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GAS CHROMATOGRAPHIC ANALYSIS OF STEROID HORMONES

PART I. PROBLEMS IN QUANTITATIVE ASPECTS OF GAS CHROMATOGRAPHY

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ABSTRACT

In an attempt to evaluate gas chromatography for quantitative use, relative peak area ratio (RPAR) was studied under various analytical conditions and several problems in the quantitative analysis were elucidated.

The loss was influenced by the sample size, structure of the steroid, retention time, operating temperature, loading effect and gas chromatographic appartaus. Problems relating to matters of instrumental design and column packing inclusive were of the most importance. In certain instances trimethylsilyl ethers were useful for avoiding these difficulties but these derivatives and even saturated hydrocarbons were not found to be recovered completely in gas chromatography. Of the various causes proposed for this phenomenon, adsorption appeared to be the most siginificant.

When precise estimation is required, it should not be extended beyond a certain limit even by employing suitable operating conditions. Relatively reliable procedures for careful control of routine estimation are outlined. As a matter of course, the necessity for proper instrumentation should also be emphasized and the properties of any given equipment, analytical conditions and the accuracy of the obtained data should be checked with the RPAR curve.

INTRODUCTION

With regard to steroid hormones and their related substances, gas chromatography has been studied by several groups of workers in recent years and the majority of the works on this subject have dealt with the qualitative aspects of these procedures. Small knowledge of various kinds of columns (liquid phase and solid supports) and the column effiiciency has also been reported. The separation, identification and estimation of certain substances of biochemical or biological interest have been explored too, in considerable detail.

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Besides the above, on account of the high temperature employed, the possibility of loss by thermal decomposition[1)2)3)4)] or by other causes[5)6)7)8)9)10)] has been recognized particularly with these complex substances.

During the course of its application in biological studies, only little attention has been paid to the problem involved in quantitative procedures and it has generally been believed that the dose-response relation of steroids is linear or at least nearly linear[9)11)12)13)14)15)16)17)] although there were differences in opinion[3)5)6)7)18)19)20)] concerning the precision and accuracy which could be attained. In various applications of gas chromatography, several studies along this line have been carried out, for the purpose of correcting observed responses with a reference substance but, up to the present, sufficient data are not available for the analyst to choose the suitable conditions for quantitative application. A study on the properties of gas chromatography was undertaken with the three aims stated below; namely, evaluating the adaptability of the equipment for quantitative use, understanding the nature of several problems involved in obtaining quantitative data, and establishing the conditions suitable for routine analysis in order to secure satisfactory quantitative result, with the belief that a discussion of these problems may contribute to a better understanding of the procedures involved. Emphasis was placed on less complicated problems in the case of C_{19} steroids which contain only few substituents in the molecule, as they are connected with the fundamental problems encountered in analysis of compounds present in biological fluid.

EXPERIMENTAL

Apparatus: A gas chromatograph GC-1 B (Shimadzu Seisakusho Co. Ltd. Japan) was used. This instrument consisted of a dual column system equipped with hydrogen flame ionization detector; Samples were not introduced directly onto the heated chromatographic column but injected (with a Hamilton Microsyringe) into the sample chamber through the silicon rubber disk. All the surfaces in contact with the samples—the sample chamber, column and detector unit—were made of stainless steel. These compartments were designed to be heated separately.

Unless otherwise stated, operating conditions were as follows: The sample chamber (flash heater) was maintained at 270°C to 300°C and the detector at 240°C to 250°C. For avoiding temperature drop in the region of the connections between these compartments, the sample chamber, column and detector bath were set directly in order and the connecting tubes between them were shielded with asbestos.

The columns were U tube in type (2.25 m long by 4 mm. in internal diam) packed with 60 to 80 mesh Chromosorb W (acid washed and silanized coated by one of the following liquids: 1.5% SE-30 (methyl siloxane), 1% SE-52 (methyl phenyl siloxane), 1.5% NGS (neopentyl glycol succinate), 1% XF-1150 (nitrile

silicone) and 1.5% DEAS (diethanol amine succinate, Shimadzu Seisakusho Co. Ltd.). These packed columns were purchased. Non-polar columns showed a stable base line with minimal noise, and with high sensitivity. With polar columns, when the system was operated at its upper thermal limit, considerable column bleeding occurred and sudden column deterioration was observed frequently.

Sample injection: Solutions of steroids were prepared in a suitable solvent (aceton or 1 : 1 chloroform-methanol up to their solubility) at concentrations of 0.01 to 1% w/v. In order to determine the linear range of the estimation, reference solutions were employed at different volumes (1 to 10 μl), and were delivered from 10 μl Hamilton microsyringe.

When a sample size below 2 μl was necessary, uniform sample injection could not be achieved by this procedure. In most experiments of this series, there was no need for precision in sample injection, but only approximate quantity. The injection of small volumes of up to 2 μl was carried out in the manner described by Bloomfield[6], as follows: Before a sample solution is drawn into the syringe, the syringe is filled with the solvent, all bubbles removed and brought to the 1 μl mark. The desired sample volume is then drawn into the syringe and the sample solution plus the wash solvent injected into the column. Because of the narrow bore of the syringe there is minimal mixing of the sample and the solvent behind it. The solvent wash allows complete injection of the sample into the column. Without this procedure variation in injected volume is observed in case of injections of small volumes. Duplicate injections by this procedure can be reproduced within 5% limits of error.

Sample handling: All steroids used were obtained commercially and produced only one peak by gas chromatography. No further precaution was taken initially with these steroids. They were used experimentally to observe minor relative losses under variable column conditions. As a rule all steroids were mixed with cholestane (in a ratio of 1 : 1 w/w). Instead of untreated steroids, sample components were often converted to more stable derivatives for use.

Acetylation was carried out as described by Wotiz and Martin[21].

Trifluoracetate was prepared according to the method of Vanden Heuvel, Sjövall and Horning[22].

Trimethylsilylation was modified from the method of Chamberlain, Knight and Thomas[23], as below:

As reaction mixture, a solution of 0.5 to 1 ml of hexamethyldisilazane and 50 to 100 μl of trimethylchlorosilane in 5 ml of pyridine was prepared. Pyridine was dehydrated with KOH pellets before use. By this dehydration trimethylsilylation of 11 β-hydroxyl group of the steroid was easily attained. To the samples which contained 200 to 400 μg of steroids 0.5 ml of this solution was added and heated at 90°C for a few hours. These procedures were carried

out in short stoppered test tubes filled with nitrogen. After the reaction excess reagents were evaporated under reduced pressure at the same temperature as mentioned above. Acetone was added to the dry residue and this solution was injected into a gas chromatograph without further purification.

Measurement of peak area on chromatogram and RPAR:

Peak area measurement was made in several ways[24]

1) by a mechanical or electronic integrator
2) by a planimeter
3) by cutting out and weighing the chart
4) by multiplying peak heights by half-width

Pprobably, a combination of relatively sharp early peaks and flat late peaks make it difficult to obtain high precision by any of these measurement procedures[25]. In this study a peak with tailing such as in Fig. 1 was often encountered, and some of the above methods were compared, as regards the following:

(A) multiply peak heights by peak width at half height, that is the square

TABLE 1. Gas Chromatographic

Sample			Columan		
Steroid	Derivative	Internal standard	Material	Length	Diam.*
11-deoxy, 17-KS	Untreated		Glass	12 ft. 6	3 mm 5
〃	〃	Cholestane		12 6	
11-deoxy, 11-oxy, 17-KS	〃		Glass (coil)	12	1/4 inch
〃	TMS		Stainless steel	6	1/8 inch(OD)
11-deoxy, 17-KS	〃	5α-Androstane-17-one	Glass (spiral)	6	3.4
〃	〃	Epicoprostanol TMS β-Sitosterol TMS	Glass Glass	12	4
11-deoxy, 11-oxy, 17-KS Correspond 17-KS	Untreated Acetate		Glass (U) 〃	6 〃	5 〃
11-deoxy, 11-oxy, 17-KS	Untreated	4-Androstene-11 ß-ol-3, 17-dione	Glass (coil)	12	
11-deoxy, 17-KS, Pd	TMS		Glass	8	4
Correspond 17-KS		Androsta-1, 4-diene-3, 11, 17-trione	Glass	132 cm	5
〃			Glass (U)	8	5
11-deoxy, 11-oxy, 17-KS Pd, Pt	TMS		Glass (spiral)	6	3.4
〃	〃	Coprostane-3α-ol TMS	Glass (U)	6	4
11-deoxy, 17-KS	〃		Stainless steel	6	3.8

* Diam: Internal diameter, OD: Outer diameter

* Liquid phase

NGSuc: Neopentyl glycol succinate polyester
XE-60: Silicone nitrile elastomer
NGSeb: Neopentyl glycol sebacate polyester
Hi-Eff 8 B: A polyester phase (Applied Science Laboratory)

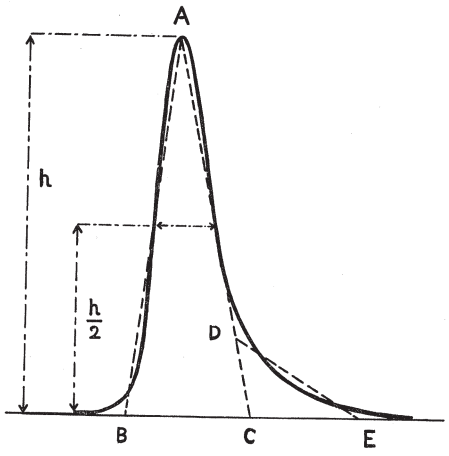

FIG. 1. Peak area measurement of peak with tailing, made as bellow:
(A) multiply peak height by peak width at half height.
(B) planimetry
(C) adjust peak area of method (A).
The straight line DE was determined so as to supply the area of the tailing part appropriately.

Analysis of 17-Ketosteroids

Liquid phase	Solid support	Mesh	Temperature		Detector	Literature
			Flash	Column		
1.5% SE-30 0.75% NGSuc	Gas Chrom P	100–140		216°C 209		Haahti *et al.* '61[26] 〃
1.5% SE-30 0.75% NGSuc	Gas Chrom P	100–140		220 220		Cooper *et al.* '61[27] 〃
3% SE-30	Gas Chrom P	80–100	290	221	FID*	Sparagana *et al.* '62[28]
3% XE-60* 1% Hi-Eff 8 B* 1% SE-30 1% NGSeb	Diatoport S 〃 Anakrom ABS 〃	80–100 〃 80– 90 〃		262 242 251 231	〃 〃 〃 〃	Hartman *et al.* '63[29] 〃 〃 〃
1% QF-1	Gas Chrom P	100–120	250	187	AID*	Kirschner *et al.* '63[30]
2% CMSi*	Gas Chrom P 〃	100–120 80–100		220	AI, FID	Horning *et al.* '63[31] 〃 [25]
1.5% SE-52	〃	〃	285	210	AID	Stein *et al.* '63[32] Patti *et al.* '63[33]
3% SE-30 1.5% NGSuc	〃	〃	290	220 210	FID	Sparagana *et al.* 63[12] 〃
2 : 1 SE-30 NGSuc	Gas Chrom S	100–120	285	205	AID	Nair *et al.* '64[34]
0.65% NGA*	Celite	100–120	280	230	AID	Bailey '64[13]
0.75% SE-30	Chromosorb W		250	217	AID	Luetscher *et al.* '64[14]
1% SE-30 2% XE-60	Gas Chrom P	60– 80	285 265	235 215	AID	Kirschner *et al.* '64[20] 〃
2% NGSuc	Gas Chrom	80–100	270	215	AID	Creech '64[35]
3.3% XE-60	Anakrom ABS	90–100	327	225	FID	France *et al.* '64[16]

CMSi: Cyanoetyl methyl siloxane polymer
NGA: Neopentyl glycol adipate polyester
* Detector
FID: Hydrogen flame ionization detector
AID: Argon ionization detector

of △ABC.

