CONTENTS

* Translated by Express Translation Service, London, S.W. 19.

PRINCIPLES OF GRADIENT ELUTION

LLOYD R. SNYDER

Union Oil Company of California, Union Research Center, Brea, Calif. (U.S.A.)

CONTENTS

1. INTRODUCTION

In elution chromatography the composition of solvent entering the column is normally held constant, whereas *gradient elution chromatography* is characterized by the intentional variation of eluent composition (so as to increase eluent strength) during the course of separation. If eluent composition V_B (volume fraction of a strong eluent component B) during separation is plotted *versus* the volume of eluate V leaving the column as in Fig. 1, the resulting gradients may be described as continuous (as in a–d) or discontinuous (as in e–h). The term *gradient elution* has customarily been reserved for separations carried out with continuous gradients, while *stepwise elution* has generally referred to separations which involve discontinuous gradients. A number

References p. 48.

of workers (*e.g.* refs. 1–4), however, have not distinguished between gradient and stepwise elution. As we shall see, there is no very fundamental distinction between these two chromatographic techniques, despite certain practical differences. From this standpoint, the basic technique of gradient elution chromatography can be considered to have originated with TSWETT (as pointed out by STRAIN[5] and SYNGE[6]),

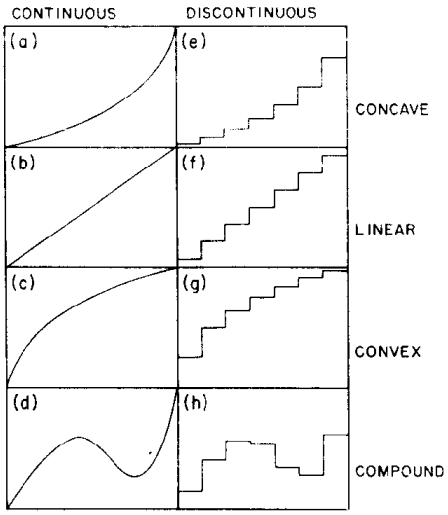

Fig. 1. Classification of gradient type and shape.

who used column development with successive changes of solvent. By the early 1940's, this technique had gradually merged into that of stepwise elution as we presently know it. Gradient elution appears to have been first carried out in deliberate fashion by MITCHELL, GORDON AND HASKINS[7] in 1949, using salt and pH gradients in "chromatopile" development. Unfortunately, the main emphasis in this initial application of gradient elution was on the chromatopile technique (essentially paper chromatography in a column), with no apparent awareness on the authors' part of the unique and general advantages of gradient elution. In 1949, SYNGE[6] referred in pessimistic tones to the possibility of gradient elution, attributing the original concept to TISELIUS. Similarly, STRAIN[8] in 1950 acknowledged the possibility of accidental "sorption gradients" in many chromatographic systems, and postulated that these might result in improved separation.

The real "discovery" of gradient elution, with appreciation of its unique possibilities in the separation of complex mixtures, occurred independently within several laboratories in the early 1950's. Within a few months of one another, HAGDAHL, WILLIAMS AND TISELIUS[9] conceived of gradient elution as a solution to certain rather general problems associated with chromatography on charcoal, while DONALDSON, TULANE AND MARSHALL[10] visualized the technique as a more practical way to carry out separations previously requiring stepwise elution. At somewhat later times, NERVIK[11] and BUSCH, HURLBERT AND POTTER[12] appear also to have independently conceived of the gradient elution technique. From this point the application of gradient elution to a large number of practical separation problems quickly followed: inorganic anions[13] and cations[14], silicone polymers[15], carboxylic acids[10,12], amino acids and

References p. 48.

peptides[16], steroids[17], lipids[18], purine bases[19], sugar derivatives and oligosaccharides[20,21], vitamins[22], alkaloids[23], enzymes[24], hemoglobin[25], serum proteins[26], hormones[27], nucleotides[28], nucleic acids[29], and miscellaneous non-biological organic mixtures[30,31]. According to LEBRETON[32], much of the credit for the rapid exploitation of gradient elution in the years immediately following its discovery belongs to WILLIAMS, by virtue of his extensive researches and publications relating to the technique.

Gradient elution has today matured to the point where most new experimental examples of its application are taken for granted. Consequently, a complete cataloging of all such cases would be pointless, as well as physically impossible. Moreover, because of the fundamental interrelationships which exist between gradient, stepwise, and conventional elution, more can frequently be learned, concerning the optimum application of gradient elution to a particular separation, by studying experimental examples of stepwise or conventional elution, than from specific instances of gradient elution itself. To be of real use, therefore, a present day review of gradient elution should emphasize those fundamental principles which either distinguish it from or relate it to other elution chromatographic techniques. Two previous review articles on gradient elution, by LEBRETON[32] and MIKĚS[33], have fallen somewhat short of this goal, while a number of subsequent publications in the field have greatly increased the prospects for a successful synthesis of gradient elution theory. The present communication is an attempt at a critical review of gradient elution, with as much emphasis as seems possible toward an integration of the first principles of gradient elution in a form useful to practical chromatographers.

