Amino acids metabolism by VO 208 hybridoma cells: some aspects of the culture process and medium composition influence

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Received 8 February 2001; accepted in revised form 27 August 2001

Key words: Amino acids, Batch culture, Continuous culture, Hybridoma, Kinetics, Medium composition, Metabolism, Serum

Abstract

In the present study an approach has been developed in order to examine the consequence of essential and non essential amino acid supplementation on VO 208 hybridoma cells behaviour. The effect of amino acid enrichment has been studied taking into account the culture process, i.e., batch or continuous culture mode and the medium composition, i.e., a home made serum-free medium or a serum containing one. A group of 4 amino acids, i.e., Ser, Pro, Gly and Arg presented a typical evolution pattern of their extracellular concentration depending on the type of the medium and on the culture mode. Some amino acids were probably involved in the limitation of the cellular proliferation. Met was one of the amino acids that appears to may have been at limiting concentration in all cases. In continuous culture mode, an enrichment of amino acids resulted in a rapid improvement of the viable cell density in both media, with or without the presence of serum. For most amino acids, supplementation during continuous culture induced an increase of the amino acid uptake rate. A comparative analysis of amino acids utilisation, depending on the culture conditions studied in the present study, has been performed in order to propose an overall picture of amino acids metabolism by VO 208 Hybridoma cell line.

Introduction

Amino acids play a diverse role within the metabolism of the cell. They participate in the synthesis of structural proteins and enzymes as well as being a source of energy. Thus their transport, catabolism, anabolism and “ultimate utilisation” potentially have an effect at all levels of cell growth and metabolism. Besides, according to Wang et al. (1996), nutrients depletion other than amino acids, glucose, vitamins, may be the possible factor limiting culture performance. Information on the requirement of other essential nutrients such as serum components or trace elements are also required to improve culture performance and to obtain a deeper understanding of animal cell metabolism *in vitro*, especially as far as

the quality of the bioproducts (such as glycosylation) are concerned. According to Seewöster and Lehmann (1995), to investigate reaction cascades that influence protein synthesis or overall gene expression, one had to look carefully at intracellular amino acids levels, because of their importance for polypeptide synthesis and energy supply, but also because of their obvious sensitivity to extracellular stresses. Extracellular limitation in most of the amino acids will initially have an important influence on intracellular amino acid pools and then also influence the whole metabolism network. It has been shown that many hybridoma cells in culture die primarily by apoptosis, which can be induced by deprivation of essential nutrients such as amino acids and glucose (Zhou et al. 1997). Franek and Sramkova (1995) also found that starvation-in-

duced apoptosis could be prevented to a significant degree by refeeding with amino acid mixture. The culture environment changes included fluctuation of the nutrient concentration, accumulation of the metabolites and increase of the osmolality. An appropriate amino acids supply is important because it is necessary to avoid complete depletion of essential nutrients in culture and it is also important to avoid excessive accumulation of nutrients and metabolites as well as an increase of osmolality late in the cultivation.

Many observations presented in the literature have been realised by manipulating the cellular metabolism of mammalian cells in culture, and most of the time, they depend on the particular culture conditions used (batch, fed-batch or in 24 well plates) and on the type of the cell line studied (hybridoma cell lines, recombinant CHO cells). Further complete studies with a same cell line reacting in different culture conditions are thus necessary to have a better insight into the relationship between nutrient limitation, cellular metabolism transition and culture process. In order to examine the influence of amino acids supplemented in a medium more precisely and therefore have a better understanding of their role and metabolism we realised a comparative study of the behaviour of an hybridoma cell line both in batch and continuous culture conditions, in a serum supplemented and also in a home-made serum-free medium. The main objective of this work was to study the amino acids metabolism according to the culture conditions, taking into account both culture process and medium composition, for VO 208 murine hybridoma cell line.

Materials and methods

Cell line

Mouse hybridoma cells (VO 208) secreting IgG1 of unknown specificity were provided by Bertin, France. The cell line resulted from the fusion of BALB/C mouse spleen cells with myeloma cells X63Ag8.653.

