Decreasing Extracellular pH Increases CD13 Receptor Surface Content and Alters the Metabolism of HL60 Cells Cultured in Stirred Tank Bioreactors

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Flow cytometry and Northern blotting were used to examine the effects of extracellular pH on CD13 receptor surface content and mRNA levels of HL60 (human promyelocytic leukemia) cells. Decreasing culture pH (7.4, 7.2, and 7.0) increased the CD13 receptor surface content of HL60 cells cultured at low agitation intensity (80 rpm) in 2-L bioreactors. Unlike our earlier findings on the effects of increasing agitation rates and serum concentrations, changes in CD13 receptor content in response to decreasing culture pH did not correlate with changes in CD13 mRNA levels. Decreasing culture pH also decreased the average HL60 cell size. HL60 cells cultured at pH 7.0 and 7.2 exhibited glucose consumption and lactate production rates that were approximately 30−40% and 20−30% lower, respectively, than values of cells cultured at pH 7.4.

Introduction

Extracellular pH is an important bioprocessing parameter in the culture of mammalian cells for the production of diagnostic and therapeutic proteins, viral vaccines, and cells for somatic and gene therapies. It must be optimized along with other culture parameters, such as medium formulation and oxygen tension, to obtain the desired cellular characteristics. Extracellular pH must also be effectively controlled in order to ensure the quality and consistency of the culture outcome, be it cells or a cell product.

Culture pH affects several cellular functions and properties including cell proliferation (*1*, *2*), cell differentiation (*3*−*5*), cell metabolism (*6*, *7*), intracellular pH (*8*), how ammonia affects cells (*9*, *10*), protein glycosylation (*11*−*13*), protein synthesis (*14*), antibody production (*6*, *7*, *10*), enzyme mRNA levels (*15*), enzyme secretion (*16*), glutamine synthesis (*17*), and glutamine decomposition (*18*). Since variations in culture pH affect several cellular functions and properties, effective pH control is necessary in industrial-scale mammalian cell culture. Unless pH is properly controlled, problems with cellular quality and/or uniformity may result. Effective pH control is not always obtainable, however, as spatial inhomogeneities in the culture system may result in pH gradients. Also, the extracellular pH may vary dramatically depending upon the culture system, especially as the cell density increases. In T-flasks, Akatov et al. (*2*) found that within 6 h of feeding, Chinese hamster fibroblasts had a local pH of 6.5, even though the bulk pH remained at 7.6. In a hollow fiber reactor system, Heath and Belfort (*19*) found gradients in nutrient concentrations, which could eventually lead to gradients in pH and dissolved oxygen. Since effective pH control is necessary to obtain culture-outcome homogeneity and consistency, but is not always obtainable, it is important to understand the effects of variations in culture pH on cellular functions and properties.

Of particular interest is the effect of extracellular pH on surface receptor expression. In the only well-documented report in the literature that we are aware of on the effects of pH on receptor levels, Katafuchi et al. (*20*) found that changes in the culture pH resulted in alterations in the surface concentration of the atrial natriuretic peptide receptor. Since surface receptors mediate several cellular functions, including viral infection (*21*) and recognition of cancerous cells (*22*), studying the effects of culture pH on receptors has applications in viral vaccine production, and in the production of cells for somatic and gene therapies. This work examines the effects of extracellular pH on the growth, viability, metabolism, and CD13 (aminopeptidase N) receptor surface content and mRNA levels of HL60 (promyelocytic leukemia) cells cultured under conditions of gentle agitation (80 rpm). This agitation intensity is necessary to achieve culture−pH homogeneity, yet gentle enough to avoid any detrimental effects on cells (*23*, *24*). Duplicate paired-bioreactor experiments, comparing extracellular pH 7.0 vs 7.4, and pH 7.2 vs 7.4 were performed. It is important to note that HL60 cells differentiate to eosinophils and eosinophilic precursors when cultured at pH 7.6−7.8 for 7 days (*3*). However, CD13 is presumably not related to this differentiation. The choice of HL60 cells and the CD13 antigen as models for this research have been discussed (*24*).

