macological Meeting . . . Effect of Drugs on Synthesis and Mobilization of Lipids," E. C. Horning, and P. Lindgren, eds., Pergamon Press, New York, 1963, pp. 77–84.
25. Avigan, J., and D. Steinberg, Lancet *1*, 572 (1962).
26. Herndon, J. H., Jr., and M. D. Siperstein, Circulation Res. *12*, 228–234 (1963).
27. Jose, A. D., and H. J. Peak, Brit. Heart J. *25*, 133–136 (1963).
28. Holmes, W. L., and J. D. Bentz, J. Biol. Chem. *235*, 3118–3122 (1960).
29. Ranney, R. E., and R. E. Counsell, Proc. Soc. Exp. Biol. Med. *109*, 820–824 (1962).
30. Counsell, R. E., P. D. Klimstra, R. E. Ranney and D. L. Cook, J. Med. Pharm. Chem. *5*, 720–729 (1962).
31. Sachs, B. A., and L. Wolfman, Metabolism *12*, 608–617 (1963).
32. Dvornik, D., and M. Kraml, Proc. Soc. Exp. Biol. Med. *112*, 1012–1014 (1963).
33. Holmes, W. L., and N. DiTullo, unpublished observation.
34. Gordon, S., E. W. Cantrall, W. P. Cekleniak, H. J. Albers, R. Littell and S. Bernstein, Biochem. Biophys. Research Communs. *6*, 359–363 (1961).
35. Phillips, W. A., and J. Avigan, Proc. Soc. Exp. Biol. Med. *112*, 233–236 (1963).
36. Cantrall, E. W., R. Littell, S. M. Stolar, W. P Cekleniak, H. J. Albers, S. Gordon and S. Bernstein, Steroids *1*, 173–178 (1963).
37. Thorp, J. M., and W. S. Waring, Nature *194*, 948–949 (1962).
38. Thorp, J. M., Lancet *1*, 1323–1326 (1962).
39. Oliver, M. F. *Ibid.* 1321–1323 (1962).
40. Anonymous, *Ibid.* 1360–1361 (1963).
41. Howard, R. P., P. Alaupovic and R. H. Furman, Circulation *28*, 661–662 (1963).
42. Best, M. M., and C. H. Duncan, Circulation *28*, 690–691 (1963).
43. Hellman, L., B. Zumoff, G. Kessler, E. Karo, I. L. Rubin and R. S. Rosenfeld. Ann. Int. Med. *59*, 477–494 (1963).
44. Merrill, J. M., Circulation Research *6*, 482–484, 1958.
45. Hardy, R. W. F., J. L. Gaylor and C. A. Baumann, J. Nutri. *71*, 159–167 (1960).
46. Duncan, C. H., and M. M. Best, J. Lipid Res. *1*, 159–163 (1960).
47. Kritchevsky, D., and S. A. Tepper, J. Nutri. *82*, 157–161 (1964).
48. Perry, W. F., Metabolism *9*,686–689 (1960).
49. Gamble, W., and L. D. Wright, Proc. Soc. Exp Biol. Med. *107*, 160–162 (1961).
50. Schade, H., and P. Saltman, *Ibid. 102*, 265–267 (1959).
51. Parsons, W. B., Jr., Circulation *24*, 1099 (1961).
52. Nunn, S. L., W. N. Tauxe and J. L. Juergens, *Ibid. 24*, 1099 (1961).
53. Goldsmith, G. A., Fed. Proc. *21* (Suppl. No. 2), 81–85 (1962).
54. Kritchevsky, D., Metabolism *9*, 984–994 (1960).
55. Thompson, J. C., and H. M. Vars, Am. J Phys. *179*, 405–409 (1954).
56. Ericksson, S., Proc. Soc. Exp. Biol. Med. *94*, 578–584 (1957).
57. Strand, O., *Ibid. 109*, 668–672 (1962).
58. Lin, T. H., R. Rubinstein and W. L. Holmes, J. Lipid Res. *4*, 63–67 (1963).
59. Strand, O., *Ibid. 4*, 305–11 (1963).

[Received April 15, 1964—Accepted July 8, 1964]

Gas Chromatography in Lipid Investigations

E. C. HORNING and W. J. A. VANDENHEUVEL, Lipid Research Center, Department of Biochemistry, Baylor University College of Medicine, Houston, Texas

Abstract

Biological problems involving long chain compounds and steroids can now be studied more effectively than ever before through use of GLC techniques. When combined with TLC methods for the separation of classes of compounds, these procedures are the most valuable analytical methods now known for lipid investigations. The fact that both qualitative and quantitative data may be obtained at the same time, and that the methods may be used to study complex mixtures at the microgram and sub-microgram level, suggests that many new applications will be found in the fields of chemistry, biology and medicine.

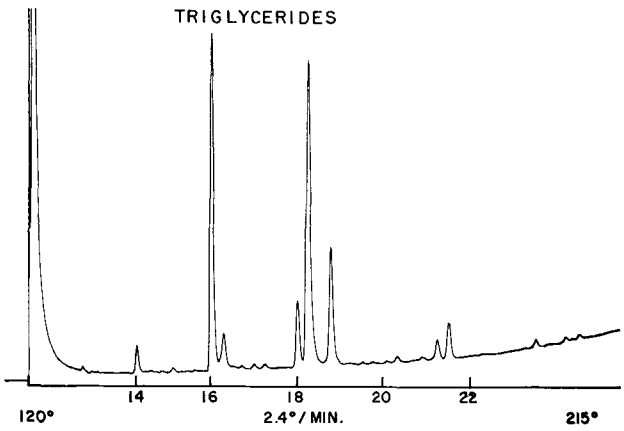

FIG. 1. GLC analysis of fatty acid methyl esters derived from triglycerides present in human arterial lesion tissue. The conditions were as follows: 12 ft x 4 mm glass coil; 1% EGSS-X (Applied Science Laboratories, Inc.) on 100–120 mesh acid-washed and silanized Gas-Chrom P; hydrogen flame ionization detector; temperature programmed separation starting at 120C. The position of saturated unbranched long chain esters is indicated on the chart. The compounds near C_{22} are C:20:3 and C:20:4 methyl esters.

The procedures described in this paper are chiefly those developed over the last few years in the laboratories of the authors. Thin-film columns prepared with diatomaceous earth supports have been used since 1960 in many studies. A new procedure, utilizing a thin-film column, was developed for the separation and estimation of long chain fatty acid methyl esters. The method has been used in conjuction with TLC separations using silica gel and silica gel-silver nitrate plates. The relationship between retention time behavior and the structure of steroids has been studied, and the "steroid number" concept has been used to described GLC properties of steroids. Procedures have been found for the inactivation of supports, for the modification of liquid phase properties, for the preparation of a variety of special derivatives useful in GLC work and for the study of several groups of steroids important in human metabolism. A number of quantitative analytical separations were also developed.

GAS-LIQUID CHROMATOGRAPHY (GLC) is the most valuable analytical technique now known for the study of long chain compounds and of steroids. When combined with TLC methods suitable for the separation of classes of compounds, these techniques provide a highly effective means of studying many biochemical and biological problems involving lipids and steroids in plants, animals and humans. In animals and in humans the effects of drugs, stress, dietary changes and many other experimentally devised circumstances, now can be studied with greater effectiveness than ever before.

At the present time, most laboratories using GLC procedures for the study of long chain compounds employ one or more variations of the polyester column separation methods developed in 1958–1959 (1–4). These methods are suitable for the study of tri-

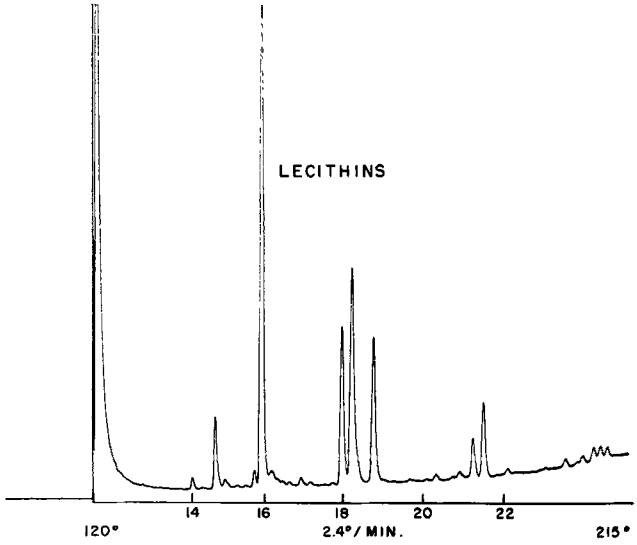

FIG. 2. GLC analysis of fatty acid methyl esters derived from lecithins present in human arterial lesion tissue. The conditions for the separation are the same as those for Figure 1. The compound eluted just before C:15 is unidentified.

glyceride fatty acids in many applications, and have also been used in work with long chain alcohols, acetals and ketones. They are not fully informative or satisfactory in several applications which are of interest at the present time. Positional and steric isomers of the common unsaturated fatty acids are not usually separated, although their presence may often be noticed through a change in peak profile. This is not a significant problem in many studies of triglycerides of plant origin, but the separation of isomers is of considerable interest in work with animals and with humans. The usual methods are also somewhat less than satisfactory when it is necessary to scan a fatty acid mixture, as the methyl esters, from ca. C_{10} to ca. C_{24}. For example, a separation of human sphingomyelin fatty acid methyl esters should be carried to nervonic acid (C-24:15-*cis*), and it is often desirable to start a separation under conditions where esters of C_8 or C_{10} acids would be seen if they are present.

