Cytotechnology **28:** 213–227, 1998.

Effects of CO_2 and osmolality on hybridoma cells: growth, metabolism and monoclonal antibody production

Vivian M. deZengotita*, Roy Kimura*,† & William M. Miller‡
Chemical Engineering Department, Northwestern University, Evanston, IL 60208-3120, U.S.A.
E-mail: wmmiller@nwu.edu

Received 14 September 1998; accepted 14 September 1998

Key words: antibody production, carbon dioxide, cell metabolism, continuous culture, inhibition

Abstract

CO_2 partial pressure (pCO_2) in industrial cell culture reactors may reach 150–200 mm Hg, which can significantly inhibit cell growth and recombinant protein production. Due to equilibrium with bicarbonate, increased pCO_2 at constant pH results in a proportional increase in osmolality. Hybridoma AB2-143.2 cell growth rate decreased with increasing pCO_2 in well-plate culture, with a 45% decrease at 195 mm Hg with partial osmolality compensation (to 361 mOsm kg^{-1}). Inhibition was more extensive without osmolality compensation, with a 63% decrease in growth rate at 195 mm Hg and 415 mOsm kg^{-1}. Also, the hybridoma death rate increased with increasing pCO_2, with 31- and 64-fold increases at 250 mm Hg pCO_2 for 401 and 469 mOsm kg^{-1}, respectively. The specific glucose consumption and lactate production rates were 40–50% lower at 140 mm Hg pCO_2. However, there was little further inhibition of glycolysis at higher pCO_2. The specific antibody production rate was not significantly affected by pCO_2 or osmolality within the range tested. Hybridomas were also exposed to elevated pCO_2 in continuous culture. The viable cell density decreased by 25–40% at 140 mm Hg. In contrast to the well-plate cultures, the death rate was lower at the new steady state at 140 mm Hg. This was probably due to higher residual nutrient and lower byproduct levels at the lower cell density (at the same dilution rate), and was associated with increased cell-specific glucose and oxygen uptake. Thus, the apparent effects of pCO_2 may vary with the culture system.

Abbreviations: CC – continuous culture; D – dilution rate; f_v – fraction of viable cells; k_d – specific death rate; pCO_2 – carbon dioxide partial pressure; q_{glc} – specific glucose consumption rate; q_{lac} – specific lactate production rate; q_{O_2} – specific oxygen consumption rate; q_P – specific protein production rate; RQ – respiratory quotient; X_d – dead cell density; X_t – total cell density; X_v – viable cell density; $Y_{lac,glc}$ – apparent yield of lactate from glucose; $Y_{n,glc}$ – apparent yield of cells from glucose; $Y_{O_2,glc}$ – ratio of oxygen to glucose consumption; μ – specific growth rate.

Introduction

CO_2 is produced via catabolic reactions, and is required for the synthesis of pyrimidines, purines, and fatty acids. Dissolved CO_2 is hydrated and is in equilibrium with H^+ and HCO_3^-:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad (1)$$

Inhibition by CO_2 is due in part to medium acidification if pH is not controlled. If pH is controlled by base addition, the equilibrium in reaction (1) is driven farther to the right, such that osmolality increases with pCO_2. High osmolality by itself may have detrimental effects on cells (Kurano et al., 1990; Ozturk and Palsson, 1991).

* VMdZ and RK contributed equally to the results in this article.
† Present address: MedImmune, Inc., 35 W. Watkins Mill Rd., Gaithersburg, MD 20878, U.S.A.
‡ Author for all correspondence.

Information on CO_2 production by cultured cells is still relatively scarce. Reported values for the respiratory quotient (RQ; mol CO_2 produced per mol O_2 consumed) range from 0.6 to 1.2 (Aunins and Henzler, 1993; Lovrecz and Gray, 1994; Gray et al., 1996). The physiological range for CO_2 partial pressure (pCO_2) is 31–54 mm Hg (Altman and Dittmer, 1971). The RQ and CO_2 transport relations (Royce and Thornhill, 1991) can be used to estimate pCO_2 for different aeration strategies. CO_2 accumulation is greatest in cultures oxygenated with a low flow rate and small bubbles of pure O_2 – with pCO_2 predicted to reach 150–200 mm Hg (Kimura and Miller, 1996; Gray et al., 1996; Zupke and Green, 1998). pCO_2 values in this range have been reported for a 200-L culture sparged with small amounts of O_2 (Aunins et al., 1993), 1800–2500-L production bioreactors (Drapeau et al., 1990; Zupke and Green, 1998), and a high-cell-density perfusion bioreactor (Taticek et al., 1998).

pCO_2 values in the range of 120–200 mm Hg have been shown to inhibit growth and recombinant protein production by CHO and other cells. CHO cell growth and cell-specific M-CSF production (q_{M-CSF}) were both ~40% lower at a pCO_2 of 165 mm Hg than at 53 mm Hg (Drapeau et al., 1990). Similarly, Gray et al. (1996) reported dose-dependent decreases in CHO cell density, viability, and specific production rate – such that total productivity of a recombinant viral antigen in a 10-L perfusion bioreactor decreased by 69% at 148 mm Hg. We have previously reported that CO_2 inhibits CHO cell growth in a dose-dependent manner, with a greater decrease when there is no compensation for increased osmolality (Kimura and Miller, 1996). The specific growth rate (μ) decreased by 43% at 250 mm Hg pCO_2 without osmolality compensation. q_{tPA} decreased to 73% of control at 140 mm Hg without osmolality compensation (Kimura and Miller, 1996). Decreases in specific productivity of BHK-21 cells have been observed beginning at 50–80 mm Hg (Taticek et al., 1998). It has also been reported that recombinant protein production by infected *Sf*-9 insect cells is markedly delayed under 115 mm Hg pCO_2 (Garnier et al., 1996). Early cell death has been observed in NS/0 myeloma cell cultures with a final pCO_2 of 120 mm Hg (Aunins and Henzler, 1993). However, there was no change in the specific production rate of these cells.

Hybridomas are used extensively for monoclonal antibody production. Industrial hybridoma cultures may reach very high cell densities in large-scale reactors, so that CO_2 accumulation is likely to be a problem. Here, we present the effects of elevated pCO_2 – with and without osmolality compensation – on hybridoma growth, death, glucose metabolism, and antibody production in well-plate cultures. Continuous culture and batch experiments in a controlled bioreactor were used to further characterize the effects of concurrent increases in pCO_2 and osmolality, including changes in oxygen consumption.

Materials and methods

Cell line and medium

The hybridoma AB2-143.2 cell line (University of California, San Francisco) is an Sp2/0-derived mouse-mouse hybridoma that produces an IgG_{2a} monoclonal antibody against benzene-arsonate (Hornbeck and Lewis, 1985). Unless noted, all reagents were purchased from Sigma (St. Louis, MO). AB2-143.2 cells were grown in a custom low-salt (35.5 mg L^{-1}) DMEM:F12 mixture with 15 mM HEPES (Irvine Scientific; Santa Ana, CA) for well-plate experiments and a standard DMEM:F12 mixture with 25 mM HEPES for bioreactor experiments. Both media were supplemented with MEM nonessential amino acids (at 1.15x), sodium bicarbonate (29 mM), glutamine (to 6 mM; Gibco; Grand Island, NY), HEPES (to 25 mM), sodium selenite (20 nM), ethanolamine (20 μm), bovine insulin (5 mg L^{-1}), bovine holo-transferrin (30 mg L^{-1}), and 2% (v/v) fetal bovine serum (Gibco).

Low-salt medium used for stock cultures, control experiments at 36 mm Hg pCO_2 (5% CO_2), and high pCO_2 experiments not corrected for osmolality was supplemented with 5.71 g L^{-1} of NaCl. Media for experiments at 140, 195, and 250 mm Hg pCO_2 with partial osmolality compensation were supplemented with 4.73, 4.17, and 3.62 g L^{-1} of NaCl, respectively. Media for control pCO_2 experiments with osmolalities of 342, 357, and 376 mOsm kg^{-1} were supplemented with 6.75, 7.24, and 7.86 g L^{-1} of NaCl, respectively. Media for well-plate experiments were supplemented with 100 U mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin sulfate. Stock cultures and reactor experiments were conducted without antibiotics. The AB2-143.2 cells tested negative for mycoplasma (Mycoplasma TC Kit, Genprobe; San Diego, CA).

