151. A NEW FORM OF CHROMATOGRAM EMPLOYING TWO LIQUID PHASES

1. A THEORY OF CHROMATOGRAPHY

2. APPLICATION TO THE MICRO-DETERMINATION OF THE HIGHER MONOAMINO-ACIDS IN PROTEINS

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Introduction

In most forms of counter-current extraction column the very small drop required for the rapid attainment of equilibrium, and hence for high efficiencies, cannot be used owing to the difficulty of preventing it moving in the wrong direction. In the case of a solid, however, for any reasonable size of particle a filter will prevent movement in any undesired direction. Consideration of such facts led us to try absorbing water in silica gel etc., and then using the water-saturated solid as one phase of a chromatogram, the other being some fluid immiscible with water, the silica acting merely as mechanical support. Separations in a chromatogram of this type thus depend upon differences in the partition between two liquid phases of the substances to be separated, and not, as in all previously described chromatograms, on differences in adsorption between liquid and solid phases.

The difficulties of using chromatograms are very greatly lessened when the substances to be separated are coloured, or if colourless can be made visible. Various methods have been used for this [cf. Zechmeister & Cholnoky, 1936; Cook, 1941], though none of these was suitable for our problems. As the substances which we desired to separate were acids, and water was one of our phases, we were able to obtain visual evidence of the presence of any of these acids by adding a suitable indicator to the water with which the gel was saturated.

In the present paper we present an approximate theory of chromatographic separations, and describe an application of the new chromatogram to the micro-determination of the higher monoamino-acids in protein hydrolysates. This method is based on the partition of acetamino-acids between chloroform and water phases, and supersedes the macro-method described by us [Martin & Synge, 1941, 1], being rapid and economical both of materials and of apparatus.

Work is in progress, using ethyl acetate as the less polar phase in the chromatogram, on the separation of the acetyl derivatives of most of the other naturally occurring amino-acids, and the method promises also to be of use in analogous separations of simple peptides.

We wish to stress, however, that the possible field of usefulness of the new chromatogram is by no means confined to protein chemistry. By employing suitable phase pairs, many other substances should be separable. Where water is suitable as one of the phases, an indicator may be used to render visible the separation of organic acids or bases. Even where this is not possible, as with

neutral substances, the theory given below will enable 'cuts' of known partition coefficient to be taken. In the ordinary adsorption chromatogram optically active adsorbents have been employed for optical resolutions [cf. Henderson & Rule, 1937; Karagunis & Coumoulos, 1938], and resolution of racemic substances may be expected in the new chromatogram when either phase is optically active [cf. Bailey & Hass, 1941].

The mobile phase need not be a liquid but may be a vapour. We show below that the efficiency of contact between the phases (theoretical plates per unit length of column) is far greater in the chromatogram than in ordinary distillation or extraction columns. Very refined separations of volatile substances should therefore be possible in a column in which permanent gas is made to flow over gel impregnated with a non-volatile solvent in which the substances to be separated approximately obey Raoult's law. When differences of volatility are too small to permit of ready separation by these means, advantage may be taken in some cases of deviation from Raoult's law, as in azeotropic distillation.

Possibly the method may also be found to be of use in the separation of isotopes, e.g. of nitrogen isotopes, by passing ammonia gas over gel impregnated with ammonium sulphate solution [cf. Urey *et al.* 1937].

1. A THEORY OF CHROMATOGRAPHY

The chromatogram has for a long time been realized to be closely analogous in its mode of operation to distillation and extraction fractionating columns. No one, however, seems to have attempted to work out in detail the theory of the chromatogram using the concepts developed for distillation. This we attempt to do here, and thus to give a picture of the concentration of solute at any time and place in the column, and of the way in which the resolution depends upon the length of the column.

