BIOCHEMICAL SOCIETY SYMPOSIA No. 3

PARTITION CHROMATOGRAPHY

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1. INTRODUCTION

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'The invention of a new, specialized laboratory procedure brings about rapid conquests in new fields of science and technology; finally, it exhausts itself and is replaced by a still more practical method. The method of chromatographic adsorption, invented by the talented Russian botanist, Prof. M. Tswett, makes possible spatial separation of the components of a mixture. It is just now at the beginning of a brilliant development; it offers a simple experimental procedure to the investigator, especially in the field of both pure and applied organic chemistry, of biochemistry and of physiology.' Thus wrote L. Zeichmeister in the preface to the first edition of his important *Principles and Practice of Chromatography* (see Zeichmeister & Cholnoky, 1938).

The separation of chemical substances by chromatographic adsorption is in many cases preceded by partial fractionation of the original mixture by partition between immiscible solvents. Adsorption chromatography has now passed into common usage for innumerable chemical and biochemical purposes, and has been supplemented by the newer technique of partition chromatography, a linear descendant of the original Tswett technique, which forms the subject of the present Symposium. Since its introduction by Martin & Synge (1941) and more recent elaboration by Consden, Gordon & Martin (1944), this new method has invaded the vast majority of chemical and biochemical laboratories in this country and elsewhere. It has invaded, too, almost every field of biochemical research. The present Symposium includes discussions of applications of the method to studies of the chemistry and metabolism of amino-acids, proteins, carbohydrates and fats, as well as to the separation of purines and pyrimidines and some large and important groups of natural pigments. These represent only some of the more important applications, for few methods, probably, have developed so rapidly and in so many diverse directions. And, as the contents of the present volume make clear, the method is still far from exhausting itself. Indeed, new applications, new ideas and new technical devices are still being introduced.

In introducing this Symposium perhaps it is not out of place to point to a few of the reasons that have made the method of partition chromatography so important. Much of the total time expended on biochemical research is devoted, in one way or another, to studies of the relationship

between chemical structure and biological activity. So intimate and so profound is this relationship that many cases are known in which a slight change in its structure—a change which from a purely chemical standpoint might seem insignificant—will totally destroy the characteristic biological activity of a given chemical compound. Perhaps these facts seem less surprising nowadays than they did a decade or two ago, for much has been learned about enzymes and enzymic specificity in the interval. But the structure/activity relationship is nevertheless still of profound and even vital importance in many fields of biochemistry, pharmacology and physiology.

Substances of similar structure, while often differing fundamentally in biological activity, tend to exhibit similar chemical properties. Much of the biochemist's time and energy have, in the past, been necessarily spent in attempts to isolate, often from mixtures containing closely related compounds, some substance of biochemical importance. The classical methods of organic chemistry—fractional distillation, fractional crystallization and the like—have yielded many notable successes in the past, but are often extremely laborious and commonly involve the loss of considerable proportions of the starting material which, when it is biological in origin, is not infrequently in short supply. But comparatively small differences of adsorbability, or of solubility in pairs of immiscible solvents, frequently suffice to allow of separation, purification, estimation, recovery and isolation of one substance from a complex mixture by suitable chromatographic procedures. By an appropriate choice of adsorbents and solvents, and by modification of the quantities of these materials, it is usually possible to work with anything from a fraction of a milligramme upwards, so that chromatographic methods are valuable not only to the research worker who first isolates a few milligrammes of some new biochemical or pharmacological principle but also to the worker who studies its chemistry and metabolism on the larger scale, and even to the manufacturing chemist who eventually puts the product on the market. Small wonder, then, that chromatography has found a warm welcome in our hearts.

