CHROM. 3815

REFERENCE HIGH-EFFICIENCY NONPOLAR PACKED COLUMNS FOR THE GAS–LIQUID CHROMATOGRAPHY OF NANOGRAM AMOUNTS OF STEROIDS

PART II. IDENTIFICATION OF STEROIDS*

F. A. VANDENHEUVEL AND A. SALLY COURT**

Canada Department of Agriculture, Animal Research Institute, Research Branch, Ottawa, Ontario (Canada)

(Received August 30th, 1968)

SUMMARY

Several methods for the identification of steroids in complex mixtures of natural origin, based on the use of corrected retention data are described and discussed. A procedure for the correction of load-induced retention time deviation is presented. The methods for identification include: (1) calculation of retention time from given additive structure-specific increments, (2) determination of temperature- and flow rate-independent, structure-specific retention constants, (3) time-lapse gas–liquid chromatographic analysis of reduction products, (4) a dilution test, and (5) peak-shape examination. Retention constants for 100 steroids are given.

INTRODUCTION

In Part I of the present series of articles[1], the packing of 9 ft., 1/8-in. O.D. stainless-steel columns with 3% JXR on 100–120 mesh GasChrom Q was described. Use of a given conditioning schedule resulted in the production of highly efficient columns (5500 theoretical plates for cholesterol) which retained constant separating power and retention characteristics for at least three years of continuous use. Because such columns were readily duplicated retention data for 140 steroids at 215, 230, and 240° were given and discussed.

The present article concerns the use of JXR columns in identifying steroids. It has been emphasized[1–3] that the efficiency of gas–liquid chromatography (GLC) in analyzing steroids is considerably enhanced by preliminary separation since mixtures of natural origin are often too complex to be resolved directly by GLC. Thin-layer chromatography (TLC), particularly with the solvent system used in this laboratory[1–9], achieves both a helpful preliminary separation and the removal of extraneous material

* Contribution No. 321 from the Animal Research Institute.

** Present address: Forest Research Laboratory, Department of Forestry and Rural Development, P.O. Box 4000, Fredericton, New Brunswick, Canada.

interfering with GLC. As shown by the diagram in Fig. 1, zones characterized by steroid molecules with specific numbers of hydroxyl and carbonyl groups are sharply separated on TLC plates developed in this solvent system; in addition, subzones characterized by specific stereoisomers can often be located in these zones. It will be shown in a forthcoming publication that by including pilot dyes in the sample, the center of any steroid band can be located within ± 1 mm (ref. 15).

The GLC analysis of steroids quantitatively removed from TLC plate zones or subzones[9] can be made readily with the present JXR columns. The presence of steroids in a specific zone gives significant information on these molecules since all should contain specific numbers of hydroxyl and carbonyl groups; as such, they belong to one of the groups listed in Tables II to IX of Part I[1]: If standard GLC conditions are used[1], their retention times could be found among listed ones. While identification can often be made on this basis, the possible occurrence of steroids not listed in the tables, presumably minor constituents not readily obtainable, invites considerable caution, particularly when the observed retention time differs by more

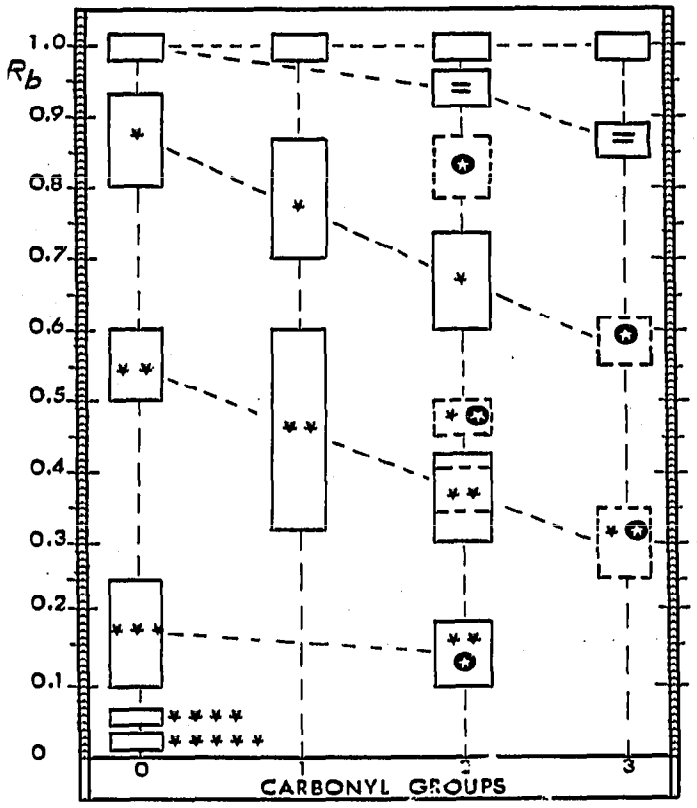

Fig. 1. Diagram showing relative migration distance R_b of steroids on TLC plates in relation to hydroxyl group (0 to 5) and carbonyl group (0, 1, 2, or 3) content. The 20 × 20 cm TLC plates were coated with a 0.25 mm layer of Adsorbosil 4 (Applied Science) and developed in $CHCl_3$–MeOH–H_2O (200:9:0.75, v/v/v). For details on coating, conditioning, loading and developing, *cf.* refs. 3, 6 and 7. R_b = 1 is the migration distance of Sudan Blue included in the samples. Rectangles indicate spread of band centers for components of specific groups of steroids; number of hydroxyl groups is indicated by number of stars, number of carbonyl groups by figure at bottom of relevant column. Dashed contours are used for corticosteroids; white star in black circle indicates 21-hydroxyl group. Two horizontal dashes indicate double bonds. Tetra- and pentahydroxysteroids (4 and 5 black stars at bottom of first column) also include 21-corticosteroids.

than 1% from a listed one. Indeed, Table X, Part I[1], concerned with a number of androstane- and pregnanediols, shows our present retention data to be still limited, and differences in the retention times of some stereoisomers to be small. Similar observations apply to log t_R' values of sterols listed in Table XII, Part I[1].

Understandably, the process of identification on the basis of retention time will gain in efficiency and reliability by the use of highly discriminating columns and care in determining retention times: replicate values for an unknown steroid, and for the standards obligatorily included in the sample mixture, must be obtained under well defined conditions. Only then can the number of possible configurations corresponding to this unknown compound be reduced to a minimum. In fact, unequivocal identification often requires the complementary use of independent methods, and in this respect, mass spectrometry offers by far the highest discriminating power when used in conjunction with GLC[10,11].