2. THEORY

(a) *Fundamental aspects of elution chromatography*

Any discussion of the gradient elution technique must begin with an understanding of the normal elution chromatographic process, where eluent composition is held fixed throughout separation. Several useful mathematical models for fixed eluent chromatography have been developed (*e.g.* see ref. 34–36). It will best suit our present purpose to choose one of the simplest and most widely known models, the "continuous transfer equivalent plate" model described by KEULEMANS[35], and to elaborate on this as necessary. This model assumes that the chromatographic column can be represented by p separate "equivalent plates", and that elution of sample through the column is equivalent to *continuous* transfer of eluent from plate to plate, with partial equilibration occurring within each plate after each transfer. For columns having large values of p, the theory predicts the development of symmetrical, Gaussian elution bands as in Fig. 2, where the elution of a component i from a column of p equivalent plates is visualized. With p given for a column, the elution band of component i is completely defined by the distribution coefficient K_i, for equilibrium partitioning of i between the stationary and moving phases within the column. If K_i is defined as $(i)_s/(i)_m$, where $(i)_s$ and $(i)_m$ are the equilibrium concentrations of i in the stationary phase (g/g) and moving phase (g/ml), respectively, and if W and V° are the total quantities of stationary phase (g) and moving phase (ml), respectively, contained within the column, then the retention volume R_i (ml) for the band i (see Fig. 2) is given as:

$$R_i = K_i W + V^\circ \qquad (1)$$

References p. 48.

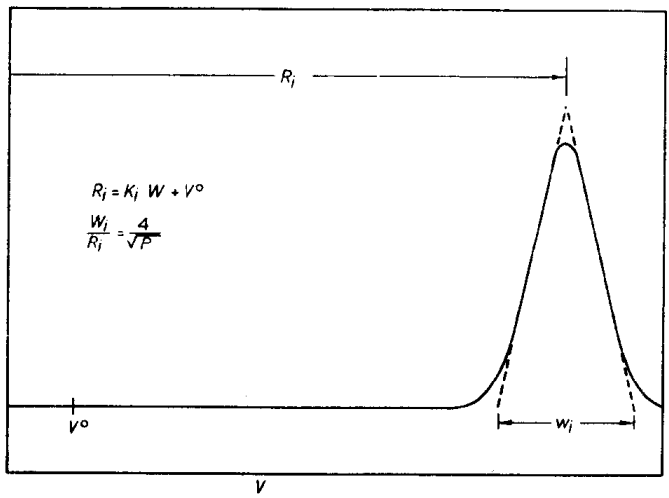

Fig. 2. A hypothetical elution band in fixed eluent elution chromatography.

For large values of p, this model predicts an approximate relationship between band width w_i, plate number p, and retention volume R_i,

$$w_i/R_i = 4/\sqrt{p} \tag{2}$$

Next consider the elution of a two component sample as in Fig. 3a. The relative separation of components 1 and 2 is obviously determined by the individual band widths w_1 and w_2, and by the separation of the two band maxima $(R_2 - R_1)$. The degree of overlap of the two bands can be quantitatively measured by the number of half widths of 1 and 2, $(w_1 + w_2)/2$, which can be accommodated in the interval $(R_2 - R_1)$. If we define a separation parameter S_r;

$$S_r = (R_2 - R_1)/(w_1 + w_2)$$

separation is seen to be good when S_r equals 2, and improves as S_r increases. Eliminating retention volumes and band widths from the above relationship by means of eqns. (1) and (2) gives:

$$S_r = \frac{\sqrt{p}\,[(K_2/K_1) - 1]}{4\,[(K_2/K_1) + 1 + 2\,(V°/WK_1)]} \tag{2a}$$

For $W K_1$ (or R_1) considerably larger than $V°$, separation is seen to be uniquely defined by column plate number p and separation factor (K_2/K_1). As R_1 and R_2 become small, however, a point is eventually reached where separation becomes poor even for large values of p and (K_2/K_1) (which would normally ensure good separation).

A major problem in the elution chromatographic separation of complex, multicomponent mixtures arises as follows. For a given separation system or column, initially eluted sample components (small R_i values) tend to be less well separated than later eluted components, as predicted by eqn. (2a). Indeed, for very readily eluted components no separation at all will be possible. Because K_i and R_i values tend

References p. 48.

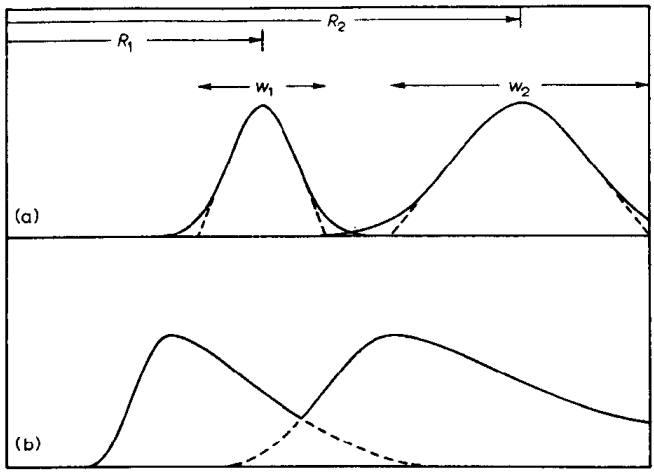

Fig. 3. (a) Hypothetical separation of two components 1 and 2 in elution chromatography; linear sorption isotherms. (b) Same with tailing of bands.

