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FINAL REPORT

on

CANDIDATE SPACE PROCESSING TECHNIQUES FOR
BIOMATERIALS OTHER THAN PREPARATIVE ELECTROPHORESIS

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A number of areas of research were examined with a view to determining, in a preliminary way, possible applications in Space Processing. Experts in four subjects of interest were identified and the following topics explored: partition and countercurrent distribution (CCD) of cells in phase separated aqueous polymer systems, freezing front separation of cells, adsorption of cells at the air-water interface, and the macrophage electrophoretic mobility test for cancer. Of these four, the only process which appeared to be of interest to the Space Processing Program was CCD of cells in two phase polymer systems. A theoretical analysis of CCD in zero g was carried out. There would seem to be three advantages to performing CCD in a low gravity environment: Cells of biomedical interest which are too large (diameter $>24\ \mu$) to partition in 1 g because of their high sedimentation rate may be readily fractionated. Secondly, large cells (diameter $15\mu - 24\mu$) which can be distributed via CCD with low resolution on earth due to partial sedimentation may be separated with higher resolution. Thirdly, if zero g will allow electric field-driven phase separation to be carried out in the absence of convective mixing, calculations indicate that significantly more rapid phase separation should occur. More transfers could thus be made per unit time, resulting in higher resolution for all cell types. It is therefore recommended that further effort be expended to test and develop the applicability of CCD in reduced gravity.

2 SCOPE OF WORK

2.1 Objectives

To make preliminary feasibility studies of the following five candidates for space processing of biological materials, emphasizing immediate elimination of those techniques showing minimum promise for specific returns in the field of zero g biomaterials processing:

- (1) Partition and counter current distribution of cells in phase separated aqueous polymer systems.
- (2) Growth of monolayer cell cultures on beads in suspension to allow higher cell densities per unit volume to be maintained and thus to increase the efficiency of production of commercially valuable metabolic products (enzymes, hormones. etc.).
- (3) Separation of cells by partition at a moving solid/liquid phase boundary (analagous to zone refining).
- (4) Separation of cells by preferential adsorption at the air/water interface (analagous to froth flotation).
- (5) Carrying out the macrophage electrophoretic mobility diagnostic procedure for early detection of malignant disease in space to avoid problems associated with sedimentation of the very large macrophages.

2.2 Approach

The outlined studies are to be divided into two separate, related tasks, portions of which can be pursued concurrently.

Task 1: Through consultation with acknowledged experts in the relevant fields, preliminary feasibility studies will be made on the following candidates for space processing of biological materials:

- (i) Partition and counter current distribution of cells in phase separated aqueous polymer systems.
- (ii) Growth of monolayer cell cultures on beads in suspension to allow higher cell densities per unit volume to be maintained and thus to increase the efficiency of production of commercially valuable metabolic products (enzymes, hormones, etc.).
- (iii) Separation of cells by partition at a moving solid/liquid phase boundary (analagous to zone refining).
- (iv) Separation of cells by preferential adsorption at the air/water interface (analagous to froth flotation).
- (v) Carrying out the macrophage electrophoretic mobility diagnostic procedure for early detection of malignant

disease in space to avoid problems associated with sedimentation of the very large macrophages.

Evaluations of the above processes will be provided which will include discussion of the relative utility of each approach, the techniques as to their difficulty and likelihood of success.

Task 2: With the cooperation of laboratories expert in the relevant areas preliminary simple experiments will be performed to provide information critical to the evaluation of the above procedures.

Included among these will be:

- (i) Experiments to determine whether or not phase-separation of two-phase aqueous polymer systems can be driven by application of an electric field.
- (ii) Experiments to examine the behavior of cells at the freezing front of an aqueous suspension. Fixed cells will be used to avoid cell damage in these first attempts.
- (iii) Experiments to determine whether or not adsorption of normal cells at the air/water interface results in gross cell damage due to high interfacial tension.

Given the short period (6 funded months) available for this study, it was not possible to carry out a detailed examination of all the areas outlined in the Scope of Work. Instead, the strategy generally employed was to contact experts in each field and discuss with them the possible utility of their technique in space processing of biological materials. The experts so consulted are listed in Appendix A. Based on these discussions, on readings of relevant literature, and on the Principal Investigator's own knowledge and experience a preliminary evaluation was made of all but one of the proposed procedures. The subject of cell culturing was not investigated because major studies in this area are reportedly being carried out by Johnson Space Center. It is the preliminary evaluations which in most cases appear in this Report. The most promising technique--countercurrent distribution of cells in phase separated aqueous polymer solutions--was then subjected to more detailed theoretical and experimental examination, with the results given in the sections immediately following.

3.1 Partition and Countercurrent Distribution of Cells in
 Phase Separated Aqueous Polymer Systems

3.1.1 Description of Technique

Much of modern biomedical research is directly aimed at defining and elucidating the normal and pathological activity of living systems. A principal problem in such work is frequently encountered when

attempts are made to prepare the specific cell population of interest in a pure, viable state. Non-specific preparation techniques based on cell size or density are seldom sufficiently sensitive, as the total range of these parameters encountered among biological organisms is relatively narrow. Separation methods based upon cell surface properties hold more promise, however, since a cell's function and its ability to interact with other cells in its immediate environment appear frequently to be reflected in characteristics of the cell membrane. One such characteristic which is being exploited for preparative purposes is cell surface charge as detected by electrophoresis. Free-flow electrophoresis is capable of spatially distributing a cell population on the basis of the net charge density located within about 10 to 30 Å of the hydrodynamic cell surface. An even more sensitive separation technique is available, however, which also depends partly on the surface charge but which has been shown to be capable of separating cell populations which are electrophoretically indistinguishable.

