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Growth and death rates of bovine embryonic kidney cells in turbulent microcarrier bioreactors

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Abstract. Bead-bead collisions have been characterized using the velocity of the smallest turbulent eddies to calculate a turbulent collision severity (defined as the energy of collisions times their frequency), but a shear-based collision mechanism with a different dependence on the system variables is also applicable. This shear-based mechanism and the ratio of smallest eddy size to microcarrier diameter can explain the beneficial effects of both smaller diameter microcarriers and higher viscosity of the medium on the growth rate of bovine embryonic kidney cells. Death rates of these cells have also been measured at several levels of agitation. The decrease in apparent growth rate from increasing agitation is caused both by a higher rate of cell death as well as a lower intrinsic growth rate.

List of symbols

B	–	unspecified biological variable
d	cm	bead diameter
d_i	cm	impeller diameter
e	–	error in estimate of power number
F_n, F_s	$(g \cdot cm)/s^2$	normal and shear forces on a cell
Fr	–	Froude number
g	980 cm/s^2	acceleration of gravity
k	k^{-1}	first order death rate constant
m	g	mass of a bead
n	s^{-1}	impeller rotational rate
n_b	–	number of impeller blades
N_p	–	impeller power number
R_i	cm	impeller leading edge radius
TCS	$(g \cdot cm^2)/s^3$	turbulent collision severity
V	cm^3	reactor volume
v_{br}	cm/s	rms relative velocity between beads
v_e	cm/s	velocity in smallest eddies
X	number of cells/cm^3	cell population

Greek symbols

α	–	volume fraction microcarriers
γ	s^{-1}	shear rate
ε	cm^2/s^3	turbulent power dissipation rate
η	cm	size of smallest eddies
μ	g/(cm · s)	dynamic viscosity
μ	h^{-1}	apparent growth rate of cells
μ_0	h^{-1}	intrinsic growth rate of cells in absence of death
ν	cm^2/s	kinematic viscosity
ϱ_b	g/cm^3	bead density
ϱ_f	g/cm^3	fluid density
τ	$g/(cm \cdot s^2)$	shear stress

1 Introduction

It has often been stated that excessive agitation is a cause of damage to mammalian cells in microcarrier culture [11, 9, 16] with the usual explanation centering on "shear" from the agitator. However, there has been relatively little quantitative work published on exactly how mechanical energy from the agitator is transmitted to the cells and how the cells in turn respond to these specific forces. A fundamental understanding of the damage mechanism(s) is essential for design and scale-up of microcarrier bioreactors.

The intent of this work is to understand the particular aspect(s) of agitation that influence the in vitro culturing of cells. We make no attempt to describe rigorously or in detail the fluid mechanics of a turbulent two-phase system, nor is it our objective to explain the cell biology of the responses to external fluid forces. In particular, such important matters as product secretion by cells (for example, urokinase and tissue plasminogen activator by the bovine embryonic kidney cells used in this work) and nutrient consumption rates were not considered.

Quantitative data on the behavior of cells under different agitation conditions in actual microcarrier reactors are not plentiful. In some of the earliest work, Midler and Finn [18] used 80 μm diameter protozoa as models of cells to study the effects of agitation on cell viability. They measured death rates of the protozoa in both a concentric cylinder viscometer and two sizes of stirred vessels, and found that the death rate in agitated vessels was first order in protozoan concentration and could be correlated by impeller tip speed. Sinskey et al. [23] correlated maximum population of chick embryo fibroblasts in a stirred microcarrier bioreactor with an "integrated shear factor" (ISF) obtained by dividing the impeller tip velocity by the distance between the impeller tip and the vessel wall. They report that neither power input per unit volume nor "shear rate" – which they did not define – correlated the observed cell populations. Hu [14] addressed a number of issues concerning the amount of agitation and the effect of bead concentration and seeding densities on FS-4 cells, a human foreskin fibroblast line. He considered

the effect of mixing in one series of tests and found that impeller tip speed, nominally a scaling parameter for agitated systems (Oldshue [21]), did not correlate his data on relative growth extent, whereas the ISF defined by Sinskey et al. [23] did.

Cherry and Papoutsakis [2] presented a conceptual analysis of the possible effects the bioreactor environment might have on a cell-covered microcarrier. They conclude that interactions of a bead with the smallest turbulent eddies and collisions with either other beads or with solid parts of the bioreactor (such as the impeller leading edge) are the most likely means of cell damage. In a later paper (Cherry and Papoutsakis [4]) they present data on the growth of bovine embryonic kidney cells that indicate that an impeller collision severity, defined as the product of the kinetic energy and frequency of bead collisions against the leading edge of the impeller blades, does not adequately correlate their results. However, turbulent collision severity (the product of bead-bead collision kinetic energy and frequency) and the ratio of smallest eddy size to microcarrier bead size do predict the correct trends for several impeller diameters and rotational speeds if one characterizes the intensity of turbulence by the higher turbulent energy dissipation rate in the immediate region of the impeller. They also discuss the phenomenon at low agitation levels of cellular bridging between beads to form large clumps, which can also lead to a reduced apparent growth rate.

