A COMPARISON OF THE BEHAVIOR OF THE HYDROCARBONS USED AS REFERENCE STANDARDS IN THE DETERMINATION OF "STEROID NUMBERS" AND "METHYLENE UNITS"; SELECTIVE RETENTION OF STEROID HYDROCARBONS BY POLAR STATIONARY PHASES

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INTRODUCTION

Gas–liquid chromatographic (GLC) techniques have provided a powerful method for the structural study and identification of a variety of natural products[1–3]. The steroid number concept[4,5] was advanced in order to describe relationships between the structure of steroids and their GLC behavior. A steroid number (SN) is defined in experimental terms as an additive (logarithmic) value determined from retention data relative to two reference steroids (androstane and cholestane; SN values of 19 and 27, respectively), rather than to a single standard. The reference substances and the compound under study are carried through the chromatographic process. Relative (to cholestane) retention times are plotted graphically as log values with respect to SN values. The SN of the compound under study is then read from the graph as the intercept on the SN axis (see Fig. 1). Such values are independent of temperature over a relatively wide range, which is often not true for relative retention times[4–6].

Perhaps the most generally useful set of reference compounds for GLC work are the straight chain aliphatic hydrocarbons. These compounds have been used as reference substances in an approach to the correlation of retention behavior and structure for long chain amines and alcohols and their derivatives[7,8]. When these compounds are used as standards the resulting values may be expressed as "methylene units" or MU values[7,8]. Such values are obtained in a manner analogous to the determination of SN values. The retention time of the compound under study is compared with those observed for the long chain hydrocarbon standards on a logarithmic basis, and the MU value is then read from the graph (see Fig. 1). An MU value is equivalent to the "retention index" as defined by KOVATS[9] divided by a factor of 100. It is also possible for purposes of identification and the study of structural relationships to use the "theoretical nonane value" or the "effective molecular weight value" of EVANS AND SMITH[10]. The use of MU values has the advantage of indicating the precise relationship between the retention behavior of the substance under study and

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that for the neighboring straight chain hydrocarbons which would be eluted just before and just after the compound of interest. As with SN values, MU values are relatively independent of temperature over a moderate range of temperature[7,8].

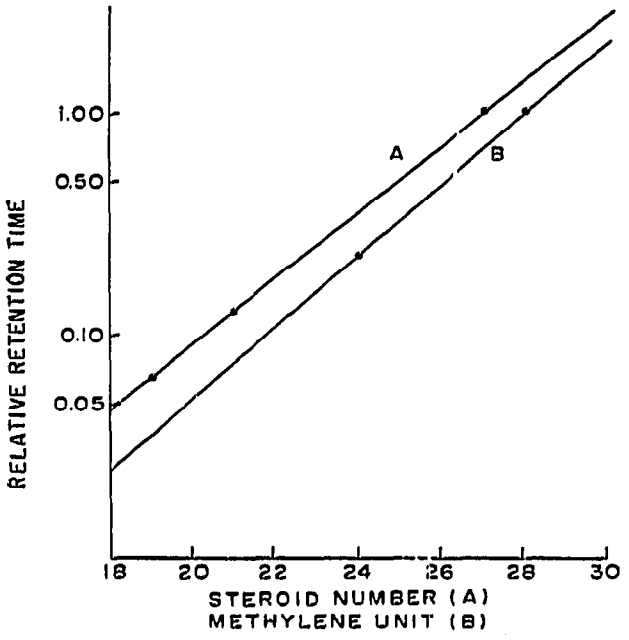

Fig. 1. Steroid number (A) and methylene unit (B) chart showing the slopes of the two lines used to determine steroid numbers (A) and methylene units (B) from relative retention times with a JXR column at 190°.

