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A Simple Device for Making Constant Density Gradients

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Summary

The possibility of adapting the well-known mixing device shown in Fig. 1 for production of density gradients with retained hydrostatic equilibrium is investigated. It is shown that a specific form of the unstirred container can be found that gives a strictly linear density course. Instead of specifically shaped containers, specific plungers can be used in cylindrical containers. All pertinent equations are given. The modified arrangement is useful for falling as well as for rising densities by use of exchangeable plungers.

The communicating duct between the containers has to have a large bore. With the aid of a V-shaped channel in a stopcock, turnable to a Λ orientation, hydrostatic stability can be retained under all circumstances.

In the experimental section it is shown that accurately constant density gradients are really obtained by using plungers constructed according to the requirements of the theory.

It is also shown that, under certain conditions, approximately constant density gradients can be obtained by the use of cylindrical containers with free cross-sectional areas proportional to the densities of the liquids in them.

A device according to Fig. 1 has been described by Parr (*1*) and is frequently used in chromatographic work for preparation of liquid gradients. If the density difference between the two liquids is negligible, and if the liquid flow is slow enough to allow hydrostatic equilibrium to prevail in each moment, then the concentration in the effluent obeys

$$c = c_0 + (c_i - c_0)(V/V_i)^{1/f} \tag{1}$$

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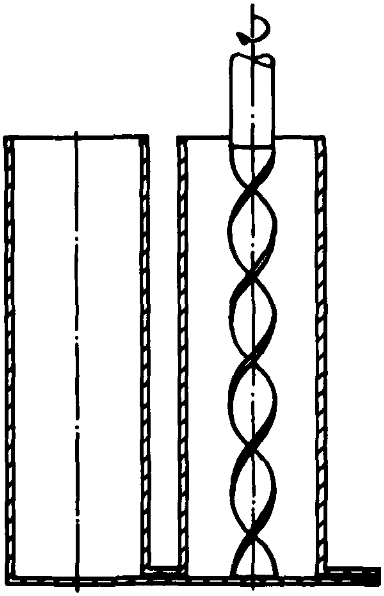


FIG. 1. Mixing device according to Parr (*1*).

where c_0 and c_i are the initial concentrations in the unstirred and stirred container, respectively; V_i the initial total volume; V the remaining volume; and f the cross-sectional area ratio between stirred and unstirred container. The c vs. V curve becomes convex upward if the bigger container has the higher concentration; in the opposite case it is convex downward. With two equal containers, the effluent concentration changes linearly with the volume; its concentration gradient is then constant.

For making density gradients, it is necessary for hydrostatic reasons to fill the two containers to different heights. Equation (1) is then, of course, no longer valid. Density gradients have become extremely important as a stabilizing principle in modern centrifugal and electrophoretic techniques, and constant density gradients are often desired. Ayad et al. (*2*) have recently shown that constant gradients can be achieved by forcibly guiding the effluent flow to a speed twice as large as that between the containers by the use of a peristaltic pump. The cross sections and the positions of the two containers are then arbitrary. In this article it will be shown that simple modifications in the device in Fig. 1 can be used for making constant density gradients without auxiliary machinery.

THEORETICAL

Basic Equations

Hydrostatic Balance. The hydrostatic law gives

$$x\rho_0 = y\rho \tag{2}$$

which can also be written in two alternative forms:

$$\rho_0(x - y) = y\,\Delta\rho \tag{3}$$

$$\rho(x - y) = x\,\Delta\rho \tag{4}$$

The differential form of (2) is

$$\rho_0\,dx = y\,d\rho + \rho\,dy \tag{5}$$

Volume Balance. The remaining volume is the sum of the volumes in each container, the duct between them being disregarded:

$$A\int_0^x dx/f + Ay = V \tag{6}$$

Volume changes on mixing are very small and will be disregarded here. The delivered volume will consequently be equal to the initial minus the remaining volume, $V_t - V$. After differentiation, Eq. (6) takes the form

$$A\,dx + Af\,dy = f\,dV \tag{7}$$

Mass Balance. The total mass in the mixer is

$$\rho_0 \int (A/f)\,dx + Ay\rho = m$$

Differentiation gives

$$\rho_0(A/f)\,dx + Ay\,d\rho + A\rho\,dy = dm$$

The change in mass is, however, identical with $\rho\,dV$, which gives the following mass balance equation:

$$A\rho_0\,dx + fAy\,d\rho + fA\rho\,dy = f\rho\,dV$$

With the aid of Eq. (7), dV and dy can be eliminated, and one gets

$$fy\,d\rho = \Delta\rho\,dx \tag{8}$$

Level Difference. If the differential equations (5), (7), and (8) are arranged in a matrix as follows:

$$\rho_0\, dx - \rho\, dy - y\, d\rho = 0 \qquad (5)$$

$$A\, dx + Af\, dy = f\, dV \qquad (7)$$

$$\Delta\rho\, dx - fy\, d\rho = 0 \qquad (8)$$

it is realized that they can be solved for *dx*, *dy*, and *dρ*, which are thus obtained in terms of the independent differential *dV*:

$$dx = \frac{f\rho\, dV}{A\rho_0(f+1)} \qquad (9)$$

$$dy = \frac{f\rho_0 - \Delta\rho}{A\rho_0(f+1)}\, dV \qquad (10)$$

$$d\rho = \frac{\rho\, \Delta\rho\, dV}{A\rho_0 y(f+1)} \qquad (11)$$

Subtraction of (10) from (9) gives the differential of the level difference:

$$d(x-y) = \frac{\Delta\rho}{A\rho_0}\, dV \qquad (12)$$

Whereas Eqs. (9)–(11) contain four variables each, Eq. (12) has only three variables: $(x - y)$, $\Delta\rho$, and V. Moreover, if $\Delta\rho$ is specified as a certain desired function of V, the number of variables reduces to two, and the equation can be integrated to yield the level difference as a function of V.

