DENSITY GRADIENT TECHNIQUES

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CONTENTS

I. INTRODUCTION

Most chemical reactions in condensed media are accompanied by a change in density. Changes in density occur with changes in bond type, formation of electrical charge, geometrical isomerism, etc. It should therefore be possible to determine the course of any chemical reaction if sufficiently sensitive means of measuring small differences in density are employed. The changes which take place in solids due to variations in the extent of crystallinity and to lattice defects can also be observed by methods which detect small changes in density. The detection of isotopic content of materials (including macromolecules) can also be carried out by sensitive density difference methods.

Perhaps the most sensitive and simplest method for determining minute changes in density is the density gradient column technique. This method is capable of detecting density differences as small as 10^{-7} g./cc. whereas the absolute values for the densities of most materials are reported only to the fifth decimal place. Furthermore, only minute samples, of the order of a milligram, are required Another feature of the density gradient technique is that samples of different densities are separated in the column.

In this review an attempt is made (1) to outline the theory of the gradient and the techniques of its formation, (2) to describe the movement of the test object in the gradient, and (3) to review applications of the technique. No attempt is made, however, to review all papers in which the density gradient could have been employed but rather to choose those works where an example of some phenomenon of particular interest to chemists has been studied.

Essentially the density gradient column consists of a liquid mixture or a solution whose density varies with height (or with horizontal position in the case of the ultracentrifuge). The test object whose density is to be determined takes on the position in the column corresponding to that position of the column where the density is exactly equal to that of the test object.

Perhaps the first written description of a density gradient and its application was given by Galileo in 1630 (published, however, after his death in 1665 (29)):

"In the bottom of a vessel I placed some salt water and upon this some fresh water; then I showed them that the ball (of wax) stopped in the middle and that when pushed to the bottom or lifted to the top it would not remain in either of these places but would return to the middle."

Fick (26) in 1855 demonstrated experimentally that if two miscible liquids which are contained in different reservoirs are allowed to interdiffuse, then eventually a linear density gradient is set up in the tube connecting the two reservoirs. Linderstrøm-Lang around 1936 (52, 49, 51, 42, 38) revived interest in the density gradient column and demonstrated its potentialities for certain chemical reactions. Recently, particularly due to the effects of Meselson, Vinograd and others (60, 59, 34), a new density gradient technique has been applied in the ultracentrifugation of macromolecules, wherein the gradient (usually of aqueous CsCl) is actually produced by the centrifugal field. As early as 1931, Harvey (32, 33, see also 14 and 39) recognized the potentialities of the centrifuge buoyancy technique using preformed sucrose density gradients.

II. THEORY AND TECHNIQUE

A. FORMATION OF THE GRADIENT BY DIFFUSION

In its simplest form a density gradient column is produced by carefully contacting a liquid (a pure liquid or a solution) with another liquid of different density and allowing the system to stand so that interdiffusion takes place. Such a system would not, however, form a linear concentration gradient even after an infinite time of standing unless there is a large

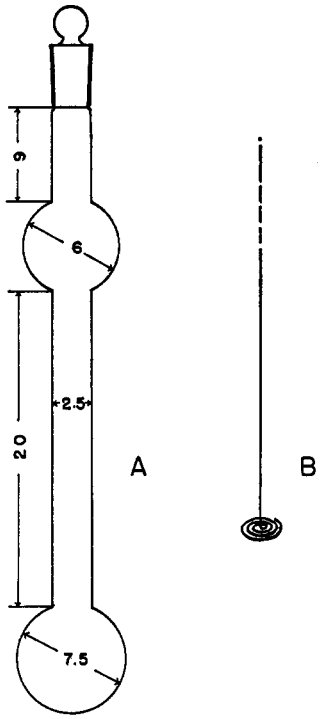

Fig. 1.—(A) Two-bulb density gradient column vessel; numbers give dimensions in centimeters. (B) Stirring spiral.

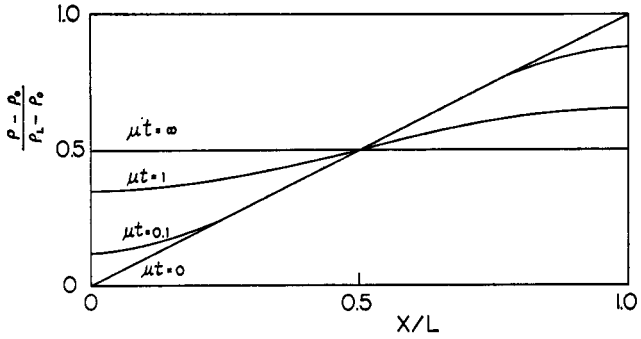

Fig. 2.—Density–distance relationships at several values of μt where $\mu = \pi^2 D/L$ (redrawn from ref. 28).

source for each of the two liquids and furthermore that the diffusion coefficient be independent of concentration.

Fick's law for one-dimensional diffusion in the steady-state condition is given by

$$\frac{d}{dx}\left(D\frac{dc}{dx}\right) = 0 \tag{1}$$

where D is the mean diffusion coefficient for the species and dc/dx is the concentration gradient. If D is a constant D_0 (independent of gradient and concentration) then, from equation 1, the gradient must be a constant for all x. The concentration is then given by

$$c = c_0 + kx \tag{2}$$

where c_0 is the concentration at an end of the gradient and k is the concentration gradient.

For non-ideal mixtures the diffusion coefficient is given by the Onsager–Fuoss relation (64)

$$D = D_0\left(\frac{d \ln a}{dc}\right) \tag{3}$$

where a is the activity of the diffusing species and D_0 is its diffusion constant at infinite dilution. For sufficiently dilute solutions equation 3 may be approximated by the linear expression

$$D = D_0(1 + Kc) \tag{4}$$

where K is related to the second virial coefficient in the osmotic pressure equation for the mixture. The constant K would normally be positive but is usually negative for aqueous solutions (53). A linear dependence of D on concentration can also arise from the effect of viscosity of the solution on the diffusion process. With this hydrodynamic correction K can have a negative value (63).

