Volume 1
Number 5

Review

Mass spectrometry in lipid research

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[Received for publication July 25, 1960]

Mass spectrometry is a very efficient tool in organic structure determination. It seems, however, that its wide applicability and power are not as yet fully appreciated among organic chemists and biochemists. The object of the present article is to demonstrate the use of mass spectrometric analysis in the lipid field.

PRINCIPLE OF THE METHOD

In the mass spectrometer the molecules of the compound studied are bombarded in the gas phase at low pressure with electrons of low energy (10 to 100 electron volts). The processes that are the result of the impact of electrons on organic molecules are exceedingly complex and are by no means fully understood (1, 2, 3). If the electron possesses an energy larger than that needed for the removal of an electron from the molecule (that is, its kinetic energy is larger than the ionization potential of the molecule), the most common primary process is the removal of one electron from the molecule

$$M + e^- = M^+ + 2e^-.$$

The molecule-ions M^+ are usually formed in excited states and decompose after a certain time (of the order of a microsecond) into a number of fragments (3). Both ionized and neutral fragments are formed. The nature of the ions and their relative abundance depend on the structure of the molecules bombarded. In general, therefore, two compounds that are structurally different will give different fragmentation patterns. Like the infrared spectrum, the mass spectrum can be considered as a "fingerprint" of the compound.

Only one negatively charged ion is formed for 10^4 positively charged ions, and only positive ions are usually measured in the mass spectrometer. The unfragmented molecule-ion M^+ is often present in the mass spectrum and may, even in the case of compounds of complex structure and high molecular weight, be the ion present in the greatest number, forming the "base peak" of the spectrum.

The mass spectrum of a complex molecule cannot be calculated by methods now available (cf. 3), and mass spectrometry in organic structure analysis is an empirical tool, the usefulness of which increases with the amount of information available for compounds of known structure.

Cleavage of carbon-carbon bonds during the dissociation of the molecule-ion is often accompanied by rearrangement, involving the transfer of the part of the fragment lost to the ionized fragment. The rearrangements are considered to proceed via cyclic intermediates (4, 5). Reactions of this type are common in the case of long-chain compounds (6, 7, 8), as discussed further below. A knowledge of such rearrangements is essential when the mass spectrum is used to deduce the structure of an unknown compound.

CONSTRUCTIONAL FEATURES AND OPERATION OF A MASS SPECTROMETER INTENDED FOR STRUCTURE ANALYSIS OF ORGANIC COMPOUNDS OF HIGH MOLECULAR WEIGHT

In order to be useful for the analysis of lipids and lipid components, the mass spectrometer must have a resolution such that it is possible with certainty to discover the rearrangement of one hydrogen atom in cleavages leading to the formation of ions having masses around 500. Furthermore, the instrument must be equipped with a heated intake system (9). Most of the work on compounds of high molecular weight so far reported has been on hydrocarbons. It has to a very

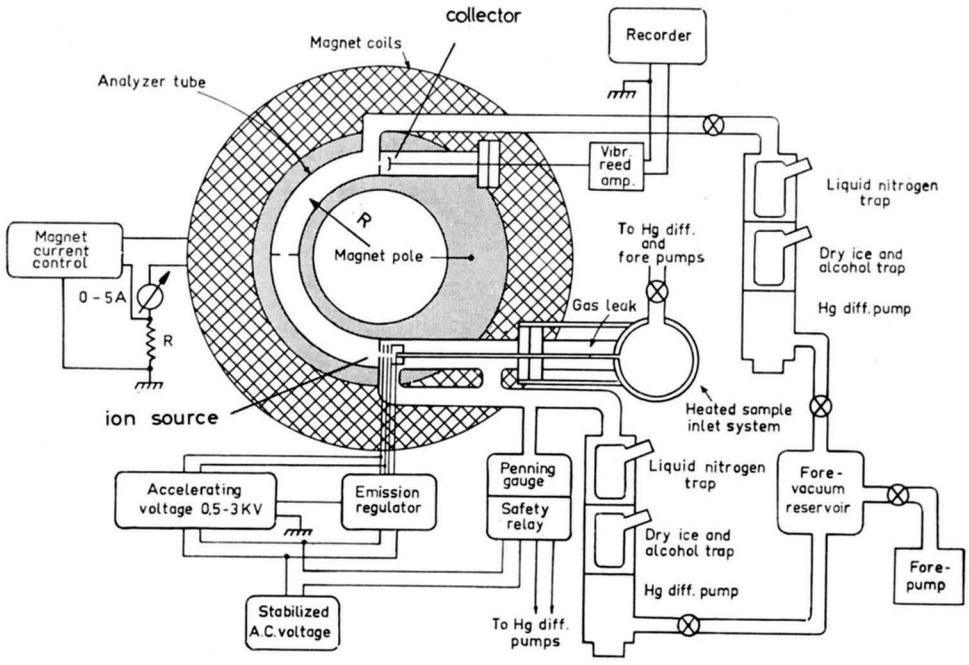


FIG. 1. Block diagram of mass spectrometer. From Ryhage (13).

large extent been performed in the laboratories of the American petroleum industry (9, 10) with the use of the CEC (Consolidated Electrodynamics Corporation) type 21-103 mass spectrometer. This instrument has a direction focusing magnetic analyzer of the semi-circular type (11).

The mass spectra used to illustrate this article have been obtained with a similar instrument built by one of the authors. Details of the construction and operation of this mass spectrometer have been published (12, 13). A block diagram of the instrument is shown in Figure 1.

The sample inlet system is shown in Figure 2. The sample (0.1 to 1.0 mg) is contained in a small glass tube open at one end. In order to introduce the sample tube, the ball and socket valve (*V1*) is closed and the upper end of tube *A* cut open. The valve (*V2*) to the vacuum line remains open. After the introduction of the sample tube, tube *A* is evacuated via an auxiliary vacuum line temporarily connected to the open end. After evacuation, tube *A* is sealed off. This glass-blowing operation quickly becomes a routine. Valve *V1* is opened, valve *V2* to the evacuation line closed, and the sample evaporated by putting a small heater around tube *A*. The pressure in the reservoir should be 10^{-1} to 10^{-2} mm Hg. Gas from the reservoir is led to the ion source via a molecular leak (consisting of a very small hole in a glass membrane). The assembly of two intake systems and the ion source is shown in Figure 3. The Bleakney-Nier ion source is shown

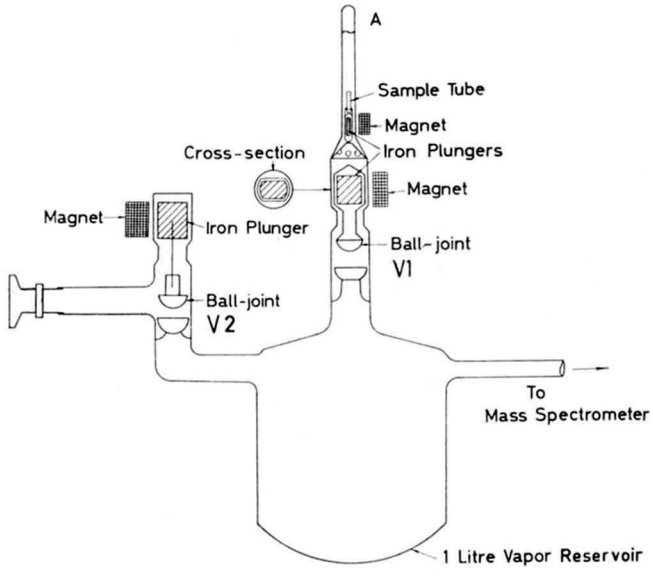


FIG. 2. Heated sample inlet system. From Ryhage (13).

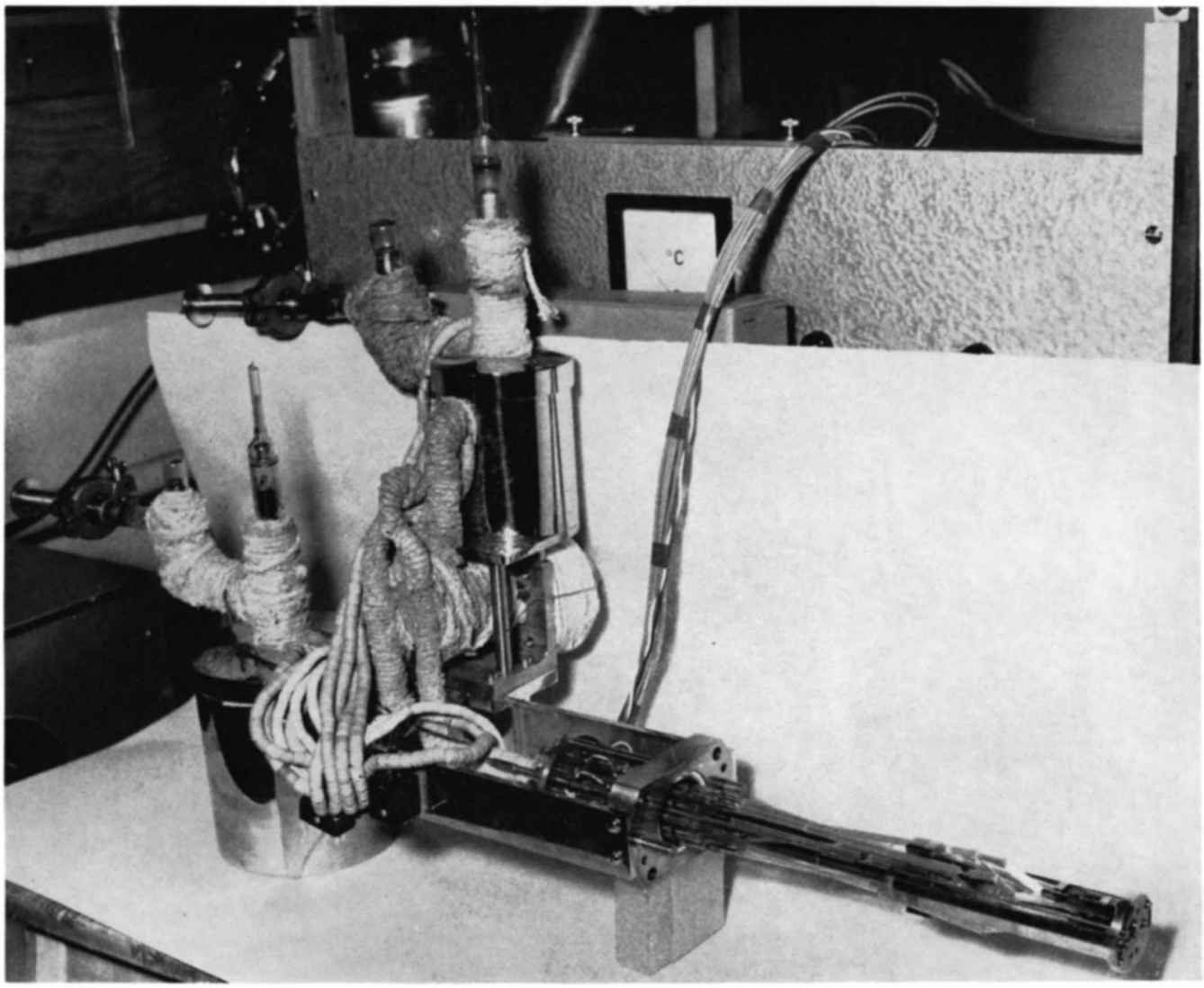


FIG. 3. Sample inlet systems and ion source assembly. From Ryhage (13).

schematically in Figure 4. Gas molecules enter the ionization chamber (*IC*) through a hole in the electrode (*Rp*) and are ionized by a transverse beam of electrons generated by a rhenium filament (*F*). The electron beam enters the ionization chamber through a slit (*S1*) and leaves it through a similar slit (*S2*) in front of electron trap *T*. The trap current is used to control the emission from the filament. The ion source lies immersed in the magnet field, and the electron beam, which runs in the direction of the lines of force, is strongly collimated by the field. The positive ions formed are repelled by a small positive potential applied to the electrode (*Rp*) and are withdrawn from the ionization chamber through the opposite slit, focused, and centered by the slits *J1* and *J2* and the

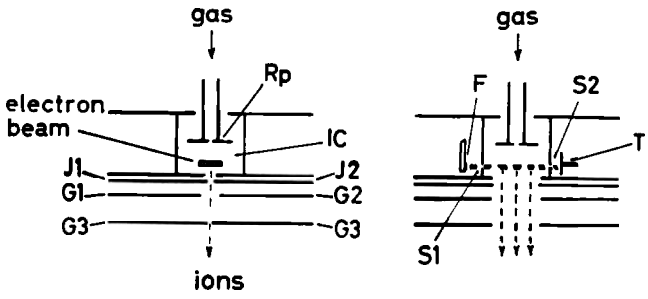


FIG. 4. Schematic drawings of Bleakney-Nier ion source. Left: View in the direction of the magnetic field. Right: Magnetic field parallel to plane of paper. For further explanation see text.

plates *G1* and *G2*, and finally accelerated by a high potential applied between the ion source exit slit (*G3*) and the ionization chamber.

Some compounds, in our experience especially bile acid derivatives, tend to give rise to conducting coatings on the insulators of the ion source structure. It is desirable, therefore, that the ion source can be easily disassembled, cleaned, and reassembled without upsetting the electrode alignment. The self-locking structure based on the use of sapphire balls as spacers and insulators (14) is particularly convenient from this point of view. Two drawings of such a source are shown in Figure 5. The end view (left) shows the device used for the adjustment of the exit slit.

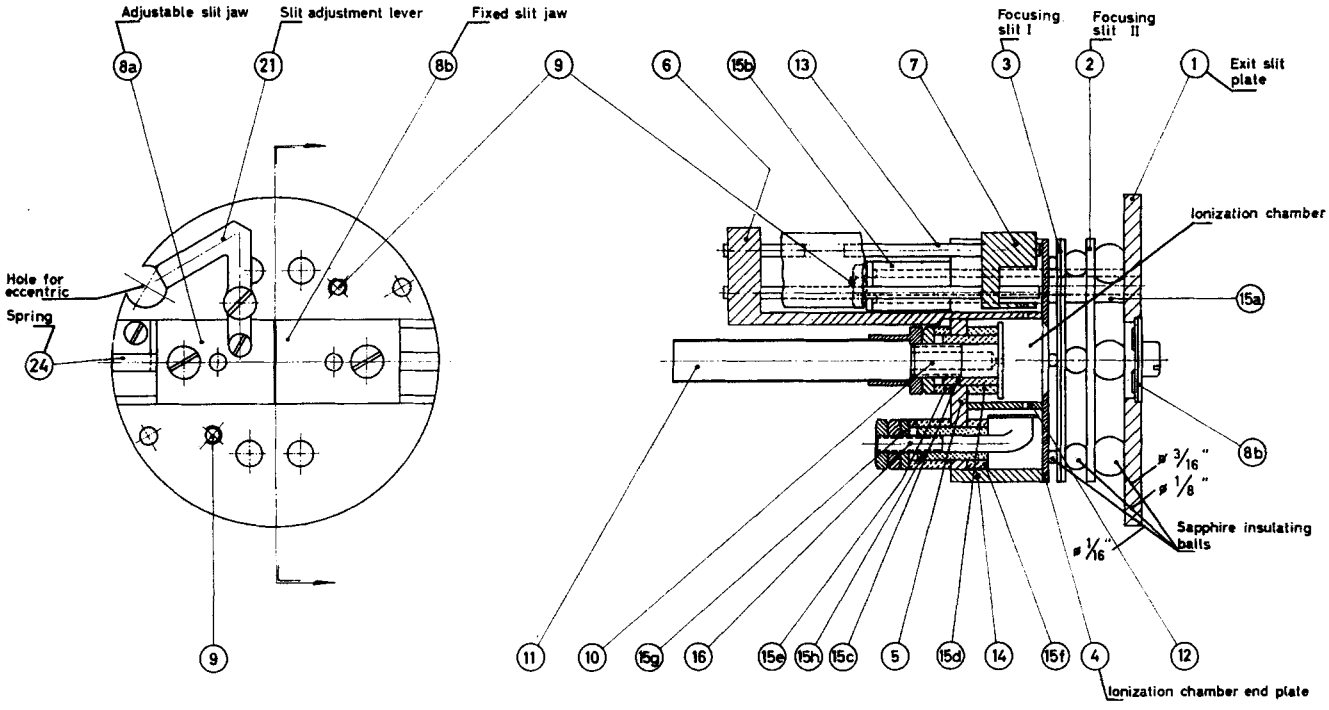


FIG. 5. Ion source. Left: End view showing mechanism for the adjustment of the exit slit. Right: Section through the source. Electron beam and magnetic field parallel to plane of paper. From Ryhage (13).

From the ion source exit slit the ions pass into the analyzer tube where they move in circular paths (of radius r cm) perpendicular to the magnetic field. In a field of H gauss an ion with a charge of e e.s.u. moving with a velocity v cm/sec is subjected to a force $\frac{H \times e \times v}{c}$ dynes (c = velocity of light in cm per sec). v is at right angles to H and this force must equal the centrifugal force. Hence.

$$\frac{m \times v^2}{r} = \frac{H \times e \times v}{c}. \qquad (1)$$

By falling through the accelerating potential V e.s.u. the ion has gained an energy $V \times e$ ergs which is equal to the kinetic energy

$$V \times e = \frac{m \times v^2}{2}. \qquad (2)$$

Elimination of v between equation (1) and equation (2) gives

$$r^2 = \frac{2V \times m \times c^2}{e \times H^2}.$$

The slightly diverging beam of ions entering the analyzer tube is brought to a focus after being deflected through 180° in the magnetic field. If a collector slit (*CS*) is placed at the distance (*2R*) across the center from the ion source exit slit (cf. Fig. 1), the following expression gives the relation between the mass over charge (m/e) ratio of the ions passing through to the collector, the accelerating potential, and the magnetic field strength

$$\frac{m}{e} = \frac{R^2 \times H^2}{2V \times c^2}.$$

Ions of different m/e's can be brought successively onto the collector either by varying the accelerating

potential V or the magnetic field strength H. Because the intensity of the ion beam increases with increasing accelerating potential, it is preferable to use magnetic scanning when a large mass range (12 to 600) is to be covered.

