THE APPLICATION OF CHROMATOGRAPHY TO THE ANALYSIS AND REACTIONS OF SOME LONG CHAIN ACIDS.

being a Thesis

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DECLARATION.

I hereby declare that the following thesis is based on results of experiments carried out by me, that the Thesis is my own composition and that it has not previously been presented for a Higher Degree.

The research was carried out in the Chemical Research Laboratories of the United College in the University of St. Andrews, under the direction of Dr. F. D. Gunstone.

CERTIFICATE.

I hereby certify that Mr. Peter Job Sykes has spent nine terms at research work under my supervision, has fulfilled the conditions of Ordinance 16 (St. Andrews), and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Research Supervisor.

UNIVERSITY CAREER

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PUBLICATIONS.

(1). Gunstone and Sykes. Vegetable Oils. Part VIII. The Separation of Fatty Acids by Reversed-phase Chromatography : An Empirical Approach and a Mathematical Treatment.
J. Chem. Soc., 1960, 5050.

(2). Gunstone and Sykes. Vegetable Oils. Part IX. The Application of Reversed-phase Chromatography to the Analysis of Seed Oils.
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(3). Gunstone and Sykes. Partial Oxidation as a Means of Determining the Structure of Poly-unsaturated Acids.
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PART I

A REVIEW OF CHROMATOGRAPHIC METHODS FOR FATTY ACID ANALYSIS

(1) Introduction

Progress in fatty acid chemistry has always been intimately linked with the development and application of more effective and accurate methods of investigation. The natural fats consist of complex mixtures of triglycerides and the analysis of these generally commences with an investigation, both qualitative and quantitative, into the component acids present after hydrolysis, followed by a study of how these acids are distributed amongst the triglycerides.

For the analysis of the component acids a technique is required that will both resolve an intricate mixture of homologous acids and allow for the estimation, description and isolation of each component. The classical analytical techniques, depending largely upon the measurement of saponification equivalent and iodine value of fractions obtained from a partial separation of the mixture by fractional crystallisation or vacuum distillation etc., gave a reasonably accurate overall picture of the acids present, but were generally insensitive to minor components ($<1\%$). Many of the newer techniques are however able to achieve such analyses, their greater resolving power readily separating substances which were generally resistant to the older methods. This great increase in resolving power permits analysis of complex mixtures even in the presence of unsuspected

components and hence these techniques are particularly suitable for identifying trace acids.

The methods used for the analysis of fatty acids have undergone rapid changes during the last decade and it is now realised that there exists no single analytical method able to deal with the whole complex range of naturally occurring acids. It is with this in mind that this short review, summarising the more important chromatographic methods available at the present time has been written.

(2) Chromatography.

Chromatography may be defined as a method of analysis in which the flow of a fluid causes the components of a mixture to migrate differentially from a narrow zone in a sorptive medium into a sequence of separated zones. Chromatographic methods are usually classified as either, adsorption, ion exchange or partition depending upon the type of stationary phase employed. Since there are many different materials which can serve as stationary and mobile phases it is possible to devise many experimental systems for chromatography, the most important of which in relation to the analysis of fatty acids, are outlined below.

(3) Adsorption in Chromatography

In adsorption chromatography the materials to be separated are applied to the top of a column of finely divided adsorbant and a solvent is allowed to percolate through the column. The components of the mixture which are the least adsorbed migrate at the greatest speed

and elution is continued until each zone is eventually washed through the column into a suitable detector. For the analysis of fatty acids the material in each eluate fraction can be estimated most conveniently by alkali titration. The method just described is called elution chromatography. Two other methods of operating such a column are however used, namely, frontal analysis, in which a solution containing the mixture is passed continuously through the column and displacement analysis, in which the mixture being analysed is displaced by a more strongly adsorbed substance. This latter method has been used by Holman & Hagdahl[1] to separate mixtures of formic to eicosanoic acids using charcoal as adsorbant and aqueous ethanol as the displacer.

Adsorbtion chromatography on silicic acid has been used to prepare pure methyl linoleate and methyl linolenate from linseed oil by Riemenschneider[2] and the separation of fatty acids on charcoal columns has been studied by Cason and Gillies[3]. Howton[4] has separated the methyl esters of fatty acids containing different numbers of vicinal bromine atoms by elution from alumina and the alumina adsorbent has found use in the chromatographic analysis of the hydroxy acids of sugar cane wax[5].

The separation of acid mixtures by adsorbtive chromatography on silica gel, in the form of a thin layer on a glass plate, has been developed by Stahl et al[6,7] and has been applied to the analysis of fatty acids by Mangold and Malins[8]. By varying the polarity of the solvent Morris et al[9] have separated saturated and unsaturated esters

as well as mono-oxygenated and di-oxygenated esters of long chain acids. The method of detection is by spraying the chromatoplate with 50% sulphuric acid and heating until all the organic constituents are charred and appear as black spots. The rate of appearance and initial colour of the spots gives qualitative information regarding the degree and type of unsaturation of each component.

Adsorbtion chromatography does not usually give a quantitative analysis, probably due to incompletely reversible adsorbtion; and partition chromatography, where quantitative work has been particularly successful, has largely taken its place.

(4) Partition Chromatography.

Partition chromatography depends, like counter-current distribution, upon the differences in concentration of solutes in two contacting fluids resulting from the differing partition coefficients of each component. The method utilises a system whereby one liquid is held stationary on an inert support whilst the other liquid or gas flows past. The most common systems are: (i) Partition chromatography itself, with a stationary polar phase and a non-polar moving phase; (ii) Reversed phase chromatography in which the polarity of the two phases is reversed; and (iii) Gas/liquid chromatography which has a stationary liquid phase and a mobile gas phase. Combinations of the many possible stationary and mobile phases with a differing number of supports and methods of detecting the eluted zones give rise to a great number of chromatographic systems. Only the more important will be

reviewed here.

(a) Paper Chromatography.

Paper chromatography is a type of partition chromatography in which the stationary phase is supported on paper, and the mobile phase is placed at the top or botton of the paper to give either a descending or an ascending solvent front. During development of the chromatogram equilibrium is maintained between the liquid phase and its vapour. Materials can be characterised by their R_f values, which express the relative rate of movement of solute and solvent. Thus in many cases the method enables the components of a mixture to be both separated quantitatively and identified individually.

Paper chromatography has been applied to the analysis of short chain fatty acids by Reid and Lederer[10]. These workers used butanol saturated with 0.15 N ammonia as a solvent and bromocresol purple and formaldehyde as a detecting reagent. The fatty acid content was estimated from the area of the yellow spots produced on a purple ground. Methyl red and bromothymol blue in formalin have been used by Duncan and Porteous[11] to detect eluted acids and the method has been used to analyse the volatile acids from wool wax by Janecke and Benft[12]. Kaufmann et al in a long series of publications on paper chromatography have discussed the application of various systems to the analysis of fatty acids among which is a method for converting the separated fatty acid spots to their copper soaps and thence to copper sulphate followed by a polarographic analysis of the copper[13]. Kaufmann and Schnurbusch[14]

have used paper chromatography to analyse the mixed acids from linseed oil and detected the separated acids as their copper or mercury soaps and mercury addition compounds of the unsaturated acids. The areas are made visible as mercuric sulphide or cupric acetate.

The paper support has been modified in various ways to enhance its sorptive powers for the higher fatty acids, thereby converting the system to reversed phase chromatography. Latex on paper was used by Ashley[15] for the analysis of the C_{12} to C_{24} acids and the impregnation of the paper with paraffin oil extended the acid range to C_{34}[16]. Ballance and Crombie[17] discussed the separation of over forty acids by reversed phase chromatography on paper impregnated with liquid paraffin or castor oil, using aqueous acetic acid as the mobile phase. The method was made quantitative by photometric estimation of the copper, as a dithio-oxamide complex, from the copper soaps. Chromatograms of the mixed acids alone, after hydrogenation and after oxidation with alkaline permanganate allowed the quantitative analysis of 5 mg. samples of acid mixtures. Paper chromatography on siliconised paper with a solvent system of aqueous acetonitrile containing 2% acetic acid has been used by Morris et al[9] to separate the mono and di-hydroxy and epoxy acids. The chromatograms were stained with iodine vapour to indicate the unsaturated compounds or α-cyclodextrin and iodine to show the saturated compounds, according to the method of Schlenk et al[18].

The inverse radial chromatographic method of Sulser[19] using paper impregnated with liquid paraffin permits the analysis of naturally

occurring acid mixtures. The circular chromatogram is cut in half and one half is sprayed with cupric acetate and potassium ferro-cyanide to detect the saturated acids whilst the other half is treated with potassium permanganate, benzidine reagent for the unsaturated acids. A matching of the two halves permits calculation of the composition of the mixture.

Both Howe[20] and Crombie[17] have discussed the relationship between R_f value and chemical structure and point out that there are pairs of acids whose R_f values are practically identical and are therefore not seperable by paper chromatography (e.g. Palmitic and Oleic). Kaufmann and Arens[21] have however separated these "critical" pairs by chromatography of their more polar thiocyanogen derivatives.

The separation of the C_{10} to C_{18} fatty acids has been accomplished by Franks[22], using paper impregnated with liquid paraffin and a mobile phase, the composition of which was altered at a constant rate. The technique is known as gradient elution.

The versatile technique of paper chromatography, which possesses an advantage over gas/liquid chromatography in that only simple apparatus is required, therefore makes an important contribution to the micro-analysis of fatty acids.

(b) Column Partition Chromatography.

In this method of chromatography a mobile phase is allowed to percolate through a column in which a second phase is held stationary on an inert support. For satisfactory chromatography the stationary phase should be a solvent in which the compounds to be separated have a

greater solubility; they are then eluted by a greater volume of mobile phase in which they are less soluble. For analysis of fatty acids the most usual method of detecting the eluted zones is by titrating small fractions of the effluent from the column. These titration values are then plotted against the fraction number and, as in countercurrent distribution, a series of Gaussian shaped curves result. The area under each curve is then a measure of the quantity of acid eluted. Quantitative recoveries of material are generally possible by this method.

The resolving power of partition chromatography is determined by the number of theoretical plates available for the particular system being used and as in countercurrent distribution a mathematical description of the technique has been formulated [23,24]. The mathematical treatments permit calculation of the number of theoretical plates and the partition coefficient of each component from the shape of the elution curves, and can therefore be used to predict possible separations of fatty acids based upon partition coefficient data.

Column partition chromatography has been applied to the separation of short-chain water-soluble acids by Marvel and Rands[25], using a silicic acid column and various mixtures of butanol /chloroform as the mobile phase. A silicic acid column, holding methanol as a stationary phase, and 2:2:4-trimethylpentane as mobile solvent was used by Ramsey and Patterson[26] to separate the C_5 to C_{10} acids and this method was developed as a semimicro technique by Nijkamp[27]. The method has

been extended to cover the range C_2 to C_{12} monobasic acids, and C_4 to C_{10} dibasic acids, by Vandenheuvel[28]; and by using aqueous ammonia in silicic acid and a mixture of petroleum ether and butyl ether as developing solvent, Zbinovsky[29] extended the range to C_2 to C_{14} for monobasic acids and from C_{22} down for dibasic acids. Glycine supported on silicic acid has been proposed as a stationary phase by Corcoran[30]. In this method the pH of the glycine determines which acids are removed by elution with butanol/chloroform solvent. A pH range of 2 to 10 is used to separate the monobasic acids C_1 to C_{10}; and using the citric acid in place of glycine the technique has been extended to the dibasic acids.

Column chromatography has been extended to the analysis of the longer-chain fatty acids by holding the non-polar phase stationary in the modification known as reversed phase chromatography. This modification was first proposed by Howard and Martin[31] who separated mixtures of C_{12} to C_{18} monobasic acids using liquid paraffin supported on siliconised kieselguhr and various concentrations of aqueous acetone as mobile phase. The scope of this method was extended by Silk and Hahn[32] and Kapitel[33] to cover the monobasic acids ranging from C_{16} to C_{24}. Other stationary phases used for reversed phase chromatography include benzene supported on powdered rubber[34], castor oil[35] and acetylated castor oil[36] supported on siliconised kieselguhr; and powdered polythene[37]. A further modification to this type of chromatography is the use of a mobile phase which alters in concentration

at a uniform rate during development of the chromatogram. The technique is known as gradient elution[38], the theory of which has been given by Warner and Lands[39].

The partition coefficients of a monoethenoid acid and the saturated acid with two more methylene groups are so similar that partition chromatography cannot separate such "critical" pairs of acids[17]. Analysis of these critical pairs is therefore generally accomplished by two chromatograms; one of the mixed acids alone and one after chemical modification. The point is illustrated by Lough and Garton[40] who studied the plasma lipids of the lactating cow by analysis of the mixed acids before and after hydrogenation and by Crombie et al[41] who studied mixtures of fatty acids by chromatograms before and after alkaline permanganate oxidation. The present work[42] describes a method for the analysis of seed oils with chromatograms of the mixed acids alone, after hydrogenation and after ozonolysis.

Reversed phase chromatography has been used to separate di- and tetrahydroxy acids by Savary and Desnuelle[35] using castor oil as stationary phase; Desnuelle and Durnet[43,44] extended the method using a powdered rubber support, to a whole range of hydroxy acids and Matic[45] used the technique to analyse the hydroxy acids of plant cuticles.

Partition chromatography is therefore seen to have extensive applications to the analysis of fatty acids and although it is not as simple a technique to operate as gas/liquid chromatography it does

have some advantages. The analysis is effected under very mild conditions on quantities of material sufficient for further investigation, it requires no elaborate equipment and it is more easily adaptable to the conjugated and less volatile oxygenated acids[45A].

(c) Gas/Liquid Chromatography.

Methods of partition chromatography have probably reached their ultimate form in the technique known as gas/liquid chromatography. In this system the fatty acid esters are partitioned between a non-volatile liquid held stationary on an inert support and a gas, which acts as the mobile phase. The method constitutes a rapid and accurate form of micro-analysis with the advantage over liquid/liquid partition that many highly sensitive methods are available for detecting solutes eluted in the gas phase. Some of the more important detection systems are thermal conductivity[46], thermistor[47], gas density balance[48], flame temperature[49], flame ionisation[50] and β-ray ionisation detectors[51]. A micro version of the latter detector has been constructed by Lovelock[52], which is reported to be able to sense 10^{-15} mole. A comparison of the performance of various detectors has been made by McWilliam[53] and Jamieson[54].

The response of a detector to eluted material is generally plotted against time by means of a potentiometric recorder and each eluted component gives rise to a Gaussian curve. Qualitative identification of the components can be made from their retention times

relative to a standard compound. The retention time is defined as the time between the emergence of the air peak or carrier gas front and the peak of the relevant component, and a method of calculating these has been given by Peterson and Hirsch[55]. Retention volume data for a number of fatty acids on both polar and non-polar stationary phases has been given by James[56], Hawke et al[57] and Farquhar et al[58]. A plot of $\log_{10}$ (Relative retention volume) vs. carbon number of the acid is linear for a homologous series and this relationship can be used to identify the components of a mixture by their carbon numbers[59]. Quantitative estimation of the esters present in a mixture is made by measuring the area under each peak either with a planimeter or by calculation[60]. It is thus desirable to use a detector whose signal output bears a definate relationship to some molecular parameter of the eluted compounds.

The theoretical treatment of chromatography by Martin and Synge[23] and Glueckauf[24] has been extended to gas/liquid chromatography by van Deemter[61]; and various workers [62,63,64] have related the number of theoretical plates to the performance of a column. A correlation between the separating power and efficiency of gas/liquid columns has been made by Purnell[65].

James and Martin[66], have described the application of gas/liquid chromatography to the separation of fatty acids from formic to stearic, iso and ante-iso acids from their straight-chain isomers, and unsaturated acids from saturated acids of the same chain length.

The separation of short chain esters was carried out using stationary phases of liquid paraffin, benzyl diphenyl or dioctylphthalate at 100°C whilst for the higher esters Apiezon M grease columns were used at 197°C. All analyses were made on 4 feet columns of 4 m.m. internal diameter. Siliconised vacuum grease was introduced as a stationary phase by Cropper and Heywood[67] and has been used by McInnes[68] to identify the short chain acids present in mutton fat. James and Webb[69] have separated mono and di-carboxylic acids and have used the technique to identify the degradation products of permanganate oxidation of some naturally occurring mono and di-unsaturated acids.

Lipsky and Landowne[70] together with Orr and Callen[71] introduced the more polar stationary phases of polydiethylene glycol adipate and polypropylene glycol adipate. These polyester phases exert specific intermolecular attractions for the unsaturated acids, causing them to be specifically retarded duringchromatography. The magnitude of the effect increases with the number of double bonds present in the molecule[72]. Lipsky et al[73] have studied the effect of varying the stationary phase on the resolution of the saturated and unsaturated acids. The sebacate and azelate polyesters of diethyleneglycol were studied but best results were to be had by using the polyester of diethylene glycol and succinic acid. Such a column working at 200°C separated mixtures of saturated and unsaturated esters from C_{12} to C_{26} in 38 minutes.

James[56] has described a method for determining the degree of

unsaturation and chain length of a fatty acid by a comparison of relative retention volumes on a polar (polyester) and a non-polar (Apiezon grease) phase. The use of two phases in the quantitative analysis of acids from vegetable oils has also been reported by Craig and Murty[74,75]. Analysis of seed oils has been reported by Hopkins and Chisholm[76,77], of epoxy acids by Morris[9] and of the fatty acids from Tubocele bacillus by Gason and Tavs[78]. A comparison of the gas/liquid chromatographic method with analysis by ultra-violet spectrophotometry has been made by Herb et al[79] and by Schlenk et al[80].

A variation in technique known as programmed heating was introduced by Desty[81]. In this modification the temperature of the column is raised in a linear fashion during the course of the chromatogram. This leads to a more equal spacing of the eluted components over a wide range of carbon numbers rather than the logarithmic scale of isothermal operation. Downing et al[82] have used programmed heated columns to separate the complex mixtures of acids, as their hydrocarbon derivatives, obtained from wool wax and Giddings[83] has given a theoretical analysis of retention times and retention temperatures in programmed gas chromatography.

A new concept of gas chromatography was proposed by Golay[84] who prepared columns by coating the inner surface of narrow bore capillary tubing with a thin layer of stationary phase. Nylon capilliary columns have been used by Scott[85]. Such columns, giving a theoretical plate efficiency of 1000 plates per foot have been

constructed in 200 ft. lengths. A microversion of the β -ray argon detector is used with these columns and the sampleload is approximately one microgramme. Lipsky et al[86,87] have used these high efficiency columns for the analysis of complex mixtures of fatty acids and to separate the cis and trans isomers of oleic acid.

The rapid development in the technology of gas/liquid chromatography has thus provided the chemist with an exceptionally effective method of separating micro-quantities of organic compounds. Applications of this technique to lipid chemistry, and especially to the more complex biochemical problems, will undoubtedly see a great expansion in the future.

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* * *

PART II

REVERSED PHASE CHROMATOGRAPHY.

(1) Introduction.

Partition chromatography separates mixtures by distributing the components between two imiscible solvents according to their several partition coefficients. It therefore differs only from counter-current distribution in that there is a continuous flow of mobile over stationary phase as opposed to the discrete number of transfers made in the latter method. A brief review of the scope of reversed phase chromatography has already been given on p9.

For the satisfactory analysis of fatty acids by partition chromatography stationary and mobile phases are chosen such that the acids to be separated are more soluble in the former; they are then slowly eluted by a greater volume of mobile phase. In the case of the longer chain fatty acids or non-polar stationary phase fulfils this condition and a solvent of decreasing polarity is used to remove increasingly non-polar material from the column. Howard and Martin[1] were the first to demonstrate the usefulness of this variation of partition chromatography by separating the C_{12} to C_{18} acids. They termed the method reversed phase chromatography. The stationary phase used was liquid paraffin supported on Kieselguhr made non-wetting by treatment with dichloro-dimethyl-silane and the mobile

phase consisted of a range of aqueous acetones of increasing acetone content.

The method has been extended by several workers[2-9] to separate acids ranging from C_8 to C_{24} using aqueous acetones from 40 - 90%. A double bond has the same effect on the column behaviour of an acid as a reduction in chain length of two carbon atoms, consequently oleic and palmitic acids are eluted together as are linoleic, hexadecenoic and myristic acids.

It is shown in the present work that the best separations are obtained when each group of acids is eluted with an aqueous acetone of optimum concentration. An acetone concentration higher or lower than the optimum leads to an incomplete separation of the next higher or lower acid group respectively. A method has been developed for determining this optimum solvent from a knowledge of the partition coefficient of the acid between the two imiscible phases. The same method has also been used to investigate the possible separations of oxygenated acids by using stationary phases other than liquid paraffin.

Finally a mathematical description of the experimental system is given, which shows results in accord with those obtained experimentally.

(2) Experimental Methods.

(i) Materials

(a) Non-wetting Kieselguhr

Hyflo Supercel is submitted to a process of

flotation in water to remove the finer particles. About 750 g. of kieselguhr are thoroughly mixed with 5 litres of water and allowed to stand for 1 hr., the suspended fines are then decanted. The process is repeated twice and the residual kieselguhr is collected on a Büchner funnel and dried at 110°C. When the material is cold it is exposed to the vapour of dichloro-dimethyl-silane for 24 hrs. by placing it in trays at a depth of 2 cm. inside a partially evacuated dessicator. During this process the kieselguhr is stirred periodically. The siliconised material is washed free of acid (to litmus) with methanol, collected on a Buchner funnel and dried at 110°C. The resultant product should be completely non-wetting to water.

(b) Stationary Phases.

(i) Liquid Paraffin

Colourless medicinal liquid paraffin is dissolved in redistilled petroleum ether (b.p.40-60°C) and is percolated through an alumina column. (Spence Grade / "H"). Vacuum evaporation of the solvent gives the required neutral paraffin.

(ii) Castor oil.

Medicinal castor oil is purified according to the method of Achaya and Saletore[10]. Castor oil (500 g.) is shaken with petroleum ether. (b.p.40-60°, 5 x 2.5 litres). The petrol extracts are rejected leaving the purified oil (350 g.) after removal of the solvent. The material is neutralised by passing through an alumina column in the same manner as for the liquid paraffin.

(iii) Acetylated Castor Oil.

Purified castor oil (100 g.) is refluxed for three hours with acetic anhydride (500 cc.). Water (300 cc.) is then added cautiously and the mixture refluxed for a further hour. The product is extracted with ether and washed acid free with sodium bicarbonate solution before drying over anhydrous sodium sulphate. Last traces of acid are removed by passing the ether solution through an alumina column and evaporation of the ether gives neutral acetylated castor oil (108 g.)

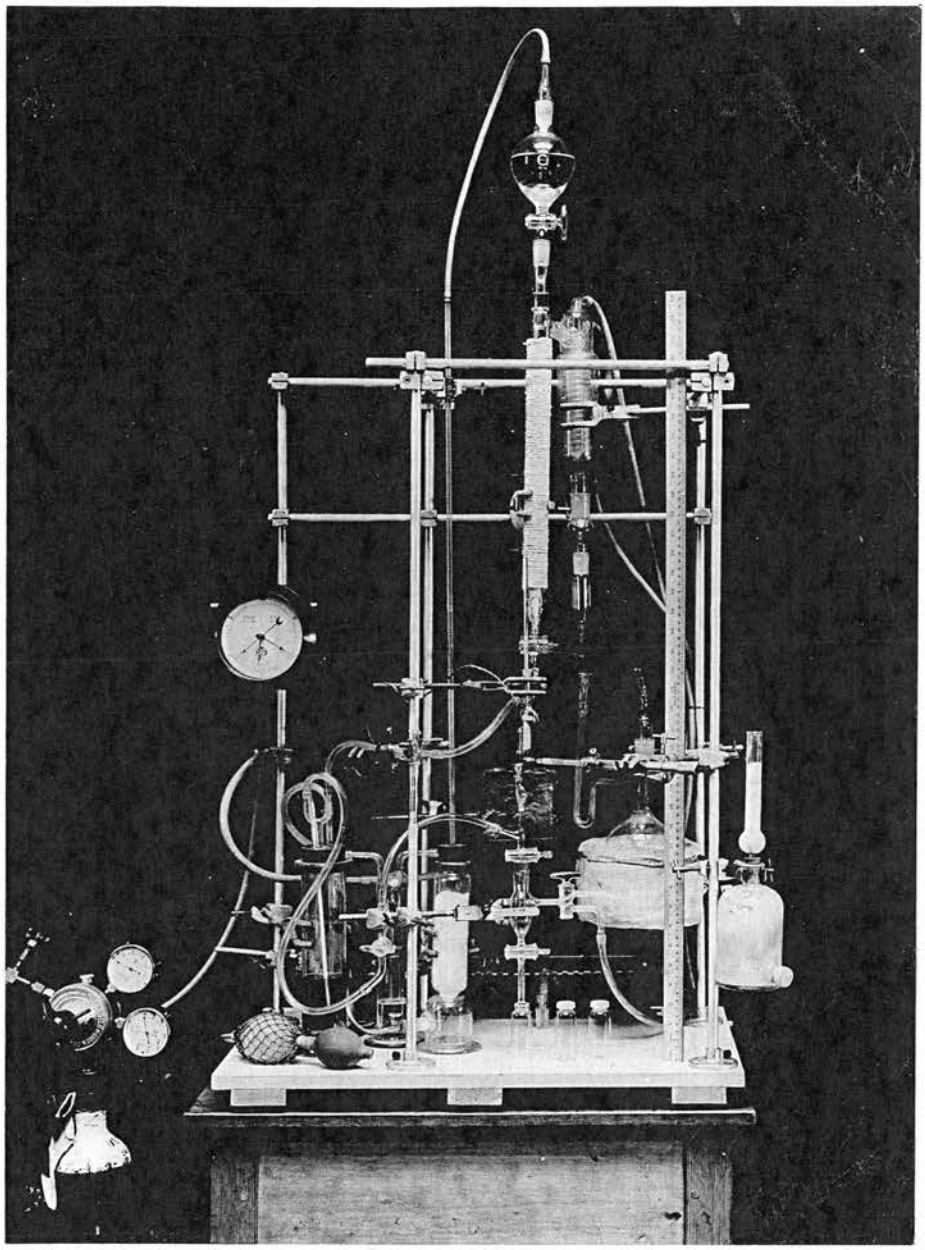
(c) Mobile Phase.

The mobile phase used for all the chromatograms consists of a range of aqueous acetones. These solutions are prepared by diluting A ml. of acetone to 100 ml. with boiled-out distilled water, and are designated A% acetone. All the solvents, which should be freshly made for each chromatogram, are equilibrated with a little stationary phase and bromothymol blue indicator added to give a concentration of 0.001% (w/v). Mobile phases to be used with liquid paraffin chromatograms are stored at 35°C. in a thermostated cupboard, and those for use with other stationary phases are stored at 20°C.

(d) Indicator Solutions.

The indicator solution used throughout these experiments is bromothymol blue which changes from yellow to blue over a pH range 6.0-7.6. It gives a satisfactory colour change when used in a concentration of 0.001% (w/v). Two solutions are made:-

(i) Strong solution. The solid indicator (0.5 g.) is titurated



with 0.2 N alkali (4 cc.) and made up to 100 cc. with 70% acetone. 1.0 cc. of this solution, which should be dark green in colour, added to 500 cc. of aqueous acetone produces the required indicator strength.

(ii) Weak Solution. 4.0 cc. of the strong solution is diluted to 100 cc. with distilled water. The resulting solution is such that 0.1 cc. added to 2 cc. of liquid in the titration cell (see p 26) produces the correct indicator strength.

(e) Column Packing Material.

The mull used for packing the columns is made up in batches; the ratio of siliconised kieselguhr to stationary phase being:-

$\frac{\text{Wt. kieselguhr}}{\text{Wt. paraffin}} = 1.4$ $\frac{\text{Wt. kieselguhr}}{\text{Wt. castor oil or acetylated castor oil}} = 1.5$

Accordingly siliconised kieselguhr (100 g.) and liquid paraffin (81 cc., density = 0.88) are together dissolved in ether (500 cc.) The solvent is then slowly evaporated to give a coarse homogeneous powder. A rotary film evaporator is ideal for this purpose. The mull is dried at 60°C. for 2 hrs. under vacuum. The other mulls are prepared in like manner using kieselguhr (100 g.) and castor oil (80 cc., density = 0.96) or acetylated castor oil (79 cc., density = 0.97).

(ii) Apparatus.

A photograph of the apparatus is shown opposite. The meter stick is inserted for scale only and the illuminated screen behind the titration cell has been blacked out for photographic

purposes.

(a) The Column.

The column used for all the chromatograms consists of a glass tube 1.3 cm. internal diameter and 45 cm. long, with a B 19 socket at the top and a tap with a burette end at the lower end. The lower 40 cm. of the column are surrounded by a jacket which enables this portion of the column to be heated during chromatography.

(b) Column Heating Unit.

Columns used with a liquid paraffin packing are operated at 55°C, according to the method of Howard and Martin[1], thereby increasing the solubility of the longer chain acids in the stationary phase.

During storage the column is kept along with the developing solvents, in a cupboard which is thermostated at 55°C. and heated with a 250 w. heating lamp.

During operation the column is heated by surrounding it with the vapour of 2-chloropropane. (b.p. 34.8°C.) A 500 ml. flask, heated by an isomantle and controlled from a simmerstat unit, contains the boiling chloropropane; the vapour is led by means of glass and P.V.C. tubing (with as little of the latter as possible) to the lower end of the column jacket. Eventually vapour issues from the top exit of the jacket where it is passed to a triple surface water condenser and the liquid chloropropane is returned to the boiler by means of a side arm with a U-bend.

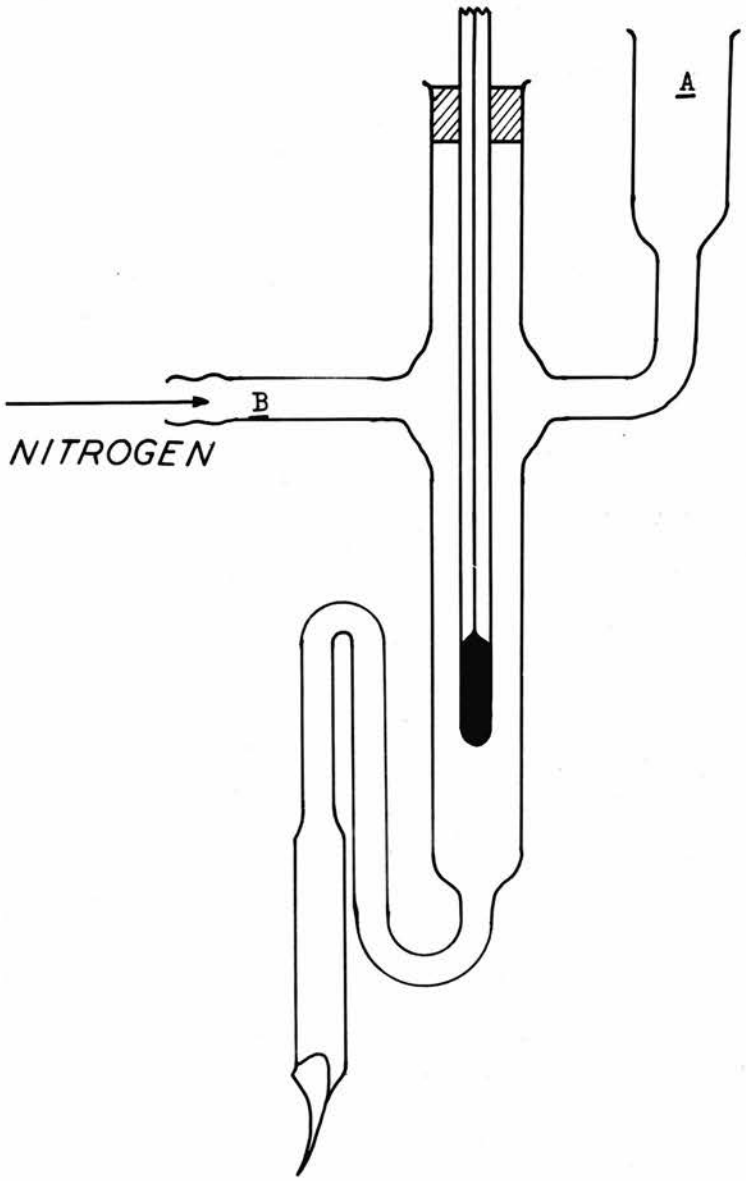


FIG 1. THE SIPHON.

The castor oil and acetylated castor oil packed columns are operated at 20°C by passing tap water through the column jacket.

