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THE PARTITION CHROMATOGRAPHY OF LIPIDS

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I. INTRODUCTION

Lipids of natural origin are usually mixtures of aliphatic compounds of different *classes*, characterised by a definite number of polar functional groups, COOH, OH, NH_2, etc. The structural differences between lipids within a given class are determined entirely by two parameters — the number of methylene groups and the number and position of double bonds. A set of lipids in which one of the parameters is constant while the other changes monotonically forms a *homologous series*. Each class may contain several such series. Their detailed analysis is of considerable theoretical and practical importance.

A mixture of lipids is separated into individual classes by adsorption chromatography; separation of the classes into homologous series and separation within the series generally does not take place in this case, since the differences in polarity among lipids within a class are small compared with the changes in polarity due to the functional groups. Even these relatively slight differences, however, are quite sufficient for the separation of lipids within classes and homologous series by the method of *partition chromatography*†.

Partition chromatography, discovered in 1941 by Martin and Synge [1], was applied in the field of lipids only in 1948, in work by Ramsey and Patterson [2] and Boldingh [3]. In the last 17 years, several hundred scientific papers and a large number of reviews [4-19] have been devoted to the partition chromatography of lipids.

The subject of the present review is the use of various types of partition chromatography in the analysis of natural

† This term, which is now firmly established, cannot be regarded as appropriate, since the mass is partitioned between solid, liquid, or gaseous phases in *any* chromatographic separation. Here and subsequently we shall have in mind *a system of two immiscible liquid phases*, one mobile and one stationary.

lipids. The theoretical principles of the method and the characteristic features of the apparatus used are not examined in detail: this information can be found in textbooks[9,17]. Little reference is made to studies on the chromatography of aliphatic compounds with short chains (up to C_8), steroids, and other isoprene derivatives, or to the extremely large number of papers in which established chromatographic methods have been applied without any fundamental modification. The present review covers the most important work on the partition chromatography of lipids published before 1st March 1964.

II. CHROMATOGRAPHY WITH A POLAR STATIONARY PHASE

Following the historical order, we shall examine first of all systems in which the stationary phase is more polar than the solvent passing through, although at the present time these forms of chromatography are used in the field of lipids much less frequently than are reversed-phase systems (see Section III).

1. Column Chromatography

Column chromatography has been used for the purification, separation, and identification of aliphatic acids and other lipids.

Columns of cellulose powder have been used to purify phospholipids from peptides and amino-acids. The lipids were eluted with a mixture of chloroform, butanol, and water[20], chloroform, ethanol, and water (80:20:2.5)[21], or chloroform and methanol[22]. In the chloroform–methanol system, the phospholipids left the column in the sequence — phosphatidic acids, Φ-choline (Φ = phosphatidyl), sphyngomyelin, Φ-aminoethanol, Φ-serine, and Φ-inositol[23]. Phospholipids have also been separated by means of acetone at -18°.[24] A mixture of methanol, hexane, water, and acetic acid (30:50:5:1) has been used for the fractionation of the hydroxamate derivatives of the "even" C_{14-22} saturated and C_{18} unsaturated acids. The hydroxamates were detected by their reaction with Fe^{3+} and identified from their melting points. The retention time increased with increase in polarity; derivatives with the same polarity were not separated. An advantage of the hydroxamates over the original acids is their greater solubility in the stationary phase[25].

In 1948, Ramsey and Patterson[2] proposed the use of silica gel as carrier for the partition chromatography of aliphatic acids. This method was standardised with ^{14}C acids[26,27] and has been used repeatedly for the separation of lipids[28-33]. For partition chromatography the silica gel was suspended in the lower phase of the system[2,29,30,33], and the column was filled under pressure[29-31,33]. The specimen (3.3–3.8 mg of lipids per g of carrier)[30,33,34] was dissolved in the upper phase and introduced into the column. The solvent (upper phase) was passed at a rate of 0.6–1.2 ml min^{-1}.[29,30] The acids were separated in the systems: furfuryl alcohol–2-aminopyridine (1:1)/hexane[2], 0.25 *N* NH_4OH in methanol/isooctane[29], 0.2 *N* NH_4OH in a mixture of 2-methoxyethanol and water (9:1)/hexane[30], and 0.3 *N* NaOH in methanol/cyclohexane[34]; thus the aliphatic acids moved along the column as the salts of organic or inorganic cations. Hydroxy-acids have been separated by means of the systems: 0.1 *N* NH_4OH in 95% methanol/petroleum ether[35] or methanol–hexane–acetone (2:10:1);[36] and dicarboxylic acids formed by the oxidative splitting of lipids have been separated using the systems: 0.2 *N* NH_4OH in a mixture of 2-methoxyethanol and water (9:1)/mixture of hexane and dibutyl ether (1:1),[30] 1 M Na citrate (pH 5.4)/chloroform–butanol[37] or methanol–water–hexane–acetone (7.7:1.9:77:34).[33] The products obtained by the oxidation of triglycerides with a permanganate–periodate mixture were separated in the 90% ethanol/petroleum ether system[38]. Neutral derivatives of the acids have been fractionated in the systems: nitromethane/petroleum ether[31,39] or 2-phenoxyethanol/heptane[32]. The time required for the analysis on columns of silicic acid was 20 h.[30] For the quantitative determination, the individual fractions of the eluate were titrated with 0.05 *N* NaOH using phenolphthalein[2,33] or Phenol Red[30] as indicator. The silica gel can be impregnated with indicator before the separation; in this case the acids migrate in the form of coloured bands[29,30]. Derivatives of aliphatic acids form coloured zones on the column in the absence of indicators[31,32,39]. The eluate contained 95–105% of the original lipids[2,29,30,33].

The lipids leave the column in order of increasing polarity. The even saturated C_{10-22} mono- and di-basic acids are readily separated[2,29,30,34,37]; a difference of one CH_2 group is sufficient for separation of the C_{6-10} dicarboxylic acids[33]. Ricinoleic and dihydroxy stearic acids are separated from one another and from other acids[35,36]. The products of the oxidation of glycerides have been separated into fractions SSS + SSA and SAA + AAA, where S and A are the acyl groups of saturated acids and azelaic acid[38]. In the series of *p*-phenylazophenacyl esters of C_{2-18} saturated acids[32] and the 2,4-dinitrophenylhydrazones of these esters[31], homologues differing by one CH_2 group were separated completely. The homologous series formed by the 2,4-dinitrophenylhydrazones of the C_{2-18} aliphatic aldehydes was fractionated by Ramsey's method using columns[39]. In agreement with Martin's fundamental equation[1] for partition chromatography, the logarithm of the retention volume V_R for these compounds in the column was found to depend linearly on chain length. This relationship was used for the identification of lipids[28,29,40].

The new method of *crystallisation chromatography* in a column has been used for the separation of the triglycerides of saturated acids. A brass column was filled with steel wool and sand. The temperature in the lower part of the column was 20 deg lower than that in the upper part; the central portion was kept at a constant temperature. The mixture of glycerides was introduced into the upper part, and the column was filled with acetone; hexane or carbon tetrachloride was then supplied at the rate of 1–2.8 ml min^{-1}. The high-melting components were retained in the column in the form of crystals of the stable β' form. The MyrMyrMyr glyceride left the column first, followed by the PalPalPal and StStSt glycerides‡. The eutectic mixture of PalStPal and StPalPal glycerides was not separated[41].

2. Paper Chromatography

In early work, *aliphatic acids* of decreasing polarity were eluted from the point of application with aqueous methanol or acetone; by selection of a suitable solvent, the mixture of acids was partly separated into two or even three

‡ Myr, Pal, and St represent the myristoyl, palmitoyl, and stearoyl radicals.

components[42-50]. The triglycerides[44] and polymerised lipids[44,45,47] remained at the starting point. The salts of aliphatic acids were separated from the other lipids by means of water[42]. Petroleum ether, ether, and dichloro--ethylene were used for the separation of mixtures of di-, tetra-, and hexa-bromostearic acids respectively[51]. Di-, tetra-, and hexa-hydroxystearic acids, obtained by the permanganate oxidation of unsaturated C_{18} acids, were separated in the butanol–ethanol–water–conc. NH_4OH (75 : 15 : 8 : 2) system[52]. Aliphatic acids containing conjugated bonds were treated with maleic anhydride vapour before separation; the products of the diene synthesis remained at the start, and the unsubstituted acids moved in the solvent front with 75–99% methanol[45,46]. Mono- and di-basic C_{2-12} acids (products of the oxidation of lipids) can be separated in 1 *N* NH_4OH in 78% ethanol[53]. This separation is more frequently carried out by chromatography of the hydroxamates in water-saturated butanol or phenol; the hydroxamates are detected with Fe^{3+}.[54-58].

