INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Studies on the characterization of the hydrodynamic environment in sparged animal cell cultures

Garcia-Briones, Miguel Angel, Ph.D.

The Ohio State University, 1994
STUDIES ON THE CHARACTERIZATION OF THE HYDRODYNAMIC
ENVIRONMENT IN SPARGED ANIMAL CELL CULTURES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By
Miguel Angel Garcia-Briones

*****

The Ohio State University
1994

Dissertation Committee:

J.J. Chalmers
S.T. Yang
K.W. Koelling

Approved by

Adviser
Department of Chemical Engineering
To Zoila Amparo
ACKNOWLEDGMENTS

I like to express sincere appreciation to Dr Jeff J. Chalmers for his guidance and insight throughout the realization of this work. Thanks go to the other members of my committee, Dr. S. T. Yang and Dr. K. W. Koelling for their suggestions and comments. Gratitude is expressed to Dr. R. Leyva for have believed in me. Thanks to the members of my research group for their encouragement and friendship. To my wife Zoila for her support and love. To my children, Miguel Angel and Zoila Anareli for understanding my frequent absences. Finally, I want to thank my parents for their love during every day of my life.
<table>
<thead>
<tr>
<th>Year Range</th>
<th>Event Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>January 8, 1962</td>
<td>Born - San Luis Potosi, S.L.P. Mexico</td>
</tr>
<tr>
<td>1982</td>
<td>B. S., Chemical Engineering, Autonomous University of San Luis Potosi, San Luis Potosi, Mexico.</td>
</tr>
<tr>
<td>1984-1988</td>
<td>Compounding Engineer, General Tire of Mexico, San Luis Potosi, Mexico</td>
</tr>
<tr>
<td>1988-1989</td>
<td>Research Associate, Department of Chemical Engineering, Autonomous University of San Luis Potosi, San Luis Potosi, Mexico.</td>
</tr>
<tr>
<td>1989-1992</td>
<td>M.S. Chemical Engineering, The Ohio State University, Columbus, Ohio</td>
</tr>
<tr>
<td>1992-Present</td>
<td>Research Assistant, The Ohio State University, Columbus, Ohio</td>
</tr>
</tbody>
</table>


FIELDS OF STUDY

Major Field: Chemical Engineering
TABLE OF CONTENTS

ACKNOWLEDGMENTS ................................................................. ii
VITA ........................................................................................ iv
LIST OF TABLES ...................................................................... x
LIST OF FIGURES ..................................................................... xi

CHAPTER PAGE

I. INTRODUCTION .................................................................... 1
  1.1 Background ................................................................. 1
  1.2 Literature review .......................................................... 3
    1.2.1 Response of cells to hydrodynamic environments .... 3
      1.2.2.1 Effects of viscometric flows and flows in parallel
               plate chambers on anchorage dependent cells ...... 3
      1.2.1.2 Effects of viscometric flows on suspended cells ... 5
      1.2.1.3 Effects of fluid flow in capillary tubing on suspended
               cells .......................................................... 6
      1.2.1.4 Effects of agitation and/or sparging on suspended
               cells ........................................................... 7
      1.2.1.5 Effects of agitation on anchorage dependent cells .. 9
    1.2.2 Mechanisms and formulation of models for cell death
        in bioreactors ......................................................... 10
      1.2.2.1 Mechanisms and models of cell death for anchorage
              dependent cells ............................................. 10
      1.2.2.2 Mechanisms and models of cell death for suspended
              cells .......................................................... 12
      1.2.3 Protective additives .............................................. 16

II. CELL-BUBBLE INTERACTIONS: MECHANISMS OF
    SUSPENDED CELL DAMAGE .......................................... 21
    2.1 Summary ................................................................. 21
2.2 Introduction ................................................................................ 21
2.3 Visual observations of cell-bubble interactions ............................24
2.4 Materials and Methods .............................................................. 27
  2.4.1 Cells and Medium ................................................................. 27
  2.4.2 Method of drop capture ...........................................................27
  2.4.3 Equipment for visualization ................................................. 28
2.5 Results ......................................................................................... 29
  2.5.1 Visualization of cells attached to the bubble film .............. 29
  2.5.2 Visualization of cells in the upward jet............................. 29
  2.5.3 Cells in the jet drops ............................................................ 30
  2.5.4 Control experiments .......................................................... 30
2.6 Discussion .......................................................................................32

III. COMPUTER SIMULATION OF THE RUPTURE OF A GAS
BUBBLE AT A GAS-LIQUID INTERFACE AND ITS
IMPLICATIONS IN ANIMAL CELL DAMAGE .................. 40
  3.1 Summary ..................................................................................... 40
  3.2 Introduction ................................................................................ 41
  3.3 Software and computer system ................................................. 42
  3.4 Initial conditions .......................................................................... 43
  3.5 Problem description ........................................................................44
  3.6 Numerical description ................................................................. 45
  3.7 Comparison of the computer solution with actual photographs 46
    3.7.1 Erratic behavior of the bubble rupture process ............47
    3.7.2 Proximity of the walls and meniscus .................................47
    3.7.3 Influence of the film rupture ...................................................48
  3.8 Resultant stresses ...........................................................................48
  3.9 Grid refinement ............................................................................ 49
  3.10 Proximity of the outer and bottom boundaries ....................... 50
  3.11 Discussion of the computer solution ...........................................52
  3.12 Analysis of the hydrodynamic information obtained from
      the computer solution ................................................................. 53
  3.13 Region of maximum values for the dissipation function ...... 58
  3.14 Comparison with data from the literature ............................ 59
    3.14.1 Well defined flow devices .....................................................59
    3.14.2 Mixed bioreactor experiments ................................. 61
  3.15 Discussion of results .....................................................................61
3.16 Conclusions ................................................................. 63
3.17 Acknowledgements ......................................................... 64
3.18 Notation ........................................................................... 65

IV. FLOW PARAMETERS ASSOCIATED WITH HYDRODYNAMIC CELL INJURY ........................................... 87

4.1 Summary ........................................................................ 87
4.2 Introduction .................................................................... 88
4.3 Relevant flow parameters .................................................. 93
4.4 Relevance of Taylor experiments to hydrodynamic related cell injury ......................................................... 96
  4.4.1 Anchorage dependent cells ........................................... 96
  4.4.2 Suspended cells ......................................................... 97
4.5 Flow classification ............................................................ 98
4.6 Flow analysis .................................................................... 101
  4.6.1 Computer solution ...................................................... 101
  4.6.2 Computation of the state of stress and flow classification parameters .................................................. 102
4.7 Results ............................................................................. 103
4.8 Discussion ........................................................................ 104
4.9 Comparison with data from the literature ......................... 105
4.10 Conclusions and closing remarks .................................... 106
4.11 Acknowledgements .......................................................... 107
4.12 Notation ........................................................................... 108

V. CHARACTERIZATION OF A SPARGED FLOW FIELD USING A PARTICLE TRACKING VELOCIMETRY ALGORITHM ................................................................. 117

5.1 Summary ........................................................................ 117
5.2 Introduction .................................................................... 117
5.3 Particle tracking velocimetry ............................................. 118
5.4 Materials and methods .................................................... 120
  5.4.1 Experimental apparatus .............................................. 120
  5.4.2 Concentration of flow markers ..................................... 121
  5.4.3 Dying of flow markers ............................................... 121
  5.4.4 Density matching ....................................................... 122
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Frequencies of cells with resultant stresses ($\tau_{re}$) in the indicated range over the calculation time of $2.0 \times 10^{-3}$ s for the 35x55 and 70x110 grids</td>
<td>82</td>
</tr>
<tr>
<td>2. Frequencies of cells with resultant stresses ($\tau_{re}$) in the indicated range over the calculation time of $2.0 \times 10^{-3}$ s for three different grid arrangements where the boundaries were set at 2, 50 and 100 bubble diameters (bd)</td>
<td>83</td>
</tr>
<tr>
<td>3. Comparison of parameters for three bubble sizes</td>
<td>84</td>
</tr>
<tr>
<td>4. Dissipation function for experiments in well defined flow devices</td>
<td>85</td>
</tr>
<tr>
<td>5. Average dissipation function for mixed bioreactors experiments</td>
<td>86</td>
</tr>
<tr>
<td>6. Times and maximum calculated values of $\Gamma^2(\bar{\tau})$, $R_D$, and $(1-R_D)\Gamma^2(\bar{\tau})$ for each frame in Figures 21-23</td>
<td>114</td>
</tr>
<tr>
<td>7. Maximum calculated values of the state stress [$\Gamma^2(\bar{\tau})<em>{\text{max}}$] and flow classification parameter ($R_D$) at $\Gamma^2(\bar{\tau})</em>{\text{max}}$ for three different bubble sizes</td>
<td>115</td>
</tr>
<tr>
<td>8. Experimental data showing the magnitude of $\Gamma^2(\bar{\tau})$ and $R_D$ that results in cell disruption</td>
<td>116</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Photographs of video images of cells attached to rising bubbles (a,b) and cells trapped in the foam layer (c,d). Arrows indicate attached/trapped cells(s)</td>
<td>34</td>
</tr>
<tr>
<td>2. Experimental apparatus used to capture jet drops from a rupturing bubble</td>
<td>35</td>
</tr>
<tr>
<td>3. Photographs of a gas bubble on the air-medium interface before bubble rupture. Cells on the bubble film can be faintly observed. Part 3a is just before bubble rupture, whereas part 3b is just after bubble rupture. An arrow in part 3b indicates the captured drop</td>
<td>36</td>
</tr>
<tr>
<td>4. Photographs of a bubble resting at the air-medium interface. Cells appear as small white spots on the bubble film. Part 4a is immediately after the bubble came to rest on the interface, whereas part 4b is one minute later. Part 4c is a bubble in serum-free medium, whereas part 4d is a bubble in medium containing 0.2% Pluronic F-68. A &quot;zone of clearing&quot; that does not contain cells is indicated by the arrows in part 4b and 4c</td>
<td>37</td>
</tr>
<tr>
<td>5. A photograph of the liquid jet that results from a bubble rupture. Cells are faintly observable in this photograph and one cell, or group of cells is indicated by an arrow</td>
<td>38</td>
</tr>
<tr>
<td>6. Part 6a is a photograph of a jet drop, which resulted from the first bubble rupture in a sample. Part 6b is a photograph of the same jet drop as in part 6a after an equal volume of 1.0% trypan blue in balanced salt solution had been added. Dead cells appear dark</td>
<td>39</td>
</tr>
<tr>
<td>7. Bubble shape solution. When properties of water and air are considered the actual diameters (maximum diameters) are 0.77, 1.70 and 6.32 mm (Medrow, 1968)</td>
<td>67</td>
</tr>
</tbody>
</table>
8. Computational grid. Thirty five cells are used in x (radial direction) and 55 in z. The bubble shape at time = 0 s is shown. The outer and bottom boundaries are set to about two bubble diameters from the still liquid level and axis of symmetry (left side), respectively ........................................ 68

9. Free surface plot at time = 0 s showing the azimuthal segment used in the computation. The bubble is 1.7 mm in diameter ................................................................. 69

10. Comparison of high-speed photographs (Woodcock et al., 1953) and computer results from the 1.7 mm diameter bubble. (Reprinted with permission from Nature 172, 1144, 1145, copyright 1953 Macmillan Magazines Limited) ...................... 70

11. Free surface plots for the 35x55 (left) and 70x110 (right) grids. Time from top to bottom is: 0.0, 8.0x10^-4, 1.4x10^-3, and 2.0x10^-3 s ................................................................. 71

12. As the total number of cells decreases the imposed condition to calculate stresses (fluid fraction equal to 1 for the cell being considered and its surrounding cells) becomes more restrictive. Most of the stresses in the higher range (>50 dyne cm^-2) are located at cells close to the bubble cavity interface where high rates of deformation occur ....................... 72

13. Free surface plots for grid arrangements where the outer and bottom boundaries were set at 2 (left), 50 (middle), and 100 (right) bubble diameters (bd) at selected times. The window used is the same to facilitate comparison. Time from top to bottom is 0.0, 8.0x10^-4, 1.4x10^-3, and 2.0x10^-3 s ..................... 73

14. Fluid fraction (left) and dissipation function distribution (right) for a 0.77 mm diameter bubble. Time from top to bottom is 0.0, 1.0x10^-4, 2.0x10^-4, and 3.0x10^-4 s ..................... 74
15. Fluid fraction (left) and dissipation function distribution (right) for a 1.7 mm diameter bubble. Time from top to bottom is 0.0, 4.0x10^-4, 8.0x10^-4, and 1.2x10^-3 s .......................... 76

16. Fluid fraction (left) and dissipation function distribution (right) for a 6.32 mm diameter bubble. Time from top to bottom is 0.0, 2.0x10^-3, 4.0x10^-3, and 5.2x10^-3 s .......................... 78

17. Fluid fraction (left) and dissipation function distribution (right) corresponding to the time when the maximum is calculated. Time, bubble size, and range for the gray scale used to plot the dissipation function are indicated .......................... 80

18. Source regions for the material going into the upward jet for a 2.07 mm bubble (MacIntyre, 1972). The downward jet is not shown ........................................................... 81

19. (a) Four-roll mill apparatus. When the rollers are rotated at the same speed in the directions indicated, an extensional flow is produced in the central area. The principal directions of the rate of strain coincide with the x and y axes. (b) Drop interactions with an extensional flow field (drop placed at center) .................................................. 109

20. (a) Parallel band apparatus. A simple shear flow is produced when the rollers are rotated as indicated. The principal directions of the rate of strain are shifted 45° with respect to the x and y axes. (b) Drop interactions with a simple shear flow field (drop placed at center) .................................................. 110

21. Distribution of the state of stress, [I_2(\vec{F})] in the flow produced when a 1.7 mm bubble breaks at a gas-liquid interface. Times and maximum calculated values for the state of stress corresponding to the frame numbers are given in Table 6 .... 111
22. Distribution of the parameter $R_D$ plotted as $1-R_D$. Times and values for $R_D$ corresponding to the frame numbers are given in Table 6 .......................................................... 112

23. Distribution of strength of the flow $(1-R_D)l'_2(f)$. Times and maximum calculated values for $(1-R_D)l'_2(f)$ corresponding to the frame numbers are given in Table 6 ........................................ 113

24. Photograph showing the experimental set up. One side mirrors was removed in this picture to allow the square column to be seen ................................................................. 145

25. Top view of the mirror arrangement used to acquire stereo images of the flow region. The enlargement shows the ground joint of the central mirror ................................................. 146

26. Processing steps involved in the PTV algorithm ........................................ 147

27. The top view of the flow system shows the positions where the targets were placed to span the region of interest. The side view shows a stereo image of the capillary with the targets inside placed at the center of the x-y plane. The z axis is perpendicular to the plane of the page ................................................................. 148

28. Formation of tracks on each of the stereo images. The search of tracks grows in a tree structure fashion with one branch for every possible displacement .................................................. 149

29. A stereo match is found as the near intersection of optical rays. The position of the particle is determined as the midpoint between the line joining optical rays ........................................ 150

30. Validation of the measured velocity field (top) involves first smoothing the velocity field (middle). Each velocity vector is then compared with the smoothed field in its immediate vicinity and vectors are discarded if large differences are found (bottom) 151
31. Raw image of the bubble-flow experiment as acquired from the video tape recorder with the frame grabber (after rotation and splitting) ................................................................. 152

32. Raw image after substraction of the background and stretching ............................................. 153

33. Sequence of left and right images analyzed with the PTV algorithm. Images were recorded at 60 frames per second. Frames 1 (top) and 2 (bottom) ..................................................... 154

34. Typical image where particles that have been identified by the PTV algorithm are shown with a cross ...................................................... 160

35. Raw velocity vectors projected on the y-z plane for frames 3 (left) and 4 (right). Measurement of the velocity field was restricted to a central region. Units of the horizontal and vertical axes are cm. Scale for velocities is given in cm sec\(^{-1}\) .................................. 161

36. Interpolated velocity vectors projected on a plane perpendicular to the x axis and passing through the origin of the coordinate system. Units of the horizontal and vertical axes are cm. Scale of velocities is given in cm sec\(^{-1}\). The velocity field shown corresponds to frames 3 (left) and 4 (right) ......................... 165

37. Distribution of the second invariant of the stress tensor for frames 3 (left) and 4 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the second invariant is given in dyne cm\(^{-2}\) ................................................ 169

38. Distribution of the rate of energy dissipation for frames 3 (left) and 4(right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the rate of energy dissipation is given in erg cm\(^{-3}\) sec\(^{-1}\) ................................................ 173
CHAPTER I

INTRODUCTION

1.1 Background

Aims of in vitro cultivation of animal cells can be broadly divided in two categories: cell research and manufacture of biological products. In the latter case the product may be the cells themselves, cell components, or metabolites which are produced naturally or by expression of recombinant DNA. Metabolites of commercial value which have been produced using animal cells include hormones, growth factors, antibodies etc. Because of their complex structure, most of these metabolites cannot be produced using bacterial systems. Cultured animal cells which require an external surface for attachment and proper propagation are called anchorage dependent cells, while those which propagate without surface attachment are called anchorage independent or suspended cells. For proper cell propagation the cell culturist needs to provide an in vitro environment as similar as possible as the natural cell environment. This includes the provision of appropriate nutrients (including oxygen) and control of physicochemical factors such as temperature and pH.
Provision of nutrients not only implies their addition to the culture medium but also the maintenance of a uniform concentration (throughout the bioreactor in which the cells are grown). Uniform conditions are also important to prevent regions with high concentration of cell waste products which may inhibit cell growth. Homogeneous conditions in the bioreactor are accomplished by mixing which results from mechanical (such in the so called mixed bioreactors) or pneumatic (such in air-lift and bubble columns) agitation. Since mixing is accomplished by deformation of fluid elements, hydrodynamic stresses are transmitted to the cells (which may be suspended or attached to a surface). It is important to point out that hydrodynamic stresses from sources other than mixing also occur in a bioreactor and will be further discussed in the following sections. External stresses elicit a variety of cell responses and in the extreme case cells may be inactivated and die. In contrast to bacteria, animal cells are more sensitive to hydrodynamic effects. This sensitivity becomes evident by the difference in maximum cell number and fraction of viable cells observed in static cultures (non agitated cultures in tissue flasks with working volume between 5 to 25 ml) as compared to bioreactor cultures (mixed or sparged containers with a working volume which may range from 0.5 to several thousands of liters). Hydrodynamic events occurring in a bioreactor or other flow devices which affect the cell integrity and lead to a reduced maximum cell concentration have been called in the literature collectively as "shear effects". Consequently the sensitivity of animal cells related to hydrodynamic factors has been referred to as "shear sensitivity" even though flows other than simple shear flows are being considered. This terminology will be used in this manuscript.
1.2 Literature review

Three areas of research can be distinguished in the literature related to hydrodynamic effects on cultured animal cells: 1) response of cells to hydrodynamic environments, 2) mechanisms and formulation of models for cell death in bioreactors, and 3) cell protective additives.

1.2.1 Response of cells to hydrodynamic environments

Because animal cells are sensitive to hydrodynamic environments, extensive work has been directed to study the response of cells to different flows such as those occurring in parallel plates, viscometers, capillary tubing and agitated and/or sparged containers. The response of cells to these flows has been observed to consist of changes in morphology, structure, metabolism (non lethal effects) and proliferation (lethal effects).

1.2.1.1 Effects of viscometric flows and flows in parallel plate chambers on anchorage dependent cells

Viscometric flows and flows in parallel plate chambers have been used to study the response of anchorage dependent cells to hydrodynamic stresses. A monolayer of cells is grown on a flat surface and subsequently subjected to laminar shear stress (only few reports have used turbulent regime). Most of the studies have been done using endothelial cells and
are contained within the biomedical engineering literature. These studies have been mainly
directed to improve the understanding of vascular diseases such as artherosclerosis. Studies
performed using endothelial cells have usually used non lethal levels of shear stress similar to
those occurring in the vasculature. Depending on the endothelial cell type, time of shear
exposure, substrate to which the cells are attached and medium composition, cells have been
observed to undergo a variety of responses. These include elongation and orientation of cells
in the direction of flow, rearrangement of cytoskeletal components, changes in extracellular
matrix organization (and adhesion properties) and signal transduction. A central question
which still remains to be answered is how the cells recognize and transduce hydrodynamic
stresses. In addition to non lethal effects, it has also been observed that at sufficiently high
levels of shear stress cells detach from their surface support and die. For a recent review in
shear effects on endothelial cells see Girard et al. (1993).

In addition to endothelial cells, other anchorage dependent cell lines used to produced
biological compounds of commercial value (so called "cells of biotechnological interest") have
also been studied in viscometric flows and flows in parallel plates. The main goal however,
is not only to measure non lethal responses such as the stimulation of cell metabolism, but
also to characterize the level of shear that the cell can withstand before its removal from its
surface support. Shear sensitivity of cells is an important piece of information for the design
of bioreactors used to grow the cells at large scales. As with endothelial cells, extensive cell
removal occurs as the shear stress is increased.
1.2.1.2 Effects of viscometric flows on suspended cells

There exist numerous reports in the biomedical literature regarding shear stress effects in blood related cells. These studies are aimed to characterize the possible effects of blood cells in artificial devices used to support human life. In such devices blood cells not only should maintain their physical integrity, but also their physiological functions. An additional complexity is that blood cells are sensitive not only to fluid flow, but also to contact with foreign materials. Studies with red blood cells (Leverett et al., 1972; Morris and Williams, 1979; Sutera et al., 1972) indicate that the extent of cell damage depends on both the level of shear and time of exposure. Other shear stress effects include platelet aggregation (Jen et al., 1984), decrease in red blood cell deformability (O'Rear et al., 1982), reduction in leukocyte phagocytic response and increase in adhesiveness (Martin et al., 1979). MacIntyre et al. (1987) reported activation of arachidonic acid metabolism in human platelets and leukocytes. Chittur et al. (1988) observed a reduction in the proliferative response of T cells at low shear stresses. Decrease in cell viability with shear stress has also been reported for tumor cells (Brooks, 1984; Koyama et al., 1987).

Hybridoma (Smith et al., 1987; Petersen et al., 1988, 1990; Schurch et al., 1988; Abu-Reesh and Kargi, 1989) and insect cells (Tramper et al., 1986; Goldblum et al., 1990) have also been studied in viscometric flows. Smith et al. (1987) correlated cell damage to shear rate. They observed exponential growth for hybridoma cells subjected to a shear rate of 420 s\(^{-1}\) for 15 hours. Cell damage, however, measured as a reduction of viable cell count and
increase in LDH activity occurred at a shear rate of 8501. Petersen et al. (1988) found that cell viability decreased with increasing levels of shear and times of exposure. Cell sensitivity was dependent on the history of agitation used to grow the cells, and batch culture phase. That is, cells in lag and stationary phase were seen to be less shear resistant. Schurch et al. (1988) found that cells which remained viable after shear exposure did not show any change in growth and antibody production rates as compared with controls. Abu-Reesh and Kargi (1989) used both laminar and turbulent flow to study cell biological responses. They found that turbulent flow caused more detrimental effects than laminar flow for the same calculated shear stress level and time of exposure. Respiration activity was affected at levels of shear lower than those required to produce cell membrane disruption. Petersen et al. (1990) showed that shear sensitivity of exponentially growing cells is not dependent on the growth rate. Growth rate was controlled by means of a continuous chemostat culture. Tramper et al. (1986) reported that values of shear stress as low as 1.5 N m⁻² caused an appreciable reduction in cell viability for insect cells. Goldblum et al. (1990) studied the protective effect of different polymers on two insect cell lines in a plate and cone viscometer. In all cases the rate of cell lysis increased with the level of shear stress.

1.2.1.3 Effects of fluid flow in capillary tubing on suspended cells

Flow in capillary tubing has been used to study the response of suspended cells to hydrodynamic stresses. In these studies the cells are subjected to intermittent hydrodynamic stresses as the cells pass through a tube section of very small diameter in a close loop. The
total cell residence time in these capillaries is only a small fraction of a second. Depending on the geometry of the contraction and volumetric flow rate the flow regime may be laminar or turbulent. Augenstein et al. (1971) used two strains of mammalian cells and showed an exponential decline in viable cell count as the number of passages through the capillary system increased. The decline in cell viability, however, was different for each cell line indicating that different cell types have different shear sensitivity. They found a good correlation of the specific death rate with two hydrodynamic parameters: energy dissipation in the fluid and average wall shear. McQueen et al. (1987) working with a similar system as that used by Augenstein et al (1971) and using a mouse myeloma cell line reported that a minimum threshold wall shear stress level of 1800 dyne/cm² applied for a total residence time of 0.02 sec was required to observe cell lysis. As the wall shear stress increased so did the extent of cell lysis. McQueen and Bailey (1989) extended their work to study the effect on myeloma and hybridoma cells of turbulent and laminar capillary flows as the medium serum content and medium viscosity was varied. The fragility of the cells was seen to increase as the serum content was decreased. Different cell lines were seen to have different hydrodynamic sensitivity. In addition no difference in cell lysis was observed when the viscosity of the media was increased.

1.2.1.4 Effects of agitation and/or sparging on suspended cells

The sensitivity of suspended cells has been studied in agitated bioreactors with and without sparging. Most of these reports have investigated the effect of different agitation
speeds in hybridoma cell metabolism and proliferation. Dodge and Hu (1986) found that the specific rate of glucose consumption and lactate production increased with the agitation speed. No difference in cell growth rate was observed at 60 and 120 rpm. A slower cell growth rate, however, was measured at 240 rpm. Lee et al. (1988) studied the shear sensitivity of cells during stationary and decline phases. Their experiments were motivated by the fact that a significant amount of MAb is produced by viable cells during stationary and decline phases. They found that exponentially growing cells are more resistant to shear as compared to cells in stationary and decline phases. In contrast to Smith and Greenfield (1992), they reported that the death rate measured during the decline phase was a function of agitation intensity. Oh et al. (1989) found no difference in cell growth, viability, antibody production, and cell metabolism for surface aerated cell cultures grown at agitation speeds from 100 to 450 rpm. When agitated cultures were sparged, however, the growth rate and maximum cell number decreased significantly. Similar results were also reported by Gardner et al. (1990). Using a continuous stirred tank reactor, Abu-Reesh and Kargi (1991) reported a drop in cell concentration and respiration activity and increased cell lysis at impeller tip speeds above 40 cm s\(^{-1}\). Lakhotia et al. (1992) showed that cells which survive high agitation had an increase in DNA synthesis. This effect being proportional to the agitation intensity. Passini and Goocchee, (1989) reported that stress proteins are not produced as a result of agitation and sparging.

