THE CHEMISTRY OF NATURALLY OCCURRING LONG CHAIN UNSATURATED COMPOUNDS

PART I - HIGHLY UNSATURATED ACIDS FROM THE BODY OIL OF THE SOUTH AFRICAN PILCHARD

by

M.H. SILK. M.Sc., A.R.I.C.

Part I of a thesis presented for the degree of Doctor of Philosophy in the Faculty of Science of Rhodes University

February 1954

PART I - HIGHLY UNSATURATED ACIDS FROM THE BODY OIL OF THE SOUTH AFRICAN PILCHARD

I N D E X

CHAPTER 1	Introduction	1
CHAPTER 2	The Nature of Marine Oils and Previous Work Concerning their Composition	5
CHAPTER 3	Preliminary Experiments on South African Pilchard Oil	13
CHAPTER 4	The Resolution of Mixtures of C_{16} - C_{24} Normal Chain Saturated Fatty Acids by Reversed Phase Partition Chromatography	25
CHAPTER 5	The Resolution of Unsaturated Acid Mixtures by Reversed Phase Partition Chromatography	34
CHAPTER 6	Comparison of Methods for the Preparation of Concentrates of Highly Unsaturated Acids from Pilchard Oil	38
CHAPTER 7	An Approach to the Analysis of Pilchard Oil	45
CHAPTER 8	The Isolation of a New Fatty Acid from South African Pilchard Oil . . .	56

EXPERIMENTAL SECTION

EXPERIMENTAL DETAILS Arrangement 63

SECTION A

1) Saponification of Crude Bleached Pilchard Oil . 64

2) Lithium Soap-Acetone Segregation of the More Highly Unsaturated Acids 64

3) Molecular Distillation of Pilchard Unsaturated Acids 65

4) Counter-Current Distribution of a Pilchard Acid Mixture 65

5) Fractional Low Temperature Crystallisation of a Mixture of Saturated Pilchard Acids 66

6) Paper Chromatography of Pilchard Acid Mixtures. 66

7) Separation of Lauric and Myristic Acids by Paper Partition Chromatography 67

SECTION B

1) Preparation of Non-Wetting Kieselguhr 68

2) Preparation of Acid-free Medicinal Paraffin . . 68

3) a) Electrolytic Synthesis of Arachidic Acid . . 69

b) Electrolytic Synthesis of Behenic Acid . . . 70

4) X-Ray Long Spacings of C_{16} - C_{24} Saturated Fatty Acids 70

5) Preparation of Hydrophobic Kieselguhr-Medicinal Paraffin Mulls, and the Packing of Chromatographic Columns 70

6) Loading of Chromatographic Columns 72

7) Siphon for Collection of Column Eluate 73

8) Titration of Chromatographic Column Eluates . . 73

9) Chromatographic Procedure 74

10) Calculation of Results 74

SECTION C

1) Preparation of Pure Oleic and Linoleic Acids . . 76

2) Reversed Phase Partition Chromatography

a) Mixture of Oleic and Linoleic Acids 76

b) Mixture of Oleic and Palmitic Acids 77

c) Mixtures of Unsaturated Pilchard Acids at Different Temperatures 77

SECTION D

1) Lithium Soap-Acetone Segregation of Total Pilchard Acids 78

2) Urea Complex Fractionation of the Lithium Soap-Acetone Concentrate 78

3) Determination of Average Unsaturation of Acid Mixtures by Micro-Hydrogenation 80

4) Chain Length Analysis of Acid Mixtures by Reversed Phase Partition Chromatography 81

SECTION E

1) Molecular Distillation of the Lithium Soap-Acetone Concentrate of Pilchard Unsaturated Acids 82

2) Reversed Phase Partition Chromatography of Pilchard Unsaturated Acid Mixtures from Molecular Distillation 83

3) Chromatographic Chain Length Analyses of Acid Mixtures from Molecular Distillation 83

4) Examination of Unsaturated Acid Mixtures for Evidence of Autoxidation

a) Qualitative Ferrous Thiocyanate Test 83

b) Ultra-Violet Examination 83

c) Infra-Red Examination and Method for Calculation of Percentage Oxidation 84

5) Alkali Isomerisation of Pilchard Unsaturated Acid Mixtures 87

a) Preparation of Reagent 87

b) Reaction Tubes 87

c) Procedure 88

6) Urea Complex Fractionation of a Mixture of Pilchard Unsaturated Acids 88

7) Determination of Average Unsaturation of Acid Mixtures by Micro-Hydrogenation 89

SECTION F

1) Preparation of a Concentrate of Pilchard Unsaturated Acids 91

2) Molecular Distillation of the Unsaturated Acid Concentrate 91

3) Determination of Average Unsaturation of Acid Mixtures by Micro-Hydrogenation 92

4) Reversed Phase Partition Chromatography of a Pilchard Unsaturated Acid Mixture (Preparative Scale) 92

5) Recovery of a Hexadecatetraenoic Acid from the Chromatographic Column Eluate

a) Carbon and Hydrogen Analysis 93

b) Equivalent Weight 93

c) Unsaturation 93

d) Hydrogenation Product 94

e) Equivalent Weight of Hydrogenation Product . 94

6) Ultra-Violet Light Absorption of the Hexadecatetraenoic Acid 94

7) Alkali Isomerisation of the Hexadecatetraenoic Acid 95

8) Infra-Red Absorption of the Hexadecatetraenoic Acid 95

9) Permanganate Oxidation of the Hexadecatetraenoic Acid 95

10) Paper Chromatography of the Permanganate Oxidation Products of the Hexadecatetraenoic Acid 96

11) Note on the Composition of an Acid Mixture Isolated from the Second Peak of the Chromatogram 97

SUMMARY

PUBLICATIONS

ACKNOWLEDGEMENTS

REFERENCES are to be found at the end of each Chapter and at the end of each Section of the Experimental Details

HIGHLY UNSATURATED ACIDS FROM THE BODY OIL OF THE SOUTH AFRICAN PILCHARD.

CHAPTER 1.

INTRODUCTION.

Ample evidence exists that the people of early civilisations were familiar with the use of fats and waxes. Klemgard (1) mentions the fact that the Egyptians used olive oil as a lubricant for moving large stones, statues and building materials, and that greases consisting of a fat and lime, together with other materials, were used for lubricating the axles of Egyptian chariots as early as 1400 B.C. Friedel (2) analysed the contents of a number of earthenware vessels found in Egyptian tombs and showed that these contained natural fats such as palm oil and mutton tallow, presumably intended as provisions for the dead. The use of soap is referred to by Pliny (23 - 79 A.D.), but was undoubtedly known long before his time. Candles made of tallow were used by the Romans, while the spreading of oil upon the surface of water appears to have been known by Greek sailors and was applied by them in subduing waves during storms.

The purposes for which fats and oils are used today in modern technology are primarily the same as those for which they were employed in antiquity. Aside from their use as foodstuffs, they are consumed in the production of paints, varnishes and lacquers, cosmetics, pharmaceuticals, soaps, lubricants, fuels and illuminants, while more recent uses have been in the manufacture of textile processing aids, synthetic resins and rubbers, insecticides, weed killers and de-emulsification agents for the production of petroleum oil. The number of products derived from natural fats and oils becomes increasingly larger each year, and nations have been more than ever concerned in taking

steps to ensure adequate supplies of these valuable raw materials.

In South Africa some two and a quarter million pounds worth of fats, oils and waxes were imported during 1951, but since then the amount has been considerably decreased owing to the expansion in our own production of these commodities. Locally produced castor oil and sunflower seed oil are being used to an increasing extent, while the expansion of the South African fishing industry has in large measure led to a replacement in the use of imported linseed oil by refined marine drying oils in the manufacture of paint.

The principal South African marine oil comes from the pilchard (Sardina ocellata Jenyns) which occurs in large shoals along the coast from Cape Town to Lamberts Bay. This area is under the influence of the cold and phosphate rich Benguella current which originates in the Antarctic and wells up against the continental shelf near the mouth of the Orange River. As this current rises into the photo-synthetic zone of the ocean, conditions are ideal for the growth of plankton, and the consequent abundance of marine life has earned description of the coastline as "The World's Richest 100 Miles of Sea" (3). The Union's Pilchard Fishery is principally centered on this area, while other rich fields occur further north, near Walvis Bay.

The fish are caught in nets from trawlers operating at night, and are delivered to the nine processing factories along the coast where they are pumped into the bins feeding the fish meal plants. After cooking under steam pressure for 20 minutes they are discharged into conical screw presses which force out the body oil and stickwater leaving the meal which is further dried and sold to balanced feed manufacturers. The liquid discharge from

the presses is passed through filtering screens to remove a certain amount of suspended solid matter and is then centrifuged to recover the oil. After washing a second time in the centrifuge, the oil is ready for despatch to the refinery either in drums or by means of 15 ton road tankers.

Raw marine oils are subject to considerable variation in composition with season and are moreover very unbalanced oils for direct use in the paint industry. Further refining is undertaken chiefly by Messrs Marine Oil Refiners of Africa Ltd., whose factory is situated at Simonstown. In their plant, marine oils and others are treated by the Solexol process (4) involving counter-current extraction of the oil with liquid propane in a tower over which a temperature gradient is maintained. The process achieves the removal of a large proportion of the relatively saturated glycerides, the natural antioxidants, and the relatively highly unsaturated components, all of which are detrimental to the eventual formation of good paint films. The segregated marine oil from the Solexol process is then bodied or polymerised at high temperatures to yield a "drying oil" which is used as a substitute for bodied linseed oil in paints.

The chemical reactions taking place during the polymerisation and drying of these oils are of an extremely complex nature, and for their understanding it is necessary to have an accurate knowledge of the chemical nature of the component fatty acids in the natural glycerides. It is to this end that a Fellowship has been endowed by Messrs Marine Oil Refiners of Africa Ltd. at the laboratories of the South African Council for Scientific and Industrial Research in Pretoria. The author has been the holder of this award for the past two years and the results of the research he has undertaken are presented in the following pages.

The term "marine oils" in this work should be understood to mean marine fish body oils, marine mammal oils being excluded from consideration.

REFERENCES.

(1) E.N. Klemgard. Lubricating Greases. Their Manufacture and Use. Reinhold. New York (1937).

(2) C. Friedel. Compt. Rend. 124, 648 (1897).

(3) W.M. Neale-May. S. African Ind. Chemist 4, 416 (1950).

(4) A.L. Stubbs. S. African Ind. Chemist 5, 8 (1951).

CHAPTER 2.

THE NATURE OF MARINE OILS AND PREVIOUS WORK CONCERNING THEIR COMPOSITION.

Marine oils are probably the most complex of all naturally occurring fats. Saponification of these oils results in the liberation of a variety of fatty acids from their combination as glycerides, and also provides a fraction of so-called "non-saponifiable" material. Common constituents of the non-saponifiable portion are cholesterol, squalene, vitamin A, α-glyceryl ethers, saturated hydrocarbons and fatty alcohols representing, together with small amounts of other substances, some 2 - 7% of the total oil. The non-saponifiable fraction of marine oils is however of little importance in regard to their use as drying oils, and their application in the paint industry depends almost entirely upon the nature of the fatty acids present in the glyceride portion.

The Analysis of Marine Oil Fatty Acids.

The isolation and identification of the acids comprising a natural fat is generally a difficult process, but is rendered extremely complex in the case of marine oils by the diverse nature of the acids present. Lovern (1) in a report on the depot fats of aquatic animals records that acids of from 12 - 28 carbon atoms in chain length have been discovered, with degrees of unsaturation ranging from one to seven double bonds per molecule. Recent experience indicates that acids with all possible degrees of unsaturation may be present in a single oil, and that while certain acids may be common to many oils, there is no uniformity in the nature of the acids found in marine oils in general.

Owing to the extreme complexity of these materials no single method is universally applicable to their analysis.

In all cases several methods of differing applicability must be used in appropriate sequence to determine the composition or to isolate individual acids.

The Isolation of Individual Acids from Marine Oils.

Since the turn of the century, Japanese workers have consistently led the field in the isolation of marine oil fatty acids. Their work has been chiefly concerned with Japanese sardine oil which is closely similar in nature to pilchard oil.

Recent investigations of marine oils have been to a large extent inspired by the Japanese workers, but many of the results are open to considerable criticism on the grounds that the methods used for the isolation of individual acids have been such as might have caused extensive modification of structure during processing. While it is not intended to discount the existence of the acids reported, the assignment of double bond positions in the carbon chains cannot be accepted unreservedly. This is chiefly because the isolation of acids from mixtures by means of vacuum distillation (at 0.1 mm. pressure) involving prolonged heating during fractionation, is known to cause polymerisation and isomerisation reactions in the case of compounds with more than three double bonds per molecule (2). In all probability, therefore, certain of the acids obtained were heat altered, and the double bond positions assigned on the basis of permanganate oxidation or ozonolysis products, may not be those of the original compounds.

In recent years the use of vacuum distillation has been superseded by that of molecular distillation where highly unsaturated acids are concerned. While molecular distillation does not afford such high fractionation efficiency, the risk of isomerisation and decomposition is

greatly diminished. The use of falling film stills in which the distilland is subject to the minimum contact time with the heated surface, has been a modern development (3) replacing the older type of pot still in which the whole bulk of the distilland was heated for a considerable time. The very thin films obtainable with these stills permit the escape of substantially all of the molecules which are distillable at a given temperature, whereas in a pot still a high proportion of these molecules are, in general, not able to reach the surface of the large bulk of distilland within a reasonably short time. Farmer and Van den Heuvel (4a, b) applied falling film molecular distillation to the unsaturated acids of fish oils and claimed to have isolated docosahexaenoic acid (C_{22} hexaene) in a high state of purity, as well as to have separated the esters of C_{16}, C_{18} and C_{20} polyunsaturated acids with considerable accuracy.

Acids Isolated from Marine Oils.

The following list of acids isolated from marine oils gives some idea as to the variety of types that are encountered.

Saturated acids.

Most of the well known normal chain saturated acids have been isolated, e.g., lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), stearic (C_{18}), arachidic (C_{20}), behenic (C_{22}) and lignoceric (C_{24}).

Monethenoid acids.

Myristoleic (C_{14}) and palmitoleic (C_{16}) acids have been reported, the latter often in high proportion. Frequent, though minor constituents are gadoleic (C_{20}), cetoleic (C_{22}) and selacholeic (C_{24}) acids. The last named is also known as nervonic acid and is a common

constituent of brain cerebrosides. It is of interest here to note that the fatty acids of brain lipids are of a very similar nature to those occurring in marine oils (5).

Diethenoid acids.

Linoleic (C_{18}) acid which is usually a major component of natural oils, appears to be absent in all but a few marine oils and, where found, is generally considered to have a different configuration to that occurring in plant and animal fats (6). A C_{20} dienoic acid has been isolated from fish oil (7), but diethenoid acids do not appear to be common.

Triethenoid acids.

Hiragonic (C_{16}) acid has been found as a minor constituent of Japanese sardine oil (8). Linolenic (C_{18}) acid and its isomers have not been reported, although there is evidence that these acids do occur in marine oils. A C_{20} trienoic acid has been reported in the oil of a Mediterranean fish (7).

Tetraethenoid acids.

Among tetraethenoid acids, moroctic (C_{18}) acid has been isolated from sardine oil (9, 10), while arachidonic (C_{20}) acid appears to be a major constituent of most marine oils (11). The isolation of an unusual C_{16} tetraenoic acid from South African pilchard oil is described in Chapter 8. The name marinolic acid has been proposed for this compound.

Pentaethenoid acids.

Timnodonic (C_{20}) acid has been obtained from sardine oil (11, 12), while clupanodonic (C_{22}) acid is a major constituent of practically all fish oils (13).

Scoliodonic (C_{24}) acid and shibic (C_{26}) acid have been found as minor constituents of tunny and other oils (14, 15).

Hexaethenoid acids.

A C_{22} hexaenoic acid has been isolated from Japanese sardine oil (16) and from cod liver oil (4a, b). Two C_{24} hexaenoic acids, thynnic and nisinic, have also been obtained from Japanese sardine oil (17, 18).

Heptaenoic acids.

Among heptaenoic acids, those with C_{24} and C_{28} carbon skeletons have been reported to occur in several fish oils (1, 19).

While the list of acids isolated from marine oils appears a fairly large one, recent experience indicates that it represents but a small number of the full range of acids which actually occur in these materials. The unsaturation found in marine oil acids is of the non-conjugated type, and no conjugated members have ever been reported.

The Analysis of Marine Oils without Isolation of Individual Acids.

A large number of papers have appeared in the literature giving the analysis of marine oils as determined by the conventional Hilditch distillation method (19). While the method is entirely satisfactory in the case of relatively simple natural fats, it should be pointed out that in the case of marine oils it affords only an empiric assignment of unsaturation between the component chain lengths and does, moreover, suffer from a disadvantage in regard to thermal modification of the more labile components.

Little has been done to overcome the disadvantages of the Hilditch method as far as marine oils are concerned,

but an attempt has now been made in the case of South African pilchard oil and will be discussed later. In any attempt to provide an analysis of marine oils without isolation of individual acids, the difficulty is not so much in the choice of mild techniques in order to obviate structural modification, but in the accurate assignment of unsaturation between the amounts of each chain length present, i.e., the postulation of the number of acids having a given chain length and the degree of unsaturation of each.

Previous Work on South African Pilchard Oil.

Little previous work has been carried out on the body oil of the South African pilchard, and the only investigations of any significance are those of Black and Schwartz (20a, b). These authors carried out an analysis of the oil by the Hilditch method and reported the composition of a typical batch as follows:

	Saturated acids	Unsaturated acids
C_{12}	trace	-
C_{14}	5.6	2.3 (-2.0 H)
C_{16}	13.1	17.6 (-3.0 H)
C_{18}	1.4	16.2 (-4.3 H)
C_{20}	0.6	26.8 (-8.8 H)
C_{22}	0.3	16.2(-10.7 H)

Such an analysis throws little light on the chemical nature of the component acids, but does serve to indicate that by far the greater proportion are of an unsaturated nature. Neale May (21) has discussed the industrial processing of pilchard oil, while Joubert and Sutton (22) have investigated the properties of stand oils (polymeric oils) derived from it. Nevertheless, until the present investigation was undertaken in Pretoria, no pure acids had been isolated from pilchard oil and practically nothing was known of its composition.

The investigation has been conducted with the dual aim of isolating individual acids, and of providing a complete and unambiguous analysis of the oil without the necessity for the isolation of any components.

REFERENCES.

(1) J.A. Lovern. "The Composition of the Depot Fats of Aquatic Animals". Food Investigation Board, Special Report No. 51, H.M. Stationery Office, London (1942).

(2) F.A. Norris, I.I. Rusoff, E.S. Miller & G.O. Burr. J. Biol. Chem. 139, 199 (1941); ibid. 147, 273 (1943).

(3) D.A. Sutton. Chem. and Ind. 1383 (1953).

(4) E.H. Farmer & F.A. Van den Heuvel.
(a) J. Soc. Chem. Ind. 57, 24 (1938).
(b) J. Chem. Soc. 427 (1938).

(5) E. Klenk & W. Bongard. Hoppe Seyler's Zeitschrift für Physiol. Chemie 291, 104 (1952).

(6) K.S. Markley. "Fatty Acids". Interscience. New York. p.31 (1947).

(7) P. Baudart. Bull. Soc. Chim. 11, 174 (1944).

(8) Y. Toyama & T. Tsuchiya. Bull. Chem. Soc. Japan 10, 192 (1935).

(9) M. Tsujimoto. J. Coll. Eng. Tokio Imp. Univ. 4, No.1 (1906).

(10) J.B. Brown & G.D. Beal. J. Amer. Chem. Soc. 45, 1289 (1923).

(11) Y. Toyama & T. Tsuchiya. Bull. Chem. Soc. Japan 10, 241, 296 (1935).

(12) G. Sanna. Chem. Zentr. II, 2616 (1937).

(13) M. Tsujimoto & K. Kimura. J. Soc. Chem. Ind. Japan 26, 1162 (1923).

(14) S. Ueno & M. Iwai. ibid. 37, Sb. 251 (1934).

(15) S. Ueno & C. Yonese. J. Chem. Soc. Japan 57, 322 (1936).

(16) Y. Toyama & T. Tsuchiya. Bull. Chem. Soc. Japan 10, 539 (1935).

(17) S. Ueno & C. Yonese. J. Chem. Soc. Japan 57, 180 (1936).

(18) Y. Toyama & T. Tsuchiya. Bull. Chem. Soc. Japan 10, 543, 547 (1935).

(19) T.P. Hilditch. "The Chemical Constitution of Natural Fats". Chapman & Hall London, p.331 (1940).

(20) M.M. Black & H.M. Schwartz.
(a) J. Sci. Food & Agric. 8, 248 (1950).
(b) ibid. 6, 182 (1950).

(21) W.M. Neale-May. S. African Ind. Chemist 4, 146 (1950).

(22) F.J. Joubert & D.A. Sutton. J. Amer. Oil Chem. Soc. 29, 287 (1952).

CHAPTER 3.

PRELIMINARY EXPERIMENTS ON SOUTH AFRICAN PILCHARD OIL.

In this chapter an account is given of certain preliminary experiments carried out on South African pilchard oil. The investigations were of great value in indicating a possible approach towards an analysis of the oil, but were intended only to provide the basis for the fuller investigations reported in subsequent chapters.

In dealing with a complex mixture of fatty acids such as that obtained on saponification of a marine oil, the first essential is a means for subdivision of the mixture into fractions of a more homogeneous nature. Segregation of a concentrate of the more highly unsaturated acids can be achieved by means of the lithium soap-acetone procedure of Tsujimoto (1). The method depends upon the fact that the lithium salts of the more saturated acids are less soluble in 95% aqueous acetone than are the lithium salts of the relatively more unsaturated acids. Equilibration of the lithium salts of total marine oil acids with 95% aqueous acetone results therefore in precipitation of the more saturated soaps, leaving a concentrate of unsaturated material in solution. Applied to pilchard oil, this procedure afforded a concentrate representing 30% of the total and having an average unsaturation of approximately four and a half double bonds per molecule. The total pilchard acids had an average unsaturation of slightly less than three double bonds per molecule, so that a reasonably efficient separation between relatively saturated and unsaturated acids was achieved.

The concentrate of unsaturated acids obtained by the lithium soap-acetone method is, in the case of marine

oils, still a very complex mixture, and other means are required for its further subdivision into fractions of simpler composition. The technique which seemed most likely to be profitable in this respect was falling film molecular distillation (2), and this was accordingly investigated in application to the unsaturated acid concentrate from pilchard oil.

A three stage falling film still was available for this purpose (3), and in it the residue from each stage of distillation was redistilled at the next. The still is represented diagrammatically in Fig. I.

In operation, the still was evacuated to a pressure of 10^{-5}mm. with an oil diffusion pump, and each of the distilling stages heated by boiling a solvent of the required b.p. under reflux using a small electrical immersion heater. The distilland was then allowed to pass slowly through a needle valve from the reservoir to the degasser, where dissolved air and low boiling solvent were flashed off as the liquid filmed over a heated surface under vacuum. On entry to the first stage of the still, the liquid was distributed over the milled glass surface of the heater by means of a small distribution cap. As filming took place down the sides of the heater, distillation occurred, and the residue from the bottom was allowed to drip onto the second heated stage. The distillate condensed on the inside of the glass surface surrounding the heater and was allowed to drain into an inclined gutter from where it was led off to the receiver. The distillates from the second and third stages were collected similarly, and the residue from the final stage allowed to drip direct into the residue receiver.

In the first experiment, the heaters of the three stages were maintained at temperatures of 96°, 108° and 130° with boiling water, toluene and xylene respectively.

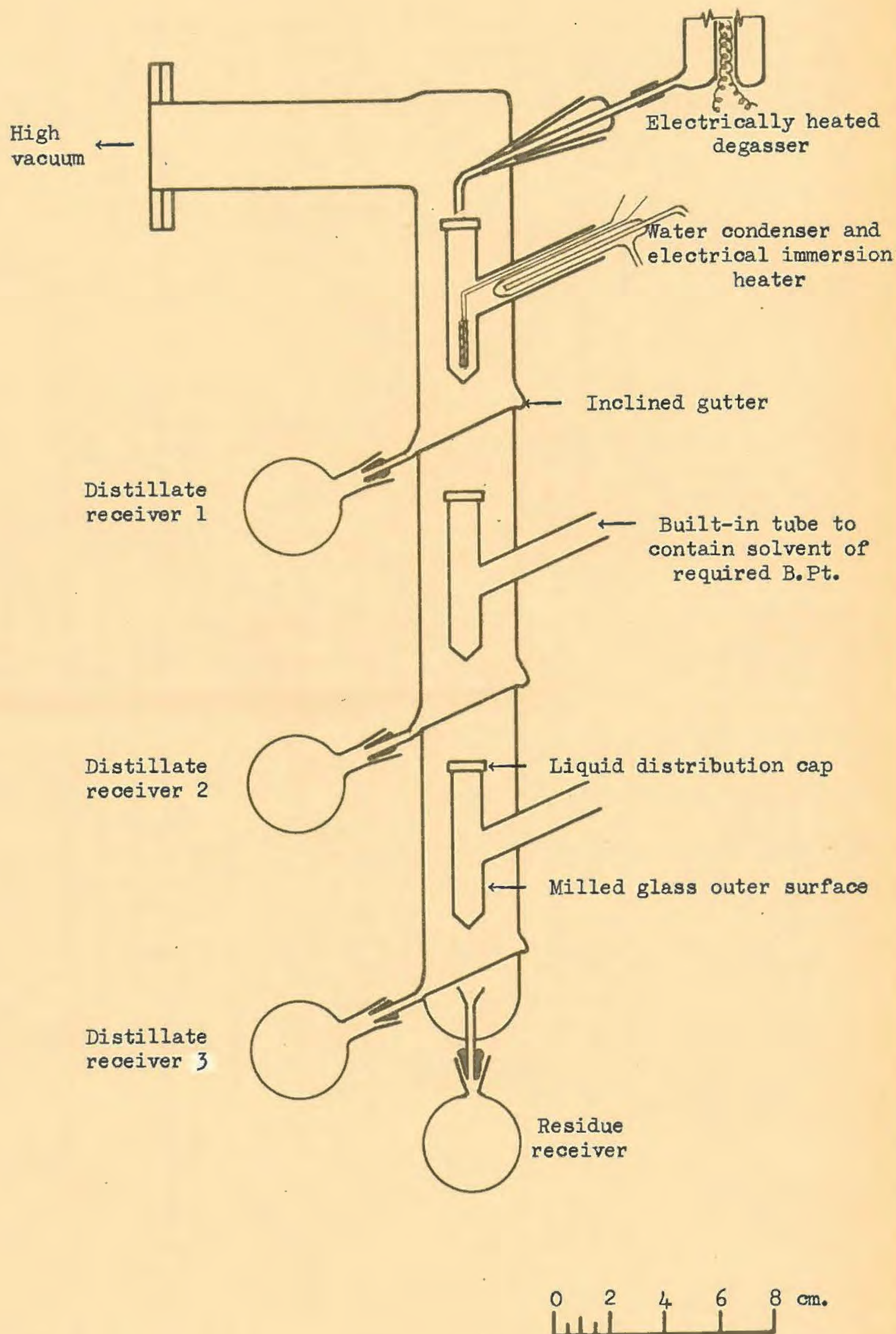


Fig. I. Three Stage Falling-Film Molecular Still - No.1.

(The residue from each stage of distillation drops on to the heated surface of the next stage and is re-distilled. The distillates drain into inclined gutters leading to the receivers.)

The concentrate of unsaturated pilchard acids was divided into three fractions and a residue as shown in Table I.

Table I. Molecular Distillation of the Concentrate of Unsaturated Pilchard Acids.

Fraction	Distillation temperature	Weight (g.)	Eq. wt.	Refractive index (30°)
Unsaturated concentrate	-	333	307	1.4857
I	96°	27	278	1.4755
II	108°	45	283	1.4785
III	130°	80	297	1.4852
Residue	-	175	320	1.4915

The encouraging fractionation into groups of acids with increasing average equivalent weight shown in this experiment, prompted an attempt at further subdivision of the fractions by molecular distillation. Accordingly Fraction III (Table I) was subjected to three additional stages of distillation with the results shown in Table II.

Table II. Molecular Distillation of Fraction III (Table I).

Fraction	Distillation temperature	Weight (g.)	Eq. wt.	Iodine value*	No. of** double bonds per molecule
Fraction III (Table I)	-	73	297	306	3.3
1	96°	26.1	269	316	3.9
2	108°	26.2	279	318	3.7
3	120°	13.9	320	400	5.1
Residue	-	1.5	348	323	4.4

* Wijs method (3 hours).

**Calculated from iodine value and equivalent weight.

A further satisfactory fractionation with regard to equivalent weight and unsaturation was achieved in this experiment. The analytical values of Fraction C were similar to those of a C_{22} pentaene acid (Theor: Eq. wt. = 330 and number of double bonds per molecule = 5.0), so with a view to determining whether or not it might be possible to achieve the isolation of a pure compound by means of molecular distillation (2), this material was subjected to further fractionation as shown in Table III.

Table III. Molecular Distillation of Fraction 3 (Table II).

Fraction	Distil- lation temp.	Weight (g.)	Eq. wt.	Iodine value*	No. of** double bonds per molecule	Refr. index (21°)
Fraction 3 (Table II)	-	13.9	320	400	5.07	-
A	96°	2.3	316	373	4.64	1.4970
B	108°	4.6	321	385	4.87	1.4993
C	120°	2.2	340	385	5.15	1.4943
Residue	-	1.0	342	379	5.10	1.5008

* Wijs method (3 hours).

** Calculated from iodine value and equivalent weight.

The results of this experiment showed that Fraction 3 (Table II) was still a complex mixture. This is particularly evidenced by the range in double bond values of the sub-fractions obtained on distillation. The distillate from the third stage (Fraction C) was by no means pure, but was nevertheless probably a relatively simple mixture of acids. This material was analysed and the results are presented for comparison with those theoretically required for a C_{22} pentaene acid (Table IV).

Table IV. Analysis of Fraction C (Table III).

Analytical value	Fraction C	Theor. for C_{22} pentaene acid
Equivalent weight	340	330
Carbon; Hydrogen percentage	C, 80.9; H, 10.61	C, 80.0; H, 10.60
Iodine value	385	384
Double bonds per molecule	5.2*	5.0
M.p. of hydrogenation product	73.5 - 75.6°	80.7°

* H_2 uptake $Pd/BaSO_4$.

While most of the analytical values of Fraction C appear to be in reasonable agreement with those required for a C_{22} pentaene acid, the clue to its state of purity is given by the melting point of its hydrogenation product. In dealing with homologous series of fatty acids, a range of 2° in the observed melting point of a given material indicates a high degree of contamination by other components. For reasonable assurance of purity, a single acid should preferably melt over a range of 0.3° or less, and certainly over not more than 0.5°.

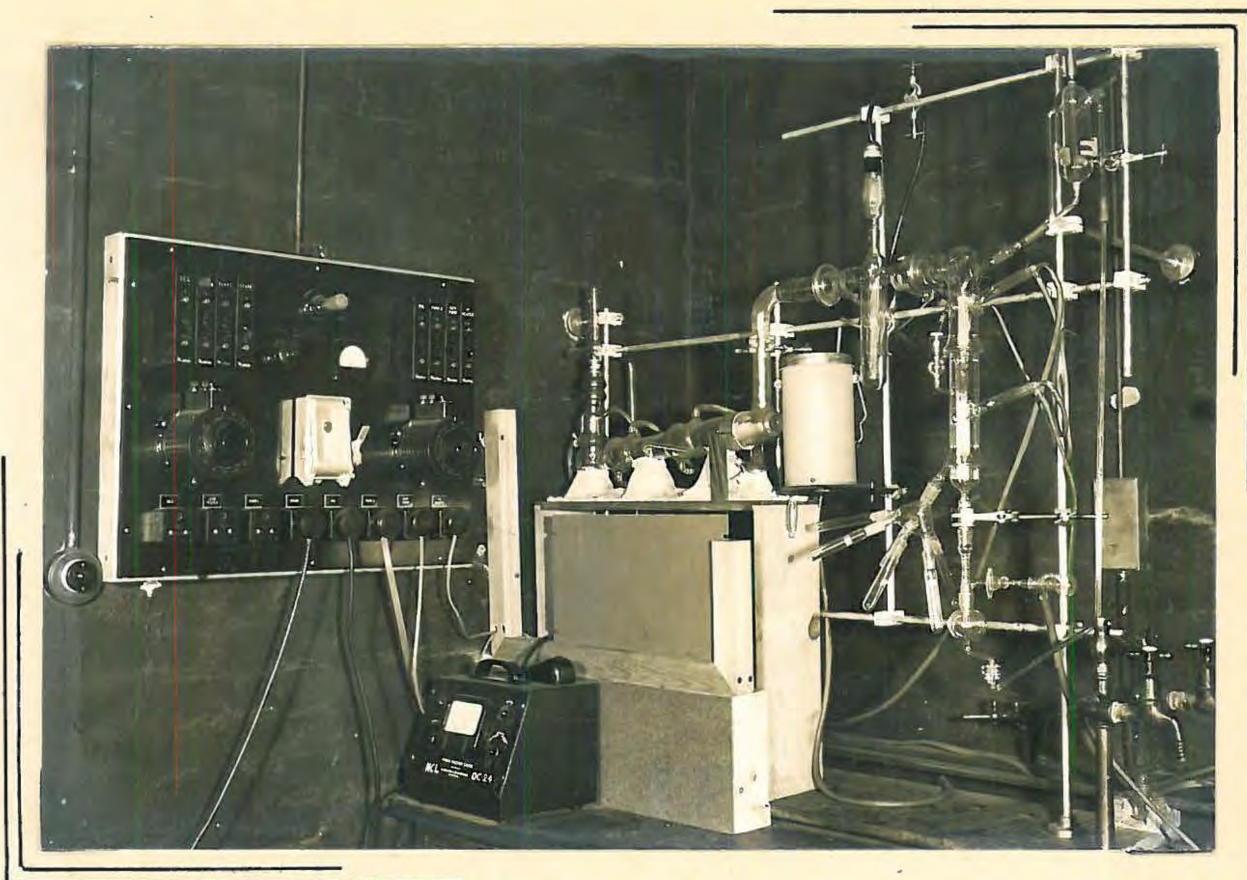
Consideration of the results obtained on successive redistillation of pilchard acid fractions shows that molecular distillation is a very efficient means of obtaining material reasonably homogeneous in chain length. Since the vapour pressure of the components in a crude mixture is chiefly dependent upon chain length, the process is not likely to afford any useful fractionation with regard to degree of unsaturation. A limiting factor in the application of molecular distillation as a means of subdividing mixtures of acids is the quantity of material available at the start. Tables I - III indicate that

333 g. of unsaturated acids yielded only 2.2 g. of the final Fraction C. The disadvantage occasioned by the labour involved in successive repetition of the distillation, has now been overcome by the construction of a still by means of which, in a single operation, each distillate is redistilled instead of each residue as previously described. In this still, designed and perfected by Dr. D.A. Sutton of the South African Council for Scientific and Industrial Research, the combined residues from all stages are continuously recycled, and each fraction collected after distillation at three successive stages. The still is diagrammatically represented in Fig. II, and photographs of a two stage prototype are shown on the following page. The details of construction and operation have now been published (3b).

Counter-current Distribution of Pilchard Unsaturated Acids.

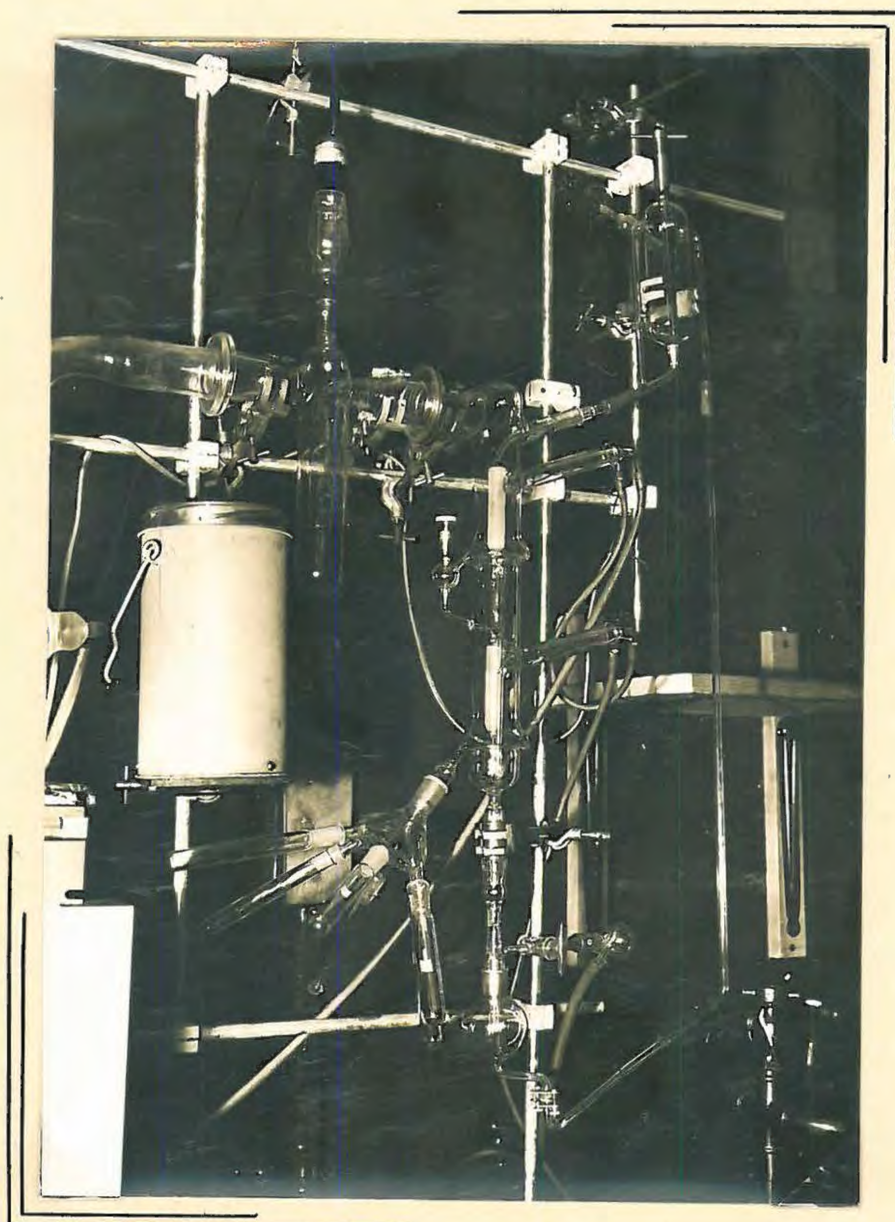
Since molecular distillation, with all its merits, did not seem to offer a means of obtaining pure compounds, an attempt was made to resolve a mixture of unsaturated pilchard acids by means of the well known Craig Counter-current Distribution technique (4). This technique, involving successive partition of the components between two immiscible phases, has achieved recognition for its remarkable efficiency in application to biological materials which are frequently complex mixtures of closely related chemical compounds.