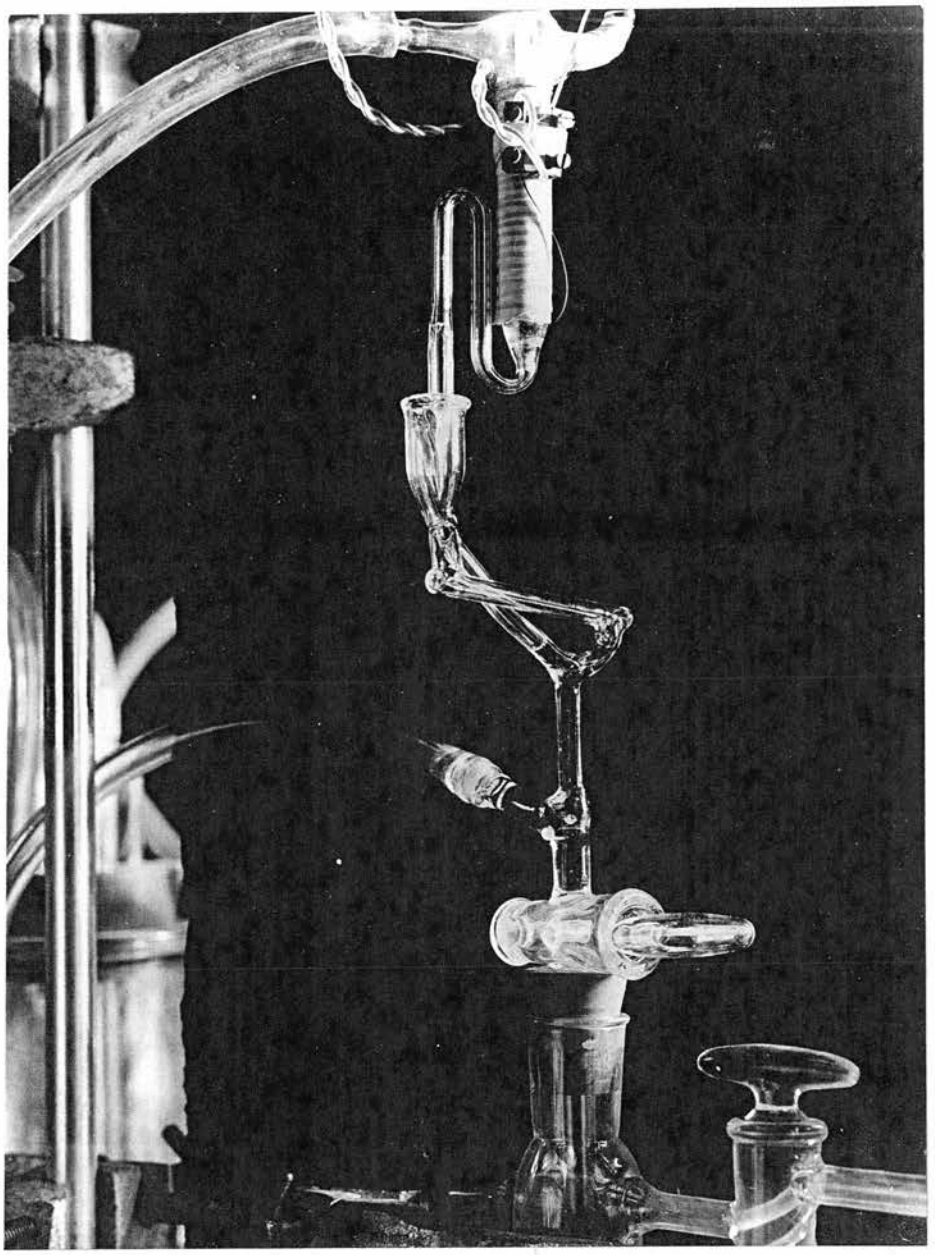
(c) Solvent Reservoir.

A B19, 250 ml. separating funnel placed at the head of the column is used as a solvent reservoir. It is sometimes advantageous to be able to apply pressure to the head of the solvent reservoir, particularly if the column elution rate is very slow. Carbon dioxide free air, obtained by passing air from rubber hand-bellows through a Carbosorb tower and thence to the head of the reservoir, is satisfactory.

(d) The Siphon.

A siphon to deliver 2 ml. portions of eluate is constructed as shown in fig. 1. The eluate enters the siphon at A and nitrogen entering at B protects it from any atmospheric carbon dioxide. The volume delivered can be adjusted by raising or lowering the thermometer; for the present work it was set so that the siphon delivered 2.0 ml. portions of 60% aqueous acetone. Calibration revealed that the volume delivered by the siphon varied with the content of acetone in the mobile phase. Over the range 40% to 80% aqueous acetone, the volume delivered was found to be 2.0 ml. ($\pm$ 0.05 ml).

To prevent precipitation of the higher acids ($>C_{18}$) when the eluate at 35°C enters the cold siphon, it is heated to 35°C by winding with nichrome tape which is connected with a series resistance to a 6 volt transformer, to give a 2 watt heater.



(e) The Titration Cell.

The titration of eluate is carried out in a cell with nitrogen circulation as described by Howard and Martin[1]. The original apparatus of Howard and Martin was modified for the present work and is shown in fig. 2 and the photograph opposite.

The cell is constructed from 0.5 cm. outside diameter tubing and the light path AB, down which the colour change during titration is observed, then measures 6.5 cm. for a total volume of 2 ml. A capilliary tube admits nitrogen to circulate the liquid during titration and is used to drain the cell when the tap is opened. It is essential for the correct functioning of the apparatus that the line of this capilliary tube is such that nitrogen bubbles from D to C rather than from D to A.

The cell is lit with a background of white light by placing a 60 w. lamp behind a 3 x 3 in. white perspex screen as shown at E. The nitrogen is saturated with acetone vapour at 35°C. to counteract evaporation from the cell during titration, by passing through a 5 cm. mercury blow-off valve, a 40% aqueous potassium hydroxide solution and finally through a sintered glass wash bottle containing neutral acetone maintained at 35°C by a 3 watt immersion heater run from a 6 volt transformer.

(f) Vacuum Drain and Fraction Reciever.

At the end of a titration the liquid is drained from the cell through a capilliary tube by means of the apparatus shown in

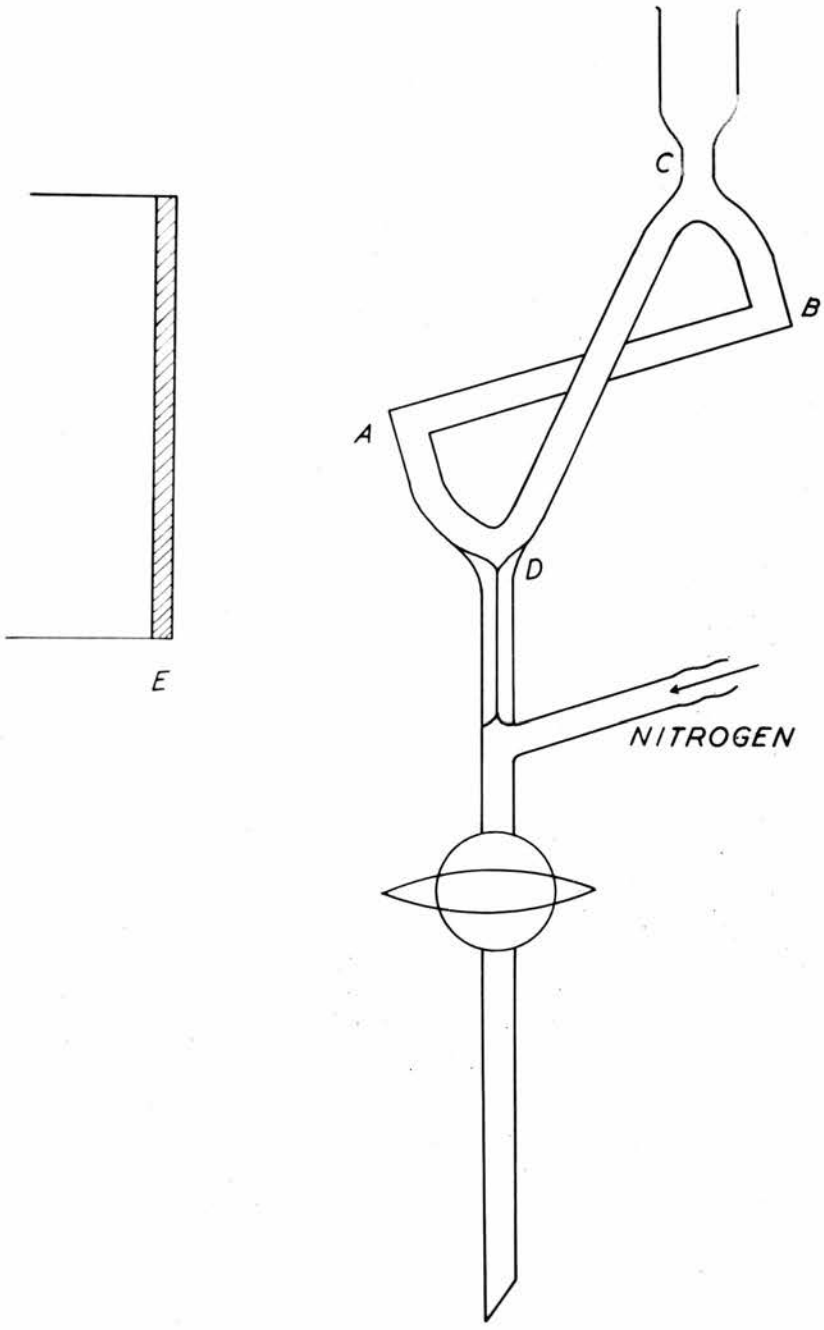


FIG 2. THE TITRATION CELL.

fig. 3. Vacuum is connected at A and can be applied to the cell by suitably setting the two-way tap. The titrated eluate is then run into 25 ml. or 10 ml. polythene capped storage bottles by turning the two-way tap to atmosphere and opening tap B.

(g) The Microburette.

The eluate from the column is titrated with 10^{-2}N. methanolic potassium hydroxide using an "Agla" micrometer syringe, which enables titrations to be done with speed and accuracy to $\pm$0.0005 ml. The syringe is mounted horisontally and the alkali delivered to the cell by means of a No. 14 Luer fitting hypodermic needle bent through a right angle, the tip of the needle dipping below the surface of the liquid in the cell. The alkali is protected at all points from atmospheric carbon dioxide and is stored in a bottle fitted with a small carbosorb guard tube. Alkali is taken from this bottle by puncturing a self-sealing serum cap with the needle of the syringe. The microburette was calibrated before use for uniformity of barrel and overall delivery. The calibration factor was found to be 1.004 for delivery from any part of the barrel.

(h) An Accurate 2 ml. Pipette.

An accurate 2 ml pipette was constructed by joining 20 cm. of pyrex capilliary tubing to 8 cm. of 0.3 cm. internal bore tubing as shown in fig. 4. The pipette was calibrated by the following method:- A length of white paper (10 cm.) is affixed to the capilliary tubing to cover half its circumference and two small

bands (about 1 mm.broad) are then cut away perpendicular to the length of the capilliary tube and 5 cm. apart. The pipette is filled with water and the meniscus in the capilliary tube is clearly visible against the white ground. The meniscus is then allowed to fall until it just "fits into" the upper cut in the paper, and the pipette is emptied into a weighing bottle. A ten-second drainage time is allowed and the tip is blown out. The procedure is repeated five times and a similar set of five weighings are taken from the lower cut in the paper.

The volume of the tubing attached to the capilliary is now adjusted until the volume delivered from the top mark is greater than 2 ml. and the volume delivered from the lower marking less than 2 ml. The difference between these two volumes is the vlume of the 5 cm. of capilliary tube between the two marks and it is possible to work out by simple proportion, assuming uniform bore tubing, where to mark the pipette to deliver exactly 2.000 ml. A cut in the paper is made at this point and the pipette is calibrated by weighing at least ten volumes of water delivered from this mark. The mean volume of water delivered and the standard deviation of the pipette can then be calculated.

All the paper except about 2 cm. on either side of the final calibration mark is then removed and the remaining paper is coated with a solution of perspex in chloroform.

The pipette constructed for the present work delivered 2.003 ml.

with a standard deviation of ± 0.001 ml. The design of the pipette makes it ideal for rapid routine measurements without loosing the accuracy quoted above.

(3) Experimental Technique.

(a) Column Packing.

Special precautions are required to obtain air-free columns, otherwise the packing breakes up during chromatography leading eventually to channeling.

Paraffin/Kieselguhr mull (25 g.) is placed in a glass mortar and ground to a fine slurry with 70% aqueous acetone (50 ml.) previously equilibrated with a little of the stationary phase. The slurry is transferred to a conical flask and boiled gently on a water bath for a short time to expel all the air. Meanwhile the column is filled with 70% acetone and a plug of cotton wool is placed at the bottom. The boiled-out slurry is then fed on to the top of the column which is set to flow. By suitably adjusting the rate at which the slurry is poured into the column and the exit flow rate, a suitable packing can be obtained. After some settling has taken place the packing is compressed with nitrogen at an excess pressure of 5 cm. mercury, the column being tapped to ensure homogeniety of packing. A neatly fitting filter paper disc (1.3 cm. diameter) is placed on top of the packing by means of a perforated stainless steel plunger. The column is wrapped in asbestos rope and stored at 35°C. The length of packing should be 35 cm.

Castor oil and acetylated castor oil columns are prepared in the same manner, except that 60% acetone is used and the columns are stored at 20°C. Each type of column can generally be used for five chromatograms, after which time it is rejected.

(b) Column Loading.

The mixture of acids to be separated is weighed accurately and dissolved in 10 ml. of ether in a short tube (5 cm. x 3.4 cm) with a B 34 neck. It is inadvisable to use sodium dried ether due to its alkali content. Paraffin impregnated kieselguhr (the same material that is used to pack the column) is added at the rate of 0.05 g. per mg. of acid mixture and the ether is evaporated with continual swirling. The resulting mull is dried for 1 hr. at 60°C under vacuum.

During preparation of the mull about 10 ml. of 35% acetone are run out on to the column. The mull is slurried with 10 ml. of 55% acetone, containing no indicator solution, whilst warming to 55°C on a water bath and the slurry is transferred quantitatively to the column by pouring the bulk of it from the tube on to the top of the column packing. The mull still adhering to the tube is washed into the column by means of a fine spray of 55% acetone from an atomiser. The whole of the mull is compressed into a band by gently lowering a close fitting filter paper disc by means of the perforated plunger.

A loaded column may be stored at 55°C until it is required for development.

(c) Column Development.

The apparatus is assembled as shown in the photograph opposite p 23 and the column is heated to 35°C by passing chloropropane vapour through the heating jacket. After temperature equilibrium has been attained the excess 35% loading solvent is removed by means of a dropping tube and the solvent reservoir containing mobile phase is fitted to the head of the column. Mobile phase is then run in to give a 5 cm. depth above the top of the mull inside the heating jacket, thereby ensuring that the mobile phase is in temperature equilibrium when it comes into contact with the column of stationary phase.

The nitrogen is turned on to supply the titration cell and the siphon; and the heaters for the siphon and the nitrogen saturation unit are started. Development is commenced by opening the taps of the reservoir and the column, simultaneously starting the clock. The eluate flow rate is adjusted by varying the air pressure above the solvent in the reservoir by means of the hand-bellows. 60 ml. per hour is found to be a suitable flow rate for most chromatograms. Should it be necessary to halt the development of a chromatogram before the completion of the analysis, the tap at the base of the column is closed and the clock is stopped. It is best to make such breaks in development in a trough between the elution of any two components.

The column eluate passes into the siphon which delivers it in 2 ml. portions into the titration cell.

(d) Titration of Eluates.

Titration of eluates is carried out in the nitrogen stirred cell using accurately standardised 0.01 N methanolic potassium hydroxide delivered from an "Agla" micrometer syringe with bromothymol blue as the indicator. Approximately the first fifteen 2 ml. fractions of eluate from the column are the 55% acetone used during the loading procedure and consequently contain no indicator. Thus when the siphon delivers a 2 ml. fraction to the cell, 0.1 ml. of the weak indicator solution (p 23) is added prior to titration. The approach of the end-point is heralded by a flickering colour change and is finally taken as a persistant green colour. (The actual shade of green at the end-point is achieved only by analysis of known compounds. When judged correctly it is possible to determine the equivalent weight of palmitic acid to $\pm$ 0.5% or better).

At the end of the titration the nitrogen flow is stopped, the cell drained by vacuum and the nitrogen restarted in preparation for the next portion of eluate. The micrometer reading is noted after each titration, and a plot of alkali per fraction vs. fraction number gives elution curves as shown in fig. 5. After about fifteen fractions the first developing solvent will appear in the titration cell and from this point onwards it is unnecessary to add indicator prior to the titration. The number of fractions between the change to developing solvent containing indicator and the appearance of this solvent at the titration cell is a measure of the column hold-up and should be noted

for each chromatogram.

The titrated eluate is stored at $0^{\circ}C$ until it is required. Material recovered from chromatograms is used to identify further the components of the acid mixture.

(e) Alkali Standardisation.

The alkali used to titrate the eluate is standardised prior to each chromatogram against an accurately standardised 2×10^{-3} N acid. Acids of this concentration were found to change normality on standing and the following procedure was therefore adopted:- 0.25 N hydrochloric acid was accurately standardised against AnalaR sodium carbonate. 2.005 ml. of this acid are diluted to 250 ml. in a standard flask with boiled-out distilled water each time a standardisation is made. The resultant acid is 2×10^{-3} N. 2.005 ml. of this diluted acid are placed in the cell and titrated with the 0.01 N alkali using 0.1 ml. bromothymol blue indicator solution. The standardisation procedure was checked at intervals against freshly prepared 2×10^{-3} N AnalaR succinic acid.

(f) Mobile Phase Changes.

A chromatogram is developed by eluting each group of acids with an appropriate solvent (see p 38). If a group of acids is known to be absent it is still advisable to pass the solvent for that group, a blank will be recorded for these fractions but better elution of the remaining acids will result.

The change in developing solvent should ideally take place in the trough between two elution maxima, but because of the column hold-up

this change has to be anticipated by some fifteen fractions. The following rules can be given for general guidance only since each chromatogram is different, depending on the load and complexity of the mixture. The solvent change following elution of a large peak (5×10^{-5} Equivs.) is made several fractions after the maximum; following a medium sized curve (1×10^{-5} Equivs.) the change is made two fractions after the elution maximum and following a small curve (0.1×10^{-5} Equivs.) the change may be made before the elution maximum is reached.

At the end of each chromatogram 85% acetone is passed through the column until a satisfactory blank titration is recorded. (<0.01 ml. of 10^{-2} N alkali per fraction). This indicates that all the acidic material placed on the column has been removed.

Castor oil and acetylated castor oil packed columns are developed in the same manner except that the column temperature is 20°C. These columns are not stable to mobile phases greater than 80% acetone.

(g) Solvent Blank Titre.

Solvent blank titres are determined by placing 2.00 ml. of each solvent in the titration cell and titrating with standard alkali. Values of 0.002 to 0.01 ml. of 10^{-2} N alkali per fraction are generally recorded.

(h) The Equivalent Weight of the Acid Mixture.

The equivalent weight of the mixed acids being analysed is required in order to compute the overall recovery of the material

placed on the column. It is measured by making up a suitable weight of the mixed acids to 100 ml. with 80% acetone in a standard flask. The normality of the acid is found by titration with 10^{-2} N alkali and the equivalent weight calculated.

(i) The Composition of the Acid Mixture.

The alkali titration for each sample delivered by the siphon is plotted against the sample number as shown in fig. 5, and the blank titrations for the acid content of the mobile phases are inserted as shown. The alkali used during the elution of an acid group is then found by adding up the titrations relevant to that particular curve and subtracting the appropriate number of blank titres. A calculating machine facilitates this work. The net alkali multiplied by its normality and any correction factors for burettes etc. gives the number of equivalents eluted under each curve. The number of equivalents of mixed acids placed on the column is known and the total % recovery can therefore be calculated. This recovery should be 97-100%, if it falls below 95% then the chromatogram is considered unsatisfactory.

(4) An Empirical Choice of Mobile Phase.

(a) Method.

Consider the reversed phase chromatographic separation of palmitic and stearic acids. Using a mobile phase of low acetone concentration both acids will be eluted very slowly and although separable, the system will be inconvenient. At higher acetone concentrations both acids will be eluted very quickly and separation

would then be impracticable. Between these two values, however, there is a range of acetone concentrations for which the palmitic acid is eluted at a convenient rate whilst the stearic acid is separated only after prolonged percolation. The solvent choice for the separation will lie within this range. A chromatogram of palmitic and stearic acids separated with the optimum solvent for palmitic acid only is shown in fig. 10. The palmitic acid is eluted quickly and gives a well shaped Gaussian curve whilst the stearic acid is eluted much more slowly and the curve shape is poor. It thus becomes apparent that there must be an optimum solvent for each acid group and by suitably changing the concentration of the mobile phase throughout the chromatogram a mixture may be separated as shown in fig. 5. In the two chromatograms illustrated in figs. 5 and 10, the concentration of acetone in the mobile phase for the elution of the palmitic acid is identical; but in the former analysis the concentration of acetone in the mobile phase is raised after the elution of the palmitic acid with the result that the stearic acid which follows, is eluted at the same speed.

There is however only general agreement among other investigators about which is the optimum eluting solvent for each acid group (Table 1).

TABLE I

Mobile Phase Concentrations used by Previous Workers (% Acetone)

ACID GROUP	REFERENCES								
	1	2	3	4	5	6	7	8	9
C8	-	45	-	55	-	50	40	45	-
C10	-		-	40/45	-	56			-
C12	55	55	-	50/55	-	65			-
C14	55/60		-	55/60	-	70			50
C16	65/68	65	70	65	-	70/75			
C18	70	75	75	70	70	75			
C20	-	-	80	75	70	80	75	80	
C22	-	-	85	-	75	85	-	-	85
C24	-	-	90	-	-	-	-	-	-

Change in 2 and 3% steps

Change in 5% steps

Gradient elution

For the present work some pure saturated and unsaturated acids were examined experimentally to determine their optimum eluting solvents. Each acid was run singly and the best eluting solvent determined from the shape of the curve; a needle shaped curve indicates % acetone too high and a low flat curve indicates % acetone too low. Binary and ternary mixtures were then chromatographed and the % acetone for the elution of each component adjusted to give the best resolution for each acid.

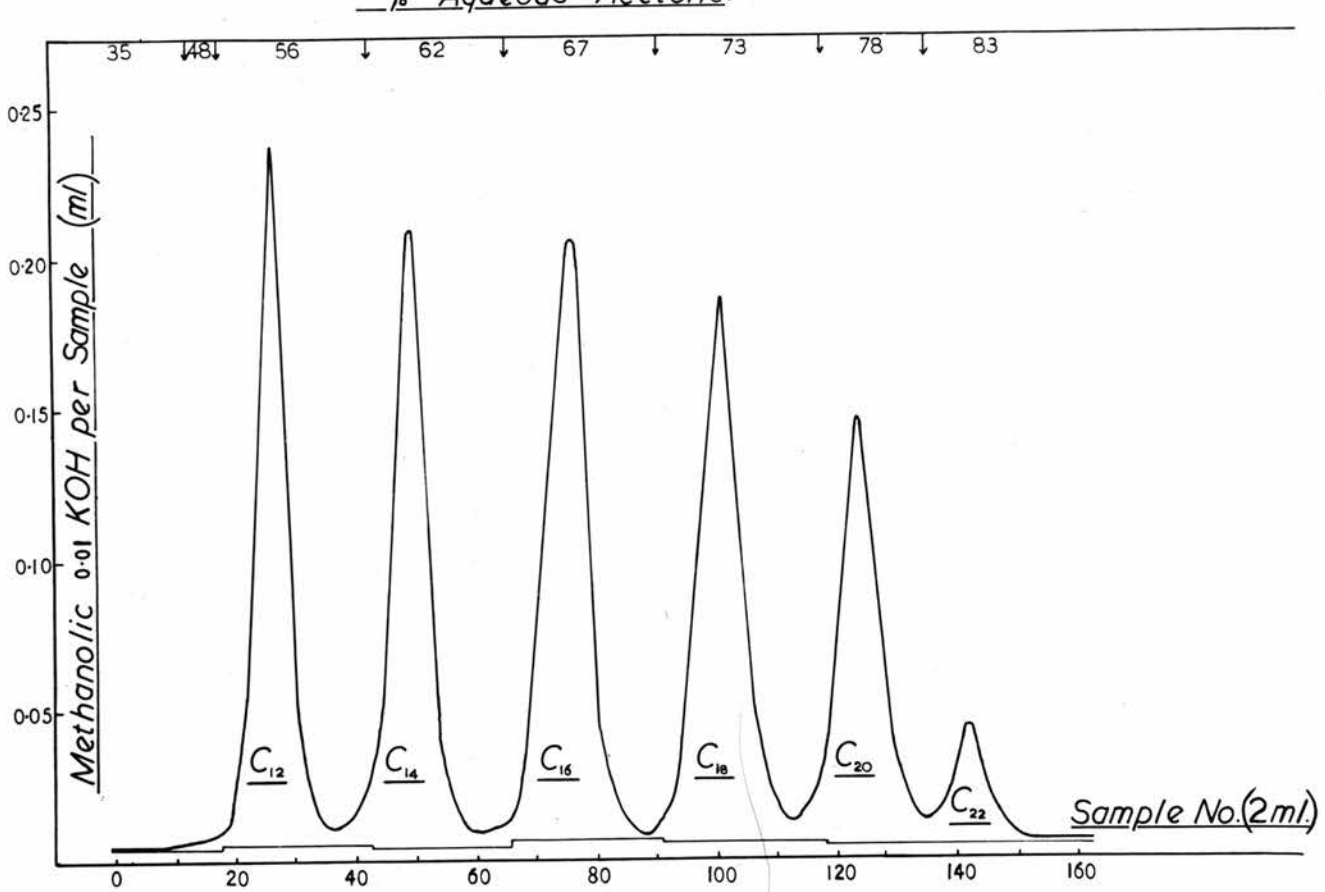


FIG 5. *Elution Curve of the Saturated Acids.*

(b) Results.

The results of these experiments are shown in Table 2, and a chromatogram of a test mixture of some saturated acids is shown in fig. 5

TABLE 2

Optimum Eluting Solvents for Liquid Paraffin/Aqueous Acetone Chromatograms.

(a) ACID	12:0	14:0,16:1, 18:2	16:0, 18:1	18:0	20:0
% ACETONE	56	62	67	73	78

(a) The shorthand terms used to designate acids indicate the number of carbon atoms and the number of double bonds per molecule, according to the method of Dole and Ahrens[11].

Analytical results from the chromatogram shown in fig. 5 and a chromatogram of some unsaturated acids are shown in Table 3.

TABLE 3

Quantitative Results from Two Test Chromatograms.

	MIXTURE 1 (Fig. 5)						MIXTURE 2			
ACID	12:0	14:0	16:0	18:0	20:0	22:0	12:0	18:0	18:1	18:2
Calc.%	15.2	17.3	20.5	21.8	25.2	0[(a)]	10.6	14.3	39.9	35.2
Obs. %	14.9	16.8	20.6	21.8	21.3	4.6	10.4	14.4	39.5	35.7

(a) The arachidic acid was known to be impure and apparently contains behenic acid.

The results shown in fig. 5 and table 3 indicate that the correct

solvents to separate an acid mixture quantitatively can be determined empirically, but this determination is both time consuming and wasteful on materials. Part of the purpose of the present work is to extend the reversed phase chromatographic technique to the separation of oxygenated acids by using other stationary phases, thus a rapid method of determining optimum mobile phase concentrations is required. It was decided to correlate the column behaviour of an acid to its partition coefficient between the mobile and stationary phases used for its chromatographic separation. Partition coefficient data for a set of acids between a stationary and various mobile phases could then be used to design an appropriate chromatographic system and to predict the possible separations obtainable by the system chosen. A similar type of approach to the design of chromatographic systems has been described by Peterson and Johnson[12] for the short chain acids (C_1 to C_{10}) using 35 N sulphuric acid as a stationary phase.

(5) Determination of Partition Coefficients.

(a) Method.

In seeking to relate the partition coefficients (K) of fatty acids to their chromatographic behaviour values of K must be measured under conditions which resemble those operating during column elution and are also practically convenient. For a liquid paraffin/aqueous acetone column with a column hold-up of 30 ml. the ration of mobile to stationary phase is 2.5:1. This ratio was increased to 10:1 in

the method for measuring K for practical reasons; the change has little effect on the results. (Table 4). The addition of kieselguhr, on the other hand, makes a marked difference in the measured values of K and has the effect of increasing the portion of acid in the stationary phase, presumably due to absorbtive forces.

TABLE 4

Partition Coefficients[a] Measured under Various Conditions.

ACID	PHASE RATIO MOBILE:STATIONARY	KIESELGUHR	AQUEOUS ACETONE.		
			62%	67%	75%
Palmitic	10:1	Present	0.10	0.16	-
	2.5:1	Present	-	0.19	-
	2.5:1	Absent	0.57	1.03	-
Stearic	10:1	Present	-	0.07	0.18
	2.5:1	Present	-	0.09	-
	2.5:1	Absent	-	0.44	0.85

(a) Partition coefficient = $\frac{\text{Concentration of acid in Mobile Phase}}{\text{Concentration of acid in Stationary Phase.}}$

The following method was used to measure the partition coefficients:-

The acid (ca. 2 mg., accurately weighed) under investigation is dissolved in the stationary phase (ca 0.5 ml., accurately weighed) by warming together in a centrifuge tube to 100°C or if this fails, by addition of a mutual solvent (ether alcohol or acetone) which is subsequently completely removed under reduced pressure leaving a super-saturated solution. Siliconised Hyflo Super-cel (1.3 to 1.4 times the weight of stationary phase) is added to absorb the stationary phase and to facilitate separation of the two liquid phases along with

the chosen acetone (5.00 ml.) The tube is corked and after equilibrium at the desired temperature (35°C with paraffin, 20°C with castor oil and acetylated castor oil) is centrifuged. Two 2.00 ml. portions are removed with a pipette and titrated against 0.01 N methanolic potassium hydroxide (A ml) using the nitrogen stirred cell and micrometer syringe burette. To the remaining mixture of acid, stationary phase, inert support and aqueous acetone (1 ml.), 95% aqueous acetone (5.00 ml.) is added and after equilibrium two further 2 ml. portions are removed and titrated. (P ml.)

The partition coefficient which is the concentration of acid in the mobile phase divided by the concentration of acid in the stationary phase is given by the following expression, in which V is the volume of stationary phase. (The density of the stationary phase is also determined).

$$K = \frac{AV}{6P-A}$$

In this calculation it is assumed that the 95% acetone, even though diluted by 1 ml. of a more aqueous acetone, removes all the acid from the stationary phase. There is good evidence that the partition coefficient under these conditions is very large and as a further check, the recovery of the acid is calculated. This should be 100 ± 5% for the value of K to be significant.

$$\% \text{ Recovery} = 100\ NE\ (2A + 5P)\ /\ W$$

where N is the normality of the alkali and W and E are the weight and equivalent of the acid used.

(b) Results.

Values of K for a number of saturated and unsaturated acids and for various oxygenated acids have been measured using several aqueous acetones as one liquid phase and liquid paraffin, castor oil or acetylated castor oil as the other liquid phase. Table 5 shows a specimen calculation for six results, which are given in their entirety in Tables 6 - 8. The partition coefficient values may be shown either by plotting log 100 K against the percentage acetone in the aqueous acetone, (one line for each acid, Figs. 6 - 8) or by plotting log 1/K against the chain length of the acid. (One line for each aqueous acetone, Fig. 6 a.) Both plots are linear and a theoretical reason for this is given later on p. 57. The lines for non-separable groups of saturated and unsaturated acids lie very close together and some of these have been omitted for the sake of clarity.

TABLE 5

Calculation of K for Stearic Acid (Paraffin / Aq. Acetone).

Wt.Acid	Vol.Par.	Solvent	Titration	Blank	A	P	%Rec.	K
1.65 mg	0.4595	67%	0.0644	0.056	0.0588		96	0.0731
		95	0.0784	0.070		0.0714		
2.27	0.4874	70	0.0976	0.058	0.0918		92	0.104
		95	0.0940	0.070		0.0870		
1.95	0.4797	73	0.1120	0.070	0.1050		98	0.193
		95	0.0680	0.070		0.0610		
2.12	0.4731	75	0.1350	0.070	0.1290		96	0.249
		95	0.0688	0.070		0.0618		
2.79	0.6110	78	0.1826	0.070	0.1756		98	0.381
		95	0.0852	0.070		0.0762		
2.07	0.5088	83	0.1690	0.090	0.1600		101	0.921
		95	0.0484	0.070		0.0414		

TABLE 6 a

K Values for Paraffin / Aq. Acetone. (Fig. 6).

	AQUEOUS ACETONE (%)												
ACID	42	48	52	56	59	62	65	67	70	73	75	78	83
D	.05	.08	.15	.28		.64		1.5					
D		.09		.28									
C			.05	.08	.11	.18	.23	.34		.69			
C				.08		.19		.34					
B				.03		.08	.12	.17	.27	.35		.91	
B						.08	.11	.17	.26	.34		.89	2.19
A								.07	.09	.18	.25	.48	.95
A								.07	.10	.19	.25	.38	.92
G			.05	.08		.15		.29					
E						.08		.18		.32		.91	
F			.03	.06		.15		.26					

Key to Tables 6 - 8 and Figures 6 - 8.

