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1965-08-28

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STABLE-FLOW FREE-BOUNDARY ELECTROPHORESIS
THEORY AND PRACTICE
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UCRL-16290

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AEC Contract No. W-7405-eng-48

STABLE-FLOW FREE-BOUNDARY ELECTROPHORESIS
THEORY AND PRACTICE
AS APPLIED TO THE STUDY OF HUMAN SERUM LIPOPROTEINS

Roger D. Tippetts
(Ph. D. Thesis)

August 28, 1965

Stable-Flow Free-Boundary Electrophoresis
Theory and Practice
As Applied to The Study of Human Serum Lipoproteins

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ABSTRACT

I have reviewed electrophoretic methods, pointing out their general inadequacy for the study of serum lipoproteins because of small sample size, instabilities for free-boundary (zone) methods, and sorption effects of those requiring solid supporting material. The Stable-Flow Free-Boundary (Staflor) Electrophoretic method is described and the Staflor theory of Howard C. Mel is reviewed. This includes aspects of density-gradient stability, self-balancing hydrostatic-hydrodynamic feedback, and the laminar flow pattern. New phenomena involving the dynamics of fluid flow are described, and their effect upon the design of Staflor apparatus and experiments are discussed. These include: the characteristic vertical and horizontal velocity profiles, solute migration profiles and the phenomena called "diffusion-convection," "laminar defocusing," and "central sharpening." Novel methods of Staflor sample handling and observation are described. These include the "thin sample" technique, the programmed electric field, and the thin-slit illumination method for observation of the Staflor sample migration profiles.

The experimental conditions necessary for Staflor electrophoresis of the low-density lipoproteins ($S_f 0-10^5$) are described. This study has coupled Staflor electrophoresis of serum lipoproteins with ultraviolet spectrophotometry, lipid analysis, preparative and analytical ultracentrifugation, and electron microscopy. The principal results of this study demonstrated that low-density lipoprotein fractions may be preparatively or analytically (or both) Staflor electrophoresed. This study

also indicates methods by which dense lipoproteins and proteins might be Staflo electrophoresed. These methods should be of more general application than only to lipoproteins. The observations and theory concerning the Staflo method should be valid for almost any soluble or suspendable solute, whether in an aqueous or other environment, and for a variety of forces causing the migration, e. g., electrical, gravitational, magnetic, and diffusional.

I. STABLE-FLOW FREE-BOUNDARY (STAFLO) ELECTROPHORESIS

A. Introduction

1. Review of Electrophoretic Methods

Human serum lipoproteins are commonly studied by ultracentrifugal methods. These methods have served to classify the lipoproteins into various flotation classes and to determine their molecular weights, molecular dimensions, and densities. It is of great importance to our understanding of these macromolecules that information be obtained regarding their electrical charge and electrophoretic mobilities. Such information might lead to an understanding of the orientation and number of charged groups that are part of the lipoprotein molecule, and also yield supplemental data for other studies of the lipoproteins. Ultracentrifugal studies cannot of themselves yield information about the electrical properties of the lipoproteins. Electrophoretic methods, potentially, can supply this information.

Electrophoresis is the movement of charged particles suspended in a fluid, under the influence of an applied electric field. The usual aims of electrophoresis experiments are to obtain information on the electrophoretic velocity (mobility), to analyze a mixture, or to separate it into components.

There are two general types of electrophoretic methods presently available for the study of soluble proteins. These are (a) supporting medium and (b) free-solution methods. Both types have been used to study the serum lipoproteins. In addition there are several methods of more limited application that have not been used in the study of lipoproteins, such as microelectrophoresis,¹⁰ barrier electrophoresis,⁵⁹ electrodialysis,^{18, 50} and electromagnetic electrophoresis.²² Since these four methods are of limited application and have not been used to study the lipoproteins, their mode of operation and potential usefulness to the study of serum lipoproteins are not discussed.

Those electrophoretic techniques which utilize any solid, gel, or similar support material are often unsuitable for electrophoresis of

the lipoproteins. Nevertheless, Bloemendal⁴ in his review indicates several methods that have been used to separate the α and β serum lipoproteins. These lipoproteins are so named because A. Tiselius found with his apparatus four fractions from human serum which he named albumin, α -, β -, and γ - globulin in order of decreasing electrophoretic mobility. The α - and β - lipoproteins are so designated because they have electrophoretic mobilities similar to the α - and β - globulins respectively.³⁶

On support medium, electrophoresis lipoproteins are found to be adsorbed, absorbed at the origin, and even possibly denatured by the support materials. One of the main reasons for using support material such as starch, agar, glass, etc. is to prevent convection during an electrophoretic run due to heat produced from the passage of an electric current. This problem of convection must be dealt with in other ways in free-solution methods.

The electroconvection method, an electrophoretic free-solution method, essentially causes proteins to migrate normal to the direction of gravity. This movement produces a density instability in the initially uniform solution and results in convection that concentrates the protein at the bottom of the cell. Such a convection separates the mobile proteins, which concentrate at the bottom of the cell if they are more dense than the background solution, from the nonmobile components, which remain unconcentrated in the bulk solution.

This method requires that at least one of the proteins to be separated from the others of a mixture have a very low or zero electrophoretic mobility. This usually requires operation near isoelectric point of that protein. Electroconvection is based upon a combination of electrophoresis and thermal diffusion² and was developed by Kirkwood and his co-workers^{6,7,20,39,48} and others.^{1,45,46} This is a preparative method and has not been used with the serum lipoproteins. The electroconvective method will not be discussed further, for it is relatively unsuited for preparative or analytical work upon the lipoproteins because taking a lipoprotein to its isoelectric point is often injurious to them.

The Tiselius-type electrophoresis is an example of moving-boundary electrophoresis in free solution. Since it is a moving-boundary method it is essentially an analytical method. Trautman⁵⁶⁻⁵⁸ discussed the problems associated with this approach to the study of serum lipoproteins. Principally these problems were local microconvections at the moving boundary between the separating protein zones, and non-uniform background fluid motion caused by migration of the proteins and electro-osmosis. This method, in addition to having the problems discussed by Trautman, is unsuitable for preparative work for three reasons:

- (a) The total quantity of material that it can handle is small.
- (b) A long time is required for a protein separation.
- (c) Only one or at most two proteins can be separated from a mixture of proteins.

Nevertheless, some information relevant to lipoproteins has been obtained by its use. For example, Blix, Tiselius, and Svensson³ showed as early as 1940 that the protein fractions designated albumin, α - β -, and γ -globulins had associated with them detectable quantities of cholesterol and phospholipids. We now know that these lipids are always associated with lipoproteins. Therefore it is reasonable that these protein fractions also had lipoproteins associated with them.

Svensson and co-workers^{52, 53} have reviewed the methods and problems of zone (narrow band or region) electrophoresis in free solution [Also known as density gradient (column) electrophoresis]. They described how this method can resolve a mixture of proteins by their respective electrophoretic mobilities. Kolin²¹ has discussed density gradient electrophoresis combined with pH or resistivity gradient or both. Bloemendal⁴ has also discussed density-gradient column electrophoresis. None of these authors indicates that zone electrophoresis in free solution has been applied to the study of serum lipoproteins. However, this method has been applied to the serum proteins, which implies that this method could be applied to the study of serum lipoproteins.

Because of the many differences in electrophoretic tools and methods presently available, there emerges only a broad characterization

of serum lipoproteins¹ electrophoretic mobilities that is related to their ultracentrifugal classes. Perhaps the best summary of electrophoresis data related to ultracentrifugal data for lipoproteins is given by Kunkel and Trautman.²³ They showed that there are three zone starch block electrophoretic classes of lipoproteins designated α , β , and γ that are related to their densities 1.035, 1.000 and (1.075 and 1.140) g/ml respectively. Not all investigators, using different electrophoretic methods, will agree in every detail with these findings.

It became evident that if an electrophoretic study (preparative and analytical) of human serum lipoproteins were to be related to other studies not limited to ultracentrifugation, then the electrophoretic method should ideally fulfill the following conditions:

- (a) It should utilize no solid or similar support material.
- (b) It should be a variation of zone electrophoresis.
- (c) It should be capable of separating the sample mixture into individual fractions according to their electrophoretic mobilities, or isoelectric points, or other measurable parameters.
- (d) It should be capable of handling a large sample (1 ml or greater).

It would also be desirable that this tool could be operated in a continuous manner, that it could be operated at any pH, and that it could handle the sample rapidly.

The Stable-Flow Free-Boundary (Staflow) method fulfills all the above conditions. This method causes free solutions of different densities to be layered in a continuous manner within a chamber at such a rate that the flow is laminar. The sample is introduced into this chamber, at some intermediate density, where it forms a layer or zone in free solution. An electric field is applied normal to the fluid flow and normal to the sample zone parallel to the direction of gravity. This method handles (at present) in a single working day (8 hr) about 30 ml of sample in a continuous manner. It is a variation of zone electrophoresis in free solution, using a density gradient for stability rather than solid support materials. It can potentially be operated at any pH, and the time for electrophoresis of any sample portion is of the order of 15 minutes.

Mel,^{27, 29, 33-35} in a series of papers, has set forth many general operational requirements dealing with the theory and practice of the Staflo method. These are discussed at length later in this thesis. In the main the requirements are:

- (a) A steady and uniform inlet flow rate for each of the various inlets of the Staflo.
- (b) A density gradient that increases from top to bottom for each stream.
- (c) A laminar flow within the Staflo Chamber.
- (d) A negative hydrostatic-hydrodynamic feedback giving rise to steady and nearly identical outlet flow rates for each of the various outlets of the Staflo Chamber.

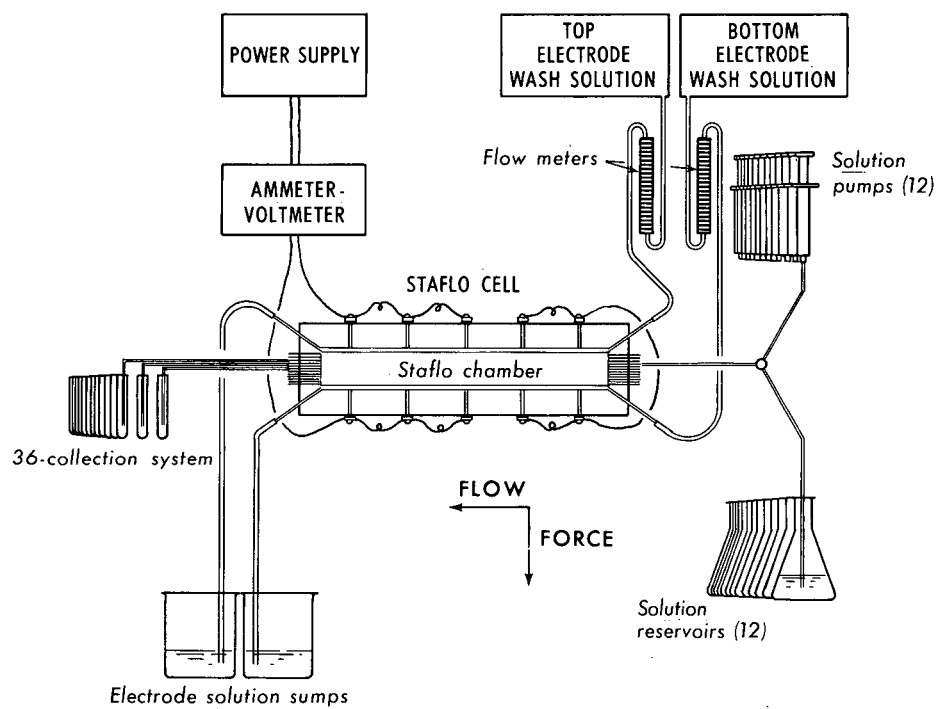
The Staflo method has been applied to systems of cell mixtures,²⁹ to an enzyme-substrate reaction,³⁰ to an attempted characterization of spermatozoa,⁴⁴ to chloroplast light-induced changes,⁴⁰ to electrophoretic concentration of dilute protein solutions,³¹ and to the electrophoretic separation of a simple protein mixture.²⁸ However, no complete analytical-preparative study has been performed by this method on a complex protein mixture, and neither has any work with it been done upon the human serum lipoproteins.

It seemed reasonable that a study of the serum lipoproteins by the Staflo method would yield additional information upon serum lipoproteins in particular, and upon the Staflo method in general as applied to other proteins and macromolecular species up to and including cells.

2. The Staflo Apparatus

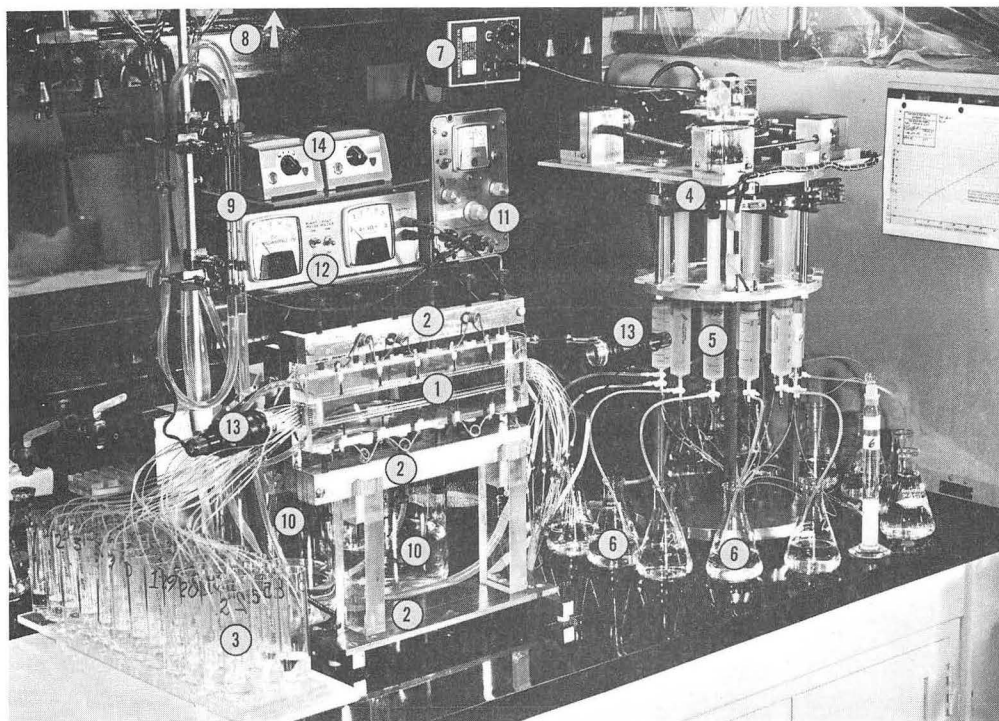
The Staflo apparatus (as designed by Mel^{27-29, 33} and modified by this author) consists of a variable-speed multiple-syringe drive pump, a multiple collection system, necessary connecting tubing, electrode wash reservoirs and sumps, a constant-voltage electrophoretic power supply, associated optical equipment, and a Stable-Flow Free-Boundary Electrophoresis Cell containing the Staflo migration chamber and the upper and lower electrode chambers (see Figs. 1 and 2).

My variable-speed multiple-syringe drive pump is designed to drive up to 12 syringes. These syringes can be (for the present design)



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Fig. 1. Schematic drawing of Staflo apparatus.



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Fig. 2. Stable-flow free-boundary Staflo apparatus.

1. Staflo cell
2. Staflo-cell clamp and supporting leveling stand
3. Collection system on leveling table
4. Syringe drive
5. Solution pump syringes
6. Solution reservoirs
7. Syringe drive power supply
8. Electrode wash solution reservoirs (located above picture)
9. Electrode wash flowmeters
10. Electrode solution sumps
11. Electrophoresis power supply
12. Ammeter-voltmeter
13. Side lights
14. Side-lighting power supplies

of any normal size up to 50 ml. It is possible to use glass syringes in this pump, but plastic syringes (Plastipak--sterile, disposable plastic syringe made by Becton-Dickinson & Co., Rutherford, N.J.) were chosen instead of glass because of their nonfragile nature, noncritical requirements of alignment of syringe barrel with syringe piston, and--most important of all--the very great uniformity from syringe to syringe within a given lot number.

The pump is powered by a 1/70-horse-power, 0 to 1725-rpm shunt-wound electric motor (Bodine Electric Co., Chicago, Illinois), having a variable-speed power supply (Minarik Electric Co., Los Angeles, California, model Sh-14). The motor has a shaft projecting from each end. The high-speed shaft (0 to 1725 rpm) drives the pump for fast flush of the Staflo chamber and for filling the syringes. The low-speed shaft reduces the speed by a factor of 36, i. e., to 0 to 48 rpm, and is used to pump the fluids at a slow constant rate during Staflo runs. The pump can also be driven in reverse. The speed output of either motor shaft is further reduced by a factor of 100 by a worm-gear drive before translation into linear motion for driving the syringes. The pistons of the syringes can then be driven at a linear rate up to 0.1175 cm/sec on high speed (fast flush or fast fill), and at a linear rate from nearly zero up to 0.00363 cm/sec on low speed. This speed in both cases is very uniform in time, since the speed of the motor is very nearly independent of load. When 50-ml syringes are used this linear piston rate delivers a liquid at a flow rate of 0.65 ml/sec on high-speed maximum, and 0.018 ml/sec on low-speed maximum.

The multiple collection system is a series of uniform containers, one for each outlet stream. For this purpose plastic syringe barrels have proved excellent.

Electrode wash reservoirs are 2000-ml separatory funnels that supply streams that can be of the same composition as or of a different composition from those of the Staflo chamber streams. The only requirement is that the density changes across the membrane into the Staflo chamber not increase the density at the top or decrease the density at the bottom of the Staflo chamber to the point of instability, for

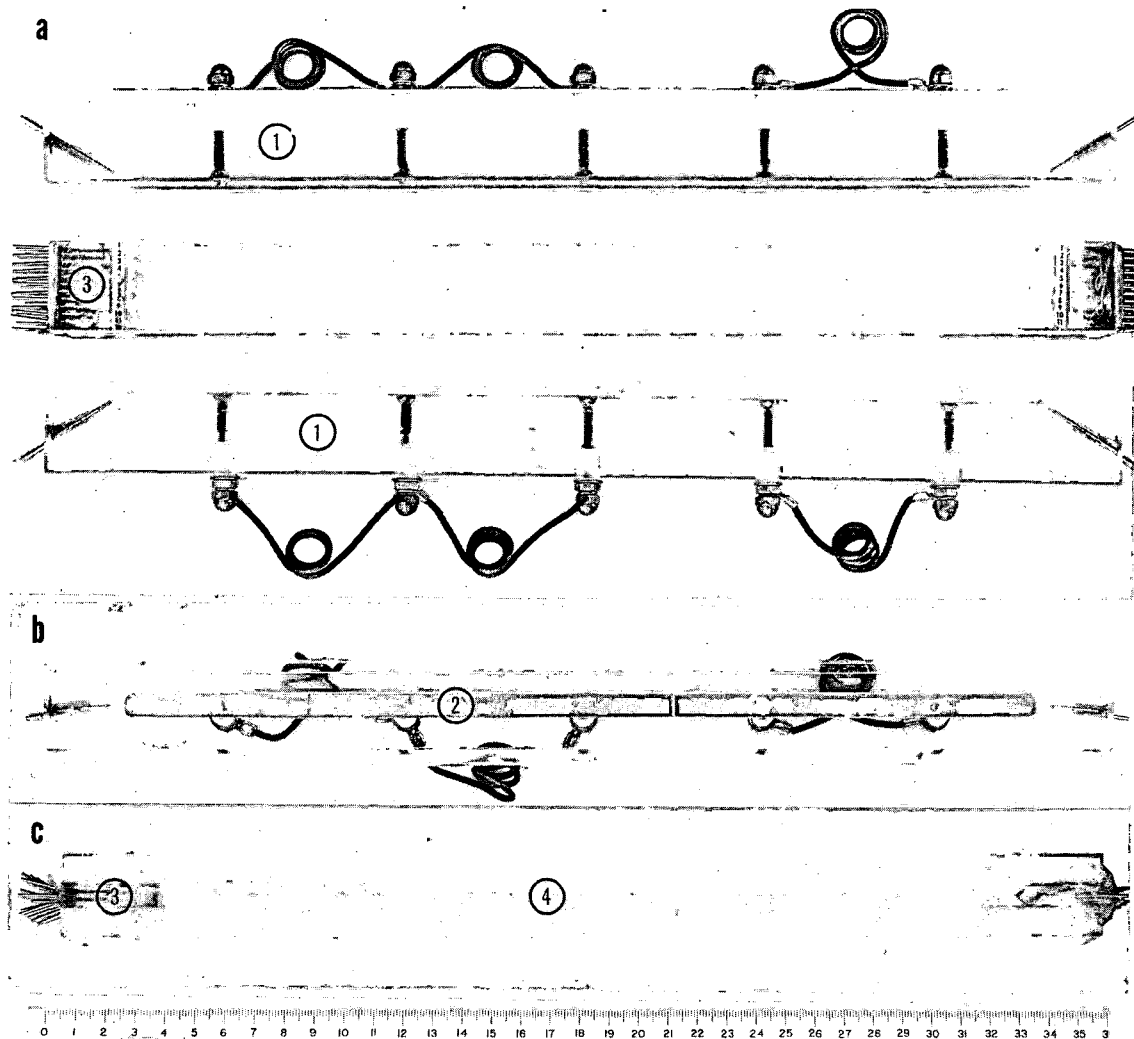
this would lead to a convection disturbance within the Staflo chamber.

The flow rate through the electrode chambers is normally 10 times as large as that of any stream within the Staflo chamber. The electrode wash solutions' flow rates are measured by flowmeters (Fischer & Porter Co. of Holbord, Pennsylvania, Tube No. 02-F 1/8-16-5/36) each containing a stainless steel ball and a sapphire ball.

The electrophoretic migration power supply is the Model 5051 A transistorized 0 to 50-Vdc, 0-1, 5A manufactured by Power Designs Inc., Westbury, N. Y. with a voltage drift of less than 100 millivolts per day at constant ambient temperature. At our normal operating voltages of 25 Vdc or 50 Vdc this is a 0.04% to 0.02% variation respectively.

The Staflo cell (see Figs. 3 and 4) is made of a central migration chamber (Staflo chamber) which consists of inlet and outlet flow dividers sandwiched between transparent plates of Lucite, or glass or quartz. The internal dimensions of this chamber are 30 cm long $\times$ 3.0 cm high $\times$ 0.8 cm wide. The upper and a lower electrode compartments are made of Lucite. Each of these matches the central chamber in length and width. The electrode chambers are 0.25 cm high. Each electrode chamber contains five platinum electrodes 5.95 cm long separated from each other by a gap of about 0.05 cm. These three cell parts are clamped together with cellophane membranes (dialysis tubing) between them, forming three physically separated chambers. These are the upper and lower electrode chambers and the central Staflo chamber in which the electrophoretic migrations or sedimentations occur.

It is of prime importance for the operation of the Staflo method that the flow rate be uniform for each inlet stream, and independent of piston position within pump syringes. Nonfulfillment of either of these conditions precludes meaningful electrophoretic or sedimentation separations by the usual Staflo methods.

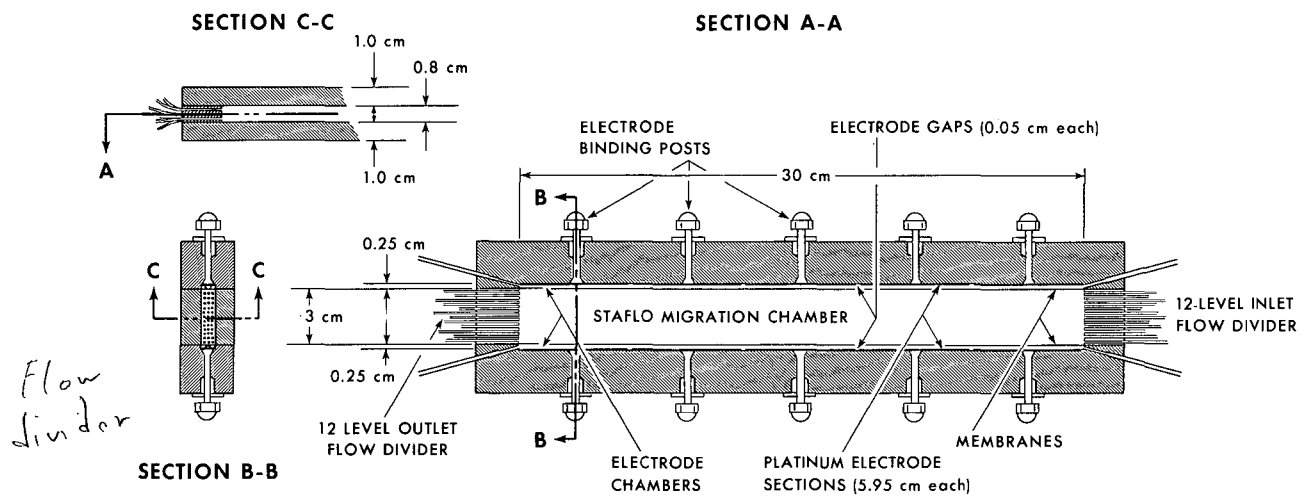


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Fig. 3. a. Exploded side view of Staflo cell, showing upper and lower electrode compartments (1) and the Staflo chamber (2) with flow dividers (3).

b. Internal detail of an electrode chamber, showing the split electrode section (5).

c. Top view of Staflo chamber (4) with flow dividers (3).



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Fig. 4. Schematic drawing of Staflo cell RDT-3.

B. Two-Dimensional Staflo Properties

1. Density Gradient Stability

In the operation of the Staflo apparatus it is essential that positive density gradient (i. e., density increasing in the downward direction) be maintained within the Staflo chamber in order to suppress convection. In some cases this gradient may be made vanishingly small. This density gradient is not initially a smooth function, but is in fact a finite step function at the inlet, becoming smoother as the outlet is approached (as will be seen in Fig. 9). This smoothing occurs because of diffusion of the density-gradient-producing molecules. (Solutions of constant composition but of varying temperatures can also be used, e. g., aqueous solutions above 4°C having warmer solutions on top.)