(B) planimetry.

(C) adjust peak area of (A), that is fhe sqaure of △ABC plus the square of △DCE. As the straight line DE is determined arbitrarily △DCE is not always reliable.

Method (A) was usually employed in the present study.

The development of an accurate gas chromatographic method requires some means of comparing the quantity injected with peak area. Although some authors reported on the linear relationship between mass chromatographed and the peak area of any given steroid, this relationship has not been accepted entirely by others. In fact, the peak area may be influenced by error in sample loading and slight fluctuations in operating condition. Since it is difficult in routine practice to inject precisely known volumes into the gas chromatograph, some complemental devices are required. The most frequently used method for quantification is the so-called internal standard added prior to injection, and this eliminates the source of uncertainty caused by trivial errors mentioned above and extends greatly the reliability of the quantitative determination by gas chromatography. Of many internal standards shown in Table 1, cholestane was employed, for the present investigation, to evaluate the so-called response—in other words, recovery. The relative recovery of a given steroid was studied by comparison of the peak area of a given steroid with that of cholestane when equal amounts of both were chromatographed in one sample. This comparison can be expressed conveniently in terms of relative peak area ratio to cholestane (RPAR), obtained by the following equation:

$$R=\frac{Ax}{Ac}$$

Where R is RPAR, Ax the peak area of a given steroid and Ac the peak area of cholestane. RPAR was influenced by the loss of steroids due to various causes in gas chromatographic technique. The variation of RPAR of a given steroid was plotted as RPAR curve as it varied with sample size.

RESULTS AND DISCUSSION

1. RPAR curve

In Fig. 2 RPAR is plotted against varying sample loads for dehydroepiandrosterone (DHEA). For this experiment a series of applications were made after presaturation, with the first application the largest sample of the series, and successive samples of decreasing size. The entire set of observations was completed during a single experimental period.

When equal amounts (1 : 1 mixture) of DHEA and cholestane were chromatographed in varying quantities, the steroids did not yield equal areas; the peak

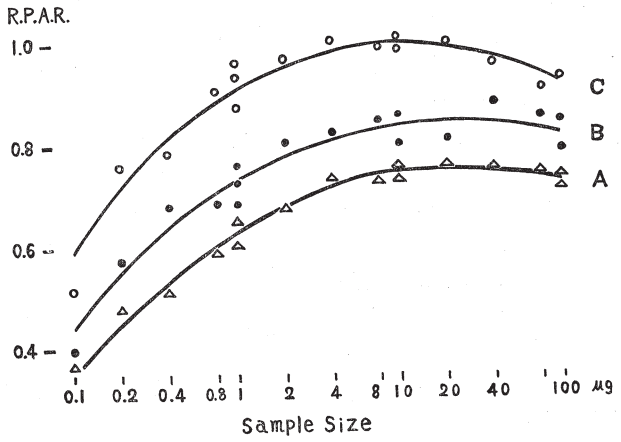

FIG. 2. RPAR curve of DHEA

Peak area ratio of DHEA to cholestane was calculated with a 1 : 1 mixture of various amounts. The sample size was plotted logarithmically. In the RPAR curve A, B and C mean the RPAR curve obtained by the peak area measurement (A), (B) and (C) respectively.

Column, 1.5% SE-30 2.25 m, Column temp., 230°C

Retention time, 7.5 min. (DHEA), 18.3 min. (cholestane)

of DHEA being always smaller than that of cholestane. Calculating the results of multiple injection, the DHEA-cholestane ratio (RPAR of DHEA) was under 1.0 and the ratio changed significantly with the difference of dose. Proportion lost was inversely related to the sample size.

Although some scattering of the points may be seen on the graph, the typical RPAR curve as in Fig. 2 was obtained. It is apparent that no straight line can be drawn and a linear correlation can be observed in only relatively short range of sample size.

As would be expected from the method of peak area measurement such as A, B and C in Fig. 1, RPAR curves differed considerably from each other. The given RPAR curves for the three measurement procedures are presented merely to illustrate the general trends of RPAR curve. The RPAR curve C obtained by method (C) was the highest, but the extreme variability in RPAR is possibly due to the arbitrary adjustment. On the other hand, the low curve A reflected the effect of tailing especially.

It is interesting to note that RPAR was not independent of the mass chromatographed, and that the range with constant RPAR was rather difficult to dfine because the curve was paraboloid in shape which was accentuated by plotting the dose logarithmically. This means the peak area did not increase linearly with quantity over a range from 0.1 to 100 μg; the peak area of any given recording could not be directly proportional to the mass of steroid giving rise to the peak. The minimum sample load for reliable measurement,

relatively free of errors due to losses, was 5 μg in this case, but this by no means was able to determine the minimum detectable quantities.

2. RPAR curves under several columns

Fig. 3 shows RPAR curves of DHEA under several columns. The effect of sample size was observed with various polar or non-polar columns, the RPAR curves showing entirely the same tendency as the curve illustrated before in Fig. 2. But the curves showed different slopes according to the column, each appearing to be gently-sloping only over a narrow range of sample size and with decreasing dose to be broken more sharply downwards. In spite of the polarity of the liquid phase, a relatively high and sharp RPAR curve is obtained with one column but with another a somewhat low and flat curve showing a relatively constant RPAR value over a wider range of sample size. In view of peak area measurement, larger peaks which result in higher RPAR values are preferred especially with smaller amounts. For the estimation of a wide variety of sample size, constant RPAR value is preferable to varying RPAR values; the deviation from a linear dose-response relationship can be easily detected on RPAR curves. Thus, when a sample contains variable

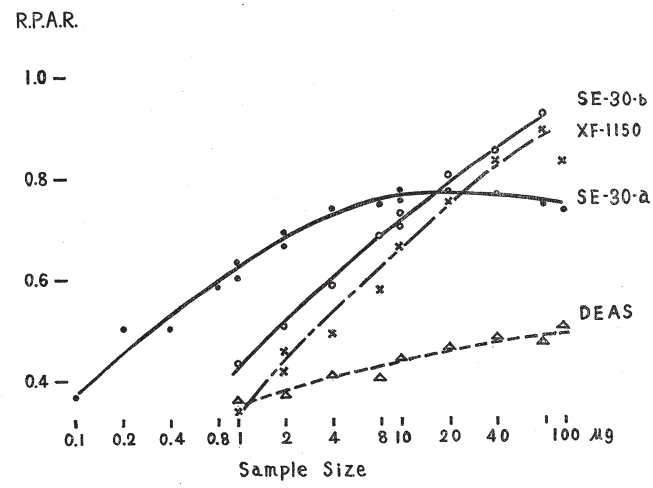

FIG. 3. RPAR curves of DHEA under several columne

Conditions were as follows:

Column	Column temp. (°C)	Retention time Cholestane (min.)	Retention time DHEA (min.)	Theoretical plate number (cholestane)
1.5% SE-30 (*a*)	230	18.3	7.5	1380
1.5% SE-30 (*b*)	230	17.6	7.4	1570
2% XF-1150	205	4.2	30.6	1370
1.5% DEAS	205	11.7	30.2	1660

amounts of steroids, it is evident that a high flat RPAR curve is suited for good quantitation.

In order to adjust the decrease of the peak area of a given steroid, such an appropriate calibration constant as described by others[12)17)] can not be adopted in the estimation of a steep RPAR curve. In this experiment the characteristics of a column expressed in terms of RPAR was not found to be dependent on the column efficiency, partly due to the difference in number of the theoretical plate being not conspicuous. Hence, this was not necessarily incompatible with the result reported by Bloomfield[7)] that higher relative response can be obtained with a higher efficiency column packed with Gas Chrom P. In spite of the theoretical plate number which expresses the resolution ability of a column, RPAR value represents the relative recovery of a steroid. If both values vary proportionally, improvement of the support material is needed, because the nature of the support material is one of the major factors providing high resolution column. In this respect Gas Chrom P may be preferred[36)] also in this case.

Fig. 4 shows the RPAR curves of DHEA acetate. It seems natural that the curve again showed a tendency similar to that of Fig. 3. In general the results indicate increasing loss with decrease in quantity injected. It is obvious that

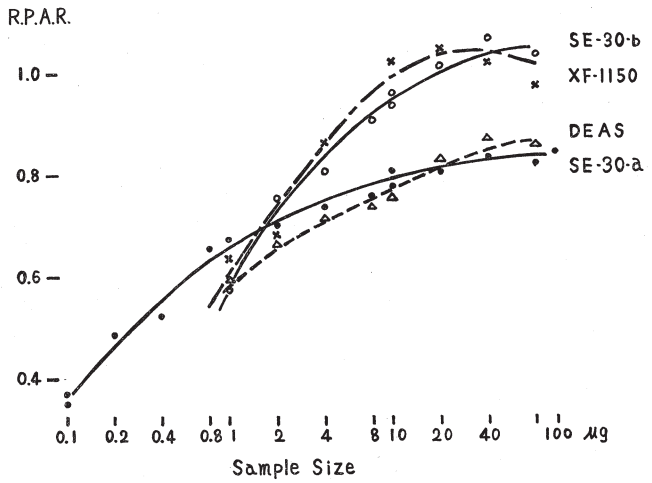

FIG. 4. RPAR curves of DHEA acetate under several columns

Conditions were as follows:

Column	Column temp. (°C)	Retention time		Theoretical plate No.
		Cholestane	DHEA acetate	
1.5% SE-30 (*a*)	230	18.3	10.7	1320
1.5% SE-30 (*b*)	230	17.6	10.4	2350
2% XF-1150	205	3 6	23.5	650
1.5% DEAS	205	12.7	26.1	1720

DHEA acetate has a higher RPAR value than DHEA, but an exception was observed with the SE-30 (a) column; the RPAR curve of acetate being as low and flat as that of DHEA. Individual RPAR cnrves may vary considerably from column to column.

3. Peak shape and RPAR

Fig. 6 demonstrates the peak shape of DHEA over a range of from 0.1 to 100 μg. As can be seen, peaks often show a pronounced tailing effect; the peak initially descends rather steeply, but in the later portions to approach the base line only asymptotically. When the sample size of DHEA decreased, lower peaks, compared with the symmetrical peaks of cholestane, were presented. They were accompanied by tailing which became also more striking with smaller sample size (submicrogram range). This characteristic of tailing suggests that the tailing in this case did not originate from non-linear adsor-

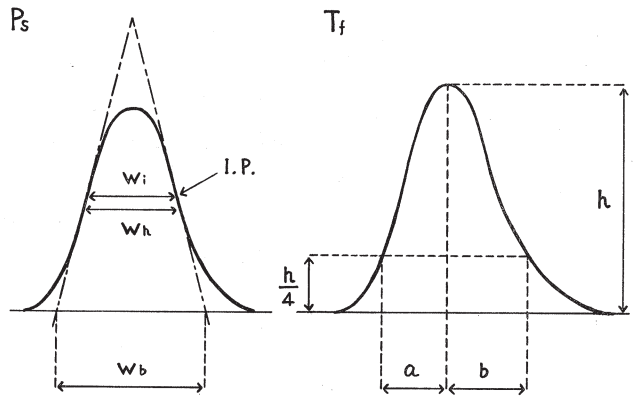

FIG. 5. Expression of peak shape

left side I.P.: inflexion point
W_h: peak width at half height
W_i: peak width at inflexion point ($=2\sigma$)
W_b: peak width at base ($=4\sigma$)

Ettre[37] stated: In case of a truly Gaussian peak, the ratio of W_b to W_h is equal to 1.698. Thus, if at an actual chromatographic peak the ratio differs from 1.698 ($\fallingdotseq$1.70), it is an indication of peak unsymmetry.

