A THESIS

entitled

"PHYSICO-CHEMICAL TECHNIQUES IN THE STUDY OF ORGANIC MOLECULES OF BIOLOGICAL IMPORTANCE"

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Finally I would like to thank Miss H. Humphrys, Miss J. Malcolm and Miss J. Johnston for recording the mass spectra.

Aim of the present Investigation

The purpose of the present work was to isolate and identify the minor lipid components of the human atheromatous plaque in the hope that the study of these compounds might help to throw some light on the pathogenesis of the disease atherosclerosis. We set out to use the recently developed micro techniques of TLC, GLC and GC-MS described later to enable each investigation to be restricted to the lesions from a single aorta and eventually to extend the work to the lipid chemistry of individual plaques at various stages of evolution. Early work on the subject had indicated that certain minor steroids were present, but the necessarily protracted nature of the investigations had led to doubts being expressed about their authenticity as arterial constituents. We hoped that we would be able to confirm or disprove the existence of these steroids and would be able to look for unidentified components in this and other lipid groups.

C O N T E N T S

PART I

THE DISEASE ATHEROSCLEROSIS

A. INTRODUCTION

THE DISEASE ATHEROSCLEROSIS

The high incidence of diseases of the arteries and particularly that of fatal illness due to the obstruction of vital blood supplies are matters of wide concern. So acute is the problem that heart disease has earned the description "common illness of our time" (1), the number of deaths in the U.S.A. in 1950 (2) from this cause being greater than the sum of deaths from the next four causes in order of frequency, namely: cancer, accidents, tuberculosis and pneumonia.

Progressive changes occur in all arteries as they age. The most important alteration of the intima, (see Fig. 1) or inner surface of the artery, consists in the accumulation of fatty materials (lipids)[x] of which cholesterol and its esters are characteristically preponderant. Lipid accumulation in the intima of an artery is thought by many investigators to start with the development of "fatty streaks" which gradually build up into progressively raised lesions. As the accumulation process goes on, the lumen, or passageway of the artery begins to narrow.

The arteries of most adults are affected to varying degrees by atheroma - the name given to the fatty deposits

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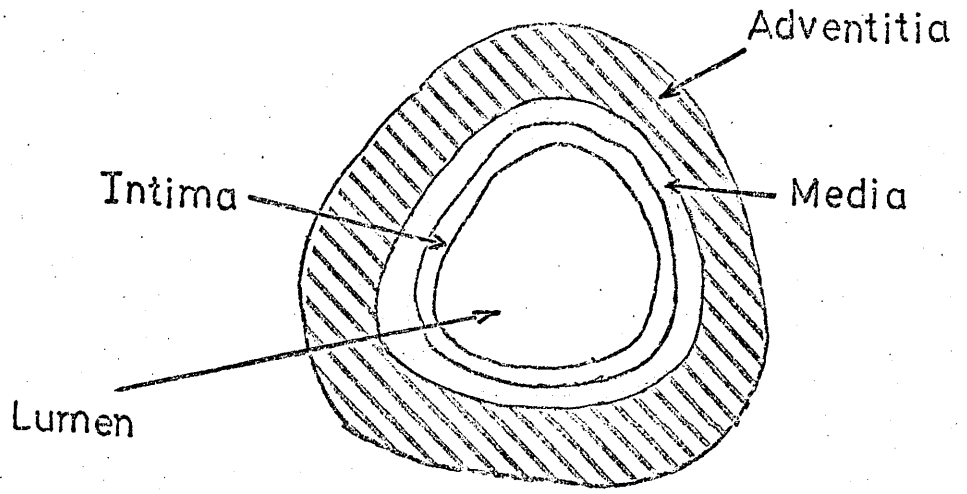
[x] For detailed comment on this description, see p. 8.

occurring as "plaques" (the raised lesions mentioned above) in the artery wall. In fact the tissue changes of this disease have been regarded by some as part of a normal ageing process of vessels (3 - 6), but according to the opinion of others (7 - 11) the lesions are caused by abnormal lipid infiltrations of the intima, which initiate other changes of the vessel walls, and should best be treated as disorders of lipid metabolism.

Figure 1. SCHEMATIC DIAGRAM OF A NORMAL ARTERY
(X-SECTION TRANSVERSE)



In the initial stages of the disease the tissue overlying the plaques is reasonably elastic (see Fig. 2), but in the later stages the lining of the artery may degenerate and rupture giving an "atheromatous ulcer" (see Fig. 3). Frequently a

Figure 2. NORMAL AORTA

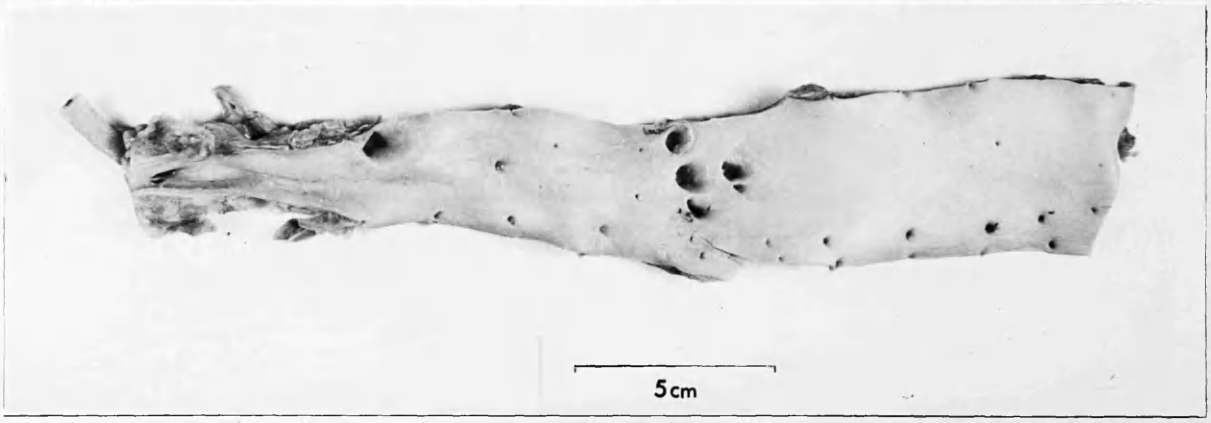
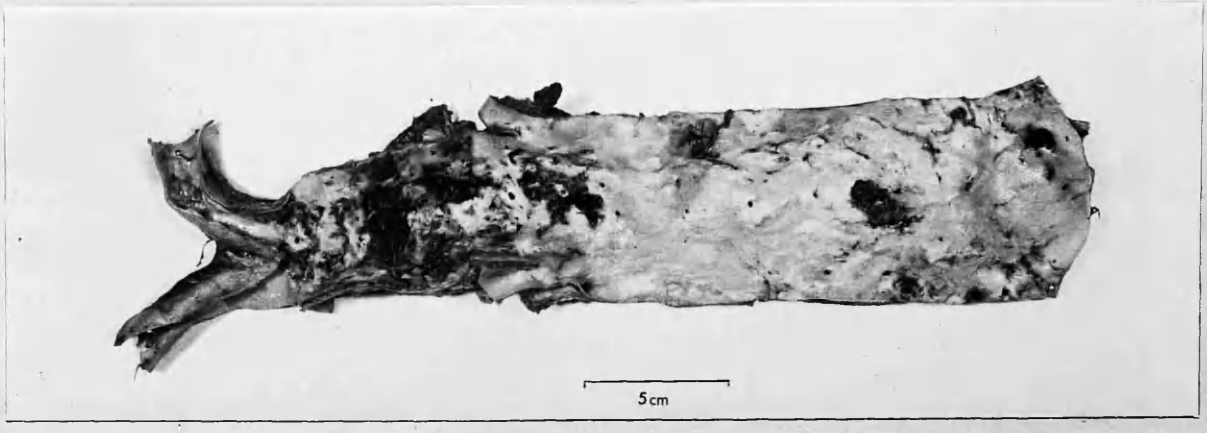


Figure 3. DISEASED AORTA



blood clot ("thrombus") forms on such a site, and if this happens in one of the smaller arteries such as the coronary artery, complete occlusion resulting in sudden death or acute myocardial infarction (localised death of tissue) may occur. Fig. 4 shows a photomicrograph of such an occluded artery.

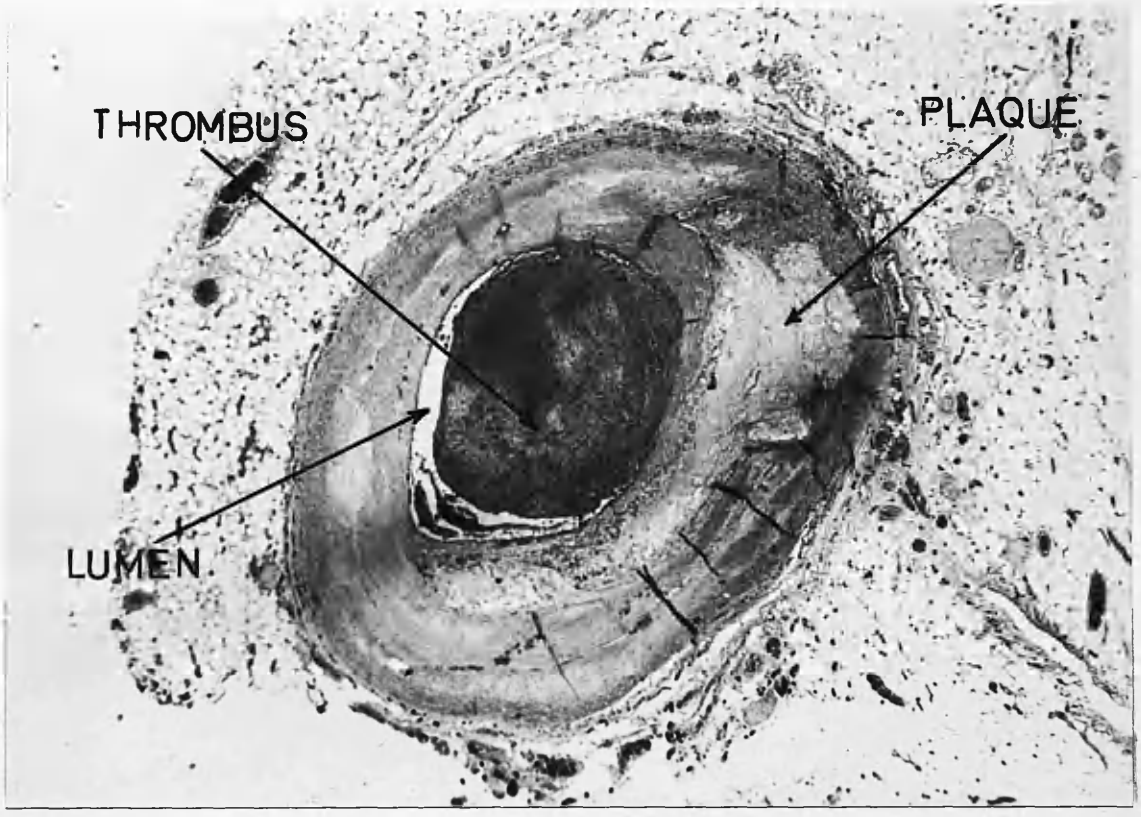
The degenerative process

In spite of a tremendous number of investigations carried out since the beginning of the century, our knowledge about the processes underlying the atherosclerotic degeneration of the human artery wall is still very limited. There are many theories as to the cause of the lipid accumulations, the chief of which is that this material is deposited by a simple infiltration from the blood flowing through the lumen (15). Another opinion is that the deposits arise by local synthesis of lipids in the vessel wall (16). The first of these hypotheses is believed to be closer to reality, although there is some evidence for *in situ* synthesis. The relative merits of these two theories have been discussed by Holman *et al.* (17). What is not clear, however, is whether this lipid accumulation is the primary cause of the degeneration of arterial structure and function, or whether it is simply a secondary process initiated by some metabolic defect.

Dietary factors in atheroma

The role of diet in the development of atherosclerosis is a very controversial subject. Opinion about the importance of this factor ranges from the view that it is trivial, to the belief

Figure 4. AN OCCLUDED ARTERY



A Photomicrograph of a cross section of a human coronary artery (stained H and E x 30).[x] The artery has been markedly narrowed by an accumulation of lipid material in the wall (atheroma). Note the slit-like spaces due to so-called crystals of cholesterol. The lipid plaque has ruptured, and the lumen or passageway of the vessel is occluded by a thrombus (black area in centre).

[x] H and E refer to the tissue stains Haematoxylin and Eosin and x 30 is the magnification.

that diet is the primary cause of atherosclerotic disease.

It is known that the incidence of myocardial infarction is greatest in subjects with an abnormally high quantity of cholesterol in the blood and that this level is directly influenced by the amount of fat in the diet. However, the exact contribution of diet in causing atherosclerosis has not been established. Dietary cholesterol has only a slight effect on the concentration of blood cholesterol, which may also be influenced by ingested complex carbohydrates. The largest effect is however produced by dietary triglycerides.

The influence of fat has been clearly illustrated during the Second World War in many areas where the amount of fat ingested dropped markedly. Autopsy findings during this period have shown that deaths ascribed to atherosclerosis began to decline in 1942 and fell to about one third of the pre-war level in the years 1943 - 1946 (12).

Other factors

It is now generally agreed that instead of a single cause of atherogenesis it is necessary to consider the contributions of a number of influences including diet. There is a wide geographical variation in diseases due to atheroma. Japan, for instance, has been shown to have a very low incidence of atherosclerotic heart diseases (13).

This, however, is not a racial peculiarity since second generation Japanese living in California tend to resemble other Americans in their susceptibility to the disease and its complications (14). A similar situation arises when Italians living in Southern Italy are compared with those living in the United States of America.

Another important factor is thought to be lack of exercise due to the increase in sedentary occupations. This factor has been found to be closely related to the development of heart disease(18) although Keys et al.(19) have collected evidence to suggest that it is less important than diet. Attempts have also been made to correlate smoking habits (20) and stress(21) with the occurrence of heart disease. Whatever may be the prime cause of atheroma, there is general agreement that the disease involves a derangement of lipid metabolism. A detailed examination of the lipids of severely diseased aortas thus appears warranted.

SECTION A INTRODUCTION

PART II

THE LIPIDS

THE LIPIDS

In living systems the structures of the cells and tissues are based on large molecules - proteins, polysaccharides and complex lipids - whilst the organisation of the systems appears to be the function of the nucleic acid complexes. In recent years, detailed studies on proteins, polysaccharides and nucleic acids have demonstrated patterns of repeating units within an almost infinite variety of molecular structures. Although most lipid molecules are relatively less complex, a large variety of compounds exists, many of them differing only in the composition of the fatty acid moieties.

Fats have been recognised as a separate group of compounds for many centuries. The substances called fats were usually glycerol esters and in this work we shall restrict the term to these. The word "lipid" (Gr. lipos, fat), although probably initially synonymous with the term "fat" is now used to cover a much wider group of compounds.

The classification of lipids has been the subject of some controversy. The early classifications were unsatisfactory as the analytical methods available to define them were crude. Nowadays with the advances in separation techniques such as chromatography there is a growing tendency to refer to lipids by names indicative of their structure, and this practice is helping to remove the vagueness which has until quite recently

dominated lipid classifications. Such vagueness has led to trivial names such as cerebroside and ganglioside, based on the biological source. Ultimately it may be possible to classify lipids according to their biological function, but this aspiration is at present far in the future. Therefore an interim classification has had to be used and the system adopted here subdivides lipids in the manner shown here and in Fig. 5.

1. Simple lipids

   (a) Glycerides:- esters of glycerol with long-chain fatty acids. These are the "fats" of the classical definition.

   (b) Sterols and their fatty acid esters.

   (c) Long-chain alcohols and their fatty acid esters which are usually called "Waxes".

   (d) Terpene alcohols.

   (e) Hydrocarbons.

2. Complex lipids

Complex lipids consist of esters which may contain phosphorus, nitrogen bases and sugars in addition to long-chain fatty acids.

   (a) Phosphoglycerides:- esters which contain a phosphate group and a nitrogen base in addition to a glycerol moiety.

   (b) Sphingolipids

      (i) Sphingomyelin

      (ii) Cerebroside

      (iii) Ganglioside

3. Miscellaneous compounds whose solubility classes them as lipids.

(a) Vitamins A, D, E and K

(b) Ubiquinones and plastoquinones

(c) Phenols (i.e. natural alkylated phenols e.g. estrogens)

(d) Squalene epoxide

(e) Steroids

(f) Bile acids

Fatty acid nomenclature

A frequently used short-hand nomenclature to define the chain length and degree of unsaturation of a fatty acid will be employed in this work and is as follows:-

The fully saturated 18 - carbon fatty acid stearic acid would be referred to as (18:0); the 18 - carbon monounsaturated acid oleic acid as (18:1), similarly

palmitic acid	(16:0)
palmitoleic	(16:1)
linoleic	(18:2)
linolenic	(18:3)

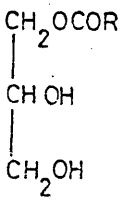
The positions of the double bonds are specified thus:-

oleic acid	18:1 - 9
linoleic acid	18:2 - 9, 12
linolenic acid	18:3 - 9, 12, 15

Figure 5.

Simple lipids

1.



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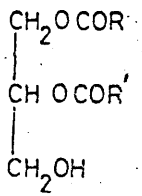

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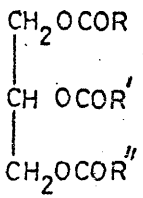

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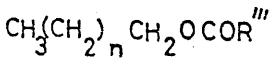

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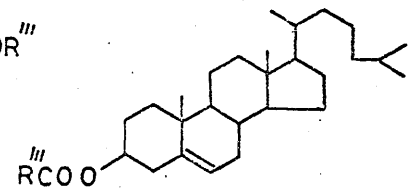

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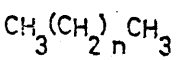

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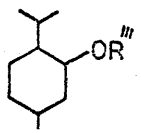

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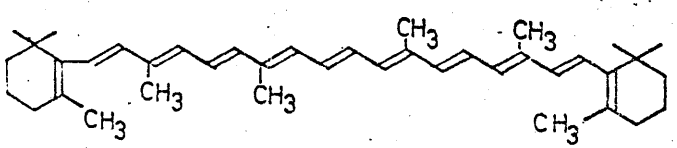
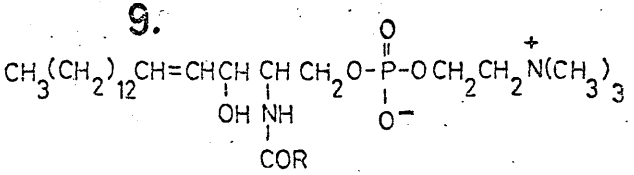


R, R′, R″ ≡ alkyl

Complex lipids

9.



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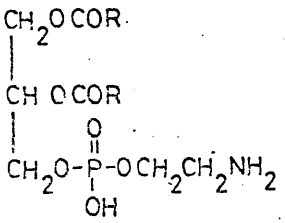

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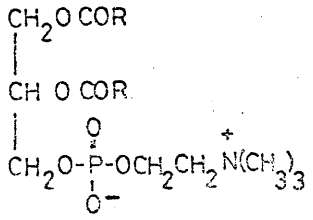
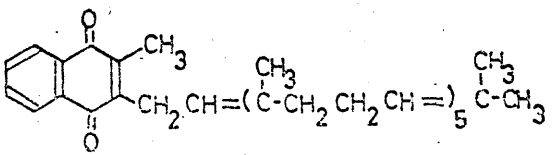


Lipid-like compounds

12.



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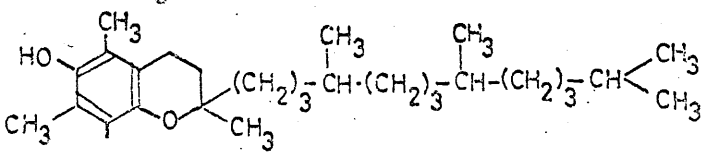

R‴ ≡ H or alkyl

Key to Figure 5.

1. Monoglyceride
2. Diglyceride
3. Triglyceride
4. Wax Ester
5. Sterol and Sterol Ester
6. Alkane
7. Terpene Alcohol
8. Carotene
9. Sphingomyelin
10. Phosphatidyl Ethanolamine
11. Phosphatidyl Choline
12. Vitamin K_2
13. Vitamin E (α- Tocopherol)

Polarity of lipids

Lipids are frequently tabulated in order of their chromatographic polarity (TLC[x] or column) on silica gel or silicic acid (22) and this sequence is given below for the major groups.

hydrocarbons
wax esters
sterol esters
triglycerides
sterols
fatty acids
diglycerides
monoglycerides
phospholipids

increasing polarity ↓

The elution order on an alumina column is basically the same except for the fatty acids which then become the most polar group of compounds (23). A similar situation occurs when Florisil is used as adsorbent and in this case the fatty acids are eluted just before the phospholipids (24).

[x] This abbreviation is defined later on page 47.

SECTION A INTRODUCTION

PART III

THE LIPID CHEMISTRY OF ATHEROMA

THE LIPID CHEMISTRY OF ATHEROSCLEROSIS

Although lipids have been recognised as a separate group of compounds for more than a century, it is only relatively recently that methods have been developed to separate them into their individual components. Methods of purification and analysis which were applicable to water-soluble compounds such as carbohydrates and proteins, and especially to their hydrolysis products, could not be applied to any great extent to lipids, which frequently occur in nature in complex mixtures in which the individual components may differ only very slightly. Classical methods in general were incapable of separating such mixtures.

Sterols in tissues

Sterols, because of their wide distribution in tissues constitute an especially interesting category of lipids. Cholesterol itself, although it cannot be regarded as the most striking member of this group on the basis of its known direct physiological action, must be regarded as a key constituent, both because of its exceedingly wide distribution in mammalian tissue and also because of its obvious significance as the parent substance of the steroid hormones.

The discovery of cholesterol as a separate compound dates back to 1769 (25), although its true nature was not recognised at this time. In 1815 Chevreul (26) demonstrated that this compound

was unsaponifiable and introduced the name "cholesterine". Its identification in human bile (27), blood (28) and brain tissue (29) soon followed. However, the modern understanding of this substance began with the demonstration of its alcoholic nature by Berthelot (30) who also prepared esters. Quickly cholesterol became recognised as a normal constituent in animal tissues and fluids as well as a component of certain pathological deposits such as the atheromatous plaque (31, 32).

Lipids of the atheromatous aorta

Early analyses of total aortas (31 - 33) demonstrated that atherosclerosis of the aorta and its branches is associated with an increased lipid content of the tissues: however, they failed to differentiate between the chemical changes which occur with age and those due to the disease. Meeker and Jobling (34) stressed this point and were able to achieve a better understanding of the problem by comparing analyses of separated lesions with those of normal tissue.

Lipid changes due to ageing

Weinhouse and Hirsch (35) also pointed out the necessity to separate normal from pathological tissue and, since atherosclerosis is principally a disease of arterial intimas, they were able to gain information about the effects of ageing by examination of the media carefully dissected from the diseased intima. They

found large increases in the quantity of cholesterol and its esters with age, and although the amount of phospholipid did not alter markedly there was a significant qualitative change within this lipid group.

The changes due to atherosclerosis

In a study of the intimal changes attributed to atherosclerosis, Weinhouse and Hirsch (35) found that the amount of cholesterol in the lipid extract rose sharply from the value in the normal intima to that found in atheromatous ulcers associated with the most advanced stage of the disease. In agreement with the findings of Hooker and Jobling, the quantity of cholesterol esters, the largest lipid group, was found to increase less rapidly. No marked quantitative change was noted in the phospholipids; however, a very significant qualitative change was found to take place in this component in lesions representing an advanced stage of the disease. It was found that in the normal intima there were approximately twice as much of the ether-soluble phospholipids (lecithin and cephalin) as ether-insoluble phospholipids (mainly sphingomyelin and cerebrosides). In severely diseased tissues the proportions of these compounds were reversed. This investigation (35) and others (34, 65) have emphasized the importance of the changing ratio between cholesterol and cholesterol ester. According to these workers the ratio of ester cholesterol to free cholesterol ($^{CE}/_{C}$) declines with advancing severity of the disease. However Zeek (53) and

Böttcher et al. (45) found that this ratio tends to be higher in early and medium grades of atheroma but tends to be lower in the most advanced lesions.

Classification of lesions

As a preliminary to any analysis it is important to classify the type of lesion being examined. It is usual to employ the system evolved by Böttcher et al. (39) which follows the principle of classification of Buck and Rossiter (40) adapted to the terminology recommended by a committee of the World Health Organisation (41). By this method (Table 1) the arteries are divided into four groups on the basis of their macroscopic appearance.

The first type of lesion visible (stage I) is of the streaky yellow fatty type in which the lipid is mainly intracellular, the intima being slightly thickened but not appreciably damaged. In stage II, the plaques are either of the tough fibrous type or of the soft lipid type known as atheromas, but with no complications such as ulceration, thrombosis or calcification present. Stage III marks the onset of the above complications in which the atheromas frequently seem to be ruptured or about to rupture and contain in addition to soft lipid, blood clots and calcified material.

The three classes of plaque outlined above are normally assumed to represent stages in the evolution of a lesion; but it is possible that this is not so and that they might represent several quite unrelated biochemical changes.

Table 1. Classification of aortas according to Böttcher (42)

STAGE 0	No lesions discernible at a magnification of 10
STAGE I	Fatty streaks and/or spots present
STAGE II	Fibrous plaques and/or atheromas present, but no evidence of ulceration or other complications
STAGE III	As above, with additional complications, e.g. ulceration, necrosis, haemorrhage, thrombosis

Recent investigations of plaque chemistry

The gross quantitative changes within the various lipid classes with the advance of atherosclerosis have recently been summarised in the work of Böttcher (42) (Table 2). He includes results from the examination of thirty-three aortas at various stages of the disease. The trends given in this table are basically the same as those found in the much earlier work of Weinhouse and Hirsch (35), although the individual values differ greatly in some cases.

Table 2. AVERAGE CONTRIBUTION OF EACH LIPID COMPONENT AS A PERCENTAGE OF THE TOTAL LIPID EXTRACTED AT VARIOUS STAGES OF DISEASE

COMPONENT	STAGE 0	STAGE I	STAGE II	STAGE III
Phospholipids	60.9	55.0	33.2	33.7
Free fatty acids	9.6	6.4	2.7	1.2
Cholesterol	8.1	12.0	19.1	19.7
Cholesterol esters	4.1	13.4	36.0	36.2
Glycerol esters	15.6	14.3	11.7	9.5

The early studies of atherosclerosis established that there was an increased lipid content of the vessel wall associated with the disease, and gave useful information especially when it was appreciated that the more general tissue changes due to ageing should be excluded from such an investigation. However, there was a limit to the amount of information, both qualitative and quantitative, which could be obtained using the then known chemical procedures. The development of micro-analytical procedures, especially those based on chromatography, opened many new approaches to the chemistry of the disease, and fractions formerly assumed to be homogeneous, were shown to contain several components. The availability of gas chromatography made it possible to study the individual fatty acid components of the major lipid sub-groups in plaques after separation of these groups by some other form of chromatography. In fact, this type of study has dominated the literature on the chemistry of the artery wall in recent years. The average fatty acid composition of the major lipid classes of the atheromatous plaques (stage II in the Böttcher classification) of four individuals were recorded by Tuna and Mangold (43). The results of this investigation and of a similar study by Lawrie et al. (44) are given in table 3.

Table 3. THE FATTY ACIDS OF THE HUMAN ATHEROMATOUS PLAQUE

(recorded as percentages of the total fatty acids of each lipid component)

Fatty acid	cholesterol esters		triglyceride		phospho-lipid	
	T	L	T	L	T	L
myristic (14:0)	1.1	2.1	2.1	5.1	1.0	2.0
palmitic (16:0)	19.1	12.5	27.2	24.7	46.9	32.1
palmitoleic (16:1)	4.5	10.0	5.1	10.1	1.7	4.7
stearic (18:0)	1.9	1.1	5.7	3.8	21.7	12.7
oleic (18:1)	32.3	23.7	43.9	32.9	19.8	18.9
linoleic (18:2)	37.7	38.7	14.3	11.3	6.3	11.3
linolenic (18:3)	-	-	-	-	-	-
arachidonic (20:4)	3.6	6.4	1.6	1.5	4.0	9.2

T represents the results according to Tuna and Mangold (43)

L represents the results according to Lawrie et al. (44)

From a comparison of these two sets of results the similarities in "fatty acid pattern" can be quickly distinguished. The predominant fatty acids found in each of the major sub-groups were basically the same in both investigations. For instance in the cholesterol esters, linoleic acid (18:2) is the acid present in the largest concentration, followed by oleic (18:1) and palmitic (16:0). On the other hand, in the triglycerides oleic acid predominates followed by palmitic and to a lesser extent by linoleic acid, while in the phospholipids the order is palmitic acid followed by stearic (18:0) and oleic acid.

Changes in fatty acid pattern with advance in severity of the disease.

Both of these investigations of fatty acid pattern in the lesion have been performed on material from one specific stage in the progress of the disease: Böttcher et al. (45) on the other hand, in a larger and more comprehensive study, attempted to relate fatty acid pattern to the development of atherosclerosis. Their findings, summarised in Table 4, are based on the classification of each selected aorta according to the scheme laid down in Table 1 on page 19. The average age of the subjects supplying the tissue in each stage of the disease is also given in this Table. The fatty acids of each lipid group were classified as saturated, mono-unsaturated or poly-unsaturated, and the composition within each of these classes was recorded as a percentage of the total fatty acids in that lipid group.

Table 4. FATTY ACIDS OF AORTA LIPIDS (Böttcher (45))

(mean total percentages)

	Stage of atherosclerosis			
	0	I	II	III
Average age of subjects	6	29	58	56
CHOLESTEROL ESTERS				
Saturated	31.2	22.9	18.7	17.0
Mono-unsaturated	39.9	39.9	35.1	36.7
Poly-unsaturated	23.9	37.2	43.2	46.3
GLYCERIDES				
Saturated	44.9	42.8	34.7	35.3
Mono-unsaturated	40.4	41.8	48.0	43.3
Poly-unsaturated	14.7	15.4	17.3	21.4
PHOSPHOLIPIDS				
Saturated	55.2	59.4	61.2	62.0
Mono-unsaturated	19.4	15.6	16.6	16.6
Poly-unsaturated	25.4	25.0	22.2	21.4

[ ] denotes a significant change

Böttcher found that when aortas were classified according to stage of development, the greatest changes in fatty acid composition from one stage to another were observed in the cholesterol ester fraction, among two of the three classes of fatty acid. With increasing atherosclerosis the poly-unsaturated acid percentage rose from 25-30% to 45-50% and the saturated acids decreased from 30-35% to 15-20%, while the mono-unsaturated acids showed no significant change. Similar but much smaller changes were found to occur in the glyceride fatty acids.

As earlier demonstrated by Weinhouse and Hirsch (35) in the phospholipids there is a large increase in the ether-insoluble sphingomyelins with the advance of the disease, and this is echoed by an increase in palmitic acid, since sphingomyelins are exceptionally rich in palmitic acid.

Origin of plaque lipids

Determinations of the composition of lipids in the intima and in the atherosclerotic lesions have added considerable support to the infiltration theory to which reference was made on page 4 . If the deposits in atherosclerosis of the aorta arise by infiltration of the blood lipids, the lipid composition of the fatty deposits should be compatible with that of the lipids in the blood plasma. The most comprehensive early study of the lipid composition of human blood plasma is that of Page et al.(46).

Comparison of the results of this investigation with later results such as those of Weinhouse and Hirsch (35) for the arterial wall affords some interesting information. The close agreement in composition between lipid extracts from the plasma and the normal intima (stage 0 of plaque formation) and the wide differences between lipid extracts from the intima and the media seem to indicate that the lipids of the intima originate in plasma rather than being synthesised *in situ*. However, this could merely indicate that some sort of an equilibration process had taken place.

More recently, Böttcher (42) also produced evidence supporting the "infiltration theory" of pathogenesis. He found that the fatty acid composition of plaque cholesterol esters and triglycerides at stage III of the disease, approached the values recorded for these lipid groups in the blood of atherosclerotic individuals (45, 47, 50).

The fatty acid pattern of the plaque phospholipids, however, differs from that of the blood phospholipids, even in the severest cases of atherosclerosis. A similar conclusion was reached by Lawrie *et al.* (44) as can be seen from a comparison of their results for plaques (Table 3) with those for the blood of atherosclerotic individuals (Table 5). This difference in phospholipid composition could either mean that these compounds do not infiltrate, or that they do infiltrate but rapidly change in composition. Zilversmit and co-workers (48) have produced evidence of phospholipid synthesis in the artery wall, which would possibly explain this difference in fatty acid composition.

Table 5. FATTY ACID COMPOSITION OF SERUM IN ATHEROSCLEROTIC INDIVIDUALS (Lawrie et al. 44)

(as percentages of total fatty acids in each lipid group)

Fatty acid	Cholesterol ester	Triglyceride	Phospholipid
14:0	1.8	4.1	1.1
16:0	12.2	27.5	29.1
16:1	8.3	10.7	5.4
18:0	2.7	4.4	9.1
18:1	20.5	35.4	16.8
18:2	36.6	8.5	20.6
20:4	6.3	1.9	8.2

Minor lipids of the atheromatous plaque

The presence of low levels of other lipids in atheromatous vessels is well recognised. These are listed in chronological order in Table 6 below. It should be emphasised that only tentative identifications were made in several instances and the possibility of gross artefact formation has to be considered in others. The significance of these findings will be discussed later in the appropriate section.

Table 6 MINOR LIPIDS OF THE HUMAN ATHEROMATOUS AORTA

DATE	COMPOUND	REFERENCE
1930	5α- cholestane - 3β- ol	Schoenheimer (54)
1932	3β- hydroxycholest-5-en-7-one	Schoenheimer (55)
1942	5α- cholestane-3β- ol	McArthur (56)
1943	cholesta-3,5-dien-7-one	Hardegger, Ruzicka and Tagmann (57)
	5α- cholestane-3β,5,6β-triol	
	cholest-5-ene-3β,7β-diol	
	batyl alcohol	
1954	5α- cholestane-3β,5,6β-triol	Henderson and MacDougall (58)
	cholest-5-ene-3β,7β-diol	
1955	cholesta-3,5-dien-7-one	Kantiengar and Morton (59) Robertson (60)

DATE	COMPOUND	
1956	cholest - 5 - ene- 3β, 7α- diol	Henderson (61)
	cholest - 5 - ene- 3β, 7β - diol	
	5α- cholestane - 3β, 5, 6β -triol	
	cholest - 5 - ene- 3β, 24 or 25 - diol	
1962	1, 3, 5 trioxanes	Tuna, Mangold, Kammerck and Louden (62)
	glyceryl ether diesters	
	aldehydogenic triglycerides	
1963	5α- cholestane - 3β- ol	Mosbach, Blum, Arroyo and Milch (63)
1964	5α- cholestane - 3β- ol	Kuroda, Werbin and Chaikoff (64)

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SECTION B

RECENT DEVELOPMENTS IN ANALYTICAL TECHNIQUES FOR ORGANIC MATERIALS

B. SOME RECENT DEVELOPMENTS IN ANALYTICAL TECHNIQUES FOR ORGANIC MATERIALS

Introduction

The ability to separate and identify the individual components of a complex mixture is an essential preliminary in the solution of many biological problems. For milligram quantities of pure compounds the choice of methods of characterisation is large and includes such widely used methods as elemental analysis, infra-red spectroscopy and nuclear magnetic resonance spectroscopy. Such methods are of limited use for microgram quantities of materials, especially when these require isolation from complex mixtures. Such is the case in respect of the minor components of the human atheromatous plaque. Hitherto the only comprehensive study on the subject has been that of Hardegger et al.(1) in 1943. These investigators did not have the advantage of the recently developed techniques (mainly chromatographic) for microanalytical separation and characterisation. Accordingly they employed pooled tissue from almost four hundred aortas, collected over a period of two years. Using this type of approach they were able to obtain a quantity of several pure compounds sufficient to allow characterisation by classical procedures based on elemental analysis, physical constants and correlation with authentic samples. This investigation provided important information on the general

chemical character of the lipid constituents of aortas (including lesions), but of course gave no information concerning individual aortas. The necessary length of the study also introduced the possibility of an extensive degree of artefact formation (50). In our work we have aimed to avoid such an approach, and using the now available microchromatographic techniques, we have been able to limit each analytical investigation to the tissue from one aorta, extracted within twenty-four hours post mortem.

Chromatographic techniques

The term chromatography denotes a procedure in which a solution of substances to be separated is passed over a finely divided solid or liquid phase resulting in retention of the individual components to different extents. Chromatographic methods can be broadly divided into three basic types namely:

(1) Adsorption (or liquid/solid chromatography), in which the moving substances are bound by reversible physical surface forces to the adsorbent.

(2) Partition chromatography, in which the compounds to be separated are partitioned between two liquid phases (or a gas and a liquid in the case of gas-liquid chromatography).

(3) Ion-exchange chromatography in which the rate of migration of a compound is mainly determined by the pH-dependent total charge.

In practice it is very difficult to decide which of these processes is actually taking place at a particular surface. Most practical procedures involve several contributory effects with one or other of the types predominating. It is unusual to achieve a "pure" partition process: reversed phase chromatography, for example, gives a fairly close approach to this ideal case, but on the other hand, some adsorption is associated with many practical partition techniques.

Chromatographic techniques represent a vast improvement over the classical procedures of separation, among which were:

(1) solvent extraction,

(2) crystallisation,

(3) distillation (plus sublimation).

Both the classical techniques and the newer separation methods provide physical constants useful in characterisation.

Early chromatographic methods

The earliest form of chromatography, adsorption chromatography, was discovered in 1903 by Tswett (2), but it was not until 1931 (3, 4) that it was recognised as a useful practical method when it enabled the separation of α- and β-carotene. In the next few years, the use of column adsorption chromatography, mainly on alumina, greatly aided the investigation of many other natural pigments. Colourless compounds were also separated using an elution technique: the various fractions obtained were

then weighed. In these early days this technique was also widely employed in the steroid field. For instance alumina was used in the first isolation of androsterone and etiocholanolone from urine (51) and in the work of Dobriner and his group (52, 53) on urinary steroids and steroid metabolism in man. Reichstein and his school (6,7,8) separated mixtures of adrenal steroids, sterols and bile acids. Where possible the separated compounds were subsequently characterised by melting point or other suitable data. Hardegger et al. (1) also used this type of chromatography extensively in their separation and identification of the minor sterols of the human atherosclerotic aorta. There has been some suggestion that alumina can give so-called "alkaline reactions (5)". Reichstein and Shoppee (6) found that on alkaline alumina, 17-hydroxy-20-keto steroids undergo molecular rearrangement during chromatography to give D-homo ketones, but this can be largely avoided by the use of neutralised alumina of low activity and completely avoided by the use of 17-acetates.

Partition chromatography

The type of chromatography described above, although suitable for lipids of low polarity, is of limited use for the separation of the highly polar lipids and other hydrophilic compounds such as proteins, polysaccharides and nucleic acids. These types of compound are best separated by some form of liquid/liquid partition chromatography. This technique was

introduced in 1941 by Martin and Synge (9) who used silica gel columns containing definite quantities of water to separate amino acid mixtures; the compounds being separated were partitioned between this aqueous "stationary phase" and an organic (11) "mobile phase".

Silicic acid column chromatography

The role of water in the chromatographic behaviour of silicic acid[x] was subjected to a study by Kay and Trueblood (10) who concluded that depending on the amount of water held, silicic acid may act as an adsorption or a partition support; usually both properties contribute to varying degrees to the separation effects obtained. Silicic acid column chromatography was introduced for lipid separations in 1940 by Trappe (5), and since then it has developed into the most widely used method for the initial class separations required in most lipid investigations. Fillerup and Mead (11) used a column of this material to separate blood lipids into their major classes, while Hirsch and Ahrens (12) and Horning et al. (13) have used the method very successfully on tissue extracts. The degree of resolution and reproducibility obtained with a silicic acid column is dependent on the regulation of the moisture content and on the use of carefully washed and graded silicic acid, and such a standardisation procedure has been described in detail by Horning et al. (13) in their work on tissue lipids.

[x] The terms silica gel and silicic acid refer to hydrated silica precipitates, the properties of which can vary according to the method of precipitation and purification.

Recent developments in detection of compounds in column effluents.

Column chromatography in its conventional form as described above is not very suitable for the separation of microgram quantities, and other forms such as thin-layer, paper and gas chromatography have been used for this purpose.

Since the development of highly sensitive detectors for gas chromatography, interest has been aroused to develop such a detection system for liquid chromatography. A method employing a hydrogen flame ionization detector was developed by Karmen, Walker and Bowman (14) in 1963 and one involving an argon ionisation detector by James (55). In principle a continuously moving chain carries a minute part of the column effluent first through a heated solvent evaporation tunnel and then into one or other of the very sensitive detectors mentioned above. Recently several commercial instruments using this principle have been developed.

Paper Chromatography

In 1944 Consden, Gordon and Martin (16) demonstrated that filter paper strips could also be used as a support for a stationary phase in partition chromatography. This technique, "paper chromatography" was developed to fill the need for a microanalytical type of chromatography. In addition to an analytical separation, this method also provides physical constants extremely useful in characterisation. Paper chromatography in its original form was ideally suited for polar water

soluble compounds such as amino acids, sugars and proteins, but unsuitable for lipophilic compounds such as sterols and steroids. Attempts to use more hydrophilic derivatives of these compounds such as the Girard hydrazones of ketosteroids have been largely unsuccessful (54) because of the overwhelming influence of the polar moiety. Paper chromatography of sterols and steroids was made possible by the introduction of special solvent systems by Zaffaroni *et al.* (17) and by Bush (18). The Zaffaroni type of paper chromatography involves impregnation of the paper with a non-volatile organic stationary phase, for example phenyl cellosolve (ethylene glycol monophenyl ether). The sample is then loaded on to this treated paper and the chromatogram developed with a volatile organic solvent such as hexane. Using this system Zaffaroni and Burton (19) were able to separate adrenal steroids from gland extracts and Burton *et al.* (20) the steroids in human urine.

The other widely applied form of paper chromatography involves the use of volatile solvents for both phases (Bush-type system). In this the paper is hung in a chamber containing both phases and allowed to equilibrate. The paper preferentially adsorbs the polar phase and the chromatogram is developed with the weakly polar mobile phase. A typical system involves aqueous ethanol as the stationary phase and petroleum as the mobile phase. Bush (21) used this system in the analysis of adrenal extracts.

It has also been successfully applied to blood steroids (22), to bile acids and esters (23) and to bile salts (24).

Accurate quantitative estimations have been obtained from paper chromatography using densitometry, and greater sensitivity is possible through the use of radioactive tracers and counting devices.

Relationship between structure and paper chromatographic mobility

The physical constant normally recorded in paper chromatography is the R_F value. This is the ratio of the distance moved by a given substance from the origin to the distance moved by the solvent front from the origin. Another extremely useful constant, the R_M value, where

$$R_M = \log (^1/_{R_F} - 1),$$

has been proposed by Bate-Smith and Westall (56). This function has additive properties. These additive factors are expressed as ΔR_M values which can be calculated from the difference between the R_M value for the parent substance (the skeleton) and a derivative, or between derivative A and derivative B, and also between the same substance in two different solvents. Using R_M values, the R_F values of simple compounds were successfully calculated (26, 27). Initial calculations with more complicated molecules such as steroids

suggested that they could not be treated in this way, but when it was realised that the effect of a substituent on the R_M value must be calculated for each position, the ΔR_M values obtained were remarkably constant. Difficulties only arise when interactions between substituents, such as vicinal effects, occur. Correction factors must be applied in these cases.

Paper Chromatography of lipids

Most successful applications of this technique in the lipid field have employed impregnated paper. The use of untreated papers has been largely unsuccessful (25), and this is especially true in the case of fatty acids where chromatography has been complicated by dimerisation caused by hydrogen bonding, by adsorption to the paper, and by incomplete dissociation in neutral or weakly acid systems. Separations have, however, been obtained on reversed phase papers impregnated with such substances as undecane (26) and kerosene (27). For lipids other than fatty acids, greatest progress has been made with the use of silicic acid - impregnated filter paper and silicate - impregnated glass paper. It was soon shown that the separations on silicic acid columns obtained for blood lipids by Borgström (28) and by Fillerup and Mead (11) could be duplicated and even improved by the use of paper. Silicic acid filter paper was used extensively by Rouser et al. (25) and by Marinetti (29) for separating both neutral lipids and phospholipids.

Glass filter paper impregnated with potassium silicate or silica gel has proved extremely useful where the use of a corrosive charring reagent is desired. Such papers are extremely sensitive and as little as 0.2 µg. of a compound may be detected. Muldrey et al. (30) have produced excellent separations of phospholipids using a solvent system including pyridine. Impregnated glass paper resembles thin-layer chromatoplates very closely and it has been shown by Hamilton (31) that solvent systems developed for the separation of bile acids can be transferred to use with thin-layer plates with equally good results.

Ion Exchange papers

This type of paper is useful for the separation of highly polar lipids such as sulpholipids. Its very low capacity can be compensated by the use of radioactive compounds and under these conditions some extremely useful separations have been obtained (32).

Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) emerged around the same time as paper chromatography to fulfil the need for a micro-analytical technique. However, owing to initial problems, such as mechanical instability of the layers, paper chromatography was the first to be successfully developed.

TLC, although described (33) as early as 1938, was not recognised as a useful general procedure until about 1956. Examination of recent literature on chromatography indicates that thin-layer chromatography is in more widespread use than paper chromatography. There are, however, certain specialised areas of paper chromatography in which its power has not yet been equalled by TLC.

Thin-layer chromatography was first described by Izmailov and Shraiber (33) in 1938. These workers dusted alumina on to glass plates and separated various substances on these loose layers. Meinhard and Hall (34) were first to employ a binding agent (starch) to give the layers mechanical stability. Kirchner et al. (35) developed the procedure further and demonstrated its applicability to the separation and identification of terpenes. Although a few compounds other than terpenes were separated at this time, the general scope of the method was not fully recognised. It was first introduced as a general procedure for analytical chromatography by Stahl (36) who in 1956 introduced a calcium sulphate binding agent which is still in use today. He described separations on layers of about 250 μ thick of a special adsorbent "Kieselgel G" consisting of silica gel ($>$ 200 mesh) with a calcium sulphate binder.

Although this type of chromatography had been widely used in different fields of research, it was not until 1959 that

Mangold (37) adapted it for the analysis of lipids. Using a petroleum ether and diethyl ether mixture he fractionated lipids on a silica gel plate, in the same way that Hirsch and Ahrens (12) had carried out fractionation of lipids on a silica gel column. Mangold (38,39) drew up general rules on the application of thin-layer chromatography to lipid analysis. The TLC methods used for lipid analysis have also been discussed more recently by Bobbitt (40) and by Padley (41), and are reviewed by Mangold (42) and Kirchner (80).

Operating characteristics of TLC

One advantage of TLC over the use of a column is that it more readily supplies physical constants useful in characterisation. In the lipid field it allows separation of lipids from extraneous materials, and permits separation of the lipids into classes of chemically similar structure as well as the subfractionation of these individual classes. A particular advantage of this method over column chromatography is that it is a technique of limited development, as distinct from elution which is usually employed with a column, and thus gives easier perception of the progress of the analytical separation. TLC is also useful in that it will provide a tentative identification through direct comparison with reference compounds in terms of mobility and colour reactions with

spray reagents. Quantitative estimations may also be obtained either by direct densitometry or by measurement of radio-activity.

Separating Characteristics of TLC

In TLC, separation takes place in basically the same order as on a column of corresponding type. According to the investigations of Brockmann and Volpers (43), hydrocarbons are adsorbed very little if at all. The adsorption of unsaturated hydrocarbons increases according to the number of double bonds they contain. If functional groups are added to the hydro-carbons, their affinity for adsorption is increased in the following order:-

O-alkyl; C = O; NH_2; OH; CO_2H

In all of these groups, short-chain compounds are more strongly adsorbed than are long-chain ones, and unsaturated more strongly than saturated.

Types of layer

Silica gel is the most widely used layer in thin-layer chromatography, probably because in the early days of TLC this was the only commercially available material. It is normally supplied containing about 10% of calcium sulphate as a binding agent but if desired it can be obtained free from this substance.

Silica gel GF_{254} (E. Merck, AG, Darmstadt, Germany) is specially useful for very small amounts of material as it contains a compound which causes substances which absorb ultra-violet light to be seen under a u.v. lamp without prior spraying.

Neutral alumina (44) has approximately the same separating characteristics as silica gel and has been used to separate such compounds as terpenes, alkaloids, steroids and aromatic and aliphatic compounds. Because of its later availability, it is not so widely used as silica gel.

Other materials such as kieselguhr, hydroxyapatite, cellulose, and Sephadex are available and are especially useful in the chromatography of polar compounds such as amino acids, proteins and nucleic acid derivatives.

Special applications of TLC involving modified layers

Acidic compounds are especially well separated on layers prepared using 0.5N oxalic acid. Since untreated silica gel is acidic, basic compounds are not transported owing to salt formation with the layers. This problem can be overcome by the use of alkaline (45) or buffered (56) silica gel layers, and these have been applied, for instance, to alkaloids.

Remarkable separations of compounds with very small differences in structure or stereochemistry can be achieved by TLC. However, natural lipid mixtures are frequently

groups of compounds differing only in chain-length, degree of unsaturation or in stereochemistry of the component fatty acids or alcohols. The composition of each lipid, with respect to these compounds, can generally be readily determined by gas-liquid chromatography (GLC) after trans-esterification or hydrolysis. However, for many biochemical studies it would be more useful to be able to fractionate each lipid class into its individual components, or at least, into simpler and more closely defined mixtures.

Chromatography on thin layers of silica gel impregnated with silver nitrate makes it possible to separate compounds on the basis of the number, type and position of the double bonds. The silver ions in the adsorbent form complexes with the double bonds in the unsaturated compound giving separations which were not previously possible. The use of silver ion chromatography in the lipid field has been reviewed in a recent paper by Morris (47).

Other useful methods for the study of unsaturated compounds in the presence of similar saturated compounds include bromination and hydrogenation of the compounds actually on the plate before development (48).

