Pharmaceutical BIOPROCESSING

Are nonessential amino acids not so redundant for Chinese hamster ovary cell lines?

"…original experiments indicate that amino acid requirements vary by cell line and that the classification of amino acids into 'essential' and 'nonessential' categories becomes context dependent."

Keywords: amino acid response pathway • bioprocess optimization • cell line development • Chinese hamster ovary cell line • essential amino acid • media development • nonessential amino acid depletion • transcriptional analysis

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The understanding of the nutritional requirements for Chinese hamster ovary (CHO) cells and other immortalized cell lines was an early milestone in developing cell culture media. A key aspect of these early studies was defining those amino acids termed 'essential' for survival and growth of different cell lines. However, the amino acids essential for growth of cells in culture differ from those defined as essential in biochemistry texts.

Definitions of amino acid 'essentiality'

Nonessential amino acids were originally defined as those that "can be manufactured in sufficient amounts to meet fully the requirements of the animal" [1]. Eight amino acids, namely tryptophan, phenylalanine, lysine, threonine, methionine, valine, leucine and isoleucine, were shown to be essential in adult men for "the maintenance of nitrogen equilibrium" [1]. Arginine and histidine were shown not to be essential in adult men [1], but previously made the list of ten amino acids essential for growth of male rats [2]. As early as 1948, Rose *et al.* wondered about the dietary role of the amino acids that can be synthesized by the organism, making them nutritionally 'nonessential'. The animals fed mixtures of 19 amino acids gained more weight than those fed mixtures of the ten essential ones [2]. Therefore, the authors concluded that the mixture of ten essential amino acids was not optimal for growth of the organism and hypothesized that "the task of synthesizing (all of the nonessential) amino acids simultaneously appears to present too great a burden upon the chemical resources of the cell to permit the latter to keep pace with the needs of the organism for optimum growth" [2].

Determination of amino acids essential for mammalian cell culture & development of serum-free chemically defined media

Around the time of experiments by Rose *et al.*, other researchers started culturing mammalian cell lines *in vitro* [3,4]. Eagle attempted to identify media components essential for growth, and concluded that 13 amino acids, including those amino acids listed above as well as glutamine, cystine and tyrosine, are essential for growth of L-fibroblasts and HeLa cells [5]. HeLa cells required 1–3-times higher concentrations of individual amino acids than L-fibroblasts, the highest difference observed in consumption of cystine and glutamine, suggesting that transformed highly proliferative cells require excess of certain nutrients compared with nonmalignant cell lines [5]. In contrast to the other essential amino acids, whose "primary function in cell cultures appears to be their utilization of incorporation into protein", glutamine is "actively metabolized" [6]. These original experiments indicate that amino acid requirements vary by cell line and that the classification of amino acids into 'essential' and 'nonessential' categories becomes context dependent.

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CHO cells were developed in the late 1950s and became widely used due to their robust growth, relatively stable chromosome number and utility for somatic cell genetic studies [7]. Although defined as a nonessential amino acid, most CHO cell lines require proline since the parental CHO cell line is a spontaneously derived proline-requiring mutant. Ham and colleagues developed and later refined chemically defined cell culture medium, F12, containing 20 amino acids, ten vitamins, 12 inorganic salts and eight other organic compounds for clonal growth of CHO and Chinese hamster lung cell lines [8,9]. Of the 20 amino acids, only glycine, alanine, glutamic acid and aspartic acid could be removed without impacting growth of CHO cells [10], indicating the other amino acids previously defined as 'nonessential' were, in fact, essential for robust growth of the CHO cell lines.

> "...levels of these nonessential amino acids and therefore the timing of their depletion during fed-batch processes can be adjusted to achieve the balance between culture growth and productivity in a variety of cell lines."

As CHO cells became more widely used for recombinant protein production, there was increased emphasis on developing media to support higher yield production processes. One approach that many groups have used is to supplement nutrients based on their depletion rates from cell culture. However, nutrient requirements during production processes differ from the nutrient requirements during growth and propagation for each cell line, as well as among the different stages of the bioprocess itself. In addition, some amino acids, such as Gln and Asn, can be used to fuel the tricarboxylic acid cycle and are, therefore, used in excess of their stoichiometric needs [11]. Fomina-Yadlin *et al.* described depletion of Gln and Asn in fed-batch production cultures utilizing three different antibody-producing cell lines and sought to clarify the biological responses to starvation for these two non-essential amino acids in both antibody-producing and parental CHO cell lines [12].

Cellular responses to depletion of individual amino acids

Appropriately responding to changes in nutrient availability is essential for growth, maintenance and homeostasis at both the organismal and cellular level. In mammalian cells, intracellular amino acid availability regulates protein turnover through the mammalian target of rapamycin (mTOR) pathway and the amino acid response (AAR) pathway [13]. Amino acid depletion downregulates global translation rates by diminishing phosphorylation of the mTOR signaling effectors, 4E-BP1 and S6, and increasing phosphorylation of the AAR sensor, eIF2α [13]. In addition to regulation of protein stability and translation rates, amino acid depletion enhances translation of a subset of mRNA molecules, including certain transcription factors such as ATF4, and induces a robust transcriptional signature of ATF4-dependent stress response [14]. The mechanistic details of the AAR have been elucidated by utilizing experimental depletion of individual essential amino acids in a variety of mammalian cell lines commonly used in scientific research [14–17]. However, cellular responses to depletion of amino acids commonly classified as 'nonessential' and especially in CHO cell lines commonly used for protein production processes remained unclear.