Shape of the Unstirred Container that Gives a Constant Density Gradient

Conditions for a Constant Density Gradient. When a total density difference of $(\Delta\rho)_t$ and a total volume of V_t are available, then a constant density gradient must have the value

$$\frac{d\rho}{dV} = \frac{(\Delta\rho)_t}{V_t} \qquad \frac{d\rho}{dv} = (\Delta\rho)_t \qquad (13)$$

which is easily integrated to give

$$\rho = \rho_0 + (\Delta\rho)_t v \qquad (14)$$

$$\Delta\rho = (\Delta\rho)_t v \qquad (15)$$

Level Difference. The density difference from (15) can now be inserted into (12), which gives

$$d(x-y)=\frac{V_i(\Delta\rho)_i}{A\rho_0}\,v\,dv \tag{16}$$

Integrations yields

$$x-y=\frac{V_i(\Delta\rho)_i}{2A\rho_0}\,v^2 \tag{17}$$

The level difference is consequently a parabolic function of the remaining volume in the mixer.

Individual Heights of the Liquid Columns. After having found the explicit solution of one variable in terms of the independent variable v, it is easy to obtain all other variables as functions of v. Thus it is now possible to introduce $(x-y)$ from (17) and $\Delta\rho$ from (15) into Eq. (3) in order to get y as a function of v:

$$y=\frac{V_i v}{2A}=\frac{V}{2A} \tag{18}$$

Since Ay is the volume of the liquid in this container, it follows that this volume is always half the remaining volume. During the whole operation, consequently, the two containers keep exactly the same volume of liquid. Differentiation of (18) gives

$$A\,dy=dV/2 \tag{19}$$

which means that the stirred container always delivers exactly half of the effluent volume. This is the principle upon which Ayad et al. (2) based their device and could be regarded as self-evident for constant gradients. This theory could thus have been started with Eq. (18) in conjunction with Eqs. (2) and (7) and could so have been a lot simpler. However, the general theory presented here can be extended to any kind of density gradient by inserting nonlinear density courses into Eq. (12).

To find the height of liquid in the unstirred container, one may add Eqs. (17) and (18), with the result

$$x=\frac{V_i}{2A\rho_0}\,[\rho_0 v+(\Delta\rho)_i v^2] \tag{20}$$

Differentiation gives

$$\frac{dx}{dv}=\frac{V_i}{2A\rho_0}\,[\rho_0+2(\Delta\rho)_i v] \tag{21}$$

Cross-Sectional Area of the Unstirred Container. The parameter f may be solved from Eq. (8) to give

$$f = \frac{\Delta\rho\, dx}{y\, d\rho} = \frac{\Delta\rho(dx/dv)}{y(d\rho/dv)} \tag{22}$$

The two derivatives are taken from Eqs. (13) and (21). Using in addition Eqs. (15) and (18), one finds for f

$$\rho_0(f-1) = 2(\Delta\rho)_i v \tag{23}$$

Solved for f, it can be brought into the form

$$f = 1 - 2(1 - s_i)v \tag{24}$$

The meniscus area A/f is thus a hyperbolic function of the remaining volume.

The surface area parameter f is by definition a positive quantity; negative values or zero cannot be allowed. This leads to the following inequality:

$$1 - 2(1 - s_i)v > 0 \tag{25}$$

that has to be satisfied for all values of v. For $v = 1$, one consequently gets the restriction

$$s_i > \tfrac{1}{2} \tag{26}$$

which may be expressed in words by saying that the unstirred liquid cannot be allowed to be twice as dense as the initial stirred liquid.

The value of f at the initial level x_i is obtained by putting $v = 1$ in Eq. (24):

$$f_i = f(x_i) = 2s_i - 1 \tag{27}$$

The free meniscus area in the unstirred container is thus positive and finite subject to condition (26), but for $s_i = \frac{1}{2}$, it rises to infinity.

The unstirred container cannot be constructed on the basis of Eqs. (23) and (24). To make it, one has to know f as a function of x. Consequently what remains to be done is to eliminate v between Eqs. (20) and (23), which gives

$$x = \frac{V_i(f^2-1)}{8A(s_i-1)} = \frac{V_i\rho_0(f^2-1)}{8A(\Delta\rho)_i} \tag{28}$$

With the substitution

$$a = \frac{V_i \rho_0}{8A(\Delta\rho)_i} \tag{29}$$

where a is a distance that can be calculated from given experimental conditions, Eq. (28) can be solved for f to give

$$f = \sqrt{1 + x/a} \tag{30}$$

This equation makes it possible to calculate various shapes of and to construct containers for the unstirred liquid which are to be used in the device in Fig. 1 in order to get constant density gradients. It should be noticed that $f = 1$ for $x = 0$; that is, the two containers must have the same free base area. It should further be noted that a changes sign with $(\Delta\rho)_i$. Consequently a is a positive distance for falling and a negative distance for rising effluent densities. A falling density is thus connected with an f function rising from $f = 1$ at the bottom of the container; for a rising density f decreases from a value $f = 1$ at the bottom.

The distance a can be given various forms other than Eq. (29). With use of s_i, the initial density ratio between stirred and unstirred liquid, a can be written

$$a = \frac{V_i}{8A(s_i - 1)} \tag{31}$$

Since $V_i = 2Ay_i$ by virtue of Eq. (18), one also has

$$a = \frac{y_i}{4(s_i - 1)} \tag{32}$$

which is the simplest possible expression for a. According to this equation, a is identical with the initial height y_i for $s_i = 1.25$ and with $-y_i$ for $s_i = 0.75$. Elimination of y_i in favor of x_i by way of Eq. (2) for initial conditions, leads to

$$a = \frac{x_i}{4s_i(s_i - 1)} \tag{33}$$

The function $a(s_i)$ is depicted in Fig. 2. In this graph the field to the right of $s_i = 1$ represents decreasing densities and the field between $s_i = 1$ and $s_i = 0$ represents increasing densities, whereas the field to the left of $s_i = 0$ lacks physical meaning since all densities and density ratios are positive.