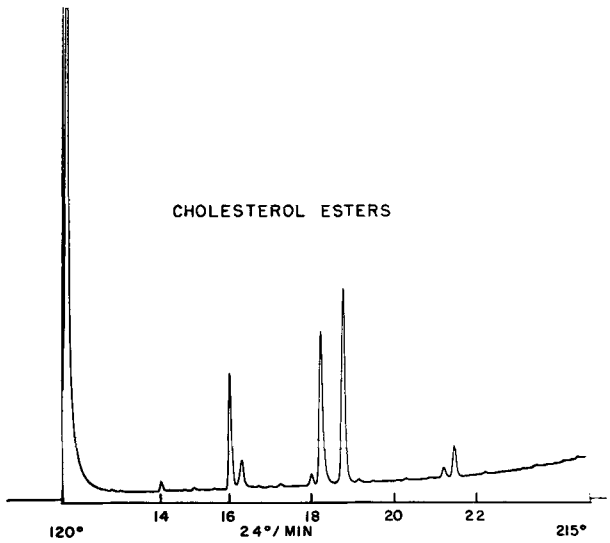

FIG. 3. GLC analysis of fatty acid methyl esters derived from cholesterol esters present in human arterial lesion tissue. The conditions were the same as for Figure 1. The identification of each methyl ester is evident from the relative position of the peaks.

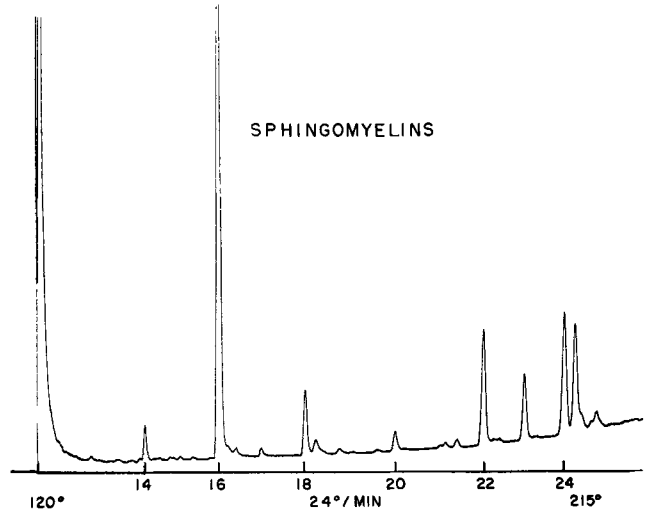

FIG. 4. GLC analysis of fatty acid methyl esters derived from sphingomyelins present in human arterial lesion tissue. The conditions were the same as for Figure 1. The major components of the mixture found in the region C_{22} to C_{24} are the methyl esters of C:22, C:23, C:24 and C:24:1 fatty acids.

The problem of scanning over a relatively large range of molecular size for methyl esters may be dealt with through use of a temperature programmed separation with 1% EGSS-X (a copolymer of ethylene glycol, succinic acid and a dimethyl siloxane monomer) liquid phase using a 12-ft packed column. This method has been in use in our laboratory for over a year in a variety of applications and the results have been entirely satisfactory. All of the commonly encountered unsaturated fatty acids may be separated in a single run. Figures 1–3 show analytical separations carried out by this procedure for the fatty acids of triglycerides, lecithins and cholesterol esters found in human arterial lesion tissue. Monoene and diene fatty acid methyl esters are separated without difficulty from the corresponding saturated esters and from each other. The separation of a triene and tetraene in the C_{20} series is shown in Figure 2. Figure 4 shows the separation of fatty acids from sphingomyelins found in human arterial lesion tissue. The methyl esters of nervonic and lignoceric acids are separated with about the same degree of resolution as found for C_{16} and C_{18} monoene fatty acid methyl esters. There is a slight rise in the base line during the separation (dual columns were not used); no correction for increased bleed at higher temp was made. Figure 5 shows a separation of the dimethyl acetals and fatty acid methyl esters derived from human serum cephalins. The C_{16} and C_{18} dimethyl acetals are evident in the normal pattern, and they are present in reduced amt but still evident in traces in the pattern shown in the upper chart.

These figures were prepared for purposes of illustration by using a very slow chart speed. Consequently, the peaks are extremely narrow, and while this is helpful for illustrations, it is not a satisfactory condition for calculation of composition. When this is required, a faster chart speed is employed, and the peaks are of about the same size as those usually measured in isothermal work. A flame ionization detection procedure was used. Table I shows typical results obtained in two routine runs with mixtures of reference compounds. Results obtained with an argon ionization detection system in this application are not satisfactory; low values for the late components are usu-

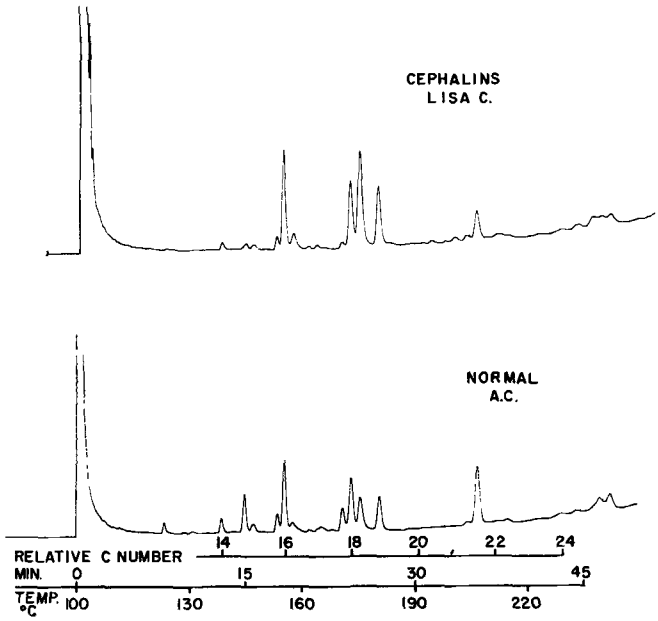

FIG. 5. GLC separation of fatty acid methyl esters and dimethyl acetals derived from human serum cephalins for two subjects. The conditions are the same as those for Figure 1. The dimethyl acetals from palmitaldehyde and stearaldehyde are eluted under these conditions just before the corresponding saturated unbranched fatty acid methyl esters.

B-TG-Me ESTERS

ally observed. A preliminary account of this method has been published (5).

Several years ago lipid classes were usually separated by column chromatography with silicic acid. These methods have now been replaced in many laboratories by TLC separations using solvent systems related to those formerly employed in column separations. In our laboratory and in others it has been found that neutral lipids and phospholipids may be eluted without loss from the silica gel employed for thin-layer separations. Since current information about thin-layer techniques will be reviewed by H. K. Mangold, it would be inappropriate to discuss these separations in detail. Nevertheless, it should be pointed out that the accuracy of the analytical separations shown in Figures 1–5 depends, for biochemical purposes, on a satisfactory extraction procedure and a satisfactory lipid class separation method as well as upon good gas chromatography. In this connection, it should be mentioned again that positional and stereoisomers of the common unsaturated fatty acids may occur in some cases in triglycerides and in other lipid classes. The presence of stereoisomers may be demonstrated through the use of TLC with silver nitrate-silica gel according to de Vries (6); this method may also be modified to permit two-dimensional chromatography. When this is done, a sample of fatty acid methyl esters from normal human serum triglycerides shows the presence of compounds containing *trans*-isomers (Figs. 6,7); the same effect may be seen in a one dimensional silver nitrate-silica gel separation of human serum triglycerides and the derived fatty acid methyl esters (7). Stereoisomeric fatty acid methyl esters are included along with the normal compounds in the separation illustrated in Figure 1. It is therefore clear that the present packed column methods for analysis of fatty acid methyl esters by GLC techniques are not satisfactory in some applications, although they are entirely suitable when isomeric fatty acids are not present. Capillary column methods may

TABLE I

Analyses of Reference Mixtures of Fatty Acid Methyl Esters with 1% EGSS-X Phase[a]

Compound	Weight %	Anal.,[b] found
Mixture 1		
C:16	15.1	15.6
C:18	20.0	20.6
C:20	20.4	20.3
C:22	20.6	20.4
C:24	23.9	23.2
Mixture 2		
C:16	24.9	25.7
C:18	24.9	24.3
C:18:1	25.2	25.1
C:18:2	25.0	24.9

[a] Conditions: 12 ft x 4 mm glass coil; 1% EGSS-SX on 100–120 mesh acid-washed and silanized Gas-Chrom P; hydrogen flame ionization detector; nitrogen carrier gas (27 psi).