Several problems confront the worker who attempts to use the partition technique for a new purpose. In his choice of solvents he may find guidance in the partition coefficients of his compounds if these happen already to be known. In his search for a supporting medium for the stationary phase of his system he will probably try silica gels, starch or cellulose first of all, or filter paper if he proposes to operate on the very small scale. There then remains the development of suitable procedures for the detection and estimation of the substance or substances under investigation, and here classical methods can usually be put to service. Recourse may be had to measurements of pH, to titration,

to spectrophotometry, to refractometry, fluorimetry or what not. But beyond this it is still up to the investigator himself to carry out preliminary trials, often in large numbers, for the theoretical background of partition chromatography, like that of its adsorption counterpart, is still too rudimentary to allow of much in the way of accurate prediction. The development of new partition methods is still essentially a problem to be tackled at the bench rather than in the library.

I therefore welcome particularly the first contribution to this Symposium. It will, I know, be valuable to all of us to have, collected together and discussed, the general principles and the theoretical background on which we may hope in due time to build. Ultimately, as we gain knowledge and understanding, we may hope to be able to choose, on purely theoretical grounds, a system appropriate for the performance of any given task and thus to avoid the preliminary phases of trial and error which at present consume so much time and labour.

REFERENCES

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Zeichmeister, L. & Cholnoky, L. v. (1938). *Die Chromatographische Adsorptionsmethode*. Wien.

2. SOME THEORETICAL ASPECTS OF PARTITION CHROMATOGRAPHY

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It is intended in this contribution to interpret rather widely the theoretical aspects and to begin with a disquisition on the problems of purification in general, in order that partition chromatography may be seen in a framework of related methods, so that possible variants, not hitherto explored, may be easier to discern.

There are two fundamental problems in purification: (*a*) the separation of substances originally present in different phases and (*b*) the separation of substances present in the same phase.

(*a*) Many physical properties are employed in the separation of already pre-existing phases: size, shape, hardness, density, adhesiveness, electrical and magnetic behaviour. In general, in the separation of solid phases from one another, great purity is difficult to achieve, and chemical methods, changing the nature of some of the phases, have to be employed.

(*b*) Where substances are present in the same phase there are two types of separation possible, separation by distributing between different phases and separation by diffusional transport. The point does not have to be laboured that all 'chemical' methods of separation are means of changing the physical properties of the substances involved so that one or both of these basic methods may be used to greatest advantage. In diffusional processes the components move differentially under pressure, concentration or temperature gradients, or in centrifugal, electric or magnetic fields. It is characteristic of diffusional processes that the degree of enrichment achieved is dependent on the magnitude of the gradient, or the distances the components move in the fields. Some diffusional processes use membranes, whose function may range from preventing unwanted movements of fluids, when the membrane is equally permeable to all components, to acting as microsieves by manifesting permeability, maintaining in effect distinct phases more or less in equilibrium with each other, which but for the membrane would be miscible. The behaviour of such diffusional systems is more characteristic of the phase-change methods discussed below. But however the relative enrichment has been achieved the final operation is removal of material from regions of high and low relative concentration.

Most of the classical methods of purifying substances present in the same phase have involved phase changes or diffusional separation with semipermeable membranes in which a very high degree of enrichment was attainable in a single operation. It was upon a high enrichment factor that attention was concentrated, for when the factor was small, purification became an intolerably tedious and laborious process. Simple distillation many times repeated, or fractional crystallization carried out in long rows of dishes, are the examples perhaps most familiar.

Much of the apparatus of a normal laboratory, e.g. for filtration, distillation, centrifugation, is concerned with the separation of phases, within one of which the desired substance has become relatively enriched. But for a long time past and increasingly in the last few years, apparatus has been devised in which equilibration between phases can be many times repeated in a countercurrent manner, so that without performing separate operations, a relatively small enrichment factor gives rise to good separation. Absorption and extraction towers, distillation columns and countercurrent liquid-liquid extraction columns are common examples of such apparatus. This countercurrent principle is equally applicable to many diffusional separations, and has been widely used in isotope separations by diffusional and thermodiffusional methods. The first demonstration of the latter method by Clusius & Dickel (1938) had a brilliant and elegant simplicity.

The chromatogram is another simple and elegant, and much older, example of the application of the countercurrent principle to purification. But, in spite of its venerable history, it is still too modern for many laboratory and particularly industrial workers, who remain convinced of the virtues of stirring up the adsorbent with the liquid to be treated.