Croughan et al. [6] have analyzed the reports by Sinskey et al. [23] and Hu [14] and have shown that the size of the smallest turbulent eddies in the system is physically better justified as a correlator of the results than is the integrated shear factor. Stronger, more vigorous turbulence extends the range of turbulent eddy sizes to smaller values, the limit of which is known from theory. They found that eddy/bead size ratios of about one or less were more damaging. In a second paper [7] they show that at low levels of agitation the bead concentration, and by implication bead-bead collisions, is not a factor in determining the growth rate of FS-4 cells, although it is important with greater agitation.

2 Theory

The approach to this problem and some early results have been previously published [2, 4]. In essence, the complex fluid dynamic environment of a stirred microcarrier bioreactor has been categorized into several representative situations such as a single bead interacting with turbulent eddies of different sizes, a bead in a boundary layer, or a bead colliding with other beads. Each mechanism is characterized by a certain parameter indicative of the presence or amount of that mechanism occurring.

In particular, the interaction of the microcarriers with turbulent eddies is described by η/d, the ratio of smallest eddy size to the bead diameter:

$$\frac{\eta}{d} = \frac{\nu^{3/4}}{\varepsilon^{1/4} d} = \frac{\nu^{3/4}}{(N_p n^3 d_i^2)^{1/4} d}, \tag{1}$$

in which ν is the fluid kinematic viscosity, ε is the turbulent energy dissipation rate in the region of the impeller, N_p is the dimensionless power number for the particular style of impeller used [20], n is the impeller rotational speed, and d_i is the impeller diameter. By definition ε is equal to $N_p n^3 d_i^5 / V$, where V is the volume in which the dissipation occurs. As shown in Cherry and Papoutsakis [4] for the same reactor configuration and cell type as in this paper, V is better described by the volume d_i^3 around the impeller than by the actual total liquid volume in the vessel. Other reactor/impeller configurations may yield other results for the dissipation volume. A larger η/d ratio, that is larger eddies, is expected to be less damaging to cells on the surface of the beads.

The turbulent collision severity *TCS*, which represents collision energy per bead per time, is defined as:

$$TCS = (\text{kinetic energy}) \cdot (\text{collision frequency/volume})/N$$

$$= \left(\frac{m v_{br}^2}{2}\right) \cdot \left(\frac{v_{br} \alpha^2}{d^4}\right) \Big/ \left(\frac{\alpha}{(\pi/6) d^3}\right) = v_{br}^3 \left(\frac{\pi^2 \varrho_b d^2 \alpha}{72}\right), \tag{2}$$

where m is the mass of a bead, v_{br} is the root mean square relative velocity of the beads, ϱ_b is the bead density, α is the volume fraction of beads in the system, and N is the number of beads per volume. Hinze [13] gives the general form of the frequency of bead-bead collisions as $v_{br} \alpha^2 / d^4$, and various specific results containing constant factors near unity are reviewed by Cherry [3]. Higher values of *TCS* are expected to be more damaging to cells.

In the previous work (Cherry and Papoutsakis [4]) the relative velocity v_{br} of the beads was characterized by the velocity $v = (\varepsilon \nu)^{1/4}$ of the smallest eddies in turbulence, since the beads have nearly the same size and density as those eddies. Using the eddy-based velocity for v_{br} gives the following result for an eddy-based *TCS*:

$$TCS = (\varepsilon \nu)^{3/4} \left(\frac{\pi^2 \varrho_b d^2 \alpha}{72}\right). \tag{3}$$

This result is somewhat different from that in Cherry and Papoutsakis [2], because here we express *TCS* on a per bead basis rather than per volume of bead slurry.

If, on the other hand, the eddies are much larger than the beads, the relative velocity between neighboring beads is not well described using this eddy velocity assumption. In that situation, collisions between beads can be caused by a shear-based mechanism. Given two beads in a shear field, the relative velocity between the beads will equal the distance between the streamlines along which the beads are each moving times the local velocity gradient across those streamlines. Beads moving on streamlines less than one bead diameter apart can therefore collide, and the velocity of that

collision can be characterized by γd, where γ is the local velocity gradient or shear rate. This situation is most easily imagined when the smallest eddies are much larger than the beads, so the beads are completely surrounded by a coherent flow field.