The lipids from skin, blood serum, and spinal fluid, and also triglycerides from vegetable oils, have been separated into several fractions in 99% methanol[59,60], a mixture of methanol with acetone[61], or a mixture of diisobutyl ketone, acetic acid, and water (8 : 5 : 1).[62] Phospholipids[7,11,17] were separated by means of a mixture of chloroform, methanol, and water (32 : 8 : 1),[63] a mixture of acetone with methanol followed by water-saturated phenol[64], aqueous ethanol[65], a mixture of lutidine with methanol (3 : 2),[66] and (with the use of aminoethylcellulose anion-exchange paper) a mixture of diisobutyl ketone, acetic acid, and water (8 : 5 : 1).[67] The efficiency of separation on untreated paper is usually low. A mixture of Φ-choline and sphyngomyelin has been separated from a mixture of Φ-aminoethanol and Φ-serine[63]. The compounds Φ-choline, Φ-serine, and Φ-aminoethanol have been separated by successive treatment with two solvent systems[64]. Saturated and unsaturated Φ-choline and lyso-Φ-choline have been separated in a one-dimensional system[65]. Acetylation of the NH_2 groups of Φ-aminoethanol leads to its complete separation from Φ-choline and sphyngomyelin[66]. A more effective separation was observed on anion-exchange paper; the R_f values at 4° were — Φ-inositol 0.29, sulpholipid 0.30, Φ-glycerol 0.42, digalactolipid 0.49, Φ-aminoethanol 0.58, Φ-choline 0.61, galactolipid 0.68, and triglycerides 0.98.[67]

Aliphatic acids and other lipids are sometimes separated with a non-aqueous polar phase. The monobasic C_{4-14} unsaturated acids (10–100 mg) have been separated on paper impregnated with potash alum, a mixture of carbon tetrachloride, methanol, and conc. NH_4OH (81 : 18 : 1) being used[68]. The same acids, in the form of their 2,4-dinitrobenzyl esters, were separated with dimethylformamide in a mixture of cyclohexane and hydrocarbons of b.p. 190–220°.[69] Glycerol and the mono- and di-glycerides of stearic acid have been separated in the system n-butanol–acetic acid–water (4 : 1 : 5)/formaldehyde[70]. The R_f value was always proportional to the chain length.

Phospholipids have been separated on paper with formaldehyde, the solvent being a mixture of butanol, acetic acid, and water (4 : 1 : 5), or by a two-dimensional system with the same mixture and a neutral solvent, tetrahydrofuran–diisobutyl ketone–water (45 : 5 : 6).[71-73] In the acid solvent the lipids were arranged in the order (from the front) triglycerides, Φ-aminoethanol + Φ-choline, lyso-Φ-choline, Φ-serine, Φ-inositol, lyso-Φ-inositol, and glycolipid[71]; in the neutral system, sphyngomyelin, Φ-choline, Φ-aminoethanol, polyglycero phosphatide and cerebroside, and also lyso-Φ-choline and lyso-Φ-aminoethanol, were separated[72,73].

Lipids are detected by the usual reactions (see Section III4E); thus phospholipids give colour reactions with molybdophosphoric acid, stannous chloride, ninhydrin, Schiff's reagent, permanganate, iodine, or Nile Blue[64,71]. For neutron-activation analysis, the dry chromatograms were irradiated in an aluminium container with slow neutrons (5×10^{11} neutrons cm^{-2} s^{-1}) for 15 h. The strip was kept for 1 week until the short-lived isotopes had decayed completely, after which the strip was exposed with X-ray film, or the radioactivity of the zones was measured with a Geiger counter. The minimum detectable quantity of lipids (z) amounted to 0.05 μg P cm^{-2}.[74,75]

III. REVERSED-PHASE CHROMATOGRAPHY

1. General Features of Reversed-Phase Chromatography

We have so far examined the chromatography of lipids in ordinary partition systems where the stationary phase is the more polar. For effective separation by partition chromatography, however, the stationary phase should be a substance in which the components to be analysed have a higher solubility; during the chromatographic process these compounds are slowly eluted by a larger volume of the mobile phase, in which they are less soluble. Since lipids containing > 10 C atoms are sparingly soluble in water but have a high solubility in non-polar substances, it is better to carry out the separation of lipids with systems in which the stationary phase is less polar than the mobile phase, so that the lipids are distributed on the chromatogram in the opposite order to that given above (see Section II), so-called reversed-phase systems. This term reflects the historical development of chromatography, since systems with the "normal" relationship of polarities for the individual phases were developed before reversed--phase chromatography (see Section I), which was first used by Boldingh in 1948 to separate the methyl esters of aliphatic acids[3], and was later developed by Howard and Martin[76], Kaufmann[7], Inoue[56], Schlenk[14], Gunstone[77], Buchanan[78], and others.

The solubility of lipids in non-polar phases is determined by the interaction between the aliphatic chains of the lipids and the solvent resulting from van der Waals forces, which increase with increasing chain length and decrease under the influence of double bonds. The chain is shortened to the minimum extent by *trans* double bonds, whose influence on the van der Waals forces is not so marked as that of *cis* bonds, which reduce considerably the effective length of the molecule; this makes it possible to separate geometric isomers (see Section III4D). The appearance of a double bond in the chain leads to an appreciable increase in the solubility of lipids in the polar phase, as a result of the occurrence of dipole interactions.

The main rule in reversed-phase chromatography is that within a given class the appearance of a double bond in the aliphatic chain of a lipid (at a sufficient distance from the ends of the chain) and shortening of the chain by two CH_2 groups usually increase the chromatographic mobility to equal extents. This relationship makes it possible to predict the relative mobility of lipids from their composition, on the basis of the polarity constant $K = 100 - m + 2e$, where m is the number of carbon atoms and e the number of double bonds: lipids with equal values of K are not

separated from one another in reversed-phase systems. Deviations from this rule are rarely observed (see Sections III3 and III4F).

It may be assumed that lipids of equal polarity have equal values for the distribution coefficient α. For the experimental determination of α for aliphatic acids in the aqueous acetone/paraffin oil system, 2 mg of the acid was dissolved in 0.5 ml of the non-polar phase, the solution mixed with 0.5 g of carrier (kieselguhr), and 5 ml of aqueous acetone added. The system was brought to equilibrium at 35° and centrifuged, and 2 ml specimens of the upper phase were titrated with A ml of 0.001 N potassium hydroxide in methanol. The residue was treated with 5 ml of 95% acetone, which eluted completely the acid from the non-polar phase, and 2 ml specimens were titrated with P ml of alkali. The value of α (the ratio of the concentrations of acid in mobile and stationary phases) was calculated from the formula $\alpha = AV/(6P - A)$, where V is the volume of the non-polar phase. The percentage accuracy T of the determination, found from the equation $T = 100NE(2A + 3P)/W$, where N is the normality of the alkali, W the weight of acid, and E the equivalent of the acid, usually amounted to 100 ± 5%. The values of $\log_{10} 100\alpha$ for different aliphatic acids, plotted against the concentration of acetone in the solvent, gave a family of parallel straight lines; these straight lines coincided for acids of equal polarity. The optimum conditions for separation were achieved at a solvent concentration at which α for the given acid was 0.16 and its value for the next higher homologue was 0.08.[79]

The following symbols were introduced for the mathematical description of the reversed-phase system for the separation of palmitic and stearic acids: α' is the distribution coefficient of the acids under the conditions of the chromatographic separation; p the number of theoretical plates in the column; a the number of the eluate fraction, where each fraction has a volume equal to the volume of the mobile phase in a theoretical plate; E_a the fraction of dissolved substance present in the eluate a; V_m the volume of mobile phase in the column; v the volume of each eluate in the experiment; f the number of the experimental eluate fraction containing the greatest quantity of dissolved substance, counted from the moment when the solvent front leaves the column; E'_{max} the fraction of dissolved substance in eluate f; $c = V_m/v$ is the volume of mobile phase in the column, expressed as the number of eluate fractions in the experiment; and $A_\epsilon\{x\}$ is the area of the normal error curve for the argument $\{x\}$.

A column containing p plates was considered. On the assumption that each plate behaves like a separating funnel in countercurrent distribution, the quantity of dissolved substance $T_{n,r}$ with distribution coefficient α in the rth plate after n transfers was determined from the formula for countercurrent distribution:

$$T_{n,r} = \frac{n!}{r!\,(n-r)!} \times \left(\frac{1}{\alpha+1}\right)^n \times \alpha^r. \qquad (1)$$

After p transfers the solvent front reaches the last pth plate, after which a known quantity of dissolved substance E_{a+1} leaves the column with the eluate:

$$E_{a+1} = \frac{(p+a)!}{p!\,a!} \times \frac{(\alpha')^{p+1}}{(\alpha'+1)^{p+a+1}} = \frac{p+a}{a(\alpha'+1)} \times E_a. \qquad (2)$$

The fraction of dissolved substance in the eluate reaches a maximum when $\alpha' = p/a$. The ratio a/p is equivalent to the total volume of eluate necessary for the attainment of the maximum, expressed in volumes of the mobile phase of the column:

$$1/\alpha' = a/p = fv/V_m. \qquad (3)$$

Eqn. (3) and data on the position and height of each peak on the experimental elution curve obtained with 67% acetone as solvent were used to calculate the actual distribution coefficient α' for palmitic (0.50) and stearic (0.22) acids. Division of α' by the ratio of the liquid phases in the column (2.5) gave the true distribution coefficients α as 0.20 and 0.09 respectively in good agreement with the experimental values of 0.16 and 0.08.

