APPLICATIONS OF GAS CHROMATOGRAPHY AND MASS SPECTROMETRY TO STEROIDS AND OTHER BIOLOGICALLY IMPORTANT MATERIALS

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C O N T E N T S

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Summary

General

Techniques have been developed for the structural analysis of microgram quantities of some biological materials using gas chromatography and mass spectrometry. The work has been mostly limited to two types of material

(i) β-hydroxy-amines and related catecholamines

(ii) natural and synthetic steroids

The latter type, the study of which constitutes most of this thesis, presents special difficulties due to the many positions on the steroid nucleus which may contain a functional group. The substituent groups studied are alkyl, olefin, hydroxyl and carbonyl. Methods are developed for obtaining structural information directly (where the course of fragmentation under electron impact is not known) and by methods which require a knowledge of ion fragmentation processes.

Considerable use is made of derivatives for hydroxyl and carbonyl modification to improve gas chromatographic properties, and to assist in functional group characterisation.

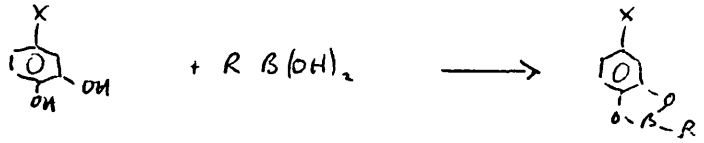
Such assistance is afforded by:-

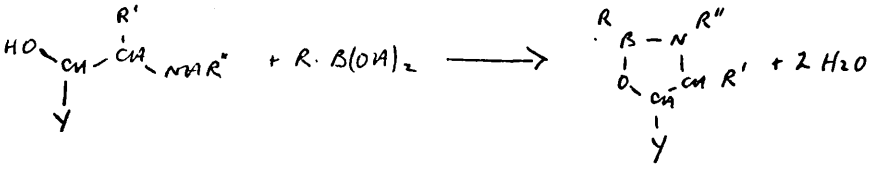
(a) molecular weight increment giving the number of functional groups capable of forming the derivative, and

(b) the ability of the derivative to direct mass spectral fragmentation to produce ions characteristic of the group's location.

PART 1 - β-HYDROXY-AMINES AND CATECHOLAMINES

A study is made of the usefulness of alkyl and aryl boronic acids as derivatives of β-hydroxy-amines and related catecholamines. This bifunctional reagent reacts with both the catechol and hydroxy-amine groups viz:-

(i) 

(ii) 

Both of these types of derivatives are amenable to gas chromatographic study, and from the mass spectra it is possible to identify the groups R' and R".

PART 2 - ALKYLATED STEROIDS

A study is made of alkylated and nor-steroids as their trimethylsilyl ethers. The location of methyl groups on steroids of the testosterone type is shown to be possible

by means of gas chromatographic retention increments and by shifts in m/e values of ions in the mass spectrum.

PART 3 - OLEFINIC STEROIDS

One of the more exacting problems inherent in steroid analysis is the locations of some olefinic groups by mass spectrometry. Although olefinic bonds may modify the fragmentation of neighbouring groups, the mass spectra of steroids often give no direct indication of the location of isolated unsaturation. A study is made of isomeric Δ^4,$\Delta^{5(10)}$, and Δ^5 estren-17-ones which give almost identical mass spectra. Chemical modification, such as oxidation of the olefin with osmium tetroxide and derivatisation of the resulting cis-diol, is shown to assist in characterising these three isomers by gas chromatography and mass spectrometry.

PART 4 - OXYGENATED STEROIDS AND TERPENOIDS

A technique of deuteriation by gas liquid chromatography is developed and applied to the examination of some steroids and terpenoids. Saturated and unsaturated ketones are shown to give satisfactory exchange of enolic hydrogen with deuterium after gas chromatography on basic columns saturated with deuterium oxide. Subsequent mass spectrometry shows the extent of deuteriation which is informative for location of the carbonyl group. Ethynyl groups (which are present in some progestational steroid drugs) and hydroxyl groups are also shown to exchange acetylenic or hydroxylic hydrogen with deuterium after deuteriation. The use of trimethylsilyl ethers and O-methyl-

oximes as derivatives for "blocking" exchange at hydroxylic and enolic positions is demonstrated, thereby permitting selective deuteriation at unprotected groups.

PART 5- STEROID DRUG METABOLISM

Some of the above techniques are applied to the study of urinary metabolites of the anabolic steroid drug "Nilevar" (17α-ethylestr-4-en-17β-ol-3-one) enabling tentative structures to be postulated.

INTRODUCTION

Gas Liquid Chromatography and Mass Spectrometry as complementary techniques

The gas chromatograph has for many years now been an extremely powerful analytical tool in its own right. It is able to separate quite complex mixtures under the appropriate conditions, and to identify the components by their retention volumes (see Introduction, "Recording of G. L. C. Data") compared with those of authentic standards. A component might be considered identified when its behaviour is indistinguishable from that of the standard on two or three different stationary phases in its free form and as one or more of its derivatives. This implies a certain amount of prior knowledge or presumption and it is clear that a method for further characterisation of components after the chromatographic separation will be of assistance whether one knows, or does not know what the components might be. The mass spectrometer is an ideal instrument for this purpose since it requires material in the vapour phase and also yields definitive information from sub-microgram quantities.

The first analyses by gas chromatography - mass

spectrometry (GC/MS) were made by simply trapping out the effluents individually from the gas chromatograph and subsequently identifying them by means of the mass-spectrometer. This method, although simple, is far from ideal since it is time-consuming, liable to lead to contamination and difficult to apply to samples of only a few micrograms. For high-resolution capillary columns, often used for flavour analysis, the quantities eluted are far too small for effective transfer by this method, and the components usually emerge from the column too rapidly for manual handling. To attach the mass spectrometer to the end of the gas chromatograph column so that the effluents pass straight into the ion source requires certain modifications to be made both to the gas chromatograph and the mass spectrometer. These have been discussed in a review by W. H. McFadden [1] and more recently by other workers [2,177,178]. The main requirement to be met by the mass spectrometer is that the time of measurement must be short so that each GLC peak can be scanned as it emerges, each scan covering only a small section of the peak while the concentration in the ion source remains approximately constant. Methods of overcoming this problem vary for different mass spectrometers but the main difficulty is amplifying the signal which might be of the order of 200 peaks per second and forming

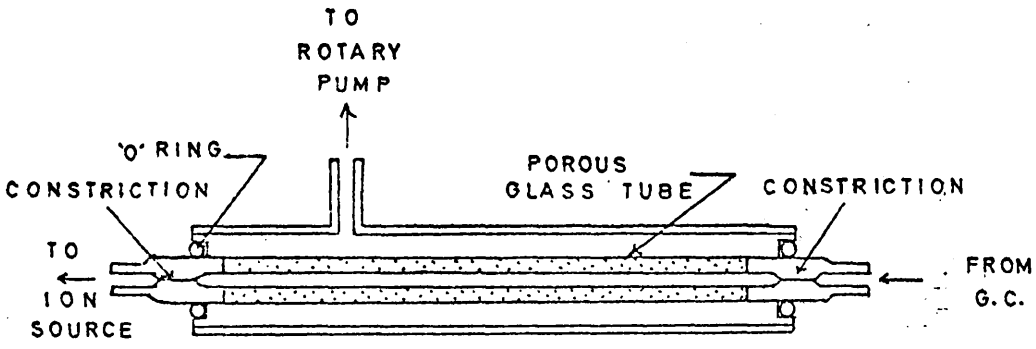
a suitable permanent record, losing as little information as possible. This is particularly important for high resolution mass spectrometry where precise mass measurements are required. Ideally the recording system should possess no inertia, thus the oscilloscope and magnetic tape recordings are ideal methods in this respect.

The pressure in the analyser of the mass spectrometer should be kept below 10^{-5} torr., and is determined by the capacity of the evacuating pumps and the rate at which the column effluent enters the ion source. Higher pressures cause impairment of the mass spectrum due to ion-molecule collision. To counteract this by increasing the pumping efficiency means that more of the sample will be pumped away and thus sensitivity is lost.

The problem is not quite so severe for capillary columns where the flow rate is 1 to 2 ml. per minute, and GC/MS analyses have been obtained using a flow splitter at the column exit which directs a suitable portion of the gas into the ion source [3]. Up to half of the total sample can be introduced into the mass spectrometer by this means. Satisfactory incorporation of capillary columns has also been achieved

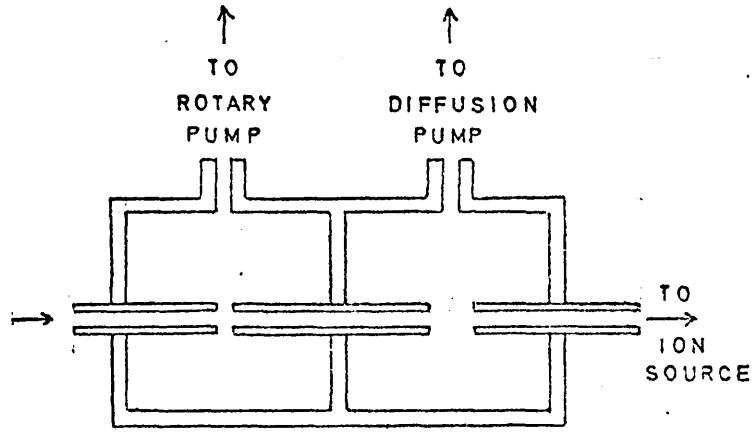
FIG. 1

Some Molecule Separators used in GC-MS



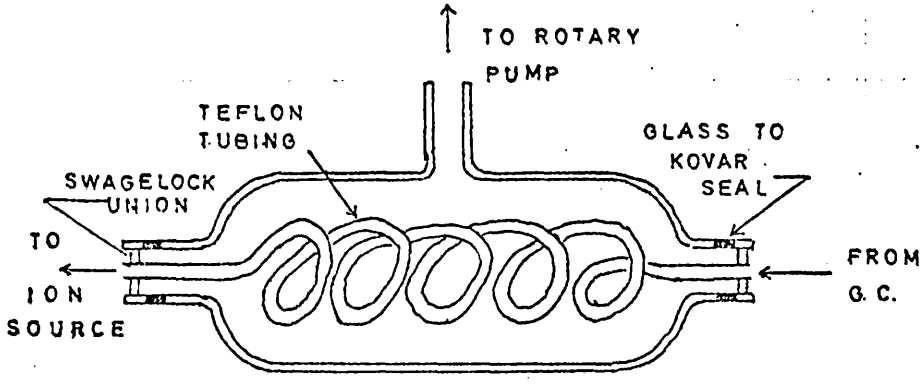
(a)

Watson, Biemann- glass frit separator



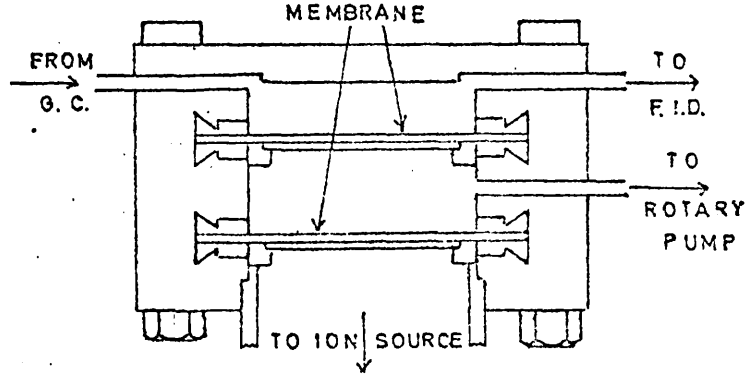
(b)

Becker, Ryhage- jet separator



(c)

Lipsky- Teflon separator



(d)

Llewellyn- membrane separator

by operating the gas chromatograph at reduced pressure[4]. In this way the whole column effluent can be fed into the ion source without any critical rise in pressure.

For packed columns where the flow rate is usually from 30 to 60 ml/min., more elaborate methods of effluent introduction are required. Simple flow splitting can be used, but clearly a much smaller fraction of the eluted sample must be fed into the ion source than is possible with capillary columns. Thus samples in the microgram range must be concentrated by selective removal of carrier gas before mass spectral analysis. Various devices are now available for this purpose and have recently been reviewed by Rees[5] and Jank[177]. Some of them are shown diagrammatically in Fig. 1.

The all glass separator[6] introduced by Watson and Biemann in 1964 comprises essentially a porous glass tube evacuated on the outside. The sample in the carrier gas (helium) passes through the tube and into the ion source. The faster diffusion of the helium through the porous glass causes an enrichment of the eluted sample. A fifty-fold enrichment was found in the case of diethyl ether, with a separator yield of 10 to 50% . We may define sample enrichment and separator yield as :-

$$\text{Enrichment} = \frac{\text{sample: carrier gas ratio after separation}}{\text{sample: carrier gas ratio before separation}}$$

$$\text{Separator Yield} = \frac{\text{amount of sample entering mass spectrometer}}{\text{amount of sample entering separator}}$$

The dimensions of the separator must be optimised with respect to the gas flow rate expected, in order to obtain maximum efficiency[7].

The method first reported by Ryhage[8] in 1964 was based on the isotope jet separator described by Becker[9]. The device is made from stainless steel and is both robust and efficient. The efficiency does vary with helium flow rate but an enrichment factor of 90 has been found for a flow rate of 40 ml/min using a two stage separator with 40 to 50% of the sample entering the ion source. A single stage Ryhage separator has been found satisfactory for work with capillary columns[10, 11]. A modification of the Watson-Biemann separator was made by Lipsky et al.[12] in 1966. Instead of the porous glass tube, Lipsky used a thin-walled Teflon capillary tube (length 2.1 m, wall thickness 0.125 mm, outside diameter 0.5 mm) through which the helium carrier gas selectively diffused. It is an apparent improvement on the Biemann separator in that it is more robust and allows 40 to 70% of the sample into

the mass spectrometer under optimum conditions. The temperature of the separator is however critical for maximum performance, and must be maintained in the 280° to 330°C temperature range. Furthermore, there is only one to fivefold enrichment of the sample in the carrier gas and the pressure in the ion source is generally higher than that obtainable with a Watson-Biemann type separator. It appears then that the Lipsky separator is an improvement on the Watson-Biemann type only when using open tubular columns, where high sample enrichment is not so important and the analyser pressure can be maintained at 10^{-7} torr.

Another principle of separation which is of increasing interest [13-16] was first applied by Llewellyn and Littlejohn in 1966.[13] The separator they devised consists basically of a silicone rubber membrane over which the gas chromatographic effluents pass. The other side of the membrane is kept under vacuum and connected to the ion source or to another separator. A two stage separator is illustrated in Fig. 1. The silicone rubber membrane allows the organic effluents to pass through into the ion source but remains relatively impermeable to the helium carrier gas. Any sample not absorbed by the first membrane can be passed at atmospheric pressure into a

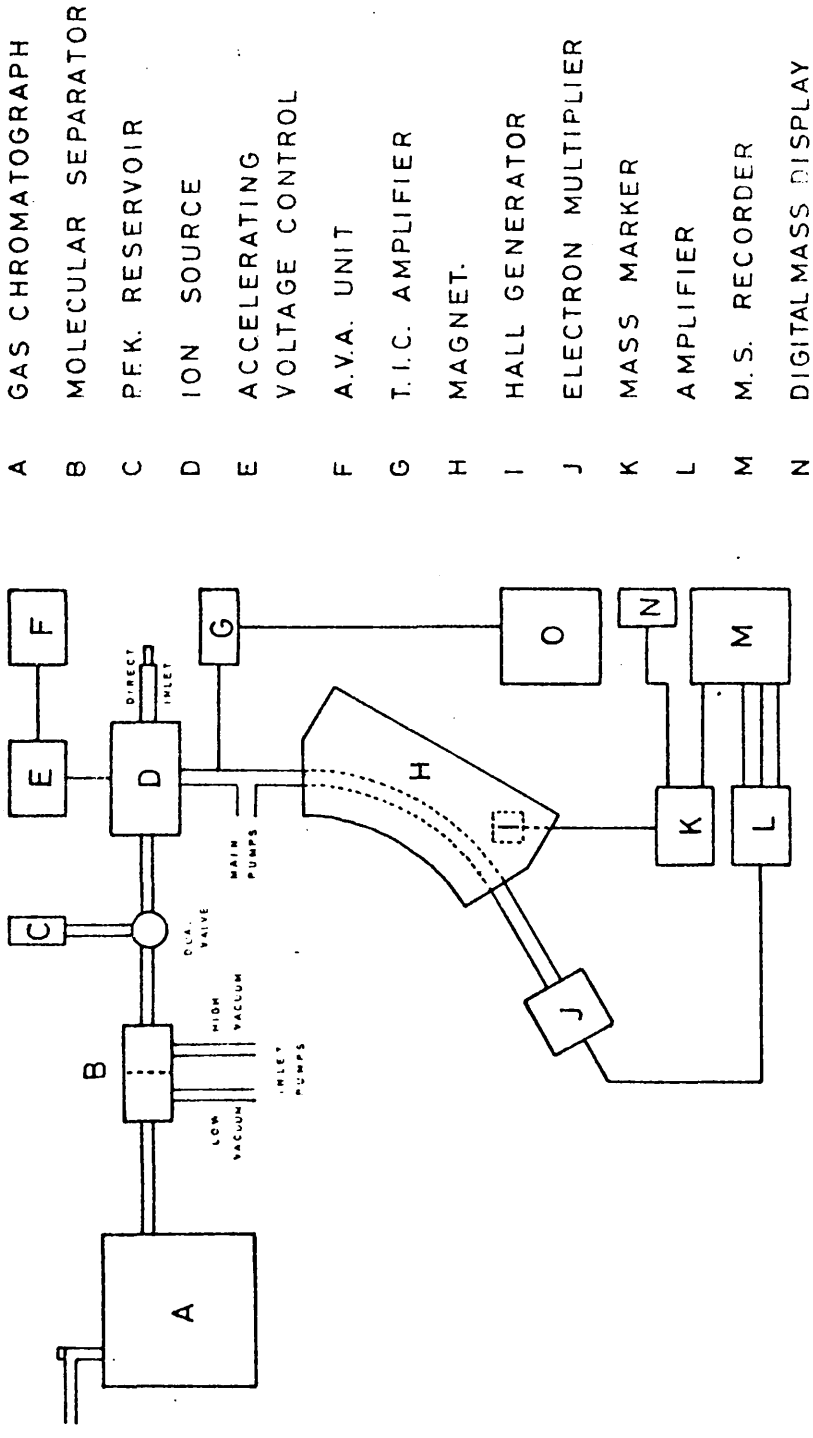
conventional gas chromatographic detector. Thus for a single stage separator no sample is wasted.

These systems, although not comprehensive, represent the principles which have been used to date in order to effect sample to carrier gas enrichment. There have been many variations of the Watson-Biemann system such as the porous metal separators described by Cree [17], Blumer[18], and Krueger and McCloskey[19], and the sintered glass disc separator described by Morin[20]. These devices however appear to offer no appreciable advantage except possibly increased robustness.

One further consideration to be taken into account for tandem GC/MS analysis is that the chromatogram obtained should reflect at any instant the sample concentration in the ion source. Although some of the methods of GLC sample introduction mentioned earlier permit the use of an auxiliary detector, some time lags are usually inevitable. It is preferable therefore to have a chromatographic detector in or near the ion source of the mass spectrometer. One method, which was used with the Bendix time-of-flight mass spectrometer, continuously recorded the signal output from a single ion common to all of the compounds being studied[21] (e. g. $C_3H_5^+$ = m/e 41 which

FIG.2

Schematic diagram of the LKB 9000 Gas Chromatograph - Mass Spectrometer



is present in most hydrocarbons with 4 or more carbon atoms). This has the obvious disadvantage that the peak height on the chromatogram will be a poor representation of the quantity of material in the mass spectrometer since the relative abundance of this fragment is very much dependent on the structure of the compound. A much superior method is to collect a proportion of the total ions produced in the mass spectrometer and feed this signal into the GLC recorder. For the magnetic-deflection mass spectrometer such a detector can be placed in a suitable portion of the ion beam between the ion source and the analyser tube.

The LKB 9000 combined instrument uses a single-focusing magnetic-deflection mass-spectrometer which is coupled to the gas chromatograph via a two stage Ryhage separator. The principal working parts of the instrument are shown schematically in Fig. 2. The mass spectrum signal is amplified using an electron multiplier and subsequent DC amplifier, the latter feeding three signals into a multi-channel high speed UV recorder in the intensity ratios 1:10:100. Simultaneously a 'mass marker' records a signal for every mass unit scanned to assist counting of the mass spectrum. This device uses a Hall generator which is placed between the

poles of the magnet and provides a signal representative of the magnetic flux density. This signal is amplified, converted to digital form using a series of pre-calibrated potentiometers and displayed both on the control panel (numerically) and as a series of lines on the UV sensitive chart. The gas chromatogram is recorded using a total ionisation current (TIC) detector of the type described above. In between scans the electron bombardment in the ion source is kept at 20 eV in order to avoid helium ionisation. At the start of each scan, however, the energy may be automatically increased to a preselected value, usually of 70 eV.

As mentioned earlier, the combined method of GC/MS is generally superior to the two separate techniques for analysis of materials in the sub-microgram range. Measurement of the mass spectra immediately after separation saves time and avoids the risk of contamination through intermediary handling. Another inherent advantage is the ability of the mass spectrometer to act as a selective GLC detector. Thus by setting the magnet current to record continuously fragments of a particular mass to charge ratio a GLC trace will be obtained showing only compounds producing a fragment of this mass. Thus for example a setting of $\underline{m/e}$ = 124 will give a strong signal for many 10 methyl steroids

with the Δ^4-3-one-structure, and a setting of m/e = 129 will give a strong signal for most trimethylsilyl derivatives of steroids with Δ^5-3-hydroxy, or 17-hydroxy groups provided that there are no other substituents in rings A or D respectively (see Fig. 2.1, part 2).

An extension of this technique of 'single ion monitoring' is the use of the Accelerating Voltage Alternator (AVA) ('Multiple Ion Detector') which will record the intensity of two or three separate fragments as the chromatogram progresses. With the LKB system this is achieved by continuously switching the accelerating voltage in the ion source between two or three values which are calibrated to allow each selected fragment to be focused successively on to the electron multiplier. Thus two unresolved components of a chromatogram can be traced individually by selecting concurrently two uncommon fragment masses of sufficient intensity. This technique was introduced in connection with studies of isotope-labelled carbohydrates by Sweeley et al[22].

There are still problems to be overcome however, before the techniques of gas chromatography and mass spectrometry work as well together as they can do apart.

Perhaps the most important of these is column 'bleed' into the mass spectrometer. This is a nuisance during interpretation of the resulting 'contaminated' mass spectra, and is also harmful to the mass spectrometer. The problems are more acute in investigations of steroids and other compounds of high molecular weight, because of the high column temperatures required to achieve practical retention times. By recording a 'background' mass spectrum at the temperature of operation it is possible to obtain mass spectra approximately representative of the pure samples by measuring and substracting the background peaks, or by using a computer to do this. However, it is obviously beneficial to reduce the level of contaminating volatile material arising from the column, and this can be achieved using low concentrations of stationary phases of types which are relatively thermostable. Until recently such phases were of limited variety and consisted of high molecular weight silicone polymers containing varying proportions of methyl and phenyl residues. Fortunately, a wider variety of selective phases is now being manufactured and some phases are stable enough to be used for GC/MS at 250^{o}C or above, thus increasing the choice for effecting suitable separation.

The problem of mass spectrum bias due to varying

concentration in the ion source has been mentioned earlier in connection with amplifier and recorder limitations. Scan times as short as three seconds however can still give spectra sufficiently biased to give an erroneous base peak (i.e. the most intense peak in the mass spectrum) and this can be a severe disadvantage when an unknown sample is to be identified by computer search techniques. In general, least distortion will occur if the mass spectrum is scanned at the apex of the peak on the chromatogram since the concentration will vary least at this point. Further improvements by shortening the scan time are limited by the inherent loss of resolution.

RECORDING G. L. C. DATA

As well as separating mixtures prior to mass spectrometry the gas chromatograph itself provides an important aid to structure identification. Indeed many compounds differing only in their stereochemistry give almost identical mass spectra, but can be distinguished by their GLC retention characteristics.

The distinguishing feature of any component when subjected to GLC is its retention volume (V_R), i.e. the volume of carrier gas required under specified conditions to elute the sample from the column. It is usual to substract from this the column 'dead volume' (V_M) so that the resulting parameter is representative of solute-solvent

and solute-vapour interactions only. Since the gas chromatograph is normally operated using a constant carrier gas flow rate, it is more convenient - especially in isothermal chromatography-to express retention volume in terms of retention time (t_R). To avoid the analogous 'dead time' resulting from the internal volume of the column, t_R is measured from the air peak to the apex of the sample peak, or, when chromatographing high molecular weight material in low boiling solvents, from the solvent front to the apex of the sample peak.

Comparatively non-volatile materials such as steroids may be chromatographed using solid injection techniques, in which the sample solution is deposited upon a small gauze (or metal spiral), the solvent evaporated and the residue introduced into the column by inserting the gauze into the injection port heater. Although no air peak or solvent front is apparent during these chromatograms, small traces of solvent dissolved in the sample often provide a convenient zero for retention measurements.

Before retention data can be useful in structure elucidation they should satisfy the following requirements :-

i) small variation with slight changes in gas flow rate;

ii) small variation with slight changes in temperature;

iii) small variation with slight differences in the quantity

of stationary phase used.

Moreover, they should be expressed in units relevant to structural properties. These requirements are important to ensure reproducibility of results from sample to sample, to allow equivalence of data obtained from different columns with similar liquid phases, and to enable structural information to be inferred from such data. Unfortunately, t_R is unsuitable in all of these respects.

One method of overcoming some sources of variation (in isothermal chromatography) is in the use of relative retention times (r_x) defined as :

$$r_x = \frac{t_{RA}}{t_{RX}}$$

(where t_{RA} = retention time of the compound (A) under investigation; t_{RX} = retention time of the standard (X)). The standard is usually structurally similar to the material under investigation, and both must be introduced into the chromatograph simultaneously in order to achieve reproducibility. A standard often used for the GLC of steroids is cholestane.

Evans and Smith in 1961 introduced the term r_{X9} which they defined as the retention time relative to n -nonane [23]. This

universal standard was related to other standards by the expression

$$r_{X9} = r_{XN} \times r_{N9}$$

(where r_{XN} = retention time relative to an internal standard (N) and r_{N9} = retention time of standard (N) relative to n-nonane.)

By using a standard which is injected simultaneously with the material under examination it is possible to reduce errors arising from small changes in operating conditions since these will presumably affect both standard and specimen. Clayton in 1962 used relative retention data to obtain structural information from steroids [24]. By assuming that each group on the steroid nucleus is associated with a specific increment in retention time, he related the relative retention time r_X (molecule) to r_X(nucleus) and r_X (groups) by the expression

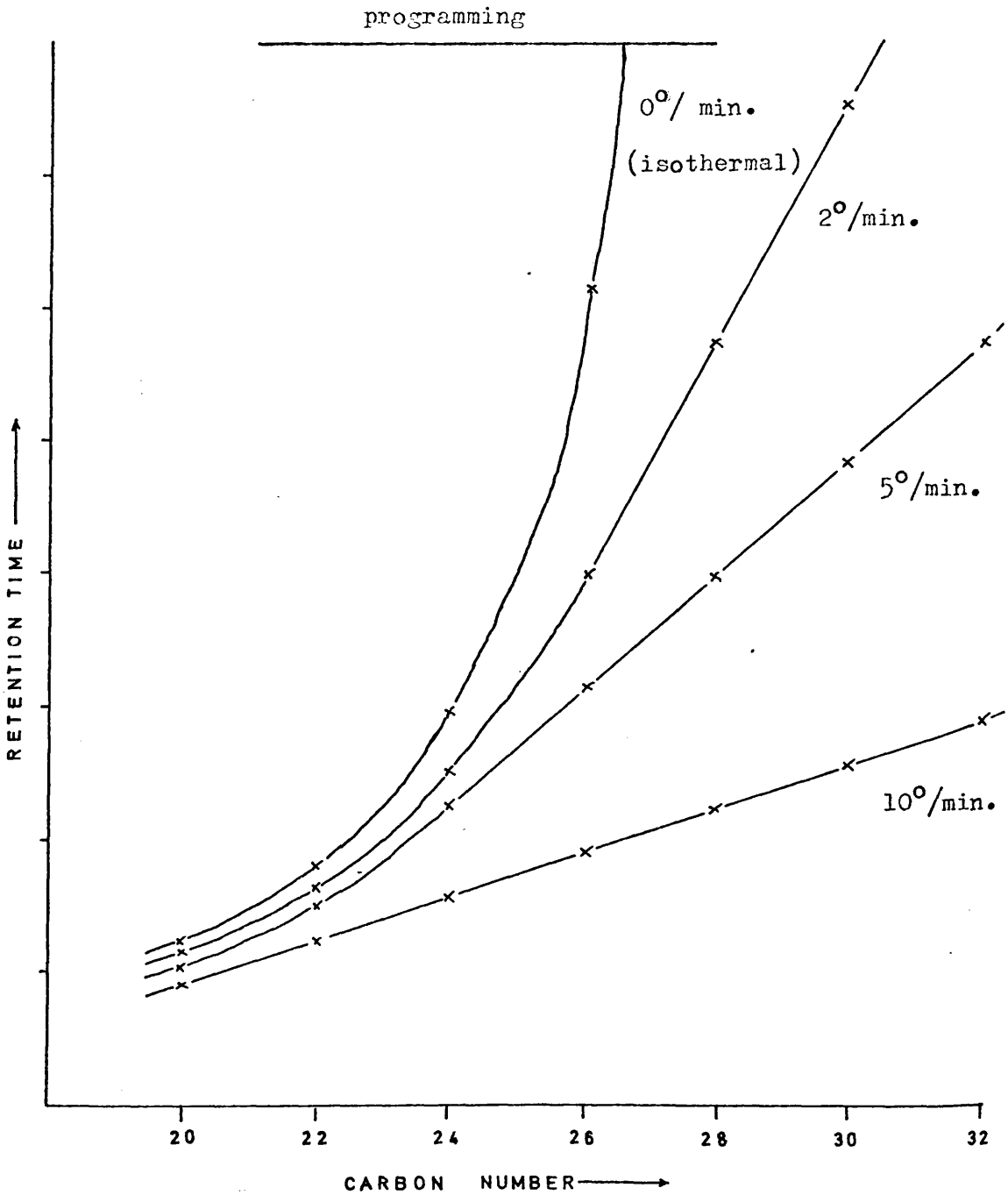
$$r_X(n+a+b+c\ ---) = r_X(n) \times ka \times kb \times kc\ ---$$

(where n = nucleus; a, b, c, etc. = groups on the nucleus; k = retention factor for each group.)

Thus a knowledge of n-1 of the above parameters will enable computation of the final one, although what is probably more useful is the ability to check that a chosen structure relates to an observed retention time. The above relationship breaks down when the groups on the molecule are situated in such proximity that they exert mutual steric or electronic effects.

FIG.3

Illustrating the relationship between retention time and carbon number for a homologous series under conditions of linear temperature programming



A different approach to recording GLC data was devised by Kovats in 1958[25]. This method makes use of the fact that for a homologous series of n-alkanes there is a linear relationship between the number of carbon atoms in the molecule and the logarithm of its retention time. The retention index(I)is defined by the expression :-

$$I_{(x)} = 100i \times \frac{R(x) - R(Pz)}{R(Pz+i) - R(Pz)} + 100z$$

Z = N^{o} carbon atoms in standard 1; Z+i = N^{o} carbon atoms in standard 2; X = specimen; R = log retention time for isothermal conditions.

Although this calculation is cumbersome it can be carried out simply by a graphic method using semi-logarithmic paper[26]. The arithmetic method involves two suitable hydrocarbon standards with retention times greater and less than that of the specimen. For linear temperature programmed runs R = retention time, since the hydrocarbon standards are eluted with retention times approximately proportional to their number of carbon atoms. This relationship is not exact however and the linearity of the relationship depends upon such factors as stability of programme rate and speed of programme (since an infinitely slow programme will produce a logarithmic relationship between retention time and carbon number). This is illustrated in Fig. 3.

The form which the retention index takes is 100 x the number of carbon atoms in the n-alkane (whole or fractional) which would be required to give the same retention time as the specimen (e.g. for n-pentane I = 500.) The term 'methylene unit' (MU) often used to record GLC retention data is related to I by the expression

$$MU = \frac{I}{100}$$

Thus for n-pentane MU = 5.00.

Since similar relationships between retention time and number of carbon atoms exist for all homologous series it is possible to use standards more directly related to the specimen. Woodford and VanGhent in 1960 used n-fatty acid methyl esters as standards to obtain the 'carbon number' of unknown fatty acids[27]. The term 'steroid number' (SN) was devised by Vandenheuvel and Horning in 1962 to correlate steroid retention times with a homologous series of steroids[28] comprising 5α-androstane (SN = 19.00) 5α-cholestane (SN = 27.00) and 5α-stigmastane (SN = 29.00). These standards, although bearing a closer structural resemblance to typical steroid samples, have the disadvantage of being limited in number and range. For retention data on selective phases it was found necessary to introduce the secondary steroid standards[29] 5α-cholestan-3-one, cholesteryl valerate and cholesteryl

heptanoate whose steroid number had been previously determined on that column using the primary standards.

Retention indices, methylene units, carbon numbers and steroid numbers can all be expressed in a form yielding structural information about the specimen, i.e.

$$X(n+a+b+c \ldots) = X(n) + ka + kb + kc \ldots$$

(where X = retention index, methylene unit, carbon number or steroid number; k = group retention factor; n = nucleus; a, b, c ... = functional groups)

The absence of inter-group effects such as hydrogen bonding and steric repulsion is important for these relationships to hold. There are some distinct advantages in this method of structure correlation over the method used by Clayton, for example group retention contributions and the nucleus retention contribution are all additive and therefore enable easier interpretation of data. The chief advantage of the method is that it is also suitable for temperature and pressure programmed GLC.

Important information may also be gained by measuring the retention increment in going from a non-selective to a selective stationary phase, or after carrying out a simple structural modification

such as Jones oxidation or derivative formation. These points can be illustrated by the following examples :

Compound	M U OV - 1	M U OV - 17
5α cholestane	27.90	29.22
5α cholestan - 3β -ol	30.05	32.65
5α cholestan - 3β -OTMS	30.90	31.92
5α cholestan - 3-one	30.34	33.20

From this we can obtain information about the 3β -OH group which will help its recognition in unknown samples i.e.

	Δ M U OV - 1	Δ M U OV - 17
3β -OH group retention factor	+ 2.15	+ 3.45
TMS formation	+ 0.85	- 0.63
Jones oxidation	+ 0.29	+ 0.55

The importance of data of this sort was illustrated by Knights (1966) using a number of different stationary phases.[30]

Other methods of data recording of less value in structure determination have been reported. Evans et al (1961) introduced the term "effective molecular weight"[31] (M_e). Knights and Thomas (1962) introduced the term R_M into gas liquid chromatography in order to

compare relative retention time with thin layer chromatography characteristics.[32]

From the systems discussed above methylene unit values were chosen as the most suitable form of retention data for the following reasons :

1) They fulfil requirements (i) to (iii) discussed earlier.

2) They can be usefully applied to a wide variety of compounds.

3) Suitable standards are available in high purity for a wide range of GLC conditions.

4) The system is self-descriptive and therefore used in preference to the alternative "retention index" values.

There is a slight disadvantage of this method over Horning's SN values in that the steroid nucleus itself posesses some "functional group" character and different MU values for steroid hydrocarbons are observed on different stationary phases. This effect, although slight, is allowed for when the steroid nucleus is known and data can be handled in the form of group retention values.

Table 1 continued

Functional Group	Derivative	Refs. - GLC	Refs. - MS
R—NH—R^1 (amine)	acetate	80	
	HFB	81, 92	
	TMS	84, 85, 91, 48	
	Schiffs base	86, 87	
	Thiocyanate	179	
	pentafluorophenyl-Schiffs base	88	
	dansylate	89	
(R)(OH)(NHR^1) (hydroxy amine)	oxazolidine	80, 87, 90	
	oxaborolidine	68, 94, 95	94, 95
R.CO_2H (carboxylic acid)	methyl ester	37, 38, 44, 59	37, 38, 59
	TMS ester	85, 98	98
(R)(CO_2H)(OH) (α -hydroxy acid; β -hydroxy acid)	boronate	68	68

TABLE 1

Some derivatives of functional groups used in gas chromatography and mass spectrometry

Functional Group	Derivative	Refs. - GLC	Refs - MS
R - OH (alcohol)	acetate	33	34, 35
	trifluoroacetate (TFA)	36	35, 37, 38
	heptafluorobutyrate (HFB)	39, 40, 41	
	trimethylsilyl (TMS) ether	42-51	33, 49, 52-57
	halomethyldimethylsilyl ether	58	
	methyl ether	59	59
	halomethyl acetonide	69	69
(R)(OH)(OH) (1, 2-diol; 1, 3-diol)	acetonide	60, 61, 62	63
	dimethyl siliconide	64, 65	64, 65
	boronate ester	66, 67, 68	67, 68
R, R^1 C = O (aldehyde; ketone)	oxime	97	70, 71
	methyloxime (MO)	43, 44, 68, 72-75	72-75
	benzyloxime	180	
	ethylene ketal		33, 76-78, 104
	ethylene thioketal		78
	enol TMS ether	181	79, 181
	enol HFB	39, 40	
	dimethylhydrazone	33, 72	33

DERIVATIVES

For the purpose of structure elucidation the principal aim behind derivative formation is to modify existing functional groups on the molecule in order to :

1) assist in their separation from each other;

2) assist in their separation or distinction from species not able to form the derivative;

3) stabilise some species ordinarily too labile for GLC, and

4) provide additional information about molecular structure.

Table 1 lists some of the derivatives which have been used for gas chromatography and mass spectrometry, together with appropriate literature citations.

Reagents capable of forming cyclic derivatives with neighbouring functional groups have been shown to enable GLC separation of diastereoisomers [67, 87, 90] which gave coincident retention times in their free form. Thin layer chromatographic separations have also been achieved for steroid alcohols as their trimethylsilyl (TMS) ethers [99-103] acetates [99] and trifluoroacetates [103] using solvent systems of low or moderate polarity. Separation is enhanced by the presence of ketonic groups or alcoholic functions too sterically hindered to form the derivative. The reduction in dipolar character of the molecule thus

achieved also enables almost quantitative elution from the preparative plate with moderately polar solvents.[102]

Derivatives with electron capture properties such as trifluoroacetates, heptafluorobutyrates and halomethyl (dimethylsilyl) ethers have been used to record selectively the presence of compounds containing hydroxyl, carbonyl and amine groups during gas chromatography using the electron capture detector. This technique is particularly useful for the detection of sub-nanogram quantities.

Thermolabile species including some corticosteroids undergo decomposition in the gas chromatograph, but may be chromatographed without decomposition after the formation of a suitable derivative. Trimethylsilyl ethers. trimethylsilyl ether-methyl oximes, trimethylsilyl ether-benzyl oximes, dimethyl siliconides and alkyl boronates have been successfully used for this purpose.

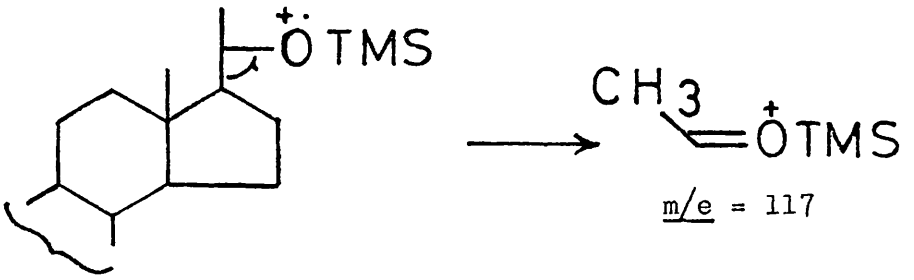
For gas chromatographic - mass spectrometric studies the main value of derivative formation is the provision of material similar in structure to the original compound but with different and preferably improved physico-chemical properties. Thus valuable information may be obtained without resorting to any other instrumental method of analysis, and with quantities therefore still at the sub-microgram level.

It has already been mentioned how changes in GLC retention values upon derivative formation can aid structure elucidation. The choice of derivative, however, is important, especially when dealing with polyfunctional compounds of high molecular weight. Classical methods such as acetylation of hydroxyl groups are of limited value when their accumulated retention increments render the compounds too non-volatile for GLC, and in these circumstances it is an obvious advantage to use derivatives with lower retention contributions. Trimethylsilyl ethers, O-methyloxime trimethylsilyl ethers, cyclic dimethylsilyl ethers, acetonides, methyl boronates, and bis-methylene-dioxy (BMD) derivatives give satisfactory retention times for the gas chromatography of corticosteroids at temperatures suitable for coupling with the mass spectrometer.

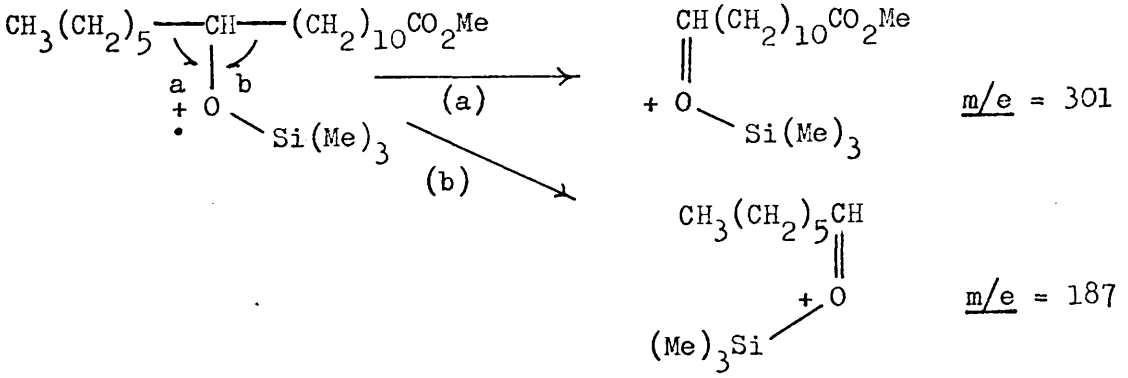
Besides modifying or improving the gas chromatographic properties, a good derivative should if possible influence the mode of fragmentation in the mass spectrometer in a way informative of the location of the original functional group in the molecule. For this purpose acetates are often unsuitable derivatives of alcohols because elimination of acetic acid occurs too readily upon electron impact. In contrast, trimethylsilyl ethers often yield fragment ions retaining silicon, which arise from fission α to the oxygen-silicon bond, and are

of diagnostic value for determining the location of the hydroxyl group

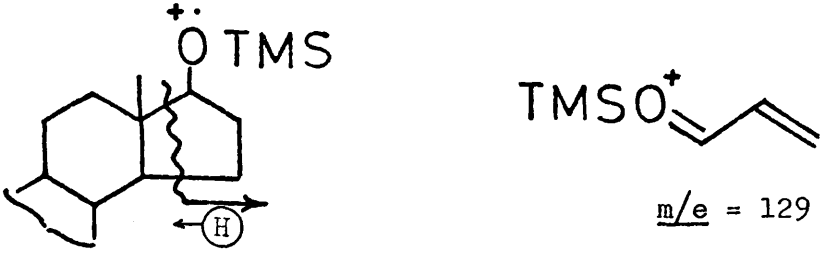
e. g. i) 20-hydroxy steroid TMS ethers



ii) hydroxy fatty acid methyl ester TMS ethers



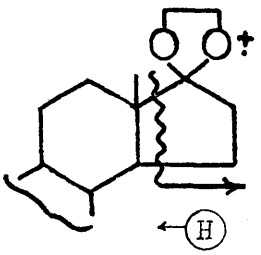
iii) 17α-hydroxy-steroid TMS ethers

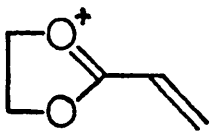


Dimethylhydrazones have been used for mass spectrometric

studies of carbonyl compounds. Their main value is the formation of an intense molecular ion, which is often the base peak, and comparatively few fragment ions.

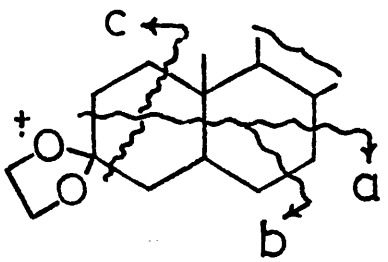
In contrast ethylene ketals give mass spectra usually preponderant in fragment ions arising out of fission of the bond β to the functional group. For 5α-androstan-17-one ethylene ketal the fragment at m/e = 99, which comprises C-15, C-16, C-17 and the ethylene ketal group, is the base peak in the mass spectrum, and no other fragment ion amounts to more than 2% of its abundance.

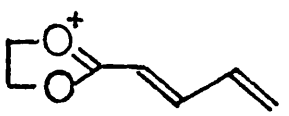




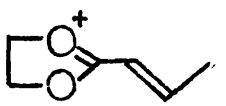
m/e =99

Steroidal 3-ethylene ketals form a similar fragment ion at m/e = 99 and also ions at m/e = 112 and m/e = 125

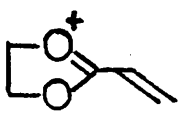




m/e = 125



m/e = 112

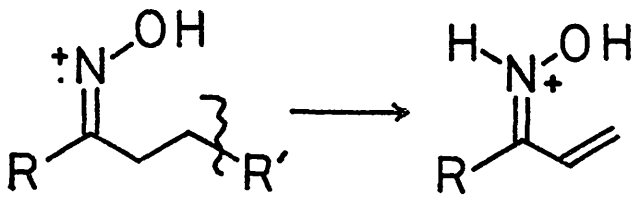


m/e = 99

Ethylene thioketals and ethylene hemithioketals have been used as alternative derivatives and result in analogous fragments under electron impact to those formed from the ethylene ketal.

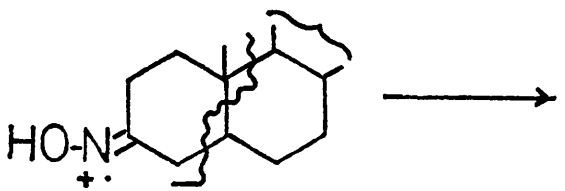
Oxime and methyl oxime derivatives, like dimethylhydrazones, fragment under electron impact giving ions initiated by cleavage of the bond $\alpha\beta$ or $\beta\gamma$ to the functional group.

e.g. i) linear aliphatic ketone oximes



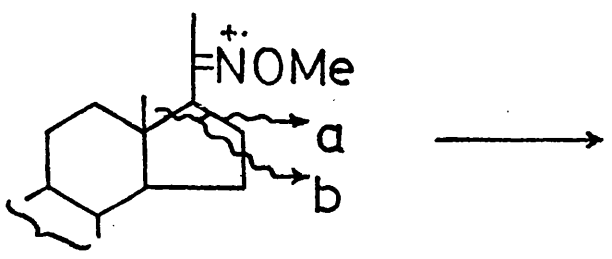
m/e = 71+R

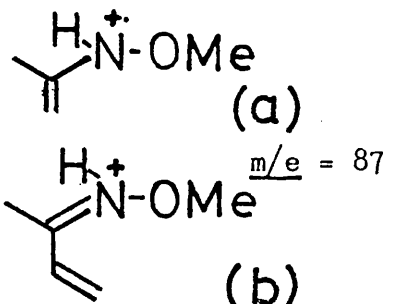
ii) steroid-3-ketone oximes



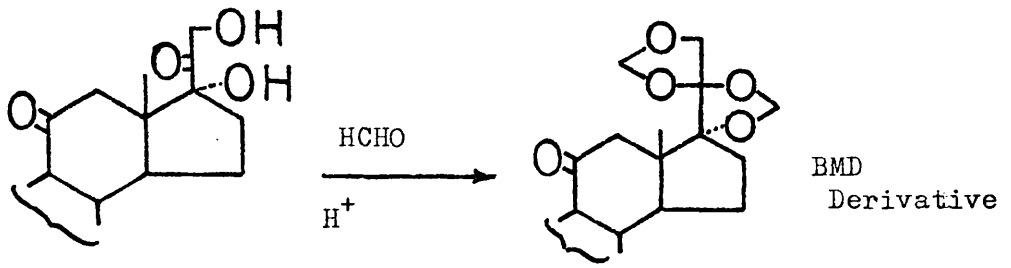
m/e = 112

iii) steroid-20-ketone, methyl oximes

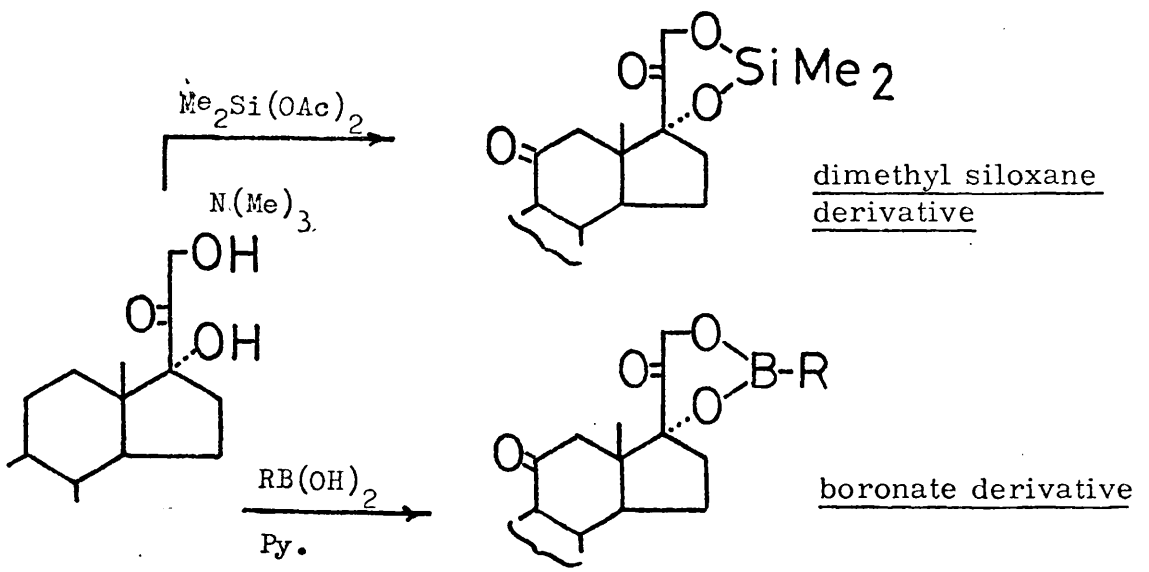




Cyclic derivatives of functional groups which lie in close proximity are valuable tools for structural analysis by GC/MS because of the specific nature of their formation and the stability many of them show to gas chromatography. Bis-methylenedioxy (BMD) derivatives were proposed in 1962 for stabilisation of the ß -hydroxy acetone side chain of corticosteroids in order to make them amenable to gas-liquid chromatography.



More recently, heterocyclic derivatives of silicon and boron have been used in corticosteroid analysis.



Acetone will also form useful cyclic derivatives with

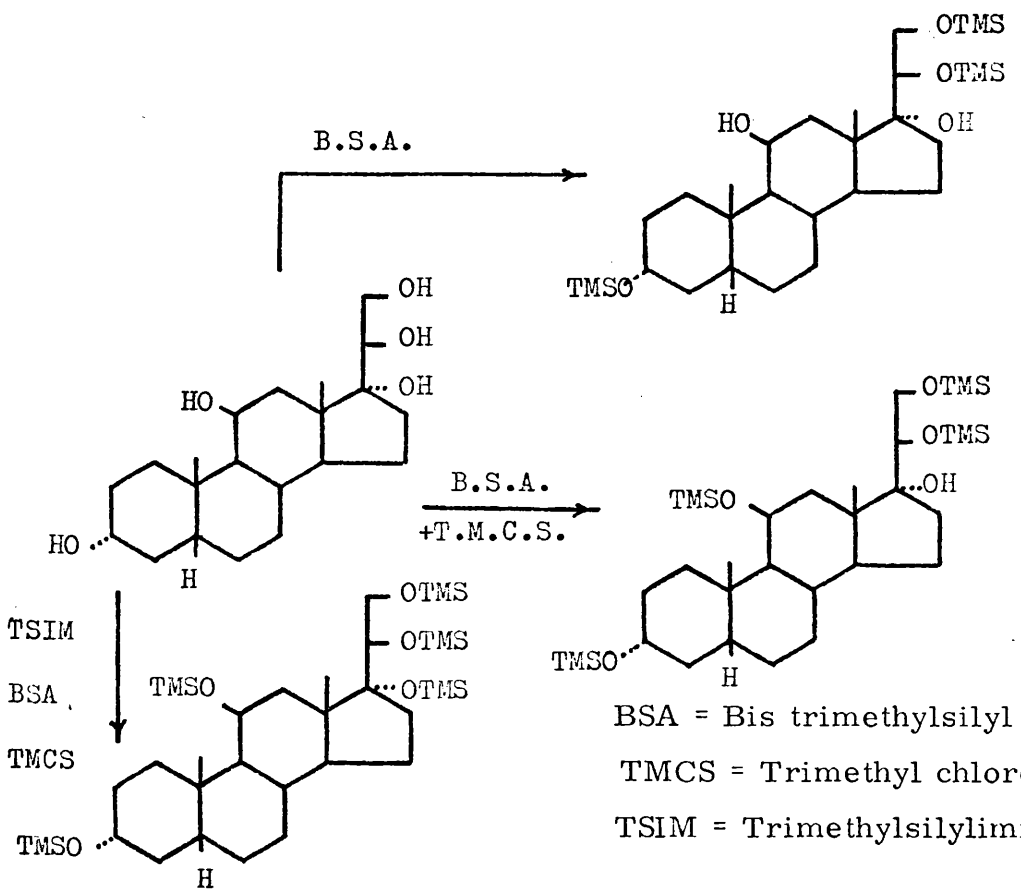
ß-hydroxy secondary amines and 1, 2-cis-diols, while alkyl and aryl boronates have been shown to form cyclic derivatives with 1, 2 cis-diols, catechols, ß -hydroxy primary and secondary amines, αß -ketols, α-hydroxy acids and ß-hydroxy acids.

Reagents

Choosing a suitable derivative for structure investigation by gas chromatography/mass spectrometry requires consideration of the suitability of the reagent. It is often important to form a derivative under conditions which are as mild and as near to neutrality as possible in order to avoid undesirable modification of other functional groups. Furthermore it is desirable that the reaction is rapid, quantitative, and that the product can be separated easily from excess reagent prior to or during chromatography. Acyl esters, trimethylsilyl ethers, cyclic dimethylsiliconides, methyl ethers, acetonides and boronate esters are all derivatives of alcohols with can be formed rapidly without the necessity for strong acid or base catalysis or high temperatures. Less desirable in this respect are bis-methylenedioxy derivatives which require catalysis, elevated temperatures and lengthy reaction times for their formation. Similarly ethylene ketals and ethylene thioketals, although useful derivatives of ketones for mass spectroscopic purposes are less convenient to prepare than methyl

oximes or dimethylhydrazones.

When several functional groups of the same type are present in a compound it may be possible to modify one or more of them by varying the reagents or reaction conditions. Thus for cortol it has been shown possible to produce a tris trimethylsilyl ether, a tetra trimethylsilyl ether or a penta trimethylsilyl ether exclusively by varying the reagents used for silylation:-[45, 46]



BSA = Bis trimethylsilyl acetamide

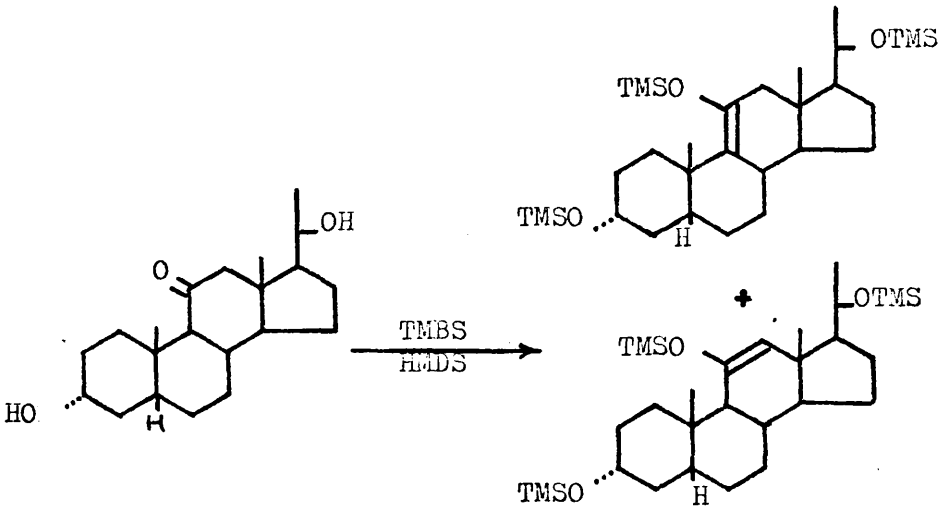
TMCS = Trimethyl chlorosilane

TSIM = Trimethylsilylimidazole

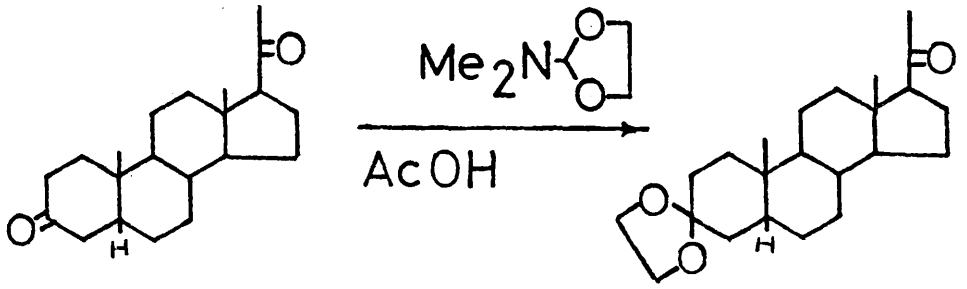
Trimethylbromosilane (TMBS) has recently been shown to have catalytic properties superior to TMCS when used with HMDS to silylate

sterically hindered groups.[47] Steroids with hydroxyl groups in positions 3, 11, 17, 20 and 21 (e.g. α and β cortol) formed fully silylated derivatives in high yields at room temperature using these reagents in pyridine. 11-keto steroids form enol TMS ethers with this reagent

e.g.



