CONTRACT NO.: DAMD17-88-C-8009

TITLE: FEASIBILITY OF AN ULTRASONIC BLOOD CELL WASHER

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REPORT DATE: February 13, 1989

TYPE OF REPORT: Final

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21701-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a REPORT SECURITY CLASSIFICATION: Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b DECLASSIFICATION / DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: Technical Research Associates; 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code): 410 Chipeta Way, Salt Lake City, Utah 84108	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING / SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-88-C-8009
8c ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10 SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M1-61102BS14	BC	002

11 TITLE (Include Security Classification)

(U) Feasibility of an Ultrasonic Blood Cell Washer

12 PERSONAL AUTHOR(S)

Stephen C. Peterson

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 10/15/87 TO 4/14/89	1989 February 13	48

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA 2, Deglycerolization, Acoustic energy, Blood cells
06	01		
06	04		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Technical Research Associates explored the feasibility of using an ultrasonic pseudostanding wave to effect the removal of glycerol from thawed frozen red cells. In the first part of this project basic data regarding the acoustic properties of prediluted thawed red cells, and the prediluted cells plus the final washing solution were obtained. These data would aid in the design of a sweeping wave acoustic separation cell. Subsequent work involved the design, construction and characterization of numerous acoustic separation cells. None of these cells showed promise for scaling to feed flow rates competitive with current washing systems. Several other approaches to the problem of low cost, low mass cell washers were suggested.

20 DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

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S. PETERSON 2.13.89

PI Signature Date

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APPENDIX

Introduction

In a potential conflict the armed forces will need large quantities of blood for transfusion. Standard blood banking procedures can provide blood storage for about thirty days after which the blood must be discarded. To provide an adequate emergency supply continual collection and replacement of blood is necessary. In the last few years it has become possible to store blood frozen. Frozen red cells can be kept for several years thus allowing buildup of a large emergency supply and eliminating the wastage associated with the conventional blood banking scheme.

The freezing procedure utilizes glycerine to prevent lysis of the cells. Upon thawing and before infusion the relatively high concentration of glycerine must be reduced to below about one percent. Because of the high concentration of glycerine and the mandated small volumes of wash solution (primarily physiological saline) simple dilution and subsequent centrifugation is inadequate in reducing the concentration of glycerine to acceptable levels; the cells must actually be washed.

Several procedures and apparatus are used or are being developed to wash the cells. The Hemonetics device and procedure is currently the simplest and probably most popular. In this procedure thawed glycerolized cells are first prediluted with two different concentrations of saline. The cells are then washed with physiological saline in a special centrifuge head. The cells are

contained in the head by centrifugal force while wash solution "percolates" through the semi packed cells. Waste solution containing the glycerine exits the head and is discarded. While this scheme works it requires constant monitoring by a technician and allows for the introduction of bacteria via a rotating seal.

A second apparatus and procedure has been developed by Cobe. This apparatus is much more complex with electromagnetic valves and sensors and a donut shaped washing bag. Prediluted cells are transferred to the toroidal bag and wash solution is added. The bag is then agitated much like clothes in a standard home washing machine. Then the bag is spun and the supernatant expelled by squeezing the bag. This procedure is repeated several times until the cells are adequately washed. This apparatus and procedure also has a rotating seal which allows for possible bacterial contamination and machine is mechanically and electrically complex.

Millipore corporation is currently developing still another approach to solve problems associated with tne washing procedure. They are using a membrane to filter red cells from the waste wash solution. To prevent the "plugging" of the membrane pores by red cells held by the transmembrane hydrostatic pressure drop they "flow" the red cells past the membrane at relatively high speed. In theory the erythrocytes flow tangentially past the pores in the membrane and are thus prevented from entering or plugging the pore. In practice the control of the flow is very critical with respect

to the transmembrane pressure and often the membrane plugs.

The purpose of the present work is to explore the feasibility of using acoustic radiation pressure to separate the erythrocytes from the wash solution. For the past several years TRA has been using acoustic radiation pressure to move in a controlled way, particles, including erythrocytes, through fluids. It is well known that in an acoustic standing wave radiation pressure can force particles, whose acoustic properties differ from that of the medium, to the nodes or antinodes of the standing wave. This property alone only allows separation of particle and fluid on a microscopic scale.

TRA has developed a technology that allows controlled large scale movement of the nodes and antinodes [1] (this paper is included in the appendix as a convenient review of the physics of the separation technique). This pseudostanding wave can trap cells and subsequently move them over long distances. TRA has used this technique to effect the separation of red cells and plasma in whole blood. Currently typical input flow rates are 1 ml/min/6 cm^2 of transducer. Cells can be concentrated to about 80 percent and plasma cleared to less than one percent of cells. The purpose of this work was to explore the feasibility of this technology to wash glycerine from thawed frozen red cells.

In order to apply our technology to glycerol containing red cells it was first necessary to examine some relevant acoustic properties of the glycerol containing cells and medium. Further additional

fundamental understanding of the separation technique was also needed. Based on this information, several small scale separators could be designed and evaluated. The best design would then be scaled to flow rates necessary for competitive washing of the thawed blood cells. Finally, if the approach proved feasible, a prototype separator would be delivered for Army evaluation.

Experimental Methods and Results

In this final report only data that are significant and germane to the problem will be presented. Data from all of the experiments were presented in the quarterly reports and will not be included in toto here. In addition, there will be new discussion of the results obtained for the purpose of evaluating future application of ultrasound separation techniques to the problem of cell washing.

During the first quarter a visit to the Naval Blood Research Laboratory was made. The PI felt that this visit was very useful in obtaining an overview of the glycerolization/deglycerolization process. During this visit the PI became acquainted with the various approaches to the deglycerolization process. Both Dr. Valeri and Mr. Gray were helpful and cooperative. One important observation was made which was relevant to the potential implementation of ultrasound to the washing process; mixing of the predilution and wash solutions with the thawed blood. Dr. Valeri admitted that the current mixing procedure, which is used by the two current procedures probably results in the loss of some cells

due to osmotic stress. TRA subsequently developed a "cut and fold" mixer. We had thought that it might be possible to use sweeping ultrasound to obtain better, more uniform mixing but opted instead for the cut and fold approach as it required no energy input. In fact that mixer could be used for the predilution and wash additions in the current procedures. Figure 1 shows recently obtained data for extracellular potassium and hemoglobin. For this experiment thawed cells were divided into two aliquot, one run through the mixer, which mixed the predilution and saline wash solutions with the cells. The control was the standard mixing procedure where predilution and wash solutions were added to the thawed cells in the bag. Samples were drawn. centrifuged and analyzed by a commercial blood laboratory (Associated Regional and University Pathologists Inc.). As can be seen there are no apparent differences between the standard procedure and TRA's cut and fold mixer at any of the mixing steps. The mixer thus allows for good mixing with an on line process such as that proposed by TRA for this project (see Figure 2 below from Figure 5 in the proposal).

Early work with properly glycerolized blood was delayed until the second quarter for two reasons. Firstly, although equipment was ordered as soon as the contract started, there were delays in delivery. Frozen glycerolized blood could not be properly stored until the minus 80 freezer arrived and set into operation. Secondly, there were delays in obtaining blood from NBRL. These delays were caused by scarcity of donors and the general demand for blood. In future projects of this type LAIR should allow

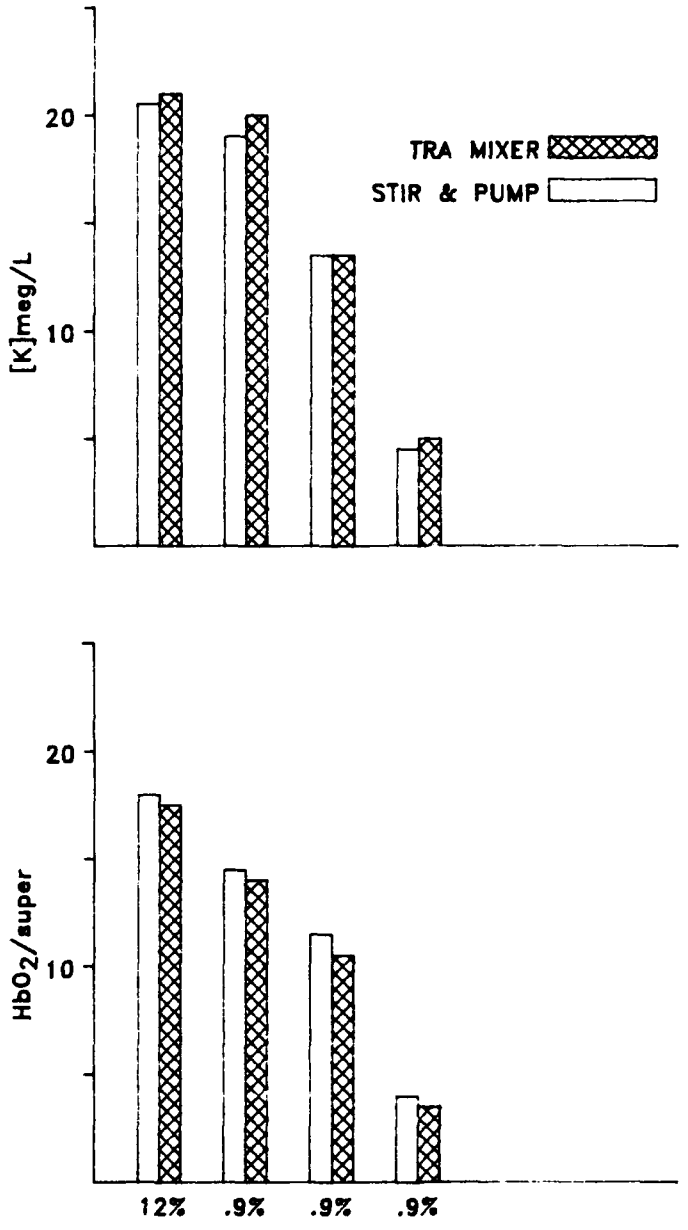


Figure 1

additional time in the schedule for similar delays. Fortunately some work deemed necessary for the understanding of the ultrasonic separation process were done which did not require glycerolized cells.

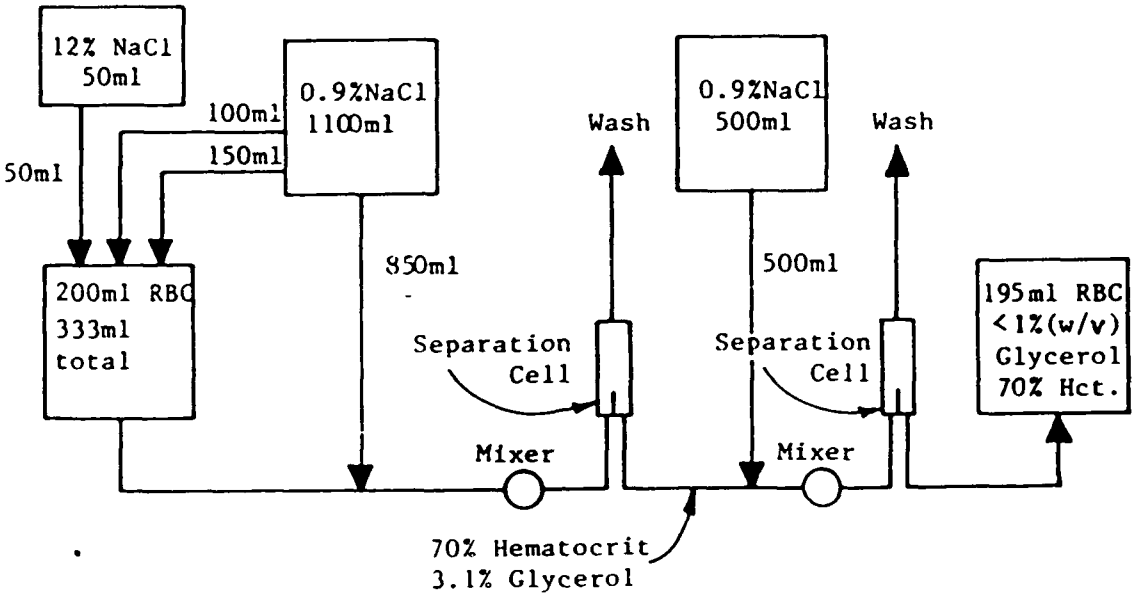


Figure 2

Figure 3 shows the speed of sound measured by the time delay of a pulse echo in a measurement cell of known length as a function of percent glycerol in water. This measurement was deemed useful for estimating the magnitude of the radiation pressure to be expected in blood at various stages in the deglycerolization process. Table 1 shows the density of different glycerol water mixtures and the experimentally determined speed of sound. With these data it is now possible to examine the force factor which determines whether the