Well-plate experiments

Three types of experiments were conducted in six-well-plates: (1) elevated pCO_2 (140, 195, and 250 mm Hg)–partial osmolality compensation (337, 361 and 401 mOsm kg^{-1}, respectively); (2) elevated pCO_2 (140, 195, and 250 mm Hg) – uncompensated osmolality (370, 415, and 469 mOsm kg^{-1}, respectively); and (3) elevated osmolality (342, 357, and 376 mOsm kg^{-1}) at 36 mm Hg pCO_2. Cells in well-plates were exposed to high pCO_2 in a modular incubator chamber (Forma Scientific; Marietta, OH). The chamber was fed with controlled amounts of air and CO_2 using flowmeters (Cole-Parmer; Vernon Hills, IL) with needle valves. pCO_2 of the gas mixture was checked using a blood gas analyzer (Instrumentation Laboratories Model 1306; Lexington, MA). The inlet gas line was sparged through a stainless steel frit into a beaker of water to keep the chamber humidified.

Medium for well-plate experiments was transferred into T-150 flasks and pre-equilibrated in a 5% CO_2 incubator or in a modular incubator chamber gassed with the appropriate CO_2/air mixture for 8–12 hr. For experiments at 140, 195, and 250 mm Hg pCO_2 the pH was then adjusted to 7.25±0.05 by adding 7.1, 12.5, and 17.8 μL, respectively, of 5 M NaOH per mL of medium, after which the medium was re-equilibrated for 8–12 hr in the high pCO_2 environment.

Mid-exponential AB2-143.2 cells from stock cultures were inoculated at a density of 5×10^4 cells mL^{-1} into 5 mL of medium pre-equilibrated at the appropriate pCO_2, after which the well-plates were placed either in a 5% CO_2 incubator or in a modular incubator chamber. These hybridomas do not grow well at seeding densities less than 1×10^4 cells mL^{-1}. The higher seeding density assured that any effects observed were due only to the test conditions. Samples were taken at 12-hour intervals. pH was measured by drawing medium from a well directly into a glass syringe, and injecting the medium into the blood gas analyzer. A 2-point calibration for pH, pO_2 and pCO_2 was performed before each sample.

Bioreactor experiments

Continuous culture and batch experiments were performed in a 3-L Applikon (Foster City, CA) bioreactor equipped with pH (Broadley-James; Santa Ana, CA) and dissolved oxygen (Ingold; Wilmington, MA) probes. The working volume was 1.5 L and the impeller speed was 175 rpm. The medium pH was controlled at 7.20±0.02 by addition of 1 M NaOH. A stainless steel mesh filter with a pore opening of 75 μm was attached to the base line so that the NaOH would be diluted with medium inside the filter and then diffuse out. There were no signs of cell damage around the NaOH filter. At elevated pCO_2 more NaOH was required to maintain constant pH. Therefore, increases in pCO_2 were accompanied by increases in medium osmolality.

Medium pCO_2 and pH were determined off-line using the blood gas analyzer. Samples were taken through a sampling system that consisted of a four-way valve (Whitey SS-43YTFS1-049, Swagelok; Solon, OH) and stainless steel HPLC tubing (Waters; Milford, MA). The system was purged with 10–30 mL of medium using a 50-mL syringe. A sample was extracted into a 2.5-mL glass syringe and immediately injected into the blood gas analyzer. Any drift in the pH probe was corrected based on the blood gas analyzer pH measurement.

For continuous culture experiments, the dilution rate was maintained at 0.022 hr^{-1} for one run and at 0.028 hr^{-1} for two others. A spin filter with a mesh size of 75 μm was attached to the impeller to be used when the cells were in danger of washing out.

Assays

The viable cell density and viability were measured with a hemacytometer using the Trypan blue dye exclusion method. Glucose and lactate concentrations were determined using a YSI model 2700 biochemistry analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). The osmolality of culture medium equilibrated at different pCO_2 values, with or without osmolality compensation, was measured using both a Wescor (Logan, UT) Model 5520 Vapro vapor pressure osmometer and an Advanced Instruments (Norwood, MA) 3D3 freezing point osmometer. Samples for osmolality measurement were pipetted into an 0.5-mL Eppendorf (Brinkman, Westbury, NY) microcentrifuge tube (completely filled), capped to minimize degassing, and analyzed. The elapsed time from sampling to analysis was less than 2 minutes. Reported osmolalities are the average of the vapor pressure and freezing point values, since both methods gave comparable results (manuscript in preparation).

Antibody concentrations were determined using a sandwich ELISA in 96-well plates coated with polyclonal goat anti-mouse IgG antibody (Pierce; Rockford, IL). Bound mouse IgG was detected using

horse radish peroxidase-conjugated goat anti-mouse IgG antibody (Pierce) and developed with an *o*-phenylenediamine dihydrochloride solution. ELISA standards were prepared from pooled hybridoma supernatant using an Affi-Prep protein A column (Pharmacia; Piscataway, NJ).

Calculation of metabolic parameters

Specific growth (μ) and death (k_d) rates for the well-plate and batch reactor experiments were based on the material balances for total and dead cells:

$$\frac{dX_t}{dt} = \mu X_v \tag{2}$$

$$\frac{dX_d}{dt} = k_d X_v \tag{3}$$

where X_t, X_v, and X_d are the total, viable, and dead cell densities, respectively. Equations (2) and (3) were integrated assuming constant μ and k_d. Thus, the values for μ and k_d were obtained by plotting the value of X_t and X_d, respectively, vs. the integral of (X_v dt) at each time point. Similarly, the specific glucose consumption rate (q_{glc}), lactate production rate (q_{lac}), and antibody production rate (q_{Ab}) were obtained by plotting the glucose, lactate, and antibody concentration, respectively vs. the integral of (X_v dt). The apparent yield of lactate from glucose ($Y_{lac,glc}$) was determined from q_{lac}/q_{glc} and the apparent yield of cells from glucose ($Y_{n,glc}$) from μ/q_{glc}. In several experiments the final data point(s) for X_t or X_d deviated significantly from the straight line for constant μ or k_d, respectively. In these cases, the final time point(s) was not used for determining culture parameters.

The volumetric oxygen uptake rate (Q_{O_2}) for batch reactor cultures was obtained from on-line measurements of the O_2, N_2, and CO_2 gas flowrates (Sierra Instruments model 820 and 840 mass flowmeters; Carmel Valley, CA) into the reactor headspace. The calculated values for pO_2 were consistent with the headspace values measured using the blood gas analyzer. The specific oxygen uptake rate (q_{O_2}) was obtained by dividing $Q_{O_2}(t)$ by a curve-fit of $X_V(t)$ and taking the average from 40–60 hr. The ratio of oxygen consumption to glucose consumption ($Y_{O_2,glc}$) was determined from q_{O_2}/q_{glc}.

The derivative method was employed to calculate the various parameters for continuous culture, as previously described (Miller et al., 1988). Derivative terms were determined after 3-point smoothing.

Statistical analysis

The Kruskal-Wallis test (Glantz, 1981) was used to determine the statistical significance of the effects of elevated pCO_2–partial osmolality compensation, elevated pCO_2–uncompensated osmolality, and constant pCO_2–elevated osmolality for the well-plate experiments. The Kruskal-Wallis test is the non-parametric analog of one-way ANOVA, and does not assume a normal distribution or constant variance.

