media at isotonic osmolarity. For a given osmolarity, the death rates evaluated in the decline phase were about one order of magnitude higher than those evaluated in the exponential phase. The decline phase death rates were also higher at high osmolarities.

The specific growth and death rates were normalized using the values obtained in an isotonic solution and presented as a function of normalized osmolarity, i.e., the ratio of media osmolarity to isotonic osmolarity. [Fig. 2(A)]. The growth and death rates obtained in sucrose-containing batches are also included in this figure. It can be seen that the results presented here are independent of the source of increased osmolarity. Both ionic (PBS and NaCl) and nonionic (sucrose) components yielded similar rate parameters.

Cell Metabolism

The specific rates of consumption of glucose and glutamine, and the rates of production of lactate and ammonia were all increased at high osmolarities (Table I). An increase in osmolarity from 290 to 435 mOsm resulted in a more than twofold increase in these metabolic rates. In Figure 2(B), the metabolic rates were normalized to the values at isotonic media and plotted as a function of relative increase in osmolarity. We see that the metabolic rates increased essentially at the same rate. The yield coefficients of lactate from glucose and ammonia from glutamine remained constant in all osmolarities (Table I).

Antibody Synthesis

Although cell growth was lower at higher osmolarities, similar antibody concentrations were obtained for all the cultures [Fig. 1(D)]. This result was due to altered specific antibody productivity. Monoclonal antibody production rates were calculated using an integral method;

$$\frac{dMAb}{dt} = q_{Ab}X_v \quad \text{or}$$

$$MAb = q_{Ab}\int_0^t X_v dt \quad \text{if } q_{Ab} \text{ is constant}$$

where MAb is antibody concentration and q_{Ab} is the specific production rate, hence obtained from a plot of antibody concentration (MAb) versus time integral of viable cells ($\int_0^t X_v dt$). Figure 3(A) shows the curves obtained from the data presented in Figure 1. For all osmolarities we obtained straight lines, indicating a constant productivity for each culture. The slopes, hence the production, rates were different. Specific antibody production rates were higher at elevated osmolarities. More than a twofold increase in specific antibody production rate was obtained at 435 mOsm osmolarity [Fig. 3(B)].

Kinetics of Cell Size Variation

When the cells were suspended in PBS at different osmolarities, cell size rapidly equilibrated to the media osmolarity. Figure 4 summarizes the cell volume data at different osmolarities. Cells showed almost ideal osmotic volume responses to the external osmotic pressures. Ideal osmotic response of the cells to the external osmotic pressure is expressed by the Boyle-vant Hoff relation[3]:

$$P(V - b) = P_0(V_0 - b) = \text{const.} \quad (1)$$

where P_0 and V_0 are the isotonic pressure and volume, respectively; P and V are the corresponding values for a given osmolarity; and b is the nonsolvent volume of the cells. When the above equation is rearranged we obtain:

$$\frac{V}{V_0} = \frac{P_0}{P}\left(1 - \frac{b}{V_0}\right) + \frac{b}{V_0} \quad (2)$$

Then the plot of V/V_0 vs. P_0/P should give a straight line. Figure 4 shows that the initial response of 167.4G5.3 hybridoma cells followed this relationship with constants of $V_0 = 1250$ fL and $b = 500$ fL. The ratio of $b/V_0 = 0.4$ shows that about 40% of the cell is "incompressible." The balance of the cell volume swells in hypotonic solutions, and shrinks in hypertonic solu-

Table I. Growth and metabolic rates at different osmolarities.

Osmolarity (mOsm)	Growth rate (h^{-1})	Death rate (h^{-1})	Glucose utilization rate	Lactate production rate	Glutamine utilization rate	Ammonia production rate	$Y_{Lac/Glu}$ (mol/mol)	$Y_{NH_4^+/Gln}$ (mol/mol)
290	0.040	0.003	0.247	0.396	0.047	0.028	1.60	0.60
362	0.033	0.004	0.342	0.553	0.061	0.040	1.62	0.64
398	0.028	0.006	0.478	0.794	0.087	0.054	1.66	0.62
435	0.024	0.008	0.579	0.949	0.104	0.069	1.64	0.66

Metabolic rates are in μmol/10^6 cell h.