[b] Calculated from areas measured as height x width at half-height; the precision of the determination was ca. 1%.

be used to separate isomers, but it would also be desirable to have a packed column method suitable for this purpose; the problem of achieving a packed column analytical separation is currently under investigation in our laboratory.

The separation of long chain aldehydes as the dimethyl acetals presents no special problem, as far as is known at the present time. Recent studies by Farquhar (8) and Gray (9) include much current information about long chain aldehydes and plasmalogens; the presence of plasmalogens in human serum cephalins is indicated by the appearance of the characteristic peaks due to C_{16} and C_{18} aldehyde dimethyl acetals in a fatty acid methyl ester mixture prepared in the usual way (Fig. 5). Hydroxy acids require modified procedures, and these were reviewed recently by Downing (10); we have not worked with these compounds.

Separations of steroids by GLC techniques are now carried out in many laboratories. The objective is usually to obtain an analytical separation, on a quantitative basis and generally with a very small sample, but GLC methods may also be used for identification purposes and for preparative separations. A discussion of current methods, and a brief description of suitable procedures for preparing and using thin-film columns may be found in a recent review (11).

It is not possible to summarize at this time all of the new developments that have occurred for GLC work with steroids, and for this reason the following discussion is limited to a summary of advances in identification techniques and in quantitative methods. Several examples of separations of current interest are also described.

The separation of steroids by gas chromatography is a partition chromatographic process, and the basic theories of partition chromatography should therefore be applicable. The special problem of steroid separations by paper partition chromatography was studied in detail by Bush (12), and the Bush monograph contains the best extant discussion of the basic theories of partition chromatography and of structure-behavior relationships for steroids. The principles were developed over a period of several years by a number of scientists, including Martin (13), Bate-Smith and Westall (14) and Bush (12). The "steroid number" concept of VandenHeuvel and Horning (15) is based on earlier work in paper partition chromatography, and on the "carbon number" concept of Woodford and Van Gent (16), and it is designed for steroid studies in gas chromatography. According to this concept a steroid number may be described by the equation:

$$SN = S + F_1 + \ldots + F_n$$

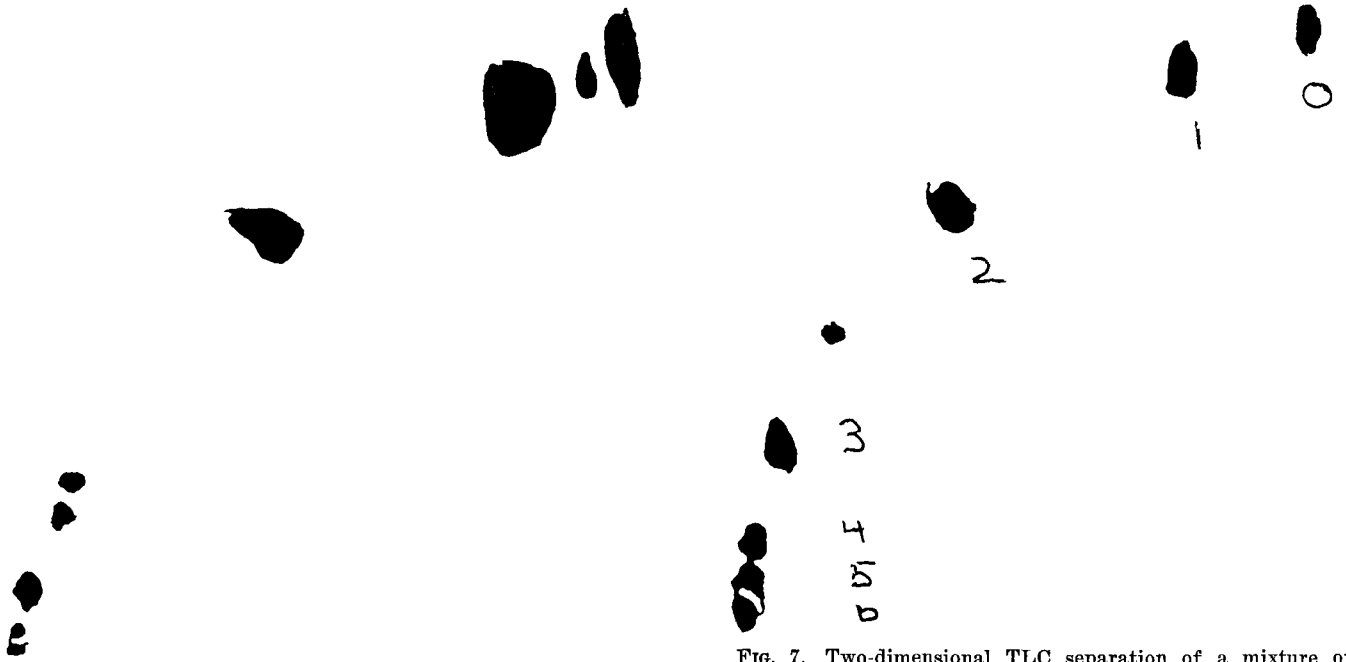

FIG. 6. Two-dimensional TLC separation of fatty acid methyl esters derived from human serum triglycerides. The silica gel-silver nitrate method of deVries (6) was used; the solvent systems were (vertical) ethyl acetate-benzene (88:12) and (horizontal) benzene. The presence of one or more *trans*-monene compounds is clearly evident in the upper right hand section of the plate.

FIG. 7. Two-dimensional TLC separation of a mixture of reference standards of fatty acid methyl esters (from C:16, C:18:1, C:18:2, C:18:3, C:20:4, C:20:5, C:22:6). The conditions of the separation were the same as for Figure 6. The numbers refer to the number of double bonds for the naturally occurring *cis* methylene-interrupted unsaturated fatty acids. The presence of a triene isomer in the mixture is evident in the region between the esters of C:18:2 and C:18:3 fatty acids.

where S is the number of carbon atoms in the steroid skeleton and $F_1 \ldots F_n$ are values characteristic of the functional groups which are present. It is evident that this relationship assumes the absence of intramolecular interaction of functional groups, and consideration should be given to intramolecular effects if an observed steroid number is greatly different from the calculated value.

This relationship will be useful and valid to the extent that F_n values are constant from one steroid to the next, and to the extent that F_n values are reproducible in interlaboratory comparisons. It is a well known fact that relative retention times are rarely reproducible on an interlaboratory basis, and it is also true that some laboratories have had difficulty in achieving satisfactory technology for steroid separations. These factors have led to the relatively widespread belief that GLC techniques are not yet at a stage where retention time data are useful in structural studies. This view is without foundation, provided that sound experimental technology is employed.

Steroid numbers are determined experimentally in the ways shown in Figures 8,9. A non-selective phase (SE-30) was employed to obtain the data in Figure 8. Androstane (SN 19.0) and cholestane (SN 27.0) were used as reference compounds in this instance, and the SN of another steroid (cholesterol, SN 29.3, is used as the example) may be determined from the reference line relating relative retention times to steroid number. It was found that SN values determined in this way are essentially independent of small changes in flow rate, amount of liquid phase, and temperature of the determination.

Relative retention times are sharply temp dependent, and it is this fact that is responsible for much of the difficulty in reproducing relative retention times in interlaboratory comparisons. Much of the com-

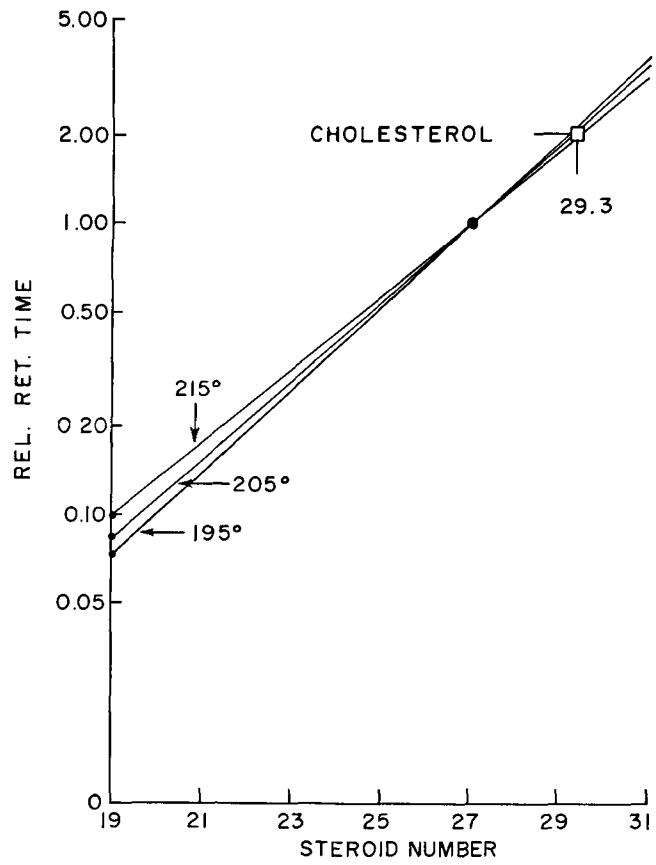

FIG. 8. Steroid number chart showing the relationship between temp and the slope of the androstane-cholestane line used to determine steroid numbers from relative retention times (1% SE-30). The mode of determination of the steroid number (29.3) of cholesterol (relative retention time, 2.06 at 205C, is shown.