Results

Well plate experiments

In order to decouple the effects of elevated pCO_2 from those of elevated osmolality, three types of experiments were performed. In the first type, the initial medium osmolality was adjusted so that the final osmolality, after equilibration and pH adjustment at the desired pCO_2, would be about midway between the control level (310 mOsm kg^{-1}) and the osmolality in uncompensated cultures. (Our intention was to achieve full osmolality compensation. However, we recently discovered that the osmolality values that we had reported for these conditions (Kimura and Miller, 1996) are not correct; see Note in Proof). In the second type, the initial medium osmolality was not adjusted. Finally, the third type of experiments evaluated the effects of elevated osmolality at control pCO_2. pH in cultures at 36 mm Hg pCO_2 remained at 7.25–7.3 for the first 36 hr, and then decreased – reaching values of 7.1–7.2 at 48 hr and 6.9–7.1 at 60 hr (Kimura, 1996). In contrast, the pH changed by only $\sim$0.1 unit during culture at elevated pCO_2. More stable pH at elevated pCO_2 has also been observed for CHO cells (Kimura and Miller, 1996), and is probably due to the greater bicarbonate concentration.

Cell growth and death

Representative growth curves for cells exposed to elevated pCO_2 (with and without osmolality adjustment) are shown in Figure 1. The effects of pCO_2 on cell viability are shown in Figure 2 for the same experiments. Higher pCO_2 was associated with greater inhibition of cell growth, as well as more extensive cell death. For both parameters, the effects were more severe when osmolality was not adjusted. There was some variability between the growth curves for replicate experiments, but large differences were only observed

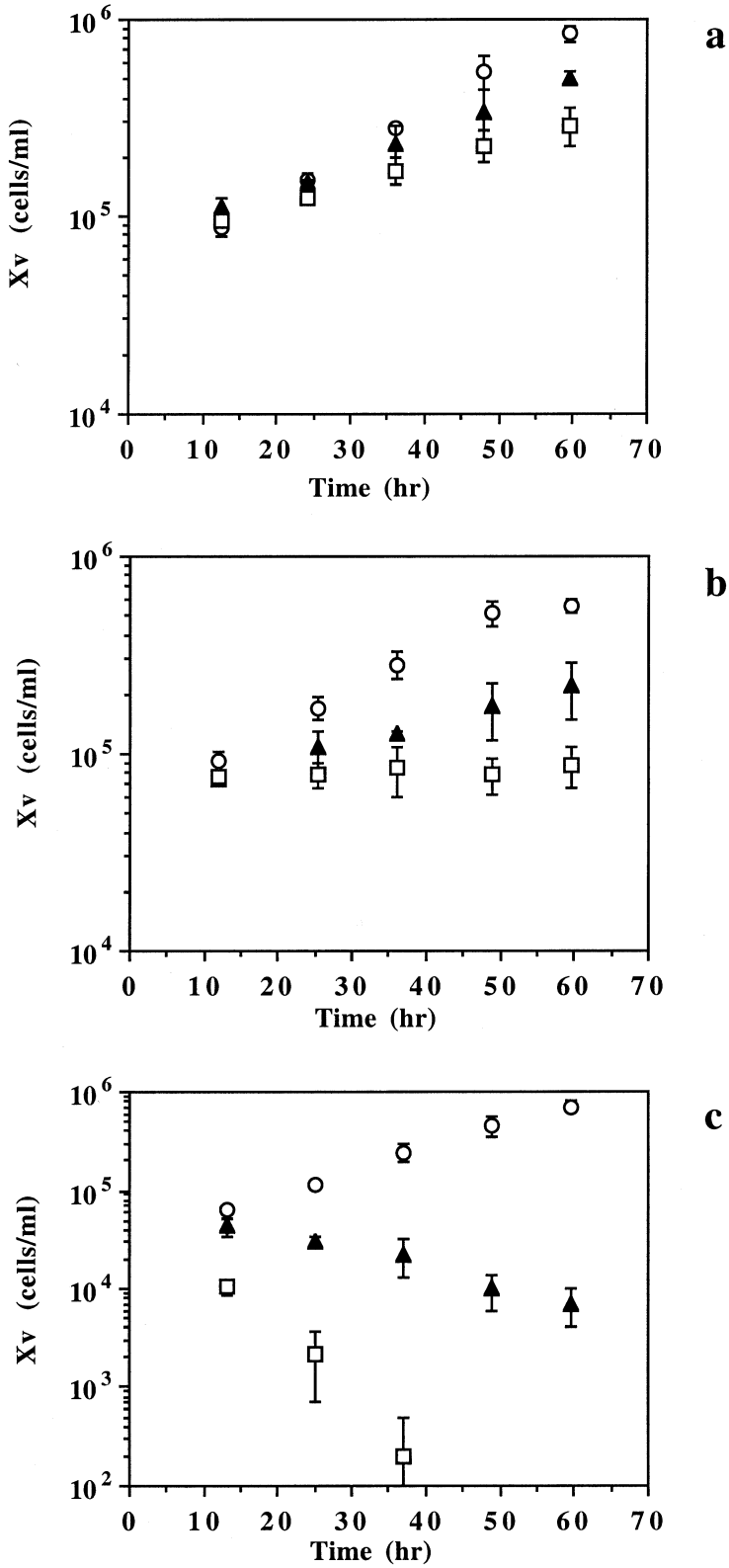

Figure 1. Growth curves for representative well-plate experiments at (a) 140, (b) 195, and (c) 250 mm Hg pCO_2 with (filled triangles) and without (open squares) osmolality adjustment. Control cultures at 36 mm Hg and 310 mOsm kg^{-1} (open circles) are shown for each experiment. Data represent the mean and standard deviation for 3 replicate wells.

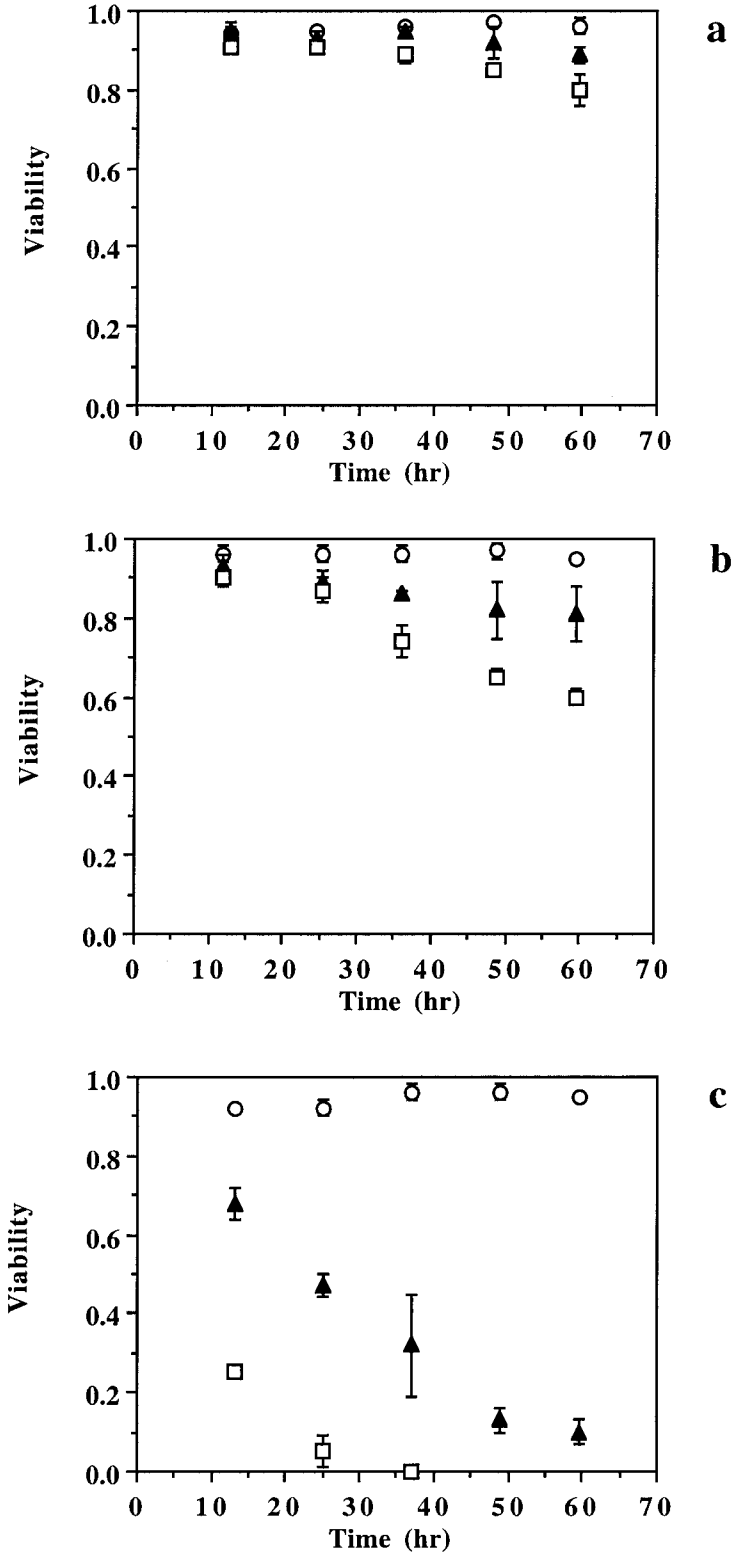

Figure 2. Cell viability for the well plate experiments shown in Figure 1. The symbols are the same as in Figure 1.