The current from the collector electrode is very small (10^{-14} to 10^{-10} amp) and must be amplified before recording. A sensitive "vibrating reed" electrometer with a reasonably fast response time (0.1 sec) is therefore interposed between the collector and the galvanometer recorder. The time needed to scan the spectrum is determined mainly by the response time of the recording system. The mass range 12 to 455 can be scanned in 7 minutes without impairing the resolution (13). Figure 6 shows the complete analyzing system withdrawn from the magnet. Part of the mass spectrum of cholesterol obtained with this analyzer is shown in Figure 7.

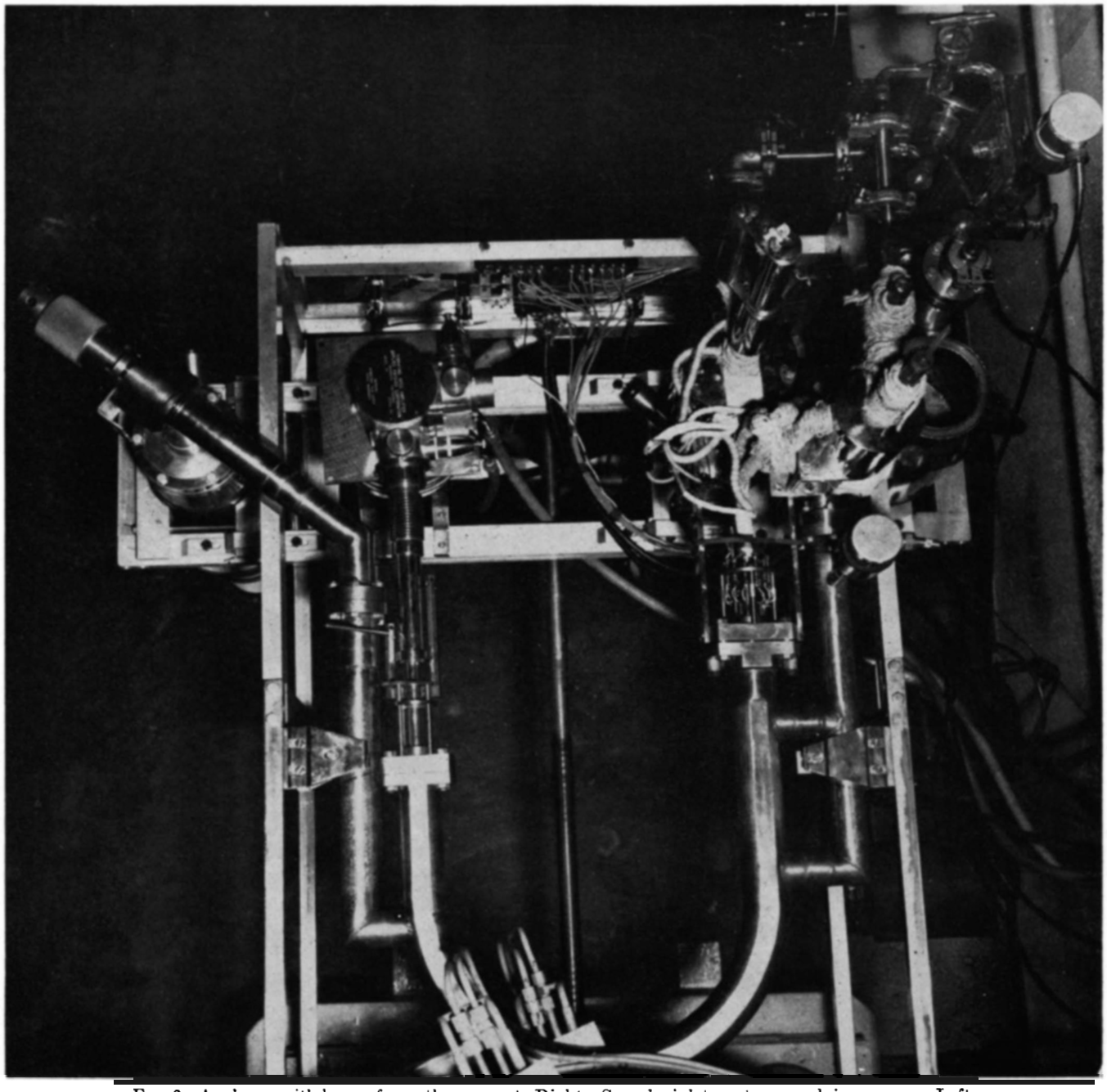


FIG. 6. Analyzer withdrawn from the magnet. Right: Sample inlet systems and ion source. Left: Collector and measuring head of vibrating reed amplifier.

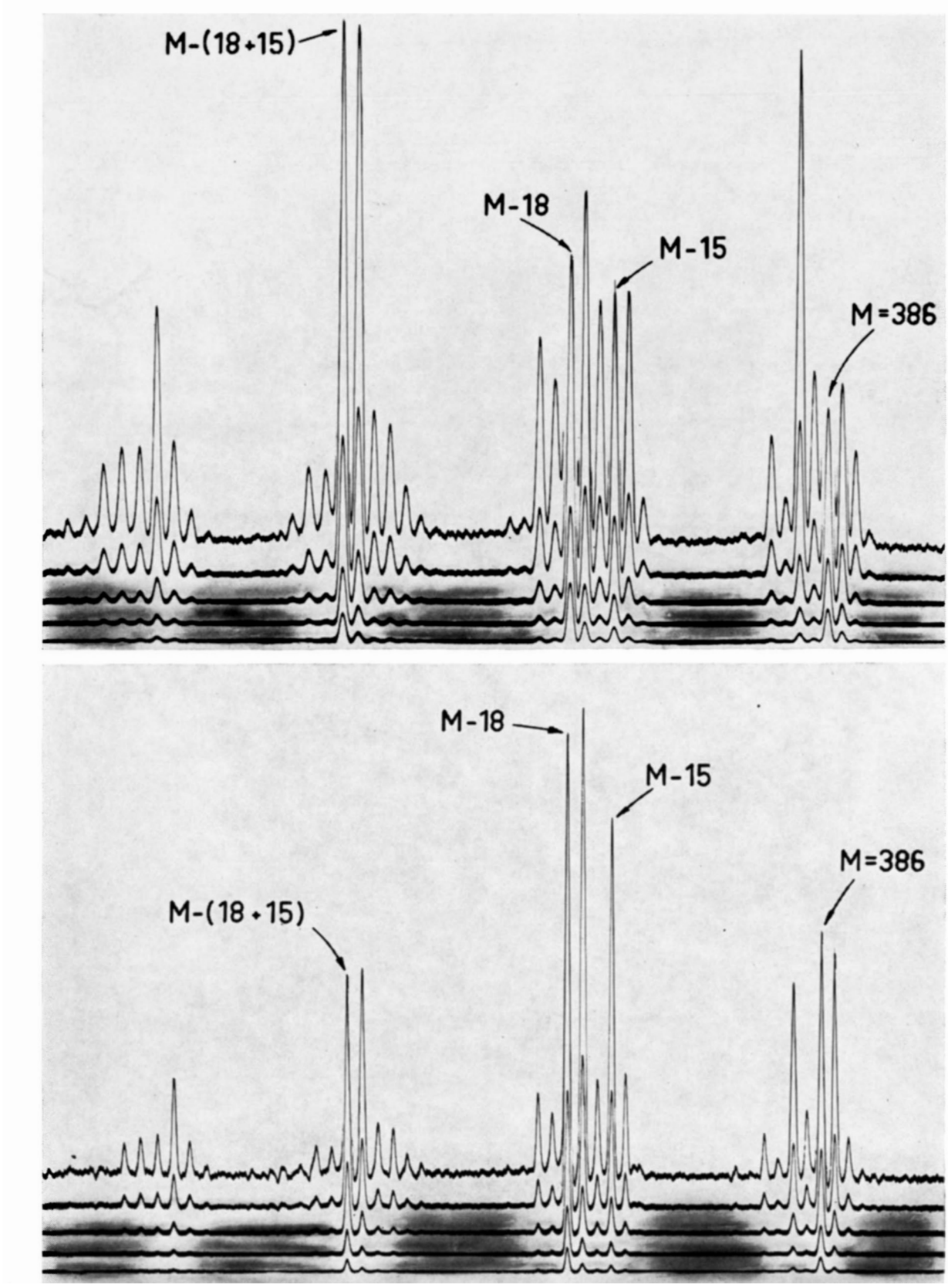


FIG. 7. High mass end of mass spectrum of cholesterol. Top: Ion source exit slit 0.1 mm and collector resolving slit 0.2 mm. Bottom: Slit widths reduced to half of those in top. From Ryhage (13). The large deflections of the most sensitive galvanometer (uppermost trace) for the three highest peaks are invisible in the reproductions.

REPRODUCIBILITY OF MASS SPECTRA; POSSIBLE DEVELOPMENTS OF THE TECHNIQUE

The mass spectra of a compound obtained with different instruments and under different experimental conditions usually do not differ to such an extent that the characteristic fragmentation pattern of the compound is not recognized in the spectra. However, the relative peak heights are influenced by a number of factors such as mass discrimination in the ion source (cf. Ref. 1, p. 12), the temperature of the ion source, and the focusing of the ion beam. When large differences are observed between mass spectra run at different temperatures of the intake system, they are usually caused by thermal cracking. In the example shown in Figure 8, the spectrum run at 100° shows characteristic peaks that make it easy to recognize the structure of the compound. At 200°, however, the glycol has lost one molecule of water and the characteristic peaks have either disappeared or are of much reduced height.

This example shows that the slow recording system, which makes it necessary to have a reservoir at rather high pressure in order to supply molecules to the ion source at a constant rate over a prolonged time, is a great limitation of the present technique. If the large reservoir and the molecular leak could be dispensed with, a great reduction in sample size would be possible. Furthermore, many compounds that decompose on heating to the temperatures needed to give a vapor pressure of 10^{-1} to 10^{-2} mm Hg would stand the temperatures needed to give a vapor pressure of 10^{-5} mm.

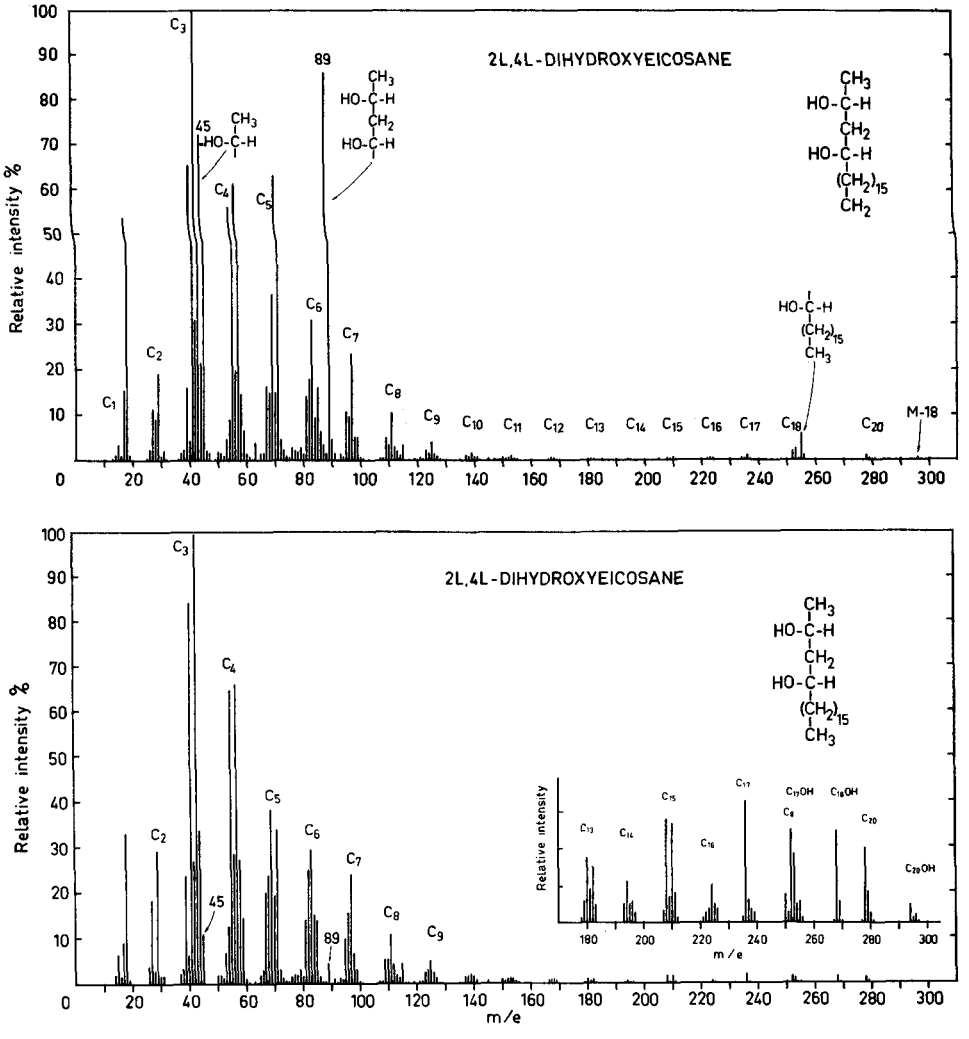


FIG. 8. Mass spectra of 2 L, 4 L-dihydroxyeicosane run at different temperatures of the intake system. Top: 100°. Bottom: 200°. From Ahlquist *et al.* (15).

A fast recording instrument with a sufficiently high resolution at high masses would thus extend the applicability of mass spectrometric structure analysis very considerably.

Because of the band width necessary for the recording of mass spectra at high speed, it is necessary to use an electron multiplier as ion detector in place of the standard ion collector. A high speed cycloidal type mass spectrometer of moderate resolving power has been described (16). Extremely high recording speed is a feature of the Bendix "time of flight" (TOF) mass spectrometer. In this instrument a pulsed ion source is used and the mass analysis performed by measuring the time taken for the ions to fly the distance between the ion source and the collector (an electron multiplier). The flight time is proportional to $\sqrt{m}$. This time-mass relation is unfavorable for the attainment of high resolution at high masses. We have had no experience of the Bendix mass spectrometer, but it appears from the published description (17) that the resolution of the instrument in its present stage of development is somewhat on the low side for structure analysis of compounds of high molecular weight.

Beynon (18) has pointed out that the use of a mass spectrometer of very high resolving power (allowing the separation of ions whose masses differ by millimass units) makes it possible to determine the empirical formulae of the various ions present. The construction of a practical double-focusing instrument with a resolving power of about one part in ten thousand is therefore an important development (19). So far, the instrument does not appear to have been used for the analysis of compounds of higher molecular weight than around 200.

THE MASS SPECTRA OF SOME TYPICAL LIPID COMPONENTS

Hydrocarbons. Although long-chain hydrocarbons themselves are perhaps not to be regarded as lipids, they are of common occurrence in nature, for instance, in the water-repellant surface coating of many leaves and fruits. Furthermore, the determination of the structure of the "parent hydrocarbon" is often an important step in the structure determination of a natural product. Because of their importance for the petroleum industry, the mass spectra of long-chain hydrocarbons have been extensively studied (9, 10).

The mass spectrum of *n*-eicosane ($C_{20}H_{42}$, $M = 282$) is shown in Figure 9. The most prominent series of ionized fragments consists of singly charged alkyl-type ions of the empirical formula $[C_nH_{2n+1}]^+$. These ions have odd mass numbers and contain an even number of electrons. A recent study (6) of a normal-chain C_{26} hydrocarbon in which both $-CH_3$ end groups had been replaced by $-CD_3$ groups showed that they are formed partly by simple cleavage of the chain, and partly from $[-(CH_2)_n- + H]$, that is, by a process involving double cleavage and capture of one hydrogen atom from one of the fragments lost, a mechanism previously suggested by Guriev and Tikhomirov (20) in order to explain the fragmentation patterns of shorter chain hydrocarbons containing heavy isotopes.

The base peak of the spectrum of Figure 9 is due to butyl ions $C_4H_9^+$. The peak heights of the alkyl-type ions decrease in a regular manner with increase in the number of carbon atoms in the ionized fragment. The molecular weight is obtained directly from the m/e of the molecule-ion, which in this case gives rise to a peak of considerable height at m/e 282 (ion of even

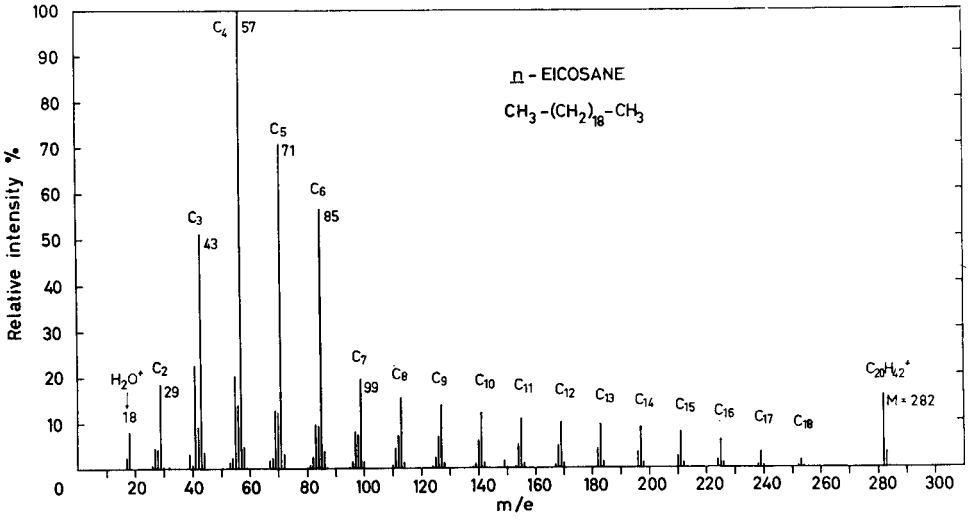


FIG. 9. Mass spectrum of *n*-eicosane. $M = 282$.