On passing from large values of s_i (in experimental practice at ordinary temperatures and pressures, s_i cannot possibly exceed 5)

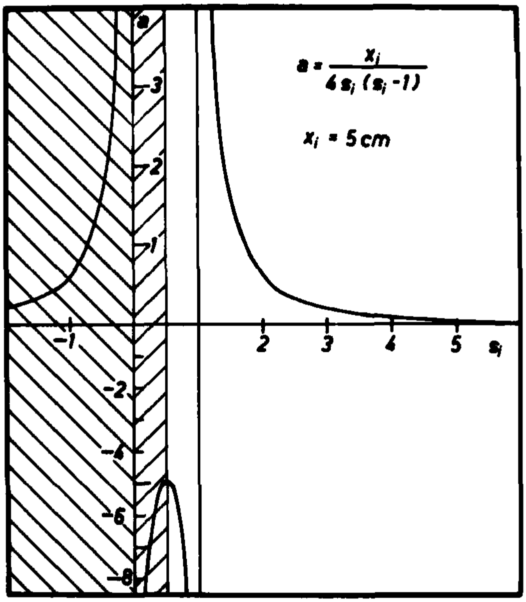


FIG. 2. The distance a defined by Eq. (29) as a function of the density ratio s_i for $x_i = 5$ cm [Eq. (33)]. The field to the left of $s_i = 0$ lacks physical meaning. The field between $s_i = 0$ and $s_i = \frac{1}{2}$ is forbidden according to condition (26).

down to $s_i = 1$, the graph shows that a increases from rather small values to the positive infinity for $s_i = 1$, that is, equal densities. At this point a shifts from positive to negative infinity, and as s_i decreases from unity, the negative a rises to a maximum: $-x_i$ for $s_i = \frac{1}{2}$. The field between 0 and $-x_i$ is inaccessible to a, which is also evident from Eq. (30). The field to the left of $s_i = \frac{1}{2}$ is not allowed in view of restriction (26). If the latter were overlooked, Fig. 2 would teach that every negative a value, that is, any specific container made for a rising density, would suit two different density ratios equally well, which is incomprehensible.

Versatility. Equation (30) together with the definition of f specifies the shape of the container without stirrer in two respects: the area at the base and the way in which it increases or decreases with height. Other properties, such as total volume (total height) and form of horizontal sections, are open to free choice. If rectangular cross sections are chosen, one side of the rectangle may be constant and the other variable as $1/f$; alternatively, both sides can be made to vary as $1/f^{1/2}$. A certain preference should be given to circular cross sections because of the possibility of turning; the radius then has to vary as $1/f^{1/2}$.

The shape of the function $f(x)$ is exclusively determined by the value of the distance a, which is connected with the total volume, the base area, the initial heights of the menisci, and the two initial densities as described by Eqs. (29), (31), (32), and (33). On consideration of the fact that volumes between 5 and 1000 ml may be of interest in chemical laboratories in connection with density gradients, and that liquid densities ranging from 0.692 (isooctane) to 3.325 g/ml (methylene iodide) are available at room temperature, it appears necessary to have access to a two-dimensional multitude of specific containers for various volumes and various a values in order to comply with all possible requirements.

This complexity is, however, grossly exaggerated. Only comparatively small density intervals have frequent application in experimental practice. Thus in general biochemists have only use of the density range between 1.00 and 1.20 g/ml, whereas earth scientists, wherever on the density scale they are operating, have to use density intervals of the same order of magnitude in order to obtain sufficiently accurate density values of particles or in order to achieve satisfactory separations of such particles. If density gradients comprising greater intervals are occasionally desired, they can be prepared in a nonlinear fashion by use of the unmodified arrangement in Fig. 1, as will be described later in this article. Consequently the two-dimensional multitude of containers first anticipated reduces to two one-dimensional multitudes adapted to various volumes but to only two a values, one positive for falling and one negative for rising densities. People who can standardize their work to one volume would thus be satisfied with only two containers of specifically programmed shape.

It should be noted that only the density ratio enters into Eqs. (31)–(33). One and the same specific container can thus be used for an unlimited number of different density intervals characterized by a constant density ratio. For instance, in powder analyses with the aid of isooctane as the least dense and methylene iodide as the densest liquid available, constant density gradients within density intervals such as 0.700–0.875, 0.800–1.000, 0.900–1.125, 1.000–1.250, etc., can be produced by using the same specific container with $a = y_i$ since the density ratio over all intervals is 1.25. All these gradients will also be confined to the same volume and can thus be fed into a series of equal columns.

Equation (29) further shows that a setup designed for certain a and A values will still function if V_i and $(\Delta\rho)_i$ are changed in direct proportion and if V_i and ρ_0 are changed in reciprocal proportion.

Numerical Calculation. For a decreasing density the distance a is positive, and the numerical calculation of f is then much facilitated by introduction of an intermediary variable α defined by

$$x = a \tan^2 \alpha \tag{34}$$

which leads to the relation

$$f = \sec \alpha \tag{35}$$

For an increasing density, the distance a is negative, and the intermediary variable β, defined by

$$x = -a \sin^2 \beta \tag{36}$$

is more suitable since it gives the relation

$$f = \cos \beta \tag{37}$$

Use of Plungers

In practice it is not feasible to make containers with specifically programmed cross sections unless large-scale manufacture is considered. It is much more practicable to use cylindrical containers and to have a stirrer with a constant cross-sectional area in one container and a plunger with a variable cross section in the other. The cross-sectional area $P(x)$ of the plunger then has to satisfy the equation

$$C - P(x) = A/f(x) \tag{38}$$

where C is the constant internal cross-sectional area of the unstirred cylinder. The stirred cylinder may, but need not, have the same internal area. The only thing that matters is the free base area, which must be the same in the two cylinders.