One of the first accounts of the detrimental effect of sparging in cell viability was reported by Kilburn and Webb (1968). More recent studies (Handa et al., 1987; Handa-Corrigan et al., 1989; Oh et al., 1989, 1992; Kunas and Papoutsakis, 1990b; van der Pol et
al., 1990; Jobses et al. 1991; and Tramper et al., 1986, 1987, 1988) indicate that these effects
are cell line dependent. A decrease in cell viability is observed as the gas flow rate is increased
(and so the bubble frequency) and bubble size is decreased. The combined effect of agitation
and sparging is stronger than the individual contributions alone. In addition to effects in cell
proliferation an increase in cell metabolic activity has been observed.

1.2.1.5 Effects of agitation on anchorage dependent cells

Cultivation of cells on microcarriers is one of the most promising strategies for large
scale cultivation of anchorage dependent cells. Excessive agitation, however, has been
reported to reduce the maximum number of viable cells. Hu (1983) working with fibroblasts
showed that the reduction in growth extent could be correlated to an integrated shear factor
defined in terms of the impeller rotation rate and the space between the impeller and the vessel
wall. Hirtenstein and Clark (1980) observed an optimum agitation rate of 60 rpm for growth
of vero cells. A reduced cell concentration was obtained at both slower and faster agitation
speeds. It was suggested that the reduced concentration at agitation speeds less than 60 rpm
was the result of mass transfer limitation effects while that observed at agitation speeds higher
than 60 rpm resulted from hydrodynamic related cell injury. Croughan et al. (1987) working
with the same cell line also reported a reduction in cell concentration as the speed of agitation
was increased from 60 to 220 rpm. Cherry and Papoutsakis (1988) studied the role of
agitation in the formation and breakup of cellular bridges between microcarriers. Formation
of cellular bridges have an effect in the overall cell performance as the cells forming the bridge
can be damaged when the bridges are destroyed by turbulent flow. In addition, diffusion of nutrients becomes limited for cells in the interior of the bridge. Croughan and Wang (1989) demonstrated that the reduction in cell growth at high agitation speeds is the result of cell death and not growth inhibition. Cell death was seen to be the result of an increase in the percentage of cell detachment as the agitation speeds were increased. Detached cells became round and lysed in a period of 1 to 2 days. They were seen to absorb trypan blue and unable to attach and grow when inoculated into new microcarrier or T-flask cultures. In contrast, cells which remained attached to microcarriers did not take trypan blue and maintained the characteristic flat shape of exponentially growing cells.

1.2.2 Mechanisms and formulation of models for cell death in bioreactors

Mechanisms and models for cell death in mixed bioreactors with and without gas sparging will be reviewed in this section. Those proposed for anchorage dependent cells will be presented first followed by those for suspended cells.

1.2.2.1 Mechanisms and models of cell death for anchorage dependent cells. Even though anchorage dependent cells can be grown in a variety of systems, the most promising alternative seems to be the cultivation of cells in microcarriers. Croughan et al (1987) have summarized the advantages of microcarrier culture: a) high surface for growth to reactor volume, b) simple cell-media separation, c) homogeneous culture environment d) high maximum cell concentrations (1x10^7 cells/ml) and, e) solid scale up potential. Accordingly,
a lot of effort has been dedicated to characterize the mechanism of cell damage in agitated bioreactors and to find the relevant hydrodynamic parameters which should be manipulated to obtain optimal results. Cherry and Papoutsakis (1986, 1988, 1990) proposed that cell damage in microcarrier culture was the result of a) interaction of turbulent eddies and beads (microcarriers) b) bead-bead collisions and c) collisions of beads with stationary and moving surfaces. Interaction of turbulent eddies and microcarriers were considered of two kinds. If a microcarrier encounters a turbulent eddy at least 10 times bigger than the microcarrier size, it will be engulfed and accelerated to the local fluid velocity. The forces experienced by the bead under this situation were considered negligible. Instead, if the turbulent eddy is of the same size as that of the microcarrier, it will dissipate its energy on the microcarrier surface and cell damage is expected to occur. The smallest turbulent eddy size was estimated using the dimensional analysis presented by Kolmogorov (1941). Bead-bead collisions were characterized using the "turbulent collision severity" parameter (TCS). Cherry and Papoutsakis (1988) correlated data presented by Croughan et al. (1988) and their own using the TCS parameter. They observed that as TCS increased the apparent cell growth rate decreased. The interaction of beads and stationary and moving surfaces (other than beads) has been suggested to be of less relevance regarding their possible effects on cell integrity. Cherry and Papoutsakis (1986) showed that microcarriers in the boundary layer of solid surfaces were unlikely to produce significant cell damage. In similar fashion, collisions with stationary and moving surfaces were seen to be not sufficiently energetic to produce detrimental effects.

Considering only bead-flow effect, Croughan et al. (1987) correlated cell growth with an integrated shear factor, time average shear rate, Kolmogorov eddy length and average
power per unit mass. No correlation was observed for the maximum time average shear rate. In a latter publication Croughan et al. (1988) found that at high level of agitation the contribution of bead-bead interactions to cell death was more important as the microcarrier concentration was increased. In contrast, no effect was observed under mild agitation conditions. Croughan and Wang (1989) showed that the reduction in cell growth as a result of agitation was not due to cell growth inhibition but because of cell death.

1.2.2.2 Mechanisms and models of cell death for suspended cells

The sensitivity of suspended animal cells became evident in the 1950's when high agitation rates were used to increase the oxygen transfer rate in surface aerated experiments, especially in those cases when free serum formulation were used (Bryant et al., 1960; Runyan and Geyer, 1963). Reduction of cell viability was also observed when air or oxygen was injected directly into the medium to enhance oxygen supply (Kilburn and Webb, 1968; Telling and Elsworth, 1965). To explain these results Kilburn and Webb (1968) suggested that cell function was altered as a result of a modification of the cell surface by surface active forces at the bubble interface. From microscopic visualization studies, Handa et al. (1987) suggested that the critical region of cell damage in sparged columns was the region of bubble disengagement. In this region cells were observed to experience violent turbulence, oscillations and surface deformations. Cells were seen to be entrained in the moving bubble surface interface and transported at high velocities in the film. These interactions were considered to be a highly shearing environment. The overall mechanism of cell damage was
explained in terms of the fact that cells would be at one time or another close to the interface and as a result of this proximity they would experience oscillations and high levels of turbulence. In further studies (Handa-Corrigan et al., 1989) found that increasing the bubble column height fully protected the cells. Retention in viability was explained to result from a reduction of the time spent by the cells close to the region of bubble disengagement. In addition, they proposed three basic mechanisms of cell damage 1) damage due to rapid oscillations caused by bursting bubbles, 2) damage due to shearing in draining liquid films in foams and finally 3) physical loss of the cells in the foam.

By estimating the levels of shear stress that cells will experience in a bubble column Tramper et al. (1986, 1987) and Jobses et al. (1991) suggested that there are three regions in bubble columns where hydrodynamic forces may be detrimental to the cells: 1) bubble formation at the sparger, 2) bubble rising at the bulk cell suspension, and 3) bubble breakup at the suspension surface. They concluded that bubble breakup is the most important factor leading to cell death. Tramper et al. (1986, 1987, 1988) proposed a model for the scale up of bubble columns for the cultivation of insect cells. This model starts with the assumption that there is a liquid volume associated with each bubble where all the viable cells are killed. They derived expressions for the first order death-rate constant and the area available for oxygen transfer in terms of the column geometry and operation parameters. According to this model positive cell growth is obtained when parameters are selected such that the first-order death rate constant is less than the growth rate constant and the specific surface area available for oxygen transfer (determined from the total bubble interface) is at least as large as the minimum specific area needed to satisfy the oxygen uptake rate. In accordance to the
results reported by Handa et al. (1987) and Handa-Corrigan et al. (1989) the hypothetical killing volume model also predicts an improvement in cell growth as the column height to diameter ratio increases.

Oh et al. (1989) studied the effect of agitation on three murine hybridomas. No reduction in cell viability was observed in surface aerated bioreactors where bubble entrainment was carefully avoided. Sparging, however, resulted in a reduction of total cell concentration and viability. This effect was even more pronounced at higher agitation speeds. Oh et al. (1992) reported that small bubbles were more detrimental to cells. In particular, bubbles bigger than 5 mm (which were not further broken down by the impeller) did not cause cell damage. They also showed that the effect of sparging is physical and not due to an increased level of dO₂. Passini and Goochee (1989) reported that sparging resulted in the disruption of hybridoma cells in a few minutes as compared with static and surface aerated cultures. Similar results were presented by Gardner et al. (1990) for hybridoma cells and Murhammer and Goochee (1990a) for insect cells. Kunas and Papoutsakis (1990a) concluded that two mechanisms of cell damage can be distinguished. The first one occurs when the agitation rate is increased so that bubbles are entrained at the bottom of a formed vortex. In the absence of a gas head space (by filling up the bioreactor completely) and thus bubble entrainment, a second mechanism of damage occurs only at high agitation speeds. At high agitation speeds and in the absence of bubbles the correlation of cell damage to the Kolmogorov eddy size first introduced for microcarriers was seen to be valid. Orton and Wang, (1990) showed that cell death in sparged columns correlates with liquid entrainment occurring in single bursting bubbles.
Chalmers et al. (1990), Bavarian et al. (1991) and Chalmers and Bavarian (1991) analyzed the hydrodynamics of a bubble breakup and its implications with cell damage. Bavarian et al. (1991) have reported that some insect cell lines attach to bubbles in their way to the interface. They also reported that significant experimental and theoretical evidence exists to explain non-specific bacterial-cell adhesion to gas-liquid interfaces. They suggested that these results can be extended to explain animal cell adhesion to gas-liquid interfaces. Chalmers and Bavarian (1991) proposed two mechanisms of cell damage in sparged and agitated bioreactors with bubble entrainment: 1) cells are killed by the rapid acceleration of the bubble film after rupture. 2) Cells attached or close to the bubble cavity are killed as a result of the levels of shear stress produced upon bubble rupture. They suggested that the hypothetical killing volume introduced by Tramper et al. (1988) may consist of the medium and cells which make up the bubble film and a thin layer surrounding the bubble cavity. Cherry and Hulle (1992) suggested that the mechanism of cell damage in sparged bioreactors results from the retraction of the bubble film when it breaks. They presented a model to correlate cell death in a foam layer. Garcia-Briones and Chalmers (1992) took a sample from the upward jet produced upon bubble breakup. They found that the majority of the cells in the sample were dead. More recently Garcia-Briones and Chalmers (1994) reported on computer simulation of the break up of a bubble at a gas-liquid interface. They found that when a bubble collapses there is a very high, localized, energy dissipation in the liquid forming the vicinity of the bubble. This energy dissipation was found to be a strong function of bubble size, increasing rapidly as the bubble size decreases. Yang and Wang (1992) proposed a model of cell death in agitated/sparged bioreactors. Their model considers breakup and coalescence of
bubbles as the primary mechanisms of cell death. Zhang et al. (1993) proposed a model to predict cell disruption in turbulent fields. The model assumes that the turbulent flow transfers energy to cells and as a result cells deform. Cell disruption occurs when the amount of energy transferred exceeds the cell bursting energy.

1.2.3 Protective additives

Medium formulation for the cultivation of mammalian cells in vitro was first reported by Earle and his associates (Earle et al., 1954). In the early attempts of in vitro cell cultivation serum from different sources was always present in the growth media (Earle et al., 1954; Kuchler et al., 1956; McLimans et al. 1957). However, to unravel the mechanisms of the immune response and in general to study the cell function it was necessary to eliminate the serum from the media formulations. In addition to its high cost and limited availability, serum has different biological activity from batch to batch. Most of the attempts to adapt cell lines to serum free medium in stationary flasks were successful, the problem came when this serum free formulations were used in agitated or sparged bioreactors. It was then recognized that serum protected cells from mechanical stress. Since then, the protective effect of serum in the cultivation of animal cells has been extensively documented in the literature. Serum has been effective to protect cells from hydrodynamic stresses in agitated and/or sparged bioreactors in a concentration dependent fashion. McQueen and Bailey (1989) observed a reduction in the specific cell lysis rate in a capillary flow device as the serum concentration was increased. Handa-Corrigan et al. (1989) reported absence of cell damage in bubble columns as compared
with surface aerated cultures when the media was supplemented with 10 % fetal calf serum. Kunas and Papoutsakis (1989) showed no effect of fetal bovine serum (FBS) on cell growth in static cultures. In agitated (surface aerated) bioreactor experiments, however, it was observed that cells could grow at higher agitation speeds as the FBS concentration was increased above 5%. Kunas and Papoutsakis (1990b) reported that serum protected cells even if it was added one hour before the cells were subjected to high agitation with bubble entrainment. They concluded that the protective effect of serum was not nutritional but physical and it was not due to an increase in the viscosity of the media. In viscometric studies, Michaels et al. (1991) found that hybridoma cells became more shear tolerant at long exposure times to FBS, while no effect on cells was observed at short term exposures. They concluded that the protective effect of serum has both a biological and a physical contribution. Ozturk and Palsson (1991) tested whether the mechanism of protection by serum comes from the serum albumin fraction (BSA) which according to the authors could form a protective layer around the cell membrane. No change in growth or death rates was found by the addition of BSA. In contrast to Kunas and Papoutsakis (1990b) they suggested that the shear protective effect of serum is nutritional in nature. Martens et al. (1992) observed a reduction in the "hypothetical killing volume" as the serum concentration was increased in an air-lift system. Ramirez and Mutharasan (1992) proposed that the mechanism of protection by serum was related to its ability to decrease the plasma membrane fluidity. Smith and Greenfield (1992) concluded that serum has a metabolic effect by reducing the rate of glycolysis in exponentially growing cells.
Pluronic F-68 has also being used as a shear protective additive. Pluronic F-68 is a nonionic surfactant block copolymer of polyoxyethylene-polyoxypropylene (POE-POP). Its protection action has been found to be concentration dependent. In most cases concentrations of 0.1% provided cell protection in agitated and sparged bioreactors as compared with controls. It has been reported that Pluronic F-68 does not affect cell growth in stationary or spinner flasks as well as metabolism and product formation at low speeds. There are some other cases however where an enhancement or reduction in cell growth has been found (Mizrahi, 1975). Mizrahi, in 1975, presented evidence that Pluronic F-68 is not metabolized. After 7 days of cell culture he measured the Pluronic F-68 concentration in the supernatant and concluded that it was not utilized. From these results he suggested that the mechanism of protection was physical rather than one associated with cell metabolism. Michaels et al. (1991) examined long and short exposure of cells to Pluronic F-68 before high agitation levels were imposed. In both cases protection to cells was observed. They also concluded that the protective effect was physical in nature. Murhammer and Goochee (1990b) studied the structural features of polyols responsible for their protective effects. They found that the protection ability of the polyols tested correlated with the hydrophobic-hydrophilic balance (HLB). The higher the HLB the better the protection characteristics. Molecular weight, relative sizes, and relative positions of the POE-POP chains did not influence protection properties.

Different mechanisms of cell protection by Pluronic F-68 have been proposed in the literature. Kilburn and Webb (1968) suggested that the mechanism of protection by Pluronic F-68 came from stabilization of the cell-liquid interface by adsorbed molecules forming a highly condensed structure. Handa et al. (1987) proposed a dampening in the turbulence at
the gas-liquid interface or a reduction in the circulation of the bubble surface. They also argued (Handa-Corrigan et al., 1989) that protection is achieved from the formation of a stable foam from which cells are excluded. Murhamer and Goochee (1988) found a protective effect in the absence of a foam layer in agitated/sparged bioreactors. They concluded that the formation of a stable foam layer is not a necessary condition for cell protection. Another interesting result reported by these workers is the fact that Pluronic F-68 affects the cells trypan blue uptake. This effect is explained in terms of an interaction of Pluronic F-68 and the cell membrane. It is suggested that this interaction plays an important role in the mechanism of protection. Michaels et al. (1991) used a combination of viscometric and agitated experiments to study the mechanism of protection of Pluronic F-68 and other additives. They proposed that an additive may act by two possible mechanisms: biological or physical. The mechanism is biological if cells are modified and become more shear resistant. This biological mechanism in turn can be fast (membrane interactions) or require metabolic events. The mechanism is physical if the cells remain unchanged but somehow the magnitude and frequency of the detrimental hydrodynamic forces is reduced. In these experiments it is indicated that the viscometric results give information about the ability of the additive to provide protection by a biological effect since the conditions in these viscometric flows (applied shear stress) remain unchanged. In contrast with Goldblum et al. (1990) they didn't find protection from the addition of Pluronic F-68 in viscometric flows. Protection however was observed in bioreactor experiments. It was concluded that the mechanism of protection is physical, which implies that somehow the intensity and frequency of the hydrodynamic forces leading to cell death are reduced. Garcia-Briones and Chalmers (1992) reported on
visualizations of the bubble film before rupture. They observed many cells (SF-9 insect cells) attached to the bubble film in media without Pluronic F-68. Upon bubble rupture a sample from the upward liquid jet was taken. When viability was measured they found that the majority of the cells were dead. When the media was supplemented with Pluronic F-68, however, cells were observed to drain from the bubble film. The liquid sampled from the upward jet contained few cells which were found viable. They suggested that addition of Pluronic F-68 modified either the bubble interface, the cell membrane or both such that attachment of cells to the bubble surface (film or lower bubble interface) was prevented.

Other protective additives which have been used with different degrees of success include methylcellulose (Kuchler et al., 1960, Bryant., 1966; Bryant, 1969; Goldblum et al. 1990), sodium carboxymethylcellulose (Telling and Elsworth., 1965, Mizrahi and Moore, 1970), dextran (Mizrahi and Moore, 1970; Papoutsakis and Kunas, 1989; Goldblum et a., 1990), Polyethylene glycols (Murhammer and Goochee, 1990b), Michaels et al., 1991; Michaels and Papoutsakis, 1991), Polyvinyl alcohol (Michaels and Papoutsakis, 1991).
CHAPTER II

CELL-BUBBLE INTERACTIONS: MECHANISMS OF SUSPENDED CELL DAMAGE


2.1 Summary

Visualization studies on cell bubble interactions and other experimental observations which demonstrate that cell damage results when bubbles break at a gas-liquid interface are presented.

2.2 Introduction

The "shear sensitivity" of animal and insect cells has been recognized and reported to be one of the most significant challenges in the scale-up of animal cell production systems. Despite this challenge, animal cells have been grown in suspension culture for over 35 years,
yet the problem of cell damage has remained and its prevention is more of an art than a science.

Contained within some of these early reports of animal cells grown in suspension are accounts of how damage to the cells seems to be the result of cells interacting with air bubbles through sparging. In addition, in many of these accounts researchers mention that "protective additives" can be added to the growth medium to protect cells from damage (Swim and Parker, 1960; Runyan and Gyer, 1963). In 1968, Kilburn and Webb (1968) were the first to suggest a mechanism for this protection - that the additive, in this case Pluronic F-68, protected cells by creating a "highly condensed interfacial structure of adsorbed molecules" at the cell-liquid interface. Unfortunately, no experimental evidence was provided for this hypothesis.

Despite this early evidence that air sparging was the mechanism of cell damage, a commonly held belief is that mechanical mixing, and the associated hydrodynamic forces (such as shear stress) damages cells, hence the name "shear sensitivity".

However, the more recent works of Kunas and Papoutsakis (1990a) and Oh et al. (1989) indicate that suspended Hybridoma cells can withstand agitation rates from 450 to 600 rpms - levels of agitation much greater than previously thought and much higher than normally used. It is important to note that these levels of agitation were obtained in systems in which no gas was sparged. In the work of Kunas and Papoutsakis (1990a), in addition to no gas sparging, the formation of a central vortex, which results from the high rpm of mixing, was inhibited.
These and other results indicate that the damage to suspended cells is predominately the result of gas sparging and lead Kunas and Papoutsakis (1990a) to state "Only when entrained bubbles interact with a freely moving gas-liquid interface, such as what exists between the culture medium and gas headspace, does significant cell damage occur."

When gas is sparged into a bioreactor, three areas have been suggested to be the regions of damage (Tramper et al., 1986, 1987, 1988) the bubble injection region, the bulk medium through which bubbles rise, and the bubble disengagement region at the air-medium interface.

Several research groups have taken a correlational and/or modeling type of approach to begin to determine the mechanism of cell damage as a result of cell-bubble interactions (Tramper et al., 1988; Handa et al., 1987; Handa-Corrigan et al, 1989). This approach, combined with experimental data lead Tramper et al. (1988) to conclude that the damage to a cell takes place in a "hypothetical killing volume" associated with each gas bubble. By varying the size and diameter of a sparged bioreactor they were able to demonstrate that this "killing volume" is not a function of the distance that a bubble travels through the bioreactor. This implies that a majority of the cell damage takes place in either the bubble disengagement region at the medium-air interface or the bubble injection region. While this approach has suggested in which region damage to cells takes place, it does not provide insight, nor a mechanistic picture of the actual mode of this cell destruction.

To begin to obtain a mechanistic understanding of the causes of cell damage in sparged bioreactors, Handa et al. (1987) and Handa-Corrigan et al. (1989) developed a microscopic-video technique which allowed them to visually observe cell-bubble interactions.
They concluded that the primary region of cell damage was at the medium-air interface and that the following mechanisms were responsible for cell damage: 

"(1) In culture medium supplemented with antifoam, cell damage is a result of oscillatory disturbances caused by rapidly bursting bubbles, (2) In medium without antifoam, cell damage is due to a physical shearing effect in the draining liquid films (lamella) around bubbles, and (3) Due to the actual physical loss of cells in the foam."

In regard to the bubble injection region, it has been proposed that the high velocity of the gas being injected into the system, and the corresponding shear stresses, result in cell damage (Tramper et al. 1987; Murhammer and Gooche, 1990a). Evidence for this hypothesis has been reported by Murhammer and Gooche (1990a). They demonstrated that large differences in cell damage can be obtained when the only difference in two similar airlift reactors, operated under the same conditions, is the design of the gas sparger. In the system with greater damage the pressure drop across the sparger was twice as great.

In this part visualization of cell bubble interactions which further extends the mechanistic understanding of cell damage as a result of gas sparging will be given as well as more recent results which confirm that bubble rupture damages cells. Finally, a mechanism will be proposed which explains how "protective additives" protect cells.

2.3 Visual observations of cell-bubble interactions

Although Handa et al. (1989) pioneered the use of visual observations to begin to understand the mechanism of cell damage, the equipment they used was limited in both the
depth and field of view and the speed by which images could be recorded.

To overcome these limitations, an Olympus BMHJ metallurgical microscope was attached to a Pulnix black and white video camera (Model TM-845, Pulnix America, Sunnyvale, CA) which has a shutter speed of 1/10,000 of a second. Visual observations, using a 5X objective, were made of cell-bubble interactions in a "two-dimensional" bubble column which had a working volume 1 mm thick, 50 cm high and 10 cm wide.

Once a bubble was injected or created through electrolysis, it was observed that individual cells or clumps of cells become attached to the rising bubble (Figure 1a and 1b). It is possible that the shear stress that a cell would experience when attached to a rising bubble is sufficient to cause cell damage/death (Bavarian et al. 1991; Chalmers and Bavarian, 1991). However, it is important to note that this proposed mechanism of damage is minor in regard to the number of cells damaged when compared to the mechanism discussed later.

Once a bubble reaches the air-medium interface, two fates await it: the bubble will either immediately rupture or it will become part of the foam layer and will eventually rupture. Visual observations of the foam layer confirm one of the mechanisms proposed by Handa et al. (1989), namely, that cells can become physically trapped in the foam layer. Examples of cells trapped in the foam layer are shown in Figure 1c and 1d. When no protective agents such as Pluronic F-68 are present, large numbers of cells become trapped in the foam layer as can be observed.

The dynamics of bubble rupture at the air-medium interface have been discussed perviously (Chalmers and Bavarian, 1991) and will only be briefly reviewed here. When a bubble reaches the air-medium interface, a thin liquid film forms, separating the air within the
bubble from the air above the interface. This liquid film evaporates/drains until a critical thickness is reached and the film ruptures.

This film then retracts by rolling up to form a toroidal ring (a donut shape) until it reaches the edge of the bubble cavity. Medium containing cells then flows down into the bubble cavity in a boundary layer flow. Once it reaches the bottom of the cavity, a stagnation point is obtained, which results from the symmetric flow into the cavity, and two jets are formed. One of these jets flows upward into the air above the medium, while the other flows down into the medium underneath the bubble cavity. This upward jet then breaks up into several drops, as a result of Rayleigh-Taylor instabilities, and it has been shown experimentally that these jet drops contain liquid which at least partially originate from the bubble film (MacIntyre, 1972).

Earlier work indicated that cells become attached to this bubble film and that the forces involved when a bubble ruptured were probably sufficient to kill suspended cells. In the work presented here, improved images of cells attached to the bubble film are presented as well as evidence that cells are contained in the liquid jets. Finally the effect that the presence of Pluronic F-68 in the medium has on these observations are given.
2.4 Materials and methods

2.4.1 Cells and medium

The Insect cell line Spodoptera frugiperda (SF-9) growing in TNM-FH and SFM-L medium was used. SFM-L is a formulation of serum-free medium supplemented with lipids (Hink, 1991). The method of cultivation has been reported previously (Bavarian et al., 1991). Cells in exponential phase of growth were used for all experiments. For some of the experiments TNM-FH medium supplemented with Pluronic F-68 was used. Viability was measured using the Trypan Blue exclusion test (Tolnai, 1975).

2.4.2 Method of drop capture

The bubble column used to obtain the results presented in this paper is shown in Figure 2. It consists of a segment of Pyrex glass of square geometry. This geometry was selected to facilitate the visualization of cell bubble interactions and prevent distortions due to round tubes. A reduction at the bottom of the column was made to hinder cell settling. Because of this reduction it was possible to produce one bubble at a time of about 3 mm in diameter.

The initial, surface life time (time that the bubble remains at the medium-air interface before it breaks) of the bubbles injected at the bottom of the column using a hypodermic needle was in the range of 10 to 20 seconds.
This time was observed to increase up to several minutes when the interface became contaminated with air-born particles, material released from lysed cells, or denatured proteins.

When the bubble collapsed the droplets ejected were collected by either setting a clean glass surface or a hanging drop of TNM-FH medium just above the bubble film. Upon bubble rupture the ejected droplets attached to the glass surface or to the hanging drop (depending on which method was used).

Figure 3 is a photograph of the system in use. Figure 3a is just before the bubble ruptured, while 3b was taken just after the bubble ruptured, with the captured drop indicated by the arrow.

2.4.3 Equipment for visualization

The system used in visualization studies was the same as that previously described in other papers (Bavarian et al., 1991; Chalmers and Bavarian, 1991) except that an Infinity CFM lens was used for magnification of the images. The Infinity lens is a continuously-focusable microscope that can be focused from infinity down to 6 mm with depths of field several orders of magnitude greater than most microscopes including the system reported earlier.
2.5 Results

2.5.1 Visualization of cells attached the bubble film

Figures 4a and 4b are photographs of the same bubble (approximately 3 mm in diameter) resting at the air-medium interface taken 1 minute apart. The cells, grown and suspended in TNM-FH medium, appear as small white spots and can clearly be seen on the bubble film. During the one minute increment between 4a and 4b significant contraction of the liquid containing the cells on the bubble film was observed. This contraction creates "zones of clearing" where few or no cells are present; one of these "zones" is indicated by the arrow in 4b.

