Cytotechnology **36:** 71–83, 2001.

Increased productivity of recombinant tissular plasminogen activator (t-PA) by butyrate and shift of temperature: a cell cycle phases analysis

V. Hendrick[1], P. Winnepenninckx[1], C. Abdelkafi[1], O. Vandeputte[1], M. Cherlet[1], T. Marique[1], G. Renemann[2], A. Loa[2], G. Kretzmer[2] & J. Werenne[1]*

[1] *Laboratory of Animal Cell Biotechnology CP160/17, Faculty of Sciences, Université Libre de Bruxelles, 50 Av. F.D. Roosevelt, 1050 Brussels, Belgium;* [2] *Institut für Technische Chemie, University of Hannover, Germany*

(Author for correspondence; E-mail: vincianne.hendrick@compaqnet.be, biocelan@ulb.ac.be; Fax: +32-2-650.32.30)*

Received 4 January 2001; accepted 1 August 2001

Key words: cell cycle, CHO cells, Kromenaker and Srienc, equations, Na-butyrate, Temperature shift, t-PA production, t-PA glycosylation

Abstract

Directed control of cell metabolism by a modification of the physicochemical conditions (presence of Na-butyrate and modification of the temperature) was used to modulate the productivity of human recombinant tissular plasminogen activator (t-PA) expressed under control of SV40 promoter in Chinese Hamster Ovary (CHO) cell lines. We showed that both by adding Na-butyrate or lowering temperature from 37 °C to 32 °C there is an increase in the amount of t-PA excreted, while cell growth is significantly reduced. The treatments also increased the intracellular amount of t-PA.

We measured the distribution of cell cycle phases by cytometry and used a modification of the equations of Kromenaker and Srienc (1991, 1994 a, b) to analyse the intracellular t-PA production rate in the different cell cycle phases. Intracellular t-PA was shown to accumulate in G1 phase in all conditions (at 37 °C, at 32 °C and in presence of butyrate). Moreover, we have shown that the distribution of the time cells treated by butyrate are maintained in the G1 cell cycle phase is significantly increased.

t-PA produced in the different cell culture conditions tested was analysed by zymogram and western blotting: neither butyrate, neither the shift of temperature changed significantly the overall quality of the protein. The N-glycan patterns of recombinant human t-PA was also analysed with carbohydrate-specific lectins. Butyrate caused a transitory increase in N-linked complex high-mannose oligosaccharides without any effect on the sialic acid content of t-PA.

Introduction

Mammalian cell culture is becoming increasingly important for the production of human recombinant proteins especially in the pharmaceutical field. The growing demand for these products prompted the development of mammalian cell culture to large scale and long term operation.

The production of therapeutic proteins in mammalian cells has advantages over the use of bacteria or yeast. Post-translational processing such as glycosylation and proteolytic cleavage of human proteins may indeed be important for their biomedical activity. These important modifications could be achieved properly only in higher eukaryotic systems. The amino-acids sequence of t-PA contains four potential N-glycosylation sites, three of them only being usually glycosylated (Asn^{117}, Asn^{448}, Asn^{184}) (Parekh et al., 1989). The N-linked complex oligosaccharides include fucose, galactose, N-acetylglucosamine and N-acetylneuraminic acid linked α(2–6) or α(2–3) to galactose while N-linked high-mannose oligosaccharides include only terminal mannose α(1–3), α(1–6) or α(1–2) linked to mannose. The natural t-PA produced

in almost all human cells predominantly contains mannose oligosaccharide structure at the 117 site, the 184 and 448 sites have a complex type oligosaccharide structure which presents differences in antennarity and sialylation according the conditions of cell culture. In contrast to the native t-PA which contains a NeuAc α(2–6) Gal link, the recombinant t-PA synthesised from mammalian cells in culture has a NeuAc α(2–3) Gal link. This difference has a relatively low importance for activity according many authors (Spellman et al., 1989; Parekh et al., 1989a, b) but glycosylation is specific to cells, tissues, cell differentiation state, and it is therefore difficult to establish a general theoretical model for t-PA glycosylation.

The yield of protein production in eukaryotic cell systems is however lower than in prokaryotic systems. Therefore adequate optimisation of the process is essential.

Any improvement of cell production may be of importance toward this goal. Stimulation of specific protein expression was also looked for. Number of stimulating agents are known for their potency to enhance protein productivity. It was shown that Na-butyrate increases the specific production in CHO cells of Factor VIII and of number of other heterologous secreted proteins (Dorner at al., 1987; Ganne et al., 1991; Gebert and Gray, 1991) including t-PA either in transfected CHO (Palermo et al., 1991) or in cultured human endothelial cells (Kooistra et al., 1987). It was shown that hybridoma cells are more productive during the G1 phase of the cell cycle (Kromenaker and Srienc, 1991, 1994a, 1994b).

It was therefore of interest to investigate if Na-butyrate is stimulating t-PA through a specific modulation of the cell cycle. As temperature is also an important factor influencing cells (Jenkins and Hovey, 1993; Kretzmer et al., 1998) and prolonging G1 cell cycle phase (Hendrick et al., 1999), the effect of temperature shift down on t-PA production was therefore further investigated in parallel to butyrate treatment. The direct comparison undertaken in the present paper on the same system of the effects of Na-butyrate and of the temperature shift permitted us to ascribe a general effect of the cell cycle on the production of a given recombinant protein.

A sequence of correlations was followed in the present study to develop a mathematical model to discriminate the individual cell cycle phases. In bacteria, protein production is correlated to the cell size and a specific equation was established by Collins and Richmond (1962) to characterise this property. This equation has been adapted by Kirkwood and Burdett (1986, 1988) to calculate the growth rate of individual cells of bacteria. Kromenaker and Srienc (1991, 1994a, 1994b) however, by isolating cells according their size demonstrated that there is no clear correlation between the cell size and the cell cycle position. Such a conclusion is however controversial at the moment.

On the basis of centrifugal elutriation to produce cell cycle enriched fractions, Lloyd et al. (2001) established a correlation of productivity and cell size.

The use of a mathematical approach as described here to analyse cytofluorimetry data avoids to select cells in different phases of the cell cycle, by using chemicals or elutriation, which causes important perturbations to the balanced cellular properties.

This paper demonstrates that Na-butyrate and temperature shift increase recombinant t-PA productivity in CHO cells in suspension culture with serum free medium. It showed also that there is a clear relation between the intracellular accumulation of recombinant t-PA in CHO and time cells are maintained in G0G1 cell cycle in the different conditions tested. t-PA productivity may therefore be improved by controlling the cell cycle.

Materials and methods

Cell lines and culture conditions

The t-PA-producing cell line (CHO Tf70R) was obtained from Pharmacia Upjohn (kind gift of T. Björing). All cells were usually grown at 37 °C in a 5% CO_2 and 95% humidity atmosphere.

The recombinant cells were grown in suspension in a serum free medium (Biopro-1: BioWhittaker) supplemented with 2 mM glutamine and 20 mM glucose either in 80 cm^2 T.C. Flasks for preliminary studies and in 250 ml or 500 ml Techne spinners (agitation: 50 rpm). The cell concentration determination was carried out manually with a haemacytometer and viability by trypan blue dye exclusion. The cells were inoculated at 37 °C at 1.5×10^5 cells ml^{-1} either treated 72 hours later with 1mM Na-butyrate or shifted from 37 °C to 32 °C.

Protein concentration determination

Protein concentration was determined by dye binding using the Bradford method with bovine serum albumin (BSA) as the standard (Bio-Rad).

t-PA concentration

A sandwich ELISA test (Immulyse t-PA kit by Diagnostic International, Germany) was used for the quantitative determination of single-chain and two-chain t-PA antigen in cell culture supernatants.