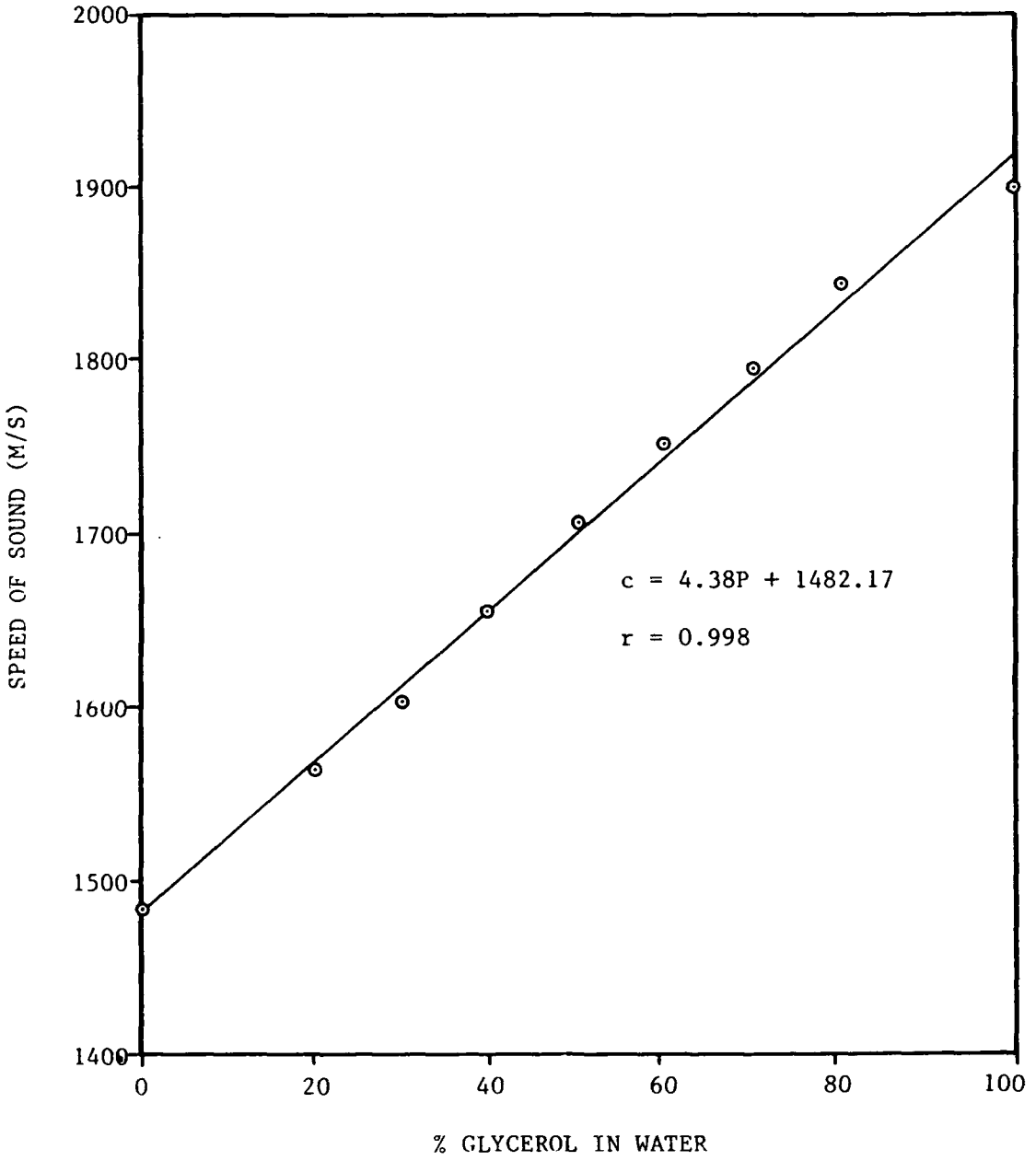

2000
1900
1800
1700
1600
1500
1400
SPEED OF SOUND (M/S)
c = 4.38P + 1482.17
r = 0.998
0
20
40
60
80
100
% GLYCEROL IN WATER

FIGURE 3

TABLE 1

Glycerol/ Water Ratio (wt%)	PM Density K m^{-3}	CM Speed of Sound m Sec^{-1}	RBC (1096*, 1600)	RBC (1164*, 1660 m/s)	N/AN
0	1000	1482	-0.293	-0.426	N
10	1021	1526	-0.200	-0.342	N
20	1046	1570	-0.100	-0.251	N
30	1072	1614	+0.004	-0.0757	N
40	1098	1658	+0.112	-0.060	N
50	1126	1701	+0.223	+0.040	N
60	1153	1745	+0.339	+0.145	No Bands
70	1182	1789	+0.462	+0.255	Weak Bands N
80	1208	1833	+0.586	+0.366	N
100	1261	1921	+0.847	+0.600	-

*Density Kg/m^3

radiation pressure will act to move and hold a particle in the node or antinode. This important information will allow us to estimate the success of an ultrasonic cell washer. The fourth column in Table 1 shows the value of the force factor assuming RBC density of 1096 Kg/m^3 and speed of sound of 1600 m/s. The values in the fifth column are our estimate for glycerol containing cells. Note that the "no force" value for the best estimate occurs at about 45 percent glycerine. At that time we felt that since we were diluting

the cells to about 20 percent with the predilutions, we would still be able to exert a substantial force on the cells.

In retrospect,because of the troubles we had in scaling the process we are no longer convinced that this is the case. Note that as the RBC's are washed they should approach their normal values for speed of sound and density and that the zero force point approaches 30 percent glycerol value. At 20 percent glycerol the force value is only -0.1 or about 1/3 that of normal cells in plasma. It may well be that part of our later inability to scale the separator was due to these small forces.

A KB Aerotech UTA-4 ultrasound analyzer was used to measure the attenuation of the glycerol/water ratio that was estimated to exist in the final washing stage (8.4 wt percent). The measurement was made at 2.25 MHz a frequency close to that used for our separation experiments. A control value of -1.134 dB/m was obtained for water (accepted calculated value is -1.099 dB/m [2]). For the 8.4 wt percent glycerol/water we obtained a value of -1.74 dB/m. This value is 1.5 times that for water and may also have contributed to our subsequent scaling problems.

Since our ultrasonic washing procedure generally used sweeping ultrasound the viscosity of the glycerol containing medium would result in lower critical velocities and hence make separation of the cells from plasma more difficult. Calculated viscosity for 10 percent glycerol/water was 0.00115 PaS a value that is 1.3 times

that of water.

Other experiments done during the first quarter included measure and verification of band (node) velocity as a function of both frequency ramp rate and distance from the reflector. These experiments corroborate our theoretical understanding of the pseudostanding wave with reality.

More important experiments were done to investigate the effects of "clustering". In our earlier work we had predicted that aggregates or clusters of red cells would form in the sound field. Cells would be forced by radiation pressure closely together, perhaps in temporary rouleaux formation. This super particle would have a much larger cross section and would be held even more strongly in the sound field than a single cell.

The calculations presented in the first quarter report need to be modified by data obtained in the second quarters work. In the second quarter we found that the capillary tube used for the clustering experiments actually stored energy. The program, presented as Figure 4, has been modified to take into account this energy storage. Figure 5 shows the results of the calculation for red cells in saline and our best estimate for cells in 20 percent glycerol. A cluster number of 50 was calculated for cells in saline and a lower number of 18 for the 20 percent glycerol/saline mixture. The smaller cluster in glycerol medium probably resulted from the higher viscosity with the concomitant higher drag thus

Figure 4

```
10 REM STANDING WAVE FORCE
20 F$=CHR$(27)&'&12J'
30 PRINT
40 PRINT DATE$&F$&TIME$
50 PRINT
60 PRINT F$;'STANDING WAVE FORCE'
70 PRINT
80 INPUT 'PARTICLE DENSITY(kg/m3)';P1
90 PRINT 'PARTICLE DENSITY (kg/m3)=';P1
100 INPUT 'PARTICLE SOS (M/S)';V1
110 PRINT 'PARTICLE SoS (m/s)';V1
120 INPUT 'MEDIUM DENSITY (kg/m3)';P2
130 PRINT 'MEDIUM DENSITY(kg/m3)';P2
140 INPUT 'MEDIUM SOS (m/s)';V2
150 PRINT 'MEDIUM SoS (m/s)';V2
160 INPUT 'FREQUENCY (MHz)';F1
170 PRINT 'FREQUENCY (MHz)=';F1
180 INPUT 'XDCR Z(OHMS)';Z1
190 PRINT 'XDCR Z(OHMS)=';Z1
200 INPUT 'V(rms)';V4
210 PRINT 'V(rms)=';V4
220 INPUT 'XDCR DIAM(m)';X1
230 PRINT 'XDCR DIAM(m)=';X1
235 REM ASSUMES 56% EFFICIENCY OF TRANSDUCER (see 420).
240 A2=2*P2*V2*V4^2/(Z1*PI*X1^2)
250 PRINT 'ACOUSTIC PRESSURE SQUARED (Pa^2)=';A2
260 INPUT ' PARTICLE RADIUS (um)';R
265 PRINT 'PARTICLE RADIUS= ';R;'um'
270 PRINT
280 REM CALC OF DELTA
290 D=P1/P2
300 REM CALC OF SIGMA SQUARED
310 S2=(V1/V2)^2
320 REM CALC OF FORCE FACTOR
330 F2=1/(D*S2)-(5*D-2)/(2*D+1)
340 PRINT 'FORCE FACTOR=';F2
350 REM CALC OF V0
360 V0=4*PI*(R*10^-6)^3/3
370 PRINT 'PARTICLE VOLUME=';V0
380 REM CALC OF WAVENUMBER
390 K=2*PI*F1*10^6/V2
400 PRINT 'WAVENUMBER=';K
410 REM CALC OF SW FORCE
420 F3=F2*1.988*V0*A2*K/(4*V2^2*P2)
430 PRINT 'STANDING WAVE FORCE (N)=';F3
440 REM CALC OF GRAVIT FORCE
450 F4=V0*9.8*(P1-P2)
460 PRINT 'GRAVITATIONAL FORCE ON PARTICLE(N)=';F4
470 REM CALC OF STOKES DRAG COEFF
480 INPUT 'VISCOSITY OF MEDIUM (Pa s)';V3
490 F5=6*V3*PI*R*10^-6
500 PRINT 'VISCOUS DRAG COEFFICIENT=';F5
510 REM CALC OF SWFORCE/GRAV FORCE
520 F6=F3/F4
530 PRINT 'SWF/GRAV F =';F6
540 REM CALC CRIT VEL
550 V5=F3/F5
560 PRINT 'CRITICAL VELOCITY (m/s)=';V5
564 I=A2/(2*P2*V2*10000)
566 PRINT 'INTENSITY W/cm^2 =';I
570 END
```

PROGRAM NOTES.

* Q of tube = 3.55 harmonics, account in step 420.

Figure 5

STANDING WAVE FORCE

RED CELLS / SALINE

PARTICLE DENSITY (kg/m3)= 1096
PARTICLE SoS (m/s) 1560
MEDIUM DENSITY(kg/m3) 1000
MEDIUM SoS (m/s) 1498
FREQUENCY (MHz)= .98
XDCR Z(OHMS)= 30
V(rms)= 50
XDCR DIAM(m)= .02866
ACOUSTIC PRESSURE SQUARED (Pa^2)= 96751636179.4
PARTICLE RADIUS= 2.7 um

FORCE FACTOR=-.248900405651
PARTICLE VOLUME= 8.2447957601E-17
WAVENUMBER= 4110.49506077
STANDING WAVE FORCE (N)=-1.8075516547E-12
GRAVITATIONAL FORCE ON PARTICLE(N)= 7.7567038511E-14
VISCOUS DRAG COEFFICIENT= 4.07150407904E-8
SWF/GRAV F =-23.3030896809
CRITICAL VELOCITY (m/s)=-4.4395182213E-5 ⇐ — 44 μm/sec.
INTENSITY W/cm^2 = 3.22936035312

experimental

$V_g = \frac{xR}{f} = 2160$ μm/s

(50 x)

89/02/0810:55:32

This force is higher due to Q of the bubble.

