0273-2289/96/6103—0211$09.75

High-Density Hybridoma Perfusion Culture

Limitation vs Inhibition

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Received June 19, 1995; Accepted October 5, 1995

ABSTRACT

Because our earlier work indicated a strong correlation between specific antibody productivity and cell density in perfusion culture, we conducted experiments to determine the optimum means of increasing cell density while mainitaining high antibody productivity. The rates of medium supply and waste removal were varied to determine whether cell density was limited or inhibited, and whether a diffusable substance could be responsible for the correlation between antibody productivity and cell density. Nutrient supply was found to be a stronger determinant of cell density than waste removal; however, the rate of waste removal had a greater effect on cell growth at lower cell densities. Even at noninhibitory levels of ammonia and lactate, cellular metabolism was regulated to minimize their concentrations at lowered rates of waste removal. Separate step changes in glucose and glutamine resulted in increased cell density and antibody concentration. Specific antibody productivity increased following the step in glutamine, but not glucose. Both steps caused changes in cellular metabolism that prevented the levels of lactate and ammonia from reaching toxic levels.

Index Entries: Hybridoma; monoclonal antibody; perfusion; nutrient limitation; waste inhibition; cell growth; metabolism; high-density culture; glucose; glutamine.

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INTRODUCTION

In recent homogeneous perfusion culture studies of hybridoma H22, we have found an interesting and potentially important phenomenon: a strong correlation between specific monoclonal antibody productivity and viable cell density, with productivity increasing at higher cell densities (*1–3*). This observation is supported by other reports of high specific productivities in immobilized cell cultures that have high local cell concentrations (*4,5*). Because of this relationship, we are interested in strategies to increase the viable cell concentration in the perfusion reactor to test the limits of specific and volumetric antibody productivity. In our earlier studies, however, cell density was inhibited and/or limited, stoichiometrically or kinetically, as has also been observed by others (*6–13*).

In this paper, we report on a systematic study of potential limitations and/or inhibitions that could be controlling cell density, and, as a result, specific and volumetric antibody productivity. In the first set of experiments, we addressed whether the cells are stoichiometrically or kinetically limited, as is believed to be the case with most systems (*6,8–11*), or whether the cells are inhibited by secreted metabolites, as has also been hypothesized (*14*). This was done by means of simultaneous step changes in the feed medium concentration (by dilution) and the perfusion rate, to examine the effects of changing the rates of substrate supply and waste removal. The step changes were conducted in such a way that the dilution rate was always halved (or held constant), since the cells had difficulty adapting to a doubling of the perfusion rate while halving the medium composition (which suggests a stoichiometric rather than kinetic limitation). In these experiments, we also hoped to determine whether a diffusable metabolite was responsible for the increase in specific antibody productivity with increased cell density. Finally, since our normal medium contained 2.5% fetal bovine serum (FBS), we also examined whether dilution (or removal) of serum and proteins could be responsible for any observed effects on metabolism.

The next set of experiments examined whether the cells were limited by glucose, glutamine, or some other compound, as has been suggested by others (*6,8–11*), using step changes in feed nutrient concentrations. The hypothesis was that if glucose (or glutamine) were the only limiting compound, then an increase in its concentration would yield stoichiometric increases in both cell density and specific antibody productivity.

MATERIALS AND METHODS

Perfusion Reactor

The three identical reactors consisted of jacketed glass vessels, inside of which were coiled six meters of hydrophilized, microporous (0.22 μm) polypropylene fibers (Akzo, Germany), used for medium exchange (2).

Three meters of hydrophobic polypropylene tubing were used for oxygenation of each reactor. The dissolved oxygen concentration was maintained between 40 and 50% by varying the ratio of oxygen to air in the gas feed stream. Reactor pH was controlled about a set point of 7.2 by supplying 5% CO_2 in the gas phase, and by adding 0.5*M* $NaHCO_3$ as needed. The reactors were inoculated with cells (2–5 × 10^5 cells/mL) from an exponentially growing batch culture and were run in batch mode until the density increased to more than 10^6 cells/mL, after which perfusion was initiated. The reactors were well-mixed with magnetic stirrers at 70–75 rpm, which also served to minimize the buildup of cells on the membrane surfaces.

A continuous flow of medium into the reactor determined the volumetric feed or perfusion rate, D (reactor vol/d). While the liquid level in the reactor was maintained at 550 ± 10 mL by a level controller (Cole Parmer, Niles, IL), the medium was removed in two ways. Part of the outlet stream consisted of cell-free broth pumped out through the microporous fibers, and the rest of the outlet stream was removed directly from the reactor, determining the cell bleed rate, B_c.

Cell Culture

The mouse–mouse hybridoma cell line H22 used in this study was NS1 and B-cell derived, producing an IgG_1 MAb. Used for all experiments was Dulbecco's (Gibco, Grand Island, NY) Modified Eagle's Medium (DMEM), containing 4.5 g/L (25 m*M*) glucose, 6 m*M* glutamine, 3.7 g/L sodium bicarbonate, 15 m*M* HEPES buffer, 0.11 g/L sodium pyruvate, 5 mg/L insulin, 5 mg/L transferin, 10 n*M* sodium selenite, and 10 μm ethanolamine. The medium (referred to hereafter as "normal"), which was supplemented with 2.5% fetal bovine serum (FBS), 10^5 U/L penicillin-streptomycin, and 20 mL of 50X essential and 10 mL of 100X nonessential amino acids (Gibco), was adjusted to a pH of 7.2. Dilute medium refers to normal medium diluted 1:1 in PBS. For the serum step change experiments, the medium composition was normal, except that no FBS, insulin, or transferrin was added.

Dilution Experiments

The bleed rate for these experiments (five cases) was kept constant at 0.5/d. When steady state was achieved at a perfusion rate of 2.9/d (case 1), the normal medium was replaced with dilute medium (case 2), halving the nutrient supply rate without changing the rate at which metabolites were removed from the reactor. Next, the medium composition was restored to normal while approximately halving the perfusion rate to 1.5/d (case 3), keeping the rate of substrate supply constant but halving the rate of metabolite removal. The next changes were to halve the medium composition (dilute) while keeping the perfusion rate constant (case 4), followed by restoring medium composition to normal while halving the perfusion rate to 0.75/d (case 5).