Brakke⁵ and Svensson and co-workers⁵²⁻⁵⁴ discussed the conditions of initial stability of a protein solution zone in a sucrose density gradient and the conditions which obtain during the electrophoretic migration. (In general their treatment should apply to solutions of any high-molecular-weight particles, perhaps up to and including cells.) These conditions must also be met in the operation of the Staflo method. If at any time the influx or efflux of the faster-diffusing molecule into or out of the protein zone stream results in a density shift such that the density gradient becomes negative downward, then convection or "droplet sedimentation" may occur. This must be prevented. Svensson⁵² has discussed the methods for preventing convection and "droplet sedimentation." I summarize here his conclusions.

(a) A solute heavier than the solvent and diffusing slower than solute outside the zone should be added to the density gradient column, but be excluded from the sample solution zone.

(b) A slowly diffusing substance lighter than the solvent should be present initially in the protein solution zone but not in the rest of the streams.

(c) There should be in the protein solution zone, but not in the other streams, a substance heavier than the solvent, and diffusing more rapidly than solute that is maintaining the density gradient outside the protein zone.

(d) All three of the above should obtain.

2. Self-Balancing Hydrostatic-Hydrodynamic Feedback

Mel³³ states that if high-resolution continuous free-solution analytical or separation processes are to occur, the fluid flow within the Staflo chamber must be UNIFORM. This uniformity of flow is related directly to self-balancing hydrostatic-hydrodynamic feedback expressed in the limiting case by his equations

$$l_2/l_1 = \frac{M}{l_1} \left(1 - \frac{\rho_1}{\rho_2}\right) + \frac{\rho_1}{\rho_2} = \frac{(h-d/2)}{l_1} \left(1 - \frac{\rho_1}{\rho_2}\right) + \frac{\rho_1}{\rho_2} \quad (1)$$

or

$$l_2 - l_1 = (h-d/2 - l_1) \left(1 - \frac{\rho_1}{\rho_2}\right) = \Delta l. \quad (2)$$

Here l_1 and l_2 are the heights of the liquid in the collection containers 1 and 2 respectively; h is the height from the bottom of the collection containers to the center of stream 1 within the Staflo chamber, d is the height of the Staflo chamber containing streams 1 and 2 with their respective densities ρ_1 and ρ_2 . (Similar but not identical equations apply for any pair among the more usual multicollection simultaneous siphon system.)

Mel³³ calls Eq. 1 the basic equation of instantaneous hydrostatic balance for the Staflo system with a linear gradient and with any given ρ_1 , ρ_2 , and M . If a fluctuation should cause a momentary deviation of l_2/l_1 from that consistent with Eq. 1 an appropriate restoring force would operate to correct the imbalance, hence the hydrostatic self-balancing feedback quality. This restoring force is caused by a small pressure-difference signal which arises whenever height differences in the collection containers are other than those given by Eq. 2. The rate of increase of this pressure-difference signal varies directly with the outlet flow rates and inversely with the cross section of the collection vessels.

Normally when one is using solutions of low density, low viscosity, and average residence time τ_{av} in excess of about 10 min (which is the usual case for steady-state operation), only hydrostatic feedback need be considered. That is, the viscosity effects due to increased solute concentration can be neglected in this limiting case. (The average residence time--previously known as "throughout time"--

is the time required for passage through the Staflo chamber of a volume of fluid equal to the volume of the Staflo chamber.)

During the setup procedure for any Staflo experiment the Staflo chamber, pump, syringes, collection apparatus, and connecting tubing are usually filled with water. Under these conditions the fluid levels in the collection apparatus are all equal, as predicted by Mel's Eq. 1. However, in my experiments after the setup of the density gradient and the attainment of the steady state these levels are observed to be no longer equal but to have changed, as predicted by Eq. 2 for the static case,³³ within experimental error.

In my experiments the density gradient due to sucrose was 0.4 g/100 ml per inlet (hence outlet), that is, for streams 1 to 12 the density change is from 0.4 to 4.8 g/100 ml. This is a change of 0.9998 to 1.0171 g/ml at 20°C. During any experiment the change in liquid levels in my collection system never exceeded 12 cm, the height of the collection containers. The parameters, h , and d had the values 25.5 and 3.0 cm respectively.

Using these values in Eq. 2, one obtains the theoretical differences between collection liquid levels at the beginning and the end of an experiment, assuming the use of all 12 cm of collection container height. My $l_{12} - l_1$ initial difference was 0.41 cm and the final difference was 0.20 cm. The change in difference over the course of an experiment was then at most 0.21 cm. For twelve "50-ml" collection syringes of 2.9 cm diameter, this represents 1.35 ml more flowing through outlet 1 than through outlet 12. (Each collection syringe holds more than 50 ml when the piston is removed.) The total quantity of fluid which flowed through any outlet during this same period would be approximately 79 ml. Thus outlet 1 delivered 1.7% more fluid than did outlet 12. Correspondingly smaller differences held for all other outlet combination pairs. The differences were also smaller if less than the full 12 cm height of the collection containers was used. The 1.71% displacement of the steady-state pattern (stream 1 relative to stream 12) is of no consequence, since a change of this magnitude at present is not detectable. Displacement and drift problems are discussed further elsewhere.³³

It is to be understood that no hydrostatic pressure error signal exists if all outlets drain into a single container or if the respective levels of the collection apparatus are externally fixed in an arbitrary fashion. It is absolutely necessary, if self-balancing hydrostatic feedback is to occur, that differences in height, however small, have the potential of occurring.

Negative hydrodynamic feedback can exist independently of hydrostatic feedback. It is usually very small, but increases when one stream's outlet flow rate exceeds another. It arises because of viscous flow that makes the faster flowing stream have a greater resistance to flow than the slower-flowing stream, which tends to decrease the flow rate of the faster stream relative to that of the slower stream.

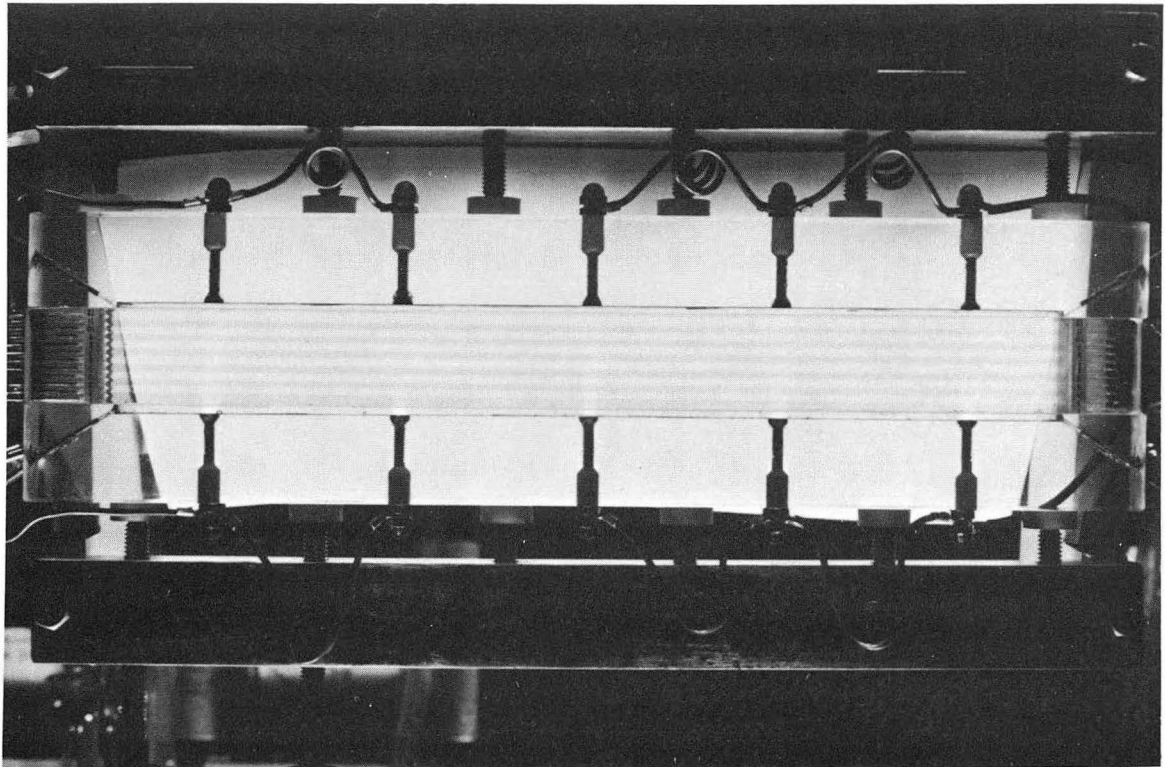
The stability of flow, most readily demonstrated in the flow pattern of solutions of alternating colors, is discussed in more detail in the following sections (see Figs. 5 and 6.)

C. Three-Dimensional Staflo Properties

1. The Flow Pattern

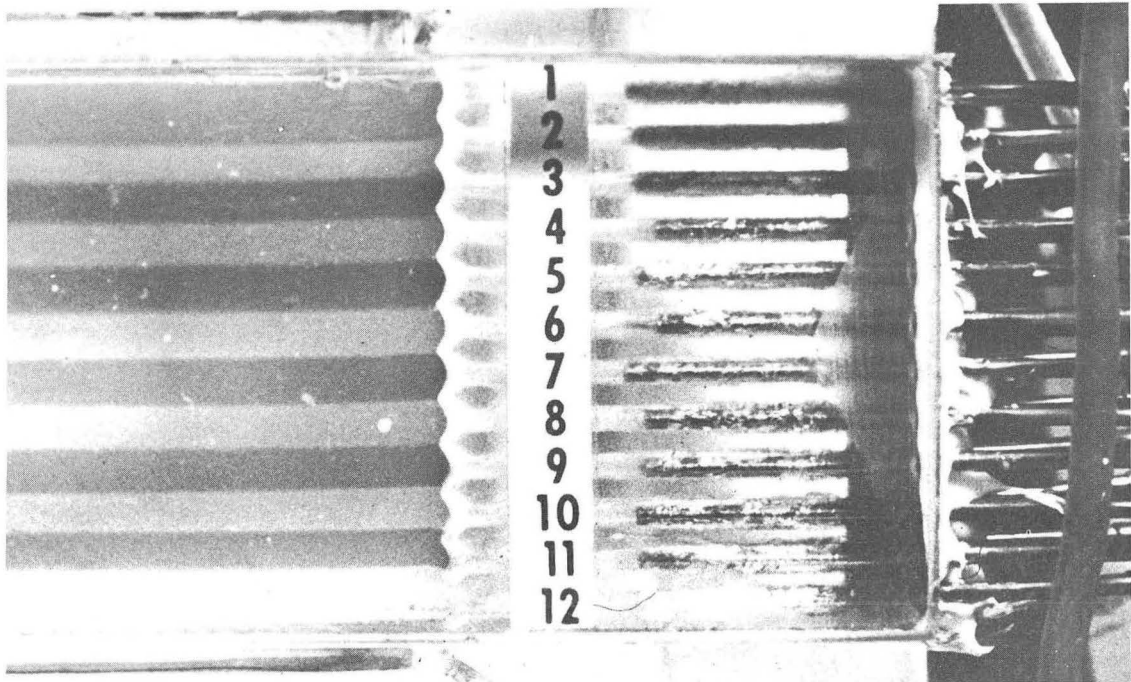
a. Vertical Velocity Profile

Figure 5 shows alternate streams of blue and colorless dextran flowing through the Staflo chamber with an average residence time $\tau_{av} = 20$ minutes. The blue dextran (mol wt $\approx 3 \times 10^6$) and colorless dextran (mol wt $\approx 6 \times 10^5$) were in equal concentrations by weight. Notice that beginning with stream 1 every other stream is blue, and that beginning with stream 2 every other stream is white (colorless). It has been previously shown by Mel²⁹ that the inlet and outlet volume flow rates must, to a very good approximation, be equal during steady-state operation. This is also true for each inlet-outlet combination, such as, for example, inlet 2-outlet 2. With this in mind it becomes apparent that the width of any stream relative to the other streams is a measure of the average linear flow rate within the Staflo chamber. This linear flow rate need not be identical from point to point within a stream or be equal to the inlet-outlet linear flow rate. However, the volume flow rate of any stream (as indicated by the colored streams, for example) must be identical at



ZN-5126

Fig. 5. "Dye stripe" experiment. Side view of Staflo cell with steady-state flow pattern established. (Odd-numbered stripes are blue.)



ZN-5106

Fig. 6. Closeup of Staflo "dye stripe" experiment at inlet end.

all points. It is readily seen that streams 1 and 12 are wider while streams 2 and 3, and 10 and 11, are narrower than the center streams 4, 5, 6, 7, 8, and 9, which have identical widths.

A careful examination of Fig. 5 will show that streams 1 and 12 become narrower as the outlet is approached and that all streams tend in thickness to that of the center streams. This phenomenon is believed to be an expression of hydrostatic-hydrodynamic negative feedback of the collection apparatus operating in the vertical sense coupled with the existence of a stable density gradient and laminar flow within the Staflo chamber. Convection from one stream to another is prevented by the positive density gradient. Notice that the stream widths are essentially independent of inlet flow divider effects (see Fig. 6) and it would seem that this is an expression of viscous flow related to the upper and lower membranes.

Mel,²⁹ in his analysis of the flow pattern, discussed his proposal of what he has termed "plug flow." He states "A study of steady-state patterns... has revealed no systematic vertical distortion... near the top or bottom, at least down to the level of a single layer thickness (though, of course, precisely at the stationary top and bottom membranes, the flow rate must be zero.)"

It would seem that he could be correct concerning the central streams (4 through 9), which have identical vertical thickness. Identical vertical thickness would mean that they have identical average linear flow rates, since all streams have identical horizontal widths (0.8 cm), which is the internal thickness of the Staflo chamber. However, streams 1 and 12 are thicker and 2, 3, 10, and 11 thinner than the central streams, and this implies that their respective linear flow rates are slower and faster than the central streams. These observations imply that there is a (systematic) vertical distortion of the linear flow rate and that this flow rate does not continuously decrease from the center of the Staflo chamber to the upper or lower membranes, but must first increase and then decrease. In addition "plug flow" would imply that the linear flow

rates at all points through a vertical x-z plane* are the same. These assumptions with regard to the linear flow rates have not been experimentally tested. If analytical measurements of electrophoretic or other separations are to be made the linear vertical and horizontal velocity profiles of the fluid within the Staflo chamber must be known, as well as other perturbations of the fluid flow.

An experiment was designed to investigate the details of these vertical and horizontal flow profiles.

b. Experimental Measurement of Linear Velocity Flow Profiles

This experiment was set up according to the following scheme: Staflo Cell RDT #2 was used. Every stream contained human blood at 5000-fold dilution in 0.9% NaCl. The density gradient was made with sucrose in steps of 0.4 g/100 ml, from 0.4 in stream 1 to 4.8 in stream 12. The upper and lower electrode compartments were filled respectively with solutions of the same composition as streams 1 and 12 but without the diluted blood. The average residence time was chosen to be $\tau_{av} = 16.6$ min.

The suspended RBC were viewed with a low-power microscope over an actual field width of 1.6 mm. Illumination was provided by a microscope lamp. The light was passed through a water bath of optical thickness greater than 11.5 cm to reduce the heating effect upon the solutions within the Staflo chamber. This light illuminated the RBC at an angle of about 150 deg to the direction of view. The RBC were seen by the light that they scattered, and they had the appearance of stars against the night sky.

* x Axis: Normal to length and width of Staflo chamber, identical to direction of gravity; positive downward.

y Axis: Normal to gravity and width of Staflo chamber, measured in same direction as fluid flow. Positive direction: inlet to outlet of Staflo chamber.

z Axis: Normal to gravity and length of Staflo chamber. Normal to face of Staflo chamber.

The time necessary for the suspended RBC to cross this 1.6-mm field of view was recorded with a stop watch. Their respective velocities were calculated, and are recorded in Table I for the location 18 cm downstream from the inlet.

Several other downstream locations were also investigated. The velocities at their respective centers are recorded in Table II.

Smoothed curves for the vertical velocity profiles are shown in Figs. 7 and 9. The slight differences between the curves for near wall, far wall, and intermediate positions are due to the error in positioning of microscope and not due to a departure of fluid flow from horizontal parabolic profile.

The curves of Fig. 7 indicate that point-to-point vertical plug flow occurs only in the central portion of the Staflo chamber, as shown by the flat central portion of the curves. In the top and bottom approximate $1/4$ of the chamber there is fluid flowing more rapidly than in the central portion. One sees two "horns" at the top and bottom, separated by the central portion of constant velocity. As the outlet is approached the width of the horns decreases, the maximum velocity in the horns increases, and this point of maximum velocity becomes nearer the membrane (see Fig. 9.)

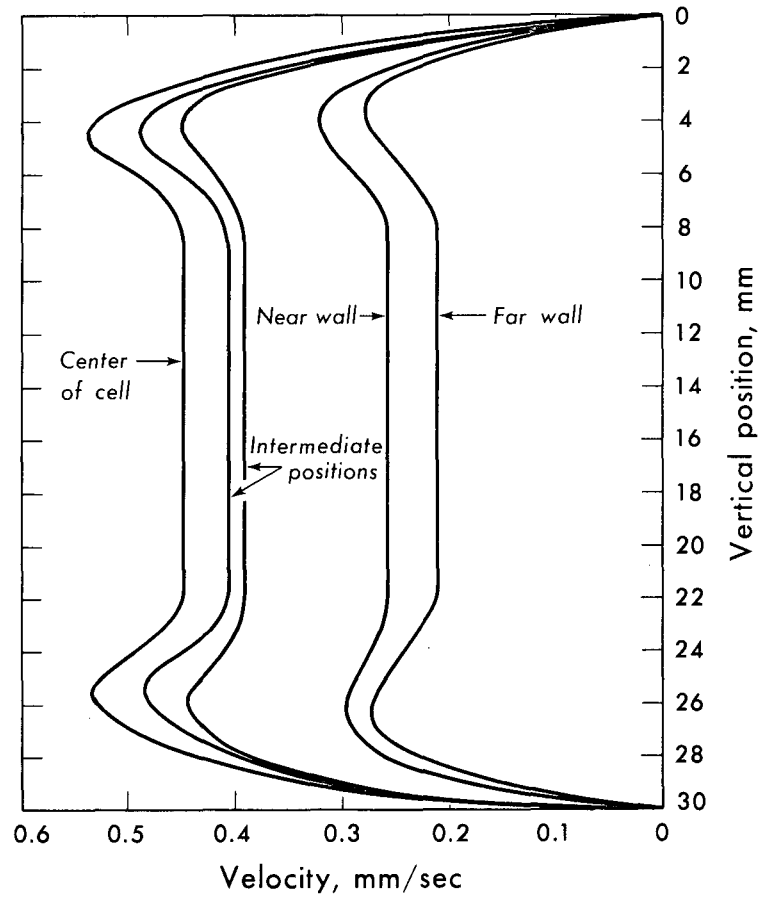
A plot of the velocities in the horizontal sense gives a parabola at all levels of the Staflo chamber with the possible exception of very near the membranes. It was not possible to investigate this region thoroughly enough to establish for certain whether or not the flow is parabolic. Murphy³⁷ indicates that it is not parabolic in this region. The maximum velocity does change in the vertical direction, as indicated before, nevertheless it remains parabolic (horizontal) throughout. Does this mean that horizontal negative hydrostatic-hydrodynamic feedback does not exist? It certainly would not be expected for a 1×12 outlet arrangement (see Sec. I. E2), but it might still exist for a 3×12 arrangement. If horizontal feedback existed one would expect a departure in the horizontal velocity profile from the parabola at large distances from the outlet.

Table I. Fluid velocity as calculated from times of flight of human RBC within Staflo chamber measured 18 cm from the inlet for an average residence time $\tau_{av}=16.6$ min. (velocities are in units of 10^{-2} cm/sec.)

Distance from upper membrane (mm)	Distance from near wall									
	(mm)									
	1.43	2.76	4.10	5.43	6.76					
0	0.80	—	—	—	—	0.76	0.76	0.70	0.80	
1	1.61	0.16	2.00	1.88	2.25	2.13	2.19	2.19	1.28	1.39
2	2.66	2.66	3.90	3.80	4.21	4.00	3.80	3.80	2.46	2.58
3	3.13	3.07	4.44	4.57	4.84	4.84	4.32	4.57	—	—
4	3.26	3.13	5.00	5.00	5.16	5.33	4.84	5.00	2.71	2.75
5	3.13	3.20	4.57	4.57	5.16	5.51	4.57	4.32	2.66	2.66
6	2.85	2.85	4.70	4.70	5.16	5.16	4.10	4.32	2.62	2.62
7	2.80	2.80	4.21	4.21	4.70	4.44	4.10	4.21	2.35	2.35
8	2.50	2.58	4.21	4.21	4.44	4.32	3.90	4.00	2.22	2.22
9	2.50	2.58	4.10	4.00	4.32	4.21	3.72	3.72	2.07	2.07
10	2.58	2.62	4.00	4.00	4.21	4.21	4.00	4.00	2.02	2.10
11	2.58	2.58	4.10	4.10	4.57	4.57	3.90	4.00	2.05	2.02
12	2.58	2.58	4.10	4.10	4.57	4.32	3.90	3.90	2.05	2.02
13	2.66	2.66	4.00	4.10	4.44	4.44	3.90	3.90	2.00	2.00
14	2.58	2.58	4.10	4.00	4.57	4.21	3.80	4.00	2.05	2.00
15	2.62	2.58	4.10	4.00	4.57	4.44	4.00	3.90	2.00	—
16	2.58	2.58	4.00	4.00	4.57	4.57	3.90	3.90	2.05	—
17	2.62	2.58	4.00	3.90	4.44	4.57	4.00	4.00	2.13	2.13
18	2.62	2.66	4.10	4.00	4.57	4.57	3.90	4.00	2.10	—
19	2.46	2.42	3.90	4.00	4.57	4.44	3.90	3.90	2.02	1.95
20	2.50	2.50	4.00	4.00	4.44	4.32	4.10	3.90	2.10	2.07
21	2.46	2.46	3.90	4.00	4.44	4.21	3.90	3.90	2.10	2.07
22	2.58	2.58	4.00	4.10	4.57	4.44	4.00	4.00	2.10	2.10
23	2.71	2.66	4.21	4.00	4.57	4.57	4.00	3.90	2.72	2.28
24	2.75	2.66	4.32	4.70	4.70	4.84	4.10	4.00	2.38	2.42
25	2.75	2.75	4.70	4.70	5.16	5.16	4.21	4.21	2.46	2.58
26	2.96	2.96	4.70	4.70	5.33	5.16	4.32	4.57	2.66	2.62
27	2.75	2.75	4.44	4.44	5.16	5.33	4.21	4.21	2.66	2.62
28	2.66	2.66	4.00	4.10	4.32	4.44	3.90	3.90	2.28	2.22
29	1.96	1.86	3.20	3.07	3.33	3.33	3.13	3.13	1.60	1.63
30	0.77	0.62	0.89	0.80	0.76	0.76	0.57	0.50	0.62	0.57

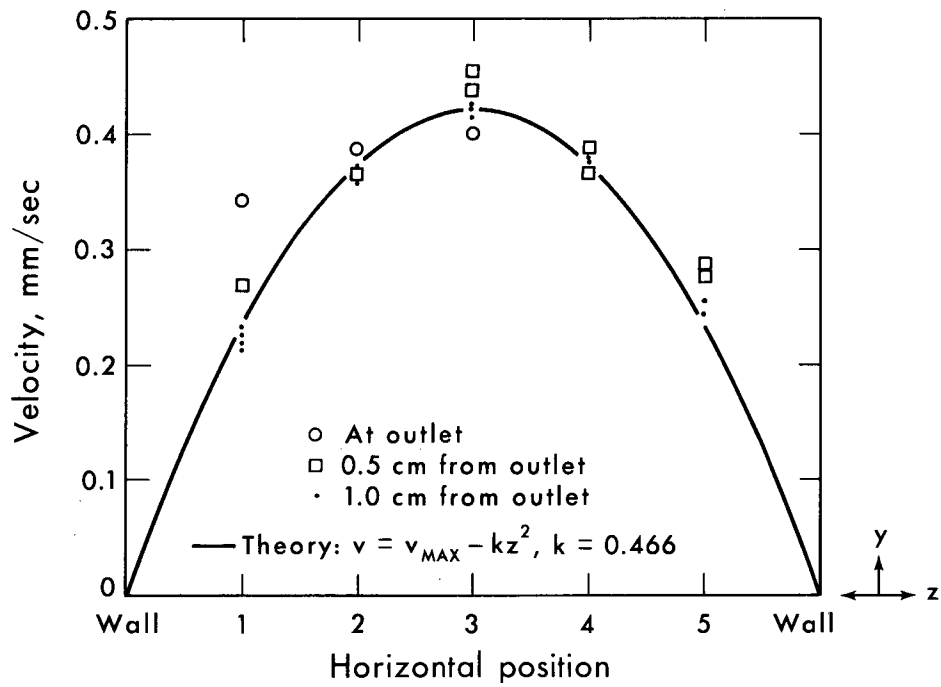
Table II. Fluid velocity at the center x-y plane calculated from times of flight of human RBC within the Staflo chamber at the distances from the inlet as indicated. Average residence time $\tau=16.6$ min. (All velocities in 10^{-1} mm/sec.)