Media formulation

The serum-free medium has been developed in our laboratory (Martial et al. 1994). The basal culture medium (50% IMDM / 50% Ham F12) and glutamine (2.5 mM) were provided by Intermed S.A. (France). Glucose (15 mM) was bought from Laboratoires Fandre, (France). The serum-free medium was supplemented with iron saturated human transferrin: 25 mg l⁻¹, polyethylene glycol PM 3000–3700: 0.2%W/V, ethanolamine: 20 μM, β mercap-

toethanol: 50 μM (Sigma, USA), sodium selenite: 12 nM, bovine insulin (24 IU mg⁻¹): 20 mg l⁻¹ (Boehringer Mannheim) and 18 ml l⁻¹ of liposomes. The stock solution of liposomes contained 35 mg l⁻¹ cholesterol, 35 mg l⁻¹ Lα phosphatidylcholine dipalmitoyl, 29 mg l⁻¹ oleic acid and 0.18 mg l⁻¹ BSA (Sigma, USA).

The serum containing medium consisted of RPMI 1640 Intermed S.A. (France) supplemented with 5% of foetal calf serum (FCS) and glutamine (2.5 mM) Intermed S.A. (France).

Depending on the cultures, the medium was supplemented with essential amino acids 2%v/v and non essential amino acids 1%v/v (Gibco, USA). Some variations in the initial concentrations of amino acids between batch and continuous cultures were observed.

Culture operating conditions

Serum-free adapted hybridoma cells were prepared from the same stock ampoule by several successive static cultures in Roux flasks. Then, the cells were inoculated at a density of 0.2×10⁶ cells ml⁻¹ in spinner flasks of 250 ml (Techne), stirred at 40 rpm, in a 5% CO₂-95% air atmosphere incubator. Otherwise, cells were inoculated at 0.2×10⁶ cells ml⁻¹ in a 2-litre stirred bioreactor with a 1.5-litre working volume (Biolafitte, France) where pH and pO₂ were respectively controlled at 7 and 40% of the air saturation. Cultures were performed in continuous mode without cell retention at a dilution rate of 0.02 h⁻¹.

During the continuous culture, amino acids concentration in the feed was varied stepwise. The composition of amino acids in the different medium used is given in Tables 1–3.

Analytical methods and calculation of specific rates

Twice a day, 8 ml aliquots were collected from the spinners or from the reactor. Cells were counted using a hemacytometer and the dead cell population was evaluated by the Trypan Blue exclusion method. Each result was the average of 6 counts. The experimental error involved was calculated to be between ±5 to 10%.

The remaining sample was centrifuged and the supernatant was aliquoted and stored at -20 °C for further analyses. Amino acids were determined by ion-exchange chromatography on a Beckman 7300 analyser. The detection threshold was 50–100 pmol l⁻¹ (Gelot et al. 1990). All results are the means of triplicate assays. The coefficient of variation for each

Table 1. Initial composition of amino acids in serum containing and serum-free medium. Mean values of 3 assays are given. The coefficient of variation for each result was less than 10%.

Amino acid concentration	serum containing medium (μM)	serum-free medium (μM)
Ala	100	160
Arg	575	405
Asp acid	170	125
Cys	365	135
Glu acid	220	255
Gly	185	185
His	115	95
Ileu	375	285
Leu	395	305
Lys	185	305
Met	85	65
Phe	95	140
Pro	210	240
Ser	285	185
Thr	175	325
Tyr	110	135
Val	235	380

assay within the range of concentration studied, was calculated to be less than 10%. Glucose and glutamine concentrations were measured by enzymatic assays

(Boehringer Mannheim). The concentration of antibodies was evaluated by an enzyme linked immunosorbent assay (ELISA), with goat anti-mouse IgG antibody and goat anti-mouse IgG-peroxidase conjugate bought from Biosoft (France). The error involved in the AB concentration determination was calculated to be $\pm 10\%$ and the error involved in π_{AB} was estimated to be $\pm 20\%$.

The measured time variations of the concentrations of the different medium components were first filtered using a moving average method (Pialkowski 1988). The time variations of the specific rates were then calculated from the derivative with respect to time of the filtered curves. For either batch or continuous cultures, the actual specific growth rate, μ , was calculated as:

$$\mu = D \frac{X_t}{X_v} + \frac{1}{X_v} \cdot \frac{dX_t}{dt}$$

where X_v is the viable cell concentration, X_t is the total viable and non viable cell concentration, t the culture time and D the dilution rate.