From Eqns. (2) and (3) we can obtain the equation

$$p = 2\pi(1+\alpha') \times \left(\frac{E'_{max} \cdot V_m}{\alpha' \cdot v}\right)^2 = 2\pi f\,(f+c)\,(E'_{max})^2, \qquad (4)$$

which makes it possible to calculate the number of plates p for each acid from the position and height of the eluate maximum on the elution curve. For palmitic and stearic acids, the values of p were 70 and 114 respectively. The calculated values of α' and p were substituted in Eqn. (2), and used to construct the theoretical elution curve; this showed satisfactory agreement with the curve obtained experimentally.

The percentage contamination of each peak by the substance corresponding to the neighbouring peak η was determined from the equation to the normal error curve

$$\eta = 100\left(0.5 - A_\epsilon\left\{\frac{\sqrt{p}(\sqrt{B}-\sqrt{A})}{\sqrt{AB}}\right\}\right),$$

where $A = 1 + 1/\alpha'_A$ and $B = 1 + 1/\alpha'_B$. Thus to obtain fractions of the required purity ($\eta \geqslant 0.2\%$) in the separation of an acid having $\alpha' = 0.50$ from another acid, in a reversed-phase system with an efficiency $p = 100$, the value of α' for the latter acid should not exceed 0.23. It can be seen that the system used by the authors cited[79] ensures almost complete separation of palmitic ($\alpha' = 0.50$) and stearic ($\alpha' = 0.22$) acids.

Mathematical treatment of a reversed-phase chromatographic system makes it possible to select the optimum conditions of solvent composition and column efficiency for the separation of a lipid mixture.

2. Column Chromatography

A. Preparation of columns. In 1950, Howard and Martin[76] proposed the use of hydrophobic Celite treated with dimethyldichlorosilane vapour as stationary-phase carrier. The Celite was mixed with neutralised (with alumina) paraffin oil[77] (1:0.8 by weight[14,80], 1:1,[76,81], or 1:1.4 [77]), suspended in the mobile phase, and transferred to the column. The paraffin oil can be replaced by silicone oil (b.p. 195–230° at 0.25 mmHg)[14,81] or acetylated castor oil[77], isooctane[82], heptane[83,84], or cyclohexane[85].

Rubber powder swollen in benzene[86], petroleum ether[35,87,88], or acetone[89] has been used simultaneously as carrier and non-polar phase. Soya-bean oil polymerised with S_2Cl_2 ("factice") has properties similar to those of rubber[90]; separation using factice is no less effective than that using Celite and paraffin oil[84]. Polyethylene powder swollen in acetone[91,92] or polyethylene on Celite[93] can be used for a separation not merely 3–8 times in succession, as in the case of the phases given above, but for an unlimited period. Use has also been made of cellulose impregnated with paraffin oil[94] and of nylon fibres, on

which 2–6 μg of acids were separated by means of a mixture of acetone, ethanol, and water and detected with Bromocresol Green[95].

B. Production of chromatogram. A specimen containing 1–10 mg of each component for columns of diameter 1–2 cm [76,77,84,93,96] or 150–250 mg of the mixture for broader columns [14,97] is introduced in the form of a solution in the mobile [76,93] or stationary [14,84,96] phase, depending on the solubility of the lipids. The volume of the solution should correspond to one theoretical plate [84]. The rate of flow of the mobile phase may vary over the range (ml h^{-1}) 4–20, [85,91,93] 35–60, [76,84,90,98] 120–180, [14,99] and up to 300, [83] the efficiency of the column being slightly higher at lower rate of flow. The temperature of the separation should be kept constant. Unsaturated acids are usually separated at 10°, [96,98] mixtures of acids on rubber and polyethylene columns at 20–25°, [86,92] and columns containing paraffin oil are kept at 35°. [14,76,80,99] The eluate is collected in an automatic collector [87], and the concentration of lipids in the fractions is determined gravimetrically [35,85] or by titration with 0.01 *N* potassium hydroxide in methanol using Bromothymol Blue as indicator [76,77,80,82,92,96,99]. In other cases two volumes of water are added to the eluate, and nephelometer readings S and S_1 for the specimen and a standard are determined. In the range 2–20 μg, the relationship between $\log_{10}(S/S_1)$ and the mass of the lipids was linear [14,81,100]. The concentration of glycerides was determined colorimetrically with hydroxylamine and Fe^{3+}. [84] A differential refractometer has been used for the continuous recording of lipids at the column outlet; the area of the peaks on the chromatogram was not proportional to the mass [90]. Between 97% and 103% of the original lipids was detected in the eluate [35,77,80,82,89–91]. The purity of the specimens isolated reached 99–100%. [14,83]

Aqueous solutions of acetone, saturated with the non-polar phase, are commonly used as solvents for the separation of lipids. The following acetone concentrations (%) have been used for the elution of saturated acids — C_6 30, C_8 40, C_{10} 50, C_{12} 53, C_{14} 58–60, C_{16} 65–67, C_{18} 70–73, C_{20} 75–78, C_{22} 80–83, and C_{24} 85–90. [14,76,77,80,89,91,96] A mixture of acetone, heptane, and water (5 : 1 : 0.629) has been used as solvent for the separation of triglycerides from coconut oil [84], and 95% acetone for that of glycerides from linseed oil [90]. Increase in the concentration of water increases V_R and the efficiency of the column [76,84]. Acids and their derivatives have also been separated by means of a mixture of methanol with acetone (3 : 1), [86] 65–100% methanol [82,98], 40–70% ethanol [93], a mixture of acetonitrile with methanol (17 : 3), [83] and a mixture of acetic and formic acids with water (2 : 2 : 1). [94] Acids and their methyl esters have been separated in the acetonitrile–water (3 : 1) system [14,81]. The use of aqueous ethanol for the fractionation of 2-mono- and 1,2-di-glycerides led to their conversion to the stable 1-mono- and 1,3-di-isomers; in aqueous acetone no migration of the acyl groups was observed [85].

C. Separation of the lipids. Saturated normal aliphatic acids differing by one CH_2 group are readily separated and leave the column in order of decreasing values of the constant K. [14,76,77,80–82,86,87,91] The value of V_R for lactobacillic acid (11,12-methyleneoctadecanoic acid) coincides with that for stearic acid [87]. Unsaturated acids are separated from one another, but not from saturated acids having the same value of K. [8,77,83,87,99]

For the quantitative analysis of a mixture, a specimen of acids is hydrogenated in the presence of 5–20% Pd on carbon for 24 h; [77,97,101] the hydrogenation products (saturated acids) are readily separated on a column [8,102–104]. Another specimen of the acids is cleaved at the double bonds by means of 5% permanganate [99,104], hydroxylated with peroxyformic acid [87,105], or subjected to ozonolysis in methyl acetate at -40° for 5 min. [77] The results of the simultaneous determination of the original acids and the products of their hydrogenation and oxidation make it possible to establish fairly precisely the composition of a given mixture [77]. Thus oleic and petroselinic acids are not separated in a reversed-phase system, but the pelargonic and lauric acids produced by their oxidation are readily separated in this system [99].

Hydroxy-, epoxy-, and keto-acids, because of their high polarity, leave well away from ordinary acids, and are separated both in unchanged form [76,86–88,99] and as their acetates on columns containing acetylated castor oil [35,77]. The value of V_R for hydroxystearic acid coincided with that for lauric acid [35,77]. The chlorophenacyl esters [93] and fluorides of aliphatic acids [94] were separated in the same way as the unsubstituted acids. Columns containing paraffin oil were used to separate mono-, di-, and tri-glycerides [85] and also triglycerides differing by four CH_2 groups or by two double bonds; glycerides differing by two CH_2 groups or by one double bond were not separated completely [84]. Methyl esters, cholesterol esters, and triglycerides were separated on a factice column into fractions having identical values of K; the values of $\log V_R$ were found to vary linearly with the number of carbon atoms in these compounds [90].

3. Chromatography on Glass Plates

Chromatography on a thin layer of adsorbent attached to a glass plate has now been widely adopted as a method for the separation of lipids by adsorption. At the same time plates carrying silicic acid are being used in the chromatography of lipids on an ever increasing scale as carriers of a non-polar phase.

For the *reversed-phase* separation, activated plates were treated with 10–15% undecane in petroleum ether [106–108], 10% dodecane in hexane [109], or 5% tetradecane [107,108,110–115], 0.5% [116,117] or 10% [118–120] paraffin oil, or 5% silicone oil [110,121] in ether. Heavy oils cannot be removed from the plate by heating [110]. The separation of lipids and the detection of the individual zones are carried out by the methods generally used in thin-layer chromatography [122].

