SHEAR AND MIXING EFFECTS ON CELLS IN AGITATED MICROCARRIER TISSUE CULTURE REACTORS

Robert S. Cherry and E. Terry Papoutsakis
Department of Chemical Engineering
Rice University, Houston, Texas

ABSTRACT

Tissue cells are known to be sensitive to mechanical stresses imposed on them by agitation in bioreactors. The amount of agitation provided in a microcarrier or suspension bioreactor should be only enough to provide effective homogeneity. Three distinct flow regions can be identified in the reactor: bulk turbulent flow, bulk laminar flow, and boundary-layer flows. Possible mechanisms of cell damage are examined by analyzing the motion of microcarriers or free cells relative to the surrounding fluid, to each other, and to moving or stationary solid surfaces. The primary mechanisms of cell damage appear to result from (1) direct interaction between microcarriers and turbulent eddies; (2) collisions between microcarriers in turbulent flow; and (3) collisions against the impeller or other stationary surfaces. If the smallest eddies of turbulent flow are of the same size as the microcarrier beads, they may cause high shear stresses on the cells. Eddies the size of the average interbead spacing may cause bead-bead collisions which damage cells. The severity of the collisions increases when the eddies are also of the same size as the beads. Bead size and the interbead distance are virtually equal in typical microcarrier suspensions. Impeller collisions occur when the beads cannot avoid the impeller leading edge as it advances through the liquid. The implications of the results of this analysis on the design and operation of tissue culture bioreactors are also discussed.

INTRODUCTION

Tissue cells, lacking a cell wall and not being evolutionary adapted to life exposed to a free-flowing liquid phase, are more sensitive to hydrodynamic forces in their environment than are fungi or bacteria. The particular problem usually cited in tissue culture work is shear from the agitator used to suspend the cells.[1-3] Various effects of shear have been reported. Most deal with cell viability,[4-8] while some show increased production rates of some excreted products.[8,9]

Shear has many manifestations within a stirred vessel contained suspended solids, not all of which would be expected to be harmful. This paper will consider the mechanisms by which hydrodynamic forces can affect cells in agitated cell culture reactors, and specifically microcarrier systems.

The effects of bulk liquid turbulence, boundary layers and shear fields, and collisions will each be considered. The results should be useful for rational reactor design and scale-up to any size.

PURPOSES OF AGITATION

Agitation of a cell culture reactor is required to keep the microcarriers from settling out and to assure a homogeneous environment for cell growth. In bacterial fermentations agitation is also used to control the amount of dissolved oxygen by affecting the oxygen transfer rate from the sparged gas into the liquid. In tissue cultures sparging may cause cell lysis and foaming, so other oxygenation systems are often used that diffuse oxygen through a tube or membrane or else oxygenate and recirculate medium from which the cells have been separated.[1,2] Agitation is not critical to oxygenation with those systems,[10] so settling and homogeneity will be considered individually to determine how much agitation is minimally required.

The first item, preventing settling, requires a negligible fluid velocity in the bulk phase. Assuming microcarrier and liquid properties as in table 5-1, Stokes' law gives a terminal velocity v_t of 0.053 cm/s. The maximum shear stress on the bead surface that results from this velocity is ~0.1 dyne/cm^2, well below the 10 dyne/cm^2 that starts to damage kidney cells.[8]

Maintaining homogeneity by minimizing variations throughout the reactor of dissolved oxygen and other nutrient concentrations or temperature is the primary reason for agitating tissue culture reactors. There will inevitably be local variations, for example higher oxygen concentration near the oxygen source, or slightly different temperatures at the wall of a jacketed reactor. We can approximate the average liquid velocity needed to give effective homogeneity by requiring that the cells move through these areas of different conditions in an amount of time that is small compared to their metabolic response time. Although there is apparently no data published for tissue cells, several references for bacteria[11,12] and yeast[13] suggest that cells do not respond to transients of two seconds or less.