The behaviour of a column consisting of a number of 'theoretical plates', within each of which perfect equilibrium between the two phases occurs, can be described with great simplicity. Peters [1922] showed that the continuous or packed type of distillation column (in which equilibrium is not established at any point) could be divided up into a number of layers each of which was equivalent to one theoretical plate, and the height of such a layer was called the H.E.T.P. or 'height equivalent to one theoretical plate'. For the present purpose the H.E.T.P. is defined as the thickness of the layer such that the solution issuing from it is in equilibrium with the mean concentration of solute in the non-mobile phase throughout the layer. It can be shown from diffusion arguments that the H.E.T.P. is a constant through a given column except when the ratio of the concentrations of the solution entering and leaving the plate differs greatly from unity [cf. Sherwood, 1937]. It may be taken as constant for the chromatogram without serious error.

For the equations to be manageable certain simplifying assumptions must be made, viz. that the diffusion of solute from one 'plate' to another must be negligible, and that at equilibrium the distribution ratio of one solute between the two phases must be independent both of the absolute value of its concentration and of the presence of other solutes.

We consider here a chromatogram of the new type, having two liquid phases. The extension of the theory to the usual adsorption chromatogram is obvious

when the adsorption isotherm is a linear function of the concentration of solute in the liquid phase.

Consider a chromatogram of many 'plates':

Let h = the H.E.T.P.,

A = the area of cross-section of the column,

A_S = the area of cross-section of the non-mobile phase,

A_L = the area of cross-section of the mobile phase,

A_I = the area of cross-section of inert solid ($A_S+A_L+A_I=A$),

v = the volume of solvent used in development of the chromatogram,

α = the partition coefficient, i.e.

$$\frac{\text{g. solute per ml. of non-mobile phase}}{\text{g. solute per ml. of mobile phase}} \text{ at equilibrium,}$$

$V = h(A_L+\alpha A_S)$,

$$R = \frac{\text{movement of position of maximum concentration of solute}}{\text{simultaneous movement of surface of developing fluid in empty part of tube above chromatogram column}},$$

r = serial number of 'plate', measured from top of column downwards,

Q_r = total quantity of solute in plate r.

Consider the case where unit mass of a single solute is put into the first plate, and is then followed by pure solvent. We can draw up a table showing the quantity of solute in each plate after successive infinitesimal volumes of mobile phase δv have passed:

Vol. of solvent passed ($x\delta v$)	Serial number of plate				
	$r=1$	2	3	4	5
0	1	0	0	0	0
1	$(1-\delta v/V)$	$\delta v/V$	0	0	0
2	$(1-\delta v/V)^2$	$2(1-\delta v/V)\,\delta v/V$	$(\delta v/V)^2$	0	0
3	$(1-\delta v/V)^3$	$3(1-\delta v/V)^2(\delta v/V)$	$3(1-\delta v/V)(\delta v/V)^2$	$(\delta v/V)^3$	0
4	$(1-\delta v/V)^4$	$4(1-\delta v/V)^3(\delta v/V)$	$6(1-\delta v/V)^2(\delta v/V)^2$	$4(1-\delta v/V)(\delta v/V)^3$	$(\delta v/V)^4$

We see that the quantity in each plate is a term of the binomial expansion $((1-\delta v/V)+\delta v/V)^n$ so that when n successive volumes of solvent δv have passed,

$$Q_{r+1}=\frac{n!\,(1-\delta v/V)^{n-r}(\delta v/V)^r}{r!\,(n-r)!}.$$

Now when n is large, this becomes

$$Q_{r+1}=\frac{1}{r!}(n\delta v/V)^r e^{-n\delta v/V}.$$

But $n\delta v$ is the volume of solvent that has been used to develop the chromatogram. Putting $n\delta v=v$,

$$Q_{r+1}=\frac{1}{r!}(v/V)^r e^{-v/V},$$

which, by Stirling's approximation, becomes

$$Q_{r+1}=\frac{1}{\sqrt{(2\pi r)}}(v/rV)^r e^{r-v/V}, \qquad \text{.....(1)}$$

when r is large (>10).