However, Delichatsios [8] states that on the basis of experiment this type of correlation applies for particle sizes up to ten times the smallest eddy size; this broader range extends well over the conditions of typical microcarrier systems. Physically this is understandable by realizing that eddies 2–5 times the size of the beads (and consequently 2–5 times the size of the smallest eddies) can cause this type of collision. In this system where the eddy size range is quite broad, from roughly the impeller blade height of 2 cm down to the Kolmogorov scale of 0.02 cm, there will be a good correlation between the characteristics of Kolmogorov scale eddies and those just somewhat larger that cause shear-based bead collisions. Therefore, the Kolmogorov scales may be used to define this variable for correlation of the biological results. The Kolmogorov shear rate γ = eddy velocity/eddy size $= (\varepsilon \nu)^{1/2}$, so we can define a distinct shear-based *TCS* as:

$$TCS = \left[\left(\frac{\varepsilon}{\nu}\right)^{1/2} d\right]^3 \cdot \left(\frac{\pi^2 \varrho_b d^2 \alpha}{72}\right) = \left(\frac{\varepsilon}{\nu}\right)^{3/2} \cdot \left(\frac{\pi^2 \varrho_b d^5 \alpha}{72}\right). \quad (4)$$

The ratio between these two expressions for turbulent collison severities produces an interesting result:

$$\frac{\text{eddy } TCS}{\text{shear } TCS} = \frac{(\varepsilon \nu)^{3/4}}{\left(\frac{\varepsilon}{\nu}\right)^{3/2} \cdot d^3}$$

$$\frac{\text{eddy } TCS}{\text{shear } TCS} = \frac{\nu^{9/4}}{\varepsilon^{3/4} d^3} = \left(\frac{\eta}{d}\right)^3. \quad (5)$$

The severity calculated for inter-eddy collisions increases faster than that for intra-eddy collisions (the shear mechanism) as η/d increases, even as that inter-eddy mechanism becomes less likely because of the increasing eddy/bead size ratio.

Which of these two calculations of *TCS* to use is difficult to state in advance, since a number of assumptions are built into each, and microcarrier systems operate in the area between the clear and exclusive validity of each set. Both are justifiable for bead/eddy size ratios near one, although for eddies much larger than the beads the eddy *TCS* is probably not valid. Furthermore, changes in the reactor system which affect the numerical value of the two *TCS*'s will also generally affect η/d and the applicability of each of the *TCS*'s. Even the eddy size η is not physically well defined, since it is a characteristic length representing the "end" of a continuum of sizes and was originally obtained by dimensional analysis. The differences between the two collision severities are the exponent on ε, a rather large difference in the exponent on bead diameter d, and an opposite trend with changing viscosity ν. If the physical situation actually lies somewhere between the two cases described, the trends with these variables might be expected also to lie between the predictions of these two equations. Therefore, both measures will be used in the remainder of this work.

This derivation of *TCS* considers only direct collisions and not near-misses that would affect the local flow fields and impose fluid stresses on the two beads. While a detailed analysis of near-miss fluid mechanics is beyond this paper, the number of those indirect interactions should be proportional to the number of direct collisions, and the maximum energy of the direct interactions dependent on the velocity hence kinetic energy of the beads involved. These latter quantities appear in the *TCS* definitions already presented. Any separate effect of the fluid forces generated in near-misses will appear as a part of the empirical correlation of *TCS* with cell behavior.

It should be reiterated here that the two *TCS*'s and η/d are intended to be variables for correlation with experimental results rather than rigorous descriptors of the physical processes occurring. Each represents the average of a spectrum of fluid interactions with cells which themselves will have a range of sensitivities to any given stimulus. The physical significance of any specific value for these parameters (as opposed to trends) would be difficult to state.

In a somewhat different, but in principle equivalent approach, the Buckingham Π theorem (Churchill [5]) is a general technique to identify dimensionless groups to be used in correlating experimental data. We use this method to try to confirm the groups obtained by the mechanistic analyses. The theorem states that the number of dimensionless groups required to describe a system in the absence of any model is equal to the number of important variables minus the greatest number of variables that will not form a dimensionless product (usually equal to the number of independent dimensions in the units of the variables). The groups obtained can be used to correlate any of the system variables against each other.

If systems of different sizes are to be compared they must be geometrically similar, or groups describing the geometry must be included. Choosing the particular variables which describe the system is not an obvious task. They must be independent of each other, yet fully characterize the behavior of the system. Missing, extraneous or dependent variables will lead to missing, extraneous or dependent dimensionless groups. One intuitive approach is to list all the variables that a careful experimenter would specify or record in designing and running the experiment, then eliminate any that are related by known functions to variables still in the list.

Applying this theorem to the basic reactor geometry used in this work, there are thirteen major experimental variables – a measure of the cellular response to fluid forces, for which we use apparent growth rate μ, ν, d, ϱ_b, ϱ_f, g, α, reactor volume V, n, d_i, N_p, number of impeller blades n_b, impeller

leading edge radius R_i, and three dimensions (length, time, mass), so that ten dimensionless groups should be needed.

The growth rate is made dimensionless using the group μ/μ_{ref}, where μ_{ref} is the reference growth rate measured under a specified and fixed set of conditions. Correlating the growth rate μ against a number of hydrodynamic variables makes the assumption that $\mu = \mu(F_s, F_n, B_1, B_2, \ldots, B_i)$ where F_s and F_n are the time-dependent shear and normal forces on the cells, which are implicitly determined by the independent correlation groups. The various B's are biological conditions such as the type of cell, the medium used, the amount of serum present, the physiological state of the cells at the time of the experiment, etc. These B's must be held constant; Sinskey et al. [23] have shown that a 24 h delay before introducing agitation, as one example, can give higher maximum cell populations at equal levels of agitation.