The determination of the intracellular t-PA amount was done after a complete lysis (five minutes treatment : 'Mammalian Protein Extraction buffer' supplied by Pierce). After centrifugation of pellets, supernatants were tested for the intracellular t-PA concentration with the ELISA kit as above.

t-PA activity

The enzymatic activity of t-PA was measured by a colorimetric method using the synthetic peptide-p-nitroanalide (Chromozym t-PA by Boehringer Mannheim).

The intracellular activity determination of the t-PA was done after a complete lysis obtained as above. After centrifugation of pellets, supernatants were tested for the intracellular activity of t-PA with the same chromogenic test.

Cell cycle analysis

The distribution of cells in the different phases of the cell cycle (G0G1, S, and G2M) was analysed by cytofluorimetry (Excalibur, Becton Dickinson). The cells were fixed in 75% PBS/25 % methanol (v/v) and then maintained at –20 °C until analysis.

Cells were centrifuged and the pellet was homogeneized in 2ml of phosphate-citrate buffer (0.2M Na_2HPO_4, 0.1M monohydrated citrate). After incubation during 30 min, 1 ml of RNase (3 mg ml^{-1}) was added to the solution. After incubation at 37 °C during 20 min, pellet was resuspended in propidium iodide (PI) (50 μg/ml).

Distinction between cells in G2 or M phase

The classical cell cycle analysis methods can not discriminate cells in G2 phase or in M phase; indeed they both contain the same DNA amount. However to apply the Kromenaker and Srienc equation (1991), it is necessary to separate the cells into two clear distinct G2 and M phases. The following procedure was therefore required to differentiate cells in G2 and M phases:

Briefly, cells were fixed in PBS-methanol and formaldehyde. RNase and HCl-Triton X-100, respectively destroying RNA and histone of DNA, were added to the solution. Cells were boiled to separate the two strands of DNA and kept after on ice to prevent renaturation of DNA. An anti-t-PA FITC antibody (labelling was operated using the "fluoresceine Labelling kit" from Boehringer Mannheim following instructions described by the firm) and PI was added to the samples before the cytometric analysis.

Flow cytometric measurements were made with a Facscalibur Becton Dickinson cytometer. The green fluorescence represented the amount of FITC bound to the t-PA protein and the red fluorescence represented the amount of PI bound to the DNA.

Determination of time passed in the different cell cycle phases

Slater et al. (1997) have defined three equations to characterise the time passed in the different cell cycle phases. The proportions of the cells in each phase of the cell cycle were obtained through the CellQuest program (Becton Dickinson). The equations involved are the following:

$$T_{G1} = Td/\ln(2).\ln[1 - f_{G1}/2]$$

$$T_S = Td/\ln(2).\ln[1 + f_S/(1 + f_{G2M})]$$

$$T_{G2M} = Td/\ln(2).\ln[1 + f_{G2M}]$$

where:

Td:	Doubling time (h)
T_{G1}:	Time passed in G1 phase (h)
T_S:	Time passed in S phase (h)
T_{G2M}:	Time passed in G_2M phase (h)
f_{G1}:	Fraction of the population in G1 phase (%)
f_S:	Fraction of the population in S phase (%)
f_{G2M}:	Fraction of the population in G_2M phase (%)

Conversion from cell percentages to t-PA production rates in the different phases of the cell cycle

The mathematical basis of this analysis were, as reported above, first developed by Kirkwood and Burdett (1986, 1988) for bacterial growth, and later extended to animal cell growth by Kromenaker and Srienc (1991, 1994). Briefly, they consider a population of hybridoma cells in exponential growth with a specific growth rate k. This population is maintained in a steady state so that the dynamics of protein products can be established between the different cell cycle phases. In other words, we convert measures of cell distribution and t-PA concentration in each phase of

the cell cycle into accumulation rates of t-PA in the same phase as a function of cell distribution. This lead to the following equation for G0G1, G2M and S phases respectively:

$$V^{G1}(p) = \frac{k}{\lambda^{G1}(p).G1}$$
$$\left[G_1.\int_p^\infty \lambda^{G1}(x).dx - 2.N.\int_p^\infty \Psi(x).dx + (N + G2 + S).\int_p^\infty \theta(x).dx\right]$$

$$V^{S}(p) = \frac{k}{\lambda^{S}(p).S}$$
$$\left[S.\int_p^\infty \lambda^{S}(x).dx + (G2 + N)\int_p^\infty \chi(x).dx - (N + G2 + S).\int_p^\infty \theta(x).dx\right]$$

$$V^{G2}(p) = \frac{k}{\lambda^{G2}(p).G2}$$
$$\left[G_2.\int_p^\infty \lambda^{G2}(x).dx + N.\int_p^\infty \varphi(x).dx + (N + G2 + S).\int_p^\infty \chi(x).dx\right]$$

where:

N :	Cell number at any time
K :	Specific growth rate
G_1 :	Cell number in G_1 phase
S :	Cell number in S phase
G_2 :	Cell number in G_2M phase
$\chi(p)$:	Frequency distribution values of intracellular amount for cells in S/G2M transition phase
$\lambda^{G1}(p)$:	Frequency distribution values of intracellular amount for cells in G1 phase
$\lambda^{S}(p)$:	Frequency distribution values of intracellular amount for cells in S phase
$\lambda^{G2}(p)$:	Frequency distribution values of intracellular amount for cells in G_2M phase
$\varphi(p)$:	Frequency distribution values of intracellular amount for mitotic cells
$\Psi(p)$:	Frequency distribution values of intracellular amount for post-mitotic cells
$\theta(p)$:	Frequency distribution values of intracellular amount for cells in G1/S transition phase

Electrophoresis analysis

t-PA protein is detected by two methods of SDS-PAGE electrophoresis analysis: a classical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% Acrylamide-bis-acrylamide gel under reducing conditions using the Laemmliþs method (1970) and a modified substrate gel method used to detect proteolytic activity (Heussen et al., 1980; Blankaert at al., 1998). This zymographic analysis differs from SDS-Laemmli gels in two aspects:

– the gel were made by incorporating the protein substrates (plasminogen and gelatin) of interest within the polymerised acrylamide matrix.
– the gel were then incubated in order to activate the action of the enzyme on the substrates.

The first substrate (plasminogen) reacts with the t-PA protein and forms plasmin which then degrades gelatin. We used in this case 15 % acrylamide gel to increase the resolution of the electrophoresis. After migration, proteins were stained with Coomassie blue and clear bands appeared on the gel where gelatin was degraded by the activated enzyme on a blue background. These degraded bands represent active t-PA chains.

t-PA Western blotting

After SDS-PAGE (12%acrylamide-bis-acrylamide), the gel content was transferred onto western blotting nitrocellulose membranes. After the transfer step, membrane was washed with PBS and saturated with BSA-PBS (0.1% W/V) for 1 hour. Later, 5μg ml^{-1} t-PA IgG antibody conjugated with biotin (The Binding Site, Birmingham, UK) was added to the membrane for 2 hours. The membrane was then washed twice with PBS-Tween 20 (0.1% V/V) and the membrane was incubated for 1 hour with 50 ng ml^{-1} streptavidin-peroxydase. The Staining was performed by the HRP conjugate Substrate kit from Biorad.

Detection of glycan structures with digoxigenin-labeled lectins

After SDS-PAGE and electrotransfer of proteins onto nitrocellulose membranes, glycoproteins were incubated with digoxigenin-labeled lectins followed by detection of glycoprotein-bound lectins with alkaline phosphatase-conjugated anti-digoxigenin immunoglobulin (Boehringer Mannheim). Fetuin and asialofetuin (Boehringer Mannheim) were used as

control glycoproteins to certify the reactivity of the lectins.