Figure 4c is another photograph of a bubble at the air-medium interface. In this case, the cells were grown and suspended in SFM-L medium. Again, "zones of clearing" are observed. These zones of clearing appear immediately before the bubble ruptures. Figure 4d is a photograph of a bubble at the air-medium interface in which the medium contained 0.2% Pluronic F-68. As can be observed there are no cells present on the bubble film.

2.5.2 Visualization of cells in the upward jet

With the large depth of field infinity lens system, it is possible to have not only the bubble film in focus, but once the film ruptures, the rising jet can also be in focus. Figure 5 is a photograph of one of these rising jets. The spots which are faintly visible in the
photograph are believed to be clumps of cells.

2.5.3 Cells in the jet drops

Figure 6a is a photograph of a jet drop (approximately 5 ml) from the first bubble injected into the column, while Figure 6b is a photograph of the same drop after an equal volume of a balanced salt solution containing 1.0% Trypan Blue was mixed with it. In this black and white photograph, the dead cells appear dark while the viable cells remain clear. As can be observed a very well-defined, circular region of cells and cell debris is visible, and almost all of the cells are dead. The initial cell viability in the bulk medium before rupture was >90%.

When 0.2% Pluronic F-68 is present in the medium, very few (less than 10) cells were observed in the captured jet drops. This result complements the observation that no cells are present on the bubble film.

2.5.4 Control experiments

While these results seem to indicate that the cells were destroyed by the process of bubble rupture, it is possible that the cells were already dead before the bubble ruptured or that these observations were artifacts of the experimental procedure used to obtain the jet drop.
Three arguments can be made against the idea that the cells were already dead before the bubble ruptured. First, as stated above, the viability of the cells in the bulk medium before any bubbles were introduced was very high (>90%). Second, the time that a bubble remained at the air-medium interface was reduced to as low as 3 seconds (a factor of 10 shorter) when serum free medium (SFM-L) was used when compared to serum containing medium (TNM-FH). This reduced the time that the cell could be damaged/killed on the bubble film before the film ruptured. Third, experiments reported by other researchers (Hulle and Cherry, 1990) show that when cells are harvested on a liquid film by dipping a copper wire loop into the medium, no loss of viability results as long as the film does not break.

It has been suggested that cells could be damaged as a result of the impact of the liquid sample on the surface of the glass used to collect the sample. Again several reasons exist which suggest that this procedure did not introduce artifacts into the data. First, the glass plate was positioned at such a height above the bubble that the drop was almost at the apex of its trajectory when it impacted the glass plate. Hence the drop velocity was relatively low. Second, in several experiments a hanging drop on the glass plate was used to collect the jet drop which would lessen the impact of the jet drop onto the plate. Using this method of drop capture, similar results were obtained, in that almost all (>95%) of the cells were dead. Third, an ejected droplet was produced by hitting the glass column containing the cells and medium with a small bar. This ejected drop was then captured using the same method as was reported earlier. The ejected drop contained the same concentration, distribution and viability as the bulk medium from which it originated.
2.6 Discussion

The presence of cells in the upward jet and in the jet drop is not surprising since similar results with bacteria and latex particles have been reported (Quinn et al., 1975; Blanchard and Syzdek, 1972). In addition to the presence of these particles in the jet drops, these researchers reported that an increase in concentration of these cells or particles from 10 to 1000 times, relative to the bulk suspension, can be observed. They concluded that this increase in concentration was the result of cells or particles adsorbing onto the air-medium interface surrounding the bubble and being swept into the liquid jet as the bubble ruptured.

While the concentration of cells in the jet drop has not been quantified, it is apparent from our observations and the photographs in Figure 6 that there is an increase in the concentration of cells in the jet drop relative to the bulk concentration. As was previously reported (Bavarian et al. 1991; Chalmers and Bavarian, 1991), SF-9 and TNM-FH cells adsorb to the air-medium interface as well as the bubble film. Consequently, as in the case of bacteria and latex particles, the increased concentration of cells in the jet drop is most likely the result of cells adsorbing to the air-medium interface.

The jet drop capture and Trypan Blue staining results demonstrate that cells are killed as a result of the hydrodynamic forces acting at the time of bubble rupture. The very high concentration of cells and cell debris in the circular region most probably originated from cells which were attached to the film; however, further work needs to be conducted to confirm this suggestion.
Finally, Figure 4 clearly indicates that at least one of the mechanisms of Pluronic F-68 protection is the prevention of cell adhesion to the bubble film. Other observation (data not shown) indicates that Pluronic F-68 also decreases the number of cells which attach to rising bubbles.

The non-specific adhesion of cells to gas-liquid interfaces is probably the result of hydrophobic-hydrophilic interactions. The adhesion of bacteria to surfaces and gas-liquid interfaces has been studied by several researchers over a period of years, and a thermodynamic relationship has been developed to explain/predict cell adhesion (Kjellelberg, 1985; Absolomon 1986). This relationship is a function of the interfacial tension between the cell and the liquid, the cell and the gas phase, and the liquid and the gas phase. Since most, if not all, of the "protective additives" such as Pluronic F-68, are surface active, it is highly probable that these additives are changing the balance of interfacial tensions. When this balance is changed in the correct manner, cell adhesion to the gas-liquid interface would be inhibited and, consequently, cell death would be prevented.
Figure 1. Photographs of video images of cells attached to rising bubbles (a,b) and cells trapped in the foam layer (c,d). Arrows indicate attached/trapped cells(s).
Figure 2. Experimental apparatus used to capture jet drops from a rupturing bubble.
Figure 3. Photographs of a gas bubble on the air-medium interface before bubble rupture. Cells on the bubble film can be faintly observed. Part 3a is just before bubble rupture, whereas part 3b is just after bubble rupture. An arrow in part 3b indicates the captured drop.
Figure 4. Photographs of a bubble resting at the air-medium interface. Cells appear as small white spots on the bubble film. Part 4a is immediately after the bubble came to rest on the interface, whereas part 4b is one minute later. Part 4c is a bubble in serum-free medium, whereas part 4d is a bubble in medium containing 0.2% Pluronic F-68. A "zone of clearing" that does not contain cells is indicated by the arrows in part 4b and 4c.
Figure 5. A photograph of the liquid jet that results from a bubble rupture. Cells are faintly observable in this photograph and one cell, or group of cells is indicated by an arrow.
Figure 6. Part 6a is a photograph of a jet drop, which resulted from the first bubble rupture in a sample. Part 6b is a photograph of the same jet drop as in part 6a after an equal volume of 1.0% trypan blue in balanced salt solution had been added. Dead cells appear dark.
CHAPTER III

COMPUTER SIMULATION OF THE RUPTURE OF A GAS BUBBLE AT A GAS-LIQUID INTERFACE AND ITS IMPLICATIONS IN ANIMAL CELL DAMAGE.


3.1 Summary

Hydrodynamic information of the flow occurring as a bubble breaks at a gas liquid interface has been obtained from computer simulations. The transient rate of energy dissipation per unit volume in the region close to the bubble interface has been calculated. It was found that when a bubble collapses, there is a very high, localized energy dissipation which can be used to explain animal cell damage in sparged suspended animal cell cultures. The rate of energy dissipation was found to be a strong function of bubble size, increasing rapidly as the bubble size decreases.
3.2 Introduction

The number of products and product applications derived from the in vitro culture of animal cells has increased in the last few years. As a result of this expansion the need of systematic methods to scale up production processes is considered a major priority. The traditional system used to grow animal cells in suspended cultures at small scales is the agitated bioreactor. Some of the advantages associated with this system are homogeneous conditions and simple control of operation parameters such as temperature, pH, oxygen and nutrient concentration. As the bioreactors are scaled up, the supply of oxygen by simple diffusion through the gas head space is insufficient; consequently, oxygen transfer is enhanced by injection of air or oxygen into the liquid phase. This operation, however, has been observed to result in cell damage. Experimental observations reported by different research groups have suggested that cell damage occurs at the region of bubble disengagement (Handa et al., 1987; Handa-Corrigan et al., 1989; Tramper et al., 1986; Tramper et al., 1987; Jobses et al., 1991; Kunas and Papoutsakis, 1990a; Oh et al., 1989; Oh et al., 1992; Garcia-Briones and Chalmers, 1992; Chalmers et al., 1990; Chalmers and Bavarian, 1991; Bavarian and Chalmers, 1991). A thorough understanding of the events at the liquid interface is needed if a systematic scale-up method is to be developed. In this report hydrodynamic information obtained from the computer solution of the flow occurring as a bubble breaks has been used to calculate the rate of energy dissipated in the liquid forming the vicinity of the bubble. These results are compared with data from experiments in well defined flow devices and
mixed bioreactors reported in the literature. Three bubble sizes are used (0.77, 1.7, and 6.32 mm in diameter) to determine the dependency of the rate of energy dissipation with bubble size.

### 3.3 Software and computer system

The numerical simulations were performed using the computer program FLOW-3D (Flow Science, Inc., Los Alamos, NM). This code uses a control volume method to produce a transient solution of the fluid conservation laws of mass, momentum and energy. Particular capabilities of FLOW-3D essential for our application are its general free surface tracking method and its incorporation of surface tension forces.

Numerical solutions are obtained using a grid of rectangular control elements of variable size. Location of fluid within this grid is accomplished with the Volume-of-Fluid (VOF) method, which allows for the coalescence and breakup of fluid masses. The basic solution algorithms used in FLOW-3D for incompressible flows are a direct descendent of the Marker-and-Cell (MAC) method developed at the Los Alamos National Laboratory in the early 1960's. The original VOF publication (Hirt and Nichols, 1981) offers an excellent overview of the solution methodology used in FLOW-3D. This commercial code has been extensively refined and validated over the past 10 years for a variety of applications. In this report we have explored its ability in solving small scale flows where surface tension forces dominate.
The code was executed on a Cray Y-MP 8/864 supercomputer at the Ohio Supercomputer Center (OSC). The numerical results were plotted using apE. This software was developed at OSC for visualization of scientific and engineering data.

3.4 Initial conditions

The initial state of the fluid was specified as the shape of the bubble resting at the liquid interface without the bubble film. The force driving the motion of the liquid close to the cavity wall is dictated by the Laplace equation:

\[
\Delta P = \sigma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)
\]

where R1 and R2 are the principal radii of curvature. According to Eq. 1 no realistic results can be expected unless an accurate bubble profile is determined.

Toba (1959) solved the equations defining the shape of floating bubbles. His model is not restricted to gas-liquid interfaces, so it applies to any two immiscible systems which may have similar densities. The solution reported by Toba (1959), however, involves semi-graphical methods. Medrow (1968) solved the governing equations proposed by Toba (1959) using a different approach. He developed an analytic solution for the shape of small bubbles and a combination of numerical and trial-and-error procedures for big bubbles. These solutions were experimentally verified for a broad range of bubble sizes. For details in the
derivation of the model used to find the bubble shape as well as the solution methodology, the reader is referred to the work reported by Toba (1959) and Medrow (1968). Figure 7 shows the bubble profiles for the bubble sizes used in this work.

3.5 Problem description

When a bubble breaks, its film retracts collecting the liquid into a growing totoidal ring. The initial condition used in this work is the instant at which the expanding toroidal rim reaches the edge of the cavity wall. At this instant the velocities of every element of fluid were taken to be zero. This also implies that the upward drift velocity of the bubble (produced by buoyancy forces) was regarded to be zero; i.e., the bubble was at rest at the interface before it ruptured. If we consider the small mass of the bubble film, and the fact that the collected liquid from the film may break into small droplets, the assumption of zero velocity at the edge of the bubble cavity seems to be a reasonable approximation. Because of this initial condition, no information will be provided about the hydrodynamics of the film rupture. Currently there is no experimental evidence that would indicate the individual contributions of the film retraction and bubble cavity collapse to cell damage.

In defining the problem, a uniform pressure above the liquid phase was specified. This condition is justified if one considers that once the bubble film breaks, pressure equilibrium inside the bubble cavity is reached in a few microseconds. The propagation of a pressure wave in air (velocity of sound) can be estimated by the following formula (Brodkey, 1967):
\[ C = 49.02 \sqrt{T} \]  \hspace{1cm} (2)

where \( c \) is given in feet per second and \( T \) is in R. If a 1.7 mm diameter bubble in sea water at 65 °F is considered, it can be calculated using eq. (2) that the pressure wave will propagate 2 bubble diameters in \( 9.9 \times 10^{-6} \) sec. As it will be shown later, the collapse of this bubble occurs in about \( 2.3 \times 10^{-4} \) sec. Then it can be safely assumed that the propagation of the pressure wave will not have any effect on the evolution of the bubble cavity collapse and that uniform pressure above the liquid phase can be specified.

3.6 Numerical description

A type of solids modeling capability is used in FLOW 3-D to define the initial fluid region. Regions are defined in terms of general quadratic shapes (e.g., spheres, cones, cylinders, rectangular blocks, etc.) that may be added or subtracted from one another. The intersection of these shapes with the computational grid is computed to determine the initial fractional volume of fluid in each grid cell. The fractional volume of fluid (or fluid fraction) is one for liquid-phase cells and zero for gas-phase cells. Intermediate values between one and zero are assigned for cells at the interface. The quadratic polynomial provided by the code to define general quadratic shapes was extended to include terms of higher order which were required to accurately define the bubble profile.

The problem was solved using cylindrical coordinates assuming symmetry in the \( \theta \) direction. Incompressible Newtonian fluid under isothermal conditions was also assumed. It
has been shown that the growth medium used for insect and animal cells is Newtonian and that the cell density normally obtained is sufficiently low to prevent non-Newtonian effects (Goldblum et al., 1990). The outer and bottom boundaries were set to about 2 times the bubble diameter. For one bubble size (1.7mm diameter bubble) the outer and bottom boundaries were also set at 50 and 100 bubble diameters to assess the influence of the proximity of these boundaries. In all cases the outer and bottom boundaries were specified as fixed hydrostatic pressure boundaries. The top boundary was defined as an output boundary, through which the fluid could freely flow.

In order to improve the numerical results, the mesh was concentrated at the center of the bubble. In this region the greatest rates of change in the flow variables were expected. Fig. 8 shows the grid used for the 1.7 mm diameter bubble where the outer and bottom boundaries were set at two bubble diameters apart. The actual azimuthal segment used in the computation is shown in Fig. 9.

3.7 Comparison of the computer solution with actual photographs

A natural way to know how close the computer solution resembles the actual process is to compare free surface plots with pictures of the bubble breakup at the same elapsed time. Fig. 10 shows a comparison of the computer solution and photographs reported by Woodcock et al. (1953). Several factors may contribute to the differences observed in Fig. 10 and are discussed below.
3.7.1 Erratic behavior of the bubble rupture process

Blanchard and Syzdek (1978) have reported on the erratic nature of the bubble rupture process in regard to drop ejection heights. They indicated that drop ejection heights often vary in the same experiment from one bubble to the next in distilled water where bubbles burst instantly. A possible explanation suggested by these authors is that this erratic behavior may be associated with the exact position of the bubble at the interface when the bursting occurs. The bubble of the photographic experiment shown in Fig. 10 was assumed to have come to static equilibrium (zero upward velocity) before rupture. Evidence supporting or disproving this assumption is not available in the reference from which these results were taken. Because of this erratic behavior in drop ejection heights it is plausible to expect an erratic behavior in the stages previous to the jet formation. In other words, the time sequence pictures of two rupturing bubbles of the same size may not be exactly equal. This points out the difficulty in arriving at an accurate judgment on the extent by which the computer simulation results presented here deviate from the experimental photographs.

3.7.2 Proximity of the walls and meniscus

No information is available on the proximity of the walls in the photographs of Fig. 10 or the exact shape of the free surface (meniscus) before the bubble was injected. In the calculation presented infinitely removed boundaries (see problem description) and a horizontal free surface (implied in the derivation of the equations governing the bubble shape at equilibrium) were used.
3.7.3 Influence of the film rupture

As indicated in the problem description, the hydrodynamics of the film rupture is not considered. Even though we believed that this simplification will not affect the motion of the bubble cavity, its exact influence is unknown.

3.8 Resultant stresses

Another important aspect of the computer solution is its numerical stability. In particular, we were interested in the effect of grid refinement and proximity of the boundaries on the rate of deformation of the fluid. The change of deformation rates was assessed by looking at the distribution of resultant stresses, \( \tau_{\text{res}} \), defined by

\[
\tau_{\text{res}} = \left( \sum \tau_{ij}^2 \right)^{\frac{1}{2}}
\]  

(3)

where \( \tau_{ij} \) represents every component of the stress tensor. The components of the stress tensor were calculated from the velocity field using linear approximations for partial derivatives. The stress tensor can be used since it is linearly related to the rate of deformation for Newtonian fluids. The value of the resultant stress at every fluid element calculated from eq. (3) is a measure of the components of the stress tensor. This resultant stress is not invariant to the coordinate system, but it can be used to assess the change in deformation rates as long as the same coordinate system is used in all comparisons.
3.9 Grid refinement

The dependency of the solution on grid refinement was determined for the 1.7 mm diameter bubble shown in Fig. 8 by increasing the number of cells by a factor of two; i.e., twice as many cells were used in both $r$ and $z$ directions. In the following discussion we will refer to the grid shown in Fig. 8 as the 35x55 grid and the refined grid as the 70x110 grid. The objective of this analysis was to assess the extent by which the numerical solution changes as the grid is refined. This will in turn determine whether further grid refinement is indeed required. Figure 11 shows the time sequence of free surface plots for the 35x55 and 70x110 grids at selected elapsed times. The differences observed result from the fact that the curvature of the free surface is more accurately calculated as the number of cells in the grid increases. The free surface plots shown in Fig. 11 show that as the grid is refined the computer results resemble the actual process to the same extent when compared to the photographic results.

The change in deformation rates with grid refinement are assessed by looking at the distribution of resultant stresses. Table 1 shows the frequency of cells with resultant stresses in the indicated range over the time domain of the calculation ($2 \times 10^{-3}$ sec) for the 35x55 and 70x110 grids. Cells with resultant stresses less than 50 dyne/cm$^2$ were not considered since we were interested only in the range of high resultant stresses. The frequencies of cells with resultant stresses are dependent on the time interval selected to obtain data outputs from the computer program of the hydrodynamic state of the flow. For this grid refinement study fifteen data outputs were used. As shown in Table 1 the highest frequencies corresponded
to cells with stresses in the range of 50 to 100 dyne/cm². The frequencies decrease rapidly as the range of stress increased. In both cases only a few cells presented resultant stresses larger than 500 dyne/cm². The frequency for all intervals is more than twice as high for the 70x110 grid when compared to the 35x55 grid. This is because of the condition imposed to calculate the stresses. For any given cell its associated stresses are calculated only if the fluid fraction for the cell and its surrounding cells is one. This condition becomes more restrictive as the total number of cells used decreases, and it is not linear with the refinement factor. This is schematically shown in Fig. 12. However, as a percentage of the total number of cells with an associated resultant stress, both the 35x55 and 70x110 grids have a similar distribution of resultant stresses (See Table 1). From the results presented in Fig. 12 and Table 1 it can be concluded that further refinement of the 35x55 grid does not produce significant changes in the distribution or the range of the calculated resultant stresses.

3.10 Proximity of the outer and bottom boundaries

The effect of the proximity of the outer and bottom boundaries was evaluated by specifying these boundaries at 2, 50 and 100 bubble diameters for a 1.7 mm diameter bubble. In expanding the grid to 50 and 100 bubble diameters, the grid of Fig. 8 was preserved and additional cells were added in both r and z directions. Beyond the grid presented in Fig. 8, the size of individual cells expanded uniformly until the required proximity of the boundaries was achieved. The grid can be expanded in r at a position far from the axis of symmetry (by far we mean beyond 2 bubble diameters) since the motion of the fluid at this distance is very
slow as compared with speeds measured close to the bubble interface and particularly at the axis of symmetry. In a similar way, the grid can be expanded in the z direction at a position far from the still liquid level since the high speed of the downward jet does not propagate more than 2.5 bubble diameters during the calculation time (2×10^{-3} sec). In addition, this speed decreases as the downward jet propagates due to viscous dissipation. Fig. 13 shows selected surface plots for grid arrangements where the outer and bottom boundaries were set at 2, 50 and 100 bubble diameters at selected times. To facilitate the comparison of these results the plots are shown using the same output window. This provides plots of identical physical dimensions. The proximity of the boundaries, however, were set as described above. Based upon comparisons of the outputs, extending the distance of the outer and bottom boundaries results in differences in the position of the free surface of at most two cells, and the free surface plots for every case resemble the actual process to the same extent. As with the grid refinement study, the change of deformation rates as the proximity of the boundaries was increased was evaluated. Table 2 shows the distribution of resultant stresses over the time domain. Again for each case, fifteen data outputs of the hydrodynamic state were used and cells with stresses less than 50 dyne/cm² were not considered. From Table 2 we see that the largest frequencies are localized in the range of 50-100 dyne cm² and then decrease for higher stress ranges. In each case only a few cells show values greater than 650 dyne cm². Figure 13 and Table 2 show that the numerical solution does not change appreciably as the proximity of the outer and bottom boundaries increase beyond 2 bubble diameters. These results are expected when one considers that the driving force is surface curvature and the displacement of the still liquid level (due to the presence of the bubble at the interface)
becomes very small at distances larger than two bubble diameters.

3.11 Discussion of the computer solution

The numerical results are sensitive to the initial conditions, boundary conditions, and numerical resolution used in the calculation mesh. However, as it was shown for the 1.7 mm diameter bubble, a grid definition can be found for which the solution no longer changes appreciably. In doing this, one has to be careful to maintain an appropriate cell aspect ratio (ratio of $\delta r$ to $\delta z$ for any cell), especially in those regions where surface curvature is being defined. That is, where there are significant surface tension forces. The evaluation of surface curvatures from data available in the VOF method is a difficult numerical problem. Using large cell aspect ratios exasperates these difficulties because it implies that curvatures can be more accurately calculated in one direction than another and this incorrectly biases the computed force.

During the calculation of the three cases presented, a point is reached at which the convergence of flow at the axis of symmetry (instant at which opposite jets are formed) results in the formation of a very small bubble. That is, as the cavity wall approaches the axis of symmetry it closes slightly above the bottom of the original bubble (data not shown). The development of this small bubble is not an unrealistic result. MacIntyre (1972) have reported a downward ejection of small bubbles upon bubble breakup. In fact, these observations corroborated the formation of the downward jet theoretically predicted. This phenomenon has also been observed in visualization studies in our laboratory. Some of the energy released
in the original bubble is conserved as surface energy as smaller bubbles are formed. These smaller bubbles eventually will break and released their energy. It is likely that a minimum bubble size exists at which smaller bubbles are not produced.

3.12 Analysis of the hydrodynamic information obtained from the computer solution

One of the goals of this work is to extract a quantity from the numeric solution which can be related to cell damage. Ideally, this parameter should be of general nature; i.e., it should be useful not only to analyze the results of our computer solution but also results from other independent experiments. If these characteristics are met, this quantity has the potential to be used as a criterion for the design of animal cell bioreactors. Inversely, given a suitable design parameter one should be able to use it to analyze results from any particular experiment.

Shear stress is the parameter that traditionally has been used to correlate cell damage. It has been used, for example, in experiments with rotational and capillary viscometers where cells are subjected to deformation of the fluid in which they are suspended.

A comparison of shear stress from two different viscometer designs such as plate and cone and concentric cylinders can be made. This is because at a given position in the flow of any of these viscometers, an orientation of a Cartesian coordinate system exists for which normal stress components, as well as two of the three different shear stress components of the stress tensor, are equal to zero. For example, for the cone and plate viscometer
for some $e_1$, $e_2$, $e_3$. These non-zero shear stress components have been associated with changes in cell physiology or death. However, for a complex flow (such as that occurring when a bubble collapses) shear stress does not completely characterize the state of stress at any point within the fluid. We conclude that shear stress by itself should not be considered as the appropriate quantity to analyze our data. Following the idea that a single, suitable design parameter should be useful to analyze different and independent experiments the following alternatives are proposed.

In trying to find a suitable parameter, it is necessary to consider some quantity which is uniquely defined in space and time and is related to cell damage. If it is assumed that cell damage results from the rate of deformation of the fluid in general (and not from shear stress only as the literature suggests), one is tempted to consider the rate of deformation itself or another quantity related to it, such as stress (not shear stress only), which for Newtonian fluids is linearly related to the rate of deformation. Since the rate of deformation and stress at a specific time and location are characterized by the rate of deformation tensor and the stress tensor respectively, and both are symmetric second order tensors, quantities which are uniquely defined to these tensors could be considered as design parameters. If the stress tensor is considered, for example, possibilities include its second or third invariants (first invariant is zero for incompressible fluids). Another parameter which is relatively easy to
understand, that is uniquely defined at a given time and position, and that can be calculated from the rate of deformation, is the rate at which work is done on an element of fluid as it moves and deforms. In this approach, as in that involving invariants, no consideration is made on the resultant forces acting on the cell surface since the cell may rotate or deform as a response to the rate of deformation of the fluid, but instead the functionality

\[
\text{Cell damage} = f(\text{rate of work per unit volume})
\]
is based on the rate of deformation of the continuum (the fluid phase). In addition, the implication is made that cells are contained within the fluid element being considered.

For an incompressible Newtonian fluid the rate at which work is done on an element of fluid (also called dissipation function) is given by

\[
\Phi = \mu \left[ (\nabla U \cdot (\nabla U)^T) : (\nabla U) \right]. \tag{5}
\]

For example, a value of \(\Phi = 2.5 \times 10^8\) dyne-cm sec\(^{-1}\) cm\(^3\) means that in one cubic centimeter of fluid the rate of energy dissipated is \(2.5 \times 10^8\) dyne-cm sec\(^{-1}\). Thus, given a velocity field obtained from the numerical solution of a complex flow such as that resulting when a bubble breaks or from experimental data from agitated containers (using techniques from which local information of the flow can be obtained) or from well defined flow devices (where the rate of deformation is fixed), the dissipation function can be calculated and compared. In this work the dissipation function is used to analyze the local hydrodynamic information obtained from the computer solution.
Dissipation of energy in the fluid has been used in the past to correlate cell damage on microcarriers (Croughan et al., 1987). It has also been used as a design parameter using a turbulent eddy model (Croughan et al., 1987; Cherry and Papoutsakis, 1986). This model, assumes that cell damage will occur if the Kolmogoroff eddy microscale calculated from

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

is of comparable size to the microcarrier size, where $\varepsilon$ is the average specific rate of energy dissipation. The turbulent eddy model does not take into account local values of the flow. Events at the liquid interface where, as it will be shown, highly localized energy dissipation takes place as sparged or entrained bubbles burst are not considered in this turbulent eddy model.