When aqueous solutions of two different polymers are mixed above certain concentrations they frequently form immiscible-liquid, two-phase solutions. Each of these phases usually consists of more than 90 percent water and can be buffered and made isotonic by the addition of low molecular weight species. If a cell or particle suspension is added to such a system, then shaken, the cells--upon re-equilibration--are frequently found to have partitioned unequally between one of the phases and the interface. This preferential

partition behavior can be used as the basis of a separation procedure for differing cell populations since partition in these systems is determined directly by cell membrane properties.^{1,2}

Cell populations which have related, but not identical surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, multiple partitions are carried out via countercurrent distribution to effect the separation. CCD in phase systems derived from dextran/polyethylene glycol (PEG) mixtures has proven to be an extremely sensitive and valuable preparation technique in cell biology. Erythrocyte populations can be separated on the basis of cell age by CCD, for instance. This separation cannot be accomplished by preparative electrophoresis.^{2,3} CCD of human lymphocytes fractionates them into subpopulations which vary markedly in their T:B ratio and in their responses to various mitogens. Again, preparative electrophoresis has not yet proven capable of producing such a separation.

The effectiveness of CCD as a separation procedure resides in the fact that the partition coefficient, K , is sensitive to a variety of cell surface properties. Moreover, K depends *exponentially* on the relevant surface properties, as may be seen from the following expression:⁴

$$K = \exp \left(\frac{1}{kT} \right) \left[Q_{\text{eff}} (\psi^{\text{II}} - \psi^{\text{I}}) + (\gamma^{\text{II}} - \gamma^{\text{I}}) A \right] \quad (1)$$

where K = ratio of concentrations of partitioned material
in phases I and II, C^I/C^{II} (or between one phase
and the interface)

Q_{eff} = effective cell surface charge

$(\psi^{II} - \psi^I)$ = electrostatic potential difference between phases

$(\gamma^{II} - \gamma^I)$ = difference in interfacial free energy of cell/
polymer solution interface in two phases

A = cell surface area

The partition coefficient, therefore, depends exponentially on the effective cell surface charge, in contrast to the linear dependence of electrophoretic mobility on cell charge. This relationship accounts in part for the relatively higher sensitivity of CCD over preparative electrophoresis.

Equation (1) also indicates that cell charge is not the only parameter determining K . The interfacial free energy of the cell/solution interface also can play a strong role. This free energy will be determined largely by the degree to which one or the other of the phase polymers adsorbs to the cell surface, thus lowering the free energy between the polymer-coated cell and the phase in which the polymer predominates. The competitive adsorption of the two polymer species depends in turn on the chemical nature of the polymers and on a variety of cell membrane properties. Few of these membrane

properties have been identified as yet, but in a PEG/dextran system having no phase potential difference there is good evidence that PEG-membrane interactions are stronger, and partition into the PEG-rich phase higher, the greater the ratio of polyunsaturated to mono-unsaturated fatty acids in the membrane lipid.⁵ The chemical composition and structure of the membrane--independent of surface charge--can, therefore, determine partition behavior as well.

A strength of the partition approach to cell separation is that the conditions which determine the value of K are mainly under experimental control. Hence, the cell characteristic on which the separation is to be based can be made the dominant determinant of K by appropriate choice of operating conditions. The bulk phase potential difference, $\psi^{II} - \psi^I$, is caused by the slight preference of some salts for one phase over the other. By manipulating the ionic species and concentrations in the system, then, the magnitude of $\Delta\psi$ can be controlled. Likewise, by varying the chemical character of the phase polymers themselves, γ^I and γ^{II} can be changed and the interfacial free energy term made dominant. In particular, if appropriate polymers are used, affinity labels such as antibodies or haptens can be covalently bonded to one of the phase polymers. Cells bearing the specific structure to which the affinity label is directed will be preferentially coated with the labeled polymer and the cells will partition into the phase rich in that species. The phase can, therefore, act as a support medium for the affinity label,

one which has much greater access to the cell surface than the gel beads commonly used.

Countercurrent distribution of cells has been applied with great success to relatively small biological cells, such as erythrocytes and lymphocytes. There are a variety of cell types such as macrophages, megakaryocytes, and some tumor cells, which are too large and/or dense to be separated successfully in the phase systems currently used, however. Such cells do not remain in suspension long enough to allow the phases to separate and permit a transfer along the countercurrent train. By performing CCD in a zero-gravity environment, this problem would be eliminated. This very powerful technique would then be made available for the separation, viable recovery, and the study of those cells which are currently too large to fractionate in suspension on earth.

3.1.2 Cell Partition in a Low Gravity Environment

Countercurrent distribution can be successfully applied to cells on earth because phase separation occurs sufficiently rapidly that the 120 transfers necessary for many separations can be accomplished in a reasonable length of time. Under the best conditions, using a thin layer CCD apparatus to minimize the phase thickness, it takes five minutes for dextran/PEG phases to separate, resulting in a ten-hour run time. Phase separation at one g is driven by two mechanisms:

- 1) bouyancy forces due to the density difference between the phases;
- 2) interfacial tension which tends to minimize the interfacial area between the phases.

In zero g the first mechanism is absent. It seems unlikely that interfacial tension forces will be sufficient to drive phase separation rapidly enough to be of use since these tensions are very small in the phase systems currently in use (10^{-3} to 10^{-4} dyne/cm). The problem cannot be overcome by increasing the interfacial tension because this will result in all the cells becoming adsorbed in the interface. Another driving force must be introduced, therefore, if CCD is to be performed in space.