Feed Glucose and Glutamine Steps

The perfusion rate for all experiments was maintained at $D = 2.0$/d and the bleed rate was maintained at 0.1/d because the cell density obtained at these conditions was fairly high (1.2×10^7 cells/mL) and the specific growth rate was very low (0.13/d). At steady state with normal medium, the glucose and glutamine concentrations in the reactor were 0.7 m*M* and 0.11 m*M*, respectively. The first step consisted of replacing the normal feed medium with medium containing 8 g/L (44 m*M*) glucose. After approx 100 h at the new steady state, the feed was replaced with normal medium. When the system had returned to the normal steady state, the feed was replaced with medium containing 10 m*M* glutamine.

Analytical Procedures

Cells, sampled at regular intervals, were counted in a hemocytometer, using erythrosin B, with an average of six determinations used to calculate the viable cell concentration and percentage viability. The remainder of the sample was centrifuged, and the supernatant was frozen at −20°C for later analysis. Glucose and lactate were measured with an analyzer (Model 2000, Yellow Springs Instrument, Yellow Springs, OH). An enzyme-linked immunosorbent assay (ELISA) was used to determine antibody concentrations. Amino acid concentrations were determined using HPLC with a C-18 column and gradient elution of the *o*-phthaldialdehyde derivatives. Because proline, cysteine, and tryptophan do not give fluorescent derivatives, their concentrations were not determined. An ion-selective electrode (Model 95-12, Orion, Boston, MA) was used to measure ammonia. Steady state was assumed when the cell count, viability, and concentrations of glucose, lactate, and dissolved oxygen did not vary appreciably over a period of 3–4 residence times. The reported steady state values are averages of 4–6 data points.

Viable (X_v) and dead cell (X_d) balances around the reactor yield the following equations at steady state:

$$\mu_{app} = B_c \tag{1}$$

$$\mu = \mu_{app}\,(X / X_v) \tag{2}$$

$$k_d = \mu - B_c \tag{3}$$

where μ_{app} and μ are the apparent and true specific growth rates (1/d), k_d is the specific death rate, and X is the total cell density (cells/mL). The specific substrate uptake (q_s) and product formation (q_p) rates were calculated using the following equations:

$$q_p = D\,(S_f - S) / X_v \tag{4}$$

$$q_p = D\,(P / X_v) \tag{5}$$

where S and S_f are the reactor and feed substrate concentrations, and P is the reactor product concentration.

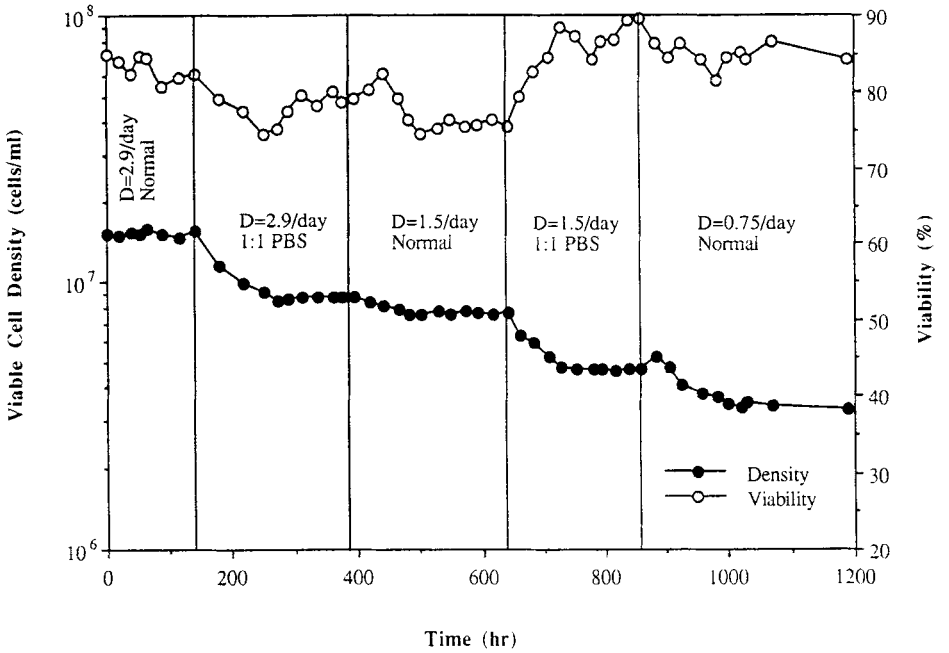

Fig. 1. Viable cell density and percent viability at different rates of medium addition and removal. The bleed rate was constant at B_c = 0.5/d; the perfusion rates and medium compositions are as indicated.

RESULTS AND DISCUSSION

Dilution Experiments

Cell Growth

The changes in cell density following medium dilution or halving of the perfusion rate (Fig. 1) indicate that, in the range of conditions studied, the rate of medium supply is a much stronger determinant of viable cell density than is the rate of metabolite removal, in agreement with de la Broise et al. (*15*). The large drops in viable cell density (cases 2 and 4) show that the cells were limited by the decreased rate of nutrient supply (and/or concentration), rather than being relieved by lower metabolite levels. Statistically, supply rate, not concentration, was limiting. Linear regression of the viable cell density, with concentrations of glucose, glutamine, lactate, and ammonia, showed no correlation, while regression of density with the medium supply rate gave a correlation coefficient (r^2) of 0.95. Regression of cell density with the waste removal rate (i.e., perfusion rate) gave r^2 equal to 0.69.

