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EDITOR'S PREFACE

THE METHODS FOR THE STUDY OF a variety of phenomena in the life sciences are multiplying at such a prodigious rate from such a variety of sources that the average investigator in the biomedical sciences is hard put to find an informative and critical review of methods for accomplishing given determinations and estimations in biologic materials. The objective of METHODS IN MEDICAL RESEARCH is to provide constructive and critical reviews of current methods for use by investigators in the life sciences that can be put to immediate use. We have tried to emphasize this principle in the production of Volume 12, which is devoted to the subject of chromatography.

I believe it may be said that chromatography in all its forms has been the single greatest system of techniques for separation and identification of natural compounds of biologic importance that has ever been devised. It stems from the initial observations of Michael Tswett, a young Russian scientist, who observed in 1906 (6) that the pigments of the green leaves, which include the chlorophylls and the carotenoids, had differential adsorptive behavior on cellulose and other adsorbent materials in the presence of different solvents. He quickly applied these observations to the separation of these pigments by devising columns of adsorbing substances and demonstrated all the features of chromatography, i.e., loading, development and elution, in this system.

It was obvious to Tswett, who used the term chromatography to denote the separation of pigments, that the principles he had observed also applied to colorless substances of different properties. The term *chromatography*, however, has persisted and has been a useful term to describe all procedures that operate to separate compounds in a heterogeneous system by the countercurrent principle.

As is true of many discoveries, there was a latent period in the application of chromatography to chemistry and biochemistry which lasted almost 25 years. This latency period was broken in 1931 by Kuhn and his associates (3, 4) who applied the method successfully to the separation of plant carotenoids.

Other forms of chromatography have subsequently been introduced. Ion-exchange chromatography was introduced in 1935 by Adams and Holmes (1). In 1941 Martin and Synge (5) developed

a theory for partition chromatography, and applied it to the separation of mono-amino acids from protein digests. In this system, the adsorbent becomes a support for 1 of 2 liquid phases. Subsequently, James and Martin (2) described a special application of this principle to gas-liquid chromatography, which has been extremely important in recent developments in biochemistry. In addition, several modifications of chromatographic systems have been devised, featuring application of electric fields with or without gradients of density and pH to aid in the separation of compounds, i.e., combined electrophoresis and chromatography.

In this volume, the present state of the art and science of chromatography is presented for the researcher in biology and medicine who needs to have at hand a simple treatise of the subject, including most of its applications. Volume 12, METHODS IN MEDICAL RESEARCH, includes five sections devoted, respectively, to adsorption chromatography, gas-liquid chromatography, ion-exchange chromatography, electrochromatography and finally a systematic review of systems for separating and identifying a variety of compounds of biologic interest.

I am very much indebted to my associate editors, Drs. Lloyd R. Snyder, Charles Sweeley, S. Jacobs, Anthony Martonosi, and Evan and Marjorie Horning, who have organized their respective sections dealing with the various applications of chromatography. I am hopeful that this volume of METHODS IN MEDICAL RESEARCH will be as useful to biologic investigators as previous volumes.

ROBERT E. OLSON

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SECTION I

Adsorption Chromatography

ASSOCIATE EDITOR—*Lloyd R. Snyder*

INTRODUCTION

ALL CHROMATOGRAPHIC methods possess certain common advantages: the ability to separate complex mixtures into their component compounds, applicability to both very small and moderately large samples, great speed and convenience relative to more traditional separation procedures and a flexibility which permits the ready application of chromatographic separation to an enormous range of possible sample types. Nowhere have these advantages been more appreciated than in the areas of biochemical and medical research. Each of the chromatographic methods—with the exception of electrophoresis—can be understood as a special case of the general chromatographic process. Accordingly, the following discussion will begin with an examination of chromatographic separation in general, without reference to those special features which differentiate individual methods (adsorption, partition, ion exchange chromatography, etc.). Then we will consider the various adsorption chromatographic procedures, along with the experimental techniques and equipment which make these procedures workable. Finally, we will turn to the principles of separation by adsorption chromatography, i.e., to an understanding of how separation can be optimized in given cases. Our discussion will be aimed at the reader with a practical interest in separation, and it will assume little or no previous experience. At the same time we hope to draw on the practical implications of recent work and to go significantly beyond previous treatments of a similar nature.