Materials and Methods

Cells and Culture Medium. HL60 cells (cell line CCL240) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS, Gibco BRL Life Technologies, Gaithersburg, MD), 3.0 g/L sodium bicarbonate (Sigma, St. Louis, MO), 1% sodium pyruvate (Sigma, St. Louis, MO), and 1% penicil-

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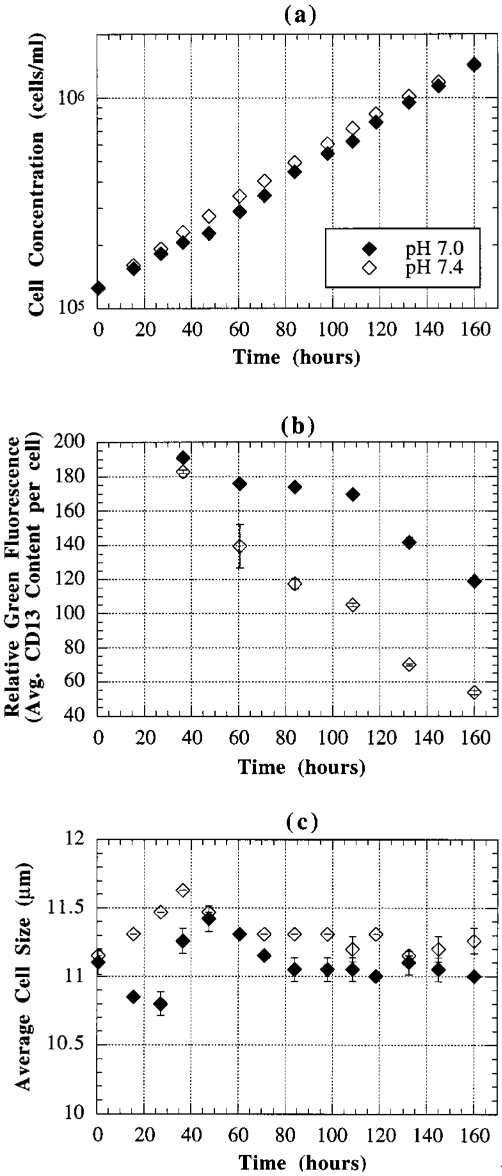

Figure 1. Time profiles of cell concentration (a), average CD13 content per cell (b), and average HL60 cell size (c) for the experiment designed to examine the effects of extracellular pH 7.0 vs 7.4 under conditions of mild agitation (80 rpm) on HL60 cell concentrations, CD13 receptor surface content, CD13 mRNA levels, and HL60 cell size. The points are averages of triplicates. The fluorescence units are arbitrary.

lin/streptomycin (Gibco BRL Life Technologies, Gaithersburg, MD). The cells were maintained in 100-mL, 250-mL, and 500-mL spinner flasks (Bellco Glass, Vineland, NJ). The flasks were agitated at 60 rpm using a Bellco low-profile multistir 4 magnetic stirrer plate (Bellco Glass, Vineland, NJ) placed in an incubator at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity.

Bioreactor Cultures. Bioreactor experiments were carried out in two Setric Genie 2-L bioreactors (Setric Genie Industrial, Toulouse, France) as described in McDowell and Papoutsakis (*24*). Briefly, the reactors were seeded at an initial concentration of approximately $(1.3-1.5) \times 10^5$ cells/mL, with an initial culture volume of 1.35 L, and grown at the nondamaging agitation rate of 80 rpm. The pH of the reactor medium was adjusted to 7.4, 7.2, or 7.0 by addition of 5 M NaOH. To ensure consistent osmolarity between the two reactors (in the initial batch culture), a volume of 5 M NaCl equal to the