Steady state cell densities decreased in close proportion (i.e., 50%) to each medium dilution (cases 2 and 4), but dropped only slightly with lowered rates of waste removal (cases 3 and 5). By diluting the medium at constant perfusion rate, the molar throughput of nutrients was reduced

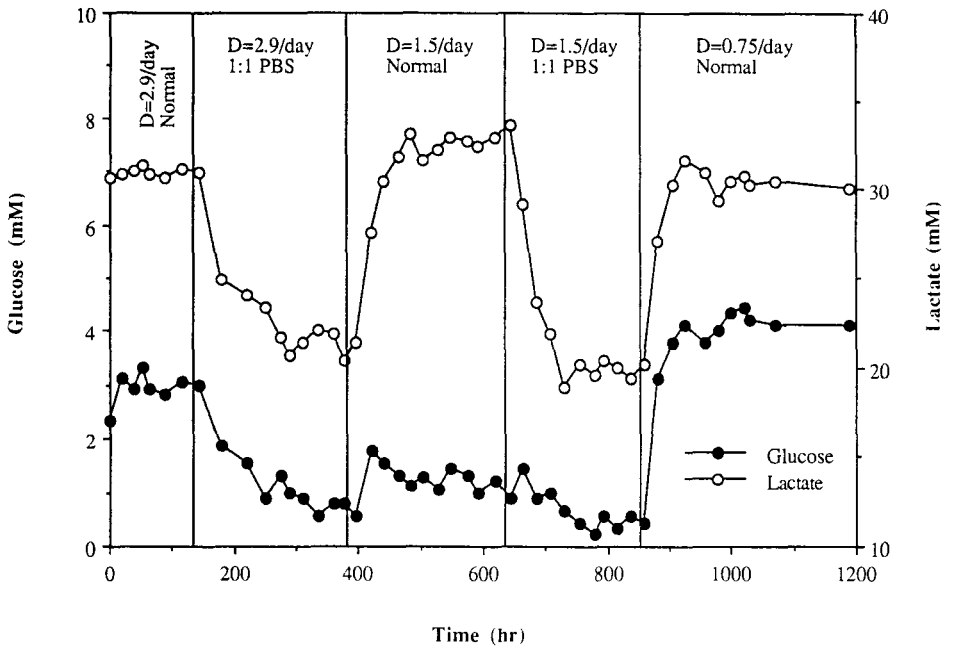

Fig. 2. Glucose and lactate concentration profiles at different rates of medium addition and removal.

by half, while the rate of waste removal was unchanged. For example, comparing cases 2 and 3 (Fig. 1) shows that the cell density (and viability) decreased only slightly when the rate of waste removal was halved at constant nutrient supply rate. The viable cell density at this steady state (case 3), however, was half that at twice the nutrient supply rate (case 1), indicative of nutrient limitation.

Waste removal, however, is more important at lower perfusion rates. More efficient waste removal at D = 1.5/d (dilute) compared to D = 0.75/d (normal) resulted in a 25% higher viable cell density, an increase twice as large as that found between cases 2 and 3. The cell viability, while decreasing less than 10% in response to each of the first drops in medium concentration and perfusion rate (cases 2 and 3), actually increased to values greater than the starting viability in both the next medium dilution and perfusion rate drop (cases 4 and 5).

Substrate and Product Metabolism

Glucose and lactate concentrations responded to changes in nutrient supply and waste removal rates in a parallel fashion: Both concentrations decreased when the medium composition was halved at constant perfusion rate and increased with halving of the perfusion rate and a return to normal medium (Fig. 2). The increase in residual glucose concentration was the most dramatic (800%) when the perfusion rate was halved from 1.5 to 0.75/d and medium was restored to normal, probably as a result of the low cell numbers under these conditions. Even though changes in lac-

Table 1
Steady-State Parameters
with Different Rates of Medium Supply and Waste Removal

Case	1	2	3	4	5
Perfusion rate (1/d)	2.9	2.9	1.5	1.5	0.75
Medium composition	normal	1:1 PBS	normal	1:1 PBS	normal
Rate of medium addition (1/d)	2.9	1.45	1.5	0.75	0.75
Rate of waste removal (1/d)	2.9	2.9	1.5	1.5	0.75
Specific growth rate (1/d)	0.60	0.63	0.66	0.57	0.59
Specific death rate (1/d)	0.10	0.13	0.16	0.07	0.09
q_{glu} (mmol/10^9 cells/d)	4.17	3.95	4.70	3.86	4.45
q_{lac} (mmol/10^9 cells/d)	5.88	7.14	6.51	6.47	6.46
q_{gln} (mmol/10^9 cells/d)	1.02	0.96	1.17	0.95	1.07
q_{amm} (mmol/10^9 cells/d)	0.63	0.67	0.65	0.53	0.57
q_{ala} (mmol/10^9 cells/d)	0.22	0.32	0.26	0.32	0.28
q_{gly} (mmol/10^9 cells/d)	0.22	0.12	0.17	0.09	0.11
$Y'_{lac/glu}$	1.41	1.81	1.39	1.68	1.45
$Y'_{amm/gln}$	0.61	0.70	0.56	0.53	0.53
$Y'_{ala/gln}$	0.21	0.33	0.22	0.34	0.27
$Y'_{gly/gln}$	0.22	0.13	0.14	0.10	0.10

tate concentration were generally greater than those of glucose, the rate of medium supply, not waste removal, was found to be the dominating factor controlling cell density.

The specific glucose consumption and lactate production rates (Table 1) were much less affected by changes in medium supply and waste removal rates than were their concentrations. Both times that the medium composition was halved by dilution, there was a sharp initial decrease in q_{glu}, resulting in an ultimate drop of about 15% in both cases. From D = 2.9/d (dilute) to D = 1.5/d (normal), the lactate concentration increased 50%, to 32.8 m*M*, yet the viable cell density decreased only 12%, indicating little effect of the increased lactate concentration. From D = 1.5/d (dilute) to D = 0.75/d (normal), the lactate concentration increased 53%, to 30.3 m*M*, while the cell viability decreased 25%, suggesting that lactate has a greater effect on cell growth at lower cell density.

Similar to the relationship between lactate and glucose, the ammonia concentration was much more responsive to changes in medium supply and waste removal than was the glutamine concentration (Fig. 3), and the specific rates of glutamine consumption and ammonia production (Table 1) were less affected than were their concentrations. As with lactate, a large increase in the ammonia concentration corresponded to only a small decrease in the viable cell density. As with glucose, there was a significant increase in the glutamine concentration after the last step change from 1.5/d (dilute) to 0.75/d (normal). The viable cell density, however, decreased 25%, to 3.5 × 10^6 cells/mL, again suggesting that