Saying that a one liter cell culture reactor has a characteristic dimension of 10 cm, the minimum liquid velocity needed is on the order of 10 cm/(2 s), or 5 cm/s. This is about one hundred times the settling velocity of the microcarriers, so mixing of the liquid that is sufficient to keep cells from lingering in areas of locally different conditions will be more than enough to keep the microcarriers or free cells from settling under gravity's influence.

In addition, mixing and its associated mechanical stresses may be beneficial in enhancing growth and/or product formation due to the physiological effect

of fluid shear stresses.[8,9] In that case, the mixing or agitation of the biorector should be designed to provide the spectrum of stresses that gives the optimal cellular response.

BULK LIQUID TURBULENCE EFFECTS

The structure of isotropic turbulence was originally formulated by Kolmogorov in 1941.[14] The kinetic energy of the velocity fluctuations in turbulent flow is passed from larger eddies to smaller ones with minimal dissipation until, in the smallest eddies, viscous losses degrade the kinetic energy to heat or in this case possibly to mechanical work in physically damaging cells.

The liquid flow in a typical stirred reactor is at least locally turbulent because of the high impeller tip speed and the various probes, thermowells, and sampling tubes that act as baffles. If the scale of the smallest turbulence is sufficiently larger than the microcarriers, the beads just follow the local flow pattern (fig. 5-1a) and move at the local liquid velocity.[15]

Turbulent eddies of the same size as a microcarrier, however, may effect cell performance in several possible ways. A single eddy cannot engulf the bead and can only act on part of the surface, causing the bead to rotate and generating a cyclic shear stress. Frangos et al.,[9] found a 1 Hz shear variation to have a significant positive effect on prostacyclin production. The general effects on cells of other frequencies are unknown. However, in one case[16] fibroblasts gave up to a 30 times increase in specific interferon production when grown on microcarriers in spinner bottles versus on the walls of roller bottles under identical conditions, although no explanation for the increase was offered.

Alternatively, several eddies the size of the microcarrier could interact with it simultaneously. If their actions are opposed to one another, the eddies cause a greater shear stress against the part of the microcarrier nearest them (fig. 5-1b) since the bead cannot rotate to cancel each of the shear forces on it.

Turbulent eddies of the same size scale as the microcarrier separation may also cause cell damage by promoting bead-bead collisions. Eddies much larger than the bead spacing can move groups of beads without causing large relative velocities between them. It is easily conceivable that eddies the size of the interbead spacing could accelerate one bead without disturbing another nearby (fig. 5-1c). The two beads then have a significant relative velocity and a finite chance of collision.

The collision frequency per unit volume N_c for suspended particles is of the order[15]

$$N_c = 0\left[\frac{v_{b,r}\alpha^2}{d^4}\right]$$

where $v_{b,r}$ is the root mean square relative velocity between neighboring particles, α is the volume fraction of beads and d is bead diameter.

Substituting for α as a function of bead spacing, d_s, and setting the relative velocity equal to that of eddies the size of the interbead spacing ($v_{b,r} = v/d_s$ by Kolmogorov's theory),[14,17,18] and since for typical conditions the bead spacing is approximately equal to the bead diameter d,

$$N_c = 0\left[\frac{vk^{7/3}}{d^{8/3}}\right] s^{-1}cm^{-3}$$

where k is proportional to the required bead surface area per reactor volume. Thus, the collision frequency is strongly dependent on the particle diameter when the smallest eddies are the size of the bead spacing. For typical conditions, $k = 2.4\ cm^{-1}$ and $N_c \sim 4{,}000$ collisions/s-cm^3, or roughly one collision per bead every five seconds.

The severity of collisions (SC), defined as the energy (E_c) times the frequency (N_c) of collison, will be of the order of

$$SC = 0\left[\left(m\ v_{b,r}^2\right)N_c\right]$$

or

$$SC = 0\left[\frac{\rho_b \alpha v^3 k^{7/3}}{6d^{5/3}}\right]$$

where m is the mass of an individual bead. The effect of severity of collision on the cells may be hard to quantitate because the cellular responses and severity are unlikely to be linear - if a certain blow kills the cell, hitting it twice as hard does not make it twice as dead - so the net effect is uncertain. It could conceivably be in either direction depending on the relative sensitivity to the frequency and energy terms.