Similarly ethylene ketals can be selectively formed from 3-oxo groups on a steroid containing also the more hindered 20-oxo group [104]



Selectivity has also been achieved with some derivatives which are normally associated with more than one type of functional group. Bis-trimethylsilyl acetamide (BSA) will react both with alcoholic functions and with amino groups to form O-trimethylsilyl ethers and N-trimethylsilyl amines respectively. Trimethylsilylimidazole (TSIM) on the other hand reacts only with alcoholic functions. It was demonstrated by Horning and others how amino groups could be selectively acylated by silylation of alcoholic functions with TSIM and subsequent acylation of the amine with N-heptafluorobutylimidazole.[75] Similarly acetone, which will form a Schiff's base with a primary amine or an oxazolidine with a β-hydroxy secondary amine at room temperature without a catalyst, usually requires a catalyst for acetal formation with 1, 2-or 1, 3-diols.

MASS SPECTRUM INTERPRETATION

There can be said to be four stages for obtaining structural information by mass spectrometry. Firstly, simple information such as molecular weight and elemental composition (high resolution MS) can in most cases be obtained directly from the mass spectrum of the sample without consideration of fragmentation processes . Compounds which are too labile to give a clearly defined molecular ion at 70 eV

often do so at lower electron energies.

Secondly, the number of various functional groups and to some extent their location on the nucleus may be determined directly by observing molecular weight increments after treatment with reagents of differing selectivity.

Thirdly, the position of these groups may sometimes be inferred from the mode of fragmentation of the molecule both in the derivatised and un-derivatised form. The use of known fragmentation behaviour may be applied with relative ease to linear molecules where simple well-defined fragmentions are prevalent, but for polycyclic molecules such as steroids, where a fragment ion often involves the fission of two or more carbon-carbon bonds, the mode of fragmentation is more complex and the mass spectrum less predictable. For this reason, group derivatives which produce recognisable fragment ions characteristic of their position on the nucleus are of particular value in the analysis of steroids by mass spectrometry.

The final stage of analysis by this and any other physico-chemical technique should be by identity of data with those obtained from a compound of known structure which has been synthesised by established chemical methods.

THE ROLE OF COMPUTERS

The computer is now an established ancillary to both gas chromatographic and mass spectrometric analysis. Computer 'hardware' and 'software' are commercially available for performing many routine tasks associated with these techniques. This is especially useful in mass spectrometry where time spent in data processing is far in excess of the time taken obtaining the mass spectrum. Systems now in use enable the following operations to be carried out automatically and quickly :

1) normalisation of the mass spectrum;

2) background subtraction;

3) accurate mass measurement (for high resolution mass spectrometers);

4) data recording as table, histogram or element map;

5) compound identification by comparing data with a library of reference spectra.

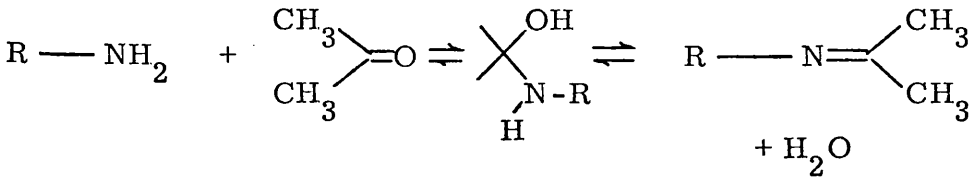
CATECHOLAMINES AND RELATED β -HYDROXYAMINES

Many biologically active amines, because of their polar nature, are particularly difficult to characterise by GLC. Amines of moderate polarity have been separated without prior modification, good resolution being obtained by coating the support with potassium hydroxide, [80, 90, 105-109] or by using high percentage of stationary phase. [110]

Biological amines of higher polarity such as catecholamines generally require the formation of a non-polar derivative before they are suitable for gas chromatography. Some of the modifications described for this purpose are as follows :

(1) Schiff's Base Formation [80, 83, 84, 86, 87, 88]

Primary amines react with a ketone to give a Schiff's base e, g., with acetone

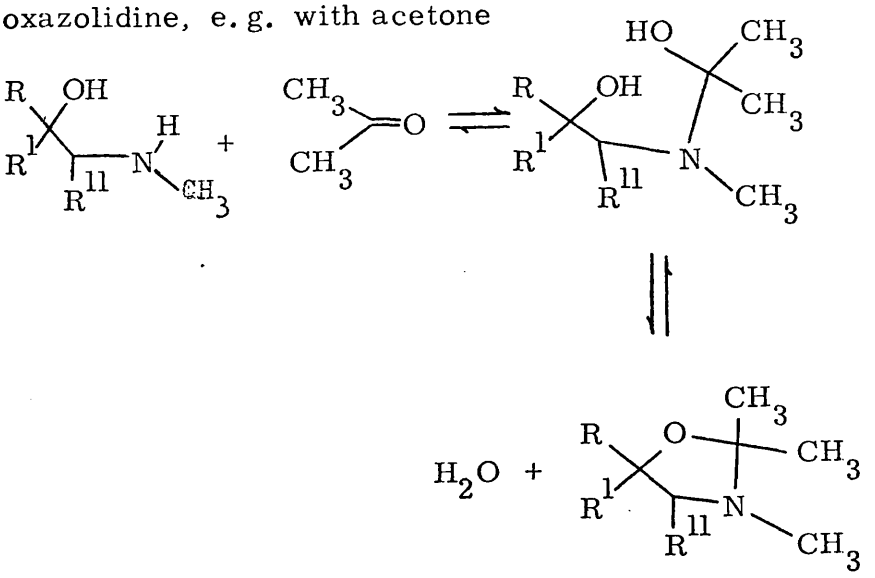


This derivative is often readily formed by dissolving the amine in dry acetone.

(2) Oxazolidine Formation [80, 87, 90]

β -hydroxy secondary amines may react with a ketone to form an

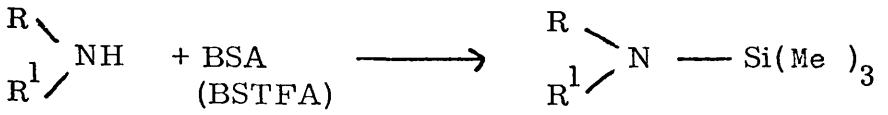
oxazolidine, e.g. with acetone



This derivative is also formed by dissolving the amine in dry acetone.

(3) Trimethylsilylation [85, 111]

Trimethylsilylation reagents such as bistrimethylsilylacetamide (BSA) and bistrimethylsilyltrifluoroacetamide (BSTFA) are able to trimethylsilylate primary and secondary amines.



When trimethylchlorosilane (TMCS) is used as catalyst it has been shown possible to form the bistrimethylsilyl derivative of a primary amine. [111]

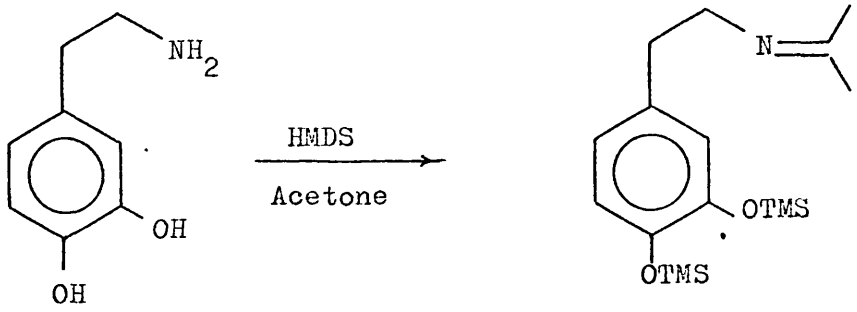
(4) Acylation [80, 81, 83, 92, 93]

Amides are conveniently formed by reaction of the primary or secondary amine with a carboxylic acid anhydride in pyridine, e.g. with acetic anhydride

N-acetyl derivatives of most amines are stable and suitable for gas chromatography. Halogenated amides such as heptafluorobutyrates [80, 81, 92] pentafluoropropionates [93] trifluoroacetates [83] pentafluorobenzoates [182] and dinitrophenyl sulphonates possess electron capture properties and are suitable for the analysis of small quantities of amines. The pentafluorophenyl Schiffs base has also been used to detect small quantities of primary amine by electron capture detection [182]

Various techniques have been used to modify amine groups in the presence of hydroxyl substituents. Thus, by reacting the biological amine with a dimethylformamide solution of hexamethyldisilazane (HMDS and then adding to a mixture of HMDS in acetone, Capella and Horning [86] were able to form derivatives with trimethylsilylated hydroxyl groups and with all primary amino groups converted to the Schiff's base. Secondary amino groups did not form the derivative.

E.g. for 3,4-dihydroxyphenylamine (Dopamine):-



Furthermore, by using cyclobutanone instead of acetone, they were able to indicate all components possessing the primary amine or β-hydroxy amine function by changes in retention time.

Horning and co-workers [48, 81] were also able to acylate selectively primary and secondary amines by trimethylsilation of hydroxyl groups with N-trimethylsilylimidazole (TSIM) followed by the addition of N-heptafluorobutylimidazole. Using an electron capture detector this method afforded selective detection of nitrogen functions with high sensitivity.

CYCLIC BORONATES AS DERIVATIVES OF CATECHOLAMINES AND β-HYDROXY-AMINES

The phenylboronate derived from catechol was one of the first cyclic esters of this type to be described, [112] and is formed under mild conditions by the reaction of phenylboronic acid with catechol. Many analogous derivatives such as the t-butylboronate are known. [113] The interaction of phenylboronic acid with β-hydroxy-

amines has been studied by a number of investigators[114-116, 184] since the phenylboronates derived from 2-amino-ethanol and o-aminophenol were reported by Sugihara and Bowman.[117] The ready formation of ephedrine phenylboronate has also been described by Pailer and others.[114]

In order to test the suitability of boronic acids as derivatives of catechol amines and related ß-hydroxy amines, n-butyl boronic acid was used to treat a number of biologically important amines or their salts at room temperature. The solvent used was usually pyridine, but dimethylformamide was used for some of the more polar compounds. Other boronic acids such as cyclohexylboronic acid and phenylboronic acid were used for a few selected amines.

The general reactions involved in the formation of boronate derivatives of catecholamines and ß-hydroxy amines were exemplified below with catechol and ephedrine.

(a) catechol

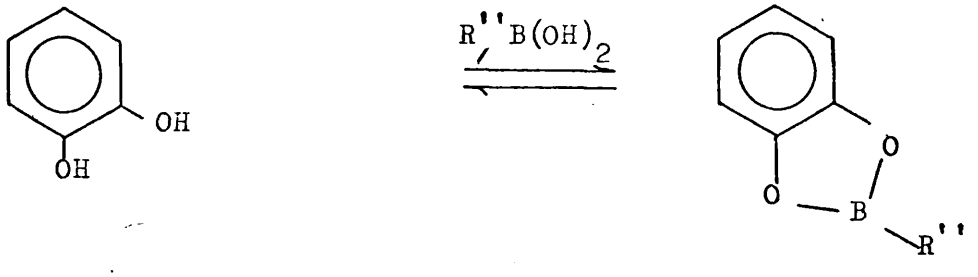
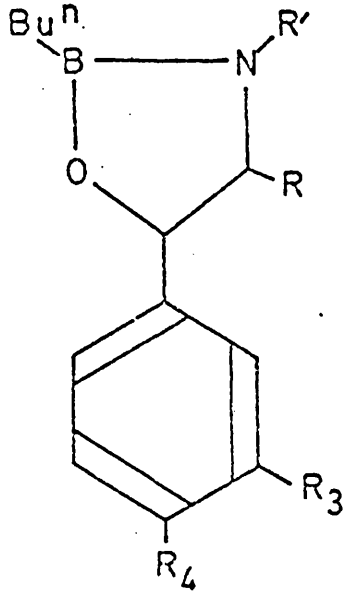


FIG. 1.1

Structures of butylboronates derived from β – hydroxyamines

GENERAL FORMULA



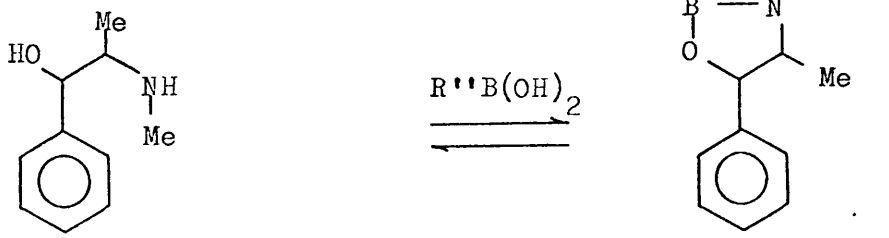
PARENT COMPOUND	SUBSTITUENTS			
	R	R'	R_3	R_4
β-HYDROXY-β-PHENYLETHYLAMINE	H	H	H	H
NORPSEUDOEPHEDRINE	Me	H	H	H
NOREPHEDRINE	Me	H	H	H
PSEUDOEPHEDRINE	Me	Me	H	H
EPHEDRINE	Me	Me	H	H
OCTOPAMINE	H	H	H	OH
4-DEOXYNOREPINEPHRINE	H	H	OH	H
SYNEPHRINE	H	Me	H	OH
NEOSYNEPHRINE	H	Me	OH	H
NORMETANEPHRINE	H	H	OH	OMe
METANEPHRINE	H	Me	OH	OMe
NOREPINEPHRINE	H	H	$Bu^n \cdot BO_2$	
EPINEPHRINE	H	Me	$Bu^n \cdot BO_2$	
ISOPRENALINE	H	Pr^i	$Bu^n \cdot BO_2$	
3,4-DIHYDROXYNOREPHEDRINE	Me	H	$Bu^n \cdot BO_2$	

FIG.1.2

Gas Chromatographs of n-butylboronate derivatives of 3,4-dihydroxy-methyl benzoate and ephedrine

1% OV-17

(b) ephedrine



The chemical structures of fifteen n-butyl boronates derived from biological amines are shown in Fig. 1.1.

Gas Chromatography

Fig. 1.2 illustrates the GLC behaviour of n-butylboronates derived respectively from methyl 3,4-dihydroxymethylbenzoate and ephedrine. Although the latter compounds can be studied in the free state by GLC, the ß-hydroxy amine side-chain is susceptible to partial decomposition. Co-occurrence of such a side-chain with a catechol grouping as in epinephrine and congeners, yields highly polar compounds which must be converted to derivatives for satisfactory GLC. In this connection it appeared possible to apply boronic acids as selective reagents to convert epinephrine (adrenaline) and its analogues to bis-boronates, while the 3-O-methylated metabolites should be transformed only to the mono-boronates. This expectation was realised as illustrated in Fig. 1.3, which depicts the GLC separation of metanephrine-mono-n-butylboronate and the bis derivatives of

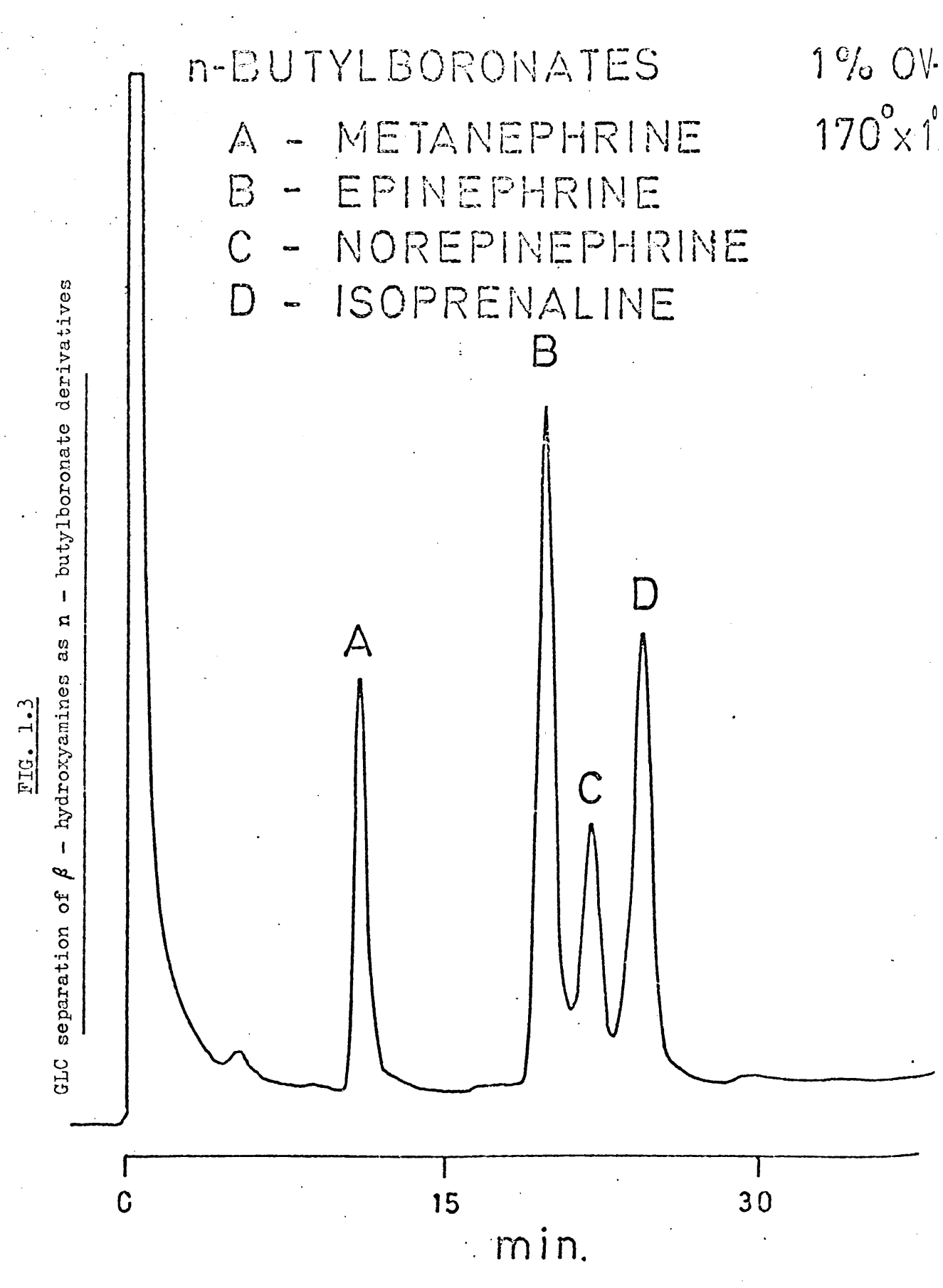


FIG. 1.3

GLC separation of β - hydroxyamines as n - butylboronate derivatives

Table 1.1. Methylene unit (MU) values for n-butylboronates of catecholamines and related compounds

Parent compound	Type of derivative	Retention data MU *	Tem
β -hydroxy- β - phenylethylamine	Mono	17.99	140
Norephedrine	Mono	17.76	140
Nor-ψ-ephedrine	Mono	17.74	140
Ephedrine	Mono	17.96	140
ψ -Ephedrine	Mono	17.82	140
Synephrine	Mono	21.85	170
Neosynephrine	Mono	21.71	170
Octopamine	Mono	22.18	170
4-Deoxynorepinephrine	Mono	22.03	170
Normetanephrine	Mono	23.15	190
Metanephrine	Mono	22.70	190
Norepinephrine	Bis	24.78	190
Epinephrine	Bis	24.38	190
Isoprenaline	Bis	25.12	190
3, 4-Dihydroxynorephedrine	Bis	24.50	190
Methyl 2, 3-dihydroxybenzoate	Mono	19.15	140
Methyl 3, 4-dihydroxybenzoate	Mono	19.01	140

*1% OV-17 on Gas Chrom Q (100/120 mesh), 6 ft column

epinephrine, norepinephrine (noradrenaline) and isoprenaline. Retention data of these and n-butyl boronate derivatives of other amines are given in Table 1.1.

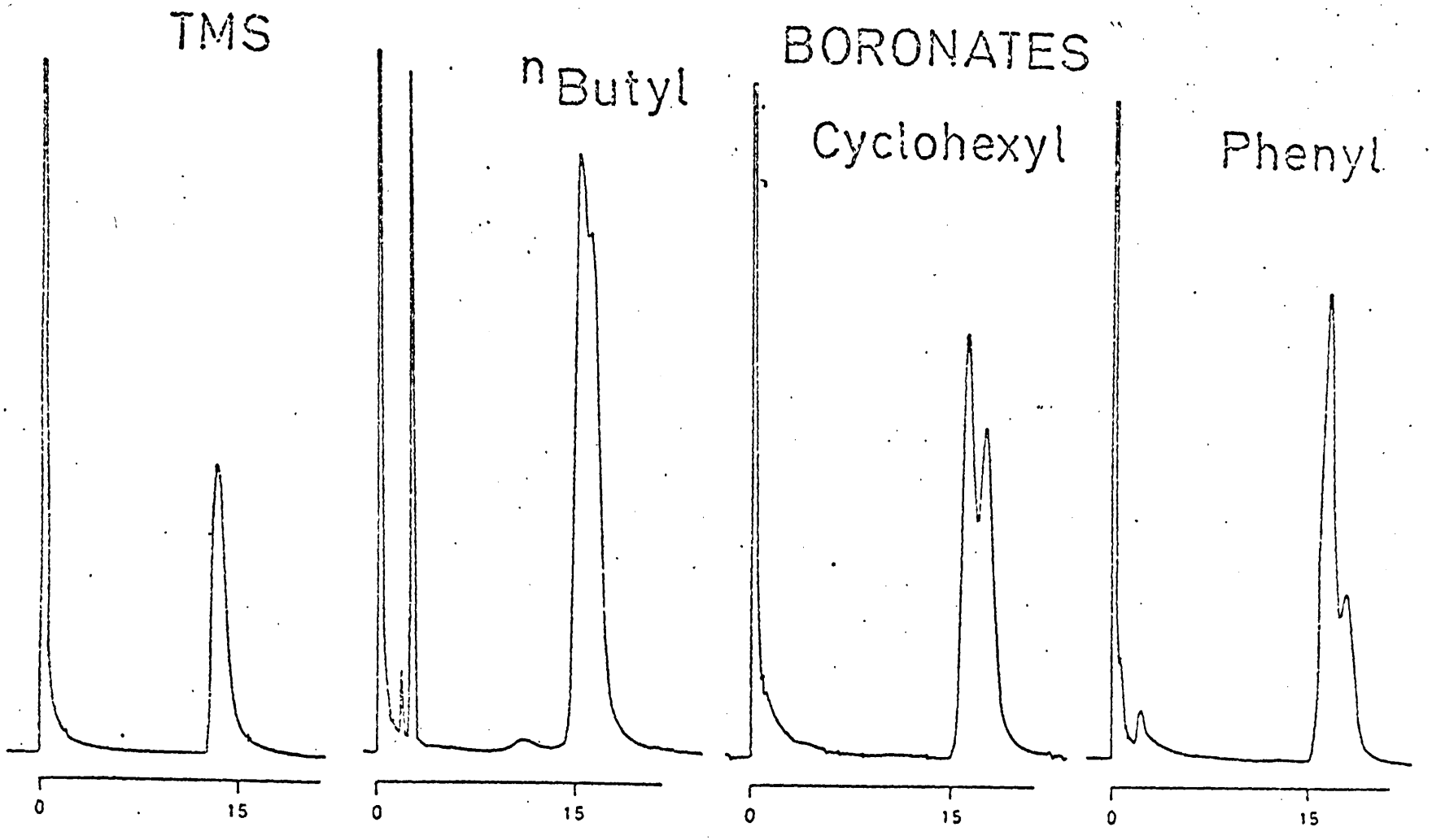
Among β-hydroxy-amines lacking a catechol grouping, the eleven examples listed in Table 1.1 yielded cyclic boronates (oxazaborolidines) characterised by their retention data and mass spectra. Synephrine and neosynephrine, possessing isolated phenolic groups gave polar derivatives showing considerable 'tailing' on GLC.

The derivatives from octopamine and 4-deoxynorepinephrine did not give a peak when the reaction mixture was injected directly but products amenable to gas chromatography were obtained when the reaction mixtures were subjected to prolonged vacuum sublimation at 250°. The principal component for both amine derivatives presented a GLC peak showing considerable tailing and gave a molecular weight (by GC/MS) corresponding to the mono boronate.

Diastereoisomeric β-hydroxy-amines such as ephedrine and ψ-ephedrine have been distinguished by selective conversion of the 'threo-' isomer by acetone to the oxazolidine.[81] They have also been separated by GLC after conversion of both isomers to this

FIG. 1.4

GLC Separation of ephedrine and ψ - ephedrine as TMS-, and boronate derivatives.



derivative. Fig. 1.4 indicates that a clear distinction can be made between the erythro and threo-isomers by GLC of their n-butyl-, cyclohexyl- or phenylboronates. The separations are enhanced in the boronates with bulkier substituents as denoted by the methylene unit values given in Table 1.2.

Table 1.2

The effect of different groups on the boron atom (R'') in resolving the diasteroisomers ephedrine and Ψ-ephedrine as their boronate derivatives by GLC on 1% OV-17

R''	Temp (°C)	MU		ΔMU
		Ephedrine	Ψ-Ephedrine	
Methyl	90	15.13	15.09	0.04
n-butyl	140	17.96	17.82	0.14
t-butyl	130	16.80	16.69	0.11
Cyclohexyl	150	20.80	20.64	0.16
Phenyl	170	22.58	22.38	0.20

Under similar conditions a single unresolved peak was observed (Fig. 1.4) for a mixture of the O-trimethylsilyl ethers prepared with BSTFA.

MASS SPECTROMETRY

The cyclic nature of boronate derivatives of bifunctional compounds in some cases directs the mode of mass spectrometric

FIG. 1.5

Postulated structures for ions resulting from electron impact on boronate derivatives of β-hydroxyamines

Table 1·3 *Mass spectral breakdown of n-butylboronates of β-hydroxy-β-arylethylamines*

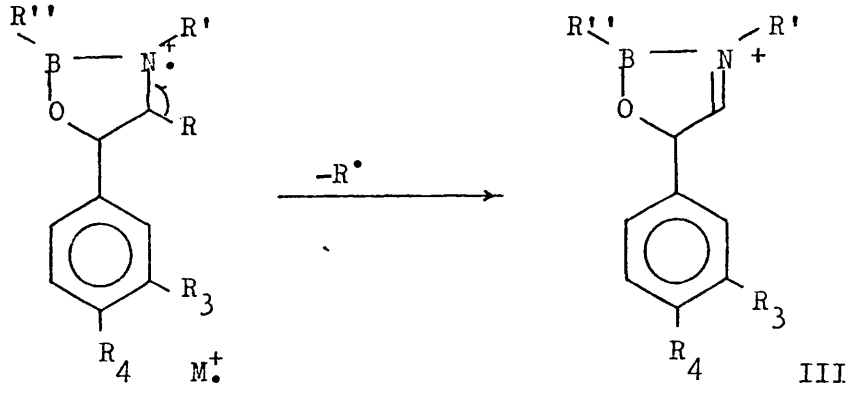
Parent compound	Molecular ion	M/e and type of major fragments*										M/e and type of other diagnostic fragments				
β-Hydroxyphenethylamine	203 (21%)	161 X	91 VI	90VIa	202 III VII	120 VIII	118 IV	89 VIb	146 II	117 V	104 —	174 I (6%)				
Norpseudoephedrine	217 (33%)	202 III	91 VI	90 VIa	160 II	118 IV	216 VII	117 V	175 X	89 VIb	188 I	134 VIII (6%)				
Phenylpropanolamine	217 (25%)	202 III	91 VI	90 VIa	118 IV	160 II	117 V	89 VIb	216 VII	175 X	132 —	188 I (6%)	134 VIII (5%)			
Pseudoephedrine	231 (9%)	216 II	91 VI	132 IV	117 V	90 VIa	89 VIb	230 VII	118 —	105 —	174 II	202 I (2%)	189 X (2%)	148 VIII (1%)		
Ephedrine	231 (16%)	216 III	91 VI	132 IV	117 V	90 VIa	230 VII	174 II	89 VIb	159 —	118 —	189 X (2%)	148 VIII (2%)	202 I (2%)		
Octopamine	219 (10%)	107 VI	218 III VII	134 IV	177 X	133 V	105 VIb	162 II	136 VIII	106 VIa	135 IX	190 I (19%)				
4-Deoxynoradrenaline	219 (10%)	107 VI	218 III VII	134 IV	177 X	133 V	105 VIb	162 II	136 VIII	106 VIa	135 IX	190 I (18%)				
Synephrine	233 (74%)	191 X	232 III VII	107 VI	150 VIII	148 IV	106 VIa	133 V	120 —	105 VIb	176 II	149 IX (5%)	204 I (7%)			
Phenylephrine	233 (8%)	191 X	232 III VII	107 VI	149 IX	148 IV	120 —	133 V	150 VIII	134 —	105 VIb	176 II (2%)	204 I (11%)			
Normetanephrine	233 (10%)	137 VI	248 III VII	219 —	218 —	163 V	232 —	135 VIb	166 VIII	136 VIa	150 —	192 II (19%)	164 IV (15%)	165 IX (15%)	220 I (14%)	207 X (9%)
Metanephrine	263 (10%)	262 III VII	137 VI	180 VIII	179 IX	221 X	163 V	164 —	246 —	145 —	136 VIa	178 IV (19%)	206 II (8%)	234 I (5%)		
Noradrenaline	301 (6%)	300 III VII	150 VI	217 IX	216 IV	218 VIII	215 V	188 VIa	244 II	259 X	272 I					
Adrenaline	315 (71%)	273 X	314 III VII	231 IX	232 VIII	189 VI	230 IV	188 VIa	258 II	202 —	215 V	187 VIb (3%)	206 I (3%)			
3,4-Dihydroxynorephedrine	315 (49%)	300 III	189 VI	314 VII	188 VIa	216 IV	215 V	230 —	273 X	258 II	231 —	285 I (5%)	232 VIII (4%)			

* In order of abundance.

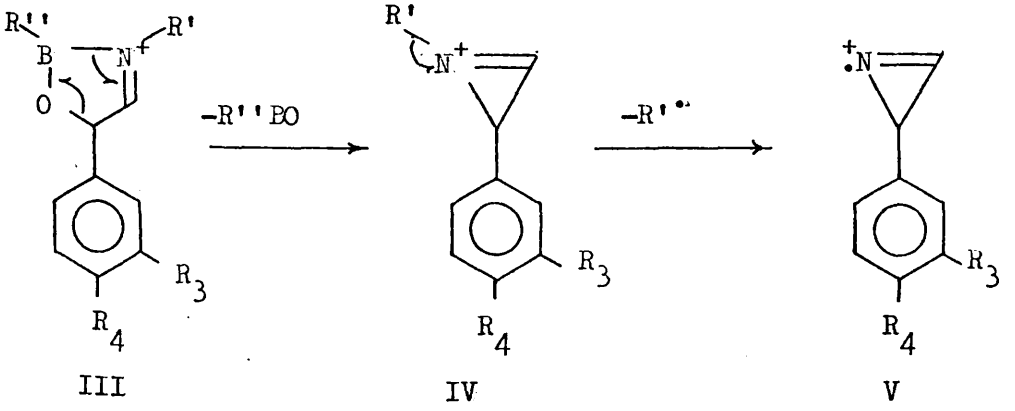
fragmentation : this is observed for the 2-substituted 1, 3, 2-oxaza-borolidines derived from ß-hydroxy-amines. Although the relative intensity of certain fragments is influenced by the substituent on boron, the general mode of breakdown is the same for the methyl-, n-butyl-, cyclohexyl- and phenyl-boronates studied. The present discussion is concerned mainly with n-butylboronates, which have useful gas chromatographic properties (e. g. capacity for resolution of diastereoisomers, combined with moderate retention times). It should be noted that certain ions observed in the mass spectra of n-butylboronates evolve from fragmentation of the n-butyl substituent.

Postulated representations of the main fragments from the mass spectra of the n-butylboronates of ß-hydroxy- ß-arylethylamines are shown in Fig. 1. 5 and the principal ions observed in the compounds studied are listed in Table 1. 3.

As can be seen in Table 1. 3 the molecular weight was easily determined in each of the compounds studied since a fairly prominent molecular ion was obtained in each case. The substituents on position 4′ of the oxazaborolidine ring (usually hydrogen or methyl) can readily be identified by their loss, principally to give ions of type III.

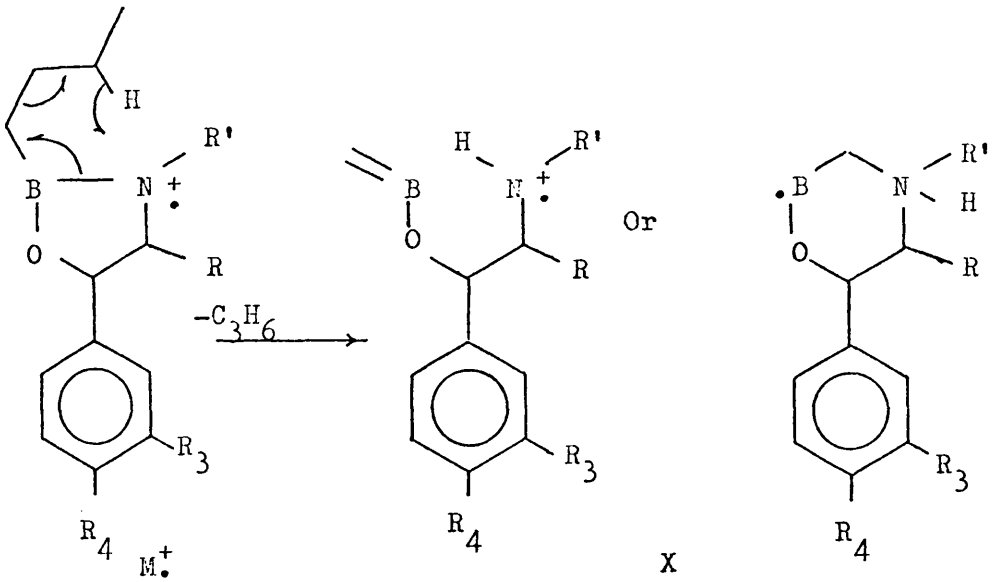


The ratio of B^{10} to B^{11} in this fragment indicates whether one or two molecules of n-butylboronic acid have been incorporated into the molecule. The nature of the substituent (H or CH_3) at position 3′ can be inferred from the transition III ⟶ IV ⟶ V where the group is eliminated as a radical. (The exceptional case of the N-isopropyl derivative, isoprenaline butylboronate, is discussed later).



In the compounds studied, the substituents on the benzene ring are retained in fragments of type V (Fig. 1.5) where the hydroxy-amine side-chains are reduced to a common moiety $(C_2H_2N)+$, and in types VI, VIa, and VIb. These relatively prominent ions readily indicate the combined molecular weights of the substituents on the benzene ring. Certain other fragments arise from the breakdown of hydroxyl and methoxyl substituents on the benzene ring. Thus synephrine gives an ion at m/e = 216 due to loss of ·OH. Metanephrine gives a similar ion at m/e = 246 and also one at m/e = 232 due to loss of ·O Me.

As noted above, the spectra of n-butylboronates contain, sometimes as major ions fragments dependent on the presence of the n-butyl substituent. Thus, the ion of type X is the base peak in the spectra of β-hydroxy-β-phenylethylamine n-butylboronate, synephrine n-butylboronate and epinephrine bis-n-butylboronate. This M-42 ion is likely to be formed via a six membered cyclic rearrangement with the elimination of propylene.



Where this fragment is predominant the two daughter ions VIII and IX can also be observed.

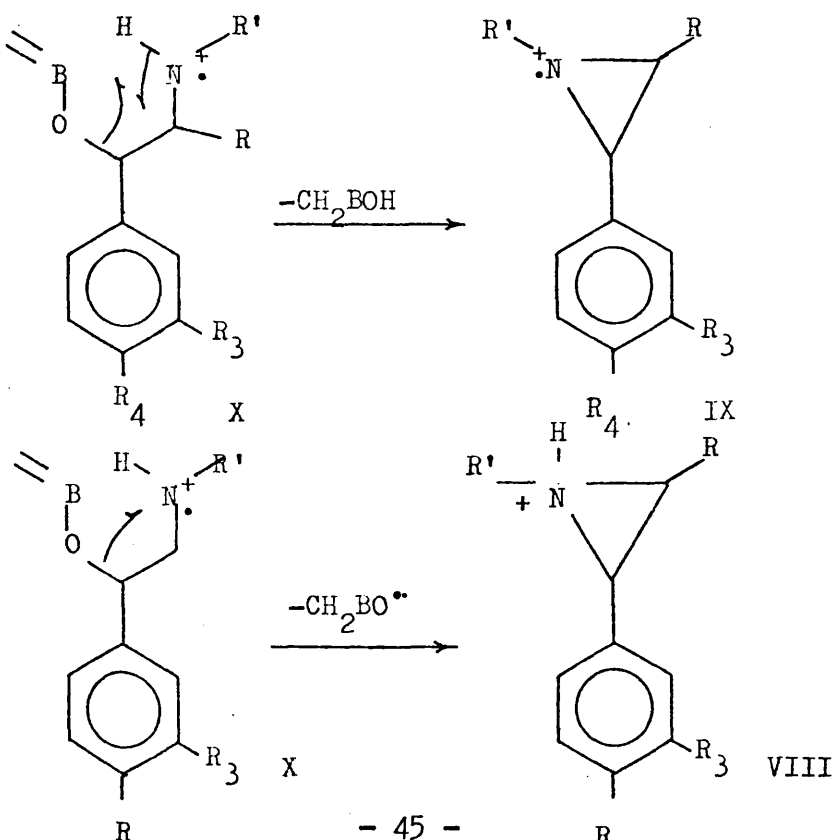
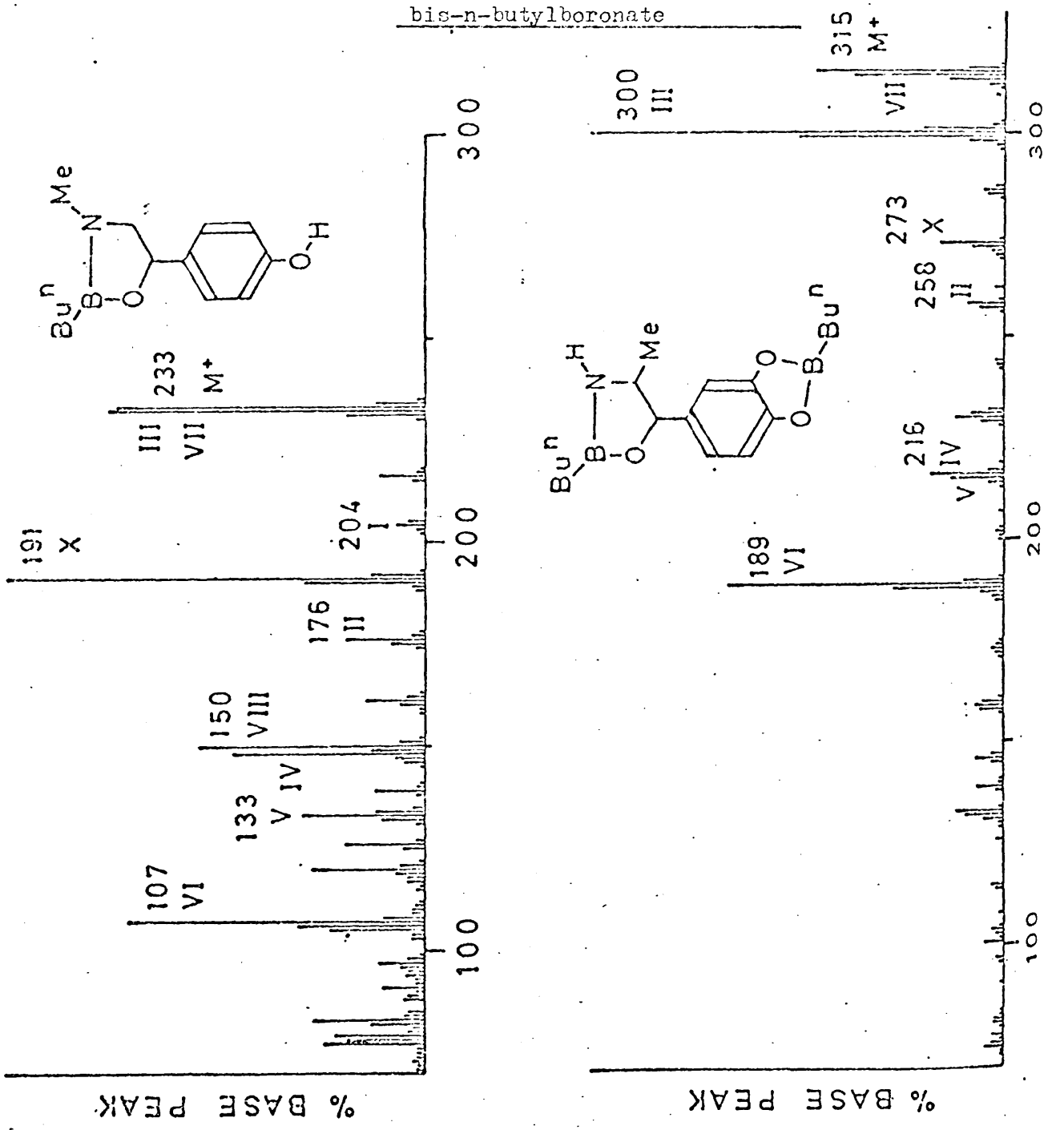
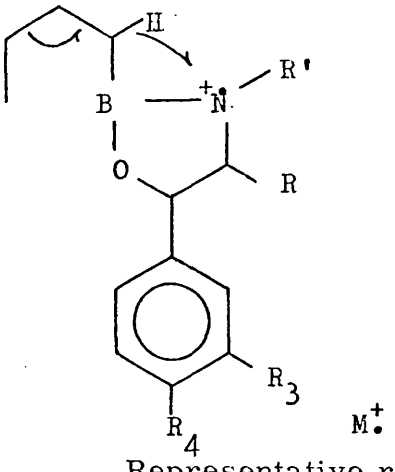


FIG. 1.6

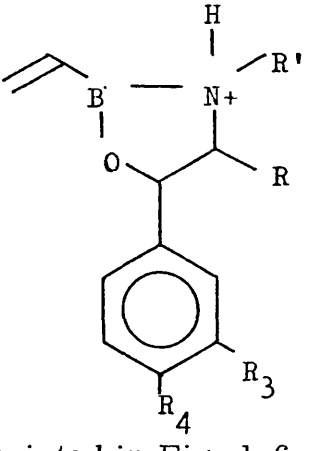
Mass spectra of synephrine n-butylboronate and 3,4-dihydroxynorepinephrine bis-n-butylboronate



The fragment I appears to arise by loss of C_2H_5 from the butyl side chain. This can occur via a 4-membered transition state.



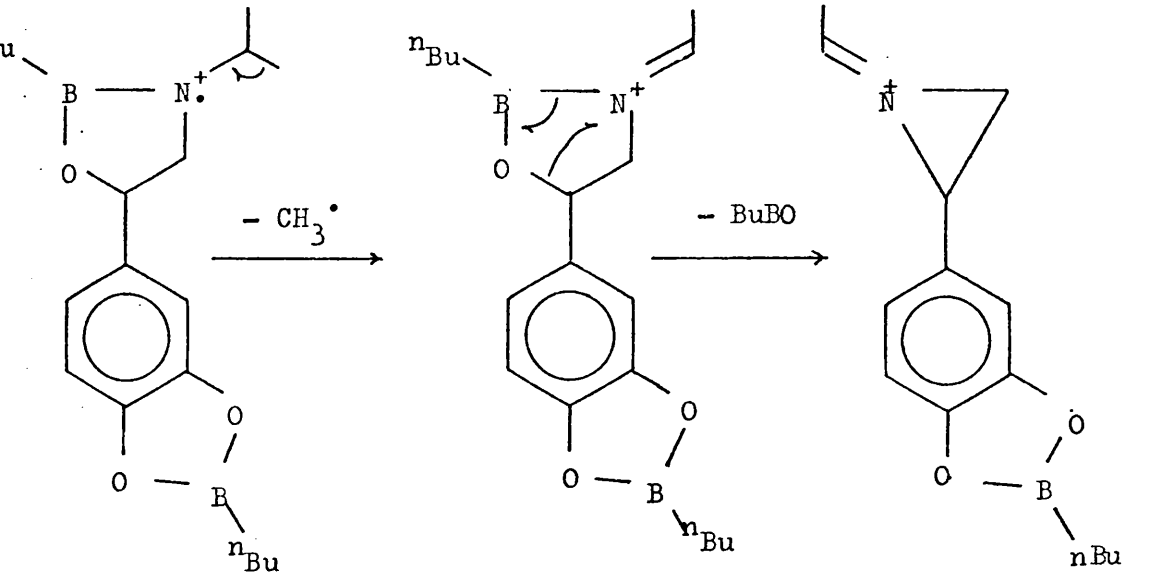
M^+



I

Representative results are depicted in Fig. 1.6, in which the mass spectra of 3,4-hydroxynorephedrine bis-n-butylboronate and synephrine n-butylboronate are given and the fragment types indicated.

Isoprenaline n-butylboronate gave only two major fragments. The first (m/e = 328) is presumably due to loss of CH_3 from the isopropyl group on nitrogen. The other predominant peak (m/e = 244) is most likely due to further loss of C_4H_9BO :



m/e = 343 m/e = 328 (100 %) m/e = 244 (66 %)

This transition is verified by a metastable peak at m/e = 181.8.

Within the group studied, substituents in the benzene ring appear to have little effect on fragmentation, which is accordingly insensitive to positional isomerism in the ring. Consequently, n-butylboronates of octopamine and 4-deoxynorepinephrine, which have a free hydroxyl group at the para and meta position respectively, cannot be effectively distinguished by their mass spectra. Their retention times are, however, sufficiently different for satisfactory characterisation as shown in Table 1.1.

Conversely, the n-butylboronates of ephedrine and

β-hydroxy-β -phenylethylamine, which cannot be separated under the conditions used, can be detected in the presence of one another by virtue of their different mass spectra. This is illustrated in Table 1.4 which shows how the peak positions of the two compounds can be located using a multiple scanning technique by measuring the heights of the respective base peaks for each scan.

Table 1.4

The effect of multiple scanning GC-MS for a mixture of ephedrine and β-hydroxy-phenethylamine as n-butylboronates (10 ft column, 1% OV-17, 130°)

Retention Index of scan	Height of peak (mm) in mass spectrum m/e = 161	m/e = 216
1784	-	30
1786	-	74
1788	1	85
1791	7	57
1793	9	30
1796	9	19
1798	8	10

SUMMARY

Qualitative analysis of catecholamines and related β -hydroxy-amines after reaction with n-butylboronic acid is

possible by the combined GC-MS technique. The boronic acid reacts with both the ß-hydroxy-amine group to form a 1, 3, 2-oxazaborolidine ring, and the catechol group to form a 1, 3, 2-dioxaborole ring, both reactions occurring under mild conditions.

Mass spectrometry gives the molecular weight, indicates the mass of substituents on positions 2 and 4 of the oxazaborolidine ring, and gives the combined molecular weights of substituents on the benzene ring. Diastereoisomers on the oxazaborolidine ring and positional isomers on the benzene ring can be distinguished by GLC using a moderately polar stationary phase.

The reaction of n-butylboronic acid with ß-hydroxy-amines as described above, is not complete, but occurs without catalyst and under mild conditions. The selectivity of the reagent also affords a clear distinction (by GLC) between catecholamines and their methylated analogues (e. g. epinephrine and metanephrine), and between compounds with and without a ß-hydroxy-amine group.

SOME MODIFIED STEROIDS USED AS DRUGS

The natural steroid hormones and their metabolites have been exhaustively studied, and convenient methods for their analytical determination have been devised. Gas chromatography has proved an extremely apt technique for such estimations. It is however, not adequate for the investigation of steroidal metabolites in situations where little other information on their structural identity is available. This problem is encountered with many of the modified steroid hormones presently used as drugs. Their well-established clinical applications, and especially their widespread general use in the control of pregnancy [118], make the development of satisfactory analytical procedures an urgent necessity. One of the most effective approaches to this problem involves the combination of the separating characteristics of gas chromatography with the structural information afforded by mass spectrometry.

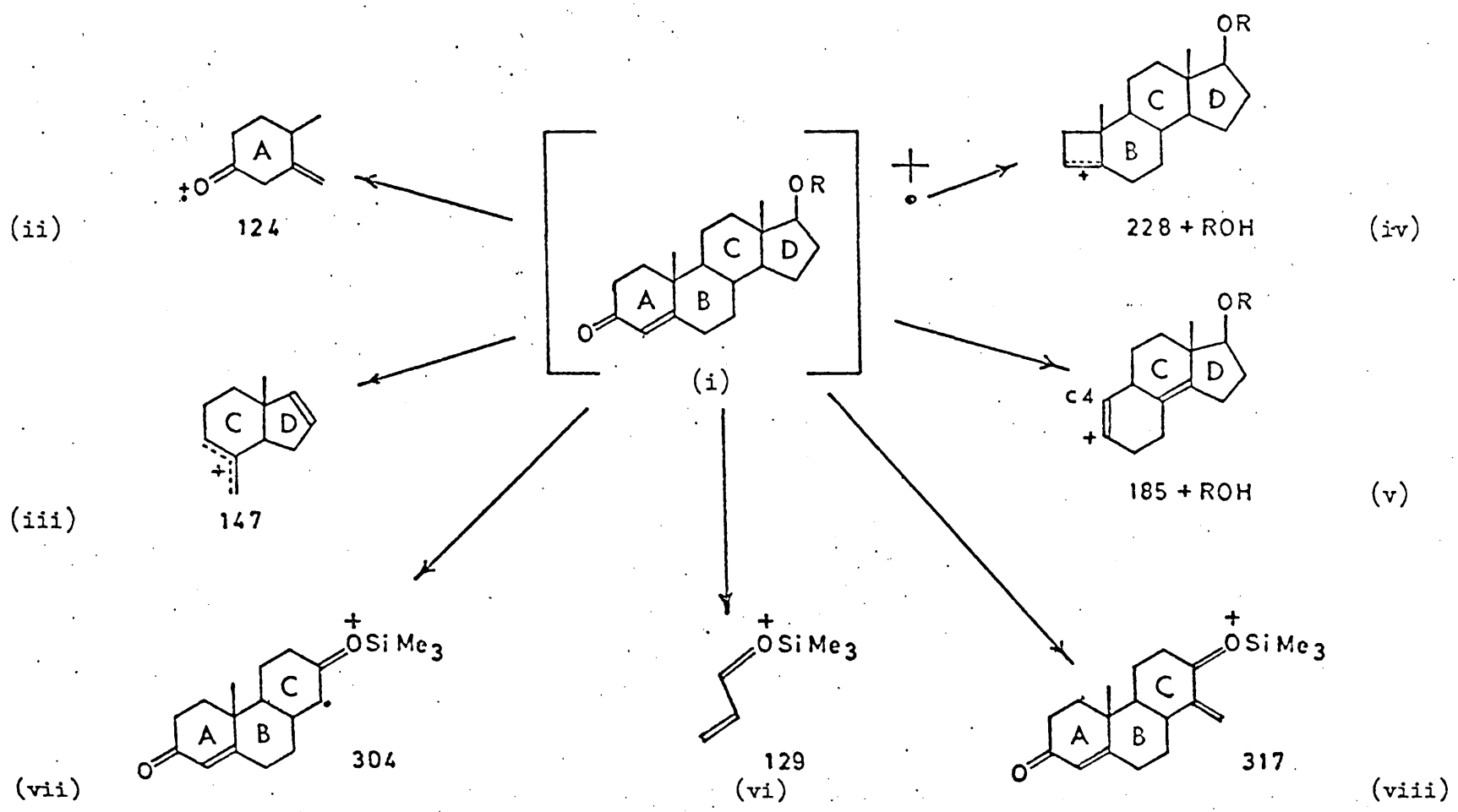
ALKYLATED TESTOSTERONES, NOR TESTOSTERONES AND THEIR TMS DERIVATIVES

Gas chromatographic data

Conditions can usually be found for the analytical

FIG. 2.1

FRAGMENTATION OF TESTOSTERONE AND TESTOSTERONE TMS.



separation of isomeric methyltestosterones or of suitable derivatives. This is illustrated by the retention index values cited in Table 2.1 for trimethylsilyl ethers of a number of methyltestosterones. The indices are of use in characterisation, but they furnish no direct information as to the positions of the methyl substituents. It may be noted that the retention index increments for the separate 6α-and 17α-methyl groups are approximately additive in the 6α,17α-dimethyl derivative.

Mass spectrometric data

The mass spectra of steroidal $\alpha\beta$-unsaturated ketones, in contrast to those of the saturated ketones, usually show characteristic fragmentation patterns: these have been elucidated for several structural classes by Djerassi and co-workers [119,120]. Table 2.2 indicates the five most intense fragment ions observed for androst-4-en-3-one [119], and the positions of corresponding peaks for testosterone, 17α-methyltestosterone and 19-norandrost-4-en-3-one. Formulations proposed by Shapiro and Djerassi [119] are shown in Fig. 2.1, Types (i) - (v).

In the analysis of hydroxysteroids by gas chromatography, their conversion to trimethylsilyl ethers is frequently advantageous[33].

TABLE 2.1

Retention index increments for methyl substituents

Testosterone trimethylsilyl ether	1% OV-1 230°C 2640	1% OV 250°C 2985
	Δ_{MU}	Δ_{MU}
2 α	+ 15	- 20
4	+ 40	+ 60
6 α	+ 55	+ 30
6 β	+ 40	+ 15
6 α,17α	+ 150	+ 125
17 α	+ 80	+ 80

TABLE 2.2

Principal ions from Δ^4-3-oxosteroids

Androst-4-ene-3-one (a)	Testosterone	17 α methyl testosterone	19-nor androst-4-ene-3-one	Nature of Ion
272	288	302	258	molecular ion
124	124	124	110	ring A + C-19, C-6
149	147	161	149	rings C and D + C-7
230	246	260	230	loss of ketone
187	203	217	187	rings C and D + C-4 to C-8

(a) from ref 119

Table 2.3

Characteristic Ions from trimethylsilyl ethers of testosterone [a] and some modified testosterones

Substituent											Origin
Testosterone							Nortestosterone				
None	2αMe	4 Me	6αMe	6βMe	6α 17αdiMe	17αMe	17αEt 19-nor	19-nor	17αMe 19-nor	17αMe A-nor	
129	129	129	129	129	143	143	157	129	143	143	C15-17 + TMS group (vi)
360	374	374	374	374	388	374	374	346	360	360	Molecular ion (i)
47%	46%	52%	37%	42%	8%	2%		24%	2%	2%	% of base peak
270	284	284	284	284	298	284	284	256	270	270	M-Me_3SiOH
147	147	147	147	147	--	--	--	147	--	--	(iii)
226	226		240	(240)[b]	--	(240)	--	--	--	--	?
124	138	138	138	138	--	--	--	(110)	--	--	(ii)
345	359	359	359	359	(373)	(359)	(359)	331	345	345	M-CH_3
304	318[c]	(318)	(318)	(318)	318	304	(290)	(290)	(290)	(290)	(vii)
228	228	--	--	--	--	--	--	(214)	--	--	(iv) - Me_3SiOH
116	116	116	116	116	130	130	144	116	130	130	$[CH_2 = CHR - OSiMe_3]^+$
(237)	(237)	237	(237)	(237)	--	--	--	--	--	--	(iii) retaining MeSiOH
(185)	(185)	--	(199)	(199)	--	--	--	--	--	--	(v) - MeSiOH
(317)	(331)	(331)	(331)	(331)	331	(317)	303	(303)	(303)	(303)	(viii)
(318)	318[c]	--	(332)	(332)	--	--	--	(304)	--	--	(iv)

a Peaks from testosterone are listed in order of abundance

b Parentheses indicate abundance 1% < 10% of base peak

c The peak at m/e = 318 may arise from (vii) or (iv)

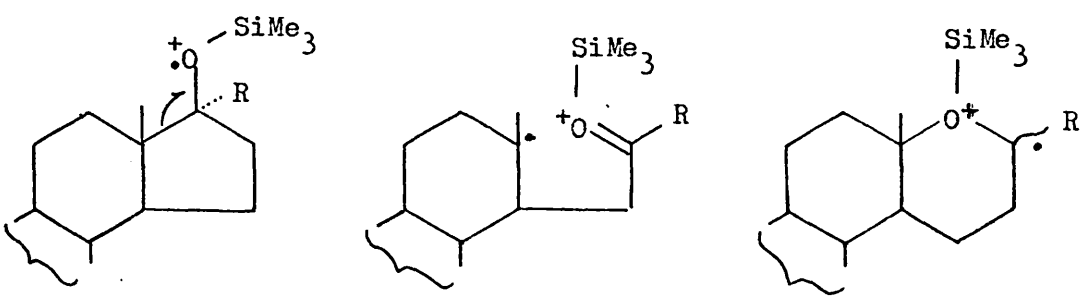
It is particularly convenient to apply these derivatives in combined gas chromatography-mass spectrometry, because their mass spectra usually afford more characteristic structural information than those of the free alcohols [33, 52-54, 73, 121]. Thus testosterone trimethylsilyl ether yields (as the most abundant ion) a fragment of m/e 129, evidently [52, 54] comprising C_{15}-C_{17} with the substituent group (cf. Fig. 2.1, Type (vi)).