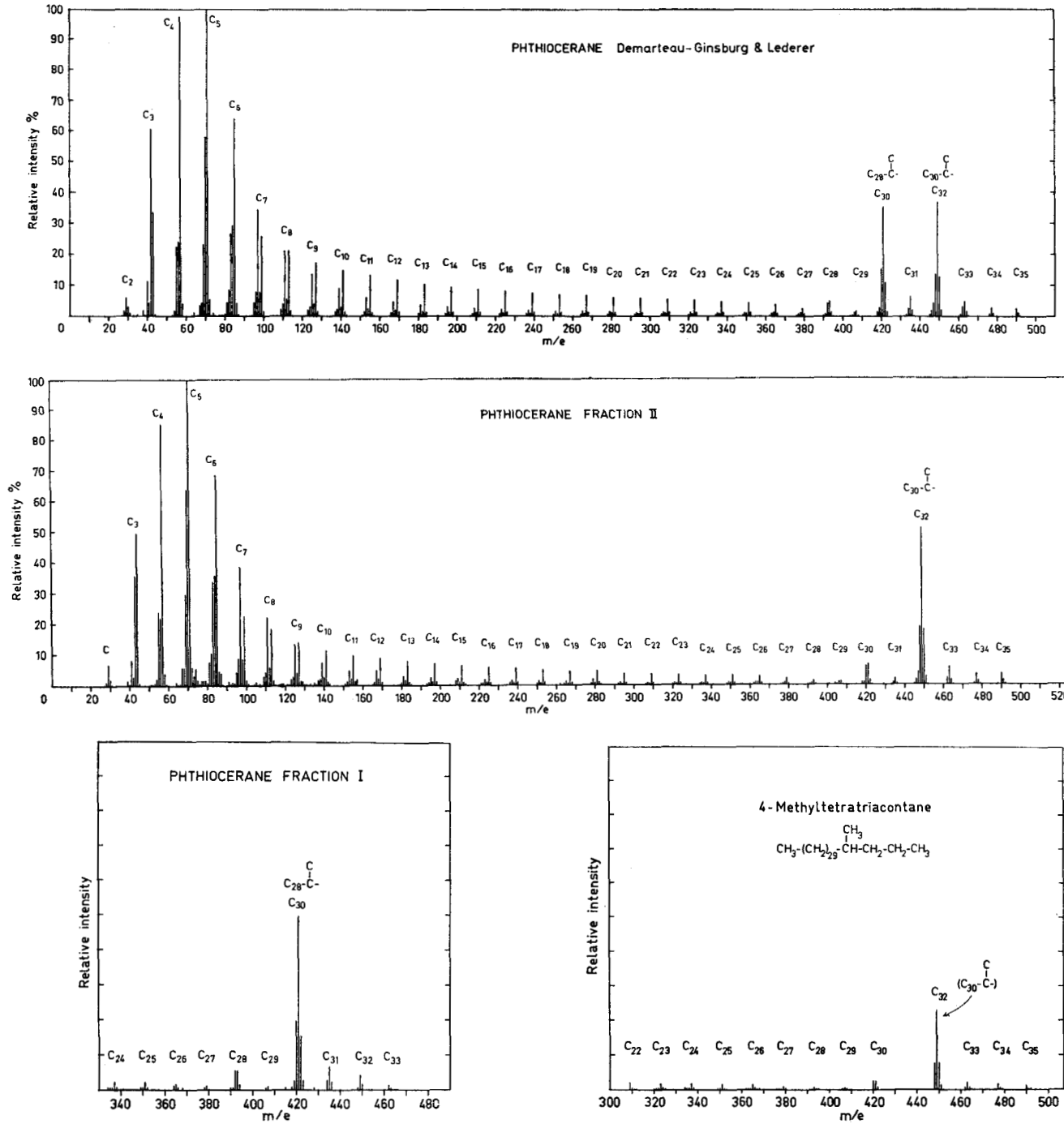


FIG. 10. Mass spectra of phthiocerane and 4-methyltetratriacontane ($M = 492$). From Ryhage *et al.* (25).

mass number containing an odd number of electrons). The molecule-ion peak (as well as all other peaks in the mass spectrum) is associated with smaller peaks of higher m/e, the strongest being that of $m/e = M + 1$. These peaks are isotope peaks caused by the presence of C^{13} and deuterium in natural carbon and hydrogen, respectively. The contribution due to C^{13}, which makes up 1.051% of natural carbon, is the most important. For statistical reasons, the isotope peak of $m/e = m + 1$ increases in height by about 1% for

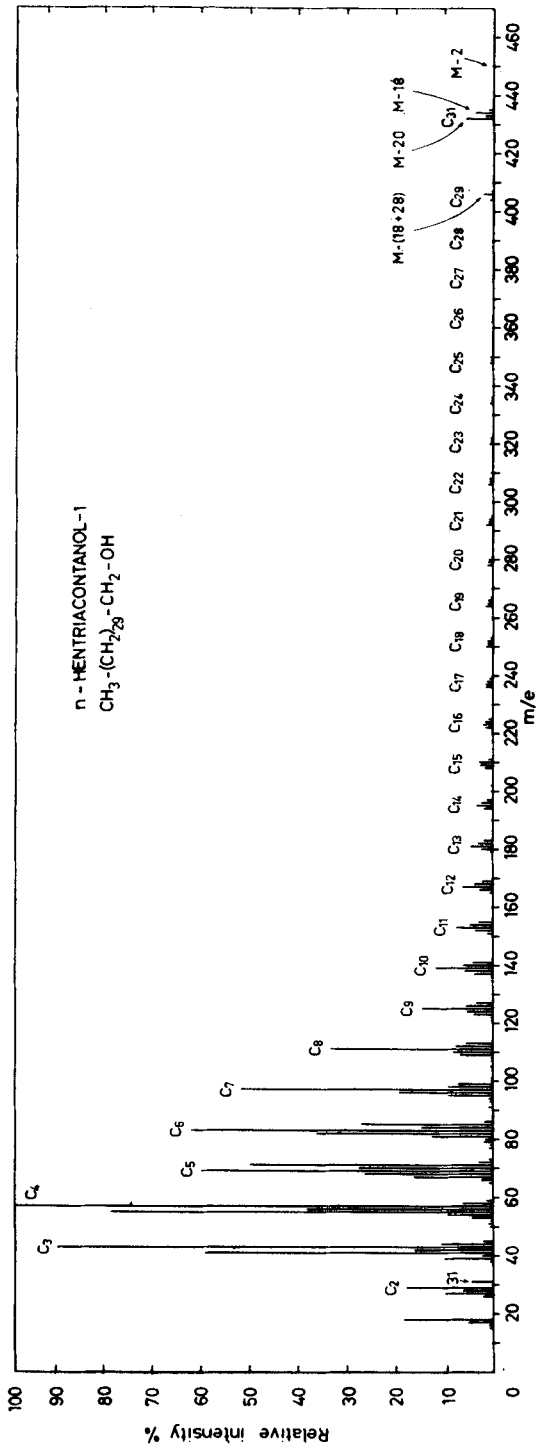


FIG. 11. Mass spectrum of *n*-hentriacontanol-1. $M = 452$.

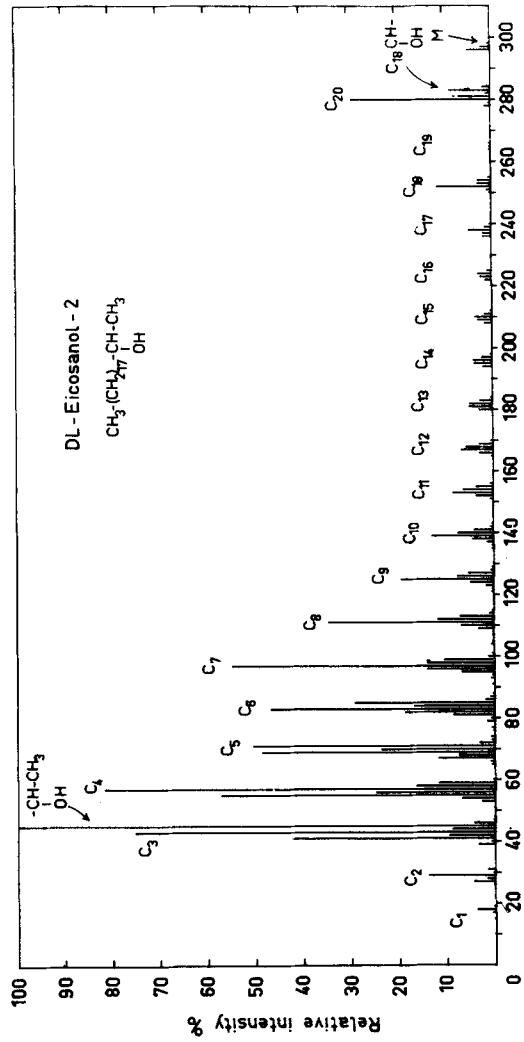
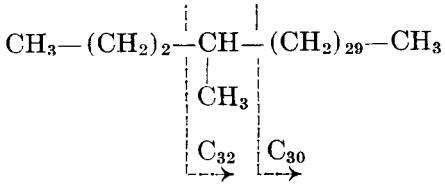


FIG. 12. Mass spectrum of DL-eicosanol-2. $M = 298$.

each carbon atom added to the ion. Series of peaks corresponding to ions of the empirical formulae $[C_nH_{2n}]^+$ and $[C_nH_{2n-1}]^+$ are also present in the spectrum of Figure 9.

Methyl-substituted, long-chain hydrocarbons show a strong tendency to cleavage of the chain on either side of the tertiary carbon atom carrying the methyl group. The molecule-ion peak is small or absent from the spectrum. As an example of a mass spectrometric identification of a hydrocarbon of this type, we may take phthiocerane, the hydrocarbon obtained on drastic reduction of the wax alcohol phthiocerol present in the human and bovine types of tubercle bacilli (19). Previous work had shown that phthiocerane had a methyl side chain at position 4 (21, 22, 23), and that the properties of the hydrocarbon did not agree completely with those of any synthetic 4-methyl-substituted hydrocarbon.

The mass spectrum of a recently prepared specimen (24, 25) is shown in Figure 10 (top). Gas-liquid chromatography showed that this specimen consisted of two apparently homologous compounds (25). These were isolated, and the mass spectrum of the higher homologue is shown in Figure 10 (middle). The peaks of the C_{30} and particularly the C_{32} group are much higher than those of the neighboring groups. It is evident that the hydrocarbon contains 35 carbon atoms. 4-Methyltetratriacontane would be expected to give rise to abundant C_{30} and C_{32} fragments,



a conclusion confirmed by the examination of synthetic material (Fig. 10, bottom, right).

Much information about mass spectra of hydrocarbons of high molecular weight is found in a paper by O'Neal and Wier (9).

Alcohols. Normal long-chain primary alcohols are important components of waxes. Their mass spectra have been studied by Brown *et al.* (26) H. Bergström *et al.* (27), and McLafferty (28). The relations between structure and mass spectrum in alcohols of different types having 13 carbon atoms or less have been studied by Friedel *et al.* (29).

The mass spectrum of *n*-hentriacontanol-1 ($C_{31}H_{64}O$, $M = 452$) is shown in Figure 11. The presence of a primary alcohol group is indicated by a small peak at m/e 31, presumably caused by the ion $—CH_2OH^+$. No peak due to the molecule-ion is observed, the heaviest ion being the one of $m/e = M - 2$. The highest peaks in the high mass range are the hydrocarbon type peaks at $m/e = M - 18$ and $M - 20$, corresponding to ions formed with loss of water, and water plus one molecule of hydrogen, respectively, from the molecule-ion. There is also a significant peak at m/e 406 ($= M - [18 + 28]$), formally corresponding to an ionized fragment formed with loss of one molecule of water and molecule of ethylene from the molecule-ion. Friedel *et al.* (29) have pointed out the great similarity between the hydrocarbon part of the mass spectrum of a primary alcohol and that of the 1-olefin obtained by dehydration of the alcohol.

The structure of the long-chain secondary alcohol eicosanol-2 is readily deduced from the mass spectrum shown in Figure 12. The dextrorotatory enantiomer of this alcohol occurs in the waxes of certain acid-fast bacteria (30). The peak at $m/e = M - 2$ is the highest in the group of three small peaks at the high mass end of the spectrum. As was the case for the primary alcohol, hydrocarbon-type peaks appear at m/e 280 ($= M - 18$) and 252 ($= M - [18 + 28]$). The position of the hydroxyl group is indicated by the ionized fragments of m/e 45 and $m/e = M - 15$, which are formed, respectively, with loss of the long alkyl group and the methyl group, cleavage taking place on either side of the carbon atom carrying the hydroxyl group.

Long-chain methyl ethers have also the base peak at m/e 45 due to ions $[—CH_2—O—CH_3]^+$ (31, 32). The ether is readily distinguished from the isomeric 2-alkanol by the absence of a peak at $m/e = M - 18$.

The branched-chain alcohol, 16-methylheptadecanol-1 (*iso*octadecyl alcohol), whose mass spectrum is reproduced in Figure 13, gives, in addition to the peaks at m/e 31, $M - 18$, and $M - (18 + 28)$ characteristic of long-chain primary alcohols, a fairly high peak at m/e 196 ($= M - [31 + 43]$). The $[C_{14}H_{28}]^+$ ion responsible for this peak is evidently formed with simultaneous loss of the $—CH_2OH$ group and the terminal isopropyl group from the molecule-ion.

Alkoxyglycerols. A series of new alkoxyglycerols have recently been reported by Hallgren and Larsson (33). The molecular weight and structure of the isolated compounds were determined by mass spectrometry after conversion into the corresponding dimethyl ethers (34). The mass spectrum of batyl dimethyl ether derived from a sample of synthetic batyl alcohol is shown in Figure 14. The fragmentation pattern is a highly characteristic one. The molecular weight is obtained from the even-numbered molecule-ion peak at m/e 372. High peaks occur at m/e 45 and 89 due to

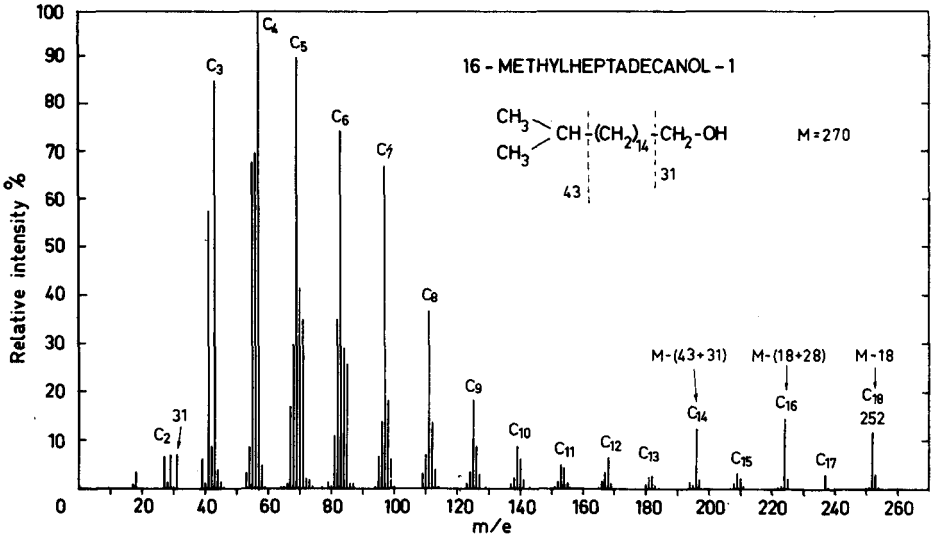


FIG. 13. Mass spectrum of 16-methylheptadecanol-1 (*iso*stearyl alcohol). $M = 270$.

ions containing one and two methyl ether groups, respectively. The alkyl peak at m/e 253 indicates the presence of a saturated C_{18} chain. The high peaks at m/e 58 (base peak) and 59 are due to rearranged ions. As indicated in Figure 14, several characteristic peaks appear in addition to those mentioned, making the identification of the compound a relatively easy matter.

Methyl Esters of Fatty Acids. The mass spectrum of a long-chain carboxylic acid is closely analogous to that of the methyl ester of the acid. As the methyl esters are more volatile than the free acids, they are usually the derivative used in gas chromatographic separations and mass spectrometric structure determinations. An extensive study of esters of lower aliphatic acids has been carried out by Sharkey *et al.* (35).