An attempt was made to resolve Fraction III (Table I) using the two phase solvent system resulting from a mixture of isoheptane : acetonitrile : methanol : acetic acid (4:1:1:1). This system had been successfully employed by Ahrens and Craig (5) for the resolution of artificial mixtures of C_{12} - C_{18} saturated fatty acids, and for the separation of fatty acids from pig mesenteric fat. The



Two Stage Falling-Film Molecular Still

(Showing Control Panel, Pirani gauge, Oil Diffusion Pump and Still)



Two Stage Falling-Film Molecular Still

(Detailed view)

partition coefficient of Fraction III in this solvent system was found to be near unity, but after 24 transfers no resolution was evidenced. The distribution curve was of a uniform nature but deviated considerably from the theoretical curve calculated for the maximum. Withdrawal of the material in the 5 tubes at each end of the curve, followed by 24 additional transfers on the material contained in the 14 central tubes, showed a distribution curve with two minor inflections on either side of the peak. The resolution obtained after 48 transfers was thus insufficient to provide a practical means of subdividing the complex mixture of pilchard unsaturated acids. The failure of the method in this instance was doubtless due to the insufficient number of transfers which it was possible to apply with the available apparatus. An apparatus designed to apply 220 transfers has now been reported in the literature (6), and it is suggested that this might afford a practical resolution of mixtures of pilchard unsaturated acids.

Fractional Crystallisation of Pilchard Acid Mixtures.

Low temperature crystallisation from solvents at temperatures between 0° and -70° is a well established technique for the separation of fatty acid mixtures into fractions of simpler composition. The extensive work of Brown and his collaborators in this field has been reviewed by Markley (7).

Application of the technique to a mixture of pilchard unsaturated acids (Fraction III (Table I)) dissolved in acetone, afforded some useful fractionation, but the results were not as encouraging as those obtained by molecular distillation of the same material. In order to gain some indication as to the nature of the chain lengths present in Fraction III, it was stabilised by hydrogenation

and the resulting mixture of saturated fatty acids submitted to low temperature fractional crystallisation from methanol solution. The results are shown in Table V.

Table V. Fractional Crystallisation of Saturated Fraction III (Table I).

Fraction	Appearance	Weight (mg.)	Melting point	Average eq. wt.
Saturated Fraction III	White micro crystals	180	57.5 - 60.5°	304
1	Fine needles	25	68.0 - 68.8°	316
2	Needles + plates	38	65.0 - 67.3°	-
3	White micro crystals	20	65.5 - 67.2°	309
4	do.	20	64.5 - 67.0°	291
5	do.	24	61.0 - 64.5°	293
6	do.	15	57.5 - 59.8°	284
7	do.	3	54.5 - 56.0°	253
8 (Residue)	do.	16	53.0 - 54.0°	254

Examination of the results in Table V shows that the longest chain material of average equivalent weight 316 was precipitated first, and that further cooling of the solution afforded material of gradually decreasing equivalent weight until a residue of equivalent weight 254 was obtained. The range of equivalent weights observed shows that acids from C_{22} (eq. wt. 340) to C_{16} (eq. wt. 256) in chain length are probably present in Fraction III. The experiment illustrates the need for a quantitative method of chain length analysis as a means for determining the chain lengths present in pilchard acid fractions from distillation and other procedures.

Paper Chromatography of Pilchard Acid Mixtures.

H.P. Kaufmann and his fellow workers have reported the resolution of a number of unsaturated acids on paper chromatograms using the technique of two dimensional development (8). The mixtures are spotted onto paper and developed in one direction with a given solvent, and then in a direction at right angles to the first, using a different solvent. Unsaturated acids have in some cases been resolved from others as their maleic anhydride adducts.

Attempts to resolve Fraction III (Table I) by means of several developing solvents, gave only diffuse and elongated streaks along the paper, indicating that the mixture was still probably too complex for analysis in this manner.

Among methods for resolving fatty acids or their derivatives on paper chromatograms (9, 10, 11), those procedures involving partition of the components between a stationary non-polar phase adsorbed on the paper, and a moving polar developer phase, seem to have proved particularly successful (12, 13). The resolution of Fraction III (Table I) was attempted using a procedure devised after consideration of published methods. Strips of filter paper were rendered non-wetting by exposure to the vapour of dimethyldichlorosilane and were then impregnated with medicinal paraffin as stationary phase. Fraction III was spotted onto these papers and the chromatograms developed with several concentrations of aqueous acetone. Only diffuse streaks were shown after spraying with indicator, showing that Fraction III acids were not resolvable, although in the case of an artificial mixture of lauric and myristic acids very clear resolution had been evidenced.

Ultra-violet and Infra-red Examination of Total Pilchard Acids.

Ultra-violet examination of the total pilchard acids in a Beckmann Model D.U. Quartz Spectrophotometer showed that no peaks were apparent in the region $\lambda 215$ - $\lambda 360$ mμ. No acids with conjugated unsaturation are therefore present in pilchard oil.

While no branched chain acids have been reported in marine oils, certain "iso fatty acids" with $-C\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$ branching at the end of the chain have recently been found to occur in natural fats (14). Normal chain acids with a terminal $C-CH_3$ group exhibit a peak at 7.25 μ in the infra-red (15), while iso acids with the $-C\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix}$ grouping exhibit an additional peak at 7.3 μ due to the combined deformation vibration of the methyl groups (16).

In order to ensure that no iso acids were present in pilchard oil, a sample of the total acids was stabilised by hydrogenation, and examined in a Perkin-Elmer single beam Infra-red Spectrophotometer as a solution in carbon tetrachloride. Only the expected peak at 7.25 μ was observed and no peak due to iso acids was apparent at 7.30 μ. A sample of the known iso acid 16-methyl-heptadecanoic, was used as a reference compound.

Examination of the infra-red absorption curve of total pilchard acids (without hydrogenation) showed no peak due to trans bonds in the region of 10.1 μ. The naturally occurring acids must therefore be all cis bonded.

Discussion of Preliminary Experiments.

Aside from the demonstration that no iso acids, trans bonded acids, or acids with conjugated unsaturation are present in pilchard oil, the preliminary experiments gave little positive information regarding its composition.

These experiments did, however, fulfil a very definite need in indicating the requirements for a successful analysis of pilchard oil. These requirements may be summarised as follows:

(1) A full investigation of the use of the lithium soap-acetone and any other methods for achieving a preliminary segregation of the relatively unsaturated from the relatively more saturated acids.

(2) More efficient and less time consuming molecular distillation technique.

(3) A method for the quantitative separation of saturated fatty acid mixtures on a micro scale to provide a means for chain length analysis of pilchard acid fractions.

(4) A method for quantitative analysis of the simpler unsaturated acid mixtures obtained by subdivision of the total unsaturated concentrate.

The requirement in respect of molecular distillation was met by the construction of a new three-stage still to provide successive redistillation of distillates in a single operation (Sutton (3) as described earlier in this chapter). In the following pages it will be shown how each of the other requirements was met, and the results applied to the analysis of pilchard oil.

REFERENCES.

(1) M. Tsujimoto. J. Chem. Ind. Japan 23, 1007 (1920).

(2) E.H. Farmer & F.A. Vandenheuvel.

(a) J. Soc. Chem. Ind. 57, 24 (1938).

(b) J. Chem. Soc. 427 (1938).

(3) D.A. Sutton.

(a) Unpublished.

(b) Chem. and Ind. 1383 (1953).

(4) A. Weissberger. Ed. "Technique of Organic Chemistry". Interscience. New York. Vol. III, p.171 (1950).

(5) E.H. Ahrens & L.C. Craig. J. Biol. Chem. 195, 299 (1952).

(6) L.C. Craig, W. Hausmann, E.H. Ahrens & E.J. Harfenist. Anal. Chem. 1237 (1951).

(7) K.S. Markley. "Fatty Acids". Interscience. New York. p.610 (1947).

(8) H.P. Kaufmann, et al. Fette u. Seifen 52, 331, 390 (1950). ibid. 53, 285 (1951). idem, ibid. 54, 10, 73 (1952).

(9) Y. Inoue & M. Noda. J. Agr. Chem. Soc. Japan 25, 496 (1952).

(10) J. Asselineau. Bull. Soc. Chim. 884 (1952).

(11) R.L. Reid & M. Lederer. Biochem. J. 50, 60 (1951).

(12) R.G. Baker. ibid. 54, xxxix (1953).

(13) J. Boldingh. Experientia 4, 170 (1948).

(14) F.B. Shorland, et al. Chem and Ind. 839 (1951). ibid. 516 (1953). Biochem. J. 50, 207 (1951). ibid. 50, 581 (1952).

(15) R.G. Sinclair, A.F. McKay & R.N. Jones. J. Amer. Chem. Soc. 74, 2570 (1952).

(16) N.K. Freeman. ibid. 74, 2523 (1952).

CHAPTER 4.

THE RESOLUTION OF MIXTURES OF $C_{16} - C_{24}$ NORMAL CHAIN SATURATED FATTY ACIDS BY REVERSED PHASE PARTITION CHROMATOGRAPHY.

In the previous chapter it was explained that one of the requirements for a successful analysis of pilchard oil, was a method suitable for the separation of mixtures of saturated and unsaturated fatty acids from 16 to 22 or possibly 24 carbon atoms in chain length. For convenience, it was desirable that such a method should be readily applicable on a semi-micro scale.

Examination of published literature suggested several chromatographic methods for the purpose, and among these, the displacement chromatographic technique of Holman and Hagdahl (1) seemed likely to be satisfactory. In this procedure the mixture of acids is adsorbed from a solvent onto a column packed with charcoal in several sections of diminishing cross-sectional area. The displacer, which may be a long chain acid ester, hydrocarbon, or higher homologue of the acids to be separated, is dissolved in the solvent, and the chromatogram is developed with this solution in the usual manner. The component acids are displaced in the order of their adsorbability on charcoal, and if the concentration of solute is plotted against the volume of effluent, a stepwise curve is obtained in which the height of each step is characteristic of an individual acid in a given chromatographic system. In the case of fatty acids it has been shown that each acid is an excellent displacer of its next lower homologue.

While this method has been successfully applied to the resolution of $C_1 - C_{20}$ fatty acids, it was felt that the elaborate apparatus and technique involved would impair its

ready applicability to the investigation of pilchard oil, where not only accuracy but speed in the analysis of numerous fractions was necessary.

Vandenheuvel and Hayes (2) have separated C_2 - C_{12} monocarboxylic acids and C_4 - C_{14} dicarboxylic acids by development of the mixture with iso-octane on a column packed with silicic acid supporting methanol as a stationary phase. The column is impregnated with ammonium ions and bromocresol green indicator to facilitate visual observation of the course of development. The same solvent system has very recently been employed by Nijkamp (3) for the separation of C_{16} - C_{20} saturated acids on a column only 9 cm. long. It did not, however, seem that the method could be readily extended to cover acids above 20 carbon atoms in chain length because of the low solubility of such higher acids in the methanol stationary phase. Ramsey and Patterson have separated mixtures of C_{11} - C_{19} fatty acids using 2-aminopyridine and fufuryl alcohol as the solvent system, but their method was deemed unlikely to be suitable on the grounds that the higher fatty acids would have partition coefficients too greatly in favour of the less polar phase.

Several elution chromatographic methods have been described for the separation of fatty acid mixtures on columns of alumina, silica gel and other supports (cf. 5, 6, 7, 8). In general, only very simple mixtures of acids have been examined, and for this reason it did not appear that elution analysis could be readily adapted to the study of the more complex mixtures obtained from pilchard oil.

The method of reversed phase partition chromatography on benzene-rubber columns using methanol-acetone (3:1) containing varying amounts of water as mobile phase, has been applied by Boldingh (9) to the quantitative semi-micro determination of saturated normal chain fatty acids

from C_6 - C_{18}. Chromatography using rubber columns has been claimed by Boekenoogen (10, not published in detail) to be satisfactory for resolution of mixtures containing saturated acids up to C_{24}, as well as for mixtures of various C_{18} unsaturated acids. Since "Mealorub" rubber powder was unfortunately not available at the beginning of this work, it was not possible to investigate the suitability of the method for application to pilchard oil analysis, and other methods were accordingly sought.

Howard and Martin (11) published in 1950 a method for the resolution of C_{12} - C_{18} fatty acids by reversed phase partition chromatography using a hydrocarbon supported on non-wetting kieselguhr as stationary phase, and either aqueous methanol or aqueous acetone as moving phase. Although this method had only been extended to C_{18} acids, and had not been investigated from a quantitative point of view, it appeared to offer several advantages in the case of pilchard oil analysis, particularly in regard to its ease of operation and the small quantities of acids required. The method has now been extended by the author to cover the resolution of mixtures of acids from 16 to 24 carbon atoms in chain length, with a quantitative accuracy of $\pm 10\%$ on a semi-micro scale.

The chromatographic procedure is analogous in principle to the Craig counter-current distribution technique, and as previously suggested (Chapter 3), the Craig method would doubtless be suitable for resolution of mixtures of pilchard acids, provided that a sufficient number of transfers could be conveniently performed. The relative merits of the chromatographic and counter-current methods for separation of fatty acid mixtures are discussed in the following chapter.

Resolution of Mixtures of Normal Chain C_{16} - C_{24} Saturated Fatty Acids by Reversed Phase Partition Chromatography.

Only acids with an even number of carbon atoms were used for the investigation, since natural fats seldom contain uneven acids. Samples of palmitic, stearic and lignoceric acids were drawn from laboratory stocks which had been prepared from natural sources by high precision distillation. Arachidic and behenic acids were synthesised from palmitic and stearic acids respectively, using a recent adaptation of the well known Kolbe electrolytic synthesis (12). All acids showed a satisfactory melting point and equivalent weight. The X-ray long spacing of each acid was determined as an additional check on the purity, and the results were found to be in close agreement with published values (13). A full discussion of the X-ray long spacings of fatty acids in their several crystalline forms has been given by Ralston (14).

The aqueous acetone-medicinal paraffin solvent system of Howard and Martin (loc. cit.) was used throughout the investigation. Kieselguhr, after flotation to remove finer particles, was rendered non-wetting by exposure to the vapour of dimethyldichlorosilane. The washed and dried powder was then suspended in an ethereal solution of medicinal paraffin, and the ether evaporated with constant agitation of the slurry. The resulting mull of paraffin and hydrophobic kieselguhr was then suspended in aqueous acetone and packed into columns using special precautions to exclude any air bubbles. In earlier experiments a column 1.3 cm. in diameter and 30 - 35 cm. in height was found satisfactory for separation of component acids in mixtures, but it was later shown that resolution could be considerably improved by use of a column 85 cm. in height

and 0.8 cm. in diameter. All columns were jacketed and maintained at 35°.

The procedure employed by Howard and Martin (loc. cit.) of loading mixtures of C_{12} - C_{18} acids dissolved in the developing solvent, was found unsuitable for mixtures of C_{16} - C_{24} acids owing to the low solubility of the longer chain acids in the initial solvent required. The acid mixtures were accordingly dissolved in a small quantity of paraffin and adsorbed onto non-wetting kieselguhr using the ether evaporation method. The acid-containing mull was then mechanically packed on top of column as a narrow band.

Development of the chromatogram was carried out with aqueous acetone. The column effluent was allowed to drip into a siphon, and the course of resolution was followed by titration of each aliquot with standard alkali using bromothymol blue as indicator. As each acid was eluted from the column, the concentration of developing solvent was increased to elute the next higher homologue. For resolution of a mixture of C_{16} - C_{24} acids, development was begun with 70% aqueous acetone and the concentration increased → 75% → 80% → 83% → 90% as each successive acid was eluted.

Full details of the preparation and purity of materials, the packing and loading of columns and the chromatographic procedure are given in the Experimental Section. Details regarding the titration apparatus, and an arrangement for changing developing solvents are also included in the Experimental Section.

A typical curve demonstrating the resolution of a mixture of palmitic (C_{16}), stearic (C_{18}), arachidic (C_{20}), behenic (C_{22}) and lignoceric (C_{24}) acids is shown in Fig. III.

The area underneath each peak was measured accurately with a planimeter, and the relative proportions

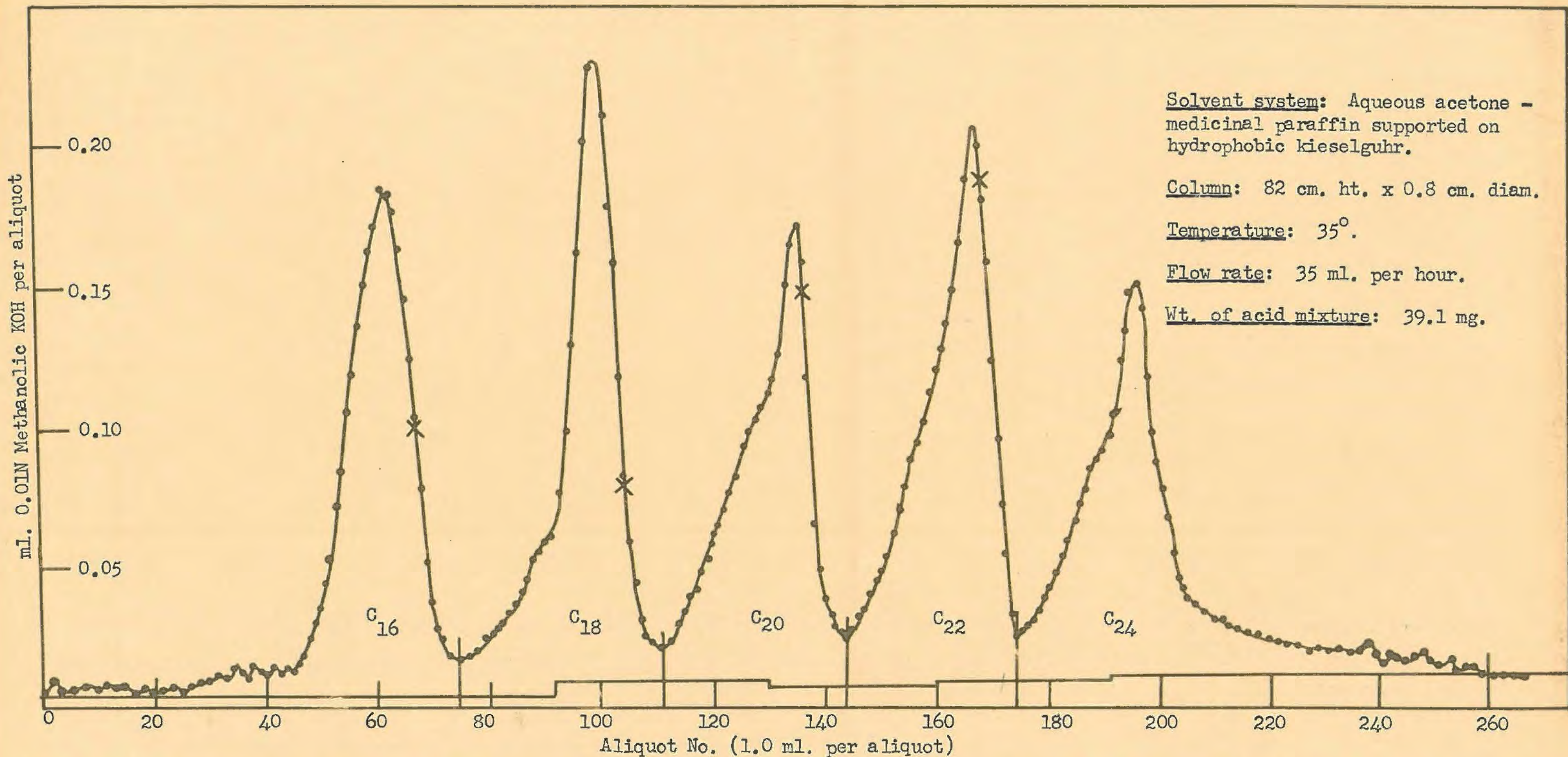


Fig. III. Chromatographic Resolution of a Mixture of Palmitic (C_{16}), Stearic (C_{18}), Arachidic (C_{20}), Behenic (C_{22}) and Lignoceric (C_{24}) Acids.

(Points marked X represent change points to higher concentrations of aqueous acetone developing solvent. Solvent changes made at these points became effective in the eluate 25 aliquots later owing to column hold-up. The blank titre of the solvent in the eluate is represented by the lower broken line; the steps in this line correspond to the effective solvent change points which are associated with steeper rises in the curve.)

Table VI. Resolution of Mixtures of C_{16} - C_{24} Fatty Acids.

(The weight percentage of each acid added in the mixture is shown in column a.
The weight percentage experimentally determined is shown in column b.
The percentage error is shown in column c.)

Expt. No.	Total wt. of acids (mg.)	Components														
		C_{16}			C_{18}			C_{20}			C_{22}			C_{24}		
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	32.20	26.2	24.4	-6.9	26.6	22.8	-14.3	24.1	26.8	+11.2	23.1	26.0	+12.5			
2	40.70	20.0	18.4	-8.0	21.0	19.5	-7.1	20.0	21.6	+8.0	19.8	19.0	-4.0	19.2	21.4	+11.5
3	39.92	17.6	16.6	-5.7	20.4	18.5	-9.3	19.5	20.0	+2.6	21.7	21.8	+0.5	20.8	23.1	+11.0
4	39.23	18.4	16.4	-10.9	20.2	19.4	-4.0	19.4	19.5	+0.5	21.4	20.7	-3.3	20.6	24.0	+16.5
5	39.07	18.7	17.6	-5.9	20.1	19.7	-2.0	19.2	20.2	+5.2	21.2	20.9	-1.4	20.8	21.6	+3.8
6	31.42	23.0	23.9	+3.9	26.4	28.0	+6.1	25.4	23.5	-7.5	25.2	24.6	-2.4			
7	66.59	18.9	18.4	-2.6	20.8	18.3	-12.0	19.9	19.2	-3.5	21.0	24.7	+17.6	19.4	19.4	0.0
8	33.82	17.9	16.4	-8.4	20.1	17.8	-11.4	18.9	17.1	-9.5	20.7	23.3	+12.5	22.3	25.4	+13.9
Average error				-5.6%			-6.8%			+0.9%			+4.0%			+9.5%

of these areas gave the molar ratio of the component acids. Multiplication of the molar ratios by the appropriate equivalent weights of the respective acids, gave the weight ratios. The results of eight chromatographic resolutions of mixtures of pure acids are illustrated in Table VI.

Reference to Fig. III shows that satisfactory qualitative resolution was obtained for mixtures of acids from 16 to 24 carbon atoms in chain length. For determination of the weight ratio or molar ratio of the component acids in a mixture, it is shown in Table VI that a quantitative accuracy of ±10% can be expected.

Low recoveries of acids did not permit of absolute weight determinations being made. In <u>individual</u> chromatograms recoveries of C_{16}, C_{18}, C_{20} and C_{22} acids were on the average 25% low, while recovery of C_{24} acid was of the order of 60%. In chromatograms of acid <u>mixtures</u>, recoveries were also low, but proportionately the same for all acids, thus permitting the ratios of each to be determined with the ±10% accuracy shown in Table VI. Efforts to improve recoveries by "saturating" columns with higher homologues before chromatography, were unavailing. Lost acid could be recovered and determined by stripping the column mull with ether, and titrating the acid-paraffin mixture in 50% benzene-neutral ethanol solution.

Adsorption effects on the non-wetting kieselguhr may have accounted for losses of acid, but attempts to find other supports were unsuccessful. Among others, cellulose powder, glass powder and silica powder were investigated. Attempts were made to render these materials non-polar by exposure to the vapour of either dimethyldichlorosilane or trimethylchlorosilane, and by use of "Quilon" (15). In no case was the treated material able to retain the paraffin phase of the solvent system.

Apart from adsorption losses, the observed low recoveries of acids may to some extent be accounted for by the use of bromothymol blue as indicator. When added during a pH titration of 0.002 N lignoceric acid with methanolic 0.01 N KOH in 90% aqueous acetone, the indicator showed a pK of 10.5 while the pH at end point was nearer 11.5. Thus bromothymol blue gave a low end point, but all acids were found to titrate low by the same amount (10%) in any concentration of aqueous acetone. Quantitatively, acid would thus appear to have been lost, but the true ratio of the peak areas could not be affected, since all acids showed a titre low by the same equivalent amount. Since the purpose of the investigation was to determine only the relative proportions of acids, rather than exact amounts, bromothymol blue was considered a satisfactory indicator.

Inspection of Fig. III shows that in chromatography of a mixture of acids, the eluate titre never quite reaches that of the solvent blank between the peaks. This was not due to the fact that the solvent concentration was increased too soon, because the minimum was passed before the next higher solvent became effective in the eluate. The lower broken line representing the blank titre in Fig. III shows this to be so, and the change points to stronger solvents were selected to give a boost to the rise of the next peak only after the previous one had been completely eluted by the solvent of lower concentration. Solvent changes made on the downward slope of each peak became effective in the eluate approximately 25 aliquots later, owing to the column hold up. The minimum was in each case passed before 25 aliquots had been eluted. It was not possible to overcome the minor defect in resolution between peaks, within the practical limit of column length obtainable (columns longer than 80 cm. gave impracticably slow flow

rates). Attempts to eliminate the solvent blank titre determination by using solvents neutralised either directly with alkali, or by percolation through beds of Amberlite IRA 400 ion exchange resin, resulted in loss of chromatographic resolution.

Reversed phase partition chromatography was thus adapted to provide a quantitative means for chain length analysis of pilchard acid fractions with as little as 6 - 7 mg. of each component. The method was developed for saturated acid mixtures, but is equally applicable to the chain length analysis of unsaturated mixtures after these have been hydrogenated. This is because the introduction of hydrogen atoms causes little change in the weight ratio of the component acids.

The method was not suitable for the resolution of normal and iso acids; for example, a mixture of stearic and 16-methyl-heptadecanoic acids gave only a single peak; but in the following chapter it is shown that the technique can be applied to the resolution of mixtures of unsaturated acids.

REFERENCES.

(1) R.T. Holman & L. Hagdahl. J. Biol. Chem. 182, 421 (1950).

(2) F.A. Vandenheuvel & E.R. Hayes. Anal. Chem. 24, 960 (1952).

(3) H.J. Nijkamp. Nature 172, 1102 (1953).

(4) L.L. Ramsey & W.I. Patterson. J. Ass. Off. Agric. Chem. Wash. 31, 441 (1948).

(5) R.W. Riemenschneider, S.F. Herb & P.L. Nichols. J. Amer. Oil Chem. Soc. 26, 371 (1949).

(6) F.E. Kurtz. J. Amer. Chem. Soc. 74, 1902 (1952).

(7) G. Di Modica & P.F. Rossi. Chim. anal. 34, 271 (1953).

(8) T. Asahara, Y. Konishi, Y. Kuroda & M. Mashino. J. Chem. Soc. Japan (Ind. Chem.) 54, 70 (1951).

(9) J. Boldingh. Rec. Trav. Chim. 69, 247 (1950).

(10) H.A. Boekenoogen. Angew. Chemie 64, 339 (1952).

(11) C.A. Howard & A.J.P. Martin. Biochem. J. 46, 532 (1950).

(12) W.S. Greaves, R.P. Linstead, B.R. Shepheard, S.L.S. Thomas & B.C.L. Weedon. J. Chem. Soc. 3326 (1950).

(13) F. Francis & S.H. Piper. J. Amer. Chem. Soc. 61, 577 (1939).

(14) A.W. Ralston. "Fatty Acids and Their Derivatives". N.Y. John Wiley & Son. 330 et seq. (1948).

(15) D. Kritchevsky & M. Calvin. J. Amer. Chem. Soc. 72, 4330 (1950).

CHAPTER 5.

THE RESOLUTION OF UNSATURATED ACID MIXTURES BY REVERSED PHASE PARTITION CHROMATOGRAPHY.

In Chapter 4 a method was described for the resolution of mixtures of C_{16} - C_{24} saturated acids. The application of this method to the separation of unsaturated fatty acid mixtures is now discussed.

As a preliminary experiment, an artificial mixture of pure oleic and linoleic acids was subjected to reversed phase partition chromatography using the procedure detailed in the preceding chapter. Clear resolution between these acids was demonstrated (Fig. IV) indicating that acids of the same chain length but differing degree of unsaturation should be easily separable. On attempted resolution of a mixture of pure oleic and palmitic acids, only a single peak was obtained, showing that where one component had a double bond and two methylene groups more than the other, resolution could not be expected.

In their studies on the resolution of fatty acid mixtures by counter-current distribution, Ahrens and Craig (1) arrived at similar conclusions. These authors were unable to separate palmitic and oleic acids after 3,000 transfers, but showed that oleic and linoleic acids were resolved after 650 transfers. The efficiency of the column used by the author to separate the pair of C_{18} acids, can therefore be considered as equivalent to that obtainable by 650 counter-current transfers. In view of the enormous labour required to perform this number of counter-current transfers with any apparatus other than the 220 tube type recently described (2), it would seem that the simpler chromatographic method should be preferred for fatty acid separations. However, one advantage inherent in the counter-current procedure is the greater amount of material

Fig. IV. Chromatographic Resolution of a Mixture of Oleic (C_{18} monoene) and Linoleic (C_{18} diene) Acids.

Solvent system: Aqueous acetone (60% v/v) - medicinal paraffin supported on hydrophobic kieselguhr. (Solvent changed to (67.5% v/v) aqueous acetone at point marked X.)

Column: 84.5 cm. ht. x 0.8 cm. diam.

Temperature: 35°.

Flow rate: 35 ml. per hour.

Wt. of acids: Oleic (12.35 mg.); linoleic (14.90 mg.).

that can be handled. Analytical scale chromatographic columns did not operate satisfactorily with loads of more than 40 mg., while the largest preparative column built (Chapter 8) could only resolve 500 mg. of a four component mixture. The advantage possessed by the counter-current method in respect of preparative work, does not in any way detract from the merits of the chromatographic method which is rapid in operation and affords accurate results where only small quantities of material are available. Although both methods are analogous in principle, the counter-current procedure is the more exactly reproducible of the two, and is more readily amenable to mathematical treatment. Given the partition coefficient of a substance in a solvent system, it is possible to calculate the position and shape of the distribution curve. Where mixtures are concerned, it is possible to calculate the number of transfers necessary for resolution of the components, provided that the partition coefficient of each is known.

The resolution of unsaturated fatty acid mixtures by partition chromatography with an aqueous acetone-paraffin system, has very recently been investigated by Savary and Desnuelle (3). These workers have confirmed the author's findings with regard to the resolvability of the oleic-linoleic acid pair, and with regard to the overlapping peak shown by an oleic-palmitic acid mixture. In the latter case they were able to show that hydroxylation of the mixture followed by chromatography with an aqueous acetone-castor oil system, afforded resolution between palmitic acid and the 9:10-dihydroxystearic acid resulting from oxidation of oleic acid.

Since the chromatographic method appeared to be suitable for resolution of acids with the same chain length and differing degree of unsaturation, although not

for mixtures where one component possessed one double bond and two methylene groups more than the other, it was decided to investigate its applicability to certain of the unsaturated acid mixtures obtained from pilchard oil. For this purpose one of the fractions (Fraction C, Table III) obtained by molecular distillation of pilchard unsaturated acids, was selected. The experiments were designed to determine the optimum temperature and solvent concentration for resolution of this material.

First results showed that at the temperature of circulating tap water (20°) resolution was ill defined on development with 55% aqueous acetone, whereas development with 65% aqueous acetone evidenced three incompletely resolved peaks. An intermediate aqueous acetone concentration of 60% was proved to be the best for resolution of Fraction C, since increasing the concentration above 65% destroyed resolution by causing the simultaneous elution of all peaks, and decreasing the concentration below 55% caused only slight development.

Investigation of the effect of temperature on the resolution of Fraction C with 60% aqueous acetone, showed that resolution was best defined at 10°. The chromatographic curve of Fraction C at this temperature (Fig. V) showed that, although no peaks were completely separated, resolution had occurred to some considerable extent. It was not possible to postulate the composition of the peaks shown in Fig. V, but it seemed likely that this could be done if an accurate chain length analysis of Fraction C were available (cf. Chapter 7).

Fraction B (Table III) showed a curve similar to that of Fraction C at 10°, and indeed it appeared likely that this temperature and solvent concentrations of approximately 60% would be generally suitable for resolution of higher unsaturated acid mixtures.

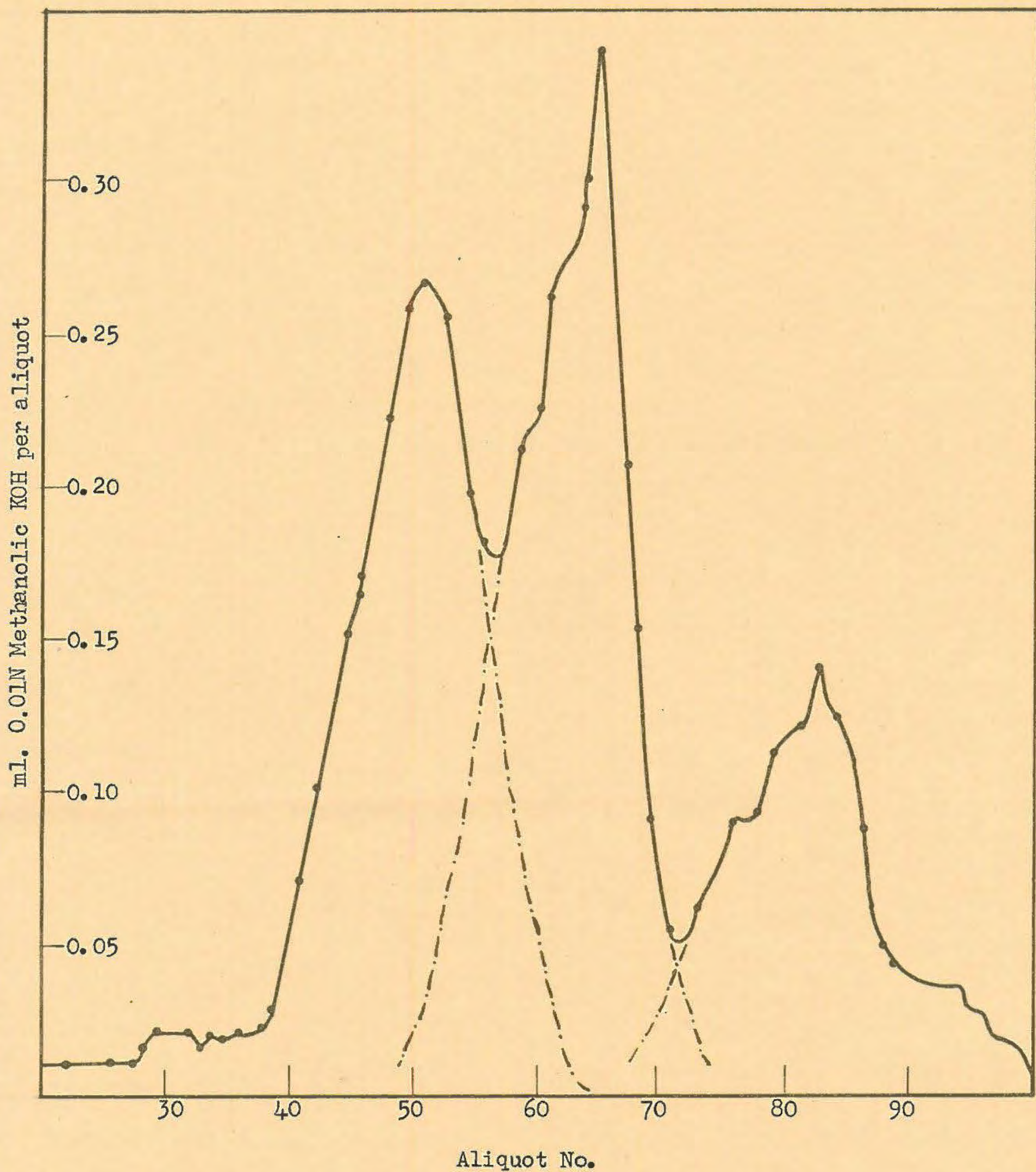


Fig. V. Chromatographic Resolution of Fraction C (Table III).

Solvent system: Aqueous acetone 60% (v/v) - medicinal paraffin supported on hydrophobic kieselguhr.

Column: 31.5 cm. ht. x 1.4 cm. diam.

Temperature: 10°.

Flow rate: 35 ml. per hour.

Wt. of Fraction C: 20.5 mg.

In later applications of the chromatographic method to pilchard acid mixtures, conditions were varied when components of a relatively low degree of unsaturation were present. Since the solubilities of these components approximated those of saturated acids, more closely than those of the highly unsaturated acids present in Fraction C, higher concentrations of aqueous acetone were used for their development and in some cases higher temperatures. Reference to Fig. IV shows that relatively saturated acids such as oleic and linoleic acid can be developed at 35°, but acids with a greater degree of unsaturation would not be resolvable at such a temperature, and could only be separated at approximately 10°.

REFERENCES.

(1) E.H. Ahrens & L.C. Craig. J. Biol. Chem. 195, 304 (1952).

(2) L.C. Craig, W. Hausmann, E.H. Ahrens & E.J. Harfenist. Anal. Chem. 23, 1237 (1951).

(3) P. Savary & P. Desnuelle. Bull. Soc. Chim. 939 (1953).

CHAPTER 6.

COMPARISON OF METHODS FOR THE PREPARATION OF CONCENTRATES OF HIGHLY UNSATURATED ACIDS FROM PILCHARD OIL.

It was pointed out in Chapter 3 that analysis of the complex mixture of fatty acids obtained on saponification of a marine oil, is greatly facilitated if preceded by a fractionation into groups of acids of largely similar properties. Preliminary segregation of a concentrate of highly unsaturated acids from the total hydrolysate has been achieved by several methods, among which are:

(1) adsorption chromatography (Kaufmann 1 and 2),

(2) low temperature crystallisation (Hilditch and Riley 3), and

(3) methods based on the relative solubility of the metallic salts of saturated and unsaturated acids in various solvents.

Among such solubility methods, the lead salt-alcohol procedure of Twitchell (4), the barium salt-benzene method of Farnsteiner (5) and the magnesium salt-alcohol method (cf. Voorhies and Bauer (6)) are well known. The lithium soap-acetone method of Tsujimoto (7, 8), has been claimed to be the most efficient in application to marine oils, and was used in the preliminary investigation of pilchard oil (cf. Chapter 3).

More recently, a new segregation procedure has been suggested by the fact that fatty acids of a high degree of unsaturation form complexes with urea less readily than do acids of lower unsaturation (cf. Schlenk and Holman, 9).

In the formation of complexes with fatty acids, urea crystallises as hollow hexagonal prisms enclosing the carbon chain. Acids with branched non-linear carbon chains

do not form complexes, because the dimensions of the molecule are too great for it to be accommodated inside the hexagonal structure of the urea prism. Normal chain saturated fatty acids form complexes readily, and unsaturated acids less readily because their dimensions are greater due to folding of the chain about the double bonds.

Autoxidised fatty acids do not form urea complexes owing to the chain branching introduced by the hydroperoxide group. Cis-trans hydroxy acids resulting from decomposition of hydroperoxides do not form complexes, but trans-trans hydroxy acids, which also result from hydroperoxide decomposition, can form complexes (10). Secondary oxidation products such as epoxy-hydroxy acids would be unlikely to do so. Only unoxidised fatty acids are therefore likely to form complexes with urea, and these complexes can be stored without danger of oxidation taking place (9).

The efficiency of the urea complex method as a means for separating relatively saturated from relatively more unsaturated acids, has been compared with the efficiency of the low temperature crystallisation technique (3 loc. cit.) by Swern and Parker (11). In the present investigation, a comparison has been made between the efficiency of the urea method and that of the lithium soap-acetone procedure.

For the purpose of this study, three concentrates of highly unsaturated acids were prepared from pilchard oil.

