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RETENTION IN SEDIMENTATION–FLOTATION FOCUSING FIELD-FLOW FRACTIONATION USING A STEP DENSITY GRADIENT

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SUMMARY

The previously developed method for the numerical calculation of the flow velocity profile formed in rectangular and trapezoidal cross-section channels when applying a step density gradient was used to calculate the positions of the boundaries between liquids of various density flowing in channels. The theoretical results are compared with experimental data obtained under various experimental conditions. Good agreement between the theoretical and experimental data was obtained, thus confirming the theoretical model used in the calculations and the reliability of the interpretation of the experimental retentions in terms of the densities of the fractionated solutes.

INTRODUCTION

Separation in sedimentation–flotation focusing field-flow fractionation (SFFFFF) is due to the differences in the densities of macromolecular or particulate solutes migrating through a density gradient in the direction of the external gravitational or centrifugal field forces and being focused at their isopycnic positions[1]. The focused solute zones are carried by the liquid flowing along the separation channel in a direction perpendicular to the direction of the focusing forces. The shape of the cross-section of the channel determines the shape of the flow velocity profile formed in the flowing liquid under isoviscous laminar conditions. The effective separation of the focused solute zones can be achieved by choosing a suitable shape of the separation channel. Channels with modulated cross-sectional permeability were proposed in order to realize different shapes of the flow velocity profile[2]. The usefulness of this concept was demonstrated when particles of density standards and various latexes were separated in a natural gravitational field[3].

A discontinuous density gradient can be pre-formed inside the separation channel for SFFFFF. A step density gradient is established provided that the liquids of different densities enter the separation channel via the individual inflow capillaries under conditions of laminar flow[4]. The form of the separation channel cross-section affects the flow velocity pattern inside.

A method for the numerical calculation of the flow velocity distributions formed in rectangular and trapezoidal cross-section channels under the conditions

of a step density gradient was developed recently[5]. It can be used if the liquids of different viscosities forming the step density gradient are applied in SFFFFF. Several typical examples of the flow velocity profiles were calculated for various conditions simulating real experiments[5].

The shape of the flow velocity profile and the position of the boundaries between the liquids of different viscosity should be known for the quantitative evaluation of the experimental retention data.

The retention ratio, R is defined as

$$R = v_R / <v> \tag{1}$$

where v_R is the average linear velocity of the longitudinal migration of the focused zone of the solute carried by flow and $<v>$ is the average linear longitudinal velocity of the flow of the bulk liquid inside the channel.

General theoretical relationships describing the retention and dispersion in focusing FFF for various cross-section channels (rectangular, trapezoidal and parabolic) were derived previously[6]. The theory assumed a constant gradient of focusing forces in the region of the focused zone and isoviscous flow conditions.

If several liquids of different densities are used to form the step density gradient inside the channel, the flow velocity profile reflects the differences in the viscosities of individual liquid layers and the gradient of focusing forces also has a more complicated shape. This has to be taken into account if the retention ratio measured experimentally for a given solute is considered as a fundamental parameter from which the density of the fractionated sample is to be evaluated.

The aim of this work was to verify experimentally the previously developed method for the numerical calculation of the flow velocity profile[5] and to establish the reliability of the interpretation of the experimental retention ratios in terms of the densities of the fractionated solutes. In order to avoid mathematical difficulties in data treatment, simplified model experimental conditions were designed. However, the experimental conditions were chosen in such a way that the resulting conclusions would be generally valid.

THEORY

Focusing forces

The force acting on the solute particles or macromolecules undergoing focusing by sedimentation flotation processes is coordinate dependent:

$$F(\varphi) = Vg(d\rho/d\varphi)(\varphi_{max} - \varphi) \tag{2}$$

where V is the molar volume of the solute species, $d\rho/d\varphi$ is the density gradient, g is the gravitational or centrifugal acceleration, φ is the dimensionless coordinate in the direction of the focusing forces and φ_{max} is the coordinate of the maximum concentration of the zone, *i.e.*, the focusing point for which $F(\varphi_{max}) = 0$. The coordinate system used in this paper is shown in Fig. 1 for both rectangular and trapezoidal cross-section channels. At equilibrium the flux of the solute due to focusing forces is just balanced by the flux due to the diffusion in the direction of the φ axis:
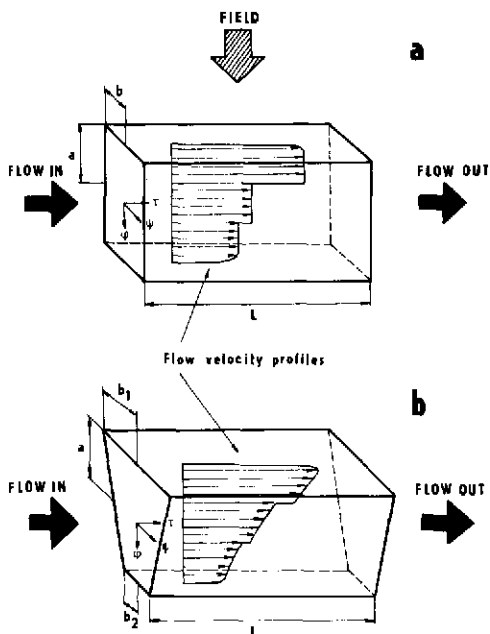
Fig. 1. Schematic representation of (a) rectangular and (b) trapezoidal cross-section channels with flow velocity profiles formed under step density gradient conditions with defined coordinate system.

$$D(\mathrm{d}c/\mathrm{d}\varphi) = u(\varphi)c \tag{3}$$

where D is the diffusion coefficient and c is concentration. The velocity of solute migration under the influence of the focusing forces, $u(\varphi)$, is given by

$$u(\varphi) = F(\varphi)/f \tag{4}$$

and the friction coefficient f is defined by

$$f = R^0 T/D \tag{5}$$

R^0 being the universal gas constant and T the absolute temperature. The gaussian distribution for the focused zone of the solute along the φ axis is obtained by substituting from eqns. 2, 4 and 5 in eqn. 3 and solving the first-order differential eqn. 3:

$$c(\varphi) = c(\varphi_{max}) \exp[-Vg(\mathrm{d}\rho/\mathrm{d}\varphi)(\varphi-\varphi_{max})^2/2R^0 T] \tag{6}$$

The first simplifying condition based on eqn. 6 can be formulated. The average velocity of movement of the focused zone due to the longitudinal flow can be approximated by the streamline velocity at the position of maximum concentration in the focused zone, φ_{max}. This approximation holds for narrowly focused solute zones.

Shape of the flow velocity profile

The most practical shapes of the flow velocity profiles are those formed in the fluid flowing inside the rectangular or trapezoidal cross-section channels under laminar and isoviscous flow conditions. The distribution of the flow velocities is influenced by non-isoviscous flow if a continuous or a step density gradient is applied. The relationship describing the mean flow velocity of the liquid averaged across the rectangular or trapezoidal cross-section channel thickness, $\bar{v}(\varphi)$, normalized relative to the velocity in the centre line $\varphi = 0$) of the channel, has been derived[2]:

$$\bar{v}(\varphi) = \frac{b^2(\varphi)L}{b^2(0)l(\varphi)} \frac{\left\{ 1 - \dfrac{\cosh[\sqrt{3}\,A(\varphi)\varphi]}{\cosh[\sqrt{3}\,A(\varphi)]} \right\}}{\left\{ 1 - \dfrac{1}{\cosh[\sqrt{3}\,A(0)]} \right\}} \tag{7}$$

where $b(\varphi)$ is a φ coordinate-dependent half-thickness of the channel (see Fig. 1), $A = a/b(\varphi)$ is an aspect ratio (see Fig. 1), L is the length of the flow streamline at the centre line of the channel and $l(\varphi)$ is the length of the flow streamline at the given coordinate φ. The actual shape of the flow velocity profile can be calculated using eqn. 7 for given geometric dimensions of the fractionation channel and for isoviscous conditions of the flow.

