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BIOLOGICAL MIXTURES, SOME BIOPHYSICAL PROBLEMS, AND THE STABLE-FLOW FREE-BOUNDARY METHOD

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Howard C. Mel

February 26, 1960

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Howard C. Mel

Donner Laboratory of Biophysics and Medical Physics
University of California, Berkeley, California

February 26, 1960

ABSTRACT

This study is concerned with the following biophysical problems:

1. Weak interactions between biological substances
2. Experimenting with cell suspensions
3. Experimenting with labile biological activities
4. Handling dilute samples in large volumes
5. Good-resolution separations
6. Controlled reaction studies
7. Analytical measurements of transport properties.

The development of a new method for dealing with these problems is discussed. It permits achieving rapid, continuous, convection-stabilized flows in free solution, either alone or in concert with transversely acting force fields. Several new approaches to some of these old problems have thus been opened up. These include (a) a new rapid, continuous "low-stress" electrophoretic method which may pave the way for certain otherwise difficult or impossible biological studies, (b) a new method for mixing and unmixing (in seconds or even less) chemical or biological reactants, with suggestions for studying mechanisms of reaction over discrete intervals and especially during the initial interval, (c) suggestion for a new method for instantaneously measuring transport properties which depend on time in an explicit manner. Some typical examples are presented of the initial experimental results in these areas with small molecules, large molecules, and cells, and future work is outlined and discussed.

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I. INTRODUCTION

As research into biological fine structure and function becomes more sophisticated, the investigator finds himself making ever more critical demands of his system of study. If his interest is directed towards the molecular and cellular levels of organization, he is anxious that the species under consideration remain, during his study, in the forms in which they truly exist in nature. Without knowing exactly what changes are taking place after removing biological material from its natural environment, he can often be quite sure that irreversible processes are in fact occurring. He may be further frustrated upon ascertaining that his own procedures are partly or largely responsible for these changes and yet be forced to settle for such altered material as the best available. His greatest chances for immediate improvement of this situation may be in finding faster ways of handling his material; i.e., speed becomes of the essence.

The investigator may also find himself increasingly dissatisfied to work with the complex mixtures so common in biology, if his object relates to the study of a particular biological activity that most surely constitutes only a small fraction of the mixture. That is, the problem of separation becomes a central one that can be the real key to understanding the process under investigation. This situation is not without analogy in the physical sciences; a successful separation process led to the discovery and understanding of fission by O. Hahn. Fermi's earlier experiment had temporarily eluded understanding precisely because of the lack of these separations. Another well-known example is the vital role played by ion exchange in the discovery of many of the transuranium elements.

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*Work presented orally at the annual meeting of the Biophysical Society, February 26, 1960, at Philadelphia, Pennsylvania.

In biology perhaps more than in any other field, however, are the new and powerful separation methods of ion exchange, chromatography, electrophoresis, etc., proving tremendously important. They permit studies of organization and dynamics that often would not otherwise be feasible. These problems of biological mixtures and some other related ones can be more explicitly stated as follows:

1. Molecules and other particles of biological interest occur naturally in complex mixtures that often act to stabilize the native qualities and biological activities of the particles. For making the most meaningful studies of these activities and relating them to fundamental physical or chemical properties, means for rapid separation of desired components from the mixture may be essential. (If separation procedures and subsequent studies can be completed in a very few minutes, then irreversible processes taking place over a longer time--perhaps a half hour or an hour--may not interfere with the studies.) The sine qua non for effective separations of any kind is means to prevent convective mixing of separated components.

2. Evidence is mounting that labile complexes and associations of molecules and subcellular particles make important if not essential contribution to the unique qualities of the fundamental unit of living machinery--the cell. Not only is speed of handling essential here, but also the procedures must be mild. The weak interactions involved may not survive competition with adsorptive forces caused by contact with supporting media (such as paper, starch, glass powder), so these latter may be ruled out. "Mild," of course, is a relative term. However, free-solution conditions in the presence of (electric) force fields that are small or comparable in size to those naturally experienced by chemical or biological species in their environment are felt to be milder than conditions inherent in many or most other handling methods.

It is desirable and necessary, when dealing with such transient associations, to have means for investigating not only the natural mixtures but also model systems of small molecules, proteins, etc., under controlled conditions, to determine their interaction rates, complex stabilities, etc. (For a discussion of some of these factors and the role of speed with relation to the cell complex, see "Labile Colloidal Complexes," Anderson (1957).)

3. The function of the biological apparatus often depends on the presence of a small number of certain critical molecules, e. g., hormones. To obtain sufficient quantity of such molecules for study may mean, in addition to the above requirements, the necessity for handling large volumes of fluids.

4. The added complexity of cells in comparison with molecules often means greatly increased fragility and attendant difficulty for the experimenter. Partly for this reason, and partly because good methods are lacking, important natural cellular mixtures consisting of many different kinds of cells (e. g., bone marrow) are often of necessity used as a group. It would be of fundamental importance in understanding the physiological relationships between these cells, and between them and the organism as a whole, if such groups of cells could be conveniently fractionated into the individual types for separate study and use.

5. Biological mixtures contain many chemically reacting systems. Detailed knowledge of the rate laws governing the reactions can lead to important insight into the mechanisms of the processes. Since these rate processes are often very rapid, ways of controlling the kinetic processes for study of the various stages (including the initial stages) are very desirable. The less these ways of control disrupt the reaction environment, the more desirable they are.

Interest in biological mixtures, and in particular in the nature and dynamics of the molecular and larger particulate components of these mixtures, has led to an examination of the biophysical means available for their study along the lines outlined above. Realization of the shortcomings of the previous approaches has in turn led to the development of a new approach and a general method suitable for attacking a number of these problem areas. It may be called the Stable-Flow-Free-Boundary method. Not only is this method of interest for the above problems, but it also suggests a possible new type of analytical determination: Measurement of time-dependent transport properties of molecules and other particles at single instants in time. The principles are explained and preliminary experimental evidence is shown in Section VII.

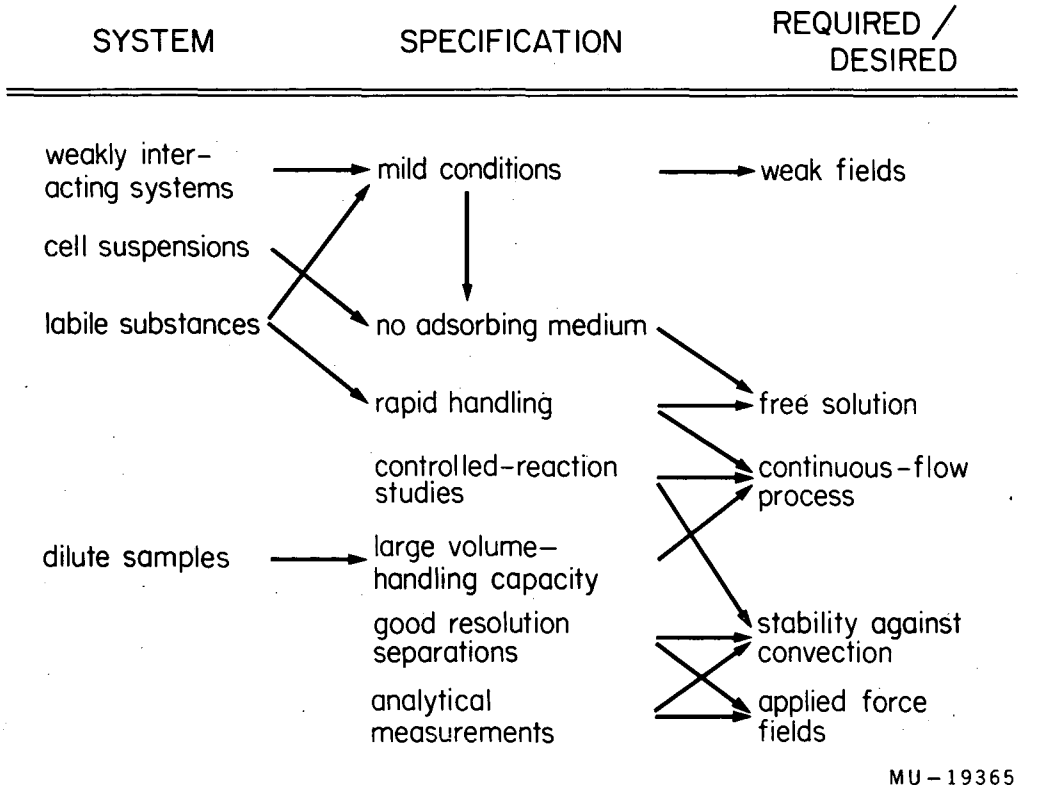


Fig. 1. Interrelations between some biological problems, processes, and requirements.