Given the mass balances, the specific rate of metabolite production π_{AB} was calculated as:

Table 2. Continuous culture in serum-free medium: amino acids concentrations in the feed with and without amino acid supplementation, residual concentration of the amino acids at each apparent stationary phase (without and with amino acids supplementation) and corresponding rates of amino acids production or utilisation by VO 208 hybridoma cells. Mean values of 3 assays of amino acids concentration are given. The coefficient of variation for each result was less than 10%.

Amino acid	concentration in the feed		stationary phase without residual conc. ¹	supplementation specific rate	stationary phase with residual conc. ¹	supplementation specific rate
	without suppl. ² (μM)	with				
			(μM)	($\times 10^{-8} \mu\text{mol cell}^{-1} \text{h}^{-1}$)	(μM)	($\times 10^{-8} \mu\text{mol cell}^{-1} \text{h}^{-1}$)
<i>Consumed</i>						
Met	90	200	33	0.15 ± 0.02	70	0.25 ± 0.03
Leu	350	805	160	0.55 ± 0.08	300	1.00 ± 0.15
Ileu	330	800	170	0.45 ± 0.06	325	0.90 ± 0.13
Val	430	900	250	0.55 ± 0.08	470	0.60 ± 0.09
Cys	160	340	150	0.02 ± 0.01	250	0.18 ± 0.02
Arg	500	1200	485	0.10 ± 0.01	832	0.75 ± 0.11
His	120	300	98	0.08 ± 0.01	245	0.20 ± 0.03
Lys	400	1000	33	0.30 ± 0.04	340	0.60 ± 0.09
Phe	160	370	125	0.10 ± 0.01	230	0.30 ± 0.04
Thr	320	740	295	0.08 ± 0.01	500	0.40 ± 0.06
Tyr	120	380	100	0.10 ± 0.01	235	0.30 ± 0.04
Pro	250	430	175	0.20 ± 0.03	250	0.35 ± 0.05
Gly	190	330	190		223	0.20 ± 0.03
<i>Excreted</i>						
Ser	180	300	350	0.45 ± 0.06	470	0.30 ± 0.04
Ala	170	260	800	1.80 ± 0.27	1230	1.80 ± 0.25
Glu.acid	300	400	370	0.20 ± 0.03	800	0.20 ± 0.03

¹: concentration; ²: supplementation

Table 3. Continuous culture in serum containing medium: amino acid concentrations in the feed with and without amino acid supplementation, residual concentration of the amino acids at each apparent stationary phase (without and with amino acids supplementation) and corresponding rates of amino acids production or utilisation by VO 208 hybridoma cells. Mean values of 3 assays of amino acids concentration are given. The coefficient of variation for each result was less than 10%.

Amino acid	concentration in the feed		stationary phase without residual conc. ¹ (μM)	supplementation specific rate ($\times 10^{-8} \mu\text{mol}$ $\text{cell}^{-1} \text{h}^{-1}$)	stationary phase with residual conc. ¹ (μM)	supplementation specific rate ($\times 10^{-8} \mu\text{mol}$ $\text{cell}^{-1} \text{h}^{-1}$)
	without suppl. ² (μM)	with				
<i>Consumed</i>						
Met	65	165	25	0.09±0.01	65	0.10±0.01
Leu	310	710	110	0.43±0.06	190	0.53±0.07
Ileu	300	700	130	0.37±0.05	220	0.49±0.07
Val	140	540	40	0.22±0.03	200	0.36±0.05
Cys	260	360	120	0.27±0.04	80	0.28±0.04
Arg	610	1330	510	0.22±0.03	870	0.47±0.07
His	100	370	50	0.11±0.01	170	0.21±0.03
Lys	180	680	60	0.27±0.04	270	0.43±0.06
Phe	70	270	30	0.10±0.01	150	0.12±0.01
Thr	160	560	90	0.15±0.02	360	0.18±0.02
Tyr	90	290	50	0.09±0.01	180	0.11±0.01
Glu.acid	570		450	0.24±0.03		
<i>Excreted</i>						
Ser	260	260	360	0.20±0.03	460	0.21±0.03
Ala	120	120	1200	2.30±0.34	2020	1.95±0.29
Gly	150	150	205	0.11±0.01	250	0.10±0.01
Pro	170	170	230	0.12±0.01	230	0.06±0.01
Glu.acid		570			620	0.04±0.01

¹: concentration; ²: supplementation

$$\pi_{AB} = \frac{D}{X_v} \cdot (AB) + \frac{1}{X_v} \cdot \frac{d(AB)}{dt}$$

where AB is the antibody concentration in the medium.