The collisions between beads can have a variety of effects on the cells covering the beads. A head-on collision flattens the cells at the point of collision, possibly rupturing them depending on the energy of collision and the elasticity of the cells. As the collision becomes more and more off-center, the cells in contact between the two beads see less comparison but a larger component of shear force, which will in turn depend on the coefficient of friction of two cells sliding over one another, the cells feel only a shear force. The gross effect of this may be either cell rupture or detachment from the bead surface. The physiological effects of nonfatal compression or mechanical shearing are not known. The analysis of collision is further complicated by any rotation the beads may have, which would in general contribute an additional shearing component to the force of the collision.

The smallest eddy size may also be calculated if the impeller geometry and operating conditions are known. There exists relationships (fig. 5-2) that relate dimensionless power consumption N_p to impeller Reynolds number N_{Re} and specific turbulent energy dissipation ε, and ε to eddy size η:

$$N_{Re} = \frac{d_i^2 n \rho_f}{\mu}$$

$$N_P = \frac{P_g}{\rho_f n^3 d_i^5}$$

$$\varepsilon = \frac{P_g}{\rho_f V} = \frac{N_P n^3 d_i^5}{V}$$

$$\eta = \left(\frac{\nu^3}{\varepsilon} \right)^{1/4}$$

where P is power consumption by the impeller, n is impeller speed in revolutions per unit time, d_i is impeller diameter, and V is the agitated liquid volume.[19] Using typical values, the predicted eddy size is 0.012 cm, which compares with a microcarrier diameter of 0.015 cm and a typical bead spacing of 0.018 cm.

To see the effect of some important reactor variables, the N_p expression for ε is substituted into the expression for eddy size η:

$$\eta = \left(\frac{v^3 V}{N_p n^3 d_i^5} \right)^{1/4}$$

Reactor volume V is fixed by production requirements. N_p varies in a relatively narrow range for reasonable values of N_{Re} so the $\frac{1}{4}$ power of it is ineffectual in significantly changing eddy size. The important factors to change eddy size are $v^{3/4}$, $n^{-3/4}$, and $d_i^{-5/4}$.

In summary then, cells on beads are most affected by turbulence of a size scale the same as the average bead spacing or bead diameter (causing collisions) or the bead diameter (causing rotation or high local shear on the bead surface). In a typical one liter reactor these dimensions are effectively the same, emphasizing the empirical significance of this eddy size. The turbulent eddies may be made larger, and cell damage presumably reduced, by increasing kinematic viscosity or reducing impeller diameter and speed. If the eddy size cannot be sufficiently increased, using a larger bead diameter may reduce the collision frequency, and may, depending on the behavior of the cells, improve the performance of the bioreactor.

BOUNDARY LAYER SHEAR FORCES

Relatively large areas of high shear rate are expected in the boundary layers around the solid objects submerged in the reactor. The moving impeller would be expected to have the highest velocity relative to the liquid, so we shall analyze it in detail to characterize the general effect of boundary layer shear forces on microcarriers. Much of this discussion can also be applied to the hydrodynamically similar case of the physically much larger shear fields expected in a non-turbulent, laminar flow reactor.

As a first approximation marine and angled flat impeller blades can be modelled as stationary flat plates with fluid moving over them. Boundary layer thickness and wall shear stress for both turbulent and laminar boundary layers are shown in figure 5-3. Up to 0.3 cm from the impeller leading edge the two types of flow give significantly different results, but this is also the area where the flat plate assumption of the calculations is least valid and the presence of a microcarrier bead causes the greatest disruption to the boundary layer. Past 1 cm, and over the majority of the blade, the results are similar: there is a boundary layer of 0.1 cm thickness ($\simeq$ 7 bead diameters) with a relatively low shear rate within it.