II. DISCUSSION OF THE PROBLEM AREAS

One can analyze and relate the problem areas mentioned above according to the scheme of Fig. 1, which should be valid for most cases. If all the requirements could be met at one time, then one could carry out all the specifications for each of the systems. The difficulty arises not in meeting the requirements individually, but rather in attempting to meet them all together. Consider a separation process for labile substances. Ignoring for the moment which force field will be chosen, we are left with requirements for a continuous-flow process in free solution, rigorously stabilized against convection. Numerous attempts to accomplish this have been made over the past 20 years, but they have not hitherto succeeded (the customary applied field has been an electric one, i.e. electrophoresis).

One of the earliest attempts was by Philpot (1940), who achieved a certain measure of success but had this to say of his method: "Considerable progress has been made in overcoming the many small difficulties involved; but at the moment of writing, the apparatus must still be classed with those aeroplanes at South Kensington which never left the ground." M. Bier (1959), in commenting on this work, remarked simply "The method never became functional."

On the same subject, Raymond (1958) has written "...experience with apparatus whose operation depends on a constant and continuous flow of solution through the apparatus has demonstrated that many difficulties are encountered in adjusting and maintaining the proper rates of flow." R. Dobry and R. K. Finn (1958), with interests in the same field, also refer to the difficulties in establishing and maintaining steady flow patterns.

Perhaps because of Philpot's lack of success and because the various requirements represented in Fig. 1 had to be met in some manner even if they could not be put all together, this whole field has fragmented enormously. There are free-solution methods such as Tiselius and gradient-type electrophoresis, forced-flow countercurrent electrophoresis, electroconvection, electrodecantation, sedimentation, thermal diffusion, etc., some of which can be operated continuously but not with stable flows; there are continuous-flow methods using paper, glass, high polymer added to solution, etc., and there are convection-stabilized methods on supporting media like starch or those mentioned above, some of which can be operated continuously but

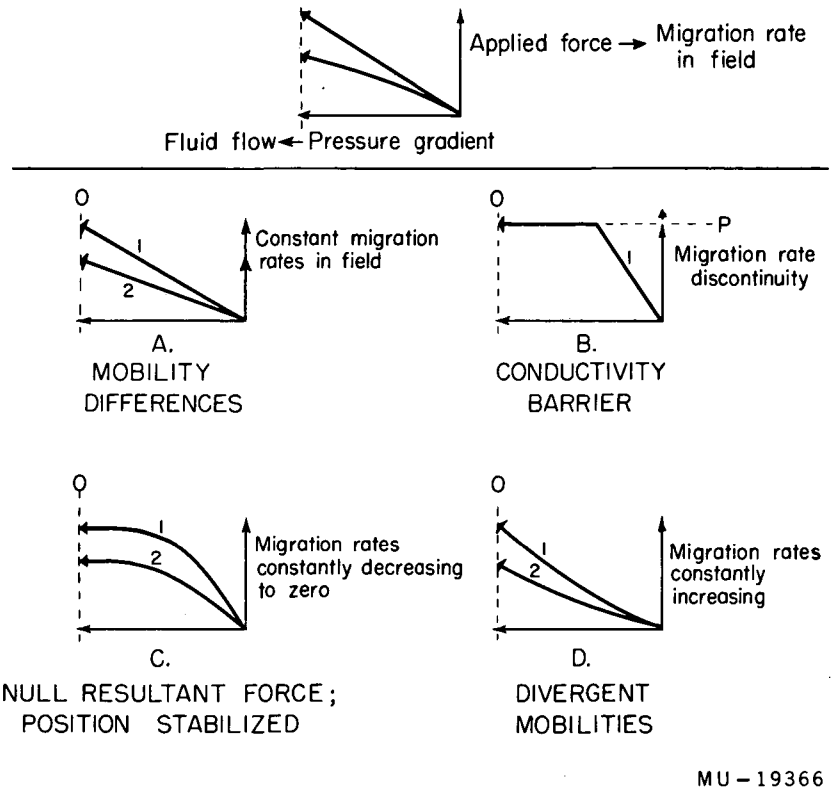


Fig. 2. Migration principles in a uniformly flowing system.

which are obviously not free-solution methods. An extensive up-to-date account of the theory and practice of different approaches using electric force fields can be found in the excellent book edited by Bier (1959).

Examples and discussion bearing on this section, are given in Section V, both with respect to work accomplished in this laboratory and to work planned or indicated.

III. MIGRATION PRINCIPLES

The simultaneous application to a solution of pressure differences (if uniform nonconvective flows can be achieved) and of other fields applied transversely leads to situations illustrated in an idealized manner in the vector diagrams of Figs. 2A, B, C and D. In each case a constant rate of horizontal fluid flow is assumed at each point in solution, but the applied fields, migration rates, or conditions in solution differ in the vertical sense for the three different cases. A system of outlets is assumed, arranged along plane O, to take off the different vertically ordered strata arriving there. For any of the cases shown, depending on the nature and concentration of solutes and solvents in the system, it may be desirable or necessary to have a density gradient(s) to prevent convective disturbance of the pattern.

Figure 2A represents two components with differing responses to the force fields (i. e. different mobilities in the general sense) migrating in a uniform field. By the time plane O is reached, the faster component 1 has moved up from component 2 sufficiently for complete separation to have occurred.

Figure 2B depicts component 1 migrating upward in a uniform field until it reaches a free-boundary "conductivity barrier" at plane P. At this point the field strength drops to a much lower value (by virtue of the increased conductivity of the solution above P), causing component 1 to proceed essentially horizontally to the outlet.

Figure 2C illustrates components moving with mobilities successively decreasing until they vanish at some points in solution corresponding to null resultant forces acting on each of them. Once having reached these positions in solution they are stabilized there and flow horizontally toward the outlets, where they may be separated. For a given sample substance the situation in Fig. 2C can result, for example, from the action of a

nonuniform net force field on particles with fixed physical properties. A simple illustration is particles of constant density suspended in a density gradient: the combination of a constant downward gravitational force (1 g, or more or less) with the varying upward buoyant force leads to sedimentation or flotation of the particles to their stable positions in solution (provided that the proper values can be chosen for the variable parameters). An alternative origin for Fig. 2C is the action of a force field (uniform or non-uniform) on sample substances with variable physical properties. An example is the electrophoretic migration of an amphoteric substance in a pH gradient. Williams and Waterman (1929) clearly stated the principle, pointing out that (with proper polarity) a migrating ampholyte is stabilized at a point in solution where the pH corresponds to that of its isoelectric point. Kolin (1954) reported an interesting rapid microelectrophoretic U-tube method using various gradients of pH, conductivity, and density. This method has remained largely a laboratory curiosity, but in combination with a stabilized flow system it may become a useful and valuable part of the electrophoretic repertoire.

Finally, Fig. 2D represents a situation like that in Fig. 2C, but with the direction of field reversed, leading to diverging mobilities and thus divergence of the streams.

It should be immediately evident that with single or multiple sample streams and single or multiple components per stream, a wide variety of combinations of these principles may be employed during a single experiment. This leads to great flexibility of experimental design. Certain of these principles are implicit in the various examples discussed later.

IV. BRIEF DESCRIPTION OF NEW METHOD

The method should really be considered from two aspects, for as is seen in Fig. 1, these can lead to either separate or combined applications. They are the achievement of uniform steady-state free-boundary flow conditions (hence the name stable-flow free-boundary method), and the nature and use of conventional applied force fields. It is beyond the scope of this paper to discuss the latter in detail. Suffice it to say that any force fields known to cause migration of solutes or suspends should potentially be applicable to the stable-flow-boundary method, either separately or in combination. The common ones that come to mind are electric, magnetic, gravitational, and thermal fields. Since almost all the work to date on the method by the author has been carried out with an electric field (or without field), the remainder of this discussion deals primarily with electrophoretic embodiments of the principles previously discussed.

Figure 3 illustrates schematically apparatus No. 1, in which the combined requirements were first successfully achieved. A preliminary report on this apparatus has already appeared (Mel, 1959), and a more complete analysis of this and later versions is appearing elsewhere, so that no detailed description is given here. It will be useful, however, to summarize some of the essential features.

