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Relationship between cell size, cell cycle and specific recombinant protein productivity

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Abstract

Centrifugal elutriation was used to produce cell cycle enriched fractions of four commercially relevant recombinant cell lines, chosen to allow for variation in properties due to construct, expression system and parent cell type, from normally growing heterogeneous batch cultures. As these fractions had identical culture histories and had not been subjected to any insult or stress which was likely to have adversely affected cellular metabolism, they were ideal for further study of cellular properties. Specific productivity, cell size and cell cycle state of replicate elutriated fractions were measured for each cell line. Results showed that cell size was the major cellular determinant of productivity for all cell lines examined. Product formation was not restricted to any particular cell cycle phase and in all cases, production occurred irrespective of cell cycle phase. Specific productivity was lowest when the majority of cells in the fraction were G_1, intermediate when the majority of cells in the fraction were S phase and greater when the majority of cells in the fraction were in G_2/M. However, the evidence suggests that size is the major cellular determinant of productivity; the apparent relationship between cell cycle and productivity is secondary and can simply be ascribed to the increasing size of cells as they progress though the cell cycle. Thus, in addition to cell density and viability cell size is the cellular parameter which should be incorporated not only into mathematical models of recombinant mammalian cell production processes but also into process monitoring and control strategies.

Abbreviations: CHO, Chinese hamster ovary; dO_2, dissolved oxygen concentration; DHFR, dihydrofolate reductase; ELISA, enzyme linked immunosorbent assay; FCS, foetal calf serum; FF, fluorosphere fluorescence; FS, forward scattered laser light; G-MEM, Glasgow minimum essential medium, also called BHK-21; hCMV, human cytomegalovirus; IFN-γ, interferon-γ; IgG, immunoglobulin G; MSX, methionine sulphoximine; MTX, methotrexate (4-amino-10-methylfolic acid); PBS, phosphate buffered saline (Dulbecco's formula without Ca^{2+} or Mg^{2+}); PI, propidium iodide; RNase, ribonuclease; SS, 90° side scattered laser light; SV40, simian virus 40; t-PA, tissue-like plasminogen activator

Introduction

One of the most important parameters in the commercial operation of recombinant mammalian cell production processes is the specific productivity of each cell in that process. If it can be shown that a particular subpopulation of cells within a process is more productive than another, processes can be designed and control strategies can be developed to favour or enhance the more productive fraction. One obvious difference between cells in a population growing normally in a batch, continuous or semi-continuous culture is the cell cycle state of each cell.

However, there is some confusion in the literature as to the effect of cell cycle phase on protein production by mammalian cells. Product expression or the maximum rate of expression, has not only been shown to be related to G_1 phase (Al-Rubeai and Emery, 1990;

Kromenaker and Srienc, 1991) but also related to S phase (Kubbies and Stockinger, 1990; Banik et al., 1996; Gu et al., 1995a; Leelavatcharamas et al., 1997) and G_2/M (Aggeler et al., 1982). Other workers have shown the relationship to be aphasic (Matherly et al., 1989; Feder et al., 1989). A more detailed description of these and other works are given in tabular form in Lloyd et al. (1999). These observations do not necessarily contradict each other and may be a true reflection of the systems used since a variety of cells, products and vectors were studied. Cell cycle dependent product expression may not be universal. Product expression could vary with cell type or cell line (Chai et al., 1996), the construction of the expression vector (Gu et al., 1996b), the product gene copy number (Gu et al., 1996b, c) or the promoter/enhancer used to drive product expression (Gu et al., 1996b; Banik et al., 1996). Other possible variables include the nature of the recombinant gene expressed, post transcription regulation (mRNA level), even the culture mode, whether the cells are attached to a substrate or free in suspension, may be relevant (Lloyd and Al-Rubeai, 1999).

Unfortunately, there are artefacts that may affect studies of cell cycle related productivity. Many studies used cells which had been synchronised by chemical blockade (Buell and Fahey, 1969; Mariani et al., 1981; Scott et al., 1987) or nutrient deprivation (Garatun-Tjeldstø et al., 1976; Leelavatcharamas et al., 1997) or had not involved normally growing cultures (Fussenegger et al., 1997, 1998a, b). The first problem comes from using cells synchronised by chemical means, such as thymidine block or nutrient starvation, the second is associated with comparing cells of different phases from the same culture in samples which are separated in time. The two problems often go together, for example in following a culture for a day after release from thymidine block. The problem with the former is that any chemical synchronisation stress may, in addition to its desired effect of cell synchrony, also directly cause perturbations in productivity. The second problem is simply that if two cell populations from a culture are separated in time, they are also separated in environmental conditions, recent work (Lloyd et al., 1999), has shown that the effect of medium condition is dominant over the cell cycle in its effect on productivity. The effect of temporal separation is made more uncertain following stress synchronisation; a population examined shortly after release from blockade may have only partially recovered from the stress, when a later population has fully recovered. In all cases the observed differences in productivity, which could be an artefact of the experimental design, could wrongfully be ascribed to the cell cycle. A third problem arises when the cells examined are not growing normally, i.e. when they are arrested, taken out of the cell cycle or effectively resting in G_0. Al-Rubeai et al. (1992) showed marked increases in specific antibody production by hybridoma cells in batch and perfusion culture after arresting cell cycle progression by thymidine blockade. However, it is important not to confuse the relationship between cell cycle phase and productivity with the relationship between cell cycle arrest and productivity. The substantial increases in productivity described in metabolically engineered transfects (Fusenegger et al., 1997, 1998a, b) are most likely to be because the arrested cells do not need to devote resources in biomass production (Fussenegger et al., 1997). In contrast, this current work addresses the situation in simple recombinant cells which may be grown in standard industrial processes in batch, continuous or draw and fill semi-continuous culture modes.

