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FINAL REPORT

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New Polymers for Low-Gravity Purification of Cells by Phase Partitioning

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ABSTRACT A potentially powerful technique for separating different biological cell types is based on the partitioning of these cells between the immiscible aqueous phases formed by solution of certain polymers in water. This process is gravity-limited because cells sediment rather than associate with the phase most favored on the basis of cell-phase interactions. In the present contract we have been involved in the synthesis of new polymers both to aid in understanding the partitioning process and to improve the quality of separations. The prime driving force behind the design of these polymers is to produce materials which will aid in space experiments to separate important cell types and to study the partitioning process in the absence of gravity (i.e., in an equilibrium state). Two additional tasks have been: (1) Examination of the Ito countercurrent chromatograph (a device on loan from NIH) to determine its suitability for performing automated cell separations by phase partitioning; and (2) design of apparatus suitable for performing simple phase partitioning space experiments, including examination of mechanisms for separating phases in the absence of gravity.



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Table of Contents

Abstract	page 1
Background	3
Goals of the Present Contract	3
New Polymers	4
Crown Ethers	4
Hydrocarbon Ethers	5
Proteins	5
Countercurrent Chromatography	6
Zero-G Apparatus	6
Contract-Supported Publications	7
Appendixes I-V	

Background

Modern biomedical research, whether molecular or cellular, depends heavily upon effective techniques for separation and purification. Separation methods for molecular biology are well developed. However, the preparation of homogeneous, functionally specific populations of living cells remains one of the chief obstacles to progress in cell biology today. Phase partitioning, a sensitive and increasingly important method for purifying samples of biological cells, involves a relatively simple process. The impure cell sample is added to a container holding two immiscible liquids (phases). The container is shaken thoroughly to disperse the cells uniformly and mix the two phases, which are insoluble in each other. The container is then set aside and gravity causes the two phases, which are of different densities, to separate into a top layer and a bottom layer. Because of its biochemical nature, each cell type has a preference to be surrounded by one phase or the other, and thus will tend to become concentrated in either the top or the bottom layer. This allows separation of the cell sample into top-preferring and bottom-preferring fractions. However, the cells are more dense than the phase solutions, and thus tend to sediment to the bottom of the container before reaching equilibrium with the preferred phase. This limits the resolution of phase partitioning, especially when the sample to be purified contains large or dense cells.

The rationale for carrying out phase partitioning experiments in microgravity is threefold: (1) It will allow theoretical examination of the complex micro-events of this process. The delicate studies needed cannot be performed on Earth because of gravity-induced movement and interaction of phase globules during the phase separation process. (2) It will allow partitioning of large cells and other particles that sediment too rapidly for purifications in unit gravity. (3) It may permit higher resolution separations of all cell partitioning procedures, since the process can be allowed to proceed to equilibrium only in the absence of gravity. However, phase partitioning in microgravity will obviously require some alternate method of separating the two phases from each other.

Goals of the Present Contract

The goals of the present contract as described in the original Scope of Work were to prepare new polymers which would provide improved separations and which would facilitate space experiments. An expansion of the scope resulted from the October 82 continuation of the contract to include: (1) examination of the Ito countercurrent chromatograph (a device on loan from NIH) to determine its suitability for performing

automated cell separations by phase partitioning; and (2) design of apparatus suitable for performing simple phase partitioning space experiments, including development of polymer-coated vessels.

New Polymers (details in Appendixes I and II)

The polymer phase systems generally used in phase partitioning are made from aqueous solutions of the polymers poly ethylene glycol (PEG) and dextran. The great majority of polymers we have made have been modified PEG's, although a few dextran derivatives have also been prepared. Our basic approach has been to attach to the polymers ligands which have high affinity for cells. The new polymers which we have synthesized in this work are as follows: (1) PEG's with attached crown ethers; (2) PEG's with attached long-chain hydrocarbons; (3) PEG's with attached proteins (lectins in particular); and (4) dextrans with attached long-chain hydrocarbons. A rationale for preparing each of the polymer types follows along with a brief summary of the work on each polymer type.

Crown Ethers. Crown ethers are cyclic compounds, composed of varying numbers of ethylene-oxide units, whose structures resemble crowns with oxygen atoms at the points of the crown. They are chemically interesting because of their ability to form complexes selectively with metal cations. This property is of interest in the present context as it introduces the ability to prepare PEG derivatives which will bind metal cations and transfer them to the PEG phase of a polymer phase system. Such a charged PEG phase would then be expected to interact with the charged portions of a cell surface. Consequently it might be expected that cell types with a larger portion of charged groups on its surface would be more strongly attracted to the PEG phase than a cell type with a lower portion of charges on its surface.