About $\pm$10% difference in the ratio was considered as normal. In the case of tailing one would rather expect a larger value. In this experiment calculated ratio was within $\pm$10% (Fig. 6) range.

right side $$T_f=\frac{b}{a}$$

Takeuchi *et al.*[38] defined tailing factor (T_f) as above.

--- a and b shown in Fig. may vary independently of various causes but the peak shape can be easily evaluated by the ratio of b and a, as follows:

$T_f=1$ symmetrical peak approximately without tailing
$T_f>1$ peak with tailing
$T_f<1$ peak with leading

ption isotherm because non-linear tailing decreases as the sample size is reduced. It seems that these asymmetric peaks correlate well with the decreasing RPAR value obtained before.

Ettre[37] proposed an expression for the characterization of peak asymmetry by the following equation.

$$\text{Peak symmetry } (P_s) = \frac{W_b}{W_h} = \frac{4\sigma}{2.355\sigma} = 1.698 \fallingdotseq 1.70$$

Where W_h is peak width at half height, W_b peak width at the base (as shown in Fig. 5), σ variation coefficient of the peak. This equation is based on a true Gaussian peak in case of non-ideal linear isotherm. Since correct measurement of the peak width value is difficult, a difference of about $\pm 10\%$ in the ratio has to be considered as normal. But as shown in Fig. 6 this failed to express the tailing effect. In spite of the existence of remarkable tailing, the large deviation from the theoretical value of W_b/W_h could not be found in the present chromatogram.

On the other hand, in an effort to study support materials, Takeuchi *et al.*[38] expressed the degree of tailing by tailing factor $\left(T_f = \frac{b}{a}\right)$ which was defined in Fig. 5. A peak of 1.0 T_f represents approximately the ideal symmetrical peak not accompanied by any tailing. While a peak with tailing results in T_f of above 1.0, a T_f of below 1.0 implies a leading phenomenon (gently-sloping on the front side of a peak compared with the back side).

As shown in Fig. 6, the T_f value varied considerably with sample size, showing certain peak asymmetry or tailing, and may serve as an indicator for peaks with relatively constant RPAR value, though a peak with T_f value of approximately 1.0 did not show a high RPAR value occasionally—with some dissociation between the two. Thus, it is evident that peak asymmetry is expressed rather by the T_f value than by the Ettre's index (P_s), and the RPAR value indicates merely relative recovery of a given steroid and nothing concerning the peak asymmetry or tailing. Nevertheless, these observations lead to the assumption that the cause of the decrease of RPAR may be related somewhat to cause of peak asymmetry or tailing.

4. RPAR values of derivatives

In view of the results obtained with acetate, it seemed reasonable that a similar effect, variation in RPAR and peak shape after converting to various derivatives, might be observed with steroids with the hydroxyl group. Several derivatives were therefore, chromatographed on 1.5% SE-30 column.

In Fig. 7, typical chromatograms of various derivatives of etiocholanolone are presented, of sample size of 1 μg level. The RPAR was found to vary appreciably with the nature of the steroid. It is apparent that the parent steriod was recovered in lower yield than the derivatives. Table 2 contains

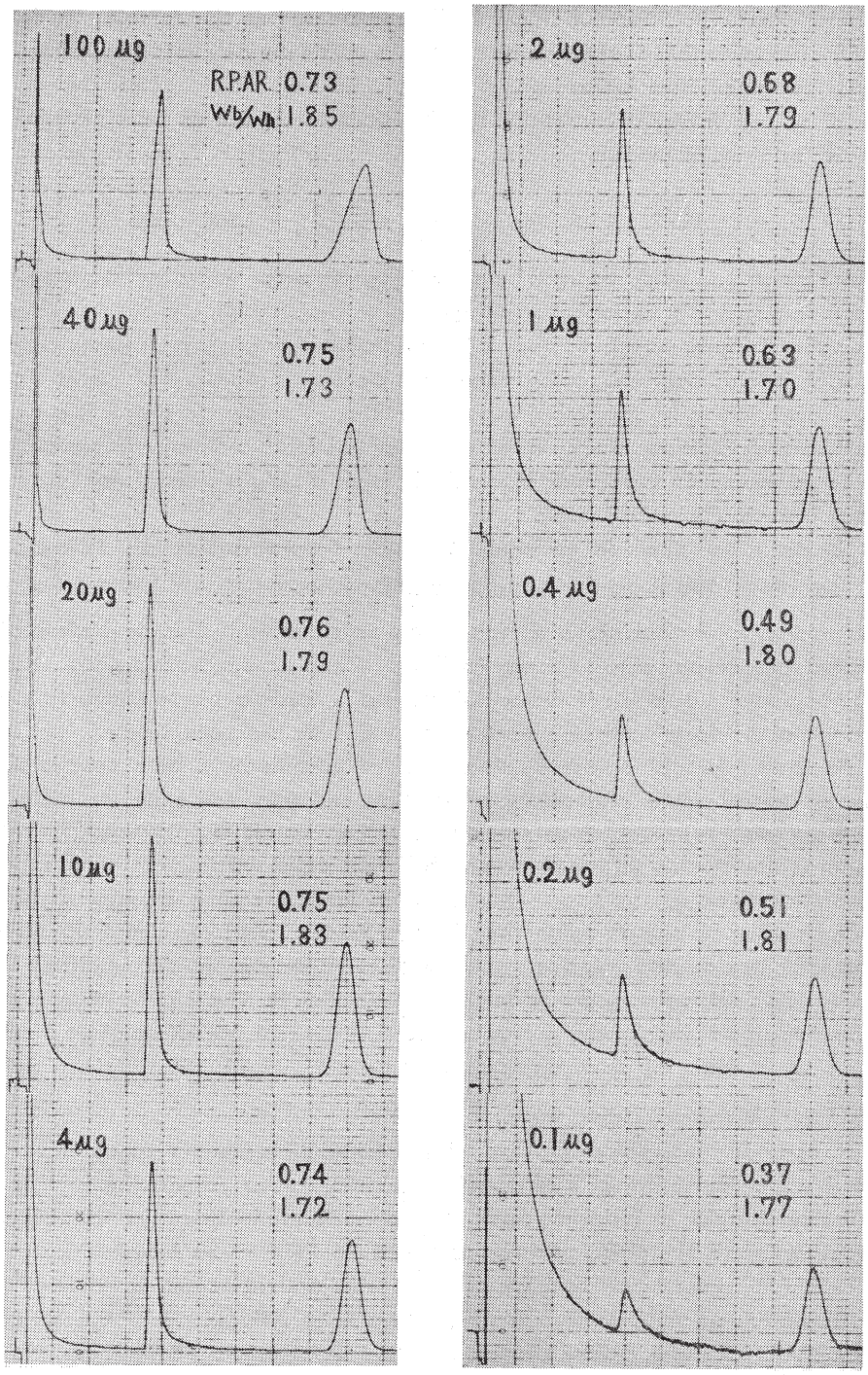

FIG. 6. Peak shape of DHEA

TABLE 2. RPAR Values of Various Derivatives of 17-Ketosteroids

	Parent[a]		Acetate[a]		TFA[b]		TMS[a]	
	RT (min.)	RPAR	RT (min.)	RPAR	RT (min.)	RPAR	RT (min.)	RPAR
Cholestane	17.0	1.00	17.3	1.00	20.4	1.00	17.4	1.00
Etiocholanolone	6.7		9.2		5.4		7.3	
5 μg		0.59		0.63		0.41		0.88
1		0.47		0.54		0.36		0.85
0.5						0.30		0.86
0.1								0.77
Androsterone	7.3		9.4		5.3		7.0	
5 μg		0.54		0.53		0.40		0.86
1		0.49		0.47		0.42		0.84
0.5						0.36		0.83
0.1								0.78
Epiandrosterone	7.4		10.6		6.7		8.8	
5 μg		0.53		0.58		0.45		0.87
1		0.43		0.44		0.37		0.85
0.5						0.25		0.84
0.1								0.76
DHEA	7.2		10.4		6.2		8.5	
5 μg		0.51		0.53		0.33		0.87
1		0.42		0.40		0.17		0.80
0.5						0.16		0.78
0.1								0.72
Pregnanediol	11.5		22.5		8.2		16.4	
5 μg		0.45		0.53		0.61		
1		0.37		0.29		0.55		
0.5						0.33		

Column, 1.5% SE-30 2.25 m
Column temp., a: 230°C, b 210°C

results for the RPAR related to sample size for several derivatives. Although the absolute RPAR value is considered to depend on the extent of the chemical conversion of the parent compound, the amount of sample loaded, the net recovery from a gas chromatograph and the detector response to derivative etc., the data can reveal certain characteristics of the derivatives.

FIG. 6. Equal quantities of DHEA and cholestane were chromatographed. The peak shape and RPAR value of DHEA varied markedly with sample size of ragne from 0.1 to 100 μg.
Column, 1.5% SE-30, Column temp., 230°C

Results were as follows

Sample size (μg)	100	40	20	10	4	2	1	0.4	0.2	0.1
RPAR	0.73	0.75	0.76	0.75	0.74	0.68	0.63	0.49	0.51	0.37
P_s (DHEA)	1.85	1.73	1.79	1.83	1.72	1.79	1.70	1.80	1.81	1.77
T_f (DHEA)	0.48	0.89	1.03	1.72	1.96	2.13	2.50	2.85	3.23	2.81
(cholestane)	0.38	0.61	0.85	0.96	0.99	1.05	1.03	1.09	1.12	1.29

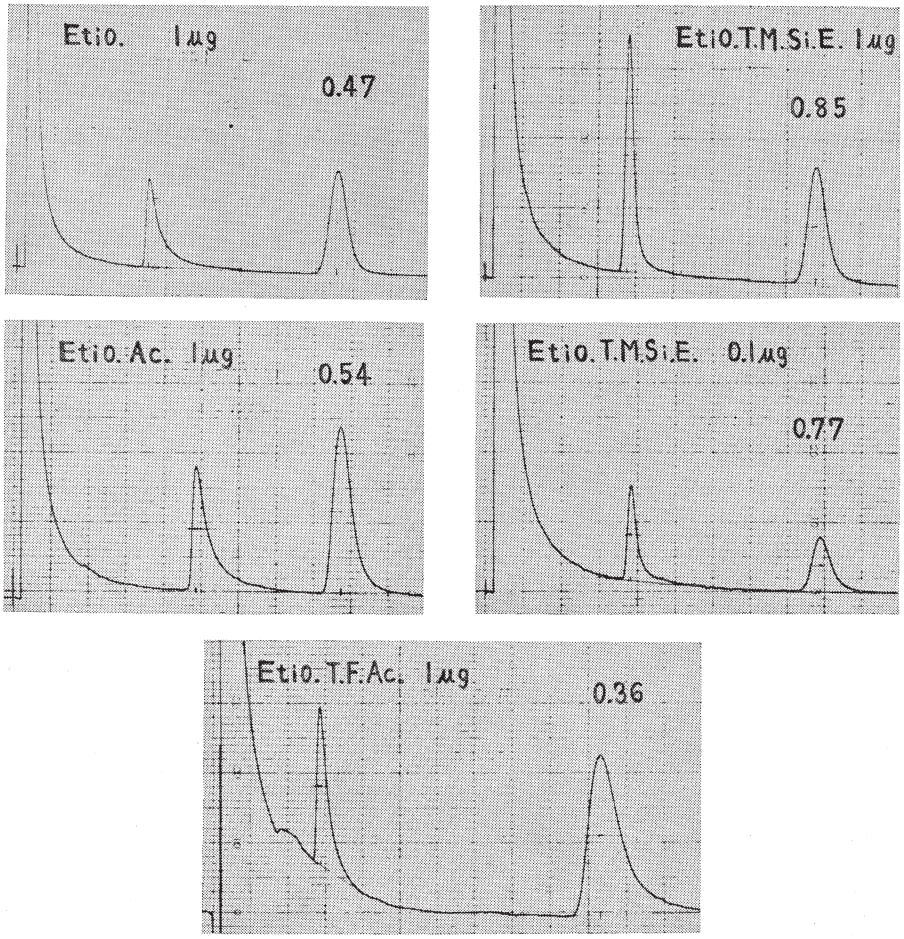

FIG. 7. Peak shapes of various derivatives of etiocholanolone

Peak shapes of etiocholanolone and its derivatives (first peak) are illustrated, compared with cholestane (second peak). The least tailing was observed with TMS, Column, 1.5% SE-30, Column temp., 230°C except for the tracing of TFA at 210°C.