STANDING WAVE FORCE

RED CELLS/20% GLYCEROL

PARTICLE DENSITY (kg/m3)= 1164
PARTICLE SoS (m/s) 1660
MEDIUM DENSITY(kg/m3) 1046
MEDIUM SoS (m/s) 1570
FREQUENCY (MHz)= .98
XDCR Z(OHMS)= 30
V(rms)= 50
XDCR DIAM(m)= .02866
ACOUSTIC PRESSURE SQUARED (Pa^2)= 106066403182
PARTICLE RADIUS= 2.7 um

$V_g = \frac{xR}{f} = 444$ μm/s

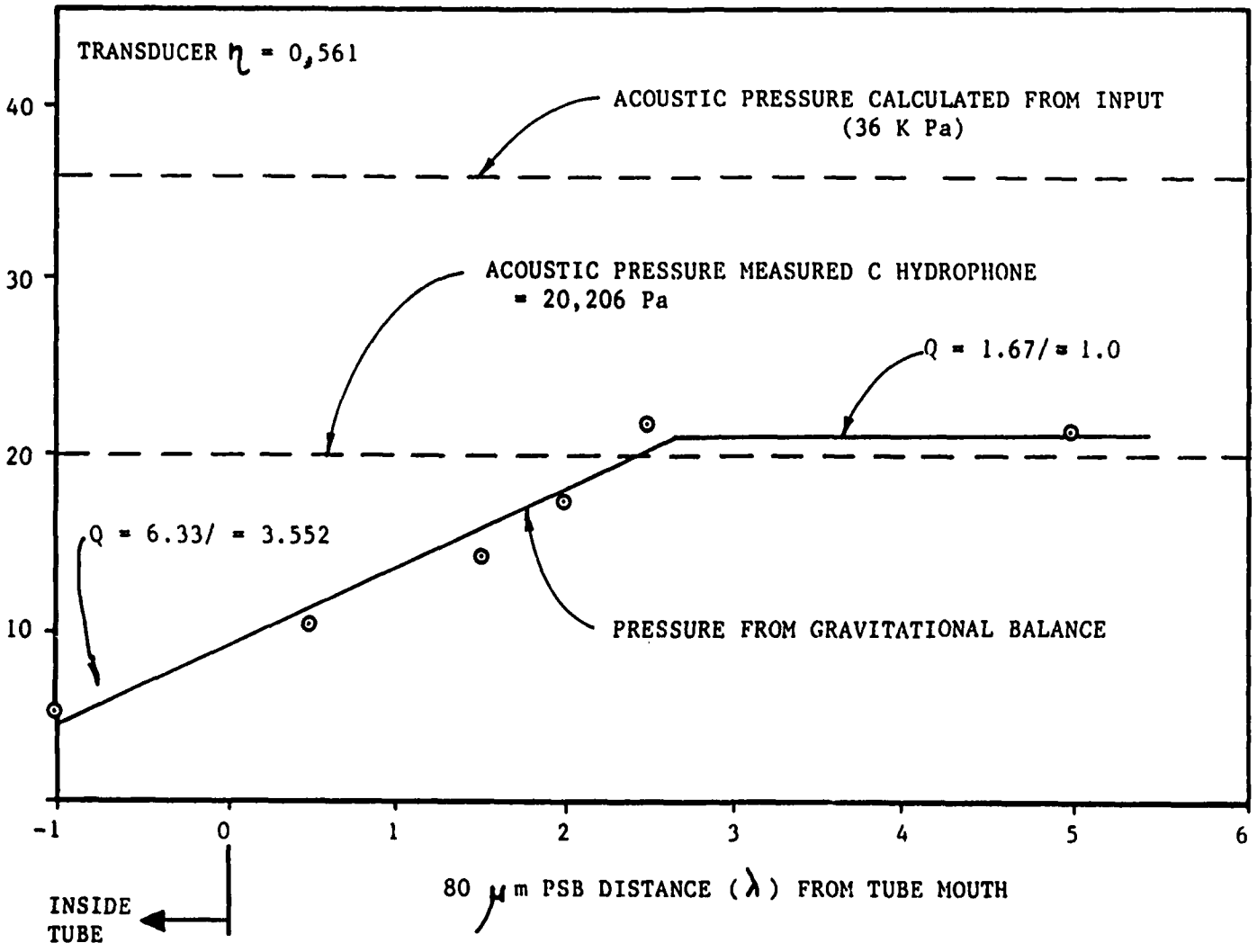
FORCE FACTOR=-.30109437703
PARTICLE VOLUME= 8.2447957601E-17
WAVENUMBER= 3921.98828092
STANDING WAVE FORCE (N)=-1.9906370348E-12
GRAVITATIONAL FORCE ON PARTICLE(N)= 9.53428181698E-14
VISCOUS DRAG COEFFICIENT= 7.83764535218E-8
SWF/GRAV F =-20.8787308054
CRITICAL VELOCITY (m/s)=-2.53984066049E-5 ⇐ 25 μm/sec.
INTENSITY W/cm^2 = 3.22936035312

(18 x)

preventing the formation of larger clusters.

The data used to determine the Q of the capillary tube are shown in Figure 6. These data were obtained from the following experiment. Our calibrated hydrophone was used to measure the acoustic pressure in a large cavity. A small capillary tube, plugged at one end and filled with a suspension of polystyrene beads, was inserted into one end of the chamber. Some particles were allowed to fall out of the tube into the cavity during the application of ultrasound. Pressure measurements were made in the larger cavity during this process. The acoustic energy was adjusted to just suspend a single polystyrene bead. The ratio of the applied force to the gravitational force was computed for particles suspended at different positions in the tube and from the mouth of the tube but in the larger cavity.

The results shown in Figure 6 suggest that the tube indeed stores acoustic energy and that its "Q" (referenced to the large cavity) is approximately 3.5. The larger cavity does not appear to store significant energy. This experiment shows that during the generation of either standing or sweeping pressure surfaces forces may exceed those calculated from energy input to the system. For some systems this amplification may be six fold. We must be careful when estimating acoustic pressure amplitudes for the purpose of establishing limits to sound levels that cause cell damage. The experiment also lets us to determine the efficiency of a typical transducer in converting electrical to acoustic energy (56%).



= X/ = Y
X = Q FROM CALCULATION
Y = Q FROM HYDROPHONE MEASUREMENT

FIGURE 6

only 1 dB of attenuation.

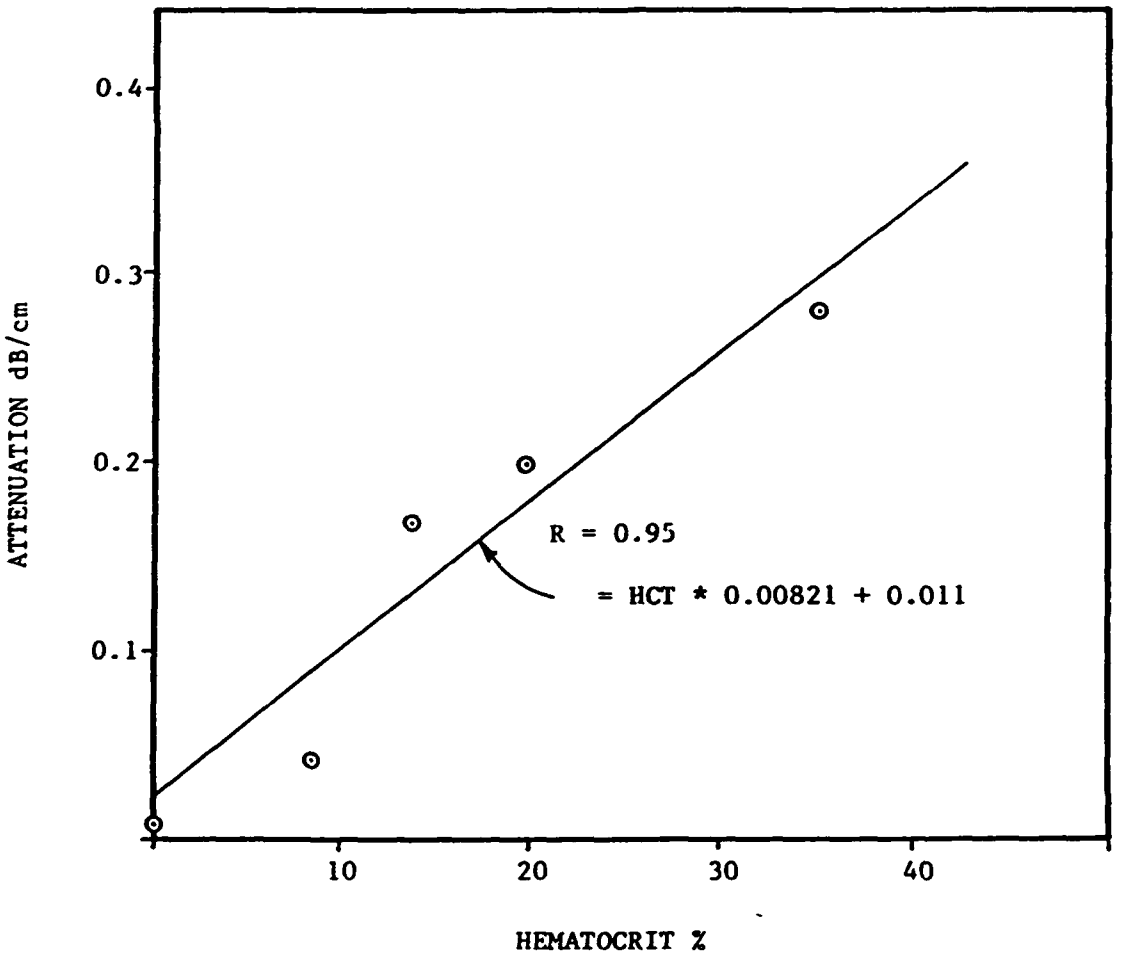


Figure 7

Could our inability to get effective separation in stacked systems be due to some surprising attenuation by banded blood? We attempted to simultaneously measure attenuation and band blood but were thwarted by swamping of the pulsed 2.25 MHz signal by the 2.5 MHz banding energy. The approach taken, though not the best, was the only compromise. We banded the blood and then turned off the standing wave field and immediately measured the attenuation. We

know that from other work the blood cells stay banded for a few seconds, hopefully long enough for us to make an approximate measurement. Measurements were made at different input power levels so that the density of the blood in the bands would hopefully vary. Figure 8 shows the result of these experiments.

From Figure 8 we can see that there is a significant decrease in attenuation at all applied power levels. The low correlation coefficient for the applied power curve suggests that attenuation and banding power are not correlated. The higher attenuation coefficient for the unbanded control blood suggests that scattering by the red cells is reduced by organizing them into more ordered bands. This explanation is consistent with scattering theory. The results, however, do not provide any explanation for our poor separation for serially stacked cells.

The remainder of the work on this project involved the construction and evaluation of a variety of acoustic separation cell configurations. The designs incorporated knowledge gained so far as well as art and craft. Our best cell consumed 4.8 ml/min of prediluted blood mixed with the final 0.9 percent wash saline. RBC and waste hematocrits of 52 percent and 2.7 percent respectively were obtained. Although this result demonstrates some scaling the final apparatus would require approximately 15 transducers to achieve a total input flow of 70 ml/min. This was deemed unacceptable for a competitive technique.

