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GAS CHROMATOGRAPHIC ANALYSIS OF STEROID HORMONES

PART II. GAS CHROMATOGRAPHIC BEHAVIOUR OF TRIMETHYLSILYL ETHERS

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ABSTRACT

The potential value of trimethylsilyl ethers has been reported in the field of gas chromatographic analysis of steroids. So far as has been known, satisfactory procedures affording trimethylsilyl ethers of corticosteroids have not been attained because of variable by-products produced but such a result was by no means incomprehensible.

In the present study, an abbreviated procedure of trimethylsilylation, especially suited for use in this country, was devised and the determining conditions for conversion to the derivative was studied; required derivatives could be obtained by selecting proper conditions although satisfactory results could not be achieved for a series of naturally occurring steroids with polyfunctional groups.

The steroid number data presented here, in agreement with the original view, substantiated the regularity and the significance of the steroid numbers for various derivatives of less polar steroids. It is of interest that the degree of irregularity in steroid number was generally related to the complexity of polar groups, the presence of reactive structures in a steroid molecule; so that steroids of corticoid type, being more polar, showed steroid numbers far from the expected values, probably due to the complicated side reaction during trimethylsilylation, on the one hand, and on the other, probably due to the interrelationship between reactive structures being much more extended than so called "vicinal effect" which has been pointed out in the gas chromatographic behaviour of polyfunctional steroids.

INTRODUCTION

Gas chromatographic techniques have made it possible to separate small amounts of various steroidal compounds for the purpose of identification and estimation. Although they have been applied currently to the assay of 17-ketosteroids, estrogens, progestogens and bile acids in the field of clinical and experimental studies, there has been no satisfactory method available for the

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analysis of closely related polyfunctional steroids which are frequently found in metabolites of steroid hormones. One of such unresolved problems was the separation of 17-hydroxy corticosteroids and their analogues.

For the first time, Vanden Heuvel and Horning[1] convincingly demonstrated that corticosteroids could not be chromatographed satisfactorily. During the vaporization process adrenal cortical steroids containing a 17α, 21-dihydroxy-20-keto structure were found to undergo an intramolecular elimination of the side chain. The products of this thermally induced reaction were corresponding 17-ketosteroids. Another specific thermal change was the conversion of a 21-hydroxy-20-ketosteroids to a mixture of a few degradation products.

Since this observation, a number of studies have been undertaken to avoid this thermally induced degradation. These attemps were carried out in the following way:

(1) estimation of corresponding 17-ketosteroids yielded by pyrolysis.
(2) oxidation to 17-ketosteroids prior to gas chromatography.
(3) conversion to appropriate derivatives. (substituting reactive functional groups with protecting groups.)

Pyrolysis was made available by Patti *et al.*[2)3)], and also by Luetscher *et al.*[4)]. Their procedures yielded varying degrees of success for the determination of the principal metabolites of cortisol in urine. Quantitative aspect of this process was studied by Gottfried[5)].

For quantitative work, however, it would seem more profitable to prepare 17-ketosteroids prior to gas chromatography than carrying out this conversion during the vaporization process. This oxidation was used in the systemic analysis by Menini[6)] and was also the basis of the analytical procedure described by Bailey[7)] as "initial oxidation" which attained greater recovery by oxidizing corticosteroids with sodium bismuthate before loading. This oxidation was also applied for the determination of urinary metabolites of cortisol by Sparagana[8)]. Similarly, for the identification of metabolites of corticosterone, Exley[9)] employed reduction by potassium borohydride and oxidation by chromic acid and sodium bismuthate. In this later oxidizing step periodate was used as well by Murphy *et al.*[10)] These degradative procedures were employed for the purpose of stabilizing corticosteroids as much as possible or obtaining quantitative data. However, the conversion to less specific derivatives inevitably neglected the difference in the side chain and, in addition, the reaction might accompany certain changes in the functional groups attached to the steroid nucleus.

Conversion to appropriate derivatives was another way attempted on polyfunctional steroids which tended to be unstable under the usual conditions of chromatography. Hydroxyl groups of cortisone and aldosterone were acetylated by Wotiz[11)]. Aldosterone-18, 21-diacetate was stable on 3% SE-30 column. Conversely, Kliman and Foster[12)] proved transformation of this derivative on 2%

SE-30 column. With regard to this discrepancy, it is of interest to refer to Wotiz's observation[13] that, as the substance chromatographed became less stable, greater concentration of the stationary phase was required. Aldosterone gave better results when chromatographed as γ-lactone[12)14)]. By means of acetylation Carr and Wotiz[15] demonstrated considerable thermal stability for ring A reduced corticosteroids such as tetrahydroaldosterone, tetrahydrocortisone, tetrahydrocostisol, cortolone, cortol etc. but the recovery was not satisfactory. On the other hand, Merits[14] degraded the side chain of 21-hydroxy-20-ketosteroids with periodic acid. The products were esterified with diazomethane and the resultant methylesters were chromatographed on relatively thin film columns.

Later this method was practically applied by Kittinger[16] to the steroids isolated from rat adrenal incubate. Bismethylenedioxy derivatives are the specific chemical products of steroids with the dihydroxyacetone moiety. Kirschner and Fales[17] adopted these derivatives for chromatography and obtained more advantages of this procedure than the acethylation or the direct pyrolysis method, although the reaction to convert bismethylenedioxy derivatives gave variable amounts of by-products except cortisone. More recently Brooks[18)19)] examined acetylated corticosteroids and their related 20-keto-pregnane derivatives on 1% SE-30 column; 17α, 21-diacetoxy-20-ketosteroids as well as 21-acetoxy-20-ketosteroids showed considerable stability giving principal peaks attributable to the unaltered compounds. On aged column the peak of a 17α, 21-diacetoxy-20-ketosteroid diminished and a new one emerged corresponding to a Δ^{16}, 21 -acetoxy-20-ketosteroid induced by elimination. Similar decomposition of acetate and chloroacetate was observed by Rapp and Eik-Nes[20]; chloroacetates of corticosteroids were less sensitive to electron capture detection than acetates and most of them led to several peaks, only some of which were quantitatable.

Most of the above methods were concerned primarily with converting polyfunctional steroids into more stable compounds and in the studies along this line they succeeded partially. Simultaneously, however, more simplified techniques, designed to increase thermal stability of steroids were being developed. Degradation of steroids under these exposed conditions was demonstrated by Wotiz[13] to be due to a unimolecular vapor phase reaction rather than to a catalytic decomposition at the active site; the thermal stability of a steroid and deactivating ability determined by the amount and polarity of a liquid phase are the determinant factor of this thermally induced phenomenon. Therefore, more stable derivatives may be required for the separation of such labile substances as corticosteroids.

The introduction of the use of trimethylsilyl derivatives (TMS) was presumed to eliminate the above obstacles and the separation which was once thought to be difficult appeared to be accomplished with ease at a much lower temperature. In addition the hydrocarbon like behaviour of these derivatives

resulted in peaks which were very nearly theoretical in shape and, therefore, more accurately quantitated by peak area measurement[21]. Anylysis of polyfunctional steroids by means of their TMS has now been proposed by a few investigators. TMS of cortol and cortolone were reported by Rosenfeld[22] to be particularly suitable for gas chromatography. Gottfried[23] observed the gas chromatographic behariour of trimethylsilylated 20β-dihydro derivatives of corticosteroids where 20β-dihydro-17-deoxycorticosteroids afforded single peak whereas 20β-dihydrocortisone yielded two peaks without any thermal decomposition. Cortisol also gave three products in relatively varied yields. Since the labile nature of Δ^4-17α, 21-diacetoxy-3-ketosteroids was previously observed by Brooks[19], it is easily understood that this structure also interferes satisfactory chromatography when attempted to increase the thermal stability by trimethylsilylation.

Therefore, of particular interest is to observe the reaction of convertion to TMS and the gas chromatographic behaviour of these derivatives, partly because it has not been reported in detail and partly because these steroids seem to have a complicated reactivity under several conditions.

General Considerations on Trimethylsilyl Derivatives. (TMS)

In organic chemistry a number of groups or radicals have been used to protect functional groups in various compounds from degradation during several procedures—oxidations, reductions, hydrolysis, distillations etc. Examples of such protecting groups are illustrated by the use of the methyl or acetyl group to protect hydroxyl groups of organic compounds. But the reaction conditions required for the removal of these protecting groups may often destroy the compounds during the removal reactions. In contrast to these difficulties, trimethylsilyl derivatives were easily prepared, were stable to heat and oxygen, had considerably lower boiling points than the corresponding polyols and were readily hydrolyzed at room temperature to yield the original materials[24]. These properties made them ideal for use in the purification or separation of hydroxy compounds sensitive to heat, acids, alkalines, air oxidation, etc.[24] As a matter of fact, trimethylsilyl group has been used as a protecting group for the hydroxyl group in phenols[24)25] polyols[26] and hydroxy aromatic acids[27]. In addition the derivatives of alcohols[28)29], amines[30], amino acids[31)32)33], carboxylic acids[34] and carbohydrates such as uronic acid[35], glucose[36] sucrose[34], dextrin[34] and pektin[36] were reported. Recent progress in gas chromatography and also in mass spectrometry has stimulated considerably greater interest in certain derivatives of organic compounds with increased thermal stability, volatility, reduced adsorption on solid surfaces and enhanced separation factor[37]. Some of these required properties were satisfied with the use of TMS. Since the first report on trimethylsilylated phenols[38], application of this derivative for gas chromatography has been demonstrated successively for monosaccharide[39], al-

cohols[37] and amino acids[40]. As the first application for steroidal compounds, Lukkainen *et al.*[41][42] chromatographed derivatives of 17-ketosteroids, progestogens and estrogens and studied their properties in detail and concluded that they were most effective derivatives in quantitative work: The reagents for the derivative formation did not affect the keto group. They could be prepared in quantitative yield and were not liable to be subjected to absorption on active centers present in the gas chromatographic column. In spite of the polarity of the liquid phase, they had shorter retention time than would be expected from their molecular weight, and did not give rise to any tailing. Accordingly, high levels of sensitivity and precision were obtainable with the ordinary apparatus. Besides these, since the earlier reports on TMS, the general properties of TMS can be mentioned to be as follows:

In general the boiling points of TMS were about 10° to 15° higher than the parent aliphatic alcohols above the C_3 and C_4 regions. For amines and mercaptanes where hydrogen bonding of the parent compounds plays a smaller role, the boiling point elevation due to formation of TMS was of the order of 60°[43] but the boiling points of polyols[26] and polyphenol[43] were lowered by formation of TMS. It was reported[39] that by comparison with the parent compound, the derivatives of alcohols, polyols, phenols, aromatic hydroxyl acids, amines and amino acids showed increased volatility coupled with thermal stability. TMS of phenoxysilane[44], glucose[36] and pektin[36] showed such increased thermal stability as silyl derivatives of cellulose that showed much greater stability for heat than its acetyl derivatives[45]. Trimethylsilyl ethers are easily hydrolyzed with water but there are exceptions with different degrees of susceptibility. Trimethylsilylated glucose was stable for water but the derivative of uronic acid was less stable. This was interpreted as follows[36]: While in the former labile Si-O-C bonding is covered by hydrophobic CH_2 groups, in the latter trimethylsilyl ester group is hydrolyzed at first as in carboxylic acid[34] or amino acids[31] and the resultant acid serves as a catalyst[35] for hydrolysis of TMS. Derivatives of other carbohydrates such as sucrose[34] dextrin[34] and pektin[36] are not so stable for water as glucose, fructose and galactose.