Stabilized free flows are achieved by virtue of laminar flow conditions, density differences between the various strata (these differences can in many cases be made vanishingly small), mechanical stabilization by the dividers (these have now been discarded for most purposes), and of the closed hydraulic nature of the entire flow system. All apparatuses used to date have 12 outlets. They now all have 12 inlets to the main chamber (only three are indicated in Fig. 3, and this was the configuration for those examples in Section V employing appartus No. 1). Apparatuses 2 and 3 provide for 30 and 12 cm, respectively, of free-boundary migration in the field.

Samples are pumped at constant rate through one or more inlets, with the remaining inlets carrying buffer or other solutions. A wide variety of operating conditions has proved satisfactory, though in this complex multivariable system the totality of combinations of workable conditions is far from delineated. Evidence of flow stability is shown in Fig. 4, which

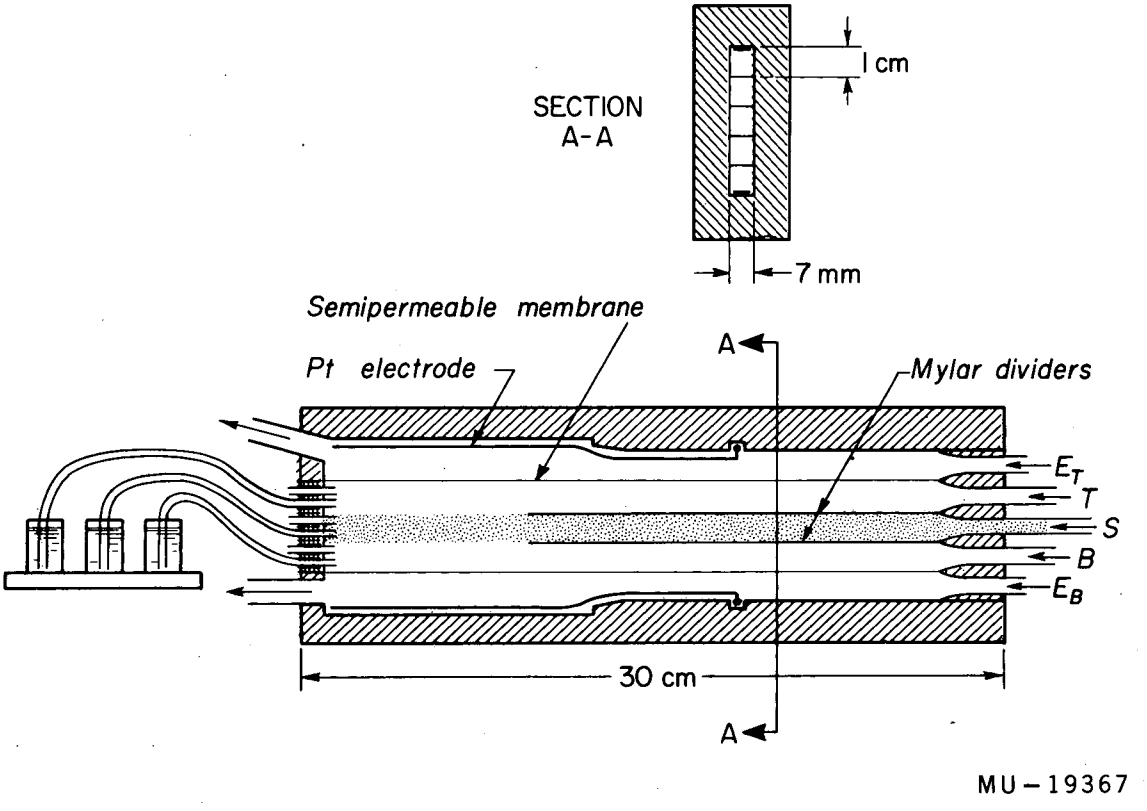
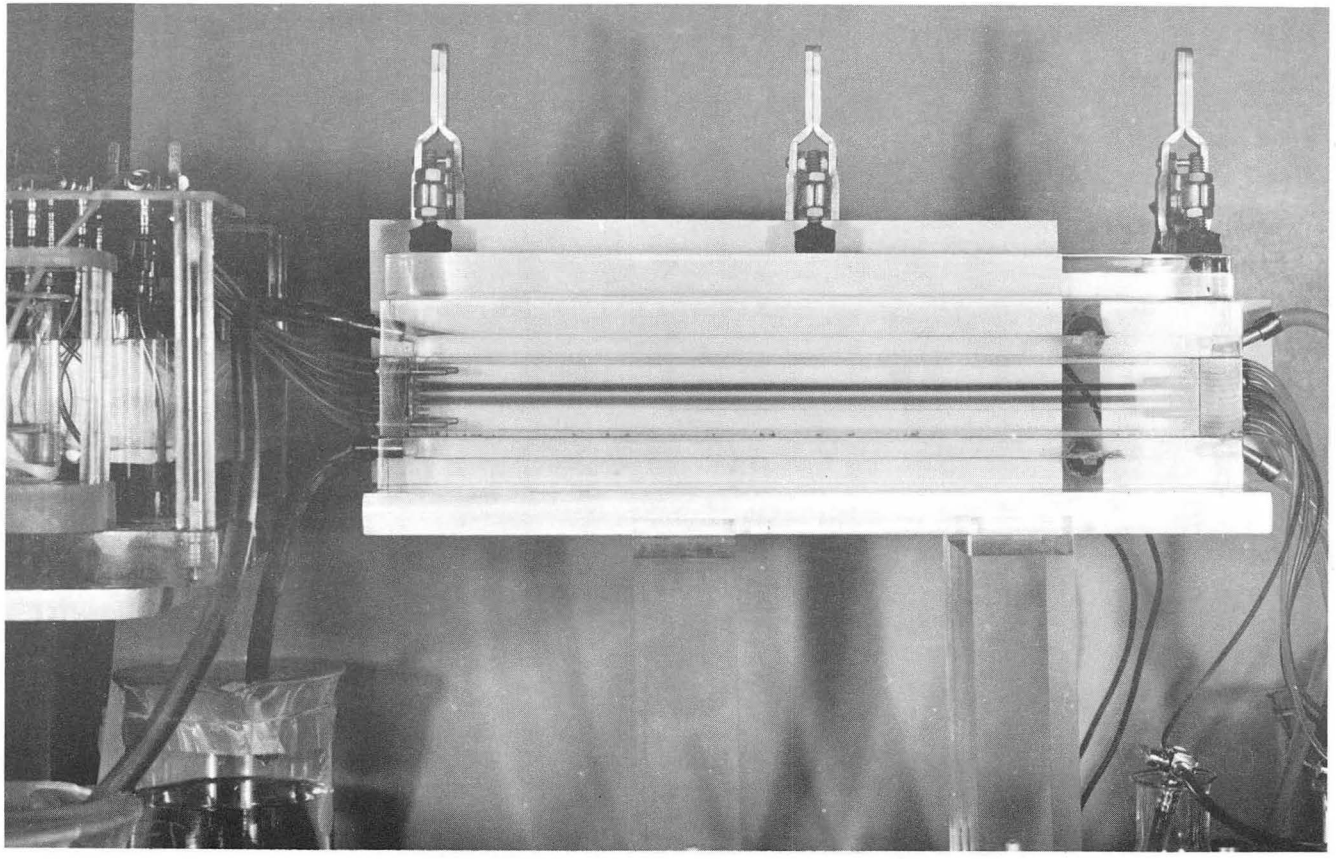


Fig. 3. Diagram of free-flow apparatus No. 1.



ZN-2336

Fig. 4. Photograph of steady-state flow pattern for two separate dye streams without transverse field, indicating stability of flow (see text).

ZN-2335

Fig. 5. Over-all photograph of apparatus and auxiliary equipment.

represents a double dye sample system in apparatus No. 2 without field, with flows of 1.2 cc/minute/outlet or 14.4 cc/minute total. (For the sake of comparison, note that a typical sample volume flow rate for a continuous paper-curtain electrochromatography apparatus is of the order of a few cc per 24 hours.) The dyes are entering via inlets 5 and 7. The bottom sample "ribbon" is bromphenol blue, and the top is cresyl violet. An analysis of the spectral curves of the collection tubes after a run of this nature has demonstrated the essentially perfect flow stability. (For a photograph indicating the uniformity of the collection during a 27-minute dye separation experiment in apparatus No. 1, see Ref. 8.)

A photograph of the entire apparatus, including auxiliary equipment, is shown in Fig. 5.

