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Growth Inhibition in Animal Cell Culture

The Effect of Lactate and Ammonia

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ABSTRACT

Eight independent cell lines accumulated ammonia in culture to concentrations between 1.3 and 2.9 m*M*. The growth inhibition of such concentrations of ammonium chloride when added to culture medium was variable. The cell lines tested could be divided into 3 groups depending on their growth response to 2 m*M* added NH_4Cl. In the first group (293, HDF, Vero, and PQXB1/2) little (<14%) or no growth inhibition occurred. In the second group (McCoy and MDCK) a reduction in final cell yield of 50–60% was observed. The third group (HeLa and BHK) was most sensitive to the effects of NH_4Cl with growth inhibition (>75%) compared to controls.

The growth inhibitory effect of added lactate up to 20 m*M* was negligible (<10%) for 3 cell lines, although one cell line (PQXB1/2) showed greater sensitivity.

The interactive effects of ammonia and lactate were determined in a matrix experiment. At lactate (>12 m*M*) and ammonia (1–4 m*M*), the growth inhibitory effects of the two components were synergistic. However, at low concentrations of lactate (<12 m*M*) the toxic effect of ammonia was reduced. A proposed mechanism for the sparing effect of lactate on ammonia toxicity is discussed. This may have importance in developing strategies for the optimal growth of ammonia-sensitive cell lines.

Index Entries: Ammonia; lactate; glutamine; growth inhibition.

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INTRODUCTION

The application of animal cell cultures for large scale processes requires the optimization of growth conditions to achieve maximum cell productivity. In batch culture the final cell density obtained is usually related to the nature of the culture medium. The depletion of nutrients and the accumulation of growth inhibitors are the two major factors that cause deterioration and the consequent cessation of cell growth. An assessment of the contribution of each of these factors is an essential step in the optimization of a cell culture system.

The potential growth inhibitors that have been reported to accumulate in cell cultures are ammonia (*1–3*) and lactate (*4–7*). The accumulation of these compounds arises from the catabolism of glutamine and glucose, which are the major substrates normally provided in culture medium (*8–10*). Some of the accumulated ammonia also occurs by the chemical degradation of glutamine in the medium.

Ammonia (mainly as ammonium ions) accumulates to concentrations typically of 2 m*M* during batch cultures and such a concentration has been shown to be significantly growth inhibitory to cells in culture (*3*). In such cultures, the toxicity of ammonia has been suggested to be the primary factor in causing the cessation of cell growth. Lactate has been found to accumulate to concentrations as high as 25 m*M* in hybridoma cultures (*7*). However, if the pH of the medium is adjusted, such a concentration of lactate may not be growth inhibitory.

In the work reported here, the toxicity of ammonia and lactate was assessed in cultures of up to eight different cell types in order to establish the general importance of these potential inhibitors in limiting final cell yields. Further, the effects of the addition of variable concentrations of ammonia and lactate on cell growth were determined in order to assess any interactive effects between the two compounds.

MATERIALS AND METHODS

Cell Lines

Seven anchorage-dependent cell lines and one anchorage-independent hybridoma were used in this study:

1. Baby hamster kidney cells—BHK 21 (*11*)
2. Human kidney embryo cells—293 (*12*)
3. Madin Darby canine kidney cells—MDCK (*13*)
4. McCoy mouse LS fibroblasts (*14*)
5. Human diploid fibroblasts—HDF, from foetal skin cells at passage 4

6. African green monkey kidney cells—Vero (*15*)
7. Human carcinoma cells—HeLa (*16*)
8. Mouse hybridoma cell line—PQX B1/2 (*17*)

The cell lines were obtained from the following sources: A. Bailey, Booth Hall Children's Hospital, Manchester (2 and 6), D. Coupes, Manchester University Medical School (1 and 4), A. R. Sattar, Christie Research Institute, Manchester (5), Flow Laboratories, Irvine, UK (3,7), and I.C.I. Laboratories, Macclesfield (8).

