Gas-liquid Partition Chromatography: the Separation and Micro-estimation of Volatile Fatty Acids from Formic Acid to Dodecanoic Acid

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Industry has long used charcoal or other solid absorbents in columns for cleaning gas streams or for solvent recovery, and more recently Turner (1943), Claesson (1946), Glueckauf, Barker & Kitt (1949), Phillips (1949) and Turkel'taub (1950) have used charcoal in gas chromatograms for the analysis of hydrocarbons and esters. Gas-liquid scrubbing columns have been used in industry for many years but, though Martin & Synge (1941) suggested the use of gas-liquid partition chromatograms for analytical purposes, no work has been reported along these lines.

This paper presents modifications necessary to the theory of Martin & Synge (1941) to allow for the compressibility of the mobile phase and describes the application of the gas-liquid partition chromatogram to the separation of volatile fatty acids. The separations obtainable by this method are essentially parallel to those obtainable by distillation, but good separations can be achieved much more easily and it is possible to work with very much smaller quantities. In fact, the lower limit of quantity of material used is determined only by the efficiency of detection. In general, far less trouble from azeotrope formation is to be expected, since the concentrations of the substances to be separated in the liquid phase are always low and it may be possible to choose a liquid phase which associates only with one component of the azeotrope. Work on a preparative scale, though theoretically possible, is likely to be inconvenient because of the bulk of the apparatus. The method of detection described here is acid-base titration, but many methods of detecting changes in the composition of a gas stream could be used and it is intended in future publications to explore some of these, which should extend the range of application to all substances capable of being distilled at the pressure of a few mm. of mercury.

In suitable cases the gas-liquid partition column has two principal advantages over the ordinary liquid-liquid partition column: (*a*) the low viscosity of the mobile phase allows relatively longer columns to be used with a corresponding gain in efficiency and (*b*) in general it is easier to detect changes in composition of a gas than of a liquid stream.

A THEORY OF GAS-LIQUID PARTITION CHROMATOGRAPHY

The theory of gas-liquid chromatography differs from that of liquid-liquid chromatography, where a constant partition coefficient is assumed (Martin & Synge, 1941), only by virtue of the fact that the mobile phase is compressible and thus produces a gradient of gas velocity down the column. It is assumed that the partial pressures of the substances to be separated are negligible in relation to that of the carrying stream of gas. Let

p_1 = pressure of gas applied to chromatogram.
p_0 = pressure of gas at outlet.
p = pressure of gas at point distant x from the outlet.
v = linear velocity of gas at point x.
l = length of column.
t = time which elapses before the centre of the zone emerges from the column.
F = volume of gas emerging from outlet in unit time.
$V_R = tF$ = the retention volume of the centre of the zone.
V_R^0 = limiting value of V_R as p_1/p_0 tends to unity.
a = the area occupied by the gas phase in any cross-section of the column.
K = column constant, a function of the viscosity of the gas phase and the tightness of the column packing.

Then
$$K\frac{\mathrm{d}p}{\mathrm{d}x}=\frac{Fp_0}{p}=av.$$

Integrating to give the pressure along the column:

$$K\frac{p^2}{p_0}=2Fx+Kp_0,$$

or
$$K=\frac{2Fxp_0}{p^2-p_0^2},$$

or
$$F=\frac{p_0K}{2x}\left[\left(\frac{p}{p_0}\right)^2-1\right]. \quad (1)$$

Now
$$t=\int_0^l\frac{\mathrm{d}x}{vR_F}=\int_0^l\frac{ap\,\mathrm{d}x}{R_F\,p_0\,F}. \quad (2)$$

Here R_F has the usual chromatographic meaning (Consden, Gordon & Martin, 1944), that is, it represents the rate of movement of the zone of the substance under consideration relative to the rate of flow of the moving phase. In the present case the rate of flow of the moving phase increases continuously as it advances down the column, but the R_F remains constant and the rate of movement of the zone increases correspondingly. The R_F is a constant depending only on the substance to which it refers, the temperature, the nature and amount of the stationary phase and, in so far as the support is not inert, on its amount.

Rewriting Eqn. 2 in terms of pressures,

$$t=\int_{p_0}^{p_1}\frac{Kap^2\,dp}{R_F F^2 p_0^2}=\frac{Ka\,(p_1^3-p_0^3)}{3R_F p_0^2 F^2},$$

$$V_R=Kap_0\frac{[(p_1/p_0)^3-1]}{3R_F F}.$$

Hence, by (1),

$$V_R=\frac{2}{3}\frac{al}{R_F}\frac{(p_1/p_0)^3-1}{(p_1/p_0)^2-1}. \quad (3)$$

As p_1/p_0 tends to unity then V_R tends to $V_R^0=al/R_F$. In fact, the flow rate V is measured at room temperature and should be corrected to the column temperature.

Since the value of R_F is not dependent on p_0 it is not possible by reducing p_0 to reduce the temperature of operation of the column. Further, since the viscosity of the gas is independent of the absolute pressure, when p_0 is low, the ratio of the pressures at the two ends of the column becomes excessive if a reasonable rate of flow is to be maintained. Under normal conditions it is convenient for p_0 to be atmospheric pressure. It might, however, be preferable to increase p_0 by a factor of approximately 10 if a very long column is to be used in order to effect a difficult separation. This is due to the fact that the absolute pressure drop from one end of the column to the other for a given retention volume will be independent of p_0; consequently it will be possible to work at a low ratio of p_1/p_0 even with a very long column.

Test of general theory

Test of relationship between pressure, flow rate and retention volume. An experimental test of the theory given above was carried out by the procedure which is described later in the paper. A 4 ft. silicone-stearic acid column was set up and a series of separations of acetic, propionic, *n*- and *iso*-butyric acids carried out at four different pressures. The flow rates were measured in each case and from the graphs the retention volume of each acid band was measured.

The theoretical ratio of flow rates is given by (1):

$$\frac{F_1}{F_2}=\frac{(p_1/p_0)^2-1}{(p_2/p_0)^2-1}.$$

In Table 1 the theoretical ratios of the flow rates are compared with those experimentally determined; agreement is within expected error.

Table 1. *Comparison of calculated with experimentally determined flow rates in a gas-liquid partition chromatogram operated at different pressure ratios*

(The rates in runs *a–d* (Table 2) are compared. For basis of calculation see text.)

