the salt in decreasing the swelling pressure. (3) The effect of the salt on cohesion. The curves shown in Fig. 3 suggest that at low concentrations of sodium chloride, increase in the sodium-ion concentration is the predominant factor, the rise in the Na^+/Ca^{++} ratio increasing the swelling pressure with the result that swelling increases. As the concentration of sodium chloride increases, the effect of salt in depressing the swelling pressure becomes apparent, and balances the effect of increase in the sodium-ion concentration, so that the swelling remains constant. The change in the shape of the curve above 0·1 M suggests that cohesion is beginning to be affected; at concentrations of this order the swelling pressure probably begins to decrease, but the decrease in cohesion balances this and the swelling remains unchanged. At concentrations of 2 M or greater, sodium chloride begins to decrease the swelling again, probably by an action similar to that occurring in the salting out of proteins (Cohn & Edsall, 1943).

SUMMARY

1. The swelling of collagen in solutions of bivalent bases was found to decrease in the order barium hydroxide ≥ strontium hydroxide > calcium hydroxide. Swelling in barium hydroxide solutions was rather less than half, and that in calcium hydroxide solutions about one-eighth of that in sodium hydroxide solutions. The small amount of swelling in calcium hydroxide solutions is attributed to combination of calcium ions with the collagen.

2. The addition of calcium or sodium chlorides increased swelling in calcium hydroxide solutions. The increase with calcium chloride is attributed to decrease in the cohesion of the collagen, and the increase with sodium chloride to the addition of a univalent cation and consequent increase in swelling pressure.

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The Separation of the C_{12}-C_{18} Fatty Acids by Reversed-phase Partition Chromatography

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Work in this laboratory (Pollock, Howard & Boughton, 1949) on inhibition and promotion of bacterial growth by fatty acids revealed the need for analytical and preparative methods adapted to handling small quantities of these substances. Chromatography seemed to offer a possible solution to these problems.

Early attempts at adsorption chromatography showed little promise until Claesson (1946) and Holman & Hagdahl (1948, 1949) developed frontal and displacement analysis methods. These, though very successful, require rather elaborate apparatus and larger amounts of material than we wished to work with. We turned, therefore, to partition chromatography.

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This method had already been successfully applied to the separation of the lower fatty acids (Elsden, 1946; Ramsey & Patterson, 1945; Peterson & Johnson, 1948; Moyle, Baldwin & Scarisbrick, 1948). Ramsey & Patterson (1948) have extended the method to the longer chain (C_{11}-C_{19}) acids using 2-aminopyridine and furfuryl alcohol as their solvent system. They were able to separate acids differing by two CH_2 groups. It seems improbable that suitable solvent systems of this type could be found for much longer-chain fatty acids or for lipophilic substances lacking acidic groups. These substances would have, in general, partition coefficients too much in favour of the less polar phase. We tried, therefore, to evolve a partition chromatogram in which the less polar phase is stationary. The choice of solvent systems

should then be very much more extensive and the method correspondingly more flexible.

Attempts have previously been made to reverse the phases. Boscott (1947) suggested the use of cellulose acetate, and Synge (1949) attempted to apply this to the separation of peptides which were too fast running for the usual type of chromatogram, but with disappointing results. Boldingh (1948) successfully separated methyl esters of long-chain fatty acids on filter paper impregnated with vulcanized rubber latex. He located the ester spots by dyeing with Sudan III. This was not very satisfactory, nor was the method well adapted for preparative work.

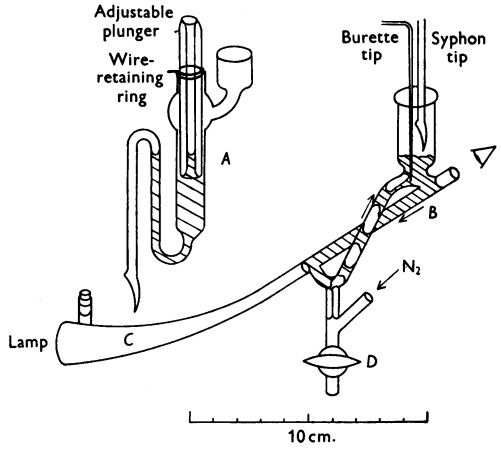

Fig. 1. Diagram of apparatus used. *A*, adjustable siphon; *B*, titration cell; *C*, tube filled with $CHCl_3$ to transmit light to titration cell; *D*, tap to empty cell.