ACID.	SYMBOL.
Stearic	A
Palmitic	B
Myristic	C
Lauric	D
Oleic	E
Linoleic	F
Hexadec-9-enoic	G
12-Hydroxystearic	H
9-Hydroxyoctadec-12-enoic	J
threo-9:10-Dihydroxystearic	L
erythro-9:10-Dihydroxystearic	M
erythro-9:10-Dihydroxypalmitic	N
threo-9:10:12-Trihydroxystearic[1]	O
threo-9:10-threo-12:13-Tetrahydroxystearic[2]	P
12-Acetoxystearic	R
threo-9:10-Diacetoxystearic	S
erythro-9:10-Diacetoxystearic	T
9:10-Epoxystearic	U
12-Ketostearic	W

1. m.p. 106-108°

2. m.p. 144-146°

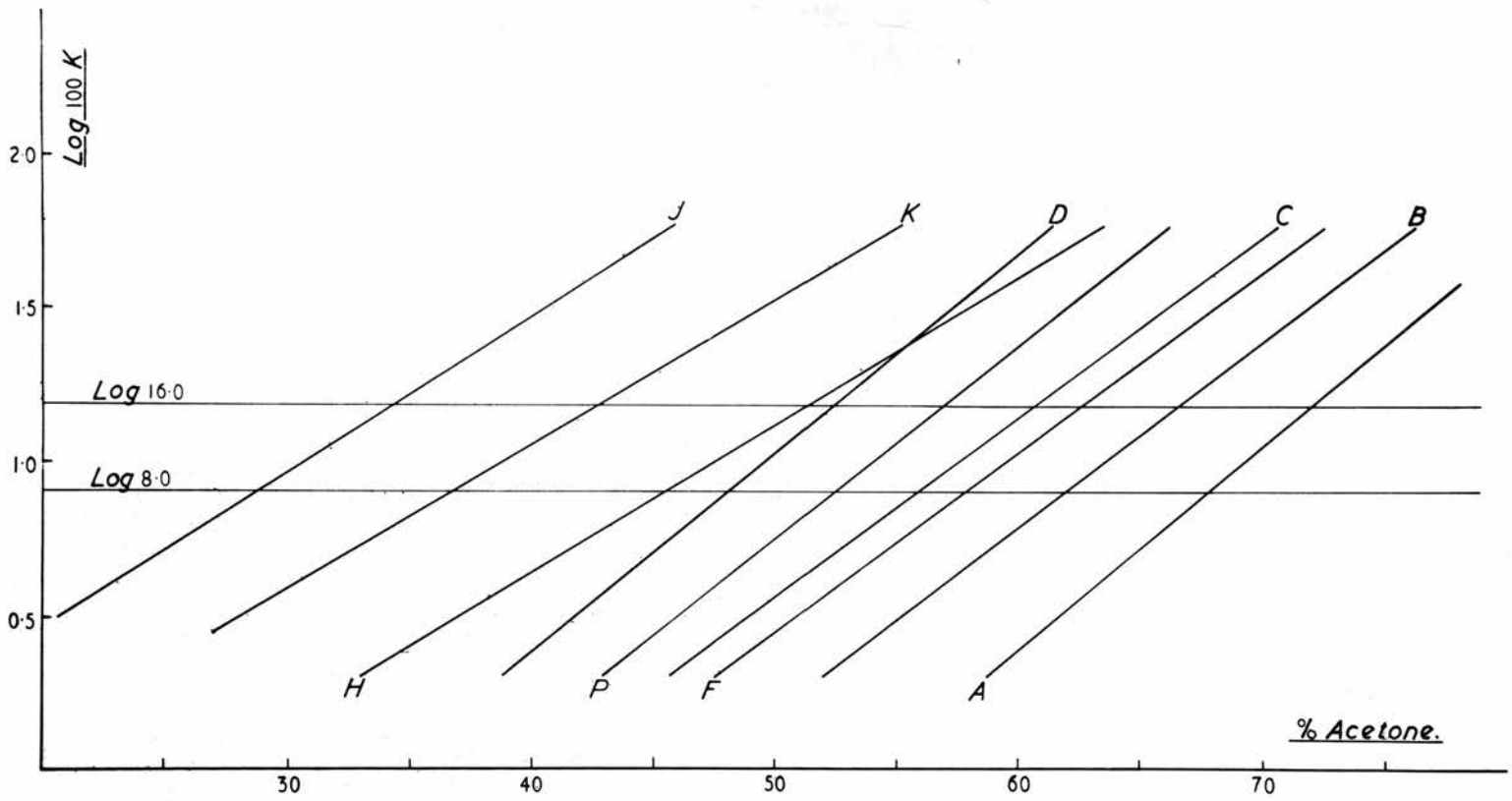


FIG 6. *Partition Coefficient for Paraffin/Aq. Acetone.*

TABLE 6 b

K Values for Paraffin / Aq. Acetone (Fig 6)

	AQUEOUS ACETONE (%)							
ACID	25	30	35	42	48	52	56	62
H	.002			.04	.11	.14	.25	
H				.05	.11		.22	
J	.05	.08	.17		.76		1.56	
J			.16					
W					.04		.12	.25
W					.05			.24
U				05	.09		.24	.48
L	.02	.04	.06	.11	.27	.56		
L			.06	.15	.26			
S			.05		.29		.61	1.38
S					.29		.69	
T			.06		.25		.66	
R					.4		.15	.31

In Table 6 a note the similarity in the data for palmitic (B) and oleic (E) acids; and for myristic (C) palmitoleic (G) and linoleic (F) acids. This bears out the empirical observation that one double bond has an effect on column behaviour equivalent to a shortening of the chain by two carbon atoms.

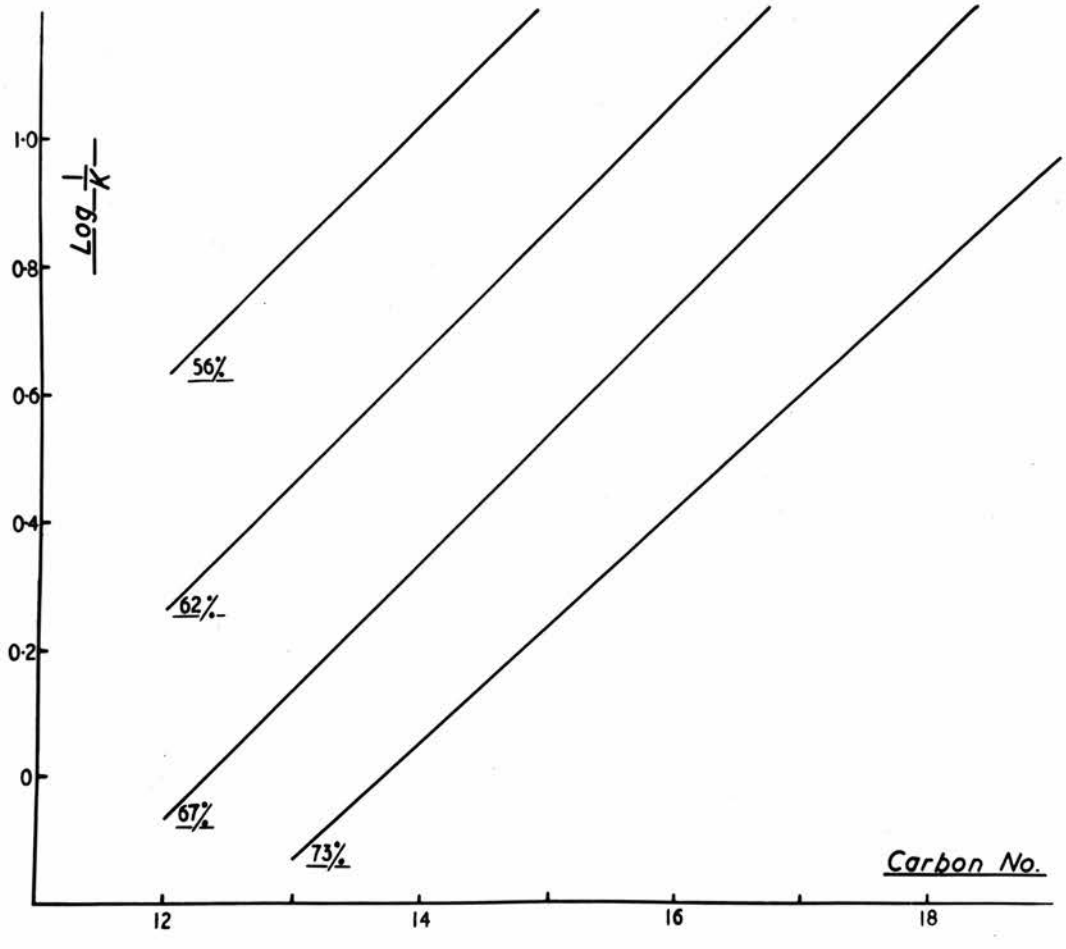


FIG 6a. Partition Coefficient for Paraffin Aq. Acetone.

TABLE 7.

K Values for Castor Oil / Aq. Acetone (Fig.7).

	AQUEOUS ACETONE (%)								
ACID	40	45	50	55	60	65	70	75	80
A							.01	.02	.05
A								.02	.04
E							.02	.05	.06
D							.05	.10	.15
H						.04	.06	.11	.15
H						.05		.10	
H						.03	.05	.08	.16
L					.05	.08	.13	.24	
M						.07	.14	.19	.53
O			.07	.13	.20		.50		
O		.04	.08	.13		.30			
P	.04	.07	.12		.48				

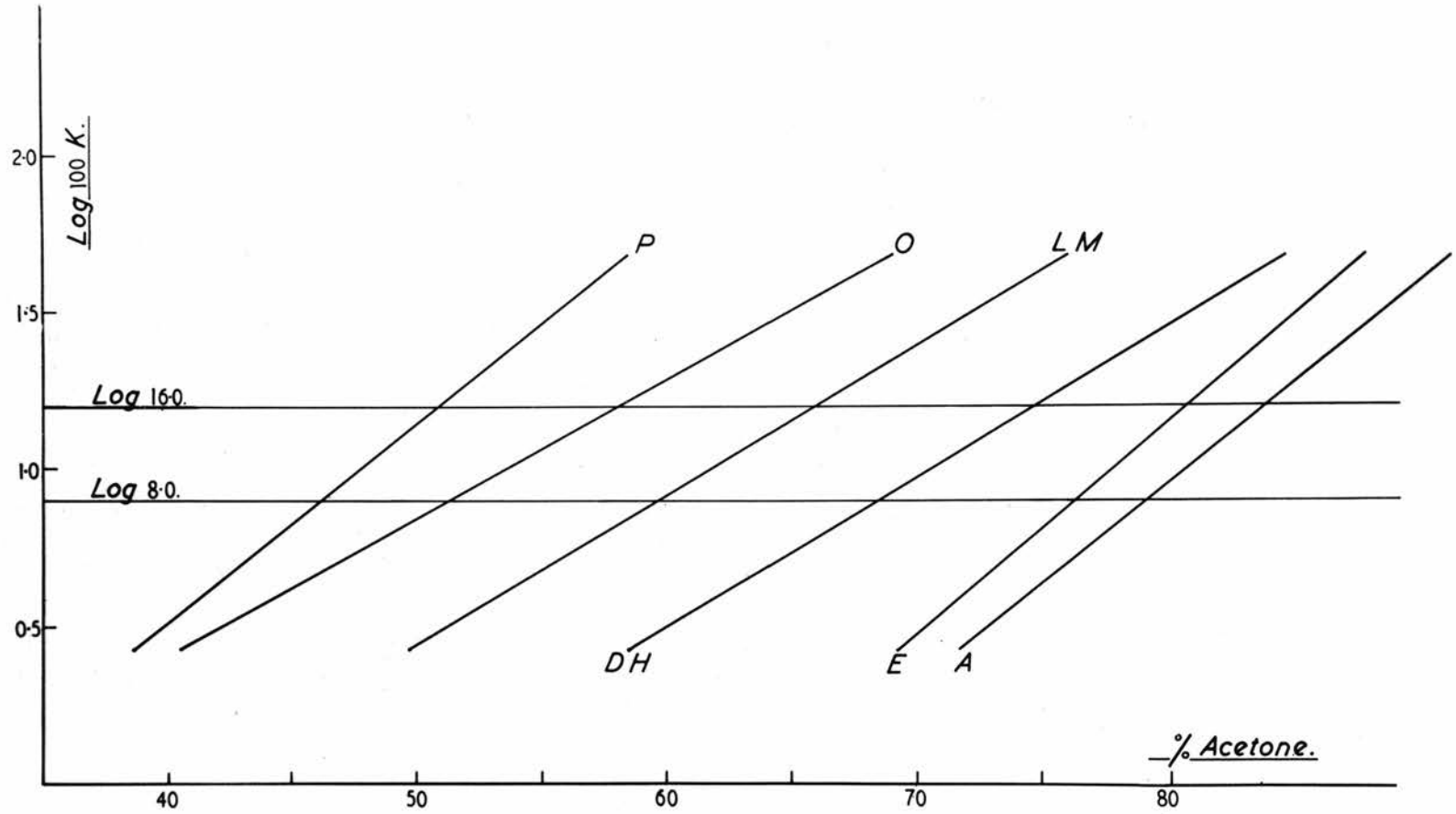


FIG 7. *Partition Coefficient for Castor Oil/Aq. Acetone.*

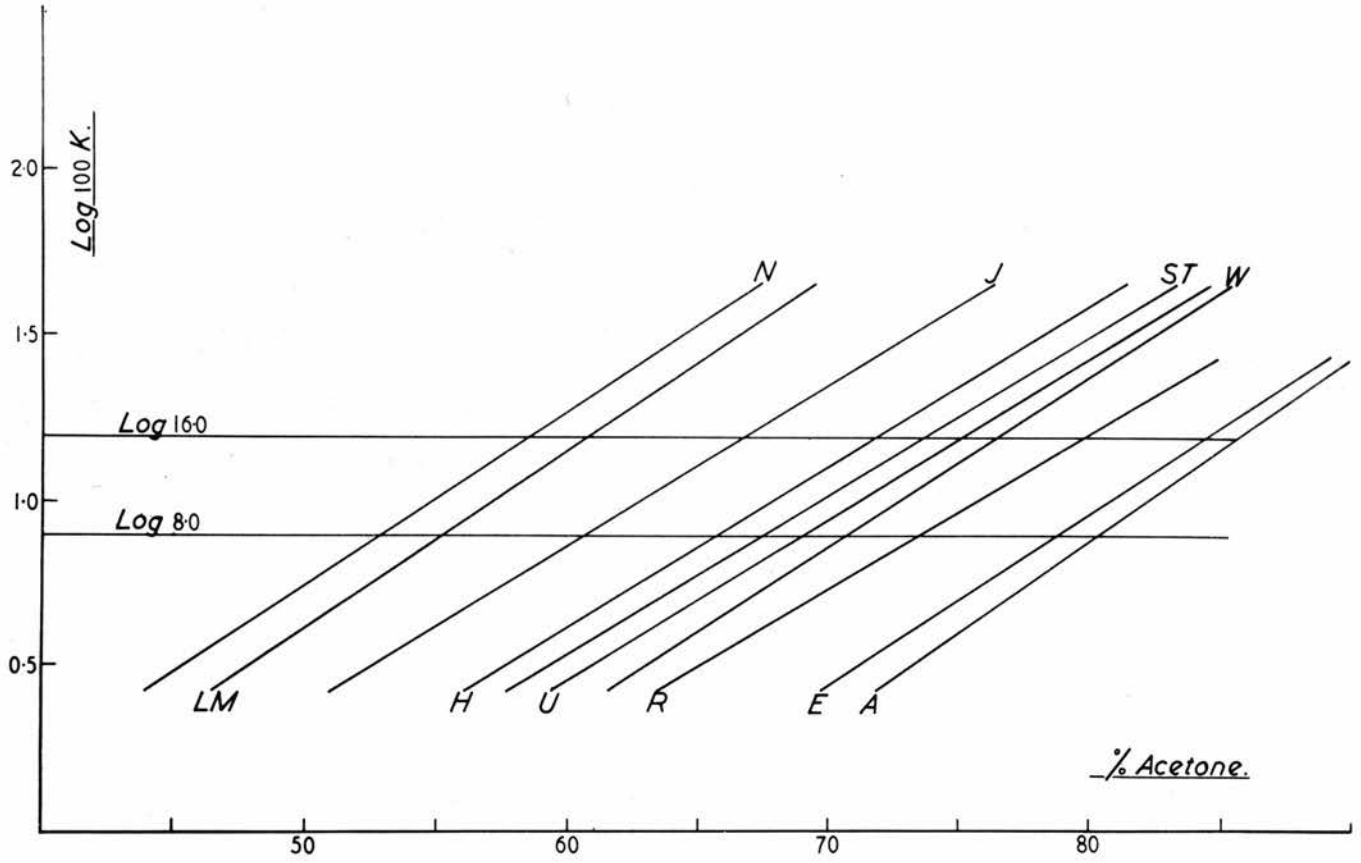


FIG 8. *Partition Coefficient for Acetylated Castor Oil/Aq. Acetone.*

TABLE 8.

K Values for Acetylated Castor Oil / Aq. Acetone (Fig.8)

	AQUEOUS ACETONE (%)							
ACID	45	50	55	60	65	70	75	80
D		.04	.27	.54		1.0	1.2	1.5
A				0.0	.01	.32	.51	.89
E						.44		.94
H		.08		.60	.85	1.1	1.4	1.6
J	.04	.37	.60	.86	.98			
W			.08		.58		1.1	
U			.19	.48	.65	.95	1.2	
L	.04	.69	.94	1.1	1.5	1.7		
M		.60		1.2		1.5		
N	.49		1.0		1.5			
R			0.0		.45		.95	
S		0.0	0.25	.49	.78	1.0		
T		0.0		.49		.98		

TABLE 9.

K Values at Optimum Solvents.

ACID	D	C	G	F	B	E	A
(a)	53*	62	62	62	67	67	73
(b)	.16	.18	.15	.15	.16	.18	.18
(c)	-	.05	.05	.04	.10	.08	.07

* The value of 56% found empirically and reported in Table 2 was changed to 53% on the basis of these results.

(a) The optimum concentration of acetone for the elution of the acid

concerned from a paraffin column.

(b) The partition coefficient of the acid for this particular acetone.

(c) The partition coefficient of the acid for the aqueous acetone used for the elution of the next lower group of acids. i.e. 55% for the myristic acid group, 62% for the palmitic acid group and 67% for the stearic acid group.

(6) DEDUCTION OF OPTIMUM MOBILE PHASE FROM K DATA.

(a) Discussion.

The optimum eluting solvents for some saturated and unsaturated acids have already been determined empirically. (Table 2). It is now seen that each acid has about the same partition coefficient at the chosen acetone concentration. (Table 9). For the paraffin system, an acid is satisfactorily eluted when its partition coefficient is equal to or greater than 0.16 and will be conveniently separated from other acids having partition coefficients not greatly above 0.08. These two values have been used to draw the horizontal lines on Fig. 6. If these two values were closer together then the separation of acids having closer values of K would be possible. The available data does not, however, allow them to be put much closer together with the present experimental system, without the loss in the high degree of resolution required for quantitative analysis. Some improvement might follow a change in the dimensions of the column.

A plot of partition coefficient data with the log 0.16 and 0.08

lines, inserted as shown in Fig. 6 therefore enables possible separations between various acids to be predicted and also indicates the optimum eluting solvent for each acid group. Other factors may however limit the practical value of the system even when the partition coefficient data indicates the possibility of separation. Mobile phase of acetone content so high that it removes stationary phase from the column cannot be used; and difficulties also arise when the solute has a very low solubility in the stationary phase. The latter leads to poor recovery of material and "tailing" of the peak.

The only stationary phases used for reversed phase chromatography are paraffin, castor oil[13,14] and mixtures of the two[14]. Mixtures are unsatisfactory because they are difficult to reproduce and are frequently unstable due to the differential removal of one of the two components by the mobile phase.

In an attempt to increase the usefulness of reversed phase chromatography some potential stationary phases have been investigated. These include dinonyl-phthalate, six polyesters of the Reoplex type and acetylated castor oil. All except the last were considered to be too soluble in the higher aqueous acetones. Castor oil may be used with aqueous acetone up to 80% and acetylated castor oil up to 85%. (Liquid paraffin has been used up to 90% acetone.) The partition coefficients measured for a number of acids using castor oil and acetylated castor oil are given in Tables 7 and 8.

Since solvents with an acetone concentration greater than 80%

cannot be used with castor oil, saturated acids higher than lauric cannot be conveniently eluted from the column without removing so much stationary phase that it is unfit for further use. Acetylated castor oil is thus considered to be a more suitable stationary phase since aqueous acetone solutions up to 85% can be used and, at the same time, the values of K are slightly higher than those with castor oil. Thus 12-hydroxystearic acid is best eluted from castor oil with 80% aqueous acetone and from an acetylated castor oil column with 72% aqueous acetone, thereby enabling the acid to be satisfactorily eluted from an acetylated castor oil column without causing the collapse of the packing.

It is of interest to note that the relative values of K for a range of acids is not the same for different stationary phases. i.e. 9:10-Dihydroxystearic acid can be separated from its diacetyl derivative on an acetylated castor oil column but not on a paraffin column. (c.f. also Palmitic and Stearic acids).

(b) Predicted Separations and Eluting Solvents.

Using partition coefficient data in the manner outlined above it is possible to construct Table 10.

Table 10 may be used to predict the possible separations between any of the given acids and the stationary and mobile phases which will effect the best separations. Four synthetic mixtures were analysed using chromatographic systems designed solely from partition coefficient data. The results were entirely satisfactory and confirm the

usefulness of the method for the design of an analytical system tailor made for a particular combination of acids.

TABLE 10.

Predicted Eluting Solvents (% Aqueous Acetone).

ACID	PARAFFIN	CASTOR OIL	ACET. CAST. OIL.
Tetrahydroxystearic	-	51	-
Trihydroxystearic	-	58	-
Dihydroxypalmitic	-	-	61
Hydroxyoleic	55	-	68
Dihydroxystearic	45	71	61
Diacetoxystearic	43	-	74
Hydroxystearic	53	80	74
Lauric	53	80	74
Epoxystearic	58	-	74
Ketostearic	58	-	74
Acetoxystearic	58	-	80
Myristic	62	-	80
Palmitoleic	62	-	80
Linoleic	62	-	80
Palmitic	67	-	85
Oleic	67	87	85
Stearic	75	87	85
Arachidic	78	-	-

Mixture 1. Separation of 12-Hydroxystearic and 9:10-Dihydroxystearic Acids.

Table 10 indicates that this separation may be achieved using a castor-oil packed column and a solvent system of 71% and 80% aqueous

acetone. A mixture was separated with the following results: 12-hydroxystearic 40.0% (38.0%); 9:10-dihydroxystearic acid 60.0% (62.0%). Figures in parentheses are the calculated molar percentages of the components.

Mixture 2. Separation of 9:10-Dihydroxystearic and 9:10:12-Trihydroxystearic acids.

Table 10 indicates that the separation can be achieved using a castor oil packed column and a solvent system 58% and 71% aqueous acetone. The separation gave the following results: Dihydroxystearic acid 53.0% (54.0%) and Trihydroxystearic acid 47.0% (46.0%).

Notes on Castor Oil Columns.

(a) The castor oil column becomes unstable with 80% acetone and thus although analyses such as mixture 1 can be achieved with this column, its use is not recommended.

(b) Attempts to chromatograph tetrahydroxystearic acid failed due to the insolubility of the acid in the stationary phase.

Mixture 3. Separation of 12-Hydroxystearic acid and 9:10-Dihydroxystearic acid.

Table 10 indicates that this mixture (same as 1) may also be separated with an acetylated castor oil column and 61% and 74% aqueous acetones. A mixture was separated with the following results: Monohydroxystearic acid 51.3% (50.7%) ; dihydroxystearic acid 48.7% (49.3%).

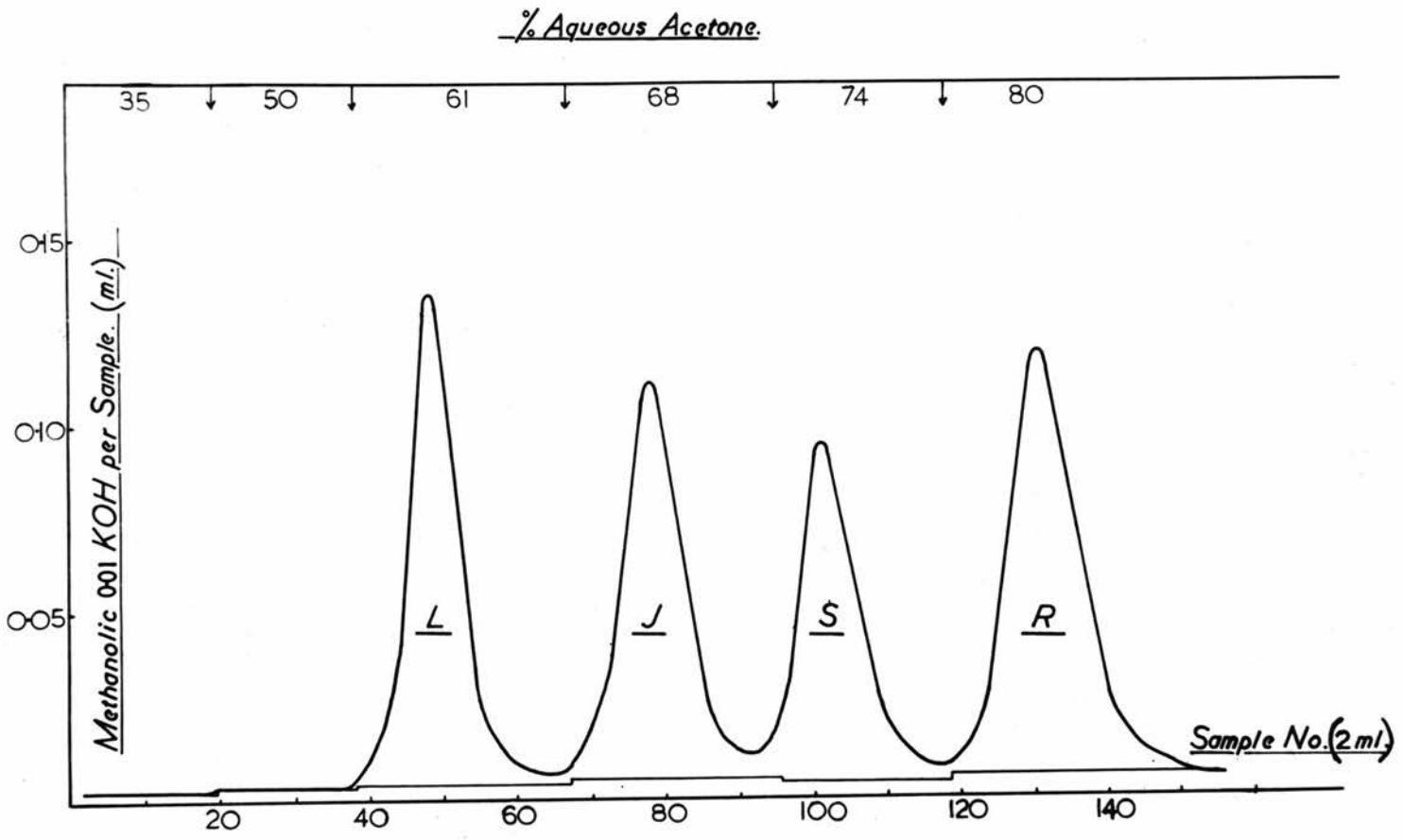


FIG 9. *Elution Curve of Some Oxygenated Acids.*

Mixture 4. Separation of 9:10-Dihydroxystearic Acid; 9-Hydroxyoctadec-12-enoic Acid; 9:10-Diacetoxystearic Acid and 12-Acetoxystearic Acid.

Table 10 indicates that this mixture may be separated using an acetylated castor oil column and a developing solvent system of 61% 68%, 74% and 80% aqueous acetone. A mixture was separated with the following results: Dihydroxystearic acid 22.9% (22.6%); Hydroxyoleic acid 21.7% (22.2%); Diacetoxystearic acid 25.2% (25.1%) and Monoacetoxystearic acid 30.2% (30.1%). The chromatogram is shown in Fig. 9.

Notes on Acetylated Castor Oil Columns.

(a) The packing has the advantage over castor oil in that it can be used up to80% acetone without serious loss of stationary phase.

The use of paraffin and acetylated castor oil packed columns provides a valuable technique for the analytical separation of a wide range of saturated, unsaturated and oxygenated acids.

(7) THEORETICAL TREATMENT OF EXPERIMENTAL SYSTEM.

The general theory of partition has been applied to counter-current distribution by Craig[15] and to chromatography by Martin and Synge[16], Mayer and Tomkins[17] and Glueckauf[18]. The following treatment provides a reasonable explanation of the results obtained in the present study. The symbols used are given in Table 11. From the experimentally determined position and height of the eluate maximum

it has been possible to derive values of K^1 and p, to plot a theoretical eluate curve and to determine the partition coefficient K^1_B which an acid B must have if it is to be separated from an acid A of partition coefficient K^1_A. Though some of the experimental results are obtained using only one solvent, rather than the usual range, the application of the theory to the more usual experimental conditions is also considered.

TABLE 11.

Symbols used in the Mathematical Treatment.

K^1 The partition coefficient of an acid under chromatographic conditions.

p The number of theoretical plates in the column.

a The number of an eluate fraction, where each fraction has the same volume as that of the mobile phase in a theoretical plate.

E_a The fraction of solute present in the ath. eluate.

V_m The volume of mobile phase contained in the column.

V The volume of each experimental eluate fraction.

f. The number of the eluate fraction containing most solute, counted from the point where the solvent front emerges from the column.

$E^1_{max.}$ The fraction of solute in the eluate f.

C The volume of mobile phase in the column, expressed as a number of experimental eluate fractions.

i.e. $C = \frac{V_m}{V}$

$A_{\{x\}}$ The area of the normal curve of error for the argument $\{x\}$

The theoretical approach described below is based on the analogy of a partition chromatographic column to a counter-current extraction process. Accordingly the column is considered to contain a number of theoretical plates (p) each of which acts as a single tube in the counter-current distribution apparatus. A theoretical plate is defined as a section of the column in which the mobile phase leaving the section has the composition that would be in equilibrium with the average concentration of stationary phase solution within the section.

When mobile phase flows through the column the amount of solute in any plate can be derived from the general formula (1) for calculating the fraction ($T_{n,r}$) of solute of partition coefficient K, in the rth. plate after n transfers:

$$T_{n,r} = \frac{n!}{r!\,(n-r)!} \left[\frac{1}{K+1}\right]^n K^r \quad - - - \quad (1)$$

The fraction in the pth. plate when the solvent front first reaches this plate, i.e. after p transfers is given by:

$$T_{p,p} = \frac{p!}{p!\,(p-p)!} \left[\frac{1}{K^1+1}\right]^p \quad (K^1)^p = \left[\frac{K^1}{K^1+1}\right]^p$$

When a further volume of mobile phase equivalent to the amount in one plate flows through the column then a portion $K^1/(K^1+1)$ of the solute in the pth plate will flow out and be present in the first eluate fraction (E_1).

$$E_1 = \left[\frac{K^1}{K^1+1}\right]^{p+1}$$

As the column is developed this fraction, $K^1 / (K^1 + 1)$, of solute in the pth plate after $p + 1$, $p + 2$, $p + 5$, etc. transfers

will appear in the 2nd, 3rd, 4th, etc. eluate fractions.

$$E_2 = \frac{(p+1)!}{p!\ 1!} \cdot \frac{(K^1)^p + 1}{(K^1+1)^{p+2}} = \frac{p+1}{K^1+1} \cdot E_1.$$

$$E_3 = \frac{(p+2)!}{p!\ 2!} \cdot \frac{(K^1)^{p+1}}{(K^1+1)^{p+3}} = \frac{p+2}{2(K^1+1)} \cdot E_2$$

and in general:-

$$E_{a+1} = \frac{(p+a)!}{p!\ a!} \cdot \frac{(K^1)^{p+1}}{(K^1+1)^{p+a+1}} = \frac{p+a}{a(K^1+1)} \cdot E_a \quad - - - (2)$$

(a) The Partition Coefficient.