Preparative TLC

Up to this point we have referred to thin-layer chromatography as used in a purely analytical manner, but it is also frequently employed to achieve preparative separations which would not be possible or as convenient by the use of a column. Normal analytical plates have layers of adsorbent of approximately 0.25 mm. thickness and are "spotted" with mixtures of the order of 20 - 40 µg of material. Using layers of between 1-2 mm. thick on a 20 x 20 cm. plate quantities as high as ten milligrams of material have been separated on one plate and collected. One of the big problems in using preparative TLC is that the plates cannot for obvious reasons be sprayed with the destructive oxidising reagents frequently used in analytical TLC, and other methods of visualising the separated bands have to be devised. For compounds which are visible under ultra-violet light there is no problem, but for others, non-destructive methods such as brief exposure to iodine vapour, or the incorporation of an easily removable dye (such as Rhodamine 6 G) have to be employed.

The TLC of trimethylsilyl ethers

The usefulness of trimethylsilyl (TMSi) ethers in thin-layer chromatography is not widely appreciated probably because of the earlier misconception that derivatives of this type undergo rapid hydrolysis even under the mildest conditions. While this

may be true to some extent of TMSi ethers of phenols and oximes, these formed from alcoholic hydroxyl groups are fairly stable at neutral pH even in aqueous media. The technique was first applied to the purification of TMSi ethers from extraneous materials for GLC purposes (74) and successive groups of workers have used the extremely low polarities of these derivatives to improve recoveries of polyhydroxy compounds in preparative TLC. Until recently, the quantitative elution of steroids containing more than one hydroxyl group has proved difficult (75), however, using TMSi ethers, Brooks and Watson (76) have demonstrated recoveries of 90 per cent or better of radioactively labelled steroids containing one to four hydroxyl groups at the 10 ng. level. Quantities as low as 1 ng. (76) or as large as 80 mg. (77) have been successfully recovered without appreciable losses due to adsorption or hydrolysis.

TLC of TMSi ethers has also proved particularly effective in group separations (78, 79) such as the isolation of purely hydroxylic steroids from those possessing ketonic groups. In our work we shall demonstrate that it can also be adapted to the separation of individual steroids and that it has proved especially useful in resolving epimeric pairs.

Gas-liquid chromatography (GLC)

Gas-liquid chromatography was introduced by James and Martin (57) in 1952 and is today probably the most important form of partition chromatography. Like most other chromatographic techniques it had its origins in the field of biochemistry, although a large part of its early development was directed towards its use in petroleum chemistry.

In GLC the volatilised sample is partitioned between a liquid stationary phase, held on an inert solid surface, and a mobile gas phase. The compounds are separated from one another in a flowing gas phase, are detected as individuals *in situ*, but are not normally recovered in the pure state, unless there is some specific motive for doing so. The use of this technique in the biological field was greatly enhanced by the introduction of highly sensitive detectors and by the availability of column packings coated with this films of thermostable liquid phases, enabling the analysis of small quantities of highly complex mixtures.

Stationary phases

In "packed-column" chromatography, the stationary phase is a non-volatile gum coated on an inert solid support, whereas in "capillary" or "open-tube" chromatography, the inner surface of the tube is coated with the stationary phase and no packing is employed. The liquid phases currently in use fall roughly

into two categories, non-selective and selective. Non-selective phases separate compounds according to their molecular weight and shape and are only slightly affected by the type of functional group present in the molecule. Such phases are normally high boiling petroleum greases, or siloxane polymers with methyl substituents. On the other hand, selective stationary phases show characteristic behaviour which may often be related to specific functional groups, and are usually polymers of the polyester type or methyl siloxane polymers with additional polar groupings such as fluorine or cyano-ethyl. An infinite variety of special separations of an intermediary nature can be obtained by careful blending of selective and non-selective phases in definite proportions.

Columns

The columns used by the early workers in this field (58) contained packings with a high percentage of liquid phase, sometimes as great as 40%. This, although not necessarily leading to low efficiencies gave very long retention times (i.e. the time taken for a sample to emerge after addition to a column) for compounds of high molecular weight such as steroids. The introduction of packings with 1 - 3% of stationary phase

greatly increased the applicability of the method by reducing retention times of high molecular weight compounds. It also helped to avoid column "bleed" when the highly sensitive detectors were employed.

The efficiency of a column is measured in "theoretical plates" and is calculated from the following formula:

$$n = 16 \left(\frac{R_T}{W}\right)^2$$

where R_T is the retention time and W is the width of the base of a peak. The first columns with a low percentage of stationary phase were about 4 ft. long and had efficiencies in the region of 2000 - 4000 theoretical plates. With the development of the theory of gas chromatography it has been possible to increase the resolving power of columns giving efficiencies up to about 40,000 theoretical plates by increasing the length of the column to between 50 and 100 ft. and decreasing the diameter to about 2 mm. High efficiency columns of this type were first developed by Scott (59).

Detectors

The types of high efficiency column described above would be ineffective if high efficiency detectors were not available to be used in conjunction with them. The first type of high sensitivity detector, the argon ionisation detector, was

developed in 1960 by Lovelock (60). Since then several other high sensitivity detectors, notably the flame ionisation and the electron capture detector have been developed. These three detectors are based on an ionisation principle and each has its own special advantage in use. The argon ionisation detector is slightly more sensitive than the flame ionisation detector permitting the detection of concentrations of solutes in carrier gas down to about 10^{-13} g/ml: on the other hand it is extremely sensitive to impurities such as oxygen and water vapour in the carrier gas which reduce its sensitivity for the detection of organic compounds. The flame ionisation detector on the other hand does not have this disadvantage and is probably more universally useful. The third type of detector, the electron capture type, is rather specialised in its application, since it detects only substances which contain atoms such as oxygen, nitrogen or halogen which capture electrons. It is the most sensitive detector of the three.

Operating techniques

A sample may be introduced into a chromatograph either as a liquid solution or/as a solid, the primary requirement being that it is vaporised as near instantaneously as possible. Syringes are available to deliver from 0.1μl. to 250μl. into the instrument, the normal injection size being between 0.2μl.

and 1µl., since larger quantities of solution produce large "solvent peaks" which can completely obscure early peaks as well as reducing the sensitivity of the detector.

The major disadvantage of liquid injection is that only a portion of the total sample is utilized. There are many instances especially in quantitative uses in biochemical systems where it is desirable to introduce the total sample. "Solid injection" has provided a means whereby an entire sample can be deposited on a wire gauze, the solvent evaporated, and the solid introduced onto the top of a chromatography column. One of the best such systems was devised by Menini and Norymberski (61). This technique is limited to samples of low volatility.

Derivative formation prior to chromatography

The amount of information gained by gas chromatography can be greatly increased by the use of suitable derivatives. There are two requirements that have to be fulfilled for such a derivative to be of use in GLC. The reaction used in formation of the derivative should be essentially quantitative, and the compound thus formed should have a retention time markedly different from the parent compound.

There are several reasons why derivative formation may be desirable in this type of chromatography. Firstly, in many instances the preparation of suitable derivatives will increase

the separation of the components of a mixture as compared with the parent compounds. Secondly, for highly polar compounds as sugars, amino acids and fatty acids, derivative preparation is absolutely essential to reduce the polarity of the parent compounds, providing substances suitable for chromatography. Thirdly, derivatives which lower the polarity of a compound, as do most of the derivatives currently used in GLC, are extremely useful for quantification purposes as they greatly reduce adverse adsorption effects. Most of the useful derivatives also increase volatility and thus aid not only chromatography but also preliminary purification, e.g. by sublimation.

The types of derivatives in general use at the present time are listed below:

FUNCTIONAL GROUP	DERIVATIVE
Hydroxyl group	Acetate, propionate trifluoroacetate trimethylsilyl ether methyl ether
Carbonyl group	N,N Dimethylhydrazone O-Methyl oxime
Carboxylic acid group	Methyl ester
Amino group	Acetyl derivative

Application of GLC

The value normally measured in qualitative GLC is the retention time, along with which the packing material being used, the temperature, the gas flow rate, and the column dimensions also have to be recorded. Because several of these factors are variable, the retention time has to be related to that of a reference compound run under identical conditions in order to obtain standardised data. Hence, a frequently calculated value is the relative retention time

$$\text{relative retention time} = \frac{t_{comp.}}{t_{ref.}}$$

$t_{comp.}$ = retention time of compound

$t_{ref.}$ = retention time of reference compound.

For steroids of the cholestane series, the reference compound normally used is the hydrocarbon 5α-cholestane. However, a superior method of compensation for temperature and gas pressure variability is available and this method of standardisation is called the "Retention Index" system. In this system the point at which a substance is eluted is measured on a scale supplied by the actual gas chromatogram itself. The fixed points are obtained by plotting the logarithm of retention time against the carbon number of the members of a homologous series of n-alkanes. Such a method gives a reasonably universal system of standards. It is also extremely useful for temperature

programmed gas chromatography where the use of a single reference compound would be inappropriate.

GLC has been used in almost every field of organic chemistry where substances suitable for chromatography are obtained, but probably its most extensive application has been in biological research. This is a field where the analysis of minute amounts of highly complex mixtures is essential to the solution of many problems. Probably the most frequent application in this context has been to natural steroids in the fields of biology and medicine. For example Sjövall (62) has made remarkable progress in the field of human bile acids by examining them by GLC as their methyl esters. One of the best examples of the quantitative use of this technique has been in the field of the steroid hormones where it has been extensively used for example in the estimation of urinary pregnanediol (63) and pregnanetriol (64).

Sugars may also be examined by GLC after conversion to volatile derivatives such as trimethylsilyl ethers, acetates or methyl ethers. The usefulness of GLC techniques in the analysis of carbohydrates from natural sources has been illustrated in the work of Aspinall and co-workers (65).

The semiquantitative application of gas chromatography is frequently demonstrated in lipid investigations, where it has been used to estimate the relative percentages of the various

fatty acids in sterol ester, triglyceride and phospholipid fractions (66).

Additional methods used in conjunction with GLC.

Although gas chromatography can provide substantial evidence for the characterisation of an unknown compound, complete identification by this method alone is seldom feasible. Elution of more than one component at the same time is common, and therefore it is advisable to use more than one column in any identification or better to employ some ancillary non-chromatographic method of characterisation.

Before any of these additional techniques may be used, the separated compounds must be trapped in some manner. The materials may either be led directly from the gas chromatography into a second analytical apparatus or they may be collected separately for subsequent analysis. Where possible the direct method is preferable as condensation of samples produces several technical problems.

Where compounds containing conjugated double bonds are being examined, ultra-violet spectroscopy is a very suitable method for use in conjunction with GLC. The sample size of 10^{-4} to 10^{-6}g. needed for an examination makes it approximately of the correct order of magnitude for use with this form of chromatography. Ultra-violet spectroscopy is of little use for compounds which do not exhibit conjugation.

Infra-red spectroscopy is however of more general use and effective techniques (67) have been developed for trapping GLC fractions. A slightly larger sample of 10^{-5} to 10^{-4} g. is necessary for adequate characterisation by this method.

Nuclear magnetic resonance (N.M.R.) has also been used in conjunction with GLC and a direct trapping cell has been described by Brane (68). A modern high resolution instrument used in conjunction with a computer can produce a spectrum from as little as 10^{-3} g. which will give considerable structural information.

The technique which is most nearly equivalent in sensitivity to GLC is mass spectrometry. This technique has the added advantage that it is also a vapour phase method and in view of its extensive use in our work it will be more fully discussed separately.

Gas chromatography - mass spectrometry (GC-MS)

Although the chromatographic methods outlined so far afford important evidence for the identification of an unknown compound, complete identification by the physical constants derived in this way alone is seldom feasible. Ideally it would be desirable to apply in conjunction some non-chromatographic method of characterisation to strengthen the certainty of each structural assignment. Where the available sample is small, few techniques are as useful as mass spectrometry in providing detailed

structural information. Its application in the study of a large number of lipid materials has been demonstrated in the work of Ryhage and Stenhagen (69). This method may be applied to less than one microgram of a pure compound, but on the other hand is of limited value in the study of mixtures.

As well as being an extremely powerful separation technique, GLC is also useful in characterisation. Therefore, a combination of gas chromatography and mass spectrometry, if possible, would represent a technique of immense use in biological studies. The individual procedures possess two important similarities which make such a combination technically feasible, namely that both are performed in the vapour phase and both are concerned with microgram quantities. There are two ways in which such a combination can be brought about: either the separated samples are individually condensed from the gas chromatographic effluent for subsequent mass spectrometry, or they are allowed to flow directly into the mass spectrometer while still in the vapour phase. The latter method is the more desirable since the collection procedure has several serious disadvantages:-

(1) There are usually considerable losses in condensing and transferring samples;

(2) the process is time consuming and requires considerable skill, especially when dealing with microgram quantities.

(3) the mass spectrum obtained usually represents the average composition of the chromatographic effluent over the time of collection which may be several minutes.

(4) There is a lack of confidence in the correspondence of a particular chromatographic peak to the sample condensed.

(5) Sometimes compounds which are perfectly stable in the inert carrier gas decompose when they are condensed in the atmosphere.

In contrast the direct technique is much faster and greatly reduces mechanical losses and the possibility of contamination. The possibility of decomposition is minimised, and the mass spectrum obtained represents the composition of the chromatographic effluent over a few seconds. This fact makes it possible to record several sequential mass spectra during the emergence of one chromatographic peak, and thus to detect impurities or unresolved mixtures.

Methods of combination

Improvements in the design of commercial mass spectrometers provided the fast scanning instruments necessary for such a combination. For packed column gas chromatographs provision has to be made for removal of the carrier gas, which would cause excessive pressures in the mass spectrometer resulting in peak broadening and lack of resolution. To remove the carrier gas

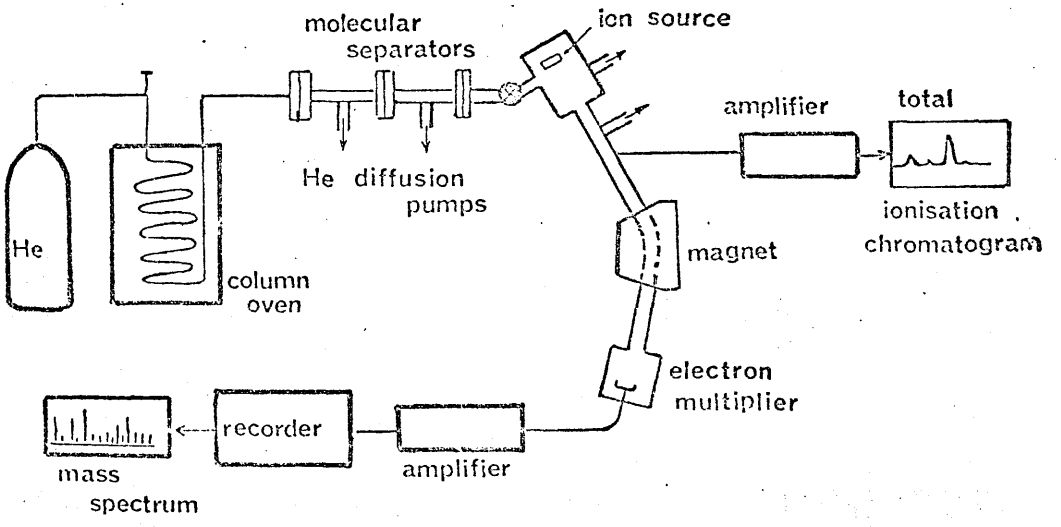
it is necessary to have some kind of a "molecular separator" between the gas chromatograph column outlet and the gas inlet of the mass spectrometer. Early combination instruments (70) were able to overcome the absence of such a device by the use of capillary gas chromatographic columns, which, by their very nature, have a very low gas flow and thus a high sample to carrier gas concentration. Such a system allows direct sampling of the GLC effluent into the mass spectrometer.

Recently an efficient molecular separator, for use with packed columns, has been developed in Sweden by Ryhage (71) and has been employed in a successful commercial instrument (see Fig.6, the LKB Model 9000 instrument). An alternative system of comparable efficiency has been developed in the U.S.A. by Watson and Biemann (62) and has also been used in commercial combination instruments.

Automation of GC-MS

A GC-MS instrument can handle complex mixtures extremely rapidly. Unfortunately, the injection of solutions requires the presence of an operator at frequent intervals and wastes almost half the operating time of the instrument, when it is out of action during the night. However, the development of automatic "solid injection" systems for commercial gas chromatographs will no doubt be extended to GC-MS instruments

Figure 6. Schematic diagram of a gas chromatograph - mass spectrometer



making 24-hour operation of such a machine possible.

The other handicap with a GC-MS instrument is that it produces mass spectral information much faster than it can be dealt with by one person. Analysis of mass spectral data is a tedious and time-consuming process. However, it is possible to deal with such information on a computer (73), and, perhaps an automatic injecting GC-MS instrument linked to a small computer will be a commercially available instrument in the near future.

Conclusion

The various techniques outlined here provide probably the most powerful method of separating and characterising microgram mixtures of unknown compounds, especially when they are used in conjunction with one another.

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SECTION C

THE ANALYSIS AND CHARACTERISATION OF STEROIDS

The General Problem

The analysis of steroids, especially those derived from natural sources, presents many problems in isolation, separation, characterisation and identification.

Steroids are usually relatively easy to isolate as a group, and in the free form they are usually extracted with a lipid solvent mixture such as chloroform-methanol (2:1 v/v). However, in aqueous media such as urine or bile, steroids exist largely in water soluble conjugated forms such as sulphates and glucuronides, or in the case of bile acids as glycine or taurine conjugates. These conjugates are relatively insoluble in lipid solvents and require a special extraction procedure frequently involving the use of such extraction mixtures as aqueous alcohol or ammonium sulphate/tertiary butanol, after which the free steroids are liberated by hydrolysis.

Many of the separation processes used in steroid chemistry depend on the chemical properties of the molecule. Such properties are essentially determined by the type and number of functional groups present. Chemical separations on the basis of functional groups are an invaluable preliminary to any investigation into steroid structure. These will generally separate one class of steroid from another (e.g. ketones from alcohols) and are usually based on differences in solubilities

of ionised and non-ionised compounds. For example, basic extraction will selectively remove acidic and phenolic compounds from a lipid extract by conversion of these compounds into their water soluble ionised sodium salts, while the neutral compounds remain unchanged in the lipid solvent. Similarly, by formation of a water soluble Girard hydrazone derivative, ketones may be preferentially removed from a lipid extract. The separation of individual members of a group is usually then accomplished by some form of preparative chromatography.

The second problem is the characterisation of the newly isolated steroid. Group separations, if employed, have solved part of this problem in that they have indicated the presence, though not the position, of a particular functional group. From this point, it is usual to assume regular nuclear structure, unless there is any evidence for any other viewpoint, and to attempt to correlate the unidentified steroid with known compounds.

The main features which have to be settled are the size of the skeleton, (since steroids may comprise eighteen to thirty carbon atoms), and the stereochemistry of the ring junctions and nuclear substituents. The most reliable, but the most tedious method for settling the stereochemistry of a steroid is a detailed X-ray analysis of the crystal structure (1). For a completely new type of steroid such an analysis may be necessary. Where

this is not possible, the stereochemistry may be assigned on the basis of chemical reactions and physical evidence.

Ring junctions B:C and C:D are trans in stereochemistry in almost all naturally occurring steroids, whereas ring junction A:B is trans in some steroids and cis in others. The spatial arrangement of nuclear substituents may also be decided from an X-ray study, but it is more usual to use chemical evidence such as reactions of known stereochemical consequence, or formation of new rings using the substituents. Among the physical techniques which may also be used to settle this problem are infrared spectroscopy and optical rotatory dispersion.

The classical approach

Early structural studies on steroids were conducted with cholesterol and the bile acid cholic acid, which were abundantly available and easy to isolate. The structures of these steroids were established mainly by exhaustive oxidative degradation. The next phase of steroid research was concerned with the isolation of the steroid hormones. These compounds differed from cholesterol and cholic acid in that they were only found in minute quantities in the body. Their isolation and identification illustrates the classical approach to the problem, and is well illustrated in the work on the hormones estrone (2) and androsterone (3). Both these steroids were isolated in small

quantities from enormous volumes of urine in which their presence had been indicated by biological assay. The pure compounds were only arrived at after extensive vacuum distillation, and repeated sublimation and crystallisation. The final product was characterised by its melting point and optical rotation. (A similar approach was used by Hardegger *et al.* (4) to the minor steroids of the human atheromatous aorta.)

After characterisation of the isolated steroids in this way, identity was established by calculation of an empirical formula from elemental analysis, followed by extensive degradation. The structures of the degradation products were established by synthesis.

The various classical identification procedures can be broadly divided into two groups:

(1) those giving contributory information, and

(2) those providing exclusive information.

Into the first category fall the determination of empirical formula and molecular formulae, the numerous methods of determination of type and number of functional groups and the determination of physical properties such as optical rotation and absorption spectra. Each of these methods contributes to an identification but rarely gives a solution exclusive of all other possible structures. In the second group the object is to show that the unknown compound is identical to a known compound.

Into this class fall the determination of melting point, both separately and mixed, and fingerprinting techniques such as infrared spectroscopy.

The present approach

At the present time we still use parts of the classical procedures, especially those which have been adapted for use on a small scale. Although, for instance, molecular rotation measurements cannot be used routinely on less than about 1 mg. of material they have largely been replaced by TLC and GLC parameters. Molecular rotation evidence, and especially rotatory dispersion, are however, still used in important identifications where a sufficient quantity of pure material can be amassed. Isolation of steroids is now usually done initially by column chromatography followed by preparative TLC for the subfractionation of this group.

Chromatographic mobilities in characterisation

In characterisation, much more use is now made of regularities in chromatographic mobilities. Much of the early work on the correlation of the chromatographic behaviour of steroids with their structure was done by Bush (5) using paper chromatography. He extensively applied the equation developed by Bate-Smith and Westall (6) for partition systems, namely

$$R_M = \log \left(\frac{1}{R_F} - 1\right).$$

Using this he was able to calculate ΔR_M values for the introduction of a specific functional group into a given position and orientation. He used the term ΔR_{MS} for the difference in R_M values of a steroid in two different solvent systems. Thus using the R_F value of an unknown steroid determined in several different solvent systems, Bush was able to identify many steroids.

A similar approach to R_M values was used by Lisboa (7,8) for TLC systems. He devised a method for the identification of microgram amounts of steroids using this technique. Unfortunately, when a steroid is chromatographed using a mixture of solvents on a thin-layer of silica gel it is subjected to an adsorption and a partition process. These differing effects interfere with the determination of ΔR_M values. Together with this, the irregular saturation of the upper regions of the chamber limit the use of these values in TLC. However, although accurate constants cannot be determined, approximate data are obtained which greatly aid any steroid characterisation. This is extremely important where the steroid is available in quantities which are insufficient to permit infrared spectroscopy. Lisboa (8) also used group colour reactions for the in situ characterisation of steroids after development. Using colour reactions, he was, for instance, able to differentiate between hydroxy and keto-steroids and even to suggest possible locations of the functional groups before undertaking a study of R_M values.

For gas-liquid chromatography, the first significant correlations between retention behaviour and structure of steroids were established by Clayton (9,10,11) for a series of sterol methyl ethers. He calculated "group retention factors" which expressed the relative change in retention time for the introduction of a particular substituent. He introduced the equation:

$$r = r_n \times K_a \times K_b \times \ldots.$$

in which the relative retention time of the steroid is approximated by the product of the retention of the parent nucleus (r_n) and the group retention factors (K_a, K_b etc.). This relationship only holds where there is no interaction of the substituents and its applicability for different types of compound was later illustrated by several other workers (12,13, 14,15).

Knights and Thomas (16) suggested that the logarithmic form of Clayton's equation was more convenient, i.e.

$$\log r = \log r_n + \log K_a + \log K_b + \ldots$$

Such values are especially useful because of their close relationship to the ΔR_M values calculated for thin-layer and paper chromatography. Using this approach these workers were, for instance, able to demonstrate the constancy of the contribution of the C-10 methyl group in a large number of steroids on QF-1. The substantial variations found in the value for

the 11β-hydroxyl group were attributed to neighbouring group interactions of this substituent with the angular methyl groups.

Brooks and Hanaineh (13) studied a much larger group of compounds than Knights and Thomas (16) and established the constancy of group retention factors for certain hydroxyl, acetoxyl and ketonic groupings and for acetylation in numerous examples. Unlike previous workers they also used standard deviations to demonstrate the validity of the experimental values.

Hartman and Wotiz (14) studied the GLC behaviour of a series of C-19 isomeric steroid pairs and calculated log r values for hydroxyl, trimethylsilyl and trifluoroacetate groupings. Using these results they were able to correlate GLC behaviour with the chemical nature of the liquid phase being used. These workers also noted that the retention time of the 5α-androstane nucleus was consistently greater than that of the 5β-isomer as had been found by earlier investigators (13,16,17).

VandenHeuvel and Horning (17) were also aware of the many regularities in the chromatographic behaviour of steroids. They introduced a new term, the steroid number (SN) for the elucidation of the structural features of the steroid nucleus. This term was derived from the work of Woodford and Van Gent on fatty acids (18) and in effect it employs the parent steroid nuclei as reference standards. Retention factors associated with particular functional groups were then expressed as characteristic contributions to the steroid number.

Mass spectrometry and steroid structure

Mass spectrometry is of great value in confirming structural assignments made on the basis of chromatographic mobilities. Its value is accentuated when it is used in direct combination with a gas chromatograph. A mass spectrum derived in this way may either be used as a "fingerprint" for comparison with that of a reference compound, or it may be analysed with the aim of relating observed fragments with fragmentation expected to occur on the basis of structural features.

Mass spectrometry was first applied to steroids by de Mayo and Reed (19) with particular reference to the determination of molecular weight. Since then many studies of the mass spectra of steroids have been carried out: for instance, Friedland *et al.* (20) discussed the principal fragmentation processes of a group of steroid alcohols, while Bergström *et al.* (21) emphasised its use in the determination of the molecular weights of bile acids. A more detailed discussion of the fragmentation pattern of steroids has been published by Ryhage and Stenhagen (22).

The fragmentation processes of the saturated skeleton have been detailed (19,20,33) in terms of the hydrocarbon cholestane. The ring system of the nucleus gives a very complicated fragmentation pattern in the mass range below about m/e 200: however, in the high mass range characteristic peaks appear which make the spectra useful for identification purposes. One of the

most common features of the spectra of C-17 substituted steroids is the elimination of the side chain plus 42 mass units derived from ring D (see Fig. 1). Such a fragmentation is represented by the wavy line in the above diagram. Another noteworthy feature of the spectra of steroidal hydrocarbons corresponds to the loss of the C-18 and C-19 angular methyl substituents. Thus in the mass spectrum of cholestane (M = 372), the base peak occurs at m/e 217 M - (C_8H_{17} - 42) and an (M-15) species is abundant. Also evident are ions arising from the loss of the side chain (i.e. M - C_8H_{17}) at m/e 259 and the side chain plus ring A at m/e 203.

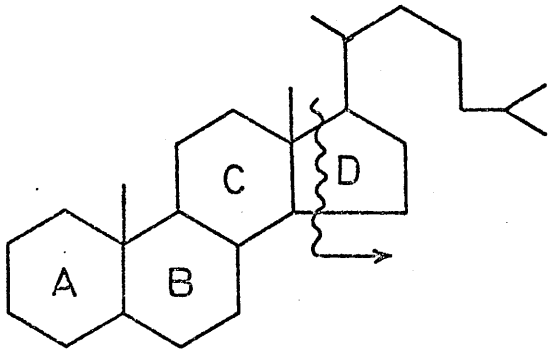
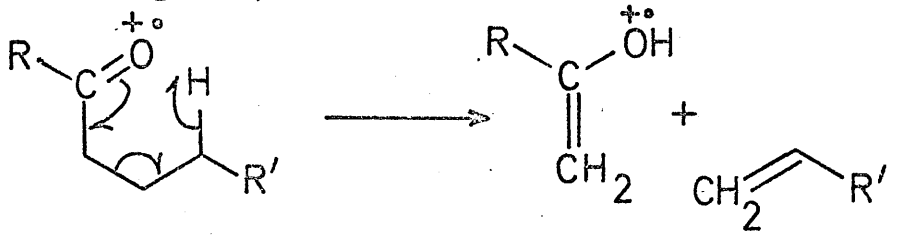


Fig. 1

Sterols tend to eliminate the elements of water in the mass spectrometer, an effect which is due partly to thermal processes and partly to electron impact. The resulting spectra closely resemble those of the corresponding olefins. Acetates and other esters undergo a similar elimination, but the process is less marked with ether derivatives. Satisfactory spectra of alcohols can, however, be obtained by their introduction into the mass spectrometer via a gas chromatograph inlet system, or better by conversion to trimethylsilyl ether derivatives (see below).

Unlike that of sterols, the fragmentation of steroidal ketones is frequently unpredictable and extremely complex. These compounds often undergo rearrangements during fragmentation. Such a process, an example of the more general "McLafferty" type of rearrangement, is illustrated below.

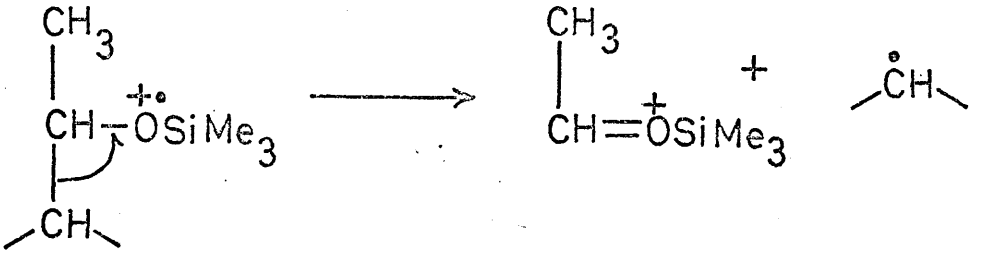


This mode of fragmentation involves fission of the $\beta - \gamma$ bond. The mass spectra of steroidal ketones have been extensively studied by Djerassi and co-workers (23,24,25).

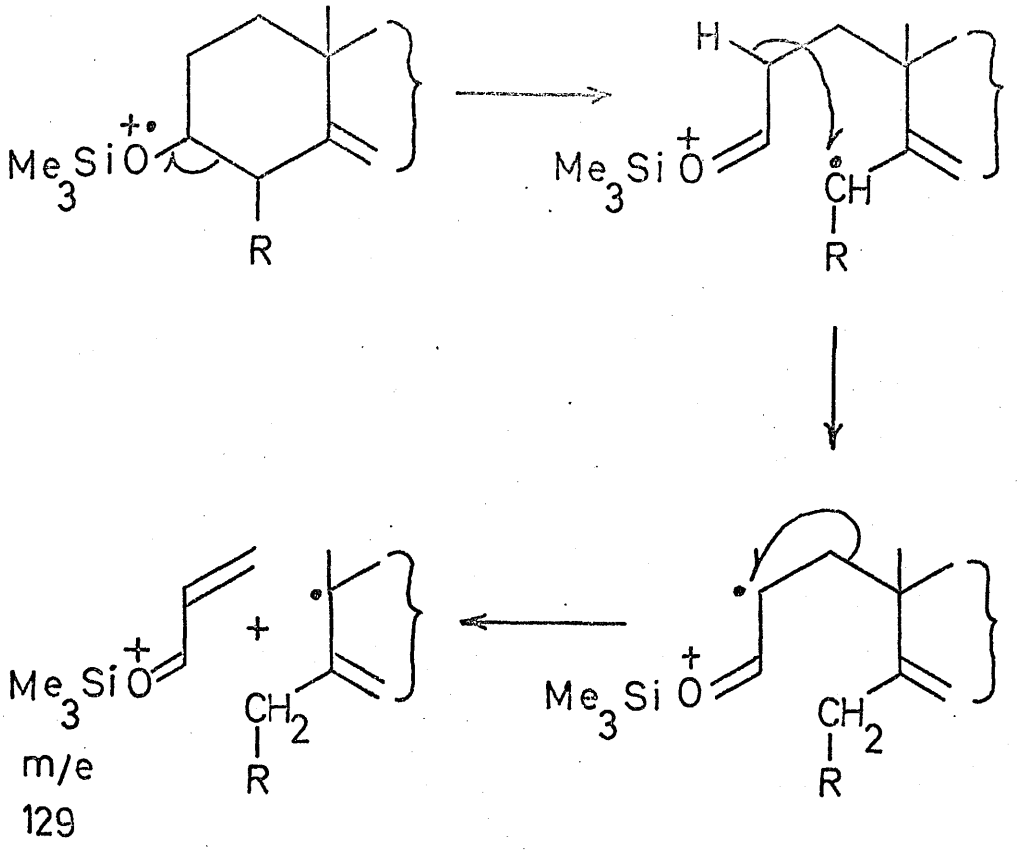
The success of mass spectrometry in the analysis of steroids has been largely due to the introduction of specific derivatives to protect labile functional groups and to promote a regular pattern of fragmentation. Frequently derivatives are chosen which also give good gas chromatographic characteristics and these are especially useful in the GC-MS combination instrument.

For sterols such a derivative is the trimethylsilyl (TMSi) ether derivative. These derivatives were introduced by Sharkey (26) in 1957 with the object of obtaining better molecular ions than were normally obtained from alcohols and their ester derivatives. Later these derivatives were shown by Luukkainen et al. (27) to have excellent gas chromatographic

separation properties, and since then they have become extensively used in this field. The mass spectra of TMSi ethers are characterised by losses of 90 mass units corresponding to trimethylsilanol $[(CH_3)_3SiOH]$ a feature easily recognised in such spectra. Perhaps the most valuable feature of TMSi ethers in mass spectrometry is their tendency to promote well-defined modes of fragmentation with the formation of rather stable ions of the trimethylsilyl oxonium type. For example, where a trimethylsilyl ether group occurs in the side chain, as in 24 and 25-hydroxycholesterol bis-TMSi ethers the following process is typical of the type of fragmentation which takes place.



One feature of the mass spectra of this type of derivative which has been especially useful in our own work is the extremely high abundance of the m/e 129 ion in the spectra of Δ^5 - 3β-trimethylsilyloxy steroids. This fragment has been established to consist of the TMSi ether grouping and carbon atoms 1, 2 and 3 of the steroid nucleus (31,28). A fragmentation of the following type has been suggested for the formation of this ion.



17-Trimethylsilyloxy steroids of the androstane and oestrane series also give an intense peak at m/e 129, but are readily distinguished from the Δ^5 - 3β-trimethylsilyloxy steroids which also give an intense peak at M-129 (31,32).

Derivatives are also extremely useful in promoting regular fragmentations of keto-steroids and for this purpose ethylene ketal (29) and O-methyloxime derivatives (30) are especially useful. The GLC behaviour of unsubstituted oxime derivatives is greatly improved by the formation of O-trimethylsilyl oximes (31). The spectra of these compounds are characterised

by the loss of 89 mass units due to elimination of the trimethylsilyloxy group.

Structural Identification

If completely new types of compound are encountered, structural identification still remains a major problem even with all the techniques described above. A possible approach in such a case is extensive characterisation and limitation of probable structures by TLC and GLC under various conditions followed by low resolution mass spectrometry of the compound and of suitable derivatives and chemical transformation products. The development of micro infrared and NMR spectroscopy has also proved extremely useful in giving structural information.

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SECTION D

EXPERIMENTAL METHODS

1. Solvents and Extracts

All solvents used in this work both in extraction and in chromatography were of analytical grade and were freshly distilled before use. Final organic extracts were dried over anhydrous sodium sulphate, except as otherwise stated.

2. Preparation of Tissue

Figure 7.

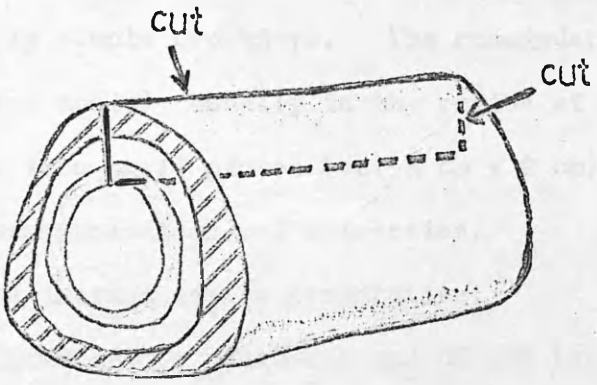
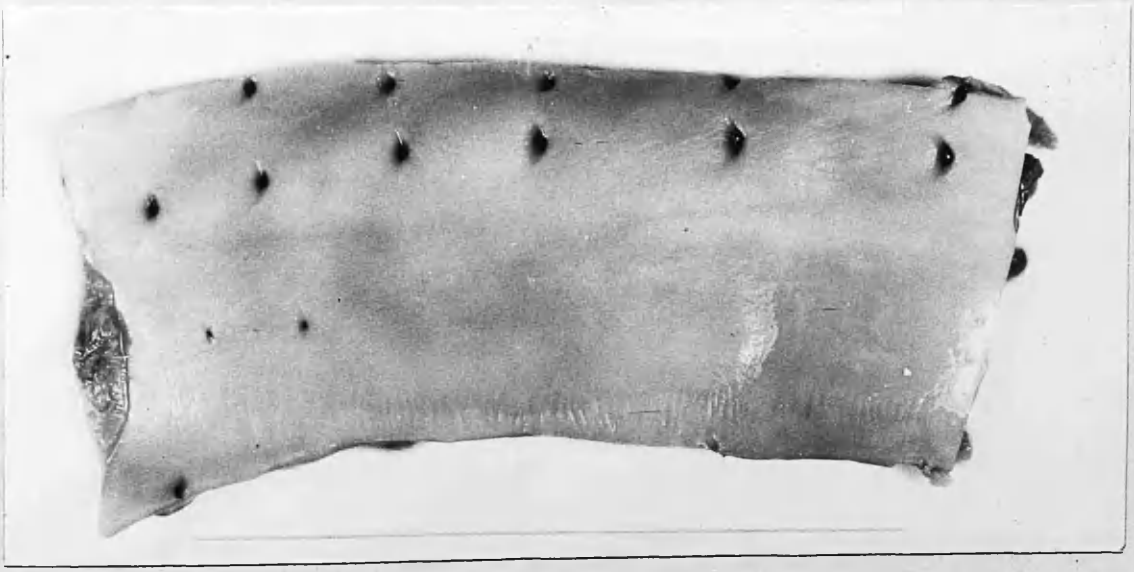


Figure 8.



(a) The initial method

All aortas were received open (see Figs. 7 and 8) within 24 hr. post mortem. They were washed in normal saline solution (150 milli-equivalents sodium chloride per litre) for a few minutes to remove adhering blood. The three layers (intima, media and adventitia) were clearly visible. The fatty outer layer (the adventitia) was peeled off lengthwise and discarded. The differing texture of the individual layers made this separation a fairly simple procedure. The remainder of the artery (intima plus media), usually in the region of 30-40 g. of tissue was cut into small pieces (ca. 2 cm x 2 cm) in preparation for homogenisation and extraction.

(b) Faults of the initial tissue preparation.

As was pointed out by Weinhouse and Hirsch (see page 17), when an intimal/medial preparation is used, undiseased tissue is included with pathological tissue. Hence lipid changes due to a normal ageing process are not distinguished from the changes caused by atherosclerosis.

(c) Improved tissue preparation.

The adventitia was peeled from the aortas in one piece as described above. The media, or central layer, was then removed in small strips across the aorta leaving the intima containing the plaques. The plaques were visible as bulges of yellow lipid material on an almost transparent background of relatively

undiseased intimal tissue. After removal, the plaques were cut into small pieces, exposing the yellow lipid material, and added to a conical flask containing the extracting solvent.

3. Extraction of Tissue Lipids

Many solvent extraction procedures have been developed for the removal of a "total lipid" fraction from disintegrated tissue and their effectiveness has been discussed in great detail by Sperry (1) and Entenman (2). The use of a mixture of chloroform and methanol is now however the generally preferred method. In the present work the lipids found in an extract made with this solvent system include hydrocarbons, cholesterol esters, cholesterol and other sterols, mono-, di- and triglycerides, wax esters, free fatty acids, most phospholipids, and fat - soluble vitamins and pigments.

(a) Initial extraction procedure

Homogenisation was carried out at room temperature in a "Paladin" mechanical liquidiser, with cutting blades driven from the bottom of the container, for five minutes at full speed with two separate 300 ml. portions of chloroform-methanol (2:1 v/v). The extracts were then filtered, combined, and washed with one fifth their total volume of distilled water. The phases were then allowed to separate overnight in a refrigerator at $4^{o}C$. The upper aqueous-methanolic layer, containing non-lipid material

was removed by suction according to the method of Folch et al.(3).

(b) Present procedure

The use of the mechanical homogeniser was abandoned, as it introduced impurities from the lubricants which were necessary on the spindle of the cutter. It was found that an equivalent or superior extraction could be obtained by manual comminution of the plaques and immersion of these tissue fragments in 600 ml. of chloroform-methanol (2:1 v/v) overnight (ca. 15 hr.) in a stoppered conical flask. To minimise the risk of artefact formation, the flask was filled with nitrogen before closure and stored at -20°C in the dark.

The extract was filtered and cautiously reduced to dryness on a rotary evaporator at 40°C. The lipids were then selectively re-extracted from the yellow semi-solid residue with 600 ml. of diethyl ether-hexane (1:1 v/v) and washed with 200 ml. of distilled water. The water washing removes water soluble non-lipid contaminants leaving the major lipid classes behind.[x] This procedure for the separation of non-lipid material seems preferable to the Folch (3) method (separation of the methanolic layer) since some of the interesting polar sterols are appreciably soluble in methanol and may be lost.

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[x] Some lipid containing compounds will be removed in the water wash, for example gangliosides and some cerebrosides. For a complete discussion of the extraction of these compounds, see Entenman (2).

Table 7. Some representative tissue weights

Total wet weight of aorta (g.)	Weight of plaque tissue (g.)	Weight of purified lipid extract (g.)
118.5	6.8	0.5
87.2	10.23	1.0
130.2	16.8	2.5
77.5	12.5	1.25
95.0	14.0	1.7
60.5	9.5	0.25
49.5	7.2	0.3

Two of the most severely diseased aortas encountered gave 3.8 and 4.5 g. of purified lipid material respectively, but these were quite unusual, and the tissue preparation probably contained some media as well as intima, since most of the plaques penetrated through the medial layer.

4. Column Chromatography

(a) Silicic Acid

Silicic acid of uniform chromatographic behaviour from batch to batch can only be obtained if its preparation is carried out in a meticulous way. The important variables are, particle size, metallic ions, pH and moisture content. The method of preparation used in our work was that described by Horning et al.(4)

which results in a material with a moisture content of 9 - 10 per cent.

Silicic acid chromatography was used to divide the total lipid extract into its major classes. It was carried out in a glass column of dimensions 60 cm. x 6 cm. (ID.) which was surrounded by a layer of aluminium foil to minimise exposure of the contents to light. The column was filled by the slow addition of a slurry of silicic acid in hexane and closed by a drying tube to prevent the entry of dust. Before loading with lipid, the column was washed with 500 ml. of hexane. An additional 200 ml. fraction of hexane was collected and examined for lipid impurities after evaporation to dryness.

The best separations on this type of column were obtained when a load of approximately 10 mg. of lipid per g. of silicic acid was used.[x] Higher loads, although giving adequate separations of some of the lipid classes, tended to cause elution of cholesterol esters together with the hydrocarbon fractions. Eluates were collected in long-neck round bottom flasks and the solvent was removed by a water pump vacuum with very gentle heating ($< 40^{\circ}C$) to prevent the formation of ice on the outside of the flask. The eluting solvents employed are shown in Table 8 and in Fig. 10. This illustrates the step-wise elution of a typical silicic acid

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[x] Horning et al. (4) recommend a load of 5 - 17 mg./g., whereas Wren (5) suggests 50 mg./g.

Table 8. Elution of a typical silicic acid column

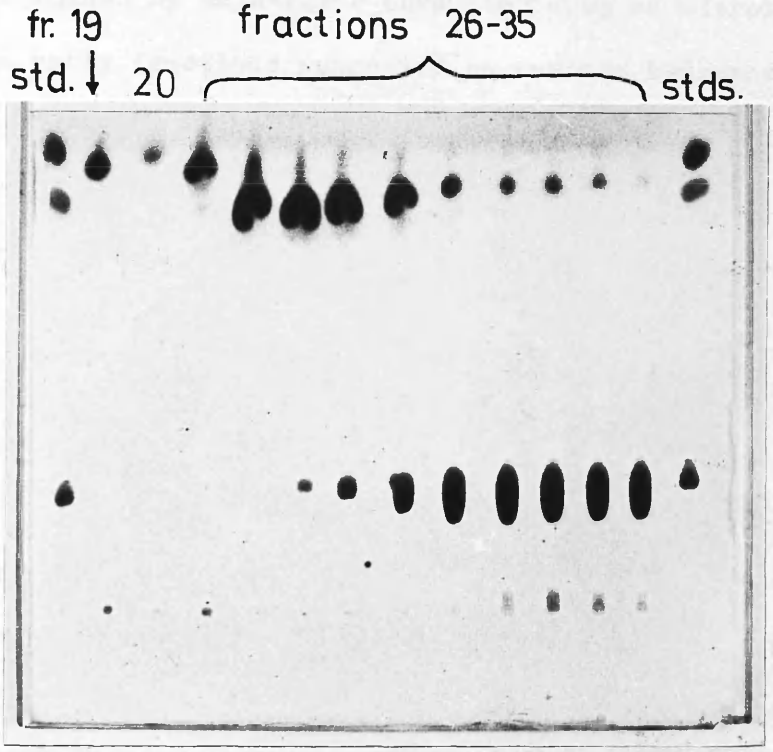
Weight of purified lipid extract = 3.8 g.

Weight of silicic acid (Horning standard) - 260 g.

Column load = 15 mg./g.

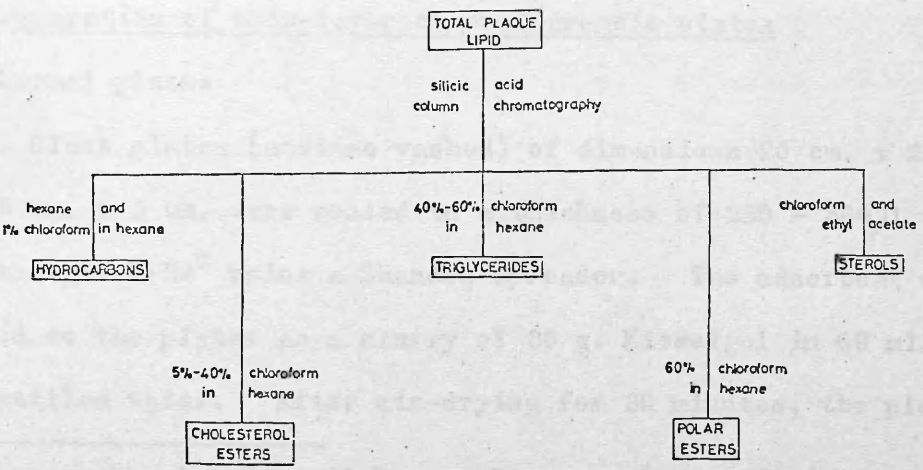
Fraction No.	Eluting Solvent	Volume ml.	Result of micro TLC examination
1 - 5	hexane	1000	hydrocarbons
6, 7	1% chloroform in hexane	300	hydrocarbons
8, 9	1% chloroform in hexane	300	sterol esters
10-15	5% chloroform in hexane	1200	sterol esters
16, 17	10% chloroform in hexane	400	sterol esters
18-22	15% chloroform in hexane	1000	sterol esters
23-25	20% chloroform in hexane	600	sterol esters (small amount)
26-29	40% chloroform in hexane	800	start of triglycerides
30-37	60% chloroform in hexane	1600	triglycerides plus sterol
38-39	chloroform	400	sterols
40-43	ethyl acetate	800	sterols

Figure 9. TLC of Silicic Acid Column Eluates



spray:- 1% ceric sulphate in 10% sulphuric acid

Figure 10. Flow diagram of Silicic Acid Chromatography



column. The qualitative content of each fraction was rapidly determined by thin-layer chromatography on microscope slides (6). The early fractions suspected to contain hydrocarbons were chromatographed in hexane:benzene (97:3 v/v) while the later fractions were examined on micro TLC plates developed in benzene:ethyl acetate (20:1 v/v). The TLC appearance of a group of successive fractions from the above silicic acid is illustrated in Figure 9.

(b) Florisil

Florisil (Koch-Light Laboratories Ltd. 60/100 mesh) was used without pre-treatment except in the case of chromatography of polar sterols where it was deactivated by the addition of water (7% v/w) (7). Columns were prepared in the normal manner by slurrying in hexane and a lipid load of approximately 20 mg./g of adsorbent was used.

5. Preparation of thin-layer chromatographic plates

(a) Normal plates

Glass plates (acetone washed) of dimensions 20 cm. x 20 cm. and 20 cm. x 5 cm. were coated to a thickness of 250 - 300μ with MN-Kieselgel G-HR[x] using a Shandon spreader. The adsorbent was applied to the plates as a slurry of 30 g. Kieselgel in 60 ml. of distilled water. After air-drying for 30 minutes, the plates

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[x] Macherey, Nagel & Co. 516 Duren, Germany, (a purified adsorbent, more suitable for preparative TLC than Kieselgel G supplied by Merck).

were activated at 100-105°C for 15 minutes and stored in a glass cabinet containing blue silica gel as drying agent.

(b) Micro TLC plates

Microscope slides (7.5 x 4 cm.) were coated with MN-Kieselgel G-HR also using a Shandon spreader. The slides were moistened and pressed on to 20 x 20 cm. glass plates held in the spreader so as to cover the entire surface. They were spread lengthwise to a thickness of 250μ and dried in the atmosphere for 15 minutes after which they were activated at 100-105°C for 5 minutes. The plates thus made were developed in screw-cap jars with metal foil seals during chromatography.

(c) Silver nitrate impregnated TLC plates

Silver nitrate plates were prepared according to the method of Claude (8): 8 g. of silver nitrate (Analar) was dissolved in 60 ml. of distilled water and made into a slurry with 30 g. of MN-Kieselgel G-HR. Five 20 cm. x 20 cm. plates of 250-300μ thickness were prepared using this mixture. After spreading, the plates were allowed to set in the dark for 30 minutes and were then activated for 2 hours at 50°C. They were stored in the darkness in a glass cabinet containing a drying agent and could be stored in this manner for about 3 days without deterioration.

