A Thesis

entitled

"Physico-Chemical Techniques in the Study of Organic Molecules of Biological Importance".

Submitted in part fulfilment of the requirements for admittance to the degree of

Doctor of Philosophy

in

The University of Glasgow

by

L. Hanaineh, B.Sc.

University of Glasgow **1964.**

ACKNOWLEDGMENTS

I would like to express my appreciation to Professor R. A. Raphael, F.R.S., University of Glasgow and Dr. B. Bronte-Stewart, Director, Atheroma Research Unit, Medical Research Council, Glasgow for the opportunity to carry out the work described in this thesis, and for their guidance. I also gratefully acknowledge the constant advice and help of Drs. C. J. W. Brooks and G. Eglinton, who jointly supervised this work.

My thanks also go to Mrs. F. Lawrie for considerable aid in infrared measurements and to Mr. T. Cairns, B.Sc. for a few measurements and the drawings of Figs. 1-4 in Part B, Section 1.

Finally, I wish to thank Miss Joan Cairns, Secretary of the Atheroma Research Unit, for her valuable help in typing this thesis.

CONTENTS

A. GAS LIQUID CHROMATOGRAPHY

Introduction.

Historical.

Gas chromatography which has only come into its own in the last two decades, has made remarkable progress since. The reason for the slow emergence of chromatographic methods as an established separatory technique lay in the needs of the times. Up till the 20th century there was need only for separating or analysing large amounts of material and therefore any advances in micromethods were neglected. Around 1900 in a period of increasing interest in natural products, Tswett (1) succeeded in separating plant pigments by adsorption on solids and coined the word chromatography. He was followed in 1931 by Kuhn, Winterstein and Lederer (2) who used the same technique of liquid-solid chromatography and showed the importance of chromatographic processes in chemical analysis. This was the beginning of interest in separating micro quantities and as a result analytical micro methods and spectroscopic procedures were invented and developed. Martin and Synge in 1941 (3) were the first to establish liquid-liquid chromatography which involved a mechanism of partition in contrast to the usual mechanism of adsorption in liquid-solid chromatography. At the same time

they showed the possibility of using partition chromatography with a gas replacing the mobile liquid phase thus laying down the basic principle of present day gas-liquid chromatography. In this type of chromatography a gas is the mobile phase and the sample, introduced into the gas phase, travels through the column in vapour form and is distributed between the liquid and gas phases according to its partition coefficient.

In the 1940's the need was still not great enough for such a micromethod to be immediately exploited and it was left to James and Martin in 1950 to begin the development of this technique (4). They successfully applied it to the microseparation of volatile acids and bases (0.02 mgs. of acetic acid were detectable). Since then the need for a micro analytical method capable of qualitative and quantitative analysis has stimulated the rapid development and practical application of gas-liquid chromatography in almost every field of organic chemistry.

Applications

The first applications of gas-liquid chromatography were mainly directed towards the chemical industries and in particular those concerned with petroleum, essential oils, flavours and perfumes. Its early use in these fields was due to the success of this

technique in the identification of hydrocarbon skeletons of organic compounds, in the location of unsaturation and chain branching and in functional group analysis. It had also proved indispensable in the separation of complex mixtures and the characterisation of the component parts by their retention behaviour. With further modifications and refinements in instrument design, detector type, column design and composition of liquid phase, applications to the biochemical and medical fields became possible.

In studies of mixtures of biological origin the frequent sparseness of material and its complex composition has hindered the success of conventional methods. The introduction of highly sensitive detectors and column packings coated with thin films of thermostable liquid phases has permitted the analysis of small amounts of such mixtures by gas liquid chromatography. The combination of small sample size, high resolving power and speed of analysis has found wide application in the study of a large variety of biologically important compounds such as steroids, alkaloids and narcotics, vitamins, carbohydrates, amino acids, herbicides and pesticides and lipids. One of the largest molecules to which the method has been successfully applied (5) is the

trimethylsilyl derivative of the tetrasaccharide stachyose (molecular weight 1676). At the other extreme the method has proved effective in the analysis of blood gases and other volatile components of blood, and in the determination of atmospheric pollution.

Instrument:

There are four basic components to a gas chromatograph, a column, a detector which responds to changes in the chemical or physical property of the gas issuing from the column and an amplifying and recording system. The standard column, which may be made of glass, stainless steel, copper, aluminium or other suitable material, varies in length from 2 to 20 feet and in internal diameter from 2 to 30 mm. This column is packed with the stationary phase which consists of an inert supporting material (grain size 0.1-0.4 mm. which approximately corresponds to a mesh range of 140-40) impregnated with the non-volatile liquid phase. One of the innovations in column design has been the introduction of capillary columns, generally 50 to 250 feet in length, which are coated internally with the liquid phase. However, for steroid work the packed columns have so far proved more satisfactory.

The sample to be analysed, which may be a gas, liquid or solid, is introduced into the top of the column by means of a suitable device and then swept through the column and into the detector by an inert carrier gas. The column is operated at a sufficiently high temperature to vaporize the sample on introduction. With compounds of high molecular weight a supplementary heater at the point of injection may be essential. In analytical instruments the fractions which issue from the end of the column pass through the detector and are allowed to escape into the atmosphere, but in preparative gas-liquid chromatography they are trapped and collected for further analysis.

With the use of an inert carrier gas as mobile phase, possible operating temperatures have ranged from -200°C to + 1000°C which necessitate substantially non-volatile and thermally stable liquid phases. In general a liquid phase should boil at 250°C to 300°C above the maximum operating temperature and therefore for column temperatures above 350°C, a limited range of liquid phases such as polyphenyl tars and eutectic mixtures of inorganic salts is available. Without the use of an inert carrier gas the chromatography of high

molecular weight compounds would not be possible. At the high temperatures employed, their stability to pyrolytic decomposition is aided by the inert atmosphere of the gas chromatograph. For steroid analysis a temperature range of 200° to 250° is normally used and the analysis can be carried out either under isothermal conditions or by temperature programming where the temperature is gradually raised during an analysis. For the separation of components of a mixture of widely varying molecular weights temperature programming is an advantage since it permits analysis of the mixture in a single run.

Before the development of highly sensitive detectors, packings containing 8 to 40% liquid phase were used in analytical instruments since relatively large samples were employed and high liquid phase concentrations were necessary to avoid overloading. This resulted in long retention times and the necessity to use high column temperatures. In addition there was a general belief that columns with less than 5% liquid phase would show low efficiencies. Columns usually employed for steroid analysis show efficiencies in the range 2000 to 8000 "theoretical plates" (i.e. between 400 and 600 theoretical plate values per foot):

the values are calculated from the following formula:

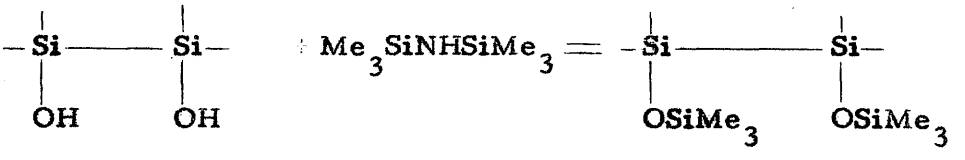
$$n = 16\left(\frac{R_T}{W}\right)^2$$

where R_T is the retention time and W the base width of the peak in the same units. At present high percentages of liquid phase are only necessary in preparative gas-liquid chromatography where the samples are collected for further analysis. In biochemical analysis where the emphasis is on small sample size, liquid phase concentrations of 0.5 to 3% are used to enable the detection of microgram and sub-microgram quantities and to permit the rapid analysis of compounds which are labile by virtue of their high molecular weight or of the functional groups present. Burchfield and Storrs (18) point out that a complete analysis can now be obtained on a 1-µg. sample containing as many as 20 components if a capillary column is used in combination with an highly sensitive (e.g. ionisation) detector. Sample volumes applied to packed columns usually vary between 0.1 and 3 µl. and are injected through a silicone rubber stopper by means of a micro-syringe. With capillary columns even smaller samples are needed and to overcome the problem of inaccuracy a large sample is introduced, only a small part of which is swept into the detector - the remaining part is allowed

to escape into the atmosphere.

Supports.

For steroid work where thin-film column packings are used particular attention is directed to the choice of support material. An important consideration is the inertness of the support material with regard to solute molecules. Diatomaceous earth-type supports of mesh size 100-120 or 100-140 (grain size 0.10 to 0.15 mm.)are most commonly used in steroid work. These supports are silicates which contain active sites on the surface and these require blocking in order to minimize surface adsorption of the solute molecules. The most widely used deactivating procedure is that of "silanizing" the support by treatment with hexamethyl disilazane or dichlorodimethylsilane:



This process eliminates the major part of the chemical reactivity of the support and is essential when dealing with hydroxy and ketonic compounds.

Phases.

One of the many advantages of gas-liquid chromatography is the ability to use a range of liquid phases which differ in chemical

structure and in their retention behaviour towards specific functional groups. Liquid phases of current use in steroid work fall roughly into two categories (i) Non-selective liquid phases which separate compounds primarily according to the molecular weight and shape of the molecule. These are non-polar liquids the most common of which are the siloxane polymers with methyl or phenyl substituents. (ii) Selective liquid phases which show characteristic behaviour towards specific functional groups. Polymers of the polyester type and methyl siloxane polymers with polar groups (fluorine or cyano ethyl groupings) fall into this category. Retention times observed for polyfunctional steroids with these polar phases are very long which somewhat limits their use to the less polar steroids.

Detectors.

When gas chromatography was first developed in 1950 by Martin and James (4) these authors used an integral detector i.e. one which measures the total amount of sample in the effluent gas collected over any specified period. Such a detector translates the data into sigmoid curves: as the sample passes through the detector the change in base line

takes the form of a step instead of a peak. Integral detectors have since been largely superseded by differential detectors of which the thermal conductivity and ionisation detectors are the most used. The thermal conductivity detector is based on measurement of the differences between the thermal conductivities of the solute and mobile phase. Although once the most widely used, it has several disadvantages, the most important of which is lack of sensitivity coupled with a ready disturbance of the base line by small changes in column temperature and flow rate. The argon ionisation detector invented by Lovelock (6) is in contrast highly sensitive and is almost unaffected by small changes in temperature and in carrier gas flow rate.

The basis of the argon ionisation detector is that the argon used as carrier gas is excited (excitation potential is 11.6 electron volts) to a metastable state by a source of β-radiation such as strontium-90. The metastable argon atoms then collide with the solute molecules entering the detector resulting in transfer of energy and ionisation of these organic molecules since their ionisation potentials are mostly less than 11.6 e.v. This increase in the number of ions i.e. the change in ionisation current is, after amplification, automatically recorded on a

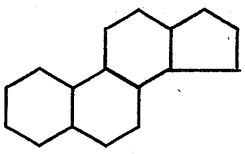
strip chart which presents the data as a series of peaks along a horizontal time axis. The area of such a peak is proportional to the quantity of the compound or mixture in the effluent gas. In the argon detector the response of the detector with respect to solute concentration is linear over a fairly wide range; moreover the presence of as little as 2×10^{-12} moles of most organic compounds may be detected. An increase in the applied potential increases the concentration of metastable argon atoms and consequently increases the response of the detector to solute molecules. For ionisation detection systems sample sizes exceeding 20 μg. are not normally used as these would lead to overloading. For larger sample sizes thick-film column packings and thermal conductivity detection systems are best employed.

The flame ionisation detector of McWilliam and Dewar (7) which is based on the measurement of the electrical conductivity of gases in a hydrogen flame is coming into wide use. It shows a larger linear concentration range than the argon ionisation detector and is considered to be more insensitive to temperature changes. Moreover it is unaffected by water vapour. Other modifications of the ionisation detector have

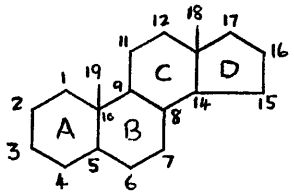
also been introduced: the differential flame ionisation detector (8) based on the same principle but utilizing dual column operation to compensate for column "bleeding" during temperature programmed or isothermal analyses, the electron capture detector of Lovelock and Lipsky (9) and the cross-section detector of Lovelock and co-workers (10). The electron capture detector exhibits a high sensitivity towards specific molecular species and atoms (eg. Halogen) some of which do not give a good response with the argon ionisation detector. The cross-section detector which is the least sensitive of the ionisation detectors has the advantage of enabling the calculation of the absolute amount of components in a mixed sample.

Steroid Analysis.

Our main interest in gas liquid chromatography has been in its application to the steroid field where a persistent need for microanalytical methods exists. Most steroids (II) are derivatives of cyclopentanoperhydrophenanthrene (I).



I



II

Except for the estrogens, the natural steroids usually contain two angular methyl groups attached to carbon atoms 10 and 13. Modifications of this basic structure (C_{19}) involve either extension of the carbon chain at C_{17} or substitution in the present carbon skeleton or both, e.g. in sterols (C_{27} - C_{30}) and bile acids (C_{24}) extension and substitution occur while in the androgens (C_{19}) only substitution in the existing carbon skeleton takes place. Among the many groups of compounds which are classified as steroids are the sterols, the bile acids, the sex hormones, the adrenocortical hormones, the vitamin D series, cardiac glycosides, toad poisons and the steroid saponins (11, 19).

SYNOPSIS OF FOLLOWING SECTIONS.

Section 1.

Of particular interest in our investigation of steroids has been the assessment of regularities achieved in the gas chromatography of androstane and pregnane derivatives (including sex hormones) and some cholestane derivatives and the correlation of their retention behaviour with particular structural aspects. This formed the basis of Section 1 of our investigation into the gas-liquid chromatography of steroids and has appeared in the Biochemical Journal (1963) 87, 151 (preliminary communication 1962, 84, 102P).

Compounds may be characterised by their retention time, that is the time between injection and elution, and such a retention time is constant for a particular liquid phase and temperature and for a given gas flow rate. Values independent of this last variable are expressed in terms of relative retention time, i.e. relative to a suitable compound used as an internal or external standard. Clayton (12) has shown that the retention time of a substituted steroid may be evaluated by compounding "group retention factors" characteristic of each substituent with the retention time of the parent steroid. Good correlations were observed where interactions between substituents could be disregarded.

This approach finds analogy in molecular rotation relationships where Van't Hoff in 1898 deduced the principle of additivity, that is that the molecular rotation of a compound containing several asymmetric centres is the sum of the individual rotation contributions of the component rotary centres. The molecular rotation of a compound is its specific rotation $(a)_D$ multiplied by its molecular weight and is expressed numerically as follows:

$$M_D = \frac{(a)_D \times \text{Mol. Wt.}}{100}$$

This permits comparison of the optical properties of a parent compound and its derivatives on a standard molecular weight basis.

Before the advent of gas-liquid chromatography molecular rotations were used in identifying steroids of unknown structure by comparison with the molecular rotations of known steroids. The rotation contribution of the functional group (Δ value) to the molecular rotation of the parent compound is characteristic of the particular functional group and of its position and configuration in the steroid nucleus. These molecular rotation contributions (where $\Delta = (M)_D$ substituted compound - $(M)_D$ parent compound) have been calculated for a large number of functional groups and have been used in the elucidation of structures of unknown steroids (13).

The Δ values are additive provided the functional groups are well separated from one another in the steroid skeleton. "Vicinal effects" were observed for compounds with highly unsaturated functional groups e.g. an $\alpha\beta$-unsaturated keto group or with substituents separated by less than three or four carbon atoms.

A major advantage of gas-liquid chromatography over molecular rotations for identification purposes is the scale on which the analysis can be carried out. Whereas in gas chromatography microgram and submicrogram amounts of steroid can be detected, the minimum amount required for molecular rotation determinations is of the order of 1 to 10 milligrams.

Section 2

Our investigation of the gas-liquid chromatography of steroids was extended to the study of cholestanols and cholestanediols. Many of these compounds were supplied by Dr. G. D. Meakins who has investigated these compounds by infrared spectroscopy (14). A correlation between the retention time and the position and type of hydroxyl group seems to exist but whereas in Section 1 it was shown that "group retention factors" were additive this does not apply to the 1, 2 and 1, 3 cholestanediols under study.