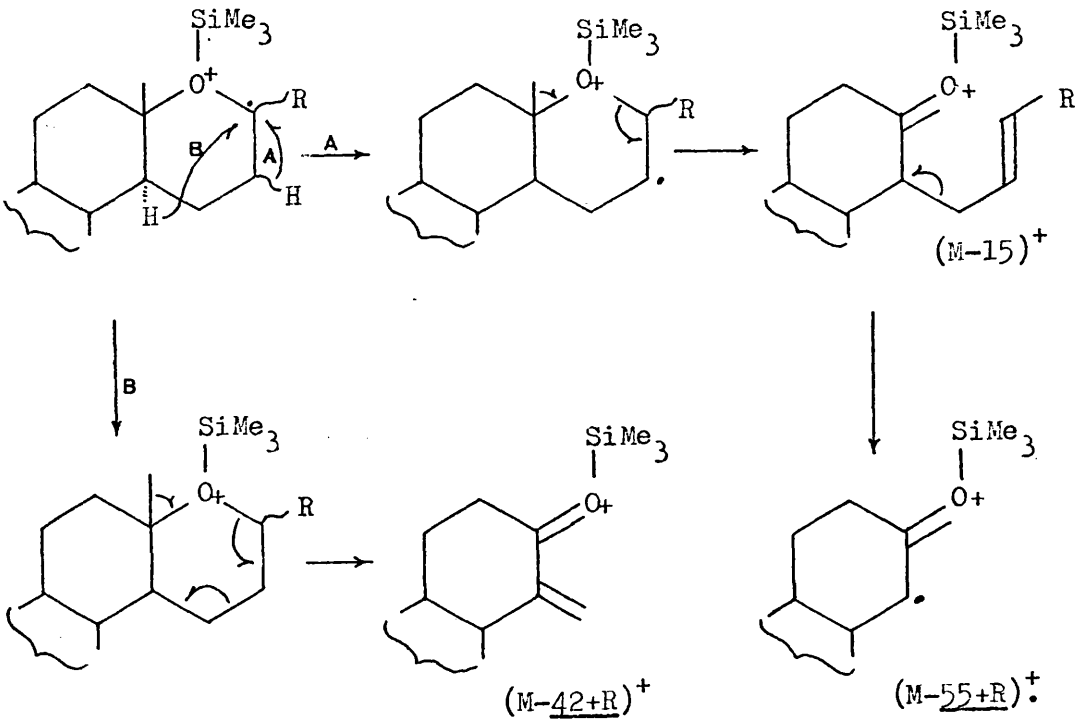
Mass spectrometric data for the trimethylsilyl ethers in Table 2.3 show how these regularities extend to alkyl substituted testosterone derivatives and thus aid the location of substituents in such modified steroids. The ion of type (vi) is uniformly the most abundant, and its importance is enhanced by the introduction of a 17α-alkyl group. Location of a methyl group on $C_{(15)}$, $C_{(16)}$ or $C_{(17)}$ is indicated by the m/e value of 143 in this instance. More precise location on $C_{(16)}$ or $C_{(17)}$, is implied by the peak at m/e 130.

When an ethyl substituent is present at the 17 α - position, ring D fragmentation is even more predominant and the group's location on ring D is indicated by the fragments at m/e 157 and m/e 144. The nature of the extra fragments

which are enhanced by substituents in the 17α-position are discussed in more detail in the section on 17α- ethynyl steroids.

Testosterone trimethylsilyl ether yields an interesting peak at m/e 304 (type (vii)), which has no analogue at m/e 232 in free testosterone, and which is prominent in 17α -methyl-testosterone trimethylsilyl ether. It appears to result from loss of a methyl group followed by a fragment comprising $C_{(15)}$-$C_{(17)}$ (with substituents if present). The trimethylsilyl ether group is retained, presumably through migration to $C_{(13)}$ or to another nuclear position. A peak observed at m/e 317 would appear also to result from a migration of the trimethylsilyl ether group, but with retention of an additional carbon atom, probably $C_{(15)}$ as in the tentative formula (viii) (Fig. 2.1). The mechanisms are unestablished, but a formal possibility is outlined below.

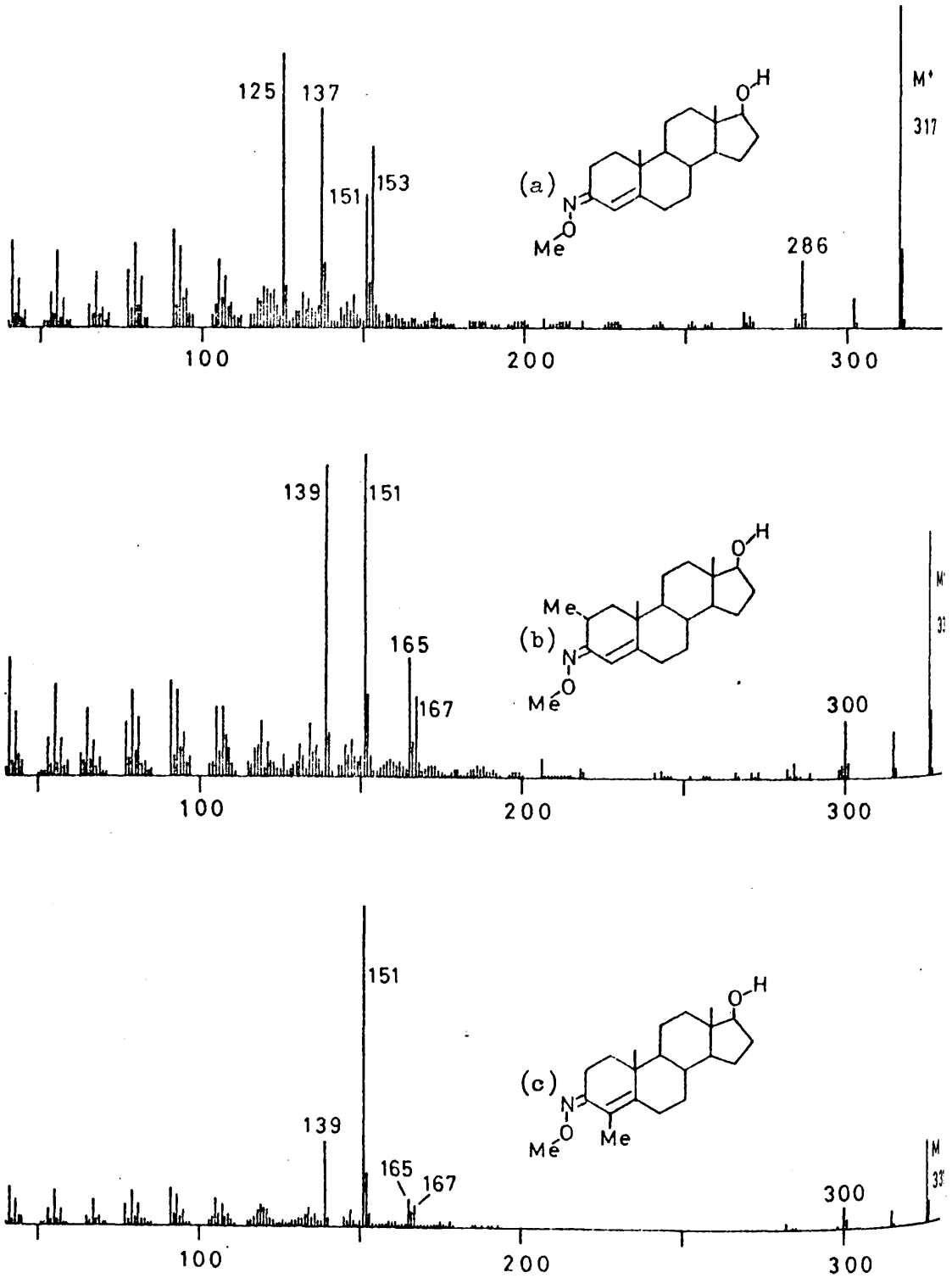




Methyl groups situated in rings A and B are disclosed by observations on other ions. The fragment of type (ii), for example, appears at m/e 138 in the 2α, 4 and 6-methyl derivatives, and at m/e 110 for 19-nor testosterone TMS ether. The 6-methyl derivative may be further distinguished by consideration of the ion of type (v), which includes $C_{(4)}$-$C_{(8)}$ and thus appears at m/e 199

FIG. 2.2

Mass spectrum of testosterone MO, 2α-methyltestosterone MO and 4-methyltestosterone MO



in the 6-methyl derivatives, though no prominent corresponding peak is observed in the 4-methyl analogue. Both 6-methyl compounds also show well-defined peaks at m/e 240, probably corresponding to those at m/e 226 in testosterone and 2α -methyl testosterone trimethylsilyl ether: the nature of these ions is however not yet established.

METHYL OXIMES OF TESTOSTERONE AND ANALOGS

Although methyl oximes generally fragment under electron impact in a similar manner to their parent ketones [71], some results (e.g. Goldsmith et al [70], and Part 4 of this thesis, "O-Substituted Oxime Derivatives") have shown in some cases that this derivative can fragment differently from its carbonyl analog. Fig. 2.2a shows the mass spectrum of testosterone methyl oxime. Table 2.4 compares some of the principal ions observed in this spectrum with ions observed in the spectra of oximes of methyltestosterones and nortestosterones.

Table 2.4

Characteristic ions from methyloximes of testosterone, methyl analogs and nortestosterones.

Parent Steroid	m/e			
	a	b	c	d
Testosterone	125*	137	151	153
2α Methyltestosterone	139*	151	165	167
4-Methyltestosterone	139	151*	165	167
6α,17α-Dimethyltestosterone	125	151	165	167
19-Nortestosterone	-	-	137	139
17α-Methyl-19-nortestosterone	-	-	137	139
17α-Methyl-A-nortestosterone	-	-	137	139

* denotes base peak.

Although the mechanism of fragmentation is difficult to postulate on this evidence alone it would appear that there is no fragmentation of ring A. It does not appear possible therefore to distinguish isomeric methyl substituents on ring A by mass spectrometry of this derivative, although the 6-methyl group is evident by its loss in fragment 'A'. 19-Nor- and A-nor-

Table 2.5

Comparative data for 2α- and 4-methyl testosterone and derivatives

	MW	M.U. (OV-1)			M.U. (OV-17)			Main MS Peaks	
		Temp. °C	2α	4	Temp. °C	2α	4	2α	4
Free	302	230	25.90	26.10	250	30.45	30.65	138, 302, 246	123, 138, 302
MO	331	200	26.20	26.10	230	30.30	30.60	139, 151, 331	151, 139, 331
TMS	374	230	26.55	26.80	250	29.60	30.45	129, 374, 284	129, 138, 147
MO. TMS	403	230	26.90	27.30	250	29.30	29.65	403, 139, 151	151, 139, 403

Table 2.6

Comparative data for 19- and A-nor-17α -methyl-testosterone and some derivatives

	MW	M.U. (OV-1)			M.U. (OV-17)			Main MS Peaks	
		Temp. °C	19-nor	A-nor	Temp. °C	19-nor	A-nor	19-nor	A-nor
Free	288	205	24.95	24.05	220	29.55	28.43	288, 110, 231	71, 109, 231
TMS	360	205	26.23	25.34	220	29.36	28.19	143, 73, 270	143, 73, 270
MO	307	205	25.46	24.12	220	29.43	28.25	317, 137, 139	317, 137, 139
				24.33		29.64	28.63		

testosterones do not give the expected fragments at m/e = 111 and m/e = 123 for a and b type ions.

DISTINCTION BETWEEN 2α- AND 4-METHYLTESTOSTERONE

A summary of gas chromatographic retention data and of the three principal mass spectrometric peaks, is given in Table 2.5 for 2α - and 4-methyltestosterone and three corresponding pairs of derivatives.

The mass spectra of the free steroids are depicted in Fig. 2.3. The strong peak at m/e 260 in the spectrum of 2α-methyltestosterone, which is not observed in any of the isomeric derivatives examined, may be confidently attributed to an ion of type (iv) resulting from the loss of methylketene. An analogous ion at m/e = 272 has been observed in the mass spectrum of 2α-methyl-4-pregnen-3,20 dione by Grostic and Rinehart.[122] It is accordingly consistent only with a 2α-methyltestosterone structure.

The mass spectra of the trimethylsilyl ethers, shown in Fig. 2.4 are dominated by the peak of type (vi) at m/e 129, and differences characteristic of the methyl group location are less striking. The ion of m/e (M-56) is more abundant

FIG. 2.4

Mass spectra of 2α-methyltestosterone TMS and 4-methyltestosterone TMS.

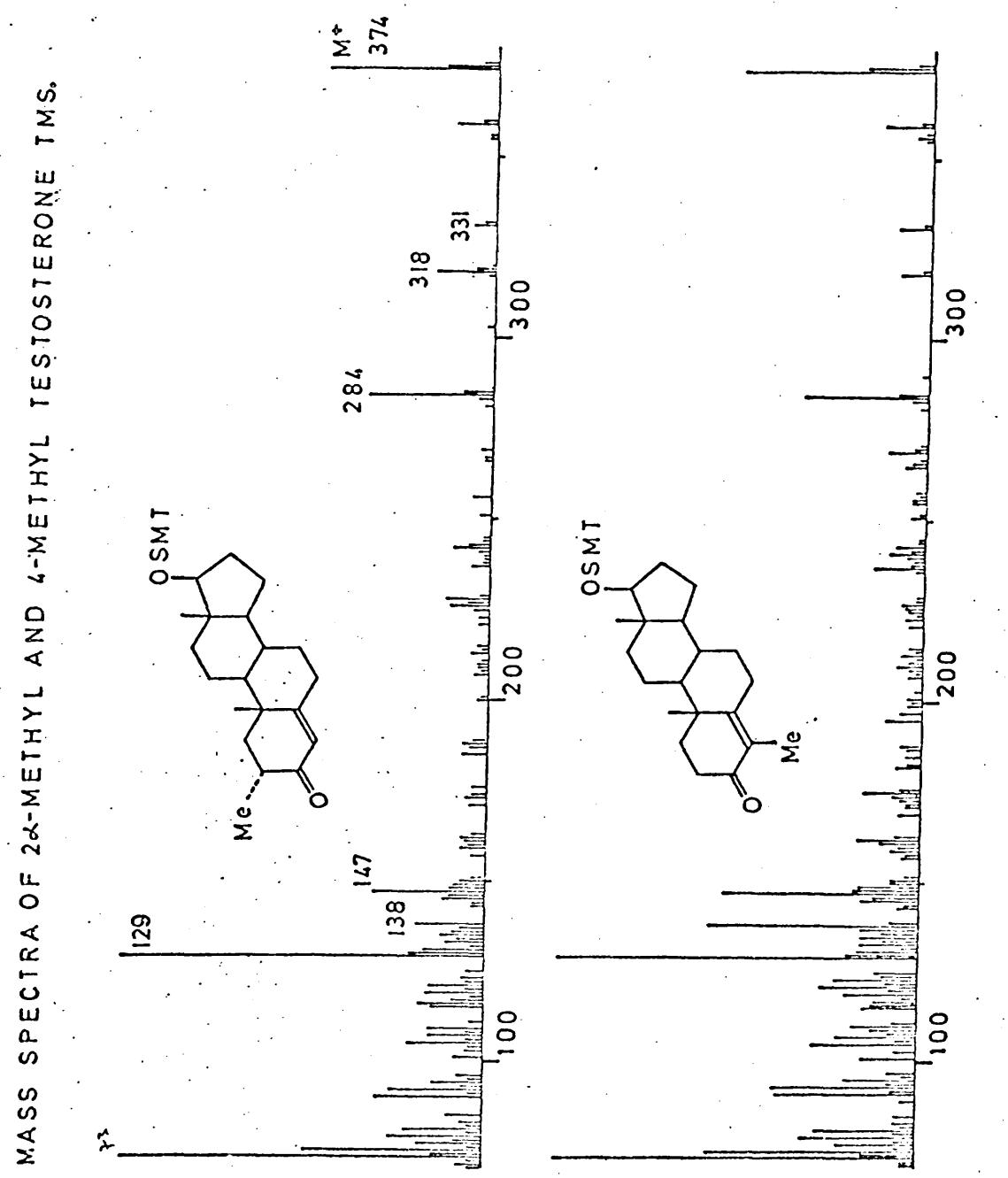


FIG. 2.5

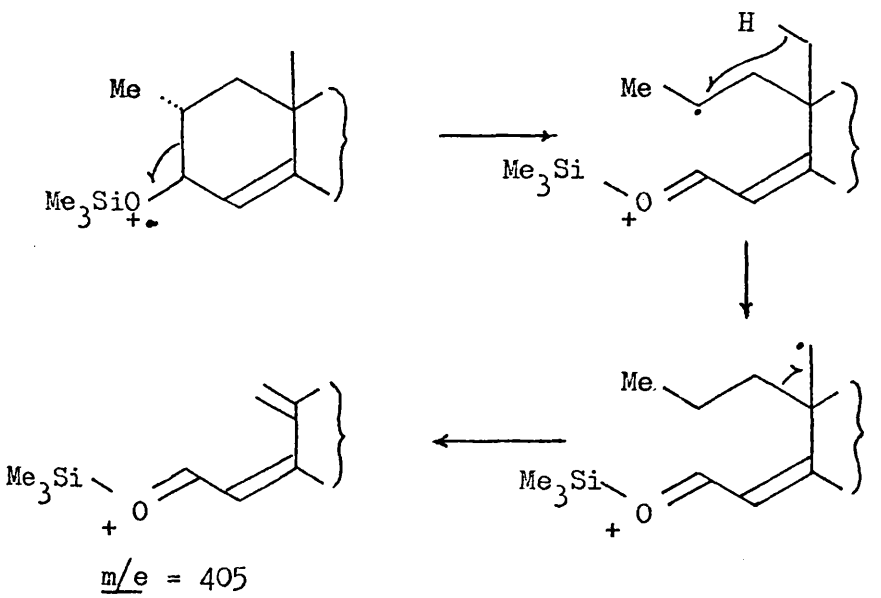
Mass spectra of TMS ether derivatives of reduced methyl testosterones

in the 2α- than in the 4-isomer, but its significance is obscured by the fact that, in the former case, it may be compounded of types (iv) and (vii).

The mass spectra of the methyloximes are shown in Fig. 2.2 (b + c). Although both steroids apparently undergo very similar fragmentation routes there is sufficient difference in the intensity ratio of the fragments of m/e = 139 and m/e = 151 to distinguish these two isomers.

Partial reduction of the methyltestosterones, using sodium borohydride, afforded mainly the 3β-hydroxy-Δ^4-steroids [123]. These are unstable towards gas chromatography, and were examined as their trimethylsilyl ethers. The characteristic peaks in the mass spectrum of reduced testosterone at m/e 143 and 142 are shifted in the 2α- and 4-methyl compounds to m/e 157 and 156 respectively. (This affords a distinction from 6-methyl derivatives (Fig. 2.5 c), which yield ions at m/e 143 and 142). Differences between the spectra of the 2α- and 4-isomers are relatively small (Fig 2.5 a + b) but a peak of diagnostic value occurs at m/e 405 in the 2α-methyl derivative, and

corresponds to a less prominent peak in the spectrum of androst-4-ene-3β,17β -diol ditrimethylsilyl ether. It is attributable to an ion arising through loss of C_3H_7 from ring A possibly as shown below



m/e = 405

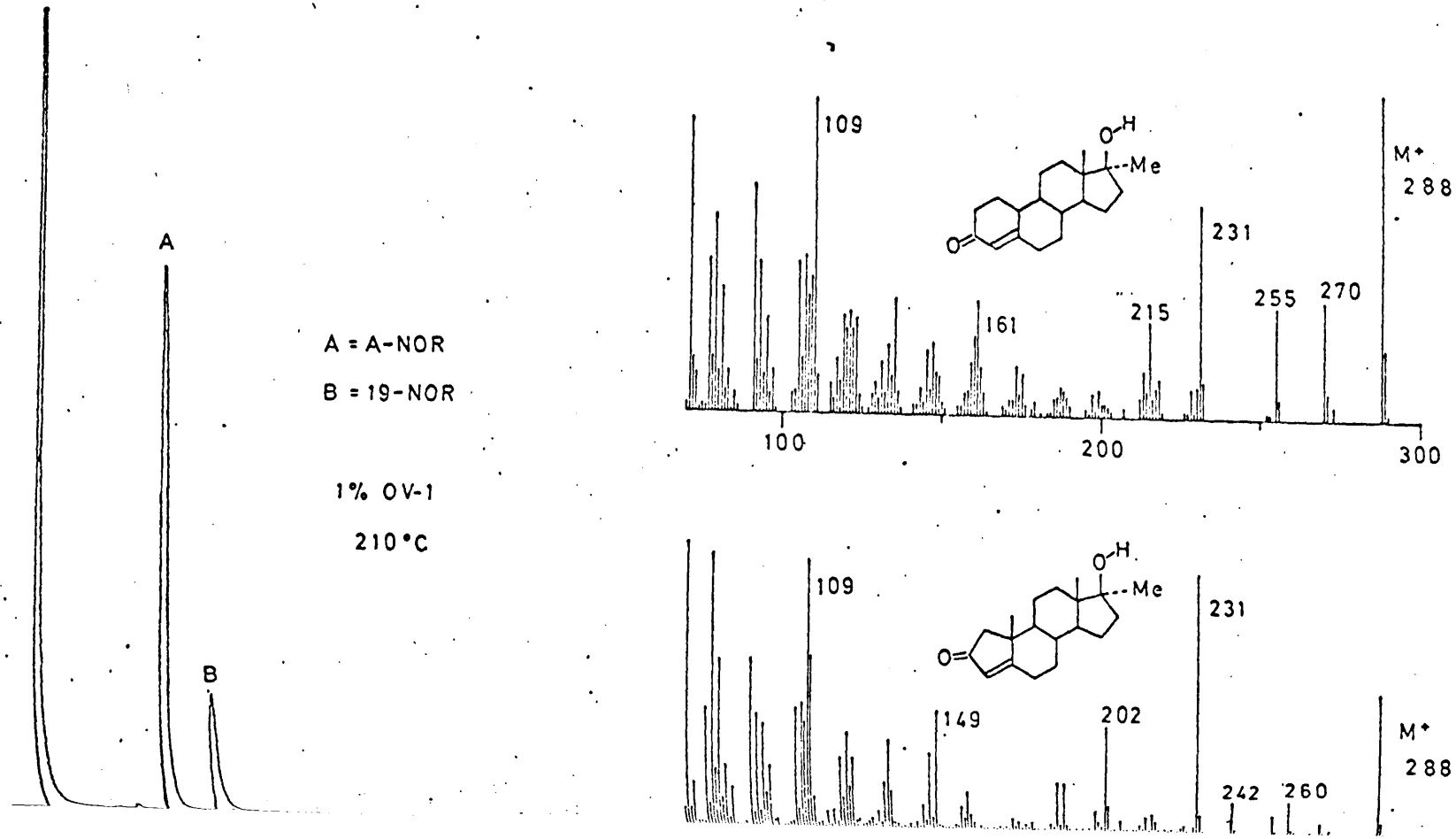
DISTINCTION BETWEEN 19- AND A-NORTESTOSTERONES

A summary of gas chromatographic and mass spectrometric data is given in Table 2.6 for 19- and A-nor-17α -methyltestosterone and two corresponding pairs of derivatives.

There are marked differences in retention time between the two steroids in their free form, and as their

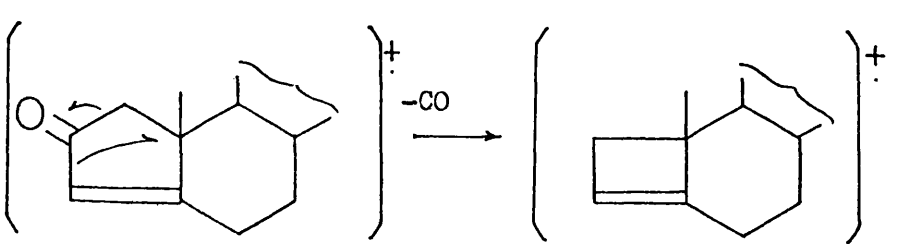
FIG. 2.6

G.L.C. AND MASS SPECTRA OF 19-NOR AND A-NOR 17α-METHYL TESTOSTERONES



TMS and MO derivatives on both OV-1 and OV-17 columns. The separation of syn- and anti- methyl oxime isomers of 17α-methyl-A-nortestosterone on the non-polar OV-1 column is unusual, but has also been noticed in this laboratory for 5α cholestan-2-one methyloxime (see part 4).

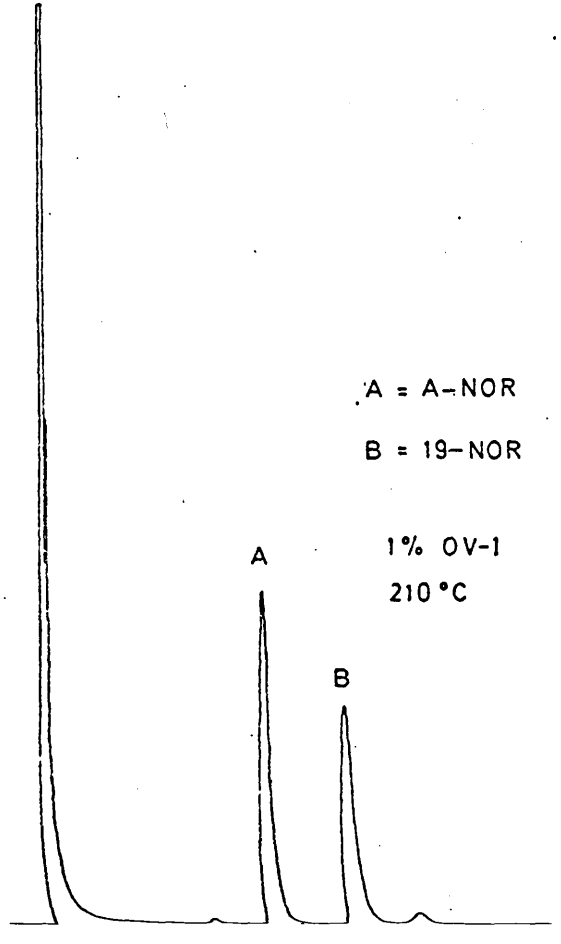
Fig. 2.6 shows a gas chromatogram of the two free steroids together with their mass spectra. The mass spectra show many differences in their fragmentation, a notable one being a loss of carbon monoxide from the molecular ion of 17α-methyl-A-nortestosterone to give an ion at m/e = 260. This fragment is unparalleled by the 19-nor-isomer but is most likely of the same structure as the ion of this mass value in the mass spectrum of 17 α-methyltestosterone (Table 2.2) which is produced by loss of ketene.

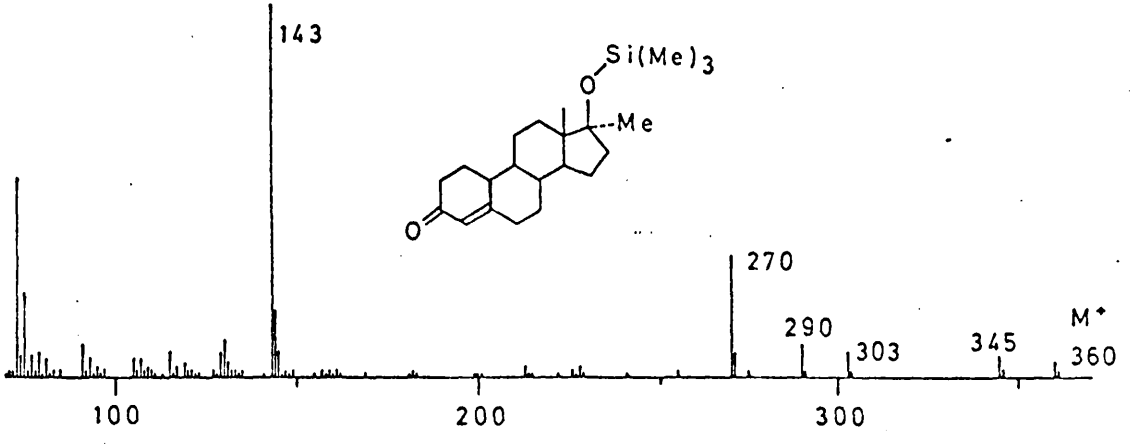


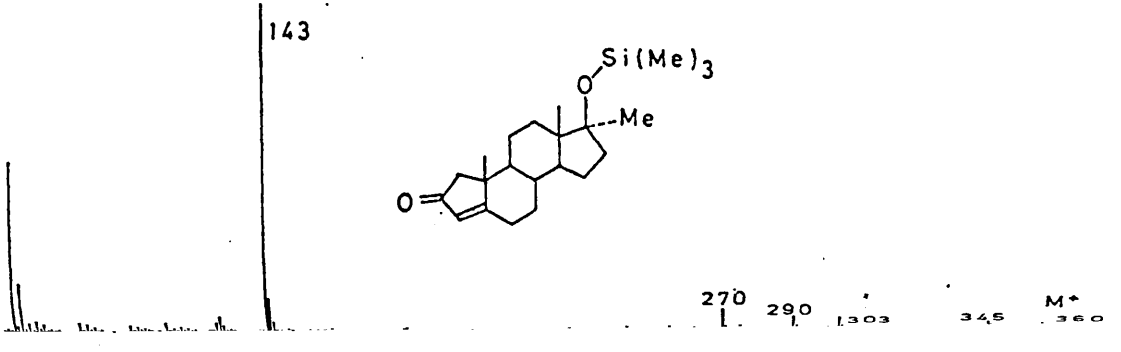
$M^{+\cdot}$ $(M-28)^{+\cdot}$

FIG.2.7

G.L.C. AND MASS SPECTRA OF 19-NOR AND A-NOR 17 α-METHYL TESTOSTERONE TRIMETHYLSILYL ETHERS



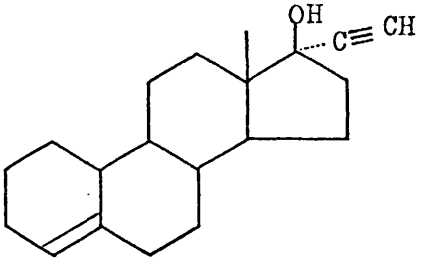




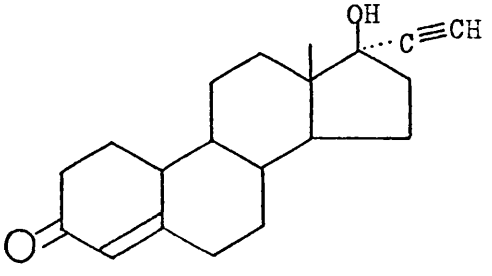
In contrast to those of the free steroids, the mass spectra of the TMS and MO derivatives show striking similarity. The former derivatives (Fig. 2.7) give mass spectra resulting almost entirely from ring D fragmentation and the methyl oximes, since they do not appear to direct fragmentation of ring A, also give very similar mass spectra for the two isomers.

ETHYNYL SUBSTITUTED STEROIDS

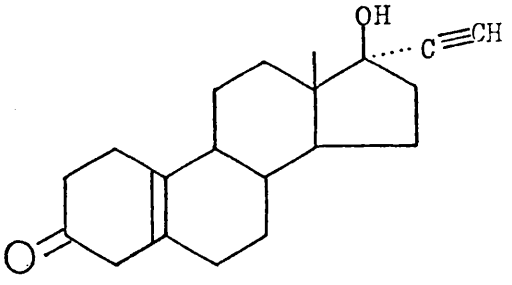
Steroid drugs containing a 17α-ethynyl 17 β-hydroxyl group constitute an important family of progestational agents. Three such steroids commonly used in oral contraceptives areLynestrenol, norethindrone and norethynodrel which are shown below.



Lynestrenol



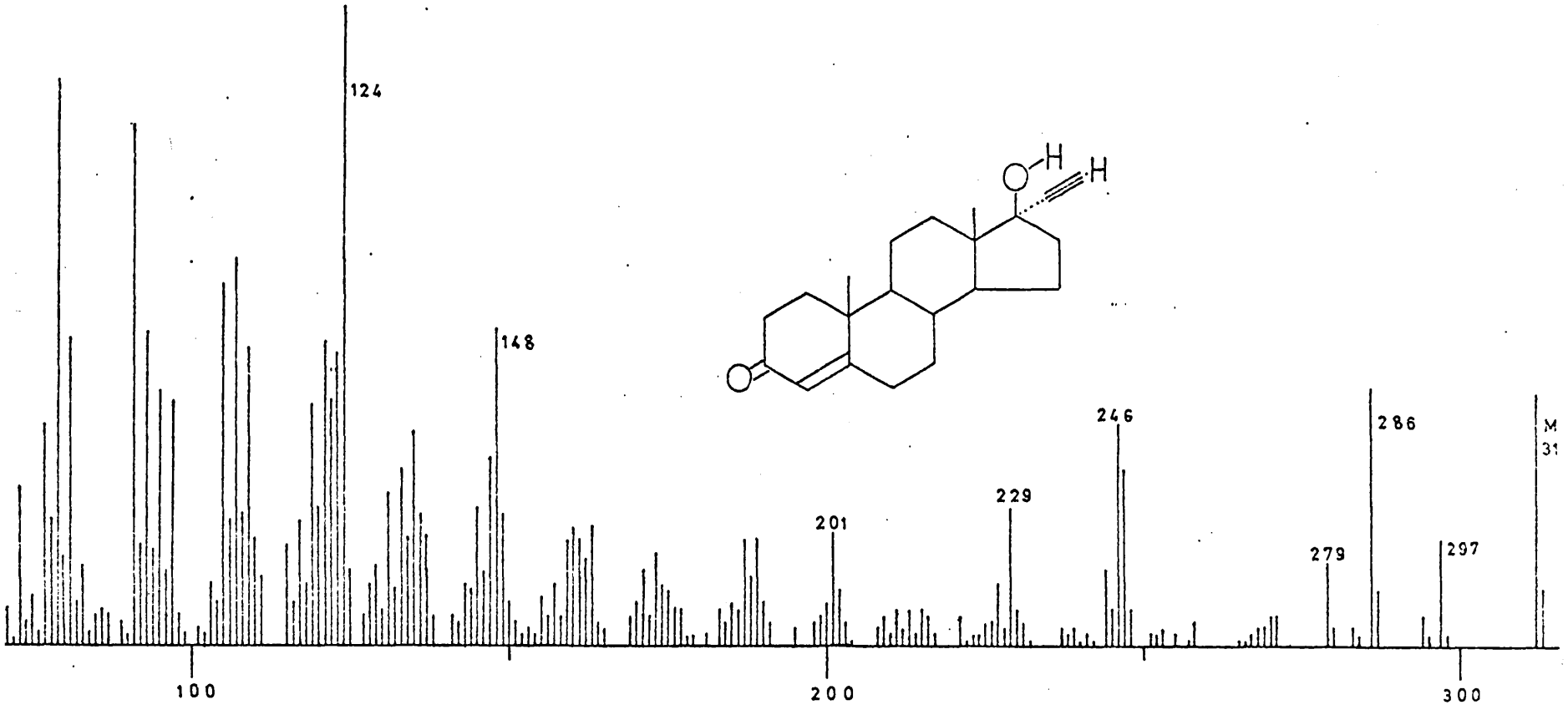
Norethisterone



Norethynodrel

FIG. 2.8

Mass spectrum of 17∝ -ethynyltestosterone



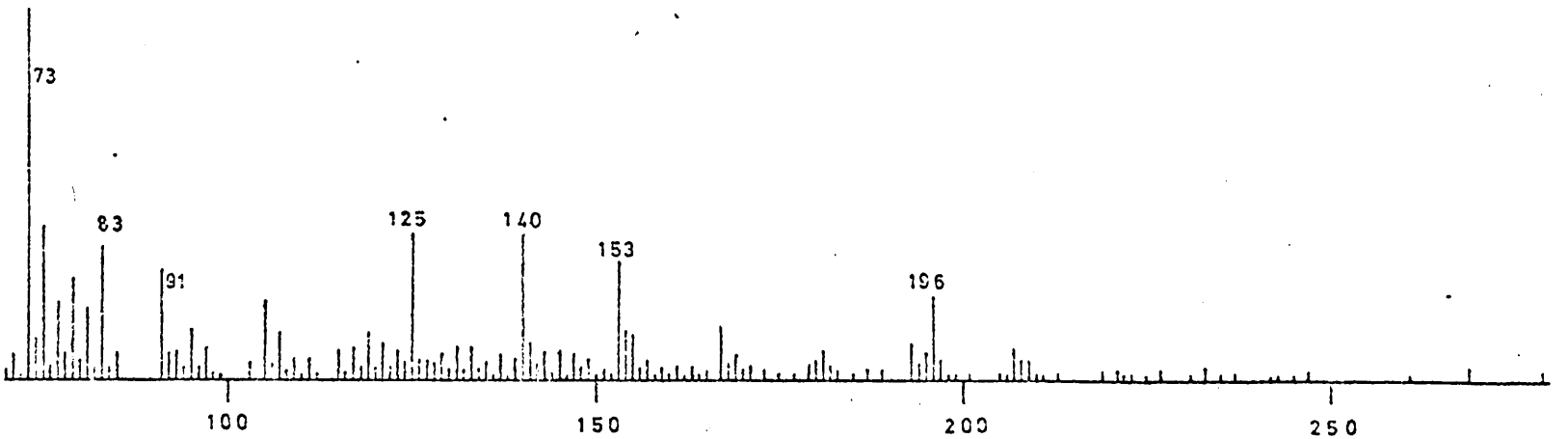
Because of the relatively small amounts of these drugs which can be tolerated by man, most metabolism studies have been confined to radio-tracer methods [124-127], and progress appears to have been made only recently [185] towards structural identification of their metabolites.

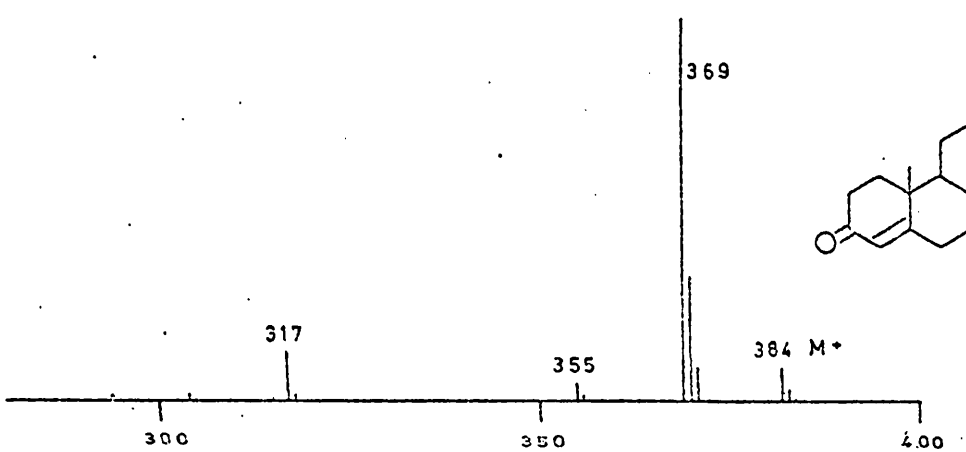
Kamyab and others by chromatographing metabolites of 4-^{14}C lynestrenol [124] on Florisil + silver nitrate established that effectively all of these metabolites contained the ethynyl group. This fact was also reported by the same authors for the metabolites of norethisterone [127].

Recognition of the 17α-ethynyl group would be very useful when examining metabolites of such steroid drugs by GC/MS. Fig. 2.8 shows the mass spectrum of 17α-ethynyl testosterone. The base peak at m/e = 124 is characteristic of the Δ4-3-oxo group involving fission of the C6-C7 and C9-C10 bond (see Fig. 2.1). No new fragmentation which is characteristic of the ethynyl group is evident except for that at m/e = 286 involving loss of acetylene from the molecular ion. Fragments containing the ethynyl group which are analogous to fragments containing ring D in testosterone occur at m/e = 171

FIG.2.9

Mass spectrum of 17α - ethynyltestosterone TMS ether





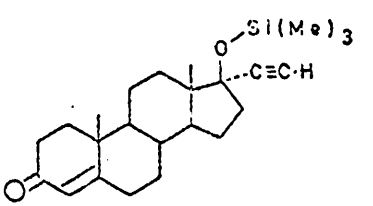
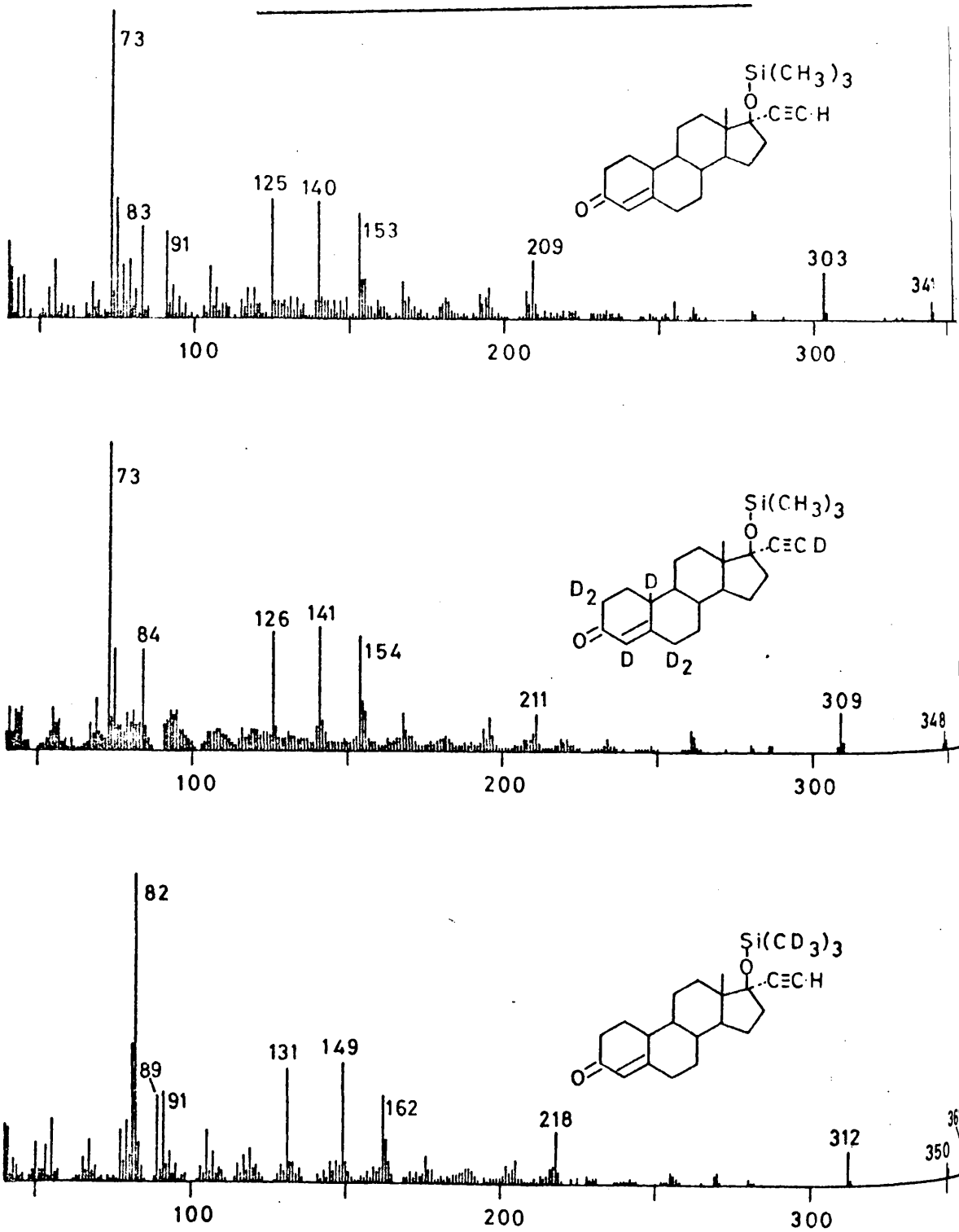


FIG. 2.10

Mass spectra of Norethisterone TMS and deuteriated analogs:
(a) Norethisterone TMS;(b) 2,2,4,5,5,10-d_6 Norethisterone TMS;
(c)Norethisterone -d_9- TMS



(cf. Fig. 2.1 type iii), m/e = 227 (cf Fig. 2.1 type v) and m/e = 270 (cf Fig. 2.1 type iv). These fragment ions are of low relative abundance and not prominent in the mass spectrum.

The trimethylsilyl ether of 17α-ethynyl testosterone however would be expected also to contain a fragment ion at m/e = 153 analogous to the ion at m/e = 129 for testosterone trimethylsilyl ether (Fig. 2.1 type vi). This fragment occurs with a relative abundance of 32% as shown in Fig. 2.9. Other prominent ions not present in testosterone trimethylsilyl ether occur at m/e = 83 (36%), m/e = 125 (40%) and m/e = 140 (40%) in the ethynyl steroid.

Fig. 2.10 shows the mass spectra of norethisterone trimethylsilyl ether compared with that of its d_7 analogue. This deuteriated derivative was obtained by gas chromatography on a column containing 0.5% barium deuteroxide. This technique which has been shown to replace hydroxylic enolic and acetylenic hydrogen on the steroid nucleus will be discussed in part 4.

The fragment ions m/e = 83, m/e = 125, m/e = 140 and

m/e = 153 which were observed in the 17α-ethynyl testosterone TMS ether are also prominent in the mass spectrum of its 19-nor analogue. Furthermore, replacement of the acetylenic hydrogen by deuterium causes a shift of one mass unit for these ions to give fragments at m/e = 84, m/e = 126, m/e = 141 and m/e = 154 respectively. It is extremely likely therefore that these ions contain the ethynyl group and are characteristic of its presence.

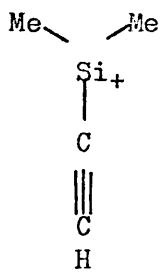
Further evidence of the nature of these ions is afforded by the mass spectrum of norethisterone tri(trideutero) methylsilyl ether (Fig. 2.10 c). This was formed by reacting norethisterone with d_{18} -bis-trimethylsilyl acetamide in pyridine. The analogous fragments give mass shifts summarised in Table 2.7.

Table 2.7

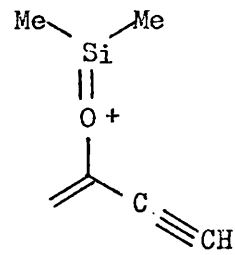
Fragment ions containing the ethynyl group in the mass spectra of norethisterone TMS and deuteriated analogues

	m/e			
	a	b	c	d
Norethisterone TMS	83	125	140	153
Norethisterone TMS after $Ba(OD)_2$ exchange	84	126	141	154
Norethisterone-d_9-TMS	89	132	149	162

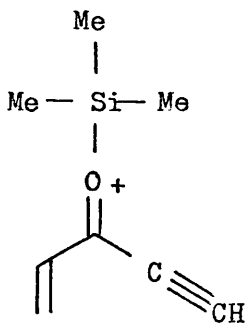
The evidence above suggests the following structures for the ions cited for norethisteroneTMS.



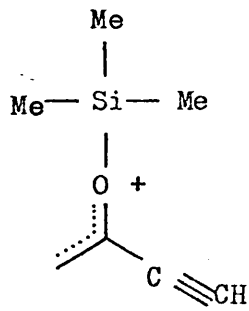
(a) m/e = 83



(b) m/e = 125

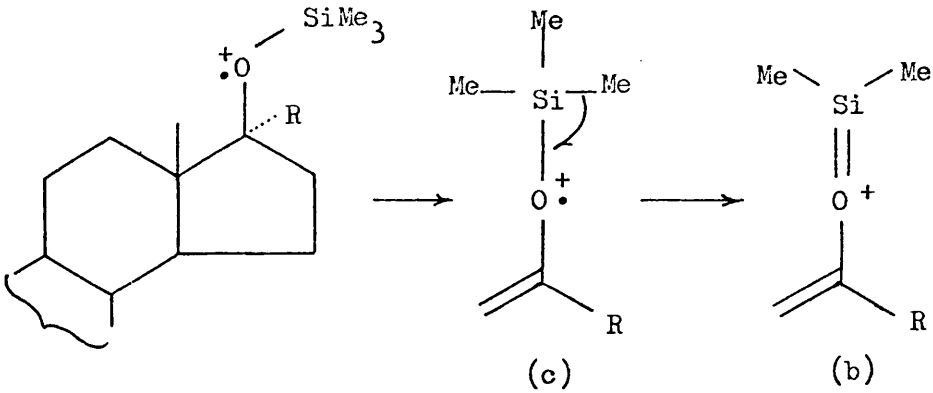


(d) m/e = 153



(c) m/e = 140

The fragment at m/e = 140 is most likely similar in structure to that at m/e = 144 in norethandrolone TMS ether and that at m/e = 130 in 17α-methyl testosterone. The loss of methyl which is observed from these fragments is verified by metastable ions. The general fragmentation sequence is postulated below.



(c) (b)

R	Steroid TMS	m/e c	m/e b	m*	c ⟶ b
C_2H_5	Norethandrolone	144	129		115.8
C_2H	Norethisterone	140	125.		111.7
CH_3	17α-methyltestosterone	130	115		101.8

Although similar fragments occur in testosterone TMS ether (where R = H) at m/e = 116 (type c) and m/e = 101 (type b) they are less easily recognised due to other more prominent neighbouring fragment ions. The fragment at m/e = 83 in norethisterone TMS ether however appears to have no counterpart in steroids with 17α-alkyl groups, e.g. there is no analogous fragment at m/e = 87 in norethandrolone TMS ether.

The above ions which have been shown to be characteristic

of the ethynyl group in norethisterone TMS ether are also prevalent in other steroids containing this functional group. They can sometimes be observed when ketonic groups have been converted to the methyloxime derivative as shown in Table 2.8

Table 2.8

Steroid derivatives showing fragmentation characteristic of the ethynyl group

Steroid	m/e			
	83	125	140	153
Lynestrenol TMS	30%	10%	24%	24%
Norethisterone TMS	30%	39%	38%	34%
17α ethynyltestosterone TMS	36%	40%	40%	32%
Norethynodrel TMS, MO	18%	9%	10%	19%
Norethisterone TMS, MO	25%	15%	11%	34%

CONCLUSIONS

The results outlined show how mass spectrometry may be used to locate alkyl and ethynyl groups on some steroids used as drugs. It has also been shown possible to demonstrate the absence of the angular methyl group at C-10 for Δ^4 -3-oxo-steroids and distinguish this type from A nor Δ4-3-oxo steroids.

In order to distinguish substituents on ring A of the steroid nucleus by mass spectrometry it is desirable that fragmentation of

this ring should occur on electron impact. Fragmentation of this sort is favoured by a Δ^4-3-oxo group or by a Δ^4-3-OTMS group. The Δ^4-3-methyl oxime group does not cause fragmentation of ring A and is not therefore a useful derivative for characterising isomeric substituents on this part of the molecule.

Another cause for failure to distinguish Δ^4-3-oxo-steroids isomeric in ring A is the presence of a strong fragment directing group at a non-proximal position in the molecule such as the 17β -OTMS group in the case of the 2 α-Me- and 4-Me-testosterone TMS derivatives, and the 17 α -methyl-17 β -OTMS group in the case of the A-nor- and 19-nor-testosterones of this type.

Substituents in the 17α-position of testosterone TMS ethers are easily recognised because of the strong fragmentation-directing properties of the 17β-OTMS group producing ions containing C-16 and C-17 of ring D. The 17 α-ethynyl group in conjunction with the 17β-OTMS group is signalised by such fragments even when they are of low abundance, since they give rise to a unit mass shift after chromatography on alkali treated columns saturated with deuterium oxide.

It is of course essential to recognise that certain other types of substituent may alter profoundly the pattern of fragmentation. The chances of erroneous assignment of the origins of observed ions are, however, greatly reduced if careful consideration is given to comparative data for several functional derivatives. Where necessary, assignment of the elemental composition of ions can be made by high resolution mass spectrometry, and their source can be established more rigorously by appropriate studies of isotopically labelled compounds.

STEROID OLEFINS

GLC AND MS OF STEROID OLEFINS

Determining the position of unsaturation in the steroid nucleus often proves particularly difficult when using gas chromatographic and mass spectrometric methods of analysis. Since olefinic bonds impart only small polarity differences isomeric steroid olefins can be difficult to separate by gas-liquid chromatography on inert supports. Large deviations in retention behaviour are usually observed only for isomers involving conformational changes (e. g. unsaturation at ring junctions), or electrochemical changes (e. g. olefin in conjugation with a carbonyl group) in the molecule.

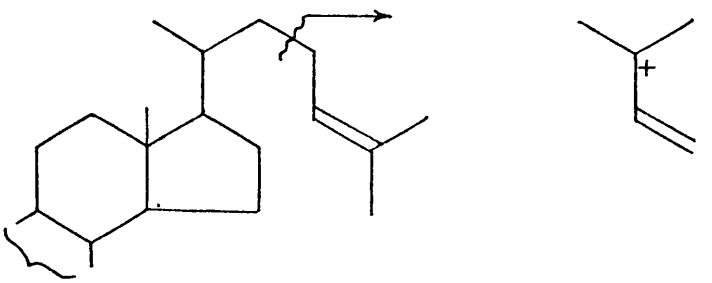
Paralleling these observations, mass spectrometry often provides little evidence of the position of isolated olefinic unsaturation, since many of these groups possess small ability to direct the mode of fragmentation. This is especially noticeable when in the presence of oxygenated substituents (not in the proximity to the double bond) which exert a stronger influence on the mode of fragmentation.

Fragmentations directed by an isolated olefinic bond have

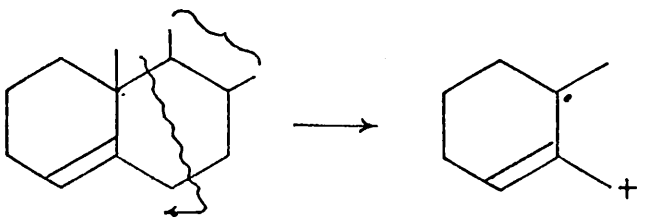
been reported and generally procede by either

(a) allylic cleavage

e. g.



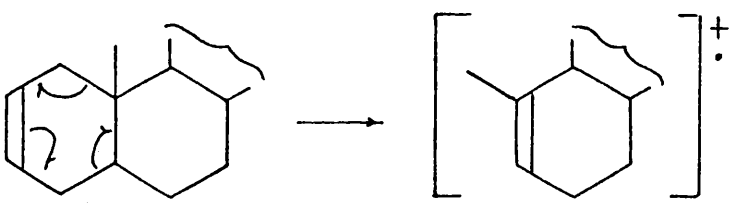
m/e = 69
(Galli et al) [34]



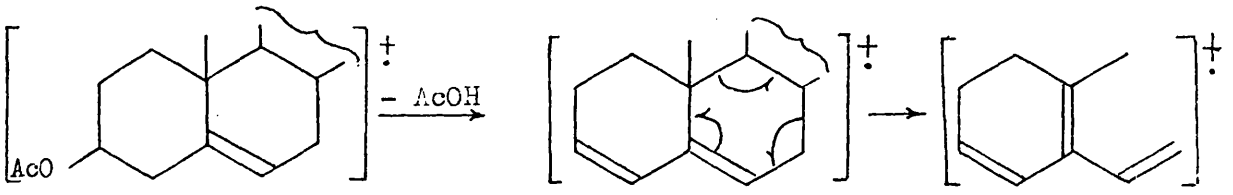
m/e = 108
(Zaretskii et al) [128]

or (b) Retro Diels Alder (RDA) elimination

e. g.