Table 1. Effects of elevated pCO_2 and/or osmolality on AB2-143.2 hybridoma cells in well-plate cultures. Based on the Kruskal-Wallis test, both elevated pCO_2-partial osmolality compensation and elevated pCO_2- uncompensated osmolality had a significant effect on specific growth rate ($P < 0.010$), specific death rate ($P < 0.025$), specific glucose consumption rate ($P < 0.025$), and specific lactate production rate ($P < 0.025$). Values are given as mean ± standard deviation, with the number of replicates indicated in parentheses

pCO_2 and osmolality	Cell growth rate	Cell death rate	Specific antibody production rate	Specific glucose consumption rate	Specific lactate production rate	Apparent yield of cells from glucose	Apparent yield of lactate from glucose
	(hr^{-1})	(10^{-3} hr^{-1})	(10^{-13}g $cell^{-1}$ hr^{-1})	(10^{-13} mol $cell^{-1}$ h^{-1})		(10^{10} cells mol^{-1})	(mol/mol)
36 mm Hg 310 mOsm kg^{-1}	0.049±0.005 (7)	2.08±1.33 (7)	7.23±2.02 (7)	5.16±0.59 (7)	10.50±0.12 (7)	9.62±1.50 (7)	2.03±0.08 (7)
140 mm Hg 337 mOsm kg^{-1}	0.038±0.000 (2)	3.70±0.12 (2)	6.29±1.25 (2)	3.17±1.77 (2)	5.14±0.62 (2)	14.2±7.92 (2)	1.79±0.82 (2)
195 mm Hg 361 mOsm kg^{-1}	0.027±0.006 (4)	7.86±0.71 (4)	6.66±1.74 (4)	2.64±0.61 (4)	4.98±0.67 (4)	11.0±4.14 (4)	1.83±0.15 (4)
250 mm Hg 401 mOsm kg^{-1}	ND	64.2 ±0.23 (2)	ND	ND	ND	ND	ND
140 mm Hg 370 mOsm kg^{-1}	0.032±0.001 (2)	4.48±3.97 (2)	9.16±4.86 (2)	3.26±1.16 (2)	5.35±2.01 (2)	10.6±4.19 (2)	1.63±0.04 (2)
195 mm Hg 415 mOsm kg^{-1}	0.018±0.007 (4)	13.0 ±12.7 (4)	9.57±4.27 (4)	2.95±0.62 (4)	5.18±1.33 (4)	6.53±3.10 (4)	1.61±0.20 (4)
250 mm Hg 469 mOsm kg^{-1}	ND	133±50.7 (2)	ND	ND	ND	ND	ND
36 mm Hg 342 mOsm kg^{-1}	0.042±0.003 (2)	2.12±0.69 (2)	10.50±4.77 (2)	6.21±0.31 (2)	12.6 ±1.06 (2)	6.78±0.80 (2)	2.02±0.03 (2)
36 mm Hg 357 mOsm kg^{-1}	0.045±0.001 (3)	2.62±1.60 (3)	8.40±2.42 (3)	5.82±0.35 (3)	12.0 ±1.18 (3)	7.74±0.32 (3)	2.06±0.08 (3)
36 mm Hg 376 mOsm kg^{-1}	0.042±0.008 (3)	3.25±0.2 (2)	9.64±4.07 (3)	5.77±1.27 (3)	11.5 ±2.79 (3)	7.56±2.76 (3)	1.98±0.11(3)

ND indicaties not determined.

for cultures at 195 mm Hg. For example, two sets of experiments at 195 mm Hg exhibited similar inhibition to that shown for 140 mm Hg in Figures 1 and 2, while another set of experiments at 195 mm Hg exhibited inhibition intermediate between those shown for 195 and 250 mm Hg in Figures 1 and 2.

The effects of elevated pCO_2 and/or osmolality on AB2-143.2 hybridoma cells are summarized in Table 1. With partial osmolality compensation, μ decreased to 78 and 55% of control at 140 and 195 mm Hg pCO_2, respectively. Without osmolality compensation, μ decreased to 65 and 37% of control at the same pCO_2 values. Growth rates were not calculated for 250 mm Hg pCO_2 because the small changes in X_t made it difficult to obtain reliable values. Elevated osmolality alone decreased μ to 86% of control at 342 mOsm kg^{-1}, with no further inhibition at 357 or 376 mOsm kg^{-1}. With partial osmolality compensation, k_d was 1.8, 3.8, and 31 times that of control cultures at 140, 195 and 250 mm Hg pCO_2, respectively. Without osmolality compensation, k_d was 2.2, 6.2, and 64 times that of control at the same pCO_2 values. Elevated osmolality alone increased k_d by 1.3 and 1.6 times that of the control cultures at 357 and 376 mOsm kg^{-1}, respectively. Thus, elevated pCO_2 and osmolality appear to have a synergistic effect on μ and k_d.

Glucose metabolism

q_{glc} was 61% of that in control cultures at 140 mm Hg pCO_2 and 337 mOsm kg^{-1} (Table 1). In contrast to what was observed for μ and k_d, there was relatively little further decrease in q_{glc} at 195 mm Hg, and little if any effect of osmolality compensation. A similar trend was observed for q_{lac}. However, the decrease in q_{lac} was 10–20% greater than that in q_{glc}, so that $Y_{lac/glc}$ was slightly lower at elevated pCO_2. Increased osmolality alone did not significantly affect q_{glc} or q_{lac}, although the values tended to be higher at elevated osmolality. Taken together, the above results suggest that elevated pCO_2 decreases glycolysis and that increased osmolality does not contribute to this effect. There was no significant difference in $Y_{n,glc}$ with pCO_2 with or without osmolality compensation. However, the lower value of $Y_{n,glc}$ at 195 mm Hg and 415 mOsm kg^{-1} is consistent with lower values of $Y_{n,glc}$ for elevated osmolality at 36 mm Hg.

Antibody production

q_{Ab} was not significantly affected when cells were exposed to elevated pCO_2 and/or osmolality (Table 1). However, q_{Ab} values tended to be higher without osmolality compensation. This is consistent with a tendency towards higher q_{Ab} values with elevated osmolality at 36 mm Hg.

Continuous flow reactor experiments

The effects of elevated pCO_2 were also evaluated in continuous culture. When pCO_2 was increased, a greater amount of NaOH was needed to maintain pH 7.2. Thus, these experiments are analogous to the well-plate experiments without osmolality compensation.