to increase exponentially as the structure of i is systematically varied or augmented, the last eluted sample components will in general have very large values of R_f. This may lead to an impossibly long analysis time, particularly where the retention volumes of the first eluted components have been made sufficiently large for their separation. In addition to excessive analysis times, large R_f values mean large peak widths and small peak heights, according to eqn. (2); the corresponding reduction in the sensitivity of peak detection may be such that small amounts of strongly held components escape notice in the final chromatogram. We will refer to this universal limitation on fixed eluent chromatographic procedures as the *"general elution problem"*. Figs. 4a–c illustrate the *general elution problem* in detail, for elution separation of a hypothetical six component mixture. In Fig. 4a, for elution by the weak eluent A, weakly held components 1 and 2 are well separated and emerge as sharp, easily detectable peaks; components 3 and 4 require a longer elution time and show reduced

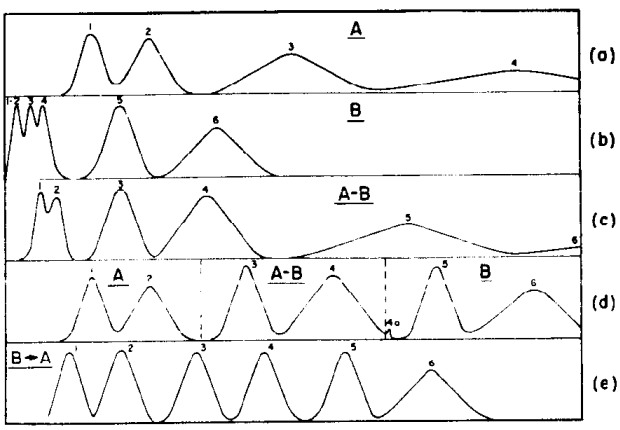

Fig. 4. (a) Hypothetical separation of a six-component mixture in fixed eluent elution chromatography; weak eluent. (b) Same, strong eluent. (c) Same, eluent of intermediate strength. (d) Stepwise elution of same mixture. (e) Gradient elution of same mixture.

References p. 48.

peak heights; components 5 and 6 require excessive elution times and give very low peak heights. In Fig. 4b for elution by the strong eluent B, components 5 and 6 emerge in a reasonable elution time as sharp peaks, but components 1 to 4 are eluted too quickly and hence show poor resolution. The mixed eluent A–B in Fig. 4c shows intermediate elution behavior, with poor resolution of peaks 1 and 2, and somewhat excessive elution times and low peak heights for peaks 5 and 6.

A second general problem in elution chromatography is associated with non-linear or semi-reversible sorption isotherms, which occur in some chromatographic systems. This problem tends to be less important today than during the 1940's and early 1950's. Isotherm non-linearity and/or irreversibility lead to elution bands which tail severely, as in Fig. 3b; the result is poorer separation of adjacent bands, excessive elution times for removal of the last bands from the column, and in some cases incomplete recovery of sample.

(*b*) *Gradient elution: some preliminary considerations*

In the elution chromatographic separations of Figs. 4a–c, it is observed that optimum elution occurs with eluent A for peaks 1 and 2, with A–B for peaks 3 and 4, and with B for peaks 5 and 6. This naturally suggests that stepwise elution, using first A to elute peaks 1 and 2, then A–B for peaks 3 and 4, and finally B for peaks 5 and 6, will give the best overall separation scheme. As illustrated in Fig. 4d, stepwise elution in this fashion does provide a number of advantages: adequate resolution of all components, reasonable elution time for the total sample, and sharp bands for the easy detection of every component. Among early workers aware of the advantages of stepwise elution as a solution to the *general elution problem* were REICHSTEIN AND VON EUW[37], who used 12 succesively stronger eluent mixtures in the separation of steroids on alumina, and MARSHALL *et al.*[38], who used three successive eluent mixtures in the partition separation of organic acid mixtures.

The gradient elution separation of the sample of Fig. 4 is illustrated in Fig. 4e, where the eluent composition is assumed to vary continuously from pure A at the beginning of elution to pure B at the end. In introducing gradient elution in 1952, DONALDSON, TULANE AND MARSHALL[10] pointed out two additional advantages of gradient over stepwise elution: (1) the change in eluent strength during gradient elution can be made automatic, thus avoiding the labor of frequent eluent changes and the uncertainty of trying to anticipate the point at which eluent changes should be made; (2) factitious peaks (as illustrated by peak 4a in Fig. 4d), which may arise in stepwise elution whenever the eluent is changed near the end of elution of a peak, are avoided in gradient elution.

The advantage of stepwise or gradient elution in separations such as that of Fig. 4 arises from the constantly decreasing K_i values of all sample components during separation. Thus, from an elution separation standpoint, there is only a small range of K_i values for optimum separation of i: K_i must be sufficiently large so that i and adjacently eluted components are not pushed off the column as a single, unresolved band, and K_i must be reasonably small if excessive elution times and band broadening are to be avoided. In a satisfactorily designed gradient elution separation, the initially eluted sample components leave the column before their K_i values are reduced enough to impair resolution, while subsequently eluted components have their K_i values reduced successively into the optimum range at the time of elution from the column.