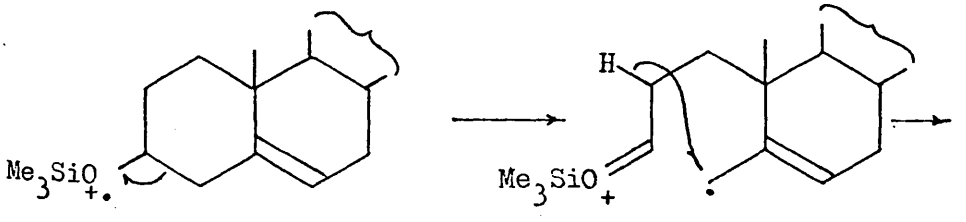
m/e = M-54
(Audier et al) [129]

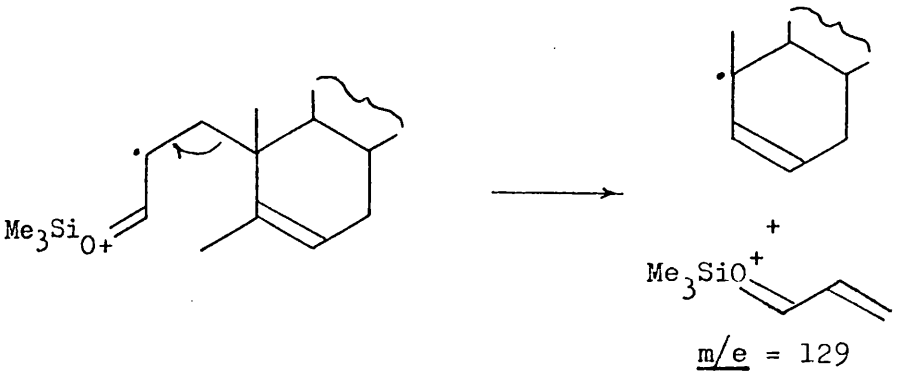


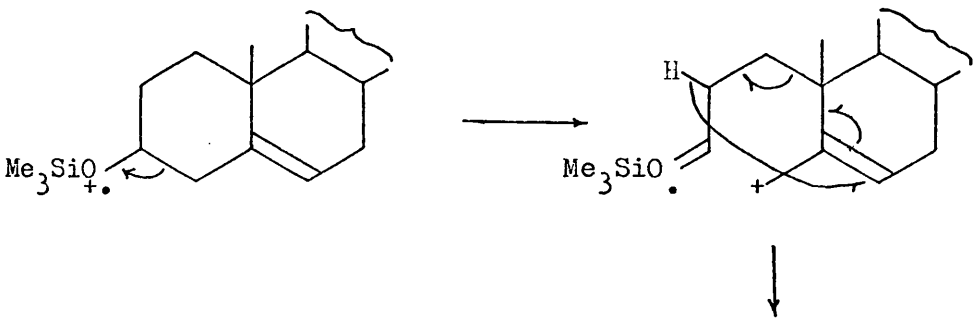
m/e = M-60 m/e = 120
(Galli et al) [34]

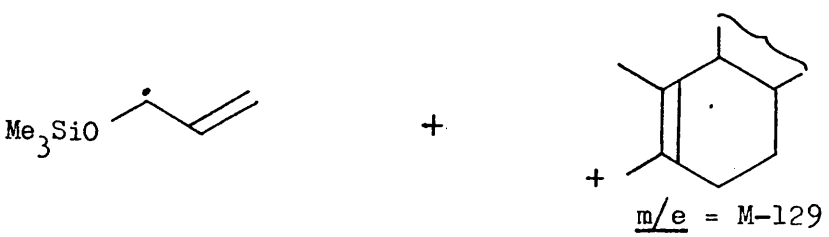
Olefins in close proximity to other functional groups may sometimes be recognised by fragment ions resulting from a combined effect of the two groups.

e.g. Steroidal Δ^5 - 3 - 0 - trimethylsilyl ethers break down under electron impact to give characteristic ions of m/e = 129 and m/e = M - 129 (where M = the molecular weight). This was shown by Diekman et al [54] as resulting from the following fragmentation :





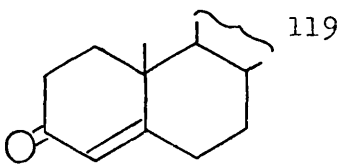




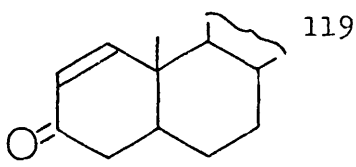
Δ^4 - 3 - 0 - trimethylsilyl ethers and Δ^3 - 3 - 0 - trimethylsilyl ethers both fragment to give ions at m/e = 142 and m/e = 143.[79] This was shown for the enolic ether by Vetter et al to result from formation of the ions :

FIG. 3.1

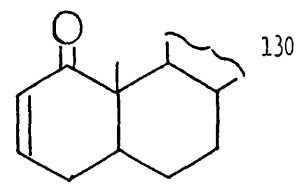
Some olefinic steroids which have been studied by mass spectrometry with literature citations



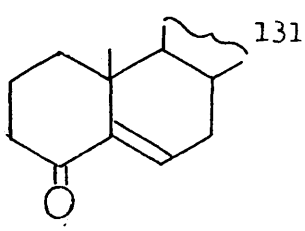
Δ^{4}-3-oxo



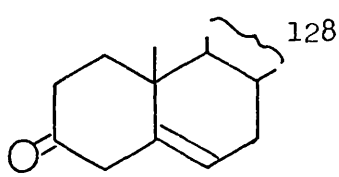
Δ^{1}-3-oxo



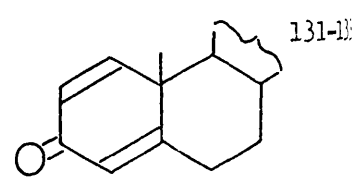
Δ^{2}-1-oxo



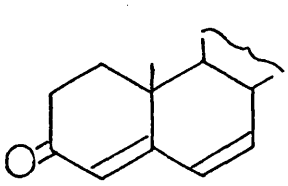
Δ^{5}-4-oxo



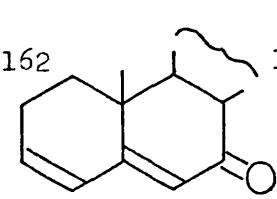
Δ^{5}-3-oxo



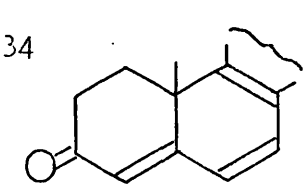
$\Delta^{1,4}$-3-oxo



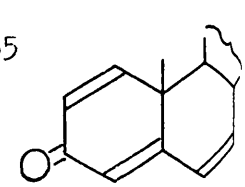
$\Delta^{4,6}$-3-oxo



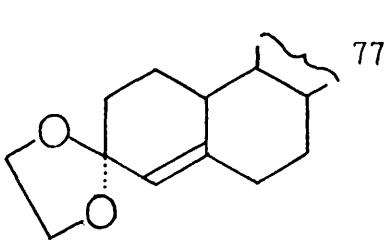
$\Delta^{3,5}$-7-oxo



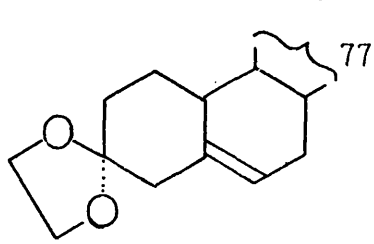
$\Delta^{4,6,8}$-3-oxo



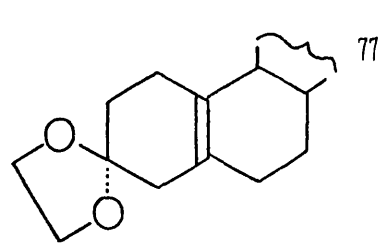
$\Delta^{1,4,6}$-3-oxo



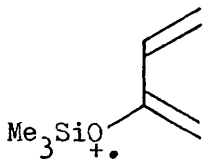
Δ^{4}-3-ethylene ketal



Δ^{5}-3-ethylene ketal

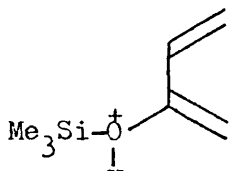


$\Delta^{5(10)}$-3-ethylene ketal



m/e = 142

and



m/e = 143

both of which comprise C-1 to C-4 from ring A. Although no isotope-labelling study has been made of trimethylsilyl ethers of allylic C-3 alcohols, results obtained for 2α-methylandrost-4-ene-3β,17β-diol bis-trimethylsilyl ether, -methylandrost-4-ene-3β,17β-diol bis-trimethylsilyl ether and 6-methylandrost-4-ene-3β,17β-diol bis-trimethylsilyl ether (Part II, Fig. 2.5) indicate that these ions comprise C-2, C-3 and C-4 and almost certainly result from fission of the C-1,2 and C-4,5 bonds in ring A.

Olefinic unsaturation α or β to ketones and derivatives of such ketones may also lead to fragmentation characteristic of the location of the groups, and many examples of this are reported in the literature. Some of the structures studied are shown in Fig. 3.1.

In Part IV it will be demonstrated how olefins which are conjugated with ketonic groups may be distinguished, using a deuterium exchange method, by the number of enolisable hydrogens in the molecule as indicated by subsequent mass spectrometry.

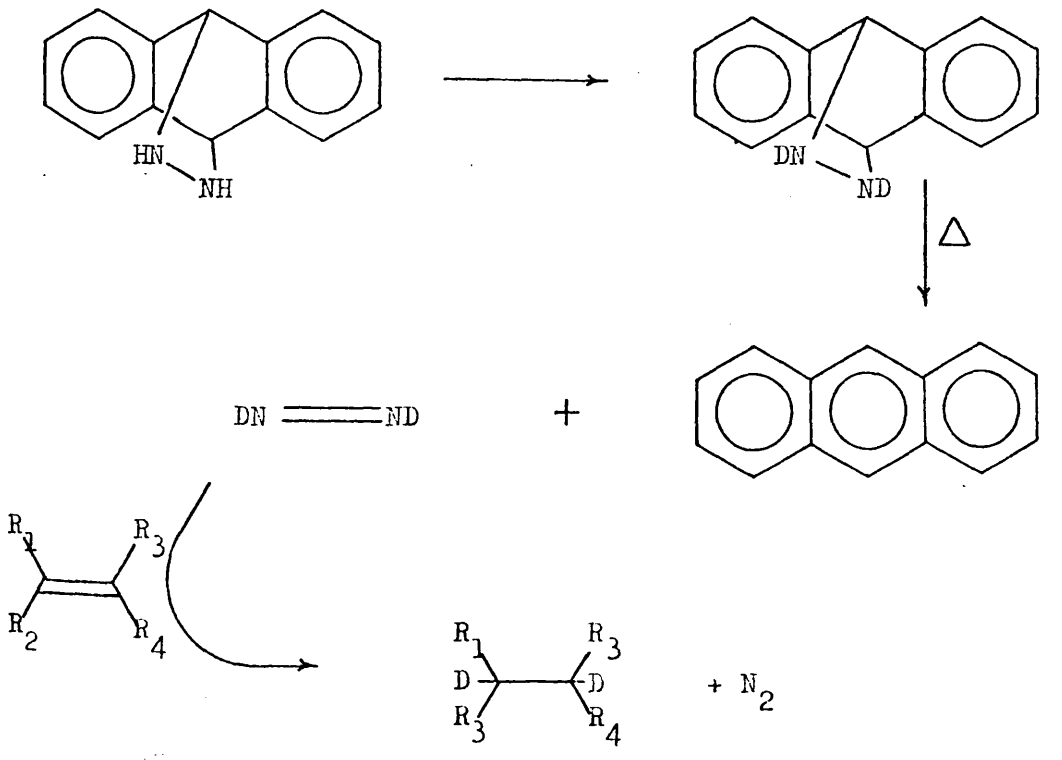
OLEFIN DERIVATIVES

For olefinic unsaturation in positions not proximal to an electronegative substituent it is often necessary to modify the group chemically in order to locate its position. Unfortunately, there are few reagents which react quantitatively with an olefin to give a single pure compound which is of diagnostic value. The following derivatives have been used successfully for the location of olefinic unsaturation in linear molecules by mass spectrometry.

Deuterio-reduction

Reagents have now been reported which permit deuterio-reduction of olefinic double bonds without causing migration or scrambling. Morandi et al [136] used tristriphenylphosphine-rhodium chloride as a homogeneous catalyst for the deuterio-reduction of olefinic n-hydrocarbons. The mass spectrum of the reduced compound gave the position of the double bond. Tetradeutero hydrazine has been used for the deuterio reduction of unsaturated esters of long chain carboxylic acids [186,187] for the purpose of determining the position of the olefinic bond

This technique obviously works well where the fragmentation of the reduced species is clearly defined and does not involve extensive hydrogen scrambling, and might well be of use for work on steroid hydrocarbons, whose breakdown under electron impact is now well documented. A more convenient reagent for this purpose however is probably d_2-diimine which is formed in situ by heating the anthracene diimide complex in dioxan + deuterium oxide, and reacts with olefins to give cis addition of deuterium [137] viz :

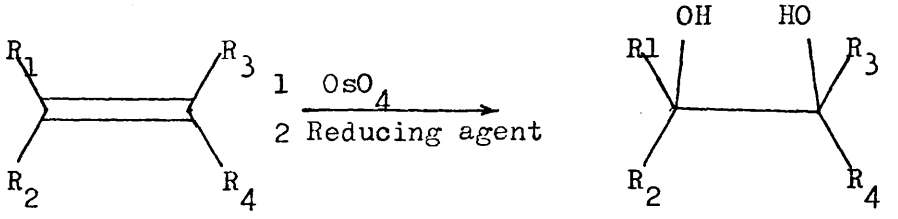


Methoxylation

Mono- and poly-enoic long chain esters may be conveniently reacted with mercuric acetate in methanol and their methoxymercuric adducts reduced with sodium borohydride to give methoxy substituted compounds. Although each olefinic bond produces two isomeric methoxy substituents, these have not been observed to separate by GLC on SE-30 or polyethylene glycol columns. More rigid molecules such as steroids however, would be expected to be disadvantageous in this respect.

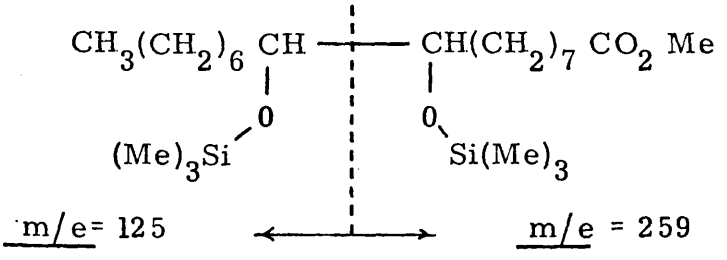
Hydroxylation

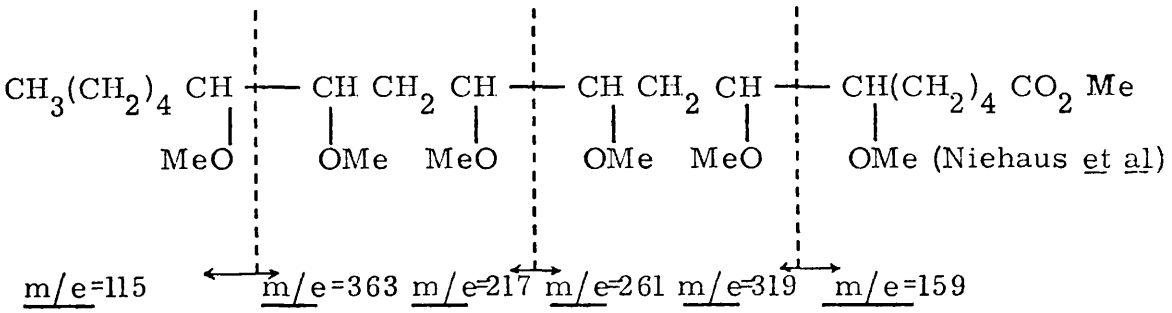
Osmium tetroxide will react cleanly and under mild conditions with olefinic material to give 1, 2 cis-diols. The cyclic osmate ester is converted to the cis-diol by mild reduction with reagents such as mannitol[138] and potassium hydroxide, or with sodium sulphite.[59]



Suitable derivatives of the cis-diol such as methyl ethers[59] or trimethylsilyl ethers[139] have been shown to direct fragmentation under electron impact to give the location of the original olefin in unsaturated fatty acids. The fragmentation is predominantly α cleavage between the two oxygen functions

e.g.



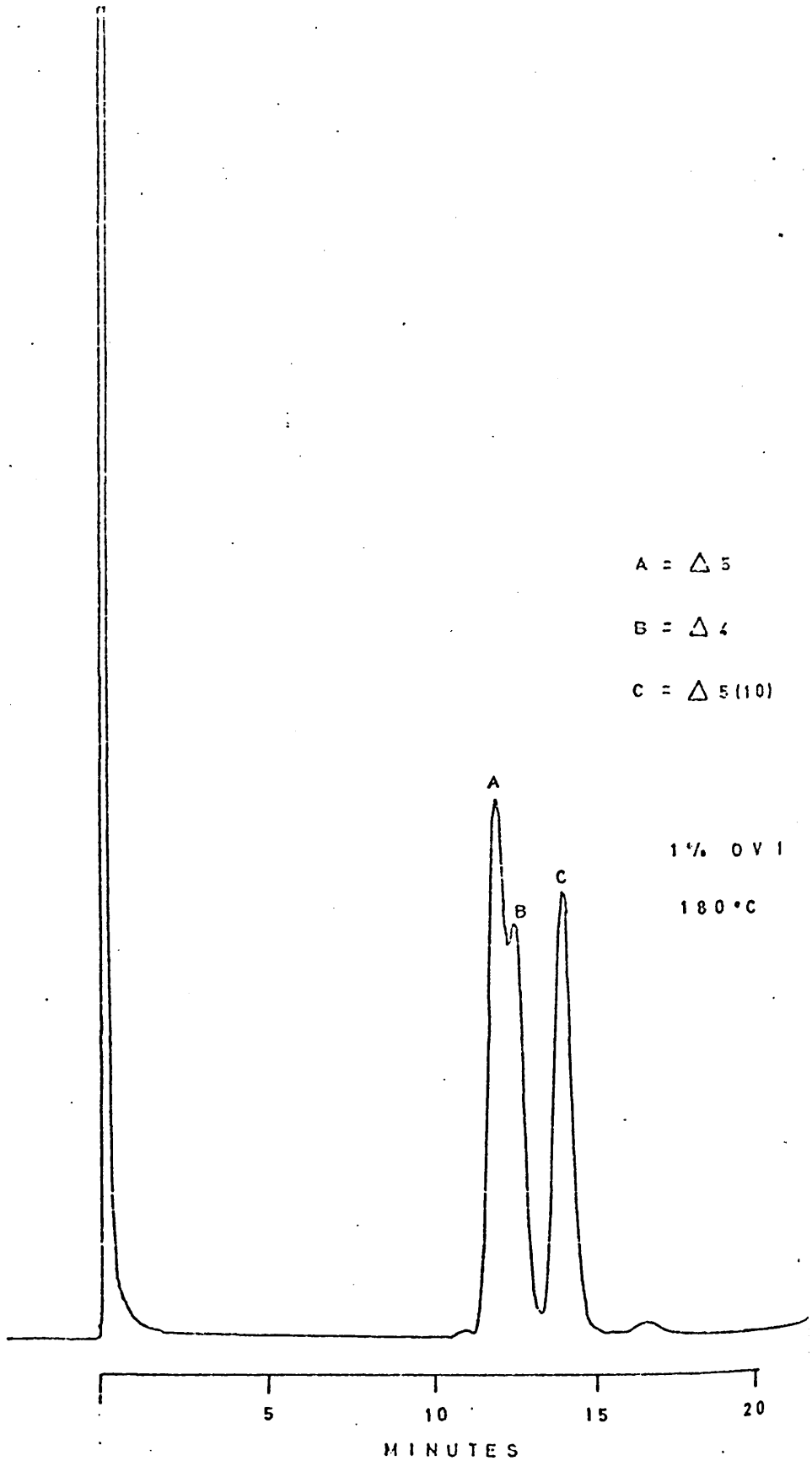


The methyl ether derivatives of polyunsaturated fatty acids studied by Niehaus and Ryhage also exhibited cleavage α to a pair of methoxyl substituted carbons.

Predictable fragmentation processes predominant in the mass spectra of these linear molecules however cannot be readily applied to cyclic structures where fission of two or more bonds is necessary to form the fragment ion. Before a steroid can be recognised in the form of a derivative based on its olefinic character it is therefore necessary to study the mode of fragmentation of the derivative at various positions in the steroid nucleus. A comprehensive survey of this sort has not to the author's knowledge been reported in the literature and is clearly beyond the scope of this thesis. A limited study of derivatives of three olefinic isomers has been undertaken.

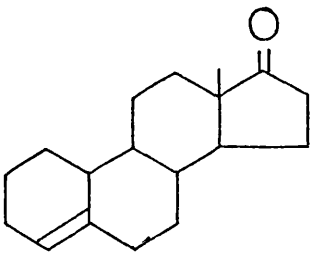
FIG.3.2

Gas chromatogram of isomeric estren-17-ones

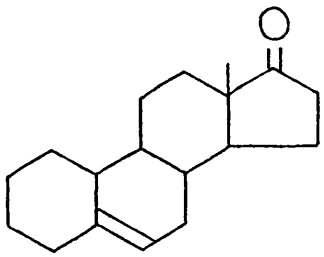


ISOMERIC ESTREN -17-ONES

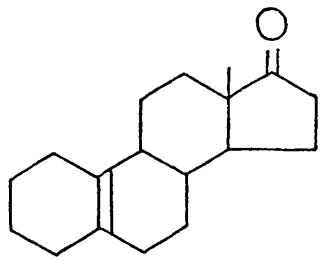
Three 17-ketosteroids (estr-4-en-17-one, estr-5-en-17-one and estr-5(10)en-17-one), which are identical except for the location of the olefinic bond, presented an interesting problem in steroid identification.



estr-4-en-17-one



estr-5-en-17-one



estr-5(10)-en-17-one

Gas Chromatography

Gas chromatographic separation on OV-1 and OV-17 separated only the $\Delta^{5(10)}$ isomer from a mixture of the three steroids; the Δ^4 and Δ^5 isomers remained unresolved as shown by the gas chromatogram (Fig. 3.2) and retention data (Table 3.1).

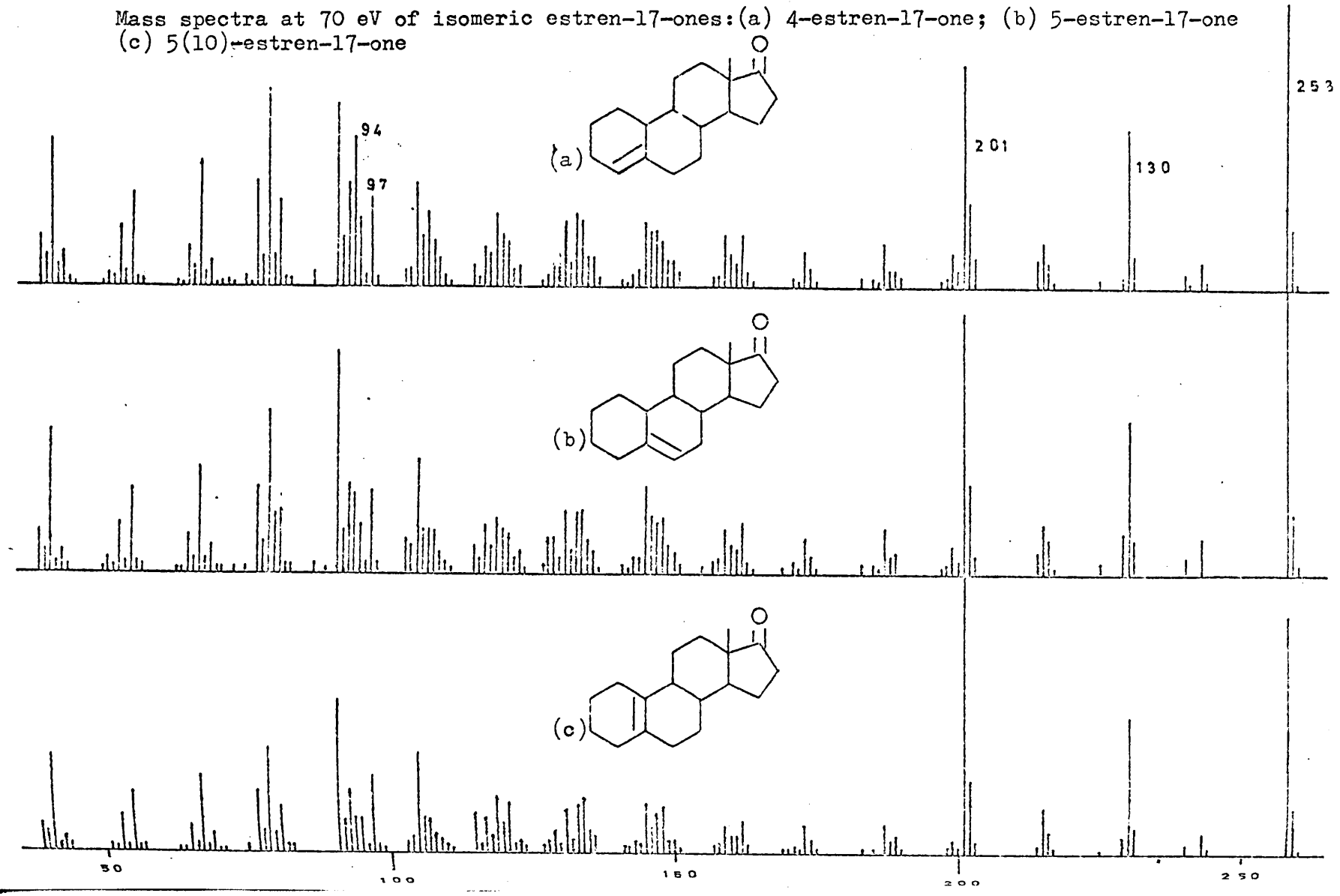
TABLE 3.1

Retention data for isomeric estren-17-ones

Isomer	MU Values	
	OV-1 (180°C)	OV-17(190°C)
Δ^4	21.05	24.86
Δ^5	21.12	24.62
$\Delta^{5(10)}$	21.29	25.54

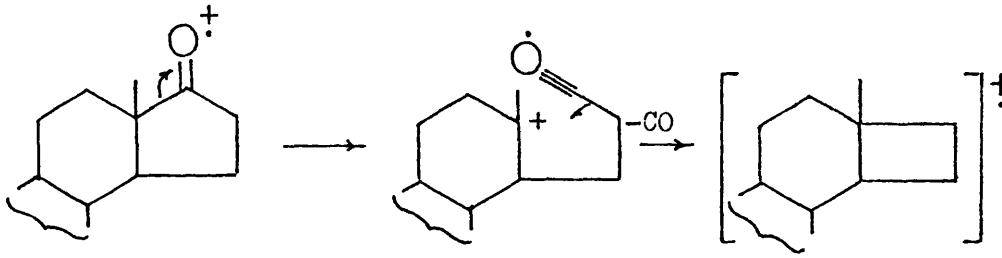
FIG 3.3

Mass spectra at 70 eV of isomeric estren-17-ones:(a) 4-estren-17-one; (b) 5-estren-17-one (c) 5(10)-estren-17-one



Mass Spectrometry at 70 eV

A notable feature in the mass spectra of these isomeric steroids at 70 eV is their similarity (Fig. 3.3.). The M-28 fragment at m/e = 230 is analogous to the fragment at m/e = 246 in 5α-androstan-17-one, which was shown by Tokés et al to result from the elimination of carbon monoxide from ring D. [140]

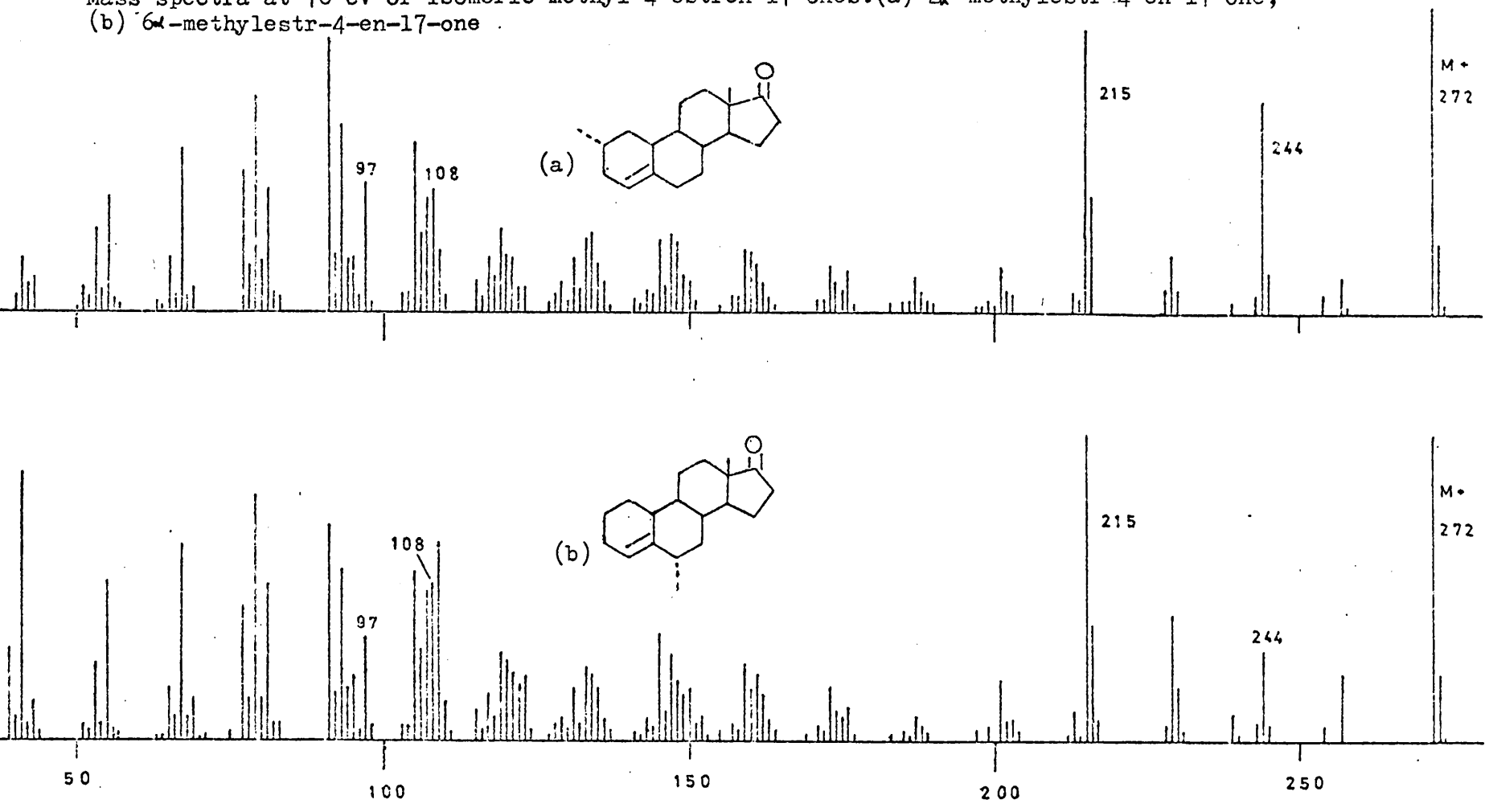


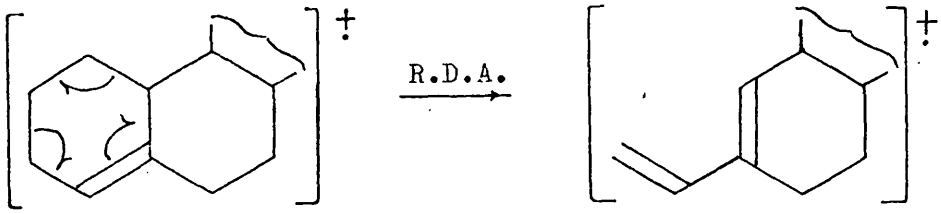
m/e = M-28

Another possible fragmentation process producing an ion of this mass would be a Retro Diels-Alder elimination from the Δ^4 or $\Delta^{5(10)}$ isomer which might be formed from the others by isomerisation in the ion source.

FIG. 3.4

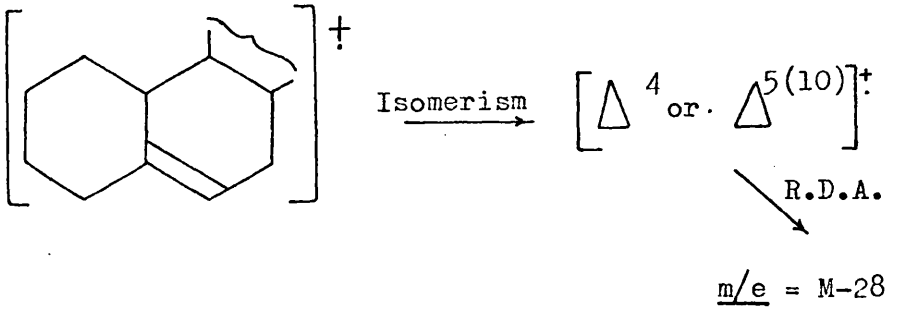
Mass spectra at 70 eV of isomeric methyl 4-estren-17-ones:(a) 2α-methylestr-4-en-17-one; (b) 6α-methylestr-4-en-17-one





m/e = M-28

m/e = M-28



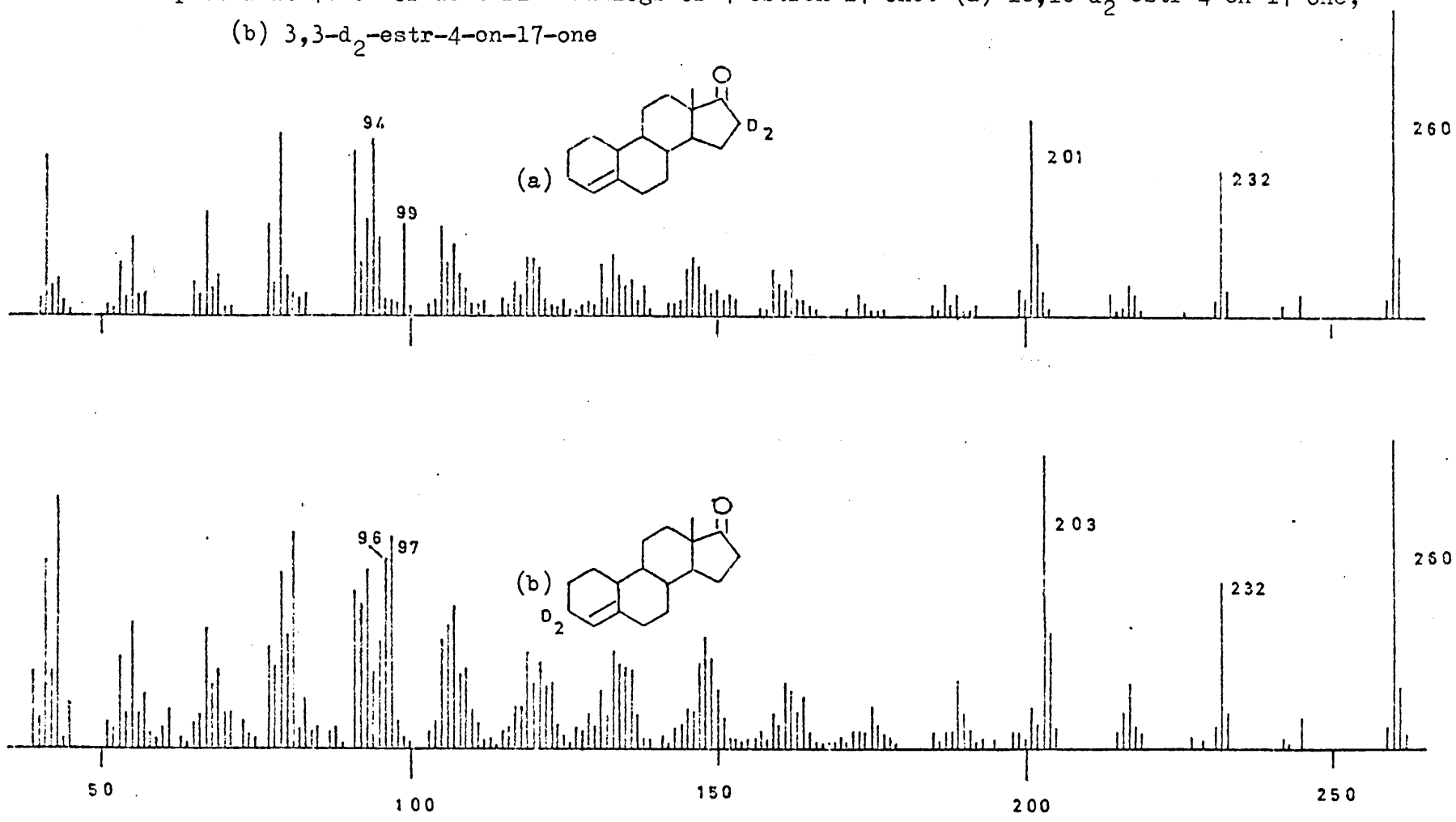
m/e = M-28

A mass shift of 14 units in the spectrum of 2 α -methylestr-4-en-17-one to give a fragment at m/e = 244 (Fig. 3. 4a) clearly shows that Retro Diels-Alder elimination does not occur for the Δ^4 isomer since C-2 is retained in the fragment.

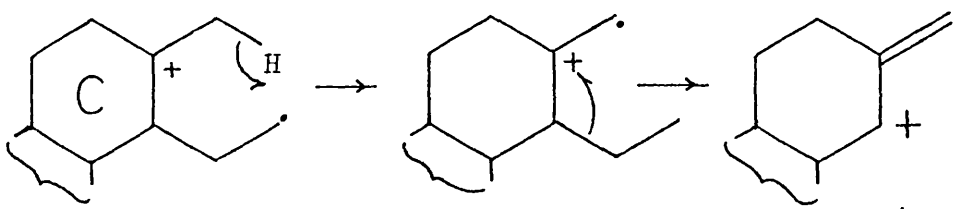
A metastable ion at m/e = 276 in the spectra of the three

FIG. 3.5

Mass spectra at 70 eV of deuterium analogs of 4-estren-17-one: (a) 16,16-d_2-estr-4-en-17-one; (b) 3,3-d_2-estr-4-on-17-one



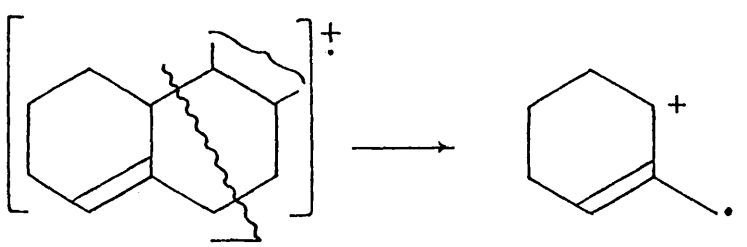
unsubstituted isomers indicates that the fragment at m/e = 201, which is the base peak for the $\Delta^{5(10)}$ isomer results at least in part from elimination of C_2H_5 from the ion at m/e = 230. This might arise as shown below :



m/e = 201

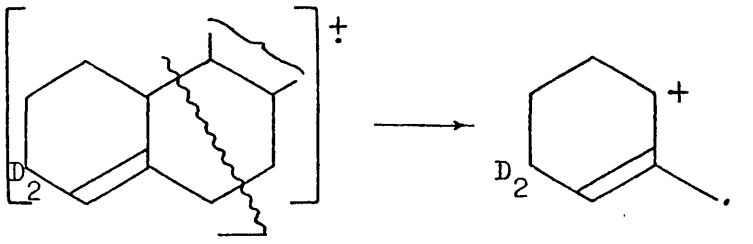
The appearance of this fragment at m/e = 201 in the mass spectrum of 16 d_2 estr-4-en-17-one (Fig. 3.5a) verifies that it arises out of ring D fragmentation.

Fragmentation directed by the Δ^4 olefinic bond as reported by Zaretskii et al for cholest-4-ene [128] should result in an analogous fragment at m/e = 94 i.e.



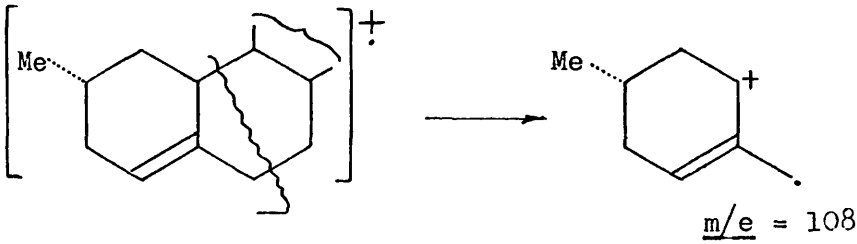
m/e = 94

A fragment of this value does have greater abundance in the Δ^4 isomer than in the Δ^5 and $\Delta^{5(10)}$ isomers, but its presence is obscured by other prominent fragments in this region of the spectrum. The mass spectrum of 3 $\underline{d_2}$ estr-4-en-17-one (Fig. 3.5b) shows clearly that this fragment does exist, by causing it to shift two mass units to $\underline{m/e}$ = 96

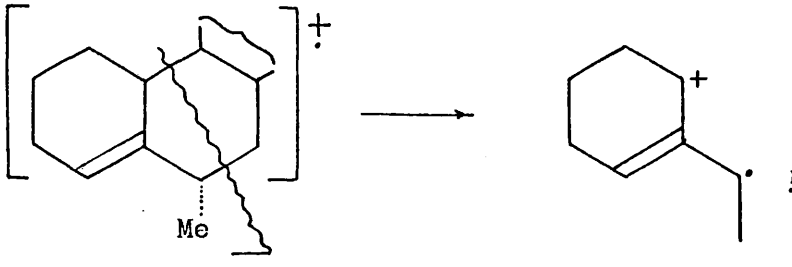


$\underline{m/e}$ = 96

2 α-methylestr-4-en-17-one (Fig. 3.4a) and 6 α-methylestr-4-en-17-one (Fig. 3.4b) give analogous fragments, both at $\underline{m/e}$ = 108

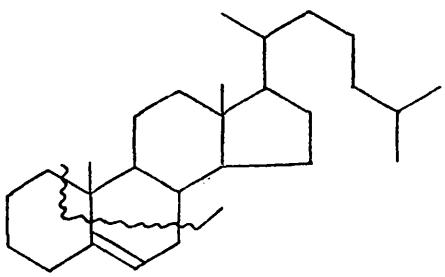


$\underline{m/e}$ = 108



$\underline{m/e}$ = 108

The Δ^5 olefinic bond in cholest-5-ene was reported by Zaretskii to induce fragmentation resulting in loss of carbons 1 to 7 [128] i.e.



m/e 275

Androst-5-en-17-one was also reported as giving an analogous fragment at m/e = 177. As can be seen from Fig. 3.3b, the expected fragment at m/e = 163 is not present in the mass spectrum of estr-5-en-17-one to any significant extent.

Mass spectrometry at 15eV

Electron impact at low energies (15-20eV) generally results in mass spectra containing fewer fragment ions than those produced at the normally used ionising energy of 70 eV. Although fewer in number, these fragment ions usually result from primary fragmentation processes. Fig. 3.6 shows the mass spectra at 15 eV of estr-4-en-17-one, estr-5-en-17-one and estr-5(10)-en-17-one respectively. Again the three spectra

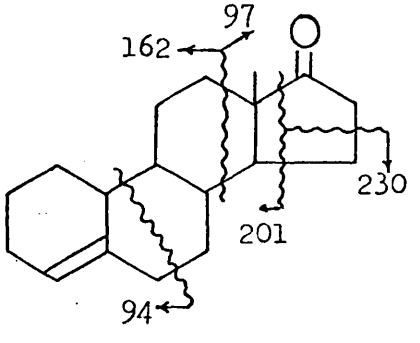
show remarkable similarity, but the extra ion at m/e = 94 for the Δ^4 isomer now shows greater prominence. By comparison with the 15 eV mass spectra of the 6α -methyl and 16 $\underline{d}_2$ - substituted analogues (Table 3.2) postulations of the origins of the other prominent fragments could be made with reasonable justification.

TABLE 3.2

Principal ions in the mass spectra at 15 eV of estr-4-en-17-one, its 16 $\underline{d}_2$ and 6αmethyl analogues

Isomer	m/e					
Δ^4	258	230	201	162	97	94
16$\underline{d}_2$ Δ^4	260	232	201	162	99	94
6α-Me Δ^4	272	244	215	176	97	108

These results can be explained by the following fragmentation of estr-4-en-17-one at 15 eV which, except for the fragment at m/e = 94, is general for all three isomers.



DERIVATIVES OF ISOMERIC ESTREN-17-ONES

In order to enhance the distinction between the three olefinic isomers by gas chromatography and mass spectrometry, chemical modifications were made to the olefin part of the molecule. These modifications comprised (i) conversion to the cis-diol (ii) formation of a non-polar derivative of the cis-diol.

A. Cis Diols

The olefinic steroids were converted to cis-diols by the method of Niehaus et al.[59]

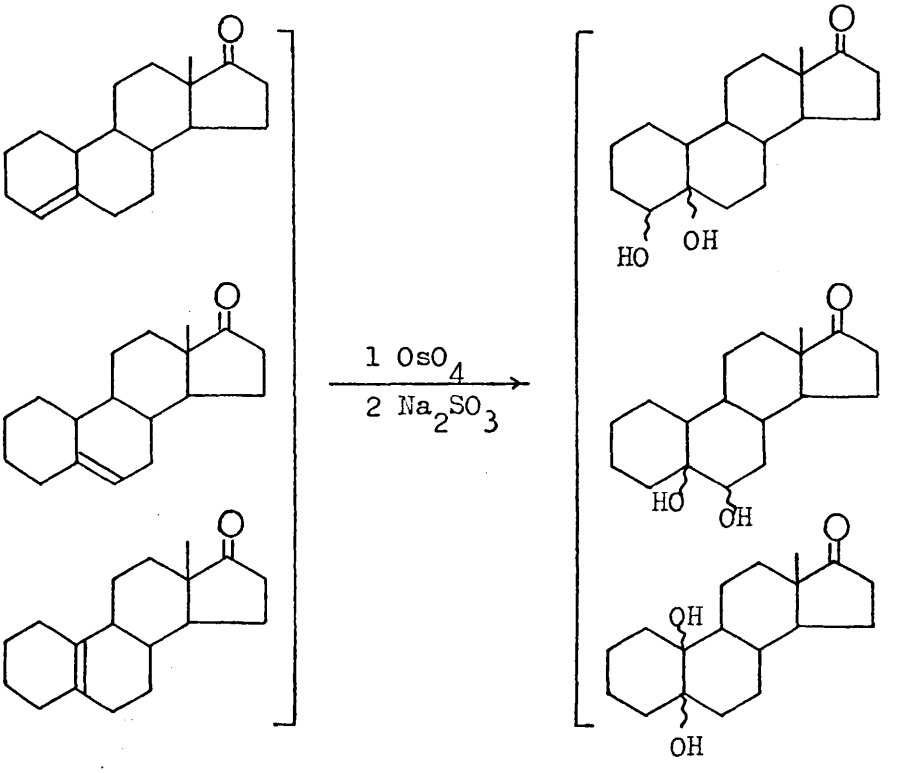
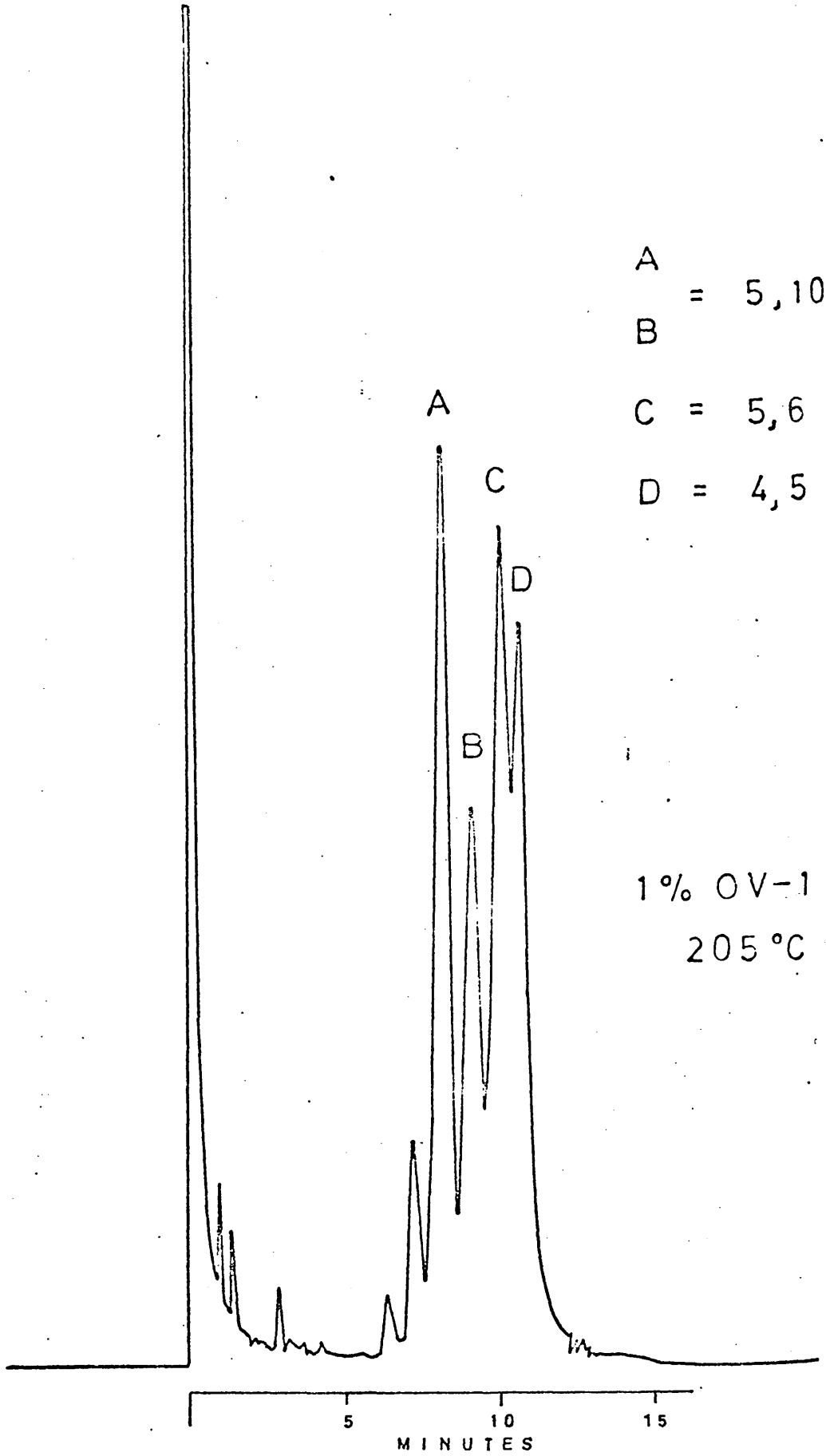


FIG.3.7

Gas chromatograph of isomeric estren-17-ones after hydroxylation with osmium tetroxide



Gas Chromatography

Gas chromatography on OV-1 and OV-17 showed increased separation of the three isomers (Table 3.2, Fig. 3.7). Both the Δ^4 and Δ^5 isomers gave only one major peak when chromatographed on these phases. The $\Delta^{5(10)}$ isomer however formed two products, in comparable amounts, which were clearly separated on both columns.

TABLE 3.3.

Retention data for isomeric estren-17-ones after oxidation with osmium tetroxide

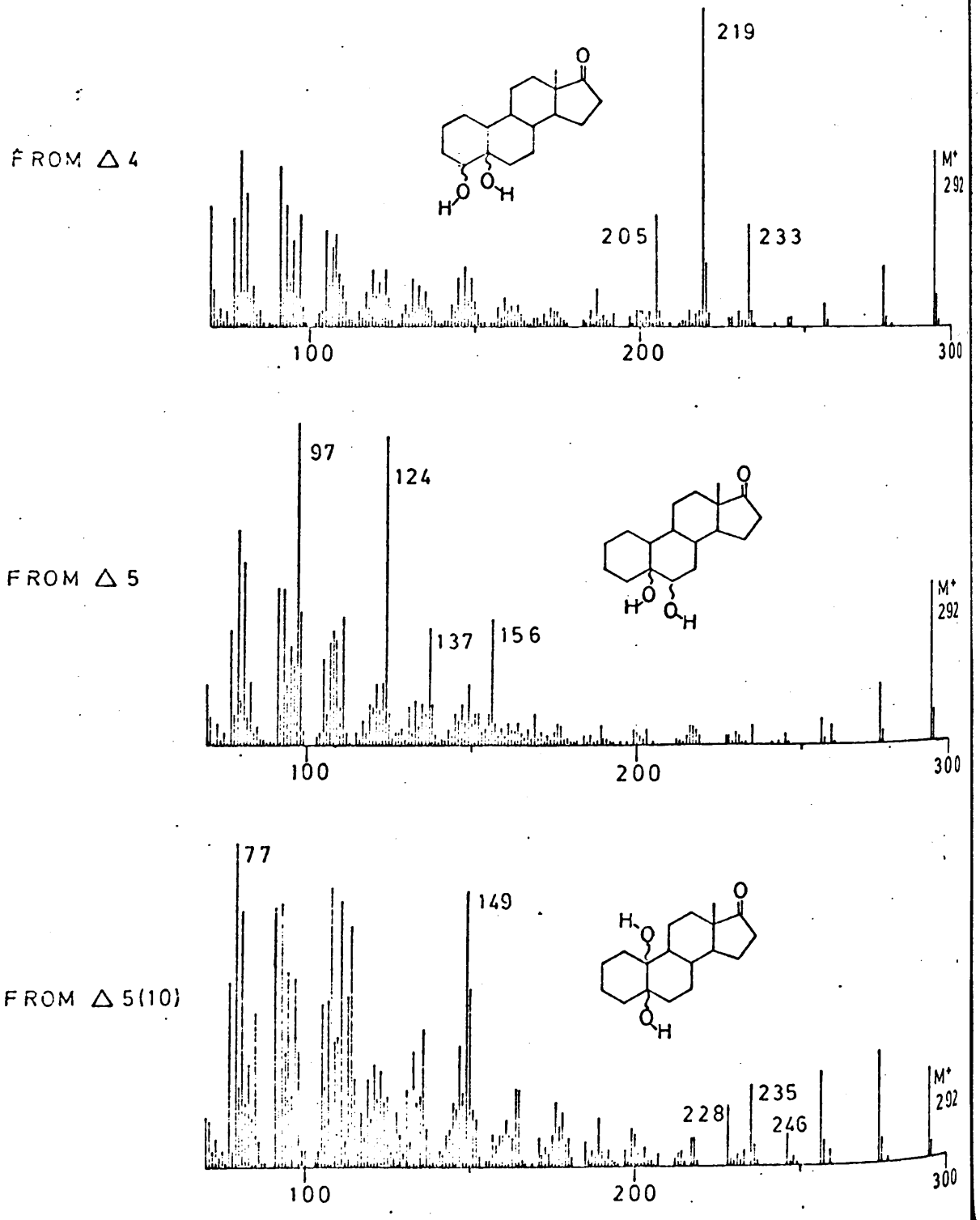
Parent Isomer	MU OV-1 (215°C)	MU OV-17 (225°C)
Δ^4	24.68	29.49
Δ^5	24.34	29.30
$\Delta^{5(10)}$	(23.91 ((24.17	(28.45 ((28.91

Mass Spectrometry

Mass spectrometry of the eluted products cited in Table 3.3. gives parent ions at m/e = 292 for each component, which is the correct value for the molecular ion of the cis-diol. The two products formed from the $\Delta^{5(10)}$ isomer therefore

FIG. 3.8

Mass spectra at 70 eV of cis diols formed by osmylation of isomeric estren-17-ones



presumably result from oxidation on the α side and the β side of the molecule. These diols although separable by GLC gave identical mass spectra.

The mass spectra of the cis-diols obtained from the three isomers are shown in Fig. 3.8, and are as striking in their difference as the steroid olefin spectra are in their similarity. It was intended to diagnose the mode of fragmentation of these diols and their derivatives by comparison with the mass spectra of a series of methyl and deuterium labelled analogues, but this work was curtailed because of the limited time available. A few labelled 4, 5 cis-diol analogues were prepared, however, and their principal ion fragments are summarised in Table 3.4.

4, 5 cis-diol

TABLE 3.4

Principal ions in the mass spectra at 70 eV of estr-4-en-17-one and substituted analogues after oxidation with osmium tetroxide

Parent isomer	m/e			
Δ^4	292	233	219*	205
2αMe Δ^4	306	233*	219	205
6αMe Δ^4	306	247	233*	219
$3\underline{d}_2$- Δ^4	294	233	219*	205
$16\ \underline{d}_1$- Δ^4	293	234	220*	206

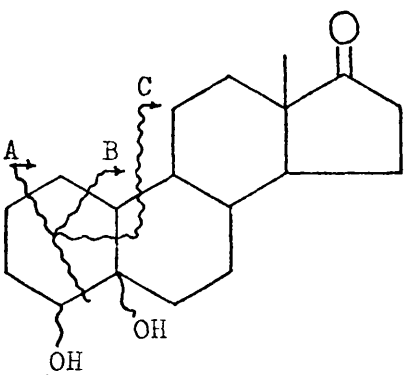
* = base peak

These results can be explained by the fragmentation shown below :

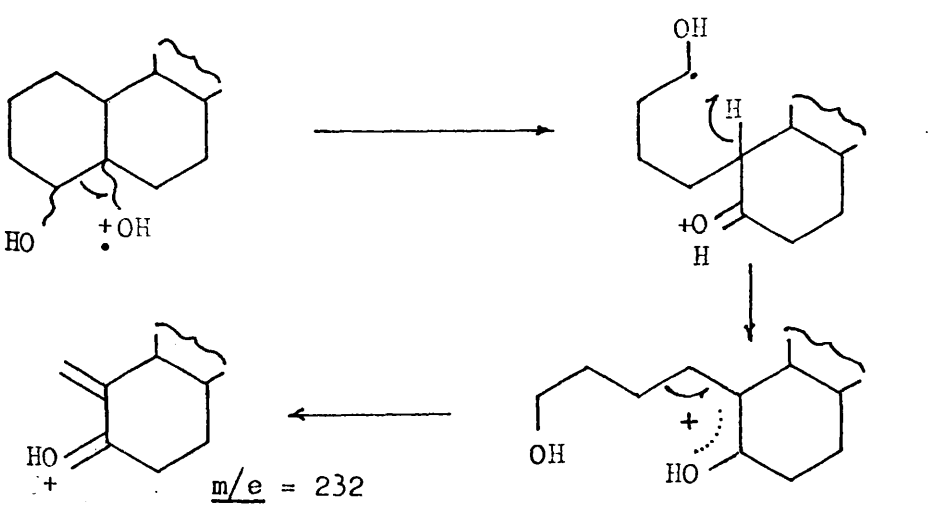
A. m/e = 233

B. m/e = 219

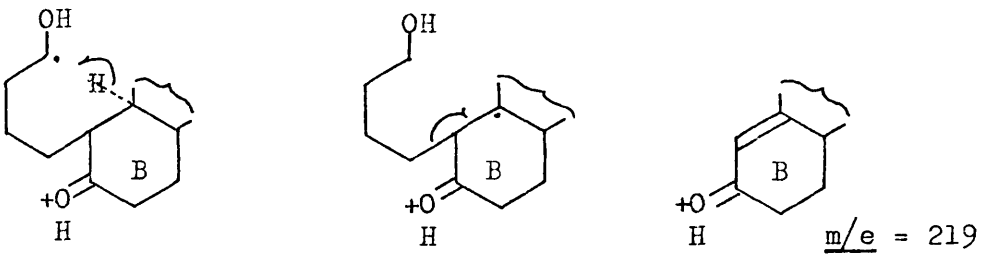
C. m/e = 205



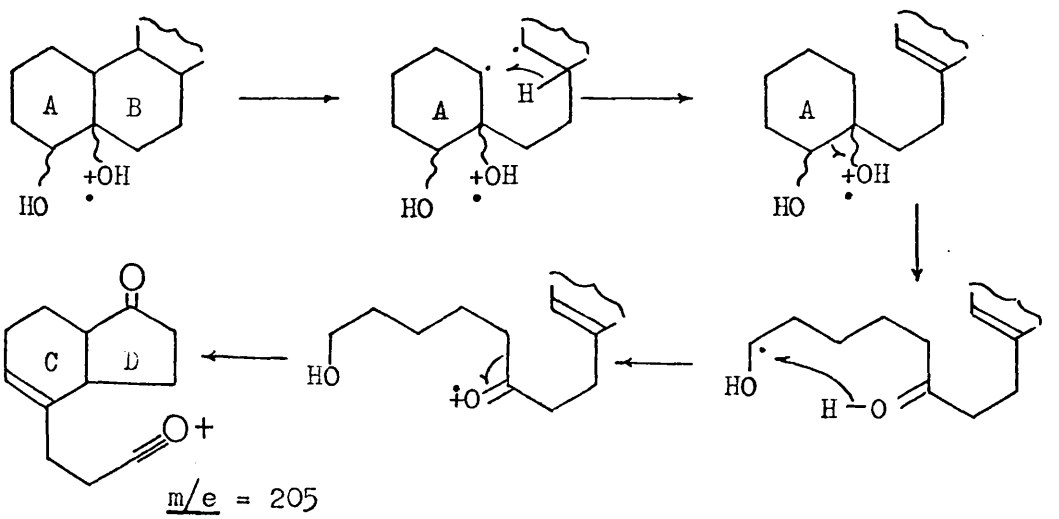
The ion at m/e = 233 is formed after one hydrogen migration to the neutral fragment. Although not confirmed by deuterium labelling, it seems probable that this transfer is from C-10.