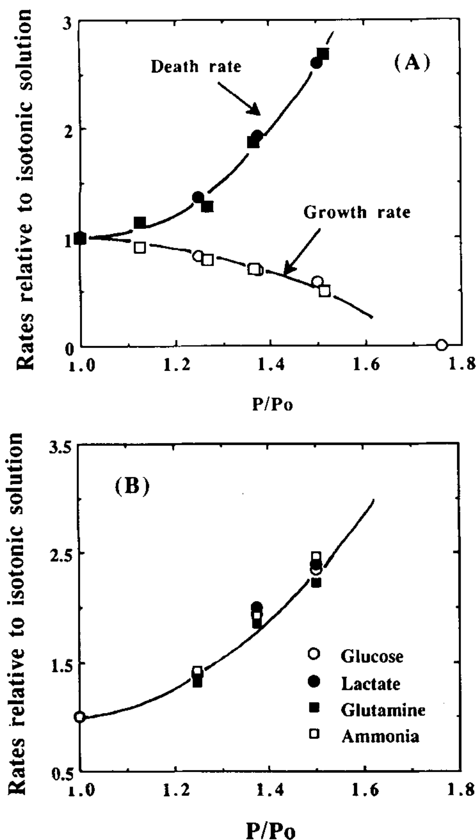

Figure 2. Effect of osmolarity on cell growth and metabolism. The rates were normalized using the values at P_0 = 290 mOsm and plotted as a function of normalized osmolarity, P/P_0: (A) growth and death rates for PBS addition (●) and for sucrose addition (square); (B) metabolic rates for glucose uptake (○), glutamine uptake (■), lactate production (closed circle), and ammonia production (□).

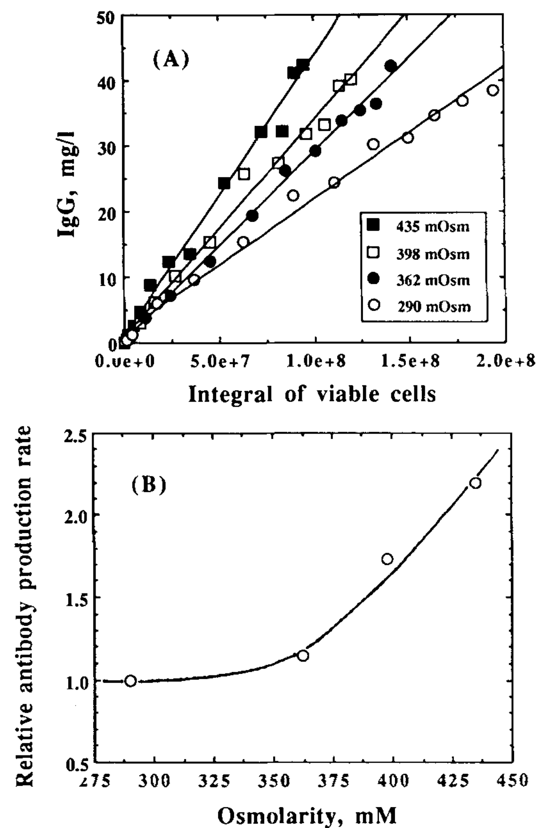

Figure 3. Effect of osmolarity on antibody production: (A) the integral method of evaluating the specific antibody production; (B) the influence of media osmolarity on specific antibody production rate.

tion in a fashion consistent with eq. (1). Similar ideal osmotic responses of living (cell type) cells were also observed.[5] Data for human lymphocytes and granulocytes is included in Figure 4 for comparison.[6] The hybridoma cells used showed similar response.