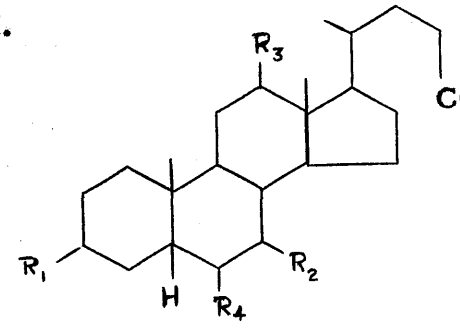
This is to be expected since vicinal effects - whether steric or hydrogen bonding - strongly influence the order and rate of elution of a compound.

Section 3

This section is devoted to the study of bile acids. A brief description of their isolation from serum and tentative identification by thin-layer chromatography is first given, followed by a more detailed examination of their gas-chromatographic behaviour.

There has been no mention in the literature so far of gas-liquid chromatography of bile acids in their conjugated form i.e. as conjugates of glycine and taurine(cf. III.)

III.



$CONHCH_2COOH$ Glycine

$H_2NCH_2CH_2SO_3H$ Taurine

$R_1 = R_2 = R_3 = R_4 = OH$ or $C = 0$

In the analysis of biological extracts (bile or serum) the procedure has been to hydrolyse the conjugated bile acids to the free acids before isolation, identification and estimation by various physical methods. Thus the proportion of bile acids conjugated to glycine and taurine in such biological extracts could only be estimated by indirect procedures. The gas-liquid chromatography of the conjugated bile acids was examined in the hope that such a method

might ultimately permit the direct analysis of free and conjugated bile acids in serum extracts where the total concentration of bile acids is 0.8-1.4 μg./ml. A preliminary communication has appeared in the Biochemical Journal (1964), 92, 9P-10P.

Unlike the other steroids, bile acids require methylation prior to gas-liquid chromatography. A number of workers have investigated the behaviour of the unconjugated bile acids on gas chromatography using a variety of silicone polymer phases of the type introduced by Horning and co-workers (15). The low concentrations of liquid phase employed (0.5-3%) have permitted the convenient chromatography of such highly polar and labile compounds as the methylated bile acids. Liquid phases of 3-5% not only result in very long retention times, but require very high column temperatures ($> 250^{\circ}$) which result in thermal decomposition of these polyhydroxy compounds. In order to reduce the retention time and minimize losses by decomposition, conversion to the less polar and more thermostable trifluoro-acetates (16) and trimethylsilyl ethers (17) was carried out. Acetates, although less polar and more thermostable than the parent hydroxy compounds, have not been widely applied to bile acids because of their normally longer retention times. However, for the gas chromatography of the conjugated bile acids the

acetates and trimethylsilyl ethers have proved the most convenient derivatives. The conjugated bile acid methyl esters and their derivatives were studied on a number of silicone polymer phases (SE-30; SE-52; F-60 and QF-1) at temperatures ranging from 235° to 250°C. Modifications of the standard 4 ft. glass column were introduced in order to shorten the retention time and allow reduction of the sample size. Under the present chromatographic conditions only the glycine conjugates as acetates and trimethylsilyl ethers have been successfully chromatographed. Analytical separations of mixtures of their derivatives have also been achieved.

(1) Tswett, M. (1906). Ber. deut. bot. Ges. 24, 318, 384.

(2) Kuhn, E., Winterstein, A. & Lederer, E. (1931). Z. phys. Chem. 197, 141.

(3) Martin, A.J.P. & Synge, R.L.M. (1941). Biochem. J. 35, 1358.

(4) James, A.T. & Martin, A.J.P. (1952). Biochem. J. 50, 679.

(5) Bentley, R., Sweeley, C.C., Makita, M. & Wells, W.W. (1963). Biochem. Biophys. Res. Comm. 11, 14.

(6) Lovelock, J.E. (1958). J. Chromat. 1, 35.

(7) McWilliam, I.G. & Dewar, R.A. (1958). In "Gas Chromatography 1958" Ed. by Desty, D.H. p. 142, London: Butterworths.

(8) Ettre, L.S., Condon, R.D., Kabot, F.J. & Cieplinsky, E.W. (1964). J. Chromat. 13, 305.

(9) Lovelock, J.E. & Lipsky, S.R. (1960). J. Amer. chem. Soc. 82, 43

(10) Lovelock, J.E., Shoemake, G.R. & Zlatkis, A. (1963). Analyt. Cher 35, 460.

(11) Fieser, L.F. & Fieser, M. (1959). Steroids. New York: Reinhold Publishing Corp.

(12) Clayton, R.B. (1961). Nature, Lond., 190, 1071.

(13) Barton, D.H.R. & Klyne, W. (1948). Chem. & Ind. p. 755.

(14) Meakins, G.D. (1963). J. chem. Soc. p. 4068.

(15) Horning, E.C., VandenHeuvel, W.J.A. & Creech, B.G. (1963). In Methods of Biochemical Analysis Vol. 11, Ed. by Glick, D. New York: John Wiley and Sons Inc.

(16) VandenHeuvel, W.J.A., Sjövall, J. & Horning, E.C. (1961). Biochim biophys. Acta, 48, 596.

(17) Makita, M. & Wells, W.W. (1963). Analyt. Chem. 5, 523.

(18) Burchfield, H.P. & Storrs, E.E. (1962). Biochemical Applications of Gas Chromatography. New York: Academic Press Inc.

(19) Klyne, W. (1960). The Chemistry of the Steroids. London: Methuen & Co., Ltd.

Section 1.

The Correlation of Gas-Liquid Chromatographic Behaviour and Structure of Steroids.

The persistent need for microanalytical methods in steroid biochemistry has stimulated many recent applications of gas-liquid chromatography in this field (1). These have been aided by technical improvements, such as the development of highly sensitive detectors and the use of low concentrations of stationary phase. Silicone polymer phases of the types introduced by Horning and co-workers (2,3) permit the convenient chromatography of a wide variety of steroids, including sterols and other hydroxysteroids which have unduly long retention times on polyester phases. The first significant correlations between retention behaviour and structure of steroids were established by Clayton (4,5,6) for a number of sterol methyl ethers (C_{19} to C_{29}). The "group retention factor" (4,5) for a particular substituent in a steroid is the relative change in retention accompanying its introduction. In general, the analytical utility of retention factors relies upon their substantial independence of molecular weight: their theoretical basis is discussed further below (p 66).

This section surveys regularities observed for a number of

steroids (C_{19}, C_{21}, C_{24}, C_{27} and C_{28}), using the silicone phases SE-30 and QF-1, principally at 200°. During the course of this work, Knights and Thomas (7,8) reported useful correlations for a variety of compounds, using the QF-1 stationary phase at 250°, while regularities observed using SE-30 at 220° were described by Tsuda, Ikekawa, Sato, Tanaka and Hasegawa (9).

The principal aim of the present investigation was to assess the regularities achieved in the gas chromatography of steroids under standard conditions, and so to assist the identification of steroids isolated in microgram quantities from biological samples.

Materials and Methods.

Steroids.

Most of the steroids were obtained commercially or were generously provided by Professor W. Klyne (M.R.C. Steroid Reference Collection): others were kindly given as specified in the footnotes to Table 1. Androst-4-ene-3,11,17-trione and 11β-hydroxy-androst-4-ene-3,17-dione were prepared by oxidation of cortisone and hydrocortisone respectively (10). Solutions were made in Analar chloroform, usually at concentrations near 2 mg./ml., and suitable portions were mixed with cholestane for chromatography. The quantity of steroid injected was generally 0.1-1.0 μg.

Apparatus.

The Pye Argon Chromatograph was modified to permit convenient injection of solutions. Glass column tubes 134 cm. long and with internal diameter 3.5-4.0 mm. were fitted with a side-arm 1 in. below the top, for admission of the argon supply, and injections (0.1-2 µl.) were made with a 10 µl. syringe (Hamilton Co., Inc., Whittier, California) through a silicone rubber "blind hole" stopper that closed the upper end of the tube. When fully inserted for injection the point of the syringe needle was 1 in. below the side-arm. The upper portion of the tube projected from the Chromatograph housing, and was packed only with a loose plug of glass wool. It was enclosed by an aluminium block which incorporated a 300-watt heating element controlled by a variable transformer. The block temperature was normally kept 25-35° above the main column temperature. Standard conditions were as follows : column 200 ± 3° (or 225 ± 3°), block 230 ± 5° (or 250 ± 5°), argon flow rate 30 ml./min. at outlet (inlet pressure 10-15 lb./sq. in.), nominal detector voltage 1250, sensitivity setting 10. The detector was the standard Lovelock argon ionisation type, fitted with a strontium 90 source. Commercial 99.95% argon was further purified by passing through a size 4A

"molecular sieve" prior to entering the chromatograph.

Preparation of columns.

The methyl siloxane polymer SE-30 (General Electric Co., Schenectady) and the methyl fluoroalkyl siloxane polymer QF-1-0065 (viscosity 10,000 cs.) (Dow-Corning Corp. Inc., ; Midland Silicones Ltd., London) were kindly provided by Dr. E.C. Horning.

Column packings were prepared on the support 'Gas-Chrom P' (Applied Science Laboratories, Inc., State College, Pennsylvania) (100-140 mesh for SE-30, 80-100 mesh for QF-1) which was pretreated according to the procedure described by Horning et al. (1). The experimental details are as follows: first the support was screened with stainless steel sieves to within the range of mesh size required. The screened support was washed repeatedly with concentrated hydrochloric acid until the extracts, initially yellow, became colourless after which it was left overnight in the acid. To remove all traces of chloride ions and any finely divided particles produced during washing, the support was washed many times with distilled water and the supernatant removed by decantation. This was followed by suspending the support in methanol to remove most of the water then in toluene to remove traces of methanol. After

preliminary drying at room temperature the drying was completed at 80°C. The support was then suspended in 5% (v/v) dimethyl dichlorosilane in toluene and a gentle vacuum applied to remove any air bubbles and aid "wetting" of the support. After 15-30 mins. the support was filtered and washed with toluene and methanol. Drying of the "silanized" support was effected first at room temperature, then in an oven at 80°C.

The lower concentration of dimethyldichlorosilane advised by Holmes and Stack (11) was not tried. Coating with stationary phase was also based on Horning's technique, as follows: 20 g. of silanized support was suspended in 100 ml. of a solution (1% w/v) of SE-30 in toluene, (or OF-1 in acetone) and a gentle vacuum applied to remove occluded air. After 15 minutes the suspension was poured in one lot on to a Buchner funnel, with gentle suction which was released as soon as the bulk of the solution had been filtered. The moist support was transferred to a filter paper, and after drying in air was finally dried in the oven at 80°. Column packings so prepared contained approximately 1% (w/v) of stationary phase: samples of the packings used in this work were examined by Soxhlet extraction and found to contain respectively 1.0% (SE-30) and 1.1% (OF-1) of stationary phase.

Glass columns, and glass wool used for packing, were first washed with hot acetone and toluene then treated with a 5% (v/v) solution of dimethyldichlorosilane in toluene, washed with toluene and methanol, and dried before use. Columns were packed by gradual addition of the coated support and repeated tapping: the uppermost inch or two was packed after applying argon pressure (20 lb./sq. in.) to ensure that undue compaction would not occur in the chromatograph. The column was not packed above the top of the column heater present in the standard Chromatograph. Before using any freshly-packed column in the Chromatograph it was heated to 300° (SE-30) or 250° (QF-1) in a slow stream of argon for 12-24 hours to remove volatile products. This process was necessary to avoid contamination of the detector and to ensure that a stable recorder baseline would be secured in the Chromatograph.

Use of columns.

All the results described in this paper were determined with the same pair of columns. The SE-30 column was also used over the same period for more than 1,000 chromatograms of other steroid samples, mainly extracts of serum cholesterol. At intervals of 1-2 months the topmost inch of the column packing and

the glass wool above it were replaced by fresh materials: this restored sensitivity and reduced "tailing" caused by the accumulation of decomposed products at the top of the column. The QF-1 column was used almost exclusively for the present work and showed no deterioration over several months. Reproducibility of data from one column to another was not checked with QF-1, except that many of the results were in general agreement with those obtained by Dr. C. J. W. Brooks, using a QF-1 column in Professor Horning's laboratory (cf. Table 9). In the case of SE-30, preliminary measurements of retention data were carried out on a separately-prepared column and showed no appreciable differences from those presented here.

Determination of retention data

Measurements of retention times were made between the first displacement of the recorder pen following injection and the point corresponding to the peak of the response to the steroid. Steroids giving asymmetric peaks are indicated in the Tables. The recorded response to the injection was observed 20-25 secs. after the moment of injection and coincided with the return of the outlet flow rate from an elevated level (due to a pressure wave from the evaporation of chloroform) to 30 ml./min. Cholestane (or

in a few instances a secondary standard sample) was included in all solutions for injection and the retention times were expressed as ratios relative to cholestane: values in Table I are the means of at least two determinations on each column.

Reproducibility of data.

The reproducibility of relative retention values measured at intervals over several months was good for most compounds: variations noted for some di- and tri-ketones were evidently due to their susceptibility to minor temperature changes (cf. Table 9). The following data are illustrative: listed in order are the mean relative retention values (Table 1), S.D. and number of determinations (in parentheses).

SE-30:- 5α-Pregnane, 0.148, 0.001 (8); 5α-pregnan-20-one, 0.278, 0.002 (10); 5β-pregnan-3α-ol, 0.283, 0.003 (5); androst-4-ene-3,17-dione, 0.452, 0.003 (9); 3α-hydroxy-5α-androstan-17-one, 0.336, 0.002 (7); 5α-pregnane-3β, 20α-diol, 0.652, 0.004 (4); 5α-pregnane-3,11,20-trione, 0.884, 0.002 (7).

QF-1:- 5α-Pregnane, 0.206, 0.003 (13); 5α-androstan-3-one, 0.805, 0.003 (14); 5α-androstan-17β-ol, 0.388, 0.005 (7); 5α-pregnane-3,20-dione, 6.30, 0.032 (6); 17β-hydroxy-5α-androstan-3-one, 2.630, 0.017 (11); 5β-pregnane-3α,20β-diol, 1.806, 0.027 (7);

androst-4-ene-3,11,17-trione, 12.64, 0.28 (7).

The efficiency of the SE-30 column was approximately 3,000 theoretical plates for cholestane, 1,700 for progesterone: neither value changed during three months' use. The QF-1 column was much less efficient: values of 1,200-1,500 were found for ketones, while cholestane afforded values as low as 600.

Results and Discussion.

Table 1 summarises retention data observed, relative to cholestane, with each of the two columns (stationary phases SE-30 and QF-1) under standard conditions at 200°. Before the results are examined in terms of "group retention factors" as defined by Clayton (4,5) the following comments are pertinent to indicate the main features of the two silicones used as stationary phases. The almost non-polar character of SE-30 is strikingly shown by the high affinity it shows for the steroid skeleton: cholestane is retained for 30 minutes as compared with 6 minutes on QF-1. Moreover, the retention of an oxygenated steroid on SE-30 is primarily dependent on its molecular weight and on the number of oxygen functions; their alcoholic or ketonic nature, and their situation (with a few exceptions) are of secondary influence. The greater selectivity resulting from the polar structure of QF-1

Table 1. Relative retention data at 200° (cholestane 1.00).

Gifts of steroids are acknowledged individually in the footnotes. Relative retention values (cholestane 1.00) are means of at least two determinations on each column and are cited to 3 decimal places to avoid rounding-off errors in the calculation of retention factors. Retention time of cholestane: 30-33 min. on SE-30, 5.5-6.0 min. on OF-1. Asymmetric peaks are indicated by asterisks.