The remaining nine groups prescribed by the Buckingham Π method may be taken to be $v^{3/4}V^{1/4}/d(N_p n^3 d_i^5)^{1/4}$ $(=\eta/d)$; ϱ_b/ϱ_f; $\varrho_f(N_p n^3 d_i^5 v)^{1/2}/\Delta\varrho\, V^{1/2} dg$ $(=\mathrm{Fr}$, a bead Froude number); α itself; d/R_i; $n^2 d_i/g$; n_b; d/d_i; and d_i^3/V. These nine groups or reactor variables should describe the effect of the fluid dynamic system on cell growth rate without having assumed any knowledge of the system.

The density ratio ϱ_b/ϱ_f is nearly one in practice, and is not expected to vary greatly from that value for obvious practical reasons. The bead Froude number Fr, which represents the ratio of fluid forces to gravity forces on the bead, can vary over a rather wide range, since $\Delta\varrho$ can have a several-fold variation, while d, ε and v are also readily manipulated. Bead-bead collisions are described by the dimensionless number α in this list. The *TCS* based on eddy velocity is equivalent to this, at least in the sense of introducing a mathematically independent variable containing bead concentration. The *TCS* based on shear rate can be obtained from the other by dividing the eddy velocity derived number by $(\eta/d)^3$, so that no additional independent groups are needed corresponding to this alternative number. The last five groups on the list above describe various aspects of the impeller geometry and should be important in correlations of impeller collision events. Those five can also be related to the turbulent power dissipation rate ε through standard curves using the power number N_p [20].

The Buckingham Π method gives the number of independent groups necessary to describe the system under the most general conditions. We have related the groups found using this method to our groups; if other experimental variables are also deemed important, corresponding dimensionless groups can be created and studied. In some cases, not all the Buckingham Π groups may be needed to adequately correlate the data. The unused groups simply do not have an important effect at the conditions of interest, although they might have one in a more general case. This method also provides no information about the functional relationship between the correlating groups; this must come from analysis of actual data.

3 Materials and methods

A description of the cells, the one dm^3 (working volume) reactors and analytical methods has been previously published [3, 4]. Briefly, primary bovine embryonic kidney cells (Whittaker M. A. Bioproducts, Walkersville, MD, USA) were grown in T-75 flasks to provide a sufficient inventory of cells and then frozen in liquid nitrogen. For each run in the one dm^3 reactors, cells were thawed and seeded at about $8 \cdot 10^4/cm^3$ into 800 cm^3 minimum essential medium – Eagle (MEME) containing 10% heat-inactivated fetal calf serum, penicillin/streptomycin/neomycin antibiotic mixture (MEME, serum and antibiotics from Sigma, St. Louis, MO, USA) and 4.8 kg/m^3 Cytodex 3 microcarriers (Pharmacia, Piscataway, NJ, USA) prepared according to the manufacturer's instructions. The cultures were grown at 37 °C, with pH controlled at 7.25 and dissolved oxygen at 75–85% of air saturation by pulsed addition of CO_2 or O_2 to the headspace. The agitator was in most runs either a 4 or 6 cm diameter, two-bladed, 45°-pitched blade turbine, although a commercially available streamlined impeller (Model A310, 6.4 cm diameter, 3 blades, Lightnin Mixing Equipment Co., Rochester, NY, USA) at 100 min^{-1} and a flat 6 cm disk at 240 min^{-1} were also tested. Cell counts were done daily using a hypotonic solution of 0.1 wt% crystal violet in 0.1 M citric acid to osmotically disrupt the cells and stain the nuclei, which were counted using a hemacytometer. Half of the medium was changed every three to five days depending on the cell count in the reactor. The low cell counts ($2 \cdot 10^4$ to $5 \cdot 10^5/cm^3$) used in these experiments to avoid contact inhibition of growth minimized the need for medium changes. There was no evidence of nutrient limitation.

Death rate data were collected in the one dm^3 reactors at the end of a growth rate run by removing the serum-containing medium, rinsing the beads with serum-free MEME and refilling the vessel with serum-free medium. The agitator was restarted at the desired speed, and cell counts were measured over several days. The lack of serum prevented cell growth, and the first order decrease in cell number over time was analyzed as a death rate due to agitation. Only growth rate runs having a low amount of bridging between beads were used for the death rate runs.