Inserting the approximation, equation 4, into equation 1 yields for the dependence of concentration on x for the steady-state

$$c = \pm\sqrt{\left(c_0 + \frac{1}{K}\right)^2 + \frac{2}{D_0}\frac{K_1}{K}x} - \frac{1}{K} \tag{5}$$

where K_1 is the steady state flux. The sign before the square root is the same sign as that for the constant K. It is clear, therefore, that the steady-state gradient can only be linear when the system is ideal or if fortuitously the hydrodynamic contributions exactly compensate the thermodynamic non-ideality contribution. It will be noticed from equation 5 that for positive values of K the gradient will be larger in one half $(0 < x < L/2)$ of the column of length L than for the other half $(L/2 < x < L)$.

In the above derivation we have assumed that the concentration at the boundary ($x = 0$) does not change with time, *i.e.*, that there is an infinite reservoir of diffusing material. If only a finite amount of material were present then at infinite time (*i.e.*, steady state) the system would be uniform with a concentration equal to the mean concentration. We can approximate infinite sources by having the volumes of the two liquids large compared with the volume of the column. In Fig. 1 is illustrated a density gradient column whose relative dimensions (volume of reservoirs is four times that of column) reasonably satisfy this condition.

When no reservoirs are present, however, one cannot achieve a linear gradient by diffusion and, furthermore, the gradient achieved is unstable and eventually disappears. The mathematical solution of diffusion in a finite cell has been solved (see also example ref. 21, Chapter IV). In Fig. 2 is illustrated how a column with no reservoirs deteriorates with time assuming that the gradient is initially linear (made, for example, by mixing methods described below) (28). Thus for $L = 70$ cm. and $D = 1.4 \times 10^{-5}$ cm.²/sec. (*e.g.*, bromo-

benzene in benzene) 40 days are required to reach the non-linear gradient $\mu l = 0.1$.

In some density gradient columns the concentrations of the diffusing species are high. Hence in such cases the approximation equation 4 and the derived expression equation 5 need not hold, especially for concentrated aqueous solutions. Furthermore, for such non-ideal solutions the density is not a linear function of concentration (Fig. 3). For several organic mixtures, however, the solutions are nearly ideal, even over the entire concentration ranges (Fig. 4) and closely linear density gradients can be achieved. In any case, if the gradient is made from two solutions with nearly the same concentration of diffusing species the variation of diffusion coefficient on concentration will be negligible and the density will vary practically linearly with concentration. It is, of course, desirable to achieve a constant density gradient, especially for purposes of interpolation, but such a special condition is not required. Any given density gradient can be calibrated with droplets of immiscible liquids or better still, with glass hollow spheres. The average density of the glass spheres will depend on the ratio of the mass of the glass to the size of the volume of the hole in the sphere. Procedures for making glass floats and for modifying them have been described in detail (30, 82). The floats should be allowed to stand for about 4 days before use to allow them to anneal at room temperature.

It is highly desirable that the gradient remains constant so that a single calibration will suffice. In practice, however, the reference density markers should remain in the column so that the density distribution can be determined just prior to using the column.*

There are a number of useful liquid mixtures which cover a wide range of densities. Organic liquid mixtures can be made from a number of completely miscible substances varying in density from 0.63 (pentane) to 3.33 (diodomethane). Other commonly used miscible liquids include hexane (0.66), benzene (0.88), butyl bromide (1.30), bromobenzene (1.50) and carbon tetrachloride (1.60). Aqueous solutions of salts vary from a density of unity to as high as 4.9 for concentrated thallium formate–thallium malonate mixtures in water (89). The more commonly used salt solutions are those containing NaCl and CsCl (see Fig. 3). Although $ZnCl_2$ is soluble in water up to 70% it is generally undesirable as a gradient medium because of its acidic character and its solubilizing effect on many materials including cellulose. Of particular use for gradients in the ultracentrifuge is CsCl since the density of its aqueous solutions at relatively low concentrations can match the effective

* A direct evaluation of the gradient can be made by the moiré technique (Y. Nishijima and G. Oster, to be published). In this method the column is viewed through two equi-spaced parallel rulings placed at a suitable angle and a straight line moiré pattern is obtained if the gradient is linear.

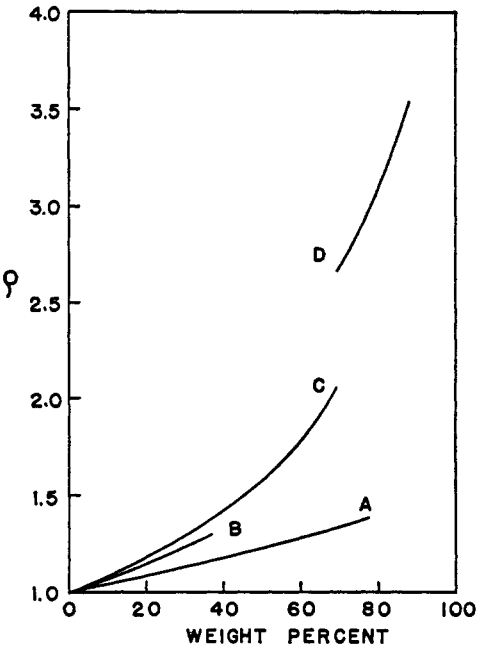

Fig. 3.—Densities at 25° of some aqueous solutions: (A) sucrose, (B) NaCl, (C) CsCl, (D) thallous formate.