Results

Batch cultures

Amino acids utilisation by VO 208 hybridoma cells cultivated in agitated batch cultures in a serum-free and in a serum containing medium

A serum-free medium has been developed and optimised in order to obtain comparable cell yield and antibody production to a serum (5% FCS) containing medium in batch culture conditions (Martial et al. 1994). Two batch cultures were realised in order to study the utilisation and metabolism of amino acids in a serum containing medium and in a serum-free medium.

Cells and antibodies

The evolution of the cell density and monoclonal antibodies are presented in Figure 1. Cells were in the exponential growth phase till 60 h in the serum containing and 70 h in the serum-free medium. The actual specific growth rate during exponential growth was around 0.04 h^{-1} and the maximum cell density achieved was around $1 \times 10^9 \pm 0.1 \times 10^9 \text{ cells l}^{-1}$ in both conditions.

The evolution of the released antibodies was similar in both media at least for the first 60 h of culture. The final concentration of monoclonal antibodies achieved was slightly higher in the serum-free medium $15 \pm 1.5 \text{ mg l}^{-1}$ compared to $12 \pm 1.2 \text{ mg l}^{-1}$ in the serum containing medium. The specific rate of antibody production calculated during the growth phase was around $0.2 \times 10^{-9} \text{ mg cells}^{-1} \text{h}^{-1}$ in both cases.

Amino acids evolution

A description of the evolution of the different amino acids during the culture is presented in Figure 2. As

Figure 1. Evolution of the cell density (A), monoclonal antibodies (B), glucose and lactate (C), glutamine and ammonia (D) in batch cultures in serum containing medium and serum-free medium. Each result of the cell density was the average of 6 counts. The experimental error involved was calculated to be 5 to 10%. The error involved in the AB concentration determination was calculated to be $\pm 10\%$.

shown in Table 1, the initial concentration of most amino acids was different in the two media.

Figure 2 shows that for most of the amino acids, the same overall profile was obtained with VO 208 cell line in batch culture condition. Nevertheless, a clear difference could be noticed concerning the evolution of 4 amino acids, i.e., serine, arginine, glycine and proline, depending on the medium. When cultivated with serum containing medium, serine and arginine were strongly utilised by these cells. Interestingly, in

the serum-free medium, serine accumulated and arginine concentration remained more or less stable during the overall culture. Another interesting result concerned the higher level of accumulation of proline and glycine in the serum containing medium. In the serum-free medium proline was poorly utilised during the growth phase and poorly accumulated during the death phase and glycine concentration in the medium increased only after 50 h of culture during the cellular death phase.

A second set of amino acids concerns those heavily consumed or released regarding their initial concentration. This is the case for leucine, isoleucine, valine, methionine, cysteine and alanine. These amino acids were strongly utilised or released during the growth phase. Especially, cysteine was completely utilised after 50 h of culture in the serum-free medium and also in the serum containing one, despite the high initial level ($370 \mu\text{M}$). Concerning glutamic acid, it appears to be released by the cells during the exponential growth phase and then consumed till the onset of the decline phase of the batch cultures. In view of the residual concentration achieved at the end of the batch culture, some amino acids, i.e., methionine and cysteine may have been at limiting concentrations at the end of both batch cultures and leucine and isoleucine especially in the serum-free medium. At the end of the exponential growth phase, lysine, phenylalanine and tyrosine may have been at limiting concentrations in the serum containing medium.

Continuous cultures

Amino acids utilisation by VO 208 hybridoma cells cultivated in continuous culture in a serum-free and in a serum containing medium

According to the results obtained with the batch cultures, it appears that this hybridoma cell line may have been limited in some amino acids in the serum-free and also in the serum containing medium.

Therefore, the consequence of amino acid supplementation on the hybridoma growth, the antibody production and on the evolution in the medium of the different amino acids was studied by performing two cultures in continuous mode. The dilution rate was maintained at 0.02 h^{-1} in both cases. This allowed us to compare the kinetics of the culture at the apparent stationary phases obtained for different concentrations of amino acids, keeping the other parameters of the culture at a constant level.

