Penicillin: Analysis of the Crude Product by Means of a Modified Partition Chromatogram

1. THEORY AND USE OF THE CHROMATOGRAM

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(Received 1 September 1947; deposited with the Royal Society 31 October 1944)

Crude penicillin isolated from solvent extracts of metabolism solutions (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941; Abraham, Chain & Holiday, 1942), as a calcium, barium or sodium salt, is a yellow to brown solid which may contain well over thirty different acidic substances, mostly coloured. Still further weakly acidic and neutral substances remain in the residual solvent phase. The physical and chemical properties of the substances are such that chromatography provides the only likely method for the separation of the components of the mixture. So far, however, few adsorbents have been found which give satisfactory chromatograms. A modified partition chromatogram has been described (Catch, Cook & Heilbron, 1942) based on that of Martin & Synge (1941), but this chromatogram is capable of little development although considerable purification can be achieved.

The present paper describes work begun in 1942 upon a modification of the Martin & Synge (1941) partition chromatogram, which has been found suitable for substances of the nature of the acids in crude penicillin, and which can be developed. This chromatogram consists of silica gel impregnated with strong solutions of potassium phosphate of a suitable acidity. With this technique it has been possible to demonstrate in crude penicillin the existence of at least five chemical individuals with varying degrees of antibacterial power, although complete purification of any one of them by this method alone has not been achieved.

In the earlier stages of this work the position of the active zones among the numerous visible coloured bands could only be located by biological activity, the test results for which were not available until 24 hr. after the column had been run. Partition coefficient determinations in tap funnels under conditions comparable to those on the columns were, therefore, carried out and the results applied to the columns according to the theoretical treatment of Martin & Synge (1941). This proved a valuable guide to the approximate position of the main active zone, but as larger columns and heavier loads were used it became apparent that this treatment was inadequate,

as indeed its authors imply (Martin & Synge, 1941). By this time experience enabled us to locate the desired bands with some certainty from the appearance of the column, and from the position of a prominent orange band associated with the colour reaction described by Catch *et al.* (1942). On our column this band was substantially inactive and occurred a little below the main active zone. Still later the polarimeter was of great assistance because the biological activity was always associated with a strong dextrorotation, other constituents being usually laevorotatory or optically inactive.

It was clearly desirable to define as closely as possible the best conditions for carrying out various types of separations, since it was obvious that there was much overlapping of bands. In particular the separations of penicillins I, II and IV proved to be very difficult (cf. Part 2, Boon, Calam, Carrington & Freeman, 1944; Boon, Carrington & Levi, 1944). An attempt was made, therefore, to apply the recent theories of de Vault (1943) and Weiss (1943) to this type of column. A practical test of the theory was then made with phenylacetic acid and benzoic acid as model substances.

THEORETICAL TREATMENT

Consider a column prepared as described below of length L cm., and cross-sectional area A sq.cm. containing M g. of silica gel (sp.gr. D) impregnated with K ml. of a buffer solution. The column is initially filled with solvent:

Area of silica phase = $M/LD = I$.

Area of buffer phase numerically equal to volume of buffer/cm. of column = $K/L = P$.

Area of solvent phase numerically equal to pore volume/cm. of column = $A - I - P = S$ (Martin & Synge, 1941).

Consider a single solute added to the column in a volume of solvent v_0 , so that its concentration is C_0 .

Let V be the total volume of filtrate at any time, and v the volume of filtrate after the commencement of development where $v = V - v_0$.

Let x be the distance of any point from the top of the column.

Let C be the concentration of the solute at any point in the solvent phase, and $B (=f(C))$ be the concentration in the buffer phase.

For the partition chromatogram the differential equation deduced by de Vault (1943) becomes

$$\frac{\partial C}{\partial x} + \frac{\partial C}{\partial v} [S + Pf'(C)] = 0, \quad \text{where } f'(C) = \frac{dB}{dC} \quad (1)$$

or
$$x = F(C) + \frac{v}{S + Pf'(C)}, \quad (2)$$

where $F(C)$ is an arbitrary function to be defined by the initial conditions.

From this equation and a detailed consideration of the processes on the column analogous to that of de Vault (1943) and Weiss (1943) we obtain the following relations.

Initial width of band (x_1). This can be found from equation (2), or more simply as follows.

Of the total quantity of solute $v_0 C_0$ the amount in the mobile phase is $x_1 S C_0$, and the amount in the stationary buffer phase is $x_1 P f(C_0)$.

