Gas-Liquid Chromatography of Sterol Methyl Ethers and Some Correlations Between Molecular Structure and Retention Data*

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A simple method for the conversion of sterols to their methyl ethers in high yield has been developed and has been shown to be applicable to representatives of all the commonly encountered classes of 3-hydroxysterols. The behavior of the sterol ethers on gas-liquid chromatography with polydiethylene glycol succinate as the liquid phase has been shown to permit the separation of many compounds of biological importance. It has been shown that the influence of substituents and other structural modifications on the retention times of these and related steroids may be described in a simple mathematical form. This finding is discussed in the light of other published work.

The purpose of the study to be described in this paper was the development of an adequate technique for the gas-liquid chromatography of sterols and, as far as possible, the correlation of the retention data so obtained with the structural features of the compounds concerned. The steroid nucleus of 17 carbon atoms is both large and rigid enough to accommodate several different functional groups with sufficient spatial separation from each other to minimize the effects of intramolecular group interactions. Under these conditions the individual contributions of the various functional groups to many of the physical properties of the whole molecule become virtually additive. The behavior of compounds on the gas-liquid chromatogram must be the resultant of the interactions of the liquid phase with all the various features of molecular structure of the solute. It was reasonable, therefore, to anticipate that this technique, as applied to the steroids, would be susceptible of some degree of systematization in terms of the correlation of retention time with modifications of molecular structure.

Such structural correlations would parallel in many respects those which have followed the application of other physical methods to the study of the steroids and related polycyclic compounds, and, if found to be sufficiently precise and reproducible, could greatly enhance the analytical value of gas-liquid chromatography.

Choice of Liquid Phase.—The major requirement was for a simple method of separating closely related and often isomeric sterols of the C_{27}, C_{28}, C_{29}, and C_{30} (lanostane) series. The published accounts of the gas-liquid chromatography of sterols using nonpolar silicone liquid phases (Beerthuis and Recourt, 1960; Vanden Heuvel *et al.*, 1960) were discouraging as to the prospect of efficiently separating such compounds under these conditions. On the other hand, it was evident from studies in the fatty acid series (Landowne and Lipsky, 1961) that the polarity of the ethylenic linkage could be exploited to great advantage by the use of polyester liquid phases. Polydiethyleneglycol succinate, one of the most polar polyester phases, which had been used with excellent results for the resolution of mixtures of saturated and unsaturated fatty acids (Lipksy *et al.*, 1959), was chosen as being most likely to elicit the maximal contribution of the steroid ethylenic linkage to the retention time. However, the high polarity of this solvent precluded its use for the separation of free sterols, since their retention times were excessive (5 hours or more). The hydroxyl group was therefore masked by conversion to the methyl ether, and it was found that in this form all the common sterols could be chromatographed in reasonably short times (1–2 hours). It will be shown below that many biochemically important separations of closely related compounds of this class could be achieved. The method is not a practical one for the separation of the common polyoxygenated steroids or bile acids, since the retention times of these compounds on this liquid phase are excessive.

While this work was in progress it was reported (Vanden Heuvel *et al.*, 1961) that sterol trifluoroacetates, chromatographed on neopentyl glycol succinate, had retention times comparable with those of the methyl ethers (an observation which has been confirmed in these laboratories: Lasser and Clayton, unpublished work). Several reports have also appeared of the use of various types of polar liquid phases for the gas-liquid chromatography of sterols and steroid hormones (Haahti *et al.*, 1961a,b; Vanden Heuvel *et al.*, 1961a,c; Lipsky and Landowne, 1961). In agreement with the findings described here, these reports all indicate the superiority of the polar phases as compared with the less polar silicone and hydrocarbon phases for effecting separations based on the more subtle structural differences between molecular species. On the other hand, there has so far been no report of a study of the gas-liquid chromatography of sterols with the aims which were kept in view throughout this work. A number of studies have,

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however, indicated close relationships between molecular structure and the retention times of steroids on the gas-liquid chromatogram, as exemplified by the sapogenins (Vanden Heuvel and Horning, 1961), the steroidal amines (Vanden Heuvel *et al.*, 1961b), the bile acids (Sjovall *et al.*, 1961) and a wide range of steroids of the pregnane and androstane series (Lipsky and Landowne, 1961).

Some of the results which have emerged from work with these different classes of steroids will be discussed in relation to those reported in this paper. Preliminary reports of this study have appeared elsewhere (Clayton, 1961a,b).

Experimental

Materials and Methods.—Cholesterol-4-C^{14}, cholesterol-7α-H^3, methyl iodide-H^3, and sodium stearate-1-C^{14} were obtained from New England Nuclear Corporation. Other radioactive compounds were synthesized from these by established literature procedures. Other steroids were pure authentic samples, some of which were synthesized in these laboratories from readily available materials by standard methods, while others, where indicated, were the generous gifts of several workers (see Acknowledgements). Methyl ethers were prepared by a method which will be described in detail. In general no attempt was made to purify and characterize the methyl ethers before they were submitted to gas-liquid chromatography. It will be shown in the first part of this paper that the methylation procedure is applicable, with good yields, to a range of structural types which make it highly unlikely that any of the substances considered in this study would give rise to abnormal products. The methyl iodide–potassium *t*-butoxide methylation method leaves carboxylic acids unesterified. In the case of the bile acid derivatives it was therefore necessary to methylate the 3α-hydroxyl group by the standard procedure to be described and, having isolated the crude 3α-methoxy-acid, to convert it to the methyl ester by treatment with diazomethane.

Chromatography on alumina was carried out with Merck "chromatographic grade" alumina which had been deactivated by treatment with 7% of its own weight of 10% acetic acid in water (Clayton, 1960). Chromatographic fractions were evaporated on the steam bath under a stream of pure nitrogen.

Solvents were analytical grade and were dried over calcium hydride and redistilled before use. Potassium *t*-butoxide was prepared by dissolving clean potassium metal (10 g) in freshly redistilled *t*-butanol (100 ml) in a 250-ml round-bottomed flask under reflux. The solution was then evaporated under vacuum to about half its volume and the remaining material frozen in liquid air and lyophilized. The resulting white powder was stored in a well-sealed bottle and remained suitable for use for several months.