EXPERIMENTAL

Gas chromatographic retention data were obtained with a Barber-Colman Model 5000 instrument equipped with a hydrogen flame ionization detection system. The column support, 100–120 mesh Gas Chrom P, was acid-washed and silanized according to procedures developed in this laboratory[11]. The stationary phases, all 1 % (w/w), were SE-30 (a methyl polysiloxane; General Electric Co.); JXR (a thermally stripped methyl polysiloxane; Applied Science Laboratories, Inc.); F-60 (a methyl polysiloxane containing a low percentage of *p*-chlorphenyl groups; Dow Corning Corp.); β-cyanoethyl methyl polysiloxanes (CNSi) containing 8, 20, 50 and 65 mole-% β-cyanoethyl substitution; (code numbers were 287–149-251, 287-149-203, 287-149-239 and 287-149-300, respectively; General Electric Co.); XE-60 (a β-cyanoethyl methyl polysiloxane containing 50 mole-% β-cyanoethyl groups; Applied Science Laboratories, Inc.); neopentylglycol succinate (NGS) (Applied Science Laboratories, Inc.); neopentylglycol adipate (NGA) (Applied Science Laboratories, Inc.); 1,4-cyclohexanedimethanol succinate (CHDMS) (Applied Science Laboratories, Inc.); 1,4-cyclohexanedimethanol adipate (CHDMA) (Applied Science Laboratories, Inc.); Versamid 900 (a polyamide; Applied Science Laboratories, Inc.); QF-1 (a fluoroalkyl polysiloxane; Dow Corning Corp.). The coatings were applied by the filtration technique[11,13]. The columns (6 ft. × 4 mm glass U-tubes) were operated at 11–13 p.s.i. and 190°.

RESULTS AND DISCUSSION

In order to achieve a degree of generality in the reporting of retention behavior in GLC, it might be best to employ a system using the straight chain hydrocarbons as reference compounds (since a number of these are readily available in high purity) and relate other retention data (such as SN values) to MU values. This approach could only be satisfactory, however, if the steroid hydrocarbons and other standards which are used as reference standards for the determination of SN and other values behave in the same way as the aliphatic hydrocarbons under a variety of GLC conditions. If this were true, then a simple arithmetic relationship would exist between, for example, SN and MU values, and the long chain hydrocarbons could be used as the primary reference substances. Such is not the case however, for as can be seen in Fig. 1, the slopes of the MU plot and the SN plot are not parallel, indicating that the effect of the extension of the side chain of the steroid nucleus is not the same as the effect observed when methylene groups are added to long chain compounds.

More striking is the observation that the retention behavior of the steroid hydrocarbons relative to that of the straight chain hydrocarbons is determined by the chemical nature of the stationary phase employed. With non-polar (non-selective)[2,3,11] polymethylsiloxanes, such as JXR, F-60 and SE-30, *n*-octacosane (C28) is eluted considerably more slowly than cholestane (C_{27}) (see Fig. 2). With a somewhat

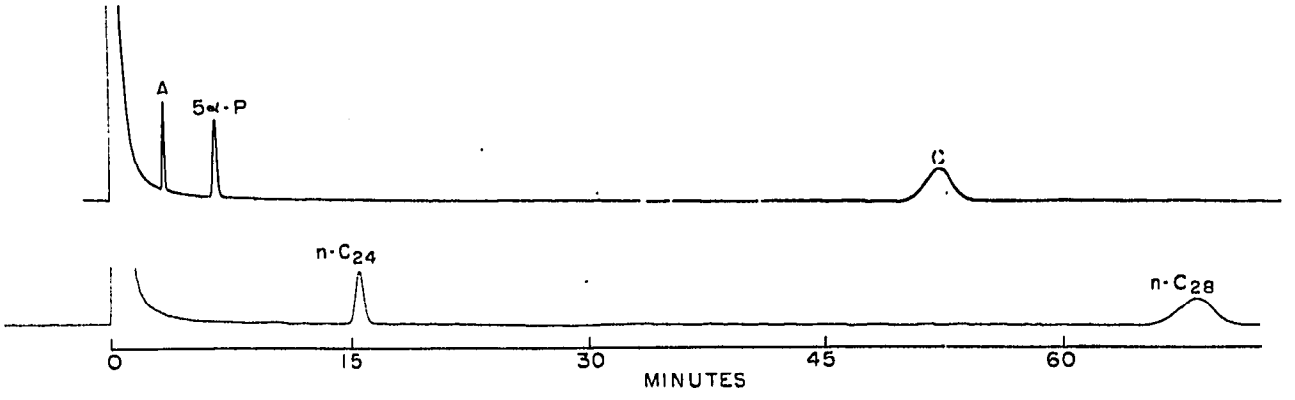

Fig. 2. Gas chromatographic separations of mixtures of androstane (A), 5α-pregnane (5α-P) and cholestane (C) (upper chromatogram) and *n*-tetracosane (*n*-C_{24}) and *n*-octacosane (*n*-C_{28}) (lower chromatogram) with JXR at 190°.