Experiments to test the effect of bead size and medium viscosity were conducted in 100 cm^3 spinner flasks (Bellco, Vineland, NJ, USA) with the large flat paddle removed from the suspended cylindrical magnetic stirrer (length 4.3 cm, cylindrical diameter 0.9 cm) to simplify power number (N_p) estimation. The cells and medium (80 cm^3 used) were as for the one dm^3 reactor. The bead size runs were stirred at 50 min^{-1}, and used 1.04 g/cm^3 density glass-coated plastic beads (Solohill Engineering, Ann Arbor, MI, USA) with size distributions 90–150 and 150–210 μm diameter. These were the only size ranges commercially available. The mean diameters, 120 and 180 μm, were used for calculation. Only 3.2 kg/m^3 of the smaller beads were used to maintain a constant ratio of bead surface area to liquid volume. In the

viscosity runs, 1.0 or 1.5 wt% dextran (J. T. Baker Chemical Co., Phillipsburg, NJ, USA), 200,000–300,000 molecular weight, was added to the medium and 0.2 μm filter sterilized. Viscosities were measured at 90 s^{-1} on a Rheometrics cone and plate viscometer, and showed no change after seven days of cell growth. These runs were stirred at 60 min^{-1}. All spinner flasks runs were incubated at 37 °C in an atmosphere of 95% relative humidity and 5% CO_2, and half of the medium was changed in the flasks after three or four days of growth.

4 Results

The growth rate data for the runs with the 4 and 6 cm impellers in the one dm^3 reactor are shown in Figs. 1–3, plotted against η/d, eddy *TCS* and shear *TCS*, respectively. Variations in these groups were achieved by operating with different impeller diameters or rotational speeds. In each of these plots ε, the turbulent energy dissipation rate, has been calculated on the basis of dissipation in the volume around the impeller (d_i^3) rather than the entire reactor liquid volume *V*. This has been justified elsewhere (Cherry and Papoutsakis [4]) and provides a much better fit of the data. At low agitation levels using the 6 cm impeller (higher η/d or lower *TCS*) the growth rate decreases due to the increased formation of bridges between beads [4]; those points are not shown. All three parameters are seen to predict the trends of both the 4 and 6 cm impeller data. The difference between the regression lines for the two impellers is most likely caused by an error in the estimation of the power number N_p for each. A value of 0.8 was used for both, since they are of the same design except for diameter. This may not be adequate, since no other dimensions were correspondingly reduced with diameter. Thus geometrical similarity has not been strictly maintained.

The data from the bead size runs are seen in Table 1. The growth rates are lower than the other reported, which may be a consequence of the conditions in the spinner flasks, the different kind of beads used, or some variation in the particular batch of cells or medium used in those runs. However, since the four runs all used the same batch of cells and medium and were conducted in parallel, the trend with bead size is considered valid. The smaller beads give a much higher growth rate. This trend is what would be predicted by the three parameters η/d (equation (1)), eddy *TCS* (equation (3)) and shear *TCS* (equation (4)). The lower concentration of the smaller beads used to maintain a constant bead area/volume does not affect the calculation of η/d, but also decreases the two *TCS* values, which should also improve growth.

The viscosity data are shown in Table 1 also. Solutions of 10% serum and 1–2 wt% dextran are still Newtonian, although viscometric data collected in standard equipment may show them to be shear-thinning because of surface tension effects caused by accumulation of serum proteins at the air-liquid interface [17]. Although there is some difference in the extent of the growth rate increase for a given viscosity increase, the clear trend is that a relatively small change in the medium viscosity (less than doubling it) causes a substantial growth rate improvement.

The first order death rate constants measured in the death rate runs are shown in Figs. 4–6 plotted against the three hydrodynamic damage parameters. As with the growth rate,

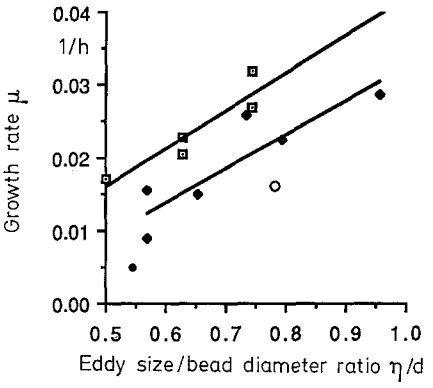

Fig. 1. Growth rate data in one dm^3 reactor plotted against η/d. (◆) 4 cm, (□) 6 cm, (○) A310, (●) flat disk impeller

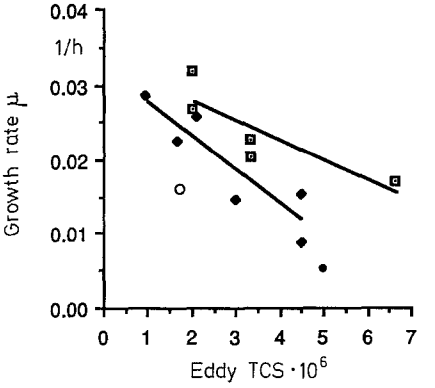

Fig. 2. Growth rate data in one dm^3 reactor plotted against eddy velocity *TCS*. (◆) 4 cm, (□) 6 cm, (○) A310, (●) flat disk impeller