Radioactivity measurements were made with a Packard liquid scintillation spectrometer. Activities are reported as disintegrations per minute (dpm) and are corrected for background.

Gas-liquid chromatography was carried out with a modular chromatogram (Research Specialties) with an ionization detector. The liquid phase was diethyleneglycol succinate polymer absorbed to a concentration of 5% on acid-washed chromosorb W (60–80 mesh) (Research Specialties "Chromatopac") and packed into stainless steel columns (6 ft × 4 mm). Conditioning was carried out by heating for 48 hours at 205° with a slow argon flow (5 psi), and then for the same length of time and at the same temperature with an argon flow rate of 220 cc per minute. The operating temperatures were as follows: column 195°, vaporizer 350°, ionization detector 212°, outlet 230°. The gas flow rate was 220 cc per minute with an inlet pressure of 30 psi. These conditions apply to the entire series of experiments described in this paper. Samples were introduced onto the column in solution in 1–3 μl of toluene by septum injection from microliter hypodermic syringes. The transfer of microgram quantities of materials to the column was carried out as follows. The whole sample was dissolved in a few tenths of a milliliter of ether and transferred with a Pasteur pipet to a micro test tube ($1^1/_2 \times {}^3/_{16}$ in.). The solvent was evaporated and the residue washed into the bottom of this tube with a few further drops of solvent, which were again evaporated under nitrogen. The tube was now cut to about half its length and the sample dissolved in a measured volume of benzene or toluene (a few microliters) introduced by means of a 10-μl syringe. The residue was completely dissolved by rotating the tube, and the solution was now taken up into a drawn-out capillary (melting point) tube into which the needle of a microliter syringe could be introduced and the sample withdrawn. If the needle of the syringe was previously filled with pure solvent, the volume of solution drawn into the syringe could be measured and finally the solution could be injected quantitatively into the gas chromatogram. With practice it was possible, by this technique, to inject 3.5–4.0 μl out of an original volume of 5.0 μl.

Standard Procedure for Methylation of Sterols on a Microgram Scale.—The sterol or sterol ester (5–500 μg) was dissolved in 1 ml dry ether in a glass-stoppered 15-ml tube. Approximately 30 mg (*i.e.*, a large excess) potassium *t*-butoxide powder was added and crushed with a glass rod to ensure thorough dispersion in the solution. The suspension was allowed to stand at room temperature for 15 minutes, and then 0.1 ml redistilled methyl iodide was added and the tube swept out with nitrogen, stoppered, and allowed to stand 4 hours. The methylated product was obtained by addition of water in the case of neutral sterols, or dilute sulfuric acid in the case of acidic steroids, followed by extraction of the aqueous phase with ether. The extraction was carried out in the reaction tube, the ether layer being removed to a second test

tube by means of a Pasteur pipet. The ether extract was washed twice with water, dried over sodium sulfate, and evaporated to dryness under pure nitrogen. To obtain a fraction containing methylated (neutral) sterols free from unmethylated material, the products were taken into petroleum ether (bp 60–80°) and chromatographed on 2 g alumina. Elution with 30 ml petroleum ether removed all the sterol methyl ether, and elution with 30% benzene in petroleum ether recovered any unchanged free sterol. Since of these two fractions only the first was of interest in this study, the free sterol fraction was in general not examined.

The chromatographic step in this procedure was carried out only in connection with the evaluation of the efficiency of methylation, to be described below. In the practical application of the method of the analysis by gas-liquid chromatography of sterol and sterol ester fractions isolated from tissues by standard chromatographic procedures, it was usually unnecessary to purify the crude methylation products further before injecting them onto the gas-liquid chromatogram.

Results and Discussion

Preparation of Cholesteryl Methyl Ether and Its Recovery After Gas-Liquid Chromatography.—Cholesterol (200 μg) was methylated by the standard procedure with methyl iodide-H^3 (0.25 mc per mmole). Chromatography yielded a fraction eluted with petroleum ether which contained 118,000 dpm. Half of this material (59,000 dpm) was combined with 20 mg pure nonradioactive cholesteryl methyl ether, mp. 84°, and the mixture was recrystallized twice from methanol, converted to the dibromide, and finally recovered by treatment with zinc dust and acetic acid. The following yields and specific activities were recorded: Crystallizations: (1) 15.2 mg, 2900 dpm per mg (calcd. 2950 dpm per mg); (2) 11.0 mg, 2970 dpm per mg. Dibromide: 6 mg, mp 72-75°, 2090 dpm per mg (calcd. 2110 dpm per mg). Recovered sterol methyl ether: 3.4 mg, 2920 dpm per mg.

The remaining half of the methylated product was dissolved in 5 μl benzene, and 3 μl of the solution was subjected to gas-liquid chromatography. The effluent gas corresponding to the single cholesterol ether peak was trapped in a glass collecting tube cooled by evaporation of dichloromethane as described by Lennarz *et al.* (1962). The condensed material was washed from the collecting tube with 10 ml benzene and two 1-ml aliquots counted for tritium, giving activities of 1400 and 1460 dpm, respectively, indicating that a total activity of 14,300 dpm (41% of the injected material) had been recovered. The remainder of the condensed material (11,440 dpm) was combined with 100 mg cholesterol methyl ether and recrystallized four times. The specific activity remained constant at 107-111 dpm per mg (calculated 114 dpm per mg) and purification via the dibromide caused no change in specific activity.

Similar recovery experiments were carried out with sitosterol methyl-H^3-ether (purified via the dibromide after recovery) and ergosterol methyl-H^3-ether. In each case only about 50% of the injected material was recovered from the column, but there was no evidence of impurities in the recovered materials as judged by constancy of specific activity on several crystallizations.

Comparison of Methylation Efficiencies for Different Classes of Sterols.—Table I shows the results of a series of experiments designed to test the general applicability of the methylation procedure described above. The sterols were dissolved in known volumes of suitable solvents, aliquots equivalent to the weights shown were evaporated to dryness in stoppered tubes and methylated by the standard method, and the methyl ether was separated from any unchanged starting material by chromatography on alumina. Duplicate sets of methylations were carried out under nitrogen and air, respectively, except in the case of epicholestanol. The yields of the methyl ethers of the isotopically labeled compounds were calculated from radioactivity measurements carried out with a suitable aliquot of the petroleum ether fraction. Cholestanol-7α-H^3 and cholesterol-4-C^{14} were methylated simultaneously in the same tubes and the isotopic assays carried out by "split channel" scintillation counting. The yield of 7-dehydrocholesterol methyl ether was assayed by UV absorption measurements at the three wave lengths of maximal absorption: 272, 282, and 294 mμ, which showed identical ratios in both methylated and unmethylated materials. The values recorded in Table I are the average yields from three experiments in each case and are expressed as percentages of the average of duplicate unmethylated controls. The homogeneity of the methylated material was tested by gas-liquid chromatography. A single, symmetrical peak, corresponding in area and retention time to the expected product, was obtained in every case.