	Flow-rate ratios	
	Calc.	Found
a/b	1·44	1·46
a/c	1·92	1·96
a/d	3·36	3·21

From Eqn. 3 it can be seen that as the ratio of the pressures at the two ends of the column tends to unity the factor $\frac{(p_1/p_0)^3-1}{(p_1/p_0)^2-1}$ tends to 3/2. At this limiting pressure ratio the retention volumes of the bands tend to a limit V_R^0. In Fig. 1 the values of p_1/p_0 are plotted against the value of the ratio V_R/V_R^0; by means of this graph the experimental value of V_R can be corrected to V_R^0. In Table 2 these values of V_R^0 are shown for four experiments carried out at different values of p_1/p_0. It can be

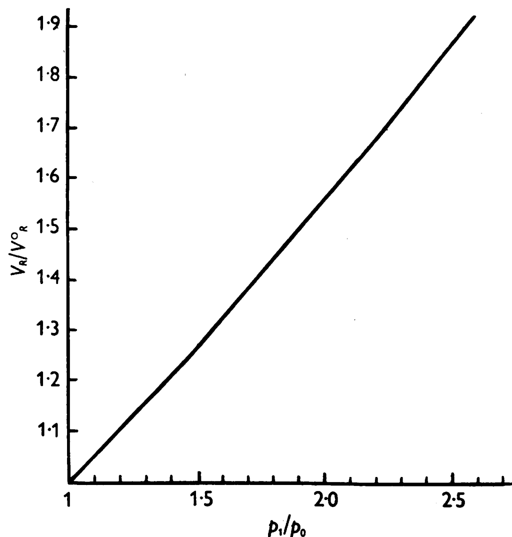

Fig. 1. The relationship between the pressure ratio across the column and the ratio between the observed retention volume and the retention volume at zero pressure difference across the column.

Table 2. *Correction of retention volumes to their limiting values* (V_R^0) *for runs at different pressure ratios*

Run	Pressure of N_2 applied to column (cm. Hg)	p_1 (N_2 pressure plus atmospheric pressure, p_0, in cm. Hg)	p_1/p_0	V_R^0 (ml.) Acetic acid	Propionic acid	*iso*Butyric acid	*n*-Butyric acid
a	76·0	150·91	2·014	203	466	758	952
b	57·1	132·0	1·762	193	457	749	932
c	45·7	120·61	1·647	194	455	740	922
d	28·9	103·81	1·385	193	464	763	954

seen that the figures agree within 5%, a fairly satisfactory confirmation of the theory, since the assumption is made that the column is uniformly packed.

Derivation of column efficiency. Two methods of calculating column efficiency from the experimental curves are given in order to allow comparison with one another and with data from liquid-liquid columns.

Method 1

Let there be r plates in the column.

Assume that all the material (M equivalents) is put on in the first plate. Then the quantity occurring in the last plate at the maximum concentration is $M/\sqrt{(2\pi r)}$ equivalents (see Martin & Synge, 1941).

Let q be the fraction of the original quantity occurring in any plate, and let the time taken for the centre of the zone to reach the last plate be t min.; then the time taken for contents of last plate to escape $=t/r$ min.

Then $Mq_{\text{max.}}=M/\sqrt{(2\pi r)}$ equivalents and $Mq_{\text{max.}}\,r/t=$ max. rate of escape = max. slope of the experimental curve $=S$ equiv./min.

Then $S=Mr/t\,\sqrt{(2\pi r)}$ equiv./min.

Therefore $r=2\pi S^2t^2/M^2$.

Thus by measuring the maximum slope of the effluent concentration curve, the time to arrive at the maximum slope and the total amount of the material in the zone, the plate number of the column and hence the height equivalent to a theoretical plate (H.E.T.P.) can be obtained. H.E.T.P. is defined for the chromatogram by Martin & Synge (1941).

Method 2

The area between ordinates one standard deviation on either side of the peak of an error curve is 68·3% of the total. From the theory given by Martin & Synge (1941) the shape of the chromatographic zone should approximate to an error curve and 68·3% of the material should be included between plates $\pm\sqrt{r}$, where r is the total plate number.

The ratio of time taken for the centre of the zone to emerge (t) to the time taken for the middle 68·3% of the zone (τ) is equal to $r/2\sqrt{r}$.

Hence $r=4t^2/\tau^2$.

A comparison of figures obtained by the two methods for the same column gives fair agreement (Table 3), the agreement increasing with the plate number.

Table 3. *Comparison of column efficiencies obtained by two independent methods*

(For methods 1 and 2 see text.)

Flow rate (ml./min.)	Plate number calculated by Method 1	Method 2
35	550	400
18·2	718	600
10·3	765	730

FACTORS INFLUENCING THE DEGREE OF SEPARATION

Column efficiency

Since diffusion in the gas is so much more rapid than in the liquid, diffusion in the liquid phase will normally be the factor limiting efficiency. At very slow rates of flow lengthways diffusion in the gas along the tube becomes relatively important, and then the efficiency is no longer increased by reducing the rate of flow. Table 4 shows the change of efficiency with flow rates above this limiting value.

Table 4. *Variation of column efficiency with flow rate*

Flow rate (ml./min.)	Efficiency (calc. by method 1) (no. of plates)
35	580
24	540
18·2	720
10	740

The H.E.T.P. shows slight variations from column to column due to differences in the uniformity of packing and of dispersion of the liquid phase. The plate number of any column is proportional to its length, the 4 ft. columns described later having plate numbers varying from 700 to 1200 plates and an 11 ft. column had a plate number of 2000, which might have been greater had it not been for the increased amount of dead space at the bends necessitated by the method of construction.

When diffusion in the liquid phase is the limiting factor, the H.E.T.P. is inversely proportional to the diffusion constant and hence an increase in temperature will lead to an increase in efficiency as the diffusion constant increases. An experimental test showed an efficiency of 365 plates at 65° and an

efficiency of 720 plates at 100° with the same column. This would suggest that in many cases the efficiency of liquid-liquid partition columns could be increased by raising the temperature (cf. Mayer & Tompkins, 1947).

Some variation of efficiency with the nature of the liquid phase will occur because the diffusion constant of the solute in the liquid phase will usually be inversely proportional to the viscosity of the liquid; hence fluids of low viscosity should in general be used.

Drake (1949) has shown that increasing the diameter of a chromatographic column leads to some loss of uniformity of front, and his results could be roughly covered by the assumption that the front made a given angle to the axis of the column irrespective of the column diameter. Since, however, with the gas-liquid columns the ratio of length to diameter is very large, moderate increases in diameter should be without measurable effect on efficiency; in fact one 4 ft. column of diameter 1·2 cm. had an efficiency of 850 plates, while with those of 4 mm. diameter the efficiency ranged from 700 to 1200 plates (at 100°).

Change of R_F

Inasmuch as in a homologous series the higher members will have larger latent heats of vaporization, their change of vapour pressure with temperature will be larger than with the lower members, so that the factor of separation will decrease as the temperature increases. This point is demonstrated in Table 5.