The mass spectrum of a normal long-chain methyl ester (36) such as methyl stearate (Fig. 15) is dominated by peaks due to ions containing oxygen. The hydrocarbon peaks of the spectrum can be almost completely suppressed by running the spectrum at reduced electron energy ($\sim$ 11 electron volts) (6). The molecule-ion peak is very prominent and increases in height relative to the base peak with increasing molecular weight of the ester, at least up to $C_{35}H_{70}O_2$ (cf. Ref. 12). The base peak at m/e 74 is due to ions

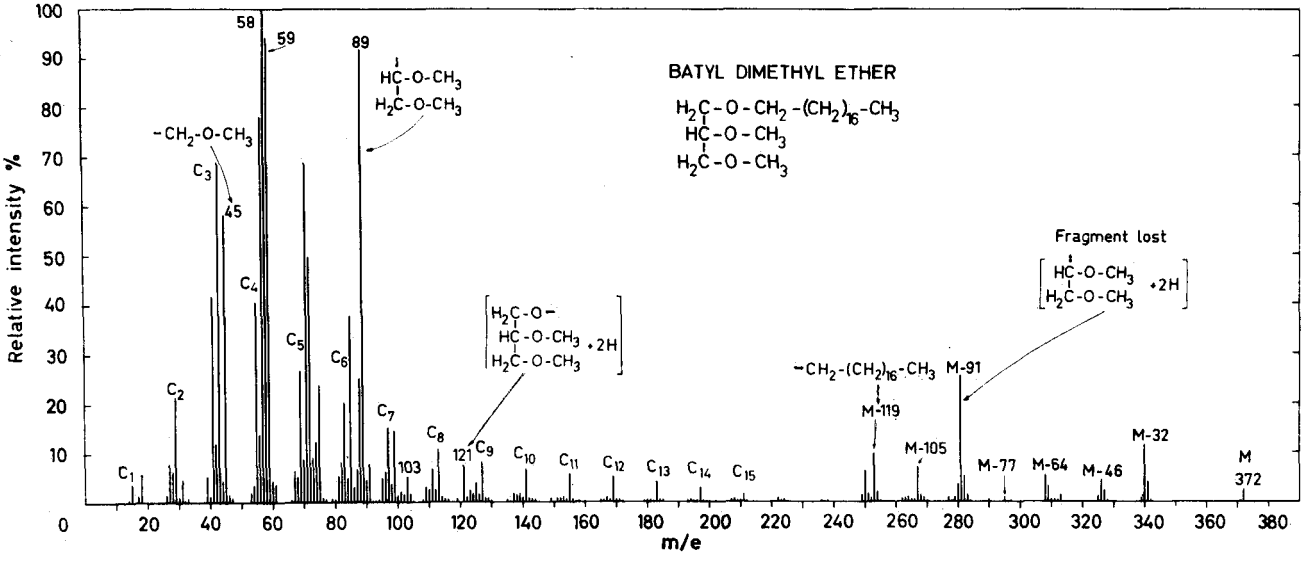
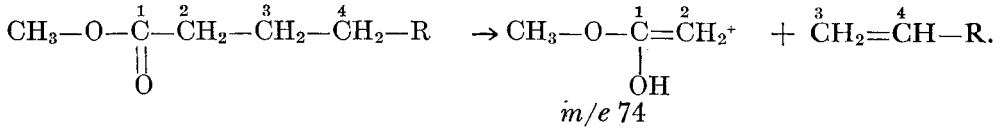


FIG. 14. Mass spectrum of batyl dimethyl ether. $M = 372$.

formed on 2,3-cleavage with simultaneous migration of one hydrogen atom from the fragment lost. Migration of two hydrogen atoms also occurs to some extent because the peak at m/e 75 is higher than expected for being solely the isotope peak associated with the peak at m/e 74. The ions of m/e 74 are analogous to the ions of m/e 60 in the case of free carboxylic acids (37). It has been suggested that the hydrogen is taken from carbon atom 4, leading to the formation of a neutral 1-olefin (38, 39, 40):



m/e 74

Direct evidence that the hydrogen atom is taken exclusively from carbon atom 4 comes from a study of a series of methyl esters with one CD_2 group instead of CH_2 at all positions from 2 through 6 (6). Measurements of the electron energy at which ions of m/e 74 first appear ("appearance potential" measurements) are considered to show that the ion has the hydroxyl form shown above rather than the alternative keto-form (41).

Apart from the base peak and the peak due to the acylium ion ($m/e = M - 31$), the mass spectrum of the methyl ester contains peaks due to oxygen-containing fragments of the general type $\left[CH_3—O—C—(CH_2)_n—\right]^+$ ($n > 1$). It has been shown (6) that these fragments are formed by two different mechanisms. The ions of lowest m/e (87, 101, 115, and 129) are formed with simple cleavage of the chain. However, from m/e 143 ions of this series are partly formed through a process involving elimination of part of the chain. One hydrogen atom is lost in addition to a number of methylene groups, the final result being an odd-numbered even-electron ion. The reaction may be looked upon as a kind of transhelical reaction in a molecule in which the hydrocarbon chain has a helical or quasi-ring conformation. The carbon atoms nearest to the carbonyl group are lost with particular ease, as the peaks at $m/e = M - 29$ and $M - 43$ are almost exclusively due to ions formed with loss of $\left[CH_2—CH_2 + H\right]$ and $\left[CH_2—CH_2—CH_2 + H\right]$ respectively, from the molecule-ion. Side chains at positions 2-4 are lost, together with the carbon atoms to which they are attached (see below).

Methyl Esters of Branched-Chain Fatty Acids. A methyl side chain at position 2 causes a shift of

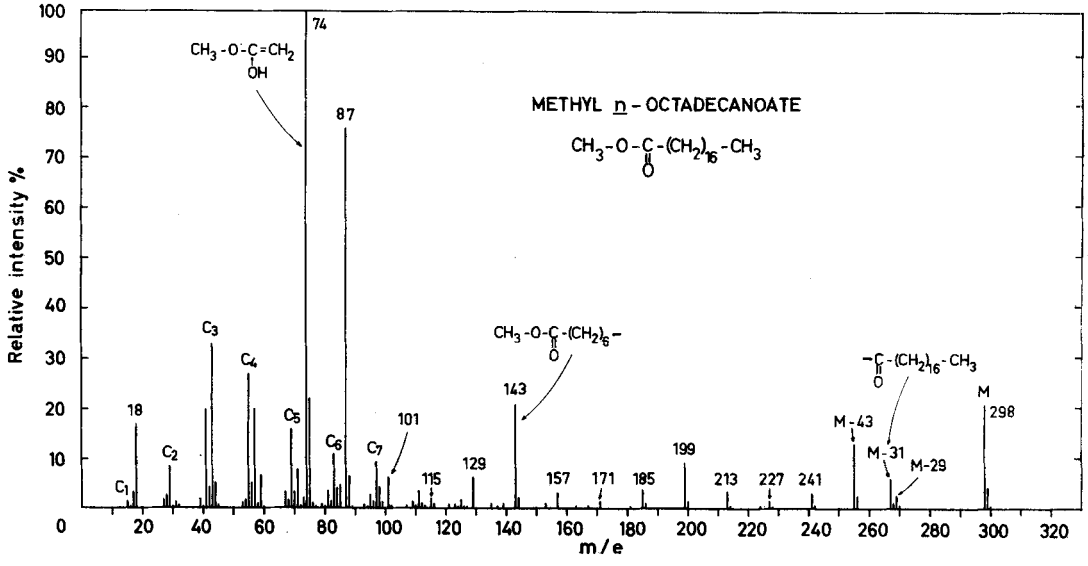
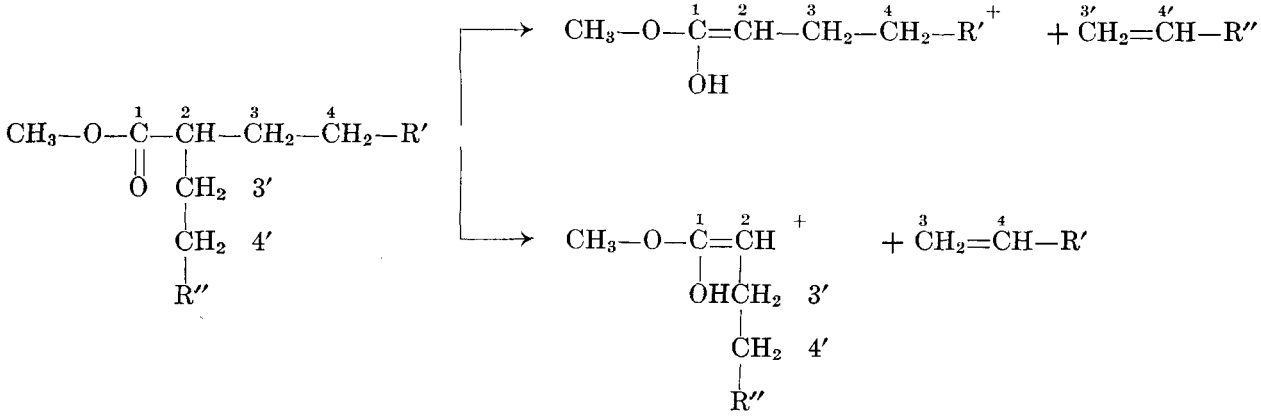


FIG. 15. Mass spectrum of methyl n-octadecanoate (methyl stearate). $M = 298$.

the base peak to m/e 88, the side chain being incorporated in the rearranged ionized fragment $\left[CH_3-O-\underset{\underset{OH}{|}}{C}=\underset{\underset{CH_3}{|}}{CH}\right]^+$. In the high mass range there appear peaks at $m/e = M - 43$ and $M - 57$ due to ions formed with loss of $\left[\overset{2}{C}H(CH_3)-\overset{3}{C}H_2 + H\right]$ and $\left[\overset{2}{C}H(CH_3)-\overset{3}{C}H_2-\overset{4}{C}H_2 + H\right]$ from the molecule-ion, whereas the peak at $m/e = M - 29$ is smaller than for normal chain esters (7).

If the side chain at position 2 is an ethyl or longer alkyl group, two rearranged ions are observed (8):



The mass spectrum of methyl tuberculostearate ([-]-methyl 10-methyloctadecanoate) (42, 43) is shown in Figure 16. The position of the side chain is indicated by the characteristic pattern consisting of the very small peak at m/e 185 and the fairly strong peaks at m/e 171 (with associated rearrangement peaks at m/e 172 and 173) and m/e 199, as well as the strong peak at m/e 167 ($= 199 - 32$). The last-mentioned peak is presumably due to a ketene-type ion (7)

$$C(=O)-CH-(CH_2)_7-\underset{\underset{CH_3}{|}}{CH}-$$

Methyl 15 DL-methylheptadecanoate (*anteiso*stearate) (Fig. 17) shows analogous peaks at m/e 269 ($= M - 29$) and 241 ($= M - 57$), as well as at m/e 237 ($= 269 - 32$). It is to be noted that in this case the peak at $m/e = M - 29$ is higher than that due to the acylium ion at $m/e = M - 31$, due to the contribution to the $m/e = M - 29$ peak from ions formed by loss of the ethyl group from the tertiary carbon atom at position 15.

Methyl esters of *iso*acids give mass spectra that are very similar to those given by normal chain esters. The former show a characteristic small peak at $m/e = M - 65$ (at m/e 233 in the mass spectrum of methyl *iso*stearate shown in Fig. 18). The *iso*structure is best recognized after reduction to the corresponding alcohol (Fig. 13).

Direct mass spectrometric structure determination is possible also in many complicated cases. The structure of the methyl ester of the major component acid of Anderson's mycocerosic acid (44) was shown, by the mass spectrum reproduced in Figure 19, to be the methyl ester of a saturated C_{32} acid ($M = 494$) (45).

The strong peak at m/e 88 indicated a methyl side chain at position 2. The occurrence of very small peaks at m/e 115, 157, and 199, together with much stronger peaks at m/e 101, 129, 143, 171, 185, and 213 suggested the presence of methyl side chains at positions 4, 6, and 8. The occurrence of peaks at $m/e = M - 43$ and $M - 71$ in the high mass range is consistent with the presence of methyl side chains at positions 2 and 4, the ions being formed through loss of $\left[\overset{2}{C}H(CH_3)-\overset{3}{C}H_2 + H\right]$ and $\left[\overset{2}{C}H(CH_3)-\overset{3}{C}H_2-\overset{4}{C}H(CH_3) + H\right]$ from the molecule-ion. The results were confirmed by a study of the alcohol obtained from the methyl ester by reduction with lithium aluminium hydride. The mass spectrum of the alcohol showed large hydrocar-

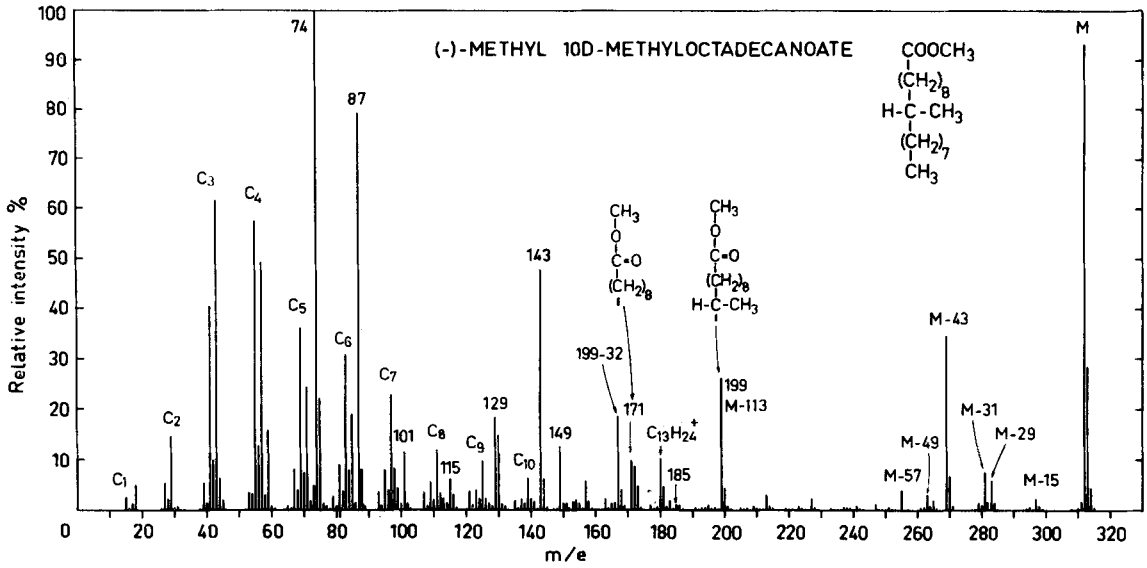


FIG. 16. Mass spectrum of (-)-methyl 10 D-methyloctadecanoate (methyl tuberculostearate). $M = 312$. From Ryhage and Stenhagen (7).

bon-type peaks at m/e's consistent with the presence of methyl side chains at positions 4, 6, and 8. As the evidence for the presence of a methyl group at position 2 is very strong from the spectrum of the methyl ester, the identification of the latter as a methyl 2,4,6,8-tetramethyloctacosanoate is beyond doubt. The mass spectra of diastereoisomeric aliphatic compounds of this type are identical (8) and the mass spectrum of Figure 19 gives no information regarding the optical configuration of the tetramethyl-substituted ester.

Methyl Esters of Oxo-, Hydroxy-, and Methoxy Acids. The presence and the position of an oxo- hydroxy-, or methoxy group can usually be readily recognized from the mass spectrum (46). The mass spectrum of methyl 8-oxoöctadecanoate reproduced in Figure 20 shows peaks due to ions formed with simple cleavage of the bonds to the carbonyl carbon atom in the chain (7,8 and 8,9 cleavage, respectively) and to ions formed through 6,7 and 9,10 cleavage, respectively, with rearrangement of one hydrogen atom. The mole-

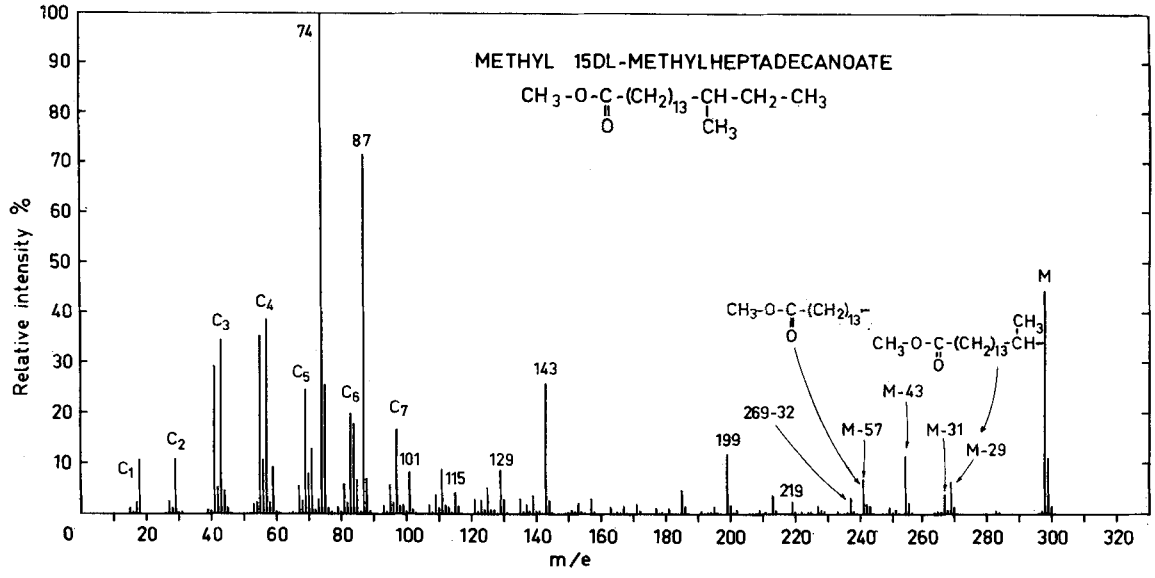


FIG. 17. Mass spectrum of 15 DL-methylheptadecanoate (methyl *anteiso*stearate). $M = 298$. From Ryhage and Stenhagen (7).

