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# "THE CHEMISTRY OF HUMAN ARTERIAL DISEASE"

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Oxygenated sterol esters of human atherosclerotic plaques, in H. Peeters (ed.), "Protides of Biological Fluids", 1971, Pergamon Press, 1972, p. 101.

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Lipids of human atheroma.
Part VII: Isolation of diesters of cholest-5-ene-3β,26-diol from extracts of advanced atherosclerotic lesions of human aorta.
Biochim.Biophys.Acta, 270 (1972) 149.

## Sources of Reference Compounds

| | |
|---|---|
| 5α-cholestan-3β-ol | B.D.H. Chemicals Ltd., Poole, England. |
| 5α-cholestan-3α-ol | Mann Research Laboratories, New York. |
| 5β-cholestan-3β-ol<br>5β-cholestan-3α-ol | K & K Laboratories Inc., Plainview, New York. |
| 7α-hydroxycholesterol<br>7β-hydroxycholesterol | By synthesis from cholesterol. |
| 26-hydroxycholesterol | By synthesis from kryptogenin (Syntex). |
| Cholest-5-ene-3α-ol | Mann Research Laboratories, New York. |
| 24-hydroxycholesterol | Dr G.F. Woods, Organon Laboratories, Newhouse, Scotland. |
| Cholesteryl linoleate | Sigma Chemical Co., St. Louis, Missouri. |
| Other cholesterol esters | Applied Science Laboratories, State College, P.A.<br>Nu-Chek-Prep.Inc. Elysian, Minnesota. |
| Fatty acid methyl esters | Applied Science Laboratories. |
| Triglycerides | Nu-Check-Prep. Inc. |
| α-dimorphecolic acid | Isolated from seed of Calendula officinalis. |
| β-dimorphecolic acid | Isolated from seed of Dimorphotheca aurantiaca. |
| Coriolic acid | From the action of soya bean lipoxygenase on linoleic acid. |
| Methyl ricinoleate | Applied Science Laboratories. |

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## SUMMARY

The work described in this thesis is concerned with the lipids present in the fatty plaques which develop in human arteries during the processes of atherosclerotic degeneration. The variations in concentration and composition of the major lipid classes as the disease progresses have received previous attention. However, other lipids present in smaller quantities have not been investigated in detail. A study of such constituents was undertaken since it seemed possible that some of them might be of significance in the pathology of atherosclerosis.

Early attempts to identify the minor steroids which accompany cholesterol in atherosclerotic lesions were hindered, and to some extent vitiated, by the lack of suitable microanalytical techniques. Since accumulation of adequate quantities of lipid necessitated collection and pooling of a large number of arteries, sometimes over a period of years, it is possible that many of the compounds identified were artefacts of the original steroids. In the present study the use of thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and combined GLC-mass spectrometry (GC-MS) has facilitated rapid and accurate analyses of minor lipids from individual arteries.

Lesions from single human aortas, obtained within 24 hours after death, were classified according to the W.H.O. convention. Following extraction, the lipids were fractionated by column chromatography. The extraction and chromatographic procedures were performed expeditiously to minimise artefact formation.

By repeated TLC on modified layers 5$\alpha$-cholestan-3$\beta$-ol (cholestanol) was isolated from both sterol and sterol ester fractions. The concentration of this compound in severely diseased tissue was estimated by GLC, and found to be considerably

less than had been reported by some earlier workers. Since recent publications suggest that the aortal concentration of cholestanol does not differ markedly from those of other tissues, and no relationship appears to exist between its aortal content and degree of atherosclerosis, it seems unlikely that this compound plays any significant role in the development of the disease.

26-Hydroxycholesterol is known to occur in both normal and diseased aortas. Although its function is unknown, the increased concentrations found in lesions suggest a possible relationship between this sterol and atherosclerosis. The present work demonstrates the occurrence in both early and advanced lesions of a diesterified form of this sterol. The distribution of the fatty acids of this unique type of ester is markedly different from that of the cholesterol esters. Whereas arterial cholesterol esters appear to be derived mainly from blood, the distinctive fatty acids of 26-hydroxycholesterol diesters may reflect a different origin for these constituents.

Previous work in Glasgow had demonstrated the presence in extracts of severely diseased aortas of a group of esters consisting largely of cholesterol esterified with 9- and 13-hydroxyoctadecadienoic acids. A survey was undertaken in an attempt to relate the quantities of these hydroxy-esters with the severity of atherosclerosis. The quantities of the hydroxy-acids derived from these esters were determined by GLC. None of the hydroxy-esters were detected in the earliest lesions, but considerable amounts were isolated from those representing more advanced atherosclerosis. These results establish what may be an important distinction between early and advanced lesions.

In order to assist the identification of other oxygenated sterol esters in diseased tissue, a study was made of the oxidation

products of cholesteryl linoleate. Those derived from hydroperoxides produced by autoxidation and photo-oxygenation of cholesteryl linoleate were dissimilar, as expected from published data on other linoleic acid derivatives. The development of improved techniques enabled the identification in arterial extracts of hydroperoxy- and keto-esters similar to those derived from autoxidised cholesteryl linoleate.

It seems probable that the keto- and hydroxy-esters found in tissue are derived from the corresponding hydroperoxides. The pathogenic character of lipid hydroperoxides is well established, and the production of hydroxy compounds has been shown to be a possible route for "detoxification". The present findings may reflect such a process. However, the full significance of oxygenated derivatives of cholesteryl linoleate to the pathogenesis of atherosclerosis requires to be ascertained. In particular, the origin of the hydroperoxides must be clearly established. At present it is not known whether these substances occur in arteries prior to death, or accumulate in the interval between death and analysis. Examination of tissues obtained during surgery may resolve this problem.

The metabolic processes whereby hydroperoxides arise and are removed are being studied in homogenates of human and animal aortas, and a method for the qualitative assessment of hydroperoxide toxicity in vivo is being developed. Whether these compounds arise by an enzymic or non-stereospecific oxidation should be determinable by resolution of the enantiomeric hydroperoxide derivatives.

# Section 1 INTRODUCTION

| COUNTRY | % Total Deaths Attributable to Coronary Atherosclerosis |
|---|---|
| UNITED STATES | 33 |
| CANADA | 32 |
| SCOTLAND | 30 |
| AUSTRALIA | 30 |
| NEW ZEALAND | 28 |
| NORTHERN IRELAND | 28 |
| ENGLAND & WALES | 27 |
| W. GERMANY | 18 |

TABLE 1. Proportion of deaths in 1966 attributable to coronary atherosclerosis in countries where the disease is most prevalent.

## 1.1. THE DISEASE ATHEROSCLEROSIS

### 1.1.1. Introduction

The World Health Organisation (WHO) defines[1] atherosclerosis as "a variable combination of changes of the intima of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcified deposits, and associated with medial changes".

The lethal sequelae of atherosclerosis, coronary heart disease and cerebral vascular accident claim more victims in the United States than any other cause of death, one-third of all deaths being attributable to coronary atherosclerosis in 1966[2]. The death rate due to this cause in Europe is only slightly lower[2] (Table 1).

Atherosclerosis involves the gradual thickening of the aortal intima by lipid deposits and fibrous tissue. The earliest recognisable manifestation of the disease is the fatty streak,[1,3] a small superficial lesion containing lipid-filled foam cells. From this the lesion is presumed to develop into a fibrous plaque or a complicated lesion.[1] Finally, rupture of the endothelial lining of some atheromatous plaques may result in thrombosis[4]. This may completely occlude a small vessel such as a coronary artery causing myocardial infarction or death.

### 1.1.2. The Processes of Atherogenesis

It is currently postulated that the initial event in the process of atherogenesis involves injury to the arterial intima followed by the accumulation of lipid in the vessel wall. Presumably vascular injury renders the intima more susceptible to permeation by lipid-containing blood constituents. Such injuries can be induced experimentally by application of heat[25]

or cold to the artery wall, by mechanical injury, by radiation, and by the use of chemical and immunological agents.[26,28,27] Shimamoto[6] has demonstrated, by use of electron microscopy, that the endothelial cells of human and rabbit aorta are equipped with intercellular bridges, which seem to control the size of clefts in the endothelial surface. When animals were treated with cholesterol (orally) or epinephrine (intravenous injection), these intercellular bridges were observed to swell and elongate. The sizes of the clefts were increased, rendering the endothelium more permeable to lipid infiltration, and possibly more susceptible to focal development of atherosclerosis.[6]

Several theories have been proposed to explain the process of lipid accumulation. The principal features of the most feasible current hypotheses are as follows.

## The Lipid Infiltration Theory

The lipid infiltration theory states that plaques evolve as a result of a reaction of the arterial wall against lipids which invade it from blood plasma. Most of the lipid in plaques is thought to originate from lipoprotein filtration,[5] possibly through clefts[6] between the endothelial cells of the intima, and evidence has recently been obtained which suggests that a basic cause of lipid deposition may be failure of the arterial wall to metabolise lipids transported as lipoprotein.[7] The initial fatty streaks are confined to the intima, the lipid being present as large intracellular droplets.[101,102]

## The Thrombogenic Theory

The thrombogenic theory states that atherosclerosis results from the initial formation of an intraluminal thrombus[8,9,10] which attaches to the arterial wall and is overgrown by endothelial

cells. Capillaries sprout from the newly grown overlying endothelium, providing a direct blood supply from the lumen.[10] The lipid in a lesion developing in this manner is likely to originate from platelets,[10] and it has been shown that foam cells characteristic of those in atherosclerotic plaques can be derived from monocytes which have phagocytosed platelets.[11] Lipid deposits may also arise by repeated capillary haemorrhages invading the thrombus from the lumen or adventitia.[12,13]

## Lipid synthesis in the artery wall

Although it is generally conceded that the greater proportion of lipid present in atherosclerotic lesions arises by deposition from blood, there is considerable evidence to suggest that the arterial wall has a certain capacity to synthesise lipids. It has been demonstrated that human[14] and rabbit[15] arterial tissues synthesise phospholipids and triglycerides, and Chobanian[16] has shown that human arterial segments obtained at post-mortem examination are capable of synthesising all the major lipid classes commonly found in lesions. The rate of lipid biosynthesis is considerably greater in fatty streaks than in normal intima.[16] The major lipid classes produced are phospholipids and triglycerides, but synthesis of significant quantities of sterols[17] and sterol esters has also been observed.

## Local factors in plaque development

Other factors may determine the site of development of an atherosclerotic lesion. These factors may involve metabolic changes, or degeneration of parts of the artery wall due to ageing or other causes.[18,19] It has been suggested that the localisation of coronary atherosclerotic plaques may be explained in terms of the variation of haemodynamic stresses within the blood vessel. Plaques are observed to develop predominantly at sites

of arterial narrowing and bifurcation,[20,21] i.e. at positions experiencing greater hydrostatic pressures. It has also been demonstrated that thrombi develop preferentially at these sites,[22-24] possibly indicating that such stresses may cause vessel injury resulting in platelet aggregation and subsequent development of thrombi.

It therefore appears that arterial accumulation of lipid and plaque development are caused by a number of factors. At the present time the infiltration mechanism is the most favoured. Although thrombus formation and subsequent incorporation into the arterial intima may account for the formation of some arterial lesions, it is probably too rare an event to account for the extensive atherosclerosis frequently observed in grossly diseased aortas. Local lipid synthesis is a minor factor in arterial lipid accumulation. The greater part of the lipid present in plaques originates from blood.

1.1.3. Epidemiology of atherosclerosis

Life style is apparently a very important factor in the development of atherosclerosis. Although the disease is universal, it is most prevalent amongst people of the affluent and technologically advanced countries.[29] It is particularly interesting that populations which migrate from a country with a low incidence of the disease (e.g. Japan) to one where the disease is a major cause of death (e.g. United States) tend to have a much higher death rate attributable to atherosclerosis than those remaining in their native land.[30,31] Keys and co-workers[30,32,33] have demonstrated a relationship between death rate from coronary heart disease, the mean serum cholesterol level and the proportion of calories derived from total dietary fat. Further striking evidence indicating a relationship between dietary fat and coronary heart disease was noted during the 1939-45 war, where, in many European countries, there was a sharp drop in the death rate from circulatory diseases. This was accompanied by a fall in consumption of animal fat.[34] Thus high fat consumption and the associated high serum cholesterol levels can almost certainly be implicated in the development of atherosclerosis, the high level of fat consumed in the more affluent countries being reflected in the geographical distribution of the disease.

Positive correlations have also been obtained between coronary heart disease and lack of exercise[35], cigarette smoking[36,37], hypertension, stress[38] and the mineral content of drinking water.[39-41] It has been suggested that deficiency in the dietary intake of linoleic acid may pertain to atheromata formation.[42] The increase in atherosclerosis in Britain is roughly paralleled by a decrease in linoleic acid consumption due to hydrogenation of vegetable

oils and eating of prepared foods. Linoleate deficiency increases the proportion of cholesterol esterified with fatty acids synthesised by the body[42] (e.g. oleate, palmitate, stearate), and it has been suggested that such esters may be preferentially deposited in tissues. Though this idea is difficult to reconcile with the fact that the predominant cholesterol ester in advanced arterial lesions is linoleate, it has been shown that the incorporation of polyunsaturated fatty acids in diets reduces the level of blood cholesterol.[42] This may possibly be due to more rapid catabolism of cholesterol when esterified with polyunsaturated acids.[43]

### 1.1.4. Cholesterol and Atherosclerosis

There is fairly general agreement that elevated blood cholesterol levels contribute significantly to the genesis and/or progression of atherosclerosis in man.[44] It is not clear, however, whether the excess cholesterol in plasma is absorbed from the diet or is synthesised by the body tissues. Although most tissues are capable of synthesising cholesterol, only in the liver, skin and intestine is there a high rate of cholesterogenesis.[45] Experiments using dogs[46,47] and rats[48-50] have demonstrated that a high dietary intake of cholesterol results in a striking suppression of body cholesterol synthesis. Since the bulk of cholesterol synthesis in these animals occurs in the liver,[46,51,52] there is apparently a control mechanism whereby a high dietary intake of cholesterol suppresses hepatic synthesis. The extrahepatic tissues lack such a feedback system and consequently synthesise cholesterol even when the animals are on high cholesterol diets[46,49].

Studies on human subjects have shown that a high dietary intake of cholesterol does not suppress synthesis to nearly the

same extent as in animals.[53-57,82] This indicates that either the human liver is not so susceptible to the type of control mechanism found in dogs and rats, or that a larger proportion of endogenous cholesterol is produced in tissues other than the liver, which are not affected by a feedback mechanism. Since *in vitro* studies have shown that human liver[56,58] is regulated in the same way as other species the latter possibility would appear the more likely.

The major catabolic pathway of cholesterol is by conversion to bile acids, and many studies have been made in attempts to relate excretion rates with serum cholesterol levels. When rats[50] and dogs[47] are fed a high cholesterol diet they are able to compensate for increased cholesterol absorption by increases in faecal bile acid output. Humans are unable to compensate in this way.[54,59,60] However, faecal bile acid output in man can be enhanced by feeding of polyunsaturated fatty acids.[61-63] The increase in cholesterol catabolism is paralleled by a reduction in serum cholesterol levels.[64,65] Boyd has studied the effect of both a high linoleate diet and orally administered ethynyl estradiol on cholesterol metabolism. Linoleate was found to reduce the quantities of plasma cholesterol in humans. The effect was accompanied by an increase in the proportions of cholesteryl linoleate in plasma relative to cholesteryl palmitate and oleate. The effect of estrogen was to reduce the relative proportion of linoleate ester from about 45% of the total cholesterol esters to 30%.[65] It has been suggested that poly-unsaturated cholesterol esters are more rapidly metabolised than the saturated esters.[43] The decrease in serum cholesterol levels when linoleate is added to the diet may reflect a greater catabolic rate of cholesterol due to an increase in the proportion of the more readily metabolised cholesteryl linoleate.[65] The effect of

estrogen may be to increase the rate of catabolism, and since cholesteryl linoleate is preferentially catabolised, its reduction in plasma might be expected. The wide fluctuation of serum cholesterol levels of woman throughout the menstrual cycle,[66,335] during pregnancy[67] and after menopause[68] may be rationalised on the basis of estrogen production.

Some substantiation for Boyd's proposals was provided by his discovery in human serum of a fatty acid esterified form of 7α-hydroxycholesterol. It seems possible that such esters may be intermediates in the catabolism of cholesterol to bile acids. If hydroxylation at $C_{(7)}$ on the cholesterol nucleus is an early step in the formation of bile acids, then this may be facilitated by the presence of polyunsaturated fatty acids esterified at $C_{(3)}$. It has been demonstrated that the relative rate of 7-hydroxylation of cholesterol esters by autoxidation increases with the degree of unsaturation of the acyl moiety.[69] The mechanism of such an autoxidation[65,69] is believed to proceed by initial formation of a hydroperoxy function on the fatty acid moiety, and an intramolecular rearrangement in which the sterol nucleus is hydroxylated at $C_{(7)}$ by cleavage of an internal peroxide. This attractive hypothesis that cholesterol may be catabolised as its linoleate ester has been challenged as a result of more recent studies. Ogura and Yamasaki,[70] having performed <u>in vitro</u> and <u>in vivo</u> studies on the metabolism of 7α-hydroxycholesterol and its esters in rats, conclude that esters of cholesterol and of 7α-hydroxycholesterol are reserve, but not activated catabolic materials.[70,71]

Human blood plasma contains a larger proportion of esterified cholesterol than any other tissue. Cholesterol esters appear to be a preferred form for cholesterol storage since an increase in hepatic storage is reflected by an increase in the ratio of

the esterified to free form,[56] and the large proportions found in plasma probably reflect an important role in cholesterol transport mechanisms throughout the body. The high proportion of cholesteryl linoleate in plasma (about 40% of total cholesterol esters) is striking when contrasted with human liver where a much higher proportion of saturated esters is found. It is thought that in humans the bulk of cholesterol ester is synthesised in plasma[56] by means of the lecithin-cholesterol-acyl transferase (LCAT) enzyme discovered by Glomset and co-workers.[73] This enzyme leads to a preferential formation of cholesteryl linoleate[74,75] and probably accounts for the preponderance of this ester in plasma. LCAT may also be involved in the transfer of cholesterol synthesised or deposited in extrahepatic tissues to the liver where catabolism takes place.[56,76,329]

Macdonald[77] has studied the effect of diet on the variations of serum lipid levels. Healthy young men were subjected to diets consisting of a mixture of fat and carbohydrate. The fats used were either cream (highly saturated), or sunflower oil (polyunsaturated) and were augmented with either glucose or sucrose. Sunflower oils caused the total cholesterol level to fall, in accord with previous findings. In the sunflower oil diet supplemented with sucrose, the fall in total cholesterol was brought about by a decrease in free cholesterol, whereas glucose reduced the serum cholesterol ester levels. When cream was fed with either sucrose or glucose the total cholesterol level remained unchanged. However, the glucose/cream diet appeared to reduce the quantity of free cholesterol in serum. Previous conflicting reports regarding the role of dietary carbohydrates,[78,79] can probably be explained by failure to appreciate that the metabolism of free and esterified cholesterol are affected differently.[72]

Macdonald has also shown that dietary sucrose is related to

an increase in serum glyceride levels, a condition associated with an increased incidence of coronary thrombosis. Since there is also close association of high serum cholesterol levels with atherosclerosis, correlations of this kind may be of considerable importance in the dietary control of coronary heart disease.

Ho and Taylor[80,82] have suggested a very plausible hypothesis relating the development of atherosclerosis to serum cholesterol levels. They showed that when blood cholesterol levels are increased by feeding a high cholesterol diet to animals, cholesterol deposits in all tissues, without any predisposing factors such as hypertension or tissue injury. By subsequent feeding of polyunsaturated fat, the cholesterol levels in almost all tissues could be reduced to normal after a fairly short period of time. The removal of cholesterol from the aorta however was very much slower. It appears possible, nevertheless, that the process of atherosclerosis is at least partially reversible and that the progression of such cholesterol induced atherosclerosis can be halted.

It is likely that when plasma cholesterol levels attain a critical level, cholesterol is deposited into all tissues, particularly the arteries. When blood cholesterol levels return to normal, the excessive cholesterol in most tissues returns to the plasma and is excreted by the liver. Since cholesterol in the aorta has a much lower rate of return to the plasma, a considerable excess still remains after reduction of the plasma cholesterol levels to normal. Subsequent influxes bring more cholesterol into the aorta before the vessel is able to clear out that already deposited. Thus progressively more lipid accumulates. Tissue reactions then ensue and typical atheromatous plaques follow. The early plaques, because of their milder tissue reactions and the smaller quantities

of cholesterol they contain, are probably reversible although the regression may take a long time. The fully developed plaques with more intensive tissue reactions and large quantities of lipid are probably reversible to a much lesser extent.

The following conclusions may be drawn. The formation of atheromatous plaques in the walls of coronary arteries initiates development of coronary heart disease. These lesions consist of lipid, primarily cholesterol which is believed to be deposited from the circulating blood. The cholesterol of atheromata is in equilibrium with serum cholesterol,[81] and infiltration of the latter is assumed to be at a rate directly related to serum cholesterol concentration.[80,82] This assumption is supported by a great many studies which have clearly shown that patients suffering with coronary heart disease have higher serum cholesterol levels than healthy subjects, and that the higher the serum cholesterol level the higher the frequency of coronary heart disease, or the greater the likelihood that it will develop. Other factors such as hypertension, greater haemodynamic stress, thrombus formation and arterial injury by chemical or immunological agents probably assist in the focal deposition of lipid, but the most common approach to preventing the development or progression of atherosclerosis is by reduction of elevated blood cholesterol levels. Serum cholesterol levels may be reduced by corresponding reduction of dietary cholesterol,[86] by increasing the proportion of dietary polyunsaturated fat or by the use of drugs which inhibit cholesterol synthesis[83,84] or stimulate excretion of bile acids.[43] Although it has been clearly demonstrated by use of animals that excessive serum cholesterol results in significant changes in arterial wall metabolism,[87] it is unclear whether such changes are due to cholesterol itself, since excessive dietary cholesterol results in changes in other lipids and proteins of plasma.

The information gained so far, relating changes of arterial wall metabolism with elevated serum cholesterol levels shows many altered metabolic activities. It remains to be seen whether the complex sequence of metabolic events in atherogenesis of experimental animals can be elucidated in the near future, and what pertinence the information has for atherogenesis in man.

### 1.1.5. Lipids of the artery wall

It has long been recognised that lipids are a major component of arterial lesions and much work has been done in attempts to determine the chemical composition of such lipids and the reasons for their accumulation. Early work in this field was hindered by the lack of suitable micro techniques enabling separation and identification of small quantities of lipids present in lesions of different types. These difficulties have been largely overcome by the use of column and thin-layer chromatography to separate the major classes of lipid, and by the advent of gas-liquid chromatography, which has enabled the further separation and identification of individual compounds within each lipid class.

#### Classification of arterial lesions

It is essential that prior to a systematic study of the lipids associated with atherosclerosis, the types of lesion should be classified in a manner that renders them consistently distinguishable. In such a classification it is usual to employ the method of Bottcher *et al.*[88,89] These workers divided their material into four groups depending upon the macroscopic appearance of the arterial wall, according to the classification of the WHO[1] as follows:

| Stage of disease | 0 | I | II | III |
|---|---|---|---|---|
| % Phospholipids | 61 | 58 | 42 | 38 |
| % Free fatty acids | 9 | 4 | 1 | 1 |
| % Cholesterol | 8 | 13 | 16 | 19 |
| % Cholesterol esters | 6 | 12 | 30 | 34 |
| % Glycerides | 17 | 13 | 10 | 9 |

TABLE 2. Average lipid content of aortal extracts at various stages of disease.

| | | |
|---|---|---|
| Stage 0 | - | no atherosclerotic lesions present at a magnification of X10. |
| Stage I | - | fatty streaks and/or spots. |
| Stage II | - | fibrous plaques and/or atheroma. |
| Stage III | - | lesions as in II, but with the added complications of ulceration, necrosis, haemorrhage or thrombosis. |

The first type of visible lesion, the fatty streak, is defined by the WHO[1] as superficial yellow or yellowish-grey intimal lesions which are selectively detected by fat stains. A fibrous plaque is an elevated intimal thickening which is firm and grey or pearly white, and an atheroma is an atherosclerotic plaque in which fatty softening is predominant. A complex lesion, Stage III, describes an atheroma with additional changes such as haemorrhage, ulceration or calcification.

## Lipid changes in arterial lesions

Using the method of classifying arterial lesions described above, Bottcher and co-workers[89] have determined the gross lipid content of undiseased and atherosclerotic aorta. The major lipid classes and the proportions in which they occur throughout the spectrum of aortal atherosclerosis are given in Table 2.

As the disease advances from fatty streak to plaques and complex lesions, the proportion of arterial total lipid made up of free cholesterol and cholesterol esters markedly increases; corresponding decreases being observed in the triglyceride, phospholipid and free fatty acid fractions.[88,89] The rate of increase in free cholesterol is considerably less than that for cholesterol esters, until well developed extensive plaques and complex lesions are present.[90] The initial high proportion of free to esterified cholesterol may be due to selective admission or retention of the free form,[91] or alternatively may reflect

| | % FATTY ACID IN TOTAL C.E.F.A. | | | | |
|---|---|---|---|---|---|
| FATTY ACID | STAGE 0 * | STAGE 1 * | STAGE 2 † | STAGE 3 † | PLASMA ≠ |
| 16:0 | 16 | 12 | 14 | 13 | 13 |
| 16:1 | 5 | 6 | 6 | 5 | 4 |
| 18:0 | 2 | 1 | 2 | 2 | 1 |
| 18:1 | 30 | 43 | 28 | 30 | 25 |
| 18:2 | 38 | 17 | 37 | 36 | 46 |
| 20:3 | trace | 6 | 2 | 3 | - |
| 20:4 | 4 | 4 | 5 | 5 | 4 |

* Geer et al. † Bottcher et al. ≠ BBang et al.

TABLE 3. Comparison of cholesterol ester fatty acids from normal aorta, diseased aorta and normal plasma.

hydrolysis of lipoprotein-derived cholesterol ester in the artery wall. As atherosclerosis progresses the increase in the proportion of cholesteryl ester possibly reflects esterification in the tissue.[92] It has been demonstrated that polyunsaturated cholesterol esters are more easily mobilised from tissue implants and exhibit relatively little irritant activity on connective tissue than either free cholesterol or its monounsaturated esters.[93] It is possible, therefore, that the arterial wall actively esterifies the sclerogenic free cholesterol. Some substantiation of this proposal is provided by the discovery that a cholesterol-lecithin fatty acid transferase is present in homogenates of arterial tissue.[94] The increase in the ratio of free to esterified cholesterol in advanced lesions may result from a decrease in the capacity of such tissue to esterify cholesterol,[94,95] combined with an increased influx of cholesterol from serum.

Comparisons of the compositions of cholesteryl ester fatty acids (CEFA) in extracts of normal tissue with those from fatty streaks show considerable differences (Table 3). The CEFA composition of fatty streaks shows considerably higher proportions of oleic and eicosatrienoic acids, and lesser quantities of linoleic acid than either normal tissue or plaques.[88,96,97] Furthermore, CEFA from plaques and undiseased tissue are similar to those of serum cholesteryl esters,[98-100] implying strongly that the former are derived from blood. The difference in the CEFA composition of fatty streaks and normal tissue can be correlated with the observation that lipid in fatty streaks is primarily intracellular, whereas that in normal intima is located predominantly in the intercellular spaces.[90,97,101,102] The implication of this correlation is that in fatty streaks intra-

cellular cholesterol esterification together with fatty acid synthesis and elongation takes place (the eicosatrienoic acid with which cholesterol is found to be esterified is predominantly the 8,11,14 isomer derived from linoleic acid).[103]

The observed differences in CEFA composition of fatty streaks and more advanced lesions can be accounted for in terms of intra- and extracellular lipid. Smith and co-workers[101,102] have been able to determine the CEFA of both types of lipid present in arterial intima. Their results demonstrate a striking difference in the proportions of oleic, linoleic and eicosatrienoic acids. The CEFA of extracellular lipid contain about 28% oleate and 40% linoleate which is comparable with the proportions in blood lipid (Table 3). The intracellular cholesteryl esters contain about 48% oleate and 14% linoleate. Plaques typically contain a large core of extracellular lipid and it is possible that the observed changes in CEFA as the lesion progresses from fatty streak to plaque are due to a large influx of serum cholesterol esters. This would result in great excesses of extracellular lipid over that produced by local synthesis which perhaps predominates in the fatty streak.

The proportion of phospholipids in arteries decreases as atherosclerosis advances (Table 2). However, the increase in the quantity of total lipid results in a net increase in the quantities of this lipid class.[89,104,105] The fatty acid content of plaque phospholipid differs from that of serum phospholipid,[100] and it has been demonstrated that the arterial wall has the capacity to synthesise phospholipid,[106] which may explain these differences. A combination of infiltration and local synthesis is the most probable explanation of arterial phospholipid occurrence.

| ISOLATED COMPOUND | REFERENCES |
|---|---|
| 5α-cholestan-3β-ol | 107-111 |
| 5α-cholestane-3β,5,6β-triol | 112-116 |
| Cholesta-5,24-dien-3β-ol | 116-120 |
| Cholesta-3,5-dien-7-one | 112,121-123 |
| Cholesta-4,6-dien-3-one | 112,123 |
| 3β-hydroxy-cholest-5-en-7-one | 107,115,124 |
| Cholest-5-ene-3β,7α-diol | 112-114,116,125 |
| Cholest-5-ene-3β,7β-diol | 114,116,125 |
| Cholest-5-ene-3β,24-diol | 114,125 |
| Cholest-5-ene-3β,25-diol | 114,115 |
| Cholest-5-ene-3β,26-diol | 124-128,111,115 |
| Cholest-5-ene | 129 |
| Cholesta-3,5-diene | 129 |
| Squalene | 124,126,129,131 |
| 1,3,5-trioxanes | 130 |
| Glyceryl ether diesters | 130 |
| Cholesterol hydroxy-octadecadienoates | 125,127 |

## Minor Lipids of Arterial Lesions

The presence of small quantities of other lipids in diseased arteries has long been recognised. The human aorta with its obvious relationship to atherosclerosis has been the subject of considerable study, much of which has been directed towards the identification of minor quantities of sterols occurring with cholesterol. Earlier workers using classical methods of separation and identification required large quantities of extracted lipid. Since accumulation of such large quantities frequently necessitated storage of tissue or extracted lipid, the possibility that several of the minor sterols identified in arterial lipid were artefacts must be considered. More recent work based on mild chromatographic techniques, has enabled both confirmation of some earlier findings and also the identification of several new constituents. The minor lipids isolated from human aorta are listed opposite. Other sterol and sterol-like components present in the aorta remain unidentified.[115] The origin of several of the trace sterols is questionable, since many of them are also products of the air autoxidation of cholesterol, and the possibility that they may be artefacts remains unsettled. Of the sterols found, desmosterol, cholestanol, 26-hydroxycholesterol and 24-hydroxycholesterol are the most likely to be genuine aortal constituents. Desmosterol has been found in substantial quantities in the arteries of patients treated with Triparanol,[117-120] a drug formerly used to control serum cholesterol levels by blocking the 24-reductase in the metabolism of desmosterol to cholesterol.[326] 24-Hydroxy-cholesterol has been identified as a minor sterol in brain tissue,[132,330] and 26-hydroxycholesterol seems to occur in human arterial tissue whether normal or diseased,[111,124-128] its quantities increasing as

atherosclerosis progresses. Cholestanol has been identified as a companion of cholesterol in many animal tissues including human arteries. The quantities in which this sterol occurs in arteries have been disputed, estimates ranging from 13% of the total sterols by early workers,[108] to 0.5% in more recent investigations.[109] The occurrence in advanced aortal lesions of hydroxylated sterol esters has been reported.[125,127] These esters may be distinguished as a) a group of hydroxycholesterols mono-esterified with normal saturated or unsatured fatty acids, and b) a group consisting of cholesterol esterified with several different hydroxy fatty acids. The origin and possible role of these esters in the pathogenesis of atherosclerosis will be discussed later.

## 1.2. TECHNIQUES OF LIPID ANALYSIS

### 1.2.1. Thin-Layer Chromatography

Since its discovery in 1938[133] and the much more recent realisation of its potential,[134-137] thin-layer chromatography (TLC) has become an invaluable technique throughout the realm of organic chemistry, and most particularly in the field of analysis of biological extracts. The use of TLC is so widespread and has so many applications that only the principles of the technique and the aspects particularly relevant to the present work can be mentioned.

TLC is used in lipid analysis mainly as an adsorption technique in which the adsorbent is coated as a thin layer (generally 100 -1000$\mu$) on an inert support. The material to be separated is applied to the adsorbent and eluted with solvent. The moving substances are separated by their differing adsorption coefficients in the equilibrium established at the adsorbent/solution interface. Pure adsorption chromatography is an ideal case. Almost invariably some degree of partition chromatography occurs, though it is dependent upon the natures of both the adsorbent and the material being chromatographed.

The types of adsorbents used in TLC vary considerably. Silica gel is the most commonly used, since it is suitable for almost all types of lipid class separations, and also because it was the first material to become commercially available. It is normally supplied containing about 10% calcium sulphate as a binder which confers on the layer considerable mechanical stability.[136] Neutral alumina has much the same separating characteristics as silica. Calcium sulphate, calcium phosphate and charcoal are amongst other materials which have also been used.[138]

In 1959 Mangold[139] first employed TLC for lipid analysis.

Using layers of silica gel he found that TLC was capable of separating the major lipid classes in a manner similar to that of silica gel column chromatography. Since then TLC has been used extensively for lipid separation and characterisation. These applications have been reviewed by Mangold,[140] Morris[141] and Kirchner.[142]

TLC has several advantages over adsorption column chromatography. The latter is usually used as an elution technique where fractions are removed stepwise from the column, and subsequently analysed. In TLC, however, the development is limited and the processes of separation are more easily observed. TLC lends itself particularly well to analyses of very small quantities of material. In the present work preparative TLC on microscope slides enabled successful separation and recovery of between 5 and 20 $\mu$g of lipid samples. The speed with which analytical chromatograms are produced can be used to monitor successive fractions eluted during column chromatography of biological extracts. A major disadvantage of TLC is that the quantity of material which may be applied to a chromatogram is limited by the thickness of the adsorbent layer. For an adsorbent of silica gel, one 20 x 20 cm plate with a layer thickness of 500 $\mu$ will accommodate 30 mg of material without impairment of separating efficiency. However, the separations on thicker layers (1000-2000 $\mu$) are distinctly inferior to those obtained with thicknesses of 100-500 $\mu$. If minor constituents are to be isolated from a complex mixture, it is advisable to pre-fractionate the mixture by column chromatography and then to purify the required fractions by TLC.[140] A form of column chromatography has been developed which uses a limited development technique on silica gel identical to that used for TLC.[143] This method permits the use of much larger loads than can be accommodated by conventional TLC, while retaining most of

the advantageous aspects of the latter. An important aspect of TLC is the use of adsorbent layers modified by the addition of complexing agents such as silver nitrate and boric acid. Unsaturated lipids form complexes with silver ions due to the interaction of the filled $\pi$ orbitals of the olefin with the empty s orbital of the silver ion, and electron transfer from the filled d orbitals of the central ion to the vacant $\pi^*$ antibonding orbitals of the olefin.[141] The presence of silver ions in the layers causes unsaturated species to be less mobile than the saturated analogues, and compounds can be separated on the basis of the number, type or position of the double bonds.[327] Hence fatty acids, cholesterol esters, triglycerides and sterols may be separated according to their degree of unsaturation. The uses of silver ion chromatography have been reviewed by Morris.[141] Boric acid impregnated silica gel has been used for the successful separation of threo and erythro dihydroxy fatty acid methyl esters,[144,145] while layers containing urea[146] enable the separation of straight-chain and branched-chain fatty acid esters.

TLC is capable, if used with care, of supplying physical data of use in the tentative identification of samples. This involves direct comparison of the mobilities of a sample with those of reference compounds, and observation of the reactions of the spots on the developed chromatograms with various detecting agents. The mobility of a spot on TLC is most commonly recorded as its $R_f$ value which is defined as the ratio of the distance moved by the material from the origin, to the distance moved by the mobile phase. Improved consistency of retention data can frequently be obtained by measuring the mobility of the sample spot relative to that of an internal reference standard.[147]

## Spot detecting agents for TLC

In a brief discussion of the techniques and reagents used to visualise spots or bands on developed chromatograms, it is advantageous to consider separately the preparative and analytical aspects of the technique.

TLC used analytically does not necessitate the recovery of the sample after development of the chromatogram. It is common practice, therefore, to spray the chromatogram with reagents which react with material on the plates to produce coloured spots. These reagents can be classified into those which react non-specifically with most compounds, and those capable of specific reaction with certain compounds or functional groups. A large number of the spray reagents used for sterol and steroid localisation on TLC have been reviewed by Lisboa.[148] The applications of the anisaldehyde-sulphuric acid reagent of Ekkert[149] have been investigated for more than 250 steroids of the estrane, pregnane and androstane series. The colours developed in the reactions of this reagent with various steroids depend on the intensity of spraying and the temperature and duration of heating, and are capable of yielding valuable information enabling the differentiation of sterol classes and stereoisomers. The reagent used most frequently in the present work was a solution of 5% ceric sulphate in sulphuric acid which was found to permit differentiation of most of the sterols and sterol esters found in arterial tissue (Appendix I). Spray reagents which react specifically with compounds containing particular functional groups are frequently of value. Reagents of this type include 2,4-dinitrophenylhydrazine in acidic ethanol for the detection of free aldehydes and ketones, aqueous ferric chloride (for phenols and carboxylic acids), potassium iodide-starch solution (for peroxides), and ninhydrin in butanol (for phosphatides

and lipids containing a free amino group). Sterols containing the 5-ene-3β-ol system may be fairly selectively differentiated from other types by exposure of developed chromatograms to hydrochloric acid vapours.[152,331] A comprehensive list of spray reagents for TLC is given by Stahl.[138]

It is normally desirable when using preparative TLC that the chromatographed material should be recovered unchanged from the developed chromatogram. Destructive methods of locating bands on preparative chromatograms are therefore unsuitable. In consequence alternative methods of detection have been developed. A solution of 2,7-dichlorofluorescein is frequently employed. After spraying, bands may be detected by their fluorescence when viewed under ultraviolet light. A modification of this method involves the use of silica gel containing a fluorescent dye. When the developed chromatogram is viewed under ultraviolet light the adsorbed material is visible due to quenching of the fluorescence in the layer.[332] Another commonly used method of band location entails exposure of the chromatogram to iodine vapour. A brown colour results with many organic compounds. This method is non-destructive with most compounds, though prolonged exposure of polyunsaturated lipids to iodine should be avoided.[140] It has recently been reported that a solution of primuline in aqueous acetone is an effective reagent for the non-destructive location of some lipids and steroids.[153]

## Use of derivatives in TLC

Until fairly recently the quantitative elution of compounds containing more than one hydroxyl function has proved difficult.[150] The use of derivatives such as acetates, propionates and trimethylsilyl (TMS) ethers can allow very high recoveries from preparative chromatograms, as a result of the much reduced polarity of such derivatives. TMS ethers deserve special mention. The usefulness

of these compounds as derivatives for TLC has not been widely appreciated, probably because of an earlier misconception that TMS ethers were very readily hydrolysed. Although the derivatives of phenols are easily hydrolysed, those of alcohols are comparatively stable. TLC of TMS ethers of poly-hydroxy sterols has enabled recoveries of better than 90% from preparative chromatograms.[151] Effective separations of individual sterols, in particular of epimeric pairs can also be obtained by TLC of their trimethylsilyl derivatives.[116]

1.2.2. Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) has become an invaluable tool in biological and medical research. This highly sensitive technique permits rapid fractionation of complex mixtures, and provides both qualitative and accurate quantitative data.

GLC involves partition of a volatilised sample between a mobile gas phase and a stationary liquid phase coated on an inert support. The components of a mixture are separated in the gas phase and individually detected. The extensive use which has been made of GLC in the biological sciences is due mainly to the development of highly sensitive detectors, and a large variety of thermally stable liquid stationary phases. The first high sensitivity detector was the argon ionisation type developed by Lovelock,[154] which is capable of detecting solute in carrier gas in concentrations as low as $10^{-13}$ g/ml. It is, however, very susceptible to impurities such as oxygen and water vapour, which reduce its efficiency in detecting organic compounds. The most common detector in use today is the hydrogen flame ionisation type which is just as sensitive as the argon type, and does not suffer from the disadvantages mentioned above. Other detectors such as the electron capture, the flame photometric and the thermionic types have been developed and each has its particular detection characteristics. In the technique of combined GLC-mass spectrometry (GC-MS), the mass spectrometer is used as a detector, and can be applied to the specific detection of compounds producing characteristic mass spectral ions.[160]

There is an extensive variety of stationary phases which may be used in GLC. Probably the most popular are those of the siloxane polymer type. These are particularly useful due to their considerable thermal stability, and because variation of the substituent in the polymer affords useful changes in the separating

characteristics of the liquid phase.

The methyl siloxane polymers such as SE-30, JXR and OV-1, are fairly "non-selective" and effect separations based more or less on differences in molecular weight. Complete or partial replacement of methyl with phenyl substituents gives stationary phases in which dipole interactions with olefinic bonds in the samples frequently permit the separation of closely related compounds. Fluoroalkyl siloxane polymers can selectively separate ketonic compounds from alcohols. The phases most commonly used in fatty acid ester analyses are the polyesters, which separate acid derivatives with the same number of carbon atoms on the basis of their multiple bond content. Though of considerable value, such phases have rather low thermal stability and separations generally necessitate the use of packings containing a rather high proportion of polyester. Several interesting thermostable stationary phases have recently become available. Poly-m-phenoxylene,[155] polyamides,[156] polyimides,[157] and carborane polymers[158] are typical examples. Among them the poly-m-carborane siloxanes exhibit thermal stability which cannot be challenged by any of the other materials which are presently available.[159]

The amount of information obtained by GLC may be greatly increased by the formation of suitable derivatives. Many substances such as sugars, amino acids and carboxylic acids are, because of their highly polar nature, unsuitable for direct analysis. In such cases derivative formation is essential. In other cases improved chromatographic resolution and greater thermal stability may be obtained by derivative formation. Some of the more common derivatives are listed below:

| Functional Group | Derivative |
|---|---|
| ALCOHOL | Acetate<br>Propionate<br>Methyl ether<br>Trimethylsilyl ether<br>Trifluoroacetate<br>Heptafluorobutyrate |
| CARBONYL | O-Methyl oxime<br>Trimethylsilyl oxime |
| CARBOXYLIC ACID | Methyl ester |
| AMINE | Acetyl derivative<br>Heptafluorobutyryl derivatives<br>Trimethylsilyl ether |

Trimethylsilyl (TMS) ethers are particularly good derivatives for GLC[161-164] and have been found especially useful in studies of sterols. They have excellent chromatographic properties, exhibiting high thermal stability and volatility, and in most cases are very easy to prepare. Moreover, TMS ethers are found generally to yield informative mass spectra,[165] in certain cases affording highly characteristic modes of fragmentation from which structural details may be inferred.[165,166-169] Some aspects of their mass spectral behaviour will be described in Section 1.2.3.

The parameters usually measured in GLC are the retention time, the temperature of separation, the carrier gas flow rate and the quantity and type of stationary phase used. Since several of these parameters are variable, it is normal to relate the retention time of a sample to that of a suitable reference standard. For steroids of the cholestane series, the reference material usually employed is the hydrocarbon 5α-cholestane. Relative retention times (at constant temperature) are expressed as the retention

time of the sample divided by that of the standard. In isothermal GLC a linear relationship exists between the number of carbon atoms in the members of a homologous series and the logarithm of their retention times. In contrast, temperature programming gives an almost linear relationship between the carbon content of homologues and their retention times. Such correlations may be applied to measurement of retention data. In fatty acid analyses retention values are generally related to those of a homologous series of fatty acid methyl esters.[170,171] Components in a mixture of sample acid esters are assigned equivalent chain length (ecl) values which are highly reproducible. Kováts[172] has devised an index scale based on the retention times of the n-alkanes. The retention times of these compounds are converted into index values according to their carbon number (i.e. methane = 100, ethane = 200, pentadecane = 1500, etc.), and samples are assigned retention index (I) values according to their retention times relative to the adjacent n-alkanes. This technique takes advantage of the high degree of repeatability of separation on a given column, and permits comparison of separations on columns prepared and used in different laboratories. The Kováts system is used exclusively in the present work.

