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Review

Hybridoma growth limitations: The roles of energy metabolism and ammonia production

M. Newland, P.F. Greenfield and S. Reid
Department of Chemical Engineering, The University of Queensland, St. Lucia, Qld., 4067, Australia

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Abstract

Energy metabolism and the production of ammonia in hybridoma cell culture and its inhibitory effects on cell growth are reviewed. The interactive roles of glucose and glutamine metabolism affect the rate of production of ammonia, and these interactions are described. It is shown that growth inhibition usually occurs between 2–4 mM ammonia although some cell lines have been shown to adapt to much higher concentrations, particularly in continuous culture. In batch cultures cell growth appears to be particularly susceptible to increased ammonia concentrations during the early stages of growth; ammonia increased the rate of cell death in the late stage of batch growth. The specific productivity of monoclonal antibodies is much less sensitive to the released ammonia than is growth; lower volumetric productivities relate to the lower viable cell concentrations which are achieved at the high ammonia levels. Techniques to prevent ammonia accumulation or remove ammonia selectively have been relatively unsuccessful to date.

Introduction

The initial impact of monoclonal antibodies from hybridoma cell lines has been in the diagnostic field. For the relatively small amounts of antibody required to produce diagnostic kits, the ascites fluid of mice has proved to be a very satisfactory production medium. Other applications, particularly those involving human and animal therapies and immunopurification, require quantities of antibodies ranging from hundreds of grams to multi kilograms. Such applications necessitate the use of cell culture (Birch *et al.*, 1987).

The standard industrial production technique currently employs a batch culture system and the use of a medium which may include foetal calf serum. Hybridoma cells typically grow to a density which ranges from $1–3 \times 10^6$ cells/ml while antibody is generally produced during both the exponential growth and stationary phases. Over the last decade, considerable effort has been devoted to understanding the nutritional aspects of hybridoma growth and antibody productivity – a recent review summarizes much of the relevant literature (Butler and Jenkins, 1989).

Despite these efforts, it is still not clear as to why hybridoma cell lines fail to grow beyond densities of the order of 10^6 cells/ml in batch culture. Studies are complicated by the interac-

tion between energy substrates (glucose, glutamine), essential nutrients (amino acids), trace factors (hormones) and inhibitory or toxic effects attributed to metabolic products (lactate, ammonia), generated chemically (hydrogen peroxide etc) or resulting from environmental stress (shear effects, bubble damage, proteolytic enzymes or other stress proteins). In batch culture there appears to be at least an indirect positive relationship between ultimate viable cell numbers and the volumetric productivity of antibody that can be achieved. While considerable effort has been directed at developing perfusion systems to achieve much higher cell densities, there remains in industry, for regulatory and operational reasons, a strong attachment to batch or fed-batch systems.

This review focusses on the role of ammonia in affecting hybridoma cell metabolism and limiting cell growth. Because ammonia is a metabolic product and because of the interactions summarised in the previous paragraph, the review considers in some detail relevant aspects of glucose and glutamine metabolism in hybridomas. In addition, it draws on related studies on the effects of ammonia in other animal cell systems; studies on hybridoma systems are relatively recent.

The term ammonia will be used throughout to refer to the sum of the ammonium ion (NH_4^+) and ammonia (NH_3) species. Although both are present the former predominates at a culture pH of around 7.0. The position of the equilibrium between NH_4^+ and NH_3 is such that the latter accounts for less than 3% of the total 'ammonia' at this pH. Table 1 lists the values of pK_a for the ammonia-ammonium ion system for temperatures of interest.

Table 1. Value of pK_a for NH_3-NH_4^+ equilibrium

Temperature °C	pK_a
10	9.20
20	9.23
25	9.25
30	9.26
40	9.27

Overview of important metabolic pathways in cultured mammalian cells

Cultured mammalian cells are grown in media containing carbohydrates, amino acids, salts, vitamins and growth factors. Except for growth factors provided by added serum the basic composition of tissue culture media has been known since the work of Eagle in the 1950s (Eagle *et al.*, 1956, 1958). The cells have two predominant energy sources available: glucose and glutamine. The consumption of either energy substrate is dependent on availability and culture conditions. Both are also important biosynthetically.

Ammonia accumulates in culture medium primarily as a result of glutamine catabolism although spontaneous glutamine decomposition to pyrrolidone carboxylic acid also contributes a small, but significant portion of the free ammonia in spent culture medium (Tristch and Moore, 1962). Negrotti *et al.* (1989) recently reported a half-life for glutamine of only 7 days at 37°C and pH 7.2 in serum free DMEM or RPMI-1640 and 90–115 days at 4°C. This value is higher than reported in earlier work (Ryan and Cardin, 1966), although the authors suggested that the half-life would increase at a pH of 7 and in the presence of serum. If correct, the implication is that a major contribution to the ammonia in serum free cell culture media is from chemical decomposition of glutamine.

Ammonia has been identified as a possible growth inhibitor in cell culture. To further understand the origin and implications of this ammonia generation it is necessary to consider the energetic and biosynthetic pathways operating in cultured cells. Figure 1 summarises current knowledge of hybridoma metabolic pathways. These are discussed in greater detail in the following sections.