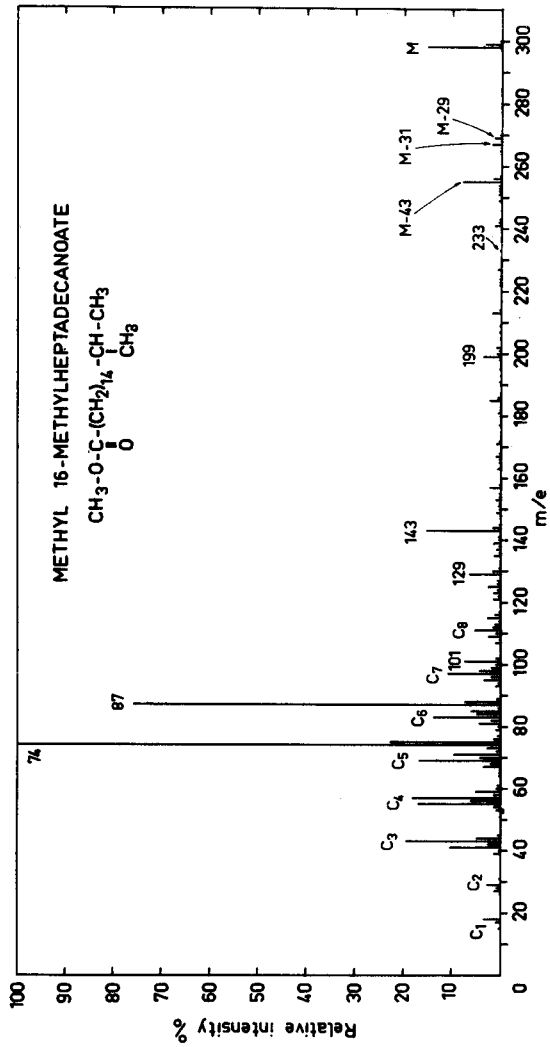


FIG. 18. Mass spectrum of 16-methylheptadecanoate (methyl *iso*stearate). $M = 298$. From Ryhage and Stenhagen (7).

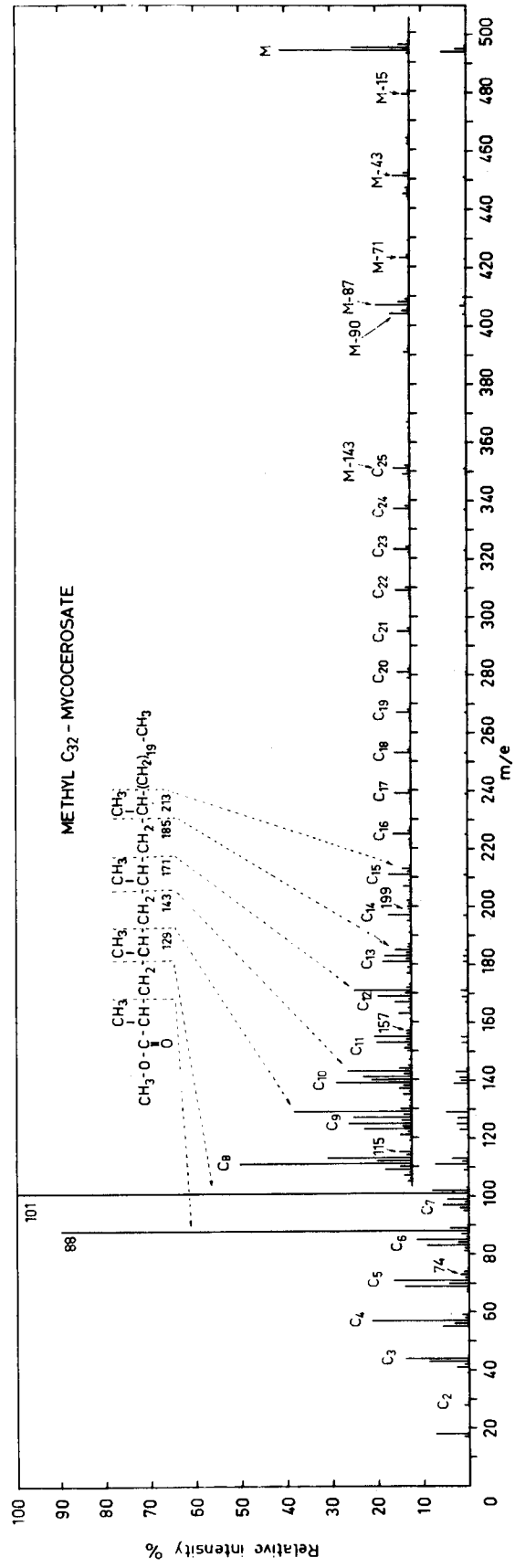


FIG. 19. Mass spectrum of C_{32}-mycocerosate. $M = 494$. Cf. Asselineau *et al.* (45).

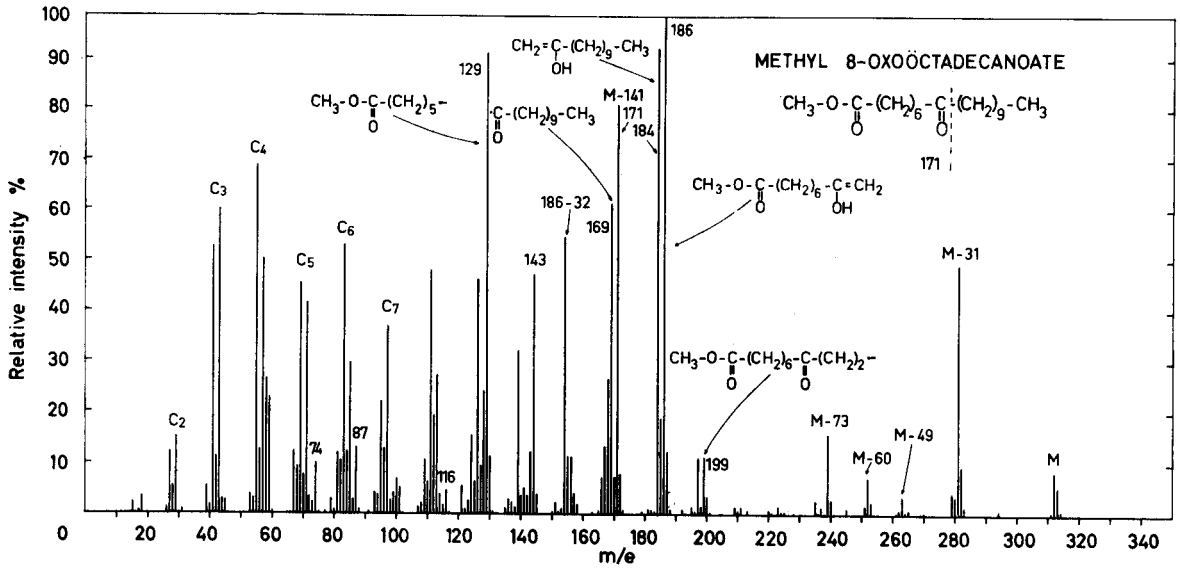


FIG. 20. Mass spectrum of methyl 8-oxoöctadecanoate. M = 312. From Ryhage and Stenhagen (46).

cule-ion peak at m/e 312 is accompanied by a peak at $m/e = M + 1$ that is much stronger than the normal isotope peak. The abnormal height of this peak is due to ions formed by secondary reactions in the ion source, involving the addition of a hydrogen atom to the molecule-ion. Peaks of this type can be recognized by altering the pressure in the ion source, as their relative heights increase when the pressure is increased. The corresponding hydroxy ester gives a simpler mass spectrum (Fig. 21) with high peaks at m/e 173 due to ions formed on cleavage of the chain on the far side of the hydroxyl group (8,9 cleavage) and at m/e 141 due to the associated ketene ion. Cleavage on the near side of the hydroxyl group (7,8 cleavage) occurs with rearrangement to give ions of m/e 144.

The hydroxy esters usually do not show a molecule-ion peak, but their molecular weight may be obtained from the peak at $m/e = M - 50$ (= −[32 + 18] [water + methanol; cf. monoethenoid esters]), which is of significant height except in the case of the 2-hydroxy esters. Methyl esters of 2-hydroxy acids give a large peak at $m/e = M - 59$ due to ions formed through the easy cleavage of the bond between carbon atoms 1 and 2. They furthermore show a very charac-

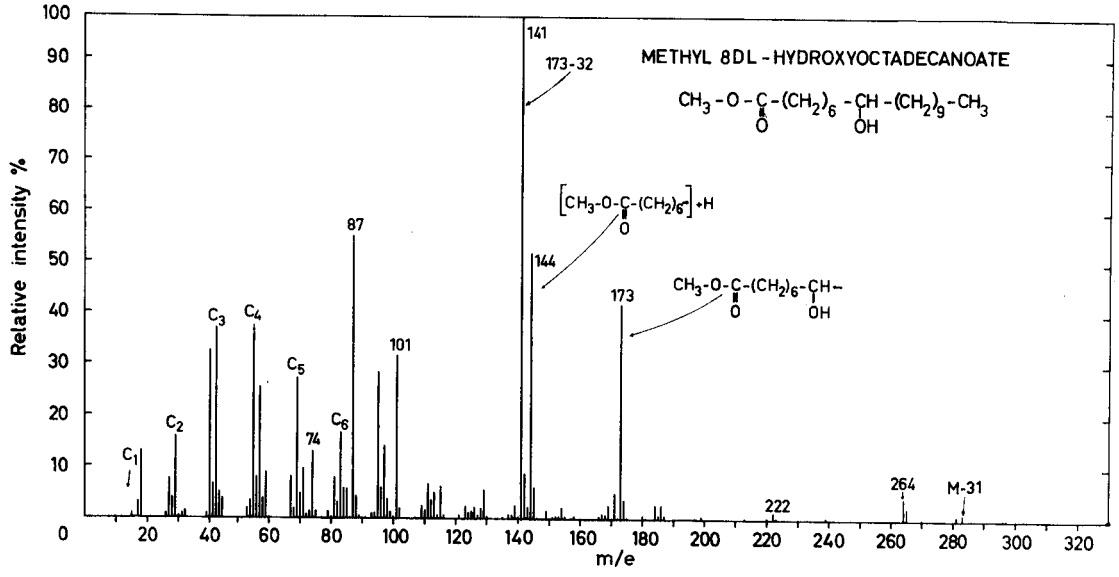


FIG. 21. Mass spectrum of methyl 8 DL-hydroxyoctadecanoate. M = 314. From Ryhage and Stenhagen (46).

teristic peak at m/e 90 corresponding to a rearranged ion $\left[\begin{matrix}CH_3-O-C=CH\\ \quad\quad\quad\quad |\quad\quad |\\ \quad\quad\quad\quad OH\ OH\end{matrix}\right]^+$.

However, the simplest mass spectra are those of the methoxy esters. The base peak of the spectrum of methyl 2-methoxytricosanoate (Fig. 22) is at $m/e = M - 59$. As in the case of the 2-hydroxy ester, cleavage readily occurs between carbon atoms 1 and 2. In the high mass range there is also observed, in addition to the base peak and the peak due to the molecule-ion, a peak at $m/e = M - 73$, which presumably corresponds to a rearranged ion formed with elimination of $\left[\begin{matrix}2\quad 3\quad 4\\ CH-CH_2-CH_2+H\\ |\\ OCH_3\end{matrix}\right]$ from the molecule-ion.

The simple mass spectra exhibited by methoxy esters with the methoxy-group higher up in the chain is exemplified by the spectrum of methyl 11-methoxyoctadecanoate shown in Figure 23. The two highest peaks in the spectrum are due to ions containing the methoxyl

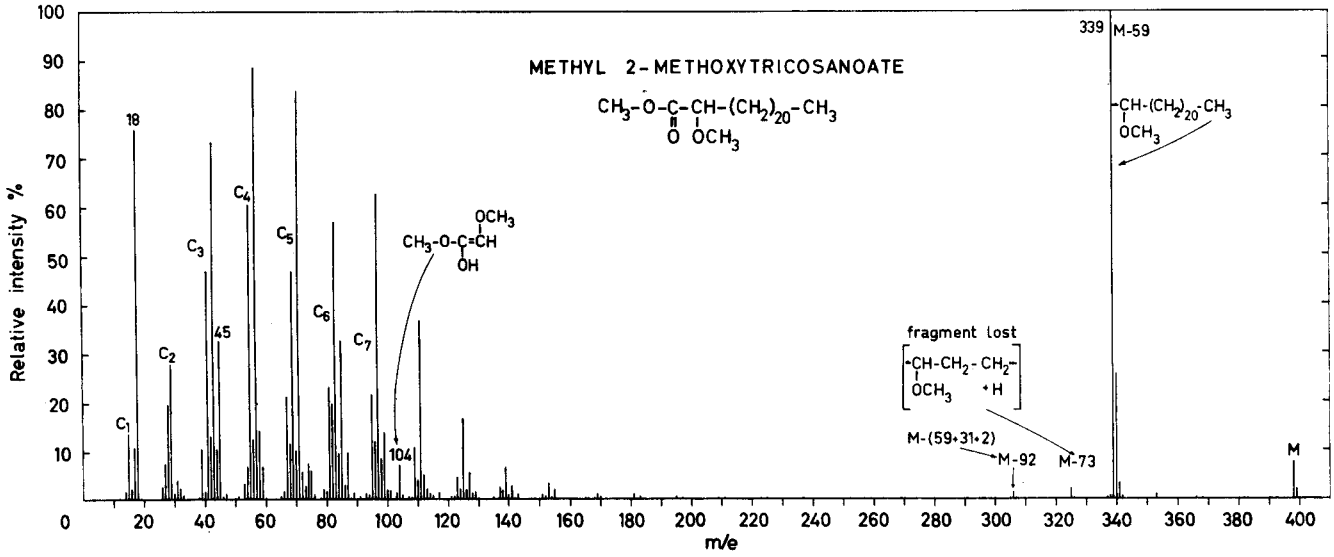


FIG. 22. Mass spectrum of methyl 2D-methoxytricosanoate. $M = 398$. From Ryhage and Stenhagen (46).

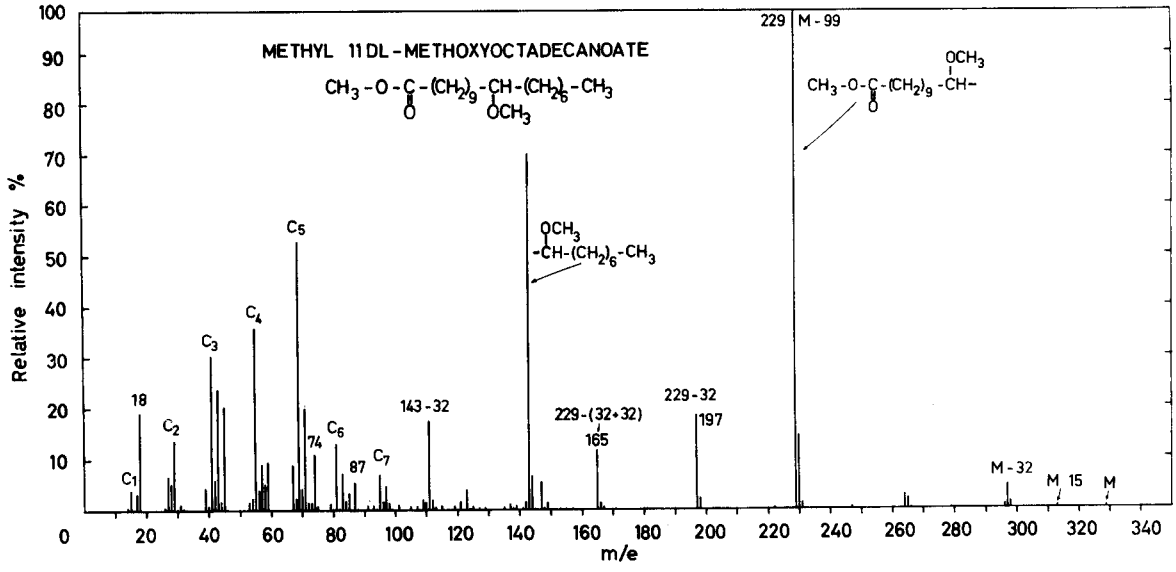


FIG. 23. Mass spectrum of methyl 11 DL-methoxyoctadecanoate. $M = 328$. From Ryhage and Stenhagen (46).

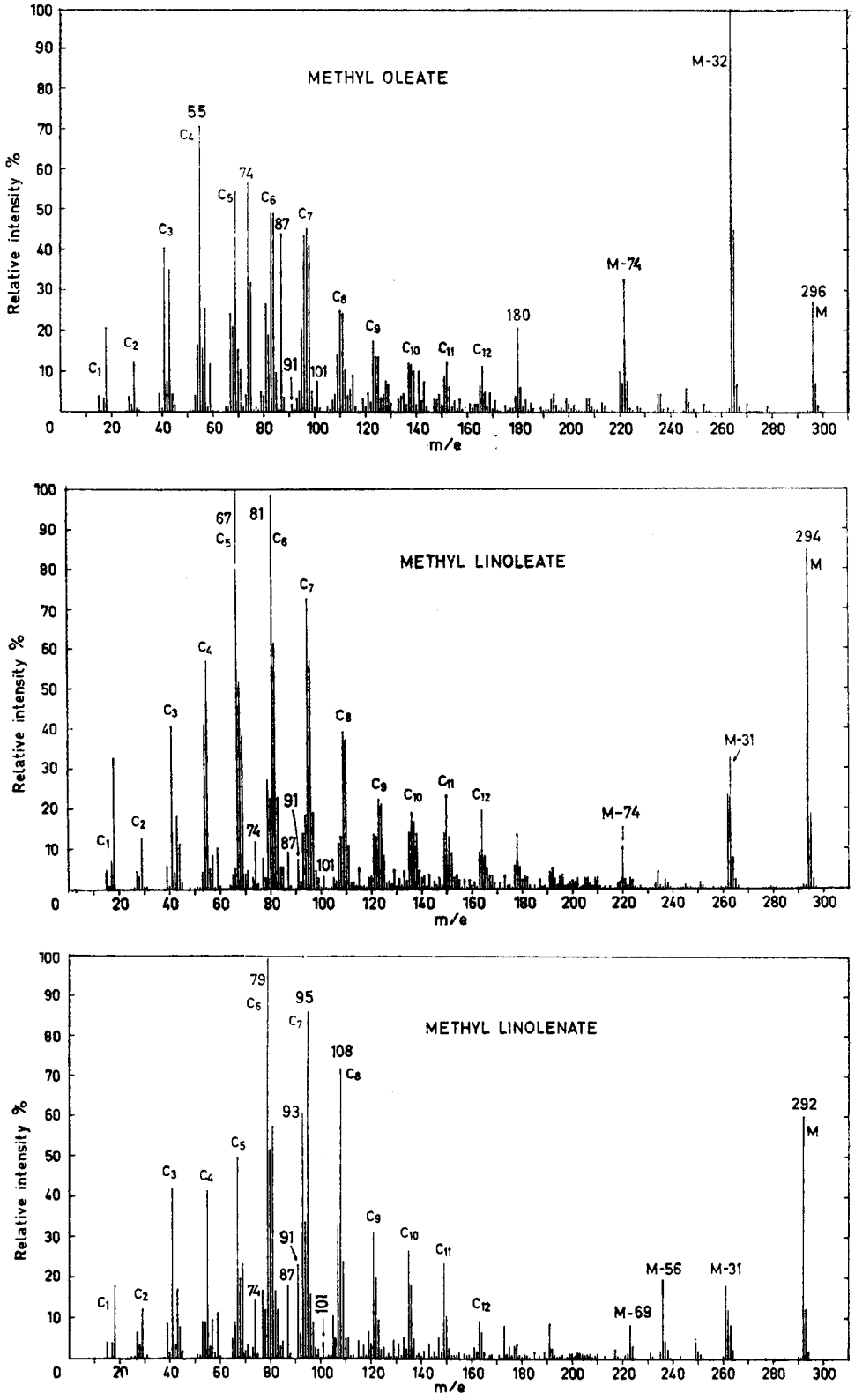


FIG. 24. Mass spectra of: (top) methyl oleate (methyl $\Delta^{9:10}$ -octadecenoate) ($M = 296$); (middle) methyl linoleate (methyl $\Delta^{9:10,12:13}$ -octadec-dienoate) ($M = 294$); (bottom) methyl linolenate (methyl $\Delta^{9:10,12:13,15:16}$ -octadec-trienoate) ($M = 292$). From Hallgren *et al.* (48).