Culture

All cells were maintained in 10 mL of medium contained in 25 cm^2 T-flasks (Corning) at 37°C in an air + 10% CO_2 atmosphere. This enabled pH to be controlled at 7.4. The anchorage-dependent cells were grown in Glasgow modification of Eagle's medium (GMEM) supplemented with 10% tryptose phosphate broth and 10% bovine serum. The growth medium for the hybridoma was RPMI 1640 supplemented with 10% newborn calf serum. The medium was obtained from Northumbria Biologicals Ltd. and the serum from Imperial Labs. The anchorage-dependent cells were harvested by exposure to 2 mL trypsin (0.25%) in PBS and incubating for 5 min at 37°C.

Cell Counting

Viable cell counts were determined by the trypan blue dye exclusion method (*18*). Equal volumes of cell suspension and reagent (0.25% trypan blue in PBS) were mixed and the cell numbers counted in a haemocytometer.

Ammonia

Media samples were treated with sodium hydroxide to convert ammonia to the gaseous form before analysis by an ammonia specific electrode (Orion 95-10). The electrode gave the characteristic response of 56 mV change per decade change in concentration.

Lactate

Lactate concentrations in the medium were determined enzymatically by monitoring the reduction of NAD during the conversion of lactate to pyruvate by lactate dehydrogenase (*19*). Media samples were deproteinized by the addition of 3% perchloric acid prior to analysis.

Table 1
The Effect of Added Ammonium Chloride on Cell Yields

Cell line	Final $[NH^+_4]$ in culture (mM)	% decrease in cell yield at 2mM NH_4Cl	IC-50 (mM)
HDF	1.55	0	>2.5
Vero	2.9	0	>2.5
293	2.35	8	>2.5
PQXB1/2	1.3	14	5.1
MDCK	1.75	50	1.8
McCoy	2.3	60	1.7
HeLa	2.65	75	0.8
BHK	2.25	80	1.3

The anchorage-dependent cells (3.5 to 5.5 × 10^5) were inoculated into 10 mL of GMEM and grown for 3 d. Final cell numbers of the controls (no added ammonia) were between 0.75 and 3.0 × 10^6 cells. The hybridoma (PQXB1/2) control culture (2 mL) was inoculated with 1.4 × 10^5 cells/mL, which increased to 1.06 × 10^6 cells/mL in 3 d.

RESULTS

The Accumulation of Ammonia in Culture

The ammonia concentration was measured in culture medium at the end of a cell growth period (Table 1). For the anchorage-dependent cells, the concentrations varied between 1.6 and 2.9 m*M*. The hybridoma culture (PQXB1/2) produced a lower concentration of ammonia (1.3 m*M*) over an equivalent culture period. This was probably attributable to the lower initial concentration of glutamine (2 m*M*) in RPMI. The concentration of the accumulated ammonia in the medium was dependent upon the cell type. Although each cell line grew at a different rate there was no simple relationship between this rate and the ammonia produced.

Ammonia can accumulate in the medium by the chemical breakdown of glutamine (*20*) or by the enzymatic activity of the serum (*21*). The ammonia produced by such means in GMEM is shown in Fig. 1. After 3 d at 37°C, the ammonia increased in concentration to 0.8 m*M*.

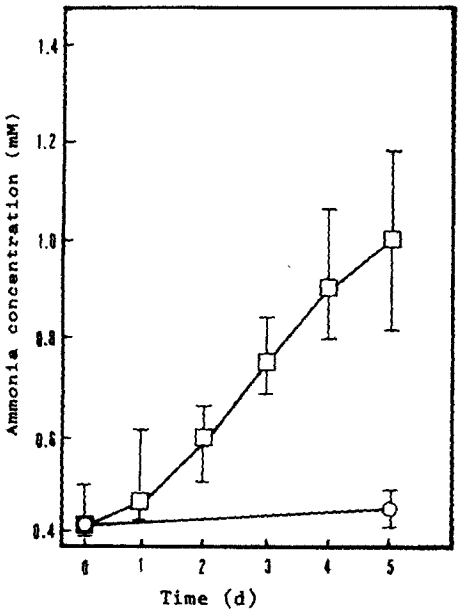

Fig. 1. Ammonia accumulation in cell free medium. GMEM (50 mL) containing 10% bovine serum was incubated at 37°C (□) and 4°C (○) for 5 d. Daily samples (5 mL) were taken and the ammonia concentration determined ($n=3$).