Glucose metabolism

Rapid glucose consumption and concomitant lactic acid production by cultured cells is well recognised, Otto Warburg noting it as a characteris-

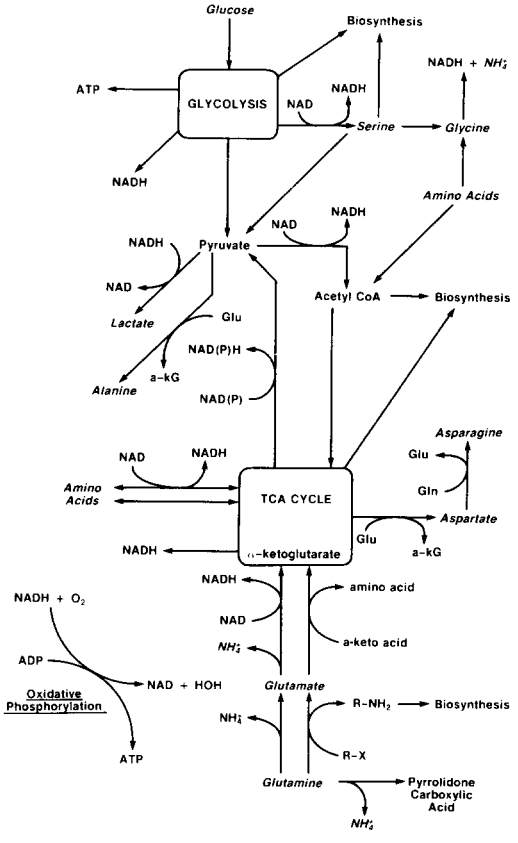

Fig. 1. Schematic diagram of hybridoma metabolic pathways (Glu = glutamate, Gln = glutamine, α-kG = α-ketoglutarate).

tic of tumour cells relative to normal cells. Levintow and Eagle (1961), while attempting to optimise culture medium composition, concluded (incorrectly) that cultured cells derived energy largely, if not entirely, through the utilization of carbohydrate. The levels of glucose in standard media formulations reflect the importance placed on glucose during this period of media development. For example RPMI contains 11 mM glucose, DMEM either 5.5 or 25 mM. Physiologically the level of glucose in human plasma is buffered at about 5.6 mM.

The rate of glucose metabolism is not directly related to cell growth, however. For example, Zielke *et al.* (1984) observed identical cell growth when human fibroblasts were subcultured in medium with 5 mM glucose or in medium with 80 μM glucose to which small aliquots of glucose were added daily. Under the low glucose conditions, the cells utilized only 7% as much glucose as was used by cells cultured in the 5 mM glucose medium.

Little glucose is oxidised to CO_2 via the tricarboxylic acid (TCA) cycle under aerobic conditions except at very low glucose concentrations. This helps explain the above observations. Reitzer *et al.* (1979) found that 80% of consumed glucose was converted to lactate by HeLa cells, 8% was metabolised via the pentose phosphate pathway and 5% or less entered the TCA cycle. Miller *et al.* (1987) observed an apparent molar yield of lactate from glucose of about 1.5 (75% of the theoretical maximum) in mouse derived hybridoma cells. This substantial conversion of glucose to lactic acid represents an apparently inefficient use of the carbon energy source. Compared with the complete aerobic breakdown of glucose via the TCA cycle, lactic acid generation results in only 2/36 of the potential ATP production. Reasons for this high rate of inefficient glycolysis under aerobic conditions in a number of cultured cells are still not clear. Recent work in our laboratory suggests that animal cells are not limited by rates of energy production but rather by rates of biosynthesis, and adjust their energy metabolism to match the rates of synthesis (Neilsen *et al.*, 1990).

Newsholme *et al.* (1985) have proposed that high rates of glycolysis and glutaminolysis are necessary in cells of the immune system to permit high rates of proliferation when required. They argue, using control theory, that high glycolytic and glutaminolytic rates are required, not for precursor/energy biosynthesis per se, but to achieve high sensitivity of the pathways involved in the use of precursors for macromolecular synthesis of specific regulators to permit high growth rates. Hume *et al.* (1978) have also suggested that a major function of enhanced aerobic glycolysis in transforming lymphocytes is to maintain higher steady-state amounts of glycolytic intermediates to act as precursors for macromolecular synthesis. Racker (1976) has postulated that, in

Ehrlich ascites tumor cells, high aerobic glycolysis is due to a lesion in the plasma membrane which causes defective operation of the Na^+ K^+ pump. Energy provision for Na^+, K^+ ATPase activities is worth consideration in explaining high glycolytic rates given that the energy cost of this enzyme is high (up to 50 percent of the total cellular energy production for some cells) and that there is evidence that the energy requirement for ATPase is compartmentalized; that is glycolysis provides all of the ATP for this ion pump in some cell types including ascites tumor cells (Glacken, 1988). However, evidence is also available that high glycolytic rates are not necessary for high rates of proliferation nor for cells to exhibit malignant properties, (Newsholme *et al.*, 1985; Morgan and Falk, 1986). Perhaps the only general conclusion available at this time is that, if hybridomas or other malignant cells in culture are presented with high glucose levels (with high glutamine levels), they will exhibit high glycolytic rates. The cells can adapt, however, to reduced glucose levels while maintaining equivalent growth rates (Morgan and Falk, 1986). At very low glucose concentrations, increased use of the TCA cycle is apparent.