Within the boundary layer a number of effects may occur (table 5-2). Considering the simpler case of a laminar boundary layer, the bead will certainly try to follow the fluid motion which has components both parallel to and perpendicular to the blade surface. Particle motion parallel to the blade is a combined result of the particle's initial velocity and fluid drag. There is also an effect due to the presence of the solid impeller surface that slows the particle's motion.[20] This retardation is particularly important when the bead is within one radius of the surface. There are two other parallel forces,[15] the Bassett force, which arises from the work necessary to establish a new fluid flow pattern when the bead is accelerated rapidly, and the added mass effect, which accounts for the behavior of the displaced fluid. These terms are negligible over most of the impeller, and are of consequence only at the leading edge.

The fluid velocity which causes the drag force normal to the impeller is a consequence of boundary layer development in an incompressible fluid and is directed away from the impeller. On the upper surface of the blade, gravity opposes the drag force of this normal flow. As with parallel motion, near the wall the hydrodynamic effect of the fixed surface damps any vertical motion.

There is also a lift force derived from the velocity gradient in the boundary layer.[21] This Saffman lift force is present only when the bead has a slip velocity relative to the fluid streamline that would pass through the sphere's center. It acts to move the bead towards the streamlines which most oppose the slip velocity, so for example, a bead moving faster than the local fluid tends to move down the velocity gradient. Near the impeller leading edge the bead will move over the impeller surface faster than the fluid because of its initial inertia, and the lift force will be toward the blade. Further back on the blade fluid drag will slow the bead and the effect of the nearby surface causes the bead to lag the fluid motion. This lag has been demonstrated by Einav and Lee,[22] and the resulting lift force is away from the impeller. There is another lift force acting, from the Magnus effect on a sphere rotating in a constant velocity field. This force is superimposed on the Saffman force in this system. However, Saffman[21] has shown that the Magnus force is negligible compared to the lift caused by the shear field.

The shear field in the boundary layer also causes the bead to rotate.[23,24] A rotational rate on the order of 20 revolutions per second is predicted, similar to the 10 Hz predicted for turbulent rotation. With 250 s^{-1} as the average shear rate in the 0.1 cm boundary layer of the example system, the maximum shear stress on the bead due to this rotation is of the order $3_{\mu\gamma f}$ or 5 dyne/cm^2, a nondestructive level.[8]

Overall, in a laminar boundary layer the microcarrier bead appears well protected from damage. The particle tends to move away from the impeller

surface (except perhaps near the leading edge), it rotates at a moderate speed, and the cells on its surface do no see excessive shear stress. There are no bead-bead collisions either.[23] In a turbulent or separated boundary layer the same basic situation holds except for the additional presence of turbulent eddies (discussed under bulk turbulence). These create the possibility of bead impact against the impeller or other beads because of randomly oriented velocity fluctuations occurring in the boundary layer or intruding from the bulk liquid.

COLLISION DAMAGE

High velocity collisions of a microcarrier against the impeller or other parts of the reactor can occur when the blade advances through the fluid or the fluid flows around a fixed object (fig. 5-4). Microcarriers flowing on a streamline that passes within one particle radius of the surface will collide with the surface, a process called interception.[25] In addition, the microcarriers, being slightly more dense than the fluid, will not follow the fluid streamlines exactly. Inertia will tend to make the microcarrier travel in a straight line rather than flow around the object with the fluid, increasing the chance of collision. The deviation from the fluid streamline will be more severe where the streamlines are most curved, as is the case at the leading edge of the impeller blade. Using potential flow theory to model the streamlines and ignoring bed inertia, one may show that any bead vertically within one bead diameter of the streamline passing through the center of the cylinder used as the leading edge model will hit the impeller.

Considering the width of this collision window, its length (impeller blade length $d_i/2$ times the number of blades n_B) and the velocity of medium through this window, one may calculate that, on average, each microcarrier hits the impeller once each 220 seconds if the entire one liter reactor is well mixed. This is 1/40 the frequency of bead-bead collisions, but the energy of collision of 2500 times greater because of the higher relative velocity.