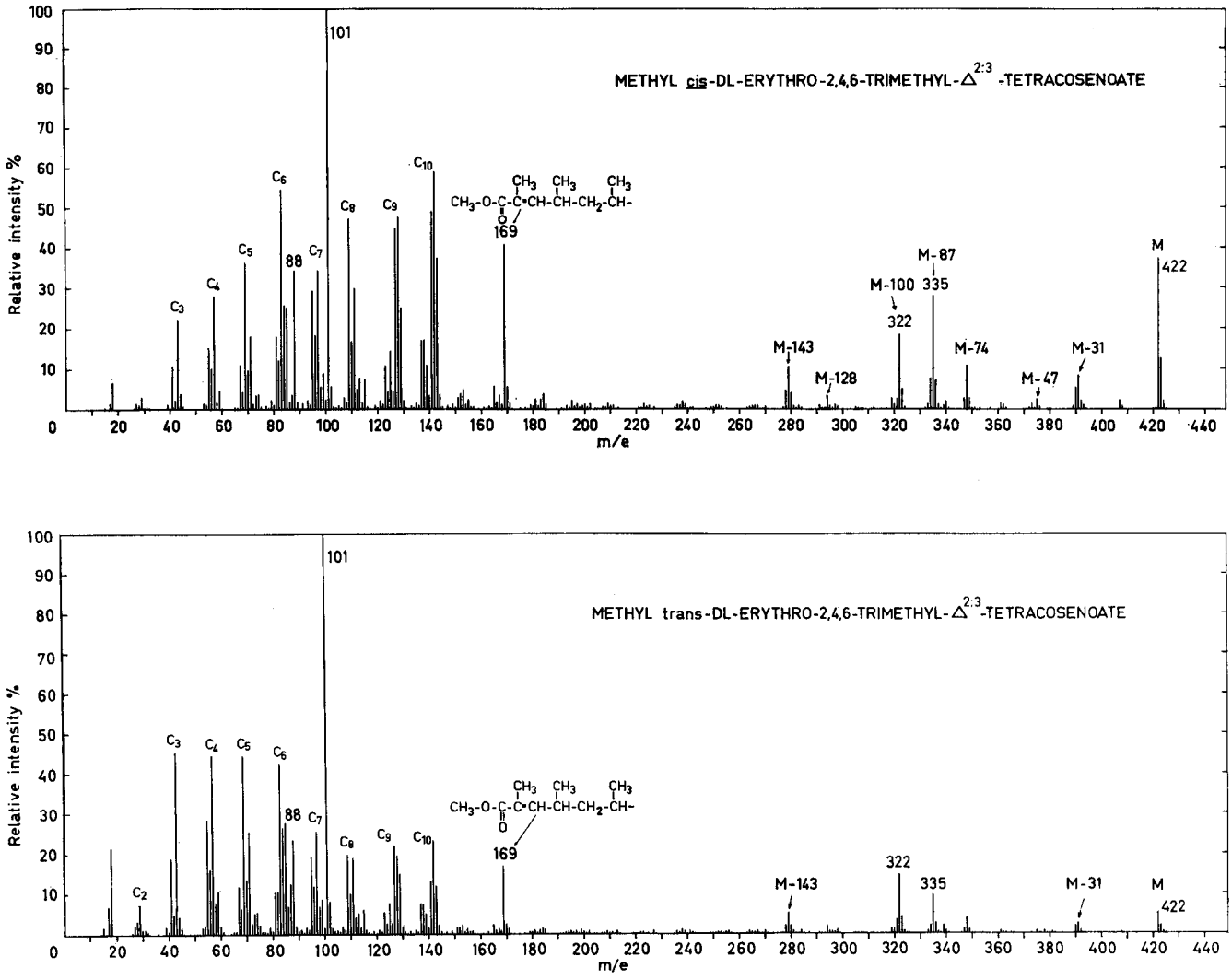


FIG. 25. Mass spectra of methyl esters of α,β-unsaturated branched-chain acids. Reproduced with minor corrections from Ahlquist *et al.* (52).

group, formed through cleavage of the chain on either side of carbon atom 11.

Methyl Esters of Dibasic Acids. Dibasic acids are often obtained in the oxidative degradation of lipids. Dimethyl esters of dibasic acids are readily recognized through their mass spectra, which show a characteristic pattern in the high mass range, consisting of a small molecule-ion peak, a high peak at $m/e = M - 31$, and moderately strong peaks at $m/e = M - 64$, $M - 73$, $M - 92$, and $M - 105$ (47). In addition, they show a series of strong peaks at $m/e = 84 + n \times 14$, which is not present in the mass spectra of methyl esters of monobasic acids.

Methyl Esters of Unsaturated Acids. The molecular weight and the degree of unsaturation of a methyl ester of an unsaturated fatty acid is immediately apparent from the molecule-ion peak, which is usually very prominent (48), as shown in Figure 24. Methyl oleate has the base peak at $m/e = M - 32$, formally corresponding to an ion formed with loss of one molecule of methanol from the molecule-ion. Further characteristic peaks at $m/e = M - 74$ and $M - 116$. The position of the double bond in a monoethenoid ester cannot be determined directly from the mass spectrum, as it has been found (48) that both *cis*- and *trans*- isomers, as well as the positional isomers so far studied, with the exception of the α,β-unsaturated esters, give identical (or practically identical) mass spectra.

The mass spectra of methyl linoleate and methyl linolenate (Fig. 24, middle and bottom) differ considerably from each other and from methyl oleate. An interesting feature of all three spectra shown in Figure

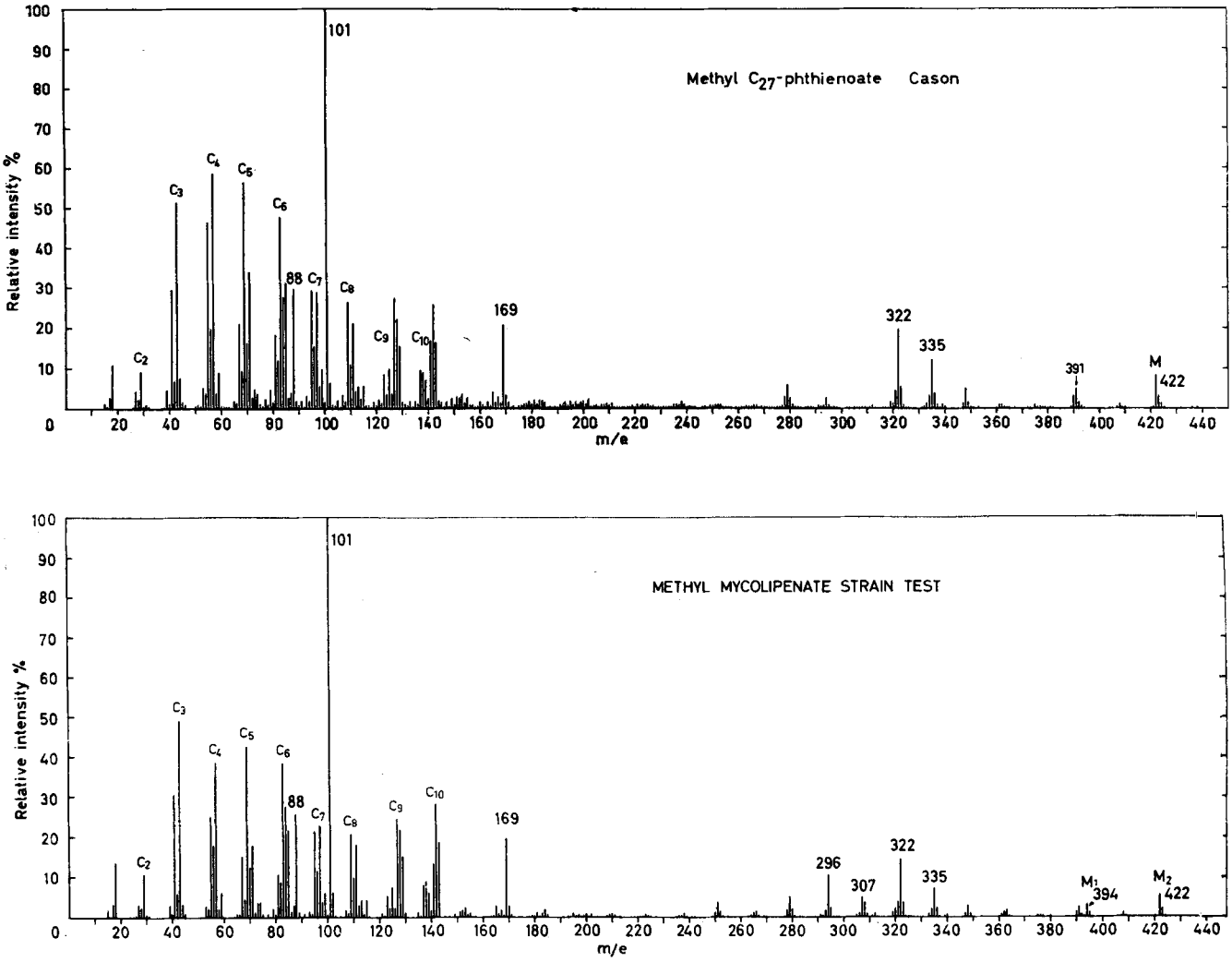


FIG. 25 (continued). Mass spectra of methyl esters of α,β-unsaturated branched-chain acids. Reproduced with minor corrections from Ahlquist *et al.* (52).

24 is the presence of a peak at *m/e* 91. The height of this peak increases with the degree of unsaturation of the ester, and has been found to be the base peak in the case of a hexaunsaturated ester (48). It seems likely that the peak at *m/e* 91 is due to tropylium ions (cf. 49) formed through extensive rearrangement and cyclization.

Methyl Esters of Branched-Chain α,β-Unsaturated Acids. Branched-chain α,β-unsaturated acids called phthienoic or mycolipenic acids are found in the lipids of the tubercle bacillus (for a recent review of bacterial lipids see Asselineau [50]). Figure 25 shows that the methyl ester of C_{27}-phthienoic acid (51) gives the same mass spectrum as synthetic methyl *trans*-2,4,6-trimethyltetracosen-2-oate, and that this spectrum is different from that of corresponding *cis*-isomer (Fig. 25, top) (52). High peaks appear at *m/e* 88 and 101, as in the case of the saturated 2,4,6-trimethyl-substituted ester (39), indicating that a double-bond shift occurs before 2,3 and 3,4 cleavage of the chain. Simple cleavage of the chain between carbon atoms 5 and 6 occurs with no double-bond shift. The ions formed in this manner give the peak at *m/e* 169, which is highly characteristic for the 2,4,6-trimethyl-2-enoate structure. The mass spectrum of a sample of methyl mycolipenate from the strain *Test* of the tubercle bacillus (52) showed the presence of the methyl esters of the C_{25} and C_{27} 2,4,6-trimethyl-2-enoic acids (Fig. 25, bottom).

The mass spectra of the synthetic *cis*- and *trans*-α,β-unsaturated isomers differ mainly with regard to the relative intensity of the ions of *m/e* 322 ($= M - 100$) and 335 ($= M - 87$) formed through 3,4 and 2,3

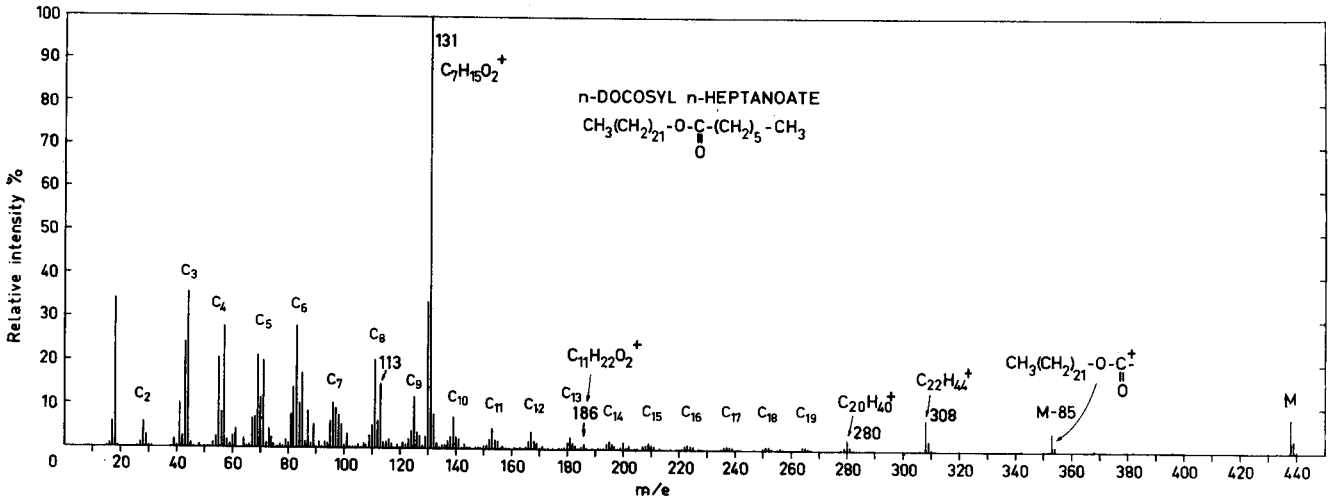


FIG. 26. Mass spectrum of *n*-docosyl *n*-heptanoate. $M = 438$. From Ryhage and Stenhagen (53).

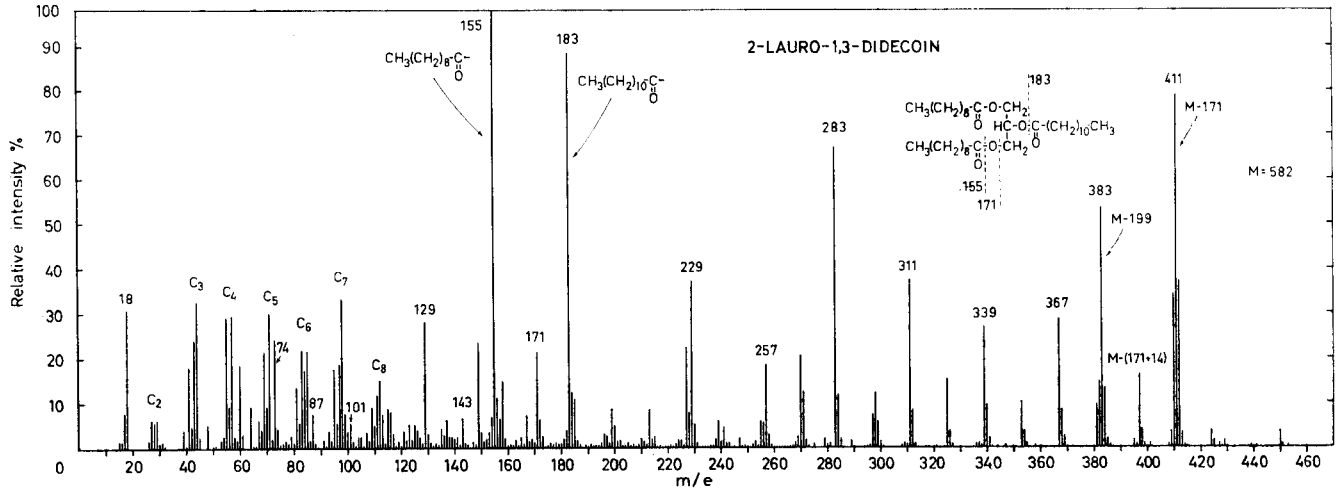


FIG. 27. Mass spectrum of 2-lauro-1,3-didecoin. $M = 582$.

cleavage (with rearrangement), respectively.

Esters of Long-Chain Alcohols and Long-Chain Fatty Acids. The typical wax ester gives a mass spectrum of the type shown in Figure 26 (53). The hydrocarbon-type peak at m/e 308 and 280 in the spectrum of *n*-docosyl *n*-heptanoate correspond to those at $m/e = M - 18$ and $M - (18 + 28)$ that would be obtained in the case of free *n*-docosanol-1. The base peak in the spectrum of Figure 26 is due to ions formed with alkyl-oxygen cleavage of the alcohol moiety and rearrangement of two hydrogen atoms, the structure of the ion being presumably

$$HO-\overset{\overset{+}{O}H}{\overset{\|}{C}}-(CH_2)_5-CH_3.$$

A distinct molecule-ion peak is present, as well as a peak at $m/e = M - 85$, the latter ion being formed through 1,2 cleavage of the acyl group. A further characteristic feature of esters of this type is the occurrence of a small peak due to ions formed through 4,5 cleavage of the alcohol part with rearrangement of one hydrogen atom, which in the present case give a peak at m/e 186.