Hence
$$v_0 C_0 = x_1 S C_0 + x_1 P f(C_0)$$

or
$$x_1 = \frac{v_0 C_0}{S C_0 + P f(C_0)} = \frac{v_0}{S + P (B_0/C_0)}. \quad (3)$$

Lower edge of constant concentration zone (x_2). Since the concentration in the solvent phase at the front of the band is still C_0 the lower edge of the zone moves as though the original solution were still being poured into the column. Hence we can write, as for x_1 ,

$$x_2 = \frac{V C_0}{S C_0 + P f(C_0)} = \frac{V}{S + P (B_0/C_0)}. \quad (4)$$

Upper edge of constant concentration zone (x_3). For the point x_3 if volumes are measured from the commencement of development $F(C) = 0$ (de Vault, 1943) and equation (2) becomes

$$x_3 = \frac{v}{S + P f'(C_0)}. \quad (5)$$

Concentration in the 'tail'. Concentrations can only be conveniently measured as the band leaves the column, i.e. when $x = L$. Measuring v from the commencement of development as before $F(C) = 0$, and

$$L = \frac{v}{S + P f'(C)} \quad \text{or} \quad f'(C) = \frac{v}{LP} - \frac{S}{P}. \quad (6)$$

From this expression concentrations at any point in the 'tail', as it emerges from the column can be determined.

Volume (v_m) at which the constant concentration zone just disappears. When this occurs $x_2 = x_3$. On equating the expressions for these we obtain

$$v_m = \frac{v_0 C_0 \{S + P f'(C_0)\}}{P \{f(C_0) - C_0 f'(C_0)\}}. \quad (7)$$

It is seen that the theory of Martin & Synge gives an equation of the same form as (3) and (4) above, the only difference being the substitution of partition ratios for partition coefficients.

EXPERIMENTAL

Distribution experiments with benzoic and phenylacetic acids

Partition coefficients of phenylacetic acid at different acidities using concentrated buffer solutions. A stock solution of phenylacetic acid (2.72 g.; 20 m-equiv.) in damp diethyl

ether (100 ml.) was prepared. Of the solution 10 ml. required 18.5 ml. of approximately 0.1 N-NaOH. A series of buffer solutions covering the approximate range pH 4.5–7.5 was prepared by mixing suitable volumes of 25% (w/w) KOH and H_3PO_4 until the desired pH was obtained after dilution 1 in 4. The solutions were saturated with ether before use. Quantities of the stock solution (2 ml.) were diluted to 20 ml. with ether, and each was shaken with 20 ml. of one of the buffer solutions. The pH of the buffer phase was measured after the partition. Sample parts of the ether phase were titrated. The concentration of acid in the buffer phase was estimated by difference. The results are shown in Fig. 1.

Fig. 1. Relation between partition ratio and pH for phenylacetic acid in ether and strong buffer solutions.

Effect of concentration on distribution between the phases.

(a) *Phenylacetic acid.* The procedure was similar to that used in the previous experiment, with one buffer solution (pH 6.84) and solutions of phenylacetic acid in diethyl ether of varying concentration. Concentrations are conveniently expressed in m-equiv./l. The results are given in Table 1.

(b) *Benzoic acid.* The experiment was carried out in the same way as the one for phenylacetic acid using buffer solutions of pH 6.72. The results are given in Table 2.

Partition chromatogram with phenylacetic acid

Silica gel (Martin & Synge, 1941; Gordon, Martin & Synge, 1943) (90 g.; 90–160 mesh) was thoroughly mixed in a mortar with concentrated potassium phosphate buffer prepared as previously described (pH 6.36; 45 ml.) and the resulting, apparently dry, powder was suspended in ethylene dichloride (500 ml.) and poured into a chromatogram tube (3.68 cm. diam.). After the column had ceased to shrink (final dimensions 27 × 3.68 cm.) the ethylene dichloride was displaced with diethyl ether. Phenylacetic acid (2 g.) in diethyl ether (200 ml.) was then applied to the top of the column, and development was effected with moist ether; 200 ml. of filtrate were collected as a first fraction followed by further fractions of 20 ml. The concentration of phenylacetic acid in the eluate was determined by titration with standard $Ba(OH)_2$. For this column the following values were calculated by the method of Martin & Synge (1941):

Pore vol./cm. numerically equal to area of solvent phase (S) = 7.49 ml.

Vol. of buffer/cm. numerically equal to area of buffer phase (P) = 1.67 ml.

Table 1. *Effect of concentration on the distribution of phenylacetic acid between ether and concentrated buffer solutions*

(Acidity of buffer solutions was pH 6.82.)