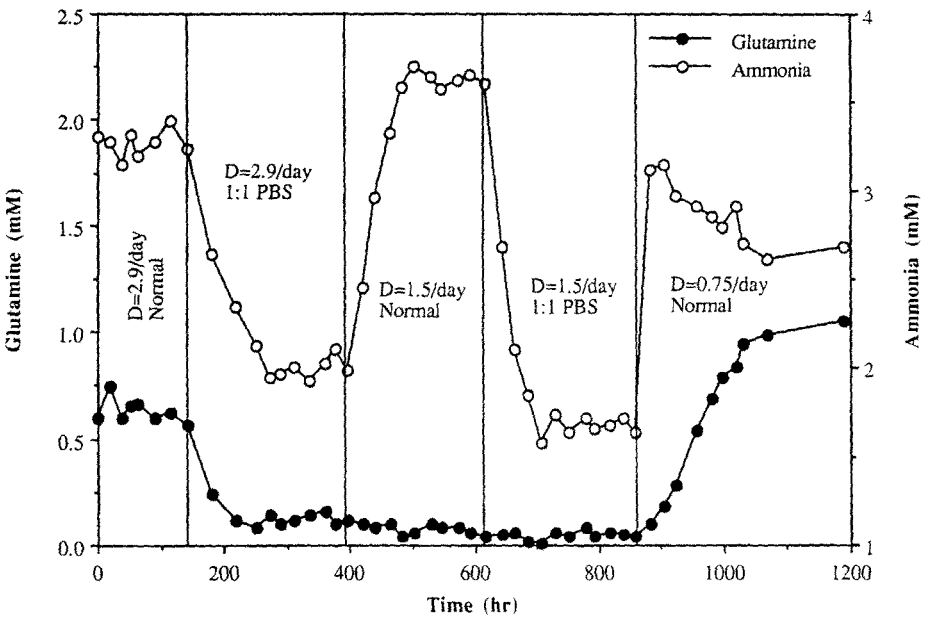

Fig. 3. Glutamine and ammonia concentration profiles at different rates of medium addition and removal.

lower levels of toxic metabolites may be capable of inhibiting cell growth at lower cell densities. A strategy that has been used with fed batch and perfusion cultures is to provide substrate at the rate at which it is consumed. By keeping reactor nutrient levels near exhaustion, the lactate and ammonia concentrations can be drastically reduced (*6,7,16,17*), which appears to be particularly important at low cell density.

As with glucose, q_{gln} with dilute medium was 15–20% lower than with normal medium at a lower perfusion rate, which may indicate either a decrease in the maintenance requirements, as a result of the lower metabolite concentrations in the reactor, and/or a reduced uptake because of its lower concentration. The specific ammonia production rate, however, was relatively constant at the higher perfusion rates, but dropped significantly after the change to dilute medium at 1.5/d.

Similar to the behavior of cells in low-density batch (*18,19*) and continuous (*18,20*) cultures, we have found that cells in high-density perfusion alter their metabolism in response to increasing concentrations of secreted metabolites to avoid toxic levels (*1,3*). The apparent yield coefficients of lactate from glucose and ammonia from glutamine were, with one exception, both slightly higher with faster waste removal and constant medium supply rate (Table 1). The effect was most prominent at the highest perfusion rate (D = 2.9/d, dilute), where $Y'_{lac/glu}$ was 30% higher and $Y'_{amm/gln}$ was 26% higher compared to the steady state at D = 1.5/d (normal). When the concentrations of lactate and ammonia are higher, which may occur at high cell density or with slower waste removal, the

cells regulate their metabolism, so that less lactate and ammonia are produced, minimizing potentially toxic effects (*9*).

Amino Acid Metabolism

As we found in our previous experiments with steady-state, partial cell-retention cultures, the amino acid consumption rates were dependent on the specific growth rate (*3*) and, since, in these dilution experiments, the specific growth rates of all five cases were similar, the specific uptake rates of most amino acids changed insignificantly and were essentially independent of the PBS dilutions. The net consumption rate decreased with medium dilution. Glycine and alanine were consistently produced; the rest, except aspartate, were consistently consumed (Table 1). At steady state, medium dilution resulted in production rates of alanine and glycine above and below, respectively, that in normal medium; halving the perfusion rate and restoring the medium to normal resulted in production rates below and above, respectively, the prior steady state levels.

As shown in Table 1, $Y'_{lac/glu}$, $Y'_{amm/gln}$ and $Y'_{ala/gln}$ increased, while $Y'_{gly/gln}$ decreased, when the waste product removal rate was high. Lower $Y'_{gly/gln}$ with dilute medium, when $Y'_{lac/glu}$ and $Y'_{ala/gln}$ are high, suggests that the pyruvate pool, under conditions of more efficient waste removal, results in more lactate and alanine rather than glycine. Although when $Y'_{ala/gln}$ increases, there is normally a concurrent decrease in both $Y'_{amm/gln}$ and $Y'_{lac/glu}$, we saw higher yield coefficients for all three metabolites when there was better waste removal.

Monoclonal Antibody Production

Slower nutrient supply resulted in a decreased q_{MAb} (Fig. 4), but, because q_{MAb} for this cell line is strongly correlated with cell density (*1,3*), this was probably a result of the lower cell density. Slower waste removal increased the MAb concentration in the reactor, as expected, but also increased q_{MAb}. Thus, it not surprising that q_{MAb} was highest at D = 2.9/d and B_c = 0.1/d, when the cell density was the highest. Other than through their effect on viable cell density, there were no deleterious effects of increased ammonia or lactate concentration on q_{MAb}. Medium dilution resulted in a lower q_{MAb} than could be predicted on the basis of density alone, which is evidence for a humoral or autocrine factor, as has also been suggested by others (*5,21,22*); however, the observed changes, though consistent, were not significant.

Effect of Serum and Proteins

To determine whether reduced serum concentration was responsible for any of the observed changes with medium dilution, the feed medium was switched to that without serum or proteins. The maximum densities at the end of batch culture for this cell line are significantly lowered by using medium containing 2% serum or less (not shown); cell growth, substrate metabolism, and product formation in perfusion culture (D = 2.0/d and B_c = 0.1/d) were all unaffected by the change to protein-free medium,