Movement of band

Now Q_{r+1} is a maximum and equals $\frac{1}{\sqrt{(2\pi r)}}$ when $v/rV=1$, so that the position of maximum concentration has moved a distance hv/V directly proportional to the volume of liquid v which has flowed through. In terms of the movement of the surface of the liquid standing above the solid in the tube, the relative rate of movement R is given by the expression

$$R=\frac{vh/V}{v/A}, \text{ i.e. } \frac{\text{movement of band}}{\text{movement of surface}}$$
$$=\frac{Ah}{V}$$
$$=\frac{A}{A_L+\alpha A_S}=\frac{A_L+A_S+A_I}{A_L+\alpha A_S},$$

or
$$\alpha=\frac{A}{RA_S}-\frac{A_L}{A_S}. \qquad \text{.....(2)}$$

Separations ideally obtainable

If we plot the concentration of solute in plate $r+1$ against v/V using equation (1) we obtain the curve shown in Fig. 1. When r is infinite this curve

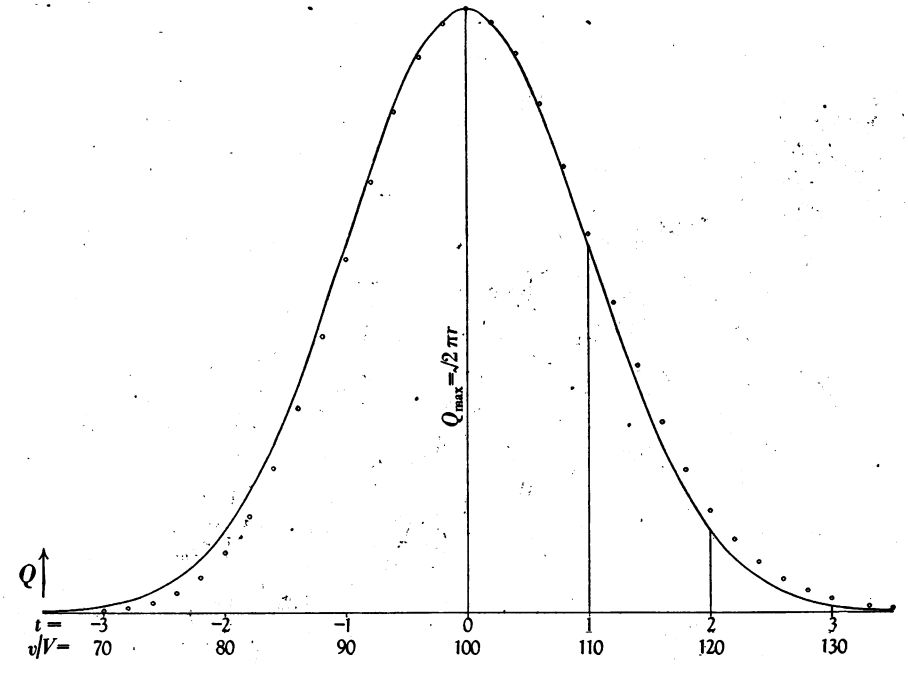

Fig. 1. The points represent the relation between Q and v/V for $r=100$. Cf. equation (1). The full line is the normal curve of error with abscissa t, i.e. $Q/Q_{max.}=\exp[-\frac{1}{2}t^2]$.

becomes the normal curve of error. When $r>100$ the ordinates of the curve can be expressed in terms of the normal curve of error by

$$\frac{v}{V}=r+t\sqrt{r}+\frac{t^2}{3}.$$

Similarly, the area under the curve can be expressed by

$$\frac{v}{V}=r+t\sqrt{r}+\frac{t^2}{4},$$

where t is the abscissa of the error curve.