When a discontinuous (*i.e.*, step) density gradient is applied, a numerical method has to be used to calculate the shape of the flow velocity profile and the positions of the boundaries between the individual liquid layers of different densities and viscosities. The method of numerical calculation, described in detail in a previous paper[5], is based on the mass balance equations. For example, the total volumetric flow-rate q of three liquids of different densities and viscosities is the sum of the flow-rates of the individual liquids:

$$q = q_1 + q_2 + q_3 \tag{8}$$

For non-isoviscous conditions a series of equations have been derived[5]:

$$\frac{\mu_1\,q_1}{\mu_2\,q_2} = \sum_{j=1}^{m'} \frac{b_{1j}^3}{l_{1j}} \bigg/ \sum_{j=m'}^{n'} \frac{b_{2j}^3}{l_{2j}} \tag{9a}$$

$$\frac{\mu_1\,q_1}{\mu_3\,q_3} = \sum_{j=1}^{m'} \frac{b_{1j}^3}{l_{1j}} \bigg/ \sum_{j=n'}^{o'} \frac{b_{3j}^3}{l_{3j}} \tag{9b}$$

$$\frac{\mu_2\,q_2}{\mu_3\,q_3} = \sum_{j=m'}^{n'} \frac{b_{2j}^3}{l_{2j}} \bigg/ \sum_{j=n'}^{o'} \frac{b_{3j}^3}{l_{3j}} \tag{9c}$$

The left-hand sides of eqns. 9a–c are known and can be designated LHS_{12}, LHS_{13} and LHS_{23}, respectively. Correspondingly, the summation terms on the right-hand sides can be designated S_1, S_2 and S_3, respectively, and rewritten

$$LHS_{12} = S_1/S_2 \tag{10a}$$

$$LHS_{13} = S_1/S_3 \tag{10b}$$

$$LHS_{23} = S_2/S_3 \tag{10c}$$

Further

$$S_1 + S_2 + S_3 = \sum_{j=1}^{m} \frac{b_{1j}^3}{l_{1j}} + \sum_{j=m}^{n} \frac{b_{2j}^3}{l_{2j}} + \sum_{j=n}^{o} \frac{b_{3j}^3}{l_{3j}} \tag{11}$$

The value of the right-hand side of eqn. 11, which describes the isoviscous flow conditions, is known. The numerical integration limits m, n and o have the meaning of the positions in φ coordinate of boundaries between different liquids but of identical viscosity. Consequently, eqns. 10a, 10b, 10c and 11 can be solved numerically to find S_1, S_2 and S_3 and the required m' and n' values that determine the numerical integration limits satisfying the above system of eqns. 10a–11. The m' and n' integration limits in this instance have the meaning of the position in φ coordinate of the boundaries between the liquids of different viscosity. The linear velocities of the two liquid layers at their boundary can be calculated for known m' and n' positions from the corresponding flow-rates at these boundaries.

Retention

For the classical field-flow fractionation method, the retention ratio, R, as defined by eqn. 1, can acquire values between 1 and 0 for unretained and completely retained solutes, respectively. This is so in view of the fact that the solutes always are or are not retarded owing to retention, and can therefore never move by flowing at an average longitudinal velocity higher than the average velocity of the fluid flow.

The situation is completely different for focusing field-flow fractionation. Here the solutes can be focused at any lateral position inside the separation channel and, consequently, can move at linear longitudinal velocities higher than the average linear velocity of the liquid flow. In these instances, the retention ratio can acquire values higher than 1, according to both the established flow velocity profile and the position of the focused zone. The relationship describing the retention ratio for isoviscous liquid flow conditions was derived previously[6] for a rectangular cross-section channel:

$$R = (3/2)(1 - \varphi_{\text{max}}^2 - \sigma^2) \tag{12}$$

where σ is the standard deviation of the gaussian focused zone expressed as a fraction of the total width of the channel in the direction of coordinate φ, which means in

the direction of the focusing forces. Similarly, the relationship for a trapezoidal cross-section channel and isoviscous conditions was derived[6]:

$$R = \frac{3}{3 + \tan^2\alpha}\left[(1 + \varphi_{max}\tan\alpha)^2 + 2\sigma^2\tan^2\alpha\right] \tag{13}$$

where α is the angle between the two opposite walls of the trapezoidal cross-section channel.