METHODS

Several methods of using precise retention data for the purpose of steroid identification are described below. Data given in Part I[1] are used to demonstrate these methods, and frequent reference is made to compounds designated by group and number as listed in Tables II to XII, Part I[1]. To simplify text and tables of the present paper, a shorthand designation essentially based on the UPAC nomenclature used in Part I will be employed. Thus, for example, 11α,17α-dihydroxypregn-4-ene-3,20-dione (compound F22, Table VII, Part I) is abbreviated to 11α,17α÷Δ^4-P÷3,20; 3β-hydroxy-4,4',14α-trimethyl-5α-cholesta-8,24-diene (lanosterol, C18, Table IV, Part I) is abbreviated to 3β÷4,4',14αMe-$\Delta^{8,24}$-5α-C. In this shorthand designation, features concerning the carbon skeleton are found behind the first ÷ sign; hydroxyl groups are shown ahead, and carbonyl groups, if any, are shown after a ÷ sign following skeleton features; the latter may include methyl (Me) and ethyl (Et) groups, and double bonds; double bond locations are preceded by Δ.

With a single exception, the bases of methods to be described were derived by mathematical manipulation of data given in Part I[1]. The procedure described immediately below derives from new experimental data.

Corrections to retention time

It is not generally known that retention times of steroids, like those of other compounds[12], vary appreciably with quantity injected. This was readily demonstrated by injecting 2 μl of a series of dilutions made from solutions containing either single steroids, or a mixture of steroids, which had been converted to TMS derivatives[1]. Injections were made into the chromatograph under standard conditions[1]. The solutions contained at least one standard to which all observed retention times were normalized[1]. As Table I shows, each group of steroids corresponded to a range of injected amount for which a constant minimum retention time (*CMt*) was obtained. Retention times increased when the quantity injected was either larger or smaller than the limits for *CMt*. For all hydrocarbons, and for all mono- and polyhydroxylated steroids the range of *CMt* was wide, approximately from 1.5 μg down to 50 ng of steroid. As seen from Table I, *CMt* ranges gradually decreased and shifted to higher levels as the number of carbonyl groups in the molecule increased. A double bond,

TABLE I

LOAD-INDUCED PER CENT DEVIATION FROM MINIMUM RETENTION TIME FOR VARIOUS STEROIDS[a] OF THE ESTRANE, ANDROSTANE, PREGNANE, AND CHOLESTANE SERIES ON JXR COLUMNS AT 230°C[b]

A = Androstane; C = cholestane; E = estrane; P = pregnane.

Group	*No.*	*Steroids*[c]	*Amount injected (ng)*									
			8 × 10³	*4 × 10³*	*2 × 10³*	*10³*	*500*	*250*	*100*	*50*	*25*	*10*
A	7	5α-C	3.8	1.6	0.6	0	0	0	0.2	0.7	1.2	1.9
	9	24βEt-5α-C	3.9	2.0	0.8	0.2	0	0	0.2	0.7	1.2	2.0
C	2	3β÷5α-A	0.8	0.5	0.3	0.1	0	0	0.2	0.5	0.7	1.1
	7	3β÷Δ^5-C	4.8	2.5	1.1	0.4	0	0	0.2	0.7	1.5	2.7
	9	3β÷$\Delta^{5,24}$-C	3.4	2.0	0.5	0	0	0	0.1	0.6	1.2	1.9
E	9	3,17β÷$\Delta^{1,3,5}$-E	3.6	2.0	0.9	0.2	0	0	0	0.1	0.3	0.6
	12	3α,20α÷5β-P	4.1	2.1	0.9	0.3	0.1	0	0.1	0.4	0.7	1.1
G	1	3,16α,17β÷$\Delta^{1,3,5}$-E	4.5	2.0	0.9	0.2	0	0	0.1	0.4	0.8	1.3
	6	3α,17α, 20α÷5β-P	4.8	2.9	1.2	0.3	0	0.1	0.5	0.9	1.4	2.2
	17	3α,11β,17α, 20β÷5β-P	3.7	1.9	0.8	0.3	0	0	0.3	0.6	0.9	1.4
H	19	3α,11β,17α,20β,21÷5β-P	6.2	3.6	1.4	0.2	0	0	0	0.3	1.0	1.9
D	2	3α÷5α-A÷17	1.4	0.7	0.2	0	0.1	0.3	0.8	1.5	2.4	3.7
	4	3β÷Δ^5-A÷17	1.5	0.7	0.1	0	0.2	0.7	1.8	2.8	4.1	6.3
	6	3÷$\Delta^{1,3,5}$-E÷17	2.4	0.8	0.1	0	0.2	0.9	2.3	3.7	5.2	7.3
	7	17β÷5α-A÷3	1.4	0.5	0.1	0	0.3	1.0	2.3	3.5	4.8	6.7
	9	17β÷Δ^4-A÷3	0.5	0.2	0	0.1	1.5	3.3	5.9	9.1	14.0	
	17	20β÷5α-P÷3	1.5	0.5	0	0.1	0.6	1.4	2.9	4.3	5.8	8.3
B	3	5β-P÷3,20	1.4	0.3	0	0	0.2	0.8	1.9	3.0	4.4	6.7
	7	5β-P÷3,20	1.7	0.4	0	0	1.0	2.6	5.7	8.5	13.4	
	9	5α-P÷3,20	1.7	0.5	0	0.6	2.6	5.5	10.5	16.0		
	11	Δ^4-P÷3,20	2.3	0.2	0.1	1.5	4.0	7.8	15.5			

[a] Hydroxylated steroids as TMS derivatives.
[b] For operational conditions, *cf.* ref. 1.
[c] Shorthand designation, *cf.* text.

when conjugated to a carbonyl group, enhanced this effect, as seen from data on compounds D9 and B11. The curves in Fig. 2 exemplify the type of plot from which corrections corresponding to any injected quantity can be determined.