Glycerides. Triglycerides have so far been investigated only in a preliminary manner.[1] One reason is that these compounds are difficult to pump out from the mass spectrometer and tend to give persistent "memory" effects. Figure 27 shows the type of results ob-

[1] R. Ryhage and E. von Sydow, unpublished data.

tained. The highest peaks are due to the two acylium ions of m/e 155 and 183, respectively. Large peaks at m/e = M — 171 and M — 199 are due to ions formed with loss of acyloxy groups from the molecule-ion. One point of interest is the existence of a peak at $m/e = M$ — (171 + 14) but no corresponding peak at $m/e = M$ — (199 + 14). Loss of acyloxymethylene thus occurs from positions 1 and 3 but not from position 2 of the glycerol moiety, which makes it possible to distinguish the acyl group attached at position 2 from those at positions 1 and 3. Tentative structures can be suggested for ions that may be responsible for the peaks at m/e 229, 283, and 311, but further experimental work is necessary before such assignments can be made with any degree of confidence.

Steroids. The mass spectra of some steroids have been studied by Reed (54), Bergström *et al.* (55) and Friedland *et al.* (56), but the information available for this important class of natural products is still rather fragmentary. In contrast to diastereoisomeric aliphatic compounds, diastereoisometric sterols usually give mass spectra that are significantly different from each other. The condensed ring systems give very complicated fragmentation patterns in the mass range below

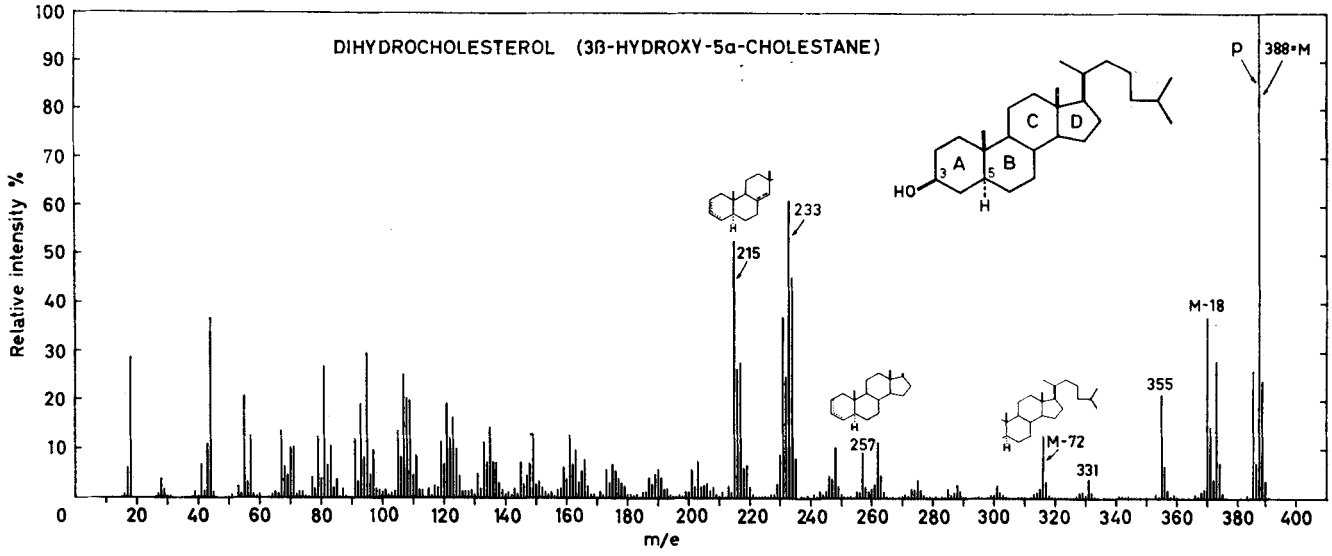


FIG. 28. Mass spectrum of dihydrocholesterol. M = 388. P = "parent ion" = M.

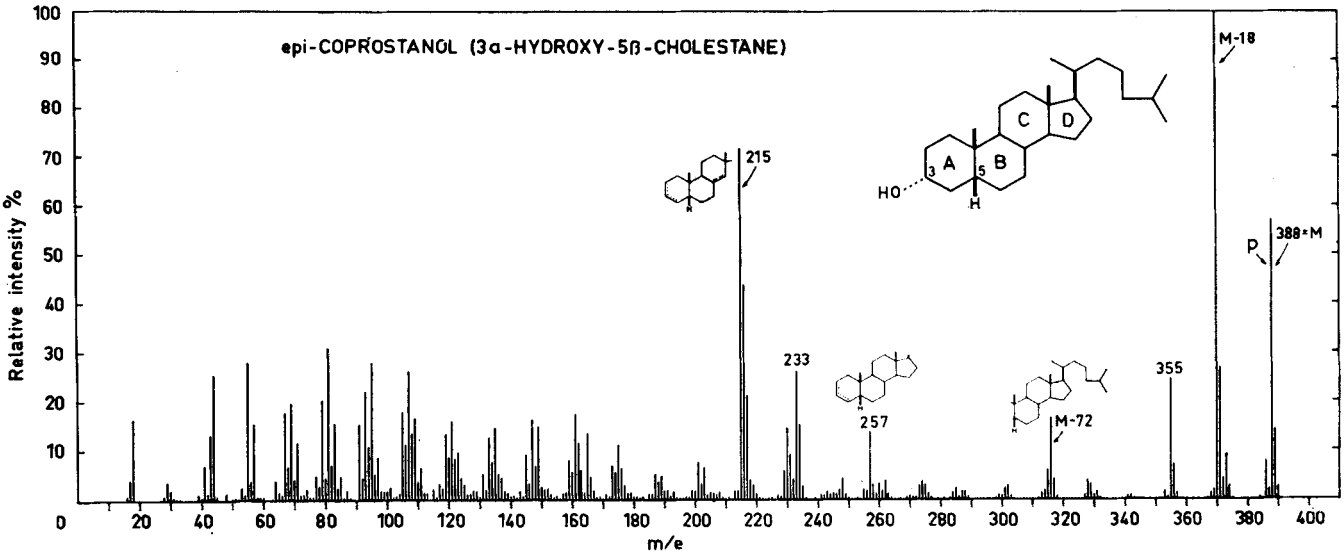


FIG. 29. Mass spectrum of *epi*coprostanol. M = 388.

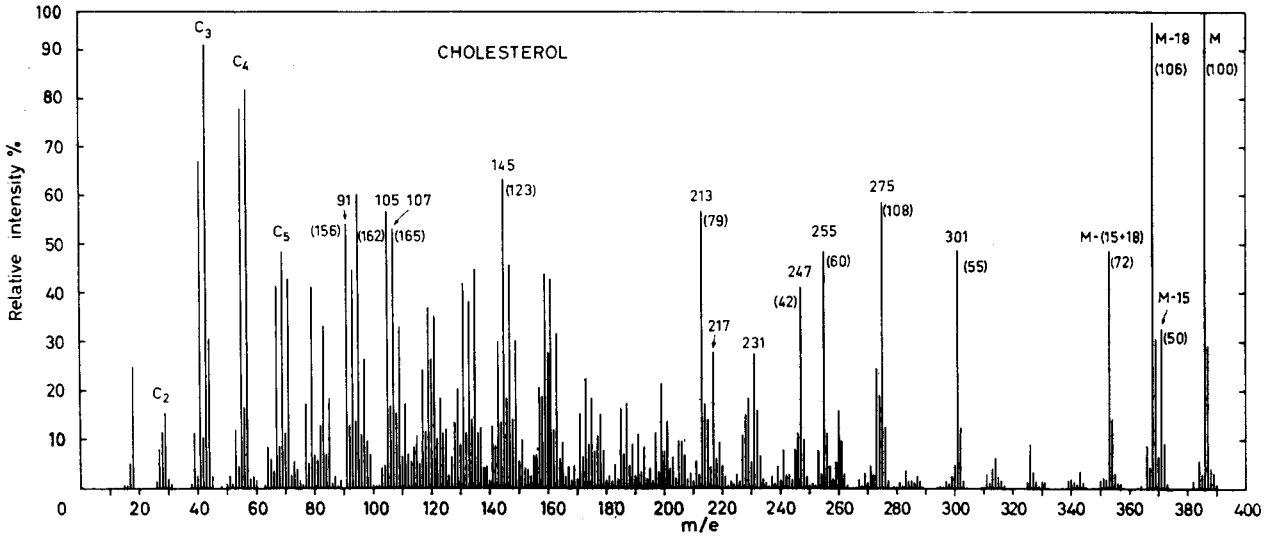
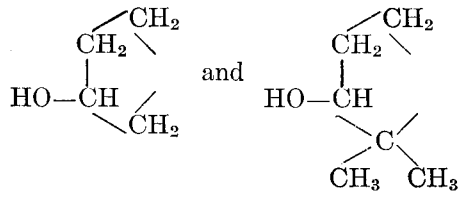


FIG. 30. Mass spectrum of cholesterol. $M = 386$. The spectrum was run under the following conditions: Temperature of inlet system, 200°. Temperature of ion chamber, ~ 220°. Electron energy, 70 electron volts. The figures within parentheses are the relative intensities (with the intensity of the molecule-ion peak set at 100) recorded by Friedland *et al.* (56), who used the following experimental conditions: Temperature of inlet system (vapor temperature), 200°. Temperature of ion chamber, 265°. Electron energy, 70 electron volts.

about m/e 200, and a full mass spectrometric study of this class of compounds requires access to a large number of synthetic compounds that contain heavy isotopes at specific sites.

In the high mass range there appear characteristic peaks, however, that make the mass spectra useful for identification purposes. The mass spectra of dihydrocholesterol (Fig. 28) and *epi*coprostanol (Fig. 29) have been run under identical conditions and the differences between the spectra must be largely due to the stereochemical differences. A striking feature of the spectra is the high peak due to the molecule-ion, which is responsible for the base peak in the case of dihydrocholesterol. This is in contrast to aliphatic secondary alcohols, which give exceedingly small molecule-ion peaks (cf. Fig. 12). In the high mass range there appear peaks at $m/e = M - 2$, $M - 15$, $M - 18$, and $M - (15 + 18)$, evidently due to ions formed with loss of a molecule of hydrogen, a methyl group, of molecule of water, and a methyl group plus a molecule of water, respectively, from the molecule-ion.

Sterols with saturated ring systems show a peak at $m/e = M - 72$. That the ions responsible for this peak are formed with loss of ring A from the molecule ion is shown by the fact that saturated sterols with *gem*-dimethyl substitution at position 4 in ring A show a corresponding peak at $m/e = M - 100$, the fragments lost in the two cases being, respectively:



The peak at m/e 257 is due to the ionized sterol nucleus formed with loss of the side chain and one molecule of water from the molecule-ion. The m/e of this peak indicates the total number of hydroxyl groups and double bonds, as well as methyl side chains present in the nucleus. For instance, cholesterol with one hydroxyl group and one double bond gives m/e 255, and 3,7,12-trihydroxycoprostane gives m/e 253 (Figs. 30 and 31). Tetrahydrodammaradienol with one hydroxyl group and three methyl groups gives m/e 299 ($= 257 + 3 \times 14$).

The mass spectra of the two stanols (Figs. 28 and 29) show a group of peaks at m/e 215 to 217, the peak at m/e 215 being the highest of the group. The saturated hydrocarbon cholestane shows a similar group at m/e 217 to 219 (A.P.I. No. 1000). In the case of the stanols, the peaks appear to be due to ions formed with loss of the side chain, water, and ring D from the mole-

cule-ion. Friedland *et al.* (56) suggest that the side chain, the angular methyl group C-18, and two carbon atoms from ring D are removed. (Rupture of both rings C and D may be involved; cf. Reed [54].) Finally, the stanols show a peak at m/e 233 ($= 215 + 18$), analogous to that of m/e 215 but with the hydroxyl group retained in the corresponding fragment.

The molecule-ion gives the largest peak in the mass spectrum of cholesterol shown in Figure 30. A comparison with the high mass end of the mass spectrum of cholesterol reproduced in Figure 7 shows that in the latter case the peak at $m/e = M - 18$ is higher than the molecule-ion peak. The difference between the spectra is to be attributed to thermal dehydration in the intake system of the mass spectrometer. The spectrum of Figure 30 was run very soon after the volatilization of the sample, whereas the spectrum of Figure 7 was run about half an hour later. The degree of reproducibility to be expected for this type of compound when the mass spectra are run by different operators using different mass spectrometers is shown by a comparison between the spectrum of Figure 30 and the data given by Friedland *et al.* The figures given within parentheses at some of the higher peaks in Figure 30 give the heights of the corresponding peaks (in per cent of the height of the molecule-ion peak) given by the authors just mentioned.

The 5:6 double bond in ring B of cholesterol stabilizes ring A, and no significant peak at m/e 314 ($= M - 72$) occurs in the mass spectrum. The double bond also makes the peak due to the ring system appear at m/e 255 and the peaks due to ions containing the rings ABC appear at m/e 213 and 231. Cholesterol also differs from its dihydro-derivative in giving high peaks at m/e 247, 275, and 301. Friedland *et al.* have suggested that the ion of m/e 247 is formed through rupture of ring B. Ions of m/e 301 ($=M - 85$) are probably formed with loss of C_6H_{13} from the side chain.

The spectrum of 3α:7α:12α-trihydroxycoprostane shown in Figure 31 contains peaks at $m/e = M - 18$, $M - 2 \times 18$, $M - 3 \times 18$, due to ions formed with loss of one, two, and three molecules of water. Significant peaks appear at $m/e = 351$ ($= M - [3 \times 18 + 15]$) and 312 ($= M - [72 + 2 \times 18]$). In the latter case, ring A is lost together with two molecules of water. The peak at m/e 253 due to triply unsaturated ring system is the next highest peak of the spectrum.

Bile Acid Derivatives. The mass spectra of the methyl esters of two saturated bile acids with no hydroxyl group attached to the ring system are reproduced in Figures 32 and 33. In both spectra the base peak is due to the molecule-ion: Ions of $m/e = M -$ 15, m/e 232, and m/e 217 appear with relative intensities almost exactly as in the mass spectrum of cholestane (A.P.I. No. 1000). Small peaks due to the acylium ions are found at $m/e = M - 31$. In the low mass region methyl cholanate shows the rearrangement peak at m/e 74 given by methyl esters of acids that possess no substituent in the alpha position, whereas methyl coprostanoate gives a small peak at m/e 74 and a much larger one at m/e 88 due to the presence of a methyl group in the alpha position. Methyl cholanate shows a very small peak at m/e 101 and a larger peak at m/e 115 due to the methyl side chain in the gamma position. The ion of m/e 115 contains all the atoms of the side chain. For methyl coprostanoate an analogous peak would be expected at m/e 157, but this peak is small in the spectrum of Figure 33.

The mass spectra reproduced in Figures 34 and 35 show the influence of positional isomerism in the case of monohydroxycholanates. Only methyl lithocholate with the hydroxyl group at position 3 on ring A shows a distinct peak at $m/e = M - 72$ due to ions formed with loss of ring A. On the other hand, the hydroxyl group at position 12 on ring C leads to a very stable ion of m/e 257 due to the sterol nucleus with a double bond on ring C. This stabilizes rings C and D as indicated by the appearance of a small peak only at m/e 215. Both monohydroxycholanates show very small peaks due to the molecule-ions. Somewhat larger peaks appear at $m/e = M - 2$, while comparatively high peaks are due to ions formed with loss of water ($m/e = M - 18$) and water plus one methyl group ($m/e = M - [18 + 15]$).