A plunger for a falling density thickens; a plunger for a rising density narrows off from the bottom upward. If they are to be used alternatively in the same setup, they must have the same base area, given by Eq. (38) with $f(0) = 1$.

A plunger for a rising density disappears at a certain level. The f value for which this occurs is obtained by putting $P = 0$ in Eq. (38). Since the arrangement can be used up to, but not above, this level, it may be identified with the initial level x_i. In this case one obtains

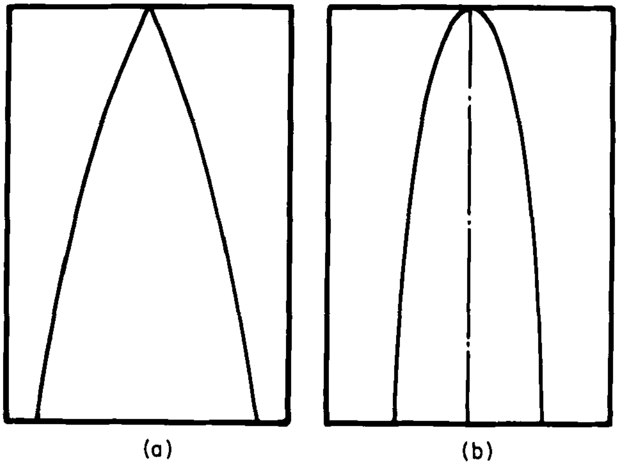
$$f(x_i) = A/C \tag{39}$$

Comparison with Eq. (27) then leads to

$$A = C(2s_i - 1) \tag{40}$$

The size of the unstirred cylinder is thus not arbitrary if one wants to use the apex of the plunger as an index for the proper volume to be used. This is not necessary, however. One can use a larger cylinder and a larger plunger and add a measured volume of liquid not reaching the apex of the plunger.

A plunger can be made either with a rectangular or, by turning, with a circular cross section. In the former case one dimension of the rectangle may be constant and the other proportional to $(C - A/f)$ in order to satisfy Eq. (38). Such a plunger is wedge-shaped and is shown in Fig. 3(a). A turned plunger with the radius $b(x)$ must, of course, obey the equation $\pi b^2 = P$. Such a plunger is shown in Fig. 3(b).



(a) (b)

FIG. 3. Plungers for rising densities, to be inserted into the unstirred container. (a) Plunger with rectangular cross section, dimension perpendicular to the paper constant; (b) turned plunger.

As a check on the calculation, one may derive the volume in the unstirred container by integration from the bottom to the apex of the plunger, making use of Eqs. (39) and (40). For the integration, one needs the differentiated form of Eq. (30):

$$2af\,df = dx \tag{41}$$

The integration then proceeds as follows:

$$\begin{aligned}\int_0^{x_i} (A/f)\,dx &= A\int_0^{x_i} dx/f = 2aA\int_1^{A/C} df = 2aA(A/C-1)\\ &= 2aA(2s_i-1-1) = 4aA(s_i-1) = 4A(s_i-1)\frac{y_i}{4(s_i-1)}\\ &= Ay_i = V_i/2\end{aligned}$$

The integrated volume comes out as half the initial volume, as it should.

Density Gradients Obtainable by Mixers with Constant Cross Sections

In many applications of density gradients, small deviations from a linear density course may be tolerated. It is therefore of great interest to know how large are the deviations from linearity that result when both containers have constant cross-sectional areas. Mathematically, this corresponds to treating the parameter f as a constant, whereas Eqs. (13)–(15) and all equations derived from them do not apply. The goal will now be to calculate the density course and its gradient.

Elimination of the density differential between Eqs. (5) and (8) gives

$$(f\rho_0 - \Delta\rho)\,dx = f\rho\,dy \tag{42}$$

If ρ and $\Delta\rho$ are taken from Eqs. (2) and (3), one gets a differential equation containing only x and y as variables:

$$\frac{dy}{dx} - \frac{f+1}{fx}\,y = -1 \tag{43}$$

It is readily solvable by standard methods with the aid of the integration factor $x^{-(f+1)/f}$. The solution is found to be

$$\frac{x-y}{x} = \frac{(\Delta\rho)_i}{\rho_i}\,(x/x_i)^{1/f} \tag{44}$$

With the aid of Eq. (4), this gives the relation:

$$\frac{\Delta\rho}{\rho} = \frac{(\Delta\rho)_i}{\rho_i}\,(x/x_i)^{1/f} \tag{45}$$

This equation describes the density course explicitly as a function of x, the height of liquid in the unstirred container; x_i is, as before, the initial height.

Equal Cross Sections, Unequal Volumes. When the two cross-sectional areas are equal, f becomes unity, and the volume of the less dense liquid necessarily becomes larger than that of the denser liquid. The resulting density course must then run entirely below the rectilinear course from ρ_i to ρ_0.

To proceed further from Eq. (45), expressions for x and x_i in terms of V and V_i are needed. For $f = 1$, Eq. (6) reads

$$Ax + Ay = V \tag{46}$$

and y can be eliminated by use of Eq. (2). One obtains

$$x = \frac{V\rho}{A(\rho + \rho_0)} \tag{47}$$

and thus

$$x_i = \frac{V_i\rho_i}{A(\rho_i + \rho_0)} \tag{47a}$$

Insertion of these equations into Eq. (45) with $f = 1$ gives an equation with only ρ and V as variables. After some manipulation, this equation can be brought into the form

$$r = \sqrt{1 - (1 - r_i^2)v} \tag{48}$$

Differentiation gives

$$\frac{dr}{dv} = \frac{r_i^2 - 1}{2r} \tag{49}$$

$$\frac{ds}{dv} = \frac{1 - r_i^2}{2r^3} = \frac{s^3(s_i^2 - 1)}{2s_i^2} \tag{49a}$$