The problem of finding a hydrophobic support for the less polar phase of a solvent system was solved by treating kieselguhr with dichlorodimethylsilane vapour and thus rendering it 'unwettable' by strongly polar solvents (cf. Martin, 1949). This treated kieselguhr successfully retains the less polar phase of numerous solvent systems, e.g. it will retain the acetone phase of an acetone-aqueous ammonium sulphate mixture and the paraffin phase of a paraffin-aqueous acetone mixture. The stability of the columns made with this support depends upon the interfacial tension of the two phases. When this is high stable columns can be made from treated kieselguhr holding as much as 1 ml. of stationary phase/g. The most suitable systems for use with fatty-acid mixtures were found to be aqueous methanol-octane and aqueous acetone-medicinal paraffin. The latter gives a very stable column which can be used repeatedly without losing the stationary phase. Using these systems, the straight-chain saturated acids from lauric to stearic have been successfully separated. Unsaturated and substituted acids behave similarly to the saturated acids. Only acids with an even number of carbon atoms have been studied.

METHODS

Preparation of kieselguhr. 'Hyflo Super Cel', obtained from British Drug Houses Ltd., was dried at 110° and, when cool, was allowed to stand in a desiccator containing dichlorodimethylsilane or stirred gently, whilst passing through it a stream of dry air previously drawn through the silane

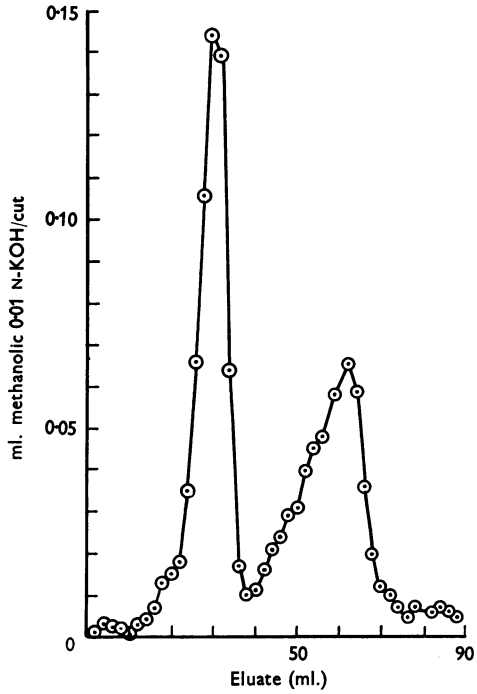

Fig. 2. The separation of a mixture of myristic and palmitic acids. Stationary phase, liquid paraffin saturated with 70% (v/v) aqueous methanol, moving phase, 70% (v/v) aqueous methanol saturated with liquid paraffin. Ambient temperature 35°.

derivative. The treated material should all float when shaken with water. The treated kieselguhr was washed with methanol until the washings were no longer acid to bromthymol blue, dried at 110° and stored for use.

Solvents. Solvents were normally used without special purification. Samples of alcohols with high acidities were distilled from solid KOH.

Columns. The standard columns used were 12 mm. in diameter. The mixture required for preparing the more polar phase, e.g. 60% (v/v) acetone in water, was equilibrated with the less polar solvent e.g. liquid paraffin. The relative proportions of the two phases equilibrated seemed to be immaterial. After equilibration, the two immiscible phases were allowed to separate and drawn off for use when required. At first, columns were packed with 4 g. of kieselguhr and 3·8–4·0 ml. of the less polar phase, but better results were

Table 1. *Behaviour of fatty acids in reversed-phase partition columns*

(The numbers indicate the start, peak and end of the bands as the total volume (ml.) of eluate collected at these points. These figures were obtained with the standard column 20 cm. long and 12 mm. in diameter made in the way indicated in the text with the solvent system quoted in the left-hand column. Solvent systems are defined as % (v/v) of the first-named substance in aqueous solution equilibrated with the second-named substance.)