more polar liquid phase (a polymethylsiloxane containing 20 mole-% β-cyanoethyl groups) *n*-octacosane and cholestane possess nearly identical retention behavior (see Fig. 3). When the amount of β-cyanoethyl substitution is increased to 50 mole-% cholestane is retained significantly longer than *n*-octacosane, and 5α-pregnane (C_{21}) is eluted at approximately the same rate as *n*-tetracosane (C_{24}) (see Fig. 4). Thus with the non-polar phase JXR the steroids are eluted at a rate which parallels their molecular weight as compared with the size of closely related long chain compounds (the MU value for cholestane is 27.3). With the polar stationary phase (50 mole-% CNSi) it is clear that selective retention exists for the steroid hydrocarbons, leading to longer retention times than would be expected on the basis of molecular size and shape alone (the MU value for cholestane is 29.4).

Such retention behavior comparisons may be reported in terms of SN and MU values (as determined from the observed retention times), and Table I shows the relationship between SN and MU values for several primary reference substances at 190° with a variety of stationary phases. A selective retention effect exists for the steroid hydrocarbons on those stationary phases which are generally considered to be polar or selective; the fused-ring system of these compounds appears to show functional

TABLE I

RELATIONSHIP BETWEEN SN AND MU VALUES FOR SEVERAL PRIMARY REFERENCE SUBSTANCES WITH A VARIETY OF STATIONARY PHASES

Compound	*Relative retention time**		*MU*	*SN*
	SE-30			
Androstane	0.07	0.05	19.95	19.0
5α-Pregnane	0.13	0.10	21.75	21.0
Cholestane	1.00	0.75	27.2	27.0
n-Tetracosane	0.30	0.22	24.0	23.4
n-Octacosane	1.33	1.00	28.0	27.8
	JXR			
Androstane	0.07	0.05	19.9	19.0
5α-Pregnane	0.13	0.10	21.75	21.0
Cholestane	1.00	0.77	27.3	27.0
n-Tetracosane	0.30	0.23	24.0	23.4
n-Octacosane	1.30	1.00	28.0	27.8
	F-60			
Androstane	0.06	0.05	20.05	19.0
5α-Pregnane	0.12	0.09	21.85	21.0
Cholestane	1.00	0.80	27.4	27.0
n-Tetracosane	0.27	0.21	24.0	23.25
n-Octacosane	1.25	1.00	28.0	27.6
	8 mole-% CNSi			
Androstane	0.07	0.06	20.15	19.0
5α-Pregnane	0.13	0.11	21.95	21.0
Cholestane	1.00	0.84	27.5	27.0
n-Tetracosane	0.27	0.23	24.0	23.15
n-Octacosane	1.19	1.00	28.0	27.55
	20 mole-% CNSi			
Androstane	0.07	0.07	20.75	19.0
5α-Pregnane	0.13	0.14	22.5	21.0
Cholestane	1.00	1.01	28.0	27.0
n-Tetracosane	0.23	0.23	24.0	22.6
n-Octacosane	0.99	1.00	28.0	27.0
	50 mole-% CNSi			
Androstane	0.09	0.15	22.0	19.0
5α-Pregnane	0.17	0.26	23.85	21.0
Cholestane	1.00	1.59	29.4	27.0
n-Tetracosane	0.17	0.28	24.0	21.15
n-Octacosane	0.63	1.00	28.0	25.45

(*continued on p. 391*)

TABLE I (*continued*)