Table I
Percentage Yields of Methyl Ethers of Various Sterols

Compound	Wt. (μg)	Percentage Yield— Nitrogen	Air
Cholestanol-7α-H^3	12.5	92 ± 2	92 ± 1
Cholesterol-4-C^{14}	16.0	92 ± 2	73 ± 1
Epicholestanol-7α-H^3	17.9	83 ± 1	
4,4-Dimethylcholesterol-4-C^{14}	250	75 ± 3	70 ± 1
7-Dehydrocholesterol	52.0	89 ± 1	73 ± 1

Simultaneous Hydrolysis of Cholesteryl Stearate and Formation of Cholesteryl Methyl Ether.—Cholesteryl-7α-H^3 stearate-1-C^{14} was dissolved in diethyl ether to give a concentration of 120 μg/ml. Two aliquots of 1 ml were taken as controls and assayed for total content of C^{14} and H^3. Three further aliquots of 1 ml were methylated by the above procedure and the sterol methyl ether fractions isolated by chromatography on alumina and assayed for C^{14} and H^3 in the usual way. The aqueous layer remaining after extraction of the

TABLE II
RECOVERIES OF STEROL METHYL ETHER-7α-H^3 AND FATTY ACID-1-C^{14} FROM CHOLESTERYL-7α-H^3 STEARATE-1-C^{14}

	dpm H^3	dpm C^{14}	% H^3 recovered	% C^{14} recovered
Controls (av. of 2)	93,500 ± 500	6130 ± 30		
Methylations (av. of 3)				
Sterol ether fraction	74,870 ± 900	130 ± 30	80 ± 1.0	2.1 ± 0.5
Fatty acid fraction	300 ± 100	5650 ± 150	0.32 ± 0.1	91 ± 2.0

sterol ether fraction was acidfied with 4 N sulfuric acid and extracted four times with ether. The ether extracts were washed twice with water, dried over sodium sulfate, evaporated to dryness, and counted for C^{14} and H^3. Recoveries of radioactivity are recorded in Table II. Gas-liquid chromatography of the sterol ether fraction gave a single peak corresponding in position and area (see below) to the calculated conversion to cholesterol methyl ether. The fatty acid fraction, on the other hand, after treatment with diazomethane, gave several unidentified peaks besides that corresponding to stearate.

The foregoing experiments show that the procedure described here is a reliable one for the micro scale preparation of sterol methyl ethers in close to quantitative yield from all the principal structural types of sterols found in biological materials. Other experiments not described here indicate that with most 3-hydroxysterols the reaction time can be reduced to 1 hour without serious loss of yield. There is good evidence that the hydroxyl groups in different positions in the molecule react at different rates. The methylation of 3,7-diols selectively in the 3-position can be achieved by cutting the reaction time to 45 minutes, and complete conversion to the 3,7-dimethoxy compounds requires two 4-hour treatments of the material. The results presented in Table I indicate the advisability of carrying out the methylation of unsaturated sterols under nitrogen to avoid loss by oxidation. It should be pointed out that the procedure is unsuitable for use with ketones, which may undergo methylation at the carbon adjacent to the keto group, or with steroids having the sensitive corticoid side-chain.

The results recorded in Table II show that a useful feature of the method is that it allows the conversion of a sterol fatty acid ester directly to the corresponding methyl ether without prior hydrolysis and isolation of the free sterol. Unfortunately, it has not yet been found possible to use the fatty acid fraction liberated in the reaction for analysis by gas-liquid chromatography owing to the presence of interfering artifacts in the chromatogram.

Behavior of Sterol Methyl Ethers on Gas-Liquid Chromatography with Polydiethyleneglycol Succinate as the Liquid Phase. General Considerations.—Figure 1 shows the separations which can be achieved under optimal conditions with mixtures of methyl ethers of several known sterols. It can be seen that symmetrical peaks are obtained with virtually no tailing and that a number of separations of biochemical significance can be accomplished within reasonably short times. Thus Δ^7-cholestenol and $\Delta^{5,7}$-cholestadienol have separation factors relative to cholesterol (absolute retention time *ca.* 55 minutes) of 1.20 and 1.41 respectively. Cholestanol and cholesterol are incompletely resolved with a separation factor of 1.08, but are sufficiently separated to allow quantitation (see below). These separations are achieved in spite of a rather low absolute column efficiency, which in fact approximates to only 1200 theoretical plates at cholesterol with a height equivalent to theoretical plate of 0.15 (Keulemans, 1957).

Recovery of Sterol Methyl Ethers from the Gas-Liquid Chromatogram.—In Table III are recorded the percentage recoveries of several sterol methyl ethers applied in isotopically labeled form to the column and trapped at the exit port by the method of Lennarz *et al.* (1961). The values represent the recovery of radioactivity corresponding to the emergent peak of the material concerned as a percentage of the total injected. They do not suggest