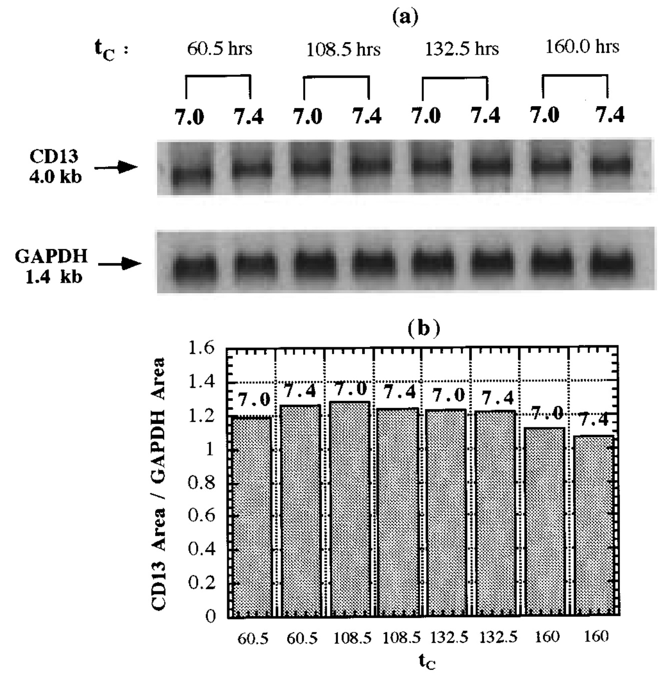

Figure 2. Northern blot (a) and normalized density plot (b) for the experiment examining the effects of pH 7.0 vs 7.4 under conditions of mild agitation (80 rpm) on HL60 cell concentrations, CD13 receptor surface content, CD13 mRNA levels, and HL60 cell size. Abbreviations: 7.0, pH 7.0; 7.4, pH 7.4; t_C, time of culture in hours.

volume of 5 M NaOH added to the first culture was added to the second culture. The temperature was maintained at 37 °C, the dissolved oxygen concentration was maintained above 70% of air saturation, and the pH was maintained at 7.0, 7.2, or 7.4 by injection of CO_2 into the headspace.

Analytical Sampling Methods. Total cell concentrations were measured using a Coulter Counter (Coulter Electronics, Hialeah, FL). Cell viability was determined by the trypan blue dye exclusion method using a Neubauer hemacytometer. For all experiments, cell samples were taken approximately every 8−12 h. For immunochemical staining of HL60 cells, triplicate samples of approximately 6×10^5 cells each were taken from the control and test cultures every 8−12 h. These samples were stained to measure the relative amount of CD13 receptor. Additional samples were taken in parallel from the control and test cultures to determine nonspecific staining. Glucose and lactate concentrations of bioreactor samples were determined using a YSI model 2700 Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH), as described in McDowell and Papoutsakis (*24*).

Surface Antigen Staining. Samples taken to determine the amount of CD13 receptor and nonspecific staining were prepared as described in McDowell and Papoutsakis (*24*). The samples were fixed with 1% formaldehyde (Sigma, St. Louis, MO), filtered through a 20 μm nylon mesh filter (VWR Scientific, McGaw Park, IL) to remove clumps, and stored at 4 °C until flow cytometric analysis. Stained samples were analyzed within a week of fixing.

Flow Cytometric Analysis of Surface Antigens. All samples from each experiment were analyzed in the same session using a Becton Dickinson FACSscan flow cytometer equipped with a 15-mW, 488-nm air-cooled argon ion laser (Becton Dickinson, San Jose, CA), as described in McDowell and Papoutsakis (*24*). For all