Such a driving force can in principle be supplied by an external electric field. It has recently been shown,⁶ using dextran/PEG phase systems commonly employed in cell partition, that drops of one phase suspended in the other have an easily measurable electrophoretic mobility. Moreover, the mobility is found to be a linearly increasing function of drop radius, as predicted by Levich's theory of the electrophoresis of conducting liquid drops.⁷ For a representative system (5 percent dextran 500, 4 percent PEG, 0.2 M K_2SO_4), the relationship found between mobility, u and drop radius, r , for PEG-rich phase drops is:

$$|u| = 0.37 r \text{ cm s}^{-1} \text{ V}^{-1} \quad (2)$$

In an applied field, taking $E = 10 \text{ vcm}^{-1}$, the drop velocity v_e is, therefore

$$v_e = |u| \cdot E = 3.7 \text{ r s}^{-1} \quad (3)$$

This can be compared with the expression for drop velocity due to sedimentation:

$$v_s = \frac{2 \Delta\rho \cdot g \cdot r^2}{3\eta_m} \frac{\eta_m + \eta_d}{2\eta_m + 3\eta_d} \quad (4)$$

where, with values given for the above system in brackets:

$\Delta\rho$ = phase density difference (0.03 g cm^{-3})

g = acceleration of gravity (980 cm s^{-2})

η_m = continuous phase viscosity (0.3 p)

η_d = viscosity of drop liquid (0.03 p)

Therefore,

$$v_s = 31 \text{ r}^2 \text{ cm}^{-1} \text{ s}^{-1} \quad (5)$$

For small drops, then, electrophoretic velocities are much greater than sedimentation velocities. For example, for $r = 5\mu$, $v_e/v_s = 240$ and the sedimentation velocity does not become greater than the electrophoretic velocity (for $E = 10 \text{ VCM}^{-1}$) until:

$$\frac{v_e}{v_s} = \frac{0.12}{r} \text{ cm} = 1 \quad (6)$$

which implies, $r = 0.12 \text{ cm}$.

Since thin layer CCD apparatuses have typical separation chamber

depths of 0.1 to 0.2 cm, it would seem on this basis that *phase separation driven by an applied electric field should proceed more rapidly than gravity-driven separation* if the electric field is applied parallel to the normal g -field.

It should be noted that the accompanying cell electrophoresis which will occur when the electric field is applied to separate the phases is unlikely to affect the final cell distribution. This is implied from the one- g results in which the cells and phase drops have greatly differing sedimentation velocities but still reach pseudo-equilibrium in which partition behavior does not correlate with sedimentation properties.

A second problem which may have to be solved if CCD is to be carried out in zero g is that of retaining the desired localization of the separated phases, after separation by the electric field, in order that efficient transfers along the countercurrent train may be made. In one g the localization of the top and bottom phases is self-maintained after separation by gravity. In space, however, a mechanism will have to be provided by which the separated phases can remain spatially localized in geometry convenient for phase transfer after separation by the field.

It should be possible to take advantage of the differential wetting behavior of the phases to achieve stable phase localization. If the

"upper" and "lower" halves of the separation chamber are coated with, for instance, different cross-linked gels or other materials having selective compatibilities with the two phase polymers, each phase ought to localize adjacent to the surface with appropriate coating. By this method opposite halves of the chamber should retain the separated phases, allowing phase transfers to be made in chambers of conventional geometry.

3.1.3 The Benefit of Low-G

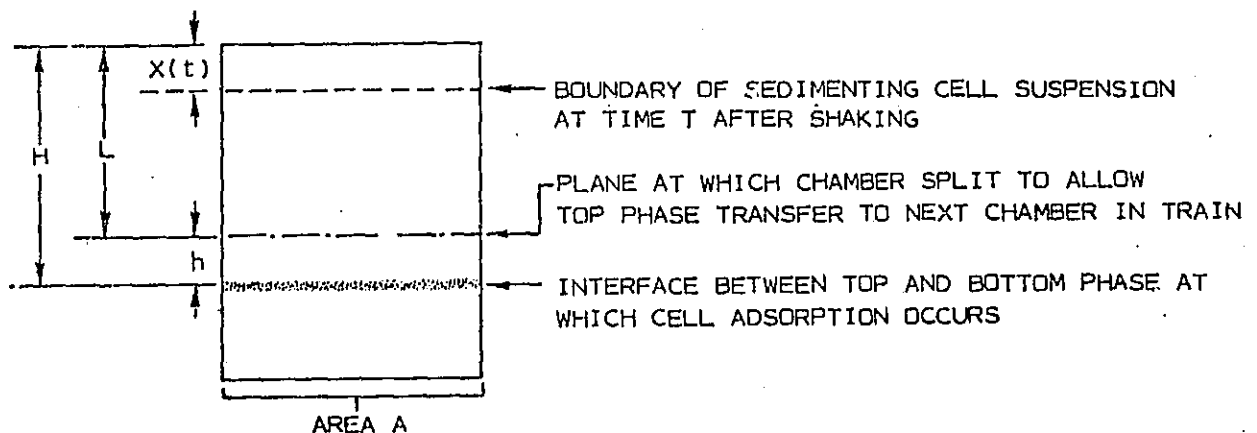
On earth, the partition of cells in phase-separated aqueous polymer systems is not an equilibrium process. Although the phases themselves can readily be brought to equilibrium, the distribution of partitioned material is time dependent due to sedimentation. Only after all the cells have sedimented to the phase boundary or the bottom of the container will the distribution be stationary--and then of no use. The degree to which the non-stationary nature of the distribution affects the usefulness of partition depends on the sedimentation rate of the cells in the appropriate phase--that is, on the cell size, shape, and density and on the phase density and viscosity. Since cell partition usually occurs between the top, PEG-rich phase and the interface, large cells or cell clumps will sediment into the interface from the top phase during the time it takes for the phases to separate, distorting the true, equilibrium distribution. If CCD is performed on such suspensions, the material transferred as the top phase will not contain all the cells that belong in that

phase and the bottom phase will contain the cells that have sedimented into the interfacial region as well as those that are truly adsorbed there. This gravity-driven accumulation has two related effects on the CCD:

- It reduces the resolution of separation for all cells with a finite sedimentation rate;
- It eliminates the use of this separation technique entirely for sufficiently large cells.

As the analysis outlined below will show, the nominal cell size above which CCD will not operate is well below the dimensions of a variety of cells of clinical interest. Developing techniques to perform CCD under low g conditions would allow both of the above limitations to be eased, thus opening up the procedure to a much wider variety of materials.