Obtained RPAR values were as follows:

	Sample size (μg)	RPAR	Tailing factor	
			Etio.	Cholestane
Parent	1	0.47	4.57	1.42
Acetate	1	0.54	4.00	1.75
TFA	1	0.36	2.65	2.10
TMS	1	0.85	2.13	1.35
	0.1	0.77	2.04	1.37

Acetate was not an useful derivative in both peak shape and RPAR, while trifluoracetate (TFA) exhibited a sharper peak. The striking properties of TFA are high volatility and relatively rapid elution from an SE-30 phase[22].

But, paradoxically, there were obtained relatively low RPAR values which can be interpreted as follows: While its peak is located on the tailing part of solvent front, a relatively short retention time for TFA may have resulted in the sharp peak. In many samples, however, the usual trifluoracetylation process yielded some by-products. Moreover this derivative shows less thermal stability[25] and the detector response to this derivative is restricted by election capture phenomena[39]. Therefore, particular attention must be paid to this derivative.

Trimethylsilylation of the hydroxyl group, which produced increased volatility and thermal stability[40], markedly changed the peak shape symmetrical, and of the derivatives studied, the trimethylsilyl ether (TMS) approximated most nearly to saturated hydrocarbon, giving rise to higher RPAR value and lower T_f value. This was also proved at the 0.1 μg level shown in Table 2 and Fig. 7.

Later, derivatives of 17-KS and pregnanediol were studied for the variation in the RPAR with various columns, providing data shown in Table 3, and the

TABLE 3. RPAR Values of Various Derivatives with Various Columns

	Parent		Acetate		TFA		TMS	
	RT (min.)	RPAR	RT (min.)	RPAR	RT (min.)	RPAR	RT (min.)	RPAR
1.5% SE-30		a		b		c		d
Etiocholanolone	6.7	0.59	9.2	0.63	5.4	0.41	7.3	0.88
Androsterone	7.3	0.55	9.4	0.53	5.3	0.40	7.0	0.86
Epiandrosterone	7.4	0.53	10.6	0.58	6.6	0.45	8.8	0.87
DHEA	7.2	0.51	10.4	0.53	6.2	0.34	8.5	0.87
Pregnanediol	11.5	0.45	22.5	0.53	8.2	0.61	16.4	—
1% SE-52		e		f		g		h
Etiocholanolone	6.7	0.40	8.8	0.60	5.0	—	6.6	0.86
Androsterone	7.2	0.36	9.0	0.49	4.8	—	6.3	0.73
Epiandrosterone	7.6	0.34	10.3	0.55	6.4	—	8.2	0.67
DHEA	7.4	0.36	10.0	0.49	6.0	—	7.9	0.74
Pregnanediol	11.6	0.19	20.8	0.51	7.0	—	13.3	0 95
1% XF-1150		i		j		k		l
Etiocholanolone	25.5	0.69	20.5	1.06	7.5	0.89	5.6	0.88
Androsterone	23.4	0.69	17.7	1.08	6.9	0.76	4.4	0.99
Epiandrosterone	27.7	0.49	20.9	1.19	8.5	0.80	6.8	1.01
DHEA	27.7	0.65	19.8	1.06	7.5	0.68	6.4	1.08
Pregnanediol	30.5	0.59	25.1	1.11	4.1	0.61	2.8	—
1.5% NGS		m *		n		o		p
Etiocholanolone	11.8	0.32	10.2	0.70	6.2	0.24	7.6	0.97
Androsterone	11.1	0.30	9.2	0.60	5.3	0.47	5.9	—
Epiandrosterone	13.0	0.36	11.4	0.74	7.2	0.22	9.1	0.72
DHEA	13.5	0.42	11.3	0.70	6.6	0.11	9.1	0.61
Pregnanediol	19.2	0.06	16.8	0.57	4.4	—	5.5	—

Various derivatives of 17-ketosteroids were chromatographed at 5 μg level (*10 μg level). RPAR values varied with daily column condition or incomplete coversion to derivatives (TFA). The data showed higher RPAR values for TMS.

Column temperature was as follows;

a, b, d, e, f, h 230°C, c, g 210°C, i, j, k, l, o 205°C, m, n, p 215°C

effectiveness of TMS was confirmed, though a marked difference in the behaviour of TMS was evident with the small dose. The high RPAR value attained by TMS may be responsible partly for the increase in carbon number, but, from a consideration of the RPAR curve and peak shape of this derivative, it is more reasonable to attribute the high value to the effect of the functional group. From these observations it is obvious that TMS, although more sensitive to water than other derivatives, is effective and therefore, valuable in conducting quantitaive analysis by gas chromatography.

In general the difference in observed peak shape seen when comparing derivatives with their parent steroid, was as distinct as the effect of sample size, emphasizing the importance of the functional group as a controlling factor in establishing the characteristics of the peak shape. On the other hand, the RPAR value appeared to be completely dependent on the functional group (or the polarity of their molecular structures[17]); the effect of which was observed easily by comparing the peak shape. Accordingly, especially in case of smaller samples, precaution should be taken against the difference of the RPAR curves between derivatives with several kinds of the functional group. This consideration will lead to selection of a suitable derivative and afford certain practical solutions for the difficulties, which have been experienced in highly oxygenated compounds, or estimations of smaller amounts of materials than are generally used.

5. Functional group effect on RPAR

In order to confirm the effect of trimethylsiloxy group on the RPAR value the following experiment was performed.

A test sample containing four steroids; equal amounts of DHEA, 11β-hydroxyetiocholanolone, pregnanediol and cholestane were chromatographed on SE-30 column after complete trimethylsilylation. The RPAR curves of these trimethylsilyl ethers are shown in Fig. 8. These ethers maintained high RPAR values, which extended over a broad range of a sample size. There seemed to occur a slight variation in their RPAR value; with diminished the RPAR value when a carbonyl group and a double bond are introduced into the molecule. The reason for this slight difference was that available data corresponded only to compounds with a few substituents such as TMS of DHEA, 11-OH-etiocholanolone and pregnanediol.

Accordingly, studies were made of the RPAR curves of a series of steroids with the carbonyl group and double bond. The experiment was made to study, in more detail, the observations above as shown in Fig. 9. It is at once apparent that the RPAR value of a hydrocarbon, such as androstane, was the highest and also that the RPAR values of closely related 17-ketosteroids decreased significantly with every carbonyl group added to the molecule. As a matter of fact, the decrease in the RPAR seemed to be an additive function

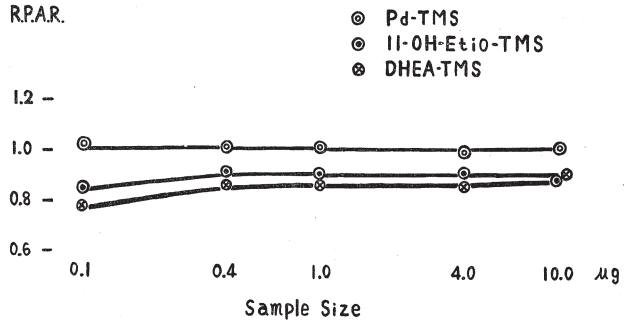

FIG. 8. RPAR curves of trimethylsilyl ethers
Column, 1% SE-52 2.25 m. Column temp., 230°C.
DHEA-TMS, 11-OH-Etio-TMS and Pd-TMS are trimethysilyl ethers of DHEA, 11 β-hydroxyetiocholanolone and pregnanediol.
These derivatives have functional groups, as follows.

	Trimethylsiloxy	Carbonyl	Double bond	R.T. (min.)
DHEA-TMS	1	1	1	12.6
11-OH-Etio-TMS	2	1	0	18.9
Pd-TMS	2	0	0	22.6
Cholestane	0	0	0	24.8

of the incremental decrease presumably attributable to each functional group. Moreover, the RPAR value was also modified somewhat by the introduction of a double bond; this was evident by the fact that the RPAR of 4-androstene-3, 17-dione was lower than that of androstane-3, 17-dione. Next, while saturated hydrocarbons such as androstane and pregnane maintained high linear RPAR curves, C_{19} carbonyl steroids did not. The effect was particularly evident for polyfunctional compounds. For example 4-androstene-3, 11, 17-trione showed the greatest decreasing tendency.

These observations were in partial agreement with the work of Sweeley and Chang[5]. In their report the correlation of molar response was made with the oxygen content; namely, the type of oxygen containing group and unsaturation. Of these the total oxygen content was regarded as having the most pronounced effect on the molar response and the effect was attributed to the response of argon ionization detector.

In the present study, without exception, the presence of certain functional groups lowered the RPAR significantly, when compared with the saturated bydrocarbon, cholestane, but prevented by inactivating the functional group. On the other hand, the fact that the RPAR value of androstane was above 1.0 suggests that RPAR is not an absolute index, but reflects the loss of a given steroid when compard with cholestane which was also lost. As will be discussed later, these results are due rather to some loss than to the difference of the detector response and influenced by several factors. Whenever an

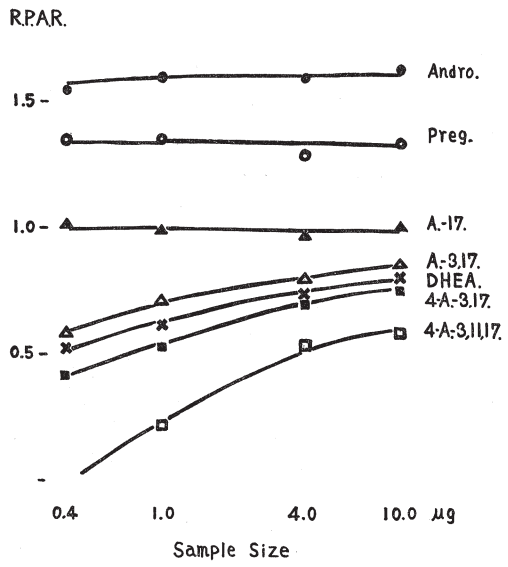

FIG. 9. RPAR curves of steroids with carbonyl group
Column, 1% SE-52 2.25 m. Column temp., 230°C
Abbreviation and retention time were as below.