The collision rate is proportional to the agitator speed since inertial effects on the width of the collision window are not included in this calculation. In addition, the kinetic energy of the collision is much higher, increasing with bead mass and the square of impact velocity which is proportional to agitator tip speed. Combining these effects,

$$SC_i = N_{c,i}\, E_{c,i} = 0\left[\frac{\pi n_B d_i dn}{2V}\right] 0\left[\frac{m}{2}\left(\pi n \frac{3}{4} d_i\right)^2\right] = 0\left[\frac{3}{128}\pi^4 \frac{\rho_b n_B n^3 d_i^4 d^4}{V}\right]$$

Severity of collisions with the impeller is proportional to the cube of the agitator speed and the fourth power of impeller diameter and bead diameter. Since tip speed equals πnd_i, note that there is also a third power dependence on tip speed. However, as with bead-bead collisions in turbulence, the effect of collision severity (as defined here) on such things as cell viability or maximum cell density is certainly not linear, and may even have a minimum or maximum within the practical range of severity values.

The nature of the surface the cell covered bead hits will affect the amount of cell damage that results. A hard surface will concentrate the total collision force on one or two cells directly in contact with the surface, and will perhaps cause the bead to distort and disrupt cell attachment. An elastic impeller coating softer than the bead could both absorb some of the collision energy and distribute the remainder over a broader area on the bead, reducing the force that indiviual cells are subjected to.

Smoothness of the impeller surface is important too, to avoid spikes or sharp-edged holes or ruts that could cause damage during what might only have been a glancing impact. Such surface roughness would be significant at the scale of the individual cells' dimension - about 10 μm. Avoiding this potential problem requires a very smooth surface, suggesting that polishing of machined, cast, or welded impellers would be of benefit.

IMPLICATIONS FOR REACTOR DESIGN

Two effects stand out as likely causes of cell damage or poor performance in microcarrier tissue culture reactors: turbulence of a size scale comparable to the microcarriers or the spacing between them, and collisions with solid objects, particularly the impeller. The smallest eddies in a turbulent flow are characterized by a length scale $\left(\frac{\upsilon^3}{\varepsilon}\right)^{1/4}$. This size has been increased empirically by reducing ε, the local energy dissipation rate, through such design changes as eliminating baffles, using marine rather than paddle impellers, reducing agitator speed, and using hemispherical rather than flat reactor bottoms. Each of these reduces turbulence, hence ε, in some part in the reactor.

Further advances in increasing the scale of turbulence can be achieved by raising the fluid kinematic viscosity. Because the turbulence scale depends on $\nu^{3/4}$ compared to $\varepsilon^{1/4}$, the effect should be much stronger. To minimize osmotic effects, high molecular weight polymers or gums are good candidates to add to the culture medium. High polymers are also known to reduce drag, and therefore agitator power consumption, further increasing eddy size. A beneficial effect of polymer addition on free-living human lymphoblastoid

cells has been reported,[26] although the effect was hypothesized to be mechanical protection of the cells by adsorbed polymer and possibly related to surface tension.

The size of the microcarrier beads should be optimized for each application. In systems where impeller collision is the primary source of damage, smaller beads have a lower collision frequency and a lower kinetic energy of collision. If bead-bead collision in turbulent eddies is the major damage mechanism, decreasing bead size lowers the collision energy, but raises the frequency. Depending on which factor is more important, the optimal bead size may be either smaller or larger.

Collision damage can be minimized by rational impeller design. The smallest impeller, in terms of both diameter and number of blades, that gives adequate mixing should be used. Streamlining the blade cross-section, and in particular rounding the leading edge, will reduce the number of collisions. As noted already, polishing any rough surface and applying an elastic coating would mitigate the effects of any collisions that do occur.

Recalling that mixing is needed primarily to prevent relatively stagnant zones from forming, it is desirable to establish a large scale circulation without extremes of velocity. This could be accomplished and impeller collision estimated by using externally recirculated liquid for mixing rather than using an agitator. The turbulence around the return jets must be considered, but could be controlled by limiting the jet velocity. The biggest problem is likely to be developing an effective way of separating the microcarriers from a relatively large flow of culture medium so that they would not be damaged in the circulation pump.