The Effect of Added Ammonium Chloride on Cell Growth

The effect of added ammonia was determined at concentrations typically found in the media at the end of cell growth. The cells were inoculated into media containing specific concentrations of ammonium chloride up to 2.5 m*M* or 20 m*M* (for PQXB1/2). The pH of the medium (7.4) was unaffected by the additions of ammonium chloride. After 3 or 4 d growth the cells were harvested and counted.

The results show the variable effect of ammonium chloride on the growth of these cell lines (Fig. 2). The eight cell lines can be divided into three identifiable groups with respect to their apparent sensitivity to the effects of ammonia (Table 1).

The first group comprises the HDF, Vero, 293, and PQXB1/2 cell lines where 2 m*M* added NH_4Cl had little (< 14%) or no effect on cell yield. The second group comprises MDCK and McCoy cells. Here, partial inhibition was observed at 2 m*M* NH_4Cl with final cell yields reduced by 50–60% of control values. The third group comprises HeLa and BHK cells where 2 m*M* NH_4Cl reduced final cell yields by >75% of the control value.

The ammonium chloride concentrations causing a 50% reduction in cell yield (IC-50) is also tabulated (Table 1). This showed that the HeLa

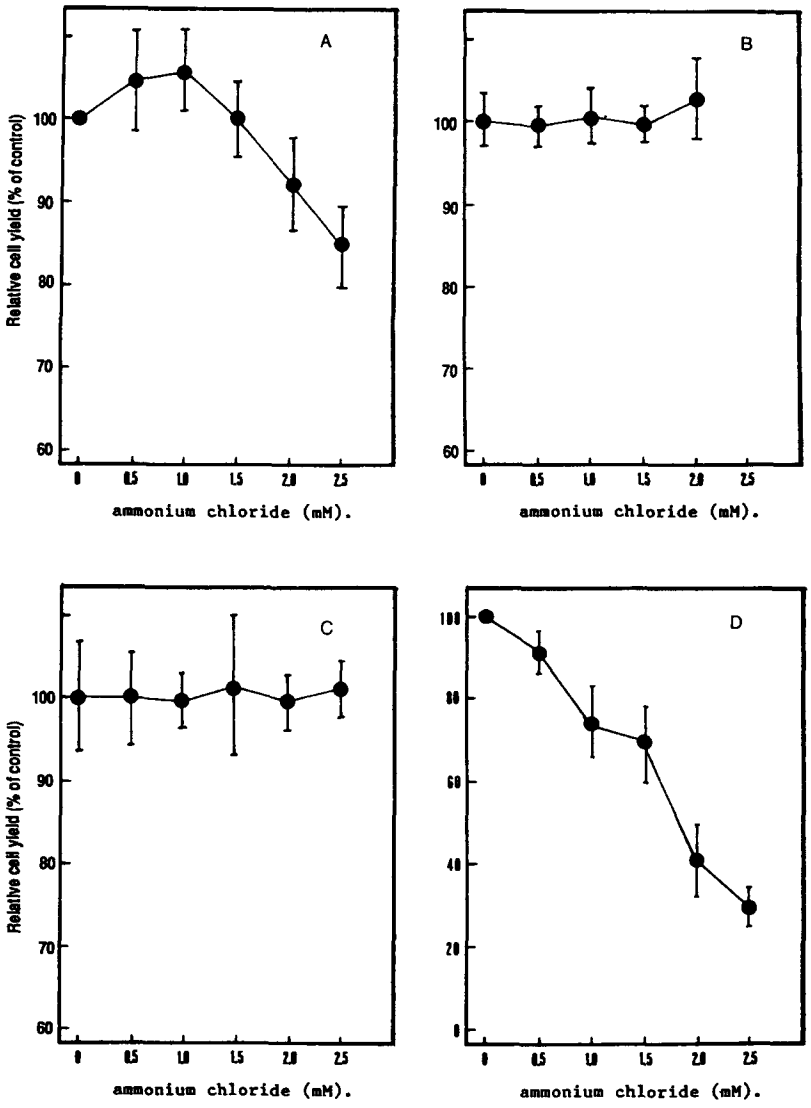

Fig. 2. Effect of ammonium chloride added to the culture medium of eight independent cell lines: (**A**) 293 cells; (**B**) human diploid fibroblasts; (**C**) Vero; (**D**) McCoy; (**E**) MDCK; (**F**) HeLa; (**G**) BHK; (**H**) PQXB1/2.