Results from experiments conducted at dilution rates (D) of 0.022 hr^{-1} and 0.028 hr^{-1} are shown in Figures 3 and 4, respectively. pCO_2 was increased in steps from 50 to 90 and then 140 mm Hg. There was little change in X_v or viability (f_v) when pCO_2 was increased from 50 to 90 mm Hg (Figures 3a and 4a). Although pCO_2 was only maintained at 90 mm Hg for 4 days in these experiments, X_v was essentially constant for 8 days at 90 mm Hg in a third experiment (also at D = 0.028 hr^{-1}; not shown). X_v decreased shortly after pCO_2 was increased to 140 mm Hg, and reached a new steady state at a lower value after 5–8 days. In both cases, k_d was slightly lower at 140 mm Hg, as evidenced by an increase in f_v. The greater decrease in X_v (40% vs. 28%) and the transient decrease in f_v for the experiment shown in Figure 4 may be due to the higher dilution rate (0.028 vs. 0.022 hr^{-1}). In this latter experiment, μ (= D/f_v) was 0.033 hr^{-1} at 90 mm Hg (f_v = 0.85) and 0.031 hr^{-1} for the new steady state at 140 mm Hg (f_v = 0.9). This is close to the value of 0.032 hr^{-1} obtained at 140 mm Hg and 370 mOsm kg^{-1} in well-plate culture. Borderline stability (D near μ_{max} at 140 mm Hg pCO_2) at D = 0.028 hr^{-1} is suggested by a continuing decrease in X_v after the increase in pCO_2 to 140 mm Hg in a second experiment at D = 0.028 hr^{-1} (not shown). However, the lack of recovery in this latter case may have been due in part to a transient increase in pCO_2 to 160 mm Hg (pCO_2 >140 mm Hg for ~24 hr).

Cell metabolic patterns are shown in Figures 3b and 4b. There was no consistent trend in q_{glc} at 90 mm Hg – with either a slight increase (Figure 3b), a slight decrease (Figure 4b), or no change (not shown). However, there was a consistent increase in q_{glc} at 140 mm Hg. Changes in q_{lac} were generally similar to those for q_{glc} for each experiment. $Y_{n,glc}$ did not change at 90 mm Hg, but decreased by ~20% at 140 mm Hg (not shown). Similarly to q_{glc}, q_{O_2} was not affected by 90 mm Hg pCO_2, and increased by 20–30% at 140 mm

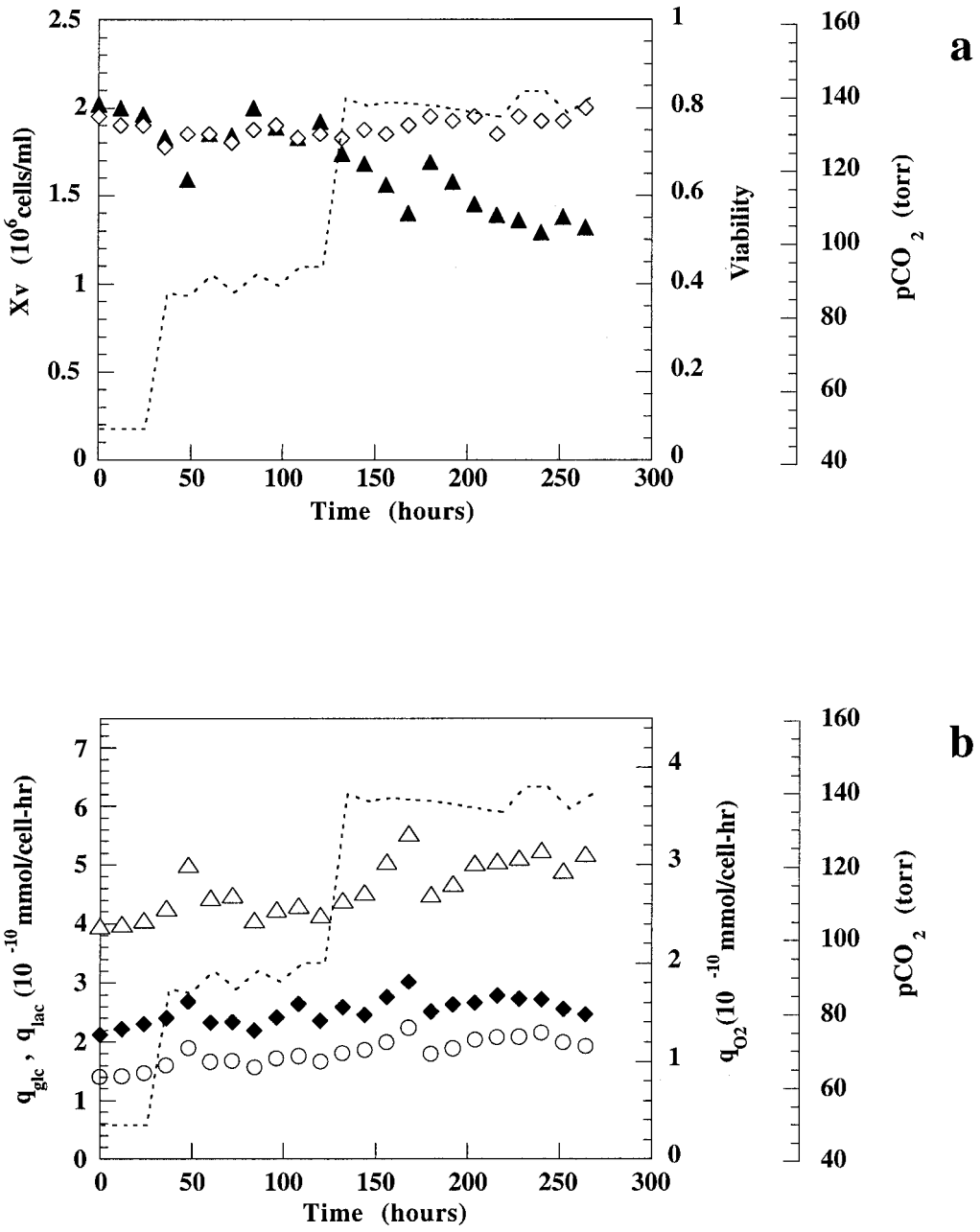

Figure 3. Hybridoma continuous culture at a dilution rate of 0.022 hr^{-1}. (a) Viable cell density (solid triangles) and viability (open diamonds). (b) Specific glucose consumption rate (open circles), lactate production rate (solid diamonds), and oxygen consumption rate (open triangles). The pCO_2 profile is indicated by a dashed line.