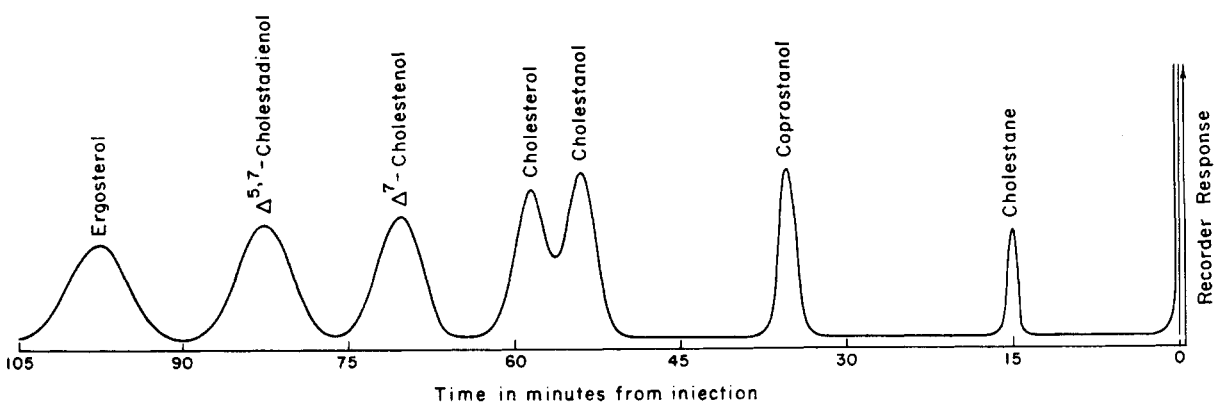

FIG. 1.—Gas-liquid chromatogram of a mixture of known sterol methyl ethers with polydiethylene glycol succinate as the liquid phase. Conditions as described in text.

any clear relationship between the structures of the compounds and the efficiency with which they may be recovered, which varies between 40 and 55%. There also does not appear to be any relationship, within the range examined, between the percentage recovery and the total quantity injected.

TABLE III
PERCENTAGE RECOVERY OF STEROL ETHERS AFTER GAS-LIQUID CHROMATOGRAPHY

	Wt. of Sterol Ether Applied to Column (μg)			
	80	60	40	20
Cholestanol ether	51	45	47	55
Cholesterol ether		50	40	40
7-Dehydrocholesterol ether	45	48	48	56
Ergosterol ether		45	49	47

Such difficulties of recovery have been noted by others in work with fatty acid methyl esters (Dr. A. J. Fulco, private communication) as well as with steroids (Dr. H. H. Wotiz, private communication). The physical state of the effluent vapor is suggested as a possible cause, and some improvement is often achieved by the use of electrostatic precipitation devices, but some loss due to irreversible binding of the solute on the column cannot at present be ruled out. In this connection it may be pointed out that the absence of hydrocarbon bands in the vicinity of cholestane is good evidence for the lack of thermal demethoxylation of almost all the 3-methoxysterols. Exceptions which have been noted are the allylic Δ^2- and Δ^4-3β-methoxy cholestenes, which gave blurred peaks early in the chromatogram, corresponding to mixtures of diene hydrocarbons.

Recorder Response to Sterol Methyl Ethers of Various Structures.—The results of a study of response (peak area in cm^2 per μg) to four different sterols, injected separately in quantities of 5 to 80 μg in solution in 2 μl benzene, are presented in Table IV. These areas were calculated by simple triangulation of the peaks. They correspond to a detector voltage of 0.95 kv (a 1-kv detector was used) and are corrected to a constant attenuation factor of 10 in each case.

These results indicate that within the limits of measurement the response of the detector is independent of the structure of the sterol ether and is proportional to the weight of material present. Since the molecular weights of these compounds are so nearly equal the data do not distinguish between proportionality of response to weight and to the number of molecules. It is clear from the range of variation of response given by the same sterol injected in different amounts that the peak areas do not represent the absolute amounts of material with an accuracy greater than about ± 11%. On the other hand, the relative amounts of substances present in the same sample may be estimated with a greater accuracy which is limited mainly by the difficulty of assessing the areas of the smaller peaks. It should be pointed out that these remarks apply only to the 3-methoxysterols. No comparisons have been made in this study between these and the more highly oxygenated steroids in relation to detector response.

Stability of the Columns and Reproducibility of Characteristics.—The stability of polydiethyleneglycol succinate falls off rapidly in the region of 200°, but it is stable enough under the conditions described here to permit accurate quantitative work with the same column for a period of 3 months. In order to extend the life of the column the temperature has been lowered whenever convenient during times when it is not in use (*e.g.*, overnight) and the gas flow reduced to about 30 cc per minute. After 3–4 months use in this way, the column characteristics begin to deteriorate; retention times and resolution efficiency fall, and the column should be replaced.

In the course of this work four different columns have been used for the collection of data on a wide series of compounds. The preparation of the columns and the operating conditions have been, as far as possible, the same throughout. It has been necessary, however, to use different batches of the packing material, and some variations have been found to occur in the absolute retention times recorded for the same compound chromatographed on different columns. Nevertheless, the retention time relationships which are discussed in the following sections have been found to hold constant for all nuclear double bonds and substituent groups with all the freshly prepared columns. The contributions of the double bonds in the side-chain, on the other hand, have been subject to some variation between one column and another, and particularly between different batches of the packing material. Thus, the retention factor for the Δ^{24}-bond, measured with different packings, has varied from 1.33 to 1.50, and that for Δ^{22}, in the ergostane series, has varied between 0.83 and 0.89. However, for a given batch of packing material, these variations have been much smaller (1.44–1.50 and 0.83–0.86 for the retention factors for Δ^{24} and Δ^{22} respectively), and for any individual column the quantitative relationships derived below have been found to be quite reproducible. The absolute

TABLE IV
RESPONSE[a] OF THE DETECTOR TO VARYING AMOUNTS OF DIFFERENT STEROL METHYL ETHERS

	Wt. of Sterol Methyl Ether Applied to Column (μg)							
	80	60	40	20	10	5	Mean	Standard Deviation
Cholestanol ether	0.29	0.31	0.26	0.28	0.32	0.24	0.28	0.032
Cholesterol ether	0.30	0.31	0.28	0.26	0.26	0.28	0.28	0.020
7-Dehydrocholesterol ether	0.30	0.26	0.24	0.25	0.25		0.26	0.021
Ergosterol ether	0.28	0.30	0.27	0.23	0.28	0.25	0.27	0.024

[a] Values in $cm^2/\mu g$ at 0.95 kv and attentuation 10.

retention times (7–25 minutes for cholestane), varied by altering the gas flow rate, have not been found to influence the double bond retention factors significantly.

Structural Correlations.—In order to minimize the effects of chance variations of such factors as temperature and gas flow rate upon the absolute retention times of the compounds considered in the following discussion, each result is reported as a "relative retention time" derived by dividing the absolute retention time of the compound by that of cholestane (12–15 minutes), which was included for reference in each run. The various factors of molecular structure which influence the retention time will be considered in turn as the results are presented.