	Relative Retention	
	SE-30	OF-1
Androstanes.		
5α-Androstane[a]	0.078	-
5α-Androstan-3-one	0.175	0.805
5α-Androstan-17-one[a]	0.161	0.622
5α-Androstan-3β-ol[a]	0.163	0.427
5α-Androstan-17α-ol[a]	0.163	0.368
5α-Androstan-17β-ol[a]	0.166	0.388
5α-Androstane-3,17-dione	0.366	4.649
3α-Hydroxy-5α-androstan-17-one	0.336	1.957
3β-Hydroxy-5α-androstan-17-one	0.338	2.249
17β-Hydroxy-5α-androstan-3-one	0.386	2.630

(continued overleaf)

5α-Androstane-3β,17β-diol	0.353	1.344
5α-Androstane-3,11,17-trione	0.475	9.036
5β-Androstane[a]	0.071	-
5β-Androstan-17-one[a]	-	0.5501
5β-Androstan-3α-ol[a]	0.147	0.388
5β-Androstane-3,17-dione	0.328	4.306
3α-Hydroxy-5β-androstan-17-one	0.306	2.051
17β-Hydroxy-5β-androstan-3-one	0.344	2.422
5β-Androstane-3α,17β-diol	0.322	1.202
5β-Androstane-3,11,17-trione[c]	0.410	8.043
3α-Hydroxy-5β-androstane-11,17-dione[c]	0.392	3.984
3α,11β-Dihydroxy-5β-androstan-17-one[e]	-	4.016
3α,17β-Dihydroxy-5β-androstan-11-one[e]	-	3.046
5α-Androst-2-en-17-one[a]	0.155	0.631
Androst-4-en-3-one[a]	0.221	1.287
Androst-4-ene-3,17-dione	0.452	7.247
17α-Hydroxyandrost-4-en-3-one[d]	0.478	3.893
17β-Hydroxyandrost-4-en-3-one	0.483	4.144
Androst-4-ene-3,6,17-trione	0.642	18.5
Androst-4-ene-3,11,17-trione	0.555	12.64
11β-Hydroxyandrost-4-ene-3,17-dione	0.822	14.14

(continued overleaf)

Androst-5-en-3β-ol[a]	0.163	0.396
3β-Hydroxyandrost-5-en-17-one	0.332	1.990
Androst-5-en-17β-ol	0.167	0.374
Androst-5-ene-3β,17β-diol[c]	0.353	1.242
Pregnanes.		
5α-Pregnane[e]	0.148	0.206
5α-Pregnan-3-one[a]	0.335	1.381
5α-Pregnan-11-one[a]	0.201	0.536
5α-Pregnan-20-one[a]	0.278	0.861
5α-Pregnan-3α-ol[a]	0.304	0.647
5α-Pregnan-3β-ol[a]	0.311	0.733
5α-Pregnan-20α-ol[a]	0.305	0.642
5α-Pregnan-20β-ol[a]	0.282	0.566
5α-Pregnane-3,20-dione	0.636	6.295
3β-Hydroxy-5α-pregnan-20-one	0.593	3.143
20β-Hydroxy-5α-pregnan-3-one	0.644	3.837
5α-Pregnane-3β,20α-diol	0.652	2.268
5α-Pregnane-3β,20β-diol	0.598	2.010
5α-Pregnane-3,11,20-trione	0.884	15.37
3β-Hydroxy-5α-pregnane-11,20-dione	0.856	8.396
5β-Pregnane[a]	0.134	0.181
5β-Pregnan-20-one[a]	0.251	0.779

(continued overleaf)

5β-Pregnan-3α-ol[a]	0.283	0.653
5β-Pregnan-3β-ol[a]	0.279	0.574
5β-Pregnane-3,20-dione	0.568	5.754
3α-Hydroxy-5β-pregnan-20-one	0.531	2.821
3β-Hydroxy-5β-pregnan-20-one	0.523	2.486
5β-Pregnane-3α,20α-diol	0.589	2.065
5β-Pregnane-3α,20β-diol	0.544	1.806
5β-Pregnane-3,11,20-trione[c]	0.769	13.38
5α-Pregn-2-en-20-one[a]	0.272	0.876
5α-Pregn-2-ene-11,20-dione[d]	0.396	2.330
11α-Hydroxy-5α-pregn-2-en-20-one[d]	0.484	2.166
16α-Methyl-5α-pregn-2-en-20-one[d]	0.275	0.832
16α-Methyl-5α-pregn-2-ene-11,20-dione[d]	0.387	2.132
Pregn-4-en-3-one[a]	0.420	2.191
Pregn-4-ene-3,20-dione	0.791	9.936
Pregn-4-ene-3,6,20-trione	1.173	-
11α-Hydroxypregn-4-ene-3,20-dione	1.510	24.51
11β-Hydroxypregn-4-ene-3,20-dione	1.469	21.47
3β-Hydroxypregn-5-en-20-one	0.585	2.831
5β-Pregn-11-ene-3,20-dione[e]	-	4.283
Pregna-4,9-diene-3,20-dione[e]	-	8.229

(continued overleaf)

Cholanes		
5α-Cholane[b]	0.403	0.475
5α-Cholan-12-one[b]	0.721	1.857
5α-Cholan-12α-ol[b]	0.706	1.096
5β-Cholane[b]	0.365	0.431
5β-Cholan-7-one[b]	0.655	
5β-Cholan-12-one[b]	0.648	1.581
5β-Cholan-7α-ol[b]	0.672	
5β-Cholan-12α-ol[b]	0.618	0.918
5β-Cholan-12β-ol	0.624	
Cholestanes		
5α-Cholestane	1.000	1.000
5α-Cholestan-3-one	2.291	6.953
5α-Cholestan-6-one	2.049	5.423
5α-Cholestan-7-one	-	4.666
5α-Cholestan-3α-ol	2.120	3.237
5α-Cholestan-3β-ol	2.135	3.662
5α-Cholestane-3,6-dione	4.223*	-
5β-Cholestane	0.903	0.897
5β-Cholestan-3-one[e]	2.055	6.277
5β-Cholestan-3α-ol[e]	1.918	3.240

(continued overleaf)

5β-Cholestan-3β-ol[f]	1.892	2.829
5α-Cholest-2-ene	-	1.006
5α-Cholest-8(9)-ene	-	1.00
Cholest-4-en-3-one	2.891*	11.25
Cholest-4-ene-3,6-dione	4.414*	-
Cholest-5-ene	0.999	0.967
Cholest-5-en-3-one	-	11.18
Cholest-5-en-3β-ol	2.120	3.327
5α-Cholest-7-en-3β-ol	-	3.888
Cholesta-3,5-dien-7-one	-	7.098

[a] Professor W. Klyne (London)

[b] Drs. M. Martin-Smith and M. Alauddin (Glasgow)

[c] Drs. J.K. Norymberski and E. Menini (Sheffield)

[d] Drs. C.L. Hewett and G.F. Woods (Organon Laboratories)

[e] Drs. E.C. Horning and W.J.A. VandenHeuvel (Houston, Texas)

[f] Dr. R.P. Cook (Dundee).

is shown by the marked variations in retention of functional group isomers and by the much stronger retention of ketones than of alcohols.

Behaviour of steroids epimeric at $C_{(5)}$.

It was expected that the difference in selectivity of SE-30 and QF-1 would be least apparent in the separation factor for epimeric pairs of 5α- and 5β-steroids since such comparisons involve no formal change in functional groups. The reactivity and accessibility of functional groups at positions 11, 17 or 20 (and of a carbonyl group at position 3) are not markedly affected by the change in nuclear configuration at $C_{(5)}$. The data for 25 pairs of saturated steroids in Table 2 bear out this view: for the pairs 1-13, comprising steroids unsubstituted in ring A and 3-oxosteroids, a mean separation factor (i.e. relative retention) of 1.12 (S.D. < 0.03) is observed on both columns. A very similar factor (1.10) has been found on SE-30 at 225°. These results are in good agreement with the values of 1.13 (on SE-30, 210°), from the data of VandenHeuvel, Sjövall and Horning (12) and 1.1 (on QF-1, 250°) found by Knights and Thomas (8). On SE-30, 3α- and 3β-hydroxyl substituents have a scarcely discernible influence (Nos. 14-19: 1.11, S.D. 0.02), but on QF-1

Table 2. Regularities in Separation Factors for 5α - and 5β - Steroids on SE-30 and OF-1 columns at 200°

The separation factor is the ratio of the retention of the 5α - steroid to that of its 5β-epimer.

5α/5β Epimers		Separation Factor SE-30	OF-1
1.	Androstane	1.11	-
2.	Pregnane	1.11	1.14
3.	Pregnan-20-one	1.11	1.11
4.	Cholane	1.10	1.10
5.	Cholan-12-one	1.11	1.19
6.	Cholan-12α-ol	1.14	1.17
7.	Cholestane	1.11	1.11
8.	Androstane-3,17-dione	1.12	1.08
9.	17β-Hydroxyandrostan-3-one	1.12	1.09
10.	Androstane-3,11,17-trione	1.16	1.12
11.	Pregnane-3,20-dione	1.12	1.09
12.	Pregnane-3,11,20-trione	1.15	1.15
13.	Cholestan-3-one	1.11	1.11
14.	3α-Hydroxyandrostan-17-one	1.10	0.95
15.	Pregnan-3α-ol	1.07	0.99
16.	Cholestan-3α-ol	1.11	1.00
17.	Pregnan-3β-ol	1.11	1.28
18.	3β-Hydroxypregnan-20-one	1.13	1.26
19.	Cholestan-3β-ol	1.13	1.29

Table 2 (continued)

5α/5β Epimers		Separation Factor SE-30	OF-1
20.	3α-Hydroxyandrostan-17-one 3-acetate*	1.02	1.06
21.	Pregnan-3α-ol 3-acetate*	1.00	1.08
22.	Cholestan-3α-ol 3-acetate*	1.00	1.08
23.	Pregnan-3β-ol 3-acetate*	1.23	1.18
24.	3β-Hydroxypregnan-20-one 3-acetate*	1.23	1.17
25.	Cholestan-3β-ol 3-acetate*	1.22	1.17

* Compounds 20-25 were prepared by acetylation of compounds 14-19 and were not isolated; relative retention data are in Table 7.

factors of 0.98 and 1.28, respectively, are observed in the presence of these groups. This ensues from the preferential retention of equatorial alcohols by QF-1, as discussed below with reference to Table 4. It is notable that on QF-1, acetylation of the 3-hydroxyl group distinctly reduces this selectivity, while on SE-30, acetylation leads to a marked divergence of retention factors. These observations again illustrate the different character of the two stationary phases, SE-30 responding essentially to molecular skeletal size and shape, and QF-1 interacting selectively with substituent groups. The exceptional elution of 3α-hydroxy-5α-androstan-17-one acetate before its 5β-isomer on a polyester column (13) may now be construed as a result of selective retention of the equatorial acetoxyl group in the latter compound.

The results in Table 2 also show that no significant difference is observed between the factors for androstane, pregnane, cholane and cholestane derivatives as expected from Clayton's work and in agreement with the data of Knights and Thomas (8). A wide range of molecular weights has been surveyed in one series of compounds, affording retention factors (on SE-30, at 200° and 225°) for the introduction of the 3β-hydroxyl group (Table 3). In

Table 3. Changes in relative retention accompanying the introduction of the 3β-hydroxyl group into steroids of the androstane, pregnane, cholestane and ergostane series.

The column used was 1% SE-30 on Gas-Chrom P. The retention data are expressed relative to cholestane (11-12 min. at 225° ; 32-33 min. at 200°) = 1.00.

	225°			200°		
	Relative retention		Factor due to	Relative retention		Factor due to
	3β-OH	3-H	3β-OH	3β-OH	3-H	3β-OH
5α-Androstane	-	-	-	0.163	0.078	2.09
5α-Androstan-17-one	0.40	0.20	1.96	0.338	0.161	2.10
5α-Androstan-17β-ol	0.41	0.21	1.93	0.353	0.166	2.12
Androst-5-en-17β-ol	0.40	0.21	1.94	0.353	0.167	2.12
5α-Pregnane	-	-	-	0.311	0.148	2.10
5α-Pregnan-20-one	0.64	0.33	1.96	0.593	0.278	2.13
5α-Pregnan-20α-ol	0.70	0.35	2.00	0.652	0.305	2.14
5α-Pregnan-20β-ol	0.65	0.33	1.95	0.598	0.282	2.12
5β-Pregnane	-	-	-	0.279	0.134	2.09
5β-Pregnan-20-one	0.58	0.30	1.90	0.523	0.251	2.09

	225°			200°		
	Relative retention		Factor due to	Relative retention		Factor due to
	3β-OH	3-H	3β-OH	3β-OH	3-H	3β-OH
5α-Cholestane	1.97	1.00	1.97	2.135	1.000	2.14
5β-Cholestane	1.76	0.91	1.94	1.892	0.903	2.10
Cholest-5-ene	1.96	1.00	1.96	2.120	0.999	2.12
5α-Cholestan-6-one	3.76	1.93	1.95	-	-	-
5α-Ergost-7-ene*	2.94	1.47	2.00	-	-	-
5α-Ergost-8(14)-ene*	2.54	1.28	1.98	-	-	-
5α-Ergosta-7,22-diene*	2.48	1.25	1.98	-	-	-
Mean values:			1.96 (S.D. 0.03)			2.11 (S.D. 0.02)

* Compounds provided by Dr. P. Bladon (Glasgow)

view of the variety of functional groups present, the results are simultaneously a test of vicinal interactions; nevertheless, it is clear that neither the molecular weights nor vicinal effects seriously impair the constancy of the retention factor for introduction of the 3β-hydroxyl group on SE-30. The mean values observed are in reasonable agreement with the comparable data for cholestan-3β-ol given by VandenHeuvel et al. (3,12). It is notable that distinctly lower retention factors are recorded by Sweeley and Lo Chang(14) and Bloomfield (15) who used Chromosorb W as support material rather than Gas-Chrom P.

Functional-group retention factors.

The main correlations arising from the data of Table 1 are summarised in Table 4* in the form of retention factors for the introduction of hydroxyl and oxo groups. Less than half the data were derived from direct comparisons of monofunctional with parent compounds; in the remainder, the presence of other substituents is indicated where they appear to have a marked effect on the retention factors. Compounds with more obvious interactions between substituents have been excluded. The principal features of the results are as follows.

* Detailed tables illustrating the derivation of the factors are given in Appendix 1.

Table 4. Retention factors due to introduction of hydroxyl and oxo groups in steroids (C_{19}-C_{27}) at 200°

Group	Other relevant groups	SE-30			QF-1		
		No. of examples	Retention factor Range	Mean	No. of examples	Retention factor Range	Mean
3α-Hydroxyl	5α-Hydrogen	3	2.06-2.12	2.09	3	3.14-3.24	3.18
	5β-Hydrogen	4	2.08-2.12	2.11	3	3.60-3.62	3.61
3β-Hydroxyl	5α-Hydrogen	8	2.09-2.14	2.12	7	3.46-3.66	3.58
	5β-Hydrogen	3	2.09-2.10	2.09	3	3.15-3.19	3.17
	Δ^5	2	2.11-2.12	2.12	2	3.32-3.44	3.38
11α-Hydroxyl	-	2	1.78-1.91	1.84	2	2.47	2.47
11β-Hydroxyl	-	2	1.82-1.86	1.84	2	1.95-2.16	2.06
17α-Hydroxyl	-	2	2.09-2.16	2.13	1	3.02	3.02
17β-Hydroxyl	-	4	2.13-2.19	2.16	3	3.10-3.15	3.13
	3-Oxo	2	2.19-2.20	2.20	2	3.22-3.27	3.24
20α-Hydroxyl	-	3	2.06-2.10	2.08	3	3.10-3.17	3.13

Table 4 (continued)

Group	Other relevant groups	SE-30 No. of examples	SE-30 Retention factor Range	SE-30 Retention factor Mean	OF-1 No. of examples	OF-1 Retention factor Range	OF-1 Retention factor Mean
20β-Hydroxyl	-	4	1.91-1.93	1.92	4	2.74-2.78	2.76
3-oxo	-	6	2.24-2.32	2.28	5	6.71-7.00	6.84
	17- or 20-Oxo	3	2.27-2.29	2.28	3	7.31-7.47	7.39
6-oxo	-	1	2.05	2.05	1	5.42	5.42
	3-oxo	1	1.84	1.84	-	-	-
	Δ^4-3-oxo	3	1.42-1.53	1.48	-	-	-
7-oxo	-	1	1.80	1.80	1	4.67	4.67
11-oxo	-	1	1.36	1.36	1	2.60	2.60
	20-Oxo	5	1.35-1.46	1.41	5	2.33-2.67	2.53
	17-Oxo	4	1.23-1.30	1.27	4	1.75-1.94	1.88
	17β-Hydroxyl	-	-	-	1	2.53	2.53
12-oxo	-	2	1.78-1.79	1.79	-	-	-

Table 4 (continued)

Group	Other relevant groups	SE-30 No. of examples	SE-30 Retention factor Range	SE-30 Retention factor Mean	OF-1 No. of examples	OF-1 Retention factor Range	OF-1 Retention factor Mean
17-oxo	-	4	2.04-2.08	2.06	3	5.02-5.29	5.19
	3-oxo	2	2.05-2.09	2.07	2	5.63-5.78	5.70
20-oxo	-	5	1.87-1.91	1.88	5	4.18-4.33	4.28
	3-oxo	2	1.88-1.90	1.89	2	4.54-4.56	4.55
Δ^4-3-oxo	-	6	2.82-2.90	2.85	5	10.64-11.65	11.2

(1) On SE-30, the retention factors for hydroxyl substitution at position 3α or 3β in both 5α- and 5β-steroids are all very similar, whereas on OF-1 the equatorial hydroxyl steroids (3α, 5β- and 3β, 5α-) show markedly longer retention than their axial epimers, and can be separated therefrom.