In the absence of other hexoses there is an absolute requirement for glucose as a precursor of ribose for nucleic acid synthesis. At low glucose concentrations (<25 μM), glucose is converted via the pentose phosphate pathway to nucleoside triphosphates and nucleic acids rather than to lactate (Renner *et al.*, 1972; Zielke *et al.*, 1976). Zielke *et al.* (1976) proposed that the cause of cell death in the absence of glucose resulted from its anabolic rather than its catabolic role. This was verified by the growth of cells in glucose-free medium substituted with low levels of nucleosides. The substitution of glucose by fructose, maltose or galactose has been shown to reduce lactic acid production and pH changes in the culture of MDCK cells grown on microcarriers (Imamura *et al.*, 1982) and other cell lines (Eagle *et al.*, 1958; Reitzer *et al.*, 1979). This effect is probably related to a lower specific uptake of the substituted carbohydrates which may be preferentially metabolised via the pentose phosphate pathway. In HeLa cells 90% of the fructose was metabolised via this pathway while only a low percentage was converted to lactate (Reitzer *et al.*, 1979). Low and Harbour (1985b) used this glucose substitution approach with two hybridoma cell lines with mixed results. They reported that one cell line produced significantly less lactate when grown on fructose compared to glucose, whereas the other cell line was able to metabolise fructose as rapidly as glucose and consequently produced equivalent amounts of lactate. Reuveny *et al.* (1986a,b) found cell yields and antibody concentrations decreased when galactose, fructose, maltose or starch was substituted for glucose. The use of glucose as a carbohydrate source continues to be standard practice.

It is now recognised that glucose is not the only major energy source present in cell culture medium and that a varying proportion of the total energy requirement is derived from the oxidation of glutamine. This is discussed in the following section.

Glutamine metabolism (Glutaminolysis)

Glutamine occurs in the blood and many other tissues at a higher concentration than any other amino acid and yet it is not an essential amino acid. Glutamine has a multiplicity of functions in the animal body (Krebs, 1980). It is required:

- as a protein and peptide constituent,
- for its role in the acid-base balance as a precursor of urinary ammonia,
- as a precursor of purines and pyrimidines of nucleic acids,
- as a nitrogen carrier between tissues, and
- as a respiratory fuel in certain tissues.

Both tumour cells and other normal cells that proliferate in culture generally exhibit a high glutamine requirement (McKeehan, 1982). Not surprisingly the concentration of glutamine in optimized medium formulations is also higher than those of the essential amino acids. Glutamine is normally included in culture media at concentrations from 2 to 5 mM. The high glutamine requirement was largely explained as a

Table 2. The deamination and transamination reactions of glutaminolysis

1	Glutamine	Glutaminase ——>	Glutamate + NH_4^+
2(a)	Glutamate	Glutamate dehydrogenase ——>	α-Ketoglutarate + NH_4^+
2(b)	Glutamate + Pyruvate	Alanine aminotransferase ——>	α-Ketoglutarate + Alanine
2(c)	Glutamate + Oxaloacetate	Aspartate aminotransferase ——>	α-Ketoglutarate + Aspartate

requirement for protein and nucleic acid biosynthesis as well as being due to the instability of glutamine under cell culture conditions (Tristch and Moore, 1962; Griffiths and Pirt, 1967). Levintow and Eagle (1961) believed glutamine was degraded to ornithine and glutamate in the medium and was thus rendered unavailable for its metabolic role. Glacken *et al.* (1986) showed that specific glutamine uptake increased as the glutamine concentration in the medium was increased.

Only over the last 10–15 years has it become clear that glutamine can be a major respiratory fuel (Donnelly and Scheffler, 1976; Zielke *et al.*, 1976, 1978, 1984; Reitzer *et al.*, 1979). Oxidation of glutamine provides up to 40% of the energy requirement for proliferating fibroblasts and HeLa cells, even when the glucose concentration of the medium is high. The major fate of the glutamine carbon is CO_2 but about 13% of metabolized glutamine is converted to lactate by the above mentioned cells. Macromolecules account for about 25% of the glutamine carbon skeleton. Glutamine can supply essentially all of the cellular requirements for energy when the supply of glucose is extremely low, or when glucose has been substituted by another hexose that does not enter the glycolytic pathway (Zielke *et al.*, 1976; Reitzer *et al.*, 1979; Imamura *et al.*, 1982).

Glutaminolysis begins with the deamination of glutamine to glutamate and ammonia. This involves a large free energy drop and is considered to be an essentially irreversible reaction (McKeehan, 1982). Removal of the 1-amino group of glutamate to form α-ketoglutarate follows. This step is catalysed by glutamate dehydrogenase or an aminotransferase. Aspartate aminotransferase and alanine aminotransferase are most likely involved. The pertinent reactions and enzymes involved in these initial steps of glutaminolysis are summarised in Table 2.

The relative contribution of each pathway depends on cell type and the conditions under which the cells are grown. As yet unpublished data from our laboratory suggests that, in SPO1 hybridoma cells, transamination of glutamate proceeds primarily via alanine aminotransferase (reaction 2(b)). Watford *et al.* (1979) found that 64% of the amino-N of glutamine could be accounted for as alanine in small intestinal mucosa. The maximum activities of several enzymes of glutaminolysis in activated lymphocytes suggested that the aminotransferases were likely to be more important than glutamate dehydrogenase for conversion of glutamate to α-ketoglutarate (Ardawi and Newsholme, 1982). Other reports suggest the glutamate dehydrogenase route (reaction 2(a)) is the most important (Butler, 1987; Miller *et al.*, 1988a,b). Butler (1987) notes that the release of specific amino acids into the culture medium during cell growth is commonly found, particularly alanine. It was suggested that

this is likely to be precipitated by a build up of excess intracellular ammonia which is sequestered by binding to available carbon metabolites. Miller *et al.* (1988b) showed the conversion of glutamate to α-ketoglutarate increased via the transamination pathway as the ammonia concentration in the medium rose. Alanine replaced ammonia as the major byproduct of glutamine metabolism at ammonia concentrations above 5 mM.