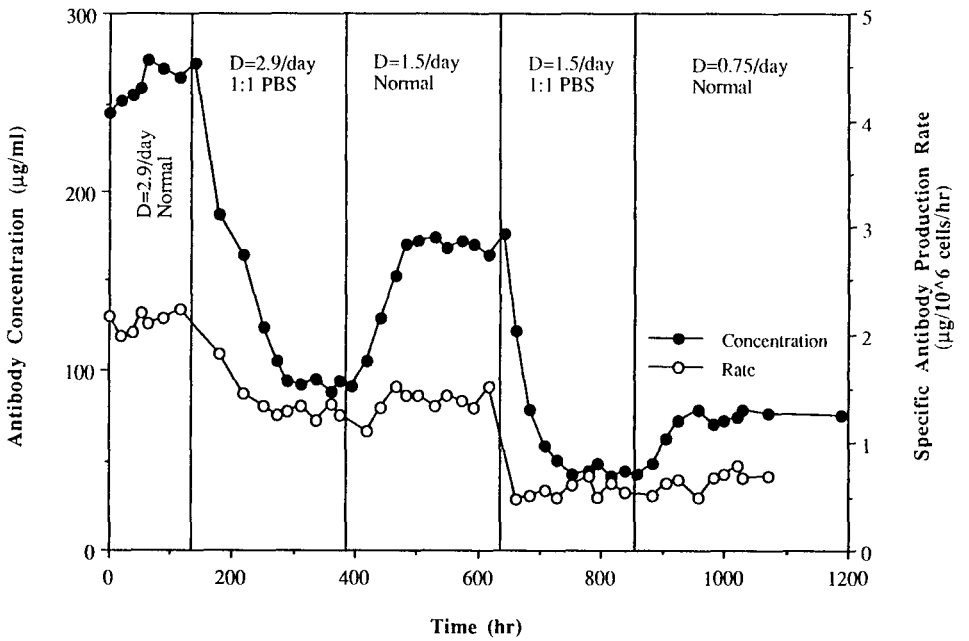

Fig. 4. MAb concentration and specific productivity at different rates of medium addition and removal.

which has also been observed with immobilized cells (5). Viable cell densities of 1.22×10^7 and 1.19×10^7 cells/mL were obtained at steady state in perfusion culture with 2 and 0% FBS, respectively. Compared to batch or low-density continuous culture, cells in high-density culture apparently have little or no requirement for serum and other added proteins, perhaps because the level of secreted factors is high enough to reduce the need for exogenous proteins.

Feed Glucose and Glutamine Step Changes

Cell Growth

Cell growth at D = 2.0/d and B_c = 0.1/d in medium with 25 m*M* glucose and 6 m*M* glutamine was limited by both substrates, or more accurately by a carbon/energy source, even with residual glucose and glutamine concentrations of 0.4 m*M* and 0.1 m*M*, respectively (K_m values vary in the literature, but are typically less than 0.5 m*M* for glucose and 0.3 m*M* for glutamine (*18*)). This is exemplified by the fact that a step change in either substrate caused an increase in cell density (Fig. 5). The glutamine step had a greater impact on the cell density than did the glucose step (40% increase, compared to 33%), possibly because the glucose step culture was still limited by glutamine, which is believed to arrest glucose utilization (23). The live-cell density and the percentage viability following the glutamine step increased more slowly than with the glucose step. The same rate difference in cell density increase following glucose and gluta-

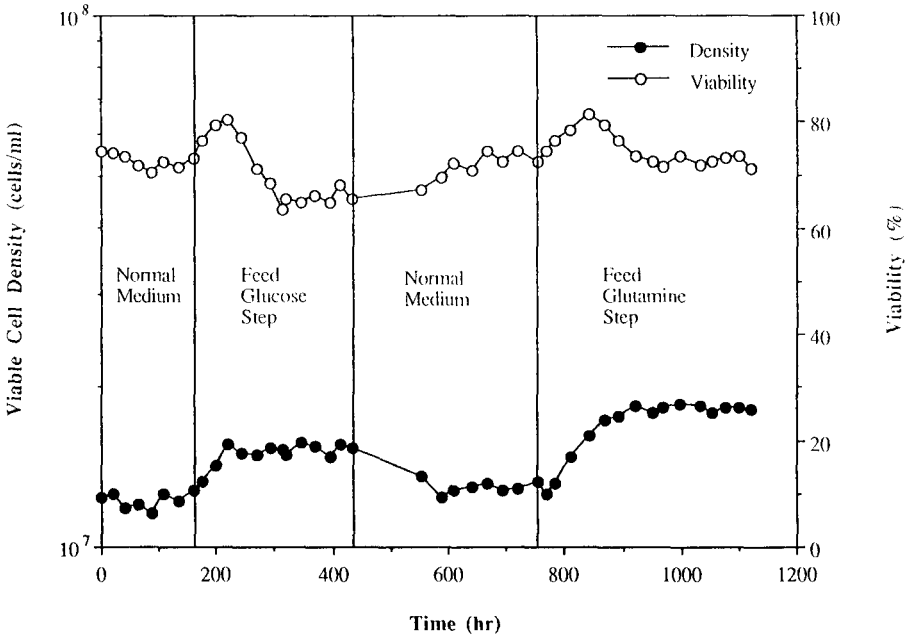

Fig. 5. Viable cell density and percent viability with step changes in feed glucose and glutamine concentrations.

mine steps in continuous culture has been attributed to a delayed increase in ATP production, with a glutamine step as a result of a high ATP/ADP ratio and buffering by the TCA cycle (24). Because of the enhanced glycolytic activity of hybridomas, the increased energy provided by glucose addition is available immediately.

Substrate and Product Metabolism

Separate step increases in the feed concentrations of glucose and glutamine produced very different metabolic responses. Following the inlet glucose step, the glucose and lactate concentrations in the reactor increased rapidly (Fig. 6). The lactate concentration remained high and leveled off at 45 m*M*, but the glucose concentration subsequently decreased to 8 m*M*, indicating that glucose was no longer limiting cell growth. The high lactate concentration could have been responsible for the drop in viability and the high residual glucose concentration. In low-density cultures, however, no changes in growth kinetics have been observed for lactate concentrations as high as 44 m*M* (25), suggesting that our system, rather than being inhibited, was limited, possibly by glutamine, which has been shown to limit glucose consumption (26). Following the glucose feed step, the glutamine concentration in the reactor increased initially, but at steady state glutamine and ammonia concentrations were essentially the same as before the step (Fig. 7).