Molecular Weight.—Table V lists the relative retention times of the methyl ethers of twelve stanols ranging from androstanol (C_{19}) to stigmastanol (C_{29}). When the logarithm of the retention time of the 3β-methoxy-5α- compounds is plotted against the number of carbon atoms in the molecule, the points so obtained give an approximately straight line (Fig. 2). That the straight line relationship is not exact is to be expected, since the compounds concerned are not members of a true homologous series. The retention factors associated with branching methyl groups would not be expected to be exactly the same as those arising from simple elongation of the side-chain (James and Martin, 1952).

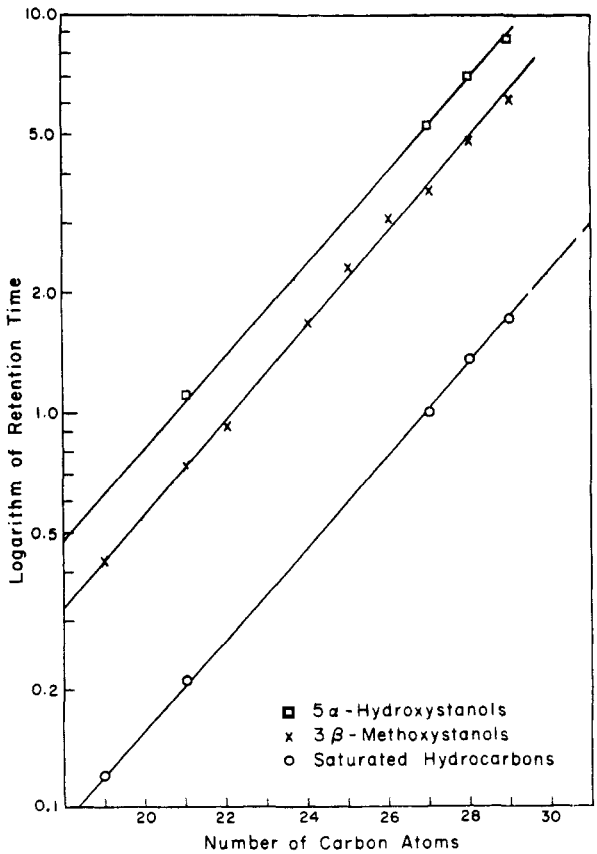

FIG. 2.—Relative retention times of some steroid hydrocarbons and monosubstituted derivatives.

TABLE V

RELATIVE RETENTION TIMES OF STANOL METHYL ETHERS

Androstan-3β-ol	0.42
Allopregnan-3β-ol	0.73
Bisnorallocholan-3β-ol	0.91
Allocholan-3β-ol	1.67
26,27-Bisnorcholestan-3β-ol[a]	2.30
27-Norcholestan-3β-ol	3.05
Cholestan-3β-ol	3.60
Cholestan-3α-ol	2.61
Coprostan-3β-ol	2.36
Coprostan-3α-ol	3.10
Ergostan-3β-ol	4.81
Stigmastan-3β-ol	6.10

[a] Gift of Professor E. D. Bergmann.

Orientation and Environment of the 3-Methoxyl Group.—Table V illustrates the influence of inversion of the 3-methoxyl group from the less hindered, equatorial configuration to the more hindered, axial configuration. The retention time of 3β-methoxycholestane exceeds that of 3α-methoxycholestane by a factor of 1.38. Similarly the separation factor between 3α-methoxycoprostane and 3β-methoxycoprostane is 1.32. The separation factor of 1.38 between the methyl ethers of ergosterol and its 10-epimer (Table VI) is no doubt mainly due to the fact that in the latter the 3β-methoxyl group is axial whereas in the former it is equatorial.

Lanosterol and the related 4:4-dimethyl sterols, the relative retention times of which are listed in Table VI, are of interest in that the weight and steric factors effectively counteract each other. Thus it can be seen that none of the C_{30} sterol ethers of this series has a retention time as great as that of the C_{29} sterol, stigmastanol. This effect is in keeping with the known hindrance of the 3β-hydroxyl group by the 4:4-dimethyl grouping of lanosterol and related compounds, which facilitates their separation from sterols unsubstituted at C_4 by conventional techniques of adsorption chromatography.

The Influence of Double Bonds.—In Table VI are listed the relative retention times of 34 unsaturated 3-methoxysteroids of the C_{19} to C_{30} (lanostane) series. Comparison of the retention times of these compounds with those of their saturated analogues shown in Table V shows that, in general, the retention time of an unsaturated sterol exceeds that of its parent stanol. This finding was fully anticipated in connection with the choice of liquid phase. There are some exceptions to this rule, however, and these will be considered below.

The magnitude of the effect of a double bond on the retention time is seen to depend upon its position in the molecule, but it is the same, within experimental limits (±2%), for any given position for each series studied. These points are illustrated in Table VII, which lists the separation factors between various unsaturated compounds and their analogues which lack some or all of the features of unsaturation. Attention is drawn to the following