L. R. SNYDER

I. THE BASIS OF CHROMATOGRAPHIC SEPARATION

L. R. SNYDER, *Union Oil Company, Brea, Calif.*

Practical separations by chromatography almost always proceed in the same general way. We begin with a porous bed, either a column filled with some granular material or a thin, rectangular film of the same material. In adsorption chromatography this granular material or *sorbent* is normally a particulate, porous solid (the *adsorbent*). In other forms of chromatography the sorbent may consist of a liquid-coated solid, porous beads of an organic polymer or even paper sheets. A small quantity of sample is applied to one end of the column or bed and then washed through the bed by flow of liquid or gas. The rate of migration of an individual sample component through the bed is determined by the distribution of that compound between the sorbent and the moving solvent or gas. Compounds which are held tightly by the sorbent move through the bed only slowly. Compounds which are held only weakly—or not at all—move through the bed rather rapidly. The result is a separation of slow-moving from fast-moving sample components. This process is illustrated in Figure 1 for different stages in the hypothetical separation of a 3-component mixture.

Figure 1 illustrates several characteristic features of chromatographic separation. The various components of the starting sample generally move through the sorbent bed at different rates. As each compound moves through the bed it spreads out on the bed to form a *band* which occupies more space than the original sample at the beginning of separation. As the movement of solvent or gas through the bed proceeds, the initially unseparated sample is gradually resolved into its individual components. We will look first at the migration of an individual compound through a sorbent bed, relating its migration rate and band shape to certain fundamental separation parameters. Then we will turn to the problem of separating 2 sample components that have similar migration rates in a given chromatographic system. Finally, we will consider the separation of complex, multicomponent samples.

The following discussion of general chromatographic theory

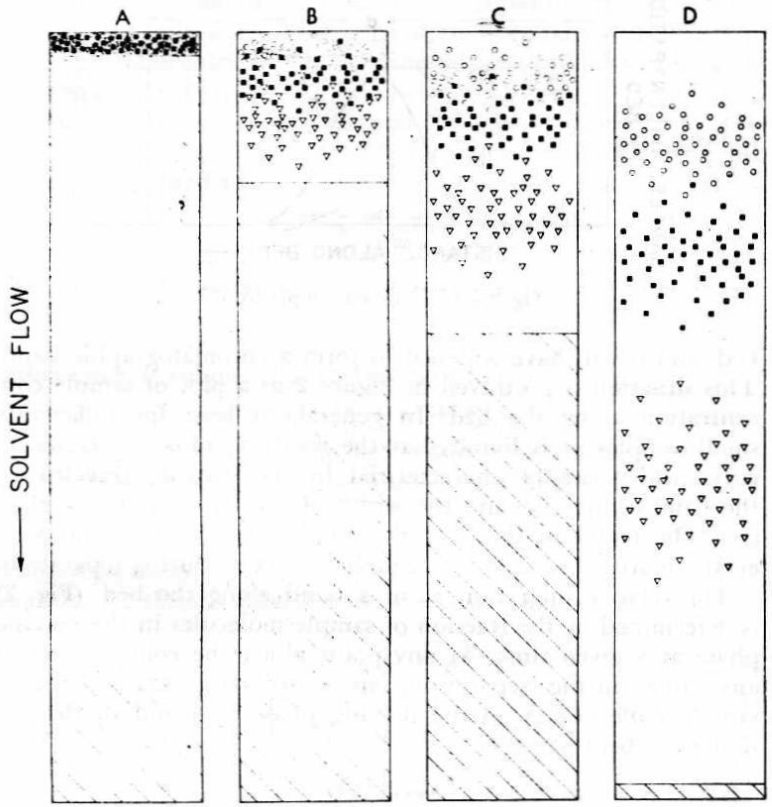


Fig. 1.—Hypothetical separation of a 3-component sample by adsorption chromatography (sample R_F values of 0.15, 0.30 and 0.60). (From Snyder [4], with permission of the publisher.)

is only a brief summary of the more important practical points. Readers interested in further details or a more fundamental understanding of the chromatographic process should consult recent books in this area (1–4).

