(NASA-CR-161487) COUNTERCURRENT DISTRIBUTION OF BIOLOGICAL CELLS Final Report, 1 Jan. 1978 - 31 Mar. 1979 (Oregon Univ. Health Sciences Center) 46 p
HC A03/MF A01
CSCL 06C G3/51
N80-27069
Unclas
27915

FINAL REPORT

on

COUNTERCURRENT DISTRIBUTION OF BIOLOGICAL CELLS

January 1, 1978-March 31, 1979

Contract No. NAS8-32817

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Prepared for:

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ABSTRACT

Basic studies have been made aimed at developing electric field driven phase separation as a method for carrying out biological cell partition studies in a reduced gravity environment. A neutral polymer phase system consisting of 7.5% dextran 40/4.5% PEG 6, 0.11 M Na phosphate, 5% fetal bovine serum (FBS), pH 7.5, has been developed which has a high phase droplet electrophoretic mobility and retains cell viability over many hours. In this and related systems the drop mobility is a linear function of drop size, at least in the range 4 μm-30 μm diameter. Drop mobility can be readily varied by fractionally replacing phosphate with chloride, with no loss of cell viability. Adsorption of cells to the phase boundary has a minimal effect on the electrophoresis of top phase, PEG-rich drops and decreases bottom phase drop mobilities by about 25%. Application of an electric field of 4.5 v cm^{-1} to a system containing 10% v/v bottom phase cleared the system more than two orders of magnitude faster than in the absence of the field. At higher bottom phase concentrations a secondary phenomenon intervened in the field driven separations which resulted in an increase in turbidity after clearing had commenced. The increase was associated with a dilution of the phase system in the chamber. The effect depended on the presence of the electric field. It may be due to electroosmotic flow of buffer through the Amicon membranes into the sample chamber and flow of phase system out into the rinse stream. Strategies to eliminate this problem are proposed.

1. BACKGROUND

1.1 Limitations of Countercurrent Distribution (CCD) on Earth

Countercurrent distribution of cells has been applied with great success to relatively small biological cells, such as erythrocytes and lymphocytes. However, there are a variety of cell types such as megakaryocytes, many tumor cell and tissue culture lines which are too large and/or dense to be separated successfully on earth. Such cells do not remain in suspension long enough to allow the phases to separate and permit a transfer along the countercurrent train.

In a one g environment, 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; the CCD curves obtained for most cell types will therefore represent only a fraction of the separation in principle possible.

- It eliminates the use of this separation technique entirely for sufficiently large cells.

1.2 Mechanism of Phase Separation

Countercurrent distribution with two phase aqueous polymer systems can be successfully applied on earth because phase separation occurs sufficiently rapidly that the 60 to 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 run times of up to 10 hours. Phase separation at one g is driven by two mechanisms:

1. convective forces caused by the density difference between the phases;
2. interfacial free energy which tends to minimize the interfacial area between the phases.

In a low g environment the convective forces will be too small to produce phase separation in an acceptable length of time ($\sim$ 5 min). Moreover, it is known that the interfacial tensions developed in these systems are also too small (10^{-2}-10^{-4} dyne cm^{-1}) to be effective in driving separation. This conclusion is based on the behavior of dextran/Ficoll phase systems which can be made with phases of equal density; such systems take many hours to separate after mixing. An external driving force must therefore be applied

if partition experiments are to be carried out in zero g.

1.3 Phase Separation via an Electric Field

The approach we have taken to producing phase separation by an external force involves the application of a small electric field across the system. This technique was suggested by the observation (1) that droplets of one phase suspended in the other had an easily measurable electrophoretic mobility. Moreover, the mobility was found to be a linearly increasing function of droplet size, up to at least 15 μm diameter. The phase systems therefore follow to some extent the behavior predicted by Levich (2) and Levine's (3) theory of the electrophoresis of mercury drops in ionic solutions which predicts such a dependence. The sign of the mobility inverts depending on which of the two phases is dispersed (i.e., depending on which side of the interface is externalized). Applying an external field will therefore drive droplets of each phase in opposite directions and, in principle, induce phase separation.

The principle of field-driven phase separation has now been verified in a series of experiments supported by the NASA SRT program. A phase separation chamber across which a known electric field can be applied was constructed by the Advanced Technology Operations Division of Beckman Instruments. The apparatus consists of two electrode chambers containing bright Pt electrodes separated from a phase chamber (5 cm L x 0.5 cm W x 0.2 cm deep) by two Amicon XM-100 membranes. Feeder ports give access to the chamber for filling and drainage. Electrode rinse buffer is circulated through the upper and lower electrode chambers to remove electrode reaction products. The chamber and electrode assembly is made of poly(methyl methacrylate) lapped and polished to provide a good seal between the upper and

lower halves. The optical system used to follow phase separation turbidimetrically consists of a small ruby laser whose beam diameter is limited to ∿ 0.03 cm by an entrance aperture. The beam traverses the width of the phase chamber at a vertical position which can be adjusted relative to the midline. The beam intensity is measured with a solid stare detector and amplifier after traversing the chamber and an 0.03 cm diameter exit aperture.

When a turbid mixed phase system is introduced into the chamber most of the light is scattered off the optical axis and the photodetector output is low. As the phases separate, the upper and lower phases clear, the scattering decreases and the detector output increases with time, reflecting the kinetics of separation. Experiments may be run in the presence or absence of the electric field and the kinetics readily compared.

The first experiments carried out to demonstrate field-driven phase separation utilized a system which exhibits very high phase drop mobilities ($\sim\pm$15-17 x 10^{-4} cm^2 s^{-1} V^{-1} for a 6.5 μm diameter drop). The polymers used were sodium dextran sulfate (8% w/w) and ®Pluronic P-104 (a block co-polymer of polyethylene glycol and polypropylene glycol; 8% w/w), their solutions containing 0.2 M K_3citrate. This phase system is not compatible with biological cells (the dextran sulfate is toxic in high concentrations) but was chosen to maximize the likelihood of demonstrating the effects of an applied electric field in the presence of gravity-driven separation.

At phase volume ratios of 9 parts top phase:1 part bottom phase the effects of the electric field, E, were dramatic: at E = 5 V cm^{-1} the initial slope of the light transmission vs time curve was up to 35 times greater than at E = 0. The phases cleared completely in 1 to 2 minutes in the field compared to 10-15 minutes in its absence. Application of the electric field was obviously successful in driving phase separation in this system.

The current contract was awarded to continue work on electric field driven phase separation, particularly to demonstrate the effect in a phase system compatible with biological cells. The specific tasks to be addressed are listed in the Statement of Work.

2. STATEMENT OF WORK

This work statement covers work to be done to develop the capability of countercurrent distribution of biological cells in space. The work continues a preliminary assessment of the feasibility of the concept during which electrophoretic separation of the phases was demonstrated in apparatus designed with flight experimentation in mind. At the same time, the advantages of the space environment for countercurrent distribution of cells was established. The following tasks should develop the capabilities of this technique for use in space on an early Shuttle mission.

Task I

Techniques to separate the phases for both manned and automated operation shall be developed. Emphasis now shall be placed on exploring simple demonstrations of phase separation in weightlessness to determine the crucial parameters of the operation, e.g. completeness of the phase and cell separations and times required, fluid handling procedures and operational measurements. The use of slow rotations to impose a small gravity force to separate the phases (analogous to the separation of phases on Earth) shall be investigated analytically and experimentally since this operation could be used during an early Shuttle mission. Any advantages of inserting the cells before or after the separation of phases by rotation should be investigated. The availability of manned operation in the various transfer steps of CCD should be examined. It must be kept in mind that the early

space experiments can be exploratory to clearly show the capabilities of CCD and answer questions raised by ground experiments.

Task II

The electrophoretic separation of phases offers different advantages and problems to CCD that have been identified during the past year. During this year:

a) Phase compositions shall be studied to maximize the phase drop electrophoretic mobility while maintaining compatibility with biological cells and a sufficient bulk phase potential difference to provide useful cell partitions. The phase forming polymers used in preliminary experiments were not compatible with living cells.

b) Droplet electrophoresis shall be carried out with cells adsorbed at the interface to determine the effects of such adsorption on droplet mobility and to allow estimation of the maximum cell number/phase volume ratio feasible.

c) Drop acceleration in an electric field should allow one to make distributions, and determine partition coefficients, on the ground by deducting a small "blank value" due to gravity phase separation from the results of rapid phase separation by electrophoresis.

d) Candidate phase systems will be allowed to phase separate in the presence and absence of an electric field, with and without cells, to attempt to estimate the effects of the field on cell partition, and the maximum feasible strength.

Task III

Chamber coatings will be tested for differential wettability by the separated phases in order to maximize the driving forces for phase localization. Polymeric coatings which in solution would phase separate

with one of the phase polymers but not the other would seem to be likely candidates as coating materials. The ridge concept as an alternative method for phase localization will have to be tested. Testing will establish if this can be done in tubes of small diameter where interfacial effects are increased and gravitational effects reduced.

Task IV

The contractor shall recommend a sequence of space and ground experiments and the rationale to pursue countercurrent distribution of cells. The space experiments should be identified to answer specific questions instead of fitting into an available schedule of flight opportunities.

3. WORK PERFORMED

3.1 Analysis of Low G Application to Drive Phase Separation

As pointed out in 1.1, the finite sedimentation rate of cells produces a non-stationary distribution of particles in the phase system during, and following, phase separation. In the absence of gravity such sedimentation will not occur, but neither can the phases be expected to separate since their density difference will no longer result in significant convective motion. It has been suggested that application of a low centrifugal acceleration might provide enough convection to cause phase separation while not producing sufficient cell sedimentation to adversely affect the partition coefficient. The following simple argument strongly suggests that this idea is not correct, however.

If a phase system is observed immediately after mixing, the entire volume is initially occupied by emulsion and appears cloudy. As separation begins, clear regions of pure upper and lower phase appear at the upper and lower boundaries of the sample. The regions increase in height at the

expense of the thickness of the emulsion region as separation progresses. Eventually the thinning emulsion contracts into a planar interface separating the upper and lower phase. If cells are present in the system, some of those which partition into the top phase will appear in the small volume of top phase present at the top of the sample volume early in the phase separation. As the emulsion interface recedes beneath these cells they will begin to sediment with a velocity which, as given by the Stokes expression, is proportional to the acceleration of gravity.