Abbreviation	Trivial name	Retention time (min.)
Andro	Androstane	2.9
Preg.	Pregnane	4.8
A-17	Androstane-17-one	5.5
DHEA	Dehydroepiandrosterone	10.8
A-3, 17.	Androstane-3, 17-dione	12.3
4-A-3, 17	4-Androstene-3, 17-dione	15.0
4-A-3, 11, 17	4-Androstene-3, 11, 17-trione	18.8
	Cholestane	24.4

With every carbonyl group added, retention time becomes longer and RPAR decreases in general.

attempt is made to improve the accuracy of a given procedure, the nature of the compounds under study and the sample size must be taken into consideration. In this regard Horning[31] stated "As far as adsorption effects are concerned allowable functional groups, which permit submicrogram sample to be used, include carbonyl and trimethylsilyl ether groups but not the free hydroxyl group."

For the quantitative estimation with gas chromatography, various internal standards were used, usually of similar structure as the sample components. Examples of internal standard substances are illustrated in Table 1. They may be introduced at the beginnings of the analysis, there by compensating for losses which occur during the preliminary isolation step or correcting the varied 'molar response' of various compounds as an appropriate calibration

constant. But when there are many carbonyl groups in a molecule, even without any hydroxyl group, it is obvious as in Fig. 9 that an appropriate calibration constant does not exist unless estimation is made only in a narrow range of sample size. The RPAR curve is variable with each component in the samples, operating column and condition. The relative concentration of component steroids should be taken into consideration as well. Accordingly, in a strict sense, it is difficult to define any correcting equation for decreased RPAR in precise mathmatical terms, and therefore to relate the RPAR of one compound to others tested.

6. RPAR and retention time

The relationship that appeared obviously constant in this series of study was that the RPAR of any given steroid generally diminished with each additional functional group accompanied by increase in retention time. The influence of retention time may be observed by comparing under varied flow rates of the carrier gas.

A similar mixture was used for this study, with the operating temparature and column packing identical with the conditions of previous anylysis. As shown later, peak area may vary but RPAR does not vary with the different flow rate of hydrogen or carrier gas. Fig. 10 shows a plot of retention time v.s. RPAR for androstane, pregnane, androstane-17-one, DHEA, 4 androstene-3, 17-dione and 4-androstene-3, 11, 17-trione. On the non-selective SE-52 phase, variation of retention time had considerable influence on the magnitude of RPAR. When observed under similar retention time, carbonyl compounds

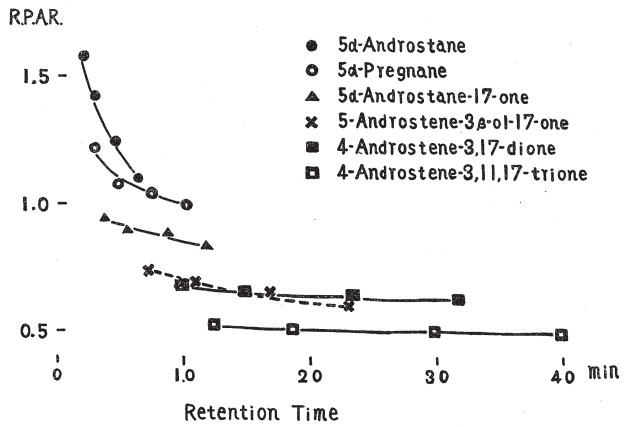

FIG. 10. Retention time and RPAR value for steroids with carbonyl group
Column, 1% SE-52 2.25 m. Column temp., 230°C
The sample (4 μg level) was chromatographed at 4 different flow rates of carrier gas: 180 ml/min., 90 ml/min., 45 ml/min. and 30 ml/min. Retention times of cholestane were 16.0 min., 23.8 min., 37.8 min. and 51.6 min. respectively. RPAR of all steroids showed decreasing tendency with longer retention time.

such as androstane-17-one showed lower RPAR than the more saturated hydrocarbon pregnane. Similar results were obtained for 4-androstene-3, 17-one and 4-androstene-3, 11, 17-trione. With each of them a progressive decrease in RPAR was noticed with increase in retention time. It is clear from Fig. 10 that for these compounds the RPAR varies remarkably with retention time of within 10 minutes and with increase in retention time the curve gradually becomes flat, giving almost constant RPAR values.

In order to study the behaviour of trimethylsilyl ethers, a test mixture was injected under varying flow rates of carrier gas and the relationship between retention time and RPAR of each compound was plotted graphically in Fig. 11. In this experiment the concentrations were chosen to give similar RPAR value. As would be expected, the data also confirmed that decreasing tendency of RPAR appears with increase in retention time. Hydroxyl group gave a somewhat more varied RPAR with retention time than the corresponding TMS, as indicated by a comparison with DHEA and its TMS.

Based on this experiment, it is obvious that the retention time did not influence markedly on RPAR, when the retention time was longer than 10 minutes, and the observed variations in the RPAR of various compound can be ascribed to the functional group and not to the retention time. However, the sharp decreasing tendency of the RPAR curve seen with short retention time can not be explained by the above, as such was also noted with saturated hydrocarbons such as androstane and pregnane. The latter phenomenon can not be explained from the present study.

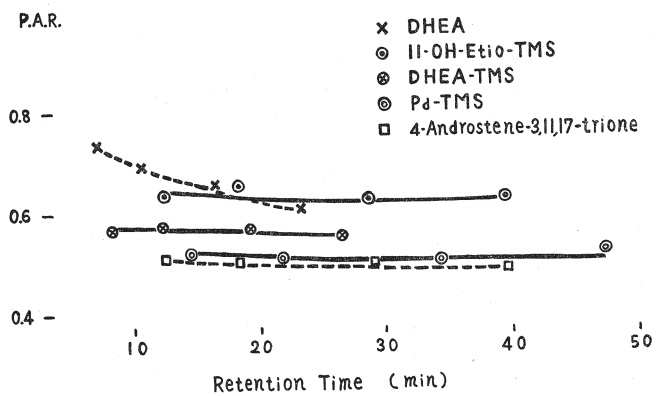

FIG. 11. Retention time and peak area ratio to cholestane of trimethylsilyl ethers

Column. 1% SE-52. Column temp., 230°C

The sample, which did not consist of equal amounts of steroids, was chromatographed under the same condition as was employed in Fig. 9. A similar decreasing tendency was observed with TMS but the tendency was less marked.

At the start of this study, it was assumed that cholestane would be stable enough to remain intact in the gas chromatograph, but the above date failed to support this assumption. Cholestane as well as androstane and pregnane is probably lost also in gas chromatograph. The loss rate of cholestane might approximate that of TMS which showed high RPAR values.

7. Gas chromatographic recovery of cholestane

In the present study the accuracy of chromatographic analysis was investigated by observing the relation of the peak area of steroids to that of cholestane. This saturated hydrocarbon was assumed to be recovered sufficiently enough from the chromatographic ccolumn, to enable detection of the difference in losses between given steroids. But in the previous observation, it was noted that the RPAR of androstane or pregnane was above 1.0 and varied with the retention time. This phenomenon, incompatible with the previous assumption, was probably due to the loss of saturated hydrocarbon, shown by Simmonds and Lovelock[10] with an ionization cross-section detector.

In order to ascertain if such occur, the peak area of cholestane per 1 μg was calculated for several sample loads and compared with the value at 8 μg. In Table 4, experiments A and B were attempted on different columns and on different days. Scattering of the value was considered to be responsible partly

TABLE 4. Gas Chromatographic Response of Cholestane of Various Doses

Dose (Sample size)	Relative response								
	A						B		
100 μg	0.91								
80	0.96								
60	0.98								
40	1.09	0.92							
20	1.07	1.08	0.98						
10	1.04	0.94					0.96		
8	1.00	0.99	0.98	0.99	1.02	1.00	0.98	0.98	1.03
6	1.02								
4	0.94	0.79					0.99	1.07	
2	0.87	0.83	0.85						
1	0.78						1.00	1.00	0.97
0.8	0.84	0.85	0.87				0.83	0.83	
0.6	0.74								
0.4	0.77						0.93	0.94	
0.2	0.97								
0.1	0.68	0.78					0.99	0.89	
0.08							0.77	0.89	
0.04							0.68	0.89	
0.01							0.77	0.82	

The peak area per 1 μg was calculated for each varied dose and compared with the value at 8 μg. In both trials (A, B), decreasing ratio was observed with decreasing dose.

Condition: 1% SE-52 2.25 m
Temp., Flash 300°C, Detector 250°C, Column 235°C
Retention time of cholestane, 20.8 min (A), 26.3 min (B) respectively.

for sample injection or loading effect. There was a slight decreasing tendency in the peak area ratio with decreasing dose and this tendency could be interpreted in the same way as mentioned above. It is concluded that cholestane is "unaffected by the loss", only in the sense that it is not markedly lost when compared with other oxygenated steroids.

8. Detector response

In the microdetermination of steroids by gas chromatography, a variation in the molecular response of the argon ionization detector to various steroids was noted by Sweeley and Chang[5]. They observed the molar response of a given steroid to be dependent on the number and nature of the functional groups present in the molecule and also dependent to a certain extent on the design of the detector. Furthermore, the total molar area was found to increase logarithmically with increased voltage and relative molar response of each steroid to be dependent on the applied potential of the detector. They stated the fact a polyketone gave a lower response than cholesterol at one potential, and an equal response at a second potential added strength to the view that this phenomenon may be attributed to the detector response rather than to the decomposition of steroids or the adsorption to the chromatographic column.

Similarly Bloomfield[7] reported that, when the column efficiency was changed, the molar responses and the limits of linearity for sterols were markedly altered. They explained this phenomenon as follows: As the number of theoretical plates increase, sharper mass peaks are presented to the detector, absolute threshold values diminished and detector overload is experienced with correspondingly smaller amounts. As the second factor which is responsible in part for this phenomenon, this was probably related to the adsorption characteristics of the solid support.

Apart from the column condition, the problem of the detector response must be taken into consideration. Various studies on the properties of ionization detectors have been undertaken by several authors. With argon ionization detector, the relative molar responses of hydrocarbons and their isomers with the same carbon number are much more spread out than in the case of flame ionization detector[41].

It is well known that the relative molar response of hydrogen flame ionization detector is related to carbon number[41][42][43]. In these papers hydrocarbons of low carbon nnmber were investigated and gave the following results: The relative molar responses of hydrocarbons are, with a good approximation, directly proportionate to the carbon number of the molecule. Response to different chemical compounds can conveniently be expressed in terms of the effective carbon number for the compound which represents the number of aliphatic carbon atom to which the response of a sample molecule is equi-

TABLE 5. Contributions to Effective Carbon Number (Sterberg *et al.* 1962)

Atom	Type	Effective carbon No. contribution
C	Aliphatic	1.0
C	Aromatic	1.0
C	Olefinic	0.95
C	Acetylenic	1 30
C	Carbonyl	0.0
C	Nitrile	0.3
O	Ether	−1.0
O	Primary alcohol	−0.6
O	Secondary alcohol	−0.75
O	Teritary alcohol, Esters	−0.25
Cl	Two or more on single aliphatic C	−0.12 each
Cl	On olefinic C	+0.05
N	In amines	Similar to 0 in corresponding alcohols

valent. Effective carbon numbers for compounds depend fairly much upon molecular structure and reasonable estimates of effective carbon number can be made from the empirical table of contributions. Table 5 was quoted from the work of Sternberg *et al.*[42].

If the difference in the contributions to effective carbon numbers would be negligible between the carbon atoms in a steroid molecule and those in an aliphatic hydrocarbon, the molar response of steroids could be calculated from contributions to effective carbon number and thus the RPAR value could be calculated theoretically also in this way. This theoretical RPAR value means the relative detector response of a given steroid compared with cholestane; therefore the influence of several factors other than detector response is neglected. Because of practical requirement, the RPAR was calculated on equal weight basis. Accordingly the relative molar response, which is based on equal molar basis, must be transformed to an equal weight basis by using the following equation:

$$R_w = \frac{M_c}{M_x} R_m.$$

Where R_w is the relative response per gram of cholestane (that is theoretical RPAR), R_m the relative molar response to cholestane ($=1.00$), M_x the molecular weight of a given steroid of interest, and M_c the molecular weight of cholestane respectively.