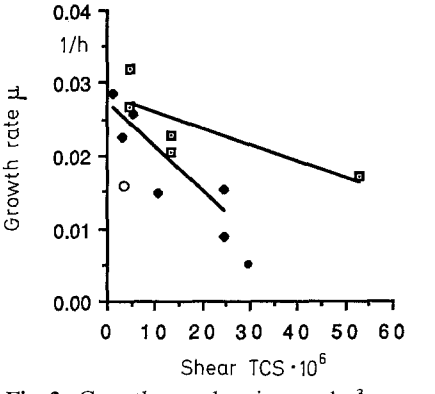

Fig. 3. Growth rate data in one dm^3 reactor plotted against shear *TCS*. (◆) 4 cm, (□) 6 cm, (○) A310, (●) flat disk impeller

Table 1. Bead diameter and viscosity data

Bead diameter μm	Impeller speed min^{-1}	Viscosity cP	Eddy size/bead diameter ratio η/d	Eddy TCS $\cdot 10^8$ $(g \cdot cm^2)/s^3$	Shear TCS $\cdot 10^8$ $(g \cdot cm^2)/s^3$	Growth rate h^{-1}
180[1]	50.0	1.3	2.10	17.8	1.92	0.0042
120[1]	50.0	1.3	3.15	5.4	0.17	0.0134
180	60.0	1.3	1.83	26.8	4.37	0.0242
180	60.0	1.6	2.14	31.3	3.20	0.0304
180	60.0	1.3	1.83	26.8	4.37	0.0227
180	60.0	1.6	2.14	31.3	3.20	0.0252
180	60.0	1.9	2.44	35.6	2.47	0.0273
180	60.0	2.2	2.72	39.7	1.98	0.0313

[1] Glass coated microcarriers used. All others were collagen coated.
All results are average of duplicate parallel runs. Sets done in parallel using the same batch of cells are grouped together. Agitation parameter calculations used impeller diameter 4.3 cm and $N_p = 0.1$ for spinner flasks

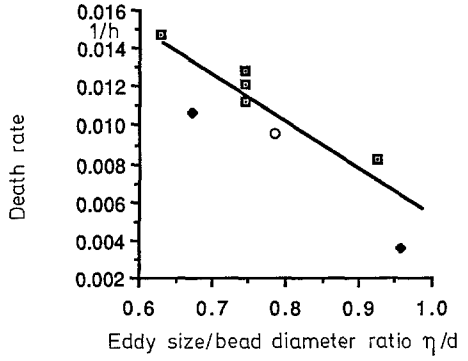

Fig. 4. Death rate data in one dm^3 reactor plotter against η/d. (◆) 4 cm, (□) 6 cm, (○) A310

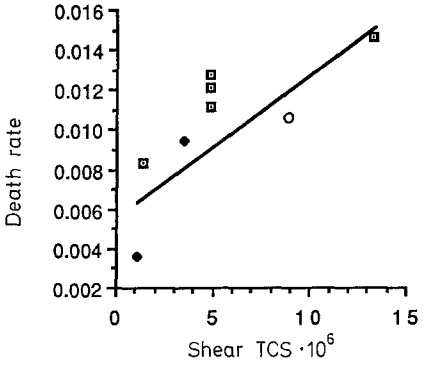

Fig. 6. Death rate data in one dm^3 reactor plotted against shear *TCS*. (◆) 4 cm, (□) 6 cm, (○) A310

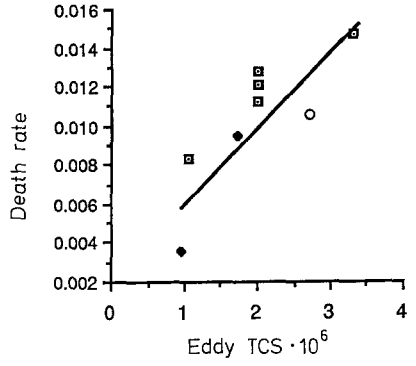

Fig. 5. Death rate data in one dm^3 reactor plotted against eddy velocity *TCS*. (◆) 4 cm, (□) 6 cm, (○) A310

all three give reasonable fits through the points. Two of the three points at $\eta/d = 0.744$ in Fig. 4 are for a duplicate set of runs with 800 cm^3 reactor volume (death rates 0.0112 and 0.0121 h^{-1}). The third run (death rate 0.0128 h^{-1}) was with identical conditions except for a reactor liquid volume of 1200 cm^3. The similarity of results confirms that the impeller local dissipation rate, rather the volume average dissipation rate, is the appropriate parameter for correlating death rate as well as growth rate data.

5 Discussion

5.1 Growth rate results

The observed result (Table 1) for runs with 120 and 180 mm diameter beads is that the smaller beads do indeed lead to higher cell growth rates. This is in line with the predictions of all three damage mechanisms under consideration here. The reduced cell growth rate when bead clumps formed at low agitation rates (data not shown) is also consistent with this, since the clumps are in essence irregularly large microcarriers. Varani et al. [24] found that in a stirred vessel KB cells (derived from a nasopharyngeal carcinoma) grew faster on 100–150 μm diamter glass beads of the type used in this study than on 180 µm diameter dextran beads, despite similar growth rates on each type bead in unagitated flask cultures. Hu [14] also looked at two bead diameters – 180 and

265 μm – although his interest was in the effect on multiplication ratio from the number of FS-4 cells seeded. The bead sizes were tested with different impellers, and the description of those runs was not clear about all the details necessary to calculate eddy size. There was no obvious pattern of better growth for either bead size in Hu's work.