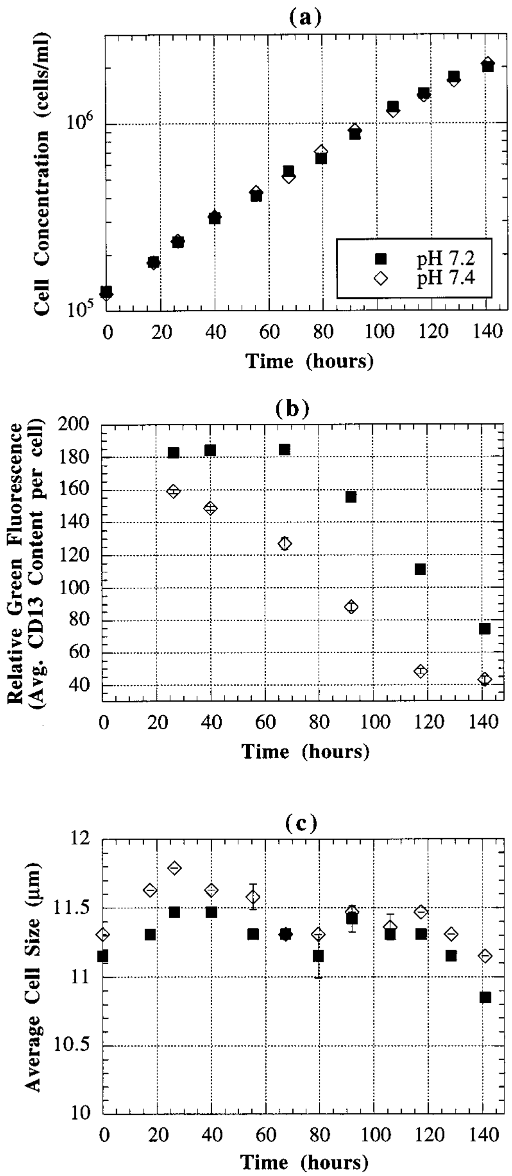

Figure 3. Time profiles of cell concentration (a), average CD13 content per cell (b), and average HL60 cell size (c) for the experiment designed to examine the effects of extracellular pH 7.2 vs 7.4 under conditions of mild agitation (80 rpm) on HL60 cell concentrations, CD13 receptor surface content, CD13 mRNA levels, and HL60 cell size. The points are averages of triplicates. The fluorescence units are arbitrary.

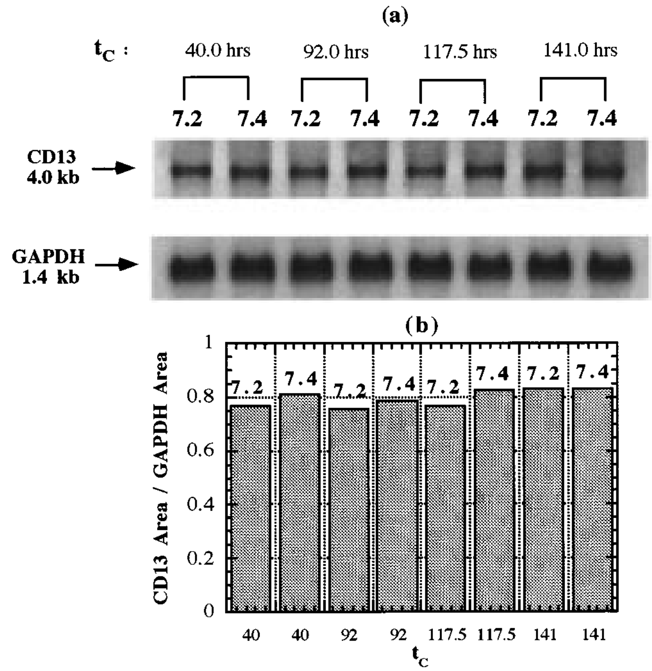

Figure 4. Northern blot (a) and normalized density plot (b) for the experiment examining the effects of pH 7.2 vs 7.4 under conditions of mild agitation (80 rpm) on HL60 cell concentrations, CD13 receptor surface content, CD13 mRNA levels, and HL60 cell size. Abbreviations: 7.2, pH 7.2; 7.4, pH 7.4; t_C, time of culture in hours.

samples, the nonspecific staining was negligible (i.e., the distribution of nonspecific staining made up less than 5% of the distribution of CD13 staining) (*25*).