The examples of countercurrent apparatus given above cover most of the possible varieties of equilibrium between gaseous, liquid and solid phases. In one most important field however, that of fractional crystallization, no apparatus giving countercurrent enrichment has been produced, at least so far as the writer is aware. The problem does not theoretically seem to be insoluble. It seems probable that it is not necessary that crystals should be completely dissolved and reprecipitated; if they are finely enough divided washing is all that is required. If this be true one of the major problems of initiating crystallization in a reproducible way could be avoided.

Countercurrent apparatus if efficient in the widest sense involves the use of many stages of equilibration, or of their equivalent in continuous columns. The time taken to pass such 'theoretical plates' is therefore important, otherwise highly efficient apparatus will be too slow to be

used. Rapid equilibration can only be obtained by reducing the distance over which the molecules must diffuse to a minimum, and increasing the interface between the phases to a maximum. In fluid phases turbulence can reduce the diffusion distance. In solid or stationary fluid phases thin films or small particles must be employed. In the chromatogram the use of fine particles offers no obstacles, and fine particles, it should be noted, reduce the diffusion distance in the mobile phase as well as in the stationary phase. If the particles are large, clear-cut zones cannot be obtained, but this point is not appreciated to a surprising extent. Manufacturers and users of ion exchange resins often recommend and use particles of excessive size. There are, of course, adsorbents in which equilibration is slow even when they are finely divided; active carbon is a good example, and if the movement of a substance from one phase to another involves an activation energy this will always limit the rate of equilibration. If, for instance, tautomerization occurs, this will probably determine how fast a column (of any kind) can be operated. It is perhaps worth pointing out here that in the case of 'Frontal Analysis' due to Tiselius (1940), slow attainment of equilibrium is relatively less important than in other ways of using a chromatogram. The front may still be readily detected, though the estimation of quantity may become more difficult. In contrast, a similar slowness in attaining equilibrium may prevent displacement development being used at all.

FACTORS GOVERNING ENRICHMENT

Let us now turn to the enrichment factor that can be obtained by passing from one phase to another in equilibrium with it. If we restrict our discussion to ideal solutions, i.e. those obeying Raoult's law,

$$\mu_A^S = \mu_A^{S_0} + RT \ln N_A^S,$$

where μ_A^S is the chemical potential of the substance A, $\mu_A^{S_0}$ is the chemical potential in some defined standard state, and N_A^S is the mole fraction of A in the phase S.

If two phases S and M are in equilibrium the chemical potential of all components is, of course, the same in each. Thus

$$\mu_A^M - \mu_A^S = 0 = \mu_A^{M_0} - \mu_A^{S_0} + RT \ln N_A^M - RT \ln N_A^S,$$

or if

$$\mu_A^{S_0} - \mu_A^{M_0} = \Delta\mu_A,$$

$$\Delta\mu_A = RT \ln \left(\frac{N_A^M}{N_A^S} \right).$$

N_A^M / N_A^S is the partition coefficient (expressed in terms of mole fractions) $= \alpha$,

$$\ln \alpha = \frac{\Delta\mu_A}{RT},$$

and $\Delta\mu_A$ is equal to the free energy required to transport one mole of A from phase S to phase M.

Now to a first approximation $\Delta\mu_A$ may be regarded as made up of

$$d\Delta\mu_{CH_2}+e\Delta\mu_{COO^-}+f\Delta\mu_{NH_3^+}+g\Delta\mu_{OH}+\ldots, \text{ etc.},$$

the sum of the potential differences of the various groups of which the molecule A is composed. That is to say, to a first approximation the free energy required to transport a given group, e.g. CH_2, from one solvent to another is independent of the rest of the molecule. Thus all isomers containing the same groups (note that the degree of ionization, etc., must not be changed) would be expected to have the same partition coefficient.