The calculation of the retention ratio for non-isoviscous flow under step density gradient conditions is more complicated. The focused zones should be so narrow in the direction of the focusing field that they will move longitudinally by the flow virtually in a single streamline. The position of the streamline is identical with a sharp boundary between the two liquids of various density and thereby various viscosity. If both diffusion and convection between the two liquids of various density are neglected, then the distribution of velocities in both rectangular and trapezoidal channels is stepwise, as illustrated schematically in Fig. 1. The linear velocity of the movement of the solute zone focused at the boundary between the layers of different densities averaged across the channel thickness, $\bar{v}_R$, will lie between the linear velocities of the streamlines adjoined from both sides to this boundary. The average linear velocity of the liquid in the channel, $<v>$, normalized with respect to the centre, can be calculated numerically from the relationship

$$<v> = \sum_{j=1}^{ot} v_j b_j \delta \bigg/ \sum_{j=1}^{ot} b_j \delta \tag{14}$$

where δ is the increment of the distance between the streamlines. The retention ratio R can be calculated by substituting these linear velocities into eqn. 1. Despite a certain indefiniteness of the mean velocities of the focused zone movement at the boundary, $\bar{v}_R$, the retention ratios R can, with application of the step density gradient, acquire only discrete values. The determination of the position of the focused zone in the step density gradient from experimentally found R values and thereby the determination of the solute density are therefore obviously possible.

The experimental part of this work was aimed at the verification of the theoretical analysis presented above.

EXPERIMENTAL

Experimental verification of a mathematical model of flow and retention was performed by using a discontinuous density gradient and a gravitational force of 1 g acting on particles.

A rectangular channel was cut in Teflon foil, 0.84 mm thick, inserted between two glass plates, 3 mm thick and 40 × 400 mm. The channel itself had the dimensions 20 × 350 × 0.84 mm. The channel was equipped with three openings with capillaries for the inlet of density media and a fourth opening with a capillary served to let the media out from the channel. The trapezoidal channel was composed of a sandwich

of two Teflon foils with rubber in the middle that was inserted between the two glass plates, 3 mm thick and 40 × 400 mm. The channel itself, cut in the sandwich, was 350 mm long and 20 mm wide. The difference in the channel thickness between the upper and lower walls was approximately 0.27 mm. The channel was again equipped with openings for the inlet and outlet of the density media. The channels were clamped mechanically between two Perspex plates with a system of screws. A complete channel of this design is illustrated in Fig. 2.

An LD 2 linear displacement feeder (Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) was used to pump simultaneously three solutions of various densities. It was equipped with three injection syringes whose pistons moved at the same linear velocity. The flow-rate of each density medium was determined by the diameter of the piston of the injection syringe used.

Solutions of sucrose (Lachema, Brno, Czechoslovakia) of various concentrations and Percoll solutions (Pharmacia, Uppsala, Sweden) diluted to the required concentrations were used as density media. Solutions of two densities were always prepared. Pure distilled water and 0.15 M sodium chloride solution were used as the topmost layers of the step density gradient in combination with sucrose and Percoll solutions, respectively. The concentrations of sucrose solutions were 6% and 14% (w/w). The 6% solution of sucrose was coloured with bromophenol blue so that the positions of individual boundaries of the density layers in the channel might be observed. The position of the boundary between the layers of different densities of Percoll solutions was determined with the aid of cross-linked dextran coloured density standards (Pharmacia). The densities of individual media were determined at a given temperature pycnometrically and the viscosities with an Ubbelohde viscometer. The heights of individual coloured density layers in the channel were read from millimetre paper fixed on the glass plate forming one of the channel walls. The measurements were performed at 22 or 23.5°C.
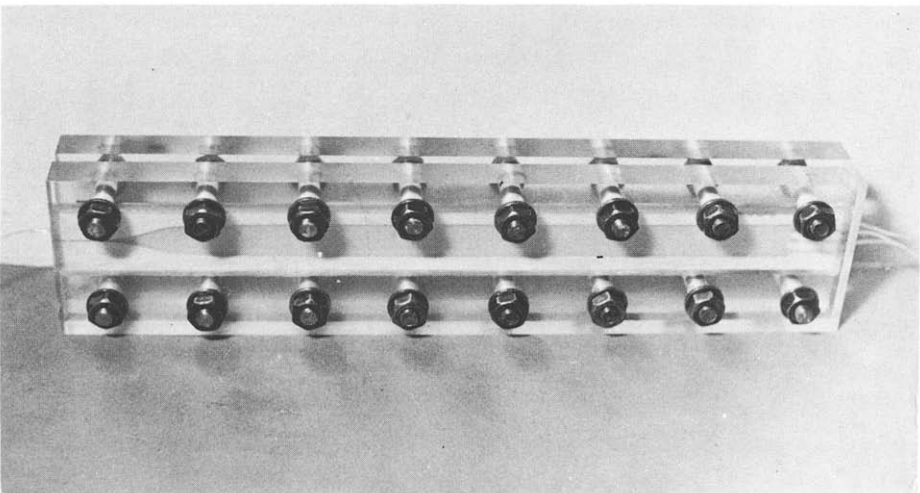