Figure 2. Detailed analysis of the evolution of the different amino acids during the batch cultures. The results are the mean of triplicate assays. The coefficient of variation for each result was less than 10%. The detection threshold was $50\text{--}100\text{ pmol l}^{-1}$.

Continuous culture in the serum-free medium

Cells and antibodies

Figure 3 represents the time change of the concentrations of viable cells and antibodies. The amino acid concentrations in both feeding media (with and without amino acids supplementation) are given in Table

2. It was checked that neither glucose, nor glutamine were at limiting concentrations during the overall continuous culture. A first apparent steady state was obtained after 300 h of culture and the second one was obtained after 500 h of culture. As seen, an enrichment of amino acids from 2- to 3-fold resulted in an increase of the viable cell density from $6.5 \pm 0.6 \times 10^8$

cells l^{-1} to $9 \pm 0.9 \times 10^8$ cells l^{-1} . For both apparent steady states, the viability was maintained at 90%. The transitory phase was characterised by a rapid increase of the cell density within 40 h. This result indicates that the amino acid supplementation suppressed a possible nutritional limitation.

The antibody concentration stabilised around

6 ± 0.6 mg l^{-1} during the first apparent stationary phase and reached 10 ± 0.1 mg l^{-1} during the apparent stationary phase obtained after amino acid supplementation. However, the specific rate of antibody production remained the same at around 0.2 mg 10^{-9} cells h^{-1} for both apparent stationary phases irrespective of the amino acids concentration. Moreover,

Figure 3. Time change of the concentration of the viable cells and antibodies (A), glucose and lactate (B), glutamine and ammonia (C) in continuous culture condition with serum-free medium: Effect of amino acids supplementation. The experimental error involved in cell density determination was calculated to be 5 to 10%. The error involved in the AB concentration determination was calculated to be $\pm 10\%$.

it is important to note that in this case, the specific rate of antibody production was comparable to the value obtained in batch culture conditions.

Evolution of amino acids

The concentrations of amino acids in both feeding media are presented in Table 2. It can be seen that the initial concentration of the amino acids is increased 1.5- to 3-fold, depending on the amino acid in the supplemented medium.

The residual concentration of the amino acids in the bioreactor and the specific rates of amino acids uptake or release by the cells, calculated for each apparent stationary phase are presented in Table 2.

A detailed study of the evolution of the amino acids during the first apparent stationary phase shows that the uptake of some amino acids was high, more than 50% of the total initial amount. Those amino acids are Leu, Ileu, Val, Met and Lys for the first apparent stationary phase and Leu, Ileu, Val, Met, Lys and Cys for the second apparent stationary phase. Only two amino acids, Met and Lys may have been at limiting concentrations for the first apparent stationary phase with residual concentration below 70 μM .

Interestingly, Gly (which was produced from the end of the exponential growth phase during batch culture) remained stable during the first apparent stationary phase and was consumed after amino acid supplementation. As Gly, Cys had a particular behaviour. The extracellular concentration of these amino acids remained more or less stable during the first apparent stationary phase and when the concentration in the feeding medium was increased, the specific rate of consumption of these amino acids slightly increased, i.e., to around $2 \times 10^{-9} \mu\text{M cells}^{-1}\text{h}^{-1}$.

It can be noticed that the amino acid Pro was utilised at a higher level than what was observed during the growth phase of the batch culture with the serum-free medium. Ser was excreted in the medium as it was produced in batch culture condition. Ala and Glu acid were excreted at the same rate in the medium for both apparent stationary phases.

Continuous culture made with serum containing medium

Cells and antibodies

Figure 4 represents the evolution with time of viable cells and antibodies during a continuous culture made

with 5% serum. As in the previous case, the dilution rate was maintained at 0.02 h^{-1} . Two apparent stationary states were obtained, one without amino acid supplementation and one with amino acid supplementation. The initial concentration of the different amino acids in both feeding media are presented in Table 3. During the first apparent stationary phase until h 210 of the culture, the viable cell concentration was $1 \pm 0.1 \times 10^9 \text{ cells l}^{-1}$, then after, when amino acids were added the viable cell concentration increased to $2.2 \pm 0.2 \times 10^9 \text{ cells l}^{-1}$, 50 h after the enrichment. As in the previous case, an enrichment of amino acids from 1.4 fold (for Cys) to 3.8-fold (for Lys and Val), depending on the amino acid, resulted in a two-fold increase of the viable cell density. With the conditions tested, even in the presence of serum these cells may have been limited in some amino acids when the serum containing medium was not supplemented with amino acids.

