Cystine/cysteine metabolism in cultured Sf9 cells: influence of cell physiology on biosynthesis, amino acid uptake and growth

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Abstract

Spodoptera frugiperda (Sf9) insect cells proliferate in a cystine-free medium, with the same growth rate, reaching the same final cell density, as in a cystine-containing medium, provided that the inoculum is taken from a pre-culture sufficiently early, at 47–53 h. With an inoculum from a 103 h culture an extended lag phase accompanied by cell death was observed during the first 50 h of cystine-free culture, even though the culture had been adapted to cystine-free conditions for 10 passages. Cystine-free cultures seeded with a 103 h inoculum had lower growth rates and reached lower final cell densities than corresponding cystine-supplied cultures. Cysteine biosynthesis occurs from methionine via the β -cystathionine pathway. More methionine was consumed by the cells in cystine-free media, and cystathionine was secreted when methionine and cystine were supplied in excess. The data suggest that cysteine biosynthesis is up-regulated in proliferating cells but down-regulated when the cells enter the stationary phase.

In cultures supplied with cystine ($10\text{--}100\text{ mg l}^{-1}$), the specific uptake rate and total consumption of cystine, as well as the uptake of glutamate, glutamine and glucose increased with increasing cystine concentrations. These results are interpreted in view of system x_c^- , a concentration dependent amino acid transporter. Similarly, the consumption of amino acids transported by system L (ile, leu, val, tyr) was enhanced in cystine-containing cultures, as compared to cystine-free cultures. Uptake of cystine, methionine and system L amino acids ceases abruptly in all cultures, even before growth ceased. The specific growth rate starts to decline early during the growth phase, but this growth behaviour could not be correlated to the depletion of nutrients. We therefore propose that the observed growth pattern is a result of (auto)regulatory events that control both proliferation and metabolism.

Introduction

Cultured insect cells, mainly used to produce heterologous protein with the baculovirus expression vector, have characteristic metabolic and physiological properties that distinguish them from other types of cultured cells. Typically, the primary metabolism is flexible in that many different substrates are readily catabolised: carbohydrates such as fructose and maltose can replace glucose, and the TCA cycle components (e.g. α -ketoglutarate, succinate, fumarate, malate) are consumed in parallel to the carbohydrates, if supplied, in the medium (Bédard *et al.*, 1993). In addition, amino acids can be channelled into the central catabolic path-

ways for energy production (Ferrance *et al.*, 1993). Insect cells have also a pattern of enzyme activities that is distinct from that of mammalian cells (Neermann and Wagner, 1996). During normal cultivation conditions when glucose (and glutamine) is in excess neither lactate nor ammonium is formed, but alanine is the only overflow metabolite. However, glucose limitation causes a switch in the metabolism, from alanine to ammonium formation (Öhman *et al.*, 1995). Lactate is normally not formed by cells growing aerobically (Rhiel and Murhammer, 1995).

We showed earlier that Sf9 cells can proliferate in a culture medium without the amino acids glutamine, glutamate and aspartate, provided that an ammonium

source was added (Öhman *et al.*, 1996). In contrast to mammalian cells, synthesis of these amino acids would occur from glucose and by incorporation of inorganic nitrogen, to a sufficient extent for normal growth to take place. In this work we present evidence that Sf9 insect cells can proliferate, and reach the same final cell density in a cystine-free medium as in a cystine-containing medium, provided that the cells are passaged early enough (i.e. 47–53 h) to seed a cystine-free culture. It has been proposed that cystine is an essential amino acid for Sf9 insect cell cultures (Tremblay *et al.*, 1992) and for other insect cell lines (Landureau and Jollès, 1969; Mitsunashi, 1982). However, the age of the inoculum can be a critical parameter for growth and final cell density in batch cultures (Kioukia *et al.*, 1995; Wu *et al.*, 1990; Neutra *et al.*, 1992).

The kinetics of cystine uptake in cystine-containing media were further studied, and a noticeable concentration dependence was found. The demand of cysteine in cultured (mammalian) cells is almost entirely fulfilled by the uptake of cystine (Bannai and Tet-suro, 1988) because cysteine oxidises spontaneously to cystine (cys₂) in the culture medium (Fedorcsak *et al.*, 1977). We therefore use the denotation cystine for the extracellular species and cysteine for intracellular. A hypothesis concerning the concentration-dependent uptake of cystine and its consequences was formulated based on the properties of the cystine specific amino acid transport system x_c⁻ (Guidotti and Gazzola, 1992). Finally, the typical growth behaviour and amino acid uptake pattern in batch cultures of Sf9 cells are considered.