For (**A**) to (**G**), the medium (10 mL) containing varying concentrations of ammonium chloride was inoculated with $0.5\text{–}2 \times 10^6$ cells and incubated for 3 d.

cells were particularly sensitive to the toxic effect of ammonia with an IC-50 value determined as 0.8 m*M*. This compared with an IC-50 for the PQXB1/2 hybridoma of 5.1 m*M*. The IC-50 values of the three other non-sensitive cell lines (HDF, Vero, and 293) were not determined although they are likely to be considerably higher than 2.5 m*M*, which was the highest ammonia concentration tested on these cells.

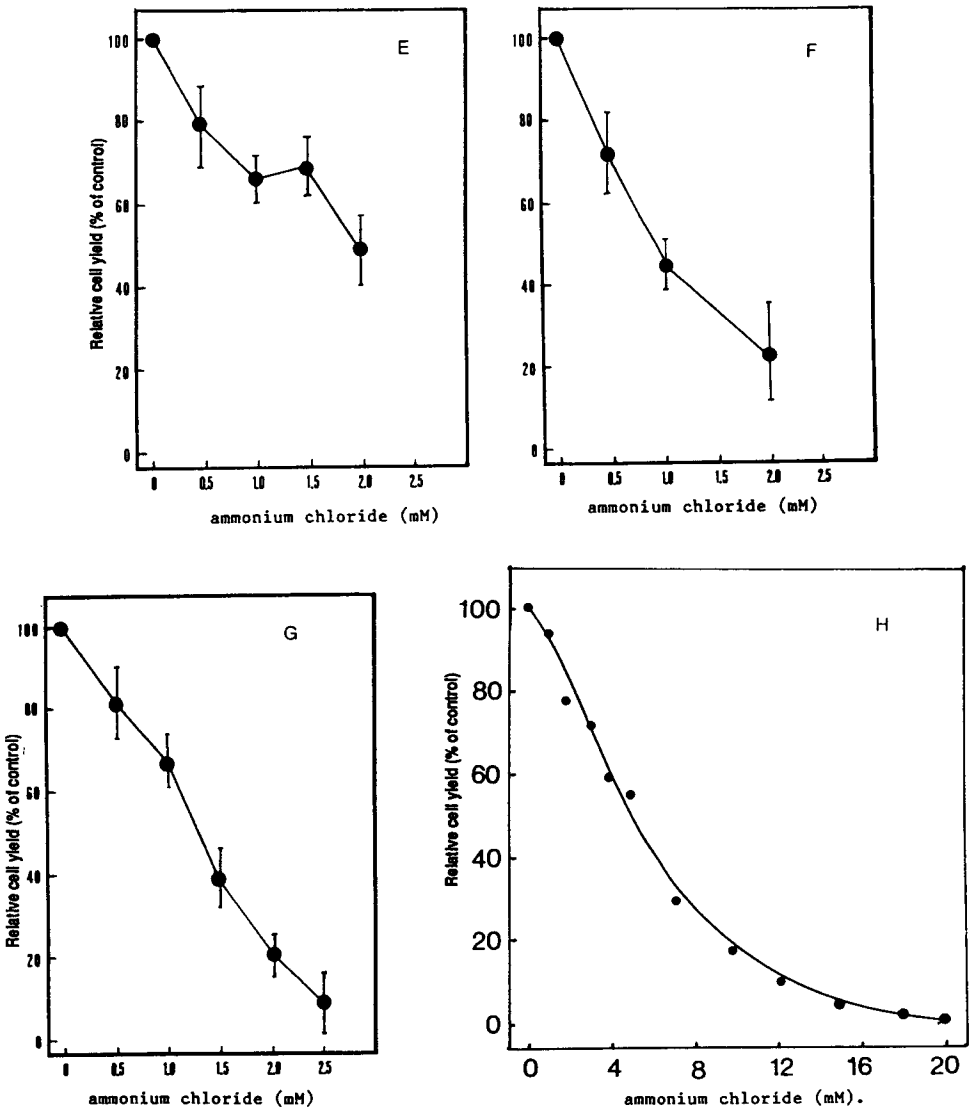

Fig. 2 (*cont'd*). The cells grew as a monolayer in 25 cm^2 T-flasks from which they were harvested by trypsinisation ($n = 3$).