References p. 48.

It is apparent that any chromatographic process where provision is made for decreasing the various solute K_i values with time will simulate the unique features of gradient elution. Since K_i is a function of the compositions of stationary and moving phases, column pressure, and column temperature, variation of any of these four quantities with time can in principle be used to control K_i. In gas chromatography, it is most convenient to vary K_i values by gradually raising column temperature ("temperature programming"), and temperature programming seems in every sense analogous to gradient elution. In liquid chromatography, temperature variation is generally less effective in decreasing K_i with time, and temperature programming in liquid elution chromatography has found little application. Pressure variation normally has very little effect on K_i, and its deliberate use in elution chromatography as a means of solving the *general elution problem* appears to be unknown*. Variation of the stationary phase composition as a function of time is normally inconvenient in elution chromatography, although this technique has been suggested[39] as a possible means of simulating the advantages of gradient elution in adsorption chromatography, without the need for the accessory equipment normally required in gradient elution. In column development chromatography, as originally practiced by TSWETT, and currently duplicated for all practical purposes in paper and thin-layer chromatography, gradient elution effects may be achieved by initially adjusting the composition of the stationary phase along the direction of solvent flow in such a manner as to cause K_i to increase with increasing migration of i. This idea was originally put forward by STRAIN[8], although no practical application of such "adsorbent gradients" has been reported in the subsequent 15 years. Difficulty in achieving the necessary compositional gradients in the stationary phase seems chiefly responsible. In the present connection, it is also appropriate to cite the technique of column fractional precipitation as originally introduced by BAKER AND WILLIAMS[40]. Solvent of gradually increasing solvent power is swept over a polymer sample and then down an inert packed column (*e.g.* glass bead packing) which has a negative temperature gradient from top to bottom. The relationships which determine the migration of sample components through the column in this procedure differ fundamentally from those appropriate to gradient elution in more conventional chromatographic systems. Finally, in the still less related technique of electrophoresis, gradient effects can be achieved by increasing the potential across the column or plate during separation. The present review will not attempt a discussion of these various techniques (*e.g.* temperature programming, stationary phase gradients, solvent precipitation, electrophoresis) which are more or less closely related to gradient elution in liquid chromatographic systems. In the case of temperature programming, however, it should be noted that this technique is basically similar to gradient elution, and is currently undergoing a detailed theoretical and experimental study for application to gas chromatography. These investigations when completed are very likely to offer greater insight into the gradient elution technique as well; a specific example is cited in a later section (Section 4,c).

A second general difficulty in conventional elution chromatography, band tailing as in Fig. 3b, has been discussed in detail by HAGDAHL, WILLIAMS AND TISELIUS[9], in their initial paper on the gradient elution technique. Noting that band tailing as in

* Note added in proof: S. A. CLARK (*Nature*, 202 (1964) 1106) has recently reported the first example of pressure programming in gas chromatography.

References p. 48.

Fig. 3b was especially severe for elution of many samples from charcoal (particularly with weak eluents), these authors showed that the technique of displacement chromatography appears in many cases superior to elution chromatography because of certain relationships between the adsorption isotherms of the sample components; frequently, in the separation of sample components 1 and 2, for certain concentrations of 1 and 2, 1 will always be displaced by 2, leading to near complete separation of the two components. Because the two sample components are immediately adjacent in displacement chromatographic separation, however, complete separation by this technique is never possible (as in elution chromatography), unless the expedient of carrier displacement is adopted, where a third component which separates between 1 and 2 is deliberately added to the sample prior to displacement separation. In a multicomponent mixture, the selection of the $(n-1)$ carriers required for n components is impossibly complex, however, and HAGDAHL *et al.*[9] visualized that an eluent gradient might in practice duplicate the operation of $(n-1)$ carriers furnishing an eluent mixture of intermediate sorption strength between each pair of sample component bands, just as required of the carrier. Additionally, these workers noted that most adsorption isotherms on charcoal become linear at sufficiently high eluent strengths, so that gradient elution should tend to linearize the adsorption isotherm of component i during the critical period when i is being eluted from the column. Similarly, the rate of sample desorption (or degree of isotherm reversibility) tends to increase as eluent strength increases, so that an eluent gradient will also reduce band tailing arising from slow desorption rates of sample components. While this analysis of band tailing and of elution *versus* displacement by HAGDAHL *et al.*[9] is a necessarily simplified one, these basic considerations led this group of workers to the independent discovery of the gradient elution technique, which does provide a satisfactory solution to the band tailing problem wherever it exists. With the advent in the last 15 years of adsorbent "saturators" or "deactivators" (*e.g.* refs. 9, 41) for the linearization of adsorption isotherms, as well as the development of improved sorbents which achieve the same result, the problem of band tailing in elution chromatography has come to be less important, and today gradient elution is more important as a solution of the *general elution problem* than for the correction of band tailing.

The major theoretical problem in gradient elution, as in other elution chromatographic techniques, is the prediction of retention volume R_i and band width w_i for each sample component as a function of separation conditions. This in turn permits the production of optimum separation conditions for a particular sample, and the formulation of general rules as guides for every separation. DRAKE[42] provided the first general, mathematical description of the dependence of R_i on the variables of separation. R_i according to DRAKE depends on:

(i) the shape of the influent gradient, V_B *versus* V;

(ii) the migration of the eluent component B through the column, with or without sorption;

(iii) the dependence of K_i on eluent composition, and for non-linear isotherms, the dependence of K_i on the concentration of i as well.