Determining the relationship between cell cycle phase and productivity is important for developing automated on line or near in line process control strategies. These strategies will be based on mathematical models which, in addition to the basic parameters of pH, dissolved oxygen concentration (dO_2), cell density etc., must incorporate a component of cell state, such as the effect of cell cycle phase or some similar variable, on productivity. By using centrifugal elutriation to overcome the flaws of previous studies we intended to examine which, if any, cell cycle phase is most productive.

Materials and methods

Cells and culture conditions

Four industrially relevant product secreting cell lines were studied. Chinese Hamster ovary (CHO) cell line CHO 320 producing human interferon-γ (IFN-γ) was kindly provided by Glaxo-Wellcome Research and Development, Beckenham, U.K. This recombinant cell line contained an insert for human IFN-γ under the control of the simian virus 40 (SV40) promoter/enhancer which was co-amplified with the dihydrofolate reductase (DHFR) gene by the presence of methotrexate (MTX). Cells were grown in stirred batch suspension cultures in RPMI 1640 (Gibco, Paisley, U.K.) with 5% foetal calf serum (FCS) (Gibco,

Paisley, U.K.) and 1 mM MTX (Sigma, Poole, U.K.) at 37 °C. CHO Tf70R cells producing human tissue-like plasminogen activator (t-PA) were kindly provided by Pharmacia & Upjohn, Sweden. This recombinant cell line contained an insert for human t-PA under the control of the SV40 promoter/enhancer. Cells were grown in stirred batch suspension cultures in a proprietary serum free medium (Biopro 1; BioWhittaker, Verviers, Belgium) with 3.6 g l^{-1} glucose (Sigma, Poole, U.K.) and 0.3 g l^{-1} glutamine (Sigma, Poole, U.K.) at 37 °C. CHO 22h11 cells producing a monoclonal mouse/human chimeric immunoglobulin G (IgG) antibody, cB72.3γ4,κ, with specificity for the human Tag72 tumour marker, were kindly provided by Lonza, Slough, U.K. This recombinant cell line contained an insert for cB72.γ4,κ under the control of the human cytomegalovirus (hCMV) promoter which was carried on the same plasmid as a glutamine synthetase gene, so that gene copy number was maintained/increased in the absence of glutamine and the presence of methionine sulphoximine (MSX). Cells were grown in stirred batch suspension cultures in Biopro 1, as before, with 20% of proprietary Supplement for Biopro 1 (BioWhittaker, Verviers, Belgium) 3.6 g l^{-1} glucose (Sigma, Poole, U.K.) and 100 μM MSX (Sigma, Poole, U.K.) at 37 °C. Mouse myeloma NS0 6A1 cells with the same insert producing the same chimeric monoclonal antibody as CHO 22h11, were also kindly provided by Lonza, Slough, U.K. These were grown in stirred batch suspension cultures in Glasgow minimum essential medium (G-MEM) (Gibco, Paisley, U.K.) with 5% FCS (Gibco, Paisley, U.K.) and 10 μM MSX (Sigma, Poole, U.K.) at 37 °C. In all cases, samples for centrifugal elutriation were taken from the mid-exponential phase of batch suspension cultures grown in stirred flasks before there was any indication of growth limitation by nutrient exhaustion or metabolite accumulation; typically 24–48 h from sub-culture, depending upon the cell line.

Centrifugal elutriation

Cells were fractionated by centrifugal elutriation on the basis of size, assuming constant density, using a Beckman J-6M/E centrifuge equipped with a JE-6B elutriation rotor and standard elutriation chamber. Centrifuge conditions were 1950 rpm (366 × g at the elutriation boundary) and 20 °C, using Dulbecco's formula (Dulbecco and Vogt, 1954) phosphate buffered saline without Ca^{2+} or Mg^{2+} salts (PBS) as eluant. Eluant flow was produced using a variable speed peristaltic pump (model 900–292, Cole-Parmer, Barrington, U.S.A.) fitted with Masterflex pump tubing number 6411–14 (Cole-Parmer, Barrington, U.S.A.), the fraction number determined by pump speed (given as arbitrary units but typically representing a flow rate in the range 5 to 80 ml min^{-1}). All cells were elutriated under aseptic conditions. Approximately 2×10^8 cells were harvested from exponentially growing cultures and loaded into the elutriation chamber with eluant running at a pump speed of 0.75 units, Pump speed was increased to 1.5 units, the first 50 ml fraction was collected and the second 50 ml was discarded. Eluant pump speed was subsequently increased in 0.2 or 0.25 units steps with fractions collected as above and kept on ice until processing for subsequent experiments was started. Elutriation took approximately one hour from loading cells into the elutriation chamber until the start of sample processing for subsequent experiments.

In preliminary studies (not shown), elutriation were examined by microscopy. The first centrifugal elutriation fraction contained many dead, trypan blue stained cells and all subsequent fractions were essentially free of dead cells. Furthermore, the majority of cell doublets and clumps were retained in the elutriation chamber and were essentially absent from centrifugal elutriation fractions in the ranges reported.

Subsequent culture of elutriated fractions

The number of cells in each fraction was counted, 1 ml was taken for size measurement and 10 ml was taken for cell cycle analysis *vide infra*. Cells were washed 3× in PBS and finally resuspended in a volume of the appropriate fresh medium to give approximately 7×10^5 cells ml^{-1}. Each fraction was then divided into replicate wells in a 24 well cluster plate (product 3829–024, Iwaki Glass, Japan). The residue of each fraction was counted again to verify the number of cells ml^{-1} in the experiment, all fractions were $6-8\times10^5$ cells ml^{-1}. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 2 h the supernatant from all wells was harvested for subsequent measurements of product concentration *vide infra*. Between 2 and 16 replicates of each fraction were studied within each subsequent experiment, depending on the amount of material available.