Fig. 2. Channel for SFFFFF using a step density gradient in a natural gravitational field.

RESULTS AND DISCUSSION

As foils or the sandwich could be pressed unequally during the assembly of the channels, the real thickness of the channels was checked by microscopic measurements. The real half-thicknesses of the rectangular channel cross-section were determined as $b_1 = 0.396$ and $b_2 = 0.405$ mm, and the half-thicknesses of the trapezoidal channel cross-section were $b_1 = 0.462$ mm and $b_2 = 0.595$ mm. These values were used in all the calculations.

Physico-chemical characteristics, *i.e.*, density and viscosity, of all the density media used are given in Table I.

Injection syringes used in the linear injector permitted the adjustment of certain discrete flow-rate ratios of the individual density media. The adjustment of the linear velocity of the movement of injection syringe pistons was continuous. In all the following experiments, the total flow-rate of all three density media in the channel and the ratio of the flow-rates of individual media from the highest density, *i.e.*, from the lowest layer, were known.

The initial experiments were designed to verify qualitatively the shape of the velocity profile formed in the channels of rectangular and trapezoidal cross-sections under isoviscous flow conditions. Distilled water was pumped through each channel only in the central inlet capillary (one of the three vertically arranged inlet capillaries). At a certain moment, water was replaced with bromophenol blue solution and the coloured zone formed in the channel indicated the shape of the velocity profile. The experiments were photographed. Fig. 3a, b and c illustrate various phases of the movement of the coloured zone in the channel of rectangular cross-section. The zone formed in this way is, in agreement with theoretical assumptions, axially symmetrical. The slight asymmetry in Fig. 3c is due to the fact that even a small difference between the densities of pure water and the dyestuff solution results in slow sedimentation of this solution. The finding that the asymmetry in the zone shape increased with time after the flow had been stopped confirmed the sedimentation of the dye solution. Fig. 4a, b and c show various phases of the movement of the coloured zone in the channel of trapezoidal cross-section. The zone formed is, in agreement with theory,