Solvent system	Lauric acid			Myristic acid			Palmitic acid			Stearic acid			Oleic acid			Dihydroxy-stearic acid			Elaidic acid		
80% Methanol-*cyclo*-hexane	—	—	—	34	48	60	70	90	110	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	28	40	52	60	78	100	—	—	—	—	—	—	—	—	—	—	—	—
60% Methanol-*n*-octane	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	10	15	24	—	—	—
70% Methanol-*n*-octane	22	34	44	48	70	80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	22	32	40	48	68	78	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	52	76	94	110	192	220	—	—	—	—	—	—	—	—	—	—	—	—
80% Methanol-*n*-octane	—	—	—	24	32	40	42	56	78	90	116	130	—	—	—	—	—	—	—	—	—
	—	—	—	24	36	44	50	64	74	—	—	—	—	—	—	—	—	—	—	—	—
50% Acetone-medicinal paraffin	14	34	48	36	78	94	—	—	—	—	—	—	—	—	—	8	12	30	—	—	—
55% Acetone-medicinal paraffin	20	32	44	48	60	68	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	14	26	32	32	44	60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	32	46	54	68	96	110	—	—	—	—	—	—	—	—	—	—	—	—
60% Acetone-medicinal paraffin	—	—	—	30	40	52	62	80	90	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	20	40	50	50	80	96	—	—	—	—	—	—	—	—	—	—	—	—
65% Acetone-medicinal paraffin	—	—	—	10	24	45	32	52	70	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	16	28	44	—	—	—	—	—	—	34	54	70	—	—	—	40	56	90
70% Acetone-medicinal paraffin	—	—	—	—	—	—	—	—	—	40	60	76	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	36	58	72	—	—	—	—	—	—	—	—	—

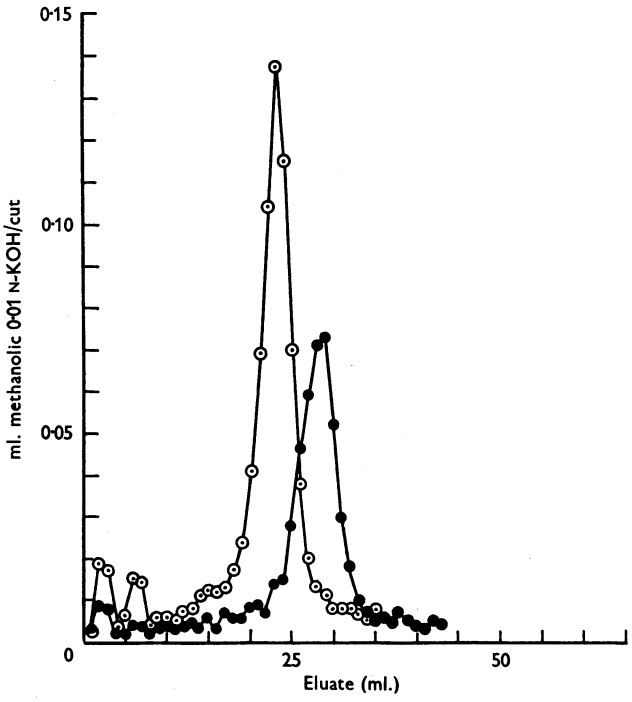

Fig. 3. Behaviour of single acids on reversed-phase partition column. ●, synthetic stearic acid; solvent system: 70% (v/v) aqueous acetone-liquid paraffin (b.p.). ⊙, synthetic myristic acid; solvent system: 60% (v/v) aqueous acetone-liquid paraffin (b.p.). Ambient temperature 35°.

obtained by using 9 g. of kieselguhr and 8 ml. of the less polar phase, giving a column 20 cm. long. The stated amounts of kieselguhr and less polar phase were stirred together in a beaker until a homogeneous powder was obtained; this was suspended in the more polar phase and the mixture was stirred by hand to remove lumps. The resulting slurry could not be packed into a column by gravity, but good columns were obtained by using a plunger. This was a perforated stainless steel disk attached at its centre to a long stainless steel rod. A portion of the stirred slurry was poured into the chromatograph tube and homogenized by rapid up and down movements of the plunger, which should fit the tube closely. The resulting fine suspension was then packed by slowly moving the plunger downwards. This procedure was repeated until all the material was packed. When the interfacial tension of the phases is low, packing cannot be effected by the plunger until the suspension has settled somewhat. It was found necessary to homogenize the kieselguhr-paraffin mixtures in a separate tube before starting to pack the column, and this was desirable on all occasions when the material was particularly lumpy. If air is inadvertently included during homogenizing, it must be removed by cautious evacuation prior to packing. Some of the solvent systems tested gave columns which tended to lose some of the stationary phase during use; aqueous methanol-$CHCl_3$ columns were particularly unstable, whereas aqueous acetone-medicinal paraffin columns have been used 14 times without changing their characteristics. The fluctuation of the room temperature during 24 hr. may so change the mutual solubilities of the less stable systems that the characteristics of the column alter. It is then advisable to maintain the column at a constant temperature if it is to be kept overnight. A constant temperature of 35° was normally used for convenience and because the increased solubility of fatty acids at that temperature was an additional advantage. There is no reason to think that other temperatures are