Figures 14-16 show a sequence of free surface plots (left) and dissipation function distribution (right) for bubbles with diameters 0.77, 1.70, and 6.32 mm, respectively. The dissipation function is shown using a gray scale that spans from white to black. For each bubble size, the corresponding range of these gray scales is presented in the second column of Table 3. Values greater than the upper limit were normalized to be shown by the maximum gray level, (i.e. black). This saturation value (the upper limit of the range) was used because only a small number of cells presented dissipation function values near the maximum value. In addition the maximum value occurs only at the instant in which the flow converges at the axis of symmetry. Had we selected saturation values equal to the maximum, only very tiny black regions would have been observed in the graphs of the dissipation function distribution.
at times before and after the maximum was detected. The corresponding time for every frame is indicated in the legend of each figure.

As it can be seen in Figures 14-16, a region of highly localized energy dissipation starts to develop in the initial stages of the bubble collapse. It is located at the edge of the bubble cavity where the fluid starts to move at high speeds due to high pressure differences located in this region. In subsequent frames the region of localized energy dissipation grows and moves towards the axis of symmetry and down into the cavity wall. When the flow converges at the axis of symmetry a maximum for the dissipation function is calculated. These maximum values and the time at which they are detected are shown on the third and fourth columns of Table 3, respectively. After the convergence of the flow upward and downward jets are observed and the dissipation function loses its intensity. The total elapsed time is shown on the fifth column of Table 3.

It is important to point out that even though similar characteristics are observed for the three bubble sizes considered, the calculated dissipation function decreases as the bubble size increases. For example, the maximum, calculated values for the dissipation function decrease three orders of magnitude when the 6.32 mm bubble diameter is compared to the 0.77 mm bubble. This decreasing relationship is nearly semi-logarithmic.

Figure 17 shows plots of the dissipation function corresponding to the time when the maximum is calculated. In this figure the range of numerical values for the gray scale is from zero to the corresponding maximum value of the dissipation function for each bubble size.
3.13 Region of maximum values for the dissipation function

Even though the maximum values of the dissipation function are concentrated in a small region, its importance becomes evident if we consider the hydrodynamics of the rupture process and animal cell-bubble interactions.

MacIntyre (1972, 1968) has described the bubble rupture process as a boundary layer "microtome". Using high-speed cinematography and dyed bubbles he observed that a very thin liquid layer next to the bubble boundary is transported towards the axis of symmetry and eventually ejected in the upward and downward jets. This is schematically shown on Figure 18. In visualization studies, Chalmers and Bavarian (1991) and Bavarian et al. (1991), have reported that some cell lines attach to rising bubbles. Garcia-Briones and Chalmers (1991), collected a liquid sample from the upward jet produced as a bubble ruptured in a suspension of insect cells. In this liquid sample they found that more than 95% of the cells were dead as compared with less than 10% in the bulk suspension.

Given the above observations, the argument can be made that even though the energy dissipation is maximum in a small region (see Fig. 17), cells attached or closed to the bubble cavity will be eventually transported to the region where high localized energy dissipation (high rates of deformation) occurs.
3.14 Comparison with data from the literature

In this section a comparison of our results with data from the literature is made.

3.14.1 Well defined flow devices

Table 4 shows the dissipation function for 4 different experiments performed in 4 different viscometers. To illustrate how the dissipation function was calculated, let us take the plate and cone viscometer. Considering spherical coordinates, the tensor $\nabla U$ for the cone and plate viscometer is given by

$$\nabla U = \begin{bmatrix}
0 & 0 & \frac{\partial U_\phi}{\partial r} \\
0 & 0 & \frac{1}{r} \frac{\partial U_\phi}{\partial \theta} \\
-\frac{U_\phi}{r} & -\frac{U_\phi}{r} \cot \theta & 0
\end{bmatrix}$$

(7)

where it has been considered that $U_r = U_\theta = 0$ and no change in the $\phi$ direction exists, thus $\partial U_\phi/\partial \phi = 0$. The product $\Phi = \mu[(\nabla U) + (\nabla U)^T]:(\nabla U)$ is then

$$\Phi = \mu \left[ \frac{\sin \theta}{r} \frac{\partial}{\partial \theta} \left( \frac{U_\phi}{\sin \theta} \right) \right]^2.$$  

(8)

We also notice that the only non-zero components of the stress tensor for this flow
become

$$\tau_{\theta \phi} = \tau_{\phi \theta} = -\mu \left[ \sin \theta \frac{\partial}{\partial \theta} \left( \frac{U_\phi}{\sin \theta} \right) \right]. \quad (9)$$

For a properly designed plate and cone viscometer where the angle of the cone is small we have

$$\tau_{\theta \phi} = \tau_{\phi \theta} = \tau_{plate} = \mu \frac{\Omega}{\theta_o}. \quad (10)$$

Comparison of eqs (8)-(10) indicates that the dissipation function can be calculated by

$$\Phi = \mu (\dot{\gamma})^2 \quad (11)$$

where

$$\dot{\gamma} = \frac{\Omega}{\theta_o}. \quad (12)$$

It can be shown that the dissipation function can be calculated with an equation of the same form as eq.(12) for concentric cylinders and double cup and bob viscometers.
3.14.2 Mixed bioreactor experiments

Even though no local values of the dissipation function are available in the literature for mixed bioreactor studies, a comparison can be made with the reported average values of the specific turbulent power dissipation rate calculated from

\[ \epsilon = \frac{N_p n^3 d_i^5}{V_d}. \]  

(13)

In this case an average dissipation function can be calculated from

\[ \Phi_{\text{ave}} = \epsilon \rho \]  

(14)

where in the absence of more information, the density can be taken to be equal to the density of water. Table 5 shows the average dissipation function for experiments in mixed bioreactors.

3.15 Discussion of results

Looking at the data presented in Table 4 we see that when the dissipation function was \(5.81 \times 10^3 \text{ erg cm}^{-3} \text{ sec}^{-1}\) no appreciable reduction in cell viability was observed. In contrast, for the experiment for which the dissipation function was \(4.8 \times 10^8 \text{ erg cm}^{-3} \text{ sec}^{-1}\) a reduction of 84.5% in viable cells was observed in 0.3 sec. This order of magnitude for the dissipation function is comparable to the maximum values calculated for the 0.77 and 1.7 mm diameter bubbles shown in Table 3.
In well-defined flow devices, time is always involved. The question arises as to what is more detrimental to cells, a low rate of energy dissipation applied for a long time or the combination of a high rate of energy dissipation applied for a very short period of time. The data in Table 3 suggests that at a sufficiently high rate of energy dissipation cell damage becomes independent of time for the time scales of hydrodynamic events common in bioreactors. This may very well be what happens when small bubbles break. The rate of energy dissipation is sufficiently high (~1x10⁹ erg cm⁻³ sec⁻¹ for 0.77 mm diameter bubbles) that the very short time of action is not important regarding its effect on cell viability. This can actually be experimentally tested by using a viscometer and subjecting the cells to high rates of deformation for very short periods of time.

Data in Table 5 shows that when the average dissipation function was in the order of 2.99x10⁴ to 2.90x10⁵ erg cm⁻³ sec⁻¹ the growth rate was seen to decrease significantly. On the other hand for an average dissipation function in the range of 2.07x10¹ to 2.97x10⁴ erg cm⁻³ sec⁻¹ no effect on cell growth and physiology was observed. The range shown in Table 5 for the dissipation function where a reduction in the growth rate was observed (2.99x10⁴ to 2.90x10⁵ erg cm⁻³ sec⁻¹) may seem to be low, however, these values are average values for the dissipation function. Higher values than the average most probably exist in the trailing vortices behind the impeller (Placek et al., 1986). These local values of the dissipation function can only be calculated if local information of the flow is known.

The magnitude of the calculated maximum value for the dissipation function decreased from 9.52x10⁵ to 9.4x10⁵ erg cm⁻³ sec⁻¹ as the bubble diameter was increased from 0.77 to 6.32 mm. The effects of sparging reported by Handa et al. (1987) show a reduction of cell
viability as the bubble size was decreased. Oh et al. (1992) found that bubbles larger than 5 mm are much less detrimental than small bubbles. Clearly the energy dissipation function helps us to understand why small bubbles are more detrimental to cells than larger ones.

In this paper, only the energy released in the boundary of the bubble cavity has been presented. It is our hypothesis that the rate of energy released as the bubble film retracts can be even greater than the calculated maximum values shown here.

3.16 Conclusions

It has been shown that as a bubble collapses at a gas-liquid interface a significant amount of energy is released in a very small region of liquid. The calculated rate of energy dissipation for a 0.77 mm diameter bubble can be as high as $\sim 1 \times 10^9$ erg cm$^{-3}$ sec$^{-1}$. As the bubble size increases the rate of energy dissipation decreases. It has also been shown that the concept of dissipation function is a general concept which is meaningful and provides a logical framework to understand animal cell damage in the region of bubble disengagement. In addition, it has the potential to be used as a criterion for the design of animal cell bioreactors. The only assumption behind this parameter is that cell damage is a function of the rate of deformation of the fluid in general and not shear stress only. If the dissipation function is to be applied in bioreactor design, details of the flow at all relevant areas in the bioreactor are needed in order to calculate local values of the dissipation function. One could then modify the reactor design (or operation) with the purpose of having a dissipation function less than a threshold value corresponding to the limit that a particular cell line can
withstand. This limit can be determined experimentally using well defined flow devices.

3.17 Acknowledgements

The authors wish to thank Dr. Tony Hirt of Flow Science Inc., Dr. Farshad Bavarian of the Department of Chemical Engineering, and Dr. F. Hink of the Department of Entomology for their technical assistance and insights. The authors would also like to acknowledge the National Science Foundation, grant number BCS-9109151, for its financial support and the Ohio Supercomputer Center for computer and consulting time. This work was partially presented at the 3rd International Conference on Bioprocess Fluid Dynamics, Cambridge, England, September 14-16, 1993.
3.18 Notation

bd  bubble diameter

\(c\)  velocity of sound

\(d_i\)  impeller diameter

\(e\)  unit vector

\(n\)  agitation rate

\(N_p\)  power number

\(R\)  radius

\(r\)  radial direction

\(T\)  temperature

\(U\)  velocity vector

\(U\)  component of the velocity vector

\(V_d\)  dissipation volume

\(V_l\)  total liquid volume

Greek letters

\(\gamma\)  shear rate

\(\epsilon\)  specific turbulent power dissipation rate

\(\eta\)  Kolmogoroff eddy microscale

\(\theta\)  theta direction

\(\mu\)  dynamic viscosity

\(\nu\)  kinematic viscosity

\(\rho\)  density

\(\sigma\)  surface tension

\(\tau\)  stress tensor

\(\tau\)  component of the stress tensor

\(\phi\)  phi direction

\(\Phi\)  dissipation function

\(\Omega\)  angular velocity

Operators

\(\nabla\)  nabla operator

Superscripts

\(T\)  transpose
Subscripts

ave    average
i      index
j      index
max    maximum
o      fixed quantity
res    resultant
Figure 7. Bubble shape solution. When properties of water and air are considered the actual diameters (maximum diameters) are 0.77, 1.70 and 6.32 mm (Medrow, 1968).
Figure 8. Computational grid. Thirty five cells are used in x (radial direction) and 55 in z. The bubble shape at time = 0 s is shown. The outer and bottom boundaries are set to about two bubble diameters from the still liquid level and axis of symmetry (left side), respectively.
Figure 9. Free surface plot at time = 0 s showing the azimuthal segment used in the computation. The bubble is 1.7 mm in diameter.
Figure 10. Comparison of high-speed photographs (Woodcock et al., 1953) and computer results from the 1.7 mm diameter bubble. (Reprinted with permission from Nature 172, 1144, 1145, copyright 1953 Macmillan Magazines Limited).
Figure 11. Free surface plots for the 35x55 (left) and 70x110 (right) grids. Time from top to bottom is: 0.0, 8.0x10^{-4}, 1.4x10^{3}, and 2.0x10^{3} s.
stresses are not calculated for the filled cell

Figure 12. As the total number of cells decreases the imposed condition to calculate stresses (fluid fraction equal to 1 for the cell being considered and its surrounding cells) becomes more restrictive. Most of the stresses in the higher range (>50 dyne cm$^{-2}$) are located at cells close to the bubble cavity interface where high rates of deformation occur.
Figure 13. Free surface plots for grid arrangements where the outer and bottom boundaries were set at 2 (left), 50 (middle), and 100 (right) bubble diameters (bd) at selected times. The window used is the same to facilitate comparison. Time from top to bottom is 0.0, 8.0x10^{-4}, 1.4x10^{3}, and 2.0x10^{3} s.
Figure 14. Fluid fraction (left) and dissipation function distribution (right) for a 0.77 mm diameter bubble. Time from top to bottom is 0.0, 1.0x10^-4, 2.0x10^-4, and 3.0x10^-4 s.
Figure 14. (Continued) Time from top to bottom is $4 \times 10^{-4}$, $4.3 \times 10^{-4}$, $4.5 \times 10^{-4}$, and $5.5 \times 10^{-4}$ s.
Figure 15. Fluid fraction (left) and dissipation function distribution (right) for a 1.7 mm diameter bubble. Time from top to bottom is 0.0 \(4.0 \times 10^{-4}\), 8.0 \(\times 10^{-4}\), and 1.2 \(\times 10^{-3}\) s.
Figure 15. (Continued) Time from top to bottom is $1.4 \times 10^{-3}$, $1.45 \times 10^{-3}$, $1.85 \times 10^{-3}$, and $2.0 \times 10^{-3}$ s.
Figure 16. Fluid fraction (left) and dissipation function distribution (right) for a 6.32 mm diameter bubble. Time from top to bottom is 0.0, 2.0x10$^{-3}$, 4.0x10$^{-3}$, and 5.2x10$^{-3}$ s.
Figure 16. (Continued) Time from top to bottom is $5.6 \times 10^{-3}$, $6.0 \times 10^{-3}$, $8 \times 10^{-3}$, and $1.0 \times 10^{-2}$ s.
Figure 17. Fluid fraction (left) and dissipation function distribution (right) corresponding to the time when the maximum is calculated. Time, bubble size, and range for the gray scale used to plot the dissipation function are indicated.
Figure 18. Source regions for the material going into the upward jet for a 2.07 mm bubble (MacIntyre, 1972). The downward jet is not shown.
Table 1. Frequencies of cells with resultant stresses ($\tau_{\text{res}}$) in the indicated range over the calculation time of $2.0 \times 10^{-3}$ s for the 35x55 and 70x110 grids.

<table>
<thead>
<tr>
<th>Range (1) (dyne cm$^{-2}$)</th>
<th>35 x 55 grid</th>
<th>70 x 100 grid</th>
</tr>
</thead>
<tbody>
<tr>
<td>at least</td>
<td>less than</td>
<td>Frequency</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>128</td>
</tr>
<tr>
<td>100</td>
<td>150</td>
<td>32</td>
</tr>
<tr>
<td>150</td>
<td>200</td>
<td>17</td>
</tr>
<tr>
<td>200</td>
<td>250</td>
<td>8</td>
</tr>
<tr>
<td>250</td>
<td>300</td>
<td>3</td>
</tr>
<tr>
<td>300</td>
<td>350</td>
<td>4</td>
</tr>
<tr>
<td>350</td>
<td>400</td>
<td>3</td>
</tr>
<tr>
<td>400</td>
<td>450</td>
<td>4</td>
</tr>
<tr>
<td>450</td>
<td>500</td>
<td>2</td>
</tr>
<tr>
<td>500</td>
<td>550</td>
<td>1</td>
</tr>
<tr>
<td>550</td>
<td>600</td>
<td>1</td>
</tr>
<tr>
<td>600</td>
<td>650</td>
<td>1</td>
</tr>
<tr>
<td>650</td>
<td>700</td>
<td>1</td>
</tr>
<tr>
<td>700</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td>750</td>
<td>800</td>
<td>0</td>
</tr>
<tr>
<td>800</td>
<td>850</td>
<td>1</td>
</tr>
<tr>
<td>850</td>
<td>900</td>
<td>0</td>
</tr>
<tr>
<td>900</td>
<td>950</td>
<td>0</td>
</tr>
<tr>
<td>950</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>1050</td>
<td>1100</td>
<td>0</td>
</tr>
<tr>
<td>1100</td>
<td>1150</td>
<td>1</td>
</tr>
<tr>
<td>1150</td>
<td>1200</td>
<td>1</td>
</tr>
<tr>
<td>1200</td>
<td>1250</td>
<td>0</td>
</tr>
<tr>
<td>1250</td>
<td>1450</td>
<td>0</td>
</tr>
<tr>
<td>1450</td>
<td>1500</td>
<td>0</td>
</tr>
<tr>
<td>1500</td>
<td>1550</td>
<td>0</td>
</tr>
<tr>
<td>1800</td>
<td>1850</td>
<td>1</td>
</tr>
<tr>
<td>1900</td>
<td>1950</td>
<td>0</td>
</tr>
</tbody>
</table>

(1) Ranges for which the frequency is zero for both cases are not included.

(2) Percentage of the total number of cells with the indicated resultant stress.
Table 2. Frequencies of cells with resultant stresses ($\tau_{\text{re}}$) in the indicated range over the calculation time of $2.0 \times 10^{-3}$ s for three different grid arrangements where the boundaries were set at 2, 50 and 100 bubble diameters (bd)

<table>
<thead>
<tr>
<th>Range (dynes cm$^{-2}$)</th>
<th>2 bd</th>
<th>50 bd</th>
<th>100 bd</th>
</tr>
</thead>
<tbody>
<tr>
<td>at least 50</td>
<td>100</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>at least 100</td>
<td>150</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>at least 150</td>
<td>200</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>at least 200</td>
<td>250</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>at least 250</td>
<td>300</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>at least 300</td>
<td>350</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>at least 350</td>
<td>400</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>at least 400</td>
<td>450</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>at least 450</td>
<td>500</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>at least 500</td>
<td>550</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>at least 550</td>
<td>600</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>at least 600</td>
<td>650</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>at least 650</td>
<td>700</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>at least 700</td>
<td>750</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>at least 750</td>
<td>800</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>at least 800</td>
<td>850</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>at least 850</td>
<td>900</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>at least 900</td>
<td>1100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>at least 1100</td>
<td>1150</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>at least 1150</td>
<td>1200</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>at least 1200</td>
<td>1250</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>at least 1250</td>
<td>1850</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

(1) Ranges for which the frequency is zero for the three cases are not included.
Table 3. Comparison of parameters for three bubble sizes

<table>
<thead>
<tr>
<th>Bubble diameter (mm)</th>
<th>Range of $\Phi$ for plots (erg cm$^{-3}$ sec$^{-1}$)</th>
<th>$\Phi_{\text{max}}$ (erg cm$^{-3}$ sec$^{-1}$)</th>
<th>Time at $\Phi_{\text{max}}$ (sec)</th>
<th>Total elapsed time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.77</td>
<td>$0 - 1 \times 10^6$</td>
<td>$9.52 \times 10^8$</td>
<td>$4.3 \times 10^{-4}$</td>
<td>$5.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>1.70</td>
<td>$0 - 1 \times 10^5$</td>
<td>$1.66 \times 10^8$</td>
<td>$1.4 \times 10^{-3}$</td>
<td>$2.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>6.32</td>
<td>$0 - 1 \times 10^4$</td>
<td>$9.40 \times 10^5$</td>
<td>$5.6 \times 10^{-3}$</td>
<td>$1.0 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

(1) Since these values are greater than the range in column 2, cells with this value appear black in plots.
Table 4. Dissipation function for experiments in well defined flow devices

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Instrument</th>
<th>Reference</th>
<th>Shear rate</th>
<th>Viscosity</th>
<th>Rate of cell damage</th>
<th>Dissipation function $\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(sec$^{-1}$)</td>
<td>(g cm$^{-1}$ sec$^{-1}$)</td>
<td>(%/min)</td>
<td>(erg cm$^{-3}$ sec$^{-1}$)</td>
</tr>
<tr>
<td>insect</td>
<td>cone and plate</td>
<td>Goldblum et al. 1990</td>
<td>5120</td>
<td>0.012</td>
<td>33.5</td>
<td>3.15x10$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8110</td>
<td>0.012</td>
<td></td>
<td>7.89x10$^5$</td>
</tr>
<tr>
<td>hybridoma</td>
<td>concentric cylinders</td>
<td>Schurch et al. 1988</td>
<td>1500</td>
<td>0.100</td>
<td>44.7</td>
<td>2.25x10$^5$</td>
</tr>
<tr>
<td>hybridoma</td>
<td>double cup and bob</td>
<td>Smith et al. 1987</td>
<td>869</td>
<td>0.008</td>
<td>3.4</td>
<td>5.81x10$^3$</td>
</tr>
<tr>
<td>mammalian</td>
<td>capillary(2)</td>
<td>Augenstein et al. 1971</td>
<td>—</td>
<td>—</td>
<td>(1)</td>
<td>4.80x10$^8$ (4)</td>
</tr>
</tbody>
</table>

(1) At 15 hrs. cell viability was 73% (78% at time=0) as compared with 85% for a control.
(2) The flow regimen was turbulent and thus non-well defined flow.
(3) 84.5% of the cells died in 0.3 sec. This is equivalent to a rate of cell damage of 16900%/min.
(4) Power dissipated is reported directly in this reference.
Table 5. Average dissipation function for mixed bioreactors experiments

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Reference</th>
<th>Reactor volume (cm³)</th>
<th>Agitation rate (rpm)</th>
<th>Impeller diameter (cm)</th>
<th>Power number (*)</th>
<th>Ave. dissipation (erg cm⁻³ sec⁻¹)</th>
<th>Effects on cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridoma</td>
<td>Kunas and Papoutsakis</td>
<td>2000</td>
<td>800</td>
<td>7</td>
<td>1.5 (2)</td>
<td>2.99x10⁴</td>
<td>1.74x10⁵ (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V_d = V_t</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V_d = d_i³</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Papoutsakis 1990</td>
<td>2000</td>
<td>800</td>
<td>7</td>
<td>2.0</td>
<td>3.98x10⁴</td>
<td>2.32x10⁵ (3)</td>
</tr>
<tr>
<td></td>
<td>Oh et al. 1992</td>
<td>1400</td>
<td>100</td>
<td>6</td>
<td>0.8</td>
<td>2.07x10¹</td>
<td>1.34x10² (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.07x10¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V_d = d_i³</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V_d = d_i³</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.07x10¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.07x10¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.07x10¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.07x10¹</td>
<td></td>
</tr>
</tbody>
</table>

(1) Values for the specific turbulent power dissipation rate calculated by taking both the dissipation volume equal to the total liquid volume and by taking a volume equal to the impeller diameter cubed.

(2) A range of power numbers was considered by these authors since the impeller used was not standard.

(3) It was found that at 800 rpm and without bubbles present in the bioreactor the cell growth rate was significantly lower than that measured under no agitation or gentle agitation.

(4) No effect on cell growth, viability, antibody production and metabolic activity was found. No sparging was used and bubble entrainment was prevented.
4.1 Summary

Two flow parameters are proposed for the analysis of flows that have potential to damage animal cells. They are the state of stress (characterized by the second invariant of the stress tensor) and the flow classification parameter $R_D$ (which is related to the possibility of stress relaxation). We consider the flow that occurs when a 1.7 mm bubble collapses at a liquid interface. Using these two parameters we show the regions in which the flow is strong in terms of high hydrodynamic stresses and elongational characteristics.
4.2 Introduction

Aims of in vitro cultivation of animal cells can be broadly divided in two categories: cell research and manufacture of biological products. In the latter case the product may be the cells themselves, cell components, or metabolites which are produced naturally or by expression of recombinant DNA. For proper cell propagation, the cell culturist needs to provide an in vitro environment as similar as possible to the natural cell environment. As the scale of cultivation increases mixing of the culture medium is usually required to maintain uniform conditions and enhance the transport of limiting nutrients such as oxygen. Since mixing is accomplished by deformation of fluid elements, hydrodynamic stresses are transmitted to the cells (which may be suspended or attached to a surface).

Lethal and non lethal effects on cells as a result of their interaction with the hydrodynamic environment have been extensively documented in the literature. These observations were obtained in flow devices such as parallel plates (Diamond et al., 1989; Eskin et al., 1984; Frangos et al. 1985, 1988; Grimm et al., 1989; Ives et al., 1983; Krueger et al., 1971; Levesque and Nerem, 1985; Viggers et al., 1986; Wechezak et al., 1985), cylindrical tubes (Augenstein et al., 1971; Goldsmith and Mason, 1975; McQueen et al., 1987; McQueen and Bailey, 1989), concentric cylinders (Abu-Reesh and Kargi, 1989; Chien et al., 1982; Drasler et al, 1987; Petersen et al., 1988; Smith et al., 1987; Tramper et al., 1986; Tran-Son-Tay et al., 1986), cone-and-plate (Brooks, 1984; Bussolari et al, 1982; Dewey, 1984; Franke et al., 1984; Goldblum et al., 1990; Tran-Son-Tay et al., 1984, 1987), and small scale agitated and/or sparged bioreactors (Abu-Reesh and Kargi, 1991; Cherry and

Even though these studies demonstrate the sensitivity of cells to hydrodynamic stresses, comparison of results from two different systems is not possible. Factors affecting the effects of fluid flow on animal cells are: flow field (flow regime, flow patterns), level of "shear", time of exposure, medium composition (protective additives), and cell line.

One of the purposes to study the shear sensitivity of cultured animal cells is to have information that will allow one to define an appropriate environment in which the cells can be cultured at large scales. This involves the identification of the relevant parameters of the flow field governing hydrodynamic related cell injury and their relationship with the equipment geometry (reactor design) and operation. In an ideal situation cell damage should be predicted by knowing the actual stresses that the cell experience and, from intrinsic cell mechanical properties, the resulting cell deformation. Should the cell deformation exceed a critical value, disruption of the cell structure would be expected.

Flows to study the cell response to hydrodynamic stresses have been characterized by different parameters. Shear stress and rate of shear were used to characterize the flow in well defined flow devices (parallel plate chambers, concentric cylinders and plate and cone viscometers). Average wall shear stress and specific power dissipation were used for
turbulent flows in capillary tubing. Impeller speed, impeller tip velocity, integrated shear factor, time average shear rate and shear stress, specific power dissipation and Kolmogorov eddy length scale have been used for agitated small-scale bioreactors. Operation parameters such as gas flow rate, bubble frequency and bubble size have also been used to characterize sparged bioreactors. Even though these parameters have proved to be useful to correlate cell damage under the conditions of the particular experiment, their application to reactor design is questionable. For example, we do not know how to use parameters that correlate cell damage in one particular system to predict cell damage in a different system; i.e. we don't know how to use the levels of shear stress that produce damage in a viscometric flow to predict cell damage in a mixed vessel, or to predict cell damage in a bubble column from capillary tube data. The problem reduces to asking what are the relevant parameters which govern hydrodynamic cell injury for any given flow. To date, they have not been identified.