## Applications of GLC

Biological research is a field in which the analysis of minute amounts of highly complex mixtures may be essential for the solution of many problems, and GLC has been used extensively to analyse natural lipids such as steroids and fatty acids. In connection with atherosclerosis, the relationships between chemical composition of the artery wall and pathological change have been largely established by comparison of the fatty acid composition of diseased and undiseased tissue.[88,96,100] GLC is particularly

applicable in such cases, since it may be used as a semi-quantitative technique to estimate the relative proportions of the individual acids in mixtures derived from glycerides, sterol esters and phospholipids. The identity of fatty acids of unknown structure can frequently be inferred by their comparison with reference compounds on several different stationary phases.

During work on small quantities of lipid, it is important to bear in mind that plasticisers and other impurities which occur in solvents,[173] plastic tubing,[174] filter paper, etc., may cause serious interference in chromatograms of fatty acid methyl esters. This problem has been encountered in the present study and is further discussed in Section 2.

The discovery that steroids could be separated by GLC was made in 1960.[175] Due to the development of the large number of thermally stable stationary phases and the application of derivatives (especially TMS ethers and O-Methyloximes), GLC has become established as virtually the only practical method of simultaneous separation, identification and quantification of the complex mixtures of steroids obtained from sources such as blood or urine. Urine contains many steroid metabolites which are the end products of a complex set of metabolic relationships. It was soon realised that "urinary steroid profiles" could be correlated with pathological change and drug administration, and many studies have been made of urinary steroids in normal and pathological cases.[176,177] Quantitative determinations of testosterone, estrogens, adrenal corticosteroids and progesterone metabolites in urine samples have been developed into routine procedures by the use of GLC. A major problem in work of this kind is the interfering compounds in the sample. The trends in recent research have generally been in the direction of developing more efficient or elaborate methods of purification, to increase the reliability

of the final analysis. The use of the relatively new single[160] ion and multiple[178,179,228] ion monitoring techniques affords a very promising method of sensitive and highly specific detection. GLC also offers a practical method for analysis of bile acids in serum and other body fluids.[180,181] The use of electron capture detectors has enabled the detection of as little as 0.1 ng of these compounds as their methyl ester trifluoroacetates.[182]

Unknown steroids may be tentatively identified by GLC. It is observed that the influence upon retention time of substituents, or other modifications of the steroid structure can frequently be described in a simple mathematical way. This makes it possible to determine with considerable precision the retention time of a sterol not yet known, provided that data are available on the effect of each substituent of the molecule as well as the basic sterol structure.[183-187] However, the use of several different stationary phases is advisable if useful identifications are to be made in this way.

Whereas earlier gas chromatographic analysis of lipids such as glycerides and sterol esters normally entailed hydrolysis of the sample and separate examination of the acyl and neutral constituents, the development of stationary phases of very high thermal stability has recently permitted direct examination of the intact lipids. Kuksis[188-190] has successfully separated triglycerides and sterol esters originating from a variety of sources on several siloxane polymers. Naturally occurring diglycerides and those derived from phospholipids may be separated as the free compounds or as TMS ethers. Analyses of high molecular weight lipids have been reported using open tubular columns coated with carborane polymers.[191]

The potency of GLC may be increased considerably if used in association with other chromatographic or analytical techniques.

Occasionally separation of closely related compounds cannot be effected by GLC, and recourse must be made to alternative chromatographic methods. The isomeric $\Delta^{8(9)}$ and $\Delta^{8(14)}$ steroids present this difficulty, but in some cases TLC on silver nitrate impregnated layers may resolve these isomers, which can be subsequently characterised by GLC. During work on mixtures of octadecadiene-1,9- and -1,13-diols, we found GLC capable of separating cis,trans from trans,trans derivatives, but unsuitable for the resolution of the positional isomers. However, the latter are readily separated by TLC, and complementary use of both techniques permitted the mass spectral analysis of the individual components. The use of GLC in conjunction with mass spectrometry has become increasingly important in lipid research, and will be discussed in the following Section.

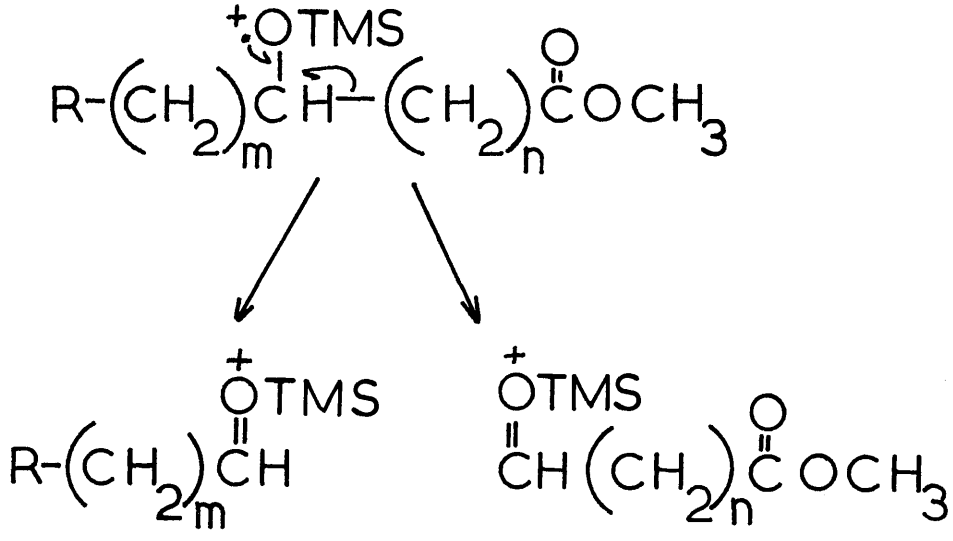
### 1.2.3. Application of Combined Gas Chromatography-Mass Spectrometry to Lipid Analysis.

Although chromatographic methods can yield important evidence for the identification of an unknown compound, complete characterisation solely by chromatographic methods is seldom feasible. Where the available sample is small, few techniques are as useful as mass spectrometry in providing detailed structural information. The use of mass spectrometry in conjunction with GLC permits direct correlation of chromatographic and mass spectral properties for individual components in very small samples of complex mixtures. Combined gas chromatography-mass spectrometry (GC-MS) is therefore particularly useful for studies of naturally occurring lipids. The potency of GC-MS may be exemplified by consideration of the complex nature of naturally occurring lipids. In most cases use of GLC alone requires very extensive and tedious calibration. Application of mass spectrometry to such mixtures can afford only generalised summaries of grouped structure. Together GC-MS techniques provide rapid and detailed composition analysis.

GC-MS has found considerable application in the analysis of mixtures of naturally occurring fatty acids[192-196] and alcohols, and is especially useful for the location of functional groups in such compounds. Studies by Odham[197-199] of the branched chain fatty acids[200,201] isolated from feather waxes represent a good example of the usefulness of mass spectrometry in the structure determination of such compounds.

The success of mass spectrometry for the analysis of lipids and in particular of steroids has been largely due to the discovery of derivatives affording protection for labile functional groups, and promoting regular patterns of fragmentation. In GC-MS derivatives are chosen which also give good gas chromatographic characteristics. For most hydroxylated lipids, and especially

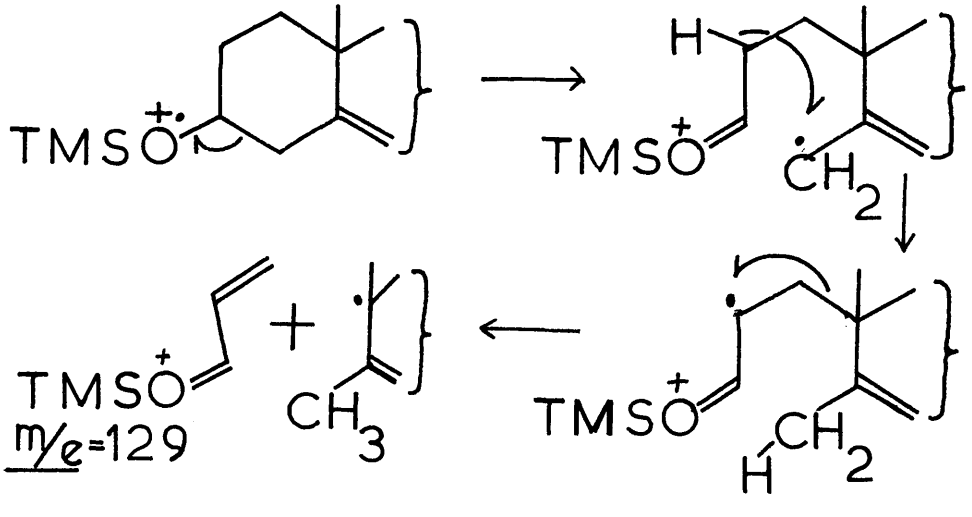
for sterols such a derivative is the trimethylsilyl (TMS) ether. These derivatives were introduced by Sharkey in 1957[165] with the object of obtaining more abundant molecular ions than were normally obtained with sterols or their esters, and were subsequently found to have very good gas chromatographic properties.[202] Probably the most valuable feature of TMS ethers is their tendency to promote well defined fragmentation modes, with the formation of rather stable trimethylsilyl oxonium ions. For example, when a TMS ether group occurs in a straight chain compound such as a hydroxy fatty acid, or in the side chain of a sterol, the following type of fragmentation is typical.[203-207]



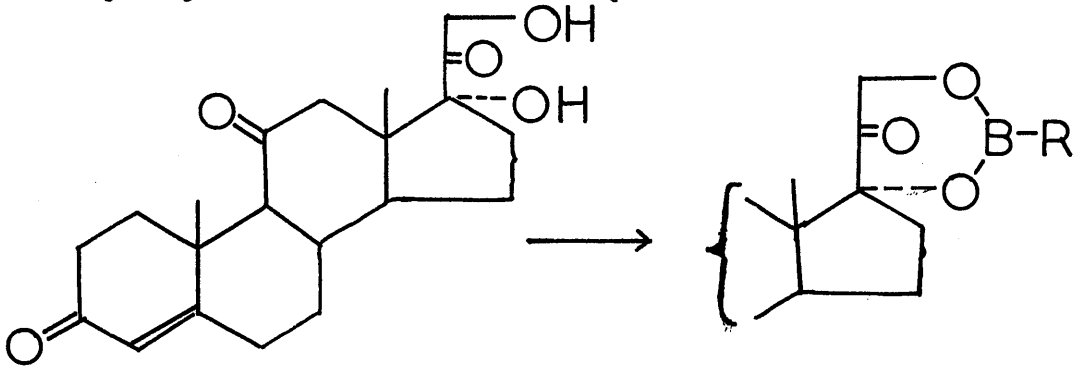
Such fragmentations are extremely useful for characterisation of long chain compounds such as fatty acids.[192] Hydroxyl functions may be located in the chain by formation of TMS ethers and subsequent mass spectral analysis.[203-207] The usefulness of TMS ethers may be extended to determine the positions of multiple bonds in fatty acids, which cannot normally be inferred from GLC or mass spectrometry of the acids themselves. Oxidation of olefinic bonds to the vicinal diols, and mass spectral analysis of the TMS ethers has been used to determine the positions of olefinic bonds in mono-[203,204,208,209] and di-unsaturated fatty[210-212] acids.

The major disadvantage of these derivatives for olefinic bond location in polyunsaturated acids is that they greatly increase the molecular weight of the compounds. Consequently the use of polymethoxy derivatives, though affording somewhat less characteristic mass spectra, is often preferred.[213] Abley et al. have described an alternative method from double bond location in mono and dienoic acid esters by reduction of methoxymercuration products with sodium borohydride and analysis of the products by GC-MS.[214] The structures of the mono-hydroperoxides of methyl oleate have been determined by mass spectral analysis of the trimethylsilylated hydroxy-esters obtained by reduction of the autoxidation products.[206] These derivatives were found to yield highly informative mass spectra which readily enabled the location of both the hydroxyl function and the double bond. In the present work mass spectrometry of the octadecadienediol bis TMS ethers[215] derived from oxygenated cholesteryl linoleate proved a satisfactory approach to their structure elucidation on a microgram scale.

The mass spectra of sterol TMS ethers are in many cases highly characteristic. Those of the $\Delta^5$-3-trimethylsilyloxy sterols contain a very abundant ion at m/e = 129. This fragment has been shown to comprise the TMS ether group and carbon atoms $C_{(1)}$, $C_{(2)}$ and $C_{(3)}$ of the steroid nucleus,[169,217] and the following mechanism has been proposed to explain its occurrence:

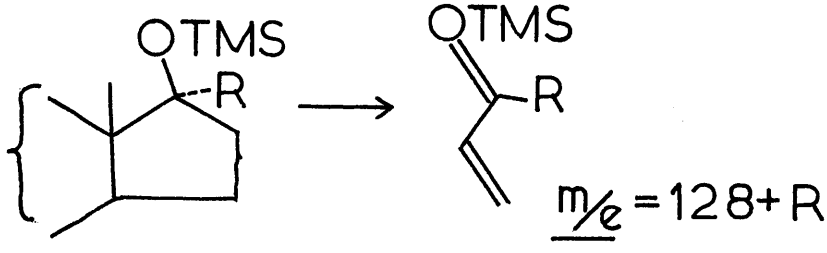


17-Trimethylsilyloxy steroids of the androstane and estrane series also give this ion, but may be distinguished from the $\Delta^5$-3-trimethylsilyloxy steroids which also give an intense ion at M-129.[169,217,218] The trimethylsilyl ether of $\Delta^4$-cholesten-3$\beta$-ol has a mass spectrum strikingly different from that of cholesterol. In this case ions are observed at m/e = 143 and 142, and may be respectively ascribed to fragments comprising the -OTMS attached to a four carbon unit from ring A, and the product of a retro-Diels-Alder fragmentation.[218] Mass spectrometry thus affords a valuable distinction, since it is difficult to separate $\Delta^4$- and $\Delta^5$-sterols by GLC. The $\Delta^{5,7}$ sterols (e.g. 7-dehydrocholesterol and ergosterol), may also be readily identified by mass spectral analysis of their TMS ethers which show characteristic ions at m/e = 131 and M-131.[218] New derivatives of steroids, affording thermal stability and characteristic modes of fragmentation useful for identification of natural steroids and drug metabolites, are being developed and evaluated. Baillie and co-workers[219] have evaluated several derivatives suitable for GC-MS analysis of corticosteroids which, when chromatographed as the free sterols, generally undergo considerable decomposition. Of particular interest are the cyclic boronic esters.[220-222] The formation of a cyclic boronic ester transforms the labile dihydroxyacetone side chain as exemplified for cortisone:



Cyclic esters with R representing methyl, n-butyl, t-butyl, cyclohexyl and phenyl have been shown to be satisfactory derivatives for GC-MS of a variety of corticosteroids.[223] Their most valuable features are the general prominence of molecular ions, and of ions characteristic of the corticosteroid side chain involved. However care must be exercised during the preparation of these derivatives, since excesses of reagent may have detrimental effects, and probably the best "all round" derivatives for corticosteroid analyses are the methyl oximes and TMS ethers.

A recent and important modification of GC-MS is the "single ion monitoring" technique whereby particular compounds may be detected by means of a small set of characteristic ions in their mass spectra.[224-228] In suitable instances the detection of one ion of particular m/e value may afford a means of detecting and estimating steroids at very low concentrations.[160,222,229,216] Application of this technique is of particular importance for studies of drug metabolites having similar structural features which are reflected by characteristic ions in their mass spectra. TMS ethers of steroid drug metabolites containing 17α-alkyl-, 17α-alkenyl-, and 17α-alkynyl-17β- ols may be detected selectively by the abundant ions at m/e = 128+R which in general do not occur in the mass spectra of natural steroids.[222]




Using these techniques a preliminary study has been made of the urinary neutral metabolites of the anabolic steroid, 17α-ethyl-17β-hydroxyestr-4-en-3-one.[222]

GC-MS has been used extensively to identify steroids of biological origin. One especially important application of the technique is the determination of urinary steroid profiles and their relationship with physiological and clinical symptoms.[176,230] Particular care must be given in such studies to accurate correlation of retention data with mass spectra, since diastereoisomers with identical mass spectra are frequently encountered. By application of microchemical techniques in conjunction with GC-MS, J. and K. Sjovall[231] identified 2$\mu$g of 5$\alpha$-pregnane-3$\alpha$,20$\alpha$,21-triol from human pregnancy plasma. No authentic reference sample was available and the configurations at $C_{(3)}$, $C_{(5)}$ and $C_{(20)}$ were elegantly deduced from gas chromatographic data.

The complexity of natural lipid samples requires that powerful tools must be available for their analysis. GC-MS is a highly efficient technique for analysing such mixtures. The application of GC-MS to analysis of complex mixtures presents new problems, in particular handling and interpretation of the large amounts of data produced. The use of computers for reading and calculation of mass spectra is being increasingly applied.[228,232]

## Section 2 EXPERIMENTAL METHODS

## 2.1. SOLVENTS

Most of the present work was carried out using "Nanograde" solvents (Mallinckrodt Chemical Works, St. Louis, Missouri.). When these were not available, solvents of analytical grade were used and these were distilled prior to use. Diethyl ether was redistilled over ferrous sulphate to remove alkyl peroxides.

## 2.2. INTERFERENCE OF PLASTICISERS DURING LIPID ANALYSIS

### 2.2.1. Introduction

The hazards attached to the use, in micro scale analysis, of solvent[174,233] and reagents[174,234] of unestablished purity are fairly well realised. The occurrence in biological extracts of plasticisers, in particular di-n-butyl phthalate has been described by several investigators,[174,235-237] and it may be concluded that the only way of ensuring that extracts remain free of such contaminants is by careful and routine examination of solvents and all other materials with which the sample may come in contact. During examination of the fatty acids occurring in diesters of 26-hydroxycholesterol, gas chromatographic analysis of fatty acid methyl esters frequently indicated the presence of a constituent whose chromatographic behaviour was inconsistent with that of any ester expected to be present in such fractions. Comparisons using GLC and mass spectrometry of this material with authentic di-n-butyl phthalate[238] indicated identical behaviour. In order to determine the mode of entry of this contaminant into our extracts, all solvents, silica gel, drying agents and boiling stones used during our chromatographic sequences were analysed to determine the quantities of plasticisers present in such materials.

2.2.2. Determination of the quantity of di-n-butyl phthalate in solvents and other materials.

100 ml of the solvent to be analysed was reduced to dryness under vacuum. The residue was dissolved in ethyl acetate, transferred to a small tapered vial, and solvent was removed in a stream of nitrogen. The residue was dissolved in ethyl acetate (10.0 $\mu$l) delivered via a Hamilton syringe. Reference solutions of di-n-butyl phthalate varying in concentration from 0.2 mg/ml to 3.0 mg/ml were prepared, and 1 $\mu$l aliquots of these were successively examined by GLC (5' column, 1% OV-17, 200°C.). Two injections were made at each concentration, the mean peak heights were measured and a graph of peak height versus quantity injected was obtained. The graph was found to be linear over the range examined (0.2-3 $\mu$g). 1 $\mu$l aliquots of the solutions of solvent residue were injected, and from the peak height (mean of two determinations) the quantity of di-butyl phthalate represented in the chromatograms, and thus the quantity/l of solvent was calculated. Samples of silica gel (both that used for TLC and column chromatography) sodium sulphate, used as a drying agent for solutions in organic solvents, and boiling stones were weighed, and refluxed for 30 min. in ethyl acetate (redistilled, 100 ml). The solutions were filtered, the filtered material was washed with hot redistilled ethyl acetate, and the filtrate and washings were combined. The ethyl acetate solutions were concentrated as described above: aliquots were examined by GLC, and the degree of plasticiser contamination was determined.

Full results are presented in Appendix II. Although the quantities of di-butyl phthalate in unredistilled analytical grade solvents are not in general excessively high (30-120 $\mu$g/l),

the use of perhaps 400 ml of total solvent during an analytical process, from the stage of tissue extraction to that of examination of fatty acid esters by GLC, is sufficient to cause serious contamination of very small quantities of sample. The use of redistilled or "Nanograde" solvents greatly reduces the risk of extract contamination. Chromatography on silica gel of high purity, and the pretreatment of drying agent, are also advantageous.

2.2.3. General precautions for the prevention of sample contamination.

(a) All containers and apparatus for storage and manipulation of sample were made of glass. Materials such as rubber or plastic were avoided.

(b) Drying of solutions in organic solvents.

Solutions were dried with analytical grade anhydrous sodium sulphate, previously washed with hot "Nanograde" ethyl acetate, and dried at 150°C in a vacuum oven.

(c) Glassware.

After washing and drying, glassware was stored with arms and necks covered by clean aluminium foil to prevent entry of dust. Prior to use glassware was rinsed twice with small quantities of "Nanograde" or redistilled solvent.

(d) Sample storage.

Samples were stored in $\frac{1}{2}$-dram screw cap vials with aluminium foil faced seals which had been rinsed with attested solvent. All lipids, both reference compounds and extracts were stored in darkness at -20°C. Solutions of lipid were in hexane or ethyl acetate.

(e) Filtration

Small volumes of solvent (0.5-5ml) containing insoluble

material were filtered through either thin glass tubes fitted with glass sinters, or through pasteur pipettes fitted with plugs of clean, solvent washed cotton wool. Larger volumes of solvent were filtered by gravity through solvent washed filter paper.

(f) Removal of solvent.

Small quantities of solvent were removed by exposure to a stream of oxygen-free nitrogen. Nitrogen was delivered to the vial or flask via a pasteur pipette plugged with a small pad of solvent washed cotton wool. Volumes of solvent greater than 10 ml were generally removed under vacuum using a rotary evaporator. No evidence was obtained to suggest that the careful use of this apparatus could contribute to sample contamination.

## 2.3. TISSUE PREPARATION AND EXTRACTION

All arterial tissue used in this work was obtained through Dr. W.A. Harland, Department of Pathology, Western Infirmary, Glasgow, G12 8QQ. Aortas were obtained at post mortem examination and were processed within 24 hours after death. The vessels were opened longitudinally and washed with water or sodium chloride solution to remove adhering blood. The three arterial layers, intima, media and adventitia were clearly visible, and the latter two were stripped from the intima. Lesions were graded according to the W.H.O. classification (Page 17), and lesions of similar grade were collectively processed. In severely diseased aortas the intima displayed considerable thickening, and the separation of intima from media presented no difficulty. Separation of the two layers in samples affected only by fatty streaks was more

difficult, and the intima obtained in such cases frequently contained small quantities of medial tissue. After collection, tissue was finely divided using scissors and extracted in chloroform:methanol (2:1 v/v) for fifteen hours at -20°C in an atmosphere of nitrogen. The solvents used for the extraction of "total lipid" from tissues have been extensively discussed[239,240] and the use of chloroform:methanol mixture appears to be generally preferred. The extract was filtered by gravity, the pieces of tissue were washed with small quantities of chloroform:methanol mixture, and the filtrate and washings were combined. The extract was reduced to dryness under vacuum using a water bath. The temperature of the latter did not exceed 40°C. Benzene: ethanol (1:1 v/v) was added to reduce frothing and aid removal of water.[241] The residue was dissolved in a mixture of diethyl ether:hexane (1:1 v/v) and the solution was washed with an appropriate volume of distilled water, to remove non lipid constituents.[240] The organic layer was dried, solvent was removed under vacuum, and the extract was weighed. The lipid residue was dissolved in hexane and subjected to silicic acid column chromatography.

## 2.4. COLUMN CHROMATOGRAPHY

Column chromatography of lipid extracts was performed initially on Mallinckrodt Silicic Acid 100 mesh, sieved to 100-200 mesh, and acid washed and dried according to the procedure of Horning.[242] Subsequently, SilicAR cc-4, 100-200 mesh (Mallinckrodt), was employed and the sieving and acid washing procedures were discontinued. The drying procedure described in Ref 242 was still used in order to obtain material of a

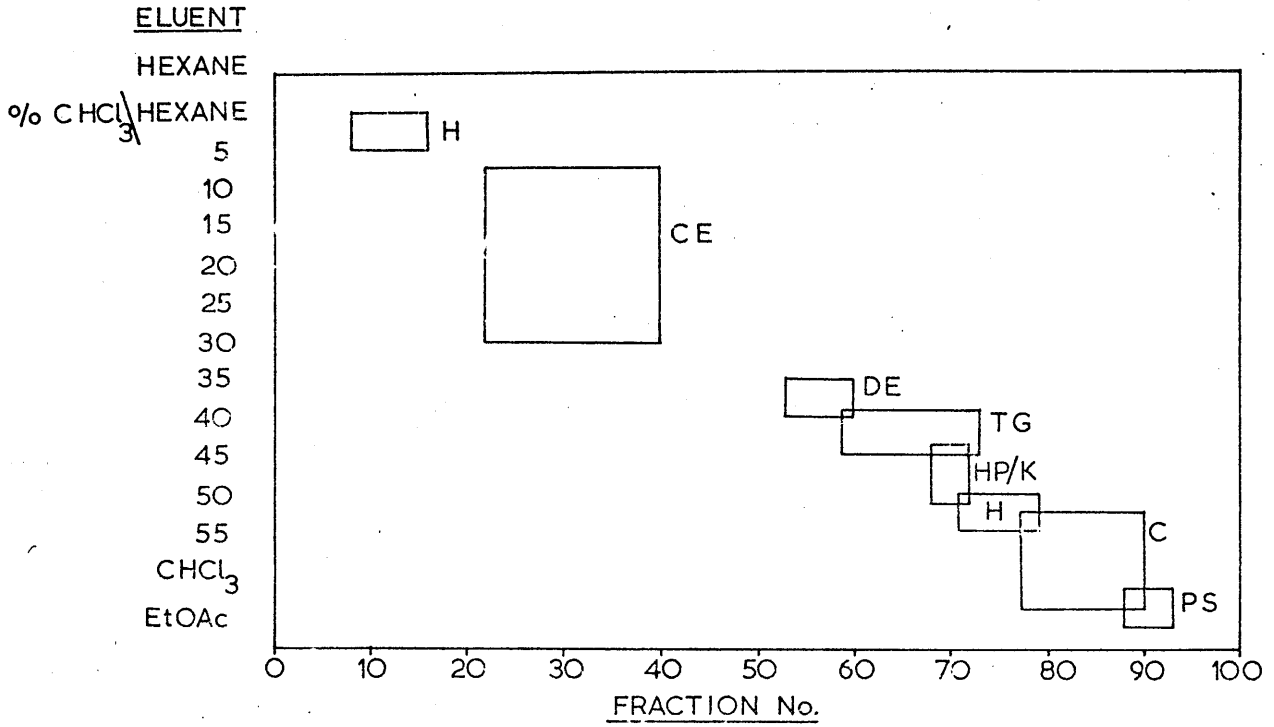



FIG. 1. Typical elution pattern obtained during silicic acid column chromatography of lipids from a severely diseased aorta.

consistent degree of activity.

Chromatography of lipid extracts was performed in glass columns fitted with glass sinters and stopcocks. The size of the column and the quantity of silicic acid used were determined by the quantity of lipid to be chromatographed, and a load of approximately 20 mg lipid/g of silicic acid was used. The column was packed by pouring a slurry of the specially treated silicic acid in hexane into the glass column which also contained hexane. The evenly packed column was washed with 500 ml hexane prior to the addition of the lipid extract. At the stage an eluate of 100 ml was collected to serve as a blank. A solution of the lipid extract in hexane was added gently via a Pasteur pipette to the top of the column, which was wrapped in aluminium foil to minimise possible light-induced artefact formation during chromatography. Chromatography was commenced by elution with hexane, continued with chloroform-hexane mixtures, and completed with ethyl acetate. In general 50 ml eluates were collected. The order of elution of different classes of neutral lipid encountered in the present study is illustrated in Fig. 1. The eluted fractions were evaporated to dryness under vacuum at 40°C. The residues were dissolved in a small quantity of hexane and ethyl acetate and stored in $\frac{1}{2}$-dram tapered screw cap vials in darkness at -20°C.

## 2.5. THIN-LAYER CHROMATOGRAPHY (TLC)

### Preparation of chromatoplates

Glass plates (20 x 20 cm or 5 x 20 cm) were coated to a thickness of 0.25 mm with MN-Kieselgel G-HR (Macherey, Nagel and Co., Duren, Germany) using a Quickfit (Quickfit and Quartz Ltd., Stone, Staffordshire) spreader. A slurry of 30 gms

adsorbent with 45 ml distilled water was used. In the case of plates for preparative TLC the layer thickness was 0.5 mm. One 20 x 20 cm chromatoplate of layer thickness 0.5 mm could accommodate a load of 40mg of lipid without noticeable deterioration of separations. After spreading, plates were air dried for ½ hour, activated at 120°C for 20 min. in a clean oven and stored in a dust-free cabinet until required.

Preparation of micro plates.

Micro plates used for the rapid monitoring of fractions from column chromatography were prepared as follows. A glass plate 20 x 100 cms was placed on the spreader. Microscope slides (7.5 x 2.5 cm) were moistened and stuck firmly on the base plate. The layer was spread in the usual way; the thickness used was 0.25 mm.

Preparation of silica layers impregnated with silver nitrate.

Silver nitrate impregnated plates were prepared by the methods of Claude[243] and Morris.[141] An aqueous silver nitrate solution of appropriate concentration is used instead of water to prepare the adsorbent slurry. In the separation of fatty acid esters by use of silver ion impregnated layers, the level of impregnation used was 12% (w/w). Separation of sterol and stanol trifluoroacetates was best effected on silica gel containing 25% (w/w) silver nitrate. After spreading, the plates were air-dried for 15 min. in darkness, and activated at 120°C for 20 min. The activated plates were stored in a dry, light-tight cabinet wrapped in aluminium foil to prevent deterioration which occurs when such chromatoplates are exposed to light or atmospheric moisture.

Methods of analytical TLC.

Analytical chromatograms were developed in Shandon tanks (Shandon Scientific Co. Ltd., London) lined with sheets of filter paper to assist atmosphere saturation, and chromatograms were developed through a distance of 15 cm from origin to solvent front. Solutions of sample were prepared in volatile solvents such as hexane, chloroform, diethyl ether or ethyl acetate, and were applied to the chromatogram by means of a Hamilton 10$\mu$l syringe. For most samples loads of between 10-30 $\mu$g were applied, resulting in symmetrical and easily detectable spots. In general spots were detected by spraying with a solution of 5% (w/v) ceric sulphate in 10% sulphuric acid. This was found to be an excellent general purpose reagent, of particular value, in view of the colour reactions produced, in the detection of sterols and sterol esters. After spraying, the chromatograms were heated in an oven at 150°C, for approximately one minute, which appeared to be the optimum time for spot colour development. If chromatograms are subjected to prolonged heating the spots turn black or brown, and the important diagnostic value of colour reactions is lost. Reagents used for the detection of specific types of compound or functional groups are given below:

2,4-dinitrophenylhydrazine for the detection of free aldehyde and keto groups - 0.5% (w/v) 2,4-dinitrophenylhydrazine in ethanol (1000 ml) plus 10 ml concentrated hydrochloric acid.

Reaction: yellow or orange spots without heating.

Saturated aqueous potassium iodide solution for the detection of hydroperoxides.

Reaction: yellow spot without heating.

Saturated sodium periodate in 10% sulphuric acid: for the detection of saturated and unsaturated fatty acid methyl esters.

Reaction: saturated fatty acid esters give light brown spots, unsaturated esters give dark brown spots. The chromatogram was heated at 150°C for 20 min. after spraying.

Methods of preparative TLC

Solutions of samples were applied to preparative chromatograms using a Hamilton syringe of 50 µl capacity. Where large quantities of material were available for TLC, micro applicators of 10 or 50 µl capacity were used (Applied Science Laboratories Inc.) These applicators enable bands to be applied evenly and very rapidly. However, some spillage was invariably found to occur, and in quantitative work, or where only small quantities of material were available, a Hamilton syringe was preferred.

Material on developed preparative chromatograms were located by brief exposure to iodine vapour. Although compounds containing polyunsaturated fatty acids are not altered by this treatment,[244] considerable losses of such compounds are encountered when plates bearing them remain exposed to air and iodine for more than a few minutes.[245,246] Bands were therefore eluted immediately after detection by this method. The use of silica gel with an added fluorescent material was found to be a very useful alternative to iodine vapour as a detecting agent. The material used was Kieselgel $HF_{254}$ (E. Merck, Darmstadt, Germany). Bands were located on chromatograms by their quenching of the fluorescence of the modified layer when viewed under an

ultraviolet lamp. Substances which could not be detected by either of these methods were located by comparison of the preparative chromatogram with a destructively sprayed analytical chromatogram of identical composition and degree of activity.

The efficiency of recovery of sample after preparative TLC is dependent upon the nature of the material adsorbed on the plate surface, and the type of solvent used for elution. The use of a mixture of chloroform:methanol (9:1 v/v) was found to give satisfactory recoveries (90-98%) of less polar lipids such as sterol esters, triglyceride and cholesterol. However, this method was not satisfactory for di- and tri-hydroxy sterols, or aromatic type compounds such as the estrogens. In such cases the silica was first deactivated with water, and the desorbed sterol was extracted with ether or ethyl acetate. If used with care this method yielded quantitative recoveries.

## 2.6. GAS LIQUID CHROMATOGRAPHY (GLC)

### 2.6.1. General methods

GLC was performed on a Pye series 104 chromatograph fitted with two flame ionisation detectors. Glass columns of 5' and 10' lengths and inside diameter 3.5 mm were used, and were treated with a 5% solution of dimethyldichlorosilane in toluene prior to use. The column packings used, with the appropriate supports and sources of supply are listed below.

1. 1% SE-30 (methyl siloxane polymer) on 100-120 mesh Gas-Chrom Q.
2. 1% JXR (methyl siloxane polymer) on 100-120 mesh Gas-Chrom Q.
3. 1% QF-1 (fluoroalkylsiloxane polymer) on 100-120 mesh Gas-Chrom Q.
4. 3% EGSS-X (ethylene glycol succinate/methyl siloxane polymer) on 100-120 mesh Gas Chrom Q.
5. 1% OV-17 (phenyl methyl siloxane polymer (50% phenyl)) on

80-100 mesh Chromosorb G HP.

6. 10% PEGA (Poly ethyleneglycol adipate) on silanised Gas-Chrom P. 80-100 mesh.

Column packings 1-4 were obtained ready made and pre-tested from Applied Science Laboratories Inc. Packing number 5 was obtained ready made from Supelco Inc., Bellefonte, Pennsylvania, U.S.A. The 10% poly ethyleneglycol adipate (PEGA) packing was obtained from Miss J.S. Young, M.R.C. Blood Pressure Research Unit, Western Infirmary, Glasgow, G12 8QQ.

Samples generally 1-3 mg/ml were injected as hexane or ethyl acetate solutions using a Hamilton syringe. The volume of solution injected ranged from 0.2 - 5 $\mu$l. The carrier gas used was oxygen-free nitrogen at a flow rate of 40 ml/min., and the injection port heaters were maintained at temperatures between 20° and 50° higher than that of the analyser oven.

2.6.2. Use of GLC for the quantitative determination of sterols and hydroxy acids in extracts of arterial tissue.

Determination of hydroxy octadecadienoic acids:

Fatty acid fractions (10-700 $\mu$g), obtained by hydrolysis of sterol esters were methylated and trimethylsilylated as described (ppp. 56-57). Quantification of hydroxy fatty acid methyl esters TMS ethers was performed using an external standard of methyl ricinoleate TMS ether. The column used was 10' long and was packed with 10% poly ethyleneglycol adipate on 80-100 mesh "silanised" Gas Chrom P. The column temperature was 180°C.

Known quantities of methyl ricinoleate TMS ether were examined by GLC and a graph was drawn of the detector response (in terms of peak area) against the quantity of standard injected. Peak areas were calculated as the height of a peak times its

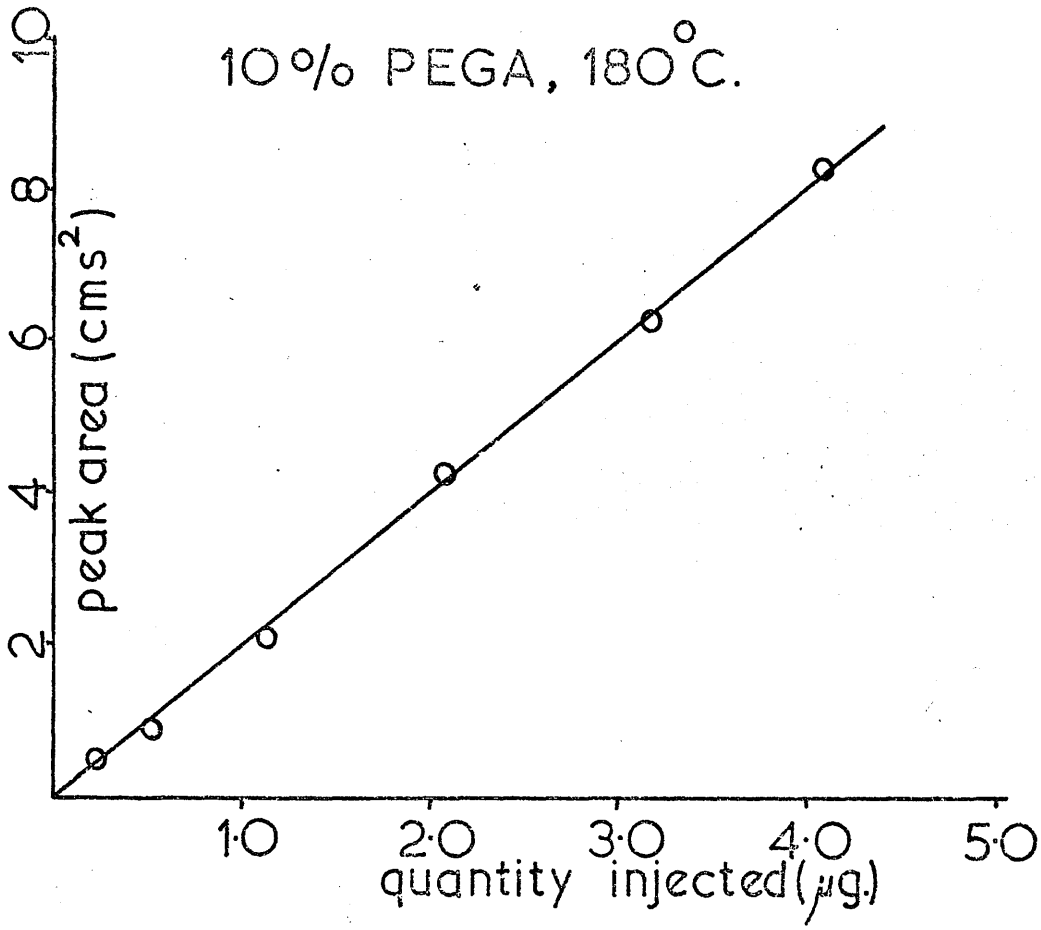



FIG. 2. Gas chromatographic estimation of hydroxy fatty acid esters: calibration graph relating detector response (in terms of peak area) against quantity injected for a reference sample of methyl ricinoleate TMS ether.

| Quantity Injected (µg) | Quantity Calculated (µg) | Quantity Injected / Quantity Calculated |
|---|---|---|
| 3.60 | 3.58 | 99% |
| 1.80 | 1.82 | 99% |
| 0.90 | 0.90 | 100% |
| 0.45 | 0.43 | 95% |
| 0.225 | 0.24 | 94% |

TABLE 4. Calculation of detector response factors in the gas chromatographic estimation of β-dimorphecolic acid methyl ester TMS ether using an external standard of methyl ricinoleate TMS ether.

width at half height. The graph (Fig. 2 ) showed good linearity over the range examined (0.2$\mu$g-4.2$\mu$g). The suitability of this standard for the analyses of hydroxy octadecadienoic acid derivatives was determined by examination of known quantities of methyl 9-hydroxy-10-trans,12-trans-octadecadienoate ($\beta$- dimorphecolate) TMS ether, and comparison of the quantities injected with those calculated from the calibration graph. The acid derivatives were examined over a range of quantities, and the mean of two values of the detector response at each quantity injected was used to calculate the required quantities from the calibration graph. Results are presented in Table 4 , and demonstrate that over a range of 1-3.6$\mu$g, methyl ricinoleate TMS ether may be successfully used as an external standard without the application of a correction factor. Aliquots of fractions containing hydroxy octadecadienoic acid derivatives were similarly examined by GLC. By use of the calibration graph, the quantity of material injected and thus the total quantity in the sample could be estimated. The standard methyl ricinoleate TMS ether was prepared from the methyl ester shortly before analysis. The calibration graph was drawn immediately before injection of the sample to be determined.

Determination of sterols:

Quantitative determination of sterols utilised a very similar technique. For the determination of 26-hydroxycholesterol, the reference standard used was the corresponding bis TMS ether. The column used was 1% SE-30 on Gas Chrom Q and analyses were performed at 240°C. Cholestanol was determined as its trifluoroacetate on a column packed with 1% QF-1 on Gas Chrom Q at 200°C.

## 2.7. GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

Mass spectra were obtained using an LKB model 9000 gas chromatograph-mass spectrometer (LKB-Produkter AB, Stockholm, Sweden). This instrument is a single focusing mass spectrometer fitted with a 60° sector, 20 cm radius magnetic analyser and a sweep generator for fast scanning of spectra. A two-stage jet separator of the Becker-Ryhage type enables removal of 99% to 99.5% of the carrier gas (helium) and retention of 50%-75% of the sample eluted from the gas chromatographic column. The instrument was fitted with a "mass marker" to facilitate counting of spectra. Spectra of samples reported in the present study were obtained using a carrier gas flow rate of 25-35 ml/min., electron energies of 70 eV - 22.5 eV, and an accelerating voltage of 2.5 - 3.0 Kv. The temperature of both the ion source and the molecular separator was 275°C.

Prior to analysis by GC-MS samples were examined on a Pye 104 chromatograph. Retention times and retention index values were determined, and the quantity of the sample to be analysed was determined by comparison with an external standard. A background mass spectrum was obtained in the absence of sample and under identical conditions to correct for spectral peaks due to gas chromatographic column packing.

## 2.8. CHEMICAL TECHNIQUES

### Hydrolysis of sterol esters

To a solution of ester in the minimum quantity of benzene, was added an ethanolic solution of potassium hydroxide consisting of 6 ml of 33% aqueous potassium hydroxide in 50 ml absolute ethanol. The mixture was heated at 40°C for 1 hour. Ether extraction yielded sterol fractions and the aqueous layer was

acidified with dilute hydrochloric acid. Re-extraction with ether yielded fatty acids.

Reduction of sterol esters with lithium aluminium hydride.

The ester to be reduced was dissolved in a small quantity of sodium-dried ether. A solution of lithium aluminium hydride in dry ether was added, and the mixture was heated at 60°C for 15 min. in a sealed tube. Excess reagent was removed by the cautious addition of dry ethyl acetate, more ether was added and the organic solution was washed with an equivalent quantity of water. Products were recovered from the separated ethereal solution.

Reduction of esters with lithium aluminium deuteride.

The method used was essentially that used for lithium aluminium hydride reduction except that lithium aluminium deuteride ($LiAlD_4$, Isotopic purity 99 Atom%D, CIBA Ltd.) was used.

Conversion of steroid and aliphatic hydroperoxides and ketones to the corresponding alcohols.

A solution of hydroperoxy or keto compound in the minimum quantity of benzene was added to an excess of a solution of methanolic sodium borohydride. The reaction mixture was stirred for 30 min. at room temperature. Methanol was removed under vacuum, and the residue was dissolved in ether and washed several times with an equal quantity of water. After drying the derived alcohol was recovered from the ethereal solution.