The results of partition chromatography on plates in general do not differ from those obtained in other forms of reversed-phase chromatography; the possibility of working on the ultramicro-scale, however, makes it possible in a number of cases to achieve higher rates and efficiencies of separation. Plates are often used for two-dimensional chromatography: the lipids are separated in one direction into classes without the application of a non-polar phase, after which part of the plate is impregnated with hydrocarbons and the lipids of each class are separated into fractions of different polarity [106,109].

The C_{10-18} aliphatic acids (0.5–20 μg) have been separated with a mixture of acetic acid and acetonitrile (1 : 1) [107,108] or 85% acetic acid [119] in 1 h. For the separation of acids having equal values of K, 0.5% bromine was added to the polar phase: the transformation of the unsaturated acids led to a marked increase in their mobility [108,112]. Lipids having the same polarity can also be separated by *hydrogenation*:

the acids on the plate are sprayed with a 1–2% palladium suspension, hydrogenated with H_2 in a desiccator, washed with 5% HCl, and separated by means of undecane [108,112,119]. The saturated C_{18-34} acids have been separated in the propan-2-ol–ethanol–acetic acid–water (8:3:4:1.3)/tetradecane system at 42°, and determined quantitatively with Cu acetate (see Section III4E*a*)[114]. The C_{13-25} 2-methyl--4-keto-acids and C_{12-22} hydroxy-acids have been separated by the use of 80% acetic acid [111]. The solvents used for the C_{12-34} aliphatic alcohols have been 85% acetic acid and 90% acetone [114,115].

Methyl esters have been separated in acetic acid–acetonitrile–water (2:14:5) [121] and acetone–acetonitrile (7:10) [109] mixtures. Saturated and unsaturated esters of equal polarity were separated at 4–6° (see Section III4D) or in the presence of peracetic acid (Section III4F). [121] Methyl and ethyl esters with different numbers of double bonds have been separated on a non-impregnated plate carrying silicic acid, by fourfold treatment with a mixture of petroleum ether and benzene (4:1); the plate was then impregnated with paraffin oil, and fractions differing in the number of carbon atoms were separated. The methyl esters were separated from the ethyl esters of the same acids [119]. An analogous method was used to separate cholesterol esters; ethyl methyl ketone–acetonitrile (7:3) [118,119] and acetic acid–chloroform (80:15) [116,117] were used as solvents.

Triglycerides have been separated by means of an acetone–acetonitrile (4:1) mixture [107,119] and acetic acid [117]; in 35–100 min, linseed oil was separated into nine fractions and soya-bean oil into six fractions. In most cases no separation of triglycerides of equal polarity took place. The mobilities of glycerides with the same values for K are not always identical, however [110]. Repeated treatment of plates with a single solvent, with drying after each treatment, leads to the separation of 1–2 μg of components of equal polarity. Thus the following glycerides are separated — LenLenLen from LauLauLau, LolLolLol from MyrMyrMyr, OlOlLol from MyrOlOl, OlOlLol from PalPalLol, OlOlOl from PalOlOl, OlOlOl from PalPalPal, PalPalOl from PalPalPal, etc. §. Threefold treatment made it possible to separate a mixture of OlOlOl, PalOlOl, PalPalOl, and PalPalPal glycerides, and also to separate the glycerides of oleic acid from the corresponding glycerides of elaidic acid. In this case linseed oil is separated into not 9 but 15 fractions, soya-bean oil into 13 fractions, ground-nut oil into 12 fractions, etc. [113]. Thus the double bond of the triglycerides is compensated for not by exactly two CH_2 groups, as in the previous case, but by approximately 2.4 groups; the Rf value for the more unsaturated component increases with increase in the difference in the number of double bonds in lipids of equal polarity. Decrease in the molecular weight of the stationary phase, an unfavourable ratio between the components of the specimen, and increase in the weight of the specimen lead to a decrease in the efficiency of separation of the glycerides [123]. A solution of 0.5% of bromine in a mixture of propionic acid and acetonitrile (3:2) is suitable for the separation of triglycerides of equal polarity; the hydrogenation method described above is also used [108,112].

§ Lau, Ol, Lol, and Len represent the acyl radicals of lauric, oleic, linoleic, and linolenic acids.

4. Paper Chromatography

A. Preparation of paper and application of stationary phase. Suitable grades of chromatographic paper for the separation are those exhibiting sufficient strength during washing in water [17]. These requirements are satisfied by the following papers: Whatman No. 1 [15,124–128], No. 3 [17,129], and No. 4 [124,130]; Schleicher and Schüll No. 595 [3,15], No. 598 [131], No. 2040*b* [132,133], No. 2043*b* [7,17,123,134,135], and No. 2043*b* Mgl [136,137]; Toyo No. 2 [138,139]; and Macherey Nagel No. 214 [134]. "B" or "M" paper from Leningrad Factory No. 2 [140–145] or No. 69 "Goznak" [146,147] have also been used; these grades are less satisfactory than those listed above [144]. The paper was purified by washing with 0.5–10% hydrochloric acid [144,148], 3% nitric acid [142], 20% formic acid [147], 95% acetic acid [127], aqueous acetone [149], ethanol [148], ether [127,146,148], or benzene [150], washed with water, dried at 90–120°, [125,128,130,135,144] and kept over ignited calcium chloride [136,150].

In early work, attempts were made to convert cellulose into its ethers in order to give the paper hydrophobic properties. To obtain the octadecyloxymethyl ether the paper was treated with 0.5% octadecyloxymethylpyridinium chloride in toluene at 90° for 3 h. [151] The hydroxamates of acids have been separated on paper acetylated with acetic anhydride in benzene at 70° [152,153] or palmitoylated in the same way [105]. Cellulose esters have not been widely used in chromatography, since hydrophobic paper is more readily prepared by impregnating ordinary paper with high-boiling lipophilic substances.

For the separation of the C_{10-20} aliphatic acids, the paper is usually impregnated with undecane saturated with the mobile phase [134,139,154–163], pressed out with filter paper under a load of 4 kg for 10–30 min, and kept in air for 10 min. [137,140,144,150,156] The optimum undecane content is 100–250 mg g^{-1}. [133,136,150,164] After the separation, the concentration of undecane in the front is double its original value, and the concentration at the point of application falls almost to zero. The flow of undecane can be decreased by decreasing its original concentration, by using hydrophobic paper as carrier, and by keeping the chromatograms horizontal during the separation. The rate of movement of the mobile phase increases appreciably if the initial section of the strip is not impregnated [159]. Undecane can be removed from the paper at 80–95°.

With decrease in polarity of the lipids to be separated, it is generally necessary to use stationary phases of higher molecular weight and lower polarity. As a result of the lower solubility of these substances in the mobile phase, the efficiency of separation and the limiting weight of the specimen increase; removal of the phase before application of the colour tests for lipids becomes impossible, however. Saturated and unsaturated acids and methyl esters have been separated using 10% tetradecane in petroleum ether [135], 5–10% liquid paraffin in benzene, ether, or carbon tetrachloride [141,142,145–147,165,166], or a 10% mixture of liquid paraffin and paraffin oil in benzene [141,145]. Saturated acids from C_{20} upwards, and also triglycerides, have been separated on paper impregnated with 5–15% paraffin oil (d = 0.885–0.890) in benzene, ether, and petroleum ether [127–131,148,149,167–174]. The paper is usually impregnated by drawing the strip through the solution or by ascending and horizontal suction [147,170,171,175]. The non-polar phase corresponds to 10–15% on the weight of the paper [165].

Paper impregnated with 10–30% rubber latex, after swelling in acetone, can be used to separate acids and methyl esters

in methanol–acetone (1 : 1) and 80% methanol–isooctane systems[3,168]. A mixture of 0.5% rubber and 30% naphthalene in benzene (4 : 1) has also been used[176]. Tetralin and decalin have been used to separate the 2,4-dinitrophenylhydrazones of *p*-bromophenacyl esters ("bromazones"), the 2,4-dinitrophenylhydrazones, acetol-, and acetoxymercurimethoxy-derivatives of unsaturated methyl esters, glycerides, and phospholipids[138,163,177-184], and also aliphatic acids[185].

Vegetable oils have been used for impregnation only in early work[124,186,187]; the efficiency of hydrocarbon phases is exceeded only by that of castor oil in the separation of hydroxystearic acids[126].

Among organosilicon compounds, use is made of silicone oil (1–5% Rodorsil 240, Dow Corning 200, Tip-2 in cyclohexane or ether), which is sparingly soluble in mobile phases[49,125,139,143].

The concentration of stationary phase in the system determines the duration of the experiment: at an undecane concentration of 150 mg g^{-1}, analysis of the acids takes 1–2 h, and at 600–700 mg g^{-1} movement of the polar phase ceases[188]. If the concentration of this phase is 0.1%, the saturated acids up to C_{26} can be separated without heating the chamber[189]. The differences in the R_f values (ΔR_f) increase with decrease in the concentration of the phase, so that the reversed-phase system is a true partition system, in which there is no adsorption[16,188]. To obtain reproducible R_f values, the concentration of the phase should be uniform along the length of the strip[5].