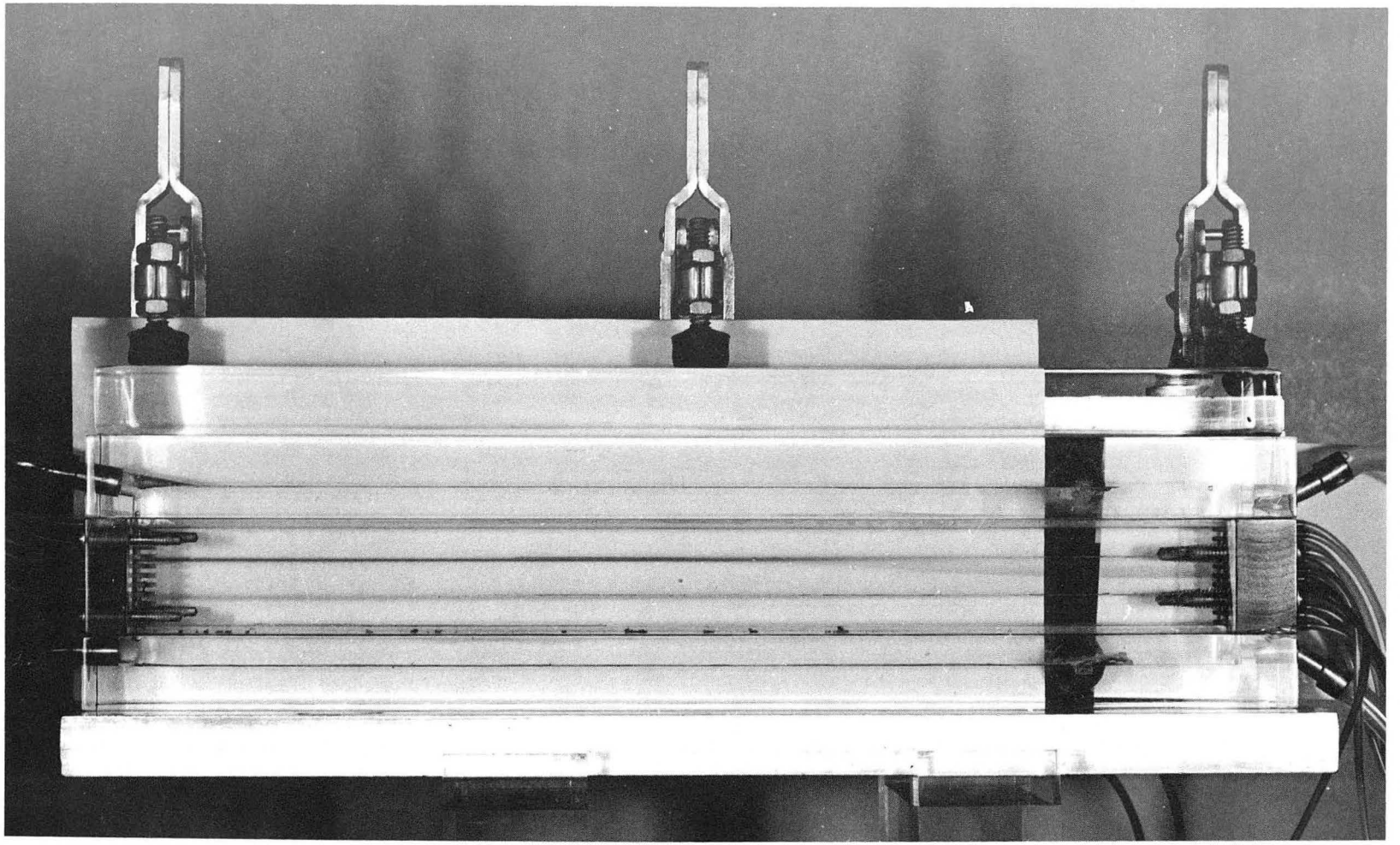


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Fig. 6. Photograph of dye mixture in the condition of steady-state flow with separation into two components at a double conductivity barrier.
(Conditions: 5 v over all, 0.9 cc/min/stream, 10.8 cc/min total.)

V. EXAMPLES

Unless otherwise stated, for all examples using apparatus No. 2 or No. 3, the following conditions obtain: Voltage is the applied voltage between the two electrodes with polarity shown for the top electrode. The top electrode compartment and inlets 1-4 contain pH4 standard phthalate buffer diluted 50-fold to ionic strength .001; bottom electrode compartment and inlets 9-12 contain standard pH7 phosphate buffer diluted 50-fold to ionic strength .002, in 2% sucrose solution. Tubes 5, 6, 7, and 8 contain 0.4%, 0.6%, 0.8%, and 1% sucrose respectively. For apparatus No. 1, top electrode compartment solution is identical with top solution and similarly for bottom electrode compartment solution and bottom solution; sample solution is 1% in sucrose, bottom solution is 2% in sucrose.

Small Molecules

Example 1. Dye-concentration experiments

Details are given in Reference 8 for a rapid continuous separation of bromphenol blue and cresyl violet, using apparatus No. 1. Figure 6 is a photograph of a similar mixture being resolved, by use of a double conductivity barrier in apparatus No. 2, with the mixture entering via inlet No. 7. Conditions: +~~5~~ 10 volt; Flow 0.45 cc/minute/tube, 5.4 cc/min total. Note that the separated dyes have reached their respective conductivity barriers before traversing half the length of the apparatus, i.e., before 5.8 minutes have elapsed. Complete separation will obviously have occurred much sooner in this case. The solutions are very dilute because of their tendency to react with each other at higher concentrations (see Section VI).

Large Molecules

Example 2. Rapid migration, concentration, preservation of activity of a dilute enzyme solution. Experiment IX-77, apparatus No. 1)
Conditions: Sample .001% lysozyme solution; pH 6.33; top and bottom solutions .001 N NaCl, adjusted to pH 6.9 and 6.0 respectively with minimum quantity of standard phosphate buffer; - 30 v, 1.5 ma; sample flow 1.2 cc/minute.

After steady-state conditions, had been achieved, samples were collected for 13 minutes. This was done "blind," as no optical system was used in this experiment and the solutions were all colorless. All 12 collection bottles were analyzed, in cooperation with Dr. Tor Brustad, by the biological-optical assay method of Shugar (1952).

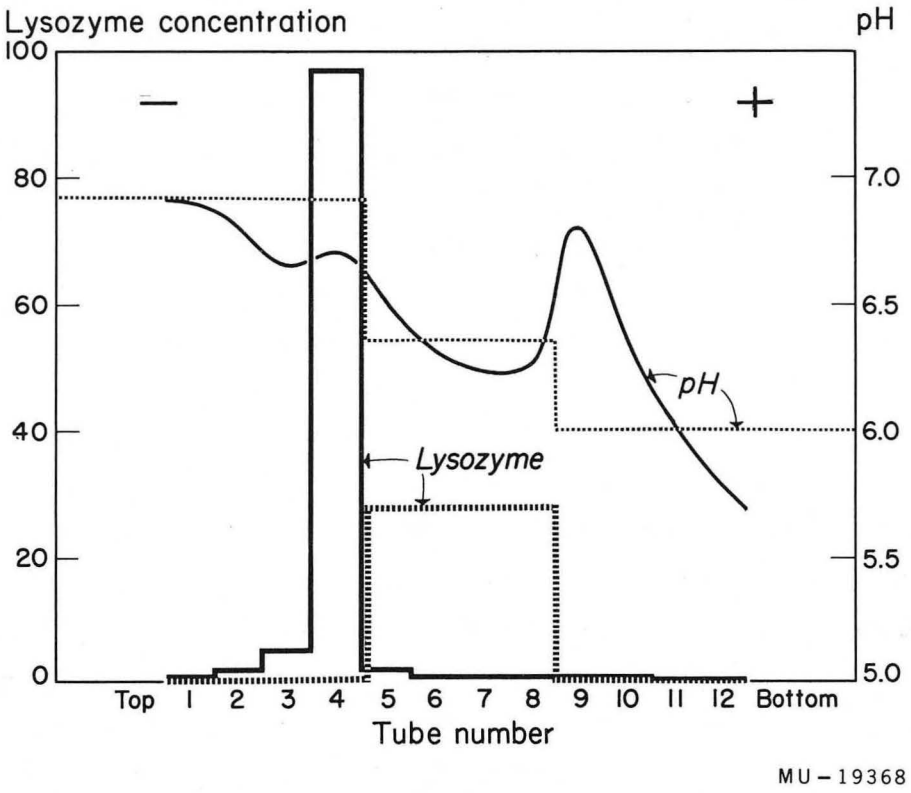

Fig. 7. Lysozyme concentration experiment.