Table 5. *Variation of relative R_F values with temperature*

Ratio of retention volumes	Temperature of column		
	65°	100°	137°
$\frac{V_R \text{ propionic}}{V_R \text{ acetic}}$	2·9	2·3	2·1
$\frac{V_R\ n\text{-butyric}}{V_R \text{ propionic}}$	2·5	2·0	1·8
$\frac{V_R\ n\text{-butyric}}{V_R\ iso\text{butyric}}$	1·34	1·27	1·20

Relative R_F values can be altered by changing the nature of the liquid phase. For example, replacement of the stearic acid in the columns described later by ricinoleic acid causes a disappearance of the α-methylbutyric-*iso*valeric acid and the $\alpha\alpha$-dimethylpropionic-*n*-butyric acid separations without markedly impairing the separation of *n*- and *iso*-acids. Incorporation of orthophosphoric or pyrophosphoric acids in the liquid phase of the silicone-stearic acid columns has a similar effect, but also decreases all R_F values, at the same time decreasing the ratio between them. This effect can no doubt be attributed to an increase in internal pressure of the liquid phase; a similar but more pronounced effect has been noticed in the separation of amines in the comparison of columns with liquid phases of hendecanol or glycerol. In this case the difference in pressure is so great that an inversion of order with length of chain is observable (James, Martin & Smith, 1952).

APPARATUS

Preparation of the column

Kieselguhr (Celite 545, Johns Manville Co. Ltd., obtainable from Imperial Chemical Industries Ltd.) is size-graded by repeated suspension in water in a beaker 18 cm. high and rejection of all fine material that does not settle out in less than 3 min. The resultant coarse-grade material is heated in a muffle for 3 hr. at 300° and then washed with conc. HCl to remove iron and basic impurities. After repeated washing with water to remove acid the kieselguhr is oven-dried at 145°. The liquid phase consisting of DC 550 silicone (Albright & Wilson Ltd., Oldbury, Birmingham), containing 10% (w/w) stearic acid, is added to the kieselguhr (0·5 g. of liquid phase to 1 g. kieselguhr) and the mixture is well stirred.

4 ft. column. A 5 ft. length of 4 mm. internal diameter glass tube is drawn down at one end to form a short length of thick-walled capillary (*F*, Fig. 2). A small wad of 'Fibreglass'

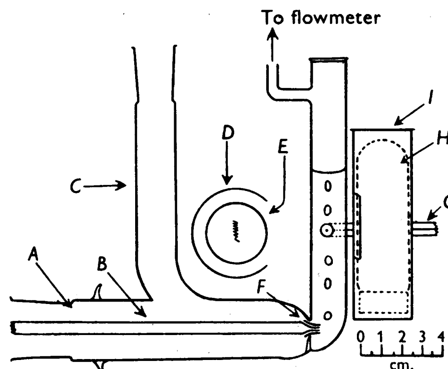

Fig. 2. Side view of titration-cell unit. *A*, vapour jacket; *B*, chromatogram; *C*, air-condenser socket; *D*, lamp house; *E*, lamp; *F*, rubber gasket on capillary; *G*, capillary lead from burette; *H*, photoelectric cell; *I*, photoelectric-cell housing with adjustable window.

yarn (900/1/41 obtainable from Fibreglass Ltd., Piccadilly, London) is pushed down the tube up to the capillary to prevent it being blocked by the kieselguhr. A small funnel is attached to the tube by a short length of pressure tubing and is then half filled with the kieselguhr mixture: with the column approximately vertical it is pressed against the rotating shaft of an electric motor (7000 rev./min.), the shaft having a flat machined on it. When all the kieselguhr has run into the column, the tube is pressed against the motor shaft until the kieselguhr packs down tightly, and this is assisted by a 'Telcothene' plunger attached to a length of steel wire. This process is repeated until 4 ft. of the column is

packed and a second wad of 'Fibreglass' is pushed down on to the packing. A short piece of cycle-valve tubing is pushed on to the capillary end of the tube and acts as a gasket to prevent leakage from the titration cell into the vapour jacket.

11 ft. column. This column is constructed of three lengths of packed tubing joined by hairpin bends of thick-walled capillary. In this way the whole column can be accommodated within the same vapour jacket as the 4 ft. column. This principle can be used for very long columns if a wide vapour jacket is used.

the bubble past the graduations is timed with a stopwatch. An error of less than 1 % is possible over a wide range of flow rates.

The manostat by-passes to the atmosphere any excess gas coming from the nitrogen cylinder through a filter paper fixed with sealing wax to the bottom of the vent tube, when the mercury level is depressed beneath it. The pressure at which this occurs can be varied by adjustment of the level of the mercury reservoir. 'Hunting' is prevented by tightening the screwclip until critical damping occurs.

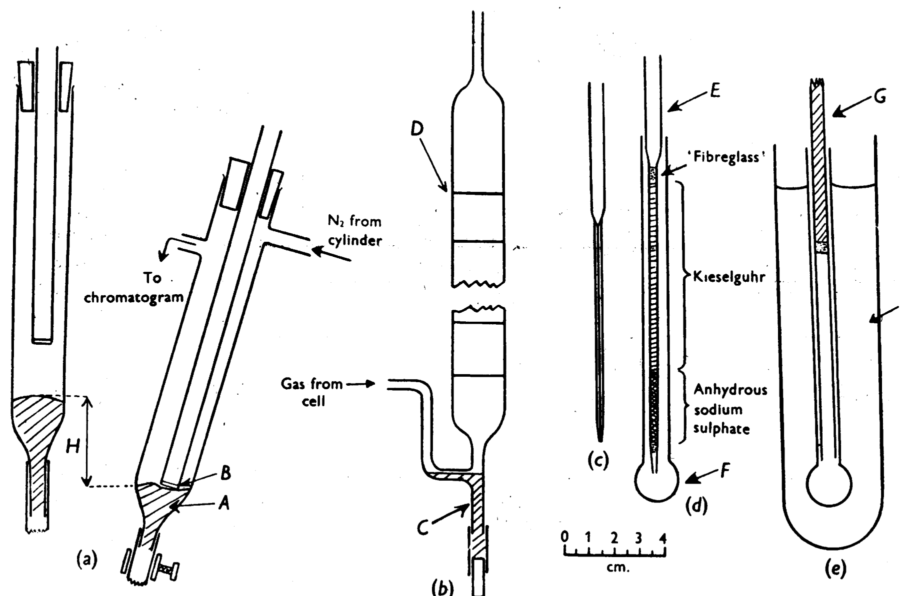

Fig. 3. (*a*) Manostat. *A*, mercury; *B*, vent tube fitted with filter-paper disk; *H*, effective nitrogen head. (*b*) Flowmeter. *C*, ammonium stearate solution; *D*, 10 ml. graduations. (*c*) Micropipette. (*d*) Extraction of free fatty acids into ether. *E*, partition column; *F*, test tube. (*e*) Displacement of fatty acids on to a gas-phase column. *G*, gas-phase chromatogram tube; *I*, heating bath.