The monoclonal antibody concentration was around $6 \pm 0.6 \text{ mg l}^{-1}$ for the first apparent stationary phase and then increased to $24 \pm 0.2 \text{ mg l}^{-1}$ after amino acid supplementation. The specific rate of antibody production slightly increased from 0.1 to 0.2 mg h^{-1} and per 10^9 cells. Unlike what was observed in serum-free conditions, in this case the specific rate of antibody production was improved.

Glutamine

Unlike what was observed with the culture made with the serum-free medium, the specific rate of glutamine consumption was lower when the medium was supplemented with amino acids.

Amino acids evolution

The evolution of amino acid concentrations with time and especially data obtained for the first and the second apparent stationary phases are presented in Table 3. The specific rates of amino acid consumption or production are presented also in Table 3. It appears that most of the amino acids were consumed as it has been observed in the culture made in the serum-free medium. For Leu, Ileu, Val, Met, Lys the residual concentration of these amino acids was less than 50% of the initial concentration. Concerning Leu, Ileu and Val this was also observed during the second apparent stationary phase.

While Ala, Gly, Pro and Ser (unlike what was observed in batch culture conditions for this latter amino acid) were produced during the overall con-

Figure 4. Time change of the concentration of the viable cells and antibodies (A), glucose and lactate (B), glutamine and ammonia (C) in continuous culture condition with serum containing medium: Effect of amino acids supplementation. The experimental error involved in cell density determination was calculated to be 5 to 10%. The error involved in the AB concentration determination was calculated to be $\pm 10\%$.

tinuous culture, glutamic acid was only produced during the second apparent stationary phase. This amino acid was consumed during the first apparent stationary phase. It is known that glutamic acid is a precursor of Pro and according to Table 3, the specific rate of Pro production was twice what was observed during the second apparent stationary phase.

As in the case of the continuous culture realised in the serum-free medium, some amino acids may have been at limiting concentrations when the medium was not supplemented with amino acids: Met, Val, Lys, Tyr, His were in concentrations around or below $50 \mu\text{M}$. Moreover, when the medium was supplemented

with amino acids, the concentrations of Met and Cys were still below $70 \mu\text{M}$.

Discussion

One of the main interests of this study is that it will be possible to evaluate the influence of the environmental composition on amino acids utilisation by VO 208 hybridoma cell line. The work has been focused both on the role of the medium components and on the influence of the mode of culture (batch and continuous) on the same cell line.

The first observation concerns the different utilisation of 4 amino acids: serine, arginine, proline and glycine. The evolution of their extracellular concentration depends on the mode of culture and on the medium composition.

The high consumption of serine observed in batch culture in the presence of serum was also noticed by Reid et al. (personal communication). According to this author the specific rate of serine consumption is increased when the serum concentration is higher in the medium. Moreover, our results show that Ser is produced in serum-free conditions, in batch and in continuous cultures. It appears that in serum-free conditions these hybridoma cells reduce their need for extracellular serine and increase its production from glucose. Another interesting result is the modification of the serine metabolic pathway according to the mode of culture, i.e., Ser is also produced during continuous culture with serum containing medium, although it was consumed in batch culture conditions. According to Miller et al. (1989a, b), a decrease in glutamine concentration may induce an increase in serine production.

Serine is also the main precursor for Gly. Gly is produced in serum containing medium in batch and in continuous culture. In the case of the batch culture with serum, we can assume that a certain amount of the high concentration of serine consumed is transformed into glycine, probably by the enzyme serine hydroxymethyltransferase. Its production is low in the serum-free medium, in batch culture mode and it occurred mainly from the late exponential phase whereas, in continuous culture conditions, its concentration does not vary during the first apparent stationary phase when there was no amino acid supplementation. Surprisingly Gly was consumed when its concentration in the serum-free medium was enriched during the second apparent stationary phase. In the fed-batch study made by Zhou et al. (1997), Ser and Gly accumulated in low levels in the late culture stage, when hybridomas were cultivated in a serum-free medium. In our case, an increase in the extracellular concentration of amino acids in the serum-free medium induced an increase of Gly consumption rate. It has been shown that when cells are actively growing in limiting conditions, Gly is utilised to produce pyruvate. This amino acid is also known to be a precursor for purines and pyrimidines.