TABLE VI

RELATIVE RETENTION TIMES OF SOME UNSATURATED STEROL METHYL ETHERS

Δ^5-Androsten-3β-ol[a]	0.455	$\Delta^{8(14)}$-Ergosten-3β-ol	5.10
Δ^5-Pregnen-3β-ol	0.805	$\Delta^{5,22}$-Ergostadien-3β-ol[b]	4.43
$\Delta^{5,16}$-Pregnadien-3β-ol	0.785	$\Delta^{7,22}$-Ergostadien-3β-ol[b]	5.55
Δ^5-Cholesten-3β-ol	3.9	$\Delta^{7,9,22}$-Ergostatrien-3β-ol	5.83
Δ^5-Cholesten-3α-ol	3.5	$\Delta^{5,7}$-Ergostadien-3β-ol[b]	7.30
Δ^6-Cholesten-3β-ol[b]	4.15	$\Delta^{5,7,22}$-Ergostatrien-3β-ol	6.52
Δ^7-Cholesten-3β-ol	4.70	$\Delta^{5,7,22}$-9β-Ergostatrien-3β-ol[d]	6.40
Δ^8-Cholesten-3β-ol[b]	3.88	$\Delta^{5,24(28)}$-Ergostadien-3β-ol[b]	6.08
$\Delta^{8(14)}$-Cholesten-3β-ol	3.73	$\Delta^{5,7,22}$-10 epi-Ergostatrien-3β-ol[d]	4.70
Δ^9-Cholesten-3β-ol[c]	3.45	$\Delta^{5,7,9,22}$-10 epi-Ergostatetraen-3β-ol	4.95
Δ^{14}-Cholesten-3β-ol	4.15	Δ^5-Stigmasten-3β-ol[b]	6.60
$\Delta^{8,14}$-Cholestadien-3β-ol[b]	4.56	Δ^{22}-Stigmasten-3β-ol[e]	5.40
$\Delta^{5,22}$-Cholestadien-3β-ol[b]	3.83	$\Delta^{5,22}$-Stigmastadien-3β-ol[b]	5.80
$\Delta^{5,7}$-Cholestadien-3β-ol	5.50	$\Delta^{5,24(28)}$-Stigmastadien-3β-ol[b]	7.86
$\Delta^{5,24}$-Cholestadien-3β-ol[b]	5.40	Δ^8-4,4,14α-Trimethylcholesten-3β-ol	4.00
Δ^5-Ergosten-3β-ol[b]	5.15	$\Delta^{8,24}$-4,4,14α-Trimethylcholestadien-3β-ol	5.32
Δ^7-Ergosten-3β-ol	6.05	$\Delta^{7,9,24}$-4,4,14α-Trimethylcholestatrien-3β-ol	5.60

[a] Gift of Professor H. B. Henbest. [b] Gifts of Professor Konrad Bloch. [c] Gift of Professor Louis Fieser. [d] Gifts of Dr. Peter Bladon. [e] Gift of Dr. Barbara Wright.

points concerning the data of Table VII. (1) The separation factor between a Δ^5-3β-methoxystenol and its 5α- saturated analogue is virtually constant, varying only between 1.07 and 1.10 from the androstane to the stigmastane series. Such a variation is within the limits of accuracy of measurement with the equipment available. (2) The separation factors associated with the Δ^7- and $\Delta^{8(14)}$-bonds are the same, within the limits of measurement, in both the cholestane and ergostane series. (3) The same is true for the separation factor associated with the $\Delta^{5,7}$-diene, taken as a unit system. (4) No monounsaturated Δ^{24}-compounds were available for testing and therefore no separation factor characteristic of this double bond could be obtained by direct comparison of a Δ^{24}- stenol with its parent stanol. However, the separation factor between lanosterol ($\Delta^{8,24}$) and dihydrolanosterol (Δ^8) methyl ethers is the same as that between the methyl ethers of desmosterol ($\Delta^{5,24}$) and cholesterol (Δ^5) (1.33 and 1.38 respectively). (5) The separation factors between the $\Delta^{5,24(28)}$- and Δ^5-compounds in the ergostane and stigmastane series are also equal. Again, no monounsaturated $\Delta^{24(28)}$-compounds were available for testing. (6) The methyl ethers of Δ^{22}-stigmastenol and stigmastanol have a separation factor of 0.89. Thus, the Δ^{22}-bond shows an anomolous effect in causing a reduction in retention time as compared with that of the stanol. The separation factors between the $\Delta^{5,22}$-dienol methyl ethers and their Δ^5-analogues is the same in both the ergostane and stigmastane series and is the same, within the limits of measurement, as that between the methyl ethers of Δ^{22}-stigmastenol and stigmastanol. (7) The separation factor between $\Delta^{5,22}$-cholestadienol and cholesterol methyl ethers has a significantly different value (0.98) from that seen in the C_{28} and C_{29} series.

TABLE VII

SEPARATION FACTORS CHARACTERISTIC OF SPECIFIC POSITIONS OF UNSATURATION IN 3β-METHOXYSTEROIDS OF DIFFERENT SERIES

Separation Factor	Androstane	Allopregnane	Cholestane	Ergostane	Stigmastane	Lanostane
Δ^5/Stanol	1.08	1.10	1.08	1.07	1.08	
Δ^7/Stanol			1.30	1.26		
$\Delta^{5,7}$/Stanol			1.53	1.52		
$\Delta^{5,7}/\Delta^7$			1.17	1.21		
$\Delta^{8(14)}$/Stanol			1.04	1.06		
Δ^{22}/Stanol					0.89	
$\Delta^{5,22}/\Delta^5$			0.98	0.86	0.88	
$\Delta^{8,24}/\Delta^8$						1.33
$\Delta^{5,24}/\Delta^5$			1.38			
$\Delta^{5,24(28)}/\Delta^5$				1.18	1.19	

If the result obtained for the Δ^{22}-bond in the cholestane series is for the moment overlooked, the above observations lead to the following generalizations.

Each isolated double bond introduced into the sterol ether molecule influences the retention time of the parent saturated structure by a factor which is constant for the particular position of the double bond, but is independent of the molecular weight. This may be expressed as:

$$r_{(s+x)} = r_s \times k_x \qquad (1)$$

where $r_{(s+x)}$ is the retention time of a sterol methyl ether with a double bond in position x, r_s is the retention time of the saturated structure, and k_x is the constant retention factor characteristic of the Δ^x- bond.

For a dienol with two non-interacting double bonds in positions x and y, the retention time $r_{(s+x+y)}$ of the total structure is given by the corresponding expression:

$$r_{(s+x+y)} = r_s \times k_x \times k_y \qquad (2)$$

where k_y is the retention factor characteristic of the second double bond, Δ^y. This relationship can be illustrated in the case of desmosterol, where r_s (cholestanol) = 3.60, k_{Δ^5} = 1.08, and $k_{\Delta^{24}}$ = 1.38. The calculated retention time, according to equation 2, is therefore 3.60 × 1.08 × 1.38 = 5.37. The observed value is 5.40. Similarly, the retention time of stigmasterol may be derived as the product of the retention time (r_s) of stigmastanol (=6.10), k_{Δ^5} (=1.08), and $k_{\Delta^{22}}$ (=0.88), giving a value of 5.80 in exact agreement with that observed.

It is clear, from the separation factors between the $\Delta^{5,7}$-dienol methyl ethers and the corresponding

Δ^7- and Δ^5-compounds, that the retention factor for a conjugated diene cannot be computed in a simple fashion from those of its constituent double bonds.