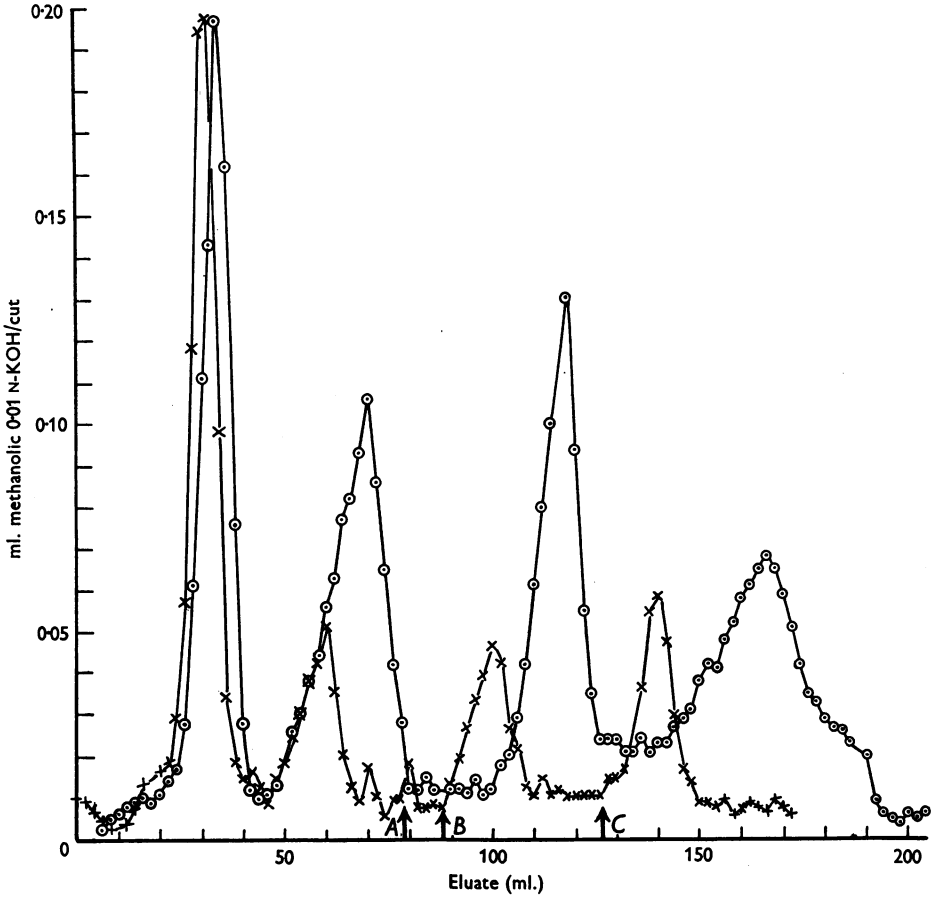

Fig. 4. The separation of a mixture of lauric, myristic, palmitic and stearic acids. ⊙, using 70 and 80% (v/v) aqueous methanol saturated with *n*-octane as the moving phase and *n*-octane saturated with 70% (v/v) aqueous methanol as the stationary phase. Moving phase changed from 70 to 80% aqueous methanol-*n*-octane at *B*. ×, using 55, 68 and 70% (v/v) aqueous acetone saturated with liquid paraffin (B.P.) as the moving phase and liquid paraffin saturated with 55% (v/v) aqueous acetone as the stationary phase. Moving phase changed from 55 to 68% aqueous acetone at *A* and to 70% aqueous acetone at *C*. Ambient temperature 35°.

unsuitable since good results have been obtained at room temperature when the column was used for a few hours only. The solvents were run down the columns at the rate of 20–50 ml./hr., but slower rates, down to 10% of this speed, give better separations. Such a slow rate is not, however, necessary in most cases.