(2) 17α-, 17β- and 20α-hydroxyl retention factors are hardly distinguishable from one another (or from axial 3-hydroxyl) on either phase.*

(3) The sterically hindered 11α- and 11β-hydroxy steroids and to a lesser extent the 20β-hydroxy steroids, show distinctly lower retention factors; 20-epimers are resolvable even on SE-30.

(4) On both phases, the retention factors for ketones

* Conversion to the acetate and propionate (16) still does not distinguish between these isomers. In the estrogen series, separation of 17α and 17β-estradiol is only effected on a phenyl silicone phase, PhSi, (separation factor 1.1) but conversion to the trimethylsilyl ethers allows their separation on SE-30, PhSi, OF-1 and NGS (17).

diminish in the sequence: Δ^4-3 > 3 > 17 > 20 > 11; the separations are small on SE-30 but are greatly enhanced on QF-1, evidently through a combination of polar and steric effects. Thus the relative retention factor of the Δ^4-3-oxo group is increased fourfold, that of the 3-oxo group threefold, and that of the 11-oxo group less than twofold on QF-1 as compared with SE-30. The relationship between structure and reactivity in steroids as shown by infrared and other physical and chemical studies is referred to below.

(5) Hydroxy and keto steroids have generally similar retentions on SE-30, but the keto steroids are retained relatively more strongly on QF-1. However, the 11-oxo steroids are eluted before the corresponding 11β hydroxy compounds (in the few examples where the latter are available for comparison) on QF-1 as on SE-30.

(6) Vicinal effects are more noticeable on QF-1, as expected from its greater reactivity towards functional groups. This is particularly illustrated by the retention factors for 17-oxo and 20-oxo groups in

the presence of a 3-oxo group. Consequently SE-30 is more suitable for the initial analysis of unknown steroids, and QF-1 for subtler investigations when a tentative structure can be entertained.

The only examples in which androstane and pregnane derivatives have yielded different retention factors are the 11-oxo-steroids (Table 5). The apparent distinction may be due to the presence of a 17-oxo group in the androstane derivatives examined because in the single example with a 17β-hydroxyl substituent the 11-oxo factor is similar to that in the pregnane series. Knights and Thomas (16, 32) have found a similar difference in 11-oxo factor for their androstane and pregnane derivatives.

This behaviour of 11-oxo steroids on gas-liquid chromatography is not unexpected in view of the different properties they have shown in other physico-chemical studies. The non-additivity of circular dichroism curves in three examples of 11-oxo steroids was interpreted as being due to interaction of the chromophore at 11 with 17-oxo and Δ^4-3-oxo groups (18). Similar behaviour was noted in rotary dispersion studies as mentioned by Giroud et al. (18). On this basis the lower 11-oxo factor for the 17-oxo androstane derivatives (Table 5) may be interpreted as resulting from interaction of the 11-oxo group with the 17-oxo group in the saturated

Table 5. Retention factors due to the introduction of the 11-oxo group into steroids of the androstane and pregnane series.

The columns were operated at 200°.

Parent compound	SE-30 11-oxo factor	OF-1 11-oxo factor
5α-Androstane-3,17-dione	1.30	1.94
5β-Androstane-3,17-dione	1.25	1.87
3α-Hydroxy-5β-androstan-17-one	1.28	1.94
Androst-4-ene-3,17-dione	1.23	1.75
5β-Androstane-3α,17β-diol	-	2.53
5α-Pregnane	1.36	2.60
5α-Pregnane-3,20-dione	1.39	2.44
3β-Hydroxy-5α-pregnan-20-one	1.44	2.67
5β-Pregnane-3,20-dione	1.35	2.33
5α-Pregn-2-en-20-one	1.46	2.66
16α-Methyl-5α-pregn-2-en-20-one	1.41	2.56

compounds and with both the 17-oxo and Δ^4-3-oxo groups in the corresponding unsaturated compound. Turner (19) has studied the condensation of benzaldehyde with 3-oxo steroids to give 2-benzylidene derivatives and has expressed his data in terms of "group rate factors" i.e. rates of reaction relative to cholestan-3-one. He noted that the presence of an 11-oxo or 11-hydroxyl group in the steroid depressed the rate of condensation of the 3-oxo group with benzaldehyde and interpreted these results in terms of "conformational transmission" i.e. the long range effects arising from the transmission of strain in fused ring systems.

The sequence of diminishing retention factors given in (4) above for SE-30 and QF-1 liquid phases is a consequence of solute-solvent (i.e. solute-liquid phase) interactions which depend on the type, position and stereochemical configuration of substituent groups in the solute molecule and on the nature of the liquid phase. This sequence for keto steroids is the same as that found by Hamilton et al. (20) for QF-1 and NGS columns. Neher (21) has found an analogous series in the sensitivity of keto steroids towards dinitrophenylhydrazine used as a specific colour reaction in paper chromatography. The sequence in decreasing order of sensitivity is: Δ^4-3-oxo > 3-oxo > 20-oxo > 17-oxo; 11-oxo steroids do not

react at all.

While investigating solvent effects on carbonyl frequencies by infrared spectroscopy (Section 2, Part B of this thesis) it was found that for keto steroids the sequence of carbonyl stretching frequencies was as follows:

17-oxo > 3-oxo ≫ 6-oxo > 7-oxo ≫ 12-oxo > 11-oxo

The order of gas-chromatographic elution of keto steroids closely parallels the order of their carbonyl stretching frequencies. For an SE-30 liquid phase, the sequence of retention factors is as follows:

3-oxo > 17-oxo > 6-oxo ≫ 7- and 12-oxo > 11-oxo

This represents the order of increasing steric congestion of the carbonyl groups.

Retention factors due to acylation

While the use of two or more different columns affords the best means of separating mixtures, in practice the changing of columns in a single Chromatograph may be inconvenient. It is then of value to effect chemical modifications, e.g. the selective conversion of hydroxyl groups into ester or ether groupings, leaving oxo groups unchanged, or the characterisation of oxo groups as N,N-dimethyl hydrazones (22). The changes in retention

characteristic of such transformations are useful for the identification as well as for the separation of steroids. Thus the retention factor accompanying trifluoroacetylation of 3β-hydroxy 5α-steroids appears to be 0.79 ± 0.02 (on SE-30 at 222°) from four examples cited by VandenHeuvel et al. (12); this derivative permits a decrease in the retention time. Simple acetylation of the 3β-hydroxyl group in the group of normal sterols listed in Table 6 is characterised by a mean retention factor of 1.47 on SE-30 at 225°; variations in substituents as close as $C_{(5)}$ cause only minor changes. The five 4,4-dimethyl sterols examined, on the other hand, afford a mean factor of 1.36, suggesting that the steric effect of the methyl groups, already well known in the retention behaviour of the free sterols (4,5,6,23) is enhanced in the bulkier acetates. The retention factors are sufficiently different to be of diagnostic value in the study of naturally occurring sterols.

The influence of the configuration of $C_{(3)}$ and $C_{(5)}$ on the retention factor for acetylation is indicated in Table 7. On the SE-30 column, axial and equatorial 3-hydroxyl substituents are distinguished by acetylation factors of 1.45 (S.D. < 0.03) and 1.58

Table 6. Retention factors due to acetylation of the 3β-hydroxyl group.

Chromatography was effected on the SE-30 column at 225°.

Sterol	Relative retention 3β-OH	Relative retention 3β-OAc	Factor due to acetylation
Cholestanol	1.97	2.91	1.48
Cholesterol	1.96	2.88	1.47
Cholest-7-enol	2.20	3.26	1.48
Desmosterol	2.17	3.12	1.44
7β-Hydroxycholestanol	3.45	5.20	1.51
7-Oxocholesterol	4.01	5.62	1.40
24-Hydroxycholesterol	3.66	5.42	1.48
Ergosterol	2.45	3.62	1.47
Stigmasterol	2.83	4.20	1.49
Mean value of factor for normal sterols:			1.47 (S.D. 0.03
4,4-Dimethylsterol			
Lanost-7-enol	3.60	4.93	1.37
Lanost-8-enol	3.06	4.16	1.36
Lanosterol	3.40	4.58	1.35
Agnosterol	3.18	4.35	1.37
Dihydroagnosterol	2.88	3.96	1.37
Mean value for 4,4-dimethylsterols:			1.36 (S.D. 0.01

The experimental work on which this table is based was carried out by Dr. C.J.W. Brooks.

Table 7. Retention factors due to acetylation of 3-hydroxysteroids*.

	SE-30, 200°			QF-1, 200°		
	Relative retention		Ac Factor	Relative retention		Ac Factor
	OH	OAc		OH	OAc	
Axial: 3α, 5α.						
Androstan-17-one	0.366	0.474	1.41	1.957	3.270	1.67
Pregnane	0.304	0.436	1.43	0.647	1.034	1.60
Cholestane	2.120	3.089	1.46	3.237	5.18	1.60
Axial: 3β, 5β.						
Pregnane	0.279	0.406	1.46	0.574	0.994	1.73
Pregnan-20-one	0.523	0.769	1.47	2.486	4.42	1.78
Cholestane	1.892	2.806	1.48	2.829	4.90	1.73
Equatorial: 3β, 5α.						
Pregnane	0.311	0.499	1.60	0.733	1.18	1.61
Pregnan-20-one	0.593	0.943	1.59	3.143	5.16	1.64
Cholestane	2.135	3.411	1.60	3.662	5.718	1.56
Equatorial: 3α, 5β.						
Androstan-17-one	0.306	0.467	1.53	2.051	3.083	1.50
Pregnane	0.283	0.437	1.54	0.653	0.961	1.47
Cholestane	1.918	3.077	1.60	3.240	4.81	1.49

* Acetates were prepared from 50 μg. of hydroxysteroid and were not isolated.

(S. D. < 0.04) respectively. The situation with QF-1 is more complex, owing to the interplay of steric effects and of the relative affinities of hydroxyl and acetoxyl groups for this phase. Thus the axial 3β-acetoxyl group in 5β-steroids is retained almost as strongly as the equatorial 3β-acetoxyl group in 5α-steroids, and yields a high and characteristic acetylation factor of 1.75. The epimeric 3-acetoxysteroids in the 5α-series afford practically identical factors on this phase. Knights and Thomas (24) using a 6% QF-1 phase at 250° show a similar order of acetylation factors for epimeric 5α and 5β 3-hydroxysteroids. It is evident from Table 7 that the stereochemistry of a 3-hydroxysteroid is assignable with high probability from a knowledge of the retention factors associated with acetylation both on SE-30 and QF-1, together with the relative retention data.

Effect of molecular weight.

As a corollary of the regularities demonstrated above, particularly with reference to 3-substituents, it is confirmed that retention factors are generally similar in androstane, pregnane, cholane and cholestane derivatives. Within each homologous series the logarithm of the relative retention time is linearly related to the molecular weight, and it thus follows that the various

sets of homologues afford lines of approximately equal slope (Figs. 1,2 and 3). For many of the steroids studied these are found to be:-

(i) on SE-30, 200° $\Delta(\log R_T) = 0.010 \times \Delta(M.W.)$

(on this phase, 11-oxosteroids behave as hydrocarbons of the same molecular weight).

(ii) on QF-1, 200° $\Delta(\log R_T) = 0.008 \times \Delta(M.W.)$

These relationships, combined with the appropriate group retention factors, permit the prediction of approximate retention data for a wide variety of steroids. For di- and trifunctional steroids the relationships may be modified by vicinal interactions.

Applications of retention data to the analysis of biological extracts

The analytical application of retention factors may be briefly illustrated by an examination of urinary steroid fractions kindly provided by Dr. J.K. Norymberski and Dr. E. Menini. The fractions had the following expected composition according to the scheme given below outlined by these authors (25):

Fraction KA : Di- and tri-oxo steroids of the androstane and pregnane series

Fraction K : Mono- and di-hydroxy-17-oxo androstanes, derived from urinary 17-hydroxycorticosteroids

Fraction KK : Fully oxidised compounds corresponding to those in K.

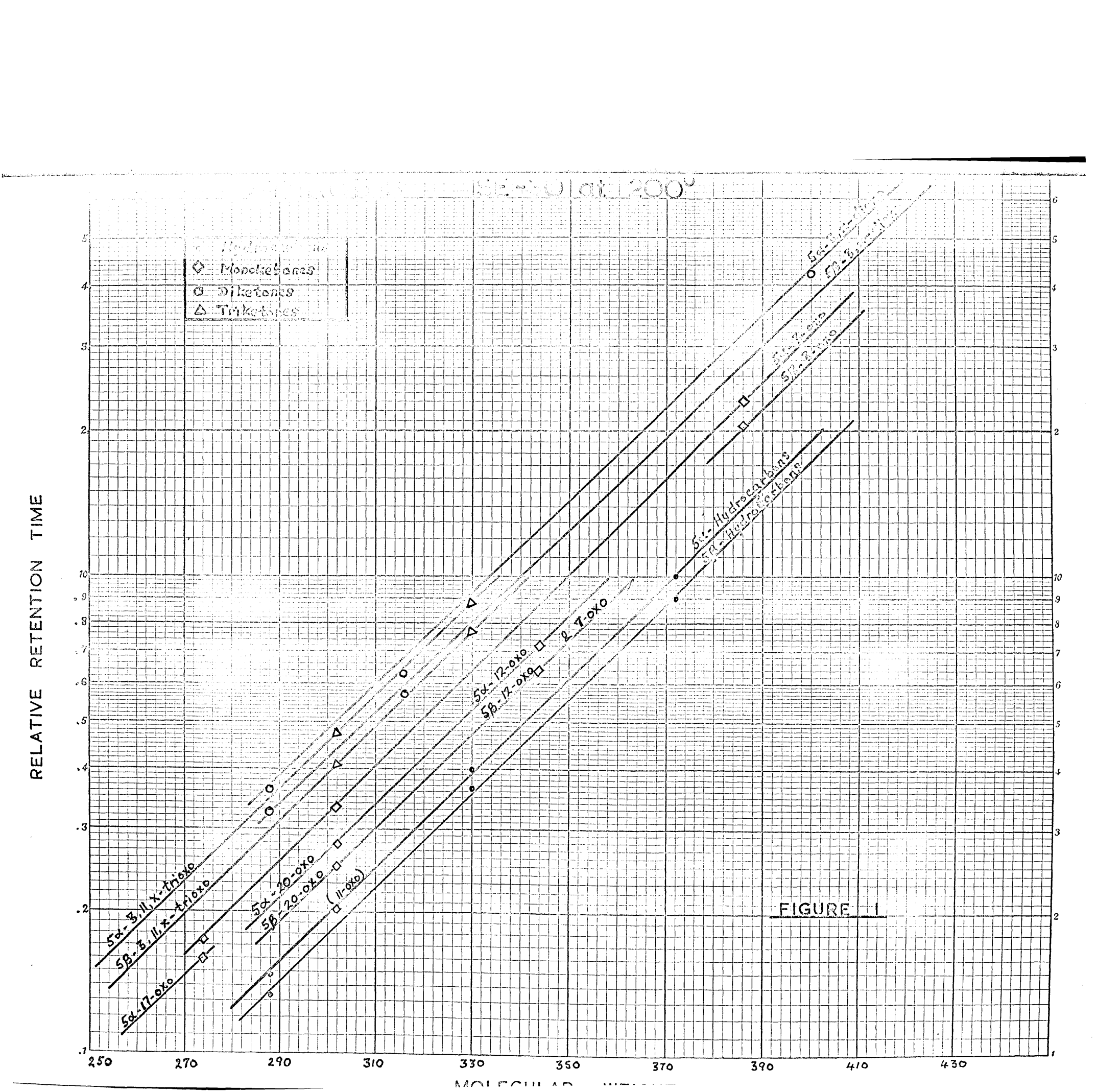

Hydrocarbons
◇ Monoketones
○ Diketones
△ Triketones
5α-Hydrocarbons
5β-Hydrocarbons
5α-3-one
5β-3-one
5α-12-oxo
5β-12-oxo
2,7-oxo
5α-3,11,x-trioxo
5β-3,11,x-trioxo
5α-17-oxo
5α-20-oxo
5β-20-oxo
(11-oxo)
RELATIVE RETENTION TIME
MOLECULAR WEIGHT
250
270
290
310
330
350
370
390
410
430
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
2
3
4
5
6
7
8
9
10