Hydrogenation of olefinic fatty acids and their derivatives.

Hydrogenation was performed in a microhydrogenation apparatus, using $PtO_2$ as catalyst. 1-2 mg of catalyst was added to a small conical flask containing 3 ml methanol and a small magnetic stirring rod. The flask was fitted to the hydrogenation apparatus and hydrogen was passed into the reaction flask for ½ hour. The sample (5-500 $\mu$g) was introduced into the reaction flask as a chloroform solution, via a rubber septum fitted to the apparatus. The hydrogen flow was stopped and the solution was stirred for two hours at room temperature. The apparatus was flushed with nitrogen, the methanol solution was filtered, evaporated to dryness and the products taken up in ethyl acetate.

Oxidation of hydroxyoctadecadienoic acid methyl esters.

Oxidations were carried out overnight at room temperature using suitable quantities of a mixture prepared from chromium trioxide (200 mg) and dry pyridine (6 ml). After the reaction the pyridine was removed in a stream of nitrogen, water was added, and the products were extracted with ethyl acetate.

Methylation of fatty acids.

Diazomethane was prepared from Bis-(N-methyl-N-nitroso) terephthalamide.[247] Fatty acids were methylated with a freshly prepared solution of diazomethane in ether at room temperature. The reagent was added until the yellow colour of diazomethane persisted. Excess reagent was removed in a stream of nitrogen and the methyl esters were dissolved in ethyl acetate.

Acetylation of sterols.

The sterol (approximately 100 $\mu$g) was dissolved in dry redistilled pyridine (20 $\mu$l). An excess of dry acetic anhydride (10-20 $\mu$l) was added, and the reaction mixture was heated at 80°C for 3 min. Excess solvent and reagent were blown away in a stream of nitrogen. The residue was dissolved in ethyl acetate (5 ml) and washed with 3 x 5 ml portions of water. Sterol acetates were recovered from the dried ethyl acetate solution.

Formation of sterol trifluoroacetates.

The sterol (3mg - 10 $\mu$g) was dissolved in the minimum quantity of chloroform and an excess (approximately 20 $\mu$l) of redistilled trifluoroacetic anhydride was added. After 10 min. at 40°C the excess reagent was blown away with nitrogen and the residual trifluoroacetates were dissolved in hexane. Trifluoroacetates prepared in this way showed no decomposition if stored as hexane solutions in tightly capped vials at -20°C.

Formation of trimethylsilyl (TMS) ethers of sterols and other alcohols.

The alcohol to be trimethylsilylated (4mg - 10 $\mu$g) was dissolved in the minimum quantity of dried redistilled pyridine. Hexamethyldisilazane (approximately 30 $\mu$l) and trimethylchlorosilane (approximately 5 $\mu$l) were added, and the reaction mixture was heated at 80°C for a few minutes. Excess reagents were blown off in a stream of nitrogen and the products were dissolved in ethyl acetate. For short term storage of TMS ethers removal of insoluble material by filtration was found to be adequate; otherwise the ethyl acetate solution was washed with water, dried and stored in darkness at -20°C.

Preparation of deuterium-labelled trimethylsilyl ethers.

The alcohol (10-100 $\mu$g) was dissolved in an excess (~5 $\mu$l) of $\underline{d}_{18}$-bis-(trimethylsilyl)-acetamide (Merck, Sharp and Dohme of Canada, Ltd.) The reaction mixture was heated at 80°C for two minutes. Excess reagent was removed in a stream of nitrogen, and the products were dissolved in ethyl acetate. Samples were stored as ethyl acetate solutions in darkness at -20°C.

Preparation of O-Methyl Oximes.

The ketone (10-100 $\mu$g) was dissolved in pyridine (~10 $\mu$l) and approximately 20 $\mu$l of a saturated solution of O-methyl hydroxylamine hydrochloride in pyridine was added. The mixture was heated at 80°C for 3-4 min. Excess solvent was removed in a stream of nitrogen, the residue was washed with water and the oximes were extracted into ethyl acetate.

## Section 3 RESULTS

## 3.1. STUDIES OF OXIDATION PRODUCTS FROM CHOLESTERYL LINOLEATE

### 3.1.1. Introduction

The monohydroperoxides obtained by oxidation of methyl linoleate have been extensively studied.[248-250] Bergström[251-253] demonstrated that autoxidation of methyl linoleate produces conjugated dienoates with hydroperoxide groups either at $C_{(9)}$ or $C_{(13)}$ of the fatty acid chain. Subsequent investigations have shown that 90% of the autoxidation products of methyl linoleate consist initially of approximately equal quantities of the 9-hydroperoxy-10-trans,12-cis-octadecadienoate and the isomeric 13-hydroperoxy-9-cis,11-trans-octadecadienoate. Appreciable quantities of the corresponding trans,trans isomers have also been detected after continued autoxidation at room temperature.[254-256]

Chlorophyll-photosensitised oxidation of methyl linoleate has been shown to produce considerable quantities of non-conjugated hydroperoxides which were initially thought to be 11-hydroperoxy-octadecadienoates.[257,258] More recent work, however, has identified the non-conjugated products as 10-hydroperoxy-8-trans,12-cis, and 12-hydroperoxy-9-cis,13-trans-octadecadienoates.[259,260] Recent work on the minor lipids of human atherosclerotic tissue has established the presence of a group of cholesterol esters in which the acid moieties are hydroxy-octadecadienoates.[125,127] Analogous hydroperoxides of cholesteryl linoleate (possible precursors of the hydroxy-esters) have also been identified in extracts from advanced aortal lesions, and in order to gain more information about the nature and origin of these compounds a study of the oxidation of cholesteryl linoleate was undertaken.

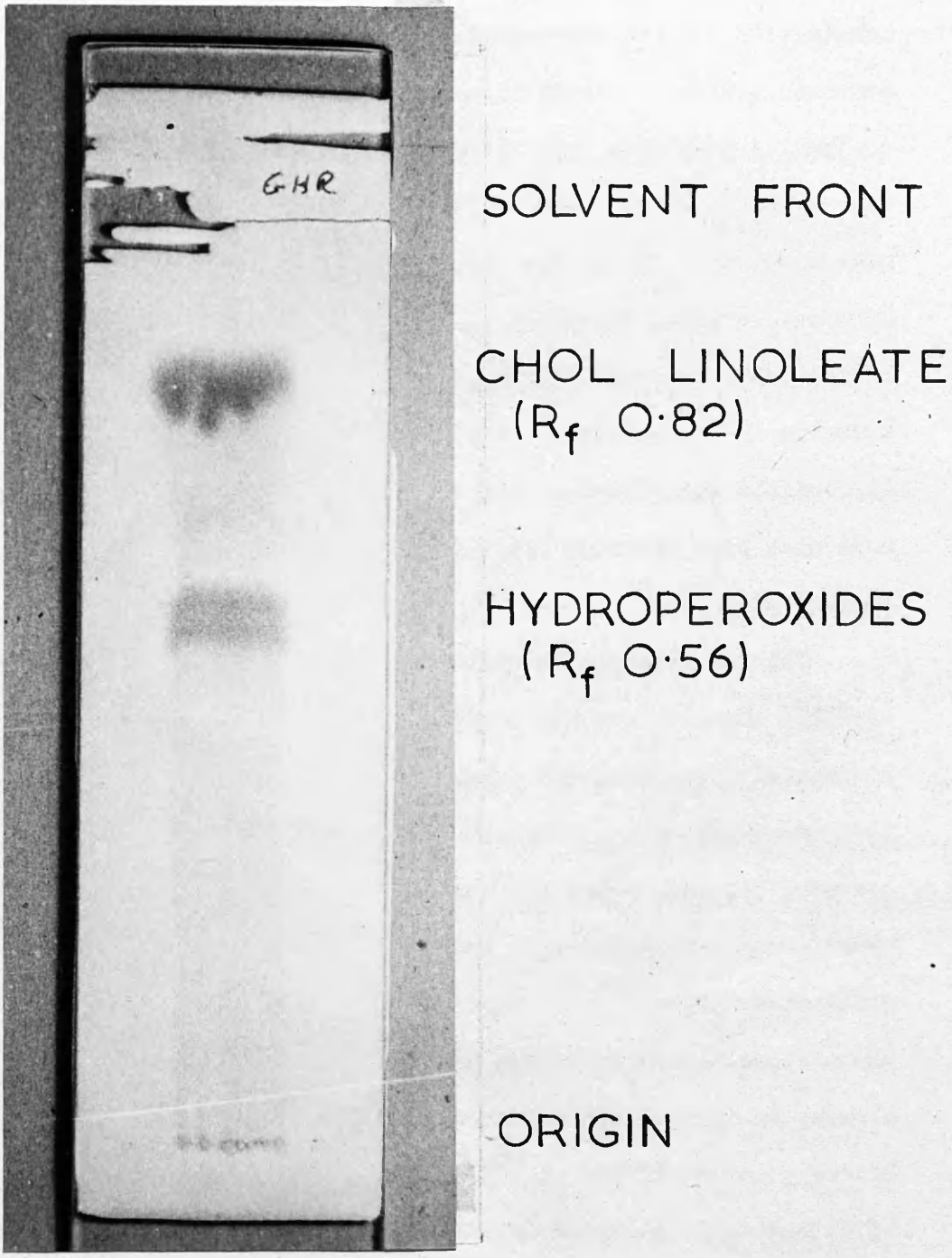



FIG. 3. Analytical thin-layer chromatogram of autoxidised cholesteryl linoleate. Mobile phase: benzene: ethyl acetate (20:1 v/v).

3.1.2. Preparation and characterisation of the hydroperoxides produced by autoxidation of cholesteryl linoleate.

A thin film of cholesteryl linoleate (115 mg) was rotated in a flask open to the atmosphere for 5 days at room temperature. Examination of the reaction mixture by TLC (mobile phase: benzene:ethyl acetate, 20:1 v/v) showed the presence, at $R_f$ = 0.56, of material more polar than cholesteryl linoleate (Fig. 3). This material coloured yellow when sprayed with potassium iodide solution. The hydroperoxides were separated from substrate by preparative TLC to yield 21 mg of a colourless oil. It was subsequently found that exposure of cholesteryl linoleate to a jet of oxygen enabled yields of 40-50% to be obtained after 4 hours.

Infrared spectroscopy of the hydroperoxide showed absorptions at 3410cm$^{-1}$ ($\nu$ O-H) and 840cm$^{-1}$ ($\nu$ O-O). Ultraviolet spectrometry of the material showed an absorption at $\lambda_{max}^{EtOH}$ = 232 nm ($\varepsilon_{max}$ = 22,000), characteristic of a conjugated diene.[261]

Treatment of the hydroperoxides with hexamethyldisilazane produced material, which on TLC examination migrated to $R_f$ 0.84 (mobile phase: benzene:ethyl acetate, 20:1 v/v) and showed signs of considerable decomposition.

In view of the instability of the hydroperoxides,[251,262] full characterisation of these compounds entailed the formation of stable derivatives which could be conveniently examined by GLC and GC-MS. Reduction of the hydroperoxy-esters with sodium borohydride yielded the corresponding hydroxy-esters. Examination of the products by TLC (mobile phase: benzene: ethyl acetate, 20:1 v/v) showed a major spot at $R_f$ 0.43 (red-brown) flanked by two lesser spots with $R_f$'s of 0.47 and 0.40

| | $R_f$ Benzene/ Ethyl Acetate 20:1 v/v | Colour reaction with Ceric Sulphate Reagent | Colour reaction with Potassium Iodide Reagent |
|---|---|---|---|
| Cholesteryl linoleate | 0.76 | Maroon | None |
| Cholesteryl linoleate hydroperoxides | 0.56 | Red-brown | Yellow |
| Hydroperoxy-esters from aortal extract | 0.54 | Red-brown | Yellow |
| Cholesteryl linoleate hydro-peroxides after borohydride reduction | 0.47<br>0.43<br>0.40 | Blue<br>Red-brown<br>Blue | None |
| Hydroxy-esters from aortal extract | 0.52<br>0.47<br>0.41 | Blue<br>Red-brown<br>Blue | None |
| Aortal hydro-peroxy-esters after borohydride reduction | 0.47<br>0.42<br>0.38 | Blue<br>Red-brown<br>Blue | None |

TABLE 5. Comparison of the thin-layer chromatographic properties of reference and isolated hydroxy- and hydroperoxy-esters of cholesteryl linoleate.

| STEROL AND ORIGIN | FREE STEROL | | STEROL TRIMETHYLSILYL ETHER | | |
|---|---|---|---|---|---|
| From hydroperoxides of cholesteryl linoleate | $R_f$ Chloroform: Ethyl acetate (3:1 v/v) | Colour reaction | $R_f$ Hexane: Benzene (2:1 v/v) | Colour reaction | Retention Index 1% SE-30 240°C |
| Sterol 1 | 0.61 | Red | 0.45 | Red | 3120 |
| Sterol 2 | 0.20 | Blue | 0.45 | Blue | 3125 |
| Sterol 3 | 0.20 | Blue | 0.12 | Blue | 3240 |
| Reference sterols | | | | | |
| cholesterol | 0.60 | Red | 0.42 | Red | 3130 |
| 7α-hydroxy cholesterol | 0.16 | Blue | 0.42 | Blue | 3130 |
| 7β-hydroxy cholesterol | 0.16 | Blue | 0.15 | Blue | 3235 |
| Sterols derived from reductive cleavage of aortal hydroperoxy-esters | | | | | |
| Sterol 1 | 0.57 | Red | 0.46 | Red | 3120 |
| Sterol 2 | 0.17 | Blue | 0.46 | Blue | 3120 |
| Sterol 3 | 0.17 | Blue | 0.17 | Blue | 3235 |

TABLE 6. Thin-layer and gas chromatographic retention data of sterols derived from synthetic and arterial hydroperoxy-esters.

Chromatograms visualised by spraying with ceric sulphate reagent.

| FATTY ACID DERIVATIVES | RETENTION INDICES | | MAJOR IONS IN MASS SPECTRUM | |
|---|---|---|---|---|
| | 1% SE-30 | 10% PEGA 178°C | $M^+$ | Other Principal Ions |
| DERIVATIVES FROM AUTOXIDISED LINOLEATE | | | | |
| Peak I | 2090 | 2560 | | |
| Peak II | 2310 | 2735 | 382 | 225, 130, 143, 155, 311, 292 |
| Peak III | 2355 | 2810 | 382 | 225, 130, 143, 155, 311, 292 |
| Peak IV | 2365 | 2830 | 382 | 225, 130, 143, 155, 311, 292 |
| REFERENCE ACID DERIVATIVES | | | | |
| Methyl linoleate | 2095 | 2565 | | |
| Methyl $\alpha$-dimorphecolate TMS | 2310 | 2740 | 382 | 225, 130, 143, 155, 311, 292 |
| Methyl $\beta$-dimorphecolate TMS | 2360 | 2830 | 382 | 225, 130, 143, 155, 311, 292 |
| * Methyl coriolate TMS | 2310 | 2735 | 382 | 225, 130, 143, 155, 311, 292 |
| DERIVATIVES FROM AORTAL HYDROPEROXIDES | | | | |
| Peak I | 2300 | | 382 | 225, 130, 143, 155, 311, 292 |
| Peak 2 | 2350 | | { 382 | 225, 130, 143, 155, 311, 292 |
| Peak 3 | 2365 | | | |

TABLE 7. Gas chromatographic and mass spectral data of hydroxy fatty acid derivatives from autoxidised cholesteryl linoleate and arterial hydroperoxides.

* Derivative of 13-hydroxy-9cis,11trans-octadecadienoic acid.

which coloured blue when sprayed with ceric sulphate reagent. This chromatogram bore a strong resemblance to those of hydroxy-esters of cholesterol previously isolated from extracts of advanced aortal lesions (Table 5). Alkaline hydrolysis of the reduced material yielded acidic and neutral fractions. Examination of the latter by TLC (mobile phase: chloroform: ethyl acetate, 3:1 v/v) showed two spots, identical in $R_f$ and colour reaction to cholesterol ($R_f$ 0.61) and either or both of the epimeric 7-hydroxycholesterols ($R_f$ 0.20). The two bands were separated by preparative TLC, and the isolated sterols were trimethylsilylated and examined by GLC. Chromatographic results are presented in Table 6. The 7-hydroxycholesterol fraction contained both the 7$\alpha$ and 7$\beta$ epimers. Analysis by GLC indicated a four-fold excess of the 7$\alpha$ epimer.

The acidic fraction from hydrolysis was methylated, trimethylsilylated and examined by GLC. Four peaks were observed in the gas chromatogram. The retention index values of the components in the mixture were compared with those of the corresponding derivatives of available acids, and results are presented in Table 7. The mass spectra of the acid methyl ester trimethylsilyl (TMS) ethers derived from the hydroperoxides and those of the appropriate derivatives of the reference acids showed considerable similarities. The major ions in the mass spectra of all these compounds are given in Table 7. The mass spectrum of the most mobile peak on GLC was not obtained. However, the chromatographic data presented in Table 7 imply that this substance is methyl linoleate, and it was estimated to account for approximately 5% of the total acids in the fraction. The mass spectra indicate that the hydroxy-acids derived from cholesteryl linoleate hydroperoxide are very

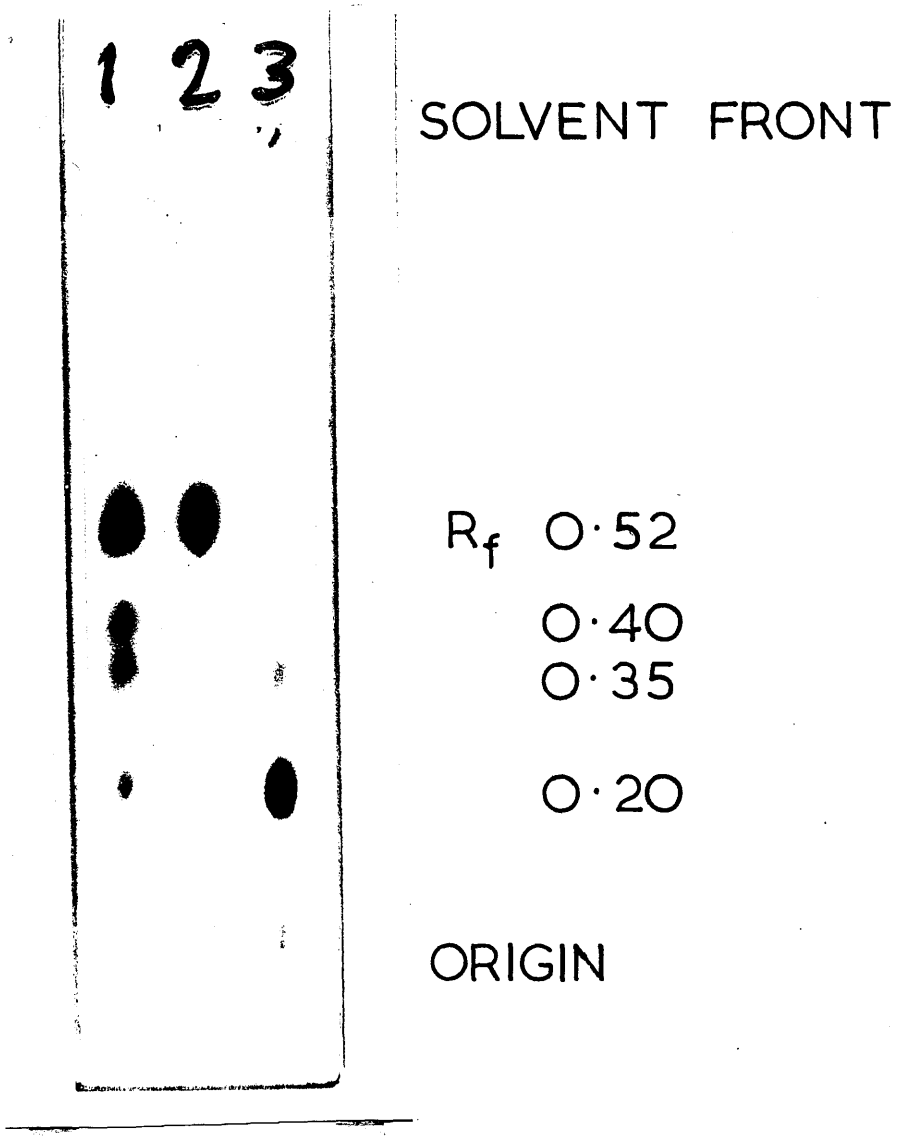



FIG. 4. Analytical thin-layer chromatogram of the products from $LiAlH_4$ reduction of cholesteryl linoleate hydroperoxides (Lane 1), and reference samples of cholesterol (Lane 2), 7β-hydroxycholesterol and octadecadiene-1,9-diol (Lane 3). Mobile phase: chloroform:ethyl acetate (3:1 v/v).

| | $R_f$ | GLC retention index of TMS ethers | | | |
|---|---|---|---|---|---|
| | Chloroform: Ethyl acetate (3:1 v/v) | 1% SE-30 172°C | 1% OV-17 172°C | 10% PEGA 169°C | 3% EGSSX 150°C |
| Linoleyl Alcohol | 0.62 | 2190 | 2165 | 2270 | 2255 |
| Material from Autoxidised cholesteryl linoleate | 0.65 | 2185 | 2165 | 2270 | 2265 |

TABLE 8. Chromatographic properties of reference linoleyl alcohol and material recovered after reductive cleavage of cholesteryl linoleate hydroperoxides.

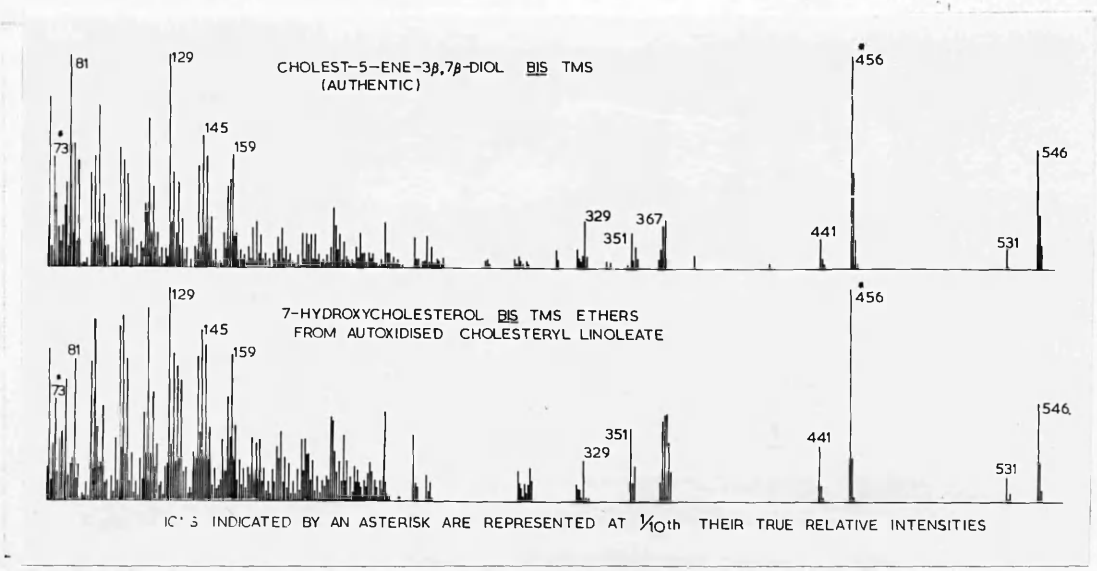



FIG. 5. Comparison of the mass spectrum of the 7-hydroxycholesterol bis TMS ethers derived from autoxidised cholesteryl linoleate with that of authentic 7β-hydroxycholesterol-bis-TMS ether.

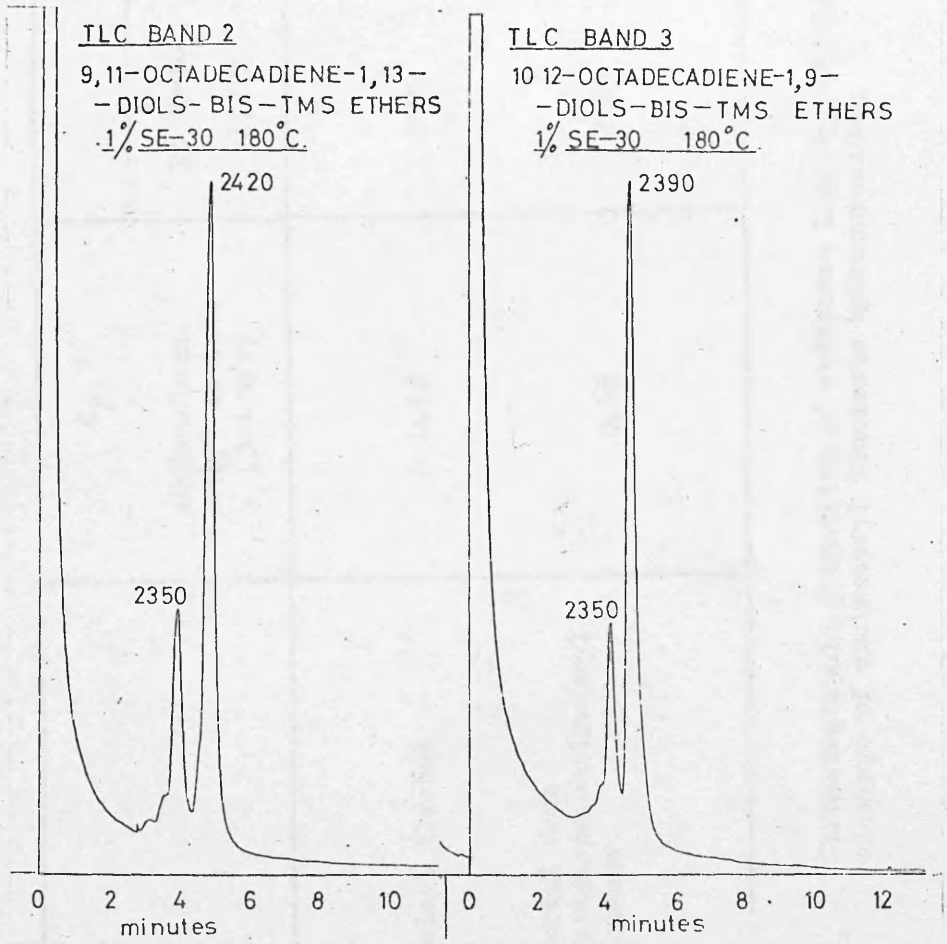



FIG. 6. Gas-liquid chromatogram of octadecadiene-1,13- and 1,9-diol-bis TMS ethers after separation of the diols by preparative TLC.

similar to those found in seed oils.[263-265] Although gas-liquid chromatographic and mass spectral results indicate that the acids derived from hydroperoxide are hydroxy-octadecadienoic acids it is extremely difficult to distinguish between the 9- and the 13-hydroxy derivatives, the mass spectra being very similar. It was desirable that derivatives of the hydroperoxy-esters with more characteristic mass spectral properties be prepared to enable full characterisation of both synthetic hydroperoxides and those occurring in aortal extracts.

Reduction of the hydroperoxides of methyl linoleate with lithium aluminium hydride,[215] and subsequent trimethylsilylation, has been shown to produce derivatives which can be readily distinguished by mass spectrometric methods. In accordance with these results a sample of cholesterol linoleate hydro-peroxides was reduced with lithium aluminium hydride as described on Page 55, and examined by TLC (mobile phase: chloroform:ethyl acetate, 3:1 v/v). Four bands were observed (Fig. 4) and were separated by preparative TLC. The most mobile band (1) at $R_f$ 0.52 corresponded to cholesterol on TLC, both as the free sterol, and as the TMS ether ($R_f$ = 0.45, mobile phase: hexane:benzene, 2:1 v/v). GLC indicated the presence of cholesterol and some linoleyl alcohol (Table 8). The least mobile band (4) ($R_f$ 0.20) was similarly identified as a mixture of the epimeric 7-hydroxycholesterols; in this case the identities were confirmed by mass spectral analysis of the TMS ethers. Mass spectra of the 7-trimethylsilyloxy cholesterol epimers were identical to those of reference samples (Fig. 5). Bands (2) ($R_f$ 0.40) and (3) ($R_f$ 0.35) were separately extracted from silica in chloroform and trimethyl-silylated. GLC of band (2) (Fig. 6) revealed the presence of

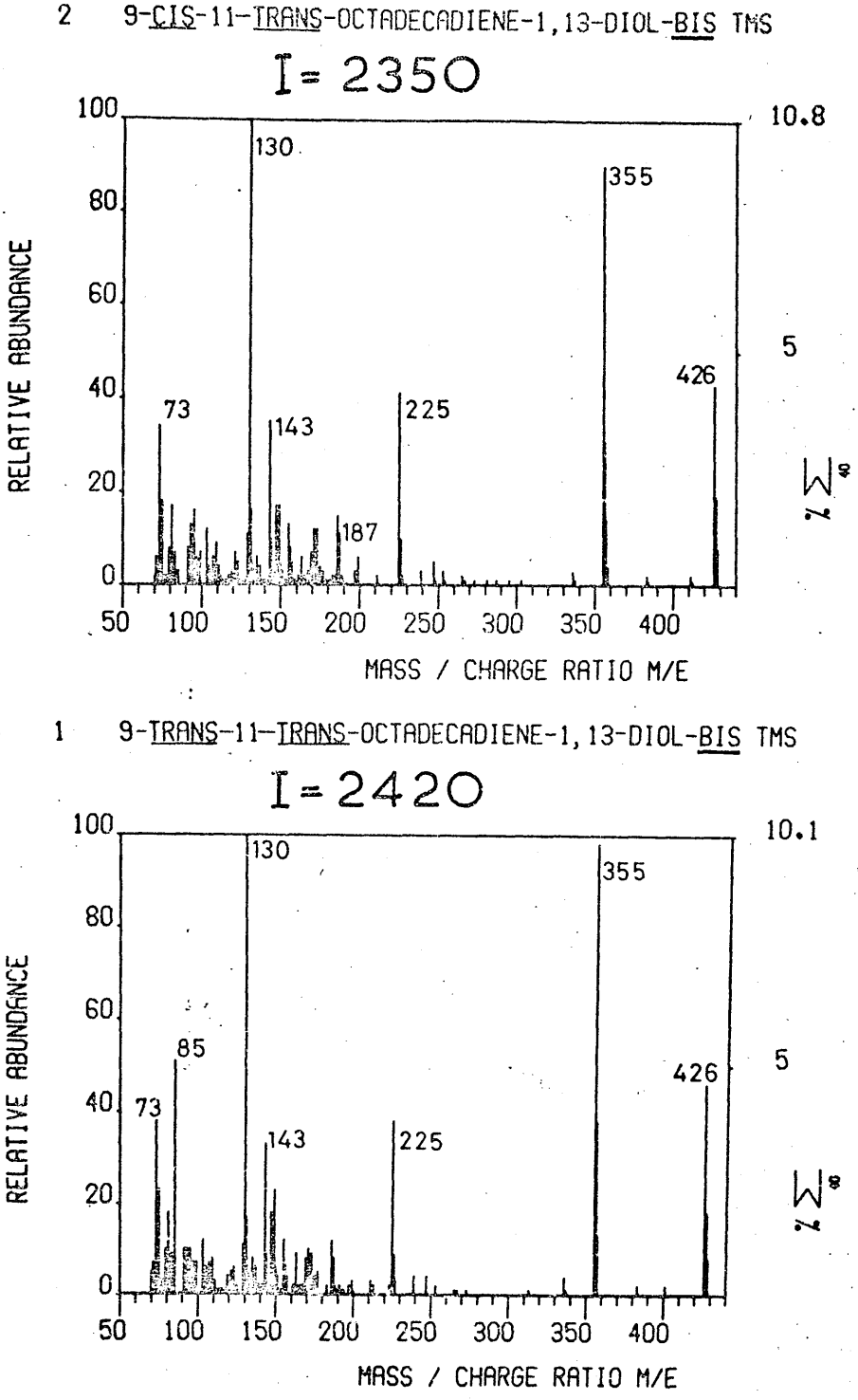



FIG. 7. Mass spectra of the octadecadiene-1,13-diol-bis TMS ethers derived from autoxidised cholesteryl linoleate.

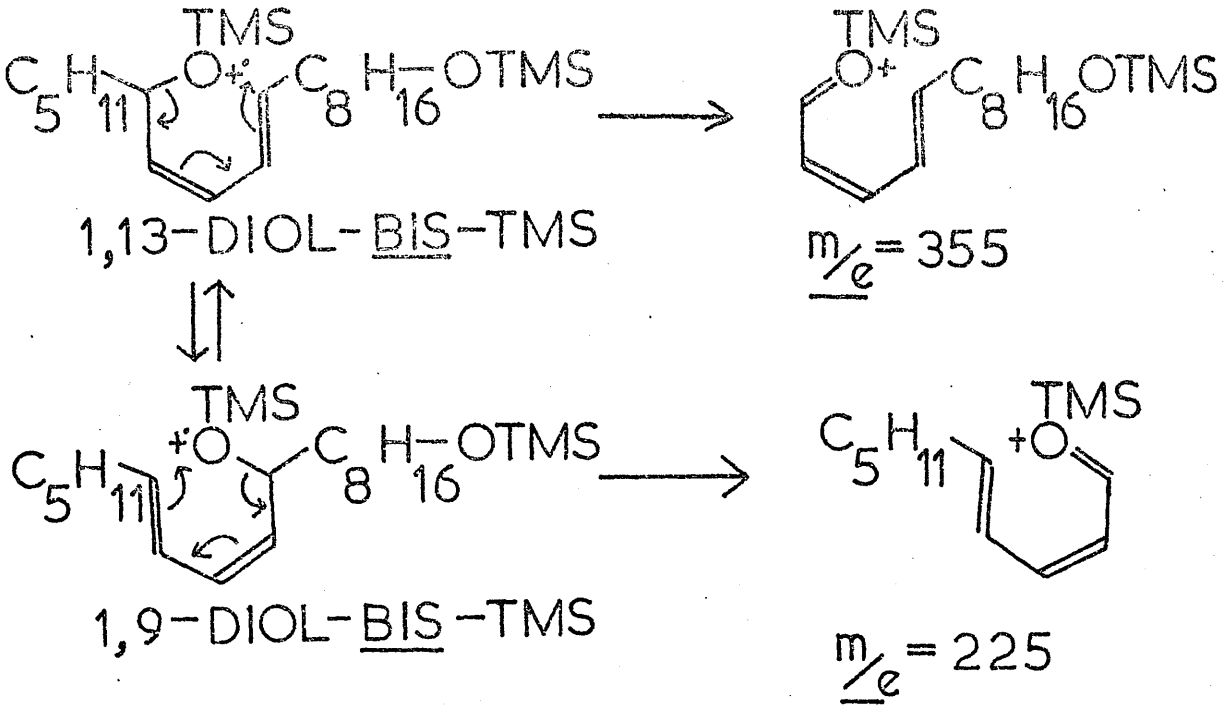



FIG. 8. Proposed rearrangement explaining the occurrence of an ion with m/e = 225 in the mass spectra of 9,11-octadecadiene-1,13 diol-bis TMS ethers.

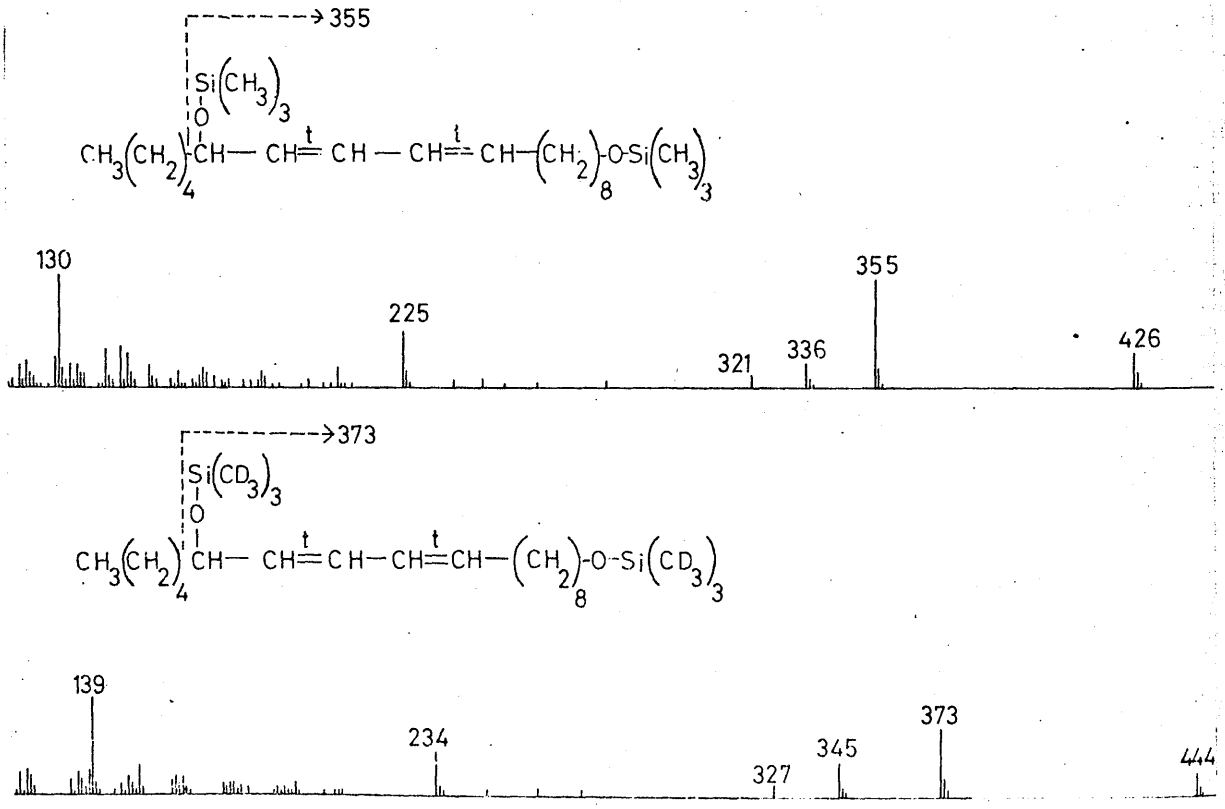


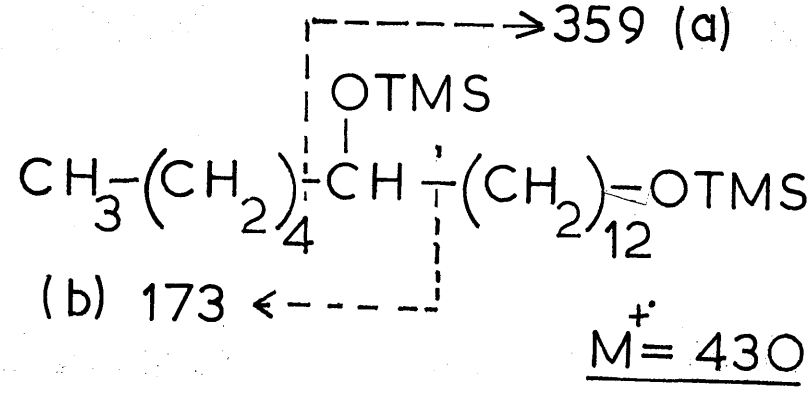

FIG. 9. Comparison of the mass spectra of the TMS and $d_{-18}$ perdeuterated TMS ethers of 9-trans, 11-trans octadecadiene-1,13-diol derived from autoxidised cholesteryl linoleate.

two major peaks with retention indices on 1% SE-30 at 180°C, of 2350 and 2420. The mass spectra of both compounds are reproduced in Fig. 7, and are consistent with those of a pair of 9,11-octadecadiene-1,13-diol bis-TMS ethers. Both mass spectra are identical and show the expected molecular ion at m/e 426. The major diagnostic ion is at m/e 355. This represents cleavage of the carbon-carbon bond between $C_{(13)}$ and $C_{(14)}$.[215,206] Cleavage is less probable at a position adjacent to the conjugated diene system and a much smaller ion is observed at m/e = 173.[205,207] The ion at m/e 225 probably arises via a cyclic rearrangement of the type shown in Fig. 8. Confirmation of the identity of the major ions in the mass spectra of the octadecadiene-1,13 diols bis TMS ethers was obtained by comparison with the mass spectra of the perdeuterated TMS ethers (Fig. 9). In the deuterium labelled molecule the molecular weight has risen by 18 mass units to 444. Similarly the ion at m/e 355 in the non-labelled molecule increases by 18 mass units to m/e 373. This demonstrates the presence of two trimethylsilyloxy groups in the original fragment. The ion at m/e 225 increases by 9 mass units to m/e 334 in the labelled molecule. Thus this fragment contains one trimethylsilyloxy group, in agreement with the fragmentation proposed in Fig. 8. The nature of the ion at m/e 130 in the non-labelled TMS ether can be inferred from data for the labelled derivatives. The rise in mass of this ion to m/e 139 reveals the presence of one trimethylsilyloxy group and indicates a molecular formula $C_3H_5OTMS^{+\cdot}$. The mechanism by which this ion is formed will be discussed later. The identity of the octadecadiene-diols in band (2) was confirmed by hydrogenation using $PtO_2$/methanol (as described on Page 56), and examination of the

| | TLC | GLC Retention data of diol TMS ethers | | |
|---|---|---|---|---|
| REFERENCE DIOLS | $R_f$ Chloroform: Ethyl Acetate (3:1 v/v) | 1% SE-30 180°C | 10% PEGA 188°C | 3% EGSSX 150°C |
| 10-trans,12-cis-1,9 diol | 0.46 | 2350 | 2415 | 2485 |
| 10-trans,12-trans-1,9 diol | 0.48 | 2390 | 2485 | 2530 |
| 9-cis,11-trans-1,13 diol | 0.53 | 2350 | 2420 | 2470 |
| 9-trans,11-trans-1,13 diol | 0.53 | 2420 | | |
| DIOLS FROM AUTOXIDISED CHOLESTERYL LINOLEATE | | | | |
| BAND 2 | 0.55 | 2350<br>2420 | 2420<br>2555 | 2475<br>2545 |
| BAND 3 | 0.49 | 2350<br>2390 | 2420<br>2485 | 2480<br>2530 |

TABLE 9. Chromatographic data for reference samples of octadecadienediols and for those derived from cholesteryl linoleate hydroperoxides prepared by autoxidation.

TMS ethers by GLC and mass-spectrometry. GLC on 1% SE-30 at 180°C afforded a single peak with retention index of 2390. The mass spectrum confirmed the molecular weight of 430. The expected cleavages adjacent to the secondary trimethylsilyloxy group yielded intense ions at m/e 173 and m/e 359, as shown below.




With bis perdeuterated TMS ethers: $M^{+\cdot}$ = 448; (a) = 377; (b) = 182

These results indicate that band (2) contains a pair of isomeric 9,11-octadecadiene-1,13-diols. The more mobile peak ($I^{180°C}_{1\%\ SE-30}$ = 2350) was found to have identical retention characteristics (Table 9) and mass spectrum to the corresponding derivative prepared from authentic methyl 13-hydroxy-9-cis,11-trans-octadecadienoate. The material of $I^{180°C}_{1\%\ SE-30}$ = 2420 is regarded as the corresponding trans,trans-isomer, which is an expected component by analogy with previous work. This assignment is supported by gas chromatographic correlations (Table 9). It is thus concluded that the material in band (2) consists of a mixture of the 9-cis,11-trans-and the 9-trans,11-trans-octadecadiene-1,13-diols.