Although it is possible to obtain the mass spectra of methyl esters of bile acids possessing more than one free hydroxyl group, the low volatility of the esters with several free hydroxyl groups makes it preferable to use derivatives with protected hydroxyl groups. The mass spectrum of the triacetyl derivative of cholic acid is shown in Figure 36. The peak of highest m/e observed in the spectrum is due to ions of m/e 428 ($= M - 2 \times 60$) formed with loss of two molecules of acetic acid. Prominent peaks are due to ions formed with loss of three molecules of acetic acid ($m/e = M - 3 \times 60$), and three molecules of acetic acid plus one methyl group ($m/e = M - [3 \times 60 + 15]$). The presence of a hydroxyl group at position 7 on ring B has no stabilizing influence on ring A as the spectrum contains a high peak at m/e 313 ($= M - 235$). This peak appears to be due to ions formed with loss of ring A with attached acetoxy group, together with two molecules of acetic acid and one hydrogen atom. The triply unsaturated ring system ion of $m/e = 253$ formed with

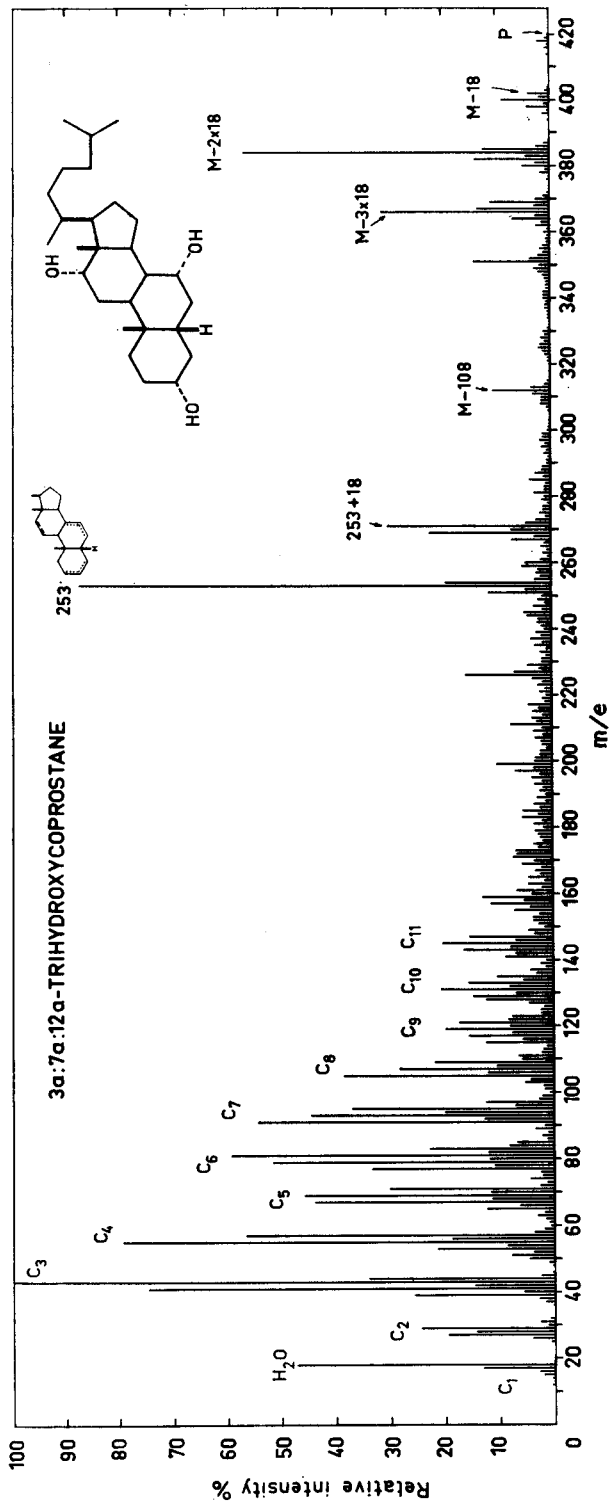


FIG. 31. Mass spectrum of 3α:7α:12α-trihydroxycoprostane. $M = 420$.

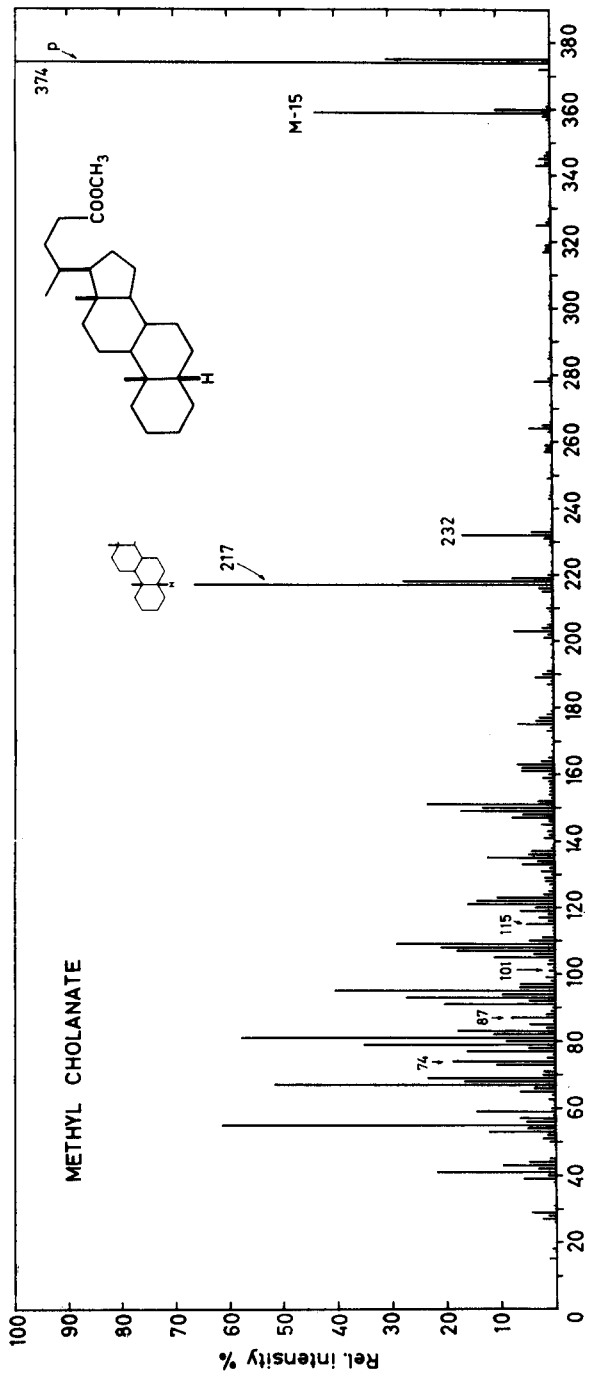


FIG. 32. Mass spectrum of methyl cholanate. $M = 374$. Cf. Bergström *et al.* (55).

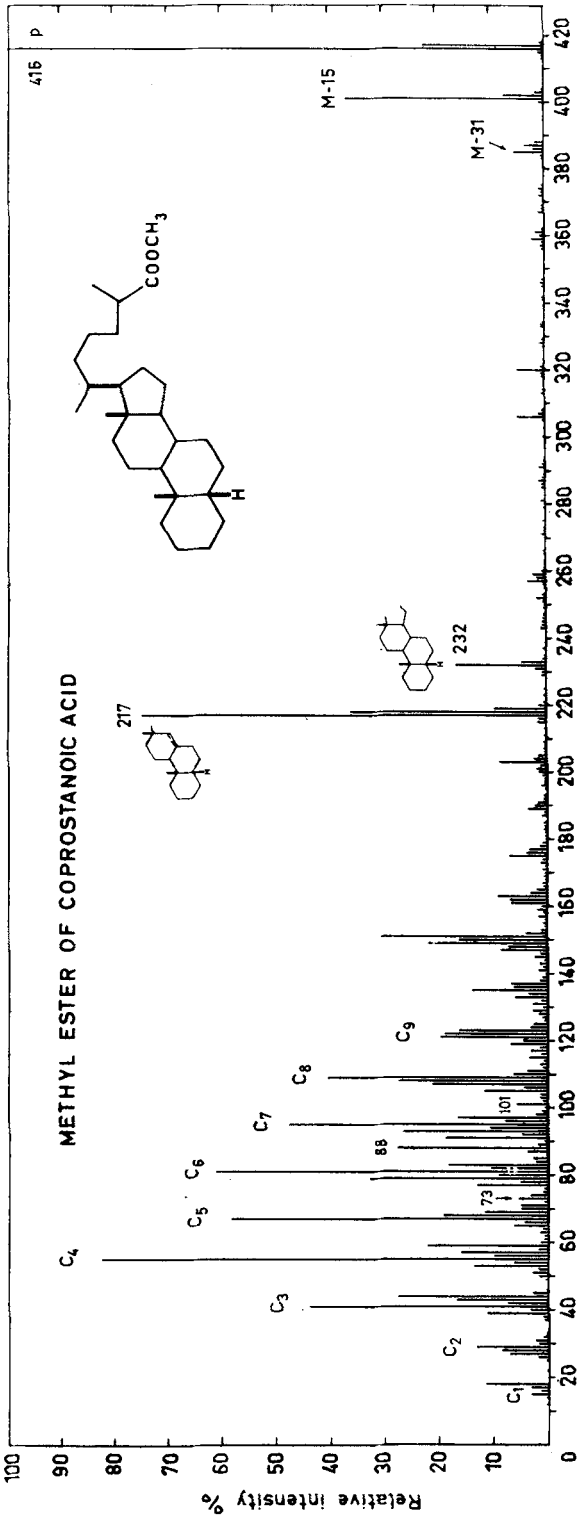


FIG. 33. Mass spectrum of methyl coprostanoate. $M = 416$. Cf. Bergström *et al.* (55).

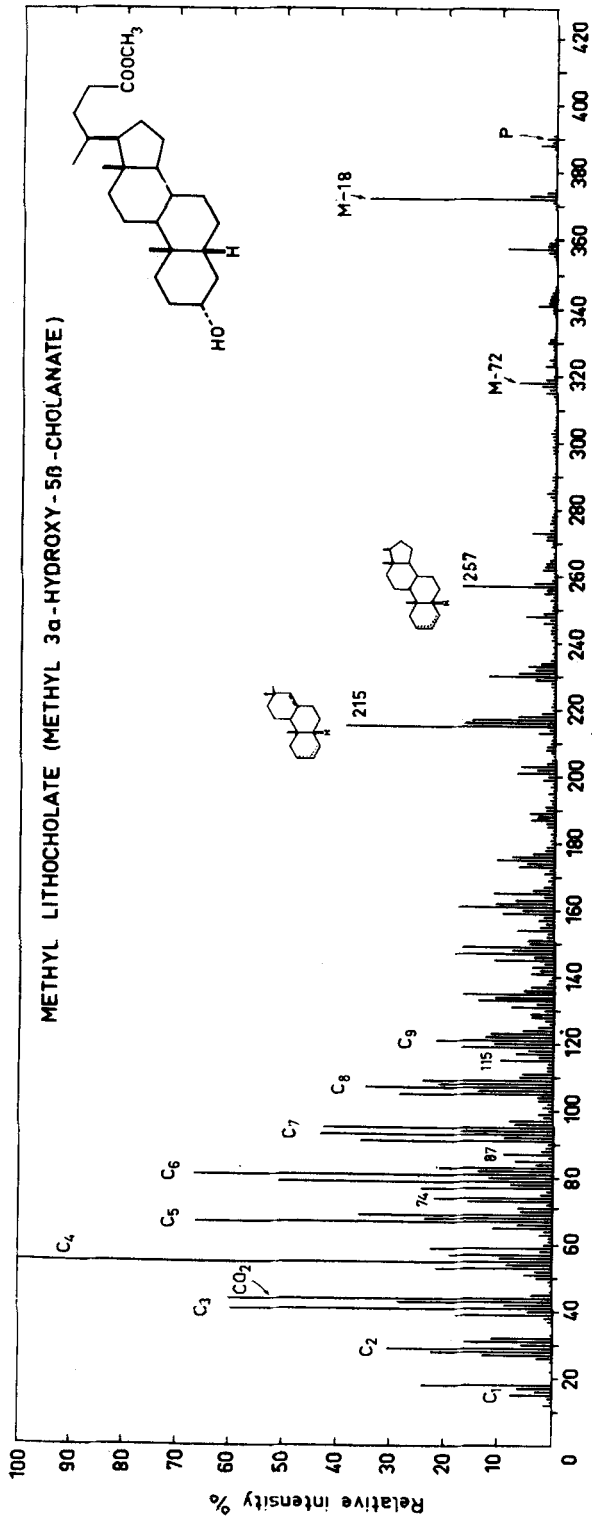


FIG. 34. Mass spectrum of methyl lithocholate. $M = 390$. Cf. Bergström *et al.* (55).

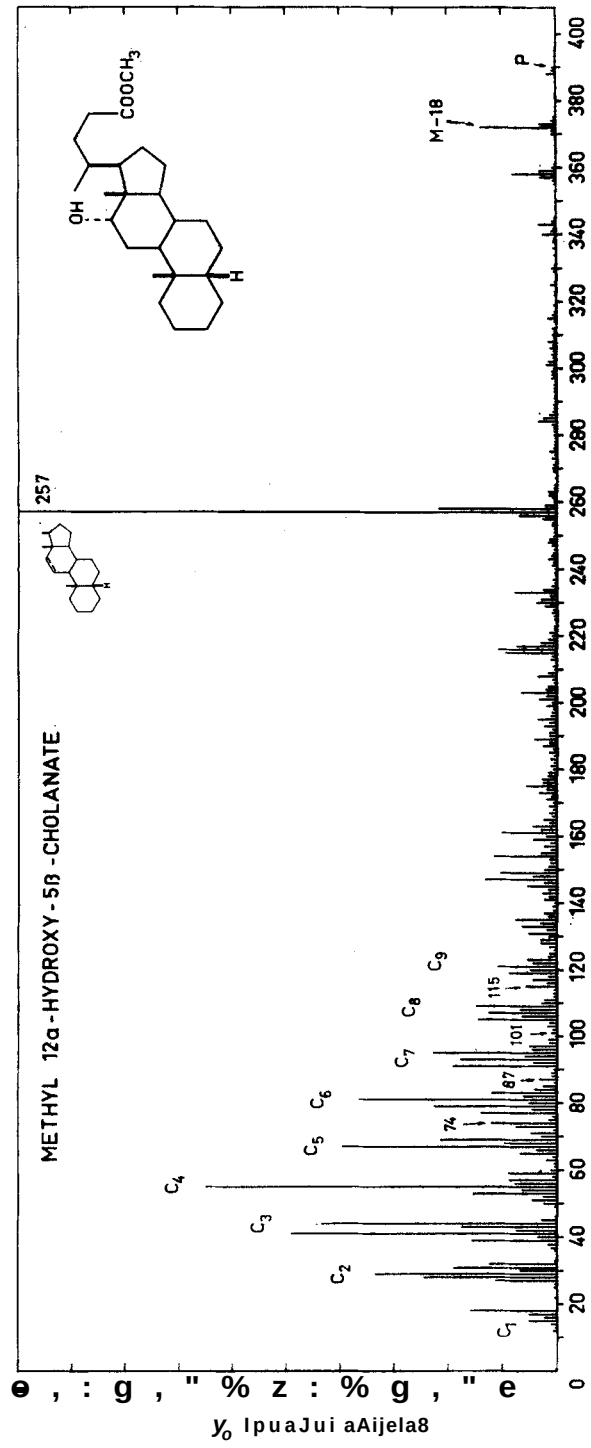


FIG. 35. Mass spectrum of methyl 12α-hydroxy-5β-cholanate. $M = 390$. Cf. Bergström *et al.* (55).

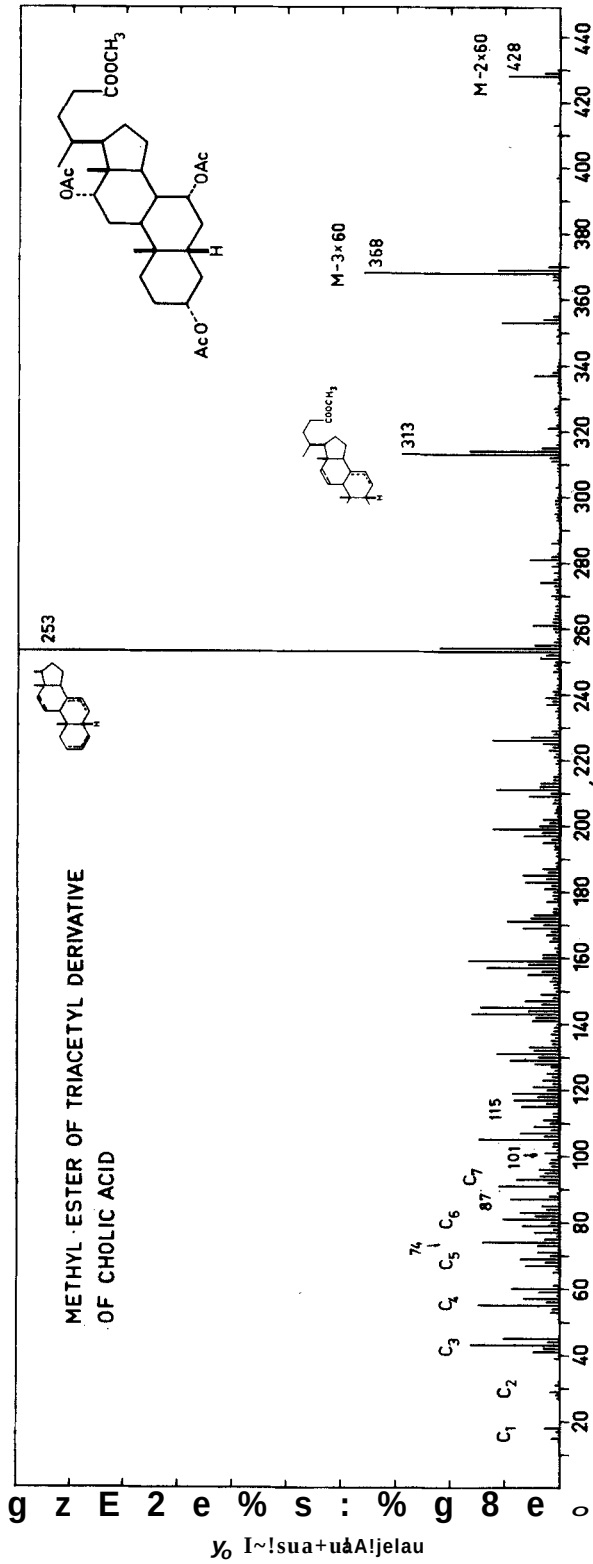


FIG. 36. Mass spectrum of methyl ester of triacetyl derivative of cholic acid. $M = 548$. Cf. Bergström *et al.* (55).

loss of the side chain and three molecules of acetic acid is very stable and gives the base peak of the spectrum. The peak at *m/e* 215 is insignificant.

The mass spectra shown in Figures 34, 35, and 36 all exhibit methoxy-carbonyl type peaks of the same *m/e* and relative intensity as shown by methyl cholanate (Fig. 32), the side chain in all four cases being the same.

It seems evident that mass spectrometry will play an important role as an analytical tool in the lipid field. Further studies of the processes involved in the fragmentation of large molecules using compounds that contain heavy isotopes at specific sites will certainly increase the analytical power of the method considerably. There is at present no method available for detailed structure determinations of ionic fragments, and many structures that have been proposed will probably have to be modified if such methods are developed.

We are indebted to Mrs. Stina Ställberg-Stenhagen for valuable suggestions, and to Mrs. Betty Ull, Miss Gunilla Norman, and Mr. L. Ahlquist for technical assistance in the preparation of this paper.

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