FIGURE 1

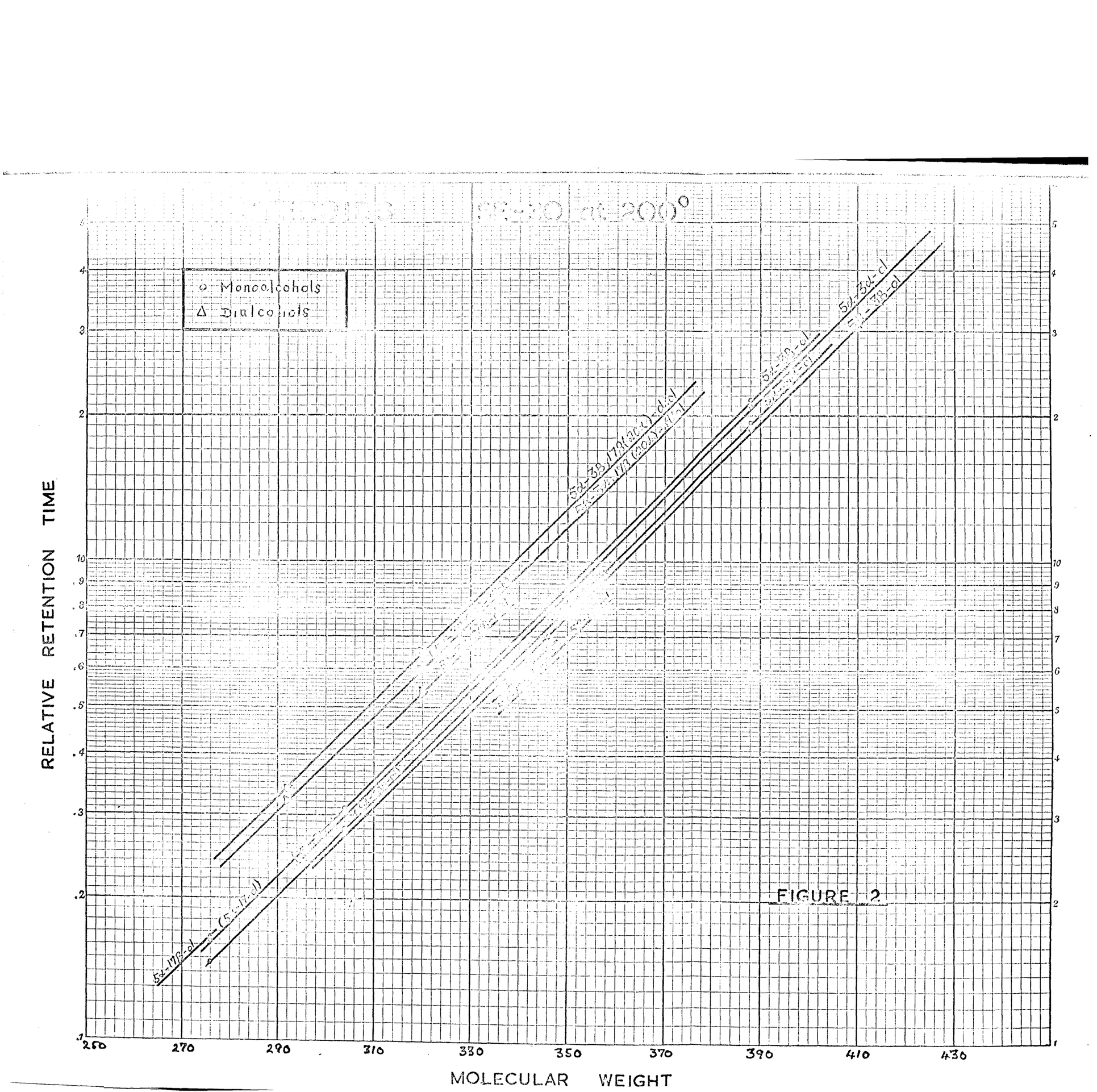

o Monoalcohols
Δ Dialcohols
5α-3β,17β(20α)-diol
5α-3β,17β(20β)-diol
5α-3β-ol
5α-3β-ol
5α-17β-ol
(5α-17-ol)
RELATIVE RETENTION TIME
MOLECULAR WEIGHT
250
270
290
310
330
350
370
390
410
430

FIGURE 2

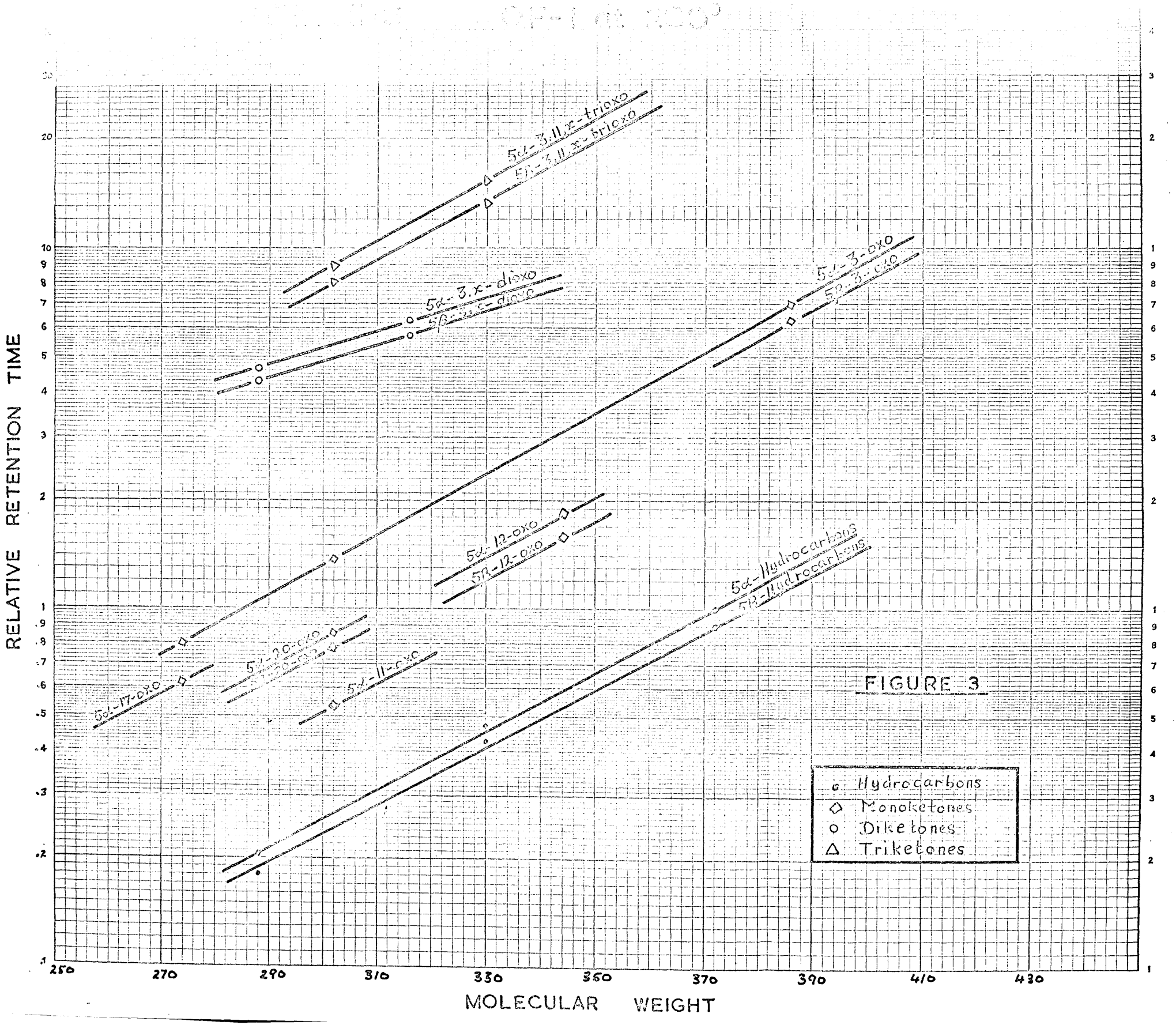

RELATIVE RETENTION TIME
MOLECULAR WEIGHT
250
270
290
310
330
360
370
390
410
430
5α-3,11,x-trioxo
5β-3,11,x-trioxo
5α-3,x-dioxo
5β-3,x-dioxo
5α-3-oxo
5β-3-oxo
5α-12-oxo
5β-12-oxo
5α-17-oxo
5α-11-oxo
5α-Hydrocarbons
5β-Hydrocarbons
Hydrocarbons
Monoketones
Diketones
Triketones

FIGURE 3

The scheme proposed by the above authors is as follows:

A urinary extract is first treated with potassium borohydride which reduces the keto steroids to the corresponding hydroxy steroids, followed by sodium bismuthate which degrades the side chain of 17-hydroxycorticosteroids to the corresponding 17-oxosteroids. The reaction products are separated into ketonic (K) and non-ketonic or alcoholic (A) fractions by the Girard process. Fractions K and A are then oxidized with tert-butylchromate to give a ketonic-ketonic fraction (KK) and a ketonic-alcoholic fraction (KA).

The results observed by chromatography of each fraction on SE-30 and on OF-1 at 200° are summarised in Table 8, and representative chromatograms are shown in Fig. 4. Most of the observed peaks could be tentatively identified by direct comparison with the data for the expected compounds. However, authentic samples of 3,11β-dihydroxy-5α- and 3β,11β-dihydroxy-5β-androstan-17-one expected in fraction K (from the 5-epimeric tetrahydro-derivatives of cortisone and hydrocortisone) were not available. The relative retentions computed for these compounds on SE-30 by using the factor 1.84 for the 11β-hydroxyl group are 0.61 and 0.56 respectively, in good agreement with strong peaks observed at 0.60 and 0.54. The assignment is strengthened by the observations of the two strongest peaks in KK (the oxidation product of K) at 0.47

Table 8. **Relative retention data and tentative structures of steroids contained in processed urinary extracts.**

Parentheses indicate less probable constituents because chemical processing should not permit them. The relative peak areas are indicated : w = weak, m = medium, s = strong, vs = very strong.

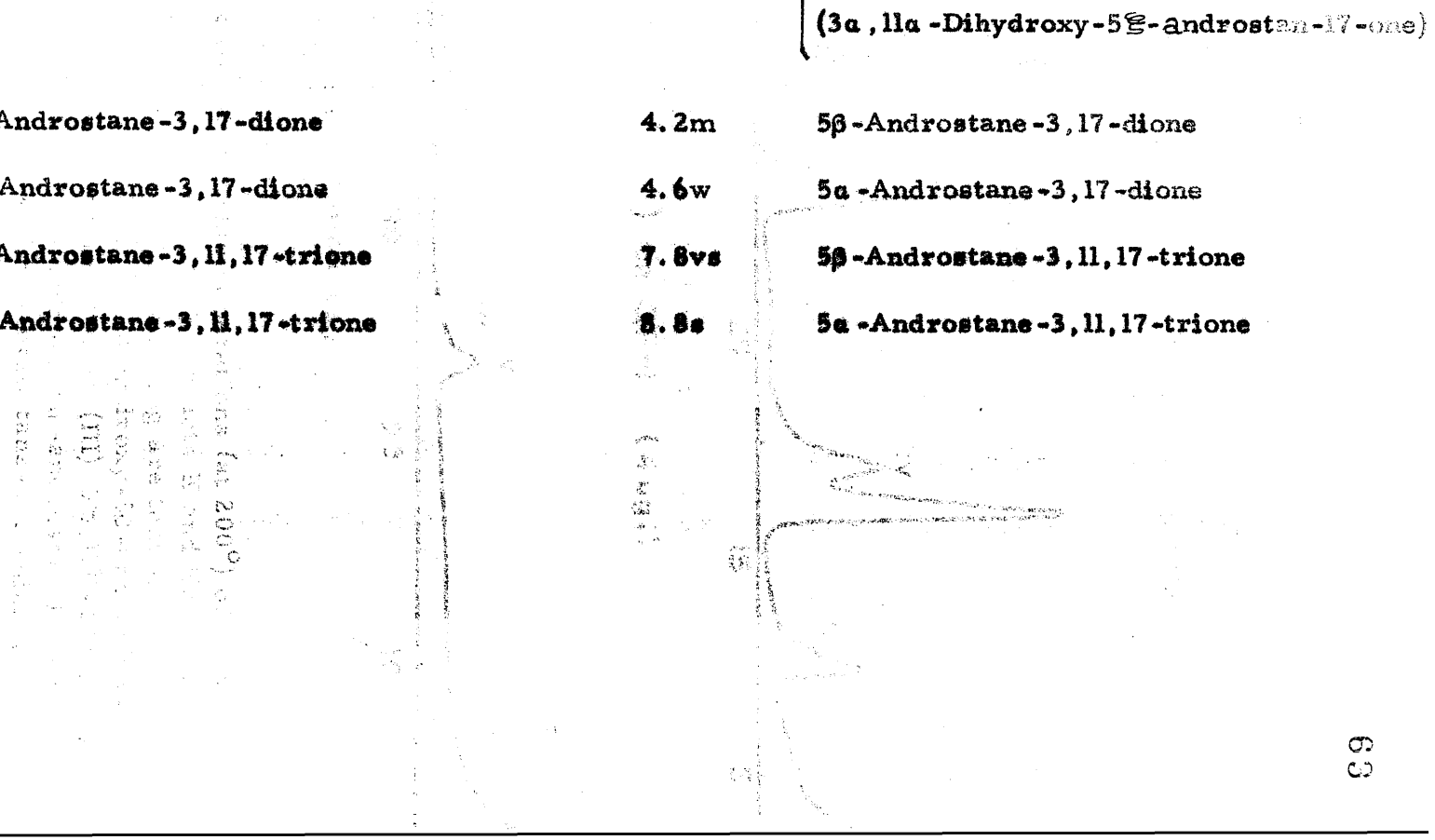
SE-30 (200°)		OF-1 (200°)	
Relative retention	Tentative structure	Relative retention	Tentative structure
Extract KA			
0.31m	(3≦-Hydroxy-5β-androstan-17-one)	1.93vs	(3≦-Hydroxy-5β-androstan-17-one) (3α-Hydroxy-5α-androstan-17-one) (3β-Hydroxy-androst-5-en-17-one)
0.33vs	(3≦-Hydroxy-5α-androstan-17-one) (3≦-Hydroxy-androst-5-en-17-one) 5β-Androstane-3,17-dione	4.23vs	5β-Androstane-3,17-dione
0.36s	5α-Androstane-3,17-dione	4.55vs	5α-Androstane-3,17-dione
0.57m	5β-Pregnane-3,20-dione	5.77m	5β-Pregnane-3,20-dione
0.41m	5β-Androstane-3,11,17-trione	8.07w	5β-Androstane-3,11,17-trione
0.47w	5α-Androstane-3,11,17-trione	9.18w	5α-Androstane-3,11,17-trione
Extract K			
0.31m	3≦-Hydroxy-5β-androstan-17-one	2.05s	3α-Hydroxy-5β-androstan-17-one 3α-Hydroxy-5α-androstan-17-one 3β-Hydroxy-androst-5-en-17-one