Vapour jacket and titration cell

The vapour jacket, a horizontal 4 ft. 1 in. diam. glass tube with a ground-glass joint fitting the titration-cell unit (Fig. 2), is attached at the other end to the boiler by a ground-glass joint on a side arm. The chromatogram is supported in the jacket by a rubber bung and in the titration cell unit by the valve-tubing gasket (Fig. 2). The gasket, being rubber, limits the choice of liquid in the boiler; methanol (b.p. 65·4°), water (b.p. 100°), ethylene glycol monoethyl ether ('cellosolve') (b.p. 137°) and ethylene glycol (b.p. 200°) are suitable liquids. An air condenser is used for the high-boiling liquids. Phenol red was found to be the most suitable indicator in the titration cell for both visual (0·001 %) and automatic (0·01 % (w/v) aqueous solution) control of the titration.

Flowmeter and manostat (Figs. 3*b*, *a*)

These are both of conventional design.

A strong solution of ammonium oleate is used in the flowmeter; when the rubber tube is squeezed the solution rises into the side tube so that a bubble is formed. The passage of

Recording burette (Fig. 4)

The burette consists of a horizontal glass tube with a 'Telcothene' (Telegraph Construction and Maintenance Co. Ltd., Telcon Works, Greenwich, London, S.E. 10) gland (*I*) at one end through which passes an 0·125 in. diam. stainless-steel rod (*E*). A swelling on the burette tube where it passes through the end plate of the apparatus is insulated with a short length of rubber tube and is held in position by a small clamp (*J*), in this way providing a firm, but slightly flexible, attachment. A similar device allows pressure to be applied to the polythene gland without danger of cracking the tube. The other end of the burette is connected to two valves (*K*) consisting of a short length of pressure tubing whose bore is blocked by a short plug of glass rod; the valves may be opened by tightening a screwclip on to the plug. These valves have been found to be more reliable than glass taps. One valve leads to a reservoir fitted with a guard tube of soda lime, and the other to the titration cell through a length of capillary tube.

The free end of the stainless steel rod is attached to a carriage (*H*) sliding on two horizontal 0·5 in. steel rods (*C*). A lead screw (*D*, 1 mm. pitch, O.B.A. thread) between these

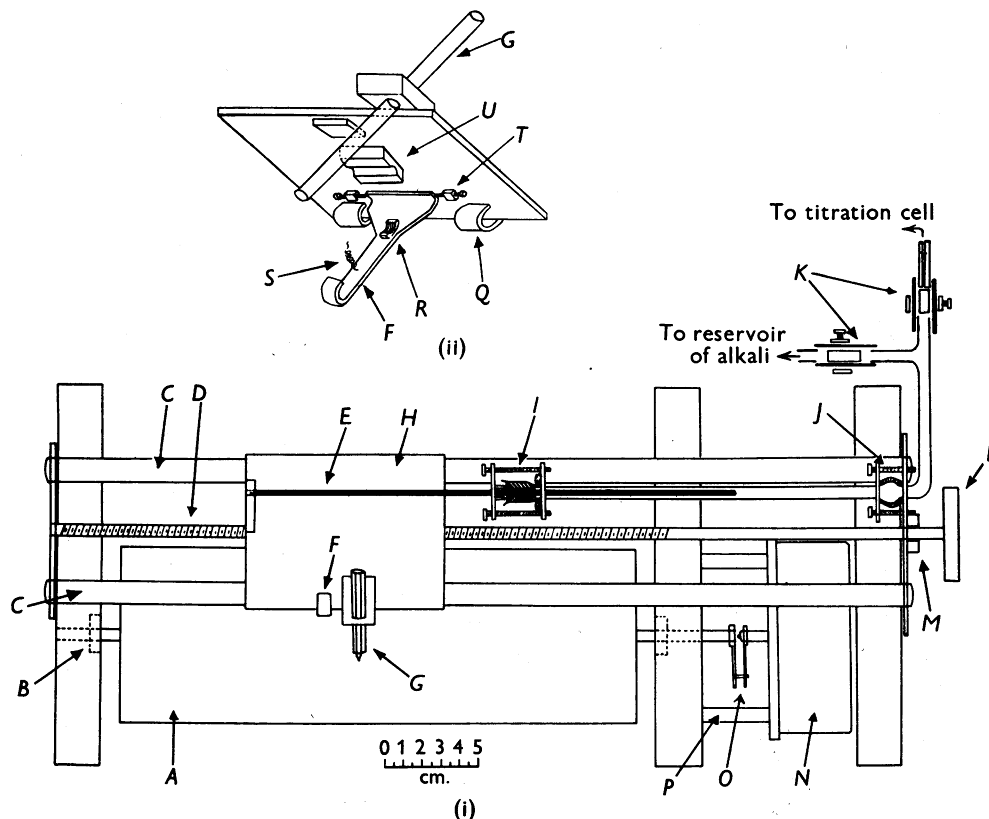

Fig. 4. (i) Plan view of automatic burette: *A*, rotating drum; *B*, drum-shaft bearing; *C*, steel rods; *D*, lead screw; *E*, stainless-steel rod; *F*, arm to disengage lead screw; *G*, pen; *H*, carriage; *I*, 'Telcothene' gland; *J*, burette-tube clamp; *K*, valves; *L*, pulley; *M*, lead-screw bearing; *N*, electric-clock movement; *O*, drum drive coupling (only one fork is shown); *P*, clock spacers. (ii) Underside view of carriage (flat springs holding front runner against rods are not shown): *F*, arm bearing half nut; *G*, pen holder; *Q*, carriage runners; *R*, half nut; *S*, spring to hold half nut against lead screw (shown disengaged from the carriage); *T*, pivots of half-nut support; *U*, 'Tufnol' (Tufnol Ltd., Perry Barr, Birmingham) support for lead screw.