Distance from upper membrane (mm)	Distance from the inlet (cm)									
	1.0		12.0		18.0		24.0		29.0	
1	3.33	3.33	3.13	3.01	2.25	2.13	2.46	2.35	3.33	3.33
2	5.00	4.84	4.00	3.90	4.21	4.00	4.00	3.90	4.44	4.44
3	5.16	4.71	4.84	4.57	4.84	4.84	5.00	5.00	5.33	5.33
4	5.00	5.16	5.00	5.00	5.16	5.33	5.16	5.33	5.16	5.16
5	5.16	5.16	5.00	5.00	5.16	5.51	5.00	5.00	4.44	4.70
6	5.33	5.16	4.57	4.70	5.16	5.16	4.70	4.70	4.57	4.57
7	5.33	5.33	4.44	4.44	4.70	4.44	4.21	4.21	4.57	4.44
8	4.84	5.00	4.10	4.10	4.44	4.32	4.21	4.21	4.57	4.44
9	4.71	4.57	4.10	4.10	4.32	4.21	4.21	4.10	4.57	4.57
10	4.71	4.71	4.00	4.00	4.21	4.21	4.21	4.10	4.57	4.57
11	4.44	4.44	3.80	3.90	4.57	4.57	4.21	4.21	4.57	4.57
12	4.44	4.71	4.10	4.10	4.57	4.32	4.32	4.21	5.00	4.57
13	5.00	4.71	4.21	4.00	4.44	4.44	4.10	4.10	4.57	4.57
14	4.21	4.44	4.10	4.10	4.57	4.21	4.00	4.00	4.84	4.84
15	4.21	4.21	4.00	4.00	4.57	4.44	4.21	4.21	4.57	4.84
16	4.21	4.32	4.21	4.21	4.57	4.57	4.21	4.21	4.44	4.57
17	4.32	4.32	4.00	4.10	4.44	4.57	4.21	4.21	4.44	4.44
18	4.44	4.44	4.21	4.10	4.57	4.57	4.10	4.10	4.44	4.44
19	4.71	4.71	4.21	4.21	4.57	4.44	4.10	4.10	4.44	4.44
20	4.71	4.71	4.44	4.21	4.44	4.32	4.10	4.10	4.44	4.44
21	4.32	4.44	5.00	4.57	4.44	4.21	4.21	4.21	4.44	4.21
22	4.57	4.57	5.16	5.33	4.57	4.44	4.21	4.21	5.00	4.70
23	5.00	5.00	5.16	5.33	4.57	4.57	4.10	4.21	4.70	4.44
24	4.57	4.71	5.00	5.33	4.70	4.84	4.57	4.57	4.44	4.44
25	5.00	4.71	5.00	5.00	5.16	5.16	4.57	4.57	5.00	5.00
26	4.57	4.57	4.10	4.00	5.33	5.16	4.70	5.00	5.00	5.00
27	4.44	4.21	4.10	4.21	5.16	5.33	4.84	5.00	5.00	5.00
28	3.33	3.47	3.13	3.07	4.34	4.44	4.57	4.44	5.16	5.33
29	1.77	2.00	—	—	3.33	3.33	3.55	3.47	4.44	4.44
30	—	—	—	—	0.76	0.76	2.66	2.66	2.00	0.16



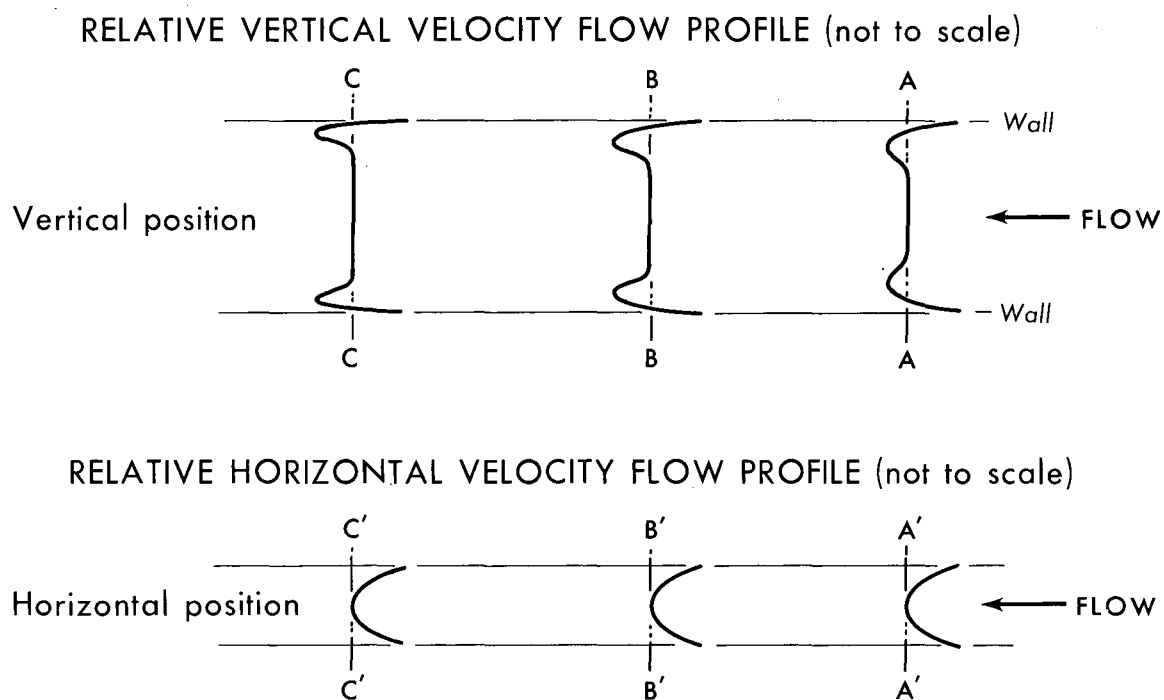
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Fig. 7. Some smoothed vertical velocity profiles at 18 cm from the inlet for an average residence time $\tau_{av} = 16.6$ min. Based on data from Table I.



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Fig. 8. Experimentally measured horizontal velocity profile in the y-z plane, for an average residence time $\tau_{av} = 17.9$ min.



MU-36567

Fig. 9. Upper schematic drawing of vertical velocity profile, showing outlet effect on the "horns" of negative hydrostatic-hydrodynamic feedback.
(Drawing exaggerated--see data in Table II)
Lower: Schematic drawing of horizontal velocity profile.

In another experiment similar to this one, the horizontal velocity profile was measured very near the outlet in this same Staflo chamber. These data are recorded in Table III and on Fig. 8 for an average residence time $\tau_{av} = 17.9$ min, with all other parameters the same. No departure from parabolic form was seen if the measurement was made more than 1.0 cm from the outlet when a 3X12 outlet arrangement was used. It was concluded that horizontal feedback does not exist (see Fig. 8).

It is considered expedient to indicate in a relative manner the changes of the vertical and horizontal velocity profiles as one progresses from inlet to outlet of the Staflo chamber. These changes are shown in Fig. 9. Notice that the position of maximum velocity of the fluid becomes nearer the membranes as the outlet is approached, while simultaneously the central portion of uniform vertical velocity widens.

c. Diffusion

Notice in Figs. 5 and 6 that the boundaries between any of the streams are very sharply delineated at the inlet and become more and more diffuse as the outlets are approached. It would appear that this increasing diffuseness would be due to the passive diffusion of the colored (or the colorless) substances across the boundaries between streams. Can this be the case? The colored streams each contained 4×10^{-4} g/ml blue dextran of nominal molecular weight of 3×10^6 . The colorless streams each contained 4×10^{-4} g/ml dextran of nominal molecular weight 6×10^5 . The background density was that of sucrose, ranging from 0.4 to 4.8 g/100 ml in 12 steps of 0.4 g/100 ml each. The average residence time was $\tau_{av} = 20$ min.

In order to make an approximate calculation of the quantity of blue dextran that passively diffuses across the boundaries I make the following simplifying assumptions:

- (i) The diffusion constant D of the protein haemocyanin (Rossia⁵¹) (mol wt = 3.3×10^6) can be used as an approximation to that of blue dextran (mol wt = 3×10^6) with $D = 1.58 \times 10^{-7}$ cm²/sec.
- (ii) The diffusion of dextran is independent of that of sucrose.
- (iii) No convective readjustment of fluid within the Staflo chamber occurs.

Table III. Fluid velocity profile as calculated from times of flight of human RBC within Staflo chamber measured 1 cm and less from outlet.

Average residence time $\tau = 17.9$ min.

(all velocities are in 10^{-1} millimeter/second.)

Distance from outlet (cm)	Distance from upper membrane (mm)	Distance from near wall (mm)									
		1.33		2.66		4.0		5.33		6.66	
1	15.0	2.33	2.26	3.68	3.59	4.38	4.38	3.89	3.89	2.80	2.80
	16.0	2.33	2.37	3.68	3.78	4.12	4.38	3.89	3.78	2.80	2.80
	17.0	2.12	2.12	3.68	3.68	4.38	4.24	3.89	3.78	2.41	2.41
	18.0	2.19	2.19	3.68	3.78	4.38	4.38	3.78	3.78	2.80	2.59
	19.0	2.12	2.15	3.68	3.78	4.38	4.38	3.78	3.89	2.80	2.80

0.5	18.0	2.69	2.69	3.89	3.68	4.52	4.38	3.89	3.68	2.74	2.69

0	18.0	3.41	3.41	3.89	3.68	4.00	4.00	---	---	---	---

(iv) The simplified form of Fick's law,

$$D = Ql/At\Delta C \quad \text{or} \quad Q = D At\Delta C/l, \quad (3)$$

is a reasonable one to use.

Here Q is the amount of material that has diffused to a distance l through an area A during time t with a concentration difference of ΔC .

For this approximate calculation,

$$\begin{aligned} D &= 1.58 \times 10^{-7} \text{ cm}^2/\text{sec}, \\ A &= 1 \text{ cm}^2 \\ t &= 20 \text{ min} = 1.2 \times 10^3 \text{ sec}, \\ l &= 10^{-2} \text{ cm (this value is } 1/50 \text{ the normal stream thickness),} \\ \Delta C &= 4 \times 10^{-4} \text{ g/cm}^3. \end{aligned}$$

Using these values in Eq. 3, I find

$$Q = 1.9 \times 10^{-6} \text{ g},$$

that is, 1.9×10^{-6} g of blue dextran could diffuse through 1 cm^2 in 20 min to a depth of 0.01 cm. If this is expressed as a percent concentration change, then I have

$$\frac{1.9 \times 10^{-6} \text{ g/cm}^2 \times 10^2}{4 \times 10^{-4} \text{ g/cm}^2} = 0.475\%.$$

A concentration change of this size would be undetectable with the eye. Furthermore (I repeat) the inlet boundaries are very sharp at the inlet end (see Figs. 5 and 6), whereas they have become diffuse very soon and to a depth greater than 0.01 cm.

I conclude, therefore, that assumption (iii) is false and that some type of convection must take place. This convection is discussed in the next section, Diffusion-Convection Theory.

2. Diffusion-Convection Theory

It was pointed out in the preceding section that passive diffusion of the blue dextran alone could not account for the degree of diffuseness of the blue dextran boundary within the Staflo chamber as the fluid nears the outlet. Neither could the passive diffusion of sucrose account for

this diffuseness of the boundaries. However, it was previously stated that diffusion could bring about the mass transfer of small fluid volumes, e.g., by a kind of local convection or by the phenomenon called "droplet sedimentation" which occurs for macromolecular zones under special conditions. Could such a droplet sedimentation be responsible for this diffuseness? Mel has shown specifically³² that indeed it can occur in the Staflo chamber. However, if a high-molecular-weight solute, such as dextran, were uniformly distributed throughout the Staflo chamber and a density gradient of dissolved sucrose superimposed, then this particular kind of boundary instability would not be expected to occur. Furthermore, I have looked for such a droplet sedimentation and have not been able to observe it for such a case.

What other phenomenon might be the cause of the observed boundary diffuseness? The requirements for the phenomenon are:

(i) It must be capable of transporting solute, such as dextran, over macroscopic distances at a rate much faster than could passive diffusion alone.

(ii) It must accomplish this without local macroscopically visible discontinuities in the boundary of the kind seen with droplet sedimentation.

(iii) It must operate approximately equally and simultaneously at the upper and lower boundaries. Droplet sedimentation (flotation) occurs only at the lower (or upper) boundary for any given set of conditions — never both.

There are also several questions related to this phenomenon:

(i) Is the diffusion of the density-gradient-causing molecules related to the phenomenon?

(ii) Is the initial finite step-function density gradient related to the phenomenon?

(iii) Is laminar flow coupled in some way to the phenomenon?

(iv) Is there a specialized local convection occurring within streams that aids in the transport of solute across the boundaries?

Again consider the fluid-flow profile in the horizontal y-z plane. This flow profile is a parabola which is expressed by the equation

$$\frac{dy}{dt} = v = v_{\max} - kz^2 \quad (4)$$

when $dy/dt = v$ is the flow velocity at any distance z from the axis where the flow velocity is $v_{\max}$ the coefficient k has the value

$$k = v_{\max}/z_{\max}^2 \quad (5)$$

The half-width of the Staflo chamber is $z_{\max}$. Integrating Eq. 4 gives, for any y and, τ

$$y = (v_{\max} - kz^2) \tau = v\tau \quad (6)$$

or

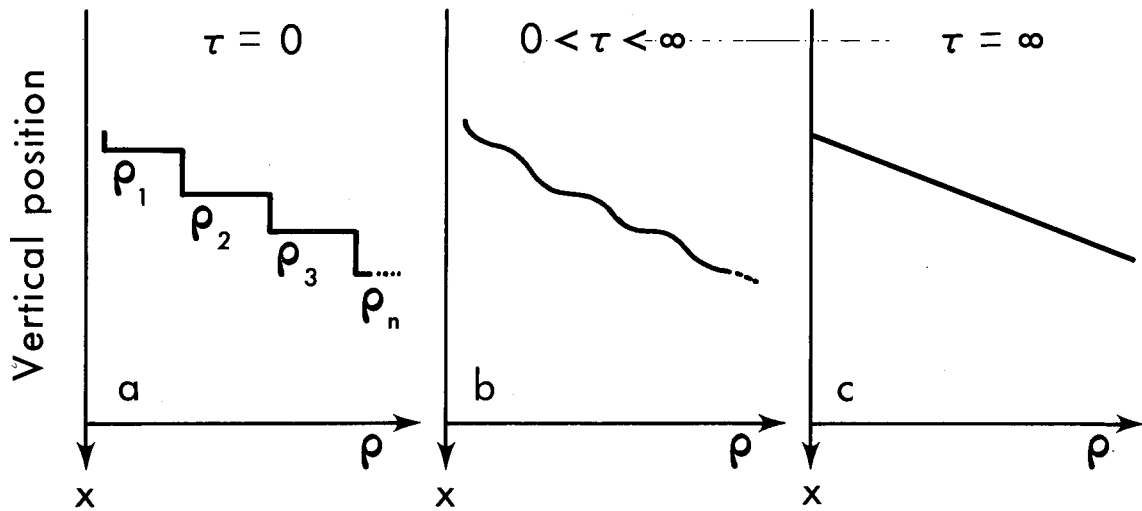
$$\tau = y/v = y/(v_{\max} - kz^2) \quad (7)$$

It is apparent that the time τ for an element of volume to flow a given distance, y from the inlet is related to the horizontal position z within the Staflo chamber. (See Fig. 8.) Obviously, τ is smallest when $z = 0$. Expressed in terms of the length of the Staflo chamber L and the minimum residence time $\tau_{\min}$,

$$\tau_{\min} = L/v_{\max} \quad (8)$$

Consider the initial density gradient in a particular x - y plane. It is a finite step function. It is gravitationally stable because the density gradient is nonnegative at all vertical depths. (I use the convention that the vertical depth direction is positive in the downward sense—hence a stable density gradient would have a nonnegative slope, i. e., $d\rho/dx \geq 0$.)

Diffusion of the solute molecules (sucrose) across the boundaries between streams will result in the boundaries becoming more and more diffuse as time for diffusion progresses. The step function will be transformed into a continuous smooth curve having a positive downward slope at all points. The density gradient will remain stable (see Fig. 10). However, will it also be stable in three dimensions if the time for diffusion is different from point to point, as is the case



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Fig. 10. "Noncompensated" density gradient profiles of fluid within the Staflo chamber in a given vertical (x-y) plane. Sketch represents the inlet-position gradient for any laminar flow rate, or the gradient approached at any position for a sufficiently fast nonturbulent flow rate.

Given the parabolic flow profile in the horizontal y-z plane, the local residence time τ increases as the wall is approached in the z direction. Hence an aged density gradient as in b would tend toward that of c as the wall is approached.

for a laminar flow system? In order to give a possible answer to this question I will use the differential form of Fick's law for diffusion as a reasonable expression for net diffusion of sucrose vertically (x direction) within the Staflo chamber:

$$\frac{dm}{dt} = DA \frac{dc}{dx}, \quad (9)$$

where dm/dt is the rate of material diffusion through an area A subjected to a concentration gradient, dc/dx , and with a diffusion constant D . However, the addition of a small quantity of material to a fixed volume will change the density. At sufficiently low solute concentrations with a solute more dense than the solvent such an addition will increase the density proportionally, i. e.,

$$dm = K dp, \quad (10)$$

where K is a constant of proportionality. (Svensson, Hagdahl, and Lerner¹⁴ have discussed this more generally.) Substituting Eq. 7 into Eq. 9 and rewriting gives, for any vertical depth x ,

$$\frac{dp}{dt} = \frac{DA}{K} \frac{dc}{dx}. \quad (11)$$

It is useful to write this equation in terms of the coordinates y and z , bearing in mind Eqs. 4 and 6. This can be done substituting dy for dt from Eq. 4, yielding

$$\frac{dp(z)}{dy} = \frac{DA}{K(v_{\max} - kz^2)} \frac{dc}{dx}. \quad (12)$$

Also, as a consequence of the horizontal laminar flow profile, it is possible to take derivative of Eq. 6 according to t and z with y constant, yielding

$$dt = 2kzt dz / (v_{\max} - kz^2). \quad (13)$$

Substituting Eq. 13 into Eq. 11 gives

$$\frac{d\rho(y)}{dz} = \frac{2DA kzt}{K(v_{\max} - kz)^2} \frac{dc}{dx}, \quad (14)$$

and replacing t from Eq. 7 gives

$$\frac{d\rho(y)}{dz} = \frac{2DA kyz}{K(v_{\max} - kz)^2} \frac{dc}{dx}. \quad (15)$$

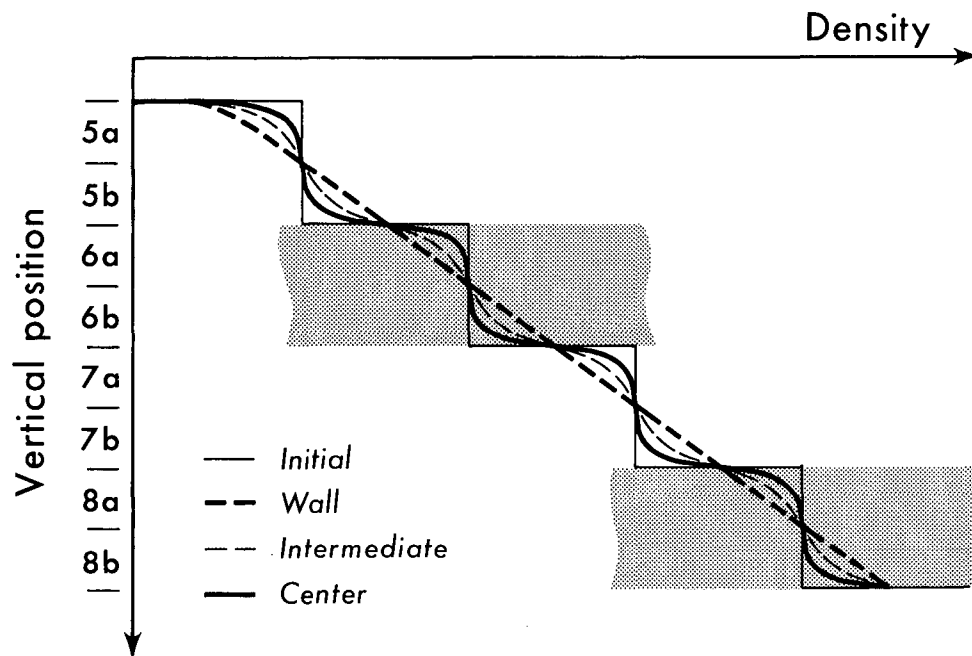
The above equations were derived on the assumption that the concentration gradient dc/dx was fixed and finite. In the Staflo chamber the initial concentration gradient at the boundaries between streams is extremely large and everywhere else the concentration gradient is zero. However, as time progresses the concentration gradient approaches the initial average concentration gradient dc/dx (average),

$$\text{i. e., } \lim_{t \rightarrow \infty} \frac{\Delta C}{\Delta x} = \lim_{y \rightarrow \infty} \frac{\Delta C}{\Delta x} = \frac{dc}{dx} \text{ (average)}. \quad (16)$$

Notice that the respective density gradients described in Eqs. 12 and 15 increase without limit as the wall is approached for any finite concentration gradient. However, the density gradient in the z direction is always greater (absolute magnitude) than in the y direction at any given vertical height x . (Compare $d\rho(y)/dz$ with $d\rho(z)/dy$.)

In a fluid along a horizontal plane, the density gradient must be zero for a condition of stability to exist. The density gradients described above are nonzero, hence the fluid must be in an unstable condition. The instability is greater the larger the absolute magnitude of the density gradient. It would seem, therefore, that the instability in the z direction would tend to cause a local fluid motion before (and hence obscure) any local fluid motion that might occur because of the instability in the y direction. Recall that the solute concentration is a step function, increasing downward in discrete steps at every stream boundary, and also that within any stream the solute concentration is constant.

Consider the boundary between two streams of different initial densities with laminar flow parallel to the boundary (see Fig. 11). Assume only diffusion transports density-causing solute molecules across



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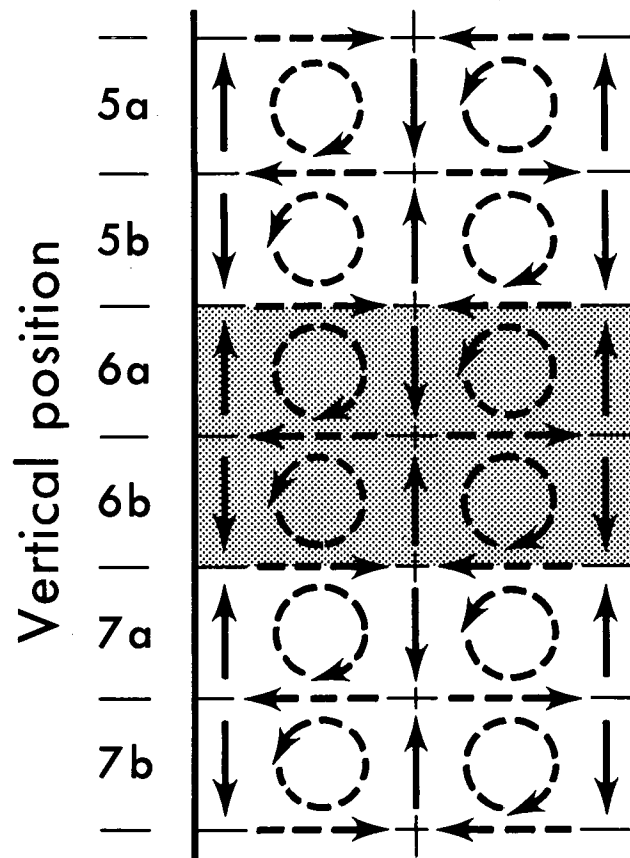
Fig. 11. Vertical density-gradient profiles at an arbitrary downstream position within the Staflo chamber, indicating the effect of diffusion upon the initial step-function density gradient at the center, intermediate, and wall positions.

the boundary. At any distance y from the inlet, as the wall is approached, the diffusion time increases. This increased diffusion time allows a greater quantity of solute to be transported across the boundary near the walls than in the center. In the upper stream this would result in a density that is greater near the walls than in the center and concomitantly in the lower stream the density would be less near the walls than in the center. However, if one considers a single stream, then at any x position in the upper half of this stream the density is less near the walls than in the center, and concomitantly at any x position in the lower half of this stream the density is greater near the walls than in the center. If conditions permit, a readjustment of this density instability would be expected in the following manner (See Fig. 12):

- (i) The fluid would flow toward the center (away from the walls) at the boundary between streams of different initial density.
- (ii) The fluid would flow away from the center (toward the walls) at the horizontal midplane of each stream.
- (iii) The fluid would flow downward in the upper half and upward in the lower half of each stream at the vertical midplane of the Staflo chamber.
- (iv) The fluid would flow upward in the upper half and downward in the lower half of each stream very near the walls of the Staflo chamber.
- (v) The net fluid flow would be somewhat like a helix (four per stream), since there is also a flow along the length of the chamber. (This helical flow may not in fact make a complete turn, but it is expected to make at least a partial turn.)

This local fluid motion would tend to make any migrating species flow at more nearly the average flow rate than laminar flow would permit. This local fluid motion would tend to cause the boundaries between layers to become diffuse at a rate more rapid than expected for diffusion operating alone. Boundary discontinuities would tend not to occur as in "droplet sedimentation," since no macromolecules are involved.

The upper and lower boundaries of a stream would appear to be very much alike.



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Fig. 12. Possible local fluid motion for a thick stream within the Staflo chamber with gross fluid flow out of the plane of the paper. At levels 5a, 6a, 7a, there is a tendency for fluid at the wall to be less dense than at the center, whereas at 5b, 6b, 7b, there is the opposite tendency. The arrows indicate the direction of local fluid motion.

Several experiments might be tried to determine the existence of this phenomenon, which I call "diffusion-convection."

(i) Recall that the boundary between streams becomes diffuse more rapidly than passive diffusion alone would permit. Therefore a diffusion experiment comparing stationary fluid boundaries with nonstationary boundaries could yield some information with regard to diffusion-convection. This type of experiment was rejected because of the difficulty of forming very sharp initial boundaries between "streams" in a stationary system.

(ii) If the Staflo streams could be sampled at various positions in the x-z plane one might obtain solutions of different density. This type of experiment was rejected because the density differences were expected to be very small and because accurate pycometry is very difficult.

(iii) If a marker solute (dye) different from the density-producing solute were placed in every other stream and then sampled at various positions in the x-z plane one might obtain solutions of different dye concentrations than expected with no diffusion-convection. This type of experiment was never considered.