Results: Figure 7 shows the relative concentration of lysoyzme, and the pH, in each tube after the experiment (solid curves), as well as the initial profiles of each of these (dotted curves). Points to note are:

a. Total enzymatic activity recovered was $\geq 95\%$ of the initial sample activity.

b. Enzyme concentration in tube 4 alone has been increased by a factor of 3.3 during the 7 minutes that any one part of it was subjected to the field.

c. The cause of the pH peak for tube 9 is unknown. It appears to be a negatively charged impurity, perhaps from the lysozyme sample, that is being concentrated at the lower conductivity barrier.

With the later forms of the apparatus it should be possible to concentrate such a substance by a factor of 10 (or 12 if migration to the membranes is permitted) in a single passage, while simultaneously separating it from other substances if desired. Of course multiple or tandem passages would compound such concentration factors. It is believed that no other mild means is available that can accomplish this.

Example 3. Protein separations, with and without interactions

Figures 8 and 9 should be considered together, for the comparison shows an interesting difference between human serum albumin and bovine gamma globulin in the presence of bovine "hemoglobin." The two experiments depicted were conducted under similar circumstances: "Hb" concentration .004% ±; albumin and γ globulin .02% ±; -35 v; in apparatus No. 3; flow rates 0.5 cc/min/inlet, 6.0 cc/min total; sample collection for 3 to 5 minutes; sample mixtures were admitted via inlet No. 7. Complete spectra were taken and analyzed, over the region 600 to 200 mμ, for all solutions.

The "hemoglobin" (Worthington) is written in quotes because examination of its absorption spectra indicates that it is probably in the methemoglobin form. The high optical density at 275 mμ for tubes 1-5 is a result of the strong absorption of the top phthalate buffer, as is the lesser absorption in tubes 6-8. The phthalate spectrum differs in shape from the characteristic protein spectrum, so that the two can be sorted out. For conducting extensive studies of this type, relying on ultraviolet spectra for analytical purposes, it probably will be more convenient to use a transparent top buffer system. For measurements in the visible, however, the phthalate is admirably suited.

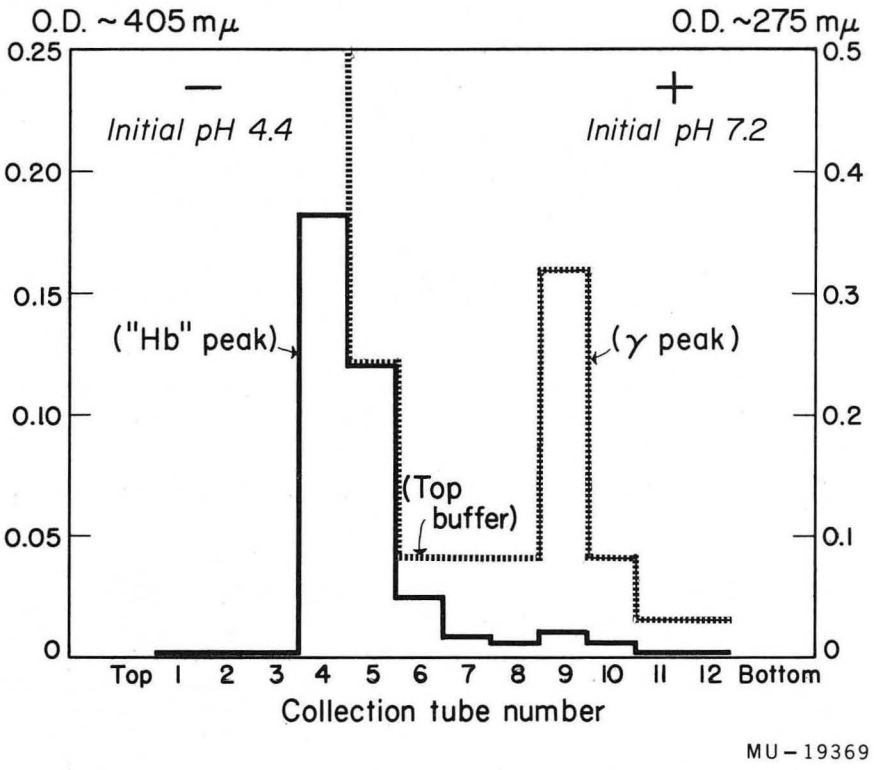

Fig. 8. "Hemoglobin"-gamma-globulin separation.

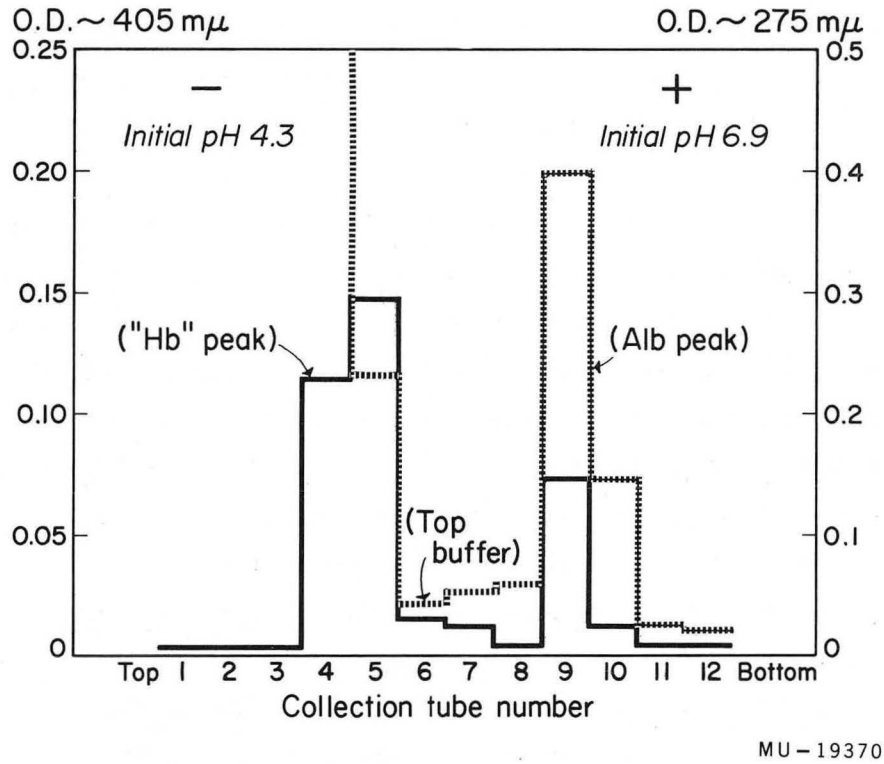

Fig. 9. "Hemoglobin"–Albumin separation, suggesting complexing.

In each case the "Hb" and serum protein have been nicely resolved. The striking feature of the curves, however, is the appearance of a real "Soret" type maximum in tube 9 with albumin, but not in tube 9 with globulin. The pH profile in solution appeared to rule out free migration of the "Hb" to tube 9. Studies on "Hb" alone under similar circumstances confirmed its migration to tubes 4 and 5 as expected. Likewise albumin and γ globulin alone follow the migration pattern indicated in Figs. 8 and 9. A further experiment was conducted with hemoglobin and albumin, but with reversed polarity. This led to reversed directions of migration and to isoelectric-point stabilization (see Section III), but about the same proportion of hemoglobin (20%) migrated with the albumin. These observations suggest strongly that "Hb" complexes with the albumin but not the globulin. The existence or nature of such complexes is a question of current research interest, and this rapid free-boundary method would seem particularly well suited for such investigations. It should be emphasized that these are preliminary results and further studies are necessary to confirm this observation; such work is planned for the near future.

Migration and Separation of Cellular Suspension

Heterogeneity of cellular suspensions in biological research is the rule rather than the exception. The design and interpretation of experiments with cell populations would be enormously simplified in almost every case if the investigator had ready access to various homogeneous subfractions of whatever cellular mixture he works with. The following features bear on the possibility of useful large-scale fractionation of cell populations.

a. Because of the physicochemical nature of cell walls, essentially all cells exhibit migration in an electric field. Different strains of cells (e.g., bacteria) exhibit large differences in mobilities. (See, e.g., Brinton and Lauffer 1959, for tabulated data.)

b. Closely related cells of a given type (e.g., human erythrocytes of different blood groups) exhibit statistically significant mobility differences, permitting their separation under a microscope. (Creger, Tulley, and Hansen, 1955).

c. Yeast cells, as an example, exhibit large and often discontinuous changes of mobility

(1) with changes of pH,

(2) with cell death,

(3) with changes in the chemical nature of the growth medium,

(4) with changes in composition or concentration of the suspending medium.