SAMPLE MIGRATION AND BAND DEVELOPMENT

Let us assume that a sample consisting of a single pure compound has been applied to a chromatographic bed, and flow of gas or solvent (the *moving phase*) has begun, as in Figure 1. We will allow the moving phase to move a certain distance through the bed, short of completely filling the bed. At this point the sample will have migrated some distance along the

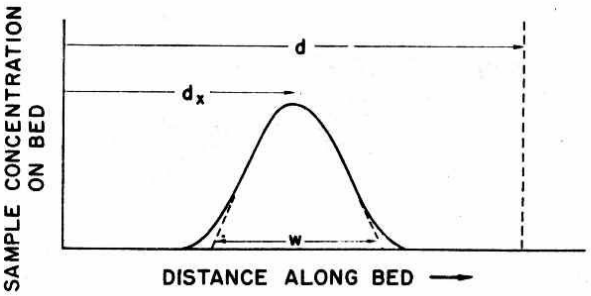


Fig. 2.—An adsorbed sample band.

bed, and it will have widened to form a chromatographic band. This situation is portrayed in Figure 2 as a plot of sample concentration along the bed. In general, at least for sufficiently small samples, it is found that the resulting plot is a Gaussian curve which can be characterized by the distance traveled by the band center (d_x) and the width of the band at the baseline (w). The Gaussian shape of the band arises from the random, erratic motion of individual sample molecules during separation.

The relative migration d_x of a band along the bed (Fig. 2) is determined by the fraction of sample molecules in the moving phase at a given time. At any point along the column, and at any time during separation, an equilibrium exists between sample molecules X in the moving phase (m) and in the stationary sorbent phase (s):

$$X_{(m)} \rightleftarrows X_{(s)}.$$

The equilibrium concentrations of X in the 2 phases, $(X)_m$ and $(X)_s$, are related by an equilibrium constant or distribution coefficient K:

$$K = (X)_s/(X)_m.$$

As long as sample concentrations are sufficiently small, K is constant for a given sorbent, solvent and temperature. Now the velocity v_x at which the sample band moves along the bed is equal to the velocity v of the moving phase (i.e., the rate of solvent or gas flow) times the fraction of total sample molecules in the moving phase at a given time. When all of the sample molecules are in the stationary sorbent phase, the band velocity is 0, i.e., the band does not move from its point of application. When all of the sample molecules are in the moving phase, the band velocity is the same as that of the moving phase, i.e., the band moves with the solvent or gas front. The distance moved by the band center relative to the distance moved by the front

of the moving phase (d_x/d) is, of course, equal to the corresponding velocity ratio v_x/v. Therefore, d_x/d is equal to the fraction of sample molecules in the moving phase. This fraction is in turn equal to $V_m(X)_m/[V_m(X)_m + V_s(X)_s]$, where V_m and V_s refer to the volumes of moving and stationary phases within the bed (when the moving phase has completely filled the bed). Consequently, we have

$$\begin{aligned} d_x/d &= V_m(X)_m/[V_m(X)_m + V_s(X)_s] \\ &= 1/[1 + (KV_x/V_m)]. \end{aligned} \tag{1}$$

The ratio d_x/d is referred to as the R_F value of the sample band. In adsorption chromatography it is more convenient to define K in terms of the weight of adsorbent within the bed (W), rather than its volume (V_s), and we then have

$$R_F = 1/[1 + (KW/V_m)], \tag{1a}$$

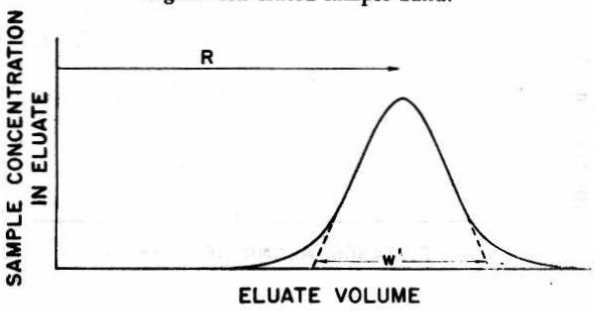
where V_m now refers to the free volume of the bed, i.e., the volume of moving phase contained in the bed when the bed is filled with solvent or gas.