Fig. 5 illustrates the operation of some of these factors. An initially linear eluent gradient is assumed: the concentration of the strong eluent component B increases in proportion to the volume of eluent that has entered the column. The solid lines of Fig. 5 show the concentrations of B in the moving phase within the column at various

References p. 48.

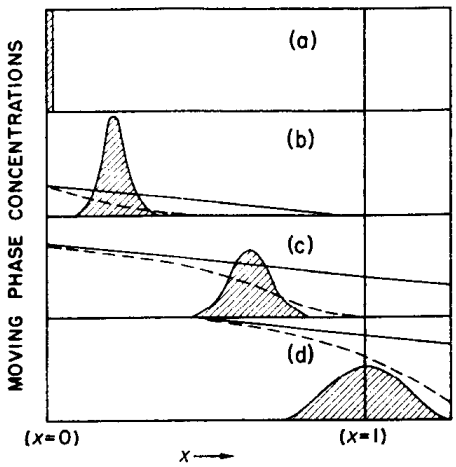

Fig. 5. Hypothetical portrayal of band elution and eluent gradient within column during gradient elution. (a) Beginning of elution. (b) Shortly after beginning of elution. (c) Later during elution. (d) Just prior to the end of elution. Cross hatching indicates sample band; — = concentration of eluent component B (no sorption); - - - = concentration of eluent component B (sorption).

times during elution of the solute band i (cross hatched), assuming no sorption of B. The corresponding dashed lines show the concentrations of B as might result from sorption of B, these curves always lying below the non-sorption curves, but approaching the non-sorption curves after enough eluent has passed through the column to permit saturation of the sorbent by B. At any point during separation, the instantaneous rate of movement of the solute band along the column depends upon the value of K_i at the band maximum, which in turn is determined by the eluent composition at that particular time and position in the column. For non-linear sorption of i, K_i will be further dependent upon the concentration of i at a particular time, and this in turn is a complex function of the elution history up to that point of the separation. Following sections will review in detail the calculation of influent gradients for various gradient devices, the effect of eluent sorption on the eluent gradient within the column, the calculation of K_i for changing eluent composition (and changing solute concentration), and finally the calculation of R_i in various gradient elution systems.

Band width in gradient elution separation is determined by three major considerations, as first noted in a qualitative fashion by ALM, WILLIAMS AND TISELIUS[43]:

(i) the normal tendency of the elution band to spread as it migrates down the column, just as in other elution separations (see Fig. 5);

(ii) the amount of eluent required to displace a band of given width from the column end, just prior to elution from the column; this depends upon the eluent strength or value of K_i at the time i leaves the column;

(iii) the variation in eluent composition *across* the band at a particular time; K_i is generally always greater for the band front than for the band tail at any given time.

In other words, in gradient elution the widening of the band during elution (i) is to some extent cancelled by the increase in K_i at the time of elution (ii), and by the gradient across the band (iii), which tends to speed up the elution of the band tail relative to the band front. Later workers have provided more precise descriptions of the effect of gradient elution on band width as will be discussed in following sections.

References p. 48.

(c) *Quantitative calculation of retention volume in gradient elution*

1. *General*

The fundamental equation for the calculation of R_i values in gradient elution can be derived without difficulty. Consider the migration of a component i down a column during gradient elution, as in Fig. 5. For the present we will fix our attention on the movement of the band maximum, ignoring the spreading of the band. At any time t, or equivalent eluate volume V, there will exist a certain eluent composition in the region of the band maximum, which determines a value of K_i and the instantaneous velocity of the band down the column. In order to emphasize the functional dependence of K_i in the region of the band maximum on eluate volume, we will refer to a value of K_i during gradient elution as $K_i\{V\}$. At any time during the elution of i, passage of an eluent volume dV through the band maximum will cause a fractional displacement dx of the band maximum along the column length, equal to $dV/W\,K_i\{V\}$. Thus, in fixed eluent chromatography, passage of $K_i\,W$ ml of eluent through the band maximum results in a displacement x equal 1 (*i.e.* the band maximum completely traverses the column), as may be seen from eqn. (1), and recalling that the total R_i value includes the V° ml of eluent initially in the column (which does not pass through the band maximum). Thus, we have:

$$\frac{dV}{WK_i\{V\}} = dx$$

The term on the right, dx, must be integrated between 0 and 1, corresponding to the positions x of the band maximum at the beginning and the end of elution of i, respectively, while the term on the left must be integrated between the initial and final elution volumes, 0 and $(R_i - V^\circ)$; (the volume of eluent on the column at the start of elution is measured as part of V, yet does not pass through the band maximum). This then gives:

$$\int_0^{(R_i - V^\circ)} \frac{dV}{WK_i\{V\}} = \int_0^1 dx = 1 \qquad (3)$$

Equation (3) is basic to the understanding of the principles of gradient elution. It was first derived by DRAKE[42] (eqn. 18), in essentially the same fashion as above. Similar derivations have also been offered by FREILING[34] (eqn. 4), SAID[44] (eqn. 10), SCHWAB *et al.*[45] (eqn. 9), and SNYDER[46] (eqn. 8).