In the frequently occurring practice of using sucrose for the production of density gradients in water solutions, ρ_i may be chosen $= 1.2$ g/cm³, whereas ρ_0 is close to 1 g/cm³ (decreasing density). This leads to the following density course:

$$\rho = (1 - 0.3056v)^{-1/2} \tag{50}$$

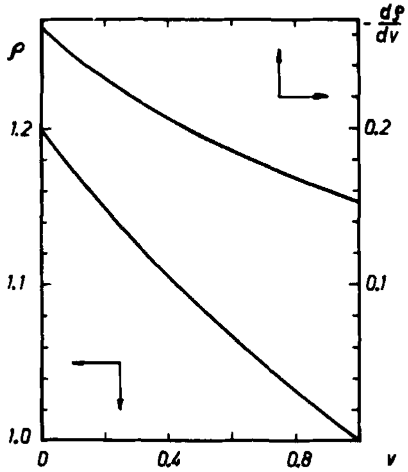


FIG. 4. Effluent density and its gradient obtained with the unmodified mixer in Fig. 1 with equal free meniscus areas and unequal initial volumes.

and to the density gradient:

$$\frac{d\rho}{dv} = 0.1528\rho^3 \tag{51}$$

Functions (50) and (51) are illustrated in Fig. 4.

Unequal Cross Sections, Equal Volumes. If the two containers have cross-sectional areas proportional to the densities of the initial liquids in them, these liquids will get the same volume at hydrostatic equilibrium. In practice this is achieved by using equal cylindrical containers in combination with suitable plungers, one for each density ratio. Quantitatively, the relations valid for this kind of mixer are given by

$$fy_i = x_i \tag{52}$$

$$f\rho_0 = \rho_i \tag{53}$$

$$fV_i = 2Afy_i = 2Ax_i \tag{54}$$

$$f = s_i = 1/r_i \tag{55}$$

Equation (45) will be used again, and expressions for x and x_i in terms of v are required. Equation (6) now takes the form

$$Ax + Afy = fV \tag{56}$$

and y can be eliminated with the aid of Eq. (2), with the result

$$x = \frac{f\rho V}{A(\rho + f\rho_0)} \tag{56a}$$

and thus

$$x_i = \frac{f\rho_i V_i}{A(\rho_i + f\rho_0)} \tag{56b}$$

Division gives

$$\frac{x}{x_i} = \frac{\rho(\rho_i + f\rho_0)}{\rho_i(\rho + f\rho_0)}\, v \tag{57}$$

If this is inserted into Eq. (45), the latter can be solved for v:

$$v = \frac{\rho_i(\rho + f\rho_0)}{\rho(\rho_i + f\rho_0)} \left[\frac{\rho_i \Delta\rho}{\rho(\Delta\rho)_i}\right]^f \tag{58}$$

With the aid of Eqs. (53) and (55), this equation can be much simplified in a variety of ways. The neatest way of presenting the solution seems to be

$$v = \frac{r + r_i}{2r_i} \left[\frac{1 - r}{1 - r_i}\right]^f \tag{59}$$

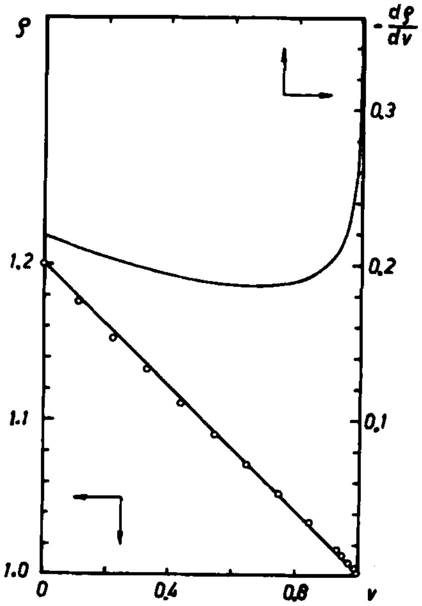


FIG. 5. Effluent density and its gradient obtained with the unmodified mixer in Fig. 1 with unequal free meniscus areas and equal initial volumes.

Differentiation gives

$$\frac{ds}{dv}=\frac{2r_i^2(1-r_i)^f}{(1+r_i)\ r^3(1-r)^{f-1}} \tag{60}$$

For the specific case of a sucrose density gradient running from 1.2 to 1.0 g/cm^3, the last two equations assume the forms

$$v=0.8586\ (6r+5)(1-r)^{1.2} \tag{61}$$

$$\frac{ds}{dv}=\frac{0.08823}{r^3(1-r)^{0.2}} \tag{62}$$

Functions (61) and (62) are illustrated in Fig. 5.

EXPERIMENTAL

Apparatus

The mixer shown in Fig. 6 comprises two cylindrical containers (a and b), one with a stirrer (c) and exit tube (d) and the other with a plunger (e) thickening upward for falling densities or a plunger (f) narrowing upward for rising densities. The cylinders are mounted on a base plate (g) on top of a chassis (h) containing a synchronous motor (i) running at a speed of 375 rpm and driving the stirrer by way of a jaw clutch and an O-ring-sealed slide bearing (j). The cylinders communicate through a stopcock (k) with a V-shaped duct (l).

Cylinders, plungers, and baseplate were made of Perspex and the stopcock of Teflon. The plungers were calculated for *a* values of 9.82 and −11.69 cm for falling and rising densities, respectively, corresponding to a total volume of 110 cm^3, a base area of 7.386 cm^2, and densities of 1.1874 and 0.9982 g/cm^3. They were turned on the lathe with a tolerance of ±0.1 mm. The construction of the stirrer as a screw with a large pitch secures a constant cross-sectional area.