(iv) If into every stream there were suspended velocity markers (RBC), as in the case when the velocity profiles were measured, one would look for a small angle change caused by the local fluid motion within a given stream. The RBC would faithfully follow any fluid motion. This experiment was tried but it was equivocal, inasmuch as the angle looked for was on the order of 1 deg or less and the measurement error was on the order of 1 deg or more.

(v) A fluorescent dye could be placed into several streams and then illuminated by the thin-slit technique (to be discussed later). The observation of the profile change, if any, along the length of the Staflo chamber would yield information pertinent to diffusion-convection. An experiment of this type was performed, and it is described in the following section.

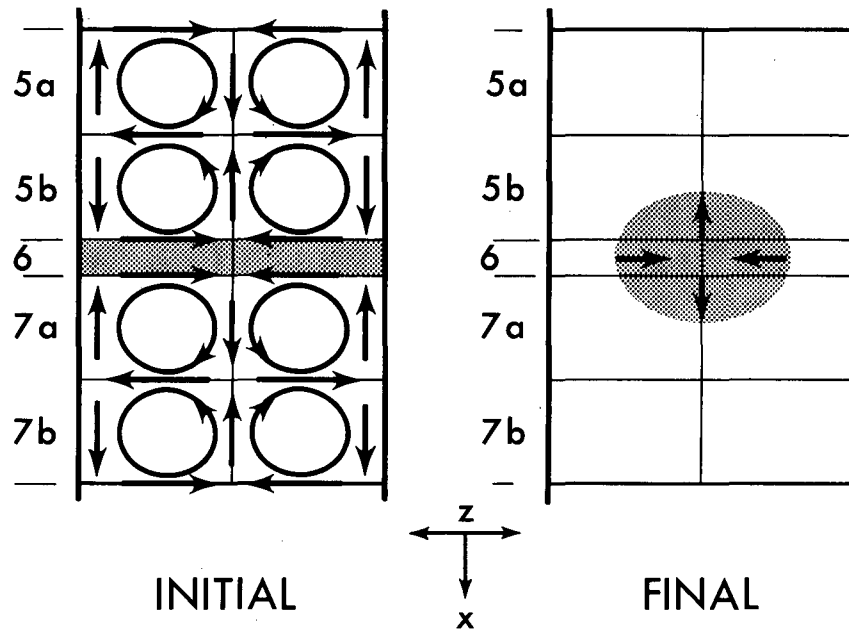
3. "Central Sharpening" Due to Diffusion-Convection

Diffusion-convection as defined and discussed in the preceding section is a specialized kind of fluid motion initiated when a fluid containing a density gradient of general step-function character is subjected to laminar flow. Notice again in Fig. 12 that the proposed diffusion-convection movement at the boundary between streams of initial density (e. g., 5b-6a and 6b-7a) is toward the center of the Staflo chamber. Such fluid movement should also prevail at the boundary for thinner streams. For such streams, the upper and lower boundaries might be sufficiently close to cause them to be coupled by viscous forces. Such coordinated coupling of the two boundaries might prevent the fluid at the midlevel of the thin stream from flowing outward toward the walls (see Fig. 13). For this case some fluid comprising the thin stream would be transported from near the walls toward the center. If this thin stream also had a small quantity of fluorescent marker dye it also would be transported from near the walls toward the center. It would be expected that the dye marker would become narrower horizontally but thicker vertically in the central region (see Fig. 13 final). This type of central sharpening would not be expected to increase the mass per unit volume of the dye transported--only the shape of its stream.

In an extreme case of diffusion-convection the sample dye might conceivably be transported to midlevel of the streams above and below and then be moved back toward the walls. (Compare Fig. 13 with Fig. 12.) This is not likely to occur, considering the relative large density increment from one stream to the next.

In thick streams, if no diffusion-convection were to occur, one would expect the sample near the walls to diffuse a greater vertical distance than in the center. Then the boundary between the sample and adjacent upper and lower streams would be curved, i. e., would then be thicker near the walls than in the center. However, if diffusion-convection did occur, then one would expect the boundary to be more or less planar.

In order to investigate the existence of diffusion-convection, an experiment was performed as described below.



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Fig. 13. Proposed effect of diffusion-convection upon a "thin" stream (designated as 6) as seen in an x-z cross section, with the fluid moving out of the plane of the paper. The arrows indicate the direction of local fluid motion.

4. Experimental Test for the Existence of Diffusion-Convection

A sucrose density gradient was established, ranging from 0.4 to 4.8 g/100 ml in twelve equal steps from streams 1 to 12. In addition, streams 2, 4, 6, 9, and 11 contained the fluorescent dye sodium fluorescein in a concentration of 4×10^{-6} g/ml. All streams were pumped at the same rate except streams 6 and 9, which were pumped at 0.23 and 0.36 times the others, respectively with the result that streams 6 and 9 were thinner than the others. The "thin-sample" technique of which these are examples, is discussed later. The average residence time $\tau_{av} = 20$ min.

The results are shown photographically in Fig. 14. These profiles in cross section (x-z plane) were obtained by the thin-slit technique. The positions of the profiles are indicated below each frame with the corresponding average residence time τ_{av} to reach these positions. The fluid flow is, as is the convention, from right to left.

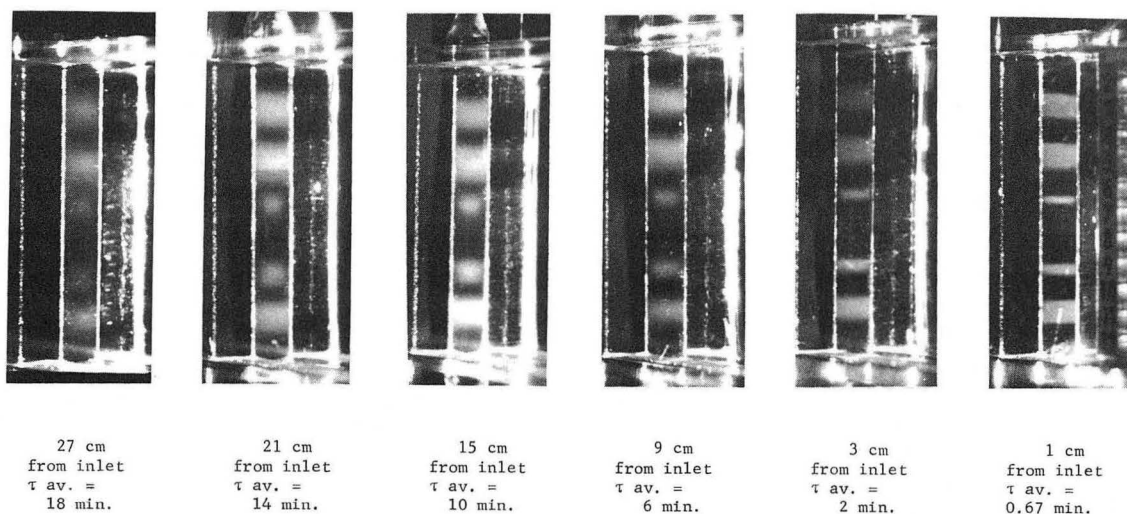
Notice that the thinnest stream, No. 6, begins to pull (or is pulled) away from the walls very soon after entering the Staflo chamber, while stream No. 9 requires a longer time. I call this effect diffusion-convection central sharpening. Furthermore, notice that the boundary between the thick streams remains planar and that there appears to be no tendency to form a curved boundary as predicted for no local fluid motion. All the findings are in accord with the concept of local fluid motion, i. e., diffusion-convection.

D. Staflo Electrophoresis

1. Theoretical Migration Profile

(Local fluid motion of the diffusion-convection type or droplet-sedimentation type is not considered in this treatment.)

Recall that in the Staflo chamber the horizontal-velocity fluid flow profile is a parabola. Any migrating species migrates normal to this flow profile. Since resolution by the Staflo method is affected by the migration profile, it is important to know the shape--i. e., the cross section--of the profile and the material distribution within the profile.



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Fig. 14. The effect of diffusion-convection upon a steady-state flow pattern of dye stripes of varying thickness at selected distances from the inlet for average residence time $\tau_{av}=20$ min for 30 cm. The time τ_{av} below each frame is the average residence time of the fluid to reach the distance indicated (illumination was by the thin-slit technique).

In order to theoretically determine its shape I use the following facts or assumptions.

(i) The horizontal fluid flow velocity profile within the Staflo chamber is a parabola.

(ii) A given species has a constant electrophoretic velocity under a given set of conditions.

(iii) The migration occurs in a fluid system that is nonturbulent and nonconvective.

Consider again the equation for the horizontal-velocity fluid flow profile,

$$\bar{v} = v_{\max} - kz^2, \quad (4)$$

with symbols as defined earlier.

I shall consider the migration profile at a particular point at a distance L' from where the electrophoresis was started [all primed (!) symbols refer to the region where electrophoresis occurs, e.g., L' , τ' , etc.]. In my Staflo chamber L' is not equal to L , the length of the Staflo chamber (see Fig. 15).

Figure 15 gives a perspective view of the Staflo chamber with the samples entering at the right and flowing 40% of the length of the chamber before the sample begins to undergo electrophoresis. As the sample proceeds further to the left, laminar defocusing occurs, giving the appearance depicted. (See also Figs. 16 and 17.)

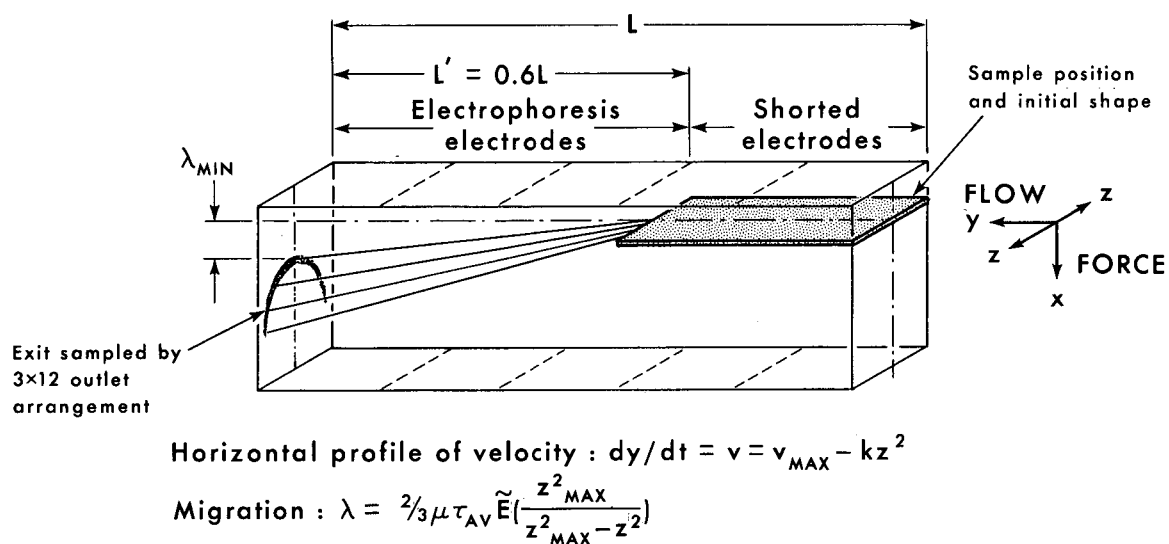
These migration profiles can be readily seen and photographed by the thin-slit technique, which is described along with other illumination methods in a subsequent section.

For all electrophoretic studies described in this thesis, we have

$$L' = 0.6 L. \quad (17)$$

Recall that τ_{av} is the average residence time (also known as the throughout time). This time is related to the minimum residence time $\tau_{\min}$, because of the parabolic flow profile, by

$$\tau_{\min} = \frac{2}{3} \tau_{av}. \quad (18)$$



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Fig. 15. Schematic drawing of the development of a Staflo electrophoretic migration profile of a single component subject to laminar defocusing. (The rays are shown only for the front half of the electrophoretic pattern.)

When the relationship of Eq. 17 is used, Eq. 18 becomes

$$\tau'_{\min} = \frac{2}{3} \tau'_{\text{av}}. \quad (19)$$

If one considers all the residence times for electrophoresis, τ' , one obtains from Eqs. 17 and 4

$$\tau' = \frac{0.6L}{v} = \frac{L'}{v} = 0.6 \tau. \quad (20)$$

When a species migrates normal to the fluid flow with a constant electrophoretic velocity $\mu \tilde{E}$, migration time τ' is proportional to the distance migrated, λ , i. e.,

$$\lambda = \mu \tilde{E} \tau', \quad (21)$$

where λ , μ , $\tilde{E}$, and τ' are measured in cm, (cm/sec)/(volt/cm), volts/cm, and sec, respectively. Substituting Eq. 20 into Eq. 21 gives

$$\lambda = \mu \tilde{E} L' / (v_{\max} - kz^2) \quad (22)$$

or

$$\lambda = \mu \tilde{E} L' z_{\max}^2 / v_{\max} (z_{\max}^2 - z^2). \quad (23)$$

It was shown earlier that

$$v_{\max} = \frac{3}{2} \frac{L}{\tau'_{\text{av}}} = \frac{3L}{2\tau'_{\text{av}}}. \quad (24)$$

Substituting this for $v_{\max}$ in Eq. 23 gives

$$\lambda = 2\mu z_{\max}^2 \tau'_{\text{av}} \tilde{E} / 3(z_{\max}^2 - z^2). \quad (25)$$

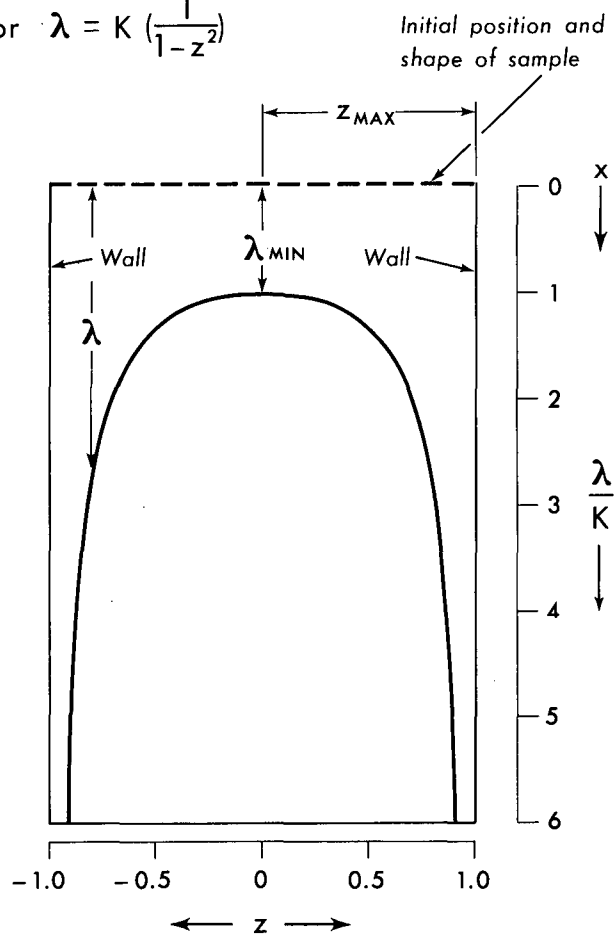
This equation gives a curve that has the general form of an arch (see Fig. 16).

It is readily seen that the minimum migration distance, $\lambda_{\min}$, occurs when $z = 0$. This migration takes place in a time of $2/3 \tau'_{\text{av}}$, i. e., $\tau'_{\min}$. It should also be noticed that the migration distance increases without bound as the wall is approached, i. e., as z approaches $z_{\max}$. Notice also that the curve for the electrophoretic migration distance does not intersect the wall, but approaches it asymptotically.

THEORETICAL MIGRATION PROFILE : $\frac{\lambda}{K}$ vs. z

$$\lambda = \frac{2}{3} \mu \tau_{AV} \tilde{E} \left(\frac{z_{MAX}^2}{z_{MAX}^2 - z^2} \right)$$

or $\lambda = K \left(\frac{1}{1-z^2} \right)$



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Fig. 16. Theoretical Stafo-electrophoretic migration profile in the x - z plane (fluid flow normal to the plane of the paper) for a monodisperse sample.

Figure 17 illustrates both the general form of the electrophoretic migration profiles, and the differences that concentration and direction of migration make upon the profile. Figure 17a' has been enlarged and an overlay of the theoretical migration profiles for selected migration distances placed over it in Fig. 24.

2. Differential-Heating "Central Sharpening" Theory

Three main factors need to be considered when a electric current is passed through the Staflo chamber during an electrophoretic run. These are

- (i) the difference in resistivity, if any, between the sample solution and the background buffer solution,
- (ii) the distribution of these resistivities from point to point within the Staflo chamber, i. e., the migration profile,
- (iii) the resistivities as functions of the parabolic fluid flow profile.

It has been observed that if the initial resistivity of the lipoprotein sample solution is much greater or much smaller than the resistivity of the background buffer solution then the electrophoretic migration of the sample is likely to be unstable. If, on the other hand, the resistivity of the lipoprotein sample solution is equal or nearly equal to the background buffer solution, then the electrophoretic migration of the sample is likely to be stable (slightly greater resistivity is much better than slightly smaller resistivity). This electrophoretic instability is related to the electric field strength, $\tilde{E}$, the electrophoretic velocity of the sample, $\mu\tilde{E}$, and the density of the solute relative to the background solution, $\Delta\rho$.

The electric field strength $\tilde{E}$ is related to the resistivities by Ohm's Law and for the respective resistivities r_s and r_b , we have

$$\tilde{E}_s = \tilde{i} r_s$$

(25)

$$\tilde{E}_b = \tilde{i} r_b,$$

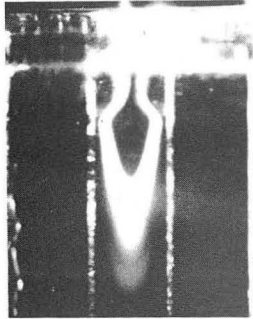
and

where $\tilde{i}$ (A/cm^2) is the current density. For the present I shall assume that the current density is constant and independent of the

Fig. 17. Photographs of Staflo electrophoretic migration profile of S_f $0-10^5$ lipoprotein taken near the outlets, with the thin-slit illumination technique (see Section IF2). Direction of migration and initial concentration given for each.

- a. Upward, 1.189 g%.
- a'. Downward, 1.189 g%.
- b. Upward, 0.549 g%.
- b'. Downward, 0.549 g%.
- c. Upward, 0.296 g%.
- c'. Downward, 0.296 g%.

Note: The narrower diffuse bands extending to the right from the regions of greatest film exposure are portions of the sample band that are visible when the whole migration pattern is viewed with broad-beam scattered light (see Fig. 21). The migration profile is very much overexposed.



a



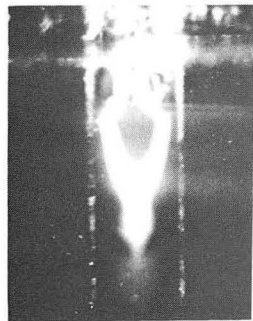
a¹



b



b¹



c



c¹

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point-to-point change in resistivity. In this case when considering Eq. 25 one would expect that the electric field strength would change proportionally for a change in the resistivity. Since the electrophoretic migration velocity contains the electric field strength as a factor, it also would change proportionally for a change in the resistivity.

Consider a solute which has an electrophoretic mobility μ and the solution zone containing this solute has a resistivity r_s that is not equal the adjacent buffer solutions' resistivity r_b ,

$$\text{i. e.,} \quad r_s \neq r_b. \quad (26)$$

Recall that a zone has an upper and a lower boundary, and that diffusion of solute and buffer across the boundaries will tend to smooth them. This results in a transition region from the buffer solution into the sample zone. That is, there is a range of resistivities across this transition region from r_s to r_b . A consideration of Eqs. 24 and 25 shows that there would be a concomitant change in the electric field strength, electrophoretic migration velocity, and migration distance.

For downward electrophoretic migration, when r_b is greater than r_s , the electrophoretic migration distance for the solute at the lower boundary of the sample zone would be greater than the electrophoretic migration distance for the upper boundary. In this case the sample zone would widen as it electrophoresed. On the other hand, if r_p is less than r_s the opposite effect would occur and the sample zone would become thinner. If the solute were more dense than the solvent then, in this latter case, the sample zone would likely sediment (or, if less dense, it would float). If, however, r_b equaled r_s , that sample zone thickness (for a monodisperse sample) would remain the same. In most of my experiments the initial resistivity r_s (ohm-cm) of the protein-containing solution is greater, by a small amount, than the resistivity r_b of the adjacent buffer solution.

Consider a single species with an electrophoretic mobility μ that is electrophoresing in a uniform current distribution and a uniform background buffer solution. If the resistivity, r_s , of the protein band

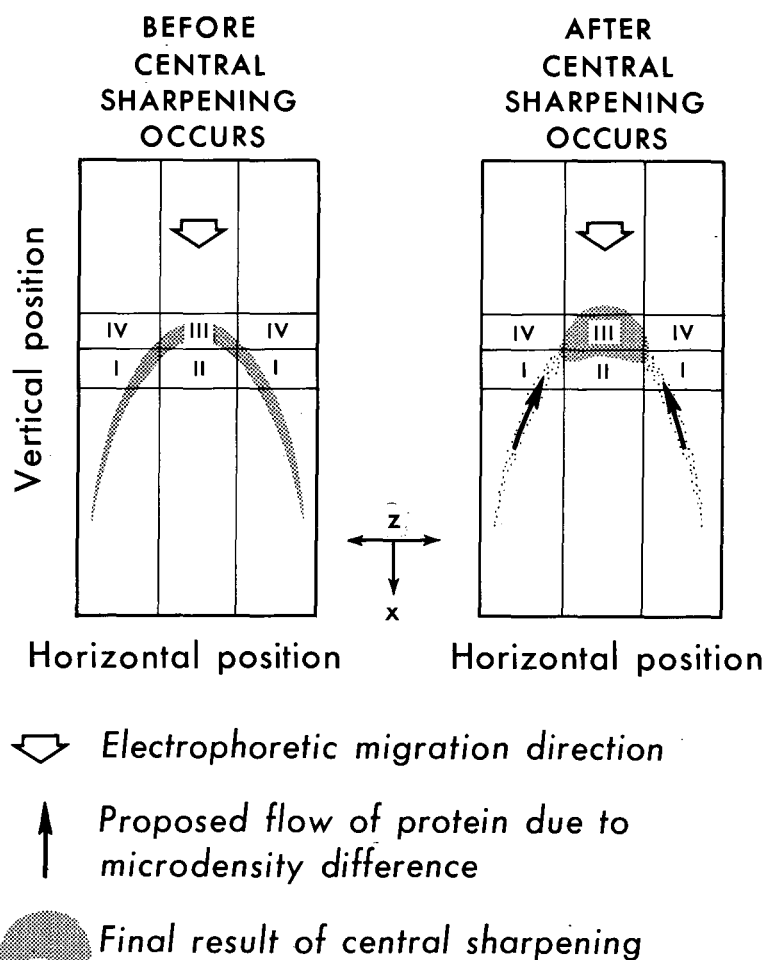
is greater than the resistivity r_b of the buffer solution, then the heat production due to the passage of electric current will be greater (if resistivity is less it will be smaller) in the protein band than in the buffer solution. This results in a decrease in density of the protein band relative to the buffer solution, i. e., the protein band would tend to float, all other things being equal. (An increase in density would make it sink.)

Recall that in the Staflor chamber "laminar defocusing" causes the migrating boundary to assume an arch-shaped profile (see Section I. D1). This assumes uniform electrophoretic velocity along the z direction of the chamber.

I have experimentally measured the resistivities of the outlet solutions in a uniform 3×12 array in the x - z plane. At this time I will discuss only those resistivities in the region of the migration profile.

In Fig. 18 there are six regions: Two numbered I, one numbered II, one numbered III, and two numbered IV. The shaded areas are the locations of the protein solute. The resistivities could be measured only over a larger region, e. g., regions I, II, III, IV. This makes it necessary to infer from the resistivity measurements what are the point-to-point resistivity, r_s , of the protein solution and the resistivity of the nearby buffer solution in the various regions. However, in all cases the resistivity of those solutions from the various regions that contained protein was smaller than for solutions from the horizontally adjacent regions. Considering Fig. 18, this would mean that regions I and III had smaller resistivities than did regions II and IV, respectively. Sometimes regions III and IV would have very nearly the same resistivity.

If the electric current is uniform then one might expect the buffer solutions to become warmer than the adjacent protein-containing solutions because of their higher resistivities. In this case the heating effect would vary according to the resistivity from point to point. However, if the electric current is not uniform, but can distribute itself according to the various resistivities as in the situation of parallel resistance, then the heating effect would vary according to the square of



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Fig. 18. Proposed modification of sample migration profile, resulting in "central sharpening" that would result from microdensity differences occurring during downward electrophoresis. Section shown is in the x-z plane. (Fluid flow is normal to the plane of the paper.) See text for explanation of Roman numerals.

the electric current from point to point. In this latter case the protein solution would be heated the most. At this time the choice between these two possibilities has not been made, indeed the electrical heating effect may be a combination of the two.

It is obvious, for a more or less uniform electric current distribution, that as the wall is approached the solutions must become warmer, hence less dense, since there is an increase in residence times τ' along the z direction as a result of the horizontal parabolic flow profile. In downward migration this would tend to cause a local fluid flow up in the region of the "legs" of the electrophoretic migration profile. Such fluid movement might tend to concentrate the sample in the center of the cell, as depicted in Fig. 18. It might also tend to suppress "laminar defocusing," which would give a more steady migration in a narrow band down the center of the chamber. This effect obviously cannot completely suppress laminar defocusing. I call the above phenomenon electrophoretic "central sharpening."

3. Solute-Solvent Differential-Density "Central Sharpening"

Theory

The foregoing argument (and discussion) is believed to be a specific case of the more general phenomenon of central sharpening related to density differences at any particular level in the Staflow chamber. These density differences have been shown to account for central sharpening by diffusion-convection, and by microheating of an electrophoresing species.

Consider a sample species that floats at a particular level (neutral buoyancy position) in the Staflow chamber. If this species is electrophoresed, or otherwise moved (but not by gravity), to a lower level the species will now have a density less than the surrounding fluid and will tend to float, i. e., there will be a density difference between the sample species and the surrounding fluid.