In earlier works[24][28][44], trimethylchlorosilane (TMCS) was used for the preparation of TMS and a slight excess of tertiary amine[26] such as pyridine[28], dimethylaniline[46] or tri-N-butylamine[26] was presumably employed to remove the hydrogen chloride[28]. With TMCS the reactive alcohols such as tertiary alcohol gave alkyl chloride and other alcohols did not give good yields of alkoxytrimethylsilane, presumably because of intervention by hydrogen chloride, but in the presence of pyridine, good yields of alkoxysilane were obtained[29]. Thus, besides a solvent, pyridine was regarded as an acid acceptor through the formation of pyridine hydrochlorid[26]. Henglein and Scheinost[36] proposed another role of pyridine as a transporter of TMCS which has already a polar

character (Si-Cl bond) before it is ionized completely through the formation of quarternary pyridinium ion. As an example hydroxymethylphenol was trimethylsilylated as belows: In the presence of pyridine TMCS was added slowly to hydroxymethylphenol in excess, while the reaction mixture was cooled so that the temperature did not exceed 55° and generally below 45°C. The reaction product was filtered and the excess solvent was distilled off by heating to a pot temperature of 160° C[24]. In case of tertiary alcohols yields from these procedures were far from quantitative preparation while olefins and halides were formed in substantial amounts; consequently hexamethyldisilazane (HMDS) in dimethyl sulfoxide or dimethyl formamide was preferred[47]. In general TMS were obtained conveniently by direct reaction with TMCS or preferably HMDS[43]. The catalytic effect of hydrogen or ammonium ion was observed on the reaction of primary amyl alcohol mixture with HMDS[43]. The reaction of alcohols with HMDS was effectively catalyzed by the availability of a proton, though it did take place in the absence of acid. Acid was generated by initial addition of a drop or two of TMCS after which precipitate (presumably ammonium salt[43]) was formed immediately. The more TMCS used initially, the greater the concentration of hydrogen chloride seemed to be generated and the reaction could be speeded[43]. Thus HMDS functions as a source of both acid acceptor (ammonia) and trimethylsilyl groups[43]. Langer *et al.* found it convenient to visualize the initial reaction of HMDS with an alcohol or amine in the presence of a proton in terms of an intermediate which may be a "pentacovalent intermediate". The low acidity of amines per se and the need for a proton in this intermediate would account for the larger accelerating effect of a small amount of acid on reaction of primary amines with HMDS[43]. On the other hand steric requirements would explain the failure of certain alcohols and secondary amines to react with HMDS[43]. All hydroxyl groups of pektin could not be converted to TMS[36]. Conversion of glucose gave penta-substitute for TMS but only tetra-substitute was obtainable for triphenylsilyl ether[34]. Amino acid containing only one amino and carboxyl group each did not react with TMCS as it was suggested that their reactivity is probably nullified by the formation of an intramolecular salt. If either of them is blocked by introduction of certain groups, they react with TMCS to give TMS: When the carboxyl group was blocked by the ethyl group, amino group reacted with TMCS to give silazane of a carboxylic acid ester (for example, N-trimethylsilyl-glycine ethyl ester[32]) and when amino group was blocked by a benzoyl group, the respective trimethylsilyl ester was obtained. (for example, N-benzoylglycine trimethylsilyl ester[31]). As amino acids, uronic acid[35] and carboxylic acid[34] reacted with TMCS or HMDS but the carbonyl group in salicylaldehyde[44], was not involved in the reaction.

As a side reaction, TMCS (b.p. 58°C) or HMDS (b.p. 126°C) reacted with water to give trimethylsilanol (b.p. 99°C) which could not be isolated because

of its tendency to dehydrate, forming hexamethyldisiloxane (b.p. 100°C) and the formation of an azeotropic mixture (b.p. 90°C) with the later compounds[28]. These could be separated from TMS by distillation[43]. Hexamethyldisiloxane did not interfere with the conversion of the alcohols to their TMS[43]. As another side reaction, it was pointed out that the lower methylketones especially acetone, undergo a variety of acid and base catalized condensation and addition reactions under the condition of conversion of alcohols to TMS[43].

As noted above, a number of studies relating to the conversion of hydroxy compounds to their TMS have been made but less is known about the silylation of steroidal compounds than about that of others. Several aspects of the reaction should be taken into consideration also in the trimethylsilylation of steroids. In the present study there were two objects in mind. First it was necessary to investigate further such reaction to establish an otherwise difficult preparation procedure for specific compounds. Second it seems desirable to observe the gas chromatographic behaviour of silyl derivatives as a prelude to further work on the more complex polyfunctional steroids seen in samples of biological origin.

EXPERIMENTAL PROCEDURE

Gas Chromatography: The equipment used was essentially the same as was used in the previous work[21] and only the major points are described. All chromatographic procedures were carried out with GC-1B (Shimadzu Seisakusho Co., Ltd., Japan) equipped with a flame ionization detector. The stationary phases were 1.5% SE-30, 1% SE-52 and 1% XE-60 adsorbed on 60-80 mesh chromosorb W. XE-60 phase was used depending on the polarity of the samples. The temperature of the column was 240°C. The flash heater and detector were held at 300 and 250°C respectively. The flow rate of carrier gas was adjusted so that these samples may elute within a reasonable period of time.

Sampling: TMS was prepared according to the method of Chamberlain *et al.*[49] but slightly modified. Not only did this save some time-consuming preparation steps in the analysis but it actually helped to assure the stability of the samples. Samples of steroids (200 μg) were treated wtih the reagent mixture (0.3 to 0.5 ml) in stoppered micro-test tubes.

Reagent mixture was prepared as follows: HMDS and TMCS (amounting to 1 ml) were added in a stoppered test tube containing 4 ml of pyridine. Pyridine was dehydrated with KOH pellet before use. The amounts of reagents were equal but the proportion of TMCS to HMDS was changed according to the respective condition.

After standing at a given temperature for a given period, pyridine and

excess reagents were removed by evaporating under reduced pressure (with water aspirator). This removed also the greater part of the non-steroidal impurities which were capable of presenting minor peaks near the solvent front on the recordings. Without further purification the residue was then dissolved in dehydrated aceton (100-200 μl) and injected into the gas chromatograph as soon as possible after evaporation since decomposition of TMS resulted from prolonged conservation. During these procedures all contact with moisture was carefully avoided by handling the samples in a N_2 tent which prevented the formation of minimal white precipitate. It may be difficult to keep the aceton free of water. Accordingly aceton was used only after storage over sodium sulfate and further precautions as was noted by Creech[50] were not taken in the present study. Hexane[51] may be preferred for this reason.

Together with an adequate quantity (5 μg level) of the samples thus prepared, cholestane was introduced into the syringe prior to injection into the gas chromatograph.

Nomenclature of Steroids: For convenience a sort of shorthand method of describing steroids was used in the present study. Steroid nucleus such as androstane, pregnane and cholestane was noted with the initial A, P, and C respectively. The configuration at C_5 and double bonds were indicated by prefixes; for example 5 β-Pregnane was "5 β-P". Other functional groups were indicated by suffixes with abbreviations of usual suffixes -ol or -one; for example, carbonyl group at C_{20} was noted by the position number 20 and 3 α-hydroxyl group as 3 α. As an exception, 21-hydroxyl group was described by the position number alone as 21. Hence 4-P-3-11 β, 17 α -20-21. implies 4-Pregnenene-11 β, 17 α, 21-triol-3, 20-dione (cortisol).

Besides these abbreviations, common trivial names were used as follows; comp. Q (desoxycorticosterone), comp. B (corticosterone), comp. A (dehydrocorticosterone), comp. S (cortexolone), comp. F (cortisol), comp. E (cortisone) etc. and similarly THE (tetrahydrocortisone), allo-THE (5 α-hydrogen equivalent compound of THE), β-cortolone (20 β-hydroxy derivative of THE) etc.

RESULTS AND DISCUSSION

I. Preliminary Experiment with Representative Steroids

As typical representatives of steroids with respective functional groups, 11-hydroxyandrosterone (5 α-A-3 α, 11 β-17.), comp. Q (4-P-3, 20-21.), 5 α-P-3 β, 17 α-20, THF (5 β-P-3 α, 11 β, 17 α-20-21.) and 4-A-3, 11, 17. were employed. These steroids were trimethylsilylated under various conditions and the course of the reaction was followed by gas chromatography. The reaction conditions employed in the preliminary expriment are given in Table 1. Reaction conditions

TABLE 1. Conditions Employed for Trimethylsilylation in Preliminary Experiment

Condition	Reagent mixture			Standing	
	Pyridine (μl)	HMDS (μl)	TMCS (μl)	Temp. (°C)	Hours
A	400	100	—	37	48
B	400	90	10	37	24
C	400	50	50	37~52	24
D	400	—	100	37	1

The conditions used in preliminary experiment are grossly classified into four groups. These conditions become severer in the order of A, B, C and D. Each reagent mixture was used for a test sample containing 200 μg of a steroid.

become severer, in the order of A, B, C and D. The difference between them chiefly depends on different concentrations of HMDS and TMCS. Although a white precipitate was formed with the reaction mixture of C or D, this did not interfere with the subsequent gas chromatographic analysis, since on dissolving TMS in aceton, the precipitate remained on the wall of vessels.

When compared by a series of samples treated under these condition the close correspondence of reaction conditions and products was usually observed although occasional discrepancies were encountered. The reaction took only 1 hour under condition A but treatment for 48 hours will point out the degree of process to further reactions. In order to observe the effect of TMCS, exposure to the severe condition D was attempted and resulted in the decomposition of steroids.

As an example of a commonly occuring 11-hydroxy steroid, 11-hydroxyandrosterone was trimethylsilylated under the conditions A, B, C and D, then the crude products were chromatographed on 1% XE-60. The resultant chromatograms are shown in A, B, C and D of Fig. 1 respectively. Under condition A, 11-hydroxyandrosterone yielded 3α-trimethylsiloxy derivative (RRT 2.55) without detectable by-products, whereas under condition B another peak was observed at a shorter retention time (RRT 1.40), consistent with its less polar characteristics of 3α, 11β-ditrimethylsiloxy derivatives. As was reported earlier[52], 11β-trimethylsiloxy derivative of 17-ketosteroid was eluted earlier on XE-60 phase than the corresponding 11-keto or 11β-hydroxy derivative. With the greater concentration of TMCS in the reaction mixture, the reaction proceeded to yield the latter derivative alone. When TMCS was used as a reagent, decomposition of steroids occurred. According to the earlier report[22], 3α-hydroxyl group was easily silylated whereas the 11β-hydroxyl group was less reactive; the silylation of the 11β-hydroxyl group needed the presence of pyridine as a

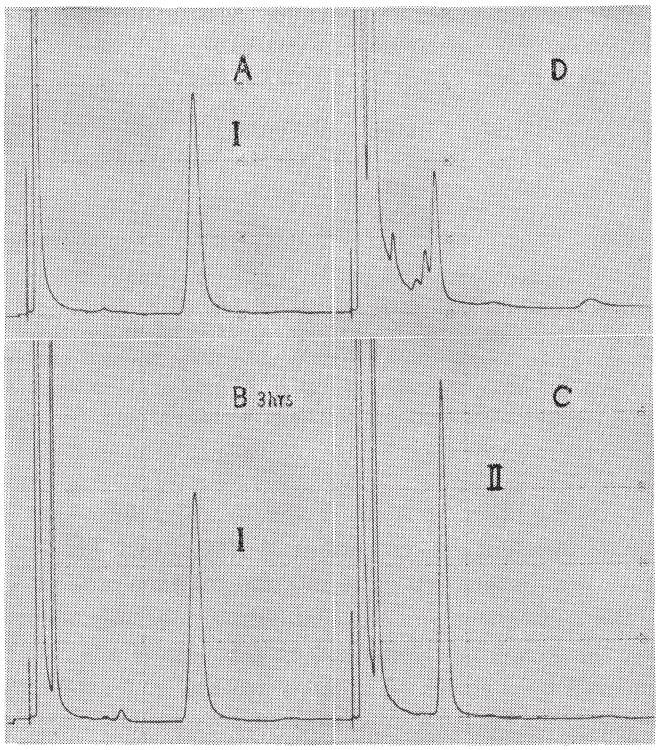

FIG. 1. Chromatogram of trimethylsilylation product of 11-OH-androsterone
Column, 1% XE-60. Column temp., 240° C.
Retention time. peak I 2.55
peak II 1.40

solvent. The result presented here, however, shows that 11 β-hydroxyl group was not necessarily sylilated even when a given steroid was derivatized in the pyridine solution; the reaction of this function depended rather on the concentration of TMCS in this case.