Nothern blotting

The plasmid t-PA probe from E. Coli bacteria used (pKGE-45), was received from Pharmacia Upjohn (Dr T. Björing). A single colony should be inoculated into 2-10 ml of LB-medium ('Luria Bertani medium') containing 10 μg ml^{-1} Ampicillin as selective agent (and grown for 8 hours : logarithmic phase). First, DNA was purified from bacteria with a Quiagen kit. After harvesting and resuspension, the bacterial cells were lysed in NaOH-SDS in presence of RNase A. The cleared lysate was loaded onto a pre-equilibrated column (anion-exchange resin) by gravity flow. The column was then washed with medium-salt buffer which removed traces of RNA and protein, without affecting the binding of the plasmid DNA.

After purification, 30 μg of pKGE-45 DNA was digested by a ECO-RI restriction enzyme for 2 hours to obtain a fragment of DNA (+/- 400 pb) and analysed by an Agarose gel electrophoresis (in 1.2% TEA: 40 mM Tris-Acetate, 1 mM EDTA; pH 8). The DNA band was then extracted from the agarose gel. A radioactive probe (dCTP P^{32}) was prepared with the Rediprime II kit from Amersham. The RNA extracted from CHO cells using TRIZol from Life Technologies-Gibco was hybridized with the radioactive probe.

The nitrocellulose membrane was incubated for 2 hours with a pre-hybridisation solution (50% formamide, 6X SSC, 100μg ml^{-1} de DNA salmon, 5X Denhaert's) to saturate the non-specific binding sites and the radioactive probe was incubated overnight in this solution at 65°C. After hybridising, the nitrocellulose was washed (twice for 20 min. with a 2X SSC buffer and again with a 0.2X SSC buffer) before autoradiographic processing and scanned for quantitative evaluation of mRNA amounts.

Results

Effect of Na-butyrate

Effect of Na-butyrate on cell growth and protein production

It is known that butyrate have some effects on cell growth and productivity. Although its precise mechanism of action is unknown, it is clear that butyrate changes chromatin structure as a result of hyperacetylation of histones, and that these changes can

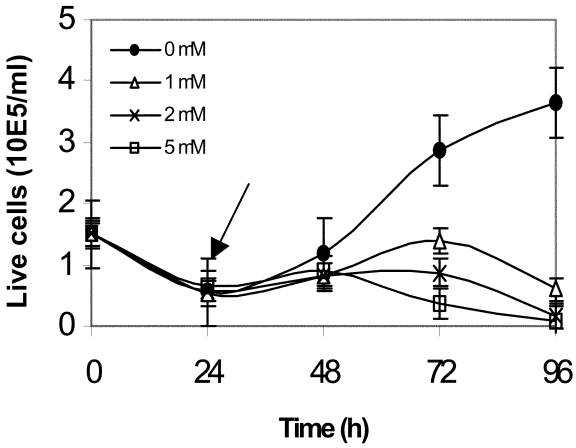

Figure 1. Cell growth kinetics for CHO-Tf70 in function of time in T.C. flasks. Arrow represented the addition of butyrate at 24 hours (● Control; Δ 1 mM; × 2mM; □ 5 mM butyrate).

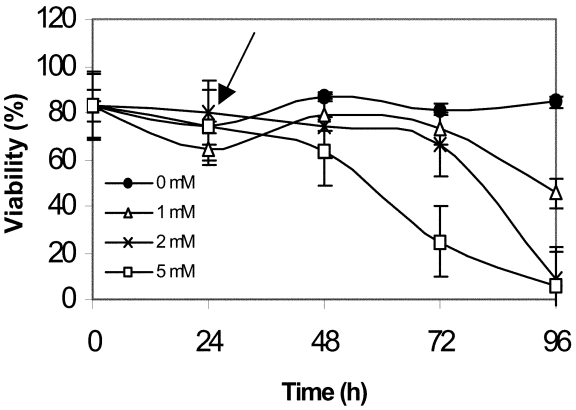

Figure 2. Viability for CHO-Tf70 in function of time in T.C. flasks. Arrow represented the addition of butyrate at 24 hours (● Control; Δ 1 mM; × 2mM; □ 5 mM butyrate).

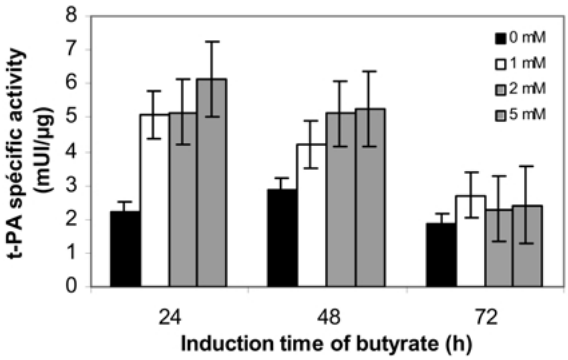

Figure 3. t-PA specific activity (mUI/μg) for CHO-Tf70 in function of time after addition of butyrate in T.C. flasks (Control, 1 mM, 2mM, 5 mM butyrate respectively as indicated).

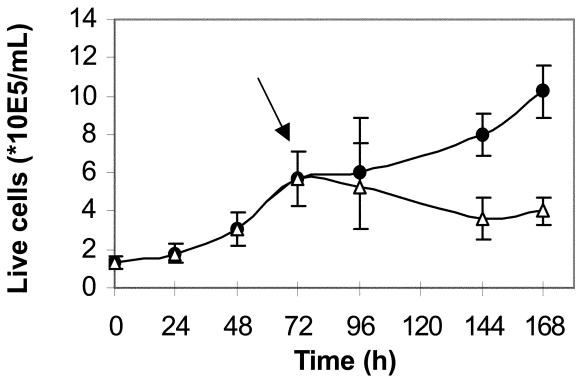

Figure 4. Cell growth kinetics (seeding at 1.5×10^5 cells in 200 ml medium) for CHO-Tf70 in function of time in mini-bioreactor 'spinner' with addition of 1 mM butyrate at 72 hours (Arrow) after splitting the culture in two separate cultures of 100 ml in 250 ml stirrers (• Control and Δ 1 mM butyrate).

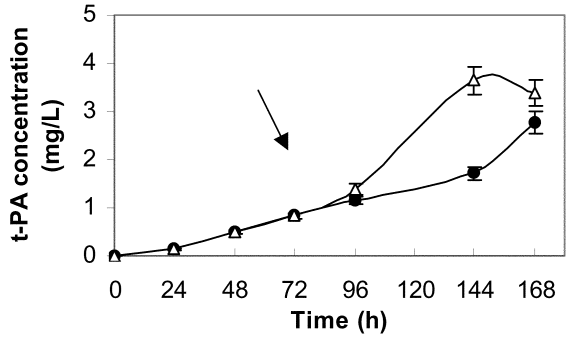

Figure 5. t-PA concentration for CHO-Tf70 in function of time in mini-bioreactor 'spinner' with addition of 1mM butyrate at 72 hours (Arrow) after splitting the culture in two cultures of 100 ml in 250 ml stirrers (Same conditions as in Figure 4) (• Control and Δ 1 mM butyrate).