We have prepared the PEG-crowns and have found that they do in fact produce a large interfacial potential in PEG-dextran phase systems (details follow in Appendix I). We have not chosen to examine the utility of these polymers for cell purifications because some of the other polymers prepared have proven to be very powerful and have thus received a higher priority. An interesting spin-off of this synthetic work has been the observation of catalytic activity for the crown polymers and also for the PEG's with hydrocarbon "tails;" this catalytic work is described in detail in Appendixes III and IV and in general below.

Hydrocarbon Ethers. The second type of polymer prepared was PEG with long (up to 18 carbons) hydrocarbon tails attached. These polymers were prepared because of the known ability of hydrocarbon tails to bind (in unknown fashion) to nonpolar regions of the cell surface. These polymers proved to be very

effective at selectively binding cells, and offer much potential for cell separations. A major contribution of ours has been to prepare such polymers with highly stable chemical linkages thus making their use much more practical than would otherwise be the case.

As noted above, the PEG's with hydrocabons attached have proven to be important catalysts. This work is presented in detail in Appendix IV.

= Proteins. PEG-proteins constitute our third polymer type, and have more potential than any of our other polymers because of the much greater selectivity for cell binding by protein ligands. The proteins examined have been of two types: lectins and antibodies. Lectins are plant-derived proteins with the surprizing ability to selectively bind to subtly different animal cell types (e.g., T and B cells can be distinguished by the lectin wheat germ agglutinin). Lectins have found many uses in biochemistry because of this cell-binding ability, and consequently can be aquired commercially. They also are in general rather durable proteins that require no unusual handling techniques. Antibodies are produced by organisms to bind to alien substances, and are rekknowned for the great selectivity of this binding.

The major barrier to our use of lectins and antibodies for affinity phase partitioning was the chemical difficulty of attaching a certain number of PEG's to a protein without destroying the activity of the protein. What is required here is mild, selective chemistry. We explored two routes, the first being preparation of a cyanuric chloride-activated PEG for reaction with the protein, and the second being reductive amination of the protein with PEG aldehyde and sodium cyanoborohydride. Both methods are effective, although the cyanuric-chloride method is preferable because it is somewhat faster.

Once we had developed our chemical methods it became necessary to determine how many PEG's were required to cleanly partition a protein to the top PEG phase of a typical phase system. This question was answered by preparing several samples of bovine serum albumin (MW 67,000; our model protein for development work) having differing numbers of PEG-5000's attached. Partitioning of these materials showed that five PEG's were sufficient for clean partitioning.

The next step was to determine if PEG substitution had an effect on protein activity. Previous work by Abuchowski with enzymes indicated that there would be little loss of activity (Appendix I). Similarly, we found that lentil lectin with 40% substitution retained at least 80% of its ability to agglutinate human red blood cells.

We are now at the point of testing wheat germ agglutin

attached to PEG and antihuman-RBC antibodies attached to PEG. Initial results are quite promising. This work will be continued under our new contract.

Countercurrent Chromatography (Appendix V)

Dr. Y. Ito of NIH has designed several versions of a device called a countercurrent chromatograph which provides countercurrent movement through a tube of two immiscible liquids. We have borrowed two of these instruments to determine the suitability of the devices for performing cell separations with the polymer phase systems. To perform this evaluation we have passed model particles (sheep, human, and dog red blood cells) through the machine and used peak shape and computer analysis to calculate the number of transfers (theoretical plates) achieved in the Ito device. To summarize, we have found that the device provides the equivalent of about twenty transfers. The two basic problems we encountered and were unable to solve were: (1) enhanced gravitational fields throw cells against the tubing walls causing them to stick; and (2) dramatic band spreading occurs during removal of cells from the column. It is our opinion that the traditional countercurrent distribution apparatus (Albertsson type) is a more effective apparatus for cell separations with polymer phase systems. On the other hand the Ito device is quite effective for molecular separations with nonpolymer phase systems.

Zero-G Apparatus

As an initial space experiment we plan to examine nongravitational mechanisms for phase separation. The two simplest mechanisms, and the first we will examine, are: (1) Use of the liquid's interfacial energies to coalesce the droplets of each phase and thus form the required separated phases. And (2) use of containers coated with the polymers themselves to cause preferential wetting of the walls with one phase, thus providing a separation.

Two things were needed. First we had to develop chemistry to attach PEG to glass, and second we needed a container which would provide mixing of the cells in micro-gravity. The first goal was achieved by reacting glass first with trimethoxyaminopropylsilane and next with PEG (Appendix I). This procedure effectively coats glass with PEG.

Two designs for apparatus have been considered. The first design is intended to fit in a locker and to have several chambers, Figure 1. The set of cells would be shaken manually, inserted in the holder, and the camera activated. Photographs would be taken at predetermined times by using the automatic timing capability of the available Nikon cameras. Mixing would be provided by steel balls, which would be removed from the

light path by attraction to a magnetic plate at the end of the chamber. This apparatus is very similar to one used on STS-8 by Milan Bier; our plan is to use this apparatus, and to modify it for our needs.