Excluding B7, B9, D6, D7, and E12, all compounds listed in Table I can be included in a single solution; they form a mixture whose components separate well enough for the purpose at hand. A1, A2, A3, B3, and C16 (*cf.* Tables II to IX, Part I), among others, may be included. Stock solutions of TMS derivatives in CS_2 keep very well under conditions already described[1]. Hence, the determination of retention time

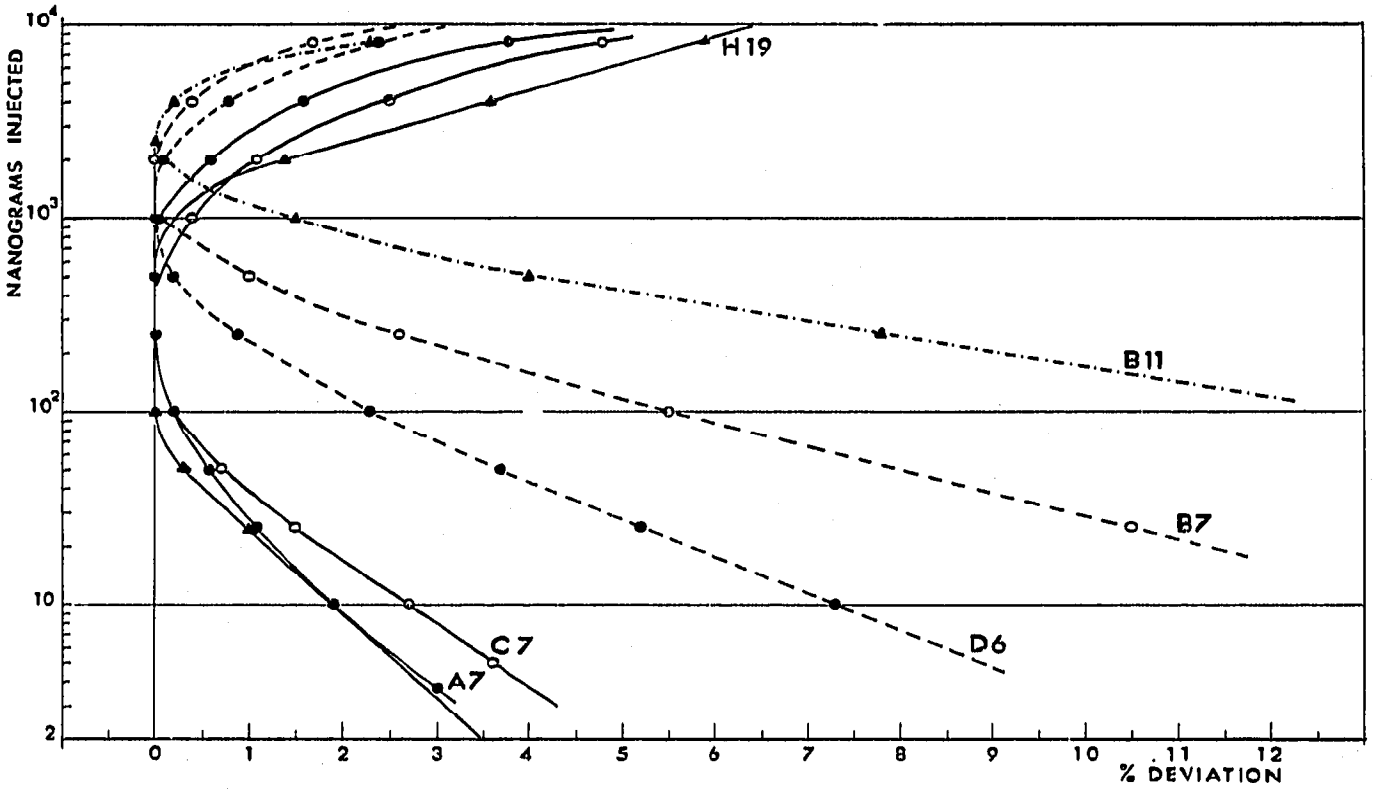

Fig. 2. Per cent deviation from constant minimum retention time as a function of quantity injected for the following steroids: (A7) 5α-cholestane; (C7) cholesterol; (H19) 3α,11β,17α,20β,21-pentahydroxy-5β-pregnane; (D6) estrone; (B7) 5β-pregnane-3,20-dione; (B11) progesterone. *Cf.* Table I.

corrections can be made for many compounds simultaneously and rapidly, by using dilutions corresponding to submicrogram levels normally met in routine analyses[1].

It was observed that corrective factors were valid over long periods, increasing very slowly with column age (use). Hence these factors should be determined every year. Data in Table I correspond to 2-year-old columns. Obviously, the corrections are similar within a given group, particularly when quantities injected do not exceed considerably the limits of *CMt* ranges; consequently, corrections are sufficiently accurate if taken as averages for groups to which steroids belong.

With a preliminary TLC separation, the injected quantity for an unknown compound can be estimated accurately enough for this purpose from peak size, attenuation setting, and average specific response for members of the relevant group;

hence, retention time corrections can be determined from plots such as shown in Fig. 2.

Normalization[1] of corrected retention times to those of standards, or any known compounds included in the mixture is, of course, mandatory.

It will be observed from Table I that in the presence of carbonyl groups the correction value increases very substantially; furthermore, a decrease in quantity injected is much more effective in increasing t'_R as the number of these groups increases. Hence observation of retention times using a 1:5 or 1:10 dilution of the sample brings additional information as to carbonyl group content of unknown compounds. This procedure will often confirm the nature of an unknown compound by indicating a shift in retention time typical of the group. Examination of Fig. 1 shows that occasional TLC zone overlap may occur. In this case, the dilution test will indicate a difference in the number of carbonyl groups for some of the compounds in the mixture. The test is sensitive since retention time shifts are obtained simultaneously for all compounds in the mixture which generally includes several known steroids.

The cause of load-induced retention time shifts is examined in the General discussion.

Retention time increments

Additive increments of log t'_R have been described and discussed to some extent (*cf.* Tables X, XI, and XII, Part I[1]).

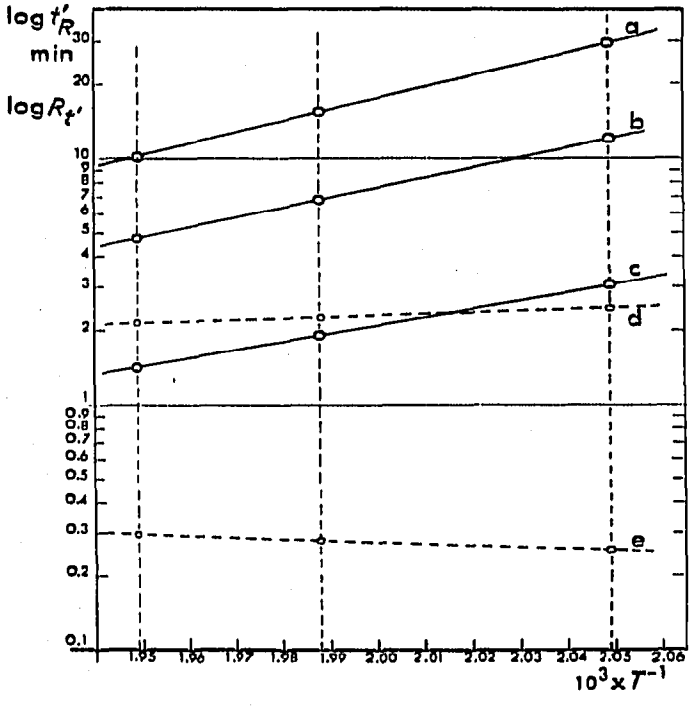

Fig. 3. Plots of log t'_R *vs.* $10^3 \times T^{-1}$ for: (a) cholesterol (TMS), (b) 5α-cholestane, and (c) 3β-hydroxy-5α-androstane (TMS). Plots of log t'_{Rt}/t'_{Rs} = log of relative retention time (s = cholestane) *vs.* $10^3 \times T^{-1}$ for: (d) cholesterol, and (e) 3β-hydroxy-5α-androstane (TMS). Note that the slope of d is positive, that of e, negative. Constants $10^{-3} \times A$ and $10^{-3} \times B$ [*cf.* expression (8)] are, respectively, intercepts and slopes of lines d and e.