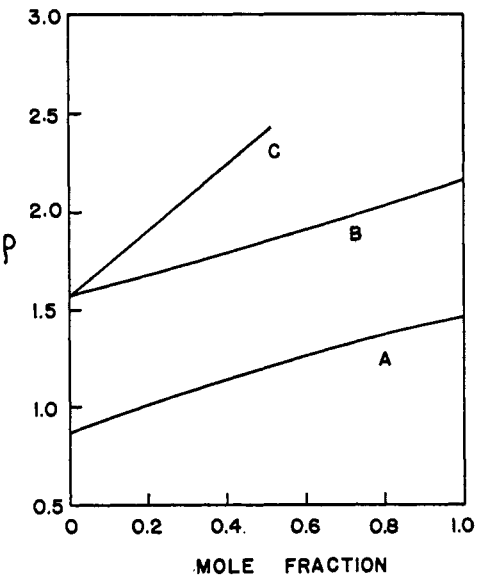

Fig. 4.—Densities at 25° of some organic mixtures: (A) benzene–bromobenzene: (B) carbon tetrachloride–ethylene dibromide; (C) carbon tetrachloride–carbon tetrabromide.

density (reciprocal of partial specific volume) of most macromolecules in aqueous solution.

Diffusion processes are, of course, extremely slow and for macroscopic systems it is obviously impractical to wait for steady-state conditions. One can, on the other hand, accelerate the approach to steady-state conditions in a density gradient by judicious stirring. In particular, for a vertical density tube as in Fig. 1A, one carefully overlays the heavier liquid, which occupies the lower bulb and half the tube, with the lighter liquid. Then using a stirrer of the form illustrated in

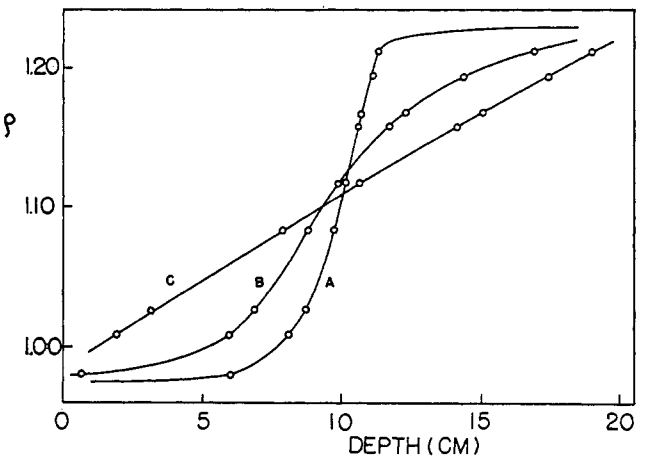

Fig. 5.—Effects of stirring to achieve linear gradient: (A) original density distribution; (B) after 25 strokes; (C) after 50 strokes and standing 10 hours. Column (L = 20 cm.) made from benzene and bromobenzene. Densities determined with calibrated glass sphere floats.

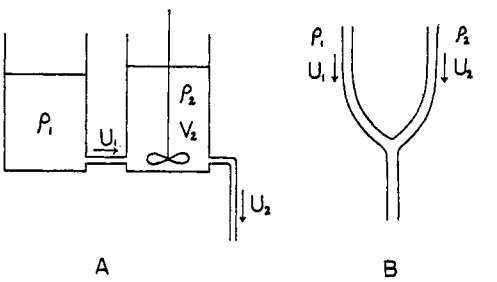

Fig. 6.—Two methods of mixing to produce a density gradient (see text for explanation).

Fig. 1B up and down slow continuous stirring about the interface is carried out. By such large convective movements the heavier liquid is dispersed into the lighter liquid portions and *vice versa*. Due to gravity, however, the system will be somewhat stabilized so that this will suppress any tendency to overshoot the desired final density distribution.

Perhaps the best method of stirring is to perform slow continuous up and down strokes about the original interface with increasing amplitudes of the strokes. In Fig. 5 are illustrated our results using this method. As seen, even 25 strokes are not sufficient to achieve the desired linear distribution. After a total of 50 strokes, however, one achieves a linear distribution which remains linear for several months but its value decreases very slowly. Another procedure is to carry out large strokes of equal amplitude running through the whole column. We have found, however, that there is a tendency by this method to overshoot the linear gradient because of mixing of the liquids in the reservoirs.

In the stirring procedure large swirling motion of the liquid should be avoided since this leads to uniform mixing. If the diameter of the gradient tube is small compared with amplitude of the movements, then the walls will break up such large undesirable swirling. Having a narrow tube is also desirable in that convection currents brought about by thermal non-uniformity will also be broken up by the walls. Thermal convection operates vertically in the direction of the gradient and gravity further stabilizes the system. The gradient tube should, however, be immersed in a constant temperature bath since fluctuations in temperature of 10^{-2} degrees make density variations of the order of 10^{-5} g./cc. Furthermore, the smaller the gradient the greater is the disruptive effect of thermal inhomogeneities. The interplay of convection currents and the geometry of the system is quite complicated (see for example, ref. 48, 27, 11).

B. FORMATION OF THE GRADIENT BY MIXING

In order to avoid the tedious procedure of repetitive stirring and to circumvent the necessity of long standing to obtain the steady-state, one can revert to mixing procedures which give practically instantaneously the desired density distribution. An instantaneous linear gradient can be achieved by one of two general mixing procedures (Fig. 6). In method A one of the liquids is introduced at a constant rate into the mixing chamber containing the second liquid and the mixture flows into the gradient tube at another rate of flow. In method B the two liquids are introduced into the gradient tube at different rates of flow.