In order to calculate gradient elution retention volumes by means of eqn. (3), $K_i\{V\}$ must be known as a function of V. In the general case, this is quite complex, as may be appreciated by itemizing the factors which determine the $K_i\{V\}$–V relationship:

(i) the composition of eluent entering the column must be known as a function of V;

(ii) K_i must be known as a function of eluent composition; if the sorption isotherm of i is non-linear, the complete isotherm for every eluent composition must be known, and the concentration of i at the band maximum must be calculable throughout separation;

(iii) if the strong eluent component B is sorbed to an appreciable extent, the effect

of sorption on the eluent composition at any point within the column and at any value of V must be calculable.

Item (i), the composition of eluent entering the column as a function of V, presents the least practical difficulty. As we shall shortly see (Section 3,a), explicit equations expressing V_B as a function of B have been derived for most of the commonly used gradient devices, and other gradient devices are available which will provide any V_B *versus* V relationship desired. In many practical cases, however, the complexity of the derived V_B function renders the explicit integration of eqn. (3) impossible.

The prediction of K_i as a function of eluent composition, item (ii), has been one of the general problems of elution chromatography, as has the prediction of the variation of K_i with other separation conditions (*e.g.* adsorbent, temperature, etc.), and the dependence of K_i upon solute structure. From the standpoint of gradient elution theory, only the effect of eluent composition on K_i is of direct importance, and we will avoid trying to relate K_i to the structure of i and to other separation variables. In the immediately following sections, where the calculation of gradient elution R_f values for various chromatographic systems is discussed, we will review what is known concerning the dependence of K_i on eluent composition in these systems.

Where K_i also varies with the concentration of i (non-linear isotherms), the calculation of R_i values appears generally unfeasible. Not only is it required that K_i be calculated for all possible values of the eluent composition and concentration of i, in itself a formidable task, but the spreading of the band as i is eluted down the column must also be taken into account, since this leads to changing concentrations of i. DRAKE[42] has been the only author attempting to treat non-linear isotherm gradient elution, and his results do not appear to be of general interest in this respect. As is true of all elution chromatographic techniques, however, the study of linear isotherm systems promises considerable insight into related non-linear systems. Non-linear isotherm systems are treated briefly in Section 2, g; unless otherwise noted, all other calculations will refer to linear isotherm systems.

With sorption of the strong eluent component B, which must occur to some extent in most gradient elution adsorbent systems, it is virtually impossible to carry out the exact calculation of $K_i\{V\}$. Little is known about the *general* behavior of the sorption isotherm (for B) over the wide ranges in eluent composition frequently encountered in gradient elution; moreover, even where the B isotherm is known in a particular chromatographic system, correction of eqn. (3) for eluent sorption greatly complicates its subsequent integration to give R_f. Fortunately, eluent sorption can quite frequently be ignored in practice, and its potential seriousness in some situations can be readily estimated. A later section will treat this topic separately (Section 2,e).

2. Ion exchange

In the ion exchange separation of an ion i which bears a net charge $\pm q$, by some single charged eluent component B of similar sign the distribution coefficient K_i for partition of i between ion exchanger and solution is given as:

$$K_i = E/[\mathrm{B}]^q \tag{4}$$

E is the equilibrium constant for the ion exchange reaction (*e.g.* see ref. 45), and [B] refers here to the molar concentration of B in the eluent. For the general case where

the eluent B bears a multiple charge r, K_i becomes equal to $(E^{1/r})/[B]^{q/r}$. Equation (4) and the gradient relationship ($[B] = f\{V\}$) can be substituted directly into eqn. (3) for calculation of R_i in gradient elution with a *salt gradient*, providing that the ion exchange equilibrium constant E remains constant as the concentration of B varies throughout separation. For the simple inorganic ions, the ion exchange equilibrium constant E is observed to be constant at low concentrations (0.5–1.0 M) of B[45,47], while for separation of the ribonucleotides COHN[48] has similarly noted K_i approximately inversely proportional to the concentration of B, in agreement with eqn. (4) for q equal 1.

FREILING[47], SCHWAB *et al.*[45], and MASLOVA *et al.*[49] have reported the comparison of experimental R_i values with values calculated from eqn. (3) for several salt gradient elution systems. Agreement between experimental and calculated R_i values averaged about $\pm$ 2 % standard deviation, verifying the applicability of eqn. (3) to such gradient elution calculations. FREILING studied the elution of Na^+ and Cs^+ by an H^+ gradient, over a range of HCl concentrations where E was *not* constant throughout, using several different gradient shapes; graphical integration of eqn. (3) was used.

SCHWAB *et al.*[45] studied the gradient elution of Cl^-, Br^- and oxalate ion by NO_3^- gradients, over concentration ranges of NO_3^- where eqn. (4) applied. R_i values were calculated explicitly from eqn. (3) by expressing K_i as a function of V from eqn. (4) and the known relationships between V and [B] for the gradient devices used. General solutions were given for the elution of monovalent, bivalent and tervalent ions i (B monovalent) by either of two gradient types: linear or "exponential" (see Section 2,h), either of which gradient shapes are readily obtainable in a simple experimental apparatus. MASLOVA *et al.*[49] studied the gradient elution of Pm and Ce ions.