Following the feed glutamine step, the reactor glutamine concentration increased initially, then slowly decreased to a steady level of 0.36 m*M*,

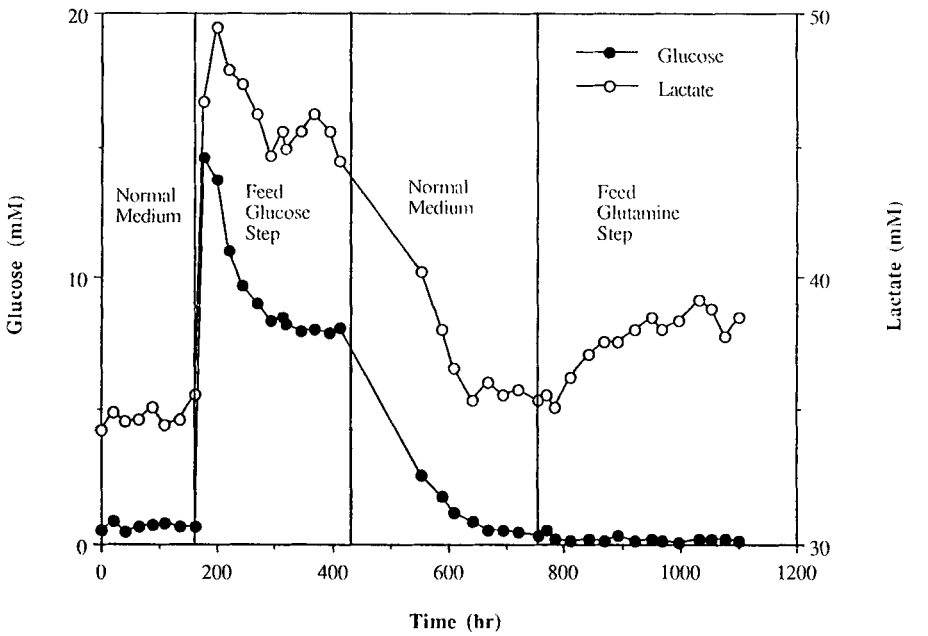

Fig. 6. Glucose and lactate concentrations with step changes in feed glucose and glutamine concentrations.

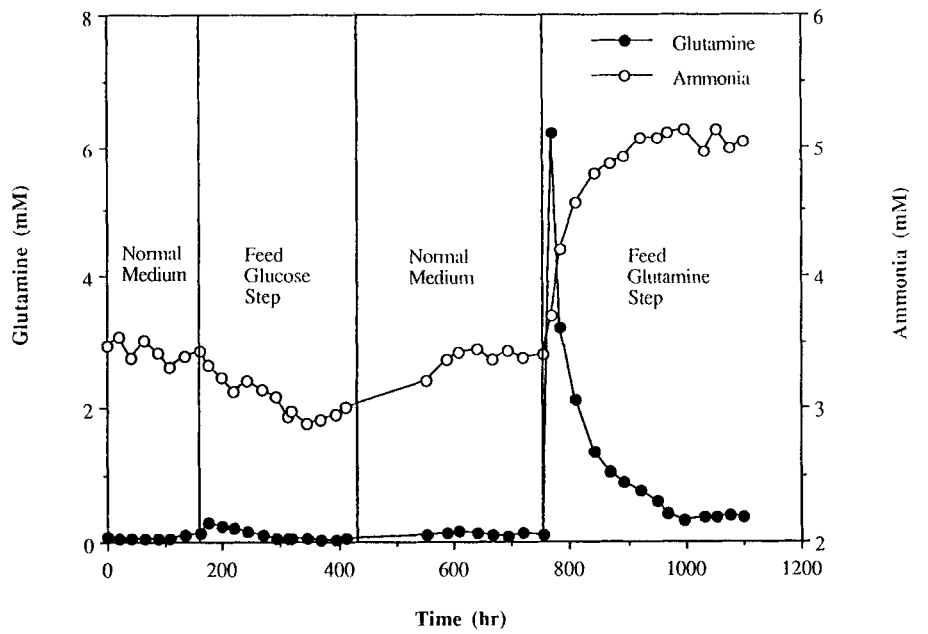

Fig. 7. Glutamine and ammonia concentrations with step changes in feed glucose and glutamine concentrations.

Table 2
Steady-State Apparent Yield Coefficients
Before and After the Feed Glucose Step

	Before	After
Perfusion rate (1/d)	2.0	2.0
Bleed rate (1/d)	0.1	0.1
Feed glucose concentration (m*M*)	25.0	44.4
Specific growth rate (1/d)	0.14	0.15
q_{glu} (mmol/10^9 cells/d)	3.98	4.46
q_{lac} (mmol/10^9 cells/d)	5.74	5.59
q_{gln} (mmol/10^9 cells/d)	0.97	0.73
q_{amm} (mmol/10^9 cells/d)	0.55	0.36
$Y'_{lac/glu}$	1.44	1.25
$Y'_{amm/gln}$	0.57	0.49
$Y'_{ala/gln}$	0.21	0.20
$Y'_{gly/gln}$	0.25	0.38
$Y'_{gly/glu}$	0.06	0.08

a value not much larger than at the initial steady state (Fig. 7). This low glutamine concentration may still have been limiting further cell growth. The increase in the ammonia concentration from 3.5 to 5 m*M* (45%) did not affect the viability appreciably (Fig. 7). The reactor glucose and lactate concentrations decreased and increased slightly, respectively, following the glutamine step (Fig. 6).

Following the glucose feed step, q_{lac} increased transiently (not shown), but, at steady state, was nearly equal to that at low glucose concentration (Table 2). Though q_{glu} slowly increased after the step change to a value slightly greater than before the step, both q_{gln} and q_{amm} decreased slowly but significantly (25 and 35%, respectively) to the new steady state. Similar delayed decreases in q_{gln} and q_{amm} have been reported for low density chemostat studies in response to a glucose step (25), since a higher glucose concentration usually results in an increased rate of glycolysis and a suppression of glutaminolysis. Normally, most of the glucose catabolized results in the formation of lactate; an increase in the medium glucose concentration in low-density culture results in higher q_{glu} and $Y'_{lac/glu}$ (25). Although we did observe a 12% increase in q_{glu}, we also observed a 15% decrease in $Y'_{lac/glu}$; this is consistent with batch studies that show little effect of added glucose on lactate production in the stationary phase when lactate concentration is already high (27). The added glucose may have been used to satisfy some of the glutamine maintenance requirements, since the glutamine maintenance coefficient, determined after the glucose step, was about 20% lower than that calculated in our earlier study (3).