A) A concentrate obtained by repeated application of the lithium soap-acetone procedure to the total acids.

B)* A concentrate (non complex forming residue) prepared by direct application of urea complex fractionation technique to the mixture of total acids.

---

* Concentrate B was prepared by Mr. H.H. Sephton of the National Chemical Research Laboratory, Pretoria, and presented to the author for comparison with his concentrates A and C.

C) A concentrate obtained by urea complex fractionation of concentrate A.

Concentrates A, B and C were compared, and the relative efficiency of the urea and lithium soap-acetone procedures assessed on the analytical results obtained.

The Lithium Soap-Acetone Procedure.

In Chapter 3 it was shown that a single application of the lithium soap-acetone procedure to total pilchard acids resulted in an unsaturated acid concentrate representing 33% of the total, and having an average unsaturation of 4.55 double bonds per molecule. In order to determine whether successive application of the process would afford further enrichment in unsaturation, a fresh sample of total pilchard acids was submitted to lithium soap-acetone segregation, and the process was repeated twice on the initial unsaturated concentrate. The results are shown in Table VII. Two segregations were carried out in 95% aqueous acetone, with a final stage in 97% aqueous acetone. The water content of the solvent was decreased in the last stage to cause precipitation of some of the moderately unsaturated soaps, as well as any of the relatively saturated components remaining after the first two treatments.

Table VII. Lithium Soap-Acetone Segregation of Pilchard Unsaturated Acids.

No. of treatments	Percentage aqueous acetone	Weight (g.)	Eq. wt.	No. of double* bonds per molecule
0		100	282	2.40
1	95	38	295	4.23
2	95	31	304	4.52
3	97	29.5	311	4.62

* H_2 uptake over $Pd/BaSO_4$

The process was not carried further than the three stages illustrated in Table VII, since the enrichment in unsaturation proved insufficient to warrant further treatments.

Urea Complex Fractionation of the Lithium Soap-Acetone Concentrate.

Fractionation of the lithium soap-acetone concentrate was carried out using the urea complex method (9). The unsaturated acids were dissolved in alcohol, previously saturated with urea, and the solution allowed to stand. The precipitated urea complexes were removed by filtration and decomposed with water to recover the acids. Further crops of complexes were obtained by cooling of successive filtrates, which were in each case saturated with urea.

Table VIII illustrates the fractionation of the lithium soap-acetone concentrate in this manner. The final non complex forming concentrate represented 20% of the total pilchard acids and had an average degree of unsaturation of 4.85 double bonds per molecule.

Table VIII. Urea Complex Fractionation of the Unsaturated Concentrate Segregated by the Lithium Soap-Acetone Procedure.

Fraction	Weight (g.)	Weight %	Eq. wt.	No. of double* bonds per molecule
Lithium soap-acetone concentrate	93.0	100	307	4.55
1	4.8	5.16	315	3.00
2	2.48	2.67	304	3.38
3	1.85	1.99	300	3.39
4	4.40	4.74	300	4.01
5	2.58	2.78	302	4.21
6	1.50	1.61	304	4.40
7	3.95	4.25	304	4.54
8	1.90	2.04	303	4.60
9	4.05	4.36	301	4.69
10	2.24	2.41	305	4.82
11	0.82	0.88	303	4.71
Non complex forming residue	55.0	59.1	308	4.85

* H_2 uptake over $Pd/BaSO_4$

Urea Complex Fractionation of Total Pilchard Acids.

Urea complex fractionation of total pilchard acids was carried out by Mr. H.H. Sephton of the National Chemical Research Laboratory, Pretoria, with the results shown in Table IX. The non complex forming residue, representing 22% of the total, had an average unsaturation of 4.89 double bonds per molecule.

Table IX. Urea Complex Fractionation of Total Pilchard Acids.

Fraction	Weight (g.)	Weight %	Eq. wt.	No. of* double bonds per molecule	Iodine** value
Total fatty acids	95.0	100.0	282	2.40	201.0
1	5.0	5.6	278	0.20	12.0
2	13.5	15.0	276	0.35	20.7
3	13.5	15.0	271	0.46	46.7
4	17.0	19.0	280	1.49	135
5	12.0	13.3	298	3.65	300
6	2.0	2.2	305	4.54	-
7	0.5	0.6	310	-	-
8	4.0	4.5	304	4.55	368
9	1.0	1.1	302	-	368
10	1.0	1.1	305	4.65	-
Non complex forming residue	20.0	22.0	304	4.89	385.7

* H_2 uptake over Adams' catalyst.

** Wijs (3 hours).

Table X. Comparison of Pilchard Unsaturated Acid Concentrates.

Concentrate	Percentage yield on total	Eq. wt.	No. of double bonds per molecule	Molar percentage			
				C_{16}	C_{18}	C_{20}	C_{22}
Total pilchard fatty acids	100	282	2.40*	18.25	23.9	39.6	18.25
A. Acids prepared by lithium soap-acetone segregation of total	32	307	4.55*	23.5	12.0	40.8	23.6
B. Non complex forming acids segregated from total by the urea method	22	304	4.88**	29.6	18.6	31.9	19.9
C. Non complex forming acids segregated from concentrate A by the urea method	20	308	4.85*	19.9	13.4	38.6	28.1

* H_2 uptake over $Pd/BaSO_4$. ** H_2 uptake over Adams' catalyst.

<u>Comparison of the Unsaturated Concentrates of Pilchard Acids obtained by the Lithium Soap-Acetone and Urea Complex Fractionation Procedures.</u>

Chain length analyses were carried out by quantitative reversed phase partition chromatography of a sample of each concentrate after hydrogenation (cf. Chapter 4). The properties of the concentrates are shown together with those of the total pilchard acids in Table X.

Direct comparison of concentrates A and B (Table X) shows that the latter is of greater average unsaturation and, moreover, contains proportionately less of the longer chain C_{20} and C_{22} acids. It is thus apparent that the urea procedure is more efficient than the lithium soap for removal of long chain acids of low unsaturation. For the purpose of preparing a concentrate rich in shorter chain highly unsaturated acids, the urea method is therefore to be preferred. The better stepwise fractionation obtainable with the urea method would also commend its use in preference to the lithium soap procedure for this purpose.

Comparison of concentrates B and C shows that both have the same average unsaturation, but that the latter is proportionately richer in the longer chain C_{20} and C_{22} unsaturated acids. Thus, if a preliminary lithium soap-acetone segregation is carried out before application of the urea method, then the final unsaturated concentrate is richer in C_{20} and C_{22} unsaturated acids than the concentrate obtained by direct application of the urea method to total pilchard acids. The lithium soap-acetone procedure is thus effective in removing an appreciable quantity of C_{16} and C_{18} acids of relatively low unsaturation which are not easily removed by the urea method. This is consistent with the first observation that the lithium soap-acetone procedure, in direct application to total pilchard acids,

affords a concentrate richer in longer chain acids than does the urea method.

Each of the procedures investigated would appear to have its respective merits depending upon the nature of the unsaturated concentrate required. Material rich in either the shorter C_{16} and C_{18} chain lengths or in the longer C_{20} and C_{22} chain lengths, can be prepared for subsequent isolation of the individual acids of each group.

Urea fractionation of acid esters was not undertaken, although this has been claimed by Abu Nasr and Holman (12) to result in the removal of material more highly unsaturated than is the case when the free acids are fractionated. The reduced tendency of the esters to associate is offered as an explanation of this behaviour.

REFERENCES.

(1) H.P. Kaufmann. Fette u. Seifen 46, 268 (1939).

(2) H.P. Kaufmann. Angew. Chemie 53, 98 (1940).

(3) T.P. Hilditch & J.P. Riley. J. Soc. Chem. Ind. 64, 204 (1945).

(4) E. Twitchell. J. Ind. Eng. Chem. 13, 806 (1921).

(5) K. Farnsteiner. Z. Untersuch. Nahrung Genussm. 6, 161 (1903).

(6) S.T. Voorhies & S.T. Bauer. Oil and Soap 20, 175 (1943).

(7) M. Tsujimoto. J. Chem. Ind. Japan 23, 1007 (1920).

(8) M. Tsujimoto & K. Kimura. ibid. 26, 891 (1923).

(9) H. Schlenk & R.T. Holman. J. Amer. Chem. Soc. 72, 5001 (1950).

(10) D.A. Sutton. S. African Ind. Chemist 7, 196 (1953).

(11) D. Swern & W.E. Parker. J. Amer. Oil Chem. Soc. 29, 431 (1952).

(12) A.M. Abu Nasr & R.T. Holman. Univ. of Minnesota Ann. Rep. of Hormel Inst. p.38 (1951-2).

CHAPTER 7.

AN APPROACH TO THE ANALYSIS OF PILCHARD OIL.

It was explained in an earlier chapter that the analysis of a marine oil is an extremely complex matter owing to the diverse nature of the component fatty acids. In the case of pilchard oil, acids of from 16 to 22 carbon atoms in chain length are present; the unsaturated members containing from one to six or probably seven double bonds per molecule.

For analysis of such mixtures without isolation of individual components, the well established Hilditch method has been almost universally employed (1). The method was discussed in Chapter 2, where it was mentioned that its chief disadvantage is the purely empiric assignment of unsaturation between the component chain lengths. The Hilditch method in fact affords no information as to the number or nature of the unsaturated acids possessing a given chain length, in cases where complex mixtures of such acids are to be analysed.

In this chapter a new approach to the analysis of marine oils without isolation of individual component acids, is discussed with reference to pilchard oil. The techniques of lithium soap-acetone segregation, molecular distillation, and urea complex fractionation have been applied in successive subdivision of total pilchard acids, to provide fractions which have been examined by the methods of reversed phase partition chromatography and alkali isomerisation (2, 3). By application of these techniques in appropriate sequence it has proved possible to obtain a fraction whose composition could be calculated unambiguously without isolation of an individual component. Although an overall analysis of pilchard oil was not undertaken in this manner, there is no reason to doubt that it would be

possible by means of the techniques mentioned.

All the procedures employed in this investigation can reasonably be expected to have caused little or no modification of the original acids.

The Analysis of Total Pilchard Acids.

As a first step in subdivision of the mixture of total pilchard acids, the lithium soap-acetone procedure was applied to prepare a concentrate of the more highly unsaturated members (cf. Chapter 6).

The unsaturated concentrate was then subjected to molecular distillation in a two stage prototype of the falling film molecular still described by Sutton (4). By this means six distillates of increasing boiling point (Fractions I - VI) were obtained as shown in Table XI.

Table XI. Molecular Distillation of the Lithium Soap-Acetone Concentrate.

Fraction	Distil- lation temp.	Weight (g.)	Eq. wt.	Refractive index (30°)	No. of* double bonds per molecule
Unsaturated acids	-	29.5	311	-	4.62
I	96°	3.40	271	1.4806	3.55
Intermediate	105°	0.72	274	1.4832	3.62
II	105°	2.84	276	1.4848	3.65
Intermediate	120°	0.59	287	1.4879	4.08
III	120°	2.51	287	1.4886	4.18
Intermediate	130°	0.47	306	1.4913	4.65
IV	130°	4.83	307	1.4933	4.86
V	135°	3.57	315	1.4950	5.20
VI	145°	2.27	327	1.4972	5.42

Residue: 7.0 g. Hold-up and loss: 1.3 g.

* H_2 uptake: $Pd/BaSO_4$.

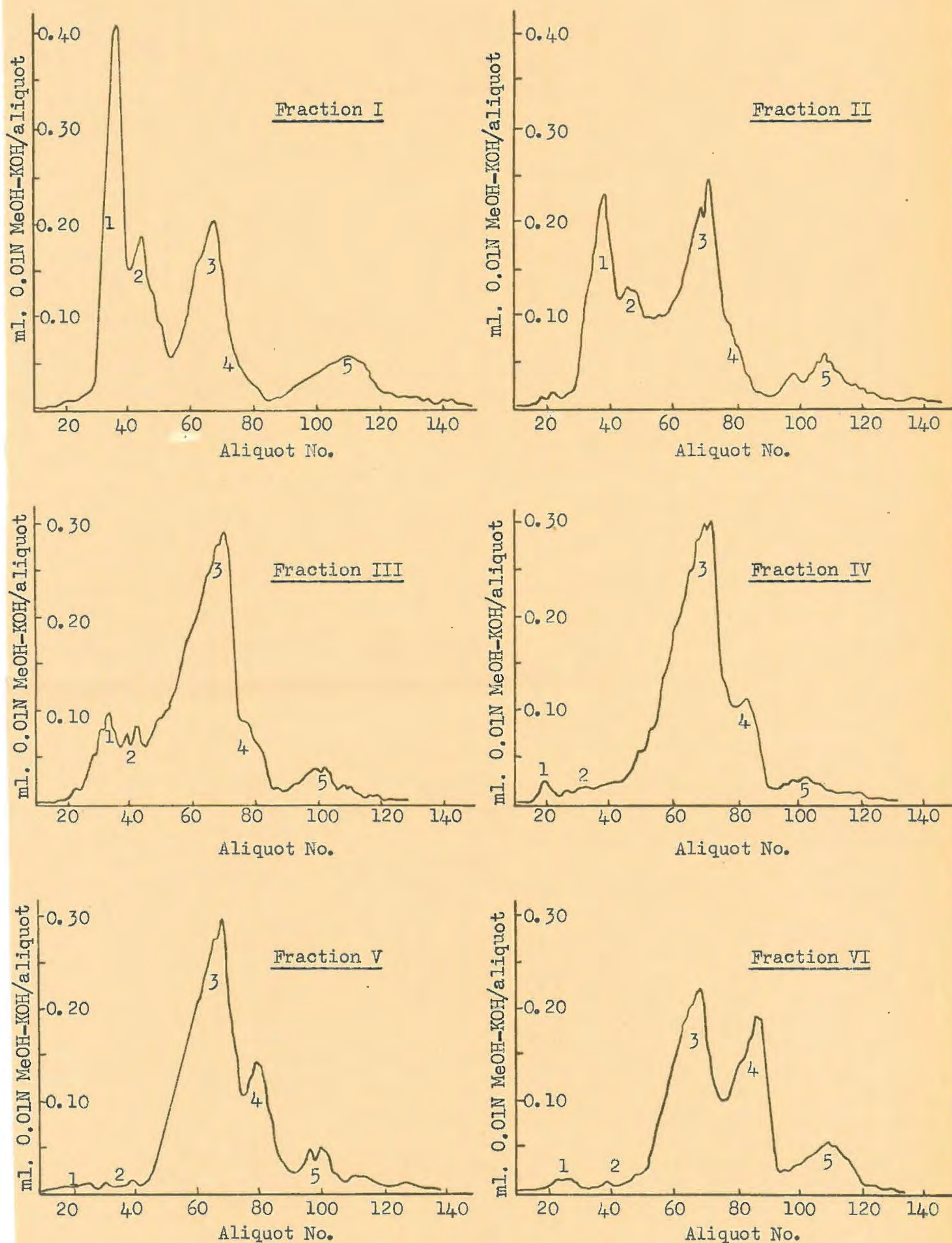


Fig. VI. Chromatographic Curves of Fractions I - VI (Table XI) from Molecular Distillation of the Lithium Soap-Acetone Concentrate.

Solvent system:	Aqueous acetone (60% v/v) - medicinal paraffin supported on hydrophobic kieselguhr.
Column:	30 cm. ht. x 1.4 cm. diam.
Temperature:	10°.
Flow rate:	35 ml. per hour.
Column load:	23 mg. each Fraction.

Each of Fractions I - VI was examined by reversed phase partition chromatography as described for unsaturated acid mixtures in Chapter 5. The curves obtained for each of the fractions are shown in Fig. VI.

Each of the fractions was also analysed for chain length distribution by the chromatographic procedure described in Chapter 4, and the results are shown in Table XII.

Table XII. Chain Length Distribution of Fractions I - VI.

Fraction	Molar percentages			
	C_{16}	C_{18}	C_{20}	C_{22}
I	62.8	13.2	17.0	7.0
II	41.3	18.2	32.1	8.4
III	19.7	20.8	49.2	10.3
IV	5.1	10.3	64.2	20.4
V	1.9	4.4	64.5	29.2
VI	2.0	2.6	44.9	50.5

Examination of Fractions I - VI for Evidence of Oxidation.

Before proceeding with the analysis of Fractions I - VI, it seemed desirable to investigate whether any oxidation of their components had taken place during processing and storage. A qualitative test of each by the ferrous thiocyanate method (5), showed that no oxidation had taken place, but although this test is extremely sensitive for minute traces of hydroperoxide oxidation products, it was supplemented by a further examination of each fraction using two other methods; one based on ultra-violet and the other on infra-red spectroscopy.

Both these methods have as their basis the fact that autoxidation of unsaturated fatty acids results in

conjugation of the methylene interrupted double bonds. The double bonds involved in migration assume a trans configuration, and naturally occurring all cis bonded acids give rise to cis-trans and trans-trans conjugated hydroperoxides on autoxidation. The conjugated double bonds exhibit a peak at ca. $\lambda 230$ mμ in the ultra-violet spectrum, which is not apparent in unoxidised acids, so that measurement of the $E^{1\%}_{1cm.}$ observed at $\lambda 230$ mμ for the material under examination and comparison with the known value for pure conjugated hydroperoxide at the same wave length (6), gives a measure of the extent to which oxidation has taken place.

Similarly, trans-trans conjugated bonds exhibit a peak near 10.15 μ in the infra-red, whereas cis-trans conjugated bonds exhibit an additional peak near 10.55 μ (7). Provided that the spectrometer has been calibrated with known similar compounds containing such bonds, it is possible to measure quantitatively the amount of each bonding and therefore the extent of oxidation of a given material. Care must be exercised in interpretation of the infra-red data since acids containing a terminal vinyl group also exhibit a peak at 10.1 μ which is not due to oxidation.

Fractions I - VI were examined by both the infra-red and ultra-violet spectroscopic methods, and shown to have suffered a negligible amount of oxidation (probably less than 0.3%). An account of these measurements is given in detail in the experimental section, together with the method of calculation.

Alkali Isomerisation of Fractions I - VI.

The technique of alkali isomerisation has been used for determination of the nature of the unsaturation present in the component fatty acids of natural oils and fats, as well as in individual acids (2, 3, 8). The method

depends upon the fact that when these compounds are heated with strong alkali at elevated temperatures, unconjugated double bonds move into conjugation. Where the double bonds are separated by more than a single methylene group in the original compounds, conjugation does not occur under the conditions usually applied (9). Ultra-violet examination of the alkali isomerised material then affords a ready means of determining quantitatively the amount of diene, triene, tetraene and higher conjugation present, and from this information it is possible, in many cases, to determine the number and nature of the double bonds present in the original material.

Quantitative application of the method requires standardised conditions suitable for the particular material under examination. Optimum conditions for the estimation of the unsaturated acids present in say linseed oil, are not suitable for materials containing acids of higher unsaturation. In all cases maximum conjugation is exhibited after a fixed time in a given strength of alkali, and thereafter decreases owing to decomposition of the material. Where mixtures of widely differing unsaturation are encountered, the method can only be qualitative in a single determination, because, while the conditions may be optimum for conjugation of certain components, they may be insufficient or excessive for others.

In order to provide information concerning the unsaturation present in Fractions I - VI and to facilitate assignment of unsaturation between the component chain lengths (Table XII), these materials were alkali isomerised using the method of Herb and Riemenschneider (8), which had been found satisfactory for acids of higher unsaturation. The results obtained are summarised in Table XIII and a typical curve showing the light absorption of the alkali isomerised Fraction I is shown in Fig. VII.

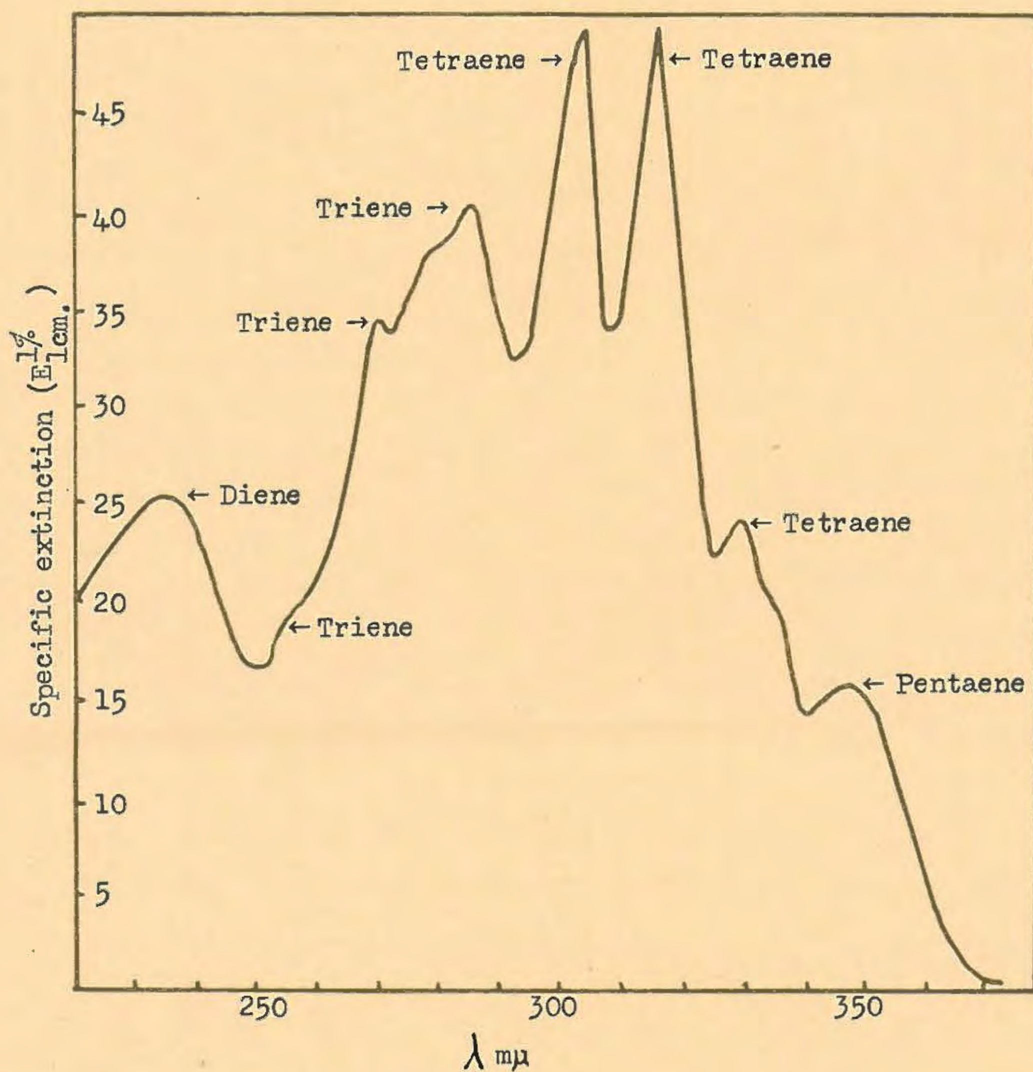


Fig. VII. Ultra-Violet Absorption of Alkali-Isomerised Fraction I (Table XI).

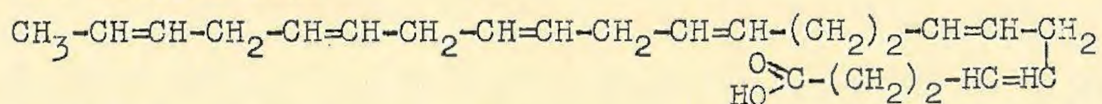
(Isomerised in 21% KOH-ethylene glycol reagent for 15 min. at 180° under N_2, acc. to Herb & Riemenschneider (8).)

Table XIII. Specific Ultra-Violet Extinction ($E^{1\%}_{1cm.}$) of Alkali Isomerised Fractions I, II, III and VI.

Fraction	Diene	Triene			Tetraene			Pentaene			
	λ234	λ259	λ270	λ278	λ283	λ303	λ317	λ303	λ317	λ330	λ347
I	25	21	34	38	40	50.4	50.5	50.4	50.5	23	15.5
II	34	30	40	41.3	40.8	42	38.4	42	38.4	17	10
III	40	36	44	42.5	43	39	37	39	37	21	13
VI	41	38	46.5	44	42	32.5	32	32.5	32	23	17

Inspection of the results in Table XIII shows an increase in the amount of diene and triene absorption in the series I - VI, but whether this is due to an increase in the actual amount of diene and triene acids or to incomplete isomerisation of higher acids cannot be ascertained. As has been pointed out, conditions which could be satisfactory for isomerisation of Fraction I, might be insufficient for complete isomerisation of Fraction VI.

Owing to considerable overlapping between tetraene and pentaene peaks and because of the compound nature of the alkali isomerisation curves in general, the results can only be accepted as qualitative. It is of interest to note that whereas Fraction VI (Table XI) has an average unsaturation of over 5 double bonds per molecule and must therefore contain hexaene or higher unsaturated acids, no peaks due to higher than pentaene conjugation were observed for the alkali isomerised material. It would thus appear possible that Fraction VI contains acids in which certain of the six (or more) double bonds are separated from the rest by more than a single methylene group. For example the hexaene acid



would show only diene and tetraene conjugation after alkali isomerisation.

The Composition of Fractions I - VI.

Inspection of the chromatographic curves for Fractions I - VI (Fig. VI) shows that as peaks 1 and 2 on the left hand side diminish in quantity, so peak 4 on the right hand side increases, while peak 3 reaches a maximum and then again diminishes. This behaviour is paralleled by the variation in chain length distribution of Fractions I - VI (Table XII), from which it is seen that as the molar percentage of lower boiling C_{16} acids shows a steady decrease, so the percentage of higher boiling C_{22} acids shows an increase, while the percentage of C_{18} and C_{20} acids reaches a maximum and then decreases. A broad assignment of chain length to the peaks shown in the chromatographic curves is thus possible. Peaks 1 and 2 may well represent C_{16} acids, peak 4 C_{22} acids, peak 3 a mixture of C_{18} and C_{20} acids, and peak 5 a complex mixture of variable composition probably consisting largely of C_{22} acid in Fraction VI.

Although such an assignment of chain length to the peaks shown in the chromatographic curves (Fig. VI) is probably correct, the limitations of the chromatographic method must at the same time be taken into account. In Chapter 5 it was shown that acids of the same chain length but differing degree of unsaturation should be easily resolvable, but that where one component has a double bond and two methylene groups more than another, resolution cannot be expected. The peaks shown for Fractions I - VI (Fig. VI) cannot therefore represent acids of a single chain length, and considerable overlapping must be expected. For example, C_{18} triene, C_{20} tetraene and C_{22} pentaene acids might all be represented by a single peak, which would be quite distinct from the peak due to say a C_{20} pentaene acid.

Examination of the alkali isomerisation data for four of Fractions I - VI (cf. Table XIII), shows that these

RHODES UNIVERSITY LIBRARY

materials are still too complex for unambiguous assignment of unsaturation between the component chain lengths. Comparison of the average unsaturation of the Fractions (Table XI) with the molar proportions of the acids present (Table XII) also shows this to be the case.

Subdivision of Fraction I by Urea Complex Fractionation.

Since all of Fractions I - VI were still complex mixtures, a further means was required for their subdivision into fractions of simpler composition.

To this end, the method of urea complex fractionation (Chapter 6) was found to be suitable, and was applied to Fraction I with the results shown in Table XIV. The chain length analyses of certain of the sub-fractions are also shown in this table, together with other relevant analytical data.

Table XIV. Urea Complex Fractionation of Fraction I.

Fraction	Weight (g.)	Eq. wt.	No. of* double bonds per molecule	Molar percentages			
				C_{16}	C_{18}	C_{20}	C_{22}
I	2.02	271	3.55	62.8	13.2	17.0	7.0
I α	0.14	281	1.66	69.0	19.0	10.5	1.5
I β	0.18	273	2.55	59.5	13.8	22.6	4.1
I γ	0.08	276	3.14	-	-	-	-
I Δ	0.04	275	3.34	-	-	-	-
Residue	1.5	263	3.68	79.3	11.0	9.7	0.0

* H_2 uptake $Pd/BaSO_4$.

The sub-fractions Iα - IΔ obtained as their urea complexes from Fraction I, and the non complex forming residue were all subjected to reversed phase partition chromatography (cf. Chapter 5) and the curves obtained are shown in Fig. VIII.

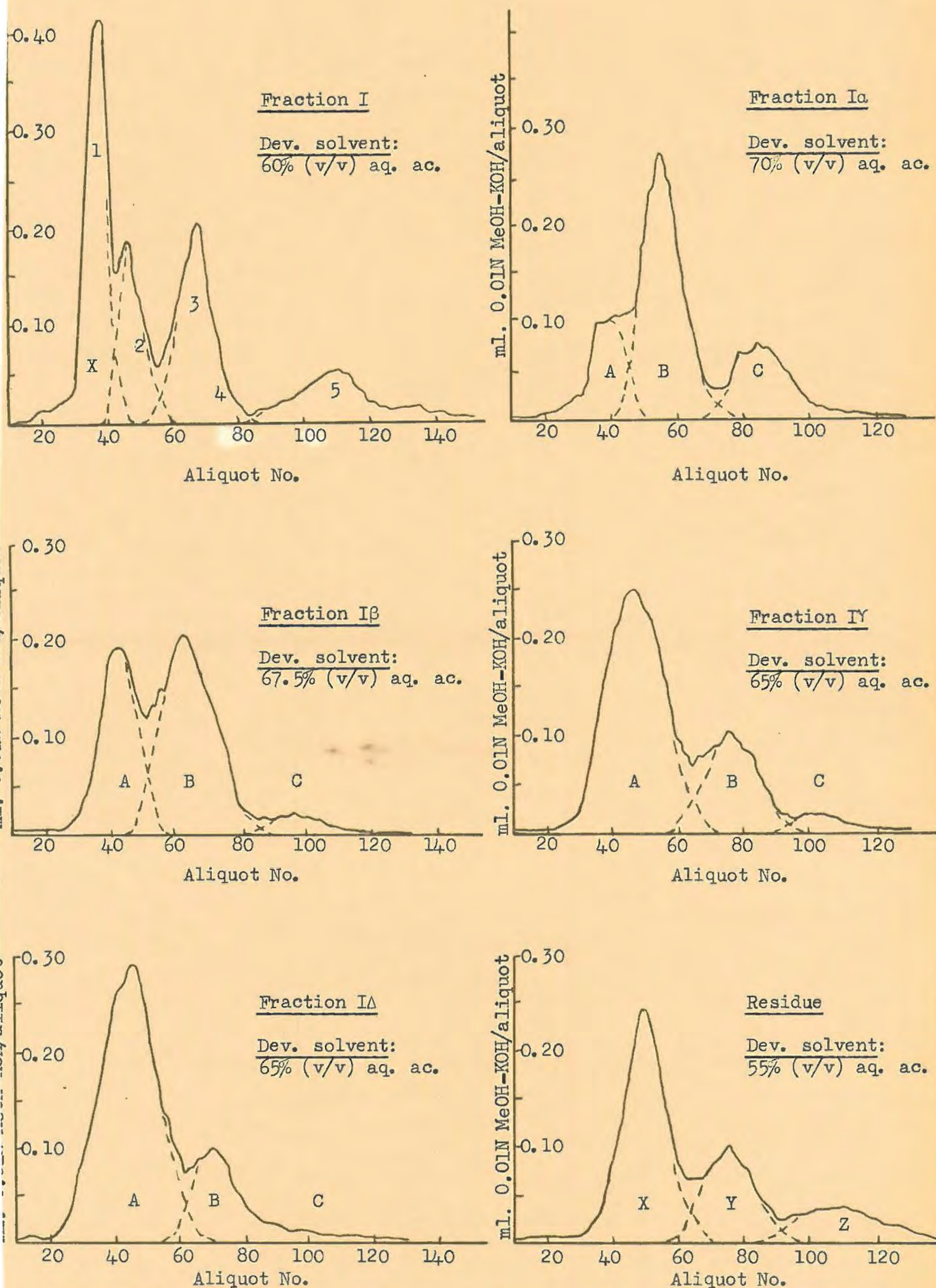


Fig. VIII. Chromatographic Curves of Fractions Iα - IΔ (Table XIV) from Urea Complex Fractionation of Fraction I (Table XI).

Solvent system:	Aqueous acetone - medicinal paraffin supported on hydrophobic kieselguhr.
Column:	35 cm. ht. x 1.3 cm. diam.
Temperature:	10°.
Flow rate:	35 ml. per hour.
Column load:	ca. 21 mg. each Fraction.

Inspection of the chromatographic curves for the urea segregates, Fractions Iα - IΔ (Fig. VIII) shows that a well defined variation of the peaks is apparent. Peak A increases throughout the series, while B and C diminish. An attempt to assign unsaturation between the component chain lengths of these fractions showed that they were, however, still too complex to permit unambiguous calculation of their composition.

The non complex forming residue obtained by urea fractionation of Fraction I was, on the other hand, a relatively simple mixture of acids whose composition could be calculated without isolation of individual components. The chromatographic curve of this material shows three peaks (Fig. VIII) whose molar percentages are 52.7%, 26.6% and 20.6% respectively. The chain length analysis of the fraction (Table XIV) shows that no C_{22} acid is present. The molar percentage of C_{16} acid (79.3%) suggests that peaks X and Y in the unsaturated curve (Fig. VIII) are both C_{16} acid, since the sum of their molar percentages (52.7 + 26.6) is in agreement with the C_{16} value determined. Peak Z is therefore likely to be a mixture of C_{18} and C_{20} acids, since its molar percentage (20.6%) is in agreement with the total of the C_{18} and C_{20} percentages determined (11.0 + 9.7, Table XIV).

In the assignment of unsaturation to the peaks of the residue curve (Fig. VIII), it is reasonable to assume that peak X is a C_{16} tetraene acid and peak Y a C_{16} triene, since resolution might be expected between such a pair of acids with the more highly unsaturated member being eluted first. Peak Z of the residue curve (Fig. VIII) is likely to be a mixture of C_{18} triene and C_{20} tetraene acids, since these would be expected to give an overlapping peak and yet both be resolvable from C_{16} tetraene and triene acids.

Assuming therefore, that the residue curve (Fig.VIII) represents a mixture of 52.7% C_{16} tetraene, 26.6% C_{16} triene, 11% C_{18} triene and 9.7% C_{20} tetraene, then the average equivalent weight calculated for such a mixture would be 259 and the average number of double bonds per molecule, 3.62. These values are in excellent agreement with the values of 263 and 3.68 experimentally determined (Table XIV), and indeed it is not possible to postulate any other composition for the residue curve such that the theoretically calculated values for equivalent weight and average unsaturation agree with those experimentally observed.

Analysis of the residue curve (Fig. VIII) shows that by successive subdivision of pilchard acid fractions it is possible to obtain a mixture of a sufficiently simple nature to permit calculation of its composition without isolation of individual components. Other fractions obtained were still too complex in nature to allow of more than qualitative analysis, but there is no reason to doubt that further subdivision of these mixtures by the techniques suggested, would result in less complex fractions amenable to quantitative analysis. An overall analysis of pilchard oil would thus be possible, and the method proposed constitutes a new approach to the analysis of marine oils without isolation of individual acids.

It is considered that the procedure overcomes certain disadvantages inherent in the Hilditch method, where the latter is applied to marine oils. The use of very mild fractionation techniques overcomes a disadvantage in regard to thermal modification of components during the extended Hilditch distillation, while the fact that relatively more simple fractions can be obtained, permits an unambiguous assignment of unsaturation between component chain lengths. Consideration of the results obtained in

this new analytical approach, illustrates that the composition of marine oils is of far greater complexity than even hitherto envisaged.

REFERENCES.

(1) T.P. Hilditch. "The Chemical Constitution of Natural Fats". Chapman & Hall, London (1940).

(2) S.F. Herb & R.W. Riemenschneider. Anal. Chem. 25, 953 (1953).

(3) F.A. Vandenheuvel & G.H. Richardson. J. Amer. Oil Chem. Soc. 30, 104 (1953).

(4) D.A. Sutton. Chem. and Ind. 1383 (1953).

(5) J.A. Bolland, A. Sundralingham, D.A. Sutton & G.R. Tristram. British Rubber Producers Assn. Publication 13 (1941).

(6) H.H. Sephton & D.A. Sutton. Chem. and Ind. 667 (1953).

(7) J.E. Jackson, R.F. Paschke, W. Tolberg, H.M. Boyd & D.H. Wheeler. J. Amer. Oil Chem. Soc. 29, 229 (1952).

(8) S.F. Herb & R.W. Riemenschneider. J. Amer. Oil Chem. Soc. 29, 456 (1952).

(9) E.H. Farmer. Trans. Farad. Soc. 38, 358 (1942).

CHAPTER 8.

THE ISOLATION OF A NEW FATTY ACID FROM SOUTH AFRICAN PILCHARD OIL.

In the previous discussion concerning a new approach to the analysis of pilchard oil without isolation of individual components, the presence of a C_{16} tetraene acid was postulated in order to account for certain observed analytical values. The isolation of such an acid is now reported, together with its characterisation by physical and chemical means.

It was earlier shown (Chapter 6) that by a combination of the techniques of lithium soap-acetone segregation and urea complex fractionation, it was possible to prepare a concentrate of pilchard acids rich in the shorter C_{16} and C_{18} unsaturated members. This concentrate (cf. Table VII, Chapter 6) was selected as the raw material for isolation of the required C_{16} acid and, as a first step, the mixture was distilled in a three stage falling film molecular still. The results of this distillation are illustrated in Table XV.

Table XV. Molecular Distillation of the Unsaturated Concentrate prepared by Urea Fractionation of the Lithium Soap-Acetone Concentrate.

Fraction	Distil-lation temp.	Weight (g.)	Eq. wt.	Refractive index $\eta^{D}_{31^{\circ}}$	No. of* double bonds per molecule
Unsaturated concentrate	-	54.3	308	-	4.85
A	85°	2.60	260	1.4801	3.67
B	90.5°	1.78	263	1.4811	3.72
C	96°	5.53	268	1.4827	3.93
Residue R (3rd stage)	-	1.18	295	1.4889	4.54
Main bulk Res.	-	41.7	325	-	5.38

* H_2 uptake $Pd/BaSO_4$.