(See, e. g., Wartenberg, 1956, and Eddy and Rudin, 1958.)

d. There exist large distributions of mobilities even for populations of a given type of cell under well-controlled conditions. These may be due in part at least to the random state of cell division of the population. [12,6]

e. Mobility differences have also been reported for malignant cells in different stages of their evolution and for male-determining vs female-determining sperm. [3]

f. Known cellular mobilities are sufficiently large to assure migration necessary for separation in short times.

g. Preliminary experiments with the present free-flow method have demonstrated its applicability to cellular separations. (See Examples 4 and 5 below.)

A problem under active consideration for investigation with the present free-boundary flow approach is the fractionation of the various classes of bone marrow cells. The precursor-follower relationships are not well known here, nor is the relative antigenicity of the different cell classes or their applicability for repairing bone marrow damaged, e. g., by ionizing radiations.

The considerations a-g above offer by implication the possibility of removing the normal heterogeneity of a pure-strain nonsynchronized cell population by carrying out sufficiently rapid separations of subfractions existing in (more nearly) synchronous conditions. Present methods of forcing synchrony generally also force some unnatural state on the population, e. g., an altered division time. If one could simply select large numbers of cells in a given division stage at any time, this would amount (to paraphrase D. Mazia) to having the biological system give up its information on its own terms, and could greatly facilitate quantitating the dynamic biochemistry and biophysics of normal or abnormal cell division.

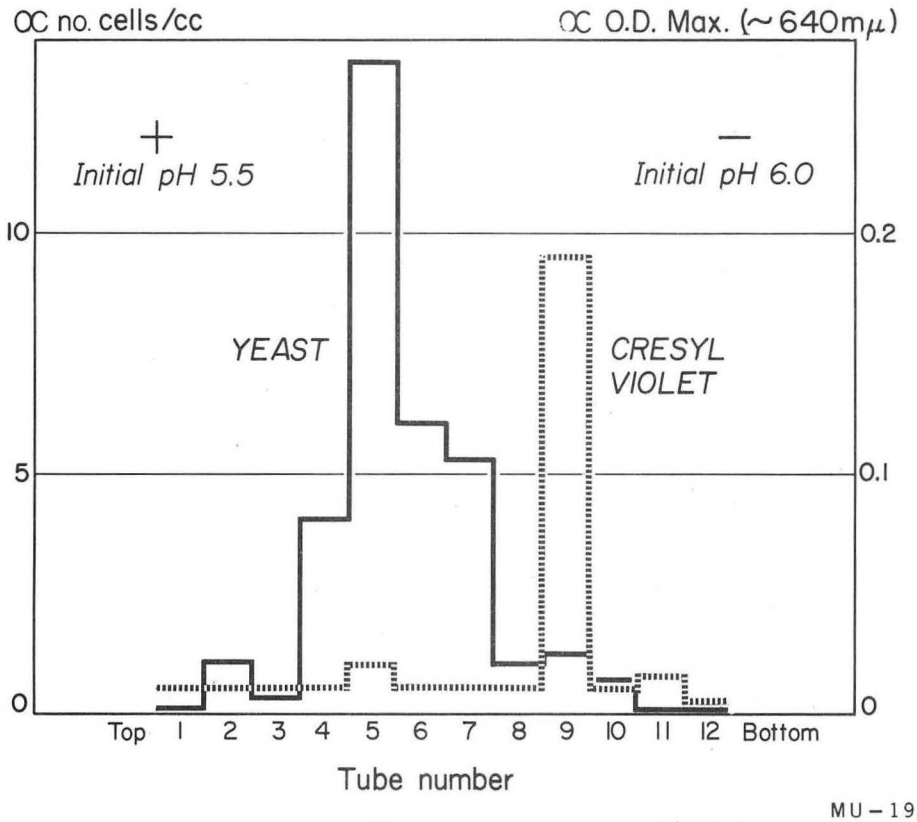

Fig. 10. Yeast cell–cresyl violet separation experiment.

Example 4. Upward migration of yeast cells, with separation from a dye (Experiment IX-74, apparatus No. 1)

Conditions: Suspension of Fleischmann commercial yeast in the starved condition (about 10^5 cells/cc), 0.001% cresyl violet; +30 v, 1.5 ma; sample flow = 1.2 cc/minute; analysis by hemocytometer counts and optical-density measurements in the visible region.

The resolution of the cells (moving against the force of gravity) and dye is evident from examination of Fig. 10. The nature of the cellular distribution pattern is as yet unknown. With the recent development of apparatuses No. 2 and No. 3, providing a significant improvement in resolution and in the facility for setting up controlled pH and conductivity subregions, it is planned to pursue more studies of such cellular distributions, and more refined studies like Example 5, with high priority.

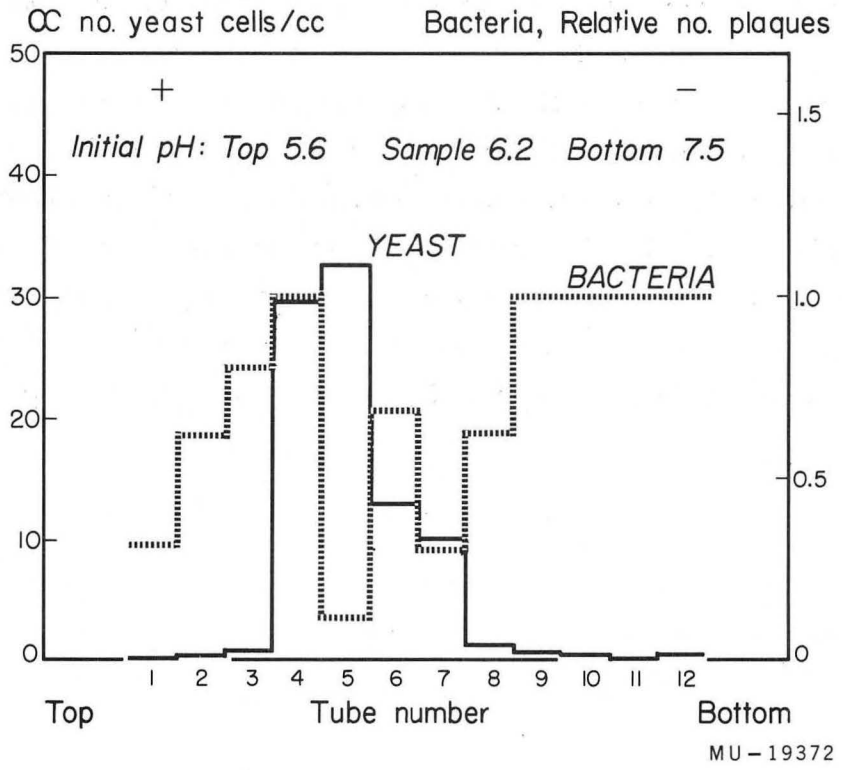

Fig. 11. Yeast-bacteria fractionation.

Example 5. Fractionation of dead yeast cells and tap-water bacteria

(Experiment X-11, apparatus No. 1)

A suspension in tap water of previously killed Fleischmann starved yeast cells (about 10^6 cells/cc) was fractionated as for Example 4, with collection for 24 minutes. Yeast cells were counted in a hemocytometer and aliquots were then pipeted onto a nutrient agar medium and incubated at 30° for 24 hours. Figure 11 shows the relative numbers of yeast cells counted microscopically and large bacterial plaques counted by eye, for each tube. The yeast behaved approximately as expected, but the cause of the curious bacterial distribution is as yet unknown. Note that tube 5 contains the maximum yeast count and minimum bacteria count.