The ion at m/e = 219 which is the base peak can be formed by hydrogen transfer from C-9 to C-4.



The ion at m/e = 205 contains C-6 and C-16 but has lost C-2 and C-3. It also loses two hydrogen atoms to the neutral fragment. One possible fragmentation sequence is shown below :



To prove the above sequences beyond doubt would require further deuterium labelling at C-10, C-9, C-8 and 4-OH.

5-6 cis diol and 5-10 cis diol

Without deuterium or other labelled analogues for comparison, it is not possible to form reliable postulations for the structure of ions produced by these isomers under electron impact. It is quite clear however that they differ quite markedly in fragmentation both from each other and from the 4, 5-diol isomer, and in this respect oxidation with osmium tetroxide may prove a useful means of olefin determination in steroids.

B. Trimethylsilyl Ethers of Cis Diols

No satisfactory method was found for the formation of trimethylsilyl ethers of the above cis-diols. After heating the sample in a sealed tube with excess of a mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane (50:20:5 v/v) for six hours at a temperature of 120°C, mostly unchanged starting material was recovered from the reaction mixture together with trace quantities of monosilylated product. The 4, 5-cis-diol and the 5, 6-cis-diol both showed three products

on OV-1 after silylation which were shown by subsequent analysis by GC/MS to comprise two monosilylated compounds and a small amount of bis-silylated material.

C. Boronate Esters of cis-Diols

Cis-diols obtained from Δ^4, Δ^5 and $\Delta^{5(10)}$ estren-17-ones were found to react completely with alkyl and aryl-boronic acids to give cyclic esters of the type shown :

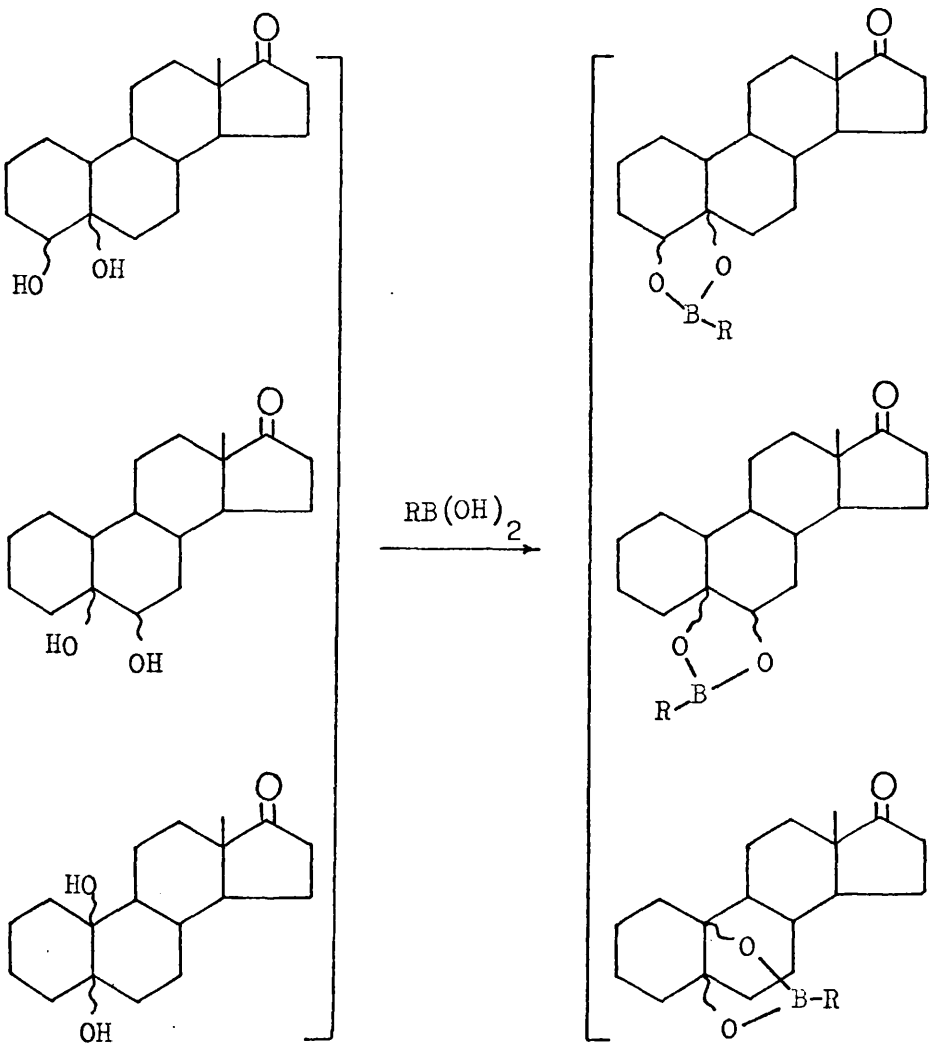
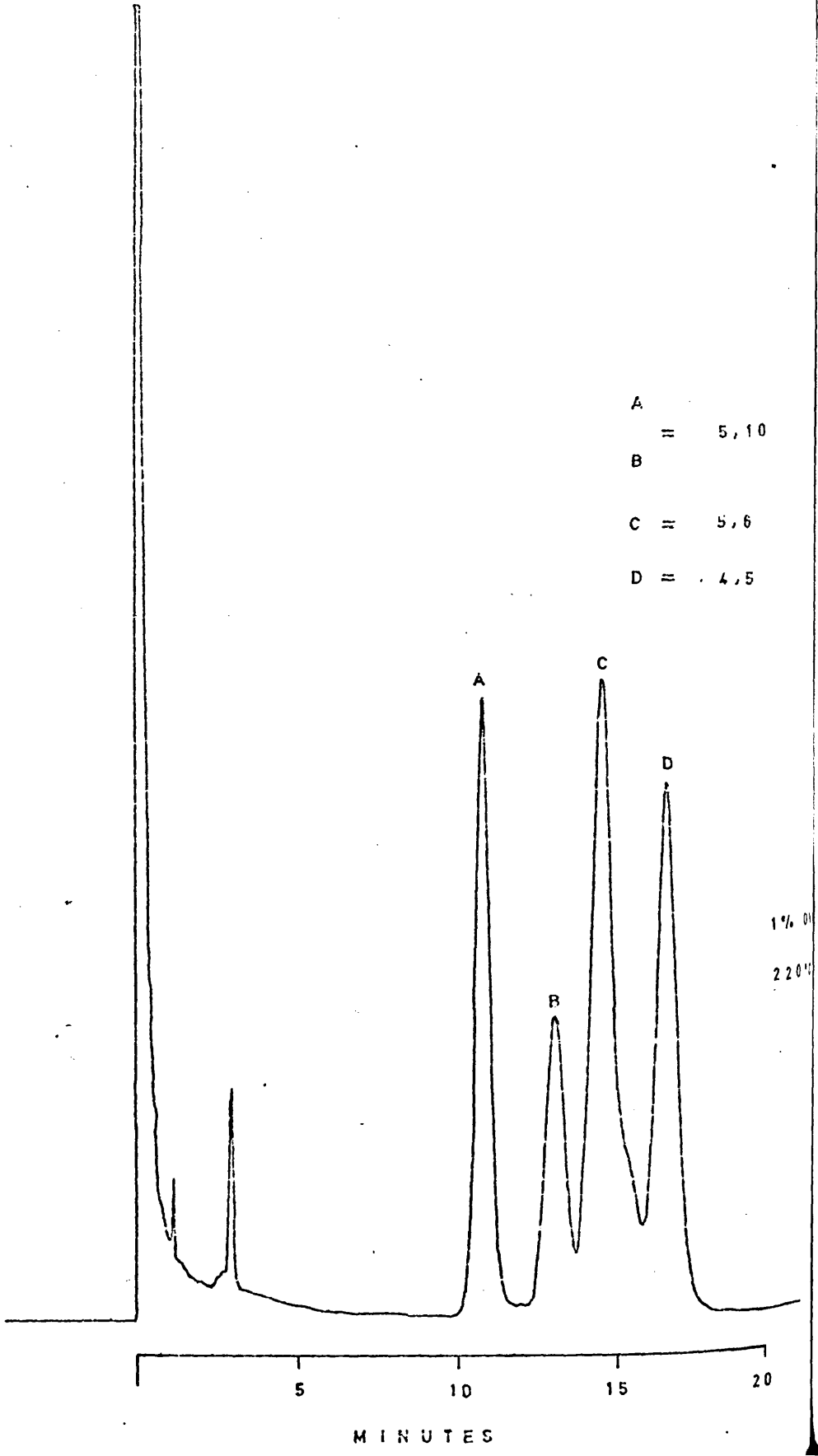


FIG. 3.9

GLC of n-butylboronate esters derived from isomeric estren-17-ones



Gas Chromatography

The boronate esters depicted above showed good gas chromatographic properties and enhanced separation of isomeric forms. (Fig. 3.9, Table 3.5).

TABLE 3.5

Retention data for isomeric estren-17-ones as boronate esters

Parent Isomer	M.U.			
	n-butyl boronate		phenylboronate	
	OV-1(215°C)	OV-17(225°C)	OV-1(230°C)	OV-17(250°C
Δ^4	26.02	29.76	29.74	35.02
Δ^5	25.60	29.38	29.32	34.62
$\Delta^{5(10)}$	(25.07	(28.66	(28.44	(33.58
	(25.28	(28.92	(29.01	(34.19

Single products were obtained from the Δ^4 and Δ^5 isomer and two products from the $\Delta^{5(10)}$ isomer in accordance with results observed from the cis-diols.

Mass Spectrometry

As was the case with boronate derivatives of β-hydroxy

FIG. 3.10

Mass spectra at 70 eV of phenylboronates derived from isomeric estren-17-ones: (a) 4,5-phenylboronate; (b) 5,6-phenylboronate

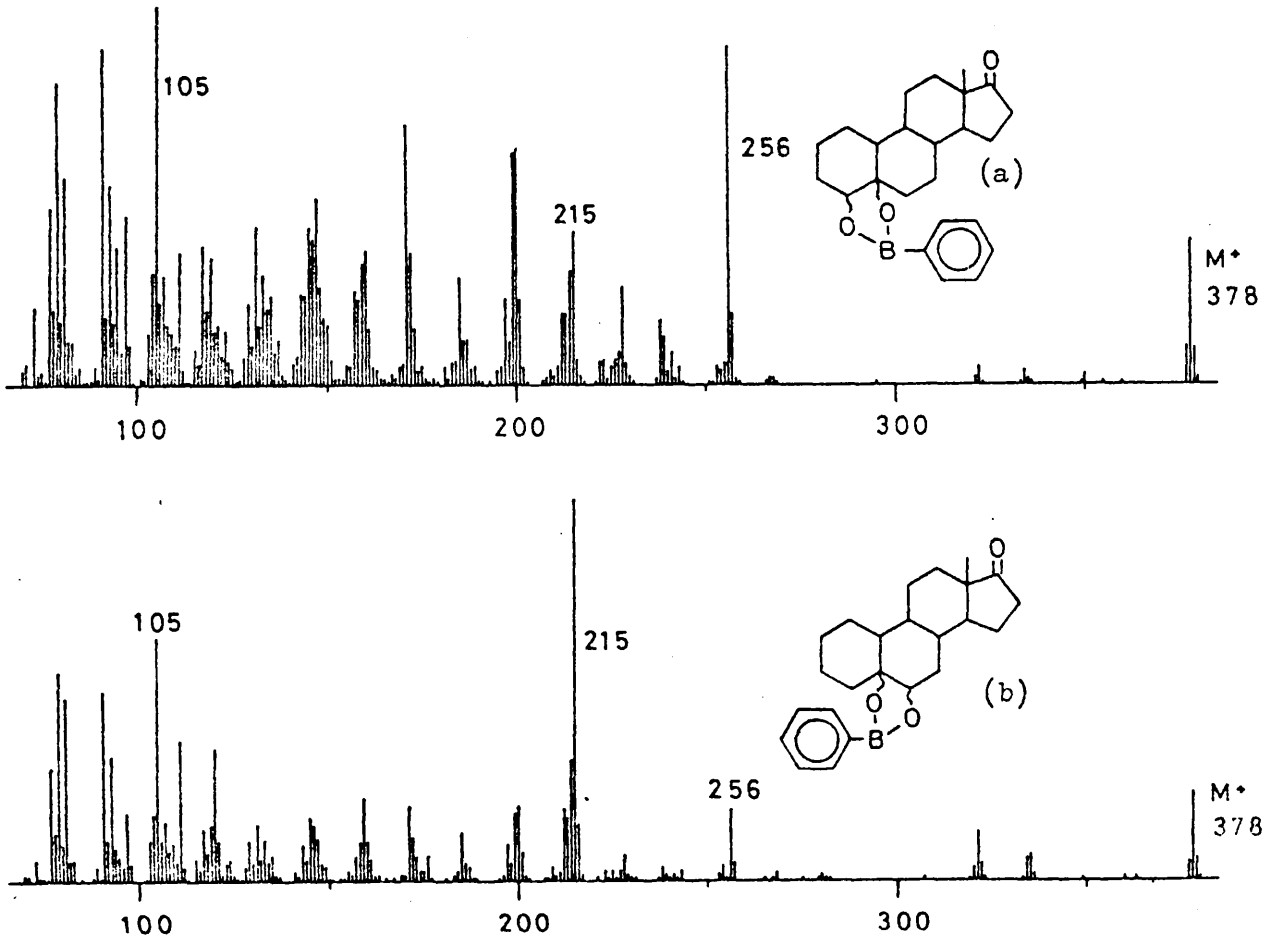
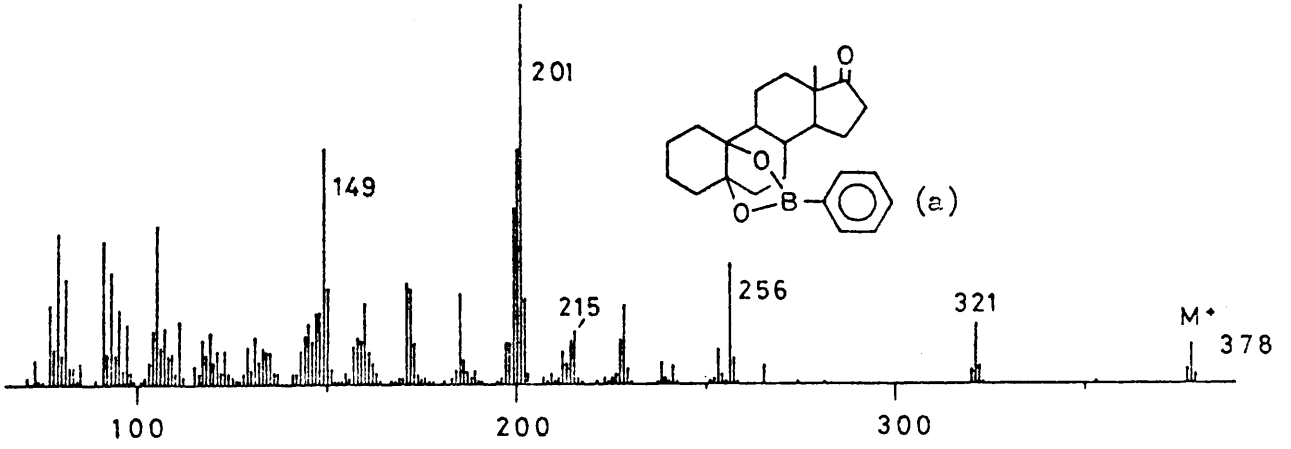
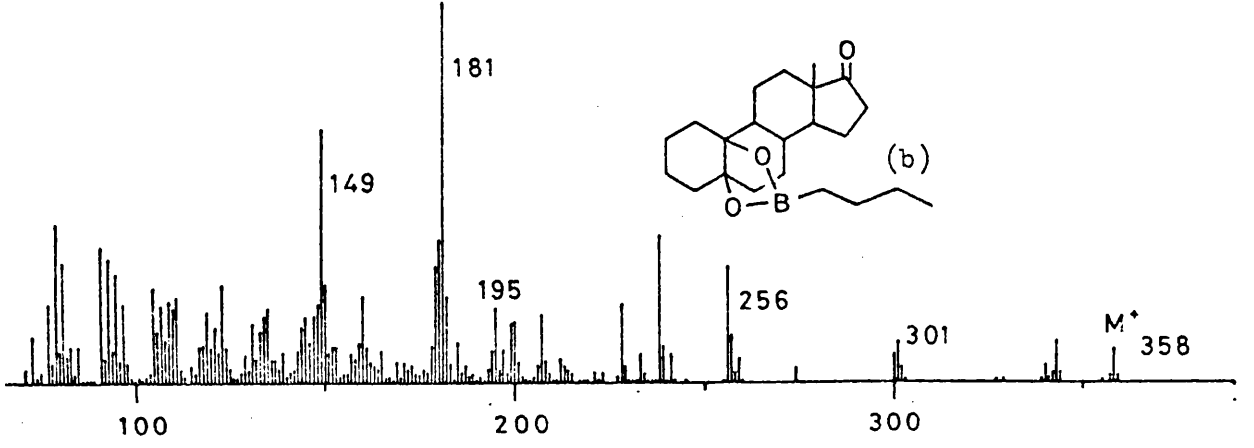


FIG. 3.11

Mass spectra at 70 eV of boronate esters derived from 5(10)-estren-17-one:
(a) phenylboronate (b) n-butylboronate





amines (Part I) it was hoped that the cyclic nature of the αß diol esters would direct mass spectrometric fragmentation to give predictable fragments which were characteristic of the location of the groups.

Ions containing boron may often be recognised by the natural abundance ratio $^{10}B:^{11}B = 1:4$, which is reflected in all fragments containing this element. Additional recognition of these fragments in the mass spectra of boronate esters is afforded by the mass shifts observed when using boronic acids of different molecular weights. Fig. 3.10 shows the mass spectra of phenylboronates of cis-diols derived from the Δ^4, Δ^5 and $\Delta^{5(10)}$ isomers of estren-17-one and Fig. 3.11b shows the mass spectrum of the n-butylboronate of the 5,6-cis diol. The butyl- and phenyl-boronates of 5,10-cis-dihydroxyestran-17-one (Fig. 3.11) demonstrate clearly the use of the mass shift technique for identifying fragments containing the boronic ester group. The m/e values of prominent boron-containing fragments observed in the mass spectra of the three isomers (Table 3.6) enable clear distinction of the 5,10 isomer and distinguish the 4,5 from the 5,6 isomer by the relative abundance of the fragment at

m/e = 215 in the mass spectra of the phenylboronates.

TABLE 3.6

Prominent boron-containing fragments in the mass spectra of n-butyl- and phenylboronates derived from isomers of estren-17-one

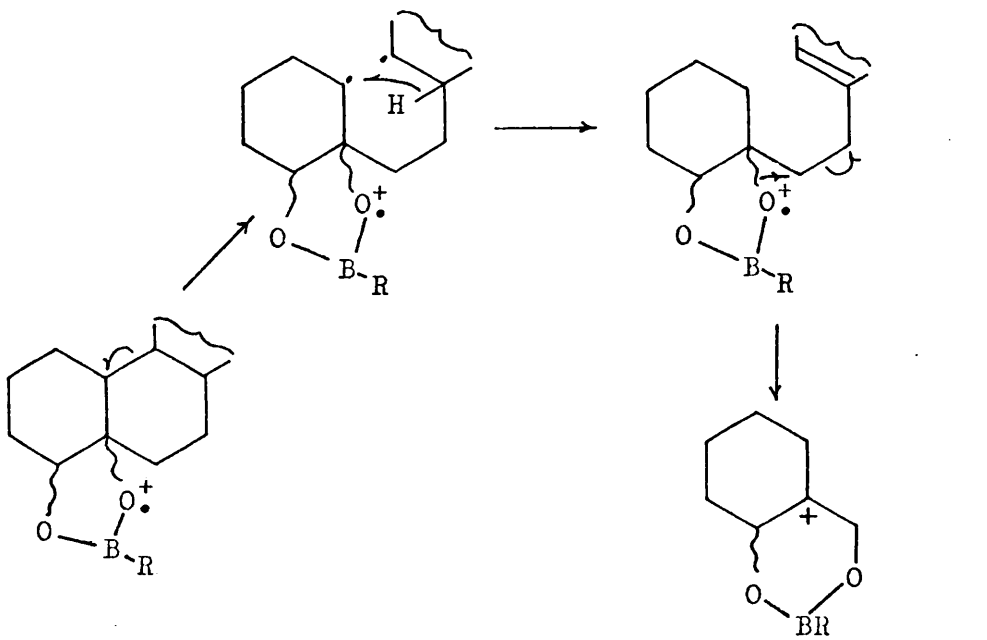
	n-butylboronate		phenylboronate	
Parent isomer	m/e	%	m/e	%
Δ^4	195	46	215	40
Δ^5	195	100	215	100
$\Delta^{5(10)}$	181	100	201	100
	195	23	215	14

Fragments not exhibiting a 20 unit mass difference for phenyl- and butylboronate esters were usually common to the three isomers and not therefore characteristic of the position of the diol groups. Exceptions were the fragments at m/e = 228 for the 4, 5 isomer, m/e = 120 for the 5, 6 isomer and m/e = 149 for the 5, 10 isomer.

4, 5-boronate esters

It is apparent from the mass spectrum of 4, 5-cis-dihydroxyestra 17-one phenylboronate that the boronate ester at this position has little influence on the fragmentation of the molecule. The principal ions in this spectrum result from common fragmentation of the

steroid nucleus (e.g. m/e = 91, m/e = 105), elimination of phenylboronic acid (m/e = 256) and elimination of phenylboronic acid with subsequent fragmentation of ring D (e.g. m/e = 228, m/e = 200, m/e = 199). The boron-containing fragment at m/e = 215 in the mass spectrum of the phenylboronate shifted to m/e = 229 for the 2 α-methyl and 6 α-methyl analogues. Similarly the boronate of the $3d_2$ analogue gave a fragment at m/e = 197 instead of 195. Knowing that these ions contain carbon atoms 2, 3 and 6, a likely fragmentation would be :

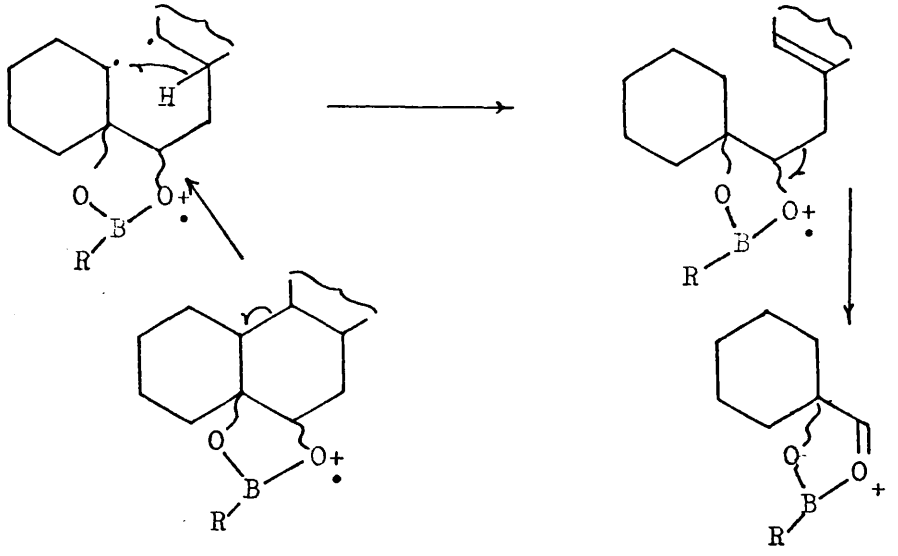


R = 77 (phenyl), 57 (n-butyl)

m/e = 138 + R

5, 6-boronate ester

In contrast to the mass spectrum of the 4, 5-phenylboronate, the 5, 6-phenylboronate ester fragments under electron impact to give a spectrum dominated by one ion at m/e = 215 which retains the boronate group. Although this was not confirmed by comparison with labelled analogues, there is little reason to doubt that this ion comprises ring A and C-6. A likely mechanism therefore would be :

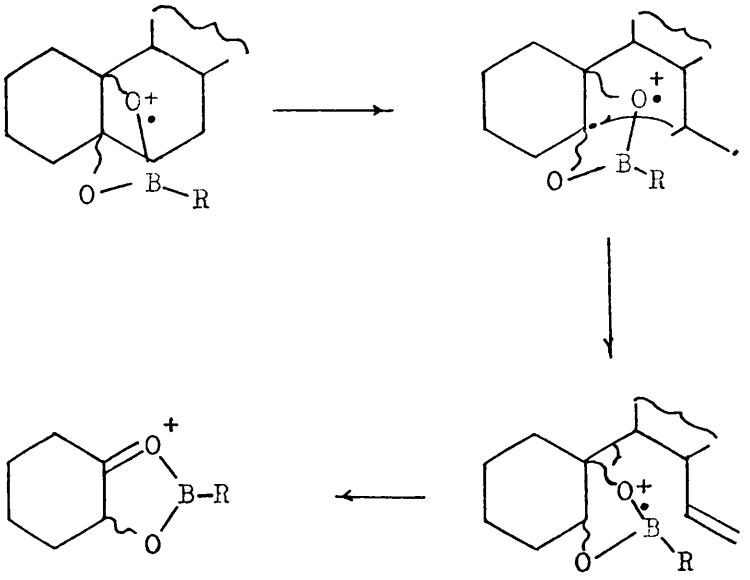


R = 77 (phenyl), 57 (n-butyl) m/e = 138 + R

5, 10-boronate esters

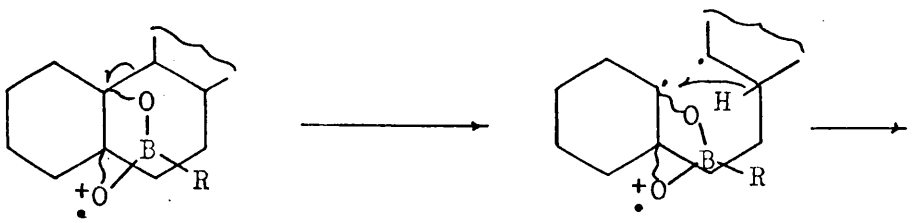
The base peak in the mass spectrum of the 5, 10 phenylboronate ester is at m/e = 201 and contains the boronate

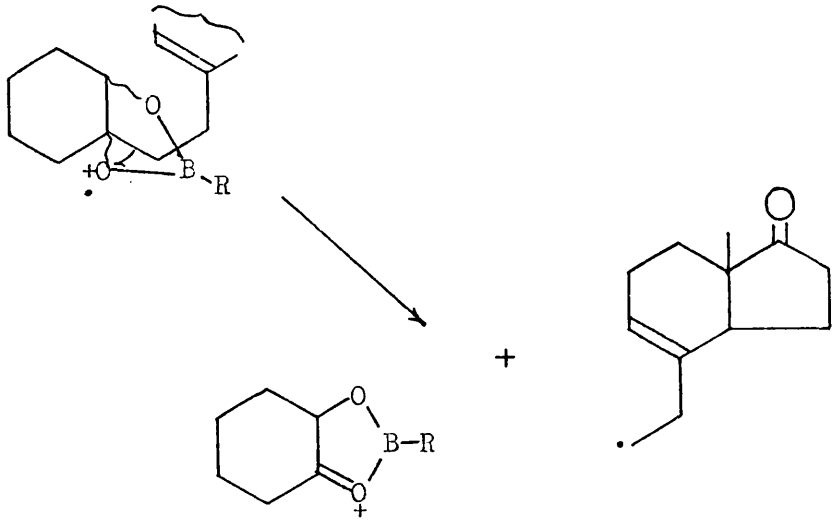
group. This fragment might well be formed by a mechanism such as the following :



m/e = 123 + R
R = 77 (phenyl), 57 (butyl)

This mode of fragmentation seemed more likely than the alternative 9,10-bond fission followed by hydrogen transfer from C-8 to C-10 since the latter necessitated the elimination of a comparatively unstable neutral species

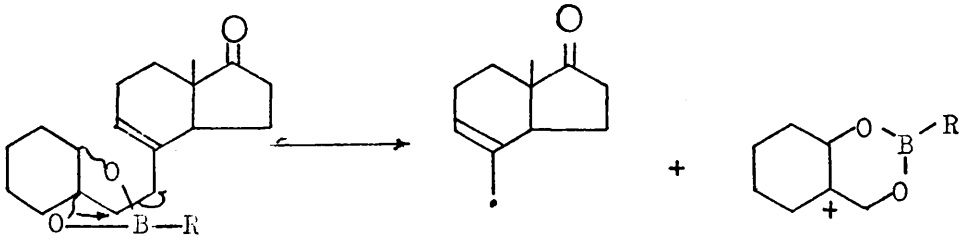




m/e = 123 + R

This would also explain the absence of a fragment at this mass value for the 4, 5-boronate ester which would also be expected to occur by fission of the 9,10 and 5, 6 bond with hydrogen transfer from C-8 to C-10.

The weaker boron-containing fragment at m/e = 215 may be formed by a mechanism similar to that proposed for the fragment at m/e = 215 in the 4, 5-phenylboronate ester.



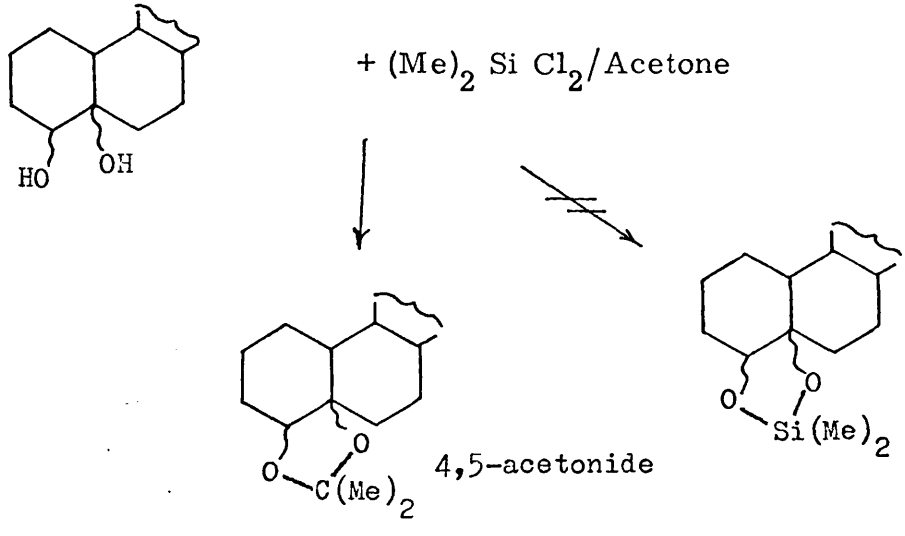
m/e = 138 + R

R = 77 (phenyl), 57 (butyl)

The prominent fragment at m/e = 149 does not contain the boronate group, but appears to be characteristic of its location. A fragment of the same mass is prevalent in the mass spectrum of the 5,10-cis-diol.

D. ACETONIDES OF CIS-DIOLS

Acetonides can be prepared from 1,2 or 1,3 diaxial steroid cis-diols by refluxing an acetone solution containing an acid catalyst such as p-toluenesulphonic acid or by using copper sulphate as catalyst. In an attempt to synthesise an analogous siliconide by dissolving the cis-diol at room temperature in an acetone solution of dichlorodimethylsilane, the acetonides of the 4, 5 and the 5, 6 cis-diol were inadvertently prepared in high yield (85% 4, 5-acetonide; 95% 5, 6-acetonide) i.e.



The isomeric 5, 6 acetonide was formed from the 5, 6 cis-diol but the 5, 10 cis-diol failed to form a derivative under these conditions.

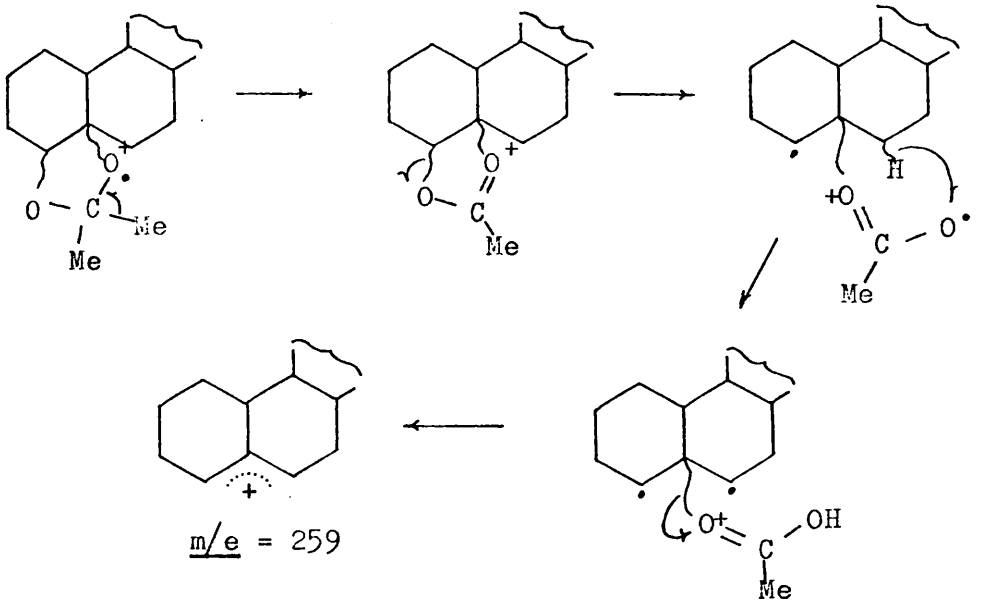
Gas Chromatography

The acetonides chromatographed well forming symmetrical peaks which were well separated from the cis-diols on OV-17 (4, 5-cis dihydroxyestran - 17-one acetonide = 26.18 MU; 5, 6-cis-dihydroxyextran - 17-one acetonide = 26.90 MU) thus enabling satisfactory distinction from the unreacted 5, 10 cis-diols (= 28.45 MU, 28.91 MU) using gas chromatography.

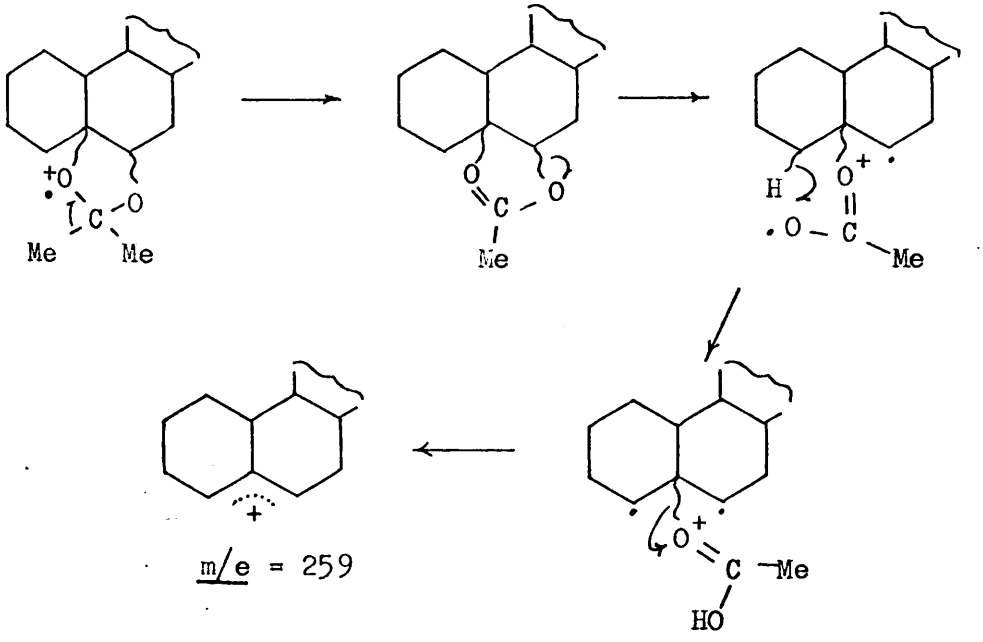
Mass Spectrometry

The mass spectra of acetonides derived from unsaturated fatty acids have been reported by McCloskey and McClelland.[63] The mass spectra of the 4, 5-acetonide and the 5, 6-acetonide were essentially identical. No molecular ion was apparent in either spectrum (at 70eV) but the ion at m/e = 317, produced by loss of a methyl group was found to be more stable. Further loss of acetic acid from this ion forms the fragment at m/e = 259 which is the base peak. Likely mechanisms are postulated below.

4, 5-acetonide



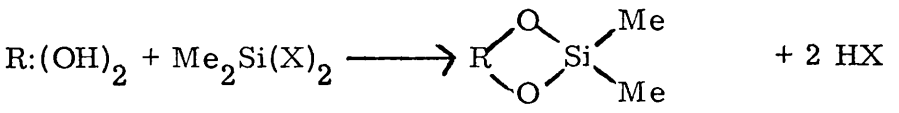
5, 6-acetonide



Since by this hypothesis the base peak at m/e = 259 has the same structure for the 4, 5- and 5, 6-acetonide, it is not surprising that further fragmentation from this ion is the same for both isomers.

E. DIMETHYL SILICONIDES OF CIS-DIOLS

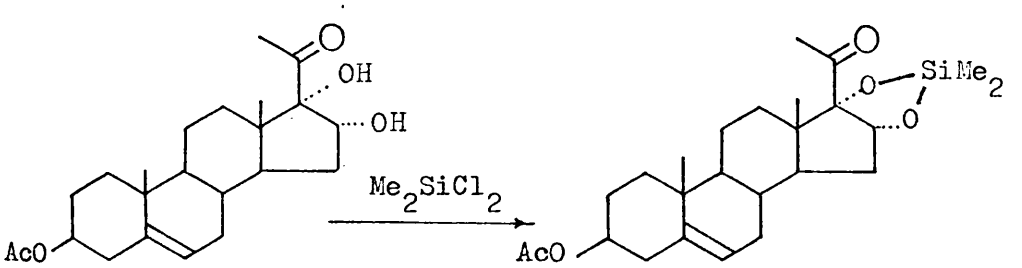
During our search for suitable derivatives of cis-diols, considerable attention was given to the possible formation of a silicon analogue of the acetonide. It was thought likely that these "dimethyl siliconides" might be formed by the general reaction



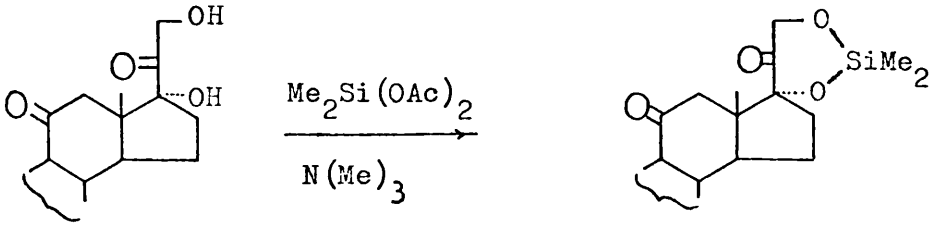
where X is an electronegative substituent and $R:(OH)_2$ is a 1, 2 cis-diol. Such derivatives it was hoped would be suitable for gas chromatography, since they are non-polar, should have retention times comparable with acetonide derivatives, and give mass spectra characteristic of their location on the steroid nucleus. In consideration of the latter we would expect a different mode of fragmentation from that of the acetonides since the elimination of $MeSiO_2H$ from the M-15 ion is less favourable than the elimination of acetic acid from the equivalent ion in the mass spectrum of the

acetonide.

This research was undertaken before publication of similar work by Kelly who succeeded in making a derivative of this type in good yield by reacting 3 ß-acetoxy-16 α 17α-dihydroxypregn-5-ene-20-one with dimethyldichlorosilane in pyridine.[64]



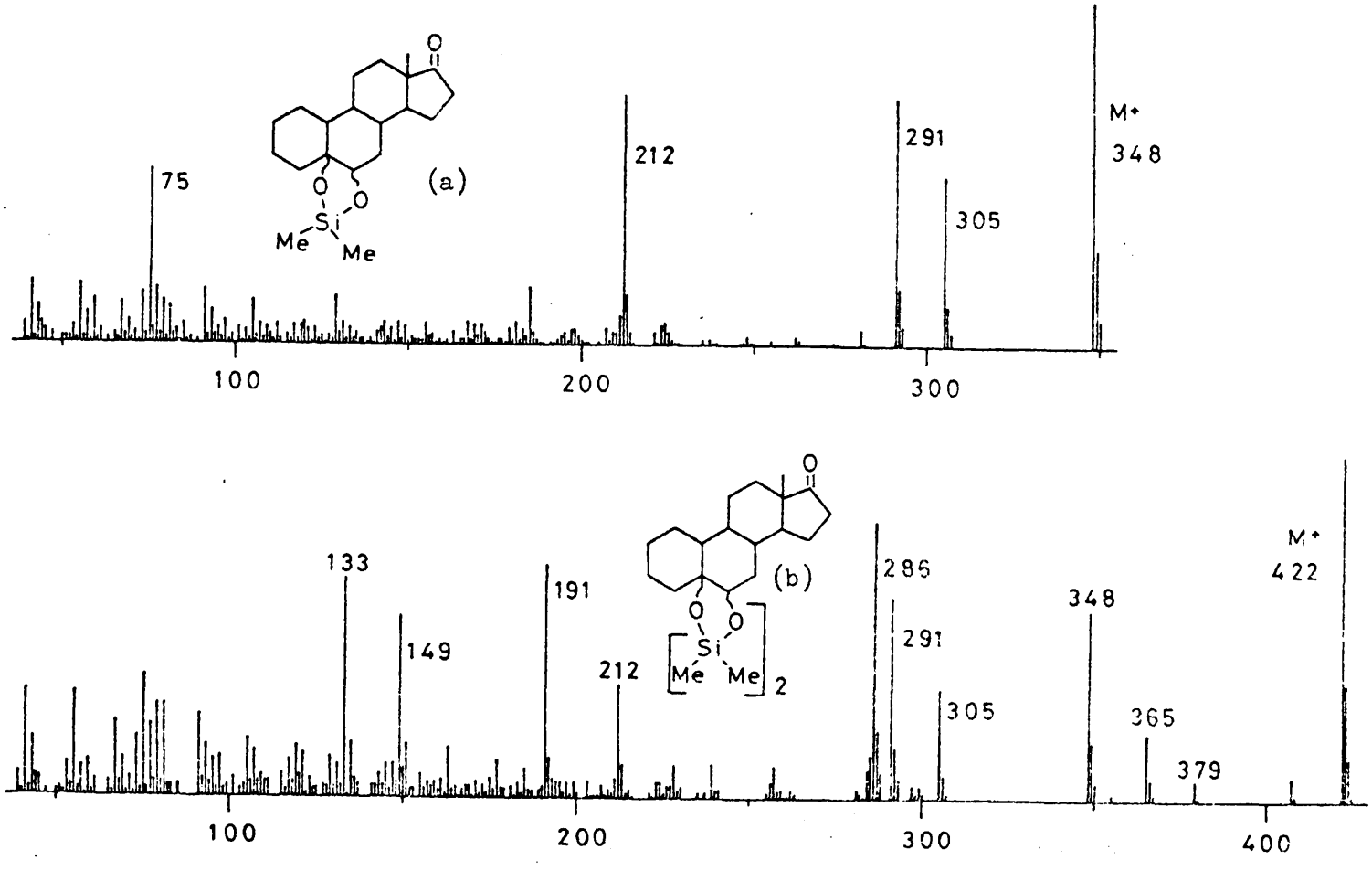
Later he reported siliconide formation of the dihydroxy acetone side chain of a corticosteroid using dimethyldiacetoxy silane in hexane solution with trimethylamine as catalyst.[65]



Three bifunctional reagents of the dimethyl silicon type : dimethylchlorosilane (Me_2SiCl_2), dimethyldiethoxysilane

FIG. 3.12

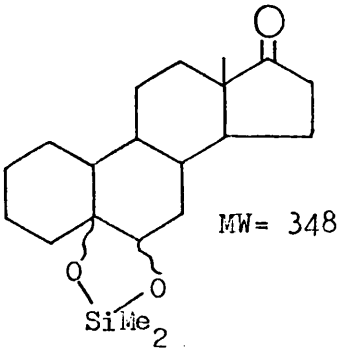
Mass spectra at 70 eV of siliconides derived from 4-estren-17-one: (a) mono-siliconide; (b) di-siliconide



($Me_2Si(OEt)_2$) and dimethylbis(dimethylamino)-silane ($Me_2Si(NMe_2)_2$), were used to treat indane-cis-diol under various thermal and catalytic conditions in order to indicate their suitability for siliconide formation. A common factor in these reactions however was the formation of polymeric silicone species, at least one of which contained the indane nucleus.

Application to 5,6-dihydroxyestran-17-one

The cis-diol obtained from estr-5-en-17-one was heated with a large excess of dimethylbis(dimethylamino)silane at 120° for 17 hours in a sealed tube. The reaction products were shown by GC/MS to comprise three components in approximately equal proportions, one of which gave a parent ion which corresponded to the molecular ion of the desired product.



The full mass spectrum (Fig. 3.12a) is characterised by a

very stable molecular ion (which is the base peak) and relatively few daughter ions. However, although fragments at m/e = 219 and m/e = 305 can be explained by ring D fragmentation, the fragment at m/e = 212 which would appear to be characteristic of the siliconide group cannot be explained by simple fission processes and probably involves a skeletal rearrangement.

The two other components of the mixture behaved almost identically on the mass spectrometer and showed molecular ions corresponding to the addition of two dimethylsiloxy groups.

SUMMARY

The following summarises the suitability of the techniques used to isolate and characterise Δ^4, Δ^5 and $\Delta^{5(10)}$ isomeric estren-17-ones.

1. Olefins

GLC on OV-1 and OV-17 separated the $\Delta^{5(10)}$ isomer from a mixture of the three compounds, but failed to resolve the Δ^4 and Δ^5 components. Mass spectrometry at 15eV distinguished the Δ^4 isomer by the presence of an extra fragment at m/e = 94.

2. Cis-diols

GLC on OV-1 and OV-17 separated all three isomers from a mixture. The $\Delta^{5(10)}$ isomer was immediately distinguished from the others by the formation of two products (presumably α and ß cis-diols) which were separated from each other on both stationary phases. Mass spectrometry at 70eV afforded excellent distinction between the three positional isomers, but the fragmentation was difficult to predict and therefore to interpret. The two diastereisomers resulting from hydroxylation of the $\Delta^{5(10)}$-olefin gave very similar mass spectra.

3. Trimethylsilyl ethers

Trimethylsilyl ethers of the cis-diols were considered unsuitable

derivatives for characterisation purposes when in the presence of other material (as is the case for extracts from urine) due to the multiplicity of products formed. However, as was exemplified by Chambaz and Horning,[45] ease of silylation of an alcohol is in itself a means of determining its position, and this may prove to be a suitable method with single substances for distinguishing between secondary-secondary cis-diols, secondary-tertiary cis-diols and tertiary-tertiary cis-diols by GLC.

4. Boronate Esters

Quantitative reaction between boronic acids and cis-diols produced cyclic esters which gave symmetrical peaks on the gas chromatograph showing no signs of decomposition. Separation was achieved on OV-1 and OV-17 for phenylboronates of a mixture of cis-diols obtained from the three isomeric olefins. The $\Delta^{5(10)}$ isomer again resulted in two products which were separable by GLC but which gave very similar mass spectra.

Mass spectral fragmentation produced only few fragments containing the boronate group. These were readily distinguished by mass shifts of 20 a.m.u. when comprising the

spectra of phenyl- and n-butylboronate esters, and afforded satisfactory distinction between the three isomers studied.

5. Acetonides

Under the conditions described, acetonides were selectively formed for the 4, 5-cis-diol and the 5, 6-cis-diol. The 5, 10-cis-diols remained completely unmodified by the reagent. Mass spectrometry however afforded no distinction between the two isomeric products, and the derivative may therefore only be used as a means of distinguishing the 5, 10 and possibly other tertiary-tertiary cis-diols by GLC.

6. Dimethyl Siliconides

It was evident from the results obtained that the bifunctional silicon reagents described are of limited use as reagents for cis-diols due to the inherent tendency to polymerise. Despite precautions taken to exclude water from the reaction mixture, polymeric material (some of which incorporated the cis-diol) was always observed whenever conditions necessary to form a diol derivative were achieved.

OXYGEN SUBSTITUENTS ON THE STEROID NUCLEUS

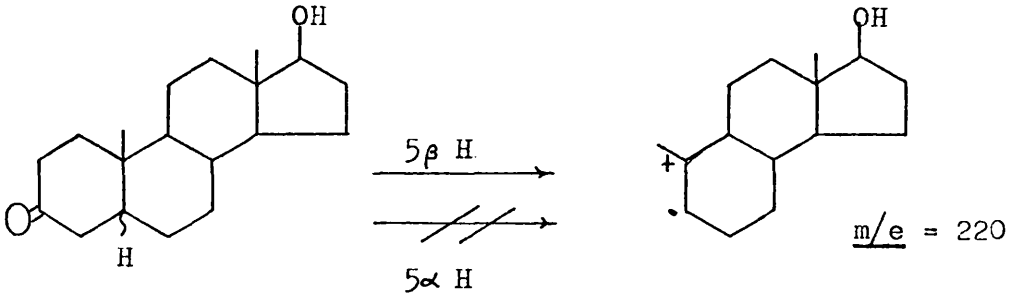
The location of a hydroxyl or ketone group on a steroid nucleus is a problem often met with in steroid metabolism studies. Unlike alkyl groups which may often be located by mass shifts of fragments characteristic of their parent steroid (see part 2), oxygen substituents often direct fragmentation along different pathways according to the position of the groups. The assignment of position of a functional group on the steroid skeleton from mass spectral data is often difficult however for reasons discussed below :

(i) Suppression of fragmentation.

When more than one functional group is present on the molecule competitive processes may suppress fragmentation characteristic of one of the groups in preference for another. A recent study by Vetter and others[141] into the factors affecting competitive fragmentation processes suggested that electron deficiency at a potentially reactive site is a prerequisite for any fragmentation to occur to an appreciable extent. The localisation of charge at a particular functional group was

thought to be related to its ionisation potential and to the possibility of charge transfer processes from other sites of the molecule after preceding fragmentation.

Configurational effects have also been reported to cause suppression of the formation of fragments associated with some stereoisomers.[142] For example 17α-hydroxy-5α-androstan-3-one does not give the fragment at m/e = 220 (due to loss of C1-C4) which is present in the mass spectrum of the 5β-isomer with a relative abundance of 45% of the base peak (m/e = 290).[143]

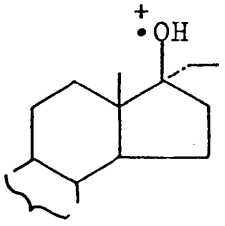


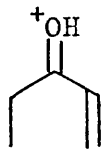
(ii) New fragmentation pathways

Alternatively the presence of two oxygen groups in the molecule may give rise to fragments which are not characteristic of either individual group. Thus, 1,6-dioxosteroids,[144] Δ^4-3,6-dioxosteroids[145] and Δ^4-3,11-dioxosteroids[119] all give rise to fragments which cannot be predicted by the behaviour of the monofunctional steroids.

The influence of an olefinic double bond on the fragmentation of proximal oxygen substituents has been discussed in part 3.

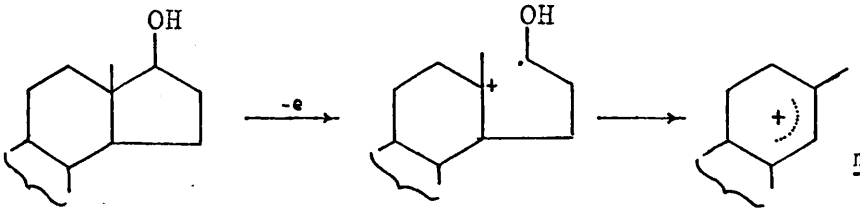
Certain substituents on the nucleus have been reported to promote fragmentation directed by other functional groups, or redirect the fragmentation normally associated with a particular group. In one investigation [143] the effect of an alkyl substituent at C-17 appeared to promote ionisation of the 17-OH group in 17α-ethyl-17β-hydroxy-5α-androstan-3-one to produce an ion at m/e = 85 resulting from fragmentation directed by this charge location.





m/e = 85

An analogous ion at m/e = 57 was not present in the non-alkylated steroid, the preferred fragmentation pathway apparently being initiated by ionisation of the C13-C17 bond leading to the elimination of C15-C17 as a neutral radical.



m/e = M-59

Charge localisation at oxygen substituted on C-17 appears to be enhanced to an even greater extent by alkyl substitution on oxygen, and by the formation of 17-OTMS ethers as evident in part 2 of this thesis, and the relevant literature citations.

Fortunately, a great deal of work by various authors has determined the mode of fragmentation for many monofunctional and polyfunctional steroids, and mass spectra for the majority of naturally occurring steroids found in plasma and urine are reported in the literature. A series of useful reviews on this matter have recently been compiled by Von Unruh and Spiteller[146-148] which relate evidence of structure (together with appropriate literature citation) to the mass of the prominent ions in the mass spectrum and of neutral species eliminated from the molecular ion.

The use of derivatives of alcohols and ketones for the characterisation of steroids by mass spectrometry provides a complementary method of locating oxygen substituents. Derivatives such as trimethylsilyl ethers (of alcohols) methyloximes and ethylene ketals (of aldehydes and ketones) often give fragments of high relative abundance which are characteristic of the groups location. The use of these derivatives can be beneficial when

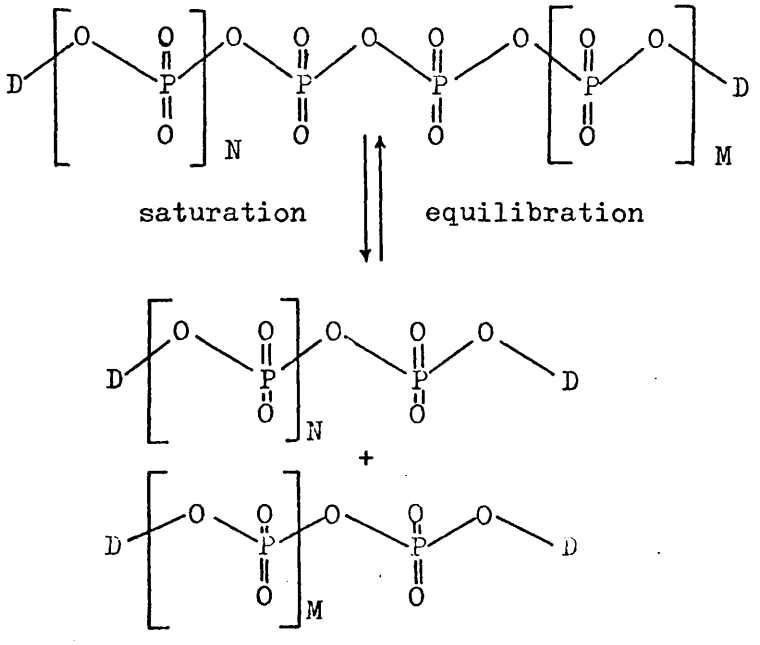
hydroxyl or ketone substituents are present which have weak fragmentation-directing properties.

The following section comprises in part examination of the utility of some oxime type of derivatives for characterising 1-, 2- and 3-oxosteroids by GC/MS and the development of gas phase deuteriation as a general method of functional group analysis.

GAS PHASE DEUTERIATION

The technique of isotope exchange during the course of gas chromatography has been described by earlier workers as a means of preparing $^{2}_{1}H$, $^{3}_{1}H$ or $^{18}_{8}O$ labelled compounds in high isotopic purity. In these studies hydroxylic phases such as polyethylene glycol [149-152] or sorbitol [153] previously saturated with deuterium oxide or tritium oxide were used to label samples by exchanges with hydrogen on electronegative substituents such as O, N or P. The incorporation of basic [149,150,152] or acidic [151,154] catalysts (e.g. potassium hydroxide, phosphoric acid) promotes exchange also of enolic hydrogen atoms. The phosphoric acid column is

superior to the basic column when chromatographing aldehydes and phenolic material, and has been shown to promote exchange of $^{18}_{8}O$ with aldehyde or ketone carbonyl after the column has been saturated with $H_2\,{}^{18}_{8}O$.[4-14] One disadvantage of this catalyst however, is the lengthy equilibration time required after saturating with the appropriate label. This equilibration time which was reported by Richter and others to be 5-10 hours for $^{2}_{1}H$ and $^{3}_{1}H$ labelling, and is most likely due to depolymerisation and reformation of polyphosphoric acid.