Loading. The acids to be analysed were dissolved in the solvent to be used for development and the solution (approx. 2 mg. of each individual acid in approx. 2 ml. of solvent) was added to the top of the column. This solution was allowed to soak in and was washed down with two 2 ml. portions of the solvent before attaching the separating funnel as in Fig. 1. Fatty acids readily form supersaturated solutions. If these crystallize when loaded on to the column, the solid acid dissolves slowly and causes a steady trickle of acid down the column. High blanks and low recoveries result. When this crystallization is avoided, the recovery is within the range of 95–100%. If a mixture of fatty acids of very different chain length is encountered, it may not be possible to dissolve it in a solvent suitable for separating the shorter-chain acids. In this case a solvent suitable for the longer-chain acids must be employed and the first runnings from the column must be refractionated on the appropriate column.

Siphon. The siphon *A* (Fig. 1) may be adjusted to deliver various volumes by moving the plunger; 2 ml. was normally delivered.

Titrations. Titrations were carried out under N_2 using cresol red or, in later experiments, bromthymol blue as indicator. It was found convenient to dissolve the indicator in the developing solvent. Standardized 0·01 N-KOH in methanol was used for the titrations and stored in a dark internally waxed bottle. A 2 ml. microburette (Fig. 1), graduated by 0·01 ml., was used to deliver the alkali to the titration cup. The alkali was protected at all points from atmospheric CO_2. The accuracy and speed of the titrations was improved by using the apparatus shown in Fig. 1. Light from the lamp is led into the titration cell (*B*) by a tube (*C*) filled with $CHCl_3$, and the colour is observed by looking endwise down the cell.

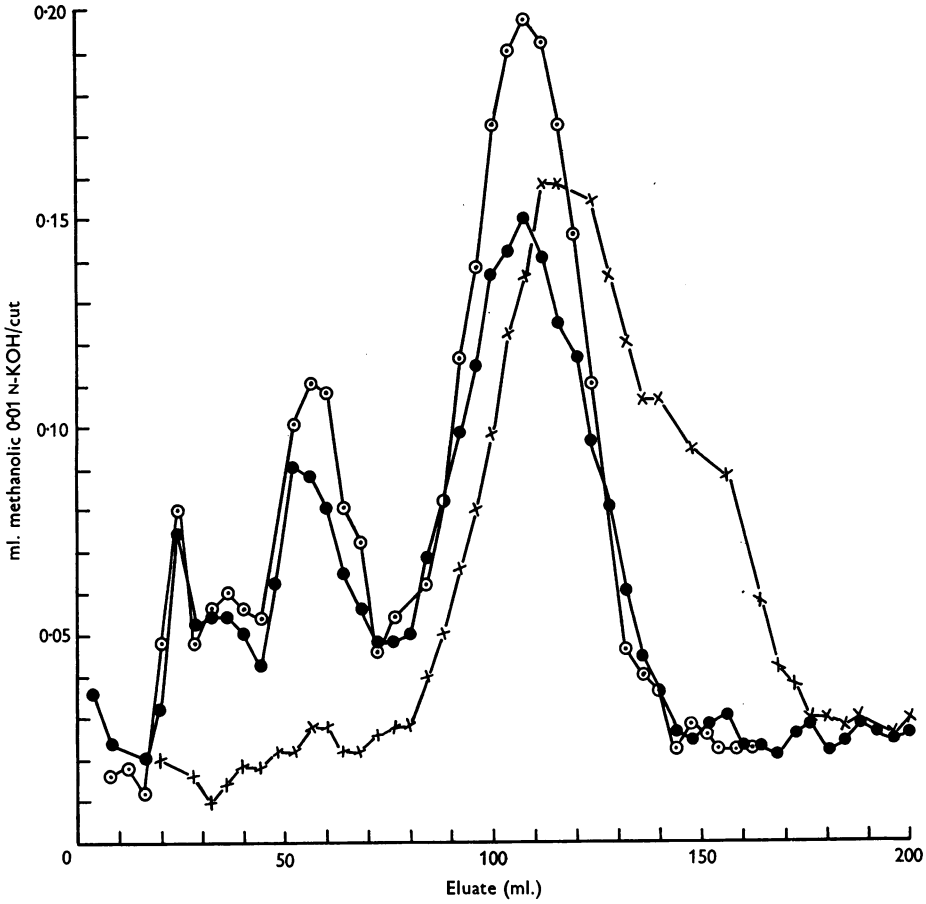

Fig. 5. Chromatographic behaviour of a commercial 'purified' oleic acid and its derivatives. ⊙, oleic acid (commercial 'purified'); ●, bromination product of above oleic acid; ×, elaidic acid prepared from commercial oleic acid. Ambient temperature 35°. Stationary phase, liquid paraffin (B.P.) saturated with 65% (v/v) aqueous acetone; moving phase, 65% (v/v) aqueous acetone saturated with liquid paraffin.