B. Production of chromatogram. If the concentration of individual lipids in the mixture is low, they must be isolated by preparative methods before separation. Higher C_{20-36} acids have been concentrated from wax by crystallisation with urea[141]. Polyunsaturated acids from fish oils have been fractionated as the Li salts[190]. Aliphatic acids from wood have been separated from resin acids by chromatography[191]. On paper impregnated with potassium hydroxide, alcohols moved with the toluene front and acids remained at the point of application[192].

A sample containing 10–100 μg of each component[7,78,141,170,174,176] or 100–500 μg of a mixture of lipids[15,126-128,134,147,161,167,193] in solution in chloroform[15,78,126], alcohol[127,167,170], ether[128], acetone[146,147], or benzene[16,179,193] is applied to a starting line at a distance of 2–6 cm from the edge[78,126,147] in the form of bands[147,193] or spots[126]. To prevent crystallisation, saturated C_{20-30} acids should be kept at 80–90° before the separation[15]. Unsaturated acids with conjugated bonds should be applied immediately after isolation, to avoid polymerisation[194]. The common unsaturated acids are also decomposed on paper in the absence of solvent[125].

The strip is treated in the vapour of the mobile phase[126,137,155,195] and developed by descending[78,126,128,140,164,179], ascending[141,144,146,167,176,184,196], horizontal[7,188,197], or circular[160,161,163,173,198] methods; the last method can be used for the preparative separation of lipids (more than 10 mg)[172]. The mobile phase usually moves a distance of 20–35 cm from the point of application. The efficiency of the separation increases if the upper part of the strip is wedge-shaped, as described by Matthias[17,132,199]. Ascending chromatography in test-tubes is used for the rapid analysis of acids[137].

C. Mobile phases. The mobile phase should have high polarity and low volatility, it should readily dissolve the lipids of the specimen, and its miscibility with the non-polar phase should be low. With increase in the polarity of the phase, ΔR_f increases and the solubility of the lipids decreases. If, however, the quantity of water is decreased, the duration and efficiency of separation are reduced. The mobile phases unavoidably contain the stationary phase of the system (80–90% saturation).

Acetic acid is almost always present in the mobile phase. The 90% acetic acid/undecane system separates the C_{16-18} saturated and unsaturated acids[15,16,137,140,144,156-158,161,163,193], cyclopentenyl aliphatic acids[200], and aliphatic alcohols[192]. Sometimes 90% acetic acid is used with paraffin oil[126,173] and liquid paraffin[146]. The 95–100% acetic acid is used for the C_{20-34} saturated acids[15,126,127,129,160,174,201,202] and polyunsaturated glycerides[132,172,198], and 50–75% acetic acid is used for the C_{10-14} unsaturated acids and C_{10-18} hydroxy-, epoxy-, and keto-acids[15,126,127,140,157,168,202], the thiocyanato-derivatives of the acids[203], monoglycerides[143], and oximes of C_{8-18} aldehydes[204]. If the specimen contains C_{10-24} aliphatic acids, separation with an acetic acid concentration gradient from 50% to 90% is used; the concentration C_t at time t is calculated from the formula $C_t = C_0 e^{-vt/V}$, where C_0 is the original concentration of acid, V the volume of the mixing chamber, and v the rate of supply of acid to the chamber[15,170]. The use of formic acid for the separation of aliphatic acids — 98% acetic acid–85% formic acid–water (30 : 10 : 1)[141,142,145,147,166] or 85% acetic acid–88% formic acid (1 : 1)[205] — increases the efficiency of separation and makes the zones more compact. Systems containing acetonitrile or acetone dissolve lipids readily, increase the rate of separation and ΔR_f, and withstand temperatures up to 45°.[7,15,188] Acetic acid–acetonitrile (5 : 7)[190], acetone–acetic acid–water (8 : 2 : 1)[7,16,133,188], acetone–acetic acid (3 : 1)[165], or 85% acetone–90% acetic acid (3 : 2)[164] mixtures are used for acids; acetone–acetonitrile (4 : 1)[131,150,172] and acetone–methanol (9 : 1[128,172] or 3 : 1[130]) mixtures are used for triglycerides; and acetic acid–acetonitrile (3 : 1)[196,204,206] is used for the acetoxymercurimethoxy-derivatives of the allyl esters of saturated acids and the allylurethanes of higher alcohols, and also for the oximes of diesters of dihydroxyacetone. Aqueous 80–98% solutions of acetone have been used to separate mono- and tri-glycerides[134] and aliphatic acids[126,207].

Systems containing methanol — methanol–acetic acid (30 : 1), 90% methanol–acetic acid (30 : 1)[179], methanol–acetic acid (5 : 1)[139,163,178,182], 90% methanol–acetic acid (5 : 1)[178,181,183], and 70% methanol–acetic acid (3 : 2)[184] — have been used for the separation of "bromazones" and the acetoxymercurimethoxy-derivatives of unsaturated lipids and phospholipids. Saturated C_{20-36} acids have been separated in the systems methyl acetate–acetic acid–water (5 : 20 : 2)[139,162], 99.5% propan-2-ol–96% ethanol–acetic acid–water (8 : 2.5 : 4 : 1.25)[135], and methanol–acetic acid–water (5 : 4 : 1)[16]. Aqueous 75–95% methanol[167,176,177] and ethanol[124,186], 55–65% propan-2-ol[156], and propan-2-ol–methanol–water (11 : 5 : 4)[208] are hardly ever used at the present time for the separation of acids, because of the low values of ΔR_f and the "mutual dissolution" taking place when zones with low lipid concentration are absorbed by larger neighbouring zones[193]. Mixtures of methanol with chloroform (1 : 3)[205] or with tetrahydrofuran and carbon tetrachloride[125] and an acetic acid–chloroform mixture (16 : 3)[209] have been used for the separation of triglycerides.

The 2,4-dinitrophenylhydrazones of C_{8-18} aldehydes have been separated in the dimethylformamide–formamide (5 : 2)/hydrocarbons of b.p. 150–180° system, and their acetoxymercurimethoxy-derivatives have been separated in the same system with tetralin[7,210,211].

D. Temperature of separation. Mutual saturation of the phases and chromatographic separation are usually carried out at 20 ± 2°. Increase in temperature increases R_f and the rate of separation, but leads to flow of the non-polar phase [16]. Cooling to 17° stops the separation of saturated acids, whose solubilities (in g per 100 g of acetic acid) have the following values at 20° and 30° respectively — C_{10} 567 and 8230, C_{12} 81.8 and 297, C_{14} 10.2 and 51.1, C_{16} 2.14 and 8.11, and C_{18} 0.12 and 1.68. [159,188]

The decrease in solubility with temperature is used for the separation of saturated and unsaturated acids of equal polarity. The saturated acids can be precipitated before separation from petroleum ether at -20°. [163,212] In the acetone–undecane system, the following saturated acids remain at the point of application — C_{18} and above at -20°, C_{16} and above at -30°, C_{14} and above at -38°, and C_{12} and above at -50° — the acids which move at a given temperature being separated from one another [188]. After application of the acids, the strip was kept at -30° and then developed for 20 h in the systems (1) acetone–propionic acid–water (4 : 3 : 0.5)/undecane at -30°, or (2) 85% acetic acid–85% formic acid–water (10 : 10 : 1)/silicone oil at -10°. The saturated acids from C_{14} did not move [15,125,188]. In two-dimensional chromatography, system (2) was used in the first direction, and 93% acetic acid at 20° in the second direction [213]. In the separation of *cis*- and *trans*-isomers, system (1) eluted oleic acid, while elaidic acid remained at the point of application [18,214].

To increase the solubility of the saturated acids, the temperature in the chamber has to be raised to 30° for the C_{20-26} acids [7,15,125,127,188,205], to 45–55° for the C_{24-28} acids [139,145,149,173], and to 55–85° for the C_{26-34} acids [129,135,141,174].

E. Detection and quantitative determination of lipids. Coloured lipids, for example the "bromazones" of aliphatic acids, can be determined quantitatively at once by photometric methods [181]. Most lipids of the aliphatic series are colourless, however. These are detected and determined quantitatively by means of the colour reactions given by the carboxyl, hydroxyl, and other groups, double bonds, or the aliphatic chain itself.

(*a*) *Reactions of the carboxy-group.* Aliphatic acids and other acidic lipids are most readily detected by a non-specific test with an indicator [7]: 0.2–0.4% Bromothymol Blue in alcohol, pH 8–8.3, [151,168,176] Bromophenol Blue [134,215,216] or Bromocresol Green [177], 0.05% Cresol Red and cresolphthalein in 0.8% NaOH, [198,217] Malachite Green [185], Nile Blue [158,217], thymolphthalein, and Phenol Red [217,218]. With Pyrogallol Red, z = 15 μg. [219] The colour produced by the acids disappears rapidly. The results obtained by titrating the acids with 0.005 *N* NaOH using Bromothymol Blue as indicator after their extraction from the paper agreed with the results of the thiocyanatometric determination [134,215].