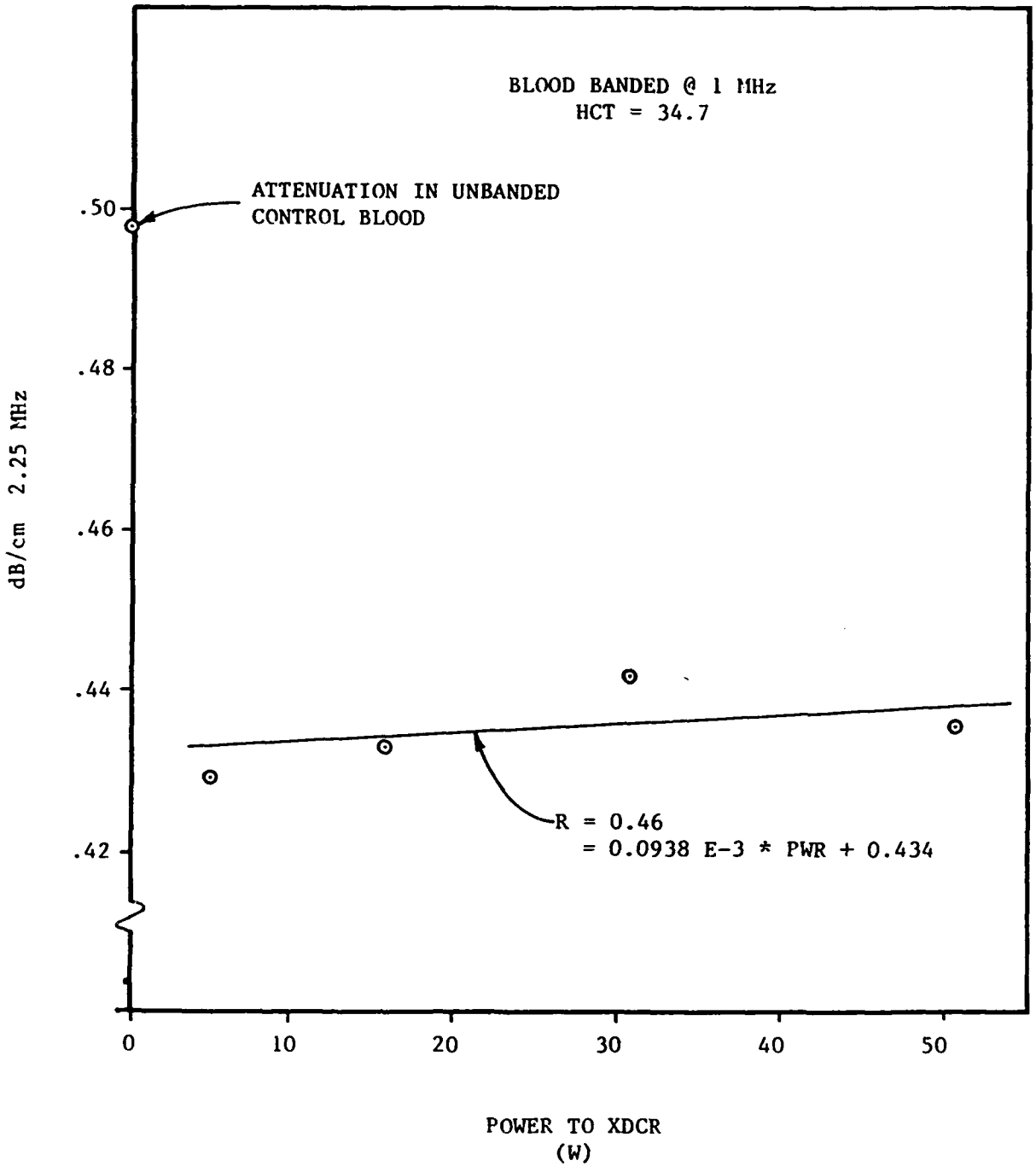
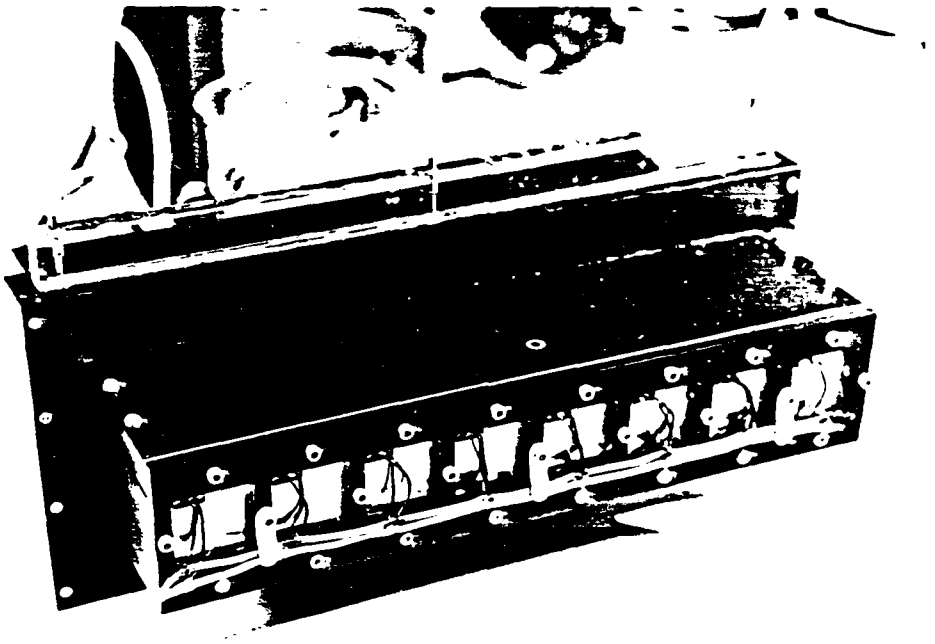

BLOOD BANDED @ 1 MHz
HCT = 34.7
ATTENUATION IN UNBANDED
CONTROL BLOOD
.50
.48
.46
.44
.42
dB/cm 2.25 MHz
R = 0.46
= 0.0938 E-3 * PWR + 0.434
0
10
20
30
40
50
POWER TO XDCR
(W)

FIGURE 8

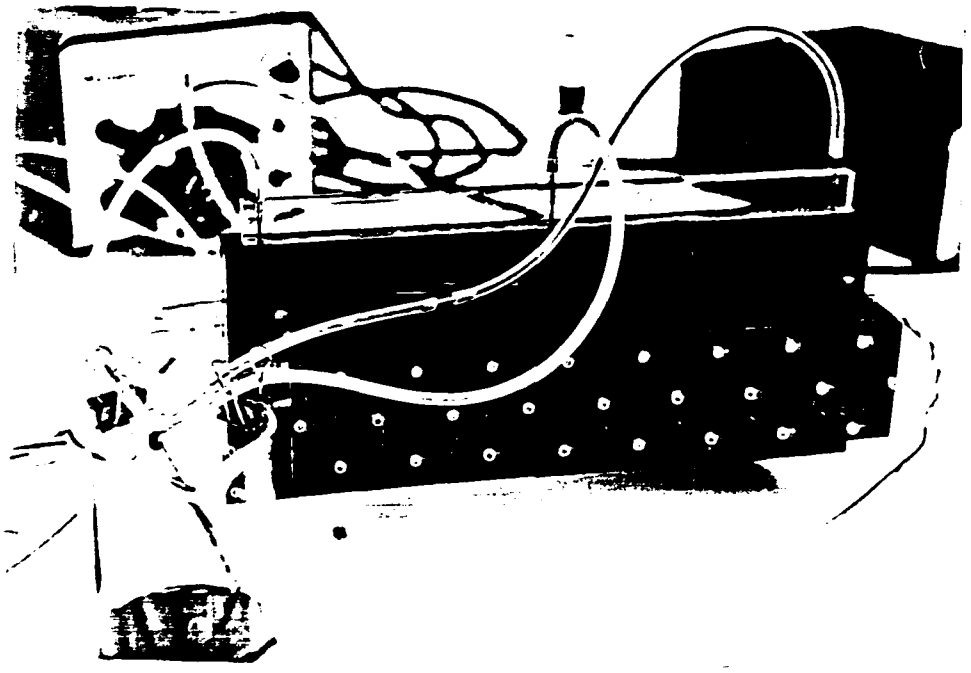
The various designs and characterization experiments are summarized in the following pages.

Photographs 1,2, and 3 show the salient features of an eight transducer attempt at scaling. Photos 1 and 2 illustrate the outside aspects of the separator and photograph 3 shows the first separation cell tried. In this scheme eight single cells were fed in parallel in order to achieve higher flow rates. Unfortunately it was virtually impossible to achieve uniform flow through the eight cells. The separation cells were of the standard up and over variety where blood flows up into the sound field whereupon it is banded and the bands are swept up and over to the other side of a partition. The cells fall down into a sump and the waste solution exits from the top of the cell. This is shown more clearly in Figure 9. Best flow achieved prior to the unit plugging was 9.25 ml/min or 1.156 ml/min per cell.

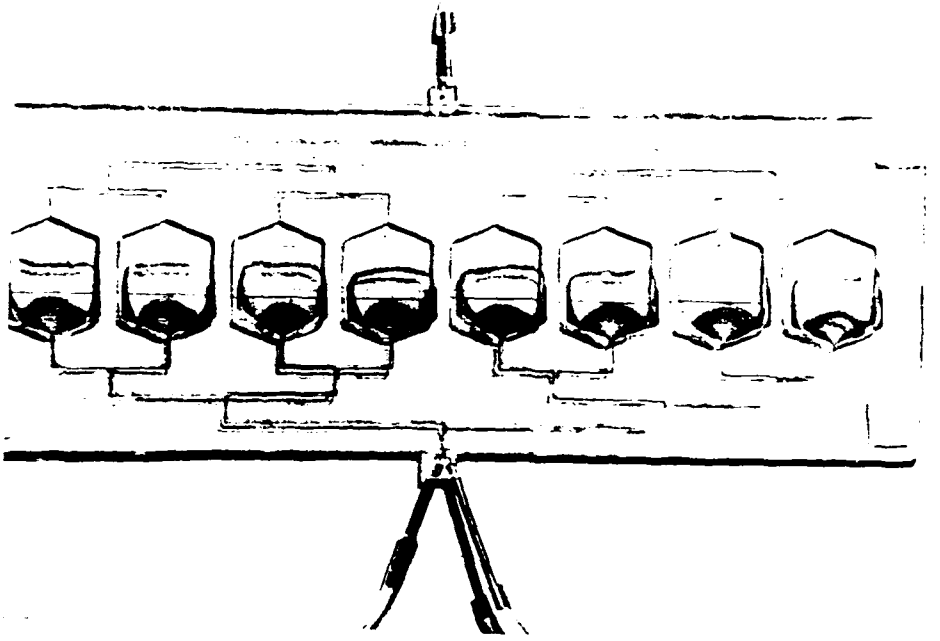
New cells were designed for this transducer assembly. Figure 10 schematically illustrates the next design. This cell is much like the first described but feeds and recovers with large collection manifolds rather than small, branching tubes. It worked no better than the unit that plugged. A maximum of 9 ml/min could be fed for the same concentrating ability (55% HCT). Figure 11 shows a completely open cell where settling alone is used to cause concentration of the red cells. Eleven ml/min flow (1.37 ml/min/unit cell) was achieved but with a loss in concentrative ability (26% from 22% feed).



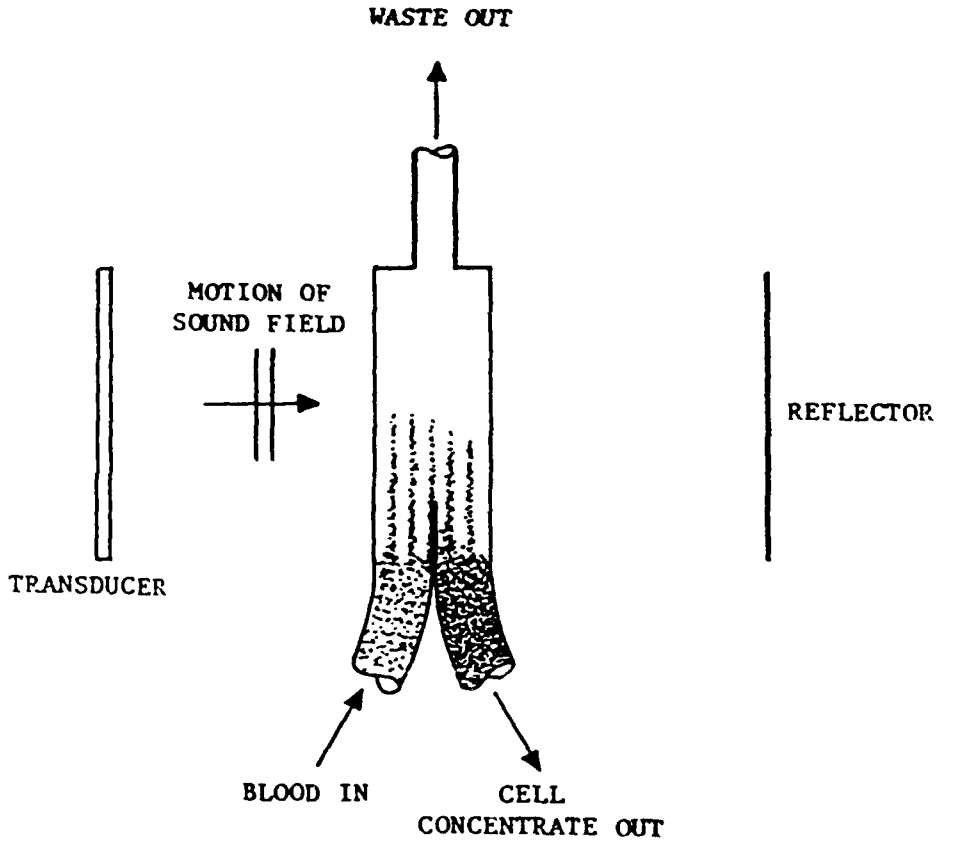
Photograph 1. Transducer side of peraration cell for 8 parallel unit cells. Plastic 8 unit up and over cells fit into fluid filled separator at top.



Photograph 2. Reflector side view of 8 unit separator. Small plastic windows are half wavelengh thick acrylic.



Photograph 3. Shows 8 up and over cells being tested for flow uniformity. A similar unit was used for actual test washing.