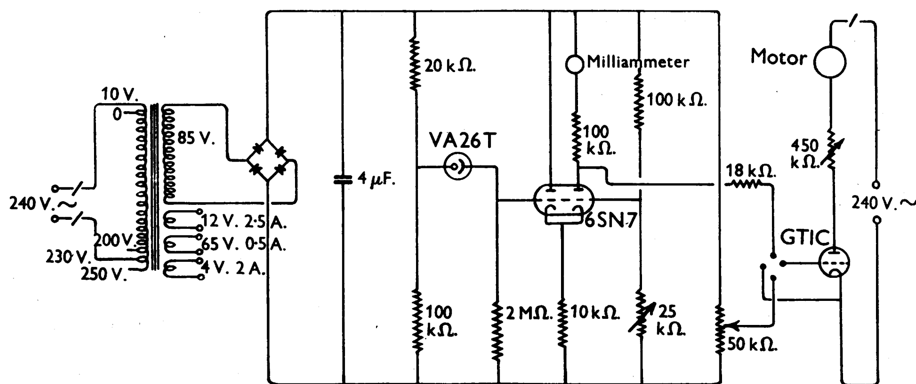

Fig. 5. Circuit of photoelectric control unit.

bars engages with a half nut on a plate pivoted at the back of the carriage (F, ii). By the depression of this plate against the action of a spring (S, ii) the half nut can be disengaged from the screw to allow rapid filling of the burette. A drum rotates on an axle driven by an electric-clock movement (N, Movement 55558/55, 10W. rating, English Clock Systems Ltd., 179 Great Portland Street, London, W. 1) parallel to the lead screw, and the position of the carriage at any time is recorded by a ball-point pen (G). The pen is held in a weighted tube (22 g.) which slides freely in a tube (G, ii) attached to the carriage, which makes an angle of 70° to the tangent at the point of contact with the drum. Round the drum, whose circumference is 30 cm., a piece of cm.-ruled graph paper is retained by means of two endless bands of 0·125 in. helical spring which can be readily rolled off the paper to the ends of the drum in order to change the paper. The drum can be rotated either once per hr. or once per 12 hr. by adjusting the position of a pin on a crank at the end of the drum shaft to engage either with a fork on the minute shaft of the clock (O) or with another fork on the hour shaft which rotates outside the minute shaft. In the diagram only the inner fork is shown.

The recording burette can be used manually by a handle on the lead screw. It is a very great convenience to use such apparatus instead of other types of burette and a stop-watch. Where the apparatus is expected to be in constant commission the photoelectric control unit will quickly repay the labour involved in its construction.

Photoelectric control unit

The circuit used is shown in Fig. 5. The tube GTIC is a gas-filled triode. The photoelectric cell (V.A. 26T Cinema-Television Ltd.) is predominantly green sensitive and consequently no filter is necessary when used with phenol red or methyl red as indicator. The photoelectric cell is provided with an adjustable window so that the area of the titration cell scanned can be varied. The lamp used to illuminate the cell is a 12V. (20W.) car bulb. The lamp and photocell are appropriately housed so that a minimum of daylight falls on to the cell, but so that visual observation of the titration is possible. The motor driving the lead screw is fitted with a reduction gear so that the maximum speed of the lead screw is of the order 30 rev./min.; a series rheostat is inserted in the motor circuit so that critical damping of the titration can be obtained.

APPLICATION TO THE SEPARATION OF THE VOLATILE FATTY ACIDS

Experimental procedure

The chromatogram supported in the vapour jacket is maintained at a temperature such that the saturated vapour pressures of the acids to be separated lie between 10 and 1000 mm. Hg. The temperatures used are 100° (boiling water) for acids from formic to valeric acid and 137° (boiling cellosolve) for acids from valeric to dodecanoic acid. The mixture of acids free from water is applied by means of a micropipette to the 'Fibreglass' plug in contact with the column packing, the N_2 supply from the manostat is attached, the electric clock driving the recording drum is started, and 8 ml. of aqueous phenol red solution are run into the titration cell. If very low rates of flow are used additional stirring is provided by a stream of N_2 through a stainless-steel capillary tube. The photoelectric control unit is then switched on. The acids emerge in the gas phase and are absorbed in the water in the titration cell where they are continuously titrated, and simultaneously the titration curve is automatically plotted. The curve obtained is the integral of the conventional chromatogram peak (cf. Moyle, Baldwin & Scarisbrick, 1948); the height of each step gives directly the amount of acid, each cm. corresponding to 3 μg. equiv. throughout these experiments, 0·038 N-NaOH being used in the burette. This method of detection of the acids by titration in an aqueous medium limits the chain length of the acids that can be separated; with acids of chain length greater than C_{12} the columns would have to be run at a temperature of 180–200° in order to obtain a reasonable retention volume. The maximum possible temperature of the titration cell is 100° (using water) and with a temperature drop of 80–100° the acids will condense inside the capillary at the end of the column and so be prevented from reaching the titration liquid. Titration in non-aqueous media has not in our experience proved satisfactory.

RESULTS

The theory given above assumes that the partial pressures of substances to be separated are negligible; this may not always be so in practice. When they are not negligible the shapes of the zones are distorted because the velocity of gas is appreciably higher in the regions where the partial pressure is high, and this results in regions of high concentration tending to overtake regions of low concentration so that the zone will have a sharp front and a correspondingly longer tail. However, in all the examples quoted below, the partial vapour pressure (estimated from the maximum slope of the recorded step) can be neglected as it does not exceed 5 mm. Hg.

When the solutes obey Henry's law the partition coefficient between the two phases will be independent of concentration and the titration curve obtained will have a symmetrical sigmoid shape. Complete resolution of two zones will result in a plateau between the two curves. Any deviation from Henry's law will cause a deviation, of step shape, from the expected sigmoid curve.

Curve A (Fig. 6) shows the curve obtained in an attempted separation of propionic, *iso*butyric and *n*-butyric, and *iso*- and *n*-valeric acids on a column at 100°, having as liquid phase the high boiling-point silicone DC 550. The breaks in the curve indicate the ends of the different zones. The extended front and sharp tail of the zones of the higher acids is presumably due to dimerization in the liquid phase causing regions of low acid concentration (relatively high monomer to dimer ratio) to move more rapidly down the column than regions of high acid concentration (relatively low monomer to dimer ratio). This is because the partial pressure of the acid in the gas is nearly proportional to the concentration of the monomer in the liquid phase.

Incorporation of a long-chain fatty acid of low vapour pressure in the liquid phase will decrease the concentration of monomer by association to such a value that the deviation from ideal zone shape caused by dimerization will be much less. Curve *B* (Fig. 6) shows the result obtained when stearic acid (10 % w/w) is incorporated in the silicone liquid phase. The acids are now completely resolved and the *iso*valeric acid zone is shown to consist of two components, the second of which proved to be α-methylbutyric acid ('active' valeric acid). This result was obtained with all commercial samples of '*iso*valeric' acid tested (cf. Conrad & Bischoff, 1880; Kahlbaum, 1894). The increased retention volumes are a measure of the effectiveness of the volatile acid-stearic acid association and indicate