Cell counting

To check the suitability of the starting material and to determine the volume of culture required to give

2×10^8 viable cells for centrifugal elutriation, viable cell density and percentage viability was determined using trypan blue exclusion and counting with a haemacytometer. All cultures used for further investigation were >95% viable. At all other times cells were counted using a flow cytometric method modified from Al-Rubeai et al. (1997). Briefly, 200 μl cell suspension was added to 20 μl calibration solution (1×10^6 ml^{-1} uniform fluorospheres (Flow-Check, Coulter Electronics, Luton, U.K.)), mixed by gentle agitation and analysed immediately. Flow cytometric analysis was performed using an EPICS Elite flow cytometer (Coulter Electronics, Luton, U.K.) equipped with an argon laser emitting 15 mW at 488 nm. Forward scattered laser light (FS) was collected using a neutral density filter and the standard cross beam mask, 90° side scattered laser light (SS) was collected using a 488 nm band pass filter, fluorosphere fluorescence (FF) was collected using a 488 nm long pass filter, followed by a 635 nm band pass filter. FS signal integral, SS signal integral log, and FF signal integral were recorded. An unknown volume of sample containing 2000 fluorospheres was analysed and the number of cells in that volume was counted. Cell number ml^{-1} was then calculated from the number of fluorospheres present, at a known concentration, in relation to the number of cells present and allowing for dilution.

Cell size measurement

Absolute size measurements; cell volume and equivalent spherical diameter; were measured using a Multisizer II and Accucomp software (Coulter Electronics, Luton, U.K.). In some cases, where Multisizer data was not available, the FS integral mean channel number of the cell population, recorded during cell counting, was used as an indicator of cell size.

Product measurement

Concentrations of secreted products were measured by enzyme linked immunosorbent assay (ELISA). IFN-γ (CHO 320 product) concentration was measured using a DuoSeT kit (Genzyme, West Malling, U.K.), as described previously (Lloyd et al., 1999) with the exception that, after the final washing, freshly prepared substrate solution (o-phenylene diamine (Sigma, Poole, U.K.) and H_2O_2 (BDH, Poole, U.K.) was used instead of the 3,3′,5,5′ tetramethylbenzidine liquid substrate system. After dark incubation at room

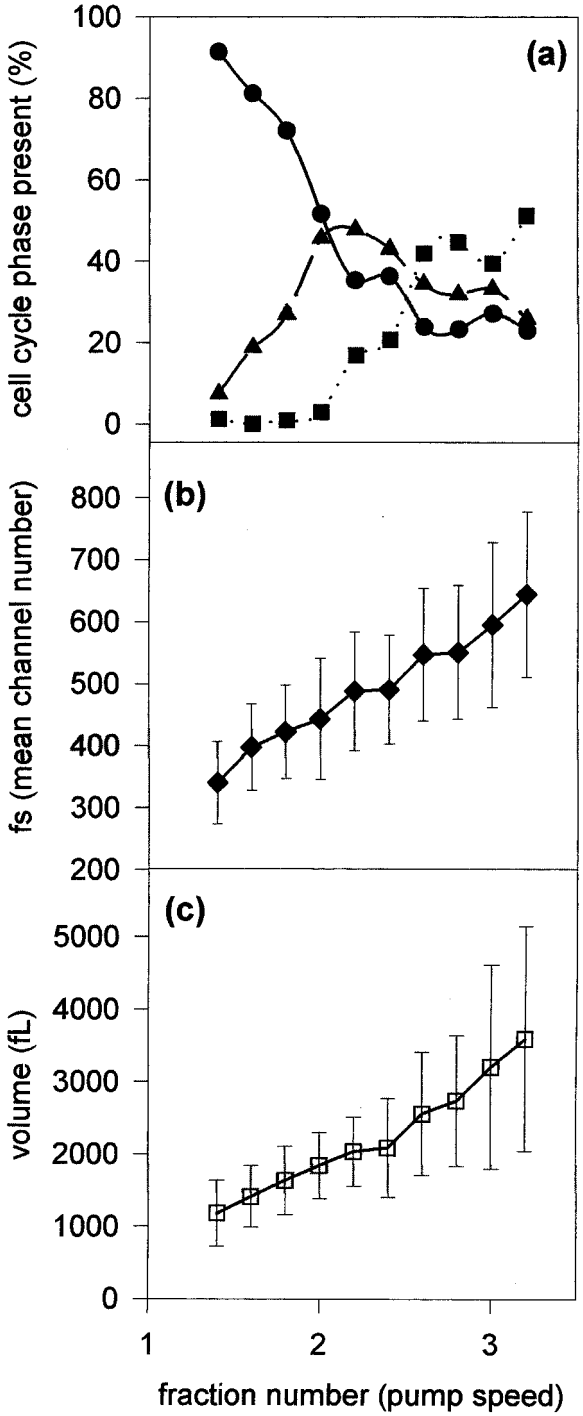

Figure 1. Elutriated CHO 22h11 cells: (a) showing proportion of cells in G_1 (●), G_2/M (■) and S phase (▲), (b) showing mean size measured by FS during the subsequent productivity experiment (♦), bars = ±1 sd. (c) Showing mean volume (□) measured by Coulter principle, bars = ±1 sd.

temperature, until the lowest standard showed an appreciable colour change, the reaction was stopped by acidification. Absorbance was measured at 492 nm, with 620 nm as reference, using an ELISA plate reader (SLT Spectra, Tecan, Reading, U.K.). Absorbance was converted to IFN-γ concentration (pg ml^{-1}) by interpolation from the standard curve.

t-PA concentration (CHO Tf70R product) was measured using a method similar to that described by Drying et al. (1994). A Biopool Immulyse t-PA kit (Bio-Stat Diagnostics, Stockport, U.K.) was used, following the manufacturer's protocol supplied with the kit. After final acidification, absorbance was measured as described above and was converted to t-PA concentration (ng ml^{-1}) by interpolation from the standard curve.