The existence of increments permitting a precise prediction of retention times from structural features can be inferred from the general linear relationship

$$10^3 \times \log t'_{Ri} = A_i + 10^3 \times B_i T^{-1} \quad (1)$$

readily demonstrated with $\log t_R'$ data given in Part I[1]. Examples showing the linearity of a plot of $\log t'_{Ri}$ *vs.* $10^3 \times T^{-1}$ are given by plots a, b, and c, Fig. 3.

Let $\Delta 10^3 \times \log t'_{Rf}$ represent an additive increment corresponding to some structural feature, f, of the molecule; then

$$10^3 \times \log t'_{Ri} = \Sigma \Delta 10^3 \times \log t'_{Rf} \quad (2)$$

expresses the additivity condition. If in addition the following relation holds:

$$\Delta 10^3 \times \log t'_{Rf} = \Delta A_f + 10^3 \times \Delta B_f T^{-1} \quad (3)$$

one must have also

$$A_i = \Sigma \Delta A_f \quad (4)$$

$$B_i = \Sigma \Delta B_f \quad (5)$$

From the above expressions one can predict the existence of constants ΔA_f and ΔB_f which, when introduced in expression (3), should enable the estimation of additive incremental factors for any structural feature at any temperature.

Such constants were determined from $\log t'_{Ri}$ data given in Part I[1]. First, incremental factors for 215 and 240° were determined by computation as were values corresponding to 230° given in Tables X and XII of the former paper. Next, linear equations of type (3) written for two temperatures were solved for ΔA_f and ΔB_f. For the purpose of steroid identification, however, incremental factors for 230° given in Tables X and XII, Part I are sufficient, since this temperature can be used conveniently with all steroids[1]. When the corrected retention time (*cf.* above) of an unknown steroid differs by more than 1 to 2% from a retention time listed in the relevant group, it must be compared to calculated retention times using the listed increments. An example of computation of this type is given in Table XI, Part I[1].

It should be emphasized that listed increments are valid only when used with retention data obtained under adjusted standard conditions[1]; though these factors are temperature- and flow rate-dependent, the ease whereby operational conditions required for their determination are established renders their use very practical.

Relative retention time and retention constants

As indicated in Part I[1], equations of type (1) can be written for a given compound, i, and a standard, s, as follows:

$$10^3 \times \log t'_{Ri} = A_i + 10^3 \times B_i T^{-1} \quad (6)$$

and

TABLE II

RETENTION CONSTANTS $\bar{A}$ AND $\bar{B}$ FOR STEROIDS[a] OF THE ANDROSTANE, PREGNANE, CHOLESTANE, AND ESTRANE SERIES ON JXR COLUMNS[b]

$10^3 \times \log$ (retention time relative to cholestane) $= \bar{A} + \bar{B} \times 10^3 \times T^{-1}$.

No.	Steroids	$\bar{A}$	$\bar{B}$
	Group A[c]		
1	5β-A	+1685	−1324
2	5α-A	+1622	−1274
3	5α-P	−2234	+ 964
4	5β-C	− 41	0
5	5α-Δ²-C	− 9	0
6	Δ⁵-C	0	0
7	5α-C	0	0
8	Δ³,⁵-C	− 77	+ 59
9	24β-Et-5α-C	− 309	+ 263
	Group B[c]		
1	5α-A÷17	−1275	+ 964
2	5α-A÷3	−1056	− 836
3	5β-P÷3	+ 739	− 580
4	5β-A÷17	+ 808	− 594
5	Δ⁴-A÷3,17	+ 750	− 500
6	Δ¹,⁴-A÷3,17	+ 692	− 455
7	5β-P÷3,20	+ 490	− 337
8	Δ⁴-A÷3,11,20	+ 459	− 310
9	5α-P÷3,20	+ 600	− 337
10	5β-P÷3,11,20	+ 575	− 328
11	Δ⁴-P÷3,20	+ 623	− 337
12	5α-P÷3,11,20	+ 612	− 319
13	5β-C÷3	− 478	+ 375
14	5α-C÷3	− 403	+ 360
15	Δ³,⁵-C÷7	− 414	+ 386
16	Δ⁴,⁶-C÷3	− 606	+ 520
	Group C[c]		
1	3α÷5α-A	+ 879	− 772
2	3β÷5α-A	+ 764	− 663
3	3α÷5β-P	+ 324	− 369
4	3β÷5α-P	+ 355	− 343
5	3β÷5β-C	− 637	+ 449
6	3α÷5β-C	− 844	+ 561
7	3β÷Δ⁵-C	− 783	+ 571
8	3β÷5α-C	− 753	+ 562
9	3β÷Δ⁵,²⁴-C	− 850	+ 624
10	3β÷Δ⁵,⁷-C	− 828	+ 614
11	3β÷Δ⁷-C	− 903	+ 660
12	3β÷Δ⁸,²⁴-C	− 853	+ 636
13	3β÷5,7,24-C	− 730	+ 585
14	3β÷Δ⁵,⁷,²²-24βMe-C	− 657	+ 555
15	3β÷Δ⁵-24βEt-C	− 940	+ 709
16	3β÷Δ⁵,²²-24αEt-C	−1000	+ 760
17	3β÷Δ⁷-4αMe-C	− 968	+ 763
18	3β÷Δ⁸,²⁴-4,4′,14αMe-C	−1111	+ 845
19	3β÷Δ⁵-24αEt-C	− 355	+ 516
20	3β÷Δ⁷-4,4′Me-C	− 879	+ 695
	Group D[c]		
1	3β÷5β-A÷17	+ 476	− 437
2	3α÷5α-A÷17	+ 421	− 408
3	3α÷5β-A÷17	+ 242	− 310
4	3β÷Δ⁵-A÷17	+ 143	− 225
5	3β÷5α-A÷17	+ 342	− 317
6	3÷Δ¹,³,⁵-E÷17	+ 88	− 177
7	17β÷5α-A÷3	+ 310	− 284
8	17α÷Δ⁴-A÷3	+ 285	− 266
9	17β÷Δ⁴-NorA÷3	+ 8	− 120
10	3β÷5β-P÷20	+ 296	− 248
11	17β÷Δ⁴-A÷3	+ 295	− 234
12	3α÷5β-P÷20	+ 8	− 92
13	17β÷Δ¹,⁴-P÷3	+ 110	− 172
14	3β÷Δ⁵,¹⁰-P÷20	− 140	0
15	3β÷Δ⁵-P÷20	− 101	0
16	3β÷5α-P÷20	− 90	0
17	20β÷5α-P÷3	− 23	0
18	20β÷Δ⁴-P÷3	− 230	+ 150
	Group E[c]		
1	3α,17β÷5α-P	+ 5	− 154
2	3α,17α÷Δ⁵-P	− 5	− 151
3	3α,6α÷5β-P	− 269	0
4	3β,17β÷Δ⁴-A	− 54	− 89
5	3β,16α÷5α-A	− 54	− 89
6	3β,17β÷Δ⁵-A	− 9	− 103
7	3,17α÷Δ¹,³,⁵-E	− 217	0
8	3β,17β÷5α-A	+ 11	− 109
9	3,17β÷Δ¹,³,⁵-E	− 171	0
10	3β,20β÷5β-P	− 229	+ 74
11	3α,20β÷5β-P	− 468	+ 202
12	3α,20α÷5β-P	− 486	+ 229
13	3β,20α÷Δ⁵,¹⁰-P	− 379	+ 163
14	3β,20β÷Δ⁴-P	− 376	+ 190
15	3β,20β÷5α-P	− 287	+ 159
16	3β,20α÷5α-P	− 400	+ 228
	Group F[c]		
1	3α÷5α-A÷11,17	− 385	+ 342
2	3α÷5β-A÷11,17	− 351	+ 322
3	12α÷5β-P÷3,20	− 162	+ 134
4	3α÷5β-P÷11, 20	− 53	0
5	3β÷5α-P÷11,20	− 100	+ 70
6	11α÷Δ⁴-P÷3,20	− 334	+ 237
7	11β÷Δ⁴-P÷3,20	− 778	+ 488
8	3α,6α÷5α-A÷17	− 242	0
9	3α,11β÷5α-A÷17	− 130	+ 162
10	3α,11β÷5β-A÷17	+ 42	+ 70
11	3β,17α÷5β-P÷20	− 163	+ 58
12	3α,17α÷5β-P÷20	− 123	+ 40
13	3α,6α÷5β-P÷20	− 240	+ 100
14	3β,17β÷Δ⁵-P÷16	− 311	+ 139
15	3β,16α÷Δ⁴-P÷20	+ 149	− 102
16	3β,17α÷5α-P÷20		[d]
17	17α,20β÷Δ⁴-P÷3		[d]
18	17α,20α÷Δ⁴-P÷3		[d]
19	3α,5α÷5α-C÷6	+1150	− 940