Let us consider the flow conditions of method A. Suppose that liquid 1 is flowing into the mixing chamber (vessel 2) with a constant rate u_1. For simplicity assume that the volumes of liquids 1 and 2 are additive (*i.e.*, ideal solutions). The density ρ_2 of the efflux from the mixing chamber with rate of flow u_2 varies with time according to

$$\frac{d\rho_2}{dt} = \frac{\rho_1 u_1 - \rho_2 u_2}{V_2} - \frac{\rho_2(u_2 - u_1)}{V_2} = \frac{(\rho_1 - \rho_2)u_2}{V_2} \qquad (6)$$

where V_2 is the volume of liquid in the mixing chamber at any time t. The first term on the right of the equality sign describes the mass accumulation in vessel 2 and the second term describes the contribution due to volume change. The time rate of change of volume of liquid in vessel 2 is given by

$$dV_2/dt = u_1 - u_2 \qquad (7)$$

and the time rate of change of the volume V of liquid in the column is, of course, simply u_2. In these equations ρ_1, u_1, and u_2 are constants independent of time while ρ_2, V_2, and V vary with time. Hence from equation 7, $V_2(t) = (u_1 - u_2)t + V_2^0$ where V_2^0 is the original volume of liquid 2. Inserting this value of $V_2(t)$ into equation 6 and integrating, yields

$$\frac{\rho_2 - \rho_1}{\rho_2^0 - \rho_1} = \left[\frac{(u_1 - u_2) + V_2^0}{V_2^0}\right]^{u_1/u_2 - u_1} \qquad (8)$$

where ρ_2^0 is the density of the liquid in vessel 2 at the

initial time. Equation 8 is valid only for $u_2 \neq u_1$ while for $u_2 = u_1$, integration of equation 6 yields

$$\frac{\rho_2 - \rho_1}{\rho_2^0 - \rho_1} = \exp\left[-\frac{V}{V_2^0}\right] \tag{9}$$

Particular interest appears when $u_2 = 2u_1$, *i.e.*, the efflux into the gradient tube is twice the rate at which liquid 1 enters the mixing chamber. Then equation 8 becomes

$$\rho_2 = \rho_2^0 - \frac{\rho_2^0 - \rho_1}{2V_2^0} u_2 t \tag{10}$$

That is, a linear gradient is produced since $u_2 t$ is the volume of liquid in the column. Equation 9 is not, however, of particular interest unless the volume of the mixing chamber is much greater than that of the column in which case, on expansion of the exponential, a linear gradient condition is approximated. Equation 10 shows that the density gradient is greater, the greater is the difference in densities, ρ_1 and ρ_2^0, of the starting liquids and the smaller is the original volume V_2^0 of liquid 2.

Essentially method A was first utilized in elution chromatography (47, 24) and then applied for the production of gradient columns (85, see also 87 and errata 86) from which the above derivations were obtained. Our equation 10 differs, however, from theirs by a factor of 2 in the second term on the right. When liquid 1 is the more dense liquid, the mixture is introduced into the column at the bottom so that as the column is being filled the less dense mixture rises. On the other hand, if liquid 1 is less dense, then the mixture is introduced along a glass rod into the column so that the densest liquid is at the bottom and lighter mixtures pile on top.

Method B (compare 11) is conceptually simple and requires no mathematical description but requires rather elaborate mechanical accessory equipment to achieve a linear gradient. One procedure (62) consists of suspending the two liquids on a pulley rotated at constant speed so that as one vessel is raised at a certain rate the other is lowered at the same rate. Normally one cannot obtain a linear gradient by such a procedure since the rate of efflux from a vessel is proportional to the square root of the height of the surface of the liquid (Toricelli's theorem) and only for extremely slow efflux is the rate proportional to the height (Poiseuille's equation). The two liquids can be introduced into the gradient column at variable rates by use of syringes whose pistons are operated through helicoidal cams of variable pitch (46, compare 1). The cams are designed so as to cause the rate of delivery of the denser liquid to increase with constant acceleration and that of the lighter liquid to decrease proportionally, and the combined output is kept constant. When used with identical syringes, the instrument produces a linear gradient.

C. FORMATION OF THE GRADIENT BY ULTRACENTRIFUGATION

Another approach to making a density gradient is to subject a solution to a high centrifugal field. Even for a pure liquid a density gradient will be set up due to centrifugal pressure effects on the compressible fluid (see, for example, 18). Thus for water rotated at 60,000 r.p.m. in a standard ultracentrifuge cell the density on the outer part of the cell will have a value about 1% greater than that at the inner part of the cell. Such a density gradient, which by the way, may be much higher for organic liquids especially as the critical temperature is approached, is produced instantaneously. With solutions, however, a considerable time of centrifugation must elapse before a steady-state gradient is achieved. The purpose of using solutions, particularly aqueous salt solutions, however, is to be able somewhere in the gradient to match the effective density (reciprocal of the partial specific volume $\bar{V}$) of the macromolecular species being studied.

In the steady-state, *i.e.*, equilibrium centrifugation, the ratio of concentrations of solute at distances x_1 and x_2 from the center of rotation is given by the Boltzmann distribution for ideal solutions in a solvent

$$\frac{c_2}{c_1} = \exp\left\{\frac{M(1 - \rho_0 \bar{V})}{2RT} \omega^2 (x_2^2 - x_1^2)\right\} \tag{11}$$

of density ρ_0 where M is molecular weight of the solute, and ω is the angular rotational velocity. For small values of the exponent (*e.g.*, sucrose solution at a rotational speed of 20,000 r.p.m. in a 1 cm. cell) the concentration gradient is approximately linear. For smaller cells, such as the 1 mm. cells which have been employed (90), the approximation is better and furthermore c_2/c_1 is closer to the ratios of the densities. In general, for dilute solutions, equation 11 correctly describes the gradient when the steady-state is achieved. For finite concentrations the activity of the solutions must be taken into account. From data on the activity coefficients as a function of concentration, the density gradient at the steady-state can be computed numerically (Fig. 7). This has been carried out for aqueous solutions of CsCl, KBr, RbBr, LiBr, and sucrose at 25° although pressure effects have not been considered (41).

To reach the steady-state distribution in an ultracentrifuge requires a long period of operation of the machine. Numerous theoretical studies have been made to describe the concentration distribution and the time to approach steady-state conditions in the ultracentrifuge (see, for example, ref. 90, 56, 4, 95. For reviews, see ref. 74 and 97).