In contrast to decreasing bead diameter, increasing medium viscosity increases the calculated values of both η/d and eddy velocity *TCS*, but decreases the shear *TCS*. This should reduce cell damage, if bead-eddy interactions or shear-caused collisions were the predominant damage mechanism, and increase it, if inter-eddy bead collisions were. These effects come about because higher viscosity leads to larger eddies with a higher characteristic velocity but lower internal shear rate. The observed beneficial effect of higher viscosity supports the eddy size and shear-based *TCS* mechanisms, at least for these cells in spinner flasks.

This viscosity increase is best achieved using high molecular weight polymers to minimize osmotic effects. The use of dextran in microcarrier culture media has been previously reported [22]. The effect seems to be physical rather than chemical, since others have reported advantage to using cellulosic polymers in media [12, 15, 19]. There was no change in the viscosity of the medium between the beginning and end of the run, as might be expected if the dextran were enzymatically attacked for nutrition. Neither have there been reports of effects related to the dextran of the microcarriers themselves.

Returning to the physical damage mechanisms under consideration, the actual means by which increased eddy/bead size ratio might reduce cell damage is not clear. The velocity v_e and the shear stress τ_e within the smallest eddies can be estimated in terms of ν and ε:

$$v_e=(\varepsilon\nu)^{1/4}. \tag{6}$$

$$\tau_e=\mu\left(\frac{v_e}{\eta}\right)$$

$$\tau_e=\varrho_f\nu\left(\frac{\varepsilon}{\nu}\right)^{1/2}$$

$$\tau_e=\varrho_f(\varepsilon\nu)^{1/2}. \tag{7}$$

Decreasing ε by reducing the impeller power input reduces both of these. Changing the bead diameter does not affect these factors in the fluid environment. Increasing viscosity would seem to intensify any interaction between an eddy and the bead. These trends are difficult to reconcile with the experimental results unless the value of η/d reflects the qualitative nature of that interaction. For a change in viscosity from 1.3 to 1.6 cP, a change that produced a significant improvement in growth rate, the calculated increase in eddy size is only 17%, so that any qualitative change must be rather sensitive to the eddy/bead size ratio in this range.

Similarly, a bead in a shear field, which is the assumed situation for the shear-based *TCS*, rotates because of the local velocity gradient and this generates a cyclic shear stress on the surface of the bead [2]. The rotational rate in radians/s is $\gamma/2$, and the bead feels an average shear stress τ_{av} on its surface of

$$\tau_{av}=\frac{1}{2}(\mu)\gamma$$

$$\tau_{av}=\frac{1}{2}(\nu\varrho_f)\left(\frac{\varepsilon}{\nu}\right)^{1/2}$$

$$\tau_{av}=\frac{1}{2}(\varrho_f)(\varepsilon\nu)^{1/2}, \tag{8}$$

where μ is the dynamic viscosity. The maximum shear stress on the bead from rotation in a shear field is six times this average value. This shear stress is similar to that calculated in equation (7), and also increases with increasing viscosity.

In resolving which mechanism is most important, one must also consider that the distinction between interactions with eddies and with other beads may be somewhat artificial. The smallest eddies, the beads, the disturbed flow fields around the beads, and the average interbead spacing are all about the same size. Each clearly has some influence on the others. A cell damage theory incorporating all these effects may prove more successful than one addressing bead-eddy or bead-bead interactions alone. However, the characterization of the structure of turbulence in the presence of a non-negligible volume fraction of large (relative to the smallest eddies) solids is a difficult question that apparently has not yet been addressed.

5.2 Relationship between growth and death rates

The plots of death rate against the parameters representing each of the three cell damage mechanisms (Figs. 4–6) all show at least some evidence of correlation. There are not enough data to choose one mechanism over another at this point.

This death rate data is significant in determining whether the effect of agitation on cells is to reduce the actual growth rate, or instead purely to kill cells that are reproducing at a normal rate, thereby reducing the apparent growth rate. If one assumes that the death rate is a first order process in cell concentration, then during the exponential phase of cell growth:

$$\frac{dX}{dt}=\mu X, \tag{9}$$

$$\mu=\mu_0-k, \tag{10}$$

$$\frac{d\mu}{d\log(\eta/d)}=\frac{d\mu_0}{d\log(\eta/d)}-\frac{dk}{d\log(\eta/d)}, \tag{11}$$

where X is cell count, μ is apparent growth rate, μ_0 is the intrinsic growth rate in the absence of death, and k is the death rate constant. Choosing η/d arbitrarily (either of the *TCS*'s could be used), using the logarithm of η/d in the