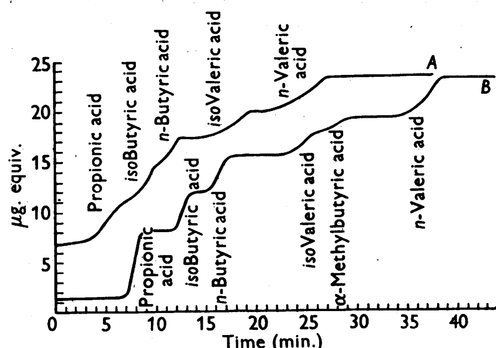

Fig. 6. The separation of propionic, *iso*butyric, *n*-butyric, commercial '*iso*valeric' and *n*-valeric acids. *A*, column length, 4 ft.; liquid phase, DC550 silicone; nitrogen pressure, 30 cm. Hg.; flow rate 23 ml./min.; column temp., 100°. Incomplete resolution of all the acids. *B*, column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 46 cm. Hg; flow rate, 45 ml./min.; column temp., 100°. The '*iso*-valeric' band has been almost completely resolved into two components.

a diminution of self-dimerization. Most commercial samples of stearic acid contain appreciable amounts of the lower homologues so that a freshly prepared column loses acid continuously to the titration cell. In so far as the rate of loss of acid is constant, it does not give rise to error in the estimation, but the step has to be measured against a sloping base-line. By running the column at a temperature 50° higher than the normal working temperature for a few hours the volatile impurities can be effectively removed. With pure stearic acid the loss of acid is very small, and in addition the liquid in the titration cell rapidly becomes saturated and thereafter no further titration of the stearic acid entering the cell occurs.

The lower curve (*A*) of Fig. 7 shows the separation of acetic, propionic, *iso*butyric and *n*-butyric acids on a similar silicone-stearic acid column at a smaller flow rate. The upper curve (*B*) is obtained by graphical differentiation of the lower curve and shows a change of zone shape along the series. It is clear that the zones still deviate from the theoretical

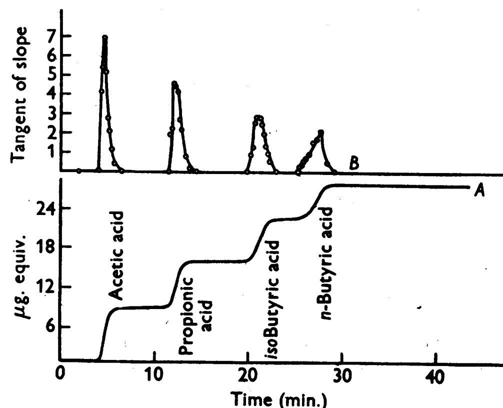

Fig. 7. The separation of acetic, propionic, *iso*butyric and *n*-butyric acids. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 48 cm. Hg; flow rate, 33 cm./min.; column temp., 100°. *A*, experimental curve. *B*, differential of experimental curve.

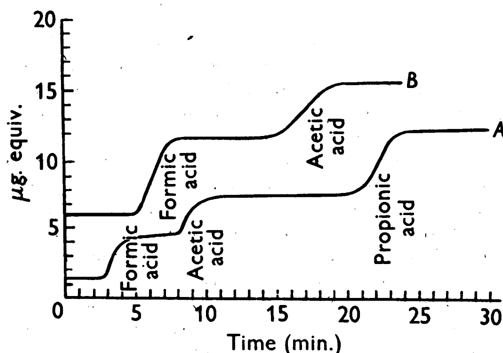

Fig. 8. *A*, the separation of formic, acetic and propionic acids showing tailing of formic acid band. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 25 cm. Hg; flow rate, 15 ml./min.; temp., 100°. *B*, the separation of formic and acetic acids, showing nearly symmetrical formic acid band. Column length, 4 ft.; liquid phase, stearic acid (10% w/w), orthophosphoric acid (10% w/w) in DC550 silicone; nitrogen pressure, 70 cm. Hg; flow rate, 50 ml./min.; temp., 100°.

shape. The acetic acid and propionic acid zones have sharper fronts than tails, the *iso*butyric acid zone is almost symmetrical and the *n*-butyric acid zone has a sharper tail than front. The sharp fronts of the acetic and propionic acid zones are to be attributed to adsorption on the kieselguhr since when the acids were run on a column containing no liquid phase

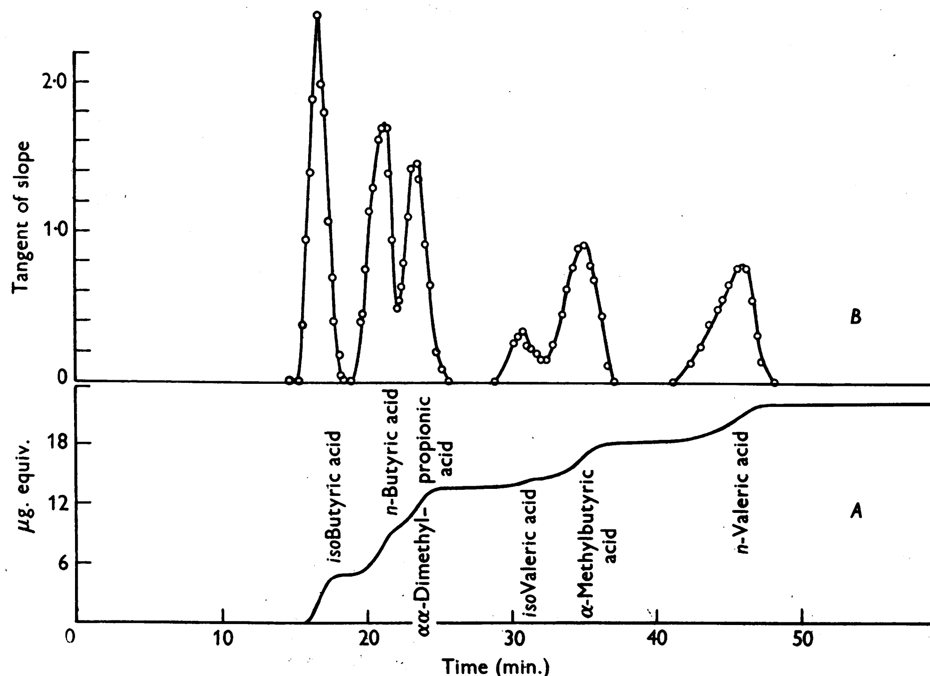

Fig. 9. The separation of the isomers of valeric acid from *n*-butyric and *iso*butyric acids, showing incomplete resolution of αα-dimethylpropionic and *n*-butyric acids and of *iso*valeric and α-methylbutyric acids. *A*, experimental curve. *B*, differential of experimental curve. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 44 cm. Hg; flow rate, 43 ml./min.; temp., 100°.