Comp. Q is the representative of steroids with the 21-hydroxy-20-keto side chain. The reaction products of this compounds were chromatographed on 1.5% SE-30 as is shown in Fig. 2. After treatment under the mild condition A, the derivative appeared to be stable to gas chromatography, giving a well-defined single peak (RRT 1.88). Since sharp peaks were normally observed for TMS, it seems probable that the reaction occurred at the hydroxyl group at C_{21}. The small broad peak which appeared at times in front of the main peak was ascribed to decomposition products of the parent steroid, because the same broad peak emerged on the chromatogram of comp. Q. Trimethylsilylation with the reaction mixture containing HMDS and TMCS, however, resulted in

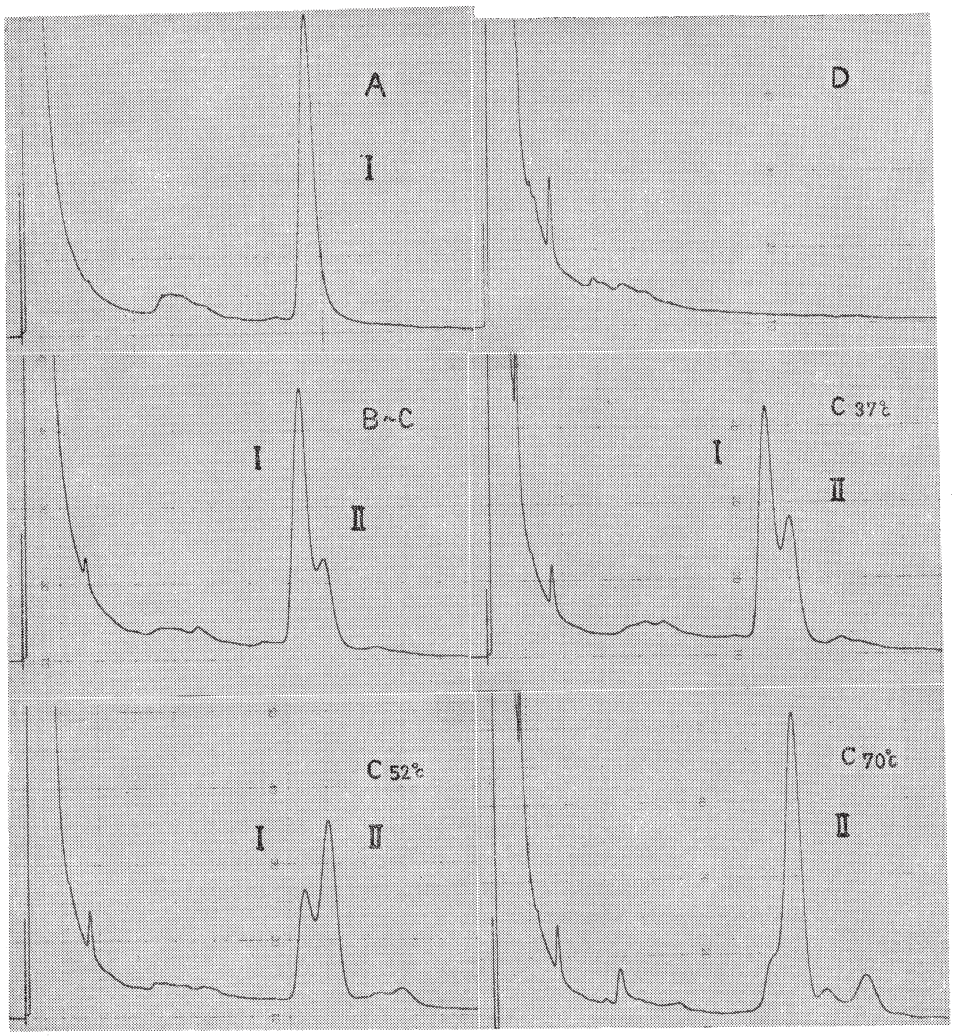

FIG. 2. Chromatogram of trimethylsilylation product of desoxycorticosterone (comp. Q).
Column, 1.5% SE-30. Column temp., 240° C.
Retention time, peak I 1.88
peak II 2.05

two distinct peaks; one was identical with the peak obtained under mild condition and another peak appeared at a retention time close to the former (RRT 2.05). Increasing the amount of TMCS in the reaction mixture or a higher temperature changed the ratio of these two peaks found in favour of the latter and, in addition, the peak was accompanied with some by-products which may be ascribed to the result of side reaction under severe condition, the products

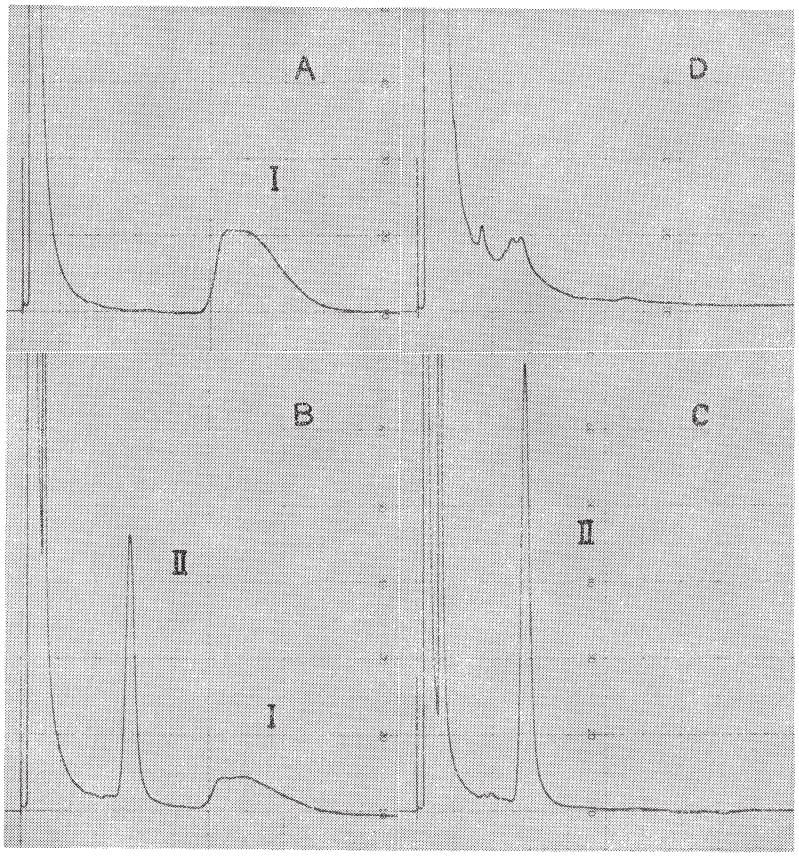

FIG. 3. Chromatogram of trimethylsilylation product of 5α-pregnane-3β, 17α-diol-20-one.
Column, 1% XE-60. Column temp., 240° C.
Retention time, peak I 3.09 (3.30)
peak II 1.61

would not be consistent with the interpretation that thermal degradation occurred also with TMS.

The steroid with a 17α-hydroxy-20-keto side chain was reported to be susceptible to thermal rearrangement with the formation of D-homosteroids[53]. The chromatogram of silylation products of 5α-P-3β, 17α-20. are given in Fig. 3. When this steroid was trimethylsilylated under condition A, a characteristic broad peak (RRT 3.09 to 3.30) was observed on 1% XE-60 phase, probably attributable to transformation occurring during chromatography. Even after the substitution of 3β-hydroxyl group, the steroid posessing the 17α-hydroxy-20-ketone structure was also altered, probably with D-homoanulation, since the similar peak as that of the parent compound emerged on the chromatogram.

After treatment under severer condition, the major peak was observed at a shorter retention time (RRT 1.61), sharp symmetrical in shape, which strongly suggests that this corresponds to the altered 17 α-hydroxy-20-ketone structure

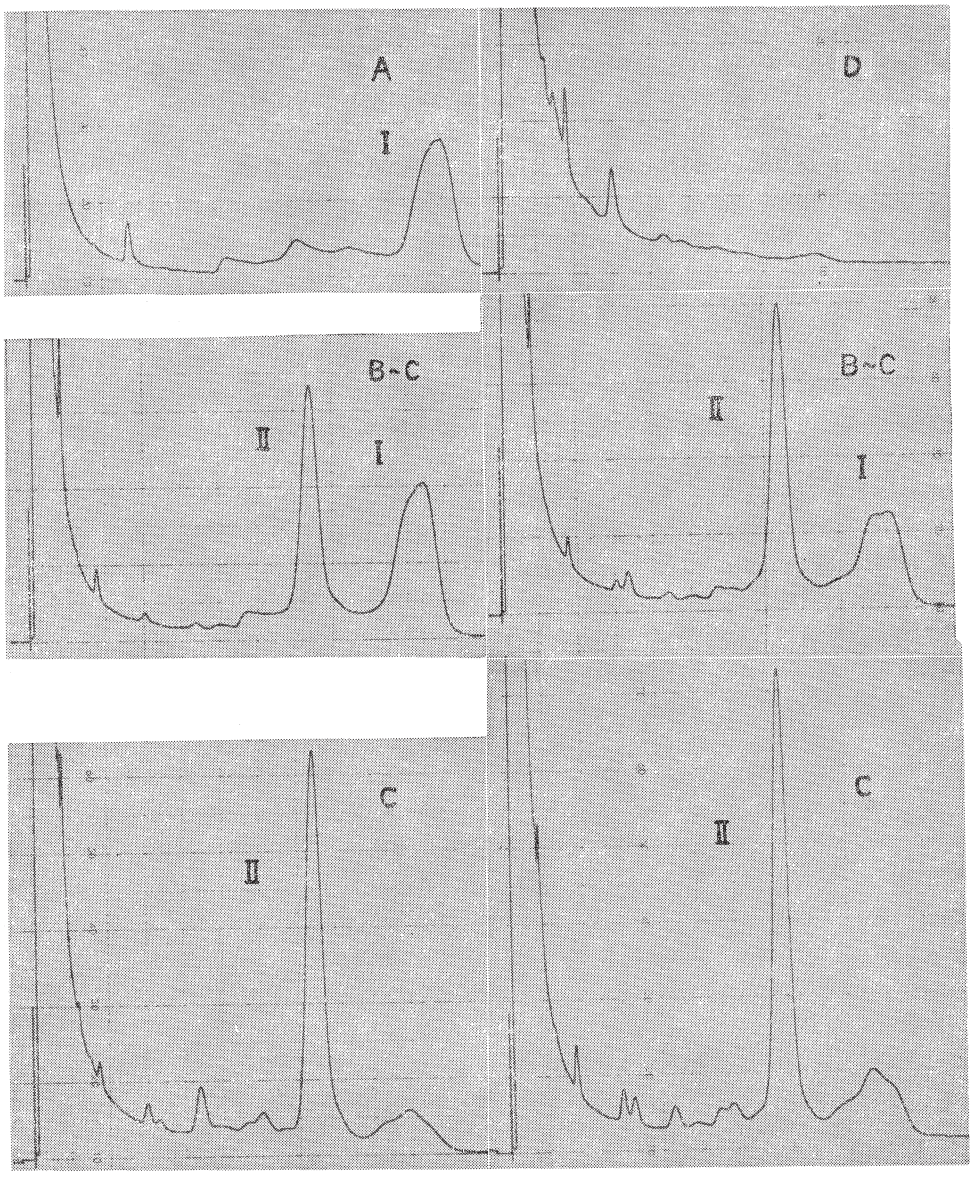

FIG. 4. Chromatogram of trimethylsilylation product of tetrahydro compound F. (THF).
Column, 1.5% SE-30. Column temp., 240° C.
Retention time, peak I 2.70
peak II 1.84

other than D-homosteroids. The 17 α-acetoxy-20-ketones were reported to be stable to chromatography, giving a well-defined single peak sharply distinguishable from the peak of 17 α-hydroxy-20-ketones[19]. Treatment under severer condition afforded a more complete change to this sharp peak but the reaction with TMCS alone caused decomposition. It was thus postulated that the reaction which affects 17 α-hydroxyl group may be caused by changing the reaction condition. The observation that this derivative yielded the main symmetrical peak on the recording without no resultant by-product, may provide the proper application of this derivative for reliable clinical estimation.