I will base the following argument--for nonthermal central sharpening for a solute particle concentration that is high enough to make the solution, to some degree, density-dependent--upon the solute concentration.

As the sample migrates downward within the Staflo chamber laminar defocusing occurs, giving rise to the "archlike" migration profile, as shown before. (The force causing the migration, other than gravity, is of no importance in this argument. It could be magnetic, for example.) Returning to Fig. 18, we see that the sample solution that has migrated into region I from region IV must be less dense than region II for solute particles having an inherent density less than that of the solvent or background solution. It follows, therefore, that the expected fluid flow would result in central sharpening and would be identical in every respect to that caused by differential heating. However, for solute particles more dense than the solvent one would expect the opposite effect, with the local fluid flow withdrawing sample from the center down the legs of the migration profile because the sample in region I would be more dense than in region II and also more dense than in region III.

Upward migration of solute particles more dense than the solvent would be expected to give rise to central sharpening because the sample in region IV would be more dense than in region I. For solute particles less dense than the solvent upward migration would not give central sharpening, because the sample in region I would be less dense than in region IV. The fluid containing the sample would be expected to flow upward, withdrawing sample from region II into region IV.

Gravity-induced flotations would not be expected to give rise to central sharpening; flotation is the result of solute particles' being less dense than the solvent which would make the sample in region IV less dense than the sample in region II because less dense solute particles would rise within the migration profile legs pointing upward.

On the other hand gravity-induced sedimentations would not be expected to give rise to central sharpening either; sedimentation is the result of solute particles' being more dense than the solvent which would make the sample in region I more dense than the sample in region II because the more dense solute particles would fall within the migration profile legs pointing downward.

Summary:

- (i) Downward migrations of solute particles less dense than the solvent are expected to give rise to central sharpening.
- (ii) Upward migrations of solute particles more dense than the solvent are expected to give rise to central sharpening.
- (iii) Gravity-induced migrations are not expected to give central sharpening.

It seems that there are three possible types of central sharpening:

- (i) diffusion-convection central sharpening,
- (ii) electrophoresis-differential-heating central sharpening,
- (iii) solute-solvent differential-density central sharpening.

What would be the expected density differences of the very low-density lipoprotein in downward migration, neglecting ohmic heating, during Staflo electrophoresis? (The hydrated density of this fraction is between 0.93 and 1.007 g/ml.). The background solution of the Staflo chamber ranges in uniform steps of 0.4 g/100 ml (0.00156 g/ml) sucrose from stream 1 to stream 12 from 0.9998 to 1.071 g/ml at 20°C. The sucrose background of density 1.007 g/ml would be located at the approximate level of stream 5. That is, the background solvent in the lower streams would always have densities greater than the hydrated densities of any of these lipoproteins. The difference in stream density without sample is 0.00156 g/ml. For this problem, we shall assume that region I contains very-low-density lipoprotein at 0.5 g/100 ml concentration at a hydrated density of 1.007 g/ml, and that region II contains none. I choose level 6, in which the solvent density (2.4 g/100 ml sucrose) is 1.00756 g/ml. This is the density of region II. I assume that the density increment of the lipoprotein is additive to that of the solvent, in which case a 0.5-g/100 ml very-low-density lipoprotein sample would decrease the solution density by 2.8×10^{-6} g/ml. The final density of region I would then be 1.007557 g/ml. This change in density is

$$(2.8 \times 10^{-6} / 1 \times 10^{-4}) 10^2 = 2.8 \%$$

of a density difference known to cause stream inversion. (If a stream is introduced into the Staflo chamber that is less than the stream above

it, stream inversion will result. This is known to occur for a density difference as small as 0.0001 g/ml.) If the lower value of these lipoproteins' density range is taken as 0.94 g/ml, then the corresponding density decrease of region I relative to region II is 3.8×10^{-5} g/ml.

This value is

$$(3.8 \times 10^{-5} / 1 \times 10^{-4}) 10^2 = 38\%$$

of a density change known to cause stream inversion.

As the migration progresses to lower and lower streams, this density difference increases with the concomitant increased tendency for solute-solvent differential-density central sharpening to occur.

I have now completed the discussion of the various types of central sharpening. I have shown that each is related to the horizontal-velocity fluid flow profile and to a local density difference that results in a local fluid motion tending to deliver the material of a thin zone toward the center of the Staflo chamber.

4. Experimental Confirmation of Staflo Electrophoretic Central Sharpening

I have observed that the sample would sometimes migrate within the Staflo chamber as a very narrow band. This band had the appearance of a pencil lead seen from the side--i.e., round--whereas the initial sample had the appearance of a thin box or sheet. This change in shape of the migrating sample was my first indication that the phenomenon now called "central sharpening" was occurring. The following experiment was repeated for further analysis because it seemed that it gave considerable central sharpening. The experimental conditions are given in Table IV.

The methylene blue sample streams entered the Staflo chamber at levels 3, 4, 5, and 6 to form a single thick stream. This wide stream remained thick until it was electrophoresed downward in the latter 60% (18 cm) of the Staflo chamber, where it became very narrow vertically and horizontally in a distance of about 3 cm. This pencil-lead-shaped methylene blue stream remained very narrow and approximately equidistant from the front and back wall throughout the

Table IV. Experimental conditions for methylene blue electrophoresis: central sharpening.

Inlet stream number	Standard ^a buffer (ml/100 ml)	Sucrose (g/100 ml)	Methylene blue (g/100 ml)
Top electrode wash	4.4	0.0	0
1	4.0	0.4	0
2	3.2	0.8	0
3	0.0	1.2	4×10^{-4}
4	0.0	1.6	4×10^{-4}
5	0.0	2.0	4×10^{-4}
6	0.0	2.4	4×10^{-4}
7	0.4	2.8	0
8	0.8	3.2	0
9	1.2	3.6	0
10	1.6	4.0	0
11	2.0	4.4	0
12	2.4	4.8	0
Bottom electrode wash	2.8	5.2	0

Total voltage impressed across the last 60% (18 cm) of the Staflo cell electrodes from top to bottom: 50 V @ 8 mA (0.55 mA/cm^2)

average residence time: $\tau_{av} = 11.0 \text{ min.}$

Electrode wash rate: 10 ml/min.

pH: 8.9

3×12 outlet arrangement: to 36 equal collection containers.

^a Standard buffer: 0.5 M tris, 0.0205 M EDTA, and 0.076 MH_3PO_4 .

remainder of the Staflo chamber.

The narrowing of the thick methylene blue stream in the vertical direction was a consequence of the resistivity gradient that began at level 7. The effect of this resistivity gradient is to progressively lower the electric field strength as the bottom of the Staflo chamber is approached. This causes the forward (bottom) boundary to be slowed relative to the backward (upper) boundary of the methylene blue sample as it electrophoreses downward. The optical density (OD) of the methylene blue solutions was measured at 649 m μ by a Beckman DK-1 spectrophotometer.

I have chosen the OD of the inlet solutions of methylene blue solute to be 100% each for a total of 400%. The outlet solutions are compared to a single inlet stream.

If there were no central sharpening or laminar defocusing within the Staflo chamber, then all the methylene blue solute would be expected to exit at three outlets side by side at a concentration no greater than 4 times the inlet concentration, i. e., 400% or less each. (Concentration in this experiment is equivalent to mass of solute, since the volume outputs from each of the 36 outlets are equal.) Central sharpening would be expected to increase the concentration of methylene blue above 400% while laminar defocusing would be expected to decrease the concentration of methylene blue below 400%. Notice that center outlet stream 8 had a concentration of 448%. It appears that central sharpening has occurred.

If the sum of the quantity of methylene blue in the front, center, and back vertical rows of outlets is totalled then (assuming only laminar defocusing), one would expect equal quantities of methylene blue in each set of outlets. However, in this experiment, if the front, center, and back outlet sets are compared, it is found that they contain 20.1%, 56.2%, and 24.0% of the total outlet quantity of methylene blue. Again, it appears that central sharpening has occurred.

If a correction for the horizontal parabolic velocity-flow profile is made for a 3 $\times$ 12 outlet arrangement when equal volumes are collected for all 36 collection containers, then one finds that the center

one-third of the volume flowed in the center 23% of the width of the Staflö chamber. (See Section I. E2, following.) Notice in Fig. 19 that there is a concentration change of about 2-1/2 times at the center outlet 8 over either front or back outlets 8. It is apparent that the center methylene blue solute must be more localized in space than Fig. 19 indicates. Again it appears that central sharpening has occurred.

It should be clearly seen that central sharpening has occurred because of

- (i) the appearance of a "pencil-lead" shape of migration profile during electrophoresis,
- (ii) the increase in concentration of the center stream at level 8 above that expected without central sharpening,
- (iii) the increase in total mass of solute in the center stream over that expected in the absence of laminar defocusing,
- (iv) the localization in space of the center stream as determined by a consideration of parabolic flow.

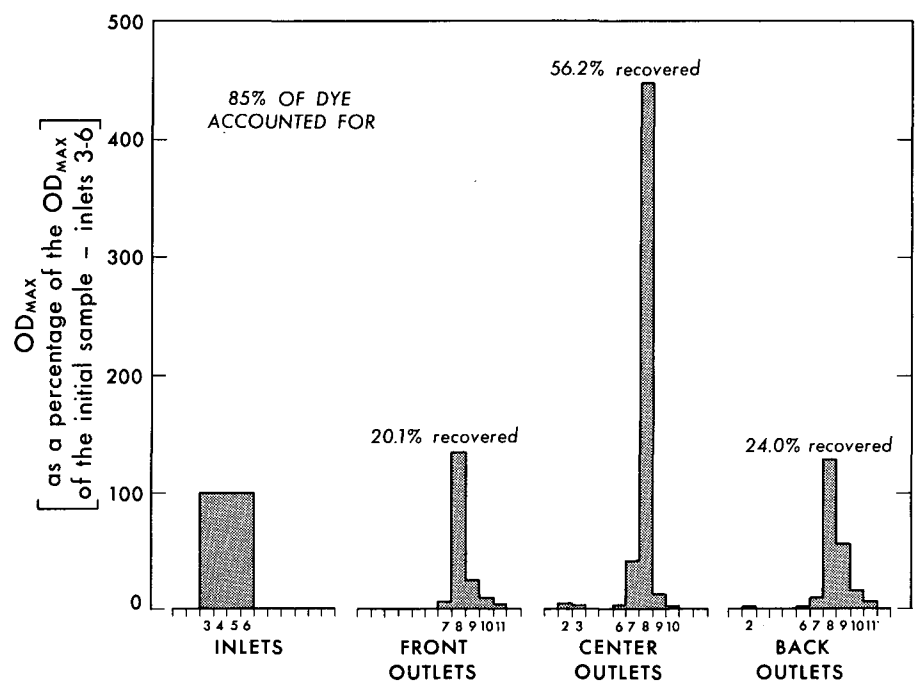
I therefore conclude that central sharpening has occurred and in this case it was accompanied by a concomitant concentration of methylene blue in the horizontal direction.

E. Linear and Volume Flow Relations

1. The Thin-Sample Technique

The methylene blue experiment (of the previous section) that was used to test for the existence of Staflö electrophoresis central sharpening has shown that thick samples can be used for some types of experiments--particularly those that involve electrophoretic concentration. However, for analytical work, a thin sample is necessary for increased resolution, as was pointed out by Philpot.⁴³

He discusses the separation of a mixture having an initial sample zone thickness p as a function of current density $\tilde{i}$, time t , resistivity r , differences in mobility (of two components 2 and 1), $\mu_2 - \mu_1$ and the differences their respective total migration distances, $\Delta\lambda = \lambda_2 - \lambda_1$, covered during this separation.



MU-36573

Fig. 19. Staflo electrophoresis of a "thick" sample of methylene blue, indicating electrophoretic "central-sharpening" effect. Optical densities were read on a Beckman DK-1 spectrophotometer at the absorption maximum at 649 mμ.

His equation for complete separation is

$$P = \tilde{i} t r (\mu_2 - \mu_1) = \lambda_2 - \lambda_1 = \Delta \lambda. \quad (27)$$

It is obvious that for any given separation $\tilde{r}$, μ_1 , and μ_2 are fixed, and that only p , t , and $\tilde{i}$ can be altered. However, the current density $\tilde{i}$ is usually limited because of the heat produced during the current flow or owing to instabilities that can develop during migration of a sample because of excessive electric field strength $\tilde{E} = \tilde{i} r$. As a practical matter, in the Staflo chamber the average residence time, $\tau_{av}^1 = t$, is limited because the distance λ_2 that the faster component of the mixture migrates before a separation is complete is greater than the practical height of the Staflo chamber. Therefore only p can be altered. It is seen that as p becomes smaller, $\Delta \lambda$ can become smaller. Hence, λ_2 and λ_1 could also become smaller, because the time t necessary to achieve the separations becomes smaller.

The thin sample in the Staflo chamber is obtained by introduction of the sample at a volume flow rate less than that of the other streams. This is possible because the viscous shear gradient at the boundary between streams is experimentally found to be zero. The sample stream thickness is related to

- (i) the ratio of the volume flow rate of the sample stream to the volume flow rate of any other stream,
 - (ii) the total volume flow rate, and
 - (iii) the position of introduction into the Staflo chamber.
- The latter two points are of minor importance, and they are related to the vertical velocity profile (see Section I. C1a). They are of minor importance except when the sample stream is introduced into stream positions 1 and 12. Then it would be expected to be thicker than if introduced elsewhere. The sample stream would be somewhat thinner if introduced into inlets 2, 3, 10, and 11.

If the sample stream has a volume flow rate of 0.2 times that of each of the other streams, its thickness is approximately 0.2 times their thickness. In the 3.0-cm-high chamber the sample stream's

thickness is ≈ 0.5 cm. For twelve inlet streams its true thickness if introduced into the vertical plug flow region--i. e., inlets 4, 5, 6, 7, 8, and 9--would be

$$P_s = P \left(\frac{12 Q}{11Q + Q_s} \right) \frac{Q_s}{Q}, \quad (28)$$

where Q is the volume flow rate of any stream when all have the same volume flow rate; Q_s is the volume flow rate of the sample stream, P_s is the sample stream's thickness, and P is the thickness of the sample stream when $Q_s = Q$.

2. Horizontal Flow Relations for the 3X12 Outlet Arrangement

Stafo electrophoretic migration and separations are not as simple as indicated by Eq. 27, but are complicated by laminar defocusing (see Section I. D1). This electrophoretic migration takes place normal to the horizontal parabolic fluid flow profile, which is described by Eq. 4. $v = v_{\max} - kz^2$. If this equation is graphed (Fig. 20) it is evident that the area under the curve is proportional to total volume flow rate for a parabolic-shaped slab of infinitesimal thickness. In addition, the area above any increment along the z axis represents a volume flow rate normal to that particular increment of Stafo chamber width.

$$\text{Let Area I} = 1/3 (\text{Area I} + \text{Area II}); \quad (29)$$

$$\text{Area I} = \int_0^{z_1} v dz. \quad (30)$$

$$\text{Area (I + II)} = \int_0^{z_{\max}} v dz = 3 \int_0^{z_1} v dz. \quad (31)$$

Substituting Eq. 4 into Eq. 31 and integrating, one obtains

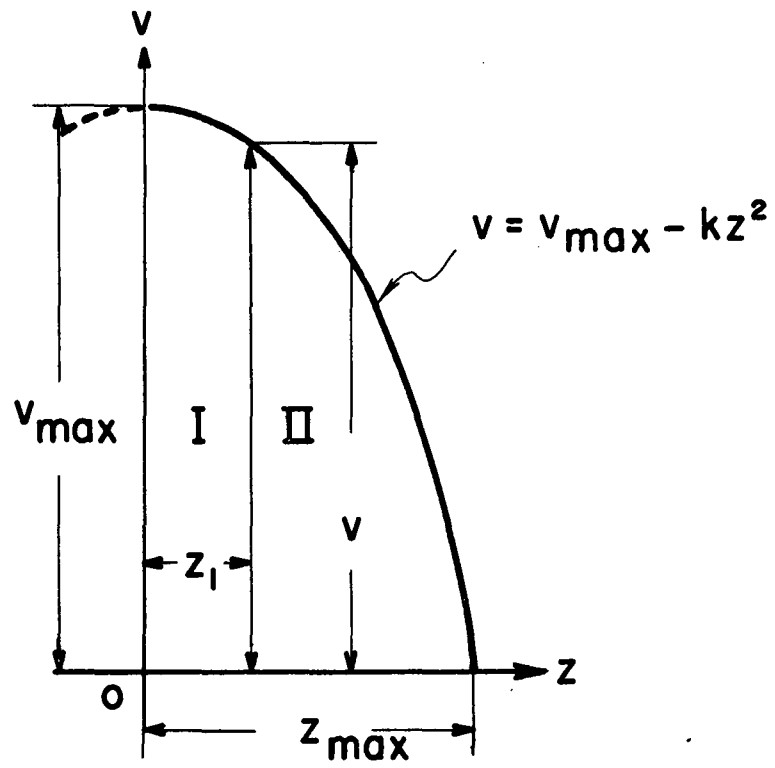
$$3z_1^3 - 9z_1 + 2 = 0, \quad (32)$$

and solving for z_1 , with $z_{\max} = 1$, we find

$$z_1 = 0.226, \quad (33)$$

or the ratio

$$\frac{z_1}{z_{\max}} = 0.226. \quad (34)$$



MU-36702

Fig. 20

This result is interpreted to mean that when the 3X12 outlet arrangement is used with equal horizontal outlet flow rates to all collection containers, $1/3$ of the volume flow goes through the center 22.6% of the chamber width. Normally, equal-sized outlet collection vessels are used for all outlets; for a sufficiently small density gradient this assures equal outflow rates from all outlets. However, different-sized collection vessels can be used. Since the collection apparatus and Staflo chamber together are a set of simultaneous siphons, the level of the fluid within each collection vessel is the same as in the others. Hence, the rate of fluid flow into any collection vessel is directly proportional to its cross-sectional area relative to the others (see Section I.B2). For example, if, in the center streams, one uses collection vessels twice the cross-sectional area of those used for the outside streams, then each of the center streams delivers twice the volume of fluid delivered by the adjacent side streams. This fluid is only from the center portion of the Staflo chamber.

If uniform collection vessels are used with the 3X12 outlet arrangement, then at any particular level the front, center, and back outlets will deliver identical volumes of fluid per unit time (see Section I.B2). What width of the Staflo chamber's central portion will deliver the same volume flow rate as the adjacent portions near the two walls? This can be determined by an integrating of Eq. 4 and solving for z_1 as shown in Fig. 20. This integration is meaningful because it was experimentally observed microscopically that the fluid in the central portion, to a good approximation, does not mix or intermingle with that adjacent near the walls except for diffusion-convection effects (see Section I.C2). Diffusion-convection is expected to weaken and become less important as the density gradient is "smoothed out." Consideration of this central 22.6% of the Staflo chamber widths compared with those portions adjacent near the walls shows that laminar defocusing of the migrating sample is at most 5.2%. That is, the sample migrates 1.052 times as far at the boundary between the central portion and the adjacent portions, where $z_1 = 0.226 z_{\text{max}}$, as at the exact center of the Staflo chamber, where laminar defocusing is absent. The average

laminar defocusing that occurs in the central 22.6% of Staflo chamber width is about 1.5%.

Thus it is seen that by sampling the center portion of the Staflo chamber most of the laminar defocusing can be avoided, but there is a concomitant loss of capacity.

However, when a thin-sample zone stream is used there is an increase in capacity and a reduction in laminar defocusing that results from the phenomenon known as central sharpening within the Staflo chamber. For a single vertical row of outlets from the Staflo chamber laminar-defocusing is likely to be severe enough to make analytical and preparative separations almost meaningless except for the phenomenon of central sharpening. Recall that all three types of central sharpening transport sample from a thin zone stream into the center of the Staflo chamber, where laminar defocusing is small.

Nevertheless completely clean separations of the slower-migrating components from the faster-migrating components is not possible with a single vertical row of Staflo outlets, because it is not possible to completely suppress laminar defocusing.

Thus for analytical and preparative separation at the greatest resolution that the Staflo apparatus is capable of it is necessary to sample in a 3x12 or similar arrangement. I believe, however, that the resolution of the Staflo can be much improved by making the Staflo chamber longer and higher. Why is this the case? Equation 24 states for $\lambda_{\min}$ that

$$\lambda_{\min} = \frac{2}{3} \mu \tau^i \tilde{E}. \quad (24)$$

That is, for a given electrophoretic mobility μ there is a certain minimum migration distance, $\lambda_{\min}$ for a fixed τ^i and $\tilde{E}$. It is to be remembered that $\lambda_{\min}$ is in the direction of migration, but that it is also in the same direction as the height of the Staflo. Therefore $\lambda_{\min}$ can never exceed the height of the Staflo chamber. Indeed only about 50% of the total height is useful, or approximately 1.5 cm out of 3 cm.

With a two-component system of two different mobilities, μ_1 and μ_2 , Eq. 24 becomes

$$\mu_2 - \mu_1 = \Delta\mu = \frac{3}{2} \left(\frac{A}{\tau^1 \tilde{E} R} \right) (\lambda_{2_{\min}} - \lambda_{1_{\min}}) = \frac{3}{2} \left(\frac{A'}{\tau^1 \tilde{E} R} \right) \Delta\lambda_{\min}, \quad (35)$$

where τ^1 and A are fixed for any experiment and $\tilde{E} = \tilde{E} R$ is nearly the same for two different components, and A is the area of the electrode.

It is apparent that if two components have a small difference in mobility but have large mobilities, no separation can be achieved. Also as τ^1 or $\tilde{E}$ (or both) become larger, smaller mobilities and smaller mobility difference can be measured. But t is directly related to the length of the Staflö chamber. If time τ^1 is to be increased, then the length L of the Staflö chamber must also be increased.

It should be possible to increase the capacity of the Staflö method to handle larger sample volume by increasing the width of the chamber (z direction) without loss of resolution at the outlet, provided the streams are sampled at three horizontal points in each level. Outlet resolution can also be improved by outlet sampling points closer together in the vertical direction.

F. Additional Experimental Techniques

1. The Programmed Electric Field

It was sometimes noticed experimentally if electrophoresis of the lipoproteins was started just beyond the inlet of the Staflö chamber that in many instances the migration would not be "well behaved," depending upon the direction of the electrophoresis. That is, it sometimes had the gross appearance of droplet sedimentation or flotation (see Section I.B1). It was felt that such instability was due to insufficient time for diffusion to "smooth" the density discontinuity between the sample and the surrounding fluid. Brakke¹⁴, Svensson,¹⁵⁻¹⁷ and Mel¹⁰ discussed the problem of density stability. Svensson discussed especially the aspects of long-term stability, and so did Philpot.¹⁹

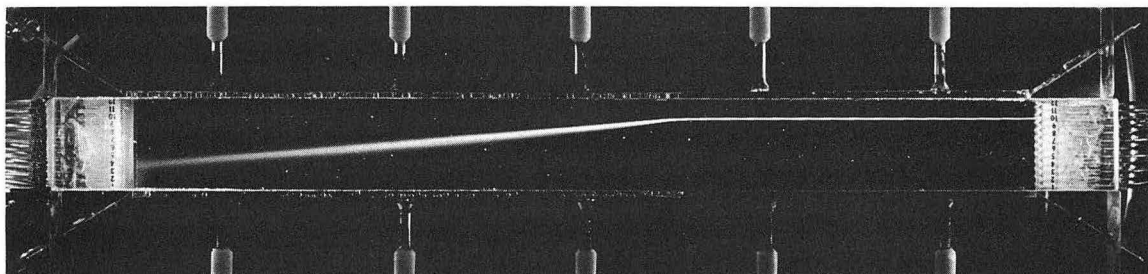
The problems relate to the density-gradient changes brought about by passage of diffusing molecules into and out of the initial zone. Sometimes when the sample zone was electrophoresed and the sample moved into another region, the density gradient would become positive downward as a result of this movement. A density instability would develop, resulting in a convectional disturbance. The result was poor migration and reduced resolution of components.

If, however, the electric field were initially reduced or absent while diffusion occurred, stable electrophoretic migration could also occur under exactly the same initial concentration, density, and fluid flow rate conditions, but at a later time.

There are three methods by which the electric field in a solution can be reduced in a portion of the Staflo chamber. They are:

- (i) The electrodes may be omitted in that portion of the Staflo cell where the electric field is to be reduced.
- (ii) The electrodes can be left unconnected to any voltage supply in that portion of the Staflo cell where the electric field is to be reduced. This causes the electrodes to "float" and leads to an effective value of voltage intermediate between the full value and zero.
- (iii) The electrodes can be unconnected to any voltage supply but connected together by a shortening wire of low resistance in that portion of the Staflo cell where the electric field is to be reduced to a very near zero.

This last method has proved to be the one of choice (see Fig. 21), because it is the easiest to effect and control and it gives the best electrophoretic migrations. This method effectively reduces the electric field between the shorted-out electrodes to zero. Actually the electric field is reduced by a factor very nearly equal to the ratio resistance between the shorting wire and the total resistance of the fluid between the electrodes in the upper and lower electrode chambers. This reduction of electric field strength can be of the order of 10^4 or greater, and is a consequence of the fortunate fact that extreme polarization occurs at the gap between the electrophoresis electrodes and



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Fig. 21. Steady-state Staflo electrophoretic migration pattern for a sample of low-density lipoprotein solution (S_f 0-10⁵), viewed by scattered light, showing the effect of programmed electric field when the first 40% of the electrodes (right end) has been shorted out. Note the electrolysis bubbles in the left 60% of the electrode chambers. These bubbles do not affect the electrophoretic migration; they are physically separated from the Staflo chamber by a cellophane (dialysis) membrane and they are continuously flushed out by the electrode wash solutions. (The numbers on the flow dividers are upside down, but the migration pattern is down as shown).

the shorted-out electrodes.