Plates with a lower percentage of silver nitrate (approx. 5%) were found to be extremely useful for the separation

of unsaturated hydrocarbons. These were prepared using 1.5 g. rather than 8 g. of silver nitrate and could be stored in the dark for several weeks without appreciable deterioration.

TLC spray reagent

Unless otherwise stated all thin-layer chromatograms were visualised by spraying with a 1% solution of ceric sulphate in 10% sulphuric acid and heating at 110°C for 5-10 minutes.

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concentrated sulphuric acid and sodium hydroxide as absorbents. The residue was then dissolved in 10-100μl. of chloroform or ethyl acetate in preparation for thin-layer or gas-liquid chromatography.

(b) Trimethylsilyl ethers (9, 10)

The sterol (10-100μ g.) was dissolved in pyridine (5μl.) in a micro test-tube and treated with 20μl. of hexamethyldisilazane (HMDS) and 2μl. of trimethylchlorosilane (TMCS). The mixture was stoppered, shaken and set aside for approximately 4 hours at room temperature. The reagents were then either evaporated in a vacuum desiccator or blown off under a stream of nitrogen. The residue was then dissolved in 0.5 ml. of chloroform and the inorganic suspension removed by filtration through a micro-sinter glass filtration tube. For long-term storage the

trimethylsilyl ethers were either dissolved in hexane or left in the reaction mixture. Trimethylsilyl ether derivatives of extracted material were normally sublimed (150°, 0.2 mm. Hg) immediately after preparation.

(c) Propionates (8)

The sterol (1-20 mg.) was treated with 0.5 ml. of propionyl chloride in a small test-tube. The reaction mixture was heated extremely briefly in a low micro bunsen flame to aid solution of the sterol, and then allowed to cool for ten minutes; 10 ml. of hexane was then added and the organic phase washed with 10 ml. of distilled water, followed by 10 ml. of 10% sodium bicarbonate solution and finally by 10 ml. of distilled water. The presence of any remaining propionic acid was easily detected at this stage by its smell and if necessary the solution was rewashed with bicarbonate and distilled water.

(d) Trimethylsilyl ether of a hindered hydroxyl group

The sterol (10-100μg.) was dissolved in 10μl. of hexamethyldisilazane in a capillary melting point tube sealed at one end; 20μl. of trimethylchlorosilane was added and the reagents were carefully mixed. The tube was sealed and heated in an oven at 150°C for 15 hours. The capillary tube was then broken inside a test-tube containing 0.5 ml. of chloroform or ethyl acetate and the solution filtered and concentrated under a stream of nitrogen as required. Pyridine was not used as a

solvent in the preparation as it appeared to cause decomposition.

Reagents.

"AnalaR" pyridine and acetic anhydride were employed in the preparation of derivatives, both being redistilled before use. The pyridine had been previously dried over sodium hydroxide pellets. Propionyl chloride (Aldrich Chemical Co.) was distilled twice before use. Hexamethyldisilazane (Koch-Light) and trimethylchlorosilane (Hopkin and Williams) were both used without further purification.

7. Preparation of thiourea adduct of squalene (11)

Squalene was dissolved in a saturated methanolic solution of a recrystallized thiourea. The solutions were set aside at room temperature for 1 day during which the thiourea adduct crystallized out. The hydrocarbon was regenerated by treating the adduct with hot water, and was extracted with hexane and washed with water after cooling. The hexane extract was dried over anhydrous sodium sulphate. The regenerated product showed no different TLC, GLC or GC-MS properties from the starting material.

8. Preparation of fatty acid methyl esters

Fatty acids were methylated by the addition of an ethereal solution of diazomethane (12). The reaction was judged to be complete when a persistent yellow colour after shaking indicated an excess of reagent. The reagent was removed by flushing with nitrogen.

9. Hydrolysis of lipid extracts

(a) Alkaline hydrolysis

Methanolic potassium hydroxide was prepared by dissolving potassium hydroxide (5.6 g.) in methanol (100 ml.). Extracts were hydrolysed by refluxing with a mixture of the alkaline solution and benzene (1:2 v/v) under an atmosphere of nitrogen for 2 hours. After completion of the reaction, the solvents were removed under reduced pressure and the residue was dissolved in water. The aqueous solution was then extracted three times with ether.

(b) Reductive hydrolysis

The extract was dissolved in anhydrous ether and a small quantity of lithium aluminium hydride added and allowed to react for 5 minutes. The excess reagent was destroyed by the addition of ethyl acetate. An equal volume of water was added and the organic layer was removed with a Pasteur pipette after shaking. The aqueous layer was re-extracted with an equal volume of ether and the organic extracts combined.

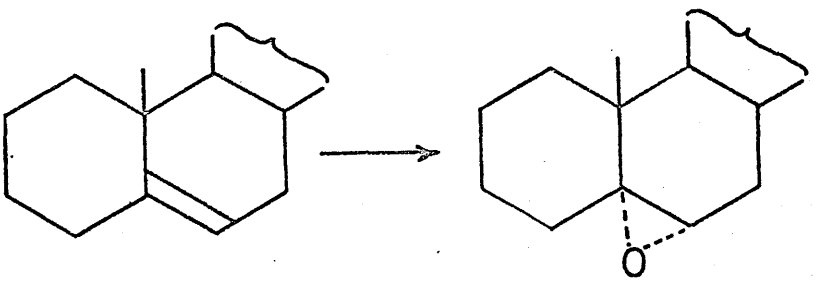
(c) Alkaline transesterification (16) p.193.

The esters were dissolved in the minimum volume of cholroform and an excess of 0.5 N solium methoxide was added. The mixture was boiled for 2 minutes on a water bath and then neutralised with 10% hydrochloric acid solution. The resulting alcohols and fatty acid methyl esters were twice extracted with ether and the extracts combined.

10. Hydroxylation of a double bond in a cholestene (13)

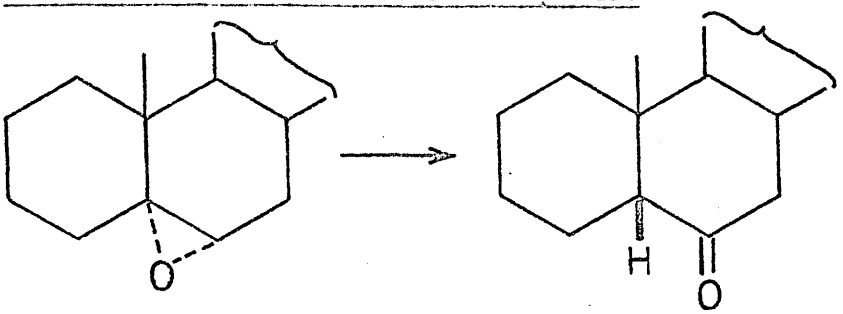
The cholestene (10-20μg.) was dissolved in dry ether (dried over sodium) (20μl.) and 5μl. of dry pyridine was added. The solution was then treated with 10μl. of a solution of 2 mg. osmium tetroxide in 1 ml. of dry ether. The mixture was set aside in a stoppered test-tube at room temperature for 3 days. The solution was then reduced to dryness in a stream of nitrogen and the residue dissolved in 1 ml. of chloroform. The chloroform solution was shaken with a 1 ml. aqueous solution containing 500μg. potassium hydroxide and 500μg. mannitol for 2 hours. The chloroform layer was then removed, washed with 1 ml. of distilled water and evaporated.

11. Epoxidation of cholest-5-ene



The epoxide of choles-5-ene was prepared according to the method of Emmons (14) using pertrifluoroacetic acid prepared from trifluoroacetic anhydride and 90% hydrogen peroxide in methylene chloride buffered with sodium carbonate.

12. Isomerisation of cholest-5-one epoxide



A benzene solution containing 100μg. of 5,6α- epoxy - 5α- cholestane was treated with one drop of boron trifluoride in ether as described by Henbest and Wrigley (15) and allowed to react for 2 minutes. Water was added and the ether layer removed and washed with 10% sodium bicarbonate solution followed by water. The resulting product 5βcholestan-6-one, was separated from other products by preparative TLC in benzene:ethyl acetate (20:1 v/v). The TLC, GLC and the GC-MS behaviour of this product will be discussed later on page 150.

13. Concentration of polar sterols relative to cholesterol

The total sterol fraction as obtained from the silicic acid column was reduced to dryness under a vacuum. The residue was dissolved in the minimum amount of chloroform and a relatively large volume of methanol added. The solution was then concentrated in vacuo in a long-neck round bottom flask with a splash-head. A water bath at 40^{o}C was used to prevent the formation

of ice on the outside of the flask. Concentration was continued until a white suspension was visible in the flask. The precipitate was filtered off using a glass filter stick, and the process was repeated using the methanolic mother-liquor. A viscous yellow oily material was obtained after approximately six crystallisations. This oil was dissolved in hexane: ether (3:1 v/v) in preparation for chromatography on a column of deactivated Florisil which was employed to remove the remainder of the cholesterol.

14. Gas-liquid chromatography

Instruments: Isothermal GLC was carried out on a Pye Argon Chromatograph using a 130 cm. straight glass column (internal diameter 3.5-4.0 mm.) and a standard Lovelock argon ionisation detector with a strontium 90 source. The instrument had been modified to incorporate a "flash heater" block in the injection zone to aid rapid volatilisation of the sample and this was maintained at 25° - 35°C above the column temperature. The argon carrier gas was normally maintained at an outlet flow rate of 40 ml./min. by an inlet pressure of 8-12 psi. and the detector was set at 1500v.

Temperature programmed GLC was carried out on a standard Pye 104 instrument employing a 160 cm. coiled glass column (internal diameter 3.5 mm.) and a flame ionisation detector. Nitrogen was used as carrier gas and was maintained at a flow

rate of 40 ml./min. with a detector voltage of 1000 v.

Columns: The glass columns were silanised with a 5% solution of dimethyldichlorosilane in toluene and dried before packing. The bulk of the work was carried out using two stationary phases, namely 1% SE - 30 and 1% QF - 1, which were coated on 80-100 mesh acid washed, silanised Gas Chrom P. These packing materials were bought ready made and pre-tested from Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A.

Samples: Solutions of known concentration (usually 1-5 mg./ml.) were prepared, and where these were extracts particular attention was paid to drying the solution. Chloroform was found to be the best solvent for the Pye Argon instrument and ethyl-acetate was used for the Pye 104. Samples of 0.2 - 2.0μl. were injected into the instruments using a Hamilton syringe.

15. GC - MS instrument

Unless otherwise stated, mass spectra were obtained using an LKB model 9000 gas chromatograph-mass spectrometer (LKB Produkter AB, Stockholm). This instrument is fitted with a 2-step jet separator of the Becker-Ryhage type. The mass spectrometer is a single focusing type equipped with a 60° sector, 20 cm. radius magnetic analyser and a sweep generator for the fast scanning of spectra. The conditions used were as follows:-

Carrier gas: helium at 25 - 30 ml./min.

Electron energy: 70 ev.

Accelerating voltage: 2.5 - 3.0 kv.

Temperature of ion source: 275°C.

Temperature of molecular separator: 275°C.

16. General working procedure for GC - MS.

1) Samples were prepared for gas chromatography in the manner described above and derivatives were chosen which produced the desired GLC separations and which gave suitably characteristic mass spectral fragmentations.

2) The GLC behaviour of an aliquot of the sample was established using a Pye Argon Chromatograph and the conditions were duplicated in the chromatograph of the LKB GC - MS instrument.

3) Solutions of suitable concentration were injected into the instrument and the approximate chromatographic behaviour of the sample was registered on the "total ion current" record which also indicated the points at which mass spectral scans were obtained.

4) Where possible, several scans were taken during the emergence of each chromatographic peak, in order to assess and to eliminate the possibility of "biased" spectra which do not represent the true relative distribution of ion abundance characteristic of a particular compound.

5) A "background" scan in the absence of sample and under identical conditions was also taken in order to correct for mass spectral peaks due to the chromatographic packing material.

6) Spectra were "counted" in the normal manner starting at the low mass numbers with easily recognisable peaks due to air background. For masses over 400, counting becomes difficult owing to an unfavourable signal-to-noise ratio and counting of the high masses is aided by bleeding in a known "marker" substance during the time that the spectrum is recorded. The usual marker is perfluorokerosene (PFK) which gives a large number of recognisable peaks. Mass values are then obtained by interpolation from marker peaks.

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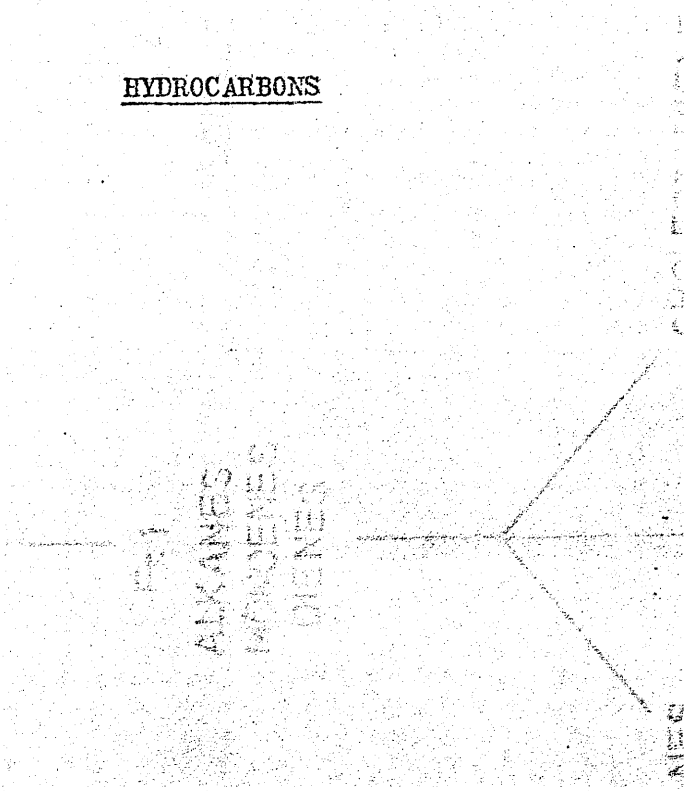
SECTION E

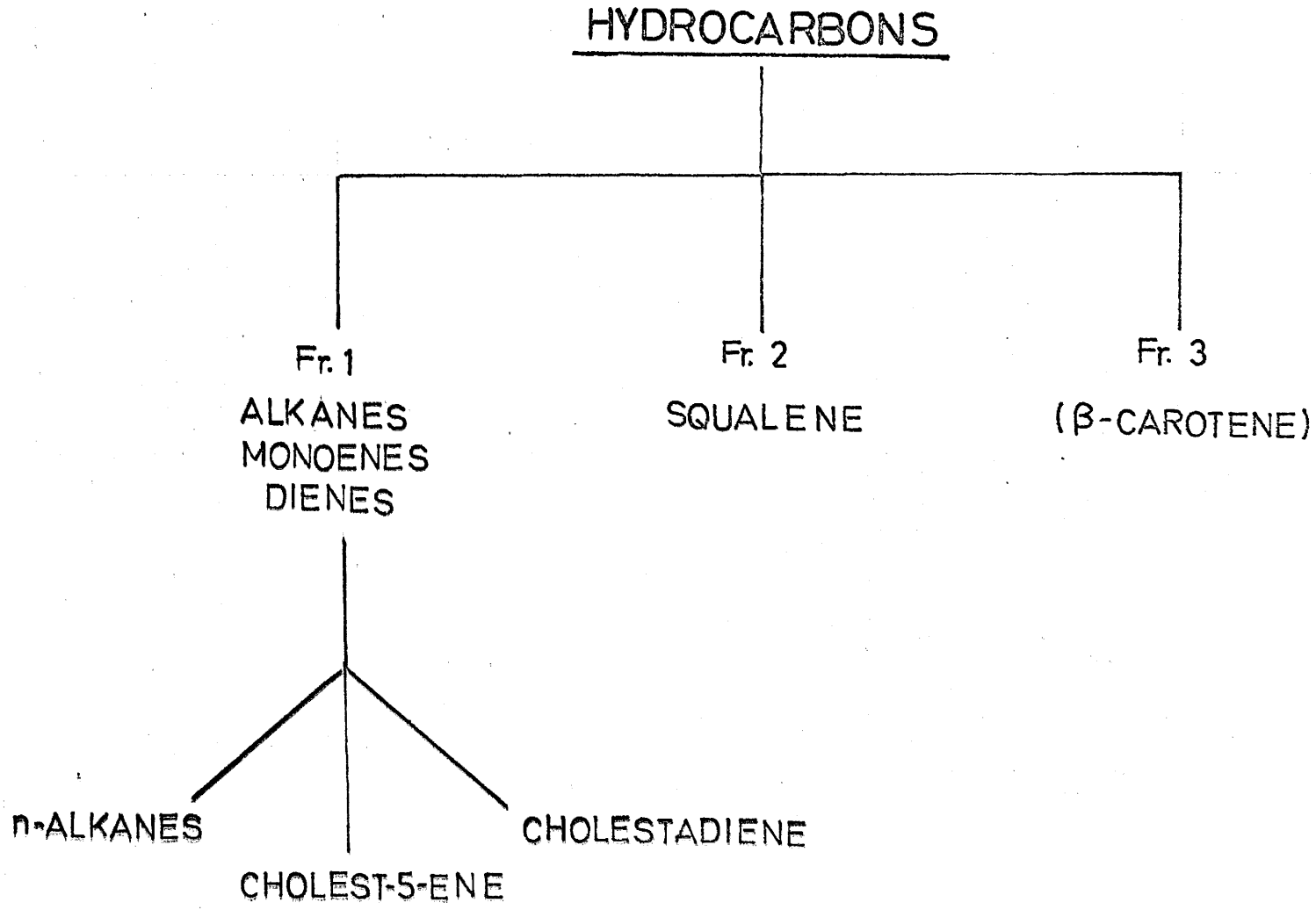
RESULTS

PART I

HYDROCARBONS



Key 1.



Brackets signify a tentative identification.

HYDROCARBONS OF BIOLOGICAL ORIGIN

Hydrocarbons are considered by many authors to be without biological significance; however, there is an increasing amount of evidence to suggest that this is not so and that they play an important role in the plant and animal kingdoms as well as in many marine forms.

Paraffinic hydrocarbons: Paraffinic hydrocarbons have been found to have an especially wide distribution in fruit and vegetable waxes (1, 2) and in insect waxes (3-5). In the animal kingdom, this type of compound has been found in butter (6), in wool wax (7) and in the human skin surface and blood (8-10). Very little is known about the origin or metabolic function of these compounds.

Isoprenoid hydrocarbons: Isoprenoid hydrocarbons are present in relatively large quantities in a wide variety of plants and trees. Farnesene for instance, has been identified in hop oil (11) and limonene in oil of lemon (12) while the hydrocarbon α- pinene has been shown to make up 78 per cent of the oil from Juniperus phoenicea (13). None of these compounds has however been shown to be present in human or animal tissue.

The C_{30} isoprenoid hydrocarbon squalene is probably the most widely studied hydrocarbon in mammalian tissue and fluids.

It is especially interesting because of the role it plays in sterol biosynthesis. It was first isolated from shark liver oil in 1918 by Tsujimoto (14). Since then its presence has been demonstrated in human hair fat (15), in human and rat blood (16) and in the brain tissue of both humans (17) and cattle (18).

Carotenes are well established components of many members of the plant kingdom (19, 20) and have also been identified in mammalian tissues (21, 22) as well as in human blood (23). Pristane (2,6,10,14 - tetramethylpentadecane) and phytane (2,6,10,14 - tetramethylhexadecane) have been identified in a large variety of marine sources (24, 25) as well as in many sites in both man and animals (26).

HYDROCARBONS OF THE ATHEROMATOUS PLAQUE

We have approached the problem of the composition of the hydrocarbon fraction with a completely open mind as no previous investigation of these compounds had been carried out. It was known, however, that silicic acid column chromatography, as well as separating hydrocarbons from other lipid classes, also gives some fractionation within this group based on the degree of unsaturation existing in the molecule. It has been shown (27,28) using a mixture of reference compounds that the following elution sequence can be drawn up for hydrocarbons on silicic acid:- acyclic alkanes; cycloalkanes; straight-chain monoenes; polyenes.

Elution of plaque hydrocarbons

The extremely delicate separation mentioned above for pure hydrocarbons could not be achieved when dealing with a total lipid extract as described earlier on page 102 . It was possible, however, to obtain three major hydrocarbon-containing fractions (fractions 1, 2 and 3) which were completely free from sterol esters. The column was monitored by TLC on microscope slides which suggested the existence of two major compounds in fraction 1 (designated A and B), one in fraction 2 and one in fraction 3 (called C and D respectively). TLC mobilities and colour reactions obtained on a full-size chromatogram (20 cm. x 20 cm.) are recorded in table 9 below.

Table 9

TLC of plaque hydrocarbons in hexane : benzene (97:3 v/v).

adsorbent:- MN - Kieselgel G - HR.

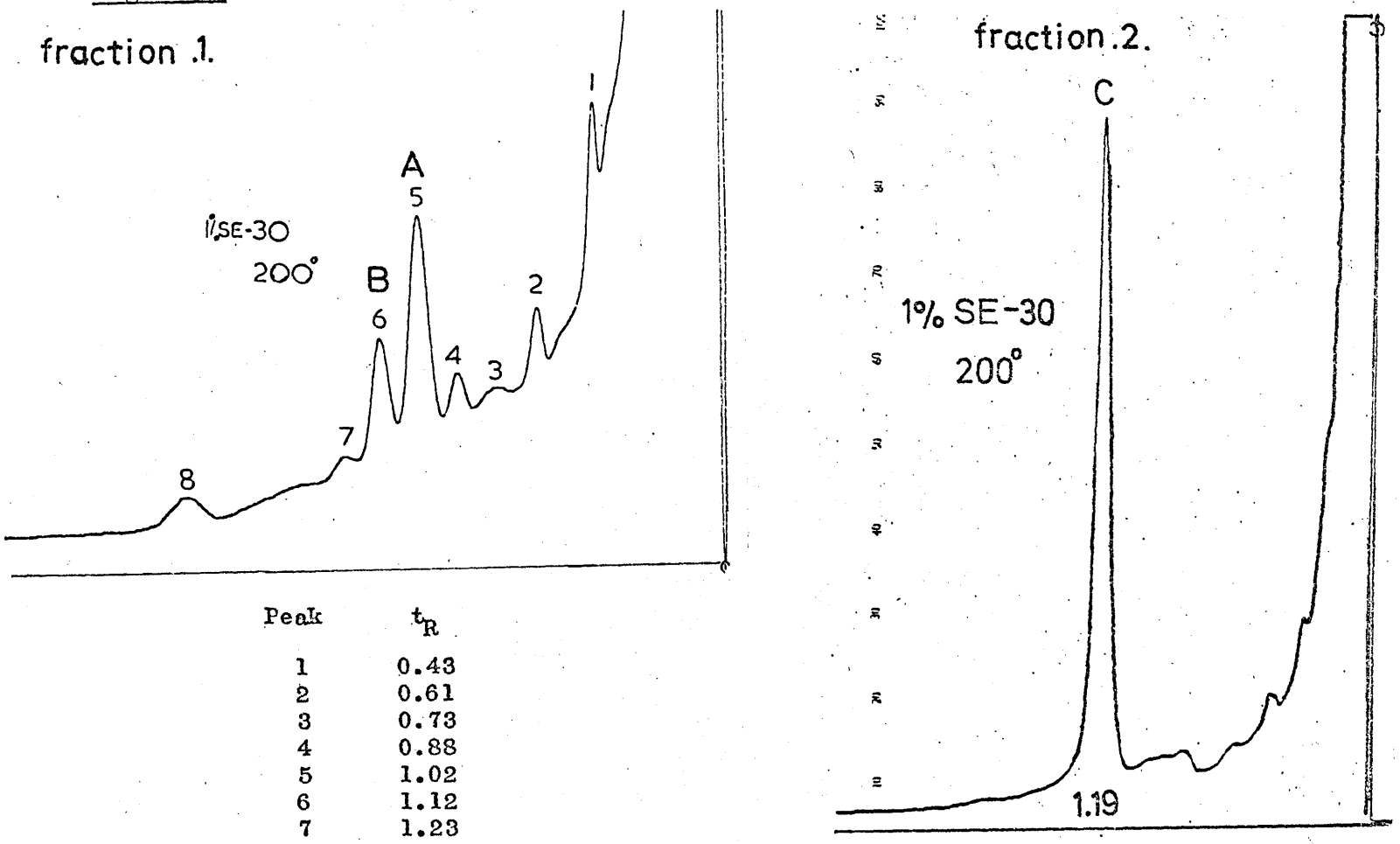
Fraction	Compound	R_F	Colour reaction with ceric sulphate
1	A	0.82	Maroon
	B	0.75	brown
2	C	0.47	Brown, becoming maroon on standing
3	D	0.23	Yellow without spraying, brown after spraying

The hydrocarbons were then examined by gas-liquid chromatography firstly on the non-selective phase SE-30 (Fig.11) and then on the polar phase QF-1 (Fig.12). Fraction 3 gave no satisfactory GLC behaviour, however, its TLC and UV characteristics closely paralleled that of β - carotene. No further characterisation was carried out.

By comparison with available reference hydrocarbons, and in the light of previous work on human blood (16) and tissues (17) we suspected that compound C in fraction 2 might be squalene and further evidence was collected to support this tentative identification. Firstly, the extracted compound was shown to form a thiourea adduct (see page 108) a reaction characteristic of the natural all-trans isomer of squalene, and of other isoprenoid hydrocarbons.

Secondly an infra-red spectrum of compound C was recorded, using a pooled sample from twelve aortic extracts, and this was compared with that of a reference sample of squalene. The spectra obtained both showed a characteristic carbon-carbon double bond stretching frequency band at 1665 cm^{-1}, and were essentially the same except for two extra peaks at 1745 cm^{-1} and 770 cm^{-1} in the extract which have been attributed to the presence of impurities. These peaks were largely removed after purification of the extracted material through the thiourea adduct (see Fig.13 (a)).

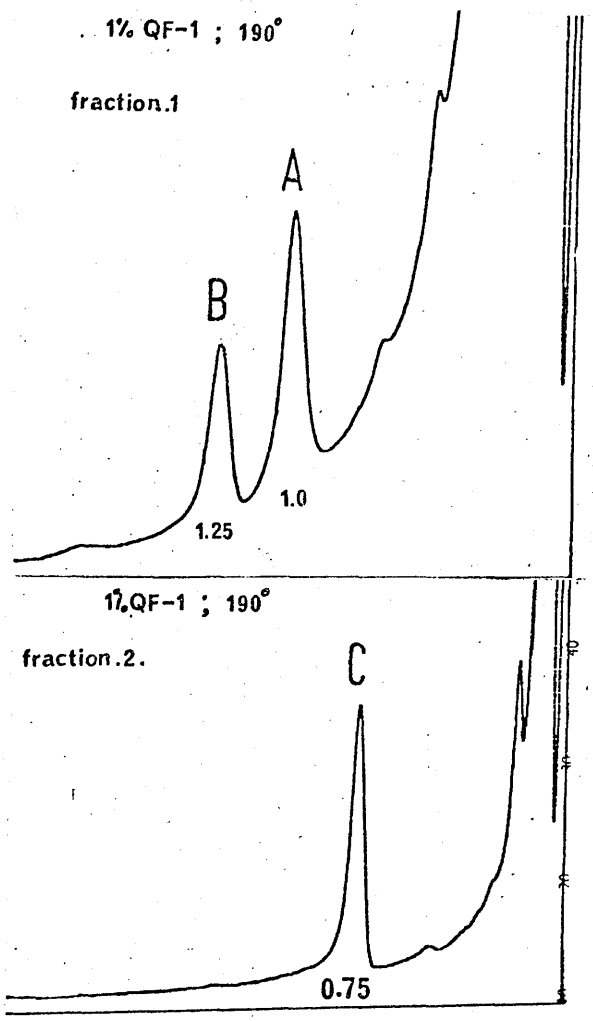
Figure 11 GLC of Plaque Hydrocarbons



Peak	t_R
1	0.43
2	0.61
3	0.73
4	0.88
5	1.02
6	1.12
7	1.23
8	1.75

t_R = retention time relative to 5α-cholestane

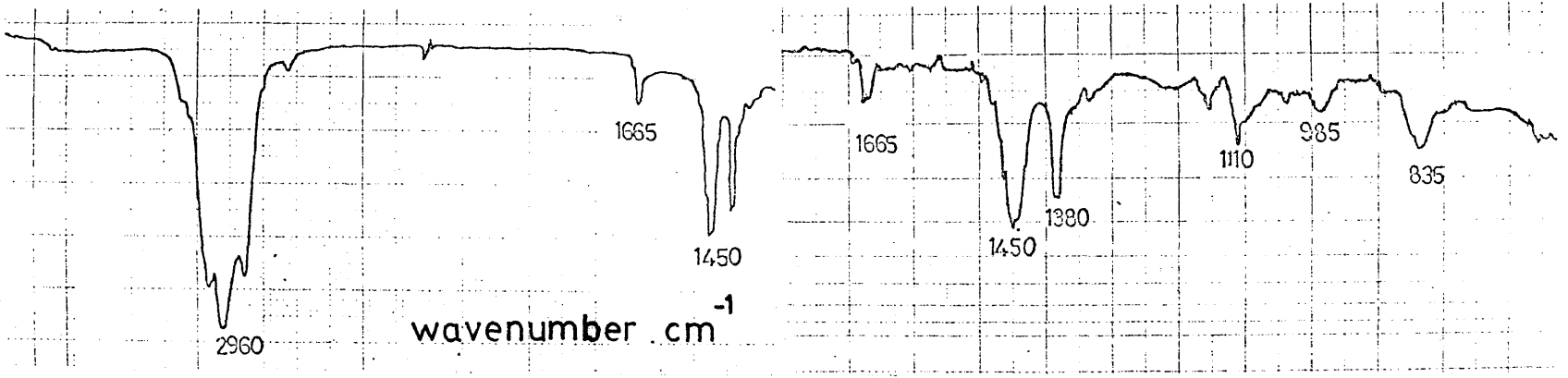
Figure 12 GLC of Plaque Hydrocarbons



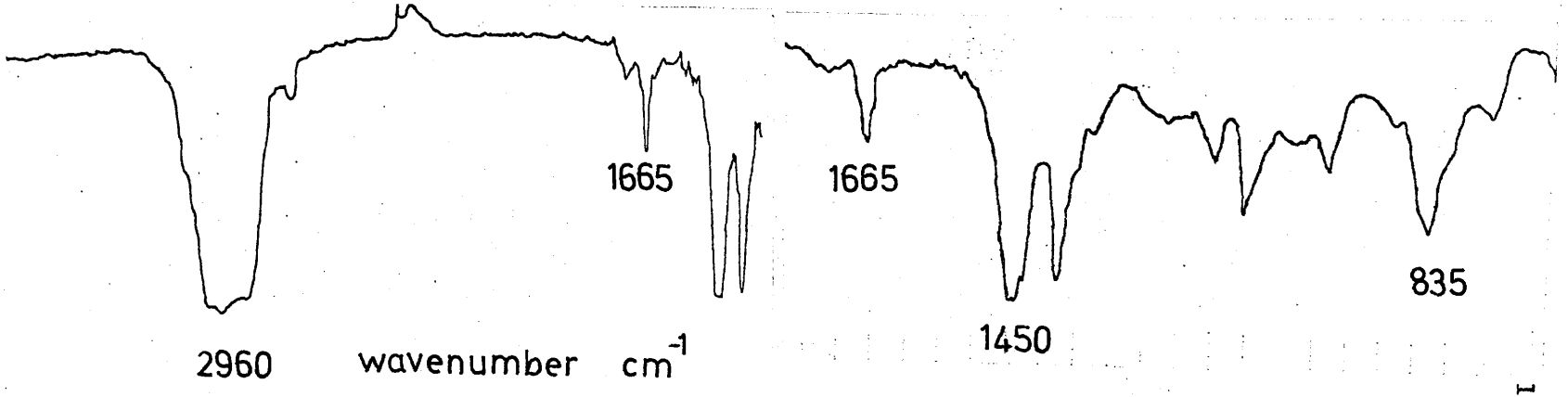
The figures recorded under the peaks are the retention times relative to 5α-cholestane.

Figure 13 Infra-red spectroscopy of squalene

(a) Purified Extract



(b) Authentic

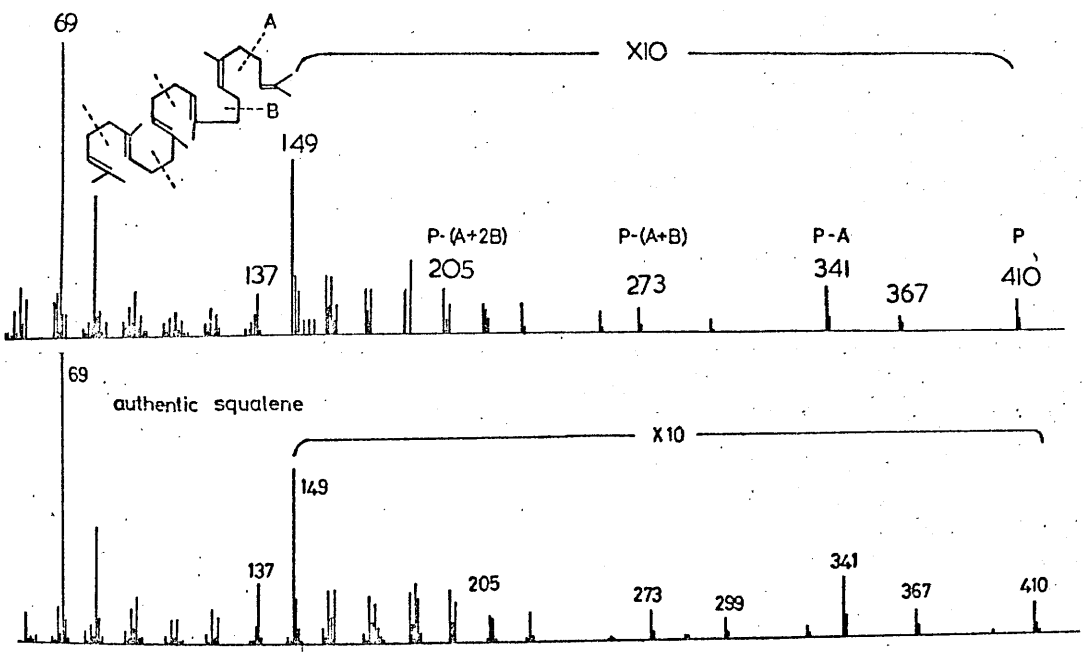


A very similar spectrum was recorded by Isler et al. (28) for purified natural squalene.

More definitive proof of the structure of compound C was obtained by mass spectrometry firstly with an MS-9 instrument using a pooled sample and later with the LKB 9000 gas chromatograph-mass spectrometer using the extracts from a single aorta. A line diagram of the mass spectrum of the extract obtained on the MS-9 instrument is shown in Fig. 14.

Figure 14

Mass spectrum of aortic squalene (MS-9 instrument)



This mass spectrum was shown to be identical to that of authentic squalene and shows characteristic fragmentation into C_5 isoprenoid moieties. This is seen in an initial loss of 69 mass

units from the molecular ion for a terminal isoprene unit and then by successive losses of 68 mass units for other C_5 non-terminal fragments. The other ions due to this mode of fragmentation can be seen at m/e 273, 205, 137 and 69 (base peak).

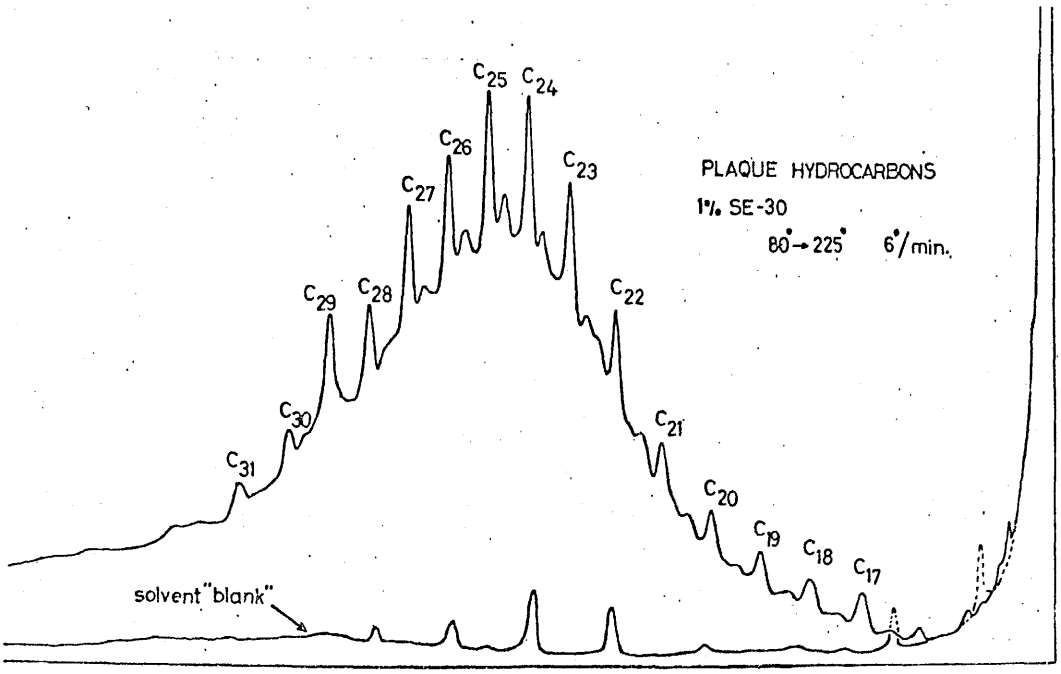
On the basis of the above evidence compound C is identified as all-trans squalene.

GC-MS examination of hydrocarbon fraction 1

The advent of gas chromatography-mass spectrometry in a combined instrument made the identification of the components of fraction 1 possible on the extracts from one aorta. Such an identification would not have been possible in the early stages of this work, where this instrument was not available.

The large "solvent peak" given by this fraction (see Figs. 11 and 12 on pages 125,126) was thought to be due to the presence of unresolved saturated hydrocarbons and was removed by preparative thin-layer chromatography on layers of silica gel impregnated with 5% of silver nitrate, using hexane : benzene (97:3 v/v) as developing solvent. The saturated hydrocarbons thus obtained were examined by gas-liquid chromatography using temperature programming to compensate for the relatively large range of molecular weights. A typical chromatogram obtained is shown in Fig. 15. The existence of two homologous series was indicated. The major of these was composed of n-alkanes

Figure 15

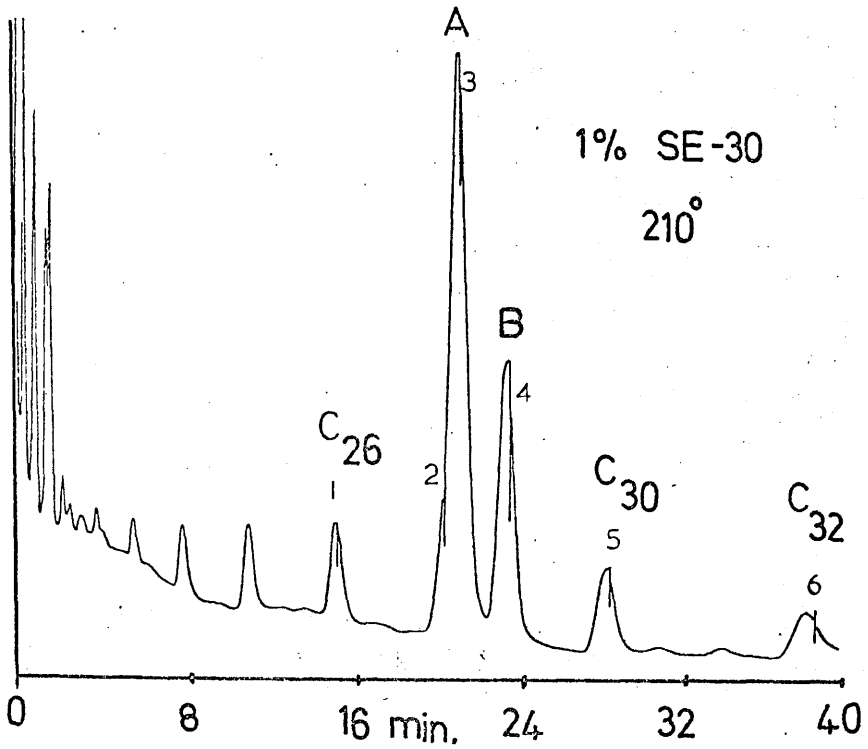


between C_{20} and C_{31}, with no particular bias for odd or even numbers of carbon atoms. The second series, present in much smaller quantities, gave small peaks intermediate between successive members of the larger series and are thought to be branched-chain hydrocarbons. All of these assignments have been made on the basis of GLC retention data. A GC-MS study has not been carried out because of the complexity of the fraction and because mass spectra of hydrocarbons of this type are not very informative. Attempts are being made to simplify this fraction by resolving it into straight-chain and branched-chain hydrocarbons using molecular sieves (29, 30) and urea inclusion complexes (31).

The possibility that these compounds came from either the silicic acid or the eluting solvent has been ruled out. Examination of solvent "blanks" taken before loading the column with lipid has revealed that although saturated hydrocarbons are present in the solvent they are present in much smaller quantities. The blanks were also qualitatively distinct since they appeared to contain only the even carbon atom hydrocarbons, C_{14}, C_{16}, C_{22}, C_{24}, C_{26} and C_{28}.

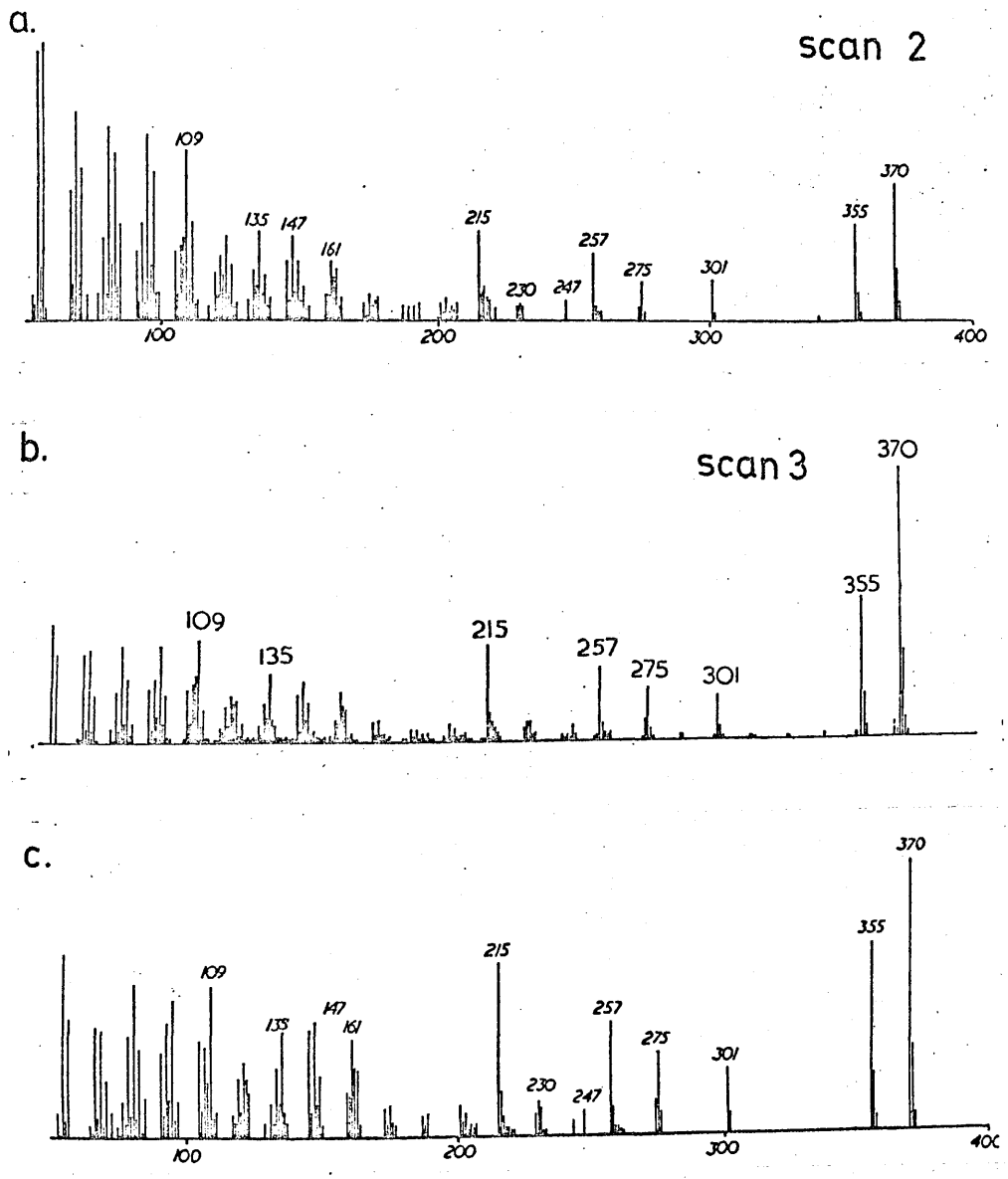
After the removal of the bulk of the paraffinic hydrocarbons, fraction 1, containing compounds A and B, was examined by GC-MS on an LKB 9000 instrument and Fig. 16 represents the "total ion current" trace of GLC behaviour thus obtained. The peak due to compound A was scanned twice to test for homogeneity (scan 2, t_R = 0.96 and scan 3, t_R = 1.01, where t_R = retention time relative to cholestane) and a comparison of the two spectra obtained is given in Fig. 17. The mass spectral behaviour of peak B is represented by scan 4, a line diagram of which is shown in Fig. 18. No satisfactory spectra were obtained from scans 1, 5 and 6 in Fig. 16, but retention data suggest that they are part of a homologous series of n - alkanes. The TLC and GLC mobilities of compound A were closely similar to those of 5α-cholestane: however, examination of its mass spectrum showed that A had a molecular weight of 370, 2 mass units less than 5α-cholestane. Similarities in the fragmentation of the two

Figure 16 Total ion current chromatogram of fraction 1.



substances suggested that the former might be a cholestene. A reference sample of cholest-5-ene was found to have similar retention data to the isolated hydrocarbon and its mass spectrum (shown in Fig. 17) is almost identical to that of the extracted hydrocarbon A. Similarly compound B, molecular weight 368, gave a mass spectral fragmentation pattern which bears a striking resemblance to that of cholesta-3,5-diene as can be seen in Fig. 18. The nature of some of the most abundant ions in the spectra of compounds A and B are discussed on page 278.