As is shown in Fig. 4, THF showed a more complicated gas chromatographic behaviour on SE-30 phase. After treatment under mild condition, the crude

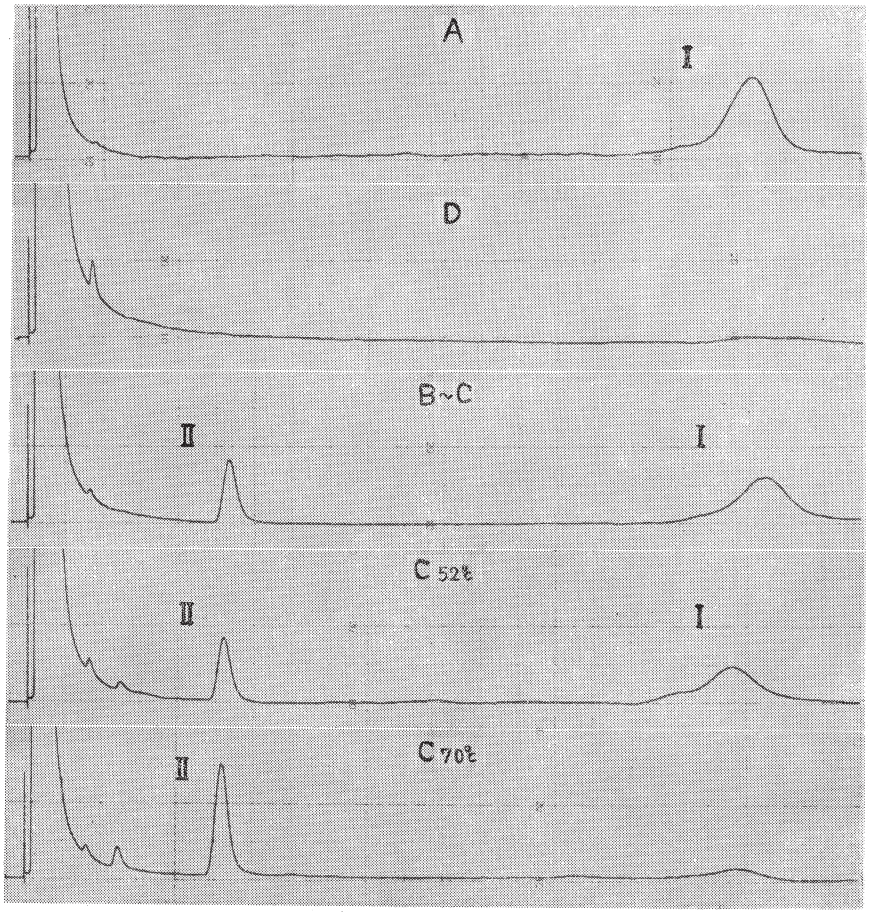

FIG. 5. Effect of trimethylsilylation on 4-androstene-3, 11, 17-trione
Column, 1% XE-60. Column temp., 240° C.
Retention time, peak I 12.7 (parent compound)
peak II 3.40 (by-product)

reaction product gave a main peak at a retention time 2.70, together with small subsidiary peaks which eluted earlier than the main peak. When TMCS was added to the reagent mixture, the major peak with a shorter retention time 1.84, appeared at the expense of the former peak. Although improved chromatographic properties were observed, the peak may represent some elimination product of smaller molecular weight or with less polar characteristics. By increasing the ratio of TMCS in the reagent mixture, the reaction apparently proceeded to completion but the conversion to a single derivative could not be attained under any condition and the yield was reduced by the formation of by-products in varying degrees. In general, mixtures of two peaks may be expected, accompanied with other subsidiary peaks. Brooks[18)19)] reported on the gas chromatographic behaviour of acetate; 17α,21-diacetoxy-20-ketones generally giving two peaks. One ascribed to the unchanged material and predominating when fresh columns were used, and the other to the Δ^{16}-21-acetoxy-20-ketone resulting from elimination especially on aged column. However trimethylsilylation with HMDS and TMCS afforded two major peaks, and the relative proportion of them depended on the reaction condition.

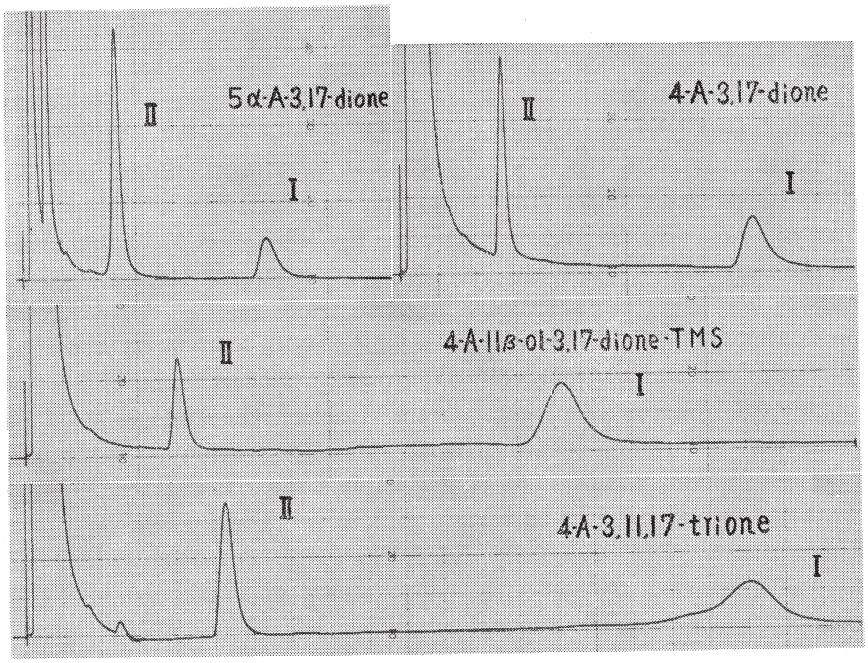

FIG. 6. Effect of trimethylsilylation on steroids with carbonyl groups.
Column, 1% XE-60. Column temp., 240° C
peak I parent compound
peak II by-product

As a representative of carbonyl compounds, 4-A-3, 11, 17. was treated with the regaent mixture and chromatographed on 1% XE-60. The results are shown in Fig. 5. Treatment with HMDS alone did not show any effect on 4-A-3, 11, 17.; this type of steroid appeared relatively stable to the reaction and to the chromatographic condition. However, in the chromatogram of products under severer trimethylsilylation, the second peak (RRT 3.40) other than the original peak (RRT 12.7) emerged. As no subsidiary peaks were obtained in case of the mild treatment, this component perhaps corresponded to the secondary by-product under the severer condition, suggesting that a highly reactive carbonyl group was involved in this case. The lack of specificity of this reaction can be seen in Fig. 6. This type of steroid, 5 α-A-3, 17., 4-A-3, 17. as well as 4-A-3-11 β-17 induced essentially the same reaction of the carbonyl group, with 3-keto structure as the most likely possibility of the carbonyl groups. According to the above observation, possible reaction of carbonyl group can also be postulated as a reason for the secondary change in the trimethylsilylation of comp. Q.

II. Certain Determining Factors in Trimethylsilylation

Before entering into a more detailed consideration of trimethylsilylated products of various steroids, it seems most desirable first to observe more precisely just what occurs under various conditions. The observations above are useful for understanding the variation in gas chromatographic behaviour of products obtained by trimethylsilylation and it may be concluded that the widely used trimethylsilylation reaction as applied to polyfunctional steroids is subjected to considerable uncertainty regarding its result and its interpretation. This uncertainty may be reduced significantly when factors influencing this reaction are elucidated and when a relatively selective reaction condition is used for steroids consisting the samples. The conditions may be difficult to differentiate at times, since the intensity of condition is dependent on a number of factors, not all of which are readily controlled. Nevertheless it is apparent that such varied factors as solvent, concentrations of reagents, quality of TMCS, moisture, temperature and period of treatment alter the nature of the product. Even though certain modifications of the original method of trimethylsilylation may be employed, there may be certain factors which are much more controllable than others. The subsequent observations are concerned with them.

As was mentioned previously, a larger amount of TMCS led to formation of a white precipitate in the reagent mixture. HMDS and TMCS, when used in high concentrations on treating converted the reagent mixture to a viscous polymer which interfered with the following procedures. For this reason adequate concentrations of reagents were required. Within such concentrations the dependence of the reaction on reagent concentration was evident as is il-

TABLE 2. Effect of Reagent Concentration on Trimethylsilylation

Conditions				Peaks resulting from reaction products																
Reagents		Temp.	Time	Comp. Q			THF			11-OH-andro.			5α-P-3β, 17α, 20.			4-A-3, 11, 17.				
HMDS	TMCS	°C	hrs.	I	II	B	I	II	B	I	II	B	I	II	B	I	II	B		
100	—	37	48	M	—	—	M	—	s	M	—	—	M	—	—	M	—	—		
90	10	〃	24	M	—	—				s	M	—	M	m	—	M	—	—		
90	10	〃	48	M	s	—	M	—	s	—	M	—	m	M	—	M	s	—		
75	25	〃	24	M	m	—	M	M	—	—	M	—	s	M	—	M	m	—		
50	50	〃	24	M	m	—	s	M	—	—	M	—	s	M	—	M	M	—		
25	75	〃	24	M	m	—	s	M	—	—	M	—	s	M	—	M	s	—		
—	100	〃	1	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+		
100	—	52	48	M	—	—	M	—	s	M	—	—	M	—	—	M	—	—		
90	10	〃	24	M	s	—				—	M	—	M	M	—					
90	10	〃	48	M	m	—	M	M	s	—	M	—	M	M	—	M	M	—		
75	25	〃	24	M	M	—	m	M	—	—	M	—	s	M	—	m	M	—		
50	50	〃	24	m	M	s	m	M	s	—	M	—	s	M	—	M	M	—		
25	75	〃	24				s	M	s	—	M	—	s	M	—	s	M	—		

The peaks I and II are the same as those seen in Figs. 1 to 5. According to the peak size, obtained peaks were classified into three; major peak (M) minor peak (m) and small peak (s) and the relation between them was M>m>s. The peaks in each chromatogram are shown by the above abbreviations and other minute peak of by-product (B) neglected in most cases.

lustrated in Table 2. Trimethylsilylation proceeded as the greater amount of TMCS was employed and the amount of TMCS had a critical effect on the reaction. On the other hand, it is evident that there was a marked difference in the reaction of the individual functional group which was treated under various conditions. Hydroxyl group at C_3 and C_{21} reacted easily with HMDS alone but the change of the hydroxyl group at 11β position or at 17α position in 17α-hydroxy-20-keto structure needed the presence of TMCS. Moreover the greater amount of TMCS induced a secondary change of comp. Q, THF and 4-A-3, 11, 17. and finally TMCS alone caused decomposition of steroids.

The data given in Table 3, shows the effect of temperature on the reaction. Although there was observed a rough relation between the rise in reaction temperature and the reaction product, temperature elevation from 37° to 90°C led to comparatively small changes in the pattern of the main product. But in case of comp. Q, THF and 4-A-3, 11, 17. the formation of by-products appeared to be much more marked with a higher temperature. It seems that one of the critical points in trimethylsilylation for the purpose of avoiding secondary by-product lies in employing a lower temperature.

Table 4 suggests the magnitude of time effect. The reaction conditions involve treatment at 37°C for a period which varied from 1 to 72 hours. The composition of the trimethylsilylation product was rather time dependent; longer treatment led to rise in the quantity of converted products in samples, that

TABLE 3. Temperature Effect on Trimethylsilylation

Conditions		Peaks resulted from reaction products														
Temp.	Time	Comp. Q			THF			11-OH-andro.			5α-P-3β, 17α,20.			4-A-3,11,17.		
°C	hrs.	I	II	B	I	II	B	I	II	B	I	II	B	I	II	B
37	1	M	—	—	M	—	m	M	M	—	M	—	—	M	—	—
52	〃	M	—	—	M	—	s	s	M	—	M	s	—	M	—	—
70	〃	M	—	—	M	—	m	m	M	—	s	M	—			
90	〃	M	—	—				s	M	—	M	s	—	M	s	—
37	48	M	s	—	M	—	s	—	M	—	m	M	—	M	s	—
52	〃	M	m	—	M	M	s	—	M	—	M	M	—	M	M	—
70	〃	m	M	—				—	M	—	—	M	s			

The steroids (200 μg) were treated with reagent mixture containing 90 μl of HMDS and 10 μl of TMCS in 400 μl of pyridine. Description the same as employed in Table 2.

TABLE 4. Time Effect on Trimethylsilylation

Conditions		Peaks resulting from reaction products														
Time	Temp.	Comp. Q			THF			11-OH-andro.			5α-P-3β, 17α,20.			4-A-3,11,17.		
hrs.	°C	I	II	B	I	II	B	I	II	B	I	II	B	I	II	B
1	37	M	—	—	M	—	m	M	M	—	M	—	—	M	—	—
3	〃	M	—	—	M	—	M	m	M	—				M	—	—
12	〃	M	—	—	M	—	s	s	M	—	M	s	—	M	—	—
24	〃	M	—	—				s	M	—	M	m	—	M	—	—
48	〃	M	s	—	M	—	s	—	M	—	m	M	—	M	s	—
72	〃	M	s	—	M	M	—	—	M	—	—	M	—	M	s	—
1	52	M	—	—	M	—	s	s	M	—	M	s	—	M	—	—
24	〃							—	M	—	M	M	—			
48	〃	M	m	—	M	M	s	—	M	—	M	M	—	M	M	—
72	〃	M	s	—	s	M	s	—	M	—	—	M	—	M	M	—

The steroids (200 μg) were treated with reagent mixture containing 90 μl of HMDS and 10 μl of TMCS in 400 μl of pyridine. Description the same as employed in Table 2.

is, to a corresponding procession of reaction and in fact the reaction proceeded even after 48 hours. But the length of time required for maximum yield of a derivative of a certain steroid could not be defined in this experiment, because the process was accompanied by successive side reactions.

In general trimethylsilylation proceeded with the greater amount of TMCS, higher temperature and longer treating time, but there were no marked difference in reaction products under various conditions. Probably they differed, in degree, in the procession of a series of reaction. Nevertheless the rate at which this reaction proceeds could be easily governed by the length of time of treatment and the main product found was rather controllable by the amount of

TMCS in the reaction mixture. Accordingly by determining the reaction condition, it is perhaps possible to obtain the required product with considerable effectiveness. These observations provide an approach to a better understanding and utilization of trimethylsilylation that is less subjected to experimental variations than alternative methods which have been described in previous work. With these background data in mind, one may understand the typical pattern of the product. The interpretation of the relationship of the reaction condition to the products and concomitant gas chromatographic behaviour of them as well as the significance of the main peak may need a further observation of the results in relation to the structure of steroids.