Characteristics of the curve for various values of t are shown below:

t	$Q/Q_{max.}$	Area under tail of curve as % of whole area under curve
1	0·605	15·9
2	0·135	2·27
3	0·011	0·13

Now if two solutes be present with partition coefficients α and β, then practically complete separation will be obtained when $t=3$; then

$$\frac{A_L+\alpha A_S}{A_L+\beta A_S}=\frac{r-3\sqrt{r}+2{\cdot}25}{r+3\sqrt{r}+2{\cdot}25},$$

since only $<0{\cdot}2\,\%$ of the substance whose partition coefficient is β has passed the $(r+1)$th plate and $>99{\cdot}8\,\%$ of the other substance whose partition coefficient is α has passed it. Fig. 2 shows graphically the relation between $\frac{A_L+\alpha A_S}{A_L+\beta A_S}$ and r for various values of t, i.e. for various degrees of separation.

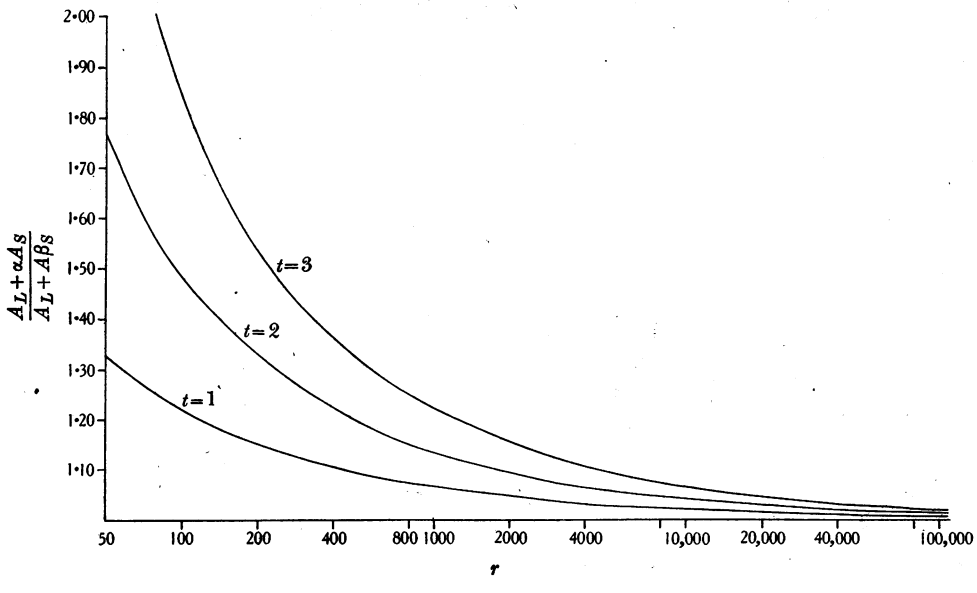

Fig. 2. Relation between difference in partition coefficient, number of plates (r) and degree of separation (t).

It follows from the assumption that the partition coefficient is a constant that there will be no interaction between various chromatograms occurring simultaneously in the same tube. Hence if, as in the practical case, the whole of the solute was not initially in the first plate, but distributed over a number, then the concentration at any plate and time will be the sum of the concentrations for the relevant number of chromatograms started successively. Thus the region of maximum concentration will be broadened, and the total width

of the band will be greater than that shown in Fig. 1 approximately by the initial width of the band before development by solvent was begun. The number of plates required for resolution will be correspondingly increased.

Factors influencing the H.E T.P.

The height equivalent to a theoretical plate depends upon the factors controlling diffusion and upon the rate of flow of the liquid. There is an optimum rate of flow in any given case, since diffusion from plate to plate becomes relatively more important the slower the flow of liquid and tends of course always to increase the H.E.T.P. Apart from this, the H.E.T.P. is proportional to the rate of flow of liquid and to the square of the particle diameter. Thus the smallest H.E.T.P. should be obtainable by using very small particles and a high pressure difference across the length of the column. The H.E.T.P. depends also on the diffusibility of the solute in the solvent employed, and in the case of large molecules, such as proteins, this will result in serious decrease in efficiency as compared with solutes of molecular weights of the order of hundreds.