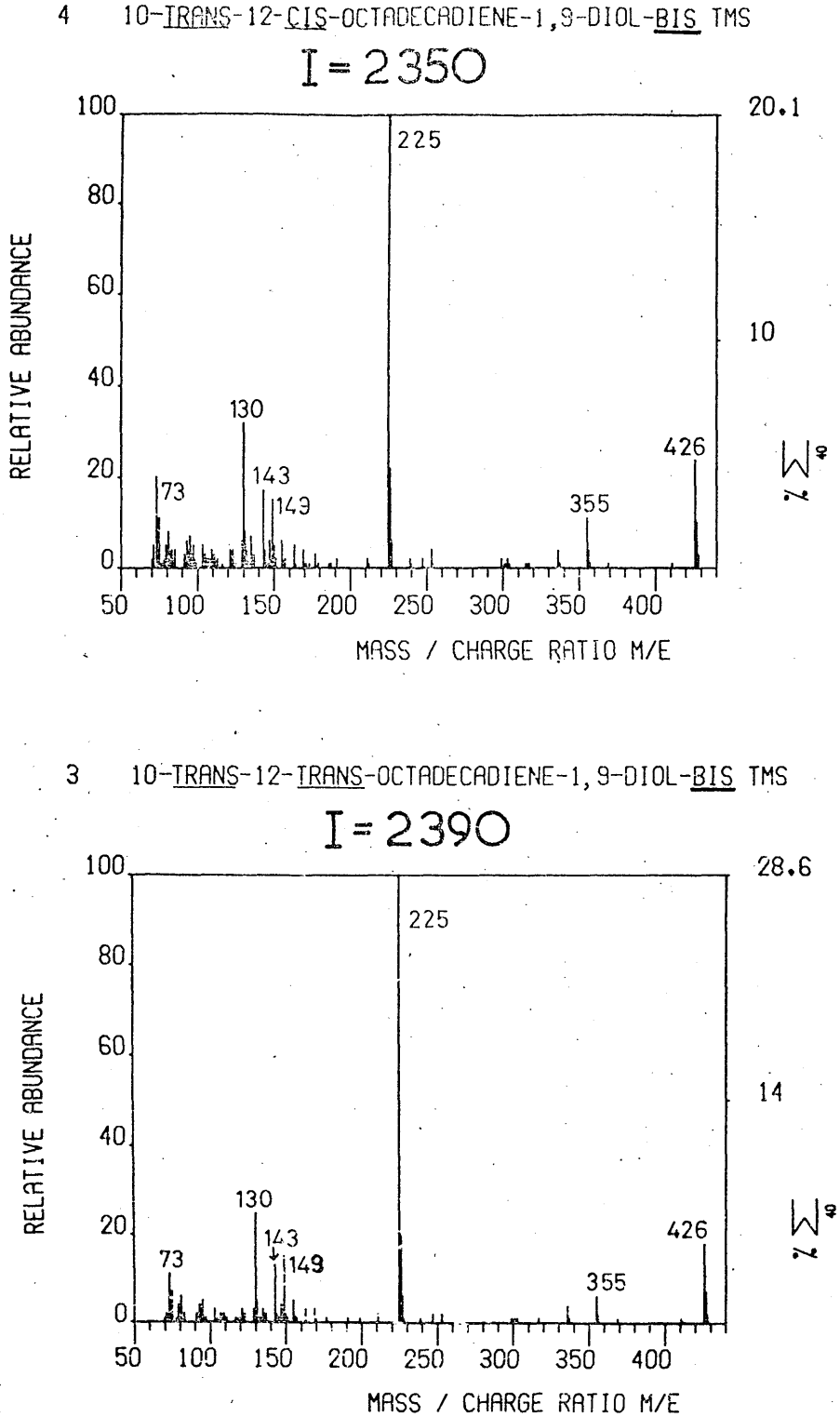
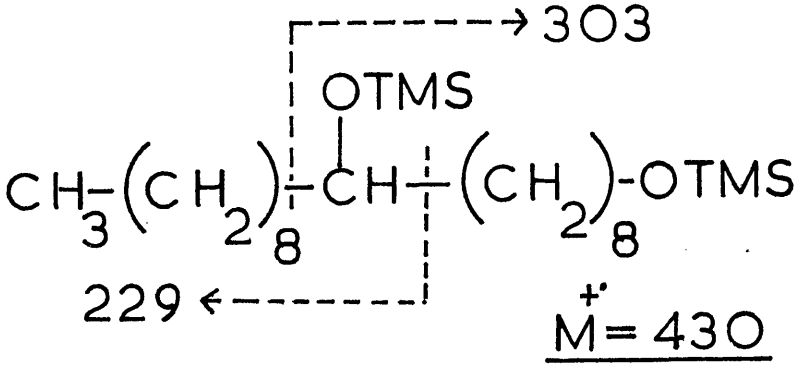



FIG. 10. Mass spectra of the octadecadiene-1,9-diol-bis TMS ethers derived from autoxidised cholesteryl linoleate.

The octadecadiendiols present in band (3) were identified by similar methods. In this case examination of the bis-TMS ethers by GLC (1% SE-30, 180°C) showed two peaks at I = 2350 and I = 2390 (Fig. 6). The mass spectra of both compounds are reproduced in Fig. 10. The spectra, which are essentially identical, show the expected molecular ion at m/e = 426. The base peak at m/e = 225, corresponds to a fragment formed by cleavage of the bond between $C_{(8)}$ and $C_{(9)}$.[215] Ions at m/e 130 and m/e 355 were observed but with much smaller relative abundances than for the 1,13-diol derivatives. The ion at m/e = 355 almost certainly arises by means of the mechanism proposed in Fig. 8. Mass spectral examination of the perdeuterated TMS ethers gave the same results as for material in band (2). Hydrogenation of material from band (3) and subsequent formation of the TMS ethers showed one peak on GLC at $I^{180°C}_{1\%\ SE-30}$ = 2375. Mass spectral analysis of this compound showed the expected increase of the molecular ion by 4 mass units to m/e = 430. The diagnostic ions are in this case observed at m/e = 229 and m/e = 303, and represent $\alpha$-cleavage of the bonds on both sides of the secondary trimethylsilyloxy group as illustrated below:



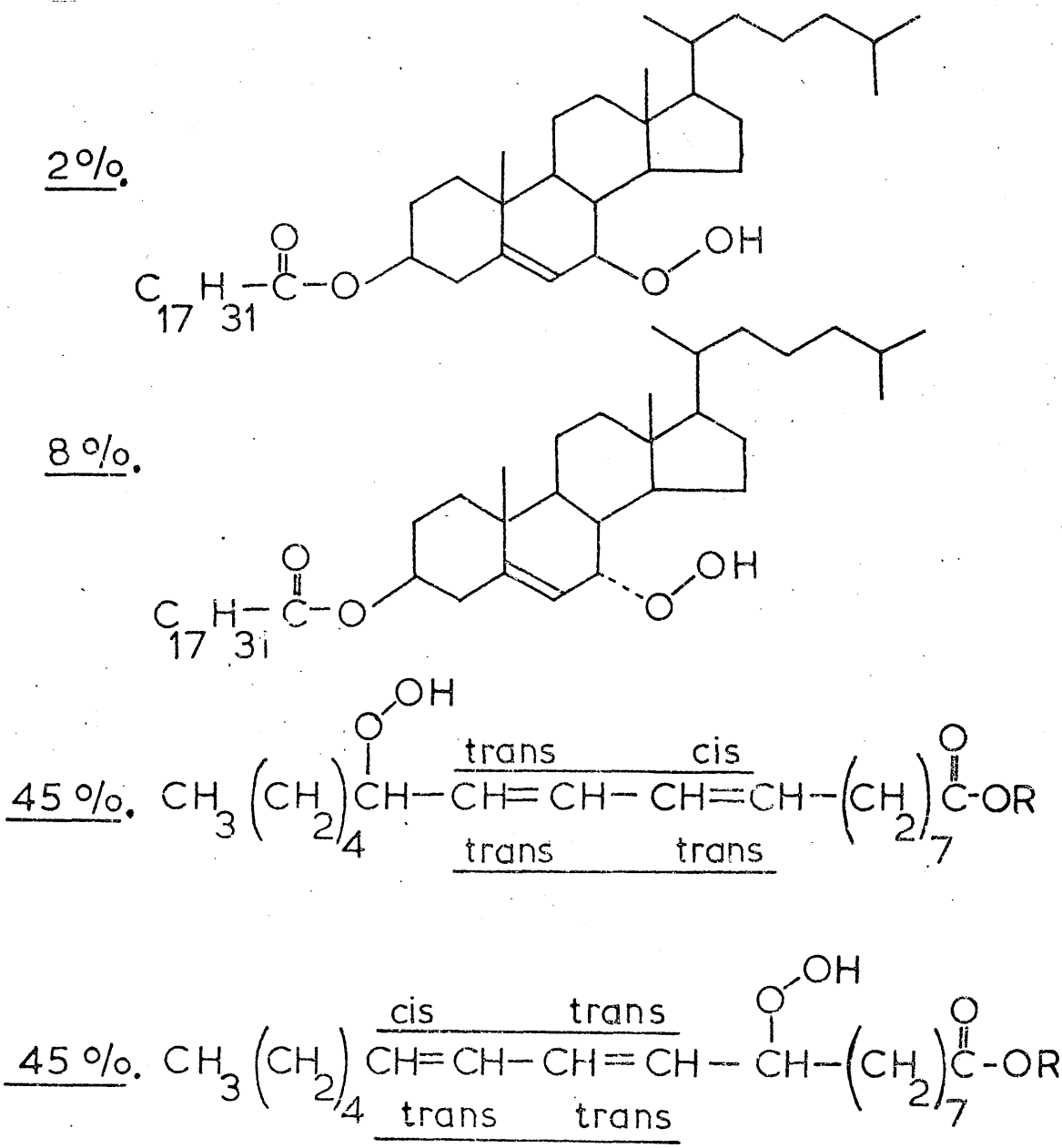



FIG. 11. Nature and relative proportions of the primary hydroperoxides produced by autoxidation of cholesteryl linoleate.

the identities of these ions were again confirmed by formation of the perdeuterated TMS ethers of the octadecanediols and subsequent mass spectral analysis. Comparison of gas chromatographic and mass spectrometric data of the TMS ethers of the octadecadienediols from band (3), with those of reference compounds (Table 9) indicates that the components in band (3) are the cis,trans- and trans,trans-1,9-octadecadienediols: the trans,trans derivative has the longer retention time.

The composition of the mixture of original hydroperoxy-esters is concluded to be as shown in Fig. 11.

The approximate relative quantities were determined by comparison of the peak areas in gas chromatograms. In the case of the octadecadienediols the ratio of cis,trans to trans,trans isomers varies according to the age of the hydroperoxide sample prior to analysis; larger quantities of the all-trans isomers were formed with increasing age. However, the relative quantities of cis,trans to trans,trans isomers in both the 1,9- and 1,13-diols produced by reduction of freshly prepared hydroperoxides were roughly 2:1 in both cases.

### 3.1.3. Preparation and characterisation of the mono hydroperoxides of cholesteryl linoleate produced by methylene blue-sensitised photo oxygenation.

A solution of cholesteryl linoleate (500 mg) in chloroform (15 ml) with methylene blue (2 mg) as sensitiser was placed in a small tapered tube. Oxygen was bubbled into the solution via a delivery tube with its outlet at the bottom of the reaction vessel. The reaction time was 2 hours, and the irradiation was provided by four 60 watt tungsten light bulbs positioned about 2" from the reaction tube. Frequent additions of chloroform were made to compensate for that lost by evaporation. The

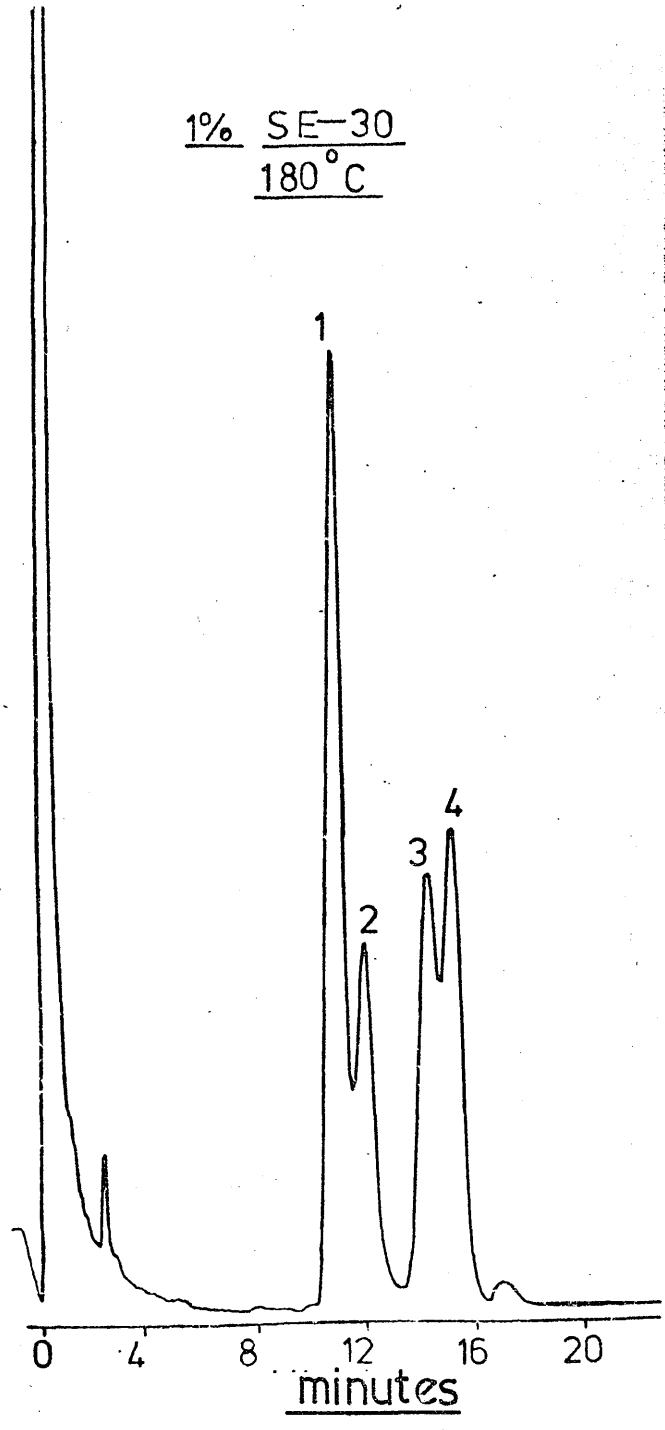



FIG. 12. Gas-liquid chromatogram of the octadecadienediol bis TMS ethers derived from hydroperoxides of cholesteryl linoleate prepared by photo-oxygenation. Peak 1 consists of a mixture of non-conjugated octadecadiene-1,10- and 1,12-diol bis TMS ethers.

reaction mixture was evaporated to dryness and the residue dissolved in a small quantity of hexane. Examination of the solution by TLC (mobile phase: benzene:ethyl acetate, 20:1 v/v) showed two spots at $R_f$ = 0.78 and $R_f$ = 0.54. The lower spot, when sprayed with aqueous potassium iodide, gave the colour characteristic of hydroperoxides. The mixture was chromatographed on 100 g silicic acid. Cholesteryl linoleate was eluted in 15-25% chloroform in hexane. Monohydroperoxides were eluted in 35-40% chloroform in hexane, and smaller quantities of more polar compounds, also present in the mixture, were discarded. The quantity of cholesteryl linoleate recovered was 215 mg. The mono-hydroperoxides were further purified by preparative TLC (benzene:ethyl acetate, 20:1 v/v), and the yield was 197 mg.

The purified hydroperoxides were characterised by reductive cleavage with lithium aluminium hydride as previously described, and the products were examined by TLC using a mobile phase of chloroform:ethyl acetate (3:1 v/v). The resultant chromatogram was almost identical to that produced by the corresponding products derived from autoxidised cholesteryl linoleate. Bands were observed at $R_f$ = 0.65 (cholesterol), $R_f$ 0.45-0.58 (octadecadienediols), and 0.20 (epimeric 7-hydroxycholesterols). Material from the central band was purified by preparative TLC, and a small quantity was trimethylsilylated and examined by GLC (1% SE-30, 180°C). The chromatogram (reproduced in Fig. 12), showed four peaks, and retention indices are given below:

| | |
|---|---|
| Peak 1 | 2330 |
| Peak 2 | 2350 |
| Peak 3 | 2395 |
| Peak 4 | 2415 |

Mass spectrometric analysis of the fraction showed Peak 2 to

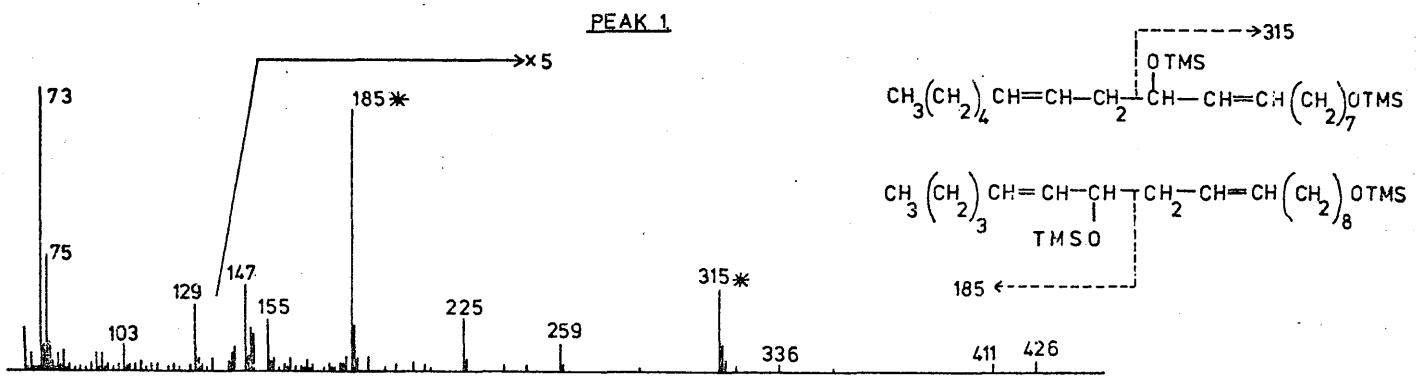



FIG. 13. Mass spectra of a mixture of octadecadiene-1,10- and 1,12-diol-bis TMS ethers derived from photo-oxygenated cholesteryl linoleate. The mixture (corresponding to peak 1 in Fig. 12) had $I^{1\%\ SE\text{-}30}_{180^{o}} = 2330$. With the exception of those indicated by an asterisk, all ions of m/e greater than 135 are represented at 5 X their true relative intensities.

consist of a mixture of a 9,11-octadecadiene-1,13 diol bis-TMS ether and a 10,12-octadecadiene-1,9 diol bis-TMS ether. GLC correlation indicated that these compounds were cis,trans isomers (Table 9). Similarly Peaks 3 and 4 were identified by GLC and GC-MS as being respectively 10-trans,12-trans-octadecadiene-1,9 diol, and 9-trans,11-trans-octadecadiene-1,13 diol bis-TMS ethers. These four isomers have been identified in the corresponding TLC fraction from reduction products of the cholesteryl linoleate hydroperoxides prepared by autoxidation. The major peak (Peak 1) in the chromatogram (Fig. 12) had not, however, been previously observed. Re-examination of the free octadecadienediol fraction on TLC revealed two bands at $R_f$ = 0.48 and 0.55 (mobile phase: chloroform:ethyl acetate, 3:1 v/v). The upper portion of the more mobile band ($R_f$ 0.57-0.60) was removed and a small quantity was trimethylsilylated and examined by GLC (1% SE-30, 180°C). At least 95% of the material chromatographed was eluted with a retention index of 2330, thus corresponding to Peak 1 in Fig. 12. Mass spectral analysis of Peak 1 (Fig. 13) revealed a mixture of two previously unencountered diols.

The mass spectrum is consistent with that expected for a mixture of an octadecadiene-1,10 diol-bis-TMS ether, and an octadecadiene-1,12-diol bis-TMS ether. The molecular is, as expected, at m/e = 426, but is of much lower relative intensity than that found in spectra of the conjugated octadecadiene-1,9- and 1,13-diols. The major ions at m/e = 185 and m/e = 315 represent cleavage adjacent to the secondary trimethylsilyloxy group as shown in Fig. 13. The lack of another ion representing $\alpha$-cleavage at the other side of this trimethylsilyloxy function suggests the presence of an olefinic bond adjacent to it.

| Reference hydroxy stearic acid methyl esters, TMS ethers and octadecanediol bis TMS ethers. | Retention Index | |
|---|---|---|
| | % SE-30 180°C | 10% PEGA 180°C |
| Methyl-9-trimethylsilyloxy stearate | 2325 | 2625 |
| Methyl-10-trimethylsilyloxy stearate | 2325 | 2625 |
| Methyl-12-trimethylsilyloxy stearate | 2335 | 2635 |
| Methyl-13-trimethylsilyloxy stearate | 2335 | 2640 |
| Octadecane-1,9-diol bis TMS | 2375 | 2425 |
| Octadecane-1,10-diol bis TMS | 2375 | 2420 |
| Octadecane-1,12-diol bis TMS | 2390 | 2425 |
| Octadecane-1,13-diol bis TMS | 2390 | 2420 |
| Corresponding derivatives from photo-oxygenated cholesteryl linoleate | | |
| Hydroxy acid methyl esters TMS ethers | 2325<br>2340 | 2625<br>2635 |
| Octadecanediol bis TMS ethers | 2380<br>2395 | 2420 |

TABLE 10. Comparison of the gas chromatographic retention data of reference saturated hydroxy acid ester TMS ethers and diol bis TMS ethers with those of the corresponding derivatives from photo-oxygenated cholesteryl linoleate.

(This phenomenon has been observed in the mass spectra of hydroxyoctadecenoic acid methyl ester TMS ethers).[206] It is also noteworthy that the mass spectrum contains no ion at m/e = 130, but that a prominent fragment exists at m/e = 129. The significance of this observation will be considered later.

The implication (drawn from mass spectral data) that material in Peak 1 consists of non-conjugated octadecadienediols was supported by the absence of significant absorption at 233 nm. The proposed structures for the non-conjugated diols were substantiated by formation of perdeuterated TMS ethers and examination of mass spectra of the derivatives. In this case the molecular ion increased by 18 mass units from m/e = 426 to m/e = 444. The diagnostic ions at m/e = 185 (1,12-diol), and m/e = 315 (1,10-diol) were found to increase to m/e = 194 and m/e = 333 indicating the presence in the fragments of one and two trimethylsilyloxy groups respectively. The positions of the hydroperoxy groups in esters prepared from photo-oxygenated cholesteryl linoleate were verified in the following way:

A sample of purified (TLC) hydroperoxy-esters was reduced in sodium borohydride/methanol solution. Alkaline hydrolysis yielded a hydroxy-acid fraction which was methylated with diazomethane, and hydrogenated as described on Page 56. A portion of the saturated hydroxyoctadecanoates was reduced with lithium aluminium hydride and both hydroxy-acid esters and octadecanediols were trimethylsilylated and examined by GLC and GC-MS. GLC retention indices of these derivatives are shown in Table 10. Two peaks were observed in the chromatogram of the octadecanediol bis-TMS ethers (1% SE-30, 180°C). The more mobile (I = 2380) had a mass spectrum consistent with that of a mixture of an octadecane-1,9-diol bis-TMS ether and an

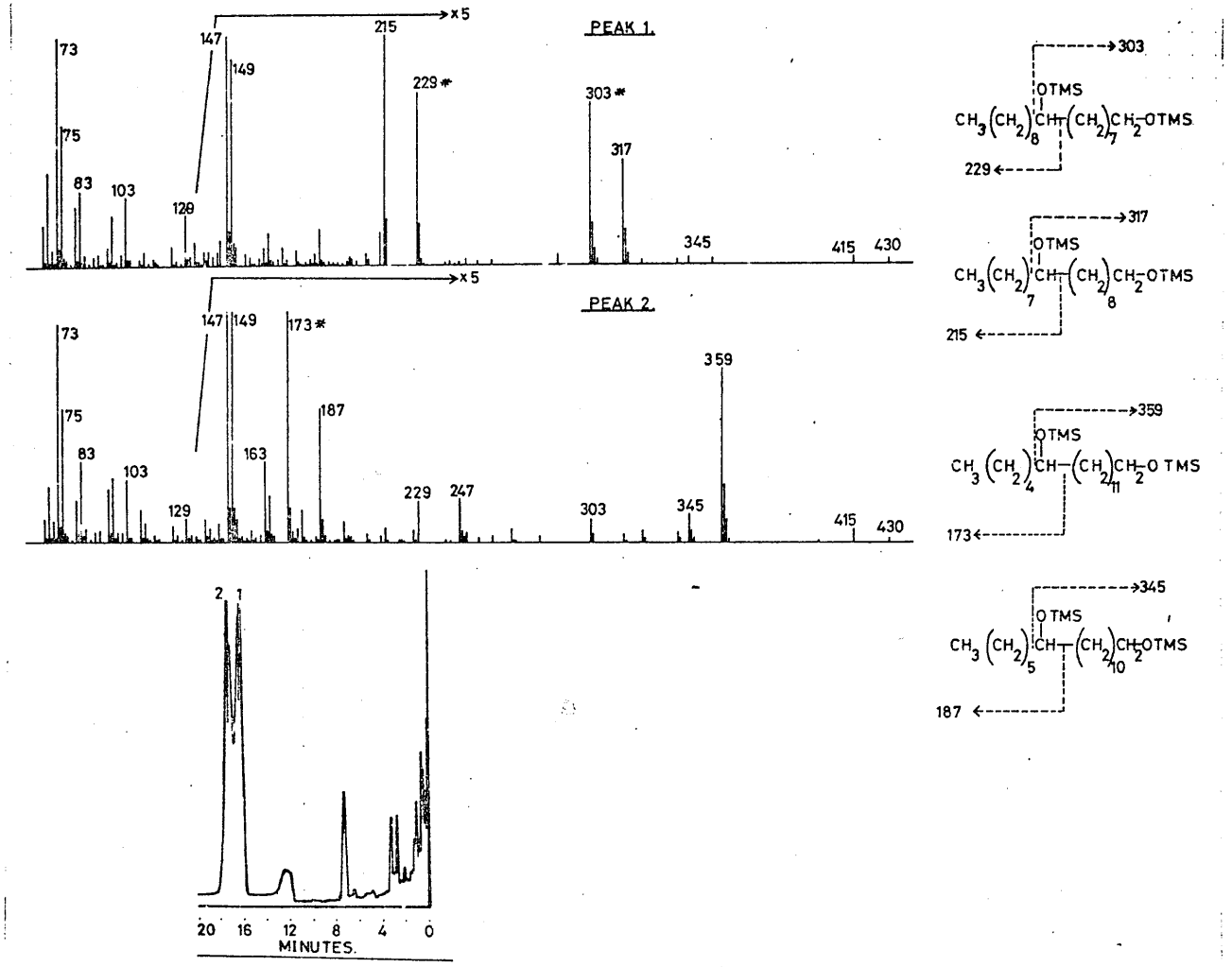



FIG. 14. Total ion current chromatogram, mass spectra and principal fragmentations of octadecanediol-bis TMS ethers derived from photo-oxygenated cholesteryl linoleate. With the exception of those indicated by an asterisk, all ions of m/e greater than 135 are represented at 5 X their true relative intensities. GLC conditions 1% OV-1, 200°C, LKB 9000, He flow rate 30 ml/min.

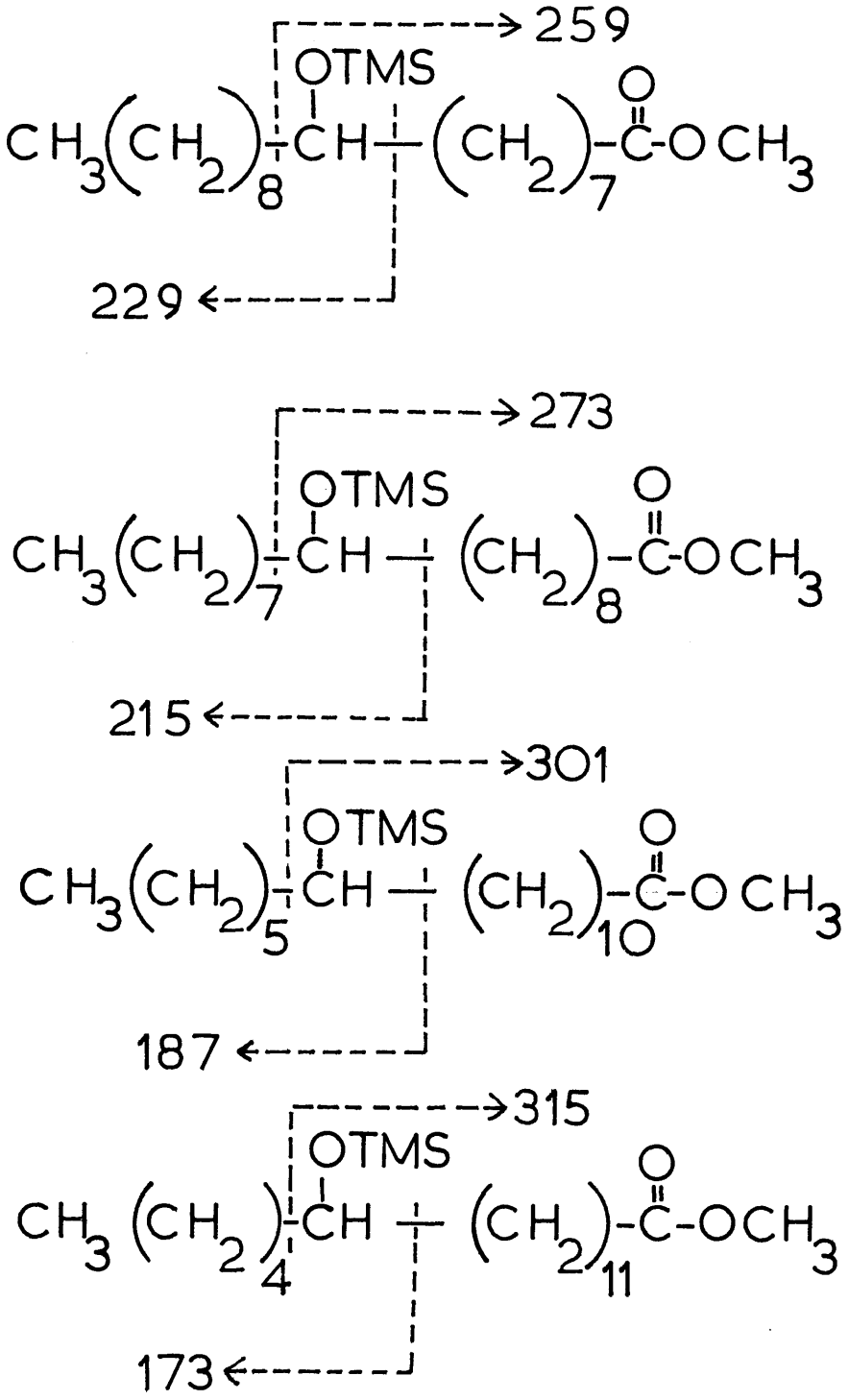
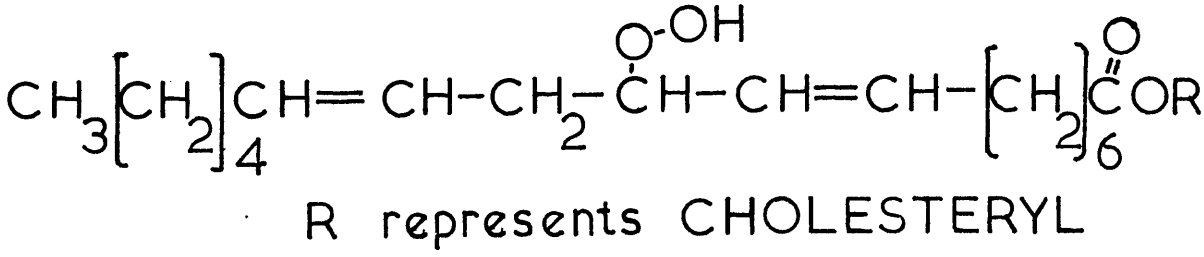



FIG. 15. Principal fragmentations giving rise to diagnostic ions in the mass spectra of hydroxy methyl stearate TMS ethers derived from photo-oxygenated cholesteryl linoleate.

octadecane-1,10-diol bis-TMS ether. The major ions can be explained by α-cleavage at both sides of the secondary trimethylsilyloxy function. The mass spectra of these compounds, with total ion current trace are shown in Fig.14. Similarly, the constituents of the second peak in the gas chromatogram (I = 2395) are identified as a mixture of an octadecane-1,12- and an octadecane-1,13-diol bis-TMS ether. Examination of the TMS ethers of the methyl hydroxyoctadecanoates by GLC (1% SE-30, 180°C) revealed two peaks (Table 10). Mass spectrometry of these compounds indicated the presence of four isomeric trimethylsilyloxy acid methyl esters. Peak 1 (I = 2325) showed ions at m/e = 386 ($M^{+\bullet}$), m/e = 229, and m/e = 259, which indicate the presence of the 9-trimethylsilyloxy derivative, and at m/e = 215 and 273 characteristic of the 10-trimethylsilyloxy isomer. All the ions in the mass spectrum of peak 1 were also present in a synthetic mixture of the 9- and 10-trimethylsilyloxy stearate derived from methyl oleate.[214]

Peak 2 (I = 2340) gave a mass spectrum showing diagnostic ions at m/e = 386 ($M^{+\bullet}$), m/e = 173 and 315 (13-trimethylsilyloxy isomer), and at m/e = 187 and 301 (12-trimethylsilyloxy isomer). The bond cleavages producing the major ions in the four isomeric trimethylsilyloxy stearates are shown in Fig. 15. The results indicate that in contrast to the air or oxygen autoxidation of cholesteryl linoleate, the products of photosensitised oxidation contain considerable quantities of non-conjugated hydroperoxy-esters of the type shown below:



The products are very similar to those found to be formed by chlorophyll-photosensitised oxygenation of methyl linoleate.[259,260]

Although a study was not made of the reaction kinetics, the quantities of non-conjugated hydroperoxides appeared to increase as the reaction time was prolonged. Since the non-conjugated octadecadienediol bis-TMS ethers are readily separated from the conjugated isomers by GLC (Fig. 12), comparison of the peak areas probably gives a good guide to the relative quantities of conjugated and non-conjugated products in the reaction mixtures. Making this assumption, the proportion of non-conjugated esters in the reaction mixture increases from approximately 33% after a reaction time of 2 hours to about 48% in 4 hours. The significance of this observation and mechanistic evidence supporting the structures postulated above will be considered later.

### 3.1.4. Preparation and characterisation of keto-esters of cholesterol.

It was observed that during purification of hydroperoxides of cholesteryl linoleate by preparative TLC on silica gel, small quantities of material were formed with a characteristic absorption in the ultraviolet spectrum at 272 nm. Material with a similar absorption has also been observed by Bergström[251] and Christophersen[262] during their respective studies on methyl linoleate hydroperoxides, and the absorption has been assigned to small quantities of a doubly conjugated ketone, presumably formed by dehydration of the hydroperoxides. During our work on polar esters of human atheroma similar absorptions have frequently been observed in fractions containing hydroperoxy-esters of cholesterol, and in order to gain information about these keto-esters, and to devise practical methods for their

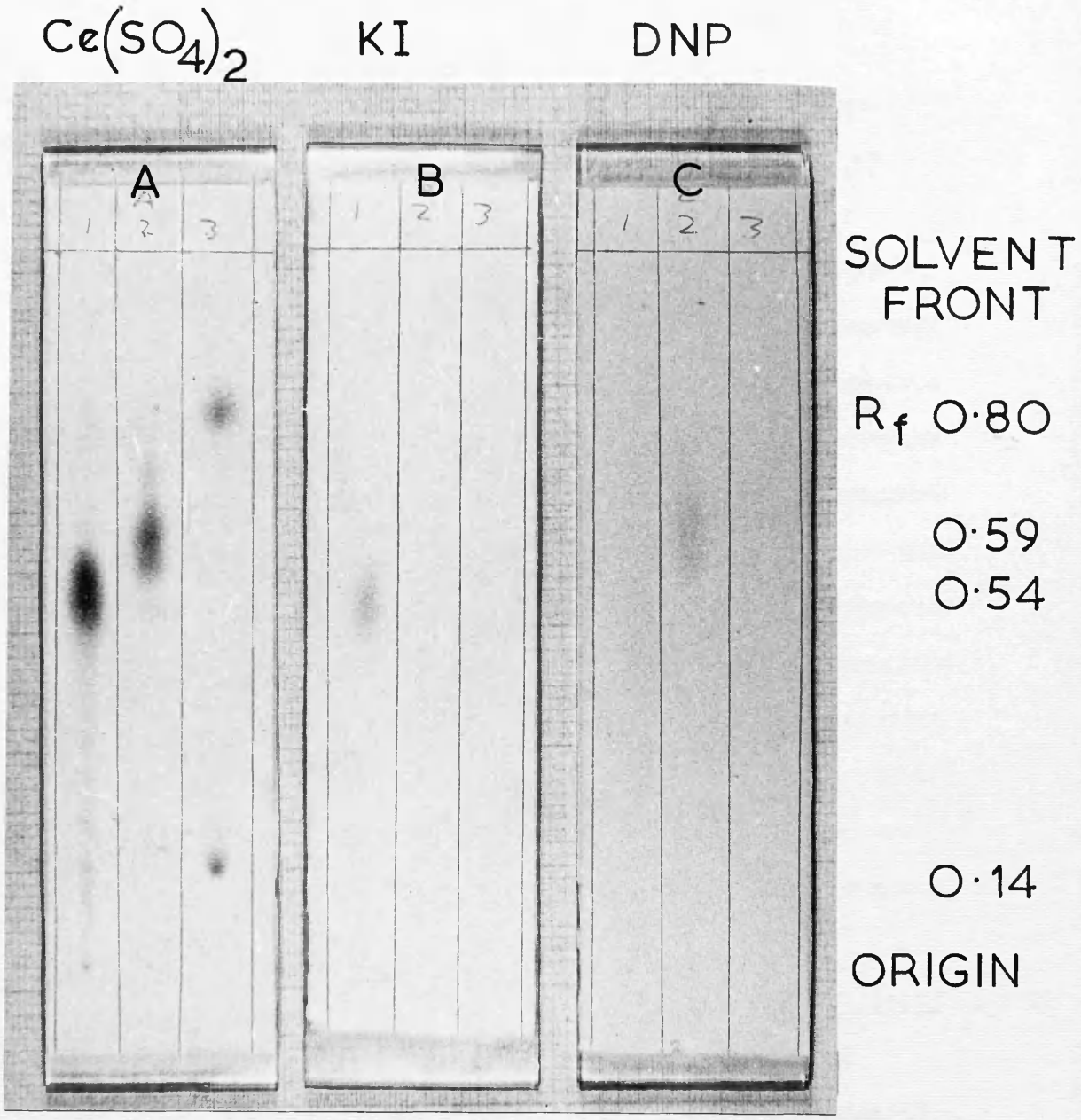



FIG. 16. Thin-layer chromatograms illustrating the use of selective spray reagents to detect hydroperoxy- and keto-esters. Mobile phase: benzene:ethyl acetate (20:1 v/v). For full explanation see text.

separation from hydroperoxides, an authentic sample of keto esters was prepared.

50 mg of freshly prepared and purified hydroperoxides (from autoxidised cholesteryl linoleate) were heated in a sealed vial at 150°C for 20 min in the presence of dry pyridine (200 $\mu$l) and triethylamine (50 $\mu$l). Solvent was blown off in a stream of nitrogen and the residue was dissolved in diethyl ether (200 $\mu$l), and subjected to preparative TLC using a mobile phase of benzene:ethyl acetate (20:1 v/v). The major band at $R_f$ = 0.57-0.64 was eluted from the silica and examined by analytical TLC using different reagents for visualisation of the spots. Fig. 16 illustrates three chromatograms (A, B and C). To lane 1 of each chromatogram a sample of purified cholesteryl linoleate hydroperoxide has been applied; to lane 2 a sample of the derived ketonic material, and to lane 3 a mixture of cholesterol and cholesteryl linoleate. After development in benzene:ethyl acetate (20:1 v/v), each plate was sprayed with a different reagent. Place A was sprayed with ceric sulphate. Plate B was sprayed with potassium iodide solution, and plate C was sprayed with 2,4-dinitrophenylhydrazine reagent. Fig. 16 shows that plate A, when sprayed with the non-selective ceric sulphate solution reveals a spot in lane 1 at $R_f$ = 0.54, corresponding to hydroperoxy-esters. Lane 2 contains a spot at $R_f$ = 0.59 (keto-esters), and lane 3 contains two spots at $R_f$ = 0.80 and 0.14 corresponding respectively to cholesteryl linoleate, and cholesterol. Plate B (sprayed with potassium iodide solution) shows only a spot in lane 1 at $R_f$ = 0.54, and plate C (sprayed with dinitrophenylhydrazine reagent) shows a spot at $R_f$ = 0.59, in lane 2.

Thus these chromatograms indicate that hydroperoxy-and

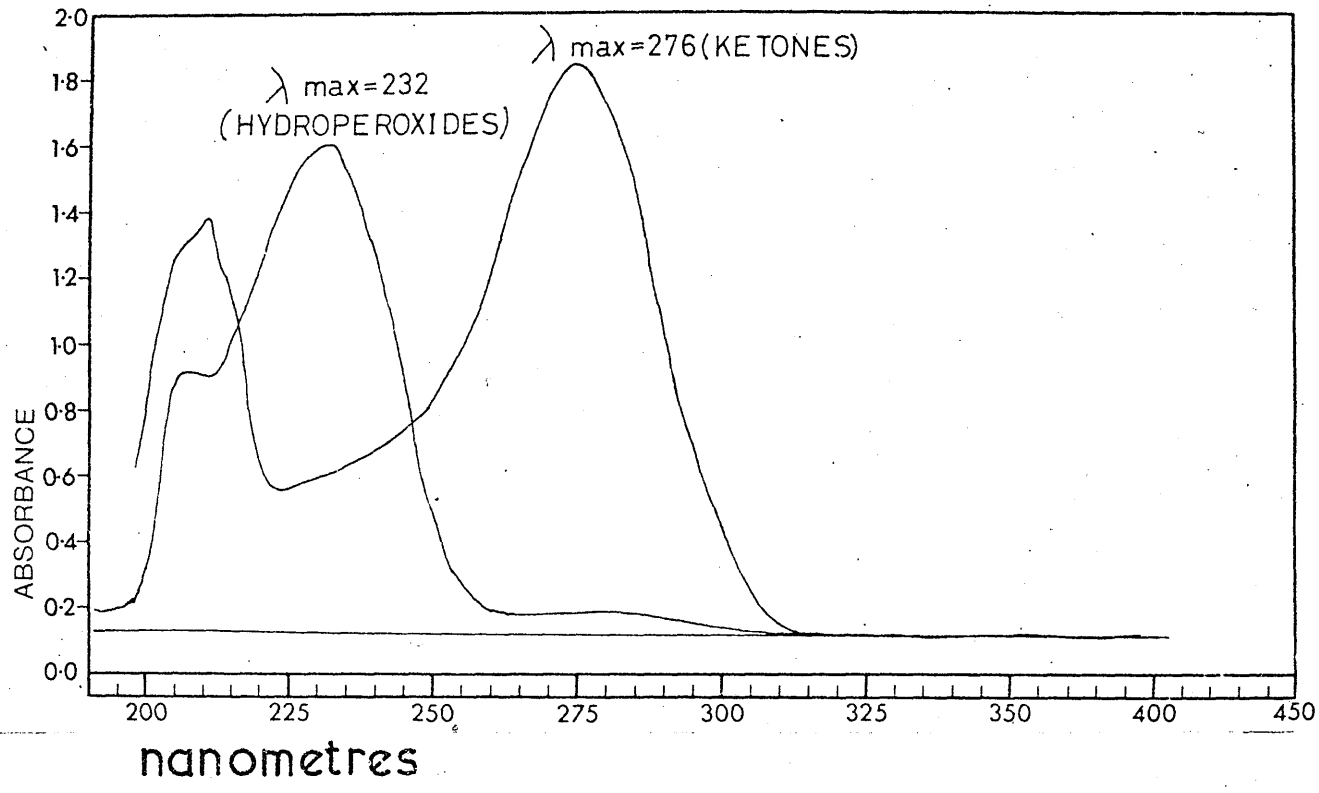



FIG. 17. Comparison of the ultraviolet spectra of hydroperoxy- and keto-esters derived from autoxidised cholesteryl linoleate. Solvent: ethanol.

keto-esters can be distinguished during TLC analysis by spraying with different test reagents. Such a distinction is valuable in view of the very similar mobilities of the two types of compound on TLC.