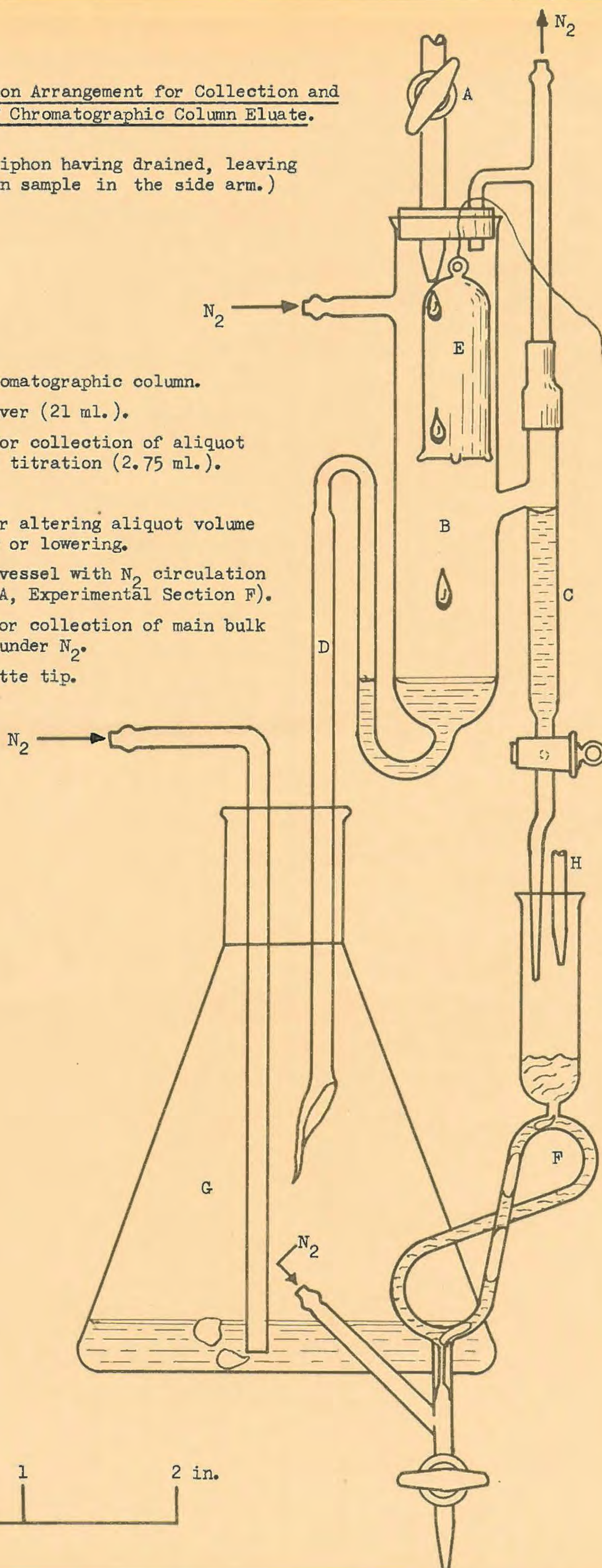


Fig. IX. Siphon Arrangement for Collection and Titration of Chromatographic Column Eluate.

(Showing siphon having drained, leaving titration sample in the side arm.)

Legend.

A = Tap of chromatographic column.

B = Main receiver (21 ml.).

C = Side arm for collection of aliquot sample for titration (2.75 ml.).

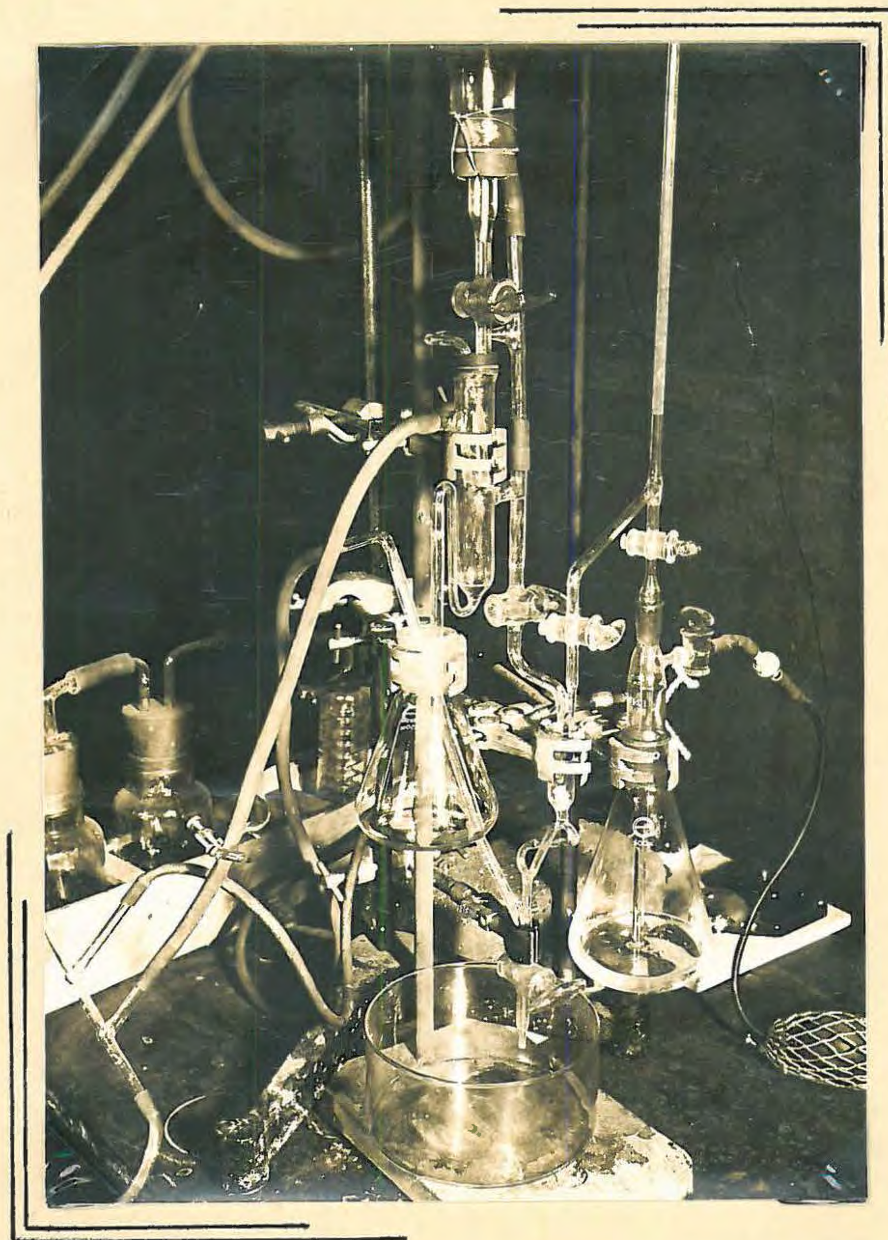
D = Siphon.

E = Plunger for altering aliquot volume by raising or lowering.

F = Titration vessel with N_2 circulation (cf. Fig. A, Experimental Section F).

G = Receiver for collection of main bulk of eluate under N_2.

H = Micro-burette tip.



Siphon Arrangement for the Collection and Titration of Preparative Chromatographic Column Eluates under Nitrogen

(Showing siphon with side-arm, burette and figure of eight titration vessel)

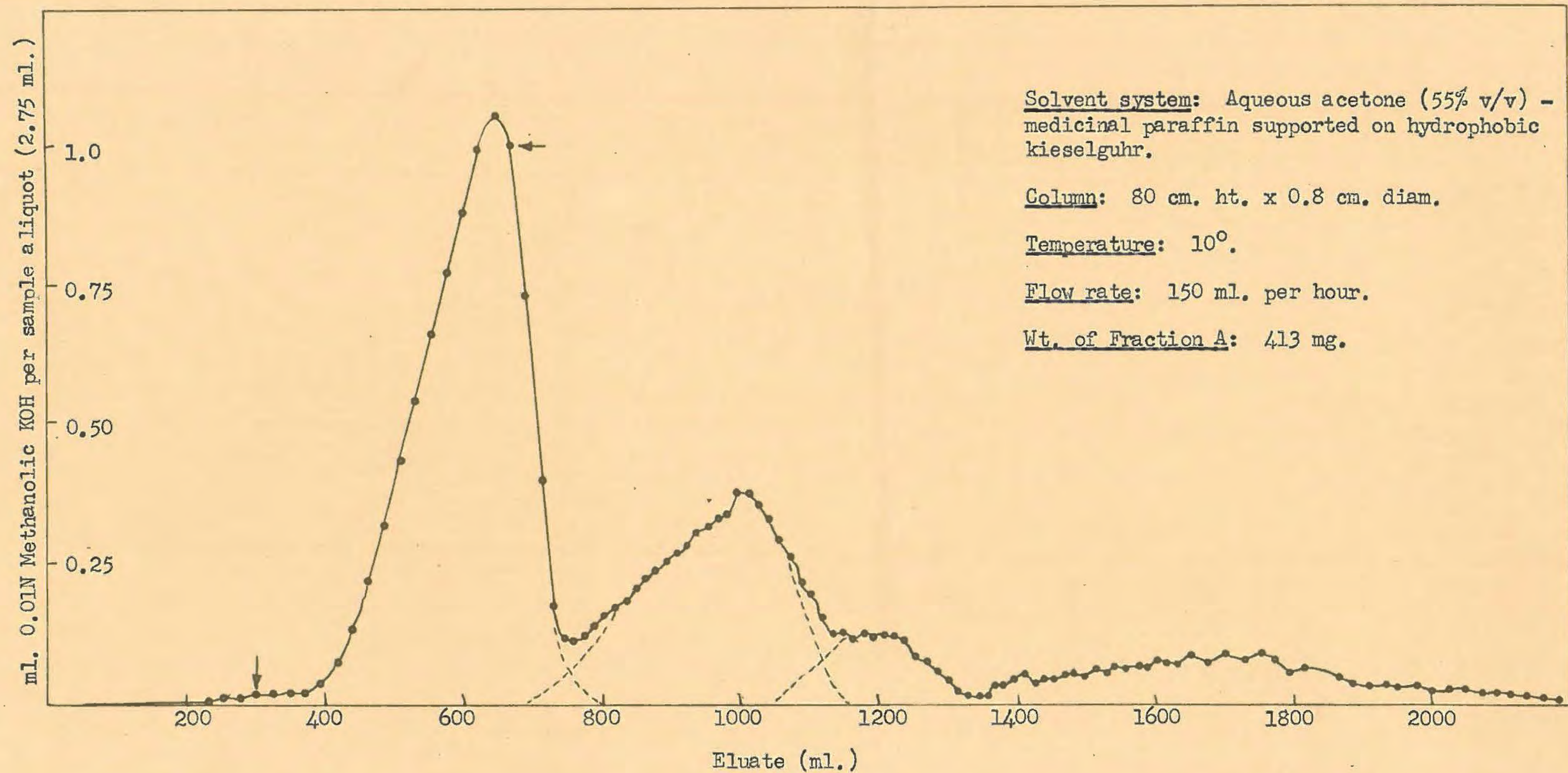


Fig. X. Chromatographic Resolution of Fraction A (Table XV) — Preparative Scale.

(The eluate between the arrows was collected separately as being representative of the first peak.)

The lowest boiling Fraction A resulting from the distillation, contained the required acid in the highest concentration and was accordingly submitted to reversed phase partition chromatography on a preparative scale. After preliminary investigation, it was found that a column 80 cm. high x 3 cm. in diameter was best suited to the purpose, and this was accordingly packed with non-wetting kieselguhr supporting medicinal paraffin as the stationary phase (cf. Chapter 4). The chromatogram was developed at 10° with 55% (v/v) aqueous acetone, and the eluate collected under nitrogen in a siphon constructed such that each aliquot was divided into two portions; the main bulk and a smaller sample which was titrated for the purpose of following the course of chromatographic resolution. This device is illustrated in Fig. IX, and photographs are shown on the following page. The chromatographic curve obtained is shown in Fig. X.

The column eluate between the arrows shown in Fig. X was collected separately as being representative of the first peak. Exhaustive extraction of this eluate with n-pentane, followed by evaporation of the solvent, gave a pale yellow liquid ($\eta^{D}_{29^{\circ}}$ = 1.4870) with analytical values corresponding closely to those required for a hexadeca-tetraenoic acid. Quantitative hydrogenation showed the presence of four double bonds per molecule. Analysis of the recovered hydrogenation product showed it to be palmitic (C_{16}) acid, and this was confirmed by a mixed melting point determination with an authentic sample of palmitic acid.

The eluate corresponding to the second peak shown in Fig. X was also collected separately, and the dissolved acid extracted with n-pentane. The peak very probably represented a mixture of incompletely resolved

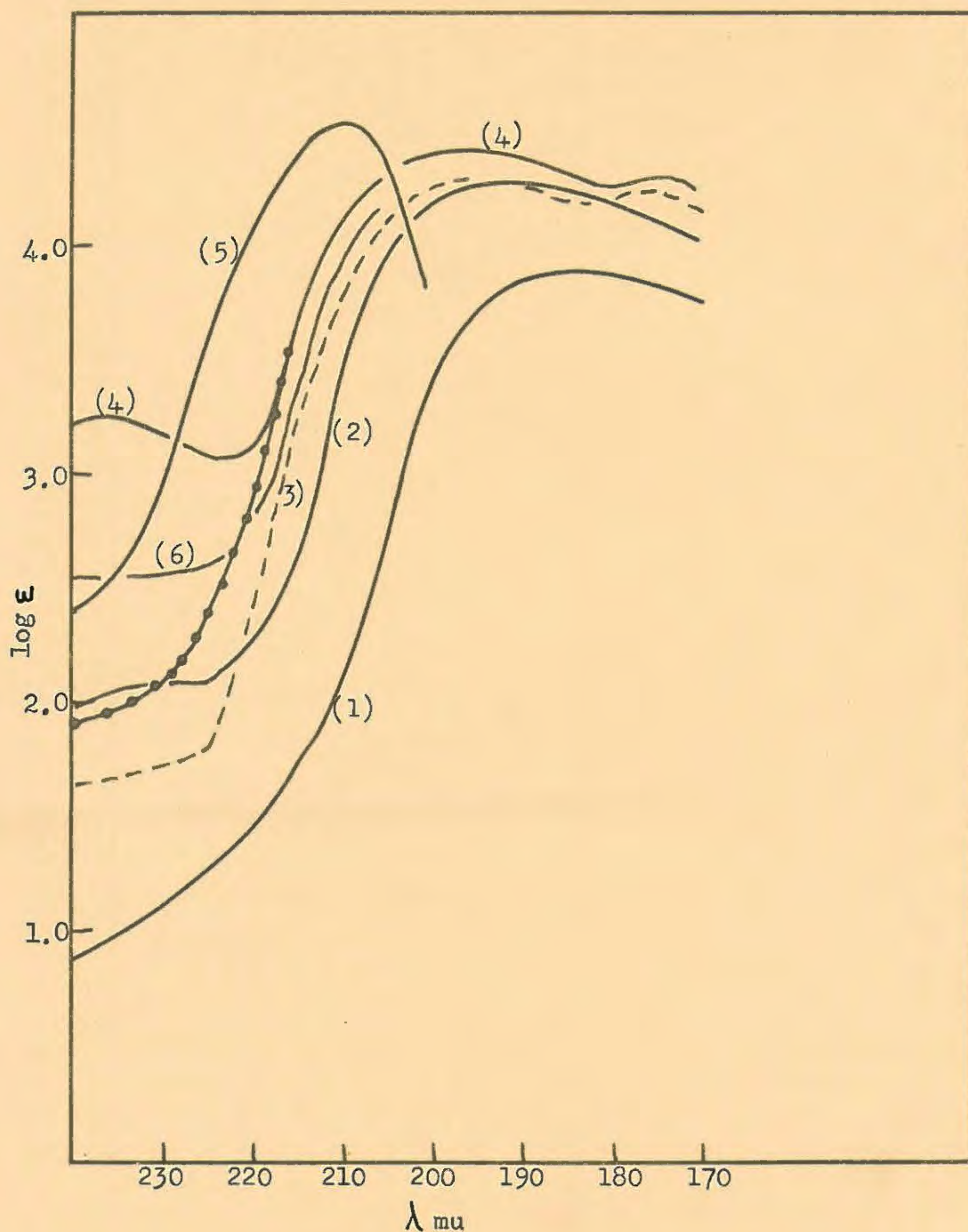


Fig. XI. Ultra-Violet Absorption of Fatty Acids in the Region λ170 - λ240 mμ.

Curve (1), oleic acid. Curve (2), linoleic acid.
Curve (3), linolenic acid. Curve (4), methyl arachidonate.

(Curves (1), (2), (3) and (4) from I.I. Rusoff, J.R. Platt, H.B. Klevens and G.O. Burr. J. Amer. Chem. Soc. 67, 675 (1945).)

Curve (4, dotted line), methyl arachidonate in the region λ220 - λ240 mμ (Taken from R.H. Barnes, I.I. Rusoff, E.S. Miller and G.O. Burr. Ind. Eng. Chem. (Anal. Ed.), 16, 385 (1944).)

Curve (5), heptadeca-2-enoic acid. (Taken from W.M. Lauer, W.J. Gensler and E. Miller. J. Amer. Chem. Soc. 63, 1153 (1945).)

Curve (6), hexadecatetraenoic acid isolated from South African pilchard oil.

hexadecatrienoic acid and eicosatetraenoic acid (see Experimental Section).

The Structure of the Hexadecatetraenoic Acid.

Ultra-violet light absorption.

The ultra-violet light absorption curve of the hexadecatetraenoic acid showed no diene, triene or tetraene peaks in the region λ220 - λ350 mμ, thus proving that no conjugated double bonds were present. A faint inflection at λ231 mμ indicated that a negligible amount of oxidation had taken place, as is usual with materials of this type. This amount of oxidation was not greater than 0.33%, as shown by comparison of the $E^{1\%}_{1cm.}$ observed at λ231 mμ (= 1.95) with that of pure methyl linoleate hydroperoxide (=780) at the same wave length (1).

The ultra-violet absorption of the hexadecatetraenoic acid in the region λ206 - λ240 mμ is shown in Fig. XI, together with the far ultra-violet absorption of several other known acids in the region λ170 - λ240 mμ.

Comparison of the curves shows that the hexadecatetraenoic acid spectrum lies in the expected position close to that of methyl arachidonate (C_{20} tetraene ester). The molar absorption observed at λ210 mμ is some twenty times less than that shown by the peak of 2-heptadecenoic acid which has unsaturation conjugated with the carboxyl group. The hexadecatetraenoic acid cannot therefore be α-β unsaturated.

Alkali isomerisation of the hexadecatetraenoic acid.

In order to gain information concerning the nature of the unsaturation present in the hexadecatetraenoic acid, it was alkali isomerised by the method of Herb and Riemenschneider (2) (cf. Chapter 7). The ultra-violet

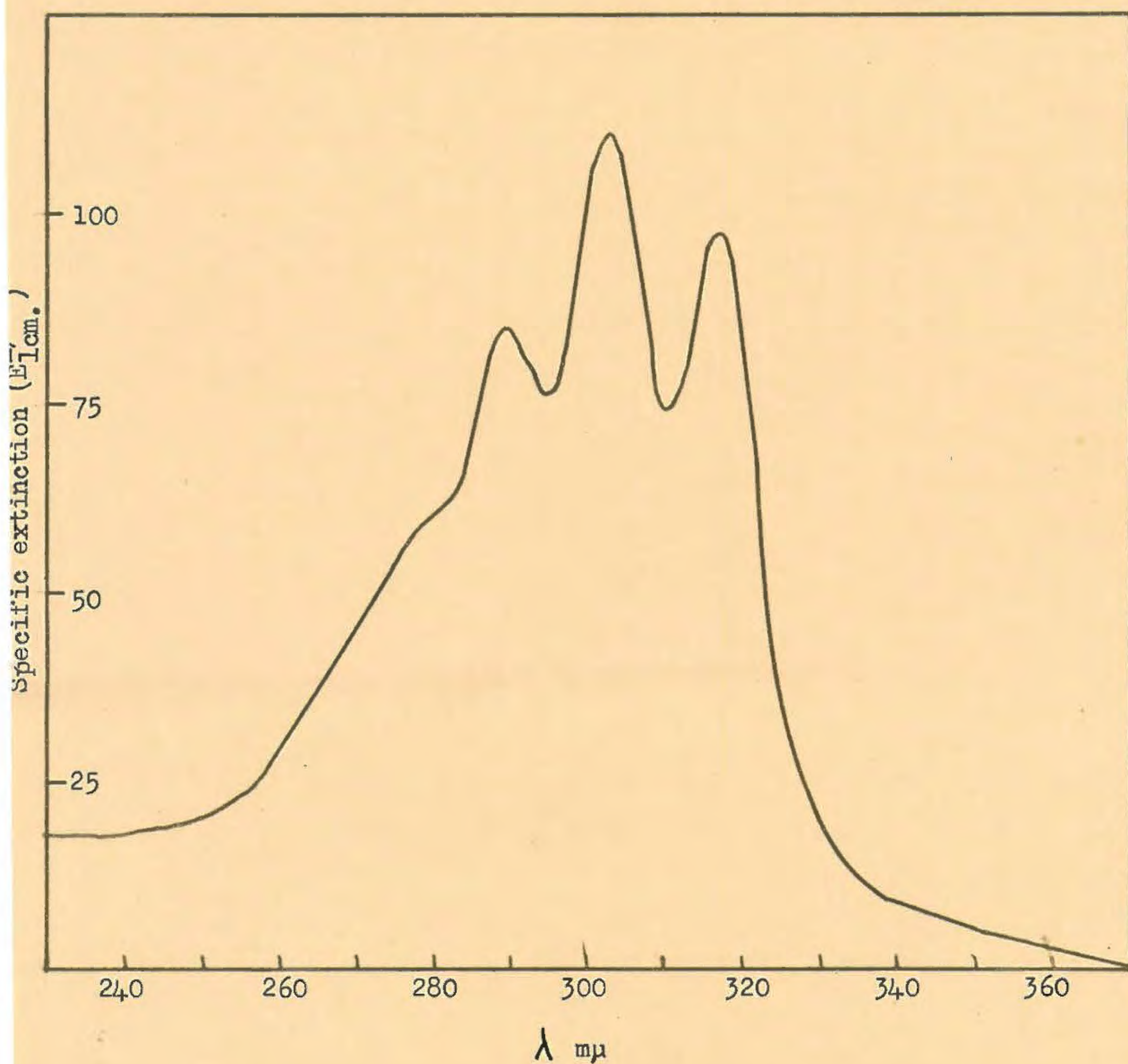


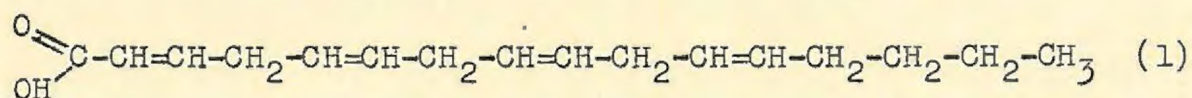
Fig. XII. Ultra-Violet Absorption of Alkali-Isomerised Hexadecatetraenoic Acid.

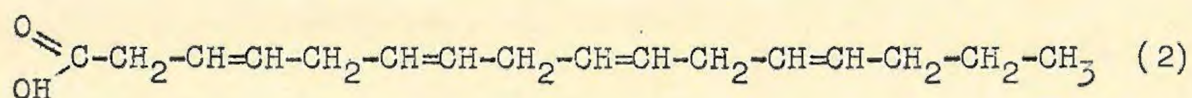
(Isomerised in 21% KOH-ethylene glycol reagent for 15 min. at 180° under N_2, acc. to Herb & Riemenschneider (2).)

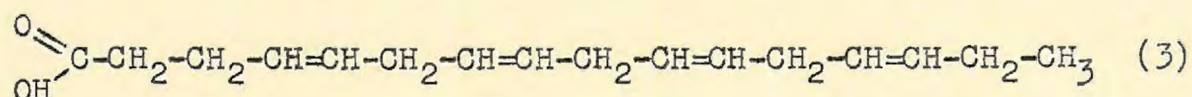
absorption curve of the isomerised material (Fig. XII) showed clearly the three peaks and one inflection due to a conjugated tetraene. No other peaks were apparent.

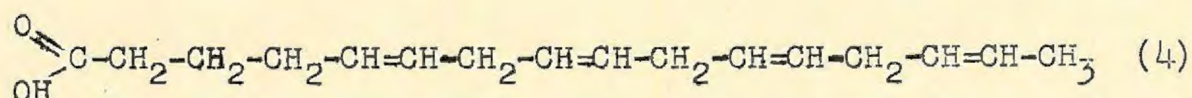
The acid is thus proved to be a tetraene with all the double bonds separated by a single methylene group. If this were not so, then tetraene absorption would not have been evidenced after alkali isomerisation because double bonds separated by more than a single methylene group do not isomerise into conjugation (3).

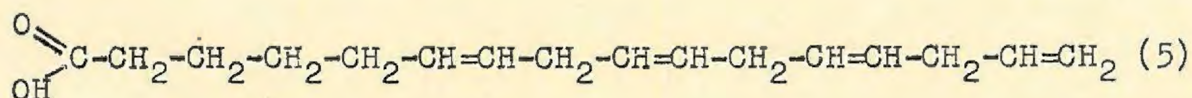
Only five structures are possible for a C_{16} tetraene acid in which the double bonds are separated by a single methylene group. These are as follows:











Of these structures (1) is α-β unsaturated and cannot obtain on the grounds of ultra-violet evidence. The choice remains therefore between structures (2), (3), (4) and (5).

<u>Infra-red spectroscopic examination of the hexadecatetraenoic acid.</u>

Structures (1), (2), (3) and (4) all contain a $C-CH_3$ group which exhibits a well defined peak at approximately 1375 $cm.^{-1}$ in the infra-red. The infra-red absorption of the hexadecatetraenoic acid was accordingly measured in the region 1325 - 1425 $cm.^{-1}$, and the curve

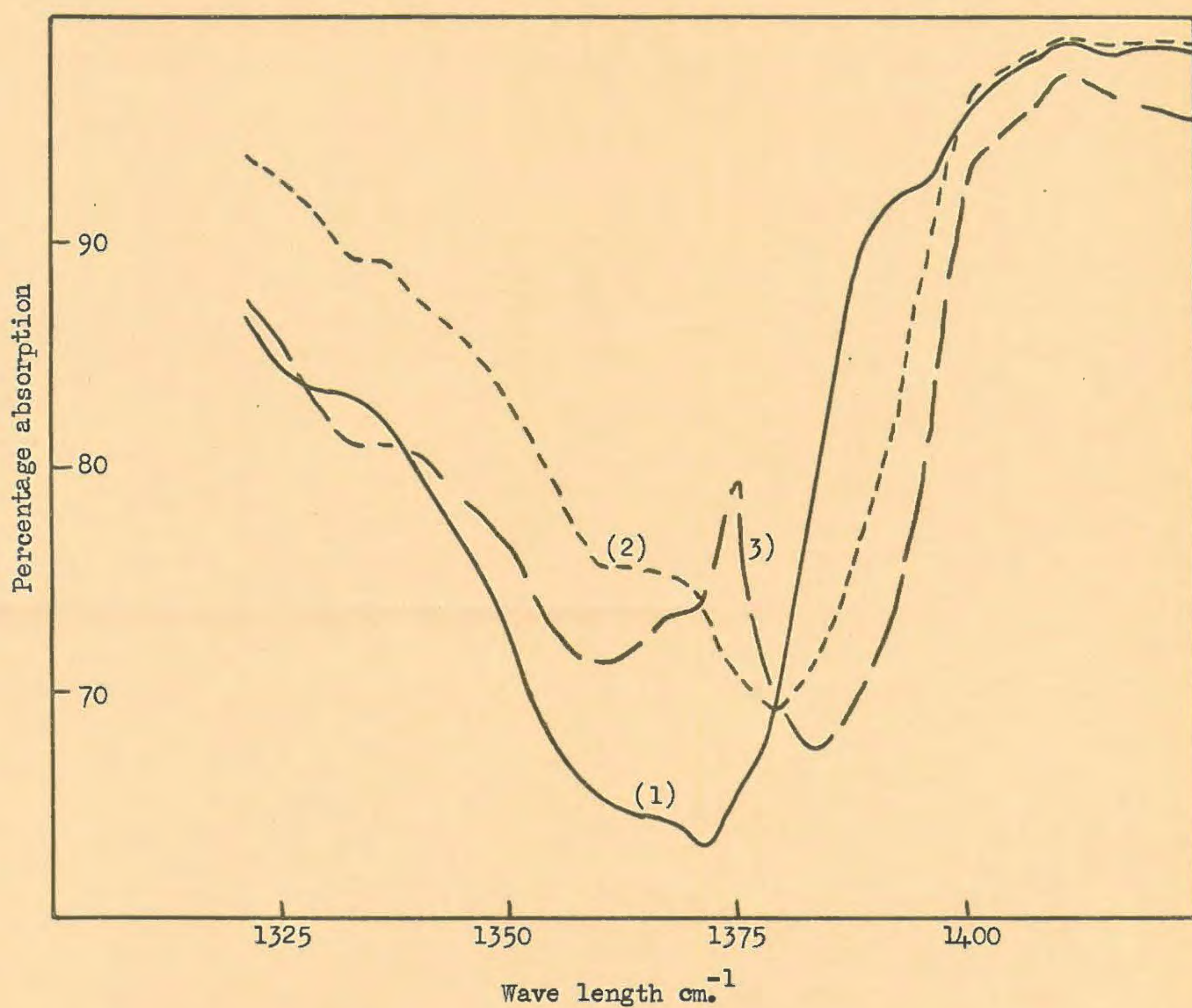


Fig. XIII. Infra-Red Absorption of Hexadecatetraenoic Acid (1), 10-Undecylenic Acid (2) and Palmitic Acid (3) in the Region 1325 - 1425 cm.$^{-1}$

(Concentrations in CCl_4: hexadecatetraenoic acid, 4.97% (w/w); 10-undecylenic acid 5.00% (w/w); palmitic acid, 4.84% (w/w).

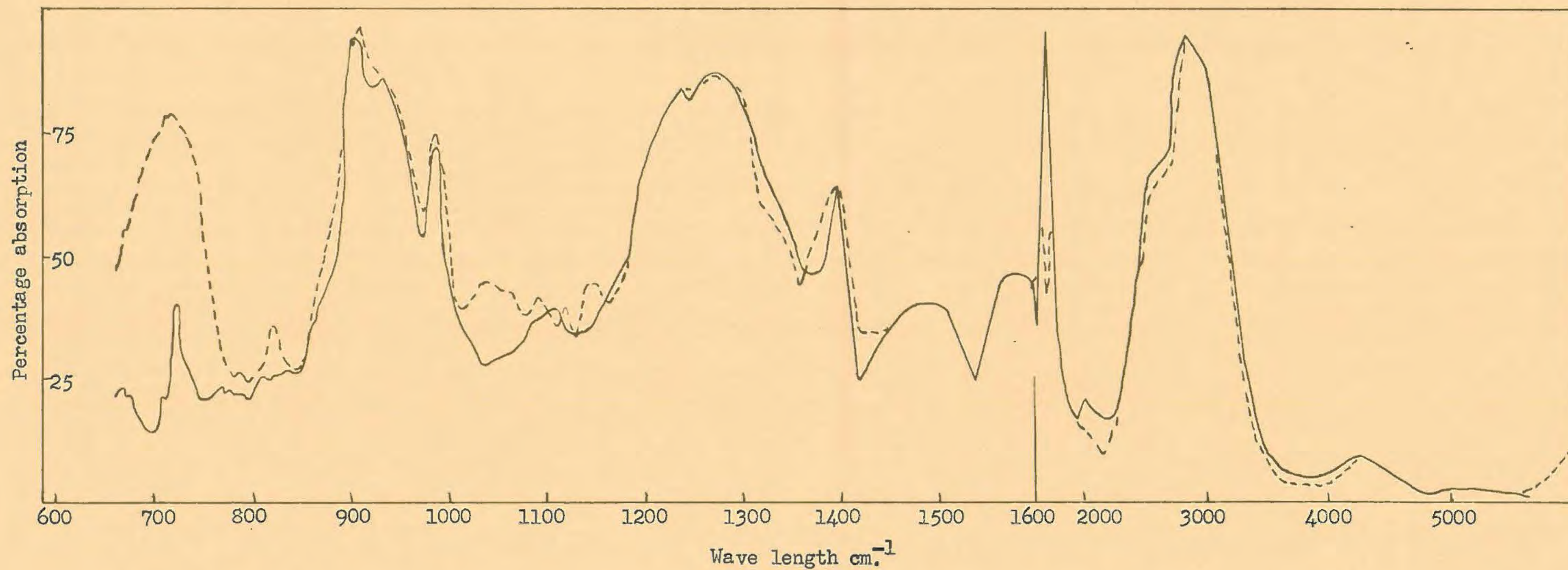


Fig. XIV. Infra-Red Absorption of Hexadecatetraenoic Acid (smooth curve) and 10-Undecylenic Acid (broken curve).

(Concentrations: Hexadecatetraenoic acid, 3.04% (w/w) in CS_2; 10-undecylenic acid 2.47% (w/w) in CS_2.)

obtained is shown in Fig.XIII, together with the curves of two reference compounds in the same region. The two compounds chosen for reference were palmitic acid which contains a $C-CH_3$ group, and 10-undecylenic acid which contains no $C-CH_3$ group, but instead a terminal vinyl group as in structure (5).

Examination of the curves shown in Fig. XIII shows that the peak due to the $C-CH_3$ absorption of palmitic acid at 1375 $cm.^{-1}$ is completely absent from the curve of the hexadecatetraenoic acid and also from that of the second reference compound, 10-undecylenic acid.

Structures (1), (2), (3) and (4) are thus clearly impossible for the isolated acid, which must therefore be represented by structure (5) containing a terminal vinyl group. This grouping exhibits intense maxima at 910 $cm.^{-1}$ and 990 $cm.^{-1}$ in the infra-red (4), and both these peaks were strongly evidenced in the full absorption curve of the hexadecatetraenoic acid shown in Fig. XIV. The spectrum of the reference compound 10-undecylenic acid which contains the $-CH=CH_2$ grouping is also shown in Fig. XIV.

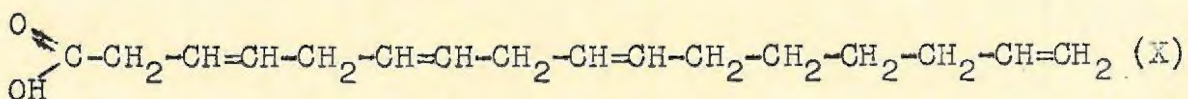
Trans-trans conjugated double bonds such as might arise from autoxidation of an unsaturated acid, also exhibit an infra-red maximum at 990 $cm.^{-1}$, but since no peak due to oxidation was shown by the ultra-violet absorption curve of the isolated acid, there is no likelihood that the observed peak at 990 $cm.^{-1}$ can have been due to oxidation. The measured intensity of this peak, if due to trans-trans conjugated bonds and not to a terminal vinyl group, would correspond to some 50% oxidation having taken place (cf. Chapter 7), which is clearly impossible.

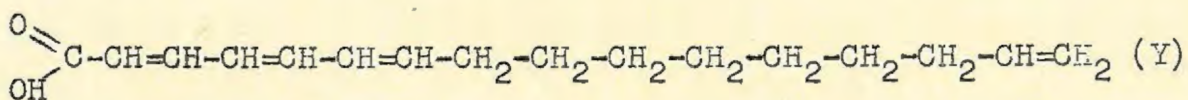
Oxidation of the hexadecatetraenoic acid.

In order to gain additional proof as to the validity of structure (5) for the isolated acid, it was

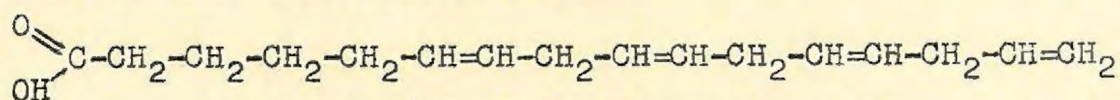
subjected to disruptive oxidation with cold alkaline permanganate (5). Separation of the dibasic oxidation products, followed by paper chromatography of the mixture with ethanolic ammonia as solvent (6), showed a spot due to adipic acid. This acid would be expected to result from oxidation of structure (5), but not from any of structures (1), (2), (3) or (4). The spot was in exact correspondence with a spot of authentic adipic acid, and well separated from spots due to other (C_2 - C_4) reference acids. Some glutaric acid was also evidenced in the oxidation products of the hexadecatetraenoic acid, but this probably arose from further degradation of the adipic acid (cf. 7).

A further structure (X) which includes a terminal vinyl group and which would also give adipic acid on oxidation, cannot be that of the isolated acid, because the conjugated triene-carboxylic acid which might arise on alkali isomerisation (structure Y) would show only one peak at approximately 300 mμ in the ultra-violet (cf. 8 and 9). Since the three peaks of a conjugated tetraene were observed for the alkali isomerised acid (Fig. XII), structure (X) cannot obtain.





Thus structure (5), hexadeca-6:9:12:15-tetraenoic acid, is clearly established as that of the acid isolated from pilchard oil. This compound has not previously been reported and the name <u>marinolic acid</u> is accordingly proposed for it. The acid occurs to an extent of approximately 1.3% of the total pilchard acids.



hexadeca-6:9:12:15-tetraenoic acid

(marinolic acid)

During the writing of this thesis it has come to the notice of the author that a C_{16} tetraene acid has recently been isolated by Toyama and Yamamoto (10) from Japanese sardine oil. The acid was insufficiently characterised and the structural investigation was based on oxidative degradation alone. However, one of the two possible structures suggested contained a terminal vinyl group.

Acids with a terminal vinyl group appear to be extremely rare in nature and only two others have been isolated to date, viz., 9-decenoic acid from butter fat (11), and erythrogenic acid, an acetylenic compound (12) from the seed oil of Ongueka Gore Engler. The latter acid is discussed in Part II of this thesis.

REFERENCES.

(1) H.H. Sephton & D.A. Sutton. Chem. and Ind. 667 (1953).

(2) S.F. Herb & R.W. Riemenschneider. J. Amer. Oil Chem. Soc. 29, 505 (1952).

(3) E.H. Farmer. Trans. Farad. Soc. 38, 358 (1942).

(4) D. Barnard, L. Bateman, A.J. Harding, H.P. Koch, N. Sheppard & G.B.B.M. Sutherland. J. Chem. Soc. 916 (1950).

(5) D.E. Dolby, L.C.A. Nunn & I. Smedley-Maclean. Biochem J. 34, 1423 (1940).

(6) A.G. Long, J.R. Quayle & R.J. Stedman. J. Chem. Soc. 2197 (1951).

(7) P.H. Begemann, J.G. Keppler & H.A. Boekenoogen. Rec. Trav. Chim. 69, 439 (1950).

(8) K.W. Hauser, R. Kuhn, A. Smakula & M. Hoffer. Z. physikal. Chemie B29, 371 (1935).

(9) B.C.L. Weedon & R.J. Woods. J. Chem. Soc. 2687 (1951).

(10) Y. Toyama & T. Yamamoto. Memoirs Faculty of Eng. Nagoya Univ. 5, No.1 (1953).

(11) A. Grün & T. Wirth. Chem. Ber. 55, 2197 (1922).

(12) H.K. Black & B.C.L. Weedon. J. Chem. Soc. 1785 (1953).

HIGHLY UNSATURATED ACIDS FROM THE BODY OIL OF THE SOUTH AFRICAN PILCHARD

EXPERIMENTAL SECTION

EXPERIMENTAL SECTION.

The experimental details are presented in six separate Sections, each relevant to a Chapter in the text.

SECTION A records the details relevant to CHAPTER 3
SECTION B records the details relevant to CHAPTER 4
SECTION C records the details relevant to CHAPTER 5
SECTION D records the details relevant to CHAPTER 6
SECTION E records the details relevant to CHAPTER 7
SECTION F records the details relevant to CHAPTER 8

EXPERIMENTAL DETAILS.

SECTION A.

The following experimental details are relevant to Chapter 3.

Saponification of Crude Bleached Pilchard Oil.