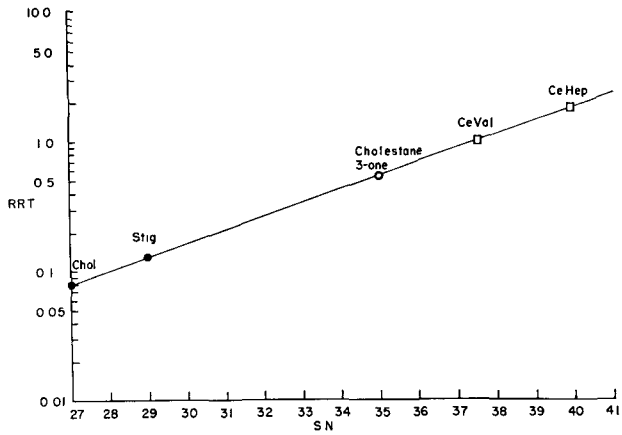

FIG. 9. Steroid number chart used to determine steroid numbers from relative retention times with a selective phase (1% NGS at 222C). The retention times of cholestane (Chol) and stigmastane (Stig) (whose SN values are 27 and 29, respectively) relative to cholesteryl valerate (CeVal) are used to determine the straight line. Using this line and the relative retention times of CeVal and cholesteryl heptanoate (CeHep) SN values are assigned to these compounds. These SN values are then used as reference points for the determination of SN values of other steroids. Thus the SN of cholestane-3-one, 35.0, can be obtained from its relative (to CeVal) retention time, 0.50, and the reference line.

monly available equipment for gas chromatographic work is constructed with relatively poor temp controls for the column compartment, and the temp readings are also frequently in error through faulty placing of thermocouples. Properly prepared thin-film columns containing different amounts of liquid phase (from 1–2%, for example) do not show different relative retention times or SN values unless the temp of the separation is changed at the same time. A variation in temp, for whatever reason, leads directly to altered relative retention times.

The effect of a temp change on the slope of the reference line for determining steroid numbers is shown in Figure 8. A temp effect is also shown in tabular form in Table II for relative retention times for a few representative steroids. Table II also shows that very little change in steroid number is found for small changes in the temp of the determination. These data are from the work of VandenHeuvel and Horning (15).

The same kind of experimental procedure may be used to determine SN values with selective phases. The experimental techniques are slightly different, since it is usually no longer possible to use the same reference compounds (androstane and often cholestane are eluted in the solvent front when polar columns

TABLE II

Determination of Steroid Number at Three Different Temp with a Non-Selective Phase (SE-30)

	RRT	Steroid number
Temperature: 195C		
Androstane	0.07	
Androstane-17-one	0.15	21.2
5α-Pregnane-3,20-dione	0.61	25.5
Cholestane	1.00	
Cholestanyl methyl ether	1.99	29.1
Temperature: 205C		
Androstane	0.08	
Androstane-17-one	0.17	21.2
5α-Pregnane-3,20-dione	0.63	25.5
Cholestane	1.00	
Cholestanyl methyl ether	1.90	29.1
Temperature: 215C		
Androstane	0.10	
Androstane-17-one	0.19	21.3
5α-Pregnane-3,20-dione	0.66	25.6
Cholestane	1.00	
Cholestanyl methyl ether	1.83	29.2

TABLE III

F. Values for Commonly Occurring Functional Groups Determined with SE-30, NGS and QF-1 Phases

Functional group	F value		
	SE-30[a]	NGS[a]	QF-1[b]
3α(ax)-ol	2.3	7.2	4.7
3β(ax)-ol	2.4	7.2	4.6
3α(eq.)-ol	2.4	7.5	5.1
3β(eq.)-ol	2.4	7.7	5.2
11β(ax.)-ol(4-ene)	1.7	5.9	2.5
17α-ol(sec.)	2.4	7.0	4.2
17β-ol(sec.)	2.4	7.3	4.3
20α-ol	2.2	6.9	4.4
20β-ol	2.0	6.3	4.0
3-one	2.6	8.0	8.1
3-one-Δ⁴	3.3	9.9	10.0
11-one(4-ene)	0 5	3.2	2.0
17-one	2.3	6.9	6 7
20-one	2.0	6.3	5.9
A/B *cis*	−0.3	−0 3	−0 3

[a] Determined with a 1% phase on acid-washed and silanized Gas Chrom S.
[b] Determined with a 2% phase on acid-washed and silanized Gas Chrom S.

are used for steroid separations). We have used cholesteryl valerate and cholesteryl heptanoate as secondary standards, and Figure 9 illustrates the determination of the SN value for cholestane-3-one with a selective phase (NGS). In order to use approx the same temp range in comparative studies, we used a 2% QF-1 column and 1% NGS column for this work. The effects of small changes in flow rate, amt of liquid phase and temp on SN values were investigated. It was found that the rate of change of SN values with temp was dependent on the type of functional group found in the steroid. No variation with temp, over a 20-degree range, was observed for a trimethylsilyl ether, although a variation of 0.3 was noted for a trifluoracetate when an NGS column was employed. Other types of compounds showed only 0.1–0.2 variation in SN value under these conditions. Very little variation in SN values were observed as a result of changes in flow rate and amt of liquid phase. From this recent investigation (17) and from earlier work it may be concluded that SN values, in contrast to relative retention times, are relatively independent of temp over the range usually employed for steroid separations, and that SN values obtained with either selective or non-selective phases should be useful both in intra- and interlaboratory comparisons. In a trial of experimental technique, a steroid number value for cholesterol was determined in Milan, Italy by Pompeo Capella of the Stazione Sperimentale per le Industrie degli Olii e dei Grassi, with a Carlo Erba gas chromatograph (flame ionization detection system) and an SE-30 column packing prepared in Milan. The observed value was 29.3; this was the same value determined in Houston, Texas, with different equipment and a different column packing.

Table 3 contains F values for a number of commonly occurring functional groups. Table 4 shows the way in which SN calculations are made for a steroid of relatively simple structure, pregnane-3α,20α-diol, and the experimentally determined values are given for

TABLE IV

Comparison of Calculated and Experimental SN Values for Pregnane-3α,-20α-diol for SE-30, NGS and QF-1 Phases

	Steroid number		
	SE-30	NGS	QF-1
C21 steroid, A/B *trans*	21.0	21.0	21.0
3α(eq.)-ol	2.4	7.5	5.1
20α-ol	2.2	6.9	4.4
A/B *cis*	−0.3	−0.3	−0.3
Calc.	25.3	35.1	30.2
Found	25.3	35.1	30.2

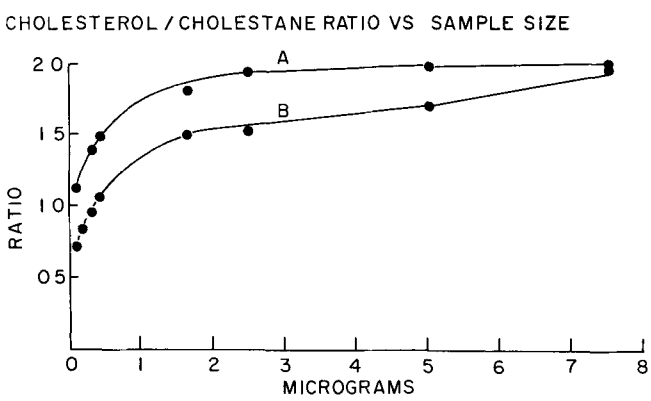

FIG. 10. Variation of response ratio (ratio of peak areas) of cholesterol-cholestane mixture with sample size (micrograms of cholesterol) with three differently prepared column packings. Liquid phase, 1% F-60. A 6 ft glass U-tube was used with an argon ionization detection system. The support was Gas-Chrom P, 100–120 mesh.