An estimate can be made of the effects of gravity on CCD in a typical phase system. The separation chamber geometry is:



$H \cdot A = V_T$ = Volume of total top phase

$L \cdot A$ = Volume of transferred top phase

$h \cdot A = u$ = Volume of top phase which remains with
interface upon transfer

$x(t) \cdot A$ = Volume of cell-free zone emptied by
sedimentation after time t

$C(o)$ = Concentration of cells in total top phase
in absence of sedimentation

$a = a_{sp} A$ = Number of cells adsorbed at interface

a_{sp} = Specific adsorption (number cells per
unit area)

G_I = Ideal distribution coefficient in absence
of sedimentation

$$G_I = \frac{C(o) V_T}{a} = \frac{C(o) H}{a_{sp}} \quad (7)$$

G_c = effective distribution coefficient during CCD in the absence of sedimentation taking into account cells left with bottom phase in volume v after transfer:

$$G_c = \frac{C(o) (V_t - v)}{a + C(o)v} = \frac{C(o) L}{a_{sp} t + C(o)h} \quad (8)$$

$G_c(t)$ = effective distribution coefficient during CCD in the presence of sedimentation at time t after shaking:

$$G_c(t) = \frac{C(o) [L - x(t)]}{a_{sp} + C(o) [h + x(t)]} \quad (9)$$

Now, $x(t)$ is determined by the cell sedimentation rate in the top phase:

$$x(t) = \dot{s}t \quad (10)$$

Here $\dot{s}$ is the sedimentation rate given by the Stokes expression:

$$\dot{s} = \frac{2 \cdot \Delta\rho \cdot g \cdot R^2}{9\eta} \quad (11)$$

where, with nominal values for the 5 percent dextran, 4 percent PEG system in brackets:

$\Delta\rho$ = density difference between cell and top phase
(0.06 gm cm⁻³)

η = top phase viscosity (0.033 p)

g = 980 cm s⁻¹

R = cell radius

The effect of sedimentation on the effective partition coefficients is therefore found to be:

$$\frac{G_s(t)}{G_c} = \frac{1 - \dot{s}t/L}{1 + \dot{s}t/h} \quad (12)$$

That is, the effective distribution coefficient of cells between the top phase and the interface is smaller in the presence of sedimentation than in its absence.

Counter current distribution can be described analytically by the binomial distribution⁸ from which the location of the peak and its width (proportional to the standard deviation) can be calculated for a species whose ideal distribution coefficient is known. After n transfers in the absence of sedimentation, the peak will occur in tube $\bar{r}_c$ which can be calculated from:

$$\bar{r}_c = \frac{n G_c}{G_c + 1} \quad (13)$$

G_c is related to the ideal distribution coefficient through:

$$G_c = \frac{G_I - \frac{C(o)h}{a_{sp}}}{1 + \frac{C(o)h}{a_{sp}}} \quad (14)$$

The peak location for $\dot{s} = 0$ is therefore given by:

$$\bar{r}_c = \frac{n G_I}{G_I + 1} (1 - h/H) \quad (15)$$

In the presence of sedimentation, the peak location $\bar{r}_c(t)$ is calculated from:

$$\bar{r}_c(t) = \frac{n G_c(t)}{G_c(t) + 1} \quad (16)$$

which leads to:

$$\bar{r}_c(t) = \bar{r}_c(1 - \dot{s}t/L) \quad (17)$$

The expression for the standard deviation of the distribution peak, σ , for any of the above cases (ideal, effective with $\dot{s} = 0$ or effective with $\dot{s} \neq 0$) is given in terms of the appropriate distribution coefficient by:

$$\sigma = \frac{(nG)^{1/2}}{G + 1} \quad (18)$$

For the case of $\dot{s} = 0$ this gives:

$$\sigma_c = \sigma_I \left[(1 - h/H)(1 + G_I h/H) \right]^{1/2} \quad (19)$$

and when sedimentation is present:

$$\sigma_c(t) = \sigma_c \left[(1 - \dot{s}t/L) \left\{ \frac{H + G_I(h + \dot{s}t)}{H + G_I h} \right\} \right]^{1/2} \quad (20)$$

The above analysis allows a quantitative estimate to be made of the effects of sedimentation on partition and resolution. It shows that:

- a. The effective partition of cells is reduced in the presence of sedimentation by a factor which depends on $\dot{s}$ and the chamber geometry.
- b. Partition will effectively not occur, and no peak will move out along the distribution train, if:

$$(1 - \dot{s}t/L) \leq 0 \quad (21)$$

In terms of cell radius, this implies the nominal upper limit on cell size for CCD at one g is:

$$R^* = \left[\frac{L}{2 \cdot \Delta \rho \cdot g \cdot t} \right]^{1/2} \quad (22)$$

For the commercial thin film CCD apparatus, $H = 0.2$ cm and $h = 0.029$ cm so $L = 0.171$ cm; settling times less than 5 minutes are not used for cells, and using the earlier values for the 5:4 dextran/PEG system:

$$R^* \approx 12 \text{ } \mu\text{m} \quad (23)$$

Therefore cells larger than about 24 μm diameter cannot be separated effectively by CCD in the systems currently in use. There are, however, a variety of cells of clinical and biological interest, such as megakaryocytes, liver cells, tissue culture lines, and some tumor cells which are considerably larger than this. These systems would be accessible for separation and examination by CCD in zero g.

- c. For cells which are near the critical size limit, a category which includes the majority of cell types, the resolution of subpopulation separation should be markedly better in the absence of sedimentation. This may be seen by calculating the resolution for some representative separations. Resolution, R_N , is defined for partition or

column chromatography by:

$$RN = \frac{\bar{r}^{(1)} - \bar{r}^{(2)}}{2 (\sigma^{(1)} + \sigma^{(2)})} \quad (24)$$

where the superscripts refer to components (1) and (2) to be resolved. $RN = 1$ implies the peaks are separated by about four standard deviations, which is virtually complete resolution.