Isotopic exchange prior to gas chromatographic

separation has also been described [151, 154] and is an advantage where incompatability exists between the stationary phase and the catalyst. The method described by Richter and others [154] used a pre-column of support material which was coated with the catalyst and connected directly to the GLC column. Disadvantages of this method are the short contact time between exchange material and the catalyst (which is also the only effective label carrier), and the apparent adsorption of some components on the exchanger column. [151]

Bearing the above discussion in mind the system most suitable for steroid analysis by deuterium exchange followed by mass spectrometry was thought to be the single column method using a stationary phase coated on base treated support material, since :

(i) the prolonged contact time between exchangeable material and catalyst would lessen the amount of catalyst required for the reaction to go to completion,

(ii) column adsorption effects would be minimised,

(iii) speed of equilibration is important for GC-MS where instrument time is expensive, and therefore polyphosphoric acid catalyst is less desirable.

Silicone type stationary phases which are traditionally used for steroids are not compatible with potassium hydroxide at temperatures required for GLC of these compounds, and initial experiments were made with a column comprising 1% Carbowax 20 M on Gas Chrom Q support which was impregnated with 1% KOH. A 2' column of the above packing material was exchanged with deuterium by passing 3 x 10 μl deuterium oxide through via the injection port. The exchange capability with some monofunctional steroids was determined (Table 4-1) by mass spectrometry.

Table 4-1

Deuterium exchange by GLC using 2' column of 1% Carbowax - 20 M and 1% KOH

Steroid	Temp (^{o}C)	Percentage Deuterium			
		d_1	d_2	d_3	d_4
5-Estren-17-one	160	7	93	0	0
5α-Androstan-17-one	160	6	94	0	0
2-Pregnen-20-one	170	0	0	29	71

This column, however, was too polar for chromatography of many bifunctional steroids and gave rise to intolerable 'bleeding' at temperatures above 200^{o}C. Furthermore, the short column which was necessary for chromatographing monofunctional steroids

would not be efficient enough for separating many steroid mixtures. Apiezon-L was chosen as a preferable alternative phase since it has low polarity and has been shown to be compatible with potassium hydroxide in the GLC of free amines.[80,106]

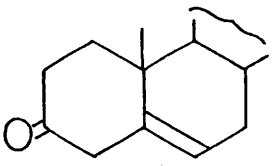
EFFECT OF BASICITY OF CATALYST

At infinite dilution, aqueous solutions of all inorganic bases have the same effective base strength, i.e. that of the solvated hydroxide ion. In the solid state, however, the strength of an inorganic base is related amongst other things to the crystal lattice energy, which is partially determined by the metal ion. In order to learn more about the exchange and chromatographic properties of base treated columns, 6' columns were prepared comprising 1% Apiezon-L coated on supports containing 1% potassium hydroxide, on supports containing equivalent amounts of barium hydroxide and strontium hydroxide and on a neutral support.

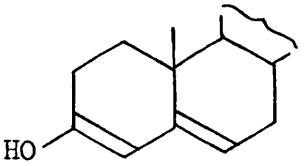
Preliminary work with monofunctional steroid ketones showed the strontium hydroxide coated support to be unsuitable for the chromatography of ketonic steroids. Whereas the non-ketonic steroid 5α-androstan-17β-OTMS showed excellent

GLC properties on the strontium hydroxide treated column, the ketonic steroid 5α -androstan-17-one gave no GLC peak, presumably due to its strong adsorption onto the base.

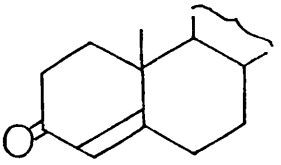
Retention data for saturated and unsaturated C-27 steroid ketones (Table 4-2) show only moderate change of MU value when chromatographing each steroid on neutral, KOH and $Ba(OH)_2$ treated supports. The poor results obtained for cholest-5-ene-3-one and cholest-3, 5-dien-7-one with the KOH treated support were not paralleled with the $Ba(OH)_2$ treated support, and in this respect it was evident that $Ba(OH)_2$ was probably a better catalyst for our purposes. Δ^5-cholesten-3-one was eluted from the $Ba(OH)_2$ treated column with the same retention value as the Δ^4 isomer. This result is hardly surprising since ketones which share a common enol structure will be expected to equilibrate to the most stable isomer, e.g.



Δ^5-3-oxo



enol



Δ^4-3-oxo

(most stable)

Table 4-2

GC retention data of 5 α-cholestane and some C-27 steroid ketones on 1% Apiezon-L used with neutral and alkali treated [a] supports.

Steroid	MU (6' x 1% Apiezon-L, 260^{0}C) Neutral	KOH	$Ba(OH)_2$
5 α-cholestane	29.03	29.04	29.03
5 α-cholestan-2-one	30.37	31.20	30.91
5 α-cholestan-3-one	31.17	31.59	31.62
5 α-cholestan-6-one	30.80	30.39	30.87
5 α-cholestan-7-one	30.60	30.26	31.05
Δ4-cholesten-3-one	31.65	32.60	32.50
Δ5-cholesten-3-one	32.73	(b)	32.50 (c)
Δ1, 4-cholestadien-3-one	31.91	32.65	32.31
Δ4, 6-cholestadien-3-one	32.00	32.77	32.63
Δ3, 5-cholestadien-7-one	31.41	(d)	31.79

(a) KOH treated support contained 1% by weight KOH, $Ba(OH)_2$ treated support contained an $(OH)^-$ equivalent of $Ba(OH)_2 . 8H_2O$

(b) No peak was observed at the expected retention time.

(c) Eluted as Δ^4-cholesten-3-one

(d) Evidence of decomposition

It was also evident that the column 'bleed' (as observed in the recorder base line) was less with the barium hydroxide support than with the potassium hydroxide support. In the light

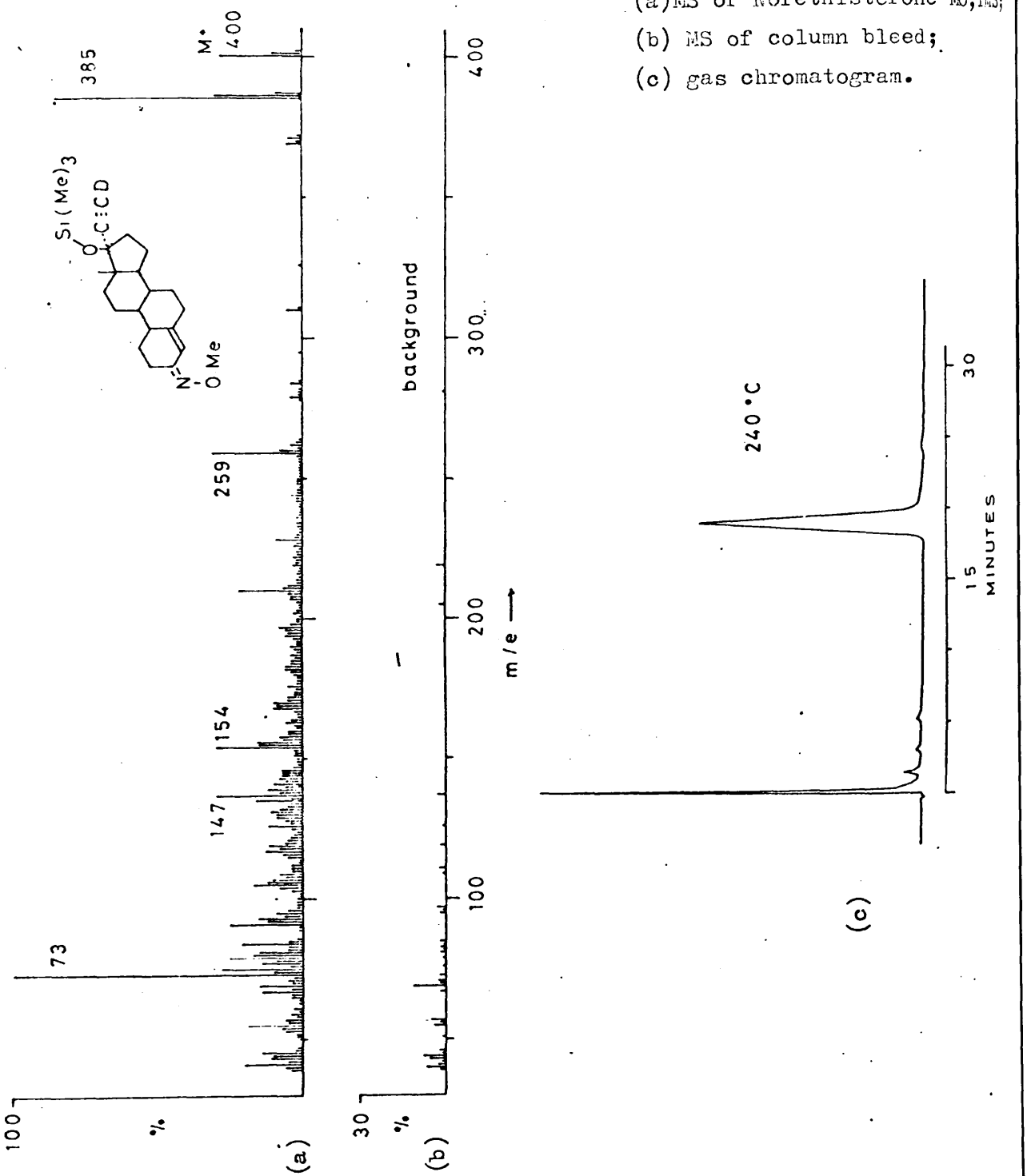
FIG. 4.1

GC-MS of Norethisterone MO,TMS on 1% Apiezon-L, 0.5% $Ba(OD)_2$:

(a) MS of Norethisterone MO,TMS;

(b) MS of column bleed;

(c) gas chromatogram.



of the above observations, all further studies of deuterium incorporation by GC-MS were made using 1% Apiezon-L on barium hydroxide treated support.

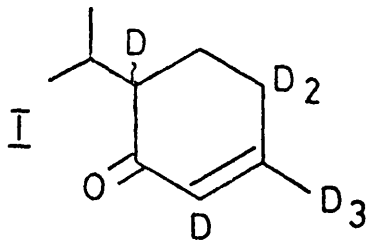
The 'tailing' of conjugated ketones on $Ba(OH)_2$ treated columns was attributed to reversible adsorption onto the base. This effect was later manifested during GC-MS of the sesquiterpenoid derivative isopetasol TMS ether (Table 4.3) where changes in retention time were evident with different sample loadings.

COLUMN TEMPERATURE LIMITATIONS FOR GC-MS

Columns comprising 1% Apiezon-L with 0.5 to 1% barium hydroxide were found to remain adequately thermostable up to 250°C using helium as carrier gas provided that the system was kept air free. The 'bleed' of material from the column into the mass spectrometer was found to be remarkably low after the column had been kept at this temperature for three days, and the mass spectra of compounds run under these conditions were almost free from contamination. Fig. 4.1a shows a line diagram drawn from the mass spectrum of a 2 μ g sample of the steroid drug Norethisterone which had been chromatographed (Fig. 4.1c) at 240°C on a 0.5% barium

FIG. 4.2

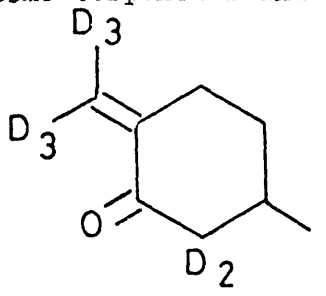
Deuterium incorporation in some terpenoids and derivatives



I

Piperitone

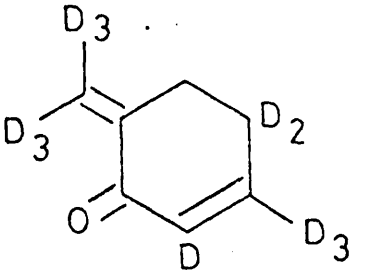
(d_7 = 8%)



II

Pulegone

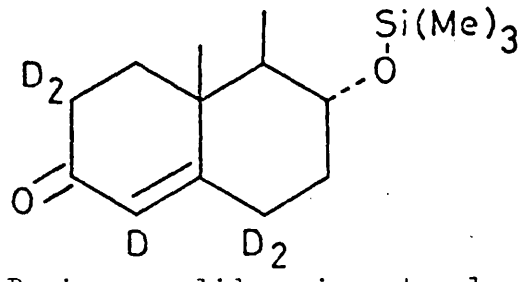
(d_8 = 48%)



III

Piperitenone

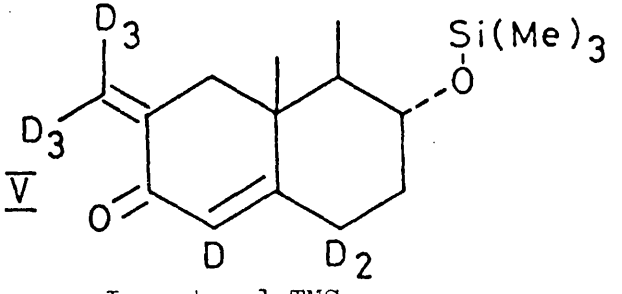
(d_{12} = 2%)



I

Desisopropylidene-isopetasol

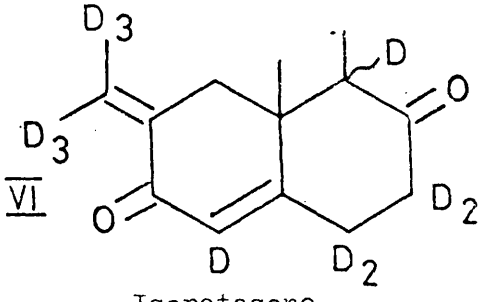
(d_5 = 83%) TMS



V

Isopetasol TMS

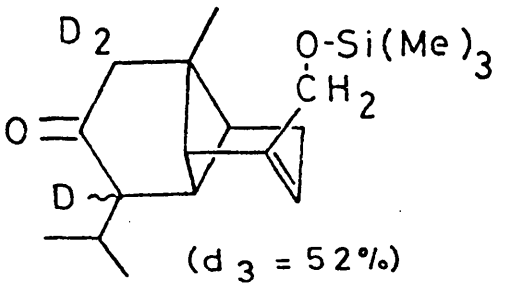
(d_9 = 70%)



VI

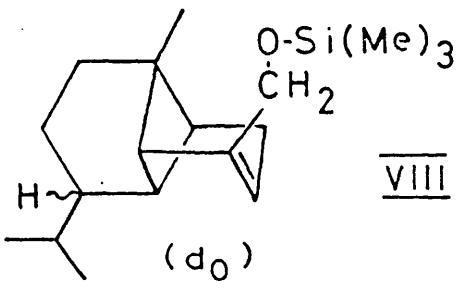
Isopetasone

(d_{12} = 70%)



VII

(d_3 = 52%)



VIII

(d_0)

Derived from Brachylaena huchinsii

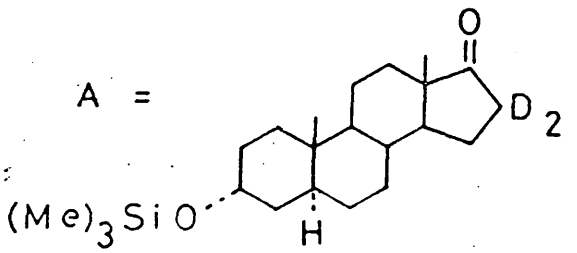
hydroxide deuteriating column as its 17-trimethylsilyl ether 3-O-methyloxime derivative. The column 'bleed' at this temperature as indicated by Fig. 4.1b (drawn to the same scale as Fig. 4.1a) is clearly tolerable and permits measurements on smaller amounts of sample. The exchange of ethynyl hydrogen with deuterium under these conditions was virtually quantitative.

A low temperature limit became apparent when studying deuterium incorporation in some monoterpenoids and sesquiterpenoids (see Fig. 4.2). The poor deuterium incorporation in piperitenone (d_{12} = 2%) which was chromatographed at 110° was not reflected in the deuterium incorporation of isopetasone (d_{12} = 70%) which contains a similar unsaturated ketone group and was chromatographed at 190°.

Samples which can exist in two epimeric forms may be eluted as a mixture of the two isomers. While this results in no change in deuterium incorporation and little difference in the mass spectrum, GLC peaks of compounds such as 5α-pregnan-20-one are noticeably broader than those from non-epimerisable saturated ketones.

FIG. 4.3

GLC of a trimethylsilylated extract of neutral urinary steroids on 1% Apiezon-L 0.5% $Ba(OD)_2$



16-d_2 Androsterone

3-TMS Ether

86% d_2 (1μg)

82% d_2 (3μg)

A

200

250

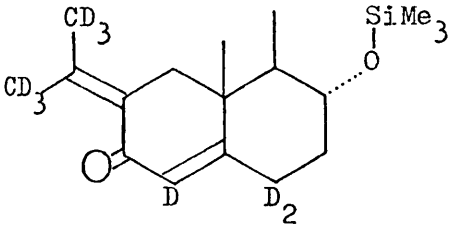
TEMPERATURE (°C)

CAPACITY AND RESOLUTION

Although primarily effective for the characterisation of single substances, the columns used for deuterium-hydrogen exchange may still retain the resolving power of conventionally packed columns. Peak tailing due to column adsorption can often be avoided by chromatographing compounds as their trimethylsilyl ethers where alcoholic groups are present or as O-Methyl oxime derivatives of $\alpha\beta$ -unsaturated ketones. Fig. 4.3 shows a chromatogram of the neutral fraction of a urinary steroid extract after enzymic hydrolysis with ß-glucuronidase [155] and trimethylsilylation. Deuterium incorporation into androsterone under these conditions (in the presence of substantial quantities of extraneous materials) was still sufficient to be of diagnostic value. The capacity of the column for deuterium exchange with single substances is illustrated in Table 4.3 for the production of d_9-isopetasol trimethylsilyl ether.

TABLE 4.3

Variation of retention time and deuterium incorporation with sample loading for isopetasol TMS ether



Loading (μg)	Retention Time (minutes)	Deuterium Incorporation (% d_9)
0.1	8.6	79
0.5	8.4	77
1.0	8.0	74
6.0	6.3	56
10.0	5.6	50

Deuterium Exchange with Monofunctional Steroid Ketones

The following results illustrate the use of deuterium exchange GC-MS as an analytical tool for the determination of carbonyl environment in the steroid nucleus. The increase in molecular weight upon deuteriation serves in itself to distinguish between certain steroid ketones, whilst knowledge of the expected mode of fragmentation under electron impact may yield supplementary structural evidence.

TABLE 4.4

Mass spectra of deuteriated steroid ketones

Steroid Ketones		Deuterium Incorporation[a]					Diagnostic Fragments[b, c, d]				
A: Saturated	MW	d_1	d_2	d_3	d_4	d_5	d_o	d_n	d_o	d_n	references
5 α-Cholestan-1-one	386	13	87	-	-	-	124	126	343	345	156
5 α-Cholestan-2-one	386	-	9	24	67	-					156
5 α-Cholestan-3-one	386	-	-	19	81	-					156
5 α-Androstan-4-one	274	-	7	93	-	-	98	100	111	114	157
5 α-Cholestan-6-one	386	3	23	74	-	-	123	123	331	334	158
5 α-Cholestan-7-one	302	-	19	81	-	-	178	181	191	194	156
5 α-Pregnan-11-one	302	-	24	76	-	-	192	195	205	208	156
5 α-Cholan-12-one	344	14	86	-	-	-	233	235			159
5 α-Androstan-17-one	274	9	91	-	-	-	230	230	218	218	(140 (160
5 α-Pregnan-20-one	302	-	4	23	73	-	217 84	217 88	43	46	)) 161
B: Conjugated											
Cholest-4-en-3-one	384	-		14	37	49	124	129	342	345	156
Cholesta-1, 4-dien-3-one	382	-	17	83	-	-	122	125			133
Cholesta-4, 6-dien-3-one	382	12	70	5	6	7	136	138	247	247	(162 (133
Cholesta-3, 5-dien-7-one	382	21	12	26	29	3	174	178	187	191	163

(a) Expressed as percentages of total deuteriated species, assessed in terms of peak heights (corrected for natural abundance of ^{13}C.)

(b) Only ions considered to be characteristic of the ketone position are cited.

(c) d_o = m/e of fragment from undeuteriated species, d_n = m/e of fragment incorporating deuterium.

(d) Deuteriated fragment quoted as m/e of most prominent ion.

Results from the mass spectra of 14 monofunctional steroid ketones after deuterium exchange on 1% Apiezon-L incorporating 1% barium hydroxide are summarised in Table 4.4. For unconjugated steroid ketones the data indicate good deuterium incorporation at all enolic sites. The αß -unsaturated ketones gave somewhat less satisfactory results but ions representing exchange of enolic states were invariably observed. The ßα-unsaturated ketone, cholest-5-en-3-one, was isomerised (by the barium hydroxide column) and the resulting mass spectrum was that of the deuteriated αß-isomer.

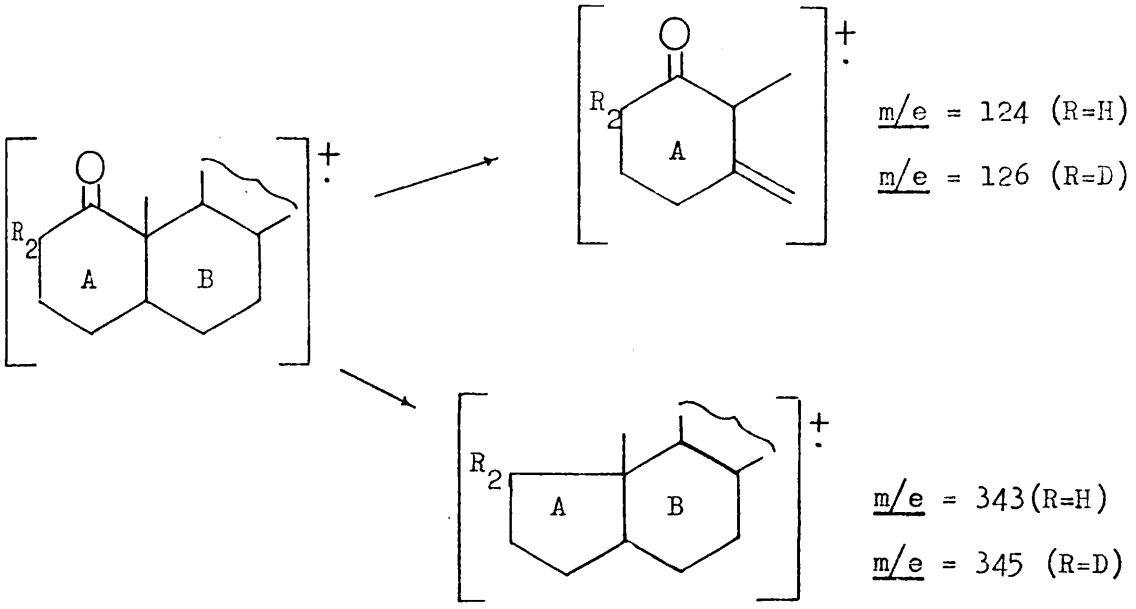
IDENTIFICATION OF FRAGMENTS

The mass spectral fragmentations of many saturated and some conjugated steroid ketones are well established and the deuterium incorporation in the principal fragments (Table 4.4) agrees with that expected. These fragments are discussed in more detail below :

(A) Saturated

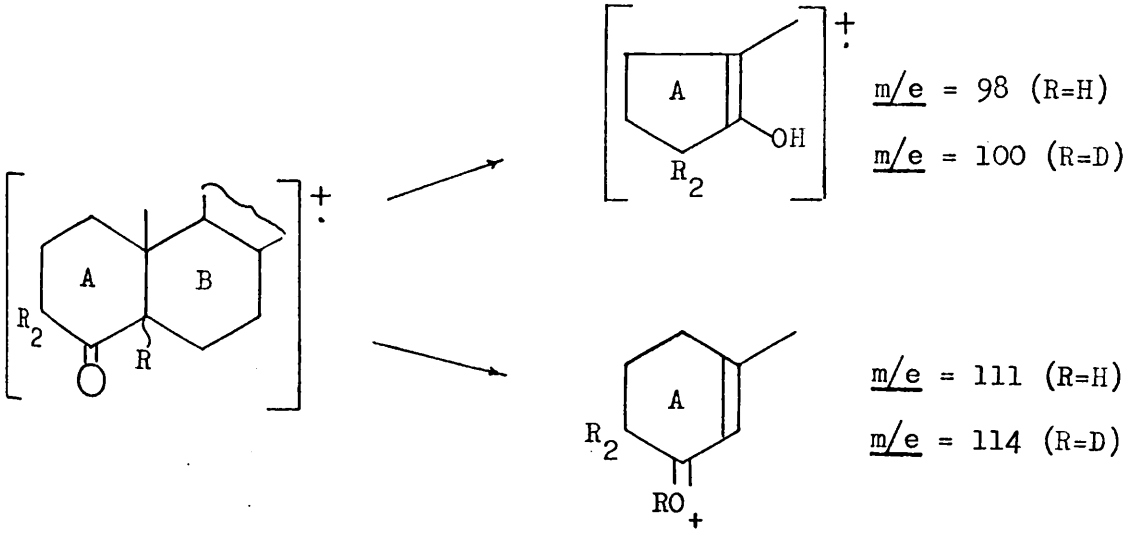
1-Oxo-steroid

1-Oxo-steroids such as 5α-cholestan-1-one give rise to an ion at m/e = 124 which contains ring A and an ion at m/e = M-43 due to loss of CH_3 and CO, i.e.



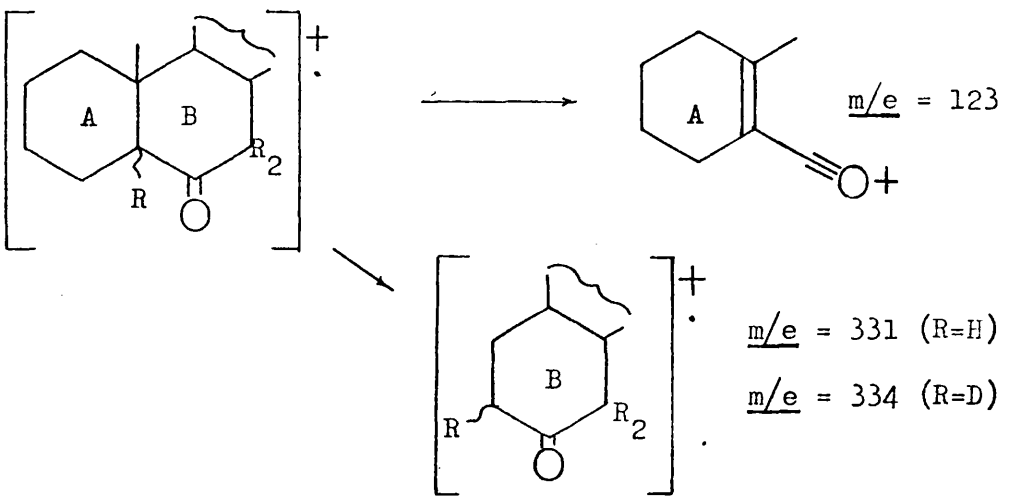
4-Oxo-steroids

The fragments at m/e = 98 and m/e = 111 in the mass spectrum of 5α -androstan-4-one have been shown to be the species below :



6-Oxo-Steroids

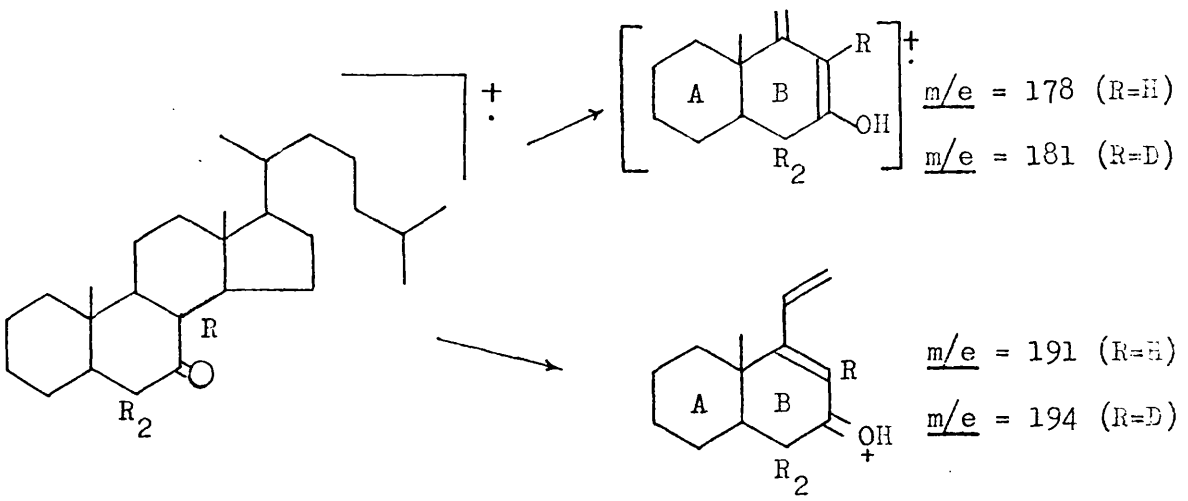
The fragments at m/e = 123 and m/e = 331 in the mass spectra of 6-oxo-steroids such as 5 α -cholestan-6-one have been shown to be the species below :



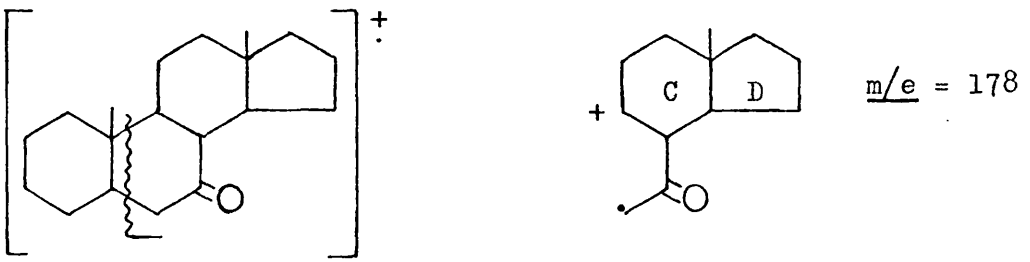
Although the fragment at m/e = 123 contains ring A the deuterium atom at C-5 in the deuteriated molecule is lost by hydrogen transfer to the neutral fragment giving therefore no mass increment to the charged species.

7-Oxo-Steroids

The fragments at m/e = 178 and m/e = 191 in the mass spectrum of 5α -cholestan-7-one have been shown to be the species below :

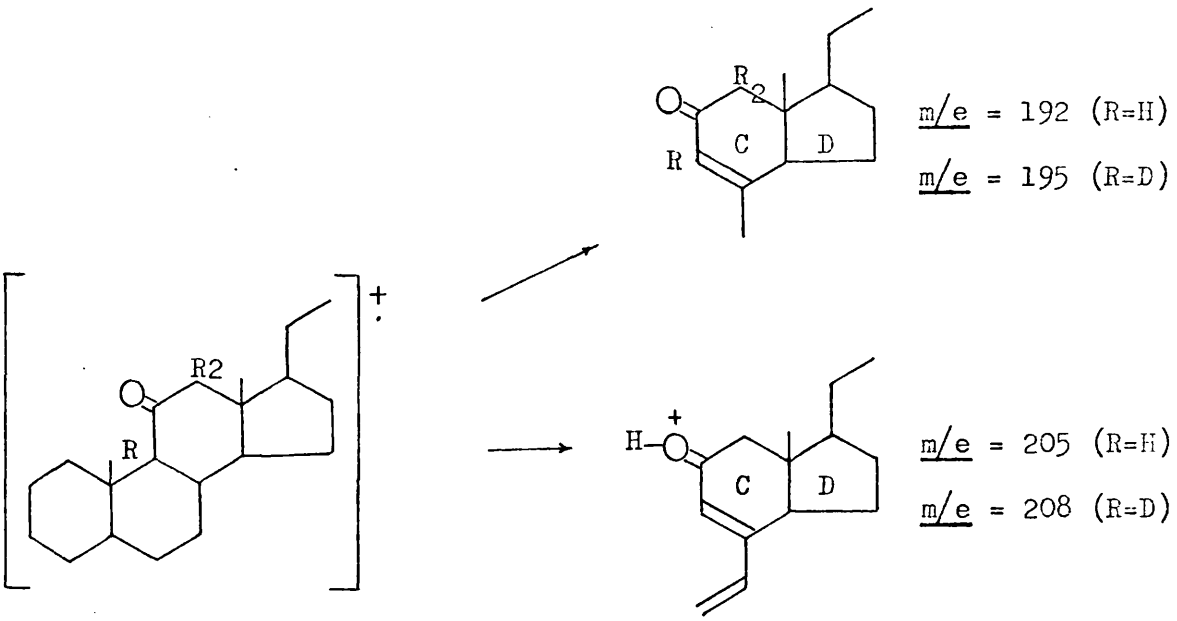


In the mass spectrum of 5α-androstan-7-one the fragment at m/e = 178 is due also to cleavage of the C9-10 and C5-6 bonds to give the fragment below:



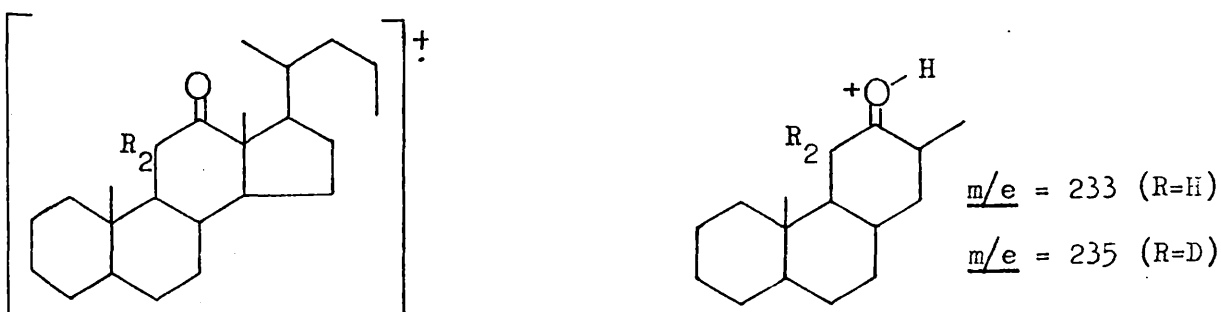
11-Oxo-Steroids

The fragments at m/e = 192 and m/e = 205 in 5α-pregnan-11-one have been shown to be the species below :



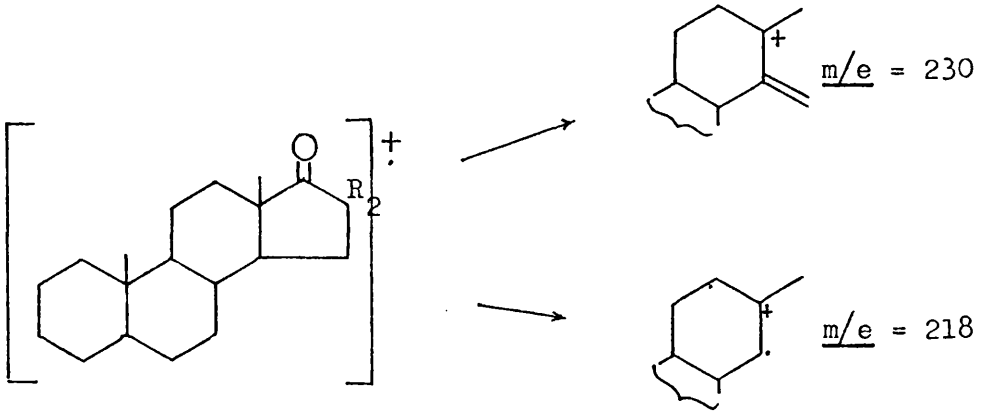
12-Oxo-steroids

The fragments at m/e = 233 in 5α -cholan-12-one has been shown to be the species below :



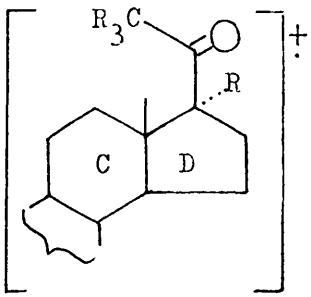
17-Oxo-steroids

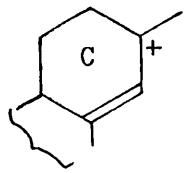
The fragments at m/e = 230 and m/e = 218 in the mass spectrum of 5 α -androstan-17-one are due to the elimination of C17-C15 and C17-C16 respectively and do not give a mass increment after deuteriation, although a mass increment of 2 a.m.u. is apparent in the molecular ion, i.e.



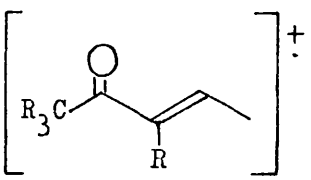
20-Oxo-steroids

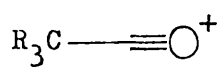
The fragments at m/e = 217, m/e = 43 and m/e = 84 in the mass spectrum of 5α-pregnan-20-one have been shown to be the species below





m/e = 230





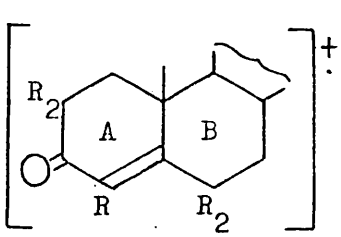
m/e = 43 (R=H)
m/e = 46 (R=D)

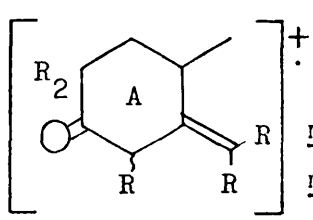
m/e = 84 (R=H)
m/e = 88 (R=D)

(B) Conjugated Steroids

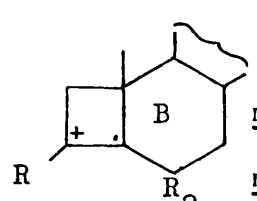
Δ^4-3-Oxo-steroids

The fragments at m/e = 342 and m/e = 124 in the mass spectrum of cholest-4-en-3-one result respectively from loss of ketene and fission of the C9-10 and C6-7 bonds, i.e.





m/e = 124 (R=H)
m/e = 129 (R=D)

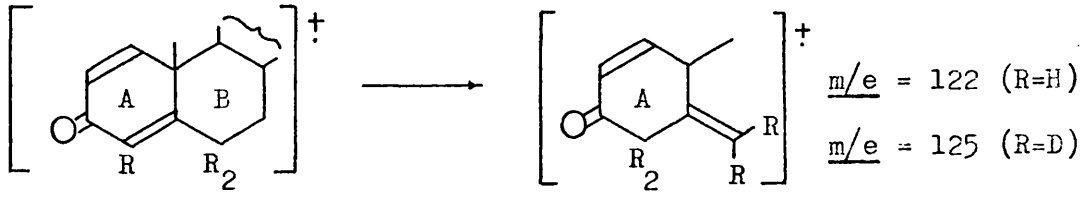


m/e = 342 (R=H)
m/e = 345 (R=D)

The two hydrogen migrations to the charged fragment which are necessary to form the fragment at m/e = 124 have been shown to originate from C-8 and C-11.

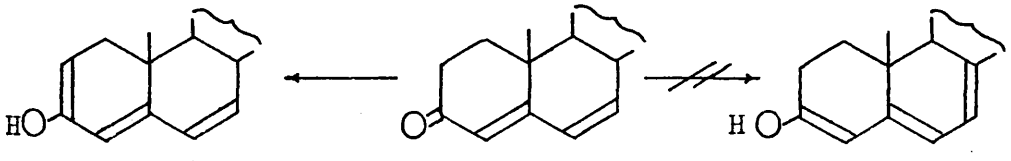
Δ^{1-4}-3-oxo-steroids

The fragment at m/e = 122 in the mass spectrum of cholesta-1, 4-diene-3-one has a structure analogous to the ion at m/e = 124 from the mass spectrum of the Δ4-3-oxo-steroid. The mass shift upon deuteriation, however, is only 3 a.m.u. since the hydrogen at C-2 is not enolic.

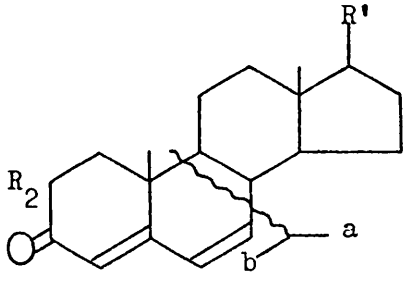


$\Delta^{4,6}$-3-oxo-steroids

The incorporation of 5 deuterium atoms with a yield of only 7% compared with a 70% incorporation of two deuterium atoms suggests that enolisation by removal of the C-8 proton does not occur readily.



The fragments at m/e = 136 and m/e = 247 in the mass spectrum of cholesta-4, 6-dien-3-one are analogous to the ions at m/e = 136 and m/e = 151 in the mass spectrum of dehydrotestosterone reported by Spiteller-Friedmann et al [133]. Although the nature of these ions was not discussed they are likely to originate as shown below :



$C_9H_{10}OR_2$ m/e = 136 (R=H)
m/e = 138 (R=D)

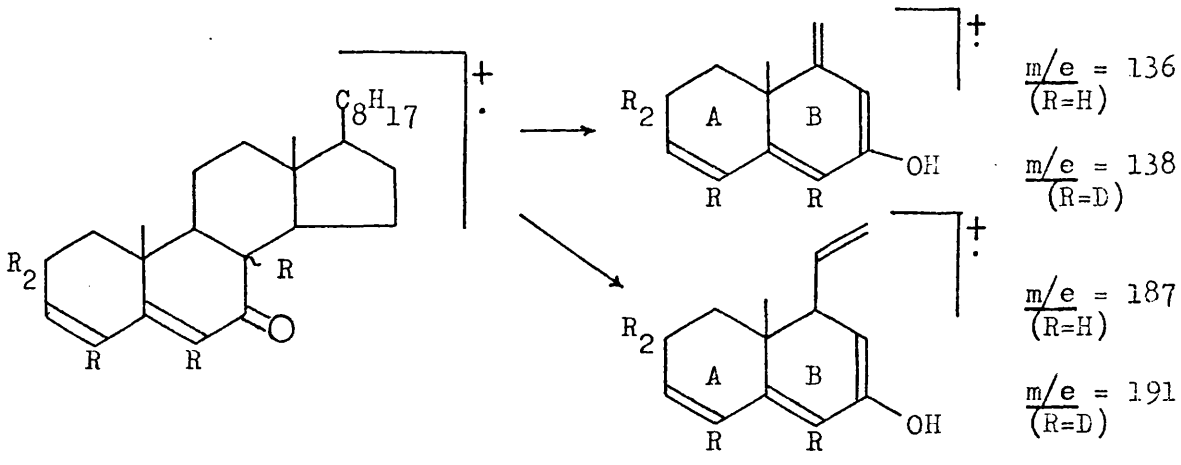
$C_{10}H_{14}R'$ m/e = 151 (R'=OH)
m/e = 247 (R' = C_8H_{17})

Equivalent ions at m/e = 136, m/e = 149 and at m/e = 137, m/e = 149 were reported by Orr and Broughton [162] for the mass spectrum of androst-4, 6-dien-3, 17-dione and its 7-d analogue respectively.

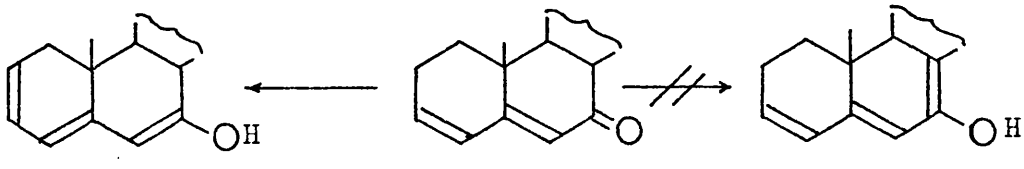
$\Delta^{3,5}$-7-oxo-steroids

The fragments at m/e = 174 and m/e = 187 in the mass spectrum of cholesta-3, 5-dien-7-one are both four mass units less than the respective ions at m/e = 178 and m/e = 191 in the saturated

7-oxo-steroid and are thought to have the following structures :



The incorporation of 5 deuterium atoms with a yield of only 3% (d5) after deuterium exchange chromatography as compared with 29% of the d4 analogue suggests that one hydrogen at an enolic site does not exchange readily with deuterium under the method used. The hydrogen most likely to resist exchange would be at C-8 assuming enolisation takes place preferentially by removal of C-2 proton, i.e.



Although prominent for monofunctional steroids exemplified above, ions characteristic of such functional groups may be considerably less significant for the mass spectra of polyfunctional steroids. Mass shifts after deuteriation, however, will considerably help the recognition of these ions when they occur at low relative abundance.

The fragment ions which contain ring D such as the ions at m/e = 192 and m/e = 205 in 5α-pregnan-11-one will of course occur at different mass values for 11-oxo-steroids with different alkyl substituents at C-17. This is generally true for fragments bearing any substituent which does not modify the skeletal fragmentation of the steroid, e.g. 3β-acetoxy-5α-cholestan-7-one contains a fragment at m/e = 236 which is analogous to that at m/e = 178 in the steroid unsubstituted at C-3.

19-nor-4-en-3-oxo-steroids may give a fragment at m/e = 110 which is 14 mass units lower than the analogous fragment for normal Δ^4-3-oxo-steroids. The 19-nor-4-en-3-oxo-group is able to exchange six hydrogen atoms with deuterium as manifested in the molecular weight increment and a mass shift of 6 a.m.u. of the peak originally at m/e = 110, i.e.

TABLE 4.5

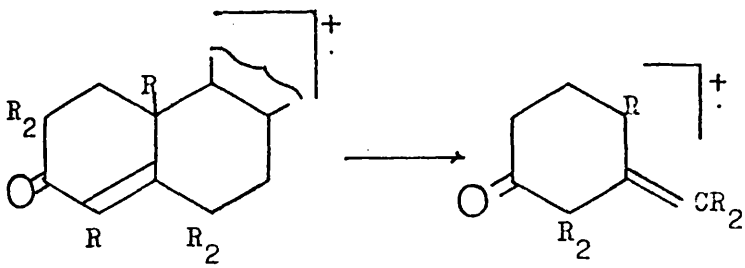
GLC conditions and deuterium incorporation for some steroids and steroid derivatives

Compounds	Column temp[a] (°C)	Maximum No. of deuterium atoms incorporated	Efficiency[b] (%)
Lynestrenol	210	2[c]	83
Lynestrenol TMS ether	210	1	89
Norethisterone	240	8[c]	80
Norethisterone TMS ether	240	7	76
Norethisterone MO	240	2[c]	90
Norethisterone MO TMS ether	240	1	100
11-Oxoandrosterone	240	6	69
11-Oxoandrosterone TMS ether	230	5	83
11-Oxoandrosterone MO	235	4	63
11-Oxoandrosterone MO TMS ether	230	3	18
Testosterone MO TMS ether	240	0	-
5α-Androstan-17-one MO	220	0	-
5α-Pregnan-20-one MO	240	0	-
20β,21-Dihydroxypregn-4-en-3-one t-butylboronate	250	5	85

a retention times were between 5 and 20 minutes for the temperature cited

b percentage of fully deuteriated molecules

c products resulting from the elimination of acetylene



m/e = 110 (R=H)

m/e = 116 (R=D)

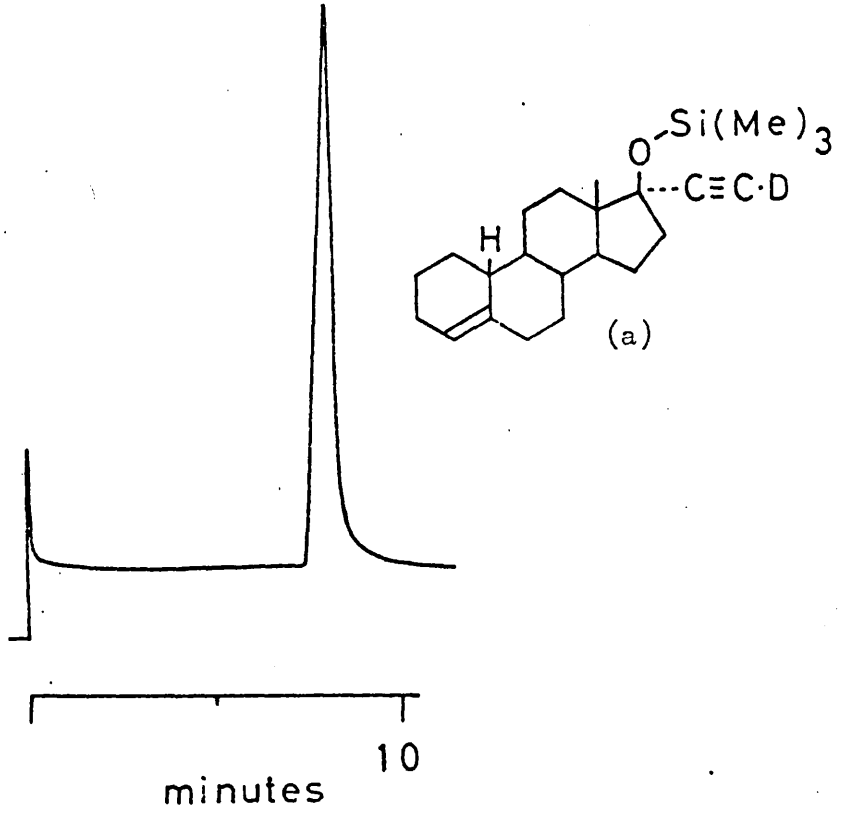
Deuterium Exchange with Polyfunctional Steroids and Steroid Derivatives

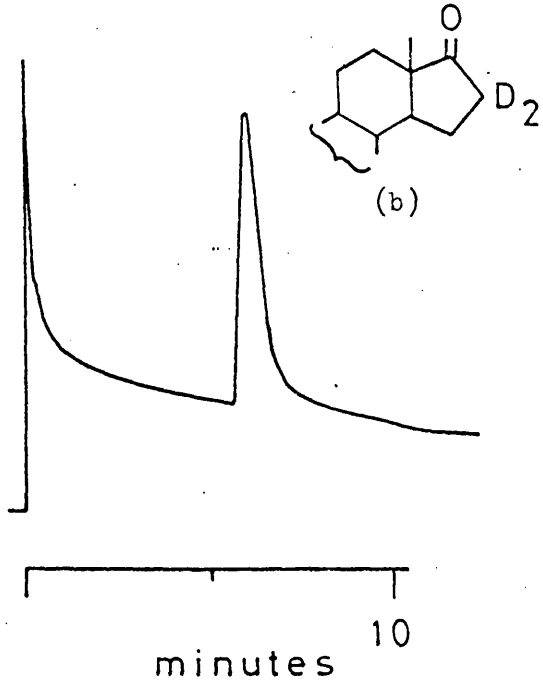
Many steroids and steroid derivatives are stable to deuterium exchange chromatography with basic columns. Certain other steroids are unstable but can still be recognised by their reaction products. Steroids and their derivatives which have been studied by this technique are listed in Table 4.5 together with GLC conditions and the extent of deuterium incorporation.

Lynestrenol is representative of a family of steroid drugs possessing a 17α -ethynyl-17 β-hydroxy group. Although stable to GLC on conventional columns, this type of molecule readily eliminates acetylene on basic columns

FIG. 4.4

GLC of Lynestrenol on 1% Apiezon-L, 0.5% $Ba(OD)_2$ at 220° : (a) as the TMS derivative; (b) as the free steroid





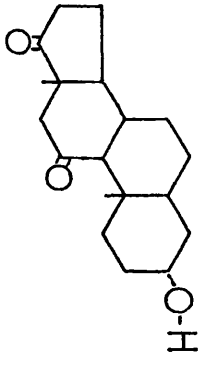
to give the ketonic moiety at C-17 which is easily recognisable by the incorporation of two atoms of deuterium (Fig. 4.4b). These observations conform with results obtained by classical 'wet' methods.[164, 165, 189] The 17α-ethynyl-17 ß-trimethylsilyl ether group, however, remains intact under these conditions, and one deuterium atom is incorporated due to exchange with acetylenic hydrogen (Fig. 4.4a). Norethisterone, which is the 3-keto analogue of lynestrenol, undergoes similar decomposition in the free form to give $\underline{d}_8$-estr-4-ene-3,17-dione which incorporates deuterium at the six enolic positions in rings A and B, and at C-16. The trimethylsilyl ether remains intact to give the $\underline{d}_7$ derivative. The presence of the 17α-ethynyl, 17ß-OTMS group is also inferred by fragment ions retaining the ethynyl group (see part 2) which show an increase of one mass unit after deuteriation (see Fig. 2.10(b)).

O-Methyloximes have been found to prevent enolisation and act as effective blocking groups for deuterium exchange with ketones at C-3 (as in the 17-trimethylsilyl ether 3-O-methyloximes of testosterone and 5α-dihydrotestosterone), at C-17 (as in 5α-

androstan-17-one 0-methyloxime), and at C-20 (as in 5α-pregnan-20-one 0-methyloxime). By sequential derivative formation from a polyfunctional steroid, it is therefore possible to demonstrate the presence of various functions in the molecule by molecular weight increments after deuterium exchange. This is illustrated in Table 4.6 for 11-oxoandrosterone which incorporates six deuterium atoms as the free steroid.

Table 4.6

Incorporation of deuterium into 11-oxo-androsterone and some of its derivatives as determined by mass spectrometry



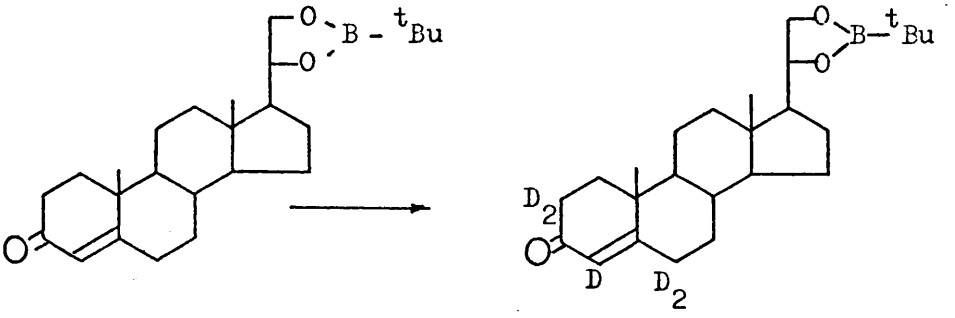
Derivative	M^+	% Base	dn	Efficiency[a] (%)
Free	304	100	6	69
TMS	376	47	5	83
17-MO	333	7	4	63
TMS,17-MO	405	15	3	18

a See footnote "a" of Table 4.4

Trimethylsilylation of the 3α-hydroxyl group selectively blocks one site of exchange, whereas the trimethylsilyl ether 17-O-methyloxime still exchanges three deuterium atoms due

to the carbonyl group at C-11 which does not form a methyloxime under the conditions employed.

Alkyl boronates have been reported as suitable derivatives of 1,2- and 1,3-diols [68] and have been recently used for the GLC of corticosteroids. [67] Boronates of steroids containing the 17α,21-dihydroxy-20-oxo side chain were found to be too labile for chromatography on basic columns. It has, however, been found possible to chromatograph certain boronates with more stable structures on columns containing barium hydroxide; thus deuteriation of 20β,21-dihydroxypregn-4-en-3-one t-butylboronate occurred without decomposition provided that sufficient time had been allowed for the column to equilibrate after deuterium oxide saturation. i.e.



Deuterium Exchange with terpenoids

The technique has been applied with varying degrees of success to several mono- and sesquinterpenoids and derivatives thereof as illustrated in Table 4.7 and Fig. 4.2

TABLE 4.7

GLC conditions and deuterium incorporation for some terpenoids and terpenoid derivatives

Compound	Column temp.[a] (°C)	Maximum No. of deuterium incorporated	Efficiency[b] (%)
Monoterpenoids			
Pulegone (cf. II Fig. 4.2)	110	8	48
Piperitone (cf. I)	110	7	8
Piperitenone (cf. III)	110	12	2
Sesquiterpenoids			
Desisopropylidine-isopetasol TMS (cf. IV)	180	5	83
Isopetasol TMS (cf. V)	190	9	70
Isopetasone (cf. VI)	190	12	70
Ketol TMS (cf. VII)	140	3	52
Alcohol TMS (cf. VIII)	140	0	-

a, b See footnotes for Table 4.4

The extent of deuterium exchange as measured by the molecular ion is dependent on structure, retention time and

column temperature. The monoterpenoids piperitone and piperitenone gave very incomplete deuterium incorporation at 110°. Consistently better results were observed for the sesquiterpenoids, a notable example being isopetasone, which possesses twelve enolisable hydrogens and which gave 70% d_{12}-isomer after exchange chromatography at 170°.

In collaboration with A. G. Mackintosh the method has been applied to a sesquiterpenoid isolated from the E. African timber Muhuhu, derived from Brachylaena hutchinsii. The incorporation of three deuterium atoms into the trimethylsilyl ether (VII) was in conformity with the proposed location of the ketonic group. The reduced compound (VIII) did not incorporate deuterium.

O-SUBSTITUTED OXIME DERIVATIVES

The mass spectral data of 5α-cholestan-2-one and 5α cholestan-3-one are insufficient to distinguish between these isomers, and deuterium exchange on base treated columns affords a molecular weight increment of four mass units for both isomers. Methyloxime (MO) and ethyloxime (EO)

derivatives may be quantitatively formed from these steroids by their respective reactions with methoxylamine hydrochloride and ethoxylamine hydrochloride in pyridine. The trimethylsilyloxime (TMSO) derivative may be formed by reacting the steroid ketone with hydroxylamine hydrochloride in pyridine and silylating the resulting oxime derivative with a reagent such as BSA. A study was made of the GC and MS properties of these derivatives of 5 α-cholestanones ketonic at C-1, C-2 and C-3 to determine their suitability for identifying these functional groups.