Ultraviolet spectroscopy of the derived ketones showed an absorption of $\lambda_{max}^{EtOH} = 276$, $\varepsilon_{max}^{EtOH} = 28,000$. Fig. 17 compares the U.V. spectra of purified samples of hydroperoxy-esters and keto-esters, and demonstrates another method of distinction between these compounds.

The structures of the derived keto-esters of cholesterol were determined by reduction with ethereal lithium aluminium deuteride ($LiAlD_4$), and examination of the TMS ethers of the reduction products by GLC and GC-MS. Reduction of hydroperoxy-esters with this reagent produces four isomeric octadecadienediols which are labelled with a pair of deuterium atoms attached to the carbon atom $C_{(1)}$ containing the primary hydroxyl function. The mass spectra of the derived trimethylsilyl ethers of these compounds show a molecular ion at $m/e = 428$, and major ions at $m/e = 225$ (indicative of 1,9-diols), and $m/e = 357$, characteristic of the 1,13-diols.

In contrast, the octadecadienediols derived from keto-esters of cholesterol by treatment with $LiAlD_4$, contain three deuterium atoms. Two of these are at $C_{(1)}$. The third deuterium atom, however, is inserted at that carbon atom attached to the secondary hydroxyl fraction (i.e. either $C_{(9)}$ or $C_{(13)}$). As a result the mass spectra of the TMS ethers of the diols produced from the different substrates are characteristically dissimilar.

The octadecadiene-1,9- and 1,13-diols from the reduced keto-esters were separately isolated by preparative TLC as

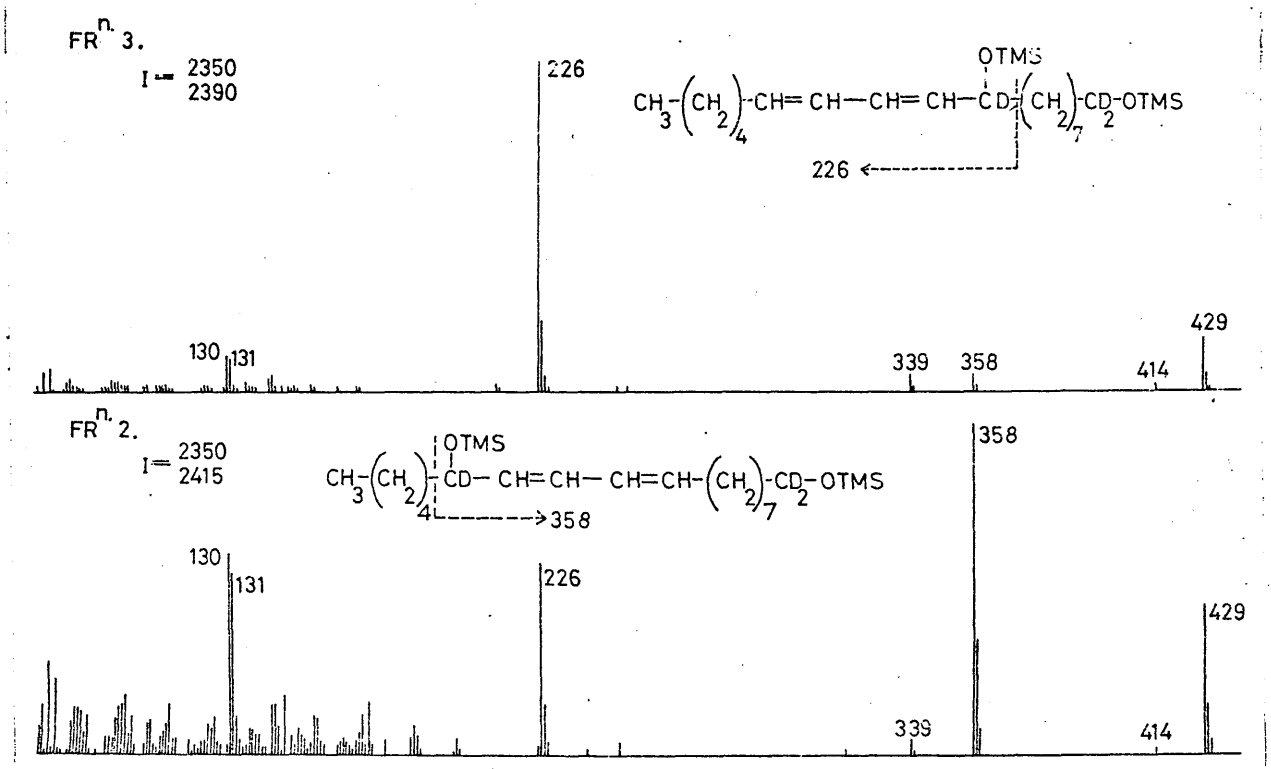



FIG. 18. Mass spectra of the TMS ethers of octadecadienediols derived from keto-esters of cholesterol by reduction with $LiAlD_4$. TLC $F_R$"3 :- mass spectrum obtained from both the 10-trans,12-cis and 10-trans,12-trans-octadecadiene-1,9 diol-bis TMS ethers. TLC $F_R$"2 :- mass spectra obtained from both the 9-cis,11-trans- and the 9-trans,11-trans-octadecadiene-1,13 diol-bis TMS ethers.

previously described (Page 63). After trimethylsilylation the products were examined by GLC (1% SE-30, 180°C). The fraction containing the 1,13-diol bis-TMS ethers contained two peaks with retention indices of 2350 and 2415. These corresponded respectively to the cis,trans-, and trans,trans-octadecadienediol derivatives and had identical mass spectra. Similarly, the fraction containing the 1,9-diol bis-TMS ethers showed two peaks at I = 2350 (trans,cis isomer) and at I = 2390 (all-trans isomer), with identical mass spectra. The proportions of the cis,trans to trans,trans isomers were in both cases much less ($\sim$1:8, as estimated by comparison of peak areas) than those encountered in the octadecadienediol mixtures from reduction of hydroperoxy-esters (cf Fig. 6). This is likely due to isomerisation of the cis,trans forms to the more favoured all-trans compounds, during the thermally induced conversion of hydroperoxy-esters to keto-esters.

The mass spectra of the TMS ethers of the major constituents (all-trans) from fractions containing the 1,9- and 1,13-octadecadienediols are reproduced in Fig. 18.

The incorporation of three deuterium atoms into the molecules is indicated by the molecular ion at m/e = 429. The fragmentations are identical to those already observed in the products of both $LiAlH_4$ and $LiAlD_4$ reduction of hydroperoxy-esters. However, the masses of the ions are different. Thus in the present case diagnostic ions are observed at m/e = 226 and m/e = 358 which are respectively characteristic of the 1,9- and 1,13- octadecadienediol bis-TMS ethers. Each of these ions ($M^{+\bullet}$ included) is, therefore, one mass unit greater than those observed in the corresponding derivatives from $LiAlD_4$ reduction of hydroperoxy-esters, and the keto- and hydroperoxy-esters can

be differentiated by mass spectral analysis of the deuterium labelled derivatives.

It has been demonstrated that hydroperoxy- and keto-esters of cholesterol may be distinguished by their spectral, mass spectral and thin-layer chromatographic properties. It seemed desirable, however, that mixtures of the two ester classes, such as those which occur in some arterial extracts, should be resolved, and the separated classes individually characterised. Both types of ester have very similar $R_f$ values on TLC, and attention was given to chemical modification of one or other type, with the aim of separation of the modified species from the unreacted ester by preparative TLC.

The keto-esters were found to react with hydroxylamine hydrochloride in pyridine to produce oximes. The oximes, due to their increased polarity on TLC, can be separated from the hydroperoxides, but the reaction was found to require heating at 70°C to enable complete conversion of ketones to oximes, and when a mixture of hydroperoxy- and keto-esters were subjected to the required conditions, the bulk of the hydroperoxy-esters were converted to ketones and thence to oximes. This method was therefore obviously unsuitable.

### 3.1.5. Selective reduction of hydroperoxy-esters in the presence of keto-esters by reaction with stannous chloride.

Sodium borohydride in methanol reduces both hydroperoxy- and keto-esters to the corresponding alcohols. Hydroperoxides of methyl oleate have, however, been reduced to the hydroxy compounds by use of acidic stannous chloride solution.[207] It appeared likely that hydroperoxides of cholesteryl linoleate should react in a similar fashion, whereas the keto-esters should remain unaltered.

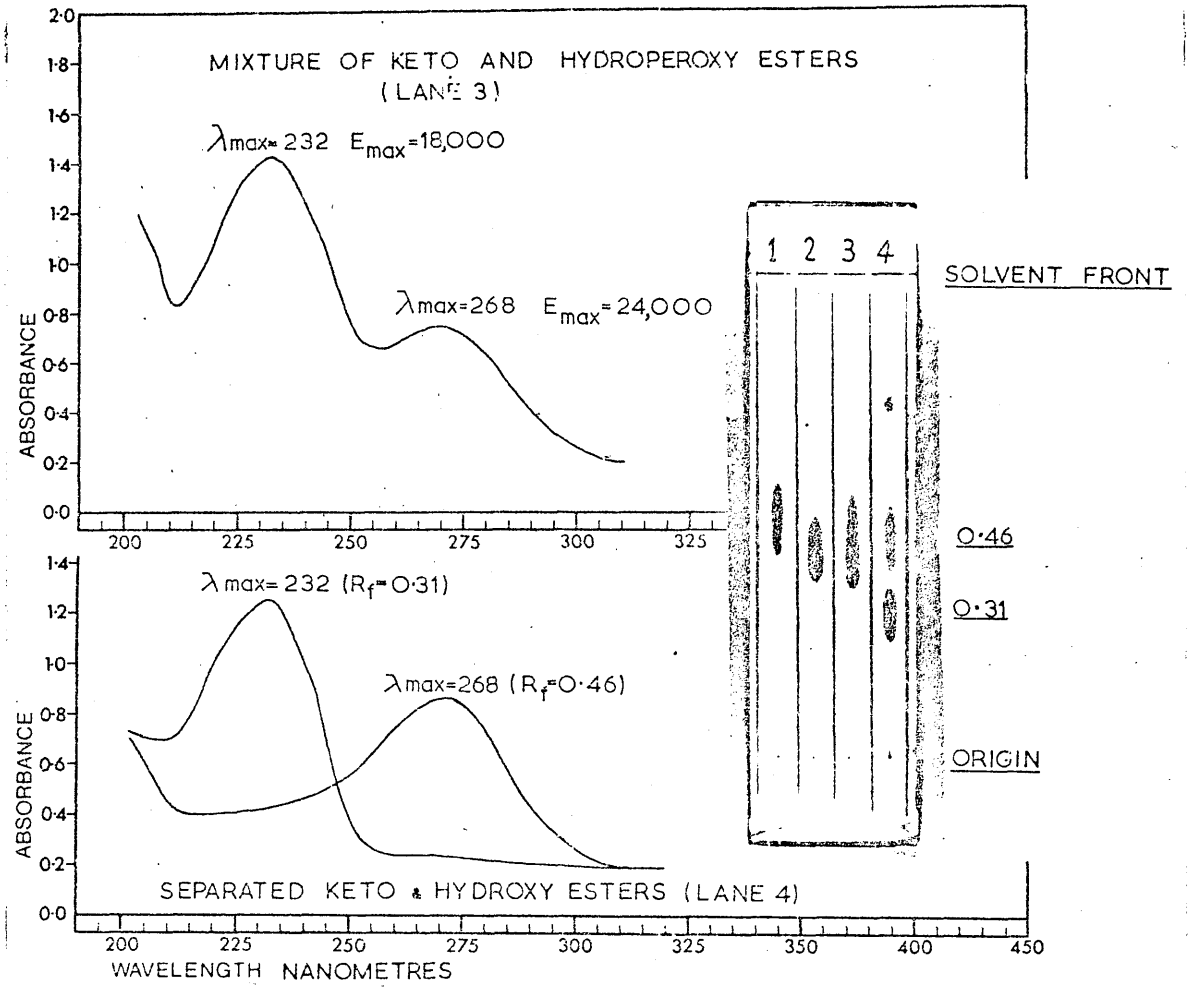



FIG. 19. Thin-layer chromatogram and ultraviolet spectra illustrating the selective reduction of hydroperoxides in the presence of keto-esters. Mobile phase: benzene:ethyl acetate (20:1 v/v). Lane 1: keto-esters, Lane 2: hydroperoxy-esters, Lane 3: mixture of both keto- and hydroperoxy-esters, Lane 4: mixture after treatment with acidic stannous chloride solution. Ultraviolet spectra (solvent: hexane) are of the mixture of keto- and hydroperoxy-esters, and of the keto- and derived hydroxy-esters after reduction and separation by preparative TLC.

To 15 ml of a solution of 0.1 N stannous chloride in hydrochloric acid, prepared according to Barnard and Hargrave[266] was added a mixture of purified hydroperoxy-esters (7.4 mg) and keto-esters (5.5 mg) in benzene (0.20 ml). Acetone (10 ml) was added to render the mixture homogeneous. The solution was stirred for 2 hours at room temperature, and the products were extracted into 3 x 10 ml portions of diethyl ether. The ethereal solution was washed with 60 ml water, dried, and the products examined by TLC. The chromatogram is shown in Fig. 19. Lanes 1 and 2 respectively contain reference samples of keto- and hydroperoxy-esters. Lane 3 contains a sample of the mixture prior to reduction, and Lane 4 shows the reduction products. The mixture of esters on the developed chromatograms were sprayed with 2,4-dinitrophenylhydrazine and potassium iodide spray reagents. Material in Lane 3 (prior to reduction) gave a positive result with both reagents. The mixture of esters in Lane 4, however, gave no reaction with potassium iodide, but the upper spots at $R_f = 0.46$ gave a positive result with 2,4-dinitrophenylhydrazine solution. The mobility of the lower band in Lane 4 was identical to that of a mixture of hydroxy-esters of cholesterol. The keto- and hydroxy-esters were separated by preparative TLC (mobile phase: benzene:ethyl acetate, 20:1 v/v). The quantities recovered were respectively 5.3 mg and 5.8 mg. The almost quantitative conversion of the hydroperoxides and retention of the ketones were confirmed by ultraviolet spectrometry. The reduction was repeated using 200 $\mu$g of a mixture of keto- and hydroperoxy-esters, and the same efficiency of the selective reduction on the smaller scale was confirmed. This method should be applicable to the characterisation of hydroperoxy- and keto-esters present in aortal tissue extracts.

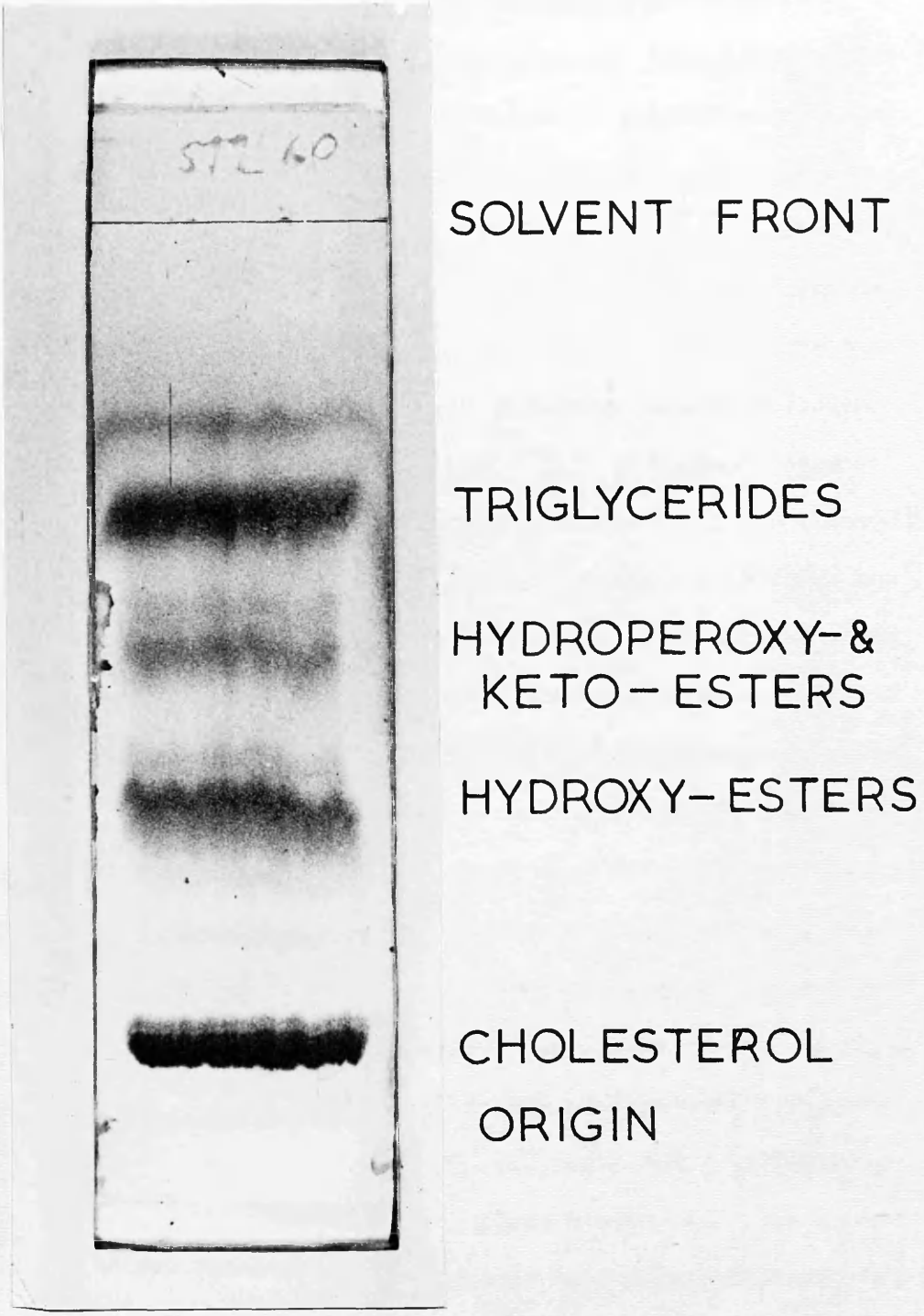



FIG. 20. Analytical thin-layer chromatogram of the fractions of an arterial lipid extract containing polar sterol esters. Mobile phase: benzene:ethyl acetate (20:1 v/v).

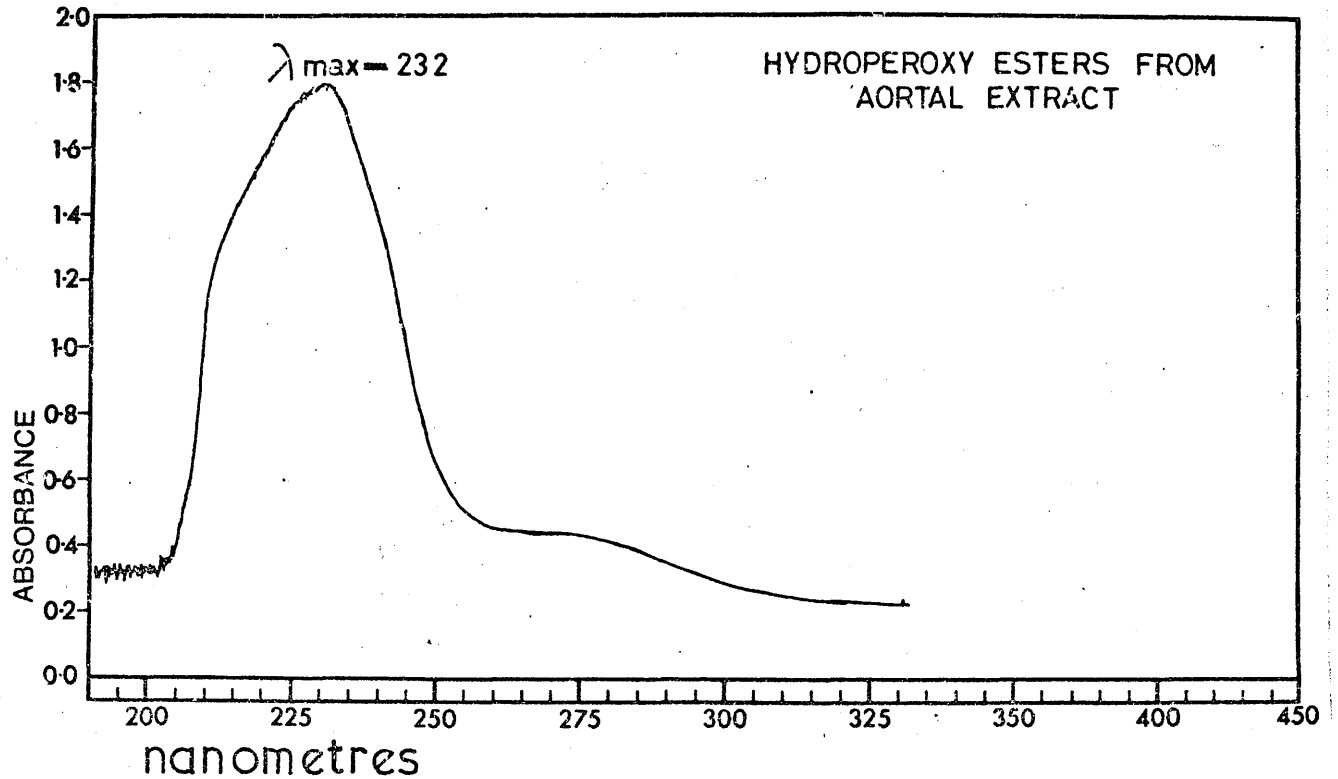



FIG. 21. Ultraviolet spectrum (solvent: ethanol) of hydroperoxy-esters of cholesterol isolated from a severely diseased aorta.

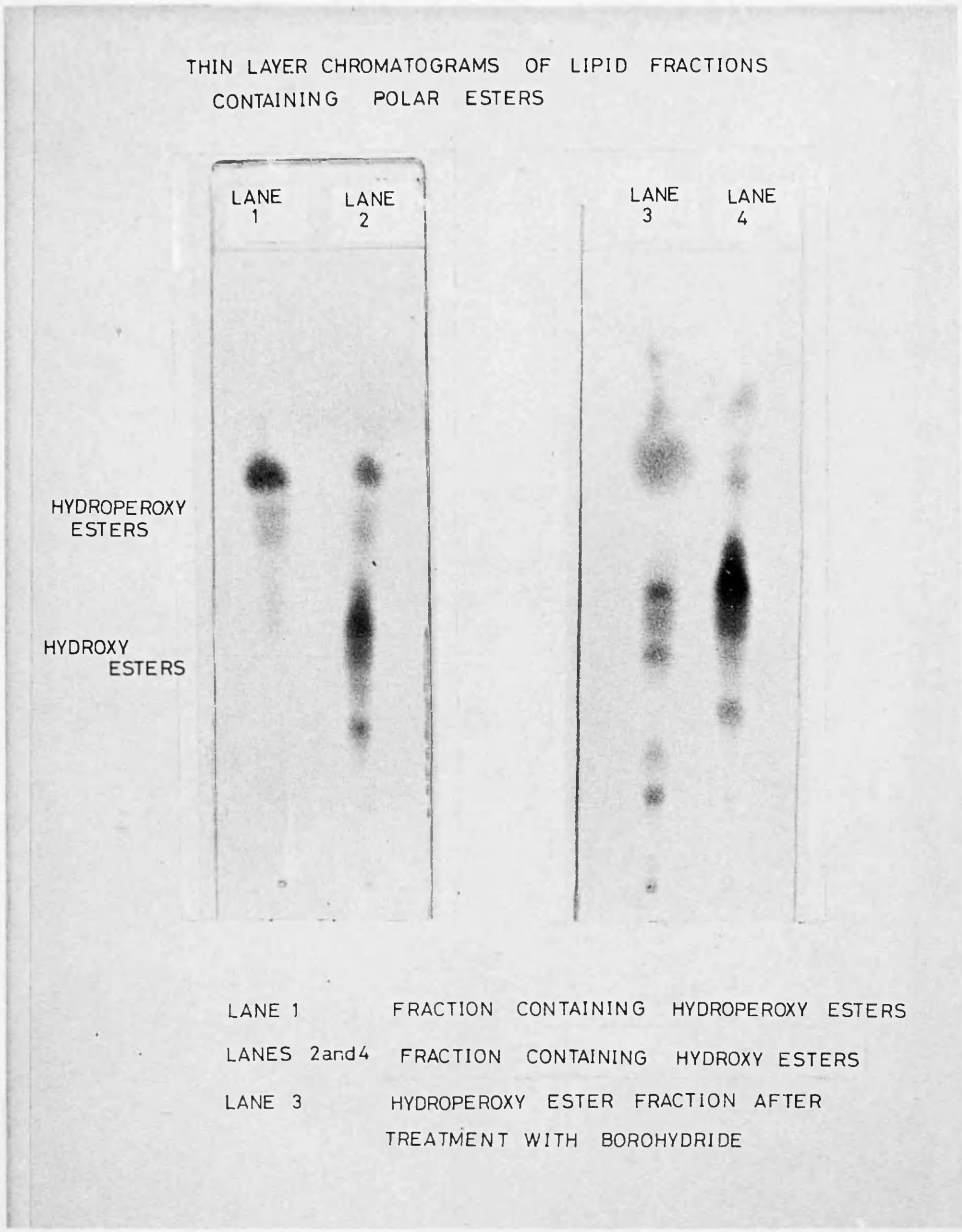



FIG. 22. Thin-layer chromatograms of fractions containing hydroxy- and hydroperoxy-esters isolated from a severely diseased aorta. The reduction of the hydroperoxides to the hydroxy-esters with sodium borohydride is also illustrated. Mobile phase: benzene:ethyl acetate (20:1 v/v).

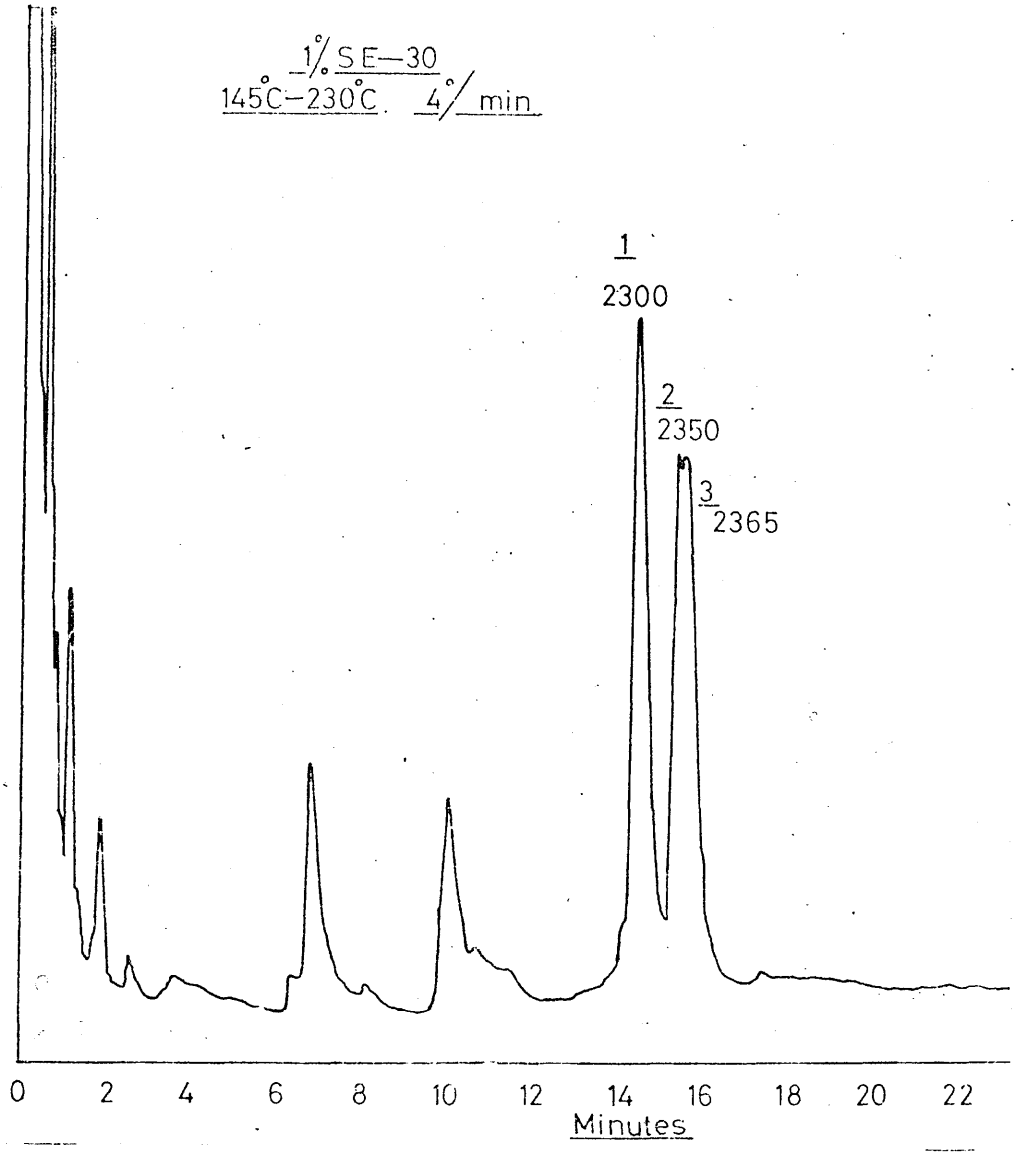



FIG. 23. Gas-liquid chromatogram of hydroxy acid methyl esters derived from aortal hydroperoxides. Peak 1 corresponds to a mixture of the 9-hydroxy-10-trans,12-cis- and 13-hydroxy-9-cis,11-trans-octadecadienoic acid derivatives. Peaks 2 and 3 (very slightly resolved) are the all-trans 9- and 13-hydroxy-acid derivatives respectively.

## 3.2. THE OCCURRENCE OF HYDROPEROXY- AND KETO-ESTERS IN ADVANCED AORTAL LESIONS

### 3.2.1. Identification of Aortal Hydroperoxy-esters.

During our work on the characterisation of hydroxy sterol esters isolated from aortal lesions we have frequently observed material with a mobility on TLC intermediate between hydroxy-esters and triglycerides. Preparative TLC (mobile phase: benzene:ethyl acetate, 20:1 v/v) of column eluates containing this material, allowed the separation of three bonds at $R_f$s = 0.67, 0.61-0.50, and 0.43-0.33, corresponding respectively to triglycerides, a group of previously unidentified compounds, and hydroxy-esters (Fig. 20). The central band was further purified by preparative TLC, and thus rendered free from hydroxy-esters and triglycerides. Examination by ultraviolet spectrometry (Fig. 21) disclosed an absorption at $\lambda_{max}$ = 233 nm ($\epsilon_{max}$ ca. 20,000) characteristic of a conjugated diene. Weak absorption at $\lambda_{max}$ = 272 nm was ascribed to small quantities of a conjugated dienone. Thin layer chromatography of the isolated material showed it to be identical to hydroperoxides of cholesteryl linoleate prepared by autoxidation, both in $R_f$ value, and in its reaction with ceric sulphate and potassium iodide spray reagents (Table 5).

Reduction of the isolated material with sodium borohydride produced a group of compounds identical in TLC properties to hydroxy-esters (Fig. 22, Table 5). Alkaline hydrolysis of the reduced material yielded an acidic fraction which was methylated, trimethylsilylated and examined by GLC. Three peaks corresponding to methyl trimethylsilyloxy-octadecadienoates were observed (Fig. 23). The retention data of the isolated derivatives were compared with those of available reference compounds. The results

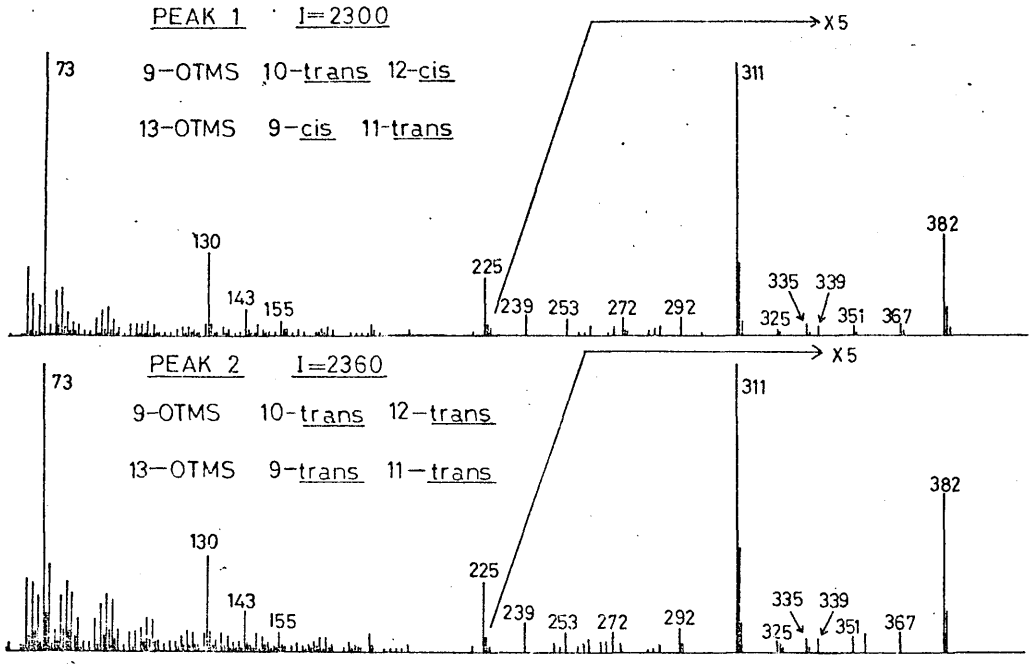



FIG. 24. Mass spectra of the hydroxy-acid methyl ester TMS ethers derived from aortal hydroperoxides. Ions of m/e greater than 230 are represented at 5 X their true relative intensity.

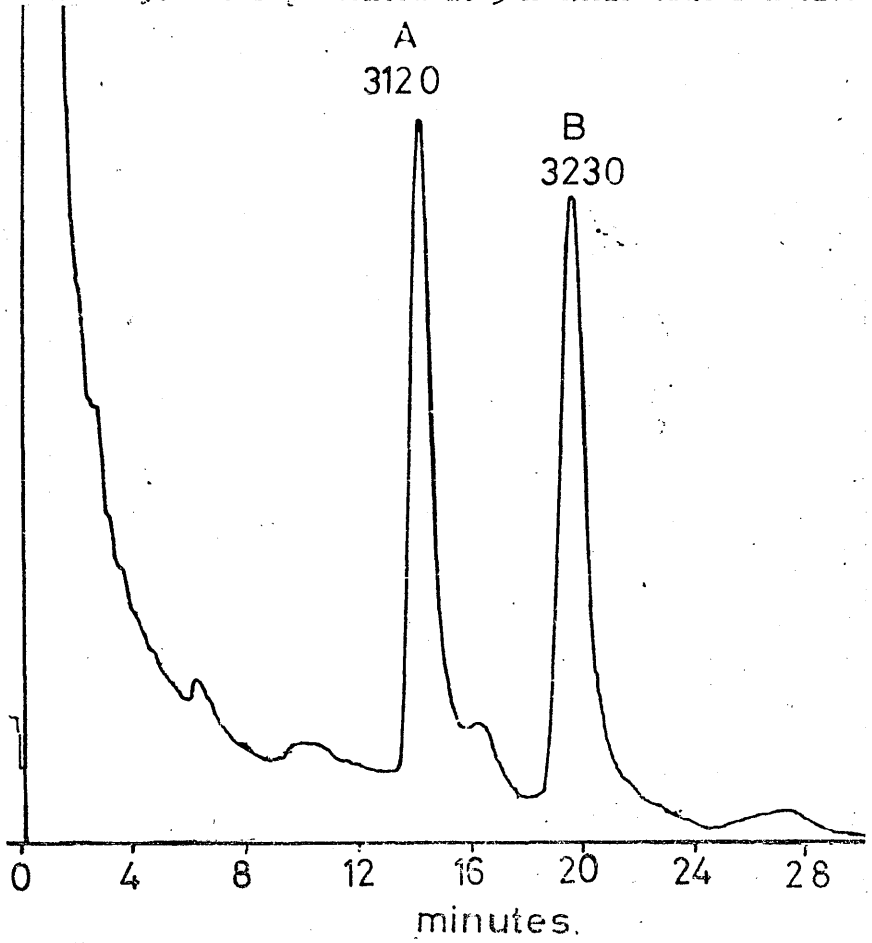



FIG. 25. Gas-liquid chromatogram of the bis TMS ethers of 7α-hydroxycholesterol (Peak A) and 7β-hydroxycholesterol (Peak B) derived from aortal hydroperoxy-esters. 1% SE-30, 240°C.

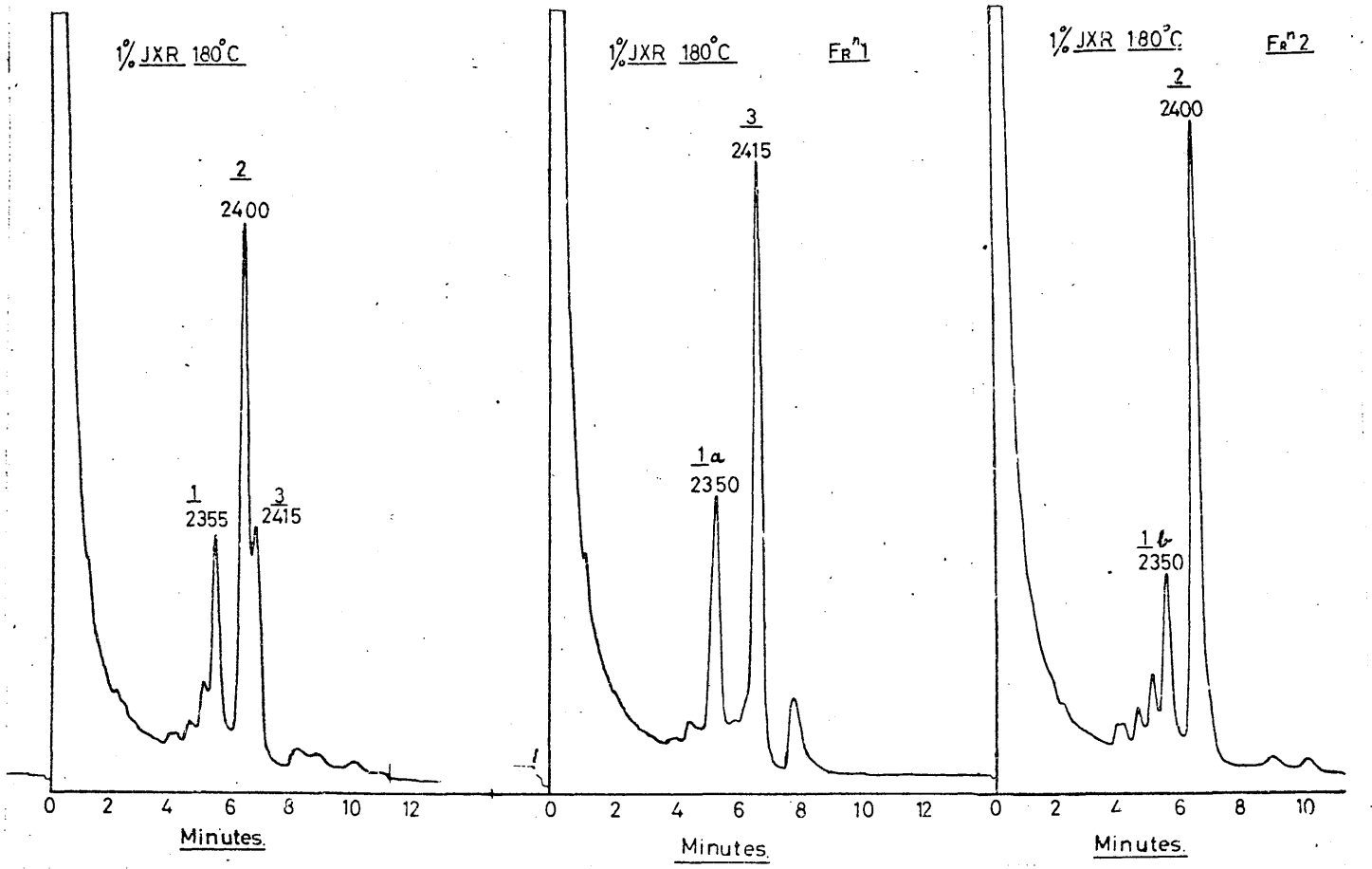



FIG. 26. Gas-liquid chromatograms of octadecadienediol bis TMS ethers derived from aortal hydroperoxy-esters. The left-hand chromatogram corresponds to the mixture of diols before preparative TLC. The middle and right-hand chromatograms refer respectively to fraction 1 (1,13-diols) and fraction 2 (1,9-diols) isolated from the mixture by preparative TLC.

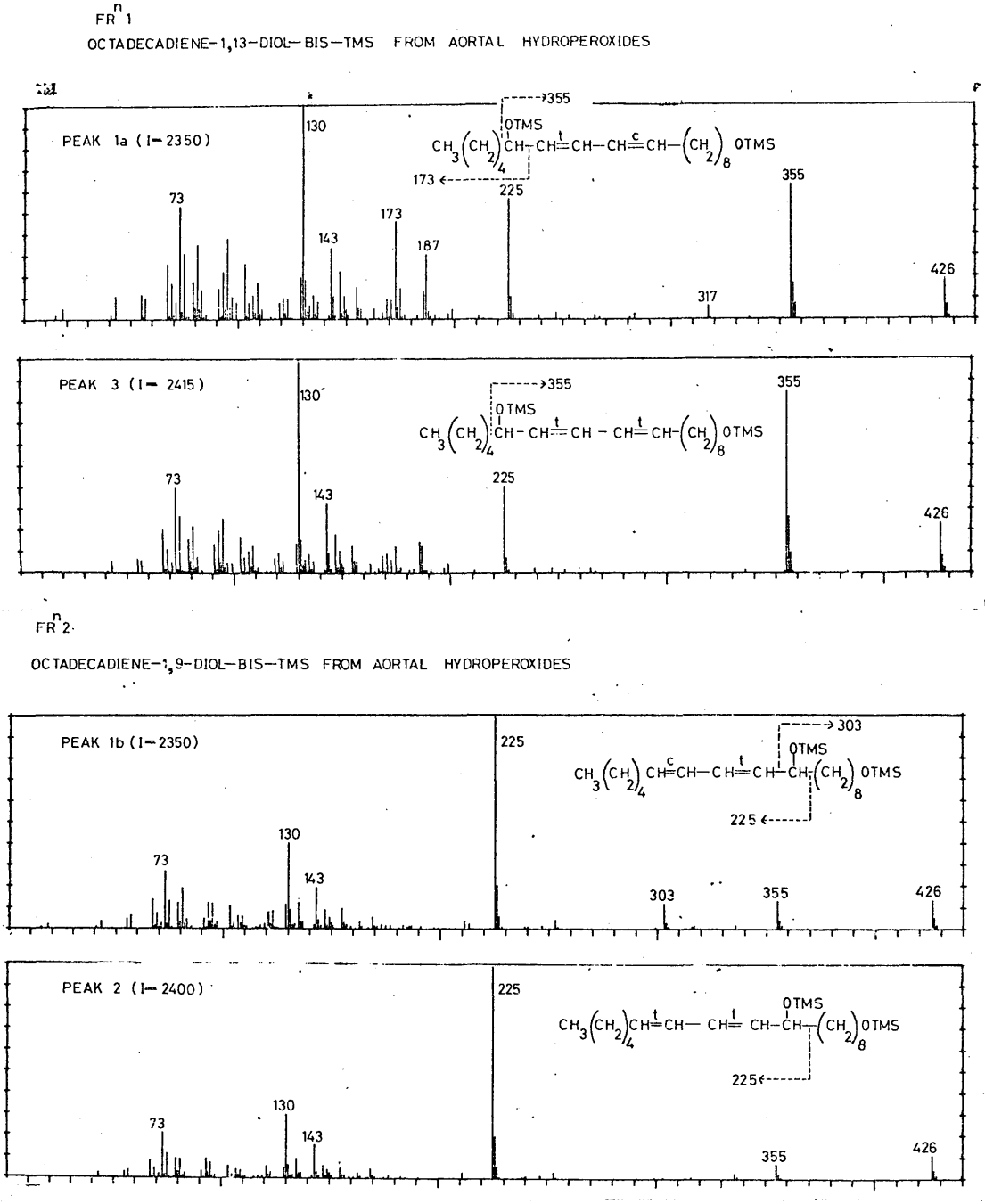



FIG. 27. Mass spectra of octadecadienediol-bis TMS ethers derived from aortal hydroperoxides. Upper pair: isomeric 1,13-diol bis TMS ethers. Lower pair: isomeric 1,9-diol derivatives. Mass spectra were scanned at the retention index values indicated.

are summarised in Table 7. Mass spectra recorded during GLC of the isolated derivatives are shown in Fig. 24. Peaks 2 and 3 were not resolved under the conditions used. The two mass spectra recorded are consistent with those of 9- and 13- trimethylsilyloxy-octadecadienoates.