For high centrifugal energy compared with thermal energy (*i.e.*, large values of the exponent in equation 11) the transient term sensibly vanishes for times greater

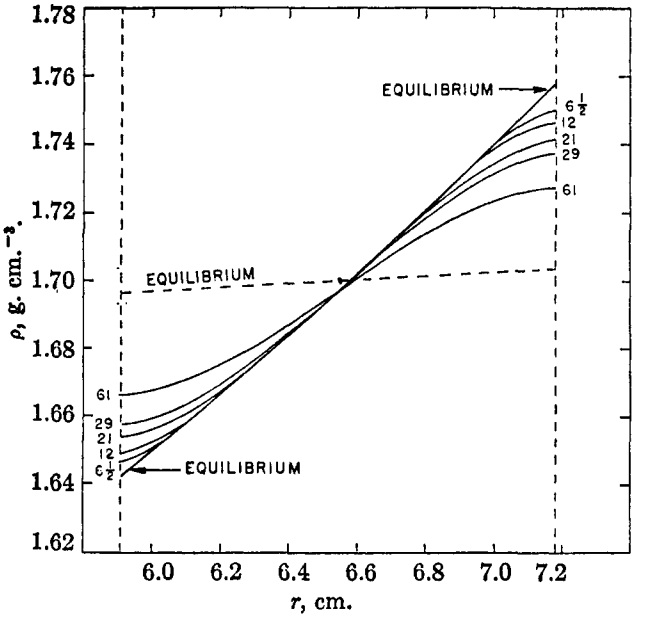

Fig. 7.—Density distribution of aqueous CsCl solution in the analytical centrifuge at equilibrium at 39,460 r.p.m. and after reducing velocity to 9,945 r.p.m. Dashed line is equilibrium distribution at 9,945 r.p.m. Numbers on the curves are minutes after reducing the velocity from the higher to the lower speed (41).

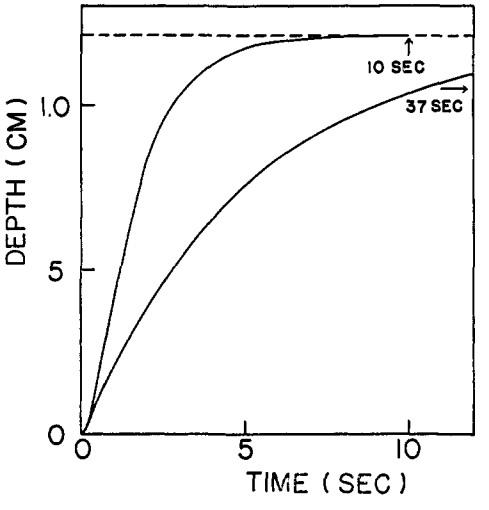

Fig. 8.—Fall of spherical test objects ($\rho^* = 1.02$) of diameters 0.2 cm.: (A) and 0.1 cm.; (B) in a gradient of $k = 2.5 \times 10^{-3}$ and uniform viscosity $\eta = 0.007$ poise with density at the top of the column $\rho_0 = 0.99$.

than the time of sedimentation of solute through the cell. For low centrifugal energies, on the other hand, the time to approach the equilibrium distribution is governed mainly by Brownian movement and hence is inversely proportional to the diffusion constant of the solute. The time for a given concentration distribution to reach the steady-state distribution is given by (90)

$$t = (L^2/D)F(\alpha) \tag{12}$$

where α is the ratio of thermal energy to centrifugal energy and L is the length of the cell. The function $F(\alpha)$ is practically independent of α for $\alpha > 1$ but drops rapidly toward zero for α less than about 0.5. This relationship has been demonstrated experimentally for sucrose and for ribonuclease where at about 60,000 r.p.m. in a 0.3 cm. column equilibrium is achieved in 3.5 hours for sucrose and about 20 hours for ribonuclease (90). For smaller cells (cells as small as 0.07 cm. have been employed (99)) equilibrium is obtained in much shorter times as given by equation 12.

D. RATE OF MOVEMENT OF THE TEST OBJECT

The speed which the object to be studied (the test object) reaches its proper density level should be great. Not only is this important for rapidity of measurement but also for cases where the object varies in density with time as in a chemical reaction. An object falling in a density gradient due to gravitational force will, after it reaches its terminal velocity, fall progressively more slowly and slowly as it approaches its proper density level. The elementary differential equation describing the movement of a particle of mass m and volume V falling in a linear density gradient of value k with the density at its top equal to ρ is given by

$$m\ddot{x} + f\dot{x} + Vg\rho_0(1 + kx) = mg \tag{13}$$

For mathematical simplicity we assume that the Stokes frictional factor f is a constant, *i.e.*, the viscosity is the same throughout the column. The solution of equation 13 for the distance of fall x as a function of time is given by

$$x = \frac{\rho^* - \rho_0}{\rho_0 k}\left\{1 - e^{-f/2mt}\left[\frac{f}{A}\sinh\frac{A}{2m}t + \cosh\frac{A}{2m}t\right]\right\} \tag{14}$$

where $A = \sqrt{f^2 - 4mVg\rho_0 k}$ and ρ^* is the density of the test object.

For a very short time of observation we can expand equation 14 to give

$$x = \frac{g}{2}\frac{\rho^* - \rho_0}{\rho^*}\left[t^2 - \frac{f}{3m}t^3 + \ldots\right] \tag{15}$$

where the inertial effect is proportional to t^2. After the initial effect is overcome, the fall is described when $f^2 \gg 4mVg\rho_0 k$ by

$$x = \frac{\rho^* - \rho_0}{\rho_0 k}\left[1 - \exp\left(-\frac{Vg\rho_0 k}{f}t\right)\right] \tag{16}$$

Hence the particle approaches its proper density level asymptotically.

In Fig. 8 is shown the time course of fall for spheres of different radii. Aside from the inertial effect which is complete after 0.2 sec. the fall is more rapid for the larger sphere. In ordinary usage of a cathetometer the position of the test object can be determined to about 0.01 cm. In the examples shown in Fig. 8 we calculate from equation 16 that the larger particle will approach its true density level within 0.01 cm. after about 10 sec. of fall whereas the smaller particle will

do so only after 37 sec. Hence for rapid measurements the particle should be large but not too large as to disturb the density gradient. Combining Reynolds number and the Ladenburg–Faxen expression relating velocity of a particle and the size of the tube, it can be demonstrated that a sphere of diameter nearly that of the tube will in its fall introduce turbulence into the system.