If the effect of cell sedimentation on the final distribution of cells between the upper phase and interface is to be small upon completion of phase separation, the cell sedimentation rate must be much less than the velocity with which the emulsion interface settles. The interface settling velocity will be determined primarily by the rate at which the individual emulsion drops sediment, although droplet-droplet interactions and secondary flocus obviously will complicate the interpretation. The emulsion drop sedimentation velocity will also be proportional to the acceleration vector, however. Hence, the ratio of cell settling velocity to interface settling velocity will be <u>independent</u> of the acceleration applied to the system, as both rates are scaled by g. Since it is this relative cell settling velocity which determines the degree to which the partition technique can be applied to cell separation, varying the acceleration vector in the system cannot provide any enhancement in performance over that available at one g. Only a force which differs in its effect on the cells and emulsion drops--such as that produced by an electric field--can provide the results sought.

3.2 Development of Biocompatible Systems Suitable for Field-driven Phase Separation

3.2.1 Optimization of Droplet Electrophoretic Mobility

Since the rate at which the phases will separate in an electric field will depend directly on the electrophoretic mobility of the phase drops, various manipulations were carried out to try and maximize drop mobility while maintaining biocompatibility and the capacity for useful partition. Earlier work by the P.I. showed a strong correlation existed between the degree to which some salts partition in the phase systems and droplet mobility. The salt partition is apparently determined largely by the solvent binding or organizing properties of the more hydrophobic phase (PEG-rich). We therefore examined the effects on droplet mobility of altering the character of the PEG-rich phase by incorporating into it molecules which are more hydrophobic than PEG. In these experiments a standard phase system composed of 5% dextran 500 (mol. wt. ≈ 500,000), 4% PEG 6 (mol. wt. ≈ 6,000) and 0.1 M K_3citrate to which the following additions were made: propylene glycol (PG, 5%-10% w/w); polypropylene glycol (PPG), mol. wt. = 400 (1%-10%); PPG mol. wt. = 1,200 (1% in presence of 5% and 10% PG to make PPG soluble). In addition the citrate was replaced in one set of measurements by 0.05 M, 0.1 M and 0.25 M HEPES (N-2-hydroxyethyldiperazine-N'-2-ethane-sulfonic acid), a molecule with a high dipole moment.

Phase systems were made from stock solutions of 40% w/w PEG 6 (Union Carbide, Batch 553 IS54489), made up by weight, 18.57% w/w dextran 500 (Pharmacia Fine Chemicals, Lot No. 7693), concentration determined polarimetrically and 2.0 M K_3citrate (reagent grade). All phase systems were made up by weight with a top loading balance to ± 0.005 g using twice pyrex-distilled water. The completed systems were shaken vigorously, incubated in a 25°C water bath for at least 20 min, shaken and allowed to re-equilibrate at 25°C for a further period of at least 30 min after which

time the system was spun at 800 g for 20 min. The phases were separated (discarding the interface region) and the suspensions for electrophoresis made up at volume concentrations of approximately 4 x 10^{-3} v/v (bottom phase drops in top phase) and 8 x 10^{-3} v/v (top phase drops in bottom). The droplet suspensions could be stored overnight at 4°C provided they were equilibrated at 25°C for more than 20 min before use.

Electrophoretic mobilities were measured in a standard analytical cell electrophoresis apparatus with Ag/AgCl electrodes and a cylindrical chamber at 25.0 ± 0.1°C, as described (4). A standard droplet diameter of 7-10 μm was chosen. The electrical conductivities of some of the systems were measured with a Radiometer Conductivity Meter.

The results of these experiments are summarized in Tables 3.2.1.1 to 3.2.1.4 below. Table 3.2.1.1 demonstrates that the drop mobilities were independent of the electric field strength applied, as expected for the low Reynold's number motion.

System	Electric field strength (v cm^{-1})	Mobility ± S.D. (no. drops) (x 10^4 cm^2 v^{-1} s^{-1})
Bottom drops in top phase	3.9	+1.16 ± 0.08 (10)
	4.3	+1.15 ± 0.08 (15)
	4.6	+1.18 ± 0.10 (10)
Top drops in bottom phase	3.9	-1.66 ± 0.15 (30)
	4.3	-1.49 ± 0.17 (30)
	4.6	-1.67 ± 0.10 (20)

Table 3.2.1.1. Effect of electric field on phase droplet mobility.

The effects of adding hydrophobic molecules to the standard system are given in Table 3.2.1.2. In all cases decreased mobilities were observed, probably due to increased phase viscosities. Table 3.2.1.3 shows that all

Mobilities of Standard Droplets of Bottom Phase in the Top Phase
A Standard Drop ≡ 7-10 μm Diameter STD = 5:4 Dex:PEG + 0.1 M K_3 Citrate

Phase System	Mobility ($x10^4 cm^2 v^{-1} s^{-1}$) $\bar{N}$ ± S.D. (n)	Phase System	Mobility ($x10^4 cm^2 v^{-1} s^{-1}$) $\bar{N}$ ± S.D. (n)	Phase System	Mobility ($x10^4 cm^2 v^{-1} s^{-1}$) $\bar{N}$ ± S.D. (n)
STD	+1.15±0.08 (15)	STD	+1.15±0.08 (15)	STD + 1%(w/w)PPG 1200	+1.19±0.12 (10)
STD + 1%(w/w)PPG 400	+1.05±0.12 (10)	STD + 5%(w/w)PG	+1.22±0.08 (22)	STD + 1%(w/w)PPG + 5%(w/w) PG	+1.19±0.10 (20)
STD + 5%(w/w)PPG 400		STD + 10%(w/w)PG	+0.89±0.10 (20)	STD + 1%(w/w)PPG + 10%(w/w)PG	+1.10±0.08 (20)
STD + 10%(w/w)PPG 400					
5/4/0 citrate	0				
5/4/HEPES*	≤ 0 (v-small)				

Mobilities of Standard Droplets of Top Phase in the Bottom Phase
A Standard Drop ≡ 7-10 μm Diameter STD = 5:4 Dex:PEG + 0.1 M K_3 Citrate

STD	-1.63±0.18 (20)	STD	-1.63±0.18 (20)	STD + 1%(w/w)PPG 1200	-1.57±0.13 (.)
STD + 1%(w/w)PPG 400	-1.58±0.12 (30)	STD + 5%(w/w)PG	-1.41±0.10 (16)	STD + 1%(w/w)PPG + 5%(w/w)PG	-1.54±0.10 (20)
STD + 5%(w/w)PPG 400	-1.34±0.07 (20)	STD + 10%(w/w)PG	-1.23±0.08 (20)	STD + 1%(w/w)PPG + 10%(w/w)PG	-1.22±0.14 (20)
STD + 10%(w/w)PPG 400	-0.95±0.08 (20)				
5/4/0 citrate	0				
5/4/HEPES*	≥ 0 (v-small)				

Table 3.2.1.2. Effects of additives on electrophoretic mobilities of 5% dextran 500/4% PEG 6, 0.1 M K_3 citrate phase systems. * No citrate.

Phase System	Specific Conductivity at 25°C millimho/cm
STD (5:4 Dex:PEG + 0.1 M K_3 Citrate)	
Top	13.86
Bottom	13.86
STD + 1%(w/w)PPG 400	
Top	13.16
Bottom	13.33
STD + 5%(w/w)PPG 400	
Top	11.05
Bottom	11.40
STD + 10%(w/w)PPG 400	
Top	8.60
Bottom	-
STD + 5%(w/w)PG	
Top	11.23
Bottom	11.58
STD + 10%(w/w)PG	
Top	9.56
Bottom	9.30
STD + 1%(w/w)PPG 1200 + 5%(w/w)PG	
Top	11.05
Bottom	11.23
STD + 1%(w/w)PPG 1200 + 10%(w/w)PG	
Top	9.30
Bottom	9.30

Table 3.2.1.3. Specific conductivities of phase systems of Table 3.2.1.2.

Phase volume ratio	Systems		
	STD	STD+5%(w/w)PG	STD+10%(w/w)PG
T:B(v/v)	1	1.18	1.24
	STD+1% PPG 1200	STD+1% PPG 1200+5% PG	STD+1% PPG 1200+10% PG
T:B(v/v)	1.24	1.38	1.53

Table 3.2.1.4. Ratio of top to bottom phase volumes (T:B) for 5% dextran 500/4% PEG 6/0.1 M K_3 citrate phase system plus additives.

the additives decreased the electrical conductivity, indicative of a viscosity increase. That the hydrophobic materials did, in fact, partition into the top phase is strongly suggested by the phase volume ratio increase, recorded in Table 3.2.1.4, observed as a function of increasing additive concentration. Apparently any mobility increase associated with increased salt partition is masked by viscosity effects in these manipulations.

A second approach to optimizing phase drop mobilities that was taken was to add electrolytes that might be expected to be more or less soluble in one or other of the phases and hence might partition more strongly than citrate. Since the top, PEG-rich drops invaribly have a negative mobility, dicarboxylic acids of variable hydrophobic chain length were tried. Amino sugars and borate ion were also used, aiming at preferential partition into the bottom, dextran-rich phase. Phosphates were also substituted for citrate, as they form a more physiological buffer. As may be seen from Table 3.2.1.5, no system tried had a higher mobility than the standard dextran/PEG/citrate, although it was found that Na_2HPO_4 could be substituted for K_3 citrate with little or no loss of mobility.