III. Gas Chromatographic Behaviour of Trimethylsilylated Stroids

As a practical application of the above observations, several steroids were subjected to treatment under various conditions. As now recognized from the preceeding observation, the condition, whether mild or severe, was influenced by the amount of TMCS, reaction temperature and incubation time. However the problem with this observation was that the difference in the result under various reaction conditions was rather a matter of degree, not an all or none change and it was necessary to establish a standard of the reaction condition which would inevitably be quite arbitrary. The standard employed in the present observation is listed in Table 5. In the following observation steroids were treated under these conditions and the products were chromatographed on two non-polar phases (1.5% SE-30 and 1% SE-52) at 240° C. In order to exclude the expected variation in retention time, the same column was used at a constant temperature (within $240 \pm 0.2°C$) and the flow rate was regulated to allow constant retention time for cholestane (10 min.). Before every determination on different days, relative retention time (RRT) of 5α-androstane was determined on both phases; the mean value of 0.147 ± 0.002 on 1.5% SE-30 and 0.135 ± 0.001 on 1% SE-52 were encountered. As is shown in Table 6, an ex-

TABLE 5. Conditions Employed for Trimethylsilylation of Various Steroids

Condition	Reagent mixture			Standing	
	Pyridine (μl)	HMDS (μl)	TMCS (μl)	Temp.	Hours.
Mild	400	100	—	52	1
Moderate	400	75	25	37	24
Severe	400	50	50	52	24

These conditions were selected arbitrarily from consideration of the results obtained in preceeding experiments. Each reagent mixture was used for a test sample containing 200 μg of a steroid.

TABLE 6. Relative Retention Times for Various Trimethylsilylated Steroids

Steroid ↓ \ Phase→ / Condition↘	Relative retention times							
	1.5% SE-30 (240°C)				1% SE-52 (240°C)			
	Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
5 α-A.	0.15				0.14			
5 α-A-17.	0.25			0.25	0.25			0.25
5 α-A-3, 17.	0.47			0.55	0.54			0.57
5 α-A-3, 16.	0.47			0.54	0.55			0.57
5 α-A-3 α-17.	0.43	0.42		0.43	0.48	0.43		0.43
5 α-A-3 β-17.	0.44	0.53		0.53	0.49	0.55		0.55
5 α-A-3 β, 16 β.	0.44	0.60		0.60	0.49	0.57		0.57
5 α-A-3 α-11, 17.	0.53	0.52		0.52	0.63	0.56		0.56
5 α-A-3 α, 11 β-17.	0.67	0.65	0.72	0.71	0.82	0.70	0.73	0.72
5 α-P.	0.23			0.23	0.22			0.22
5 α-P-3, 20.	0.73			0.85	0.84			0.89
5 α-P-3 β-20.	0.67	0.80		0.80	0.75	0.84		0.84
5 α-P-3-20 β.	0.74	0.94		1.12	0.84	0.99		1.09
5 α-P-3 β, 20 β.	0.69	1.06		1.07	0.76	1.04		1.05
5 α-P-3 β, 17 α-20.	0.94	1.11	1.11	1.11	1.11	1.19	1.11	1.11
allo-THB		2.18	2.03	2.02		2.41	2.05	2.06
allo-THF		2.75	2.55 (1.87)	1.88		3.01 (1.94)	2.58 (1.85)	1.88
5 α-C.	1.00			1.00	1.00			1.00
5 α-C-3.	1.97			2.34	2.22			2.38
Estrone		0.56		0.56		0.61		0.62
Estradiol		0.68		0.69		0.69		0.70
Estriol		1.23		1.23		1.27		1.27
5 β-A.	0.14				0.12			
5 β-A-3 α-17.	0.40	0.44		0.43	0.44	0.45		0.45
5 β-A-3 α-11, 17.	0.49	0.53		0.53	0.57	0.57		0.57
5 β-A-3 α, 11 β-17.	0.63	0.66	0.75	0.75	0.74	0.73	0.76	0.76
5 β-P-3, 20.	0.67			0.80	0.75			0.84
5 β-P-3 α-20.	0.62	0.68		0.68	0.69	0.70		0.70
5 β-P-3 β-20.	0.61	0.61		0.65	0.68	0.68		0.66
5 β-P-3 α, 20 α.	0.67	0.94 (0.73)		0.94	0.74	0.89 (0.74)		0.89
Pt.		1.37		1.36		1.35		1.35
11-keto-Pt.		1.77		1.78		1.82		1.82
THA		1.71	1.71	1.70		1.85	1.84	1.84
THB		2.09	2.01	2.00 (2.32)		2.30	2.03	2.03 (1.88,2.18)
THS		1.80		1.40		1.86		1.41
THE		2.18		1.76 (2.22)		2.38		1.81
THF		2.70	2.58 (1.83)	1.84		2.84, 2.99 (1.91)	2.46, 26.4	1.81
Cortolone		3.01	2.98	2.99		3.03	3.04	3.02
β-Cortolone		3.16	3.16	3.19		3.30	3.21	3.28 (2.00)
Cortol		3.49	3.89	3.85 (2.34)		3.52	3.70	3.68 (2.16)
β-Cortol		3.59	3.93	3.88		3.68	3.75	3.74 (2.00)
β-Cholestanol	1.87	2.24		2.26	2.02	2.29		2.29
4-A-3, 17.	0.55	0.56		0.58	0.66	0.66		0.62
4-A-3, 16.	0.56			0.58	0.67			0.62
4-A-3-17 α.	0.58	0.58	0.63	0.63	0.67	0.67	0.61	0.61
4-A-3-17 β.	0.59	0.67	0.71	0.71	0.69	0.71	0.70	0.69
4-A-3, 11, 17.	0.64	0.65		0.70	0.81	0.82		0.80
4-A-3-11 β-17.	0.89	0.90	1.01	1.02	1.15	1.15	1.07	1.08

TABLE 6. (*Continued*)

Phase→ / Condition↘ / Steroid↓	Relative retention times							
	1.5% SE-30 (240°C)				1% SE-52 (240°C)			
	Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
4-P-3, 20.	0.86			0.91	1.02			1.00
4-P-3-17 α-20.	1.22	1.22	1.27 (1.13)	1.27	1.45	1.45	1.29	1.30
Comp. Q		1.88		2.05		2.21		2.20
Comp. A		2.24	2.23	2.58		2.73	2.76	2.83
Comp. B		3.03	2.95	3.08		3.78	3.38	3.29
Comp. S		2.41		2.60 (2.33)		2.87 (1.90)		2.65
Comp. E		1.82,2.77 (2.24)	2.77	3.37 (2.79)		2.27, 3.39 (2.82)	3.22 (2.48)	3.51 (3.21)
Comp. F		2.26, 2.46	3.12 (3.73)	3.38 (3.18)		2.86, 3.19 (2.58,4.07)	3.40	3.41 (2.85)
Aldosterone		2.48		2.84 (2.50)		2.85		2.96
4-C-3.	2.45			2.56	2.74			2.69
1, 4-C-3.	2.70			2.65	3.02			3.03
5-A-3 β-17.	0.43	0.50		0.51	0.47	0.52		0.52
5-P-3 β-20.	0.66	0.80		0.80	0.75	0.84		0.83
5-P-3 β, 17 α-20.	0.91	1.06	1.09	1.09	1.06	1.13	1.09	1.09
Δ^5-Pt.		1.63		1.62 (1.52)		1.65		1.65 (1.46)
5-C-3 β.	1.83	2.23		2.21	1.99	2.28		2.28
5-C-3 β-7.	3.62	4.29		4.28	4.32	4.70		4.70

Several steroids were treated under different conditions (mild, moderate and severe) as shown in Table 5 and the products were chromatographed on 1.5% SE-30 and 1% SE-52 column at 240°C.

Relative retention times (RRT) of major and minor peaks were listed and those of small peaks shown in the parenthesis.

tensive experiment resulted in the accumulation of considerable RRT data for the main products of all compounds studied. The chromatogram of derivatives thus prepared frequently showed some small peaks aside from the main peak. The small peaks were neglected. Similarly certain corticosteroids were found to give more than one peak; the data for minor peaks were noted in parenthesis. But in any case it coule not be found that steroid possesing specific side chain underwent usual thermal degradation into 17-ketosteroids since the peaks of corresponding 17-ketosteroids were not yielded except when hydrolysis of TMS was suspected. These data were used for studying the relationship between structure and gas chromatographic behaviour.

Although there are several ways of expressing the relationship between the structure of a steroid and its RRT, the steroid number concept proposed by Vanden Heuvel and Horning[54] possesses greater convenience and validity than other expressions. A particular advantage lies in the relative constancy of

these values with respect to changes in the condition of determination. At first steroid number (SN) was obtained by employing the reference line or reference equation for calculation. The equation used in the present study was obtained as follows:

Steroid number was defined as a value based on carbon content relationship for steroid skeltons which was found by comparing (in logarithmic relationships) the retention time of a steroid with that of cholestane and androstane under the same condition. Steroid number of androstane was determined to be 19 while that of cholestane to be 27. Thus as shown in Fig. 7, SN for a given steroid was obtained from its RRT to cholestane by the following relationship:

$$\frac{\log 1 - \log R_a}{27-19} = \frac{\log 1 - \log R_n}{27-N}$$

where N is SN for a given steroid, R_n, its RRT and R_a, RRT for 5α-androstane. The equation will then be:

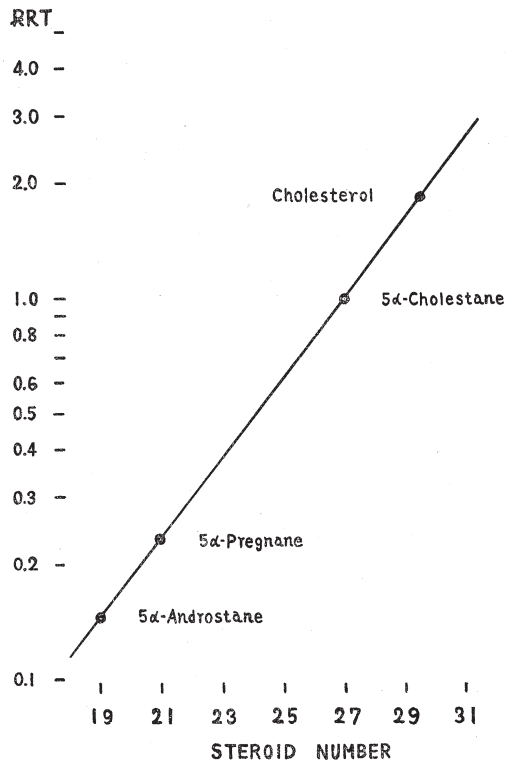

FIG. 7. The reference line (the androstane-cholestane line) used to determine steroid numbers from relative retention times.

$$-\frac{\log R_a}{8}=\frac{-\log R_n}{27-N} \qquad N-27=-8\times\frac{\log R_n}{\log R_a}$$

$$N=27-8\times\frac{\log R_n}{\log R_a}$$

R_a was 0.147 and 0.135 on 1.5% SE-30 and 1% SE-52 respectively, hence

$$N=27+0.61\times\log R_n \quad \text{on 1.5\% SE-30}$$
$$N=27+9.20\times\log R_n \quad \text{on 1\% SE-52.}$$

As has been referred to before, the relative retention time observed in the determination of a steroid number was determined with the same column and under the same condition, although steroid numbers were independent of small changes in experimental variables which were difficult to control with high precision in ordinary gas chromatographic apparatus. Under these circumstances there was a striking constancy observed for steroid numbers. Originally steroid numbers were not different in principle for relative retention times, but the corresponding steroid numbers were constant to a much greater degree, and inferences drawn with regard to structure were on a sounder experimental basis.

Next, it has been postulated[54] that steroid number may be expressed as a summation of terms dependent on the nature of the carbon skelton and on the functional group present in the molecule as follows:

$$N=S+F_1+\cdots+F_n.$$

Where N is the steroid number, S the carbon content of the steroid skelton and $F_1, \ldots, F_n$ the values determined with the respective functional groups of the steroid. Thus SN were found to be useful in defining relationships based primarily on molecular weight and molecular shape. It is obvious that an additive expression relating steroid numbers to structure presupposes an absence of intramolecular interaction between groups. Unusual or unexpected SN relationship should be examined to elucidate if intramolecular effects are involved.