[a] The TMS derivatives of hydroxylated steroids were used.

[b] For preparation, conditioning of columns and conditions of use, *cf.* ref. 1.

[c] Listing corresponds to that in Tables II–VII of ref. 1, where UPAC nomenclature was used. The present shorthand designation is explained in text.

[d] Incomplete data, *cf.* Table VII, ref. 1.

$$10^3 \times \log t'_{Rs} = A_s + 10^3 \times B_s T^{-1} \quad (7)$$

Subtracting (7) from (6) yields

$$10^3 \times \log t'_{Ri} - 10^3 \times \log t'_{Rs} = A_i - A_s + 10^3 \times B_i T^{-1} - 10^3 \times B_s T^{-1}$$

hence,

$$10^3 \times \log t'_{Ri}/t'_{Rs} = A_i - A_s + 10^3(B_i - B_s)T^{-1} \quad (8)$$

In linear expression (8), t'_{Ri}/t'_{Rs} is the relative retention time of compound i with standard s. Linearity of the $\log t'_{Ri}/t'_{Rs}$ *vs.* $10^3 \times T^{-1}$ plots, which is obtained with all steroids is exemplified in Fig. 3, d, e. In expression (8) constants

$$\bar{A}(i, s) = A_i - A_s \text{ and } \bar{B}(i, s) = B_i - B_s \quad (9)$$

are independent of temperature. These constants are also independent of carrier gas flow rate since relative retention times at a given temperature are constant over a wide range of flow rates.

Specific constants $\bar{A}(i, s)$ and $\bar{B}(i, s)$ exist for any steroid in relation to any selected standard. Chosing cholestane as a convenient primary standard, s, we define as *retention constants* (*RC*), values of $\bar{A}(i, s)$ and $\bar{B}(i, s)$ obtained by solving for $\bar{A}(i, s)$ and $\bar{A}(i, s)$ two equations of type (8), namely

$$10^3 \times \log_{T_1} t'_{Ri}/t'_{Rs} = \bar{A}(i, s) + 10^3 \times \bar{B}(i, s)T_1^{-1} \quad (10)$$

and

$$10^3 \times \log_{T_2} t'_{Ri}/t'_{Rs} = \bar{A}(i, s) + 10^3 \times \bar{B}(i, s)T_2^{-1} \quad (11)$$

Clearly, all that is required to obtain retention constants is to determine the relative retention time of a steroid with cholestane as a standard at two different temperatures. When operating under standard conditions[1] this is achieved simply by changing the temperature setting of the instrument, first to one temperature, then to another, injecting the test solution and observing the retention times of both steroid and cholestane at each temperature. A temperature difference of 20 to 30° is sufficient. It is, however, necessary to know the two temperatures to $\pm$ 0.2° or better, and therefore, use of a calibrated oven thermometer is recommended.

Examples of simple calculations leading to *RC* values are given in Appendix I.