Case 1: $R = 10 \mu m$ $G_I^{(1)} = 1.25$ $G_I^{(2)} = 0.75$ $n = 100$

All other parameters have previous values.

a. In the presence of sedimentation:

$$\begin{aligned} \bar{r}_c^{(1)}(t) &= 14.5 & \bar{r}_c^{(2)}(t) &= 11.2 \\ \sigma_c^{(1)}(t) &= 3.52 & \sigma_c^{(2)}(t) &= 3.15 \\ RN(t) &= 0.247 \end{aligned}$$

b. No sedimentation:

$$\begin{aligned} \bar{r}_c^{(1)} &= 47.5 & \bar{r}_c^{(2)} &= 36.6 \\ \sigma_c^{(1)} &= 4.99 & \sigma_c^{(2)} &= 4.82 \\ RN &= 0.556 \end{aligned}$$

Therefore, the resolution at zero g is improved by a factor:

$$RN/RN(t) = 2.25 \text{ for } 20 \mu\text{m diameter cells.}$$

Case 2: $R = 7.5 \mu\text{m}$; all other parameters as above

a. In the presence of sedimentation:

$$\begin{aligned} \overline{r}_c^{(1)}(t) &= 28.9 & \overline{r}_c^{(2)}(t) &= 22.3 \\ \sigma_c^{(1)}(t) &= 4.53 & \sigma_c^{(2)}(t) &= 4.17 \\ RN(t) &= 0.380 \end{aligned}$$

b. No sedimentation:

$$\begin{aligned} \overline{r}_c^{(1)} &= 47.5 & \overline{r}_c^{(2)} &= 36.6 \\ \sigma_c^{(1)} &= 4.99 & \sigma_c^{(2)} &= 4.82 \\ RN &= 0.556 \end{aligned}$$

Here the improvement factor at zero g is, for $15 \mu\text{m}$ diameter cells,

$$RN/RN(t) = 1.47$$

Case 3: $R = 5 \mu\text{m}$; all other parameters as above:

a. In the presence of sedimentation:

$$\begin{aligned} \overline{r}_c^{(1)}(t) &= 39.3 & \overline{r}_c^{(2)}(t) &= 30.2 \\ \sigma_c^{(1)}(t) &= 4.88 & \sigma_c^{(2)}(t) &= 4.60 \\ RN(t) &= 0.480 \end{aligned}$$

b. No sedimentation: as Case 1 and 2:

$$RN/RN(t) = 1.16$$

There is therefore only slight gain in resolution at zero g for cells 10 μm in diameter.

Further calculations have shown that these improvement factors depend only very weakly on the values of G_I used so the above results apply reasonably well to all useful values of G_I .

There is a further possible advantage to performing CCD at low g if field-driven phase separation is employed. The calculations outlined in 3.1.2 suggested that field-driven separation might well be more rapid than that driven by gravity. In this event, more CCD transfers could be carried out in a given run time than is currently feasible on the ground, since adding Joule heating to a terrestrial phase system is likely to produce phase mixing due to unstable thermal convection. This implies that higher resolution would be obtained for *any* mixed cell population, since substitution of (16) and (18) into (24) shows that RN is proportional to $\eta^{1/2}$. Therefore, if the field-driven phase separations possible in low g turn out to separate faster than 1 g systems by a factor m , the resolution in such a system will, in principle, be increased by a factor $m^{1/2}$. Under zero gravity conditions, then, the resolution of CCD could be markedly improved for all cell types.

3.1.4 Experimental Results

Experimental studies were carried out in three areas in an attempt to verify the applicability of field-driven phase separation to the problem of performing CCD in a low g environment.

3.1.4.1 Electrophoretic Mobilities of Droplets of Phase Systems

In order for field-driven phase separation to occur the electrophoretic mobilities of droplets of one of the phases suspended in the other must be maximized. To this end, the 5% w/w dextran ($\bar{M}_w \approx 500,000$)/4% PEG phase system was examined with respect to phase droplet mobilities when a variety of different salts were incorporated into the polymer solutions. The phase systems were made up¹ and the droplet electrophoretic mobilities measured⁹ using standard techniques. Droplets of the same size were measured in all cases (diameter $\approx 10\mu$). The results are presented in Table 3.1.4.1.1.

It is seen that the mobilities increase in the order

$$\text{Cs} > \text{Rb} > \text{K} > \text{Na}$$

$$\text{citrate} > \text{oxalate} > \text{molybdate} \approx \text{SO}_4 > \text{succinate} > \text{acetate}$$

at concentrations of 0.1 M. We have found in earlier work that phosphate is roughly equivalent to sulphate in its effect, and that Cl^- and NO_3^- produce very low droplet mobilities. Hence, it would seem that near physiological ionic strengths Cs citrate is the salt of choice, but at higher concentrations the cation is likely to have little effect so long as citrate, phosphate or sulphate are the anions

5% dextran T500/4% PEG 6,000 Containing:	Mobility of Bottom Phase Droplets in Top Phase ($\mu \text{ sec}^{-1} \text{ v}^{-1} \text{ cm}$)	Mobilities of Top Phase Droplets in Bottom Phase ($\mu \text{ sec}^{-1} \text{ v}^{-1} \text{ cm}$)
0.10M K_2SO_4	+ 0.362 $\pm$ 0.021 (10)	- 0.858 $\pm$ 0.067 (12)
0.30M K_2SO_4	+ 2.087 $\pm$ 0.234 (22)	- 3.112 $\pm$ 0.285 (22)
0.10M Rb_2SO_4	+ 0.434 $\pm$ 0.042 (20)	- 0.913 $\pm$ 0.062 (24)
0.30M Rb_2SO_4	+ 2.106 $\pm$ 0.236 (24)	- 3.153 $\pm$ 0.153 (20)
0.10M Cs_2SO_4	+ 0.458 $\pm$ 0.044 (22)	- 0.969 $\pm$ 0.083 (22)
0.30M Cs_2SO_4	+ 2.090 $\pm$ 0.200 (22)	- 3.134 $\pm$ 0.216 (20)
0.1M Potassium Oxalate	+ 0.424 $\pm$ 0.061 (28)	- 0.956 $\pm$ 0.070 (20)
0.1M Sodium Acetate	very slow	very slow
0.1M Sodium Succinate	+ 0.259 $\pm$ 0.031 (20)	- 0.589 $\pm$ 0.057 (20)
0.1M Potassium Citrate	+ 1.149 $\pm$ 0.094 (30)	- 1.594 $\pm$ 0.122 (20)
0.2M Potassium Citrate	+ 2.037 $\pm$ 0.194 (22)	- 2.983 $\pm$ 0.267 (20)
0.1M Sodium Molybdate	+ 0.394 $\pm$ 0.041 (20)	- 0.930 $\pm$ 0.052 (20)

Notes: (i) Phase systems unbuffered; pH ranges from 4.5 to 8.8

(ii) Top phase is PEG-rich, bottom is dextran-rich.