SIDE VIEW SEPARATION CELL (UP & OVER)

FIGURE 9

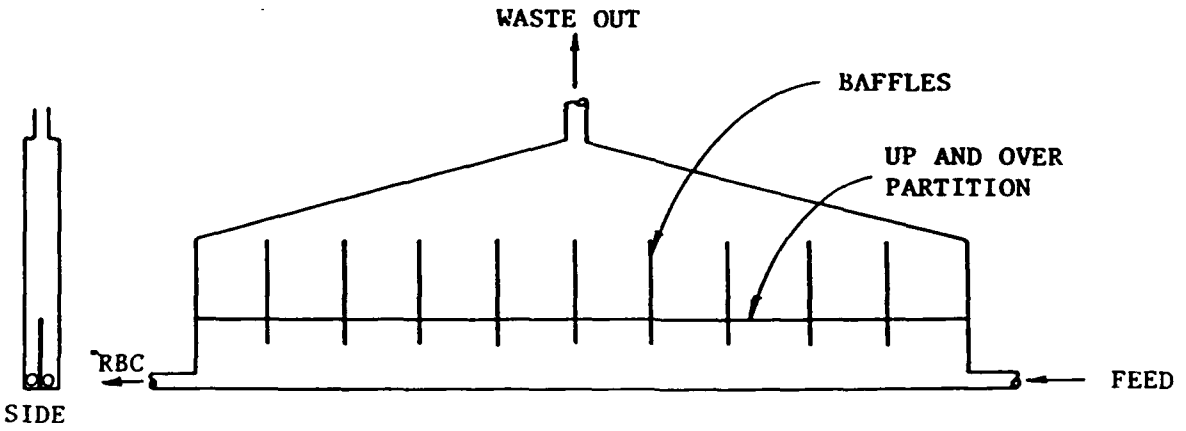


FIGURE 10

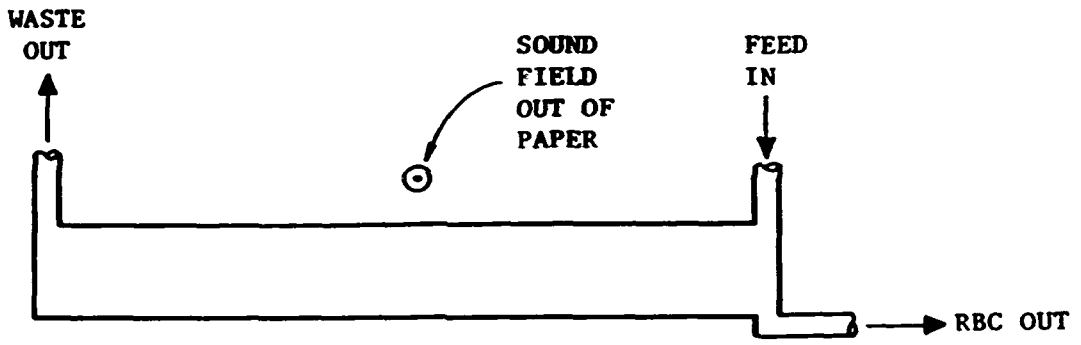


FIGURE 11

Several different approaches were studied using smaller single or double cells. These cells are about one square inch in area and are lower in cost to construct. Figure 12 shows schematically TRA's mixer being used to mix the diluted glycerolized blood with the 0.9% saline wash solution. This is then fed into an up and over

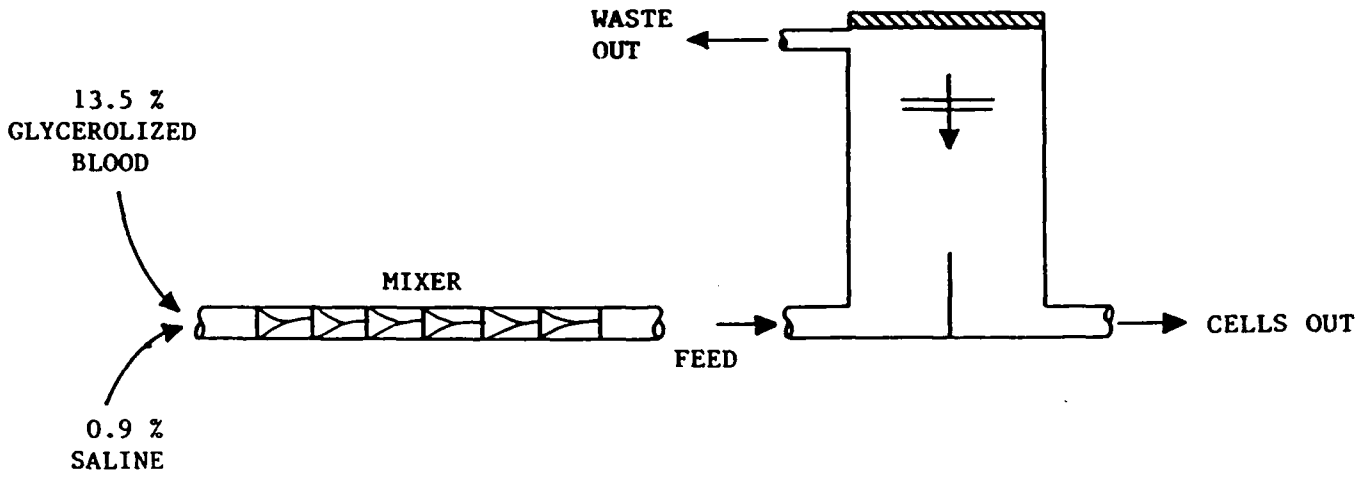


FIGURE 12

cell but with the sound field orthogonal to previous cells. Here the blood is forced over the partition and then driven to the bottom of the separation cell. This cell could concentrate cells from 13.5% feed to 27.4% at a flow of 4 ml/min, our most successful cell so far although the concentrative ability is still poor.

Figure 13 sketches still another design, this one incorporating Saran baffles. Saran is used because it is thin and absorbs little sound. In this device the glycerolized blood channeled in the relatively long blood space (4 cm) and bands were observed for only about 1 inch into the blood. We were unable to concentrate blood with this device. A similar cell without the baffles was constructed and it too failed to concentrate cells.

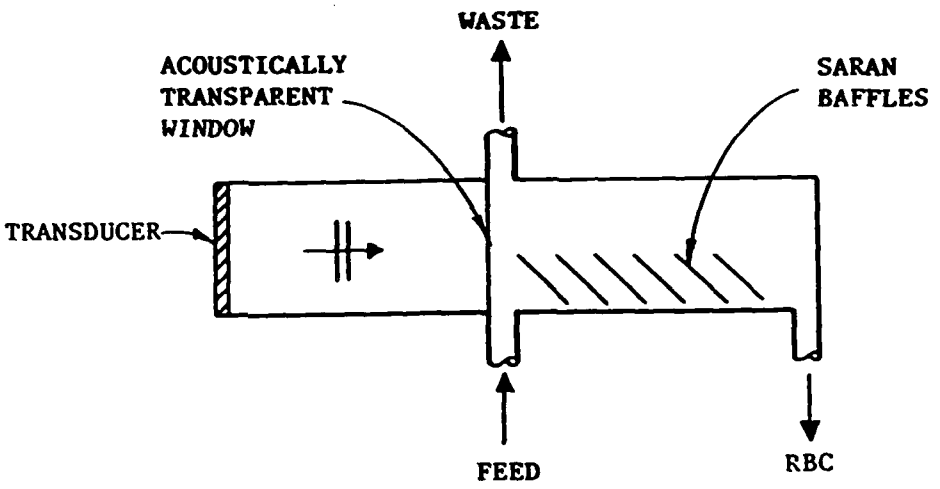


FIGURE 13

Figure 14 shows an attempt to scale by the use of series chambers. An 'A 'chamber was designed to allow flow of blood into the up and over cell and waste out of the cell, 'B' chambers were stacked and separated by Saran, and a 'C' chamber was used to carry away the concentrated cells. This unit ran at 3 ml/min but concentrated poorly and heat denaturation of blood protein (from lysed cells) was noted. Apparently heat built up in the center of the stack and could not be carried away.

Figure 15 shows two cells in flow series but acoustically in parallel. This cell was run both horizontally as shown and vertically. In neither configuration did it concentrate well.

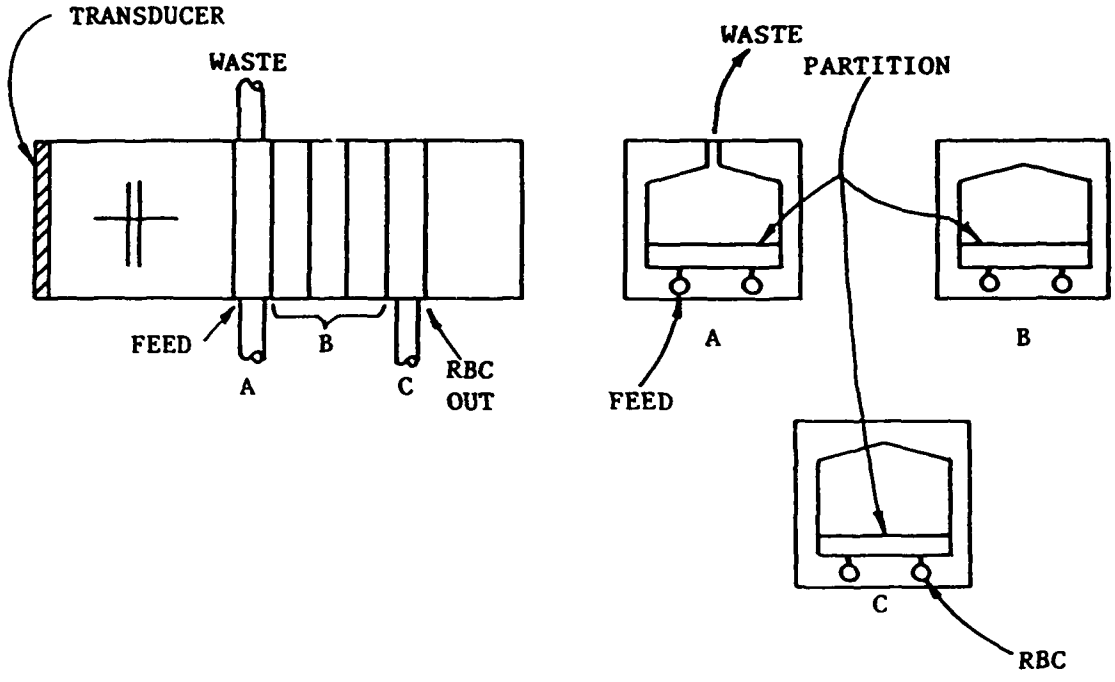


FIGURE 14

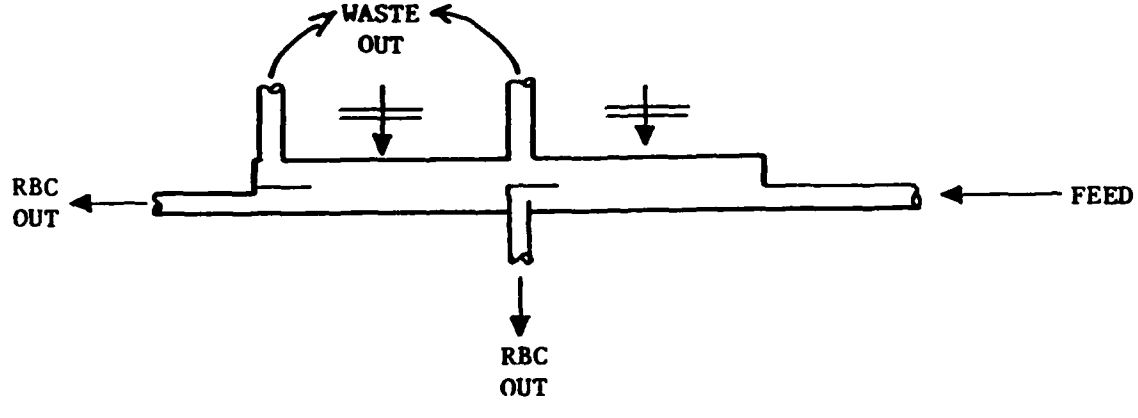


Figure 15

Figure 16 shows schematically a cylindrical "constricting" ultrasound cell concentrator. A transducer and associated cooling chamber are shown at the top end of a hollow cylinder. The other end of the cylinder is terminated in a 45 degree cone shaped reflector whose apex contains an exit port. Sound waves from the transducer are reflected twice from the 45 degree surfaces of the cone and superpose with sound from the "other side" of the transducer. Sound from that side of the transducer similarly superpose after reflection from the cone with sound directly from the first "side" of the transducer. The result of this superposition is that disk shaped nodal bands are formed in the cylindrical portion of the separation cell and these bands move towards the base of the cone. In the cone itself sound reflected from the 45 degree surfaces superpose forming cylindrical nodal annuli which constrict towards the cone axis.