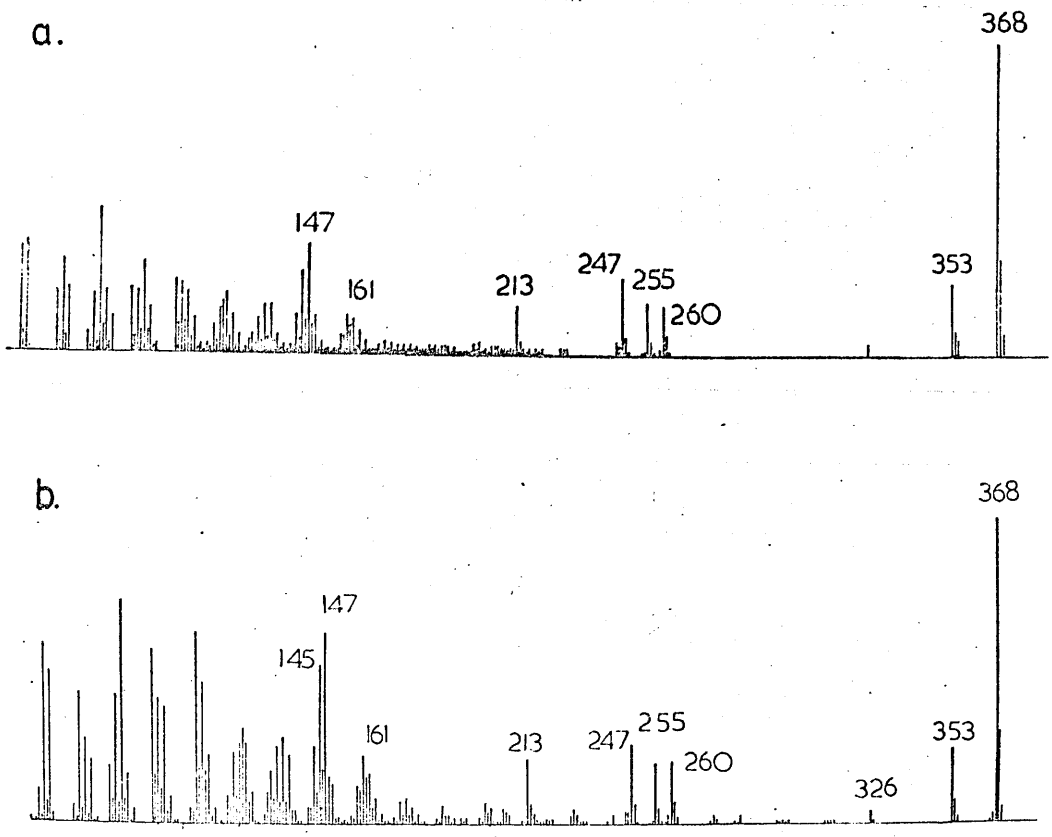
Figure 17



Mass spectra (LKB 9000 GC-MS instrument) of

(a) compound A (scan 2)
(b) compound A (scan 3)
(c) cholest - 5 - ene

Figure 18

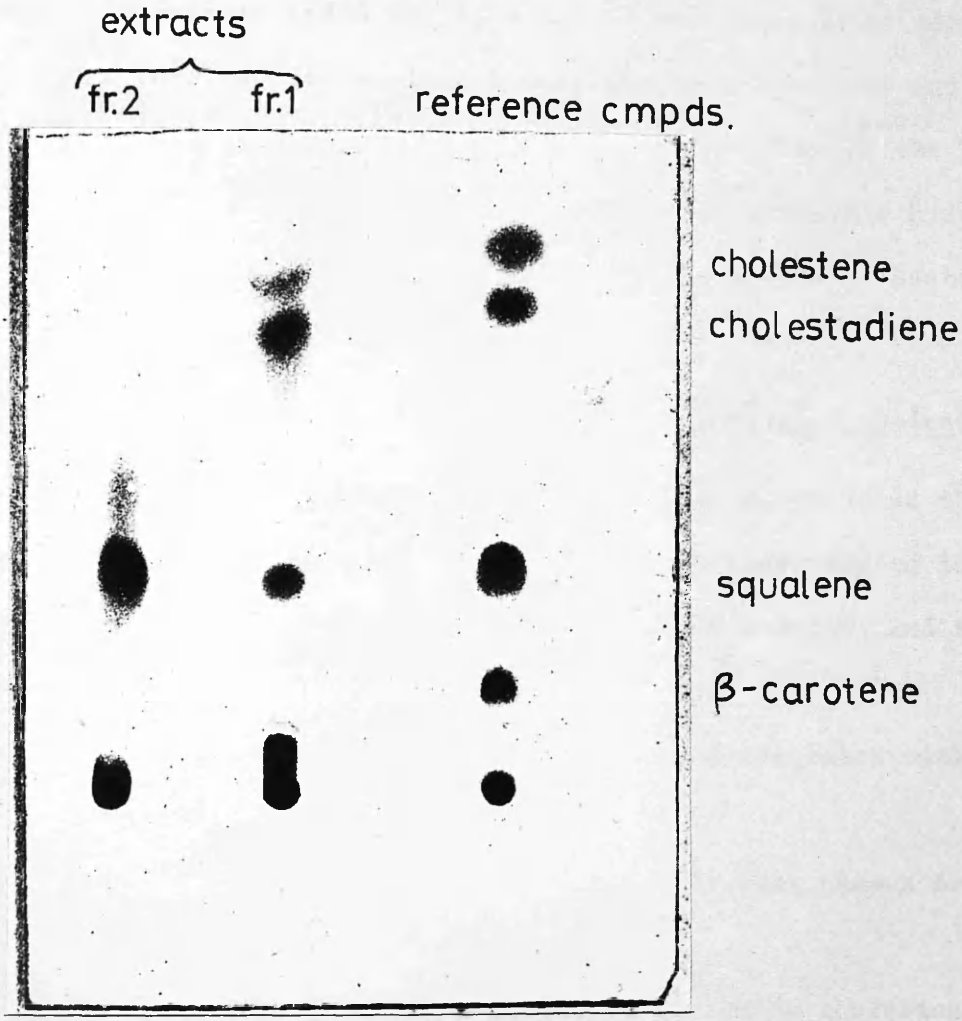


Mass spectra (LKB 9000) of

(a) compound B (scan 4)

(b) cholesta - 3, 5 - diene

Figure 19. TLC of extracted hydrocarbons



solvent system:- hexane : benzene (97:3 v/v)
adsorbent:- MN - Kieselgel G - HR.
spray:- 1% ceric sulphate in 10% sulphuric acid

The extracted compounds were then re-examined by TLC and GLC and compared directly with authentic compounds. The GLC retention data recorded for A, B and C were found to be identical to those recorded for cholest-5-ene, cholesta-3,5-diene and squalene respectively. Fig. 19 shows a comparison of the TLC behaviour of the extracted compounds with the authentic hydrocarbons mentioned above and strengthens the tentative identifications of A and B.

Characterisation of the double bond in the extracted cholestene.

After the use of mass spectrometry had established that compound A was a cholestene, four approaches were adopted to locate the position of the double bond in the molecule and these were as follows:-

1. Thin-layer chromatography on silica gel impregnated with silver nitrate;
2. gas-liquid chromatography on several different phases both selective and non-selective;
3. mass spectrometry, using a series of authentic cholestenes for comparison, and
4. specific reactions of the double bond to produce oxygenated compounds which give more characteristic mass spectral fragmentation.

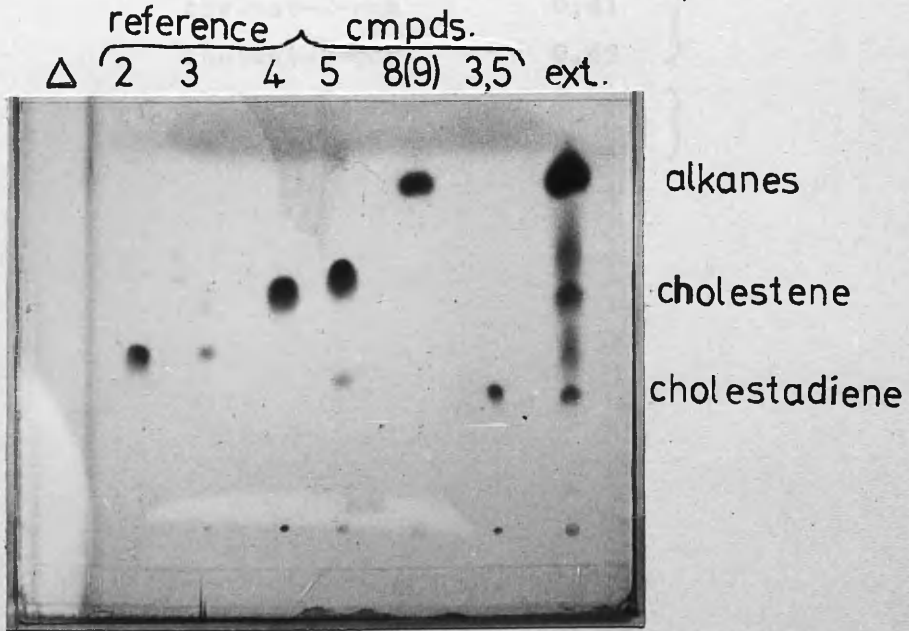
Method 1:- Thin-layer chromatography.

TLC on silica gel using hexane : benzene (97:3 v/v) as solvent system was largely unsuccessful in separating a mixture of reference cholestenes and consequently was of little use in locating the double bond in the extracted cholestene. However, good separations have been demonstrated for sterols differing only in double bond position, using layers of silica gel impregnated with silver nitrate (32, 33). A hydrocarbon extract was compared with a series of known cholestenes, on TLC plates which had been impregnated with 5% of silver nitrate, using hexane : benzene (97:3 v/v) as solvent system. A typical result obtained is shown below in Fig. 20.

Figure 20. TLC OF CHOLESTENES.

adsorbent:- MN - Kieselgel GHR/5% $AgNO_3$

solvent:- hexane : benzene (97:3 v/v)



spray:- 1% ceric sulphate in 10% sulphuric acid

From this chromatogram we can see that the reference hydrocarbons fall into three mobility groups and their R_F values are recorded in Table 10. The mobility differences obtained for these compounds would appear to be explained by proposing that the ions in the adsorbent would complex to the greatest extent with the least sterically hindered double bonds making these compounds the least mobile. However, on the other hand, consideration of the electron density about the double bonds would suggest that cholest-8(9)-ene should be the most polar which is the reverse of what was found experimentally. The validity of these theoretical considerations remains to be determined.

Table 10. TLC OF CHOLESTENES

(MN - Kieselgel G - HR/5% $AgNO_3$ - hexane:benzene 97:3 v/v)

COMPOUND	R_F	
cholest-2-ene	0.41	}
cholest-3-ene	0.42	}
cholest-4-ene	0.57	}
cholest-5-ene	0.59	}
cholest-8(9)-ene	0.82	
cholesta-3,5-diene	0.33	
EXTRACT		
cholestene A	0.57	
cholestadiene B	0.33	
alkanes	0.85	

The cholestene in the extracted material (see Fig. 20, extreme right-hand lane) is clearly distinct from cholest-8(9)-ene, -2-ene and -3-ene but is not differentiated from the Δ^4 and Δ^5 isomers.

Method 2:- Gas-liquid chromatography.

Since no separations could be demonstrated between the five available reference cholestenes on the non-selective phase SE - 30, several selective phases namely QF - 1, OV - 17 and OV - 22 were tested. These also failed in most instances to produce separations useful in characterisation as can be seen from the retention data presented in Table 11.

Table 11. GLC OF CHOLESTENES

	QF - 1		OV - 17		OV - 22		
Δ	I	t_R	I	t_R	I	t_R	
2	2950	1.02	2910	1.03	2600	1.02	
3	2930	1.05	2920	1.07	2605	1.05	
4	2925	0.94	2910	1.02	2300	1.02	
5	2930	0.96	2935	1.12	2610	1.06	†
8(9)	2935	1.00	2930	1.10	2615	1.07	

I = retention index

t_R = retention time relative to 5α-cholestane

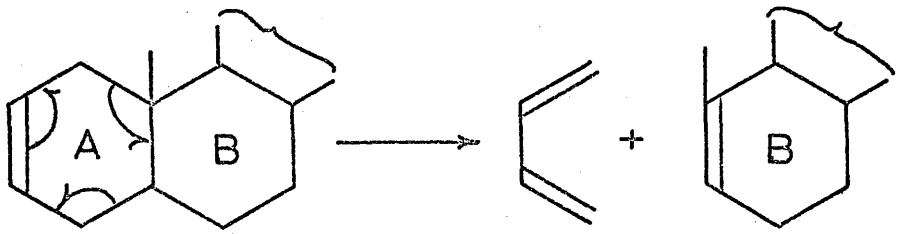
GLC conditions:- 1% QF - 1 (5 ft.) at 180°C [x]
1% OV - 17 (4 ft.) at 200°C
1% OV - 22 (5 ft.) at 195°C [x]

[x] Pye 104 instrument.

† The data recorded for the extracted cholestene were identical to those of authentic cholest-5-ene.

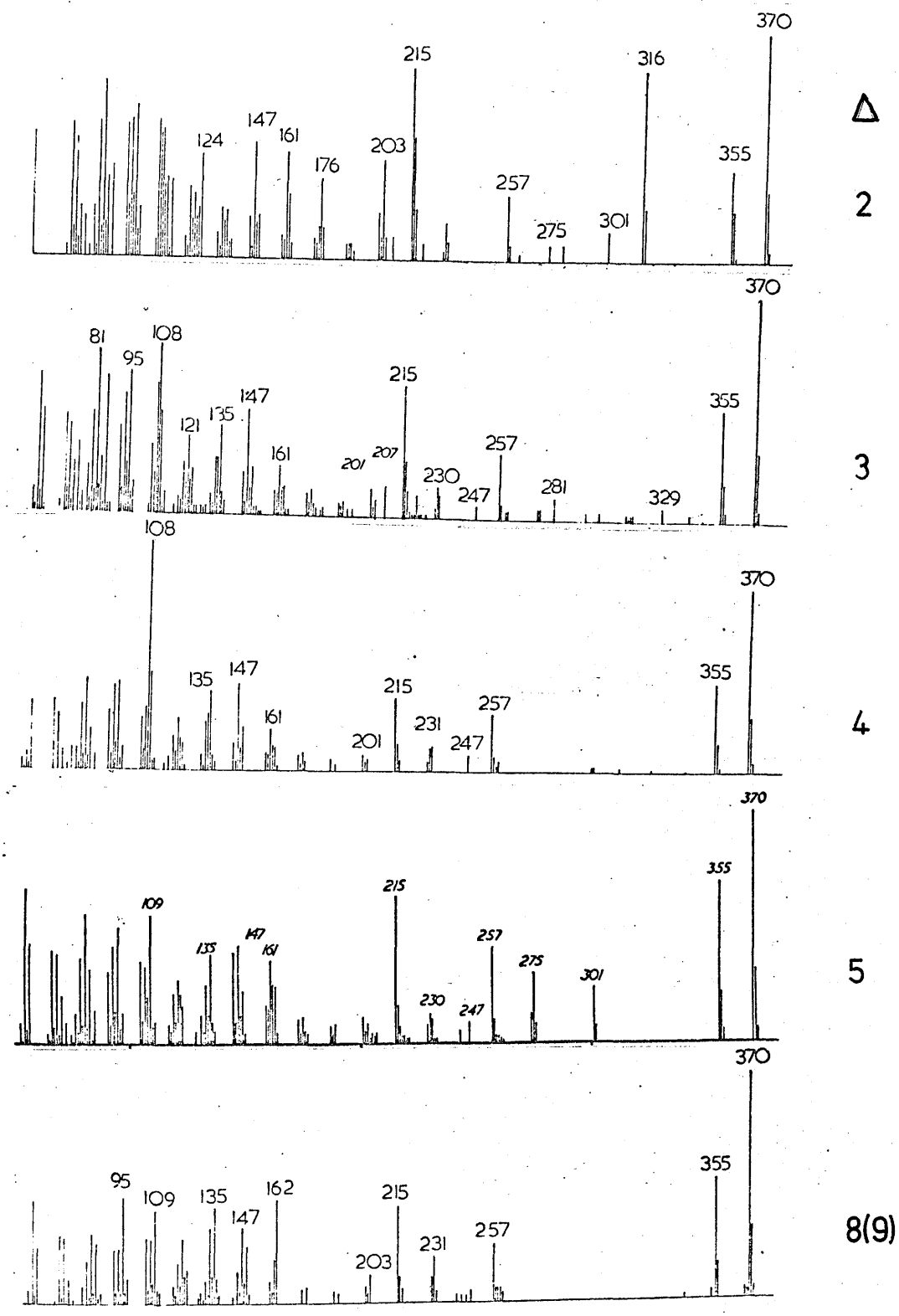
Method 3:- Mass spectrometry.

Examination of the mass spectra of the available pure reference cholestenes (Fig. 21) shows that they can all be adequately differentiated. Cholest - 2 - ene, because of its ability to undergo a retro - Diels Alder fragmentation process yields a major ion at m/e 316 due to the elimination of butadiene (54 mu.) as shown below:-



This ion was completely absent from the spectra of all other cholestenes examined, including that of the extract. The isolated cholestene (Fig. 17), cholest - 5 - ene and cholest - 2 - ene all possess abundant ions at m/e 275 and m/e 301 which are not present in any of the other spectra studied. Since we have already eliminated cholest - 2 - ene from consideration, cholest - 5 - ene is left as a possible identity. The mass spectrum of this compound is indeed closely similar to that of the aortic cholestene as can be seen in Fig. 17. A certain amount of caution must however be exercised in this assignment since the superimposition of the fragmentation of two or more cholestenes as a consequence of unresolved

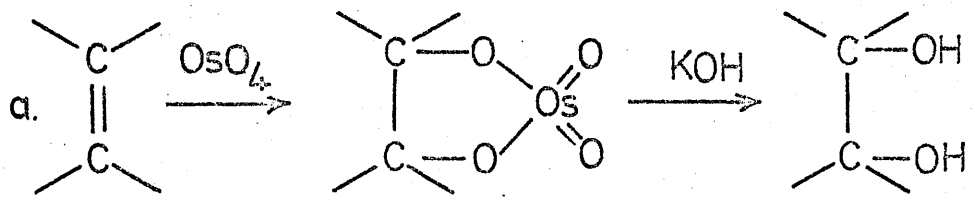
Figure 21. Mass spectra of authentic cholestenes

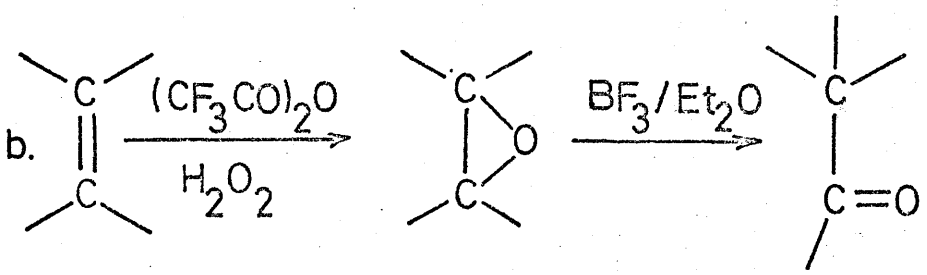


GLC peaks could build up a composite spectrum not unlike that of one of the pure cholestenes. It is also important to consider that one of the unavailable isomers might give a mass spectrum which cannot be distinguished from that of cholest - 5 - ene.

Method 4:- Specific reactions of the double bond.

Two reactions were attempted and these are outlined below:-



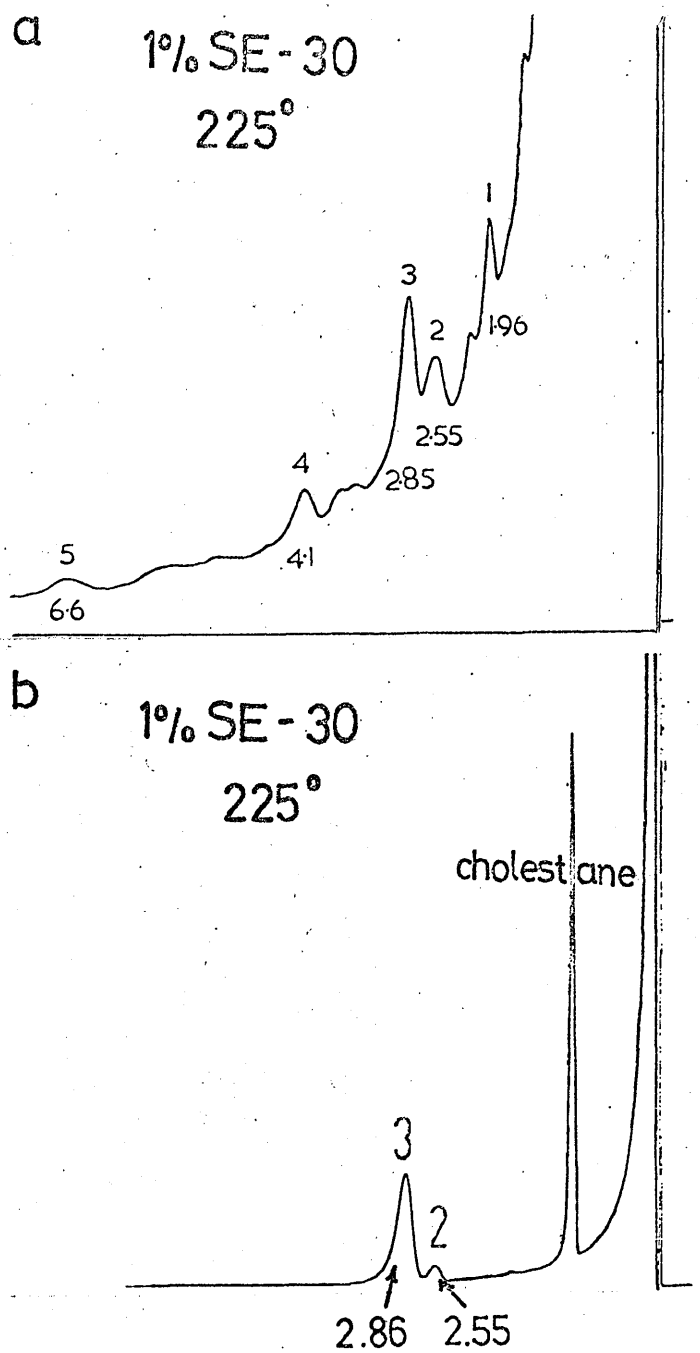


4.(a) Hydroxylation:- Crude aortic cholestene was treated with osmium tetroxide and the resulting ester hydrolysed to form a cholestane diol in the manner described on page 110 . Trimethylsilyl ethers were prepared from the resulting diol and

purification was effected by sublimation (140°C; 0.1 mm). The GLC behaviour of this fraction and that of the TMSi ether of 5α- cholestane - 5, 6α - diol produced from authentic cholest - 5 - ene by the same reaction sequence is shown in Fig. 22.

Unfortunately, the product obtained from the extract was insufficiently pure for direct GC-MS examination. However, gas chromatography gave additional evidence for the identification of the extracted material as cholest - 5 - ene since the retention times of the principal peaks of the products from extract and authentic were identical. The isolated cholestene was purified by preparative TLC on silica gel impregnated with silver nitrate with the aim of obtaining a fraction which would be suitable for GC-MS after treatment with osmium tetroxide. Fig. 23 shows the result of a GLC examination of a purified cholestene containing fraction and Fig. 24 that of the resulting diol (as TMSi ether). It was found that the GLC behaviour of the major peak of this sample could not be distinguished from that of the TMSi ether of 5α- cholestane - 5, 6α- diol.

A series of cholestane diol TMSi ethers was examined by GLC in order to establish that the possible diols produced by the action of osmium tetroxide on a group of cholestenes (2 - ene; 3 - ene; 4 - ene; 5 - ene; 6 - ene; 7 - ene and 8(9) - ene) could all be distinguished by GLC mobility, although little or no



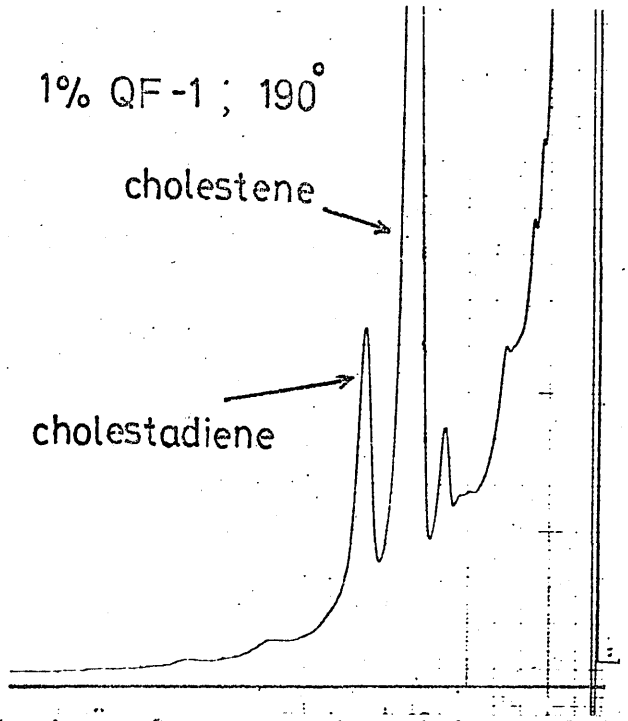
GLC of

(a) the TMSi ethers of the mixture produced by the action of osmium tetroxide of a crude extract containing cholestene and

(b) the TMSi ether of 5α- cholestane - 5, 6α- diol.

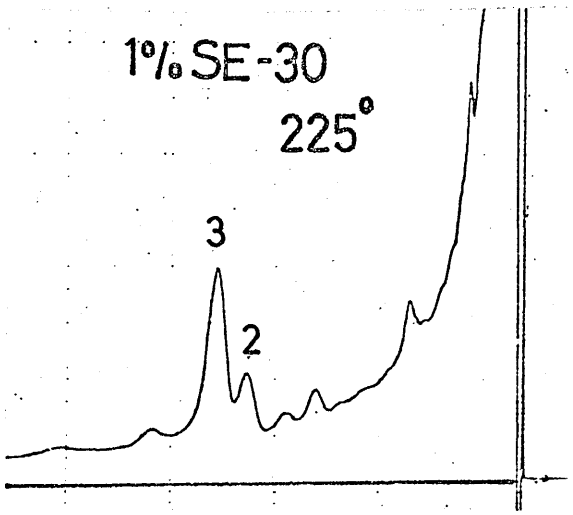
Retention times relative to cholestane are given under the peaks.

Figure 23



The GLC behaviour of an extract containing cholestene after purification by TLC on layers impregnated with silver nitrate.

Figure 24



GLC of TMSi ethers of purified cholestene extract after treatment with osmium tetroxide. The retention times under the peaks are relative to cholestane.

Table 12. GLC of TMSi ethers of cholestane diols

(1% SE - 30 ; 225°C)

COMPOUND	t_R	I
5α- cholestane - 2α, 3α- diol TMSi	3.40	3220
5α- cholestane - 2β, 3β- diol TMSi	4.47	3310
5α- cholestane - 3α, 4α- diol TMSi	3.85	3250
5α- cholestane - 3β, 4β- diol TMSi	5.11	3350
5α- cholestane - 4α, 5 - diol TMSi	2.47	3120
5β- cholestane - 4β, 5 - diol TMSi	3.42	3220
5α- cholestane - 5, 6α- diol TMSi	2.86	3160
5α- cholestane - 6α, 7α- diol TMSi	2.23	3080
5α- cholestane - 7β, 8β- diol TMSi	1.73	2990
5α- cholestane - 8ε, 9ε- diol TMSi	1.95	3025

t_R = retention time relative to cholestane

I = retention index

separation exists between the parent hydrocarbons. Table 12 shows that considerable separations do exist between the majority of the derivatives and that 5α- cholestane - 5, 6α- diol TMSi corresponds in retention time to that formed from the extracted cholestene and is markedly different from most other isomers. However, since only a very limited number of the possible cis- glycols was available we cannot say with certainty that some other diol would not give the same retention time under these conditions. Nevertheless the evidence to date suggests that the diol produced from the extracted cholestene on treatment with osmium tetroxide is 5α- cholestane - 5, 6α- diol which is known to be formed from cholest - 5 - ene.

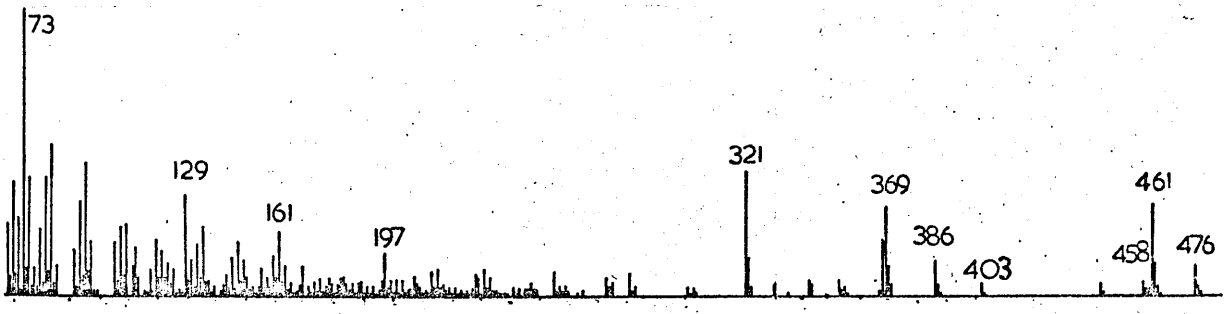
The reference sample of 5α, 6α- diol and the diol produced from the aortic cholestene were then both examined by GC-MS as their TMSi ethers and this quickly established that both compounds had formed mono- rather than bis-derivatives. This result was not completely unexpected since previous work on 5α- cholestane - 3β, 5, 6β- triol had shown that 5α- hydroxyl groups do not etherify under normal conditions. Line diagrams of the mass spectra obtained are shown in Fig. 25. The spectra are almost identical and are compatible with the fragmentation of 6α-trimethylsilyloxy-5α-cholestan-5-ol. The presence of a free hydroxyl and a trimethylsilyl ether group in both compounds is indicated by the elimination of water (P-18)

and trimethylsilanol (P-90) respectively from the molecular ion, the latter elimination being attested by the presence of a strong metastable ion at m/e 295.5 corresponding to the transition m/e 458 to m/e 368.

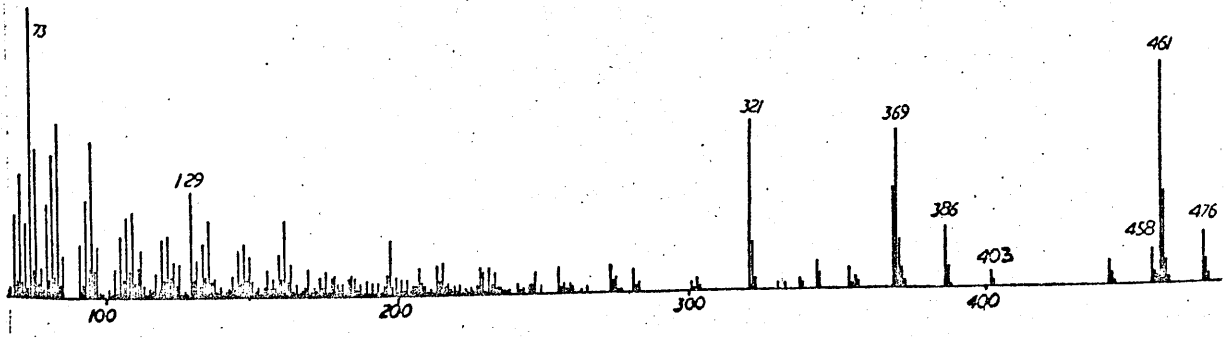
The bis-TMSi ether of 5α-cholestane-5,6α-diol can be formed under stronger etherification conditions (see page 107) and this compound, in agreement with the results for 5α-cholestane-3β, 5, 6β-triol, was found to have a shorter GLC retention time than the mono-TMSi ether.

Figure 25. Mass spectra of 5α-cholestane-5,6α-diol 6-TMSi ether

(a) Extract



(b) Authentic



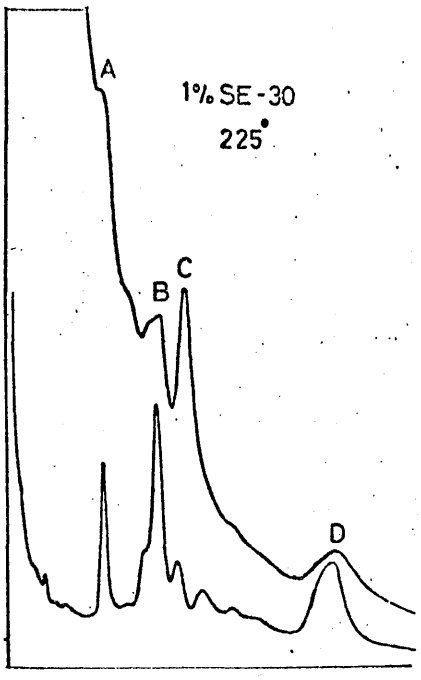
A small quantity of the bis-derivative is formed under normal etherification conditions and this appears as peak 2 in the chromatogram shown in Fig. 22(b). The corresponding, but proportionally larger peak in the chromatogram derived from the extracted material appears to represent a mixture of compounds suggested to be the bis-TMSi ether of 5α- cholestane - 5, 6α- diol plus that of another unidentified cholestane diol. This suggests that the extracted cholestene was not entirely homogeneous.

This is considered to be fairly conclusive evidence of the presence of cholest - 5 - ene in the extract since a combination of gas chromatographic and mass spectrometric information has allowed positive identification of any of the sterols so far examined in our work. A second double bond reaction was attempted to establish this identification beyond doubt.

4.(b) Isomerisation of cholestene epoxide:-

Pertrifluoroacetic acid was used to form epoxides from the extracted cholestene and a reference sample of cholest - 5 - ene. These compounds were treated with boron trifluoride in ether in the manner described on page 110. Using this reaction Henbest and Wrigley (34) were able to form coprostan - 6 - one (5β- cholestan - 6 - one) from cholest - 5 - ene. The products obtained in the present work were examined by GLC and the

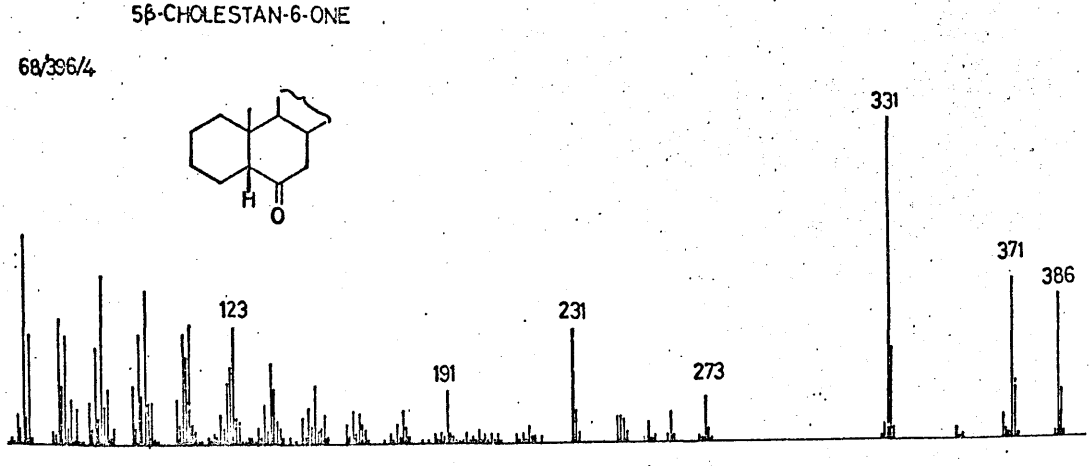
Figure 26. GLC of Coprostan - 6 - one



Upper chromatogram derived from the products of the extracted cholestene.

Lower chromatogram derived from the products of authentic cholest - 5 - ene.

Figure 27. Mass spectrum of Coprostan - 6 - one



chromatograms obtained are shown in Fig. 26. Peak B in the GLC trace of the authenticated material has been shown by GC-MS to be coprostan - 6 - one and peak C to be cholestan - 6 - one. A line diagram of the mass spectrum of peak B is illustrated in Fig. 27 and this has been shown to be identical to a literature mass spectrum of coprostan - 6 - one (35). The fraction produced from the extracted cholestene is as yet insufficiently pure for GC-MS examination, however, the retention times of the major peaks correspond to those produced from cholest - 5 - ene. It is hoped that purification by preparative TLC might enable a GC-MS study.

This additional evidence again suggests that the bulk of the extracted cholestene is almost certainly cholest - 5 - ene.

Assignment of the position of the double bonds in the extracted cholestadiene.

The extracted cholestadiene has been shown to be conjugated since it possesses a strong ultra-violet absorption. Early determinations were hampered by an inability to remove other hydrocarbons from the fraction being examined. Development of purification methods involving the use of silver nitrate TLC has recently enabled more satisfactory measurements to be made. The results obtained are shown below:-

λ max (approx.)	ε (approx.)
227 - 230	$17 - 20 \times 10^3$
234 - 239	$18 - 20 \times 10^3$

Although only approximate, these values are fairly close to the literature values for cholesta - 3,5-diene (38). However, because of the ranges of wavelength and intensity recorded cholesta - 4,6-diene cannot be excluded from consideration. It is hoped that this problem will be solved by the use of molecular rotation since it is known that the isomers mentioned above have widely different optical rotations (39). This technique however, requires a fairly large pure sample and so work is in progress to collect such a sample.

Quantities of hydrocarbons

Squalene was the major hydrocarbon in all plaque tissue examined. The quantities of this compound obtained varied quite markedly from sample to sample, but appeared to be greatest in the severest cases of the disease. However, this increased amount might have resulted from the inclusion of parts of medial as well as intimal tissue, since in these cases plaques frequently extended through the media. In these examples the media could not be separated from the intima. An average value was of the order of 50 - 100μg. of squalene per g. of lipid.

Cholest - 5 - ene was present at concentrations of about 20 - 30μg./g. of lipid and cholestadiene at about 10μg./g.

During the course of our work two publications (36, 37) have emerged relating to the hydrocarbons of the human artery

wall. The first of these articles has confirmed the presence of squalene in the aorta using thin-layer chromatography while the other describes an investigation of the hydrocarbons of human femoral arterial plaques. Using TLC the authors have tentatively identified n - octadecane and n - octadecene. However, since only one method of characterisation was used it is possible the compounds actually present were a series of alkanes and a cholestene.

A summary of the compounds identified in this section is given in Key 1 on page 120.

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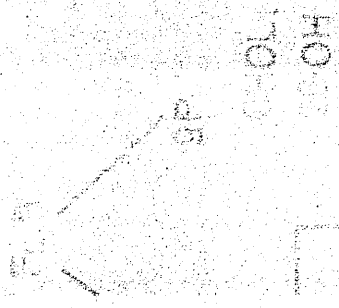
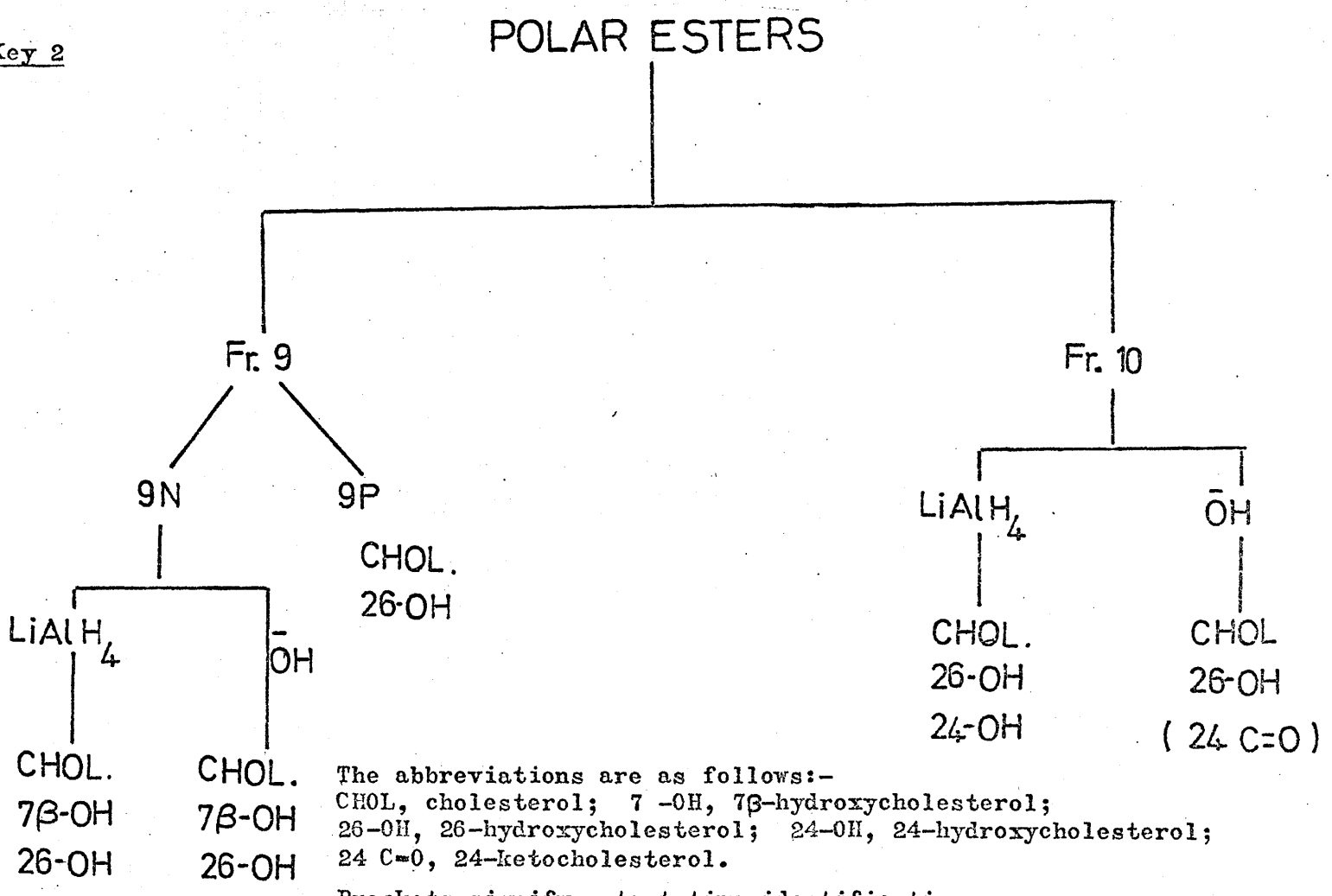
PART II

POLAR ESTERS



Key 2



The abbreviations are as follows:-
CHOL, cholesterol; 7 -OH, 7β-hydroxycholesterol;
26-OH, 26-hydroxycholesterol; 24-OH, 24-hydroxycholesterol;
24 C=O, 24-ketocholesterol.

Brackets signify a tentative identification.

"POLAR ESTERS"

In column chromatography of lipids, there is a grave danger that minor lipid groups may remain unresolved, unless the various fractions obtained are subjected to further chromatography. The necessity for cross-checking is illustrated by the detection, in extracts of the atheromatous plaque, of glyceryl ether diesters (1) in the so-called "triglyceride" fraction as isolated by column chromatography. Minor lipid classes, possibly of great metabolic significance, can frequently be demonstrated by thin-layer chromatography although not apparent in column elution histograms. The common practice of examining total lipid extracts by TLC alone also frequently leads to certain groups of compounds escaping detection although they are present in appreciable quantities.

At the start of our search for minor lipids in the atheromatous plaque we were greatly encouraged by a recent identification of a new type of sterol ester in blood by Crastes de Paulet and Crastes de Paulet (2). This new type of lipid was detected in the blood of patients suffering from hyper-cholesterolemia,[x] but could not be demonstrated in normal blood. Having regard to the "infiltration theory" of plaque formation,

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[x] A disease involving an exceptionally high concentration of cholesterol in the blood and closely related to atherosclerosis.

it seemed possible that this type of ester might be of importance in the atheromatous plaque.

On examination of column eluates of hypercholesterolemic blood lipid by paper chromatography Crastes de Paulet (2) demonstrated the presence of several previously unidentified compounds of polarity intermediate between cholesterol ester and cholesterol. One of these compounds gave the characteristic bright blue colouration of a 7-hydroxycholesterol when sprayed with a chloroform solution of antimony trichloride. This compound was isolated and its infrared spectrum examined. This spectrum was very similar to that of a cholesterol ester, except that it indicated the presence of a free hydroxyl group. Hydrolysis of the extracted ester yielded a compound which was chromatographically identical with a sample of authentic 7α-hydroxycholesterol. Undepressed mixed melting points were carried out both as the free sterol and as the dibenzoate. The authors were aware of the possibility that such a compound might be formed by autoxidation of a cholesterol ester, but ruled this out, since if the ester isolated was an artefact its formation might be expected during extraction and chromatography of normal blood extracts, however, the compound was found only in cases of hyperlipemia.

Boyd (3), using lipid extracts of human and rat blood made under strictly anaerobic conditions, was able to identify the

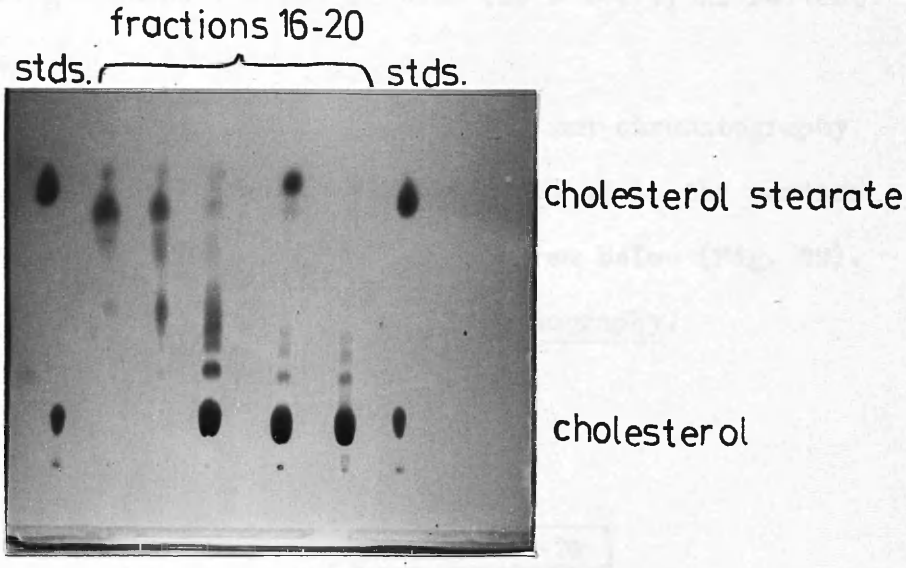
same compound. He also prepared a series of 3β acyl esters of 7α-hydroxycholesterol and demonstrated that these compounds were chromatographically identical to the extracted material. Using infrared spectroscopy he was able to establish that it was the 3- rather than the 7-hydroxyl group that was esterified.

Encouraging results on polar esters of this type obtained in our own laboratory (4) using hypercholesterolemic blood also added impetus to our plaque study.

The present work

On the basis of the findings described above, we set out to look for esters of this type in extracts of the atheromatous plaque. It had been shown by previous workers (2) that the "polar" esters were eluted from a silicic acid column after the normal cholesterol esters but before cholesterol. We therefore carefully monitored silicic acid column eluates in this chromatographic region by TLC, and Fig. 28 illustrates a typical chromatogram obtained. It shows traces of unidentified compounds in the R_F range 0.3 to 0.7 in fractions 16 to 20. The extreme right and left-hand lanes of the chromatogram show a reference mixture of cholesterol stearate (R_F = 0.85) and cholesterol (R_F = 0.19). In addition to "polar" esters, fractions 16 and 17 contain an appreciable quantity of

Figure 28 TLC of silicic acid column eluates.



spray:- 1% ceric sulphate in 10% sulphuric acid
Solvent:- benzene : ethyl acetate (20 : 1 v/v)
adsorbent:- MN-Kieselgel G-HR.

triglyceride, whereas fractions 18 to 20 contain cholesterol with little or no triglyceride.

At this point we had two alternative methods of concentrating these minor compounds,

(1) by preparative thin-layer chromatography, or

(2) by column chromatography.

In most instances column chromatography was the method chosen since the quantity of material involved (as high as 1 g. in some instances) was usually rather large for TLC. Fraction 18 in the example shown in Fig. 28 was however considered to be an

exceptional case and was separately purified by preparative TLC using benzene : ethyl acetate (20 : 1 v/v) as solvent system.

Florisil was chosen for the column chromatography (see page 104) and the solvents used to elute the various components are shown in the flow diagram below (Fig. 29).

Figure 29 Florisil column chromatography.

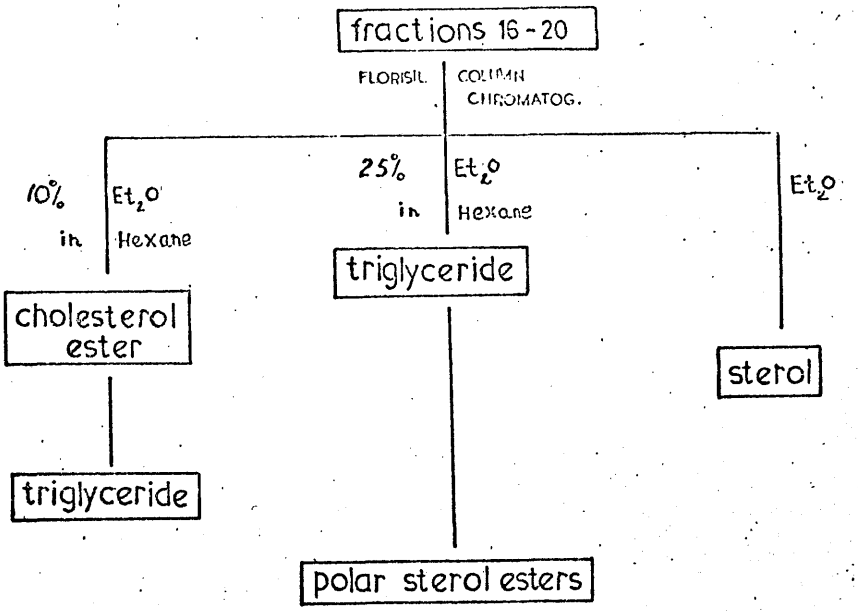


TABLE 13. Florisil column chromatography

50 g Florisil and 1.04 g lipid

Fraction No.	Eluting solvent	Volume ml.	Result of micro TLC examination
1 - 6	10% Et_2O: Hexane	300 (6 x 50)	cholesterol esters
7	25% Et_2O: Hexane	50	two spots in cholesterol ester region and a little triglyceride
8	25% Et_2O: Hexane	50	triglycerides and start of "polar esters"
9	25% Et_2O: Hexane	50	triglycerides and "polar esters"
10	25% Et_2O: Hexane	50	"polar esters" and free sterols
11	25% Et_2O: Hexane	50	sterols
12	25% Et_2O: Hexane	50	sterols
13	Et_2O	100	sterols

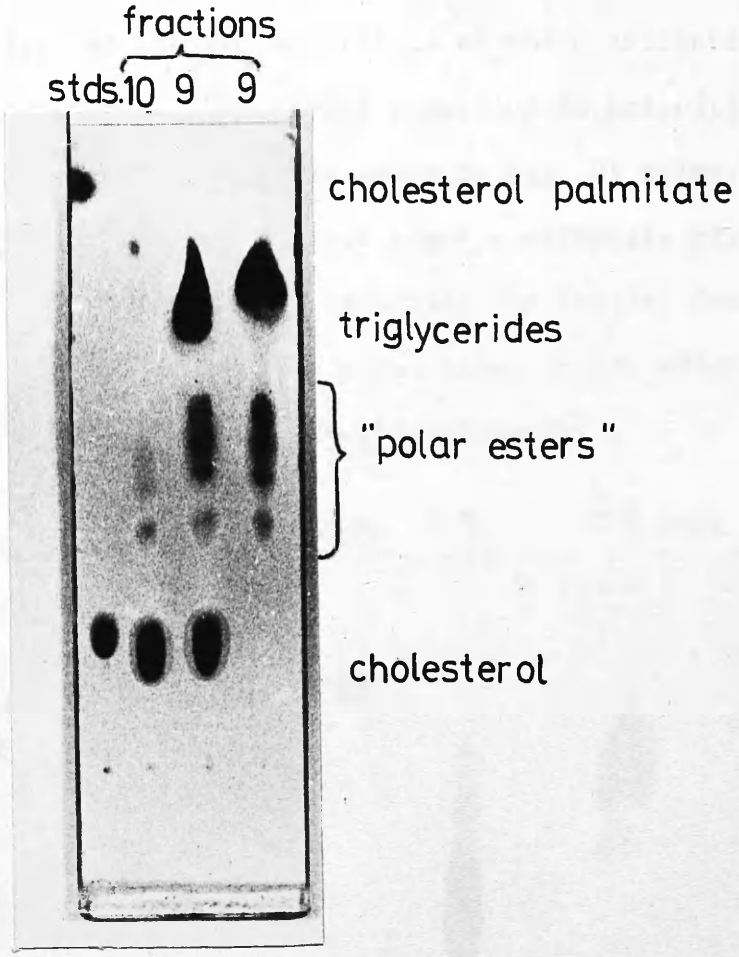
Table 13 illustrates typical solvent volumes used in the elution of a Florisil column and also the results of a micro TLC examination of each fraction. Elution of sterols was quickly completed with ethyl acetate when TLC showed that all of the "polar ester" material had been removed from the column.