A capillary tube at the lowest part of the cell admits a nitrogen stream to circulate the liquid during the titration, and drains the cell when the tap (*D*) is opened. With this apparatus the colour change in a 6·5 cm. depth of liquid can be observed with a total volume of 2 ml. The approach of the end point is heralded by a flickering colour change. With bromthymol blue, the end point was taken to be the first persistent green colour. Attempts were made to follow the behaviour of the columns by a conductimetric method, but were abandoned because of erratic behaviour of the electrodes and the satisfactory nature of the titration method.

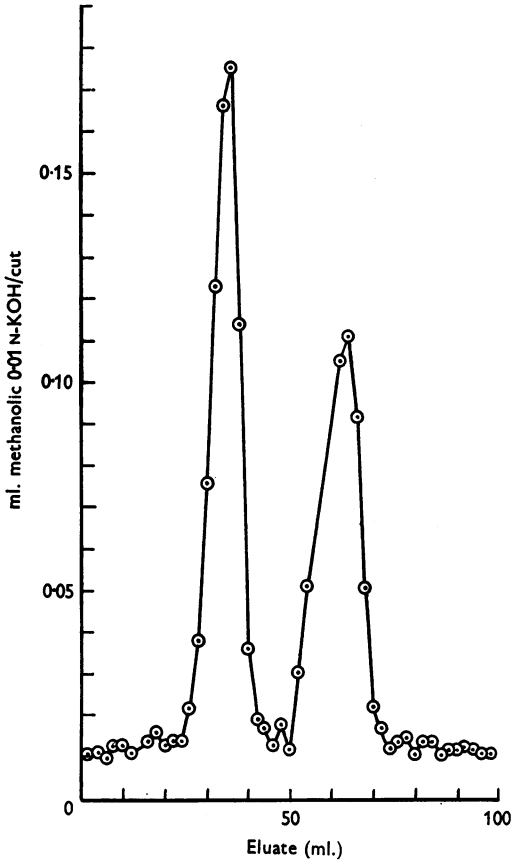

Fig. 6. The separation of a mixture of myristic and palmitic acids. Moving phase, 80% (v/v) aqueous methanol saturated with *n*-octane; stationary phase, *n*-octane saturated with 80% (v/v) aqueous methanol. Ambient temperature 35°.

Solvent systems. The most satisfactory systems tested were aqueous acetone-medicinal paraffin, and aqueous methanol-*n*-octane or *cyclo*hexane. The proportion of water in the solvent was chosen to give the most rapid development consistent with good separation. Increasing the proportion of water decreased the rate of development and improved the separation, but increased the width of the band. The most satisfactory procedure with a complicated mixture of acids was to change the solvent after the shorter-chain acids had emerged. The change was to a solvent containing a smaller proportion of water. The points at which such changes should be made can be decided readily on the basis of the titrations.

In addition to the solvent systems listed in Table 1, others were tested, tentatively in most cases. These were: aqueous