Turning next to the widening of a sample band during a given separation, it is convenient to define the experimental quantity N, the so-called theoretical plate number of the separation system:

$$N = 16\,(d_x/w)^2. \tag{2}$$

Here we assume that the band center has moved across the entire length of the bed. For partial migration of the band across the bed (Fig. 2), we can define the number of theoretical plates N' traversed by the band center. Since the number of theoretical plates per unit of bed length tends to remain constant, N' equals N times the fractional distance migrated by the band center (d_x divided by bed length). When the moving gas or solvent is allowed to flow all the way through the bed (just filling the bed), N' equals $N \cdot R_F$. N is approximately independent of sample

Fig. 3.—An eluted sample band.



type and can be regarded as a characteristic property of the bed for a given set of operating conditions.

If we do not stop the flow of gas or solvent through the bed (as in Fig. 2), so that the moving phase leaves the other end of the bed, the sample band will eventually be washed or *eluted* from the bed. This separation mode is referred to as *elution* chromatography. A plot of sample concentration in the leaving gas or solvent (eluate) versus the total volume of the eluate then gives a plot similar to that of Figure 3. A Gaussian sample band is again observed, just like that found on the bed (Fig. 2). This band may be characterized by the eluate volume required to wash the band center from the bed—the retention volume R—and the width of the band w'. It can easily be shown that R is equal to $[(V_m/R_F) - V_m]$ for the case of an initially dry bed, or

$$R = WK. \tag{3}$$

If the bed is filled with moving phase before the sample is applied to the bed, then

$$R' = WK + V_m. \tag{3a}$$

Similarly it may be shown for elution chromatography that band width w' is related to R' and the theoretical plate number of the bed N by means of

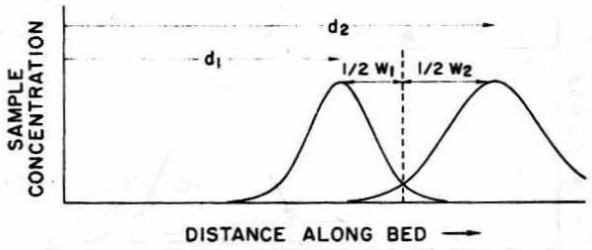
$$N = 16\ (R'/w')^2. \tag{1}$$

The similarity of Equations (2) and (4) should be noted.

SEPARATION OF ADJACENT SAMPLE BANDS

The object of chromatographic separation is the resolution of individual sample components. That is, we wish as little overlap of adjacent sample bands as possible, once the separation is completed. In order to understand the various factors which affect separation in a given case, let us consider the 2 adjacent sample bands of Figure 4. Here we assume that the 2 bands are

Fig. 4.—Resolution of two adjacent sample bands on the adsorbent bed.



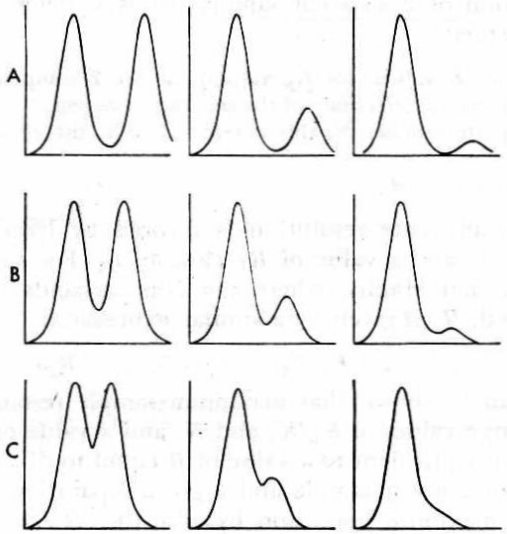


Fig. 5.—Separation of adjacent sample bands for different values of R_s: **A**, $R_s = 1.5$, **B**, $R_s = 1$, **C**, $R_s = 0.8$. (From Snyder [4], with permission of the publisher.)