This extreme polarization results in a small bubble to be formed and to be maintained at this gap. This bubble acts as an insulator, and it causes the current between the electrophoresis electrodes and the shorted-out electrodes to take a longer path through the solutions within the Staflo chambers than if the bubble were not there. This results in a considerable reduction in the electric current that passes from the electrophoresis electrodes to the shorted-out electrodes. This electric current is at most only a few milliamperes or a few percent of the total current. During steady-state operation of the Staflo apparatus the total and shorting currents are constant.

The use of several electrodes (see Fig. 3b) permits different voltages to be impressed across the Staflo chamber at various places along the length of the cell, and their polarity can be reversed from place to place. Thus it becomes possible to program the electric field to suit the needs of any particular experiment.

2. Optical Observation and Photography

The Staflo cell was constructed of transparent materials, (Lucite, glass, epoxy resins, and quartz) because of the greater information potentially available by direct viewing or by photographic methods than by indirect methods. The Staflo chamber was designed to maximize viewing and illumination of events that occur within it. There are several examples of different lighting and observation techniques in this thesis. Figures 5 and 6 are examples of back lighting for a colored sample. Figure 21 is an example of side lighting for a light-scattering sample. Figures 17 and 23 are examples of illumination of the sample by the "thin-plane-illumination" technique for a light-scattering sample. Figure 14 is an example of this last technique for a fluorescent sample. These various techniques may be categorized as follows:

(i) The Backlighting Technique

In the backlighting technique light is transmitted towards the observer through the sample within the Staflo chamber. If the sample (a) is colored or (b) has a sufficient refractive index increment

relative to the background solution, then the sample can be "seen" by this technique.

(ii) The Sidelighting Technique

In the sidelighting technique light illuminates the sample within the Staflo chamber at a grazing angle by light directed nearly parallel to the length of the Staflo chamber. If the sample (a) scatters light or (b) fluoresces, it can be seen.

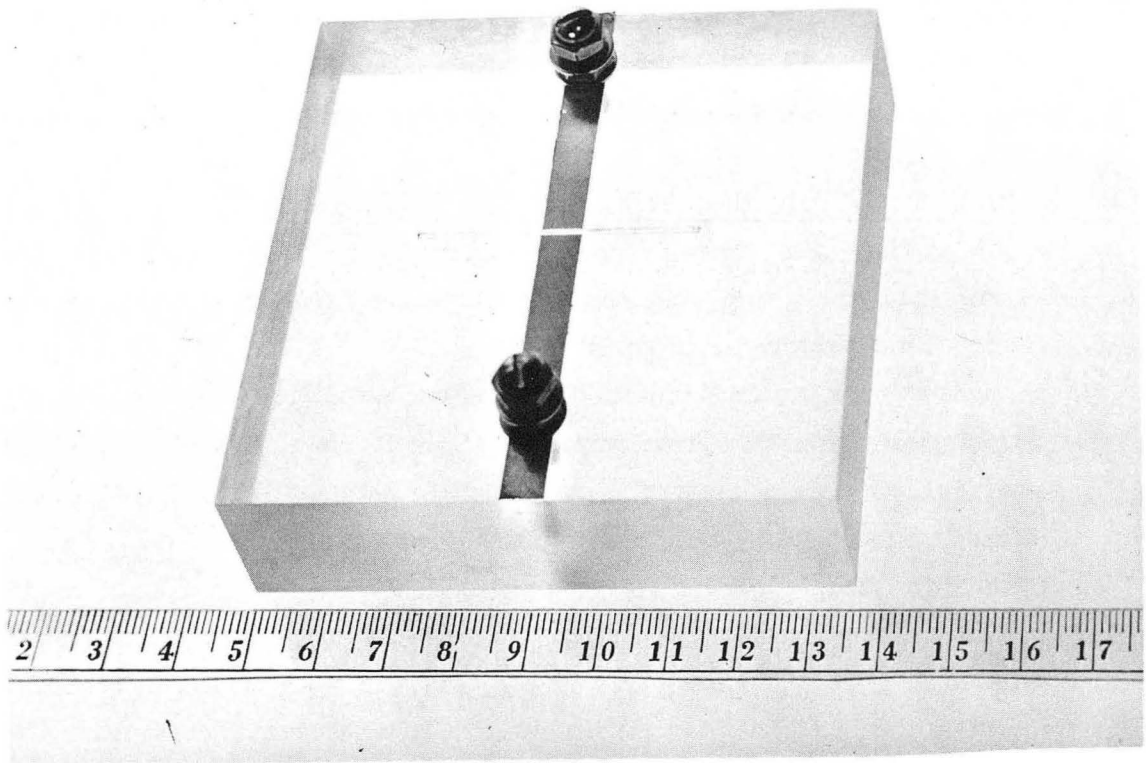
(iii) The Thin-Plane-Illumination Technique

When the sample is illuminated by a thin plane of light and observed obliquely one observes the cross section of the migrating species. This technique is most effective when the light is transmitted from the back to the front of the Staflo chamber. This technique will not give the cross section of the sample if the sample has no light-scattering centers or if it does not fluoresce. The delineation of the sample cross section is best if this plane of light is normal to the length and height of the Staflo chamber. This is because imperfections in the walls of the Staflo chamber cause less problem under these conditions than they do for oblique lighting. Also a cross section that is normal to the fluid flow within the Staflo chamber is taken at a particular distance, y , from the inlet and for a characteristic average residence time, τ_{av} . Any other cross-section type has neither.

3. The-Micro Resistivity Cell

The total volume of lipoprotein sample generally available for a Staflo electrophoresis run was about 5 to 30 ml. However, the resistivity cell available to me required at least 10 ml for a single resistivity measurement with none of the sample recoverable in an unaltered condition. Therefore it was apparent that a resistivity cell of much smaller sample volume requirements was needed. It was also very desirable that the sample be recoverable in an unaltered condition after the resistivity measurement.

A micro resistivity cell (see Fig. 22) was constructed to fulfill the requirements of small sample size and recovery of unaltered sample. The resistivity cell consisted of a block of Lucite into which was embedded a single strip of platinum approximately 0.2 in. wide



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Fig. 22. Micro resistivity cell.

and 0.010 in. thick. A single long groove was milled at right angles to and across this platinum strip. This long groove was 0.031 in. wide by approximately 0.04 in. deep. This forms a 0.031-in. gap between the electrodes. The entire surface of the resistivity cell was lapped in such a way that the upper surfaces of the Lucite and of the platinum were identical. Finally, binding posts were attached for connection of the leads from a Wheatstone bridge.¹³

How is it that any meaningful resistivity measurement can be made by such a device when there seems to be no control upon the volume of the sample?

It is important to remember that whenever small air-liquid interfaces are present the surface tension of this surface can be very important, and this is just the situation in this case. The long groove of the resistivity cell is filled with liquid from a micropipette as the micropipette is drawn along the groove. At the end of the groove a small pool of liquid (about 4 to 10 drops) is placed. This pool should have only its edge over the end of the groove, and it must be large enough to have a flat (not rounded) upper surface. If this filling procedure is done carefully the surface tension of the liquid within the groove will withdraw any small spillage over the edges of the groove and expel an equal amount into the small pool at the end of the groove. At this point there is a balance of pressures--the pressure due to the surface tension of the liquid in the groove is just balanced by the hydrostatic pressure of the small pool at the end of the groove. Furthermore, for every aqueous sample the interface of the liquid in the groove is flat and in the same plane as the surface of the resistivity cell. This simply means that the sample volume is constant from sample to sample except for the liquid in the small pool at the end of the groove. The size of the pool is of very minor importance, as long as it fulfills the above conditions. The electrical path from one electrode to the pool and back to the other electrode is long (high resistance) compared with the short path (low resistance) across the gap between electrodes. Recall that, for parallel resistances, a very high resistance relative to a low resistance affects the total resistance

but little. The resistance of this small pool has been estimated to affect the overall measured resistivity of the sample by less than 1%, i. e., it is neglected.

A much more important factor than this pool size is evaporation of the sample. However, if the resistivity measurement is made quickly, changes in resistivity due to evaporation are very small. The RDT #1 resistivity cell is used to make only relative measurements. These are performed in the following manner.

- (i) A "resistivity" measurement of the sample is made with the RDT #1 resistivity cell.
- (ii) Similar measurements are made with salt or buffer solutions that bracket the "resistivity" measurement of the sample in Step 1.
- (iii) The resistivity of the salt or buffer solutions is measured with a standard conductivity cell. It is then presumed that the absolute resistivity of the sample (if it could be measured on the standard conductivity cell) would bear the same relationships to the absolute resistivities of the salt and buffer as when the measured "resistivities" of the RDT #1 resistivity cell are compared.

II. SERUM LIPOPROTEIN STUDIES

A. Introduction

1. Physical-Chemical Properties of the Serum Lipoproteins

The serum lipoproteins are macromolecules containing between 1 and 99% lipid by weight. They have estimated hydrated densities from about 1.32 to 0.95 g/ml. Their molecular weight ranges from 7×10^5 to 10^{11} , but by far the greatest part present in the serum ranges from about 2×10^5 to 10^7 molecular weight and contain 40 to 95% lipid, respectively.

Not only do the lipid and protein contribute to the density of lipoprotein, but water does also. The lipoproteins are probably hydrated to the extent of 0.2 to 0.6 g H₂O per g protein. Much of the surface of a lipoprotein must be exposed lipids, and hydration of certain of the more polar lipids, such as the phospholipids, also may be involved.

Most of the lipoproteins are of sufficient size to allow evaluation of their molecular dimensions and shape directly by visualization with the electron microscope. The large low-density lipoproteins of $S_f 400$ and greater appear to be nearly spherical in shape and to range in size from 800 to 10 000 Å. [When molecules more dense than the solvent are subjected to a centrifugal field they tend to sediment at a certain rate. The unit for this sedimentation is named in honor of Svedberg. One Svedberg = $1S = 10^{-13}$ (cm/sec)/(dyne/cm). The unit for lipoprotein flotation is $S_f = -S$.¹⁴] Lipoproteins of $S_f 20-400$ also appear spherical but somewhat flattened. The lipoproteins of $S_f 0-20$, as well as the high-density lipoproteins, appear definitely as flattened structures. The lipoproteins of the $S_f 6-8$ appear to be prolate ellipsoids of 150×350 Å.

The lipoproteins have a variable electrophoretic mobility as the result of variable amounts of unesterified fatty acids that may be present in any class of lipoproteins studied. They also contain varying quantities of cholesterol esters, cholesterol, glycerides, unesterified fatty acids, and phospholipids. The ratios of these also

change from one lipoprotein class to another.

Since these molecules contain protein they can be characterized by their amino acid composition. The N-terminal amino acid composition of each of the major lipoprotein classes differs significantly from others. However, each class appears to be characterized by a complicated but relatively distinct protein moiety containing more than one N-terminal amino acid. The S_f 3-20 fraction has glutamic acid as the principal terminal amino acid. Lipoprotein fractions of higher S_f rate contain a decreasing content of N-terminal glutamic acid and an increasing content of serine, threonine, and aspartic acid end groups. The principal high-density lipoproteins (HDL_2 and HDL_3) contain aspartic as the chief N-terminal amino acid, with small amounts of threonine and serine.²⁶

The serum lipoproteins have been studied by many techniques. Searcy and Bergquist⁴⁷ have summarized in broad outline the classification of serum lipoprotein fractions according to the techniques used to study them. This information appears in Table V.

Notice that each of the various techniques appears to give a unique characterization according to a specific physical parameter, e.g., density, flotation rate, etc. However, closer inspection of the pertinent experiments reveals that the classification of the serum lipoproteins is somewhat arbitrary. For example, Searcy and Bergquist⁴⁷ state that electrophoresis of the serum lipoproteins supporting medium yields two major species known as alpha and beta lipoproteins, but that this classification is governed by the type of supporting medium employed.

Levy and Fredrickson²⁵ have shown recently that the high-density lipoproteins (HDL) show heterogeneity the use of immunochemical techniques. They found, reproducibly, that human serum alpha lipoprotein from all sources except fresh plasma and HDL_2 exists in two forms which they call alpha LP_A and alpha LP_B . These two forms have different electrophoretic mobilities. Alpha LP_B contains relatively less lipid and has a greater density than the alpha LP_A , which is presumably the native form in which these lipoproteins

Table V. Classification of serum lipoprotein fractions.

Lipoprotein fraction	Ultracentrifugation			Electrophoresis		
	Density (g/ml)	S_f	-S	Paper	Starch	Ethanol precipitation
Chylomicron	< 1.006	400-40,000	> 70	Origin lipid	Beta	I + III
Very low density	< 1.019	10-400	40-70	Beta	Alpha-2	I + III
Low density	1.019-1.063	0-10	25-40	Beta	Beta	I + III
High density	1.063-1.21	HDL ₂	20-25	Alpha	Alpha	IV + V
		HDL ₃	0-10			
Albumin-UFA						V

From Searcy and Bergquist, Ref. 47.

circulate. Both alpha LP_A and alpha LP_B contain protein of the same amino acid composition and amino terminal groups.

Gustafson, Alaupovic, and Furman¹⁷ have recently developed a method for the fractionation of the very-low-density (VLD) human serum lipoproteins (S_f 20-10⁵) by angle-head preparative ultracentrifugation. They say that the lipoprotein particles that comprise these fractions (S_f 20-50, S_f 50-100, S_f 100-400, S_f 400-5000, S_f > 5000) are inhomogeneous in respect to their physical properties and protein moieties, as they certainly are with respect to flotation rate.

Lindgren and Nichols²⁶ have proposed a model that indicates the continuous conversion of lipoproteins of higher S_f rates into those of successively lower S_f rates. Nelson,³⁸ in his Ph. D. dissertation, has expanded their concept in more detail, and he indicates the relationship of fat ingestion and tissue metabolism to this continuous conversion.

It can be concluded that the human lipoproteins exist in a broad spectrum of characteristics such as molecular weight, size, density, flotation or sedimentation rates, electrophoretic properties, and N-terminal amino acid composition.

The variableness of the concentration of the various lipoproteins in the serum lipoprotein spectrum, and also their concentration ratio to one another, has been related to the state of health or disease, to fasting or feasting, to the time of day, whether male or female, and to the age of the individual.²⁶

2. A Short Review of Recent Electrophoretic Research on Human Lipoproteins

A review of electrophoretic studies of the serum proteins, including the serum lipoproteins, to 1960 is given by Cooper,⁸ but the review is too extensive to be discussed here.

Laurell²⁴ made the observation that the S_f 20-10⁵ class in free electrophoresis migrates with an α_2 mobility, but Oncley et al.⁴² observed that in paper electrophoresis they usually have a β_1 mobility but with considerable tailing if it moves at all. Kunkel²³ observed that the S_f 20-10⁵ class has an α_2 mobility, whereas the S_f 0-20 class

usually exhibits a β_2 mobility but does occasionally show an α_2 mobility. The reasons for these different relative mobilities of the various lipoprotein classes seem to be related to support materials upon which electrophoresis is occurring and to the particular lipoprotein which is being electrophoresed.

It seems reasonable that, if relative or absolute mobility measurements or definitive separations of the lipoproteins (or other proteins) are to be made, support materials should probably be avoided.

It was observed by Trautman⁵⁸ in moving-boundary electrophoresis that the presence of lipoproteins caused difficulties other than those inherent in the method itself. The chief difficulty was due to the density instability caused by the presence of low-density (β) lipoproteins, which gives rise to the β anomaly on descending electrophoresis. The β anomaly represents an irregular scanning or cylindrical lens pattern which averages out any distribution across the free-solution electrophoresis cell. It is also a definite nonplanar concentration region in the cell. The β anomaly is due to the presence of β lipoproteins in the serum undergoing electrophoresis, and can be removed by removal of the lipoproteins.

Trautman makes the recommendation that (a) serum should be separated into two parts, one containing lipoproteins and the other free from lipoproteins; (b) the lipoprotein-containing fraction should be studied with the ultracentrifuge; (c) the lipoprotein-free fraction should be studied with electrophoresis. He says that this results in simpler patterns than would be obtained if whole serum were run in the ultracentrifuge or electrophoresis apparatus and [it would give] quantitative data on many [of the] serum components.

It would seem that if electrophoretic mobility characterization or definitive separations of the lipoproteins are ever to be performed unambiguously, the free-solution methods will have to be used, for these alone will remove the very serious problems of solid support. Furthermore, a variation of zone electrophoresis in free solution should probably be best of all, because this method can potentially separate a multicomponent sample into its various fractions and also

simultaneously make it possible to measure their respective electrophoretic mobilities free from interference of other lipoproteins.

Lindgren and Nichols state²⁶ "Electrophoresis of lipoproteins, though providing a measure of the net electrical charge carried by a given lipoprotein under the conditions of electrophoresis, unfortunately does not lead to a definitive or reproducible lipoprotein characterization." This is because of

- (i) the variable mobility of lipoproteins that results from variable amounts of unesterified fatty acid that may be present on any class of lipoproteins studied,¹⁵
- (ii) the differing basic methods of electrophoresis (free, paper, starch block), which do not yield similar characterization for the same lipoprotein classes (as characterized by ultracentrifugal and chemical separation studies).

Point number one above can be answered by stating that that which is to be characterized may not be a pure lipoprotein even if it can be isolated by other procedures. With regard to point number two, it would be a fortuitous circumstance indeed if three basically different electrophoretic methods would yield identical results for a lipoprotein that had been characterized by an ultracentrifugal method. It is to be understood that electrophoresis and ultracentrifugal methods operate upon different properties of the lipoprotein and these properties can never be treated in isolation.

3. Objectives of Staflo Electrophoresis Study

Although ultracentrifugal studies can yield information on molecular weights, hydrated densities, sedimentation or flotation rates, and molecular size and shape, they can yield little if any information on electrical charge, isoelectric points, and electrophoretic mobility of the serum lipoproteins.

Trautman⁵⁷ recommended that the serum lipoproteins be investigated ultracentrifugally and not electrophoretically. (In the context of this work and other research of his it would seem that his recommendation applied to preparative studies upon the lipoproteins and not necessarily to analytical studies.)

It was pointed out in the introduction to this thesis that preparative Staflo electrophoresis of the serum lipoproteins should be possible. This preparation would likely be very gentle and rapid, and yield sufficient lipoprotein for further studies, even as the preparative ultracentrifuge has yielded.

Furthermore, it seemed that a measurement of the electrophoretic mobility of the lipoproteins simultaneously with their preparation by Staflo electrophoresis should be possible.

It also seemed likely that a study of ultracentrifuged or Staflo-electrophoresed lipoproteins (or both) would yield additional information upon the lipoproteins that independent studies could not give, and that such experiments would perhaps give samples of lipoproteins having narrow bands of flotation rates and electrophoretic mobilities.

B. Staflo Studies: Experimental Conditions

1. Lipoprotein Sample Preparation

The patients were nonpregnant married females, between the ages of 21 and 31, and presumably in good health. These patients were divided into four classes:

- (i) nonfasting,
- (ii) nonfasting followed by fatty meal [Fatty meal: 1.5 g/kg polyunsaturated oil (corn or safflower), 0.15 g/kg sucrose, 3.0 ml/kg low-fat milk, vanilla or chocolate to flavor. This mixture was blended for 1 to 2 min in a Waring blender],
- (iii) fasting,
- (iv) fasting followed by a fatty meal.

A total of 250 to 300 ml of whole blood was taken from each patient in the morning. The fasted group had fasted at least 12 hours (overnight), whereas the nonfasted group had had a breakfast. Those patients who had received the fatty meal had their blood taken 3 hours following this meal. The blood sample was taken by vacuum into stoppered 40-ml glass tubes and allowed to stand and to clot for 1 hour. After this time the clot was loosed from the wall of the tubes with a glass rod and then centrifuged for 30 min at 1500 rpm (International centrifuge,

model EXD) to sediment the clot and the suspended cells.

The above centrifugation normally yielded 130 to 140 ml of serum from 300 ml of whole blood.

Subsequent preparative ultracentrifugal fractionations were performed, using 6-ml Lustroid tubes in the Spinco 40.3 rotor. Normal runs were for 24 hr at 40 000 rpm. The procedural steps for obtaining the serum lipoprotein sample of particular ultracentrifugal flotation fractions are as follows (the method described is a slight modification of that of deLalla and Gofman⁹):

(i) The S_f 20-10⁵ fraction was obtained from normal serum by means of a 24-hr preparative ultracentrifugal run. This fraction formed a milky band at the very top of the preparative ultracentrifuge tube and was pipetted off quantitatively. All other lipoproteins and serum protein fractions would be at or near the bottom of the centrifuge tube.

(ii) The S_f 0-20 fraction, $1.006 \text{ g/ml} < d < 1.063 \text{ g/ml}$, was obtained from the residue of step (i) by adjustment of the background density with NaCl to a nominal value of 1.063 g/ml or with sucrose to 1.070 g/ml . This solution was subjected to preparative ultracentrifugation for 24 hr at 40 000 rpm in the Spinco 40.3 rotor. At the end of the run the S_f 0-20 fraction formed an intense yellow band at the very top of the centrifuge tube. The residue at the bottom of the centrifuge tube contained the HDL₂ and HDL₃ fractions, and other serum proteins.

(iii) The HDL₂ and the HDL₃ fractions, $1.063 \text{ g/ml} < d < 1.200 \text{ g/ml}$, was obtained from the residue of step (ii) by adjustment of the background density to 1.200 g/ml with NaBr. This solution was subjected to preparative ultracentrifugation for 24 hr or longer at 40 000 rpm in a Spinco 40.3 rotor. This floated the HDL₂ and HDL₃ into a pale yellow band at the very top of the centrifuge tube. The residue was then discarded.

If the entire low-density lipoprotein (S_f 0-10⁵) fraction was desired, then an initial background density adjustment of the serum to 1.070 g/ml (with sucrose) could be made before preparative

ultracentrifugation. Preparative ultracentrifugation at this density would float these low-density lipoproteins into a milky yellow band at the very top of the centrifuge tube following a 24-hr run at 40 000 rpm in a Spinco 40.3 rotor. If, in addition, the HDL₂ and HDL₃ fraction was desired, it could then be obtained by following step (iii) above.

In every case the electrical resistivity of the lipoprotein sample was too low for immediate Staflor electrophoresis. The resistivity was raised by dialysis. The buffer used for dialysis was LKB 3276-GB High Resolution Buffer Salt (8×10^{-3} M tris, 3.3×10^{-4} M EDTA, and 1.22×10^{-3} M H₃BO₄) in distilled-deionized water. The lipoprotein resistivity was increased to about 0.9×10^4 ohm-cm by this dialysis. It varied somewhat, depending upon the lipoprotein sample. The resistivity of the lipoprotein samples was measured with the Resistivity Cell RDT #1 constructed specifically for that purpose (see Section IF3 and Fig. 22).

If the sample is to be introduced into the Staflor chamber at level 3 then its density must be intermediate between the densities of streams 2 and 4. In order to determine the approximate density of the sample a series of solutions of varying density is used. The tip of a micropipette containing the sample is carefully lowered below the surface of one of the solutions whose density is known. A very small quantity of sample is gently expelled into the solution. If it tends to float then it is less dense than the solution (if it tends to sink it is more dense). By repeating the process with other solutions one can determine quickly with a fair degree of accuracy the density of the samples. When the sample's density is not correct (in the usual case it is too low) it may be increased by adding sucrose. The density of the sample is again checked by the above method. Exact measurement of the various sample densities is not necessary for stable and reproducible Staflor electrophoretic experiments. For initial stability the sample may have any intermediate density between the upper and lower streams between which it is to float. However, a diffusion-induced instability may develop, resulting in droplet sedimentation or droplet flotation (see section I B1). It is necessary that this be

prevented for high-resolution work. The usual method is to choose the density of the sample nearer that of the upper stream than of the lower stream in the case of droplet sedimentation, or in the opposite sense for droplet flotation. If it still occurred it would then be necessary to lower the lipoprotein concentration of the sample (at constant background density) until the instability was avoided.

It was a fortunate circumstance that a 0.4-g/100 ml sucrose density gradient per stream would support in long-term stability a 1.5-g/100 ml sample of the S_f 0-20 lipoprotein fraction. However, this same density gradient would support only 0.2 g/100 ml of the S_f 20-10⁵ lipoprotein fraction. It was fortunate because complementary studies--such as analytical ultracentrifugation, lipid extraction, and electron microscopy--were facilitated by the extra quantity of lipoprotein available as a result of the relatively large capacity of the Staflo method for this fraction. The Staflo method can handle such high lipoprotein concentrations because of the closeness of the lipoprotein density to that of water. On the basis of this observation the more dense proteins and lipoproteins could probably be Staflo electrophoresed at higher concentrations in a more dense medium, e. g., heavy water.

2. Staflo Chamber Solution Gradients

a. The Density Gradient

The density gradient in all experiments reported in this thesis was constructed by using increments of 0.4 g/100 ml sucrose per stream, beginning with none in the upper electrode wash solution and increasing to 5.2 g/100 ml sucrose in the lower electrode wash solution. This gradient amounts to an increase of 0.00156 g/ml per stream. The viscosity of the streams also increases in the downward direction from 0.894 to about 1.119 centipoise.