Further purification of "polar esters"

Fractions 9 and 10 from the Florisil column cited above contained the bulk of the "polar ester" material and their TLC behaviour is shown in Fig. 30. From this chromatogram it can be seen that fraction 9 contained a considerable amount of cholesterol and triglycerides in addition to polar esters, whereas fraction 10 showed only cholesterol as impurity and contained a smaller quantity of "polar esters". Both samples were further purified by preparative TLC in benzene:ethyl acetate (20:1 v/v).

Figure 30 TLC of "polar ester" fractions 9 and 10

(after Florisil column chromatography)



spray:- 1% ceric sulphate in 10% sulphuric acid

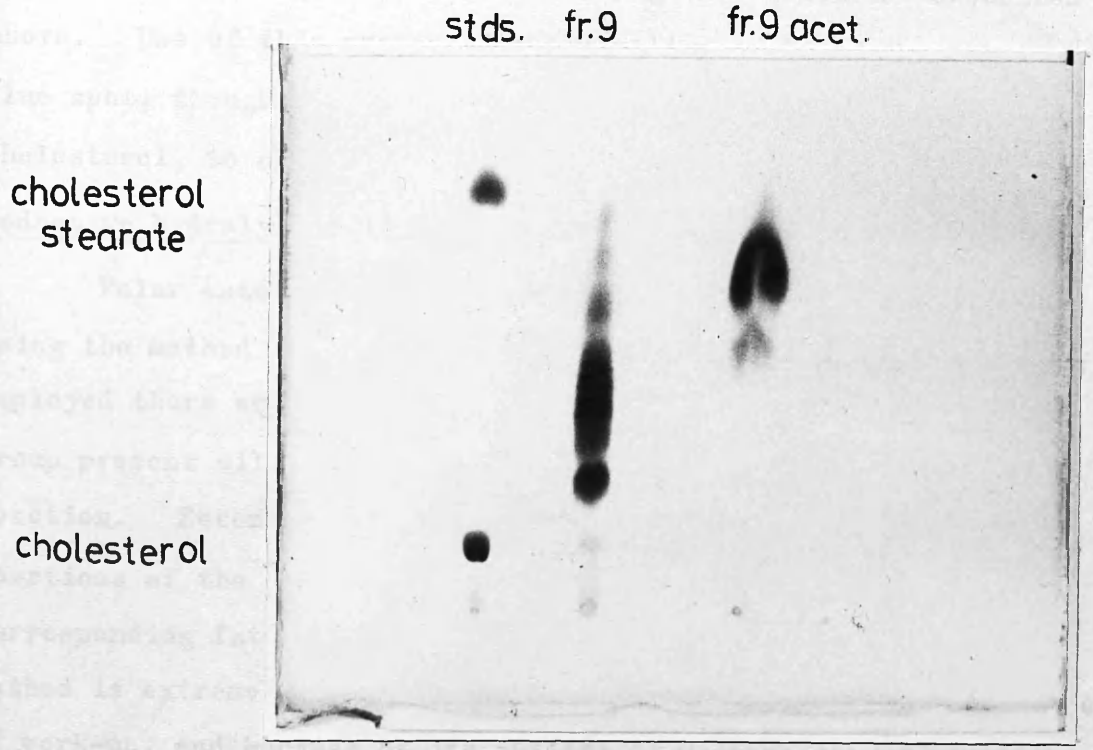
solvent:- benzene : ethyl acetate (20 : 1 v/v)
adsorbent:- MN-Kieselgel G-HR

The extreme right-hand lane shows fraction 9 after removal of cholesterol by preparative TLC.

Free hydroxyl groups in the "polar esters"

The presence of unesterified hydroxyl groups in the bulk of the material in purified fractions 9 and 10 was demonstrated by comparison of the TLC mobilities of these extracts before and after acetylation. The marked reduction in polarity of fraction 9 following esterification is shown in Fig. 31 below. In this chromatogram the left-hand lane shows a reference mixture of cholesterol and cholesterol stearate, the centre, fraction 9 and the right-hand lane the same polar ester sample after acetylation.

FIGURE 31 TLC of "Polar ester" fraction 9



solvent:- benzene: ethyl acetate (20:1 v/v)

spray:- 1% ceric sulphate in 10% sulphuric acid

When run on TLC and sprayed with an acidic solution of ceric sulphate, fraction 9 showed a series of predominantly red-brown spots broken by two spots with an intense blue colouration (R_F values of 0.50 and 0.38 in benzene:ethyl acetate 20:1 v/v). The more mobile of these two blue coloured components was present in a considerably larger quantity. In contrast, fraction 10 appeared to contain only the esters which gave the red-brown colouration.

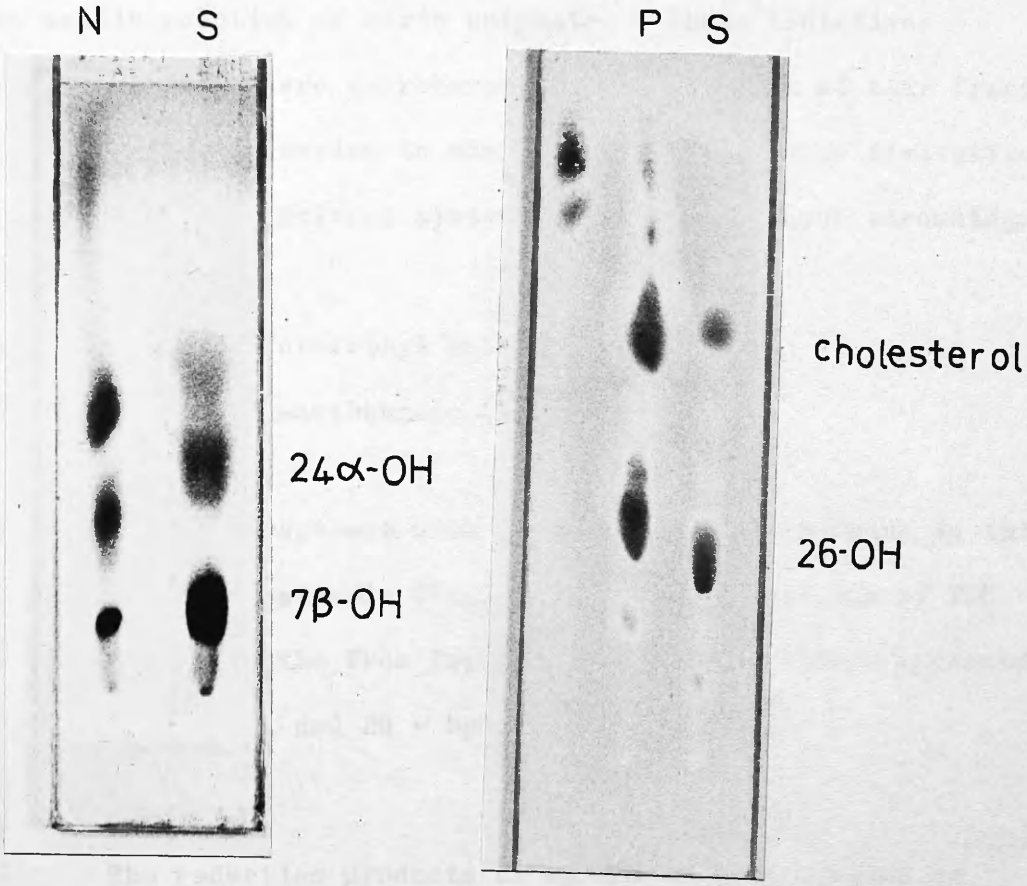
In an attempt to simplify matters an aliquot of fraction 9 was divided into two portions, designated 9N (less polar) and 9P (more polar) by preparative TLC using the conditions described above. Use of this preparative separation confined the larger blue spot, thought to be due to the esters of 7α-hydroxy-cholesterol, to aliquot 9N.

Reductive hydrolysis

Polar ester aliquots 9P and 9N were reductively hydrolysed using the method described on page 109. When such a procedure is employed there are two problems which arise. Firstly, any keto group present will be converted into a hydroxyl group during the reaction. Secondly, after reductive hydrolysis, the fatty acid portions of the ester molecules will exist in the form of the corresponding fatty alcohols. Apart from these disadvantages the method is extremely attractive because of its rapidity and the ease of work-up, and because of its ability to prevent the elimination of labile hydroxyl groups (e.g. the 7α-hydroxyl group of 7α-hydroxy-cholesterol) which might ensue from the use of strong alkali.

Thin-layer chromatograms obtained from the products of the treatment of 9N and 9P with lithium aluminium hydride are shown in Fig. 32.

FIGURE 32



Solvent system:- chloroform:ethyl acetate (3:1 v/v)
N = fraction 9N after treatment with Li Al H_4
P = fraction 9P after treatment with Li Al H_4
S = reference sterols
spray:- 1% ceric sulphate in 10% sulphuric acid

Fraction 9N:- Fraction 9N yielded compounds which corresponded to cholesterol (R_F = 0.66, maroon); 26-hydroxycholesterol (R_F = 0.28, maroon) and 7β-hydroxycholesterol (R_F = 0.18, blue) in both mobility and colour reaction, when the chromatograms were developed in chloroform:ethyl acetate (3:1 v/v) and sprayed with an acidic solution of ceric sulphate. These tentative identifications were corroborated by examination of this fraction by TLC after conversion to the trimethylsilyl ether derivatives. (Fig. 33). The solvent systems used in thin-layer chromatography were as follows:

sterols - chloroform:ethyl acetate (3:1 v/v)

TMSi ethers - hexane:benzene (2:1 v/v).

Fraction 9P:- Treatment with lithium aluminium hydride in this case yielded two sterols (Fig. 32) which on the basis of TLC evidence both in the free form and as the TMSi ethers appeared to be cholesterol and 26 - hydroxycholesterol.[x]

GLC Examination

The reduction products of 9N and 9P were studied by gas-liquid chromatography (1% SE-30; 225°C) as TMSi ethers and the chromatograms obtained are illustrated in Fig. 34, where

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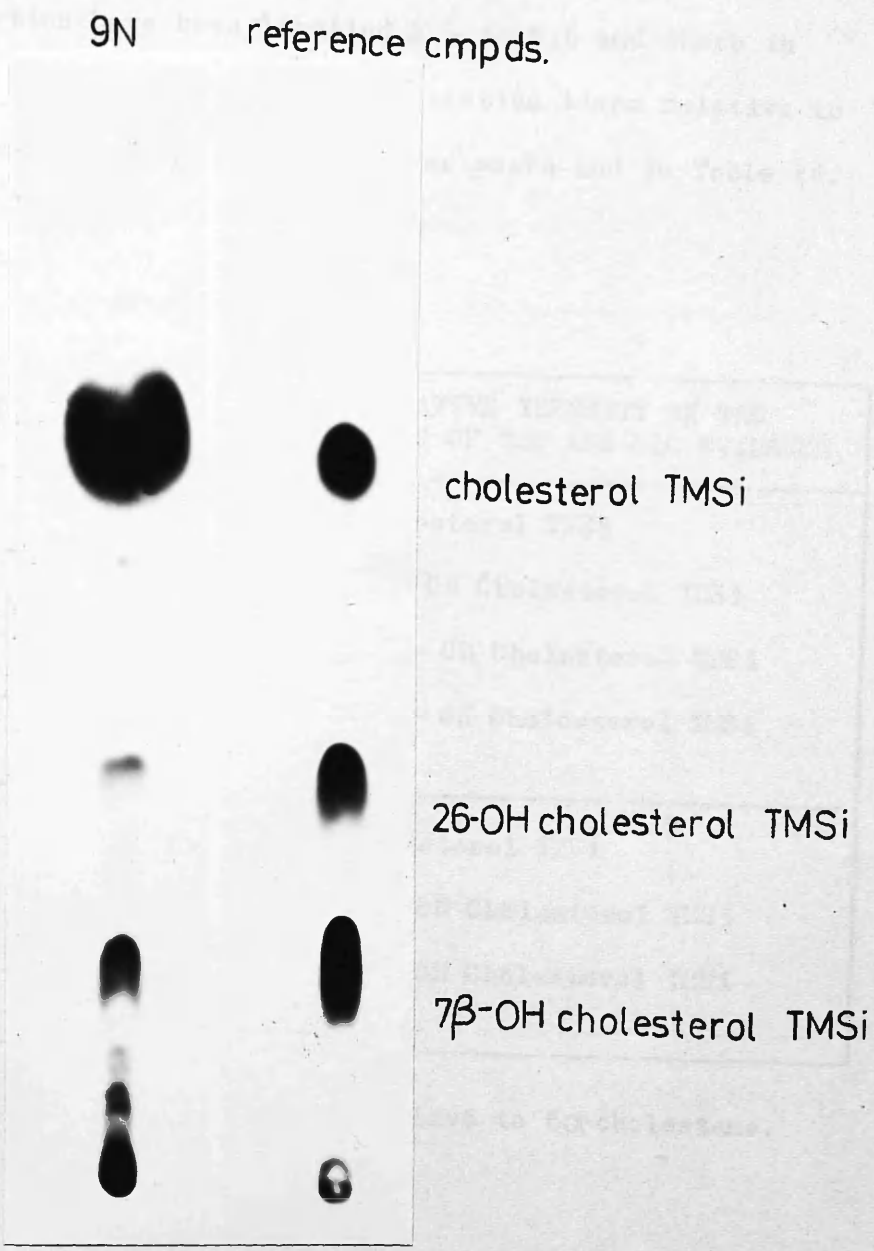
[x] 26 - Hydroxycholesterol was being concurrently studied in relation to the unesterified sterols present in the atheromatous plaque.

FIGURE 33 TLC of fraction 9N after treatment with lithium aluminium hydride (TMSi ethers).



Solvent:- hexane : benzene (2:1 v/v)
adsorbent:- MN-Kieselgel G-HR.
spray:- 1% ceric sulphate in 10% sulphuric acid

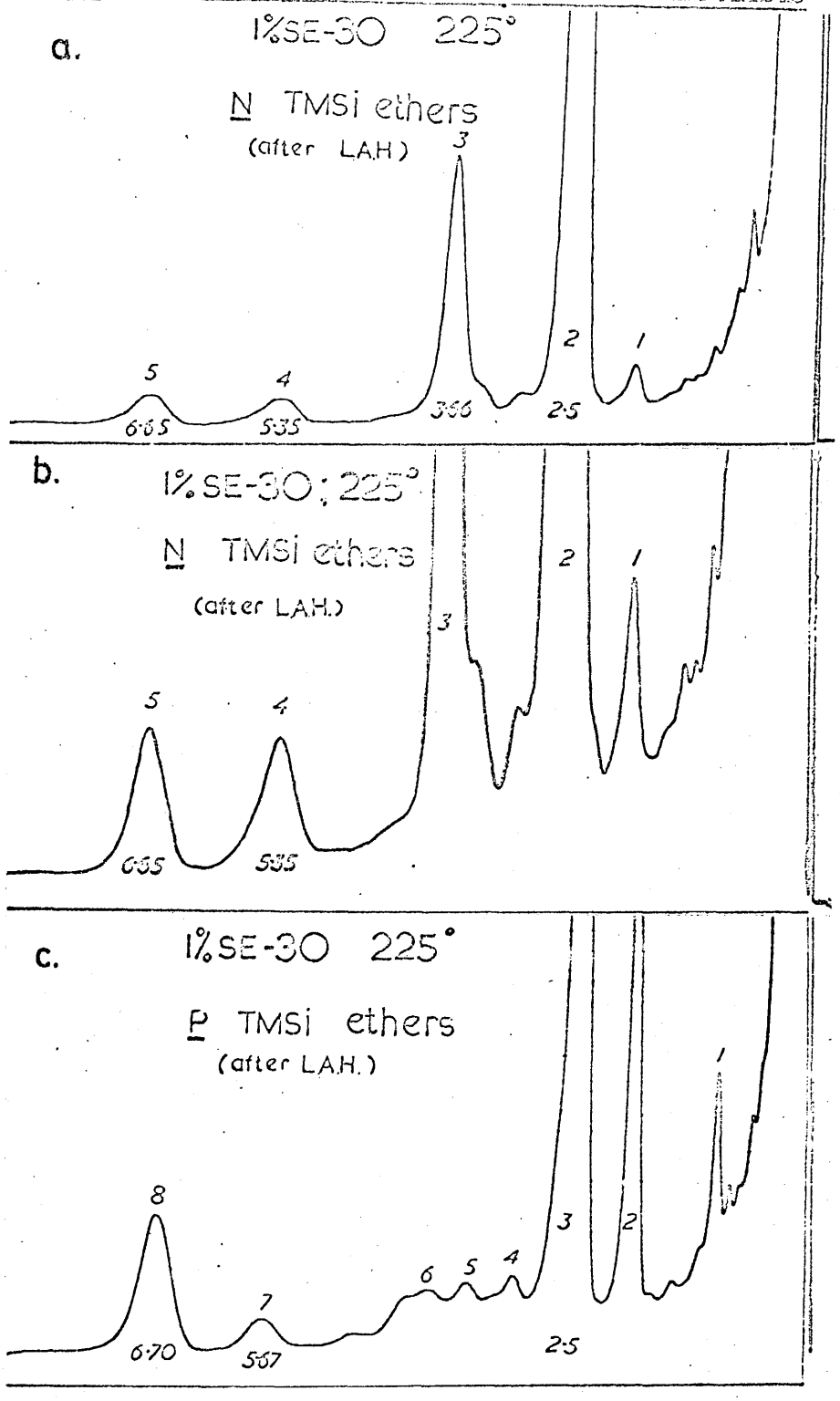
(a) and (b) record the behaviour of 9N at two different concentrations under the same conditions. The peaks in this fraction have been labelled N.1 to N.5 and those in 9P (Fig. 34 (c)), P.1 to P.8. Retention times relative to cholestane are given under the major peaks and in Table 14.

Table 14.

COMPOUND	t_R	TENTATIVE IDENTITY ON THE BASIS OF TLC AND GLC EVIDENCE
N.2	2.50	Cholesterol TMSi
N.3	3.66	7β- OH Cholesterol TMSi
N.4	5.35	24α- OH Cholesterol TMSi
N.5	6.65	26 - OH Cholesterol TMSi
P.3	2.50	Cholesterol TMSi
P.7	5.67	25 - OH Cholesterol TMSi
P.8	6.70	26 - OH Cholesterol TMSi

where t_R = retention time relative to 5α-cholestane.

FIGURE 34 GLC OF 5N AND 5P AFTER REDUCTIVE HYDROLYSIS



retention times relative to cholestane are given under the major peaks.

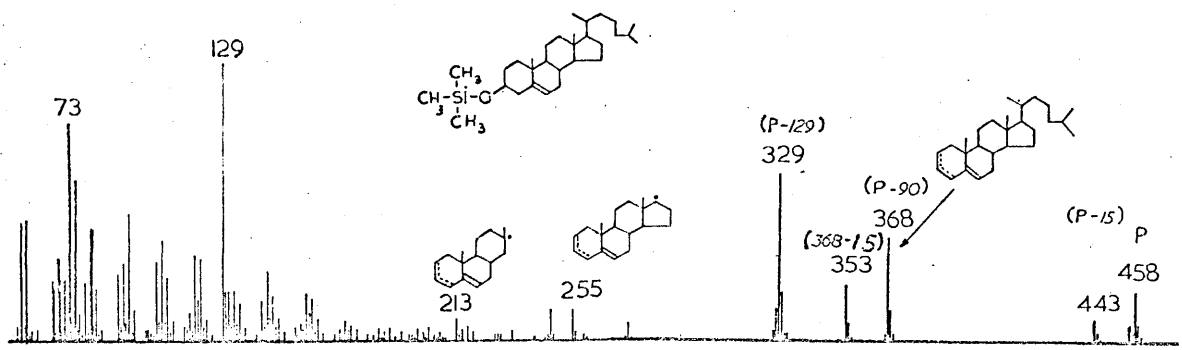
GC-MS Examination

Mass spectra were recorded for the major peaks in the chromatograms shown in Fig. 34 using an LKB 9000 instrument.

Fraction 9N

Peak N.2:- A line diagram of the mass spectrum of peak N.2 is shown in Fig. 35.

FIGURE 35. MASS SPECTRUM OF COMPOUND N.2



In agreement with chromatographic evidence this mass spectrum was found to be identical to that of cholesterol (cholest-5 - en - 3β- ol) TMSi ether as recorded by ourselves and other workers (6, 7). The identification of cholesterol in this fraction poses a new problem. It finally dispels any idea that the major sterol in this fraction could be 7-dehydro-cholesterol resulting from dehydration of an ester of 7α-hydroxycholesterol during hydrolysis. No detectable quantity of 7 - dehydrocholesterol trimethylsilyl ether could be demonstrated in this fraction. On the other hand, all

unesterified cholesterol had been removed from the original polar ester fraction before treatment with lithium aluminium hydride. We therefore have to postulate the presence in the extract of a "polar ester" which will yield cholesterol on hydrolysis. Two types of compound which could possibly yield cholesterol on reductive hydrolysis and have the correct TLC mobility are:-

(a) a hydroxy fatty acid ester of cholesterol, or

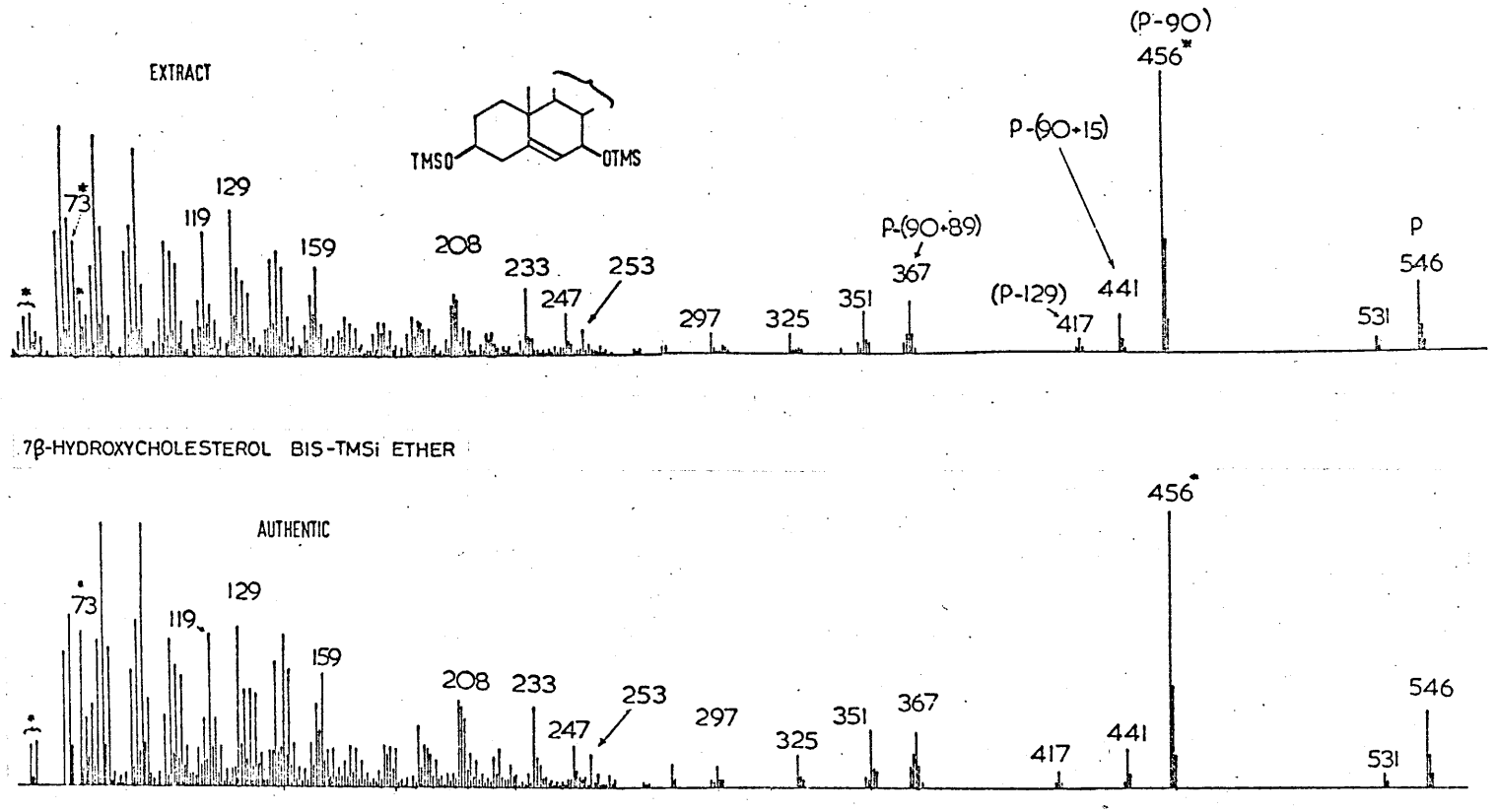
(b) a normal fatty acid ester of a compound such as cholestane-3β,5(or 6) - diol, which might eliminate water to form a double bond in the 5-6 position of the steroid nucleus.

Hypothesis (b) seems unlikely since 5α-cholestane-3β, 5-diol, 5α-cholestane-3β,6β-diol and 5α-cholestane-3β,6α-diol all fail to dehydrate to cholesterol on treatment with lithium aluminium hydride. It was thus important to examine the fatty acids produced by alkaline hydrolysis or the fatty alcohols formed by reductive hydrolysis of a polar ester fraction. Work of this kind is described later on page 186.

Peak N.3:- The mass spectrum obtained for peak N.3 is shown below in Fig. 36. Since the fragments at m/e 456 and 73 dwarf all other ions, the line diagram has been drawn with these peaks at one tenth of their true intensity to bring out the details of the fragmentation. The spectrum represents a dihydroxy cholestene bis-TMSi ether (molecular weight 546). On the basis of its TLC behaviour and intense blue colour reaction with ceric

FIGURE 36

Compound N.3



peaks marked with an asterisk are 1/10th of true intensity

sulphate this compound was thought to represent a trimethylsilyl ether of a 7-hydroxycholesterol. Comparison of the spectrum of compound N.3 with those of 7α- and 7β-hydroxycholesterol trimethylsilyl ethers tends to further the view expressed above since all three spectra are very similar. The most striking feature is the preponderance of the m/e 456 fragment, and also the relatively small intensity of the m/e 129 fragment in contrast to most other Δ^5-3β-ol trimethylsilyl ethers. Unfortunately, mass spectrometry is unable to differentiate between the epimeric 7α- and 7β-hydroxycholesterols: however, they can be distinguished by TLC and GLC in the form of their TMSi ethers as is shown in Table 15 and Figs. 37 and 38.

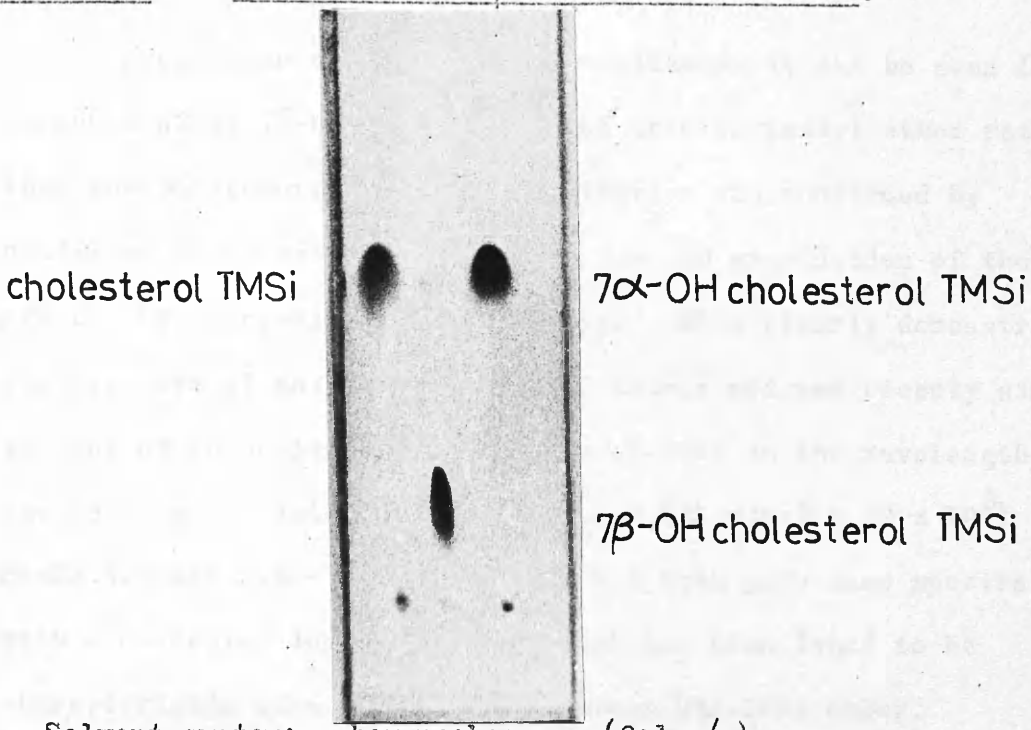
Table 15

COMPOUND	t_R	R_F	Colour reaction with ceric sulphate
7α-OH cholesterol bis-TMSi	2.62	0.53	blue
7β-OH cholesterol bis-TMSi	3.69	0.16	blue
N.3	3.66	0.17	blue

t_R = retention time relative to cholestane on 1% SE - 30 at 225°C.

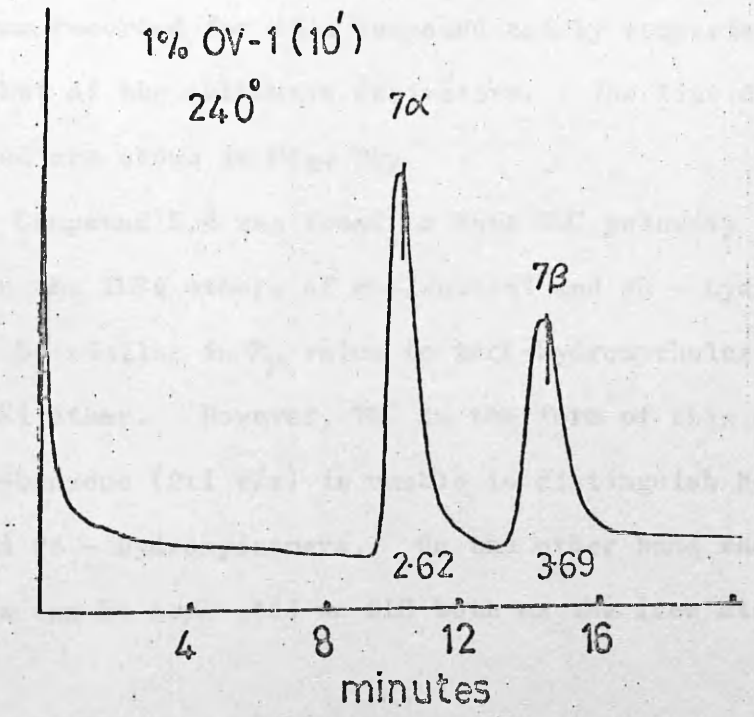
R_F values were recorded using hexane:benzene (2:1 v/v) as solvent system.

FIGURE 37 TLC OF 7α AND 7β-OH CHOLESTEROL TMSi.



Solvent system:- hexane:benzene (2:1 v/v)
spray:- 1% ceric sulphate in 10% sulphuric acid

FIGURE 38 GLC OF 7α AND 7β-OH CHOLESTEROL TMSi.

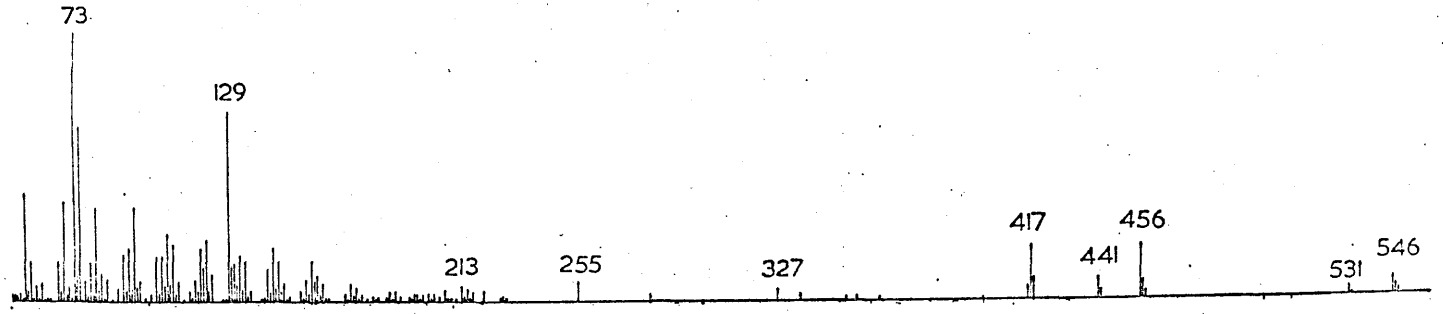


From these chromatographic mobilities it can be seen that compound N3 is 7β-hydroxycholesterol trimethylsilyl ether rather than the 7α-isomer. This identification was confirmed by oxidation of N3 with manganese dioxide and examination of the product by ultra-violet spectroscopy. This clearly demonstrated the presence of an α,β-unsaturated ketone and was closely similar to that of authentic 7-ketocholesterol both in the wavelength of the maximum and intensity. ($\lambda_{max.}^{EtOH}$ = 237 mμ; ε = 12 x 10^3)

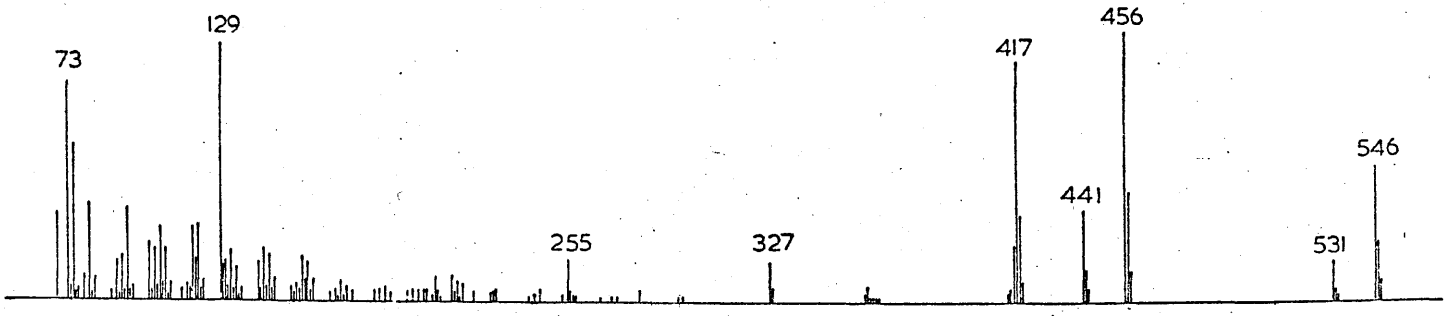
Peaks N.4 and N.5:- Peaks N.4 and N.5 both gave mass spectra with a molecular ion at m/e 546 which has been found to be characteristic of a dihydroxycholestene bis-TMSi ether. TLC and GLC evidence suggested that compound N.5 might be 26 - hydroxycholesterol bis-TMSi ether. This tentative identification was confirmed by examination of the mass spectrum recorded for this compound and by comparison of this with that of the authentic derivative. The line diagrams thus obtained are shown in Fig. 39.

Compound N.4 was found to have TLC polarity intermediate between the TMSi ethers of cholesterol and 26 - hydroxycholesterol and to be similar in R_F value to 24α-hydroxycholesterol bis-TMSi ether. However, TLC in the form of this derivative in hexane-benzene (2:1 v/v) is unable to distinguish between the 24α and 25 - hydroxyisomers. On the other hand these isomeric sterols can be separated on GLC both as the free sterol and as

FIGURE 39 Peak N.5



Authentic 26-hydroxycholesterol bis-TMSi ether



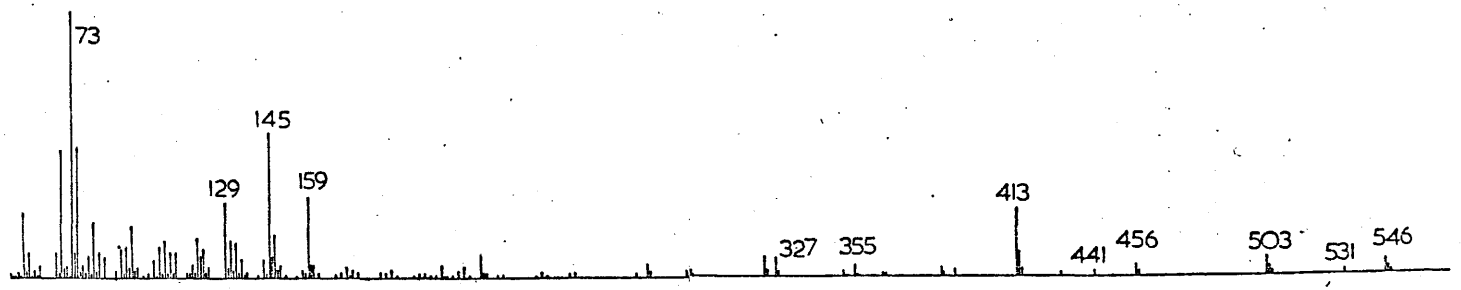
the TMSi ether (see Appendix I, page 269).

This latter fact, used in conjunction with mass spectrometry, enabled the identification of peak N.4 as 24α-hydroxycholesterol bis-TMSi ether, and line diagrams of extracted and reference derivatives are illustrated in Fig. 40. Since no reference sample of the 24β-hydroxyepimer (cerebrostendiol) was available we cannot say with certainty that we could differentiate between this compound and the 24α-hydroxy compound. Nevertheless, our experience with epimeric pairs of sterols shows that they are almost invariably separable by TLC or GLC in the form of the TMSi ethers. This effect probably results from the fact that this bulky ether grouping greatly enhances the stereochemical differences between epimers. Examples from our own experience can be seen in the GLC separations (1% SE 30) of the TMSi ethers of the following pairs: 7α and 7β-hydroxycholesterol; cholest-5-en-3α-ol and cholest-5-en-3β-ol; 5α-cholestan-7α-ol and 5α-cholestan-7β-ol; 5α-cholestan-5,6α-diol and 5α-cholestan-5,6β-diol. (see Appendix I, page 269).

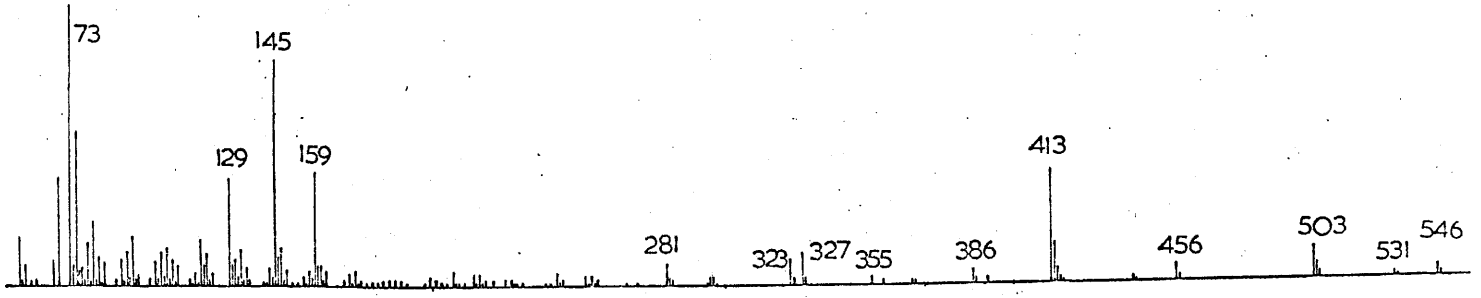
Fraction 9P

In a similar manner, GC-MS used in conjunction with TLC and GLC established that the main components of fraction 9P were the TMSi ethers of cholesterol and 26 - hydroxycholesterol with traces of 7β- and 25 - hydroxycholesterols.

FIGURE 40 Peak N.4.



Authentic 24α-hydroxycholesterol bis-TMSi ether.



Alkaline hydrolysis of "polar ester" fraction 9.

Alkaline hydrolysis is a necessary sequel to reductive hydrolysis, since it is possible that some of the sterols (e.g. 7β-hydroxycholesterol and 24α-hydroxycholesterol) found following the use of the latter technique could have resulted by reduction of the corresponding keto-compounds. An additional advantage resulting from the use of the alkaline method is that the sterols and fatty acid portions of the ester molecules are isolated separately.

Bearing in mind the facile dehydration of 3β-acyl esters of 7α-hydroxycholesterol observed by Boyd (3), a very mild alkaline hydrolysis was carried out on an aliquot of fraction 9 in an attempt to minimise the risk of such an elimination (see page 109). The neutral reaction products were examined by TLC in chloroform:ethyl acetate (3:1 v/v) and the three main sterols found had identical behaviour to cholesterol, 26-hydroxy-cholesterol and a 7-hydroxycholesterol (see Appendix I on page 267). Fig. 41 shows a thin-layer chromatogram of the same saponified polar ester fraction in the form of the trimethylsilyl ether derivatives. Clearly visible in this chromatogram are the TMSi ethers of the sterols tentatively identified by TLC in the free form. However, what cannot be seen in this figure is the important colour reaction which took place during the development of the spot corresponding to cholesterol TMSi ether.

FIGURE 41. TLC OF TMSi ETHERS (FROM FRACTION 9)

Solvent:- hexane:benzene (2:1 v/v)

spray:- 1% ceric sulphate in 10% sulphuric acid

During heating a brilliant blue spot emerged at this position and could still be clearly seen after the full colour development of cholesterol TMSi ether. This is thought to be due to the presence of 7α-hydroxycholesterol bis-TMSi ether which has been found to have a TLC mobility closely similar to the corresponding cholesterol derivative. (see Fig. 37 on page 178). Hydrolysis, followed by separation of the 7-hydroxycholesterols by preparative TLC and repreparation of the TMSi ethers has, however, yielded an unexpectedly small spot for 7α-hydroxycholesterol TMSi on TLC.

Study of Florisil column fraction 10.

The work on "polar esters" outlined above relates to the study of a Florisil column fraction number 9, whereas the following section describes the analysis of the next fraction (No. 10) from the same column. As expected, this sample contained a preponderance of esters which resemble those in fraction 9P. TLC, GLC and GC-MS was carried out on aliquots of this sample after alkaline and reductive hydrolysis. The major compounds were found to be cholesterol and 26-hydroxycholesterol with a much smaller quantity of 7β-hydroxycholesterol. A trace of 24α-hydroxycholesterol which appeared after treatment with lithium aluminium hydride is thought, on the basis of chromatographic evidence, to have resulted from reduction of an ester of 24-ketocholesterol. This has not been confirmed by mass spectrometry owing to the very limited supply of this relatively minor component.

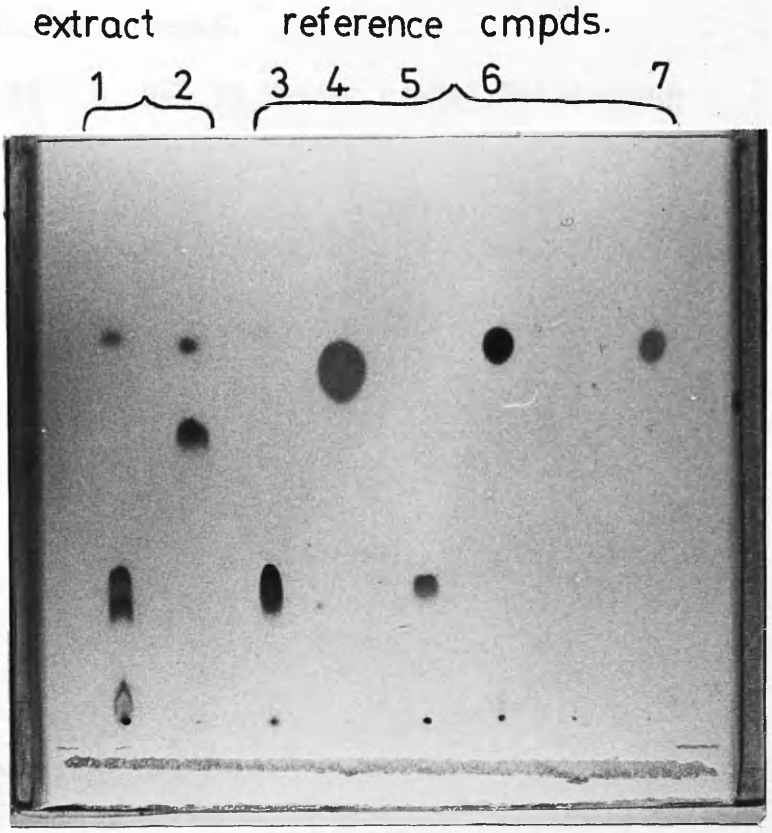
A summary of the sterols identified after hydrolysis of the various fractions is given in Key 2 on page 158.

"POLAR ESTER" FATTY ACIDS

The necessity to examine the fatty acid moieties of the "polar esters" has already been stressed in connection with the proposed existence of a hydroxy fatty acid ester of cholesterol. Three approaches to the problem of the analysis of these acids were employed, and these were dictated by the methods of hydrolysis.

Method 1. Alkaline hydrolysis:- A portion of "polar ester" representative of the total range of compounds present (cf. fraction 9) was hydrolysed under alkaline conditions in the manner described on page 109. After extraction of the alcohols the aqueous layer was acidified and the acids were extracted with ether. The acids were methylated with diazomethane (page 108) and were scrutinized by TLC using benzene:ethyl acetate (20:1 v/v) as solvent system. A typical chromatogram obtained in this manner is shown below in Fig. 42. This indicates that the main components from the extracted acids resemble reference samples of mono-hydroxy fatty acid methyl esters in chromatographic mobility. The existence of a hydroxyl group in the extracted acids was indicated by a marked reduction in TLC polarity following acetylation. A corresponding increase in mobility was observed for the authentic mono-hydroxy fatty acid methyl esters.

FIGURE 42. TLC of fatty acid methyl esters



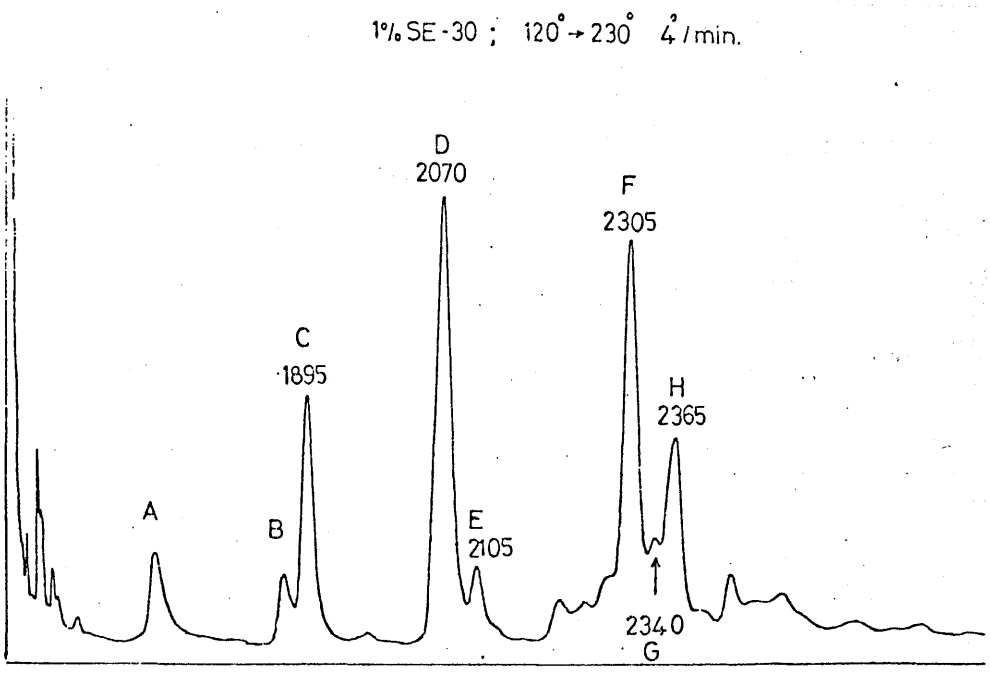
Solvent:- benzene : ethyl acetate (20:1 v/v)

Spray:- Saturated sodium periodate in 10% sulphuric acid.

1. Methyl esters of fatty acids formed on alkaline hydrolysis of "polar esters".
2. Acetylation products of fraction in lane 1.
3. 12-OH Stearic acid methyl ester.
4. Methyl laurate.
5. α-OH Myristic acid methyl ester.
6. Methyl linolenate.
7. Methyl laurate.

Figure 43 shows a GLC study of the fatty acid methyl esters derived from the polar esters, after treatment with hexamethyldisilazane.

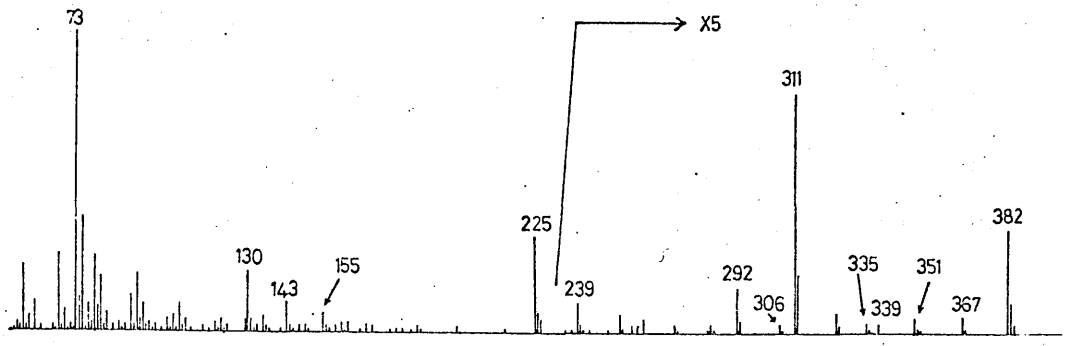
FIGURE 43. GLC of "polar ester" fatty acids



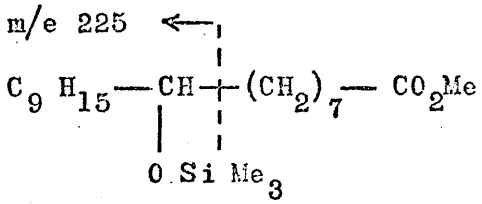
Retention data suggest that peaks A, B, C, D and E represent conventional fatty acid methyl esters and these compounds were unaffected by the attempted trimethylsilylation. However, peaks F, G and H showed a marked sharpening and increased retention times suggestive of the etherification of hydroxyl groups. GC-MS examination confirmed that peaks C and D represented normal fatty acids (C was found to be methyl palmitate and D a mixture of methyl oleate and methyl linoleate).