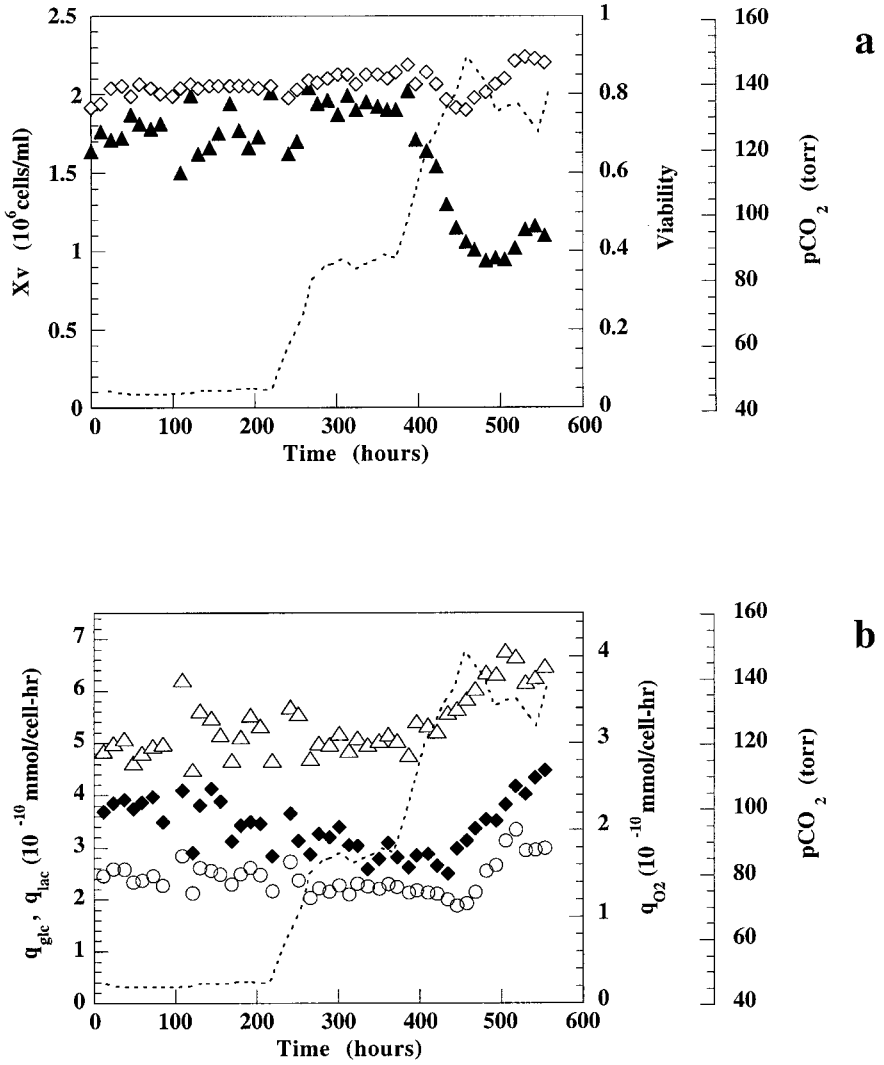

Figure 4. Hybridoma continuous culture at a dilution rate of 0.028 hr^{-1}. The symbols are the same as those shown in Figure 3.

Hg. q_{O_2} was not likely affected by changes in the glucose concentration, which never dropped below 3 mM. $Y_{O_2,glc}$ was very steady at a value of ~1.4 mol/mol, except for a transient 35% increase during the increase in pCO_2 to 140 mm Hg for the experiment shown in Figure 4. There was no consistent effect of 140 mm Hg pCO_2 on q_{Ab} – with a 100% increase (with considerable scatter) for the experiment shown in Figure 3 and a slight decrease for the experiment shown in Figure 4 (not shown).

Batch bioreactor experiments

Several batch reactor experiments were performed at 140 mm Hg pCO_2 and 370 mOsm kg^{-1} to determine whether the differences observed between well-plate and continuous culture experiments (see Discussion) were due in part to the presence of pH and DO control in the continuous culture experiments. Representative growth curves for control and 140 mm Hg pCO_2 cultures are shown in Figure 5. Although the decrease in viability at 140 mm Hg (Figure 5b and results not shown) is not as great as that shown in Figure 2a, it is similar to that observed in a replicate well-plate culture (Kimura, 1996). Apoptosis was identified as the primary mechanism of cell death in the controlled batch cultures, with somewhat more extensive apoptosis in cultures at 140 mm Hg pCO_2 (not shown).

Table 2. Effects of elevated pCO_2 and osmolality on AB2-143.2 hybridoma cells in batch reactor cultures. Values are given as mean ± standard deviation (n = 2 for each condition)

pCO_2 and osmolality	Cell growth rate	Cell death rate	Specific antibody production rate	Specific glucose consumption rate	Specific lactate production rate	Specific oxygen consumption rate	Apparent yield of cells from glucose	Apparent yield of lactate from glucose
	(hr^{-1})	(10^{-3} hr^{-1})	(10^{-13}g $cell^{-1}$ hr^{-1})	(10^{-13} mol $cell^{-1}$ h^{-1})			(10^{10} cells mol^{-1})	(mol/mol)
36 mm Hg 310 mOsm kg^{-1}	0.051±0.005	1.71±0.65	19.8±6.29	4.91±1.17	9.94±2.35	6.8±2.8	10.50±1.48	2.03±0.01
140 mm Hg 370 mOsm kg^{-1}	0.037±0.001	4.17±1.03	33.4±5.09	3.89±0.27	7.11±0.33	3.3±0.2	9.41±0.83	1.83±0.21

The effects of 140 mm Hg pCO_2 and 370 mOsm kg^{-1} in batch reactor culture are summarized in Table 2. The 27% decrease in μ and the 2.4-fold increase in k_d were similar to those in well-plates (Table 1). In contrast, the 21% decrease in q_{glc} and 28% decrease in q_{lac} in the bioreactor were only about 60% as extensive as those in well-plates. As for the well-plates, there was no significant change in $Y_{n,glc}$. q_{O_2} was 51% lower at 140 mm Hg. The larger difference in q_{O_2} relative to q_{glc} resulted in a 38% lower value of $Y_{O_2,glc}$ at 140 mm Hg. Specific antibody production was about 70% greater at elevated pCO_2.

Discussion

AB2-143.2 hybridoma cells exposed to elevated pCO_2 in well-plates experienced both an inhibition of growth and an enhancement of cell death, with greater detrimental effects without osmolality compensation. Cell growth was also inhibited by pCO_2 in continuous culture (CC). The decrease in X_v at 140 mm Hg was due to a transient decrease in μ, and is consistent with the 35% decrease in μ at 140 mm Hg and 370 mOsm kg^{-1} in well-plates and the 27% decrease in μ in batch reactors (Table 3). There was also a trend towards higher q_{Ab} at 140 mm Hg and 370 mOsm kg^{-1} in all 3 culture systems. In contrast, k_d decreased slightly at 140 mm Hg in CC (after a transient increase for the experiment shown in Figure 4), while in well-plate and batch reactor culture k_d was more than twice as great as that for the control at 140 mm Hg. While q_{glc} and q_{lac} were 40–50% lower than control at 140 mm Hg in well-plates and 20–30% lower in batch reactor culture, q_{glc} and q_{lac} increased by 20–30% at 140 mm Hg in CC. Similarly, q_{O_2} was 50% lower at 140 mm Hg in batch culture, while CC q_{O_2} increased by ~30% at 140 mm Hg. Further, $Y_{O_2,glc}$ was ~40% lower at 140 mm Hg in batch culture, while $Y_{O_2,glc}$ remained the same or transiently increased at 140 mm Hg in CC. Finally, there was a consistent decrease in $Y_{n,glc}$ at 140 mm Hg in CC, while $Y_{n,glc}$ was not significantly affected by pCO_2 in batch culture.

Although differences between the responses in batch culture and those in CC may be partially explained by cell adaptation to elevated pCO_2 in CC, they can also be explained by differences in growth limitation for the two systems. Cell growth in CC is limited by the concentrations of nutrients and/or byproducts. Thus, k_d for control cultures was greater in CC (0.006–0.008 hr^{-1}) than in well-plates or batch reactors. The dilution rates of 0.022 and 0.028 hr^{-1} are lower than μ of 0.032 hr^{-1} for cells exposed to 140 mm Hg and 370 mOsm kg^{-1} in well-plates (or 0.037 hr^{-1} for cells in batch reactors). Since D is less than the effective μ_{max} for cells exposed to 140 mm Hg and 370 mOsm kg^{-1}, the cells in CC were able to compensate for inhibition by elevated pCO_2 and reach a new steady state at lower X_v due to the increase in nutrient levels and decrease in byproduct levels that accompanied the decrease in X_v. This explanation is consistent with the decrease in k_d at 140 mm Hg in CC. However, even after the decrease, k_d in CC at 140 mm Hg (0.003–0.006 hr^{-1}) was similar to that in well-plates and batch reactors at the same pCO_2. Also, even after the increase at 140 mm Hg, q_{glc} in CC was less than or equal to that in well-plates or batch reactors. Glucose utilization is generally more efficient at lower glucose consumption rates (Miller and Blanch, 1991). This is consistent with the observation that, even after the decrease, $Y_{n,glc}$ in CC was greater than or equal to that in well-plates or batch reactors (not shown). The lower q_{O_2} at 140 mm Hg in batch culture is consistent with an overall inhibition of cell metabolism. While the increase in q_{O_2} with pCO_2 in CC is consistent with an overall increase in cell metabolic activity, the increase in q_{O_2} cannot be attributed to an increase in DO, as was the case for higher q_{glc} with higher residual glucose concentration. Rather, the similar increases in q_{glc} and q_{O_2}, together with the fact that the glucose concentration was always $\geq$3 mM, suggest that cell metabolism was limited by depletion of another nutrient(s) or by a metabolic byproduct(s) not monitored in these experiments. Glutamine was not limiting for the experiment shown in Figure 4 (0.96 mM before the shift to 140 mm Hg and 3.2 mM after the decrease in X_v at 140 mm Hg), but other amino acids may have been depleted.