Concentration in ether phase (m-equiv./l.)	log	Buffer phase (m-equiv./l. observed)	log	Buffer phase calculated from regression equation (m-equiv./l.)
2.66	0.4249	14.3	1.1553	15.2
5.8	0.7634	30.8	1.4886	27.6
15.2	1.1818	56.1	1.7490	57.4
15.3	1.1847	57.8	1.7619	57.7
19.7	1.2945	69.6	1.8426	70.0
44	1.6435	134	2.1271	128
104	2.0170	242	2.3838	245

Table 2. *Effect of concentration on the partition of benzoic acid between ether and concentrated buffer*

(Acidity of buffer solutions was pH 6.72.)

Concentration in ether phase (m-equiv./l.)	log <i>C</i>	Concentration in buffer phase (m-equiv./l.)	log <i>B</i>
27.6	1.44	69.3	1.84
59.8	1.78	123	2.09
168	2.23	239	2.38

Chromatograms with mixtures of benzoic and phenylacetic acids

Results of four experiments are illustrated in Fig. 2. The experiment illustrated in Fig. 2A was carried out with the same column as used for the above experiment with phenyl-

Fig. 2. Mixtures of phenylacetic and benzoic acids; partition chromatogram.

acetic acid. The other three were carried out with a single new column differing only slightly in dimensions but with a buffer solution of higher pH (6.7).

DISCUSSION

In order to evaluate the expressions obtained in the theoretical portion it was first necessary to evaluate $f(C)$, i.e. to determine the effect of concentration on the partition ratio. For benzoic and phenylacetic acids the experimental results can be satisfactorily expressed in the form $f(C) = B = aC^b$ resembling the Freundlich adsorption isotherm where a and b are constants for the particular substance and pair of phases. It probably has little theoretical significance, and only holds for a comparatively narrow range of conditions since at low concentrations the value of B/C should approach a constant value whereas the above expression would make B/C continue to increase with decreasing C , i.e. b is not truly constant, but tends to unity as the concentration decreases. Nevertheless, over the range of concentrations normally employed in chromatography, the expression is sufficiently accurate for most purposes.

The calculated regression line for the results given in Table 1 is $\log B = 0.86 + 0.76 \log C$, i.e. $B = 7.2 C^{0.76}$ at pH 6.84. At pH 7.4, $a = 16.98$, $b = 0.76$. The corresponding figures for benzoic acid at pH 6.72 are $a = 7.24$, $b = 0.68$. The value of b will probably normally lie well within the limits 0.5 and 1.0 (Note 1). The value of a is the value of B when $C = 1$, and approximates to the limiting partition ratio if suitable units are chosen.

The best fitting equation for the line shown in Fig. 1 is $\log (C/B) = 7.32 - 1.20 \text{ pH}$. By means of this result and the value $b = 0.76$ obtained above for phenylacetic acid it is possible to calculate $f(C)$ and $f'(C)$ for the chromatogram using phenylacetic acid. The appropriate expressions are $f(C) = 2.82 C^{0.76}$;

$f'(C) = 2.14C^{-0.24}$, and from these the following may be calculated:

Position of front edge of band: 250 ml. (from equation (4)),

End of constant concentration band: 436 ml. (from equation (5)).

These are in reasonably good agreement with the experimental results, particularly at the front edge of the band, as the small overlap shown in Fig. 3

Fig. 3. Phenylacetic acid on a partition chromatogram. Full line, observed; dotted line, calculated.

is due to the fact that fractions were taken at 240 and 260 ml., and thus would include some eluate containing no acid. This sharpness of the front edge of the band indicates that other factors (diffusion, non-attainment of equilibrium, etc.) are relatively unimportant. The observed tail end of the constant concentration zone appeared a little earlier, and the fall off in the tail was slower than anticipated. It is thought that this is almost entirely due to the practical impossibility of achieving sharp boundary conditions when changing from solution to pure solvent. Thus, even if there is little mixing of the layers of liquid where they enter the column, some solute will be washed down from the walls of the tube above the column, especially with volatile solvents. In earlier experiments with this technique it was usual to add washings from the various vessels to the column, after the bulk of the charge had been added, for the sake of a good recovery. This was clearly a mistake and made the banding unnecessarily diffuse.

The results for the chromatograms of mixtures of benzoic and phenylacetic acids are of particular interest since the difficulty in separating acids was of about the same degree as the separation of penicillins I and II. In experiment A not only is

the pH low, but the initial volume is too great so that the bands largely overlap. The higher pH and lower concentrations of experiments B, C and D proved much more effective (Fig. 2). These experiments were not carried further as ample examples of the practical application of the technique were obtained during the course of its use with crude penicillin (see Boon, Calam, Gudgeon & Levi, 1948).