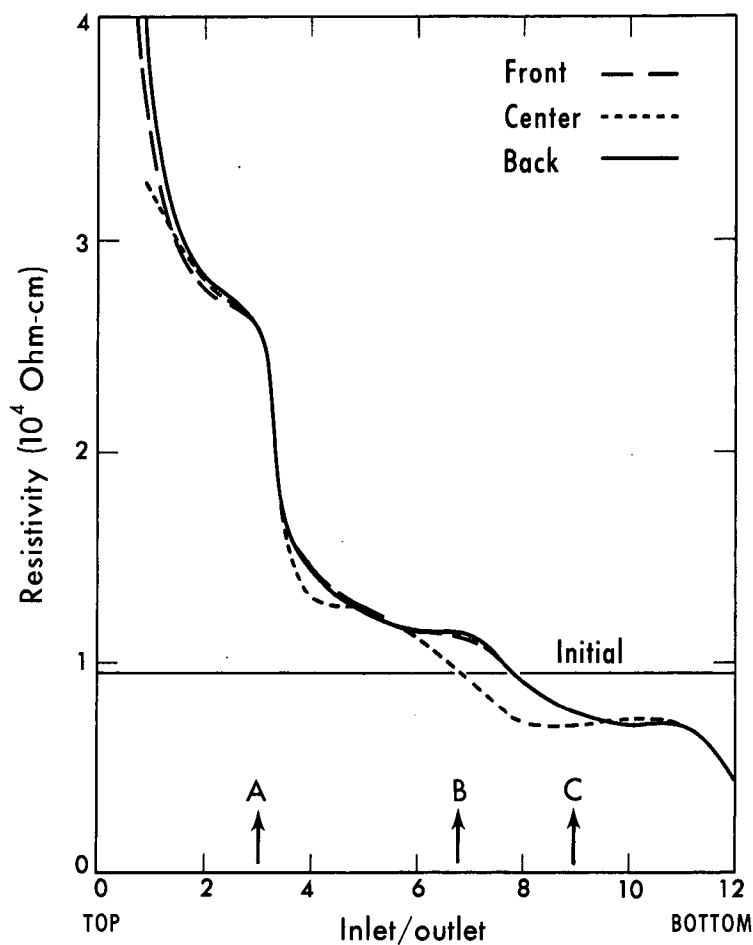
b. The Resistivity Gradient

The kind of restrictions that apply to the density gradient do not apply to the resistivity gradient. This gradient may increase, decrease, remain constant, be large, be small, or vary in an arbitrary way. However, not all resistivity gradients give good

electrophoretic migration, and the optimum gradient depends somewhat upon the experiment. For a sufficiently dilute buffer a small change in the concentration of the buffer produces a proportional change in the resistivity of the buffer solution. There is also a change of similar nature when the ratio of buffering salts is altered.

The resistivity and pH were the same for all Staflo inlet solutions. The values were normally within $\pm 5\%$ of 0.9×10^4 ohm-cm and 8.8 pH respectively. Figure 23 shows typical 3×12 specific resistance curves of the outlet solutions during downward migration. It is to be noticed that the curves marked front and back (of Staflo) are nearly identical at all points throughout, but that the center curve has marked deviations, especially between outlets 6 to 10. This is at just the positions that the electrophoresing lipoproteins are exiting from the Staflo chamber. This phenomenon is considered in the discussion of central sharpening (see Section I. D. 2). The higher resistivity in the upper part of the Staflo chamber (left side of Fig. 23) results from selective permeability of the dialysis membrane that separates the Staflo chamber from the upper electrode chamber to the buffer ions diffusing into and out of the Staflo chamber during electrophoresis. This higher resistivity would cause greater ohmic heat production during current flow in the upper part of the Staflo chamber relative to the lower levels, and thereby make the density gradient even more stable! A similar phenomenon occurs in the lower part of the Staflo chamber but in the opposite direction, but again in the direction of increased density stability! Of course, if the current were reversed the resistivity gradient would also reverse and the ohmic heating would operate to give a lower density gradient and tendency toward instability.

In the central region (outlet 3 to 10) the pH is about 0.1 pH unit higher at outlet 3 and 0.1 pH unit lower at outlet 10 during downward migration than initially, with approximately a linear slope in between. During upward migration the reverse is true. That is, in the central part of the Staflo chamber the pH is 8.6 ± 0.1 . The pH decreases or increases very rapidly the closer the respective electrode chambers are approached, depending upon the direction of



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Fig. 23. Resistivity profile of a typical Staflo electrophoretic run of the S_f 0-10⁵ lipoprotein class, showing the change that has occurred within the Staflo chamber from the uniform initial inlet resistivities to the altered outlet resistivities. The position marked A is that of the sample at the inlet. The positions marked B and C are those for the S_f 0-20 and S_f 20-10⁵ lipoprotein classes, respectively, at the center outlets of the Staflo chamber (upper boundary in each case).

current flow. The pH increases at the anode and decreases at the cathode.

The resistivity was measured as described earlier. The pH was measured by using the Leeds & Northrup 7664 - A1 pH indicator, according to their manual.

c. pH Gradient

The pH of the initial solution depends upon the particular buffer chosen and upon its concentration. In all Staflo electrophoresis experiments of the lipoproteins all initial pH's were in the range 8.6 to 8.8. The buffer was 1.2×10^{-2} M tris, 5×10^{-4} M EDTA, and 1.83×10^{-3} M H_3BO_4 (LKB 3276-GB High Resolution Buffer Salt). This concentration of this buffer had a resistivity of 0.9×10^4 ohm-cm. The buffer concentration was initially uniform throughout all streams except for the lipoprotein sample, where it was about 66% of this value. The exact value of sample buffer concentration depended upon the lipoprotein concentration and upon the completeness of the dialysis.

The ionic strength of this buffer is unknown because it consists of multibasic weak acids and a weak base which are only partly dissociated.⁴¹

3. The Average Residence Time

One of the basic parameters by which one Staflo electrophoresis or sedimentation experiment is compared to another is the average residence time or throughput time, τ_{av} . A residence time, τ , is the time required for a small element of volume to cross the Staflo chamber from the inlet to the outlet. This time is inversely related to the fluid flow velocity. A consideration of the horizontal velocity profile (parabolic) in the vertical center half of the Staflo chamber will show that the residence times range from $2/3 \tau_{av}$ to infinity.

The average residence time τ_{av} is determined by the internal dimensions of the Staflo chamber and a direct measurement of the rate from the inlet syringes at a given setting of syringe pump motor. The average residence time, τ_{av} , is defined as the time necessary to deliver a total volume of fluid, equal to the internal volume of the Staflo chamber, V , at a constant rate dQ/dt from the inlet syringes,

i. e.,

$$\tau_{av} = V/(dQ/dt), \quad (36)$$

where

$$dQ/dt = \sum_{i=1}^{12} \frac{dq_i}{dt}; \quad (37)$$

that is, the total flow rate is just the sum of the individual flow rates of all 12 inlets. Normally for lipoprotein experiments reported in this thesis the average residence time was 16 to 20 minutes.

4. Establishment of the Steady State

At the beginning of an experiment and prior to the establishment of the density gradient within the Staflo chamber, the tubing leading from the sample syringe to the Staflo chamber is filled with a solution of the appropriate density. The density gradient is established by flowing solutions from each of the other syringes into the Staflo chamber in a nonturbulent manner. This washes out previously used solutions (usually H₂O) and replaces them with solutions for the given experiment.²⁹ The total volume of fluid required for establishment of a steady state is about 4 to 6 Staflo chamber volumes. The density gradient may be almost correct for any experiment even if very rapid nonturbulent flow is used followed by about one throughput volume at the correct average residence time τ_{av} before introduction of the sample. When all the procedures discussed previously with regard to the sample preparation and experimental setup are followed, the sample will upon introduction float at the correct level in the Staflo and the sample zone will have a stability sufficiently long for electrophoretic migrations or separations to occur.

C. Steady-State Migration and the Migration Profile

1. Experimental Resolution

Steady-state electrophoretic migration never occurs if the total width of the Staflo chamber is considered (see Section ID 1). However, if only the center 1/3 of the fluid volume flow is considered, then steady state is virtually complete after slightly more than 2/3

an average residence time. The theoretical migration profile is shown in Fig. 16, and the laminar defocusing which occurs in this center 1/3 volume flow is at most 5.2% of the migration distance, but averages about 1.5%. Figure 24 over-lies the theoretical curve on an actual photograph of the migration profile. The fit is quite good. The resolution shown in Fig. 16 is much better than can be fully taken advantage of because the outlet flow divider is 8.34% the total height of the cell for a 3×12 arrangement outlet. However, with a two-component sample, the migration pattern can be made to strike a flow divider between streams by adjusting either or both the average residence time or the electric field. [Even a third component could be separated provided that it had a migration (mobility) distance $\lambda_{\min}$ such that it was separated from the nearest of the other two by at least 0.25 cm.]

The average residence time and electric field (current) are chosen to optimize the operation of the Staflo.

It is readily seen that the electrophoretic mobilities can be calculated if the minimum migration distance $\lambda_{\min}$ is measured. A sample calculation is made from experimental data in the following section.

2. Electrophoretic Mobility of the S_f 0-20 and S_f 20-10⁵

Lipoproteins

With the use of Eq. 24 with $z = 0$, one can calculate the electrophoretic mobility μ of a phoresing species within the Staflo chamber. For this research I was more interested in separation of various components than in their respective mobilities, and therefore I chose buffer solution of very low ionic strength. The lowering of ionic strength increases the electrophoretic mobility.⁵⁵ The exact ionic strength is unknown, but is known to be lower than $\Gamma/2 = 0.1$.

The electrophoretic mobility μ may be obtained as follows:

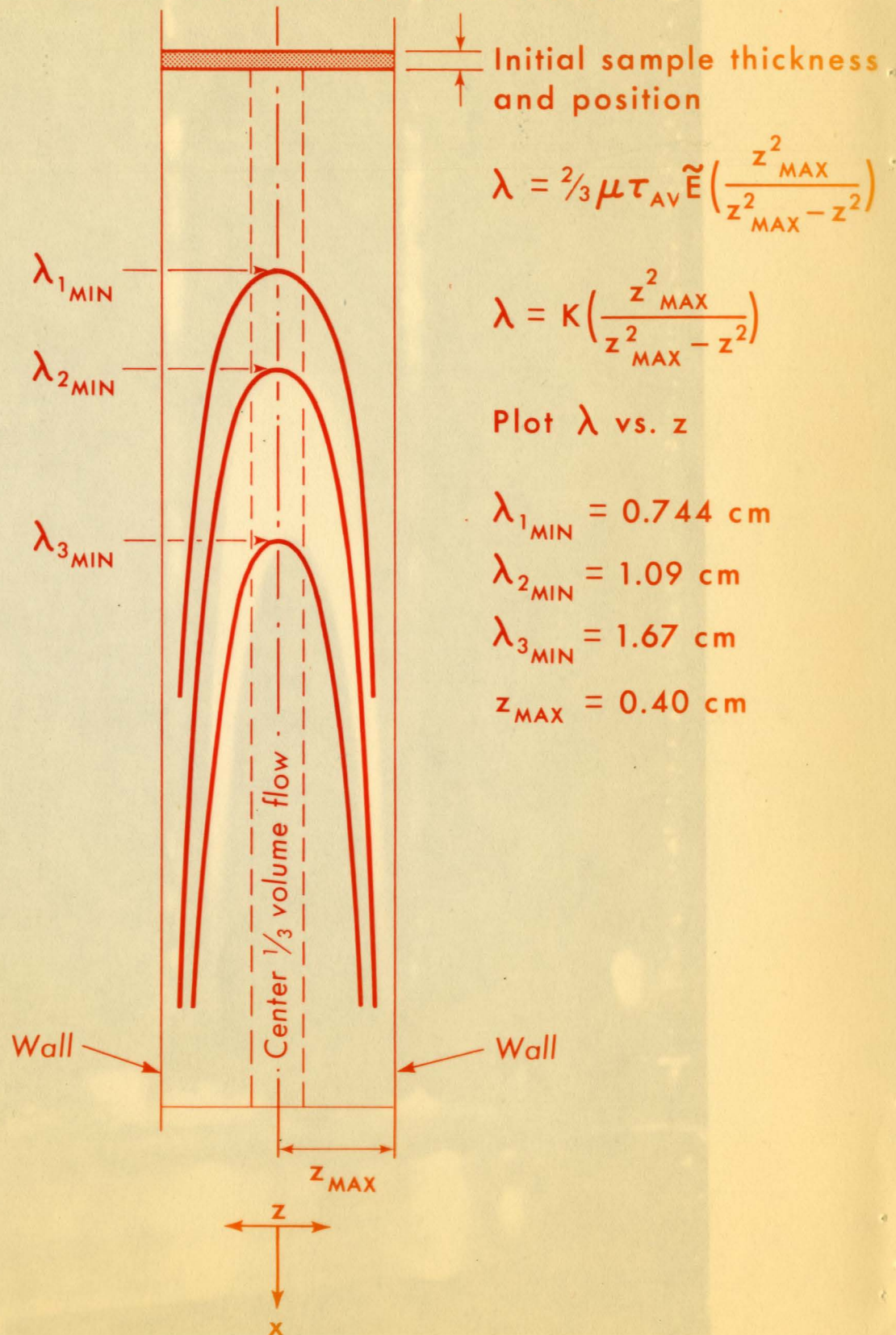
$$\mu = 31A/2TIR = 3\lambda_{\min} A/2 \tau_{av} i r, \quad (38)$$

where for the S_f 0-20 fraction

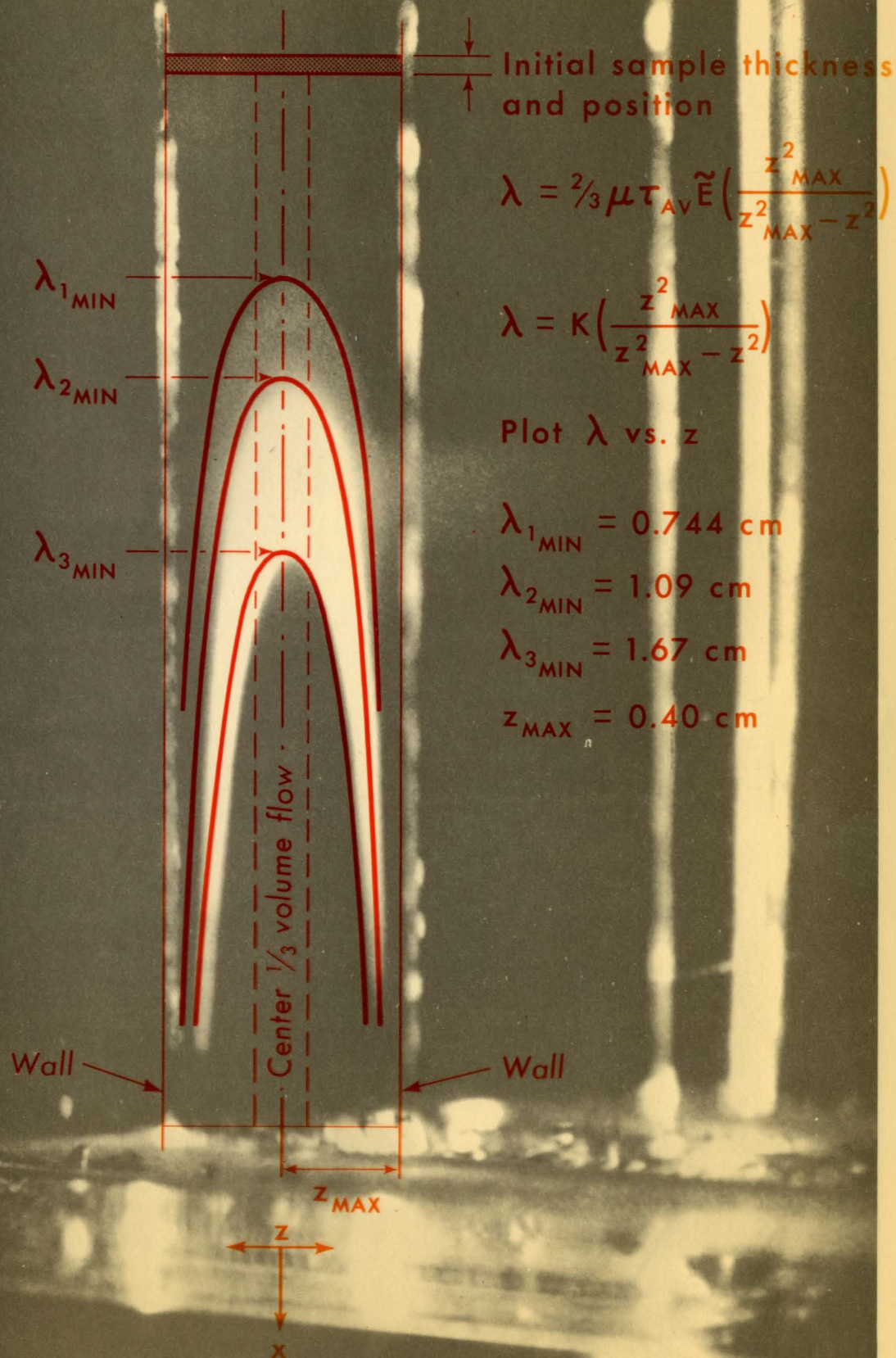
$$\begin{aligned} \lambda &= 1.0 \text{ cm}, \\ A &= 14.4 \text{ cm}^2, \end{aligned}$$

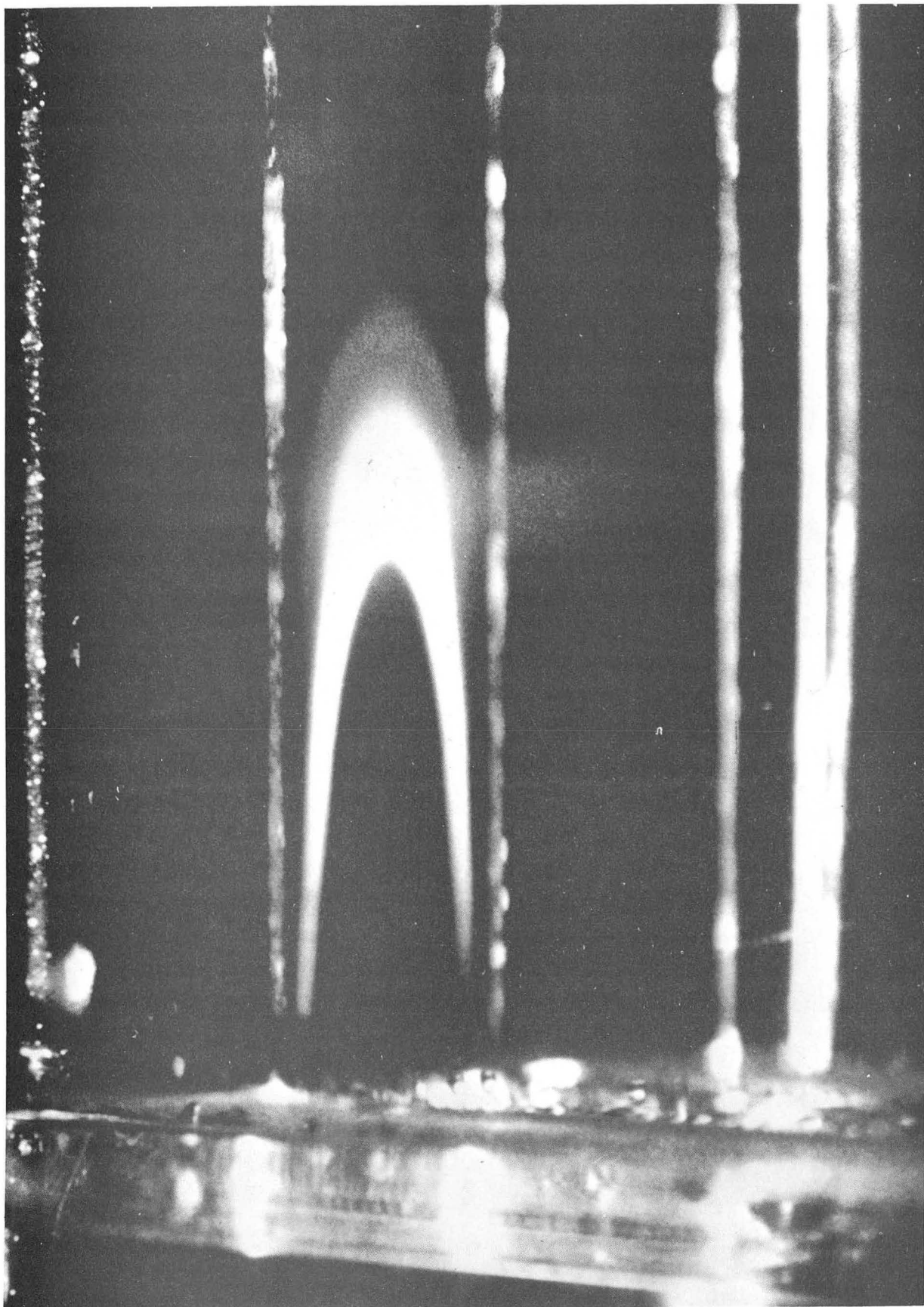
Fig. 24. Experimental migration profile (enlargement of Fig. 17C') with overlay of theoretical noncompensated laminar defocused migration profile.

OVERLAY OF THEORETICAL MIGRATION PROFILE



OVERLAY OF THEORETICAL MIGRATION PROFILE





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$$\tau_{av} = 1224 \text{ sec,}$$

$$i = 0.015 \text{ amperes,}$$

$$r = 9500 \text{ ohm-cm,}$$

and μ , as calculated, is $2.07 \times 10^{-4} \text{ (cm/sec)/(volt/sec)}$.

The electrophoretic mobility of the $S_f 20-10^5$ fraction found by using this same equation with

$$\lambda_{min} = 1.375 \text{ cm,}$$

$$A = 14.4 \text{ cm}^2,$$

$$\tau_{av} = 1224 \text{ sec,}$$

$$i = 0.015 \text{ amperes, and}$$

$$r = 6800 \text{ ohm-cm}$$

gives μ as $4.70 \times 10^{-4} \text{ (cm/sec)/(volt/cm)}$.

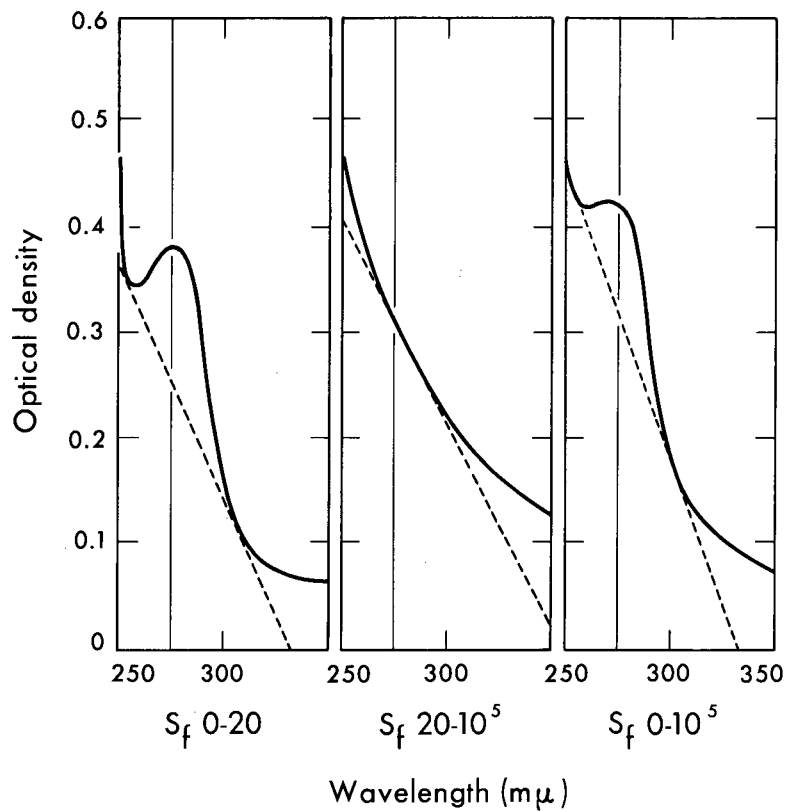
D. Studies on Staflo Electrophoresed Human Serum Lipoprotein Fractions

1. Ultraviolet Spectrophotometry

In the research reported in this thesis the lipoprotein samples contained either the $S_f 0-20$ fraction or the $S_f 20-10^5$ fraction or both. It was desirable to have a rapid, quantitative, nondestructive means of measuring the concentrations of these two lipoprotein fractions in the presence of each other.

It is known that most soluble proteins have a strong uv absorption band between 270 and 280 $m\mu$. The absorption is believed to be due to the tyrosine and phenylalanine residues within the protein moiety. The $S_f 0-20$ lipoprotein fraction was not an exception, for it has an absorption maximum at $275 \pm 1 m\mu$. The $S_f 20-10^5$ fraction does not have a maximum at this point, but rather has an absorption that decreases monotonically from about 255 to about 305 $m\mu$ (see Fig. 25).

Each of the Staflo outlet fractions had its respective optical density (OD) spectrum measured from 250 to 350 $m\mu$ with the Beckman Instruments Recording Spectrophotometer Model DK-1 (hydrogen lamp, automatic scan and slit width, scanning time, 350 to 250 $m\mu$, is 40 sec at a chart speed 3 in/min.)



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Fig. 25. Representative examples of chart recordings (solid curve) of spectra of the human serum lipoprotein classes S_f 0-20, S_f 20-10⁵, and S_f 0-10⁵. Note: These are representative examples of the uv spectrum of the human serum lipoproteins of the classes shown. It is not to be inferred that the left and center spectra can be added to give the right-hand one.

Figure 25 shows typical examples of the OD spectra of the two lipoprotein classes. The diagonal line drawn tangent to each of the curves from near 255 to near 305 mμ is a reference line for the purpose of calculating the respective concentrations of S_f 0-20 and S_f 20-10⁵ fractions in the presence of each other. A vertical line is drawn at 275 mμ. The combined OD spectra of a mixture of S_f 0-20 and S_f 20-10⁵ lipoproteins can be analyzed for these two components by the following method:

A tangent is drawn to the OD-vs-wavelength curve as recorded from 250 to 350 mμ. This tangent touches the curve at approximately 255 and 305 mμ. Multiplying by 2.56 the OD difference between the OD-vs-wavelength curve at 275 mμ and the diagonal tangent curve gives the corrected OD of the S_f 0-20 fraction. The OD(S_f 20-10⁵) is found by subtracting the OD (S_f 0-20) from the total OD at 275 mμ.

The concentration (mg/ml as dry weight) of each fraction is obtained by the empirical formula

$$(S_f \text{ 0-20})\text{mg/ml} = \frac{\text{Corrected OD units } (S_f \text{ 0-20})}{0.0235 \text{ (OD units)/(mg/ml)}}, \quad (39)$$

$$(S_f \text{ 20-10}^5)\text{mg/ml} = \frac{\text{Corrected OD units } (S_f \text{ 20-10}^5)}{0.302 \text{ OD units/(mg/ml)}}. \quad (40)$$

The concentrations of S_f 0-20 and S_f 20-10⁵ were obtained by drying a sample of known OD at 107°C for at least 12 hr and then weighing. This ultraviolet spectrophotometric method is relatively rapid. It is semiquantitative and it is nondestructive. By the use of this method it is possible to make a reasonable estimate of the concentrations of the S_f 0-20 or the S_f 20-10⁵ fractions separately or in the presence of each other.