The final approach which was taken to maximize phase drop mobility involved lowering the molecular weights of the dextran and PEG fractions used. While there is no theory available which adequately models the behavior of these two phase polymer systems undergoing electrophoresis, one can argue on fairly general grounds that the dependence of mobility on the continuous and drop phase viscosities, η_m and η_d respectively, should vary as $[2\eta_m + 3\eta_d]^{-1}$ (3). That is, assuming the charging mechanism is not affected, the mobility should increase if the viscosities of the two phases are decreased. A simple way to achieve such a reduction is to reduce the

Phase System	Mobility of Bottom Phase Droplets† in Top Phase ($\mu m\ sec^{-1}\ V^{-1}\ cm$) ±S.D.(*)	Mobility of Top Phase Droplets† in Bottom Phase ($\mu m\ sec^{-1}\ V^{-1}\ cm$) ±S.D.(*)
5:4% (w/w) Dextran 500:PEG 6		
+ 0.10 M Na_2 Malonate	+0.45 ± 0.06 (6)	-0.93 ± 0.06 (9)
+ 0.10 M Na_2 Succinate	+0.45 ± 0.04 (10)	-0.89 ± 0.10 (5)
+ 0.10 M Na_2 Malonate + 0.05 M K_3 Citrate	+0.85 ± 0.07 (10)	-1.40 ± 0.08 (10)
+ 0.10 M Na_2 Succinate + 0.05 M K_3 Citrate	+1.00 ± 0.07 (10)	-1.38 ± 0.12 (10)
+ 0.10 M Na Glucuronate + 0.05 M K_3 Citrate	+0.13 ± 0.01 (4)	-0.35 ± 0.05 (10)
+ 0.10 M Glucosamine HCl	+ ≈ 0	≈ -0.16 ± 0.05 (3)
+ 0.10 M Glucosamine HCl + 0.05 M K_3 Citrate	+ < 0.14	-0.28 ± 0.04 (3)
+ 0.10 M Na_2HPO_4	+1.05 ± 0.18 (30)	-1.58 ± 0.21 (10)
+ 0.10 M Na_2HPO_4 + 0.05 M K_3 Citrate	This phase system formed an irreversible gel after stored overnight at 4°C	
+ 0.10 M Na_2 Borate	+ ≈ 0	-0.25 ± 0.03 (5)
+ 0.10 M Na_2 Borate + 0.05 M K_3 Citrate	+0.45 ± 0.09 (5)	-1.00 ± 0.11 (20)
+ 0.10 M K_3 Citrate	+1.15 ± 0.08 (15)	-1.63 ± 0.18 (20)

Table 3.2.1.5. Electrophoretic mobilities of phase drops of 5% dextran 500/4% PEG 6 to which electrolytes indicated have been added. †Standard droplets are 7-10 μm diameter. *Number of droplets timed.

polymer molecular weights. A price is paid for doing so, however, since the concentrations required for phase separation are higher the lower the polymer molecular weight. These higher concentrations mean higher viscosities, so a trade off between the two effects can be expected.

Table 3.2.1.6 gives an idea of the variation in composition required to produce phase separation as the PEG and dextran molecular weights are varied. These values can be compared with those for the standard dextran 500, PEG 6 system in which phase separation occurs above about 4.4% dextran/3.6% PEG at room temperature. The results of phase drop mobility determinations in some of these systems, all containing 0.15 M K_3 citrate, are shown in Table 3.2.1.7. It is seen there is a clear advantage in using lower molecular weight dextran fractions, as mobilities more than double the standard 5% dextran 500/4% PEG 6 are obtained using 7.5% dextran 40/4.5% PEG 6.

Dextran 70	4.0	5.0	7.0	9.0	
PEG 6	3.5	4.0	4.5	5.0	
Ratio of phase volumes (top/bottom)	NS	NS	0.90	0.85	
Dextran 40	6.0	7.5	8.0	10.0	
PEG 6	7.5	4.5	5.0	6.5	
Ratio of phase volumes (top/bottom)	NS	1.11	0.90	1.06	
Dextran 70	8.0	9.0	10.0	10.5	11.0
PEG 4	4.0	5.0	5.0	6.0	7.0
Ratio of phase volumes (top/bottom)	NS	NS	0.90	0.65	0.95
Dextran 40	10.0	10.5	11.0	11.5	
PEG 6	5.0	6.0	7.0	8.0	
Ratio of phase volumes (top/bottom)	NS	0.73	1.06	1.06	

Table 3.2.1.6. Compositions of phase systems containing dextran 70 (Pharmacia, mol. wt. ≈ 70,000), dextran 40 (Pharmacia, mol. wt. ≈ 40,000), PEG 6 and PEG 4 (Union Carbide, mol. wt. ≈ 6,000 and 4,000). Numerical values given are the total phase compositions in % w/w. NS implies no phase separation occurred.

Phase System	Mobilities (x 10^4 cm^2 v^{-1} s^{-1})±S.D.(*)		Viscosity (mPa s) at 25°C
5:4 (% w/w) Dextran 500:PEG 6 + 0.10 M K_3 Citrate (Standard System)			
Bottom Drops in Top Phase	+1.15 ± 0.08 (15)	Top	3.2 (approx.)
Top Drops in Bottom Phase	-1.63 ± 0.18 (20)	Bottom	28.4 (approx.)
7.5:4.5 (% w/w) Dextran 70:PEG 6 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+1.95 ± 0.35 (20)	Top	4.3 ± 0.2
Top Drops in Bottom Phase	-3.43 ± 0.44 (20)	Bottom	17.6 ± 1.1
9:5 (% w/w) Dextran 70:PEG 6 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+2.39 ± 0.29 (20)		
Top Drops in Bottom Phase	-3.28 ± 0.29 (20)		
7.5:4.5 (% w/w) Dextran 40:PEG 6 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+3.12 ± 0.28 (20)		
Top Drops in Bottom Phase	-3.87 ± 0.35 (20)		
8:5 (% w/w) Dextran 40:PEG 6 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+2.93 ± 0.31 (20)	Top	5.1 ± 0.1
Top Drops in Bottom Phase	-3.61 ± 0.40 (20)	Bottom	14.2 ± 0.4
10:6.5 (% w/w) Dextran 40:PEG 6 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+3.02 ± 0.35 (20)		
Top Drops in Bottom Phase	-3.44 ± 0.29 (20)		

Table 3.2.1.7. Electrophoretic mobilities of 7-10 μm droplets and phase viscosities of various phase systems at 25.0 ± 0.1°C. *Number of droplets timed.

Phase System	Mobilities (x 10^4 cm^2 v^{-1} s^{-1})±S.D. (*)		Viscosity (mPa s) at 25°C
10:5 (% w/w) Dextran 70:PEG 4 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+1.82 ± 0.35 (20)	Top	4.2 ± 0.2
Top Drops in Bottom Phase	-3.02 ± 0.32 (20)	Bottom	25.0 ± 1.0
10.5:6 (% w/w) Dextran 70:PEG 4 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+1.41 ± 0.20 (20)		
Top Drops in Bottom Phase	-2.90 ± 0.33 (20)		
10.5:6 (% w/w) Dextran 40:PEG 4 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+1.97 ± 0.26 (20)	Top	4.0 ± 0.1
Top Drops in Bottom Phase	-2.52 ± 0.33 (20)	Bottom	25.7 ± 0.7
11:7 (% w/w) Dextran 40:PEG 4 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+2.40 ± 0.23 (20)	Top	4.2 ± 0.1
Top Drops in Bottom Phase	-2.24 ± 0.18 (20)	Bottom	35.5 ± 0.9
11.5:8 (% w/w) Dextran 40:PEG 4 + 0.10 M K_3 Citrate			
Bottom Drops in Top Phase	+2.30 ± 0.32 (20)	Top	4.6 ± 0.1
Top Drops in Bottom Phase	-2.41 ± 0.29 (20)	Bottom	49.8 ± 0.9

Table 3.2.1.7. (Continued)

Because of the high drop mobilities obtained, it was decided to utilize the 7.5% dextran 40/4.5% PEG 6 system as the basis for future work. The final experiments carried out to maximize drop mobility involved examination of the effects of utilizing various proportions of 0.11 M Na_2HPO_4 buffer, pH 7.5, and 0.15 M NaCl to make phase systems of roughly constant ionic strength which varied in their PO_4/Cl ratio. The systems contained 5% fetal bovine serum (FBS; Grand Island Biological Co.) as it was known that such an addition markedly improved cell viability (vide infra). The results, shown in Figure 3.2.1.1, indicate that at constant pH and ionic strength the droplet mobility increases roughly linearly with phosphate concentration. Varying the PO_4/Cl ratio therefore provides a convenient technique for controlling the phase drop mobility without drastically changing most of the other physical characteristics of the system.

3.2.2 Biocompatibility of Dextran/PEG System

In order to examine the biocompatibility of the dextran 40/PEG 6 system, the viability of a typical nucleated mammalian cell type was determined under conditions that approximated those present during CCD. Spleens were removed from LAF_1 mice (Jackson Laboratories) which had been ether-anesthetized and sacrificed by cervical dislocation. Splenic cell suspensions were prepared and washed three times in 0.01 M phosphate-buffered saline containing 5% v/v heat-inactivated fetal calf serum (PBS-FCS), pH 7.2, by centrifugation at 490 g for 10 min at 4°C. To 10 ml of each phase system 0.1 ml of packed cells was added, the system shaken, then allowed to stand at room temperature. Every 15 min each system was shaken to re-disperse the phases and cells; a control suspension of cells

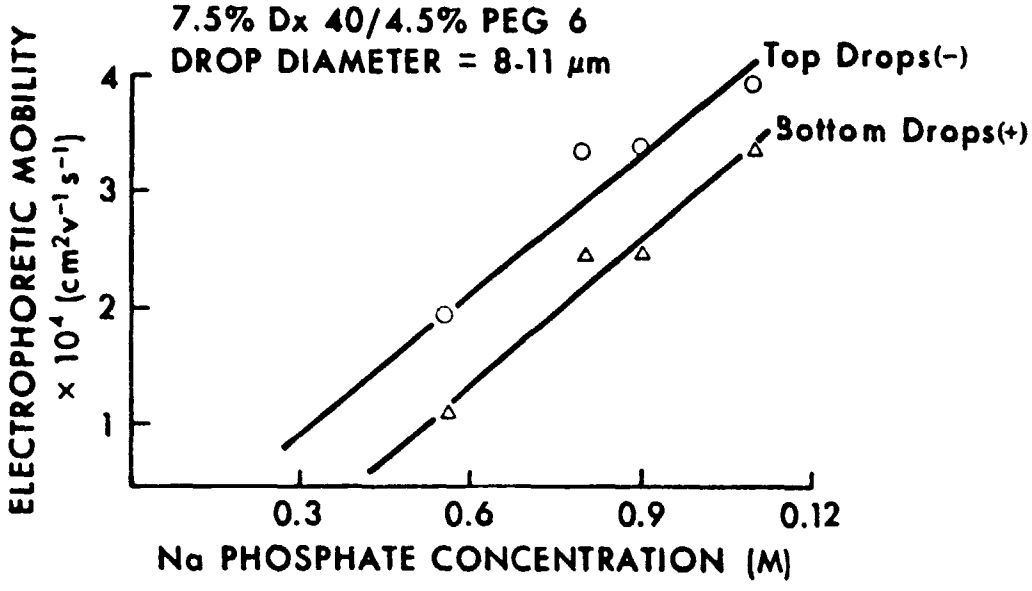


Figure 3.2.1.1. Electrophoretic mobility of droplets as function of concentration of Na_2HPO_4/NaH_2PO_4 buffer (0.11 M stocks of each mixed to give pH 7.5) in phase systems containing 7.5% dextran 40/ 4.5% PEG 6, 5% FBS and, for points from low to high phosphate, 0.05 M, 0.04 M, 0.03 M and 0 NaCl; drop diameter 8-11 μm; T = 25.0 ± 0.1°C. Top drops have a negative mobility, bottom drops a positive mobility.

in phosphate-buffered saline (PBS) + 5% FBS was treated similarly. At the intervals noted in Table 3.2.2.1, 1.0 ml aliquots were removed from each system, 4 ml of PBS + 5% FBS added, the suspension centrifuged for 10 min at 300 g and the supernatant discarded. To the cell button 6 drops of 0.1% filtered trypan blue in PBS was added. At least 100 cells were counted for each sample, the viability being identified with the fraction of cells counted which excluded the dye. All phase systems were made up of 7.5% dextran 40/4.5% PEG 6, 5% FBS as earlier work had shown (D.E. Brooks and H. Walter, unpublished) that cell viabilities were invariably low after CCD if serum was not present. The effect on viability of diminishing the concentration of chloride was examined, with the results shown in Table 3.2.2.1.