Northern Blot Analysis. Approximately 1×10^7 HL60 cells were lysed in RNA STAT-60 (Tel-Test Inc., Friendswood, TX), and the total RNA was purified according to the manufacturer's instructions. Eight micrograms of total RNA (for each sample) was separated by electrophoresis on a 1.0% MOPS−formaldehyde agarose gel. The transfer of RNA to a nylon membrane, membrane hybridization with the [^{32}P]dCTP labeled CD13 cDNA probe, autoradiography, and quantitation were carried out as described in McDowell and Papoutsakis (*24*). The quality and amounts of RNA were controlled by rehybridization of the blots with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

Probes were labeled with [^{32}P]dCTP (3000 Ci/mmol) (NEN Research Products, Boston, MA) using the Prime-It II Random Primer Labeling Kit from Stratagene (La Jolla, CA). CD13 cDNA on pBluescript SK$^+$ was provided by Dr. A. Thomas Look of St. Jude's Children's Research Hospital in Memphis, TN. GAPDH cDNA on pBR322 was received from the American Type Culture Collection (Rockville, MD, deposited by R. Wu).

Results

We examined the effects of extracellular pH 7.0 vs 7.4, and pH 7.2 vs 7.4 on CD13 receptor surface content and mRNA levels of HL60 cells in paired-bioreactor experiments. The cells were cultured in two identical bioreactors (initial culture volume = 1.35 L) with an agitation rate of 80 rpm. Samples were periodically taken for flow cytometry and mRNA analysis. Cell concentration profiles of the pH 7.0 and 7.4 cultures were similar (Figure 1a), as were the profiles of the pH 7.2 and 7.4 cultures (Figure 3a). The apparent growth rates of the pH 7.0 and 7.4 cultures, and the pH 7.2 and 7.4 cultures, were nearly identical (data not shown). The viability of all cultures remained at or above 88% for the duration of all experiments (data not shown).

The CD13 receptor surface content per cell was higher in the pH 7.0 culture as compared to the pH 7.4 culture (Figure 1b), and higher in the pH 7.2 culture as compared to the pH 7.4 culture (Figure 3b). Decreasing the pH from 7.4 to 7.0 increased the CD13 receptor surface content, on average, by ~70%, while decreasing the pH from 7.4 to 7.2 increased the CD13 receptor surface content, on average, by ~60%. In contrast, the CD13 mRNA levels of the pH 7.0 and pH 7.4 cultures were similar for each time point (Figure 2), as were the CD13 mRNA levels of the pH 7.2 and pH 7.4 cultures (Figure 4). Thus, changes in CD13 receptor surface content due to changes in extracellular pH (7.0 vs 7.4 and 7.2 vs 7.4) *do not* correlate with changes in CD13 mRNA levels.

The mean HL60 cell size of the pH 7.4 culture was slightly larger than that of the pH 7.0 culture (Figure

Table 1. Specific Glucose Consumption Rate (a) and Lactate Production Rate (b) for the Experiments Examining the Effects of Culture pH 7.0 vs 7.4, and pH 7.2 vs 7.4 under Conditions of Mild Agitation (80 rpm)

a. Glucose Production Rate

	$q_{glucose}$ (mmol cell^{-1} h^{-1})			$q_{glucose,pH7.0}$/$q_{glucose,pH7.4}$	$q_{glucose,pH7.2}$/$q_{glucose,pH7.4}$
	pH 7.4	pH 7.2	pH 7.0		
	1.05×10^{-10}		0.83×10^{-10}	0.79	
	0.93×10^{-10}		0.65×10^{-10}	0.70	
	1.01×10^{-10}	0.76×10^{-10}			0.75
	1.10×10^{-10}	0.99×10^{-10}			0.90
mean	1.02×10^{-10}	0.88×10^{-10}	0.74×10^{-10}	0.75	0.83

b. Lactate Production Rate

	$q_{lactate}$ (mmol cell^{-1} h^{-1})			$q_{lactate,pH7.0}$/$q_{lactate,pH7.4}$	$q_{lactate,pH7.2}$/$q_{lactate,pH7.4}$
	pH 7.4	pH 7.2	pH 7.0		
	1.60×10^{-10}		1.04×10^{-10}	0.65	
	1.69×10^{-10}		0.92×10^{-10}	0.54	
	1.51×10^{-10}	0.87×10^{-10}			0.58
	1.67×10^{-10}	1.26×10^{-10}			0.75
mean	1.62×10^{-10}	1.07×10^{-10}	0.98×10^{-10}	0.60	0.67

1c), and of the pH 7.2 culture (Figure 3c). Obviously, if the CD13 content is normalized per unit surface area, the difference between the low and high pH values would be further amplified.