derivative eliminates the effect of errors in the estimation of N_p for the variety of impellers used. If N_p is the estimated value, $N_p = (1+e)N_p^*$ where N_p^* is the actual value and $(1+e)$ is the multiplicative error. Substituting this into the expression for η/d (equation (1)) gives:

$$\frac{d\mu}{d\log(\eta/d)} = \frac{d\mu}{d\log[(1+e)^{-1/4}(\eta^*/d)]}$$
$$= \frac{d\mu}{d[\log(\eta^*/d)+\log(1+e)^{-1/4}]}$$
$$= \frac{d\mu}{d\log(\eta^*/d)}, \quad (12)$$

where η^*/d is the actual size ratio (disregarding the error from e), that is the hypothesized actual damage predictor. This correction works only when e is constant for all the data whose slope is being calculated, i.e. only for data trends obtained using one type of impeller throughout the series of points.

If the intrinsic growth rate μ_0 is not a function of agitation, the first term on the right hand side of equation (11) is zero and the negative derivative of the death rate constant with $\log(\eta/d)$ should equal the derivative of the growth rate constant with respect to the same variable. The death rate data of Fig. 4 may be regressed against the logarithm of η/d to give a slope of $-0.047\ h^{-1}$, with 95% confidence limits of $\pm 0.0050\ h^{-1}$ on this estimate. The regressions of the growth data with the four and six cm impeller runs at various speeds give slopes of 0.077 and $0.074\ h^{-1}$, respectively. As explained in the derivation of equation (12), these data must be regressed separately because of possible different errors in the power number estimates. The two data sets have a mean slope of $0.0754 \pm 0.0078\ h^{-1}$ (95% confidence limits, combining the variances of the two individual estimates [1]). The 95% confidence ranges differ appreciably and a comparison of means test [1] indicates that the negative slope of the death rate is different from the slope of the growth rate at more than the 98% confidence level.

Since the numerical values of these derivatives are statistically different, one concludes that the intrinsic growth rate is affected by agitation. However, the death rate is still an important contributor to the change in the apparent growth rate μ. This conclusion is reinforced by the increase in free nuclei in the medium over the course of a run, presumably from cell death [3, 4].

The use of serum-free medium to prevent cell growth in the death rate runs introduces some complicating factors such as possible reduced cell adhesion in serum-free medium and residual growth from adsorbed serum components. Prevention of mitosis (by any means) changes the apparent death rate caused by detachment of weakly attached, "rounded up" dividing cells. Because of this, the relative amounts of death and growth inhibition we report here should be considered approximations. However, these results are internally consistent, and the conclusion that the intrinsic growth rate is reduced is what would be expected over the opposite conclusion that cells grow at the same rate regardless of the physical environment.

This result that cells are actually being killed has great practical importance and provides a real economic incentive to design for the optimum level of agitation. Cell death constitutes a waste of nutrients supplied to the reactor. The biggest problem in this regard is likely to be oxygen, which can be difficult to supply to vessels much above 1–5 dm^3 working volume [10]. More importantly, cell death will cause the release of the intracellular contents into the medium. This at best aggravates the down-stream separation and purification problem, and could possibly lead to increased enzymatic degradation of a desired product.

The fact that a typical death rate measured here is $0.01\ h^{-1}$ indicates that a death event occurs to a particular cell in the order of every 100 h. This seems too infrequent to be a purely fluid mechanical phenomenon in a turbulent system. It most likely represents a balance between a fluid mechanical rate and a biological rate of some kind. Under typical reactor conditions the cells are somewhere between the extreme case of slow growth but no fatal damage, and the other pole of maximum vigor with random death events occurring to otherwise undisturbed cells. The presence of a reduced intrinsic growth rate suggests that manipulation of the cells' biochemistry could improve their resistance to agitation, at least as expressed by the growth rate. The problem is not one of purely hydrodynamic concern, although that is certainly the easier aspect to tackle.

6 Conclusions

The eddy/bead size ratio and turbulent collision severity (*TCS*) were found earlier to correlate growth rate data for bovine embryonic kidney cells in a one dm^3 reactor. A second calculation of *TCS* was derived here to account for a shear mechanism of bead-bead collisions.

Experimental data confirmed that smaller diameter microcarriers give a higher growth rate as predicted by all three of these agitation parameters. Increased medium viscosity also caused higher growth rates. This supports the eddy/bead size ratio and shear-based *TCS* mechanisms, but is opposite to the prediction of the eddy velocity based *TCS*.

The death rate of cells on beads was measured separately, and could be correlated with each of the three agitation parameters. A comparison of measured growth and death rates shows that a significant part of the growth rate change at various levels of agitation can be ascribed to concurrent death of cells, as distinct from changes in the intrinsic growth rate, although both seem to be occurring. The available data do not allow a specific quantification of each individual effect. Death of cells, as opposed to reduced growth, is undesirable because it leads to reduced net cell yields from nutrients and contamination of the growth medium with intracellular material.

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