TABLE I

DENSITIES AND VISCOSITIES OF THE DENSITY MEDIA USED AT DIFFERENT TEMPERATURES

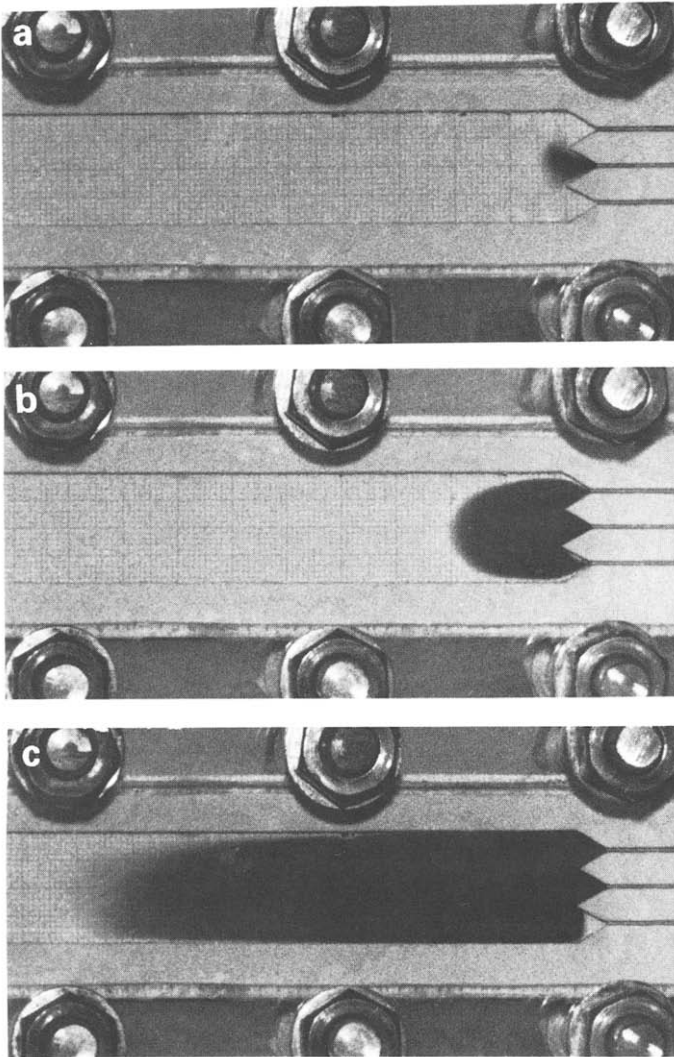| Density medium	Density (g/cm³)		Viscosity (mPa s)	
	22°C	23.5°C	22°C	23.5°C
Water	0.9978	0.9974	0.960	0.927
0.15 M NaCl	1.0027	1.0016	1.031	0.928
6% sucrose (coloured)	1.0220	—	1.123	—
14% sucrose	1.0460	—	1.445	—
Percoll I	1.1023	1.1020	1.596	1.535
Percoll II	1.1223	1.1220	1.845	1.776

Fig. 3. Various phases of the movement of the coloured zone in a rectangular cross-section channel under isoviscous conditions.

axially asymmetrical, the most rapid streamlines being in the upper and wider section of the channel.

The aim of the following experiments was to determine the positions of the boundaries in both channels with the flow of liquids of different viscosities and at various flow-rate ratios and to compare the experimental values with the theoretical calculation. All the results obtained with the use of sucrose and Percoll solutions as density media in the channels of both rectangular and trapezoidal cross-sections are presented in Table II. For illustration, the formation of sharply separated layers of sucrose solutions in the channels of rectangular and trapezoidal cross-sections is

TABLE II

EXPERIMENTAL AND THEORETICAL POSITIONS OF THE BOUNDARIES BETWEEN THE LAYERS OF DIFFERENT DENSITY MEDIA FLOWING IN CHANNELS OF RECTANGULAR AND TRAPEZOIDAL CROSS-SECTIONS UNDER VARIOUS CONDITIONS