The above arguments also apply to irregularly shaped particles. There is a practical difficulty with irregularly shaped particles which should be kept in mind, the problem of wetting the solid. If occluded air bubbles are not removed the apparent density will be considerably lower than the true density.

Perhaps the greatest limitation of the density gradient method is that the test object may interact with the column. For example, if the material to be tested is an aqueous system some of the water may dissolve in the organic liquid column. Thus it has been demonstrated that the density of droplets of salt solutions in a kerosene–bromobenzene column will increase with time due to the removal of water (61). Instead of the asymptotic approach to the stable density position the drop gradually falls approximately linearly with time. Since the rate of removal of water is determined by the surface-to-volume ratio of the droplet, the rate of variation in density is less for big droplets than for small ones. The dehydration effect can be diminished by saturating the column with water, or better still with a salt solution. Apparently equilibrium saturation of the column is achieved in a matter of minutes. On the other hand a water-insoluble test object will, because of its small size, rapidly reach equilibrium with the salt density gradient as it is falling to its proper density level. Here the gradient will withdraw rapidly any small amount of water which might have been in the droplet.

Sedimenting macromolecules in an ultracentrifugal density gradient column will have an effective density given by the value of the reciprocal partial specific volume of the macromolecule in the particular solution in the gradient. The partial specific volume of a macromolecule in a salt solution diminishes with increasing salt concentration. Hence in a salt density gradient the effective gradient will be diminished by the counteracting increase in the density of the macromolecule (34, 35). On the other hand, difference in compressibilities of solvent and solvated polymer can, due to the pressure effect of centrifugal action, have an opposite effect on the gradient (36).

The problem of effective partial specific volume of the macromolecular species is difficult to solve in practice. Here we are concerned with the interaction of a polymer molecule with a binary solvent mixture whose composition varies with the height of the column. In a polydispersed mixture of polymers of one type the solvation of the macromolecules causes the entire polymer band at equilibrium in the centrifuge to be shifted in position (37). This does not affect, therefore, conclusions regarding the molecular weight distribution but gives the partial specific volume of the polymer–solvent complex rather than that of the unsolvated polymer. In a mixture of polymers that differ chemically, the shifts are different for different polymers, and it is clear that this sets limitations to the practical application of the ultracentrifugation density gradient method because the variations in chemical potentials with concentration for the three components is usually not available. A detailed theory has been worked out, however, (37) with the assumptions that in the comparatively narrow region where the polymer collects the density gradient is constant and the deviations from ideality are relatively small and are linear functions of the position. Under these conditions one obtains an expression for the excess refractive index as a function of position over the distribution with respect to the partial specific volume. By a Fourier transformation of the integral one obtains the distribution over the partial specific volumes.*

For a monodispersed polymer system in a gradient considered as a single component the distribution is gaussian with respect to position (60) but this theory is limited to low polymer concentrations. For finite polymer concentrations, however, one must consider second-order virial terms in the expansion for the chemical potentials. This leads to the result (37) that the reciprocal of the apparent number average molecular weight is a linear function of polymer concentration while the apparent weight average molecular weight is a direct linear function of polymer concentration. The intercepts of such curves give the true molecular weight averages and the slopes are determined by the Flory–Huggins constants for the polymer–solvent system in question.

If a mixture of polymers of the same chemical nature and molecular weight but of varying densities (assumed gaussian in distribution) reaches the equilibrium condition, the width of the band will be increased. Neglect of such a factor will underestimate the molecular weight if it were calculated from the width of the band (6). Such a case might arise with branched polymers.

Of some interest is the effect of variation in time of the density on the rate of fall of a test object in a gravity density gradient. Increased density would be accompanied by a decrease in volume and frictional constant of the test object. Hence, in equation 13 the coefficient of the second and third terms will be time dependent. This problem can be solved, at least for very short times, but the general solution is an in-

* Theory of ultracentrifugation in a density gradient is treated in detail by J. J. Hermans and H. A. Ende in "Newer Methods of Polymer Characterization" (B. Ke, editor), Interscience, John Wiley and Sons, New York, N. Y., in press.

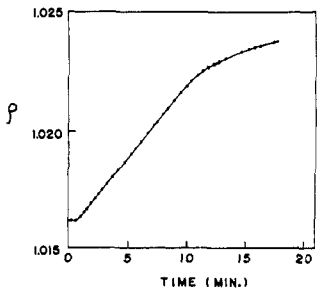

Fig. 9.—Dye-sensitized photopolymerization of acrylamide carried out in a density gradient column (benzene–bromobenzene): monomer concentration 30% in water; dye (riboflavin) concentration 1.1×10^{-5} *M*. Illumination with a 500 watt tungsten lamp. Droplet diameter was 0.34 cm.

tractable complicated algebraic expression. In general, for chemically reacting systems it is best to have changes with time which are slow relative to the rate with which the test object reaches its proper density level. In Fig. 9 is illustrated a photochemical reaction carried out in a density gradient column. After an initial induction period the position of the test object varies linearly with time, indicating a steady rate of polymerization. After about 10% conversion the rate diminishes. There is a decrease in density on long standing which can be attributed to influx of water into the test droplet from the gradient column which had been saturated with water. Despite this complication, the method is nevertheless accurate for the determination of initial rates of chemical reactions.

III. Applications

A great number of chemical reactions are accompanied by a large change in molal volume. Perhaps the most striking cases are those involving electrostriction of water. When an ion or an ion dipole is produced, the aqueous solution contracts due to hydration of the ions with a local destruction of the open structure of liquid water (9, 20). This effect has been utilized to follow the enzymatic hydrolysis of polypeptides by means of the density gradient column (49, 51, 42, 38). When a peptide bond is broken in water there is an increase in density due to electrostriction by the carboxyl and amino groups which is far in excess of the increased molal volume expected for the breakage of the bond. The value of the decrease in molal volume (about 20 cc. per mole of peptide bond) depends on the presence of residues on the polypeptide chain near the broken bond. A further factor, of importance for small peptides, is the proximity of the ionized residues and end groups. Naturally, the effect also depends upon pH, the electrostriction being smaller for the un-ionized pH condition. Salt effects as they may influence the ionization are also of importance. All these factors have been considered in detail (50). By means of the density gradient technique enzymatic hydrolysis of proteins can be followed even for very low conversions where only one peptide bond per protein molecule is split. It has been observed that the contraction of solutions of lactoglobulin during digestion are greater than those expected from calculations of electrostriction suggesting that more than peptide bond splitting is involved. On the other hand, clupein behaves normally (50).