2. Lipid Extraction

It is a reasonable assumption that since there is a low-density spectrum of lipoprotein molecules there also may coexist in them a spectrum of lipid molecules. In order to determine if there is or is not a spectrum of lipid molecule it is necessary to chemically

separate the lipids from their respective protein moieties and to determine their types, e.g., glycerides, fatty acids, cholesterol and cholesterol esters, or phospholipids.

Before lipid extraction could be performed upon the Staflo outlet collection fractions, it was necessary to concentrate the lipoproteins in these fractions. The outlet samples were ultracentrifuged for 24 hr at 40 000 rpm at the density of each Staflo outlet collection fraction. In all cases the S_f 0-20 sedimented and the S_f 20-10⁵ floated. This ultracentrifugation concentrated these samples sixfold to tenfold. One milliliter of each of these concentrated Staflo outlet fractions was then extracted by a modified Sperry extraction technique.⁴⁹ This extraction was performed in the following manner.

(i) Each of the 1 ml aqueous lipoprotein samples was placed in a 40-ml vial.

(ii) 8.5 ml of methanol was added to each sample while the sample was gently swirled.

(iii) 8.5 ml of CHCl_3 was then added to each sample while the sample was gently swirled.

(iv) Each sample-containing vial was capped.

(v) Each vial was placed in a heating block at 68 to 70°C for 20 minutes. Each was cautiously swirled at 5-min intervals.

(vi) At the end of this 20-min interval the vials were removed and allowed to cool for 20 min.

(vii) Following the cooling, CHCl_3 was added to each sample to bring the volume nearly to 25 ml.

(viii) The vials were allowed to stand for a few moments to permit the protein precipitate to settle, and then the volume of each was brought to 25 ml by addition of CHCl_3 .

(ix) These 25-ml solutions were filtered through preextracted 41-H Whatman filter paper.

(x) The volume in excess of 20 ml in each was pipetted off.

(xi) To each vial was added 4 ml of distilled water; the vials were capped and shaken vigorously for 30 strokes.

(xii) The vials were balanced against each other and centrifuged for 10 minutes at 1000 rpm.

(xiii) Following centrifugation the aqueous phase was siphoned off to within 2 mm of the organic phase in each vial.

(xiv) To each vial 4 ml of Sperry wash was "layered" over organic phase. (Sperry wash: 200 mg CaCl_2 /liter distilled H_2O , 400 ml CHCl_3 , and 200 ml methanol.)

(xv) The vials were again balanced against each other and centrifuged for 10 min at 1000 rpm.

(xvi) Following this centrifugation the aqueous phase of each sample was as completely as possible removed.

(xvii) Each sample was dried at about 55°C under streaming N_2 .

(xviii) Each sample was then transferred quantitatively to 9-ml vials, again dried and weighed.

The weight of the lipid extracted could then be referred back to the concentration in the Staflo outlet collection fractions or serum by applying the appropriate dilution and concentration factors. This referral was normally not done, since only relative weights of total lipid were of interest.

The left-hand column of Table IV gives as an example the concentration of the total lipid in the Staflo samples (inlet and outlet).

Aliquots of lipoprotein from the concentrated Staflo outlet samples were dialyzed against LKB 3276-GB high-resolution buffer salt (8×10^{-3} M tris, 3.3×10^{-4} M EDTA, and 1.22×10^{-3} M H_3BO_4) in distilled-deionized water for at least 8 hr, with several changes of buffer, at a temperature between 0° and 10°C . These dialyzed samples were reserved for OsO_4 fixation and electron microscope characterization, which is discussed later.

3. Silicic Acid Chromatography and Infrared Spectrophotometry

The total quantity of extracted lipid from the lipoproteins yields some information related to the possible existence of a lipid spectrum within the lipoprotein. However, additional information related to the possible lipid spectrum could be obtained if the various types and quantities of the extracted lipids could be determined.

It was possible to make infrared (ir) measurements upon the total lipid extract. However, this would not have yielded much useful information because of the severe overlapping of ir absorption bands of the various serum lipids.¹¹ Therefore it was necessary to fractionate the extracted lipids according to a modification of the Bergstrom method.¹² This modification yields four lipid fractions. These are

- (i) cholesterol esters;
- (ii) glycerides, unesterified fatty acids;
- (iii) glycerides unesterified fatty acids, unesterified cholesterol esters;
- (iv) phospholipids.

If ir spectrophotometric measurements are not to be made shortly after the silicic acid chromatography the lipid samples are dissolved in CHCl_3 under dry nitrogen. Prior to ir measurement the samples are dried by blowing dry nitrogen over each with the temperature not exceeding 50°C . Following drying and just prior to ir readings the samples are dissolved in a quantity of CCl_4 calculated to give optional readings with the Perkin-Elmer double-beam ir spectrophotometer. In all cases with my samples this amount of CCl_4 was 0.4 ml - the minimum quantity that could be used. Freeman gives the methods for reading the ir spectrographs and for calculation of the concentration of the various lipids in the samples.^{11, 12} The results for one such ir spectrophotometric analysis of lipid extract fractionated by silicic acid chromatography is given in Table VI.

The specific concentrations and types were determined by infrared spectrophotometry by the method of Freeman.¹¹ Table VI gives a typical result of a Staflo-fractionated lipoprotein of the $S_f 0-20$ class using the 1×12 outlet arrangement. It is apparent that there are no systematic composition changes in the lipid classes from one outlet to the next. This was expected from Staflo electrophoresis with a 1×12 outlet arrangement, since "laminar defocusing" was expected to limit resolution to a very large extent, even if a heterogeneous sample were used. This experiment will have to be repeated, but

Table VI. Extracted lipids by modified Sperry extraction technique of
Stafl-o-electrophoresed human serum lipoproteins of the S_f 0-20 class
(1×12 outlet arrangement, -25 V, 8 mA,
average residence time of 20.4 min, sample introduced at level 3).

Total lipid concentration (mg/ml)	Total lipid silic acid chromatographed (mg)	Particular lipid classes ^a (infrared) (mg/ml)					Total recovery (%)	
		CSE	TG	UFA	CS	PL		
<u>Inlet sample</u>								
12.54	8.36	4.024	0.728	0.070	0.070	1.964	82.0	

<u>Outlet samples</u>								
<u>outlet</u>								
4 ₁	0.233	2.80	1.464	0.309	0.018	0.022	0.820	94.0
4 ₂	0.257	3.09	1.490	0.318	0.022	0.023	0.582	87.5
5 ₁	0.690	8.29	4.000	0.745	0.092	0.071	2.236	85.2
5 ₂	0.694	8.33	4.000	0.717	0.097	0.059	1.964	82.1
6 ₁	0.201	2.41	1.180	0.331	0.063	0.019	0.744	97.0
6 ₂	0.190	2.28	1.130	0.249	----	0.012	0.605	87.6

a. CSE: cholesterol esters, TG: triglycerides, UFA: unesterified fatty acids, CS: cholesterol,
PL: phospholipids

with a 3X12 or better arrangement, on the Staflo chamber. In this way most of the laminar defocusing can be avoided.

4. Electron Microscopy

Very early in this research it was discovered that in many cases only low concentrations of the serum lipoproteins would be available for analysis following Staflo electrophoresis, even when a rather large sample was used to begin the experiment. This meant that adequate lipid extraction and analytical centrifugation of the lipoproteins would be limited, since both require a concentration of lipoprotein near to or greater than that found in normal whole blood. For the electron microscope, however, a very low concentration is not the severe handicap that it is for the other methods.

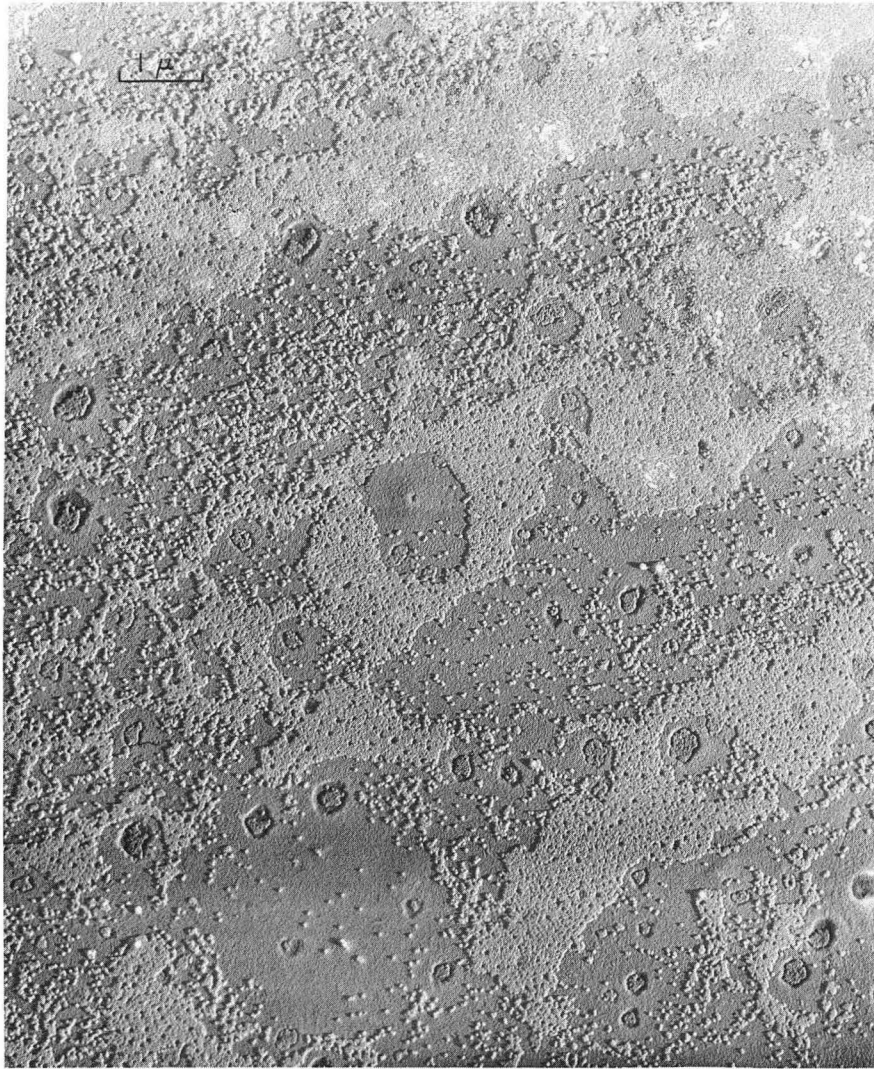
The lipoprotein samples from the Staflo-electrophoresed fractions were fixed with OsO_4 , according to the method developed by Hayes,¹⁹ and then they were chromium-shadowed.

The electronmicrograph of the S_f 0-20 fraction is shown in Figs. 26 and 27. Notice the very uniform size of the individual particles and the apparent sheet formation of this fraction. It may be that this sheet could be considered a pseudomembrane. It has been observed that many living biological membranes contain lipoproteins in their structure,¹⁶ e.g., the cell membrane, the mitochondrion outer membrane and the crista membranes, and the nuclear membrane. It would seem that here is an area that needs intense future study.

5. Analytical Ultracentrifugation

Throughout this entire study all lipoprotein samples were first separated from serum by preparative ultracentrifugation according to their respective densities. This separation served to classify them. Since within each class there is a specific range of S_f rates, a test of separation efficiency or the effect of Staflo electrophoresis upon the sample can be performed. This is done by checking the sedimentation rates of the inlet sample against the same material following Staflo electrophoresis by analytical ultracentrifugation.

The sample chosen was a S_f 0-20 fraction isolated ultracentrifugally. After Staflo electrophoresis, the outlet samples were



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Fig. 26. Electron micrograph of Staflo-electrophoresed sample of Sf 0-20 lipoproteins (OsO₄ fixation, Cr shadowing).



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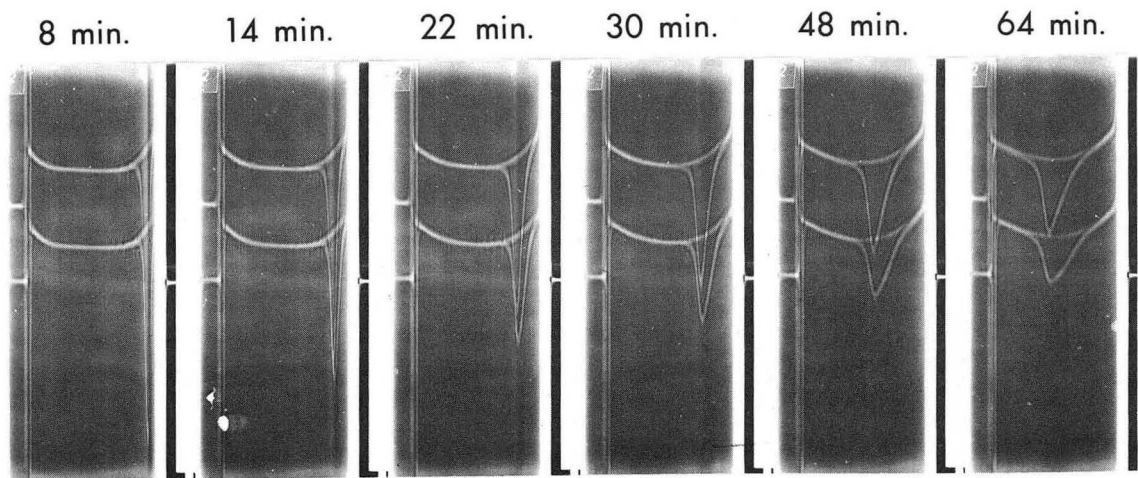
Fig. 27. Enlargement of a portion of the same lipoprotein fraction as in Fig. 26.

concentrated by preparative centrifugation at their respective solution densities. Following preparative centrifugation, each concentrated sample was located in the bottom 1 ml of the preparative centrifuge tubes. The samples were dialyzed against a NaCl solution of density 1.063 g/ml for 24 hr with several changes of dialyzing solution. It was assumed that the sample solutions after dialysis had a salt background of this same density.

The analytical ultracentrifugation was performed according to the method of de Lalla and Gofman.⁹ The sedimentation patterns (Schlieren optics), following attainment of 52 640 rpm, are shown in Fig. 28. The upper pattern is the inlet sample at 980 mg/100 ml and the lower pattern is the outlet sample of 770 mg/100 ml dry weight. After correction for the Ogston-Johnson effect, the inlet and outlet peaks have S_f rates of 8.122 and 7.961 respectively. Table VII summarizes the ultracentrifugal data for these samples.

It would appear that the outlet sample has a small enrichment of lipoproteins of slightly lower S_f rate. The cause for the enrichment is, as yet, unknown. However, this could be due to the extra handling that the outlet sample experienced. This extra handling involved Staflo electrophoresis and one additional concentration step. At present it is not possible to determine whether the change is due solely to Staflo electrophoresis or due to the concentration step of preparative ultracentrifugation or both. (I think that the concentration step is probably the more damaging of the two.)

Staflo electrophoresis of the S_f 0-20 fraction does not adversely affect this fraction to any large extent.



UPPER : S_f 0-20 sample (inlet) (980 mg/100 ml)

LOWER : S_f 0-20 "Same sample" after STAFLO-electrophoresis (770 mg/100 ml)

ZN-5118

Fig. 28. Schlieren patterns of the S_f 0-20 lipoprotein class before and after Staflo electrophoresis.

Table VII. Ultracentrifugal analysis of Staflo-electrophoresed S_f 0-20 lipoproteins.

S_f Interval	Inlet sample		Outlet sample		Ratio (% outlet/% inlet)
	Concentration (mg%)	Part of total (%)	Concentration (mg%)	Part of total (%)	
0.0 - 1.0	5.441	0.576	7.073	0.926	1.608
1.0 - 2.0	13.171	1.394	18.181	2.380	1.707
2.0 - 3.0	14.233	1.506	20.693	2.709	1.799
3.0 - 4.0	21.687	2.295	32.476	4.251	1.852
4.0 - 5.0	39.505	4.181	54.606	7.148	1.710
5.0 - 6.0	73.981	7.830	84.055	11.003	1.405
6.0 - 7.0	120.659	12.770	118.728	15.542	1.217
7.0 - 8.0	167.556	17.733	150.969	19.762	1.114
8.0 - 9.0	223.175	23.619	158.684	20.772	0.879
9.0 - 10.0	166.458	17.617	83.024	10.878	0.617
10.0 - 12.0	90.033	9.528	31.970	4.185	0.439
12.0 - 14.0	8.882	0.940	3.475	0.455	0.484
14.0 - 16.0	0.101	0.011	0.000	0.000	0.000
16.0 - 20.0	0.000	0.000	0.000	0.000	----
0.0 - 20.0	944.881	100.000	763.934	100.000	0.808
Peak S_f Rate:	Uncorrected		0 - J Corrected		Ratio Un/0-J
Inlet sample	7.341		8.122		1.1063
Outlet sample	7.234		7.961		1.1004
Ratio: Inlet/outlet	1.015		1.020		

III. CONCLUSIONS

The velocity profile within the Staflo chamber has been shown to be neither plug-shaped (for most of the height of the Staflo chamber) nor parabolic, but rather it has two horns separated by a region of vertical plug flow. Furthermore, the shape of this vertical profile is not affected either by (a) the shape of the inlet flow divider, (b) the horizontal position of the inlet tubes, or (c) their respective flow rates after about the first half centimeter. The horizontal velocity profile is truly parabolic at all levels, from within a half centimeter of the outlet. Negative hydrostatic-hydrodynamic feedback operates only vertically and not horizontally.

Fluid flow is stable from an average residence time of 4 min to at least 90 min. There are no indications, either theoretical or practical, that a 90-min average residence time is near the upper limit of operation. In fact, it should be possible to operate the Staflo continuously at any average time within the above time limits for an indefinitely extended period. Experiments lasting as long as 60 throughput volumes have been performed without development of long-term drift instability or other type of instability.

It has been shown that in some cases during Staflo electrophoresis the passage of electric current actually makes the fluid more stable gravitationally. In this case the density gradient becomes larger with the passage of an electric current.

Phenomena leading to a net transfer or displacement of sample away from the walls and toward the center of the Staflo chamber of a thin initial zone which extends completely across the width of the Staflo chamber have been shown to occur. This net transfer or displacement is called "central sharpening." It is the result of micro-density differences that occur within the Staflo chamber, for any of the following:

- (i) Diffusion acting upon a finite step-function density gradient in a laminar flow system. I call this diffusion-convection central-sharpening.

(ii) Differential heating of the solvent relative to the solute during electrophoresis. I call this differential heating central sharpening.

(iii) A density difference between the solute and the solvent (the usual case), I call this solute-solvent differential density central sharpening.

The effects of these differences upon capacity and resolution of the Staflo have been discussed. Methods of sampling of the Staflo chamber have shown how the severe problem of "laminar defocusing" can be significantly avoided. Optical techniques such as the new thin-slit method yield information upon the sample migration that no other method can currently provide.

It has been established that the low-density lipoproteins can be studied by the Staflo method. This method can separate the S_f 0-20 class from the S_f 20-10⁵ class. There is also an indication that sub-fraction may take place within the S_f 20-10⁵ class. Staflo electrophoresis and electron microscopy of the S_f 0-20 fraction indicate that this fraction is homogeneous.

A method is described whereby ultraviolet spectrophotometry can be used to determine the concentration of S_f 0-20 and S_f 20-10⁵ lipoproteins in the presence of each other.

The Staflo method can at present handle only low concentrations (less than 0.1 wt%) of albumin, the globulins, or the lipoprotein classes HDL₂ and HDL₃. Long-term density instability results at high concentrations. The problem of long-term density instability for proteins (or other high-mol-wt polymers) can very likely be solved by the judicious use of heavy water (D₂O) in place of sucrose for density adjustment of the inlet sample, or by the use of dextran in the other streams, or both.

The Staflo method has been shown by this author to work well for the lipoproteins of S_f 0-10⁵ class. Mel has shown that it also works for other proteins such as hemoglobin,²⁸ enzyme substrate reaction components,³⁰ and bone marrow cells. Noble has shown that the Staflo method can be used to study chloroplasts.⁴⁰ Pistemna is presently using the method to characterize rabbit spermatozoa.⁴⁴

It seems that if the sample to be studied or separated by the Staflo method--whether it be electrophoretically, by flotation, by sedimentation, or whatever--the only requirement is that the sample zone be stable for an appropriate period of time.

The author has built or aided in building other Staflo cells. These Staflo cells have all been smaller than the ones described in this thesis. At least two of these are 1.5 cm high instead of 3.0 cm high. All other dimensions and the number of streams remain the same. These Staflo cells seem to operate as well as the one used in the thesis research. This author has also built and preliminarily tested a Staflo cell 1.1 cm high $\times$ 6 cm long $\times$ 0.015 cm thick having six inlets and outlets. This Staflo cell evidenced stable flow similar to the larger Staflo chambers. Such a small cell should require less sample and permit high-power microscopic measurements of the migrating particles within it.

ACKNOWLEDGMENTS

I wish to express my deepest thanks to the members of my thesis committee, Professor Alexander V. Nichols, Professor Howard C. Mel, and Professor A. Douglas McLaren, for their helpful suggestions, advice, and encouragement extended to me. Thanks are also due to Professor Thomas Hayes, for his assistance in the use of the electron microscope; to Mr. Robert C. Bigelow of Technical Photography and Mr. John Flambard for their able assistance in the photography and the presentation of the drawings that appear in this thesis.

My gratitude goes also to Mr. Edward F. Dowling and Mr. Henry L. Minassian for their assistance in building a Stable-Flow Free-Boundary Apparatus, without which this research would not have been possible, and to many others too numerous to mention.

Deep appreciation goes to my wife Kathryn, not only for her help in typing of the many drafts of this thesis, but primarily for the long years of her patience.

I thank the Oak Ridge Institute of Nuclear Studies for fellowship support during my graduate studies.

This research was supported in part under the auspices of the U. S. Atomic Energy Commission and the U. S. Public Health Department.

GLOSSARY OF SYMBOLS AND TERMS

Symbol or term	Meaning	Units	Where first used
A	Area	cm ²	Eq. 9
A'	Area of electrophoretic electrodes	cm ²	Eq. 35
central sharpening	The phenomenon that results in the solute's flowing toward the center of the Stafo chamber	...	IC3
ΔC	Concentration gradient	...	Eq. 3
D	Diffusion coefficient for principal solute	...	Eq. 3
d	Vertical height of Stafo chamber	cm	Eq. 1
$\tilde{E}$	Electric field strength	V/cm ²	Eq. 24
$\tilde{E}_b, \tilde{E}_s$	Electric field strength in background and sample, respectively	V/cm	Eq. 25
h	Apparatus parameter	cm	Eq. 1
$\tilde{i}$	Current density	A/cm ²	Eq. 27
ir	Infrared	...	ID3
K	Ratio of mass to density	g/(g/ml)	Eq. 10
k	Apparatus parameter	(cm/sec)/cm ²	Eq. 4
L	Length of Stafo chamber	cm	Eq. 17
L'	Length over which electrophoresis takes place	cm	Eq. 17
l	Diffusion distance	cm	Eq. 3
l_1, l_2, l_{12}	Collector container liquid height	cm	Eq. 1
Δl	Difference in collection container heights	cm	Eq. 2
laminar defocusing	The phenomenon that results when a solute migrates normal to laminar fluid flow	...	ID1

Symbol or term	Meaning	Units	Where first used
M	Apparatus parameter ($=h - d/2$)	cm	Eq. 1
OD	Optical density	...	Eq. 39
P, P _s	Sample zone thickness	cm	Eq. 27
Q	Volume flow rate of any stream stream	ml/sec	Eq. 28
Q _s	Volume flow rate of sample stream	ml/sec	Eq. 28
r _b	Resistivity of background solution	ohm-cm	Eq. 25
r _s	Resistivity of sample	ohm-cm	Eq. 25
S _f	Flotation coefficient	svedbergs	IIA1
Staflor	Short for "Stable-flow free-boundary"	...	...
Staflor cell	Consists of upper and lower electrode chambers and Staflor chamber	...	Fig. 1
Staflor chamber	Chamber in which electro- phoretic or sedimentation migrations occur	...	Fig. 3
t	Time	sec	Eq. 6
v	Linear fluid flow velocity within Staflor chamber	cm/sec	Eq. 4
v _{max}	Maximum fluid flow velocity within Staflor chamber	cm/sec	Eq. 4
x	Vertical height coordinate	cm	IC1a
y	Horizontal length coordinate	cm	IC1a
z	Horizontal depth coordinate	cm	IC1a
z _{max}	Half horizontal depth of Staflor chamber	cm	Fig. 20

<u>Symbol or term</u>	<u>Meaning</u>	<u>Units</u>	<u>Where first used</u>
λ	Electrophoretic migration distance	cm	Eq. 21
λ_1, λ_2	Electrophoretic migration distances for solutes 1 and 2, respectively	cm	Eq. 27
$\lambda_{\min}$	Electrophoretic migration distance at center of Stafo chamber	cm	ID1
$\Delta\lambda$	Difference in total migration distances of two solutes	cm	Eq. 27
μ	Electrophoretic mobility	(cm/sec)/(V/cm)	Eq. 21
μ_1, μ_2	Electrophoretic mobilities of solutes 1 and 2 respectively	(cm/sec)/(V/cm)	Eq. 27
$\Delta\mu$	Difference in electrophoretic mobility	(cm/sec)/(V/cm)	Eq. 35
ρ_1, ρ_2	Densities of individual streams 1 and 2	g/ml	Eq. 1
$\Delta\rho$	Difference or change in density	g/ml	ID2
τ	Residence time	sec	Eq. 6
τ_{av}	Average residence time	sec	IB2
$\tau_{\min}$	Minimum residence time	sec	Eq. 18
$\tau'_{\min}$	Minimum residence time for electrophoresis	sec	Eq. 19

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