The specific glucose consumption and lactate production rates are shown in Table 1. The pH 7.0 cultures exhibited glucose consumption and lactate production rates that were 33% lower (on average) than those of the pH 7.4 cultures, while the pH 7.2 cultures exhibited glucose consumption and lactate production rates that were 25% lower (on average) than those of the pH 7.4 cultures. The lactate yields on glucose for the pH 7.0 and 7.2 cultures were ~20% lower than those of the pH 7.4 cultures (data not shown). Since decreasing the culture pH led to a reduction in cell size (diameter), the HL60 glucose consumption and lactate production rates were recalculated on a per cell volume basis. The results were largely the same, as the pH 7.0 and 7.2 cultures exhibited glucose consumption and lactate production rates that were, on average, 30% and 22% lower than those of the pH 7.4 cultures. The lactate yields on glucose for the pH 7.0 and 7.2 cultures remained ~20% lower than those of the pH 7.4 cultures (data not shown). Each experiment was done in duplicate, and each showed similar trends in cell concentration, viability, CD13 receptor surface content, CD13 mRNA levels, HL60 cell size, and HL60 metabolism. These experiments show that in the pH range of 7.4 to 7.0, the effects of increasing CD13 receptor surface content and decreasing HL60 cell metabolism are dose-dependent.

In all Northern blots, GAPDH was used as an internal standard of mRNA amount and quality. Since GAPDH is a glycolytic enzyme (it catalyzes the reaction of glyceraldehyde-3-phosphate to glycerate-1,3-bisphosphate), it is possible that it could be affected by changes in extracellular pH. To examine if GAPDH mRNA levels were affected by changes in pH, the ratios of ($GAPDH_{pH7.0}/GAPDH_{pH7.4}$) and ($GAPDH_{pH7.2}/GAPDH_{pH7.4}$) peak areas were calculated for the Northern blots of Figures 2 and 4 and their respective duplicate experiments. As shown in Table 2, for each time point, these ratios were near 1.0 and did not vary in a systematic way (i.e. were not always greater than 1.0), as would have been the case if GAPDH mRNA were upregulated at lower pH. The observed variations (~5%) can be explained by the expected variability in the loading of the gel lanes. These data, combined with the data of Figures 2b and 4b (whereby the ratios of CD13 to GAPDH mRNA are virtually identical at each time point with no systematic trends) suggest that GAPDH mRNA levels were not affected by changes in extracellular pH from 7.4 to 7.0, or 7.4 to 7.2.

Table 2. Ratio of GAPDH (Internal mRNA Standard) Peak Areas from Densitometry Plots of pH 7.0 vs 7.4 (a) and pH 7.2 vs 7.4 (b)

a. pH 7.0 vs 7.4 (from Figure 2 and Duplicate Experiment)

time of culture (h)		$GAPDH_{pH7.0}$/$GAPDH_{pH7.4}$	time of culture (h)	$GAPDH_{pH7.0}$/$GAPDH_{pH7.4}$
60.5		1.10	61.0	1.11
108.5		1.05	134.5	0.97
132.5		1.04	159.5	1.07
160.0		0.98		
	mean ± std	1.04 ± 0.05		1.05 ± 0.07

b. pH 7.2 vs 7.4 (from Figure 4 and Duplicate Experiment)

time of culture (h)		$GAPDH_{pH7.2}$/$GAPDH_{pH7.4}$	time of culture (h)	$GAPDH_{pH7.2}$/$GAPDH_{pH7.4}$
40.0		0.98	44.0	0.96
92.0		1.02	85.5	1.14
117.5		1.10	110.0	1.04
141.0		0.99	134.0	1.02
	mean ± std	1.02 ± 0.05		1.05 ± 0.09