(continued overleaf)

SE-30 (200°)		QF-1 (200°)	
Relative retention	Tentative structure	Relative retention	Tentative structure
0.54vs	3ξ,11ξ-Dihydroxy-5β-androstan-17-one	4.0vs	3α 11β-Dihydroxy-5β-androstan-17-one
0.60s	3α,11ξ-Dihydroxy-5α-androstan-17-one		3α,11β-Dihydroxy-5α-androstan-17-one
	3β,11ξ-Dihydroxy-5α-androstan-17-one	4.8m	3β-11β-Dihydroxy-5α-androstan-17-one
			(3α,11α-Dihydroxy-5ξ-androstan-17-one)

Extract KK

0.33m	5β-Androstane-3,17-dione	4.2m	5β-Androstane-3,17-dione
0.36w	5α-Androstane-3,17-dione	4.6w	5α-Androstane-3,17-dione
0.40vs	5β-Androstane-3,11,17-trione	7.8vs	5β-Androstane-3,11,17-trione
0.47s	5α-Androstane-3,11,17-trione	8.8s	5α-Androstane-3,11,17-trione



SEPARATION OF OXO-STEROIDS DERIVED FROM A URINARY EXTRACT AT 200°

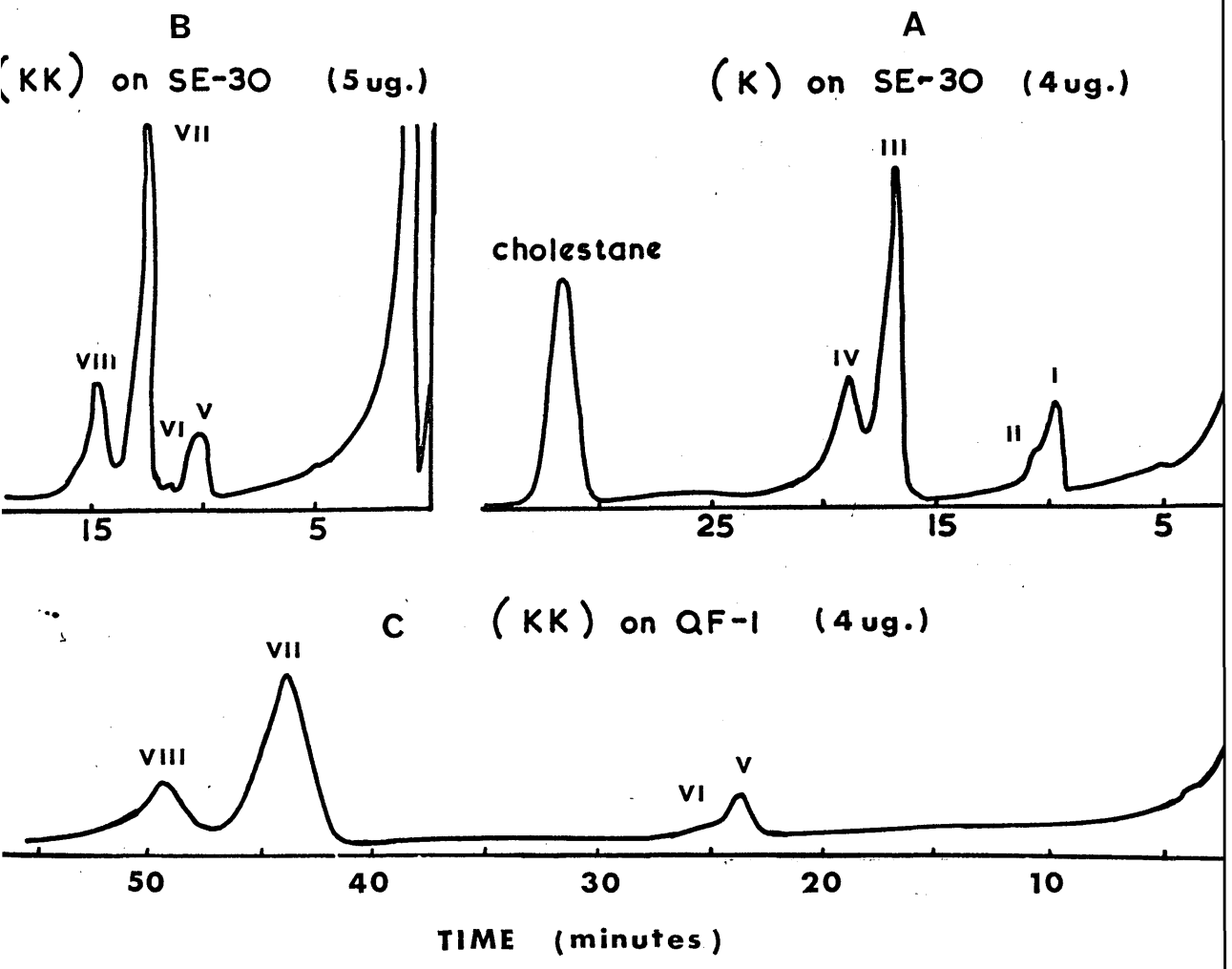


Fig. 4. Gas-liquid-chromatographic separations (at 200°) of keto steroids derived from a urinary extract. Extracts K and KK were prepared as indicated in the text. The following are tentative structures of the separated steroids: (I) 3ξ-hydroxy-5β-androstan-17-one; (II) 3ξ-hydroxy-5α-androstan-17-one; (III) 3ξ,11ξ-dihydroxy-5β-androstan-17-one; (IV) 3ξ,11ξ-dihydroxy-5α-androstan-17-one; (V) 5β-androstane-3,17-dione; (VI) 5α-androstane-3,17-dione; (VII) 5β-androstane-3,11,17-trione; (VIII) 5α-androstane-3,11,17-trione.

and 0.40: the conversion of the 11β-hydroxyl group into the 11-oxo group is exceptional in causing a reduction in retention time (cf. Fig. 4, A & B), and these values are in good agreement with the recorded values (Table 1) of 0.475 and 0.410 for 5α- and 5β-androstane-3, 11,17-trione respectively.

The complementary data for the OF-1 column strengthen some configurational assignments, as shown in Table 8. The strong peak observed at 4.0 in fraction K is probably 3α, 11β-dihydroxy-5β-androstan-17-one in comparison with the authentic sample observed at 4.02 (Table 1). On the whole, the retention values and the relative peak areas are satisfactorily accommodated by the interpretations given. In a few instances the assignments appear chemically implausible, but are worth recording to assist more detailed gas-chromatographic analysis. Rigorous identification of the components of the mixtures would of course demand their isolation: however, the gas chromatographic data alone may be sufficiently characteristic in many instances. Thus in the fully ketonic fraction KK (cf. Fig. 4, B & C), the data for the two columns are in complete agreement in confirming the expected composition, and also permit the approximate estimation of the relative proportions of each constituent.

Reliability of retention data.

The variability of relative retention data between laboratories is clearly important, and has been briefly discussed (23) for SE-30 columns. In the case of QF-1, the data collected in Table 9 indicate fair agreement except for di- and tri-oxosteroids, where the retention relative to cholestane is extremely temperature-dependent. The discrepancies arise from the expected large differences in the temperature - partition coefficient relationships for compounds of widely differing constitution. Retention factors for polyfunctional steroids should accordingly be employed with reference to model compounds of related structure, as is customary in the application of other empirical physico-chemical information. The separation factors for 5α - and 5β-steroids strikingly illustrate this principle: thus for SE-30 at 225°, the factors for six 5α/5β pairs (Nos. 3,7, 13,16,18 and 19 in Table 2) are within 1% of those at 200°. Similarly on QF-1 the separation factors for the 5α - and 5β-androstane-triones, pregnanediones and pregnanetriones (Table 9) at 225° and (with the Barber-Colman chromatograph) ca. 210° are within 2% of the values at 200° given in Table 2, despite the gross changes in retention relative to cholestane.

Following Clayton's correlations between retention behaviour and structure of steroids where he introduced "group retention factors"

Table 9. Comparative data for the relative retention of steroids on various QF-1 columns.

Steroid	Column and relative retention data: 1% QF-1 on Gas-Chrom P				6% QF-1 on Celite
	I 200°	I 225°	II ca. 210°	III 195°	IV 250°
3α-Hydroxy-5α-androstan-17-one	1.96	-	1.88	-	2.18
5α-Androstane-3,11,17-trione	9.04	7.48	8.04	-	-
5β-Androstane-3,11,17-trione	8.04	6.65	7.12	-	7.41
5α-Pregnane-3β,20β-diol	2.01	1.89	1.88	1.94	2.02
5α-Pregnane-3β,20α-diol	2.26	2.10	2.08	2.16	2.25
3β-Hydroxy-5α-pregnan-20-one	3.14	2.82	2.88	-	-
20β-Hydroxy-5α-pregnan-3-one	3.84	3.43	3.60	-	-
5α-Pregnane-3,20-dione	6.30	5.35	5.72	5.93*	5.90
5α-Pregnane-3,11,20-trione	15.4	12.1	13.08	-	12.42

(continued overleaf)

Steroid	Column and relative retention data 1% QF-1 on Gas-Chrom P I 200°	I 225°	II ca. 210°	III 195°	6% QF-1 on Celite IV 250°
5β-Pregnane-3,20-dione	5.75	4.99	5.32	-	5.37
5β-Pregnane-3,11,20-trione	13.4	10.8	11.52	-	11.10
5α-Cholestan-3β-ol	3.66	-	3.16	3.70	3.03
Cholest-5-en-3β-ol	3.33	-	3.00	3.39	-

I : Present work.

II : Results obtained by C. J. W. Brooks in Dr. E. C. Horning's laboratory at Bethesda : Barber-Colman chromatograph, 6 ft. column.

III : Luukkainen, VandenHeuvel, Haahti & Horning (31): Barber-Colman chromatograph, 6 ft. column.
* VandenHeuvel, Haahti & Horning (3).

IV : Knights & Thomas (7,8): Pye Argon chromatograph, 4 ft. column.

(4, 5, 6). Knights and Thomas (7) pointed out the correspondence between the logarithms of relative retention times and the R_M values defined by Bate-Smith and Westall (26) and extensively applied by Bush (27) in the paper chromatographic identification of steroids. Clayton expressed his correlations by the general equation:

$$r(n+a\ b+c....) = r_n x k_a \times k_b \times k_c .....$$

where $r(n + a + b + c...)$ is the retention time of the substituted steroid, r_n the retention time of the parent compound and k_a, k_b, k_c ..... are constant factors characteristic of the type and position of the substituent groups a, b, c .....i.e. "group retention factors". Knights and Thomas (7) expressed the same relationship in logarithmic terms where they showed that the logarithm of the relative retention time of a substituted steroid (log r) is made up of the additive contributions of the individual groups (ΔR_{Mg}) and the logarithm of the relative retention time of the steroid nucleus (log r_N):

$$\log r = \Sigma \Delta R_{Mg} + \log r_N$$

ΔR_{Mg} denotes the change in log r of a steroid on introduction of a group g. The equivalence of the two approaches is shown by the expression:

$$\log k = \Delta R_{Mg}$$

Yet another approach in establishing relationships between structure and gas-chromatographic behaviour is that of Haahti, VandenHeuvel and Horning (28) who defined T values which are based on relative retention times observed with two phases used in equal concentrations and at the same temperature. They defined T values as:

$$T = \frac{t's - t'n}{t'n}$$

where t's and t'n are the relative retention times observed with a selective and non-selective phase respectively. T values, like the factor k_n and Δ RMg, are characteristic of the number, type and position of the substituent groups in the steroid and like the latter are derived from relative retention data which are dependent on temperature, amount of phase and flow rate as shown by VandenHeuvel and Horning (1962). The variations with amount of phase and flow rate are not significant but the marked temperature dependence of these entities led VandenHeuvel and Horning (29) to introduce the "steroid number" concept which is based on the work of Woodford and Van Gent (30) in the fatty acid field. Although steroid numbers are calculated from relative retention times they are less temperature dependent because they are determined relative to two or more reference compounds. The expression relating structure to retention behaviour for a polyfunctional steroid is as follows:

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

where SN is the steroid number, S is the number of carbon atoms in the steroid skeleton and $F_1 \ldots\ldots\ldots\ldots F_n$ are the additive contributions characteristic of the functional groups in the steroid. The determination of steroid numbers is more time consuming than that of relative retention times which have proved adequate for tentative structural assignments and identification purposes when a sufficient number of authentic samples are available for comparison. The suggested advantage of steroid numbers (29,33) is in facilitating comparisons of data from various laboratories. However, simple retention data recorded under similar conditions in different laboratories are often found to agree, or to differ by an approximately constant factor and by a suitable choice of reference compound, comparable relative retention values are generally obtained.

A summary of the different structural correlations is given in Table 10.

Nelson (34) erroneously treated retention factors as arithmetically additive. However, it is of interest to note that the relative retention values he gave for a number of pregnane derivatives on SE-30 are in excellent agreement with those found by Dr. C. J. W. Brooks and the writer, supporting the conclusions reached above on the reliability of such data.

Table 10.

Authors	Compounds studied	Conditions	Correlation	Reference
Clayton	C_{19}-C_{27} sterols & sterol methyl ethers	Polydiethylene glycol succinate, 195°	Relative retention factor k_n	Biochemistry 1, [illegible] (1962)
Knights & Thomas	C_{19}-C_{27} steroids	OF-1-0065, 250°	ΔRMg	Nature, 194, 833 [illegible] and subsequent publications
Haahti, Vanden-Heuvel & Horning	C_{19}-C_{27} steroids	SE-30; OF-1-0065 and mixture of SE-30 & EGIP, 203°-215°	T values	Analyt. Biochem. [illegible] (1961)
VandenHeuvel & Horning	C_{19}-C_{27} steroids	SE-30, OF-1-0065 NGS, 195°-225°	Steroid Numbers	Biochim. biophys. [illegible] 64, 416 (1962); 7[illegible] (1963)
Nelson	C_{21} steroids Pregnenes & Pregnanes	SE-30; OF-1-0065 265°	Erroneous calculation of retention factors	J. Gas Chromato[illegible] (1963)

It is concluded that gas-liquid-chromatographic retention data, when carefully interpreted, permit the tentative identification of steroids in samples amounting to a few micrograms. Applications of the method in the steroid field have been of two kinds: (i) the identification of known compounds in biological extracts by their retention behaviour as demonstrated by Makita and Wells (35) and Danielsson et al. (36), among others, in the characterisation of faecal sterols and bile acids from biological extracts respectively; (ii) the characterisation of unknown compounds by their retention behaviour in conjunction with other physico-chemical methods. For example, Danielsson et al. (37) have reported the presence of a new bile acid in rabbit faeces found to correspond to 3α, 12α-dihydroxy-5α-cholanic acid from gas-chromatographic and mass spectrometric data. More recently Eneroth and co-workers (38) have carried out a detailed examination of neutral faecal steroids by gas-liquid chromatography and mass spectrometry and have been able to characterise a number of new steroids (derivatives of cholestane and coprostane) by a combination of data from both methods. Where rigorous characterisation is necessary, the isolation of separated steroids may still be required, but it seems probable that the combined use of gas chromatographic and mass-spectrometric methods will in many instances suffice for identification.