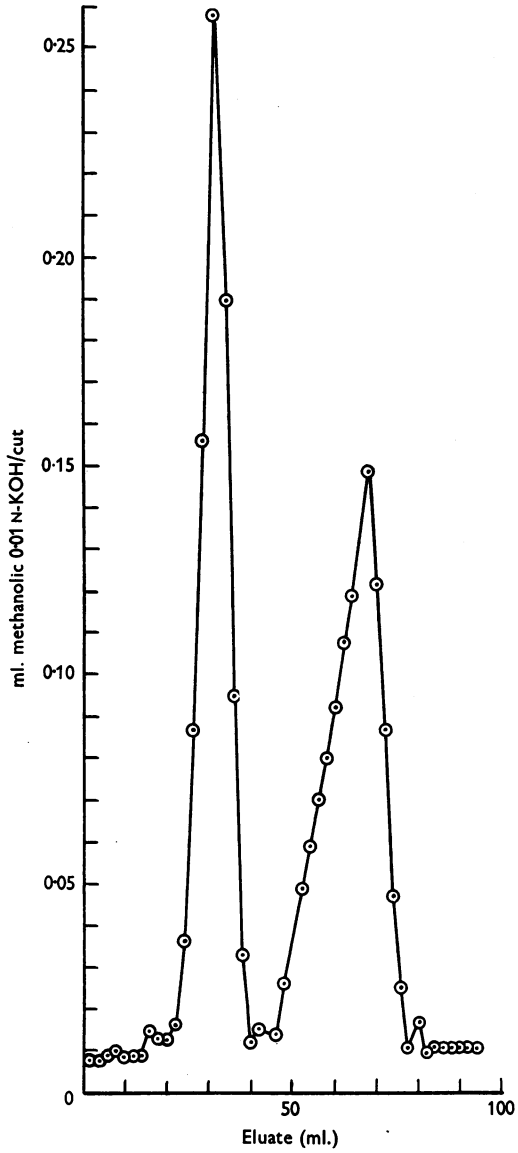

Fig. 7. The chromatographic separation of lauric and myristic acids. Moving phase, 70% (v/v) aqueous methanol saturated with *n*-octane; stationary phase, *n*-octane saturated with 70% (v/v) aqueous methanol. Ambient temperature 35°; recovery 98%.

methanol-toluene, aqueous methanol-$CHCl_3$, aqueous methanol-undecanol, aqueous ethylcellosolve-$CHCl_3$ and aqueous methanol-paraffin. The use of the last is exemplified in Fig. 2, but in view of the suitability of the aqueous methanol-octane and aqueous acetone-paraffin systems the use of the others was not pursued.

RESULTS AND DISCUSSION

The small amounts (about 2 mg.) of material, used on the columns precluded any identification of the acids in the eluate by normal physical means such as melting points. However, acids of authentic purity were employed in the work described here and single acids applied to the columns gave single peaks in the titration curve. There can thus be no doubt that the peaks observed represent the acids applied. The position of the peak is characteristic for an acid in a particular solvent system and the acids present in a mixture may be identified by comparing the position of their peaks with those given by known acids in the same solvent system.

Figs. 2–7 indicate that lauric, myristic, palmitic and stearic acids may be separated readily and the proportion of each in the mixture determined. Aqueous methanol-*cyclo*hexane or *n*-octane and aqueous acetone-medicinal paraffin have been used effectively as the solvent systems. No saturated acids with more than 18 carbon atoms and no acids with an odd number of carbon atoms have been available for testing, but brassidic and erucic acids behave well and no doubt the method could be applied to acids having more than 22 carbon atoms.

Table 1 shows some of the results obtained with various acids and solvent systems. The effect of lowering the percentage of water in the developing solvent on the rate of movement of any acid is evident from the data; the more water there is present the more slowly the acid travels down the column. The band formed by any given acid tends to spread more widely when the water content of the solvent is too high; the behaviour of palmitic acid on the 70 % (v/v) aqueous methanol-octane column is an example of this (Fig. 2). It will be noted that on the 65 % (v/v) aqueous acetone-paraffin column oleic, elaidic and palmitic acids all travel at similar rates. Earlier experiments with oleic, petroselenic, linoleic, linolenic and eleostearic acids made it clear that with constant chain length increasing the degree of unsaturation increases the rate of movement down the column. Acids with different degrees of unsaturation but the same chain length should be separable, as the behaviour of a commercial 'purified' oleic acid (Fig. 5) indicates. It is also interesting to note from this figure that oleic acid behaves in a similar way to the dibromostearic acid derived from it. Elaidic acid has always been found to travel down a column slightly more slowly than oleic acid, and the erucic-brassidic acid pair behaves similarly. This is in agreement with the general idea that the *trans* acids are more like the saturated acids than the unsaturated acids in their physical behaviour. The hydroxyacids are readily separated from both saturated and unsaturated acids by reversed-phase partition chromatography. Ricinoleic acid was readily purified in this way and the dihydroxystearic acids derived from oleic acid travel very quickly down the columns. Saturated and unsaturated acids could be readily separated by oxidizing the mixture before chromatography and the hydroxyacids themselves are being studied as a basis for analysing mixtures of unsaturated fatty acids.

SUMMARY

1. A new method of partition chromatography has been devised in which the less polar of the two phases is the stationary phase.

2. This reversed-phase method has been applied to the separation of the long-chain fatty acids.

3. Saturated acids having 12 to 18 carbon atoms have been separated, and mixtures of saturated, unsaturated and substituted acids have been analysed.

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