Chimeric monoclonal cB72.2γ4,κ antibody (CHO 22h11 and NS0 6A1 product) concentration was measured using a triple sandwich ELISA based on that of Whittle et al. (1987). Briefly, 96 well plates (product 442404A, Life Technologies, Paisley, U.K.) were coated with capture antibody (product I-6260, Sigma, Poole, U.K.) overnight. Plates were washed, standards or unknowns were added. After incubation and further washing, peroxidase conjugated secondary antibody (product A-7164, Sigma, Poole, U.K.) was added. Following incubation and further washing, freshly prepared substrate solution was added and plates were developed and measured as described above for IFN-γ. Absorbance was converted to antibody concentration (pg ml^{-1}) by interpolation from the standard curve.

Cell cycle analysis

1×10^6 cells were harvested, washed with PBS, fixed with cold 70% ethanol and stored at –20 °C until analysed. Cells were washed in PBS, then resuspended in ribonuclease (RNase) solution (250 μg ml^{-1} RNase (Sigma, U.K.) in PBS) and incubated at 37 °C for 30 min. Propidium iodide (PI) (Sigma, Poole, U.K.) was added to a final concentration of 50 μg ml^{-1} and the preparation was incubated at room temperature for a further 10 min. The relative cellular DNA content of stained cells was measured using an EPICS Elite flow cytometer described above. FS and SS were collected as before, PI fluorescence was collected using a 488 nm long pass, followed by a 635 nm band pass filter. FS signal integral, SS signal integral, PI fluorescence signal integral and PI fluorescence signal peak were recorded. Single cells were selected for analysis by using the distribution of PI fluorescence signal integral against PI fluorescence signal peak to discriminate doublets and debris (Al-Rubeai et al., 1995). The relative size of PI fluorescence signal integral, proportional to the DNA content in single cells, was plotted as a frequency histogram. The proportions of cells in phases G_1, S and G_2/M were determined from the latter by cell cycle analysis using Multicycle software (Phoenix Flow Systems, San Diego, U.S.A.).

Results

Fractionation of heterogeneous cell population by centrifugal elutriation

Although centrifugal elutriation is well established in basic biomedical research, (McEwen et al., 1968; Suzuki et al., 1977; Van Es and Bont, 1980; Brandt et al., 1988; Lee et al., 1992; Ghosh et al., 1996) there is much less literature on its application to the recombinant cell lines used in process biotechnology (Feder et al., 1989). The results of a typical elutriation are shown in Figure 1. The horizontal axis (fraction number) represents the effect of increasing the flow rate of eluant opposing the centrifugal retention of cells in the elutriation chamber. Therefore a higher flow rate will elute larger cells. Also, in the cell cycle analysis graph (Figure 1a) the numbers represent the proportion of cells in each fraction which are in each cell cycle phase, not the absolute number of cells in each phase in each fraction. The cell cycle composition of fractions exhibits marked variation while cell size increases significantly with fraction number. These results confirm that, compared with the yield from flow cytometeric cell sorting, centrifugal elutriation can be used to separate large numbers of cells relevant to bioprocess engineering into cell cycle enriched fractions on the basis of their size. Although, in the example shown, no elutriation fractions were purely of any single cell cycle fraction, substantial enrichment was made. Early, late and mid fractions containing maxima of 92% G_1, 51% G_2/M and 48% S phase, respectively, were obtained from an original culture comprising 63% G_1, 28% S and 8% G_2/M phase cells. The centrifugal elutriation conditions used were a compromise between cell yield in each fraction and cell size/cell cycle state in each fraction. By using smaller pump speed increments between fractions it should have been possible to obtain purer fractions, in terms of cell cycle composition. However, to do