(1) Horning, E.C., VandenHeuvel, W.J.A. & Creech, B.G. (1963). In Methods of Biochemical Analysis, Vol. 11, Ed. by Glick, D. New York: Interscience, John Wiley.

(2) VandenHeuvel, W.J.A., Sweeley, C.C. & Horning, E.C. (1960). J. Amer. chem. Soc. 82, 3481.

(3) VandenHeuvel, W.J.A., Haahti, E.O.A. & Horning, E.C. (1961). J. Amer. chem. Soc. 83, 1513.

(4) Clayton, R.B. (1961). Nature, Lond., 190, 1071.

(5) Clayton, R.B. (1961). Nature, Lond., 192, 524.

(6) Clayton, R.B. (1962). Biochemistry, 1, 357.

(7) Knights, B.A. & Thomas, G.H. (1962). Nature, Lond., 194, 833.

(8) Knights, B.A. & Thomas, G.H. (1962). Analyt. Chem. 34, 1046.

(9) Tsuda, K., Ikekawa, N., Sato, Y., Tanaka, S. & Hasegawa, H. (1962). Chem. Pharm. Bull. 10, 332.

(10) Brooks, C.J.W. & Norymberski, J.K. (1953). Biochem. J. 55, 37

(11) Holmes, W.L. & Stack, E. (1962). Biochim. biophys. Acta, 56, 16

(12) VandenHeuvel, W.J.A., Sjövall, J. & Horning, E.C. (1961). Biochim. biophys. Acta, 48, 596.

(13) Lipsky, S.R. & Landowne, R.A. (1961). Analyt. Chem. 33, 818.

(14) Sweeley, C.C. & Lo Chang, T.-C. (1961). Analyt. Chem. 33, 1860.

(15) Bloomfield, D.K. (1962). Analyt. Chem. 34, 737.

(16) Knights, B.A. & Thomas, G.H. (1963). Chem. & Ind. p. 43.

(17) Luukkainen, T., VandenHeuvel, W.J.A. & Horning, E.C. (1962). Biochim. biophys. Acta, 62, 153.

(18) Girard, A.-M., Lacroix, A. & Lull, T. (1963). Bull. Soc. chim. France II, 2363.

(19) Turner, R.B. (1961). In Mechanism of Action of Steroid Hormones Ed. by Villee, C.A. & Engel, L.L., Oxford: Pergamon Press Ltd.

(20) Hamilton, R.J., VandenHeuvel, W.J.A. & Horning, E.C. (1963). Biochim. biophys. Acta, 70, 679.

(21) Neher, R. (1963). In Physical Properties of the Stercid Hormones Ed. by Engel, L.L., Oxford: Pergamon Press Ltd.

(22) VandenHeuvel, W.J.A. & Horning, E.C. (1963). Biochim. biophys. Acta, 174, 560.

(23) Brooks, C.J.W. Monograph No. 2: The Determination of Sterols. London: The Society for Analytical Chemistry (in press).

(24) Knights, B.A. & Thomas, G.H. (1963). J. chem. Soc., p. 3477.

(25) Menini, E. & Norymberski, J.K. (1962). Biochem. J. 83, 31P.

(26) Bate-Smith, E.C. & Westall, R.G. (1950). Biochim. biophys. Acta, 4, 427.

(27) Bush, I.E. (1961). The Chromatography of Steroids, p. 68. Oxford: Pergamon Press Ltd.

(28) Haahti, E.O.A., VandenHeuvel, W.J.A. & Horning, E.C. (1961). Analyt. Biochem., 2, 344.

(29) VandenHeuvel, W.J.A. & Horning, E.C. (1962). Biochim. biophys. Acta, 64, 416.

(30) Woodford, F.P. & Van Gent, C.M. (1960). J. Lipid Res. 1, 188.

(31) Luukkainen, T., VandenHeuvel, W.J.A., Haahti, E.O.A. & Horning, E.C. (1961). Biochim. biophys. Acta, 52, 599.

(32) Chamberlain, J., Knights, B.A. & Thomas, G.H. (1963). J. Endocrin. 26, 367.

(33) Hamilton, R.J., VandenHeuvel, W.J.A. & Horning, E.C. (1963). Biochim. biophys. Acta, 70, 679.

(34) Nelson, J.P. (1963). J. Gas Chromatog. 1, 27.

(35) Makita, M., & Wells, W.W. (1963). Analyt. Biochem., 5, 523.

(36) Danielsson, H., Eneroth, P., Hellström, K., Lindstedt, S. & Sjövall, J. (1963). J. biol. Chem., 238, 2299.

(37) Danielsson, H., Kallner, A. & Sjövall, J. (1963). J. biol. Chem., 238, 3846.

(38) Eneroth, P., Hellström, K. & Ryhage, R. (1963). J. Lipid Res., 5, 245.

APPENDIX 1.

Tables of Retention Data

Temperature 200°

Introduction of 3α-Hydroxyl group

1% SE-30 on Gas Chrom P.

	Relative Retention		Factor
	3α-OH	3-H	3α-OH
5α-Androstan-17-one	0.336	0.161	2.094
5α-Pregnane	0.304	0.148	2.056
5α-Cholestane	2.120	1.00	2.120
5β-Androstane	0.147	0.071	2.084
5β-Pregnane	0.283	0.134	2.120
5β-Pregnan-20-one	0.531	0.251	2.118
5β-Cholestane	1.918	0.903	2.124

1% QF-1 on Gas Chrom P.

5α-Androstan-17-one	1.957	0.622	3.146
5α-Pregnane	0.647	0.206	3.142
5α-Cholestane	3.237	1.000	3.237
5β-Androstane	0.388	-	-
5β-Pregnane	0.653	0.181	3.599
5β-Pregnan-20-one	2.821	0.779	3.623
5β-Cholestane	3.240	0.897	3.612

(Column: 1% SE-30 on Gas Chrom P)

Temperature 225°

Introduction of 3β-Hydroxyl group

	Relative Retention[a]		Factor
	3β-OH	3-H	3β-OH
5α-Androst-17-one	0.395	0.202	1.96
5α-Androstan-17β-ol	0.407	0.211	1.93
Androst-5-en-17β-ol	0.400	0.207	1.94
5α-Pregnan-20-one	0.640	0.327	1.96
5α-Pregnan-20α-ol	0.702	0.352	2.00
5α-Pregnan-20β-ol	0.651	0.334	1.95
5β-Pregnan-20-one	0.577	0.304	1.90
5α-Cholestane	1.97	1.00	1.97
5β-Cholestane	1.76	0.91	1.94
Cholest-5-ene	1.96	1.00	1.96
Cholestan-6-one	3.76	1.92	1.96
5α-Ergost-7-ene	2.94	1.47	2.00
5α-Ergost-3(14)-ene	2.55	1.29	1.98
5α-Ergosta-7,22-diene	2.51	1.27	1.98

a) Cholestane (11.7 min.) = 1.00.

The experimental work for this table was carried out by

Dr. C.J.W. Brooks.

Temperature 200°

Introduction of 3β-Hydroxyl group

1% SE-30 on Gas Chrom P.

	Relative Retention		Factor
	3β-OH	3-H	3β-OH
5α-Androstane	0.163	0.078	2.085
5α-Androstan-17-one	0.338	0.161	2.104
5α-Androstan-17β-ol	0.353	0.166	2.120
5α-Pregnane	0.311	0.148	2.101
5α-Pregnan-20-one	0.593	0.278	2.134
5α-Pregnan-20α-ol	0.652	0.305	2.139
5α-Pregnan-20β-ol	0.598	0.282	2.123
5α-Cholestane	2.135	1.000	2.135
5β-Pregnane	0.279	0.134	2.086
5β-Pregnan-20-one	0.523	0.251	2.087
5β-Cholestane	1.892	0.903	2.096
Androst-5-en-17β-ol	0.353	0.167	2.114
Cholest-5-ene	2.120	0.999	2.121

Temperature 200°

Introduction of 3β-Hydroxyl group

1% OF-1 on Gas Chrom P.

	Relative Retention		Factor
	3β-OH	3-H	3β-OH
5α-Androstane	0.427	-	-
5α-Androstan-17-one	2.249	0.622	3.614
5α-Androstan-17β-ol	1.344	0.388	3.461
5α-Pregnane	0.733	0.206	3.559
5α-Pregnan-20-one	3.143	0.861	3.652
5α-Pregnan-20α-ol	2.268	0.642	3.534
5α-Pregnan-20β-ol	2.010	0.566	3.553
5α-Cholestane	3.662	1.000	3.662
5β-Pregnane	0.574	0.181	3.166
5β-Pregnan-20-one	2.486	0.779	3.194
5β-Cholestane	2.829	0.897	3.154
Androst-5-en-17β-ol	1.242	0.374	3.324
Cholest-5-ene	3.327	0.967	3.441

Temperature 200°

Introduction of 11α -Hydroxyl group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	11α -OH	11-H	11α -OH
5α -Pregn-2-en-20-one	0.484	0.272	1.776
Pregn-4-ene-3,20-dione	1.510	0.791	1.910

1% QF-1 on Gas-Chrom P.

5α -Pregn-2-en-20-one	2.166	0.876	2.473
Pregn-4-ene-3,20-dione	24.51	9.936	2.467

Temperature 200°

Introduction of 11β-Hydroxyl group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	11β-OH	11-H	11β-OH
Androst-4-ene-3,17-dione	0.822	0.452	1.820
Pregn-4-ene-3,20-dione	1.469	0.791	1.858

1% QF-1 on Gas-Chrom P.

3α-Hydroxy-5β-androstan-17-one	4.016	2.051	1.957
Androst-4-ene-3,17, dione	14.14	7.247	1.951
Pregn-4-ene-3,20-dione	21.47	9.936	2.161

Temperature 200°

Introduction of 17α -Hydroxyl group

1% SE-30 on Gas-Chrom P

	Relative retention		Factor
	17α -OH	17-H	17α -OH
5α -Androstane	0.163	0.078	2.089
Androst-4-en-3-one	0.478	0.221	2.163

1% OF-1 on Gas-Chrom P.

5α -Androstane	0.368	-	-
Androst-4-en-3-one	3.893	1.287	3.024

Temperature 200°

Introduction of 17β-Hydroxyl group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	17β-OH	17β-H	17β-OH
5α-Androstane	0.166	0.078	2.128
5α-Androstan-3β-ol	0.353	0.163	2.163
5β-Androstan-3α-ol	0.322	0.147	2.191
Androst-5-en-3β-ol	0.353	0.163	2.173
5α-Androstan-3-one	0.386	0.175	2.204
Androst-4-en-3-one	0.483	0.221	2.185

1% OF-1 on Gas-Chrom P.

5α-Androstan-3β-ol	1.344	0.427	3.147
5β-Androstan-3α-ol	1.202	0.388	3.097
Androst-5-en-3β-ol	1.242	0.396	3.134
5α-Androstan-3-one	2.630	0.805	3.268
Androst-4-en-3-one	4.144	1.287	3.220

Temperature 200°

Introduction of 20α -Hydroxyl group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	20α -OH	20α -H	20α -OH
5α -Pregnane	0.305	0.148	2.063
5α -Pregnan-3β -ol	0.652	0.311	2.100
5β -Pregnan-3α -ol	0.589	0.283	2.080

1% QF-1 on Gas-Chrom P.

5α -Pregnane	0.642	0.206	3.118
5α -Pregnan-3β -ol	2.268	0.733	3.095
5β -Pregnan-3α -ol	2.065	0.653	3.165

Temperature 200°

Introduction of 20β-Hydroxyl group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	20β-OH	20β-H	20β-OH
5α-Pregnane	0.282	0.148	1.905
5α-Pregnan-3-one	0.644	0.335	1.923
5α-Pregnan-3β-ol	0.598	0.311	1.926
5β-Pregnan-3α-ol	0.544	0.283	1.920

1% QF-1 on Gas-Chrom P.

5α-Pregnane	0.566	0.206	2.749
5α-Pregnan-3-one	3.837	1.381	2.778
5α-Pregnan-3β-ol	2.010	0.733	2.744
5β-Pregnan-3α-ol	1.806	0.653	2.768

Temperature 200°

Introduction of 3-oxo group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	3-oxo	3-H_2	3-oxo
5α-Androstane	0.175	0.078	2.241
5α-Androstan-17β-ol	0.386	0.166	2.320
5α-Pregnane	0.335	0.148	2.266
5α-Pregnan-20β-ol	0.644	0.282	2.286
5α-Cholestane	2.291	1.000	2.291
5β-Cholestane	2.055	0.903	2.277
5α-Androstan-17-one	0.366	0.161	2.281
5α-Pregnan-20-one	0.636	0.278	2.290
5β-Pregnan-20-one	0.568	0.251	2.267

Temperature 200°

Introduction of 3-oxo group.

1% QF-1 on Gas-Chrom P.

	Relative Retention		Factor
	3-oxo	3-H_2	3-oxo
5α-Androstane	0.805	-	-
5α-Androstan-17β-ol	2.630	0.388	6.775
5α-Pregnane	1.381	0.206	6.710
5α-Pregnan-20β-ol	3.837	0.566	6.782
5α-Cholestane	6.953	1.000	6.953
5β-Cholestane	6.277	0.897	6.998
5α-Androstan-17-one	4.649	0.622	7.471
5α-Pregnan-20-one	6.295	0.861	7.314
5β-Pregnan-20-one	5.754	0.779	7.391

Temperature 200°

Introduction of 6-oxo group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	6-oxo	6-H_2	6-oxo
Androst-4-ene-3,17-dione	0.642	0.451	1.421
Pregn-4-ene-3,20-dione	1.173	0.790	1.486
Cholest-4-en-3-one	4.414	2.891	1.526
5α-Cholestane	2.049	1.000	2.049
5α-Cholestan-3-one	4.223	2.291	1.844

Temperature 200°

1% SE-30 on Gas-Chrom P

Introduction of 7-oxo group.

	Relative Retention		Factor
	7-oxo	7-H_2	7-oxo
5β-Cholane	0.655	0.365	1.795
5α-Cholestane		1.000	

Introduction of 12-oxo group.

	Relative Retention		Factor
	12-oxo	12-H_2	12-oxo
5α-Cholane	0.721	0.403	1.788
5β-Cholane	0.648	0.365	1.775

Temperature 200°

Introduction of 11-oxo group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	11-oxo	11-H_2	11-oxo
5α-Androstane-3,17-dione	0.475	0.366	1.299
5β-Androstane-3,17-dione	0.410	0.328	1.251
3α-Hydroxy-5β-androstan-17-one	0.392	0.306	1.280
Androst-4-ene-3,17-dione	0.555	0.452	1.229
5α-Pregnane	0.201	0.148	1.357
5α-Pregnane-3,20-dione	0.884	0.636	1.390
3β-Hydroxy-5α-pregnan-20-one	0.856	0.593	1.444
5β-Pregnane-3,20-dione	0.769	0.568	1.353
5α-Pregn-2-en-20-one	0.396	0.272	1.455
16α-Me-5α-Pregn-2-en-20-one	0.387	0.275	1.409

Temperature 200°

Introduction of 11-oxo group.

1% QF-1 on Gas-Chrom P.

	Relative Retention		Factor
	11-oxo	11-H_2	11-oxo
5α-Androstane-3,17-dione	9.036	4.649	1.944
5β-Androstane-3,17-dione	8.043	4.306	1.868
3α-Hydroxy-5β-androstan-17-one	3.984	2.051	1.942
Androst-4-ene-3,17-dione	12.640	7.247	1.745
5β-Androstane-3α,17β-diol	3.046	1.202	2.534
5α-Pregnane	0.536	0.206	2.604
5α-Pregnane-3,20-dione	15.370	6.295	2.442
3β-Hydroxy-5α-pregnan-20-one	8.396	3.143	2.672
5β-Pregnane-3,20-dione	13.380	5.754	2.326
5α-Pregn-2-en-20-one	2.330	0.876	2.661
16α-Me-5α-Pregn-2-en-20-one	2.132	0.832	2.563

Temperature 200°

Introduction of 17-oxo group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	17-oxo	17-H_2	17-oxo
5α-Androstane	0.161	0.078	2.052
5α-Androstan-3β-ol	0.338	0.163	2.070
5β-Androstan-3α-ol	0.306	0.147	2.082
Androst-5-ên-3β-ol	0.332	0.163	2.040
5α-Androstan-3-one	0.366	0.175	2.089
Androst-4-en-3-one	0.452	0.221	2.045

1% QF-1 on Gas-Chrom P.