Hydroxamic acids have been detected with acidic 1.5–2% $FeCl_3$ in a mixture of ethanol and butanol (1 : 4) in the form of purple zones [152,153]. The sensitivity of this reaction is lower than that of the reaction with indicators [176]. The methyl esters of aliphatic acids, obtained with ^{14}C-diazomethane, were determined quantitatively by scanning a radio autograph of the chromatogram on an MF-4 microphotometer [165,220].

Aliphatic acids are usually determined as the heavy-metal salts [7]. To obtain the Co salts, the strip was held in NH_3 vapour, immersed for 1 h in 0.03–2.5% ^{59}Co acetate at pH 5.8–6.0, containing 2–3 μc ^{60}Co ml^{-1}, treated with ammonium sulphide to develop the colour, and measured on a Geiger counter with an accuracy of 2.5%. [221,222] The ^{59}Co salts alone are not suitable [43].

Basic copper(II) acetate is used more frequently than other salts [7]. The strip is immersed in a mixture of saturated copper acetate and water (1 : 49) for 20 min, and washed with water acidified with acetic acid to reveal the pale green zones due to the acids [186]. Potassium cyanoferrate(II) (1–1.5%) is used to obtain the dark brown colour of copper cyanoferrate(II) (z = 5 μg). [155,156,158,167] If this solution is acidified, the salts are converted into the free aliphatic acids. Thus the reaction with copper acetate and potassium cyanoferrate(II) can be carried out repeatedly, to increase the sensitivity [188]. Under the influence of 5% ferric chloride, copper cyanoferrate(II) gives blue zones of $FeCu[Fe(CN)_6]$. [146]

In the reaction of copper salts, treated with ammonia vapour [133], with a basic 0.03% alcoholic dithiooxamide (rubeanic acid) solution, z = 0.1 μg Cu. [126,223,224] By repeated development of the colour, the C_{30-36} acids from wax, which can be separated only in very small quantities, have been detected [224]. The same reaction is given by aldoximes and ketoximes [204].

Copper salts can be detected from the liberation of oxygen and foaming with hydrogen peroxide and NH_4OH (z = 5–10 μg). [225] When Cu acetate solution moves along the paper, the aliphatic-acid zone brings about distortion of the front [59]. The colour of the copper salts of aliphatic acids is intensified by 0.5% Rhodamine B [43,49] and aniline–ethanol (1 : 1). [137]

The stoichiometric reaction leading to formation of the copper salts of acids from C_{12} upwards is often used for quantitative determinations. For an approximate estimate, measurements are made of the area of the spot T', and the quantity of acid C' is calculated from the formula $T' = k \log C' + b$, where for oleic acid k = 182 and b = 166, and for linoleic acid k = 134 and b = 189. [193] The ratio of the zones is usually determined quantitatively with a photoelectric densitometer. The coloured chromatograms or their photographs are drawn past the 1 mm slit of the photoelement, and the light absorption is recorded. The area of the peaks on the curve is proportional to the molar concentration of the acids [144,146,155,223]. To obtain a uniformly low light-absorption background, it is necessary to purify the paper (see Section III4A) and to remove as far as possible the stationary phase, acetic acid (without heating), and the copper salt solution, whose pH should not exceed 5. [53,126,204,226] If the non-polar phase cannot be removed from the paper, acids with low values for K are not converted completely into the copper salts, and a correction must be introduced into the calculation [126]. When the light absorption between the peaks does not return to zero, the area for each component is found by means of supplementary lines [126,227]. Beer's law is obeyed over the range 1–60 μg of the acids [126,226]. The accuracy of the determination of acids whose concentration exceeded 2% [7] reached ±3%. [228]

Polarographic and colorimetric methods have proved that the reaction is stoichiometric. Copper ions were eluted with 0.06–1 *N* H_2SO_4. A linear relationship between the height of the step on the polarogram and the quantity of copper was observed for 1–7 μg copper ml^{-1}. [227,229,230] In the colorimetric estimation with 0.1 M tetraethylthiuram

disulphide [231] or 0.001% dithizone [232], Beer's law was obeyed over the range 10–30 μg ml^{-1}. The accuracy of both methods was ±5%. [230,231]

Silver salts were obtained in 1% ammoniacal silver nitrate within 5–30 min; excess of the salt was washed out, and the Ag was fixed with dilute HCl. [186] For the C_{20-34} acids, the solution was heated to 55–85°. [129,174,186] The silver salts were coloured by heating to 140–150° for 10 min [129,194] or by treating with ammonium sulphide [167,186], sodium sulphide [147], hydrogen sulphide [156], phenylhydrazine [43], and 0.03–0.5% *p*-diethylaminobenzylidenerhodanine [43,174,233]. The accuracy of the densitometric determination of the C_{16-18} acids on an MF-4 apparatus with a supplementary projecting lens was adequate [147]; photographs of chromatograms of the C_{24-34} acids were scanned on an MF-2 apparatus with unsatisfactory results [174]. After the paper had been ashed, the accuracy of the determination of 0.2–2 μmole of silver with dithizone was ±5%. [233] Aliphatic acids can also be determined radiometrically with ^{110}Ag. [234]

The mercury salts (0.1% Hg^{2+} acetate in 0.5% acetic acid for 15 min) were more stable to hydrolysis by water than were the copper salts, and the paper background was lighter. The salts were coloured with 0.2% 1,5-diphenylcarbazone [78,173], the colour was eluted with methanol and toluene (1 : 1), and measurements were made at 530 mμm. [15,173] The radiometric determination was carried out with ^{203}Hg. [234]

The lead salts (1–3% lead acetate, pH 5.3–5.7, 20–40°) were coloured with hydrogen sulphide, 1.5% ammonium sulphide, or a solution of 2 g of sodium sulphide, 100 g of ammonium nitrate, and 30 ml of hydrogen peroxide per litre (z = 0.05 μg of acid). 2-Hydroxy-acids are not detected by means of lead [139,164,168]. After extraction from the paper with 10% nitric acid, the lead was determined with dithizone at 520 mμm. Satisfactory accuracy in the measurements was achieved by thorough purification of the paper, reagents, and the quartz vessel from traces of heavy metals [142]. The reaction of the acids with bismuth nitrate (at 70–80°) and ammonium sulphide gave values of z equal to those for reactions with the lead salts [127,141,148].

(*b*) *Reactions of the hydroxy-group.* The vicinal OH groups of monoglycerides and polyhydroxystearic acids have been detected by Schiff's reaction with 1% periodate, sulphur dioxide, and pararosaniline (z = = 10–20 μg) [70], or with a solution containing 35% borax, 0.8% potassium iodide, 0.9% boric acid, and 3% starch (the lipids gave white spots on a blue background) [52]. For radiometric determination, mono- and di-glycerides were acetylated with $(^{14}CH_3CO)_2O$. [220]

(*c*) *Reactions of the double bond.* Unsaturated lipids are detected by oxidation or replacement of the double bonds. On oxidation in 0.1–1% alkaline permanganate, unsaturated lipids after 1.5–5 min give brown spots of manganese dioxide [48,132,139,141,166]; acid permanganate gives white spots on a pink background [7]. Densitometric measurement of the colour produced by the action of alkaline permanganate has been carried out in reflected light; the concentration of aliphatic acids, glycerides, and sterol esters was determined from the height [209] or the area of the peaks with an accuracy of 3–5%. [166] Unsaturated acids were oxidised with sodium periodate and 1% potassium permanganate, and scanned on a microphotometer [235].

In the ozonisation, the band was treated with sodium hydrogen sulphite, then with oxone for 20–60 min, and finally with Schiff reagent [208,236].

Unsaturated lipids on a moist strip without hydrocarbons or with silicone oil give brown or yellow spots in iodine vapour at different pH values [125,132,150,220]. The colour was scanned with a blue filter [205]. The iodine reacting with the double bonds was detected with 0.5–1% starch in the form of blue spots on a light background (z = 3–4 μg) [127,128] or with 0.2 *N* silver nitrate and the photographic developer Eukobrom [237]. Bromine and thiocyanogen may be partly liberated from the double bonds in ammonia vapour: Br^- was detected with Phenol Red and chloramine (z = 0.1 μg) [238], and CNS^- with Fe^{3+} salts [203]. The vapour and solutions of ^{131}IBr have been used for the radiometric microdetermination of the mass and iodine number of lipids [222,234,239,240].