Crude pilchard oil (2.5 kg.) was added in small portions with shaking to aqueous alcoholic potassium hydroxide (KOH, 1500 g.; EtOH 9.0 l., H_2O 1.5 l.). The solution was left under N_2, 19 hours at 30° and then 47 hours at 4°, after which it was diluted with water (25 l.). Portions (6 l.) of the solution were extracted with n-hexane (4 x 3 l.) to remove the "non-saponifiable" product. The aqueous layer was then acidified with 10% aqueous sulphuric acid solution (12 l.), and the precipitated (liquid) acids removed in a separating funnel. The acids were then dissolved in n-hexane (5 l.) and washed with water (4 x 2 l.). Evaporation of the hexane gave the total pilchard acids (2.17 kg., 87% of total) having an equivalent weight of 282 and an average number of double bonds per molecule = 2.40 (H_2 uptake Pd/$BaSO_4$). These acids were sealed in glass bulbs (500 g. in each) at 10^{-4}mm., and stored for further use.

Lithium Soap-Acetone Segregation of the more highly Unsaturated Acids.

Total pilchard acids (1 kg.) in acetone (2 l.) were neutralised at 15° with saturated aqueous LiOH solution (500 ml.). Acetone (7.5 l.) was added to adjust the solvent concentration to 95%. The mixture was thoroughly shaken and allowed to equilibrate 4 hours at 30° and then overnight at 15°. The precipitated lithium soaps (relatively saturated) were filtered off, and the more highly unsaturated acids recovered from the filtrate by

acidification followed by extraction with n-pentane. After washing of the pentane solution with water, the solvent was evaporated yielding a concentrate of unsaturated acids (333 g.) having an equivalent weight = 307 and average number of double bonds per molecule = 4.55 (208.5 mg. absorbed 86.1 c.c. H_2 at 19.5° and 655 mm. (corr.) over $Pd/BaSO_4$ in HOAc).

Molecular Distillation of Pilchard Unsaturated Acids.

The falling film still illustrated in Fig. I was used to fractionate the mixtures of unsaturated acids. The distilling stages were heated by suitable solvents maintained at boiling point by means of a small electrical immersion heater. The results are shown in Tables I, II and III.

Counter-current Distribution of Pilchard Acid Fraction III (Table I).

A 24-tube stainless steel counter-current apparatus was used. The solvent system iso-heptane : actonitrile (redist.) : methanol : acetic acid (4:1:1:1) was previously equilibrated before use. Polar phase (6.8 ml.) and hydrocarbon phase (9.05 ml.) were placed in each tube, and Fraction III (0.26 g.) introduced into tube 0*. After each equilibration, the layers were allowed to separate (1½ minutes) before rotating the upper barrel for transference of the hydrocarbon phase to the next tube. After completion of the transfers, aliquots (1.0 ml. lower phase + 1.33 ml. upper phase) were extracted from each tube and evaporated in tared basins. A curve of tube number versus weight of material obtained on evaporation was plotted.

For redistribution of the material centered round the peak of the curve, the experiment was performed again

* Numbering of tubes follows the accepted Craig procedure (1).

and after 24 transfers, the contents of ten tubes centered round the minimum of the distribution curve were removed. These tubes were refilled with the appropriate amounts of pure upper and lower phases, and 24 additional transfers performed. The distribution curve was plotted after evaporating aliquots from each tube and weighing the residue.

Fractional Low Temperature Crystallisation of Saturated Fraction III (Table I).

Fraction III (183 mg.) in absolute methanol (35 ml.) was completely hydrogenated over $Pd/BaSO_4$ catalyst (2.5%). The catalyst was removed by filtration, and on standing the solution deposited fine needles which were removed manually and recrystallised once ex MeOH (Fraction 1). On further standing more crystals deposited and were filtered off (Fraction 2). The filtrate was cooled to $+4^{\circ}$ and the precipitated crystals removed (Fraction 3). On cooling of the filtrate to -4° another batch of crystals was obtained (Fraction 4). Further crops of crystals (Fractions 5, 6 and 7) were obtained by cooling successive filtrates to -10°, -20° and -37° respectively. The final filtrate was evaporated to yield the residue (Fraction 8). (The weights and properties of Fractions 1 - 8 are recorded in Table V.)

Paper Chromatography.

All chromatograms were run at a constant temperature of 30°, and the paper allowed to equilibrate with solvent vapour before starting development.

For paper partition chromatograms, strips of filter paper (Whatman No.1, 2 x 13 in.) were exposed (2 hours) to the vapour of dimethyldichlorosilane in a partially evacuated desiccator. The strips were washed with methanol, and after drying (110°, 2 hours) were dipped

in an ethereal solution of medicinal paraffin (10%). Evaporation of the ether gave strips impregnated with paraffin as stationary phase.

Separation of a mixture of lauric and myristic acids:

A strip of paper was spotted with 60γ of a mixture of lauric and myristic acids (1:1), and with 40γ of lauric acid as a separate reference spot. Development of the chromatogram with aqueous acetone (60% v/v, previously equilibrated with paraffin) was carried out for 20 hours at 30°. The paper was allowed to dry somewhat, sprayed with bromothymol blue indicator (0.2% in aqueous acetone 70% v/v), and exposed to ammonia vapour. Acid spots (dark blue against faint blue background) showed clear resolution between lauric (RF 0.6) and myristic (RF 0.65) acids.

REFERENCES.

(1) A. Weissberger. Ed. "Technique of Organic Chemistry". Interscience. New York. Vol. III, p.186 (1950).

EXPERIMENTAL DETAILS.

SECTION B.

The following experimental details are relevant to Chapter 4.

Aqueous acetone concentrations are expressed on a v/v basis (eg. 70% (v/v) acetone in water).

Preparation of Materials.

(1) Non-wetting kieselguhr. "Hyflo Super Cel" obtained from Messrs Johns Manville, U.S.A., was submitted to a process of flotation in water to remove the finer particles. Kieselguhr (ca. 5 lb.) was thoroughly mixed with water (ca. 3 gal.) in a tall narrow vessel and allowed to stand 1 hour, after which the suspended particles were decanted. The process was repeated 3 times on the sediment, and the final product so obtained was dried at 110°. When cool, the material was exposed to the vapour of dimethyl-dichlorosilane in a partially evacuated desiccator. After 2 hours exposure, the kieselguhr was washed free of acids with absolute methanol and dried at 110°. All the particles in a sample of this material were found to float upon the surface when shaken with water, thus ensuring that the kieselguhr had been rendered completely hydrophobic.

(2) Medicinal paraffin. Colourless liquid paraffin (S.G. 0.880 - 0.895) was dissolved in pure n-pentane and percolated through a column of Brockmann Grade I alkaline alumina, to ensure removal of any acidic material. Evaporation of the pentane gave pure paraffin used as stationary phase.

(3) Acids. Samples of palmitic, stearic and lignoceric acids were drawn from laboratory stocks which had been prepared from natural sources by high precision distillation. The acids were further purified by crystallisation before use.

Arachidic and behenic acids were synthesised using an adaptation of the electrolytic method of Greaves, Linstead, Shepheard, Thomas and Weedòn (1).

Arachidic acid: Ethyl hydrogen adipate (17.6 g., 2 mols, prepared according to Swann, Oehler and Buswell (2)) and palmitic acid (13.0 g., 1 mol) were dissolved in methanol (150 ml.). Sodium metal (0.07 g.) was added to neutralise ca. 2% of the acids present. The solution was electrolysed (22 hours) with a current of 0.6 to 0.7 amp. in a tube of 2 in. diameter fitted with a cooling coil, thermometer, reflux condenser, and a pair of platinum electrodes (3.5 x 2.5 cm. spaced ca. 1 mm. apart). The vessel was surrounded by a cooled bath, and the flow of water through the condenser adjusted to maintain a temperature of 40 - 50°. At the end of the electrolysis a large precipitate of crystalline n-triacontane was observed to have formed and the solution was found to be weakly alkaline. The mixture was rendered faintly acid with acetic acid (0.2 ml.) and the solvent evaporated. Aqueous alcoholic KOH solution was then added (KOH, 10 g.; H_{2O}, 7 ml.; and abs. EtOH (93 ml.)), and the mixture refluxed 2½ hours on the water bath to saponify the ethyl esters. Water (55 ml.) was then added and the solution extracted with n-pentane (4 x 100 ml.) to remove hydrocarbon. The aqueous layer was then acidified with cold aqueous H_2SO_4 solution (20%) and extracted with ether (6 x 200 ml.). Washing of the ethereal extract with water (4 x 100 ml.) followed by evaporation of the solvent gave a mixture of arachidic and sebacic acids. The solid acids were equilibrated between aqueous methanol (200 ml. 80% v/v) and n-hexane (200 ml.) at 60°. Removal of the hydrocarbon layer followed by its evaporation together with further hexane extracts (3 x 100 ml.) of the aqueous layer, gave crude arachidic acid. Recrystallisation (5 times)

from methanol gave arachidic acid (5.37 g., 40% yield) of m.p. 75.2 - 75.8° (Found: C, 77.05; H, 12.92%; eq. wt., 309. Arachidic acid requires C, 77.0; H, 12.82%; eq. wt., 312 and m.p. 76.3°).

Behenic acid: Ethyl hydrogen adipate (12.25 g., 2 mols) and stearic acid (10.0 g., 1 mol) were dissolved in absolute methanol (100 ml.). Sodium metal (0.05 g.) was added to the solution which was then electrolysed 30 hours at 40 - 50° with a current of 0.5 - 0.8 amp. A heavy crystalline precipitate of n-tetratriacontane formed during the reaction.

Treatment of the electrolysis solution in the same manner as for the arachidic acid synthesis furnished crude material recrystallised from methanol (4 times) to yield behenic acid (4.76 g., 40% yield) of m.p. 80.2 - 80.7° (Found: C, 77.7; H, 12.93%; eq. wt., 342. Behenic acid requires C, 77.6; H, 12.92%; eq. wt., 341 and m.p. 80.7°).

X-Ray Long Spacings of the Fatty Acids.

Measurements were made on a Philips Geiger Counter Spectrometer, and at least seven well defined "reflections" were observed for each acid. Filtered Cu-K_α radiation (λ = 1.541 Å) was used, and the specimens examined in the α form by allowing them to solidify slowly after melting between microscope slides. The long spacing values determined were as follows (literature values (3) in parenthesis): palmitic acid, 35.5 (35.6), stearic acid 39.4 (39.8), arachidic acid 43.6 (44.2), behenic acid 47.9 (48.3), lignoceric acid 51.8 (52.6).

Preparation of Columns.

The mull used for packing columns was prepared in batches. Non-wetting kieselguhr (93 g.) was stirred

with a solution of paraffin (75 ml.) in pure anhydrous ether (500 ml.). The ether was slowly evaporated with constant agitation of the slurry, until a coarse homogeneous powder was obtained which was then dried for 2 hours at 60° in a vacuum oven.

Owing to difficulty in preparing air-free columns, special precautions were taken during packing. The mull (19.5 g.) was placed in a top-drive macerator together with 83% aqueous acetone (200 ml.) previously equilibrated with paraffin. After thorough agitation, the slurry was transferred to a 500 ml. separating funnel constructed with a wide bore stopcock and a ground glass neck into which a tap was fitted after introduction of the material. To expel all air, the slurry was thoroughly boiled in the separating funnel by holding it in a horizontal position on a water bath.

Next, the column was completely filled with boiled 83% aqueous acetone to give a positive meniscus at the top. The stem of the separating funnel was then closed with the tip of the forefinger and filled with aqueous acetone by inclining the vessel, which was attached to the column by judiciously sliding the stem over the liquid meniscus and withdrawing the finger at the same time. With the column and separating funnel stem completely full of liquid, the mull was allowed to fall into the column by setting it to flow. After some settling had taken place, the contents were pressurised with air at an excess pressure of 50 mm. During subsequent settling, the column was vigorously tapped and shaken to ensure homogeneity of packing. A neatly fitting disc of filter paper was then placed on top of the packed mull. Satisfactory air-free columns were obtained by this method.

All columns were jacketed and maintained at a constant temperature of 35°.

Loading of Columns.

The mixture to be chromatographed (containing 6 - 8 mg. of each component, accurately weighed) was dissolved in paraffin with gentle warming (0.2 ml. paraffin to each 7 mg. of acids). When cool, the solution was taken up in anhydrous ether (15 ml.). Non-wetting kieselguhr was then added (0.26 g. for each 0.2 ml. paraffin), and the ether was evaporated with continuous stirring. The powdery mull was then dried overnight under vacuum at 60°.

For loading, the mull was thoroughly slurried with 50% aqueous acetone (10 - 15 ml.) previously equilibrated with paraffin. The mixture was boiled to expel air and poured on the column. After some settling, the solid mull was gently bedded by the slow downward motion of a plunger fitted with a perforated stainless steel disc. The supernatant 50% aqueous acetone solution was run into the column, after a filter paper disc had been placed on top of the acid containing band. To prevent disturbance of the band and loss of acids during changes of developing solvents, a small protecting band of acid-free mull was placed on top of the column. For this purpose, a small quantity (ca. 1 g.) of mull was boiled with 50% aqueous acetone and packed with the plunger, as above, to give a height of approximately 2 cm.

Developing Solvents.

Ordinary commercial acetone of low acidity was used to prepare developing solvents. Before use, all solvents were equilibrated with paraffin, and clarified of suspended oil droplets by percolation under pressure through a 3 cm. bed of kieselguhr-paraffin mull. The clear solutions were placed in separating funnels fitted with stopcocks and connected to a manifold of capillary tubing.

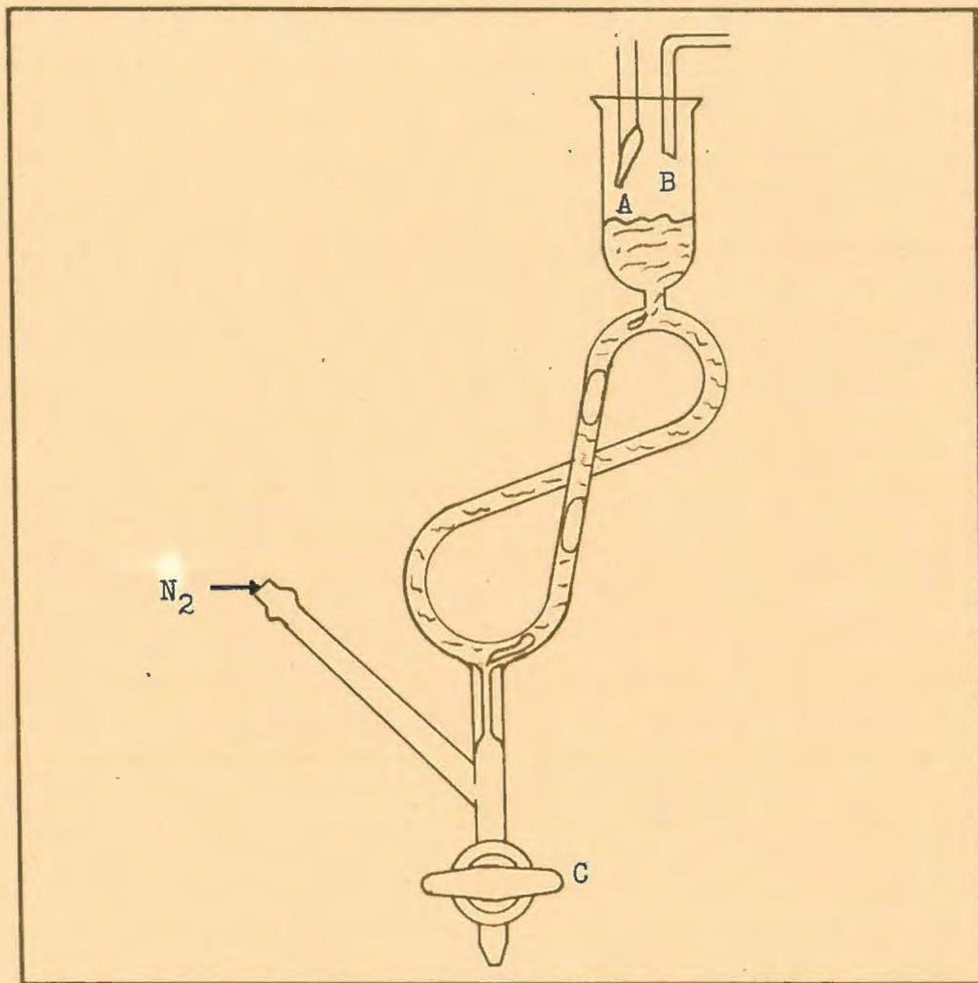


Fig. A. Titration Vessel with Nitrogen Circulation.

A = Siphon tip.
B = Micro-burette tip.
C = Stopcock: a) When closed, aliquot circulated by N_2 stream.
b) When open, liquid allowed to drain.

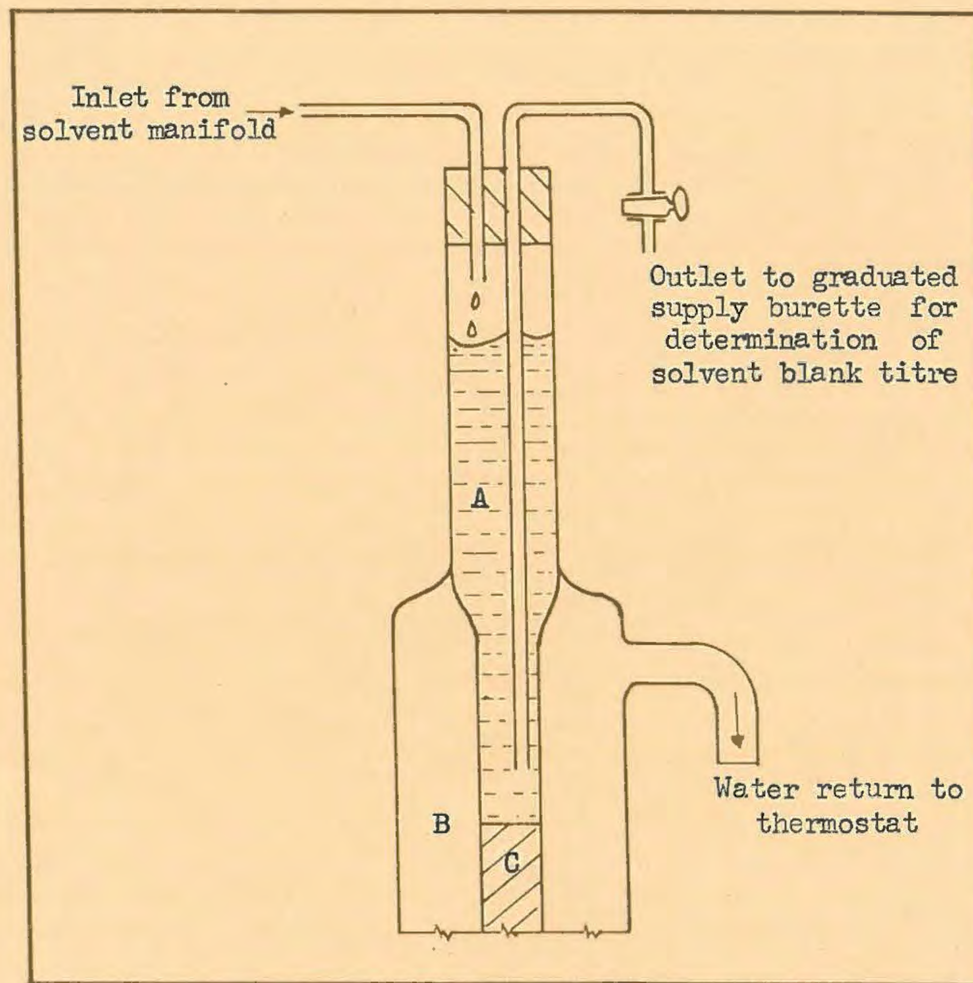


Fig. B. Diagram of Column Head.

A = Developing solvent.
B = Column jacket.
C = Top of protecting band (kieselguhr-paraffin mull).

To facilitate the process of changing the developing solvents and also to provide a means of obtaining aliquots for blank titre determination, the column top was fitted as in Fig.B.

While in use the supply funnels were fitted with soda lime guard tubes. The column effluent was allowed to run into a siphon, and was protected from contact with atmospheric CO_2 by means of a rubber collar and guard tube placed between the column tip and the siphon cup.

Siphon.

The siphon was constructed to deliver 1.0 ml. aliquots with efficient drainage. To prevent the precipitation of higher acids in the siphon during cooler weather, it was wound with a 15 ohm length of nichrome wire and heated with a current of 0.35 amp.

Titration of Eluates.

A titration vessel with nitrogen circulation was constructed according to the design of Howard and Martin (4). The chloroform tube and light system described by these authors was replaced by an ordinary 25 watt globe built into a lamp housing which surrounded the titration vessel, and was fitted with suitable filters to give a background of white light. The vessel is illustrated in Fig. A.

It was found necessary to saturate the nitrogen stream with acetone vapour to counteract evaporation effects in the titration vessel. The nitrogen was accordingly passed over copper turnings at 700°, then through 40% aqueous KOH solution and finally through a sintered glass wash bottle containing neutral acetone maintained at 30° by means of a small 1.5 watt immersion heater.

Standardised methanolic 0.01 N KOH was delivered for titration from a 5 ml. microburette graduated in

0.01 ml., and was protected at all points from contact with atmospheric CO_2. Bromothymol blue as a 0.2% neutral solution in 70% aqueous acetone was used as indicator.

Procedure.

For chromatography of a mixture of C_{16} - C_{24} acids, development was begun with 70% aqueous acetone. This was continued to just over the C_{16} peak, when a change was made to 75% aqueous acetone. The solvent was changed to 80% aqueous acetone on the downward slope of the C_{18} peak, to 83% aqueous acetone on the downward slope of the C_{20} peak and finally to 90% aqueous acetone on the downward slope of the C_{22} peak. The hold up was determined by running a narrow band of $KMnO_4$ solution down the column after the experiment, and observing the number of aliquots required for its elution. After each solvent change the blank titre of the previous solvent was determined by titration of 10 ml. samples under N_2 without boiling. Individual acids were chromatographed with solvent concentrations corresponding to those used for their elution from a mixture. A flow rate of 35 ml. per hour was used in all chromatograms.

Calculation of Results.

Curves were plotted showing the number of aliquots of column effluent versus the quantity of 0.01 N alkali required to neutralise each aliquot. The area underneath each peak was measured with a planimeter. Multiplication of the areas by the equivalent weights of the respective acids, followed by proportionation, gave the weight ratios of the components (cf. Table VI, Chapter 4).

REFERENCES.

(1) W.S. Greaves, R.P. Linstead, B.R. Shepheard, S.L.S. Thomas & B.C.L. Weedon. J. Chem. Soc. 3326 (1950).

(2) S. Swann, R. Oehler & R.J. Buswell. Org. Synth. Coll. Vol. 2, 276 (1946).

(3) F. Francis & S.H. Piper. J. Amer. Chem. Soc. 61, 577 (1939).

(4) G.A. Howard & A.J.P. Martin. Biochem. J. 46, 532 (1950).

EXPERIMENTAL DETAILS.

SECTION C.

The following experimental details are relevant to Chapter 5.

Pure oleic and linoleic acids.

These acids were obtained by saponification of their pure esters supplied by the Hormel Institute (University of Minnesota, U.S.A.). Thus for example:

Methyl linoleate (1.13 g.) was added to alcoholic potash solution (KOH, 0.35 g., water 0.3 ml. abs. EtOH, 15 ml.) and left to stand 30 hours under N_2 at 30°. Water (10 ml.) was added to the solution and unchanged ester extracted with n-pentane (3 x 50 ml.). The soap solution was acidified with 10% aqueous sulphuric acid (20 ml.), and extracted with n-pentane (4 x 25 ml.). Washing of the pentane extracts with water (4 x 20 ml.), followed by evaporation of the solvent gave pure colourless linoleic acid.

Reversed Phase Partition Chromatography of Acid Mixtures.

The aqueous acetone-paraffin system was used as described in Chapter 4.

(1) Oleic and linoleic acids.

Oleic acid (12.35 mg. 45.5 mol %) and linoleic acid (14.9 mg. 54.5 mol %) were dissolved in 60% aqueous acetone (7.0 ml.) and poured onto a column 85 cm. high x 0.8 cm. in diameter. The chromatogram was developed with 60% aqueous acetone (105 ml.) and then with 67.5% aqueous acetone (95 ml.) at a temperature of 35° and a flow rate of 35 ml. per hour. Two peaks were shown (Fig. IV). Measurement of the area underneath each peak followed by proportionation, showed oleic acid, 48.0 mol % and linoleic acid, 52.0 mol %. Experimental accuracy $\pm 5\%$.

(2) Oleic and palmitic acids.

Oleic acid (11.20 mg.) and palmitic acid (9.6 mg.) were dissolved in 67.5% aqueous acetone (5.0 ml.) and poured onto a column 85 cm. high x 0.8 cm. in diameter. The chromatogram was developed with 67.5% aqueous acetone (150 ml.) at a temperature of 35° and a flow rate of 35 ml. per hour. Only one peak was observed, the slope being greater on the right hand side than on the left.

(3) Fraction C (Table III) at different temperatures.

Fraction C (20 mg.) was dissolved in 60% aqueous acetone (7 ml.), and chromatographed on column 32 cm. high x 1.4 cm. diameter, using 60% aqueous acetone (120 ml.) as the developing solvent. Flow rate, 35 ml. per hour.

At 20° three incompletely resolved peaks were observed. No sign of resolution was apparent at 30°. Resolution was ill defined at 19°, poor at 16° and 14°, good at 10° and not improved at temperatures between 0° and 10°.

(4) Fractions B and C (Table III) at 10°.

These fractions were chromatographed at 10° using the conditions described in the preceding experiment (3). The curve of Fraction C is shown in Fig. V, Chapter 5.

EXPERIMENTAL DETAILS.

SECTION D.

The following experimental details are relevant to Chapter 6.

Unsaturated materials were sealed under high vacuum and stored at 0°. Wherever possible, operations with these materials were carried out under nitrogen atmosphere. Evaporations were carried out under water pump vacuum at a temperature not greater than 35°.

Total Pilchard Fatty Acids.

Material of average equivalent weight = 282 and average number of double bonds per molecule = 2.40 (H_2 uptake $Pd/BaSO_4$), was prepared as described in Chapter 3.

Lithium Soap-Acetone Segregation Procedure.

The process was carried out as described in Section A, and was repeated a second time in 95% aqueous acetone. A final treatment in 97% aqueous acetone yielded a concentrate of unsaturated acids (29.5% of total) having an average equivalent weight = 311 and an average number of double bonds per molecule = 4.62 (H_2 uptake $Pd/BaSO_4$).

Urea Complex Fractionation of the Lithium Soap-Acetone Concentrate.

(1) The lithium soap-acetone concentrate of unsaturated acids (93 g.) was dissolved in absolute ethanol (1 l.) containing AR urea (50 g.). After standing 14 hours at 4° the precipitated complexes were filtered off, washed with cold ethanol and sucked dry. The complexes (21 g.) were decomposed with water (100 ml.) and the solution extracted with n-pentane (3 x 50 ml.). After washing of the pentane extracts with water (3 x 20 ml.), and

evaporation of the solvent, Fraction 1 (4.80 g.) was obtained and sealed under high vacuum.

(2) The filtrate from (1) was evaporated to a volume of 850 ml. and left to stand 14 hours at 4°. Complexes (10 g.) were obtained and decomposed as above to yield Fraction 2 (2.48 g.).

(3) The filtrate from (2) was evaporated to 750 ml. and left to stand 12 hours at 4°. Complexes (7.0 g.) yielded Fraction 3 (1.85 g.) after decomposition.

(4) The filtrate from (3) was concentrated to 550 ml. Urea (10 g.) was added and the solution stood 14 hours at 4°. Complexes (17.0 g.) yielded Fraction 4 (4.40 g.) after decomposition.

(5) The filtrate from (4) was concentrated to 470 ml. and stood 14 hours at 4°. Complexes (9.60 g.) yielded Fraction 5 (2.58 g.) after decomposition.

(6) The filtrate from (5) was concentrated to 400 ml. and urea (20 g.) added. After standing 2 hours at 20° the precipitated urea and complexes (14 g.) yielded Fraction 6 (1.5 g.) after decomposition.

(7) The filtrate (410 ml.) from (6) was stood 14 hours at 4°. Complexes (20.0 g.) yielded Fraction 7 (3.95 g.).

(8) The filtrate from (7) was concentrated to 250 ml. and stood 14 hours at 4°. Complexes (9.0 g.) yielded Fraction 8 (1.90 g.) after decomposition.

(9) Urea (9.0 g.) was added to the filtrate (260 ml.) from (8). After standing 14 hours at 4°, complexes (16.8 g.) yielded Fraction 9 (4.05 g.) after decomposition.

(10) The filtrate from (9) was concentrated to 200 ml. and urea (5.0 g.) added. After standing 14 hours at 4°, complexes (9.0 g.) yielded Fraction 10 (2.24 g.) after decomposition.

(11) The filtrate from (10) was evaporated to dryness. The mixture of solid urea, complexes and acids was shaken

with n-pentane (500 ml.). The solid urea and complexes (4.60 g.) were filtered off and decomposed to give Fraction 11 (0.82 g.).

(12) The pentane filtrate from (11) was washed with water (4 x 100 ml.) and evaporated to give the Non Complex Forming Residue (55 g.) representing ca. 20% of total pilchard acids.

Fractions 1 - 11 were all pale yellow oils, and the non complex forming residue somewhat darker in colour, probably owing to a certain amount of oxidation during processing.

Average Unsaturation of Materials obtained by the Lithium Soap-Acetone and Urea Complex Procedures.

Final concentrates and segregated fractions were all hydrogenated in glacial acetic acid (15 ml.) over palladium-barium sulphate (2.5%). The volume of hydrogen absorbed was measured together with the barometric pressure and the temperature. The equivalent weights were determined by titration of ca. 10 mg. samples with standardised alkali, and the number of double bonds per molecule calculated from the data obtained. The results were as follows:

Hydrogenation of Acid Mixtures obtained by Lithium Soap-Acetone Segregation of Total Pilchard Acids.

Concentrate (cf. Table VII)	Wt. taken (mg.)	Barometric pressure (mm. corr.)	Temp. gas °C.	Vol. H_2 absorbed (c.c.)	Eq. wt.	No. of double bonds per molecule
Total pilchard acids	96.4	652.8	24.0	23.3	282	2.40
1st stage	102.1	654.0	20.0	41.0	295	4.23
2nd stage	99.4	654.0	20.0	41.3	304	4.52
3rd stage	106.7	649.2	22.0	44.8	311	4.62

Micro-hydrogenation of Acid Mixtures obtained by Urea Complex Fractionation of the Final Lithium Soap-Acetone Concentrate.

Fraction (cf. Table VIII)	Wt. taken (mg.)	Barometric pressure (mm. corr.)	Temp. gas ^oC.	Vol. H_2 absorbed (c.c.)	Eq. wt.	No. of double bonds per molecule
1	14.170	649.2	28.0	3.900	315	3.00
2	14.870	649.6	28.0	4.762	305	3.38
3	10.165	646.4	28.0	3.334	300	3.39
4	9.691	649.5	28.0	3.755	300	4.01
5	9.195	652.2	28.0	3.690	302	4.21
6	9.050	652.9	28.0	3.740	304	4.40
7	9.267	647.8	28.0	4.000	304	4.54
8	9.102	649.7	27.9	3.995	303	4.60
9	9.077	650.4	27.8	4.075	301	4.69
10	8.965	649.2	28.0	4.084	305	4.82
11	9.159	649.8	28.1	4.113	303	4.71
Non complex forming residue	9.557	650.4	28.4	4.347	308	4.85

Chain Length Analyses of Unsaturated Concentrates.

Concentrates of unsaturated acids were completely hydrogenated over Adams' catalyst, and the molar percentages of the chain lengths in the saturated material determined by quantitative reversed phase partition chromatography as described in Chapter 4, and Section B.

Samples (ca. 35 mg.), dissolved in paraffin and adsorbed on non-wetting kieselguhr, were chromatographed on columns ca. 80 cm. high x 0.8 cm. diameter. The chromatograms were developed with increasing concentrations of aqueous acetone (70, 75, 80, 83%) at a flow rate of 35 ml. per hour, and a temperature of 35°. The results are shown in Table X.

EXPERIMENTAL DETAILS.

SECTION E.

The following experimental details are relevant to Chapter 7.

Wherever possible, operations with unsaturated materials were carried out under nitrogen atmosphere. Such materials were stored at 0° after sealing under high vacuum in glass ampoules. Evaporations were carried out at the water pump and at a temperature not greater than 35°.

Molecular Distillation of the Unsaturated Concentrate of Pilchard Acids.

A concentrate of unsaturated acids (eq. wt. = 311 and number of double bonds per molecule = 4.62 (H_2 uptake $Pd/BaSO_4$)) was prepared by use of the lithium soap-acetone procedure (cf. Section A). The concentrate was fractionated in a two stage prototype of the three stage falling film molecular still described by Sutton (1). The distilland was recycled twice with all the distilling stages at the same temperature before removal of each fraction. The temperature of the stages was increased by use of a higher boiling solvent after each successive fraction had been removed. Six Fractions (I - VI) were obtained, as well as three smaller intermediate fractions (Table XI).

Fraction I was removed with the distilling stages heated by boiling water (96°). Fraction II was distilled at the temperature of boiling toluene (105°), while Fractions III and IV were distilled at 120° and 130° respectively, using constant-boiling mixtures prepared from toluene and xylol. Fraction V was distilled at the temperature of boiling xylol (135°), and Fraction VI at the temperature of a constant-boiling mixture of xylol and anisole (145°).

Reversed Phase Partition Chromatography of Fractions I - VI from Molecular Distillation.

Fractions I - VI were chromatographed (23 mg. each) on a column 31.5 cm. in height x 1.4 cm. diameter packed with non-wetting keiselguhr supporting liquid paraffin as the stationary phase (cf. Section C). Aqueous acetone (60% v/v) was used to develop the chromatograms at a flow rate of 35 ml. per hour and a temperature of 10°. Curves are shown in Fig. VI.

Chain Length Analysis of Fractions I - VI.

Fractions I - VI were completely hydrogenated in methanol solution over $Pd/BaSO_4$ catalyst, and the resulting mixture of saturated acids analysed for chain length distribution by reversed phase partition chromatography (cf. Section B). The results are shown in Table XII.

Examination of Fractions I - VI for Evidence of Autoxidation.

(1) Qualitative test.

Each of Fractions I - VI (0.5 mg.) was dissolved in absolute methanol (1 ml.) and added to methanolic ferrous thiocyanate solution (AR ammonium thiocyanate (0.13 g.) and H_2SO_4 (0.12 ml.) in absolute methanol (25 ml.), previously saturated with AR ferrous ammonium sulphate). After standing 20 minutes only the faintest trace of red colour was observed for Fractions I - VI, indicating a negligible amount of oxidation.

(2) Ultra-violet examination.

Solutions of Fractions I - VI (ca. 5.0 mg.) in 96% ethanol (25 ml.) were examined in a Beckmann Model D.U. Quartz Spectrophotometer. After extropolation of the curve to make allowance for background absorption, the $E^{1\%}_{1cm.}$ value was measured at λ234 mμ, and calculated as a

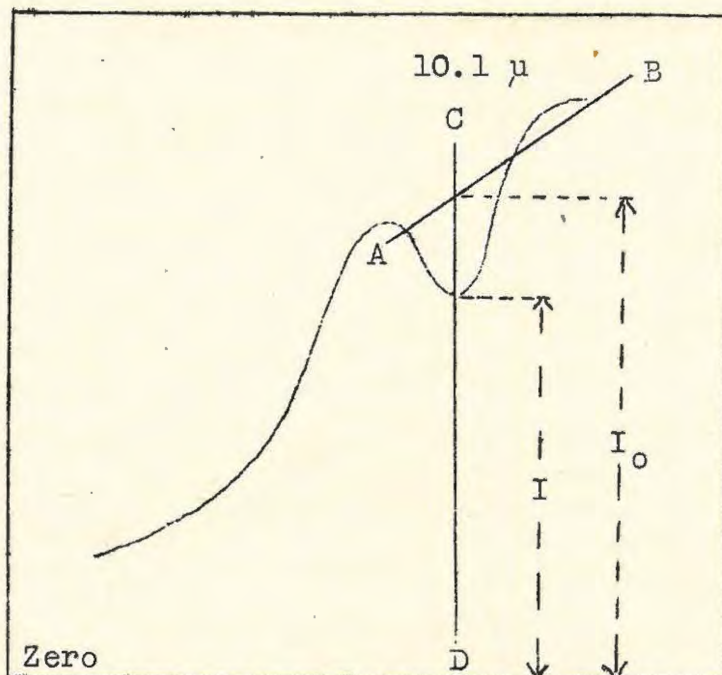
percentage of the $E^{1\%}_{1cm.}$ value for pure methyl linoleate hydroperoxide (= 780) at the same wave length (2). The values obtained showed not more than 0.5% oxidation for each of Fractions I - VI.

(3) Infra-red examination.

The double bonds which migrate into conjugation during autoxidation of a naturally occurring cis bonded fatty acid assume a trans configuration, so that measurement of the percentage of trans bonds in an oxidised acid gives a measure of the degree of autoxidation.

Conjugated trans-trans bonds give rise to a peak near 10.15 μ in the infra-red and this peak was investigated for Fractions I - VI. Measurements were made on a Perkin-Elmer single-beam infra-red spectrometer previously calibrated with mixtures of oleic acid (cis) and β-elaeostearic acid which is known to contain three conjugated trans-trans-trans double bonds.

The "absorptivity" was calculated for each artificial mixture of oleic and β-elaeostearic acids by measurement of the 10.1 μ peak in the manner shown below.



A line AB was drawn through the peak parallel to the general slope of the curve. A vertical line CD was then drawn through the apex of the peak to intersect AB and join the zero co-ordinate at D. The distances I_o and I were then measured, and the "absorptivity" calculated as $\log \frac{I_o}{I}$.

A straight line calibration graph was then obtained by plotting the "absorptivity" versus the concentration of β-elaeostearic acid (i.e. concentration of trans bonds) for each mixture.

The "absorptivity" was measured for the 10.1 μ peaks shown by Fractions I - VI, and the concentration of trans bonds read off from the calibration graph. The results are shown in the following table.

Fraction	Concentration (wt. % in CS_2)	I_o	I	$\log \frac{I_o}{I}$	Concentration of trans bonds from calibration graph	No. of isolated trans bonds per molecule
I	2.34	883	793	0.0464	0.038	0.048
II	2.43	901	840	0.0315	0.027	0.033
III	2.44	945	918	0.0123	0.010	0.012
IV	2.37	Negligible absorption				
V	2.91					
VI	2.70					
Total pilchard acids	4.19	755	743	0.0068	0.007	0.005

In calculating the number of isolated trans bonds per molecule (last column of Table) it was assumed, as a fair approximation, that all Fractions had the same molecular weight as β-elaeostearic acid. Thus for each Fraction, the concentration of trans bonds from the calibration graph was multiplied by 3 (since β-elaeostearic acid contains 3 trans

bonds per molecule) and divided by the concentration used for measurement, i.e. for Fraction I, $\frac{0.038 \times 3}{2.34} = 0.048$ trans bonds per molecule. The number of conjugated pairs of trans bonds is one half the total number of trans bonds, i.e. for Fraction I, $\frac{0.048}{2} = 0.024$ conjugated trans-trans bonds per molecule.

The approximate percentage of oxidation of each Fraction is then obtained by expressing the number of trans-trans conjugated pairs per molecule, as a percentage of the total number of double bonds per molecule (determined by hydrogenation (cf. Table XI)). The results were as follows:

Fraction	No. of conjugated trans-trans double bonds per molecule	Total No. of double bonds per molecule (Table XI)	% Oxidation
I	0.024	3.55	0.67%
II	0.016	3.65	0.46%
III	0.006	4.18	0.14%
IV	Nil	4.86	Nil
V	Nil	5.20	Nil
VI	Nil	5.42	Nil
Total pilchard acids	0.0025	2.40	0.01%

Note. In assessing the accuracy of the results shown for the percentage oxidation, two factors must be considered.