| Channel	Density medium	Temperature (°C)	Total flow-rate, q ($\mu l/min$)	Relative flow-rates, $q_1:q_2:q_3$	Relative positions of boundaries			
					Theory		Experiment	
					Lower	Higher	Lower	Higher
Rectangular	Sucrose solutions + water	22	1500	1:1:1	0.41	0.71	0.36	0.71
Rectangular	Sucrose solutions + water	22	1360	1.54:1.54:1	0.31	0.67	0.30	0.66
Trapezoidal	Sucrose solutions – water	22	1500	1:1:1	0.49	0.77	0.50 0.45	0.75 0.77
Trapezoidal	Sucrose solutions – water	22	488	1.54:1:1	0.43	0.69	0.40	0.65
Trapezoidal	Sucrose solutions – water	22	1360	1.54:1.54:1	0.39	0.73	0.35 0.40	0.72 0.70
Rectangular	Percoll solutions + 0.15 M NaCl	22	122	1.54:1:1	0.37	0.68	0.36	0.64
Trapezoidal	Percoll solutions + 0.15 M NaCl	23.5	122	1.54:1:1	0.46	0.75	0.47	0.70
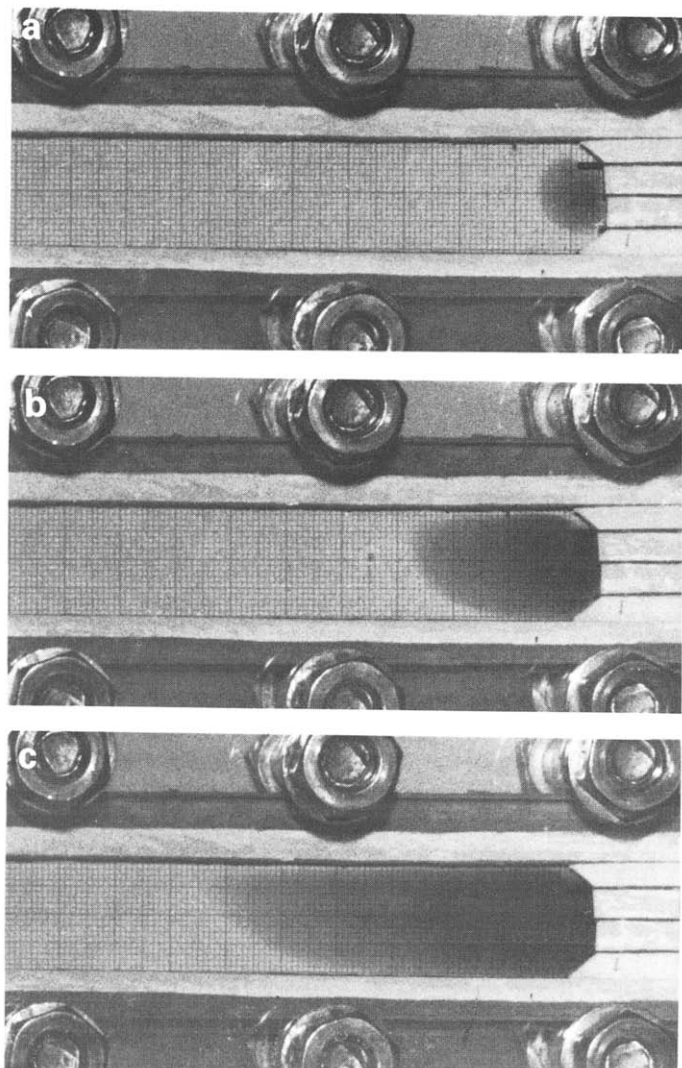
Fig. 4. Various phases of the movement of a coloured zone in a trapezoidal cross-section channel under isoviscous conditions.

shown in Figs. 5 and 6, respectively. In both instances the sucrose solution in the middle layer is coloured.

The relative position of the boundary was calculated as the ratio of the distance of this boundary from the lower channel wall to the total channel width in the vertical direction.

It follows from both the experimental results and the calculated boundary positions presented in Table II that very good agreement was obtained between the theoretical and experimental data in the channels of both types under various experimental conditions. The average deviation of the experiment from the theory,
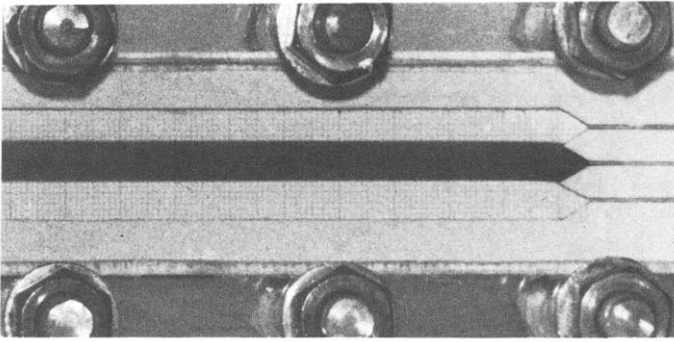
Fig. 5. Coloured layers of sucrose solutions of different density formed in a rectangular cross-section channel ($q = 1500\ \mu l/min$; $q_1 : q_2 : q_3 = 1 : 1 : 1$).

calculated from all the experimental and theoretical data, is 4.4% relative. Most of the experimental data show, within the given range, a negative deviation from the theoretically calculated values of boundary positions. The deviation, probably systematic, cannot be explained.