As long as the value $\frac{p+a}{a(K^1+1)}$ is greater than one, the fraction of solute in successive eluates increases and the maximum value is reached when $\frac{p+a}{a(K^1+1)} = 1$. i.e. when $K^1 = p/a$. Though both p and a are as yet unknown the ratio a/p, the number of eluate fractions to the maximum divided by the number of theoretical plates in the column is equivalent to the total volume of eluate required to attain the maximum expressed in volumes of mobile phase on the column.

$$\frac{1}{K^1} = \frac{a}{p} = \frac{fv}{V_m} \quad - - - - - - - (3)$$

It has been shown by van Duin[19] that, during partition chromatography of a homologous series, the logarithm of the retention volume plotted against the chain length of the members of that series is a straight line.

i. e. Log (retention volume) = log (2fv) = const. (chain length)

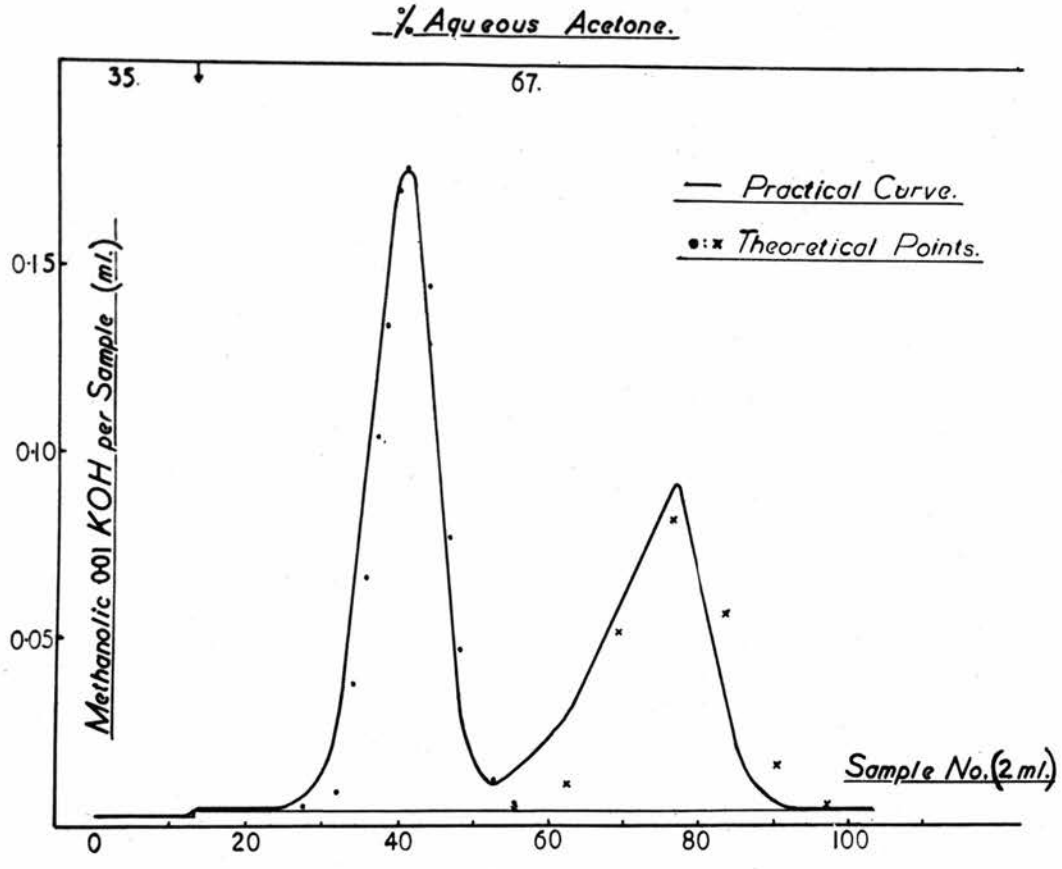


FIG 10. *Elution Curve of Palmitic & Stearic Acids.*

From (3) $2fv = \frac{2V_m}{K^1}$ Log $\frac{2V_m}{K^1}$ = const. (chain length)

A plot of log $1/K^1$ against chain length should therefore be linear. Fig. 6a shows such a linear plot for the partition coefficients of the homologous series C_{12} to C_{18} monobasic acids measured between liquidparaffin and aqueous acetone.

From eluate curves obtained with palmitic and stearic acids using only 55% and 67% acetone, palmitic acid has an operating partition coefficient of 0.50 and stearic acid of 0.22 (Fig. 10). It is considered that the 55% acetone has practically no effect on the subsequent behaviour of the acids. These values are not directly comparable with recorded earlier but have to be modified because the volumes of the two liquid phases in the column are not the same. (Ref 15 p.200). After making allowance for this ratio of 2.5:1, partition coefficients of 0.20 and 0.09 are obtained. These are to be compared with experimentally measured values of 0.16 and 0.07 (Table 9). Absorbtive forces due to the kieselguhr which have been neglected in the theoretical treatment are in fact included in the values for partition coefficients measured experimentally. (Table 4)

(b) The Number of Plates.

Equation (2) gives the fraction of solute in any eluate fraction. At the maximum $a = p / K^1$ and by inserting this value and using the approximation $p! = e^{-p}p^p\sqrt{2\pi p}$ the following equation may be derived:-

$$E_{max} = K^1 \left[2\pi p \ (1 + K^1)\right]^{-\frac{1}{2}}$$

This eluate fraction, however, has a volume equal to that of the mobile phase in one plate whereas the titrated fractions have some arbitrary value (V, in this case v = 2.0 ml). If the equation is rewritten:-

$$p\,E_{max} = K^1(p)^{\frac{1}{2}}\left[2\pi(1+K^1)\right]^{-\frac{1}{2}}$$

the value of $p\,E_{max}$ can be calculated from the experimentally observed maximum (E^1_{max}) by multiplying by V_m/v whence:-

$$p = 2\pi(1+K^1)\left[\frac{E^1_{max}\,V_m}{K^1\,v}\right]^2 = 2\pi f\,(f+c)\,(E^1_{max})^2 \quad - - -(4)$$

Using equation (4) it is possible to calculate the value of p from the position and height of the maximum in Fig. 10; values of 70 and 110 are obtained, and many other curves give values of around 100. These values are particularly sensitive to changes in E^1_{max} and since the observed value is the total eluate in 5-8 plates the value per plate will be slightly lower than the true eluate maximum.

(c) Theoretical Eluate Curve.

By substituting derived values of K^1 and p in equation 2, it is possible to plot a theoretical elution curve. This has been done for some selected points in Fig. 10. and the agreement is considered to be satisfactory.

(d) Separation of Two Acids.

The equation of Glueckauf[19] may be used to discuss the separation of two solutes:-

$$\% \text{ Impurity} = 100\left[0.5 - A_{\epsilon}\left\{\frac{\sqrt{p}\,(\sqrt{B}-\sqrt{A})}{4\sqrt{AB}}\right\}\right]$$

where $A = (1 + \frac{1}{K^1_A})$ and $B = (1 + \frac{1}{K^1_B})$. This equation relates the efficiency of separation to the K^1 values of the two solutes and to the number of theoretical plates in the column. To separate a solute of $K^1 = 0.5$ from a second solute in a system having $p = 100$ so that the two overlap by less than 0.2%, the second solute must have K^1 less than 0.25. These K^1 values of 0.50 and <0.25 correspond to 0.20 and <0.09 for the experimentally determined values and are in agreement with the empirically selected values of 0.16 and <0.08.

When the column is developed with a single solvent acid of K^1 ca 0.5 reaches its maximum eluate after 2 V_m of eluate has been collected, in line with the requirement that $\frac{1}{K^1} = \frac{a}{p} = \frac{fv}{V_m}$

Under normal running conditions the acids which have this K^1 value for the optimum solvent appear to reach their maximum eluate after about 1 V_m. (Fig. 5 and 9). This apparent anomoly is related to the use of a range of solvents to elute the acid mixture. Those preceeding solvents which are not ideal for the elution of a particular acid will cause it to move slowly down the column, so that when the correct solvent is applied the acid is already part way down the column. It is therefore eluted more quickly, generally after $1.0V_m$ or less.

* * *

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PART III

APPLICATION OF REVERSED PHASE CHROMATOGRAPHY TO SEED OIL ANALYSIS

(1) Introduction.

The saturated acids usually encountered in Triglyceride studies (C_{12} to C_{24}) are readily separated using the liquid paraffin/aqueous acetone chromatographic system as described in Part II. The unsaturated acids are eluted along with the saturated acids in groups, under conditions in which one double bond is equivalent to a reduction in chain length of approximately two carbon atoms. Various methods have been used to determine the amount of unsaturated acids in any eluted group.

Boldingh[1], using a column packed with porous rubber, chromatographed the mixed acids only after complete hydrogenation or after removal of the unsaturated acids by oxidation with alkaline permanganate. Crombie et al[2] chromatographed the mixed acids alone and after oxidation with alkaline permanganate. The data obtained by both of these methods does not give a completely unambiguous solution with complex mixtures and suffers from the disadvantage that the oxidation procedure is known to degrade the saturated acids to some extent, especially those below myristic. Popják and Tietz[3] and Lough and Garton[4] have tried to overcome these difficulties by collecting the eluate from a chromatogram of the mixed acids, recovering the group of acids and rechromatographing after complete hydrogenation. The oxidation

procedure was thus avoided and additional data, if required, was obtained by alkali isomerisation. Savary and Desnuelle[5] have hydroxylated the unsaturated acids and subsequently separated the di - and tetra - hydroxy acids from oleic and linoleic acids respectively, on another column using castor oil as stationary phase. This system has not, however, found any general use for the analysis of mixed acids.

A method has now been developed whereby chromatograms of the mixed acids (i) alone, (ii) after hydrogenation and (iii) after ozonolysis yield sufficient information to determine the molar percentage of complex acid mixtures. The method has several advantages: (a) The results follow from chromatographic data alone and other determinations such as saponification equivalent and iodine value of the mixed acids can be used to confirm the accuracy of the chromatographic results. (b) The unsatisfactory permanganate oxidation procedure is replaced by ozonolysis, and (c) The difficulty of obtaining a quantitative recovery of eluted acids, as required in the method of Popjak[3] and Garton[4] is avoided. Further, by the use of acetylated castor oil packed columns the procedure has been satisfactorily extended to a wide range of oxygenated acids.

Some disadvantages associated with the method are: (a) The technique can not be operated with less than 75 mg. of the mixed acids, (b) It does not readily resolve very small impurities from adjacent large fractions, (c) it is completely non - automatic and (d) it

requires a degree of skill and practice above those required for gas/liquid chromatographic analysis.

(2) Experimental

(a) The Mixed Acids.

The specialised technique described below was developed in order to preserve intact any epoxy acids which may be present in the mixed acids.

Fatty material is extracted from the crushed seeds with petroleum ether (b.p. 40/60°). The triglycerides are hydrolysed by treatment with N alcoholic potassium hydroxide at room temperature for 24 hours, and the unsaponifiable material is extracted[6]. The soap solution and washings are acidified with an ion exchange resin (Zeo-karb 225) and a trace of sodium acetate. This last is to facilitate the subsequent ether extraction of the liberated acids. Any acetic acid extracted along with the mixed acids is removed by washing the extract with water, prior to drying with anhydrous sodium sulphate. Evaporation of the ether extract then gives the mixed acids. The Iodine value[6], saponification equivalent[6] and, if necessary, the epoxide[7] and glycol[8] values are then measured on the mixed acids.

(b) Hydrogenation.

Complete hydrogenation is effected by shaking an ethanolic solution of the mixed acids (ca.100 mg.)with 20% palladium charcoal catalyst[9] in an atmosphere of hydrogen for 24 hours. The catalyst is removed by centrifuging and the hydrogenated acids recovered by evaporation of the ethanol.

(c) Ozonolysis

Ozonolysis of the mixed acids is carried out in a methyl acetate solution at -40° (Solid carbon dioxide/acetone) using a 100% excess of ozone. With 50 - 100 mg. of mixed acids the reaction takes about 5 mins. using a stream of ozonised oxygen (2.5% O_3) obtained by silent high tension discharge[10]. The reaction is carried out in an all-glass apparatus and the effluent gas is bubbled through a solution of potassium iodide and starch in dilute sulphuric acid. Under the reaction conditions described above the ozone reacts quantitatively with the unsaturated acids[11] and the completion of the reaction is indicated by the iodine/starch indicator. Ozonolysis is continued for an equal time thereafter to obtain a 100% excess of the reagent.

The ozonides are decomposed according to the method of Cason and Tavs[12]. The reaction mixture is allowed to warm to 20° and, after boiling with water (5 ml.) for three hours, all the solvent is removed by vacuum distillation. The ozonised product is then loaded quantitatively on to the column.

(d) Acetylation.

The mixed acids are refluxed with acetic acid, freshly distilled acetic anhydride and pyridine (1:5 by vol.) for two hours, and then with water. The pyridine and acetic acid are removed by co-distillation with acetone, the last traces by vacuum distillation. (0.1 mm./60°for 6 hrs.). This procedure effects the complete acetylation of hydroxy and epoxy groups.

(e) Qualitative Identification of Acids.

Appropriate eluate fractions are collected and combined for identification of the acids therein. The fractions containing the potassium salt of a monoethenoid acid are firstly concentrated by vacuum distillation and then hydroxylated with 1% potassium permanganate according to the method of Lapworth and Mottram[15]. The di-hydroxy acid so produced is refluxed with pet. ether (b.p. 40/60°) to remove any saturated acids and is crystallised from ethanol. It is identified by its melting point and mixed melting point with an authentic sample.

The fractions containing the potassium salt of a diethenoid acid are acidified (dil. HCl.) and the precipitated acids extracted with ether. The ether solution is evaporated to a small volume, cooled to 0° and a weak solution of bromine in ether added until the bromine colour just persists. The reaction mixture is allowed to stand overnight and the ether is then evaporated. The tetrabromo-acid is crystallised from pet. ether, (b.p. 80-100°), any resinous material being rejected. It is identified by its melting point and mixed melting point with an authentic sample.

(3) Analytical Results for Synthetic Mixtures

Each step in the analytical process was carefully checked by the separation of synthetic mixtures prepared from pure acids. A selection of results is given below. In all cases chromatographic recoveries were 100±3% and the agreement between observed and calculated results is satisfactory.

(a) Isolation of Epoxy Acids.

Because of the reactivity of the oxirane group, epoxy acids have generally been converted to dihydroxy acids prior to alkaline hydrolysis of the glycerides[14]. The presence of a dihydroxy acid in the oil is then demonstrated by the difference between the mole. % dihydroxy acid present after ring opening of the epoxide and the mole. % of the epoxy acid[15]. The method suffers from two disadvantages; (i) Should the quantity of dihydroxy acid be small compared with the amount of epoxy acid present, then the accuracy of the difference between the glycol and epoxide determinations may be seriously affected by experimental errors and (ii) any glycerol or α- monoglyceride present records as dihydroxy acid.

These difficulties can, however, be overcome by using the chromatographic technique already described, since it has been shown that when mixed acids are separated with an acetylated castor oil column, epoxy and dihydroxy acids are well resolved, (Table 10). The usual hydrolytic procedure for the glycerides can not be used since mineral acid opens any oxirane rings which are present. Methods for the isolation of epoxy acids have been described recently by Hopkins and Chisholm[16] and Smith et al[17] using a cold alkaline hydrolysis followed by acidification to pH 4 - 5 and immediate extraction. In the present work a cold hydrolysis is followed by liberation of the fatty acids with an ion exchange resin (p 63).

A sample of epoxy-stearic acid (m.p. 55.5-56°) was put through

the saponification and recovery procedure. The product was recovered in 96% yield, had a melting point of 56° and gave a single peak corresponding to epoxy-stearic acid in a chromatogram using an acetylated castor oil column and a solvent system of 55, 61 and 75% acetone. The experiment shows that no ring opening takes place during the isolation of the epoxy acid.

(b) Hydrogenation Procedure. (p 63).

Chromatographic analysis of oleic and linoleic acids after hydrogenation indicated less than ½% of residual unsaturated material, thus the reduction procedure was considered to be quantitative. Analysis of a mixture of oleic (43%) and palmitic acids (57%) after hydrogenation gave results of:- stearic acid 44% and palmitic acid (56%). The analytical results for a more complicated mixture, found by chromatography before and after hydrogenation, are shown below:-

ACID	12:0	14:0	16:0	16:1	18:0	18:1	18:2	?
Calc. %	4.4	8.4	23.9	6.2	15.9	22.6	18.6	0
obs. %	4.5	8.4	25.1	6.2	15.8	21.8	17.1	1.1

The results were obtained by hydrogenation of material recovered from the eluates of the chromatogram of the mixed acids - a procedure which is not now recommended. The poor results for palmitic and oleic acids are probably related to a poor recovery of these acids from the eluate fraction. The 1.1% of an unknown component is possibly some product formed from the deterioration of the linoleic acid.

(c) Ozonolysis Procedure, (p 64).

Chromatographic analysis of the unsaturated acids oleic and linoleic after ozonolysis indicated that they are completely degraded by the reagent. The results of two synthetic mixtures given below indicate that there is no degradation of the saturated acids during the ozonolysis.

ACID	12:0	14:0	16:0	18:0	18:1	18:2	MIXTURE
Calc. %	-	18.8	-	19.3	61.9	-	1
obs. %	-	18.4	-	19.0	0	-	
Calc. %	31.8	-	18.0	-	-	60.2	2
obs. %	21.4	-	18.4	-	-	0	

A chromatographic analysis of mixed acids after ozonolysis records only the saturated acids present.

(d) Reduction of Epoxy Acids.

The catalytic reduction of epoxy acids has been studied by Mack and Bickford[18] and Figulevskii and Rubashko[19]. These workers consider that a unidirectional opening of the oxirane ring occurs during hydrogenation to give a monohydroxy saturated acid. Later work by Jullietti et al[20], however, seems to indicate that reductive fission of an oxirane ring produces a mixture of the two possible monohydroxy acids.

During the present work a sample of epoxystearic acid was reduced in ethanol at 30° for 24 hours with a 20% palladium charcoal catalyst in an atmosphere of hydrogen. Chromatographic analysis of the product (m.p. 60-71°) indicated 14% stearic acid

and 86% monohydroxystearic acid.

This complete loss of oxygen from epoxy acids during catalytic reduction has not previously been reported.

It is therefore apparent that a chromatogram of the hydrogenated mixed acids from an oil containing an epoxy acid will give false results. The mole. % of epoxy acid will be underestimated whilst the mole. % of the corresponding fully saturated acid will be overestimated. These difficulties may be overcome if the epoxy acid is converted to its diacetoxy derivative prior to hydrogenation (p64). The results from analysing a mixture of epoxystearic acid and oleic acid are given below. They confirm that no reduction of the diacetoxy group occurs during hydrogenation and that the procedure does not affect the unsaturated acids.

Synthetic mixture Calc: Epoxystearic : 81.5% oleic : 18.5%

obs: Diacetoxystearic :80.8% Stearic : 19.2%

Conversion of an epoxide to its corresponding glycol is unsatisfactory since, although the glycol group is stable towards hydrogenation, dihydroxy acids "tail" very badly during chromatography[2].

(4) Seed Oil Analysis

(a) Analysis of Gmelina asiatica (Linn) Seed Oil

This investigation was carried out on a sample of seeds obtained from Singapore. A second sample from India contained the same acids in very similar proportions.

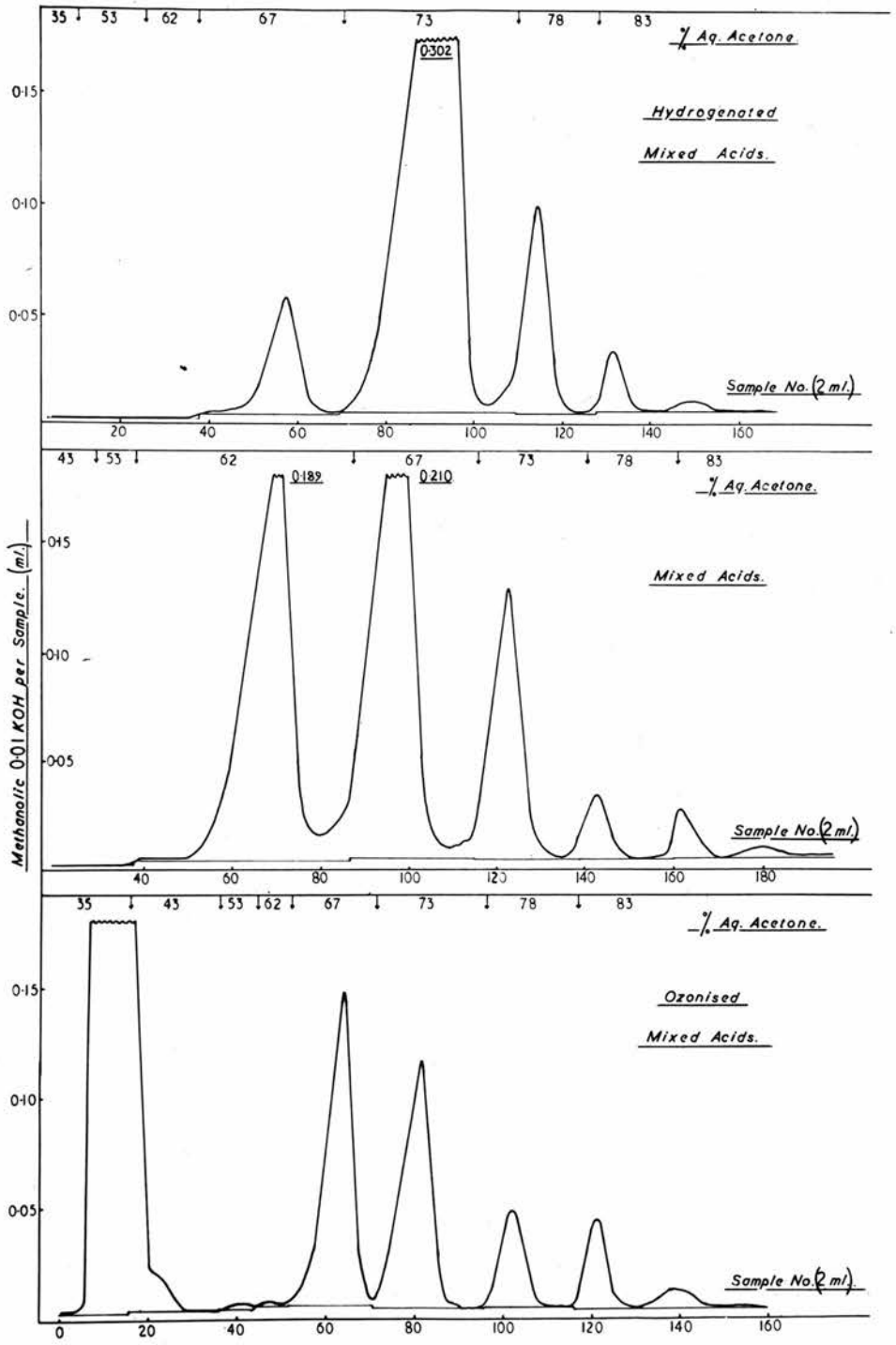

FIG 11. *Gmelina asiatica* Acids

The seeds (43.6g.), of average weight 0.28g. were shelled to give the kernels, (4.68g. 10.7%) which were extracted with light petroleum (b.p. 40/60°) in a Soxhlet extractor to give the oil (2.79g. 59.8% on the kernels). An infra-red spectrum of the oil indicated the absence of any hydroxy or epoxy acids.

The mixed acids were obtained as outlined on p 63 , and the unsaponifiable material determined, (2.8%). Chromatograms of the mixed acids (i) alone, (ii) after hydrogenation and (iii) after ozonolysis were run and these elution curves are shown in Fig. 11. By summing the titres under each peak, correcting for the small acidity of the developing solvent, and comparing with the total titre, it is possible to determine the mole. % of each acid group. These results are given in Table 12.

The figures in Table 12 provide a number of simultaneous equations from which the mole. % of each component acid may be calculated.

Table 12. Analysis of Gmelina Asiatica Mixed Acids.

GMELINA ASIATICA (LINN).

HYDROG.ACIDS(24.4mg.) S.E.200.0 Recov.99.5% Alkali $1.6877X10^{-2}$N

ACID	FR.No	Tot. Alk.	Blank	Alk. Cor.	Eq.$X10^{-5}$	Mol. %
16:0	59-66	0.571	0.140	0.431	0.75	8.8
18:0	70-105	5.898	0.170	3.728	6.32	75.7
20:0	104-122	0.730	0.108	0.622	1.05	12.6
22:0	127-137	0.196	0.078	0.120	0.20	2.4
24:0	144-154	0.102	0.077	0.025	0.042	0.5

MIX.ACIDS(28.5mg.) S.E.207.0 Recov.90.6% Alkali $1.694X10^{-2}$N

ACID.GRP.	FR.No	Tot. Alk.	Blank	Alk. Cor.	Eq.$X10^{-5}$	Mol. %
14:0	42-79	2.281	0.152	2.129	3.59	56.3
16:0	80-110	2.518	0.147	2.371	4.00	40.4
18:0	111-155	1.158	0.132	1.026	1.75	17.5
20:0	155-151	0.294	0.102	0.192	0.324	5.3
22:0	158-172	0.220	0.102	0.118	0.199	2.0
24:0	175-186	0.126	0.098	0.028	0.047	0.5

OZON.ACIDS(51.06mg.) S.E.207.0 Recov.22.1% Alkali $1.694X10^{-2}$N

ACID	FR.No	Tot. Alk.	Blank	Alk. Cor.	Eq.$X10^{-5}$	Mol. %
16:0	51-70	1.061	0.120	0.941	1.59	8.8
18:0	71.91	0.977	0.105	0.872	1.48	8.2
20:0	95-111	0.365	0.091	0.274	0.464	2.6
22:0	116-130	0.296	0.090	0.206	0.349	1.9
24:0	132-147	0.160	0.096	0.064	0.108	0.6

Total alkali, alkali blank and corrected alkali are given in ml.

Calculation of Results

Hydrogenated Acids.

12:0	+	12:1	-		-	-	=	0%	- (1)
14:0	+	14:1	-		-	-	=	0	- (2)
16:0	+	16:1	+	16:2	-	-	=	8.8	- (3)
18:0	+	18:1	+	18:2	+	18:3	=	75.7	- (4)
20:0	+	20:1	+	20:2	-	-	=	12.6	- (5)
22:0	+	22:1	+	22:2	-	-	=	2.4	- (6)
24:0	+	24:1	+	24:2	-	-	=	0.5	- (7)

Mixed Acids.

12:0	+	14:1	+	16:2	+	18:3	=	0%	- (8)
14:0	+	16:1	+	18:2	+	20:3	=	36.3	- (9)
16:0	+	18:1	+	20:2	+	22:3	=	40.4	- (10)
18:0	+	20:1	+	22:2	+	24:3	=	17.5	- (11)
20:0	+	22:1	+	24:2	-	-	=	3.3	- (12)
22:0	+	24:1	+	26:2	-	-	=	2.0	- (13)
24:0	+	26:1	-		-	-	=	0.5	- (14)

Ozonised Acids.

12:0	=	0	- (15)
14:0	=	0	- (16)
16:0	=	8.8	- (17)
18:0	=	8.2	- (18)
20:0	=	2.6	- (19)
22:0	=	1.9	- (20)
24:0	=	0.6	- (21)

Results. (After each solution is given the equation number from which it is derived).

12:0	=	0	(1;15)	20:0	=	2.6	(19)
14:0	=	0	(16)	20:1	=	9.3	(11)
14:1	=	0	(2)	20:2	=	0.7	(5)
16:0	=	8.8	(17)	22:0	=	1.9	(20) or 2.0 (15)
16:1	=	0	(3)	22:1	=	0.7	(12)
16:2	=	0	(5)	22:2	=	0	(6)
18:0	=	8.2	(18)	24:0	=	0.5	(21)
18:1	=	30.9	(10)	24:1	=	0	(7)
18:2	=	36.3	(9)	26:1	=	0	(14)
18:3	=	0	(8)	26:2	=	0	(13)

Equation (4) has not yet been used in the above solution and may therefore be used as a check on the general accuracy of the calculations.

18:0 + 18:1 + 18:2 + 18:3 = 75.4% Equation (4) = 75.7%

Note on Solution of Equations.

It may sometimes be necessary to assume that an acid, which is not likely to be present, is in fact absent. As in the above case, there is frequently one equation which is not used to obtain the results and this provides a check. The agreement at this check is generally good, but not exact, and some minor modification of the results may be made to obtain the best possible agreement.

Weight % of Gnelina Asiatica Acids. (Excluding Nonsaponifiables)

Palmitic	7.9%	Eicosenoic	10.1%
Stearic	3.2	Eicosadienoic	0.8
Oleic	30.6	Behenic	2.4
Linoleic	33.7	Docosenoic	0.8
Arachidic	2.9	Lignoceric	0.6

From the weight % of the mixture the saponification equivalent and iodine value may be calculated and compared with the measured values.

Saponification Equivalent:- Obs. 287.0 Iodine Value:- Obs. 104.2

Calc. 285.2 Calc. 102.0

The agreement between these figures is considered to be satisfactory.

The C_{18} monoethenoid acid, isolated from fractions 80 - 111 of the mixed acid chromatogram was shown to be oleic acid by characterisation as erythro-9:10-dihydroxystearic acid. (m.p. 129-130° ; mixed m.p. 129-130°). The C_{20} monoethenoid acid, isolated from fractions 111 - 133 of the mixed acid chromatogram was shown to be eicos-cis-11-enoic acid by conversion to erythro-11:12-dihydroxyarachidic acid. (m.p. 129-130° mixed mp. 130-131°.) The C_{18} diethenoid acid, isolated from fractions 42 - 79 of the mixed acid chromatogram was converted to 9:10:12:13:tetrabromostearic acid, (m.p. 113-115° : mixed m.p. 113 - 114°) and was thus shown to be linoleic acid.

The only detailed report of this oil is by Aggarwal and Soni[21] who found it to contain palmitic (9.6%), stearic (19.7%), oleic (33.6%) linoleic (25.8%) and ricinoleic acids. (11.3%) The present study gives very different results; there is no evidence of any hydroxy acid and, in addition to the usual C_{16} and C_{18} acids, there are several C_{20} and C_{22} acids, (total 17%). In this respect it is alsodifferent from Teak nut oil[22], the only other verbenaceae seed fat to have been examined in detail, for this contains only the usual range of C_{16} and C_{18} acids.

(b) Analysis of Cephalocroton Peuschelii (Pax.) Seed Oil.

The investigation was carried out on a sample of seeds from Pretoria. The seeds (29.81g.) of average weight 0.078g. were extracted with light petroleum (b.p. 40/60°) in a Soxhlet

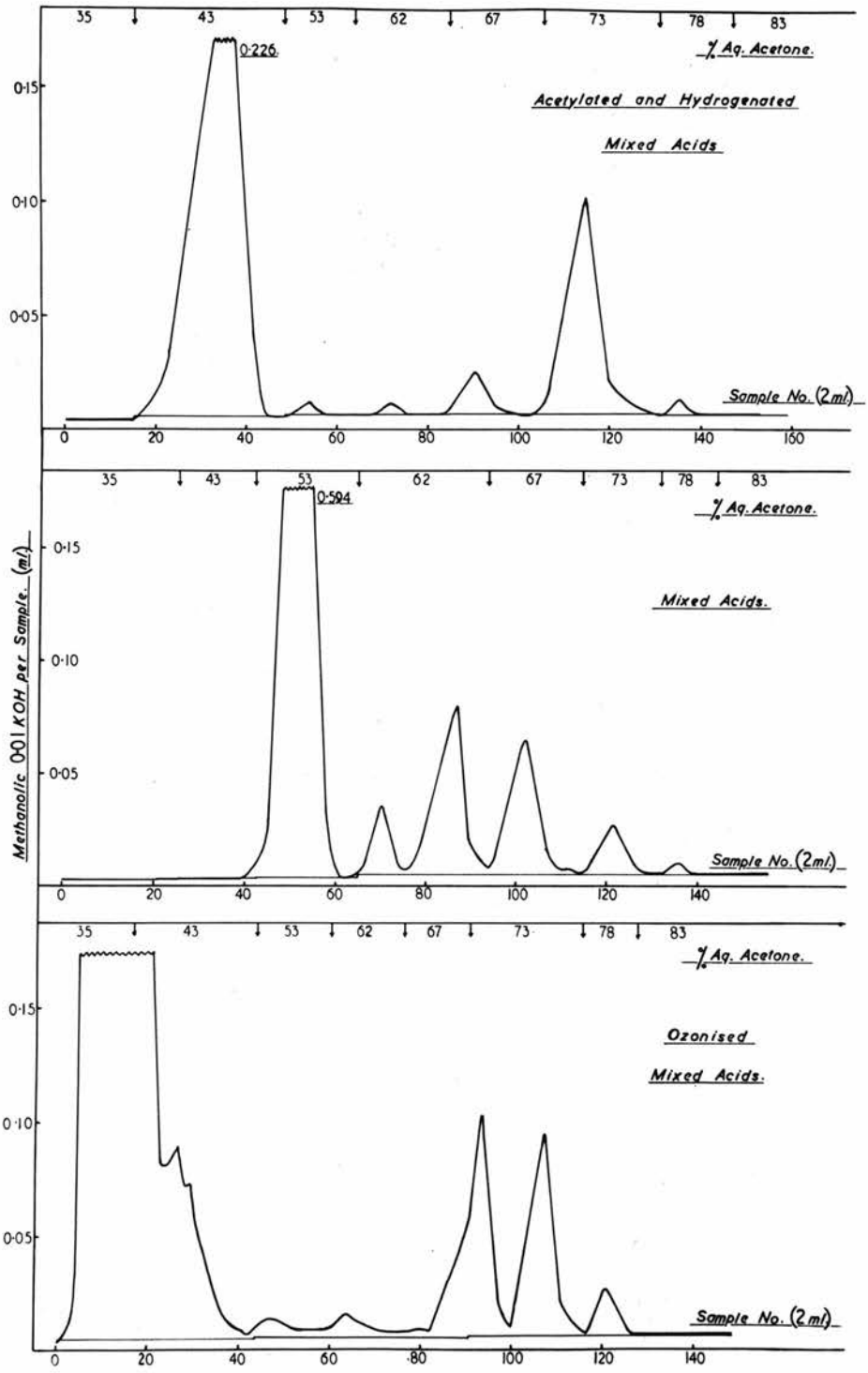

FIG 12. Cephalocroton Peuschelli Acids.

extractor to give a pale yellow oil, (8.82g. 29.6%) which was solid at 0°. An infra red spectrum of the oil showed peaks characteristic of the cis-epoxide group at 11.85 and 12.17 $m\mu$ [25]. An epoxide value[7] measured on the oil indicated 74.1% of epoxy-oleic glyceride.