Practical limitations of theory

The separations obtainable in practice are less than the theory predicts for two principal reasons. First, the partition coefficient is seldom a constant, usually decreasing as the solution becomes stronger. This results in the front of the band becoming steeper, and the back flatter and, more importantly, in the band becoming wider, since the concentrated part moves faster than the dilute part. This effect can sometimes be diminished by working with initially dilute solutions.

Interaction between two solutes often, however, leads to an increase in separation over the theoretical, the more strongly absorbed solute 'eluting' the less strongly absorbed, and thus tending to cause a sharp boundary between the two, reminiscent of the behaviour of ions in the moving boundary in transport number determinations.

The other great source of loss of efficiency lies in lack of uniformity of flow through the column. This lack of uniformity often prevents good separations being realized even though the solutes be separated in the column itself, as the cut cannot follow the required surface. In striving for conditions for uniformity of flow, the high pressure and small particle size desirable for smallest H.E.T.P. have to be abandoned.

Discussion

Apparently the only attempt at any theory of the chromatogram so far published is that of Wilson [1940]. He neglects, in his treatment, all diffusion and lack of equilibrium, i.e. in our nomenclature, assumes that h is zero. He attempts a general solution for the case that the partition coefficient (adsorption isotherm) is not constant (linear).

Unfortunately the discontinuous solution at which he arrives is in fact true only in the case in which the partition coefficient is a constant, and hence the greater part of his paper is invalidated. His equation for the rate of movement of the band reduces to the same as our equation (2) when the partition coefficient is constant; but as it stands it implies the absurd result that a square-fronted band is propagated unchanged while a band of any other shape changes shape during development.

It is undoubtedly possible to make closer allowance for the effects of diffusion than we have done here, but the simple picture of the working of the chromato-

gram is apt to be lost, and no very close approximation to practice can be obtained owing to the lack of constancy of the partition coefficient.

By employing the equivalent theoretical plate concept we are enabled to compare the chromatogram with other 'column' devices, and the astonishingly high efficiency of the chromatogram is made apparent. The H.E.T.P. is of the order of 1 cm. for the best distillation columns [cf. Podbielniak, 1941] and about the same for extraction columns [cf. Ney & Lochte, 1941]. As shown below, the H.E.T.P. in our chromatograms is of the order of 0·002 cm.

2. APPLICATION TO THE MICRO-DETERMINATION OF THE HIGHER MONOAMINO-ACIDS IN PROTEINS

Description of the chromatogram

Silica ('pure precipitated' B.D.H.) was boiled repeatedly with conc. HCl (which removed much iron etc.), then washed with distilled water and finally extracted with warm 97 % alcohol, which completed the removal of iron and removed some fatty material. The gel was then dried at 110° and was stored in a closed vessel.[1]

The gel was prepared for the chromatogram by adding 70 % *w/w* of a saturated solution of methyl orange in water. The solution is added to the gel at once, and thoroughly mixed in. A dry pink powder results, which barely adheres to the walls of the vessel in which it is mixed. An amount of this material containing 5 g. of silica is then suspended with stirring in about 35 ml. of chloroform saturated with water and containing 1 % *v/v* of *n*-butyl alcohol. At this point the colour of the gel changes from pink to yellow. The suspension is poured into a chromatogram tube (int. diam. 1 cm.; length 30 cm., furnished at its bottom with a double layer of filter paper mounted on a perforated silver plate). The gel packs down as the chloroform flows out at the bottom of the tube; being lighter than the chloroform it floats in it. When it has packed down to its final position, the top surface appears to be dry, and if evaporation is prevented, no further chloroform leaves the column, capillary forces preventing the entry of air. The gel does not float up again when fresh chloroform is carefully added at the top of the column. The solvent emerging at the bottom of the column is almost free from indicator, the methyl orange being firmly held in the aqueous phase.

The substances for analysis are dissolved in a little chloroform-butyl alcohol, and this solution is carefully added to the top of the column by allowing it to run from a pipette down the side of the chromatogram tube. When this addition and any washings have drained into the gel, the chromatogram is developed by adding fresh solvent at the top of the column.