A. Support acid-washed and silanized with dichlorodimethylsilane in toluene.
B. Support acid-washed and oven-dried (80C) before coating.

comparison with the calculated values. Similar calculations may be made for other steroids and for steroid derivatives. Many specialized derivatives can be used to good effect in both qualitative and quantitative work, and some of these are mentioned later. It is not suggested that GLC behavior be accepted as a final or definitive proof of structure, but rather that this approach provides a way for obtaining much significant structural information for compounds available in microgram or sub-microgram quantity. This information is frequently sufficient for identification purposes if an authentic sample is at hand, since highly exact comparisons for steroids and their derivatives may be made by GLC techniques.

Much earlier work has of necessity been omitted from this summary of structure-retention time relationships. Several attempts have been made to establish a universal approach for all volatile substances, and in other instances relationships have been established for relatively small numbers of closely related compounds. The early work of Kováts (18) is an example of a study in which generality was sought. For steroids, the studies of Clayton (19), Knights and Thomas (20) and Brooks and Hanaineh (21) are important in showing that the gas chromatographic separation of steroids is a partition chromatographic process. These approaches are based upon consideration of relative retention times, and the proposed relationships have the same inherent disadvantages in interlaboratory comparisons as relative retention time data.

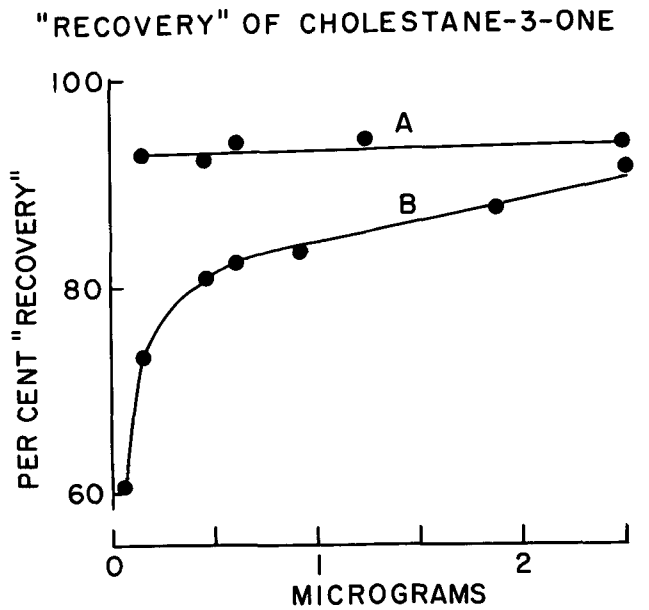

FIG. 11. Variation of recovery of cholestane-3-one with sample size and with two differently prepared column packings. The columns were the same as in Figure 10.

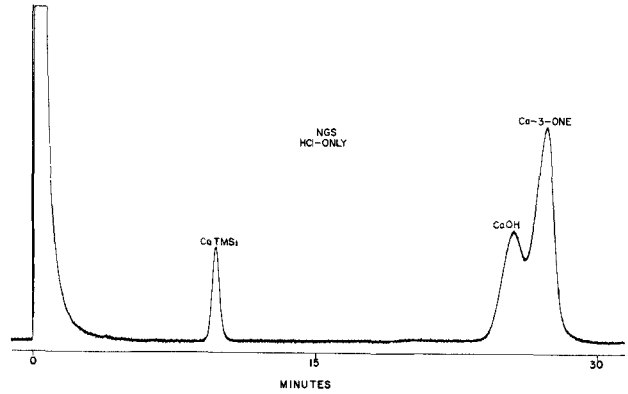

FIG. 12. Gas chromatographic separation of a mixture of cholestanol trimethylsilyl ether (CaTMSi), cholestanol (CaOH), and cholestane-3-one (Ca-3-one) with 1% NGS on acid-washed 80–100 mesh Gas Chrom P. Column conditions: 6 ft x 4 mm glass U-tube; 216C; 60 ml/min argon.

The advantages of GLC methods in many biochemical and biological applications are obvious; the sample size is small, the separation and measurement operations are combined, and many components of the mixture may be estimated in a single run with high precision and accuracy. The latter part of this statement is sometimes challenged, and the issues that are involved frequently arise from inadequate or unsatisfactory technology rather than from inherent limitations of GLC work. The following discussion is directed to a better understanding of the factors that are involved in establishing good quantification. Satisfactory column technology is the most important of these factors.

One of the ways of studying column properties is to compare the extent to which compounds with different functional groups are lost during the separation process. A very simple way of doing this is to use a mixture of components of known composition, one of which is a hydrocarbon, and to measure peak area relationships as the sample size is reduced. It is usually assumed that none of the hydrocarbon is

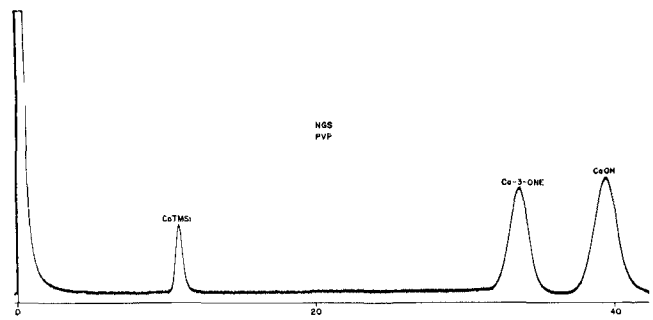

FIG. 13. Gas chromatographic separation of the same mixture of steroids as in Figure 12, but with 1% NGS on PVP-treated (2%) acid-washed 100–120 mesh Gas Chrom P. Column conditions: 6 ft x 4 mm glass U-tube; 216C; 60 ml/min argon.

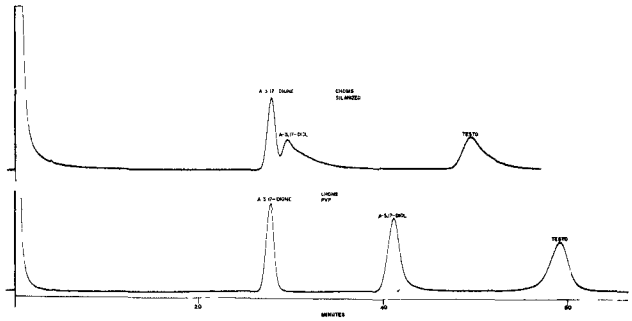

FIG. 14. Gas chromatographic separation of a mixture of steroids (androstane-3,17-dione (A-3,17-DIONE); androstane-3β, 17β-diol (A-3,17-DIOL); testosterone (TESTO) on two columns differing only in the method of deactivation of the acid-washed support used to prepare the packings. The support used to give the upper chromatogram was silanized with a 5% solution of dimethyldichlorosilane in toluene prior to coating with the stationary phase CHDMS; the support used to give the lower chromatogram was treated with a 2% solution of PVP in methanol prior to coating with CHDMS. Notice the greatly improved peak shape (marked reduction of "tailing") for the hydroxy-containing compounds on the PVP-treated packing, and also the significantly increased separation of the diol from the dione, due to a selective increase in the retention time of the former. Column conditions: 6 ft x 4 mm glass columns; 1% CHDMS on 100–120 Gas Chrom P with and without PVP-treatment; 60 ml/min; 216C.

lost in a GLC system, but in fact the measurement is only one of comparative loss. However, for practical purposes, amt of hydrocarbons may be measured down to about 0.01 μg with ordinary ionization measurement techniques, and alterations in quantitative relationships are often seen above this level. Figure 10 shows the alteration in ratio observed for a cholesterol/cholestane mixture as the sample size is decreased. It is clear that the quantitative relationships are greatly altered with sample sizes below ca. 2 μg cholesterol; the chromatography column used in this experiment was glass and the packing was prepared with an inactivated support (11), but selective loss of the sterol is clearly evident. Ketones do not show this effect. Figure 11 contains information obtained with the same column used for Figure 10. No selective loss of the ketone occurred with sample sizes down to ca. 0.1 μg. In additional experiments no selective loss was found for a sterol trimethylsilyl ether down to ca. 0.02 μg (5). These observations indicate obvious hazards when quantitative work is attempted with free sterols, but for ketones and trimethylsilyl ethers it is evident that good column behavior can be expected when satisfactory technology is employed.