Almost identical spectra were obtained from peaks F and H suggesting that these might be epimeric compounds. A line diagram of the mass spectrum of peak F is shown in Fig. 43a.

FIGURE 43a. MASS SPECTRUM OF PEAK F.



Fragments at m/e 292 (P - 90), m/e 351 (P - 31) and at m/e 73 indicate that this is the spectrum of a trimethylsilyl ether of a hydroxy acid methyl ester. A possible formal structure is shown below:-



where C_9H_{15} represents a diunsaturated alkyl group and where the abundant fragment of m/e 225 represents cleavage of the ester α to the trimethylsilyl ether group as is indicated above. This argument is based on the assumption that the ion of m/e 225 contains the TMSi ether grouping. Examination of the mass

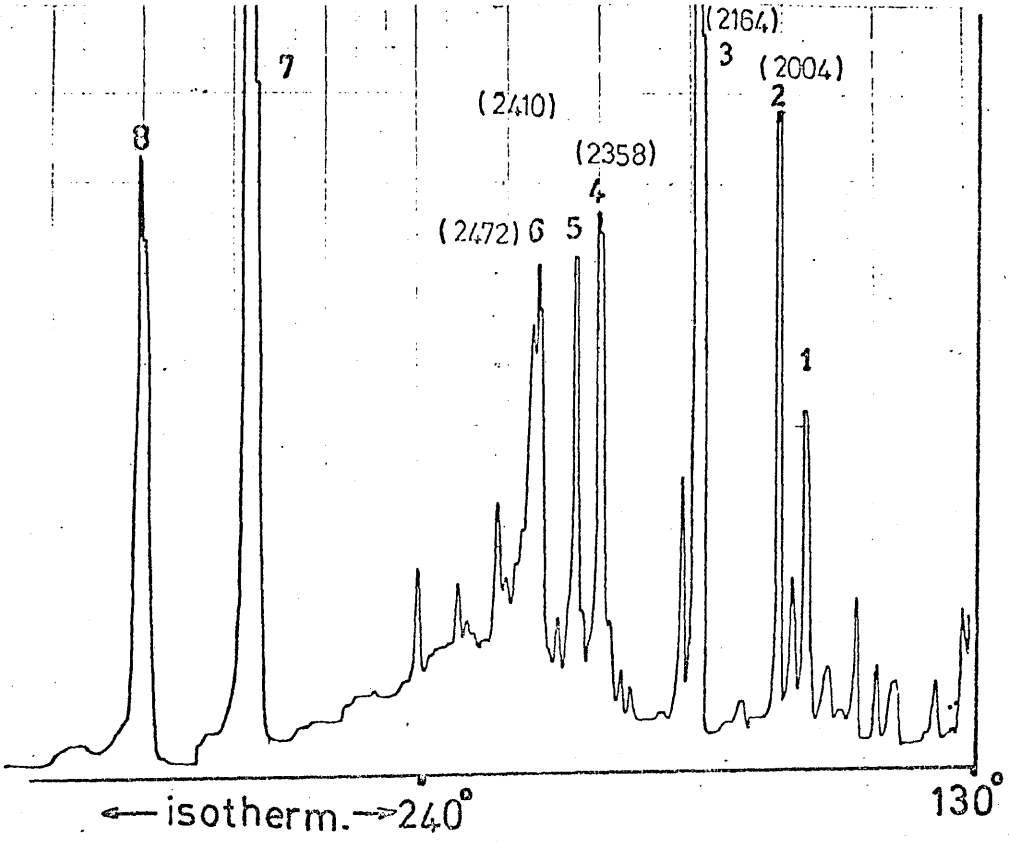
spectrum of the perdeuterated TMSi ether derivative (i.e. R - O $Si(CD_3)_3$) of this molecule shows a shift of + 9 mass units of the m/e 225 ion which indicates that this fragment contains the ether grouping (8). The location of the proposed double bonds in the $C_9 H_{15}$ alkyl group has still to be settled. It is proposed to attempt this by the procedure of Niehaus and Ryhage (9) viz. by hydroxylation with osmium tetroxide and conversion compound to the polymethoxy methyl ester. Examination of the mass spectrum of this ester should indicate the location of the double bonds.

Method 2. Reductive hydrolysis.

A study was made of the alcohols produced on reductive hydrolysis of a type N polar ester fraction. The work already described has shown that this type of fraction (cf. fraction 9N) yields cholesterol and 7β-hydroxycholesterol as the major sterols after reduction. Since the reduced fractions contain fatty alcohols as well as sterols, they show a relatively large range of molecular weights and hence are best examined by GLC using temperature programming. Fig. 44 shows a total ion current record of the chromatographic behaviour of the liberated compounds as their trimethylsilyl ethers.

Peaks 7 and 8 in Fig. 44 are on the isothermal part of the chromatogram and were shown by GC-MS to represent the TMSi ethers of cholesterol and 7β-hydroxycholesterol respectively.

FIGURE 44. TOTAL ION CURRENT CHROMATOGRAM

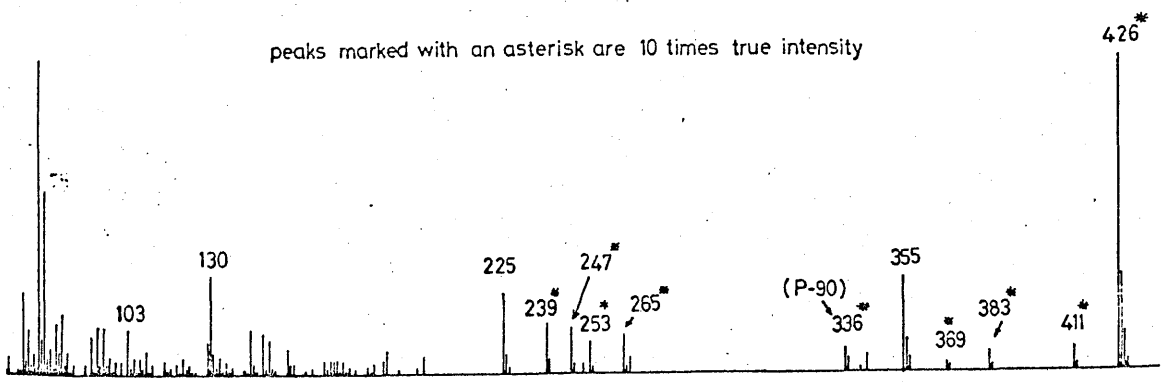


1% SE-30 (9 ft.) temperature programming from 130° to 240° at 3°/min.

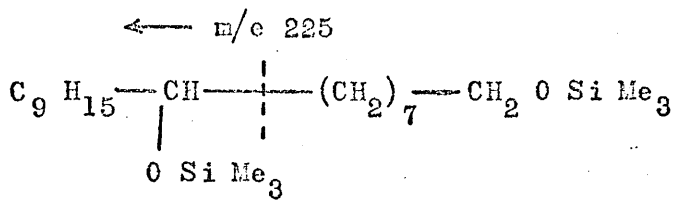
Retention indices given in brackets over the major peaks.

The mass spectra of these compounds have been discussed earlier on pages 174 and 175. Compounds 1 to 6 represent TMSi ethers of the reduced fatty acid portions of these sterol ester molecules. Peak 2 was found to be palmityl (16:0) alcohol TMSi and peak 3 a mixture of the TMSi ethers of oleyl (18:1) and linoleyl (18:2) alcohols. Compounds 4 and 5 in Fig. 44 gave almost identical mass spectra and are thought to be reduction products of compounds F and H in Fig. 43. A line diagram of the mass spectrum of peak 4 is shown in Fig. 45. As can be seen from the diagram, compound 4 contains an important ion at m/e 225 which has already been discussed with respect to compound F in Fig. 43. The change in molecular weight in going from compound F to compound 4 (+ 44 mass units) corresponds exactly to the expected change for the conversion of a methyl ester grouping (- CO_2Me) to the TMSi ether derivative of a primary alcohol (- CH_2SiMe_3). Thus, a possible

FIGURE 45. MASS SPECTRUM OF PEAK 4



structure for compound 4 is as follows:-



Method 3. Alkaline transesterification

A similar, but not identical type N fraction to the one described above was subjected to alkaline transesterification with sodium methoxide (see page 109), a reaction expected to produce fatty acid methyl esters and sterols from "polar esters". Again due to the large range of molecular weights expected, the products were examined by GLC using temperature programming. Fig. 46 shows the chromatogram obtained. Retention data suggested that peaks A and B in the chromatogram corresponded to normal fatty acid methyl esters. GC-MS confirmed this, showing that peak A is methyl palmitate and peak B is a mixture of methyl oleate and linoleate. Attempts to trimethylsilylate peaks C and D were unsuccessful, which was unexpected, since these were considered to arise from hydroxy fatty acid methyl esters. A mass spectrum of compound C is shown below in Fig. 47. From this it would appear that we have formed a methoxy fatty acid methyl ester during the transesterification. Assuming this the results can then be related to those from the other two methods of hydrolysis.

FIGURE 46. GLC of polar esters after alkaline transesterification.

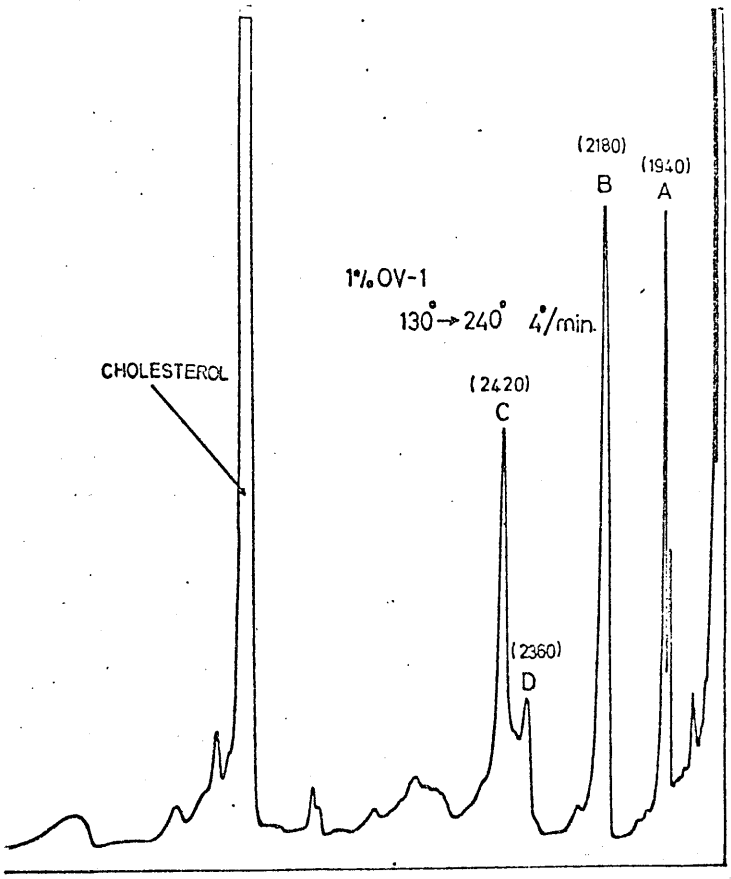
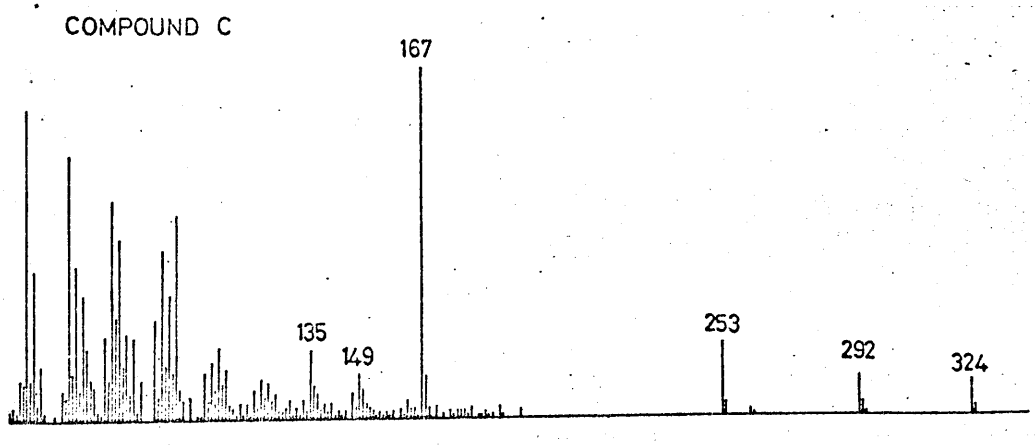
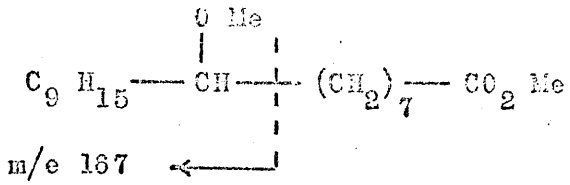


FIGURE 47. Mass spectrum of peak C

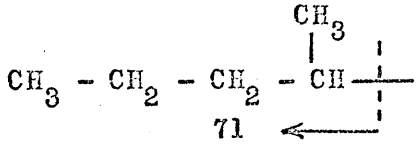


A tentative structure for the molecule is shown below:-

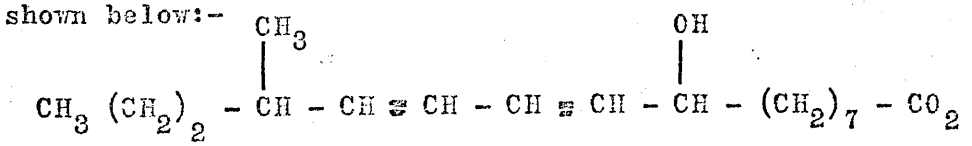


Fragmentation of the molecule ∝ to the methoxyl group as shown in the diagram would account for the base peak (m/e 167) of the spectrum. The presence of a methoxyl group in the molecule is suggested by the loss of 32 mass units (m/e 292) from the molecular ion, corresponding to a loss of methanol, an established fragment for methoxy fatty acid esters (10).

Examination of the mass spectra in Figs. 43a, 45 and 47 shows that in each case there is a loss of 71 mass units from the molecular ion which might correspond to rupture of the molecule adjacent to a site of branching (10) a possible fragment of 71 mass unit is as follows:-



If this hypothesis is correct a tentative structure of the original hydroxy fatty acid methyl ester can be drawn up and is shown below:-



This structure however, remains to be verified.

Although hydroxydienoid fatty acids of this type are unknown as constituents of mammalian tissue, they are well established components of seed oils. Dimorphecolic acid (9-hydroxy-trans, trans - 10,12 octadecadienoic acid) has been identified as the chief constituent fatty acid of Dimorphotheca Aurantiaca seed oil (13). The corresponding 10 - trans, 12-cis has been identified in Calendula Officinalis seed oil (14) and a possible biosynthetic route from linoleic acid has been suggested for both compounds (15). The allylic position of the hydroxyl group in these two plant acids was demonstrated by rapid conversion to the corresponding methoxy compounds in the presence of methanol and dilute acid (16). A similar rapid methoxylation of the acid in the present work during transesterification with dilute alkaline methanol suggests that it might also contain an allylic hydroxyl group. It should be possible to establish this by oxidation and examination of the U.V. spectrum of the resulting keto acid. Preliminary attempts have, however, failed to produce the required absorption for an α, γ-unsaturated ketone but this might be explained by the strong tendancy of such an allylic alcohol to dehydration during oxidation.

Synthetic polar esters.

The work on polar esters in the atheromatous plaque has shown that two broad types of ester exist:-

(a) a hydroxycholesterol with one of its hydroxyl groups esterified with a fatty acid and,

(b) an ester of cholesterol with a polar acyl group presumed to be from a hydroxy fatty acid.

Synthetic polar esters representative of (a) and (b) were prepared in order to check that such compounds were in the correct chromatographic range to correspond to the isolated material.

7α-Hydroxycholesteryl palmitate (type a) was prepared from cholesteryl palmitate as described by Henbest and Jones (11). Reaction of cholesterol acetate with 12-hydroxystearic acid methyl ester in the presence of sodium ethylate (12) enabled the preparation of cholesteryl 12-hydroxystearate (type b). The TLC mobilities of these synthetic esters are given in Table 16.

Table 16. TLC of synthetic polar esters

COMPOUND	R_F	Colour reaction with ceric sulphate
cholesteryl 12-OH stearate	0.43	Maroon
7α-OH cholesteryl palmitate	0.40	Blue
cholesteryl acetate	0.65	Maroon
cholesterol	0.16	Maroon

R_F values were recorded in benzene:ethyl acetate (20:1 v/v)

The extracted esters ran in the R_F range 0.3 to 0.65 in the TLC conditions described above. Thus the synthetic lipids are in the correct mobility zone to correspond to compounds in the isolated material.

Quantities of polar esters isolated

Typical quantities range from 0.5% to 1% of the total purified lipid material. A recent extract has given approximately a 20 mg. mixture of polar esters from 3.9 g. of purified lipid.

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PART III

STEROLS CLOSELY RELATED TO CHOLESTEROL

PLAQUE STEROLS CLOSELY RELATED TO CHOLESTEROL
(a preliminary study)

In 1930, cholestanol (5α-cholestane - 3β-ol) was reported to make up 5-6% of the sterols present in the atheromatous aorta (1). Later work (2) suggested that the quantity of this compound was nearer 12% of the sterol in the diseased artery wall. Recently cholestanol has been confirmed as an arterial component by Mosbach et al. (3) and by Kuroda et al. (4)

The object of our work in this field was to scan plaque extracts in search of this and other close relatives of cholesterol such as 7 - dehydrocholesterol (cholesta - 5, 7 - diene - 3β - ol), which has been reported to be present in various animal tissues (5, 6) and in human brain (7). Similarly, desmosterol (cholesta - 5, 24 - diene - 3β - ol) has been identified in human brain (7) as well as skin (8). Perhaps relevant to the present investigation is the fact that traces of 7 - dehydrocholesterol and desmosterol have been detected in the blood of patients suffering from hypercholesterolemia (9).

The present work

The main problem in this study has been to detect very small percentages of sterols in the presence of an overwhelming mass of their close relative cholesterol. Small separations

between the latter sterol and the compounds mentioned above are possible by conventional methods when equal proportions exist; however, when the quantity of cholesterol is perhaps one hundred times greater, new methods have to be sought. Such a method, which enables the differentiation of sterols differing only in the number and position of their double bonds, involves impregnation of the silica gel adsorbent used in TLC with silver nitrate (10). Further separation improvements ensue from the use of this system with acetate (11, 13) or propionate (12) derivatives. The work described below relates to the use of sterol propionates in conjunction with modified thin layers of silica gel.

TLC of plaque sterols on modified layers

The material used in this investigation was a total sterol fraction from the initial silicic acid column (see page 102) after the polar sterols had been selectively removed in methanol. TLC examination suggested that the only sterol present was cholesterol. Propionate derivatives were prepared (see page 107) and the products purified by sublimation. The purified material was chromatographed preparatively on 20 cm x 20 cm. TLC plates impregnated with silver nitrate, using hexane: benzene (5:1) v/v as developing solvent. Four areas were scraped from the preparative plates after location of the various

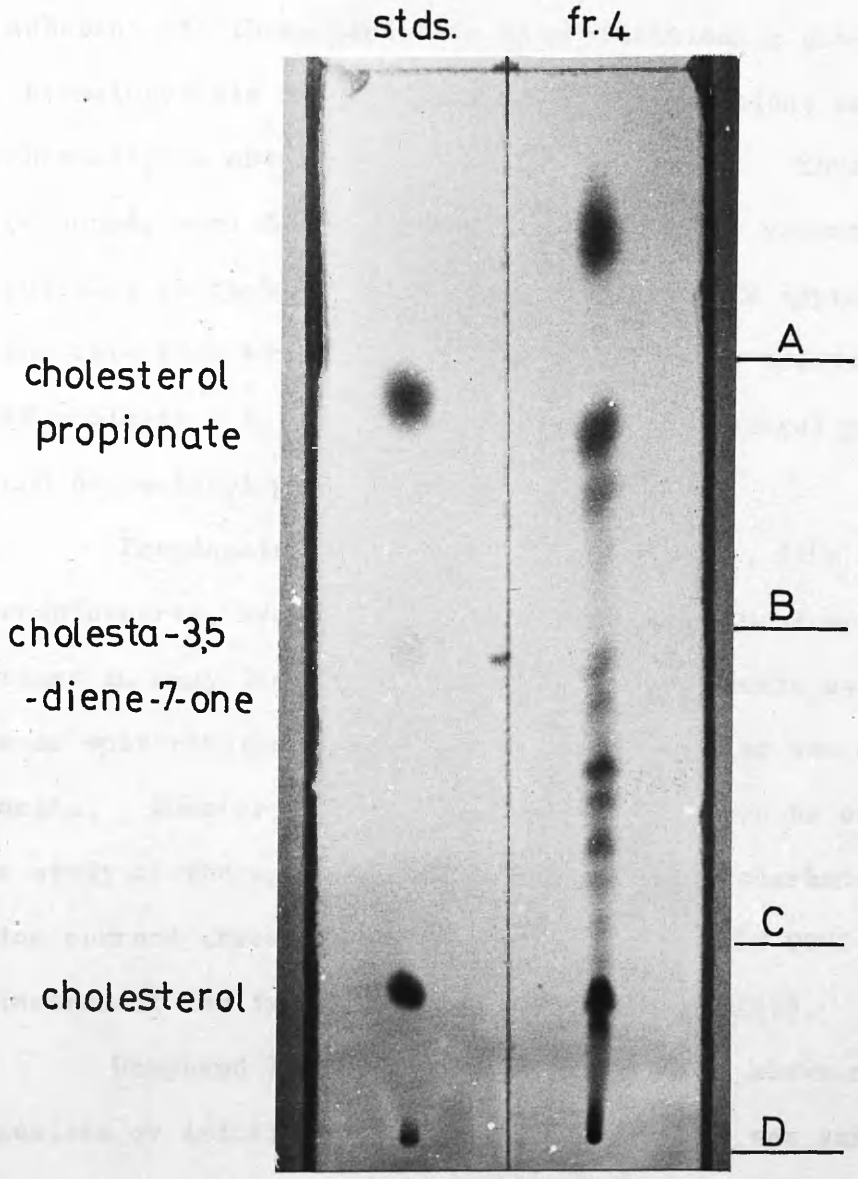
bands by comparison with a similarly run analytical plate. Two bands more polar than cholesterol propionate (band 2) and one less polar were isolated. So far only band 4, (R_F = 0 - 0.3), has been examined.

Band 4 (Area R_F = 0 - 0.3 including the origin of the chromatogram)

After elution from the silica gel the esters contained in this fraction were examined by analytical TLC on unmodified silica gel using benzene:ethyl acetate (20:1 v/v) as developing solvent. Fig. 48 shows the chromatogram obtained, the left-hand lane of the TLC plate containing reference compounds (cholesterol propionate, cholesta - 3, 5 - diene - 7 - one and cholesterol), while the right-hand lane shows band 4. Compounds were found in the extract which corresponded to cholesterol propionate, desmosterol propionate, cholesta - 3, 5 - diene - 7 - one and cholesterol in mobility and colour reaction. Apart from these there were several unidentified compounds which had polarity intermediate between cholesterol and cholesterol propionate. Band 4 was split into four sub-fractions A, B, C and D (see Fig. 48), by preparative TLC on layers of silica gel, using benzene:ethyl acetate (20:1 v/v) as solvent system. Areas B and C were found to contain the compounds of greatest interest.

Fraction 4B:- This fraction contained compounds which judging

Figure 48 . TLC of band 4 (propionates)



Adsorbent:- MN-Kieselgel G-HR (unmodified)
solvent:- benzene : ethyl acetate (20:1 v/v)
spray:- 1% ceric sulphate in 10% sulphuric acid

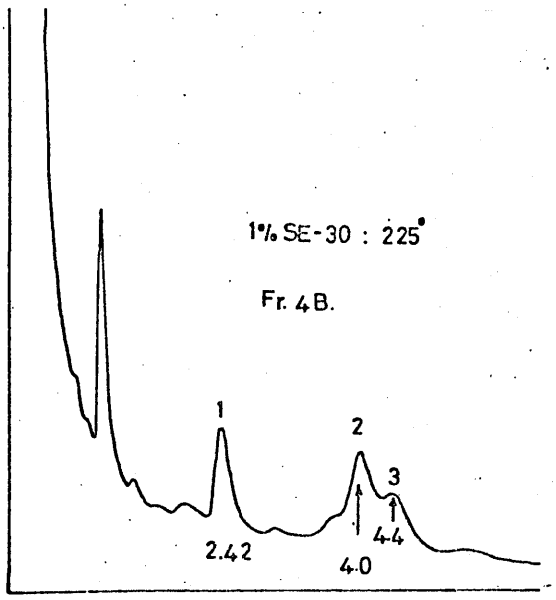
from their TLC behaviour appeared to be mono-hydroxy steroid propionates: the two major compounds corresponded in behaviour with propionates of cholesterol and desmosterol. In order to substantiate these tentative identifications a gas-liquid chromatographic study was made of this fraction, and the chromatogram obtained is shown in Fig.49(a). Three major compounds were found to be present and their retention times relative to cholestane are recorded under the appropriate peaks. The retention times of peaks 1, 2 and 3 were identical to those of cholesta - 3, 5 - diene - 7 - one, cholesterol propionate and desmosterol propionate respectively.

Propionate esters of Δ^5 - 3β-sterols, like their acetate counterparts, are not ideal derivatives for mass spectrometry since in many instances they eliminate propionic acid in the mass spectrometer giving an apparent molecular ion at P-74 mass units. However, considerable information can be obtained from a study of the spectra of the resulting hydrocarbons. The total ion current chromatogram obtained from the LKB 9000 GC-MS instrument for fraction 4B is shown in Fig.49(b).

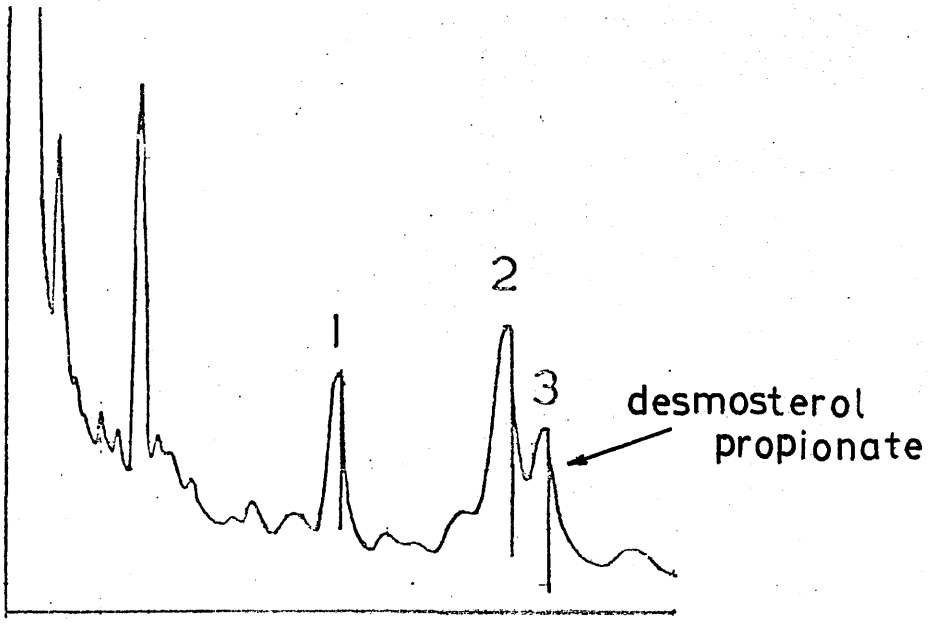
Compound 1, which had previously been shown not to form acetate or trimethylsilyl ether derivatives, was suspected to be the unsaturated ketone cholesta - 3, 5 - diene - 7 - one from TLC and GLC mobilities. The mass spectrum obtained for the isolated compound was identical to that of a reference sample of cholesta - 3, 5 - diene - 7 - one (Fig. 50).

Figure 49 GLC of fraction 4b.

(a)

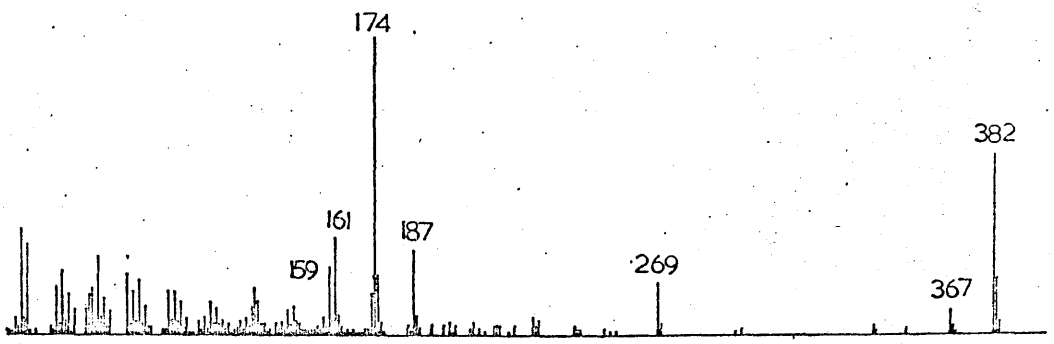


(b) TIC TRACE of fraction 4b.



retention times relative to 5α-cholestane are given under the peaks.

Figure 50 COMPOUND 1, fraction 4B



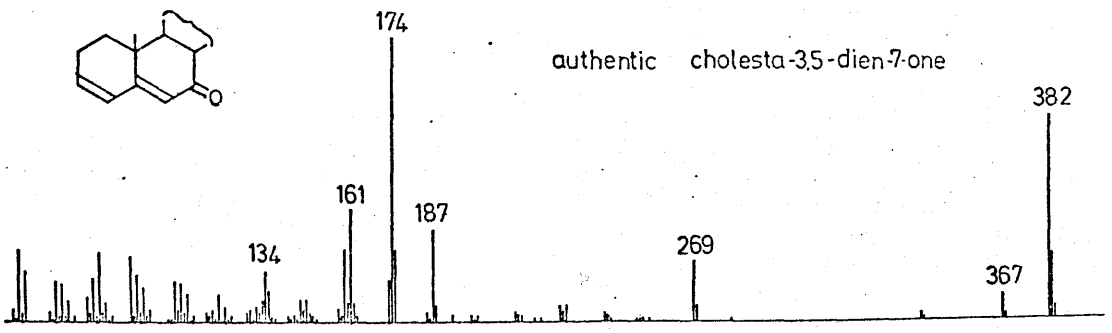
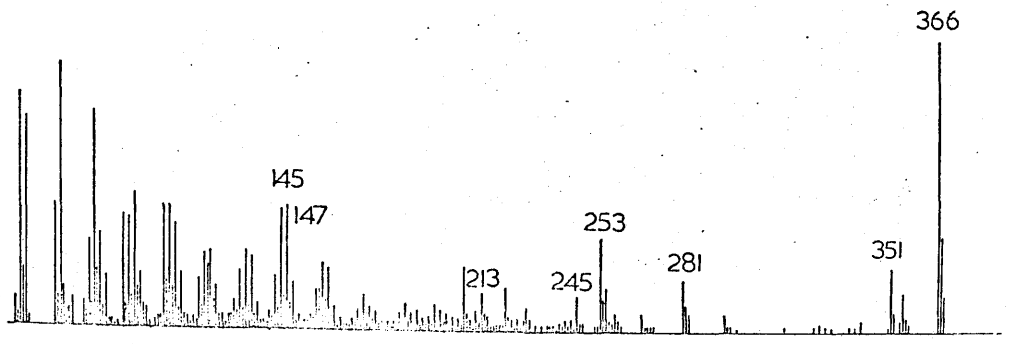
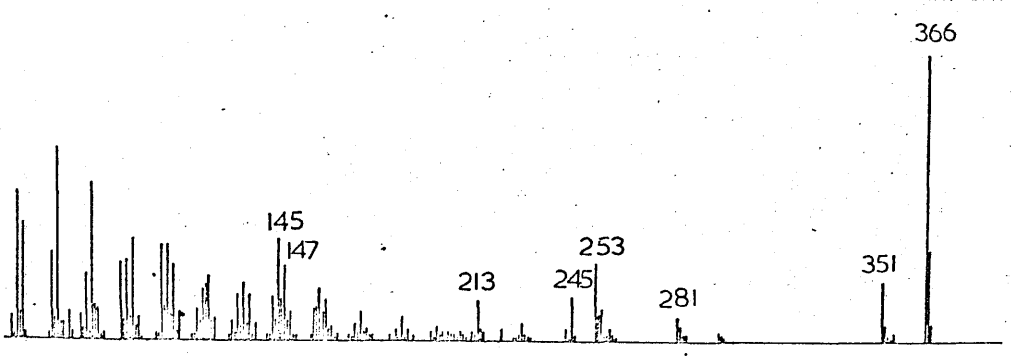


Figure 51 Mass spectra of Sterol propionates

Fraction 4B, peak 3



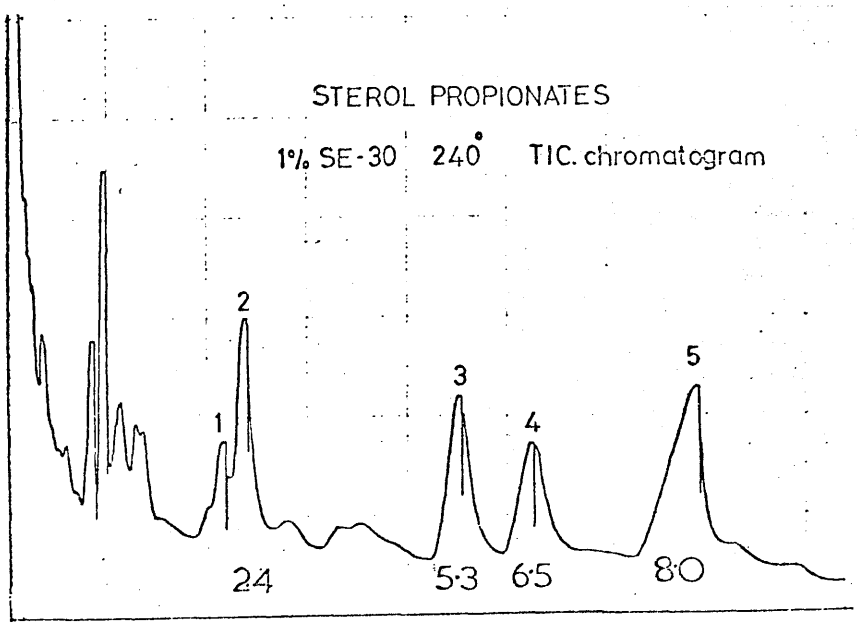
Desmosterol propionate



Peak 2 gave a mass spectrum similar to that of the hydrocarbon cholesta - 3, 5 - diene (see Fig. 18 on page 134), and is considered to result from cholesterol propionate which has eliminated propionic acid in the mass spectrometer. In a similar manner peak 3 has been shown to give a spectrum typical of a cholesta-triene or trienes (Fig. 51) as would be expected if this compound were desmosterol propionate. Almost identical spectra have been recorded by Brooks et al. (14) and by Galli and Maroni (15) for desmosterol acetate.

Additional proof of the presence of desmosterol propionate was obtained by analytical TLC of fraction 4B on layers of silica gel impregnated with silver nitrate. The chromatogram was developed using hexane:benzene (5:1 v/v) as solvent system and the two major compounds in the extract had the same R_F values as cholesterol propionate (R_F = 0.41) and desmosterol propionate (R_F = 0.22).

Figure 52 GLC behaviour of fraction 4C



Fraction 4C:- The total ion current representation of GLC behaviour of this fraction is shown in Fig. 52. Peak 2 and peak 5 from this chromatogram gave identical mass spectra which were quickly recognised as the fragmentation of cholesta - 3, 5 - diene - 7 - one (see Fig. 50). Peak 2 has the correct GLC retention time to be this unsaturated ketone but to explain the same mass spectrum's being obtained for peak 5, coupled with the long retention time of this compound (t_R = 8.00) we have to postulate the existence of a keto-sterol propionate which undergoes an elimination of propionic acid in the mass spectrometer to give a diene - one. 7-Ketocholesterol propionate was found to give the same GLC and GC-MS data, and on TLC corresponded to a previously unidentified component in the extract (see Fig. 48, page 203).

Peaks 1, 3 and 4 all gave identical spectra with an apparent molecular ion at m/e 384. The presence of a peak at m/e 366 representing a loss of 18 mass units is thought in this case not to be due to a loss of water since this should eliminate before rather than after propionic acid. It is therefore possible that it results from a ketonic grouping. Peaks 3 and 4 with relative retention times of 5.3 and 6.5 respectively therefore might represent epimeric ketosterol propionates since their retention times are considered to be too long for simple sterol propionates but too short for diol propionates. If this theory were correct peak 1 could then represent the corresponding

unsaturated ketone resulting from thermal elimination in the gas chromatograph. The structures of these unidentified compounds remain to be elucidated.

Work is in progress to modify the present method for use with derivatives other than propionates. Special attention is being paid to derivatives which have more suitable TLC, GLC and mass spectral characteristics. Examination of the properties of TMSi ethers has shown them to be unsuitable for silver nitrate TLC under the presently known conditions: however, preliminary work with trifluoroacetates has proved more successful and has enabled the identification of cholestanol in plaque extracts (16).

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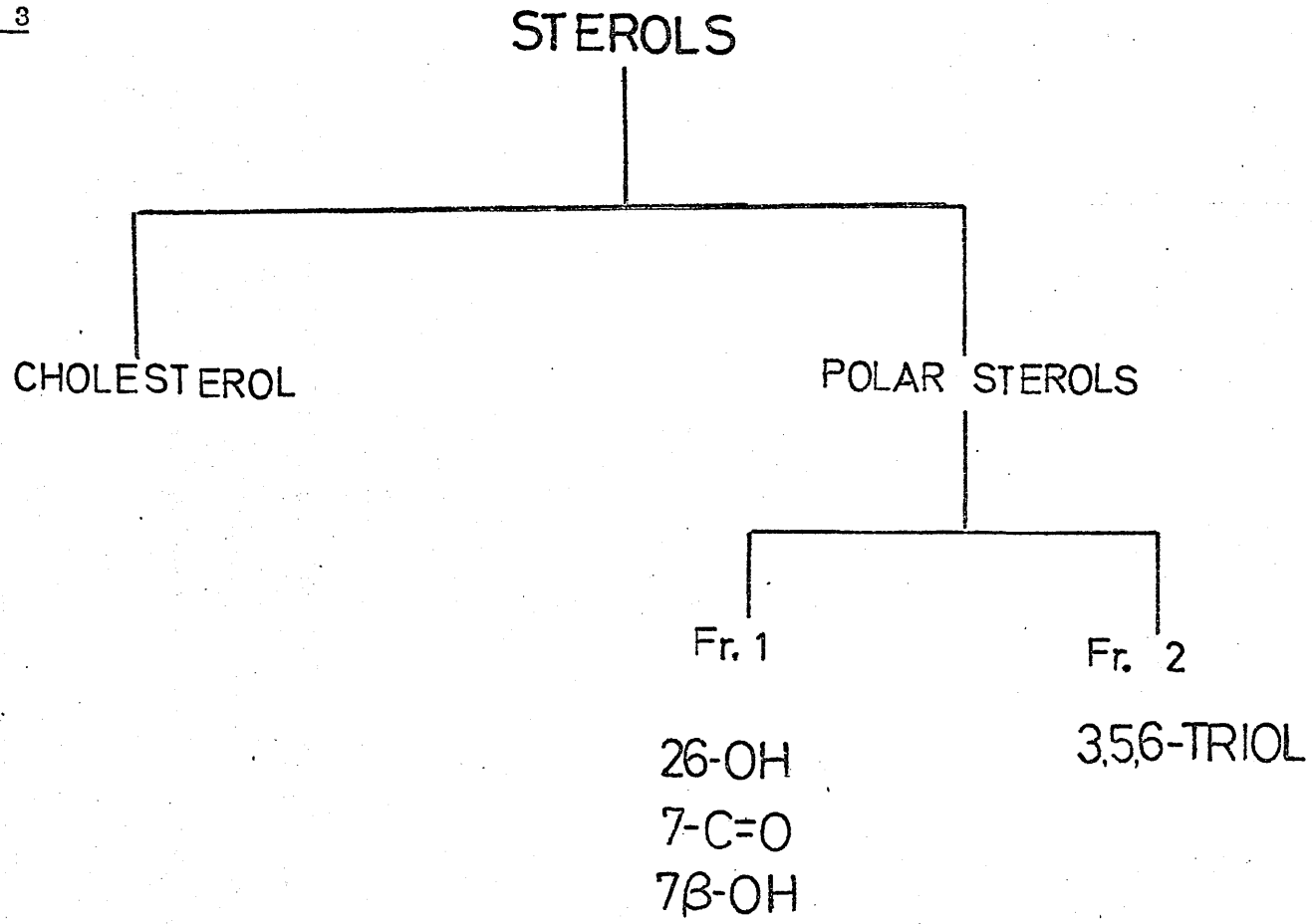
PART IV

POLAR STEROLS

Key 3



The symbols are as follows:-
26-OH, 26-hydroxycholesterol; 7-C = O, 7-ketocholesterol;
7β-OH, 7β-hydroxycholesterol; 3,5,6-triol, 5α-cholestane - 3β,5,6β-triol.

POLAR STEROLS

So far, we have discussed the lipid groups examined in order of their increasing chromatographic polarity, however, this bears no relationship to the actual chronological sequence in which the various fractions were studied. Although "polar sterols" are discussed last, they were the first type of minor component for which we searched, and are the class most fully documented by previous workers. By "polar sterols" we mean sterols which are chromatographically more polar than the major sterol cholesterol.

At the start of our work there had been three important studies on minor steroidal components in the human atheromatous aorta, namely those by:-

(a) Hardegger *et al.*, 1943 (1)

(b) Kantiengar and Morton, 1955 (2)

(c) Henderson, 1956 (3).

Each of these studies was severely handicapped by the absence of suitable techniques for the identification of microgram quantities of steroids. The work of Hardegger *et al.* (1) illustrates the classical approach to the problem, where huge amounts of tissue (almost four hundred aortas) had to be used in order to obtain sufficient quantities of the minor steroids to permit identification by the then available methods.

The autopsy material used in that investigation was collected over a period of two years, stored in ethanol, but not extracted until the collection of tissue was complete. Whole aortas were used rather than plaques, thus including material characteristic of ageing rather than atherosclerosis. The total extract was saponified and the bulk of the cholesterol removed from the unsaponifiable fraction by crystallisation from methanol. The methanolic mother liquor was then chromatographed on a column of deactivated alumina. Each minor component was then identified on the following evidence:-

(a) A carbon and hydrogen elemental analysis to establish the empirical formula.

(b) Melting point and mixed melting point, where a reference compound was available.

(c) Chromatographic mobility on an alumina column.

(d) Derivative formation (including analysis of the derivative, and a mixed melting point with the authentic derivative).

(e) Molecular rotation.

(f) Ultraviolet spectroscopy.

Thus each compound was identified on sound chemical and physical evidence: however, the necessarily prolonged nature of the investigation introduces the possibility that some of the compounds thus obtained are artefacts rather than genuine

components of the atheromatous aorta. The study of pooled aortas also loses the individual information potentially obtainable from the examination of single arteries.

The steroidal compounds identified in the work of Hardegger *et al.* were:-

(a) cholesta - 3, 5 - diene - 7 - one,

(b) cholesta - 4, 6 - diene - 3 - one,

(c) 5α - cholestane - 3β, 5, 6β - triol,

(d) 7β - hydroxycholesterol.

The second study, that of Kantiengar and Morton (2), used a much smaller scale. Preliminary work was carried out on plaques dissected from one aorta, a larger quantity of minor components being obtained later using pooled plaque extracts. Only one compound, cholesta - 3, 5 - diene - 7 - one, was identified using ultra-violet spectroscopy after repeated chromatography on alumina.

Henderson (3) used an intimal-medial preparation of the aorta and also of arterial tissue from several other common sites of atherosclerosis, such as the coronary and cerebral arteries. Tissue extracts were split into four fractions by column chromatography on silica gel, after which they were saponified. The steroids were characterised by R_F values and colour reactions on paper chromatography.

The compounds tentatively identified were:-

(a) 7α and 7β - hydroxycholesterol,

(b) 5α - cholestane - 3β, 5, 6β - triol,

(c) a compound suggested to be 24- or 25-hydroxy-cholesterol.

In our work we aimed to retain individual information, to process extracts as quickly as possible, and to avoid drastic conditions liable to initiate the formation of artefacts. At the start of our work we planned to use recently developed techniques of thin-layer and gas-liquid chromatography to identify the separated steroids. The availability of the LKB 9000 gas chromatograph - mass spectrometer during the later stages of the work greatly aided these identifications.

The present work on polar sterols

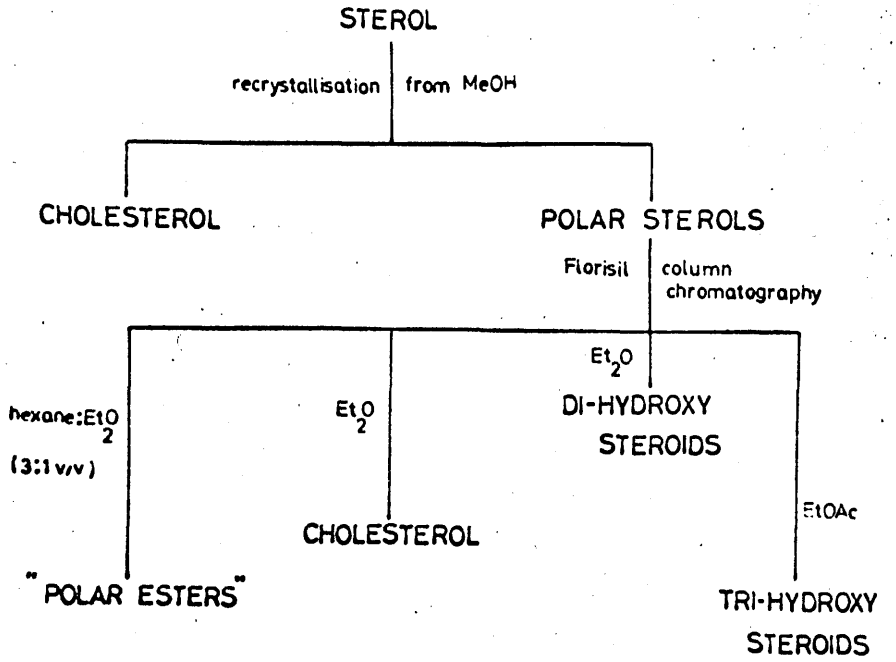
The material used in this part of the investigation was the entire sterol fraction eluted from the initial silicic acid column chromatography of the plaque lipid extract (see page 102). In addition to sterols, each fraction of this type probably contains diglycerides and monoglycerides. The basic problem has been to concentrate the polar sterols relative to an overwhelming mass of cholesterol. Fieser (4) and Cargill and Cook (5), also faced with this problem, employed repeated crystallisation of the sterols from boiling acetic acid, the cholesterol

(and perhaps other mono-hydroxy steroids), crystallising out as the 1:1 acetic acid complex and leaving the more polar sterols in the acetic acid mother liquor. Bearing in mind the risk of artefact formation, we have avoided the use of boiling acetic acid and have favoured crystallisation from methanol as suggested by Hardegger et al. (1). This solvent was thought to be especially suitable since it is known to stabilise certain labile steroids such as 7 - dehydrocholesterol (6).

Concentration of polar sterols

The total sterol fraction was repeatedly crystallised from methanol as described in the experimental methods section, page 111. The mother liquor, after approximately six successive crystallisations, was reduced to dryness under a water pump vacuum, and the oily residue dissolved in hexane:ether (3:1 v/v) and chromatographed on a column of deactivated Florisil (7) (see page 104). A flow-diagram giving the solvents used in the elution is shown in Fig. 53. The bulk of the cholesterol remaining was removed from the column by elution with ether, which also eluted later a yellow oily material containing polar sterols (polar sterol fraction 1). A second oily material was obtained using ethyl acetate and was designated polar sterol fraction 2.