The conversion of α-ketoglutarate to malate most likely occurs via the normal reactions of the TCA cycle. These reactions generate 5 mol ATP/mol α-ketoglutarate. Malic enzymes are then responsible for generation of pyruvate from malate. A major fate of the pyruvate so formed may be as a substrate for the alanine aminotransferase reaction, facilitating entry of further glutamate into cellular energy metabolism. Some of the glutamate derived pyruvate also exits the mitochondria and is excreted into the medium as lactate.

The importance of glutaminolysis to cell energy supply is clearly evident; at typical glucose concentrations found in cell culture, glutaminolysis becomes an important additional energy producing pathway in cultured cells. Ammonia is generated as a byproduct of the glutaminase and glutamate dehydrogenase reactions, and its growth inhibitory effects (discussed later) are quite possibly centred around these initial steps of glutaminolysis.

Regulation of glucose and glutamine metabolism

As noted earlier, high rates of glycolysis and glutaminolysis are generally characteristic of cultured cells. In traditional media, glucose uptake is rapid during the lag and early logarithmic growth phases while little glutamine is consumed. A considerable amount of the glucose utilized is converted into lactate. Hu *et al.* (1987) reported increased oxygen uptake rates by swine testicular cells in microcarrier culture when the glucose concentration dropped below 2 mM. Batch hybridoma cultures in our laboratory often show a belated rise in glutamine and oxygen consumption rates as glucose levels fall. This could indicate more glutamine (or possibly glucose) entering the TCA cycle. Addition of glucose to a culture results in an immediate decrease in oxygen uptake (Frame and Hu, 1985).

Zielke *et al.* (1978) found that at very low glucose concentrations utilization of glutamine by cultured human diploid fibroblasts is regulated by glucose, and vice versa. Total glutamine utilization increased by at least 30% in a medium with 25–70 μM glucose compared to a medium with 5.5 mM glucose. A concentration of 2 mM glutamine inhibited glucose oxidation via the TCA cycle by 88%. Hu *et al.* (1987) reported increased ammonia production in programmed feeding experiments, where low glucose concentrations were maintained. These effects have not been found at higher glucose concentrations (e.g. 4 mM). Luan *et al.* (1987) reported that glutamine oxidation was constant even when the glucose concentration was changed from greater than 1 g/l to less than 0.2 g/l.

A mixed type inhibition kinetic equation was proposed by Sumbilla *et al.* (1981) to describe the effects of the glucose and glutamine concentrations on glutamine consumption. Miller *et al.* (1988a) suggest that catabolic competition is responsible for the reciprocal inhibition between these two important nutrients.

Nielsen *et al.* (1990) have proposed an alternative hypothesis, namely that glucose uptake is controlled by growth rate. Under normal conditions, hybridomas use glycolysis to avoid overproduction of ATP but possess the ability to provide energy through oxidative phosphorylation if the glucose uptake is limited by its low concentration or if a more slowly metabolised sugar is substituted. The high level of glutamine consumption reflects, to a large extent, its role in providing all non-essential amino acid requirements and the importance of the glutamine amido group as a nitrogen donor to nucleotides, asparagine, NAD and amino sugars.

Culture pH has a marked effect on glucose and glutamine utilization rates. At low pH (6.8) an SP2/0 mouse hybridoma minimised lactate production by lowering the fraction of glucose meta-

bolised to lactate and reducing the amount of glucose consumed. Higher glutamine consumption occurred, possibly to compensate for energy no longer derived from glucose (Miller *et al.*, 1988a). Glucose uptake increased with increasing pH while glutamine utilization decreased. Some uncertainty exists as the data collected by Miller *et al.* at pH 6.8 does not appear to represent true steady state. Similar trends, however, were noted by Barton (1971) and Birch and Edwards (1980) with HeLa and lymphoblastoid cells, respectively.

Growth limitation by ammonia

An early recognition of the importance of ammonia in animal cell culture was made by Ryan and Cardin (1966). Investigating the stability of foetal calf serum (FCS) during storage, they drew a parallel between a sharp rise in ammonia concentration and decreased activity of the FCS (FCS is often added to cell growth medium at up to 10% v/v concentration). Deliberate addition of up to 2 mM NH_4Cl to tissue culture medium containing fresh 10% FCS demonstrates a progressive increase in toxicity. Visek *et al.* (1972) observed changes in morphology and a reduction in cell multiplication of 3T3 and SV-40 transformed 3T3 mouse fibroblasts after addition of NH_4Cl at less than 2 mM on the initial day of culture. Holley *et al.* (1978) identified ammonium ions as a growth inhibitor of monkey epithelial cells (BSC-1). Levels of 2 mM were described as 'growth reducing' with the effect being less significant in medium of higher serum content.