The position of the acids is revealed by the indicator, which turns from yellow through orange to pink. As development proceeds the single pink band

[1] Owing to the variability of the B.D.H. silica, difficulty has been found in repeating the preparation given above, but the following method gives a reproducible and satisfactory material. Commercial waterglass (140° Tw—Jos. Crossfield, Ltd., Warrington) is diluted with 2 vols. water, and 10 *N* HCl is stirred in, with methyl orange as internal indicator. After standing several hours (more acid being added if required) it is filtered and washed with distilled water on a Buchner till washings are free from indicator. The gel is allowed to age, wet, for 1–2 days on the filter, and after further washing dried at 110°. For use in the chromatogram 50 % *w/w* of indicator solution is added to the gel.

at the top of the column resolves into constituent bands which move down the column at characteristic rates.

The movement of the acetamino-acids studied is powerfully affected by minor constituents of the solvent mixture. If the chromatogram is run with pure chloroform, the acids move extremely slowly and show little separation. This may be attributed to adsorption on the silica. When B.P. chloroform (containing 1 % of ethyl alcohol) is used, the acids move readily, presumably because they are eluted from the silica by the alcohol. The separation and recovery of acetamino-acids depend on the nature and quantity of the alcohol added to the chloroform. We have had our best separations with chloroform containing 0·5 % of *n*-butyl alcohol, but the improved recovery obtained when the butyl alcohol content is raised to 1 % has caused us to use this mixture for routine work. With B.P. chloroform, recoveries of about 70 % were obtained, with good separation.

It is possible, using 0·5 % butyl alcohol, to separate as their acetyl derivatives phenylalanine, *nor*leucine, *iso*leucine, leucine, proline and valine. With 1 % butyl alcohol the naturally occurring amino-acids separate into three groups, within which separation is imperfect: (i) phenylalanine; (ii) leucine-*iso*leucine; (iii) proline-valine-methionine.

The acetyl derivatives of alanine, glycine etc. remain at the top of the column, as slowly moving bands separated by a wide space from the substances mentioned above.

Test of relationship between rate of movement of band and partition coefficient

A column was started using a solution of 2 mg. each of acetyl-*l*-proline hydrate and acetyl-*dl*-phenylalanine. The column had 1 cm. diameter and length 20 cm. The column was developed with chloroform + 1 % *v/v* of *n*-butyl alcohol until the bands were well separated. The movement of the centre of each band for a given movement of the liquid surface above the column was then measured.

The column contained 5 g. of dry silica gel, 3·5 ml. of water and 10 ml. of chloroform phase. Hence, assuming the density of silica to be 2·3 g./ml., $A_I = 0{\cdot}11$ cm.^{2}, $A_S = 0{\cdot}175$ cm.^{2}, and $A_L = 0{\cdot}5$ cm.^{2}

From equation (2) the value of the partition coefficient may be calculated:

$$\alpha = \frac{A}{R A_S} - \frac{A_L}{A_S} = \frac{4{\cdot}5}{R} - 2{\cdot}8.$$

In Table 1 the values of α found thus, and by direct titration of the two phases after shaking together in a separating funnel are given for the two acetamino-acids:

Table 1

Acetamino-acid	R	α (determined from band rate)	α (determined directly)	c (mg. solute per ml. aqueous phase in direct determination)
Acetylproline	0·37	9·4	9·5	10
Acetylphenylalanine	1·07	1·4	1·3	2

The values for α as determined by the two methods are in good agreement. A comparison of the values given here with those given by Synge [1939] and by Martin & Synge [1941, 1] shows the great effect on the partition coefficient of the addition of 1 % of butyl alcohol.

In all cases so far investigated the rate of movement of the band becomes less the more dilute the solution, i.e. the partition coefficient becomes smaller

in strong solution [cf. Martin & Synge, 1941, 1]. The concentration must of course be similar to obtain concordant results by the two methods of determining the partition coefficient.