Reductive cleavage of the isolated hydroperoxy-esters gave a mixture of sterols and octadecadienediols. Preparative TLC on silica gel (mobile phase: chloroform:ethyl acetate, 3:1 v/v) revealed four bands at $R_f$ = 0.70, 0.54, 0.47 and 0.27. Material in the most mobile band was identified as cholesterol by TLC and GLC. Trimethylsilylation of the least mobile band, and subsequent analysis by TLC and GLC (Fig. 25) indicated the presence of two sterols whose chromatographic behaviour was consistent with the epimeric 7-hydroxycholesterols (Table 6).

Fractions 1 ($R_f$ = 0.54) and 2 ($R_f$ = 0.47), containing octadecadienediols were trimethylsilylated and examined by GLC. Chromatograms of the TMS ethers of the diols, before and after separation by TLC are shown in Fig. 26.

Fraction 2 contained two principal components with retention indices of 2350 and 2400, corresponding respectively (Table 9) to 10-*trans*,12-*cis*- and 10-*trans*,12-*trans*-octadecadiene-1,9 diol *bis*-TMS ethers. The derivatives from Fraction 1 also contain two major components with retention indices of 2350 and 2415. The former value corresponded to that of standard 9-*cis*,11-*trans*-octadecadiene-1,13 diol *bis*-trimethylsilyl ether, and the latter is most likely the corresponding *trans*,*trans* derivative. The mass spectra and significant fragmentations of the components of Fractions 1 and 2 are shown in Fig. 27. The mass spectra of both peaks in Fraction 1 are identical, and correspond to the *cis*,*trans*- and *trans*,*trans*- octadecadiene-1,13 diol *bis* TMS ethers (*cf.* Fig. 7).

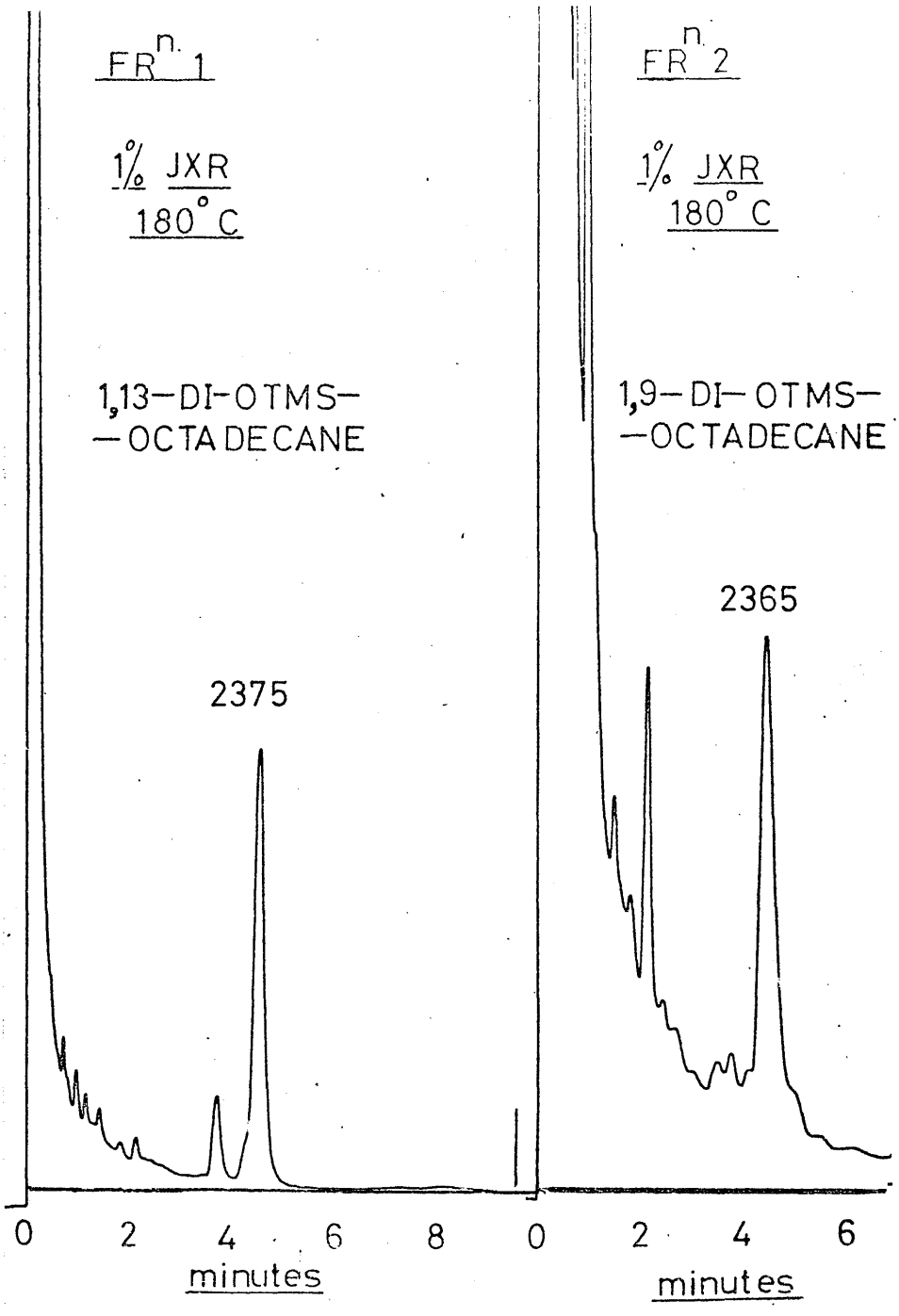



FIG. 28. Gas-liquid chromatograms of octadecanediol-bis TMS ethers prepared by hydrogenation of the dienes from fractions 1 and 2.

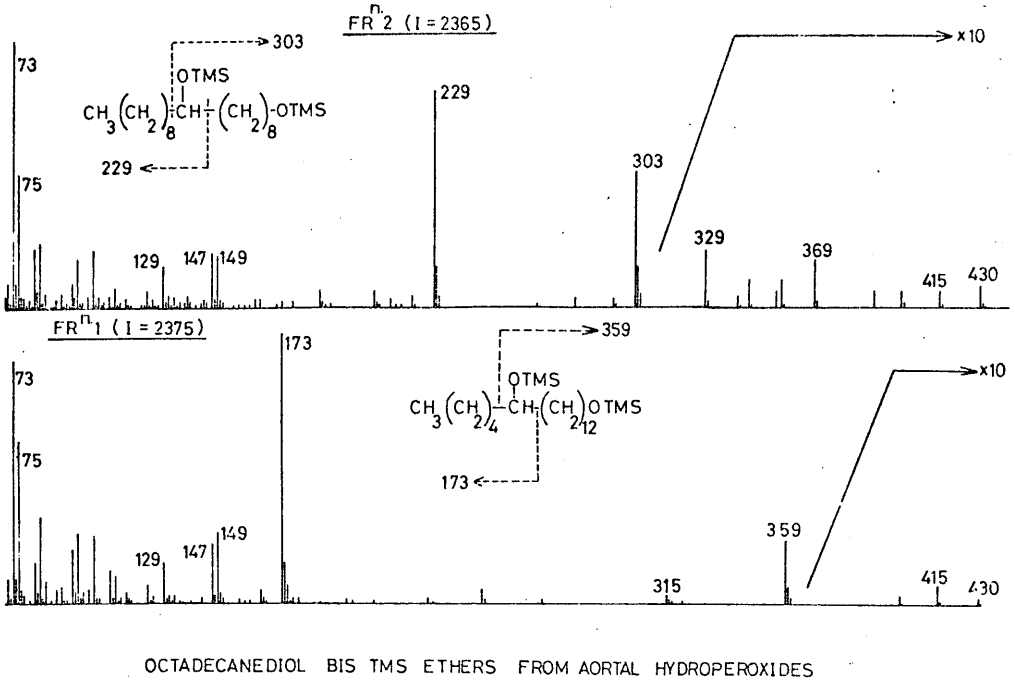



FIG. 29. Mass spectra of octadecanediol-bis TMS ethers prepared by hydrogenation of the dienes from fractions 1 and 2.

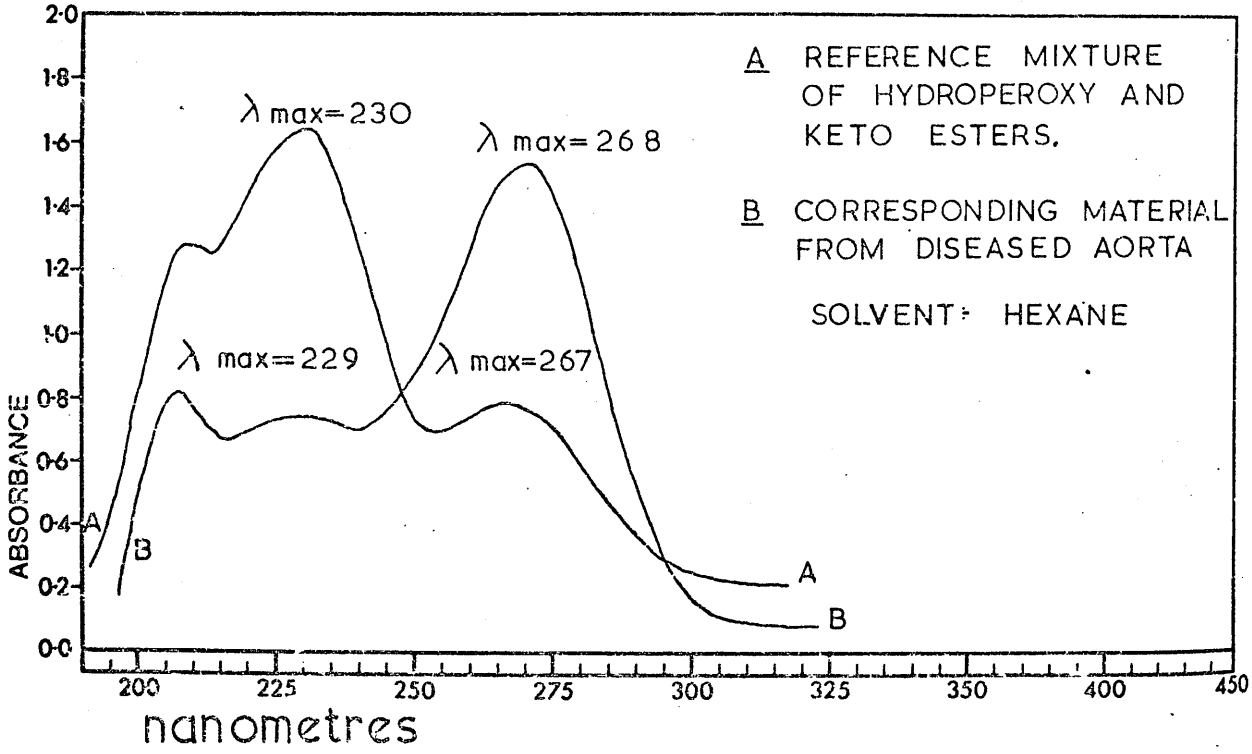



FIG. 30. Ultraviolet spectra of reference and arterial mixtures of hydroperoxy- and keto-esters of cholesterol. The spectrum of the isolated sample indicates an excess of keto-esters.

Similarly the TMS ethers of material from Fraction 2 have mass spectra characteristic of the isomeric 10,12-octadecadiene-1,9-diol bis-TMS ethers (cf. Fig. 10). Hydrogenation of Fractions 1 and 2 each resulted in one major peak on GLC of the TMS derivatives (Fig. 28). The mass spectra of the tetrahydro TMS ethers confirm the presence of octadecane-1,9-diol and octadecane-1,13-diol as the major products of hydrogenation (Fig. 29).

The quantities of cholesteryl linoleate hydroperoxides present in the extracts were determined by GLC of the derived hydroxy-acid methyl ester TMS ethers, as described on Page 52. In two separate cases the quantities of hydroperoxides were determined to be 55 $\mu$g. and 178 $\mu$g. per g. of total lipid. The quantities of hydroxy-esters in advanced lesions are between 5 and 10 times greater than the quantities of the corresponding hydroperoxides.

### 3.2.2. Identification of Aortal keto-esters.

During examination of plaque hydroperoxy-esters, ultraviolet spectra were obtained of purified material from several different aortal extracts. It was observed that quantities of ketonic material with an absorption at $\lambda_{max}^{EtOH}$ = 272 nm (268 nm in hexane) varied considerably from one extract to another. In most cases there was a large excess of hydroperoxy-esters (Fig. 21), however occasionally the quantities of keto- and hydroperoxy-esters were similar, and in the instance of one extract described below there was a large excess of keto-esters.

After isolation of the appropriate material corresponding by column and thin-layer chromatography to hydroperoxy- and/or keto-esters, a sample was examined by ultraviolet spectroscopy. The solvent used was hexane, since it had been found that small quantities of lipid were more easily recovered from this solvent

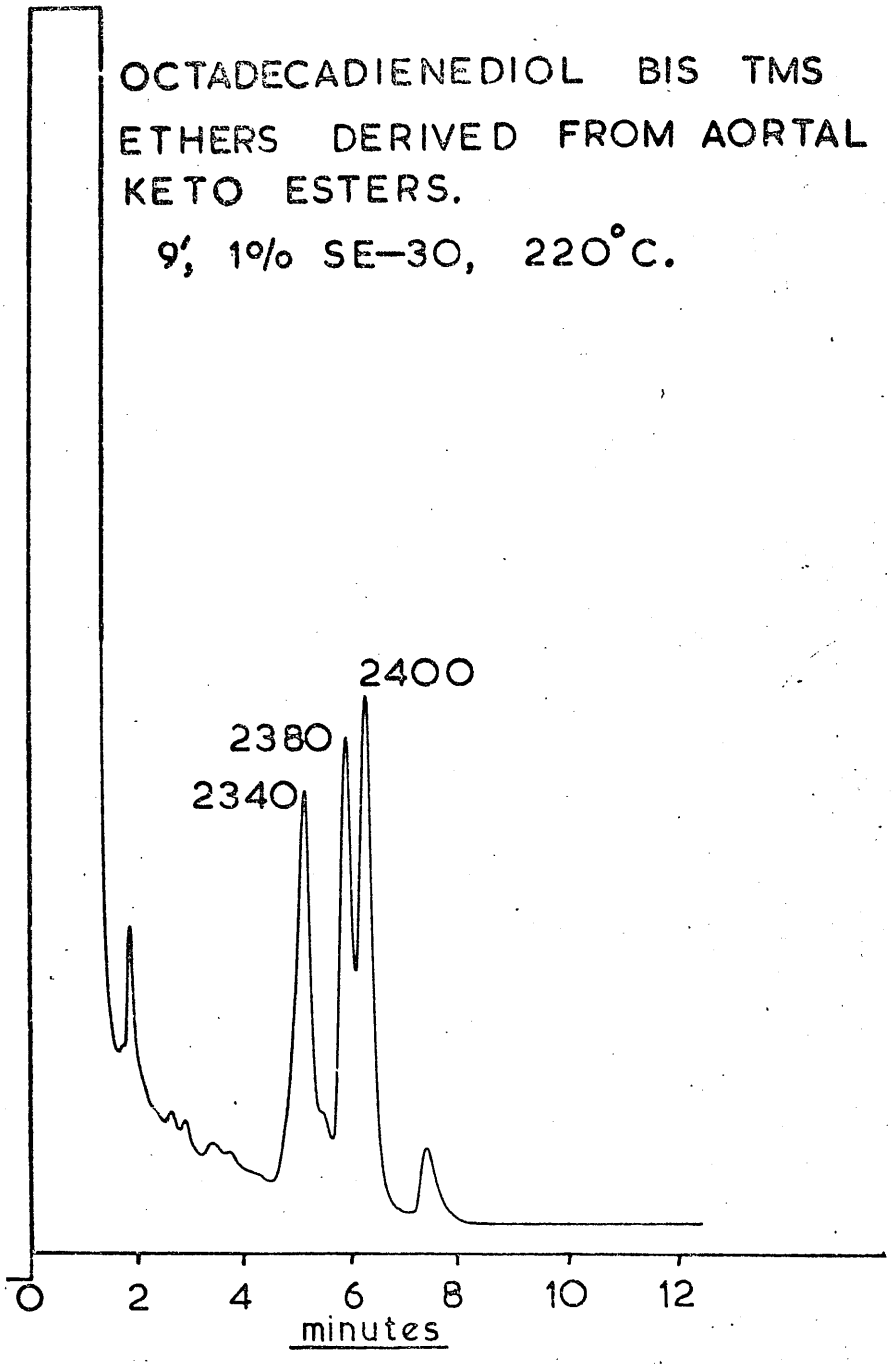



FIG. 31. Gas-liquid chromatogram of octadecadienediol-bis TMS ethers derived from aortal keto-esters by treatment with $LiAlD_4$. The peak with I = 2340 is a mixture of 10-trans,12-cis-octadecadiene-1,9- and 9-cis,11-trans-octadecadiene-1,13 diol-bis TMS ethers. The peaks with I = 2380 and 2400 are the trans,trans isomers of the respective 1,9- and 1,13-diol derivatives.

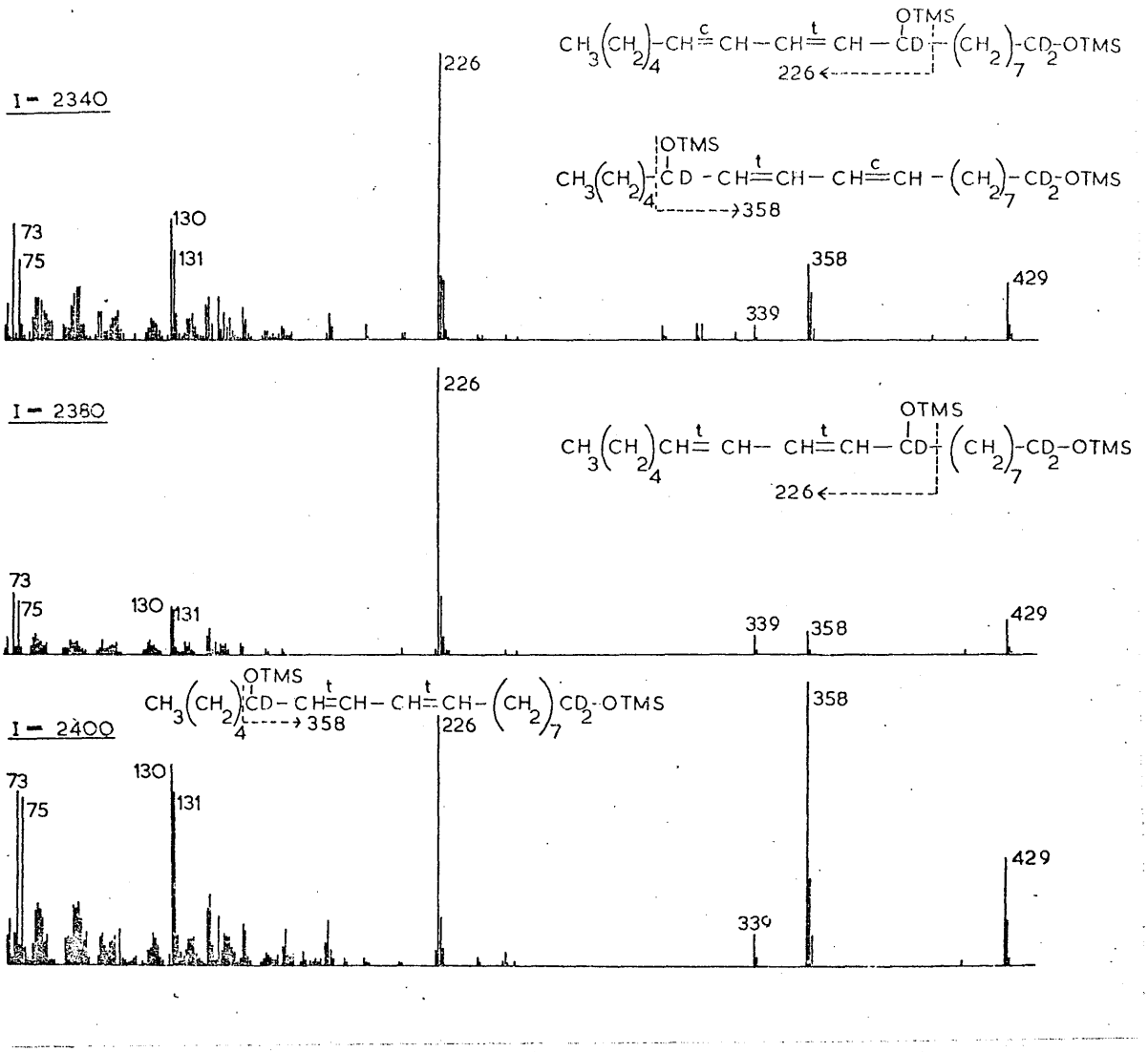



FIG. 32. Mass spectra of the octadecadienediol-bis TMS ethers derived from aortal keto-esters by $LiAlD_4$ reduction. The mass spectra were scanned at the retention indices indicated (c.f. Fig. 31).

than from ethanol. The resultant spectrum is shown in Fig. 30 and implies a large excess of keto- over hydroperoxy-esters in the sample. Re-examination of the material by analytical TLC showed that it gave only a very slight reaction when sprayed with potassium iodide solution, but reacted strongly when sprayed with 2,4-dinitrophenylhydrazine reagent. It appeared, therefore, that the bulk of this sample consisted of keto-esters. Since the sample was so small (approx. 80 $\mu$g, as determined by U.V. spectroscopy), the selective reduction of the hydroperoxy-esters described on page 76 was not attempted. The material was reduced with lithium aluminium deuteride as described for keto-esters (page 55 ) and the octadecadienediols and sterols thus produced were separated by preparative TLC (mobile phase: chloroform:ethyl acetate, 3:1 v/v) on a micro plate. After separation from cholesterol ($R_f$ = 0.68) and the 7-hydroxycholesterols ($R_f$ = 0.25), the band at $R_f$ = 0.48 corresponding to octadecadienediols, was trimethylsilylated and examined by GLC. The chromatogram is reproduced in Fig. 31 and shows the familiar pattern of peaks corresponding to a mixture of octadecadienediol bis-TMS ethers. Mass spectra of the three major peaks were obtained and line diagrams are shown in Fig. 32. As previously mentioned, formation of the deuterium labelled octadecadienediols can permit differentiation of keto- and hydroperoxy-esters. The mass spectra reveal deuterium labelling at the carbon atom attached to the secondary trimethylsilyloxy function, indicated by the ions at m/e values = 226, 358, 429 ($M^{+\cdot}$), and the doublet at m/e = 130 and 131 (cf. Fig. 18). Since the 1,9- and 1,13-octadecadienediols were not in this instance separated by preparative TLC, that peak with retention index I = 2340 is a mixture of the 10-trans,12-cis-octadecadiene-1,9-diol and the 9 cis,11-trans-octadecadiene-1,13-diol bis-TMS ethers. The all-

*trans* isomers are however, resolved, mass spectra being scanned at I = 2380 (1,9-diol-*bis* TMS) and I = 2400 (1,13-diol-*bis* TMS). The mass spectra substantiate the ultraviolet spectral evidence that the original keto-esters were almost completely free of hydroperoxy-esters. Had significant quantities of the latter been present, the ratio of the ions *m/e* = 130:*m/e* = 131 would have been expected to be much greater, since *m/e* = 131 is characteristic only of such derivatives from keto-esters.

| Group | Sex | Age (years) | Diagnosis |
|---|---|---|---|
| Group 1, Cases 1-5 (Stage I of disease) | | | |
| 1 | Male | 67 | Peritonitis; strangulated hernia. |
| 2 | Female | 37 | Myocardial infarction. |
| 3 | Male | 72 | Head injury. |
| 4 | Male | 40 | Haematemesis; gastric ulcers. |
| 5 | Female | 57 | Acute pyelonephritis: septicaemia. |
| Group 2, Cases 6-10 (Stage II of disease) | | | |
| 6 | Female | 61 | Carcinoma of bladder. |
| 7 | Female | 78 | Cerebral haemorrhage. |
| 8 | Male | 65 | Cerebral infarction. |
| 9 | Male | 73 | Carcinoma of stomach. |
| 10 | Male | 74 | Myocardial infarction. |
| Group 3, Cases 9 and 11-14 (Stage III of disease) | | | |
| 11 | Male | 61 | Mitral stenosis. |
| 12 | Female | 90 | Perforated gastric ulcer, aortal aneurysm. |
| 9 | Male | 73 | Carcinoma of stomach. |
| 13 | Female | 83 | Myocardial infarction. |
| 14 | Male | 54 | Carcinoma of stomach. |

TABLE 11. Data for cases from which aortas were obtained.

## 3.3. THE OCCURRENCE OF HYDROXY STEROL ESTERS IN VARIOUS STAGES OF HUMAN ATHEROSCLEROSIS

The presence, in severe atheroma, of a group of hydroxy sterol esters has been recently described.[125,127] Certain of these esters have been shown to consist of mono-esters of hydroxy-sterols , viz., 7α-hydroxycholesterol, 7β-hydroxycholesterol, 24-hydroxycholesterol and 26-hydroxycholesterol. A second class of hydroxy sterol esters, which comprises the larger part of the group, is composed of cholesterol esterified with hydroxy-acids. Three of these acids have been identified , viz., 9-hydroxy-10-trans,12-cis-octadecadienoic acid, 9-hydroxy-10-trans,12-trans-octadecadienoic acid, and 13-hydroxy-9-cis,11-trans-octadecadienoic acid. Previous work[125,127] was concerned with the isolation and identification of these acids from severely diseased arteries. The present study compares the quantities of the various hydroxy-octadecadienoic acids derived from hydroxy-esters isolated at various stages of the disease.

Human aortas were collected within 24 hours of death. The age, sex and principal cause of death of each subject are given in Table 11. The degree of arterial disease was graded, I, II or III according to the classification described on Page 17. Plaques were disected, and those of similar grade were collectively extracted as previously described, page 45. Fractions containing hydroxy-esters were obtained by silicic acid column chromatography, and the esters were purified by preparative TLC (Fig. 20). The weights of total lipid, and the percentage of sterols and sterol esters isolated by column chromatography are given in Table 12. The mean weight ratios of free sterol to sterol ester were 0.52 in Stage I, 0.42 in Stage II, and 1.06 in Stage III. Hydrolysis of the hydroxy sterol ester fractions from both Stage II and Stage III yielded a mixture of sterols (mainly cholesterol, together

| Stage of atherosclerosis | Case No. | Weight of total lipid (mg) | Sterol esters in total lipid (%) | Free sterols in total lipid (%) | Acids I and II in total lipid (μg/g)[a] | Acid III in total lipid (μg/g)[a] | Total hydroxy acids in total lipid (μg/g)[a] | Cholesteryl hydroxy octadecadienoates in sterol esters (μg/g)[a] |
|---|---|---|---|---|---|---|---|---|
| I | 1 | 200 | 33 | 14 | - | - | - | - |
| | 2 | 410 | 49 | 18 | - | - | - | - |
| | 3 | 428 | 37 | 17 | - [b] | - [b] | - [b] | - [b] |
| | 4 | 290 | 30 | 18 | - | - | - | - |
| | 5 | 95 | 34 | 28 | - | - | - | - |
| | | mean 285 | mean 37 | mean 19 | | | | |
| | | S.D. ± 140 | S.D. ± 7.5 | S.D. ± 5.5 | | | | |
| II | 6 | 380 | 47 | 24 | 37 | 10 | 47 | 220 |
| | 7 | 520 | 38 | 11 | 42 | 15 | 57 | 340 |
| | 8 | 385 | 42 | 13 | 81 | 13 | 94 | 510 |
| | 9 | 820 | 52 | 26 | 150 | 23 | 173 | 750 |
| | 10 | 1280 | 58 | 25 | 67 | 9 | 76 | 290 |
| | | mean 675 | mean 47 | mean 20 | mean 75 | mean 14 | mean 89 | mean 420 |
| | | S.D. ± 380 | S.D. ± 8 | S.D. ± 7 | S.D. ± 42 | SD ± 5 | SD ± 50 | S.D. ± 420 |
| III | 11 | 2550 | 40 | 46 | 140 | 81 | 220 | 1200 |
| | 12 | 2060 | 34 | 45 | 160 | 45 | 205 | 1300 |
| | 9 | 1650 | 49 | 41 | 240 | 89 | 330 | 1500 |
| | 13 | 3750 | 33 | 41 | 260 | 78 | 340 | 2300 |
| | 14 | 1860 | 39 | 34 | 410 | 170 | 580 | 3400 |
| | | mean 2370 | mean 39 | mean 41 | mean 240 | mean 93 | mean 335 | mean 1950 |
| | | S.D. ± 840 | S.D. ± 6 | S.D. ± 5 | S.D. ± 110 | SD ± 6 | SD ± 150 | S.D. ± 900 |

a Values have been rounded off in accordance with the estimated degree of precision of gas chromatographic measurement

b There were no detectable hydroxy acids in Stage (I) lesions.

TABLE 12. Free sterols, sterol esters, and hydroxy acids in extracts obtained from different stages of aortic atheroma

with hydroxycholesterols), and fatty acids. The hydroxy-acids were methylated, trimethylsilylated and examined by GLC. Three hydroxy-acids were identified, viz., Acid 1 (13-hydroxy-9-cis, 11-trans-octadecadienoic acid), Acid 2 (9-hydroxy-10-trans,12-cis-octadecadienoic acid), and Acid 3 (9-hydroxy-10-trans,12-trans-octadecadienoic acid). The identities of these acids have been rigorously determined during previous work.[125,127] Hydrolysis of fractions from Stage I cases, corresponding in chromatographic behaviour to hydroxy sterol esters, yielded neither sterols nor hydroxy-acids in detectable amounts. The minute amounts of saturated and unsaturated fatty acids found in such fractions were probably present in the tissue as free fatty acids. The quantities of hydroxy-acids isolated from Stage II and Stage III type tissue were determined as their methyl ester trimethylsilyl ethers as described on page 52. Table 12 shows the amounts of hydroxy-acids determined per g of total lipid in each case. The quantities of cholesteryl hydroxyoctadecadienoates, expressed as a proportion of the total sterol esters, are indicated in the final column of Table 12. Individual quantities of Acids 1 and 2 were not determined, because their derivatives were not effectively separated by GLC. The results indicate a statistically significant rise from 89±50 g/g (hydroxy-acids/total lipid; mean ± standard deviation) in extracts representing Stage II of atheroma, to 335±150 g/g in the Stage III extracts ($t = 3.47$, $p = 0.004$). The concentration of the cholesteryl octadecadienoates rose from 420±210 g/g (hydroxy-esters/total sterol esters) in Stage II to 1950±900 g/g in Stage III ($t = 3.58$, $p = 0.004$). There is virtually no overlap in the ranges of individual results for the three classes of lesion.

## 3.4. THE ISOLATION AND IDENTIFICATION OF 5α-CHOLESTAN-3β-OL FROM THE HUMAN ATHEROMATOUS AORTA

A preliminary study[116] of the companion sterols of cholesterol occurring in advanced atheroma has demonstrated the presence of small quantities of cholesta-5,24-dien-3β-ol (desmosterol) and cholesta-3,5-dien-7-one. The purpose of the present work was to determine the nature and quantities of saturated close relations of cholesterol reported by several workers to be present in extracts from human atheroma.

5α-Cholestan-3β-ol (cholestanol) has been identified as a companion sterol of cholesterol in several human tissues[109,110,267] including arteries.[111,268] There is, however, considerable doubt as to the quantities of this sterol present in the total sterol fractions. The older literature on atheroma[107,108] contains reports of proportions of cholestanol as high as 13% of the total sterols. Recent reports[109,111] however, based on improved analytical methods, suggest that it amounts only to about 0.5 - 1% of the total sterol in tissue. Cholestanol and cholesterol are very difficult to separate by conventional column or thin-layer chromatography. While they may be separated by GLC[269] the fact that cholestanol is often present in quantities of around 0.1% of the sterol mixture renders the analysis difficult. One method of circumventing this difficulty involves oxidation[270] of cholesterol to cholestane-3β,5α6βtriol which is readily separated from unoxidised cholestanol by solvent extraction[271-273] or silicic acid chromatography.[109] Saturated sterols may then be analysed by GLC,[271] or by precipitation with digitonin and the quantities determined either gravimetrically or via the anthrone method.[109] The outstanding disadvantage of this method is that minor quantities of olefinic sterols which may also be present in the sample would

also be irreversibly altered. An alternative to oxidation is removal of cholesterol by chromatographic methods prior to analysis by GLC. Thin-layer chromatography over silica gel impregnated with silver nitrate[141,274] offers this alternative. In this method sterols are differentiated according to the number and position of the olefinic bonds in the molecule, and thus chromatographic separations can be effected. Separations can be enhanced by the preparation of derivatives such as acetates[275,276] and propionates,[243] but in this study it was desirable that such derivatives should also have informative gas chromatographic and mass spectral properties.

Preliminary investigations showed that although acetates and propionates were satisfactory derivatives for thin-layer chromatographic separation of cholesterol and cholestanol, the mass spectra of such compounds did not normally show molecular ions. Conversely use of trimethylsilyl ethers (which have excellent GLC and mass spectral properties ) demonstrated their unsuitability as derivatives for effecting separation of cholestanol and cholesterol on silver nitrate impregnated layers, poor separations and considerable decomposition being shown to occur.

The use of trifluoroacetylation afforded derivatives which yielded clean separations on TLC, convenient retention times on GLC and good mass spectral properties.[278] Accordingly, this study of the companion sterols of cholesterol was initiated using trifluoroacetates.

Plaques from grossly diseased aortas were processed and sterol and sterol ester fractions were isolated as described on page 46. Polar sterols were removed from the total sterol fraction by repeated crystallisation from methanol. Sterol esters were subjected to alkaline hydrolysis in methanolic KOH for 15 hours.

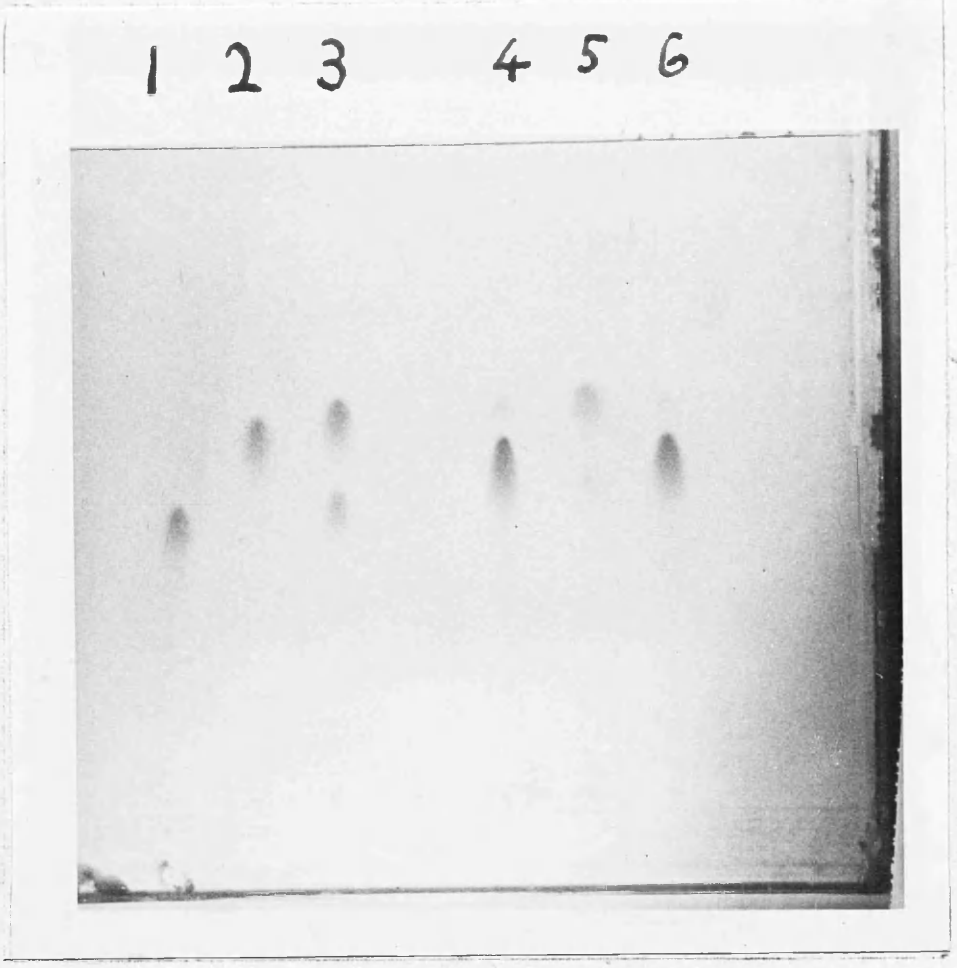



FIG. 33. Thin-layer chromatogram of reference cholesteryl and cholestanyl trifluoroacetates, and the corresponding derivatives of sterols isolated from arterial lesions. Lane 1: cholesteryl TFA; Lane 2: cholestanyl TFA; Lane 3: mixture of cholesteryl and cholestanyl TFAs; Lane 4: TFAs of a mixture of sterols isolated from arterial lesions; Lanes 5 and 6 respectively show cholestanyl and cholesteryl TFAs after separation of the mixture shown in Lane 4.
Silica gel (Adsorbosil 1) impregnated with 26% (w/w) $AgNO_3$. Mobile phase: hexane:benzene (97:3 v/v).

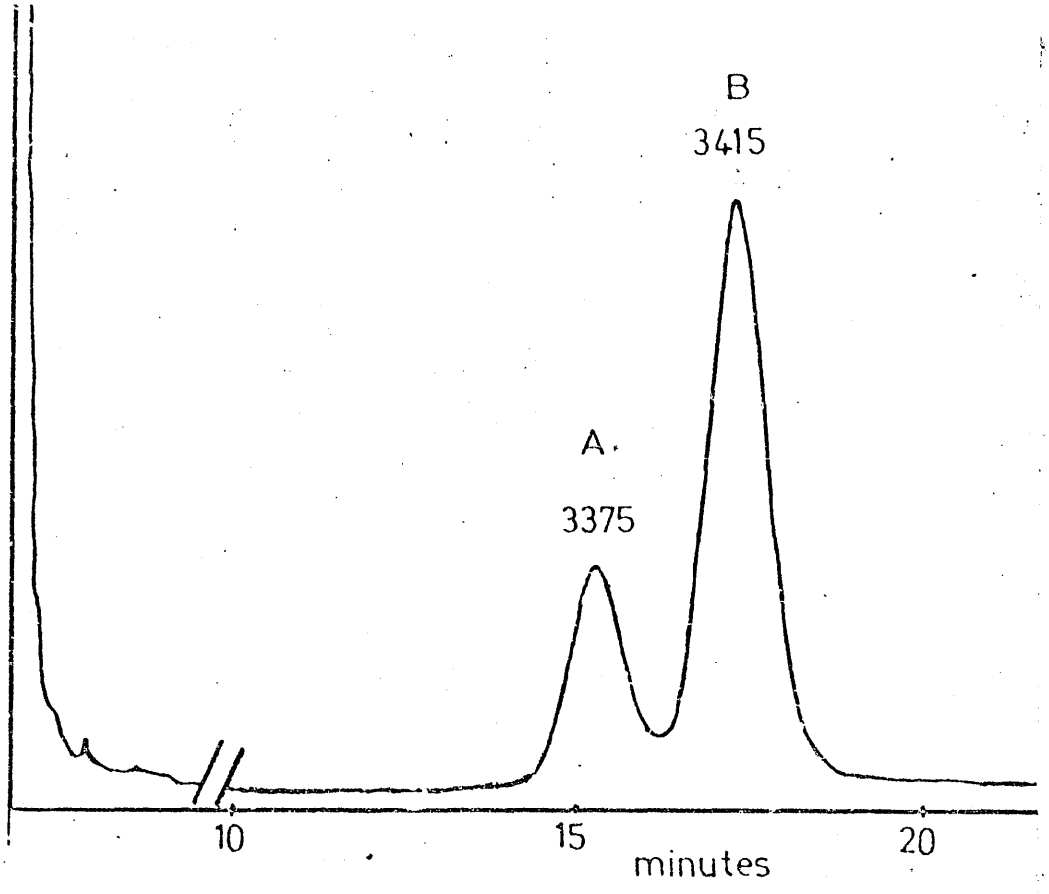



FIG. 34. Gas-liquid chromatogram of a fraction (c.f. Fig. 33, Lane 5) largely comprising the trifluoroacetates of cholesterol (A) and cholestanol (B), isolated by TLC from an extract of the saponified sterol esters of human atheromatous plaques. 1% QF-1, 200°C.

| STEROL | $R_f$ Trifluoroacetate Silica Gel ($AgNO_3$) | RETENTION INDEX | | | | |
|---|---|---|---|---|---|---|
| | | Sterol 1% SE-30 225°C | Trifluoroacetate 1% SE-30 225°C | Trifluoroacetate % QF-1 200°C | Trimethylsilyl ether 1% SE-30 225°C | Trimethylsilyl ether 1% QF-1 200°C |
| Cholest-5-en-3β-ol | 0.45 | 3005 | 2935 | 3375 | 3080 | 3315 |
| 5α-Cholestan-3α-ol | 0.42 | 2995 | 2880 | 3340 | 3005 | 3215 |
| 5α-Cholestan-3β-ol | 0.56 | 3005 | 2935 | 3415 | 3085 | 3345 |
| 5β-Cholestan-3α-ol | 0.51 | 2965 | 2890 | 3355 | 3026 | 3250 |
| 5β-Cholestan-3β-ol | 0.56 | 2965 | 2870 | 3325 | 3000 | 3220 |
| Material isolated from sterol fraction | 0.55<br>0.45 | 3000 | 2935 | 3415<br>3375 | 3080 | 3345<br>3315 |
| Material isolated from sterol ester fraction | 0.55<br>0.45 | 3000 | 2935 | 3415<br>3375 | 3080 | 3350<br>3320 |

TABLE 13. Chromatographic data for sterols and derivatives. Thin-layer chromatography was carried out in hexane:benzene (97:3 v/v).