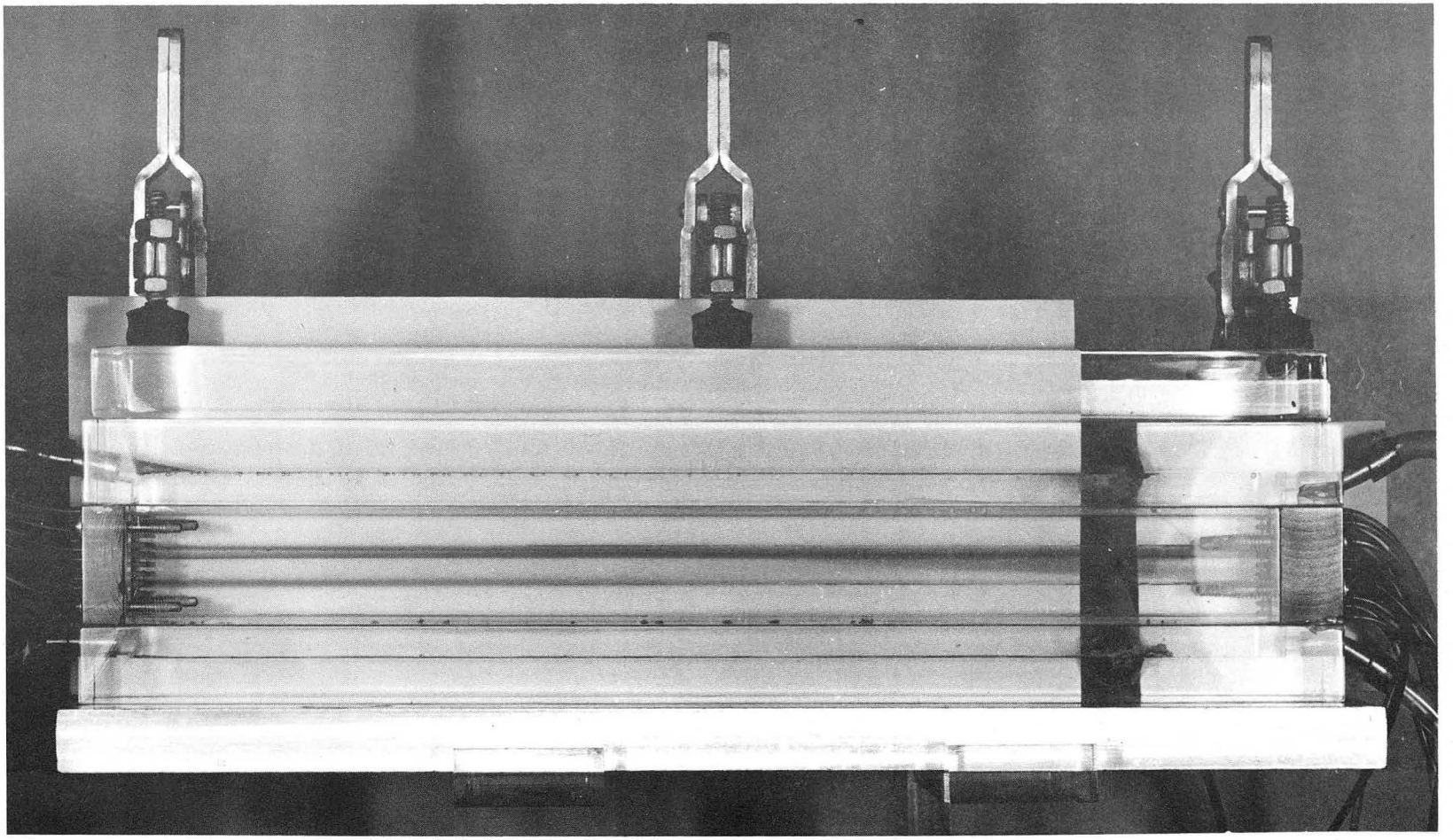

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Fig. 12. Photograph of reaction taking place in "cross-over" experiment; third component separated at outlet. (See text.)

VI. A NEW METHOD FOR CONTROLLING AND STUDYING REACTIONS IN SOLUTION

Model Study (Dye System)

An examination of Fig. 4 and consideration of the migration principles described in Section III points up some interesting implications of independently manipulating several separate sample streams. One such example that comes to mind is to adjust the conditions of polarity, solution pH, etc. relative to the properties of the substances involved, to cause transverse cross-migrations of the separate streams. (It had already been observed that the dyes bromphenol blue and cresyl violet react if present together at much higher concentration than shown in Fig. 6.) This experiment was then tried, electrically forcing the top cresyl violet stream down to a lower free-boundary conductivity barrier while simultaneously bringing the lower bromphenol blue stream up to an upper conductivity barrier. The result is shown in the photograph, Fig. 12. Fluid flow was 1 cc min/stream or 12 cc/min over-all; +~~5~~ 10 v, 1.2 ma. In response to this low field and relatively fast flow the two streams move upward and downward respectively at low angles till contact is established. (The bromphenol blue is clearly seen rising on a bias.) Where the streams contact each other the following appears to take place: Some reaction commenences, including formation of precipitate (shown on the bottom membrane and in the stream). By the time the outlet is reached a reaction product (the middle stream) is resolved from the top (formerly the bottom) sample ribbon. A color photograph taken at the same time as Fig. 12 shows clearly the characteristic colors of the dye streams. If the reactants are mixed outside the apparatus and the mixture admitted through a single inlet, one often gets (depending upon the conditions) a picture such as in Fig. 6 but with an added component or components traveling up the middle. For certain concentration conditions a soluble reaction product is formed in addition to or instead of the precipitate. It appears that the precipitated reaction product can have little or no electrophoretic mobility and still maintain its stable flow position by virtue of the (small) density gradient in the solution. Considering the basic nature of the one dye (cresyl violet) and acidic nature of the other (bromphenol blue) such a neutralization-coagulation reaction appears quite reasonable. The soluble reaction product shows isoelectric-point stabilization between the 2 reactant streams.

By increasing the field or slowing the flows or both, the lines can be made to cross at a sharp angle or in fact at virtually any angle desired. Thus we have a steady-state free-boundary reaction system permitting easy sampling of the various streams. It is suggested for the future to study

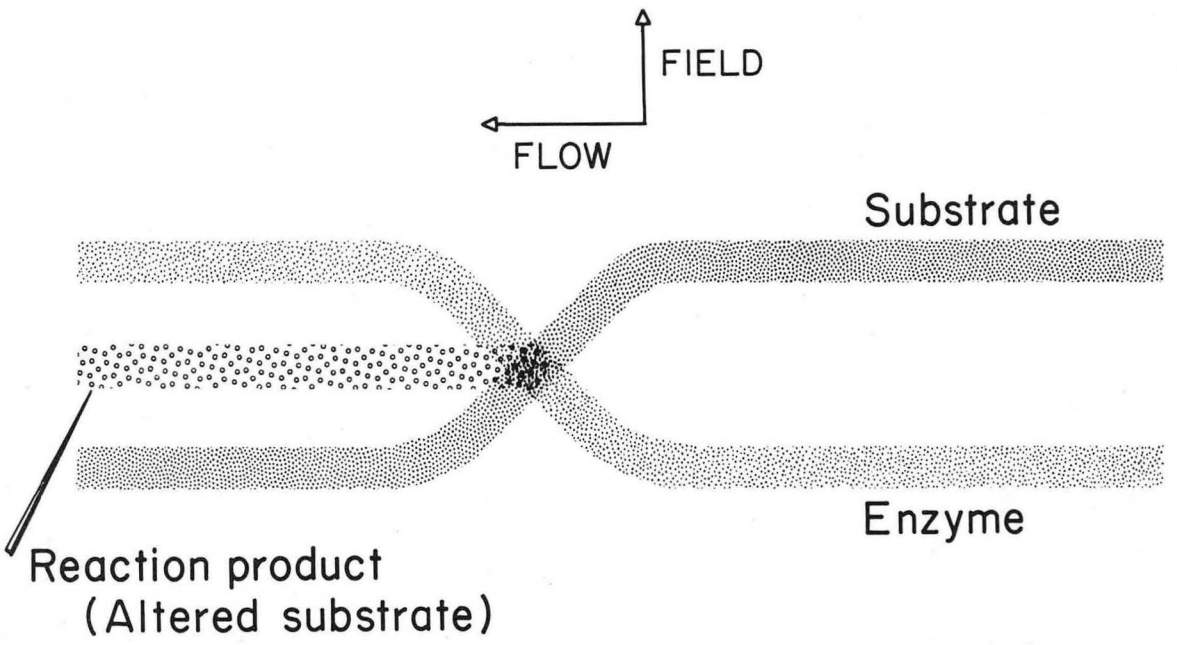

Fig. 13. Diagram of possible enzyme reaction study by "cross-over" method.

this type of interaction with more interesting biological substances, e.g., enzymes as proposed below. As can be seen, such dye experiments can serve very useful functions as model systems for indicating pathways most worthy of follow-up study.