Blood cells are trapped and moved by these nodal pressure surfaces towards the axis of the cone where upon they are removed from the separation cell. It was hoped that the concentrative ability of this cell design would allow us to highly concentrate the nodally trapped blood cells. A cell of the configuration shown in Figure 16 was built and used with very dilute blood (so that individual bands could be visualized) to verify the constrictive concentrative effect. Band formation and movement occurred as predicted. At this time a second cell was constructed by "rectangularizing" the cylindrical cell. This cell was constructed (Figure 17) using 2 square inches of transducer. Such a rectangular design could be

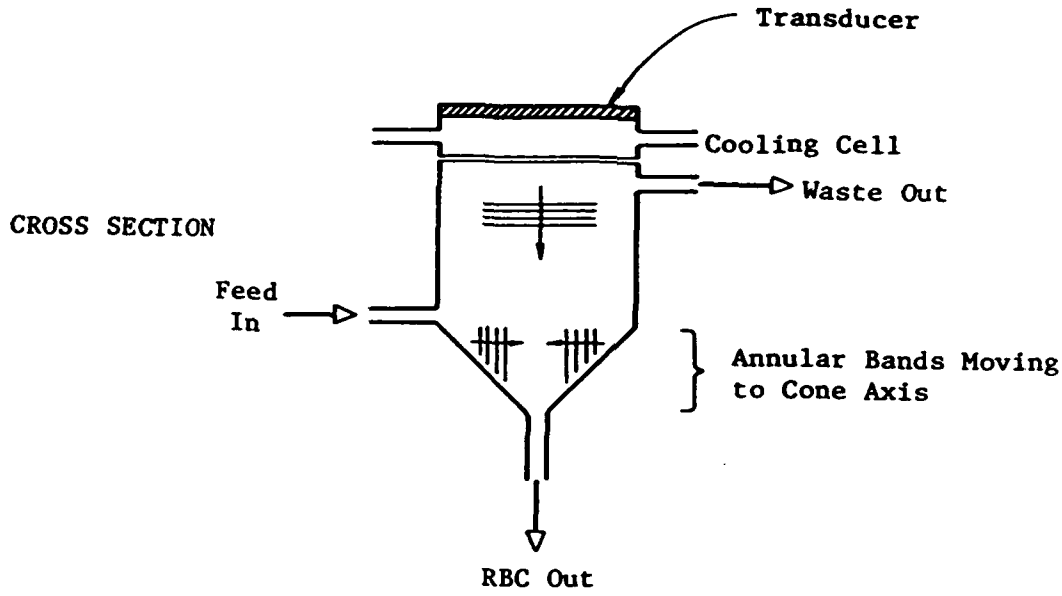


FIGURE 16

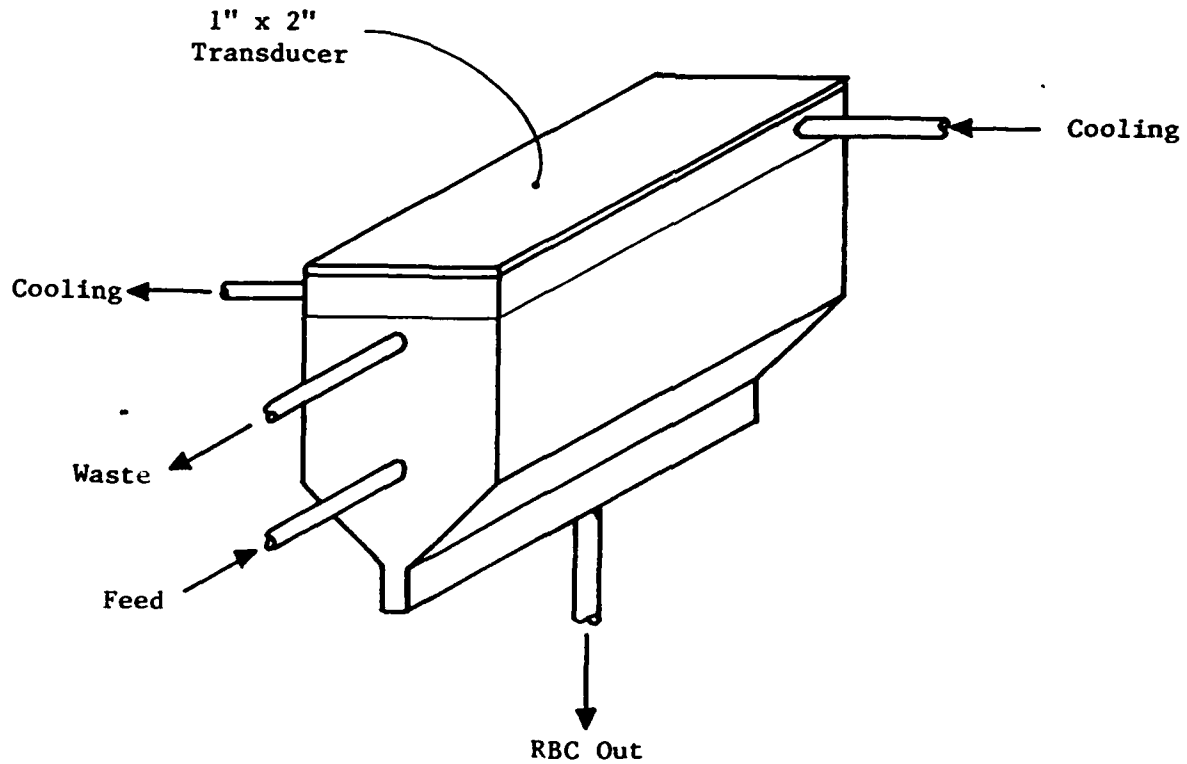
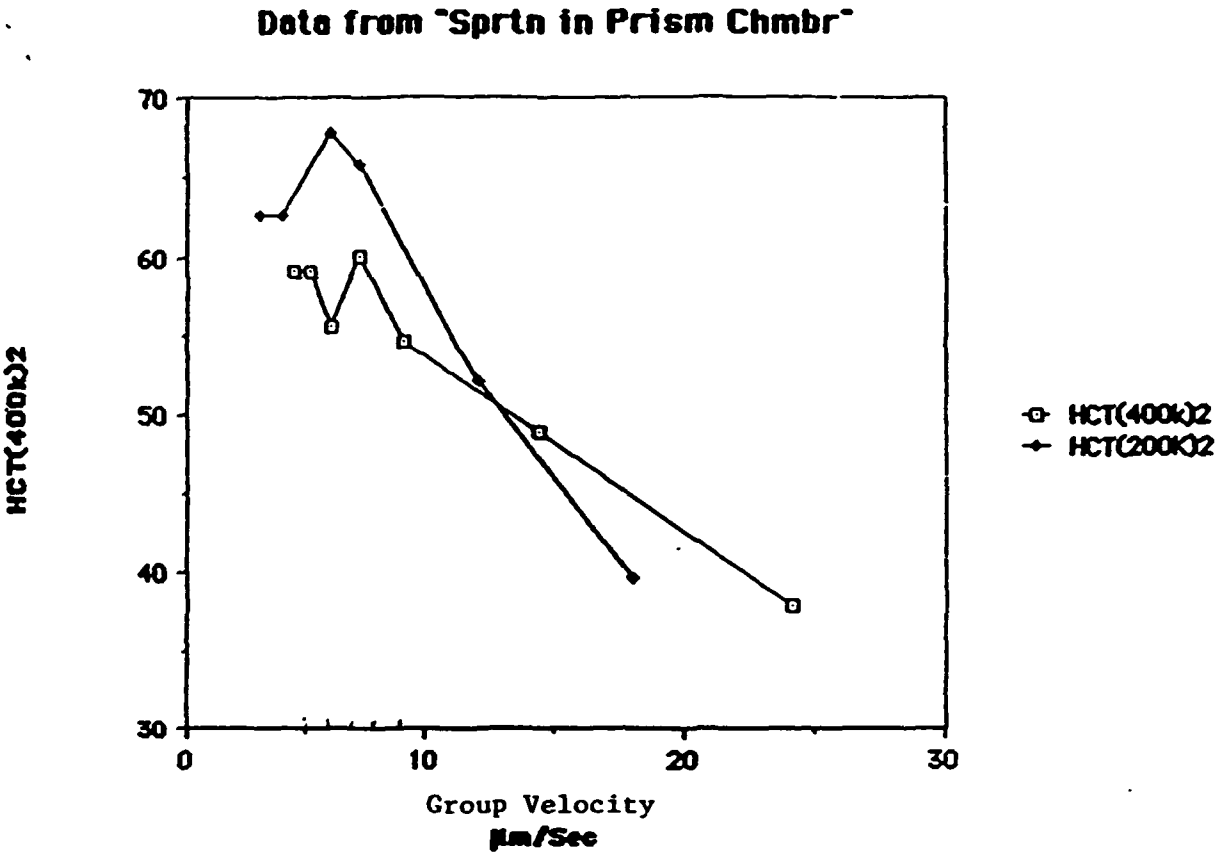


FIGURE 17

more efficiently scaled.

The rectangular or "prism" cell was tested with a feed of prediluted thawed blood. For a feed hematocrit of 25.4% and a feed flow rate of 1.5 ml per minute we were initially able to concentrate RBC's to only about 50%. A series of experiments were then done in order to determine the optimum sweep rate for a given acoustic power level. Figure 18 shows the results of these experiments. Two different sweep ranges were investigated, 200 and 400 kilohertz. Sweep times were varied to obtain different sweep rates. The feed hematocrit in this experiment was 30 percent. The hematocrit obtained for these different sweep rates is shown in Figure 18. Note that as the group velocity decreases the hematocrit rises to a peak value of 60 to 70 percent at a group velocity of 6 to 7 microns per second. Below 6 microns per second the hematocrit stabilizes at approximately peak value. These conclusions hold for either sweep range. What this experiment tells us is that we obtain maximum separator effectiveness at group velocities near the critical velocity. The critical velocity calculation (Table 2) uses parameter values from the experiment. A calculated critical velocity of 6.7 microns per second results. Although some parameter values are estimates, the theory and experimental values are close, suggesting that our understanding of the process is not too far astray. Note also that the cell was able to concentrate the blood from 30 to 65 percent.



V_g range = 6 7 μm/sec

V_g = Vertical = 6.7 μm/sec (see calculation Table A)

FIGURE 18

TABLE 2

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STANDING WAVE FORCE

PARTICLE DENSITY (kg/m3)= 1096
PARTICLE SoS (m/s) 1600
MEDIUM DENSITY(kg/m3) 1000
MEDIUM SoS (m/s) 1480
FREQUENCY (MHz)= 2.75
XDCR Z(OHMS)= 26
V(rms)= 27
XDCR DIAM(m)= .0405
ACOUSTIC PRESSURE SQUARED (Pa^2)= 16105936121.5
PARTICLE RADIUS= 2.7 um

FORCE FACTOR=-.309545819383
PARTICLE VOLUME= 8.2447957601E-17
WAVENUMBER= 11674.837564
STANDING WAVE FORCE (N)=-3.06722947311E-13
GRAVITATIONAL FORCE ON PARTICLE(N)= 7.7567038511E-14
VISCOUS DRAG COEFFICIENT= 4.52954828795E-8
SWF/GRAV F =-3.95429493247
CRITICAL VELOCITY (m/s)=-6.77160122406E-6

Standing waves were evaluated for their usefulness in separating RBC's from wash solution in another set of experiments. The eight transducer separator unit described earlier was used for the following experiments. A 2.54 X 20.32 X 0.4 cm acrylic separation cell was built to fit into the 8 transducer power unit. A standing wave (no nodal band movement) was used to attempt to "flocculate" the erythrocytes in an attempt to hasten their sedimentation. Four of the eight transducers were powered so as to provide a sump for the aggregated cells. At a feed flow rate of 8.8 ml/min (24.6% feed Hct) RBC's were concentrated to 38.4% with a waste Hct of 13.7%. At a lower flow rate (5.4 ml/min) RBC's concentrated to about the same (34%) but the waste crit was less (4.1%). When all 8 transducers were driven at the same unit power level, feed at 20% Hct and 10 ml/min was concentrated to 45% with a waste crit of 7%.

A thicker (one cm) cell was also built and tested. Fifteen ml/min of 27% feed was concentrated to 40% with a waste crit of 1.3% with power to all 8 transducers. This experiment allowed a flow rate of almost two ml/min/square inch of transducer but only with only a 47% increase in concentration.

Another set of experiments was carried out to evaluate the effect of alternately turning on and off the standing wave field. This was not as effective as the previous technique, producing 46.5% RBC concentrate from 23.3% feed at a flow rate of 4 ml/min. With 2.9 ml/min we were able to concentrate to 65.7% but with a 5.7% waste crit. In summary slow feed flow rates allow higher

concentration of cells in the output. When we achieve adequate concentrative ability we sacrifice speed.

Some additional experiments were done with 5 small (one inch square, four mm thick) up and over cells in series acoustically (shown in Figure 19). Blood coagulation occurred probably because of the lack of cooling of the inner cells. The acrylic walls used to separate the individual separation cells absorb energy and their temperature would increase without cooling. The cells were cleaned and another experiment run at lower power. The best result was 2.2 ml/min feed at 28% Hct concentrated to 64% with 10% crit in the waste line. A similar single cell (see Figure 20) was built with internal baffles but it too produced equally poor results.