For example, the theoretical RPAR values were calculated and presented in Table 6. Theoretical RPAR showed not necessarily good agreement with the empirical RPAR obtained experimentally in the present study. This data shows that certain differences always exist between the theoretical RPAR and the empirical one which varies with sample size. It can not be attributed to experimental error also for the following reasons.

First, as has been shown in Fig. 9 and Fig. 10, the peculiar characteristics

TABLE 6. Theoretical RPAR Value

Compound	Formula	No. of functional group			
		OH	C=0	$-COOCH_3$	$-C=C-$
Androstane	$C_{19}H_{32}$				
Androstane-17-one	$C_{19}H_{32}O$		1		
Androstane-3, 17-dione	$C_{19}H_{28}O_2$		2		
4-Androstene-3, 17-dione	$C_{19}H_{26}O_2$		2		1
4-Androstane-3, 11, 17-trione	$C_{19}H_{24}O_3$		3		1
Androsterone	$C_{19}H_{30}O_2$	1	1		
DHEA	$C_{19}H_{28}O_2$	1	1		1
DHEA acetate	$C_{21}H_{30}O_3$		1	1	1
Accetylated DHEA	$C_{21}H_{30}O_3$		1	1	1
Pregnane	$C_{21}H_{36}$				
Pregnanediol	$C_{21}H_{36}O_2$	2			
Pregnanediol acetate	$C_{25}H_{40}O_4$			2	
Acetylated Pregnanediol	$C_{25}H_{40}O_4$			2	
Cholestane	$C_{27}H_{48}$				

* This is the value which would be expected in the recording after equal amounts of DHEA and cholestane are acetylated. Accordingly, theroretical RPAR of acetylated steroid (R) was calculated as follows:

$$R=\frac{M_a}{M_p}\cdot R_a$$

where M_p is molecular weight of parent steroid, M_a that of acetate and R_a theoretical RPAR of acetate.

of RPAR was found. The empirical RPAR values of androstane and pregnane were well above the theoretical value of 1.00 and varied with retention time. Moreover, it could not be recognized that the standard substance, cholestane, maintains constant recovery regardless of its sample size. These phenomena are probably related to other losses rather than to the detector response.

In relation to this, attention must be paid to Clayton's report[8] of several sterol ethers applied in isotopically labelled form to the column and trapped at the exit post. The recovery of radioactivity corresponding to the emergent peak of the material concerned varied between 40 and 55% of the total injected. However, the recovery did not show any clear relationship with structure of compounds and sample sizes, because the study was based on large sample sizes (20 to 80 μg) of steroids with a little structural difference. In the present study, however, RPAR decreased in general with decreasing sample size. This is not connected with deviation from the linearity of detector response.

The linear range of hydrogen flame ionization detector was found to exceed 10^6 fold, extending from beyond the highest sample introduction to the detectable limit (3×10^{-12} g/sec propane) taken as a signal equal to twice the random noise level[39]. This has also been confirmed by the work of Fowlis *et al.*[44], where commercially available detectors were examined critically, and the flame ionization detector was seen to have a linear response over a wider range of dose than the argon ionization detector and the response to be independent of

and Empirical RPAR Value

Effective No. of C atom in a molecule	Relative molar response	Molecular weight	Theoretical RPAR	Empirical RPAR A (10 μg)	B (10 μg)	C (5 μg)	D (5 μg)
19	0.704	260	1.01	1.69		1.58	
18	0.667	274	0.91	1.01		0.96	
17	0.630	288	0.82	0.86		0.81	0.55
16.9	0.626	286	0.82	0.76		0.72	0.56
15.9	0.589	300	0.73	0.59		0.57	
17.25	0.639	290	0.82				0.55
17.15	0.635	288	0.82	0.81	0.73	0.75	0.51
18.65	0.691	330	0.78				
		288+42	0.89*		0.87		0.53
21	0.778	289	1.00	1.34		1.27	
19.5	0.722	321	0.84		0.58		0.45
22.5	0.833	405	0.77				
		321+84	0.97*		0.87		0.53
27	1.000	373	1.00				

applied voltage over a certain range[39) 44)]. Considerable changes in design and operating conditions may have little effect on the linear dynamic range and sensitivity of the flame ionization detector[39)]. It has been reported, however, that changes in geometry and in the flow rate and composition of the gases supplied to the flame may alter the relative molar response to different compounds[39)].

The response of hydrogen flame ionization detector was observed to depend chiefly on hydrogen flow rate, carrier gas flow rate and scarcely on air flow rate to a certain extent[42)]. Thus, for precise analysis with a given detector, it is necessary to calibrate peak area per unit for each compound and then to ensure that the operating conditions are maintained throughout the analysis. Fig. 12 demonstrates that the peak area of cholestane was dependent on the flow rate of hydrogen and carrier gas. From this it follows that for quantitative analysis not only the hydrogen flow rate but also flow rate of the carrier gas must be accurately stabilized. In view of the striking result obtained with varied peak area under various hydrogen flow rate, it will be of interest to observe the effects of different hydrogen flow rates on RPAR. The result of such a comparison of 6 different steroids at the eight levels of applied flow rate are presented in Fig. 13. Injections of equal amounts of the same mixture were made sequentially, leading to progressive saturation of the active sites within the column. Apart from this precaution little variation in RPAR was found under these experimental conditions. At any optional hydrogen flow rate RPAR of each steroid was seen to be consistently dependent upon the number and type of functional group. Since the peak area for each steroid is also a function of the applied hydrogen and carrier gas flow rate, it is necessary, after analysis has been made of the standard, to maintain the conditions of calibration throughout the analysis. In fact it is difficult to regulate the flow rate daily in such a reproducible manner to assure a constant peak area

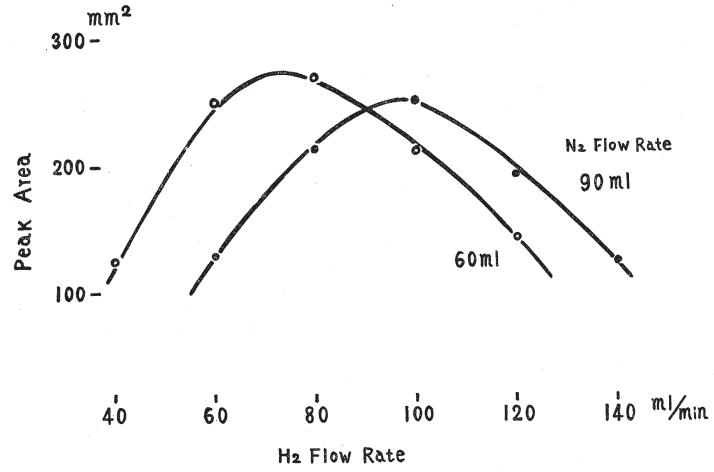

FIG. 12. Effect of flow rate on peak area

Column 1%SE-52 2.25 m.

Peak area of cholestane varied with H_2 flow rate in two different carrier gas flow rates: for 60 ml/min and 90 ml/min, the retention time of cholestane being 32.5 min and 25.2 min respectively.

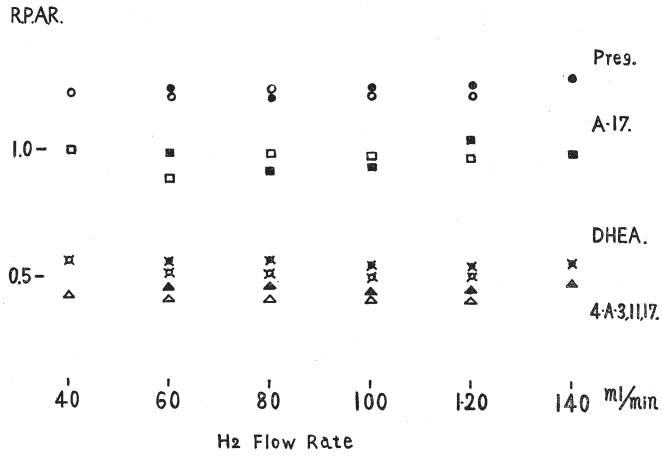

FIG. 13. Effect of flow rate on RPAR

Conditions identical with those of FIG. 11. Black marks represent RPAR obtained at carrier gas flow rate of 90 ml/min and empty marks 60 ml/min.

RPAR did not vary with flow rate of hydrogen or carrier gas and relatively constant value was maintained.

to be obtained. The RPAR value is less affected than peak area by the daily variation in flow rate due to temperature change or other causes. Consequently the use of internal standard and isothermal condition is recommended for quantitation.

9. Effect of temperature on RPAR

Gas chromatographic analysis of steroids requires the use of a high temperature for their separation in a reasonable time. As steroids are relatively unstable substances particularly at a high temperature, care must be taken to avoid decomposition due to overheating. Since the first report of Horning[1], several steroids of adrenocortical type such as cortisol and deoxycortisone are known to undergo thermal degradation or rearrangement during gas chromatography. Lipsky *et al.*[2] noted structural transformation in flash heater such as degradation of the hydroxyl group (in cholesterol, coprosterol and desmosterol) and transformation of 1,4-dienone ($\Delta^{1,4}$ androstadienolone) and 5,7-diene (7-dehydrocholesterol) group.

In all these reports including the later reports were observed change of peak shapes; virtually thermal demethoxylation of allylic Δ^{2}- and Δ^{4}-3 β-methoxycholestenes[8] and rearrangement of 3 α, 17 α-dihydroxypregnane-20-one[3] gave blurred peaks in the chromatogram and elimination of one acetoxy group of 17 α, 21-diacetoxy-20-keto pregnane derivatives yielded a small peak of transformation product (21-acetoxy-Δ^{16}-20-ketones) other than the principal peak attributable to the unaltered compound[45]. These peaks may appear on the resulting chromatogram due to partial degradation or transformation, but the peak of breakdown product is not always observed obviously.

Kirschner and Fales[9] reported that the peak area of adrenosterone (4-androstene-3, 11, 17-trione) resulting from pyrolysis of cortisone was smaller than the peak area obtained from a corresponding mass of adrenosterone. This low yields of thermal degradation product, also observed by Bailey[46], was probably responsible for complete decomposition which seemed to occur at the same time with partial decomposition.

As an obvius example of thermal effects, trifluoracetate can be illustrated. Horning *et al.*[25] pointed out that this derivative is not thermostable. In the present experiment, it decomposed above a temperature of 220°C partially to give some peaks of by-product with diminished main peak.

In certain cases androsterone produced a little peak in front of its main peak on SE-30 column, especially when a flash heater was above a temperature of 300°C.

In these series of experiments a marked decrease in RPAR was most frequently encountered with relatively polar steroid.

Although a peak of decomposition product was not found on the resulting chromatogram, these results in general were in good agreement with the previous report[10], and suggestive of an assumption that the increase in the number of oxyganated functional group in the steroid molecule leads to an increase in susceptibility to decomposition.

Therefore, the effect of flash temperature on RPAR was studied and the results are summarized in Table 7. Temperature change, within the range of 250

TABLE 7. Effect of Flash Temperature on the RPAR Value

Compound	Sample	Flash temperature			
		250°C	300°C	350°C	400°C
Pregnane	A	0.96	0.98	0.97	0.97
	B	1.06	1.07	1.04	1.08
Androstane-17-one	A	0.80	0.82	0.84	0.81
	B	0.91	0.87	0.89	0.91
DHEA	A	0.54	0.56	0.53	0.44
DHEA-TMS	B	0.77	0.76	0.79	0.79
4-Androstene-3, 17-dione	A	0.63	0.63	0.63	0.45
	B	0.45	0.42	0.45	0.44
4-Androstene-3, 11, 17-trione	A	0.45	0.46	0.45	0.27
	B	0.33	0.35	0.32	0.33
Pregnanetriol-TMS	B	0.69	0.64	0.68	0.65

* Sample A (4 μg level) and sample B (3 μg level) were not chromatographed on the same day. The steroids in the sample B were trimethylsilylated.