It is probable that it will seldom be of value to apply the above equations in practice (especially since no account has been taken of the effect of one band on another) except in the case of colourless substances, or for the purposes of identification, and indeed our main interest in the theory was to define the best conditions for the treatment of the very complex mixture in crude penicillin. In this case, before the isolation of the pure substances, $f(C)$ cannot be determined so that a rigid application of the theory is impossible. Thus, as will be shown in later papers, many of the constituents occur close together on the column, and the theory was of assistance in defining conditions under which they could be separated.

Some of the practical consequences of the theory are set out below:

(1) On a given column the initial width of a band depends mainly on the total volume of the starting solution (equation (3)) relative to the size of the column. If the equation is written in the form

$$x_1 = \frac{v_0}{S + (PB_0/C_0)} = \frac{v_0}{S + PaC_0^{b-1}},$$

and taking the values quoted above for b , it is seen that for a given v_0 the band width will vary only slowly with change of concentration. The band width will be less at higher pH values (increasing a).

(2) As a corollary to this, to separate two substances whose partition relations lie close together on a given column, a small initial volume and a high pH will give the best conditions. It will be preferable that the constituent in greater amount should be the less soluble in the buffer phase, since the rate of movement of the band is increased by increased concentration.

(3) It is desirable that the material in a band should be mainly concentrated at the head of the band, and not too spread out into the 'tail', i.e. v_m in equation (7) should be a maximum. (It is not, however, essential that v_m should not be exceeded.) Substituting the values for $f(C)$ and $f'(C)$ in equation (7) we have:

$$v_m = \frac{v_0 C_0 S}{PB_0(1-b)} + \frac{v_0 b}{(1-b)}.$$

This expression is a maximum when v_0 is large, and when the pH is low. These conditions are the opposite of those in (2) above, so that in practice compromise conditions must be sought. Prolonged

development will give a wider separation at the front edges of the bands, but owing to the increased diffuseness the total separation may not be greatly increased in some cases. In any case it is clear that development should not be unduly prolonged.

An interesting feature of the above expression is that it is independent of the diameter of the column. This gives a definite advantage to a short wide column over a long narrow one owing to its greater practical convenience in working. This must not be overdone, however, as too rapid a solvent flow gives diffuse columns, due mainly to the fact that attainment of equilibrium is not instantaneous.

(4) Provided suitable conditions of quantity, concentration, partition ratio and degree of development are chosen, a single solute should give a band after development on a column with

- (a) a sharp front edge, followed by
- (b) a constant concentration zone, and
- (c) the concentration in the solvent phase in equilibrium with the constant concentration zone should be equal to the concentration of the initial solution.

If a substance is found to give, for example, a band with a maximum near its top edge then it is not homogeneous. It is assumed, of course, that the banding on the column is regular.

Practical illustrations of the above considerations will be found in the subsequent papers of this series. They will apply *mutatis mutandis*, to all types of chromatogram except that, in the case of partition

chromatograms of this type, the effect of one band on another will always be considerable because solution of the acids in the stationary phase will lower the pH. On the other hand, in many cases the direct adsorption of one substance by, for example, alumina is apparently not greatly affected by the adsorption of another.

Partition chromatograms of this type are clearly not limited to acids. With suitable buffers, bases should be separable (cf. Catch *et al.* 1942). It might also be possible, for example, to separate mixtures of aldehydes and ketones, using columns impregnated with bisulphite solution. The necessary conditions for a separation are clearly an adsorbed stationary phase and a moving phase, between which some or all of the substances to be separated can be differentially and reversibly distributed.

SUMMARY

1. A modified partition chromatogram for the separation of acids is described. It is based on that of Martin & Synge (1941), but concentrated potassium phosphate buffer solutions replace water as the stationary phase.

2. The theories of de Vault (1943) and Weiss (1943) are shown to give an adequate account of the behaviour of a single solute on such a chromatogram.

3. The effect of concentration on the distribution of phenylacetic acid or benzoic acid between diethyl ether and concentrated buffer solutions is expressible by an equation of the same form as the Freundlich adsorption isotherm.

Addenda (1947)

(1) The limits suggested for likely values of b in the equation $B = aC^b$ are too narrow. Acids have been encountered for which values of $b < 0.5$ have been found.

(2) The work originally intended to be published as part 3 in this series will be published in a forthcoming monograph on penicillin chemistry.

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