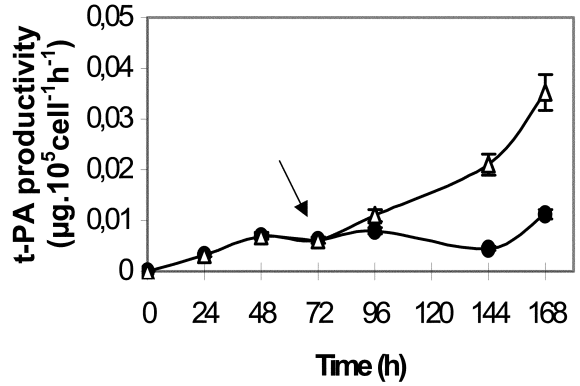

Figure 6. t-PA productivity for CHO-Tf70 in function of time in mini-bioreactor 'spinner' with addition of 1 mM butyrate at 72 hours (Arrow) (Same conditions as in Figure 4) (• Control and Δ 1 mM butyrate).

be correlated with a modulation of gene expression (Kruh, 1982). Palermo et al. (1991) have tested 5 mM of butyrate on different CHOt-PA clones and they have shown that at this concentration, the activity per cell is increased as is the specific activity of the protein produced. As for our own experiments the overall conditions and in particular the cell culture media and the transgenic construction were not the same, it was necessary to test a panel of concentrations to confirm the sensitivity of our CHO cells to butyrate. After 24 hours of cell culture, the effect on the growth of CHO cells of different butyrate concentrations were therefore tested first in T.C. flasks. We observed that for the control by seeding 1.5×10^5 cells ml^{-1}, a cell density of 3.5×10^5 cell ml^{-1} was reached after 96 hours of cell culture while the cell density decreased below 1×10^5 cell ml^{-1} for the different concentrations of butyrate tested at this time (Figure 1). Cell viability decreased also somewhat when the butyrate concentration increased and at 5mM, clear cytotoxic effects were readily observed (Figure 2). Moreover, the t-PA specific activity (UI activity/mg protein) increased for all butyrate concentrations used (Figure 3).

The increase is 3-fold in 5 mM butyrate compared to the control after 48 hours of cell culture. For the control culture, t-PA specific activity remained more or less at the same level, indicating that the quality of the protein was maintained during the culture.

However, for the butyrate treatment, the t-PA specific activity increased 24h and 48h of cell culture after the addition of the agent and then decreased at 72 hours to reached the same value than the control (Figure 3). It appeared that butyrate treatment exerted an important but transitory effect on the specific activity of t-PA produced. It should however be pointed out that butyrate concentration in the culture medium decreased during incubation indicating some metabolism by hybridoma cells (Cherlet et al., 2000).

To limit deleterious perturbations of the CHO cells by butyrate, the concentration of 1 mM butyrate was first selected for further experiments in spinner flasks. In a second step, bi-phasic growth conditions were applied: first the cells were grown without treatment and in its mid-exponential phase of growth, 1mM butyrate was added to the cell culture.

The viable cell density increased from an initial value of $1.5 \times 10^5 ml^{-1}$ to a maximum of 1×10^6 ml^{-1} after 168h for the control culture and to 4×10^5 ml^{-1} with Na-butyrate (Figure 4).

t-PA production in CHO cells was measured and the productivity (μg cell^{-1} hour^{-1}) was calculated

in function of time. t-PA concentration (Elisa essay) increased to a maximum value of 3.65 mg l^{-1} with 1mM butyrate and 1.72 mg ml^{-1} for the control. Butyrate reduced significantly cell growth (Figure 4) but increased t-PA concentration, up to two folds after 144 hours of cell culture (Figure 5) while cell growth is significantly slowed down. t-PA productivity is increased by butyrate as shown in Figure 6. The maximal productivity for the control is 0.011 μg $cell^{-1}$ h^{-1} and 0.035 μg $cell^{-1}h^{-1}$ for the treatment with Na-butyrate. t-PA productivity of CHO cells is increased about 3 folds by butyrate 1mM.

These results indicated that both t-PA concentration and t-PA productivity increased after butyrate addition in CHO cell culture. The decrease of t-PA titer at the end of cell culture (Figure 5) in presence of butyrate might be related to the cytotoxic effect of butyrate, cell lysis probably increasing t-PA degradation.

Those results indicate that Na-butyrate increased recombinant t-PA production in CHO cells ; the amount, the activity and the specific activity of t-PA are positively affected by the treatment.

Effect of Na-butyrate on cell cycle

In order to further discriminate cell growth from t-PA production we have studied the distribution of CHO cells in the cell cycle both in absence and in presence of butyrate. We observed a 10% increase in the proportion of cells in G0G1 after 1mM butyrate addition during cell culture. This increase is clearly marked 24 hours after addition of butyrate (direct data from cytometry not shown). The small difference observed between the control and the butyrate cultures may be explained by the fact that control cells maintained an active cycling throughout all the experimentation while in presence of butyrate part of the cells stopped their division process and accumulated into the G0G1 cell cycle phase, some of the cells undergoing a cell death process. Butyrate exerted most of its transitory effect on the cell cycle phase after 24 hours of addition. Butyrate disappeared during the cell culture as a consequence of cell metabolism (Cherlet et al., 2000).

In order to further characterise this situation, we have calculated here, from these raw cytofluorimetry data, the time during which cells are maintained in each cycle phases, according to the relation of Slater et al. (1997) as indicated in materials and methods.

An increase in the proportion of the time in which cells remained in the G1 phase after 24 hours butyrate addition (54%) compared to the control (43%) could be derived (Figure 7).

The intracellular t-PA content being an interesting parameter to evaluate the cell behaviour after butyrate addition, a detailed analysis was provided by the observation of the evolution of the intracellular t-PA quantity during cell cycling as adapted from the relations derived by Kirkwood and Burdett (1988) and Kromenaker and Srienc (1991, 1994 a and b) (as described in 'materials and methods'). Flow cytometric measurements, provided data on intracellular t-PA distribution in different cell cycle phases (G0G1, S and G2M).

Cells in G2 and M phases can be isolated by the treatment described in 'materials and methods' and analysed individually by cytometry : the membrane of mammalian cells nucleus was dissolved during mitosis (M phase) and the DNA of mitotic cells are more readily denatured than the DNA of the cells in the G2 phase. Acid and heat treatments of the sample provided modification of the right angle light scattering. The two different cell populations may be clearly distinguished. Moreover, as mitotic cells have more DNA in single stranded form and as propidium iodide intercalating only in double stranded DNA, the distinction is even more easy due to this effect. The treatment induced thus a lower red fluorescence with mitotic cells than with G2 cells even with the same DNA content.

After cell cycle analysis and separation by appropriate gate selection, one can obtain the cell populations in the different cell cycle phases (G0G1, G2, M and S) as well as the t-PA intracellular amounts in those cell cycle phases. These data were introduced in the mathematical model, with which one can calculate the different rates of t-PA accumulation in the different cell cycle phases.

Very different patterns for t-PA production rates in the different cell cycle phases were derived from the model (Figure 8).

The interpretation of the curve corresponding to the distribution in G1 cell cycle phase, indicated that cells with low concentration of t-PA accumulated strongly the recombinant protein.