Pro is consumed in the serum-free medium in continuous culture mode and during the growth phase in batch culture. It is interesting to note that in the

serum containing medium, the concentration of Pro increased significantly during the batch culture decline phase. In the same way, Pro was also produced in continuous culture condition and its production was higher during continuous culture with no amino acid supplementation. Phang (1985), showed the essential role of Pro in the regulation of nucleotide synthesis and amino acid metabolism. The decrease of the level of nucleotides at the end of the exponential growth phase in batch culture or the lower maximal cell density during the first apparent stationary phase of the continuous culture may explain the accumulation of Pro which is less utilised. In the serum-free medium Pro is consumed. This amino acid has been shown to be essential for the growth of CHO cells (Gottesman 1982).

Concerning Arg, it is known as an essential amino acid for *in vitro* cell proliferation (Butler et al. 1983). In batch culture conditions, Arg is intensively used in serum containing medium, whereas, its concentration remains more or less stable in serum-free conditions. Arg is known to be incorporated in the composition of enzymes and proteins involved in the cell cycle. This amino acid is also used for the production of ornithine and proline; Cells cultivated with serum containing medium excreted a high level of Pro. Nevertheless according to Phang (1985), we should take into account that excess Arg may also be degraded by arginase which may be present in serum. It was also emitted the hypothesis that serum could act on cell membranes and thus modify the transport of some amino acids and especially Arg (Phang 1985). In continuous culture with the serum-free medium, Arg is consumed more significantly when the medium is supplemented with amino acids and for both apparent stationary phases, Pro is also consumed.

The results obtained in batch culture conditions also show that the consumption of some amino acids is coupled with cell growth. This is the case for Leu, Ileu, Val, Met, Cys, Lys, Phe, Thr and Tyr.

The effect of amino acid enrichment of a medium can be observed with the results obtained in continuous culture mode and especially during the stationary phases when all the other parameters are maintained to a constant level. The increase in viable cell density is probably due to a suppression of nutritional limitation, although we should not neglect the effect of the additional amino acids on the reduction of cellular death or cell lysis (Geaugey et al. 1989; Wagner et al. 1990), especially with our serum-free, low protein, medium. The work of Franek and Sramkova (1995),

Table 4a. Summary of the amino acids (AA) network for VO 208 hybridoma cells in batch culture conditions. Bold face type written AA are those having a typical behaviour according to the medium composition and culture mode.

		Growth phase	Death phase
serum containing medium	AA consumed	Leu, Ileu, Val, Met, Cys Lys, Phe, Thr, Tyr, His Arg, Ser	Ser, Arg, Glu acid
	AA excreted	Glu acid, Gly, Ala	Pro, Gly, Ala
	AA stable conc. ¹	Pro	
serum-free medium	AA consumed	Leu, Ileu, Val, Met, Cys Lys, Phe, Thr, Tyr (Arg)	Glu acid
	AA excreted	Ser	Ser, Gly, Ala
	AA stable conc. ¹	Gly, Arg, (Pro), (His)	Arg, Pro, (His)

Table 4b. Summary of the amino acids (AA) network for VO 208 hybridoma cells in continuous culture conditions. Bold face type written AA are those having a typical behaviour according to the medium composition and culture mode.

		No amino acids supplementation (first stationary phase)	amino acids supplementation (second stationary phase)
serum containing medium	AA consumed	Leu, Ileu, Arg, Lys, Val, Cys His, Thr, Phe, Met, Tyr, Asp Glu acid	Leu, Ileu, Arg, Lys, Val, Cys, His, Thr, Phe, Met, Tyr, Asp
	AA excreted	Ser, Gly, Ala, Pro	Ser, Gly, Ala, Pro, Glu acid
	AA stable conc. ¹		
serum-free medium	AA consumed	Met, Leu, Ileu, Val, Cys, Arg, Lys, Phe, Tyr, Pro (His), (Thr)	Met, Leu, Ileu, Val, Cys, Arg, Lys, Phe, Tyr, Pro, His, Thr Gly
	AA excreted	Ser, Ala, Glu acid	Ser, Ala, Glu acid
	AA stable conc. ¹	Gly	

conc.¹: concentration

on cells cultivated in 24 well plates, shows that some amino acids (Gly, Ala, Ser, Thr, Pro) not only act as nutrients but also as specific signal molecules in order to survive. According to Baydoun et al. (1990), under conditions of limited amino acid supply, cells may switch from utilising extracellular amino acids for synthesis to using amino acids from an intracellular pool derived from protein breakdown. This was probably the case in serum-free conditions when the medium was not supplemented with amino acids.