In an attempt to obtain superior column packings for both qualitative and quantitative work, an investigation of the "two-coat" technique was carried out. A diatomaceous earth support was acid-washed and dried, and coated with 1 or 2% of polyvinylpyrrolidone (PVP) by the usual slurry technique. In separate experiments it was determined that this organic film had no useful properties for gas chromatography when used alone, presumably because it was not a liquid under these conditions. A coat of a polar polyester phase was then applied in a different solvent, again by the slurry technique, and packings prepared in this way were found to be different in at least two respects from those prepared without the primary PVP coat. Figures 12 and 13 show a change in selective properties that occurs when a PVP coating technique is used. For Figure 12, an ordinary column packing containing 1% NGS on an

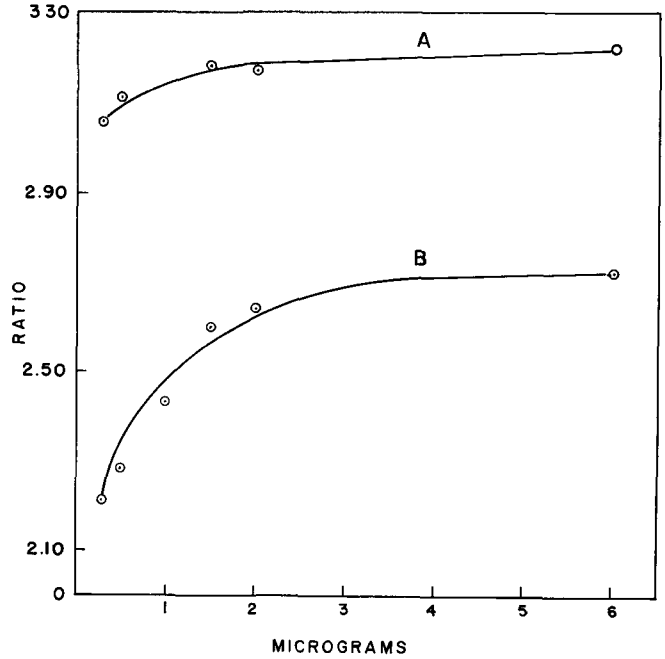

FIG. 15. Variation of response ratio (ratio of peak areas) of cholestanol-tetratriacontane mixture with sample size (μg of cholestanol) with two differently prepared column packings (A, PVP-treated; B, silanized). Column conditions: Both columns (6 ft x 4 mm glass U-tubes) were operated in same column oven with same detector (argon ionization) and conditions (216C, 100 ml/min). The support (100–120 mesh Gas Chrom P) was acid-washed. One portion was then silanized and coated according to (11); the other portion was coated with 1% PVP before application of the polyester. Elution times for cholestane were approx the same.

acid-washed diatomaceous earth support was used, and under these circumstances alcohols and ketones of corresponding structure were found to have relatively low separation factors. A 1% NGS phase on 2% PVP-coated diatomaceous earth was used to obtain the separation illustrated in Figure 13. The alcohol and ketone are now well separated, with a marked change in the direction of increased selective retention of the alcohol (in fact, the order of elution is reversed in this instance). Figure 14 shows another kind of effect as well. A silanized column packing containing 1% cyclohexanedimethanol succinate (CHDMS) was used to obtain the separation in the upper chart. The marked trailing exhibited by the diol peak indicates relatively severe adsorption effects for this compound; some trailing is also seen for testosterone. When a 1% CHDMS-2% PVP column

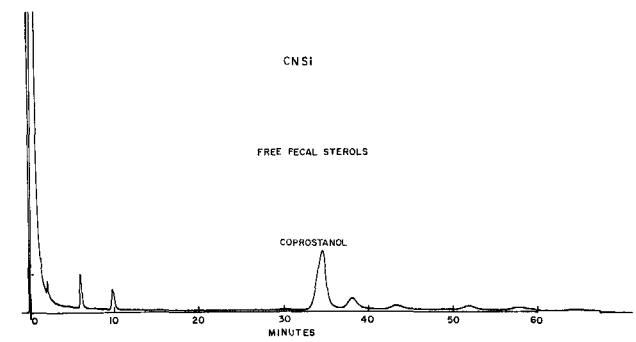

FIG. 16. Gas chromatographic separation of a sample of human fecal sterols. Column conditions: 9 ft x 4 mm glass column; 2% 65 mole-% cyanoethyl methyl silicone (CNSi) on 100–140 mesh Gas Chrom P; 205C; 22 psi. We are indebted to S. Hashim for this sample.

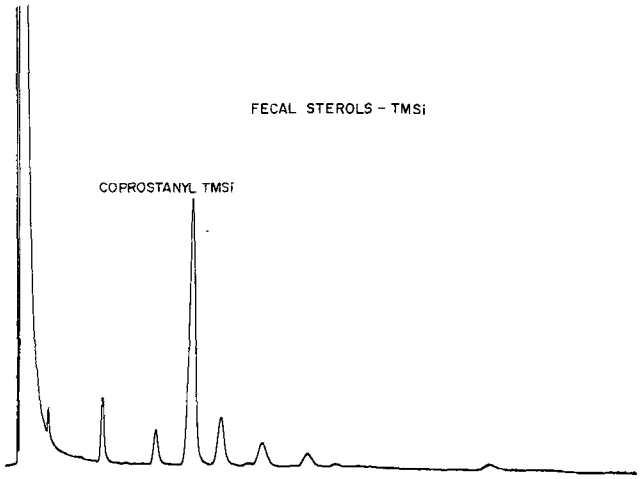

FIG. 17. Gas chromatographic separation of the same sample used for Figure 16 after conversion to the trimethylsilyl ether derivatives. Column conditions: same as in Figure 16.

was used, the trailing of the diol and of testosterone is almost entirely eliminated. This suggests that an experiment similar to that described in Figure 10 should show less selective loss for a sterol when a PVP packing is employed, and Figure 15 contains the results of a study of this kind. It is evident that Curve A, for a 1% NGS-1% PVP column, shows far less selective adsorption of cholestanol than Curve B, which was obtained with an ordinary 1% NGS column. Still another effect that is not widely recognized is clearly shown in this figure. Most current discussions of the relationship between ion yield (or peak areas) and structure for ionization detectors convey the impression that the observed differences are due to detector properties alone. This is of course true if the column is omitted, but in GLC work carried out for analytical purposes a column is part of the system. Curves A and B were obtained with the same instrument and detection system, and with samples

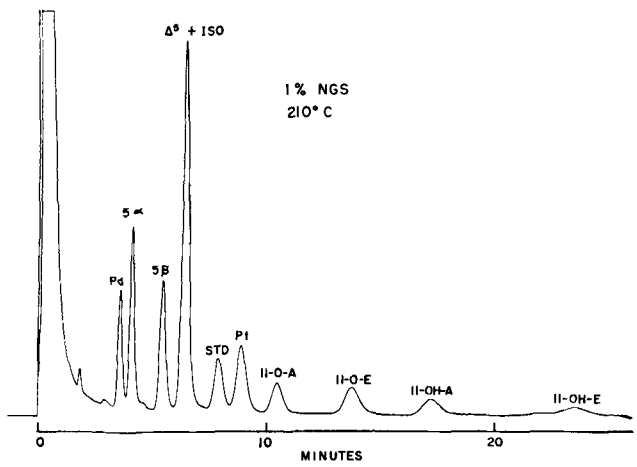

FIG. 18. Gas chromatographic separation of a mixture of steroids. The compounds are pregnanediol ditrimethylsilyl ether (Pd); androsterone trimethylsilyl ether (5α); etiocholanolone trimethylsilyl ether (5β); dehydroisoandrosterone trimethylsilyl ether (Δ^5); isoandrosterone trimethylsilyl ether (ISO); epicoprostanol trimethylsilyl ether (STD); pregnanetriol 3,20-ditrimethylsilyl ether (Pt); 11-ketoandrosterone trimethylsilyl ether (11-O-A); 11-ketoetiocholanolone trimethylsilyl ether (11-O-E); 11-hydroxyandrosterone 3-trimethylsilyl ether (11-OH-A); 11-hydroxyetiocholanolone 3-trimethylsilyl ether (11-OH-E). Column conditions: 6 ft x 4 mm glass column; 1% NGS on 80–100 mesh Gas Chrom P; 210C; 18 psi.

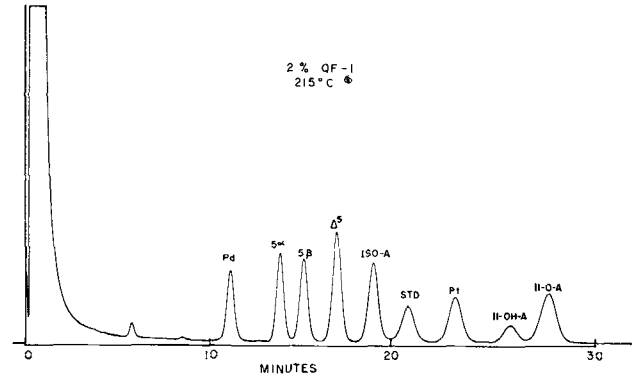

FIG. 19. Gas chromatographic separation of the same mixture of steroids as in Figure 18. Column conditions: 12 ft x 4 mm glass column; 2% QF-1 on 80–100 mesh Gas Chrom P; 215C; 22 psi.

of identical composition, yet the ion yield ratio for cholestanol-tetratriacontane is quite different in the two instances. Although there is no immediate explanation for this effect, it may be suggested tentatively that the nature and extent of column bleed modified the response seen with an argon ionization system.