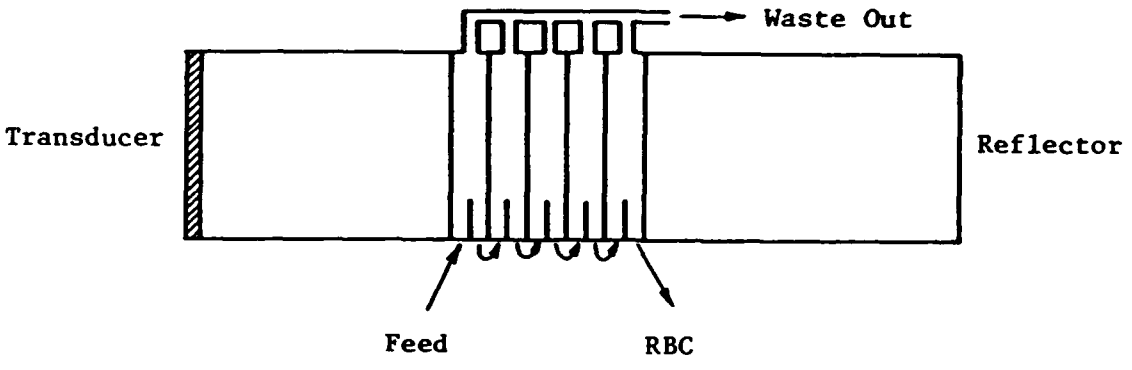


FIGURE 19

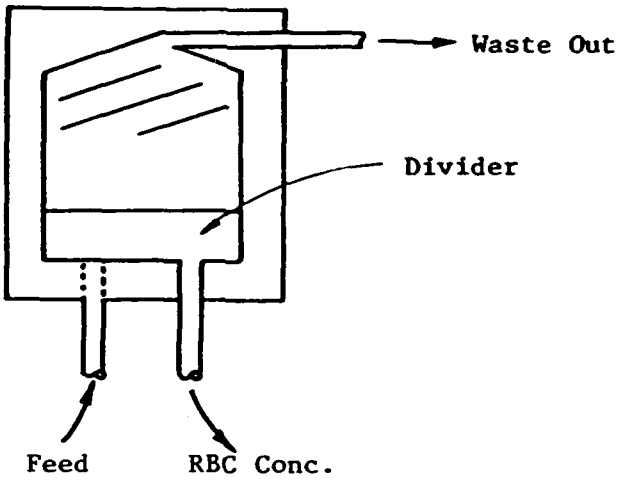
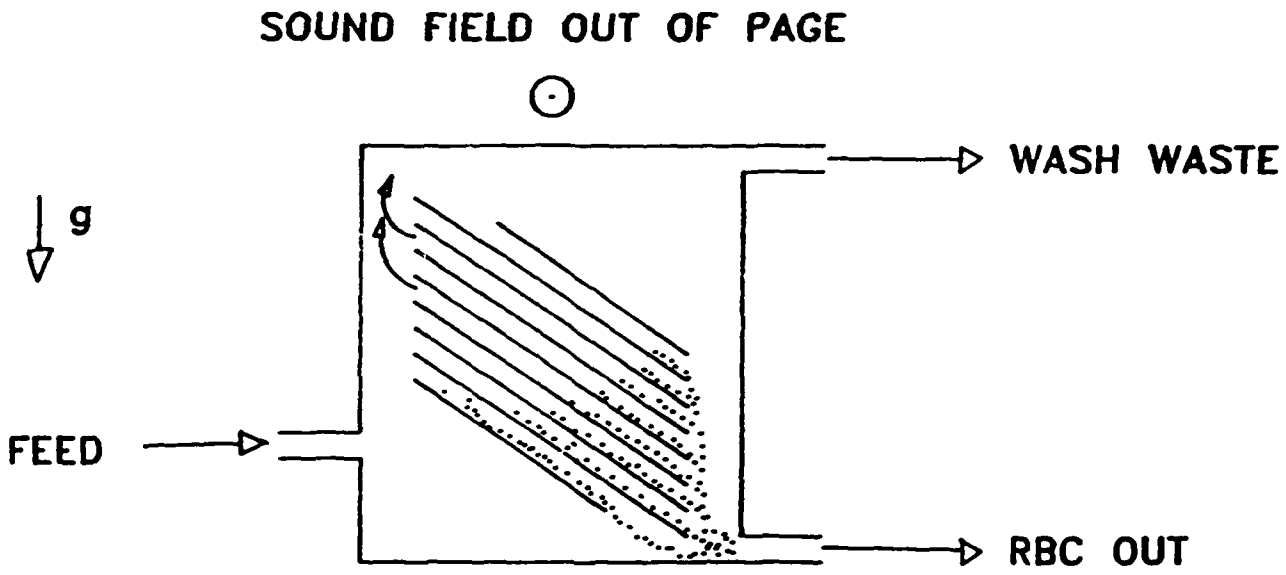


FIGURE 20

Two chamber designs were evaluated for washing glycerolized, thawed blood. The first design was basically a separation cell of one cubic inch volume containing six baffles (Figure 21). The purpose of the baffles was to provide inclined plates to hasten the sedimentation of the ultrasonically aggregated erythrocytes. Cells would settle on the top surface of the baffle plate and fall away from the downward facing surface of the plate forming a cell free channel for the displaced washing solution to rise. This channel hastens settling of the cells by preventing mixing of the wash solution and glycerol with the sedimenting cells.

Pre diluted glycerolized blood was mixed with wash saline to hematocrit 8.3 percent. For a feed of 1.43 ml/min cells were concentrated to an hematocrit of 75 while the waste contained cells at hematocrit of 0.13 percent. Osmolality of 1126 mOsm, was measured in the washed cells. This corresponds to a glycerol concentration of about 3 percent. At a somewhat higher feed flow rate (2.0 ml/min) RBC and waste hematocrits were 54% and 1%

respectively.

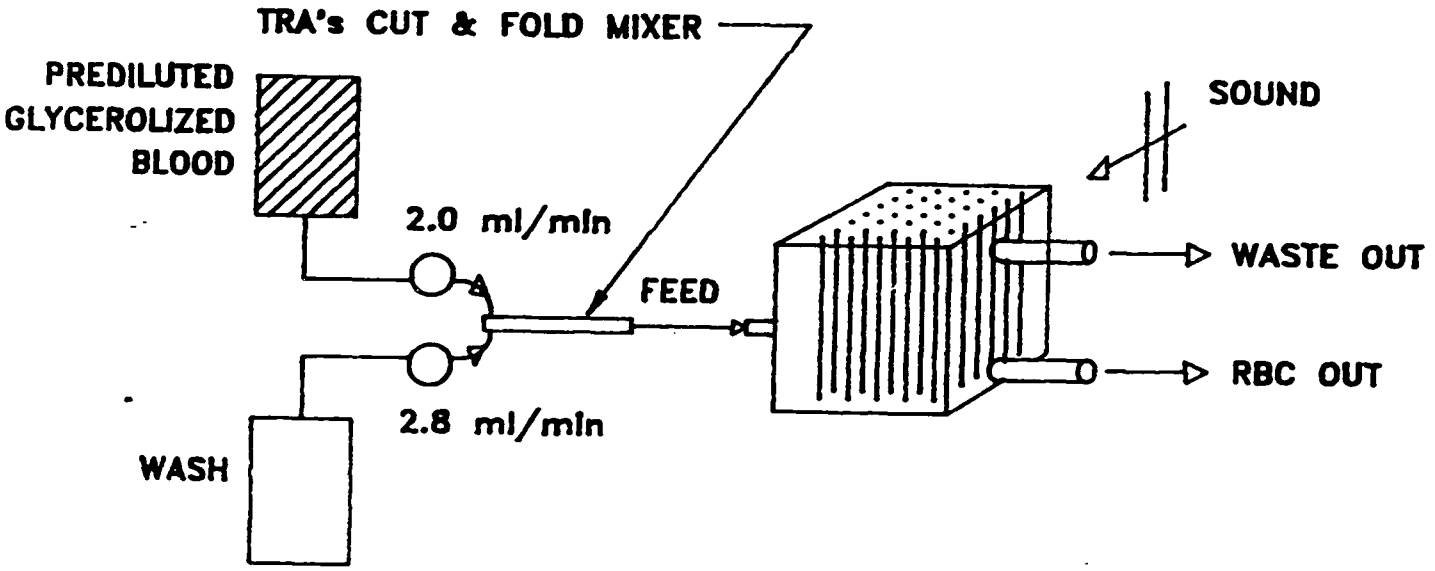


SECTION OF SEPARATION CELL

FIGURE 21

A second separation cell again about one cubic inch in volume was filled with 10 layers of monofilament line spaced 1/24 th inch apart and in 10 layers about 1/10 th inch apart (Figure 22). We had a theory (derived from other work in progress) that cell sedimentation occurs more rapidly when vertical surfaces are present in the sedimentation cell. The monofilament would thus increase the surface area without markedly disturbing the 2.7 MHz sound field. Usual sound intensity of about 1.5 watts per square centimeter was used. Our best flow rate of 4.8 ml/min was achieved at prediluted glycerolized blood flow of 2 ml/min and wash saline flow of 2.8 ml/min. RBC and wash waste hematocrits of 52% and 2.7%

respectively were obtained. washed cell glycerol was about 3% (1206 mOsm).



STRING CELL

FIGURE 22

Both of the above experiments show that scaling to 70 ml/min is not quite possible, although the latter experiment comes close if 14 transducers and cells were used. Note though that the glycerol still needs to be reduced to 1 percent or less.

Conclusions and Recommendations

The primary conclusion that can be drawn from this work is that at the present stage of development TRA's ultrasound technology is not capable of competing with current cell washing techniques. The work did increase our understanding of the physics of ultrasound separation and points toward applications that combine ultrasound with other washing systems.

In the early part of this work experiments were carried out that gave us insight into the mechanism that introduced losses into our system. Glycerol absorbs ultrasound in excess of the absorption by water alone. At concentrations that would be found in thawed glycerolized blood being mixed with the final wash saline, losses are about 1.5 times larger than if the glycerol was not present.

Similarly, the radiation pressure that we can exert on glycerolized red cells in the washing process are about one third that could be exerted on non-glycerolized cells. This is because the glycerine alters both the speed of sound and the density of both the cells and the suspending medium. That is their acoustic properties become more alike and the force that can be exerted on the cells is less.

Additionally, the glycerine increases the viscosity of the medium through which the cells must be moved. Equation (1) in [1] in the appendix, shows that the critical or limiting velocity is inversely proportional to the kinematic viscosity. As the viscosity is

increased by the glycerine, it takes either more force (higher acoustic pressures) or higher frequencies to move the cells at the same velocity. If these factors do not increase then the separation must occur more slowly.

All of these factors, increased viscous drag, lower radiation pressure due to more similar acoustic properties and reduction in acoustic pressure by absorption of sound combine to reduce the effectiveness of this technology to compete with current techniques.

During this work a "cut and fold" mixer was developed and tested. This mixer was a component in the proposed ultrasound separator/cell washer. The mixer allows for a more automated mixing scheme than is currently in use. Data shows that the mixer works at least as well as the current shaker mixers. Should further research produce a more automated washer this mixer could be incorporated into the apparatus. Only minor further development is needed.

During the bulk of this work TRA designed, built and tested numerous separation cells in an effort to scale the washing process. At best we were able to process about 2.8 ml of feed per minute. In a washing procedure a total of about 2000 ml would need to be processed. For a single separation cell this would take about 12 hours to process. Even with 12 separation cells in parallel one hour would be required. From other experience TRA has had with

manifolding flow into multiple cells, and from energy input considerations such a cell washing apparatus would consume too much energy, and be difficult to maintain in operating condition.

One of the difficulties that has become apparent in this work is the harvesting of cells from the bands. With our technique it is easy to force the red cells into bands at the node. Even with glycerol present at 20 wt. percent cells band in about 2 seconds and the band is consolidated in less than 40 seconds (see Table 4 first quarterly report). It is alsc relatively easy to move the bands using the pseudostanding wave technique. The primary difficulty occurs when we try to harvest the cells from the band which has been moved to the cell exit port or region. Discussion of this problem may give insight into the inherent limitations of the sweeping wave technique.

Radiation pressure seems to hold the cell in the nodal region by "squeezing", much like holding a piece of paper between thumb and index finger. Contrary to what we originally thought, there seem to by no lateral forces within the band caused by the radiation pressure. Only gravitational force or fluid forces caused by acoustic streaming seem to operate within the plane of the band. What is needed is another force that would allow one to "milk" the cells out of the translocated band into the collection region or sump. Gravity works but not very well since it is a weak force relative to the radiation pressure force. If we reduce the radiation pressure to equal that of gravity the cells fall but, as

they fall, remix with the suspending fluid.

To provide a "milking" force one might propose a second orthogonal sweeping force. This orthogonal force would squeeze the cells down (with gravity) into a collection sump. TRA has tried such a technique (in other research work) and it does not materially enhance separation. The reason seems to be that the cells in the sump are also banded and hence are necessarily diluted by antinodal fluid bands. That is one can not have a band of cells without a band of fluid. This necessary band of fluid seems to prevent concentration of cells greater than 60 to 70 percent. In the originally proposed cell washing scheme the 30 to 40 volume percent of fluid would contain waste glycerine which would subsequently have to be removed thus reducing washing efficiency.