Parameters used to characterize hydrodynamic related cell injury should be of general nature. These parameters cannot come from the geometry of the system producing the flow but from "intrinsic" characteristics of the flow itself. Any consideration of the geometry of the system producing the flow will limit their generality. Once "intrinsic" characteristics of the flow have been identified, their relationship with the geometry and operation of a given system can then be considered for optimization purposes. A second requirement is that we should consider local and not "average" parameters. Cell injury in a hydrodynamic environment (assuming no nutrient and mass transfer limitations) must result from the application of external stresses. Consequently, it must be a function of the local intensity of these stresses. Average values prevent the identification of those regions in the flow where
cells can be killed. A flow may injure a cell at one point and have no effect in cell viability at a different location. Thus, the values of the selected parameters must be local if general application is to be accomplished.

Laminar shear stress and rate of shear used in viscometric and parallel plate flow devices are local flow values and not system dependent when different viscometer designs are considered. That is, we can have the same level of shear stress in different types of viscometers. This bulk shear stress is a parameter proportional to the actual external stresses that the cell experience and at first seems to be an appropriate parameter. However while shear stress completely characterizes the local state of stress in these flows, that is not the case for a more complex flow (See Appendix A). Consequently, shear stress or rate of shear are not by themselves parameters of general applicability.

Turbulent shear stress or rate of shear used in flows in capillary tubing, viscometers (operated at high enough rotation speeds to have turbulent flow) and mixed bioreactors are only global average estimates and system dependent. Impeller speed (rpm), impeller tip velocity and the integrated shear factor are not flow parameters but operation parameters and obviously system dependent. Their usefulness is limited even for a defined system geometry. Specific power dissipation used in mixed/sparged bioreactors is a flow parameter but not a local one (at least in the way it has been calculated). Even though the energy dissipated in the fluid can be estimated from correlations (for some standard impeller configurations) or by direct measurement, the volume of liquid in which the energy is dissipated is an educated guess. For example, for agitated vessels this dissipation volume has been approximated to be equal to the impeller diameter cubed. However, even this approximation averages the
energy dissipation over a significantly large volume. Kolmogorov eddy length correlation used for turbulent flows is different from the specific energy dissipation correlation only in that it provides a physical picture of how cell damage in turbulent flows can occur.

Prediction of cell damage based on the Kolmogorov eddy length approach was originally proposed for microcarrier cultures. It was intended to consider flow-bead interactions in turbulent flows such as those occurring in mixed containers. The physical picture of cell damage proposed in this approach is that only when cells attached to microcarriers encounter an eddy of size comparable to the microcarrier size, cell damage will occur. The microcarrier size then becomes a correlation factor neglecting cell mechanical and adhesion (to the microcarrier) properties. The correlations of cell damage with eddy size so far reported in the literature do not validate the proposed mechanism. That is, no evidence exists that eddies of comparable size as the microcarrier size are responsible of the observed cell damage. Since the calculation of the Kolmogorov eddy length scale is based on isotropic turbulence, its application to flow regions close to the impeller where most of the cell damage is expected has not been demonstrated. The Kolmogorov eddy size approach has also been applied to explain hydrodynamic related injury for suspended cells. In this case cell size becomes a correlation parameter neglecting cell mechanical properties. It has been extensively documented in the literature that most of the damage in sparged systems results from the break up of bubbles at the liquid interface. For these systems the Kolmogorov eddy size approach cannot be used.
In this section, flow parameters of general nature that can be used to study hydrodynamic related cell injury are suggested. The first step is to propose how an individual cell or a cell attached to a surface of a microcarrier can be damaged as a result of fluid flow. Cell damage for suspended cells is defined as the disruption of the cell structure (possibly the rupture of the cell membrane) that results from cell deformation. For anchorage dependent cells, cell damage is defined as the detachment of cells from the surface support. This definition is motivated by the fact that anchorage dependent cells cannot divide when suspended. In microcarrier cultures, Croughan and Wang (1989) observed that once cells detach from their surface support they lysed in less than two hours.

An idea of how a suspended cell can be deformed (and disrupted) by a flow field and which flow parameters govern cell deformation can be obtained from the classic experiments on deformation of liquid drops by shearing flows reported by Taylor (1934). In interpreting the results of Taylor (1934), the crucial assumption is whether a cell behaves as a liquid droplet. This assumption is discussed later.

Taylor (1934) studied the formation of emulsions in two defined flow fields. The first one was an extensional flow and it was produced in a four roll mill shown schematically in Figure 19a. The velocity field is defined by

\[ v_x = C x, \quad v_y = -C y \]  

(15)
The second flow was a simple shear flow and was reproduced by a parallel band device shown in Figure 20a. This flow is defined by

\[ v_x = \alpha y, \quad v_y = 0 \]  \tag{16}.

The constants \( C \) and \( \alpha \) depend on the physical dimensions and operation of the instruments. Once the instruments are built (dimensions fixed), these constants only depend on the speed of the rollers. It can easily be shown that the rate of strain tensor for the four roll mill and parallel band instrument are given by

\[
\begin{bmatrix}
2C & 0 & 0 \\
0 & -2C & 0 \\
0 & 0 & 0
\end{bmatrix}, \quad \text{and} \quad \begin{bmatrix}
\alpha & 0 & 0 \\
0 & -\alpha & 0 \\
0 & 0 & 0
\end{bmatrix} \quad \text{at } 45^\circ \tag{17}
\]

respectively. Here, the rate of strain tensor for the parallel band instrument is given for the axes \( x, y \) in Fig. 20a rotated 45°. Clearly, when \( 2C = \alpha \) the rate of strain tensor (and thus the state of stress, if a Newtonian fluid is considered) is the same for both flows. If it were found that the deformation of liquid drops is the same in both systems when \( 2C \) is made equal to \( \alpha \), then for a given viscosity of the suspending fluid the rate of strain would be enough to characterize the effects of shearing flows on liquid droplets. Taylor (1934) found that while drops broke at a low speed in the four roll mill they could not be broken no matter how fast
the parallel band instrument was run. In other words, while a drop broke in the four roll mill for a given value of $2C$, it could not be broken in the parallel band device at $\alpha = 2C$ (same rate of strain for the shearing flow) and in some cases for values much greater than $2C$. Taylor (1934) explained the above results as follows (See Figures 19b, 20b):

"The explanation of this phenomenon is simple. In the four roller apparatus, which produces the field of flow (1) (Eq. 15 in this paper), the lines of particles which are extending at the greatest rate, namely, those parallel to the axis of $x$, remain in the direction of the maximum rate of elongation as long as the flow continues. The disruptive stress due to the viscous drag of the syrup (the suspending liquid used by Taylor, 1934) is therefore always tending to extend the drop in the same direction. As soon as this stress is able to overcome the cohesive effect of surface tension the drop burst.

In the parallel band apparatus the lines of particles which lie in the direction of the maximum rate of elongation, namely, at $45^\circ$ to the bands, are continually being rotated away from that position towards the line parallel to the bands which is neither elongating nor contracting. After attaining this position further rotation brings the line of particles into an orientation where they are contracting."

These results demonstrate that the state of stress of the suspending liquid alone should not be used to predict drop deformation, but the possibility of internal stress relaxation by rotation (of the suspending particle) should also be considered. This internal stress relaxation by rotation is directly related to the vorticity of the suspending fluid.

Motivated by Taylor's experiments we propose that two relevant flow parameters to study hydrodynamic related cell injury are (at least for the flows so far considered): 1) the local state of stress (characterized by the stress tensor) and 2) the vorticity of the flow (note
that we have selected stress rather than rate of strain since it is obvious that the viscosity of the suspending fluid should be considered).

4.4 Relevance of Taylor experiments to hydrodynamic related cell injury

4.4.1 Anchorage dependent cells

In microcarrier cultures, cells attach to the microcarrier surface and assume a flat shape. In this case what is of importance is to determine the characteristics of a flow that makes it more effective for cell removal. Croughan and Wang (1989) studied the removal of cells in microcarriers. They found that cell detachment increased with the level of agitation and thus it was the result of hydrodynamic forces. Since mitotic cells round up (while non-mitotic cells remain flat), they hypothesized that cell removal would be selective for mitotic cells if shear forces were the main mechanism of cell removal. In contrast, if normal forces were more important than shear forces for cell removal, no selectivity would be observed since mitotic cells do not present greater probability of detachment than flat cells. This is due to the fact that both the resisting cell-solid surface adhesive force and the normal force pulling the cell, are proportional to the cell contact area. Croughan and Wang (1989) found no selectivity for removal of mitotic cells indicating the importance of normal forces. If we place a microcarrier in a pure extensional flow (zero vorticity) it would not rotate and normal stresses pulling the cells from the surface will be acting continuously along the direction of maximum rate of strain (the x axis in Taylor's four roll mill). In contrast, if we place the
microcarrier in a simple shear flow (Taylor's parallel band instrument), it would rotate and normal stresses (along the x axis rotated 45° in Taylor's parallel band) will be relaxed.

4.4.2 Suspended cells

The behavior observed in the experiments presented by Taylor (1934) for liquid drops would apply for suspended cells if suspended cells have similar flow characteristics as liquid droplets. That is, if they can be modeled as incompressible fluids having a cortical tension (cell membrane tension). Using the micropipet aspiration method, Evans and Kukanr (1984) reported on the large deformation and recovery (after deformation) characteristics of granulocytes. They observed that upon application of a suction pressure in excess of a threshold value, the deformation of granulocytes was a continuous-flow process. Furthermore, flow ceased when the suction pressure was decreased to the threshold value. Once the cell was released from the pipet the granulocyte always recovered its spherical shape independent of the extent of deformation. These results led Evans and Kukan (1984) to propose that the rheologic behavior of granulocytes could be described as a liquid of high viscosity surrounded by a cortical shell under tension ("contractile surface carpet"). Using this model Yeung and Evans (1989) and Evans and Yeung (1989) presented an analysis to determine the apparent viscosity of the cytoplasm core. They reported an apparent cytoplasmic viscosity of 2000 poise at 23° C. Further evidence that granulocytes behave as a liquid drop has been reported by Needham and Hochmuth (1990, 1992), and Tran-Son-Tay et al. (1991). Needham et al. (1991) have reported that hybridomas also behave as liquid
drops in micropipet aspiration tests.

The fact that some experimental evidence exist that cultured cells may behave as viscous liquid droplets does not imply that these cells have the same dynamics as true liquid droplets in an imposed flow. Since the cell interior is not a continuum, the measured viscosity is an apparent viscosity and the cortical tension is not a true interfacial tension. Nevertheless, considering a general deformable particle having a cortical tension and viscous interior, it is easy to see that a pure extensional flow will be more effective than a rotational one in deforming the particle. Flow characteristics associated with this "effectiveness" to produced deformation should be considered in our analysis of hydrodynamic related cell injury.

4.5 Flow classification

In this section, we elaborate on the general applicability of the proposed parameters to characterize any flow in terms of its potential to produce cell damage. This is in fact a problem similar to the determination of the rheological behavior of elastic liquids in viscometric and extensional flows. It has been experimentally observed that an elastic liquid accumulates stresses if the direction of maximum strain is applied to the same material line (line of particles). However, stress accumulation is less severe if the material rotates such that the same material line is not always aligned to the direction of the maximum rate of strain. Viscometric and extensional flows have also been observed to have a different effect in the apparent viscosity of polymer solutions. Most polymer solutions undergo shear thinning (viscosity falls) with increasing shear rate and extension thickening (viscosity climbs) as the
rate of extension increases. Since these effects result from the dynamic behavior of polymer molecules with the flow field (Schunk and Scriven, 1990), similar to those discussed for liquid drops, it is of interest to have a general criteria for flow classification which will allow one to anticipate the effects of the flow on the stresses accumulated by elastic liquids and to predict the viscosity of polymer solutions.

Astarita (1979) proposed a flow classification which is a) local, b) objective (invariant under a change of reference frame), c) general (not restricted to a particular class of flows), and d) kinematic (only depends on the fluid kinematics). He pointed out that what distinguishes a viscometric flow from an extensional flow is not the magnitude of the vorticity but the relative rate of rotation of the axes of the principal rates of strain with respect to the liquid itself (given by the vorticity).

The criteria for flow classification proposed by Astarita (1979) and used by Schunk and Scriven (1990) to construct a constitutive equation for polymer solutions is given by

\[ R_D = \frac{W_{rel}}{I_2(\bar{V})} \]  \hspace{1cm} (18)

\( W_{rel} \) is the magnitude of the relative rotation vector \( \vec{W}_{rel} \) given by

\[ \vec{W}_{rel} = \vec{W} - \vec{\omega}/2 = \bar{e}_I \times \left( \frac{\partial e_I}{\partial t} + \nabla \cdot \vec{e}_I \right) - \frac{1}{2} \nabla \times \bar{v} \]  \hspace{1cm} (19)
and \( I_2(\mathbf{\gamma}) \) is the second invariant of the rate of strain tensor \( \mathbf{\gamma} \) given by

\[
I_2(\mathbf{\gamma}) = \sqrt{tr((\mathbf{\gamma})^2)}
\]  

(20).

\( \mathbf{W}_{rel} \) is the relative rotation vector given by the difference between the rate of rotation of the principal straining directions \( \mathbf{W} \) and the rotation of the liquid \( \mathbf{\omega}/2 \). The principal straining directions \( \mathbf{e}_i \) are given by

\[
(\mathbf{\gamma} - \gamma_i \mathbf{e}_i) \cdot \mathbf{e}_i = 0
\]  

(21).

\( \mathbf{W}_{rel} \) becomes equal to half the vorticity \( (\mathbf{\omega}/2) \) for the four roll mill and parallel band apparatuses previously discussed. This is because the rate of strain's principal axes are fixed (they don't rotate) for the flows produced by these two instruments. While the internal stress relaxation by rotation in the four roll mill and parallel band devices is given by the vorticity \( (\mathbf{\omega}/2) \), for any given flow one must consider \( \mathbf{W}_{rel} \) since the possibility exists that the principal straining directions rotate. Dividing \( \mathbf{W}_{rel} \) by \( I_2(\mathbf{\gamma}) \), a non-dimensional quotient \( R_D \) (Eq. 18) is obtained. \( R_D \) ranges from infinity for rigid body rotation, unity for viscometric flows and zero for extensional flows. It is important to emphasize that this criteria is only based on kinematics and it is not restricted to any particular class of flows.

We close this section proposing that two local "intrinsic" flow characteristics of general nature which affect cell deformation (or detachment) are the state of stress
(proportional to the actual stress that cells experience) and the flow classification criteria $R_D$ (related to the possibility of stress relaxation by rotation).

4.6 Flow analysis

As it was mentioned previously, the injection of air bubbles into the liquid phase of suspended cell culture systems has been associated with a reduction of total viable cell concentration. Recently, Boulton-Stone and Blake (1993) and Garcia-Briones et al. (1994) have reported on computer simulations of bubbles breaking at a gas-liquid interface. These results showed that high rates of strain occur in the vicinity of a collapsing bubble. The associated flow was analyzed in terms of the rate of energy that is spent in fluid deformation (and thus dissipated by viscosity). They found that the rate of energy dissipation rapidly increases as the bubble size decreases. In this work this flow will be analyzed in terms of the state of stress and flow classification criteria $R_D$ to look at its potential to damage suspended cells. This new approach can be considered as a refinement of our previous effort since specific characteristics of the flow field related to cell deformation are taken into account.

4.6.1 Computer solution

Numerical solution of the flow that occurs when a bubble breaks at a liquid interface was obtained using FLOW-3D (Flow Science, Inc., Los Alamos, NM). The solution scheme uses a control volume method to produce a transient solution of the Navier-Stokes equations.
Since FLOW-3D can not account for the hydrodynamics of collapsing thin films (such as the bubble film) only the flow that results when the bubble cavity collapses is considered. More on the numerical approach, convergence and validation of the solution has been reported previously (Garcia-Briones et al., 1994).

4.6.2 Computation of the state of stress and flow classification parameters

The state of stress everywhere in the flow field was characterized by the product of the second invariant of the rate of strain defined by Eq. 20 times the dynamic viscosity (0.01 P):

\[ I_2' = \mu I_2 \]

The second invariant of the rate of strain (calculated from the velocity field using linear approximations for partial derivatives) is a scalar quantity and its magnitude will be high for high rates of strain. The flow classification parameter \( R_D \) was obtained from Eqs. 18 to 20. The principal directions and principal values of the rate of strain were calculated from Eq. 21 using the Jacobi's method (Atkinson et al., 1989).
4.7 Results

Figure 21 shows the distribution of the state of stress, \( I'_2(\mathcal{T}) \). The bubble is 1.7 mm in diameter. A linear "gray" scale that spans from 0 for white to 200 dyne cm\(^{-2}\) for black is used to show the stress distribution. The corresponding times and the maximum calculated value at each time is shown in Table 6. In this plot values greater than 200 dyne cm\(^{-2}\) were normalized to be shown in black. This saturation value (200 dyne cm\(^{-2}\)) was used because only a small number of computational cells presented stresses near the maximum calculated value (1998 dyne cm\(^{-2}\)). This maximum value occurs at the instant in which the flow converges at the axis of symmetry (1.4 msec, frame 5). Had we selected saturation values equal to the maximum, only very tiny black regions would have been observed on Figure 21 for times before and after the maximum is detected.

On Figure 22 the possibility of stress relaxation by rotation is shown by plotting the distribution of the flow classification parameter \( R_D \). The value of \( R_D \) was restricted between zero and one. That is, only flow strengths between purely extensional and simple shear are considered. If at a particular location in the flow field \( R_D \) is equal to zero, the flow is locally an extensional flow. In similar fashion if \( R_D \) is equal to one the flow is locally a simple shear flow. Flows for which \( R_D \) is larger than one are not of interest since they are weaker than a simple shear flow and approach solid body rotation as \( R_D \) increases. In order to be consistent in using dark regions to represent locations where cells can be damaged, \((1-R_D)\) is actually plotted on Figure 22. As before, a gray scale which spans from white (zero) to black (one) was used. With this transformation, zero (or white) corresponds to simple shear flow, and
one (or black) to purely extensional flow. Values for $R_D$ at different times are given in Table 6. On Figure 23 the state of stress and the parameter $R_D$ are combined; i.e. the stress, $I^2(\bar{\tau})$, is weighted with $(1-R_D)$. The same gray scale as that of Figure 21 was used.

Table 7 shows the maximum calculated values of the state of stress $(I'_{2}(\bar{\tau})_{\text{max}})$ and flow classification parameter $(R_D)$ at $I'_{2}(\bar{\tau})_{\text{max}}$, for three different bubble sizes. Even though the flow is always extensional in the region where $I'_{2}(\bar{\tau})_{\text{max}}$ is calculated, the magnitude of $I'_{2}(\bar{\tau})_{\text{max}}$ decreases as the bubble size increases. In order to see if cells will be disrupted in flow fields having the same characteristics as those in Table 7 (i.e. $I'_{2}(\bar{\tau}) = 4797$ dyne cm$^{-2}$ and $R_D = 0.02$) a basis for comparison is needed. To this end our data and data from others taken from the literature, that shows the magnitude of $I'_{2}(\bar{\tau})$ and $R_D$ that results in cell disruption is shown in Table 8.

### 4.8 Discussion

As it can be seen in Figure 21, high rates of strain (high stresses) start to develop in the liquid region close to the collapsing bubble interface. High rates of strain are produced as a result of the large pressure difference dictated by the curvature of the collapsing bubble. The flow eventually converges at the axis of symmetry and two opposite jets develop. Figure 22 shows that small regions with extensional characteristics develop around the collapsing bubble cavity and eventually concentrate in the regions of the upward and downward jets. What is of interest, however, is to see the regions in which the flow is strong not only because high rates of strain and thus high hydrodynamic stresses $(I'_{2}(\bar{\tau}))$, but also because elongational
characteristics ($R_D = 0$). The second invariant of the stress tensor and the flow classification parameter $R_D$ have been combined on Figure 23 in such a way that the parameter $R_D$ (used as $1 - R_D$) becomes a weighing factor of the state of stress in the fluid. Figure 23 shows that the regions in which suspended cells can be damaged are located close to the collapsing bubble cavity and in the upward and downward jets.

4.9 Comparison with data from the literature

Table 8 contains experimental data taken from the literature that shows the magnitude of $I_2(\bar{\tau})$ and $R_D$ that results in cell disruption. With the exception of the work reported by McQueen and Bailey (McQueen and Bailey, 1989) the experiments in laminar flow were performed in viscometric flows for which $R_D=1$. For the experiment in laminar regime in converging diverging tubes and those in turbulent regime performed in capillary tubing a local value of $I_2(\bar{\tau})$ and $R_D$ cannot be calculated from the information given in the references. However, it is known that the second invariant of the stress tensor is of the same order of magnitude as that reported for the average wall shear stress. It is also known that because of the geometry of the experimental devices, the flow in these experiments has extensional characteristics. Extensional forces are produced as a result of the acceleration of the fluid flowing through the restriction (capillary).

Data in Table 8 shows that cells are completely disrupted in a fraction of a second in flows with extensional characteristics with an average wall shear stress of several thousands of dyne cm$^2$. The order of magnitude of these stresses can be compared with the calculated
values of $I_2' (\tau)$ for breaking bubbles shown in Table 7 ($I_2' (\tau) \approx 2000 - 5000$ dyne cm$^{-2}$).

Even though the data in Table 8 helps to motivate the idea that extensional flows can be detrimental to cells, it does not show conclusively that extensional flows are more effective in disrupting cells. The exact contribution of $I_2' (\tau)$, and $R_D$ to cell disruption needs to be further investigated under controlled experimental conditions.

4.10 Conclusions and closing remarks

It has been proposed that two relevant flow parameters to study hydrodynamic related cell injury are the state of stress (characterized by the second invariant of the stress tensor) and the flow classification parameter $R_D$. Analysis of the flow that occurs when a bubble collapses at a gas-liquid interface using these two flow parameters showed the regions in which the flow is strong in terms of high hydrodynamic stresses and elongational characteristics. The lack of data on the effects of extensional flows in cell viability prevents a direct comparison with the numerical values calculated from our computer results. This points to the need of further experimental work in flow systems in which the magnitude and form of the velocity field can be can fixed and controlled.

The analysis presented here can be extended to turbulent flows in mixed bioreactors. The requirement, however, is that the fluid velocity should be known in space and time. In the last 15 years different experimental techniques (such as particle image velocimetry) have been developed and improved to measure the displacement of particle markers and thus the velocity field in turbulent flows. Once the velocity field is known the flow produced in a
mixed/aerated bioreactor can be analyzed and the areas in which cell damage is likely to occur identified.

4.11 Acknowledgements

The authors thank Tony Hirt from Flow Science Inc. and Robert Brodkey from the Department of Chemical Engineering of the Ohio State University for their technical assistance and insights. The authors also acknowledge the National Science Foundation, grant number BCS-9109151, for its financial support and the Ohio Supercomputer Center for computer and consulting time. This work was presented at the Annual AIChE Meeting, St. Louis, MO, November 7-12, 1993.
4.12 Notation

C \quad \text{four roll mill apparatus constant}
\bar{e}_i \quad \text{principal straining directions}
I_2 \quad \text{second invariant}
R_D \quad \text{flow classification criteria}
t \quad \text{time}
tr \quad \text{trace of a tensor}
\nabla \quad \text{fluid velocity vector}
\mathbf{W} \quad \text{rate of rotation of the principal straining directions vector}
\mathbf{W}_{\text{rel}} \quad \text{relative rotation vector}
\mathbf{W}_{r_{\text{el}}} \quad \text{magnitude of the relative rotation vector}

\text{Greek letters}

\alpha \quad \text{parallel band apparatus constant}
\Psi \quad \text{rate of strain tensor}
\gamma_{ij} \quad \text{principal values of the rate of strain tensor}
\delta \quad \text{unit tensor}
\mu \quad \text{dynamic viscosity}
\tau \quad \text{stress tensor}
\tau_{ij} \quad \text{stress components}
\omega \quad \text{vorticity vector}

\text{Operators}

\nabla \quad \text{nabla operator}

\text{Superscripts}

T \quad \text{transpose}
a Four roll mill apparatus

![Diagram of four-roll mill apparatus with principal strain direction indicated]

b Drop placed at center

![Diagram showing drop interaction with extensional flow field]

Figure 19. (a) Four-roll mill apparatus. When the rollers are rotated at the same speed in the directions indicated, an extensional flow is produced in the central area. The principal directions of the rate of strain coincide with the x and y axes. (b) Drop interactions with an extensional flow field (drop placed at center).
Figure 20. (a) Parallel band apparatus. A simple shear flow is produced when the rollers are rotated as indicated. The principal directions of the rate of strain are shifted $45^\circ$ with respect to the $x$ and $y$ axes. (b) Drop interactions with a simple shear flow field (drop placed at center).
Figure 21. Distribution of the state of stress, $[\Pi_2(x)]$ in the flow produced when a 1.7 mm bubble breaks at a gas-liquid interface. Times and maximum calculated values for the state of stress corresponding to the frame numbers are given in Table 6.
Figure 22. Distribution of the parameter $R_D$ plotted as 1-$R_D$. Times and values for $R_D$ corresponding to the frame numbers are given in Table 6.
Figure 23. Distribution of strength of the flow \((1-R_D)l_2(t)\). Times and maximum calculated values for \((1-R_D)l_2(t)\) corresponding to the frame numbers are given in Table 6.
Table 6. Times and maximum calculated values of $I'(\bar{r})$, $R_D$, and $(1-R_D)I'(\bar{r})$ for each frame in Figures 21–23.