If peak interference prevents inclusion of cholestane in the test mixture, a more convenient compound, s', is used as a standard and relative retention times are calculated in relation to this secondary standard. Retention constants are then obtained simply by adding the resulting $\bar{A}(i, s')$ and $\bar{B}(i, s')$ values to the retention constants of secondary standard, s', in relation to cholestane. This follows from

$$10^3 \times \log t'_{Ri}/t'_{Rs'} = \bar{A}(i, s') + 10^3 \times \bar{B}(i, s')T^{-1} \quad (12)$$

$$10^3 \times \log t'_{Rs'}/t'_{Rs} = \bar{A}(s', s) + 10^3 \times \bar{B}(s', s)T^{-1} \quad (13)$$

Adding (12) and (13) yields

$$10^3 \times \log \frac{t'_{Ri}\, t'_{Rs'}}{t'_{Rs'}\, t'_{Rs}} = A(i, s') + \bar{A}(s', s) + 10^3[\bar{B}(i, s') + B(s', s)]T^{-1}$$

hence,

$$10^3 \times \log t'_{Ri}/t'_{Rs} = \bar{A}(i, s) + 10^3 \times \bar{B}(i, s)T^{-1}$$

with

$$\bar{A}(i, s) = \bar{A}(i, s') + \bar{A}(s', s) \tag{14}$$

and

$$\bar{B}(i, s) = \bar{B}(i, s') + \bar{B}(s', s) \tag{15}$$

Advantages of retention constants are numerous. Their independence from both flow rate and temperature over the practical range of column utilization makes them highly suitable parameters from which unknown steroids can be identified. Their specificity is evident from Table II showing a great sensitivity of $\bar{A}$ and $\bar{B}$ values to variations in structural features which affect both the sign and size of these factors. Furthermore, relative retention times calculated accurately from $\bar{A}$ and $\bar{B}$ values for any temperature are valid within a relative wide range of flow rates.

Since the problem of identification mostly concerns a discrimination among possible configurations corresponding to closely similar retention times, a comparison of the discriminating power of retention constants with that of steroid numbers proposed by VANDENHEUVEL AND HORNING[13] is of interest. It has been shown by one of us[2,3] that the steroid number, SN_i, of a steroid, i, could be expressed by the following relation

$$SN_i = 19 + 8 \log Rt'_i/Rt'_c \tag{16}$$

where Rt'_i and Rt'_c are relative retention times, both to androstane, of steroid, i, and cholestane, respectively.

Understandably, expression (16) permits a more accurate estimation of SN values than the graphical procedure of the proponent authors[13]. Use of this expression with retention data obtained under controlled conditions given in Tables II to IX, Part I[1] for a wide variety of compounds, allowed a precise and comprehensive survey of SN values to be made.

In Table III, last column, the largest difference, ΔSN, between SN values listed in columns 4, 5, and 6 for 215, 230, and 240°, respectively, is small compared to corresponding SN values. One reason for this is the presence in expression (16) of a large constant term, 19, masking to a certain extent fluctuations due to temperature and systematic errors affecting the second, sensitive term. Relative deviations, when expressed for the second term, appear much larger. The average of this term for compound C1, for example, is 2.24 since the average SN is 21.24 (21.24 − 19 = 2.24); hence the relative value of ΔSN = 0.15 to this term is 6.7%. For compound C4, this relative value is 4%; it is 4.3% in the case of E1, etc. The temperature dependency

TABLE III

RETENTION CONSTANTS, $\bar{A}$ AND $\bar{B}$, AND STEROID NUMBERS, SN, FOR STEROIDS OF THE ANDROSTANE, PREGNANE, AND CHOLESTANE SERIES FROM RETENTION TIMES[a] ON JXR COLUMNS

Group[a] *and No.*	*Steroids*[b]	$10^2 \times t'_R$[a] *(min)*	*Retention constants*		*SN data*				ΔSN[c]
		230°	$\bar{A}$	$\bar{B}$	*215°*	*230°*	*240°*	*Average*	
C1	3α÷5α-A	150	+ 879	−772	21.310	21.230	21.180	21.240	0.15
B1	5α-A÷17	155	−1275	+964	21.345	21.340	21.410	21.365	0.07
C4	3β÷5α-P	321	+ 355	−343	24.182	24.300	24.080	24.189	0.22
D4	3β÷Δ5-A÷17	337	+ 143	−225	24.393	24.310	24.290	24.331	0.10
E1	3α,17β÷5α-A	339	+ 5	−154	24.480	24.340	24.250	24.360	0.23
B11	Δ4-P÷3,20	613	+ 627	−337	26.465	26.620	26.680	26.566	0.22
F14	3β,17β÷Δ5÷16	614	− 311	+139	26.780	26.690	26.640	26.700	0.14
B12	5α-P÷3,11, 20	649	+ 612	−319	26.660	26.720	26.920	26.767	0.26
E14	3β,20β÷Δ4-P	684	− 396	+190	27.100	27.040	27.060	27.066	0.04
A7	3α-C	680	0	0	27.000	27.000	27.000	27.000	0.00
C7	3β÷Δ5-C	1536	− 783	+571	30.150	30.110	30.080	30.113	0.07
C8	3β÷5β-C	1574	− 753	+562	30.230	30.220	30.190	30.213	0.04
C13	3β÷Δ5,7,24-C	1841	− 730	+585	30.830	30.810	30.840	30.827	0.03
C14	3β÷Δ5,7,22-24βMe-C	1885	− 657	+555	30.910	30.890	30.960	30.920	0.07
C15	3β÷Δ5-14αEt-C	1998	− 940	+709	31.170	31.120	31.110	31.133	0.06

[a] *cf.* Tables II to VII in ref. 1.
[b] Shorthand designation, *cf.* text.
[c] Largest difference between the *SN* values at 215, 230, and 240°.

of *SN* values has been recognized by the originators of the *SN* concept[14]. A very important point, however, is that the discriminating value of steroid numbers is impaired by the occurrence of ΔSN values of the size shown in the last column. This is evident from a comparison of ΔSN with differences in *SN* values for several steroid pairs. The difference in *SN* value (average) for steroid C1 (t'_R = 1.50 min) and B1 (t'_R = 1.55 min), 0.12, is not significantly different from ΔSN for C1, for example.

Similar observations can be made with any of the following pairs listed in Table III: C4/D4, D4/E1, B11/F14, F14/B12, E14/A7, C7/C8, C13/C14, C14/C15, and with many others not listed. Note that in some cases, F14/B12 and C14/C15 for example, the difference in retention time is so large that the pair is distinctly separated on JXR columns. ΔSN values observed with the graphical method and with the use of retention data obtained under less precisely controlled conditions are even larger; they are by no means smaller with SE-30 or other nonpolar columns. Steroid numbers, therefore, are not very useful criteria in identifying steroids.

In sharp contrast, retention constants for members of steroid pairs listed above show considerable differences; values listed for $\bar{A}$ and $\bar{B}$ in columns 6 and 7, Table III differ, often both in size and sign, well enough to make confusion impossible. These differences express the fact that retention times of steroids do not vary similarly with temperature; retention constants reflect this sensitivity to structural differences while steroid numbers do so but poorly.