More recently Butler *et al.* (1983) noted coincidence between accumulation of ammonia to 2.3 mM and cessation of cell growth of MDCK cells. Cell densities reached 10^7 cells/ml in a perfusion culture, which exceeded the 10^6 cells/ml achieved in earlier batch cultures (Butler and Thilly, 1982). However, it appears the rate of medium perfusion (medium dilution rate approaching 3 vol/day) was not sufficiently high to prevent accumulation of ammonia in the cultures.

Butler and Spier (1984) report on more detailed investigations of the effects of glutamine utilization and ammonia production in batch cultures. Ammonia accumulated to 2 mM during the culture of Baby Hamster Kidney (BHK) cells; this concentration of ammonium chloride (2 mM) was growth inhibitory if added to the medium before cell inoculation. Partial growth inhibition occurred at 1 mM. Cell density reached 2×10^6 cells/ml. In experiments with diluted medium, ammonia concentration and cell density reached the same levels as previously. Cessation of cell growth always seemed to occur at a level of ammonia approaching 2 mM. A similar pattern was observed with MDCK cells in batch culture (Butler, 1985). Furthermore, the ammonium ion concentration had reached 2.3 mM in perfused cultures after 6 days when cell growth ceased. The medium dilution rate was 3 vol/day and final cell concentration 1×10^7 cells/ml, almost an order of magnitude higher than in batch culture. Thus, despite perfusion, the level of ammonia increased to a concentration previously shown to be inhibitory.

Reuveny *et al.* (1986a) cultured a hybridoma (designated VIII H-8) in shake flasks. A level of 4.5 mM ammonia ion was recorded after 4 days at which time significant cell death was occurring. Direct addition of NH_4Cl elucidated an inhibitory effect at 2 mM and a toxic effect at 4 mM in line with earlier reports on different cell lines. Reuveny *et al.* (1986b) propagated the same cells in semi-continuous and perfusion modes. In the perfusion culture ammonia levelled out at 2.1 mM with 2×10^7 cells/ml. It was concluded that this level of ammonia, being lower than that observed in earlier batch culture, was 'probably not toxic to the cells'. Nutrient limitation was probably a dominant factor limiting cell density in their perfusion culture. Nahapetian *et al.* (1986) attempted to optimize the environment for high density Vero cell culture. In batch culture cessation of cell growth coincided with around 2 mM ammonia accumulation, with cell density reaching 2×10^6 cells/ml. Perfusion cultures (4 and 8 vol/day) enabled 3×10^7 cells/ml to be attained whilst ammonia reached only 1–1.5 mM, below the 2 mM considered toxic. An

important factor in these studies, however, appeared to be a limitation of available oxygen.

In other studies where ammonia has been measured, cessation of cell growth is coincident with an ammonia ion concentration of 2 mM (Imamura *et al.*, 1982; Murdin *et al.*, 1987). Of significance is the fact that higher cell densities are achieved in perfusion systems. By virtue of medium replenishment these systems appear to delay the onset of growth inhibition until significantly more cells are present. Kimura *et al.* (1987) found specific cell growth rates were strongly affected by ammonia. The inhibition constants for HL-60 and RPMI-8226 human cells were 10 and 12 mM, respectively.

The most recent and extensive report on investigations into NH_4^+ growth inhibition is that by Miller *et al.* (1988b). They looked at the transient responses of mouse hybridomas to pulse and step changes in ammonia concentration in continuous culture. Cell growth was first inhibited by 5 mM ammonia but cells were able to adapt to concentrations as high as 8.2 mM. Other conclusions to come from this work, which was the first to link aspects of ammonia inhibition and energy metabolism, were:

- ammonia addition reduces ammonia production, probably due to a decrease in the flux from glutamate to α-ketoglutarate via glutamate dehydrogenase (reaction 2a, Table 2);
- the conversion of glutamate to α-ketoglutarate increases via the transamination pathway as ammonia concentration increases (reaction 2b, Table 2);
- glucose consumption and lactate production increase dramatically when the growth rate is reduced by ammonia;
- oxygen consumption also appears to be inhibited by ammonia. At high ammonia concentrations (10–17 mM) the combined inhibition by ammonia and the increased rate of glycolysis caused the specific oxygen consumption rate to decrease by an order of magnitude. Under these conditions over 90% of the estimated ATP production was due to glycolysis.

Glutamate is known to inhibit glutamine deamination (McKeehan, 1986). Miller *et al.* (1988b) suggest ammonia inhibition of glutamine consumption may result from an increased intracellular glutamate concentration if the transamination reactions (Table 2) are not able to compensate for the decreased flux via glutamate dehydrogenase. Glacken *et al.* (1988), however, hypothesise that the inhibitory effect of ammonia may be the result of the destruction of electrochemical gradients and a subsequent increase in cytosol and subcellular vesicle pH.

McQueen and Bailey (1989) postulated that ammonia inhibition of cell growth was caused either by:

(i) uptake of the weak base NH_3 into the lysosomes, leading to an increase in pH in this region; or
(ii) uptake of the weak acid NH_4^+ into the cytoplasm, leading to an intracellular pH decrease.

By measuring intracellular pH of cells at different external pH values and NH_4Cl concentrations using flow cytometric measurements of cell fluorescence, they concluded that the second mechanism was more significant leading to growth inhibition and an accumulation of cells in G_1 phase.