Table 7 shows steroid numbers derived from the relative retention data in Table 6. Discussion below will be resricted chiefly to the data obtained on 1.5% SE-30 because SN was originally based on the data on this non-polar phase. When compared further, these data may be capable of giving highly useful structural information. Accordingly, the following observations were carried out to arrive at a tentative structural inference for unidentified TMS and to clarify the experimental limitations inherent in trimethylsilylation. Changes in SN led to information about the molecular size and molecular shape of the product, for example the great difference in SN for 5 β-P-3 α-20. and 5 β-P-3 α, 20 α. after mild silylation, may be ascribed to the difference in the molecular shape (or size) of these derivatives. However, considering this prob-

TABLE 7. Steroid Number Values for Various Trimethylsilylated Steroids

Steroid ↓ / Phase → / Condition ↘	Steroid number values							
	1.5% SE-30				1% SE-52			
	Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
5 α-A.	19.0				19.0			
5 α-A-17.	21.1			21.2	21.5			21.4
5 α-A-3, 17.	23.8			24.5	24.6			24.8
5 α-A-3, 16.	23.8			24.5	24.6			24.7
5 α-A-3 α-17.	23.5	23.4		23.5	24.1	23.6		23.6
5 α-A-3 β-17.	23.5	24.3		24.3	24.1	24.6		24.6
5 α-A-3 β, 16 β.	23.5	24.9		24.9	24.2	24.8		24.7
5 α-A-3 α-11, 17.	24.4	24.2	24.2	24.2	25.1	24.7	24.7	24.7
5 α-A-3 α, 11 β-17.	25.4	25.2	25.6	25.6	26.2	25.6	25.7	25.7
5 α-P.	20.9			20.8	20.9			20.9
5 α-P-3, 20.	25.7			26.3	26.3			26.5
5 α-P-3 β-20.	25.3	26.1		26.0	25.9	26.3		26.3
5 α-P-3-20 β.	25.7	26.7		27.5	26.3	27.0		27.3
5 α-P-3 β, 20 β.	25.5	27.3		27.3	25.9	27.2		27.2
5 α-P-3 β, 17 α-20.	26.7	27.4	27.4	27.4	27.4	27.7	27.4	27.4
allo-THB		30.3	30.0	29.9		30.5	29.9	29.9
allo-THF		31.2	30.9 (29.5)	29.7		31.4 (29.6)	30.7 (29.5)	29.5
5 α-C.	27.0				27.0			
5 α-C-3.	29.9			30.6	30.2			30.5
Estrone		24.5		24.6		25.0		25.1
Estradiol		25.4		25.4		25.5		25.5
Estriol		27.9		27.9		28.0		28.0
5 β-A.	18.9				18.6			
5 β-A-3 α-17.	23.2	23.5		23.5	23.7	23.8		23.8
5 β-A-3 α-11, 17.	24.0	24.3	24.3	24.3	24.7	24.7	24.7	24.7
5 β-A-3 α, 11 β-17.	25.0	25.3	25.8	25.8	25.8	25.7	25.9	25.9
5 β-P-3, 20.	25.3			26.1	25.9			26.3
5 β-P-3 α-20.	25.0	25.4		25.4	25.5	25.6		25.6
5 β-P-3 β-20.	24.9	24.9		25.2	25.5	25.5		25.3
5 β-P-3 α, 20 α.	25.3	26.7 (25.7)		26.7	25.8	26.5 (25.8)		26.5
Pt.		28.3		28.3		28.2		28.2
11-keto-Pt.		29.4	29.4	29.4		29.4	29.4	29.4
THA		29.2	29.2	29.2		29.5	29.4	29.4
THB		30.1	29.9	29.9 (30.5)		30.3	29.8	29.8 (29.5,30.1)
THS		29.5		28.4		29.5		28.4
THE		30.3	30.3	29.4 (30.3)		30.5	30.5	29.4
THF		31.2	30.9 (29.5)	29.6		31.2, 31.4 (29.6)	30.6, 30.8	29.4
Cortolone		31.6	31.6	31.6		31.4	31.4	31.4
β-Cortolone		31.8	31.8	31.9		31.8	31.7	31.7 (29.8)
Cortol		32.2	32.7	32.6 (30.6)		32.0	32.2	32.2 (30.1)
β-Cortol		32.4	32.7	32.7		32.2	32.3	32.3 (29.6)
β-Cholestanol	29.6	30.4		30.4	29.8	30.3		30.3
4-A-3, 17.	24.5	24.6		24.7	25.3	25.4		25.1
4-A-3, 16.	24.5			24.7	25.4			25.1
4-A-3-17 α.	24.7	24.7	25.1	25.1	25.4	25.4	25.0	25.0
4-A-3-17 β.	24.8	25.3	25.6	25.6	25.5	25.6	25.6	25.5
4-A-3, 11, 17.	25.2	25.2	25.2	25.5	26.2	26.2	26.2	26.1
4-A-3-11 β-17.	26.5	26.5	27.0	27.1	27.6	27.6	27.2	27.3

TABLE 7. (*Continued*)

Phase→ / Condition↘ / Steroid ↓	Steroid number values							
	1.5% SE-30				1% SE-52			
	Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
4-P-3, 20.	26.4			26.6	27.1			27.0
4-P-3-17 α-20.	27.8	27.8	28.0	28.0	28.5	28.5	28.0	28.0
Comp. Q		29.6		30.0		30.2		30.1
Comp. A		30.4	30.4	31.0		31.0	31.0	31.2
Comp. B		31.6	31.4	31.7		32.3	31.9	31.8
Comp. S		30.7		31.0		31.2		30.9
				(30.5)		(29.6)		
Comp. E		31.3, 29.5	31.3	32.1		31.9, 30.3	31.7	32.0
		(30.4)		(31.3)		(31.1)	(30.6)	(31.7)
Comp. F		30.8, 30.4	31.7	32.1		31.6, 31.2	31.9	31.9
			(32.5)	(31.8)		(30.8, 32.6)		(31.2)
Aldosterone		30.8		31.4		31.2		31.3
				(30.8)				
4-C-3.	30.7			30.9	31.0			31.0
1, 4-C-3.	31.2			31.1	31.4			31.4
5-A-3 β-17.	23.4	24.1		24.1	24.0	24.4		24.4
5-P-3 β-20.	25.3	26.0		26.0	25.8	26.3		26.3
5-P-3 β, 17 α-20.	26.6	27.2	27.4	27.4	27.2	27.4	27.3	27.3
Δ^5-Pt.		29.0		29.0		29.0		29.1
				(28.8)				(28.5)
5-C-3 β.	29.5	30.4		30.3	29.8	30.3		30.3
5-C-3 β-7.	32.4	33.1		33.1	32.8	33.2		33.2

Steroid numbers were calculated with RRT in Table 6.

lem, it is easier, at the out set to observe the behaviour of a single functional group than the changes of SN for a more complicated polyfunctional group is studied.

The functional group at C_{11} was suitable for such a representative. Table 8 (A) shows SN variations which means the changes in SN accompanying trimethylsilylation of 11-hrdroxyl group under various conditions. The marked difference in SN value for 11-hydroxyl steroid between mild and moderate treatment means the change accompanying conversion of a 11-hydroxy to a 11-trimethylsiloxy group and also shows the potentialities of silylation for gas chromatographic separation, although the values were not necessarily in excellent agreement according to their parent compounds. On the other hand, with the exception of certain steroids, SN variation between moderate and severe treatment was almost zero, indicating the absence of secondary changes under the severe condition. But exceptions were found with comp. B comp. F, allo THF and THF.

Similar phenomenon was also observed with 11-carbonyl compounds. Table 8 (B) which is analogus to Table 8 (A) shows the behaviour of the 11-carbonyl group. SN variation between mild and moderate treatment was sufficiently

TABLE 8. Steroid Number Variations due to Trimethylsilylation under Different Reaction Conditions

	Steroid number variations					
A. 11β-Hydroxyl compound	1.5% SE-30			1% SE-52		
	Parent→Mild→Moderate→Severe			Parent→Mild→Moderate→Severe		
5α-A-3α, 11β-17.	−0.2	0.4	0.0	−0.6	0.1	0.0
5β-A-3α, 11β-17.	0.3	0.5	0.0	−0.1	0.2	0.0
4-A-3-11β-17.	0.0	0.5	0.1	0.0	−0.4	0.1
allo-THB		−0.3	−0.1		−0.6	0.0
allo-THF		−0.3	−1.2		−0.7	−1.2
THB		−0.2	0.0		−0.5	0.0
THF		−0.3	−1.3		−0.4	−1.2, −1.4
					−0.6, −0.8	
Cortol		0.5	−0.1		0.2	0.0
β-Cortol		0.3	0.0		0.1	0.0
Comp. B		−0.2	0.3		−0.4	−0.1
Comp. F		0.9, 1.3	0.3		0.3, 0.7	0.0
B. 11-Carbonyl compound						
5α-A-3α-11, 17.	−0.2	0.0	0.0	−0.4	0.0	0.0
5β-A-3α-11, 17.	0.3	0.0	0.0	−0.1	0.0	0.0
4-A-3, 11, 17.	0.0	0.0	0.3	0.0	0.0	−0.1
11-keto-Pt.		0.0	0.0		0.0	0.0
THA		0.0	0.0		0.0	0.0
THE		0.0	−0.9		0.0	−1.1
		(−0.9)			(−1.1)	
Cortolone		0.0	0.0		0.0	0.0
β-Cortolone		0.0	0.1		−0.1	0.0
Comp. A		0.0	0.6		0.0	0.2
Comp. E		0.0	0.8		−0.2	0.3
		(0.8)			(1.4)	

SN variation means the difference between SN for products obtained under different trimethylsilylation.

close to the expected value of zero and SN variation between moderate and severe was also zero except for certain kinds of steroid; 4-A-3, 11, 17. comp. A, comp. E and THE. When a relevant comparison was made, these exceptions provided a clue to further understanding of them; the distinction between 4-A-3, 11, 17. and the corresponding 3α compound (5α-A-3α-11, 17. or 5β-A-3α-11, 17.) or between comp. A and THA may suggest the participations of Δ^4-3-keto structure.

A similar secondary reaction was manifest in certain steroids containing 17α, 21-dihydroxy-20-keto structure when comparison was made between THA and THF, between allo-THB and allo-THF, and between THB and THF. Therefore, it can readily be understood that multiple peaks were demonstrable when comp. E or comp. F was chromatographed even after mild silylation. This may be interpreted as indicating that the introduction of Δ^4-3-keto and 17α, 21-dihydroxy-20-keto structure into a steroid molecule leads to complicated reacti-

TABLE 9. Steroid Number Contributions for 11-Oxy Group

		Steroid number contributions							
11β-Hydroxy 11-Desoxy⟶11-Hydroxy		1.5% SE-30				1% SE-52			
		Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
5α-A-3α-17.	5α-A-3α, 11β-17.	1.9	1.8	2.2	2.1	2.1	2.0	2.1	2.1
5β-A-3α-17.	5β-A-3α, 11β-17.	1.8	1.8	2.3	2.3	2.1	1.9	2.1	2.1
4-A-3, 17.	4-A-3-11β-17.	2.0	1.9	2.4	2.4	2.3	2.2	1.8	2.2
THS	THF		1.7	1.4	1.2		1.7	1.1	1.0
							(1.9)	(1.3)	
Comp. Q	Comp. B		2.0	1.8	1.7		2.1	1.7	1.7
Comp. S	Comp. F		0.1	1.0	1.1		0.4	0.7	1.0
			(−0.3)				(0.0)		
11-Carbonyl 11-Desoxy⟶11-Carbonyl									
5α-A-3α-17.	5α-A-3α-11, 17.	0.9	0.8	0.8	0.7	1.0	1.1	1.1	1.1
5β-A-3α-17.	5β-A-3α-11, 17.	0.8	0.8	0.8	0.8	1.0	0.9	0.9	0.9
4-A-3, 17.	4-A-3, 11, 17.	0.7	0.6	0.6	0.8	0.9	0.8	0.8	1.0
Pt.	11-keto-Pt.		1.1	1.1	1.1		1.2	1.2	1.2
THS	THE		0.8	0.8	1.0		1.0	1.0	1.0
Comp. Q	Comp. A		0.8	0.8	1.0		0.8	0.8	1.1
Comp. S	Comp. E		0.6	0.6	1.1		0.7	0.5	1.1
			(−1.2)				(−0.9)		
Comparison between 11-keto and 11-hydroxy steroids 11-Cardonyl⟶11-Hydroxy									
5α-A-3α-11, 17.	5α-A-3α, 11β-17.	1.0	1.0	1.4	1.4	1.1	0.9	1.0	1.0
5β-A-3α-11, 17.	5β-A-3α, 11β-17.	1.0	1.0	1.5	1.5	1.1	1.0	1.2	1.2
4-A-3, 11, 17.	4-A-3-11β-17.	1.3	1.3	1.8	1.6	1.4	1.4	1.0	1.2
THA	THB		0.9	0.7	0.7		0.8	0.4	0.4
THE	THF		0.9	0.6	0.2		0.7, 0.9	0.1, 0.3	0.0
Cortolone	Cortol		0.6	1.1	1.0		0.6	0.8	0.8
β-Cortolone	β-Cortol		0.6	0.9	0.8		0.4	0.6	0.6
Comp. A	Comp. B		1.2	1.0	0.7		1.3	0.9	0.6
Comp. E	Comp. F		−0.8, −0.5 0.9, 1.3	0.4	0.0		−0.7, 1.3	0.2	−0.1

SN contribution means the change in SN induced by the addition of a functional group. The value was obtained by comparing SN of two steroids as shown in left side of the Table.

vity of the molecule.