Whereas the bore of the exit tube is of capillary dimension, that of the communicating duct is much larger. This is necessary in order to secure a sufficiently low resistance to flow even when the duct is filled with the highly viscous, concentrated sucrose solution, since the theory assumes hydrostatic equilibrium to be reached instantaneously. We have chosen a bore of 3 mm for this duct, which in addition is rather short. A large duct cannot be allowed to run horizontally between the containers because it would result

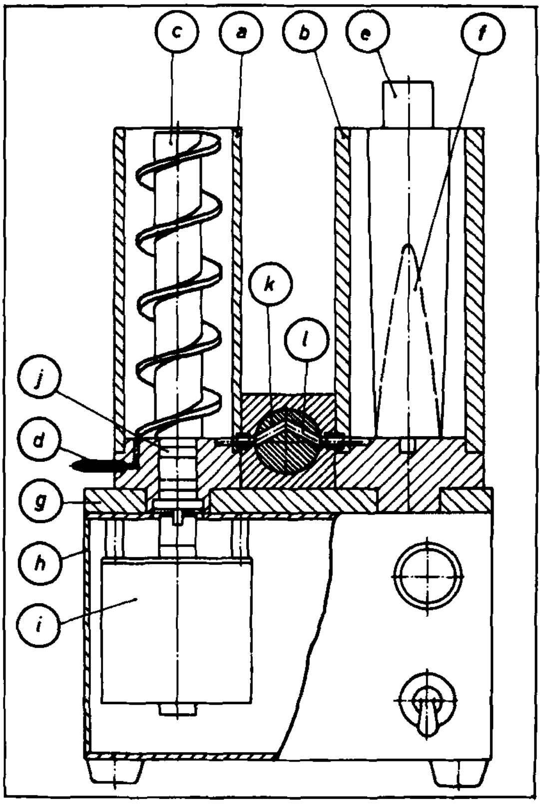


FIG. 6. Design of modified mixer with cylindrical containers and exchangeable plungers.

in an appreciable exchange of solutions between them. The denser solution would flow along the bottom and the less dense one along the ceiling of the duct until a new equilibrium was established. This undesired exchange of liquid is prevented by the V-shaped duct in the stopcock, being kept in the V position for increasing and in the Λ position for decreasing densities. Hydrostatic stability then prevails under all circumstances.

Experimental Arrangement and Procedure

The dense solution contained 500 g of sucrose (Mallinckrodt, St. Louis, Miss., analytical reagent grade), 2.002 mmoles of potassium chromate (pro analysi), and 1.030 mmoles of alkali per liter, whereas the less dense solution consisted of pure water.

The exit tube of the mixer was connected to the inlet of a UV light absorptiometer, model Uvicord 4701 A (LKB-Produkter AB, Stockholm-Bromma, Sweden), and its exit tube was attached to the suction side of a low-speed peristaltic pump driven by a synchronous motor. The photoelectric signal from the absorptiometer was fed to a potentiometric recorder, model Moseley Autograph 7100 A (F. L. Moseley Co., Pasadena, Calif.). The transmittance values recorded by the Uvicord were recalculated to absorbance, which was used as a measure of the concentration of solutes (essentially chromate, but the high concentration of sucrose also contributed to the absorbance). Since the chromate and sucrose concentrations were necessarily mutually proportional in the effluent, and since the density increment is proportional to the sucrose concentration, the density can in principle be measured by way of the absorbance. The alkali was added in order to remove any possibility of partial conversion of chromate to biochromate, but its presence involves an obvious risk of slow hydrolysis followed by reduction of the chromate. Storage of a sucrose–chromate solution during 30 days resulted in a 4% decrease in the absorbance. This slow change cannot affect our experiments, completed in 1 or 2 days.

The experiments to be reported here were done in order to test the linearity of density courses given by the mixer. A comparatively low pumping speed was therefore used: 38.6 ml/hr. An experiment thus lasted for 2 hr and 51 min. Because of the constant rate of flow, the time axis of the recorder could be recalculated to volume of effluent liquid, $V_t - V$, or to remaining volume, V.

Preparation for Rising Densities. With the plunger narrowing off upward in place in the unstirred cylinder and with the stopcock in the V position, about 5 ml of the dense solution was poured into the mixer. It was then allowed to drain off by keeping the exit tube (d) open. The stopcock, still filled with dense solution, was then closed (position < or >). The dense solution remaining in the unstirred cylinder by the action of capillary forces was not removed, whereas the portion left in the stirred cylinder was rinsed away by repeated washings with less dense solution (distilled water in our experiments). After having completed the capillary tube connections between mixer, Uvicord cell, and pump, at least 5 ml of the less dense solution was introduced into the stirred cylinder. By starting pump and recorder, the capillary tubes became filled with less dense solution, and the recorder started to register its constant light transmittance. The machinery was stopped exactly at the moment where the exit tube (d) was about to take in air. The small quantity of solution kept by capillary forces on the bottom of the cylinder was not removed.

Fifty-five milliliters of dense solution was now introduced into the unstirred cylinder, and the same volume of less dense solution was poured into the stirred one. On opening the stopcock to the V position, the two liquid columns were found to be in hydrostatic equilibrium and kept their levels unchanged. The whole machinery with stirrer, pump, and recorder was finally started and was allowed to continue until the exit tube took in air.

Preparation for Falling Densities. When a decreasing density was desired, the two solutions were exchanged in every step of the above procedural description. In addition, the other type of plunger, thickening upward, was applied in the unstirred cylinder, and the stopcock, prefilled with less dense solution, was turned to the Λ position during operation (but to the V position during filling).

Analysis of the Linearity of the Density Course

Experiments conducted as described above revealed a definitely nonlinear course of the absorbance. We then carried out extinction measurements in a Beckman spectrophotometer on a dilution series of our sucrose–chromate solution at 253.7 nm, the wavelength of the resonance line of the mercury lamp in the Uvicord. The solution was found to obey Lambert-Beer's law accurately and thus cannot be blamed for the failure of getting a constant gradient. If the mixer really delivers linear concentration courses, the nonlinear behavior of the absorbance must evidently be due to some systematic error in the recording system.