Table 3
Steady-State Apparent Yield Coefficients
Before and After the Feed Glutamine Step

	Before	After
Perfusion rate (1/d)	2.0	2.0
Bleed rate (1/d)	0.1	0.1
Medium glutamine concentration (m*M*)	6.0	10.0
Specific growth rate (1/d)	0.14	0.15
q_{glu} (mmol/10^9 cells/d)	3.78	3.24
q_{lac} (mmol/10^9 cells/d)	5.74	5.03
q_{gln} (mmol/10^9 cells/d)	0.91	1.26
q_{amm} (mmol/10^9 cells/d)	0.52	0.66
$Y'_{lac/glu}$	1.45	1.55
$Y'_{amm/gln}$	0.58	0.52
$Y'_{ala/gln}$	0.25	0.38
$Y'_{gly/gln}$	0.34	0.26
$Y'_{gly/glu}$	0.08	0.10

Both q_{glu} and q_{lac} decreased (15%) following the glutamine step (Table 3), accompanied by a concomitant increase in q_{gln} (40%) and q_{amm} (25%), agreeing with trends reported for glutamine steps in chemostat culture (*24*). A higher glutamine concentration typically causes an increase in the glutamine metabolism, reducing q_{glu}, which in this study was about 20% lower than the glucose maintenance coefficient (*17*) calculated previously for high-density perfusion culture (*3*), which again shows the substitutability of glucose and glutamine.

Contrary to what has been shown in studies of low-density chemostat cultures (*24,25*), the glutamine step did not affect the apparent yield coefficients $Y'_{amm/gln}$ and $Y'_{lac/glu}$ appreciably, since both changed by less than 10% (Table 3). The apparent yield coefficients $Y'_{lac/glu}$ and $Y'_{amm/gln}$ are calculated based on the assumptions that all lactate is formed from glucose and all ammonia is formed from glutamine. This might be because of the high ammonia concentration following the glutamine step, causing the cells to channel glutamine to other byproducts that are less toxic (*20,28*). $Y'_{lac/glu}$ decreased following the glucose step and increased following the glutamine step; $Y'_{amm/gln}$ decreased for both, suggesting that a low ammonia concentration is more important than keeping lactate levels low, particularly if reactor pH is controlled, as it was in these experiments. The inhibitory concentration of ammonia is at least an order of magnitude lower than that of lactate, making ammonia control more critical (*29*).

Amino Acid Metabolism

The consumption rates of several amino acids changed after the feed glucose step (Table 4). The consumption rates of isoleucine (70%), phenylalanine (44%), and lysine (32%) increased significantly. The production

Table 4
The Steady-State Specific Amino Acid Consumption Rates (mmol/10^9 cells/d) Before and After the Feed Glucose Step

	Before	After
Aspartate	−0.003	−0.005
Glutamate	0.015	0.003
Asparagine	0.005	0.002
Serine	−0.005	−0.011
Glutamine	0.969	0.729
Glycine	−0.238	−0.276
Histidine	0.010	0.016
Arginine	0.032	0.004
Threonine	0.045	0.004
Alanine	−0.201	−0.147
Tyrosine	0.036	0.032
Valine	0.062	0.069
Methionine	0.037	0.021
Isoleucine	0.071	0.120
Leucine	0.124	0.141
Phenylalanine	0.036	0.052
Lysine	0.069	0.091
Net	1.064	0.845

rate of alanine decreased by 36%; that of aspartate, serine, and glycine increased by 73%, 120%, and 16%, respectively. Although $Y'_{amm/gln}$ and $Y'_{lac/glu}$ both decreased as a result of the step change, $Y'_{ala/gln}$ was nearly constant (Table 2). A decrease in $Y'_{amm/gln}$ is typically accompanied by an increase in $Y'_{ala/gln}$, since transamination of pyruvate occurs through glutamate and, hence, lower amounts of ammonia are formed from glutamine. Instead, the production of glycine, via formation of serine from 3-phosphoglycerate, increased. The increases in $Y'_{gly/gln}$ (54%) and $Y'_{gly/glu}$ (34%) after the step change suggest that pyruvate, which can be formed from both glucose and glutamine, was probably diverted to form glycine (and serine), rather than alanine, because excess glutamate was not available. The net rate of amino acid consumption decreased (20%) as a result of the step.

The glutamine step resulted in changes in most amino acid consumption rates (Table 5). Histidine, arginine, valine, methionine, leucine, and lysine consumption rates changed by less than 15%, and the consumption rates of threonine, tyrosine, isoleucine, and phenylalanine all decreased by about 20%. After the glutamine step, aspartate, serine, and alanine production rates increased by a factor of two. The glycine production rate increased by only 5% after the glutamine step, which may have been because the glycine concentration did not return to its initial concentration when the glucose level was restored to 25 m*M*. All other measured variables showed excellent repeatability between the steps. Whether the subse-

Table 5
Steady-State Specific Amino Acid Consumption Rates (mmol/10^9 cells/d) Before and After the Feed Glutamine Step

	Before	After
Aspartate	−0.004	−0.007
Glutamate	0.015	0.014
Asparagine	0.004	0.001
Serine	−0.007	−0.014
Glutamine	0.908	1.260
Glycine	−0.312	−0.326
Histidine	0.014	0.013
Arginine	0.004	0.004
Threonine	0.042	0.003
Alanine	−0.230	−0.479
Tyrosine	0.038	0.031
Valine	0.063	0.058
Methionine	0.041	0.036
Isoleucine	0.075	0.061
Leucine	0.129	0.118
Phenylalanine	0.042	0.033
Lysine	0.076	0.072
Net	0.899	0.850

quent decrease in glycine concentration occurred as a result of the glutamine step, or because a steady state glycine concentration was not reached prior to the step change, it not known.

Glutamate was produced, rather than consumed, after the glutamine step. If ammonia concentrations are high, glutamate accumulates in the cell, reducing $Y'_{amm/gln}$. Glutamate can either form alanine through the transamination of pyruvate or be secreted. The lower values of $Y'_{amm/gln}$, higher values of $Y'_{ala/gln}$, and the secretion, rather than the consumption, of glutamate support this observation, as do batch studies (*20,29*).