FIGURE 53 FLORISIL COLUMN CHROMATOGRAPHY



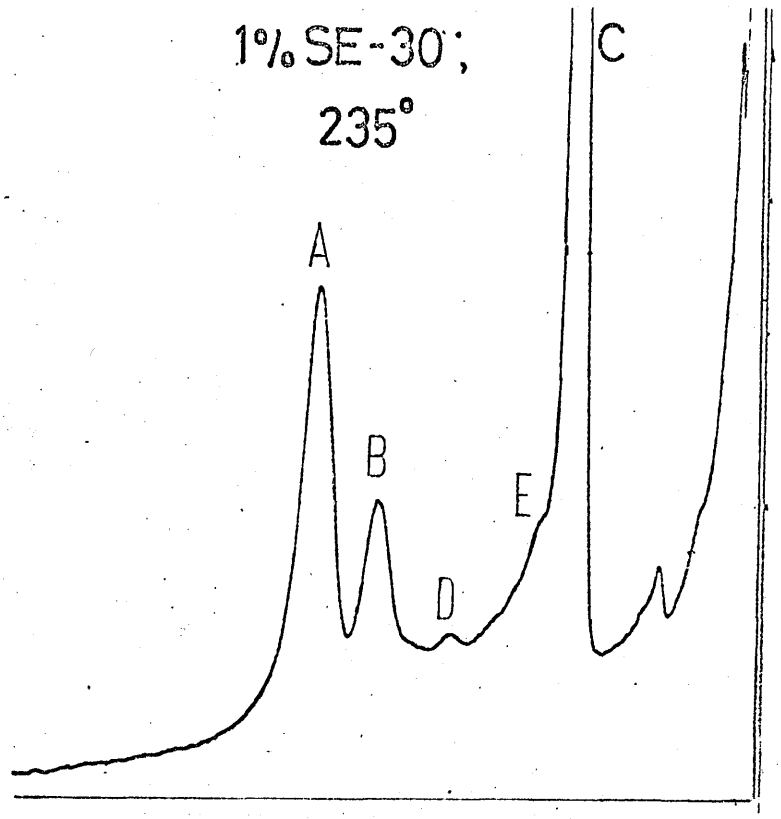
Analysis of fraction 1

Preliminary examination was carried out by TLC using chloroform : ethyl acetate (3:1 v/v) as solvent system. An especially prominent polar sterol (A at R_F = 0.30) gave a maroon colouration similar to cholesterol (R_F = 0.66) when sprayed with an acidic solution of ceric sulphate. Other less intense spots were indicated at R_F = 0.47 (dark brown) and R_F = 0.19 (blue). All spots on the chromatogram were partially obscured by a general brown streak which accompanied all fractions at this stage of purification. On the suggestion of the work of Henderson (3),

24 - and 25 - hydroxycholesterol were tested in the above TLC system as possible identities of polar sterols in the extracted material. 25 - Hydroxycholesterol (R_F = 0.33) was found to have an R_F value slightly greater than the major polar sterol in the plaque extract, whereas 24α- hydroxycholesterol (R_F = 0.45) was markedly less polar. Both of these reference compounds gave different colour reactions from the unknown when sprayed with an acidic solution of ceric sulphate. A number of other sterols were also tested under these conditions and their TLC mobilities and colour reactions are recorded on page 267 in Appendix I.

The results expressed above suggested that the major polar sterol in the plaque was neither 24- nor 25-hydroxycholesterol. This non-identity was further emphasized in a gas-chromatographic examination. Fig. 54 illustrates the chromatogram obtained in this investigation. TLC had suggested that cholesterol was still present in the extract and GLC gave a peak (peak C in Fig. 54) which corresponded to this sterol in retention time. The other major peaks which appeared were labelled A and B, A being the larger of the two. The retention times of components A, B and C relative to cholestane are shown in the figure, also recorded are the relative retention times of 24 - and 25 - hydroxycholesterol chromatographed under identical conditions. From these results it can be seen that neither

FIGURE 54 GLC OF ISOLATED POLAR STEROLS (free form)



COMPOUND	t_R
A	4.71
B	4.08
C	1.99
D	3.30
E	2.50
cholesterol	2.00
24α - OH cholesterol	3.68
25 - OH cholesterol	3.34

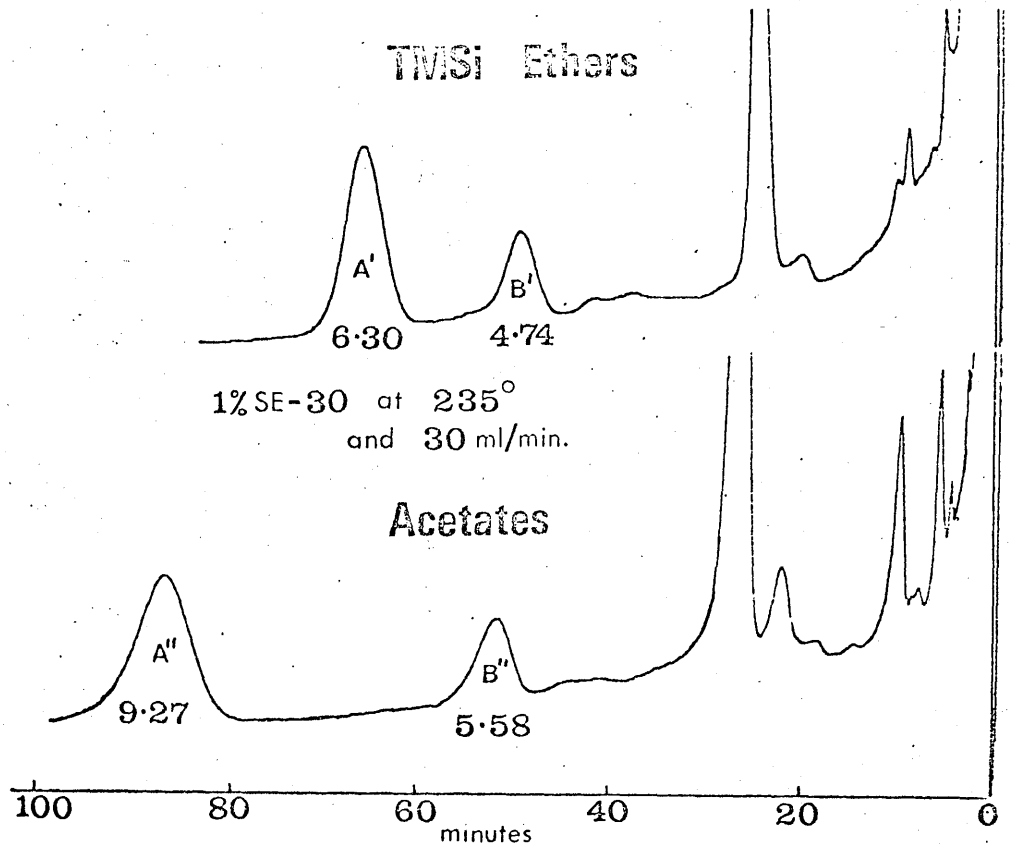
t_R = retention time relative to cholestane

compound A nor B corresponds to 24 or 25 - hydroxycholesterol.

Purification of polar sterol extracts

TLC of polar sterol extracts clearly demonstrated that although considerable quantities of polar sterols were present, they were in a very impure state, possibly contaminated with diglycerides and monoglycerides as well as pigments. A fraction containing polar material was split into two equal portions, one of which was converted to the trimethylsilyl ether derivatives and the other into acetate derivatives (see page 106), after which both were sublimed (140° - 150°; 0.2 mm Hg). After sublimation some yellow oily material was left behind in the sublimation tube in both cases. TLC examination of the hydrolysed acetate derivatives after sublimation showed that considerable purification had taken place. The GLC traces (1% SE-30; 235°C) obtained for the trimethylsilyl ether and acetate derivatives are shown below in Fig. 55. In this diagram the major polar sterols have been labelled A and B (' for TMSi ethers and " for acetates). When the relative retention times obtained here for the acetate derivatives are compared with those of the original free sterols (page 222) an important conclusion results. If we apply a retention factor of approximately 1.4 for acetylation (Brooks and Hanaineh (8)), compound A on

FIGURE 55



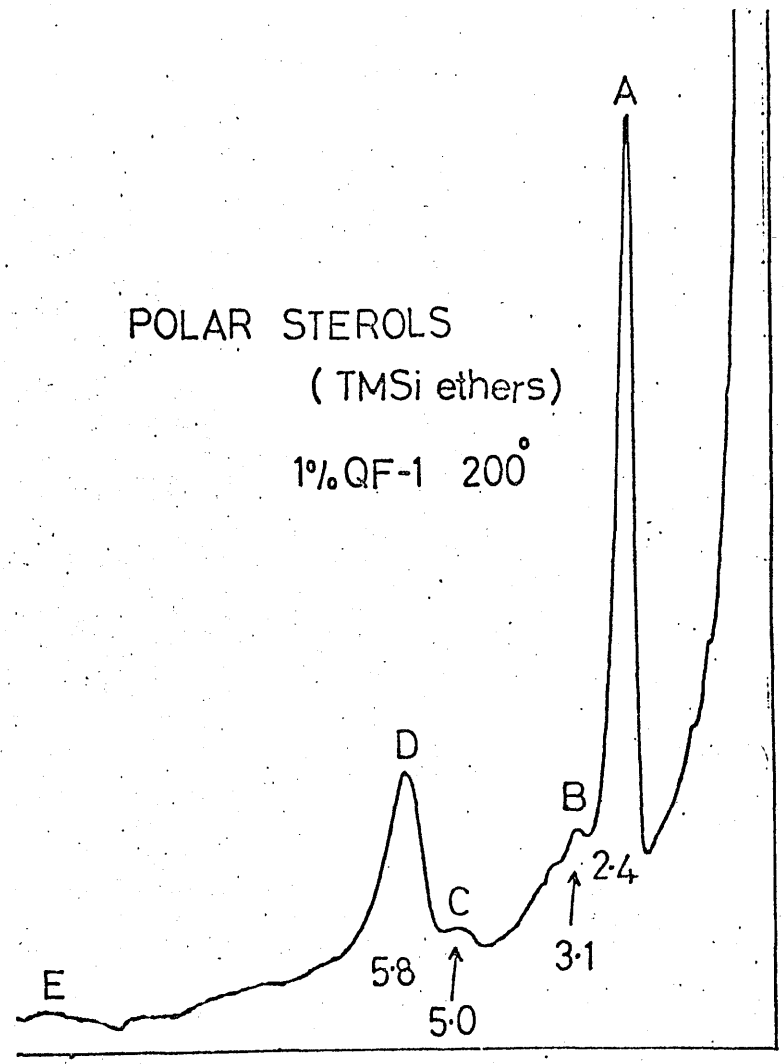
Retention times are relative to 5α-cholestane.

acetate formation shows an increase in retention time consistent with the formation of a diacetate as expected, whereas compound B formed only a monoacetate. The possibility that B was a monohydroxy steroid was unlikely in view of its chromatographic isolation, and its retention time.

The chromatographic data might have fitted a steroid diol with one of its hydroxyl groups sterically hindered preventing acetate formation, but drastic acetylation conditions produced no change in retention time. It thus seemed more probable that compound B was a keto-sterol. Two readily available keto-sterols, namely 24 - ketocholesterol (3β-hydroxy-cholest-5-en-24-one) and 7 - ketocholesterol (3β-hydroxy-cholest-5-en-7-one) were examined. 7 - Ketocholesterol was found to have identical GLC behaviour to extracted compound B as the free sterol, as the acetate and as the trimethylsilyl ether derivative.

Compound A was tentatively identified as 26-hydroxycholesterol (cholest - 5 - en - 3β, 26 - diol) on the basis of its chromatographic mobility and colour reactions. Preparative TLC as the trimethylsilyl ethers in hexane:benzene (2:1 v/v) has shown that the isolated sterol which gives a large maroon spot more polar than cholesterol TMSi is the compound which gives peak A' on GLC. It should also be noted that although 25 - and 26 - hydroxycholesterol are not well separated on TLC as the free sterols, they are clearly separated as TMSi ethers and give significantly different retention times on GLC as this derivative (see Appendix I, Table 20, on page 269): thus they can be readily differentiated. Further confirmation of the identity of compound A and 26 - hydroxycholesterol was obtained when they

FIGURE 56 GLC OF POLAR STEROL FRACTION 1 (TMSi ethers)



Compounds A and D were subsequently identified as cholesterol TMSi ether and 26-hydroxycholesterol TMSi ether respectively. (retention times recorded under the major peaks are relative to cholestane).

were compared as their TMSi ethers by GLC on the polar selective phase QF-1. The chromatogram obtained for the extract is shown in Fig. 56.

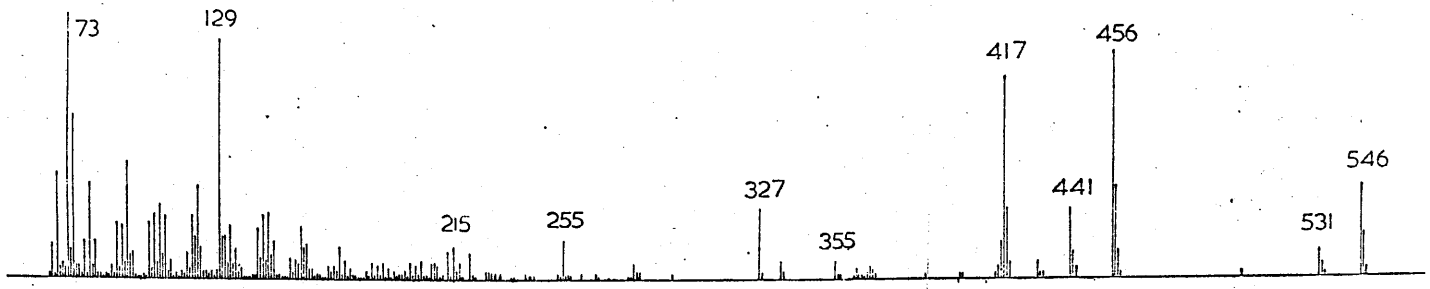
GC-MS examination of polar sterol fraction 1

Fraction 1 was examined by GC-MS in the form of the trimethylsilyl ether derivatives, and mass spectral scans were obtained of the major components in the extract. Fig. 57 shows line diagrams of the scans obtained for peak A' and for authentic 26 - hydroxycholesterol bis-TMSi ether. The close similarity of these fragmentation patterns confirms the identity of compound A as 26 - hydroxycholesterol. It is noteworthy that 24α-, 25 - and 26 - hydroxycholesterol, as well as being clearly differentiated by their GLC retention times, all give strikingly different mass spectra as their TMSi ethers (cf. Appendix 2 on page 274).

A similar approach confirmed that compound B was 7 - ketocholesterol, and the spectra obtained for extracted and reference material in a GC-MS investigation are illustrated in Fig. 58.

Our next step was to substantiate further the identification of compound B as 7 - ketocholesterol. Unlike most sterols, 7 - ketocholesterol is not well visualised on a thin-layer chromatogram by spraying with ceric sulphate, and its presence

FIGURE 57 POLAR STEROL FRACTION 1, COMPOUND A' (TMSi)



Authentic 26 - OH cholesterol bis-TMSi ether

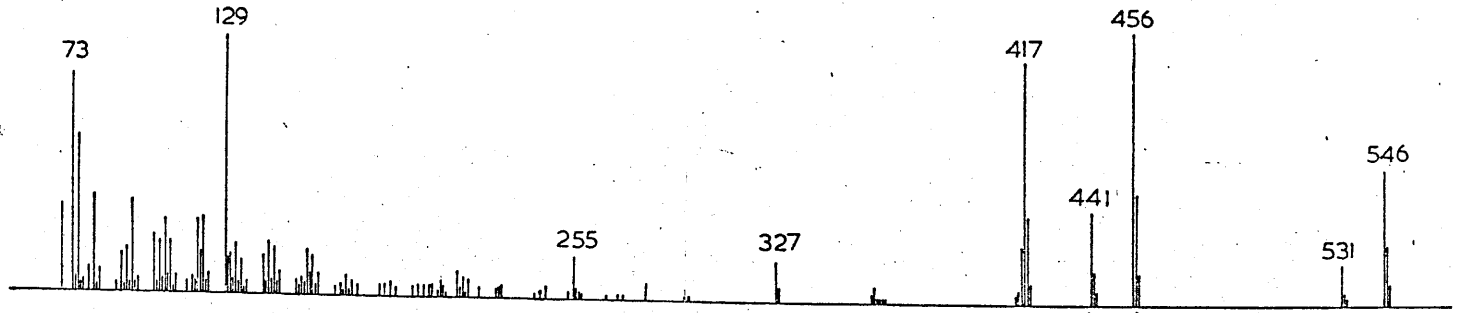
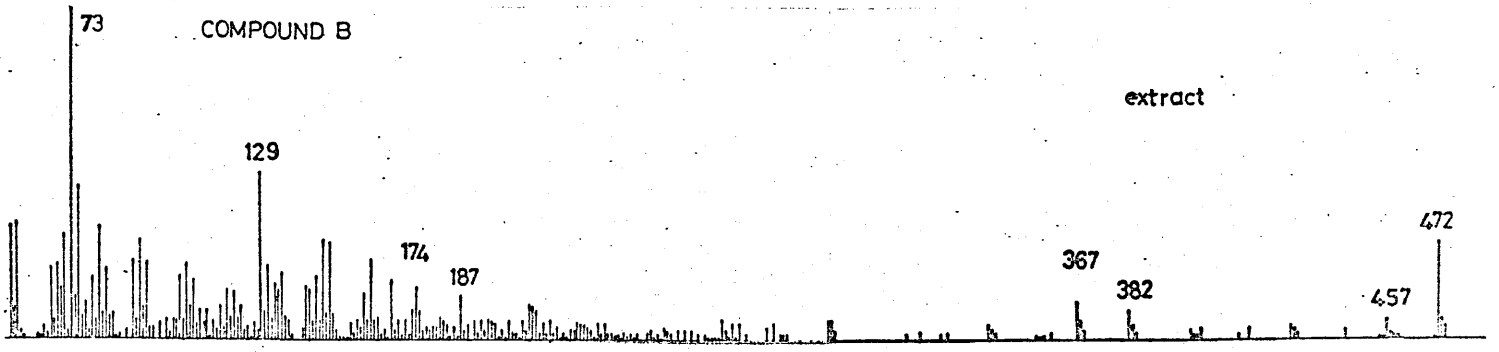
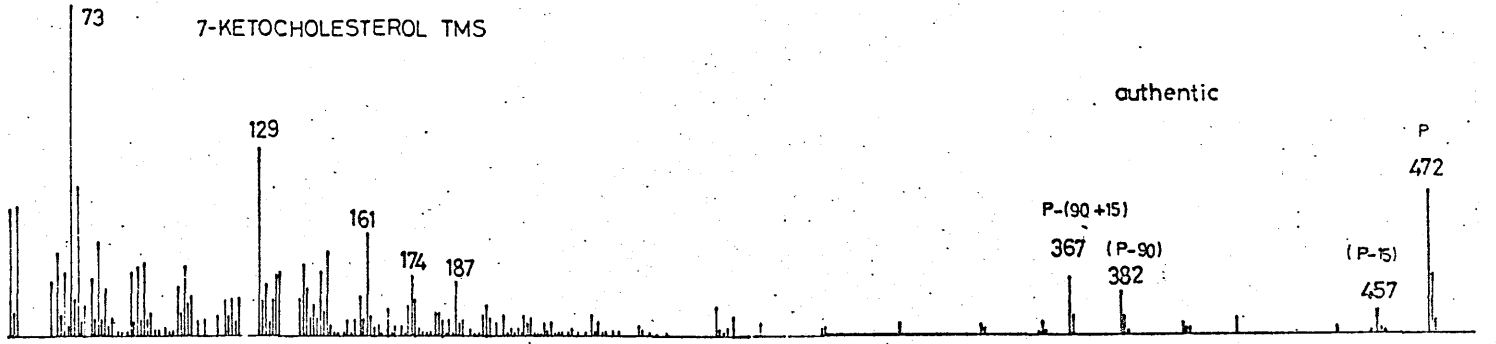


FIGURE 58 POLAR STEROL FRACTION 1, COMPOUND B' (TMSi)



Authentic 7 - Ketocholesterol TMSi ether

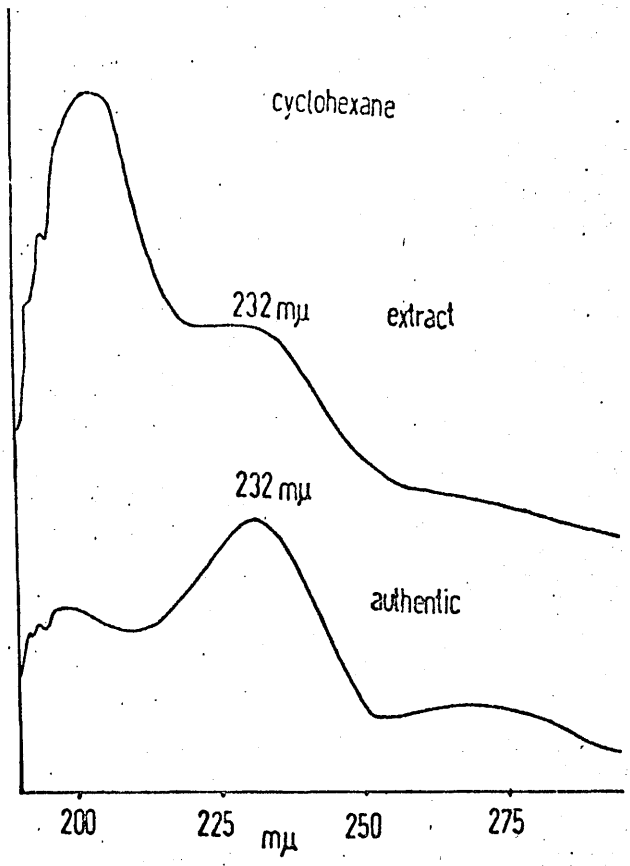


is frequently masked by the presence of impurities. However, its ketonic nature can be readily demonstrated by spraying with 2:4 - dinitrophenylhydrazine which yields an orange colouration. The best and most specific method for the detection of 7 - keto-cholesterol on a TLC plate is, however, to spray the plate with a 1% solution of sodium borohydride in methanol (9). If, after a few minutes, the chromatogram is then sprayed with ceric sulphate and heated, the characteristic intense blue colouration of a 7 - hydroxycholesterol develops at the position for 7 - ketocholesterol.

The presence of the α,β-unsaturated ketonic system in compound B was demonstrated by ultraviolet spectroscopy of polar sterol fraction 1 as the TMSi ether derivative. The U.V. spectra obtained for compound B' and authentic 7 - ketocholesterol TMSi ether are shown in Fig. 59. The literature value for the absorption maximum of 7 - ketocholesterol is 239 mμ in ethanol (10). The value found for the extracted material was 232 mμ in cyclohexane: however, applying a solvent correction of + 9 mμ takes this to 241 mμ which is close to the figure in the literature. It is difficult to calculate an accurate extinction coefficient from this ultraviolet spectrum since the fraction contains several compounds. However, if we assume that the total optical density at 232 mμ is due to 7 - ketocholesterol and obtain the weight of this compound present from a comparison of

GLC peak heights, an approximate intensity value can be calculated. Using this approach a value of 10×10^3 was obtained.

FIGURE 59 UV. SPECTRUM OF 7 - KETOCHOLESTEROL TMSi ETHER.



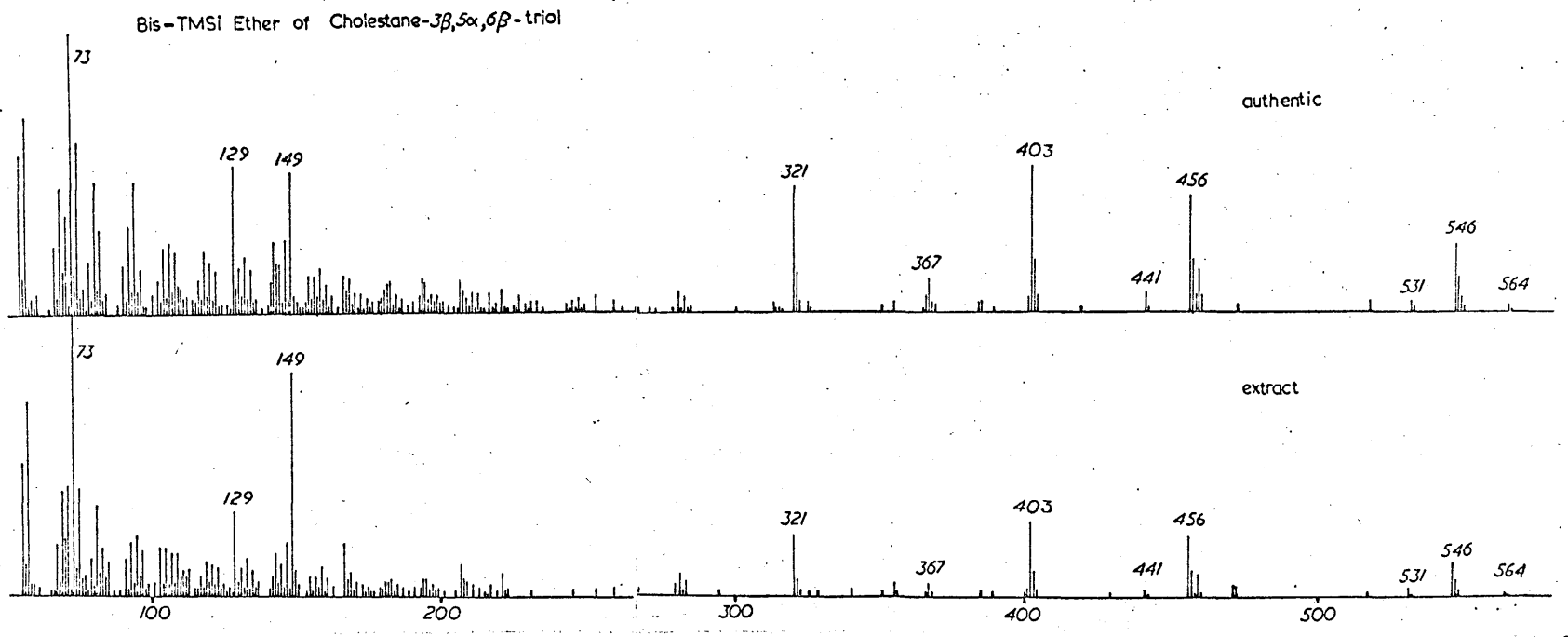
The literature value for pure 7 - ketocholesterol is 12×10^3. It is proposed to obtain an accurate value by formation of a 2:4-dinitrophenylhydrazone derivative which would give an absorption clear of the troublesome region of "end absorption", which is a feature of the spectrum in Fig. 59.

Initial difficulties caused by the masking effect of the absorption of the 2:4-dinitrophenylhydrazine reagent have been overcome by the use of preparative thin-layer chromatography. Preliminary results using this approach have produced an absorption for 7-ketocholesterol 2:4-dinitrophenylhydrazone which appears to be complicated by the absorption from other ketonic compounds and further TLC will be necessary to produce a unique absorption spectrum.

Analysis of polar sterol fraction 2

When the yellow oily material designated "polar sterol fraction 1" ceased to be eluted from the Florisil column, the eluting solvent was changed to ethyl acetate which on evaporation yielded a small quantity of another oily material designated "polar sterol fraction 2". Analysis of this fraction by TLC in chloroform:ethyl acetate (3:1 v/v) showed that this fraction contained two compounds, one of which appeared to be cholesterol. The other, on spraying with ceric sulphate, gave a light brown colouration, and moved only very slightly from the origin (R_F = 0.02). Polar sterol fraction 2 was then purified by preparation of trimethylsilyl ethers and sublimation. Examination of these ethers by TLC in hexane:benzene (2:1 v/v) showed that a considerable purification had taken place. The two components of the mixture appeared at R_F = 0.57 and 0.13 in this solvent system. The less polar of these compounds was shown to correspond to cholesterol trimethylsilyl ether in R_F value and colour reaction, while the more polar was found to correspond to the trimethylsilyl ether of 5α-cholestane - 3β,5, 6β-triol. GC-MS examination of the extract and authentic triol revealed that in both cases a bis- rather than a tris-TMSi ether had been formed. The mass spectra of extract and authentic derivatives are shown in Fig. 60: they almost certainly represent the spectra of the 3, 6 bis-TMSi ether

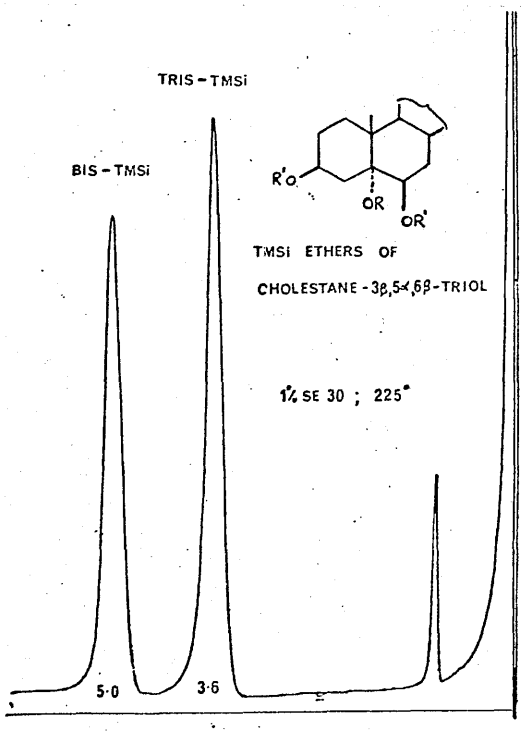
FIGURE 60



m/e 149 is thought to arise from incomplete removal of the reagents.

derivative. [The 5α- hydroxyl group has already been shown to form a TMSi ether compound only with difficulty in the case of 5α- cholestane - 5, 6α- diol studied in the investigation into plaque hydrocarbons (page 148).] It was found, however, that the tris-TMSi ether could be formed under stronger etherification conditions (page 107), and the mass spectrum of this compound is shown in Appendix 2 on page 281. Formation of the tris-TMSi ether was found to reduce rather than increase the retention time over the bis-derivative (see Fig. 61). A similar result was found by Brooks et al. (11) in the trimethylsilylation of 5β - cholanetetrols.

FIGURE 61



The formation of the tris-TMSi derivative was also clearly indicated on TLC where a striking reduction in polarity over the bis-ether was observed, as shown in Table 17 (cf. Appendix I, Fig. 66, page 272).

Table 17 Chromatographic data for 5α-cholestane-3β,5,6β-triol derivatives.

COMPOUND	t_R	R_F	Mol. wt.
bis-TMSi	4.99	0.13	564
tris-TMSi	3.60	0.75	636

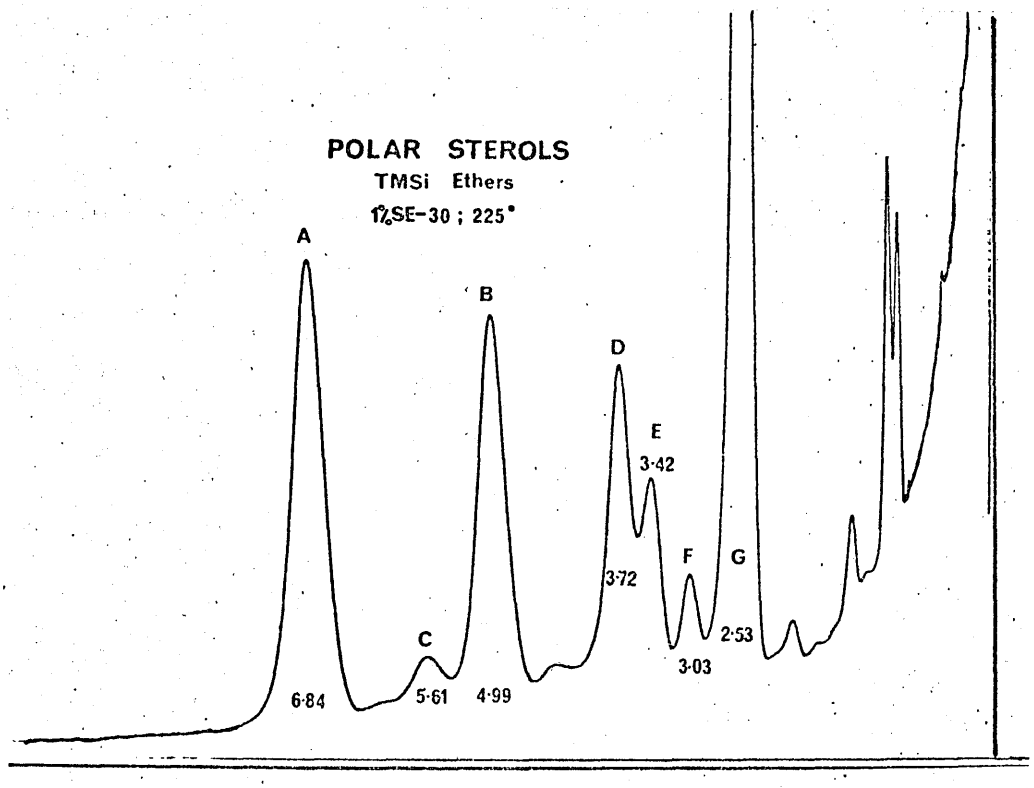
t_R - retention time relative to cholestane on 1% SE - 30 at 225°C.

R_F values were recorded using hexane-benzene (2:1 v/v) as solvent system.

Recent work on polar sterols

The work on polar sterol fraction 1, so far described, represents the results of early studies (carried out before the separation methods were fully developed) which do not reflect the true complexity of the fraction. A gas-liquid chromatogram obtained more recently from a similar investigation is shown in Fig. 62. In agreement with the earlier findings, 26-hydroxy-cholesterol TMSi ether (peak A) was found to be the most predominant polar sterol derivative. Peak B would appear to represent a rather larger proportion of 7 - ketocholesterol TMSi ether than had been previously indicated: however, scrutiny of the mass spectrum obtained for this peak revealed that it was composed of a mixture of 7 - ketocholesterol TMSi ether and 5α-cholestane-3β,5,6β-triol bis-TMSi ether. Previous work with authentic derivatives had shown that these compounds are very poorly separated on the non-selective phase SE-30. (see Appendix I, Table 20, on page 269). Peaks C, D, E and F have retention times relative to cholestane of 5.61, 3.72, 3.42 and 3.03 respectively on 1% SE-30 at 225°C. Peak C was identified as 25 - hydroxy-cholesterol TMSi ether from its chromatographic mobility and mass spectrum. This is the only polar sterol fraction where this compound has appeared and it is at present considered to be an artefact derived from cholesterol. A similar approach established

FIGURE 62



The peaks in the chromatogram were subsequently identified as the TMSi ethers of the following compounds:-

A 26 - OH cholesterol

B a mixture of 7 ketocholesterol and 5α-cholestane-3β,5,6β -triol.

C 25 - OH cholesterol

D 7β - OH cholesterol

G cholesterol

E and F have not yet been identified.

that peak D was 7β-hydroxycholesterol bis-TMSi ether: the chromatographic characteristics of this compound have already been discussed on pages 177 and 178. The structures of the compounds represented by peaks E and F have not been elucidated. GC-MS has indicated that the compound which gives peak E is a cholestene diol mono-TMSi ether, but further work will be necessary before this compound is identified.

Quantities of polar sterols

In all plaque extracts examined 26 - hydroxycholesterol was by far the major polar sterol. Its concentration varied from aorta to aorta but was estimated by GLC to be of the order of 50μg/g. of purified lipid. 7 - Ketocholesterol, although present in every extract studied, was found in smaller quantities, usually about one tenth of the concentration of 26 - hydroxycholesterol. Previous workers (1, 3) have suggested that 7α- and 7β-hydroxycholesterol are present in plaque extracts, although no adequate separation method was applied for these epimers. We have shown that these compounds are well separated as their TMSi ethers on TLC and GLC, and using this information we have been able to demonstrate the presence of the 7β-isomer in the lesion at a level of approximately 20-30μg/g. of lipid.

Although 5α- cholestane-3β,5,6β-triol was only found in some of the extracts, where its level was of the order of

30-40 μ g/g. it is not thought to be an artefact, and its absence in some instances may have been due to the extreme polarity of this compound on silicic acid and Florisil columns.

Additional proof of the presence of 26 - hydroxycholesterol

The identification of 26 - hydroxycholesterol as the TMSi ether derivative by TLC, GLC and GC-MS has been described. Early work on this identification involved the use of the diacetate derivative which behaved well on TLC, but gave an inconveniently long GLC retention time (10.10 relative to cholestane on 1% SE-30 at 225°C.). Although not giving a true molecular ion in its mass spectrum, this derivative gives a characteristic fragmentation pattern with an apparent molecular ion at m/e 426 corresponding to the elimination of the elements of acetic acid (60 mass units). Line diagrams of the spectra of isolated and authentic 26 - hydroxycholesterol as the diacetate derivative are shown on page 281 in Appendix 2.

Another approach, which makes use of the rather unusual primary hydroxyl group in 26 - hydroxycholesterol, is being attempted at present. This method involves hydrogenation followed by chromic acid oxidation and gives rise to 3 - ketocholestanoic acid.

It might be argued that the compound which we have identified as 26 - hydroxycholesterol (cholest-5-en-3β,26-diol) might equally well be the corresponding 3α-hydroxy epimer

(i.e. cholest-5-en-3α,26-diol). This compound would be expected to give an almost identical mass spectrum to 26 - hydroxycholesterol, but on the other hand it has been shown (see Appendix I on page 269) that cholesterol (cholest-5-en-3β-ol) and epicholesterol (cholest-5-en-3α-ol) are well separated on TLC and GLC as their trimethylsilyl ethers. The chromatographic data for the isolated diol (as its TMSi ether) are thus quite incompatible with the 3α- configuration.

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SECTION F

D I S C U S S I O N

DISCUSSION

We must now consider the biological significance of the compounds which we have identified in the atheromatous plaque in the light of the current theories of pathogenesis. However, since we have at present only studied one stage of the disease, the ideas expressed here must be considered as speculative, having regard to the limited evidence obtained. Nevertheless, it is felt that the methods developed in this work will form the basis of a wider approach to the problem, and will facilitate comparative studies of plaques selected as being representative of the steps of evolution of the lesion.

Hydrocarbons

The presence of squalene might suggest that some sterol synthesis takes place in the plaque and this hypothesis is consistent with the fact that desmosterol, another cholesterol precursor was also found. However, the bulk of the evidence on pathogenesis suggests that squalene is probably selectively removed from the blood plasma and deposited in the atheroma with other plasma lipids. To support this proposal there is the fact that this hydrocarbon is known to be present in human blood (1), but it is not known whether it is generated in some active site of sterol synthesis (such as the liver) or whether it is of purely dietary origin.

The paraffinic hydrocarbons found in the lesion are much more difficult to account for since very little is known about the origin or function of saturated hydrocarbons in man. In an attempt to account for the presence of n-alkanes in the human skin surface, Nicolaides (2) fed rats C^{14} octadecane and was able to demonstrate that some of this labelled material was excreted unchanged into the skin surface via the sebaceous glands. He also found activity in fractions other than hydrocarbons, showing that some octadecane must have been oxidised and then incorporated into other lipids, most likely fatty acids. These results tend to suggest that n-alkanes are not without biological importance. This viewpoint was held by Chibnall and Channon (3), who postulated that the n-nonacosane which they identified in cabbage leaves was formed by the condensation of two fatty acid molecules to yield a ketone which was subsequently reduced to a hydrocarbon. More recently Kaneda (4) has accumulated evidence to substantiate this "condensation" mechanism in a study of the formation of long-chain hydrocarbons in the tobacco plant, but has thrown doubt on the existence of a ketone intermediate. It has been suggested (5) that n-nonacosane, the major hydrocarbon of Brassica oleracea, is formed by an alternative process involving elongation of the common fatty acids. Either of these mechanisms might account for the plaque alkanes which could be formed in situ or could be transported from some other synthetic

site by the blood. Alternatively these hydrocarbons might be of purely dietary origin: however, this is thought to be unlikely since (contrary to the present findings) most dietary hydrocarbons contain an odd number of carbon atoms.

The presence of cholest-5-ene in the plaque is unexpected in terms of sterol metabolism. This steroidal hydrocarbon has not been identified in tissue or indeed in any biological material, and to involve this compound in the biosynthesis of cholesterol would necessitate the postulation of a biogenetic route very different from that currently accepted (6). Two groups of workers (7, 8) have however produced evidence of the existence of a nonoxidative cyclisation of squalene in the formation of lanosterol and the triterpene tetrahymanol. Barton and Moss (7) have shown that in a cell free yeast system the cyclisation of squalene is initiated by the biological equivalent of H^+ resulting in the generation of lanosta-8,24-diene. This compound is thought to be enzyme bound and hydroxylated to lanosterol at some later stage in the biogenesis. The conversion of lanostadiene into cholest-5-ene would require a mechanism different from that recognised for the conversion of lanosterol to cholesterol, and it is difficult to account for the removal of the 4,4-dimethyl groups in the absence of the 3-oxygen which is required to activate the process. However, we have found no evidence to suggest that lanostadiene is present in plaques.

We therefore have to consider other possible origins of cholest-5-ene. There is some evidence to suggest that lipid material undergoes changes after accumulation in the intima and it is therefore possible that this hydrocarbon is produced by reduction of cholesterol or Δ^4 - cholestenol. Such a reductive process is known to exist in the intestine where cholic acid is converted to deoxycholic acid (46), and in atherosclerosis might result from micro-organisms infecting the plaque. A more likely explanation of the presence of this hydrocarbon in the arterial lesion is that it and the other hydrocarbons identified were deposited from the blood by an infiltration process. In order to substantiate this theory we examined lipid extracts from two samples of hypercholesterolemic human blood and using TLC, GLC and GC-MS were able to identify cholest-5-ene, a cholestadiene, squalene and a series of n-alkanes (47). These results suggest that the theory expressed above is substantially correct.

The cholestadiene characterised in this work is at present thought to be a genuine component of the atheromatous plaque, in spite of the fact that cholesta-3,5-diene isolated by other workers from brain lipids (9), was considered to be an artefact produced during chemical manipulations. A corresponding $\Delta^{3,5}$ diene isolated after distillation of Tall oil rosin (10) was shown to result from dehydration of β -sitosterol. Although the techniques employed by these other groups of authors were

much more drastic than those described in our work, the possibility that this hydrocarbon results from sterol dehydration has not been ruled out at present.

"Polar Esters"

Although not previously identified in plaque extracts, "polar Esters" have been found to be present in relatively large quantities. Boyd (11) suggested that the 3β- fatty acid esters of 7α-hydroxycholesterol which he identified in blood might be intermediates in the catabolism of cholesterol. He further suggested that 7α-hydroxylation of the sterol nucleus, thought to be an early step in the formation of bile acids, might be brought about by the enzymatic formation of a hydroperoxide of an unsaturated fatty acid (12) followed by the transfer of a hydroxyl group from the fatty acid to the sterol nucleus by an internal rearrangement of the molecule (see Fig. 63). Such an explanation might apply to the formation of esters of 7β-hydroxycholesterol found in the present investigation, and also to the esters of 26-hydroxycholesterol identified. A suggestion of the possible hydroperoxide intermediate of the latter compounds is given below:-

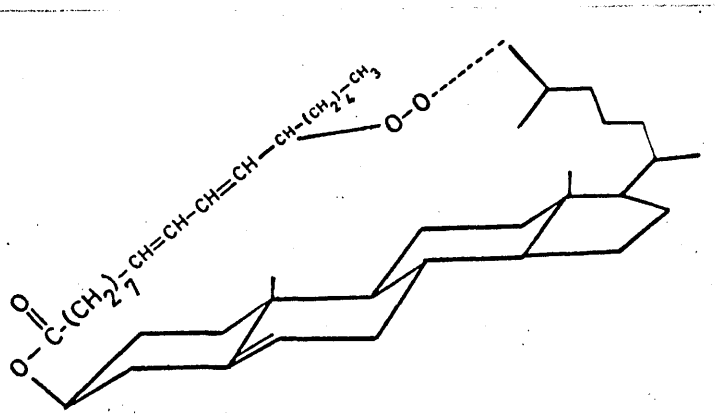
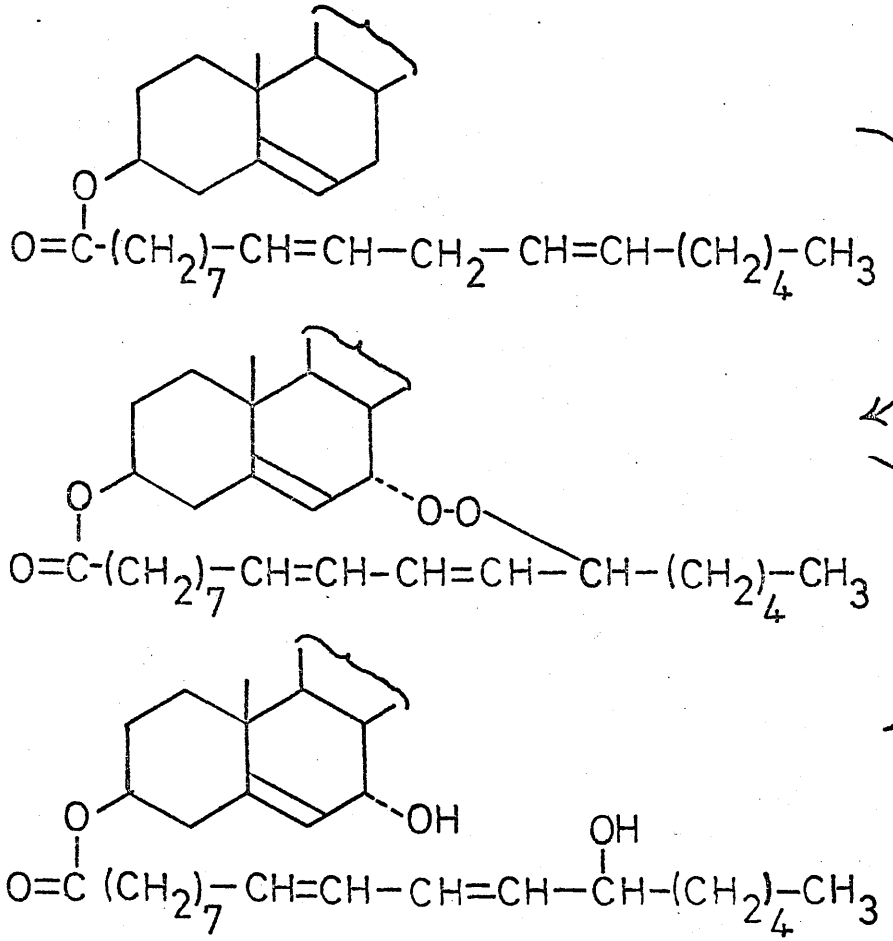


FIGURE 63. HYDROXYLATION OF CHOLESTEROL (Boyd 11)



The kinds of "polar ester" discussed above are present in smaller quantities than the predominating type characterised as cholesterol esterified with a hydroxy fatty acid. Structures of this type are almost completely unknown in nature and might constitute an extremely important group metabolically. Perhaps relevant are the results obtained by Swell and Law (13), which demonstrated that when 4 - C^{14} - cholesterol arachidonate was injected into rats, a considerable proportion of the liver C^{14} activity (25-37%) was recovered in the form of acetylatable polar lipids. On hydrolysis, cholesterol was found to account for 65% of their C^{14} activity and the authors suggest that this sterol must have been esterified with hydroxy fatty acids. Although the esters described above appear from these authors' results to be more polar than those isolated in our work, they might nevertheless be related, and together constitute evidence for a metabolic pathway of cholesterol not previously recognized.

Hydroxy fatty acids themselves are not uncommon in nature, one of the best known examples being ricinoleic acid (12-hydroxy-oleic acid) which makes up 90% of the component fatty acids of castor oil (14) and occurs in smaller quantities in other seed oils (15). In mammals, α-hydroxy acids are well established components of brain cerebrosides (16), and ω-hydroxy acids are found in wool wax (17). It was formerly suggested that hydroxy acids were intermediates in the conversion of saturated into

monounsaturated acids, but this theory has been shown to be incorrect (18): nevertheless the function of β-hydroxy acids in the biosynthesis of saturated long-chain fatty acids is well established (19). It will, however, be necessary to elucidate the structure of the acids esterified with cholesterol in the lesion before further suggestions as to origin and function can be made.

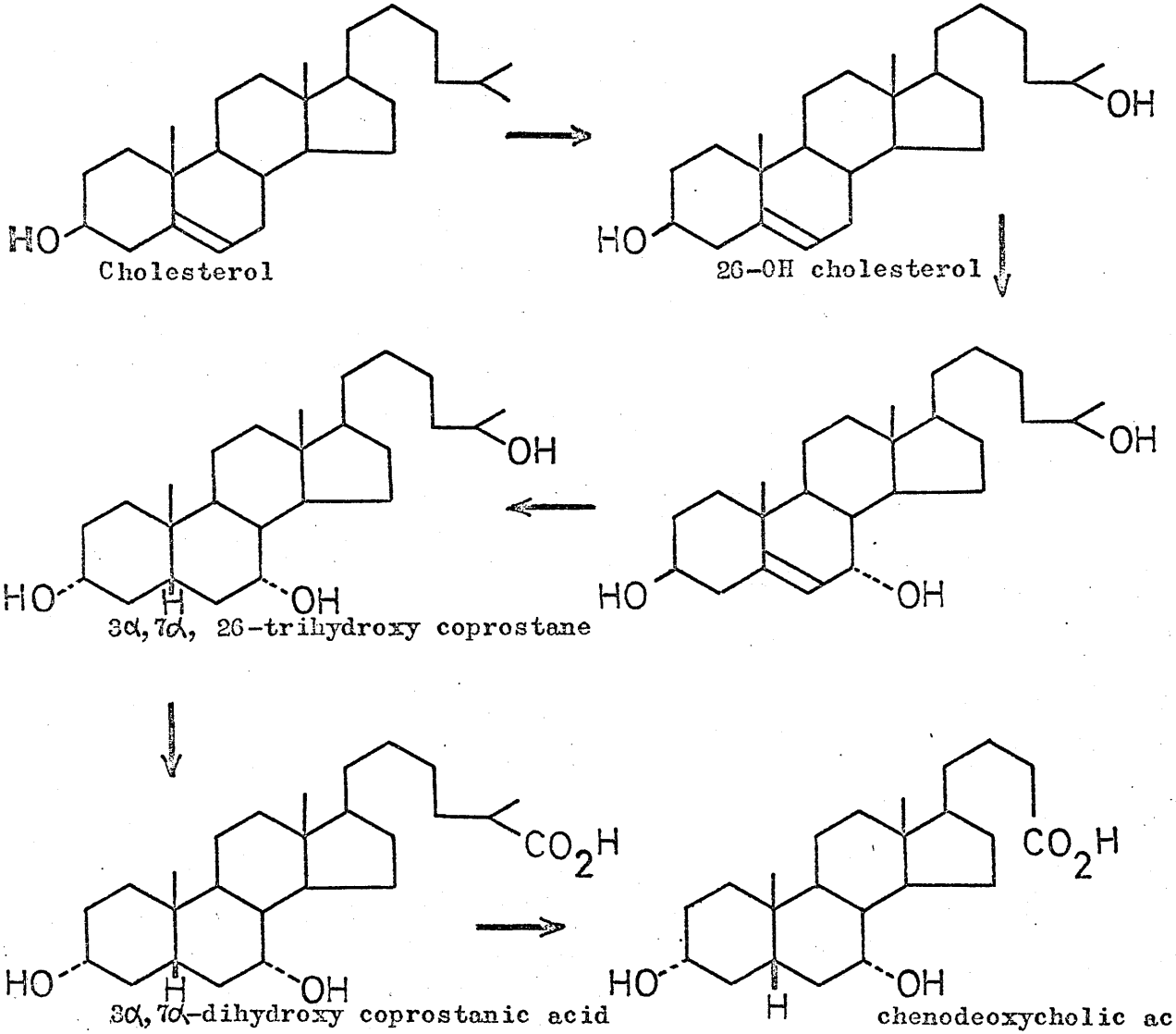
Polar Sterols

The presence of polar sterols in the atheromatous plaque must be carefully considered since, with the important exception of 26-hydroxycholesterol, each of those identified has been associated with the air oxidation of cholesterol. The susceptibility of cholesterol to autoxidation during chemical manipulations is well recognised (20-24): however, 26-hydroxy-cholesterol, the major sterol in all plaque extracts in our work, was not detected in any of these autoxidation studies. Fieser et al. (25) examined a large sample of cholesterol which had been stored in light and air for more than twenty years and were able to demonstrate a 0.34% conversion to 25-hydroxy-cholesterol, but there was no suggestion of the existence of the 26-hydroxy isomer. Considering the speed of the present analysis, and the precautions taken against alterations of the lipids, it is thought unlikely that the latter compound is an

artefact. Our identification of this sterol in the atheromatous plaque (26,27) has been recently confirmed by Smith (28) and by Van Lier and Smith (29) who also regard it as a genuine and important component of the atherosclerotic lesion. They were also able to demonstrate that this sterol is only present in the intima of the aorta, and is absent in medial lipid, again suggesting that it is unlikely that this substance was produced during isolation.