Compound	*Relative retention time**		*MU*	*SN*
	65 mole-% CNSi			
Androstane	0.10	0.17	22.3	19.0
5α-Pregnane	0.18	0.29	24.1	21.0
Cholestane	1.00	1.67	29.65	27.0
n-Tetracosane	0.17	0.29	24.0	20.85
n-Octacosane	0.60	1.00	28.0	25.20
	XE-60			
Androstane	0.09	0.14	22.0	19.0
5α-Pregnane	0.16	0.25	23.85	21.0
Cholestane	1.00	1.54	29.30	27.0
n-Tetracosane	0.17	0.27	24.0	21.1
n-Octacosane	0.65	1.00	28.0	25.5
	NGA			
Androstane	0.07	0.13	22.15	19.0
5α-Pregnane	0.14	0.24	24.0	21.0
Cholestane	1.00	1.68	29.4	27.0
n-Tetracosane	0.14	0.24	24.0	21.0
n-Octacosane	0.59	1.00	28.0	25.35
	NGS			
Androstane	0.08	0.16	22.7	19.0
5α-Pregnane	0.15	0.30	24.5	21.0
Cholestane	1.00	1.97	30.0	27.0
n-Tetracosane	0.13	0.26	24.0	20.45
n-Octacosane	0.51	1.00	28.0	24.8
	CHDMA			
Androstane	0.07	0.13	22.7	19.0
5α-Pregnane	0.13	0.25	24.4	21.0
Cholestane	1.00	1.89	29.65	27.0
n-Tetracosane	0.11	0.22	24.0	20.5
n-Octacosane	0.53	1.00	28.0	25.1
	CHDMS			
Androstane	0.07	0.15	22.8	19.0
5α-Pregnane	0.14	0.30	24.65	21.0
Cholestane	1.00	2.09	30.05	27.0
n-Tetracosane	0.11	0.24	24.0	20.25
n-Octacosane	0.48	1.00	28.0	24.7
	Versamid 900			
Androstane	0.07	0.12	22.35	19.0
5α-Pregnane	0.13	0.24	24.2	21.0
Cholestane	1.00	1.79	29.5	27.0
n-Tetracosane	0.12	0.22	24.0	20.8
n-Octacosane	0.56	1.00	28.0	25.3
	QF-1			
Androstane	0.10	0.15	21.75	19.0
5α-Pregnane	0.18	0.26	23.65	21.0
Cholestane	1.00	1.45	29.2	27.0
n-Tetracosane	0.20	0.29	24.0	21.4
n-Octacosane	0.69	1.00	28.0	25.7

* All columns (1 % stationary phase) were operated at 190°.