Materials and methods

Cell line

Spodoptera frugiperda clonal isolate 9 (Sf9) was a generous gift from KaroBio AB, Novum Research Centre (Huddinge, Sweden). Stock cultures were stored in liquid nitrogen and working cultures were maintained as suspension cultures in shake flasks at 27 °C. The cells were routinely passaged every third day. No antibiotics were used and periodic sampling for mycoplasma using the fluorescent DNA-staining method described by Del Giudice and Hopps (1978) were negative.

Medium

The standard medium used (KBM 10) had the following composition, in g l⁻¹: glucose, 5.0; KCl, 2.87; CaCl₂, 1.0; MgCl₂, 1.07; MgSO₄, 1.36; NaHCO₃, 0.35; NaH₂PO₄, 0.88; choline chloride, 0.02; L-glutamine, 1.0; L-arginine-HCl, 0.7; L-asparagine, 0.35; L-aspartic acid, 0.35; L-glutamic acid, 0.6; glycine, 0.65; L-histidine-HCl, 0.2; L-isoleucine, 0.5; L-leucine, 0.075; L-lysine-HCl, 0.625; L-methionine, 0.05; L-phenylalanine, 0.15; L-proline, 0.35; L-serine, 0.55; L-threonine, 0.175; L-valine, 0.1; L-cystine-2HCl, 0.05; L-tryptophan, 0.1; L-tyrosine, 0.072; and in µg l⁻¹: para-amino benzoic acid, 320; biotin, 160; D-calcium pantothenate, 8; folic acid, 80; myo-inositol, 400; niacin, 160; pyridoxine-HCl, 400; riboflavin, 80; thiamine-HCl, 80; vitamin B-12, 240; CoCl₂ 6H₂O, 50; FeSO₄ 7H₂O, 550; MnCl₂ 4H₂O, 20; (NH₄)₆(Mo₇O₂₄ 4H₂O), 40; ZnCl₂, 40; and CuCl₂, 158. The medium was supplemented with cholesterol (4.5 mg l⁻¹), Pluronic F-68 (25 mg l⁻¹), cod liver oil (10 mg l⁻¹), α-tocopherol (2.0 mg l⁻¹) and Yeastolate (4 g l⁻¹) (Gibco). All chemicals used were from Sigma (Insect cell culture grade) if not otherwise mentioned. The pH of the medium was 6.2.

Experimental conditions

All experiments (and precultures) were performed in shake flasks (100 ml of culture in a 1-litre glass-baffled, siliconised (Sigmacota, Sigma) Erlenmeyer glass bottles) incubated on a rotary shaker (100 rpm, 27 °C). The age of the inoculum (47–103 h) and cystine concentration (0–100 mg l⁻¹) varied depending on the experiment. The inoculum was gently pelleted in a swing-out centrifuge (Wifug clinic, Wifug) at 1000 rpm (200g, 4 °C, 6 min) and resuspended in fresh medium to an initial cell density of 3.0 10⁵ cells ml⁻¹. pH was not adjusted during the experiments as it remained almost constant. Samples for viable and total cell count, glucose and amino acid concentrations were taken at regular intervals and analysed as described below.

Analytical

Cell counting was performed using a Bürker counting chamber and cell viability was determined by the trypan blue exclusion test. To clarify the results, the data for cell density (Figures 1–4) are presented as mean values without error bars. Mean values were calculated from 5 independent data points (n = 5) and the rela-

tive confidence interval at the 95% level was calculated from the absolute confidence limit (from the beginning to the end of the culture) as ± 13.8 –2.2% of mean. Samples for amino acids and glucose were centrifuged and filtered through a $0.45 \mu\text{m}$ cellulose acetate filter and stored frozen until analysed. The glucose concentration was measured enzymatically (hexokinase, glucose-6-P dehydrogenase, Boehringer-Mannheim) ($n = 4$, 95% confidence interval, ± 2.0 –13.4% relative to mean). Amino acids were analysed by the Pico-Tag system using a reversed-phase HPLC column (Waters) and the results are presented as mean values ($n = 2$, relative standard error < 10% of mean). The method was previously described by Bidlingmeyer *et al.* (1985). Samples were derivatised with phenylisothiocyanate reagent containing norleucine as internal standard.