The mixed acids were obtained as described on p 63 and the unsaponifiable matter (1.5%) removed. An epoxide value carried out on the mixed acids indicated 75.3% of epoxyoleic acid. The value indicates that there has been no ring opening of the epoxide during hydrolysis of the oil and recovery of the mixed acids. The mixed acids were chromatographed (i) alone, (ii) after hydrogenation and (iii) after ozonolysis to give the eluate curves shown in Fig. 12 and the results shown in Table 13.

Note on Fig. 12

The chromatogram of the mixed acids indicates two components eluted with the 62% acetone, which must be a partial resolution of myristic and linoleic acids. (Fig. 6 indicates that this might be possible under ideal conditions).

The results from the three paraffin chromatograms give the amounts of the usual saturated and unsaturated acids by a calculation similar to that for the Gmelins acids and indicate the presence (71%) of one or more oxygenated acids. The agreement between this and the measured epoxide value and the eluting solvent (55%) required for the original acid compared with the solvent (45%) required for the acid after acetylation and hydrogenation, suggests one or more mono-epoxy acids. (Table 10). The acid present is mainly

cis-12:13-epoxyoleic , since threo-12:13-dihydroxyoleic acid (m.p. 54 - 55°)[24] could readily be isolated in good yield from the seed oil after acetolysis and hydrolysis. The calculated iodine value and saponification equivalent provide further evidence of this, though they do not preclude the presence of small amounts of epoxy acids differing in chain length and/or degree of unsaturation. (Epoxy acids, unlike some hydroxy acids,[24] do not interfere with the measurement of the iodine value (Wijs); as confirmation of this the iodine value of epoxystearic acid was shown to be nil.)

The chromatographic behaviour of the acid on paraffin and acetylated castor oil columns both before and after acetylation provides further evidence that it is entirely epoxyoleic acid. The oxygenated acid is eluted from the acetylated castor oil column with 75% acetone before and after acetylation. In all cases a symmetrical elution peak is obtained giving the same value (71-72%). Although there is no evidence at the moment of how other possible epoxy acids would behave, it is likely that a mixture of two such acids differing in chain length and/or unsaturation would lead to distorted elution curves.

The difference in values between the hydrogenated 16:0 group and the 16:0 group from oxonolysis would imply a o.3% of palmitoleic acid. This value is on the border-line of experimental errors and it is therefore disregarded in order to obtain a better fit on the C_{18} figures. The C_{18} group calculated = 24.4% and the observed value is 23.3%.

Table 15. Analysis of Cephalocroton neuschilii Mixed Acids.

CEPHALOCROTON NEUSCHELKI (PAX).

HYDROC/ACET.ACIDS(21.75mg.) S.E.362.1 Rec.97.8% Alk.=1.712X10^{-2}N

ACID GRP.	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.X10^{-5}	Mol. %
OXY	15-45	2.611	0.155	2.456	4.295	71.6
12:0	49-57	0.070	0.054	0.016	0.027	0.5
14:0	60-76	0.071	0.054	0.017	0.029	0.5
16:0	84-101	0.232	0.108	0.124	0.212	5.6
18:0	104-130	0.959	0.162	0.797	1.364	25.2
20:0	133-139	0.064	0.042	0.022	0.038	0.6

MIX.ACIDS(23.55mg.) S.E.291.8 Rec.98.7% Alk.=1.712X10^{-2}N

ACID GRP.	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.X10^{-5}	Mol. %
12:0+OXY	40-61	3.396	0.085	3.311	5.668	71.2
14:0	63-94	0.880	0.160	0.720	1.233	15.5
16:0	95-114	0.571	0.100	0.471	0.806	10.1
18:0	115-150	0.227	0.096	0.131	0.224	2.8
20:0	152-159	0.066	0.048	0.018	0.031	0.4

OZON.ACIDS(102.4mg.) S.E.291.8 Rec.7.2% Alk.=1.711X10^{-2}N

ACID	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.X10^{-5}	Mol. %
12:0	48-55	0.134	0.063	0.071	0.121	0.5
14:0	56-75	0.190	0.090	0.100	0.171	0.5
16:0	74-100	0.826	0.145	0.681	1.165	5.5
18:0	101-116	0.655	0.096	0.559	0.922	2.6
20:0	117-127	0.176	0.077	0.099	0.169	0.5

Total alkali, alkali blank and corrected alkali are given in ml.

Composition of Cephalocroton peuschelii Acids, (Excluding Non-saponifiable)

	CEPHALOCROTON PEUSCHELII		C. Cordofanus[15]
ACID	MOLE. %	WEIGHT %	WEIGHT %
Lauric	0.3	0.2	-
Myristic	0.5	0.4	-
Palmitic	3.3	2.9	3.9
Stearic	2.6	2.5	2.8
Oleic	6.8	6.6	9.8
Linoleic	15.0	14.5	17.1
Arachidic	0.5	0.5	0.7
Epoxyoleic	71.0	72.4	62.0
Dihydroxyoleic	NIL	NIL	3.7

Satisfactory agreement is also obtained between the calculated saponification equivalent, iodine value, epoxide value and the observed values.

Saponification Equivalent	Observed	: 291.8
	Calculated	: 292.2
Iodine Value	Observed	: 93.96
	Calculated	: 94.15
Epoxide Value	Observed	: 73.3
	Calculated	: 72.4

The C_{18} diethenoid acid, isolated from fraction 65-90 of the mixed acids chromatogram was shown to be linoleic acid by conversion to 9:10:12:13 - tetrabromostearic acid (m.p. 110-111° mixed m.p. 111-112°). The C_{18} monoethenoid acid was present in too small a quantity (1.2mg.) to be identified by hydroxylation. It was reported as oleic acid.

Previous studies[15] have shown that Cephalocroton cordofanus seed oil contains 12:13 - epoxyoleic acid and a small amount of 12:13 - dihydroxyoleic acid. It is possible that the latter may have been formed from the epoxy acid during storage of the seeds or during investigation of the seed oil, though it is relevant that Tulloch[25] considers that 9:10 - dihydroxystearic acid and 9:10 - epoxystearic acid are both present in the oil of wheat stem rust. It was of interest therefore to examine other seed oils of the Cephalocroton genus and it is now apparant that C. peuschelii seed oil is very similar to that of C. cordofanus. The main difference between the two is that C. cordofanus may contain 12;13 - dihydroxyoleic acid which is definately absent from C. peuschelii seed oil.

(c) Analysis of Vernonia camporum (A. Cheval) Seed Oil

The investigation was carried out on a sample of seeds from Sierra Leone. The seeds (20.62g.) of average weight 0.016g. were extracted with light petroleum (b.p. 40/60°) in a Soxhlet extractor to give a yellow oil (1.725g. 8.4%) which solidified at 0°. An epoxide determination made on the oil indicated the absence of the oxirane group, which was confirmed by an infrared spectrum.

The mixed acids were obtained as described on p 63 and the unsaponifiable matter (2.5%) removed. The mixed acids were chromatographed (i) alone, (ii) after hydrogenation and (iii) after osonolysis to give the eluate curves shown in Fig. 13 and

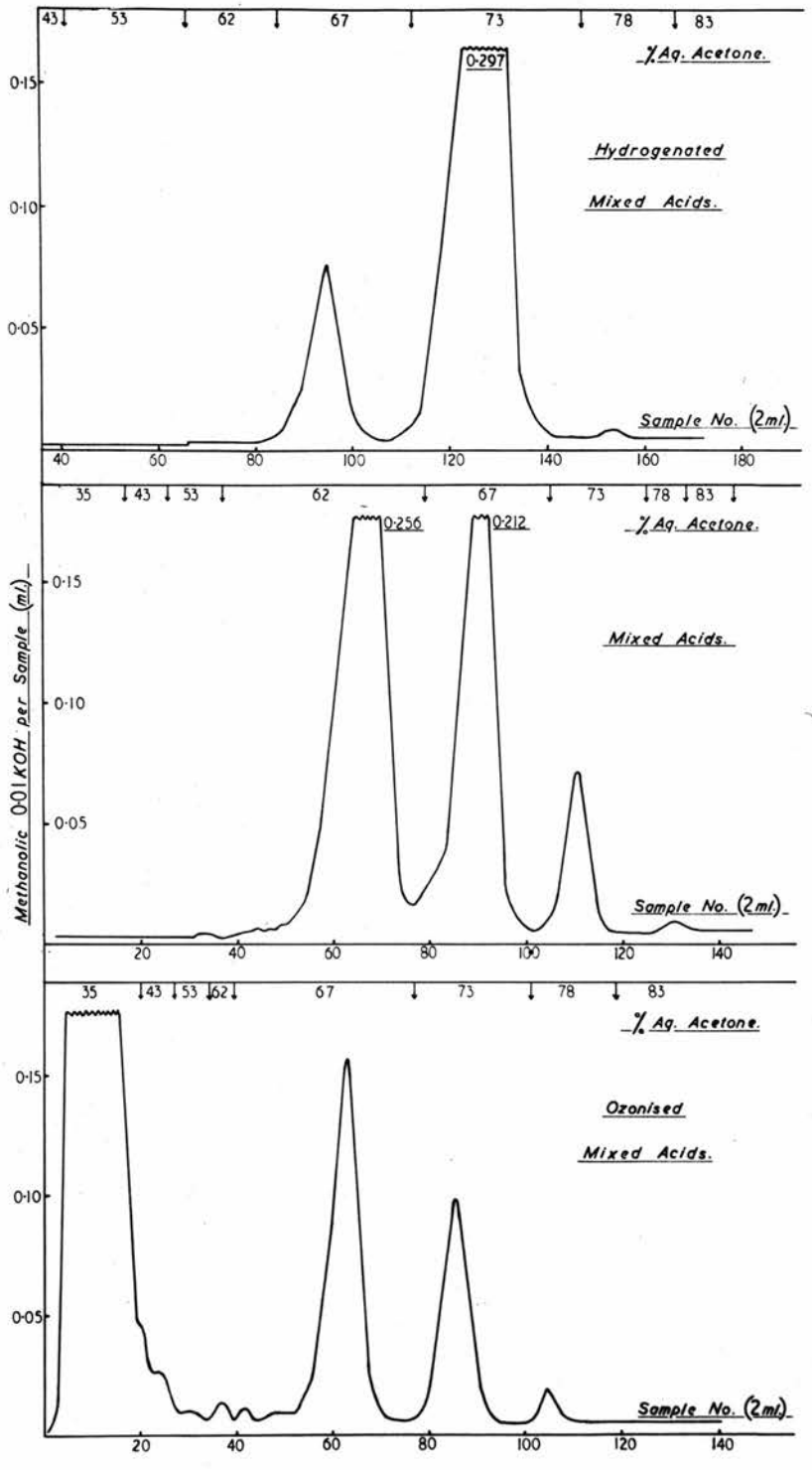

FIG 13. Vernonia Camporum Acids.

the results shown in Table 14.

Table 14. Analysis of Vernonia camporum Mixed Acids

VERNONIA CAMPORUM (A. Cheval)

HYDROG.ACIDS(17.81mg.) S.E.281.9 Rec.98.6% Alk. 1.6905×10^{-2} N

ACID	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.$\times 10^{-5}$	Mole. %
16:0	81-107	0.678	0.130	0.548	0.926	14.8
18:0	108-145	5.350	0.210	5.129	5.290	84.7
20:0	151-157	0.051	0.035	0.016	0.027	0.5

MIX.ACIDS(22.53mg.) S.E.277.6 Rec.98.6% Alk. 1.6917×10^{-2} N

ACID GRP.	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.$\times 10^{-5}$	Mole. %
12:0	26-32	0.034	0.028	0.006	0.010	0.1
14:0	43-72	2.764	0.180	2.584	4.371	54.6
16:0	73-97	1.870	0.150	1.720	2.910	36.4
18:0	98-116	0.404	0.101	0.303	0.685	8.5
20:0	122-131	0.077	0.050	0.027	0.046	0.6

OZON.ACIDS(34.75mg.) S.E.277.6 Rec.24.1% Alk. 1.6952×10^{-2} N

ACID	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.$\times 10^{-5}$	Mole. %
16:0	51-75	1.236	0.150	1.086	1.841	14.7
18:0	76-98	0.767	0.118	0.649	1.100	8.8
20:0	100-110	0.106	0.065	0.041	0.069	0.6

Total alkali, alkali blank and corrected alkali are given in ml.

The chromatographic evidence indicates the absence of any oxygenated acids. The simultaneous equations indicate a trace of palmitoleic (0.1%) and a trace of linolenic (0.1%) acids, these small quantities most likely arise from experimental errors and are therefore not included in the final results. A more serious discrepancy occurs in the C_{18} group: the stearic acid recorded by the ozonolysis chromatogram is 8.8%, yet from the mixed acids chromatogram the stearic acid is 8.3%. The latter value is taken since it provides the more consistent results and the best agreement between the calculated and observed saponification equivalent and iodine values.

Composition of Vernonia cammorum Acids (Excluding Nonsaponifiables)

ACID	MOLE. %	WEIGHT %
Palmitic	14.7	15.6
Stearic	8.3	8.5
Oleic	21.7	22.1
Linoleic	54.7	55.2
Arachidic	0.6	0.6

The C_{18} group is calculated as 84.7% and is found to be 84.7%

Saponification Equivalent: Observed : 277.6

Calculated : 277.8

Iodine Value: Observed : 118.7

Calculated : 119.8

Concentration of the C_{18} diethenoid and C_{18} monoethenoid acids

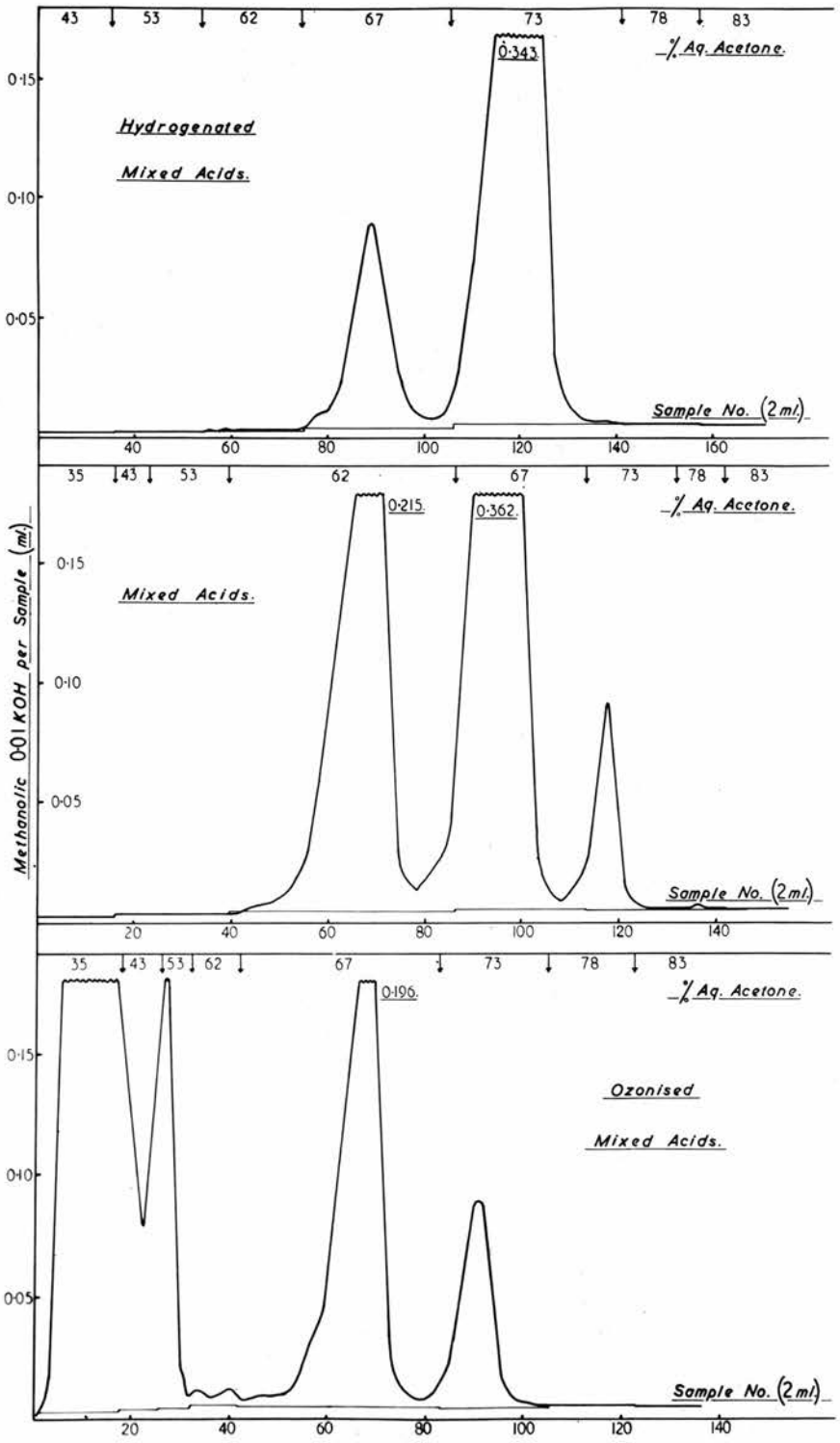

FIG 14. *Jatropha curcas* Acids.

were obtained from fractions 50-70 and 75-95 respectively of the mixed acids chromatogram. 9:10:12:13 - Tetrabromostearic acid was prepared from the diethenoid acid (m.p. 112-115°, mixed m.p. 112-115°), and the mono-ethenoid acid was converted to erythro - 9:10 - dihydroxy-stearic acid (m.p. 129-130° mixed m.p. 130-131°). The acids, therefore are linoleic and oleic acids respectively.

Six Vernonia seed oils have previously been examined, two of these, V. anthelmintica[14] and V. colorata[15] contain high proportions of epoxyoleic acid, whilst the remaining seeds[26] contain little or none of this acid. It is therefore desirable to investigate other seed oils of this genus to see how general is the occurence of this acid. The above analytical results for V. camporum clearly indicate that this genus produces no epoxy acid in its oil.

(d) Analysis of Jatropha curcas (Linn) Seed Oil

The investigation was carried out on a sample of seeds from Ibadan. The seeds (78.5g.), of average weight 0.785g. were shelled to give the kernels (51.5g. 65.6%) which were extracted with light petroleum (b.p. 40/60°) in a Soxhlet extractor to give the oil (35.37g.) 40% on the kernels). An infra-red spectrum of the oil indicated the absence of any hydroxy or epoxy acids.

The mixed acids were obtained as outlined on p63 and the unsaponifiable material removed (0.5%). Chromatograms of the mixed acids (i) alone, (ii) after hydrogenation and (iii) after ozonolysis gave the elution curves shown in Fig. 14 and the results shown in Table 15.

Table 15. Analysis of Jatropha curcas Mixed Acids

JATROPHA CURCAS (LINN).

HYDROG.ACIDS(21.18mg.) S.E.281.0 Rec.99.2% Alk. 1.6830X10^{-2}N

ACID	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.X10^{-5}	Mole. %
16:0	74-102	0.904	0.116	0.788	1.55	17.8
18:0	105-140	3.874	0.220	3.654	6.15	82.2

MIX.ACIDS(32.16mg.) S.E.277.0 Rec.98.7% Alk. 1.687X10^{-2}N

ACID GRP	FN.No.	Tot. Alk.	Blank	Alk. Cor.	Eq.X10^{-5}	Mole. %
14:0	42-78	2.649	0.185	2.464	4.157	56.5
16:0	79-108	4.067	0.172	3.895	6.571	37.5
18:0	109-127	0.549	0.114	0.435	0.734	6.4

OZON.ACIDS(48.95mg.) S.E.277.0 Rec.24.1% Alk. 1.688X10^{-2}N

ACID	FN.No	Tot. Alk.	Blank	Alk. Cor.	Eq.X10^{-5}	Mole. %
16:0	51-79	1.993	0.145	1.848	3.12	17.7
18:0	80-102	0.784	0.115	0.669	1.13	6.4

The chromatographic evidence indicates the absence of any oxygenated acids. The simultaneous equations indicate a trace (0.1%) of palmitoleic acid, this figure probably arises from experimental errors and is therefore neglected. The C_{18} group is calculated as 82.3% and is found to be 82.2% from the chromatogram of the hydrogenated acids.

Saponification Equivalent:	Observed	: 277.0
	Calculated	: 277.3
Iodine Value	Observed	: 102.9
	Calculated	: 102.6

The agreement between the saponification equivalent and the iodine value calculated from the weight % of the mixed acids and the observed values is remarkably good for this simple mixture.

Table 16. Composition of Jatropha curcas Seed Oil

	REFERENCES.						PRESENT WORK	
ACID	28	29	30	32	33	34	Mole.%	Weight %
Myristic	5.2*	-	-	1	1	-	-	-
Palmitic	4.8	14	14	12	16	17	17.7	16.4
Stearic	1.9	-	10	5	10	6	6.4	6.6
Oleic	16.7	36	55	65	41	37	39.6	40.3
Linoleic	26.1	50	25	19	32	40	36.3	36.7
Ricinoleic	12.9	-	-	-	-	-	NIL	NIL

* Also contains : Caproic 1.5%, lauric 8.9%, palmitoleic 15.7% and linolenic 8.0%.

Concentrates of the C_{18} diethenoid and C_{18} monoethenoid acids were obtained from fractions 52-74 and 80-104 respectively from the chromatogram of the mixed acids 9:10:12:13-tetrabromostearic acid (m.p.113-114, mixed m.p. 114°) was prepared from the diethenoid acid and *erythro* - 9:10-dihydroxystearic acid (m.p. 128-130°, mixed m.p. 130-131°) was prepared from the monoethenoid acid. The acids were therefore linoleic and oleic respectively.

This seed oil has been the subject of several investigations (Table 16). Two reports[27,28] suggest that a hydroxy acid is present whilst several others claim that acids of this type are absent[29-35]. Investigation by reversed phase chromatography has also shown that hydroxy acids are absent from the sample under study.

(5) General Comments.

Although reversed phase chromatography is being used more widely as paper chromatography, its use in columns provides a convenient and accurate method for the quantitative study of mixtures of saturated acids. This has been extended to include unsaturated acids by the use of oxidation and hydrogenation procedures and also oxygenated acids by the use of other stationary phases. By using ozone instead of permanganate as oxidising agent and by hydrogenating the mixed acids rather than acids recovered from other chromatograms the disadvantages of earlier methods are overcome. By suitable attention to details and careful choice of the correct eluting solvents it is possible to get good resolution between subsequent fractions and consistently high recoveries of acids from the columns. The satisfactory results obtained with model mixtures and the excellent agreement between calculated and observed iodine values and saponification equivalents (better than 1%) also indicate the general accuracy of the results. It is considered that the results reported herein are most probably correct to within $\pm 0.5\%$ on each component; an accuracy which compares favourably with the ester fractionation technique[36,37]. The agreement between iodine values is considerably

better than some recently reported for analysis by gas/liquid chromatography[38,39,40] where discrepancies of 2-4% occurred between the calculated and observed values.

Though not as simple to operate as gas/liquid chromatography the techniques here described may have some advantages in particular circumstances. The analysis is effected under very mild conditions, it is easily carried out on quantities sufficient for further investigation, it requires no elaborate or expensive equipment and it may be more easily adaptable to the less volatile oxygenated acids, which have rather long retention times during gas chromatography.

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PART IV.

GAS/LIQUID CHROMATOGRAPHY

(1) Introduction.

Gas/liquid chromatography is an extension of liquid/liquid chromatography and is closely analogous to it in principle. Fatty acid esters which are volatile at the temperature of the chromatographic column are partitioned between a moving gaseous phase and a stationary liquid phase held on an inert support. Differences in the distribution coefficients cause a separation between the volatile components which consequently move through the column at different rates. By means of a suitable detection system the concentration of eluted material in the effluent gas is plotted against time. A brief review of some chromatographic systems and their application to the analysis of fatty acids has already been given (p.11).

(2) The Pye Argon Chromatograph.

This instrument incorporates an argon ionisation detector designed by Lovelock[1]. The β-ray ionisation detector has a high degree of sensitivity to organic molecules yet is relatively insensitive to minor changes in temperature, pressure and argon flow-rate. During operation, argon flows through the chamber of the detector and radiation from a radioactive source (Radium D.) partially ionises the gas and produces a steady and stable ionisation current when a voltage is applied across the chamber. In addition to the ionised argon molecules excited (but not ionised) argon atoms are also

formed whose concentration depends on the strength of the source and the applied voltage. Most organic molecules have ionisation potentials less than the excitation potential of an argon atom (11.6 ev.) and will, on entering the chamber, accept sufficient energy by collision with the metastable argon atoms, to lead to ionisation. The resulting increase in ionisation current is then amplified and recorded.

Commercial argon (99.95% pure) is used as the moving phase. This supply contains sufficient water to affect the sensitivity of the detector and is therefore dried by passing through a trap cooled by solid carbon dioxide. The pressure of argon is regulated by a British Oxygen BOR. 12 valve and is supplied to the top of the column via ¼" diameter copper tubing and a spring loaded pyrex tap. The latter is required for closing the argon supply during the loading of the column. The argon flow-rate from the detector is measured by means of a soap-bubble flow meter.

The columns are constructed from pyrex glass and are 4' long and 4 mm. internal diameter, constricted at the lower end and fitted with a B 7 cone and socket. A leak-free seal is made into the detector by means of a B 7 tapered silicone rubber seat.

The temperature of the column and detector is held constant throughout any chromatographic run by placing both within an aluminium heat reservoir bar, heated by an iso-jacket. The temperature control system consists of a mercury-in-glass thermometer with platinum inserts which activates a transistorised relay circuit. The relay, in turn,

operates the electrical heaters. It was found that at 200° the control allowed a fluctuation of ± 2°, and it was consequently replaced by a Variac transformer when steady temperatures were required.

The high voltage supply enables voltages of 750 to 2000 v. in 250 v. intervals to be applied to the detector cell, and the amplified ionisation current is displayed on a Sunvic 10 mv. potentiometric recorder. The steady ionisation current due to pure argon in the detector is balanced electrically to give a zero base line on the recorder at 5% full scale deflection. A sensitivity switch (X1:3:10) provides the means of increasing the output of the amplifier to the recorder.

Samples are loaded on to the column by means of a micropipette (0.1, 0.05 and 0.025 μ l.) which is constructed from precision bore capilliary tubing and is filled by capilliary attraction. The flow of argon is interrupted and when the pressure has fallen to about 5 p.s.i. the column is opened at the B.7 joint. The loaded pipette is discharged on to a pad of glass yarn at the head of the column packing and then withdrawn. The column head is closed and the argon flow re-established..

(5) The Chromatographic Column.

The success of a chromatographic separation depends ultimately upon the efficiency of the column, thus the procedure for column preparation will be described in full. Columns are prepared from Apeezon L grease and celite 545 (Mesh No. 80-100) and a column con-

taining 10% grease is found convenient for most analyses.

(a) Preparation of Materials.

Celite 545 is alkali treated according to the method of James, Martin and Smith[2], with 50% methanolic sodium hydroxide. The product is washed free of alkali with methanol and dried at 110°.

Apiezon L grease (2.00g.) is dissolved in light petroleum (500 ml. b.p. 40/60°) and added to celite 545 (18.00g.). The petrol is evaporated whilst agitating the mixture. It is inadvisable to swirl the mixture since this results in excessive production of fines, which adversely effect the flow rate of carrier gas through the column with consequent lowering of the efficiency. Final evaporation of the petrol is carried out at 100° under a vacuum of 0.1 mm. for 4 hrs. The final product is a dry, pale yellow, powder. 5% and 20% Apiezon L grease columns were also prepared by suitably adjusting the ratio of grease to celite.

(b) Column Packing

About 2" of glass tape are inserted to form a wad at the bottom of a 4' column which is then filled with the coated celite and packed by vibration. Vibration is obtained by holding the glass column firmly against an eight-sided, 1 cm. diameter, Tufnol rod which is rotated at 5000 r.p.m.. A fine stream of coated celite is poured into the column and vibration is started at the base of the column and proceeds towards the top, alongside the ascending level of celite. The column is filled to within 5" of the top B 7 joint and a wad of glass tape is placed on top of the packing. A column packed

in this way contains about 5g. of coated celite and requires a pressure of 15 p.s.i. to produce an argon flow rate of between 30-35 ml. per min.. Each column is labelled and a record kept of its history and performance.

(c) Column Conditioning.

A certain quantity of stationary phase inevitably "bleeds" from a new column when it is heated to the temperature used for chromatography. It is thus necessary to allow the column to remain slightly above its maximum specified operating temperature with argon passing through to remove any low boiling materials. This must not be carried out in the machine but in an auxilliary heater. A pyrex tube (4.5ft by 1 cm.) wound with nichrome tape and placed inside a 2" diameter pyrex tube was found to be satisfactory for the purpose. The chromatographic column is placed inside the inner tube which is heated by means of a Variac transformer.

Apiezon columns are conditioned by passing argon through at a slow rate (10ml. per min.) for 12 hrs. at 220°. Replacing the argon with nitrogen was found to be unsatisfactory, perhaps due to the small content of oxygen in this gas. When not in use, the columns are stoppered at both ends and stored at room temperature.

(d) Column Efficiency.

The number of theoretical plates with which a column chromatographs a particular substance can be calculated from formula (1), (Desty[3]).

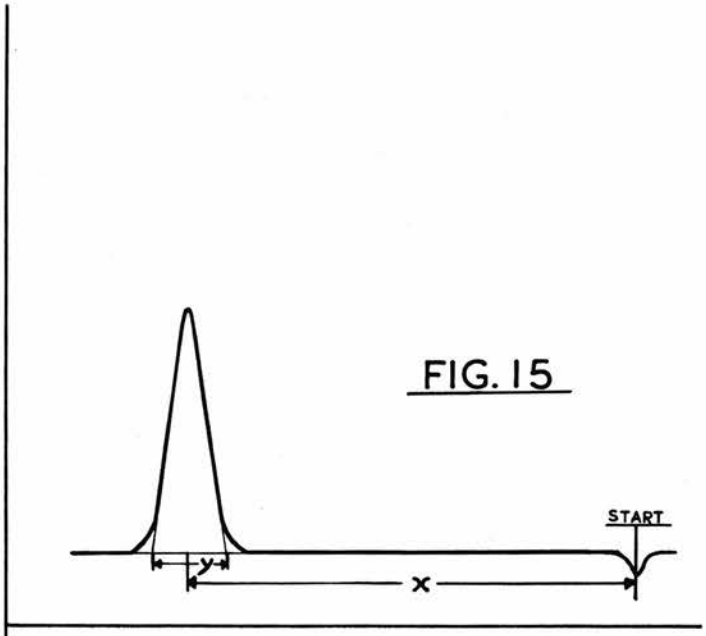

START
y
x

FIG. 15

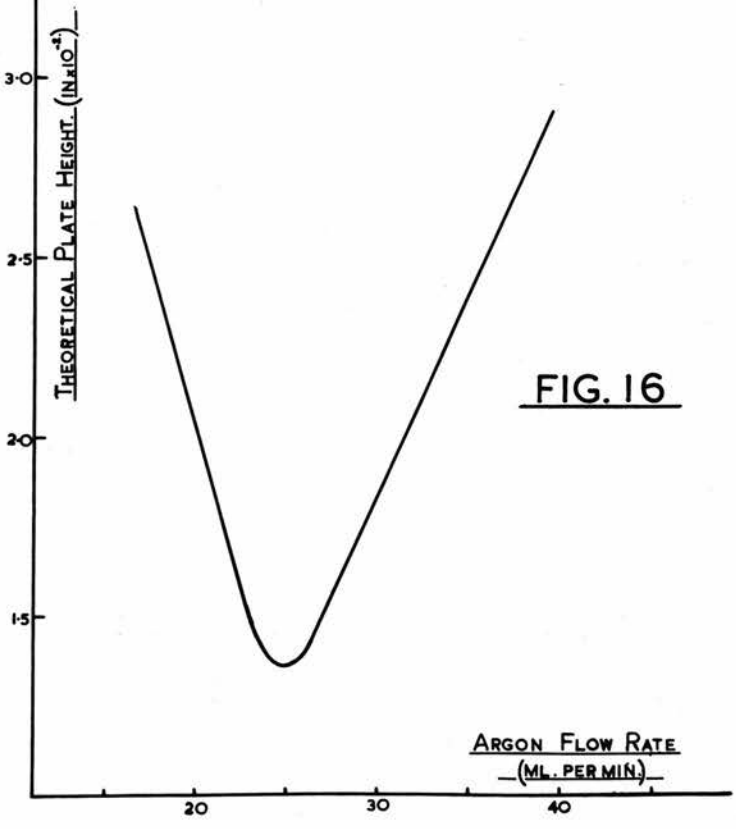

THEORETICAL PLATE HEIGHT. (IN x10⁻²)
3·0
2·5
2·0
1·5
ARGON FLOW RATE
(ML. PER MIN.)
20
30
40

FIG. 16

$$\text{No. of theoretical plates} = \frac{16x^2}{y^2} \text{ - - - - - (1).}$$

Tangents are drawn to the peak at the points of inflexions. The length of the base line cut by these two tangents (y) is measured and the length from the start of the run to the middle of this base line (x) is also determined. (Fig. 15).