Column, 1% SE-52. Column temperature, 230°C.

Retention time of cholestane, 25.2 min (A),36.2 min (B).

TABLE 8. Effect of Column Temperature on RPAR

Compound	Column temperature					
	220°C		235°C		250°C	
	RT (min)	RPAR	RT (min)	RPAR	RT (min)	RPAR
Pregnane	4.8	1.46	5.4	1.00	5.8	0.95
Androstane-17-one	5.6	1.25	6.2	0.84	6.6	0.79
DHEA TMS	13.7	1.06	13.7	0.96	13.0	0.95
4-Androstene-3, 11, 17-trione	21.0	0.46	20.6	0.46	19.4	0.45
Pregnanetriol-TMS	40.0	0.71	36.1	0.78	30.9	0.81
Cholestane	27.4	1.00	26.2	1.00	23.8	1.00

* Condition

Column, 1%SE-52 2.25 m.

Flash, 300°C. Detector, 250°C.

Flow rate of carrier gas, 160 (220°C), 60 (235°C), 30 (250°C) ml/min.

Sample, 3 μg level.

to 350°C, had no apparent effect on the RPAR value but, at a temperature of 400°C, the RPAR value for polyoxygenated steroids decreased occasionally in a degree indicating a possible catalytic effect accelerating their thermal decomposition.

Next, the results of chromatographic runs at different column temperatures are presented in Table 8. Flow rate of carrier gas was adjusted to obtain similar retention time and the flash heater was kept constant. Over the temperature range of 220 to 250°C the covered column temperature appeared to

have a negligible effect on RPAR. Pregnane, androstane-17-one and DHEA-TMS, which had relatively short retention time, gave higher RPAR at 220°C. This is believed to be the effect of retention time as shown in the foregoing observations; namely, the effect of retention time surpassed the effect of temperature and the possibility that thermal decomposition is responsible for the decrease in RPAR could not be ruled out.

From the start of this study, it was assumed that steroids would be decomposed under higher temperature and that the decrease in RPAR would be due to thermal decomposition rather than to other losses; for example, irreversible adsorption which could not be discerned from complete thermal decomposition in the present experiment. However, the fact that no peak other than the main peak appeared with the relatively constant RPAR value may suggest that in most cases no structural changes occur at this higher temperature and that the degree of thermal decomposition, if related to RPAR in any degree, did not vary appreciably over a certain temperature range. Accordingly, this makes the previous explanation unlikely, though the thermal decomposition could not be disregarded entirely, since there was no conclusive evidence regarding this point.

As to the thermal decomposition of steroids Wotiz[4] observed that it was dependent on the amount of column coating, but independent of column length, temperature and the particle size of the solid support, and suggested that thermal decomposition is an unimolecular vapor phase reaction. Another aspect of thermal decomposition process has been pointed out by Horning *et al.*[31]; namely, wide variation in temperatu e along the column may very well result in "hot spot" which leads to localized deterioration of the column packing and any alteration of the packing by thermal effects will lead to unnecessary decomposition or adsorption of the sample component under study.

Taking these observations into consideration, no reason for the thermal effect on RPAR could be known beyond the fact that RPAR is relatively constant under varying temperatures, and it may only be concluded that the causative mechanisms of the decrease in RPAR, say thermal decomposition, adsorption or any others, were rather indifferent to temperature change in these studies. Nevertheless, it was apparent that the functional group and its derivatives play a variable role in the effect on RPAR. This together with the previous data on the characteristics of the RPAR curve, may give a clue to important factors related to RPAR.

10. Loading effect

It has frequently been experienced that repeated sample injection usually lead to an observed increase in response during the course of experiment which could easily be overcome by overloading the column prior to the first injection of a series of samples. This had led some laboratories to practice

presaturation of sample so that subsequent responses may be at or near the maximum level likely to be observed for the system and the sample questioned. However, with this practice it is assumed that the condition at which the initial standard is measured is maintained constant during subsequent chromatography.

In an effort to confirm the effect of presaturation, the same sample was studied as follows: After analysis was made of a series of samples, the gas chromatograph was not used at room temperature for 40 hours and four consecutive injections of identical amounts of the same sample were compared for their RPAR. The results are shown in Table 9 (A). The first injection gave a lower RPAR than the injections which followed. Thus, presaturation prior to quantitative studies was considered to be a satisfactory procedure for routine operation.

TABLE 9. RPAR Values of the Series of Injections

A. RPAR Values of Four Consecutive Injections

Trial	Retention time of DHEA	Peak area		RPAR
		DHEA	Cholestane	
1	7.2 min.	158	251	0.63
2	7.2	175	261	0.67
3	7.1	189	267	0.71
4	7.1	182	258	0.71

Column, 1.5% SE-30, Column temp., 230°C.
Sample, 10 μg level.

B. RPAR Values during Serial Use

Run. No.	Time hr. min.	Column temp.	Flow rate (ml/min.)	4-Androstene-3, 17-dione		4-Androstene-3, 11, 17-trione	
				RT (min)	RPAR	RT (min)	RPAR
1	0	235	60	17.0	0.71	21.2	0.54
2	44			17.2	0.68	21.4	0.53
3	1 22			16.6	0.70	20.7	0.54
4	1 58			16.6	0,70	20.6	0.54
5							
6							
7							
8		250	30				
9	4 57			15.6	0.68	19.1	0.53
10							
11							
12	6 39			16.0	0.69	19.6	0.51
13	7 49	220	160	16.8	0.68	21.3	1.51
14							
15	9 15						
16	16 30						
17							
18							
19							
20	19 12			16.6	0.69	21.4	0.54
21	20 00	235	60	17.1	0.68	21.4	0.50
22							

Column, 1% SE-52 2.24. Sample, 3 μg level.

During a period of continuous use of a column for 20 hours, Bloomfield[7] observed that response increased gradually with time and was accounted for both by the increased column efficiency and gradual filling of the binding sites open on the adsorbent. In spite of this explanation the phenomenon which may be responsible for the loading effect is more complicated.

In similar experiments in which a column was used for various samples under varying conditions, the same sample was repeatedly chromatographed at different intervals. The data shown in Table 9 (B) indicate that all RPAR values vary merely within the range of experimental error, with no increasing tendency noted during the 20 hours.

Moreover, an extreme example which may be related to this phenomenon was observed. Fig. 14 presents the records of four injections (a mixture of pergnanediol and cholestane) made at a equal intervals apart. Three injections (I, II and IV) were repeated almost at equal intervals but one injection made soon after injection II. The peak of pregnanediol and cholestane of the second injection was almost equal to that of foregoing injections, while the inserted peak next following resulted in a higher RPAR value though the RPAR of the last peak returned to the previous value. This was found despite routine presaturation prior to experiment.

In this case the injections of test substances were made sequentially, so as to lead to progressive saturation of active sites within the column. However, this may be reversible since a progressive increase in RPAR could not be observed; the very slow elution of adsorbed material would not give rise to a signal from the detector great enough to be distinguished from drift or low efficiency noise. As a matter of fact, in low efficiency column, a very broad peak with persistent tailing was experienced. This persistent tailing may also exist so slightly that it is not evident on a chromatogram and the inserted peak when located on this tailing part may result in a higher peak and increased RPAR. The finding that the RPAR value, even after the higher one obtained with inserted injection, returned to the former value in the next injection, can not be accounted for by the concept "irreversible adsorption" which can not be distinguished from complete decomposition.

As pointed out already, the decrease in RPAR appeared to be related rather to the tailing in a linear range than to the tailing in a non-linear range of adsorption isotherm. On the basis of the above observations it seemed interesting to recognize this phenomenon in relation to the mechanism of tailing. The kinetic mechanism of tailing in the linear range of adsorption isotherm was postulated by Gidding[47] as follows:

1. A few active sites, such as silanol (or siloxane) group and mineral impurities (acidic hydroxyl), on the solid support may lead to tailing but this may occur reatively scarce.

2. Adsorption and hold up of solute molecule in excess droplets of liquid

phase in elongated pores (micro or macropore) and caverns with restricted entry. Any unit of liquid phase held in, say, a long narrow pore will equilibrate slowly with external solute and the slowness of desorption is caused by an excessive diffusion distance.

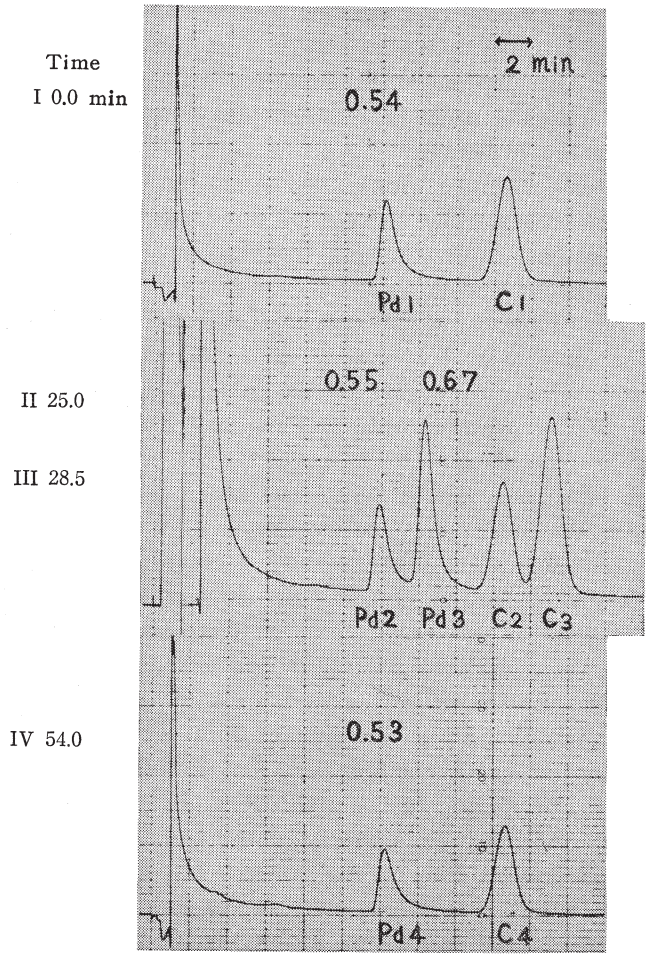

FIG. 14. An extreme exmple of loding effect

Column, 1.5% SE-30 2.25 m.

Equal amounts (3 μg) of sample were chromatographed successively. Sample contained equal amounts of pregnanediol (Pd) and cholestane (C).

Retention time: Pd 12.2 min., cholestane 18.7 min.

Result as follows.

Trial	Injecting time	RPAR value for Pd.
I	0 min.	0.54
II	26	0.55
III	28.5	0.67
IV	54.0	0.53

The peaks of trial III (Pd_3, and C_3) were greater than others. The higher RPAR value which was obtained in trial III was not necessarily attributable to sample injection, because it appears to be caused by the increase in peak area of pregnanediol.