Data analysis

All kinetic analyses were performed by curve fitting technique using Excel 5.0 and Kaleidagraph 3.1 software. A polynomial function, fitting to the mean concentration data-set (least squares method), was obtained by the software. The first time derivative of this function was then calculated and specific consumption rates could be calculated by division of the time derivative with the viable cell concentration (N_v) at the same time point. The specific growth rate (μ , h^{-1}) and death rate (k_d , h^{-1}) were calculated in the same way according to equations 1 and 2 (batch cultures), where N_t is the total cell concentration, N_v the viable cell concentration and N_d the concentration of dead cells:

$$\mu = \frac{dN_t}{dt} \frac{1}{N_v} \quad (1)$$

$$k_d = \frac{dN_d}{dt} \frac{1}{N_v} \quad (2)$$

Results

Growth in cystine-free medium – effects of the age of the inoculum and number of passages

Growth curves of Sf9 cells in our standard medium with $50 \text{ mg cystine l}^{-1}$, and after the first transfer to a medium without cystine (0 mg l^{-1}) are shown in Figure 1. The inoculum for each culture was collected from a presuspension culture at 103 h. The cystine-free culture clearly grew slower and to a lower final cell density, obviously affected by the lack of the nutrient.

Figure 1. Viable cell density of Sf9 cells grown in cystine-free and cystine containing-medium, 0 (—●—) and 50 (—○—) mg l^{-1} . Age of inoculum, 103 h.

However, when passaged earlier (53 h), cell growth (Figure 2a) and growth rate (Figure 2b) of Sf9 cells were very similar in both media.

To investigate if the latter result was a transient phenomenon due to the intracellular pool of cystine not being depleted (even though the intracellular pool of cystine is normally negligibly small (Bannai and Tetsuro, 1988)) or a persistent property, Sf9 cells were sub-cultured 10 times in cystine-free medium. To maintain a high specific growth rate (μ) of the cultures during this long-term experiment, sub-culturing into fresh medium was performed every 47–53 hours, i.e. at the time of maximum specific growth rate (μ_{max}) for each sub-culture (Figure 2b). No significant change of the cell growth in cystine-free medium was observed during these 10 passages (data not shown). The growth curves of the 11th sub-culture in cystine-free medium and for another sub-culture, transferred to a cystine-supplemented medium (50 mg l^{-1} of cystine) are shown in Figure 3a. As the growth really is indistinguishable in both media initially, and the cystine-free culture even reached the highest cell density, we can conclude that Sf9 cells can grow in a cystine-free medium provided that the inoculum is collected during an early growth phase (47–53 h) before the specific growth rate declines.

The cystine-free culture used to inoculate the flask for the experiment shown in Figure 3a was incubated another two days and used for another inoculum

Figure 2. Viable cell density (a) and specific growth rate (b) of Sf9 cells in medium containing 0 (—●—) and 50 (—○—) mg l^{-1} cystine. Age of inoculum, 53 h.

preparation, after 103 h of culture. The cells were inoculated into both cystine-free and cystine-containing media. The growth curves (Figure 3b) show that these cells, from the late growth phase, were adversely affected in a cystine-free medium as compared to a cystine-containing medium. This effect was equally pronounced whether the inoculum was adapted to a cystine-free medium or not, as was the case in Figure 1.

The difference in growth behaviour between the two cultures in Figure 3b can be explained by the viability analysis (Figures 3a and b) and the kinetic analysis in Figure 3c. The viability of the cultures seeded with a 47 h inoculum was approximately 90% dur-

Figure 3. Viable cell density (—●—, —○—), viability (—■—, —□—), specific growth rate (—▲—, —△—) and specific death rate (—◆—, —◇—) of Sf9 cells at the 11th passage in cystine-free medium. Age of inoculum 47 h (a) and 103 h (b and c) in medium containing 0 (—●—, —■—, —▲—, —◆—) and 50 (—○—, —□—, —△—, —◇—) mg l^{-1} cystine.