The maximum efficiency of a column may be determined by making use of the rate theory of van Deemter, Zuiderweg and Klinkenberg[4]; equation (2).

$$\text{Height equivalent to a theoretical plate} = A + \frac{B}{u} + Cu \text{ - - - (2).}$$

where u = flow rate of a carrier gas.

The maximum effeciency attainable by a 10% Apiezon column was determined in the following manner:- Methyl myristate was chromatographed on a 4', 10% Apiezon column at 200°, with a load of 0.025 µl. and a detector voltage of 1000v. The chromatogram was repeated at five different flow rates, and the results are given in Table 17.

Table 17. Theoretical Plate Height Variation with Flow Rate

x.	y.	No.T.P.	H.E.T.P.	Argon Flow Rates
1.78in.	0.16in.	1980	2.42×10^{-2}in	17.0 ml. per. min.
1.44	0.105	3007	1.60	22.2
1.28	0.09	3236	1.48	27.0
1.75	0.15	2128	2.35	35.3
1.48	0.15	1788	2.68	37.9

A plot of these results is shown in Fig. 16, and is the

expected shape for the van Deemter equation (2). The curve shows that the column efficiency increases with the time that a zone takes to pass through the column, up to point at which longtitudinal diffusion exerts an opposing effect.

Fig. 16 indicates that at a flow rate of 25 ml. per min. the theoretical platage of the column is at a maximum and is 3550. The value compares well with the range 3200-4000 reported for the chromatography of methyl myristate on this type of column by James[5]. For routine analysis a flow rate of 25 ml. per min. is more usually used. This means that the efficiency of the column is then only about 2200 theoretical plates but this lowered efficiency is quite sufficient to separate mixtures encountered in the present work. The column efficiency does not affect the relative position of the eluted zones, but only their width.

(4) Calibration for Qualitative Analysis.

Components of a mixture may be identified by their retention times (or volumes) relative to some internal standard, generally methyl myristate. These values are independant of gas flow rate and the quantity of liquid phase on the column provided that these remain constant during the determination. The values are also insensitive to small changes ($< 2^{\circ}$) in temperature. They are measured in the following manner:-

The straight sections of the peak sides are extrapolated to the base line and the distance from the midpoint of the intersected base line to that of the air peak, similarly measured, is determined.

The air peak is negative and follows a depression in the base line due to interuption of the argon supply. If the air peak is not fully recorded, then an alternative method of calculation has been given by Peterson and Hirsch[6]. The distance of each peak is then expressed relative to methyl myristate.

The straight chain mono-basic acids $C_6 - C_{10}$, C_{12}, C_{14}, C_{16}, C_{18} and C_{20} and the dibasic acids $C_5 - C_6$, $C_8 - C_{10}$ were esterified by refluxing with dry methanolic hydrochloric acid (0.8N) for 2 hrs., and the esters recovered by ether extraction. Chromatography of various mixtures of these acids on Apiezon L columns at different temperatures gave the data in Table 18. Most determinations were made on a 20% column since this permitted more accurate measurements to be made for the retention times of the shorter chain acids. It was also verified that changing the weight of stationary phase to 10% or 5% did not alter the relative retention times as measured on a 20% column, but merely changed the absolute retention times. Chromatographic separations of the saturated monobasic esters, the dibasic esters, and the mono-basic and dibasic esters together are shown in Figs. 17, 18 and 19 respectively.

The separation factor $\triangle CH_2$ for a homologous series has been defined by James and Martin[7] as:-

$$\triangle CH_2 = \frac{\text{Retention volume of straight chain acid with n carbon atoms}}{\text{Retention volume of straight chain acid with } (n-1) \text{ carbon atoms.}}$$

The average value for $\triangle CH_2$ for both the mono and dibasic esters computed from the data in Table 16 is 1.54, which is just below the value of 1.56 quoted by James[5]

for the chromatographic separation of the straight chain monobasic esters on an Apiezon column at 197°.

Fig. 19 indicates that a dibasic acid with (n) carbon atoms emerges just before the monobasic acid with (n+5) carbon atoms which is in agreement with the findings of James and Webb[8]. The acids are sufficiently well resolved to make identification easy.

The relationship between $\log_{10}$ (time of emergence) and number of carbon atoms in the molecule is shown in Fig. 20. The graph is based on data taken from Table 18. Excellent straight lines are obtained for each homologous series.

Table 18. Relative Retention Times for Mono and Di-basic Esters

Monobasic	150° 1	200° 2	Dibasic	200° 2
C_5	0.096	-	C_4	0.050
C_6	0.166	0.035	C_5	0.076
C_7	0.302	0.051	C_6	0.115
C_8	0.550	0.079	C_7*	0.178
C_9	1.00	0.120	C_8	0.268
C_{10}	1.75	0.186	C_9	0.407
C_{11}*		0.282	C_{10}	0.63
C_{12}		0.422		
C_{13}*		0.661		
C_{14}		1.000		
C_{15}*		1.532		
C_{16}		2.351		
C_{18}		5.50		
C_{20}		12.80		

Notes to Table 18

*These acids appear as impurities in the acid mixtures.

1. Measured relative to methyl nonanoate.

2. Measured relative to methyl myristate.

3. The values represent the mean of five or six separate determinations with standard deviations ranging from 0.001 to 0.005.

FIG 17.

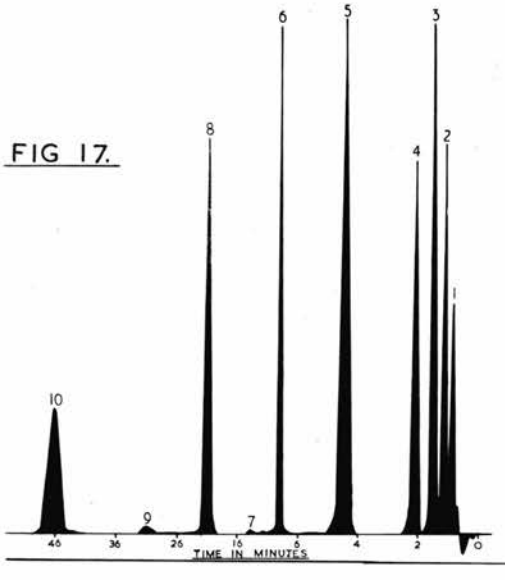


FIG 18.

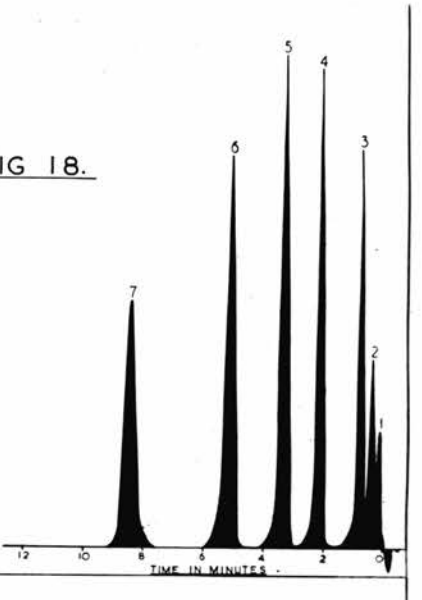
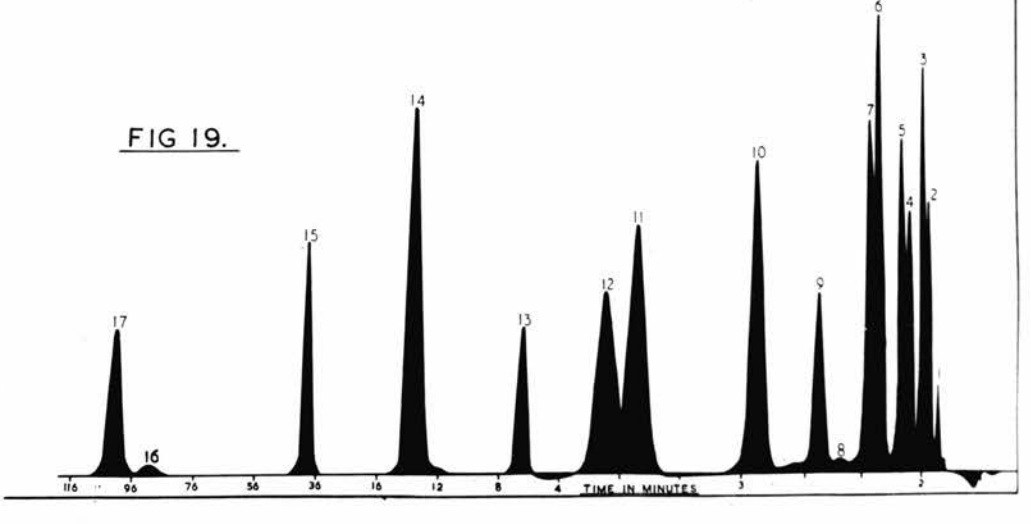


FIG 19.



Legends to Figures 17, 18 and 19

Fig. 17 Separation of monobasic esters on a 10% Apieson L column. Column length 4ft; temperature 200°; argon 34.1 ml. p. min. detector voltage 1000v; load 0.1. μl. Peaks in order of appearance; (1) methyl n-heptanoate, (2) methyl n-octanoate, (3) methyl n-nonanoate, (4) methyl n-decanoate, (5) methyl laurate, (6) methyl myristate, (7) methyl n-pentadecanoate, (8) methyl palmitate, (9) methyl n-heptadecanoate, (10) methyl stearate.

Fig. 18 Separation of dibasic esters on a 10% Apieson L column. Column length 4ft; temperature 200°; argon 34.1 ml. p. min. detector voltage 1000v; load 0.1 μl. Peaks in order of appearance; (1) dimethyl succinate, (2) dimethyl glutarate, (3) dimethyl adipate, (4) dimethyl suberate, (5) dimethyl azela-ate, (6) dimethyl sebacate, (7) methyl mysistate.

Fig. 19 Separation of mono and dibasic esters on a 20% Apieson L column. Column length 4ft: temperature 200°; argon 28.6 ml. p. min.; detector voltage 1000v; load 0.1 μl. Peaks in order of appearance (1) methyl n-hexanoate, (2) dimethyl succinate, (3) methyl n-heptanoate, (4) dimethyl glutarate, (5) methyl n-octanoate, (6) dimethyl adipate, (7) methyl n-nonanoate, (8) dimethyl pimelate, (9) methyl n-decanoate, (10) dimethyl suberate, (11) dimethyl azela-ate, (12) methyl laurate, (13) dimethyl sebacate, (14) methyl myristate, (15) methyl palmitate, (16) methyl oleate, (17) methyl stearate.

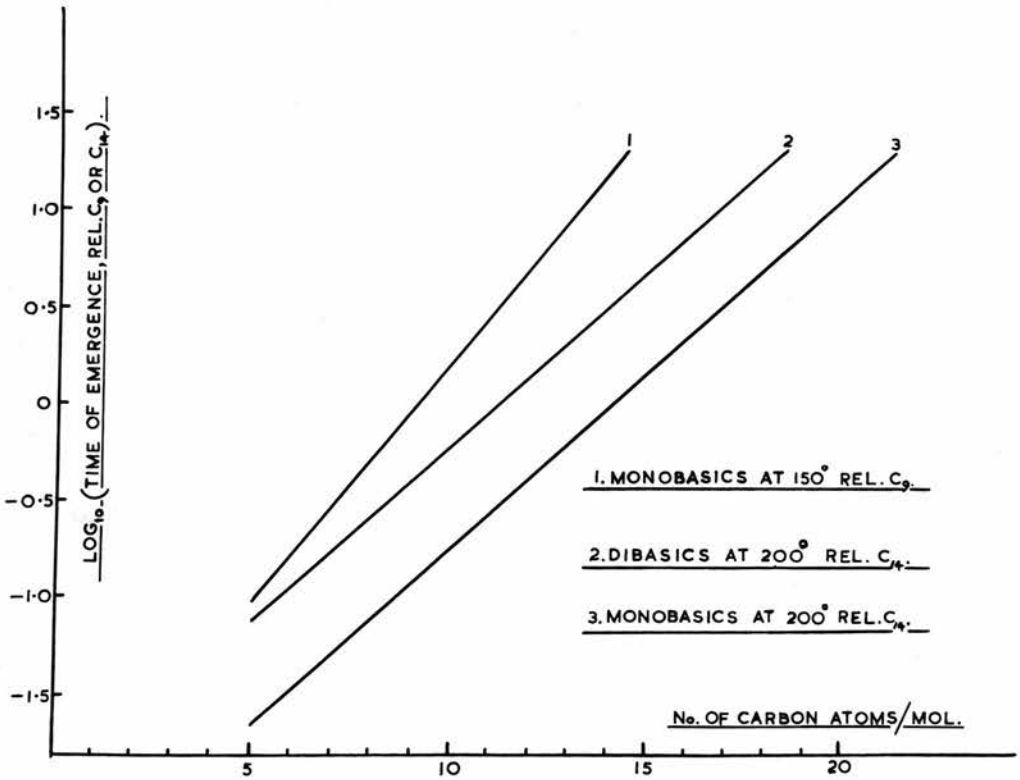

LOG10 (TIME OF EMERGENCE, REL. C9 OR C14)
1·5
1·0
0·5
0
−0·5
−1·0
−1·5
5
10
15
20
1
2
3
1. MONOBASICS AT 150° REL. C9.
2. DIBASICS AT 200° REL. C14.
3. MONOBASICS AT 200° REL. C14.
No. OF CARBON ATOMS/MOL.

FIG. 20

(5) Calibration for Quantitative Chromatography

Before quantitative analysis can be undertaken the relationship between the signal output of the detector and some molecular parameter of the mixture must be determined. The output of a β-ray/argon detector has been shown by Lovelock[1] to be a function of the molecular weight of the separated component. Hartman and Shorland[9], Bishop and Cooper[10] and Garton[11] have all shown that this detector responds on a molar basis to eluted components. Bottcher et al[12], however, claim that the detector responds to the weight of the component present and constant output on a weight basis has been found for the methyl esters of the higher fatty acids (mol. wt. $>$ 300) by McWilliam[13]. Lovelock et al[14] in a later publication state that the detector (Sr^{90} emitter) gives a response to fatty acid esters (mol. wt. $>$ 150) directly proportional to the mass of substance separated; whilst Farquhar et al[15], also using a Sr^{90} emitter, report that linearity of response against weight percentage of a mixture varied with the applied voltage across the cell, electronic circuitry and the geometry of the detector.

It is thus apparent that linearity of response must be evaluated by chromatography of standard mixtures for each particular ionisation detector.

It was found that with the two detectors used for the present work, the area under each peak taken as a percentage of the combined area of all the peaks represented the molar percentage of the mixture; provided that the following conditions were strictly observed:-

(i) The quantity of substance applied to the column should not be too large, since this results in overload of the detector and eventually leads to "double-peaking". The fault may be avoided by using 0.1 μl. of a mixture containing four or more components or a smaller quantity when only one major component is present.

(ii) Detector voltages below 1000v. should not be used. At 750v. the early components of a chromatogram (low mol. wt.) are underestimated.

(iii) Components should be run at a suitable temperature. Recommended temperatures are:- 150° for the saturated monobasic esters C_6-C_{12} and 200° for the esters C_9 to C_{20}. At 200° the esters C_6 and C_8 are seriously underestimated, although they may still be identified correctly from their relative retention times. A mixture containing the range C_6-C_{18} thus requires two chromatograms for quantitative analysis, a peak common to each chromatogram is then used to correlate the two analyses (Table 20).

(iv) Peak areas are not quantitative when they are too small. It is thus necessary to carry out two runs on a mixture containing a high proportion of one component and small proportions of others. The two chromatograms, differing only in applied load, are then correlated by a common peak.

(v) The sensitivity of the machine is best controlled by the use of the detector voltage switch. The amplifier sensitivity is kept at a minimum (X10) but may be raised to (X5) to bring up a

small component during a chromatogram. The maximum sensitivity (X1) is rarely used due to excessive amplifier noise.

Areas under the peaks are measured by planimetry or by calculation. The method of calculation used in due to Jaulmes and Mestres[16] and consists of multiplying the height of the curve by its width, measured at a point 45.4% of the height from the base line. Areas computed in this way were found not to differ significantly from the areas determined by planimetry, provided the curve was Gaussian shaped. The area of distorted curves was always measured by the planimeter.

The analysis indicating the molar percentage of a mixture may still be incomplete even after all the above precautions have been taken. The most general causes for this are:- (a) some component(s) may not have emerged from the column when the analysis was discontinued and (b) some non-volatile component which will not come through the column at all.

The present machine was calibrated by analysing standard mixtures of fatty acid methyl esters (each component was 98-100% pure). Analytical results are given in Tables 19 and 20.

Table 19. Gas Chromatographic Analysis of Standard Mixtures

Mix.[a]	Mol%	Wt.%	Found[b]	S.D.	Mix.[c]	Mol%	Wt.%	Found[d]	S.D.
12:0	10.5	8.5	10.1	±1.5	8:0	15.1	10.5	15.0	±0.8
14:0	32.2	20.0	21.1	±1.4	10:0	32.8	30.9	33.1	±0.9
16:0	30.5	30.7	32.5	±0.5	12:0	54.1	58.6	53.9	±1.4
18:0	36.8	40.8	36.3	±1.4					

S.D. = Standard deviation.

Notes to Table 19.

(a) Chromatography on a 10% Ap. L. column, at 200°, Argon flow rate = 33.3 ml. p. min. Detector voltage 1000v. or 1250v.

(b) Mean results from ten determinations, load varying between 0.025 and 0.1 µl.

(c) Chromatography on a 10% Ap. L. column, at 150° Argon flow rate = 33.3 ml. p. min. Detector voltage 1000v. or 1250v.

(d) Mean results from six determinations, load varying between 0.025 and 0.1 µl.

Table 20. Gas Chromatographic Analysis of a Standard Mixture

Acid Mixture[a]	Mol.%	Wt.%	Area[b] at 150°	Area[b] at 200°	Found
C_6 monobasic	7.4	5.7	35.9	-	7.0 [d]
C_9 monobasic	5.9	3.6	19.1 [c]	4.0 [c]	5.7
C_9 dibasic	8.9	6.5	46.0	9.4	8.8
C_{14} monobasic	14.1	15.3	-	15.3	14.2
C_{12} dibasic	8.7	7.8	-	9.6	8.9
C_{18} monobasic	57.0	66.1	-	61.7	57.4

Notes to Table 20.

(a) A mixture similar to that obtained by the partial oxidation of linoleic acid with myristate standard.

(b) Mean results of three determinations. Chromatography on a 10% Ap. L. column, argon flow rate = 35 ml. p. min., detector voltage 1000v. at 200° and 1250v. at 150°, each analysis with a sample load of 0.1 μ l.

(c) The ratios of C_9 monobasic to dibasic are 2.36 (150°) and 2.35 (200°).

(d) The agreement between the C_9 acid ratios enables the areas of the two sets of chromatograms to be correlated and leads to the molar % composition of the complete mixture.

Inspection of Tables 19 and 20 indicates that the response of the detector is to the molar percentage of the mixture and not to the weight percentage. The standard deviations reported in Table 19 are rather above those reported by other workers:- e.g. S.D. $\pm$ 0.5 on a 5 component mixture[10]; S.D. $\pm$ 0.3 on a 5 component mixture[15]; and S.D. $\pm$ 0.95 on a 6 component mixture[17], and did not improve with experience in using the machine.

James[18] has pointed out that the use of an integrating amplifier would improve the accuracy of the determination of any single acid from 3-4% to less than 1%.

The calibration of this instrument for quantitative analysis indicates that:-

(i) The detector responds to molar percentage of a mixture

(ii) The mean of at least three determinations should be used to calculate the composition of a mixture.

(iii) The final results for molar percentage composition will probably have a coeficient of variation* of 5% on each component.

* coefficient of variation = $\left[\frac{\text{standard deviation}}{\text{mean}}\right] \times 100$

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PART V.

THE PARTIAL OXIDATION OF POLY-ETHENOID ACIDS

(1) Introduction.

The Structure of Unsaturated Fatty Acids by Oxidation.

The recent application of gas chromatography to lipid chemistry, especially to the animal fats, has revealed the existence of a large variety of hitherto unknown unsaturated fatty acids. The number of double bonds and carbon atoms of such unknown acids may be determined by gas chromatography using polar and non-polar stationary phases[1] but further investigation is required to fix the position of the unsaturated centres in the molecule. An accurate method of determining double bond position is also required for experiments involving a shift in the site of an unsaturated centre such as partial reduction, alkali isomerisation and conjugation of unsaturated acids.

The classical method for determining double bond structures is by oxidative degradation followed by identification of the mono and di-carboxylic acids so produced. Recent work has been mainly directed towards obtaining more selective oxidation of the unsaturated centres, freedom from secondary degradation fragments and better methods for the analysis of the oxidation products.

Armstrong and Hilditch[2] were the first to use potassium permanganate in dry acetone or acetic acid to oxidise unsaturated esters.

Inadequate methods of separating the acids from the oxidation and the presence of fragmentary bi-products enabled only the positions of major unsaturation to be determined accurately. Hegemann et al[5] introduced column partition chromatography for the quantitative identification of the acids produced by permanganate cleavage of unsaturated acids. Both Keppler[4] and Hegemann[5] have shown that permanganate causes considerable secondary degradation of the higher homologues of the dicarboxylic acids, thus leading to uncertaintity in the positions of minor unsaturation. James and Webb[5], analysing the products from the permanganate oxidation of unsaturated acids by gas chromatography have shown the presence of homologous series of acids of shorter chain length in the reaction mixture.

Lemieux and von Rudloff[6] proposed a method oxidation applicable to mono and di-unsaturated acids in which a mixture of permanganate and periodate oxidises the soaps in an aqueous medium, maintained at pH 7.9-9.0 with carbonate, at room temperature. The method centres around the continuous regeneration of the permanganate by the periodate under alkaline conditions. The acidic reaction products were separated by Hegemann's[5] chromatographic method. A modification to this procedure by Jones and Stolp[7] (sodium hydroxide replacing potassium carbonate) enabled high yields of dibasic acids to be obtained, but isomeric impurities of 1% were consistently found. von Rudloff[8] noted that the reagent attacked linoleic acid very rapidly and that the structure of this acid had to be deduced from the recognition of caproic and azelaic acids only. Permanganate/periodate cleavage has been

applied to the mixed acids of plant rusts by Tulloch and Ledingham[9] to show that only the common C_{18} unsaturated acids were present. The method depends on obtaining azelaic acid as the sole dibasic acid experimental results however show that suberic acid is also produced (up to 15% of the total dibasics) which introduces an element of uncertaintity into the deductions made from the experiment. Hopkins and Chisholm[10] have successfully used the reagent to determine the structure of 12:13-dihydroxyoctadec-9-enoic acid by cleavage before and after hydrogenation. A variation on the procedure using osmium tetroxide catalysed periodate was introduced by Pappo et al[11] but was found to be unsatisfactory for unsaturated esters by Gason and Tavs[12]

The structure of several octadecenoic acids has been determined by Huber[13] by hydroxylating the double bonds with peracid according to the method of Swern et al[14] followed by cleavage of the α-glycol by periodate. Identification of the oxidation products was by the melting point of the dinitrophenylhydrazone of the aldehyde and the melting point of the dibasic acid produced by permanganate oxidation of the aldehydo-acid from the primary cleavage. Application of chromatographic analysis to the degradation fragments produced by this procedure both by James[5] and von Rudloff[8] indicated the presence of many secondary acids which obscured the main fission products.

Ozonolysis has proved itself especially useful for the analysis of unsaturated acids although this method is again beset with difficulties arising from undesireble side reactions. High yields of both mono and di-carboxylic acids are reported for the ozonolysis of unsaturated acids,

by Asinger[15] and Klenk and Langard[16]. Keppler[4] has applied ozonolysis to the reaction products from the isomerisation of unsaturated acids and states that ozonolysis at -5° in chloroform followed by decomposition of the ozonides by alkaline silver oxide gives the least quantity of secondary products. Several chromatographic systems are also reviewed in this paper and ozonolysis is compared with permanganate oxidation. Ozonolysis in a reactive solvent (methanol or acetic acid) followed by reductive cleavage of the ozonide is recommended by Pryde et al[17] to give high yields of the required fission products.

Ozonolysis, as a method for double bond location, has been critisised by James and Webb[5] and by Benton et al.[18] When oleic acid was ozonised relatively large fractions corresponding to C_7, C_8 and C_{10} mono and di-carboxylic acids were produced along with the expected C_9 pair. This type of evidence suggests the presence of double bonds which do not exist. Cason and Tavs[12] also report large amounts of lower homologues but indicate that these are most probably unoxidised aldehydes. They therefore suggest that ozonides should be boiled with water to decompose them, thereby giving high yields of aldehydes and only traces of acids.

Many of the oxidative methods outlined above do not, in fact, lead to an unequivocal structure for an unsaturated acid even when freed from the uncertainties of secondary reactions. The recognition of caproic and azelaic acids from a von Rudloff oxidation or ozonolysis of linolei acid defines this acid as:- (i) pentadec-9-enoic acid or,

if the chain length or number of double bonds is known, as (ii) octadeca-3:12-dienoic acid or (iii) octadeca-9:12-dienoic acid. A decision between these possibilities can be made by labelling the carboxyl group and oxidising in such a way as to retain the label in one of the oxidation products. This has been done by degradation of the ester under non-hydrolytic conditions[18], degradation before and after chain extension[19] or by degradion of the alcohol formed by lithiumaluminiumhydride reduction of the acid[20]. Stoffel and Ahrens[21] have determined the structure of the unsaturated acids in menhaden body oil by gas chromatographic analysis of the ozonolysis products but they also require to know (i) the number of double bonds (ultra-violet absorption after alkali-isomerisation) and (ii) the number of carbon atoms in the acid. (hydrogenation followed by gas chromatography). The validity of deductions from this method then depends on assuming that all the double bonds are methylene interupted.

Part of the present work describes a method for locating the positions of double bonds in polyethenoid acids[22], the only additional information required being the approximate molecular weight of the acid. The polyunsaturated acid is treated with insufficient performic acid to oxidise one double bond to give a mixture of all the possible dihydroxyacids, a small amount of more extensively hydroxylated material and unreacted starting material. After complete hydrogenation the reaction mixture is oxidised with periodate and permanganate and the fission fragments examined by gas chromatography. This procedure is

particularly advantageous in that it gives practically no secondary products and will distinguish between olefinic and acetylenic centres since the latter react extremely slowly with performic acid.

(3) Partial Ozonolysis of Unsaturated Acids.

Some examples of the application of ozonolysis to the analysis of unsaturated acids have been mentioned in the introduction, a more extensive review of the reaction of ozone with organic molecules has been given by Bailey[23]. The following section describes an unsuccessful attempt to determine the structure of unsaturated acids by partial ozonolysis.

When insufficient ozone is added to a polyethenoid acid to react with one double bond each unsaturated centre is partially oxidised. Cleavage of the ozonides, either combined with or followed by reduction furnishes a mixture of mono and di-carboxylic acids and residual unoxidised material, which can be analysed by gas chromatography. The position of each unsaturated centre may then be found by pairing off monobasic and dibasic fragments so that the carbon content of the pair is equal to the number of carbon atoms in the residual unoxidised material.

Three experimental procedures were investigated.

(a) Addition of ozone to an unsaturated acid.

(b) Ozone decomposition.

(c) The effect of the decomposition procedure upon residual unoxidised acid.

(.) Addition of Ozone to an Unsaturated Acid

Ozone has been used as a titrimetric reagent for the quantitative estimation of olefinic unsaturation by Boer and Hoyman[24], the ozone is obtained from a constant output generator and is dispersed in to the unsaturated material by means of a high speed stirrer. A simpler procedure however is suggested by the solubility of ozone in organic solvents (8m. moles per litre of carbontetrachloride[25]), such a solution could be standardised by estimating the iodine liberated from acidic potassium iodide and could then be used to titrate the unsaturated material. The amount of ozone required to fully oxidise 50m. of ethyl oleate is 1.66×10^{-4} mole. or 20ml. of the above solution.

Ozone, obtained by passing oxygen through a silent discharge ozoniser, was bubbled through dry chloroform at atmospheric pressure at 20° for 15 mins. after which time it was found that the solvent took up no further ozone and contained 8.35 m. moles. per litre. Approximately 22 ml. of this reagent would be required to oxidise 50mg. of methyl oleate. This volume was considered to be too great and a solution of ozone was prepared in dry chloroform at -20°. Estimation of the ozone content of this second solution indicated that it contained 8.4 m. moles per litre.

Further experiments on the use of standardised ozone/chloroform solutions prepared at -20° however soon indicated that they were too unstable for quantitative analysis. Some experiments also showed that there was an incomplete transference of the dissolved ozone to the unsaturated material. Attention was therefore turned to a method of

adding ozone to a chloroform solution of the unsaturated acid by measuring the time of passage of ozone through the solution. The generator was calibrated before and after each experiment for uniform ozone production.

During a 45 min. period ozone was bubbled through a solution of potassium iodide for six 5 min. periods. After acidification the liberated iodine was estimated with standard thiosulphate solution. This calibration indicated that the generator produced ozone at the rate of $8.2(\pm.1)X10^{-5}$ moles per min. The full ozonolysis of 50mg. of methyl oleate could therefore be effected in 2 mins. and partial ozonolysis by proportionately cutting the reaction time.

(b) Ozonide Decomposition.

The reaction product from the partial ozonolysis of an unsaturated acid contains residual unoxidised material thus oxidising agents such as peracids or permanganate cannot be used to decompose the ozonides. Two suitable decomposition procedures were investigated, (i) ozonide cleavage with alkaline silver oxide and (ii) ozonide cleavage by catalytic reduction.

(i) Ozonide Cleavage by Alkaline Silver Oxide.

Preliminary experiments indicated that refluxing the chloroform solution of the ozonide with water and silver oxide gave a mixture of the required acids but these were contaminated by appreciable quantities (up to 80) of unoxidised aldehydes. Replacing the water with 10 sodium hydroxide did not improve the yield of acids significantly.

Acid yields of over 95% could however be obtained by using the procedure suggested by Keppler[4]. The chloroform solution of the ozonides was added to boiling water and the chloroform allowed to evaporate. Alkali and silver oxide were then added and the suspension kept at 100° for 1 hour. The acids were liberated with dilute sulphuric acid and extracted with ether.

(ii) Ozonide Cleavage by Catalytic Reduction

The cleavage of ozonides by catalytic hydrogenation produces a mixture of aldehydes and aldehydo-acids and as such is unsatisfactory for the present study since these latter are not readily available for chromatographic calibration. It has also been stated that during reductive cleavage some of the corresponding acids are also produced by spontaneous decomposition of the ozonides. Both of these objections can be overcome by oxidising the aldehyde groups to acid functions prior to analysis. Dilute acidic potassium permanganate may be used for this since double bonds present after the partial ozonolysis have been hydrogenated.

The following procedure was adopted:- The bulk of the chloroform from the ozonide solution was removed under reduced pressure at room temperature and the ozonides taken up in methanol. Reductive cleavage was accomplished according to the method of Henne and Perilstein[26] by shaking the methanolic solution of the ozonides with a 1% palladium on barium carbonate catalyst[27] in an atmosphere of hydrogen for two hours. Subsidiary experiments indicated that under these conditions methyl

oleate was fully reduced to methyl stearate but no reduction of the carbonyl group of nonanal took place[28]. The highest yield of acids was obtained by adding just enough powdered potassium permanganate to a solution of the aldehydes in 1 N sulphuric acid at 10° to maintain a pink colour for a period of 10 mins. Nonanal oxidised in this manner gave pelargonic acid (98%) and either octanoic acid or unoxidised nonanal (2%).