Gas Chromatography

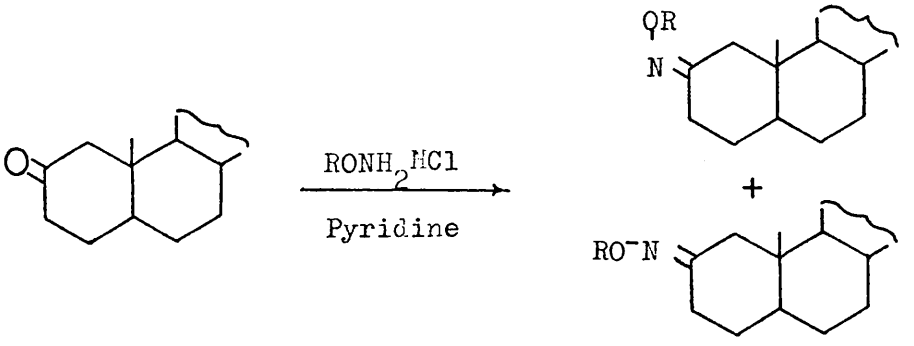
The retention data for MO, EO and TMSO derivatives of 1-oxo. 2-oxo and 3-oxo-5α-cholestane on 1% OV-1 is given below in Table 4.8

TABLE 4.8

Retention data (methylene units) for O-substituted oximes of some isomeric 5α-cholestanones

Parent Steroid	MU (1% OV-1)		
	MO (240°C)	EO (240°C)	TMSO (240°C)
5α-cholestan-1-one	29.73	30.02	30.10
5α-cholestan-2-one	30.44 30.69	31.02 31.43	31.11 31.81
5α-cholestan-3-one	31.02	31.75	32.02 32.15

The twin peaks given by the MO, EO and TMSO derivatives of 5α -cholestan-2-one were all shown by mass spectrometry to be O-substituted oximes, and are undoubtedly syn and anti isomers of this derivative.



It is evident from Table 4.8 that the degree of separation of syn and anti isomers for the 2-oxosteroid derivatives increases as TMSO > EO > MO and appears to be related to the size of the group R in the formula above. In a study of steroids with carbonyl groups at C-3, C-11, C-16 and C-20, Horning and others [166] found that 5α-3-oxo-, Δ^4-3-oxo, and 16-oxo-groups formed isomeric syn and anti MO derivatives and that these isomers could be separated by GLC on OV-17 and by TLC. Only the 5α-3-oxosteroid derivatives however were reported to give two peaks during GLC on OV-1 columns and the separation

of syn and anti isomers (e. g. 0.08 MU for 5α-cholestan-3-one methyloximes) was too small for good resolution on many packed columns. The separation of syn and anti isomers of methyl oximes on methyl silicone phases is not common but was also observed for A-nor-testosterone MO (Part 2) where the oxime group shares a similar proximity to the angular methyl group at C-10 on the steroid skeleton. MO and EO derivatives of the 3-ketosteroids gave only one peak on OV-1, although these peaks were considerably broader than normal, but the TMSO derivative enabled partial separation of syn and anti isomers.

Mass Spectrometry

A study of the fragmentation of some oximes under electron impact has been made (by Goldsmith and others [70]) which included 2-ketosteroid oximes and 3-ketosteroid oximes. Although in general this derivative did not appear to possess better fragmentation directing properties than the equivalent ketone, a fragment at m/e = 112 in 5α-cholestan-3-one oxime and fragments at m/e = 124 and m/e = 138 in 5α-cholestan-2-one oxime enabled distinction between these two steroids by mass spectrometry. They were shown by deuterium labelling studies and accurate mass measurement to have the likely structures below :

FIG. 4.5

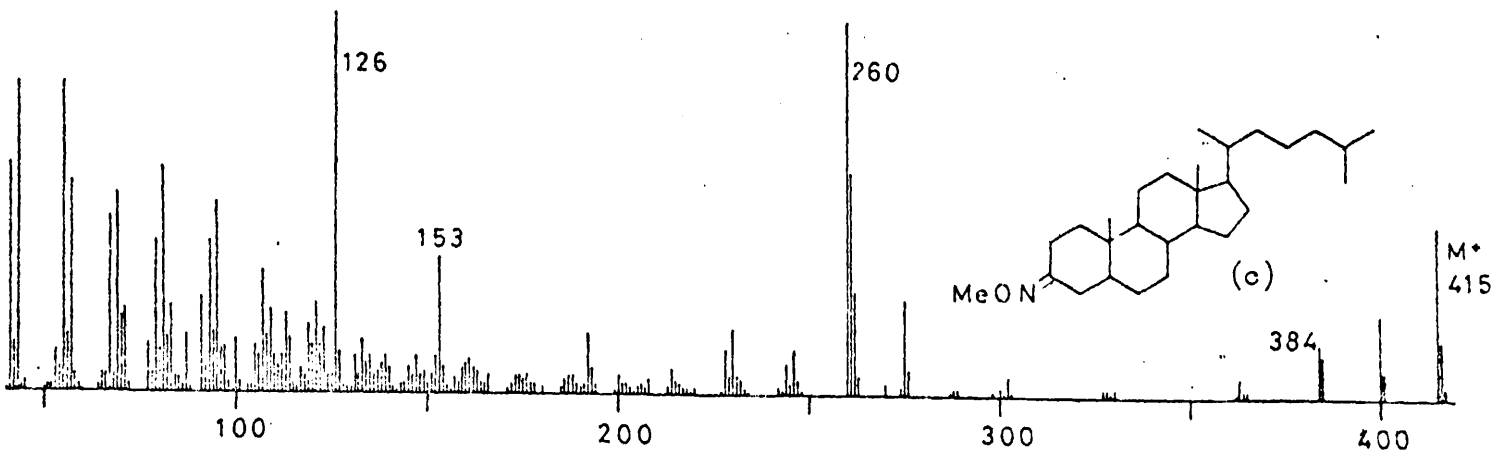
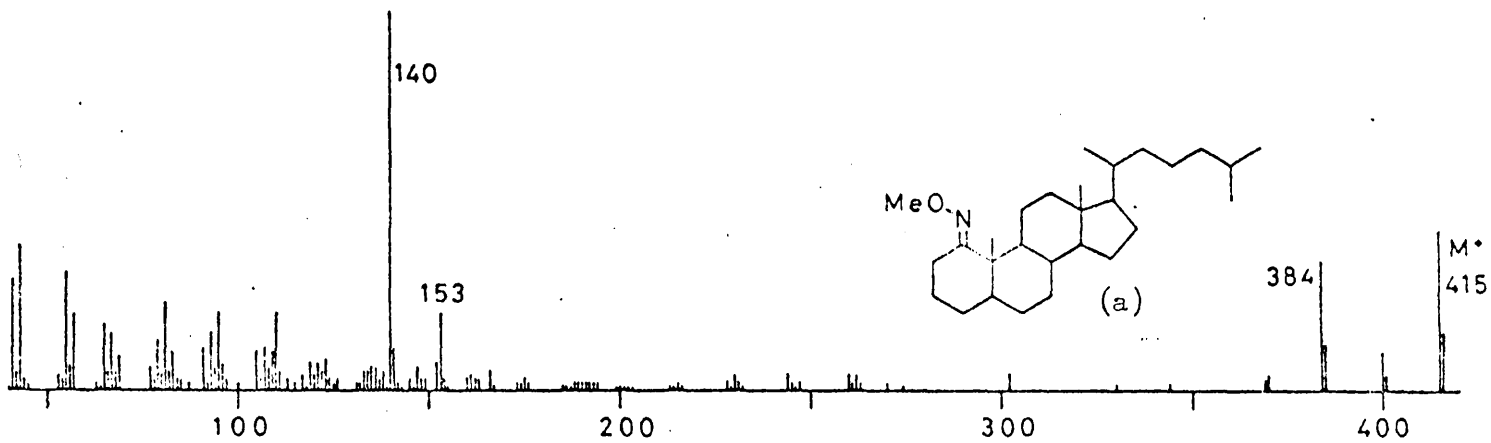
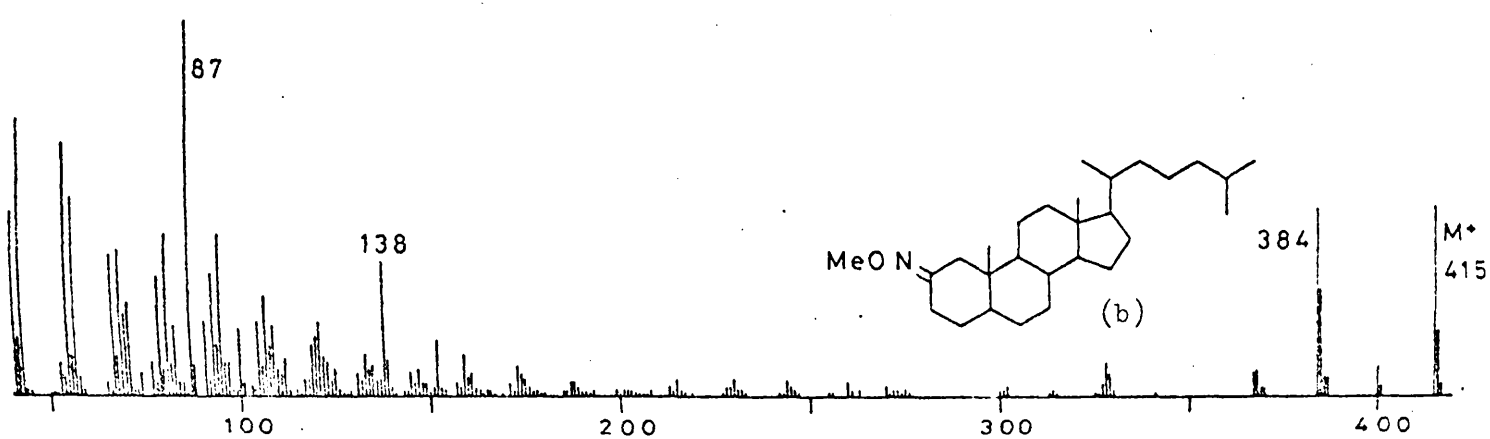
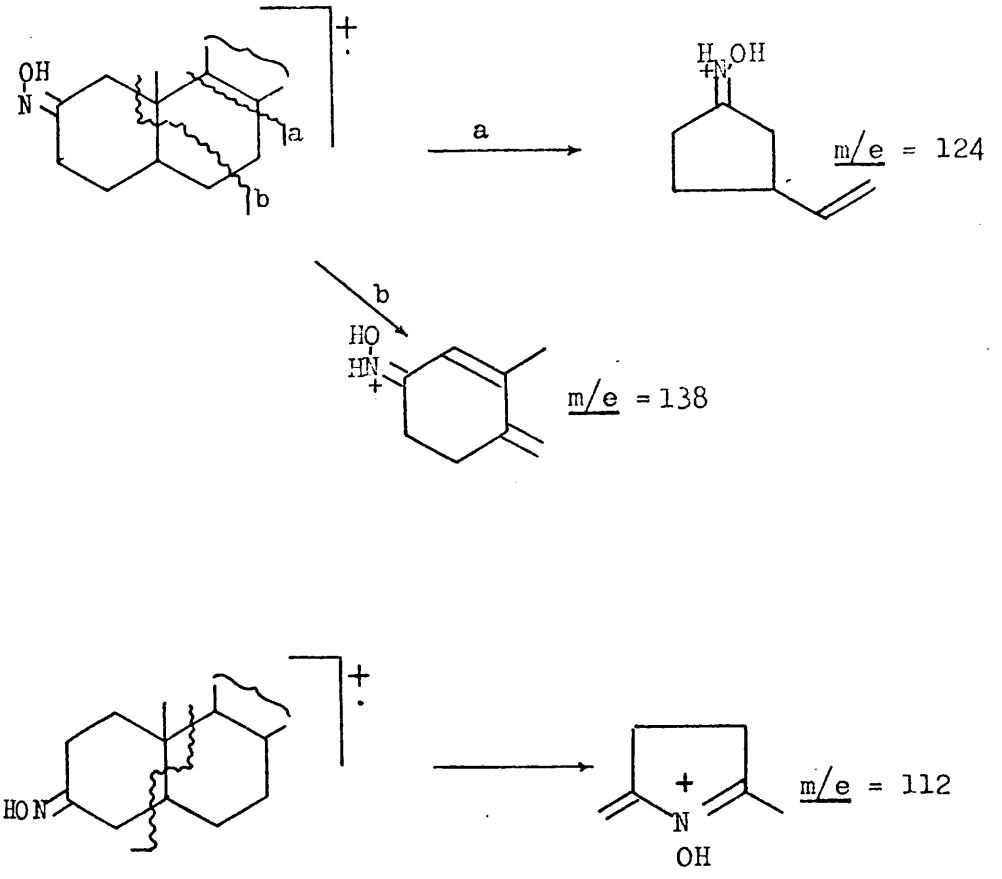


FIG. 4.5

Mass spectra at 70 eV of cholestanone methyloximes : (a) 5α - cholestan-1-one MO; (b) 5α - cholestan-2-one MO; (c) 5α - cholestan-3-one MO.







These fragments, however, were of low abundance and of no great significance compared with neighbouring hydrocarbon ions.

In order to examine further the usefulness of this type of derivative, O-methyl, O-ethyl and O-trimethylsilyl oximes of 5 α-cholestanone steroids with ketone groups at C-1, C-2 and C-3 were examined by mass spectrometry. The mass spectra for the three O-methyloximes are shown in Fig. 4. 5 and some

TABLE 4.9

Characteristic fragments in the 70 eV mass spectra of three 5α-cholestanone oximes and analogous O-substituted oximes

Parent Ketone	O-substitutent	M^+	(%)	m/e	(%)	m/e	(%)	m/e	(%)	m/e	(%)	[C]/[A]
				A		B		C				
5 α-Cholestan-1-one	Me	415	(42)	140	(100)	153	(20)	43	(38)			0.38
	Et	429	(40)	154	(100)	167	(15)	43	(47)			0.47
	TMS	473	(82)	198	(100)	211	(13)	43	(56)			0.56
				D		E		F		C		[C]/[D]
5α-Cholestan-2-one	H	401	(92)[a]	73	(13)[a]	124	(28)[a]	138	(18)[a]	43	(100)[a]	7.69
	Me	415	(52)	87	(100)	138	(37)	152	(16)	43	(74)	0.74
	Et	429	(30)	101	(100)	152	(26)	166	(13)	43	(69)	0.69
	TMS	473	(48)	145	(100)	196	(12)	210	(9)	43	(38)	0.38
				G		H		I		C		[C]/[G]
5α-Cholestan-3-one	H	401	(84)[a]	112	(37)[a]	139	(37)[a]	246	(100)[a]	43	(46)[a]	1.27
	Me	415	(45)	126	(100)	153	(36)	260	(98)	43	(81)	0.81
	Et	429	(93)	140	(100)	167	(27)	274	(84)	43	(75)	0.75
	TMS	473	(100)	184	(10)	211	(4)	318	(17)	43	(32)	3.20

a from Goldsmith et al ref. 64

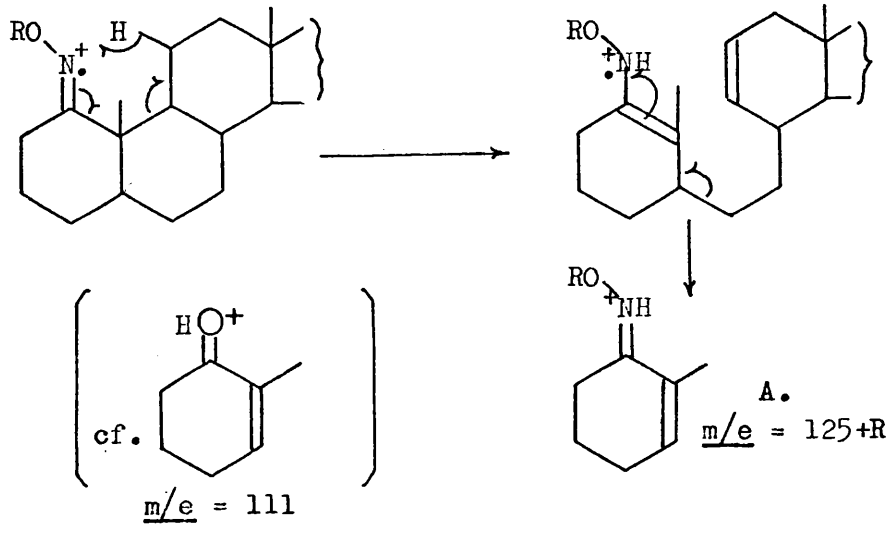
A. B. C..... etc. = classes of fragment ion as discussed in the text.

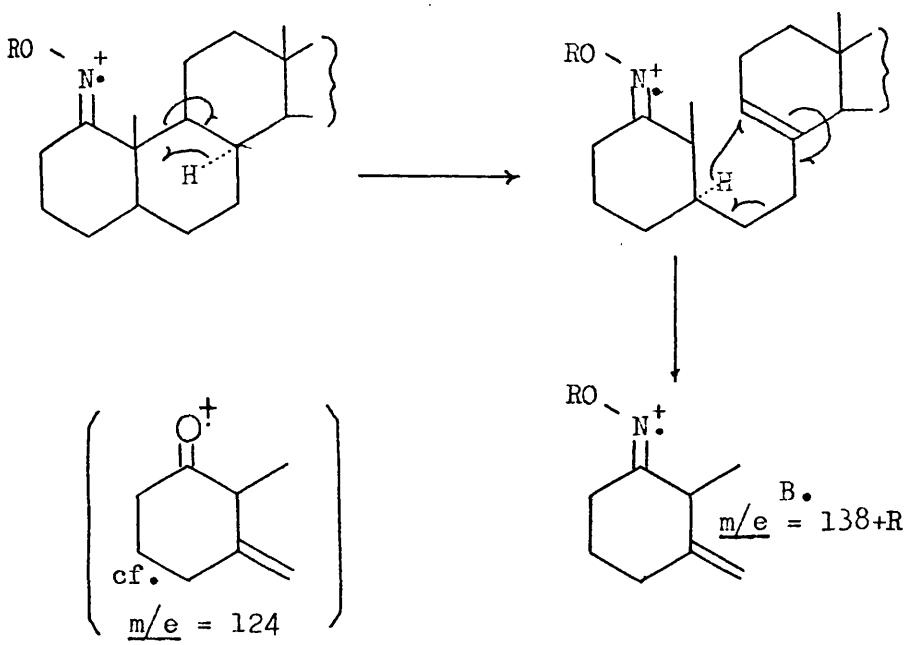
significant fragments shown in Table 4.9 compared with analogous ions resulting from the ethyl and trimethylsilyl oximes.

The fragmentation of oxime type derivatives of the three steroid ketones are now considered separately :

1. Oximes derived from 5α-cholestan-1-one

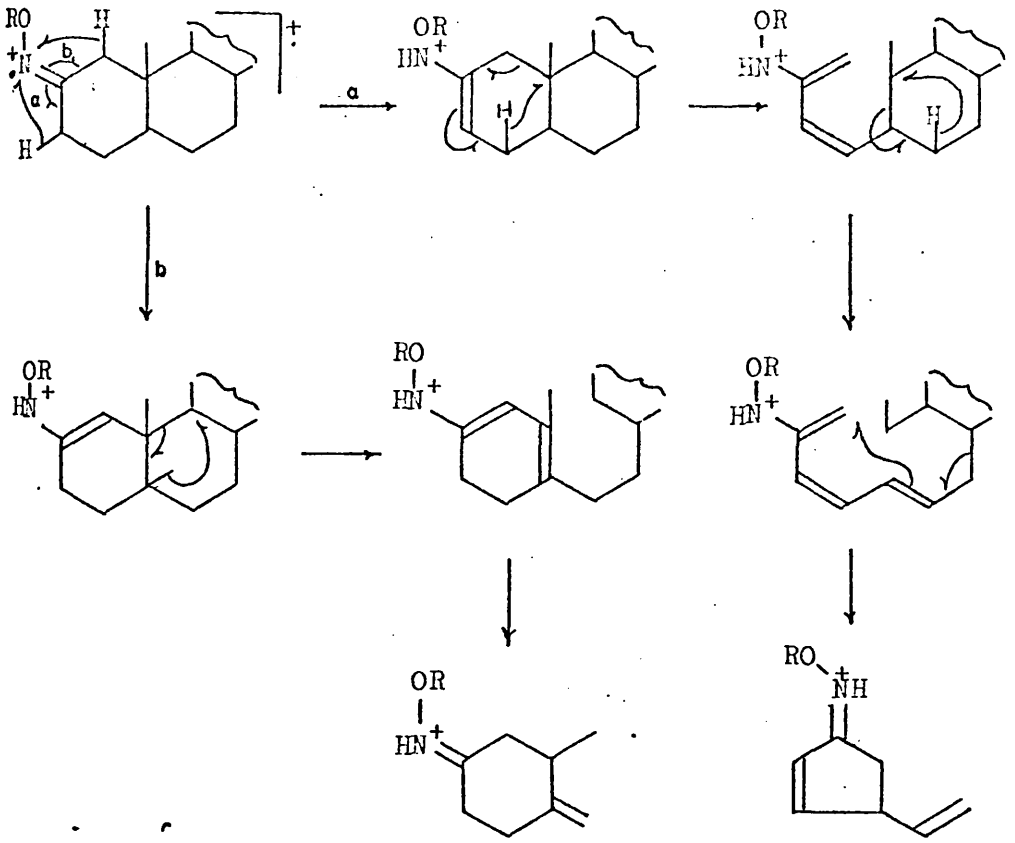
The fragmentation of 5 α-cholestan-1-one oxime was not discussed in Goldsmith's paper but the ions of types A and B appear to contain the substituted oxime group and 7 and 8 carbon atoms respectively. Their structures may convceivably resemble those of the ions at m/e = 111 and m/e = 153 in the mass spectrum of the parent ketone and a suggested mechanism for their formation is given below :





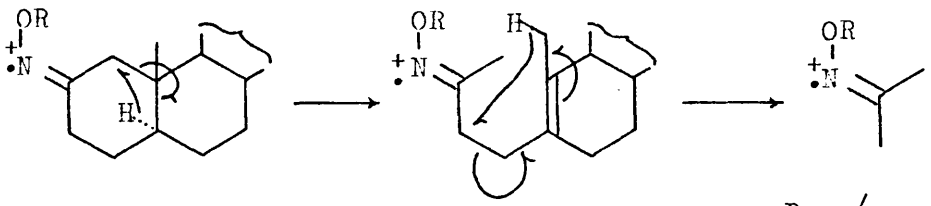
2. Oximes derived from 5α-Cholestan-2-one

The mechanisms of formation of the ions designated E and F in Table 4.9 were discussed by Goldsmith for 5α-cholestan-2-one oxime, and represented below :



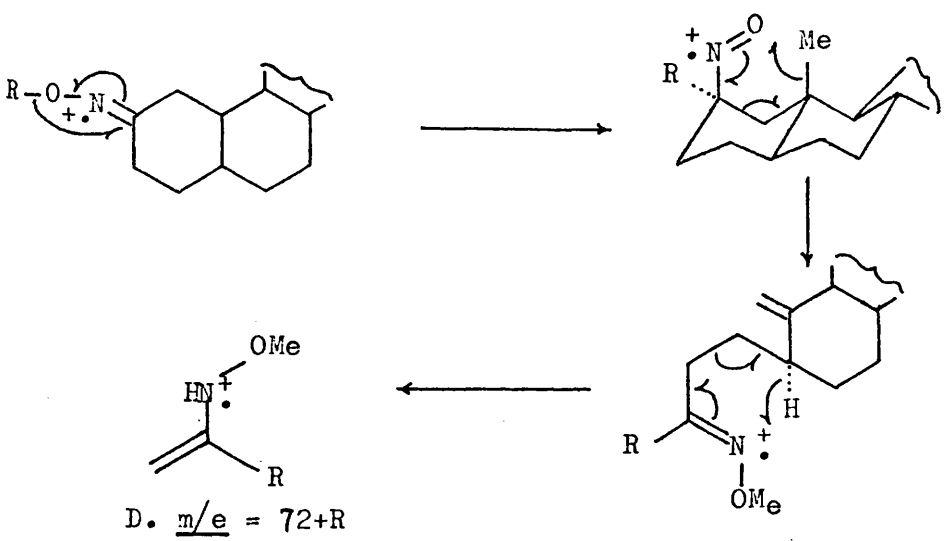
F. m/e = 137+R E. m/e = 123=R

The ions designated D in Table 4.9 represent the base peak for the methyl-, ethyl- and trimethylsilyl- oximes of 5 -cholestan-2-one. An ion at this mass has also been reported in the mass spectra of methyl oxime of some 20-oxo-steroids [190]. This fragment, which is less significant in the oxime mass spectrum, was not discussed in Goldsmith's paper but would appear to comprise the oxime group $+C_3H_6$. An ion of this sort including C_1, C_2 and C_3 of ring A is mechanistically feasible and might involve two hydrogen transfers to the charged species as shown below :



D. m/e = 72+R

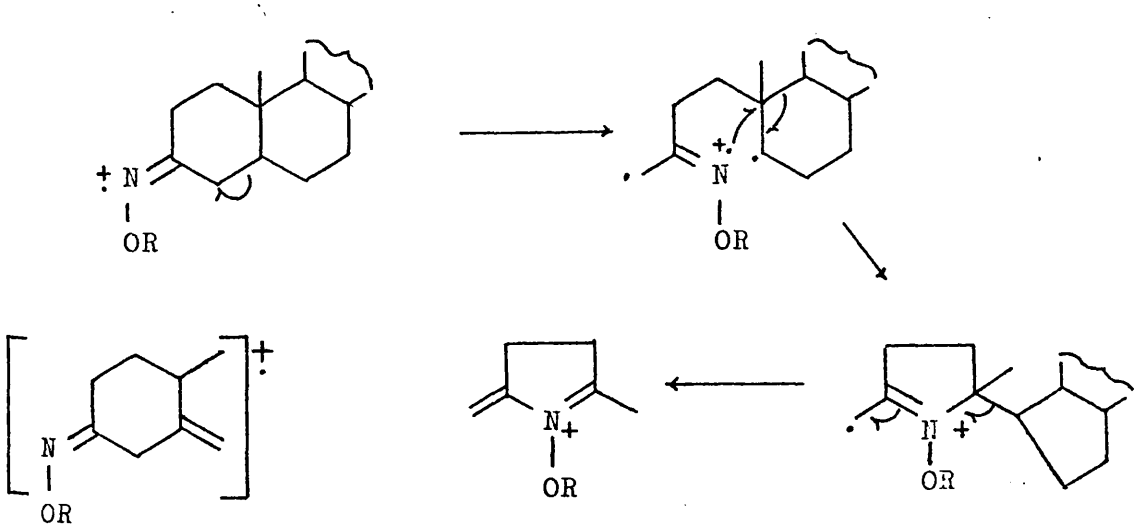
Further labelling studies are necessary to establish the exact nature of this ion. The above explanation is not entirely satisfactory since it does not explain the absence of an analogous ion at m/e = 58 in the mass spectrum of the steroid ketone. An alternative mechanism, preferable in this respect, and similar to one proposed for the formation of the ion at m/e = 73 in the mass spectrum of di-n-propylketoxime [70] involves migration of the angular methyl group :



D. m/e = 72+R

3. Oximes derived from 5α-cholestan-3-one

The ions designated G in Table 4.9 represent the base peak in the mass spectra of the methyl-, ethyl-, and trimethylsilyl-oximes of 5α-cholestan-1-one. This fragment, which is less significant in the mass spectrum of the oxime, has been shown to have the structure below. The ions designated H were not discussed in Goldsmith's paper but most likely originate by a similar mechanism to that producing type B ions in the C-1 ketoximes.



H. m/e = 138+R

G. m/e = 111+R

The ions I are caused by loss of ring D and side chain and are therefore not characteristic only of C-3 ketoximes.

The ion C (= C_3H_7)$^+$ is common to all steroids with the cholestane nucleus and its intensity relative to the most abundant fragment which is characteristic of the functional group (i.e. ions of types A, D and G) may be taken as an inverse measure of the ability of the functional group to direct fragmentation. It is evident from the values in Table 4.9 that methyloximes give a greater abundance of ions D and G than do the oxime derivatives and are better derivatives in this respect for characterising 2-oxo and 3-oxo steroids. Ethyloxime and trimethylsilyloxime derivatives appear to be less predictable in their fragment ion intensities.

SUMMARY AND CONCLUSIONS

Two approaches to the characterisation of oxygen substituents have been examined with particular emphasis on their application to steroid ketones. The first technique of deuterium exchange with gas chromatography - mass spectrometry provides a useful method of carbonyl group location. It is most powerful when used in conjunction with derivatives such as trimethylsilyl ethers (for alcohols) and methyloximes (for ketones) which effectively block enolisation and prevent deuterium exchange. Although this method can only indicate directly the

number of hydroxyl groups on the molecule, information relating to their location can be obtained after oxidation to the aldehyde or ketone.

More specifically the technique can be used in the analysis of steroids to verify the presence of the following groups :

1. Conjugated 3-ketones

Conjugated 3-ketones usually show relatively large deuterium incorporation. Natural Δ^4-3-oxo-steroids possessing a 10ß-methyl group are characterised by the exchange of five deuterium atoms which is indicated by a contribution to the molecular weight increment and by a shift in the ring A fragment ion (where this is sufficiently abundant to be recognisable) from m/e = 124 to m/e = 129. The corresponding 19-norsteroids contribute a molecular weight increment of 6 and can sometimes be further identified by a fragment shift from m/e = 110 to m/e = 116.

2. 17ß-hydroxy, 17α-ethynylsteroids

Steroid drugs such as norethisterone, which contain the above group, may be characterised as their 17-O-trimethylsilyl ethers by a contribution to the molecular weight increment of one mass unit after deuterium exchange and by shifts of one mass unit in ions containing the ethynyl group (such as m/e = 83, m/e = 125

m/e = 140 and m/e = 153 in the mass spectrum of undeuteriated norethisterone TMS ether). Some of these effects are still prevalent in the norethisterone TMS ether after formation of the 3-methyloxime. Further evidence for the presence of this group is provided by the free steroid or steroid methyloxime which eliminates acetylene on the basic column, producing the 16-d_2-17-ketone analogue.

3. 11-Oxosteroids

The ketonic group at C-11 is readily distinguished by the incorporation of three deuterium atoms at C-9 and C-12 after forming TMS ethers or methyloximes of the remaining oxygen functions. The 11-oxo group, although apparently too sterically hindered to form a methyloxime derivative, will still exchange its three enolic hydrogen with deuterium and, in the absence of other exchangeable functions may be recognised by molecular weight increment only.

For steroid ketones where the carbonyl group does not direct fragmentation, or give characteristic ions in low relative abundance, the O-substituted oxime derivative is often superior in this respect. This has been demonstrated for O-methyl-,

O-ethyl and O-trimethylsilyloximes of 5α-cholestan-2-one and 5 α-cholestan-3-one. These steroids, which are not distinguishable from each other by mass spectrometry in their free form, may be recognised individually as their oxime derivative. Ethyloxime and methyloxime derivatives have been shown to possess fragmentation-directing properties superior to the oxime derivatives for these two steroids. For 1-oxo- and Δ^4-3-oxo-steroids (see part II) where stronger fragmentation properties are associated with the carbonyl group, the corresponding oxime derivative does not appear to fragment very differently from its parent ketone.

STEROID DRUG METABOLISM

Although modified steroids have been used as drugs for more than 20 years it is only within the last decade that much progress has been made towards identifying some of their metabolites in the human. Due partially to reliance on optical spectroscopic and chemical methods of preliminary identification, earlier workers were able to obtain structures only for metabolites present in the relatively large amounts necessary for these techniques. Alternatively, identification was made entirely by comparing chemical and physiochemical properties with those of chemically synthesised materials [126]. The latter method, although implying presupposition of structure, has allowed the identification of metabolites by more sensitive techniques such as TLC [126] and paper chromatography [167].

The identification of steroid drug metabolites involves four basic processes as discussed below :

(A) EXTRACTION FROM THE BIOLOGICAL MEDIUM [127]

This process involves both hydrolysis and extraction since the majority of steroidal material exists as the sulphate or glucosiduronate conjugate. Enzymic hydrolysis enables separation of sulphate and glucosiduronate bound steroid

fractions from each other and from unconjugated material. Separation may also be achieved on a pH basis, and it is convenient at this stage to remove all biological amines and to separate phenolic steroids from neutral steroids.

(B) SEPARATION OF STEROID DRUG METABOLITES

Various techniques have been used for separating the steroid metabolites from each other and from natural steroids. Often it has been necessary to employ more than one technique. Countercurrent distribution has been used recently for the separation of metabolites from 17α-ethynyl-estradiol.[168] Perhaps more common methods however are column chromatography, TLC and GLC. Chromatography on argentous stationary phases such as silvered Florisil[124] or argentous silica gel[169] has proven to be a useful method of separating metabolites containing an ethynyl group. Separation has also been accomplished by virtue of chemical reaction with functional groups, e. g. the use of Girard's reagent for separating ketonic steroids.[124, 127]

(C) DETECTION OF STEROID DRUG METABOLITES

The most common method of detecting steroid drug metabolites is the use of $^{14}_{6}C$ or $^{3}_{1}H$ radio-labelling. This technique has been

used for studying metabolite distribution in the faeces, urine, blood, bile [125,126] etc., and for detecting individual metabolites after chromatography [124,127] or countercurrent distribution. [168] The technique is both sensitive and selective and can be used for quantitative estimation of metabolites. Other methods of detection such as flame ionisation (as in GLC) and charring (as in TLC) are sensitive to microgramme quantities but are not selective to the drug metabolites. In some cases single and multiple ion detection may be a useful means of selectively detecting drug metabolites by GC-MS, [170,171] and the electron capture (EC) detector might be used for metabolites containing halogen.

(D) IDENTIFICATION OF STEROID DRUG METABOLITES

The process of identifying drug metabolites generally involves structure elucidation by chemical and physio-chemical methods followed by comparison of the physio-chemical properties of the metabolite with those of a compound of established structure (e. g. one obtained by chemical modification of the drug). GLC and TLC although useful for the latter process, seldom provide sufficient information within themselves to enable the prediction of structure. The

introduction of GC-MS into drug metabolism study now enables the prediction of structures for metabolites present only in microgramme quantities, thus enabling a more complete study of steroid drug metabolism than was possible with other less sensitive techniques.

EXAMINATION OF THE URINARY METABOLITES OF NILEVAR

The metabolism in humans of the anabolic steroid drug 17 α-ethyl - 17 β-hydroxyestr -4-en-3-one (Nilevar) has been under investigat ion recently. The main metabolite in the neutral urinary steroid fraction had been identified as 17 α -ethyl-5 β-estran-3 α, 17 β-diol by GLC comparison of urine obtained before and after drug administration from the same volunteer, and a standard obtained by chemical reduction of Nilevar. Identification was made by comparing GLC retention times on OV-1 and OV-17 columns of the steroids in their free form and as their TMS derivatives.

At this stage the author assisted in a closer examination of the two urine extracts in the hope of detecting and identifying minor metabolites not obvious from GLC comparison of the (+) and

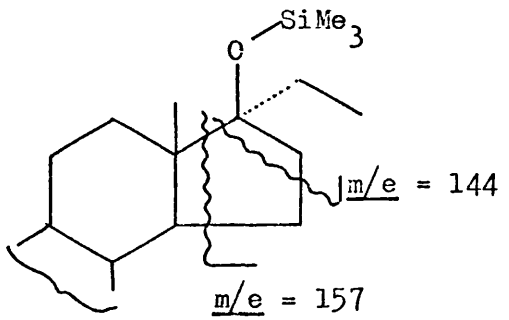
(-)* urine extracts. Two complementary techniques were employed to detect new metabolites :

(a) Single ion monitoring (SIM)

(b) Separation by preparative thin layer chromatography (PTLC)

SIM OF 17-ETHYL-17-TRIMETHYLSILYLOXYSTEROIDS IN URINE EXTRACTS

TMS derivatives of Nilevar and its ring A reduction products all exhibit mass spectra with intense peaks at m/e = 157 and m/e = 144 which result from fragmentations of ring D (Fig. 5.5 and part (II) of this thesis) and which constitute a high proportion of the total ion current. Accordingly, these ions are suitable for the selective monitoring of extremely small amounts of metabolites with the ring D structure below.

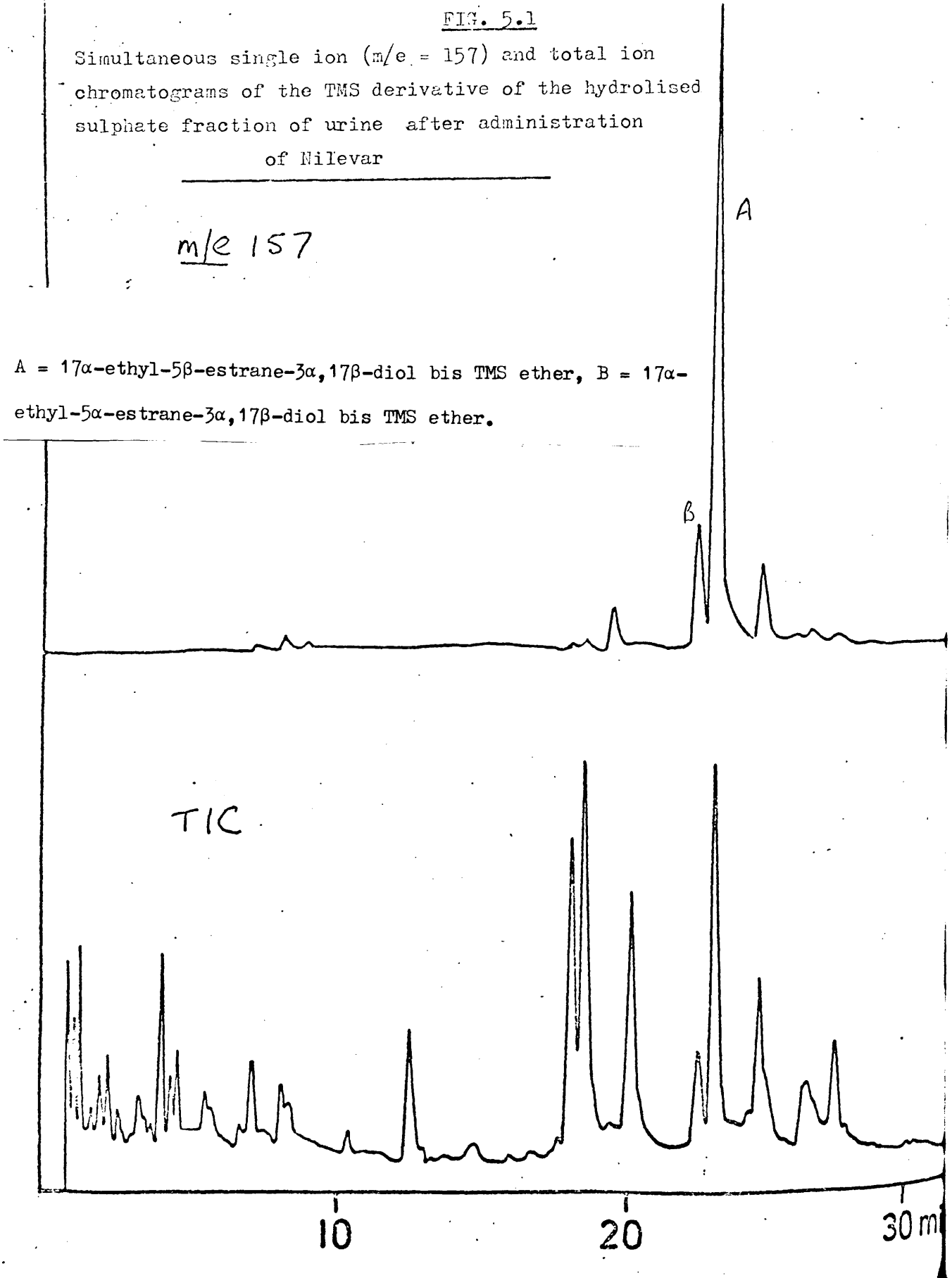


To achieve single ion monitoring an output from the galvanometer amplifier of the mass spectrometer was connected

*The terms "(+)" and (-)" used throughout this section refer to materials obtained after and before ingestion of the drug.

FIG. 5.1

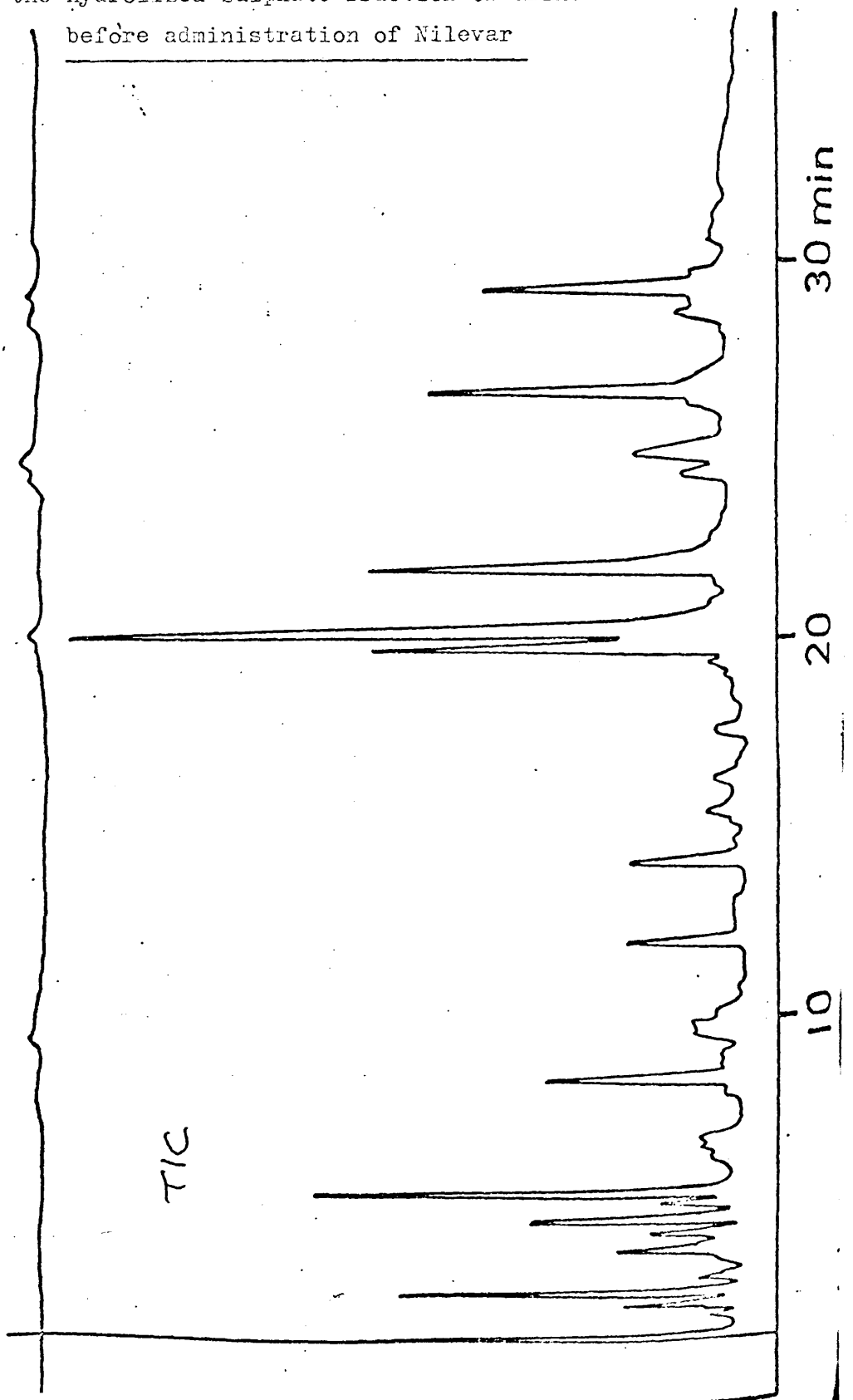
Simultaneous single ion (m/e = 157) and total ion chromatograms of the TMS derivative of the hydrolised sulphate fraction of urine after administration of Nilevar



A = 17α-ethyl-5β-estrane-3α,17β-diol bis TMS ether, B = 17α-ethyl-5α-estrane-3α,17β-diol bis TMS ether.

FIG. 5.2

Simultaneous single ion (m/e = 157) and total ion chromatograms of TMS derivatives of the hydrolised sulphate fraction of urine before administration of Nilevar



to one channel of a dual-pen potentiometric recorder. Into the other channel was fed the output from the total ion current (TIC). Fig. 5.1 and Fig. 5.2 show the resulting chromatograms for the TMS ethers of urinary steroid extracts obtained after (+) and before (-) drug administration respectively with the mass spectrometer focused continuously on m/e = 157. The TIC "chromatogram" resembled a conventional GLC trace produced by a non-selective detector, whereas the SIM trace gave clear indication of four components in the (+) fraction which were possible metabolites. Accordingly, the chromatograms were re-run this time obtaining full mass spectra for peaks suspected of containing a metabolite and at corresponding positions in the chromatogram of the (-) fraction. Retention data for suspected metabolites were also obtained using SIM by co-injecting a suitable series of n-hydrocarbons with the steroid TMS ethers. As saturated n-hydrocarbons do not give MS fragmentation at m/e = 157, these components are only detected by the TIC. By comparison of GC and MS properties with those of chemically synthesised material two of these components were shown to be 17α-ethyl-5α-estrane-3α,17β-diol bis TMS ether and

* P.70 P162

FIG. 5.3

Simultaneous single ion (m/e = 245) and total ion chromatograms of TMS derivative of the hydrolised sulphate fraction of urine after administration of Nilevar

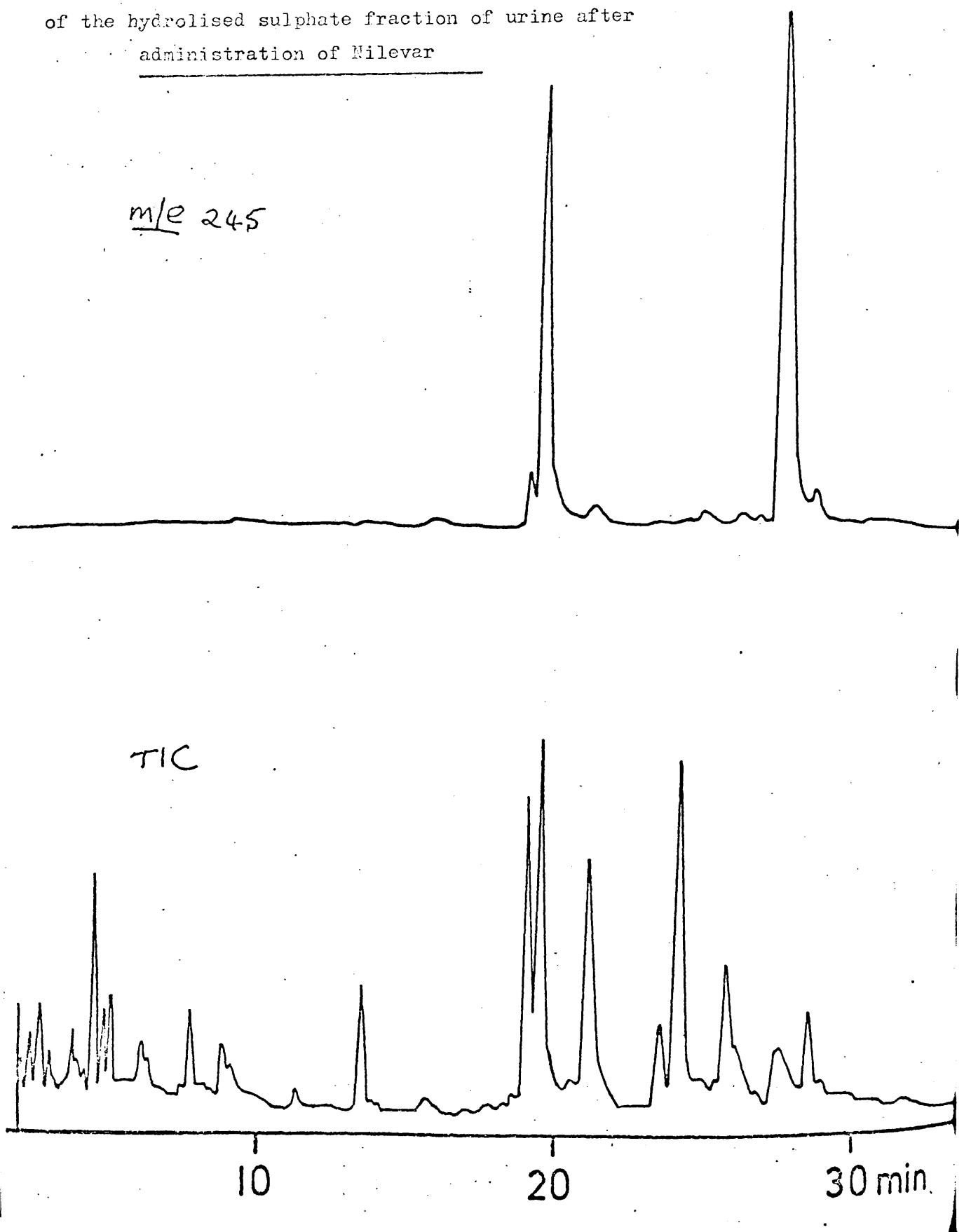
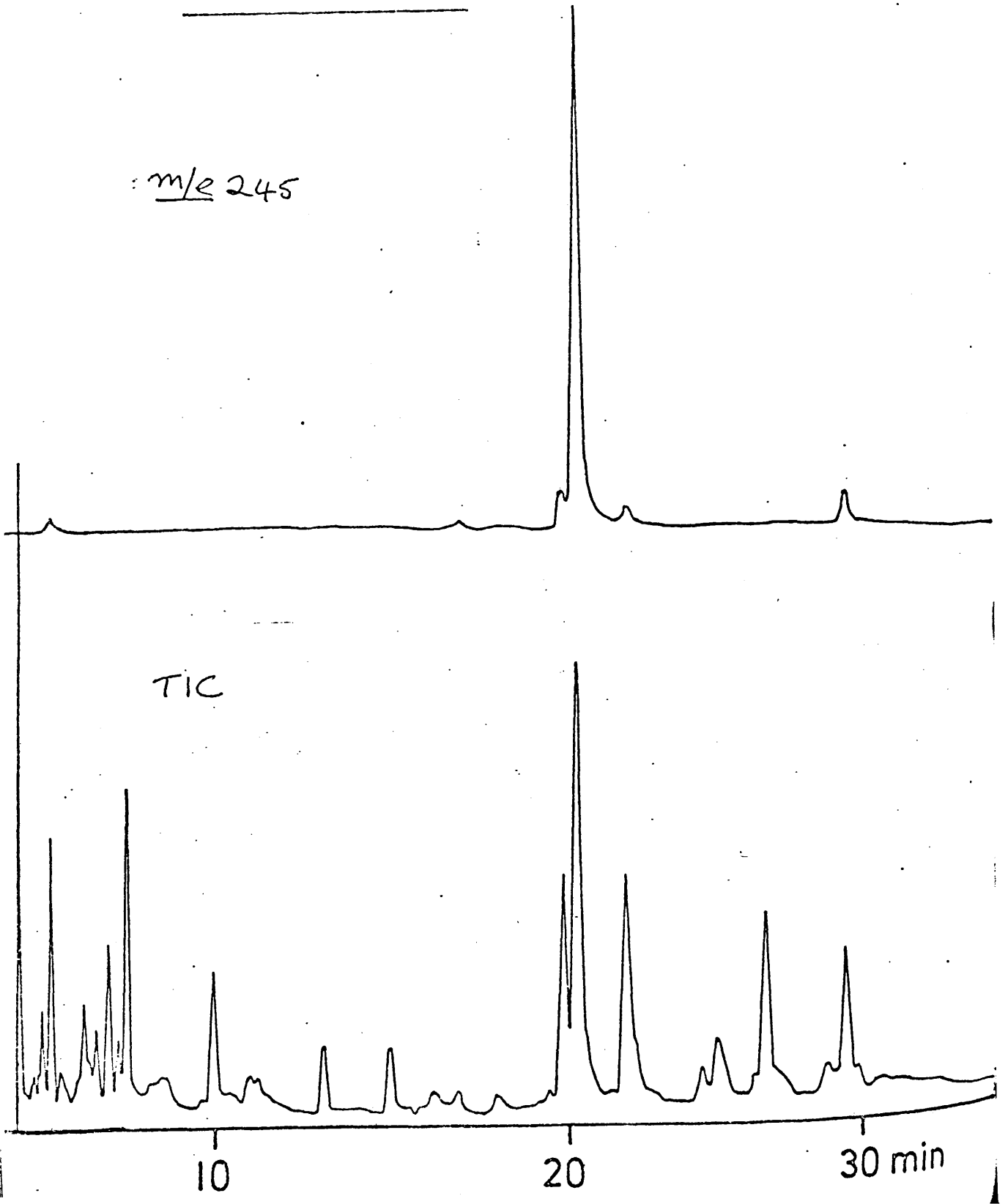


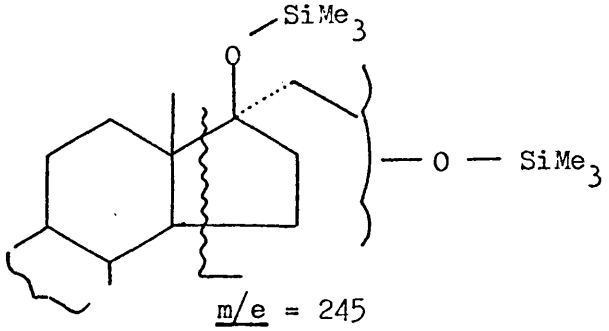
FIG. 5.4

Simultaneous single ion (m/e = 245) and total ion chromatograms of TMS derivatives of the hydrolised sulphate fraction of urine before administration of Nilevar



17α-ethyl-5β-estrane-3α,17β-diol bis TMS ether.

Metabolites of Nilevar which contained an additional hydroxyl group on ring D or on the ethyl side chain would not be expected to fragment as the silyl ether to give an ion at m/e = 157. However, assuming silylation of both groups is complete, an analogous ion at m/e = 245 might be anticipated.



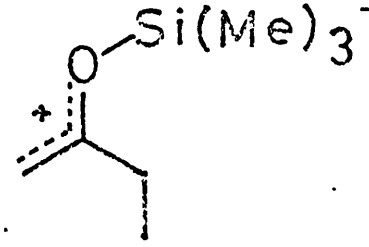
m/e = 245

SIM at m/e = 245 did detect one component in the (+) urine (Fig. 5.3) which appeared to be absent in the (-) urine (Fig. 5.4). The full mass spectrum of this component showed that ions at m/e = 103 (85%) m/e = 155 (62%), m/e = 217 (52%) m/e = 232 (34%), m/e = 245 (100%) and m/e = 421 (68%) were peculiar to the metabolite. These fragments can be rationalised by the presence of an OTMS group at the terminal position of the ethyl side chain, i.e.

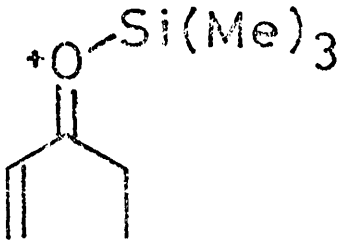
* Synthesised by Mr. A. Thawley by sterioselective reduction of Nilevar.

FIG. 5.5

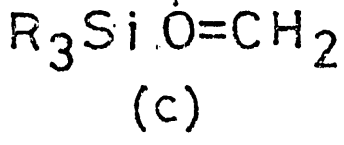
Postulated structures for ions occurring in the mass spectra of TMS and d_9-TMS derivatives of Nilevar metabolites



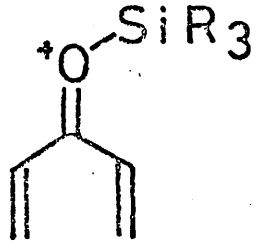
(a)



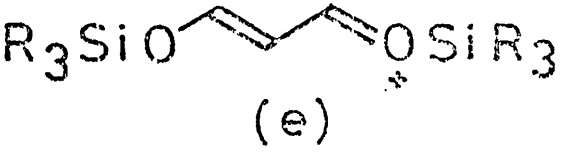
(b)



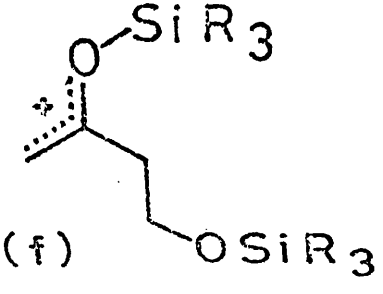
(c)



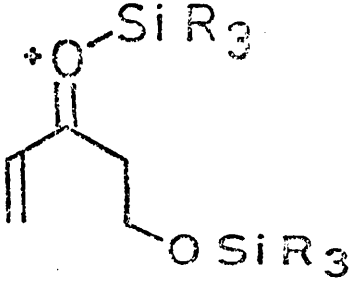
(d)



(e)



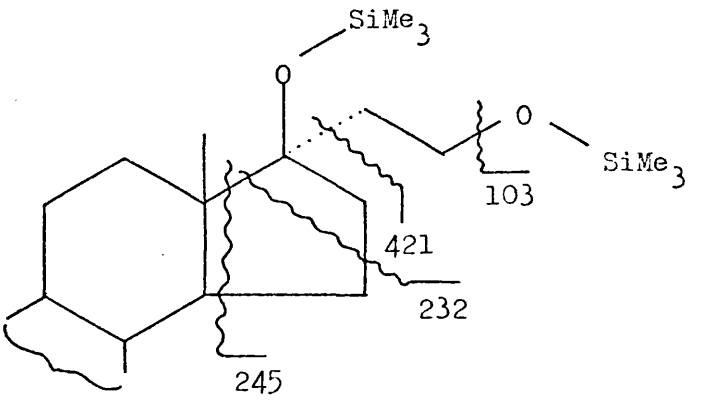
(f)



(g)

R = CH_3

R = CD_3



155 (245-Me_3SiOH)

The ion at m/e = 103 occurs in TMS ethers of primary alcohols and is due to the fragment (Fig. 5.5c R = CH_3.) The ion at m/e = 155 (Fig. 5.5d, R = CH_3) is most likely derived from loss of TMSOH from the ion at m/e = 245 (Fig. 5.5g R = CH_3). The ion at m/e = 217 (Fig. 5.5e, R = CH_3) is a rearrangement ion similar to one reported in the mass spectra of other steroidal 1,3-diol bis TMS ethers.[57] The ion at m/e = 232 (Fig. 5.5f R = CH_3) is due to fission of the C13-17 and C15-16 bonds and is analogous to the ion at m/e = 144 in the TMS derivatives of the tetrahydro reduction products of Nilevar. The proposed structures for these fragments were later substantiated by fragmentation of the d_9 TMS derivative of another metabolite

with similar ring D structure (see Fig. 5.7). The fragment at m/e = 421 is probably due to loss of the side chain bearing the OTMS group which is in accordance with a saturated triol structure. The expected molecular ion at m/e = 538 was too weak to be significant amongst the neighbouring contaminant ions.