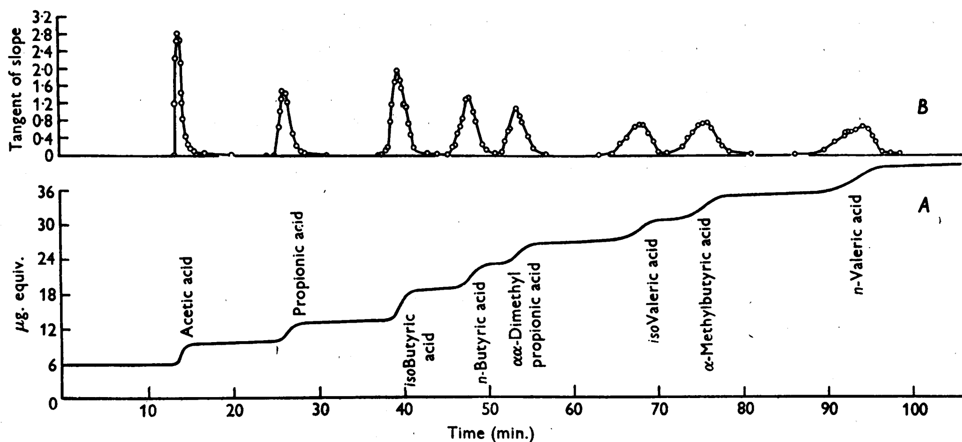

Fig. 10. The separation of acetic, propionic, *n*-butyric and *iso*butyric acids and the isomers of valeric acid, showing the complete resolution of all bands, and change in band shape in ascending the series. *A*, experimental curve. *B*, differential of experimental curve. Column length, 11 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 74 cm. Hg; flow rate, 18·2 ml./min.; temp., 137°.

they showed a non-linear adsorption isotherm with the tail of the acetic zone delayed by some 50 ml. at 100°. This delay increased with the chain length of the fatty acid. In the case of acetic and propionic acids the distortion caused by the adsorption is sufficient to mask completely the effect of the dimerization which has doubtless occurred. With the *iso*butyric acid the two effects are in approximate balance and a nearly symmetrical zone results; with *n*-butyric and higher acids the dominant effect is that of dimerization. Increasing the temperature appears to have a greater effect on dimerization than adsorption, since the symmetrical zone is then given by an acid higher than *iso*butyric acid (see also Fig. 10).

a 4 ft. silicone-stearic acid column at 100°. The upper differential curve (obtained as described earlier) shows that complete resolution of *n*-butyric and $\alpha\alpha$-dimethylpropionic acids and *iso*valeric and α-methylbutyric acids is not attained. To improve this separation a longer column was constructed. Curves *A* and *B* (Fig. 10) show the separation of acetic, propionic, *iso*butyric, *n*-butyric, $\alpha\alpha$-dimethylpropionic, *iso*valeric, α-methylbutyric and *n*-valeric acids on an 11 ft. silicone-stearic acid column at 137°. Complete resolution of all the acids is obtained. The marked change in zone shape with molecular weight is well shown in this series.

Fig. 11 shows the separation of acetic, propionic, *iso*butyric, *n*-butyric, commercial '*iso*valeric', *n*-

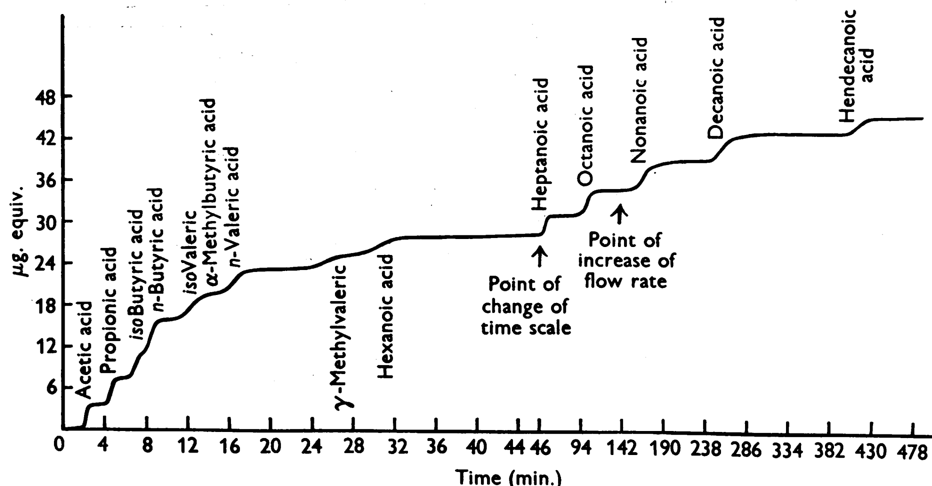

Fig. 11. The separation of acetic, propionic, *n*-butyric, *iso*butyric, *n*-valeric, commercial '*iso*valeric', γ-methylvaleric, hexanoic, heptanoic, octanoic, nonanoic, decanoic and hendecanoic acids. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 47 cm. Hg; flow rate, 18·2 ml./min.; temp., 137°. At the time indicated by the first arrow the time scale was changed to 24 min./cm. At the time indicated by the second arrow the pressure was increased to 76·5 cm. Hg and the flow rate to 35 ml./min.

Curve *A* (Fig. 8) shows the separation of formic, acetic and propionic acids on a silicone-stearic acid column. The formic acid zone shows marked tailing and extends backwards into the acetic acid zone so that complete resolution is not obtained. Curve *B* shows the separation of formic and acetic acids when orthophosphoric acid is incorporated in the column liquid phase. The shape of the formic acid zone now approximates to that of the acetic acid zone and indicates that the phosphoric acid has effectively suppressed adsorption. The greater retention volume for each zone also indicates greater association in the liquid phase. A similar effect on tailing is produced by washing the kieselguhr with dilute aqueous phosphoric acid (1 % v/v) before the final drying.

Curve *A* (Fig. 9) shows the separation of *iso*butyric, *n*-butyric, $\alpha\alpha$-dimethylpropionic, *iso*valeric, α-methylbutyric and *n*-valeric acids on

valeric, γ-methylvaleric ('*iso*caproic'), hexanoic, heptanoic, octanoic, nonanoic, decanoic and hendecanoic acids. Dodecanoic acid is not shown as it takes a longer time to emerge than can conveniently be shown on the graph (817 min.). The rate of flow is too high for complete resolution of the lower acids, but it can be seen that a rough quantitative analysis of *n*- and *iso*-acids from C_2 to C_6 can be carried out in 36 min.

All the columns used are highly reproducible; V_R^0 even at different rates of flow varies by less than 5 % for a given acid (Table 2) and even the most difficultly separable acids with less than five carbon atoms, namely, $\alpha\alpha$-dimethylpropionic and *n*-butyric acids, have retention volumes differing by 15 % (Table 6). It is thus possible to identify any of these acids by retention volume alone. No marked fall in efficiency with repeated use takes place, the factor

limiting the life being breakage and not deterioration. Recoveries are quantitative within the error of the titration and no indication of incomplete absorption in the titration cell has been found. We have not striven to reduce the errors below a few per cent, though we are of the opinion that the method is essentially a highly accurate one.