It is seen that in all the phase systems tested, shaking the suspensions every 15 min for up to 5 hr at room temperature had very little effect, all samples showing > 90% viability. There was no obvious advantage to adding chloride to the systems over this period. In samples that were incubated 24 hr at 4°C (infrequent shaking) the viability in the phase systems decreased about 15% relative to the 4° control, but again little effect of chloride was found. Only in suspensions left 24 hr at room temperature was there any improvement in viability in systems containing chloride. The effect in this case was too small to render the viabilities acceptable, however.

The results demonstrate that the system with the highest drop mobility, that containing pure 0.11 M phosphate buffer as the electrolyte, is able to retain greater than 90% of a population of murine splenic lymphocytes viable through 20 shake/settle cycles in 5 hr at room temperature. At 4°C, but not room temperature, viabilities remained acceptable (> 75%) after 24 hr

Hours after start	PERCENT OF VIABLE CELLS Each phase system contains 7.5% Dextran 40, 4.5% PEG 6 + 5% FBS in addition to:				
	Control	0.11 M Na phosphate	0.09 M Na phosphate + 0.03 M NaCl	0.08 M Na phosphate + 0.04 M NaCl	0.05 M Na phosphate + 0.05 M NaCl
0-1	94	92	-	97	97
1-2	98	95	94	96	95
2-3	98	93	96	96	93
3-4	-	93	90	-	-
4-5	98	91	94	91	95
∿24 at 4°C	94	77	81	79	85
∿24 at 22°C	91	42	42	51	58

Table 3.2.2.1. Viability of murine splenic lymphocytes in various phase systems. Except where noted, all incubations at 22°C. For explanation, see text.

in the phase systems. The 7.5% dextran 40/4.5% PEG 6, 5% FBS, 0.11 M Na phosphate system, pH 7.5, therefore was considered to be a biocompatible system well suited for studies on field-drive phase separation.

3.2.3 Cell Partition Studies in Biocompatible Systems

Phase systems based on dextran low molecular weight and PEG fractions have been used for several years in cell partition work in Sweden, particularly where partition is determined by the interaction with the cell surface of PEG to which an affinity ligand has been attached (5). In such affinity procedures, the cells which partition into the top phase are those which interact most strongly with the ligand since they will be preferentially coated with PEG bound to the affinity group and will therefore partition into the top, PEG-rich phase. In the absence of an affinity ligand, erythrocytes, at least, do not partition appreciably into the top phase, presumably because low molecular weight dextran adsorbs more strongly to red cells than does dextran 500 (6).

In the 7.5% dextran 40/4.5% PEG 6, 5% FBS, 0.11 M Na phosphate system, the partition of fresh, thrice PBS-washed human red cells was negligible, consistent with the Swedish results. Addition of 0.01% w/w PEG-palmitate, a lipid affinity ligand consisting of the PEG 6 ester of palmitic acid (Chem Services Inc.) increased the partition of red cells to 50% (50% of the cells added found in the top phase) only in the absence of FBS, however. The FBS produced a white precipitate which collected at the interface (in the presence or absence of PEG-palmitate) and was discarded before the phases were used. The nature of this material was not investigated, but it is possible that it bound a significant fraction of the ester. Certainly there are components in serum (lipoproteins, albumin) which are capable of

binding fatty acids, effectively removing them from the system. This seems a likely explanation for the results obtained.

The ability of the dextran 40 system to support cell partition was not pursued further. It was felt that once a specific cell type to be examined by partition was identified that appropriate phase systems, probably including affinity ligands, could be developed. It should be possible, for instance, to remove albumin and other serum substituents with hydrophobic binding sites from FBS via hydrophobic affinity chromatography, if PEG-fatty acid esters were to be used. Affinity ligands based on non-lipid membrane proteins would be less likely to interact with serum components and could probably be used in the present system. Finally, if no other approach proved superior, the dextran molecular weight could be increased until the desired partition behavior was obtained. There was not time to pursue these possibilities during the present effort. As noted, work in this direction would be somewhat unfocussed without a specific cell type to work on. It seems likely, however, based on the above reasoning and the personal experience of the P.I., that for any particular cell type conditions could be found that would produce useful partition behavior.

3.2.4 Size Dependence of Drop Electrophoretic Mobilities

A remarkable characteristic of the electrokinetic behavior of the phase droplets is the increase in electrophoretic mobility with drop size. This characteristic is important for field-driven phase separation because it implies that separation ought to accelerate in an electric field as droplets coalesce and their average diameter increases. The only system examined in detail before the present work was carried out on a dextran 500/ PEG 6 system (1). It was found that the mobility increased linearly with

drop diameter over the range accessible. Similar measurements were carried out on a number of dextran 40/PEG 6, 0.11 M Na phosphate systems and their mobility-diameter relationships determined.

Suspensions of one phase in the other were made up as described in 3.2.1. For the size-mobility measurements the eyepiece of the electrophoresis microscope was equipped with a filar micrometer (American Optical Co.) which allowed drop diameters to be measured to ± 0.4 μm. Drops whose centers lay within ± 30 μm of the stationary layer were so identified (by focussing in and out and noting the location of drops in focus) and their diameters measured with the micrometer. The field was then turned on and the mobility determined. Because of the time required to carry out the two measurements on each drop it proved very difficult to obtain data on large drops; the practical limit was 30 μm diameter. Because of the limited accuracy of the filar micrometer the lower size limit was about 4 μm. Four phase systems were examined, all containing 0.11 M Na phosphate buffer, pH 7.5: 7.5% dextran 40/4.5% PEG 6 with and without 5% FBS, 8% dextran 40/5% PEG 6, 5% FBS and 9% dextran 40/6% PEG 6, 5% FBS. The results are displayed in Figures 3.2.4.1 to 3.2.4.4.

It is seen that in all cases the data can be fit adequately by a straight line over the diameter range accessible. Linear regressions were carried out on all sets of data; the results are given in Table 3.2.4.1.

There are several features of this data which are worthy of comment. Firstly, it is apparent that including 5% FBS in the system has only a small effect on the u(D) relationship, decreasing the mobilities of the bottom, dextran-rich drops more than the PEG-rich drops for all but the smallest diameters. The mobility is still a linear function of drop diameter over the

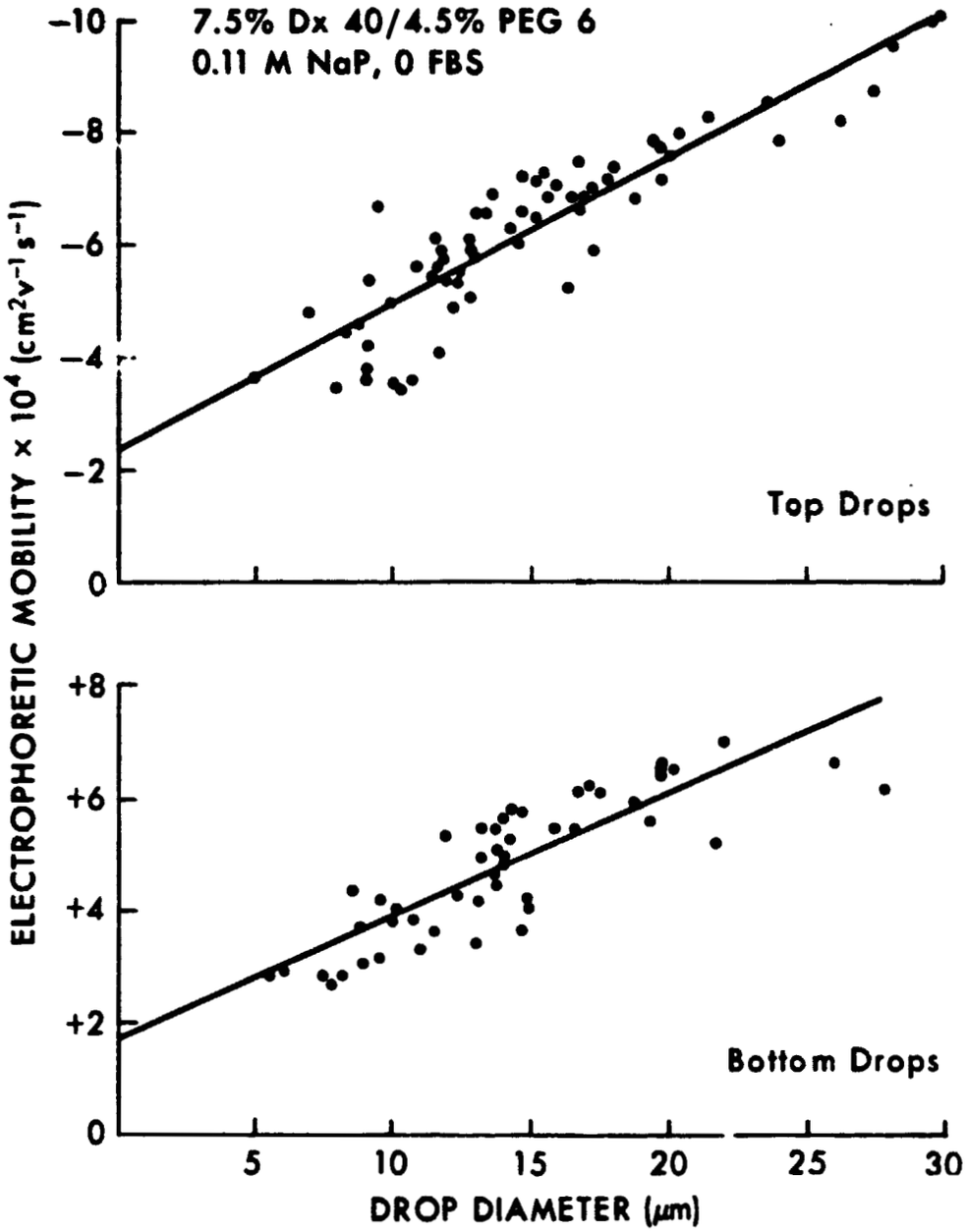


Figure 3.2.4.1. Drop mobility vs diameter for the system 7.5% dextran 40/4.5% PEG 6/0.11 M Na phosphate (0 FBS).