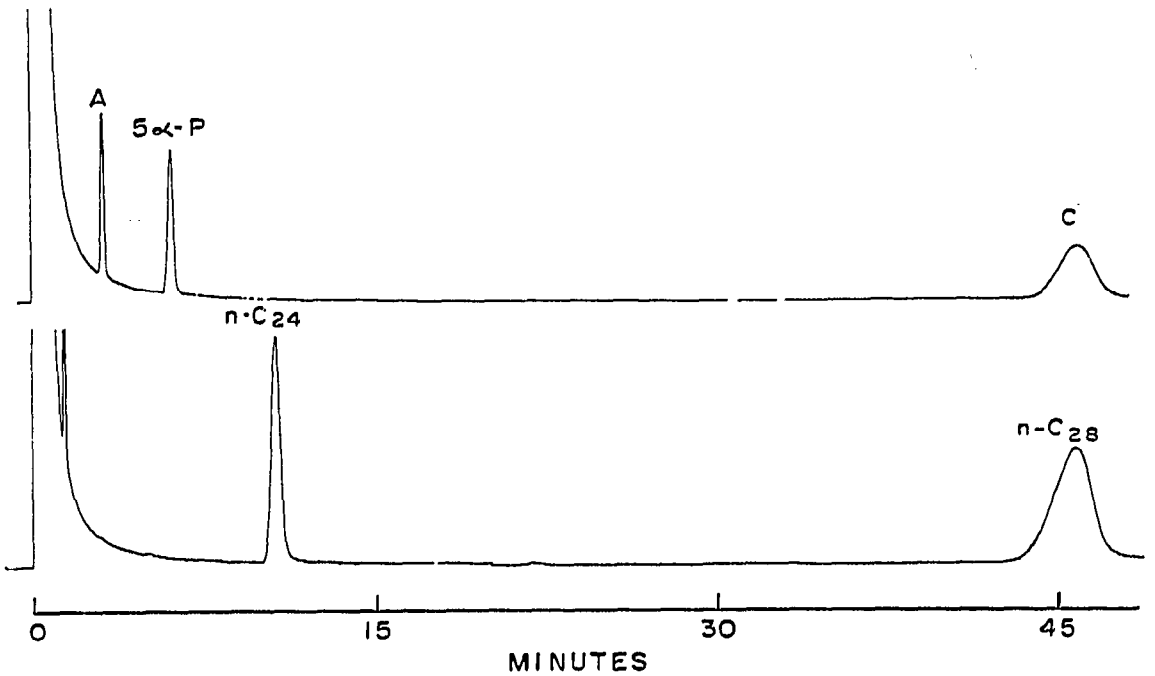

Fig. 3. Gas chromatographic separations of mixtures of androstane (A), 5α-pregnane (5α-P) and cholestane (C) (upper chromatogram) and *n*-tetracosane (*n*-C_{24}) and *n*-octacosane (*n*-C_{28}) (lower chromatogram) with 20 mole-% β-cyanoethyl methyl polysiloxane at 190°.

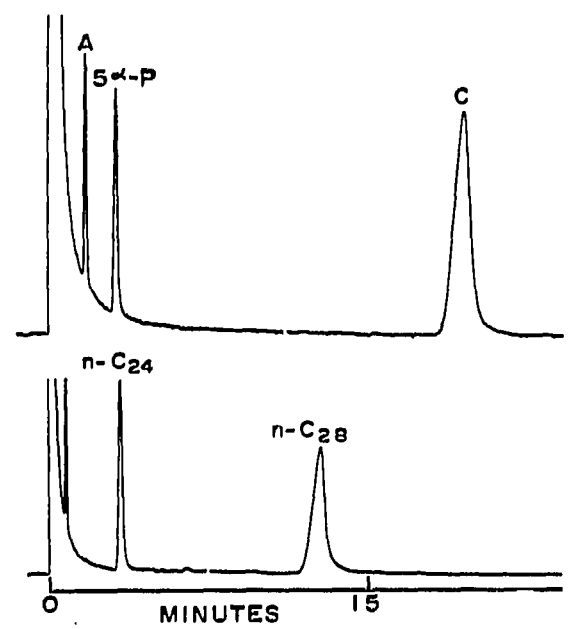

Fig. 4. Gas chromatographic separations of mixtures of androstane (A), 5α-pregnane (5α-P) and cholestane (C) (upper chromatogram) and *n*-tetracosane (*n*-C_{24}) and *n*-octacosane (*n*-C_{28}) (lower chromatogram) with 50 mole-% β-cyanoethyl methyl polysiloxane at 190°.

group character. Fortunately a family of β-cyanoethyl-substituted polymethylsiloxanes of varying percentage substitution was available to us*, and it is evident (Table I) that as the percentage of nitrile group in the polysiloxane is increased the MU values of the steroid hydrocarbons increase relative to those of the long chain hydrocarbons. Furthermore, as is widely recognized, for a given glycol a polyester prepared from condensation with succinic acid serves as a more polar stationary phase than the corresponding polyester obtained by condensation with adipic acid. As can be seen in Table I, the selective retention of the steroids is more pronounced with NGS** than with NGA, and the same pattern holds for the polyesters CHDMS and CHDMA. In N.M.R. circulation of the bonding electrons has been invoked to explain the ring current effect which appears to be operative in saturated cyclic hydrocarbons[12]. If the bonding electrons of steroidal tetracyclic ring systems are polarizable by the functional groups of stationary phases, this effect would be expected to increase with the polarity of the stationary phase. Selective retention for steroids, when compared with aliphatic hydrocarbons, should thus be greatest with the most polar stationary phases. Fig. 5 shows graphically the relationship between SN and MU values for three steroid hydrocarbon reference standards with a number of stationary phases; the shift of the plots toward the right with increase in polarity of stationary phase is striking. It should be possible to characterize stationary phases as to their polarity or selectivity through the use of relationships such as those discussed in connection with Table I and Fig. 5.