Table 6. *Retention volumes of the fatty acids relative to that of* n-*butyric acid on silicone-stearic acid columns*

Acid	B.p. (°)	Column temp. 100°	137°
Formic	107·7	0·076	—
Acetic	118·1	0·20	0·26
Propionic	141·1	0·47	0·54
*iso*Butyric	154·4	0·77	0·81
n-Butyric	163·5	1·0	1·0
αα-Dimethylpropionic	163·8	1·15	—
*iso*Valeric	176·7	1·51	1·48
α-Methylbutyric	177	1·70	—
n-Valeric	187	2·17	1·91
γ-Methylvaleric	199·1	—	2·94
Hexanoic	205	—	3·58
Heptanoic	223·5	—	6·55
Octanoic	237·5	—	12·0
Nonanoic	254	—	22·0
Decanoic	268–70	—	40·5
Hendecanoic	225*	—	72·8
Dodecanoic	225†	—	138·5

* 100 mm. Hg. † 40 mm. Hg.

A steady increase of retention volume with chain length is found to occur with the straight-chain acids, the retention volume increasing by a factor approx. 1·9 for each increase of chain length of one CH_2 group, formic acid showing the maximum deviation. Fig. 12 shows a plot of log V_R^0 against chain length. It can be seen that the curve joining the *iso* acids runs parallel to that joining the normal acids as would be expected.

The maximum permissible loading of the 4 mm. columns is approximately 1 mg. of each of the lower acids, but if the cross-section area of the column be increased the loading can be increased in the same ratio. The minimum amount of an acid detectable (using 0·038 N-base in the burette) is 0·3 μg. equiv., i.e. 0·02 mg. for acetic acid. As explained earlier, this lower level is determined only by the method of detection used.

The determination of fatty acids occurring as their sodium salts

In most biological experiments the volatile fatty acids are isolated as their sodium salts, and a simple method of obtaining the acids free from water is necessary before they can be applied to the column. The presence of even a small percentage of water in the fatty acid sample is sufficient to upset the separation, presumably owing to azeotrope formation.

The method adopted consists in drawing up a small sample of an aqueous solution of the sodium salts (30 μl. of an approx. 2 N solution) into a micropipette (Fig. 3*c*) that has been pretreated with a silicone (by washing with a 1 % (w/v) solution of DC 1107 in chloroform followed by heating at 140° for 30 min.), to render it unwettable by water. A small bubble of air is drawn in and an aqueous

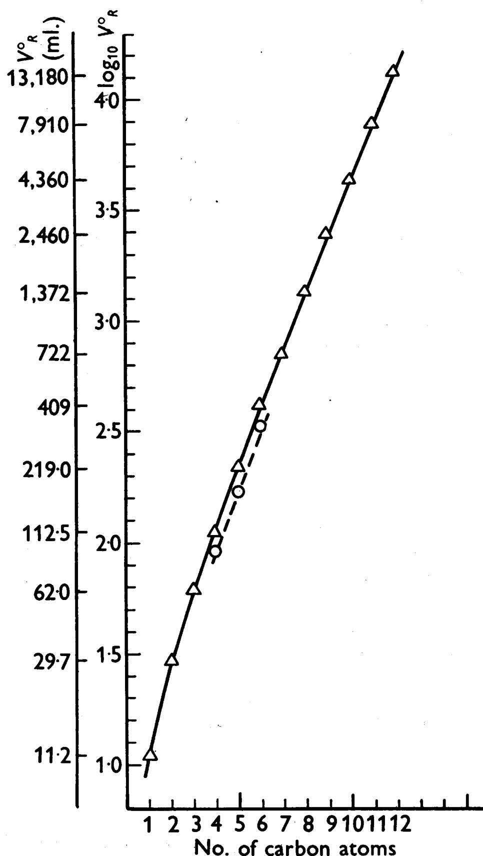

Fig. 12. The relationship between the retention volume at zero pressure difference (V_R^0) and the number of carbon atoms in the lower fatty acids. △, *n*-Acids; ○, *iso*-acids. Data are for a 4 ft. silicone-stearic acid column at 137° (cf. Fig. 11).

solution of orthophosphoric acid (60 % w/v) is drawn up and the two solutions mixed in the wide part of the pipette. The acidified mixture is then run on to the top of a micro-partition column (Fig. 3*d*), the lower part of which is packed with anhydrous sodium sulphate and the rest with kieselguhr (Celite 545). The aqueous solution of the acids is taken up by the kieselguhr. Ether (0·6 ml.) is then run through the micropipette on to the column and passed through the column under pressure. The eluate is collected in a long narrow test tube

(Fig. 3*d*), whose diameter is slightly greater than that of the chromatogram tube. When all the ether has been collected the chromatogram tube is removed from its vapour jacket and inserted in the test tube so that the end of the column is just above the surface of the ether. The test tube is immersed in warm water and air is drawn through the column until all the ether has evaporated. The test tube is then immersed in a heating bath (100° for acids up to valeric, 150° for acids up to dodecanoic acid) and air is drawn through the column for 15 min. The acids move up into the column and condense there. At the end of this time the column is replaced in the vapour jacket, the nitrogen gas turned on and the separation carried out as already described.

SUMMARY

1. The theory of the partition column has been extended to cover a compressible mobile phase. Satisfactory agreement of experiment with theory has been found.

2. Two methods of calculating the height equivalent to a theoretical plate in such columns are derived and compared.

3. Factors influencing the degree of separation are discussed.

4. Gas-liquid partition columns are described for the separation of volatile fatty acids from formic acid to dodecanoic acid.

5. Complete resolution is obtained of all normal acids (and *iso* acids up to at least C_6) on a 4 ft. column and of all the isomers of valeric acid on an 11 ft. column.

6. A method is described for the micro-estimation of volatile fatty acids isolated as their sodium salts.

7. A recording burette is described which may be coupled to a photoelectric control circuit to render the analysis automatic.

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Quantitative Studies of the Avidity of Naturally Occurring Substances for Trace Metals

2. AMINO-ACIDS HAVING THREE IONIZING GROUPS

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The fact that glycine can successfully compete for copper ions against six times its weight of serum albumin (Klotz & Fiess, 1951) suggests that the metal-binding powers of the amino-acids are of a relative magnitude sufficient to play an important part in the metabolism of trace metals.

The constants governing equilibria between the ions of heavy metals and those amino-acids that have only two ionizing groups were presented in Part 1 (Albert, 1950). The present paper deals with the constants of amino-acids having three such groups. The only relevant constants in the literature are for aspartic acid with copper (Li & Doody, 1950) and magnesium (Greenwald, 1939) and for histidine

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