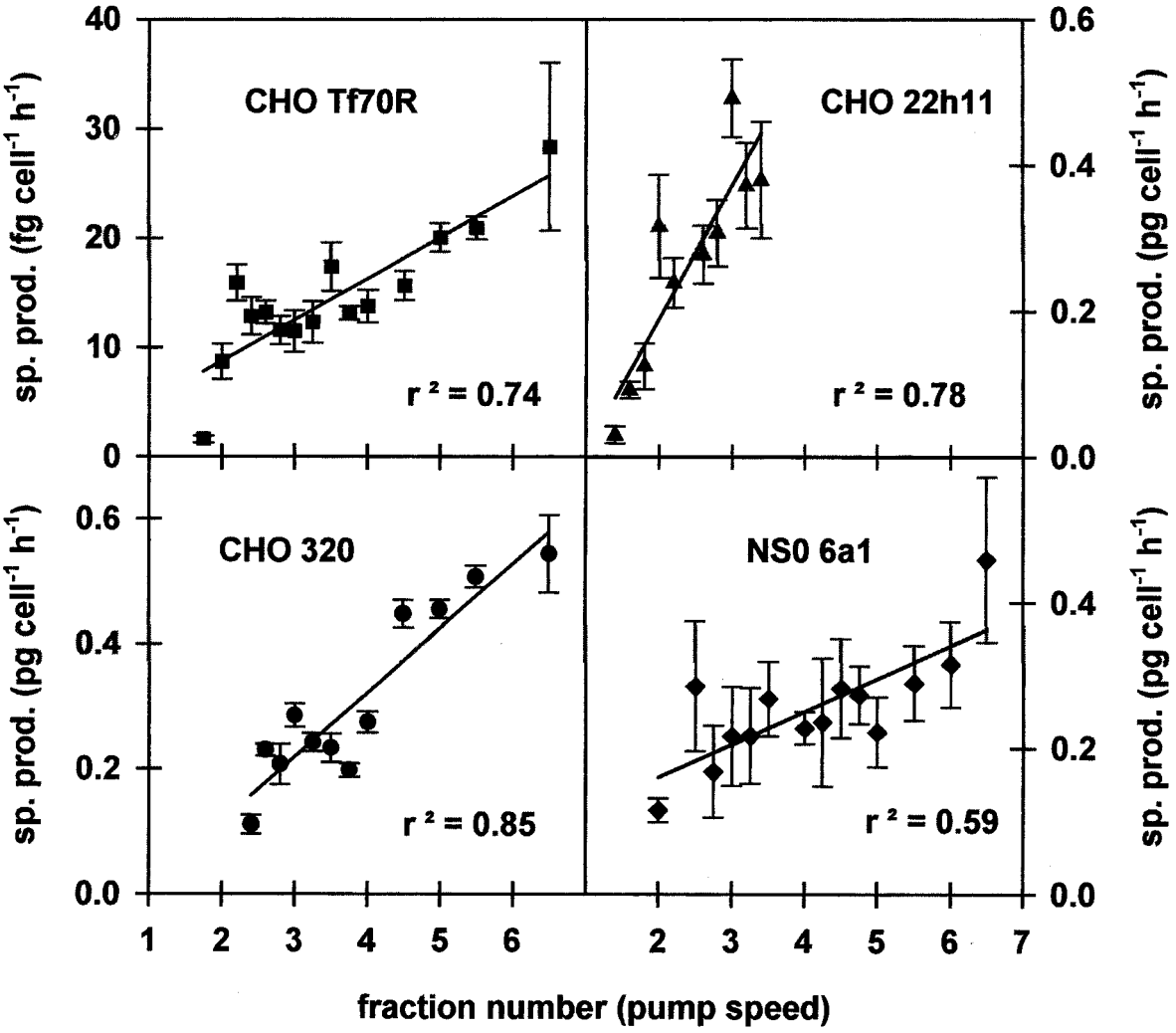

Figure 2. Variation of specific productivity with fraction number; CHO 320 (●), CHO Tf70R (■), CHO 22h11 (▲), NS0 6a1 (♦); bars = ±1 sd.

so would have given more fractions, each of which would have contained too few cells to be of any use for subsequent investigations. Similarly, despite the noticeable overlap between consecutive fractions, mean, median and modal cell size increased consistently with fraction. This observation was true not only when cell volume was determined as an absolute value by Coulter principle but also when cell size was determined only as a relative value by flow cytometry. As with the cell cycle it should have been possible, using smaller pump speed increments between fractions, to obtain purer fractions with respect to cell size range but to do so would have given more fractions, each of which would have contained too few cells to be of any use for subsequent investigations.

Specific productivity of elutriated fractions

The specific productivity of sequentially elutriated fractions in single experiments with each of the four cell lines is shown in Figure 2. Despite different specific productivity values in different experiments and different slopes to the regression lines, specific productivity increased with elutriation fraction in all cases.

Relationship between specific productivity, cell size and cell cycle phase

Figure 3 shows the relationship between specific productivity and cell size for four cell lines. In all cases specific productivity increased in linear correlation