still on the bed (as in Fig. 2) and that they are reasonably close together. The relative separation of the 2 bands obviously increases with increasing separation of their band centers $(d_2 - d_1)$. Separation is also better as the band widths w_1 and w_2 become smaller. We can define a resolution function R_s as follows:

$$R_s = \frac{(d_2 - d_1)}{\frac{1}{2}(w_1 + w_2)} \qquad (5)$$

When R_s is equal to 1, as in Figure 4, separation of the 2 bands is reasonably complete. Separation improves for larger values of R_s and becomes worse for smaller values. Figure 5 illustrates the resolution of 2 adjacent bands for different values of R_s and changing relative concentrations of the 2 compounds. Combination of Equations (1a), (2) and (5), with the assumption that the R_F values of the 2 bands are similar (i.e., $[d_2 - d_1]$ is small), leads to a more useful expression for R_s:

$$\begin{aligned} R_s &= \tfrac{1}{4}\,[(K_1/K_2) - 1]\,\sqrt{N'}\,\{K_2/[(V_m/W) + K_2]\} \\ &= \underset{(a)}{\tfrac{1}{4}\,[(K_1/K_2) - 1]}\,\underset{(b)}{\sqrt{N \cdot R_F}}\,\underset{(c)}{(1 - R_F)}. \end{aligned} \qquad (5a)$$

Here R_F refers to the value of R_F for either band 1 or 2 (the two R_F values are about the same). According to Equation (5a),

the resolution of 2 adjacent sample bands is determined by 3 separate factors:

a) the ratio of K values (or R_F values) for the 2 compounds; this is referred to as the *selectivity* of the separation system.
b) the bed plate number N; this is referred to as the *efficiency* of the bed.
c) the quantity $(1 - R_F)$.

In general, adequate resolution is favored by large values of K_1/K_2 and N, and a value of R_F close to 1/3. For resolution in elution chromatography, where the sample bands are washed from the bed, R_s is given by a similar expression:

$$R_s = \tfrac{1}{4}\,[(K_1/K_2) - 1]\,\sqrt{N}\,(1 - R_F). \qquad (5b)$$

Again it can be shown that maximum sample resolution is favored by large values of K_1/K_2 and N, and a value of R_F equal to 1/3 (this is equivalent to a value of R equal to $2V_m$). As a general rule, for a given sample and a given separation system, we will try to maximize resolution by adjusting R_F to a value of $\sim$ 1/3. In adsorption chromatography this is normally accomplished by a change of solvent (p. 13, 47), although changes in adsorbent or separation temperature are also capable of controlling R_F. If resolution is still inadequate after the capacity factor has been optimized, we are then forced to increase (K_1/K_2) (see Chap. III) or N (see Chap. IV).

SEPARATION OF COMPLEX MIXTURES

We have just seen that optimum separation of 2 adjacent bands is favored by an R_F value of about 1/3, or an R value of about $2V_m$. Often, however, we are confronted by a sample which contains many components, and in the general case these sample components will exhibit a wide range of K values (and a corresponding range of R_F or R values). This leads to a situation which has been referred to as the "general elution problem" (5), although it applies equally to separations on the bed or by elution from the bed. The general elution problem is illustrated in Figure 6 for the elution separation of a hypothetical 6-component mixture. In Figure 6, *A*, we have chosen separation conditions to maximize the resolution of bands *1* and *2;* i.e., the R values of these bands are close to 2 V_m. As a result, these 2 bands issue from the bed within a reasonable time as well-separated, sharp bands. However, the same set of separation conditions results in R values for bands *3* and *4* which are somewhat too large. These bands take an excessive time to clear the