This accumulation gradually dropped while t-PA concentration reached high level. During the S phase a similar trend was followed, t-PA accumulation dropped until reaching a very low value, ending to zero level. In G2M on the contrary, we observed a negative rate of accumulation which resulted from the fact that rates of secretion and degradation exceed the rate of synthesis. This result indicated that the overall beha-

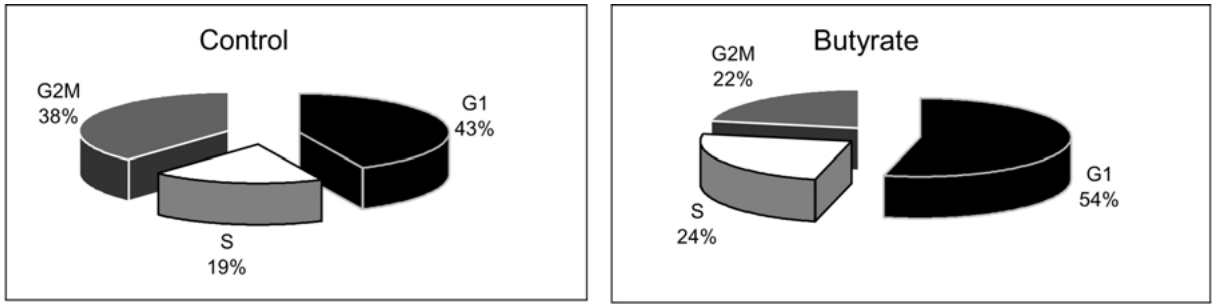

Figure 7. Proportion of the time cells are maintained in different cell cycle phases (G0G1, S and G2M) function of time in mini-bioreactor 'spinner': Control (left)- Addition of 1 mM butyrate at 72 hours (right).

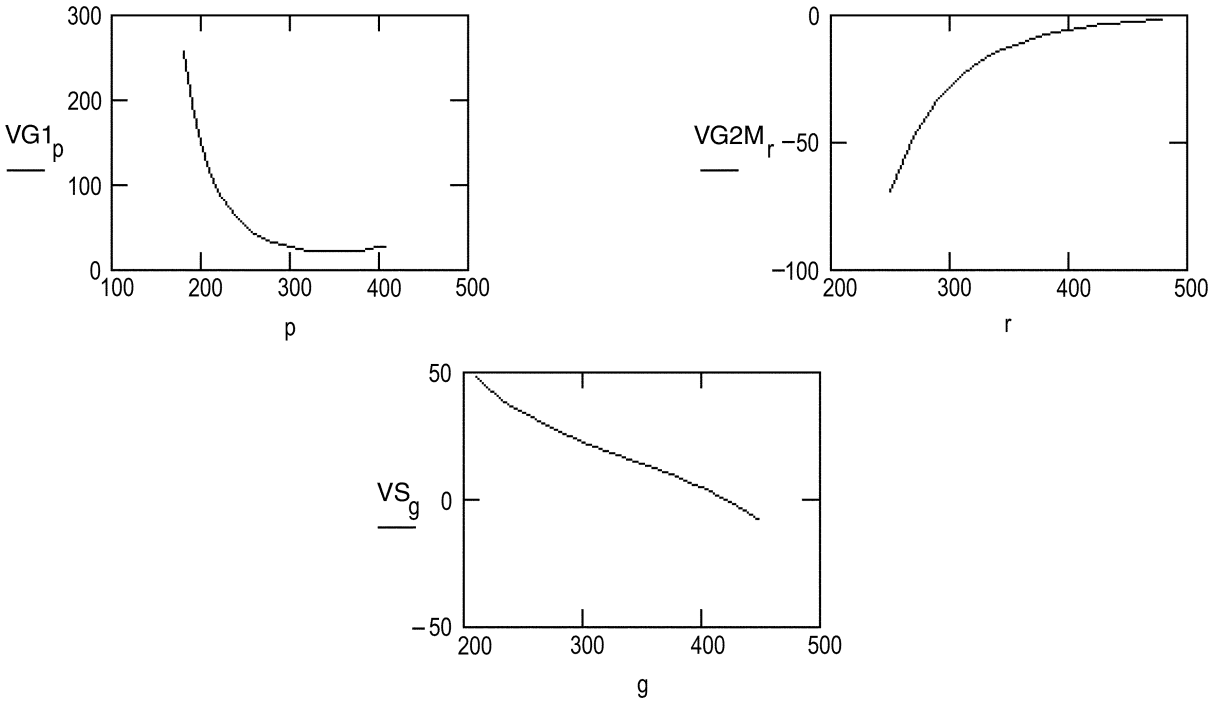

Figure 8. The intracellular t-PA rate value in function of the different cell cycle phases (G0G1, S and G2M) obtained by Kromenaker and Srienc equations described as in material and methods.

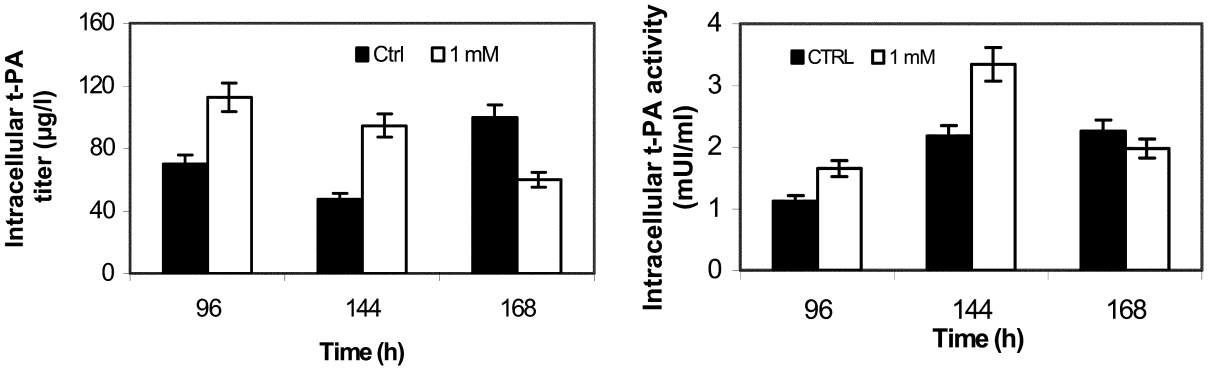

Figure 9. Intracellular t-PA in Tf70R cells in function of time in mini-bioreactor 'spinner' for control and with addition of 1mM butyrate: intracellular t-PA concentration (left) and intracellular t-PA activity (right) (Same conditions as in Figure 4).

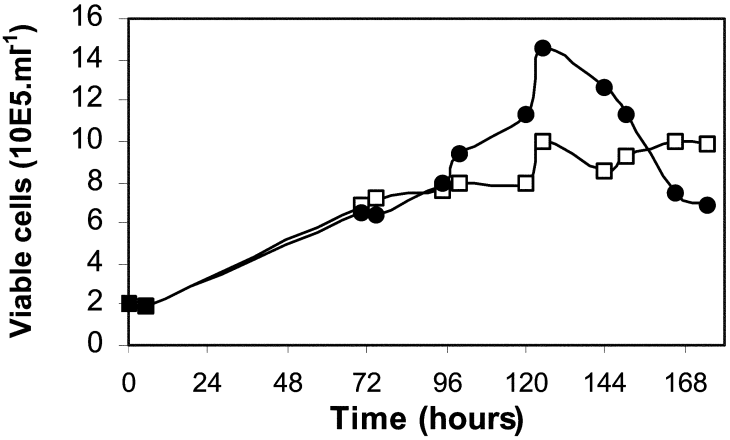

Figure 10. Cell growth kinetics for CHO-Tf70 in function of time in spinner flasks (• Control and □ Temperature shift at 32°C at 72 hours).

viour may be directed mainly by secretion properties of t-PA.

In another experiment, we measured directly the intracellular concentration and activity of t-PA in CHO cells after addition of 1 mM butyrate. We observed that both intracellular t-PA activity and concentration were higher in presence of butyrate except at the end of the culture (Figure 9).