A number of authors have reported data showing that hybridoma cells are more susceptible to equivalent ammonia concentrations during the early stages of growth in batch culture rather than during log phase (Newland, 1989; Negrotti *et al.*, 1989). Cell growth shows a higher tolerance to ammonia under the conditions of continuous culture as compared to those in batch culture (Miller *et al.*, 1988a,b; Newland *et al.*, 1990), suggesting that adaptation can play a major role in alleviating the inhibitory effects.

Recent observations are consistent with reports on the physiological effects of ammonia, which is extremely toxic to man and animals. Normal blood ammonia levels are in the order of 0.05 mM. Increased levels of blood and brain ammonia are associated with drowsiness and coma. Some two to three decades ago work was being carried out to elucidate the mechanisms underlying these effects. It was established (both *in vivo* and *in vitro*) that in the ammonia intoxicated

brain, levels of free glutamine increase while, after a transient increase, levels of glutamate and α-ketoglutarate decrease. Changes in carbohydrate metabolism also occur, consisting of increased glucose consumption and accumulation of pyruvate and lactate (McKann and Tower, 1961).

Although the evidence is somewhat circumstantial and not well quantified, it seems reasonable to conclude that growth of mammalian cells is, under certain conditions, limited by ammonia accumulation. The inhibitory concentration appears to differ between cell types but is generally between 2 and 5 mM. Certain cell lines, particularly in continuous culture, can adapt to higher ammonia concentrations.

Specific rates of production of monoclonal antibodies are much less affected by ammonia than is growth. McQueen and Bailey (1990) have reported that at 10 mM NH_4Cl, where growth was severely limited, there was no change in the specific antibody production rate. The lower volumetric productivity of antibody simply reflects the lower numbers of viable cells. Negrotti *et al.* (1989) found for another hybridoma cell line that, while volumetric antibody productivity was reduced at 8 mM ammonia, specific productivity remained relatively constant. Even 12 mM NH_4Cl had little effect on the specific antibody productivity of the same cells at a concentration of 10^6 cells/ml. It appears that the major effect on overall antibody productivity (grams/litre/hour) results from the lower rates of growth and, in particular, from the lower cell numbers which can be caused by elevated ammonia concentrations.

Approaches to overcome ammonia inhibition

Given the key role of glutamine in animal cell metabolism, preventing the accumulation of ammonia is difficult. Removing it from the medium is also a problem because of its high solubility and its ion exchange characteristics. Use of a growth substrate other than glutamine which may produce less ammonia is one possibility. As early as 1956, Eagle *et al.* investigated substitution of glutamine by glutamate. With HeLa cells the optimum concentration of glutamate was 20 mM, approximately ten times the optimal concentration of glutamine. Glutamate failed to substitute for glutamine in cultures of mouse fibroblasts, however, even at concentrations of 40 mM. It was suggested that glutamine is an essential metabolite and that the growth promoting activity of high concentrations of glutamic acid for the HeLa cell reflected its conversion to glutamine (at this time the role of glutamine in energy metabolism had not been appreciated). While no work has been done to investigate glutamine and glutamate transport across hybridoma plasma and mitochondrial membranes, such research with other cell types would suggest that glutamine can enter animal cells more quickly than can glutamate, (Kovacevic and McGivan, 1984; Kilberg *et al.*, 1985). This fact alone could explain why high concentrations (20 mM) of glutamate are needed to substitute for glutamine (2–4 mM). Other workers, however, have managed to grow cells glutamine free with the addition of only 4 mM glutamate. Griffiths (1973) was able to adapt two lines of human diploid cells to utilize glutamic acid in place of glutamine (MEM plus 10% FCS with 4 mM glutamic acid being used), although the cells grew more slowly.

Progressing the work of Butler and others (1982, 1984, 1985) on ammonia accumulation from glutamine catabolism, Hassell *et al.* (1987) substituted glutamate or α-ketoglutarate for glutamine in serum supplemented medium. The standard medium (GMEN plus 10% FCS) contained 4 mM glutamine and 25 mM glucose. The following observations were made with McCoy cells:

- substitution of glutamine with glutamate (4 mM) or α-ketoglutarate (4 mM) led to much lower ammonia accumulation (reduced by 50%) without affecting cell doubling times;
- glutamate supplemented medium led to a cell yield which was 15% greater than that found in controls grown with glutamine;
- a further 20% increase in cell yield was obtained by daily feeding of glutamate;
- substitution with α-ketoglutarate resulted in poorer cell yields.

Although this technique reduced ammonia accumulation significantly, the improvement in cell yield was less than might have been expected if glutamate completely substituted for glutamine and ammonia was the sole cause of cessation of cell growth. A major problem with the work reported by Griffiths (1973) and Hassell *et al.* (1987) is that they worked with MEM and GMEM basal media, respectively. These media have low concentrations of amino acids (except for glutamine) and vitamins compared to, for example, DMEM medium. Work from our laboratory suggests that in such media, as cells reach their peak cell density, very low concentrations of key amino acids will be present and so may be limiting further cell growth (Reid *et al.*, 1988, 1989). Thus, although Hassell *et al.* (1987) achieved a reduction in ammonia levels by substituting glutamate for glutamine, improvements in cell growth may have been limited due to the low levels of amino acids in the basal medium used. To clarify the role of ammonia inhibition in limiting cell growth in batch culture not only requires a reduction in ammonia levels by the substitution of glutamate for glutamine but also the use of a basal medium, where it can be clearly demonstrated that limitations of amino acids and vitamins do not occur as the cells reach $1–2 \times 10^6$ cells/ml. If no obvious limitations are present and cultures continue to peak at around 2×10^6 cells/ml, despite reduced ammonia (and lactate) levels, then clearly the search for other inhibitors needs to be intensified.