Discussion

In all reactor runs, the culture with the lower pH exhibited a higher CD13 receptor surface content (Figures 1b and 3b). Furthermore, the differences in CD13 content between the pH 7.0 and 7.4 cultures were greater than the differences between the pH 7.2 and 7.4 cultures. These results are consistent with studies done by Katafuchi et al. (*20*), who examined the effects of extracellular pH on the density of the atrial natriuretic peptide (ANP) receptor present on bovine aortic endothelial cells. ANP is a cardiac hormone involved in the regulation of blood pressure (*26*). Katafuchi et al. (*20*) cultured the cells in three different pH environments (pH 7.0, 7.4, and 7.7) and determined the ANP receptor content using a binding assay and ANP receptor affinity labeling. Their results showed that a *decrease* in pH from 7.4 to 7.0 resulted in a substantial increase (~100%) in ANP receptor content, while an *increase* in pH from 7.4 to 7.7 resulted in a significant decrease (>100%) in ANP receptor content.

Even though the pH 7.0 and 7.2 cultures exhibited a higher CD13 receptor surface content as compared to the pH 7.4 culture, the mRNA levels were largely the same for each time point (Figures 2 and 4). These results are unlike our previous experiments, which showed that increases in CD13 receptor surface content in response to increasing agitation rates (300 and 400 rpm) and increasing serum concentrations (2.5%, 5%, and 10%) were correlated with increases in CD13 mRNA levels (*24*, *27*). Since extracellular pH affected CD13 receptor content without affecting CD13 mRNA levels, pH must affect another processing step, namely CD13 receptor: protein synthesis, trafficking to the cell membrane, internalization, degradation, or recycling. Further experiments must to be done to determine the mechanism by which extracellular pH affects CD13 receptor surface content.

As shown in Table 1, the pH 7.0 and 7.2 cultures exhibited glucose consumption and lactate production rates that were from 20% to 40% lower than those of the pH 7.4 cultures. Although there was a 10−25% variability in the $q_{glucose}$ or $q_{lactate}$ values for duplicate experiments, the trends were consistent and reproducible, whether one compares the paired (7.0 vs 7.4, 7.2 vs 7.4) $q_{glucose}$ or $q_{lactate}$ values or variations in the $q_{glucose}$ or $q_{lactate}$ values with decreasing pH. While obviously the history of the culture inoculum or some other culture parameter results in some variability in the metabolic behavior of a specific experiment, these data show clearly the profound effect of culture pH on cell metabolism. This trend of decreasing glucose consumption and lactate production rates with decreasing culture pH was also observed in experiments done with HeLa cells (*28*) and hybridoma cells (*6*, *7*). McQueen and Bailey found that as the culture pH decreased from 7.6 to 6.8, hybridoma cells exhibited a decrease in growth rate, as well as in glucose consumption and lactate production rates (*7*). This is in contrast to our results, where as the culture pH decreased from 7.4 to 7.0, the HL60 growth rate was largely unaffected, while we *still* observed a 20−40% decrease in glucose consumption and lactate production rates. Possible reasons for the observed overall better yields (i.e., more cells per mole of glucose) could be due to utilization of a higher percentage of other nutrients at lower pH, a decrease in metabolic energy requirements (including cell maintenance), or more likely, complex events involving several metabolic and membrane processes.

Clearly, pH is an important bioprocessing parameter in the large-scale culture of mammalian cells, as it affects cell physiology, protein expression and quality, and cell differentiation. Not only must pH be optimized for the particular system, but the possible existence of pH gradients must be taken into consideration. Due to the nature of the culture system, it is likely that pH gradients may exist in T-flasks (*2*) and hollow fiber reactors (*19*). However, it is important to note that pH gradients may also exist in stirred tank reactors due to factors such as poor mixing, cell aggregation, or growth of cells to high densities. The present study showed that even a relatively small change in pH from 7.4 to 7.2 resulted in a significant increase (∼60%) in CD13 receptor surface content. Thus even slight pH gradients could have a significant effect on receptor content and therefore culture uniformity.

Therefore, pH gradients must be avoided to the extent possible to achieve a consistent and reproducible culture outcome.

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