Although results for well-plate and batch reactor cultures were qualitatively similar, the decrease in μ and especially the decrease in the extent of glycolysis (q_{glc}, q_{lac}, and $Y_{lac,glc}$) at 140 mm Hg and 370 mOsm kg^{-1} were less extensive in batch reactor culture. These differences cannot be attributed to a lack of pH control in the well-plates because well-plate cultures at 140 mm Hg showed very little change in pH. If anything, one would expect that well-plate control cultures would exhibit lower growth rate and glucose conversion to lactate than in control batch reactor cultures due to the lower pH in the well plates. However, control cultures for the two systems had similar μ, q_{glc} and q_{lac} values (Tables 1 and 2). Sim-

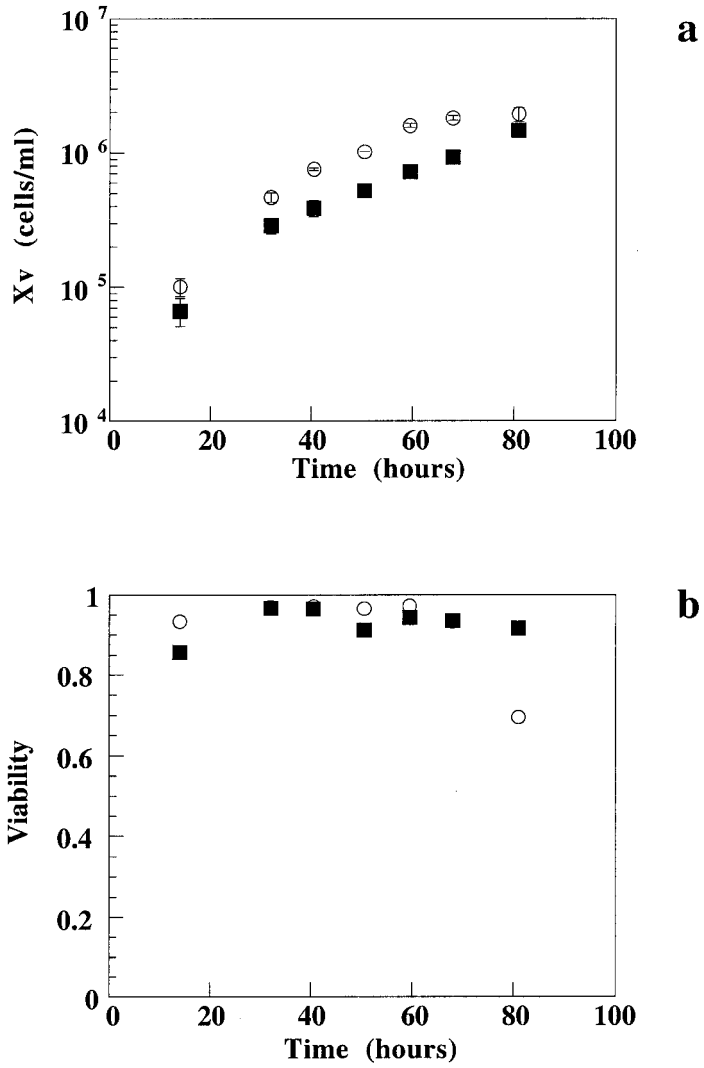

Figure 5. Cell growth (a) and viability (b) for representative batch bioreactor experiments at control conditions (open circles; 36 mm Hg pCO_2, 310 mOsm kg^{-1}) and 140 mm Hg pCO_2 and 370 mOsm kg^{-1} (solid squares). Cultures were seeded with 5×10^4 cells mL^{-1} at zero hr. Data represent the mean and standard deviation for 2 cell counts.

Table 3. Normalized values of various culture parameters (relative to those at 36 mm Hg and 310 mOsm kg^{-1}) at 140 mm Hg and 370 mOsm kg^{-1} in different culture systems. The respective control conditions are used for each culture system

Culture	μ	k_d	q_{Ab}	q_{glc}	q_{lac}	q_{O_2}	$Y_{n,glc}$	$Y_{lac,glc}$	$Y_{O_2,glc}$
Well-plates	0.65	2.2	1.27	0.63	0.51	ND	1.10	0.80	ND
Batch reactor	0.73	2.4	1.69	0.79	0.72	0.49	0.90	0.90	0.62
Continuous culture	Transient decrease; 0.94 @ SS	Slight decrease	1.4	1.3	1.2	1.3	0.8	0.9	Same or transient increase

ilar μ and q_{glc} values in well-plate and batch reactor control cultures also argue against the possibility that oxygen was depleted in the control well-plates.

Hybridoma AB2-143.2 cell growth in well-plates is more sensitive to elevated pCO_2 and osmolality than is that of CHO MT2-1-8 cells. Hybridoma μ decreased by 63% at 195 mm Hg and 415 mOsm kg^{-1}, while that of CHO MT2-1-8 cells decreased by only 24% (Kimura and Miller, 1996). (The osmolality values reported in Kimura and Miller, 1996 are in error; the correct osmolality values are essentially the same as those shown in Table 1 for the same conditions). In addition, the hybridoma k_d increased by 64-fold at 250 mm Hg pCO_2 and 469 mOsm kg^{-1}, while there was no increase in the (low) CHO MT2-1-8 cell k_d. Caution must be used when comparing results obtained in different culture systems. However, since we obtained similar qualitative results in well-plates and batch reactors, we feel that it is appropriate to directly compare results for different cell lines in batch culture. Several reports indicate that some CHO cell lines are more sensitive to pCO_2 than are MT2-1-8 cells. Drapeau et al. (1990) reported that μ for M-CSF-producing CHO cells in batch culture with pH and DO control decreased to 86 and 59% of control at 114 and 167 mm Hg pCO_2, respectively, when osmolality was controlled. Interpolation of the values in Table 1 suggests that this CHO cell line is similar in sensitivity to pCO_2 as are AB2-143.2 hybridomas. Also, another CHO cell line exhibited a dramatic decrease in shake-flask batch growth rate even at 76 mm Hg pCO_2 when osmolality was maintained between 310–340 mOsm kg^{-1} (Gray et al., 1996).

Hybridoma AB2-143.2 cell q_{Ab} did not decrease significantly at elevated pCO_2 with partial osmolality compensation and even increased slightly without osmolality compensation. This increase in q_{Ab} is similar to that observed for increased osmolality at control pCO_2, which is consistent with reports that q_{Ab} for these cells is increased by elevated osmolality in both batch and continuous culture (Reddy and Miller, 1994). As for hybridoma AB2-143.2 cells, recombinant NS/0 cells showed no decrease in q_{Ab} at 120 mm Hg pCO_2 when osmolality was allowed to increase with pCO_2 (Aunins and Henzler 1993). Recombinant protein production by CHO cells appears to be more sensitive to elevated pCO_2. At constant osmolality, CHO cell q_{M-CSF} decreased by 29% at 114 mm Hg and 44% at 167 mm Hg (Drapeau et al., 1990) in batch culture. Without osmolality compensation, CHO cell q_{tPA} decreased by 27% at 140 mm Hg and 21% at 195 mm Hg in batch culture (Kimura and Miller, 1996). Similarly, in high-density perfusion it has been shown that CHO cell q_P for a viral antigen decreased by 26% at 103 mm Hg and 44% at 148 mm Hg (Gray et al., 1996), while BHK-21 cell q_P for Factor VIII decreased by 33% at 130 mm Hg and 50% at 210 mm Hg (Taticek et al., 1998).