Alternatively, it is possible to utilize secondary flows generated by a very low speed agitator to provide reactor mixing,[27] although this may not be sufficient as reactor size increases. This may be the operating principle behind the successful use of soft spiral vanes for agitation.[2]

CONCLUSIONS

By analyzing the phenomena involved in agitation of microcarrier suspensions, harmful effects on cell cultures that have been attributed to "shear" are found to be better explained as effects of turbulence or collision. Changes meant to reduce shear have also reduced turbulence and collision, leading to improvements in the practice of cell culture. Other new approaches to reducing turbulence and collision are proposed. Studies to determine the response of cells to intermittent cyclic shear stress at frequencies in the range of 5 to 30 Hz are suggested.

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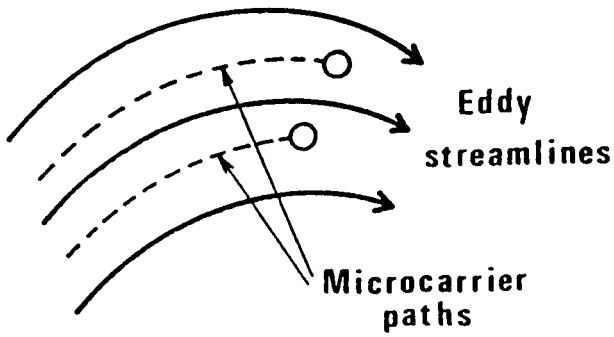
TABLE 5-1.- REPRESENTATIVE MICROCARRIER REACTOR SPECIFICATIONS

Liquid	
Volume, V	1 liter
Density, ρ_f	1.0 g/cm^3
Viscosity, μ	0.007 cP
Microcarrier beads	
Shape	Smooth spheres
Radius, R	75 μm
Density, ρ_b	1.03 g/cm^3
Concentration, α	
- dry basis	5 g/liter
- hydrated	7 vol %
Impeller	
Configuration	4 rectangular blades at 45° angle
Diameter, d_i	8 cm
Blade width, w	3 cm
Leading edge radius, R_i	0.1 cm
Rotational speed, n	60 rpm
Tip speed, v	25 cm/s

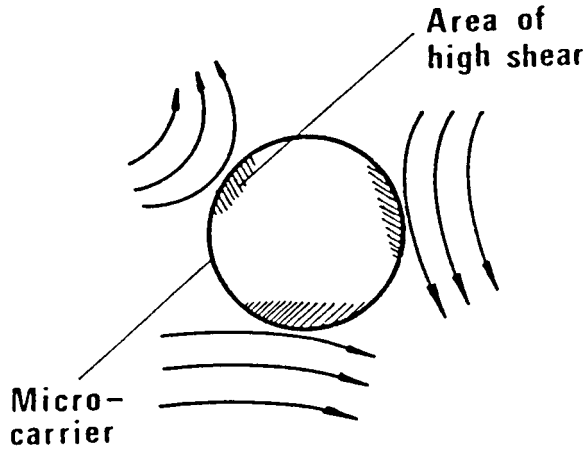
TABLE 5-2.- BOUNDARY LAYER FORCES

Force	Resultant bead motion relative to surface
Fluid drag	Parallel, normal, and/or rotational
Gravity and buoyancy	Normal
Effect of pressure gradients*	Parallel, and/or normal
Saffman lift force[21]	Normal
Added mass effect**[15]	Parallel
Bassett force**[15]	Parallel
Magnus force**[21]	Normal

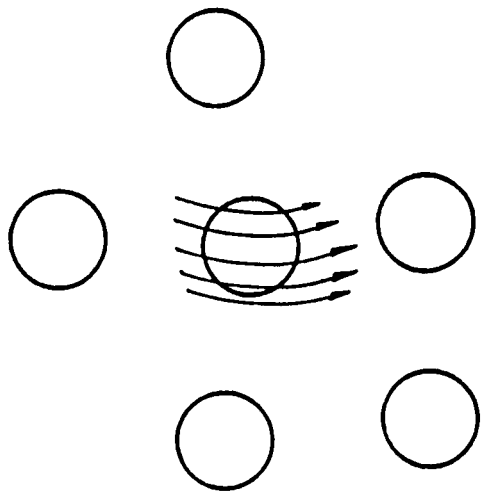
* Important only in turbulent boundary layers
** Not important in this system



(a) Eddies much larger than the beads.