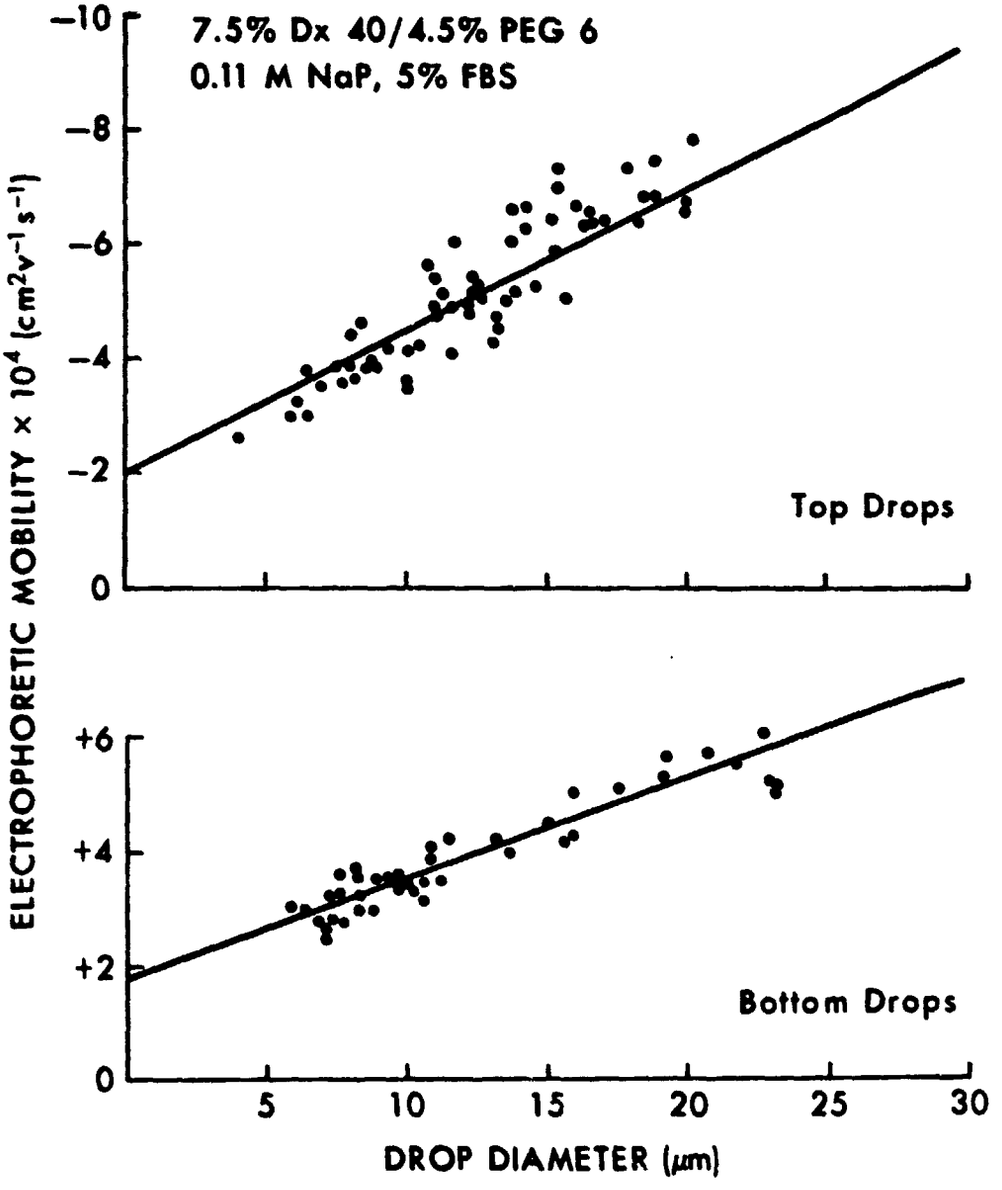


Figure 3.2.4.2. Drop mobility vs diameter for the system 7.5% dextran 40/4.5% PEG 6/0.11 M Na phosphate + 5% FBS.

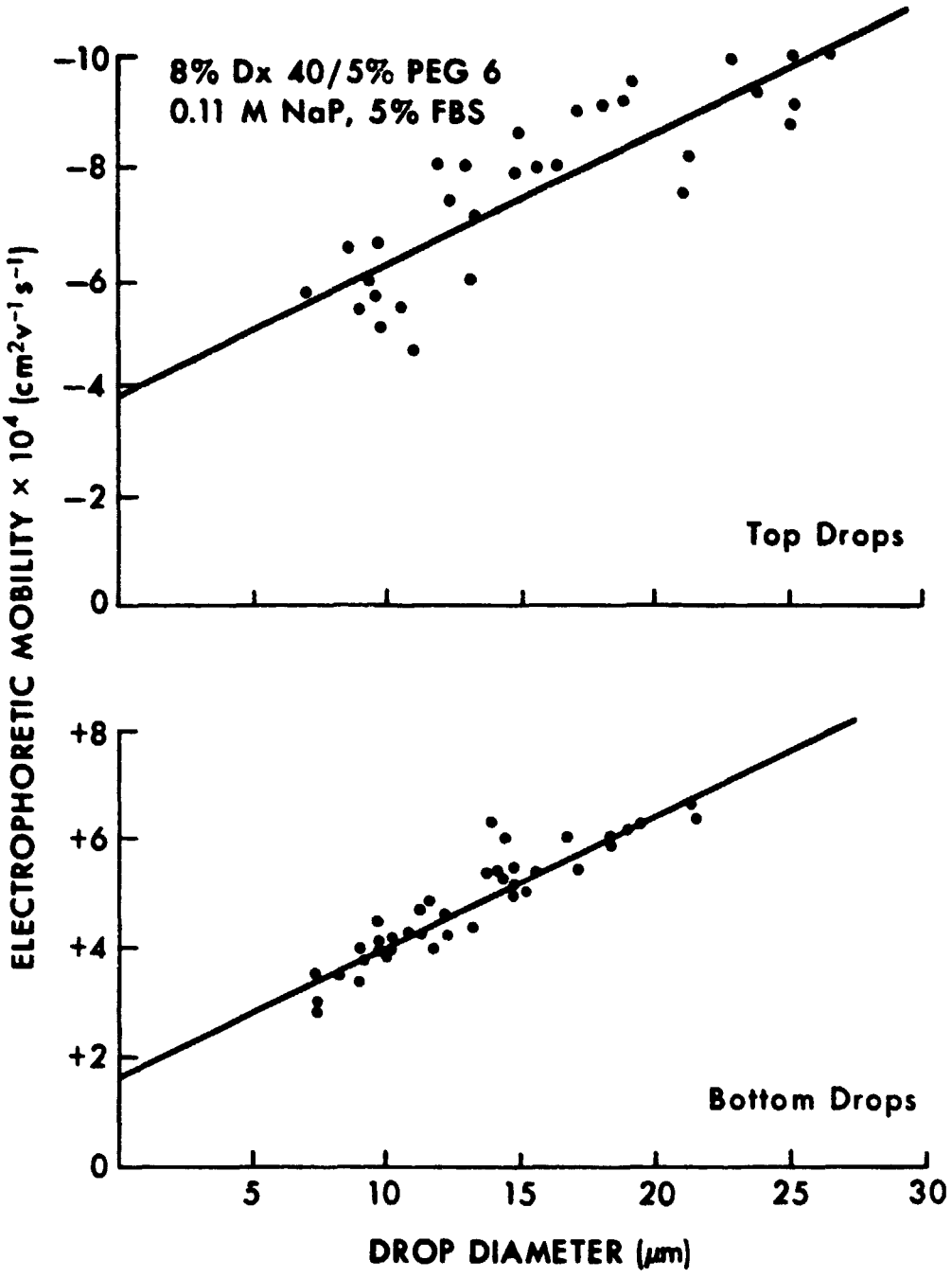


Figure 3.2.4.3. Drop mobility vs diameter for the system 8% dextran 40/5% PEG 6/0.11 M Na phosphate + 5% FBS.