The above data were obtained when the cells were kept in a nongrowth medium. The transient changes in cell volume during cultivation at various osmolarities were very different [Fig. 5(A)]. Cell sizes were measured during batch culture. Under normal conditions, we observed a constant cell volume in the exponential phase followed by a decrease in the late exponential and death phase.[10] The cells when grown in hypertonic media exhibited significant change in the cell volume during the first 10 h of cultivation. Cells were smaller at higher osmolarities only at the beginning of the culture. This initial response was similar to that obtained in the nongrowth PBS media outlined above. The cell size increased during growth and the cells attained *larger* sizes at high osmolarities, which they kept for the rest of the experiment. This interesting behavior is illustrated in Figure 5(B). At the beginning of the experiment, cell size decreased after being introduced to high osmolarities, but after 24 hours of incubation, cell size had actually increased.

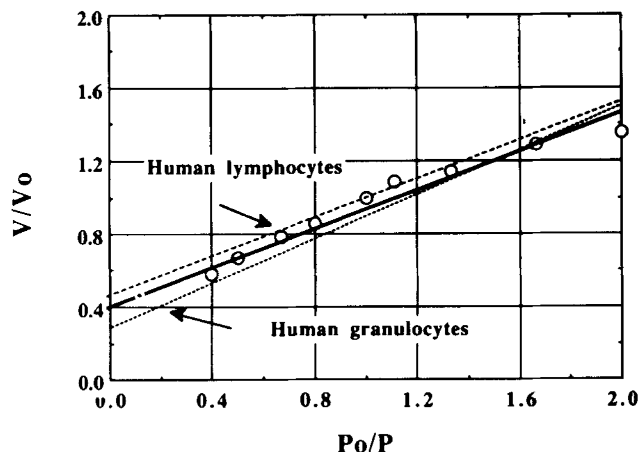

Figure 4. Effect of osmolarity on cell/cell size. Hybridoma cells were equilibrated with PBS at different osmolarities and the cell size was measured as Coulter volume. The volumes were normalized using isotonic volumes. The abscissa represents the isotonic osmotic pressure (P_0 = 290 mOsm) divided by the osmolarity of PBS solution. The data on human lymphocytes and granulocytes was taken from ref. 6.

DISCUSSION

Growth depression in hypertonic medium has been reported for MCL1 cells and for other hybridoma cells.[4,7,12] This decrease in growth rate is expected since hypertonic medium represents stressful conditions. Lower cell

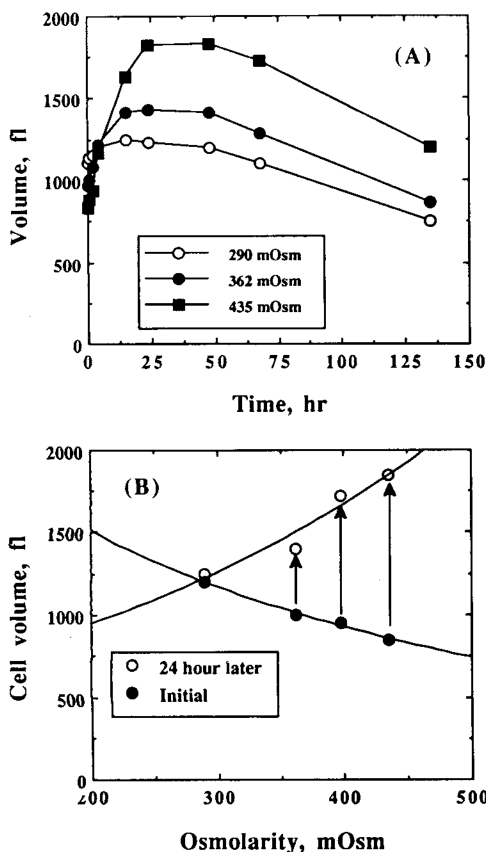

Figure 5. (A) Time profile of cell size during the batch culture; (B) the cell size at different osmolarities.

concentrations were obtained per batch because of lowered growth rates and increased death rates in hypertonic media.

The increase in specific (per cell) antibody production rate under hypertonic conditions may be important for cost-effective production of antibodies. We have also observed an increase in specific antibody productivity under non-optimal pH conditions.[11] The increase in the specific antibody productivity induced by elevated osmolarity and lower pH may be related to the environmental stresses placed on the cells.