Hybridoma AB2-143.2 cell q_{glc} and q_{lac} decreased by 40 and 50%, respectively, at 140 mm Hg in well plates at both 337 and 370 mOsm kg^{-1} – with little further decrease at 195 mm Hg (Table 1). q_{glc} and q_{lac}, as well as q_{O_2}, also decreased at 140 mm Hg in batch reactor culture. Taticek et al. (1998) observed a steady decrease in BHK-21 cell q_{glc} and q_{lac} as pCO_2 was increased in high-density perfusion culture with a constant cell-specific perfusion rate – with 25 and 33% decreases in q_{glc} at 150 and 200 mm Hg, respectively, and 28 and 38% decreases in q_{lac} at 150 and 200 mm Hg, respectively. q_{gln} decreased with increasing pCO_2 in a similar manner (Taticek et al., 1998). Perfusion culture with a constant cell-specific perfusion rate is similar to batch culture – and different from CC – in that nutrient levels will not increase due to inhibition by CO_2.

One possible mechanism through which CO_2 may affect cell growth and metabolism is by changes in the intracellular pH (pH_i), which affects the activity of various enzymes involved in metabolism and protein production (Madshus, 1988). CO_2 is a weak acid that diffuses across cell membranes so rapidly that pCO_2 inside cells is only rarely different from that outside (Thomas, 1995). Once inside, CO_2 reestablishes equilibrium with H^+ and HCO_3^-, so that pH_i initially decreases in response to an increase in pCO_2. However, the decrease in pH_i is counteracted by ion transport systems. Depending on the cell type and conditions, the steady-state pH_i may be less than (Simchowitz and Roos, 1985), the same as (Krapf et al., 1988; Mellergård et al., 1993), or greater than (Putnam and Grubbs, 1990; England et al., 1991) pH_i prior to the increase in pCO_2. Of particular interest for recombinant protein production, Taticek et al. (1998) reported that the steady-state pH_i of BHK-21 cells in perfusion culture at pH 6.8 decreased steadily with increasing pCO_2 from 7.1 at 35 mm Hg to 6.92 at 270 mm Hg. We are currently investigating in more detail the mechanisms through which CO_2 affects the intracellular environment.

Note in Proof

Well-plate experiments at elevated pCO_2 with full osmolality compensation, and at control pCO_2 (36 mm Hg) with osmolality values similar to those in uncompensated cultures at elevated pCO_2, are now in progress. Preliminary results demonstrate a continuing trend of less extensive effects of elevated pCO_2 with greater osmolality compensation. Also, cell growth is significantly inhibited by 403 and 454 mOsm kg^{-1} at 36 mm Hg, although to a slightly lesser extent than by exposure to 195 and 250 mm Hg, respectively, with full osmolality compensation. Thus, elevated pCO_2 and elevated osmolality appear to have a similar and synergistic contribution to inhibition of cell growth by elevated pCO_2 without osmolality compensation.

Acknowledgments

Custom low-salt DMEM:F12 medium was a generous gift of Irvine Scientific. Funding for this work was provided by National Science Foundation grant BES-9402030. V. M. deZengotita was partially supported by a Predoctoral Biotechnology Training Grant (NIH GM08449). We are grateful for the use of osmometers loaned to us by Joanne Bradna (Rochelle Scientific, distributor for Wescor) and Rick Zampa (Advanced Instruments).

References

Altman PL and Dittmer DS (1971) Respiration and circulation. Federation of American Societies of Experimental Biology, MD.

Aunins JG and Henzler H-J (1993) Aeration in Cell Culture Bioreactors. In: Stephanopoulos G, Rehm H-J, Reed G, Puhler A and Stadler PJW (eds.) Biotechnology: A Multi-volume Comprehensive Treatise (Vol. 3), Bioprocessing. VCH Verlag, Weinheim, Germany.

Aunins JG, Glazomitsky K and Buckland B (1993) Animal Cell Culture Reactor Design: Known and Unknown. In: Proceedings of 3rd Intl. Conference on Bioreactor and Bioprocess Fluid Dynamics,Kluwer Academic Publishers, p. 175.

Drapeau D, Luan Y-T, Whiteford JC, Lavin DP and Adamson SR (1990) Cell culture scale-up in stirred tank reactors. Presented at The Annual Meeting of the Society of Industrial Microbiology, Orlando, FL.

England BK, Chastain JL and Mitch WE (1991) Abnormalities in protein synthesis and degradation induced by extracellular pH in BC_3H1 myocytes. Am J Physiol 260: C277–C282.

Garnier A, Voyer R, Tom R, Perret S, Jardin B and Kamen A (1996) Dissolved carbon dioxide accumulation in a large scale and high density production of TGFβ receptor with baculovirus infected Sf-9 cells. Cytotechnology 22: 53–63.

Glantz SA (1981) Primer of biostatistics. McGraw-Hill, New York.

Gray DR, Chen S, Howarth W, Inlow D and Maiorella BL (1996) Carbon dioxide in large-scale and high density CHO cell perfusion culture. Cytotechnology 22: 65–78.

Hornbeck PV and Lewis GK (1985) Idiotype connectance in the immune system. II. A heavy chain variable region idiotype that dominates the antibody response to the p-azobenzenearsonate group is a minor idiotype in the response to trinitrophenyl group. J Exp Med 161: 53–71.

Kimura, R (1996) Effects of elevated carbon dioxide partial pressure on recombinant Chinese hamster ovary and hybridoma cell cultures. PhD Dissertation, Northwestern University.

Kimura R and Miller WM (1996) Effects of elevated pCO_2 and/or osmolality on the growth and recombinant tPA production of CHO Cells. Biotechnol Bioeng 52: 152–160.

Krapf R, Berry CA, Alpern RJ and Rector Jr, FC (1988) Regulation of cell pH by ambient bicarbonate, carbon dioxide tension, and pH in the rabbit proximal convoluted tubule. J Clin Invest 81: 381–389.

Kurano N, Leist C, Messi F, Kurano S and Fiechter A (1990) Growth behavior of Chinese Hamster Ovary cells in a compact loop bioreactor: 1. Effects of physical and chemical environments. J Biotechnol 15: 101–112.

Lovrecz G and Gray P (1994) Use of on-line analysis to monitor recombinant mammalian cell cultures. Cytotechnology 14: 167–175.

Madshus IH (1988) Regulation of intracellular pH in eukaryotic cells (Review Article). Biochem J 250: 1–8.

Mellergård P, Ouyang Y and Siesjö BK (1993) Intracellular pH regulation in cultured rat astrocytes in CO_2/HCO_3^--containing media. Exp Brain Res 95: 371–380.

Miller WM and Blanch HW (1991) Regulation of Animal Cell Metabolism in Bioreactors. In: Animal Cell Reactors. Ho CS and Wang DIC (eds.) Butterworth-Heinemann, Stoneham, MA.

Miller WM, Blanch HW and Wilke CR (1988) A kinetic analysis of hybridoma growth and metabolism in batch and continuous culture: Effect of nutrient concentration, dilution rate, and pH. Biotechnol Bioeng 32: 947–965.

Ozturk SS and Palsson BO (1991) Effect of medium osmolarity on hybridoma growth, metabolism, and antibody production. Biotechnol Bioeng 37: 989–993.

Putnam RW and Grubbs RD (1990) Steady-state pH_i, buffering power, and effect of CO_2 in a smooth muscle-like cell line. Am J Physiol 258: C461–C469.

Reddy S and Miller WM (1994) Effects of abrupt and gradual osmotic stress on antibody production and content in hybridoma cells that differ in production kinetics. Biotechnol Prog 10: 165–173.

Royce PNC and Thornhill NF (1991) Estimation of dissolved carbon dioxide concentrations in aerobic fermentations. AIChE J 37: 1680–1686.

Simchowitz L and Roos A (1985) Regulation of intracellular pH in human neutrophils. J Gen Physiol 85: 443–470.

Taticek R, Petersen S, Konstantinov K and Naveh D (1998) Effect of dissolved carbon dioxide and bicarbonate on mammalian cell metabolism and recombinant protein productivity in high density perfusion culture. Presented at Cell Culture Engineering VI, San Diego, CA.

Thomas RC (1995) Bicarbonate briefly CO_2-free. Nature 374: 597–598.

Zupke C and Green J (1998) Modeling of CO_2 concentration in small and large scale bioreactors. Presented at Cell Culture Engineering VI, San Diego, CA.