Now, if we consider the partition coefficients α_A and α_B of two substances A and B which differ in that B contains, in addition to those contained in A, a group X, we have,

$$\ln\alpha_A=\frac{\Delta\mu_A}{RT}, \quad \ln\alpha_B=\frac{\Delta\mu_B}{RT}+\frac{\Delta\mu_X}{RT}, \quad \ln\left(\frac{\alpha_B}{\alpha_A}\right)=\frac{\Delta\mu_X}{RT}.$$

Thus the addition of a group X changes the partition coefficient by a given factor depending on the nature of the group, and on the pair of phases employed, *but not on the rest of the molecule.*

This is a prediction contrary to usual expectation. It is usually felt that the formation of a derivative of greatly increased molecular weight will 'swamp' any differences that exist and will render separation more difficult. This, however, is not to be expected if such a derivative be chosen that the same pair of phases can be employed while still maintaining convenient values for the partition coefficients.

Let us apply this rule to amino-acids and peptides. On the formation of a dipeptide molecule from two amino-acid molecules, or a tripeptide from an amino-acid and a dipeptide, one —CONH— group is created and one COO^- and one NH_3^+ are destroyed. Let the amino-acids be $A^{NH_3^+}_{COO^-}$ and $B^{NH_3^+}_{COO^-}$ and the peptide $NH_3^+.A.CO.NH.B.COO^-$, and let the partition coefficients be α_A, α_B and α_{AB} respectively,

$$RT\ln\alpha_A=\Delta\mu_{NH_3^+}+\Delta\mu_A+\Delta\mu_{COO^-},$$

$$RT\ln\alpha_B=\Delta\mu_{NH_3^+}+\Delta\mu_B+\Delta\mu_{COO^-},$$

$$RT\ln\alpha_{AB}=\Delta\mu_{NH_3^+}+\Delta\mu_A+\Delta\mu_{CONH}+\Delta\mu_B+\Delta\mu_{COO^-},$$

$$RT\ln\left(\frac{\alpha_A\alpha_B}{\alpha_{AB}}\right)=\Delta\mu_{NH_3^+}+\Delta\mu_{COO^-}-\Delta\mu_{CONH},$$

i.e. the product of the partition coefficients of the constituent amino-acids divided by the partition coefficient of the dipeptide is a constant for any given phase pair.

The predictions of this rule hold nearly as well as that for isomers. Thus a peptide *AB* should have the same value as peptide *BA*, and, in fact, they seldom differ by as much as 30 %, while the range of partition coefficients covered by the rule may be very large indeed (factors of thousands).

The ratio of solubilities of amino-acids in water and in various organic solvents has been treated similarly by Cohn & Edsall (1943). They extend the treatment to non-ideal solutions and give much experimental data.

It follows from the argument above that the chemical potential for a large molecule is correspondingly large. Hence the difference in potential in moving from one solvent to another, or to a crystal of the substance, will be very sensitive to changes in the solvent. This does not mean that it is easy to separate large molecules differing slightly by salting-out techniques where small changes in the solvent suffice to cause precipitation, but that where such techniques do succeed in purification, it is because there are large differences in the molecules.

A large enrichment ratio of one substance relative to another, on moving from one phase to another, implies a large difference in the changes of chemical potential of the two substances. If the molecules are closely similar such a difference can occur only if the two phases themselves are dissimilar in composition. In general, therefore, phases should be chosen as far as possible from critical solution composition; this approach is in most cases limited by insolubility of the substances under investigation or inconveniently high or low values of the partition coefficient.

It is possible in many cases to make a plausible guess as to the nature of the forces which are important in determining the partition coefficient. If we consider, say, a CH_2 group passing from water to *cyclo*hexane, the CH_2 group will have van der Waals forces between it and the water and between it and the *cyclo*hexane, probably not greatly different in magnitude, and it will also, while in the water, separate water molecules and in the *cyclo*hexane separate *cyclo*hexane molecules. Whereas the *cyclo*hexane molecules are attracted together only by van der Waals forces, the water molecules are attracted also by the relatively powerful hydrogen bonds. The hydrogen bonding of the water molecules to each other will thus be responsible for the high partition in favour of the *cyclo*hexane.