The second apparatus is designed to be hand carried by an astronaut, and consists simply of two tubes with a magnetic plate at the bottom to remove the mixing ball. Such a simple tube with Velcro on the back could be shaken, attached to a wall, and photographed. The information provided would be of great assistance in designing a more sophisticated experiment for the first apparatus.

In order to facilitate ready visualization of the mixing and separation of phases, polymer-dye conjugates will be used. Commercial blue dextran is available. A colleague from West Germany, Werner Muller, has donated samples of red and green PEG. If further variation in color is needed, it can be obtained by synthesis of the PEG-dye conjugates in our laboratory.

Contract-Supported Publications

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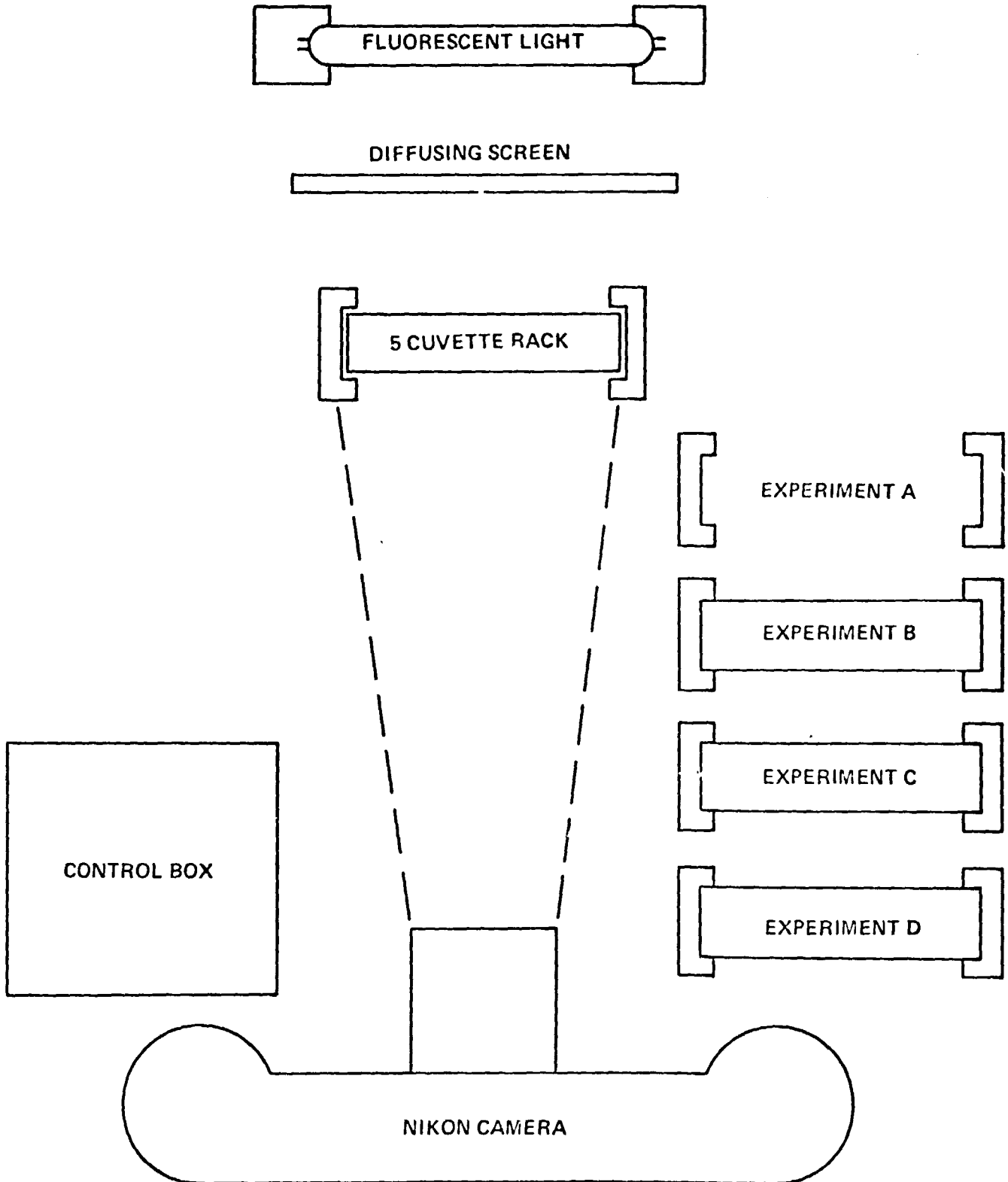
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Harris, J.M., Chenault, A.A., Snyder, R.S., and Case, M.G. "Cell Separations on the Countercurrent Chromatograph," J. Liquid Chrom., in press.

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FIGURE 3. Schematic diagram of apparatus for nongravitational phase separation.



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