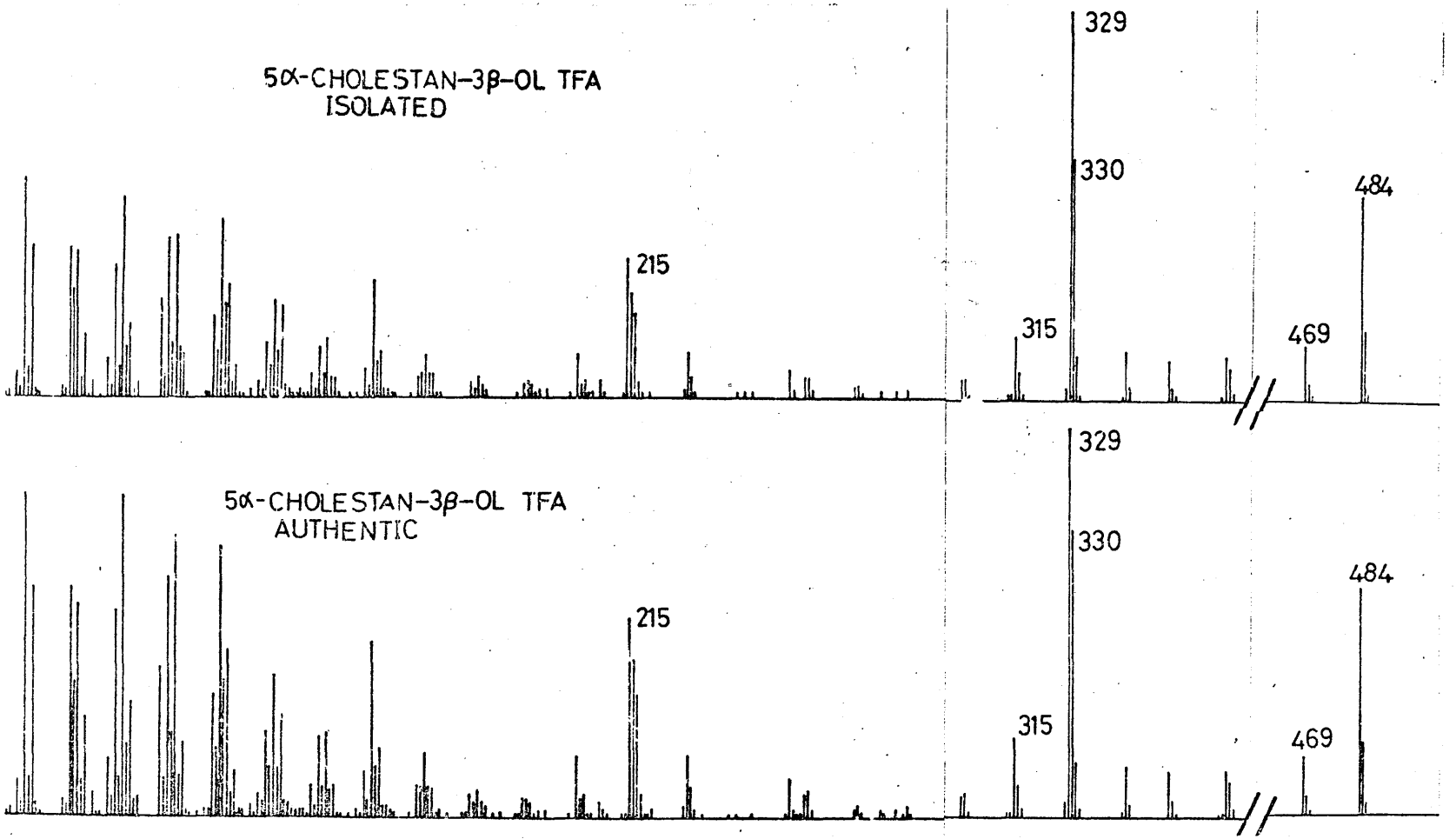



FIG. 35. Mass spectra of trifluoroacetates derived from authentic and isolated cholestanol.

Sterol trifluoroacetates (prepared as described on page 57 ) were subjected to preparative TLC on silver nitrate impregnated layers using a mobile phase of hexane:benzene (97:3 v/v). In a typical example the cholesteryl trifluoroacetate band ($R_f$ = 0.45) was located with iodine vapour, and the area immediately above was removed from the plate and extracted with hexane. After concentration the eluted material was examined by analytical TLC (Fig. 33). Two spots were obtained which were found to correspond to cholesteryl and cholestanyl trifluoroacetates in both $R_f$ values and in colour reaction. Examination by GLC demonstrated that approximately 90% of the sample was cholesteryl trifluoroacetate. A second thin-layer chromatographic separation reduced the proportion of cholesteryl trifluoroacetate to about 35% as indicated by the gas-liquid chromatogram (Fig. 34). Peak B corresponded in retention time to cholestanyl trifluoroacetate. In one instance the bulk of the fraction containing purified cholestanyl trifluoroacetate was subjected to alkaline hydrolysis and the free stanol and its trimethylsilyl ether were examined by GLC. Comparison of retention times with those of reference compounds gave satisfactory correlations (Table 13). The identification was confirmed by GC-MS of the trifluoroacetates and trimethylsilyl ethers of the isolated material. The mass spectra were found to be identical to those of the corresponding derivative of cholestanol (Fig. 35). The stereoisomers 5α-cholestan-3α-ol, 5β-cholestan-3β-ol and 5β-cholestan-3α-ol all gave very similar mass spectra as their trifluoroacetate, but are clearly distinguished by their retention characteristics on GLC (Table 13). 5α-cholestan-3β-ol is the only isomer for which the derivatives have longer retention times than those of cholesterol.

There was no evidence for the presence of other stanols

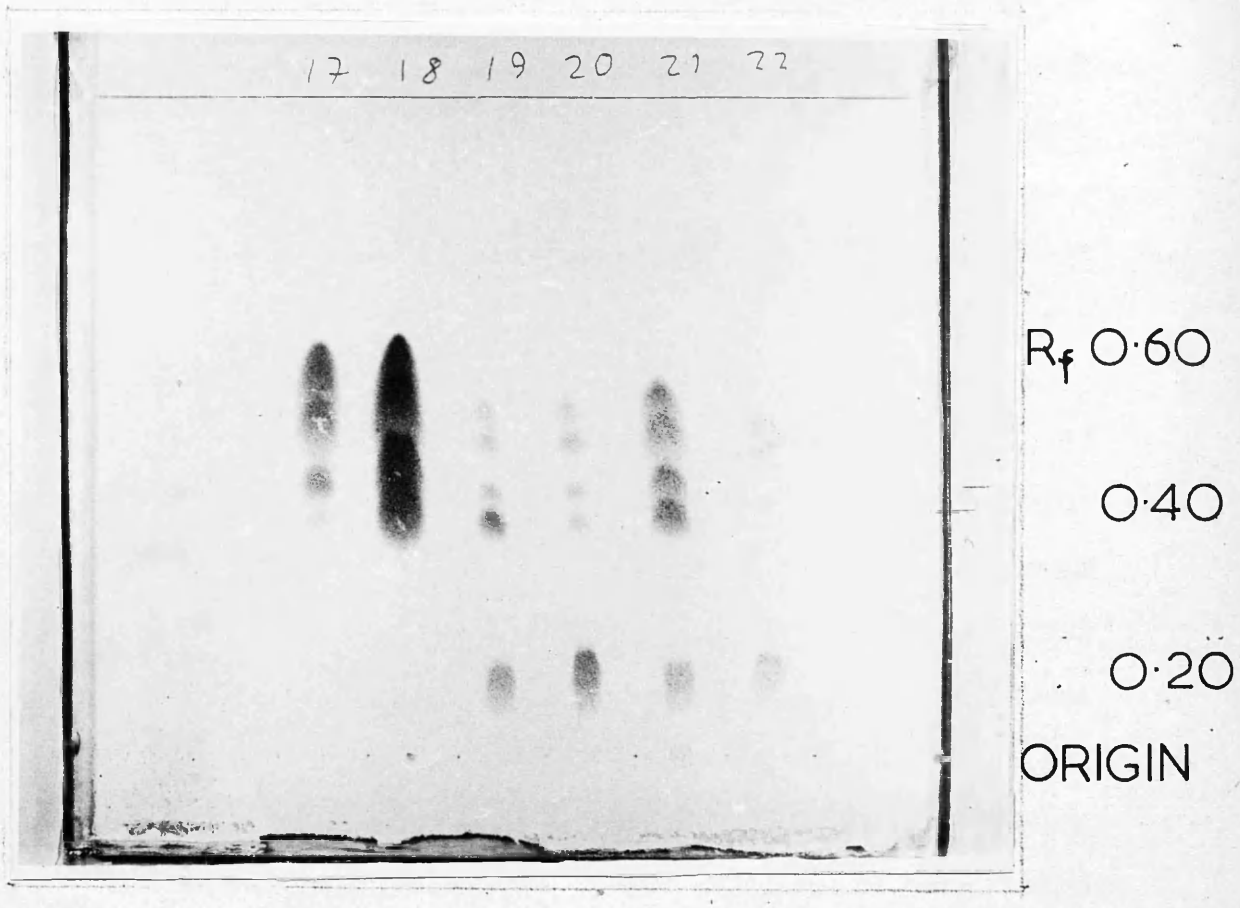



FIG. 36. Thin-layer chromatogram of fractions (numbered 17-22) eluted in 15-20% chloroform in hexane during silicic acid column chromatography of an extract from advanced aortal lesions. Mobile phase: hexane:benzene (2:1 v/v). Region at $R_f$ 0.39-0.58 contains cholesterol esters. Region at $R_f$ 0.1-0.24 contains sterol diesters.

either in the free or esterified sterol fractions from the extracts which were studied. The amounts of cholesterol isolated were estimated by gas-liquid chromatography to represent between 0.3 and 0.5% of the total in both free and esterified sterols isolated from plaques.

## 3.5. THE ISOLATION AND IDENTIFICATION OF DI-ESTERS OF 26-HYDROXYCHOLESTEROL FROM HUMAN AORTAL LESIONS.

The sterol ester composition of human arterial lesions has been extensively investigated; see reviews by Böttcher and Woodford,[89] and Swell and Treadwell.[279] The advent of gas chromatography has enabled rapid and accurate identification of the individual components in mixtures of fatty acids, and much attention has been devoted to comparative studies of the nature and quantities of fatty acids present as cholesterol esters in varying types of lesions.[88,100-103] More recently it has been shown that small quantities of esters of sterols other than cholesterol also occur in diseased aortal tissue.[125-127] Besides esters of cholestanol (a very frequent companion of cholesterol in many tissue extracts), other esters have been found[125-127] and shown to consist of mono-esterified hydroxy sterols, namely, 7$\alpha$-hydroxycholesterol, 7$\beta$-hydroxycholesterol, 24-hydroxycholesterol and 26-hydroxycholesterol. The present work demonstrates the occurrence and describes the identification of a hitherto unknown type of sterol ester. Examination by TLC of the later of the column fractions containing cholesterol esters, using a mobile phase of hexane:benzene (2:1 v/v) revealed material of slightly greater polarity than the cholesterol esters (Fig. 36). The material of $R_f$ range 0.08 to 0.21 was partially freed from cholesterol esters ($R_f$ 0.35-0.55) by preparative TLC, and hydrolysed to yield acid

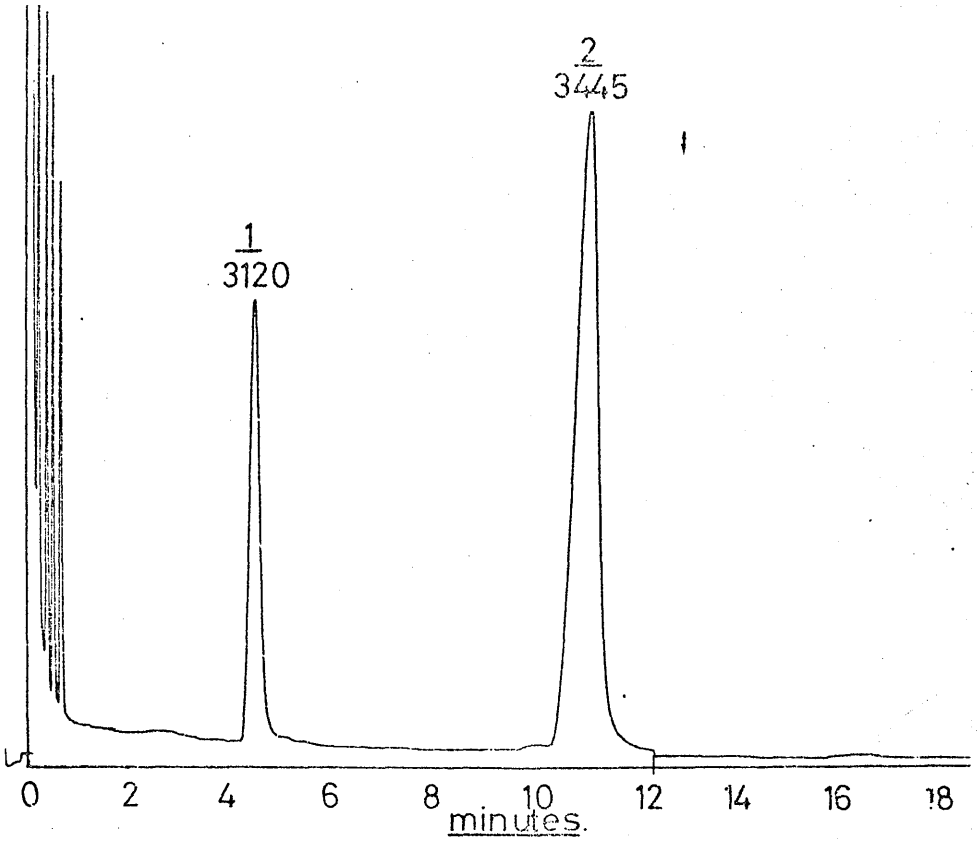



FIG. 37. Gas-liquid chromatogram of TMS ether of cholest-5-ene-3 , 26-diol obtained from aortal diesters. Peak 1, cholest-5-ene-3 -ol TMS ether: Peak 2, cholest-5-ene-3 ,26 diol bis-TMS ether. 1% SE-30, 240°C.

| | $R_f$ of Free Sterol ($CHCl_3$:EtOAc 3:1 v/v) | $R_f$ of TMS Ether (Hexane:Benzene 2:1 v/v) | I 240°C 1% SE-30 |
|---|---|---|---|
| 26-hydroxy-cholesterol | 0.37 | 0.31 | 3445 |
| Sterol isolated from di esters | 0.37 | 0.33 | 3445 |

TABLE 14. Comparison of chromatographic data of the sterol isolated from diesters with those of authentic 26-hydroxycholesterol. Gas chromatographic data refer to the sterol bis-TMS ethers.

| Acid methyl ester | 1% SE-30 172°C | 1% OV-17 172°C | 10% PEGA 180°C | % composition in di esters * | | % composition in cholesterol esters † | |
|---|---|---|---|---|---|---|---|
| | | | | Mean | Range | Mean | Range |
| Myristate | 1700 | 1820 | 2070 | 5 | 4-7 | 1 | 0.8-1.2 |
| Palmitate | 1900 | 2040 | 2270 | 29 | 26-34 | 15 | 14-17 |
| Palmitoleate | 1860 | 2040 | 2315 | 8 | 6-11 | 6 | 5-8 |
| Stearate | 2100 | 2250 | 2475 | 9 | 6-13 | 1 | 0.7-1.4 |
| Oleate | 2080 | 2250 | 2505 | 28 | 22-36 | 28 | 27-29 |
| Linoleate | 2080 | 2250 | 2560 | 15 | 11-21 | 45 | 45-46 |
| Linolenate | 2080 | 2250 | 2655 | 2 | 1-3 | 1 | 0.3-1.5 |

TABLE 15. Retention indices and relative proportions of fatty acid methyl esters derived from di esters of cholest-5-ene-3 ,26-diol, and esters of cholesterol isolated from extracts of advanced aortal lesions.

* Mean of 4 analyses

† mean of 2 analyses

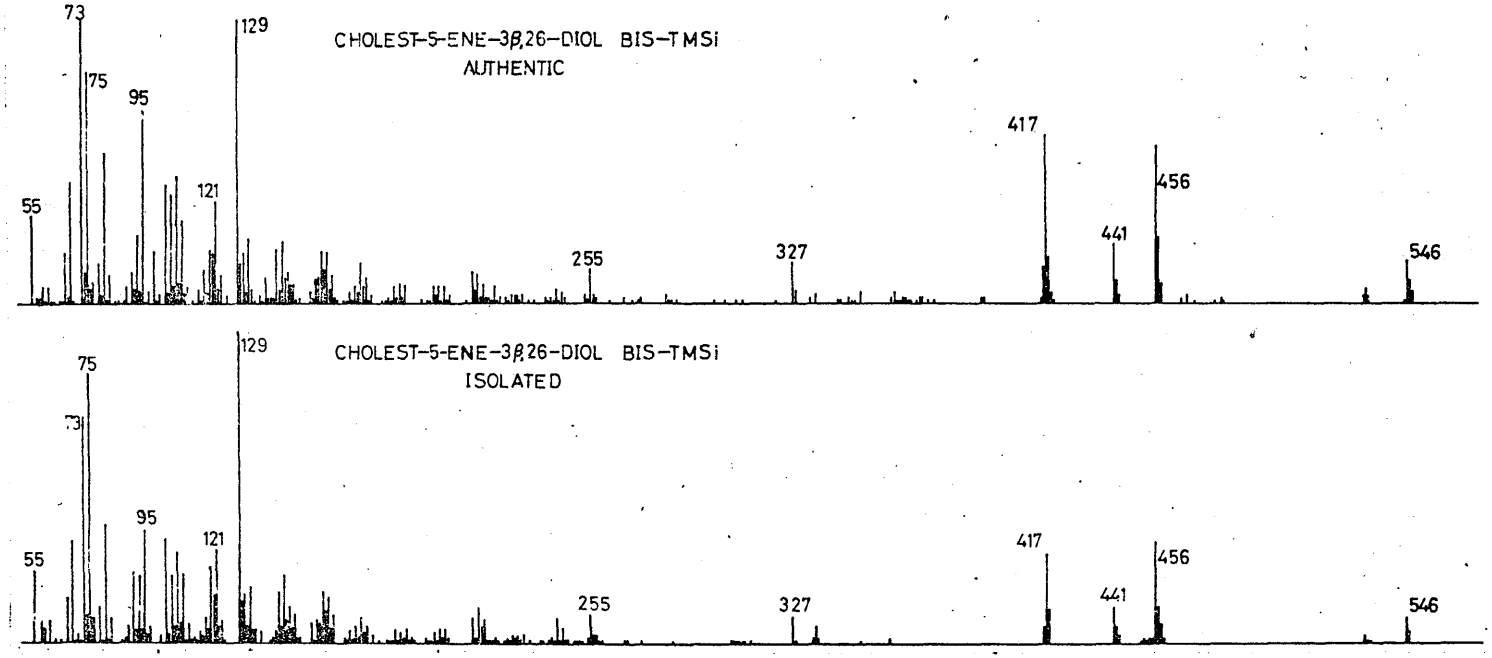



FIG. 38. Mass spectra of the TMS ethers of authentic 26-hydroxycholesterol and the sterol isolated from arterial diesters.

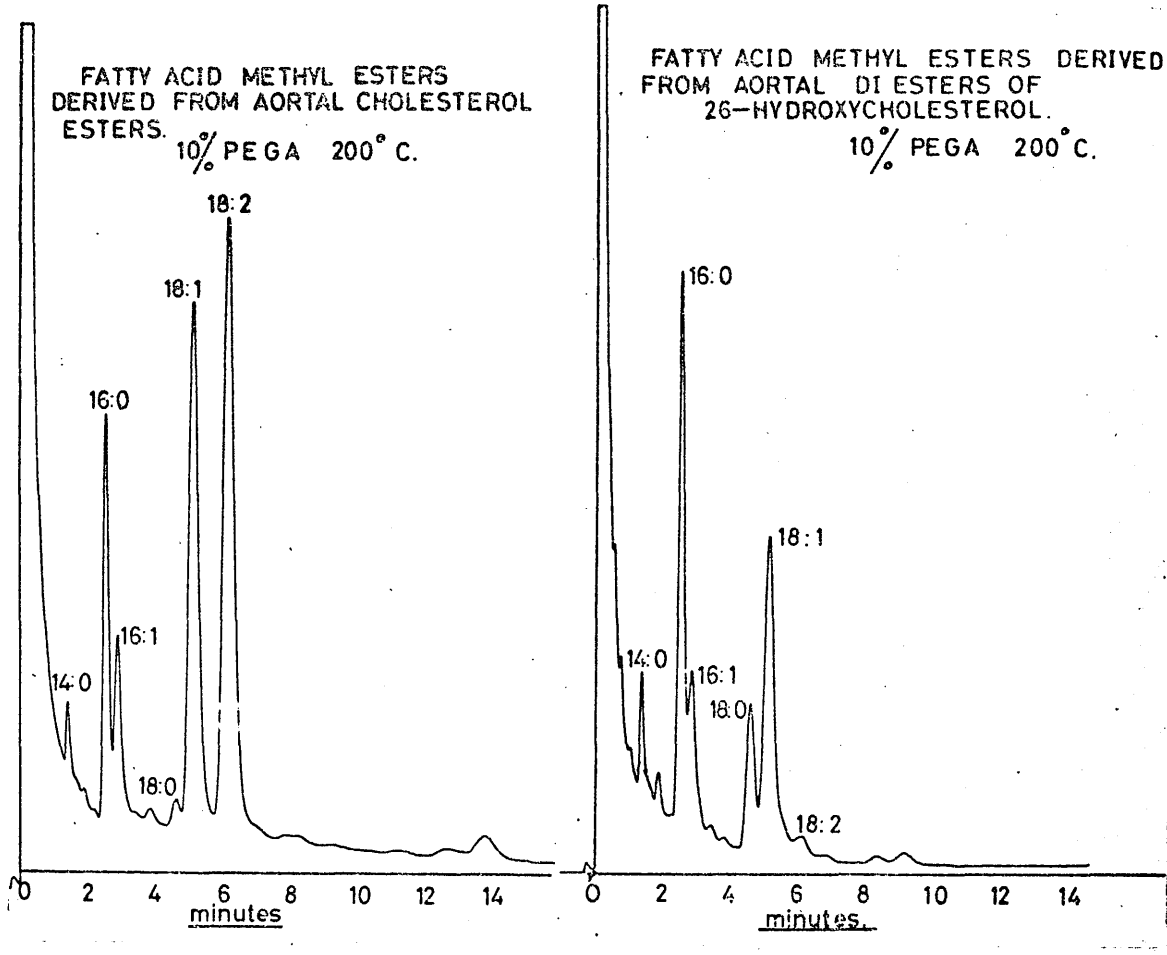



FIG. 39. Comparison of gas chromatograms of the fatty acid methyl esters derived from cholesterol ester and diester fractions from the same severely diseased aorta.

and neutral fractions. Examination of the latter on TLC both as the free sterols and as the trimethylsilyl ethers showed the major component to be indistinguishable from 26-hydroxycholesterol (Table 14). The trimethylsilyl ether of the hydroxy sterol was examined by GLC and mass spectrometry and thus positively identified as 26-hydroxycholesterol (Figs. 37 and 38).

For a study of the acids esterified with 26-hydroxycholesterol the di-ester fraction was purified twice by TLC, thus reducing the quantity of cholesterol ester present to less than 5% (as judged by GLC of the sterols liberated on hydrolysis). The acid fraction from hydrolysis was methylated with diazomethane and examined by GLC on several different stationary phases. The fatty acids characterised and their relative proportions (means of five determinations) are given in Table 15. The major acids (palmitic and oleic) account for approximately 57% of the total. Comparative data are included for the fatty acids isolated from the cholesterol esters of 3 of the lipid extracts studied. Figure 39 compares the fatty acids esterified with 26-hydroxycholesterol with those of cholesterol in the same extract of a badly diseased aorta. The virtual absence of linoleate and the large quantities of palmitate, stearate and oleate in the former are contrasted with the expectedly high linoleate concentration in the cholesteryl esters.

In order to identify further the diesterified 26-hydroxycholesterol present in aortal extracts, the synthesis of a model compound was undertaken.

Preparation of 26-hydroxycholesterol dipalmitate:

26-hydroxycholesterol was prepared by the method of Scheer et al.[281]: the corresponding diacetate, recrystallised from ethyl

| Quantity of ester hydrolysed | | Material recovered | | | | | |
|---|---|---|---|---|---|---|---|
| | | Palmityl Alcohol[a] | | | 26-hydroxycholesterol[b] | | |
| mg | M | mg | M | % recovery | mg | M | % recovery |
| 2.70 | 3.1 | 1.32 | 5.45 | 88% | 1.12 | 2.8 | 91% |
| 5.21 | 5.9 | 2.87 | 11.70 | 99% | 2.28 | 5.6 | 96% |

TABLE 16. Analytical data for synthetic 26-hydroxycholesterol dipalmitate

a Estimated as TMS ether $(I^{180^{\circ}C}_{1\%JXR} = 1965)$

b Estimated as bis TMS ether $(I^{240^{\circ}C}_{1\%\ SE-30} = 3445)$

acetate, had m.p. 127-128°. The dipalmitate was prepared using the method of Mahadevan and Lundberg[280] for preparation of cholesterol esters. A mixture of 26-hydroxycholesterol diacetate (100 mg), methyl palmitate (135 mg), and sodium ethoxide (5 mg; prepared *in situ* from clean sodium and freshly dried ethanol) was heated at 90°C under vacuum (20 mm Hg) for 1 hour. The residue was extracted 3 times with 10 ml portions of benzene, and the filtered extract was concentrated to small volume. The product was purified by preparative TLC using benzene:ethyl acetate (20:1 v/v) as mobile phase. The $R_f$ of the dipalmitate was 0.75. Recrystallised from ethyl acetate, it had m.p. 56-57°C (Found: C,80.57; H,12.10. $C_{59}H_{106}O_4$ requires C,80.57; H,12.15). The infrared spectrum showed $\nu_{C=O}$ 1740 $cm^{-1}$, $\varepsilon_{max}$ 970 (*cf.* cholesteryl palmitate $\nu_{C=O}$ 1740 $cm^{-1}$, $\varepsilon_{max}$ 520), and no hydroxyl absorption. The yield was 28 mg (17%).

The constitution of the dipalmitate was confirmed by reductive cleavage, followed by estimation of the products by gas chromatography as follows: a weighed sample (2-6 mg) of the ester was reduced with lithium aluminium hydride. The products were extracted with 5 x 10 ml portions of ethyl acetate, trimethylsilylated and examined by GLC. The retention indices of the derivatives of the reduction products were compared with those of standards and the quantity of each component was determined. The results are given in Table 16.

The chromatographic behaviour of the isolated and synthetic esters was compared. The right-hand side of Fig. 40 shows the edge of a preparative thin-layer chromatogram which has been destructively sprayed and charred. The isolated di-esters were well separated from cholesterol esters ($R_f$ 0.89-0.95) and triglycerides ($R_f$ 0.27). The left-hand side of Fig. 40 demonstrates


FIG. 40. Thin-layer chromatograms comparing synthetic and extracted diesters of 26-hydroxycholesterol.
R.H.S.: Edge of preparative plate which has been destructively sprayed and charred after removal of the bulk of the diesters isolated from an aortal extract. $R_f$ cholesterol esters 0.93, diesters 0.54, $R_f$ triglycerides 0.27. Mobile phase: benzene: hexane (2:1 v/v).
L.H.S.: Analytical chromatogram comparing purified material from extract with authentic 26-hydroxycholesterol dipalmitate.
Lane A, authentic cholest-5-ene-3$\beta$,26 diol dipalmitate ($R_f$ 0.56);
Lane B, purified diesters from aortal extract ($R_f$ 0.54);
Lane C, standard cholesterol ($R_f$ 0.03) and triolein ($R_f$ 0.27).
Mobile phase: benzene:hexane (2:1 v/v).

the identical chromatographic behaviour of isolated and synthetic di-esters of 26-hydroxycholesterol. Quantitative analysis of the lipid extract derived from plaques from a severely diseased aorta, using the method described on page 53, demonstrated the presence of 120 ± 6 $\mu$g of 26-hydroxycholesterol di-esters per g of total lipid. A similar extract from an aorta affected only by "fatty streaks" yielded 63 ± 3 $\mu$g of di-ester per g. of total lipid.

## 3.6. ANALYSIS OF INTACT CHOLESTEROL ESTERS BY COMPLEMENTARY USE OF TLC AND HIGH TEMPERATURE GLC.

Detailed studies of cholesterol esters and triglycerides occurring in aortal tissue have hitherto involved hydrolysis of the esters and separate characterisation of the products. The principal advantage of this approach is the ease with which fatty acid esters may be separated and identified by GLC. However, for very small quantities of lipid, such as are obtained from cerebral and coronary arteries, such a procedure may result in contamination of the hydrolysis products with organic impurities such as plasticisers.[233-237]

The occurrence throughout the spectrum of arterial disease, of varying quantities of cholesterol esterified with polyunsaturated $C_{20}$ fatty acids may be of importance as a reflection of the changes in mural metabolism as atherosclerosis advances.[101] We have experienced some difficulty in identifying such esters because of their rather small abundance in the total cholesterol ester fraction, and have noted that use of the polyester phase PEGA, does not achieve gas chromatographic separation of methyl 8,11,14-eicosatrienoate from the ubiquitous plasticiser di-n-butyl phthalate.[237]

A convenient alternative to the hydrolysis procedure is direct examination of the cholesterol esters by purely chromatographic methods. The use of GLC at high temperature can effect separation of cholesterol esters and glycerides containing different numbers of carbon atoms.[188-191] TLC on layers impregnated with silver ion permits separation of lipids according to the number, position and configuration of their olefinic bonds.[141] It seemed likely that a combination of these techniques might be advantageous for characterisation of arterial cholesterol esters.

The major disadvantage in the use of GLC at temperatures in

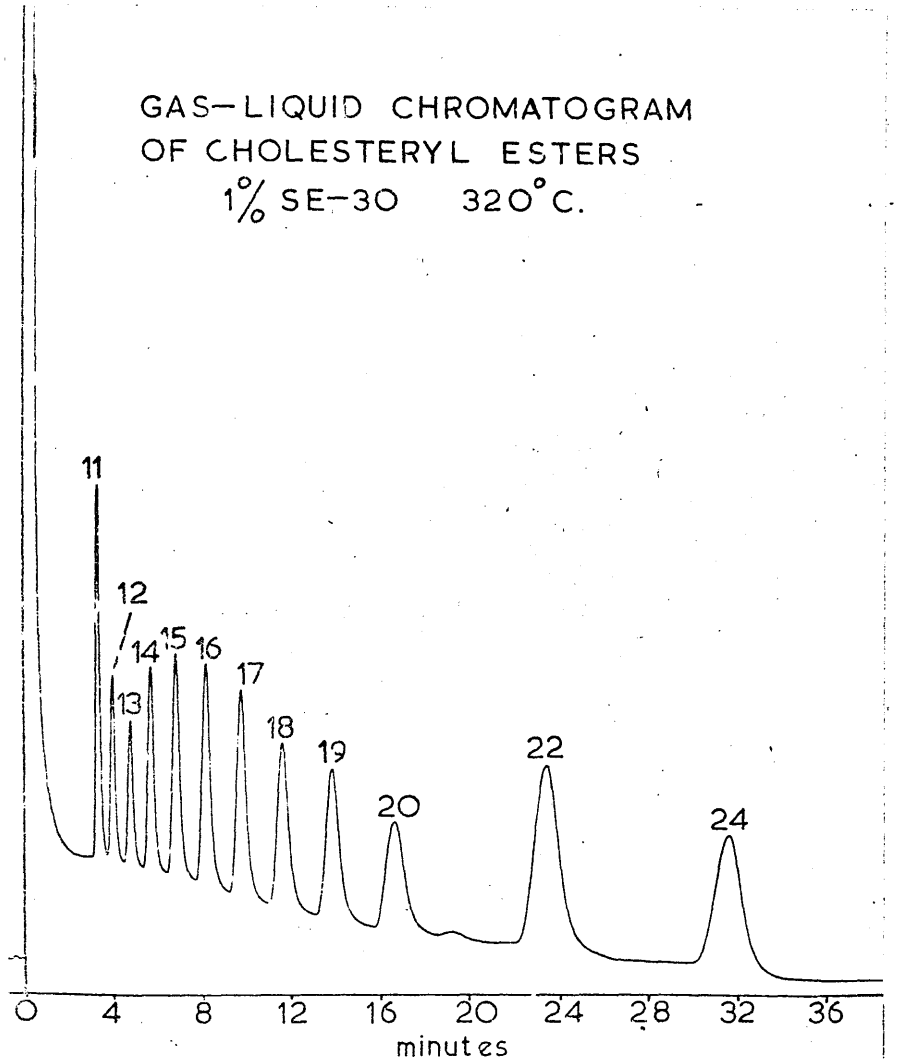



FIG. 41. Separation by GLC of a reference mixture of cholesterol esters of saturated straight chain fatty acids. The figures above each peak indicate the carbon content of the fatty acids in each ester.

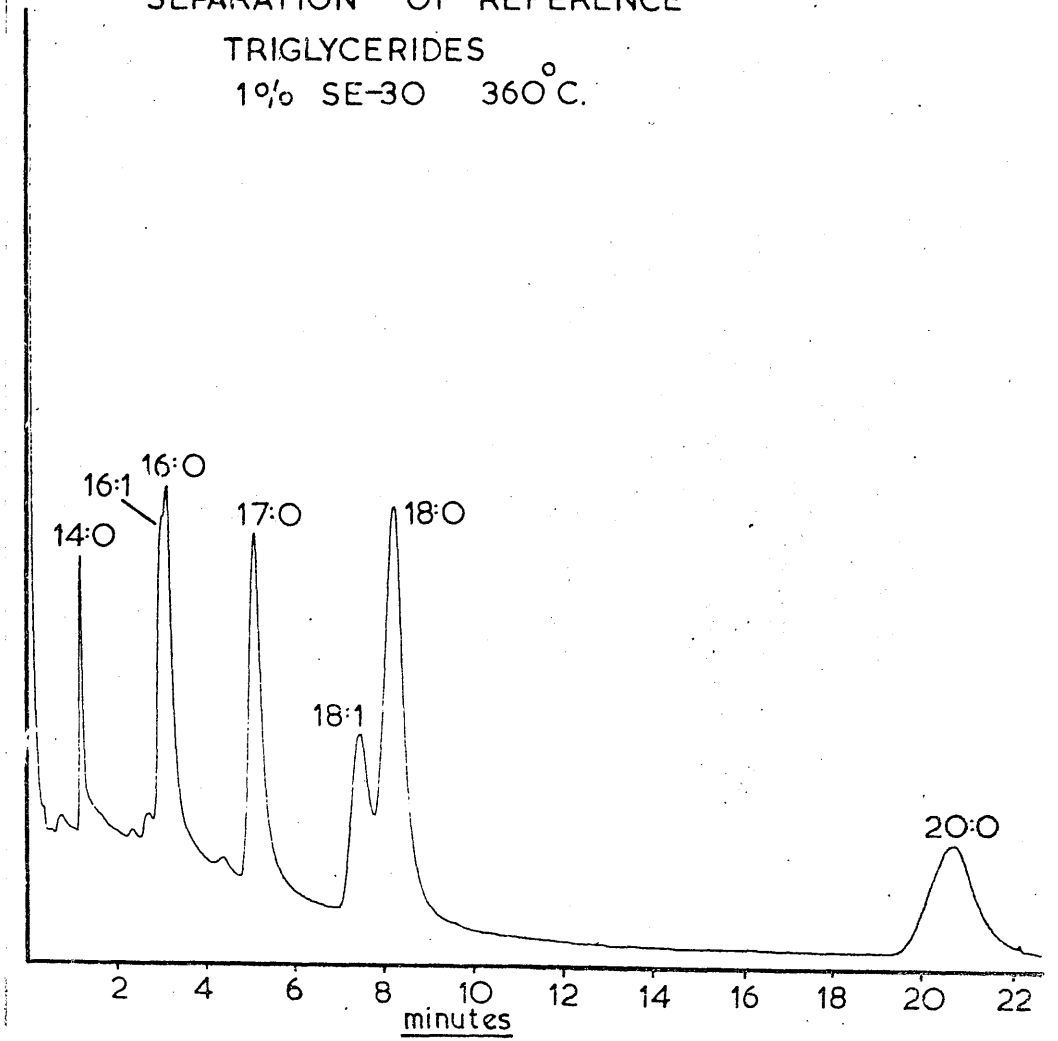



FIG. 42. Gas-chromatographic separation of a mixture of reference triglycerides. The figures above each peak designated the number of carbon atoms and the degree of unsaturation of the fatty acid with which glycerol is triply esterified.

excess of 250°C is the limited number of thermally stable liquid stationary phases, and most work of this type has utilised the siloxane[188-190] or poly-carborane siloxane[191] type polymers. In the present work the thermal stability of the methyl siloxane polymer SE-30 was investigated.

Column packings of 1% SE-30 on Gas Chrom Q were purchased from Applied Science Laboratories. The packed columns were conditioned by gradual increase in temperature from 100° to 360°C over a period of 5 days. The thermally "stripped" SE-30 was tested by analysis of a series of saturated cholesterol esters with acid chain lengths ranging from 11 to 24 carbon atoms. A typical chromatogram obtained by isothermal analysis at 320°C is reproduced in Fig. 41. Analysis of a limited number of triglycerides at 350°C demonstrated equally satisfactory results (Fig. 42).

There is a limited availability of suitable reference hydrocarbons for retention data standardisation of cholesterol esters. The esters may be assigned relative retention times with respect to an arbitrary standard, but inaccuracies in such a system frequently occur when standard and sample have considerably different retention times. However, a plot of Log retention time against the number of carbon atoms in the acid moieties of a series of saturated esters was found to be satisfactorily linear. On this basis retention data for other cholesterol esters may be standardised relative to the series of saturated esters, the samples being given index values analogous to the "equivalent chain length" values so widely used in GLC of fatty acid methyl esters. Retention data for available unsaturated cholesterol esters are given in Appendix I, and indicate that esters differing only by their degree of unsaturation may not be separated on this stationary phase. The usefulness of such a gas chromatographic system was demonstrated by its application to the analysis of aortal cholesterol esters.

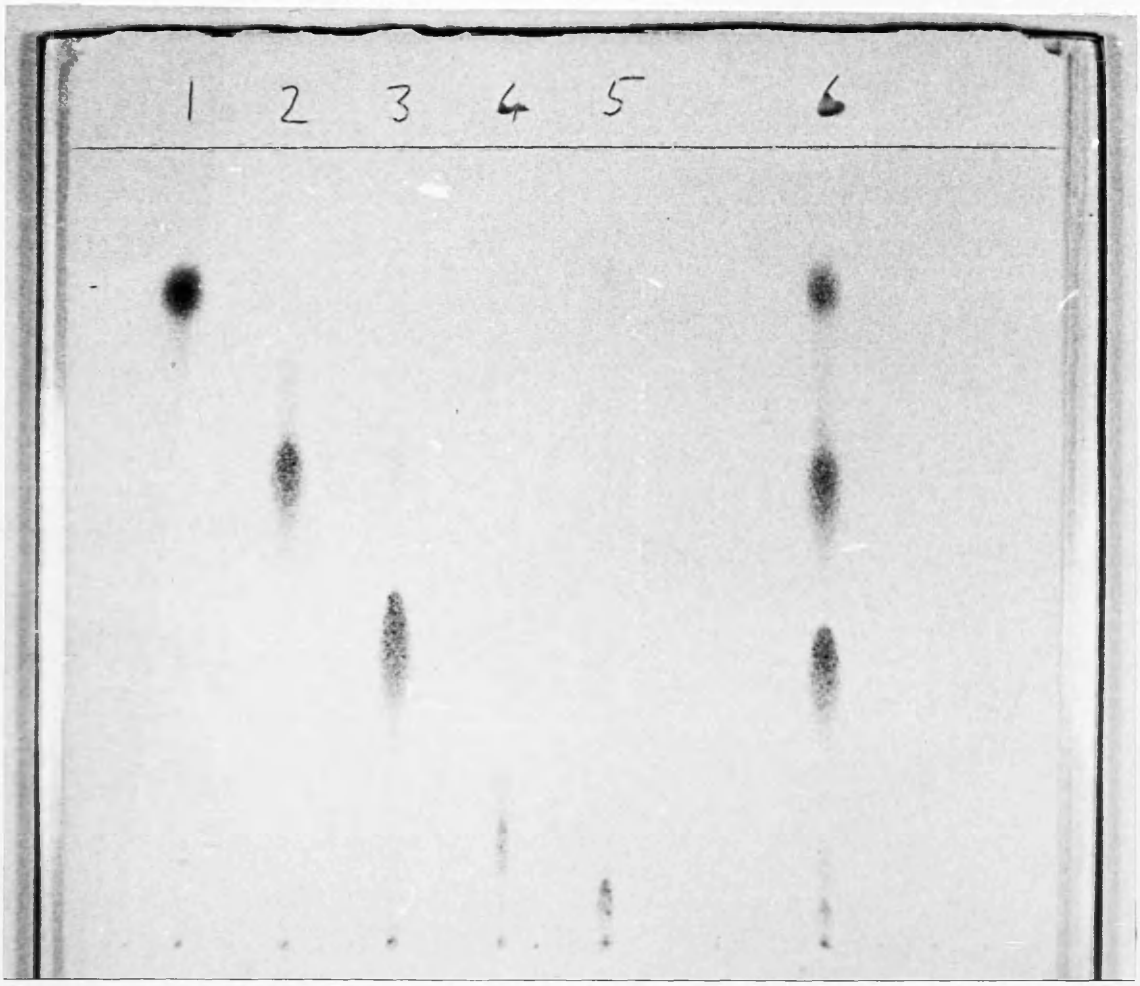



FIG. 43. Thin-layer chromatogram illustrating separation of arterial cholesterol esters on silver nitrate impregnated layers. Lane 1, cholesterol esters of saturated fatty acids; Lane 2, esters of monoenoic acids; Lane 3, esters of dienoic acids; Lane 4, esters of trienoic acids; Lane 5, esters of tetraenoic acids; Lane 6, total mixtures of esters. Kieselgel G-HR impregnated with 12% (w/w) $AgNO_3$. Mobile phase: benzene:hexane (2:1 v/v).

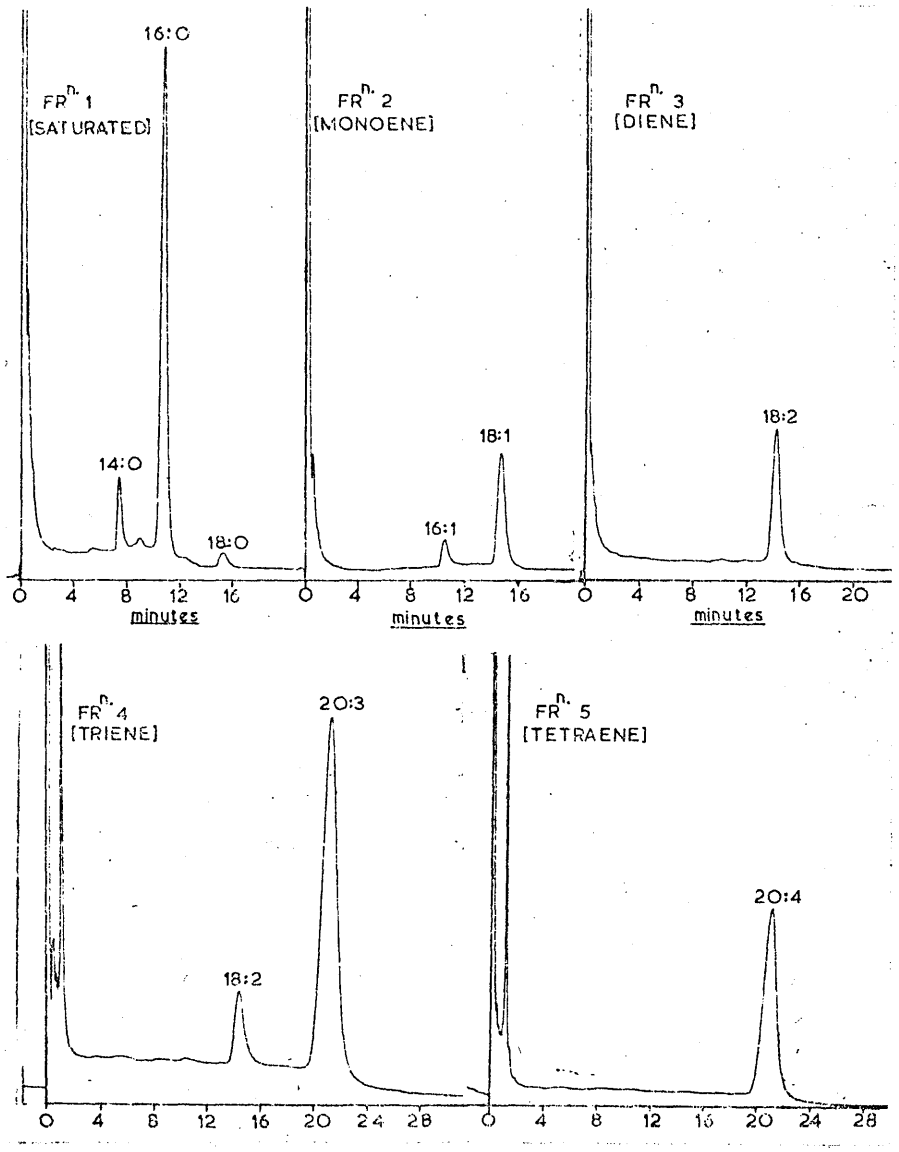



FIG. 44. Gas-liquid chromatograms of aortal cholesterol esters after fractionation by preparative TLC on silver nitrate impregnated layers. The figures above each peak designate the number of carbon atoms and the degree of unsaturation of the fatty acids with which cholesterol is esterified. 1% SE-30, 320°C.