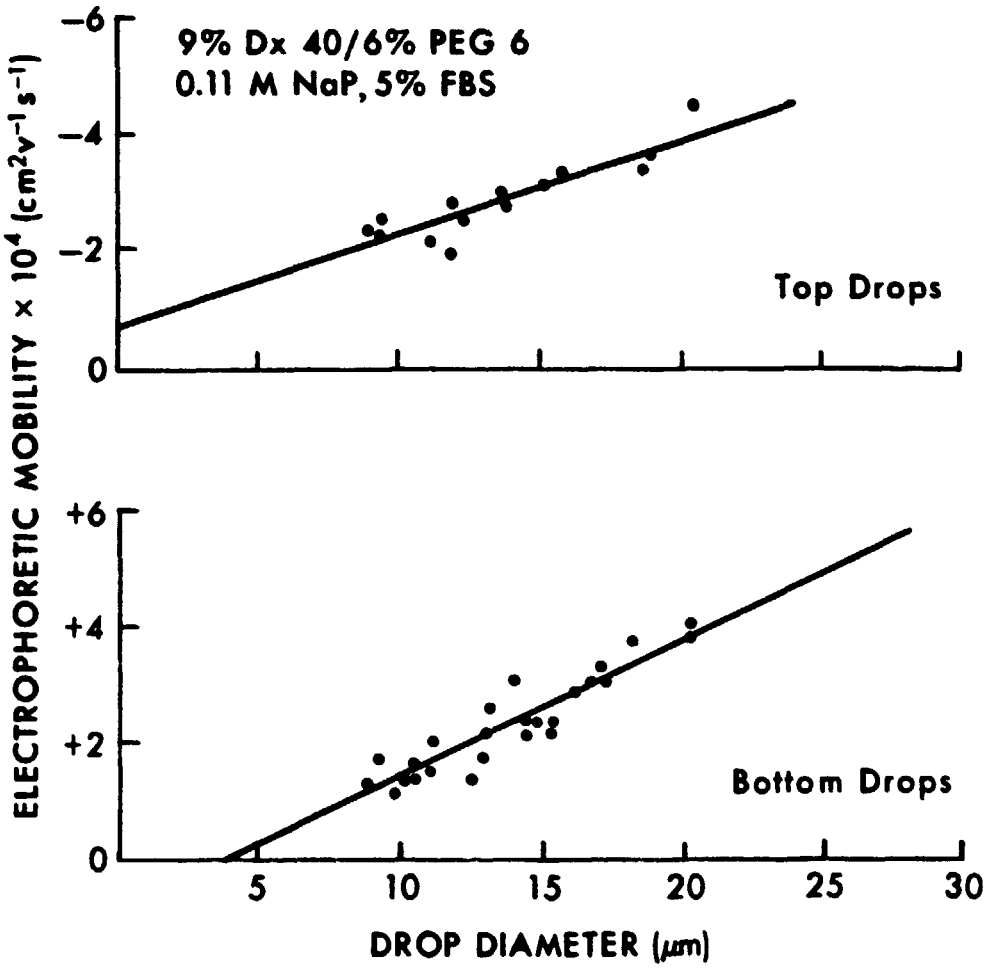


Figure 3.2.4.4. Drop mobility vs diameter for the system 9% dextran 40/6% PEG 6/0.11 M Na phosphate + 5% FBS.

System	Regression Equation and Coefficient	
7.5/4.5, 0 FBS		
Top Drops	$-u = 0.26\ D + 2.37$	$r^2 = 0.76$
Bottom Drops	$u = 0.22\ D + 1.63$	$r^2 = 0.65$
7.5/4/5, 5% FBS		
Top Drops	$-u = 0.25\ D + 2.00$	$r^2 = 0.81$
Bottom Drops	$u = 0.17\ D + 1.81$	$r^2 = 0.87$
8/5, 5% FBS		
Top Drops	$-u = 0.24\ D + 3.60$	$r^2 = 0.74$
Bottom Drops	$u = 0.23\ D + 1.66$	$r^2 = 0.91$
9/6, 5% FBS		
Top Drops	$-u = 0.16\ D + 0.70$	$r^2 = 0.87$
Bottom Drops	$u = 0.23\ D - 0.8$[illegible]	$r^2 = 0.80$

Table 3.2.4.1. Results of linear regression analysis of mobility-diameter data. u = drop electrophoretic mobility; D = drop diameter; r = regression coefficient.

range examined. This is an encouraging result since it implies that a wide variety of additives may be included in the systems with little effect on the transport properties of the emulsions.

Secondly, while in all cases the mobility was found to fit well to an equation linear in diameter, in no case was direct proportionality obtained. In all but one case, bottom drops in the 9/6 system, a finite intercept on the mobility axis resulted from the regression analysis. In the exceptional case a change in sign of mobility was predicted at about D = 4 μm. No actual mobility reversal was observed, however, and the source of the effect is obscure at present. The finite mobilities predicted at D = 0 could represent a transition to solid-like behavior as the drop size diminishes and surface tension effects become dominant. Solid particles do not exhibit a diameter dependence in the microscopic size range under the present conditions. Alternatively, a linear relationship may not be the

true dependence of u on D, the fit obtained being only apparent due to the scatter in the data. (The scatter could have been reduced by timing drops only within ± 10 μm of the stationary level rather than ± 30 but such a procedure would have been too time consuming in the limited period available). It is therefore entirely possible that the u(D) plots approach zero at diameters below the limits of the measurement technique used.

The third point of interest in this data is that there is no simple relationship between the u(D) behavior and the concentration of polymers used to make up the phase systems, i.e., the distance the systems exist above the critical point. Two competing effects would be expected as the phases became more concentrated. The increased phase viscosities will tend to reduce the mobility as discussed earlier. Farther from the critical point the salt partition increases, however, and the potential difference between the phases increases, suggesting a mobility increase as well. Apparently these two competing effects produce the complex results obtained.

There are two other points to be made regarding this data which are not illustrated by the Figures. In all the measurements made to date, if, as is usual, the sign of the mobility is identified with the sign of the zeta potential and this sign is compared with that expected from the known salt partition, it is found that the results are <u>opposite</u> to expectation (1). There has been no satisfactory explanation put forward to date for this striking observation. While it can be rationalized in principle by positing the presence of a dipole of appropriate orientation at the phase boundary, no physicochemical explanation for its presence has been presented. Since there is no theory currently available which describes any of the electrokinetic properties of these systems the sources of most of the effects observed remain obscure.

The remaining interesting discovery made during the course of this work relates to the kinetic behavior of the phase systems when the electric field was suddenly removed. In both the 7.5/4.5 and 8/5 systems, instead of the drop velocity rapidly going to zero as is found for solid particles, it initially decreased rapidly but slow motion continued in the direction of electrokinetic motion for times of the order of a minute. More remarkably, drops of the 9/6 system, with the highest phase polymer concentrations, actually reversed their motion when the field was turned off. This motion did not seem to be due to convective flow, to leaks or to a shift in the stationary level (it was observed at all locations in the chamber). Presumably the residual motion is associated with the slow relaxation of fluid circulation inside the drop. Because of the free boundary at the interface between the drop and its suspending medium the tangential fluid stresses are continuous across the phase boundary, hence the damping of circulation inside the drop might not be particularly severe. In the absence of a detailed theory for the flows involved, however, this explanation must be considered pure speculation.

3.2.5 Electrophoresis of Drops Carrying Adsorbed Cells

Utilizing the 7.5% dextran 40/4.5% PEG 6, 0.11 M Na phosphate, 5% FBS system it was possible to observe the mobility behavior of phase drops with washed human red cells adsorbed to the drop surface. The system was made up as described earlier with red cells included in the dispersed phase. Again it was possible to measure the u(D) behavior by the technique used previously, although the measurements were more difficult than those involving pure systems because of the enhanced sedimentation rate of drops to which cells were adsorbed. The results are shown in Figure 3.2.5.1.

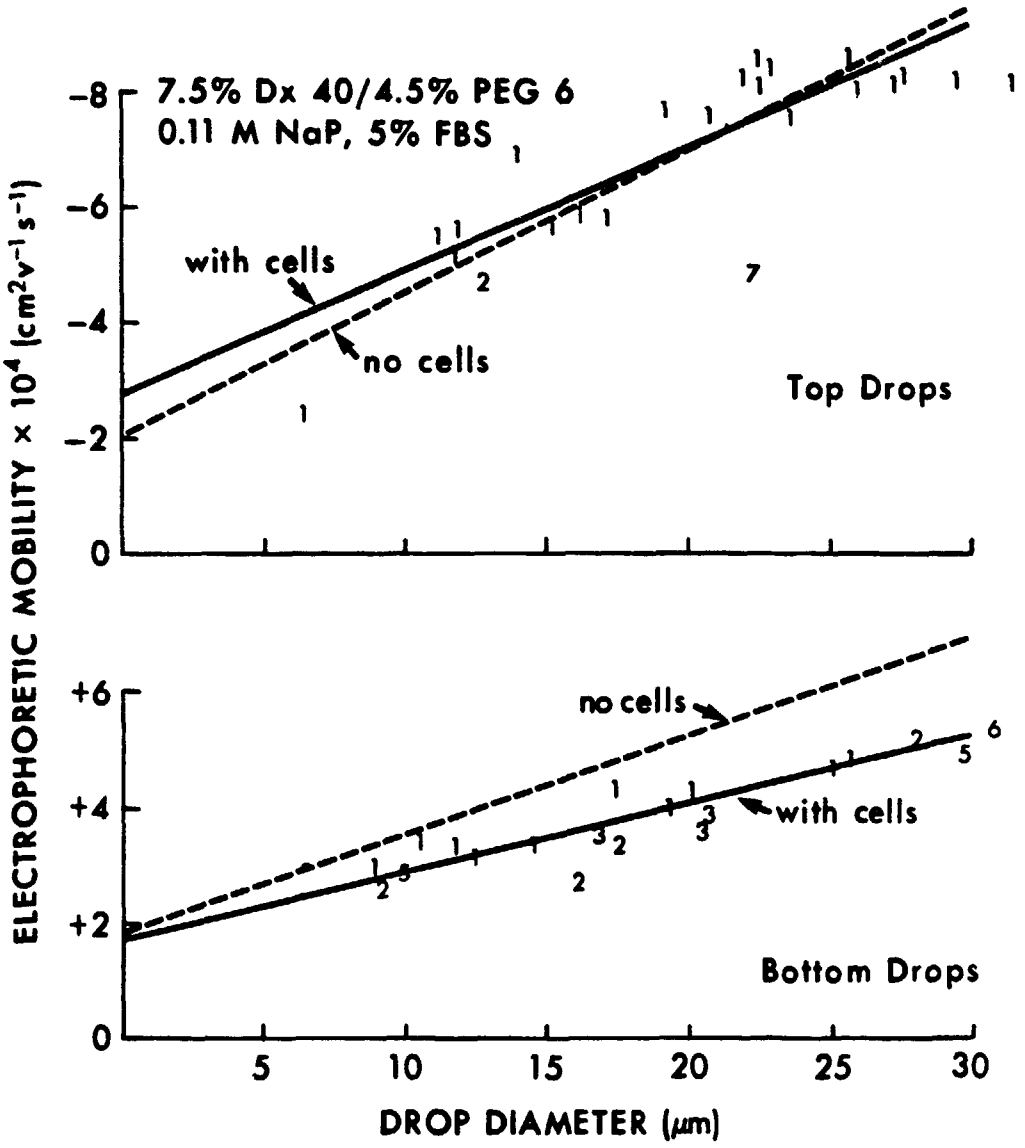


Figure 3.2.5.1. Phase drop mobilities as a function of drop diameter for the system 7.5% dextran 40/4.5% PEG 6/0.11 M Na phosphate, 5% FBS + human red cells. Dotted lines indicate data with no cells present. Numbers refer to numbers of red cells adsorbed to drop. Solid lines indicate best fit to data for one cell per drop.