Reduction of carbonyl groups

A kinetic study of the reduction by sodium borohydride of carbonyl groups in steroids will be described in a forthcoming paper. Primarily intended to determine conditions under which this reduction can be used as a simple preliminary step in the analysis of fragile corticosteroids[1-3], this study has also provided information of interest to the present problem. Indeed, structural details of an unknown steroid may be revealed by analyzing the reaction mixture by GLC at two or three successive stages of reduction. A lack of carbonyl groups is immediately indicated, of course, by peak stability as to size and position. The presence of a slowly-reduced 11-keto group is indicated by a characteristic delay in final product formation. Since the major terminal product corresponds in most cases to the β form of reduced carbonyl groups, and since the retention time of polyhydroxylated steroids is predictable from log t'_R increments[1], identification of the major reduction product, and by inference, of the original compound, can be attempted. Though suffering from limitations when dealing with complex initial mixtures, the simple reduction step always provides some useful structural information.

GENERAL DISCUSSION

The belief that without preliminary separation, total steroid mixtures of natural origin can be analyzed by differential GLC analysis on columns of different polarity is still widespread. That this is seldom possible is becoming evident from the considerable complexity of many of these mixtures, the limited number of peaks any column will clearly separate, and the confusion that arises from multiple steroid–liquid phase interactions on polar GLC columns. On the other hand, the large proportion of extraneous substances crude natural mixtures contain, and undesirable effects these substances have on the state of cleanliness, stability and retention characteristics of GLC systems, argue most strongly in favor of some preliminary cleanup separation that will not lead to steroid loss or structural alteration. TLC with the solvent system shown in the caption of Fig. 1 is very effective, not only in removing interfering substances, but also in effecting a preliminary separation which is complementary to that achieved by GLC on JXR columns. On these columns separations are induced by differences in molecular size and differences in molecular configuration; the preliminary TLC separation is based mostly on differences in polarity which, in contradistinction with separations on polar GLC columns, are not influenced, and therefore, not confused by molecular size and other factors to which polar GLC columns are variously and unpredictably sensitive.

The presence of an unknown steroid within a specific TLC zone or subzone establishes the number of hydroxyl and carbonyl groups in its molecule; as shown above, this knowledge permits a correction for load-induced increase in retention time to be made, and consequently, makes further process of identification more reliable. The necessity for this correction should be evident from appreciable deviations listed in Table I for low levels of ketonic steroids.

Increases in retention time above and below the *CMt* range can be explained from the general appearance of peaks obtained at these levels. While peaks in the *CMt* range have the generally symmetrical appearance of normal distribution curves (peak R1, P1, F1, Fig. 4), peaks corresponding to levels above the *CMt* range have a

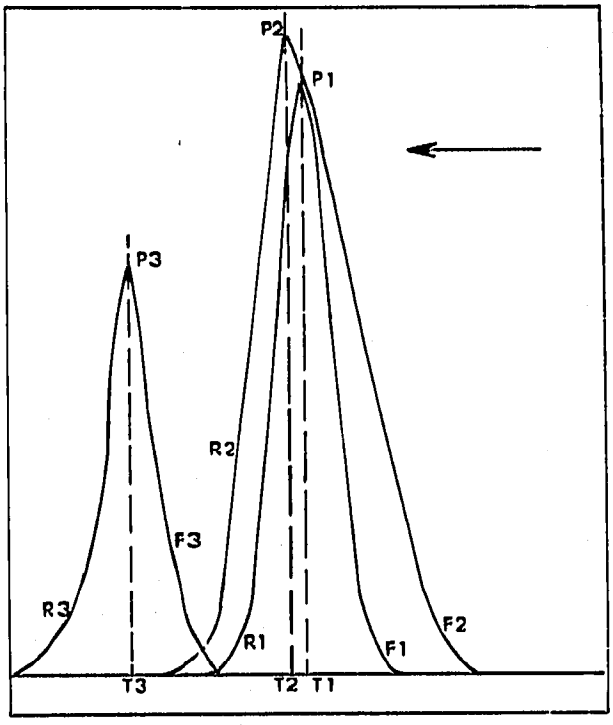

Fig. 4. Diagram illustrating three different peak slopes for the same compound depending on quantity injected. Peaks would have been recorded at widely different attenuation settings and relative size as shown bears no relation to quantity; differences in shape are exaggerated. Peak R1, P1, F1: symmetrical peak in the *CMt* range; R2, P2, F2: front F2 is more slanted above *CMt* range; R3, P3, F3: rear R3 is skewed and trailing below *CMt* range. Arrow indicates direction of increasing retention times, $T_1 > T_2 > T_3$.

skewed appearance, indicative of column overloading, illustrated in peak R2, P2, F2, Fig. 4, where frontal branch F2 is more oblique than rear branch R2. At levels below the *CMt* range, skewness is reversed and some trailing is manifested (peak R3, P3, F3, Fig. 4). This behaviour may be explained as follows. If the distribution curve for adsorption site activity of the packing material were slightly skewed, *i.e.*, if a very small number of sites had appreciably higher adsorptive activity, adsorption on the few most active, retention-increasing sites would not become significant until very low levels of steroid were injected, and a substantial proportion of total molecules would be induced to trail. At *CMt* or higher levels the same absolute number of steroid molecules would be trailing as a result of interaction with the more active sites but a much higher proportion of molecules interacting with normal sites would dwarf the trailing effect. Hence significant trailing and attendant increase of retention time would be apparent below the *CMt* range only.

An increase in retention time from overloading above the *CMt* range is understandable since the viscosity of the gas–vapour mixture is increased with increased vapour load and consequently is maximum at the peak of the travelling steroid band. This induces a slight decrease in velocity of the band maximum density region in relation to frontal velocity, *i.e.*, a displacement of P2, Fig. 3, with attendant increase in retention time. The magnitude of this effect would increase with increasing load.

In addition, a higher capacity of the column packing for carbonyl groups as opposed to trimethylsilyl groups is indicated by the data. Higher load levels are

required for overloading by carbonyl groups; furthermore, the capacity of more active sites for these groups seems larger also since the *CMt* range for carbonyl group-rich compounds is somewhat shorter. As a consequence of this difference, peaks of carbonyl group-rich steroids always show some trailing at low level of injected compound. Hence it is generally possible to detect peaks of ketonic steroids by their slightly asymmetrical appearance.

Examination of Fig. 1 shows that the retention times of steroids, with the exception of polyketonic species, deviate much more rapidly from *CMt* as the result of overloading than they do in the low-level range. Apparently, the maximum operating load for 1/8 in. O.D. columns is about 2 μg per steroid. An increase in column diameter would increase the limiting load in proportion to the square of internal diameter increase; however, deviation from *CMt* in the low-load region would occur much sooner and increase much faster with decreasing load than with 1/8 in. O.D. columns.