The inverse of electrostriction, namely, a decrease in density, might be expected when ions in solution complex with charged chelating agents such as EDTA. It is significant in this regard that EDTA derivatives in complexation with metal ions are soluble in some organic solvents.

Another type of chemical reaction which involves large density changes is vinyl polymerization. The contraction of solutions of polymerizing vinyl monomers has been known for many years and, in fact, is often used to follow the reaction, especially by means of a dilatometer (see, for example, 77, 75, 58). The van der Waals covolume of the pi bond of the vinyl group is larger than the sigma bond of the polymer product and hence the density of the monomer is less than that of the resultant polymer. On complete conversion of vinyl chloride there is a contraction (at 25°) of 35%. The contractions for other monomers are smaller and is 26% for vinyl acetate, 25% for isoprene and 23% for methyl methacrylate. Large substituents decrease the contraction, thus 14% for styrene and 12% for *p*-methylstyrene. On the basis of the above figures the density gradient method easily could detect monomer conversions as low as 0.001%. A good example of the use of the density gradient column is that for the polymerization of *p*-chlorostyrene (96). Here the polymerization is carried out in the normal fashion and from time to time droplets of the solution are introduced into a sodium chloride density gradient. If polymerizations proceed under conditions compatible with the gradient, namely, at the temperature of the gradient system and where neither the monomer solution nor the polymer dissolve in the gradient, then the reaction can be observed continuously directly in the gradient. This procedure is particularly convenient for certain photochemical reactions (*e.g.*, that of Fig. 9) as long as the constituents of the gradient do not appreciably absorb the actinic radiation.

The inverse of vinyl polymerization, namely, degradation to give monomer (*e.g.*, the thermal degradation of polystyrene or of polymethylmethacrylate) would of course show a decrease in density. Removal of HCl from halogenated polymers (by heat treatment or radiation) does not necessarily decrease the density, however, since the counteracting effects of crosslinking and of crystallization could be more important.

Although there are appreciable density changes associated with isomerization (*e.g.*, *cis–trans*, and ring

formation) these reactions have not been examined by density gradient techniques. It is further known that solutions of *ortho* isomers have a higher density than the *para* isomers, the *meta* isomers being intermediate. Density gradient techniques could be used to determine the extent of such isomerization.

The partial specific volume of a branched polymer is greater than that of a linear polymer. Thus it was demonstrated with polyacrylonitrile in an ultracentrifugal density gradient (dimethylformamide and bromoform solution) that two bands appeared corresponding to a difference of partial specific volume of only 6×10^{-4} (16). Similarly a mixture of atactic and stereospecific polystyrene was separated in a bromoform–benzene gradient. Obviously such a method could be used for the characterization of copolymers including the block and graft types.* Such polymer species are often difficult to separate from the homopolymers by precipitation techniques. Desoxyribosenucleic acid (DNA) samples from various bacteria are separable in an ultracentrifugal density gradient due to their differences in density arising from their varying purine and pyrimidine content (80, 72). Native and renatured DNA have lower specific volumes than denatured DNA and can be separated in the gradient (80, 23, 73).

The ultracentrifugal density gradient column has been used to separate DNA molecules of different isotopic content (59). Bacteria uniformly containing N^{15} were grown in a medium containing N^{14} and the DNA was extracted from the growing bacterial population. Three types of DNA were separated using the column, namely, types N^{14}–N^{14}, N^{15}–N^{15}, and N^{14}–N^{15}. The last combination had the isotope distribution equally divided, suggesting that bacterial duplication involves two physically continuous subunits. The result is compatible with the Watson and Crick reduplication theory (94, see also 66). The DNA of hybrid isotope content, *i.e.*, type N^{14}–N^{15} (density 1.717) when denatured with formamide separates in the column into pure N^{15} and N^{14} types (densities of 1.745 and 1.729, respectively) (55) demonstrating the presence of two separable units in the original material (compare 93 and 71). Ultraviolet irradiation of the hybrid DNA gives a species of density 1.723 which is believed to arise from cross linking (55) such as occurs with other polymers when treated with ultraviolet light (67).

The isotopic content of substances containing other isotopes can be determined by density gradient techniques. In fact, the ordinary density gradient tube is a simple and sensitive instrument for determining the deuterium content of water droplets (3). The deuterium content of insoluble samples also can be determined with the ordinary density gradient. Although O^{18} is usually determined by mass spectrometry with the isotope in the form of carbon dioxide, the density gradient column can be more accurate than most commercially-available mass spectrometers. Furthermore, there is no special requirement as to the chemical nature of the isotopic substance.

The density of crystalline solids is altered by physical treatment. Treatment which involves phase transformations and changes in stoichiometry, and defects can be observed in a density gradient column. Thus, density variations due to the production of F-centers by X-ray irradiation of KCl crystals results in a decrease in density (25, 98) which could be easily observable in a tetrabromoethylene–benzene gradient. Furthermore, by use of a fine net one could pick out from the column those crystals whose densities are different. Incidentally, mixtures of crystals of KCl (density 1.984) and NaCl (density 2.165) are readily separated and retrievable from a density gradient column. It has been demonstrated from density studies that X-ray irradiation of crystals results in Frenkel defects since the crystal lattice parameter increases with such treatment (10). Neutron irradiation of LiF crystals results in a profound decrease in density (10, 76). Plastic deformation of KCl crystals results, after about 10% deformation, in a considerable decrease in density due to the production of vacancies (91).