The overall transitory increase of t-PA production by butyrate treatment observed in all experiments reported above, indicated that the transcriptional effect of the agent showed by others previously (Ganne et al., 1991) should be well terminated before the end of the culture. Indeed a preliminary analysis of the t-PA mRNA by Nothern blot showed that 72 hours after addition of butyrate only 51% of the specific message remained compared to the control. However, as some cytotoxic effect of butyrate is observed even at the low concentration used, a more careful kinetic study of the t-PA mRNA should be undertaken in order to fully discriminate the relative importance of transcriptional and post-transcriptional effects of butyrate in t-PA expression.

Effect of temperature

Effect of temperature on growth kinetics

We have studied in another set of experiments operated in 'stirrers', the effect of a temperature shift from 37 °C to either 32 °C or 29 °C. As viability is too poor at 29 °C we selected a shift of temperature from 37 °C to 32 °C for further experiments reported here.

A bi-phasic growth procedure was undertaken as in the experiment with butyrate addition: first, cells were grown for 72 hours at 37 °C and then a temperature shift to 32 °C was operated. For the experiment with temperature down shift, the density of the live cell increased to a maximum of 1×10^6 cells ml^{-1} while in the control it reached a value of 1.5×10^6 ml^{-1} after 120 hours of cell culture (Figure 10). As expected substrate use and general cell metabolism appeared to be slowed down at 32 °C (data not shown). But, this slower growth at 32 °C allowed a prolonged active life time of the culture during which a higher amount of t-PA was produced as described below.

Effect of temperature on t-PA produced

t-PA production in CHO cells was followed at 37 °C and 32 °C in function of time. t-PA concentration produced increased with cell density to a final value of about 7.5 μg ml^{-1} for the control and about 13 μg ml^{-1} for the temperature shift from 37 °C to 32 °C. A clear increase in t-PA concentration after the shift of temperature operated at 72 hour was observed (Figure 11) while the cell concentration decreased at the same time compared to the control.

The temperature down shift caused a significant but transitory increase of the amount of intracellular t-PA (observed 24 hours after temperature shift and not after 48 hours) while no difference in intracellular t-PA activity could be detected 24 hours after temperature shift.

The increase in intracellular activity is however well observed 48 hours after the temperature shift (Figure 12). A maturation process could therefore be involved.

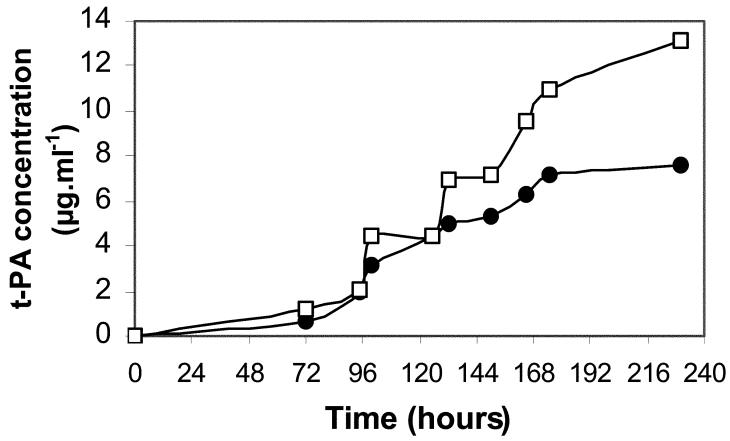

Figure 11. t-PA concentration for CHO-Tf70R in function of time in mini-bioreactor 'spinner' with shift of temperature to 32 °C at 72 hours after splitting the culture in two separate cultures of 100 ml in 250 ml stirrers. (• Control and □ Temperature shift at 32 °C).

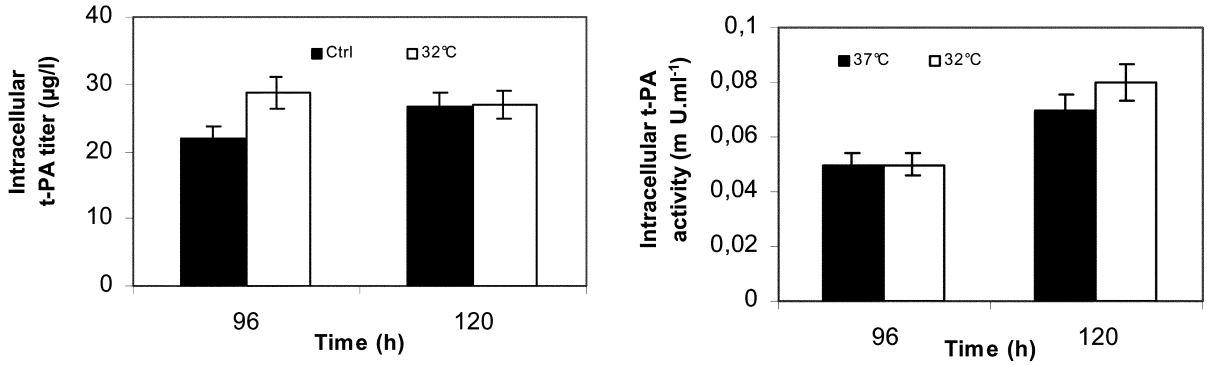

Figure 12. Intracellular t-PA for CHOTf70R in function of time in mini-bioreactor 'spinner' for control and with shift of temperature to 32 °C at 72 hours: intracellular t-PA concentration (left) and intracellular t-PA activity (right).

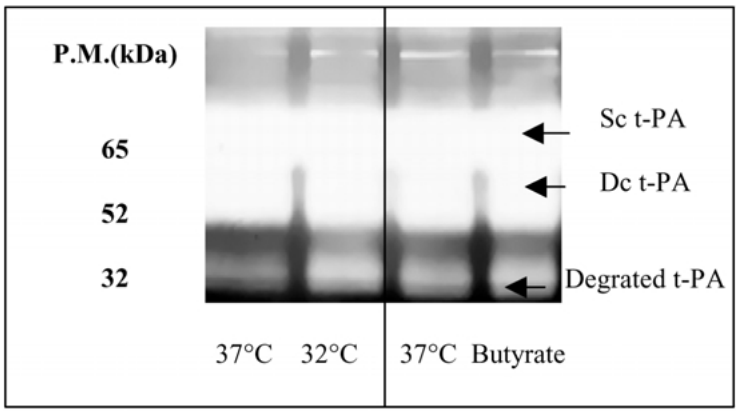

Figure 13. Zymographic analysis of t-PA produced: Temperature shift experiment (32 °C) compared to the control (37 °C) (left) and Addition of 1mM butyrate compared to the control (37 °C) (right).

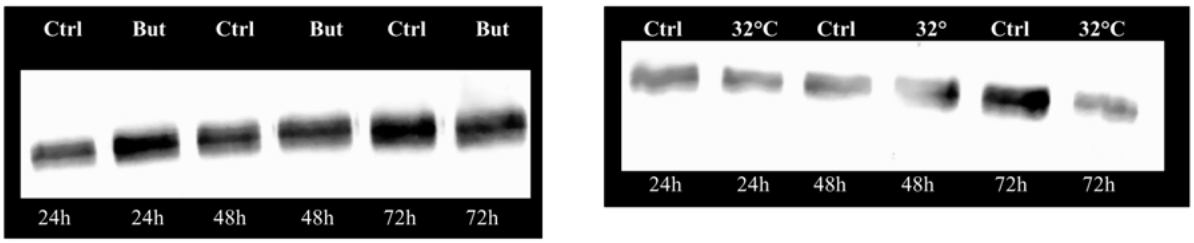

Figure 14. Western blotting with Antibody FITC-t-PA. Experiment with addition of 1mM butyrate (left) and temperature shift at 32 °C at 72 hours of cell culture (right). The same amount of 10 μg protein was loaded in each well.