ing the growth phase in both cystine-free and cystine-containing medium, the viability curves being almost identical Figure 3a). However, the viability of the cystine-free culture in Figure 3b, with an age of inoculum of 103 h, decreased to a minimum of 71% after 48 h. During the first two days of culture the specific death

rate (k_d) of this culture was significant (Figure 3c). In contrast, the viability of the cystine-supplemented culture (Figure 3b) was equally high (90%) as in the cultures seeded with a 47 h inoculum (Figure 3a). It is also apparent that the cystine-free culture with an age of inoculum of 103 h, had a lower specific growth rate throughout the cultivation ($\mu_{\max} = 0.023 \text{ h}^{-1}$, Figure 3c) and reached a lower final cell density ($2.8 \times 10^6 \text{ cells ml}^{-1}$) than the corresponding cystine supplemented culture ($\mu_{\max} = 0.033 \text{ h}^{-1}$; final cell density $4.3 \times 10^6 \text{ cells ml}^{-1}$).

Thus, cells inoculated from the late growth phase (103 h) appear less capable of proliferating in a cystine-free medium, resulting in an increased specific death rate and a decreased viability during the first 48 h of cultivation, and in a lower specific growth rate and a lower final cell density.

Growth, metabolism and cystine utilisation in media with different cystine concentrations

Although Sf9 cells are capable of proliferating in a cystine-free medium, could cystine be a growth limiting factor if exhausted from a cystine-containing medium during growth? To investigate this possibility Sf9 cells were cultivated in standard medium with cystine in the range of $10\text{--}100 \text{ mg l}^{-1}$, using an age of inoculum of 103 h (Figure 4). All cultures reached the same final cell density (Figure 4a), but $\mu_{\max}$ appears somewhat lower at the higher cystine concentrations (Figure 4b). Interestingly, the kinetic analysis shows that $\mu_{\max}$ is reached at an early stage of the growth phase, whereafter μ declines. This phenomenon, which was also observed in the previous cultures (Figures 2b and 3c), could mean that some medium component(s) became growth rate limiting already at a culture time of 50–90 h. However, neither cystine (Figure 5a) nor glucose (data not shown), glutamine or other amino acids are even depleted at the end of the cultures. (The amino acid analysis from the cultures in Figure 4 are not shown but these data are very similar to the data presented in Table 3). Nor had addition of yeastolate or lipids, at any time of culture, any growth stimulatory effect (data not shown). Other possible causes to the observed growth kinetics will be discussed.

Cystine was not totally depleted in any of the cystine-containing cultures (Figure 5a). The concentration profiles in Figure 5a level off when growth stops at 143–167 h indicating a relationship between cystine uptake and proliferation. It is also apparent that more cystine was consumed by the culture at higher initial

Figure 4. Viable cell density (a) and specific growth rate (b) in Sf9 cell cultures with varying concentrations of cystine: 10 (—●—) 50 (—○—), and 100 (—■—) mg l^{-1} . Age of inoculum, 103 h.

cystine concentrations, an observation correlating the increase in the specific uptake rate (q_s) to cystine at increasing cystine concentrations (Figure 5b).

To quantify the consumption of cystine and other substrates (at different cystine concentrations) the cellular yield coefficients (Y) were calculated (Table 1). The yield coefficient for glucose was significantly higher in the culture with 10 mg cystine l^{-1} than in the cultures with 50 and 100 mg cystine l^{-1} . Similarly, the amount of cells produced per consumed glutamine, glutamate and, most significantly, cystine, was also the highest for the culture with 10 mg cystine l^{-1} . The differences in Y between 50 and 100 mg l^{-1} are less pronounced. These results will be discussed in view of the amino acid transport systems involved.

Figure 5. Concentration profiles (a) and specific consumption rates (b) of cystine in Sf9 cultures with varying initial concentrations of cystine: 10 (●), 50 (○), and 100 (■) mg l⁻¹.