The "two-coat" technique and the use of PVP-treated column packings were described recently by VandenHeuvel, Gardiner and Horning (22). The PVP method is not useful for non-selective phases (SE-30, F-60 and other non-selective siloxane polymers), and a silanizing treatment is still the best way for preparing non-selective column packings with the usual diatomaceous earth supports. However, in our experience PVP-treated thin-film column packings with polyester phases are superior to ordinary or silanized column packings prepared with the same liquid phase.

Steroid fractions isolated from plant or animal tissues, with or without modification or change from normal through applied experimental conditions or other circumstances, may be relatively simple or highly complex in composition. The sterol fraction of human blood serum, for example, under normal conditions contains cholesterol in a high state of purity. The composition of this fraction may be altered during administration of certain agents which block the biosynthesis of cholesterol, and additional sterols may be seen under this condition. A human fecal sterol fraction is somewhat more complex in nature, and a human urinary steroid fraction, obtained after hydrolysis of the steroid conjugates, contains many compounds only a few of which have been identified with certainty. In order to secure a satisfactory separation for purposes of identification or estimation of individual compounds, it is necessary in each case to choose an appropriate column and detection system, an appropriate derivative (or, the mixture may be run directly) and an appropriate procedure for quantification. Several illustrations may serve to indicate the nature of the problems which are encountered in typical applications.

Figure 16 shows a separation of human fecal sterols, without derivative formation, with a CNSi column. It is evident that a number of compounds are present in addition to coprostanol. This phase is useful in identification studies because of its ability to separate stereoisomers, and a separation of free sterols in this way is highly desirable for identification purposes. Figure 17 shows a separation of the same mixture

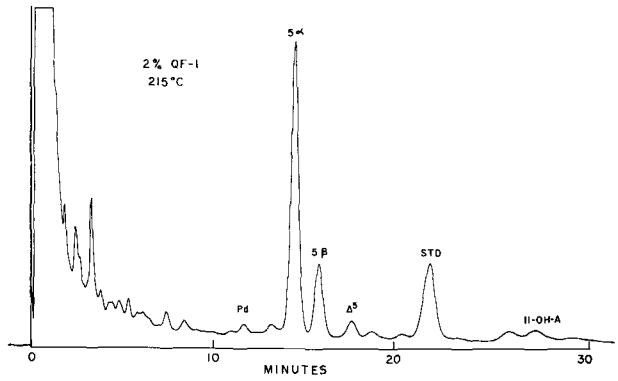

FIG. 20. Gas chromatographic separation of a urinary 17-ketosteroid fraction (as trimethylsilyl ethers) during oral administration of dehydroisoandrosterone (normal male subject). Column conditions the same as for Figure 19.

after trimethylsilyl ether formation; excellent resolution of these derivatives may be obtained, and good quantification may also be expected (23).

The separation of steroid intermediates in the pathway of cholesterol biosynthesis will be discussed by W. L. Holmes; these sterols may be separated as the free compounds or as derivatives.

A steroid fraction isolated from human urine after hydrolysis of the steroid conjugates contains many compounds including substances other than steroids. The principal 17-ketosteroids are androsterone, etiocholanolone and dehydroisoandrosterone. Isoandrosterone is sometimes present in relatively small amt. Four 11-substituted steroids are usually seen: 11-hydroxy- and 11-ketoandrosterone, and 11-hydroxy- and 11-ketoetiocholanolone. Testosterone is normally present in small amt, and pregnanediol is usually seen in varying amt, depending upon the origin of the

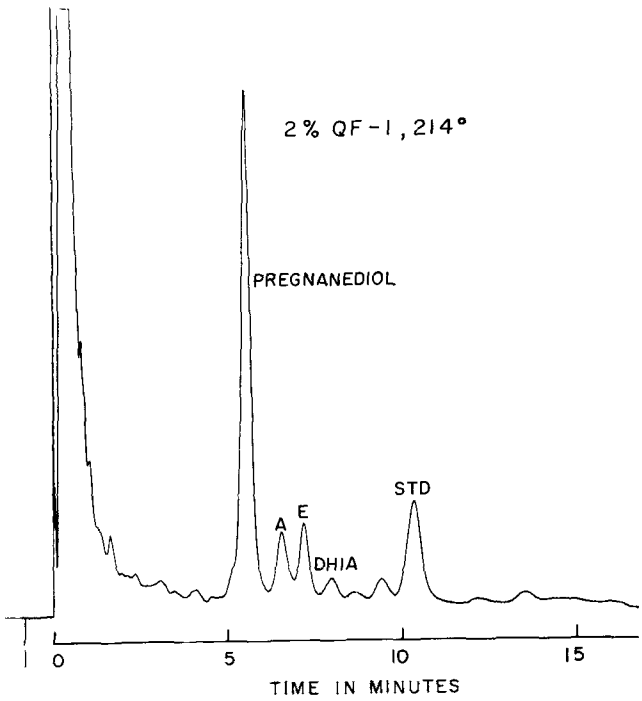

FIG. 21. Gas chromatographic separation of a urinary steroid fraction (as trimethylsilyl ethers) from a 24-hr sample of human urine obtained during early pregnancy. The compounds are the trimethylsilyl ether derivatives of pregnanediol; androsterone (A); etiocholanolone (E); dehydroisoandrosterone (DHIA) and epicoprostanol (STD). Column conditions: 6 ft x 4 mm glass column; 2% QF-1 on 80–100 mesh Gas Chrom P; 214C; 20 psi.

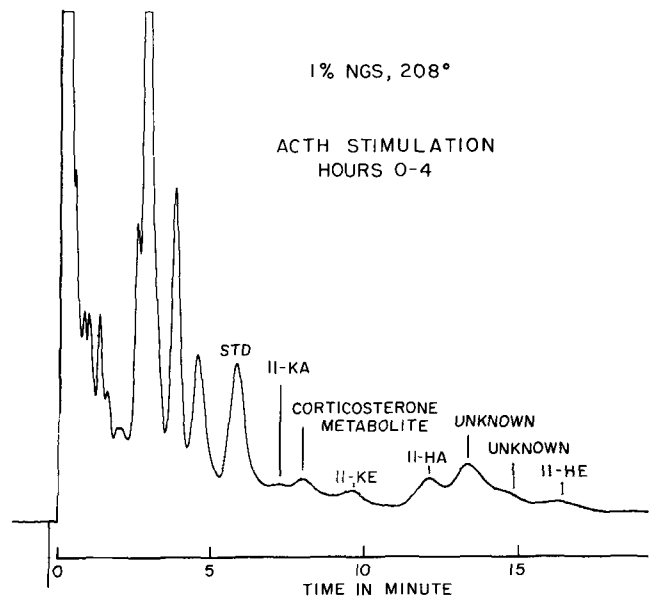

FIG. 22. GLC analysis of human urinary steroids as the *trimethylsilyl ethers*. The sample was obtained by the Vestergaard hydrolysis method applied to urine obtained during the first four hr of depot ACTH stimulation. Column conditions: same as for Figure 18, but at 208C and 28 psi.

sample. While it is perhaps theoretically possible to achieve a separation of all of the known and unidentified compounds with a single GLC run, a different approach is followed in practice. A separation condition, together with an appropriate hydrolysis method, is chosen for the estimation of a specific compound or a group of compounds. For example, Figure 18 shows the separation of a group of 17-ketosteroids and pregnanediol, as the trimethylsilyl ether derivatives, with an NGS column. The separation is an excellent one for the four 11-substituted 17-ketosteroids shown in the illustration, and it is also satisfactory for the estimation of androsterone and etiocholanolone, and

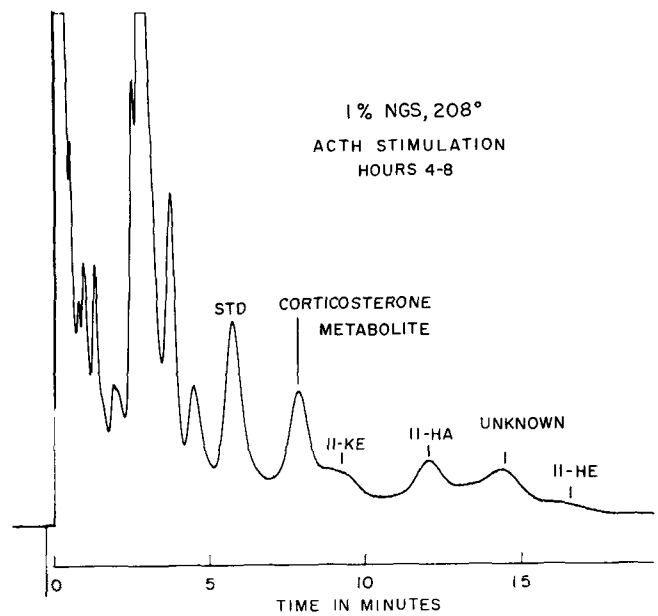

FIG. 23. GLC analysis of human urinary steroids as the trimethylsilyl ethers. The sample was from the second four-hr period of ACTH stimulation, and the conditions were those of Figure 22. The "corticosterone metabolite" is shown again in Figure 24; this compound was altered during the hydrolysis procedure.