The van der Waals forces can be to some extent specific. Thus benzene will favour aromatic more than aliphatic substances, and in *cyclo*hexane this is reversed. Now the similarity of the melting- and boiling-points of these solvents show that the van der Waals forces are very similar in magnitude in them, but because of similarity of shape of some part of

it, an aromatic molecule may be expected to fit closer to a benzene than a *cyclo*hexane molecule and hence the van der Waals forces will be greater. The same argument applies, *mutatis mutandis*, to aliphatic solutes. Benzyl alcohol, compared to butanol, shows a similar preference for aromatic substances. The great affinity of active carbon for aromatic rings is noteworthy, the C—C spacings in graphite being very similar to those in benzene.

This specificity is a kind of pale reflexion of that so common in crystallization. When crystallization is a useful method of purification, it is because a foreign molecule cannot fit into or on to the crystal so closely, and hence the van der Waals or other forces holding the foreign molecules are not so great as those holding the molecules of the crystal proper. (As a kind of converse of this, crystal specificity might be used chromatographically; a column of some finely divided more or less soluble crystal, which could form mixed crystals with one of the substances to be isolated, being employed.)

For steric reasons the solvent molecules will in many cases be unable to approach closely, and this will reduce the energy of association of the solute with the solvent below that expected from consideration of the sum of the various chemical groups of the solute molecule. Large compact molecules, e.g. native proteins, are extreme examples. Such is, no doubt, the explanation of many of the deviations from the rules discussed above; various isomeric sugars or peptides can be separated on partition chromatograms which would be expected from the rules to be inseparable. Steric factors will be even more important in adsorption, where only one side of the molecule can be expected to be in contact with the adsorbent. It is therefore to be expected that adsorption chromatography will, in general, be more successful in the separation of isomers than partition chromatography.

Hydrogen bonds (Pauling, 1945) between the solute and the solvents are of great importance in determining the partition coefficient. They are far stronger than van der Waals forces (unless many atoms are concerned in the latter). Phenol and collidine, when saturated with water, are excellent solvents for substances capable of forming hydrogen bonds.

They are of opposite character in that phenol is a proton donor, while collidine is a proton acceptor. Water, of course, is both a proton donor and acceptor. Thus we find that the addition of an amino group has little influence on the partition between phenol and water, while it greatly changes the partition between collidine and water, in favour of the water. The amino group is a proton acceptor. Collidine, on the other hand, can accept a proton from an hydroxyl group, and thus the addition of an hydroxyl group makes little difference to the partition

between collidine and water, whereas an hydroxyl group displaces molecules from phenol to water. The carboxyl group contains both the proton-donating hydroxyl and the proton-accepting carbonyl group, and behaves, if the effects of ionization be allowed for, as would be expected, the hydroxyl character predominating.

In partition chromatograms using phenol as solvent, proline runs faster than valine. Using butanol, valine is faster than proline. Here the imino group of proline is a stronger proton acceptor than the amino group of valine, and phenol is a stronger proton donator than butanol. The more acidic character of phenol than of butanol and the more basic character of the nitrogen of proline than that of valine are further examples of the proton-donating or accepting character of these molecules. A similar phenomenon is shown by acetylproline and acetylvaline in chloroform and in *cyclo*hexane. Here the chloroform is the proton donor (cf. its formation of a maximum boiling azeotrope with the proton-accepting acetone) and acetylproline and acetylvaline run at the same speed. In *cyclo*hexane (or ethyl acetate) the acetylvaline runs very much faster.

Ionic bonds are stronger than hydrogen bonds. Where substances are weak acids or bases the degree of ionization is under control by changing the pH; since the partition in most solvent pairs for ionized molecules is very heavily in favour of the more polar phase, the overall partition coefficient is under control simply by changing the pH. When two substances of different pK are concerned the enrichment ratio is similarly a function of the pH. Partition chromatograms utilizing buffers as the stationary phase are now well established, and will be considered further below.