Table 1. Cellular yield ratios (Y) of consumed glucose (Glc), glutamine, glutamate, and cystine in cultures of Sf9 insect cells at initial cystine concentrations of 10–100 mg l⁻¹. (Calculated from the beginning of the culture to the formation of 2.6 10⁶ cells ml⁻¹, Figure 4)

Mode of cultivation	Y _{Glc}	Y _{Gln}	Y _{Glu}	Y _{Cys}
	(10 ⁶ cells μmol ⁻¹)			
10 mg cys l ⁻¹	0.35	1.32	2.98	95.2
50 mg cys l ⁻¹	0.24	0.82	1.73	21.0
100 mg cys l ⁻¹	0.25	0.91	1.77	15.9

Table 2. Consumption of methionine in Sf9 cultures with varying cystine concentrations

Mode of cultivation	Consumption of methionine (μmol (10 ⁶ cells) ⁻¹)
0 mg cys l ⁻¹	0.098 ^a
10 mg cys l ⁻¹	0.069 ^b
50 mg cys l ⁻¹	0.060 ^b
100 mg cys l ⁻¹	0.056 ^b

^a From Figure 2. (Calculated from the beginning of the culture to the formation of 3.5 10⁶ cells ml⁻¹).

^b From Figure 4. (Calculated from the beginning of the culture to the formation of 2.6 10⁶ cells ml⁻¹).

As cysteine is synthesised from methionine in eukaryotic cells (Flavin, 1971) it would be appropriate to quantify the consumption of methionine in the cultures with varying cystine concentrations as well as in cystine-free cultures. A higher methionine consumption would be expected when the cystine level was low or equal to zero. Indeed, the data in Table 2 indicate a reverse relation between methionine consumption and the medium concentration of cystine.

The data on methionine consumption for the cystine containing cultures (Table 2) were derived from the experiments shown in Figure 4 and for the culture with 0 mg cystine l⁻¹ from the experiment in Figure 2. However, it was not possible to estimate the concentration of methionine in samples from the culture in Figure 2 supplemented with both methionine and cystine, due to the appearance of an interfering peak in the chromatograms. We qualitatively identified this peak as cystathionine, an intermediate in the biosynthesis of cystine (Figure 7), using dilution series of spiked growth medium for HPLC peak identification. This peak was not present in chromatograms from the culture without cystine in Figure 2 (47–53 h inoculum), nor in the cultures seeded with a 103 h inoculum and containing both cystine and methionine (Figure 4).

Amino acid metabolism

Since cystine is known to be a key component that influences uptake and metabolism of other amino acids (Bannai and Tetsuro, 1988; Finkelstein *et al.*, 1988), it would be interesting to investigate if the absence of cystine in the culture medium influenced amino acid metabolism in general. The change in concentration of all added amino acids was therefore analysed in cultures with and without cystine (Table 3). None of the measured amino acids was totally depleted but remained

Table 3. Amino acid utilisation in batch cultures of Sf9 insect cells at 0 and 50 mg cystine l^{-1} respectively. Values correspond to initial concentrations and concentrations at the maximum viable cell density at 146 h. (Data from Figure 2)

Cystine conc	0 mg l^{-1}		50 mg l^{-1}		0 mg l^{-1} 50 mg l^{-1}	
	Initial	146 h	Initial	146 h	% Remaining	
Amino Acid	(mM)	(mM)	(mM)	(mM)		
Aspartate	0.84	0.62	0.79	0.55	74	69
Glutamate	2.57	1.33	2.48	1.28	52	52
Asparagine	2.19	2.02	2.25	2.04	92	91
Serine	4.68	3.10	4.72	2.80	66	59
Glutamine	6.23	2.49	6.18	1.34	47	22
Glycine	7.70	7.98	7.91	7.30	104	92
Histidine	1.40	0.99	1.12	0.99	95	88
Arginine	3.26	2.72	3.36	2.55	83	76
Threonine	1.95	1.47	2.10	1.36	75	65
Alanine	1.89	6.83	2.38	9.6	361	403
Proline	3.25	2.69	3.38	2.61	83	77
Tyrosine	0.54	0.20	0.60	0.14	37	23
Valine	1.67	1.01	1.88	0.91	60	48
Methionine	0.56	0.14	0.65	–	25	–
Isoleucine	1.14	0.62	1.27	0.52	54	41
Leucine	1.61	0.74	1.76	0.59	46	33
Phenylalanine	1.33	1.03	1.47	0.96	77	65
Tryptophan	0.63	0.55	0.68	0.54	87	79
Lysine	3.39	3.05	3.72	2.83	90	76
Cystine	–	–	0.12	0.028	–	23

Table 4. Consumption of some system L amino acid in Sf9 cultures with 0 and 50 mg cystine l^{-1} respectively. (Calculated from the beginning of the culture until the formation of 3.5×10^6 cells ml^{-1} , Figure 2)

Mode of cultivation	Consumption of amino acids ($\mu mol (10^6 \text{ cells})^{-1}$)			
	Ile	Leu	Val	Tyr
0 mg cys l^{-1}	0.119	0.204	0.156	0.078
50 mg cys l^{-1}	0.163	0.267	0.207	0.104

at levels sufficient to support growth throughout the cultivations.