The relationships derived by SCHWAB *et al.*[45] for *linear* salt gradient are of general interest, and will find application in the following discussion of certain general aspects of gradient elution. They are as follows:

$$q = 1, r = 1 \qquad R_i - V^\circ = \sqrt{\frac{2\,WE}{C}} \tag{5a}$$

$$q = 2, r = 1 \qquad R_i - V^\circ = \sqrt[3]{\frac{3\,WE}{C^2}} \tag{5b}$$

$$q = 3, r = 1 \qquad R_i - V^\circ = \sqrt[4]{\frac{4\,WE}{C^3}} \tag{5c}$$

In the above expressions, C refers to the steepness of the salt gradient ($[B] = C\,V$).

Equation (4), and the use of salt gradients, assumes that the dependence of K_i on [B] is a purely mass action effect, arising from the competition between i and B for oppositely charged sites within the ion exchanger. Alternatively, B may have an additional effect on K_i, or even a primary effect, by reaction of B with i in the eluent phase to change the net charge on i. The commonest of such reactions of significance to ion exchange gradient elution involve the dissociation and neutralization of various acidic and basic solutes:

$$H^+ + i^- \rightleftharpoons Hi$$

$$H^+ + i \rightleftharpoons Hi^+$$

References p. 48.

If a single ion B accounts for most of the mass action effect in competitive sorption with either i^- or Hi^+ (because the concentration of B is kept large and/or B is quite strongly sorbed), and if eqn. (4) holds, then K_i for acidic and basic species Hi and i, respectively, will be given by:

$$K_i = \frac{(E/[B])\ [i^-]}{[i^-] + [Hi]}$$ for sorption of i on an anion exchanger

or:

$$K_i = \frac{(E/[B])\ [Hi^+]}{[i] + [Hi^+]}$$ for sorption of Hi on a cation exchanger

The various bracketed quantities $[x]$ refer to the molar concentration of x. If the acid dissociation constant K_a is defined, equal $[H^+]\,[i^-]/[Hi]$ or $[H^+]\,[i]/[Hi^+]$, respectively, then:

$$K_i = \frac{E K_a}{[B]\ (K_a + [H^+])}$$ for elution of an acid Hi from an anion exchanger (6a)

and:

$$K_i = \frac{E[H^+]}{[B]\ (K_a + [H^+])}$$ for elution of a base i from a cation exchanger (6b)

For a purely pH gradient, where [B] is held effectively constant during elution, the normally desired reduction in K_i (with increasing V) can only be the result of decreasing the fraction of i in the ionized form as described in eqns. (6a) and (6b), or in the case of polybasic acids and bases, of reducing the net charge on i (if K_i decreases greatly for a unit reduction in the charge on i, as is frequently the case, eqns. (6a) and (6b) will still apply to the K_i values of polybasic molecules over certain ranges in pH). For most of the components in a sample to be separated by gradient elution, K_i should be initially large, so that during pH gradient elution the fraction of i in the charged form must decrease quite substantially. As a first approximation, this means that $[H^+]$ will be significantly larger than K_a for the elution of acids Hi, and significantly smaller than K_a for elution of bases i; this then leads to the approximate relationships:

$$K_i \cong \frac{E K_a}{[B]\ [H^+]}$$ for elution of an acid Hi from an anion exchanger (6c)

and:

$$K_i \cong \frac{E[H^+]}{K_a\ [B]}$$ for elution of a base i from a cation exchanger (6d)

For monovalent anions and cations (q equal 1), and monobasic acids and bases, eqns. (4), (6c) and (6d) are seen to be of the same form, providing that $10^{-14}/[OH^-]$ is substituted for $[H^+]$ in eqn. (6d); in every case K_i is inversely proportional to the concentration of the gradient substance: B, H^+ or OH^-. Consequently, the calculation of R_i in both salt and pH gradient elution of such ions, acids or bases involves identical integrations, and the principles governing their separation are essentially the same.

The derivation, for pH gradient elution, of R_i values by integration of eqn. (3) has been reported only for one, fairly untypical case[45]; no correlation of calculated *versus* experimental R_i values has yet been attempted for pH gradient elution, al though there is no reason to doubt the success of such comparisons should they be

References p. 48.

attempted. Relative separation order in the ion exchange separation of the amino acids has been observed[50] to fall in approximately the same sequence as the K_a values, as predicted by eqns. (6c) and (6d) if E is assumed constant. Similar relationships between R_i and K_a have been cited in the ion exchange separation of the ribonucleotides[48], although frequent exceptions are noted in both cases. HURLBERT *et al.*[28] have noted that pH gradients have a greater tendency than salt gradients to change relative separation order.

The gradient elution separation of the proteins has been adequately reviewed by PETERSON AND SOBER[51,52], and the same general principles are presumed to apply to the separation of other biological macromolecules, such as the nucleic acids. The principal differences between the sorption on ion exchangers of the proteins, and small organic molecules such as the amino acids, is the extremely large number of potentially charge-bearing groups on a typical protein molecule, with the possibility of forming multiple bonds with the sorbent. Unless all but a very small fraction of these bonds are broken, the protein will tend to be held on the ion exchanger quite strongly indeed. Consequently, the proteins should tend to elute in the order of and near to their isoelectric points (the pH of zero net molecular charge), and this is in fact experimentally observed[27,53]. BROWN AND WATSON[54] have expressed this somewhat differently in the case of nucleic acid separations, referring to a separation order based on molecular "base ratios".