The cells adsorbed to the interface were always found on the outside of the phase drops regardless of which phase was dispersed. Cells were not found suspended inside the drops, but it should be noted that partition coefficients are low for erythrocytes in this particular system, as discussed earlier. When the field was turned on the adsorbed cells were swept to the trailing edge of the drops but in no case were any cells dislodged from the interface, even when the drop diameter was less than that of the adsorbed cell.

Figure 3.2.5.1 shows that for the top drops with negative mobility, adsorbing one or two cells to the surface has very little effect on the mobility. Cell adsorption to the bottom drops, which have a positive mobility, produced a more significant effect, depressing the mobility by about 25% on the average. There did not seem to be a strong dependence of mobility decrease on the fraction of the surface covered once the first cell was present, particularly with the bottom drops.

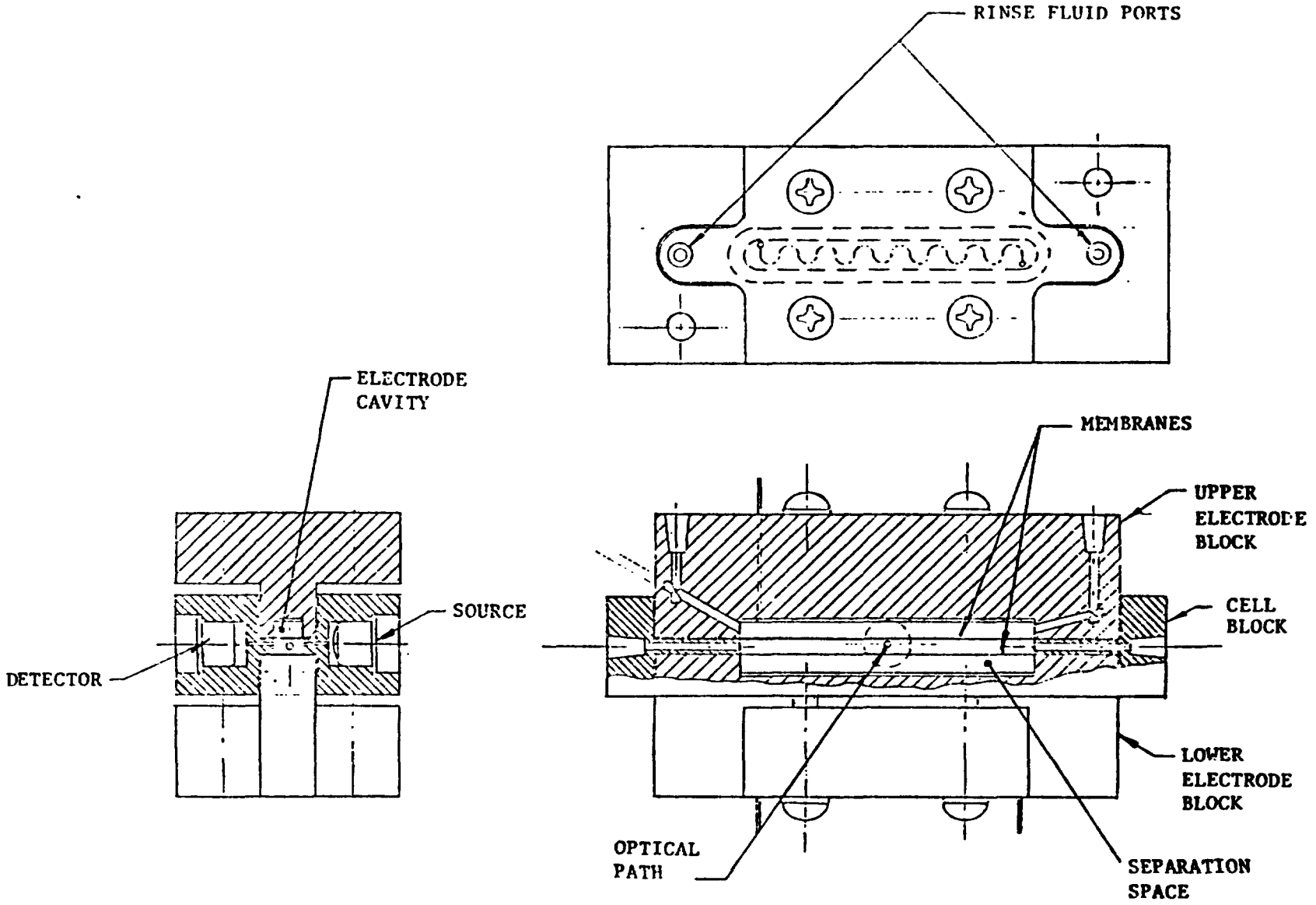
It seems likely that the differential effect of cell adsorption on top and bottom drops is due to the sign of the red cell zeta potential. Erythrocytes have a negative mobility which is at least an order of magnitude lower than the top drop mobilities. Partially covering the surface of the negative mobility drop with cells would therefore be expected to reduce its mobility less than that of a bottom phase drop with positive mobility, in agreement with the observations. It should be noted that it is not necessarily the magnitude of the cell zeta potential which is responsible for the reduction in drop mobility. It is known in other systems that immobilizing the free liquid interface of drops with adsorbed material reduces the rate of transport of such drops (2), since tangential shear can no longer be transmitted across the boundary. Hence adsorbing even a totally

neutral material to the drop surface could be expected to reduce the mobility. That the effect of covering up to approximately 20% of the drop surface with cells has such a small depressing effect on the mobility is very encouraging with regard to the prospect of field driven phase separation in cell partition experiments.

3.3 Studies on Electric Field Driven Phase Separation

In the time remaining in the contractual period following completion of the mobility studies a series of experiments on field driven phase separation of the 7.5% dextran 40/4.5% PEG 6, 0.11 M Na phosphate, 5% FBS system was carried out. The apparatus used, shown schematically in Figure 3.3.1, is that constructed by Beckman Instruments described in Section 1.3.

Measurements were made as follows. The test system was made up at a particular volume concentration of bottom fraction. It was introduced into the sample chamber through the inlet port utilizing a syringe, which contained a small pellet, and a short length of tubing that allowed the syringe to be shaken and the system mixed. The inlet and outlet lines were then clamped off to prevent redistribution of the phase volumes via exchange with material in the tubing. In some cases a T was put into the outlet tube on the chamber side of the clamp and a syringe introduced which could be used to apply approximately 6 cm H_2O pressure to the phase system in the chamber. The same phosphate buffer as was used in the phase systems was passed through the upper and lower electrode chambers to rinse out electrode products at a pressure of about 60 cm of water and flow rates up to 300 ml/min. The effluent was collected without recirculation. The output from the photo-detector circuit was displayed on a 100 mv full scale Beckman chart recorder, the maximum output being set with pure top phase in the sample chamber.



ASSEMBLED CCD CELL

Figure 3.3.1. Electric field driven phase separation apparatus. In the present configuration the light source indicated has been replaced with a ruby laser external to the chamber.

Voltage was applied to the electrodes, top electrode positive, via a Hewlett Packard Model 6271B DC power supply. The applied voltage and resulting current were monitored with digital multimeters. In general the parameters which were varied were the volume fraction of bottom phase present, the magnitude of the applied voltage and rate of buffer flow through the electrode chambers.

Figure 3.3.2. shows the trace of the photodetector output as a function of time for a system containing 10% v/v bottom phase, in the absence of an electric field and, in the insert, the effect of applying an electric field E of about 4.5 v cm^{-1} (calculated from $E = i/\kappa A$ where i = 130 to 160 ma, κ = specific conductivity $\sim 1.4 \times 10^{-2}$ mho cm^{-1}, buffer flow $\sim$ 300 ml min^{-1}). It is seen that over the first minute the optical clearing of the system, which is a measure of phase separation, is dramatically increased by the field. The time taken to reach 10 mv, an arbitrary measure of the separation rate, is over two orders of magnitude shorter in the presence of the field than in its absence. The increased amplitude of oscillation in the trace as the system clears is presumably caused by the increase in droplet size as the phases coalesce and migrate towards the upper and lower boundaries of the separation chamber.

It is noteworthy that the optical clearing in the absence of a field takes a rather long time, an hour or more at low bottom phase concentrations. This time is reduced somewhat as more bottom phase is added, as Table 3.3.1 shows, but even in a 50% mixture the trace takes from 30 to 60 min to reach 50 mv. This can be compared with the approximately 6 min allowed for phase separation in a CCD apparatus with sample chambers of approximately the same dimensions, a time which is generally sufficient to provide acceptable isolation of the phases.