SEPARATION OF METABOLITES BY PTLC

Because of the complexity of the steroid gas chromatogram and because of the apparent low relative concentration of the more polar metabolites in the urine extracts it became necessary to achieve some other means of separation prior to GC-MS. Preparative thin layer chromatography is perhaps the most convenient complementary technique for this purpose since it will readily separate the majority of trifunctional steroids from abundant bifunctional steroids such as androsterone and etiocholanolone. Procedures have been recently reported for the almost quantitative extraction of polyfunctional steroids from silica gel [172-174]. For example, a solvent mixture of methylene chloride, methanol 9:1 was reported [173] to extract microgram quantities of cortisol from silica gel with an efficiency of more than 80%.

Development of Technique

(a) Solvent System

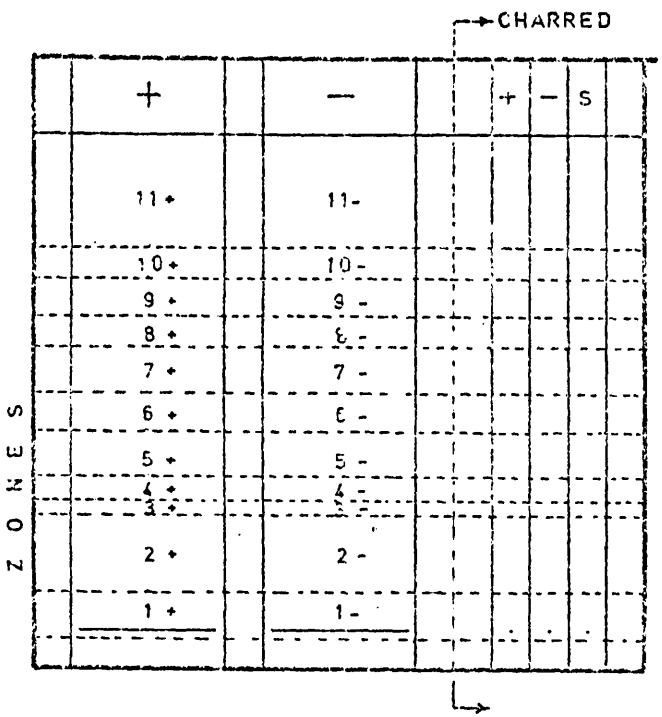
In order to develop a solvent system to effect sufficient separation of bifunctional from polyfunctional steroids a mixture of 5α-pregnane-3β, 21β-diol, 5α-pregnane-3α, 20α, 21β-triol and 5β-cholane-3α, 12α, 24-triol was used as a test mixture. Ethyl acetate was found to be a satisfactory solvent for this purpose giving R_f values of 0.51 (pregnanediol) 0.26 (pregnanetriol) and 0.17 (cholanetriol).

(b) Spot Location

Several non-destructive and destructive methods of spot and band location were tried for the above test mixture after separation using ethyl acetate. The three non-destructive methods (fluorescein spray, iodine vapour and chromatography on UV sensitive Merck GF 254), which were tried did not give satisfactory results with these steroids even for 100 μg quantities. Of the two destructive methods tried (saturated $SbCl_3$ in chloroform and 1% $Ce(SO_4)_2$ in 10% H_2SO_4), the ceric sulphate spray appeared to give the better results and as little as 5 μg of each of these steroids was clearly detected by spraying with this reagent, heating for one minute at 110° and viewing under a 300 nm ultra violet lamp.

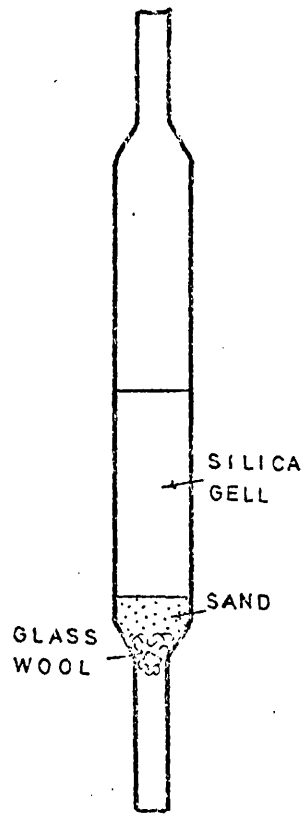
FIG. 5.6

Preparative TLC of Nilevar metabolites from urine :-



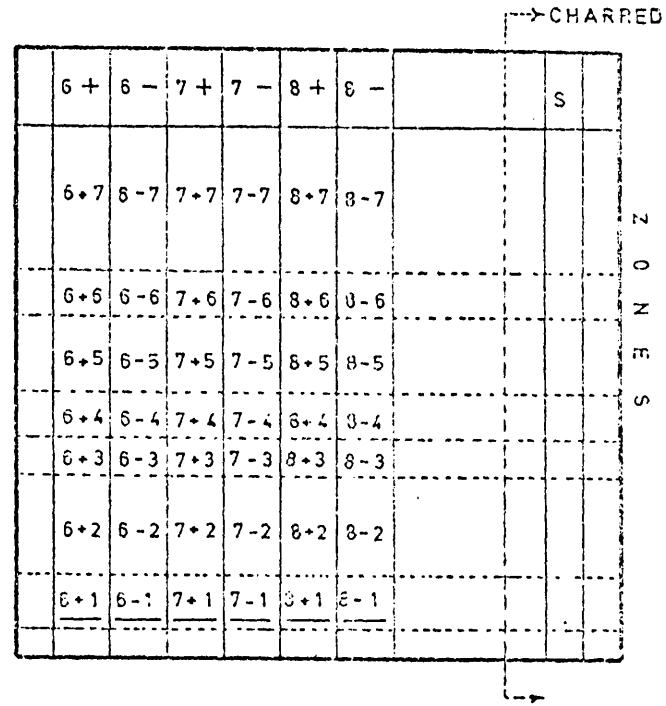
(a)

Illustrating zone separation of the free steriods



(b)

Illustrating method of steroid extraction from silica gel using a drawn out Pasteur pipette



(c)

Illustrating zone separation of the TMS ethers

(c) Sample loading

In order to find a suitable plate loading, the neutral steroid fraction extracted from urine after hydrolysis with hydrochloric acid was applied in varying amounts to 6 cm x 0.5 cm sections of a 0.5 mm thick TLC plate. Components were detected using the ceric sulphate method after the plate had been run using ethyl acetate. No loss in separating efficiency was apparent when using extracts representing up to 20 ml urine per centimeter of plate. Since enzyme hydrolysed urine usually contains less extractable material than the above, an equivalent plate loading should be adequate.

Plate Preparation and Development

A 20 cm x 20 cm plate coated 0.5 mm thick with Merck G silica gel was divided into vertical sections as shown in Fig. 5.6a. In the two 5 cm strips at a distance of 17 cm from the top of the plate were applied extracts from the neutral steroid fraction of 100 ml of "(+)" and "(-)" urine after enzymic hydrolysis. At the right hand side of the plate were applied a series of analytical spots comprising "(+)" urine extract, "(-)" urine extract and a mixture of androsterone and etiocholanolone, the two principal steroids of normal male urine.

After ethyl acetate had been allowed to run up to 1 cm from the top of the plate the preparative bands were covered over and the analytical bands sprayed with ceric sulphate solution. The analytical bands were charred by projecting the appropriate portion of the plate over the edge of the bench and heating the underside with a hot air dryer. A later examination of the PTLC sections by gas chromatography did not give any evidence of sample loss or decomposition using this technique.

Examination of Plate

(a) Visual

There were few visible differences between the "(+)" and "(-)" analytical bands when viewed under UV light. Where differences did occur (see Table 5.1) the appropriate zones of the preparative bands were removed from the plate for further examination by other techniques. The remaining preparative bands were arbitrarily divided into zones (see Fig. 5.6a and Table 5.1) for further examination.

TABLE 5.1

R_f Values of Zones from PTLC of urinary steroid extracts illustrated in Fig. 5.6a

Zone	R_f	Visual Examination
1	0 - 0.08	Brown stain in (+) and (-) [a]
2	0.08 - 0.24	
3	0.24 - 0.31	New component in (+) [b, c]
4	0.31 - 0.35	
5	0.35 - 0.40	
6	0.40 - 0.48	
7	0.48 - 0.57	Pink pigment [a]
8	0.57 - 0.63	Yellow pigment [a], etiocholanolone [b]
9	0.63 - 0.71	Androsterone [b]
10	0.71 - 0.77	New component in (+) [b]
11	0.77 - 1.00	

a observed in PTLC bands

b observed in analytical bands after charring with $Ce(SO_4)_2$ and viewing at 300 nm

c observed in PTLC bands at 240 nm

(b) Examination of Extracted Zones

Removal of the silica gel from the plate was accomplished using a suction technique. The silica was sucked into drawn out Pasteur pipettes as illustrated in Fig. 5b, and the components

extracted by passing 4 ml of eluting solvent (CH_2CL_2, MeOH, 9:1) through the pipette into a glass vial. After each fraction had been evaporated (under nitrogen) it was treated with HMDS, TMCS (10:1) in pyridine according to the method of Makita and Wells [175].

Each zone was examined as the TMS ether by GC-MS using SIM at m/e = 157 and m/e = 245 as described earlier. The saturated diol metabolites found in the unfractionated urinary steroids were found in PTLC zones 8 + (R_f = 0.57 - 0.63). After suitable concentration, six more metabolites were found in more polar zones as summarised in Table 5.2

TABLE 5.2

Retention data on 1% OV-17 for polar metabolites of Nilevar after SIM at m/e = 157 and m/e = 245

Metabolite	PTLC Zone	MU (OV-17)	SIM
A	3+	27.10	157
B	3+	27.90	157
	2+	27.80	157
C	4+	28.95	245
D	3+	29.00	157
	2+	28.85	157
E	3+	29.20	245
	2+	29.20	245
F	3+	32.35	245

CHARACTERISATION OF POLAR METABOLITES

The full mass spectra for the TMS ethers of metabolites found in zones 2, 3 and 4 were obtained by normal GC-MS. In some cases this information was supplemented by mass spectra of deuteriated derivatives. The mass spectra and conclusions therefrom are discussed below for each metabolite.

Metabolite A

This metabolite was detected in zone 3 of the PTLC plate. Characteristic ions (i.e. those not observed in the 'undrugged' urine fraction) were observed at m/e = 131 (30%) m/e = 144 (86%) and m/e = 157 (100%) indicating that the metabolite contained no further substitution on ring D. Although no molecular ion was observed in the spectrum, evidence that the compound is a saturated triol tri-TMS (MW = 534) was afforded by ions at m/e = 509 (48% = M - 29), m/e = 419 (18% = M - 29, 90) and m/e = 329 (7% = M - 29, 2 x 90). The position of the third OTMS group is not known but fragments thought to be characteristic of this group occur at m/e = 147 (40%) and m/e = 191 (11%). By observing mass shifts after forming the d_9TMS derivative the structures below may be postulated for these ions.

$(R)_3Si - \overset{+}{O} = Si(R)_2$ $(R)_3Si - O - CH = \overset{+}{O} - Si(R)_3$

m/e = 147 (R = CH_3) m/e = 191 (R = CH_3)

m/e = 162 (R = CD_3) m/e = 209 (R = CD_3)

Although the fragment at m/e = 147 occurs in many "open chain" diol bis TMS ethers [55] where the two functional groups may even be ten methylene groups apart, it is unlikely that TMS migrations of such magnitude are likely on the more rigid steroid nucleus. This assumption is substantiated in recent work by Sloan, Harvey and Vouros [176] who, after studying the mass spectra of forty six polyhydroxy steroid TMS ethers, demonstrated that the existence and abundance of this ion is dependent on the proximity of the functional groups. The ion at m/e = 191 has been observed for the 1, 2 diol derivative 16 α-hydroxy testosterone bis TMS[57] and the 1, 3 diol derivative 19 hydroxytestosterone bis TMS and the 15α-hydroxytestosterone bis TMS[57].

The presence of the two rearrangement fragments at m/e = 147 and m/e = 191 in the tris trimethylsilyl ether of this metabolite suggests therefore that the extra OTMS group is in close proximity to the OTMS group at C-3. This theory is substantiated by the small MU value relative to the other

tri hydroxy metabolites (see Table 5.2). The absence of a prominent ion at m/e = 217 in the mass spectrum suggests that an $\alpha\beta$ diol may be a stronger possibility than a $\beta\gamma$ -diol on ring A [57].

Metabolite B

This metabolite was detected in zones 2 and 3 of the PTLC plate. Characteristic ions were observed at m/e = 131 (12%), m/e = 144 (76%) and m/e = 157 (100%) indicating that the metabolite contained no further substitution on ring D. A molecular ion was observed at m/e = 538 (4%) indicating that the metabolite is a saturated triol. Ions at m/e = 448 (M - 90), m/e = 358 (M - 2 x 90) and m/e = 268 (M - 3 x 90) support this evidence.

Metabolite C

This metabolite was detected in zone 4 of the PTLC plate. Characteristic ions were observed at m/e = 103 (71%), m/e = 155 (49%), m/e = 217 (44%), m/e = 232 (25%), m/e = 245 (100%) and m/e = 421 (68%). These fragments, as was demonstrated earlier, can be rationalised by a 3,17β ,21 saturated triol, tri-TMS ether.

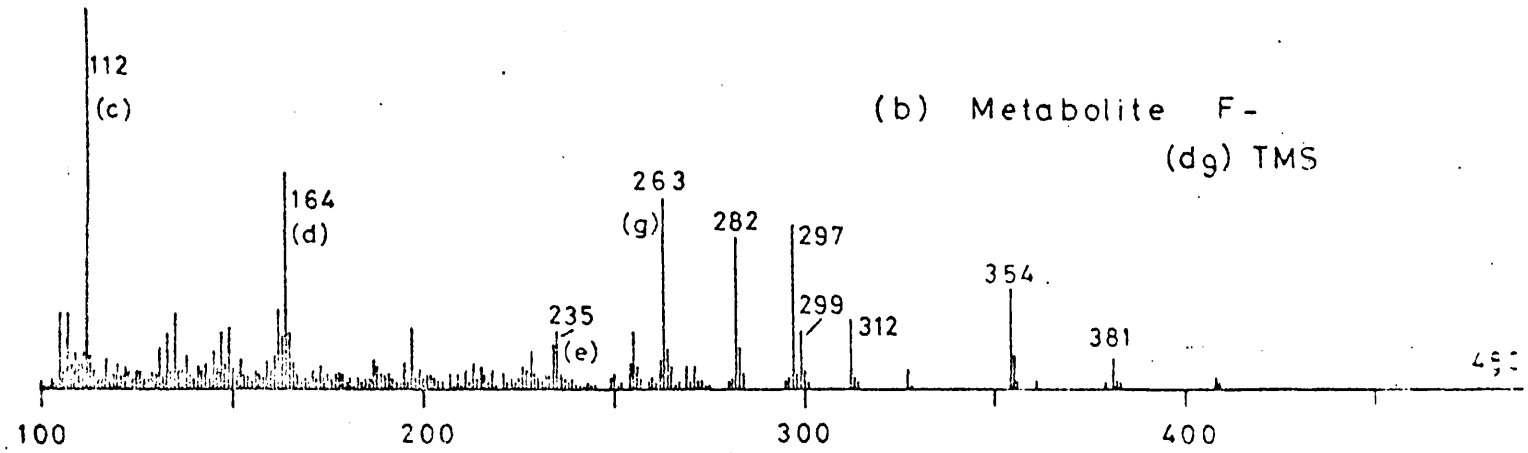
Metabolite D

This metabolite was detected in zones 2 and 3 of the PTLC plate. The GLC peak overlapped with metabolite E (MU = 29.20) and evidence of a pregnane triol tri-TMS ether was also noted in the mass spectrum. Characteristic ions were observed at m/e = 157 (80%) and m/e = 144 (47%), indicating there were no extra substituents on ring D and at m/e = 538 (M^+), m/e = 358 (M - 2 x 90) and m/e = 268 (M - 3 x 90) showing the metabolite was a saturated triol tri-TMS ether. There were no ions in the mass spectrum which were diagnostic of the position of hydroxylation.

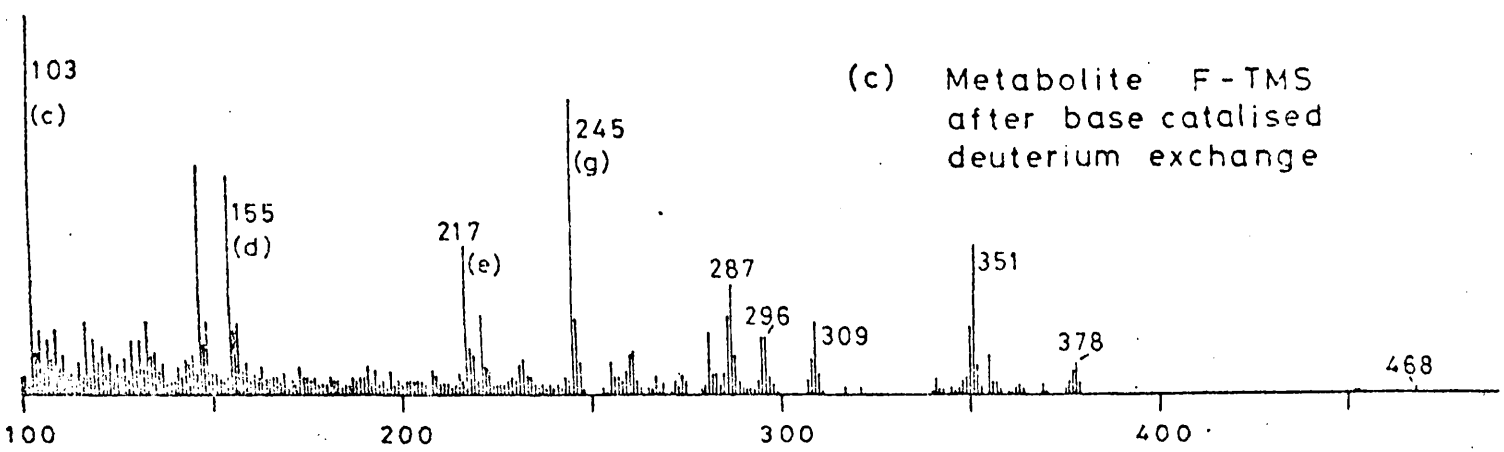
Metabolite E

This metabolite was detected in zones 2 and 3 of the PTLC plate. Characteristic ions were observed at m/e = 103 (67%), m/e = 155 (50%), m/e = 217 (49%), m/e = 232 (33%) and m/e = 245 (100%) which indicate a 17 ß, 21 di-TMS ether structure. A molecular ion at m/e = 538 (3%) supported by ions at m/e = 448 (M - 90), m/e = 358 (M -2 x 90) and m/e = 268 (M - 3 x 90) show the metabolite derivative to be a 3, 17ß, 21 saturated triol tri-TMS ether.

FIG. 5.7



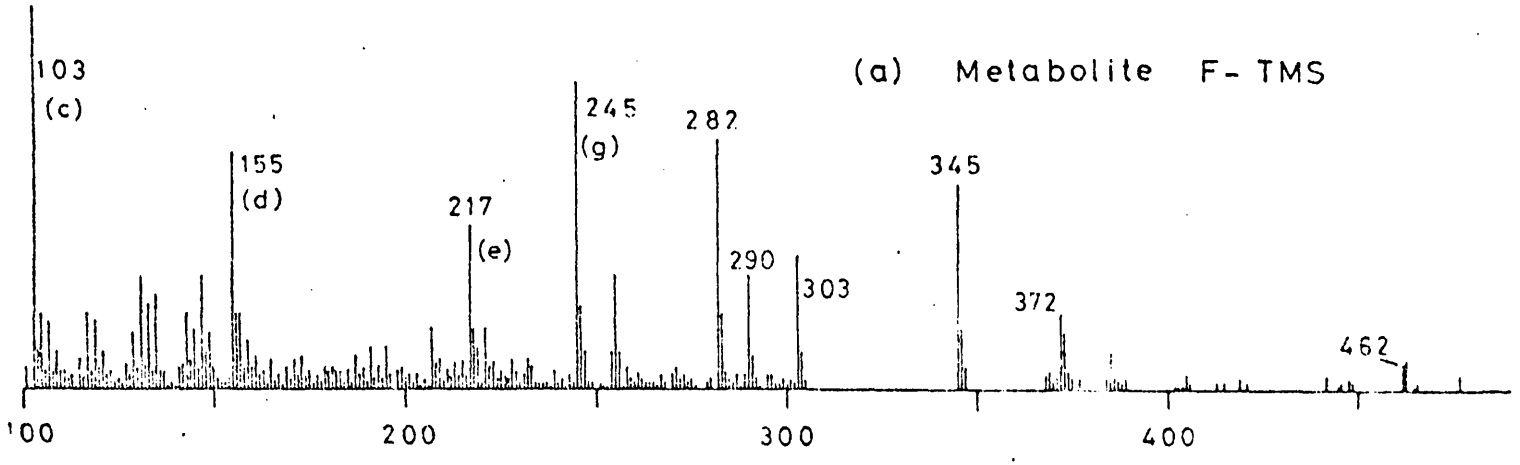
(b) Metabolite F-(d_9) TMS



(c) Metabolite F-TMS after base catalised deuterium exchange

FIG. 5.7

Mass spectra of metabolite"F" TMS and deuteriated analogs.(See Fig. 5.5 for ion structures)



Metabolite F

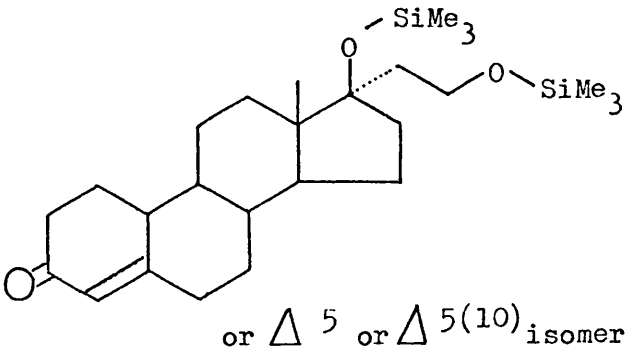
This metabolite was present in zone 3 of the PTLC plate. Ions were observed at m/e = 103 (100%), m/e = 155 (62%), m/e = 217 (43%) and m/e = 245 (80%) in the mass spectrum of the TMS derivative (Fig. 5.7a). The proposed structures of these ions illustrated in Fig. 5.5 (ions c, d, f and g respectively: R = CH_3) are verified by the expected mass increments in the mass spectrum of the analogous d_9-TMS derivative (Fig. 5.7b) and indicate a metabolite hydroxylated at C-21.

Ions observed at m/e = 282, 290, 303, 345 and 372 (Fig. 5.7a) were shown to contain 0, 1, 1, 1 and 1 TMS groups respectively by their appearance at m/e = 282, m/e = 297/299, m/e = 312, m/e = 354 and m/e = 381 in the mass spectrum of the d_9-TMS derivative (Fig. 5.7b)

Further evidence of the nature of these ions was afforded by the mass spectrum of the TMS ether after chromatography on 1% Apiezon-L, 0.5% $Ba(OD)_2$ (see part 4 of this thesis). The ions at m/e = 290, 303, 345 and 372 in Fig. 5.7a, all showed mass increments of 6 a.m.u. after such deuteriation (Fig. 5.7c). The exchange of six deuterium atoms under these conditions has

been shown (in part 4) to be characteristic of a 19-nor- Δ4-3-oxo group and it is reasonable to assume therefore that the metabolite contains this group, the Δ5-isomer or the Δ5(10) isomer (since the latter two form the Δ4-isomer under the conditions used for deuterium exchange.)

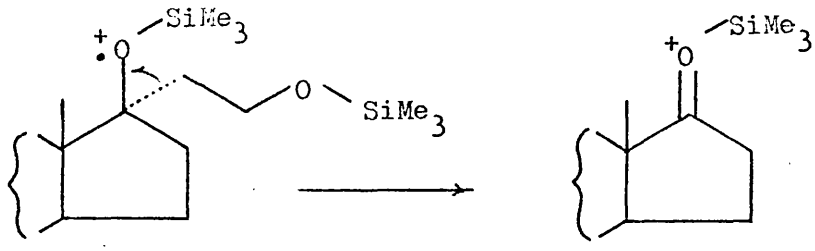
These results may be rationalised by the structures represented below :



or Δ^5 or $\Delta^{5(10)}$ isomer

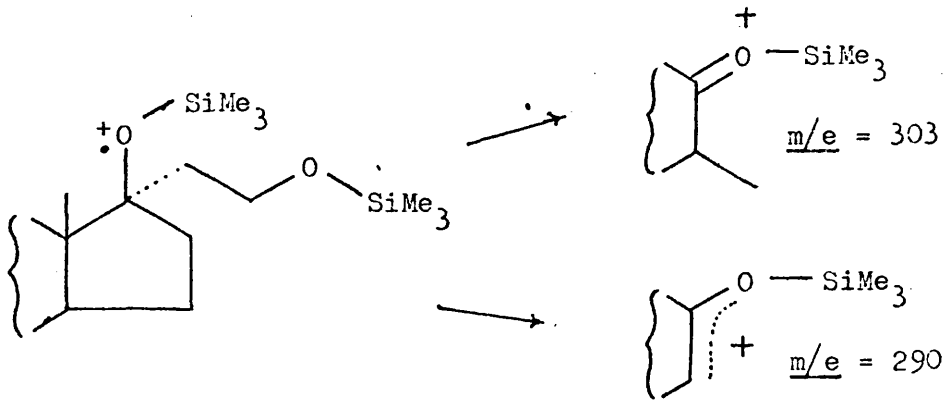
A molecular ion for this metabolite derivative is evident at m/e = 462 (Fig. 5.7a) at m/e = 480 for the d_9-TMS (Fig. 5.7b) and at m/e = 468 in the product from deuterium exchange (Fig. 5.7c).

The ions at m/e = 372 and m/e = 282 in Fig. 5.7a are due to elimination of one and two molecules of Me_3SiOH from the molecular ion. The ion at m/e = 345 is due to elimination of the side chain.



m/e = 345

The ions at m/e = 290 and m/e = 303 both involve migration of the OTMS group at C-17 and are equivalent to the M-(55 + R) and M-(42 + R) ions (where R = the mass of the substituent on C-17) which have been observed in the mass spectra of testosterone-17-OTMS and analogues (see part 2 of this thesis).



Further Separation of Bifunctional Metabolites

PTLC zone 8+ (Fig. 5. 6a) which contains the major

metabolite contains also an appreciable amount of etiocholanolone and other components of similar polarity. PTLC of TMS derivatives has been reported as a useful method of hydroxy steroid separation [101,102] and is ideal for separating ketonic steroids from non-ketonic steroids. Accordingly the zones 6, 7 and 8 were re-chromatographed as their TMS ethers. The six fractions were applied to 2 cm x 0.5 mm sections of the plate and a mixture of standards (17 α-ethyl-5α-estrane-3β,17β-diol, 17α-ethyl-5β-estrane-3α,17β-diol, 17α-ethyl-5 α-estrane-3α,17β-diol, androsterone and etiocholanolone) as their trimethylsilylation products was applied as a spot near the right edge of the plate. The solvent mixture used for developing the plate was cyclohexane:benzene 2:1. This system was found to suppress the mobility of the two ketonic steroids below Rf = 0.1. The reference samples were sprayed with ceric sulphate, and charred as described earlier. The preparative bands were divided into zones, removed from the plate by the suction technique and extracted from the silica gel with ether. The zones are defined in Table 5.3, and illustrated in Fig. 5.7c.

FIG. 5.8

GLC of principal Nilevar metabolite (17α -ethyl-5β -estran-3α ,17β -diol) as the TMS derivative after purification by TLC. The metabolite is chromatographed with n-hydrocarbon references

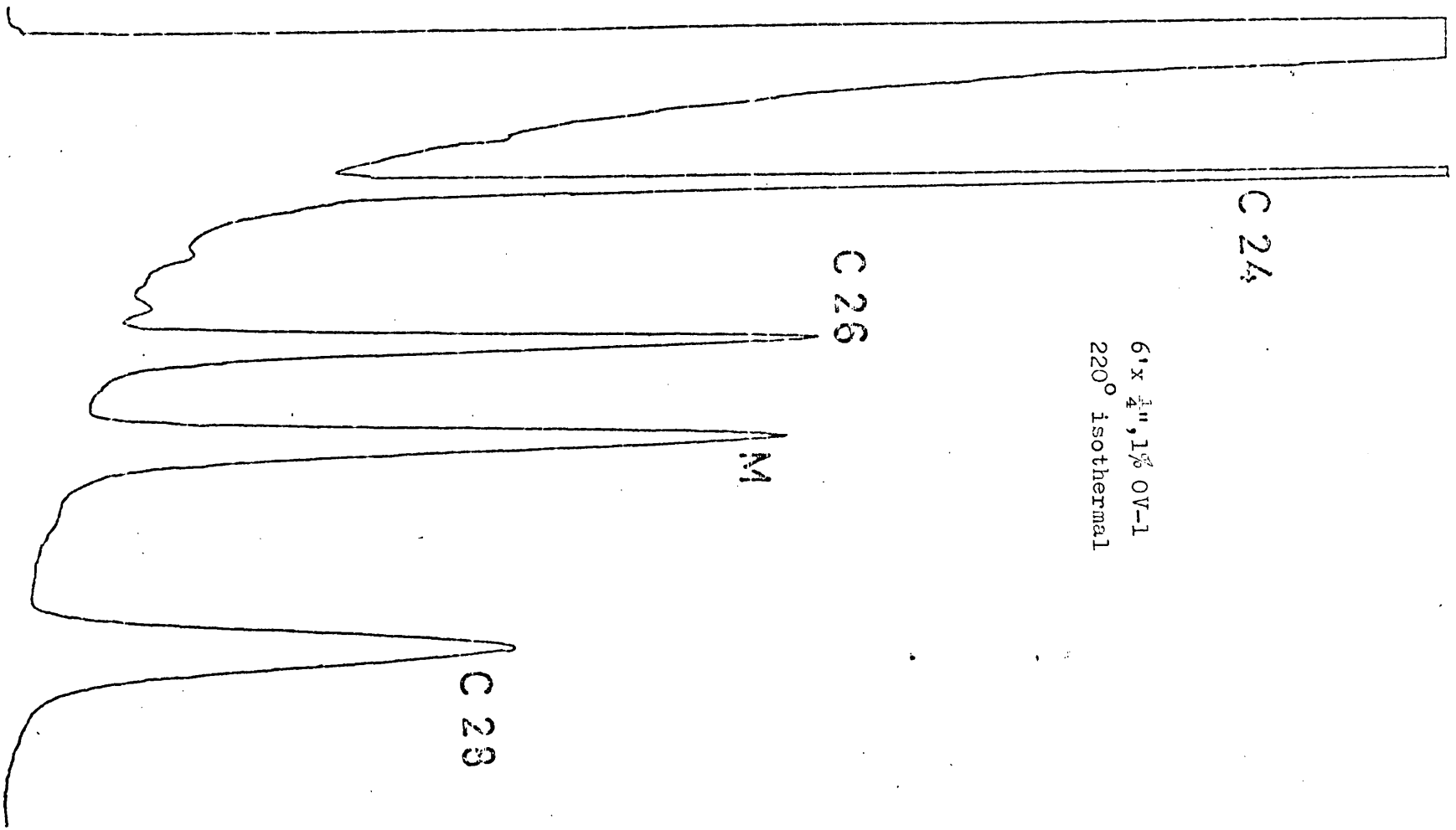


FIG. 5.9

Mass spectrum of the principal metabolite from Nilevar (17α-ethyl-5β-estran-3α,17β-diol) as the TMS derivative. (See Fig. 5.5 for ion structures.)

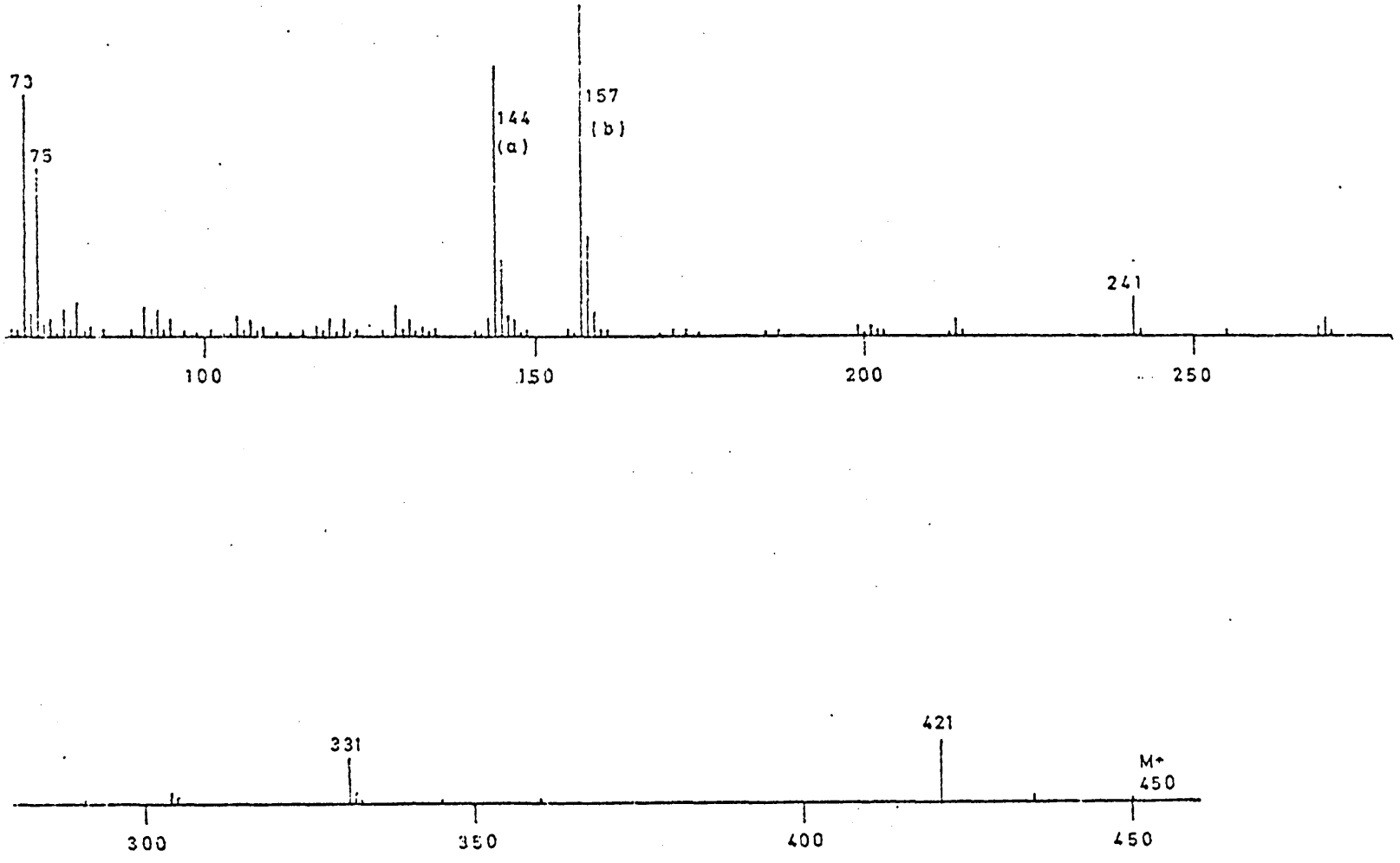


TABLE 5.3

Rf values of zones from PTLC of urinary steroids as TMS ethers

Zone	Rf	Standards
1	0.00 - 0.10	Ketonic steroids
2	0.10 - 0.29	
3	0.29 - 0.37	5α-H-3β,17β-diol
4	0.37 - 0.46	5β-H-3α,17β-diol
5	0.46 - 0.61	
6	0.61 - 0.71	5α-H-3α,17β-diol
7	0.71 - 1.00	

Each fraction was numbered 8 + 1, 8 - 1....etc., according to its Rf zone as the free steroid (first digit) and its Rf zone as the TMS derivative (last digit). The fractions were resilylated and examined by GLC.

The only fractions showing dissimilarity between "drugged" and "undrugged" urine were 8 + 4 and 8 - 4. The fraction at 8 + 4 gave a single peak on a 1% OV-1 column at 26.80 MU (Fig. 5.8) and at 27.57 MU on 1% OV-17. The standard for this Rf value (17α-ethyl-5β-estran-3α,17β-diol-bis TMS, gave retention values of 26.80 MU (1% OV-1) and 27.60 MU (1% OV-17). The mass spectrum of the component in 8 + 4 (Fig. 5.9) was also in conformity with this structure.

Ions at m/e = 157 (Fig. 5.5b) and m/e = 144 (Fig. 5.5a) result from ring D fragmentation as discussed earlier. A molecular ion of low relative abundance occurs at m/e = 450. Ions also occur at m/e = 421 (M - C_2H_5), m/e = 331 (M - C_2H_5, Me_3SiOH) and m/e = 241 (M-C_2H_5, $2Me_3SiOH$) indicating that the compound is a saturated diol bis TMS ether.

CONCLUSIONS

The above study on the in vivo metabolism of the steroid drug "Nilevar" in humans has involved the combination of complementary techniques which are all applicable to microgram quantities of material. The techniques found most useful in this study are as follows :

1. PTLC AND GLC

Separation of urinary steroids on a polarity basis by PTLC or liquid chromatography is a useful preliminary to GLC. Drug metabolites which differ in molecular weight from natural steroids may often be separated from others in the group by GLC on non-polar or moderately polar columns. Group separation also simplifies characterisation by ancillary techniques such as derivative formation, oxidation and reduction studies where changes in retention behaviour are anticipated.

Further separation of steroids in each group may also be achieved by PTLC of their TMS derivatives. This is especially useful when the group contains a mixture of ketonic and non-ketonic species and has been used to obtain a gas chromatographically pure sample of the main metabolite from urine.

2. GLC AND MS

When the mass spectra of metabolites, or one of their derivatives, are expected to contain an ion of high relative abundance which is not common for natural steroids (or the appropriate derivative thereof) selective ion detection may be a useful means of indicating their position in a complex gas chromatogram. This technique has been fruitful in the metabolite study of "Nilevar" and may have application to other 17α-substituted steroids.

3. OTHER TECHNIQUES

The technique of gas phase deuteriation prior to mass spectrometry has been usefully applied to a ketonic metabolite of "Nilevar" and may be of further benefit in oxidation studies (using Jones' reagent or Sarett's reagent) on groups of steroids separated by PTLC. The use of boronate derivatives for the

recognition of 1, 2 or 1, 3 diols amongst the polar metabolites is envisaged as another extension of this work which was unfortunately left unfinished.

CONCLUSIONS

The preceding parts of this thesis have been concerned with the development and application of techniques designed to aid the identification of unknown materials of biological interest by the combined techniques of gas chromatography and mass spectrometry. Supplementary information afforded by techniques such as derivative formation and deuterium exchange developed in parts 2 and 4 greatly assisted the identification in part 5 of the 21-hydroxy-metabolites of the anabolic steroid drug "Nilevar".

The use of derivatives in structural analysis is useful for two reasons. Firstly, when the mode of fragmentation is not understood it is possible to obtain important information about the number and type of functional groups on the molecule by changes in GLC retention time and molecular weight after the derivative has been formed. Secondly, when the derivative has strong fragmentation directing properties, a knowledge of its behaviour under electron impact may enable prediction of the group's position in unknown material.

In part 1 an examination was made of the effectiveness of boronate derivatives of β-hydroxy amines and β -hydroxy-

catacholamines. Alkyl and aryl boronic acids were found to react rapidly with the catechol group to form a cyclic ester in near quantitative yields, whereas the reaction with the β hydroxyamine group was slower and the yield of the 1, 3, 2 oxazoborolidine formed depended on the substituents on the heterocyclic ring. Nevertheless, the fact that these cyclic derivatives could be formed demonstrated the proximity of the functional groups concerned, and the fragmentation of the oxazaborolidine ring enabled the position and nature of the substituents on the ß-hydroxyamine to be determined. Another advantage of these cyclic derivatives was their ability to separate diastereoisomers (such as ephedrine and ψ-ephedrine) by GLC.

Parts 2 to 5 were concerned mainly with the structural identification of steroids and explored some ways of determining the position of various groups and substituents on the steroid nucleus. Methyl substituted and nor-testosterones were examined in part 2 as underivatised steroids, as their O-TMS ether derivatives and as their O-methyl-oxime derivatives. It was found that although methyl groups did not usually direct

the fragmentation of the molecule after electron impact, the position of these groups could be inferred by mass shifts in fragments directed by other groups in the molecule. It is interesting to note that formation of the 17 ß - OTMS ether hindered the recognition of substituents on ring A.

The problem of determining the position of an olefinic bond in a steroid was considered in part 3. The difficulty of distinguishing between olefinic isomers was demonstrated with a series of isomeric estren-17-ones, which gave almost identical mass spectra and similar GLC retention times. The weak fragmentation directing properties of these olefinic bonds were improved upon by the cis-diol formed after hydroxylation of the olefin with osmium tetroxide and after forming various derivatives of the resulting cis-diol. Boronate esters were found to be excellent derivatives in this respect since fragment ions containing boron were easily located by the ^{10}B, ^{11}B isotope ratio and by mass shifts obtained when using different substituents on boron. The boronate esters were found to have good GLC properties and enabled good separation of the isomeric estren-17-ones on OV-1 and OV-17. The cis-diols themselves were amenable to GLC

and mass spectrometry afforded good distinction between the positional isomers.

In part 4 the position of oxygen substituents on the steroid nucleus were determined by supplementary methods involving GC-MS. Since most hydroxy groups on primary and secondary carbon atoms can be conveniently oxidised to an aldehyde or ketone respectively, emphasis was placed on determining the carbonyl position on the steroid nucleus. Deuterium exchange during gas chromatography and subsequent mass spectrometry proved to be a useful technique for ketone location in steroids and terpenoids. Such ketones, when chromatographed on base treated columns which had been saturated with deuterium oxide or deuterium methoxide, exchanged hydrogen at hydroxylic and enolisable positions with deuterium to give the per deuteriated analog usually in good yield. This provided a simple criterion for determining carbonyl environment. Derivative formation such as the TMS ether of alcohols, the boronate ester of cis-diols or the 0-methyloxime of ketones successfully blocked these groups from deuterium exchange so that a TMS-MO derivative of a steroid containing an 11- keto

group (which does not form an 11-methoxime derivative) still exchanged hydrogen atoms at C-9 and C-12 with deuterium.

Saturated 2-oxo steroids and 3-oxo-steroids which could not be distinguished by their mass spectra and both incorporated 4 deuterium atoms after chromatography on the above column were differentiated by the mass spectra of their 0-methyloxime, 0-ethyloxime and 0-TMS-oxime derivatives.

In part 5 an attempt was made to utilise some of the above techniques to the study of the urinary metabolites of the anabolic steroid drug "Nilevar" (17α -ethyl estr-4-ene-3-one-17β-ol). This compound as its 17β-OTMS ether gave a very intense fragment ion at m/e = 157 which (in part 2) was shown to comprise the OTMS group, ethyl side chain and C15-C17 of ring D. Since the metabolites were not very prominent in the GLC of the steroids extracted from the urine the technique of single ion monitoring was used to pick out all metabolites containing an unmodified ring D and side chain. Metabolites hydroxylated on the side chain (or ring D) were detected by single ion monitoring at m/e = 245. Since some metabolites were

present only in very small quantities it was necessary to use another separatory technique to enable them to be concentrated prior to further study. Preparative thin layer chromatography (PTLC) was found to be an excellent method in this respect and by combining PTLC of the free steroids with PTLC of their TMS ethers it was found possible to isolate the main metabolite free from all other contaminants.

Only one keto steroid metabolite of Nilevar was found and deuterium exchange chromatography indicated that this containeda 19-nor- Δ^4-3-keto group (by an incorporation of 6 deuterium atoms) although the Δ^5 or $\Delta^{5(10)}$ isomers must also be considered as possible alternatives.

A logical progression of this work would have been further isolation of metabolites by PTLC methods and the location of hydroxyl groups by oxidation studies (to ketone or aldehyde) and the use of deuterium exchange chromatography and derivative formation. Final confirmation of structure would require synthesis of the proposed structures.

EXPERIMENTAL

GENERAL

Gas Chromatography

Gas chromatograms and retention data were obtained on a Carlo Erba "Fractovap GB" gas chromatograph with 6' x $\frac{1}{4}$" OD silanised U tube columns. Column packings of OV-1 and OV-17 stationary phase were prepared by the filtration technique using toluene as solvent and 100-120 mesh "Gas-Chrom Q" (Applied Science Laboratories Inc.) support. n-Alkanes were used as standards.

Mass Spectrometry and GC-MS

Mass spectra were obtained using an LKB 9000 combined gas chromatograph mass spectrometer. Spectra were calibrated with an LKB 9010 mass marker which had previously been calibrated with perfluorokerosene (PFK). Ionising voltages were 70 ev unless otherwise specified. Source temperatures were normally between 250 and 300^{o}C.

Trimethylsilyl (TMS) ether formation

TMS derivatives of alcohols for study by GLC or GC/MS were normally prepared by dissolving the alcohol (1 mg) in dry pyridine (1 ml) which had been distilled over sodium hydroxide, and hexamethyldisilazane (HMDS) (50 μl) added followed by trimethylchlorosilane (TMCS) (5 μl). The solution was allowed to stand at room temperature overnight and the solvent and excess reagents removed by evaporation under nitrogen. The TMS ether was taken up in a suitable solvent - usually ethyl acetate.

Methyl Oxime (MO) and Ethyl Oxime (EO) formation

MO and EO derivatives of ketones were prepared by dissolving the ketone in dry pyridine and adding an excess of methoxylamine hydrochloride or ethoxylamine hydrochloride, and leaving the solution overnight at room temperature. The pyridine was evaporated under nitrogen and the residue extracted with a suitable solvent - usually ethyl acetate. When it was necessary to make the double MO (EO) TMS derivative, this solution was evaporated, the residue taken up in pyridine and silylated as described above.

Trimethylsilyloxime formation

The oxime derivative of the ketone was prepared by the reaction of hydroxylamine hydrochloride with the ketone in pyridine using a method similar to that described above for MO and EO formation. After evaporating the pyridine and extracting the oxime with ethyl acetate the trimethylsilyloxime was prepared by silylation with HMDS and TMCS in pyridine.

PART 1

Preparation of boronate derivatives of ß-hydroxy-amines and catecholamines

Methyl boronates, n-butylboronates, t-butylboronates, cyclohexylboronates and phenylboronates of ß-hydroxyamines and catecholamines were prepared by treating the ß-hydroxyamine, or catecholamine (1 mg) in the form of its free base, hydrochloride, sulphate or tartrate with the appropriate boronic acid, (1-1.5 molar equivalents) in pyridine (1 ml) which had been dried and distilled over sodium hydroxide. The free base could be conveniently prepared from the hydrochloride by exposing the pyridine solution of the salt to ammonia vapour and separating the precipitated ammonium chloride before derivative formation. For hydroxy amines, such as isoprenaline sulphate, which were not

sufficiently soluble in pyridine, a suitable reaction solvent was dimethylformamide which had been dried by azeotropic distillation with benzene and further distilled over anhydrous sodium sulphate.

In most cases aliquots of the reaction mixture were injected directly on to the GLC column. In the reactions involving octopamine and 4-deoxynoradrenaline, cyclic derivatives appeared to be formed in low yield and vacuum sublimation (250^{o}/0.01 mm Hg) was used to separate the derivative (in its free base form) from non-volatile material.

PART 2

Reduction of Testosterone, 2 α-Methyltestosterone and 4-Methyltestosterone

The sterol (2 mg) was dissolved in methanol (0.25 ml) cooled to 0^{o} and added to sodium borohydride (2 mg), washing the container with more cold methanol (0.25 ml). The solution was allowed to reach room temperature and allowed to proceed until hydrogen gas ceased to be evolved (about 1 hour). Glacial acetic acid (10 μl) was added and the solvent evaporated without heating under nitrogen. The reduced

steroid was taken up in ethyl acetate (1 ml) for further studies after silylation. GC/MS of the silyl derivatives showed that the reduction products were mostly a mixture of 3α-and 3β -hydroxy androst-4-en-17-ols or their methyl analogues.

PART 3

Preparation of $\alpha\beta$ cis diols from steroid olefins

The steroid olefin (20 mg) was dissolved in dioxan/pyridine 8:1 (1 ml) and a 5% solution of OsO_4 in dioxan (0.2 ml) added dropwise and the mixture allowed to stand for 17 hrs at room temperature. To this solution was added methanol (2.5 ml) and a 16% w / v aqueous solution of sodium sulphite (8.5 ml) and the solution allowed to stand a further hour. The solid was removed by centrifugation, the liquor diluted X4 with methanol and re-centrifuged. The liquor was evaporated to dryness and the residue extracted with ethyl acetate.

Preparation of Boronate esters

The steroid cis diol (1 mg) was mixed with n-butylboronic acid or phenylboronic acid (2 mg) and dissolved in pyridine (1 ml). After standing at room temperature for 1 hr, injections were made directly into the gas chromatograph.

Preparation of 3 3-d_2-estr-4-en -17-one

This synthesis was in two stages.

(a) 4-estren-3-one-17 ß-ol (100 mg) in dry ether (10 ml) was added dropwise to a suspension of aluminium trichloride (170 mg) and lithium aluminium deuteroxide (27 mg) in dry ether (5 ml). The mixture was refluxed $4\frac{1}{2}$ hrs, hydrolysed with water and then dilute sulphuric acid, ether extracted, washed and dried with anhydrous magnesium sulphate.

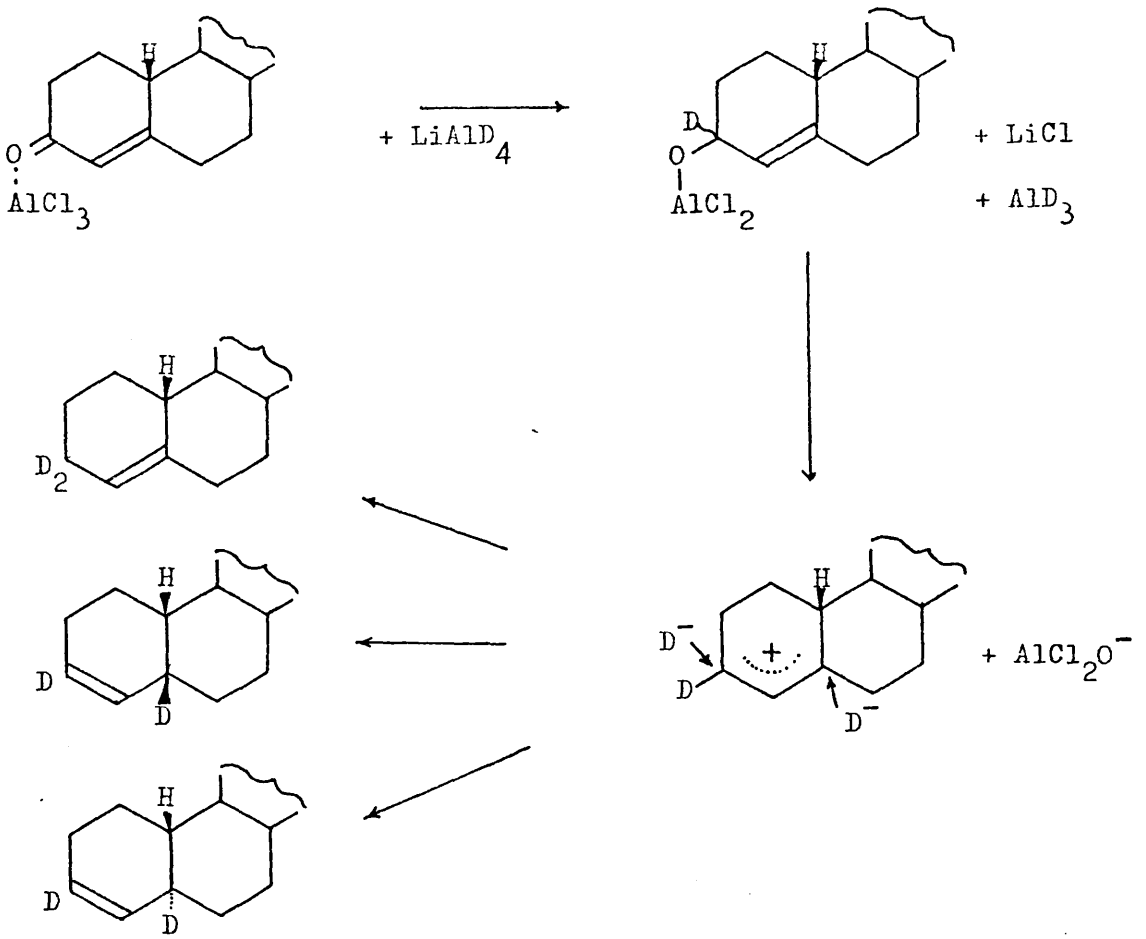
(b) The products of the above reaction were taken up in acetone (5 ml) and Jones reagent added dropwise until the orange colour persisted. After 30 minutes at room temperature the excess reagent was destroyed with isopropanol and the product ether extracted.

GLC (1% OV-1) and GC/MS showed a mixture of at least six products comprising isomeric estren-17-ones and di-ketones with one or two deuterium atoms. The mono-ketones were separated by chromatography on alumina after elution with benzene. Further separation of the deuteriated mono olefins was achieved by preparative thin layer chromatography on 1 mm thick 8" x 2" plates of silica gel containing 5% by

weight of silver nitrate. The positions of the bands were indicated by spraying with fluorescein, extracting with carbon tetrachloride and filtering through neutral alumina (Brockman 1).

Two mono olefinic fractions were obtained by the above procedure. Comparisons of these two fractions with 4-estren-17-one on an analytical argentous TLC plate showed the fraction with the higher Rf value to be of the same polarity as the standard. Infrared analysis showed the high Rf fraction to contain a $-CD_2-$ group by adsorption at 2099 cm^{-1} and 2182 cm^{-1} and also a =CD- group by adsorption at 2241 cm^{-1}. This fraction was shown by GLC on OV-1 to contain two isomeric estren-17-ones, one of which had the correct retention data for the Δ^4 isomer (21.01 mu). Infrared analysis showed the lower Rf fraction to contain a =CD- group by adsorption at 2071 cm^{-1}.

The isomeric estren-17-one reaction products described above can be explained by the reactions shown below :



Preparation of 16-$\underline{d}_2$-estr-4-en-17-one

4-Estren-17-one (5 mg) was heated for one hour at 120° in a sealed tube containing sodium (2 mg) dissolved in deuterium methoxide (0.3 ml) and deuterium oxide (0.03 ml). After evaporating the solvent and extracting the residue with ethyl acetate a white solid was obtained (5.02 mg). GC-MS showed 92% $\underline{d}_2$ isomer, I R showed vicinal -CD_2- absorption at

2145 cm^{-1} and 2220 cm^{-1}.

PART 4

Preparation of basic columns for deuterium exchange

(a) 1% Carbowax 20M, 1% KOH

A solution of Carbowax 20M (1 g) and potassium hydroxide (1 g) in methanol (100 ml) was used to coat 100-120 mesh Gas Chrom Q (25 g) by the filtration method.

(b) 1% Apiezon L with KOH, $Ba(OH)_2$ and $Sr(OH)_2$ bases

A solution of potassium hydroxide (1 g) or an equivalent (OH^-) amount of barium hydroxide or strontium hydroxide in methanol (100 ml) was used to coat 100-120 mesh Gas Chrom Q (25 g) by the filtration method. For the barium hydroxide and strontium hydroxide solutions it was necessary first to remove undissolved carbonate before coating. After drying overnight at 110^{o} the alkaline support was re-coated by a similar process using a toluene solution of Apiezon L. A 0.5% $Ba(OH)_2$ 1% Apiezon L column was later prepared by a similar process using a solution of barium hydroxide octahydrate (0.92 g) in methanol (100 ml) to coat the support (25 g).

PART 5

Single Ion Monitoring (SIM)

An output from the galvanometer amplifier of the mass spectrometer was connected via a 2.5 cps filter to one channel of a dual pen potentiometric recorder. The TIC was simultaneously recorded by the other channel. The TIC attenuation and electron multiplier voltage were selected to give recordings of suitable amplitude.

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