For (**H**), the medium (2 mL) was inoculated with 2×10^5 cells/mL. Cells grew in suspension for 4 d ($n = 4$).

The Accumulation of Lactate in Culture

The lactate concentration was measured in the medium of three of the anchorage-dependent cell cultures after 3 d growth. The values determined were 3.7 m*M* for the BHK cells, 4.8 m*M* for the McCoy cells, and 6.7 m*M* for the Vero cells. For the hybridoma culture after 4 d growth the determined lactate concentration was 14 m*M*.

Table 2
The Effect of Added Lactate on the Final Cell Yield of Three Cell Lines

	Final cell count (x 10^6)				
Lactate added (mM)	0	2.5	5.0	10.0	20.0
Cell line					
BHK	2.96	2.65	2.87	2.35	2.85
McCoy	4.00	3.78	3.93	3.96	3.78
Vero	3.86	3.97	4.01	3.80	3.59

Cells (10^6) were inoculated into 10 mL of medium containing varying concentrations of neutralized sodium lactate. The final cell yield was determined after 3 d growth. The standard deviation was less than 10% of the mean in all cases ($n = 3$).

The Effect of Added Lactate on Cell Growth

The effect of added sodium lactate at a concentration range up to 20 m*M* was determined for three anchorage-dependent cell lines. The cell types chosen represent each of the 3 groups with respect to sensitivity to ammonia toxicity. The pH of the medium was adjusted to 7.4 in all cases by the addition of sterile sodium hydroxide. The cells were inoculated at a concentration of 10^5 cells/mL in 10 mL of medium contained in T-flasks. The results of this experiment are shown in Table 2. The lactate addition up to 20 m*M* had a negligible effect on cell yield for the three cell lines—McCoy, Vero, and BHK.

The effect of lactate on the hybridoma cell line was tested in 24-well plates in which 2 mL samples of media were inoculated with 2×10^5 cells/mL. Neutralized sodium lactate was added to cultures at specific concentrations up to 50 m*M*. The results showed (Fig. 3a) that this cell line was more sensitive to the effect of lactate. Cell growth was reduced by 19% in 10 m*M* added lactate and the apparent IC-50 was determined as 22 m*M*.

The Interactive Effect of Lactate and Ammonia on Cell Growth

Hybridoma cell growth was examined in medium containing a range of concentrations of both additional ammonia and lactate. A matrix of 2 mL cultures was established in which concentrations of ammonium chloride (0, 1, 2, 3, 4, 5, and 6 m*M*) in one direction were combined with concentrations of lactate (0, 4, 8, 12, 16, 20, and 24 m*M*) in the perpendicular direction. The 49 cultures (2 mL each) were established in three 24-well culture plates. Each culture was inoculated with 0.9×10^5 cells/mL and incubated for 4 d at 37°C under a 10% CO_2 atmosphere. At the end of the culture period two viable cell counts were performed on each culture.

The results of this experiment are shown in Fig. 3. Final cell yields are expressed as percentage of control values. Analyses of these results shows that at high added lactate concentrations (>12 m*M*) and ammonia concentrations (1–4 m*M*), the observed inhibitory effect significantly exceeded the values that could be predicted by single additions of the two components. Thus, within this concentration range the toxic effects of the two components act synergistically. However, at low concentrations of lactate (4–12 m*M*) the toxic effect of ammonium chloride was reduced particularly at concentrations of 4–12 m*M*. This observed reduction in the toxic effect is of particular significance as it occurs at concentration ranges that are found typically in culture.

DISCUSSION

Glucose and glutamine are the major energy sources for animal cells in standard culture medium. The glutamine concentration (2–4 m*M*) is considerably higher than that of the other amino acids. Some of the glutamine consumed by cells may be used for synthesis of proteins, nucleotides, or lipids (*22*). However, a large proportion of the glutamine is utilized in aerobic energy metabolism. Its metabolic deamination leads to the production of 2-oxoglutarate and ammonia (*8,10*).