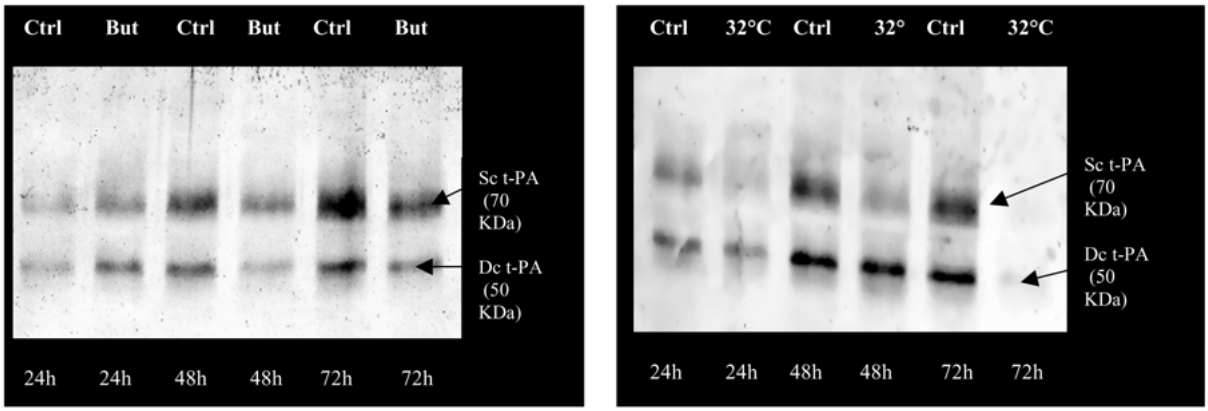

Figure 15. Western blot with GNA lectin specifically recognising mannose core structures. Experiment with 1mM butyrate (left) and temperature shift (32 °C) at 72 hours of cell culture (right). The same amount of 10 μg protein was loaded in each well.

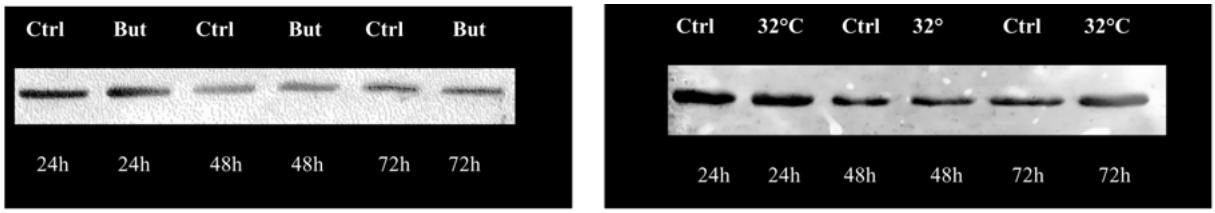

Figure 16. Western blot with SNA lectin specifically recognising terminal sialic acid. Experiment with 1mM butyrate (left) and temperature shift (32 °C) at 72 hours of cell culture (right). The same amount of 10 μg protein was loaded in each well.

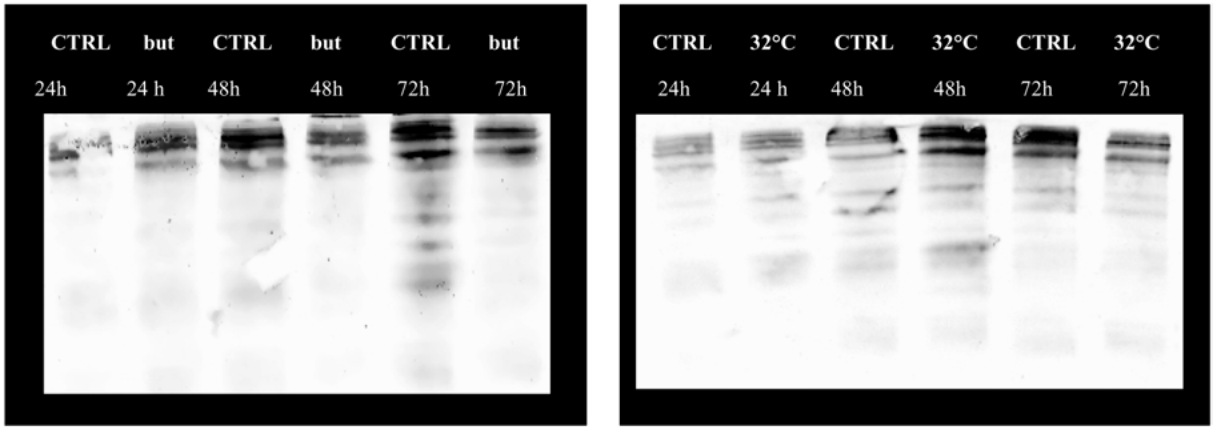

Figure 17. Western blot with MAA lectin specifically. Experiment with 1mM butyrate (left) and temperature shift (32 °C) at 72 hours of cell culture (right). The same amount of 10 μg protein was loaded in each well.

Effects of physico-chemical conditions on the quality of t-Pa

The different patterns of active t-PA

Activity of the different excreted t-PA molecules produced in the different conditions as shown by zymograms indicated that a good protein quality is produced at 37 °C and in presence of 1 mM butyrate (Figure 13). In all conditions single chain t-PA (Sc t-PA) and two chains t-PA (Dc t-PA) were observed. An extra band of 32 kDa appears at the end of the process indicating partial degradation of t-PA, the degraded protein maintaining however part of its biological activity.

Both the presence of butyrate or shift of temperature to 32 °C caused an accumulation of the activity of this lower molecular weight (M.W.) partially degraded t-PA. The high M.W. activity shown in the zymograms corresponded to t-PA bound to an inhibitor. Part of the activity of t-PA bound to the inhibitor is maintained in the zymographic conditions.

The western blot demonstrated that the antibody against t-PA recognized a main band of 55 kDa corresponding to t-PA double chain (Figure 14) in all conditions.

t-PA glycosylation

As zymograms demonstrated that both single chain and double chain t-PA are the main products observed after addition of 1mM butyrate or after shifting down of the temperature. Further characterization of the glycoprotein produced was undertaken using specific lectins.

The lectins conjugated with the steroid hapten digoxigenin enabling immunological detection of the specific binding to t-PA produced in the different experimental conditions were leading to the following general observations:

– The Galanthus nivalis agglutinin lectin (GNA) blot detection showed that butyrate (1mM) transiently increased the amount of the N-linked high-mannose oligosaccharides (24h after butyrate addition but not at 48h or at 72h where these high mannose structures are less abundant) (upper band : t-PA single chain with 70 kDa and lower band : double chain with 50 KDa). It was mainly the quantity of unfinished glycoproteins which was increased by butyrate treatment. The temperature shift caused also a significant modification of the amount of high mannose containing t-PA relative to the control, we observed a decrease of terminal mannose in t-PA single chain but not in t-PA double chain except at 72 hours after the temperature shift down at 32 °C (Figure 15). Butyrate and temperature shift down at 32 °C don't seem have the same effect on the terminal N-linked high-mannose oligosaccharide.

– In comparison during the same periods of culture there was, compared to the control, no modification by butyrate of the terminal sialic acid structures (N-Acetylneuraminic acid α(2–6) linked to galactose) produced (Sambucus nigra agglutinin lectin blot). The shift down of temperature had no significant effect either (Figure 16).

– The Maackia amurensis agglutinin (MAA) lectin blots showing the presence of a number of bands (N-Acetylneuraminic acid α(2–3) linked to galactose), with minimal positive signal at the M.W. of t-PA however, indicate a microheterogeneity of glycoproteins produced in these experimental conditions (also in presence of butyrate or after temperature shift) (Figure 17).