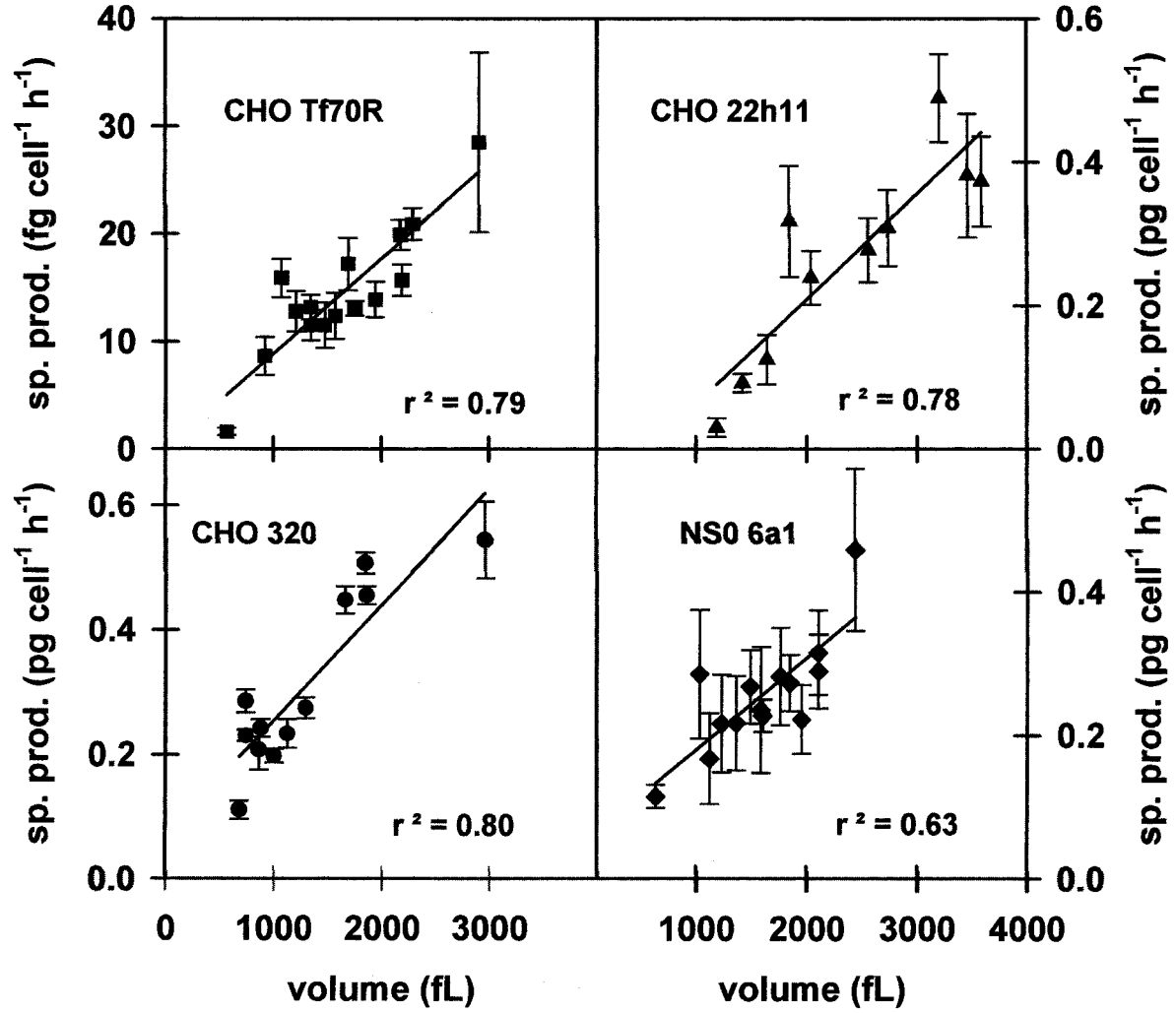

Figure 3. Variation of specific productivity with mean volume; CHO 320 (●), CHO Tf70R (■), CHO 22h11 (▲), NS0 6a1 (♦); bars = ±1 sd.

with cell size. Figure 4 shows the relationship between specific productivity and cell cycle phase composition from exemplary elutriations for all four cell lines. In all cases it is apparent that product was formed by all fractions irrespective of the cell cycle phase composition of the fractions. Specific productivity increased with fraction number, as the major phase in each fraction changed from G_1 to S phase to G_2/M. However the recurrence of G_1 cells in some later fractions, without a corresponding reduction in productivity (Figure 4), suggests that it was the size of the cells rather than their cell cycle phase *per se* which was the major determinant of specific productivity. Thus there is no evidence for product formation being restricted to any particular cell cycle phase and it is justifiable to conclude not only that product formation occurs throughout the cell cycle but also that big cells are more productive than small cells.

Discussion

It is accepted that recombinant protein production by mammalian cells can be maximised using bioreactor design, operating and control strategies appropriate for the system in question. Many intrinsic cellular properties may affect a culture's performance and cell cycle phase is one parameter which has been intensively investigated. However, as indicated in the introduction, there is uncertainty in the literature not only about the relationship between cell cycle phase and productivity but also about its importance in cell culture