It is premature at the present time to attempt to give quantitative data for free-energy changes on moving various groups from one phase to another for the solvents commonly used in partition chromatography. Cohn & Edsall (1943, p. 212) have given values for the change between water and various liquids, e.g. formamide, methanol, ethanol, butanol and acetone. The values for the CH_2 group for, say, the collidine/water equilibrium phases (which are very temperature-sensitive, being near the critical solution temperature) will be smaller than those in the table given by Cohn & Edsall because of the high solubility of water in the collidine. The same is true to a less extent of the phenol/water equilibrium phases. Rough values for ΔF can readily be deduced from the table of R_F values given by Consden, Gordon & Martin (1944) if the assumption of a given ratio of solvent and water on the paper is made.

When silica gel, cellulose, starch or a synthetic resin is used to support the stationary phase of a partition chromatogram, it will only

in special cases be possible to eliminate adsorption on the molecules of the supporting solid. Thus in the first work with acetylamino-acids on silica gel (Martin & Synge, 1941), it was found that an alcohol of some kind had to be present to the extent of a few per cent in the chloroform before even approximate agreement could be obtained between the expected and the experimental R values. In the case of acetylphenylalanine and acetylleucine, it was very difficult to prepare sufficiently non-adsorptive precipitated silica.

In the case of starch and cellulose, Moore & Stein (1948) have demonstrated that adsorption of various amino-acids is appreciable and does, in fact, at least largely account for the deviations of the R values from those expected from the partition coefficients between water and the mobile solvent. Since hydrogen bonds play so large a part in determining the partition, it is to be expected that hydrogen bonds between the hydroxyl groups of the carbohydrate of the supporting solids and the solute will also contribute. The question as to whether or not adsorption occurs in a given case is, of course, of academic interest; it is not necessarily of practical interest. If the adsorption isotherm is linear no extra spreading of the zones will occur, and the adsorption may lead either to an increase or to a decrease of the separation of the zones.

It is possible to use kieselguhr instead of the supporting solids mentioned above. For very many substances kieselguhr is practically non-adsorbent, the skeletons of the diatoms consisting of relatively dense silica, not much more adsorbent than powdered quartz. The stationary phase is held between the spines and in the holes, essentially in droplet form, not as in silica gel in a network of submicroscopic crystals. It is possible that in most cases in which kieselguhr is the supporting solid for partition chromatograms they will behave pretty accurately as pure partition chromatograms. Further, and by far more important, their use should render possible partition chromatography of large molecules, proteins, polysaccharides and synthetic polymers, which could not be expected to be able to enter silica gel, or starch. It would not of course be surprising if, because of the large size of the molecule, adsorption were appreciable.*

* Kieselguhr columns cannot be packed as a slurry in the mobile phase as silica gel can, by simply pouring it into the chromatogram tube. The kieselguhr after mixing with half its weight of stationary phase (with a spatula in a beaker), can be packed with a ramrod into the tube. This method gives indifferent columns. A much better method is to slurry the mixed kieselguhr and stationary phase with enough mobile phase to give a creamy consistency. The slurry is poured into the tube and thoroughly homogenized by a few rapid strokes of a perforated disk, which is mounted by its centre on a long wire handle. The disk should fit the tube closely. The disk is brought to within about an inch of the bottom of the tube and then moved slowly downwards. This causes the kieselguhr to pack beneath it. Rapid strokes followed by a slow packing stroke are repeated until

Tiselius (1940) was the first to show clearly that chromatograms may be run in three distinct ways, viz. frontal analysis, elution development and displacement development. These methods of running are as applicable to partition as to adsorption chromatograms.

In elution development the permissible loading of the column is usually rather low, as the partition coefficient does not often remain constant over a wide range of concentration, and any change in the partition coefficient leads to a corresponding increase in the widths of the zones.

Displacement development can only occur when changes in partition coefficient occur in the presence of the solute of the following zone. In columns loaded with buffer or strong acids or bases a gradient of pH leads to displacement of weak bases or acids.

Since constancy of partition coefficient is not desirable in displacement columns high concentrations can be used, and the amount of material that can be handled on a column is very large. Adsorption is usually quite unimportant, even where in the corresponding elution column it is troublesome.