However, some differences in consumption of individual amino acids could be seen in Table 3. If calculated on a per cell basis it became clear that significantly less of isoleucine, leucine, valine and tyrosine was consumed in the cystine-free medium (Table 4). These results will be further discussed in view of the amino acid membrane transport systems involved.

Further, the typical time dependent decrease of isoleucine, leucine and methionine in both cultures is shown in Figure 6. At 121 hours of cultivation, the

uptake of these amino acids ceases abruptly, whether the medium contained cystine or not. Uptake of cystine follows the same pattern (Figure 5a). This sharp change occurs about 30 hours before cells enter the stationary phase (Figure 6).

Discussion

Proliferation in cystine-free media

Our results clearly show that Sf9 cells are able to proliferate in a cystine-free medium and reach the same final cell density as in a cystine-containing medium (Figures 2, 3a). As the medium contained yeastolate, one might suspect trace amounts of cystine to be present. However, no cystine peak could be detected in the chromatograms from HPLC analyses of cystine-free medium or pure yeastolate, a result in agreement with the observation of Mitsuhashi that yeast extract lack cystine (Mitsuhashi, 1982). Hence, Sf9 cells are able to synthesise cysteine in amounts sufficient to support growth, since cysteine is an essential amino acid *per*

Figure 6. Concentration of methionine (—●—), isoleucine (—○—), leucine (—■—); and viable cell density (—□—) in cultures of Sf9 cells with 50 mg cystine l^{-1} (a) and 0 mg cystine l^{-1} (b).

se, needed for synthesis of proteins and glutathione (Bannai, 1986).

Cysteine biosynthesis

The route for biosynthesis of cysteine, used by animal tissue (and fungi), starts from methionine, as schematically illustrated in Figure 7 (Finkelstein *et al.*, 1988; Flavin, 1971; Mehler, 1986). Our data clearly show that the consumption of methionine per cell increases as the cystine concentration decreases (Table 2). A culture grown in cystine-free medium consumes about 40% more methionine than a culture supplied with 50 mg cystine l^{-1} . This result is in accordance with the methionine-sparing effect of cystine in mammals, where cystine can replace 70% of the dietary requirement of methionine (Finkelstein *et al.*, 1988). Serine is also involved in cysteine biosynthesis (Figure 7) but we have not been able to detect any differences in

Figure 7. Pathway of cysteine biosynthesis in animal cells. 1. methionine adenosyltransferase; 2. representative transmethylation reaction; 3. adenosylhomocysteinase; 4. cystathionine β -synthase; 5. methyltetrahydrofolate-homocysteine methyltransferase (and betaine-homocysteine methyltransferase); 6. cystathionase.

serine consumption, probably due to the high initial serine concentration in the medium (Table 3). However, the data above and the observation that cystathionine, an intermediate in the biosynthesis of cysteine from methionine, was secreted by the cells supplied simultaneously with cystine and methionine support the view that cysteine is synthesised from methionine also in Sf9 cells.

Regulation of cysteine biosynthesis

The fact that cells taken from the early growth phases (47–53 h) are capable of rapid proliferation without any lag phase in the first transfer to a cystine-free medium (Figure 2), while in the older cells (103 h) an extended lag phase accompanied by cell death occurred during these conditions (Figures 3b and c), even though the culture had been sub-cultivated in cystine-free medium 10 times, suggests that the biosynthetic pathway is expressed only in proliferating cells but down-regulated in cells entering the stationary phase. The culture seeded with a 103 h inoculum resumed growth later on, an observation indicating that cysteine biosynthesis was up-regulated after inoculation into fresh medium.