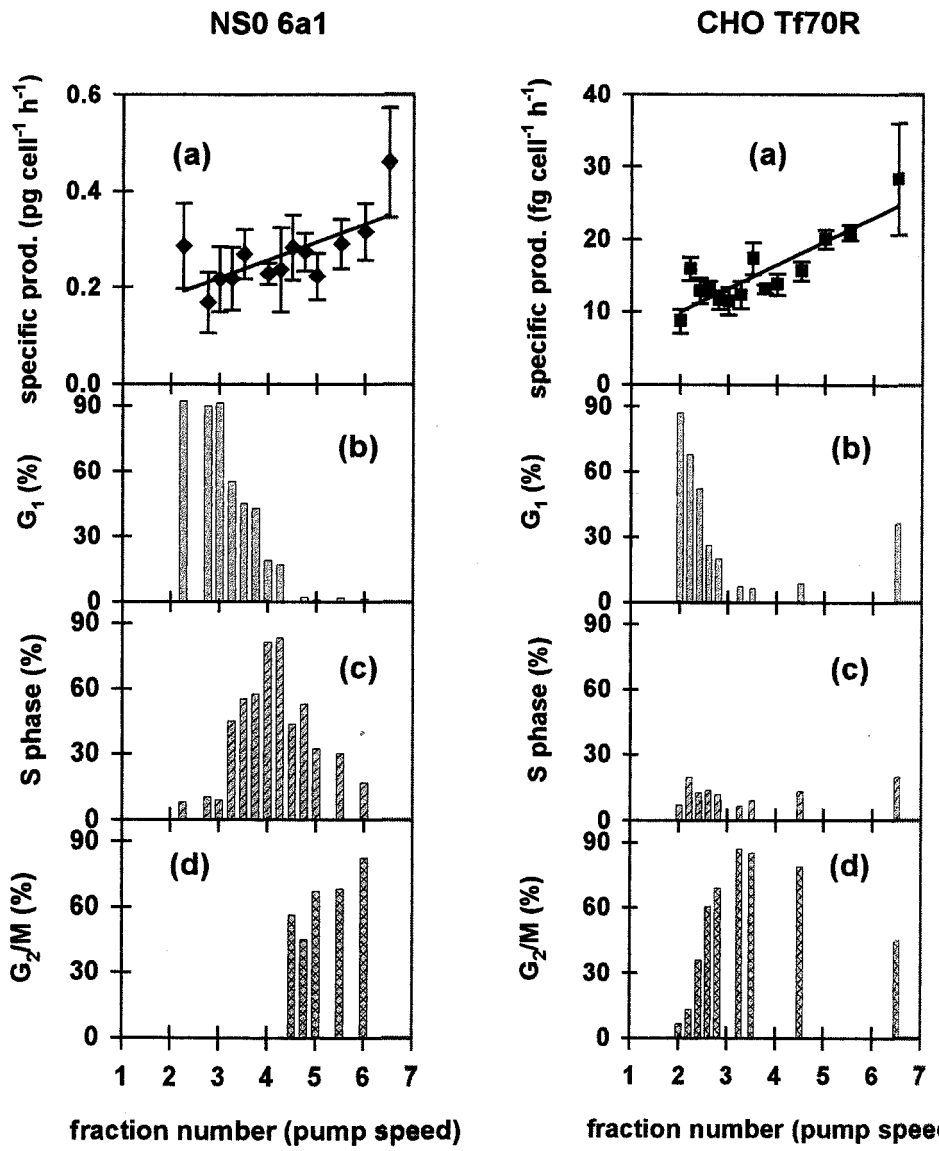

Figure 4. Variation of specific productivity and cell cycle with fraction number: panel (a) = specific productivity, CHO 320 (●), CHO Tf70R (■), CHO 22h11 (▲), NS0 6a1 (♦); bars = ±1 sd; panel (b) = proportion of cells in G_1; panel (c) = proportion of cells in S phase; panel (d) = proportion of cells in G_2/M.

management. By adopting the technique of centrifugal elutriation and by careful experimental design we believe that we have overcome the artefacts which may have affected previous studies.

Only commercially relevant recombinant cell lines were investigated, all of which secreted their product into the culture medium. Cells were grown in normal heterogeneous batch culture and cells were taken from a single point in that culture so that, within each experiment, all cells had an identical culture history and there was no scope for culture history to have caused observed differences between fractions. Cells were taken from mid exponential culture where there is known to be no nutrient limitation or toxic metabolite accumulation, so they were not subject to nutrient stress which could have affected their physiology. Furthermore, the cells had not been synchronised by a chemical insult, such as thymidine block, which could also have affected their metabolism.

It is possible that the process of centrigugal elut-

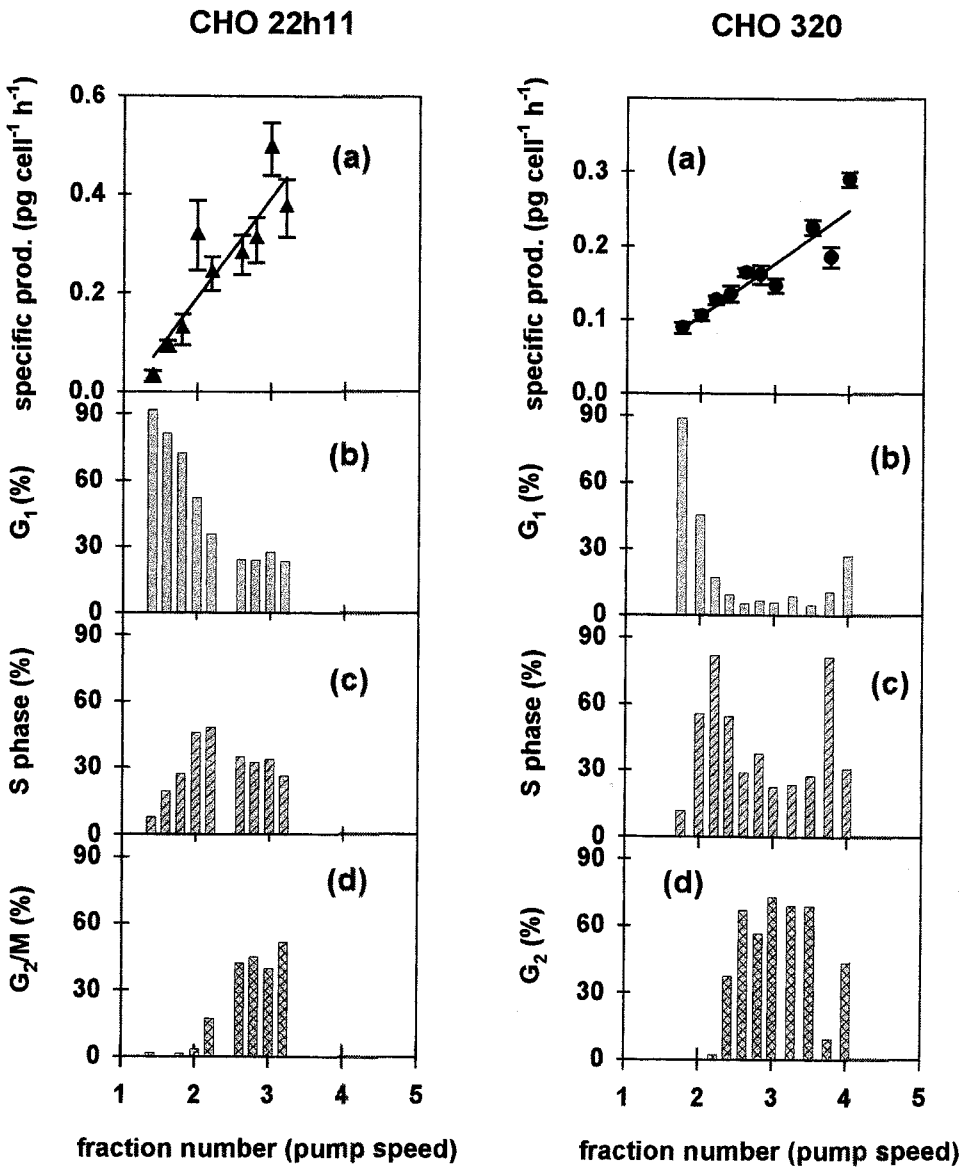

Figure 4. (continued).

riation itself had a physical effect on cell metabolism or physiology. However, since the cells are not compressed into a pellet during centrifugal elutriation, it is unlikely that any greater adverse effect would have occurred than that which may be caused by the simple centrifugation steps which are used (though the possible effects are rarely mentioned) in almost all cell experiments. However, any effect which centrifugal elutriation may have had on the cells has been controlled for in this series of experiments simply because all fractions and all cells have been exposed to the same effect at the same time. Thus, as far as was possible, we have eliminated all confounding variables from our experiments so that any differences seen between cells from different fractions can be rightly and only attributed to the differences in the properties of the cells in different fractions.