Proposed Enzyme Study

In effect the facility of independent manipulation of the flowing streams allows us to mix and unmix substances in solution in controlled and short periods of time. With the present apparatus and modes of operation the time the components are in the mixed condition can be varied between a few seconds and any arbitrary long time. A lower limit on this "mixed time" that could be achieved by modifying the apparatus is not known at present, but it would appear to be considerably less than a few seconds. A simple example proposed is that of an enzyme interacting with its substrate. The details of a possible process are indicated schematically in Fig. 13. The two streams are made to "cross over" under field and flow conditions such that they are mixed for 10 seconds before unmixing. Presumably some of the substrate is altered, leading to a new product or products (only one is shown in the figure) with different physical properties such as charge, isoelectric point, or mass. On this basis, solution conditions are sought to effect separation of the product(s) from the other streams. The different streams are then collected and analyzed. Such a procedure could provide a simple means of gaining information on the mechanism of the early stages of action of the enzyme. If it started by splitting all the substrate molecules in half, for example, the product(s) should be uniform and reflect this. On the other hand, if it started with random destruction, the product would be polydisperse. By repeating the experiment with different "mixed" times, concentrations, etc., the picture of the enzymatic action at each stage of its action could be developed. (A wide variety of results would of course be possible, depending on the nature of the enzyme-substrate interaction.)

To a certain degree, chemical or physical means of inactivitating the enzyme short times after mixing it with substrate in a test tube could lead to this same type of information. However, the "stress" to the system would almost certainly be greater than for the solution cross-over method, separation procedures would probably have to be carried out anyway, and the presence of the inactivated enzyme and poisoning agent (if chemical means are used) would very likely lead to uncertainty in interpretation of results.

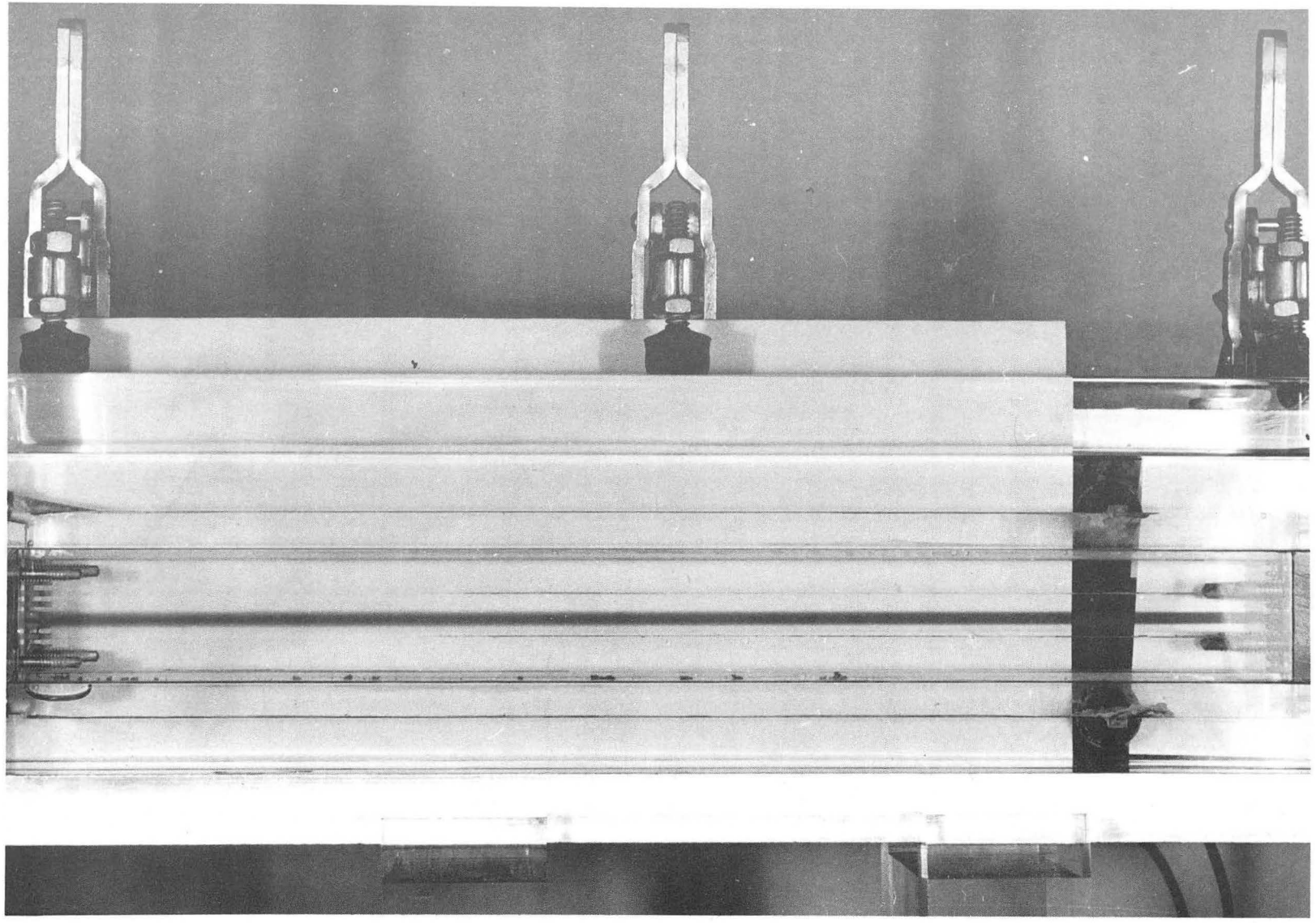

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Fig. 14. Photograph of single dye stream in free-boundary flow without field, indicating diffusion broadening toward the outlet.

This crossing-over process can of course be carried out on the surface of a supporting medium (such as paper), but the times required are much longer and the results may not be at all representative of free-solution behavior.

VII. DETERMINATION OF MOLECULAR TRANSPORT PROPERTIES AT A SINGLE INSTANT OF TIME

Knowledge of the basic physical properties of molecules and larger particles is of fundamental importance for successful investigation and understanding of matter in its larger aggregate forms, be it in physics, chemistry or biology. A number of transport properties are of particular interest to the biophysicist working at the molecular and cellular levels of organization. These include diffusion coefficients, electrophoretic mobilities, and sedimentation constants, among others. In this section a brief proposal is given for what is believed to be a new and unique method of making such time-dependent determinations at a single instant in time by optical examination of a steady-state flowing liquid system.

Figure 14 is a photograph of a flow-stabilized liquid system containing a single sample stream with no other applied force field (except normal gravity). The essential feature is that positions downstream (for the different molecules arranged along the stream) play the same role as positions for the same molecules at successively later times after formation of a fresh boundary in a nonflow system. An examination of Fig. 14 reveals how the boundary downstream (corresponding to later times) becomes more "fuzzy," as indicated. In principle such a single photograph would appear to contain, according to the degree of blackening of the film, the necessary information for determination of diffusion coefficients. For colorless substances a more elaborate optical system would be required.

In a related manner, electrophoretic mobilities should be measurable in a time-independent steady-state condition. For a given field strength, migration of transverse distance, dx, per unit of time, dt (represented by longitudinal distance)--i.e., the slope of a line of constant concentration--should be proportional to the electrophoretic mobility. Point-to-point changes in mobility resulting from changes in field strength, for example,

would be represented by changes of slope of these lines. Such lines can be seen in both Figs. 6 and 12 in the upward-inclined boundaries of constant film density. Note that very near the inlet the slope is nearly horizontal, i. e., the mobility is very small. This reflects the fact that the electrodes in this apparatus do not extend quite to the inlet. A detailed account of this matter is beyond the scope of the present paper and will be made elsewhere.

VII. FUTURE WORK

The emphasis up to this point has been on conducting type experiments in the different problem areas to demonstrate the feasibility of the approaches and to show that the principles could be put into practice. These experiments have also been valuable in defining the possibilities of the free-boundary method and indicating improvements in the equipment, which has been one of the major preoccupations up to this time. The main emphasis in the future will shift toward studies in more depth as indicated above.

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Subject: "Biological Mixtures, Some Biophysical Problems, and the Stable-Flow Free-Boundary Method" by Howard C. Mel, dated February 26, 1960.

Please make changes to your copies of UCRL-9108 as indicated below:

Page 18 -
Line 5 of Example 1 should read:
"Conditions: + 10 volt; ----------"

Page 20 -
Line 5 of Example 3 should read:
"---------and γ globulin .02% ±; -35 v; -------"

Page 30 -
Line 12 of first paragraph should read:
"---------- or 12 cc/min over-all; + 10 v, "