(iii) The number of droplets timed is in parentheses.

Table 3.1.4.1.1 Electrophoretic mobilities of 5% dextran T500/4% PEG 6,000 phase droplets containing various salts.

predominantly present.

These measurements are rather time consuming and since only very limited funds were made available for technical assistance no further examinations of the system could be made. Other salts should be examined, however, and the effects of varying the polymer concentrations studied in some detail if the optimal system is to be identified.

3.1.4.2 Studies on Isopycnic Phase Systems

Albertsson has reported¹ and confirmed in private conversation that over a certain range of concentrations the dextran-ficoll (a poly (sucrose)) phase system separated into two phases of equal density. This system was investigated as a possible model for the behavior of phase systems in zero g since phase separation in this case must be driven by other than gravitational potential energy. If the system had exhibited favorable droplet zeta potentials it would have been the best system on which to examine the effects of an applied electric field. The isopycnic composition was found to be approximately 12% dextran:13.8% ficoll (M.W. $\sim 400,000$). A 15:12 system was used for mobility studies to allow pure separated phases to be obtained via centrifugation. Because of the high concentrations and molecular weights both phases were extremely viscous and difficult to work with. The isopycnic system took several days to equilibrate. The 15:12 system exhibited essentially a zero

electrophoretic mobility when droplets of either phase in the other were run with 0.1 M concentrations of the following salts included: K_2SO_4 , Rb_2SO_4 , Cs_2SO_4 , NaOAc, NaMolybdate, KCl, K_3 Citrate, K_2HPO_4 and NaCl. In addition, doping the system with 0.2% Na Dextran SO_4 or DEAE-Dextran gave systems with mobilities $< 0.1 \mu m s^{-1} v^{-1} cm$ (compared to mobilities of the order of 1.5 for dextran-PEG-citrate). Apparently the phases are too similar chemically to produce the assymetrical salt distribution which we had found earlier correlated with the presence of high droplet mobilities. The isopycnic system is therefore unsuitable for studies of field-driven phase separation.

3.1.4.3 Studies on Electric Field-driven Phase Separation

An attempt was made to demonstrate the effect on phase separation of an electric field applied parallel to the $\underline{g}$ vector. A simple phase separation chamber was constructed at minimum cost; the result was a decidedly unsophisticated piece of apparatus. The sample chamber consisted of a cylindrical cuvette wrapped in small diameter copper tubing through which thermostatted water (25.0°C) was pumped to maintain an approximately constant wall temperature. A spiral of platinized platinum wire was sealed into the bottom of the cuvette to act as the bottom electrode. A second Pt spiral was fixed about 3 mm above the bottom electrode and held in place by running its rigid lead through a rubber stopper which in turn was used to seal the cuvette. A phase system was introduced into the cuvette with the phase volumes adjusted so the interface at equilibrium occurred

midway between the electrodes. The outside of the cuvette was masked except for a 5 mm x 10 mm window on the detector side and a narrow (< 1.0 mm) slit on the source side which could be moved vertically. The slit was positioned so light entered close to the upper electrode surface when the cuvette assembly was introduced into the sample chamber of a Beckman DB-G spectrophotometer. Its intensity at the detector reflected the loss due to scattering by the droplets of bottom phase suspended in the top. Variation of intensity as a function of time after mixing the phase system occurs because as the phases separate they clear optically due to migration of light scattering centers.

The system chosen for study of field effects was 5% dextran (M.W. $\sim 500,000$), 4% polyethylene glycol (M.W. $\sim 6,000$), 0.1 M K_3 citrate, since this mixture had an appropriately high phase droplet mobility and a reasonably low electrical conductivity (desirable to minimize Joule heating and convection). Experiments were carried out by introducing the phase system into the cuvette-electrode assembly, shaking it to mix the phases, then placing the cuvette in the spectrophotometer and recording the optical density (O.D.) at 500 nm as a function of time with and without application of the electric field. With the field off satisfactory traces were obtained. They were similar in appearance to a simple adsorption isotherm with an initial steep decrease in O.D. which plateaued to a nearly constant value within 10 to 15 minutes. The reproducibility was rather poor because

of the difficulty of reproducing the initial conditions from run to run. There was no obviously linear initial portion of the O.D.-time curve. It would be useful in future work to attempt to fit the data to an analytical expression in order to obtain descriptive parameters for each set of conditions.

When an electric field $E \leq 7.5 \text{ v cm}^{-1}$ was applied to a shaken system a trace with the general qualitative character as that for $E = 0$ was obtained but quantitative comparisons were not possible for the following reasons:

- (i) Gas evolution at the electrodes produced bubbles whose presence caused large random trace deflections.
- (ii) Joule heating due to the high currents being passed apparently caused considerable convection in the chamber that caused trace fluctuations.
- (iii) The electrodes polarized rapidly so that, since the power was supplied at constant voltage, the field applied across the phase system decreased rapidly to a low value.

The general impression gained was that with $E \leq 7.5 \text{ v cm}^{-1}$ the initial slope of the O.D.-time curve was steeper than with the field off. At higher applied fields the traces were very badly disrupted, presumably due to a combination of gas production and convection, so no information

could be obtained. Some representative data is given in Table 3.1.4.3.1 where the increase in optical density (in arbitrary units) occurring in the first two minutes after mixing is recorded as a function of applied voltage (anode at top) in the dextran/PEG/citrate phase system. The trend is for more rapid clearing at higher field strengths, but considerably more work would have to be done to establish this point, particularly considering the problems with electrode polarization and gassing.