(c) The Effect of Alkaline Silver Oxide upon an Unsaturated Acid

Samples of methyl oleate and linoleate were dissolved in 10% sodium hydroxide, silver oxide was added and the mixture kept at 100° for 1 hour. The acids were liberated with dilute acid, recovered by ether extraction and analysed by their iodine values. (Wijs). The Iodine value of oleate did not change significantly as a result of the above treatment and the iodine value of linoleate dropped only by 5%. The reagent has little effect on unsaturated centres.

(d) Partial Ozonolysis Results.

The results given in Table 21 were obtained by the ozonolysis of pure methyl oleate in chloroform at -40° followed by decomposition of the ozonides either by alkaline silver oxide at 100° (expts. 1-3) or by reductive cleavage and permanganate oxidation (expts. 4-6).

Table 21. Decomposition Products (mole %) from the Ozonolysis of Methyl Oleate.

EXPT.	MONOBASICS			DIBASIC			UNKNOWN	RESIDUAL
	C_8	C_9	C_{10}	C_8	C_9	C_{10}	FRACTIONS	OLEATE
1	0.7	10.1	-	1.3	21.1	-	1.9	64.9
2	8.4	62.5	-	5.4	23.8	-	1.9	-
3	2.3	39.3	0.3	1.9	54.7	1.3	-	-
4	4.0	41.0	-	2.5	52.5	-	-	-
5	0.8	35.6	1.3	1.3	63.0	-	-	-

The results given in Table 21 are similar to those reported by Benton et al[18] for the ozonolysis of methyl oleate followed by decomposition of the ozonides by hydrogen peroxide. Since it is impossible to decide whether the minor components in the degradation products are real or reaction artefacts it is not possible to determine the positions of minor unsaturation with any degree of certaintity. This serious limitation of the method arises from the secondary reactions undergone by the ozonide during cleavage.

The investigation into the partial oxidation of polyethenoid acids by ozone was therefore terminated.

(3) Partial Oxidation of Polyethenoid Acids by Performic Acid.

(a) Introduction

The application of gas chromatographic analysis to the permanganate/periodate cleavage products[6,8] of the naturally occuring dihydroxy acid in castor oil indicated pelargonic and azelaic acids only,

thus confirming the structure of this acid as 9:10-dihydroxystearic acid[29]. Similar analysis of the cleavage products from 12:13-dihydroxystearic acid both alone and mixed with the 9:10-isomer indicated clearly each time the position of the α-glycol groups. This procedure can be extended to determine the structure of polyethenoid acids by turning some of each unsaturated centre into an α-glycol group by peroxid oxidation. The freedom from secondary degradation products enables the position of minor unsaturation to be fixed with certainty.

The hydroxylation of a double bond is most readily effected by means of performic acid. Swern et al[14] have shown that performic acid, which is prepared and utilised *in situ*, undergoes a rapid and quantitative reaction with a monoethenoid acid to give a high yield of the dihydroxy compound. The reaction takes place under mild conditions (40°) and the quantitative uptake of hydrogen peroxide makes it an ideal procedure for partial oxidation. The oxidation of linoleic acid by performic acid has been studied by Swern and Dickel[30] and by McKay et al[31] and is reported to give poor yields of the expected tetrahydroxy acid. Swern and Dickel[30] found that the expected hydroxyformoxy compound was obtained but hydrolysis gave only a poor yield of the tetrahydroxy acid. McKay et al[31] explained this poor yield by suggesting that a 3:4-dihydroxypyran derivative could be formed under the reaction conditions.

It is thus apparent that for the successful application of performic acid to the oxidation of polyethenoid acids the amount of

peroxide must be restricted so that only one double bond in each molecule is hydroxylated. The reagent is thus effectively being used under conditions of monoepoxidation. In the present work the quantity of peracid was cut still further to 0.6 mole per mole of polyethenoid acid as a safety margin against poly-hyroxylation.

(b) Experimental Procedure.

The unsaturated acid (1m. mole), 30% hydrogen peroxide (0.6 m. mole) and 98% formic acid (6 ml. per g. of acid) are shaken together for 15 mins. at 30°. Any excess peroxide is then destroyed with sulphur dioxide, the formic acid is removed under reduced pressure at 50° and the oxidation product is hydrolysed by heating with an excess of 2N aqueous sodium hyroxide at 100° for one hour. The dihydroxy acids are liberated with dilute sulphuric acid and extracted with ether. Residual unsaturated acid is removed by complete hydrogenation of a methanol solution of the acids in an atmosphere of hydrogen over a 20% palladium / charcoal catalyst for two hours. The solvent and catalyst are removed and the product oxidised with potassium periodate (8m. mole), potassium permanganate (o.134 m. mole) and potassium carbonate (3m. mole) in a dilute aqueous solution, (200 ml.) by shaking at room temperature overnight. The resulting solution is acidified, decolourised with sulphur dioxide' saturate with salt and extracted (x6) with ether. The acids are methylated with methanolic hydrogen chloride and analysed by gas / liquid chromatography.

Notes

(i) Performic acid is unstable and looses oxygen rapidly, it must therefore be freshly prepared from 30% hydrogen peroxide and 98% formic acid for each experiment. Partial oxidations are usually carried out on 100 mg. of the acid and the oxidising agent made up on a ten-fold scale. e.g. for the 60% oxidation of 100mg. of oleic acid 0.62 ml. of a reagent prepared form formic acid (6.0 ml.) and peroxide (0.24 ml.) is used. Sufficient accuracy is obtained by measuring these quantities from a 1 ml. burette.

(ii) It is often necessary to add more formic acid (1ml.per 100mg. of acids) to obtain a homogeneous reaction mixture during oxidation.

(iii) The reagents for the von Rudloff[8] oxidation are weighed for each experiment. It is important not to exceed the given concentrations of oxidants, increase in the permanganate results in the presence of secondary degradation fragments.

(iv) Solvent ether is removed through a 15cm. Vigreux column in order to minimise the loss of volatile components.

(v) Analysis of the degradation fragments is by gas/liquid chromatography using the Argon chromatograph and the associated techniques as described in Part IV. All analyses were performed on 10% Apiezon L columns and each mixture was analysed three times and the mean result taken as the molar percentage composition. Many of the degradation mixtures range from hexanoic to stearic acids and thus require to be analysed at 150° and 200°, a peak common to both

analyses is then used to correlate the chromatograms.

(vi) The recovery of material from the above experimental procedure (estimated chromatographicaly) is generally between 70 and 90%. The losses are due to the volatility of the shortchain monobasic esters and to the water-solubility of the shortchain dibasic acids.

(c) Materials

All spectral data are obtained from ethanolic solutions and maxima are reported in mμ with extenction coefficients in parentheses.

Oleic Acid A pure sample obtained from Unilever Ltd.

Linoleic Acid A pure sample obtained from Unilever Ltd.

γ-Linolenic Acid A pure sample obtained from Roche Products Ltd.

Arachidonic Acid a pure sample obtained from Roche Products Ltd.

Tetradec-7-enoic Acid obtained by synthesis (Part VI)

Octadeca-7:11-dienoic Acid obtained by synthesis (PartVI)

Hexadeca-8:10-dienoic Acid obtained by synthesis (Part VI)

Nonadeca-10:13-dienoic Acid Prepared from linoleic acid (Part VI)

Santalbic Acid. (OctadecaSyne-llenoic acid)

The acid had previously been extracted from Santalbum album seed oil and showed ultraviolet absorbtion at 229 (16,000), 240 inflexion. (lit.[32] 229(16,600), 240 inflexion).

Octadeca-9:11-dienoic Acid The acid was obtained by reducing santalbic acid with Lindlar's catalyst. The diene acid showed ultraviolet absorbtion at 251 (22,000), 229 in 259 inflexions. (lit[32] 251 (24,000) 240 inflexion).

Elaeostearic Acid Octadeca-cis-9:trans-11:trans-13-trienoic acid The acid was obtained from Tung oil by the method of Crombie and Jacklin[32]. The sample melted at 46-47° and had an ultraviolet absorbtion at 261(35,000), 271 (46,000), 282 (56,500). (Lit[33] m.p. 48° 261 (36,000). 271 (47,000), 281 (38,000).

β-Elaeostearic Acid (Octadeca-trans-9:trans-11:trans-13-trienoic acid)

The acid was obtained from Tung oil by the method of Crombie and Jacklin[33]. The sample melted at 70.5-71° and had an ultraviolet absorbtion at 258 (46,000), 268(59,000), 279 (47,000), (Lit[33] m.p. 72°, 259 (47,000), 268 (61,000), 279 (49,000).

Punicic Acid (Octadeca-cis-9:trans-11:cis-13-trienoic acid)

The acid was obtained from pomegranate seeds oil according to the method of Crombie and Jacklin[33]. The sample melted at 41-42° and had an ultraviolet absorbtion at 264 (35,200), 274 (46,500), 285 (36,400). (Lit[33] m.p. 43.5-44°, 264 (55,000), 274 (46,000), 285(36,500)

Octadeca-trans-8:trans-10:trans-12-trienoic Acid

The acid was obtained from Scots marigold seed oil (Calendula officinalis) by the method of McLean and Clark[34]. The sample melted at 75-76° and had an ultraviolet absorbtion at 258 (44,500), 268 (59,000) 279 (47,200). (Lit[34] m.p. 77-78°, 258 (44,100), 268 (59,100), 280 (46,600)

Vernolic Acid (octadec-12:13 epoxy-9-enoic Acid)

The acid was obtained from the seed oil of Vernonia anthelmintica by the batch adsorption method of Morris, Hayes and Holman[35]. The oil was saponified by refluxing with 0.5 N methanolic potassium hydroxide for half an hour and the unsaponifiable matter was removed. The acids were liberated from their potassium soaps by Zeo-Karb 225 ion exchange resin (p.63) and extracted with ether. The mixed acids (4.0)g. were dissolved in petroleum ether (b.p. 40/60°) containing 5% ether (400 ml.) and silicic acid (80g. specialy prepared for chromatography) was added. After stirring the silicic acid was filtered off and the saturated acids removed by stirring it with petrol containing 5% ether. The vernolic acid (2.9g.) was finally removed by stirring the silicic acid with petrol containing 25% ether. The sample melted at 24-28° (Lit[36] m.p. 25-28°) and contained 5.2% oxirane oxygen (5.4%) estimated by King's[37] method.

(d) Results

1. Partial Oxidation of Single Acids.

(i) Oleic Acid

	Reaction products (mole %)
Monobasic:	C_8 trace, C_9 16, C_{18} 64
Dibasic:	- C_9 20

These are the expected degradation products from octadec-9-enoic acid. The trace (½%) of octanoic acid must occur from the secondary degradation of pelargonic acid. It does not indicate a positional isomer of the double bond at Δ8 since it is unpartenered by the C_{10} dibasic acid.

(ii) Tetradec-7-enoic Acid — Reaction products (Mole %)

Monobasic: C_7 28, C_{14} 44

Dibasic: C_7 28

These are the expected products from degration of this acid

(iii) Linoleic Acid — Reaction products (Mole %)

Monobasic: C_6 4, C_9 6, C_{18} 76

Dibasic: C_{12} 6, C_9 8

These are the acids expected from the degradation of octadeca-9:12-dienoic acid. The figures given above indicate a slight preferential attack of the performic acid at 9 but this difference is comparable with the experimental errors and thus there is no evidence of preferential attack.

(iv) Nonadecadienoic Acid — Reaction products (Mole %)

Monobasic: C_6 3.5, - C_9 5.5, C_{18} 5.8, C_{19} 69.1

Dibasic: C_{12} 0.8, C_{15} 8.0, C_9 1.5 C_{10} 9.2

These results are the expected products from the degradation of nonadeca-10:13-dienoic acid, but they also indicate that the acid contains 10% of octadeca-9:12-dienoic acid. (p.149)

(v) Octadecadienoic Acid — Reaction products (Mole %)

Monobasic: C_7 9, C_{11} 10, C_{18} 57

Dibasic: C_{11} 17, C_7 7

The degradation products are those expected from octadeca-7:11-dienoic acid.

(vi) γ- Linolenic Acid — Reaction products (Mole %)

Monobasic: C_6 4, C_9 6, C_{12} 7, C_{18} 66

Dibasic C_{12} 5, C_9 6, C_6 6 (C_4 and C_8 traces)

The products are those expected from the fission of octadeca-6:9:12-trienoic acid. The succinic and suberic acids are present only in trace amounts and are not partenered by their corresponding monobasic acids, they are thus secondary reaction products and do not represent double bond isomers. As with linoleic acid there is no evidence for preferential oxidation of the unsaturated centres.

(vii) Arachidonic Acid — Reaction products (Mole %)

Monobasic: C_{15} 4.4 C_{12} 2.2 C_9 2.7, C_6 1.0, C_{20} 80.0

Dibasic: C_5 1.2 C_8 3.4 C_{11} 2.5 C_{14} 1.6 C_9 1.0

These acids are those expected from degradion of eicosa-5:8:11:14-tetraenoic acid. The azelaic acid (1.0) is unpartenered and thus does not represent a double bond isomer at Δ 9, the product arises from terminal bisoxidation of the 1:4:7:10-undecatetraene system present in the arachidonic acid. Again there is no evidence of preferential attack of the oxidising agent on the double bonds. A chromatogram of the methylated degradation mixture is shown in Fig. 21 Peak (8) is methyl myristate introduced as an internal standard and peak (10) is methyl palmitate, an impurity in the starting material.

(viii) Octadecadienoic Acid — Reaction products (Mole. %)

Monobasic: C_7 2.2, C_8 0.4 C_9 3.8 C_{10} 0.1 C_{18} 79.4

Dibasic: C_{11} 6.3 C_{10} 0.6 C_9 6.8 C_8 0.4

The degradation fragments denote an acid with 18 carbon atoms cleaved (i) mainly at Δ 9 and Δ11, and to a lesser extent at (ii) Δ10 and (iii) 8. This pattern is quite different from linoleic acid and indicates a cumulene structure, the minor components cannot be rejected as secondary degradation fragments since each is correctly partenered. Further evidence is therefore required before the structure of the acid can be determined and this is provided by the ultraviolet absorbtion spectrum. The spectrum, in this case, indicates a conjugated diene chromophore to be present. The structure of the acid is therefore octadeca-9:10-dienoic acid, cleavage at (i) being expected, at (ii) arising from a conjugative shift of the double bonds and at (iii) still being unexplained.

This minor cleavage of the bond just outside the conjugated system at the end nearest to the carboxyl group seems to be characteristic of this type of acid. (see below). Whilst it would appear to be an inherent part of the reaction, the possibility of double bond isomerism cannot finally be ruled out.

(ix) Elaeostearic Acid — Reaction products (Mole %)

Monobasic: C_5 2.0, C_6 1.1, C_7 1.9, C_8 2.0, C_9 4.0, C_{10} 1.7, C_{18} 52.1.

Dibasic: C_{13} 4.0, C_{12} 2.3, C_{11} 2.5, C_{10} 1.3, C_9 23.2, C_8 1.9.

The pattern of this degradation mixture is characteristic of a conjugated acid. An ultraviolet spectrum of the acid indicates the presence of a conjugated triene group, thus the acid is octadeca-9:11:13-trienoic acid. Cleavage at Δ 9,11, and 13 is expected cleavage at Δ10 and 12 is ffrom a conjugative double bond movement

and cleavage at $\Delta 8$ is again unexplained. These results, therefore, do not preclude a trace of the $\Delta 8:10:12$ isomer being present in the elaeostearic acid. The overwhelming proportion of the shortest of the expected dibasic acids is also characteristic of the partial oxidation of a cojugated acid. The most probable reason for this large recovery is that this acid is not subjected to further oxidation, which is not the case for the other dibasic acids. The corresponding effect for the shortest monobasic acid (in this case C_5) will not generally be apparant since the volatility of the ester always results in a poor recovery of the short chain monobasics.

(x) β -Elaeostearic Acid — Reaction products.(mole%)

Monobasic: C_5 1.6, C_6 1.2, C_7 1.6, C_8 2.3, C_9 3.0, C_{10} 1.9, C_{18} 54.6.

Dibasic: C_{13} 3.0, C_{12} 2.5, C_{11} 2.1, C_{10} 1.5, C_9 21.8, C_8 2.9.

The interpretation of these results is analogous to that used for α-elaeostearic acid and leads to the same structure, namely octadeca-9:11:13-trienoic acid.

(xi) Punicic Acid — Reaction products (Mole %)

Monobasic: C_5 1.2, C_6 0.7, C_7 1.2, C_8 2.8, C_9 2.5, C_{10} 1.2 C_{18} 48.1

Dibasic: C_{13} 4.3, C_{12} 5.1, C_{11} 3.5, C_{10} 2.4, C_9 25.5, C_8 1.7

The structure of punicic acid is similarly shown to be octadeca-9:11:13-trienoic acid.

(xii) Octadecatrienoic Acid — Reaction products (Mole)

Monobasic: C_6 1.5, C_7 0.6, C_8 1.1, C_9 2.4, C_{10} 1.5, C_{11} trace, C_{18} 87.2

Dibasic: C_{12} 1.0, C_{11} 2.4, C_{10} 1.5, C_9 2.3, C_8 18.6 C_7 trace

The pattern of the degradion products and the ultraviolet spectrum of the acid are again characteristic of a conjugated triene. The structure is therefore octadeca-8:10:12-trienoic acid with the possibility of a trace of the 7:9:11 isomer. The structure agrees with the previous workers[54] who investigated this acid obtained from Scots marigold seed oil.

(xiii) Santalbic Acid — Reaction products (Mole)

Monobasic: C_7 0.4, C_8 0.5, C_9 0.5, C_{18} 94.6

Dibasic: C_{11} 3.7, C_{10} 0.5, C_9 0.8

The degradation fragments denote a conjugated acid but major cleavage occurs only at Δ 11. The ultraviolet spectrum of the acid indicates the ene-yne chromophore to be present and thus the acid is octadeca-8-yne-11-enoic acid.

The triple bond has been reported[58] to react with peracids but with a reaction rate only one thousandth that of an ethylenic bond (see also p133). In santalbic acid, however the triple bond reacts with

FIG. 21

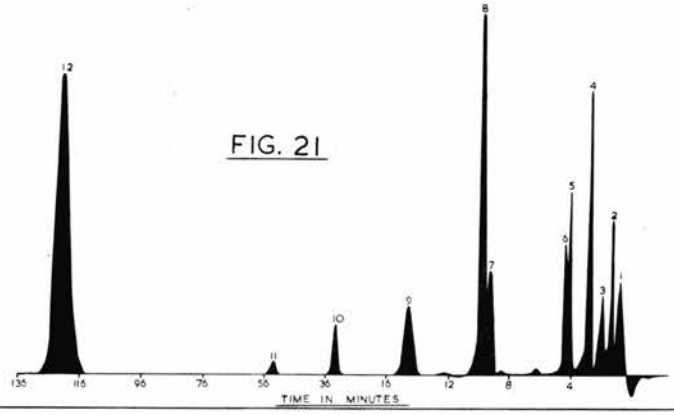


FIG. 22

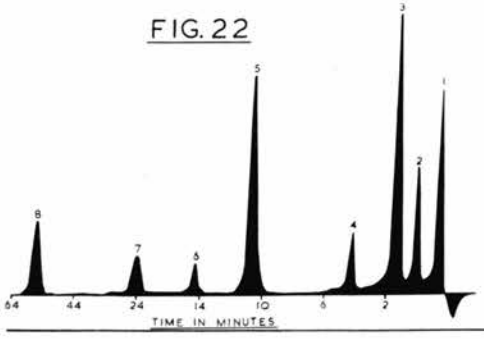
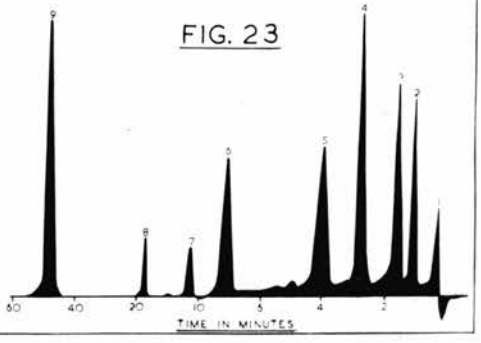


FIG. 23



Legends to Figures 21, 22 and 23

Fig. 21 Separation of the partial oxidation products from arachidonic acid. 10% Ap. L column; column length 4ft; temperature 200° argon flow 32.2 ml. p. min; detector voltage 1250v; load 0.1 μl. Peaks in order of appearance: (1) methyl n-hexanoate, (2) dimethyl pimelate, (3) methyl n-nonanoate, (4) dimethyl suberate, (5) dimethyl azela-ate, (6) methyl laurate, (7) dimethyl undecanedioate, (8) methyl myristate, (9) methyl n-pentadecanoate, (10) methyl palmitate, (11) dimethyl tetradecanedioate, (12) methyl arachidate.

Fig. 22 Separation of the partial oxidation products from a mixture of linoleic acid (0.35 m. mole) and tetradec-7-enoic acid (0.91 m. mole) 10% Ap. L. column: column length 4ft; temperature 200°; argon flow 32.2 ml. p. min; detector voltage 1000v; load 0.1 μl. Peaks in order of appearance (1) methyl n-heptanoate, (2) methyl n-nonanoate, (3) dimethyl pimelate, (4) dimeth l azela-ate, (5) methyl myristate, (6) dimethyl dodecanedioate, (7) methyl palmitate, (8) methyl stearate.

Fig. 23 Separation of he partial oxidation products from a mixture of linoleic acid (0.175 m. mole) and octadeca-7:11-dienoic acid (0.185 m. mole) 10% Ap. 1 column; column length 4ft. temperature 200°; argon flow 55.0 ml. p. min; detector voltage 1000v; load 0.1 μl. Peaks in order of appearance: (1) methyl n-heptanoate, (2) methyl n-nonanoate, (3) dimethyl pimelate, (4) methyl n-undecanoate, (5) dimethyl azela-ate, (6) dimethyl undecanedioate, (7) dimethyl dodecanedioate (8) methyl palmitate, (9) methyl stearate.

about a third of the rate of the ethylenic bond, this must be due to the conjugation between the two centres.

(xiv) Octadeca-7:11-dienoic Acid

The partial oxidation of this acid was repeated on a larger scale (0.5g.) and the reaction products examined for any cyclic material. A discussion of the experiment is reserved until p 139

Cyclic material resulting from the action of a peracid on the 1:5-hexadiene system should be a 1:2-disubstituted-3:6-cyclohexanediol. It was thus verified that cyclohexane-1:4-diol did not undergo fission when oxidised with the von Rudloff reagent.

The partial oxidation of the acid was carried out as usual except that after the von Rudloff oxidation the aqueous solution of products was concentrated to 50 ml. in a rotary-film evaporator prior to extraction with ether and chloroform. The chloroform extract contained no material. The material extracted with ether was esterified (MeOH/HCl) to give the mixed esters, (0.3784g.) which were subjected to a urea adduct separation to give two fractions; (i) U.F.A. = 0.2127g. and (ii) U.A.F. = 0.1126g. (Total recovery 86).

The fractions were analysed by gas chromatography. Fraction (i) contained C_{11} monobasic and dibasic and C_{18} monobasic acids only (98% of mixture accounted for) and fraction (ii) contained only C_7 dibasic and C_{11} mono and dibasic acids. (95% of the mixture accounted for) The reaction products therefore contain no cyclic material.

(2) Discussion of Results from Single Acids.

The degradation fragments from the partial oxidation of monothenoid

and polyethenoid acids of the methylene interupted type indicate clearly in all cases the chain length of the acid and the number and position of the double bonds. Secondary degradation is very slight and leads to products which are not paired by their corresponding fragments and can therefore be rejected. The method is also applicable to determining the structure of acids containing the epoxide group (p.135) or the diol group. The differential rate of reaction between ethylenic and acetylenic centres also enables the latter group to be detected.

The degradation fragments from a conjugated acid present a characteristic pattern for this type of molecule but spectral data is also required as additional evidence before the structure can be determined. The results consistently show a trace of positional isomerism in the double bond structure, but this is most probably a reaction artefract. The cis/trans configuration of the double bonds in conjugated acids does not have any effect upon the partial oxidation reaction.

There is little or no evidence for the preferential attack of performic acid on the double bonds in a polyethenoid acid.

(3) Partial Oxidation of Acid Mixtures

Several workers [30,38-42] have studied the rates of oxidation of unsaturated molecules by peracids. The most common procedure has been to measure the rate of disappearance of the peracid by titrating aliquot portions of the reaction mixture. This method is not however applicable to studying rates of oxidation with performic acid since this compound is relatively unstable and looses oxygen rapidly[43]. The method developed for the present work which deduces the extent of oxidation

from the products of the reaction can however be adapted to studying the relative rates of oxidation.

Each experiment consists of a competition reaction between two types of molecule, thereby ensuring that each entity is given exactly the same reaction conditions for the same reaction time. The rate of oxidation of each molecular species is then proportional to the percentage oxidation of each acid. The percentage oxidation is computed in the following manner:-

$$\% \text{ Oxidation} = \frac{\text{(Mole. \% degradion fragments from each d.b.) X100}}{\text{(above expression + \% unoxidised acid)}}$$

Note When the mole % of the mono and dibasic fragments from one double bond do not agree then the greater value is taken for the % oxidation calculation.

Results

(i) Tetradec-7-enoic Acid (0.51 m. mole)/Linoleic Acid (0.55 m. mole).

Reaction products (mole %)

Monobasic: C_6 2.5, C_7 15.0, C_9 3.5, C_{14} 22.5, C_{18} 36.6

Dibasic: C_{12} 3.7, C_7 12.1, C_9 4.5

A chromatogram of these degradation fragments is shown in fig. 22, The methyl palmitate (peak 7) was introduced as an internal standard. The results for the C_6 and C_7 monobasics come from a chromatogram run at 150° (not shown).

% Oxidations: Monoethenoid 40%; diethenoid 18%

(ii) Oleic Acid (0.18 m. mole)/Nonadeca-10:13-dienoic acid (0.17 m. mole)

Reaction products (mole%)

Monobasic : C_6 not recorded C_9 18, C_{18} 32, C_{19} 28.

Dibasic : C_{13} 3.0, C_{12} trace C_9 16, C_{10} 3.0.

Calculations are made from the dibasic acids only since the same monobasic acids arise from cleavage of both molecules. The % oxidations are calculated from corrected figures, allowing for the 10% linoleic acid in the homo-linoleic acid

% Oxidations: Monoethenoid 35%; diethenoid 17%.

(iii) Oleic Acid (0.176m. mole)/ Octadeca-7:11-dienoic Acid (0.197m.mole

Reaction products (mole %)

Monobasic : C_7 3.9, C_9 8.7, C_{11} 3.9, C_{18} 66.0

Dibasic : C_{11} 5.5, C_9 8.7, C_7 3.3.

The calculations of the % oxidations are made on the assumption that the stearic acid is recovered to the same extent from each source.

% Oxidations Oleic acid 23%, iso-linoleic acid 21%.

(iv) Oleic Acid (o.155 m.mole)/ Arachidonic Acid (0.137 m. mole)

Reaction products (mole %)

Monobasic: C_6 - , C_9 10.0, C_{12} 1.3, C_{15} 1.5, C_{18} 38.0, C_{20} 32.0

Dibasic : C_{14} 1.0, C_9 14.0, C_{11} 0.9, C_8 1.3, C_5 - .

The symbol - signifies that the acid was recorded qualitatively only. Calculations for the % oxidations are corrected for the azelaic acid which comes from the bis-oxidation of the arachidonic acid.

% Oxidations : Oleic Acid 28%; arachidonic acid 15%.

(v) Oleic Acid (0.137 m.mole)/Hexadeca-8:10-dienoic Acid (0.159 m.mole)

Reaction products (mole %)

Monobasic: C_6 1.1, C_7 0.9, C_9 19.4, C_8 2.0, C_{16} 14.0, C_{18} 33.0

Dibasic : C_{10} 3.9, C_9 21.0 C_8 4.7

% Oxidations: Oleic acid 39%; conjugated diene 38%.

(vi) Oleic Acid (0. 485m.mole)/Tetradec-7-ynoic Acid (0.49 m.mole)

Reaction products (mole %)

Monobasic : C_7 trace, C_9 17.5, C_{14} 37.0, C_{18} 26.7 .

Dibasic : C_7 1.0, C_9 17.8

% Oxidations = Oleic acid 40%; acetylenic acid 8.6%

(vii) Oleic Acid (0.37 m. mole)/Hexadeca-8:10-diynoic Acid (0.39 m. mole)

Reaction products (mole %)

Monobasic: C_9 10.5, C_{16} 45.0, C_{18} 54.0

Dibasic: C_9 12.5

% Oxidations = Oleic acid 27%: diacetylenic acid 0%

(viii) Tetradec-7-enoic Acid (0.204 m. mole)/α-Elaeostearic Acid (0.182,m. mole)

Reaction products (mole %)

Monobasic: C_7 5.4, $(C_5+C_6+C_8+C_9)$ 1.5, C_{14} 55.0, C_{18} 55.0

Dibasic: C_7 12.0, C_9 11.0, $(C_{13}+C_{12}+C_{11}+C_{10})$ 4.1

% Oxidations = Tetradecenoic acid 27% elaeostearic acid 30%

(ix) Linoleic Acid (0.185 m. mole)/Octadeca-7:11-dienoic Acid (0.173 m. mole)

Reaction products (mole %)

Monobasic: C_6 1.5, C_7 4.6, C_9 2.7, C_{11} 5.8, C_{18} 65.5

Dibasic: C_{12} 4.2, C_{11} 7.5, C_9 4.2, C_7 4.2

A chromatogram of the above degradation fragments is shown in fig. 23, the methyl palmitate (peak 8) was introduced as an internal standard and the results for the monobasic acids C_6 and C_7 are calculations for the % oxidations are made on the assumption that the stearic acid is recovered to the same extent from each source.

% Oxidations = Linoleic acid 20%; iso-linoleic acid 29%

(x) Linoleic Acid (0.174 m. mole)/Arachidonic Acid (0.175 m. mole)

Reaction products (Mole %)

Monobasic: C_6 1.8 C_9 8.5 C_{12} 1.1, C_{15} 1.4 C_{18} 36.0 C_{20} 43.0

Dibasic: C_{12} 8.9, C_{14} 1.6 C_9 5.0, C_{11} 1.1 C_8 1.2, C_5 0.6

The calculations of the % oxidations are based on figures corrected for the azelaic acid which is produced by the bis-oxidation of the arachidonic acid.

% Oxidations = Linoleic Acid 17%; arachidonic acid 16%

(xi) Vernolic Acid (0.167 m. mole)/Tetradec-7-enoic Acid (0.172 m. mole)

Reaction products (mole %)

Monobasic: C_6 1.4, C_7 9.7, C_9 2.4, C_{14} 10.4

Dibasic: C_{12} 27.0, C_7 34.2, C_9 14.9

The epoxide group of vernolic acid is completely cleaved by the partial oxidation reaction, vernolic acid not oxidised at Δ 9 is therefore measured by the quantity of C_{12} dibasic recovered.

% Oxidations = Tetradecenoic acid 77 ; vernolic acid 55%

(xii) Linoleic Acid (0.172 m. mole)/Tetradec-7-enoic Acid (0.160 m. mole)

Partial oxidation with peracetic acid.

The acids were oxidised with 0.185 ml. of a reagent prepared from glacial acetic acid (1.6 ml.), 30% hydrogen peroxide (0.25 ml.) and concentrated sulphuric acid (40 mg.), otherwise the experimental procedure was identical to that used for the performic acid oxidations.

Reaction products (mole %)

Monobasic: C_6 - C_7 8.1, C_9 5.2, C_{14} 32.0, C_{18} 25.7

Dibasic: C_{12} 9.8, C_7 13.3, C_9 11.9

% Oxidations = Tetradecenoic acid 28 %; linoleic acid 46%

Table 22 contains a summary of the results obtained from the partial oxidation of acid mixtures.

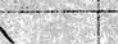
(4) Discussion of Results from the Partial Oxidation of Acid Pairs

The results obtained from the partial oxidation of unsaturated fatty acids with performic acid indicate that the rates of oxidation of the polyethenoid acids relative to a monoethenoid acid fall into three groups: (i) The acetylenic acids, relative rate nil; (ii) the linoleic acid type, relative rate 0.5 (independant of the number of double bonds) and (iii) the iso-linoleic, conjugated diene and trienoic acids, relative rate 1.0. The figures given in Table 22 probably have a standard error of 5% but the cross-check experiments (see notes to the table) indicate the overal correctness of the results.

All the experiments consistently show a surprising low relative rate of oxidation for the polyethenoid acids - results which differ markedly from those obtained with other peracids. Swern et al[30, 39] have shown that linoleic and linolenic acids oxidise faster than oleic acid during their reaction with peracetic acid and Suhara[42] states that linoleic acid oxidises 30% faster than does oleic with the same reagent. These observations are born out by experiment (xii). The substantial difference between the reactions of performic and other peracids has previously been noted by Swern et al[44, 45] as has the fast reaction between oleic and performic acids[14].