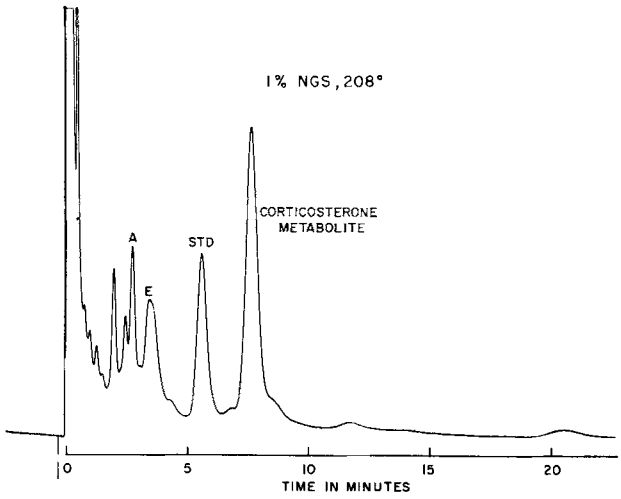

FIG. 24. GLC analysis of human urinary steroids as the trimethylsilyl ethers. The sample was obtained after oral administration of corticosterone, and the conditions were those of Figure 22. The "metabolite" was altered during the hydrolysis procedure.

for dehydroisoandrosterone except when isoandrosterone is present. We are currently interested in the estimation of dehydroisoandrosterone in urine and in blood, and this procedure is therefore not entirely satisfactory for our work, although it is useful for other purposes. When a QF-1 column is used, dehydroisoandrosterone is separated without interference from isoandrosterone, and the other principal compounds are also separated (Fig. 19). This separation is not satisfactory, however, for the usual 11-substituted 17-ketosteroids. While both methods provide satisfactory quantitative results for specific compounds, they are not interchangeable and one or the other may be used for specific purposes. An illustration of this is given in Figure 20; this is a record of analysis for 24-hr human urinary steroid excretion products found during administration of dehydroisoandrosterone. A QF-1 column was used, and the steroids were separated as the trimethylsilyl ethers (24). Epicoprostanol was used as the internal standard (25).

Figure 21 shows a similar analysis for a 24-hr sample of human urine obtained during early pregnancy. Pregnanediol is present as a major component of the steroid mixture and the GLC procedure illustrated here may be used for its estimation.

Studies of adrenal steroid secretion in the human during ACTH administration may be carried out with aid of GLC procedures. Figure 22 shows an analytical record for a urinary steroid fraction, prepared by the Vestergaard hydrolysis method and showing the 17-ketosteroids obtained during the first four hr after ACTH administration to a normal human. This analysis was carried out with a NGS column in order to study the specfic compound noted as "corticosterone metabolite"; a similar analysis was carried out with a QF-1 column (not illustrated) for estimation of the principal 17-ketosteroids. Figure 23 shows the "corticosterone metabolite" present in appreciable amt during the 4–8 hr period of the ACTH action. Figure 24 shows an analysis of the corresponding fraction of human urine obtained after oral administration of corticosterone. It should be pointed out that while this method may be used as an indication of enhanced corticosterone secretion during a specific period of adrenal stimulation, it should not be regarded as a direct measurement of a metabolite of known structure. The structure of this substance is unknown at the present time, and since the Vestergaard procedure is known to produce altered compounds in some instances it is likely that this material is a chemically altered metabolite.

Human urinary testosterone may be estimated by a GLC procedure according to R. Dorfman et al. (26), and following the work of Luukkainen, VandenHeuvel and Horning (27) and Wotiz and Martin (28) a number of estrogen estimation methods have appeared. No agreement has yet been reached with respect to GLC procedures for the estimation of adrenal cortical hormones and their metabolites.

Many steroids used for therapeutic purposes, and their metabolites, may also be identified and estimated by GLC techniques. These methods are not, of course, limited to naturally occurring steroids.

These examples of current work in the development and use of GLC techniques in the lipid and steroid field should not be taken as a final account of methods for work with long chain compounds and with steroids. This summary is a report of work in progess and it is likely that many other new or modified procedures will be developed during the next few years. The potential usefulness of GLC methods for lipid and steroid work in the fields of chemistry, biology and medicine is now clearly established, and much new knowledge may be expected to result from the use of these analytical techniques.

ACKNOWLEDGMENT

This work was aided by grant HE-05435 of the National Institutes of Health

REFERENCES

1. Orr, C. H., and J. E. Callen, J. Am. Chem. Soc. *80*, 249 (1958); Orr, C. H., and J. E. Callen, Ann. N.Y. Acad. Sci. *72*, 649 (1959)
2. Lipsky, S. R., and R. A. Landowne, Biochim. Biophys. Acta *27*, 666 (1958); Lipsky, S. R., and Landowne, R. A., Ann. N.Y. Acad. Sci. *72*, 666 (1959).
3. Craig, B. M., and N. L. Murty, JAOCS *36*, 549 (1959).
4. James, A. T., "Methods of Biochemical Analysis," Vol. VIII, ed. D. Glick, Interscience Publishers, New York, N.Y., 1960, p. 1.
5. Horning, E. C., K. C. Maddock, K. V. Anthony and W. J. A. VandenHeuvel, Anal. Chem. *35*, 526 (1963).
6. de Vries, B., Chem. Ind. (London), 1049 (1962).
7. Horning, E. C., K. V. Anthony, K. C. Maddock, E. R. Rabin, H. E. Garrett and M. E. DeBakey, "Fundamentals of Vascular Grafting," ed. S. A. Wesolowski and C. Dennis, McGraw-Hill Book Co., New York, N.Y., 1963, p. 63.
8. Farquhar, J. W., J. Lipid Res. *3*, 21 (1962); Biochim. Biophys. Acta *60*, 80 (1962).
9. Gray, G. M., Biochem. J. *77*, 82 (1960), J. Chromatog. *6*, 236 (1961).
10. Downing, D. T., Rev. Pure Appl. Chem. *11*, 196 (1961).
11. Horning, E. C., W. J. A. VandenHeuvel and B. G. Creech, "Methods of Biochemical Analysis," Vol. XI, ed. D. Glick, Interscience Publishers, New York, N.Y., 1963, p. 69.
12. Bush, I. E., "The Chromatography of Steroids," Pergamon Press, Oxford, 1961.
13. Martin, A. J. P., Biochem. Soc. Symposia *3*, 4 (1949); Ann. Rev. Biochem. *19*, 517 (1950).
14 Bate-Smith, E. C., and R. G. Westall, Biochim. Biophys. Acta *4*, 427 (1950).
15 VandenHeuvel, W. J. A., and E. C. Horning, *Ibid.* *64*, 416 (1962).
16. Woodford, F. P., and C. M. Van Gent, J. Lipid Res. *1*, 188 (1960).
17. Hamilton, R. J., W. J. A. VandenHeuvel and E. C. Horning, Biochim. Biophys. Acta *70*, 679 (1963).
18. Kováts, E., Helv. Chim. Acta *41*, 1915 (1958).
19. Clayton, R. B., Biochemistry *1*, 357 (1962); Nature *192*, 523, (1961); *Ibid.* *190*, 1071 (1961).
20. Knights, B. A., and G. H. Thomas, J. Chem. Soc *1963*, 3477; Chem. Ind. (London) *1963*, 43; Anal. Chem. *34*, 1046 (1962).
21. Brooks, C. W. J., and L. Hanaineh, Biochem. J. *87*, 151 (1963); *Ibid.* *84*, 102P (1962).
22. VandenHeuvel, W. J. A., W. L. Gardiner and E. C. Horning, Anal. Chem. *35*, 526 (1963).
23. Wells, W. W., and M. Makita, Anal. Biochem. *4*, 204 (1962).
24. VandenHeuvel, W. J. A., B. G. Creech and E. C. Horning, *Ibid.* *4*, 191 (1962).
25. Creech, B. G., and E. C. Horning, unpublished.
26. Futterweit, W., N. L. McNiven, L. Narcus, C. Lantos, M. Drosdowsky and R. I. Dorfman, Steriods *1*, 628 (1963).
27. Luukkainen, T., W. J. A. VandenHeuvel and E. C. Horning, Biochim. Biophys. Acta *62*, 153 (1962).
28 Wotiz, H. H., and H. F. Martin, Anal. Biochem *3*, 97 (1962); J. Biol. Chem. *236*, 1312 (1961).

[Received April 15, 1964—Accepted July 8, 1964]