Mercury(II) acetate in methanol is also used for the replacement of double bonds in *salts and esters* of aliphatic acids [7,15,229]:

$$-CH{=}CH- \xrightarrow{Hg(OCOCH_3)_2,\ CH_3OH} -CH(OCH_3)-CH(HgOCOCH_3)- .$$

The acetoxymercurimethoxy-derivatives of the esters are synthesised before separation [178,184,196]; the derivatives of the salts are prepared on the paper in 2% mercury(II) acetate containing 1% acetic acid only after the separation [241]. The combined mercury is detected with 0.1–0.2% alcoholic 1,5-diphenylcarbazone and ammonia vapour [78,196]; hydrogen sulphide colours mercury after its displacement from the derivatives by hydrogen chloride vapour (z = 1 μg of linolenate) [184,188]. Colorimetric determination with 1,5-diphenylcarbazone [178] or dithizone [196], polarography [229], and densitometry [184,196] are used for the quantitative determination. Kaufmann used the acetoxymercurimethoxy-derivatives to determine saturated and unsaturated acids having equal values of K, for example palmitic and oleic acids. Parallel chromatograms were coloured with copper or mercury acetate. Since the carboxy-group is equivalent to one-half of a copper or mercury atom, and the double bond to one mercury atom, the value of y (the percentage concentration of the saturated acid) was calculated from the formulae

$$y = [(2e + 1 - x)/2e] \times 100;\ x = F_{Hg}^{corr}/F_{Cu};\ F_{Hg}^{corr} = F_{Hg}(F_{Cu}^{stand}/F_{Hg}^{stand}),$$

where e is the number of double bonds in the unsaturated acid which is not separated from a given saturated acid, F_{Cu} and F_{Hg} are the areas of the peaks for a non-separating mixture of the saturated and unsaturated acids, obtained by scanning chromatograms coloured with copper and mercury salts respectively, and F_{Cu}^{stand} and F_{Hg}^{stand} are the areas for the standard (stearic acid) [241].

Osmium tetroxide vapour detects 5 μg of oleic and 2.5 μg of linoleic acids as grey spots on a light background [7,161].

(*d*) *Reactions of the aliphatic chain.* A number of methods for the detection of lipids are based on the selective adsorption of lipophilic dyes and other substances from aqueous–alcoholic solutions or on the formation of inclusion compounds [11]. Of the fluorescent dyes, use is generally made of 0.05% Rhodamine B, [7] which detects mono-, di-, and tri-glycerides and aliphatic alcohols as white spots, which exhibit blue fluorescence, on a pink background (z = 2 μg) [7,134,192]. Under the influence of permanganate, the colour of the spots changes to yellow [130]. The colour of Rhodamine is intensified by Nile Blue, quinine, Acridine Orange, etc. [43,61,242]. Rhodamine B has been used for the approximate determination of monoglycerides from the area of the spot [134,215]. Protoporphyrin detects phospholipids, acids, and glycerides as fluorescent red spots on a light background (z = 1 μg) [243].

The complexes of lipids with fat-soluble dyes, readily detectable in visible light, are formed only after the

removal of hydrocarbons from the paper [158,167]. Sudan IV, [3] Oil Red O, [62] and other dyes have been tested; the best was found to be Sudan Black B, [150] which detects the esters of polyhydric alcohols and stearins, but which, unlike Rhodamine, does not colour the esters of monohydric alcohols [244]. Unsaturated lipids combine with Sudan more readily than do saturated lipids; in direct light the colour changes to pinkish brown [245]. Solutions of fat dyes in 50% diacetin have been used for the densitometric determination of glycerides; the accuracy of the measurements was improved by impregnating the strip with paraffin oil, 1-bromonaphthalene, and an emulsifying agent [246]. Because of their surface-active properties, lipids can be detected from the change in the hydrophilic nature of separate sections of the strip after the separation. On paper carrying paraffin oil, the esters of aliphatic acids and sucrose and triglycerides gave transparent spots in water and potassium hydroxide solution respectively [132,247]. Triglycerides can be hydrolysed on paper with 10–20% potassium hydroxide at 95°, and then detected by means of copper salts. On complete hydrolysis, no colour is produced by Sudan [131,150,248,249].

α-Cyclodextrin, combined with lipids by inclusion reactions, is not coloured by iodine vapour and gives white spots on a blue ground. Di- and tri-glycerides, which do not form inclusion compounds, must be hydrolysed beforehand on the paper by means of pancreatin [125,220]. The inclusion reaction has been used for the densitometric determination of triglycerides and methyl esters [205].

F. Separation of lipids. Homologues differing by a CH_2 group and, in series of equal chain length, homologues differing by a free or substituted double or triple bond, hydroxy- or keto-group, etc., are separated in a reversed-phase system. *Homologous series* which have now been separated include the normal saturated C_{10-36} aliphatic acids, even and odd [78,127,141,201], mono-, di-, tri-, and poly-unsaturated C_{11-28} acids [126,141,157,190], mono-, and di-acetylenic acids [126], cyclopentenyl unsaturated acids [200], C_{14-24} normal saturated 2-hydroxy-acids [127], C_{30} mycolic acids [169], saturated and unsaturated hydroxy-, dihydroxy-, and keto-acids [126,158,177,202], C_{14-26} saturated 2-methyl-4-oxo-acids [222], di-, tetra-, and hexa-bromo-derivatives of stearic acid [158], adducts of maleic anhydride with acids containing conjugated double bonds [216], the 2,4-dinitrophenylhydrazones of C_{2-22} normal saturated acids, even and odd [178], the "bromazones" of C_{2-26} normal saturated and unsaturated acids, aldehydes, and ketones, and their acetoxymercurimethoxy-derivatives [139,178,182], the acetoxymercurimethoxy-derivatives of unsaturated acids [179] and the allyl esters of C_{20-30} saturated acids [196], C_{10-18} saturated and unsaturated alcohols [192] and their allylurethanes [206], the acetoxymercurimethoxy-derivatives of the allylurethanes of C_{10-30} saturated and unsaturated alcohols [206], the 2,4-dinitrophenylhydrazones of C_{8-18} saturated and unsaturated aldehydes and their acetoxymercurimethoxy-derivatives [210], the oximes of C_{8-18} esters of dihydroxyacetone and higher aldoximes [204], the esters of saturated and unsaturated acids with mono- and poly-hydric aliphatic and aromatic alcohols [244], mono-, di-, and tri-glycerides of the same or different acids [125,134,162,205,208], the triglycerides, of synthetic [125,132,144,162,183,205] and natural origin, containing residues of C_{8-24} saturated and unsaturated acids [128,130-132,134,150,162,183], and also the acetoxymercurimethoxy-derivatives of the glycerides [183].

Tuberculostearic and nonadecanoic acids have equal R_f values, so that chain branching does not influence mobility [126,200]. If the double bond is sufficiently far from the ends of the chain, the polarity rule applies (see Section III1). A double bond next to a carboxy-group, however, does not alter R_f values: thus octadec-2-enoic and stearic acids are not separated [126]. Mobilities are the same for cycloalkene and alkene double bonds [200], isolated and conjugated double bonds [126], and double bonds in the *cis*- and *trans*-positions [126,157]. Replacement of a double bond in a chain by a triple bond increases the R_f value [126,157]. The polarity of the OH group is greater than that of the CO group, so that 12-hydroxy- and 12-oxo-stearic acids are separated from one another [126]. In a series of hydroxy- and keto-acids, the chromatographic mobility decreases as the functional group approaches the carboxy-group [158,202].

The acetoxymercurimethoxy-derivatives of Φ-cholines have been separated into five fractions of different acid composition, the degree of unsaturation of the acyls increasing with increase in the R_f value [182]. Soya-bean Φ-cholines have been hydrogenated and hydrolysed on paper in a study of the acid composition [250].

If members of different homologues series have the same values for K and the same R_f values, separation of these lipids requires either elimination of the unsaturated components or displacement of the polarity equilibrium in the mixture by saturation of the double bonds with hydrophilic or hydrophobic substituents. Before the chromatographic separation, the aliphatic acids were separated into saturated and unsaturated acids by the early method of Twichell and Bertram [7,155], by crystallisation of the copper salts from ethanol [186], or by crystallisation of the acids from 55% acetonitrile [251]. The unsaturated components were detected and determined by parallel separation of the original lipids and the lipids oxidised by 1% alkaline permanganate [126,141]. For complete decomposition of the unsaturated acids during the separation, use was made at 37° of the mobile phase acetic acid–formic acid–hydrogen peroxide (9 : 1 : 1 or 6 : 1 : 1), containing peroxyformic acid; the saturated acids were not oxidised in this phase [78].

On hydroxylation with permanganate or addition of thiocyanato-groups to the double bonds, the R_f values for the acids increased, and on halogenation with iodine bromide the R_f values decreased [252,253]. The mobility of unsaturated acids increased after bromoacetoxylation and bromomethoxylation with *N*-bromosuccinimide and also after formation of the acetoxymercurimethoxy-derivatives of the "bromazones" of these acids. The diastereoisomers of the bromoacetoxy- and bromomethoxy-derivatives of polyunsaturated acids each form two zones on the chromatograms [136,139,254]. Aliphatic acids with equal R_f values have been separated by *hydrogenation* of the double bonds, with the formation of corresponding saturated acids of lower polarity [15,125]. In some cases the acids were separated before and after hydrogenation in the presence of palladium oxide on barium sulphate in ethanol [197], or of platinum oxide by Adams' method in acetic acid [126]. If the lipids contain palmitoleic acid, the complete mixture of acids is not hydrogenated, but only the fraction containing the palmitoleic, myristic, and linoleic acids; the hydrogenation products are subjected to further separation [191,224,255]. In other cases the hydrogenation was carried out directly on the paper (see Section III3), after which the hydrogenated and non-hydrogenated mixtures were separated [256-259]. For the determination of linolenic and α-elaeostearic acids, which have identical R_f values, adducts of elaeostearic acid with maleic anhydride are prepared in the presence of iodine, and the mixture of acids is hydrogenated and subjected to further separation [260,261]. Carbonyl groups in lipids are

reduced with a solution of sodium hydroborate in tetrahydrofuran[262]. For the determination of acids of equal polarity, use has also been made of low-temperature chromatography (see Section III4D) and the reaction of the double bonds with mercury salts (Section III4E*a*).