In other work TRA is trying to combine ultrasound banding with other non-acoustic forces including centrifugation. The plan here is to band the cells, effecting a local separation of cell and suspending fluid. Then using centrifugal force sediment the band in its nodal plane, laterally into a sump. In this scheme the sound field is excluded from the sump and the clustered cells in the nodal band (because they form a larger effective mass) fall more easily into the sump. This scheme could be adapted to a continuous flow centrifuge head. TRA with other research funds, is developing such a head. Additionally, TRA has designed and patented a dual axis centrifuge that has no unsterile rotating seal. This device coupled with a continuous flow, low cost disposable head could

provide sterilely washed red cells.

TRA feels that there are several approaches the Army could take to enhance the washing of thawed red cells. One approach that TRA looked at (in internally funded work) was the use of hollow fiber dialysis membrane. In preliminary experiments glycerine was easily removed by a counter current washing solution. TRA also found that cells could be concentrated by increasing the hydrostatic pressure on the cell side of the fiber. A special large pore membrane (available in Japan) should make a workable system. This system could be made to work without pumps or other power consuming apparatus.

Another approach was the one discussed above; that is the combination of ultrasound and gravity. A third approach, which TRA is considering, is the combination of ultrasound with Millipore's membrane filtering system. That system, as discussed in the introduction, has the drawback that the red cells tend to plug the membrane pores. It may be possible to use the sweeping ultrasound to move the cells away from the pore and keep them in the flow stream thus increasing the life of the membrane and making control of the flow rate and pressure gradient less critical.

References

[1] Peterson, S.C. et al., p 154-156, Proceedings of the Eighth Annual Conference of the IEEE/Engineering in Medicine and Biology Society, 1986

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[3] Gross, S.A. et al., J. Acoust. Soc. Amer. 64(2), 1978

APPENDIX

DEVELOPMENT OF AN ULTRASONIC
BLOOD CELL SEPARATOR

Reprinted from PROCEEDINGS OF THE EIGHTH ANNUAL CONFERENCE OF THE IEEE ENGINEERING IN MEDICINE AND BIOLOGY SOCIETY, Fort Worth, Texas, November 7-10, 1986

DEVELOPMENT OF AN ULTRASONIC BLOOD CELL SEPARATOR

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Abstract

A new technique utilizing the superposition of two oppositely traveling acoustic pressure waves produces pressure maxima and minima which move in space. Particles, whose acoustic properties differ from those of the suspending medium, are forced by the local pressure gradient into regions of high or low pressure depending on their compressibility relative to that of the suspending fluid. Since these regions of high or low pressure are moving in space, they may be utilized to transport the particles from one side of a chamber to the other. This phenomena is used as the basis for the design and construction of a continuous flow plasma/erythrocyte separator. One unique feature of the separation technique is that particles of the same density but of different size can be easily separated from each other.

Introduction

It has been known for some time that particles, whose acoustic properties differ from those of the medium in which they are suspended, can be forced into pressure nodes or antinodes formed by an acoustic standing wave [1,2]. During experiments designed to utilize this phenomena to separate the formed elements of blood from plasma a new approach was devised to effect particle movement relative to the supporting fluid. This new technique produces pressure surfaces, analogous to nodes and antinodes, which move in space and time. These pressure surfaces, which we call a pseudo-standing wave, are, in the first-order approximation, formed by the superposition of two oppositely traveling acoustic waves of slightly different frequency.

Background

The force on a compressible particle caused by a standing acoustic pressure wave has been worked out by several investigators (See [3] for a succinct review.) and is given by:

$$F = \frac{VP^2 k \sin(2kx)}{4\rho_o c_o^2}\left[\frac{1}{\delta\sigma^2} - \left(\frac{5\delta-2}{2\delta+1}\right)\right]$$

where V =particle volume, P=acoustic pressure, k= wavenumber, $\delta = \rho/\rho_o$, $\sigma = c/c_o$, ρ =particle density, ρ_o = medium density, c=speed of sound in particle and c_o =speed of sound in medium. Note that the particle must be small with respect to one half the wavelength and that the force is proportional to the acoustic pressure squared, the volume of the particle and the wavenumber. Some of these parameter values are fixed because of other constraints. For example the wavenumber is largely determined by the power level required to band the cells while avoiding cavitation. The practical lower frequency limit is thus about 1 MHz, below which cavitation becomes a problem at the power levels required for cell separation. Scattering losses become large as the frequency exceeds an upper limit of about 5 MHz for suspensions of blood cells.

In a system of moving particles other forces come into play. For very dilute suspensions of particles, or for particles tightly packed, Bjerknes and Bernoulli forces are significantly smaller than the pseudo-standing wave force, (approximated by the standing wave force), and Stokes' drag force [4]. Due to Stokes' viscous drag force there will be a critical velocity at which the pseudo-standing wave force will no longer be able to hold the particle. This velocity, estimated by equating the standing wave force and the Stokes' drag force, is

$$v = \frac{r^2 P^2 k \sin[(k_1+k_2)\bar{x}]}{18\rho_o c_o^2 \eta}\left[\frac{1}{\delta\sigma^2} - \left(\frac{5\delta-2}{2\delta+1}\right)\right] \quad (1)$$

where η is viscosity of the fluid, and r = particle radius. Note that we have written the particle volume as $4\pi r^3/3$ and that the force estimate should be approximately valid for low particle velocities. This relation suggests that for particles with identical densities but of different radii in dilute

solutions there will be different critical velocities. This velocity difference allows the separation of particles, such as the class of white blood cells, on the basis of size rather than the slight density differences. (See the section on applications.)

Generation of pseudo-standing wave

If two waves, Yi, of slightly different frequency traveling in opposite direction superimpose (first-order approximation) we have,

$$Y_1 + Y_2 = a\cos(\omega t_1 - k_1 x) + a\cos(\omega_2 t + k_2 x)$$

Using the trigonometric identity

$$\cos\alpha + \cos\beta = 2\cos\tfrac{1}{2}(\alpha+\beta)\cos\tfrac{1}{2}(\alpha-\beta)$$

and rearranging we have

$$Y_1 + Y_2 = 2a\cos\tfrac{1}{2}[(\omega_1-\omega_2)t - (k_1+k_2)x]\cos\tfrac{1}{2}[(\omega_1+\omega_2)t - (k_1-k_2)x]$$

Note that the first term represents the envelope of the beat frequency wave formed and the second term is the carrier. The group velocity of the beat pressure wave is in general

$$v_g = \frac{\omega_1 - \omega_2}{k_1 + k_2} = \frac{\omega_1 - \omega_2}{\omega_1 + \omega_2} c \qquad (2)$$

This is the velocity at which the pressure surfaces move through the fluid.

There are several ways to generate oppositely traveling acoustic waves. The most obvious is to excite two opposed piezoelectric transducers at different frequencies. There are several waves set up in the intervening fluid space. Each transducer will generate a traveling wave that will reflect from the other transducer face, due to the impedance difference between the transducer and the propagation medium. This reflected wave will superimpose with the incident wave and generate a true standing wave. Two such standing waves will be generated because of the different excitation frequencies. In addition a pseudo-standing or beat wave will be formed as indicated above.

In order to utilize the moving pressure surfaces formed by the beat wave it is necessary to minimize the true standing waves by impedance matching the fluid to the transducer and on the other side of the transducer impedance match the transducer to an acoustic absorber. Finding the appropriate materials out of which to construct the required quarter wave matching section is difficult as is machining the matching section to the correct quarter wave thickness. Nonetheless the apparatus was built and indeed worked. The velocity of the pseudo-standing pressure surfaces is given by equation 2.

A second way to generate oppositely moving acoustic waves of different frequency is to excite a single transducer with a sweeping frequency input and reflecting the traveling wave from a fixed reflector. Due to the finite travel velocity of the sound wave there will be a frequency difference between the incident and reflected waves everywhere in the propagation medium except at the reflector. The velocity of the pressure surfaces thus formed will be

$$v_g = -\frac{Rxc}{c\omega_0 - Rx}$$

where R= ramp rate in radians/sec**2, x= distance from reflector, and ω_0=radian frequency of carrier referenced at x.

A third approach may be used to generate oppositely traveling waves of different frequency. The transducer is excited by a fixed frequency and the traveling wave is reflected from a moving reflector. The reflector, moving at velocity u referenced to x "sees" a wave whose frequency is Doppler shifted by

$$\omega_i = \frac{\omega_0}{c}(c+u)$$

and as a moving source reflects a wave that is additionally Doppler shifted by

$$\omega_r = \omega_i c/(c-u)$$

thus the group velocity is

$$v_g = \frac{\omega_0 - \omega_i}{\omega_0 + \omega_i} \cdot c = u$$

Frequency differences of only a few Hz are required to generate pressure surface velocities of the order of millimeters per second.

The latter two methods of beat wave generation present a changing acoustic load to the transducer that results in impedance bumping as the power amplifier drives the transducer. These bumps reflect the fact that the cavity changes from resonance to anti-resonance during the frequency sweep or as the physical length of the cavity changes. For an actual system operating at several MHz these fluctuations occur every few thousand Hz.

A fourth approach to producing moving pressure surfaces combines both a frequency sweep with a moving reflector. Here a control system adjusts the excitation frequency to maintain a resonant condition as the cavity length changes due to reflector movement. The pressure surfaces formed change their spacing as the frequency changes. Particles forced into the pressure surfaces move with the spacing change and thus move relative to the fixed transducer.

Particle separation from the medium is achieved by placing a flow cell in the

acoustic propagation medium. As particles and fluid flow into the separation cell the particles are forced into the moving pressure surfaces. Fluid flow will carry the particles across the cell as the moving pressure surfaces transports them from one side of the cell to the other where they are separated from the clarified fluid by a physical splitter just prior to exiting the separation cell, see Figure 1.

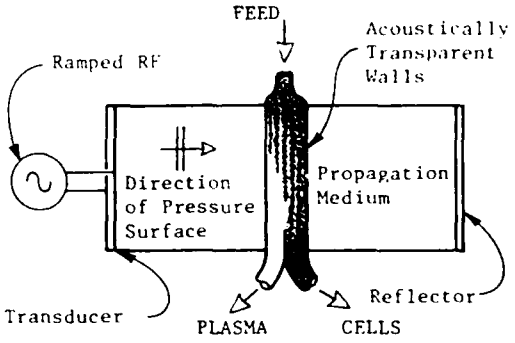

Figure 1

Blood separator design

The most effective design for the blood cell separator appears to be an up and over approach shown in Figure 2.

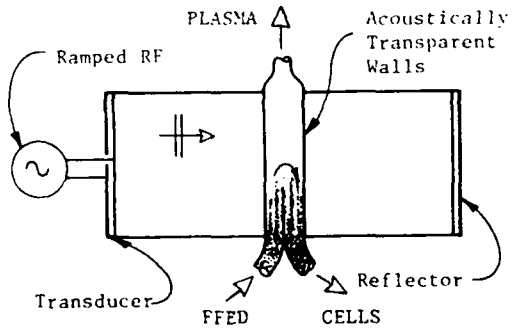

Figure 2

Here whole blood enters the separation cell at the bottom and flows up where upon it is forced into bands by the beat wave. Typical forces exerted on the cells are about 100 gravities, causing the blood cells to pack into relatively thin bands which are transported by the moving pressure surfaces to the other side of the chamber where upon the highly concentrated cells are pumped down and out of the cell. The clarified plasma flows up through the separation cell and exits at the top. This scheme works better than the approach suggested in figure 1 because there is a stronger field in the center of the chamber than he edges due to pressure losses through the propagation chamber walls. This pressure gradient within each pressure surface forces the cells to the region of highest pressure gradient, an additional force that can be utilized to increase separation efficiency. This intra surface force can be enhanced by applying different quantities of excitation power to a bank of phased transducers. In addition, the pressure surface seems to compact the individual cells into aggregates. This effectively increases the particle volume and thus a larger net force is exerted on the aggregate.

At date of writing, a single separation cell with a flow path of 7.5 cm length driven at an average power of 2 W/cm**2 has been able to separate a feed hematocrit of 40% into packed cells of 80% and plasma containing 1.5% cells at a feed flowrate of 2 ml/min. Serial and parallel combinations of such cells will comprise the final separator design tailored to a particular need.

Applications

There are several applications of the ultrasonic particle separator. These include a rapid method for determining packed cell volume by measuring the ratio of packed cell bandwidth to clear plasma as the cells are swept past an optical sensor. The separator can be modified to wash "on line" deglycerolized thawed blood cells. Blood and other structures can be moved noninvasively within the eye using this technique. Finally, the different classes of white blood cells, whose densities are very similar may be separated according to their cross section since their critical velocities differ greatly. Critical velocities for three types of cells calculated from equation 1 are shown in Table 1.

Table 1

Particle/plasma	Critical velocity (micrometers/sec)
platelet	12.5
RBC	121
neutrophil	1250

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This work was supported, in part, by an SBIR grant, R44-HL31890, from the National Heart,Lung and Blood Institute