Since in displacement columns one zone follows immediately upon the heels of another, good separation demands that the column runs with the utmost uniformity. Complete separation on the column is valueless if the zones are so distorted that they cannot be cleanly separated. In elution development a zone of zero concentration can cover a certain amount of distortion. But Claesson (1947) and Hagdahl (1948) of the Tiselius school have worked out a technique whereby excellent separation may be achieved. The column is divided into several sections connected in series by short fine-bore tubes. Any irregularity in one section (within limits) can be corrected in the section below.

the whole column is packed. If the kieselguhr and stationary phase mixture is heavier than the mobile phase, a funnel with a wide tube attached to the top of the chromatogram tube will save constant refillings. If the mobile phase is the heavier a cork in the top of the chromatogram tube with a hole in it just large enough to provide easy passage for the handle of the disk is a useful aid. The columns when packed are robust and free from gas. Excess liquid may be poured off without fear of disturbance, and their regularity is at least as good as a precipitated silica column, and their efficiency in terms of a height of equivalent theoretical plate is probably higher. There is more resistance to flow of solvent through the column and a few pounds pressure is usually desirable. Whether vacuum or pressure be employed it must be emphasized that the solvent must be kept degassed if trouble with the separation of gas in the column is to be avoided. With thoroughly degassed solvents, gas always disappears from the column even if originally present.

The size of the holes in the perforated disk should be governed by the interfacial tension between the phases. Where this is high $\frac{1}{16}$ in. holes are satisfactory, when low smaller holes should be used, or packing becomes extremely slow. The interfacial tension probably governs also the proportion of stationary phase to kieselguhr that is satisfactory. When the interfacial tension is high more than one of stationary phase to two of kieselguhr may be used.

Possibly kieselguhr can also be used for reversed phase columns if it can be made 'unwettable' by treatment with a suitable silicone, or in some other way.

This technique is available only for frontal analysis and displacement development. If applied to an elution development column it would result merely in substituting a uniform column with poor separation for a non-uniform column with good separation without altering the degree of separation actually obtained. Displacement of material that has lagged behind is the essential part of the technique. The extent of the correction that can be made in each section decreases as the substances themselves become more difficult to separate.

THEORY OF PARTITION DISPLACEMENT CHROMATOGRAMS

The theory of displacement development has been developed only for adsorption columns. A rather different treatment seems desirable for partition columns, and though they could be considered as essentially continuous, the theoretical plate method is easier to understand (cf. Martin & Synge, 1941; Mayer & Tompkins, 1947).

We shall consider below displacement chromatograms loaded with NaOH or buffer. We shall assume that weak acids A, B, etc., are on the column which is being developed with another weak acid D, and that development has proceeded far enough for a steady state to have been reached. It is assumed that no ionized acid is present in the mobile phase.

Nomenclature

A^M = concentration of acid in mobile phase.

HA = concentration of unionized acid in stationary phase.

A^- = concentration of ionized acid in stationary phase.

$A = A^- + HA$ = concentration of total acid in stationary phase.

$\alpha = HA/A^M$ = partition coefficient of unionized acid. Similarly for various forms of acids B and D, and buffer acids P_1, P_2 assumed insoluble in the mobile phase.

H^+ = concentration of hydrogen ions in stationary phase.

$K_A = H^+A^-/HA$ = dissociation constant in acid.

M = cross-sectional area of mobile phase in column.

S = cross-sectional area of stationary phase in column.

T = cross-sectional area of total area of column.

R = ratio of rate of movement of zones divided by rate of movement of developing liquid in tube above column.

V_M = volume of mobile phase which passes a given zone in unit time.

V_S = volume of stationary phase which is passed by given zone in unit time $V_M/V_S = (T - RM)/RS$.

x = number of equivalent theoretical plates from top of column.

A_x = concentration of acid A in stationary phase leaving plate x.

$A^M_{(x-1)}$ = concentration of acid A in mobile phase leaving plate $(x-1)$.