In order to confirm the secondary change discussed above, SN contributions for 11-hydroxy and 11-carbonyl group were calculated by subtracting from the SN values of given 11-oxy steroids, the SN values of the corresponding 11-deoxy steroids. A consequence of this calculation is collected in Table 9. Introduction of a functional group, in general, was accompanied by a well defined relative change in SN, conveniently expressible as an increment of SN which was characteristic of the functional group and its location. The data in the upper part of Table 9 are SN contributions of the 11-hydroxyl group and illustrate the influence of trimethylsilylation on the SN contributions.

The data in the middle part shows the contributions for 11-carbonyl group

after various trimethylsilylations. As expected, the SN contributions for 11-hydroxy group both in parent steroid and in TMS prepared by mild method differed from the values of those yielded under moderate and severe conditions; presumably the former means SN contribution for 11-hydroxy group and the latter the value for 11-trimethylsiloxy group. The contributions for the functional group at C_{11} in comp. B, THF and comp. F were lower than the expected order of magnitude especially under the severe condition, so that this can not be attributed to such secondary change as was observed with 4-A-3, 11, 17. in Fig. 6 or in Table 8. It would seem, therefore, that there is a certain interrelationship between the 17α-21-dihydroxy-20-keto side chain and 11β-hydroxyl group under these conditions. These compounds are of particular interest in this respect. In general SN contributions for 11-hydroxyl group were higher in comparison with those of 11-carbonyl group, whereas, in case of steroids with the dihydroxy acetone side chain, contributions for 11-hydroxyl group were diminished considerably.

For most of the 11-carbonyl group, the relative constancy of the contributions was found, regardless of the condition of trimethylsilylation, suggesting that 11-carbonyl group was not affected during trimethylsilylation. But exceptions were also found with comp. E. The data found were not those expected on the basis of the SN hypothesis. The SN contribution for this steroid tended to show a behaviour similar to those of 11-hydroxy steroids which would not be expected from those of simpler steroids.

In the lower part of Table 9, there is shown the result of comparisons between the contributions for 11-hydroxy and 11-carbonyl group. SN contribution of the 11-carbonyl group was lower than that of 11-hydroxyl group whereas the difference was diminished between THE and THF, between comp. E and comp. F or between cortolone and cortol after severe treatment. In these latter cases, it is by no means acceptable that SN contributions for substituents remote from the labile portion of the molecule agree closely with standard value derived from simpler steroids. It may be necessary to postulate another structural change which is different from the gas chromatographic behaviour observed with simpler steroids. Although the effect of 11-oxy groups in facilitating certain secondary changes is of mechanistic interest in these cases, the explanation for this is not at present clear. At least the changes which arise as a result of severe condition are not likely to be caused by such an intramolecular process as the vicinal effect between functional groups.

Similarly SN for steroids with different functional groups at C_3 and different A/B ring relationships were compared, as shown in Table 10. In this comparison, one steroid is coupled with a steroid which has the same structure other than the structure of A ring; therefore the difference between their SN is a reflection of SN contribution to the structure of ring A and the configuration

TABLE 10. Comparisons of Steroid Numbers for Steroids with Different Functional Groups at C_3 and Different A/B Ring Relationship

Difference in strucutre		Difference in steroid numbers							
		1.5% SE-30				1% SE-52			
5α-A-3α ⟶ 4-A-3		Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
5α-A-3α-17.	4-A-3, 17.	1.0	1.2		1.2	1.2	1.8		1.5
5α-A-3α-11, 17.	4-A-3-11, 17.	0.8	1 0	1.0	1.3	1.1	1.5	1.5	1.4
5α-A-3α, 11β-17.	4-A-3-11β-17.	1.1	1.3	1.4	1.5	1.4	2.0	1.5	1.6
allo-THB	Comp. B		1.3	1.4	1.8		1.8	2.0	1.9
allo-THF	Comp. F		−0.4	0.8	2.4		0.2	1.2	2.4
			(−0.8)	(1.6)			(−0.2)		
5α-A-3β ⟶ 4-A-3									
5α-A-3β-17.	4-A-3, 17.	1.0	0.3		0.4	1.2	0.8		0.5
5α-P-3β-20.	4-P-3, 20.	1.1	0.3		0.6	1.2	0.8		0.7
5α-P-3β, 17α-20.	4-P-3-17α-20.	1.1	0.4	0.6	0.6	1.1	0.8	0.6	0.6
5β-A-3α ⟶ 4-A-3									
5β-A-3α-17.	4-A 3, 17.	1.3	1.1		1.2	1.6	1.6		1.3
5β-A-3α-11, 17.	4-A-3, 11, 17.	1.2	0.9	0.9	1.2	1.5	1.5	1.5	1.4
5β-A-3α, 11β-17.	4-A-3-11β-17.	1.5	1.2	1.2	1.3	1.8	1.9	1.3	1.4
5β-P-3α-20.	4-P-3, 20.	1.4	1.0		1.2	1.6	1.5		1.4
THA	Comp. A		1.2	1.2	1.8		1.5	1.6	1.8
THB	Comp. B		1.5	1.5	1.8		2.0	2.1	2.0
THS	Comp. S		1.2		2.6		1.7		2.5
THE	Comp. E		1.0	1.0	2.7		1.4	1.2	2.6
			(−0.8)				(−0.2)	(0.1)	(2.3)
THF	Comp. F		−0.4	0.8	2.5		0.4	1.3	2.5
			(−0.8)	(1.6)	(2.2)		(0.2,0.0,−0.2)	(1.1)	
5β-A-3β ⟶ 4-A-3									
5β-P-3β-20.	4-P-3, 20.	1.5	1.5		1.4	1.6	1.6		1.7
5-A-3β⟶4-A-3									
5-A-3β-17.	4-A-3, 17.	1.1	0.5		0.6	1.3	1.0		0.7
5-P-3β-20.	4-P-3, 20.	1.1	0.4		0.6	1.3	0.8		0.7
5-P-3β, 17α-20.	4-P-3-17α-20.	1.2	0.6	0.6	0.6	1.3	1.1	0.7	0.7
5-C-3β.	4-C-3.	1.2	0.3		0.6	1.2	0.7		0.7

The comparison above was made between the coupled steroids as shown in the left side of the Table.

at C_5 inclusive. A conjugated ketone structure (Δ^4-3-keto) showed a relatively high SN value and this was taken as standard. In this data, however, the comparison essentially involves four changes; first, the difference betweem the hydroxyl group at C_3 in saturated A ring and Δ^4-3-keto structure for parent steroids; second, the difference between the trimethylsiloxy group at C_3 in saturated A ring and Δ^4-3-keto structure after mild treatment; third, in addition to the second result, the influence of trimethylsilylation of 11-hydroxyl or 17-

hydroxyl group under moderate condition; and fourth, the effect of secondary change of a certain structure such as Δ^4-3-keto structure which was sufficiently reactive to be affected under the severe condition compared with 3-trimethylsiloxy derivatives.

Although exceptions were found with allo-THF and THF, the obtained values were not so variable in the same group, modified slightly by the substitute at C_{11}, that the stereochemical relationship of A/B ring and the effect of substitute at C_3 could be easily distinguished each other. Moreover the result obtained indicates that even the value for the product under severe condition fairly coincided with each other in the same group. But the group containing a specific side chain did not lead to equal value: For example, the difference between THA and comp. A was 1.8, while that between THE and comp. E was 2.7. Accordingly, at least from the present data, it can not be approved that the structure of ring A and side chain are quite unrelated under these conditions. Rather, as has been referred to before, such structures as Δ^4-3-keto, 11-hydroxyl and specific side chain seem to be by no means independent of each other, because even after mild treatment, the values concerned with comp. F were so much varied from others in the same group. Therefore, these results should be accounted for with rather greater caution.

SN contributions for side chains which are frequently present in naturally occuring steroids were particularly interesting when a comparison was made with each other. In this respect, however, SN of C_{21} steroids were compared with the corresponding 17-ketosteroids and the difference of two SN values was regarded, for convenience, as an indication of SN contribution of the side chain. The result is shown in Table 11.

The small variations in the contributions found for the 20-ketosteroid was negligible. As would be expected, SN contributions for 20-hydroxy steroids were greater after trimethylsilylation (mild or severe) than the value for parent steroids. But epimers of 20-hydroxyl group were not clearly distinguished from each other in this data.

Steroids with the 17α-hydroxy-20-keto side chain did not have different SN contributions between the products after two trimethylsilylations (mild and severe), although they were easily distinguished from the peak shape as was illustrated in Fig. 3.

After mild treatment the steroid group with 21-hydroxy-20-keto structure had nearly the same SN contributions, but after severe treatment steroids with 11-hydroxy substitution or saturated ring A led to a relatively low contribution: For example, contribution in comp. B was 4.6 while that in comp. Q was 5.3 and contribution in THA was 4.9 while that in comp. A was 5.5.

Similarly, steroids with 17α-21-dihydroxy-20-keto side chain, especially when possessing Δ^4-3-keto structure, gave more variable results even though the prin-

TABLE 11. Steroid Number Contributions for Side Chains

Difference in side chain		Steroid number contributions							
		1.5% SE-30				1% SE-52			
A 17-one⟶20-one		Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
5α-A-3, 17.	5α-P-3, 20.	1.9			1.8	1.7			1.7
5α-A-3β-17.	5α-P-3β-20.	1.9	1.9		1.9	1.8	1.9		1.9
5β-A-3α-17.	5β-P-3α-20.	1.8	1.9		1.9	1.8	1.8		1.8
4-A-3, 17.	4-P-3, 20.	1.9			1.9	1.8			1.9
5-A-3β-17.	5-P-3β-20.	1.9	1.9		1.9	1.8	1.9		1.9
B 17-one⟶20-ol									
5β-A-3α-17.	5β-P-3α, 20α.	2.1	3.2		3.2	2.1	2.7		2.7
5α-A-3, 17.	5α-P-3, 20β.	1.9	2.9		3.0	1.7	2.4		2.5
5α-A-3β-17.	5α-P-3β, 20β.	2.0	3.0		3.0	1.8	2.6		2.6
C 17-one⟶17-ol-20-one									
5α-A-3β-17.	5α-P-3β, 17α-20.	3.2	3.1	3.1	3 1	3.3	3.1	2.8	2.8
4-A-3, 17.	4-P-3-17α-20.	3.3	3.2	3.4	3.3	3.2	3.1	2.6	2.9
5-A-3β-17.	5-P-3β, 17α-20.	3.2	3.1	3.3	3.3	3.2	3.0	2.9	2.9
D 17-one⟶17, 20-diol									
5β-A-3α-17.	Pt.		4.8		4.8		4.4		4.4
5β-A-3α-11 17.	11-keto-Pt.		5.1	5.1	5.1		4.7	4.7	4.7
5-A-3β-17.	Δ^5-Pt.		4.9		4.9		4.6		4.6
E 17-one⟶21-ol-20-one									
5α-A-3α, 11β-17.	allo-THB		5.1	4.8	4.3		4.9	4.2	4.2
5β-A-3α-11, 17.	THA		4.9	4.9	4.9		4.8	4.7	4.7
5β-A-3α, 11β-17.	THB		4.8	4.1	4.1		4.6	3.9	3.9
4-A-3, 17.	Comp. Q		5.0		5.3		4.8		5.0
4-A-3, 11, 17.	Comp. A		5.2	5.2	5.5		4.8	4.8	5.1
4-A-3-11β-17.	Comp. B		5.1	4.4	4.6		4.7	4.7	4.7
F 17-one⟶17α, 21-diol-20-one									
5α-A-3α, 11β-17.	allo-THF		6.0	5.7 (4.3)	4.1		5.8	5.0 (3.8)	3.8
5β-A-3α-17.	THS		6.0		4.9		5.7		4.6
5β-A-3α-11, 17.	THE		6.0	6.0	5.1		5.8	5.8	4.7
5β-A-3α,11β-17.	THF		5.9	5.1 (3.7)	3.8		5.5 (5.7)	4.7, 4.9	3.5
4-A-3, 17.	Comp. S		6.1		6.3		5.8		5.8
4-A-3, 11, 17.	Comp. E		6.1 (4.3)	6.1	6.6		5.7 (4.1)	5.5 (4.4)	5.9
4-A-3-11β-17.	Comp. F		4.3 (3.9)	4.7 (5.5)	5.0		4.0 (3.6)	4.7	4.6
G 17-one⟶17, 20, 21-triol									
5β-A-3α-11, 17.	Cortolone		7.3	7.3	7.3		6.7	6.7	6.7
5β-A-3α-11β-17.	Cortol		6.9	6.9	6.8		6.3	6.3	6.3
5β-A-3α-11, 17.	β-Cortolone		7.5	7.5	7.6		7.1	7.0	7.0
5β-A-3α, 11β-17.	β-Cortol		7.1	6.9	6.9		6.5	6.4	6.4