Measurement of H.E.T.P. for column

An estimate of the value of h was obtained from the width of an acetyl-*dl*-valine band in the course of its development. Fig. 3 shows the width of the band as measured, and the width calculated, assuming that $h=0{\cdot}002$ cm. and that the visual edge of the band occurred at $r \pm 2(\sqrt{r+w})$, where $2w$ is the initial width of the band as measured before development. The curves have been fitted to the appropriate time base to show the constancy of the rate of travel of the band.

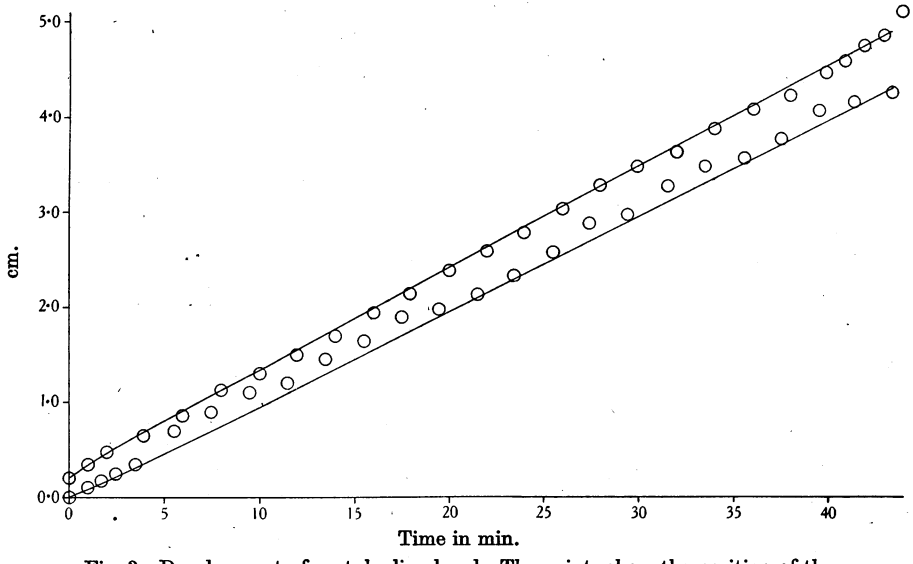

Fig. 3. Development of acetylvaline band. The points show the position of the band edges during development. The lines show calculated width.

There are three sources of error to be remembered here. The edge of the band is really measured at a definite concentration. This tends to make the width as measured less than the true value at high dilutions. Variation of the partition coefficient with concentration and diffusion from plate to plate increase the width of the band over that predicted by theory.

Though the value of h given above cannot be claimed as accurate, it is clearly of the right order.

Use of chromatogram for quantitative analysis

Tests with individual acetamino-acids showed that good recoveries were obtained from the chromatogram when used as described above, and it has therefore been adapted to the analysis of protein hydrolysates for these amino-acids. The preparation of the extract for the chromatogram is based on our earlier work [Martin & Synge, 1941, 1].

About 100 mg. of protein are hydrolysed as usual by refluxing for 24 hr. in 6*N* HCl, and the bulk of the HCl is removed by repeated evaporation with water *in vacuo*. The material thus obtained is taken up in water and made

alkaline to thymolphthalein by addition of 6*N* NaOH and is then concentrated *in vacuo* to a thin syrup. This is acetylated by addition of 10 ml. of 2*N* NaOH and 1 ml. of acetic anhydride in five equal portions in the course of 15 min. Between each addition the reaction vessel is thoroughly shaken and cooled with ice-water. After this the mixture is allowed to remain alkaline to thymolphthalein for 10 min.; 1 ml. of 10*N* H_2SO_4 is then added, and the solution is concentrated *in vacuo* below 40° to about 5 ml. It is then acidified to thymol blue by careful addition of further 10*N* H_2SO_4 (strong red colour with indicator). The solution is transferred to a separating funnel at a volume of 10 ml. with water, and is extracted with five successive batches of 50 ml. of chloroform. The chloroform extracts are filtered through paper into a distilling flask and are concentrated first at atmospheric pressure and finally *in vacuo* leaving a residue of acetamino-acids which is made up in alcohol to 10 ml. 3 ml. of this alcoholic solution (corresponding to about 30 mg. of protein) are evaporated to dryness *in vacuo* in a small Erlenmeyer flask, and stored overnight over H_2SO_4 and soda-lime in a vacuum desiccator, to effect removal of free acetic acid. The material in the flask is then transferred quantitatively to the chromatogram column in a minimum of chloroform-butyl alcohol, and the chromatogram is developed as described above with chloroform saturated with water and containing 1 % of butyl alcohol.