Both the glucose and glutamine steps caused an increase in the serine and aspartate production rates, suggesting that both substrates can serve as a source for these amino acids. The steps show that there is a decrease in q_{ala} (36%) with an increase in glucose, while there is an increase in q_{ala} (300%) with an increase in glutamine concentration. At increased concentrations, more glutamine is diverted toward the formation of alanine, which has been shown to replace ammonia as the major byproduct at ammonia concentrations above 5 m*M* (*20,28*).

The consumption rates of isoleucine, phenylalanine, and lysine show opposite trends in response to each step change: The consumption rates increased significantly following the glucose step, but the rates decreased in response to the glutamine step. At a sufficient concentration, glutamine can be used to maintain appropriate levels of the TCA cycle intermediates.

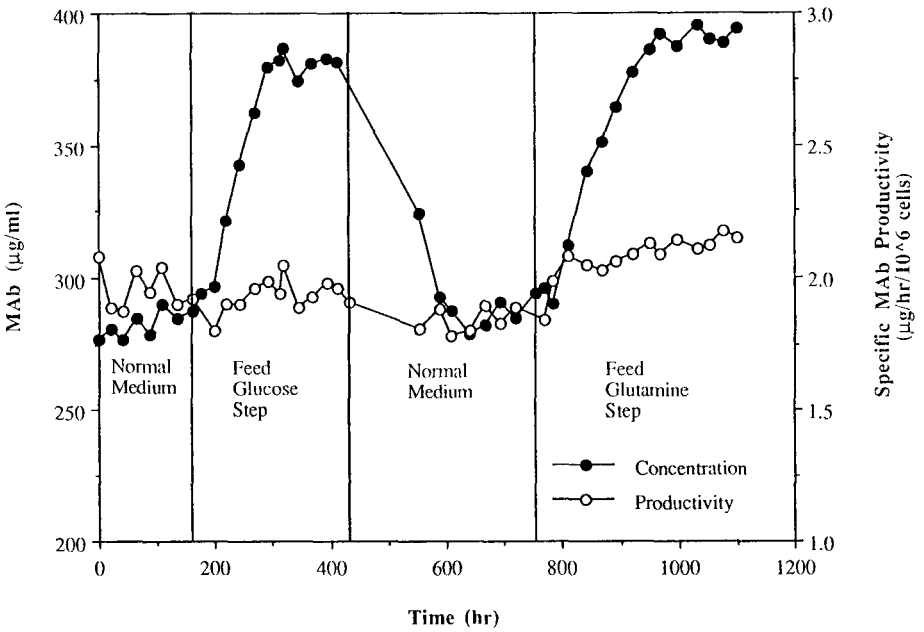

Fig. 8. MAb concentration and specific productivity with step changes in feed glucose and glutamine concentrations.

Acetyl CoA is one such intermediate, and it is the starting point for the catabolism of the carbon from isoleucine, phenylalanine, and lysine. Decreases in the consumption rates of these amino acids indicate a reduced need for them when adequate glutamine is present.

Monoclonal Antibody Production

Although the MAb level in the reactor increased after the glucose step, q_{MAb} was relatively constant (Fig. 8). A higher glucose concentration affected the MAb levels only because it resulted in an increase in cell density. The increase in viable cell density did not, however, result in a higher q_{MAb}, perhaps as a result of glutamine limitation. The MAb concentration increased significantly as a result of the glutamine step (Fig. 8), in part because of the increased cell density, but also because of a higher q_{MAb} (17%) with increased cell density, as has been shown with this hybridoma (*1,3*). Although the maximum error associated with the q_{MAb} values is nearly 15%, the trend appears to be significant. Because q_{MAb} did not increase with increased cell density following the feed glucose step, an adequate glutamine concentration (i.e., a stoichiometric limitation) is shown to be critical for high q_{MAb}, as also seen by others (*9,30*). Higher glutamine concentrations have also been shown to increase the MAb secretion rate from cells in the stationary growth phase in low-density batch cultures, possibly by stimulation of a posttranscriptional step (*31*). More experiments are needed to determine the exact nature of glutamine's influence on q_{MAb}.

CONCLUSIONS

A strategy for increasing viable cell density (and as a consequence, q_{MAb}), should focus on increasing the level of specific substrates, especially glutamine, rather than an increased perfusion rate, which results in insufficient use of medium components by the cells, low concentrations of products in the outlet stream, and potential failure of the cell retention apparatus. Nutrient supply was found to be a stronger determinant of cell density than waste removal over the range of conditions studied, because the cells adapt to higher concentrations of waste metabolites, and because they alter their metabolism to minimize the concentration of toxic waste products. Efficient metabolite removal, however, did result in higher cell yields on substrate, particularly at lower viable cell densities. Even at relatively low concentrations, potentially toxic metabolites had a greater inhibitory effect on cell growth at lower cell densities. The specific MAb production rate, however, was relatively unaffected by metabolite concentrations, and was instead primarily correlated with cell density. There was inconclusive evidence supporting the presence of an autocrine factor that enhances q_{MAb}. Although serum and proteins are important for cell growth at low densitites, their concentrations may be reduced or even eliminated in high-density culture for the cell line under study, since a change to protein-free medium had no effect on cell growth, substrate and product metabolism, or MAb production.

Step changes in glucose and glutamine at steady state resulted in increases in cell density, indicating that the culture was stoichiometrically limited by a carbon/energy source, and, since each step change caused an increase in cell density, both substrates met this need. Glucose and glutamine are interchangeable as energy and carbon sources, except as essential precursors for some biomolecules. Contrary to what is usually seen in low-density cultures, the glucose step caused an increase in q_{glu}, no change in q_{lac}, and a dramatic decrease in $Y'_{lac/glu}$, demonstrating a feedback mechanism for metabolic regulation. Despite the increase in cell density, the glucose step did not produce any change in q_{MAb}. The glutamine step caused a greater increase in live cell density than was produced by glucose; the cell density increased until limited by glutamine, and was apparently not limited by ammonia. The glutamine step caused an increase in both antibody productivity and concentration. Because the step change in glutamine, but not glucose, resulted in an increase in q_{MAb}, the presence of adequate glutamine appears to be crucial to increasing q_{MAb} as cell density increases for this hybridoma.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the National Science Foundation (BCS-915781) and by matching funds from Merck. We thank Roland Buchele of Akzo (Germany) for the membranes, and Dr. Michael Fanger (Dartmouth Medical School) for the hybridoma.

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