3. Presence of excess dead volume in the system usually in the injection or detection unit.

Concerning the phenomenon in Fig. 14, the mechanism can be understood as follows. So far as the result of this study is concerned, the first is most likely to be mechanisms of tailing noted above; because the increase in RPAR of the inserted injection, which was caused chiefly by the increase in peak area of hydroxyl containing steroid, pregnanediol, seemed rather attributable to a masking effect on the active site by the precedent injection than to additional effect of the retained molecule of the precedent injection. The high RPAR and symmetrical peak of steroids after trimethylsilylation also seem to support this point, though it is highly possible that both high volatility and thermal stability of this derivative participate too.

But these data do not suggest that the adsorption on the column packing is involved to the most prominent degree; thermal decomposition, condensation and any other form of losses in sample chamber, column and detector unit may occur to such a substantial degree that considerable loss of steroids is also brought about by them. This view was strengthened by the previous experiment which demonstrated the variability of RPAR for saturated hydrocarbons and trimethylsilyl ethers even in the absence of any conspicuous tailing.

Although the problem of column support material has been summarized in detail by other authors[25) 31) 48)], an appropriate indication for the loss of steroid has not been proposed, except for some phenomena which appeared with poor column support materials[31)].

The absolute RPAR values may often vary considerably with operating conditions but are relatively reproducible during consecutive study on a single day, provided constant operating conditions are maintained, especially when similar amounts of samples are injected at regular intervals throughout the series of determination. These characteristics have to be meticulously taken into account in estimation of steroids.

11. Chromatographic column and RPAR

It would appear that the column is most likely the site of sample losses and that these probably occur by adsorption onto the so called "inert support". But the decrease in RPAR depends on other factors which were suggested in the following observations.

During the preceding experiment while collecting quantitative data on RPAR, it was observed that the same column did not give similar RPAR when connections of columns were exchanged in the dual column system. This result was surprising in view of the relatively uniform RPAR value of a column and prompted a closer examination of the RPAR with changed column connections. Fig. 15 presents the scheme of the dual column system employed in this study.

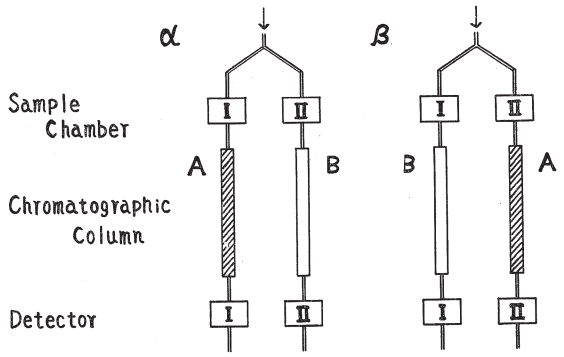

FIG. 15. Column connection in dual column system
Columns were connected as follows:
α connecting Series I Sample chamber I—Column A—Detector I
Series II Sample chamber II—Column B—Detector II
β connecting Series I Sample chamber I—Column B—Detector I
Series II Sample chamber II—Column A—Detector II

On the day first, the columns were connected as in Fig. 15 (α). Without presaturation 5 μg level of DHEA and cholestane (1 : 1) were injected and an injection of 1/10 amount was followed (trial I). Then the column connection was changed as in Fig. 15 (β) and the sample was injected in the same order (trial II). On the second day, similar trials were attempted, first with the connection of both column as in Fig. 15 (β) (trial III), then the connection was changed as in Fig. 15 (α) (trial IV). In every trial RPAR and ΔRPAR were calculated. ΔRPAR was defined as follows:

$$\Delta\text{RPAR} = \frac{R_{0.5}}{R_5}$$

Where ΔR is ΔRPAR, R_5 RPAR in 5 μg level and $R_{0.5}$ RPAR in 0.5 μg level respectively. The results shown in Table 10 refer to RPAR and ΔRPAR of this experiment.

When the RPAR values of trial I were compared with those of trial III for each connection, column A showed greater RPAR value than column B, that is, in series I column A in trial I gave RPAR value of 0.39 but column B in trial III gave RPAR value of 0.30 and in series II column A in trial IV of 0.58, column B in trial I of 0.41 respectively; and these difference are responsible for the column characteristics. Next, comparing both series of each trial, it can be clearly seen that series I gave always a lower RPAR than series II; accordingly the effect of column characteristics could not be found in this comparison. This observation added strength to the view that this phenomenon must be attributed to the gas chromatographic apparatus rather than to the chromatographic column alone. On the other hand ΔRPAR represents the gradient of the RPAR curve in the region of 0.5 μg to 5 μg level.

TABLE 10. Column Connection and RPAR Value of DHEA

A. RPAR

	Trial	Connection	Series I			Series II		
			Column	RT	RPAR	Column	RT	RPAR
The day first	I	α	A	7.9 (11.1	0.39 0.09)	B	7 2 (9.8	0.41 0.14)
〃	II	β	B	7.2 (8.6	0.34 0.27)	A	7.3 (7.9	0.56 0.42)
The day second	III	β	B	7.8 (10.9	0.30 0.09)	A	7.6 (8.3	0.58 0.36)
〃	IV	α	A	8.3 (9.9	0.43 0.18)	B	9 3 (10.6	0.54 0.34)

B. ⊿RPAR

Trial	Series I		Series II	
	⊿RPAR	Theoretical plate*	⊿RPAR	Theoretical plate*
I	0.23	1145	0.32	1274
II	0.79	1256	0.74	1230
III	0.29	1246	0.62	1238
IV	0.41	1102	0.62	1350

Column, 1.5% SE-30 2.25 m., Column temp., 230°C.
Series was for example as follows:
Series I means the connection of sample chamber I, column and detector I.
The theoretical plate number was calculated with cholestane.
The data in parentheses mean values obtained by the injection of 1/10 amount.

A similar relation as that seen between column connection and RPAR in the above observation was noted also in ⊿RPAR. When compared with similar connections, trial I showed lower ⊿RPAR than trial IV and trial III also showed lower ⊿RPAR value than trial II; the difference indicates the loading effect which could not be appreciated easily in a study of theoretical plate and also indicates the variation of loading effect with column and series.

From these observations it was concluded beyond any doubt that adsorption on the apparatus including the column would affect the loss of sample component considerably, even if thermal degradation or condensation in connecting tube might occur. These losses of steroids occurring between injection onto the column and arrival at the detector were clearly noted by Simmonds and Lovelock[10]. Since the beginning of successful application of steroid to gas chromatography, the question of instrumentation or material of the column tubing has been the subject of considerable controversy[31)49)]. As can be seen in Table 1, metal column[4)16)29)49)] and metal sample chamber[12)28)49)] have been used in some laboratories but there is seen an increasing tendency to use glass columns and on column injection[31)49)] which are considered to result in safer

operation. Virtually losses of steroids can not be prevented completely even when an glass system is used[10]. On the other hand, Arnold and Fales[50] reported that stainless steel and glass can be used for steroids as well if properly designed as sample chamber, column and connecting tube but no further quantitative investigation has yet been made with them.

Gas chromatography has provided an efficient means for separation of complex steroid mixtures. However, because of the experimental problem involved, most works with steroids have been made under various arbitrary and specific conditions and it is important that problems encountered with this method should be clarified as there is the fear of conflicting results to be obtained due to inadequate application (technique or poor instrument design).

At present no set of operating conditions has been found by which gas chromatography can be made to give uniform response to various steroids independent of their structure. When samples containing less than about 0.5 μg of each component were employed, the loss emerged as a cause of erratic and inaccurate estimations.

As a result of this study several points were made clear regarding the RPAR value. RPAR was influenced by the functional group, sample size, column and apparatus and less so by retention time, temperature, loading effect when the sample was analyzed under a limited condition. There is little doubt that RPAR for a given steroid varies from laboratory to laboratory, depending on instrumentation, operating conditions and other individual variations, so that these experimentally derived RPAR should not be regarded as absolute. It is difficult to estimate exactly the magnitude of error which may occur by the loss of sample, and all internal standard used were not acceptable especially when a relatively flat RPAR curve could not be maintained. Accordingly, it is probably too early to formulate a definite correction factor or equation for the conversion of observed peak area into quantity. Simple reliance upon relationship of linearity seen between dose and observed area will lead to great error, as a correction factor or equation turns the effects of various cause into a single parameter. In this respect Horning[31] stated: Correction factor only converts basically inaccurate analytic data into "quantitative result" and shortcoming arising from inadequate technique or poor instrumentation are not likely to be overcome successfully by this approach.

The most pronounced characteristics, that of decreasing RPAR with decreasing sample size and increasing oxygen content, as well as that of varied RPAR observed with variation in the nature of functional groups, may be attributable rather to some losses than to the response of a flame ionization detector. For the most part, the minimum amounts quantitatively detectable by gas chromatography, appear to be determined not by the detector response but by these losses or background noise brought about by the drift from the

column.

Possible factors leading to losses may be given as follows:

1. Thermal decomposition and transformation under the condition employed.
2. Condensation in gas chromatograph.
3. Reversible and irreversible adsorption in gas chromatograph.

These factors have not been explored in detail since direct and individual assessment or their control is difficult. Regardless of the other two mechanisms, considerable influence of adsorption was demonstrated in this paper and at times, the losses in conversion to derivatives must be regarded.

Functional group derivatives were preferred as they aid in the identification of specific compounds by providing better separation. For quantitative use it was preferable to mask hydroxyl groups and to convert them to their more stable derivatives prior to analysis. As was already noted by Lukkainen *et al.*[19], TMS was the most effective of the derivatives studied, even when the use of small sample load was required. The gas chromatographic properties of trimethylsilyl ethers[19] resembled those of hydrocarbons so far as the RPAR curves were concerned; on account of high RPAR value and a wide range of sample size falling relatively in the plateau region, they were usually eminently satisfactory derivatives for quantitative work. An additional advantage of this derivative was the improved peak shape (T_f value) resulting from reduced tailing even with a low efficiency column. Certain successful results by means of trimethylsilylation and the limitation of this procedure will be demonstrated in the next paper.

Now, the question of practical importance remains to be considered. It may be difficult to attain precise quantitative work by gas chromatography, because, as these data show, there was no completely satisfactory method for correction, nor were absolute standards available. Nevertheless these results suggest that with proper technique, the errors due to losses can be eliminated as much as possible for most steroids. Any analysis can not be accurate unless the following procedures are regarded.

Instrumental design and chromatographic conditions must be so chosen that relatively constant and flat RPAR curves are obtained with the components to be determined. As is the case with some reports[17)20)] estimation should not be extended beyond certain a range of sample size. Steroids with free hydroxyl groups should be trimethylsilylated. After presaturation analytical samples should be injected as much as possible at regular intervals throughout the series of determination and the calibration of RPAR repeated by running a standard mixture at least with three different amounts. If precise determination is required, the RPAR curve must be determined with each component. Finally, although it may be possible to reduce the effect of losses by estimation under the above condition, this generally will not be the best suited for precise determination, only these empirical procedures may ensure better application

in routine analysis.

In many biochemical and medical studies, the steroids under investigation are polyfunctional, moreover it is often necessary to work with samples of a few micrograms or smaller. Under these circumstances it is desirable to avoid difficulties arising from elementary problems in design or use of materials. With poor instrumentation, as was proven in the present investigation, these shortcomings become too exaggerated to estimate highly oxygenated compounds. It is obvious, with improved column or instrument, that the RPAR value for highly oxygenated compounds become higher. Under these situations application and limitation of the method can be appreciated by means of the RPAR curve. Although the variability of the RPAR curve makes it difficult to use certain internal standards routinely to validate day to day results, the RPAR value which is relatively reproducible during consecutive studies seems to suitable to evaluate accuracy of data. This does not make perfect comparison possible between laboratories, but, on a relative basis, RPAR is much more satisfactory for comparing different systems or checking analytical conditions routinely than the theoretical plate height.

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