<table>
<thead>
<tr>
<th>Frame number</th>
<th>Time (msec)</th>
<th>$I'(\bar{r})$ (dyne cm$^{-2}$)</th>
<th>$R_D$</th>
<th>$(1-R_D)I'(\bar{r})$ (dyne cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>-----</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>2</td>
<td>0.40</td>
<td>76</td>
<td>0.42</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>0.80</td>
<td>133</td>
<td>0.37</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>1.20</td>
<td>135</td>
<td>0.20</td>
<td>108</td>
</tr>
<tr>
<td>5</td>
<td>1.40</td>
<td>1998</td>
<td>0.02</td>
<td>1958</td>
</tr>
<tr>
<td>6</td>
<td>1.45</td>
<td>1176</td>
<td>0.02</td>
<td>1141</td>
</tr>
<tr>
<td>7</td>
<td>1.85</td>
<td>362</td>
<td>0.00</td>
<td>362</td>
</tr>
<tr>
<td>8</td>
<td>2.00</td>
<td>308</td>
<td>0.00</td>
<td>308</td>
</tr>
</tbody>
</table>
Table 7. Maximum calculated values of the state of stress \([\Gamma'_{2}(\overline{\tau})_{\text{max}}]\) and flow classification parameter \((R_D)\) at \(\Gamma'_{2}(\overline{\tau})_{\text{max}}\) for three different bubble sizes

<table>
<thead>
<tr>
<th>Bubble diameter (mm)</th>
<th>(\Gamma'<em>{2}(\overline{\tau})</em>{\text{max}}) (dyne cm(^{-2}))</th>
<th>(R_D) at (\Gamma'<em>{2}(\overline{\tau})</em>{\text{max}})</th>
<th>Time at (\Gamma'<em>{2}(\overline{\tau})</em>{\text{max}}) (s)</th>
<th>Total elapsed time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.77</td>
<td>4797</td>
<td>0.02</td>
<td>4.3x10(^{-4})</td>
<td>5.5x10(^{-4})</td>
</tr>
<tr>
<td>1.70</td>
<td>1998</td>
<td>0.02</td>
<td>1.4x10(^{-3})</td>
<td>2.0x10(^{-3})</td>
</tr>
<tr>
<td>6.32</td>
<td>175</td>
<td>0.01</td>
<td>5.6x10(^{-3})</td>
<td>1.0x10(^{-2})</td>
</tr>
</tbody>
</table>
Table 8. Experimental data showing the magnitude of $I'_2(\overline{\tau})$ and $R_D$ that results in cell disruption

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Instrument (regimen)</th>
<th>Reference</th>
<th>$\tau$</th>
<th>Rate of cell damage (1)</th>
<th>$I'_2(\overline{\tau})$</th>
<th>$R_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$T$</td>
<td>(%/min)</td>
<td>(dyne cm$^{-2}$)</td>
<td></td>
</tr>
<tr>
<td>Laminar flow:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insect</td>
<td>Conc. Cylinders</td>
<td>Tramper et al. 1986</td>
<td>550</td>
<td>0.6</td>
<td>778</td>
<td>1</td>
</tr>
<tr>
<td>Hybridoma</td>
<td>Double cup and bob</td>
<td>Smith et al. 1987</td>
<td>7</td>
<td>0.03</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Hybridoma</td>
<td>Conc. Cylinders</td>
<td>Schurch et al. 1988</td>
<td>150</td>
<td>3.4</td>
<td>212</td>
<td>1</td>
</tr>
<tr>
<td>Hybridoma</td>
<td>Conc. Cylinders</td>
<td>Petersen et al. 1988</td>
<td>50</td>
<td>7.0</td>
<td>71</td>
<td>1</td>
</tr>
<tr>
<td>Hybridoma</td>
<td>Conc. cylinders</td>
<td>Abu-Reesh and Kargi 1989</td>
<td>860</td>
<td>1.5</td>
<td>1216</td>
<td>1</td>
</tr>
<tr>
<td>Myeloma</td>
<td>Conv./Diver. tube</td>
<td>McQueen and Bailey 1989</td>
<td>2000</td>
<td>2.3x10$^5$</td>
<td>(2)</td>
<td>(3)</td>
</tr>
<tr>
<td>Insect</td>
<td>Cone-and-plate</td>
<td>Goldblum et al. 1990</td>
<td>73</td>
<td>33.5</td>
<td>103</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>131</td>
<td>44.7</td>
<td>185</td>
<td>1</td>
</tr>
<tr>
<td>Turbulent flow:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>Capillary tube</td>
<td>Augenstein et al. 1971</td>
<td>10000</td>
<td>2.5x10$^4$</td>
<td>(2)</td>
<td>(3)</td>
</tr>
<tr>
<td>Myeloma</td>
<td>Capillary tube</td>
<td>McQueen and Bailey 1987</td>
<td>2900</td>
<td>1.6x10$^5$</td>
<td>(2)</td>
<td>(3)</td>
</tr>
<tr>
<td>Hybridoma</td>
<td>Capillary tube</td>
<td>McQueen and Bailey 1989</td>
<td>1800</td>
<td>1.2x10$^5$</td>
<td>(2)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

(1) The rates of cell damage presented in this column are average values. The rate of cell damage from different sources was brought to the same units to facilitate comparison.
(2) A local value for the second invariant of the stress tensor cannot be calculated from the information given in the reference. However, its order of magnitude is the same as that reported for $\tau$.
(3) The flow in these experiments has extensional characteristics. A local value for $R_D$ cannot be calculated from the information given in the reference.
CHAPTER V

CHARACTERIZATION OF A SPARGED FLOW FIELD USING A PARTICLE TRACKING VELOCIMETRY ALGORITHM

5.1 Summary

The velocity field, energy dissipation and state of stress associated with the wake of a rising bubble are experimentally measured using particle image velocimetry. These flow characteristics are compared with those associated with the region of bubble disengagement.

5.2 Introduction

A considerable amount of effort has been devoted to understand the interaction of suspended cells and bubbles in sparged systems. As a result of this effort, a number of cell damage mechanisms have been proposed (Kilburn and Webb, 1968; Handa et al., 1987; Handa-Corrigan et al., 1989; Tramper et al., 1986, 1987, 1988; Jobses et al. 1991; Oh et al. 1989, 1992; Kunas and Papoutsakis, 1990a; Orton and Wang, 1990; Chalmers et al., 1990;
Bavarian et al., 1991; Chalmers and Bavarian, 1991; Cherry and Hulle, 1992; Garcia-Briones and Chalmers, 1992; Garcia-Briones et al., 1994). For sparged systems, interactions of cells and the flow field at the injection region, the region where bubbles rise and the gas-liquid interface where bubbles break have been suggested to be responsible for the observed reduction in cell viability. Our present understanding of the mechanisms of cell damage has come from visualization and other laboratory studies. However, very little quantitative information of relevant flow field characteristics related to cell performance is available.

We have previously reported on computer simulations of bubbles breaking at a gas liquid interface (Garcia-Briones et al., 1994). These results showed that very high hydrodynamic stresses are associated with the region around a collapsing bubble cavity. In this report, the characterization of the flow field around a rising bubble is determined experimentally using three-dimensional particle tracking velocimetry (PTV). The measured flow characteristics are compared with those found for the region of bubble disengagement obtained from our computer simulations.

5.3 Particle Tracking Velocimetry

Research in the area of fluid mechanics and in particular in the field of turbulence has produced a number of techniques for the measurement of flow field properties. Particle imaging techniques have received a lot of attention in the last 15 years, mainly because of their potential to resolve instantaneous three-dimensional velocities without disturbing the flow. The basic principle behind these techniques involves the measurement of the position
of particle markers that follow the fluid motion. The velocity field from which other important flow properties are derived is then determined from the basic definition of velocity. Even though this principle is conceptually simple, its application has proved to be a formidable task. Most of the research has been directed towards automating and improving the efficiency of image analysis and reducing of the errors involved.

In this work we used a particle tracking velocimetry (PTV) algorithm to determine flow characteristics associated with cell damage for a flow induced by rising bubbles. This algorithm was developed at The Ohio State University by Dr. Yann G. Guezenec and coworkers. This code has been used in the past in some of the NASA's microgravity experiments and in the study of internal combustion engines.

The determination of the velocity field using the PTV algorithm begins with the acquisition of images of two different views of the region of interest containing flow markers. Typically, these stereo images are acquired using two video cameras which record a sequence of two-dimensional views of the flow field. Alternatively, an arrangement of mirrors can also be used to obtain the stereo images of the region of interest on the same frame of the recording media. The acquired images are then digitized and fed into the PTV algorithm which first determines two dimensional positions of the markers on each view. In the next step, tracks of particles are formed (for each view) and subsequently stereo matched to reconstruct three dimensional particle positions (and thus velocities). A final step involves the validation of the measured velocities.
5.4 Materials and Methods

5.4.1 Experimental Apparatus.

A schematic of the experimental setup is shown in Fig. 24. It consists of a square column made of glass. The column had a cross section of 5.25 cm² and was filled with water to a height of 7 cm. To prevent undesired reflections the column was painted black and only a small window of about 7.25 cm² was left unpainted on each side for visualization of the flow field. Air was injected at the bottom of the column through a small glass cylinder with a restriction which allowed the intermittent production of air bubbles of about 3 mm in diameter. An arrangement of first surface mirrors was used to produce the stereo images. A schematic of the mirror arrangement is shown in Fig 25. The use of first surface mirrors was necessary to prevent double reflections from the covering glass present in conventional mirrors. The stereo image was magnified by approaching the lens to the central mirrors thus making an efficient use of all video pixels available for recording. As the lens approached the central mirrors, however, the mirror's joint was magnified more than the image of interest because the central mirrors were always closer to the lens than the glass column. To reduce this effect as much as possible, the edges of the central mirrors shown on Fig 25 were ground at 45 degrees to reduce the width of the joint. SoloHill P102-1521 polystyrene microcarrier beads of 150-212 micron nominal size were used as flow markers (SoloHill Engineering Inc., Ann Arbor, MI). These particles were dyed using a fluorescent yellow dye to improve their detection.
5.4.2 Concentration of flow markers

Images of the bubbling flow using different particle concentrations (0.2, 0.15, 0.1, 0.05, 0.025 and 0.0125 % w/v) were recorded. The particle concentration used in particle image techniques determines the minimum scale of the flow field that can be measured. This scale is of the same order of magnitude as the nearest neighboring particles in the fluid volume which can be estimated by $r = 0.55C^{1/3}$. Thus, if small scales are involved, one would attempt to use a number density of particles as high as possible. However, as the concentration of particles increases the ambiguities between particle correspondence in consecutive frames also increases. This results in an overall decrease of particle tracking efficiency. The results presented in this report correspond to a particle concentration of 0.025 % w/v. This concentration was a compromise between the scale of the flow being measured and tracking efficiency.

5.4.3 Dying of flow markers.

Three grams of Pylakrome oil fluor yellow LX-8248 (Pylam Products Company, Inc., Garden City, NY) was dissolved in a solution of 10 ml methanol and 100 ml toluene. To this solution, 1.0 g of SoloHill microcarriers were added and shaked for 24 hours. The solvent was then recovered by vacuum filtration and the dyed microcarriers were collected on a Whatman filter paper.
5.4.4 Density matching

In order to have neutrally bouyant particles, the density of the working liquid (water) was matched to that of the flow markers. Solutions of Zinc Chloride (ZnCl₂) at concentrations of 2, 2.5, 3.0, 3.5 and 4.0 % w/v were prepared. Turbidity in these solutions was prevented by adding a small amount (drops) of hydrochloric acid. About 0.5 g of SolHill microcarriers was added to each solution of ZnCl₂ and then centrifuged at 3000 rpm for five minutes. The microcarriers were seen to be nearly bouyant when suspended in the 3.5 % ZnCl₂ solution.

5.4.5 Illumination, lens and recording system

The illumination system consisted of three Fiber-Lite (Dolan-Jenner Industries Inc., Lawrence, MA) units equipped with 150 W light bulbs. Fiber optic light guides were used to illuminate the system from the top. The column and calibration system (described below) were mounted in a two dimensional manipulator to facilitate the alignment of the column and mirror arrangement with the lens used to record the images. The images were acquired using an Infinity CFM lens. This is a continuously focusable microscopic lens with depths of field greater than those found in most conventional microscopes. The Infinity lens can be focused from infinity down to a few millimeters. In preliminary trials, the maximum depth of field attained (at the required magnification) with the infinity lens was measured to estimate the volume of observation and dimensions of the flow system. The lens was attached to a Cohu RS-170 & CCIR CCD monochrome camera (Cohu Inc./Electronics Division, San Diego,
CA). The combination of Infinity lens and Cohu camera produced images with a resolution of 450 lines of 624 picture elements having up to 256 gray level discrimination. The images were recorded using a Panasonic video tape recording system model AG-7355.

5.4.6 PTV software and processing steps

The PTV software used in this work consisted of three C binding libraries that contain user-callable functions. The user is responsible for the experimental set up (including a robust calibration system) and the appropriate selection of experimental variables including: image resolution, framing rate, particle size and density, illumination, magnification and field of view. Some of these variables are fixed by the structures of the flow field to be measured and their evolution in time. The processing steps involved in the PTV algorithm are schematically shown in Figure 26. Each of these steps is described below.

5.4.6.1 Calibration

Determination of the position of flow markers in a 3-D space requires the establishment of a relationship between image coordinates (from two different views) and real world coordinates. This calibration, called camera calibration, accounts for optical aberrations from the lenses used to acquired the stereo images. It also accounts for camera misalignment and changes in the index of refraction which cause the light reflected by the flow markers to bend. The association between physical coordinates and image coordinates
is made by constructing a calibration data base. This data base contains world coordinates (x, y, z) of targets and their corresponding positions (x_1, y_1, and x_2, y_2) on the image plane of each of the stereo images. Particles of about 300 to 500 microns vertically aligned inside a glass capillary tube were used as calibration targets. The capillary tube was filled with microscope immersion oil to facilitate the detection of particles. These targets were placed at different locations along the x-y plane (see Figure 27) using a 3-D manipulator to completely span the area of interest. The manipulator had two calipers attached to it which allow the measurement of displacements with an accuracy of .001 in. Images of the targets at each position were recorded and digitized for further processing. Figure 27 shows the targets placed at the center of the region of observation as well as the x-y plane with the positions used to calibrate the flow system. To speed up the computing time of the subsequent steps involved in the PTV algorithm, the calibration data base is transformed into a series of curve fits consisting of third order polynomials in three variables. Polynomial fitting is done using least square procedures. These polynomials are used during stereo matching (explained later) to define optical rays used to find the 3-D locations of particle markers. Camera calibration was made just before images from the bubble-flow experiment were recorded.

5.4.6.2 Image acquisition

Stereo images were digitized using the P360F Power Grabber board (Dipix Technologies Inc., Ottawa, Ontario, Canada). This board has the circuitry to convert the
analog signal coming from the video tape recorder or video camera into a digital representation that ranges between 0 to 255. The software accompanying the frame grabber consists of C binding libraries (also referred as DIPIX functions) that can be used to perform basic non image processing manipulations such as grabbing, displaying and storing images. Other C binding libraries are also available that provide functions to develop more complicated image processing algorithms. Alternatively, the PTV software provides a library of functions, called DPX.LIB, where more basic DIPIX functions have been conveniently packed. Use of the functions provided in the DPX.LIB library requires less programming effort. The user, however, can always go back to the more basic DIPIX functions to develop specific algorithms the meet his needs. The source code of the program acqimg.c used for image acquisition is shown in Appendix B.

5.4.6.3 Image pre-processing

The images acquired using the frame grabber are usually not ready for the detection of the flow markers on the image planes. Preliminary processing is needed to standardize the images and enhance the contrast between particles and their background. It may also be necessary to eliminate undesired reflections coming from entities other than the flow markers.

The mirror arrangement and infinity lens used for the acquisition of images produced an inverted stereo image. This image was rotated 180 degrees and split in two halves using the program inspim.c. The source code of the program inspim.c is included in Appendix B. This manipulation produced two images which are hereafter referred to as the left and right
At the beginning of each experiment, a background image was recorded. This image corresponds to the flow system filled with the working liquid without particles and bubbles. This background was subtracted from every frame of the bubble-flow experiment to eliminate reflections from objects other than flow markers. Before the background was subtracted it was stretched. Stretching is an image processing operation in which every pixel intensity is changed in a particular way. The background image was stretched using the program cng_grey.c (See Appendix B). This program brings all pixels with intensity less than a specified threshold value to zero (black) while pixels with intensities equal or larger than the specified threshold remain unchanged. The stretching of the background in this way prevents the loss of particle marker intensity when the background image is subtracted from images of the actual experiment. Undesired reflections that could be identified as flow markers in subsequent processing are, however, eliminated in the subtraction process.

The program subimg.c (See Appendix B) was used for the subtraction of the background. This program performs the subtraction of images using the DIPIX board buffer memory. In this regard, a note of caution is in order. For the program subimg.c to work properly, the images involved in the subtraction should be of a size that fits the DIPIX board buffer memory partition. The partition of the buffer memory can be set by the user. However, the specified image width must be a multiple of 4. If the image width is not a multiple of 4, it is converted to the highest multiple of 4 which is less than the specified width. Thus, if the stereo image is 628x450, for example, the left and right images (assuming that the stereo image is divided in half) will be 314x450. These images cannot be loaded into the
DIPIX board buffer memory since their width (314) is not a multiple of 4. In this case one should start with a stereo image either 624x450 or 632x450. The program subimg.c can be used in two modes. If the user does not specify the "-c" ('c' stands for continuous execution) flag as part of the command line arguments, the program shows the original and subtracted image for every frame. If the "-c" flag is specified, the program runs continuously without displaying any image.

To facilitate the identification of particles from the background the subtracted images were standardized by stretching them. The program stretch.c (See Appendix B) was used in this step. The stretching is made by considering two threshold values (min and max). The formula used in stretch.c for stretching brings all the pixels with intensity less than the minimum threshold to zero (black) and those with intensity greater than the maximum threshold to 255 (white). Pixels with intensities in the range defined by the minimum and maximum thresholds are stretched proportionally to their original intensity. The minimum and maximum thresholds are selected so that the image histogram consists of two separate peaks corresponding to the particles and background. Two separate peaks result when particles and background have been successfully separated. The selected threshold values should also preserve most of the original number of particles. Similar to subimg.c, stretch.c can be executed in two modes. The non continuous mode ("-c" flag not specified in the command line arguments) is typically used with a few number of frames to find the optimum threshold values. In this mode, the user can adjust the threshold values interactively and see the results on the monitor. Once suitable threshold values have been found the program can be executed in continuous mode to process a large number of images without displaying intermediate
5.4.6.4 Flow marker detection

Extraction of 2-D particle coordinates on the right and left images is accomplished using a collection of user-callable functions available in the second module of the PTV software: PTV.LIB. This library also contains a number of functions to override default values of parameters used by the algorithm. This allows the user to customize this module to his particular experimental conditions. Image resolution, minimum and maximum particle size, and maximum particle aspect ratio (used as a criteria for particle identification) are some of the parameters that can be specified by the user.

Identification of particles to extract their 2-D locations is done using a fast scan and labelling approach. In this method, each row of pixels is scanned once with only two consecutive pixel rows being processed at a time. Details of this approach can be found elsewhere (Haralick and Shapiro, 1992; Guezennec and Kirtsis; 1990).

Because of the discrete nature of the pixel array where particle images are recorded, particles are defined as a group of connected pixels whose intensity exceeds a given threshold. Only particles with sizes within the limits specified by the minimum and maximum particle size are considered. The position of the particles is given by the position of their intensity weighted centroid. If the concentration of particles is high, overlapping becomes an important factor which reduces the overall efficiency of the algorithm. In this case, the function piv_find() in the PIV.LIB can be called with a flag that activates the decomposition
of particles with large aspect ratios. For completeness, the program ex_piv.c containing the function calls to the PIV.LIB library is included in Appendix B.

Identification of particles can be restricted to a certain region of the flow field. The program find_box.c (included in Appendix B) was used to restrict the region of interest to that most affected by the rising bubbles.

5.4.6.5 Tracking, stereo matching and validation

The processing of 2-D particle positions from the left and right views to reconstruct their 3-D locations is accomplished by a user-written program that makes calls to the functions provided on the third module of the PTV software: VEL.LIB. As with the previous module, a number of functions are available to customize this module to the user particular needs. The function vel_f5_track() finds particle tracks on each of the 2-D views using the concept of path coherence. The idea behind this concept is that position, velocity and acceleration of fluid elements can be described by well behaved functions of time. Thus, the displacement of particles at a given frame is predicted using the velocity and acceleration of the particle calculated from previous frames. The area of search for possible displacements (that define the tracks) is fixed by the maximum distance a particle can move in two consecutive frames. This piece of information should be supplied by the user and is determined by the physics of the flow being considered. For the problem at hand the maximum distance that the flow markers can move is given by the displacement of the bubbles. The search of tracks grows in a tree structure fashion with one branch for every
possible displacement. The most correct track is that for which a penalty function that compares the predicted and real position of particles is minimum. These process is illustrated in Fig. 28. Tracks of particles are formed for five consecutive frames. The use of 5 frames to form tracks is a compromise between avoiding a large number of wrong tracks and keeping the computational effort under certain limits.

The function vel_st_match() performs the stereo matching of particle tracks found in the previous step. Stereo matching tracks (2-D objects) rather than particles, eliminates most if not all the ambiguities that result from stereo matching individual particles. Every particle on each view has associated with it an optical ray that extends from the image plane to the three dimensional space of interest. The intersection of two optical rays determines the 3-D locations of particles and constitutes a stereo match. This is schematically shown on Figure 29. Because of errors involved in locating particles, two optical rays corresponding to the same particle may not intersect. In this case, stereo matching reduces to consider the minimum distance between optical rays. The stereo matching of tracks is then made by minimizing the distance between optical rays of particles of a given track and particles of every stereo track candidate. Once the stereo match is found, the 3-D position of the particle in the central frame (of the 5 frame considered) is determined as the midpoint between the line joining optical rays. The velocity for this particle is then calculated using a five point central difference approach. The optical rays associated with each particle are determined using the calibration data base (see calibration), the camera orientations, and the 2-D positions of particles in each stereo view.
The function vel_validate() validates the measured velocity field. This procedure begins by smoothing the velocity field using an interpolation scheme based on a Gaussian window. The raw velocity values of each particle are then compared with the smoothed field in its immediate vicinity and rejected if a significant difference is found. This is illustrated in Figure 30. Again, for completeness, the program ex_vel.c containing the function calls to the PIV.LIB library is included in Appendix B.

5.4.7 Selection of experimental variables

We have used a particle tracking velocimetry algorithm as a tool to study the evolution of a sparged flow. The performance of this code, however, depends on the selection of a number of experimental variables which are discussed below.

5.4.7.1 Particle size

Three issues should be considered in the selection of particle size. First, the accuracy of particle identification improves as the particle size increases. This improvement comes from the fact that the image of larger particles is defined with a larger number of pixels. The discretization effects on the particle image boundaries are then weighted less heavily. However, the velocity lag between particle and fluid increases with particle size. In addition, particle overlapping also increases. These opposite trends suggest the use of the smallest possible particle size which after magnification can still be detected by the recording media.
with a minimum of 5 to 7 pixels. After magnification with the infinity lens used in our experiments, our flow markers (SoloHill particles with a nominal size of 150-212 micron) were imaged with a radius of 2 to 3 pixels (this translates to about 4 to 9 pixels per particle). The velocity difference between the flow field and its markers can be estimated following the analysis presented by Adrian (1991) and Malik et al. (1992). For small particle Reynolds numbers, the velocity difference $|v_p - u|$ between the velocity of the particle, $v_p$, and the velocity of the fluid, $u$, is given by:

$$|v_p - u| = \frac{\rho_p \sigma_p^2 |v_p|}{36 \rho v}$$

Assuming that the maximum change of velocity occurs when a stagnant particle is accelerated to the maximum expected velocity (given by the upward bubble velocity = 0.19 m/s), and that this acceleration occurs over a distance of 0.001 m, then the acceleration time scale is 0.011 s. For neutrally buoyant particles and taking the kinematic viscosity of our working liquid equal to that of water, the velocity difference $|v_p - u|$ is less than 0.8% of the fluid velocity $u$.

### 5.4.7.2 Particle concentration

The sampling frequency is directly related to the minimum length scale of the flow field that can be measured. However, the particle concentration used in particle tracking techniques is fixed by the flow structures under study. In this regard, and especially if
instantaneous structures of the flow field are to be measured, one would attempt to use particle concentrations as high as possible. However, as the concentration of particles is increased, the ambiguities in tracking also increase. This eventually results in a fewer number of measured velocity vectors available to reconstruct the flow field. The particle concentration used in our experiments was a compromise between these two opposite trends.

5.4.7.3 Image resolution and framing rate

The resolution of our stereo images was 624x450. Image resolution limits the minimum particle size that can be identified and the maximum particle density before particles cannot longer be resolved as individual entities. As pointed out before, the resolution used in this study produced particle images ranging from 4 to 9 pixels. The images were recorded at a frequency of 60 frames per second. This gave us 10 to 11 frames to follow the bubble as it passed by the observation window. The framing rate determines the maximum velocity of particles that can be measured. For our bubble-flow experiment, the maximum expected velocity was estimated to be that of the rising bubbles. At a frame rate of 60 frames per second, the maximum expected displacement was only 10% of our field of view. Therefore, a frequency of 60 frames per second was appropriate to study the flow under consideration.
5.4.7.4 Lens, field of view, depth of field and illumination

In preliminary trials, a number of different lenses available in our laboratory were used to determine the dimensions of the volume of observation. The goal was to have a sufficiently large field of view to track the bubbles and particles along a minimum vertical distance, and at the same time to have an appropriate magnification that would allow particle identification on the video array with a minimum number of pixels. The infinity lens had the largest depth of field at the required magnification and was selected on these grounds as the best option.

Continuous high intensity cool illumination directed from the top of the column was sufficient for particle detection. Initially attempts were made using a copper laser with an output intensity of 1.0 mJ (at 10 kHz). The dying of particles however, improved the reflection of light coming from the particles to the extent that high intensity laser light was no longer required.

5.5 Results

It has been speculated in the literature that the stresses associated with the rising of bubbles could be sufficiently high to kill suspended animal cells. Even though gross estimates of these hydrodynamics stresses have being reported (Tramper et al. 1986) no quantitative information is available. In this report we present full-field measurements of relevant characteristics associated with cell damage for a sparged flow.
5.5.1 Image Processing

Figure 31 shows a raw image of the bubble-flow experiment as acquired from the video tape recorder and frame grabber. Figure 32 shows this raw image after subtraction of the background and stretching. Figure 33 shows the sequence of 16 frames which were analyzed with the PTV algorithm. These images were recorded at a rate of 60 frames per second. The bubble upward velocity estimated from this sequence of frames is 19 cm/sec.

5.5.2 PTV Processing

An average of 415 particles were located by the PTV algorithm in the central region of the window of observation. A number of different thresholds were tried to locate the maximum number of particles. Figure 34 shows a typical image where a cross sign is overlaid on each particle that has been located. As illustrated in Figure 34, more than 95% of the particles in the central region were identified by the code. Identification of most of the particles on each image is important to increase the efficiency of tracking. Only particles with sizes between 4 to 10 pixels and a maximum aspect ratio of 3.0 were considered as valid. Overlapping particles were resolved using the particle decomposition option available on the PIV.LIB module. This increased the number of located particles by about 10%. Particle tracks for about 90% of the located particles were found. Of these, about 73% were successfully stereo matched. On average 99% of the velocities calculated after the stereo matching step were validated. Figure 35 shows the raw velocity vectors projected on the y-z
plane for some selected frames. The circle indicates the approximate position of the bubble.