Cholesterol esters were isolated from the total lipid extract of a severely diseased aorta, as described in Section 2.4. The esters were fractionated according to their degree of unsaturation by TLC on silica layers impregnated with silver nitrate (Section 2.5). Two mobile phases were employed for TLC. The first, benzene:hexane (2:1 v/v) affords separation of unsaturated, mono- and di-unsaturated esters (Fig. 43). Polyenoic esters were separated using a mobile phase of benzene:ethyl acetate (20:1 v/v).

Five fractions corresponding respectively to saturated esters, monoenes, dienes, trienes, and tetraenes were obtained. Each fraction was examined by GLC, and the chromatograms are presented in Fig. 44. The identity of the esters isolated in the triene and tetraene fractions were established by alkaline hydrolysis and examination of the acid methyl esters by GLC and GC-MS. Two peaks were observed in the chromatogram of methyl esters derived from the triene fraction. The smaller peak with $I^{10\%\ PEGA}_{200^{\circ}C} = 2570$ corresponded to methyl linoleate both by retention index and mass spectral correlations. The major peak $I^{10\%\ PEGA}_{200^{\circ}C} = 2805$ had a molecular weight of 320 indicative of a tri-unsaturated $C_{20}$ ester. The retention index of this compound did not coincide with that of reference methyl 11,14,17-eicosatrienoate, and is most probably the 8,11,14 isomer.[103] The single peak in the methyl ester fraction derived from tetraenoic cholesterol esters was identical both in retention index ($I^{10\%\ PEGA}_{200^{\circ}C} = 2840$) and molecular weight ($M^{+\cdot} = 318$) to methyl arachidonate. Cholesterol esters of arachidonic and eicosatrienoic acids have been identified by several investigations.[101,103] While we cannot claim that the chromatographic methods described above afford any considerable advantages over conventional analysis of mixtures of cholesterol esters, they may find application in studies of esters consisting of more than one sterol type. Further applications of sterol ester gas chromatography are being investigated.

# Section 4. DISCUSSION

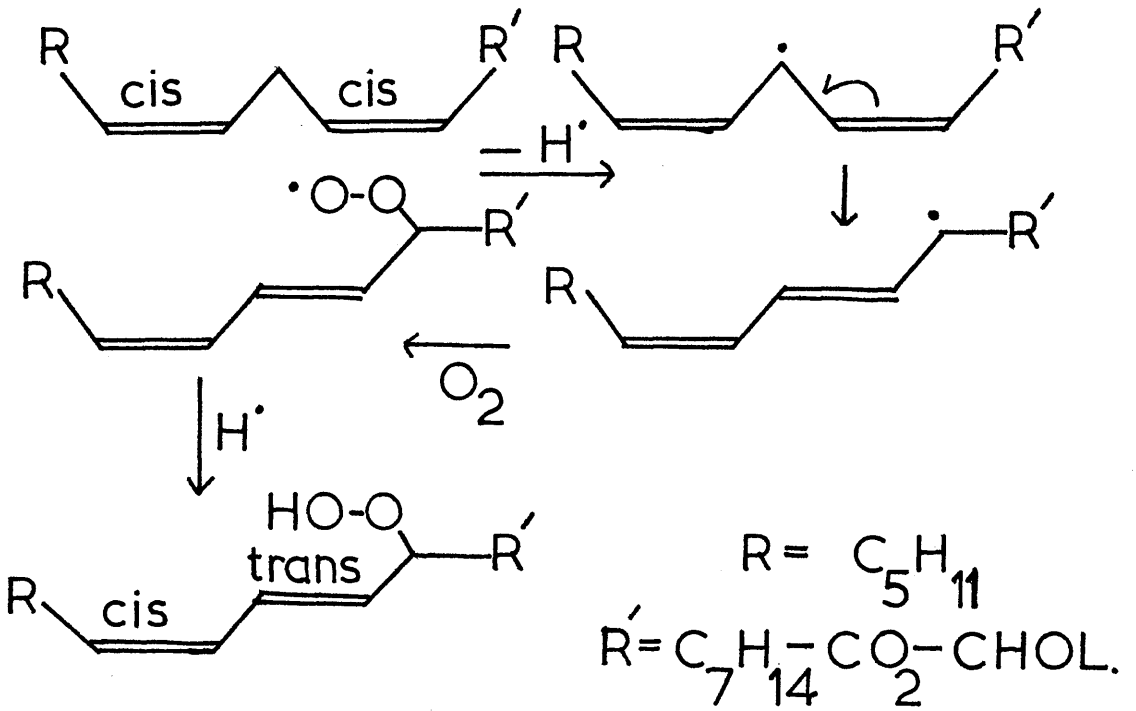



FIG. 45. Formation of linoleate hydroperoxides via autoxidation.

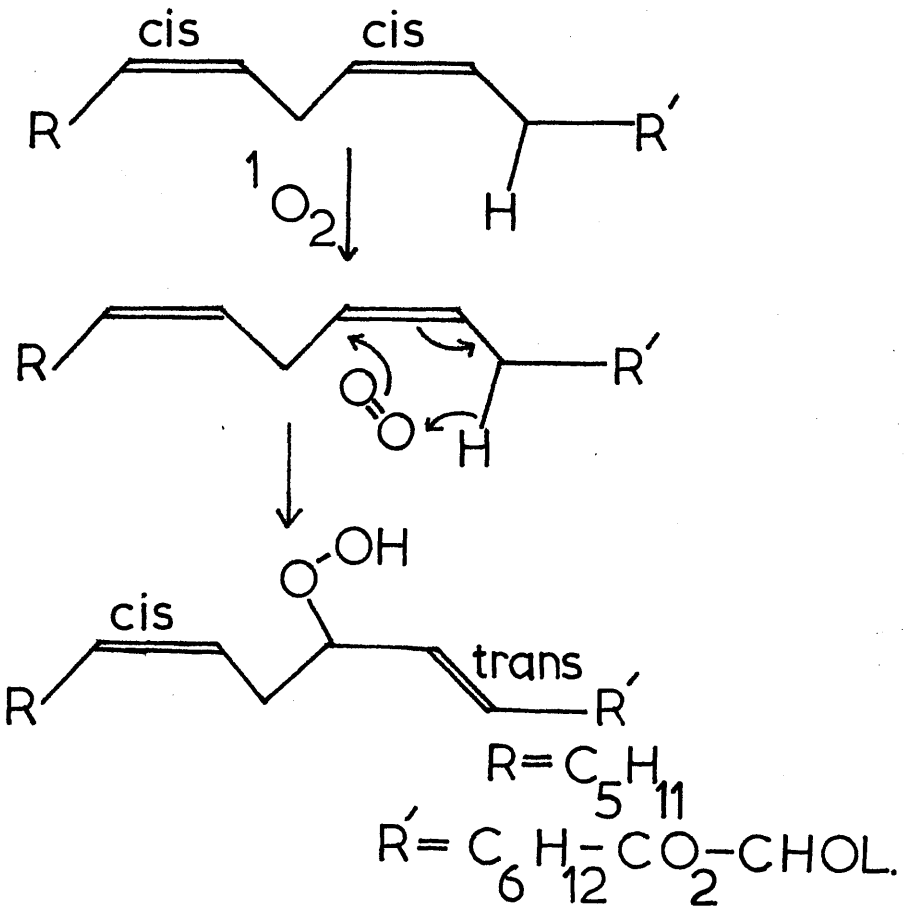



FIG. 46. Formation of linoleate hydroperoxides by the concerted 1,4 addition of singlet oxygen.

## 4.1. THE OXIDATION OF CHOLESTERYL LINOLEATE

Although studies of the oxidation of linoleic acid and methyl linoleate have been extensive, little work has been done on the oxidation of the cholesterol ester. Kaufmann and co-workers[282] prepared cholesterol esters of hydroxy- and keto-acids by autoxidation of cholesteryl linoleate in the presence of haemoglobin, and noted that in the production of these compounds, hydroperoxides exhibiting diene conjugation were produced. The products of oxidation were not fully characterised. Although the present reason for the preparation of oxygenated derivatives of cholesteryl linoleate was to obtain reference compounds with which we could compare material isolated from tissue extracts, a brief discussion of the mechanisms whereby the identified products are formed is relevant.

The products of cholesteryl linoleate autoxidation and photo-oxidation are dissimilar, but their formation may be rationalised on the basis of the mechanisms proposed for related oxidations of linoleic acid. Autoxidation of linoleate[283] proceeds via a free radical mechanism as shown in Fig. 45. Following radical formation by hydrogen abstraction, double bond migration occurs to give a conjugated diene system. Subsequent attack by molecular oxygen produces a peroxy radical, and abstraction of hydrogen from another molecule results in hydroperoxide formation at either $C_{(9)}$ or $C_{(13)}$ of the fatty acid chain. Hydroperoxides of linoleate produced by autoxidation all contain a conjugated diene system. In contrast, the hydroperoxides arising by photosensitised oxidation have been shown to arise in a completely different way. This reaction involves a concerted 1,4 addition of singlet oxygen ($^1O_2$) to the olefin (Fig. 46). Singlet oxygen may be produced by the action of a sensitiser (methylene blue in the present work) on ground state triplet oxygen. Since there are four olefinic carbon atoms in the acid, four positional isomers may occur. The concerted "ene"

mechanism has been questioned in favour of a two step perepoxide reaction[284,285] but more recent work[286] substantiates the former: the products expected from linoleate photo-oxygenation would be the same in both cases. As illustrated in Fig. 46, the migrating double bond may or may not give rise to a conjugated system, and a mixture of conjugated and non-conjugated hydroperoxides will be produced.

The structures predicted by the above mechanisms agree very well with those assigned in Section 3.1. The trans,trans isomers of the 9- and 13-hydroperoxy-esters, although not predictable by mechanistic considerations are to be expected by analogy with previous work on linoleic acid, in which appreciable quantities of these isomers result from prolonged autoxidation at room temperature. It is known that conjugated acids stereomutate more readily than the non-conjugated compounds. The former react readily with iodine in the presence of light at room temperature, whereas in the case of the latter stereomutation generally requires heating with selenium (~200°C) or with a mixture of nitric acid and sodium nitrate (~65°C). Accordingly, the non-conjugated 10- and 12-hydroperoxy octadecadienoates obtained by photo-oxidation of cholesteryl linoleate are almost certainly the cis,trans isomers analogous to those reported by Cobern[259] et al. and Hall et al.,[260] and would not be expected to exhibit stereomutation at room temperature. The stereochemistry of the olefinic bonds follows from the proposed mechanism (Fig. 46), if it is assumed that a cis olefinic bond which has migrated adopts a trans configuration.

Photo-sensitised linoleate oxidation should produce equal quantities of conjugated and non-conjugated hydroperoxides. In the present work, however, GLC of the reduction products indicated an excess of the conjugated species, implying that a free radical reaction also occurs. It seems likely that the hydroperoxides arising via photo-oxygenation may be the initiators of the free

radical process.[316]

Both autoxidation and photo-oxidation of cholesteryl linoleate yielded small quantities of epimeric 7-hydroperoxycholesterol linoleates. 7-Hydroperoxycholesterols may be readily obtained by photo-oxygenation of cholesterol,[287] but it has been demonstrated that polyunsaturated acids esterified with cholesterol give rise to a higher rate of steroid oxidation than either free cholesterol or its saturated or mono-unsaturated esters.[69] An intramolecular reaction has been proposed,[65,69] in which a free radical present on the linoleate moiety of the ester abstracts hydrogen from the allylic methylene at $C_{(7)}$ in the steroid nucleus. Subsequent attack by oxygen and hydrogen abstraction by the resultant peroxy radical yields 7-hydroperoxycholesteryl linoleate.

Recently other examples of a similar type of remote intramolecular oxidation of the steroid nucleus have been described.[288,289,333] Breslow and co-workers[288] have shown that photolysis of the p-benzoyl-β-phenyl propionic acid ester of 5α-cholestan-3α-ol introduces functional groups into the stanol at $C_{(12)}$ or $C_{(14)}$.

## 4.2. OXYGENATED STEROL ESTERS IN TISSUE

### 4.2.1. The occurrence of hydroxy sterol esters.

It has now been shown that three distinct, though structurally similar types of oxygenated sterol esters occur in severely diseased human aorta. These esters comprise cholesterol esterified with hydroxy-, hydroperoxy- and keto-octadecadienoic acids, and small quantities of esterified oxygenated sterols are found associated with each group. We have demonstrated that similar compounds may be prepared *via* cholesteryl linoleate autoxidation.

If these compounds play any pathogenic role in atherosclerosis, variations in their concentrations throughout various stages of the disease might be expected. Accordingly a survey was undertaken to establish whether such a relationship exists. The hydroxy-esters were chosen as representatives of the oxygenated esters, since they are the predominant type, and because at the time the structures of the hydroperoxy- and keto-esters had not beeen fully determined. Prior to the survey, doubts that the hydroxy-esters isolated in extracts might arise by oxidation of cholesteryl linoleate during the extraction and chromatographic procedures were dispelled by an experiment in which cholesteryl 1-$^{14}$C -linoleate was added to an extract, and the activity of the isolated hydroxy esters was determined. It was concluded that not more than 2% of the isolated hydroxy-esters could have been derived from cholesteryl linoleate during the course of the experiment.[54]

Aortas collected within 24 hours of death were visually graded into three categories: fatty streaks, fibrous atheromas and ulcerated plaques. It will be noted (Table 11) that the subjects in all groups covered a wide range of age and clinical diagnosis. In most cases only plaques of a single type were collected from one aorta, but Case 9 afforded enough fibrous atheromas and ulcerated

plaques to permit measurements on both types. The observed differences in concentration of the hydroxy-esters of cholesterol in lesions representing different stages in the progression of atherosclerosis are quite striking (Table 12). None of these compounds was detected in fatty streaks: this indicated that the concentration was less than 1 $\mu g/g$ of lipid. In fibrous plaques the concentration averaged 89 $\mu g/g$, and in ulcerated plaques this rose to 335 $\mu g/g$. It is of interest that in Case 9 the hydroxy-ester concentration in fibrous plaques was higher than in other cases, but still only half those of ulcerated lesions.

The significance of these observations must be speculative. If ulcerated plaques merely represent atheromas that have been developing for a long time, then higher concentrations of these compounds might be explained by accumulation over a number of years. However, the rate of accumulation would have to be considerably greater than those of the major lipid classes, and it may be relevant to consider possible processes whereby such relative increases of hydroxy-esters could occur, for example:

(1) The hydroxy-esters form a constant proportion of the lipids deposited into arterial tissue from blood, but are metabolically more inert than the major lipid classes, and hence their relative concentrations rise with increased deposition.

(2) The hydroxy-esters in blood increase in parallel with arterial degeneration, and are consequently deposited in larger quantities in advanced lesions.

(3) The hydroxy-esters are formed gradually <u>in situ</u>. Their higher concentrations in advanced lesions may result from higher rates of production in severely diseased tissue, or because their rates of metabolism are less than those of the other lipid classes.

(4) The hydroxy-esters are formed episodically as a result of thrombosis or other metabolic derangements.

The present work has not as yet been extended to analysis of blood, and we are unable to say whether the hydroxy-esters arise by infiltration or are produced by local synthesis. The close association of these compounds with cholesteryl linoleate may explain why detectable quantities of hydroxy-esters do not occur in fatty streaks, since most of the cholesteryl esters in the latter probably arise by local synthesis and contain little linoleate.[101,102] In contrast, advanced lesions contain large quantities of extracellular cholesteryl linoleate (presumably derived from blood), which may be the substrate for the in situ synthesis of the hydroxy-esters.

4.2.2. The possible role of Hydroperoxides.

Although we can offer no direct evidence, it seems probable that the hydroperoxy-esters isolated from diseased arteries are derived from cholesteryl linoleate, and that the hydroperoxides are in turn the precursors of the hydroxy- and keto-esters. In accordance with the ease with which the latter compounds may be prepared chemically from the hydroperoxides, it has been found that methyl linoleate hydroperoxides administered to rabbits are metabolised with the formation of esters of hydroxy-acids.[290,291,325]

Christophersen[262] and O'Brien and Little[292] have demonstrated that hydroperoxides of linoleic acid and methyl linoleate may be reduced to the corresponding hydroxy compounds by a glutathione peroxidase isolated from rat liver. It has been suggested[262] that a rapid enzymic reduction of hydroperoxides breaks the catalytic chain reaction of autoxidation and so protects the vital cellular compounds from the effect of peroxides.

The origin and significance of hydroperoxides in aortal tissue is a matter of conjecture. Glavind and co-workers[293] demonstrated increasing amounts of hydroperoxides with advancing atherosclerosis.

Subsequent work has indicated that most of the peroxides observed in arterial tissue after dissection probably occur as artefacts, but does not exclude the possibility that lipid peroxides play a role in atherogenesis.[294] Studies on the toxicity of lipoperoxides suggest that only trace quantities in the tissues are tolerated. Fatty acid hydroperoxides are highly toxic when administered intraperitoneally,[295,296] though they appear to be less harmful when administered orally.[297,298] During carbon tetrachloride and yellow phosphorus[299] intoxication experiments in rats, peroxidative decomposition of hepatic microsomal lipid occurs, and may be an important step in the abnormal accumulation of fat. Since the membrane systems of the cell contain large quantities of polyunsaturated fatty acids, lipid peroxidation can obviously have detrimental effects. Besides undergoing the autocatalytic reaction with unsaturated fatty acids,[283] the highly reactive peroxide may react with other intracellular substances such as proteins[300,301] and enzymes.[302,303]

Atherogenesis may be enhanced by substances capable of irritating the arterial wall, and it has been suggested that irritants may arise by the reaction of molecular oxygen with serum or arterial lipids.[304] Experiments on animals have shown that the severity of induced atherosclerosis is increased by feeding of cupric acetate[304,306] (a good catalyst for the oxidation of lipids by molecular oxygen), and decreased by oxidation inhibitors such as ascorbic acid, thiamine,[305] $\alpha$-tocopherol and thyroxine Analyses of serum in patients with and without history of myocardial infarction has indicated a correlation between heart disease and elevated serum copper levels.[306,307]

In a study of the possible role of peroxides in atherosclerosis, Johnson[308] concluded that most, if not all, of the peroxides detected in atherosclerotic plaques were formed in the interval between

death and analysis. It was also indicated that whereas arterial tissue obtained during surgery demonstrated peroxides associated only with the phospholipids, peroxides of all the major lipid classes were present in arterial lesions of tissue obtained at post mortem examination. Although the structures of the peroxidised cholesterol esters were not elucidated, it seems likely that they are similar to those found in the present study. As yet we have been unable to detect peroxides of the other lipid classes. However we have recently observed that diseased arterial tissue obtained during surgery and examined analytically within 12 hr of dissection contains no detectable hydroperoxides but substantial quantities of hydroxy-esters. It seems probable that hydroperoxides occurring in arterial lesions are reduced to the corresponding hydroxy-esters, thereby keeping the concentrations of the toxic hydroperoxides at very low levels. Those isolated from aortas obtained at post mortem examination are almost certainly formed in the interval between death and analysis. It is tempting to suggest that the mechanism whereby hydroperoxides are reduced does not function after death, and that this results in their accumulation. However, peroxidation of plaque lipid occurs readily in vitro, and some of the isolated hydroperoxides almost certainly occur as artefacts between post mortem examination and tissue extraction. (See footnote on page 104)

4.2.3. General significance of the present observations.

There is a view of the pathogenesis of atherosclerosis which holds that the disease should be considered as a mixture of processes, each with distinguishable morphology, natural history and biochemical abnormalities.[309] The observed differences in concentration of the hydroxy-esters may be significant in this context. In particular, they indicate what may be an important chemical distinction between fatty streaks and plaques.

The toxic properties of peroxides are well established. Infusion of small quantities of methyl linoleate hydroperoxides induces symptoms of vitamin E deficiency in chicks[310] and haemolysis in rabbits.[311] Lipid peroxides have also been implicated in ageing, carbon tetrachloride hepatotoxicity[297,298] and ionising radiation damage,[300,312,323,324] and a recent publication indicates a relationship between peroxide formation and inflammation.[313] It is possible that arterial hydroperoxides or their derivatives may be concerned with the biochemical changes which result in necrosis, inflammation and ulceration of lesions, features which distinguish fibrous plaques from fatty streaks.[4,314,315]

Recent work indicates that as much as 20% of the hydroperoxides isolated from severely diseased aortas may be formed from cholesteryl linoleate during the extraction and chromatographic procedures.

## 4.3. CHOLESTANOL AND ATHEROSCLEROSIS

The biological significance of cholestanol is still obscure. This compound is able to meet or partially replace the requirements of cholesterol for growth in various insects and micro-organisms,[317,334] and cholestanol formation has been shown to occur in a variety of mammalian tissues. Rat liver homogenates convert 5α-cholestan-3-one to cholestanol,[318] and the demonstration that the adrenals, liver and intestine of germ-free guinea pigs convert cholesterol to cholestanol rules out the possibility that this conversion could occur solely through intestinal flora.[319] Human subjects are also able to transform cholesterol to cholestanol[320] and human aortic segments removed at surgery or post mortem have been shown capable of synthesising cholestanol from acetate or mevalonic acid.[268]

The earlier hypothesis that this sterol could be involved in the pathogenesis of atherosclerosis was based on the apparent presence of relatively large concentrations of cholestanol in diseased human aorta.[107,108] With the development of more sensitive analytical techniques, the actual concentrations of cholestanol in arteries has been shown to be much lower.[104] The present study is the first in which the presence of cholestanol in diseased arterial tissue has been rigorously proved by combined gas chromatography-mass spectrometry. The quantities of cholestanol isolated were found to be in the range 0.3-0.5% of the total sterols in the extracts. A recent paper by Fumagalli and co-workers[111] has shown that there is no correlation between the aortal concentrations of cholestanol and the degree of atherosclerosis. The quantities found add confirmation to our own and to previous work. Despite the fact that cholestanol was one of four sterols found particularly toxic towards organ cultures of rabbit aorta,[321] there seems to be no substantial evidence that this stanol plays a significant part in the pathogenesis of atherosclerosis.

## 4.4. DIESTERS OF 26-HYDROXYCHOLESTEROL

The cholesterol esters from advanced aortal lesions are characteristic in that the major fatty acid to which cholesterol is esterified is linoleic acid, which accounts for 35-45% of the total esterified acids.[100] In the present case, comparison of the fatty acids esterified with cholesterol and those in diesters of 26-hydroxycholesterol has shown that the latter contain a strikingly lower proportion of linoleic acid (12 ± 8%).

It has recently been shown that unesterified 26-hydroxycholesterol occurs both in normal aorta and in aortas representing all stages of atherosclerosis.[111,128] It was also noted that the concentration of 26-hydroxycholesterol appeared to rise in parallel with that of cholesterol, and it has been suggested[128] that the accumulation of this minor sterol may be a result of the progression of atherosclerosis. The biosynthesis of 26-hydroxycholesterol from cholesterol has been demonstrated in liver tissue preparations.[277,322] The present work has not been extended to investigate the occurrence of 26-hydroxycholesterol or its esters in tissue other than aortal intima: however, other investigators[115] have recorded the absence of detectable quantities of this sterol from human serum and human liver tissue.

The cholesterol esters from extracellular lipid of the earliest lesions contain large quantities of linoleate, and are similar in this respect to the cholesterol esters of serum lipid. In intracellular cholesterol esters the major ester is oleate and much smaller quantities of linoleate are found.[90,101,102] This implies that extracellular lipid arises largely by infiltration from blood, whereas intracellular lipid is of different origin and may perhaps be produced by local synthesis.[90,101,102] Lipids of advanced lesions of the type examined in the present study are predominantly extracellular and the cholesterol esters contain the expected large proportions of linoleate. In

contrast the major acids represented in the diesters of 26-hydroxycholesterol are palmitate and oleate. It is possible, therefore, that these diesters are present mainly as intracellular constituents formed by local synthesis.

The recent surveys[111,128] in which the aortal concentrations of 26-hydroxycholesterol were shown to be correlated with increasing severity of atherosclerosis possibly suggests that this sterol plays a role in human atherogenesis. The nature of such a role will no doubt be the subject of further investigation.

# APPENDICES

APPENDIX I. Chromatographic Data

| Acid methyl esters | Δ | 10%PEGA, 180°C | | 1%SE-30, 172°C | | 1%OV-17, 172°C | |
|---|---|---|---|---|---|---|---|
| | | I | ecl | I | ecl | I | ecl |
| MYRISTATE | | 2085 | 14.00 | 1705 | 14.00 | 1810 | 14.00 |
| PALMITATE | | 2280 | 16.00 | 1910 | 16.00 | 2020 | 16.00 |
| PALMITOLEATE | 9 cis | 2325 | 16.45 | 1880 | 15.70 | 2110 | 16.85 |
| STEARATE | | 2480 | 18.00 | 2115 | 18.00 | 2225 | 18.00 |
| OLEATE | 9 cis | 2510 | 18.30 | 2065 | 17.50 | 2225 | 18.00 |
| LINOLEATE | 9,12 all cis | 2570 | 18.90 | 2065 | 17.50 | 2230 | 18.05 |
| LINOELAIDATE | 9,12 all trans | 2565 | 18.85 | 2080 | 17.65 | 2240 | 18.15 |
| LINOLENATE | 9,12,15 all cis | 2650 | 19.70 | 2065 | 17.50 | 2250 | 18.25 |
| ARACHIDATE | | 2680 | 20.00 | 2320 | 20.00 | 2425 | 20.00 |
| EICOSENOATE | 11 cis | 2710 | 20.30 | 2280 | 19.60 | 2420 | 19.90 |
| EICOSADIENOATE | 11,14 | 2760 | 20.90 | | | | |
| EICOSATRIENOATE | 11,14,17 | 2850 | 21.70 | 2270 | 19.50 | 2455 | 20.20 |
| ARACHIDONATE | 5,8,11,14 | 2840 | 21.60 | 2240 | 19.10 | 2420 | 19.90 |
| BEHENATE | | 2880 | 22.00 | 2525 | 22.00 | 2630 | 22.00 |
| LIGNOCERATE | | 3080 | 24.00 | 2730 | 24.00 | 2835 | 24.00 |

TABLE 17. Gas chromatographic retention data for reference fatty acid methyl esters.

| Cholesteryl Ester | Relative Carbon No. 1% SE-30, 320°C | $R_f$ Benzene:Hexane (2:1 v/v) |
|---|---|---|
| MYRISTATE | 14.00 | 0.69 |
| PALMITATE | 16.00 | 0.70 |
| PALMITOLEATE | 15.95 | 0.47 |
| STEARATE | 18.00 | 0.68 |
| OLEATE | 18.00 | 0.45 |
| ELAIDATE | 17.95 | 0.57 |
| LINOLEATE | 17.80 | 0.20 |
| LINOELAIDATE | 17.75 | 0.47 |
| LINOLENATE | 17.80 | 0.03 |
| ARACHIDATE | 20.00 | 0.69 |
| EICOSATRIENOATE | 19.70 | 0.06 |
| ARACHIDONATE | 19.60 | 0.01 |
| BEHENATE | 22.00 | |
| ERUCATE | 21.70 | |
| LIGNOCERATE | 24.00 | |
| NERVONATE | 23.60 | |
| 14-METHYL HEXADECANOATE | 16.95 | 0.71 |

TABLE 18. Thin-layer and gas chromatographic retention data for reference cholesteryl esters. The TLC adsorbant was Kieselgel G-HR impregnated with 12% (w/w) silver nitrate.

| Sterol TMS ether | $I^{240^{\circ}}_{1\%\ SE-30}$ | $R_f$ Benzene: Hexane (1:2 v/v) | Colour with ceric sulphate reagent |
|---|---|---|---|
| CHOLEST-5-EN-3β-OL | 3090 | 0.53 | Red |
| CHOLEST-5-EN-3α-OL | 2995 | 0.66 | Red |
| 5α-CHOLESTAN-3α-OL | 3020 | 0.85 | Lt. Brown |
| 5α-CHOLESTAN-3β-OL | 3090 | 0.54 | Lt. Brown |
| 5β-CHOLESTAN-3α-OL | 3020 | 0.78 | Lt. Brown |
| 5β-CHOLESTAN-3β-OL | 3015 | 0.83 | Lt. Brown |
| CHOLEST-5-ENE-3β,7α-DIOL* | 3090 | 0.55 | Blue |
| CHOLEST-5-ENE-3β,7β-DIOL * | 3220 | 0.18 | Blue |
| CHOLESTANE-3β,6α-DIOL * | 3250 | 0.33 | Brown |
| CHOLESTANE-3β,6β-DIOL * | 3200 | | |
| CHOLESTANE-5α,6α-DIOL * | 3140 | 0.65 | Brown |
| CHOLESTANE-5α,6β-DIOL * | 3105 | | |
| CHOLESTANE-3β,5α,6β-TRIOL * | 3340 | 0.10 | Brown |
| ** | 3225 | 0.68 | Brown |
| CHOLEST-5-EN-3β-OL-7-ONE | 3220 | 0.26 | Yellow |
| CHOLEST-5-ENE-3β,20α-DIOL * | 3275 | 0.63 | Lt. Brown |
| CHOLEST-5-ENE-3β,24-DIOL * | 3360 | 0.43 | Pink-Brown |
| CHOLEST-5-ENE-3β,25-DIOL * | 3390 | 0.41 | Brown |
| CHOLEST-5-ENE-3β,26-DIOL * | 3440 | 0.31 | Pink |

TABLE 19. Thin-layer and gas chromatographic data for reference sterol TMS ethers.

* bis-TMS

** tris-TMS

APPENDIX II. Relevant data for di-n-butyl phthalate.

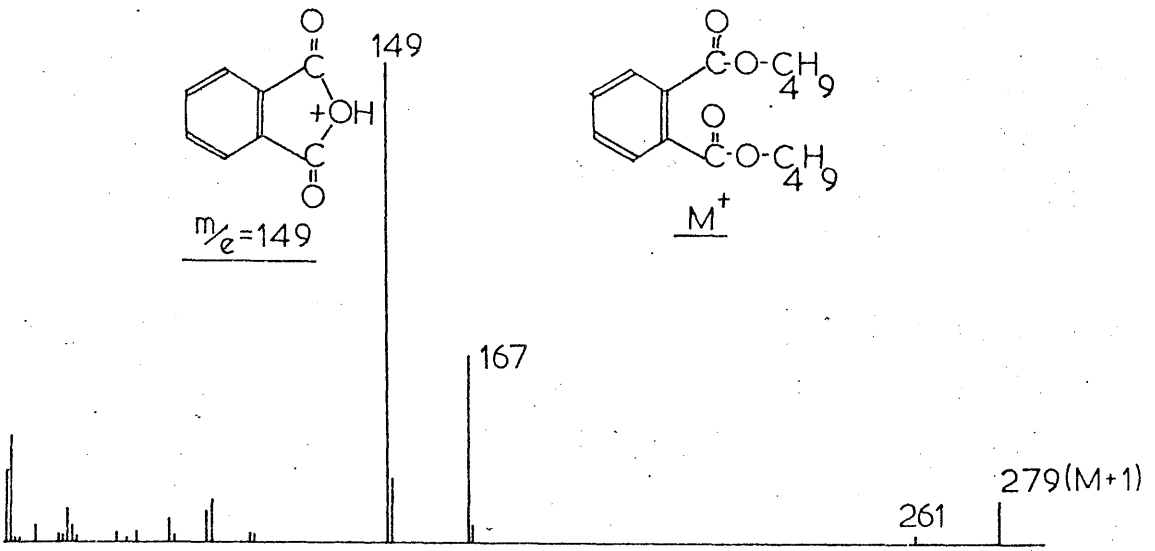



FIG. 47. Mass spectrum of Di-butyl phthalate.

| Retention Index | Column | Temperature (°C) |
|---|---|---|
| 2820 | 10% PEGA | 200 |
| 2225 | 1% OV-17 | 200 |
| 1910 | 1% SE-30 | 160 |

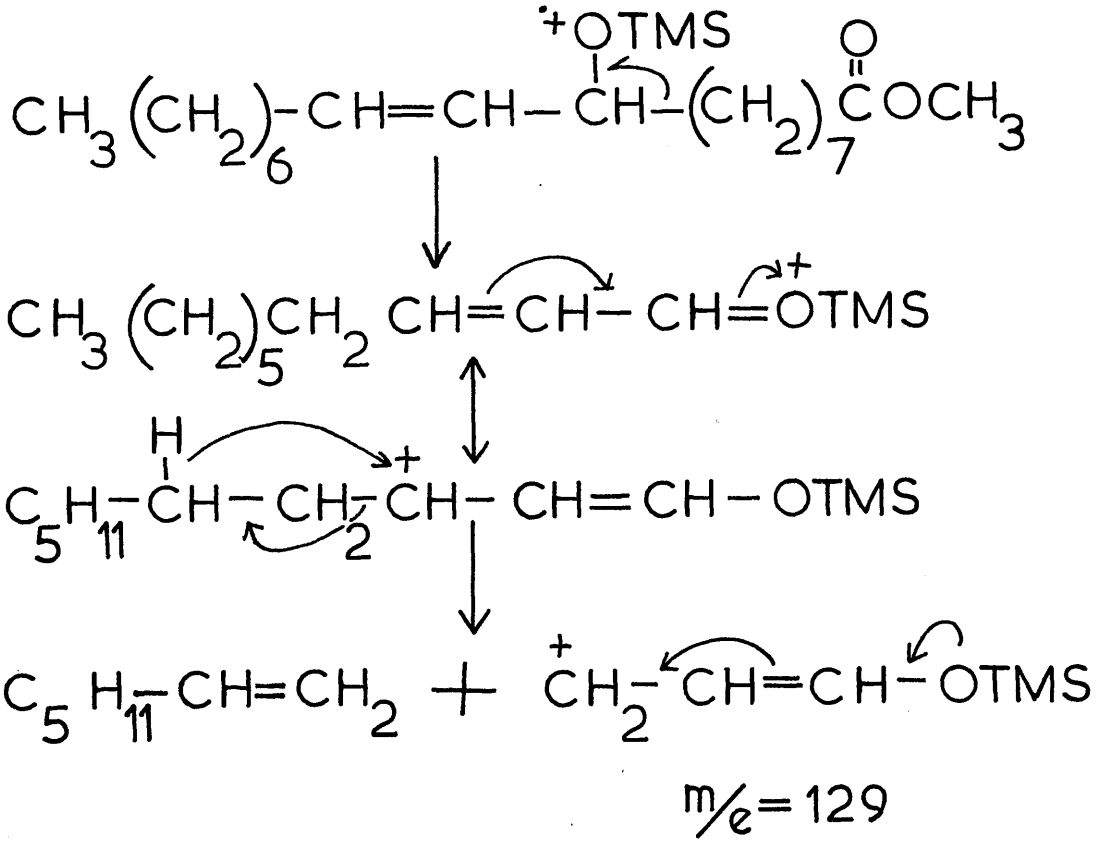
| Material tested | Quantity of di-n-butyl phthalate |
|---|---|
| Diethyl ether (reagent grade) | 16 µg/l. |
| Diethyl ether (anhydrous) | 70 µg/l. |
| Diethyl ether (Pronalys) | 82 µg/l. |
| Diethyl ether (anhydrous) redistilled | 2 µg/l. |
| Ethyl acetate (AnalaR) | 28 µg/l. |
| Ethyl acetate redistilled | n.d. |
| Ethyl acetate "Nanograde" | n.d. |
| Chloroform (AnalaR) | 25 µg/l. |
| Chloroform (Nanograde) | n.d. |
| Hexane redistilled | n.d. |
| Hexane Nanograde | n.d. |
| Ethanol Absolute | 2 µg/l. |
| Silicic Acid 100 mesh | 15 µg/100g |
| M N-Kieselgel GHR | 10 µg/100g |
| $Na_2SO_4$ anhydrous AnalaR | 63 µg/100g |

TABLE Proportions of di-n-butyl phthalate in solvents and reagents used in the present work.

n.d. indicates undetectable quantities of di-butyl phthalate, i.e. concentrations of less than 0.1 µg/l.

## APPENDIX III. The origin of the ion m/e = 130 in the mass spectra of conjugated octadecadienediol bis TMS ethers.

The mass spectra of octadecadienediols bis TMS ethers derived from autoxidised cholesteryl linoleate contain an ion of high relative abundance at m/e = 130. This ion does not occur in the mass spectra of trimethylsilylated octadecanediols or non-conjugated octadecadienediols. However, the mass spectra of the latter show an intense ion at m/e = 129 which has been previously observed in the mass spectra of trimethylsilylated hydroxy acid esters derived from autoxidised methyl oleate, and the following mechanism has been proposed.[206]



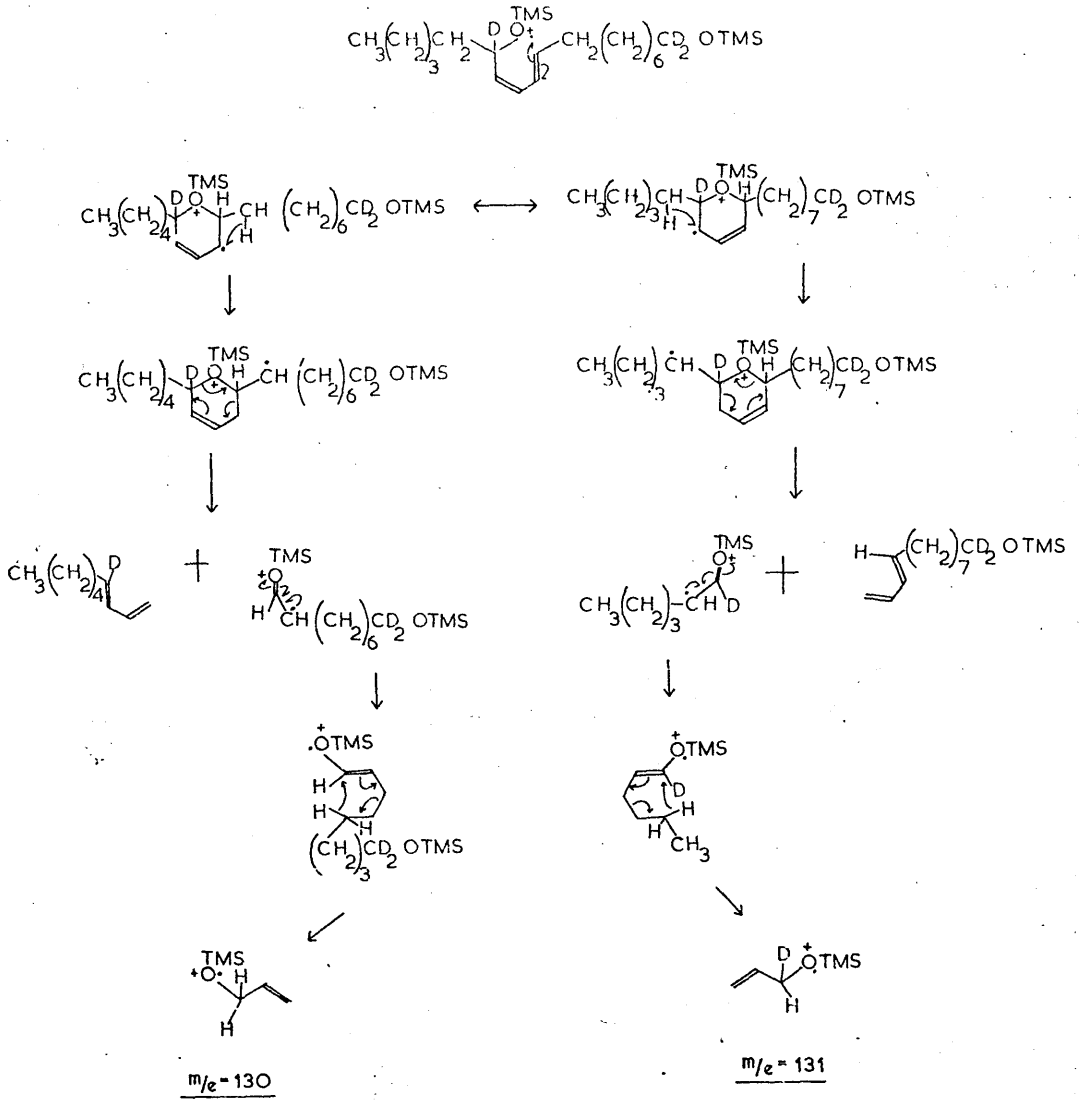



FIG. 48. Postulated mechanism accounting for the occurrence of the ion of m/e = 130 in the mass spectra of conjugated octadecadiene-diol bis TMS ethers.

In the present work labelling of 9,11-octadecadiene-1,13-diols with perdeuterated bis-trimethylsilyl acetamide resulted in an increase of m/e = 130 to m/e = 139 in the mass spectra of the resultant TMS ethers (Fig. 9). This indicates the presence in the fragment of an intact trimethylsilyloxy group, and requires a molecular formula of $C_3H_5OTMS^{+\bullet}$. Deuterium labelling of the $C_{(1)}$ methylene attached to the primary hydroxyl function by reduction of hydroperoxy-esters of cholesteryl linoleate with lithium aluminium deuteride, and subsequent mass spectral analysis of the TMS ethers gave no rise in the mass of m/e = 130.

Thus it seems probable that the occurrence of this ion requires a system comprising a trimethylsilyloxy function allylic to a pair of conjugated olefinic bonds.

Reduction of keto-esters derived from autoxidised cholesteryl linoleate with lithium aluminium deuteride produces octadecadienediols with a deuterium atom attached to the $C_{(9)}$ or $C_{(13)}$ carbon atom holding the secondary alcohol group. The mass spectra of the TMS ethers of these compounds (Fig. 18) shows not only an ion at m/e = 130 but also an ion of almost equal intensity at m/e = 131. In the mass spectra of non-labelled 9,11-octadecadiene-1,13-diol bis TMS ethers m/e = 130 is the base peak, with m/e = 355 having a relative abundance of about 90%. However, the mass spectra of the labelled derivatives from keto-esters have a base peak of m/e = 358, with m/e = 130 having a relative abundance of 63%. If the relative intensities of the ions at m/e = 130 and 131 are added, then the intensity of m/e = 358 relative to this sum is 85%. The results strongly suggest that incorporation of a deuterium atom in the derivatives from keto-esters results in a "splitting" of the ion at m/e = 130 in the non-labelled compound into a pair of ions at m/e = 130 and m/e = 131. A mechanism rationalising these findings is proposed in Fig. 48.

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