The Uvicord was then calibrated with the same dilution series of the dense sucrose–chromate solution. Due to its high viscosity, pipets were not trusted to give a good enough accuracy in the dilutions. Consequently the desired volumes of the dense solution were recalculated to weights by use of its known density, and the dilutions were made with the aid of an analytical balance and volumetric flasks. Each dilution was introduced into the Uvicord cell, and the recorder was allowed to draw a line for its transmission, which was subsequently recalculated to absorbance. This calibration gave the primary data presented in Table 1, the first two

TABLE 1

Calibration of the Uvicord with a Dilution Series of the Stock Solution of Sucrose and Potassium Chromate

	E			
c	Obs.	Calc.	$\frac{1000\Delta E}{0.8459}$	Difference
0.0000	0.0009	0.0012	−0.4	0.0528
		0.0540		0.0516
0.2002	0.1073	0.1056	2.0	0.0504
		0.1560		0.0491
0.4004	0.2041	0.2051	−1.2	0.0480
		0.2531		0.0469
0.6006	0.2984	0.3000	−1.9	0.0457
		0.3457		0.0446
0.8008	0.3893	0.3903	−1.2	0.0436
		0.4339		0.0424
1.0010	0.4776	0.4763	1.5	0.0414
		0.5177		0.0404
1.2012	0.5607	0.5581	3.1	0.0394
		0.5975		0.0383
1.4014	0.6364	0.6358	0.7	0.0373
		0.6731		0.0364
1.6016	0.7077	0.7095	−2.1	0.0355
		0.7450		0.0345
1.8018	0.7773	0.7795	−2.6	0.0336
		0.8131		0.0328
2.0020	0.8477	0.8459	2.1	

columns. It is easily seen that the extinction E is a nonlinear function of the concentration.

Since the concentrations in the dilution series formed an arithmetic series, the abbreviated least-squares treatment described by Svensson (3) could be applied in order to adapt a third-order polynomial to the E vs. c data. The best-fitting polynomial was found to be

$$E = 0.001244 + 0.533536c - 0.062001c^2 + 0.003116c^3 \qquad (63)$$

The E values calculated from this polynomial are given in the third column. The deviations between observed and calculated extinctions in relation to the maximum extinction are given in the fourth column. The mean deviation amounts to 0.2%. The fifth column contains differences facilitating calculation of concentrations from observed extinctions by linear interpolation.

The nonlinear relationsship between observed extinction and concentration, as described by Eq. (63), is due to the insufficient monochromaticity of the Uvicord lamp. According to the manual of the Uvicord, the mercury lines at 312.6–313.2 nm have an intensity of 8–10% of that of the resonance line at 253.7 nm. In addition, the lamp emits light at 296.7 and 334.1 nm with intensities of 2 and 1%, respectively, of the resonance line intensity. Unfortunately, chromate has an absorption minimum close to the ghost lines at 313 nm, which enhances the deviations from Lambert-Beer's law. Kortüm (*4*) has given a formula for correcting extinctions obtained in insufficiently monochromatic light. Insertion of available data for the emission spectrum of the lamp and for the absorption spectrum of chromate into this formula reveals that the deviations in question can be fully accounted for by the presence of the ghost lines at 313 nm. For concentration measurements by way of absorbance, however, direct calibration as shown in Table 1 is much to be preferred. According to Wegstedt (5), the ghost lines can be eliminated by use of an extra filter containing CS_2 in ethanol. Our instrument lacked this facility.

In a test run with a rising density, the primary data given in the first two columns of Table 2 were obtained. The observed extinction values in the second column were converted to concentrations by linear interpolation in Table 1, and these concentrations are presented in the third column of Table 2. They should be a linear function of the effluent volume. To test that, a least-squares treatment was applied to the data in the first and third columns. The best-fitting straight line was found to be

$$c = -0.002315 + 0.018382(V_i - V) \qquad (64)$$

The concentrations calculated from this equation are given in the fourth column of Table 2, and the relative deviations between observed and calculated concentrations are given in the last column. Except the last reading, which was excluded from the least-squares treatment, the deviations are smaller than 0.6%, the mean deviation being 0.34%.

Before a similar test run with a decreasing density could be done, another stock solution had to be prepared, the Uvicord lamp had to be exchanged, and the peristaltic pump had to be fitted with a new rubber hose. It was then necessary to make a new calibration with a dilution series of the sucrose–chromate solution as described before. This gave

$$E = -0.004084 + 0.555505c - 0.066611c^2 + 0.003116c^3 \qquad (65)$$

with a mean deviation of 0.37%. The test run with a falling density gave the primary data given in the first two columns in Table 3. The concentrations corresponding to the observed extinction values were calculated by using Eq. (65) and have been introduced into the third column. They should be rectilinear with the effluent volume. To test that, the following straight line was calculated by a least-squares treatment of the data in the first and third columns:

$$c = 2.024831 - 0.017793(V_i - V) \tag{66}$$

Concentrations calculated from this equation are given in the fourth column, and the relative deviations between observed and thus calculated concentrations are given in the last column. In this case the last two readings, which were excluded from the treatment by least squares, show a 1% deviation, whereas the rest of the concentration course shows a mean deviation from linearity of only 0.17%.

TABLE 2

Linearity Analysis on a Rising Density Course

		c		
$V_i - V$	E	Interpol.	Linear	$\frac{1000\Delta c}{2.0197}$
0.00	0.0000	−0.0022	−0.0023	0.1
4.42	0.0458	0.0845	0.0789	2.8
11.77	0.1107	0.2103	0.2140	−1.7
19.13	0.1772	0.3434	0.3493	−2.9
26.49	0.2418	0.4767	0.4846	−3.9
33.85	0.3063	0.6144	0.6199	−2.7
41.20	0.3696	0.7541	0.7550	−0.5
48.56	0.4306	0.8932	0.8903	1.4
55.92	0.4881	1.0293	1.0256	1.8
63.28	0.5452	1.1691	1.1609	4.1
70.63	0.6003	1.3086	1.2960	6.2
77.99	0.6498	1.4388	1.4313	3.7
85.35	0.6968	1.5663	1.5666	−0.1
92.71	0.7471	1.7075	1.7042	1.6
100.07	0.7878	1.8262	1.8372	−5.4
107.42	0.8327	1.9614	1.9723	−5.4
110.00	0.8416	1.9885	2.0197	−15.4