As each of the three main bands passes out of the bottom of the tube, the chloroform receiver is changed. The solution collected is evaporated *in vacuo*, and the residue is taken up in a little water and titrated with 0·01*N* $Ba(OH)_2$, using phenolphthalein as indicator. Care is taken to exclude CO_2 during the titration.

As a test of the method we employed the artificial mixture of ammonia and 14 amino-acids previously used by us [Martin & Synge, 1941, 2: Table 2]. This mixture had been refluxed with HCl, and was worked up exactly as if it were a protein hydrolysate. In a second experiment *l*-proline was added to the mixture. The results of these experiments are shown in Table 2.

Table 2. *Determination of amino-acids in artificial mixtures*

(Figures are N in % of total N of original mixture)

	Exp. I			Exp. II		
Amino-acid	Found	Calc.	Recovery %	Found	Calc.	Recovery %
Phenylalanine	2·1	2·6	81	1·9	2·4	79
Leucine	9·6	10·3	93	9·5	9·7	98
Proline	6·6	0·0	110	11·4	5·7	101
Valine		4·7			4·4	
Methionine		1·3			1·2	

In Exp. I material corresponding to 7·6 mg. N of the original mixture was put on the column. This quantity was excessive and caused broadening of the bands, with imperfect separation, which is reflected in the raising of the valine-methionine figure at the expense of the leucine figure. In Exp. II, material corresponding to 5·0 mg. N was put on, giving visually satisfactory separation of the bands, and this is reflected in the improved accuracy of the results.

We have also tested the method on a hydrolysate of the same sample of Merino 64's wool which we have previously analysed [Martin & Synge, 1941, 1] for the amino-acids in question. An amount of extract derived from wool containing 4·8 mg. of N was used for the determination. The results are compared with our previous figures in Table 3.

The reason for the lower recovery of phenylalanine than of the other amino-acids has not yet been explained. The control experiments described above were carried out under the conditions giving the best yields which we have so far obtained, i.e. with 1% of butyl alcohol in the chloroform. When the chromatogram is being used for the qualitative investigation of preparations, e.g. for homogeneity, and in preparative work, the use of chloroform containing a lower percentage of butyl alcohol gives superior separations.

Table 3. *Determination of higher monoamino-acids in Merino 64's wool*

(Figures are N in % of total N of wool)

Amino-acid	Present determination	Previous determination
Phenylalanine	1·9	1·9
Leucine-*iso*leucine	7·4	7·2
Proline	8·2	4·9
Valine		3·4
Methionine		0·4

The above determinations have been carried out on quantities of material corresponding to about 30 mg. of protein. By using a narrower chromatogram tube and micro-titration technique it is obvious that smaller quantities could be analysed with equal accuracy.

Summary

1. A new form of chromatogram is described, depending not on adsorption on a solid phase, but on partition of solutes between two liquid phases.

2. Visual detection of colourless acids in this chromatogram can be achieved by adding an indicator to one of the phases.

3. A general theory of chromatography applicable to substances with linear distribution isotherms is developed. This theory is based on the 'theoretical plate' concept, thus directly relating chromatography to fractional distillation and extraction.

4. The theory is checked experimentally in the new chromatogram.

5. The new chromatogram is applied to the micro-determination of the higher monoamino-acids in protein hydrolysates.

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