(b) Multiple eddies same size as bead.



(c) Eddy size same as interbead spacing.

Figure 5-1.- Bead-eddy interactions.

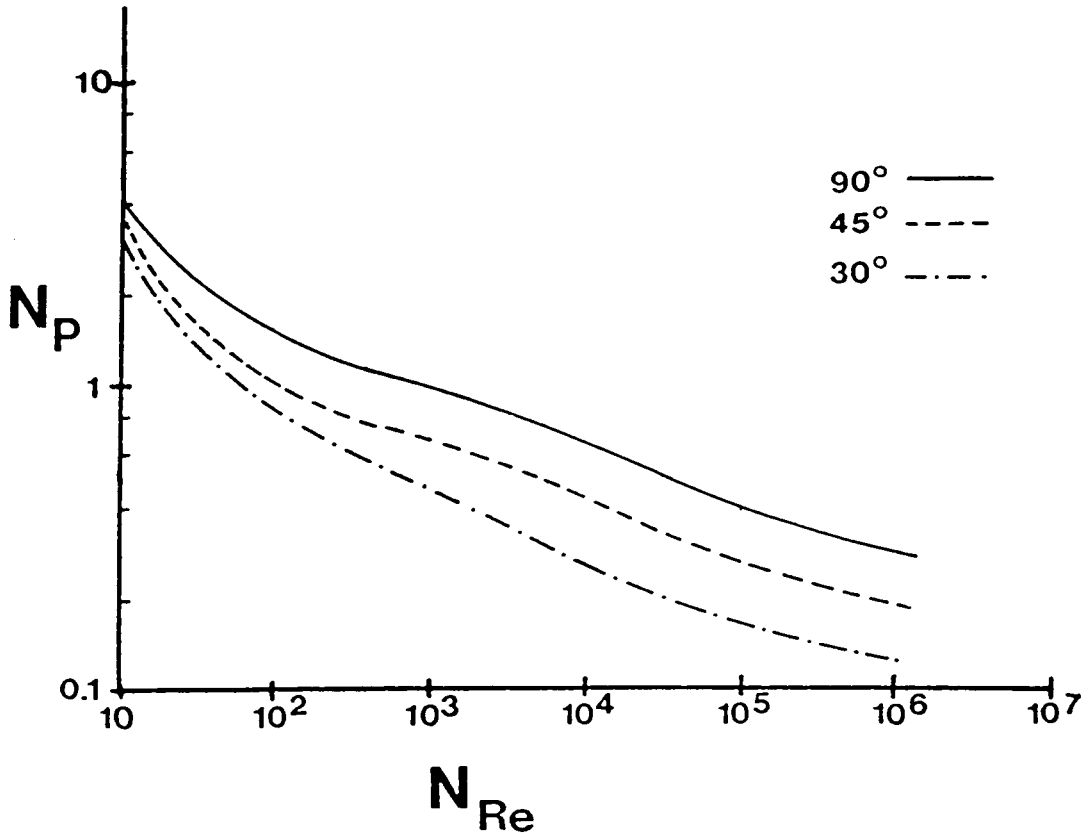


Figure 5-2.- Power number correlation for two-bladed impeller at various blade angles. Adapted from Nagata (fig. 1.21).[18]