(1) Fractions I, II and III were later shown (Chapter 8) to contain an acid which exhibits a natural peak near 10.1 μ, not attributable to trans bonding. The percentages of oxidation shown for these fractions are thus probably too high.

(2) Cis-trans bonds arising from oxidation also show a peak near 10.1 μ in the infra-red. If only cis-trans conjugated bonds are produced, then the number of pairs of

cis-trans conjugated double bonds is equal to the total number of isolated trans bonds per molecule, and not equal to one half as required for trans-trans conjugated bonds. Thus, if the intensity of the 10.1 μ peak shown by Fractions I - VI was due partly to cis-trans bonds, and not to trans-trans bonds alone, then the percentages of oxidation shown are too low.

Taking these factors into consideration, the percentage oxidation of all Fractions is probably less than 0.3%.

Alkali Isomerisation of Fractions I - VI.

Preparation of 21% KOH-ethylene glycol reagent: Ethylene glycol (100 g. distilled over Zn dust and KOH pellets) was heated under a blanket of nitrogen at 190° for 10 minutes, allowed to cool to 150°, and the calculated amount of potassium hydroxide pellets added to give a solution 21% by weight (ca. 28 g. of 85% KOH pellets). The solution was again heated at 190° for 10 minutes, and then allowed to cool to room temperature. The strength of the reagent was checked by titration of a weighed aliquot with standard alkali, and the concentration adjusted to 21 ± 0.1% by addition of ethylene glycol previously dried at 190° for 10 minutes. The pale straw coloured reagent was stored under nitrogen at 0°.

Reaction tubes: Glass tubes 1 inch in diameter and 5 inches long were fitted with necks ⅝ inches in diameter and 5½ inches in length. Each vessel was fitted with a detachable glass cap having a nitrogen inlet and a nitrogen exit tube. The latter was situated centrally in the cap and designed to dip into the reaction vessel to within 1½ inches of the bottom.

Procedure: Ethylene glycol-KOH reagent (5.0 g.) was weighed into a reaction tube fitted with a cap through which oxygen-free nitrogen was passed to displace the air. While continuously blanketed with nitrogen, the tube was heated at 180 ± 0.1^{o} in a paraffin wax bath for 10 minutes. An accurately weighed (10 - 14 mg.) sample of the Fraction contained in a glass capsule (8 mm. diameter x 6 mm. height), was then added to the reaction tube by inclining it in the bath and allowing the capsule to slide in gently. The reaction tube was then removed from the bath and shaken vigorously for 5 seconds before being replaced. This shaking operation was repeated twice at 30 second intervals. The reaction was accurately timed with a stop watch from the moment of addition of the sample, and after the tube had been heated for 15 minutes, it was removed from the bath and cooled rapidly in cold water. The isomerised mixture was then accurately diluted with distilled water and 96% ethanol (5:1) to a suitable optical density for ultra-violet examination in a Beckmann Model D.U. Quartz Spectrophotometer. Duplicate determinations were made for each of Fractions I - VI and a blank run at the same time. Both isomerisation solutions were compared directly against the blank after dilution. The results are shown in Table XIII.

Urea Complex Fractionation of Fraction I.

Urea (3 g.) was dissolved in absolute ethanol (80 ml.) and the solution cooled to 15°. Fraction I (2.02 g.) was added to the solution and the whole left to stand 14 hours at 4°. The crystalline precipitate was removed by filtration and washed with cold ethanol. The filtrate and washings were concentrated to a volume of 65 ml. and left 12 hours at 4° to deposit a second crop of complexes which were filtered off as before. Urea (0.5 g.)

was added to the filtrate and washings which were allowed to deposit a third crop of complexes on standing. A fourth crop was obtained by evaporation of the filtrate from the previous crop to a volume of 30 ml. and again allowing to stand 14 hours at 4°. The acids which did not form urea complexes were recovered from the final filtrate by evaporating it to dryness, adding water and extracting with n-pentane. The complexes precipitated at each stage in the fractionation were decomposed with water, and the acids recovered by extraction with n-pentane. The results are shown in Table XIV.

Average Unsaturation of Acid Mixtures obtained from Molecular Distillation and Urea Complex Fractionation.

Determination of average degree of unsaturation was carried out by micro-hydrogenation in glacial acetic acid (15 ml.) over palladium-barium sulphate (2.5%). The volume of hydrogen absorbed was measured together with the barometric pressure and the temperature. The equivalent weights were determined by titration of ca. 10 mg. samples with standardised alkali, and the number of double bonds per molecule calculated from the data obtained. The results were as follows:

Micro-Hydrogenation of Fractions I - VI and Intermediate Fractions obtained by Molecular Distillation of the Lithium Soap-Acetone Concentrate.

Fraction (cf. Table XI)	Wt. taken (mg.)	Barometric pressure (mm. corr.)	Temp. gas °C.	Vol. H_2 absorbed (c.c.)	Eq. wt.	No. of double bonds per molecule
I	7.070	656.0	30.0	2.65	272	3.55
Int.	9.020	654.4	28.7	3.42	274	3.62
II	8.841	654.4	28.7	3.36	276	3.65
Int.	9.345	654.4	28.7	3.82	287	4.08
III	8.847	656.5	28.5	3.68	288	4.18
Int.	7.176	658.5	28.5	3.11	306	4.65
IV	7.816	658.5	28.0	3.53	307	4.86
V	7.652	656.0	28.5	3.60	315	5.20
VI	7.423	660.0	29.0	3.52	326	5.42

Micro-Hydrogenation of Fractions Iα - IΔ and the Non Complex Forming Residue obtained by Urea Complex Fractionation of Fraction I.

Fraction (cf. Table XIV)	Wt. taken (mg.)	Barometric pressure (mm. corr.)	Temp. gas °C.	Vol. H_2 absorbed (c.c.)	Eq. wt.	No. of double bonds per molecule
Fraction I	7.070	656.0	30.0	2.650	272	3.55
Iα	12.013	659.5	28.2	2.014	281	1.66
Iβ	13.517	658.3	28.1	3.606	273	2.55
Iγ	8.955	656.6	28.0	2.920	276	3.14
IΔ	9.000	657.0	28.3	3.130	275	3.34
Non complex forming residue	8.913	649.5	28.0	3.536	269	3.68

Reversed Phase Partition Chromatography of Fractions Iα - IΔ and the Residue obtained from Urea Fractionation of Fraction I.

These materials were chromatographed (21 - 23 mg. each) on columns 32 cm. in height x 1.3 cm. in diameter, packed with non-wetting kieselguhr supporting medicinal paraffin as the stationary phase. The chromatograms were developed with aqueous acetone; Fraction Iα, (70%, v/v); Fraction Iβ, (67.5%, v/v); Fractions Iγ and IΔ, (65%, v/v); Residue, (55%, v/v). Flow rate = 35 ml. per hour and temperature 10°. The chromatographic curves are shown in Fig. VIII.

For chain length analysis, Fractions Iα, IΔ and the Residue were completely hydrogenated over Adams' catalyst, and the resulting saturated material chromatographed on columns 85 cm. in height x 0.8 cm. in diameter using a flow rate of 35 ml. per hour and a temperature of 35°. (cf. Section B). The results are shown in Table XIV.

REFERENCES.

(1) D.A. Sutton. Chem. and Ind. 1383 (1953).

(2) H.H. Sephton & D.A. Sutton. ibid. 667 (1953).

EXPERIMENTAL DETAILS.

SECTION F.

The following experimental details are relevant to Chapter 8.

Unsaturated materials were stored at 0° after sealing under high vacuum. Wherever possible, operations with these materials were carried out under nitrogen atmosphere. Evaporations were carried out at the water pump with a temperature not higher than 35°.

Preparation of a Concentrate of Unsaturated Acids.

The concentrate of unsaturated acids used as raw material for the isolation of the C_{16} tetraene acid, was that prepared (as shown in Table VII, Chapter 6) by urea complex fractionation of the lithium soap-acetone concentrate. This material which had an average equivalent weight = 308 and average number of double bonds per molecule = 4.85 (H_2 uptake $Pd/BaSO_4$), was rich in the C_{16} and C_{18} unsaturated acids.

Molecular Distillation of the Unsaturated Acid Concentrate.

The three stage falling film molecular still described by Sutton (1) was used to distil the concentrate of unsaturated acids. The distilland was recycled twice, with all the distilling stages at the same temperature, before removal of each fraction. Successive fractions were obtained using solvents of increasing boiling point to heat the distilling stages. The lowest boiling Fraction A which distilled at 85°, contained the required C_{16} tetraene acid in the highest concentration and was reserved for subsequent chromatography. The results of the distillation are shown in Table XV.

Analysis of the Unsaturated Fractions from Molecular Distillation.

Fractions A, B and C and the residues from the distillation were all quantitatively hydrogenated over palladium-barium sulphate (2.5%) catalyst. After determination of their equivalent weights by titration, the average unsaturation of these materials was calculated in the usual manner. The results were as follows:

Micro-Hydrogenation of Fractions obtained by Molecular Distillation of the Unsaturated Concentrate.

Fraction (cf. Table XV)	Wt. taken (mg.)	Barometric pressure (mm. corr.)	Temp. gas °C.	Vol. H_2 absorbed (c.c.)	Eq. wt.	No. of double bonds per molecule
Unsaturated concentrate	9.557	650.4	28.4	4.347	308	4.85
A	9.105	652.0	28.3	3.692	260	3.67
B	8.953	652.0	28.6	3.640	263	3.72
C	8.993	651.3	28.3	3.784	269	3.93
Res. R. (3rd stage)	8.945	652.0	28.4	3.963	295	4.54
Main bulk Residue	9.057	652.8	28.3	4.307	325	5.38

Reversed Phase Partition Chromatography of Fraction A (Preparative Scale).

A portion of Fraction A was chromatographed on a column 80 cm. high x 3 cm. diameter, which was maintained at 10° and packed with non-wetting kieselguhr supporting medicinal paraffin as stationary phase (cf. Section B). The mixed acids (413 mg.) were dissolved in aqueous acetone (55%, v/v, previously equilibrated with paraffin), and the solution was allowed to run into the top of the column. Last traces of acid were washed in with 20 ml. of 55% aqueous acetone. The chromatogram was then developed at a flow rate

of 150 ml. per hour with 55% aqueous acetone (pre-cooled to 10°). The column eluate was collected under nitrogen in the special receiver illustrated in Fig. IX. The chromatographic curve obtained is shown in Fig X, and the eluate between the arrows marked on this curve was collected separately as being representative of the first peak. The solution (350 ml.) was reserved for isolation of the dissolved acid.

Recovery of Hexadecatetraenoic Acid from the Column Eluate.

The column eluate (350 ml.) representative of the first peak shown on chromatography of Fraction A, was evaporated to approximately half its volume on a water bath at 38° and under a vacuum of 15 mm. Hg. Nitrogen was bubbled through the flask during evaporation. The resulting aqueous suspension of fatty acid was then saturated with AR sodium sulphate and exhaustively extracted with distilled n-pentane under nitrogen. The pentane extract (ca. 1 l.) was washed with water (4 x 100 ml.) and evaporated to yield the fatty acid (144 mg.) as a pale yellow (almost colourless) oil of refractive index $\eta^{D}_{29^{o}}$ = 1.4870.
Carbon and Hydrogen: Found: C, 77.13%; H, 9.87%.
Hexadecatetraenoic acid requires C, 77.32%; H, 9.73%.
Equivalent Weight: The acid (10.724 mg.) required 3.20 ml. of 0.01 N NaOH (f. = 1.310), showing equivalent weight = 250.
Hexadecatetraenoic acid requires equivalent weight = 248.5.
Unsaturation: The acid (9.180 mg.) absorbed 4.198 c.c. H_2 in glacial acetic acid (15 ml.) over Adams' catalyst at a temperature of 28.9^{o} and a corrected pressure of 652.25 mm.
Hence number of double bonds per molecule = 3.96.
Hexadecatetraenic acid requires number of double bonds per molecule = 4.0.

<u>Hydrogenation product</u>: The isolated acid (50.5 mg.) was hydrogenated in absolute ethanol (60 ml.) over Adams' catalyst under 30 lb. per square inch pressure of hydrogen for 3 hours in a Parr apparatus. Removal of the catalyst and evaporation of the solvent gave a waxy solid, crystallised from 96% ethanol to show a m.p. of 62.0 - 62.5°. (Palmitic (C_{16}) acid requires m.p. 62.8°). Mixed melt with an authentic sample of palmitic acid (m.p. 62.3 - 62.6°) showed m.p. 62.1 - 62.6°, confirming that the hydrogenation product of the material isolated from Fraction A was palmitic acid.

<u>Equivalent weight of the hydrogenation product (m.p. 62.0 - 62.5°)</u>: The hydrogenation product (9.990 mg.) required 2.92 ml. of 0.01 N NaOH (f. = 1.323), showing equivalent weight = 258. Palmitic acid requires equivalent weight = 256.4.

<u>Ultra-Violet Light Absorption of the Isolated Hexadecatetraenoic Acid.</u>

A solution of hexadecatetraenoic acid (5.115 mg.) in 96% ethanol (25 ml.) was examined in a Beckmann Model D.U. Quartz Spectrophotometer. No diene, triene, or tetraene peaks were apparent in the region $\lambda 220$ - $\lambda 350$ mμ, showing that no conjugated unsaturation was present. After extrapolation of the curve to allow for background absorption, the $E^{1\%}_{1cm.}$ value for an inflection at $\lambda 232$ mμ was measured = 1.95. Comparison with the $E^{1\%}_{1cm.}$ value of 780 for pure methyl linoleate hydroperoxide at this wave length (2), showed that the maximum amount of oxidation of the hexadecatetraenoic acid was 0.33%.

For measurement of light absorption in the region $\lambda 206$ - $\lambda 220$ mμ, a solution of hexadecatetraenoic acid (0.411 mg.) in 96% ethanol (25 ml.) was used. A discussion

concerning the accuracy of determinations in this region (cf. Fig. XI) has been published (3).

Alkali Isomerisation of the Hexadecatetraenoic Acid.

Hexadecatetraenoic acid (13.10 mg.) was isomerised 15 minutes in 21% KOH-ethylene glycol reagent at 180°. The reaction was carried out under nitrogen according to the procedure detailed in Section E. Dilution of the isomerised solution, followed by ultra-violet examination in a Beckmann Model D.U. Quartz Spectrophotometer gave the curve shown in Fig. XII.

Infra-Red Examination of the Hexadecatetraenoic Acid.

Measurements were made on a Perkin-Elmer single beam infra-red spectrophotometer. For determination of the full absorption curve (Fig. XIV), hexadecatetraenoic acid was examined as a 3.04% (w/w) solution in CS_2, and the reference compound, 10-undecylenic acid, as a 2.47% (w/w) solution in CS_2. For determinations in the region 1325 - 1425 $cm.^{-1}$ (Fig. XIII) carbon tetrachloride was used as solvent, because CS_2 exhibits absorption in this region. Hexadecatetraenoic acid was examined as a 4.97% (w/w) solution in CCl_4, palmitic acid as a 4.84% (w/w) solution in CCl_4 and 10-undecylenic acid as a 5.00% (w/w) solution in CCl_4.

Permanganate Oxidation of the Hexadecatetraenoic Acid.

Hexadecatetraenoic acid (145.4 mg.) was dissolved in water (80 ml.) containing potassium hydroxide (1.32 g.) and the solution cooled to 0°. A solution of potassium permanganate (2.2 g.) in water (60 ml.) was also cooled to 0°. The soap solution in a beaker (400 ml.) was surrounded by ice and stirred mechanically. After addition of chips of ice to the solution, the permanganate was added over a

period of 10 minutes. The solution was stirred ½ hour at 0° and then overnight at room temperature. Excess permanganate was then destroyed by passing in SO_2 gas until the solution became colourless. The solution was then strongly acidified with concentrated HCl and evaporated to a volume of 15 ml. After further acidification with HCl the aqueous solution was exhaustively extracted with ether in a continuous ether extractor. Evaporation of the ether extract gave the dibasic oxidation products as a crystalline mass.

Paper Chromatography of the Dibasic Permanganate Oxidation Products from the Hexadecatetraenoic Acid.

Whatman No.1 papers were spotted with the dibasic permanganate oxidation products, and with pure C_2 - C_5 dibasic acids as reference compounds. The papers were allowed to equilibrate overnight with solvent vapour in a chromatographic tank, before being developed 10 hours with ethanolic-ammonium hydroxide (ethanol, 80 : conc. NH_4OH, 20) in a descending direction. The papers were dried ¾ hour at 80°, and then sprayed with B.D.H. universal indicator to contrast the spots. Adipic acid was shown to be the major oxidation product of the hexadecatetraenoic acid, being evidenced by a spot in exact correspondence with that due to an authentic sample of adipic acid. Some glutaric acid was also evidenced together with a faint trace of oxalic acid. Succinic and malonic acids were clearly not present in the dibasic permanganate oxidation products. The glutaric acid and trace of oxalic acid, may have arisen from further degradation of the adipic (cf. 4). All spots due to both the unknown mixture and the reference compounds were clearly resolved, being separated by at least 2 cm.

Note on the Isolation of the Acid Represented by the Second Peak Shown on Chromatography of Fraction A (Fig. X).

The eluate between 743 and 1148 ml. (Fig. X) was collected separately as being largely representative of the second peak shown. Extraction of the acid from this eluate in the manner described for the isolation of the hexadecatetraenoic acid, furnished a pale yellow (almost colourless) acid (41 mg. $n^{D}_{29.5} = 1.4781$), having an equivalent weight = 258, and number of double bonds per molecule = 3.15. (Acid (9.606 mg.) absorbed 3.398 c.c. H_2 in glacial acetic acid (15 ml.) over $Pd/BaSO_4$ at 28.4^{o} and at a corrected pressure of 650 mm. Hg.). C_{16} triene acid requires equivalent weight = 250.4 and number of double bonds per molecule = 3.0.

The material isolated appeared to consist largely of C_{16} triene acid but was obviously impure. The impurity could not have been C_{18} triene acid since the average unsaturation would then have been 3.0 exactly, but was probably C_{20} tetraene acid. A mixture of C_{16} triene acid (85.5%) and C_{20} tetraene acid (14.5%) would have an average equivalent weight = 258 and average number of double bonds per molecule = 3.15, in exact correspondence with the observed values. A C_{16} triene and a C_{20} tetraene acid might be expected to give an overlapping peak, and yet both be resolvable from the hexadecatetraenoic acid of the first peak (cf. Chapter 5). The efficiency of the preparative column in resolving the mixture of C_{16} tetraene and C_{16} triene acids, was equivalent to that obtainable by 650 transfers of counter-current distribution (cf. also Chapter 5).

REFERENCES.

(1) D.A. Sutton. Chem. and Ind., 1383 (1953).

(2) H.H. Sephton & D.A. Sutton. <u>Chem and Ind</u>. 667 (1953).

(3) P. Bladon, H.B. Henbest & G.W. Wood. <u>J. Chem. Soc.</u> 2741 (1952).

(4) P.H. Begemann, J.G. Keppler & H.A. Boekenoogen. <u>Rec. Trav. Chim.</u> <u>69</u>, 439 (1950).

PART I. HIGHLY UNSATURATED ACIDS FROM THE BODY OIL OF THE SOUTH AFRICAN PILCHARD.

S U M M A R Y.

(1) Requirements for the analysis of a marine oil have been discussed.

(2) The method of reversed phase partition chromatography has been applied to the resolution of normal chain C_{16} - C_{24} saturated fatty acids, in fulfilment of the need for a quantitative semi-micro method for chain length analysis of fatty acid mixtures.

(3) Reversed phase partition chromatography has been investigated as a method for the resolution of unsaturated fatty acid mixtures. The scope and limitations of the method have been discussed.

(4) The lithium soap-acetone and urea complex fractionation techniques have been investigated for the preparation of concentrates of unsaturated acids from South African pilchard oil. The relative efficiencies of these methods have been discussed.

(5) A new approach to the analysis of marine oils without isolation of individual components, has been suggested. The method involves the use, in appropriate sequence, of the techniques of lithium soap-acetone segregation, molecular distillation, urea complex fractionation, reversed phase partition chromatography and alkali isomerisation.

(6) A new and unusual fatty acid has been isolated from South African pilchard oil and characterised by physical and chemical means as <u>hexadeca-6:9:12:15-tetraenoic acid</u>. The name <u>marinolic acid</u> has been proposed for this compound.

PUBLICATIONS CONCERNING THE WORK PRESENTED IN PART I OF THIS THESIS.

(1) "The Resolution of Mixtures of C_{16} - C_{24} Normal Chain Fatty Acids by Reversed Phase Partition Chromatography" by M.H. Silk and H.H. Hahn. Biochemical Journal - in the press (1954).

(2) "Concentrates of Highly Unsaturated Fatty Acids and Alcohols Derived from South African Pilchard Oil" by M.H. Silk, H.H. Sephton and H.H. Hahn. Biochemical Journal - submitted for publication (1954).

(3) "The Fatty Acid Composition of South African Pilchard Oil" by M.H. Silk and H.H. Hahn. Biochemical Journal - submitted for publication (1954).

(4) "The Isolation and Structure of a Hexadecatetraenoic Acid from South African Pilchard Oil" by M.H. Silk and H.H. Hahn. Biochemical Journal - submitted for publication (1954).

ACKNOWLEDGEMENTS.

(1) The award of the Marine Oils Research Fellowship endowed by Messrs Marine Oil Refiners of Africa Ltd., Simonstown, C.P., at the laboratories of the South African Council for Scientific and Industrial Research in Pretoria, is gratefully acknowledged. The author is indebted to the Council for the use of the technical facilities available in the National Chemical Research Laboratory.

(2) Thanks are due to both Messrs Marine Oil Refiners, and the Council for Scientific and Industrial Research for permission to include the research as part of a thesis.

(3) The author wishes to express his thanks to the following persons for their generous help.

Professor W.F. Barker, Head of the Department of Chemistry at Rhodes University, for his kindness in making possible the presentation of this thesis.

Dr. D.A. Sutton, Principal Research Officer of the National Chemical Research Laboratory, for his extremely valuable encouragement and scientific advice in acting as supervisor throughout the undertaking of the work.

Dr. W.S. Rapson, Director of the National Chemical Research Laboratory, for the encouragement offered and the interest he has taken in the work.

Mr. H.H. Hahn, Assistant in the Marine Oils Research Fellowship, for invaluable co-operation and scientific assistance.

THE CHEMISTRY OF NATURALLY OCCURRING LONG CHAIN UNSATURATED COMPOUNDS

PART II - THE STRUCTURE OF CICUTOL

by

M.H. SILK. M.Sc., A.R.I.C.

Part II of a thesis presented for the degree of Doctor of Philosophy in the Faculty of Science of Rhodes University

February 1954

PART II - THE STRUCTURE OF CICUTOL

I N D E X

CHAPTER 1 Introduction 1

CHAPTER 2 Previous Work on the Toxic Principle of Cicuta 8

CHAPTER 3 The Isolation of Cicutol 13

CHAPTER 4 The Structure of Cicutol 17

CHAPTER 5 Further Chemical Investigation of Cicutol and Cicutoxin 24

CHAPTER 6 Naturally Occurring Polyene-ynes Related to Cicutol and Cicutoxin . . . 28

EXPERIMENTAL SECTION

CHAPTER 7 Experimental Work on <u>Cicuta virosa</u> . . 34

1) Ether Extraction of the Plant Material 34

2) Ultra-Violet Examination of the Crude Ethereal Extract of <u>C. virosa</u> 34

3) Fluorescence-quenching Chromatography of the Crude Ethereal Extract. The Isolation of Cicutol and Crude Cicutoxin 35

4) Isolation of a Further Quantity of Cicutol from the Plant Residue after Ether Extraction . 37

5) Analysis of Pure Cicutol

a) Carbon and Hydrogen 38

b) Unsaturation 38

c) Ultra-Violet Light Absorption 38

d) Infra-Red Absorption 39

6) The Hydrogenation Product of Cicutol

a) Isolation of Heptadecan-1-ol 39

b) Oxidation of Heptadecan-1-ol to Heptadecan-1-oic Acid 40

7) Partial Hydrogenation of Cicutol over Lindlar's Catalyst 40

8) Derivatives of Cicutol

a) Cicutol p-nitrobenzoate 41

b) Cicutol acetate 42

9) Examination of the Hydrogenation Products of Crude Cicutol obtained by Chromatography of the Methanolic Plant Extract 42

10) Examination of the Crude Cicutoxin Concentrate obtained by Chromatography of the Plant Ethereal Extract 43

a) Ultra-Violet Light Absorption 43

b) Infra-Red Absorption 43

c) Chromatography of Hydrogenation Products . . 44

11) Synthetic Experiments

A) Preparation of Hendeca-5:7:9-triene-2-yne-1:4-diol 44

B) Preparation of Nona-2:4-diene-8-yn-6-ol . . 45

C) Preparation of the α-Naphthyl Urethane of Nona-2:4-diene-8-yn-6-ol 46

D) Dehydration of Nona-2:4-diene-8-yn-6-ol to Nona-3:5:7-triene-1-yne 46

E) Preparation of Ethyl-3-hydroxy-octa-4:6-dienoate 48

F) Dehydration of Ethyl-3-hydroxy-octa-4:6-dienoate to Ethyl-octa-2:4:6-trienoate 49

SUMMARY

PUBLICATIONS

ACKNOWLEDGEMENTS

REFERENCES are to be found at the end of each Chapter

Part 2

THE STRUCTURE OF CICUTOL.

CHAPTER 1.

INTRODUCTION.

Since the middle of the 16th century the plants belonging to the genus Cicuta have been known and recognised as probably the most violently toxic of all growing in temperate regions. While the symptoms of poisoning and the botanical features of the genus have been accurately described many times, little has, until recently, been known about the chemical nature of the toxin. The failure of earlier workers to isolate pure compounds suitable for structural characterisation was due both to the lack of adequate techniques, and to the extreme lability of the unusual molecules occurring in these plants.

The investigation reported in the following pages was carried out on a variety of the genus known as Cicuta virosa or "water hemlock", which is widely distributed in Europe and has been responsible for numerous fatalities. From this plant two compounds were isolated which proved to be of considerable chemical interest as members of a new class of natural products. One of these was a pure non-toxic compound named cicutol, and the other was the impure toxin named cicutoxin. Both compounds proved to be closely related, but only cicutol was investigated by the author. The toxin was subsequently purified and characterised by other workers (cf. Chapter 5).

The Genus Cicuta.

The following description is taken largely from the excellent bibliography of the genus compiled by Marsh, Clawson and Marsh of the U.S. Department of Agriculture (1).

The Flowers and Stem of Cicuta vagans,
a Typical Member of the genus Cicuta

These authors state that the term Cicuta occurs frequently in Latin literature and has as its Greek equivalent the word Κωνειον . Although certain varieties of Cicuta are today known as "water hemlock", it is evident that the hemlock used by the Greeks and Romans for the punishment of criminals and for suicidal purposes, was not an extract of Cicuta. The symptoms produced by the hemlock are described in detail by Plato in connection with the death of Socrates, and are very different to those resulting from ingestion of Cicuta; instead they suggest poisoning by another genus known as Conium. Inasmuch as species of Cicuta are not found in any abundance in Greece and Italy, it may perhaps be fairly questioned whether the Greeks and Romans had any knowledge of the plants.

The first recorded facts concerning poisoning by Cicuta are contained in a book called "Cicutae Aquaticae Historia et Noxae" published by J.J. Wepfer in 1679 (2). This work records the symptoms of poisoning, the physiology and pharmacology of eight cases and also details of the post mortems. Numerous deaths have been reported since this record of the 17th century, and almost every year further cases occur, particularly among children.

Botanical.

A complete botanical description of the genus Cicuta is available in Gray's New Manual of Botany (3) and also in a reported investigation of the plant by Jacobson (4). The following is a brief summary.

"A perennial umbellifer growing two to five feet tall from a bulbous rootstock. The leaves are lanceolate and pinnately compound with serrate leaflets. The flowers are greenish white to white and dispersed in series of

A Plant Belonging to the genus Cicuta

(Showing the rootstock which contains
a poisonous yellow-brown oil)

clusters, while the fruit is ovoid and glabrous with strong flattish corky ribs. The stem is smooth and hollow with nodes 4 - 8 inches apart, and the rootstock from which the stem or stems grow, varies in size from that of a hazel nut to that of a mans fist. The rootstock has one to four or five tapering roots covered with fine white root hairs, and is hollow inside with transverse chambers. Ducts in the fibro-vascular region of the rootstock exude a poisonous yellow-brown oil."

The plant is distributed in the northern continents, and is generally found growing along the edges of streams, ditches and lakes or in marshy ground. A large number of species have been described, most of which are closely related. The following varieties have been reported as poisonous: C. maculata, bulbifera, vagans, bolanderi, occidentalis, californica, curtisii, douglasii, purpurea, tenuifolia and virosa.

The last named, which was selected for study because of its ready availability, is a strong smelling plant flowering in July. A vesicant and highly toxic yellow oil is present in the roots, which are on occasion mistakenly eaten as parsnips. This fact probably accounts for the numerous fatalities attributed to poisoning by Cicuta (5).

Popular Names.

Among English speaking people the Cicuta is most commonly known as "water hemlock" or "cowbane". Other names are "parsnip" (or "wild parsnip"), "snakeroot", "spotted hemlock", "spotted parsley", "snakeweed", "beaver poison", "musquash root" and "muskrat weed".

In New Mexico it is known as "pecos", while an Indian name is "utcum". German names are "Wasserschierling"

or "giftiger Schierling", "Wüterich" or "giftiger Wüterich", "Parzenkraut", "Tollkraut" and "Tollrübe", while it is known in France as "cigue vireuse".

Uses of Cicuta.

The violently toxic nature of Cicuta extracts has attracted medical attention, and several instances are recorded of the use of the poison in treatment of a variety of diseases. More frequent are reports of its use for criminal and suicidal purposes.

Rafinesque (6) says "A few grains have been given in schirrose and scrofulous tumours and ulcers with equal advantage, but a larger dose produces nausea and vomiting. The doses should be small, often repeated and gradually increased. It has been used as a gargle for sore throat, but safer substances ought to be preferred."

In Siberia the crushed nut has been used for syphilitic symptoms and in Norway for gout, while the seeds are known to have a duiretic action.

In Wood and Bache's Dispensatory of the U.S.A. (7) it is stated that the plant is never used internally and rarely externally as an anodyne poultice for local pains. Dragendorff (8) states that Cicuta extracts have been used for arrow poison.

Feeding Experiments.

Experiments carried out in the United States (1), have shown that the stems, leaves, seeds and flowers are non-toxic to sheep, but that the roots are highly poisonous. It seems probable that the roots are toxic at all times of the year, but that the virulence is diminished during the growing season of the plant. It is generally stated that the plant is most poisonous in the Spring.

General Symptoms of Poisoning.

The symptoms of Cicuta poisoning are so positive that a diagnosis is easily made. Pain is experienced, especially in the region of the stomach, but may be quite general in character. Nausea is always apparent, leading sometimes to violent vomiting, and at other times to spasmodic attempts at vomitation without result. Diarrhoea and polyuria are generally observed, while the breathing becomes laboured, stertorous and at times irregular. The pupils of the eyes dilate and the pulse becomes weak, intermittent and rapid. As the effect of the poison increases, the victim is overcome by violent convulsions of both a tetanic and clonic nature. These are generally accompanied by frothing at the mouth, gnashing of the teeth and trismus, and in extreme cases by opisthotonos. The convulsions may be accompanied or followed by unconsciousness, and in fatal cases grow more violent until ended by death.

Post Mortem Findings.

Hyperemia of the brain and central nervous system have been observed, while many writers have recorded inflammation of the larynx, trachea, lungs, bronchial tubes and stomach. The blood is generally found to have lost its property of coagulation, and in the kidneys the red corpuscles are largely broken down.

From his experiments on the poisoning of frogs, Wikzemski (9) has concluded that the effect of the poison is chiefly on the central nervous system, while the activity of the heart and respiratory organs is influenced in a secondary way. The upper part of the brain is not affected, but the "convulsion centre" at the end of the medulla oblongata is profoundly activated. Terminal paralysis of the spinal cord apparently results from complete exhaustion

following the convulsions. Trojanowsky (10) says that the toxin has antiseptic properties, as evidenced by the delayed process of decay after death.

Toxic Dose.

Feeding of animals with either the whole or portions of the plant has been carried out, but little exact experimental work is recorded. All that is known definitely is that a very small quantity of the root of Cicuta may produce death, but the amount varies with the season and also with the period of time during which it is eaten. In an experiment with the pure toxin, however, it was shown that 0.2 mg. suspended in lecithin-saline caused death when administered intraperitoneally to a mouse weighing approximately 20 grams.

Animals Poisoned by Cicuta.

Wepfer (2) showed experimentally that dogs, wolves, and birds could be poisoned. Gadd (11) says that horses, oxen, cows and goats are susceptible, while Scholler (12) gives specific instances of the poisoning of swine. It is, however, very probable that most, if not all, of the higher animals may be poisoned.

Remedies for Cicuta Poisoning.

An emetic is generally given and followed by a cathartic to facilitate elimination. When this is done promptly the prognosis is favourable, because the poison is dissolved only very slowly in the stomach. Opium is occasionally administered to control the convulsions. In the light of recent experience concerning the extreme lability of the toxin towards oxidising agents, it is probably not unreasonable to suggest that a dose of potassium permanganate might nullify its effects if administered without delay.

REFERENCES.

(1) C. Dwight Marsh, A.B. Clawson & Hadleigh Marsh. "Bulletin of the U.S. Dept. of Agriculture" No. 69, May 28th (1914).

(2) J.J. Wepfer. "Cicutae Aquaticae Historia et Noxae" 336 p., 5 figs. (1679).

(3) A. Gray. "New Manual of Botany" Ed. 7, 614, fig. 825, New York (1908).

(4) C.A. Jacobson. J. Amer. Chem. Soc. 37, 916 (1915).

(5) Editorial article. Lancet II, 485 (1947).

(6) C.S. Rafinesque. "Medical Flora I" 107-110, pl. 22, Philadelphia (1828).

(7) G.B. Wood & F. Bache. "The Dispensatory of the United States of America" 1449, 19th Ed., Philadelphia (1907).

(8) G. Dragendorff. "Die Heilpflanzen" p. 487, Stuttgart (1898).

(9) A. Wikzemski. "Beiträge zur Kenntnis der Giftigen Wirkung des Wasserschierlings (Cicuta virosa). Inaugural dissertation, Dorpat (1875).

(10) C. Trojanowsky. Dorpater Medicinische Zeitschrift Bd. 5, Heft 3, 181 (1874).

(11) P.A. Gadd. Der Königl Schwedischen Akademie der Wissenschaften Abhandlungen Bd. 36, 1774, 236-248 (1781). German translation.

(12) Scholler. Magazin für die Gesammte Thierheilkunde Jahrg 19, Quartalheft 2, 262 (1853).

CHAPTER 2.

PREVIOUS WORK ON THE TOXIC PRINCIPLE OF CICUTA.

It has long been recognised that the toxin of Cicuta is present in the yellow-brown oil exuded from the rhizome, but while several investigations of this oil have been published, only those of Boehm (1), Pohl (2) and Jacobson (3) are of any significance from the chemical point of view. None of these authors was successful in isolating pure compounds from Cicuta, and, moreover, the degradation products of their crude preparations were insufficiently characterised. Nevertheless, much useful information regarding methods for extraction of the plant and the preparation of toxic concentrates is contained in their reports.

The first chemical investigation of Cicuta was undertaken in 1876 by Professor Boehm of Dorpat, Russia, who noted that the pharmacological action of the poison was similar to that of other compounds such as picrotoxin, coriamyrtin and oenanthotoxin. The observation was of no chemical value at the time since the structures of these molecules were unknown, but it has since been shown that cicutoxin and oenanthotoxin are indeed very closely related compounds.

Boehm gave three methods for the isolation of the poison, but expressed doubt as to the purity of his preparations.

(1) In the first method the dried and finely ground tubers were extracted with ether. The residue after removal of the solvent was extracted with 70% aqueous alcohol and then with petrol ether leaving an insoluble material which was called cicutoxin.

(2) In the second method the dried and ground roots were extracted with boiling water. The plant residue was then extracted with 95% aqueous alcohol, and removal of the solvent from this extract gave a material which was leached with petrol ether to leave a concentrate of cicutoxin.

(3) The third method involved extraction of the plant material with 70% aqueous alcohol containing 10% concentrated ammonium hydroxide. The poison was precipitated from the extract with lead acetate, and recovered by subsequent removal of the lead.

The work of Boehm provided useful information concerning the solubility of the toxin in various solvents, but in view of the extreme lability of the molecule towards oxidation, it is hardly surprising that the methods used for its extraction failed to yield any but crude preparations. Drying of the roots before extraction undoubtedly caused considerable degradation, while treatment with relatively high boiling solvents such as water and alcohol might also have caused some modification. Extraction with aqueous alcohol removes sugars from the plant material (3), and these would have been present as contaminants in Boehm's cicutoxin concentrates.

Following on the work of Boehm, the next attempt to isolate the toxin of Cicuta was made by Pohl in 1894 (2). The method used was, in effect, a combination of all three of Boehm's procedures, but additional precautions were taken to remove sugars and other unwanted constituents.

Briefly summarised, the method was as follows. The Cicuta roots were sliced, air dried and extracted with 96% ethanol. The syrup obtained on evaporation of the solvent was dried at 40° in an oven to remove all alcohol and then leached with water to remove sugars. The residue from this treatment was again dissolved in ethanol and the poison precipitated with lead acetate. Removal

of the lead with sodium sulphate left a toxic concentrate which was dissolved in ether and shaken out with strong alkali. This had the effect of removing a large quantity of resinous acids, but left almost all the toxin in the ethereal layer. Evaporation of the ether gave a syrupy material which was leached with petrol ether to remove an inactive phytosterol. The phytosterol tended to crystallise in needles from the syrup, and many extractions with petrol ether were required before microscopic examination of the residue showed it to be homogeneous and without any tendency to deposit crystalline material. The syrups obtained in this way from several batches of plant material, showed identical elementary analyses, and Pohl was thus led to believe that the products were probably pure cicutoxin.

The vigorous treatments involved in this procedure place it open to the same criticisms as apply to the earlier methods of Boehm, but nevertheless, the preparations were almost certainly purer than any previously obtained.

Pohl stated that cicutoxin was soluble in chloroform ether, acetone, glacial acetic acid, acetic anhydride, ethyl acetate, benzene, phenol and pyridine, but was insoluble in petrol ether, water and dilute cold acids and alkalis. The chemical investigations reported were of little value except for the observation that permanganate oxidation afforded oxalic acid together with several unidentified volatile acids. Treatment with alcoholic potash caused modification of the toxin to a resinous alkali soluble material, as did prolonged drying, while distillation with zinc dust in an atmosphere of hydrogen yielded an unidentified oil with the odour of guiacol.

In 1915, some twenty years after the work of Pohl, cicutoxin was again investigated by Jacobson in America (3).

Jacobson's method involved ether extraction of the fresh tubers after maceration to a fine pulp. The ether was evaporated in a stream of dry air and when, after several weeks, the residue no longer lost weight it was analysed as cicutoxin.