TABLE 3

Linearity Analysis on a Falling Density Course

$V_i - V$	E	c		$\frac{1000\Delta c}{2.0092}$
		Interpol.	Linear	
0.88	0.8665	2.0032	2.0092	−3.0
9.68	0.8153	1.8502	1.8526	−1.2
18.48	0.7620	1.6970	1.6960	0.5
27.28	0.7055	1.5422	1.5394	1.4
36.08	0.6459	1.3854	1.3829	1.2
44.88	0.5834	1.2282	1.2263	0.9
53.68	0.5186	1.0720	1.0697	1.1
62.48	0.4522	0.9183	0.9131	2.6
71.28	0.3799	0.7581	0.7565	0.8
80.08	0.3054	0.5990	0.6000	−0.5
88.88	0.2269	0.4386	0.4434	−2.6
97.68	0.1481	0.2837	0.2868	−1.5
106.48	0.0545	0.1068	0.1302	−11.6
110.00	0.0218	0.0471	0.0676	−10.1

DISCUSSION

This investigation was initiated after discussions between the senior author and Dr. O. Vesterberg, Department of Bacteriology, the Karolinska Institute, Stockholm. In his exploration of the isoelectric focusing method for proteins in density gradients (6), he had, by a skillful combination of qualitative arguments and experiments, realized that density gradients obtained from a device according to Fig. 1 could be much improved by using some sort of plunger in the less dense solution. He reported considerable advance with a plunger essentially conical in shape. The senior author became interested in the problem and decided to deduce the theoretical shape which a plunger should have in order to give a constant density gradient. For a turned plunger, it turned out that the radius has to be a fourth-root function of the height.

The relatively time-consuming turning of plungers with specifically programmed radii raises the question of the magnitude of the deviations from linearity that are to be expected when both containers have constant cross-sectional areas, that is, when no plungers or cylindrical plungers are used in cylindrical containers. At hydrostatic equilibrium equal cross sections give rise to a larger volume of the less dense solution and comparatively severe deviations from linearity, as shown by Fig. 4. The density gradient obtained varies as the cube of the density.

On the other hand, unequal cross sections chosen so as to give equal initial volumes at hydrostatic equilibrium give rise to much more constant density gradients, as shown by Fig. 5. As a matter of fact, the deviation from a straight line is everywhere smaller than 1.6% of the total density difference, although the density gradient goes to infinity toward the end of the flow due to the presence of the factor $(1 - r)^{-0.2}$ in Eq. (62). The numerical data underlying Fig. 5 refer to sucrose solutions in water, density difference 0.2 g/cm^3. For such solutions the use of equal volumes in unequal cylinders can be regarded as a practical possibility for making almost constant density gradients. When greater density intervals are involved, the arrangement gives density courses that are far from linear.

Experimental tests of the theory have been limited to plungers of the theoretical shape for constant gradients. Disregarding the 1–1.5% deviations that have been encountered at the end of the flow, the density courses obtained have been found to be so accurately linear that graphs would completely fail to show any deviations at all. For that reason the deviations from linearity have been given in the last columns of Tables 2 and 3 as parts in 1000 of the maximum concentrations. The mean deviations amount to 0.2–0.3% and fall entirely within the experimental uncertainty.

Surface tension effects have not been included in the theory. The latter can therefore be expected to lose in accuracy and applicability when such effects become important. We believe this to be the case toward the end of the flow when the menisci begin to touch the bottom of the respective containers. This is probably the reason for the comparatively large deviations from linearity at the end of the flow.

Increased surface tension effects are also to be expected in mixers for very small volumes, e.g., for density gradient runs in small centrifuge tubes. The use of plungers must probably be abandoned in mixers for small volumes. One will then have to make containers of the proper shape or to use unequal cylinders with equal initial volumes, giving approximately constant density gradients.

There is in general an optimum speed for the stirrer, too low a speed causing insufficient mixing and too high a speed giving rise to a paraboloid deformation of the liquid surface. We have handled this problem in an entirely empirical way. The relatively thick stirrer shown in Fig. 6 and used because of its compatibility with both types of plungers can be run at a rather high speed without causing any difficulties due to swirling.

Salo and Kouns (7) have described simple equipment for making constant density gradients in small volumes (5 ml) of liquid. They paid considerable attention to the design of the intercompartment duct and preferred to operate with the less dense solution in the stirred container (rising density), whereby complete hydrostatic equilibrium within the duct was achieved. Much less interest was devoted to the hydrostatic balance between chambers, but they seem to have attained initial hydrostatic equilibrium more or less fortuitously, by the volume occupied by the stirrer and by the added volume used to compensate for the volume of the exit tube. In this way their procedure becomes closely related to our suggestion of using equal volumes of liquid in unequal cylinders.

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List of Symbols

A	constant free cross-sectional area in the stirred container
A/f	the variable free cross-sectional area in the other container
C	constant internal cross-sectional area of unstirred container with plunger
i	(subscript) means "initial"
P	variable cross-sectional area of plunger in the unstirred container
$r = 1/s = \rho_0/\rho$	density ratio between unstirred and stirred liquid = reciprocal specific gravity as defined below
$s = \rho/\rho_0$	density ratio between stirred and unstirred liq-

	uid = specific gravity of effluent liquid with the unstirred liquid as the standard
V	volume remaining in the mixer
$v = V/V_t$	reduced volume, dimensionless, running from unity to zero
x	height of liquid column in the unstirred container
y	height of liquid column in the stirred container
ρ	variable density in the stirred container = effluent density
ρ_0	constant density in the unstirred container
$\Delta\rho = \rho - \rho_0$	density difference between stirred and unstirred container

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