5.5.3 Flow properties associated to hydrodynamic cell injury

In previous publications (Garcia-Briones et al., 1994; Garcia-Briones and Chalmers, 1994) we have discussed a number of hydrodynamic parameters of general nature that can be used to study hydrodynamic related cell injury. Two of these parameters are the state of stress and the energy spent in fluid deformation. These two hydrodynamic parameters were used for the characterization of the flow field considered here.

The state of stress was characterized by the second invariant of the stress tensor given by:

\[ I_2(\bar{\gamma}) = \mu \sqrt{tr(\bar{\gamma}^2)} \]  \hspace{1cm} (24)

\[ \bar{\gamma} = [ \nabla \vec{U} \cdot (\nabla \vec{U})^T ] \]  \hspace{1cm} (25).

Where \( \mu \) is the viscosity of the working liquid, \( \gamma \) is the rate of deformation tensor, and \( \vec{U} \) is the velocity vector. The dissipation function is given by:

\[ \Phi = \mu [ \nabla \vec{U} \cdot (\nabla \vec{U})^T ] : (\nabla \vec{U}) \]  \hspace{1cm} (26).
In order to calculate the components of the tensor $\nabla \mathbf{U}$ the raw velocity data was interpolated onto a regular grid using the following interpolation scheme:

$$F(x, y) = \frac{\sum_{k=1}^{n} w_k(x, y) f_k}{\sum_{k=1}^{n} w_k(x, y)}$$

(27).

Where $F(x, y)$ is the interpolation function at each node of the interpolation grid, $f_k$ represents the known data points, and $w_k$ is the Gaussian weighing function given by

$$w_k = e^{-\left(\frac{d_k^2}{\sigma^2}\right)}$$

(28).

In this equation $d_k$ is the distance between the interpolation point and the location of the known data points and $\sigma$ is the width of the Gaussian window.

The grid size was selected to be twice the magnitude of the mean nearest neighbor distance between particles:

$$r = 0.55 C^{-1/3}$$

(29)

where $C$ is the mean number of particles per unit volume. This grid size preserves the flow structure as determined by the known velocity vectors. Figure 36 shows interpolated velocity vectors projected on a plane perpendicular to the $x$ axis that passes through the origin of the coordinate system shown in Figure 27.
The components of the tensor $\nabla U$ were calculated at each internal node of the interpolation grid using the following differentiation formula:

$$
\frac{df(x_i)}{dx} = \frac{1}{2h} \left[ f(x_2) - f(x_0) \right]
$$

(30)

Here $h$ is the grid spacing. The distribution of the second invariant and energy dissipation was determined from equations 24-26. Figure 37 shows the distribution of the second invariant of the stress tensor. Figure 38 shows the distribution of the energy dissipation function.

5.6 Discussion

5.6.1 Reconstruction of the velocity field

The technique used in this study effectively measures the velocity of the flow field at locations randomly distributed. It remains to be demonstrated whether or not we have been successful in reconstructing the velocity field from the measured velocities. This issue involves the comparison of the smallest scale of the flow field at which velocity gradients occur with the mean space between samples. Following Nyquist's criterion which applies for regularly spaced data, the velocity field can be successfully reconstructed if the sample spacing is less than one half of the relevant flow scale. Furthermore, the differentiation of velocity is
accurate if the data density defined as \( N_\lambda = C\lambda \gg 1 \) (Adrian, 1991). Where \( C \) is the number particle density and \( \lambda_T \) is the relevant scale associated with velocity gradients.

For the case of a bubble moving through a liquid we can distinguish two relevant scales: the scale associated with the boundary layer and that associated with the wake of the bubble. In the region of the boundary layer, the effects of viscosity are more important and we expect the largest hydrodynamic stresses to occur in this region. Boltze (1908) have computed the process of boundary layer formation for a sphere accelerated impulsively. The boundary layer thickness for this case was calculated to be of the order of 3% of the sphere radius. For the bubble size used in our measurements (3-4 mm in diameter), this corresponds to a scale of 60 \( \mu \text{m} \). In order to resolve the flow field inside the boundary layer, it would be necessary to have at least five flow markers over this distance. That is a mean distance between samples of about 12 \( \mu \text{m} \) is needed to make measurements inside the boundary layer. Outside the boundary layer, in the bubble wake, the characteristic length is of the order of the bubble diameter. The mean space between samples (calculated from eq. 29) at the concentration used was about 1200 \( \mu \text{m} \) (particle size 120-212 \( \mu \text{m} \)). Clearly, under the conditions of our experiments, only the stresses associated with the wake of the bubble were measured since the mean space between particles is about one forth the bubble diameter.
5.6.2 Errors involved in the measurement of the velocity field

5.6.2.1 Camera calibration

For the camera calibration, the maximum curve fit errors ranged from 0.467 to 1.798% while the average curve fit error ranged from 0.121 to 0.452%. These errors translate into a maximum 3-D position error of 2.09% and an average of 0.64%.

5.6.2.2 Particles identification

The process of locating particles on an image plane has an intrinsic error associated with it. This error results from the discretization of the particle image into the finite resolution of the media used for recording. The image of a spherical particle appears as a series of connected pixels of different intensities. However, subpixel accuracy is obtained if the intensities of the pixels representing the particle are used to calculate the particle centroid. Another possible source of error results from non-uniform illumination. In this case, the determination of particle centroids using illumination intensity gives less accurate results. When displacement of particles is considered, however, differences in location attenuate the errors involved.
5.6.2.3 Tracking and stereo matching

Tracking may affect the efficiency of the algorithm if wrong tracks are formed during this step. Because the PTV algorithm forms tracks by considering velocity and acceleration information from previous frames, the probability of forming wrong tracks becomes very small. Even if a wrong track is formed on one of the views, no corresponding stereo match will exit in the other view. Testing of the software with synthetic simulated data has indicated that the tracking and stereo matching steps of the PTV algorithm contribute to about 2-3% to the overall error.

5.6.2.4 Particle overlapping

Overlapping of particles on the image plane increases with particle concentration. Simulated data runs have indicated that low concentrations of particles produce errors (in the measured velocity) of less than 5%. The errors are less than 10% if the number of imaged particles is less than 400.

5.6.2.5 Interpolation errors

Interpolation of the randomly spaced velocity vectors onto a regular grid may have introduced errors as large or even larger than those involved in the image processing steps. The Gaussian window interpolation scheme used to grid the raw velocity data has the width
of the Gaussian window as an adjustable parameter which influences the errors involved in interpolation. Agui and Jimenez (1987) and Spedding and Rignot (1993) have reported an optimum value for the Gaussian width given by \( \sigma/\delta = 1.24 \), independent of \( h/\delta \), and \( L/\delta \). Where \( \sigma \) is the width of the Gaussian window, \( \delta \) is the mean nearest neighbor distance between samples, \( h \) is the grid spacing, and \( L \) is the characteristic length of the flow. We used this optimum relation to reduce the randomly spaced velocity data to a regular grid. Using the performance analysis of the adaptive Gaussian window (AGW) scheme presented by Spedding and Rignot (1993) for a Burgers' vortex, the lower limits of the expected interpolation errors can be estimated. According to this analysis, minimum errors of about 4% and 10% should be expected for the interpolated velocity and vorticity fields, respectively. The upper limits, however, cannot be estimated from the graphs presented in the mentioned reference.

5.6.3 Velocity field

Interpolated values of the velocity field shown in Figure 36 show the structures of the flow field in a region restricted to the center of the flow system. The y-z plane shown is perpendicular to the x axis (see Figure 27) and passes through the origin of coordinates. The circle on each frame indicates the approximate position of the rising bubble. Because the plane shown is centered on the origin of coordinates and not on the center of the rising bubble, velocity vectors on some of the frames in Figure 36 actually point downwards. As the bubble rises, some liquid is entrained in its wake and moves upwardly. By continuity, some
liquid close the bubble must go in the opposite direction.

5.6.4 State of stress and energy dissipation

The distribution of the state of stress for the region outside the boundary layer (bubble wake) is shown in Figure 37. As expected, the highest values for the state of stress are located in the vicinity of the rising bubble. The magnitude of the state of stress, however, is significantly lower than that associated to the region of bubble breakup at the gas-liquid interface. In a recent publication (Garcia-Briones and Chalmers, 1994), we have reported on computer simulations of bubbles breaking at a gas-liquid interface. The maximum calculated values for the state of stress (characterized by its second invariant) for bubbles of 0.77, 1.7 and 6.32 mm in diameter was found to be 4797, 1998, and 175 dyne cm\(^2\) respectively. These values are three to four orders of magnitude higher than the maximum measured value (0.25 dyne cm\(^2\)) for the region of the bubble wake.

Figure 38 shows the distribution of the energy dissipation. As with the state of stress, the maximum values are found in the vicinity of the rising bubble. However, the order of magnitude of the measured energy dissipation (~4.0 erg cm\(^{-3}\) s\(^{-1}\)) is significantly lower than that calculated for the region of bubble disengagement. Boulton-Stone and Blake (1993) modelled the bubble bursting process using a boundary integral method. They reported maximum energy dissipation rates in the range of \(1 \times 10^{11}\) to \(1 \times 10^{5}\) erg cm\(^{-3}\) s\(^{-1}\) for bubble sizes in the range of 1.2 to 6 mm in diameter. In a related work, we have reported on the energy dissipation for three bubble sizes: 0.77, 1.7 and 6.32 mm. The maximum calculated energy dissipation
dissipation was $9.52 \times 10^8$, $1.66 \times 10^8$ and $9.40 \times 10^5$ erg cm$^{-3}$ s$^{-1}$, respectively. Clearly, the rate of energy dissipation associated with the breakup of bubbles is far greater than that measured for the region of the bubble wake.

5.7 Conclusions

A particle image velocimetry algorithm has been used for the characterization of a gas agitated mixing flow. The resolution of our measurements was not appropriate to resolve the hydrodynamics inside the bubble boundary layer where the maximum stresses are expected to occur. However, flow properties associated with the region outside the boundary layer have been measured. Unless cells are attached to the rising bubbles, the hydrodynamic stresses associated with the bubble wake are those that the majority of the cells will experience more frequently. The accumulated errors involved in the measurement of the velocity field, state of stress and energy dissipation are in the range of 20 to 80\%. However, the order of magnitude of the state of stress and energy dissipation for the bubble wake region is far lower than the values previously reported for breaking bubbles. These results indicate that the contribution to cell damage associated with hydrodynamic stresses produced by rising bubbles is negligible compared to that related to bubble breakup.
Figure 24. Photograph showing the experimental set up. One side mirror was removed in this picture to allow the square column to be seen.
Figure 25. Top view of the mirror arrangement used to acquire stereo images of the flow region. The enlargement shows the ground joint of the central mirror.
Figure 26. Processing steps involved in the PTV algorithm.
Figure 27. The top view of the flow system shows the positions where the targets were placed to span the region of interest. The side view shows a stereo image of the capillary with the targets inside placed at the center of the x-y plane. The z axis is perpendicular to plane of the page.
Figure 28. Formation of tracks on each of the stereo images. The search of tracks grows in a tree structure fashion with one branch for every possible displacement.
Figure 29. A stereo match is found as the near intersection of optical rays. The position of the particle is determined as the midpoint between the line joining optical rays.
Figure 30. Validation of the measured velocity field (top) involves first smoothing the velocity field (middle). Each velocity vector is then compared with the smoothed field in its immediate vicinity. Vectors are discarded if large differences are found (bottom).
Figure 31. Raw image of the bubble-flow experiment as acquired from the video tape recorder with the frame grabber (after rotation and splitting).
Figure 32. Raw image after substraction of the background and stretching.
Figure 33. Sequence of left and right images analyzed with the PTV algorithm. Images were recorded at 60 frames per second. Frames 1 (top) and 2 (bottom).
Figure 33 (Continued). Frames 3 (top) and 4 (bottom).
Figure 33 (Continued). Frames 5 (top) and 6 (bottom).
Figure 33 (Continued). Frames 7 (top) and 8 (bottom).
Figure 33 (Continued). Frames 9 (top) and 10 (bottom).
Figure 33 (Continued). Frames 11 (top) and 12 (bottom).
Figure 34. Typical image where particles that have been identified by the PTV algorithm are shown with a cross.
Figure 35. Raw velocity vectors projected on the y-z plane for frames 3 (left) and 4 (right). Measurement of the velocity field was restricted to a central region. Units of the horizontal and vertical axes are cm. Scale for velocities is given in cm sec$^{-1}$. 
Figure 35 (Continued). Raw velocity vectors projected on the y-z plane for frames 5 (left) and 6 (right). Units of the horizontal and vertical axes are cm. Scale for velocities is given in cm sec$^{-1}$. 

$\overrightarrow{v} = 8.339 \times 10^0$  \hspace{1cm}  $\overrightarrow{v} = 8.378 \times 10^0$
Figure 35 (Continued). Raw velocity vectors projected on the y-z plane for frames 7 (left) and 8 (right). Units of the horizontal and vertical axes are cm. Scale for velocities is given in cm sec$^{-1}$. 
Figure 35 (Continued). Raw velocity vectors projected on the y-z plane for frames 9 (left) and 10 (right). Units of the horizontal and vertical axes are cm. Scale for velocities is given in cm sec\(^{-1}\).
Figure 36. Interpolated velocity vectors projected on a plane perpendicular to the x axis and passing through the origin of the coordinate system. Units of the horizontal and vertical axes are cm. Scale of velocities is given in cm sec$^{-1}$. The velocity field shown corresponds to frames 3 (left) and 4 (right).
Figure 36 (Continued). Interpolated velocity vectors projected on a plane perpendicular to the x axis and passing through the origin of the coordinate system. Units of the horizontal and vertical axes are cm. Scale of velocities is given in cm sec$^{-1}$. The velocity field shown corresponds to frames 5 (left) and 6 (right).
Figure 36 (Continued). Interpolated velocity vectors projected on a plane perpendicular to the x axis and passing through the origin of the coordinate system. Units of the horizontal and vertical axes are cm. Scale of velocities is given in cm sec$^{-1}$. The velocity field shown corresponds to frames 7 (left) and 8 (right).
Figure 36 (Continued). Interpolated velocity vectors projected on a plane perpendicular to the x axis and passing through the origin of the coordinate system. Units of the horizontal and vertical axes are cm. Scale of velocities is given in cm sec$^{-1}$. The velocity field shown corresponds to frames 9 (left) and 10 (right).
Figure 37. Distribution of the second invariant of the stress tensor for frames 3 (left) and 4 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the second invariant is given in dyne cm$^2$. 
Figure 37 (Continued). Distribution of the second invariant of the stress tensor for frames 5 (left) and 6 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the second invariant is given in dyne cm$^2$. 
Figure 37 (Continued). Distribution of the second invariant of the stress tensor for frames 7 (left) and 8 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the second invariant is given in dyne cm$^{-2}$. 
Figure 37 (Continued). Distribution of the second invariant of the stress tensor for frames 9 (left) and 10 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the second invariant is given in dyne cm$^2$. 
Figure 38. Distribution of the rate of energy dissipation for frames 3 (left) and 4 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the rate of energy dissipation is given in erg cm$^3$ sec$^{-1}$. 
Figure 38 (Continued). Distribution of the rate of energy dissipation for frames 5 (left) and 6 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the rate of energy dissipation is given in erg cm$^{-3}$ sec$^{-1}$. 
Figure 38 (Continued). Distribution of the rate of energy dissipation for frames 7 (left) and 8 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the rate of energy dissipation is given in erg cm$^3$ sec$^{-1}$. 
Figure 38 (Continued). Distribution of the rate of energy dissipation for frames 9 (left) and 10 (right) on a plane perpendicular to the x axis and passing through the origin of coordinates. Units of the horizontal and vertical axes are cm. The scale for the rate of energy dissipation is given in erg cm$^3$ sec$^{-1}$.


Appendix A

PARAMETERS TO CHARACTERIZE THE LOCAL STATE OF STRESS

The state of stress at a point of an incompressible fluid is completely characterized by the stress tensor

\[ \bar{\sigma} = -\mu \bar{\nabla} = -\mu \left( \nabla \bar{V} + (\nabla \bar{V})^T \right) \]  \hspace{1cm} (i).

With a matrix representation

\[
\begin{pmatrix}
\sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\
\sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\
\sigma_{zx} & \sigma_{zy} & \sigma_{zz}
\end{pmatrix}
= -\mu
\begin{pmatrix}
\frac{\partial \nu_x}{\partial x} & \frac{\partial \nu_x}{\partial y} & \frac{\partial \nu_x}{\partial z} \\
\frac{\partial \nu_y}{\partial x} & \frac{\partial \nu_y}{\partial y} & \frac{\partial \nu_y}{\partial z} \\
\frac{\partial \nu_z}{\partial x} & \frac{\partial \nu_z}{\partial y} & \frac{\partial \nu_z}{\partial z}
\end{pmatrix}
\]  \hspace{1cm} (ii).
For a simple shear flow such as that occurring between parallel plates at a distance far from the entrance, Eq. (ii) reduces to

\[
\begin{pmatrix}
0 & \tau_{xy} & 0 \\
\tau_{yx} & 0 & 0 \\
0 & 0 & 0
\end{pmatrix} = -\mu
\begin{pmatrix}
0 & \frac{\partial v_y}{\partial y} + \frac{\partial v_y}{\partial x} & 0 \\
\frac{\partial v_x}{\partial y} + \frac{\partial v_y}{\partial x} & 0 & 0 \\
0 & 0 & 0
\end{pmatrix}
\]  

(iii).

For this simple shear flow, the measured shear stress \(\tau_{xy}\), completely characterizes the state of stress everywhere in the flow field. This is because the other components of the stress tensor are equal to zero. \(\tau_{xy}\) has been used to correlate hydrodynamic cell injury. However, for a more complex flow (for which Eq. (ii) is not reduced to a simple form) and following the assumption that hydrodynamic cell injury is a function of the state of stress in the flow field, we see from Eq. (ii) that shear stress is not the appropriate parameter to correlate cell damage. Shear stress in this case does not characterize the state of stress. Furthermore, since the magnitude of the components of the stress tensor depends on the orientation of the coordinate system being used, we need to consider a quantity independent of the coordinate system related to the state of stress. One possibility, for example, is the second invariant of the stress tensor given by

\[
I_2(\overline{\tau}) = \sqrt{\text{tr}(\overline{\tau}^2)}
\]  

(iv).

The second invariant of \(\overline{\tau}\) is a scalar quantity with magnitude proportional to the state of stress.
Appendix B

SOURCE CODE FOR IMAGE PRE-PROCESSING

/*
   *
   * ACQIMG.C
   * This program grabs and stores stereo images.
   * Frames are advanced manually.
   *
   * Written by: Miguel A. Garcia-Briones Sep, 4 1994
   *
   */

/* standard include files */

#include <stdio.h>
#include <stdlib.h>
#include <conio.h>
#include <graphics.h>

/* DPX specific include files */

#include "dpx/dpx.h"
#include "dpx/dpxproto.h"
#include "dpx/stdlib.h"
#include "dpx/ptype.h"

/* CS specific include files */

#include "cserrno.h"
#include "pubproto.h"
#include "colors.h"
#include "csscreen.h"
#include "utils.h"

#define DBL_DPX_WIDTH (2*DPX_WIDTH)

/* Global variables */
char dpx_name[6];
int dpx_frame_s;
int dpx_frame_e;
char dpx_imgpath[80];
char dpx_datpath[80];

void main(int argc, char **argv)
{
    int i;
    int image_buffer = 1;
    int input;
    int gain = 30;
    int offset = 50;
    char string[80];

    /* Initialize to default values */

    if( dpx_init() != CS_SUCCESS ) {
        printf("Error initializing DPX \n");
        exit(CS_USERERR);
    }

    /* Get arguments from the command line */

    if(dpx_set_file(argc, argv) != CS_SUCCESS ) {
        printf("Usage: executable case_name(1-5) first last \n");
        exit(CS_USERERR);
    }

    #ifdef DEBUG
    printf("dpxjname = %s\n", dpx_name);
    printf("dpx_frame_s = %d\n", dpx_frame_s);
    printf("dpx_frame_e = %d\n", dpx_frame_e);
    #endif
}
printf("dpx_imgpath = %s\n", dpx_imgpath);
printf("dpx_datpath = %s\n", dpx_datpath);
#endif

/* double check if all the VIDEO equip. is ready */
printf("Make sure all the VIDEO equipment is connected. \n");
printf("Press any key to continue or ESC to abort. \n");
if( read_kb() == ESC ) {
    printf("\n");
    printf("process aborted by the user! \n");
    exit( CS_USERERR );
}

/* Initialize "left" Dipix grabber and
set gain and offset to default values */

if( dpx_init_boards(DPX_LEFT) != CS_SUCCESS ) {
    printf("Error initializing Dipix board \n");
    exit(CS_HWINITFAIL);
}

/* Initialization for VESA display (800x600) */

if( dpx_init_display(DPX_800X600) != CS_SUCCESS ) {
    printf("Error initializing VESA display mode \n");
    exit(CS_HWINITFAIL);
}

/* Select "left" Dipix board */

select_board(DPX_LEFT);

dpx_set_directory(dpx_imgpath);

/* Set parameters for grabbing */

set_grab_parameters(2,2,1,TRUE); /* grab parameters for CCIR camera */
set_grab_size(DBL_DPX_WIDTH, DPX_HEIGHT, TRUE);

/* set and display gain and offset */
set_gain_and_offset( gain, offset, TRUE );
setcolor( YELLOW );
sprintf( string, "GAIN = %d OFFSET = %d", gain, offset);
outtextxy( DPX_B_X4, DPX_B_Y1, string );

/* Loop for frame grabbing */

for(i = dpx_frame_s; i <= dpx_frame_e; i++) {

   /* Print ready-to-grab message */
   setcolor( YELLOW );
   sprintf( string, "PGUP:INC OFFSET PGDN:DEC OFFSET\n" );
   outtextxy( DPX_B_X1, DPX_B_Y1, string );
   sprintf( string, "INS :INC GAIN DEL:DEC GAIN\n" );
   outtextxy( DPX_B_X1, DPX_B_Y2, string );
   sprintf( string, "ESC: SAVE IMG\n" );
   outtextxy( DPX_B_X1, DPX_B_Y3, string );
   sprintf( string, "ANY OTHER KEY TO GRAB/UPDATE IMG\n" );
   outtextxy( DPX_B_X1, DPX_B_Y4, string );

   /* loop to adjust gain and offset or advance frame */
   while( TRUE){

      /* grab and display frame */
      frame_grab( image_buffer, TRUE);
      VESA_display_buffer( image_buffer, DPX_X, DPX_Y );
      dpx_draw_histogram( image_buffer );

      /* check which key is pressed */
      input = read_kb();
      if( input == ESC ) {
         break;
      }
      else if( (input == PGUP) || (input == PGDN)
               || (input == INS ) || (input == DEL ) ){

         /* gain or offset was modified, erase old values */
         setcolor( BLACK );
         sprintf( string, "GAIN = %d OFFSET = %d\n", gain, offset);
         outtextxy( DPX_B_X4, DPX_B_Y1, string );

         /* modify gain and/or offset accordingly */
         if( input == PGUP ) {
            offset++;
         }
else if( input == PGDN ) {
    offset--;
}
else if( input == INS ) {
    gain++;
}
else if( input == DEL ) {
    gain--;
}

/* gain: 63 minimum gain; 0 maximum gain */
/* offset: 0 no offset; 63 maximum offset */
CLAMP(63, gain, 0);
CLAMP(63, offset, 0);

/* set gain and offset to the new values */
set_gain_and_offset(gain, offset, TRUE);

/* display new values */
setcolor( YELLOW );
sprintf( string, "GAIN = %d OFFSET = %d", gain, offset );
outtextxy( DPX_B_X4, DPX_B_Y1, string );

else {
    /* do nothing */
}

} /* end of while */

/* Erase ready-to-grab message */

setcolor( BLACK );
sprintf( string, "PGUP:INC OFFSET PGDN:DEC OFFSET" );
outtextxy( DPX_B_X1, DPX_B_Y1, string );
sprintf( string, "INS :INC GAIN DEL:DEC GAIN" );
outtextxy( DPX_B_X1, DPX_B_Y2, string );
sprintf( string, "ESC: SAVE IMG "
    "ANY OTHER KEY TO GRAB/UPDATE IMG" );
outtextxy( DPX_B_X1, DPX_B_Y3, string );
sprintf( string, "READY TO GRAB FRAME %d", i );
outtextxy( DPX_B_X1, DPX_B_Y4, string );

/* Print saving message */
setcolor( YELLOW );
sprintf( string, "SAVING FRAME %d", i );
outtextxy(DPX_B_X1, DPX_B_Y1, string);

/* Write raw image in buffer image_buffer to disk */
sprintf(string, "%s\%s_is.%03d", dpx_imgpath, dpx_name, i);
copy_image_buffer_to_disk(image_buffer, string);

/* erase processing message */

setcolor(BLACK);
sprintf(string, "SAVING FRAME %d", i);
outtextxy(DPX_B_X1, DPX_B_Y1, string);
}

text_mode();
printf("Task Completed ...");

exit(0);
/*
 * INSPIIMG.C
 *
 * this program splits an image in half. The resulting
 * images are turned upside down
 *
 * Miguel Angel Garcia Sep. 4, 1994
 *
 */

#include <stdio.h>
#include <stdlib.h>
#include <string.h>
#include <fcntl.h>
#include <dos.h>
#include <conio.h>

#include "DPX\dpx.h"
#include "pubproto.h"
#include "cserrno.h"

#define DBL_DPX_WIDTH (DPX_WIDTH * 2)

unsigned char huge data[DPX_HEIGHT][DBL_DPX_WIDTH];
unsigned char huge inv_spl_data[DPX_WIDTH];

void main(int argc, char **argv)
{
    unsigned unb;
    long lnb;
    int fd_i, fd_ol, fd_or;
    int nx, ny, nxby2;
    long image_size;
    char case_name[5];
    char string[80];
    int i, j, k;
    int first_frame;
    int last_frame;

    /* get command line parameters */
    if(argc == 4) {
        strcpy( case_name, argv[1]);
    }
first_frame = atoi( argv[2] );
last_frame = atoi( argv[3] );
}
else {
    printf( "Usage: executable case_name first last\n");
    exit( CS_USERERR );
}

/* for each frame */
for ( j = first_frame; j <= last_frame; j++ ) {

    /* display image ID being processed */
    printf("Processing image: %s_is.%03d\n", case_name, j);

    /* open input and output files */
    sprintf( string, "..\IMG\%s_is.%03d", case_name, j);
    if (_dos_open( string, O_RDONLY, &fd_i ) != 0 ) {
        printf( "FAIL TO OPEN %s\n", string );
        exit(CS_FOPENERR);
    }

    sprintf( string, "..\IMG\%s_il.%03d", case_name, j);
    if (_dos_create( string, _A_NORMAL, &fd_o ) != 0 ) {
        printf( "FAIL TO OPEN %s\n", string );
        exit(CS_FOPENERR);
    }

    sprintf( string, "..\IMG\%s_ir.%03d", case_name, j);
    if (_dos_create( string, _A_NORMAL, &fd_o ) != 0 ) {
        printf( "FAIL TO OPEN %s\n", string );
        exit(CS_FOPENERR);
    }

    /* read image width (first 2 bytes) */
    if (_dos_read( fd_i, (void *)nx, 2, &unb ) != 0 ) {
        printf( "FAIL TO READ FILE\n" );
        exit(CS_FREADERR);
    }

    /* calculate width of splitted images */
    nxby2 = nx / 2;

    /* read image height (next 2 bytes) */
    if (_dos_read( fd_i, (void *)ny, 2, &unb ) != 0 ) {
        printf( "FAIL TO READ FILE\n" );
    }
exit(CS_FREADERR);
}

/* write image dimensions in left and right images */
if( _dos_write( fd_ol, (void far *)&nxby2, 2, &unb ) != 0) {
    printf( "FAIL TO WRITE OUTPUT FILE\n" );
    exit(1);
}
if( _dos_write( fd_ol, (void far *)&ny, 2, &unb ) != 0) {
    printf( "FAIL TO WRITE OUTPUT FILE\n" );
    exit(1);
}
if( _dos_write( fd_or, (void far *)&nxby2, 2, &unb ) != 0) {
    printf( "FAIL TO WRITE OUTPUT FILE\n" );
    exit(1);
}
if( _dos_write( fd_or, (void far *)&ny, 2, &unb ) != 0) {
    printf( "FAIL TO WRITE OUTPUT FILE\n" );
    exit(1);
}

/* read image to be splitted and turned upside down */
image_size = (long)nx * (long)ny;
if( blk_read( fd_i, (void far *)data, image_size, &lnb ) != 0 ) {
    printf( "FAIL TO READ INPUT FILE \n" );
    exit(CS_FREADERR);
}

/* split and turn resulting images upside down */

/* first, the left image */
for (k = 0; k < ny; k++ ) {
    for(i = 0; i < nxby2; i++ ) {
        inv_spl_data[i] = data[ny-1-k][nx-1-i];
    }
    if( _dos_write( fd_ol, (void far *)inv_spl_data, nxby2, 
                    (unsigned *)&lnb ) != 0 ) {
        printf( "FAIL TO WRITE OUTPUT FILE \n" );
        exit(1);
    }
}
/* second, the right image */
for (k = 0; k < ny; k++ ) {
for(i = 0; i < nxby2; i++ ) {
    inv_spl_data[i] = data[ny-1-k][nxby2-1-i];
}
if( _dos_write( fd_or, (void far *)inv_spl_data, nxby2,
   (unsigned *)&lnb ) != 0 ) {
    printf( "FAIL TO WRITE OUTPUT FILE \n" );
    exit(1);
}

_dos_close( fd_i );
_dos_close( fd_ol );
_dos_close( fd_or );

} /* end of for loop */
printf("\n\n Task completed ..."),
exit(0);
CNG_GREY.C

This program gives black (grey = 0) to the background by
thresholding value manually but foreground has original grey level.