3.2 Freezing Front Separation of Cells

Freezing-front separation is one of several fractionation procedures based on the partition behavior of particles exposed to phase boundaries. In this technique, a particle suspension is directionally frozen from one end of the container. If the freezing rate is sufficiently low, particles contacted by the advancing solidification front will be either engulfed or rejected by the solid phase. Providing conditions are such that kinetic and fluid mechanical effects are negligible, and it is not at all clear just what these conditions are,¹¹ thermodynamic equilibrium considerations should in principle determine particle partition between the solid and liquid phases.¹² Hence, the particle will be engulfed or rejected depending on the sign of the net interfacial free energy change associated with replacement of the particle-liquid phase interface by the particle-solid phase interface. Particles whose surface properties are such that they exist in a lower free energy state in the liquid phase will be

Voltage Applied (v)	Increase in O.D. in 2 min. (arb. units)
0	23.5
0.25	25
0.5	22
0.75	19
1.0	25
1.25	27
1.50	37
1.75	27
2.0	34

Table 3.1.4.3.1 Optical clearing of 5% dextran/
4% PEG/0.1 M K_3 citrate in 2 minutes at various
applied voltages.

pushed by the advancing freezing front and physically separated from those frozen in place.

It has been proposed that the freezing front approach could be applied to biological cell separation problems. There are a number of major difficulties in its utilization for this purpose, however, even in a terrestrial laboratory.

- 1) The freezing and subsequent revival of biological cells is not a well understood process. There are no procedures known which allow survival of all the cells of any one type subjected to the freeze-thaw procedure. Some type of cells cannot be frozen under any conditions with subsequent retention of viability.
- 2) Successful preservation of those cell types which have been found to be capable of surviving a freeze-thaw cycle depends on the presence of cryoprotective agents such as glycerol or dimethyl sulfoxide as well as on strict adherence to an empirically determined temperature-time profile. It is not at all clear that the conditions necessary for cryopreservation and for thermodynamically-determined partition at the freezing front are compatible.
- 3) For freezing front separation to occur as described, the advancing solidification front must be smooth and free of dendrites. Aqueous salt solutions do not in general

freeze with a smooth boundary, however. Additives would therefore have to be developed which would eliminate dendrite formation. These additives would have to be compatible with biological cells over a wide temperature range. Development of such materials could be extremely difficult.

- 4) Very large electric fields generally are found at the solid-liquid interface of freezing aqueous salt solutions due to differences in ion activities in the two phases. It is unlikely that differential partition would occur in the presence of large electrostatic fields since in liquid two-phase systems a phase boundary potential difference of 2mv is sufficient to completely eliminate differential partition effects for cells.
- 5) Using conventional geometries the capacity of a freezing-front separation device will be rather low since once the interface is covered by a monolayer of cells no further interaction between suspended cells and the solid-liquid interface will occur and all cells will be pushed regardless of surface properties.
- 6) Many biological cell separation problems require fractionation of the parent population into a number of subpopulations, each characterized by a set of unique properties.

Since freezing-front separation can only divide a sample into two fractions it is inherently unsuited to problems of this kind.

- 7) It has not been demonstrated that the difference between the free energy of the cell-frozen solution interface and that of the cell-solution interface varies sufficiently among various classes of cells to allow separations to be made on this basis. A great deal of work would have to be done to prove this point. It was hoped initially that someone working with the freezing front technique would have available an apparatus in which some simple experiments could be carried out on cell suspensions to examine this fundamental point. Discussions with experts in the area revealed no appropriate equipment was available, however, so no experiments were attempted.

It has further been suggested that there could be an advantage to performing freezing front separation of cells in zero gravity to avoid sedimentation problems. There would seem to be little to gain by such a procedure, however, since rotation of the suspension about a horizontal axis can be used to cancel out cell settling. Moreover, cell sedimentation velocities are generally lower than the critical velocities for solidification front advancement (above which all particles are engulfed.¹¹) It should therefore be possible

to freeze cell suspensions vertically upward in many cases providing the freezing front is advanced sufficiently slowly that the impact velocity does not exceed the critical velocity,

Considering the above points, it would seem that freezing front separation is unlikely to become a useful fractionation procedure for biological cells, although some experimental work would have to be done before more positive statements could be made. Moreover, there would seem to be little benefit to be gained, providing the technique did prove useful on the ground, by carrying out freezing front separations on cells in a low gravity environment.

3.3 Adsorption of Cells at the Air-Water Interface

One separation procedure which had been suggested as worthy of consideration for biological space processing involved cell separation via adsorption of cells at the air/water interface, in analogy to the froth flotation procedures widely used in mineral separations.¹² Although such procedures can in principle be carried out at one g, where rising bubbles would carry the collected cells to the solution surface for recovery, an argument can be made for its use in space where purely surface free energy effects would determine adsorption at the interface undisturbed by gravity-driven perturbations. Since there appears to be considerable interest in cell separations based on adsorption at phase boundaries (two-polymer phase systems, freezing front separations) an experiment was carried out to determine

the effect of an interface with a high interfacial tension - the air/water interface - on cell membrane integrity.

Two experiments were carried out using fresh washed human red cells as the test population. Suspensions containing 5% v/v red cells in buffered saline were bubbled with O_2 through a sintered glass aeration nozzle (mean porosity $\sim 60 \mu m$) at rates of 17.3 and 260 cm^3/min .

Aliquots were removed at intervals and analyzed for hemolysis (cell content leakage). Significant hemolysis was observed at both O_2 flow velocities, the rates being 12% of total cells lysed/hr at 260 cm^3/min , and 1.3% lysed/hr at 17.3 cm^3/min ($T = 28.5 \pm 1.5^\circ C$, pH 7.4).