Results in Fomina-Yadlin *et al.* help explain cellular behavior during fed-batch processes by clarifying the biological responses of the cells during starvation for the two nonessential amino acids that are frequently depleted [12]. Specifically, we demonstrate that depletion of either Asn or Gln leads to induction of AAR on the transcriptional level. Furthermore, depletion of either Asn or Gln leads to cell cycle arrest while maintaining viability and high levels of protein translation. The magnitude of the cellular response as well as the transcriptional response differs depending on the cell line, and whether the cells are starved of Gln or Asn. By contrast, depletion of an essential amino acid (leucine) also arrests the cell cycle, but quickly leads to a reduction in protein translation and culture viability. These findings are consistent with utilization of Asn [18] or Gln [19] depletion for growth arrest of cancer cell lines and can explain, at least in part, the observation that during fed-batch processes, depletion of Asn or Gln directly precedes the stationary phase of the culture, characterized by cell cycle arrest, high viability and high protein production rates. Consequently, levels of these nonessential amino acids and therefore the timing of their depletion during fed-batch processes can be adjusted to achieve the balance between culture growth and productivity in a variety of cell lines.

Applications of cellular responses to nonessential amino acid depletion to cell line development & bioprocess optimization

Findings described by Fomina-Yadlin and colleagues enhance our biological understanding of the fed-batch process as a whole, describe a mechanistic way to shift the balance between growth and productivity in CHO production cultures, and will help provide some guideposts in developing future protein production processes. For example, ensuring amino acid excess during the growth phase of cell culture will allow rapid

growth, but utilizing controlled limitations of nonessential amino acids will halt culture growth during production phase. Furthermore, our results support previous suggestions that cell lines with high ATF4 levels [20] and robust AAR are good candidates for production cell lines, and that cellular engineering of this pathway could provide a method to fine tune culture response to nonessential amino acid depletion. Altogether, these findings can be applied to optimization of production conditions and during cell line development to achieve better control of the production process and greater consistency across cell lines.

These types of studies are a small step towards a more mechanistic understanding of the cell biological processes that influence protein production. Ultimately, we would like to employ a systems biology approach to bioprocess development rather than relying on empirical studies to advance bioprocess performance. Early studies with animals and cell lines demonstrated complexities in the amino acid requirements for growth and maintenance. Examination of industrial bioprocesses utilizing CHO cell lines revealed additional complexities in amino acid requirements for exogenous protein production. As a more complete understanding of the cellular responses and signaling pathways involved in the response to depletion of amino acids develops, these findings can be exploited to enhance the productivity, robustness and control of bioprocesses.

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References

1 Rose WC. Amino acid requirements of man. *Fed. Proc.* 8(2), 546–552 (1949).

2 Rose WC, Oesterling MJ, Womack M. Comparative growth on diets containing ten and 19 amino acids, with further observations upon the role of glutamic and aspartic acids. *J. Biol. Chem.* 176(2), 753–762 (1948).

3 Sanford KK, Earle WR, Likely GD. The growth *in vitro* of single isolated tissue cells. *J. Natl Cancer Inst.* 9(3), 229–246 (1948).

4 Scherer WF, Syverton JT, Gey GO. Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J. Exp. Med.* 97(5), 695–710 (1953).

5 Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science* 122(3168), 501–514 (1955).

6 Eagle H. Amino acid metabolism in mammalian cell cultures. *Science* 130(3373), 432–437 (1959).

7 Puck TT. Development of the Chinese hamster ovary (CHO) cell. In: *Molecular and Cell Genetics.* John Wiley & Sons, NY, USA, 37–64 (1985).

8 Hamilton WG, Ham RG. Clonal growth of chinese hamster cell lines in protein-free media. *In Vitro* 13(9), 537–547 (1977).

9 Ham RG. Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proc. Natl Acad. Sci. USA* 53, 288–293 (1965).

10 Kao FT, Puck TT. Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. *Proc. Natl Acad. Sci. USA* 60(4), 1275–1281 (1968).

11 Xie L, Wang DI. Material balance studies on animal cell metabolism using a stoichiometrically based reaction network. *Biotechnol. Bioeng.* 52(5), 579–590 (1996).

12 Fomina-Yadlin D, Gosink JJ, McCoy R *et al.* Cellular responses to individual amino-acid depletion in antibody-expressing and parental CHO cell lines. *Biotechnol. Bioeng.* 111(5), 965–979 (2013).

13 Fafournoux P, Bruhat A, Jousse C. Amino acid regulation of gene expression. *Biochem. J.* 351(Pt 1), 1–12 (2000).

14 Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Ann. Rev. Nutr.* 25, 59–85 (2005).

15 Palii SS, Kays CE, Deval C, Bruhat A, Fafournoux P, Kilberg MS. Specificity of amino acid regulated gene expression: analysis of genes subjected to either complete or single amino acid deprivation. *Amino Acids* 37(1), 79–88 (2009).

16 Jousse C, Bruhat A, Ferrara M, Fafournoux P. Evidence for multiple signaling pathways in the regulation of gene expression by amino acids in human cell lines. *J. Nutr.* 130(6), 1555–1560 (2000).

17 Harding HP, Zhang Y, Zeng H *et al.* An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* 11(3), 619–633 (2003).

18 Patterson MK Jr, Maxwell MD. Effects of L-asparagine deprivation on the cell cycle of the Jensen sarcoma. *Cancer Res.* 30(4), 1064–1067 (1970).

19 Wasa M, Bode BP, Abcouwer SF, Collins CL, Tanabe KK, Souba WW. Glutamine as a regulator of DNA and protein biosynthesis in human solid tumor cell lines. *Ann. Surg.* 224(2), 189–197 (1996).

20 Haredy AM, Nishizawa A, Honda K, Ohya T, Ohtake H, Omasa T. Improved antibody production in Chinese hamster ovary cells by ATF4 overexpression. *Cytotechnology* 65(6), 993–1002 (2013).