Clearly then, small-diameter packed columns should be used in low-load work in preference to columns of larger diameter. Small-diameter columns also limit carrier gas flow rates to levels compatible with a normal behaviour of hydrogen flame detectors ($<$ 100 ml/min) while permitting much higher carrier gas velocity in the column. Therefore, a much faster elution at the same carrier gas flow rate is possible. Duplication of our retention data with 1/4 in. JXR columns would prove very difficult since flow rates of about 200 ml/min would be needed to obtain the required gas velocity in the column. With 60 ml/min, corresponding to standard flow rate conditions with our 1/8 in. JXR columns[1], comparable retention times could not be obtained with 1/4 in. columns unless higher temperatures were used; however, this would lead to appreciable loss in resolution.

It should be clear from the retention data given in Part I[1], in Table X, for instance, that even the present highly efficient JXR columns could not separate completely all possible stereoisomers. In fact, little help could result from the use of any other type of column of comparable resolving power. However, as previously pointed out[1], preliminary TLC separation results in the segregation into different TLC subzones of stereoisomers that otherwise would form difficultly separable pairs[1]; few problems cannot be solved in this manner[15].

The possible complexity of unknown mixtures of biological origin points to the necessity of using highly efficient columns. Routinely used 1000 to 1500 theoretical-plate columns are but poorly discriminating. Several samples of steroids which had been sent to this laboratory in the belief that they represented "chromatographically pure" material proved, upon analysis with our JXR columns to contain two or three extraneous components which in some cases represented as much as 30% of the total. In each case, use of a "low-power" column had resulted in a "single peak" which, except for some skewness and trailing, gave no indication of the presence of impurities in the sample.

As already pointed out[1], the list of log t'_R increments given in Tables X and XII, Part I[1] is still incomplete. This does not argue against their use in the manner suggested since they will lead to many, if not all the possible structures for unknown steroids. Efficiency of this basically simple method should increase as more incremental factors become known.

Methods of discriminating among possible configurations have been discussed.

Undoubtedly, the determination of retention constants, the dilution test, the reduction test, and even an estimation of peak symmetry can, with the present JXR columns, bring considerable help in solving this problem.

APPENDIX: CALCULATION OF RETENTION CONSTANTS

Let R_1 and R_2 be the relative retention times of a steroid using cholestane as a standard at absolute temperature T_1 and T_2, respectively; then, according to expressions (10) and (11):

$$10^3 \times \log R_1 = \bar{A} + 10^3 \times \bar{B} T_1^{-1}$$

$$10^3 \times \log R_2 = \bar{A} + 10^3 \times \bar{B} T_2^{-1}$$

When $10^3 \times \log R_1$, $10^3 \times \log R_2$, $10^3 \times T_1^{-1}$, and $10^3 \times T^{-1}$ are determined, the above expression can be solved for $\bar{A}$ and $\bar{B}$, yielding

$$\bar{A} = \frac{10^3 \times T_2^{-1} \times 10^3 \times \log R_1 - 10^3 \times T_1^{-1} \times 10^3 \times \log R_2}{10^3 \times T_2^{-1} - 10^3 \times T_1^{-1}} \quad (17)$$

$$\bar{B} = \frac{10^3 \times \log R_2 - 10^3 \times \log R_1}{10^3 \times T_2^{-1} - 10^3 \times T_1^{-1}} \quad (18)$$

Note that

$$10^3 \times \log R_1 = 10^3 \times \log t'_{Rt}\,(\text{steroid}) - 10^3 \times \log t'_{Rs}\,(\text{cholestane}) \quad (19)$$

Values of $10^3 \times \log t'_R$ are listed in Tables II to IX of Part I[1].

Values of $10^3 \times N^{-1}$ are listed in *Handbook of Chemistry and Physics*.

Example

Assume that the steroid is the trimethylsilyl derivative of 3β-hydroxy-5α-androstane (*cf.* Table IV, C2, Part I). and that $T_1 = 215 + 273 = 488$ and $T_2 = 240 + 273 = 513$, then $10^3 \times T_1^{-1} = 2.0492$ and $10^3 \times T_2^{-1} = 1.9493$.

Using expression (19) with cholestane $10^3 \times \log t'_{Rs}$ values from Table II (A7), Part I[1],

$$10^3 \times \log R_1 = 2484 - 3079 = -595$$

and similarly

$$10^3 \times \log R_2 = 2149 - 2678 = -529$$

Hence from (17)

$$\bar{A} = \frac{-1.949 \times 595 + 2.049 \times 529}{1.949 - 2.049} = +760$$

and from (18)

$$\bar{B} = \frac{-529 + 595}{1.949 - 2.049} = -660$$

The values listed for $3\beta \div 5\alpha$-A in Table II, C2: $\bar{A} = +764$, and $\bar{B} = -663$, are more accurate averages of values calculated as above for the ranges 215°/230°, 230°/240°, and 215°/240°.

REFERENCES

1 F. A. VANDENHEUVEL AND A. S. COURT, *J. Chromatog.*, 38 (1968) 439.
2 F. A. VANDENHEUVEL, *Symposium on Quantitative Methodology in Lipid Research, Pennsylvania State University, Aug. 3–7, 1964.*
3 F. A. VANDENHEUVEL, G. J. HINDERKS AND J. C. NIXON, *J. Am. Oil Chemists' Soc.*, 42 (1965) 283.
4 F. A. VANDENHEUVEL, *Symposium on Modern Lipid Methodology, Claremont, Calif., Aug. 9–13, 1965.*
5 F. A. VANDENHEUVEL, *Second International Course on Methods for Lipid Research, Milan, Italy, Sept. 16–25, 1965.*
6 F. A. VANDENHEUVEL, *Can. J. Biochem.*, 45 (1967) 191.
7 F. A. VANDENHEUVEL, *J. Chromatog.*, 25 (1966) 102.
8 A. KUKSIS, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. XIV, Interscience, New York, 1966, p. 442–447.
9 F. A. VANDENHEUVEL, *J. Lab. Clin. Med.*, 69 (1967) 343.
10 H. BUDZIKIEWICZ, C. DJERASSI AND D. H. WILLIAMS, *Structure Elucidation of Natural Products by Mass Spectrometry*, Vol. II, Holden-Day, San Francisco, 1964.
11 J. A. VÖLLMIN, I. OMURA, J. SEIBL, K. GROB AND W. SIMON, *Helv. Chim. Acta*, 49 (1966) 1768.
12 M. J. E. GOLAY, *Nature*, 202 (1964) 489.
13 W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochim. Biophys. Acta*, 64 (1962) 416.
14 W. J. A. VANDENHEUVEL, W. L. GARDINER AND E. C. HORNING, *J. Chromatog.*, 26 (1967) 387.
15 F. A. VANDENHEUVEL, *J. Chromatog.*, 38 (1968) 373.