Glucose can be catabolized by various metabolic routes but a significant proportion is degraded by anaerobic glycolysis leading to the formation of lactic acid. Both lactic acid and ammonia are released as metabolic byproducts into the culture medium and their accumulation has been implicated in the limitation of final cell yields. In the experimental work described here, we have investigated the growth inhibitory effects of ammonia and lactic acid on a variety of cell types.

Ammonia concentrations of 1.6 to 2.9 m*M* were measured in cultures of different cell lines under apparently identical conditions and similar growth rates. Some of the ammonia originates from the chemical breakdown of glutamine to pyrrolidone carboxylic acid and this can be affected by pH, temperature, and the serum content of the medium (*23*). However, a significant proportion of accumulated ammonia arises from metabolic deamination and thus some of the variation observed here must reflect inherent differences in the metabolism of the cell lines tested.

The final level of accumulated ammonia does depend upon the glutamine content of the media with extremely low levels occurring in glutamine-free cultures (*24*). The hybridoma cells grown here were cultured in RPMI, which contains half the glutamine content of GMEM and this may account for the lower levels accumulated in these cultures.

The growth inhibitory effect of such concentrations of ammonia was investigated for eight independent cell lines. These cell lines showed considerable variation in response. At 2 m*M* ammonia, the growth of some

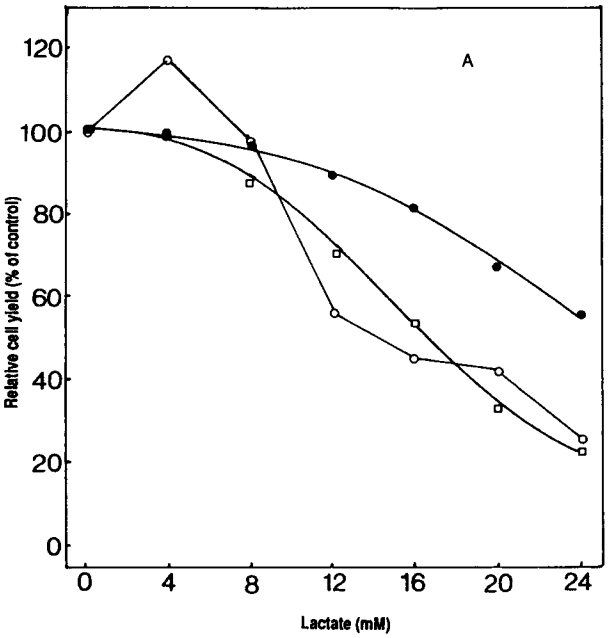

Fig. 3. The interactive effect of added lactate and ammonium on the final cell yield of PQXB1/2 cells.

Medium (2 mL) containing varying concentrations of ammonium chloride was inoculated with PQXB1/2 cells (2×10^5 cells/mL). After 4 d growth viable cells were counted by use of a haemocytometer. The final cell yield of the control (no additions) was 1.1×10^6 cells/mL. The final cell yields were calculated as relative values compared to controls at each added ammonium concentration ($n=2$).

A: The added ammonium chloride concentrations were 0 (●); 1 m*M* (□); and 2 m*M* (○).

cell lines was unaffected whereas in one case (BHK cells) the final cell yield was below 10% of the control value. The cell lines were categorized into 3 groups related to their growth response to added ammonia.

It has been shown previously that the toxic effect of ammonia in culture is related to the concentration of NH_3 rather than NH_4 and thus the toxicity of dissolved ammonia is pH dependent (*25*). A range of cell lines have been shown to be sensitive to the concentration of ammonia that normally accumulates in culture (*7,26,27*). However, we have shown here that such a sensitivity is not universal in all cell lines. In fact the growth of certain cell lines (human epithelial 293, human diploid fibroblasts, and Vero cells) was unaffected by the addition of 2.5 m*M* ammonium chloride. The nature of such tolerance to ammonia is unknown but may well be related to the ability to these cells to sequester ammonia by metabolic amination reactions.