In direct contrast to these results are the relative reaction rates measured during the autoxidation of the polyethenoid acids. With respect to oleic acid the relative autoxidation rate of linoleic acid is 12 and for linolenic acid 25[46]. This reaction has been shown[47] to

Table 22. Partial Oxidation of Acid Mixtures.

EXPT.	ACID PAIR		TOTAL OXIDN.		OXIDN.
	1	11	1	11	RATIO 1 & 11
(i)			40	18	1:0.45
(ii)			35	17	1:0.48
(iii)			23	21	1:0.91
(iv)			28	15	1:0.54
(v)			39	38	1:0.97
(vi)			40	2.6	1:0.06
(vii)			27	0	1: 0
(viii)			27	30	1:1.11
(ix)			20	29	1:1.45
(x)			17	16	1:0.94
(xi)			77	35	1:0.45
(xii)			28	46	1:1.64

Notes to Table 22

(a) Experiments (ix) and (x) function as checks on the consistency of the results, since these oxidation ratios may be computed from earlier data.

(b) The ratio of the oxidation rates of linoleic acid to iso-linoleic acid calculated from experiments (ii) and (iii) is 1:1.88 and measured is 1:1.45.

(c) The ratio of the oxidation rates of linoleic acid to arachidonic acid calculated from experiments (ii) and (iv) is 1:1.1 and measured is 1:0.94

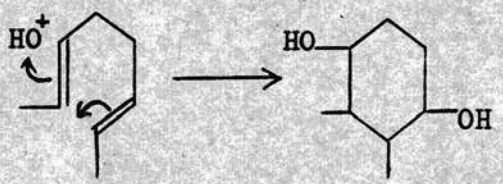
proceed by a free radical mechanism and the increased relative reaction rates of the polyethenoid acids are explained by the fact that these acids contain the 5 carbon system (CH:CH.$\dot{C}$H.CH:CH) which has a higher resonance energy than the 3 carbon system (CH:CH.$\dot{C}$H) found in oleic acid. These high oxidation rates for the polyethenoid acids would not necessarily be expected to be paralleled for the oxidation by peracids since these latter are electrophilic reagents (attacking entity OH^{+}[43]) and not free radical. The relative oxidation rates with peracids for the polyethenoid acids containing 2, 3, etc. double bonds might however have been expected to be in the ratio 1:2:3 etc.

This is however not the case, the relative rate for linoleic acid and peracetic acid is 1:1.6 and with performic acid is 1:0.6. It has been shown that the introduction of an oxygen function into the molecule prior to peracid oxidation lowers its reaction rate. Swern[40] has confirmed that ricinoleic acid oxidises 30% slower than does oleic acid with peracetic acid and Suhara[42] reports that monoepoxyoctadecenoic acid reacts at about 50% of the rate of oleic acid with the same peracid. These lowered rates have been put down to a lowering in the nucleophilic properties of the double bond by the electron attracting properties of the oxygen atom. These observations, whilst explaining the reduced rate of oxidation of linoleic acid with peracetic acid, do not explain the greatly reduced rate with performic acid.

The apparent lowering of the nucleophilic properties of the double bonds in linoleic acid might be explained by the fact that in this acid the 4π electrons are shared over 5 carbon atoms, which is a reduction

in the electron density of the double bond carbon atoms compared with oleic acid. Some support to this idea might come from the results with the conjugated acids where the interaction of the π-electrons gives the same ratio of electrons to carbon atoms as in oleic acid and the oxidation rates are all approximately the same (experiments (v) and (viii)

The double bonds of the iso-linoleic type molecule are in the 1:5 positions and thus it is difficult to explain deactivation by interaction of the π electrons. The lowered reaction rate for this acid might possibly be explained by a secondary reaction involving cyclisation:-



The 1:5 double bond spacing occurs in squalene and such cyclisation reactions have been shown to take place under the influence of electrophilic reagents[41,49]. Attempts to find such a cyclic product in the partial oxidation reaction mixture however failed (p130) and the cyclisation is now thought not to occur.

Attempted explanations of this difference observed between performic and peracetic acid towards OH^+ must surely fail since both of these reagents attack with the same entity. One important difference does however occur between the two acids, namely that whilst epoxidation is the main reaction of peracetic acid the reaction of performic acid is hydroxyformylation. The difference in the reaction rates of linoleic

acid and monoepoxyoleic acid towards further epoxidation and the rates of linoleic acid and hydroxyformoxyoleic acid towards further hydroxyformylation might explain the reduced reactivity of polyethenoid acids towards performic acid. The results obtained for the performic acid oxidation of monoepoxy oleic acid do not however bear this theory out. The low relative oxidation rate of linoleic acid (0.5) could not be adequately explained even if the relative rate for monoepoxyoleic acid were nil, in fact the relative oxidation rate for vernolic acid is 0.5.

At the present time therefore there seems to be no satisfactory explanation for the high rate of oxidation of the monoethenoid acids with performic acid.

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PART VI

THE PREPARATION AND SYNTHESIS OF SOME LONG CHAIN ACIDS

(1) Preparation of 9:10-Epoxystearic Acid.

Pure oleic acid was prepared from a commercial sample of the acid by the low-temperature crystallision technique of Brown and Shinowara[1]. Perbenzoic acid was prepared by ultra-violet catalysed autoxidation of benzaldehyde according to the method of Swern[2]. The epoxidation of the oleic acid was carried out using directions given by Swern[3] to give 9:10-epoxystearic acid. (53% yield).

$CH_3.(CH_2)_7.CH{:}CH.(CH_2)_7.COOH \xrightarrow{1} CH_3.(CH_2)_7.CH\text{-}O\text{-}CH.(CH_2)_7.COOH.$

Reagents:- 1. $Ph.CO_3H$.

(2) Preparation of Nonadeca-10:13-dienoic Acid.

The acid was prepared by the method of Whittcut and Sutton[4]. Linoleic acid chloride was prepared from pure linoleic acid and oxalyl chloride and was treated with an ethereal solution of diazomethane. To a methanolic solution of the diazoketone was added a solution of silver benzoate in triethylamine. The methylnonadecadienoate obtained was saponified to give nonadeca-10:13-dienoic acid. (75%). Gas chromatographic analysis of the fully reduced ester indicated that the product contained 10% of unreacted linoleic acid.

$R.COOH \xrightarrow{1} R.COCl \xrightarrow{2} R.CO.CHN_2 \xrightarrow{3} R.CH_2.CO_2CH_3 \xrightarrow{4} R.CH_2.COOH.$

$R = CH_3.(CH_2)_4.CH{:}CH.CH_2.CH{:}CH.(CH_2)_7-$

Reagents :- 1. $(COCl)_2$, 2. CH_2N_2, 3. $Ph.CO_2Ag/Et_3N$, 4. KOH/EtOH.

(3). Synthesis of Tetradec-7-enoic Acid.

This acid has previously been synthesised by Taylor and Strong[5]. 1:5-Dichloropentane, prepared from pentamethylene glycol and thionyl chloride in the presence of pyridine, was refluxed with sodium iodide in dry acetone to give 1:5-chloroiodopentane. n-Hexyl bromide was reacted with sodium acetylide in liquid ammonia to give oct-1-yne which when coupled, via its sodium derivative, to 1:5-chloroiodopentane yielded 1-chlorotridec-6-yne. This latter, when refluxed with alcholic sodium cyanide and sodium iodide, afforded 1-cyanotridec-6-yne which was converted to tetradec-7-ynoic acid by the action of alkali. The acetylenic acid was reduced by Lindlar's catalyst to give tetradec-cis-7-enoic acid.

$HO.(CH_2)_5.OH \xrightarrow{1} Cl.(CH_2)_5.Cl \xrightarrow{2} Cl.(CH_2)_5.I$

$CH_3.(CH_2)_5.Br \xrightarrow{3} CH_3.(CH_2)_5.C{\equiv}CH \xrightarrow{4} CH_3.(CH_2)_5.C{\equiv}C.(CH_2)_5.Cl \xrightarrow{5}$

$CH_3.(CH_2)_5.C{\equiv}C.(CH_2)_5.COOH \xrightarrow{6} CH_3.(CH_2)_5.CH{=}CH.(CH_2)_5.COOH$

Reagents :- 1. $SOCl_2/C_5H_5N$, 2. KI/Me_2CO, 3. $CH{\equiv}CH/NaNH_2$, 4. $Cl.(CH_2)_5.I/NaNH_2$, 5.(i)NaI/NaCN (ii)NaOH, 6. H_2/ Lindlar's Catalyst.

(4). Synthesis of Octadeca-7:11-dienoic Acid.

This acid which has not previously been synthesised contains a 1:5-hexadiene group and is thus similar to the iso-linoleic type of molecule observed by Riley[6] during the partial hydrogenation of methyl parinarate. A synthesis of an acid containing the 1:5-hexadiene system, trideca-5:9-dienoic acid, has previously been reported by de Surville,

Rivett and Sutton[7].

Hexa-1:5-diyne was prepared according to the method of Raphael and Sondheimer[8] by the bromination and dehydrobromination of 1:5-hexadiene. Dodeca-1:5-diyne was prepared by coupling n-hexyl bromide to the mono-sodium derivative of 1:5-hexadiyne in liquid ammonia and was isolated as a pleasant smelling liquid.(25% yield) This was then coupled to 1:5-chloroiodopentane, via its sodium deriative, to give 1-chloroheptadeca-6:10-diyne which was converted to octadeca-7:11-diynoic acid by the usual sequence of reactions involving sodium iodide, sodium cyanide and sodium hydroxide. Partial reduction of the diyne acid with Lindlar's catalyst gave octadeca-cis-7:cis-11-dienoic acid as a low melting oil.

$CH_2{:}CH.(CH_2)_2.CH{:}CH_2 \xrightarrow{1} CH_2Br.CHBr.(CH_2)_2.CHBr.CH_2\,Br \xrightarrow{2}$
$CH{:}C.(CH_2)_2.C{:}CH \xrightarrow{3} CH_3.(CH_2)_5.C{:}C.(CH_2)_2.C{:}CH \xrightarrow{4}$
$CH_3.(CH_2)_5.C{:}C.(CH_2)_2.C{:}C.(CH_2)_5.Cl \xrightarrow{5}$
$CH_3.(CH_2)_5.C{:}C.(CH_2)_2.C{:}C.(CH_2)_5.COOH \xrightarrow{6}$
$CH_3.(CH_2)_5.CH{:}CH.(CH_2)_2.CH{:}CH.(CH_2)_5.COOH$

Reagents :- 1. Br_2/Et_2O, 2. $NaNH_2/liq.NH_3$, 3. $CH_3.(CH_2)_5.Br/NaNH_2$, 4. $Cl.(CH_2)_5.I/NaNH_2$, 5.(i)NaCN/NaI (ii)NaOH, 6. H_2/Lindlar's cat.

(5) Synthesis of Hexadeca-8:10-dienoic Acid.

This conjugated acid has not previously been prepared. n-Pentyl bromide was coupled to sodium acetylide in liquid ammonia to give hept-1-yne which was converted to 1-bromohept-1-yne by the

action of sodium hypobromite. 1:6-chloroiodohexane was prepared from hexamethylene glycol by the method already outlined for chloroiodopentane (p144) and was coupled to sodium acetylide to give 8-chloro-oct-1-yne. This latter was converted to nonan-8-ynoic acid by the usual sequence of reactions with sodium iodide, sodium cyanide and sodium hydroxide. Hexadeca-8:10-diynoic acid was then prepared by coupling together 1-bromohept-1-yne and nonan-8-ynoic acid in methylamine with a cuprous chloride catalyst according to the method of Chodkiewicz[9]. (86% yield) The diynoic acid has a melting point of 32-34° and an ultra-violet spectrum as reported in Table 23. The spectrum shows four peaks and is similar to that reported for undeca-3:5-diynoic acid by Meisters and Wailes[10], but differs from the spectra rported for the C_{17}, C_{18} and C_{19} diynoic acids by Weedon[11].

Table 23. Ultra-violet Spectra of some Diynoic Acids.

ACID	REF.	λÅ	ε	λÅ	ε	λÅ	ε	λÅ	ε
Hexadeca-8:10-diynoic[1]	-	2150	358	2260	419	2390	413	2540	238
Undeca-3:5-diynoic[2]	10	2135	500	2245	490	2380	400	2525	230
Heptadeca-10:12-diynoic[2]	11	-		2280	575	2350	445	-	
Octadeca-9:11-diynoic[2]	11	2230	430	2280	430	2360	320	-	
Nonadeca-10:12-dinoic[2]	11	-		2270	470	2370	320	-	

1. In cyclo-hexane. 2. In alcohol.

Crystals of the hexadecadiynoic acid exhibit photolability, previously observed with other diacetylenes by Jones and Whiting[12]. On exposure to daylight the crystal surface becomes covered in a

vivid red polymer insoluble in the liquid acid and the more common organic solvents. Partial reduction of the diyne acid with Lindlar's catalyst gave hexadeca-cis-8:cis-10-dienoic acid as a low melting oil. The ultra-violet of the dienoic acid in methanol showed a single peak at 2340 Å with $\epsilon = 3.2 \times 10^5$.

$CH_3.(CH_2)_4.Br \xrightarrow{1} CH_3.(CH_2)_4.C{:}CH \xrightarrow{2} CH_3.(CH_2)_4.C{:}C.Br$ ..I

$HO.(CH_2)_6.OH \xrightarrow{3} Cl.(CH_2)_6.Cl \xrightarrow{4} Cl.(CH_2)_6.I \xrightarrow{5} Cl.(CH_2)_6.C{:}CH \xrightarrow{6} HOOC.(CH_2)_6.C{:}CH$ ...II

$I + II \xrightarrow{7} CH_3.(CH_2)_4.C{:}C.C{:}C.(CH_2)_6.COOH \xrightarrow{8}$

$CH_3.(CH_2)_4.CH^{c}{:}CH.CH^{c}{:}CH.(CH_2)_6.COOH$

Reagents :- 1. $CH{:}CH/NaNH_2$, 2. NaOBr, 3. $SOCl_2/C_5H_5N$, 4. KI/Me_2CO, 5. $CH{:}CH/NaNH_2$, 6. (i)NaI/NaCN (ii)NaOH, 7. $MeNH_2/H_2O/CuCl/NH_2OH$, 8. Lindlar's catalyst / H_2.

EXPERIMENTAL.

(1) Preparation of 9:10-Epoxystearic Acid[3].

Purification of Oleic Acid[1].

Oleic acid (150g.) was dissolved in methanol (1.5 l.) and allowed to stand at -20° for 24 hrs. (Dry ice / acetone). The white precipitate (7.5g.) was removed by suction at a cooled Büchner funnel and remained solid at room temperature. The filtrate was cooled to -50° for 24 hrs. and a further crop of crystals (92g.) was collected on the Büchner funnel. This precipitate melted to a pale yellow oil at room temperature and was taken as the purified oleic acid. The residue (49g.) remained a dark brown liquid at 0°.

Preparation of Perbenzoic Acid[2].

In a 1 litre, three-necked flask fitted with a thermometer, a sintered-glass gas bubbler and a reflux condenser cooled with dry ice and leading to a vacuum line, benzaldehyde (52g.) was dissolved in acetone (400ml.) The flask was partially immersed in an ice-bath and irradiated with a 1000w. mercury lamp, whilst a constant stream of dry air was drawn through the solution for 6hrs. Reaction temperature 5-10°. The peroxide content of the solution was determined at intervals by withdrawing 5ml. samples and estimating the iodine liberated from acetic acid/potassium iodide solution, with standard sodium thiosulphate. The content of perbenzoic acid was 36%.

Epoxidation of Oleic Acid.

Purified oleic acid (35g.) was dissolved in perbenzoic acid solution (400ml.,0.178 mole. active oxygen.) at 0°. After standing at room temperature for 40 hrs. the peroxide content of the reaction mixture was determined and it was found that 93% of the peroxide had reacted. The solution was cooled to -50° for 6 hrs. ,filtered and the precipitate washed with cold acetone, to give crude epoxystearic acid. (36.7g.) Two crystallisations of this material from acetone (10ml. per g.) at -25° yielded the pure epoxy acid. (19.4g. 53%.,m.p.55.5-56°, %oxirane oxygen 5.37. calc. 5.36.)

(2) Preparation of Nonadeca-10:13-dienoic Acid[4].

Pure linoleic acid (800mg.) was mixed with oxalyl chloride (10g.)

at -20° in an atmosphere of nitrogen, and was occasionaly swirled during a period of 72 hrs. at 4°. Excess of oxalyl chloride was removed under vacuum at room temperature. The product was taken up in dry ether (10ml.) and added to a large excess of diazomethane[13],(prepared from N-nitrosomethylurea 2.0g.) in dry ether at -75°. The mixture stood under nitrogen for 2 hrs. at 0° and the ether and excess diazomethane were then removed under vacuum at room temperature. The diazoketone was taken up in methanol (10ml.) and dry ether (8ml.) and added dropwise to a solution of silver benzoate (200mg.) in triethylamine (2ml.)[14] The black suspension, from which nitrogen was evolved, was allowed to stand under nitrogen for 2 hrs. at room temperature. The solution was then taken up in light petroleum (b.p. 40/60°) and was washed twice with 2N hydrochloric acid, twice with 2N sodium hydroxide and finally with water and dried over anhydrous magnesium sulphate. The petrol was removed under vacuum and an ether solution of the product allowed to percolate through a column of charcoal (15cm. x 0.5cm.) supported on a thin bed of siliconised Hyflo super cel. Evaporation of the eluate gavethe ester as a colourless oil, (658mg.,75%), which was saponified by refluxing with 0.5N alcoholic potassium hydroxide for ½ hr. The acid was recovered by ether extraction and stored at 0° under nitrogen until required. Gas chromatographic analysis of the fully reduced ester indicated that the homo-linoleic acid contained 10% of unreacted linoleic acid.

(3). Synthesis of Tetradec-7-enoic Acid.

1:5-Dichloropentane.

Penta methylene glycol (100g.) and dry pyridine (14ml.) were placed together in a 1 litre flask and redistilled thionyl-chloride (290ml.) was added slowly to the well cooled and stirred mixture, at such a rate that the temperature remained at 25°. After the addition, the flask and contents were heated to 100° on a steam bath for 2 hrs. Ice and water were carefully added and the precipitated oil taken up with light petroleum. (b.p.40/60°). The extract was washed twice with 50% sulphuric acid and once with sodium hydrogen carbonate solution, dried over anhydrous sodium sulphate and evaporated. The residue on distillation gave 1:5-dichloropentane. (119.5g.,87%.,b.p.78-81°/20mm., lit.[15] b.p. 79-80°/21mm., n_D^{20} 1.4555, lit.[16] n_D^{20} 1.4563.)

1:5-Chloroiodopentane.

1:5-Dichloropentane (119.5g.) was added to a solution of sodium iodide (127g.) in dry acetone (780ml.) and heated on a steam-bath for 6 hrs. During the latter stages of the reaction violent bumping occured as the precipitate of sodium chloride formed. Water (900ml.) was then added and the precipitated oil was taken up in light petroleum. After being washed with water and dried over anhydrous sodium sulphate the extract was evaporated and the residue distilled through a 1m. helix-packed fractionating column to give (i) unchanged dichloropentane (b.p. 79-80°/20mm.), (ii) 1:5-chloro-

iodopentane (b.p. 75-77°/4mm., lit.[15] b.p. 75.8/4mm., n_D^{20} 1.5280, lit.[16] n_D^{20} 1.5304, 100g., 51%.) and (iii) 1:5-di-iodopentane which was not distilled.

Oct-1-yne

Liquid ammonia (500ml.) was placed in a 1 litre three-necked flask fitted with a stirrer and a gas inlet tube, and was cooled to -35° by placing in a Dewar containing dry-ice and acetone. A rapid stream of acetylene, which was purified by passing through a dry-ice trap and two wash-bottles containing concentrated sulphuric acid, was passed through the ammonia. It is advisable to place also in the acetylene line a mercury blow-off valve and an empty gas wash-bottle, in reverse prior to the reaction vessel, in case of a suck-back. Sodium (11.5g., 100% excess) was then added according to the method of Campbell and Campbell,[17] at such a rate that the solution did not turn blue. After the addition of all the sodium the acetylene was shut off and 1-bromohexane (41.3g.) was added during ½ hr. and the mixture stirred for an additional 3 hrs. Ammonium chloride (15g.), water (150ml.) and ether were added and the ammonia allowed to evaporate. The aqueous layer was extracted with ether and the combined organic extracts were washed with 2N sulphuric acid, sodium hydrogen carbonate solution and dried over anhydrous sodium sulphate. The solvent was removed through a 1 m. fractionating column and the fraction b.p. 75-78°/150mm. was collected as oct-1-yne. (9.9g., 36%., n_D^{20} 1.4200., lit.[17] b.p. 76-77°/150mm., n_D^{20} 1.4157.)

1-Chlorotridec-6-yne

Oct-1-yne (10g.) in dry ether (25ml.) was slowly added to a stirred suspension of sodamide in liquid ammonia (250ml.) prepared from sodium (2.1g.) in the presence of ferric nitrate catalyst (50mg.) by the procedure of Vaughn, Vogt and Nieuwland,[18] and cooled with dry-ice and acetone. After stirring for 3 hrs. 1:5-chloroiodopentane (21g.) in dry ether (25ml.) was added dropwise and the reaction mixture stirred for a further 9 hrs. The reaction mixture was allowed to stand overnight without external cooling when most of the ammonia evaporated, and the sodium-complex was then decomposed with ammonium chloride (10g.) and water. The product was extracted with ether and the organic extract washed with 2N sulphuric acid, sodium hydrogen carbonate solution and water before being dried over anhydrous sodium sulphate. Removal of the ether and fractional distillation of the residue gave (i) a fraction b.p.68-70°/0.7mm., n_D^{20} 1.5020. (unreacted iodo-compound) and (ii) 1-chlorotridec-6-yne (b.p.106-108°/1mm., n_D^{20} 1.4625, 8.25g. 42%. lit.[5] b.p. 112-113°/2mm., n_D^{20} 1.4593.)

Tetradec-7-ynoic Acid.

1-Chlorotridec-6-yne (8.25g.) was dissolved in 80% aqueous ethanol (85ml.) containing sodium iodide (8.5g.) and sodium cyanide (8.5g.). The mixture was refluxed for 48 hrs. Potassium hydroxide (21g.) in water (73ml.) was then added and the mixture was refluxed for a further 48 hrs. The reaction mixture was diluted with an

equal volume of water and unwanted neutral material extracted with light petroleum. 30% Sulphuric acid was then added whilst maintaining the temperature below 25° and the liberated acids were extracted with light petroleum. The extract was washed with water, dried over anhydrous sodium sulphate and evaporated to give the crude acid (7.34g., 86%.) which was distilled through a vacuum-jacketed Vigreux column to give pure tetradec-7-ynoic acid.(b.p. 126-128°/10^{-4}mm., m.p. 30-31°. lit.[5] m.p. 29.5-30°.)

Tetradec-cis 7-enoic Acid.

Tetradec-7-ynoic acid (2.00g.) was dissolved in ethyl acetate (25ml.) and quinoline (0.25g.) and was semi-reduced by shaking in an atmosphere of hydrogen with Lindlar's catalyst.[19](2g.) The hydrogen uptake was 225.0 ml. at 736mm./20° corresponding to 1.02 mole. The catalyst was removed by centrifuging and the quinoline by washing with 2N hydrochloric acid. The tetradec-cis-7-enoic acid was recovered as a low melting oil and was stored under nitrogen at 0° until required. (Iodine value 111.3, calc. 112.3. Partial oxidation of the acid gave heptanoic, pimelic and myristic acids only).

(4). Synthesis of Octadeca-7:11-dienoic Acid.
1:2:5:6-Tetrabromohexane.

Bromine (97.5g.) was added slowly to a well cooled and stirred solution of diallyl (25g.) in ether (50ml.) Removal of the solvent gave 1:2:5:6-tetrabromohexane. (118g.,96%., m.p. 51-52°, lit.[21] m.p. 53-54°).

Hexa-1:5-diyne.

A suspension of sodamide in liquid ammonia (500ml.) was prepared from sodium (54g.) in the presence of the ferric nitrate catalyst.[18] Tetrabromohexane (118g.) in dry ether (300ml.) was added dropwise during 2 hrs. to the cooled and stirred suspension, which was stirred for a further 3 hrs. The reaction mixture was set aside overnight without cooling when most of the ammonia evaporated. Ether, ice and water were added to the residue, the aqueous layer was washed with ether and the combined organic extracts washed with 2N sulphuric acid, sodium hydrogen carbonate solution and water and dried over anhydrous sodium sulphate. The ether was removed through a 1 m. fractionating column followed by distillation of the dipropargyl as a colourless liquid. (13.6g.,58%.,b.p. 86-88°/760mm., n_D^{20} 1.4385. lit.[22] b.p. 85-86°/760mm.).

Dodeca-1:5-diyne.

Hexa-1:5-diyne (13.6g.) in dry ether (15ml.) was slowly added to a cooled and stirred suspension of sodamide in liquid ammonia (250ml.) prepared from sodium (4.4g.) in the presence of ferric nitrate catalyst.[18] After stirring for 1 hr. n-hexyl bromide (39g.) in ether (40ml.) was added during 30 mins. and the reaction mixture was stirred for a further 6 hrs. Ammonium chloride (15g.) was then added, the ammonia evaporated and the product isolated by ether extraction. Distillation of the extract through a 1 m. fractionating column gave (i) a mixture of dipropargyl and hexyl bromide (b.p.

68-70°/20mm.) and (ii) dodeca-1:5-diyne, a pleasant smelling mobile liquid. (6.81g., 25%., b.p. 100°/20 mm., n_D^{20} 1.4545.)

1-Chloroheptadeca-6:10-diyne.

Dodeca-1:5-diyne (6.81g.) in dry ether (10ml.) was slowly added to a stirred suspension of sodamide in liquid ammonia (200ml.) prepared from sodium in the presence of ferric nitrate catalyst.[18] After stirring for 2 hrs. 1:5-chloroiodopentane (10g.) in dry ether (10ml.) was added dropwise and the solution stirred for 8 hrs. Ammonium chloride (10g.), water and ether were added and the product extracted with ether. The combined organic extracts were washed with 2N sulphuric acid, sodium hydrogen carbonate solution and water and dried over anhydrous sodium sulphate. Removal of the ether followed by distillation through a short Vigreux column gave two fractions ; (i) a mixture of dodecadiyne and chloroiodopentane (b.p.58-60°/0.7mm. n_D^{20} 1.4680) and (ii) 1-chloroheptadeca-6:10-diyne.(2.2g., 20%., b.p. 140-142°/0.5mm., n_D^{20} 1.4725.)

Octadeca-7:11-diynoic Acid

1-Chloroheptadeca-6:10-diyne (2.2g.), sodium iodide (2.3g.) and sodium cyanide (2.3g.) were refluxed together in 80% aqueous ethanol (25ml.) for 48 hrs. Potassium hydroxide (7.7g.) in water (20ml.) was then added and the mixture refluxed for a further 48 hrs. The mixture was diluted with an equal volume of water and unwanted neutral material extracted with light petroleum. 30% Sulphuric acid was then added

keeping the temperature below 25° and the liberated acids were extracted with light petroleum. The extract was washed with water, dried over anhydrous sodium sulphate and evaporated to give the crude octadecadiynoic acid. (1.65g.,72%.) The acid was distilled through a 10cm. vacuum-jacketed Vigreux column to give octadeca-7:11-diynoic acid.(1.46g.,64%., b.p. 158-160°/10^{-4}mm., m.p. 48-48.5°.) Micro-hydrogenation of the acid indicated 8.1 hydrogens short of saturation: whilst gas chromatography of the methylated, hydrogenated acid indicated a single peak at stearic acid.

Octadeca-cis-7:cis-11-dienoic Acid.

Octadeca-7:11-diynoic acid (1.00g.) was dissolved in ethyl acetate (15ml.) and quinoline (0.1g.) and was semi-hydrogenated by shaking in an atmosphere of hydrogen with Lindlar's catalyst.(1.0g.) The hydrogen uptake was 172 ml. at 749mm./20° corresponding to 1.95 moles. Removal of the catalyst and solvent gave octadeca-cis-7:cis-11-dienoic acid as a low melting oil which was stored at 0° under nitrogen until required. (Iodine value 178.9, calc. 181.0). Partial oxidation[20] of the acid gave a mixture of heptanoic, pimelic, undecanedioic and stearic acids only.

(5). Synthesis of Hexadeca-8:10-dienoic Acid.

Hept-1-yne.

Hept-1-yne was prepared by reacting n-pentyl bromide (76g.) with sodium acetylide prepared from sodium (11.5g.) in liquid ammonia (500ml.) and acetylene using the method reported for the

preparation of octyne on p.151. The ether was removed from the extracted product through a 1 m. fractionating column followed by distillation of the hept-1-yne.(12g.,67%., b.p.95-105°, n_D^{20} 1.4080, lit.[17] b.p.99°, n_D^{20} 1.4088.)

1-Bromohept-1-yne.

Preparation according to the method of Strauss, Kollek and Heyn.[23] Ice (50g.) was placed in a 250 ml. flask fitted with an efficient stirrer. 10N Sodium hydroxide (25ml.) and bromine (5.5ml.) were then added followed by heptyne (9.6g.) and stearic acid. (300mg.) The emulsion was stirred violently for 18 hrs. after which time the organic material was extracted with ether. Ether and unreacted heptyne were removed by fractional distillation and the residue distilled to give 1-bromohept-1-yne. (16.6g.,95%.,b.p.55°/10.5mm., n_D^{20} 1.4598, lit.[24] n_D^{20} 1.4625.)

1:6-Chloroiodohexane.

1:6-Chloroiodohexane was prepared in an analogous manner to 1:5-chloroiodopentane (p. 150) starting from hexamethylene glycol.

8-Chloro-oct-1-yne.

To a stirred and cooled solution of sodium acetylide prepared from sodium (5.5g.) in liquid ammonia and acetylene as described on p. 152, was added 1:6-chloroiodohexane (59.1g.) over a period of ½ hr. The mixture was stirred for a further 4 hrs. and ammonium chloride (20g.), water and ether were added and the ammonia allowed to boil off. The aqueous layer was extracted with ether and the combined

organic extracts washed with 2N sulphuric acid, sodium hydrogen carbonate solution and dried over anhydrous sodium sulphate. The ether was evaporated and the residue distilled to give 8-chloro-Oct-1-yne. (28.5g., 83%., b.p. 73-76°/10mm., n_D^{20} 1.4560, lit.[25] b.p. 73-76°/10mm., n_D^{20} 1.4590.)

Nonan-8-ynoic Acid.

The 8-chloro-oct-1-yne (28.5g.), sodium iodide (30g.) and sodium cyanide (30g.) wererefluxed together in 80% aqueous ethanol (300ml.) for 48 hrs. Potassium hydroxide (100g.) was then added in water (250ml.) and the mixture refluxed for a further 48 hrs. The mixture was then diluted with an equal volume of water and unwanted neutral material extracted with light petroleum. 30% Sulphuric acid was then added whilst maintaining the temperature below 25° and the liberated acids extracted with light petroleum. Evaporation of the petrol gave nonan-8-ynoic acid which crystallised in large needles. (28.5g., 98%., m.p. 18-20°. lit.[26] m.p. 19°.)

Hexadeca-8:10-diynoic Acid.

The synthesis is by the method of Chodkiewicz.[9] A solution of 1-bromohept-1-yne (1.75g.) in methanol was added during 15 mins. to a solution of nonan-8-ynoic acid (1.54g.) and cuprous chloride (60mg.) in 30% aqueous methylamine (25ml.) under nitrogen. Hydroxylamine hydrochloride was added in small portions as the reaction proceeded to ensure that the copper remained in its reduced form. After the addition was completed stirring was continued for a further 5 mins.

then aqueous potassium cyanide was added and the mixture extracted with ether; this extract being rejected. Theacid was liberated by adding 2N sulphuric acid and extracted with ether, The extract was washed with water, dried with anhydrous sodium sulphate and evaporated to give the crude acid (2.48g.,81%.) which was distilled to give hexadeca-8:10-diynoic acid as a white crystalline solid. (m.p.32-34°) The crystalline acid turned red on exposure to daylight.[12] A micro-hydrogenation of the acid indicated 7.7 hydrogens short of saturation, whilst a gas chromatogram of the fully reduced methylated acid indcated a major peak at palmitic acid and a minor peak at nonanoic acid. (3%.).

Hexadeca-cis-8:cis-10-dienoic Acid.

Hexadeca-8:10-diynoic acid (297.0mg.) was dissolved in ethyl acetate (10ml.) and quinoline (50mg.) and was semi-hydrogenated by shaking in an atmosphere of hydrogen with Lindlar's catalyst. (300mg.) The hydrogen uptake was 58 ml. at 745mm./15° corresponding to 1.01 mole. The solvent and catalyst were removed to give hexadeca-cis-8:cis-10-dienoic acid as a low melting oil which was stored at 0° under nitrogen until required. Partial oxidation[20] of the acid gave the following products ; hexanoic, octanoic, suberic, decanedioic and palmitic acids, with a trace of nonanoic acid.

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