Addition of an alcoholic solution of the ethereal extract to stirred petrol ether gave an amorphous precipitate of the toxin, but the analysis of this substance was substantially the same as that observed for the ethereal extract itself, so that Jacobson considered no advantage to be gained by the additional step.

Jacobson recorded that cicutoxin was a yellowish liquid resin which was neutral to indicators. He stated that it was not an aldehyde, ketone, acid, ester, carbohydrate, anhydride or hydrocarbon, but that it exhibited weak alcoholic properties. The unsaturated nature of the molecule was indicated by its explosive reaction with nitric acid, its ready absorption of bromine or iodine, and by the marked tendency to polymerise and decompose under a variety of conditions. No absorption bands were exhibited in the visible spectrum, but a general absorption was observed in the ultra-violet which was not examined in detail.

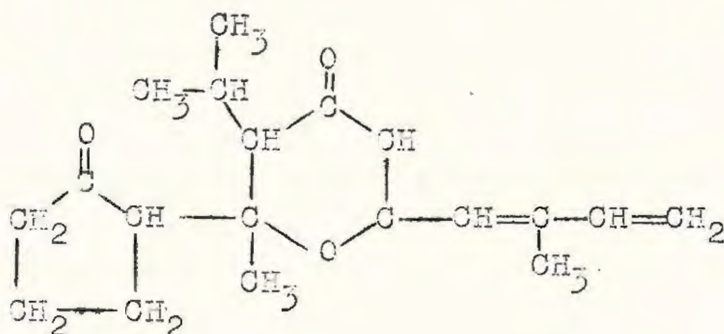
Dry distillation of cicutoxin yielded a variety of unidentified oils, while steam distillation yielded a colourless oil assigned the formula $C_{14}H_{22}O$ on the basis of its elementary analysis. Cicutoxin itself was assigned the empiric formula $C_{19}H_{26}O_3$.

Permanganate oxidation was found to give oxalic acid and carbon dioxide, while nitric acid was stated to yield oxalic acid, hydrocyanic acid, isobutyric acid, and acetyl-2-cyclopentanone. The hydrogen cyanide was identified by combustion of a silver salt, the isobutyric acid by its odour and elementary analysis, while the last

named product was characterised on the grounds that its boiling point and empiric formula corresponded to those of acetyl-2-cyclopentanone.

Numerous other chemical reactions were applied to cicutoxin, and the products examined but not identified. Metallic derivatives were prepared and also an impure acetate, iodide, bromide and hydrochloride.

In reviewing his results, Jacobson stated that the properties of cicutoxin showed a marked similarity to those of γ-pyrone. Assuming the toxin to be a derivative of this substance, he proposed the following structural formula.



The isoprene group was included to account for the lability and ease of polymerisation, while the cyclopentanone and isobutyl groups accounted for the supposedly identified oxidation products. The alcoholic nature of the molecule was stated to arise from enolisation of the carbonyl groups.

The possible reactions of this molecule were ingeneously discussed by Jacobson in relation to the observed reactions of cicutoxin, but since the proposed structure was founded on insufficiently characterised degradation products, it has rightly never been accepted.

REFERENCES.

(1) Boehm. Archiv. für Exp. Path. und. Pharm. 5, 279 (1876).

(2) J. Pohl. ibid., 34, 259 (1894).

(3) C.A. Jacobson. J. Amer. Chem. Soc. 37, 916 (1915).

CHAPTER 3.

THE ISOLATION OF CICUTOL.

The chemical investigation of Cicuta virosa described in the following pages was carried out at the University of Cambridge during 1951 and part of 1952.

Fresh rhizomes of the plant were collected in East Anglia during February, and were macerated in a mincing machine after washing with water. The pulped roots were continuously extracted with ether, and after removal of an aqueous layer and a considerable amount of greenish slime, the ethereal layer was evaporated at a low temperature under oxygen-free nitrogen. A dark viscous orange-brown oil was obtained by this process in a yield of approximately 0.7% on the weight of undried plant material.

Examination of the crude extract in an ultra-violet spectrophotometer showed intense and well defined absorption bands in the region between 230 and 350 mμ. Such marked absorption indicated the presence of one or more substances with a high degree of conjugated unsaturation, and also suggested a convenient means of following the isolation and purification of the material containing the chromophore. It was realised that in the event of the toxin being responsible for the absorption, the necessity for following its isolation by means of laborious and empiric toxicity tests would be obviated.

In early attempts to obtain pure compounds from C. virosa the crude ethereal extract was chromatographed on columns of aluminium oxide, and the eluates examined spectrographically after evaporation of the solvent. In this way concentrates rich in ultra-violet light absorbing material were obtained, but these products were oily syrups which failed to crystallise.

The chromatographic method used in the preliminary

experiments had the disadvantage that with colourless and neutral compounds it was not possible to know with certainty when an adsorbed band had been completely eluted. The somewhat arbitrary eluate fractions collected probably contained material representing more than a single adsorption band, and this would have accounted for the failure to obtain crystalline compounds.

A solution to the problem of obtaining better defined chromatographic fractions, was found in the use of the method of fluorescence-quenching chromatography on aluminium oxide impregnated with morin. In this elegant procedure developed by Brockmann and Volpers (1) the columns of impregnated adsorbent fluoresce when irradiated with light of approximately 360 mμ, and substances having marked absorption in the near ultra-violet region are contrasted as dark zones on a luminous background when adsorbed on such columns. It thus becomes possible to follow visually the development of a chromatogram, even though the chromophoric material may appear colourless in ordinary daylight.

Three distinct light absorbing zones were observed when the crude ethereal extract of C. virosa was chromatographed on a column of Brockmann Grade II neutral morin-alumina. The least strongly absorbed band was readily eluted with methanol-benzene (1:100), while the second band moved slowly down the column and the third remained firmly adsorbed at the top. Continued development with the same solvent resulted in elution of the second band, leaving the third stationary as before. Evaporation of the filtrate corresponding to the least strongly adsorbed band gave a brown oil which partly solidified at room temperature. A brown gum was obtained from the eluate of the second band, and a similar, though darker, material was obtained by elution of the third band from the

top portion of the column with absolute methanol. The material representing the third band was found to be without the detailed light absorption characteristic of the other two zones, and was discarded. In all probability it was a polymeric substance resulting from the decomposition of the compounds present in the lower bands.

The brown semi-solid oil obtained from the least strongly adsorbed band was re-chromatographed on a column of Brockmann Grade II neutral morin-alumina. Adsorption of the material from benzene solution followed by development of the chromatogram with methanol-benzene (1:100), showed a single broad dark band in ultra-violet light. In visible light this band was seen to consist of leading and trailing edges as narrow brown bands enclosing a colourless zone. The eluate corresponding to the colourless zone was collected separately, and on evaporation gave a pale yellow oil which solidified completely at room temperature. Repeated crystallisation* of this material from benzene-light petroleum and from carbon tetrachloride-light petroleum furnished an alcohol, <u>cicutol</u> as large, almost colourless, plates of m.p. 66° and $[\alpha]_D^{15}$ 0 (c. 1.5 in ethanol).

The brown gum obtained from the eluate corresponding to the second light absorbing band was also re-chromatographed on Brockmann Grade II neutral morin-alumina. A single dark zone was observed under ultra-violet light and thus was found to have a narrow brown leading edge in visible light. The eluate containing the narrow coloured band was discarded, and that corresponding to the colourless light absorbing zone was evaporated separately. The product was a yellow gum which failed to solidify on cooling. Attempts to induce crystallisation from solvents proved unavailing and further chromatography yielded only gums.

---

* Recrystallisation was carried out by Dr. (now Professor) B. Lythgoe.

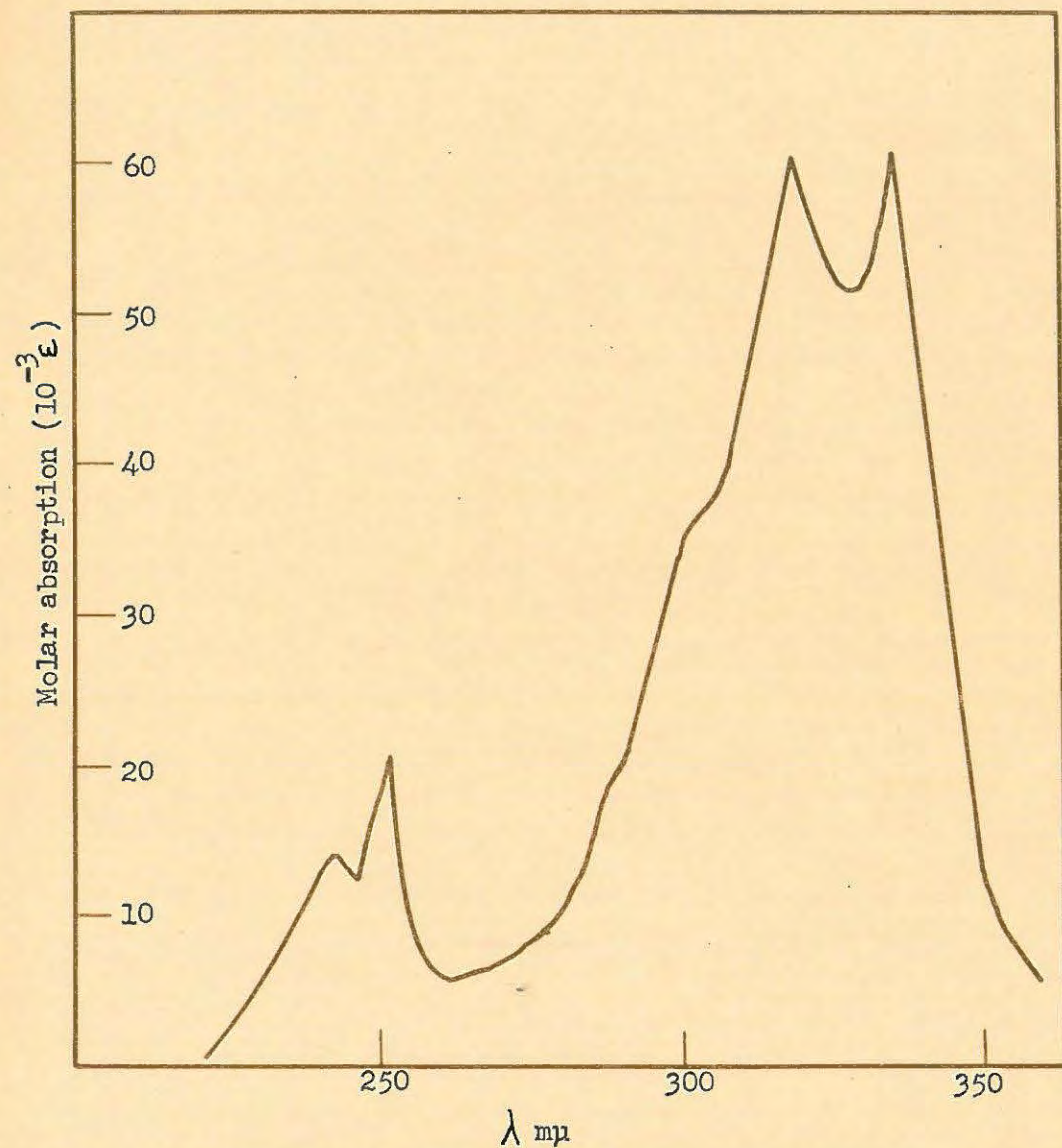


Fig. I. Ultra-Violet Light Absorption of Pure Cicutol in Alcohol.

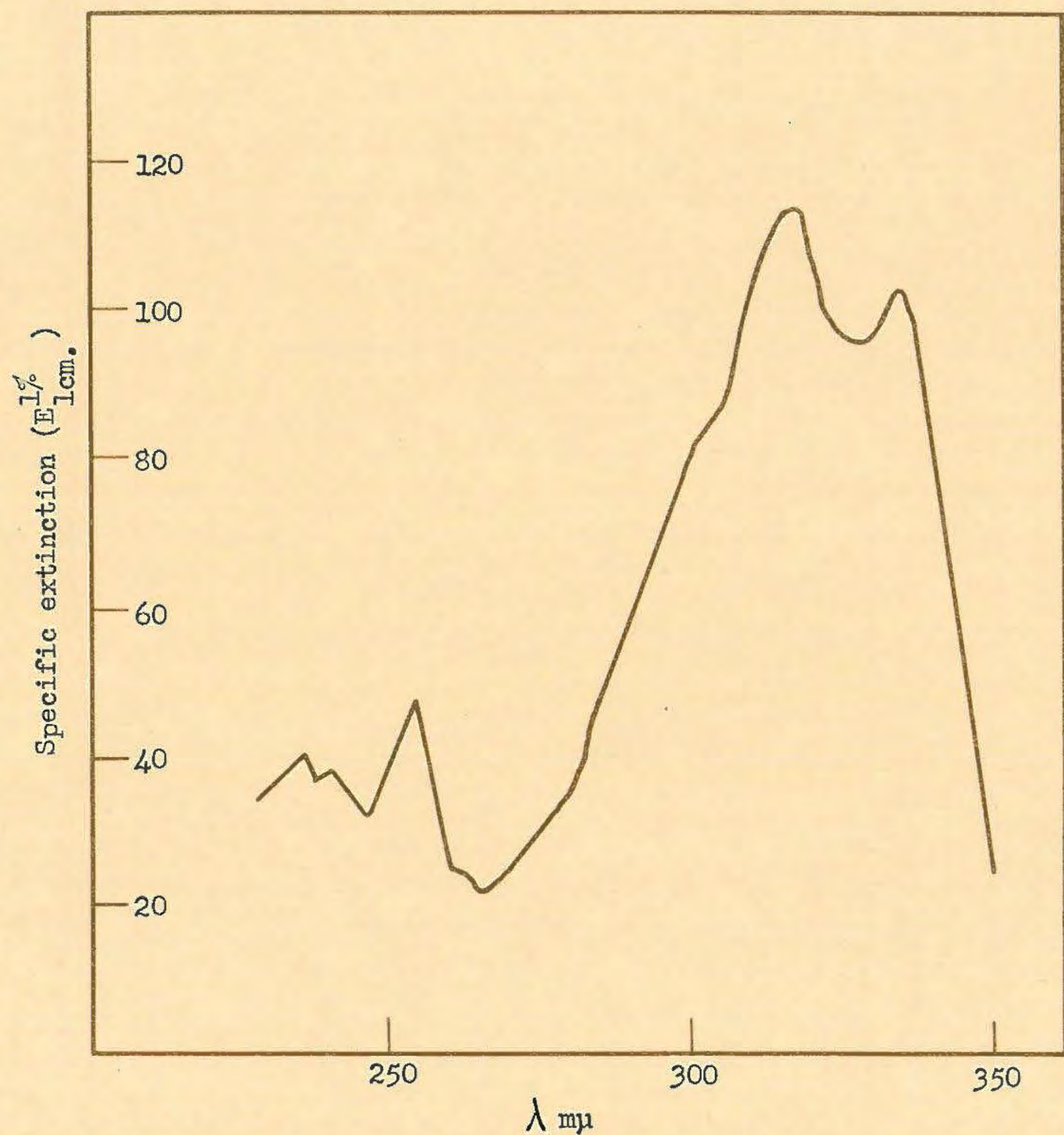


Fig. II. Ultra-Violet Light Absorption of Crude Cicutoxin (yellow gum) in Alcohol.

Cicutol and the more strongly adsorbed brown gum showed almost identical ultra-violet absorption curves (Figs. 1 and 2), but only the latter was found to be toxic towards mice. Although cicutol was evidently not the compound responsible for the poisonous nature of <u>C. virosa</u>, it was readily obtainable in a pure condition and was thus investigated first. Both products decomposed to brown insoluble resins in air and light, but could be stored unchanged for some weeks under oxygen-free nitrogen below $0^{o}C$. Cicutol was best stored in a pure condition.

<u>REFERENCES</u>.

(1) H. Brockmann & F. Volpers. <u>Chem. Ber</u>. <u>80</u>, 77 (1947).

CHAPTER 4.

THE STRUCTURE OF CICUTOL.

Elementary analysis of pure crystalline cicutol (m.p. 66°) showed the empiric formula $C_{17}H_{22}O$.

Hydrogenation over palladium resulted in the absorption of 7 mols of hydrogen, and the product was shown to be heptadecan-1-ol by analysis and oxidation to heptadecan-1-oic acid. A long chain carbon skeleton with an hydroxyl group in the 1 position and a high degree of unsaturation, was thus indicated as the basic structure of cicutol. Additional evidence for the presence of a primary hydroxyl group was obtained from the infra-red spectrum which showed a strong absorption band at 3333 $cm.^{-1}$. No other oxygen functions were indicated.

The Chromophore.

Seven double bond units of unsaturation were shown present by hydrogenation of cicutol, but it seemed unlikely that the chromophore was of a simple polyene type containing seven ethylenic bonds in conjugation. Such compounds absorb at longer wave lengths in the ultra-violet than cicutol (1).

The nature of the chromophore was eventually ascertained from infra-red spectroscopic evidence, which showed that di-substituted acetylenic links and trans-ethylenic links of the type R-CH=CH-R' were present. In addition to the strong primary hydroxyl absorption at 3333 $cm.^{-1}$ the infra-red spectrum showed bands at 2212 $cm.^{-1}$ (medium), 2128 $cm.^{-1}$ (weak), 1634 $cm.^{-1}$ (weak), 1603 $cm.^{-1}$ (medium) and 996 $cm.^{-1}$ (strong). The 2212 $cm.^{-1}$ band was undoubtedly due to R-C≡C-R' stretching while those at 1634 $cm.^{-1}$ and 1603 $cm.^{-1}$ were due to conjugated -C=C-C=C- stretching (2). The band at 996 $cm.^{-1}$ was due to the

out-of-plane C-H vibrations of the trans R-CH=CH-R' groups; the normal value for this mode is about 965 $cm.^{-1}$ but the shift to a higher frequency observed in cicutol might well be due to the effect of considerable conjugation. The weak band at 2128 $cm.^{-1}$ was not easily explained, although its presence suggested that cicutol might contain a conjugated di-acetylene system. Dimethyldiacetylene shows bands at 2264 and 2157 $cm.^{-1}$ due respectively to the symmetrical and unsymmetrical stretching modes of the acetylene links (3), so that the band at 2128 $cm.^{-1}$ could possibly be due to the unsymmetrical stretching mode of a diacetylene system.

Absorption bands characteristic of allene systems, cis disubstituted ethylenic groups (R-CH=CH-R'), and terminal ethylene groups ($R\text{-}CH{=}CH_2$), were all absent from the infra-red spectrum of cicutol. The compound contained no terminal ethynyl groups as evidenced by its failure to give a silver derivative.

Infra-red evidence was thus of great value in excluding certain structural possibilities and establishing beyond doubt the presence of ethynyl and trans ethylenic bonds. Moreover, it seemed likely from the spectrum that cicutol contained two ethynyl groups.

Further information regarding the nature of the chromophore was obtained by partial hydrogenation over Lindlar's catalyst (4) which is selective in reducing acetylenic to ethylenic linkages, and is specific even for acetylenic bonds which form part of a conjugated system. The reduction of cicutol over Lindlar's catalyst was halted after the absorption of 2.2 mols of hydrogen* and the product found to have the ultra-violet absorption spectrum of a conjugated pentaene (Fig. 3).

* Experiment carried out together with Dr. B. Lythgoe and Dr. E.F.L.J. Anet.

Fig. III. Ultra-Violet Absorption of the Product of Partial Reduction of Cicutol over Lindlar's Catalyst.

(The reduction was halted after the absorption of 2.2 mols of hydrogen. The ultra-violet absorption curve is that of a conjugated pentaene system.)

Comparison of the curve shown in Fig. 3 with that of known unbranched polyenes, is complicated by lack of uniformity in the solvents for which measurements are available. The shifts in positions of the maxima in various solvents are nevertheless unlikely to be large and reference to Table I shows that the partial hydrogenation product of cicutol exhibits the expected maxima of a conjugated pentaene. An attempt to isolate the product of semi-hydrogenation failed owing to polymerisation.

Table I.

Light Absorption of Unbranched Conjugated Polyenes

(wave lengths in mμ).

	λmax	$10^{-3}ε$	λmax	$10^{-3}ε$	λmax	$10^{-3}ε$	λmax	$10^{-3}ε$
$Me-[CH=CH]_4-Me$ (a)	272	18	282	36	296	52	320	52
$Me-[CH=CH]_6-Me$ (b)	328	25	340	46	360	69	375	50
$Me-[CH=CH]_5-Me$ (c)	302	-	312	-	330	-	349	-
Pentaene from cicutol (d)	302 (inf.)		315		330		347	

(a) In hexane (1), loc. cit. (b) In chloroform (idem ibid.)

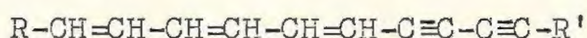
(c) Approximately calculated by interpolation.

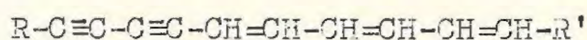
(d) In alcoholic solution used for semi-hydrogenation.

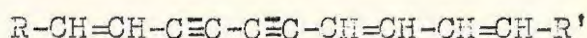
The behaviour of cicutol on partial hydrogenation suggested that the chromophore contained two acetylenic and three ethylenic links in conjugation, since reduction of the acetylenic links to ethylenic in such a system would yield a conjugated pentaene. The possibility that the original unsaturation was not conjugated, but that the double bonds moved into conjugation during hydrogenation is a remote one in view of the extremely mild nature of the catalyst. The intense ultra-violet absorption shown by cicutol is in itself evidence of conjugation in the original molecule.

The Structure of the Chromophore.

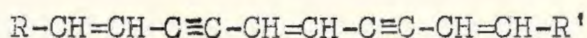
A chromophore having two acetylenic and three ethylenic links in conjugation may have any one of a number of structures. Two main structural types may be defined; one in which the two acetylenic bonds occur as a diacetylenic group having either a terminal or a central position in the chain, i.e.

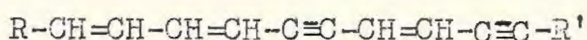

R-CH=CH-CH=CH-CH=CH-C≡C-C≡C-R'

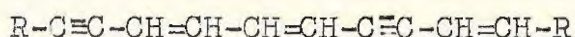

R-C≡C-C≡C-CH=CH-CH=CH-CH=CH-R'


R-CH=CH-C≡C-C≡C-CH=CH-CH=CH-R'

and the other in which the two acetylenic groups are not adjacent and occupy various positions in the chain, i.e.


R-CH=CH-C≡C-CH=CH-C≡C-CH=CH-R'


R-CH=CH-CH=CH-C≡C-CH=CH-C≡C-R'


R-C≡C-CH=CH-CH=CH-C≡C-CH=CH-R

The synthetic approach was favoured in attempting to decide the nature of the chromophore, and in view of the occurrence of the band indicative of a diacetylene system at 2128 $cm.^{-1}$ in the infra-red spectrum, it seemed likely that one of the structures in the first group would be the most reasonable to select. A structure in which the diacetylene system occupied a terminal position in the chromophore was chosen after consideration of the ultra-violet spectrum in the light of measurements made on analogous compounds by Heilbron, Jones and their collaborators (5, 6, 7). These authors compared the spectra of polyene-ynes of the types $R-[CH=CH]_n-C\equiv C-H$ and $R-[CH=CH]_n-C\equiv C-[CH=CH]_n-R$ with those of polyenes having the same number of unsaturated centres.

Their results showed that the acetylenic group causes a diminution in intensity and a shift towards shorter wave lengths of the main absorption band; the reduction in intensity being greater when the acetylenic

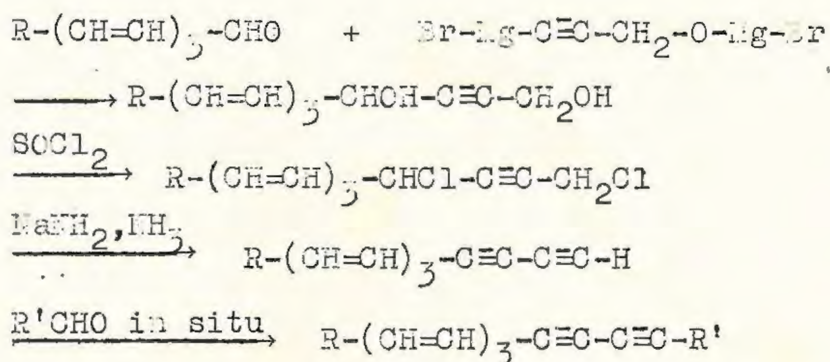
link is centrally disposed than when it is terminal in the chromophore. Since the effect of a diacetylene system appeared to be similar (8), the high intensity long wave length absorption exhibited by cicutol was taken to indicate a terminal disposition of the acetylenic groups.

<u>The Synthetic Approach Towards the Structure of the Chromophore.</u>

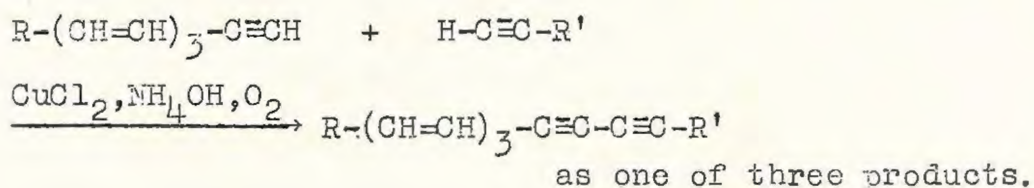
On the basis of the foregoing evidence it seemed likely that the chromophore of cicutol was of a triene-diyne nature with the diyne group in a terminal position. Accordingly, attempts were made to synthesise such a system for comparison of its ultra-violet and infra-red absorption spectra with those of the naturally occurring cicutol. The methods chosen were suggested by the work of Bohlman (8) and of Jones and his co-workers (cf. 9, 10, 11).

Three general procedures were available:

A. Reaction of a trienal with the Grignard derivative of propargyl alcohol, followed by conversion of the resulting mono-acetylenic compound to a diacetylene

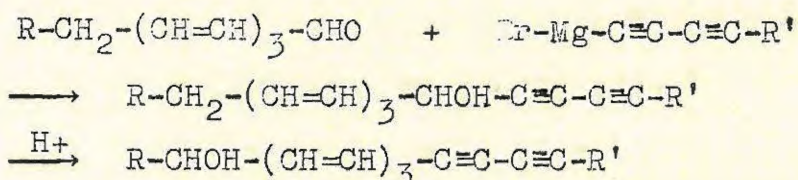


B. Oxidative coupling of two suitably substituted mono-acetylenic units.

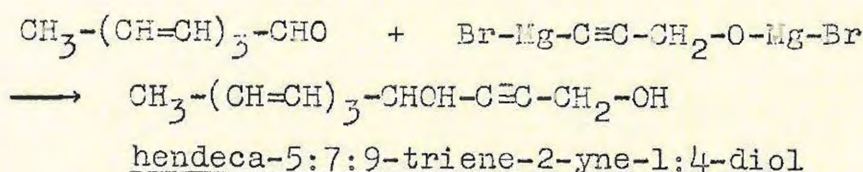


as one of three products.

C. Reaction of a trienal with the Grignard derivative of a diacetylene followed by anionotropic rearrangement of the product.

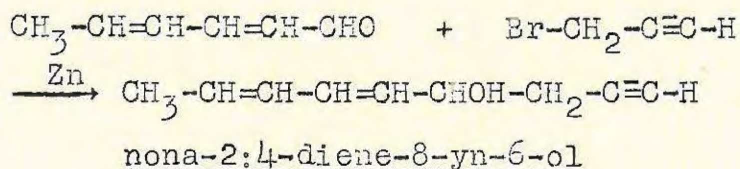


In an attempt to synthesise the chromophore by method A, the di-Grignard complex of propargyl alcohol was reacted with octatrienal to yield the required diol, i.e.

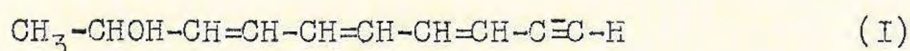


Conversion of this compound to a diacetylene proved impossible owing to decomposition during attempted replacement of the alcoholic groups with halogen.

In the attempted synthesis of the chromophore by oxidative coupling (Method B) difficulty was experienced in preparing a suitable trieneyne as starting product. The Reformatsky reaction between sorbaldehyde and propargyl bromide furnished nona-2:4-diene-8-yn-6-ol, but it proved impossible to dehydrate this to the corresponding trieneyne in satisfactory yield with any of the usual reagents.

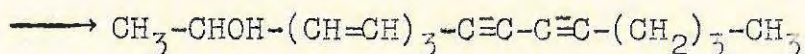


An attempt to achieve oxidative coupling between the known compounds I and II (7, 12) had to be abandoned owing to the author's departure from Cambridge. The trieneyne (I) is prepared by anionotropic rearrangement of the reaction product between octatrienal and sodium acetylide, and dehydration is not involved.



CH_3-CHOH-CH=CH-CH=CH-CH=CH-C≡C-H (I)

+ H-C≡C-$(CH_2)_3$-CH_3 (II)



⟶ CH_3-CHOH-$(CH=CH)_3$-C≡C-C≡C-$(CH_2)_3$-CH_3

The third general synthetic method (C) has subsequently been shown suitable for the preparation of triene-diyne systems.

Other Investigations.

Several minor investigations of cicutol are reported in the Experimental Section (Chapter 7) where mention is also made of a number of other compounds isolated from the hydrogenation products of crude cicutol concentrates.

REFERENCES.

(1) R. Kuhn. Angew. Chemie 50, 703 (1939).

(2) H.M. Randall, R.G. Fowler, N. Fuson & J.R. Dangl. Infra-Red Determination of Organic Structures, D. van Nostrand Inc. N.Y. (1949).

(3) N. Sheppard & D.M. Simpson. Quart. Reviews 6, 1 (1952).

(4) H. Lindlar. Helv. Chim. Acta 35, 446 (1952).

(5) E.R.H. Jones & J.T. McCombie. J. Chem. Soc. 261 (1943).

(6) I.M. Heilbron, E.R.H. Jones & R.A. Raphael, ibid., 268 (1943); idem ibid., 136 (1944).

(7) I.M. Heilbron, E.R.H. Jones & J.T. McCombie, ibid., 134 (1944).

(8) F. Bohlman. Chem. Ber. 84, 545, 785 (1951).

(9) E.R.H. Jones. Tilden Lecture. J. Chem. Soc. 754 (1950).

(10) E.R.H. Jones, M.C. Whiting, J.B. Armitage, C.L. Cook & N. Entwhistle. Nature 168, 900 (1951).

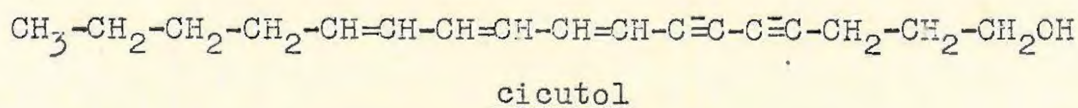
(11) E.R.H. Jones, et al. J. Chem. Soc. 1993-2014 (1952).

(12) T.L. Jacobs. Organic Reactions 5, 48 (1949).

CHAPTER 5.

FURTHER CHEMICAL INVESTIGATION OF CICUTOL AND CICUTOXIN.

Further work was carried out by Drs Lythgoe and Trippett on the structures of cicutol and cicutoxin subsequent to the author's departure from Cambridge. As a result of this cicutol was proved to have the general formula proposed, and was characterised as all-trans heptadeca-8:10:12-triene-4:6-diyne-1-ol.

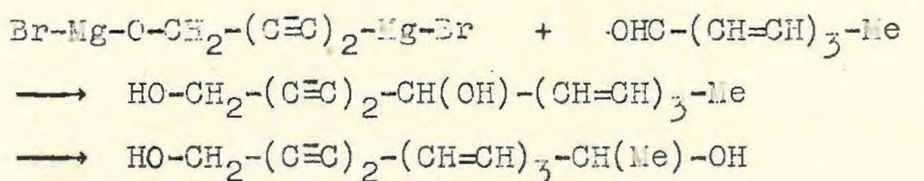


cicutol

Cicutoxin was also isolated in a pure condition from the crude extract (Chapter 3) and was shown to be 14-hydroxy cicutol.

Proof of the Structure of Cicutol.

(1) The triene-diyne nature of the chromophore was proved by its synthesis using method C (Chapter 4). Thus, penta-2:4-diyn-1-ol obtained by interaction of formaldehyde and monosodio-diacetylene (Bohlman (1)) was converted into its dimagnesium bromide derivative and condensed with octatrienal to give a crude diol which, when shaken in ether with dilute acid, rearranged to give crystalline trideca-6:8:10-triene-2:4-diyne-1:12-diol. This compound exhibited infra-red and ultra-violet absorption almost identical with that of cicutol, thus confirming that the same chromophore was present in both.



trideca-6:8:10-triene-2:4-diyne-1:12-diol

(2) The position of the chromophore was established by permanganate oxidation of cicutol which yielded n-valeric and succinic acids, thus fixing the unsaturation between C_4 and C_{13} inclusive.

(3) A crystalline acetate of cicutol was prepared and analysed.

The Structure of Cicutoxin.

(1) The more strongly adsorbed band found on chromatography of the crude ethereal extract of C. virosa (Chapter 3), was, after much difficulty, obtained crystalline. The product was cicutoxin ($C_{17}H_{22}O_2$ of m.p. 54° and $[\alpha]_D$ -14.5 in EtOH).

(2) Hydrogenation of cicutoxin gave heptadecan-1:14-diol characterised by Schmidt degradation of its oxidation product, 13-ketohexadecane-1-carboxylic acid.

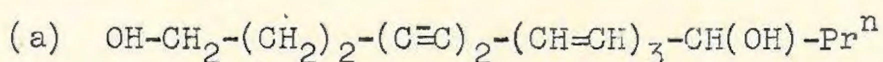
(3) Both cicutol and cicutoxin showed almost identical ultra-violet and infra-red absorption indicating that the same chromophore was present in each. Thus cicutoxin appeared to be 14-hydroxy cicutol.

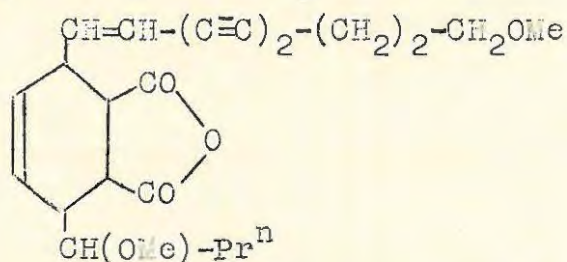
(4) Hydrogenation of cicutoxin over large amounts of platinum in acetic acid containing a trace of hydrochloric acid, caused hydrogenolysis of the C_{14} hydroxy group as well as saturation of the molecule; the product being heptadecan-1-ol. Hydrogenolysis of the OH group at C_{14} makes it probable that this group was adjacent to the chromophore which would thus extend from C_4 to C_{13} inclusive as in cicutol.

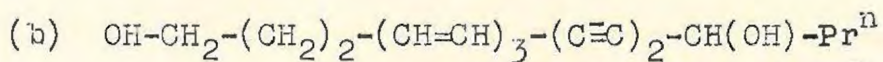
(5) The maleic anhydride adduct of cicutoxin dimethyl-ether exhibited the ultra-violet absorption of a conjugated ene-diyne system, thus proving that the chromophore contained a conjugated diene system situated in a terminal position.

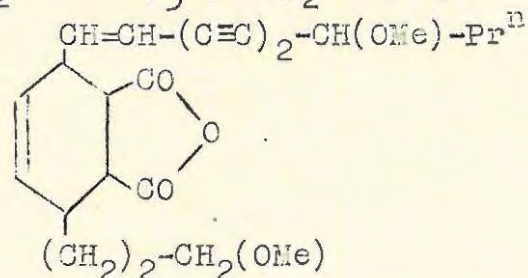
Cicutol and cicutoxin have the same unsaturation extending between C_4 and C_{13} inclusive, but since the chromophore is unsymmetrical, two orientations are possible; one with the diacetylene system nearest the 1-OH group, and the other in which the chromophore is reversed and the diacetylene system is near the opposite end of the chain.

The orientation of the chromophore in cicutoxin was determined by oxidation of the maleic anhydride adduct of its dimethylether. The two possible structures of cicutoxin (a) and (b) would give different maleic anhydride adducts, e.g.









Permanganate oxidation of the adduct with structure (a) might be expected to yield γ-methoxy n-butyric acid as the monobasic product, whereas the adduct with structure (b) would be expected to yield α-methoxy n-valeric acid. In actual fact γ-methoxy n-butyric acid was isolated and characterised as its piperazine salt, thus demonstrating that cicutoxin had structure (a). In all probability, the orientation of the chromophore in cicutoxin is the same as in cicutol, although the same formal proof was not undertaken for the latter compound.

The results of the investigation of cicutol and cicutoxin have been presented in the form of a publication by Anet, Lythgoe, Silk and Trippett (2).

REFERENCES.

(1) F. Bohlman. Chem. Ber. 84, 545, 785 (1951).

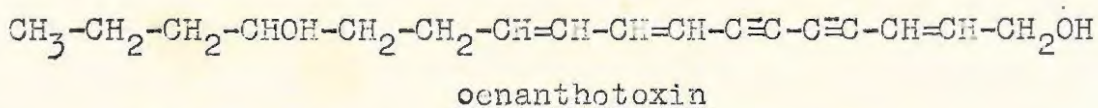
(2) E. Anet, B. Lythgoe, M.H. Silk & S. Trippett. J. Chem. Soc. 309 (1953).

CHAPTER 6.

NATURALLY OCCURRING POLYENE-YNES RELATED TO CICUTOL AND CICUTOXIN.

The compounds isolated from Cicuta virosa belong to a new and unusual, though rapidly growing class of naturally occurring polyene-ynes. It is therefore of interest to make some brief mention of other recently discovered members of the group.

Concurrently with the investigation of C. virosa at Cambridge, research was undertaken on a plant of similar toxicity known as Oenanthe crocata (1). From this plant the toxic principle oenanthotoxin, and two other related compounds oenanthetol and oenanthetone, were isolated and characterised. Oenanthotoxin was shown to be isomeric with cicutoxin and was assigned the structure,



$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CHOH\text{-}CH_2\text{-}CH_2\text{-}CH{=}CH\text{-}CH{=}CH\text{-}C{\equiv}C\text{-}C{\equiv}C\text{-}CH{=}CH\text{-}CH_2OH$$

oenanthotoxin

Oenanthetol was shown with a high degree of probability to be 14-deoxyoenanthotoxin, while oenanthetone was shown to be the 14-ketone from 1-deoxyoenanthotoxin.

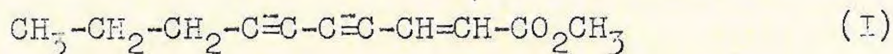
Cicutoxin and oenanthotoxin differ only in the nature of the chromophore, and the same relationship is shown between cicutol and oenanthetol. In the compounds from C. virosa the diacetylenic group is terminal in the chromophore, while in those from O. crocata it is situated between ethylenic groups. The two groups of compounds exhibit different ultra-violet absorption, but show similar peaks in the infra-red. The nature of the chromophore in oenanthotoxin was proved by synthesis of the system (1), while its position and orientation were determined as for cicutoxin (Chapter 5).

An interesting feature regarding the chemistry of these compounds is the lack of toxicity of the monols when compared with the violently toxic nature of the corresponding diols. Moreover, the n-heptadecane skeleton possessed by the compounds from both C. virosa and O. crocata is very seldom encountered in natural products.