There is no doubt that the ability to grow hybridomas glutamine free would represent a significant step forward, in the batch, fed batch, and perfusion culture of these cells. The increasing awareness of the important role of glutamine in biosynthesis implies that such a step will not be readily achieved; hence, it is likely that ammonia will be present as a metabolic product for the immediate future. A cell must have a supply of glutamine to grow for protein and nucleotide production (Meister, 1984), but it is not necessarily needed in large amounts, as in the above cases where it was substituted by glutamate. In theory the anabolic needs of the cell for glutamine can be satisfied by glutamic acid as long as glutamine synthetase is active in the cell. Glutamine synthetase is a complex enzyme catalysing the production of glutamine from glutamate + ammonia + ATP (Meister, 1984); its activity is inhibited by glutamine (Griffiths, 1973; Jourlink, 1987). Hybridomas (or at least some hybridoma cell lines) grown for so long in the presence of high glutamine levels may have irreversibly repressed glutamine synthetase activity. It is encouraging that the weaning of hybridoma cells off glutamine has been reported (Broad, 1988), but no details of how this was achieved have been published. Weaning of cells from glutamine could well lead to reduced cell growth rates if the ideas of Newsholme *et al.*, (1985) are correct, but this would not necessarily be detrimental for the production of monoclonal antibodies, provided a balanced set of nutrients and growth factors is provided in the medium.

Using higher rates of perfusion may improve cell densities and reduce ammonia concentration but it rapidly becomes expensive and impractical. Total medium replacement results in inefficient use of expensive medium components such as serum and thus increases production costs. Controlled addition of glucose or glutamine or choice of cell lines more resistant to ammonia have been suggested by Glacken *et al.* (1985) and Hu *et al.* (1987). Both of these approaches have considerable potential.

The idea of incorporating an appropriate ammonia degrading enzyme system in the culture has also been floated (Butler, 1985). One example might be that proposed by Chang and Malour (1978) as a novel approach to effective removal of ammonia in artificial kidneys (Fig. 2).

Iio *et al.* (1985) showed that a reduction in ammonia concentration could be achieved by use of an adsorbent ZCP-50 packed in dialysis tubing. Ammonia levels were reduced by half from around 1.5–2 mM. For a mouse myeloma cell line MPC-11 grown in a serum free medium, the maximum cell concentration increased from 1.6×10^5 cells/ml to 2.8×10^5 cells/ml. This corresponded to other results showing that ammonia was particularly toxic to the myeloma cell line in

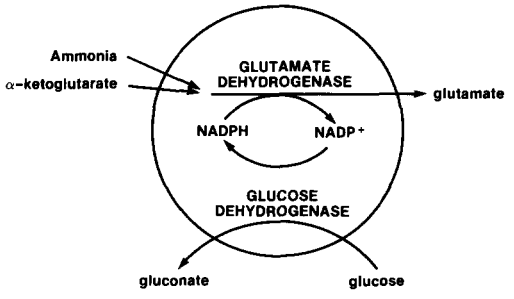

Fig. 2. Schematic representation of a multi-enzyme system in an artificial cell for the conversion of ammonia into glutamate with simultaneous regeneration of NADPH (after Chang and Malouf, 1978).

the serum free medium. While a similar reduction in ammonia concentration was obtained with a human lymphoma-human lymphocyte B hybridoma, no significant effect was found in the presence of 10% FCS and only a small increase in cell number was found in the serum free medium.

Interesting parallels can be drawn between removal of ammonia from culture medium and the use of artificial kidneys to remove urea, as ammonia, from blood dialysate. Standard artificial kidneys contain a cation exchange resin, zirconium phosphate (ZP), sandwiched between layers of immobilized urease and alumina for removal of phosphate.

The system functions by exchanging Na^+ and H^+ ions for NH_4^+. Ammonium ions are not preferentially exchanged however. ZP has the following affinity series for cations found in culture medium (Amplett *et al.*, 1957):

$$Na^+ < K^+, NH_4^+ < Mg^{2+} < Ca^{2+}$$

Hence preferential removal of Mg^{2+} and Ca^{2+} will occur when the medium is contacted with ZP. Infusion of salts is necessary when these cartridges are used for dialysis.

Tertiary wastewater treatment is another area where removal of ammonium ions is practised. Cation exchanges (usually natural or synthetic zeolites) are employed to remove ammonia prior to discharge of the wastewater into receiving waters. Clinoptilolite is an example of a natural zeolite used for this purpose. It selectively removes ammonium over calcium, magnesium and sodium ions but not potassium. The capacity of the zeolite decreases with increased mineral composition of the wastewater, however (Eckenfelder, 1980).

Reverse osmosis is another technique that may offer some promise. However specificity for NH_4^+ over other low molecular weight components of the medium is likely to be a problem.

Generally, specific removal of NH_4^+ is a problem in all the examples cited above. The requirement for non-destructive removal from complex cell culture medium has not yet been addressed in the literature.