The second major byproduct of energy metabolism is lactic acid, which arises from anaerobic glycolysis. The production of lactic acid often ex-

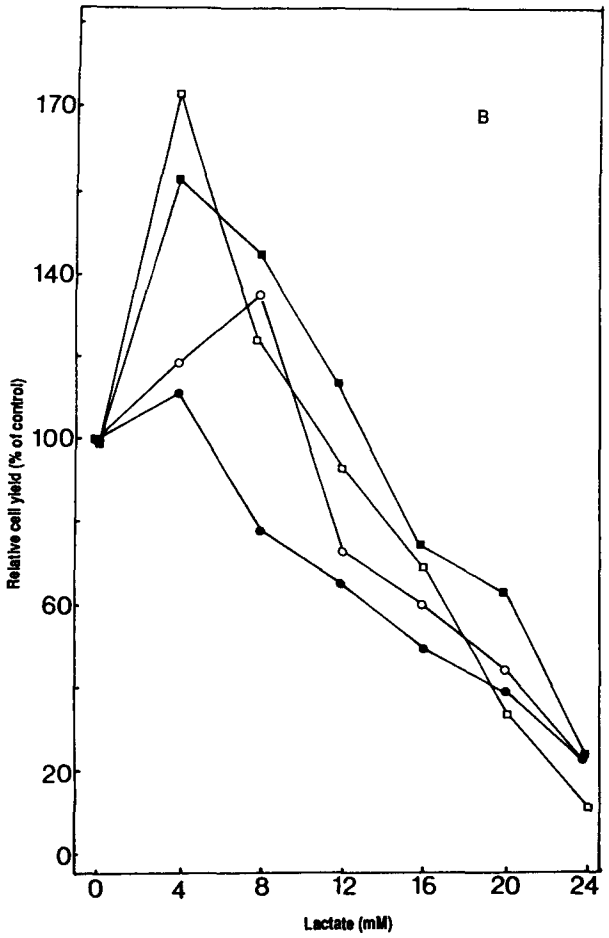

Fig. 3. (*cont'd*). B: The added ammonium chloride concentrations were 3 m*M* (●); 4 m*M* (○); 5 m*M* (□); and 6 m*M* (■).

ceeds the buffering capacity of the culture medium and this may cause undesirable effects on cell growth, as mammalian cells can only tolerate a narrow range of pH. Various reports have analyzed the toxicity of neutralized lactate and have found insignificant growth inhibition at concentrations <25 m*M*. Reuveny et al. (*7*) showed that additions of lactate up to 20 m*M* apparently stimulated hybridoma growth and antibody production over a 6 d period. However, Glacken et al. (*28*) reported that lactate at similar concentrations causes the inhibition of ammonium secretion and antibody production from another hybridoma. In this case, lactate up to 40 m*M* did not significantly affect growth but this was dependent upon the serum used.

In our study of 3 anchorage-dependent cell lines, no growth inhibition was found at initial lactate concentrations up to 20 m*M*. However, the hybridoma PQXB1/2 appeared to be more sensitive to lactate with an ap-

parent IC-50 determined at 22 m*M*. It has to be noted however that this cell line produces up to 14 m*M* lactate over 4 d when grown in RPMI. Thus, the final lactate concentrations at the end of the growth period would have been considerably higher than those added initially. At these high concentrations of lactate the osmolarity of the medium could increase by 10–20%, which may contribute to the observed decrease in cell yields (*29*).

The analysis of the interactive effects of lactate and ammonia was of particular interest. At high added lactate concentrations (>12 m*M*), the observed growth inhibition of ammonia was enhanced. However, at lower lactate concentrations (<12 m*M*), the apparent toxicity of ammonia was reduced. This result is consistent with the enhancement of hybridoma cell yield observed by Reuveny et al. (*7*) on addition of lactate.

Glacken et al. (*28*) reported that lactate could inhibit ammonia secretion in a hybridoma and this was explained by two possibilities. Either lactate may chelate calcium ions and so decrease membrane exocytosis or lactate may inhibit glutaminase, which would decrease the ammonia release from glutamine. We would like to offer a third possible explanation. Lactate may be metabolically converted to pyruvate, which could provide the means of metabolic sequestration of ammonia resulting in the formation of alanine. This is consistent with several reports showing that alanine increases in culture medium during cell growth (*30,33*).

This phenomenon of decreased ammonia toxicity in the presence of lactate is noteworthy as it appears to occur at concentrations normally found in the medium of growing cells. Thus in cultures of those cells identified as being sensitive to ammonia toxicity, the production of lactate may be beneficial in terms of maximizing cell yields.

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