Apparently adsorption at the air/water interface is injurious to erythrocytes. Although it is not known whether or not the results indicate that all adsorbed cells lyse, certainly a large proportion of them do. The conclusion is that adsorption of erythrocytes at the air/water interface can cause severe cell damage. Since the erythrocyte is one of the strongest mammalian cells in a mechanical sense, it seems likely that adsorption of nucleated cells at this interface would be even more damaging. Probably because of the high interfacial tension (72 dynes/cm), then, cell adsorption at an air-water interface must be considered to be an unsafe procedure with respect to the maintenance of cell viability. It is therefore not suitable as the basis for a cell fractionation procedure.

3.4 The Macrophage Electrophoretic Mobility Test for Malignant Disease

The macrophage electrophoretic mobility (MEM) test is a procedure based upon the detection of a change in the electrophoretic mobility of a population of macrophages which has been claimed to detect the presence of malignant disease in an individual at a very early stage.^{13,14} Briefly, the test consists of exposing a population of the patient's lymphocytes to myelin basic protein (EF) derived from human brain for 90 minutes at room temperature. The supernatant from this incubation is then mixed with a suspension of oil-induced guinea pig peritoneal macrophages for 90 min. at 37°C. The electrophoretic mobility distribution of the macrophage population exposed to supernatants from lymphocytes stimulated by EF is then compared to a population exposed to lymphocyte supernatants to which EF is added after removal of the cells. Significant electrophoretic slowing of the test population has been found by several laboratories to be an indication of the presence of cancer in the patient from whom the lymphocytes were obtained. On the other hand there have been reports published by groups who were unable to distinguish cleanly between normal and cancer populations.^{15,16} Since there was considerable question a priori as to the ease with which mobility determinations could be made on rapidly settling macrophages, the MEM test was investigated to determine if mobility measurements in zero g would be expected to improve the reliability and applicability of the procedure.

Discussions with a number of experts in the field were held. The Cardiff and Bristol groups have had extensive experience with the test and are convinced it provides a method for the early diagnosis of cancer. The Roswell Park group have apparently successfully carried out the procedure on 50 patients with colonic cancer and 50 age-matched normal controls. They found that on the average an 11% mobility decrease occurred in the cancer group upon exposure of macrophages to supernatants from patient's lymphocytes incubated with human encephalitogenic factor (EF) relative to control macrophage mobilities exposed to lymphocyte supernatants with no EF. They found that non-cancer controls produced on the average little mobility change in the macrophages, but that a significant number did exhibit mobility decreases that overlapped with the disease group. It was not clear if this overlap was due to technical difficulties with the test or if it was a true biologically false positive result. Apparently they had difficulty making the mobility measurements accurately as the cells were so large that they seldom remained unaffected by electroosmotic flow in the chamber sufficiently long for accurate mobility measurements to be made. Dr. Harlos stated that typically only about 20 of 50-60 timings showed enough stability to be considered as representative of the true mobility distribution. It seems likely, if macrophage mobility distributions are anything like those of other leukocytes, that many more than 20 determinations would be necessary to accurately describe the population.

The Chester Beatty group have been unable to perform the test successfully in their own laboratories although they were able to obtain positive results in Cardiff. World wide there are reports of ten labs which find the test works, and four which have had little success with it. Apparently a critical requirement is that the guinea pigs from which the macrophages are obtained be healthy and free of any infection. None of the groups in which the test is run routinely employ an automated electrophoresis apparatus, although the Cardiff and Bristol groups both use vidicon cameras and electronic timer/printers to ease the strain of data collection. None of the groups contacted found macrophage sedimentation to be an insurmountable problem, and none could see any significant advantage in performing the test under conditions of zero gravity. On the other hand, everyone to whom I spoke was extremely enthusiastic about the applicability of an automated analytical electrophoresis facility (AAEF) in the MEM test. Both the Bristol and Cardiff groups felt the use of the AAEF would be invaluable in proving the applicability of the test and in determining its true characteristics, unbiased by cell selection, operator error, etc. Dr. Pritchard in particular (he currently holds a National Cancer Institute contract to examine the validity of the MEM test) was forthcoming in his remarks about the AAEF and volunteered to assist in testing and applying it in his area of expertise if the opportunity arose. I was left with the clear impression from these talks that the MEM test would be an extremely useful area of application for the AAEF.

3.5 References

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RECOMMENDATIONS

- (i) Partition and countercurrent distribution of cells in phase separated aqueous polymer systems could benefit significantly from a low gravity environment since larger cells could be partitioned than is presently possible. Furthermore, if it proves possible to drive phase separation with an electric field there is a good possibility of greatly increasing the resolution of the technique in zero g by shortening the separation time and thus allowing more transfers per unit time to be carried out. It is therefore recommended that further effort be expended to develop field-driven phase separation and CCD of cells in a low gravity environment.

- (ii) There would appear to be little justification for attempting to develop adsorption at an air/water or solid(ice)/liquid interface as a cell fractionation procedure either on the ground or in space. It is therefore recommended that no further research be supported by NASA in this area.

- (iii) As workers in the area appear to have little difficulty with cell sedimentation in the macrophage electrophoretic mobility test it is recommended that no effort be expended in developing this test in the Space Processing program. It was the unanimous opinion of the experts consulted in this field that use of the AAEF, currently being developed

under NASA support, would be invaluable in unambiguously determining the limitations and applicability of the MEM test. This would be an extremely useful development in cancer research and therapy since there is currently no general procedure available which is capable of detecting the presence of neoplastic disease in its early stages.

Appendix A

<u>Field of Investigation</u>	<u>Experts Consulted</u>
Cell Partition and CCD	Dr. Per-Ake Albertsson Department of Biochemistry University of Lund, Sweden Dr. Harry Walter V.A. Hospital Long Beach, California
Freezing Front Separation	Dr. A.W. Neumann Department of Mechanical Engineering University of Toronto, Canada Dr. W. Wilcox Clarkson College of Technology Clarkson, New York
Macrophage Electrophoretic Mobility Test	Dr. Dorothy Glares and Dr. J. Harlos Department of Experimental Pathology Roswell Park Memorial Institute Buffalo, New York Dr. Anne Light and Dr. Alan Preece Radiotherapy Center University of Bristol, U.K. Dr. J.A.V. Pritchard Velindre Hospital Cardiff, Wales