Further experiments were carried out to verify whether the experimentally found retentions lie within the range of the theoretically calculated values. This task was made difficult by two factors. The first complication, of experimental character, was due to the fact that the movement of density standard particles had to be evaluated visually directly in the channel. The use of the photometric detector was prevented by the sedimentation of the density standard particles (the average particle size was in the range 100–200 μm) in the detector cell, which resulted in its plugging.

Particle movement was thus evaluated in such a manner that the relative axial position in the direction of the flow of the most rapid particles focused at one of the boundaries was measured with respect to the axial position in the direction of the flow of the most rapid particles focused at the other boundary. The ratio of these
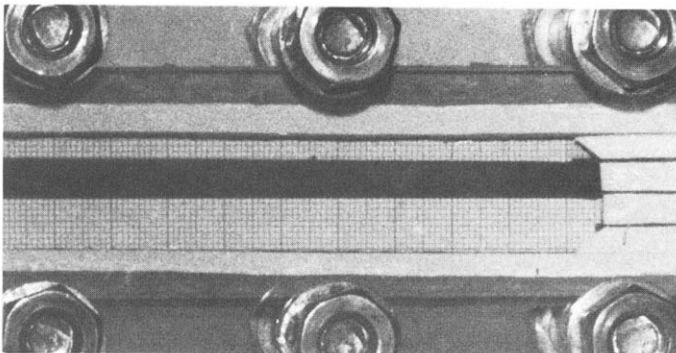

Fig. 6. Coloured layers of sucrose solutions of different density formed in a trapezoidal cross-section channel (flow-rates as in Fig. 5).

distances then specified the relative retention, $r_{1,2}$, defined for two solutes 1 and 2 by the relationship

$$r_{1,2} = R_1/R_2 \tag{15}$$

The second complication follows from the accepted simplification by which the velocity of the two streamlines adjoining the boundary changes stepwise. This transition will obviously be continuous. The particles of finite dimensions will reach the streamlines of various linear velocities. The real longitudinal velocity of the focused zones will therefore lie between the two extreme velocities at the boundary, as mentioned above. From the quantitative viewpoint, it is therefore expedient to establish whether the experimentally found relative retentions correspond to the relative retentions calculated theoretically from linear velocities lying in the middle of the interval at the boundary of the layers of various densities. The experimental and the theoretical results presented in Table III indicate the relative retentions of the both studied density standard particles. In agreement with theory, higher relative retentions were obtained by the fractionation in the trapezoidal cross-section channel than in the rectangular type. It is further obvious from the experimental values of the relative retentions that the relative velocity of the movement of the both focused zones is constant along the channel. Further experiments not shown in Table III also confirmed these findings. It will undoubtedly be possible to achieve a better agreement if analytical separations by SFFFF are performed in a classical arrangement, *i.e.*, with the detector at the end of the separation system, thereby permitting the exact determination of the retention volumes of individual focused zones.

It can be concluded that this study has demonstrated good agreement between the experimental results and the theoretical calculations, particularly with regard to the position of the boundary between the layer of the liquids of various densities

TABLE III

RELATIVE RETENTIONS OF TWO SAMPLES OF DENSITY STANDARDS (ρ_1 = 1.076 g/cm^3, ρ_2 = 1.120 g/cm^3) IN PERCOLL (q = 122 μl/min, 1.54 : 1 : 1, 22°C) FOUND EXPERIMENTALLY AND CALCULATED THEORETICALLY FOR CHANNELS OF RECTANGULAR AND TRAPEZOIDAL CROSS-SECTIONS

Channel	Focussed zone	Position of the focused zone front in axial direction (mm), and relative retention				
Rectangular	Upper	50	100	150	200	250
	Lower	48	93	140	187	231
	Relative retention	1.04	1.08	1.07	1.07	1.08
	Theoretical average relative retention			1.38		
Trapezoidal	Upper	50	100	150	200	250
	Lower	47	89	132	177	222
	Relative retention	1.06	1.12	1.14	1.13	1.13
	Theoretical average relative retention			1.63		

flowing through the channel in SFFFFF under conditions of the application of a step density gradient.

Subsequent studies wil be aimed at the applications of SFFFFF using a step density gradient to smaller particles and to particles of different characters.

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