TABLE 11. (*Continued*)

Difference in side chain		Steroid number contributions							
		1.5% SE-30				1% SE-52			
H 17-one⟶	C_{27}	Parent	Mild	Moderate	Severe	Parent	Mild	Moderate	Severe
5α-A-3, 17.	5α-C-3.	6.1			6.1	5.6			5.7
4-A-3, 17.	4-C-3.	6.2			6.2	5.7			5.9
5-A-3β-17.	5-C-3β.	6.1	6.3		6.2	5.8	5.9		5.9

SN contribution for side chain represented by the value obtained by subtracting SN value of the corresponding 17-ketosteroid from SN value of the steroid concerned.

cipal peak in each case was compared. After mild treatment these steroids showed SN contributions of similar value except for comp. F but after severe treatment characteristic coincidence in SN contributions was not observed, indicative of secondary change during the trimethylsilylation. The scatter of SN contributions in this group was so large that they seemed rather to belong to a different group, although certain regularities were found with them. As stated above steroids with saturated ring A structure showed somewhat lower contributions than Δ^4-3-keto structure. The values which were considered to be the contributions for side chain increased in the order of 11-hydroxy steroid, 11-deoxy steroid and 11-keto steroid in this group. So far as the change in SN is concerned, these results are comparable with those observed for 21-hydroxy-20-keto steroids.

A similar difference in contributions for side chain was suggested by the fact that cortols had lower contributions than the corresponding cortolones notwithstanding the stereochemical relationship of the 20-hydroxyl group. The reasons for such wide discrepancies have not been determined but were probably multiple.

The result of the present investigation tends to suggest that the magnitude of SN contributions might be a function of the number, nature, position and stereochemical relationship of the functional group they contain. These contributions, found in so many and varied functional groups are, in general, non-specific and are essentially the same, irrespective of the kind of steroid. When the side chain was observed as one substituent including the functional groups, these contributions were so different in magnitude that erroneous conclusions are unlikely to be drawn; side chains of low polarity were sharply distinguished from those of high polarity. Nevertheless, the value for 11-hydroxy steroids did not correspond to the order of magnitude which would be expected from the other. The considerable variation in the contributions of an 11β-hydroxy and 11-keto group may demonstrate that these functional groups are influenced by neighbouring structures. Moreover these differences may be interpreted as

implying that 11-hydroxy steroid is more reactive than others. As stated elsewhere, substances having these structures may give a strong reaction in the presence of concentrated TMCS and HMDS, although the mechanisms leading to this phenomenon are obscure. While there is good evidence for this, yet there is always the possibility that other factors than the silylation process are responsible for SN contributions in this case. The data available in the present study is quite inadequate to reveal these factors.

CONCLUSION

The first objective of this work concerns with the possible stabilization of corticosteroids for gas chromatography by complete trimethylsilylation of the side chain. TMS of steroid were prepared by reacting steroid with HMDS in tetrahydrofuran[41)55)], chloroform[56)57)] acetone[57)] or hexane[51)] in the presence of TMCS as a catalyst. Even if reagents and other factors were determined, the nature of the solvent remained to be of great importance. Thus the conditions in the tetrahydrofuran solution which was described for the trimethylsilylation of estrogens by Lau[55)] may be different from others according to the solvent and the functional groups concerned. With the steroids studied in the present investigation the reaction in pyridine employed by Chamberlain *et al.*[49)] was more preferable. Their method, as was pointed out by Ibayashi *et al.*[52)], afforded 11 β-etherification and was a valuable step forward in the development of a quick and reliable trimethylsilylation, whereas in the former method substitution of hydroxyl groups occurred merely at C_3, C_{20} and C_{21}. These two methods were distinguished from the behaviour of 11 β-hydroxy-17-ketosteroids on XE-60 phase [52)]; 11 β-trimethylsiloxy TMS eluted earlier than 11-keto TMS but 11 β-hydroxy TMS eluted with a longer retention time than others.

In the present study, an abbreviated procedure suitable for use with many samples was devised. The reaction was carried out in tightly capped test-tubes to prevent the entrance of moisture. In the presence of moisture the reaction retarded as if TMCS were not present in the reaction mixture. Nevertheless, in this country as it is very moist in summer, the reaction was accompanied by much white precipitate. This difficulty could be most readily overcome by using dehydrated pyridine and by decreasing the amount of reagent especially TMCS. In view of several properties of TMS, evaporation of excess reagents was applied under reduced pressure, to reaction products as an effective procedure for purification. Reextraction with a proper solvent as in usual methods was omitted, because TMS is unstable to moisture, inducing hydrolysis into parent compound. In fact decomposition of varying amounts was an obvious source of error. This procedure permitted satisfactory yield in trimethylsilylation of 17-ketosteroids to the same degree as the hydrocarbon, cholestane[21)].

In any event the procedure should be a rapid and reliable one reproducible to a high degree and permitting the detection and qualitative evaluation of TMS of hydroxy steroids. But their ready hydrolysis in atmospheric moisture renders characterization difficult. In addition, convenient means of collecting products eluted from the chromatograph were desirable for confirmatory characterization of TMS but were not available during this work. Therefore, the interpretation of results presented here may remain a matter for speculation with certain reservations. The limitation falls broadly into two kind:

As has been referred to above, the present data is confined to the gas chromatographic behaviour of the trimethylsilylation products. The gas chromatographic behaviour of materials is dependent largely upon size, shape and polarity of the molecules and the detection of materials in the gas chromatographic effluent is based on the measurement of electrical conductivity of gases in a hydrogen flame, nearly every organic substance will give a signal, resulting in a complex assay of major and minor peaks, among which are the materials of interest. Therefore, as in usual colorimetry, where various non steroidal metabolites produce colored complexes with the reagent to cause interference, the data in gas chromatography lacks in specificity, reflecting only a part of the chemical properties of the products.

The second question to be answered at this point is concerned with the cause of the behaviour of trimethylsilylated steroids. Trimethylsilylation of polyfunctional steroids often resulted in variable products, whereby it is not always obvious whether the emerged peak was due to the alteration of the respective silylation products during preparation or to transformation in the gas chromatograph. This was only roughly assessed. In general the by-products of a steroid with a few substituents were not found when the steroid was derivatized under mild condition. Conceivably this may suggest at least in part the mechanism whereby this phenomenon occurs. When considering the thermal stability of TMS or marked irregularities observed only under severe condition, the changes lie, perhaps in most part, in the process of preparating the derivatives rather than in the process in chromatographic separation.

It has been reported that hydroxyl groups at C_3, C_{20}, C_{21} and C_{11} were easily substituted with protecting groups to improve gas chromatographic stability of a steroid. With regard to corticosteroids, most of these works have been attended with difficulties, due to their thermal lability. Trimethylsilylation was reported to give thermal stability to a steroid, was not accompanied with any by-product and showed diminished adsorption on the solid support when applied to steroids with a few substituents. On the other hand there are several articles in the literature which report the generally beneficial effect of trimethylsilylation under a wide variety of reaction condition. In most case, however, the results for corticoid type of steroids were rather variable[22)23)] and difficult to interpret.

In the preliminary experiment, it was found that steroids with the polyfunctional group was converted to variable products according to the reaction condition and that these processes could be predictably brought about with ease. When a systemic analogs which possesses different chemical functional groups are reacted, the effect may be more demonstrable. In fact there was no detailed information available on the effect of trimethylsilylation at C_{17} with certain side chains and it was also found that carbonyl compounds were associated with secondary reaction under severe condition. Steroids possessing polyfunctional group did not yield single product. Of the derivatives examined, 11-oxygenated steroid with dihydroxy acetone side chain was not converted to satisfactory derivatives suitable for chromatography, even when treated under mild condition. In this respect acetylated corticosteroid was reported to be quite different[18)19)]; the fully acetylated corticosteroids possessed considerable thermal stability, in addition to their general chemical stability and decomposed only on aged column. This would not be in accord with the present findings. The results now presented indicate that 11-oxy steroids with Δ^4-3-keto or 17 α, 21-dihydroxy-20-keto structure all show greater reactivity than their 11-desoxy analogs, revealing various attendant peaks on the chromatogram. Of course, many details of the reaction described here were not completely elucidated and it is therefore quite possible that any interpretation proposed at this time may later prove somewhat misleading. They need confirmation and further investigation.

Trimethylsilylation of hydroxyl group, though it was sometimes disappointing, still retains a useful place in the analysis of steroidal compounds with a few substituents. In case of polyfunctional steroid, with the increasing knowledge of the variable reactivity of the individual functional group or specific reactive structure to the reagents, this method proved to be far more available for chromatography because of the multiple peaks they revealed. The conversion to this derivative tends to loss its usefulness and certain supplemental procedures, for example such as protecting of carbonyl group[53)] is likely to be required. Only for rough screening purposes the value of TMS persists and is likely to be utilized but in making a choice of a suitable condition it is necessary to specify the purpose in view.

Apart from reaching definite conclusions on the structural aspect, a further study was carried out by means of SN. Extensive but unsuccessful effort resulted in elucidating the relationship among them. These various findings may be suggestive and may eventually prove to have significance. Through the use of SN which was calculated from RRT it was evidently possible to arrive at correlations between the structure of steroids and their gas chromatographic behaviour. Originally it was not proposed that SN will provide acceptable proof of structure but rather that they may be used for arriving at a tentative conclusion which may be confirmed by other structural studies[51)]. With these

reservations considered, the data revealed a striking feature in the present observation.

SN obtained with two phases were relatively in good agreement and varied with the number, nature positional and stereochemical arrangement of the functional groups. Comparison of SN contributions observed for various steroids suggests that certain changes with regularity occurred fairly uniformly among similar compounds. As was already reported[54], SN contributions for a functional group were modified by the steroid nucleus which is attached to it. The data presented here provides evidence for the influence of this kind of steroid nucleus and stereochemical relationships.

The second aspect of this investigation was concerned with an hypothesis on which SN contributions as well as retention factors are based on. These contributions were determined when intramolecular interactions between functional groups are negligible and a certain deviation of contribution which was found for a functional group with different adjacent substitute was considered as vicinal effect. It is reasonable that the effect of a functional group is influenced by the adjacent functional groups. Accordingly the contribution for side chain with polyfunctional groups was not likely to be estimated predictably from contributions for respective functional groups consisting the side chain. This shortcome is avoided when several side chains are regarded as one functional group. The result generally substantiated this view and useful SN regularities were discernible even for steroids with 17 α-hydroxy-20-keto side chain which were expected to undergo transformation during chromatography; that is, similarly transformed compounds showed uniform behaviour. Nevertheless, no systemic correlation of this kind was found for a series of derivatives of certain steroids which belong to a class of compounds generally considered to be of biological activity. The marked deviation of SN contribution for the side chain may be attributed rather to alteration during silylation than to unstability under the usual conditions of gas chromatography, because it was observed chiefly on products under severe conditions. In any event, such reactive functional group as Δ^4-3-keto, 11 β-hydroxyl and specific side chains may be too remote from each other to maintain such influence as the vicinal effect. Accordingly these deviations are not attributable to the infiuence among remote functional groups during gas chromatographic separation. But it is too early to draw such a conclusion on the behaviour of side chains, because it was also observed that SN contribution for side chain was different between cortols and cortolones even after mild treatment. The peculiar behaviour of these steroids suggests that these reactive functional groups have a relation to each other under certain conditions in silylation reaction or during chromatography. Thus SN data obtained may be a result of these two factors. Many interesting results presented are still full of many obscurities and too complex for a clear

interpretation of their significance to be made; a wide variety of alternative explanations are possible. Much remains to be learned before these speculations can be assessed factually.

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