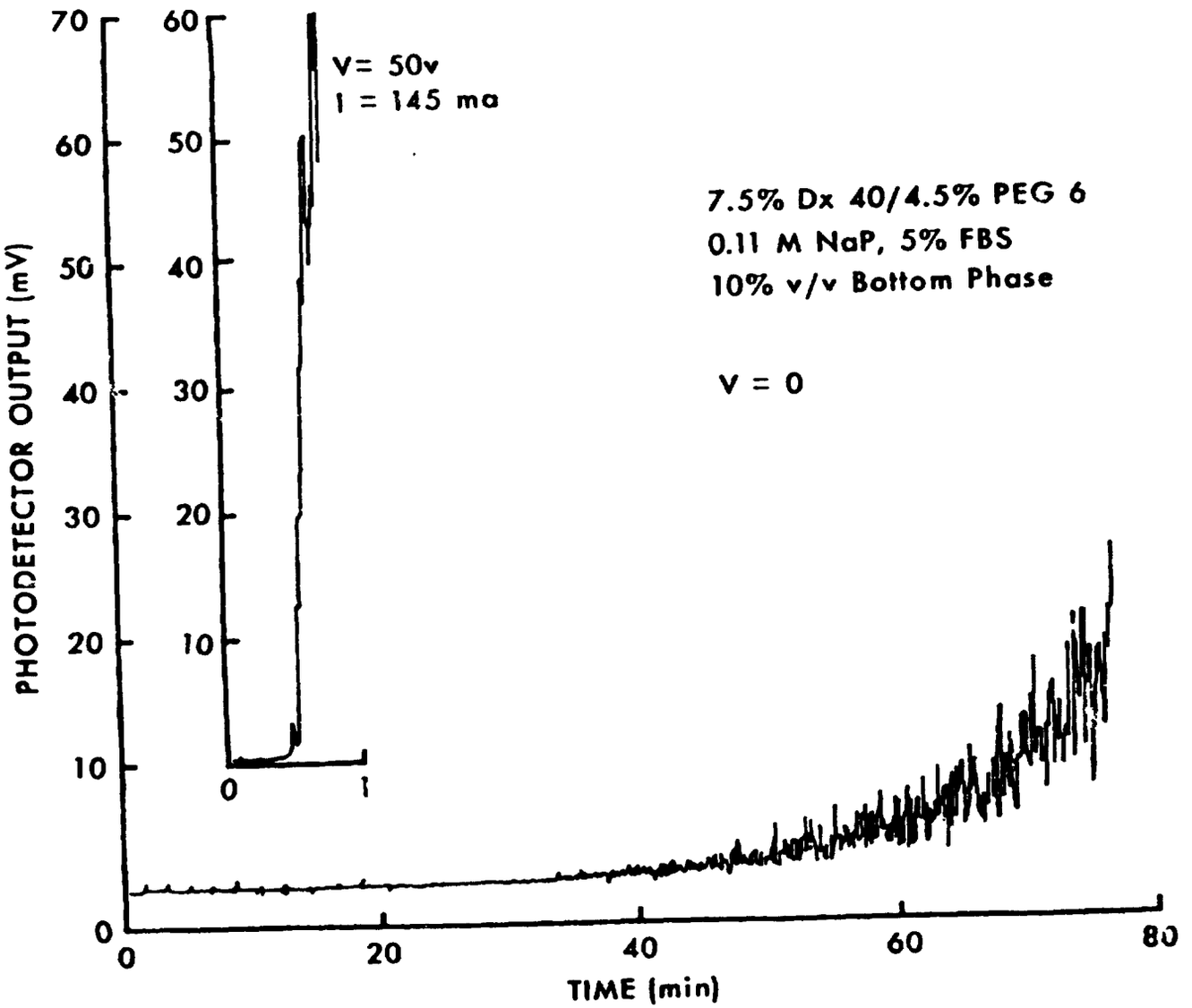


Figure 3.3.2. Optical clearing of 7.5% dextran 40/4.5% PEG 6, 0.11 M Na phosphate, 5% FBS phase system in absence (long trace), and presence (inset), of an electric field of 4.5 v cm^{-1}. Note expanded time scale in inset.

Volume fraction of bottom phase in system	Time for output to reach 50 mv (min)
10%	> 80
20%	66
30%	34

Table 3.3.1. Approximate time required for photodetector output to reach 50 mv (50% of maximum) for various phase volume ratios. E = 0; no buffer flow.

The discrepancy could be due to at least two factors. First, the exact relationship between the photodetector output and the degree of phase separation (fraction of total bottom phase present in top) is not yet known. It is likely that a system which for practical purposes seems separated would in fact retain considerable optical density due to the presence of very small, slowly settling drops of bottom phase suspended in the top volume; such drops can often be seen if the top phase is examined via microscopy. Hence, a system which appears to scatter considerable light might still be effectively separated.

The second factor which could be relevant is the presence of the upper boundary of membrane in sample chamber of the apparatus being used. This boundary, the Amicon membrane separating the sample from the top electrode chamber, has no counterpart in the normal CCD apparatus chamber as an air space is left above the sample to allow efficient mixing when the machine shakes. It is possible that the presence of a material top boundary sufficiently retards convective flows associated with phase separation that the separation takes considerably longer than when the upper surface is free.

The flow of rinse buffer did not appear to have any significant effect on the optical clearing traces when no electric field was applied. Buffer

flow was always necessary when the voltage was turned on, however, in order to remove gas bubbles from the electrodes and maintain the passage of current. With the buffer flow on full ($\sim$ 300 ml min^{-1}) no difficulty was experienced with maintaining current at any desired level (up to at least 160 ma) although there was considerable fluctuation about the mean value at any voltage setting, presumably due to fluctuations in resistance associated with the process of bubble generation and removal.

The major problem with the field driven separation experiments occurs when the field is left on for more than about a minute at field strengths of the order of 4 v cm^{-1}. Although a rapid initial clearing was observed with all systems examined, including those containing 50% v/v bottom phase, the trace generally reached a peak then decreased again with time, sometimes to baseline. When samples were removed from the apparatus following this decline in signal a single phase was obtained. It was not possible to determine the composition of this solution due to its small volume. As may be seen from Table 3.3.2 which gives the results of a single experiment, the peak appears more rapidly the higher the applied field and, although no specific studies were made, appears to occur more readily in systems containing higher volume fractions of bottom phase.

Voltage (v)	Current (ma)	Electric field (v cm^{-1})	Time to 10 mv (min)	Time to peak (min)
0	0	0	20	no peak
5	9	0.26	3.5	4.4
10	24	0.69	1.4	1.7
20	60	1.7	0.5	1.8
30	75-86	2.3	0.7	0.7
35	90	2.6	0.3	0.5
40	105	3.0	1.2	1.3
50	155	4.4	1.0	1.0

Table 3.3.2. Characteristics of the optical clearing traces for a series of samples to which different voltages were applied.

Applying hydrostatic pressure to the sample in the chamber (∿ 6 cm H_2O) had no significant effect on the appearance of the peak, nor did varying the buffer flow. Care was taken to ensure the height of the buffer reservoir above the sample chamber equalled the distance below the chamber the outlet tube hung so that the buffer in the electrode chamber was at approximately ambient pressure.

The system behaves as if, once current is passed, either polymer selectively passes through the Amicon membranes or, more likely since both polymers are neutral, electroosmosis through the Amicon membranes drives phase solution out of the chamber and rinse buffer in to such an extent that the polymer concentrations drop below the critical point and a single phase solution results once the system is removed from the chamber and mixed. An electroosmotic mechanism is consistent with the shortened time of appearance of the peak with higher fields as under these conditions flow in and out of the chamber would be more rapid. Presumably the secondary increase in optical density is associated with the concentration gradients which would be expected to appear during the postulated process.

It seems very likely, given the system behavior over short times, that the initial increase in photodetector output is a reflection of field driven phase separation. A second phenomena, dependent on the presence and magnitude of the electric field, intervenes however which results in an alteration in the sample such that a single phase eventually is formed. Neither the nature of this secondary event nor strategies to overcome its consequences could be examined due to time and effort limitations in the current contract. Further work in this area, along the lines suggested below, is clearly indicated.

4. DISCUSSION AND RECOMMENDATIONS

The work described above has added considerably to our knowledge of the behavior of phase systems in an electric field, of the effects of adsorbed cells on the electrokinetic transport of phase drops and to some of the practical problems associated with field driven phase separation. A biocompatible phase system has been developed which bears high droplet electrophoretic mobilities and the effect of an applied electric field in accelerating the initial stages of phase separation has been demonstrated.

Within the limits of the present effort it has not been possible to examine the effects of varying the surface free energies of the two halves of the sample changer. However, since the interfacial free energy between the phases is very low the differential wetting effects of each will be small and it seems highly unlikely that surface effects would be large enough to significantly effect phase separation over times of the order of a few minutes.

It is recommended that future work be carried out in the following areas.

1. Theoretical investigations need to be carried out with a view to understanding in detail the electrophoretic behavior of isolated phase drops and the dependence of the mobility on system parameters. The physicochemical mechanisms responsible for the electrokinetic behavior ought to be examined and tied in with those factors believed to be responsible for the partition of cells in these systems. The effects of including solid particles in the phase drops and at the interface need to be understood, as do droplet concentration effects on the overall electrokinetic transport of a phase drop emulsion.

2. The process of electric field driven phase separation needs further investigation. In particular, the phenomenon described in Section 3.3 leading to the dilution of the phase system when the electric field is turned on must be understood and the effect corrected. There would seem to be a number of approaches that could be taken. Electroosmotic flow through the Amicon membranes should be measured. If significant effects are found, neutral membranes should be sought which would not produce electroosmosis. Alternatively, it might be possible to replace one of the two limiting membranes with a type which produced equivalent electroosmosis of the opposite sign to the Amicon membranes. Appropriate arrangements of the two could cancel out electroosmotic effects. Another possibility would be to use membranes with such a small effective pore size that neither polymer could pass through, thus eliminating the possibility of changing the concentration of either in the sample chamber.

3. Techniques should be examined for rendering neutral polymer phase systems capable of supporting cell viability for the order of hours at 4°C without the necessity of including serum in the systems. Since serum contains so many complex materials it can be expected to interact with cell surfaces and affinity ligands incorporated in phase systems in ways which will degrade the behavior of the systems as separation media. The utility of adding specific growth factors (insulin; platelet-derived fibroblast growth factor) to maintain the viability of particular systems of interest ought to be explored.

4. Simple experiments to examine phase separation in a low g environment ought to be flown in the Fluids Experiment System in Spacelab, both in the absence and presence of electric field. The basic separation kinetics of, for instance, the 7.5% dextran 40/4.5% PEG 6 system ought to be examined as

a function of applied field strength, droplet mobility, and bottom phase volume concentration, first in the absence, then in the presence of a population of stabilized cells such as aldehyde-fixed erythrocytes. In order to do this simply an understanding of the relationship between the optical clearing of a system and the concentrations of both polymers in the top and bottom volumes must be gained. This can most easily be done by utilizing polymers labelled with two different isotopes, such as ^{14}C and ^{3}H, and correlating the distribution of the two labels in the top and bottom volumes with the optical characteristics of the system as a function of time.

5. ACKNOWLEDGEMENTS

The experimental data owes its extent and accuracy to the diligence and talent of Ms. Cherry Tamblyn and particularly Charles Zukoski IV who carried out the measurements described in this report and to whom I am deeply indebted. I thank also Ms. Janet Cowan for meticulous secretarial and accounting services.

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