Thus the polarity rule introduces considerable difficulties in the analysis of lipids. On the other hand, the identification of complex mixtures, e.g. of triglycerides, where a fraction of given polarity may contain six, seven, or more components, is sometimes greatly facilitated, since lipids of known structure are correlated with a given chromatographic zone on the basis of a strict relationship.

Reversed-phase systems are seldom used for the fractionation of lipid mixtures into classes: the differences in polarity are so great that separation in any one system becomes impossible (see Section I). Moreover, lipids from different classes, with different values for K, often have the same Rf values. Thus octadec-*cis*-9-en-1-ol is not separated from myristic acid, and diglycerides are superimposed on the stearic acid zone[192,208]. The concept of a "universal" polarity constant K_{univ} has been introduced to compare classes on the basis of their absolute polarity. A standard of reference is provided by the homologous series of esters of monohydric alcohols and even normal aliphatic acids: for these the values of K and K_{univ} coincide. For lipids of another homologous series, K_{univ} is equal to the value of K for the substance of the reference system which has the same Rf value as this lipid. The increment i, i.e. the difference between K_{univ} and K for a given lipid, is constant for a given series. The additive value of i depends on the number of CH_2, COOH, and OH groups, double or ester bonds, the position of the functional groups, and other factors. The value of i is 14 for triglycerides, 12 for the esters of unsaturated acids, 10 for 2-hydroxy--acids, 2 for the esters of aromatic alcohols, etc. This absolute polarity scale is extremely useful for the identification of lipids according to their classification in a specific homologous series[202,244].

It can be seen that the mobility of lipids during separation depends on their composition. The relationship between the Rf value in different systems and the parameters of the molecule have been described by *empirical equations*: $Rf = 1.42 + (-0.062)m$; $Rf = 1.671 - 0.1326m + 0.0028m^2$; and $Rf = 1.50 - 0.0625m$ for saturated aliphatic acids, or $Rf = 1.42 - (-0.062)(m-2)$ and $Rf = 1.57 - 0.06m$ for unsaturated acids[124,133,195]. The dependence of the Rf values on m for aliphatic acids or their *p*-phenylazophenacyl esters was linear[32,201]. This relationship was not observed for triglycerides[150]. The Rf values for C_{18} unsaturated acids was found to be a linear function of the number of double bonds in the chain[134].

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The methods of partition chromatography are now being widely used for the separation of lipids of natural origin. The various methods are not all equivalent, however.

Column chromatography with a polar stationary phase is used for the analysis of an individual homologous series of aliphatic compounds or their polar derivatives; more modern methods are necessary for the separation of mixtures of several series of lipids. The analysis of lipids on paper with a polar stationary phase has now been largely replaced by other forms of chromatography, which give much more effective separations. Of considerable practical interest is the application of this procedure to the analysis of the oxidation products of lipids and the separation of phospholipids; ion-exchange paper or paper impregnated with hydrophilic organic phases such as formaldehyde or formamide must be used.

Of much wider application are reversed-phase systems, which are used in column chromatography for the preparative separation of lipids, in particular of lipids with oxygen--containing functional groups in the chain. This method does not require complicated and expensive equipment, and makes it possible to obtain several grammes of pure specimen in sufficiently high yield under mild conditions. This form of chromatography is less suitable for analytical separations, since it requires several centigrammes of material and the actual operations are fairly tedious[8].

Reversed-phase chromatography on glass plates is now being used on an ever increasing scale for analytical separations. In combination with the highly effective methods of thin-layer chromatography, in particular with separation on silica gel impregnated with silver nitrate, lipids of a given class can be characterised from the values of m and e on a single plate. The possibilities for the preparative separation and quantitative determination of lipids are apparently more restricted in this case, although work of this nature has recently been reported[123].

Reversed-phase chromatography on paper continues to occupy a leading position among partition-chromatographic methods for the study of lipids. By changing the compositions of the stationary and mobile phases, the temperature, the duration of the experiment, and other factors, the optimum separation conditions for a specific mixture of lipids are rapidly found. The time required for the separation (several hours) and the sensitivity of the detection of the lipids (fractions of a microgramme) approach in a number of cases of paper chromatography, those characteristic of thin-layer and gas–liquid chromatography. An extensive range of qualitative reactions which can be carried out directly on the paper is available for the identification of lipids. The analytical separation is readily combined with densitometric quantitative determination and radiographic measurement of the activity of individual zones.

The simplicity and reproducibility of the separation are two of the most important advantages of paper chromatography: the analysis can be carried out without great expenditure of labour or money, in any laboratory equipped with the minimum of apparatus and reagents. The method is not only very convenient for the rapid estimation of the composition of individual fractions of the eluate from a chromatographic column, but can also be used by itself in preparative work for the isolation of lipids in quantities of 1–50 mg.

Thus reversed-phase partition chromatography on paper is entirely suitable for the analysis of classes and homologous series of lipids of simple composition. For the identification of multicomponent natural mixtures of lipids and the identification of individual compounds, this method must be combined with adsorption and gas–liquid chromatography, spectroscopy, and other modern methods for the analysis of lipids.

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THE CHROMATOGRAPHY OF BIOPOLYMERS ON CALCIUM PHOSPHATE GELS

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I. INTRODUCTION

An important factor determining the success of a chromatographic separation is the choice of a suitable adsorbents. Progress in this field in recent years has led to the appearance of two main types or groups of adsorbent[1], which are now widely used for the chromatography of the most varied biopolymers.

One of these groups — calcium phosphate sorbents or gels — consists of molecular adsorbents. The other group consists of various ion exchangers, based chiefly on cellulose[2].

Both groups of adsorbent have an extensive, well hydrated surface, but the adsorption of biopolymers on these materials takes place in different ways. The principles of the action of these adsorbents also differ[3] and their chromatographic selectivity with respect to biopolymers is probably not the same[1]. Both groups of adsorbents are now widely used for the chromatography of the most varied biopolymers.

A number of reviews have been published[4-6] on ion exchangers based on cellulose. No reviews have been published on the use of calcium phosphate gels for the chromatography of biopolymers.

The aim in the present review has been to collect and summarise available published data on the chromatography of biopolymers on calcium phosphate sorbents.

II. METHODS OF PREPARATION AND CHARACTERISTICS OF CALCIUM PHOSPHATE SORBENTS

Until recently, only tricalcium phosphate $Ca_3(PO_4)_2$ and calcium hydrogen phosphate or brushite $CaHPO_4.2H_2O$ were generally employed in chromatographic practice. In recent years, a particular form of calcium phosphate — hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ — has begun to be used extensively in the chromatography of proteins. Tricalcium phosphate was at one time used fairly extensively as an adsorbent for the chromatography of proteins. Mention may be made, for example, of the isolation and purification of catalase by the use of tricalcium phosphate[8,9].

Tricalcium phosphate is now seldom used for the chromatography of proteins in column experiments. We shall therefore not deal further with this type of calcium phosphate, except to point out that $Ca_3(PO_4)_2$ usually contains a considerable quantity of hydroxyapatite[10]. This is due to the fact that the primary product formed in the preparation of tricalcium phosphate is pseudoapatite[11], which is subsequently converted into one modification of tricalcium phosphate and partly into hydroxyapatite[12].

A second example of a calcium phosphate gel, which is still used in the chromatography of proteins, in calcium hydrogen phosphate or brushite. The structure of brushite obtained by mixing solutions of $CaCl_2$ and Na_2HPO_4 depends to a marked extent on the pH. Formation of the precipitate usually takes place at pH 6.3–6.5. At the same time, it is known[10] that at pH 6.2 and above, calcium hydrogen phosphate may undergo slow hydrolysis into another form of calcium phosphate with the structure of hydroxyapatite.

Since the chromatography of proteins and other biopolymers is usually carried out in the approximate pH range 6.8–7.2 (i.e. above pH 6.2), the experimenter is in fact concerned with the unstable changing structure of an adsorbent containing a considerable proportion of hydroxyapatite.

In this respect, an undeniable advantage is shown by another calcium phosphate gel, which is now widely used in the column chromatography of biopolymers, namely hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. This material has a high stability over a wide pH range.

The modern chromatography of biopolymers on calcium phosphate columns may be taken as dating from 1956, when Tiselius, after a number of preliminary experiments[13-16], published a method for the preparation of hydroxyapatite suitable for the chromatography of proteins[10]. In this