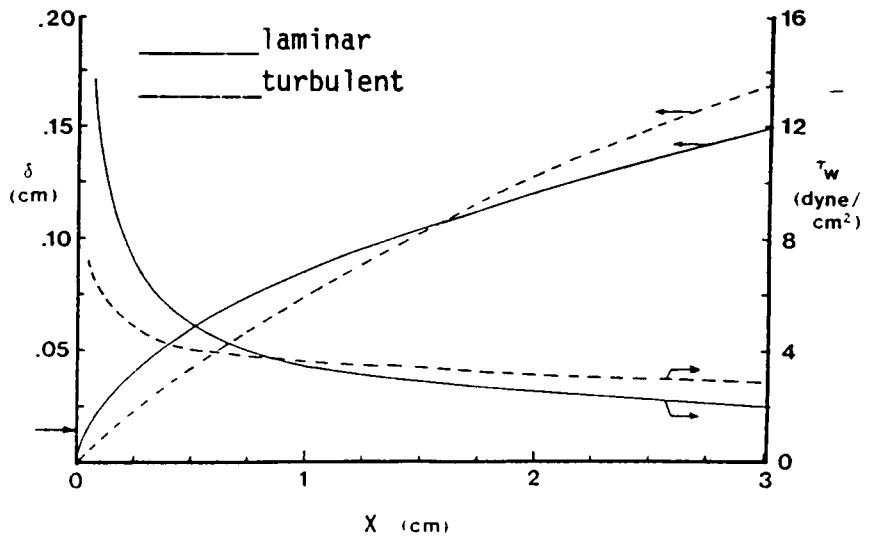


Figure 5-3.- Boundary layer thickness and wall shear stress on the impeller. Arrow indicates microcarrier diameter.

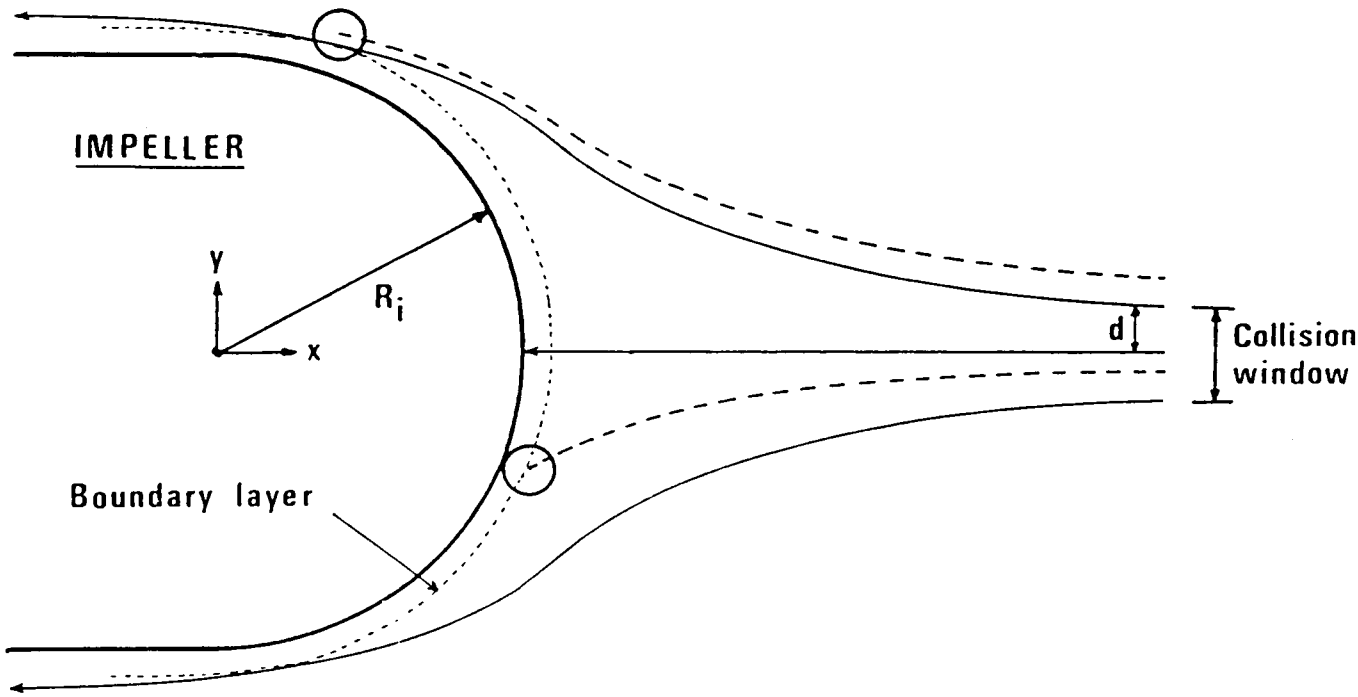


Figure 5-4.- Streamlines around impeller leading edge.
Beads inside the collision window strike the impeller.