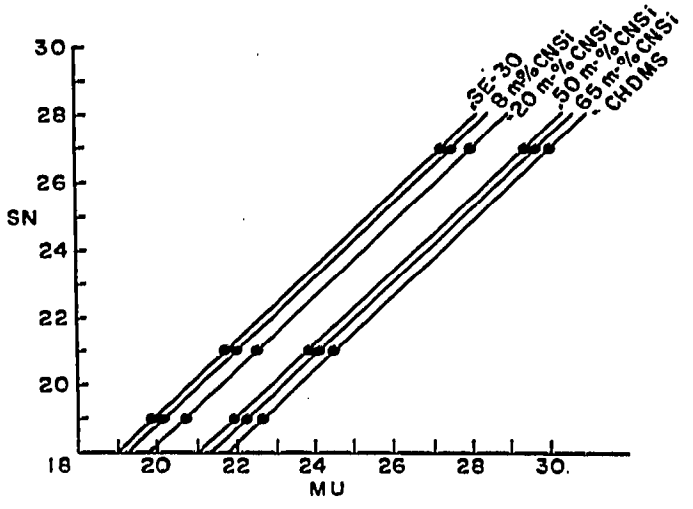

Fig. 5. Comparison of steroid number (SN) and methylene unit (MU) values for the hydrocarbons androstane, 5α-pregnane and cholestane (determined at 190°) for the liquid phases SE-30; 8, 20, 50 and 65 mole-% β-cyanoethyl methyl polysiloxanes; and CHDMS.

These data suggest that GLC techniques might be used as an additional form of measurement or comparison procedure for the determination of "partial double bond character" ascribed to ring systems. Differences in structure which result only in a difference in size or shape, and not in functional character, will lead to retention time changes which are the same when observed with either polar or non-polar

* We are grateful to Dr. A. MARTELLOCK of the General Electric Company for samples of these experimental silicones.

** A list of abbreviations is found in the experimental section.

phases; if a structural change results in a change in functional character, the retention time changes will not be the same for non-polar and for appropriate polar phases.

SN values for steroids and MU values for long chain compounds are relatively temperature independent[4,5,7,8]. The data presented in Table II, however, clearly disclose that MU values obtained for steroids and SN values obtained for long chain compounds are temperature dependent; as the temperature of the separation is increased, the retention times of the long chain hydrocarbons decrease relative to those of the steroid hydrocarbons. With SE-30 at 190° the MU value for cholestane is

TABLE II

VARIATION OF MU AND SN VALUES WITH TEMPERATURE WITH QF-1

Compound	*MU*	*SN*
	170°	
Androstane	21.25	19.0
5α-Pregnane	23.1	21.0
Cholestane	28.8	27.0
n-Tetracosane	24.0	21.95
n-Octacosane	28.0	26.1
	180°	
Androstane	21.4	19.0
5α-Pregnane	23.25	21.0
Cholestane	29.0	27.0
n-Tetracosane	24.0	21.7
n-Octacosane	28.0	25.9
	190°	
Androstane	21.75	19.0
5α-Pregnane	23.65	21.0
Cholestane	29.2	27.0
n-Tetracosane	24.0	21.4
n-Octacosane	28.0	25.7

0.8 units less than that observed for *n*-octacosane, whereas at 230° these two hydrocarbons possess equal volatility. The effect upon retention behavior of change in temperature, like change in stationary phase, is not the same for steroid hydrocarbons as it is for long chain hydrocarbons. The observations reported in this paper thus suggest that for the purpose of studying the GLC behavior of a restricted group of compounds it may be desirable to use reference standards which are of the same structural type as the compounds under investigation.

ACKNOWLEDGEMENT

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SUMMARY

During an investigation designed to compare the gas–liquid chromatographic behavior of two sets of compounds used as reference standards in structure–retention

behavior studies it was observed that with polar stationary phases selective retention exists for steroid hydrocarbons when compared to straight chain hydrocarbons of similar carbon content. With nonpolar stationary phases the steroids are eluted relative to the paraffins on the basis of molecular weight. Such behavior differences can be used to classify the polarity or selectivity of stationary phases.

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