In order to deepen the analysis, specific rates have been calculated. According to the results presented in Tables 2–3, the specific consumption rate of most amino acids is improved 1- to 2-fold after enrichment in serum containing medium, whereas in serum free conditions, we noticed a very consequent increase in the specific rate of some amino acids: for Cys 9-fold, for Thr 5-fold and for Arg 7-fold. If the cells are consuming more amino acids at a given growth rate, presumably the extra amino acids are either being used to generate waste products or larger cells. According to our results, the generation of amino acid by-products (e.g. alanine) cannot explain the increased amino acid uptake. The specific production rates of lactate or ammonia (waste products) did not increase significantly. Alternatively amino acids could have been accumulating intracellularly. To

further understand our results these kind of data are useful. According to the results obtained by Bibila et al. (1994) with concentrated medium shots, most amino acids transiently accumulate to 2 to 3 times their initial level following the shots but the specific amino acid uptake rate remained constant for the majority of the amino acids. On the other hand, according to Reddy et al. (1992) an increase in specific amino acid uptake rate was observed for cells grown in concentrated or nutritionally enriched media. The increase in transport activity may also be the result of synthesis of additional transporters. According to the results obtained by Häüssinger et al. (1992), growth arrest by removal of serum from sub confluent cells caused a reduction in system A transport activity. On the other hand, the activity of system A also decreased with increasing cell density. All these observations suggest that cells growth rate as well as cell density have an impact on the regulatory signals governing the expression of the transport system genes in response to substrate availability.

Some amino acids appeared to be at limiting concentrations at the end of the exponential growth phase in batch culture conditions. This may have been the case for Met, Cys, Leu, Ileu in both media and Lys, Phe, Tyr in the serum containing medium. The extracellular concentrations of these amino acids were

below 50 μM . In serum-free continuous culture conditions, Met may have been in limiting concentration even after amino acids supplementation, whereas, in the serum containing medium, in the case of no amino acids supplementation, both Met and Phe may have been in limiting concentrations. Nevertheless, for Lys, Tyr, Val and His, the extracellular concentration is around 50 μM . According to Zhou et al. (1997), in fed-batch culture realised with hybridoma cells in a serum-free media, three amino acids were found to be consumed at a rate faster than at which they were fed: Tyr, Leu and Met. As it was observed for branched amino acids (Duval et al. 1989), sulphurated amino acids are also known to be consumed at a high rate. Blasey and Winzer (1989), observed a rapid drop of the viable hybridoma cell density in bioreactors, when a limitation in Met occurred.

Concerning the specific rate of antibody production, unlike what was observed in serum-free conditions, in the presence of serum containing medium and in continuous culture conditions, the rate of production was improved when the medium was supplemented with amino acids. According to Borth et al. (1992), substrate limitation may in itself have a definite effect on productivity. It has also been observed that in situations of stress such as low pH, hyperosmolarity or substrate limitation hybridomas will produce more antibodies on a per cell basis than under normal conditions (Miller et al. 1988). Step changes in essential amino acids during continuous cultivation of hybridoma cells have also been investigated by Reddy et al. (1992). According to their results, only when non essential amino acids were also supplemented did an increase in antibodies appear.

One of the outcome of the present study is the data summarised in Tables 4a and 4b. Our results give an idea of the importance of the culture process, i.e., the components of a medium and the mode of culture used, on amino acid utilisation by a cell line. The approach we used gives an indication of the amino acids which may have been limiting. It also leads to the identification of those amino acids (Ser, Gly, Pro . . .) for which the metabolic pathway in the cells is modified, depending on the culture conditions used. It is important to gather all this information in order to better understand how the environment affects the cell metabolism and product secretion. The results we obtained concern VO 208 hybridoma cell line. By comparison with the data from the literature, some general findings were obtained. Nevertheless, an anal-

ysis of such data, coupled with the analysis of the intracellular levels of amino acids is not a simple task and it should be realised for each cell line intended for an optimal production of bioproducts.

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