This program is created by Miguel A Garcia Sep 10, 1994

#include <stdio.h>
#include <stdlib.h>
#include <string.h>
#include <fcntl.h>
#include <dos.h>
#include <conio.h>

#include "DPX\stddefs.h"
#include "DPX\dsploc.h"
#include "DPX\ptype.h"
#include "DPX\dpx.h"
#include "pubproto.h"

void main( int argc, char *argv[] )
{
    unsigned char data[DPX_WIDTH];
    long len;
    char fname_i[15];
    char fname_o[15];
    char string[80];
    int fd_i, fd_o;
    int i;
    int k;
    int grey1, grey2;

    if (argc == 5) {
        /* input image file name */
        strcpy( fname_i, argv[1] );
        /* output image file name */
        strcpy( fname_o, argv[2] );
        /* source grey */
        grey1 = atoi( argv[3] );
        /* destination grey */
grey2 = atoi(argv[4]);
}
else {
    printf( "Usage: executable input_file output_file source dest\n" );
    exit(1);
}

/* This section performs opening the input and output files. */
sprintf( string, "..\IMG\%s", fnamen );
if( _dos_open( string, O_RDONLY, &fd_i ) != 0 ) {
    printf( "FAIL TO OPEN %s\n", fnamen );
    exit(1);
}

sprintf( string, "..\IMG\%s", fnamen_o );
if( _dos_creat( string, _A_NORMAL, &fd_o ) != 0 ) {
    printf( "FAIL TO OPEN %s\n", fnamen_o );
    exit(1);
}

/* Define first 4 bytes as width & height */

if( _dos_read( fd_i, (void far *)data, 4, (unsigned *)&len ) != 0 ) {
    printf( "FAIL TO READ BACKGROUND FILE\n" );
    exit(1);
}

/* Write the first 4 bytes as width & height */

if( _dos_write( fd_o, (void far *)data, 4, (unsigned *)&len ) != 0 ) {
    printf( "FAIL TO WRITE OUTPUT FILE\n" );
    exit(1);
}

/* This section performs reading binary data from two input files. */

for ( i = 0; i < DPX_HEIGHT; i++ ){
    if( _dos_read( fd_i, (void far *)data, DPX_WIDTH, (unsigned *)&len ) != 0 ) {
        printf( "FAIL TO READ BACKGROUND FILE \n" );
        exit(1);
    }
    for( k = 0 ; k < DPX_WIDTH ; k++ ) {
if (data[k] < grey1) {
    data[k] = grey2;
}

} /* Write the filtered data into the output data file. */

if( _dos_write( fd_o, (void far *)data, DPX_WIDTH, (unsigned *)&len ) != 0 ) {
    printf( "FAIL TO WRITE OUTPUT FILE \n" );
    exit( 1 );
}

} /* close the files */

_dos_close( fd_i );
_dos_close( fd_o );

/* display and reset the thresholding value */

VESAGraphicsMode (0x103);
load_vga_palettes();
select_vga_palette(0);
sprintf(string, "..\\IMG\\%s", fname_i);
VESADiskToVGA(0, 0, string);
getch();
sprintf( string, "..\\IMG\\%s", fname_o);
VESADiskToVGA(0, 0, string);
getch();
text_mode();
/* SUBIMG.C */
* This program subtracts a background image
* from a series of images
* *
* Written By: Miguel Garcia-Briones
*/

/* standard include files */
#include <stdio.h>
#include <string.h>
#include <stdlib.h>
#include <graphics.h>
#include <conio.h>
#include <dos.h>

/* definitions in the dpx include files */
#include "dp\stddefs.h"
#include "dp\ptype.h"
#include "dp\errors.h"
#include "dp\dp.h"

/* CS specific include files */
#include "cserrno.h"
#include "pubproto.h"
#include "colors.h"
#include "csscreen.h"
#include "utils.h"

void main (int argc, char **argv)
{
  /* variables */

  char org_img_name[80];
  char bac_img_name[80];
  char sub_img_name[3];
int continuous_flag;  
int first_frame;  
int last_frame;  
i;  
int palette_num = 0;  
char imgpath[80] = "..\IMG";  
char string[80];  
int org_img_buffer = 1;  
int bac_img_buffer = 2;  
int sub_img_buffer = 3;  
int img_width = DPX_WIDTH;  
int img_height = DPX_HEIGHT;

/* get the command line parameters */
if(argc == 6){
    if(strlen(argv[1]) > 8){
        printf(" image name too long \n");
        exit(CS_USERERR);
    }
    if(strlen(argv[2]) > 12){
        printf(" background image name too long \n");
        exit(CS_USERERR);
    }
    if(strlen(argv[3]) > 8){
        printf("substracted image name too long \n");
        exit(CS_USERERR);
    }
    strcpy(org_img_name,argv[1]);
    strcpy(bac_img_name,argv[2]);
    strcpy(sub_img_name, argv[3]);
    first_frame = atoi (argv[4]);
    last_frame = atoi (argv[5]);
    continuous_flag= FALSE;
}
else if(argc ==7){
if(strcmp(argv[1], "-c") && strcmp(argv[1],"-C")){
    printf("invalid flag set \n");
    exit (CS_USERERR);
}
if(strlen(argv[2]) > 8){
    printf("image name too long \n");
    exit (CS_USERERR);
}
if(strlen(argv[3]) > 12){
    printf("background image name too long \n");
    exit (CS_USERERR);
}
if(strlen(argv[4]) > 8){
    printf("subtracted image name too long \n");
    exit (CS_USERERR);
}
strcpy (org_img_name, argv[2]);
strcpy (bac_img_name, argv[3]);
strcpy (sub_img_name, argv[4]);
first_frame=atoi (argv[5]);
last_frame=atoi (argv[6]);
continuous_flag= TRUE;
if((last_frame > 999) || (first_frame < 0)){
    printf("number of frame is out of range! \n");
    exit (CS_USERERR);
}
else {
    printf("Usage: executable [-c] org_img, bac_img.ext, sub_img, first, last \n");
    exit (CS_USERERR);
}
*/ initialization of the board */

init_dram();
if(load_tag_file("..\ETC\A1_32.TAG") == -1){
    printf("error loading [..\ETC\A1_32.TAG] file \n");
    exit (CS_HWINITFAIL);
}
run_dsp();
delay (100);
init_cbinding();

/*/ initialization for VESA display 800x600 */
if(init_vga_graphics (0x103) !=0){
    text_mode();
    printf("VESA display mode initialization fail. \n");
    exit (CS_HWINITFAIL);
}
sprintf(string,"..\ETC\PALETTE.FLP");
modify_palette_list(string,0);
modify_palette_list(string,1);
modify_palette_list(string,2);
load_vga_palettes();
select_vga_palette(palette_num);

for (i=first_frame;i<=last_frame;i++){

    if(continuous_flag == FALSE){
        /* display instructions and original image name */
        setcolor(YELLOW);
        sprintf(string, "Press any key to continue...");
        outtextxy(DPX_B_X1, DPX_B_Y3,string);
        sprintf(string,"Original img: %s.%03d",org_img_name,i);
        outtextxy (DPX_B_X4, DPX_B_Y3,string);

        /* display original image from disk */
        sprintf (string, "%s\%s.%03d",imgpath,org_img_name,i);
        VESADiskToVGA (DPX_X,DPX_Y,string);
        getch();
    }
    else {
        /* display image name being processed */
        setcolor(YELLOW);
        sprintf(string,"Processing img: %s.%03d",org_img_name,i);
        outtextxy(DPX_B_X1,DPX_B_Y3,string);
    }

    /* load original and background image into board memory */
    set_grab_size(img_width, img_height, TRUE);
    sprintf(string, "%s\%s.%03d",imgpath,org_img_name,i);
    ifl[copy_disk_to_image_buffer(org_img_buffer,string) != P_SUCCESS]{
        text_mode();
        printf("Image loading into Dipix board fail [%s] \n",string);
        exit (CS_FOPENERR);
    }
    sprintf(string, "%s\%s",imgpath,bac_img_name);
    ifl[copy_disk_to_image_buffer (bac_img_buffer,string)!=P_SUCCESS]{
        text_mode();
        printf("Image loading into Dipix board fail [%s] \n",string);
        exit(CS_FOPENERR);
    }
}
/* Substraction */

arith((int)org_img_buffer,(int)bac_img_buffer,(int)sub_img_buffer,P_ARITH_SUBTRACT,1.0,0.0,0.0,1,TRUE);

/* Save substracted image to disk */
sprintf(string,"%s\%s.%03d",imgpath,sub_img_name,i);
copy_image_buffer_to_disk(sub_img_buffer,string);

if(continuous_flag == FALSE){
    /* Purge original image name*/
    setcolor(BLACK);
    sprintf(string,"Original img: %s.%03d",org_img_name,i);
    outtextxy(DPX_B_X4,DPX_B_Y3,string);

    /* Display substacted image name */
    setcolor(YELLOW);
    sprintf(string,"Substracted img: %s.%03d",sub_img_name,i);
    outtextxy(DPX_B_X4,DPX_B_Y3,string);

    /* Display substracted image */
    VESA_display_buffer (sub_img_buffer,DPX_X,DPX_Y);
    getch();

    /* Purge substracted image name */
    setcolor(BLACK);
    sprintf(string,"Substracted img: %s.%03d",sub_img_name,i);
    outtextxy(DPX_B_X4,DPX_B_Y3,string);
}
else {
    /* Purge image name being processed */
    setcolor(BLACK);
    sprintf(string,"Processing img: %s.%03d",org_img_name,i);
    outtextxy(DPX_B_X1,DPX_B_Y3,string);
}
} /* End of loop */
text_mode();
printf("Task completed...");
exit(0);

} /* end of main */
STRETCH.C

This program stretches the grey level of input images.

This program was written by Miguel A. Garcia Sep 5, 1994

#include <stdio.h>
#include <stdlib.h>
#include <string.h>
#include <fcntl.h>
#include <dos.h>
#include <conio.h>
#include <graphics.h>
#include "DPX\stddefs.h"
#include "DPX\dpx.h"
#include "DPX\ptype.h"
#include "DPX\errors.h"
#include "pubproto.h"
#include "colors.h"
#include "cserrno.h"
#include "utils.h"
#include "csscreen.h"

unsigned char huge data[DPX_WIDTH][DPX_HEIGHT];

void main(int argc, char **argv)
{
    long len;
    long image_size = (long)DPX_WIDTH * (long)DPX_HEIGHT;
    char fname_i[15];
    char fname_o[15];
    char string[80];
    int fd_i, fd_o;
    int j;
    int i;
int k;
int x, y;
int min = 0;
int max = 0;
int continuous_flag;
int first_frame;
int last_frame;
int palette_num = 0;
int input;

/* get the command line parameters */
if(argc == 7){
    strcpy( fname_i, argv[1] );
    strcpy( fname_o, argv[2] );
    min = atoi( argv[3] );
    max = atoi( argv[4] );
    first_frame = atoi( argv[5] );
    last_frame = atoi( argv[6] );
    continuous_flag = FALSE;
}
else if (argc == 8){
    if(strcmp(argv[1], "-c") && strcmp(argv[1], "-C")){
        printf("invalid flag set 
");
        exit(CS_USERERR);
    }
    strcpy( fname_i, argv[2] );
    strcpy( fname_o, argv[3] );
    min = atoi( argv[4] );
    max = atoi( argv[5] );
    first_frame = atoi( argv[6] );
    last_frame = atoi( argv[7] );
    continuous_flag = TRUE;
}
else {
    printf("Usage: executable [-c] org_img str_img min max first last 
");
    exit( CS_HWINITFAIL );
}

/* initialization for VESA display 800x600 */
init_vga_graphics(0x103);
sprintf(string, ..\ETC\PALETTE.FLP);
modify_palette_list(string,0);
modify_palette_list(string,1);
modify_palette_list(string,2);
load_vga_palettes();
select_vga_palette(palette_num);

/* for each frame ... */

for (j = first_frame; j <= last_frame; j++) {

    /* display original image if "-c" is not set */
    if (continuous_flag == FALSE){
        setcolor(YELLOW);
        sprintf(string, "Press any key to continue ... ");
        outtextxy(DPX_B_X1, DPX_B_Y1, string);
        sprintf(string, "org. image: %s.%03d", frame_i, j);
        outtextxy(DPX_B_X1, DPX_B_Y2, string);

        sprintf(string, ..\IMG\%s.%03d", frame_i);
        VESADiskToVGA(0, 0, string);
        getch();

        setcolor(BLACK);
        sprintf(string, "Press any key to continue ... ");
        outtextxy(DPX_B_X1, DPX_B_Y1, string);
        sprintf(string, "org. image: %s.%03d", frame_i, j);
        outtextxy(DPX_B_X1, DPX_B_Y2, string);
    }
    else {
        setcolor(YELLOW);
        sprintf(string, "Processing image: %s.%03d", frame_i, j);
        outtextxy(DPX_B_X1, DPX_B_Y2, string);
    }

    /* while loop to adjust max and min used in stretching */
    while(TRUE){
        /* This section performs opening the input and output files. */
    }
}
211

```c
sprintf( string, "..\IMG\%s.%03d", fnam_ej );
if( _dos_open( string, O_RDWR, &fd_i ) != 0 ) {
    text_mode();
    printf( "FAIL TO OPEN %s\n", string );
    exit( 1 );
}

sprintf( string, "..\IMG\%s.%03d", fnam_o );
if( _dos_creat( string, _A_NORMAL, &fd_o ) != 0 ) {
    text_mode();
    printf( "FAIL TO OPEN %s\n", string );
    exit( 1 );
}

/* Define first 4 bytes as width & height */

if( _dos_read( fd_i, (void far *)data, 4, (unsigned *)&len ) != 0 ) {
    text_mode();
    printf( "FAIL TO READ INPUT FILE\n" );
    exit( 1 );
}

/* Write the first 4 bytes as width & height */

if( _dos_write( fd_o, (void far *)data, 4, (unsigned *)&len ) != 0 ) {
    text_mode();
    printf( "FAIL TO WRITE OUTPUT FILE\n" );
    exit( 1 );
}

/* stretch step */
if( blk_read( fd_i, (void far *)data, image_size, &len ) != 0 ) {
    text_mode();
    printf( "FAIL TO READ FILE \n" );
    exit( 1 );
}

for ( i = 0 ; i < DPX_WIDTH ; i++ ){
    for( k = 0 ; k < DPX_HEIGHT ; k++ ) {
        x = (int)data[i][k] - min;
        y = x * (255/(max - min));
        if(y > 255) {
```
```c
y = 255;
}
if (y < 0) {
    y = 0;
}
data[i][k] = (char)y;

/* Write the filtered data into the output data file. */
if (blk_write(fd_o, (void远 IData, image_size, &len) != 0) {
    text_mode();
    printf("FAIL TO WRITE OUTPUT FILE \n");
    exit(1);
}

/* close the files */
_dos_close(fd_i);
_dos_close(fd_o);

if (continuous_flag != FALSE) {
    break;
}

/* show info and instructions */
setcolor(YELLOW);
sprintf(string, "ESC : NEXT FRAME ANY KEY : TO UPDATE "
    "INS : INC MIN DEL : DEC MIN");
outtextx(DPX_B_X1, DPX_B_Y1, string);
sprintf(string, "str. image: %s.%03d "
    "PGUP: INC MAX PGDN: DEC MAX", fname_o, j);
outtextx(DPX_B_X1, DPX_B_Y2, string);

/* show current values of min and max */
sprintf(string, "MIN = %d MAX = %d", min, max);
outtextxy(DPX_B_X1, DPX_B_Y3, string);

/* display stretched image */
sprintf(string, ".\\IMG\\%s.%03d", fname_o, j);
VESADiskToVGA(0, 0, string);

/* while loop to increase or decrease min and max in one unit */
while (TRUE) {
    /* see which key is press */
    input = read_kb();
```
if (input == ESC ) {
    break;
}
else if (input == PGUP) || (input == PGDN) ||
    (input == INS ) || (input == DEL ) ) {
    /* min or max has been modified, erase old values */
    setcolor(BLACK);
    sprintf( string, "MIN = %d  MAX = %d", min, max);
    outtextxy( DPX_B_X1, DPX_B_Y3, string );

    /* indicate that min or max was changed */
    setcolor(BLACK);
    sprintf( string, "stretched image with displayed min and max"");
    outtextxy( DPX_B_X1, DPX_B_Y4, string );

    setcolor(YELLOW);
    sprintf( string, "min or max was modified press any key to"
            " update image ... ");
    outtextxy( DPX_B_X1, DPX_B_Y4, string );

    /* modify min and max accordingly */
    if( input == PGUP ) {
        max++;
    }
    else if( input == PGDN ) {
        max--;
    }
    else if( input == INS ) {
        min++;
    }
    else if( input == DEL ) {
        min--;
    }
    /* braket the new values to the range 0 to 255 */
    /* program does not check if min > than max */
    /*
    CLAMP(255, max, 0);
    CLAMP(255, min, 0);
    */
    /* display new values */
setcolor(YELLOW);
sprintf( string, "MIN = %d MAX = %d", min, max );
outtextxy( DPX_B_X1, DPX_B_Y3, string );
} else {
    /* update image with current values of min and max */
    setcolor(BLACK);
    sprintf( string, "min or max was modified press any key to"
            " update image ..." );
    outtextxy( DPX_B_X1, DPX_B_Y4, string );

    setcolor(YELLOW);
    sprintf( string, "stretched image with displayed min and max" );
    outtextxy( DPX_B_X1, DPX_B_Y4, string );
    break ;
}
} /* end of while */

if( input == ESC ) {
    /* will work with next frame erase everything */
    setcolor(BLACK);
    sprintf( string, "ESC : NEXT FRAME ANY KEY : TO UPDATE "
            " INS : INC MIN DEL : DEC MIN" );
    outtextxy( DPX_B_X1, DPX_B_Y1, string );
    sprintf( string, "str. image: %ds.%03d "
            " PGUP: INC MAX PGDN: DEC MAX", fname_o, j );
    outtextxy( DPX_B_X1, DPX_B_Y2, string );
    sprintf( string, "MIN = %d MAX = %d", min, max );
    outtextxy( DPX_B_X1, DPX_B_Y3, string );
    sprintf( string, "min or max was modified press any key to"
            " update image ..." );
    outtextxy( DPX_B_X1, DPX_B_Y4, string );
    sprintf( string, "stretched image with displayed min and max" );
    outtextxy( DPX_B_X1, DPX_B_Y4, string );
    break ;
}
} /* end of while */

if( continuous_flag != FALSE ){
    setcolor(BLACK);
    sprintf( string, "Processing image: %s.%03d", fname_i, j );
...
outtextxy(DPX_B_X1, DPX_B_Y2, string);
}
} /* end of "for" loop */

text_mode();
printf("Task completed ...");
extit(0);
}
FIND_BOX

This program is a modification of CAM_INI.C.

This program can be used to specify the processing windows from images previously stored on disk.

#include <stdio.h>
#include <stdlib.h>
#include <string.h>
#include <alloc.h>
#include <conio.h>
#include <graphics.h>

#include "colors.h" /* This color table overrides the generic BGI colors */

#include "path.h"
#include "pubproto.h"
#include "cserrno.h"
#include "csscreen.h"
#include "utils.h"

/* local CS include files */
#include "dpx/dpx.h"
#include "dpx/dpxproto.h"

/* include files for DIPIX frame grabber */
#include "dpx/stddefs.h"
#include "dpx/ptype.h"
#include "dpx/errors.h"

#define DEFAULT_GAIN 30
#define DEFAULT_OFFSET 50

#define INITIAL_THRESH 50
#define NBR_IMG_SIDES 2

cchar dpx_name[6];
int dpx_frame_s;
int dpx_frame_e;
char img_path[80];

void main( int, char** );

void main( argc, argv )
int argc;
char **argv;
{
    char case_name[6];
    char string[80];
    char img_side[2][2] = { "L", "R" };
    int frame_nbr;
    int theta[2], phi[2];
    int gain[2], offset[2];
    int i;
    FILE *fp;
    DPX_BOUNDING_BOX *box;

    if( argc == 3 ) {
        if( strlen( argv[1] ) > 5 ) {
            printf( "case name too long
" );
            exit( CS_USERERR );
        }
        strcpy( case_name, argv[1] );
        frame_nbr = atoi( argv[2] );
    } else {
        printf("\nUsage : executable case_name(1-5) frame_nbr \n" );
        exit( CS_USERERR );
    }

    sprintf( string, "%s\%s_FC.DAT", dpx_datpath, case_name );
    fp = fopen( string, "w+t" );
    if( fp == NULL ) {
        printf( "fail to open file[%s]\n", string );
        exit( CS_FOPENERR );
    }

    for( i = 0 ; i < NBR_IMG_SIDES; i++ ) {
        printf( "INPUT FRAME IMAGE SIDE [%d] ORIENTATION :\n", i );
        printf( "THETA [%d] ( x -> y ) = ", i );
        printf( "\n" );
scanf("%d", &theta[i]);
printf("PHI [%d] ( z -> x ) = ", i);
scanf("%d", &phi[i]);

/* FC.DAT file update */
fprintf(fp, "%d %d\n", theta[i], phi[i]);
}

/ * initialize the memory on the P360 */
for(i = 0 ; i < DPX_FRAME_GRABBER ; i++) {
  if( dpx_init_boards( i ) != CS_SUCCESS ) {
    fclose(fp);
    exit(CS_HWINITFAIL);
  }
}

/* initialization for VESA display (800x600) */
if( dpx_init_display(DPX_800X600) != CS_SUCCESS ) {
  text_mode();
  printf("VESA display mode initialization fail.\n");
  fclose(fp);
  exit(CS_HWINITFAIL);
}

for( i = 0 ; i < NBR_IMG_SIDES ; i++ ) {
  gain[i] = DEFAULT_GAIN;
  offset[i] = DEFAULT_OFFSET;
  fprintf(fp, "%d %d\n", gain[i], offset[i]);
}

box = (DPX_BOUNDING_BOX *)farmalloc( sizeof(DPX_BOUNDING_BOX) );
if( box == NULL ) {
  fclose(fp);
  text_mode();
  printf("malloc fail for BOUNDING_BOX setup\n");
  exit(CS_HWINITFAIL);
}

for( i = 0 ; i < NBR_IMG_SIDES ; i++ ) {
/* load image into board buffer */
sprintf(string, ".\\IMG\%s_i%s.03d", case_name,
if( copy_disk_to_image_buffer(DPX_IMG_BUFFER, string) != P_SUCCESS) {
    text_mode();
    printf( "image loading into Dipix board failed [%s]n", string );
    exit( CS_FOPENERR );
}

select_board( DPX_LEFT );
box = dpx_set_bounding_box( DPX_IMG_BUFFER );

/* _FC.DAT file update */
fprintf( fp, "%d %d %d %d\n",
    box->tl_x - DPX_X, box->tl_y - DPX_Y,
    box->br_x - DPX_X, box->br_y - DPX_Y );

/* display information line */
setcolor( WHITE );
sprintf( string, "PRESS ANY KEY FOR NEXT IMAGE SIDE" );
outtextxy( DPX_B_X1, DPX_B_Y1, string );
getch();

/* purge information line */
setcolor( BLACK );
sprintf( string, "PRESS ANY KEY FOR NEXT IMAGE SIDE" );
outtextxy( DPX_B_X1, DPX_B_Y1, string );

} /* release the memory */
farfree( box );

/* output default values for threshold values.
   These values will be updated in ex_pre.exe */
/* _FC.DAT file update */
fprintf( fp, "%d %d\n", INITIAL_THRESH, INITIAL_THRESH );
fclose( fp );
text_mode();
printf( "Task Completed ... \n" );
}