Anomalous Effects of Double Bonds.—Three double bonds—Δ^9, Δ^{16}, and Δ^{22}—have been found to shorten, rather than to increase, the retention times of the parent saturated structures (Table VI). The probability that the effect of the Δ^{22}- bond is the result of conformational restriction, which is more marked in the Δ^{22}-C_{24}-substituted series ($k_{\Delta^{22}} = 0.88$) than in the Δ^{22}-cholestene series ($k_{\Delta^{22}} = 0.98$), has been discussed in an earlier report (Clayton, 1961). Effects of the Δ^9 and Δ^{16} bonds similar to those observed in this study ($k_{\Delta^9} = 0.96$; $k_{\Delta^{16}} = 0.97$) have also been noted by Lipsky and Landowne (1961), but at the present time no fully satisfactory explanation for these observations can be offered.

In Dreiding models the Δ^9- bond deflects C_{11} and the general plane of ring C toward the α-side of the molecule with a probable reduction of molecular volume. Like the conformational restriction effects noted in relation to the Δ^{22}- bond, this could lead to a limitation of solute-solvent interaction and, hence, to shorter retention times. The well-known steric hindrance of the C_9 and C_{11} positions would, in any case, be consistent with a much smaller polarity contribution by the Δ^9- bond than by most other nuclear double bonds. As might be expected, in the bile acid series, in which the molecule is already more hindered on its α-side and restricted in volume due to the *cis*-fusion of rings A and B, the negative effect of the Δ^9- bond is found to be greater (*cf.* Table X).

The explanation of the effect of the Δ^{16}- bond is more obscure. In three-dimensional models this linkage deflects the side-chain away from the vicinity of the C_{13} angular methyl group. From this effect alone it might be expected that the resulting increase in effective molecular volume would lead to increased retention time rather than to the observed decrease. However, other effects of the Δ^{16}- bond which are measurable in the models are a reduction (*ca.* 10%) in the distance of separation of the angular methyl groups at C_{10} and C_{13} and a small (*ca.* 5%) reduction in the length of the nucleus (C_3–C_{15}).

Some General Group Retention Factor Relationships.—The observations summarized in equation (2) suggested that, by analogy, the retention time of a polysubstituted steroid with non-interacting substituent groups, *a*, *b*, *c*, . . . etc., might be expressed by the general equation:

$$r_{(n+a+b+c\ldots)} = r_n \times k_a \times k_b \times k_c \times \ldots \text{ etc.} \quad (3)$$

where $r_{(n+a+b+c\ldots)}$ is the retention time of the total structure, r_n is the retention time of the unsubstituted nucleus, and k_a, k_b, k_c . . . etc. are constant factors characteristic of the substituent groups *a*, *b*, *c*, . . . etc., and their individual positions in the molecule. Equation (3) was tested by comparing the retention times of suitably selected compounds, and the results are presented in Tables VIII, IX, and X.

In Table VIII are listed the retention times of ten steroid hydrocarbons and their corresponding 3β-methoxy- and 5α-hydroxy- derivatives. It can be seen that the sterically hindered 5α-hydroxyl group does not increase the retention time prohibitively. The last two columns show the separation factors between the hydrocarbons and their derivatives, characterized as the retention factors for the 3β-methoxyl and 5α-hydroxyl group respectively. The retention factor for the 3β-methoxyl group maintains a near-constancy over the whole range of compounds examined. The retention factor for the 5α-hydroxyl group shows a similar degree of constancy in four of the six cases listed. These results are shown graphically in Figure 2. Two of the values for $k_{5\alpha\text{-OH}}$, those derived for 5α-hydroxy-Δ^2-cholestene and 5α-hydroxy-Δ^7-ergostene, are significantly lower (4.4 and 4.1, respectively, compared with 5.2 for the saturated compounds). It has been pointed out previously (Clayton, 1961a) that hydrogen bonding between the hydroxyl group and the ethylenic bond may be responsible for the low values obtained for $k_{5\alpha\text{-OH}}$ with the Δ^2- and Δ^7-5α-hydroxy compounds. The effect (a mutual partial saturation of the dipolar solvent-bonding capacity of the two groups) is analogous to that observed by DePuy and Story (1959) in a study of the gas-liquid chromatography of some norborneol and dehydronorborneol derivatives in which intramolecular hydrogen bonding was also suggested as an explanation for a lowering of retention time in certain structures. In the present case the effect may in part be due to simple steric hindrance by the 5α-hydroxyl group to the interaction between the double bond and the solvent molecules. Some further observations relevant to this point are discussed in connection with the data for the Δ^7-bile acid derivatives (Table X).

Table IX lists the relative retention times of some mono- and bifunctional steroids. The observed values for the latter are compared with calculated values derived by means of equation (3). It is seen that there is agreement within the range $\pm 4\%$.

Table X contains the retention times of a series of bile acid derivatives with and without a 3α-oxygen function. Group retention factors, calculated as the separation factors between appropriate pairs of compounds, are also listed. The following points are significant. (1) The retention factor for a 3α-methoxyl group in this series is almost identical with that of the 3β-methoxyl group in the 5α-(cholestane) series. This is doubtless a reflection of the fact that the orientation of the methoxyl group is equatorial in both. (2) The retention factor for the Δ^9- bond is less than unity (0.90–0.93), the Δ^9- compounds having retention times lower than those of their saturated analogues. This is true also of the Δ^9- bond in the cholestane series ($k_{\Delta^9} = 0.96$) (*cf.* Table VI), and has been

TABLE VIII

RETENTION FACTORS FOR 3β-METHOXYL AND 5α-HYDROXYL GROUPS

Hydrocarbon	Relative Retention Times			Retention Factors	
	Unsubstituted	With Substituent Groups: 3β-methoxyl	With Substituent Groups: 5α-hydroxyl	$k_{3\beta}$-OMe	$k_{5\alpha}$-OH
Androstane	0.12	0.42		3.5	
Allopregnane	0.21	0.73	1.10	3.5	5.3
Cholestane	1.00	3.60	5.24	3.6	5.2
Δ^2-Cholestene[a]	1.20		5.34[a]		4.4
Δ^3-Cholestene[a]	1.10	3.88		3.5	
Ergostane	1.36	4.81	6.98	3.5	5.1
Δ^7-Ergostene[a]	1.78	6.05	7.26	3.4	4.1
$\Delta^{5(14)}$-Ergostene[a]	1.40	5.10		3.6	
$\Delta^{7,22}$-Ergostadiene[a]	1.57	5.55		3.5	
Stigmastane	1.72	6.10	8.70	3.6	5.1

[a] Gifts of Professor H. B. Henbest.