N.A. Sörensen and his collaborators in Norway have established the wide occurrence of polyene-ynes in plants of the Compositae family, but while the isolation of such compounds from natural sources is indeed remarkable, their chemistry does not warrant particular discussion. Most have been synthesised by standard acetylenic syntheses involving oxidative coupling, and their spectra exhibit no unusual features.

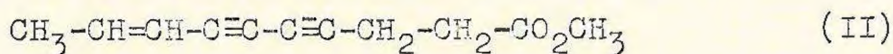
The compound cis-lachnophyllum ester (I) has been obtained from the essential oil of Lachnophyllum gossypinum Bge (2, 3), while an isomeric compound cis-dihydromatricaria ester (II) was isolated from Matricaria inodora L. (4) and characterised primarily by the similarity of its ultra-violet absorption to that of cis-lachnophyllum ester (I).

The cis-cis and cis-trans forms of the corresponding matricaria ester (III) have been obtained from Matricaria inodora L. (4, 5, 6, 7), and the closely related dehydromatricaria ester of probable structure (IV) has been found as a constituent of Artemesia vulgaris L. (8, 9).



$CH_3-CH_2-CH_2-C\equiv C-C\equiv C-CH=CH-CO_2CH_3$ (I)

cis-lachnophyllum ester

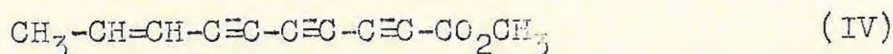


$CH_3-CH=CH-C\equiv C-C\equiv C-CH_2-CH_2-CO_2CH_3$ (II)

cis-dihydromatricaria ester

$CH_3-CH=CH-C\equiv C-C\equiv C-CH=CH-CO_2CH_3$ (III)

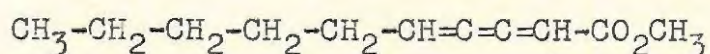
matricaria ester



$CH_3-CH=CH-C\equiv C-C\equiv C-C\equiv C-CO_2CH_3$ (IV)

dehydromatricaria ester

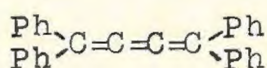
In the course of their studies on the matricarias, Sörensen and Stavholt (10) isolated a remarkable compound from Matricaria inodora L. which, although not a polyene-yne, is worthy of mention for its unusual nature. The compound was named composit-cumulene-I and ascribed the structure,



$CH_3-CH_2-CH_2-CH_2-CH_2-CH=C=C=CH-CO_2CH_3$

composit-cumulene-I

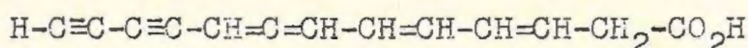
The cumulene structure was identified by the fact that its ultra-violet absorption corresponded with that of tetraphenyl butatriene synthesised by Kuhn et al. (11, 12).



$Ph_2C=C=C=CPh_2$

tetraphenyl butatriene

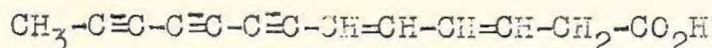
The unstable antibiotic mycomycin isolated by Celmer and Solomons (13) from the elaboration products of Norcardia acidophilus, is without doubt the most remarkable of the polyene-ynes so far characterised. This compound incorporates every known type of carbon-carbon unsaturation having the diyne-allene-diene structure,



$H-C\equiv C-C\equiv C-CH=C=CH-CH=CH-CH=CH-CH_2-CO_2H$

mycomycin

Treatment of mycomycin with normal aqueous potassium hydroxide at 27° causes its rearrangement to isomycomycin with the triyne-diene structure,



$CH_3-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH=CH-CH_2-CO_2H$

isomycomycin

In mycomycin the van't Hoff prediction of the resolvability of asymmetric allenes is exemplified for the first time by a natural product. The optical activity disappears when the allene function is destroyed either by complete hydrogenation to n-tridecanoic acid or by alkaline rearrangement to isomycomycin.

The infra-red spectrum of mycomycin exhibits bands at 3180, 2200, 1930 and 1730 $cm.^{-1}$ attributed to $\equiv C-H$,

disubstituted -C≡C- , CH=C=CH- , and unconjugated $-CO_2H$ functions respectively. A terminal acetylenic group is indicated by reaction with acetylenic hydrogen reagents such as alcoholic silver nitrate.

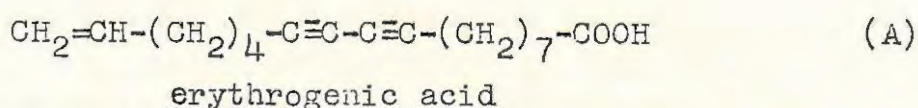
The ultra-violet absorption spectrum of mycomycin is of interest in that it shows only three maxima corresponding to those of a conjugated triene system. The spectrum is quite unlike that of cicutoxin which shows evidence of complex conjugation, but is explicable when account is taken of the existence of an allene group in the molecule. Since the double bonds of the allene group are at right angles, no conjugation is possible throughout the unsaturation as a whole, and it is thus apparent that two discreet non-interacting chromophores are present. Both the enediyne and the triene systems would show the ultra-violet absorption of a conjugated triene.

The nature of the chromophore in isomycomycin was established by the correspondence of its ultra-violet absorption with that of known diene-triyne systems (8, 14). The infra-red spectrum of isomycomycin exhibited bands at 2200 and 1730 $cm.^{-1}$ attributed to disubstituted -C≡C- and and unconjugated $-CO_2H$ respectively, while monosubstituted acetylenic and allenic bands were absent. The absence of ≡C-H function was indicated by its failure to react with alcoholic silver nitrate.

Compounds which have a stability and ultra-violet absorption similar to Sörensen's matricaria esters have been isolated from the culture liquids of various species of <u>Basidiomycetes</u> (15), and among other types, ene-diyne and ene-triyne chromophores appear to be present in these compounds.

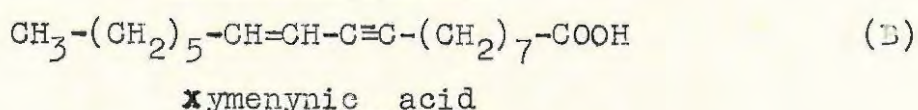
The herb <u>Centaurea cyanus L.</u> has been shown to contain at least two polyene-ynes with ultra-violet

absorption related to that of a synthetic ene-triyne-ene system (16). Two polyene-ynes have recently been isolated from natural seed fats; the compound erythrogenic (isanic) acid (17, 18) from the seed oil of Ongueka Gore Engler has been shown to have structure A recently confirmed by synthesis (19), while a related acid, xymenynic, from the seeds of three species of Ximenia genus, has been shown to have structure B (20).



$$CH_2{=}CH{-}(CH_2)_4{-}C{\equiv}C{-}C{\equiv}C{-}(CH_2)_7{-}COOH \qquad (A)$$

erythrogenic acid



$$CH_3{-}(CH_2)_5{-}CH{=}CH{-}C{\equiv}C{-}(CH_2)_7{-}COOH \qquad (B)$$

xymenynic acid

The class of naturally occurring polyene-ynes is thus a small though growing one, and the discovery of new members will be awaited with interest. The unusual nature of the chromophores in these compounds naturally prompts enquiry into the mechanism of their biogenesis, but as yet no theories have been advanced on this score.

REFERENCES.

(1) E. Anet, B. Lythgoe, M.H. Silk & S. Trippett. J. Chem. Soc. 309 (1953).

(2) W. Wiljams, V.S. Smirnov & V.P. Goljmov. J. Gen. Chem. U.S.S.R. 5, 1195 (1935).

(3) T. Bruun, C.M. Haug & N.A. Sörensen. Acta. Chem. Scand. 4, 850 (1950).

(4) K.S. Baalsrud, D. Holme, M. Nestvold, J. Pliva, J.S. Sörensen & N.A. Sörensen. ibid., 6, 883 (1952).

(5) N.A. Sörensen & J. Stene. Annalen 549, 80 (1941).

(6) R.T. Holman & N.A. Sörensen. Acta. Chem. Scand. 4, 416 (1950).

(7) T. Bruun, P.K. Christensen, C.M. Haug, J. Stene & N.A. Sörensen. ibid., 5, 1244 (1951).

(8) K. Stavholt & N.A. Sorensen. ibid., 4, 1567 (1950).

(9) N.A. Sorensen & K. Stavholt. ibid., 4, 1575 (1950).

(10) N.A. Sorensen & K. Stavholt. ibid., 4, 1080 (1950).

(11) R. Kuhn & K. Wallenfels. Chem. Ber. 71, 783, 1510 (1938).

(12) R. Kuhn & G. Platzer. ibid., 73, 1410 (1940).

(13) W.D. Celmer & I.A. Solomons. J. Amer. Chem. Soc. 74, 1870, 2245, 3838 (1952), ibid., 75, 1372 (1953).

(14) E.R.H. Jones, M.C. Whiting, J.B. Armitage, C.L. Cook & N. Entwhistle. Nature 168, 900 (1951).

(15) M. Anchel. J. Amer. Chem. Soc. 74, 1588 (1952).

(16) B. Hellström & H. Löfgren. Acta. Chem. Scand. 6, 1024 (1952).

(17) A. Steger & J. van Loon. Rec. Trav. Chim. 59, 1156 (1940).

(18) J.P. Riley. J. Chem. Soc. 1346 (1951).

(19) H.K. Black & B.C.L. Weedon. ibid., 1785 (1953).

(20) S.P. Ligthelm, H.M. Schwartz & M.M. von Holdt. ibid., 1088 (1952).

CHAPTER 7.

EXPERIMENTAL WORK ON *CICUTA VIROSA*.

The experimental work on *Cicuta virosa* was carried out at the University of Cambridge with plant material collected in East Anglia during February. All operations with the plant extracts and unsaturated compounds were conducted in diffused light under oxygen-free nitrogen. Materials were stored under nitrogen at 0°C. when not required for immediate use. Evaporations were carried out at temperatures not greater than 36°, and under reduced pressure where necessary. Solvents were carefully purified before use.

(1) Ether Extraction of the Plant Material.

Fresh rhizomes of *Cicuta virosa* (17 kg.) were washed free of mud and minced without drying. Portions (6 kg.) were extracted in a large Soxhlet apparatus with ether (8 l.) for 16 hours. After separation from an aqueous layer and a considerable amount of greenish slime, the combined ethereal extracts were dried over anhydrous sodium sulphate and evaporated. A brown pungent smelling oil (120 g.) was obtained by this process.

(2) Ultra-violet Examination of the Crude Ethereal Extract of *C. virosa*.

Ultra-violet examination of the crude ethereal extract in a Beckmann Model D.U. quartz spectrophotometer showed the presence of material with the following absorption maxima and minima ($E^{1\%}_{1cm.}$ values in parenthesis).
Maxima 335 mμ (72), 315 mμ (82), 252 mμ (29), 237 mμ (24).
Minima 327 mμ (65), 262 mμ (13), 246 mμ (17).

The light absorbing material was sought using chromatographic methods.

(3) Chromatography of the Crude Ethereal Extract of *C. virosa*.

In a number of early attempts to obtain pure compounds from *C. virosa*, the crude ethereal extract was chromatographed on columns of aluminium oxide and the eluates examined spectrographically. Concentrates rich in light absorbing material were obtained, but these were oily syrups which failed to crystallise. The method of fluorescence-quenching chromatography (1) was eventually found to yield better defined chromatographic fractions from which pure compounds could be obtained. The procedure was as follows:-

A portion of the crude ethereal extract (15 g.) was absorbed from benzene solution (50 ml.) onto a column (6 x 22 cm.) packed with Brockmann Grade II morin-alumina (600 g.) prepared according to Brockmann and Volpers (1) and standardised according to Brockmann and Schodder (2). The chromatogram was developed with methanol-benzene (1:100) and when ca. 2.5 l. of eluate had been collected, examination under ultra-violet light (ca. λ 360 mμ) revealed three dark zones. The top one was stationary close to the top of the column, the central moved slowly and the lowest (10 cm. from the bottom) moved rapidly. The filtrate (1 l.) corresponding to the lowest band was collected separately and evaporated yielding a brown oil (Fraction I, 5.0 g.) which partly solidified at room temperature. After a further 500 c.c. of eluate had been collected, the next dark band began to emerge. The filtrate (2.5 l.) corresponding to the second band was also collected separately and evaporated yielding a brown gum (Fraction II, 2.5 g.). The third dark band adsorbed near the top of the column, was eluted with absolute methanol. Evaporation of the methanol yielded a dark and dirty gum without the detailed light absorption of the other two zones. This

gum was discarded as being in all probability a polymerisation product of the materials present in Fractions I and II.

Fraction I was adsorbed from benzene (50 ml.) onto a column (2.9 x 32 cm.) packed with morin-alumina (200 g. Brockmann Grade II), and the chromatogram was developed with methanol benzene (1:100). In ultra-violet light a single dark zone was observed, but in visible light this zone appeared to consist of leading and trailing edges as narrow brown bands enclosing a colourless portion. The eluate corresponding to the colourless zone was collected separately and evaporated yielding a pale yellow oil (3.4 g.) which solidified completely at room temperature.

*Crystallisation from benzene-petrol ether (40 - 60°) gave crude material (2.5 g.) of m.p. 53 - 57°. Repeated recrystallisation from benzene-petrol ether, and from carbon tetrachloride-petrol ether gave cicutol as large, almost colourless, plates of m.p. 66° and $[\alpha]_D^{15}$ 0° (c. 1.5 in EtOH).

Fraction II was adsorbed from benzene (50 ml.) onto a column (2.5 x 20 cm.) packed with morin-alumina (100 g. Brockmann Grade II), and the chromatogram developed with methanol-benzene (1:100). Ultra-violet light revealed a single broad dark zone, the leading edge of which appeared in visible light as a narrow brown band. The filtrate corresponding to the latter was discarded, and that corresponding to the rest of the zone collected separately (ca. 2.5 l.) and evaporated. The yellow gum (1.5 g.) so obtained appeared to be spectroscopically 70% pure but failed to crystallise (see Section 10).

(Note: This gum was, with great difficulty, subsequently induced to crystallise by Drs Lythgoe and

---

* Recrystallisation of the crude cicutol was carried out by Dr. B. Lythgoe (now Professor at Leeds University).

Trippett (3). It was shown to contain cicutoxin, the poisonous principle of C. virosa (m.p. 54°, $[\alpha]^{15}_{D}$ -14.5° (c. 1.7 in EtOH)). (Found: C, 78.8; H, 8.7. $C_{17}H_{22}O_2$ requires C, 79.1; H, 8.6%). Light absorption in EtOH: max. at 242, 252, 318.5, 335 mμ: inflections at 287 and 303 mμ; $10^{-3}\epsilon$ 13, 22, 63.5, 65, (17), (38.5). The infra-red spectrum showed, inter al., bands at 3226 (strong), 2212 (weak), 2128 (very weak), 1634 (weak), 1603 (medium) and 996 $cm.^{-1}$ (very strong).)

(4) Isolation of a Further Quantity of Cicutol from the Plant Residue after Ether Extraction.

In trial experiments it was shown that the plant residue after ether extraction still contained a considerable amount of the light absorbing compounds which were required. The ether extracted material (6 kg. batches) was accordingly re-extracted with absolute methanol (8 l.) until a colourless extract was obtained. The combined (now aqueous) methanolic extracts deposited a dark brown oil and a light brown amorphous precipitate on standing. Filtration of the extracts through Hyflo Super Cel, caused retention of the dark oil and the precipitate, yielding a filtrate which was discarded after being shown spectroscopically to contain a negligible amount of the light absorbing material. Elution of the Hyflo Super Cel with ether, followed by evaporation of the solvent, yielded a dark greenish brown oil (210 g.) rich in light absorbing material.

For chromatography, a portion (4 g.) of the methanolic extract was repeatedly vacuum dried by azeotropic distillation with benzene to remove traces of methanol, and then adsorbed from benzene (20 ml.) onto a column (2.9 x 37 cm.) packed with morin-alumina (280 g. Brockmann Grade II). Development of the chromatogram with benzene (300 ml.) caused the elution of a narrow yellow band of

material which was not investigated further. Continued development with methanol-benzene (1:100) caused a yellow band with a narrow green leading edge to move down from the top of the column. This band appeared as a single dark zone under ultra-violet light, and was eluted after the passage of 650 ml. solvent through the column. The eluate corresponding to the narrow green leading edge was discarded. Evaporation of the main bulk of eluate corresponding to the yellow band yielded crude cicutol (940 mg.) which crystallised partially at 0°.

Continued development with methanol-benzene (1.5:100) resulted in the elution of a second greenish yellow light absorbing band, as observed during the chromatography of the plant ethereal extract (Section 3). This band did not crystallise and was reserved for further investigation.

A green band remained firmly adsorbed at the top of the column after elution of the light absorbing bands. This was not investigated further.

(5) Analysis of Pure Cicutol (m.p. 66°)

(a) Carbon and hydrogen analysis showed the empirical formula to be $C_{17}H_{22}O$. (Found: C, 84.4; H, 9.5%. $C_{17}H_{22}O$ requires C, 84.3; H, 9.2%).

(b) Hydrogenation of cicutol over palladium-charcoal catalyst caused the absorption of 7.2 mols hydrogen. Cicutol (5.364 mg.) in abs. EtOH (12.5 ml.) took up 3.84 c.c. H_2 in 36 minutes at 21° and 762 mm. pressure.)

(c) Ultra-violet light absorption of cicutol in alcohol (Figure I, Chapter 3).

λ_{max} 242, 252, 287 (inf.), 302 (inf.), 318.5, 335 mμ

$10^{-3}\varepsilon$ 14.1, 20.8, (17.5), (35.6), 60.6, 61.2 respectively.

(d) The infra-red absorption spectrum of cicutol was determined on a Perkin-Elmer double-beam infra-red spectrophotometer and showed, inter al., bands at 3333 (strong), 2212 (medium), 2128 (weak), 1634 (weak), 1603 (medium) and 996 $cm.^{-1}$ (very strong).

(6) The Hydrogenation Product of Cicutol.

The hydrogenation product of cicutol was first isolated by saturation of the crude cystalline material obtained on chromatography of the plant ethereal extract, followed by chromatography of the products on a column of alumina.

(a) Thus, crude cicutol (100 mg.) was completely hydrogenated over palladium-barium sulphate catalyst (2.5%). The products were dissolved in petrol ether (20 ml., 40 - 60°) and adsorbed onto a column (0.8 x 12.5 cm.) packed with neutral alumina (Brockmann Grade III). Development of the chromatogram with petrol ether (50 ml.) followed by benzene (50 ml.) caused the elution of a small quantity (5 mg.) of an oil. Continued development with chloroform-benzene (10:90, 100 ml.) yielded white crystals (57 mg.) which, after three recrystallisations from petrol ether (40 - 60°), yielded heptadecan-1-ol (35 mg. plates), m.p. 54.5 - 55.0°. (Found: C, 79.6; H, 14.2%. $C_{17}H_{34}O$ requires C, 79.6; H, 14.1%).

A further quantity of white crystals (22 mg.) was obtained by development of the chromatogram with methanol-benzene (1:100), while subsequent development with absolute methanol yielded an oil (5 mg.). The recovery of hydrogenation products was 90%.

The same heptadecan-1-ol as obtained on hydrogenation of the crude cicutol, was obtained by hydrogenation of the pure compound (m.p. 66°).

(b) Oxidation of heptadecan-1-ol.

The cicutol hydrogenation product was characterised by its oxidation to heptadecan-1-oic acid. Thus, heptadecan-1-ol (41.0 mg., m.p. 54.5 - 55.0°) was dissolved in A.R. acetone (20 ml.). A solution (3 ml.) of chromic acid (chromic oxide (3.5 g.): conc. sulphuric acid (28 ml.): water (210 ml.)) was added with vigorous shaking over a period of 5 minutes. After standing 6 hours, the solution was diluted with water (60 ml.) and extracted with ether. The ether extracts were washed with aqueous potassium hydroxide solution (5%) to remove the acid product, which was recovered in the usual manner. Three recrystallisations of the product from petrol ether (40 - 60°) furnished heptadecan-1-oic acid (10.4 mg.), m.p. 60.5 - 61°. (Found: C, 75.4; H, 12.55%. $C_{17}H_{34}O_2$ requires C, 75.5; H, 12.59%).

(7) *Partial Hydrogenation of Cicutol over Lindlar's Catalyst.

Cicutol (16.5 mg., m.p. 66°) in absolute ethanol (5 ml.) was shaken over Lindlar's catalyst (ca. 3 mg.) in the micro-hydrogenation apparatus. The hydrogenation was stopped after the absorption of 2.2 mols hydrogen, and the product examined spectroscopically (Figure 3, Chapter 4). An attempt to isolate the product of partial hydrogenation failed owing to polymerisation. Light absorption of the partial hydrogenation product:-

Maxima	302 (inf.),	315,	330,	347	mu.
$10^{-3}\varepsilon$	(26.5),	46.2,	62.6,	55.3.	
Minima	255,	322,	339.		
$10^{-3}\varepsilon$	3.31,	37.8,	33.05.		

---

* Experiment carried out together with Dr. B. Lythgoe and Dr. E.F.L.J. Anet.

(8) Derivatives of Cicutol.

Owing to the instability of cicutol under any but the mildest conditions, derivatives proved extremely difficult to prepare. A crude p-nitrobenzoate (m.p. 81 - 83°) was obtained, and also a crude acetate which polymerised on attempted recrystallisation.

(a) Cicutol p-nitrobenzoate.

Crude cicutol (60 mg.) was dissolved in anhydrous pyridine (3 ml.) and p-nitrobenzoyl chloride (140 mg., m.p. 73°) added. The solution was stoppered under nitrogen for two days, after which water (1 ml.) was added and the solution left for a further day. The whole was then taken up in ether and washed with potassium bicarbonate, and then with water. Evaporation of the ether and removal of the remaining pyridine under vacuum gave the crude p-nitrobenzoate (92 mg., partly solid).

The crude p-nitrobenzoate was purified chromatographically by adsorption from benzene-petrol ether (40 - 60°) solution (35:65, 70 ml.) onto a column (0.8 x 10 cm.) packed with neutral morin-alumina (Brockmann Grade III). On development of the chromatogram with benzene under ultra-violet light, a dark zone was observed to move down the column. The eluate corresponding to this zone (40 ml.) was collected separately and evaporated to yield waxy yellow plates (40.2 mg.). Recrystallised twice from methanol, the cicutol p-nitrobenzoate (7 mg.) had a m.p. 81 - 83°.

A second polymeric light absorbing band was eluted from the column with benzene-chloroform (80:20).

Light absorption of cicutol p-nitrobenzoate in alcohol.

$\lambda_{max.}$	237	253,	(261),	(263),	(265),	317,	335 mμ.
$10^{-3}\varepsilon$	21.0,	24.5,	(18.85),	(19.1),	(19.0),	40.5,	35.3.
$\lambda_{min.}$	245	(260),	(262),	(264),	(266),	328 mμ.	
$10^{-3}\varepsilon$	17.6,	(18.8),	(18.6),	(18.9),	(18.6),	32.65.	

The light absorption is the same as that of cicutol, but with fine structure in the region λ260 - 265 mμ due to the phenyl group.

(b) Cicutol acetate.

A crude acetate was prepared in the usual manner by acetylation of crude cicutol with acetic anhydride in pyridine. Subsequent chromatographic purification on neutral morin-alumina (Brockmann Grade III), yielded crystalline material which decomposed on attempted recrystallisation.

(9) Examination of Hydrogenation Products of the Crude Cicutol obtained by Chromatography of the Methanolic Plant Extract (see Section 4).

The crude cicutol obtained by chromatography of the methanolic extract of the plant material (Section 4), was completely hydrogenated over palladium-barium sulphate (2.5%), and the products (4.55 g.) chromatographed from benzene solution (50 ml.) on a column (3.5 x 41 cm.) packed with neutral alumina (440 g. Brockmann Grade III). Development of the chromatogram with benzene (2 l.) followed by benzene-chloroform (90:10, 2 l.), furnished a large quantity (3.62 g.) of heptadecan-1-ol characterised by m.p. and mixed melt with an authentic sample. Continued development with methanol-benzene (0.2:100, 1.5 l.) furnished needles (0.44 g.) of m.p. 153 - 158° (after crystallisation from petrol ether 40 - 60°). Further development with methanol-benzene (1:100, 1 l.) furnished material (0.15 g.) which,

after 3 crystallisations from petrol ether (40 - 60°)-chloroform (1:1), was obtained as plates of m.p. 67.5 - 68.5°. (Found: C, 74.46; H, 11.8. Infra-red absorption showed strong peaks at 3425, 1738, 1712, 1200 and 1183 $cm.^{-1}$, and minor peaks at 787, 1137, 1213, 1238, 1242, 1255, 1273, 1290 and 1301 $cm.^{-1}$).

Final development of the chromatogram with methanol-benzene (1.5:100, 500 ml.) yielded a semi-solid yellow oil (0.19 g.).

The crystals of m.p. 67.5 - 68.5° were in all probability a phytosterol (cf. 4, 5).

(10) Examination of the Second Light Absorbing Band Eluted after Cicutol on Chromatography of the Plant Ethereal Extract (Fraction II, Section 2).

This material, which was subsequently shown by Drs Lythgoe and Trippett to contain cicutoxin, the poisonous principle of *C. virosa* (27), did not crystallise.

(a) Ultra-violet light absorption in ethanol (Figure 2, Chapter 3).

$\lambda_{max.}$	236,	254,	317,	334.
$E^{1\%}_{1cm.}$	40.9,	48.0,	113.5,	102.8.
$\lambda_{min.}$	246,	265,	327.	
$E^{1\%}_{1cm.}$	31.8,	21.3,	95.3.	

The material exhibited the same general absorption as cicutol (Figure 2, Chapter 3), and by comparison of the $E^{1\%}_{1cm.}$ value at 317 mμ with that of cicutol, appeared to be approximately 70% pure.

(b) Infra-red Absorption.

A large number of peaks were observed. Those better defined were as follows: 3326 (strong), 2212 (weak), 2128 (very weak), 1634 (weak), 1603 (medium) and 996 $cm.^{-1}$ (very strong).

(c) Chromatography of Hydrogenation Products.

Fraction II (200 mg.) was completely hydrogenated over palladium-barium sulphate (2.5%), and the products adsorbed from benzene solution (50 ml.) onto a column (1.5 x 9.0 cm.) packed with neutral alumina (14 g., Brockmann Grade III). Development of the chromatogram with benzene (200 ml.) furnished a yellow oil (25 mg.). Further development with chloroform-benzene (10:90, 300 ml.) yielded crystals (72 mg.) which, after 3 recrystallisations from petrol ether (40 - 60°)-chloroform (3:1), showed m.p. 66.5 - 67.5^{o}. (This material was later shown by Drs Lythgoe and Trippett to be impure heptadecan-1:14-diol (m.p. 71°)).

Final development with methanol-benzene (1:100) yielded waxy crystals (100 mg.) which could not be purified; the m.p. being 73 - 76° after five recrystallisations from chloroform and petrol ether.

(11) Synthetic Experiments.

A. Preparation of hendeca-5:7:9-triene-2-yne-1:4-diol.

Propargyl alcohol (6.0 g. re-distilled) in anhydrous AR benzene (20 ml.) was added slowly to a stirred solution of ethylmagnesium bromide (from magnesium (5.3 g.); ethyl bromide (23.5 g.) and anhydrous ether (50 ml.) according to Ziele and Mayer (6)). Near the end of the addition a further 30 ml. benzene were added and the precipitate broken up into a slurry. The mixture was refluxed with vigorous stirring at 30° for 2 hours, after which the condenser was removed and the ether allowed to boil off. A solution of octatrienal (13.1 g. prepared according to Kuhn and Grundmann (7)) in benzene (20 ml.) was then added slowly, and stirring continued for 3 hours at 35°. The cooled mixture (0°) was decomposed by the addition of ice cold saturated ammonium chloride solution (500 ml.)

over a period of 20 minutes. Ether (1 l.) was added and the organic layer washed, dried and evaporated. The residue, recrystallised once from benzene, yielded the diol (5.49 g.) of m.p. 97 - 103°. Further crystallisation from chloroform gave material of m.p. 103 - 109° (decomposition over melting range). (Found: C, 74.0; H, 7.4%. $C_{11}H_{13}O$ requires C, 74.2; H, 7.85%). Light absorption in EtOH, $\lambda_{max.}$ 260, 267.5, 278 mμ. $10^{-3}\varepsilon$ 43, 55, 44.5. (Comparison of the molar absorption with that of octatrienal at λ267.5 mμ showed the compound 96% pure.)

B. Preparation of nona-2:4-diene-8-yn-6-ol.

Zinc dust (38 g.) was activated with 2N HCl, washed, and dried by azeotropic distillation with benzene (C). The zinc was covered with anhydrous AR benzene in a flask equipped with efficient stirring and reflux condenser. A mixture of sorbaldehyde (58 g.) and propargyl bromide (58 g.) in anhydrous ether (200 ml.) was then added slowly. After ca. 10 ml. had been run in, iodine (1 cryst.) and mercuric chloride (50 mg.) were added to the mixture, and the reaction started by gentle warming. Thereafter, the reactants were added over 30 minutes at a rate sufficient to maintain reflux. The mixture was refluxed for a further 30 minutes, cooled, and poured into ice cold acetic acid solution (1%, 450 ml.). The whole was then extracted with ether (3 x 250 ml.); the combined ethereal extracts being washed, dried and evaporated.

The residue was then distilled, first at the water pump with a bath temperature of 50° to remove benzene, water and unchanged propargyl bromide, and then at 0.1 mm. with the receiver surrounded by a CO_2-freezing mixture. The bath temperature was gradually increased to 150° as the distillate changed in appearance from a colourless liquid to a thick viscous oil. The total distillate collected

(60 ml.) was then fractionated through a column (1½" x 6½") packed with Dixon gauge rings. The required nonadienynol (15 g.) was obtained as a fraction of b.p. 62 - 65° (0.5 mm.); unchanged sorbaldehyde and a mixed fraction of b.p. 33 - 60° (0.1 mm.) being first removed.

Redistillation of the crude alcohol furnished pure nona-2:4-diene-8-yn-6-ol of b.p. 62.5° (0.5 mm.) and $\eta^{D}_{19.2}$ = 1.5136 (Found: C, 78.1; H, 8.78%. $C_9H_{12}O$ requires C, 79.6; H, 8.88%). Light absorption in ethanol; $\lambda_{max.}$ 229 mμ. $10^{-3}\epsilon$ = 22.1. (Henbest, Jones and Walls (9) state that low carbon values are always found for propargyl carbinols.)

C. Preparation of the α-naphthyl urethane of nona-2:4-diene-8-yn-6-ol.

The alcohol (300 mg.) was heated with α-naphthyl isocyanate (463 mg.) for 10 minutes on a water bath. The solid mixture was extracted with petrol ether (80 - 100°) and the solution filtered. Evaporation of the filtrate followed by 3 recrystallisations from petrol ether (80 - 100°) gave the crude urethane (140 mg.) of m.p. 98 - 103.5°. This material was adsorbed from benzene solution (20 ml.) onto a column (1.5 x 8.5 cm.) packed with neutral alumina (14 g. Brockmann Grade III). Development of the chromatogram with benzene (100 ml.) and evaporation of the eluate, furnished white needles, twice recrystallised from petrol ether (100 - 120°), to give nonadienynol α-naphthyl urethane of m.p. 104 - 106° (Found: C, 78.2; H, 6.07%. $C_{20}H_{19}O_2N$ requires C, 78.8; H, 6.22%).

D. Dehydration of nona-2:4-diene-8-yn-6-ol to nona-3:5:7-triene-1-yne.

This reaction was carried out using several reagents. The yields were determined by measurement of the

ultra-violet absorption of the product at λ 305, 292 and 279 mμ and comparison of the values with those given by Jones et al. (10) for deca-3:5:7-triene-1-yn-9-ol. In no case were the yields sufficient to warrant isolation of the nonatrienyne for preparative purposes.

(a) <u>With phosphorus oxychloride in pyridine.</u>

The nonadienynol (103 mg.) was refluxed (1½ hours) with anhydrous pyridine (2 ml.) containing phosphorus oxychloride (46 mg.). The reaction mixture was taken up in n-pentane (5 ml.) and washed thoroughly with 6N HCl solution, potassium bicarbonate solution and water. Ultra-violet examination of the dried pentane solution showed a yield of 9.9% nonatrienyne.

(b) <u>With p-toluene sulphonic acid in cyclohexane.</u>

Nonadienynol (100 mg.) was refluxed 30 minutes with p-toluene sulphonic acid (3 mg.) in cyclohexane (5 ml.). The solution was washed with aqueous potassium bicarbonate and water and then dried. Yield of nonatrienyne = 13%.

(c) <u>With p-toluene sulphonic acid in S-free toluene.</u>

The experiment was carried out as with cyclohexane. Yield of nonatrienyne = 16%.

(d) <u>Dehydration of crude nonadienynol p-toluene sulphonate.</u>

Nonadienynol (3.1 g.) was reacted with p-toluene sulphonyl chloride (4.7 g.) in AR acetonitrile (8.6 ml.) containing potassium hydroxide (1.5 g. in 2.7 ml. water). The solution was stirred 12 hours and the crude derivative (1.71 g.) isolated by ether extraction in the usual manner.

The nonadienynol tosylate (1.42 g.) was dissolved in ethanol (2 ml.) and added to potassium hydroxide solution (5 ml. 30% w/w). The mixture was refluxed for

10 minutes and extracted with ether. The ethereal extract was examined spectroscopically after washing with sodium bicarbonate solution and water. Yield of nonatrienyne = 3%.

Attempts to dehydrate the nonadienynol tosylate with sodamide in liquid ammonia and with sodium n-butoxide, yielded none of the required nonatrienyne.

E. Preparation of ethyl-3-hydroxy-octa-4:6-dienoate.

This compound was prepared as an intermediate for a proposed synthesis of octatrienal from sorbaldehyde.

Zinc powder (8 g.) was activated with 2N HCl, washed with ethanol and dried by azeotropic distillation with benzene (8). The zinc was covered with anhydrous AR benzene (150 ml.) in a flask fitted with efficient stirring and reflux condenser. A mixture of sorbaldehyde (48 g. b.p. 62 - 64°/11 mm.) and ethyl bromoacetate (90 g. b.p. 158 - 159°) in benzene (50 ml.) was then added slowly. After addition of ca. 5 ml. of this solution the mixture was gently warmed. A vigorous reaction ensued and the reactants were added at a rate sufficient to maintain reflux. The mixture was refluxed for 20 minutes after addition was complete. The products were taken up in benzene (400 ml.) and washed with acetic acid solution (1N, 3 x 300 ml.). The benzene was removed under vacuum and the product distilled through a fractionating column. Yield, 28.6 g. ethyl-3-hydroxy-octa-4:6-dienoate as a colourless pleasant smelling oil of b.p. 85.5°/0.1 mm. $\eta^{D}_{18°}$ 1.4896. (Found: C, 65.2; H, 8.7%. $C_{10}H_{16}O_3$ requires C, 65.6; H, 8.8%).

The α-naphthyl urethane of the compound was prepared as described for nondienynol (Section 11 C). After recrystallisation from petrol ether (40 - 60°)-benzene and aqueous alcohol, the α-naphthyl urethane of ethyl-3-hydroxy-octa-4:6-dienoate showed m.p. 98.5 - 99.5°.

(Found: C, 71.8; H, 6.8%. $C_{21}H_{23}O_4$ requires C, 71.4; H, 6.52%).

F. Dehydration of ethyl-3-hydroxy-octa-4:6-dienoate to ethyl-octa-2:4:6-trienoate.

Ethyl-3-hydroxy-octa-4:6-dienoate (27 g.) in anhydrous AR benzene (400 ml.) was refluxed 6 hours with AR p-toluene sulphonic acid (0.25 g.). The yield of ethyl-octa-2:4:6-trienoate estimated spectroscopically by its ultra-violet absorption at λ 299 (cf. 11) was 33%.

REFERENCES.

(1) H. Brockmann & F. Volpers. Chem. Ber. 80, 77 (1947).

(2) H. Brockman & H. Schodder. ibid., 74, 73 (1941).

(3) E. Anet, B. Lythgoe, M.H. Silk & S. Trippett. J. Chem. Soc. 309 (1953).

(4) J. Pohl. Archiv. für Exp. Path. und Pharm. 34, 259 (1894).

(5) R.N. Jones, P. Humphries & K. Dobriner. J. Amer. Chem. Soc. 72, 956 (1950).

(6) K. Ziele & H. Mayer. Chem. Ber. 75B, 361 (1942).

(7) R. Kuhn & C. Grundmann. ibid., 70, 1318 (1937).

(8) C.W. Greenhalgh, H.B. Henbest & E.R.H. Jones. J. Chem. Soc. 1193 (1951).

(9) H.B. Henbest, E.R.H. Jones & I.M.S. Walls. ibid., 2698 (1949).

(10) I.M. Heilbron, E.R.H. Jones & J.T. McCombie. ibid., 135 (1944).

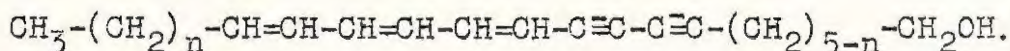
(11) B.C.L. Weedon & R.J. Woods. ibid., 2687 (1951).

PART II. THE STRUCTURE OF CICUTOL.

S U M M A R Y.

(1) A remarkable compound cicutol was isolated from the poisonous "water hemlock" (*Cicuta virosa*), by use of the technique of fluorescence-quenching chromatography.

(2) The compound was shown to be a member of the new class of naturally occurring polyene-ynes, and a type structure was proposed on the basis of its observed physical and chemical properties, viz:



$CH_3-(CH_2)_n-CH=CH-CH=CH-CH=CH-C\equiv C-C\equiv C-(CH_2)_{5-n}-CH_2OH.$

(3) Three new compounds were prepared in the course of synthetic experiments concerning the chemistry of cicutol. These were:

(1) hendeca-5:7:9-triene-2-yne-1:4-diol.

(2) nona-2:4-diene-8-yn-6-ol.

(3) ethyl-3-hydroxy-octa-4:6-dienoate.

(4) Other recently discovered compounds related to cicutol have been reviewed in Chapter 6.

PUBLICATIONS CONCERNING THE WORK PRESENTED IN PART II OF THIS THESIS.

(1) "The Chemistry of Oenanthotoxin and Cicutoxin"
by E. Anet, B. Lythgoe, M.H. Silk and S. Trippett.
Chemistry and Industry, 757 - 8 (1952).

(2) "Oenanthotoxin and Cicutoxin - Isolation and Structures"
by E. Anet, B. Lythgoe, M.H. Silk and S. Trippett.
Journal of the Chemical Society, 309 - 322 (1953).

ACKNOWLEDGEMENTS.

(1) The award of the Permutit Senior Research Studentship by the Master and Fellows of St. Catharine's College, Cambridge University, is gratefully acknowledged. The author is indebted to the Board of Directors of the Permutit Company, Gunnersbury Avenue, London, W.4, for the endowment of the scholarship.

(2) The author wishes to express his thanks to the following persons for their generous help.

Professor W.F. Barker, Head of the Department of Chemistry at Rhodes University, for his kindness in making possible the presentation of this thesis.

Professor A.R. Todd. F.R.S., for permission to use the facilities of the Cambridge University Chemical Laboratory.

Dr. B. Lythgoe (now Professor at Leeds University) for his direction of the work, and for much valuable training in methods of research.