Because two of the four cell lines used the SV40 promoter/enhancer, which is said to be S phase specific (Banik et al., 1996), it would not have been surprising if S phase cells or S phase enriched fractions had been shown to be most productive, if not the sole

producers. This was proved not to be the case. However, there are other intrinsic cellular properties which may have given rise to the disparate reports of cell cycle and productivity relationships in the literature. One factor to consider is the model system used. Some authors (Banik et al., 1996; Gu et al., 1996) used the *Escherichia coli lac-Z* gene product β-galactosidase in their model systems. Although β-galactosidase is a well defined and relatively stable molecule, which has many advantages as a marker molecule and may be very useful in other situations, it is not secreted by mammalian cells. This last property immediately poses two problems. The first, a theoretical problem, is that product can only accumulate intracellularly which may inhibit product synthesis; because of product toxicity or simply due to product accumulation; which would not occur if the product was secreted. The second problem is of practical relevance, a bioprocess engineer is interested not just in quantity of protein synthesised but also in its quality as recovered. The full product formation process includes; transcribing the appropriate gene to mRNA and translating that into the polypeptide chain which forms the primary protein structure and together can be called synthesis (i.e. gene expression), followed by post translational modifications, including peptide cleavage, protein folding, glycosylation and perhaps formation of multimeric proteins in the golgi apparatus, then finally secretion of the active finished product into the culture medium so that it can be recovered. Thus a model system which uses a non secreted marker is less representative of the 'real life' situation than one which makes a secreted product. Because we examined commercially relevant cell lines which secrete their products into the culture medium, our results address the whole product formation process rather than just the first step (gene expression) and so are more directly relevant to bioprocess engineering and may well give different results from studies which have investigated non secreting model systems.

To allow for the possibility that intrinsic cellular properties which affect productivity vary between cell lines, we chose to investigate the properties of four cell lines which would enable us to test whether the construct itself, the expression vector or the parent cell line was an important variable. Comparing CHO 320 with CHO Tf70R, both DHFR- parent cells having been co-transfected with DHFR+ and product expression vectors but with different products, would demonstrate any difference due to construct/product molecule. No difference was seen. Product formation was not specific to any cell cycle phase but increased with cell size. Comparing CHO 320 and CHO Tf70R with CHO 22h11; the two former used DHFR-parent cells co-transfected with DHFR+ and product expression vectors with the product gene under the control of the SV40 promoter/enhancer, while the latter used a GS- parent cell transfected with a GS+ and product bearing vector with the product gene under the control of the hCMV promoter. This comparison should demonstrate any difference due to promoter or expression vector. No difference was seen. In all cases product formation was not specific to any cell cycle phase and increased with cell size. Comparing CHO 22h11 with NS0 6A1, using the same GS+ and product bearing vector but in different GS- parent cell types, would demonstrate any difference due to cell type. No difference was seen. In both cases product formation was not specific to any cell cycle phase and increased with cell size, although it could be argued that in NS0 there was a lesser effect of cell size on specific productivity than in CHO cells.

It was a universal finding that product was made by all fractions and specific productivity increased with cell size, irrespective of construct or host cell line. In all cases, specific productivity was greater in G_2/M cells than in S phase cells which in turn was greater than in G_1 phase cells. However, there was no suggestion that product formation was limited to any cell cycle phase. In addition the reoccurrence of large G_1 cells in some later fractions, without a concomitant reduction in productivity, suggested that it was the size of the cells rather than their cell cycle phase *per se* which was the major determinant of specific productivity. These findings fit with anecdotal observations, made when following batch cultures, that early-mid exponential phase cells, a day or two after inoculation, tend to look bigger than cells from earlier or later phases. Furthermore it has been shown previously (Leelavatcharamas et al., 1997), using synchronised cells, that the majority of the cells in the early-mid exponential phase were in S and progressing to G_2/M phase so it was these cells, either due to their size or cell cycle state, which were the most productive in batch culture. Subsequent work suggested that these early-mid exponential phase cells showed an increased productivity due to a favourable culture environment (Lloyd et al., 1999). Thus it may be that the particular environment found in the early-mid exponential phase of batch cultures allows the appearance of larger cells, irrespective of their cell cycle phase, which are more productive simply due to their increased size. If this

is true, there is scope for further detailed development of both medium formulation and process strategy to increase productivity by extending the duration of that part of the process where environmental conditions are favourable for the formation of larger and more productive cells.

Conclusions

Using cell cycle/size enriched fractions with an identical culture history and which had not been adversely affected by major chemical or nutrient stress, we have shown that the size of a cell is an important determinant of that cell's specific productivity. Product formation is not restricted to any particular cell cycle phase and any apparent relationship between cell cycle phase and specific productivity is simply a secondary relationship due to cells getting bigger as the cell cycle progresses from G_1 to S to G_2 and M.

Thus bioreactor design and production strategies should be optimised to favour or enhance larger cells. Furthermore, when developing automated process control or modelling production processes to predict culture performance, the parameter 'cell size', which may also vary with culture condition, must be incorporated in the productivity determination. The observation of a small number of large, high producing G_1 phase cells (Figure 4) is interesting. Though it needs to be emphasised that this represents only a small proportion (<1%) of the original total of G_1 cells in the sample. It will be interesting to identify the origins of such cells and then to question whether their numbers could be beneficially enhanced.

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