The results on cystathionine excretion support further this theory of regulation of cysteine biosynthesis. Overproduction of cystathionine could occur in cells capable of cysteine biosynthesis, supplied with both methionine and cystine if cystathionine synthase, the regulatory enzyme of the pathway (Finkelstein *et al.*, 1988), is not (totally) repressed by cystine as our results indicate. It has been shown that methionine excess (likely to be the case in the medium used) enhances cystathionine formation by increasing the levels of adenosylmethionine which is an activator of cystathionine synthase (Figure 7) (Finkelstein and Martin, 1986; Finkelstein *et al.*, 1988). Cystathionine is converted to cysteine by cystathionase, but as this enzyme is feed-back inhibited by an excess of cysteine (Flavin and Slaughter, 1971), cystathionine could accumulate during these conditions. However, in cultures without an external supply of cystine, cystathionine should not accumulate because it is further converted to cysteine which in turn is incorporated into cellular constituents. In cultures not capable of cysteine biosynthesis, cystathionine should neither accumulate because cystathionine synthase should not be expressed. Indeed, cystathionine overproduction was only observed in the culture seeded with a 53 h inoculum, and supplied with both methionine and cystine (Figure 2) but not in the cystine-supplemented cultures with an age of inoculum of 103 h (Figure 4). (Whether cystathionine was produced in the cystine-free, slowly growing cultures with an age of inoculum of 103 h (Figure 1 and Figure 3b) has not been determined). Very speculatively, our data suggest that the expression of cystathionine synthase is controlled in conjunction with proliferation, and that cystathionine synthase is not feed-back inhibited by cysteine but that cystathionase is. However, it has been reported that the level of cystathionine synthase is decreased by cysteine in mammalian liver cells (Finkelstein *et al.*, 1988). Clearly, further experimental work is needed to clarify the mechanisms of regulation.

The regulation of cysteine biosynthesis in relation to proliferative status may be one reason why Sf9 (Tremblay *et al.*, 1992) and other insect cell lines (Mitsuhashi, 1982) have been considered unable to grow in cystine-free media. If cultures are passaged only once a week, then cysteine biosynthesis may be down-regulated and cells transferred to a cystine-free medium do not readily grow.

Cystine uptake in cystine-containing media

The specific consumption rate (Figure 5b) and total consumption (Figure 5a, Table 1) of cystine increased in proportion to increasing medium concentrations of cystine, without any corresponding increase in the final cell yield (Figure 4a). Consequently, cystine uptake is not stoichiometrically coupled to biomass formation but some mechanism, other than the cellular need for anabolic precursors, seems to influence cystine consumption. The answer to this behaviour might be sought in the function of the cystine transport system.

Over 90% of cystine in culture media is present in the zwitterionic form (without a net charge), cys_2 , at pH between 6 and 7 (Makowske and Christensen, 1982). The remaining fraction occurs as the tripolar ion with a net negative charge (cys_{2-1}). In spite of the zwitterion being the dominant species, transport appears to occur mainly through the tripolar ion ((Makowske and Christensen, 1982). The transporter, x_c^- , is an antiport system with high specificity for cys_2^- and the negatively charged glutamate ion, glu^- , (Figure 8) (Bannai and Tetsuro, 1988; Makowske and Christensen, 1982). The driving force for this transport system is solely the concentration gradients of the substrates over the plasma membrane, a fact that likely explains the concentration dependent increased uptake of cystine (Figure 5). An increased uptake of cystine should cause a simultaneous increase in export of glutamate (Figure 8) (Bannai, 1986). The intracellular glutamate pool may be restored through reassimilation of glutamate by the glutamate (and aspartate) specific transport system X_{AG}^- (Guidotti and Gazzola, 1992), or indirectly through glutamine uptake and metabolism (Öhman *et al.*, 1995). All these transport processes are energy demanding. In our experiments, high concentrations of cystine led to an increased consumption of the energy yielding substrates glucose, glutamine, glutamate, in addition to cystine (Table 1) and a slight decrease of μ_{max} . These results may be interpreted as an increased demand for maintenance energy at increasing cystine concentrations. Surplus intracellular cysteine may also lead to the formation of inhibitory byproducts, including H_2S (Mehler, 1986).