The elution of biological fractions such as the polynucleotides and peptides represents a case intermediate between elution of macromolecules such as the proteins, and elution of small molecules such as the amino acids which bear a net charge of only 1 or 2 under normal elution conditions. Generally, some mobility of the polynucleotides and peptides is possible, even when the solute has a substantial number of its groups ionized, and there is a tendency for elution order to fall in the same sequence as molecular size[52], rather than in the order determined by K_a values or isoelectric points.

PONTIS AND BLUMSON[55] have suggested, in the salt and pH gradient elution of the nucleotides, that the dissociation and solubility (in the eluent phase) of the calcium and sodium salts of these solutes plays a key role in determining relative separation order. Not enough appears to be known about the general importance of such effects, however, to estimate their significance in most gradient elution separations.

In the ion exchange separation of ions by salt or pH gradients, these gradients generally show the concentration of some effective eluting agent B (which may be hydrogen or hydroxide ion, respectively, in the case of pH gradient elution of acids and bases) increasing during separation. The separation of anions on a cation exchange resin, or of cations on an anion exchange resin, however, involve complex ion equilibria of the sort:

$$i^+ + n\,X^- \rightleftharpoons iX_n^{-(n-1)}$$

and:

$$i^- + n\,X^+ \rightleftharpoons iX_n^{+(n-1)}$$

with sorption of the complex of i in each case. Here, sorption of the complex is *promoted* by increased concentrations of the ion X, and the gradient must normally show a *decreasing* concentration of X. An example is provided by MARCUS AND NELSON'S separation[56] of the rare earth ions over an anion exchange resin, using a decreasing nitrate ion gradient.

References p. 48.

3. Adsorption

The dependence of K_i on eluent composition is quite similar for both adsorption chromatography, and ion exchange at constant pH. In each case, there is a competition between solute molecules and strongly sorbing eluent molecules B for a limited number of sorption sites: charged groups within the ion exchanger, or active surfaces within the adsorbent. If m eluent molecules are required to desorb one solute molecule, we have within the chromatographic column the general distribution equilibrium:

$$i_{(\text{soln})} + m\ \text{B}_{(\text{sorb})} \rightleftharpoons i_{(\text{sorb})} + m\ \text{B}_{(\text{soln})}$$

from which eqn. (4) follows directly. In the case of ion exchange, m is equal to the ratio of ion charge values for solute and eluent, q/r. In adsorption chromatography, where solute and eluent compete for a given position on the adsorbent surface, m is usually equal to the ratio of areas required by solute and eluent, respectively, on the adsorbent surface[41] (in some cases, m is somewhat larger than calculated in this fashion because of the operation of other factors which affect adsorption). As regards the dependence of K_i on eluent composition, a major difference between ion exchange and adsorption chromatography arises as follows. In ion exchange, when no ions B are present in the eluent, i cannot be displaced from the sorbent, and K_i becomes effectively infinite, as predicted by eqn. (4) for [B] equal zero. In adsorption chromatography, while B will generally be much more strongly sorbed than other eluent components, other eluent components will still be effective in displacing i from the adsorbent surface. That is, although K_i frequently becomes large in adsorption chromatography as [B] becomes small, there will be much less tendency for K_i to become very large (or effectively infinite) for [B] equal zero. Equation (4), or the related expression $K_i = (E^{1/r})/[\text{B}]^{q/r}$, is therefore not generally a good approximation in adsorption chromatography.

The actual relationship between K_i and eluent composition in adsorption chromatography has been described by SNYDER[39,41,57] for linear isotherm systems. For adsorption of a solute i, whose relative area (for adsorption) is A_s, from an eluent whose "eluent strength" is ε°, onto an adsorbent of surface energy α, K_i is given by:

$$K_i = K_p\ 10^{-\alpha\varepsilon^\circ A_s} \tag{7}$$

K_p is the value of K_i for the standard eluent, pentane. Equation (7) has been shown to be accurate for the adsorption of a wide range of solute types on alumina, silica and Florisil. Values of the adsorbent surface energy α[41] and eluent strength ε°[39,41] have been tabulated for various chromatographic systems based on the latter three adsorbents, and the calculation of A_s for any solute has been discussed in detail[41]. Relative eluent strength values for some other adsorbents (magnesia[58], carbon[59]) can be inferred from so called "elutotropic series".

For the case of binary eluents, such as occur in gradient elution, values of ε° (and hence K_i) can be readily calculated from the ε° values of the constituent eluents A (weakly adsorbing) and B (strongly adsorbing) ε°_A and ε°_B, respectively:

$$\varepsilon^\circ_{AB} = \varepsilon^\circ_A + \frac{\log_{10}\left[X_B\ 10^{\alpha n_b(\varepsilon^\circ_B - \varepsilon^\circ_A)} + 1 - X_B\right]}{\alpha n_b} \tag{8}$$

References p. 48.