TABLE IX

OBSERVED AND CALCULATED[a] RELATIVE RETENTION TIMES OF SOME MONO- AND BIFUNCTIONAL STEROIDS

Monofunctional Steroids		Bifunctional Steroids	
17β-Methoxyandrostane	0.333	3β,17β-Dimethoxyandrostane	1.19 (1.15)
7α-Methoxycholestane	1.69	3β,7α-Dimethoxycholestane	5.90 (6.10)
7β-Methoxycholestane	2.30	3β,7β-Dimethoxycholestane	7.90 (8.30)
		3β,7α-Dimethoxyallopregnane	1.19 (1.22)
		3β,7β-Dimethoxyallopregnane	1.60 (1.68)
7α-Hydroxycholestane[b]	6.83	3β-Methoxy-7α-hydroxyallopregnane	5.20 (5.00)

[a] Calculated values in parentheses. [b] Gift of Professor Louis Fieser.

TABLE X

RELATIVE RETENTION TIMES OF SOME BILE ACID DERIVATIVES[a]

Compound	Rel. retention time	$k\Delta^7$	$k\Delta^9$	$k\Delta^{7,9}$	$k_{3\alpha}$-OH
Methyl cholanate	3.81				
3α-Methoxymethyl cholanate	12.8				3.40
Methyl Δ^7-cholenate	4.31	1.13			
3α-Methoxymethyl Δ^7-cholenate	14.85	1.16			3.45
Methyl Δ^9-cholenate	3.41		0.90		
3α-Methoxymethyl Δ^9-cholenate	11.85		0.93		3.48
3α-Methoxymethyl $\Delta^{7,9}$-choladienate	14.0			1.09	

[a] All compounds (unmethylated) were the gift of Professor Louis Fieser.

discussed above. (3) The retention factor for the Δ^7- bond in the bile acid series is lower (1.13–1.16 as compared with 1.30) than in the cholestane and ergostane series. A difference of this type might be expected from a comparison of the environment of the Δ^7- bond in the A:B-*trans*- and A:B-*cis*- series. In the latter the position of the 5α-hydrogen atom in the A:B-*trans*- structure comes to be occupied by a methylene group (C_4) which is probably sufficiently close to the Δ^7- bond to cause some shielding from dipole interaction with the solvent. (4) The Δ^9- bond in conjugation with the Δ^7- bond in the bile acid series causes a slight lowering of the retention time, whereas in the A:B-*trans*- (ergostane) series (Table VI) it causes a small increase. The difference is probably related to the molecular volume effect which has been discussed above.

The results presented in this paper give strong support to the validity of the general equation, (3). The compounds tested so far are limited in number and in the types of functional groups which they contain, and only one liquid phase has been used throughout. The limits of accuracy with which equation (3) may be applied to compounds of different types chromatographed on other liquid phases therefore remain to be determined. There is evidence from the work of Vanden Heuvel and Horning (1961) that equation (3) is valid for sapogenins undergoing gas-liquid chromatography on the silicone liquid phase, S.E. 30. It is possible, by calculating the separation factors for suitably selected pairs of sapogenins from among those listed by the authors, to derive retention factors for the 12-keto- group (=1.83), the 2α-hydroxyl group (=1.77 in presence of 3β-hydroxyl), the Δ^5- bond (=0.96), and the inversion (neo:iso) at C_{25} (=1.02). These factors are found to be reproducible to within ±2% when derived as ratios of two different pairs of compounds in each case. The results obtained by Sjovall *et al.* (1961) with a wide range of bile acid derivatives chromatographed on silicone columns lend themselves to a similar analysis with results which are in substantial agreement with equation (3). On the other hand, it should be pointed out that the data of Vanden Heuvel *et al.* (1961) and of Haahti *et al.* (1961a) are not in exact agreement with equation (3). For example, the latter authors record values for the relative retention times of cholestan-3-one and androstan-17-one, from which the relative retention times of androstan-3,17-dione may be calculated. The observed retention times of this dione, on five different polyester phases, differ from the calculated values by 11% to 33%. These divergent

results require further investigation before equation (3) can be unconditionally accepted.

The relationships expressed in equation (3) are analogous to those which apply to members of the same and closely related homologous series. In these cases it is well established that substitution of a particular group into a molecule will enhance its retention time by a factor which remains constant for the liquid phase employed, provided the environment of the substituent group remains equivalent in all cases. The most familiar example is the substitution of a hydrogen atom in the terminal methyl group of an aliphatic chain by a further methyl group (James and Martin, 1952; Ray, 1954). The constant factor by which this structural modification augments the retention time is referred to (James, 1960) as the "—CH_2— retention factor," but in relation to the influence of substituent groups it might rather be termed the "terminal —CH_3 retention factor." The replacement of a hydrogen atom with a methyl group at the penultimate carbon atom in an aliphatic chain is also well known to increase the retention time by a constant factor (James and Martin, 1956).

Considered together with these and similar observations in the literature, the results obtained in the present study suggest that to a close approximation, a particular substituent group will always influence the retention time of any compound undergoing gas-liquid chromatography on a given liquid phase and under fixed conditions, by the same retention factor, provided that the intramolecular stereo-electronic environment of the group is the same. Such a generalization would be expected to hold, at least approximately, on the basis of the semi-empirical and theoretical treatments of Pierotti *et al.* (1956) and Martire (1961) of the factors involved in solute-solvent interactions on the gas chromatogram. The analysis of "retention dispersion" data by Wehrli and Kovats (1959) also lends support to this view.

The practical application of this generalization seems likely to be twofold. Under circumstances where intramolecular group interaction is negligible, and as an adjunct to established chemical methods, it is of considerable analytical value. This is especially true in relation to the chemistry of the natural products which occur as structurally closely related "families." Conversely, its judicious use may yield further insight into the nature and degree of intramolecular group interactions in their own right.

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