Other growth inhibitory substances

Complicating an elucidation of the possible inhibitory effects of ammonia on hybridoma cell growth is the fact that a number of other low and high molecular weight products are generated together with the ammonia as a result of cell metabolism.

Lactate is another metabolic product that has been suspected of playing an inhibitory role in cell culture. Lactic acid, generated as a result of glucose metabolism, can with inadequate pH control decrease the medium pH below the optimal range. It is possible that early implications of lactate being inhibitory were in fact a reflection of a drop in pH (Imamura *et al.*, 1982). Indeed Velez *et al.* (1986) showed that lactate was used by VIII H-8 hybridoma cells after most of the glucose had been consumed. Lactate accumulated to 1.9 mM before slowly subsiding. In a further experiment, (Reuveny *et al.*, 1986a), after spiking 5.5–28 mM of lactate into shake flasks inoculated with 1×10^6 cells/ml, did not elucidate any toxic effect. Surprisingly cell yield and antibody production improved. Concentrations of lactate above 28 mM, and thus above that expected in batch culture, did exhibit toxic effects. Nahapetian *et al.* (1986) also found lactate levels declined late in batch cultures of Vero cells after an initial rise of 4.5 mM, while lactate did not exceed 1.5 mM

in perfusion culture. Thorpe *et al.* (1987) found that lactate began to retard cellular growth and division of hybridoma cells when introduced at concentrations above 4 mM. Generally, as long as pH is controlled, lactate is not a problem in typical cultures but may become so in extended fedbatch systems or perfusion systems.

The notion that a more complex component is responsible for growth inhibition has been addressed by a few researchers. Holley *et al.* (1978) described the presence of an unidentified inhibitor in monkey epithelial cell culture. The inhibitor, apparently formed by the cells, was thought to be an unstable protein. Merten *et al.* (1985) studied monoclonal antibody production kinetics with high and low producing hybridomas. What appeared to be feedback inhibition was observed when spent culture supernatant was included in fresh medium. Emery and colleagues (1987) reported that investigations of spent culture medium fractions were being carried out in an attempt to identify cytotoxic components. As yet no conclusive results have been reported. Dodge *et al.* (1987) feel that as yet unidentified inhibitory substances exist and suggest that process optimization will require removal of such components from the culture fluid, or prevention of their formation by some means. Glacken *et al.* (1989) present results that indicate thiol compounds may be growth rate limiting in low serum cultures. This limitation is diminished with increasing cell concentrations, implying that the cells themselves may be acting to reduce their environment thereby minimizing the spontaneous oxidation of serum thiols. It is hypothesised that the actual rate-limiting moieties in low-serum culture are dithiols. The possibility that the observed inhibition is multi-component in nature (perhaps synergistic), just as the supply of nutrients is, has not yet been addressed.

Cell culture is rapidly moving towards serum-free or low serum-containing media. Although the role of serum remains incompletely understood it undoubtedly has a protective effect. Increased toxic effects have been reported as serum concentration is lowered (Holley *et al.*, 1978; Thorpe *et al.*, 1987). Darfler and Insel (1983) suggest that H_2O_2 is an important toxic agent in serum free medium. Addition of low concentrations (5–20 μg/ml) of catalase facilitated long term growth and cloning of lymphoid cells. Kay and Ellen (1986) found H_2O_2 accumulated in low serum media under normal laboratory lighting conditions to levels that were shown to be sufficient, when added to freshly prepared media, to explain depressed cell performance. Cell growth rates have been reported to decrease with decreasing percentage of FCS in the medium (Low and Harbour, 1985a) although the importance of an appropriate weaning protocol has not always been recognised, thereby rendering some conclusions of limited value.

Conclusions

This review has attempted to bring together aspects of cell energy metabolism and possible growth limitations caused by ammonia, a byproduct of this metabolism. Much is yet to be understood about the mechanisms by which ammonia affects hybridoma metabolism.

- Glucose and glutamine are important energetic and biosynthetic substrates for cultured mammalian cells.
- Glucose uptake is rapid, but not directly related to cell growth. Lactate is a major product of glucose metabolism.
- Glutaminolysis represents a major source of nitrogen for biosynthesis and can supply a major fraction of cellular energy requirements.
- Ammonia is a byproduct of glutamine metabolism and results also from glutamine degradation. It is implicated in growth inhibition the extent of which depends on the cell line. It appears that cells can adapt to moderate levels of ammonia, particularly in a continuous culture environment. In batch culture, cells appear more susceptible to the effects of ammonia at an early stage of growth when viable cell numbers are low.
- Specific monoclonal antibody productivity is less sensitive than growth to the effects of

ammonia. Volumetric productivity may be reduced because of lower numbers of viable cells.

- Preventing accumulation of, or removing ammonia selectively from, culture media is difficult. Attempts have been made to substitute glutamic acid for glutamine or to limit ammonia production by programmed feeding of glucose and glutamine, with limited success. The latter approach appears most promising as it minimises the ammonia resulting from chemical degradation of glutamine. Greater understanding of the biosynthetic roles of glutamine is required.
- Other growth limiting substances may be generated during cell culture, either by outside influences or by normal cell metabolism. These may act independently or in concert with ammonia to inhibit cell growth and antibody production.

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Address for offprints: S. Reid, Department of Chemical Engineering, The University of Queensland, St. Lucia, Qld., 4067, Australia