Conclusions and discussion

The preliminary experiments in T.C. flasks confirmed the numerous studies showing that butyrate treatment in the range of 1-5 mM, increased t-PA activity produced in CHO cells. In order to limit the negative effect resulting from cytotoxic effect of butyrate, a biphasic strategy to produce more efficiently t-PA using this additive was designed as following : first biomass was produced at 37 °C during the exponential growth of the cells, and then addition of butyrate was operated to enhance the t-PA production. A similar biphasic procedure involving a shift down of temperature could also be proposed : biomass accumulation at 37 °C and t-PA production at 32 °C.

We have studied then the relations between cell productivity and the cell position in the different cell cycle phase by cytometry and we have shown that the distribution of cells in G0G1 phase of the cycle is increased by both treatments : cells are maintained for a longer period in this phase of the cell cycle after butyrate addition or shift down of temperature. Using the mathematical model derived from the data published by Kromenaker and Srienc (1994a, b) and by labelling t-PA for cytofluorimetry, we demonstrated that it was in this GoG1 phase that cells produced more t-PA. Thus, the increase in t-PA production by butyrate induction is directly related to the fact that cells are maintained in the G1 phase longer and that this phase is the t-PA accumulation phase for CHO Tf70R cells.

Electrophoretical analysis of the t-PA produced showed that neither butyrate addition at 1mM, neither the shift down of temperature caused any significant deleterious modification of the active structure of the protein. The N-glycosylation structures of recombinant t-PA were investigated by binding of specific lectins. The amino-acids sequence of t-PA has four potential N-glycosylation sites : Asn^{117}, Asn^{448}, Asn^{184} and Asn^{218} (Parekh et al., 1989). Lectin glycosylation analysis of t-PA indicated that we are dealing with a complex type carbohydrate structure containing terminal linked mannose units and sialic acid linked to galactose. Our data indicate that butyrate treatment caused a transitory accumulation of high mannose active precursor structures.

In conclusion, the direct comparison of the effect of butyrate addition and shifting down of the temperature, showed that despite minor differences in their action on t-PA produced, situations may be of interest to improve t-PA production process.

Acknowledgements

This work was supported by the European Union Biotechnology program Framework IV 'Cell Factory'.

References

Blankaert D, Simonart T, Van Vooren JP, Parent D, Liesnard C, Farber CM, Marique T & Wérenne J (1998) Quantitative release of metalloprotease -9(92 kDa type IV collagenase) by Kaposi's sarcoma cells. J AIDS Hum Retro 18: 203–209.

Cherlet M & Marc A (2000) Stimulation of monoclonal antibody production of hybridoma cells by butyrate: evaluation of a feeding strategy and characterization of cell behaviour. Cytotechnology 32 (1): 17–29.

Collins JF & Richmond MH (1962) Rate of growth of Bacillus cereus between divisions. J Gen Microbiol 28: 15–33.

Dorner AJ, Wasley LC & Kaufman RJ (1987) Increased synthesis of secreted proteins induces expression of glucose regulated proteins in butyrate-treated Chinese Hamster Ovary. J Biol Chem 264, 20.602–20.607.

Ganne V, Guerin P, Faure T & Mignot C (1191) Increased expression of factor VIII by butyrate in Chinese Hamster Ovary cells. In: Spier RE, Griffiths JB and Meignier B (eds) Products of Biologicals from Animal Cells in Culture. Oxford, pp. 104–106.

Gebert C & Gray PP (1991) A screening experiment of media supplements on CHO cells. In: Spier RE, Griffiths JB and Maignier B (eds) Products of Biologicals from Animal Cells in Culture, pp. 76–78.

Hendrick V, Vandeputte O, Raschella A, Marique T, Cherlet M, Abdelkafi C & Wérenne J (1999) Modulation of cell cycle for optimal recombinant protein production. In: Bernard A, Griffiths B, Noé W and Wurm F. (eds) Animal Cell Technology: Products from Cells, Cells as Products. Kluwer Academic Publishers, pp. 179–181.

Heusen C & Dowle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Analyt Biochem 102: 196–202.

Jenkins N & Hovey A (1993) Temperature control of growth and productivity in mutant Chinese Hamster Ovary cells synthesizing a recombinant protein. Biotechnol Bioengin 42: 1029–1036.

Kirkwood IDJ & Burdett TBL (1986) Growth kinetics of individual *Bacillus subtilis* cells & correlation with nucleoid extension. J Bacteriol 167 (1): 219–230.

Kirkwood IDJ & Burdett TBL (1988) Estimating the growth pattern of micro-organisms in distinct stages of the cell cycle. J Theor Biol 130: 255–273.

Kooistra T, van den Berg J, Tons A, Platenburg G, Rijken DC & van den Berg E (1987) Butyrate stimulates t-PA synthesis in cultured human endothelial cells. Biochem J 247, 605–612.

Kretzmer G, Buch T, Konstantinov K & Naueh P (1998) The temperature effect in mammalian cell culture: An Arrhenius interpretation. In: Merten OW, Perrin P & Griffiths B (eds) New Developments and Applications in Animal Cell Technology. Kluwer Academic Publishers, pp. 363–366.

Kromenaker SJ & Srienc F (1991) Cell-cycle-dependent protein accumulation by producer and nonproducer murine hybridoma cell lines: a population analysis. Biotechnol Bioeng 38: 665–677.

Kromenaker SJ & Srienc F (1994a) Effect of lactic acid on the kinetics of growth and antibody production in a murine hybridoma: secretion patterns during the cell cycle. J Biotechnol 34: 13–34.

Kromenaker SJ & Srienc F (1994b) Cell cycle kinetics of the accumulation of heavy and light chain immunoglobulin proteins in a mouse hybridoma cell line. Cytotechnology 34: 205–218.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lloyd DR, Holmes P, Jackson LP, Emery AN & Al Rubeia M (2001) Relationship between cell size, cell cycle and specific recombinant protein productivity. Cytotechnology 34, 597.

Ozturk SS & Palsson BO (1991) Growth, metabolic and antibody production kinetics of hybridoma cell culture: 1. Analysis of data from controlled batch reactors. Biotechnol Prog 7: 471–480.

Palermo DP, De Graaf ME, Marotti KR, Rehmerg E & Post LE (1991) Production of analytical quantities of recombinant proteins in Chinese Hamster Ovary cells using sodium butyrate to elevate gene expression. J Biotechnol 19: 35–47.

Parekh RB, Dwek RA, Rudd PM, Thomas JR, Rademacher TW, Warren T, Wun TC, Hebert B, Reitz B, Palmier M, Ramabhadran T & Tiemeier DC (1989a) N-glycosylation and in vitro enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese Hamster Ovary Cells and a murine cell line. Biochemistry 28: 7670–7679.

Parekh RB, Dwek RA, Rudd PM, Thomas JR, Rademacher TW, Warren T, Wittwer A, Howard SC, Nelson R, Siegel NR, Jennings MG, Harakas NK & Feder J (1989b) Cell-type specific and site-specific N-glycosylation of type I and type II human Tissue Plasminogen Activator. Biochemistry 28: 7644–7662.

Slater ML, Sharrow SO & Gart JJ (1997) Cell cycle of saccharomyces cerevisiae in population growing at different rates. Proc Natl Acad Sci USA 74 (9): 3850–3854.

Spellman MW, Basa LJ, Leonard CK, Chakel JA, O'Connor JV, Wilson S & van Halbeek H (1989) Carbohydrate structures of human tissue plasminogen activator expressed in Chinese Hamster Ovary cells. J Biol Chem 264 (224): 14100–14111.