However, the increase in specific antibody productivity may not result in a substantial difference in the antibody concentration obtained due to decreased cell growth rate and increased death rate. In culture systems where cell growth and production are separated, the environmental stress effects on antibody productivity can be utilized for a higher antibody concentration. In immobilized systems, such as hollow fibers, or beads, the cells are maintained at minimal growth rates. In these systems, media osmolarity can be increased to stimulate the productivity.

The single volume of 167.4G5.3 hybridoma cells initially exhibited an ideal response to changes in medium osmolarity in non-growth medium (PBS). These changes in cell volume were successfully described by eq. (1), and these volume changes were comparable to those observed in other cells such as lymphocytes and granulocytes. However, changes in cell volume under non-growth conditions may be misleading. At elevated osmolarities, actively dividing cells actually increase their volume with time. The mechanism underlying this regulation is not known but this behavior may influence the way the specific (per cell) metabolic rates are interpreted. The increase in metabolic activities and the antibody production rate at high osmolarities can be partially explained by the increase in the cell volume. These rates, calculated based on cell numbers (Table I), increased by a factor of 2.5 (Fig. 2). The cell volume increased by a factor of 1.5. Hence, a part of the increase in the metabolic rates and the antibody production rate may be attributed to changes in cell volume.

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References

1. Adams, R. B., Voelker, W. H., Gregg, E. C. 1967. Electrical Counting and Sizing of Mammalian Cells in Suspension: An Experimental Evaluation, Phys. Med. Biol., **12**: 79–92.
2. Briles, D. E., Forman, C., Hudak, S., Claflin, J. L. 1984. The Effects of Idiotype on the Ability of IgG_1 anti-phosphorylcholine Antibodies to Protect Mice from Fatal Infection with *Streptococus pneumoniae.* Eur. J. Immunol. **14**: 1027–1030.
3. Lucke, B., McCutcheon, M. 1932. The Living Cell as an Osmotic System and Its Permeability to Water. Physiol. Rev. **12**: 68–139.
4. Maiorella, B. 1988. paper presented at the 196th National Meeting of American Chemical Society in Los Angeles, CA.
5. Mazur, P., Schneider, U. 1979. Osmotic Responses of Preimplantation Mouse and Bovine Embryos and Their Cryobiological Implications. Cell Biophys. **8**: 259–284.
6. McGann, L. E., Walterson, M. L., Hogg, L. M. 1988. Light Scattering and Cell Volumes in Osmotically Stressed and Frozen-Thawed Cells. Cytometry, **9**: 33–38.
7. Oyaas, K., Berg, T. M., Bakke, O., Levine, D. W. 1989. "Hybridoma Growth and Antibody Production Under Conditions of Hyperosmotic Stress," in *Advances in Animal Cell Biology and Technology for Bioprocesses.* Spier, R. E., Griffiths, J. B., Stephenne, J., Crooy, P. J., Eds. Butterworth, London.
8. Ozturk, S. S. 1990. "Kinetic Characterization of Hybridoma Growth, Metabolism, and Monoclonal Antibody Production Rates," Ph.D. Thesis, University of Michigan, Ann Arbor.
9. Ozturk, S. S., Meyerhoff, M., Palsson, B. O. 1989. Measurement of Ammonia and Glutamine in Cell Culture Media by Gas Sensing Electrodes, Biotechnol. Techniques. **3**: 217–222.
10. Ozturk, S. S., Palsson, B. O. 1990, submitted. "Growth, Metabolic, and Antibody Production Kinetics of Hybridoma Cell Culture: I. Analysis of Batch Reactor Operation."
11. Ozturk, S. S., Palsson, B. O. 1990, submitted. "Growth, Metabolic, and Antibody Production Kinetics of Hybridoma Cell Culture: II. Effects of Serum, Dissolved Oxygen, and pH."
12. Passini, C. A., Goochee, C. F. 1989. "The Response of a Mouse Hybridoma Cell Line to Environmental Stress," paper presented at AIChE 1989 Annual Meeting, San Francisco, CA, November 5–10.