The density gradient column has been employed to determine trace amounts of boron in silicon (40). In this work an ordinary gradient column consisting of iodobenzene and methylene iodide (also trimethylene diiodide) was used and boron concentrations (as interstitial atoms) as low as 0.00002% were detectable. The density observed for pure silicon agreed with X-ray diffraction unit cell determinations. For crystalline proteins where the number of molecules (an integral number) in the unit cell and the unit cell dimensions are known with precision, the molecular weight cannot be established with a precision greater than that determined for the density. Here the density of the protein crystals dry or hydrated, can be determined in the density gradient column. The crystals actually employed in the X-ray diffraction studies are often very small and under such circumstances it might be impractical to use the gradient column in the usual way. The rate of settling can be accelerated by low-speed centrifugation of the preformed density gradient (54) and this, furthermore, allows for easy separation of crystals of differing degrees of hydration.

The ordinary density gradient column is commonly being employed to determine the degree of crystallinity in plastics. Crystalline linear polyethylene has a density of about 0.96 at room temperature whereas the completely amorphous material (density of molten

* See, for example, S. E. Bresler, L. M. Pyrkov, and S. Ya. Frenkel, *Vysokomolek. Soed.*, **2**, 216 (1960); M. Wales, *J. Appl. Phys.*, **7**, 203 (1963).

paraffins corrected for thermal expansion) is 0.84 (88). It has been established from the sharpness of X-ray powder diagrams of samples of various densities that the density difference between these two extreme forms of polyethylene is a measure of degree of crystallinity (see for example, ref. 57). Since the mechanical properties of polyethylene are closely correlated with degree of branching of the molecules, and hence with the degree of crystallinity (for review, see ref. 70), density determinations, particularly by means of the gradient technique, are employed in industrial control. Here methanol salt solution density gradients are used where swelling of the plastic is minimal. The degree of crystallinity of other plastics such as nylon (78), polyvinyl alcohol (83), and polyethylene terephthalate (19, 43, 31, 45) has been determined by means of the ordinary density gradient column. We have noticed with films of this last polymer (Mylar) that the freshly cut end of the sample has a slightly higher density than that of other portions of the film. Apparently shearing forces on cutting the sample make for local orientation of the macromolecules resulting in a greater density. Drawing of the films usually results in greater molecular orientation and increased density (for review, see for example ref. 17). Textural inhomogeneities along fibers can be observed in the gradient column (84). Saran films which have been heated and then suddenly chilled exhibit a slow increase in crystallinity with time and the rate of crystallization can be determined by means of the density gradient column (13).

Cellulose-degrading reagents attack cellulose fibers and the attack is more rapid in the amorphous regions than in the crystalline regions. This is shown by the increased density of the fibers as measured in a density gradient column (84). Acetylation of cotton fibers, however, results in a decrease in density corresponding to the low density of the esterified cellulose as compared with the unmodified cellulose (69, 65). The density gradient column is also useful in textile chemistry for the routine determination of concentration of sizing and other additives (79) as well as for studying the effects on industrial treatment of the fibers (5).

Textiles often appear as mixtures of synthetic and natural fibers. By density gradient techniques one can usually separate the fibers (84, 69) and remove the fractions from the gradient for further analysis. In order to avoid occlusion of air bubbles to the fiber bundle the material should be deaerated by, for example, boiling in a non-solvent (69). The gross wool structure can be destroyed by mechanical working to yield spindle structures which are separable in the density gradient from other structures in the fiber. Two main fractions are obtained which differ in sulfur content as well as density (92).

The density gradient column has proven useful for the separation and recovery of cellular structures (8, for reviews see ref. 2 and 22). The broken cells are subjected to low speed centrifugation in a preformed density gradient column. The gradient employed is often made from sucrose–water mixtures but this has osmotic effects on the particles and, hence, in some cases a carbon tetrachloride–benzene gradient is used instead. By these means cellular constituents such as nuclei and microsomes (including isotopically labeled ribosomes using the ultracentrifuge with CsCl gradient (15)) can be separated easily. Furthermore, such particles can be recovered, for example, by pouring into the column liquid mercury and thereby pushing up the layers which then can be collected at the top. Alternatively, one can pierce the bottom of the centrifuge tube and collect drops of the desired fraction.

Due to the stabilizing influence of the density gradient, sedimentation rate studies of cellular debris are often carried out in a gradient column (2, 22). A density gradient also provides a convenient nonconvecting medium in which to carry out electrophoresis (68). In this case a skewed density distribution may be desirable since the stability of the electrophoretic moving boundary is greatest at the inflection point (81).

IV. CONCLUSIONS

In this age of complicated instrumentation it is refreshing to know that a simple and inexpensive device, namely, the ordinary density gradient column, exists which is capable of measuring a fundamental property of matter to differences of one part in ten million. Furthermore, only a milligram of material, in any geometrical form, is required. Still further, the method allows for the recovery as well as the separation of samples whose densities differ only very slightly.

There are, of course, other sensitive methods for measuring small density differences, notably the falling drop method and the dilatometric method (for reviews, see for example ref. 44 and 7). The former method is restricted to spherical liquid droplets whose size must be known precisely. The dilatometric method requires a considerably larger sample and is particularly responsive to small temperature changes since, after all, the dilatometer is also a very sensitive thermometer. Its advantage is, however, that reactions can be carried out in the instrument itself whereas this is not generally the case with a density gradient column.

The applications of the density gradient technique described in this review by no means exhaust its possibilities. Surely such a low-cost instrument should become a part of every chemical laboratory. An ordinary gradient column with large reservoirs as illustrated in Fig. 1 and properly thermostated ($\pm 0.1°$) will retain a linearity in density for several months. Scientists should consider its use for a particular problem before passing on to more complicated instruments or to more elaborate separation techniques. As for the

ultracentrifugation density gradient, its potentialities have only just begun to be realized and it may well prove to be one of the most valuable tools of the polymer chemist.

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