26-Hydroxycholesterol has been identified as a metabolite of 4 - C^{14} - cholesterol in <u>in vitro</u> incubations with mouse liver mitochondrial preparations (30,31). It has also been shown (31,55) that 4 - C^{14} - 26 - hydroxycholesterol is metabolised <u>in vivo</u> by the bile fistula rat predominantly to chenodeoxycholic acid. Studies on the conversion of cholesterol to bile acids have indicated that hydroxylation at the C-26 position is the first step in the degradation of the side chain (32,33). The catabolism of cholesterol by this route is thought to follow the sequence given in Fig. 64. These results, obtained using rats and mice, may have no relevance to any metabolic process, normal or abnormal, taking place within the human body. On the other hand they may be a pointer to the fact that hydroxylating enzymes exist within the liver, and that hydroxycholesterols produced at this site could be released into the bloodstream and hence deposited in the aorta by an "infiltration" process.

FIGURE 64. A PROPOSED SCHEME FOR THE CONVERSION OF CHOLESTEROL TO BILE ACIDS (31)



An alternative theory would be that hydroxylation takes place after the deposition of cholesterol and its esters in the artery wall. At present we have no results to favour either of these hypotheses.

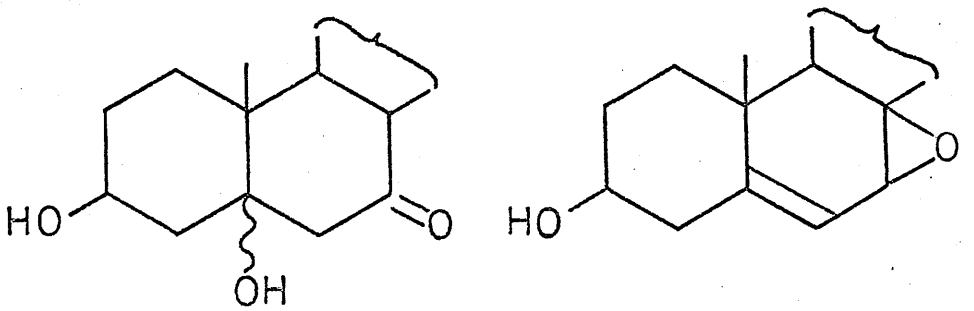
The existence of a toxic factor within the artery wall has been postulated to account for the necrosis which is common in advanced plaques (34). MacDougall et al. (35), in a study of the effects of one hundred and three compounds on animal organ cultures, have demonstrated that thirty-six steroids exhibited toxic effects. Of these, four were found to be "highly" toxic at the microgram level, viz. 5α-cholestane-3β-ol, 5α-cholestane - 3β,5,6β-triol, 5α-cholest-7-en-3β-ol and 26-hydroxycholesterol. Toxicity was judged by histological and histochemical criteria and the four compounds mentioned above were shown to cause gross derangement of the structure of the cells in the artery wall. These results suggest that it is possible that 26-hydroxycholesterol may be involved in the necrosis found in most of the aortas examined in our work.

7-Ketocholesterol (3β-hydroxycholest-5-en-7-one) was found in all extracts examined but its concentration relative to 26-hydroxycholesterol varied markedly. Since this compound is not much more polar than cholesterol it is possible that a certain amount might have crystallised out with cholesterol during the concentration of "polar" sterols. This theory is backed by

the fact that 7-ketocholesterol was detected when these same cholesterol fractions were examined by TLC on silica gel impregnated with silver nitrate, in a search for unsaturated monohydroxy steroids.

This ketosterol is one of the most frequently encountered autoxidation products of cholesterol (36). It has been detected as such or as its dehydration product, cholesta-3,5-dien-7-one, in aortal sterols (37); in swine and bull testis (38); in sheep wool sterols (39) and in human urine (45). Doubts have however been expressed about the authenticity of this compound as a natural sterol. Results have been recently obtained which show that in rat liver at least, 7-ketocholesterol can be formed enzymatically (40) and a possible metabolic role in the in vivo formation of bile acids has been demonstrated. It is therefore possible that this sterol has some metabolic function in the human atheromatous lesion or other site in the human body, and should not necessarily be regarded only as an artefact of cholesterol.

It is also necessary to consider the possibility that 7-ketocholesterol is an artefact of another sterol or sterol ester and two such hypothetical compounds are as follows:-



Like 7-ketocholesterol, 7β-hydroxycholesterol has until recently only been discussed in terms of the autoxidation of cholesterol. Recent observations have shown however that 7β-hydroxycholesterol (like its 7α-epimer) can be formed enzymatically in rat liver homogenates (40). 7α-Hydroxy-cholesterol was first identified in ox liver (41) and since then has become widely accepted as the first compound on the main metabolic pathway of the conversion of cholesterol to bile acids (42, 43). Although we have not yet been able to demonstrate free 7α-hydroxycholesterol in the atheromatous plaque, its tentative identification as a mono fatty acid ester in the "polar ester" fraction suggests that a closer investigation of the sterol fraction is warranted.

Very little is known about the metabolism of 5α-cholestane-3β,5,6β-triol, the other "polar" sterol identified in plaques. In a recent experiment (44) this compound was fed to rats, and was shown to be rapidly metabolised to bile acids as well as to neutral compounds. Toxicity under these conditions, however, appeared to be low.

So far in this discussion we have summarized the very limited existing knowledge on the metabolism of the compounds identified in our work, mainly with respect to their function in sources other than the atheromatous plaque. We must now consider their significance in this context in the light of the findings

to date. It is important to realize from the start that not all of these substances need fit into one scheme and indeed it is believed by most workers that the lipid material which we have examined results from the culmination of several processes.

According to several authors (48-50) atheromata result from the perfusion of blood lipids into the artery wall where they incite a reactive proliferation that ultimately results in the plaques of atherosclerosis. It thus seems possible that many of the compounds which we have identified might have resulted from the alteration of blood lipids after deposition in the arterial intima. This generalisation might not apply for instance to squalene, cholest-5-ene and cholestadiene which have also been shown to be present in hypercholesterolemic blood (47). On the other hand these hydrocarbons could have been formed in the intima and "leached" out by the blood flowing through the lumen.

Formation in situ would seem to be the likely explanation for the existence of hydroxycholesterols in the plaque, since there is little evidence for this type of compound in human blood. Esterification of the diols thus formed would produce the "polar esters"; alternatively these esters could have been formed directly from the normal cholesterol esters by enzymic hydroxylation.

Although esters of 7α-hydroxycholesterol have been shown to exist in hypercholesterolemic blood, no mention has ever been

made of esters composed of cholesterol and a hydroxy fatty acid which were found in the present investigation. It is conceivable, however, that these compounds could have been overlooked by earlier workers, in view of their close chromatographic similarity to esters of 7α-hydroxycholesterol. It seems likely that if these rather unusual esters of cholesterol are present in blood, they cannot be the predominant type in contrast to the present findings in the plaque.

At present we consider that most of the compounds identified in our work are not involved in the initial plaque formation, but that they might be intimately concerned in the degeneration of the arterial intima, which is a feature of the late stages of atherosclerosis. The rupture of the intima overlying the lesion has been suggested (34, 51) to initiate the formation of thrombi which may prove fatal when they occur in smaller vessels, such as the coronary and cerebral arteries.

FUTURE WORK

The proposed scheme of future work can be divided into three groups:-

1. Completion of the identification of compounds being studied at present.
2. Work on fractions or parts of fractions not yet examined.
3. A comparative study of different types of plaques characteristic of earlier stages of the disease and possibly an examination of plaques in sites other than the aorta.

Group 1.

(a) hydrocarbons:- It is proposed to examine the alkanes found in the plaque by GLC and GC-MS after separation of straight and branched chain types either by molecular sieves or by complexing with urea. The identification of cholest-5-ene will be completed by obtaining a mass spectrum of the ketone produced after isomerisation of its epoxide with boron trifluoride in ether. Work is in progress to collect enough purified cholestadiene to enable its optical rotation to be measured, which should aid the assignment of the double bond positions.

(b) "Polar esters":- The outstanding problem in this group of esters is the identification of the major pair of isomeric hydroxy fatty acids which are esterified with cholesterol. Now that the molecular weight and the probable position of the hydroxyl group

are known it remains to elucidate the nature of the skeleton and the position of the double bonds (cf. page 195).

(c) Sterols closely related to cholesterol:- The use of propionate derivatives has been abandoned because of their poor mass spectral behaviour and inconveniently long GLC retention times. Recent work with TMSi ethers on silver nitrate impregnated TLC plates has shown that these derivatives are generally unsuitable, owing to decomposition on the layer. Work with trifluoroacetates has however proved more successful and has enabled the identification of 5α-cholestan-3β-ol (52). It is intended to extend the method to look for other sterols such as desmosterol.

(d) Polar sterols:- Work is in progress to confirm the identification of 26-hydroxycholesterol by oxidising it to 3-ketocholestanoic acid.

Group 2

(a) Interesting compounds of chromatographic polarity intermediate between "polar esters" and triglycerides have appeared in several extracts and it is intended to isolate these substances by preparative TLC and to attempt to determine their structure.

(b) Previous investigators (53) have made tentative identifications of minor lipids such as glycerol ether diesters in plaque extracts and have suggested the possibility of several

other types of lipid with mobilities between those of triglycerides and cholesterol esters. A re-examination of this chromatographic region might therefore be fruitful. A recent communication (54) on the isolation of cholesterol hexadecyl ether (slightly less polar than cholesterol esters on TLC) from bovine cardiac muscle has provided another new lipid type which may also exist in the human atheromatous plaque.

Group 3

An extension of the work on the chemistry of the atheromatous plaque should include examination of more than one type of plaque. For instance, hard fibrous plaques and early fatty types should be closely studied for qualitative and quantitative differences in the minor components identified so far in the late soft ulcerated lesions.

It would also be useful to examine hypercholesterolemic blood with special reference to "polar esters" and to determine whether it contains the same range of esters as found in the plaque, or whether this is only one such type, viz. the 7α-hydroxycholesterol esters as described by earlier workers (11).

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APPENDIX

APPENDIX I. CHROMATOGRAPHIC DATA.

Table 18 TLC OF STEROLS

Solvent system:- chloroform : ethyl acetate (3:1 v/v)
adsorbent:- MN - Kieselgel G-HR.

COMPOUND	R_F	COLOUR REACTION WITH CERIC SULPHATE
cholesterol	0.65	maroon
cholestanol	0.65	pink
desmosterol	0.64	dark brown
7 - ketocholesterol	0.38	pale yellow
24α-OH cholesterol	0.45	brown
25 -OH cholesterol	0.33	dark maroon
26 -OH cholesterol	0.30	maroon
5α-cholestane -3β,5,6β -triol	0.02	light brown
7β -OH cholesterol	0.18	blue
7α-OH cholesterol	0.16	blue

Table 19. TLC OF STEROL TMSi ETHERS

Solvent system:- hexane : benzene (2:1 v/v)
Adsorbent:- MN - Kieselgel G-HR.

COMPOUND	R_F	COLOUR REACTION WITH CERIC SULPHATE
cholesterol	0.58	maroon
epicholesterol	0.80	red-brown
cholestanol	0.58	maroon
desmosterol	0.55	dark brown
7α-OH cholesterol (bis)	0.53	blue
7β -OH cholesterol (bis)	0.16	blue
24α-OH cholesterol (bis)	0.47	dark brown
25 -OH cholesterol (bis)	0.47	brown
26 -OH cholesterol (bis)	0.35	maroon
5α-cholestane -3β,5,6β -triol (bis)	0.13	light brown
5α-cholestane -3β,5,6β -triol (tris)	0.75	light brown
5α-cholestane -5,6α-diol (mono)	0.34	maroon
5α-cholestane -5,6α-diol (bis)	0.77	maroon

Table 20 GLC OF STEROL TMSi ETHERS

(1% SE-30; 225°C)

COMPOUND	t_R	I
cholesterol TMSi	2.38	3090
epicholesterol TMSi	1.74	2985
5α-cholestane-3β-ol TMSi	2.44	3095
desmosterol TMSi	2.59	3115
7-dehydrocholesterol TMSi	2.65	3125
Lanosterol TMSi	3.95	3230
24-dihydrolanosterol TMSi	3.55	3215
24-ketocholesterol TMSi	4.30	3255
7-ketocholesterol TMSi x	5.10	3325
7β-OH cholesterol bis-TMSi x	3.68	3220
7α-OH cholesterol bis-TMSi x	2.62	3120
24-OH cholesterol bis-TMSi x	5.42	3370
25-OH cholesterol bis-TMSi x	5.69	3385
26-OH cholesterol bis-TMSi x	6.75	3430
5α-cholestane-3β,5,6β-triol bis-TMSi x	5.01	3320
5α-cholestane-3β,5,6β-triol tris-TMSi	3.60	3220
5α-cholestane-5,6α-diol mono-TMSi	2.86	3140
5α-cholestane-5,6α-diol bis-TMSi	2.55	3105

Where t_R = retention time relative to cholestane and I = retention index.

x Similar retention values have recently been recorded for these compounds by J.E. Van Lier and L.L. Smith, Anal.Biochem., 1968, 24, 419.

Table 21 GLC OF STEROL PROPIONATES

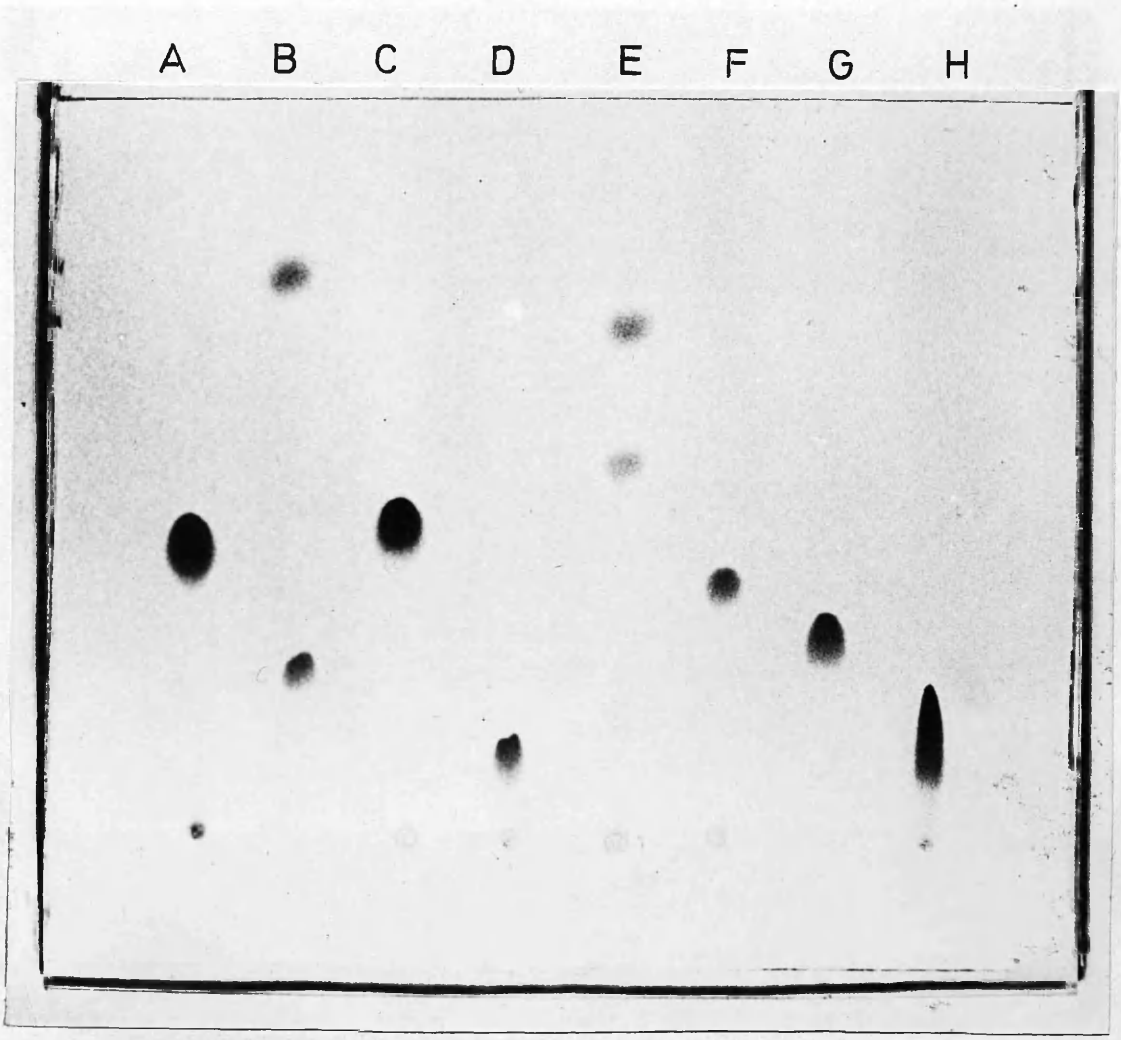
(1% SE-30; 225°C)

COMPOUND	t_R	I
cholesterol propionate	4.05	3250
desmosterol propionate	4.35	3280
cholestanol propionate	3.95	3230
coprostanol propionate	3.28	3185
24-dihydrolanosterol propionate	5.80	3370
lanosterol propionate	6.42	3405
7-ketocholesterol propionate	8.04	3480

t_R = retention time relative to cholestane

I = retention index.

FIGURE 65. TLC OF STEROL TMSi ETHERS.

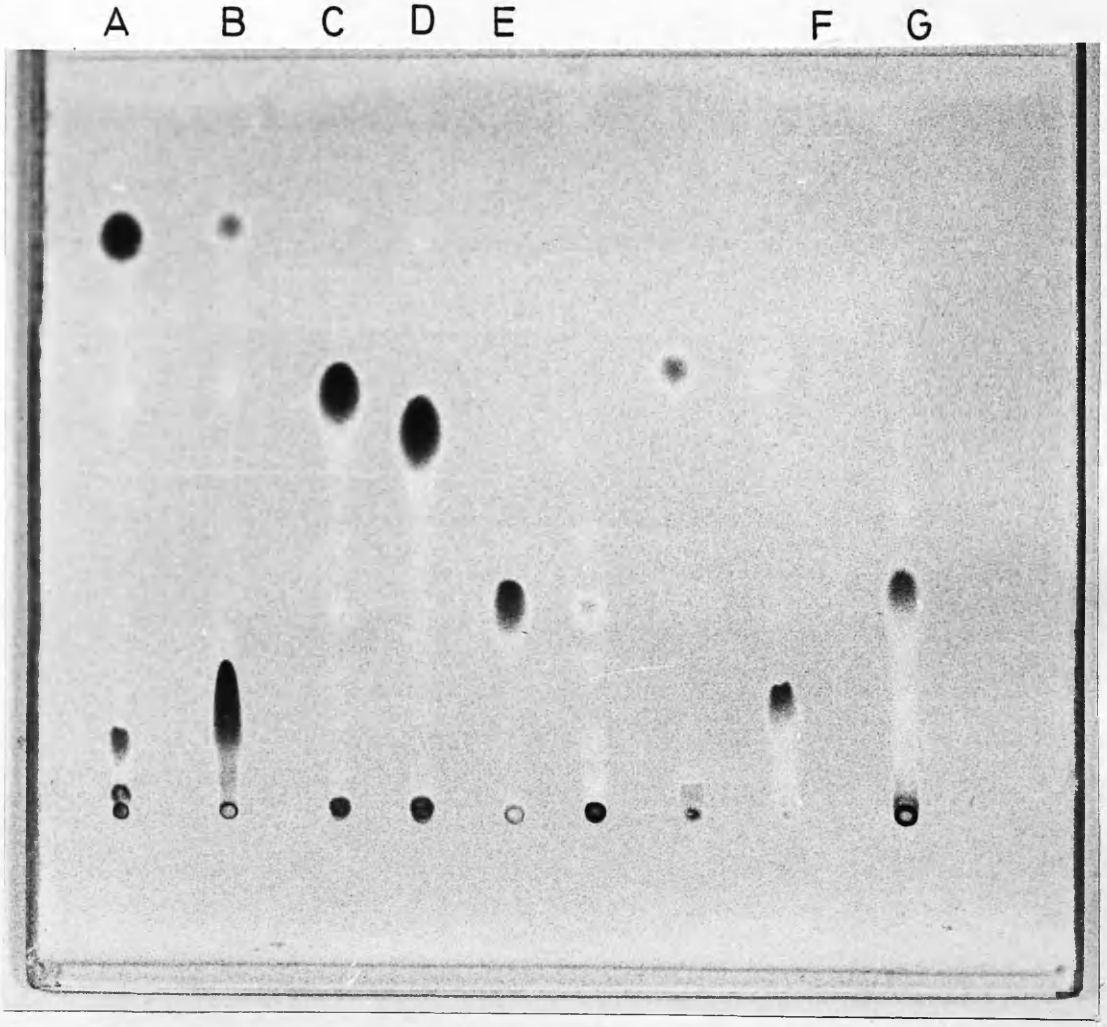


A - cholesterol; B - 5α-cholestane-5,6α-diol (mono and bis);
C - 7α-OH cholesterol (bis); D - 7β-OH cholesterol (bis);
E - cholest-5-en-3α-ol (plus more polar impurity);
F - 25 -OH cholesterol (bis); G - 26 -OH cholesterol (bis);
H - 5α-cholestane-3β,5,6β-triol (3,6-bis).

solvent:- hexane:benzene (2:1 v/v)

spray:- 1% ceric sulphate in 10% sulphuric acid

FIGURE 66 TLC OF STEROL TMSi ETHERS



A - 5α-cholestane-3β,5,6β -triol (tris mainly);
B - 5α-cholestane-3β,5,6β triol (bis mainly);
C - cholesterol; D - desmosterol;
E - 5α-cholestane-5,6α-diol (mono);
F - 7β -OH cholesterol (bis);
G - 26 - OH cholesterol (bis).

solvent:- hexane:benzene (2:1 v/v)
spray:- 1% ceric sulphate in 10% sulphuric acid

APPENDIX 2

FRAGMENTATION OF STEROL TMSi ETHERS

The principal ions in the mass spectra of sterol TMSi ethers examined in connection with the atheroma project are tabulated below and the source of some of the general fragments has been indicated. Some of the spectra on the other hand possess unique fragments and the possible structure of some of these has been discussed separately.

STEROL TRIMETHYLSILYL ETHERS (70 ev.)

	Mol. wt.	Principal ions in the mass spectrum					
cholesterol	458	129 (100%)	329[C] (60%)	368[B] (38%)	119 (31%)	121 (29%)	145 (25%)
epicholesterol	458	129 (100%)	121 (39%)	119 (35%)	329[C] (34%)	145 (27%)	368[B] (24%)
7α-OH cholesterol	546	456[B] (100%)	129 (8%)	119 (6%)	145 (6%)	143 (5%)	147 (4%)
7β-OH cholesterol	546	456[B] (100%)	129 (7%)	119 (5%)	145 (4%)	143 (4%)	131 (4%)
24α-OH cholesterol	546	145 (100%)	159 (51%)	413 (50%)	129 (43%)	503 (15%)	327 (14%)
25-OH cholesterol	546	131 (100%)	129 (19%)	147 (18%)	366[E] (10%)	271 (9%)	456[B] (6%)
26-OH cholesterol	546	456[B] (100%)	129 (96%)	417[C] (89%)	546[P] (50%)	441[D] (34%)	121 (29%)

	Mol. wt.	Principal ions in mass spectrum					
5α-cholestane-5 ,6α-diol mono-TMSi	476	461[A] (100%)	321 (76%)	369[D] (73%)	129 (48%)	368 (45%)	161 (34%)
5α-cholestane-5 ,6α-diol bis-TMSi	548	533[A] (100%)	321 (79%)	368[E] (57%)	147 (51%)	369 (49%)	548[P] (42%)
5α-cholestane-3β,5,6β-triol bis-TMSi	564	129 (100%)	403 (88%)	321 (73%)	456 (71%)	177 (64%)	546[F] (40%)
5α-cholestane-3β,5,6β-triol tris-TMSi	636	456[E] (100%)	546[B] (63%)	321 (58%)	129 (57%)	403 (50%)	217 (43%)

P = molecular ion

A = P − CH_3 = P − 15

B = P − Me_3SiOH = P − 90

C = P − 129

D = P − 105 (90, 15)

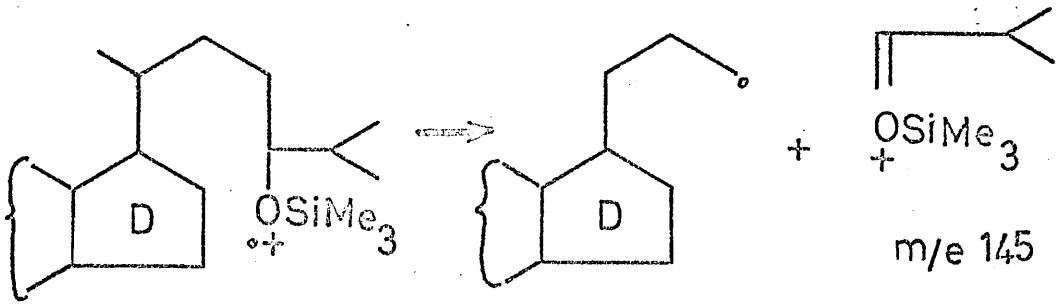
E = P − 180 (2 × 90)

F = P − 18

[only ions above m/e 110 have been considered.]

Cholest-5-en-3β,24-diol (24α-hydroxycholesterol)

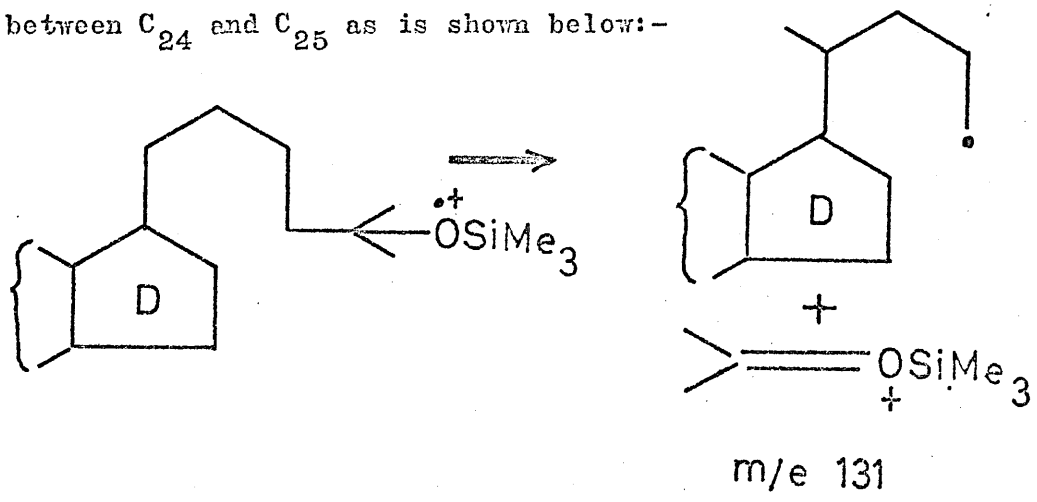
The spectrum of the bis-TMSi ether of this compound (fig. 40 page 182) contains an important fragment at m/e 145 thought to be due to an α-cleavage of the side-chain ether group as shown below:-



The alternative α-cleavage at C_{24} - C_{25} involves the loss of an isopropyl group to give an ion at m/e 503. Loss of trimethylsilanol from this latter ion results in a peak at m/e 413. These two proposed losses are affirmed by metastable peaks at 463 (546 → 503) and 339 (503 → 413).

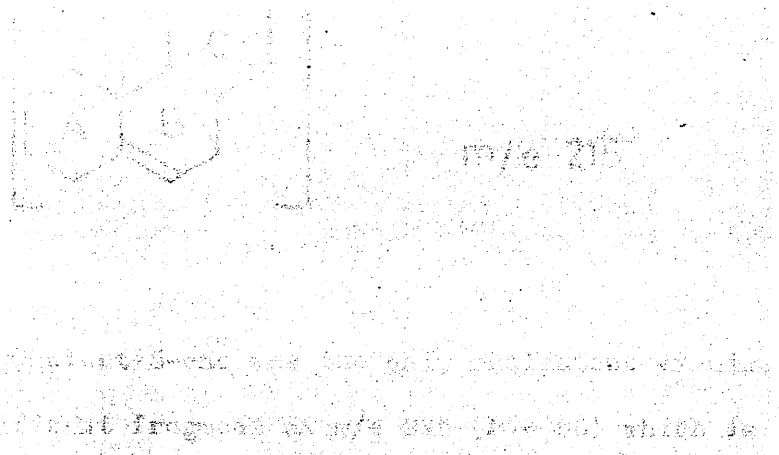
Cholest-5-en-3β,25-diol (25-hydroxycholesterol)

The base peak in this spectrum (m/e 131) can again be explained by postulating an α-cleavage of the side chain between C_{24} and C_{25} as is shown below:-



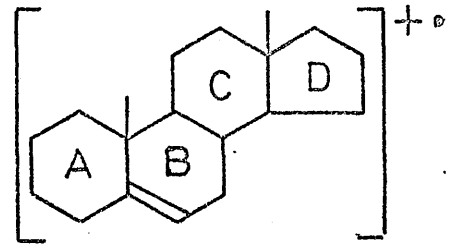
TMSi ethers of sterols containing a 6-hydroxyl group.

The trimethylsilyl ethers of four compounds containing a 6-hydroxyl group have been studied by GC-MS, and all give a characteristic fragment at m/e 321 which has not yet been assigned. It is hoped that work with deuterium-labelled TMSi ethers will allow the solution of this problem.

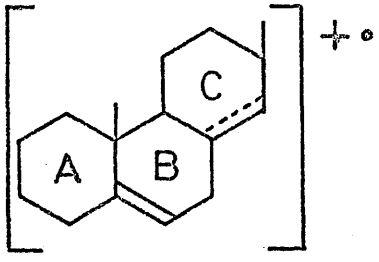


Mass spectral fragmentation of cholestenes.

The mass spectra of all of the cholestenes studies show an abundant molecular ion P(m/e 370) and a fragment at m/e 355 (P - 15), due to the elimination of an angular methyl group from the molecule. Each compound examined gave ions at m/e 257 and m/e 215 due to the loss of the side chain and the side chain plus 42 mass units from ring D respectively, as is shown below for cholest-5-ene:-



m/e 257



m/e 215

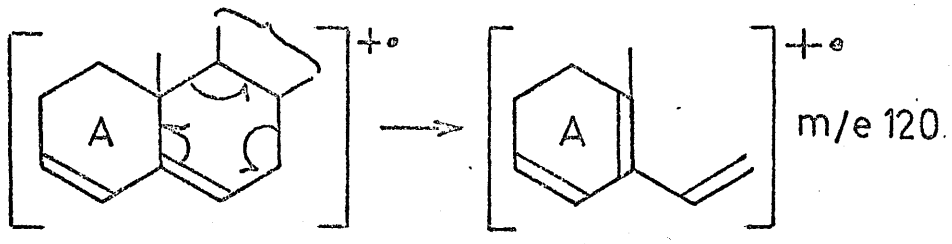
Cholest-5-ene was the only cholestene examined which gave a significant fragment at m/e 275 (P - 95) which is thought to be due to the loss of ring A plus an angular methyl group. Another ion characteristic of this compound is found at m/e 301. It is possible that this results from the elimination of part of ring A,

but there is no evidence for this other than the fact that it also occurs in the mass spectrum of cholesterol (cholest-5-en-3β-ol).

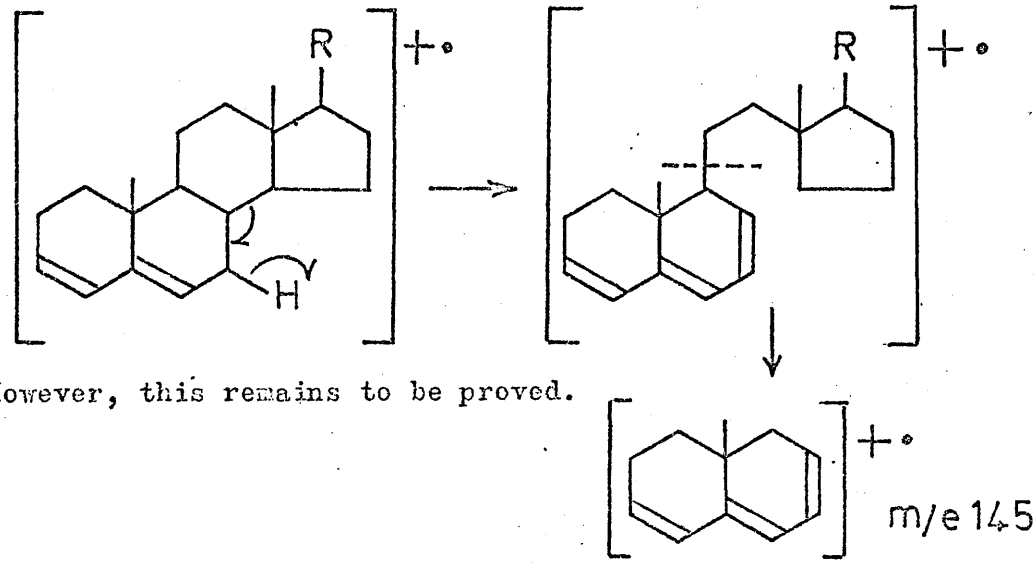
As has been already stated (page 140), cholest-2-ene gives a unique fragment at m/e 316 due to the loss of the elements of butadiene by a retro-Diels-Alder mechanism. Elimination of the side chain (C_8H_{17}) from the ion of m/e 316 gives a fragment of m/e 203 also characteristically preponderant in the spectrum of cholest-2-ene.

Fragmentation of cholesta-3,5-diene.

The mass spectrum of cholesta-3,5-diene (Fig. 18) shows a high intensity molecular ion at m/e 368 and an ion due to loss of an angular methyl group at m/e 353. The loss of the side chain and the side chain plus 42 mass units from ring D give fragments at m/e 255 and 213 respectively. The fragment at m/e 120 is thought to result from a retro-Diels-Alder reaction of the following type:-

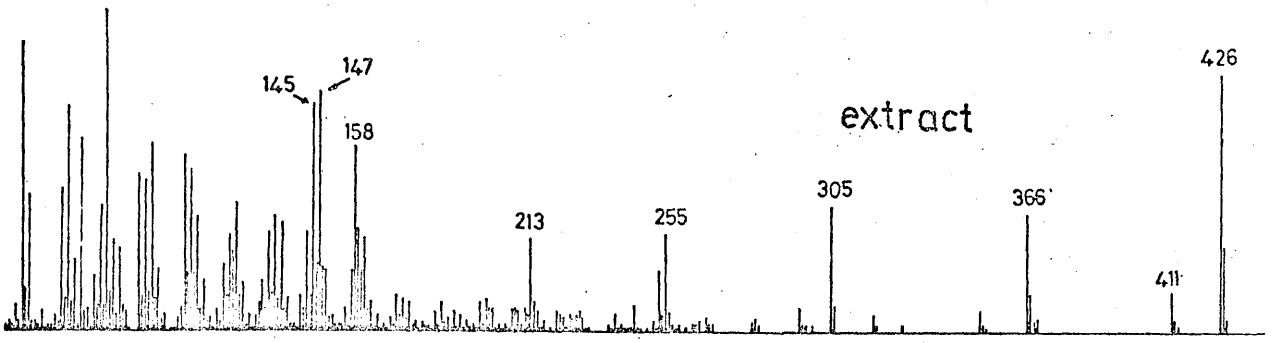


This process results from the rupture of the C7 - C8 and the C9 - C10 bonds and with transfer of two hydrogen atoms also gives the ion at m/e 247 which is found in the mass spectrum. It seems likely that m/e 145 results from a fragmentation of the type shown below:-



However, this remains to be proved.

FIGURE 67. MASS SPECTRUM OF 26-HYDROXYCHOLESTEROL DIACETATE.



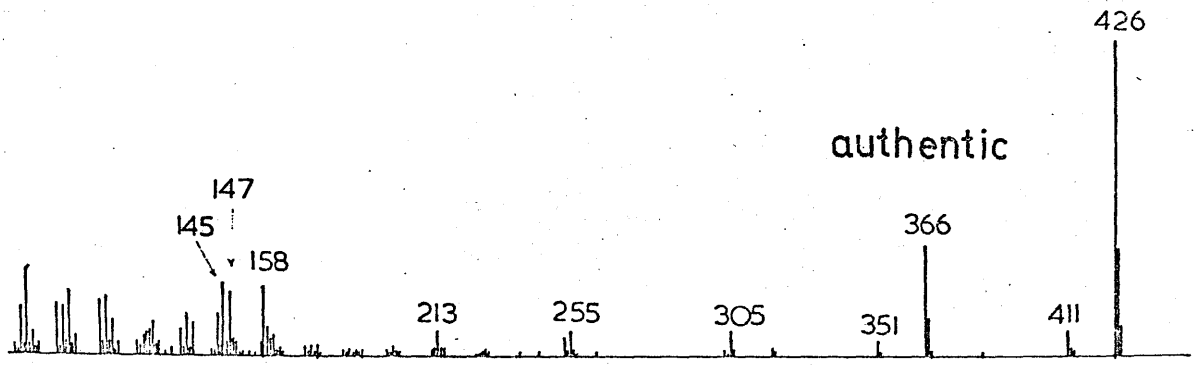


FIGURE 68.

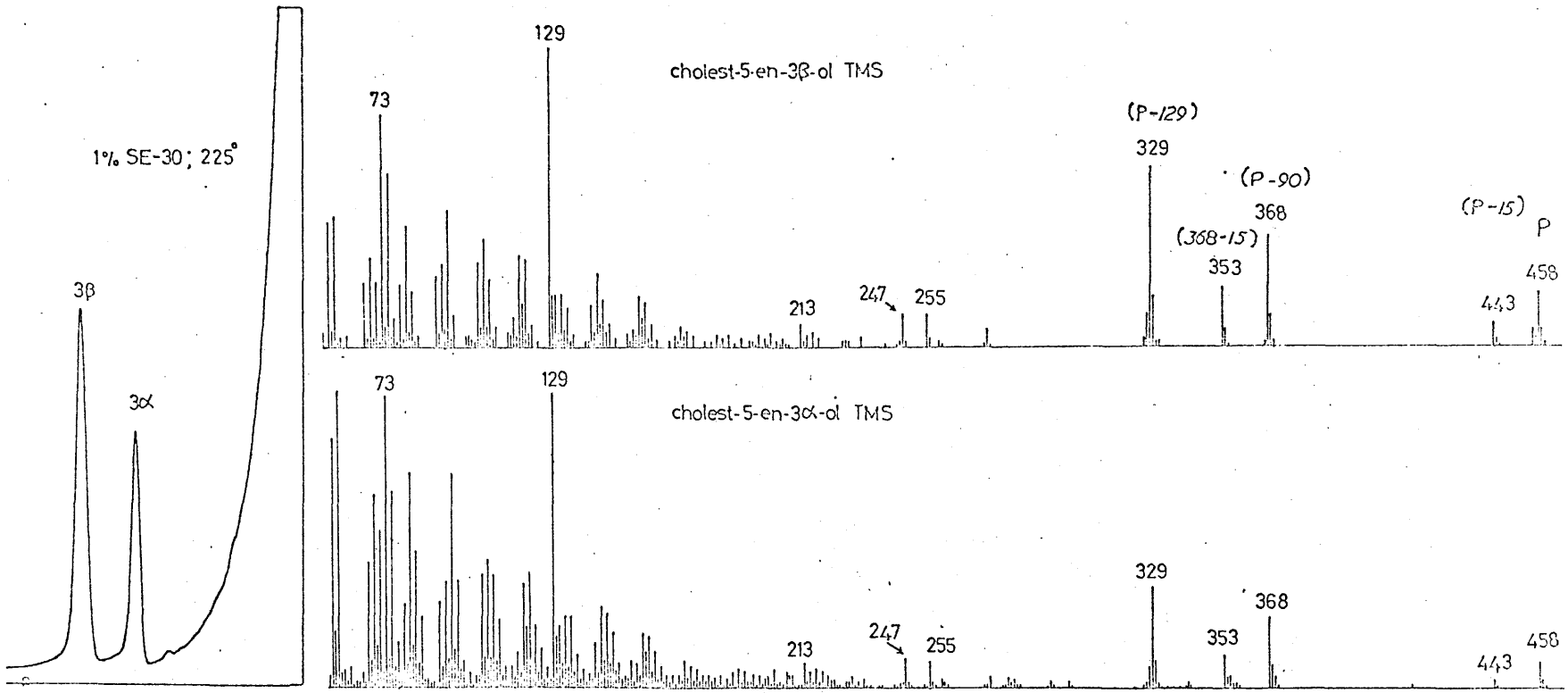
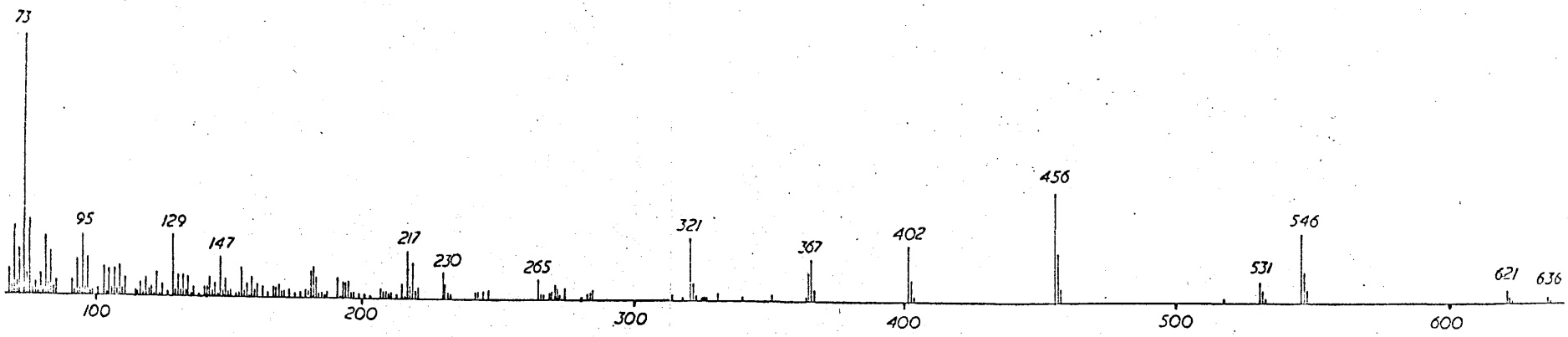

1% SE-30; 225°
3β
3α
cholest-5-en-3β-ol TMS
73
129
213
247
255
(P-129)
329
(P-90)
368
(368-15)
353
(P-15)
443
P
458
cholest-5-en-3α-ol TMS
73
129
213
247
255
329
368
353
443
458

FIGURE 69 Mass spectrum of 5α-cholestane-3β,5,6β triol tris-TMSi ether.



Sources of Reference Compounds

5α-cholestan-3β-ol	British Drug Houses, Poole, England.
desmosterol 24-ketocholesterol 24-hydroxycholesterol	Dr. G.F. Woods, Organon Laboratories, Newhouse, Scotland.
7-dehydrocholesterol lanosterol dihydrolanosterol	Koch-Light Laboratories Ltd., Colnbrook, England.
26-hydroxycholesterol	Drs. H. Danielsson and J. Sjövall, Karolinska Instituet, Stockholm, Sweden; and by synthesis from kryptogenin (Koch-Light)
epicholesterol 7-ketocholesterol 7α-hydroxycholesterol 7β-hydroxycholesterol	by synthesis from cholesterol
5α-cholestane-5,6α-diol	by synthesis from cholesterol chloride
5α-cholestane-3β,5,6β-triol cholest-2-ene	Steraloids Ltd., Croydon, England.
cholest-5-ene	Ikapharm, Ramat-Gan, Israel.

cholest-3-ene cholest-4-ene cholest-8(9)-ene	Dr. P. Bladon, University of Strathclyde, Glasgow.
cholesta-3,5-diene	Applied Science Laboratories Inc., Pennsylvania, U.S.A.
squalene	Calbiochem, Los Angeles, U.S.A.
Fatty Acid Methyl esters	Applied Science Laboratories Inc., Pennsylvania, U.S.A.

"PHYSICO-CHEMICAL TECHNIQUES IN THE STUDY OF ORGANIC MOLECULES OF BIOLOGICAL IMPORTANCE"

G. STEEL 1969

A SUMMARY

The work described in this Thesis consists of a study of the lipids present in the fatty nodules (plaques) which occur during the fatty degeneration of the human aorta associated with the disease atherosclerosis. For many years studies of the chemistry of these lesions have concentrated on the major classes of lipid constituents, viz. cholesterol, cholesterol esters, phospholipids and triglycerides: the minor lipid groups have been largely neglected. We have attempted to remedy this omission, since quantity is not necessarily a measure of biological significance.

Early studies on the subject were severely handicapped by the absence of suitable techniques for the separation and identification of microgram quantities of material and as a result had to use large quantities of tissue requiring, in some cases, several years for collection. The necessarily prolonged nature of these investigations introduced the possibility of gross artefact formation and cast doubt on the authenticity of certain compounds identified as arterial constituents.

In the present work, thin-layer and gas-liquid chromatography (TLC and GLC) and gas chromatography-mass spectrometry (GC-MS) were used to enable each investigation to be restricted to plaque tissue from one aorta. Lesions from severely diseased aortas were dissected and solvent extracted within 24 hours post mortem. Division of the extract into its major components was effected by column chromatography on silicic acid. Repeated crystallisation of the sterol fraction from methanol enabled the concentration of several sterols chromatographically more polar than cholesterol. The most prominent of these was tentatively identified on the basis of its chromatographic behaviour as 26-hydroxycholesterol (cholest-5-ene-3β,26-diol), a previously unknown tissue constituent. Confirmation of this structure was obtained by GC-MS, making use of the ability of trimethylsilyl ether derivatives to promote characteristic mass spectral fragmentations. Using a similar approach, 7-ketocholesterol (3β-hydroxycholest-5-ene-7-one); 7β-hydroxycholesterol (cholest-5-ene-3β,7β-diol) and 5α-cholestane-3β,5,6β-triol were confirmed as constituents of this fraction as had been suggested by earlier investigators.

Examination of the hydrocarbons, which had been neglected by previous workers, revealed that the major component was the isoprenoid hydrocarbon squalene. The initial identification was

made by TLC, GLC and GC-MS and was corroborated by infrared spectrometry. Preparative TLC on silica gel impregnated with silver nitrate enabled the separation of two unsaturated steroidal hydrocarbons, later shown by mass spectrometry to be a cholestene and a cholestadiene, from a series of n-alkanes. Hydroxylation of the cholestene and GC-MS of the resulting diol in the form of the trimethylsilyl ether derivative has established that the compound present is largely cholest-5-ene. Work is in progress to determine the double bond positions in the cholestadiene.

TLC analysis of silicic acid column eluates of polarity intermediate between triglycerides and cholesterol revealed the existence of several unidentified esters which were further purified by extensive chromatography. Acetylation demonstrated that these esters contained unesterified hydroxyl groups. The principal neutral compounds generated on hydrolysis were shown to be cholesterol, 26-hydroxycholesterol and 7β-hydroxycholesterol. The presence of the latter two sterols was not unexpected: however, the identification of cholesterol as a hydrolysis product necessitated the postulation of the existence of an ester of cholesterol with a hydroxy fatty acid. TLC and GLC studies on the fatty acid portion of the molecule have confirmed this and GC-MS examination has shown that the major compounds are isomeric C_{18} - monohydroxy acids each with two double bonds.

TLC of sterol fractions (as propionate esters) on silica gel impregnated with silver nitrate has permitted the separation of desmosterol from an overwhelming mass of cholesterol. The relatively unsatisfactory behaviour of sterol propionates on GLC and GC-MS has led to the preferred use of trifluoroacetates. Using this approach we have been able to identify cholestanol in plaque extracts.

It is difficult to assess the significance of these findings in relation to the development of human atherosclerosis, as only one stage of the disease has been examined. However, the methods developed will form the basis of a further investigation which will involve the study of plaques at various stages of evolution in an attempt to relate chemical changes within the artery wall to the pathogenesis of atherosclerosis.