Uptake of other amino acids

Uptake of the amino acids leucine, isoleucine and methionine ceased even before growth ceased in cultures with cystine (Figure 6a) and in cultures without cystine (Figure 6b). This uptake pattern is the same

Figure 8. The x_c^- amino acid transporter.

for valine and tyrosine (not shown) and for cystine, in cultures supplied with cystine (Figure 4a and Figure 5a). Therefore it appears that these events are subjected to physiological control mechanisms that, in turn, may even be the cause of the cessation of growth. Again, information can be obtained from the function of the amino acid transport systems. System A transports methionine, system x_c^- cys_2^- , and system L the other amino acids mentioned above. System L is an antiport system driven by the concentration gradient of its substrate amino acids (Kilberg and Christensen, 1980; Collarini and Oxender, 1987). System L imports leucine, isoleucine, and other amino acids (e.g. valine, tyrosine) in exchange for intracellular methionine and/or cysteine. The activity of system A is regulated in a complex manner and both system A and x_c^- are up-regulated by growth factors and hormones (Guidotti and Gazzola, 1992; Shotwell *et al.*, 1983). System A is also thought to be a key factor in cell cycle regulation in that it contributes to the increase in cell volume (together with the Na^+ , K^+ , 2Cl^- -cotransporter), by accumulating amino acids, necessary for the cell to enter the S-phase (Bussolati *et al.*, 1996). If the activities of system A and x_c^- are down-regulated, then uptake of amino acids by system L should cease as a consequence of the decrease in intracellular pools of methionine and cysteine. This may be the explanation to what our experimental data show.

Another interesting observation is that the uptake of system L amino acids is enhanced in cystine-containing cultures compared to cystine-free cultures (Table 4). It is difficult to understand why these amino acids, generally considered as used only for protein synthesis and consumed in stoichiometric amounts to cell mass, should be needed in higher quantities during these conditions. Rather, a high cystine concentration may force

transport through system L, due to an increased intracellular cysteine pool (Guidotti and Gazzola, 1992).

Regulation of proliferation and metabolism – a hypothesis

From the kinetic analyses in Figures 2b, 3c and 4b, and our previous studies in serum-containing medium (Öhman *et al.*, 1995), it is clear that the specific growth rate in cultures of Sf9 cells deviates from exponential course early during the growth phase, a circumstance seldom mentioned in literature. When done so, it has been reported to correlate with the depletion of nutrients, e.g. glucose (Drews *et al.*, 1995). In our cultures it is obvious that the decrease in growth rate is independent of the cystine concentration, as all μ -curves follow the same course in cultures with different cystine concentrations (Figure 4b) as well as in cultures without cystine (Figure 2b). The glucose concentration was in the range of 25 mM when μ started to decrease and none of the measured amino acids were consumed to any greater extent. Even at the end of the growth phase, sufficient amounts of the measured amino acids remained (Table 3). Adding more Yeastolate or lipids had also no effect on the specific growth rate pattern (data not shown). Thus, we have not found any indications that this growth behaviour could be correlated to the depletion of nutrients.

We therefore propose that the observed growth pattern is a result of (auto)regulatory events that control both proliferation and metabolism. Cysteine biosynthesis is only up-regulated in cells with a high growth rate and amino acid uptake in general is down-regulated even before growth ceases. The cessation of growth is a consequence of changes in cell physiology having occurred 80–90 hours earlier, at the time when μ starts to decline.

A similar growth pattern was earlier found in hybridoma cells cultivated in a serum-containing medium (Ljunggren and Häggström, 1995). Components in serum were identified as the growth rate limiting factors. By intermittent additions of serum or insulin, the culture could be kept in exponential growth (constant μ) until a critical nutrient was exhausted. However, the Sf9 cell cultures described here were carried out in a serum-free medium without any added growth factors. Hence, Sf9 cells are either able to proliferate without external growth factors or they produce their own, autocrine growth factors. Our preliminary experiments indicate the latter being the case (Doverskog *et al.*, 1997).

Concluding remarks

Sf9 cells are clearly able to proliferate normally in cystine-free culture media and in media lacking glutamine, glutamate and aspartate (Öhman *et al.*, 1996). However, our data also shown that cysteine biosynthesis is down-regulated in slowly growing cells. Such mechanisms must be considered in determining optimal conditions for production of recombinant proteins by baculovirus infection. As virus infection causes dramatic changes in cell physiology, a medium developed for optimal (and cheap) cell growth does not necessarily fulfil the requirements for high productivity during the production phase.

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