5α-Androstane	0.622	-	-
5α-Androstan-3β-ol	2.249	0.427	5.265
5β-Androstan-3α-ol	2.051	0.388	5.285
Androst-5-en-3β-ol	1.990	0.396	5.023
5α-Androstan-3-one	4.649	0.805	5.776
Androst-4-en-3-one	7.247	1.287	5.630

Temperature 200°

Introduction of 20-oxo group

1% SE-30 on Gas-Chrom P.

	Relative Retention		Factor
	20-oxo	20-H_2	20-oxo
5α-Pregnane	0.278	0.148	1.880
5α-Pregnan-3β-ol	0.593	0.311	1.910
5β-Pregnane	0.251	0.134	1.876
5β-Pregnan-3α-ol	0.531	0.283	1.874
5β-Pregnan-3β-ol	0.523	0.279	1.877
5α-Pregnan-3-one	0.636	0.335	1.900
Pregn-4-en-3-one	0.791	0.420	1.882

1% QF-1 on Gas-Chrom P.

5α-Pregnane	0.861	0.206	4.184
5α-Pregnan-3β-ol	3.143	0.733	4.289
5β-Pregnane	0.779	0.181	4.294
5β-Pregnan-3α-ol	2.821	0.653	4.323
5β-Pregnan-3β-ol	2.486	0.574	4.332
5α-Pregnan-3-one	6.295	1.381	4.558
Pregn-4-en-3-one	9.936	2.191	4.535

Temperature 210°

Introduction of Δ^4-3-oxo group

1% SE-30 on Gas-Chrom P.

	Relative Retention Times	Δ^4-3-oxo Factor
5α-Androstane	0.078	2.824
Androst-4-en-3-one	0.221	
5α-Androstan-17β-ol	0.166	2.899
17β-Hydroxyandrost-4-en-3-one	0.483	
5α-Androstan-17-one	0.161	2.815
Androst-4-ene-3,17-dione	0.452	
5α-Pregnane	0.148	2.843
Pregn-4-en-3-one	0.420	
5α-Pregnan-20-one	0.278	2.844
Pregn-4-ene-3,20-dione	0.791	
5α-Cholestane	1.000	2.891
Cholest-4-en-3-one	2.891	

Temperature 200°

Introduction of Δ^4-3-oxo group

1% CF-1 on Gas Chrom P.

	Relative Retention Times	Δ^4-3-oxo Factor
5α-Androstan-17β-ol	0.388	10.69
17β-Hydroxyandrost-4-en-3-one	4.144	
5α-Androstan-17-one	0.622	11.65
Androst-4-ene-3,17-dione	7.247	
5α-Pregnane	0.206	10.64
Pregn-4-en-3-one	2.191	
5α-Pregnan-20-one	0.861	11.54
Pregn-4-ene-3,20-dione	9.936	
5α-Cholestane	1.00	11.25
Cholest-4-en-3-one	11.25	

Temperature 200°

1% SE-30 on Gas-Chrom P.

Introduction of Δ^4-3,6-dioxo group

	Relative Retention Times	Δ^4-3,6-dioxo Factor
5α-Androstan-17-one	0.161	3.99
Androst-4-ene-3,6,17-trione	0.642	
5α-Pregnan-20-one	0.278	4.22
Pregn-4-ene-3,6,20-trione	1.173	
5α-Cholestane	1.000	4.41
Cholest-4-ene-3,6-dione	4.414	

Introduction of $\Delta^{3,5}$-diene group

	Relative Retention Times	$\Delta^{3,5}$-diene Factor
5α-Androstan-17-one	0.161	1.13
Androsta-3,5-dien-17-one	0.123	
5α-Pregnan-20-one	0.278	1.11
Pregna-3,5-dien-20-one	0.309	

Separation Factor for 5α - and 5β - Steroids

1% SE -30 on Gas-Chrom P - 200°

5α - and 5β - Epimers	Relative Retention 5α	5β	Separation Factor
Androstane	0.078	0.071	1.108
Androstane-3,17-dione	0.366	0.328	1.118
17β-Hydroxyandrostan-3-one	0.386	0.344	1.122
Androstane-3,11,17-trione	0.475	0.410	1.160
Pregnane	0.148	0.134	1.106
Pregnan-20-one	0.278	0.251	1.109
Pregnane-3,20-dione	0.636	0.568	1.120
Pregnane-3,11,20-trione	0.884	0.769	1.150
Cholane	0.403	0.365	1.104
Cholan-12-one	0.721	0.648	1.112
Cholan-12α-ol	0.706	0.618	1.142
Cholestane	1.000	0.903	1.108
Cholestan-3-one	2.291	2.055	1.114
3α-Hydroxyandrostan-17-one	0.336	0.306	1.097
Pregnan-3α ol	0.304	0.283	1.073
Cholestan-3α-ol	2.120	1.918	1.105
Pregnan-3β-ol	0.311	0.279	1.114
3β-Hydroxypregnan-20-one	0.593	0.523	1.133
Cholestan-3β-ol	2.135	1.892	1.128

Separation Factor for 5α - and 5β - Steroids
1% SE-30 on Gas-Chrom P - 200°

5α - and 5β - Epimers	Relative Retention		Separation
	5α	5β	Factor
3α -Hydroxyandrostan-17-one 3-acetate	0.474	0.467	1.015
Pregnan-3α -ol 3-acetate	0.436	0.437	0.999
Cholestan-3α -ol 3-acetate	3.089	3.077	1.004
Pregnan-3β -ol 3-acetate	0.499	0.406	1.228
3β -Hydroxypregnan-20-one 3-acetate	0.943	0.769	1.227
Cholestan-3β -ol 3-acetate	3.411	2.806	1.216
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	
[illegible]	[illegible]	[illegible]	

Separation Factor for 5α - and 5β - Steroids

1% OV-1 on Gas-Chrom P - 200°

5α - and 5β - Epimers	Relative Retention 5α	Relative Retention 5β	Separation Factor
Androstane	-	-	-
Androstane-3,17-dione	4.649	4.306	1.079
17β-Hydroxyandrostan-3-one	2.630	2.422	1.086
Androstane-3,11,17-trione	9.036	8.043	1.123
Pregnane	0.206	0.181	1.135
Pregnan-20-one	0.861	0.779	1.105
Pregnane-3,20-dione	6.295	5.754	1.094
Pregnane-3,11,20-trione	15.370	13.380	1.149
Cholane	0.475	0.431	1.100
Cholan-12-one	1.857	1.581	1.194
Cholan-12α-ol	1.096	0.918	1.174
Cholestane	1.000	0.897	1.114
Cholestan-3-one	6.953	6.277	1.108
3α-Hydroxyandrostan-17-one	1.957	2.051	0.954
Pregnan-3α-ol	0.647	0.653	0.991
Cholestan-3α-ol	3.237	3.240	0.999
Pregnan-3β-ol	0.733	0.574	1.276
3β-Hydroxy pregnan-20-one	3.143	2.486	1.264
Cholestan-3β-ol	3.662	2.829	1.294

Separation Factor for 5α - and 5β - Steroids

1% QF-1 on Gas-Chrom P - 200°

5α - and 5β - Epimers	Relative Retention		Separation Factor
	5α	5β	
3α -Hydroxyandrostan-17-one 3-acetate	3.270	3.083	1.060
Pregnan-3α -ol 3-acetate	1.034	0.961	1.076
Cholestan-3α -ol 3-acetate	5.18	4.81	1.076
Pregnan-3β -ol 3-acetate	1.175	0.994	1.182
3β -Hydroxypregnan-20-one 3-acetate	5.16	4.42	1.167
Cholestan-3β -ol 3-acetate	5.718	4.90	1.167

Section 2

Correlation of Gas-Liquid Chromatography and Structure of Steroid Diols and Triols

As discussed in Section 1, the retention time of a monosubstituted steroid can be closely approximated by the product of the retention time of the unsubstituted compound and of a retention factor characteristic of the substituent. The computation can be extended to two substituents that are sufficiently far apart, and data indicating agreement between calculated and observed retentions has been presented for many difunctional steroids (1,2,3,4,9). The correlations have not been extensively tested for three or more substituents. Knights and Thomas (2,4) noted marked variations in the retention factor (on OF-1) for introduction of the 11-oxo and 11β-hydroxyl groups into a variety of androstane and pregnane derivatives, mostly 3,17- or 3,20-diones respectively. Different factors for introducing the 11-oxo group in these two types of compounds were also found for SE-30 as mentioned in Section 1, but the two examples of 11β-hydroxyl factors showed no significant difference.

Where the independence of retention factors is spoiled by the proximity of substituents, or by related conformational changes, it is convenient to describe the perturbations as "vicinal effects" by analogy with the use of this term in the application of molecular

rotation contributions. Indeed, the mode of employment of retention factors in the assignment of steroid structures closely parallels that of molecular rotation data, but has the practical advantages of being applicable to mixtures and of requiring only a few micrograms of material. Knowledge of the occurrence of vicinal effects and their relative importance is accordingly essential to the proper application of this method of characterisation.

In this Section, comparative retention data are discussed for two groups of steroid diols and triols:

(a) Hydroxy and acetoxy steroids (not vicinally substituted)

(b) Vicinal diols of the cholestane series.

Materials and Methods

Steroids

Cholestanediols and a number of cholestanols were generously provided by Dr. G.D. Meakins (The Dyson Perrins Laboratory). Other steroids were obtained commercially mainly from Southeastern Biochemicals Inc. or were kindly given as specified in the footnotes. 5α-Androstane-3β,7α,17β- (and 3β,7β,17β-) triol and 5α-androstane-3β,7α- (and 3β,7β-) diol were prepared by alkaline hydrolysis of the corresponding 3,17-diacetates and 3-acetates respectively.

Acetylation of the majority of the steroids was carried out by

treating a solution of the hydroxy compound (100-200 µg.) in dry pyridine (20 µl.) with acetic anhydride (50-100 µl.) and leaving overnight in a vacuum desiccator. In the instances when due to steric hindrance the above method failed, acetylation was carried out by refluxing the reaction mixture for 1 to 2 hours or by catalysing the acetylation with p-toluenesulphonic acid instead of pyridine.

In the few cases where the retention times or derived retention factors on SE-30 were anomalous, the acetylation products were examined on a QF-1 column and by thin-layer chromatography. The relevant data are summarised in Table A which shows that although the retention times of some hydroxy and acetoxy compounds are not markedly different on SE-30, their retention times on QF-1 and their Rf values are sufficiently distinct to suggest that acetylation has occurred. The anomalously low retention on SE-30 of the diacetates of 5α-cholestane-3β,7α-diol and 5α-cholestane-3β,7β-diol compared to the monoacetate value is unlikely to be due to elimination in view of the normal behaviour shown on a QF-1 column at the same temperature. These results parallel the acetylation data for the bile acid methyl esters on SE-30 and QF-1 (Table 6 of Part A, section 3 (ii)).

Gas-liquid chromatography

The Pye Argon Chromatograph used for this investigation has

Table A. Additional Retention Data for Some Anomalous Hydroxy and Acetoxy Steroids

Compound	Relative Retention Times, 225°		R_f values	
	SE-30	QF-1	Benzene	Benzene:ether (8:2)
5α-Cholestan-1α-ol	1.80	2.38	0.57	-
5α-Cholestan-1α-ol acetate	1.86	3.18	0.79	-
5α-Cholestan-7α-ol	1.81	2.38	0.57	-
5α-Cholestan-7α-ol acetate	1.79	3.04	0.67	-
5β-Cholan-7α-ol	0.71	1.02	0.60	-
5β-Cholan-7α-ol acetate	0.77	1.51	0.66	-
5α-Cholestane-3β,7α-diol	3.58	7.70	-	0.08
5α-Cholestane-3β,7α-diol 3-acetate	5.49	12.15	-	0.62
5α-Cholestane-3β,7α-diol diacetate	5.12	14.25	-	0.86
5α-Cholestane-3β,7β-diol	3.58	7.45	0.00	0.04
5α-Cholestane-3β,7β-diol 3-acetate	5.50	12.66	0.06	0.53
5α-Cholestane-3β,7β-diol diacetate	5.20	14.10	0.15	0.75

been fully described in the Introduction and in Section 1. For this study the standard operating conditions were: column temperature 225 ± 3° (or 200 ± 3°); injection block temperature 250 ± 6°; argon flow rate at outlet 30 ml./min.: detector voltage 1250V and sensitivity setting 10.

Chromatography was effected on 4 ft. columns of SE-30 and OF-1 as described in the previous section. Cholestane was included in all solutions before chromatography and the retention times were expressed as ratios relative to cholestane.

Thin-layer chromatography

The preparation of thin-layer "chromatoplates" followed the method of Stahl (5): Silica Gel G (30 g: E. Merck AG, Darmstadt) was slowly mixed with 40 ml. distilled water to a smooth slurry. Distilled water (20 ml.) was then added and the suspension poured into the applicator ("Desaga" Spreading Device, Desaga AG, Heidelberg). The slurry was spread over 20 glass plates (20 x 5 cm.) giving a thin layer of silica gel (about 250 μ). The operation from the first addition of water to application of the slurry was completed in four minutes. The chromatoplates were then placed on a rack and allowed to dry at room temperature.

The compounds were dissolved in chloroform and aliquots were applied to the chromatoplates with a 10 μl. Hamilton syringe.

Development times for a 15 cm. rise ranged from 20 to 50 minutes depending on the solvent system employed. Ascending chromatography was carried out in glass cylinders sealed with ground glass covers and lined with filter paper to assist solvent equilibration. The chromatoplates were dried at room temperature, sprayed with 1% ceric sulphate in 10% sulphuric acid and heated in an oven at 130-140° for 5-10 minutes. The cholestanediols exhibited characteristic colours in visible and ultraviolet light after this treatment.

Chromatography was carried out in solvent systems of different polarities in order to give a wide range of mobilities and facilitate the resolution of closely related compounds. The following eight solvent systems, listed approximately in order of increasing polarity, were used:

(1) chloroform:benzene = 6:4

(2) chloroform

(3) toluene:ethyl acetate = 9:1

(4) cyclohexane:ethyl acetate = 7:3

(5) benzene:ethyl acetate = 6:3

(6) chloroform:acetone = 9:1

(7) chloroform:ethyl acetate = 3:1

(8) chloroform:ethyl acetate = 3:2

For thin-layer chromatography AnalaR solvents were employed where available, and solvent mixtures were prepared by volume unless otherwise stated.

Results and Discussion

(a) Non-vicinal hydroxy and acetoxy steroids

Table 1 gives retention data (for SE-30) and factors for the introduction of hydroxyl and acetoxyl groups at positions 6α, 7α, 7β, 11β and 12α in a variety of steroids substituted to different degrees. It is clear that variations in the hydroxyl factors are small, indicating that there is no marked interaction between the introduced hydroxyl group and the other substituents even in the tri- and tetrafunctional examples. Factors for the 7α and 7β positions are indistinguishable and close to that for the 11β-position, but the 6α- and 12α-hydroxyl factors differ distinctly from the others both at 200° and 225°. The mean values of the factors derived from the miscellaneous compounds of Table 1 at 225°, without classification according to vicinal effects, are collected in Table 1A. In order to avoid unduly long retention times, further examination of hydroxy and acetoxy steroids was carried out at 225°.

An application of the mean retention factors is illustrated in Table 2. Several triols and one dihydroxy ketone, which were available in

Table 1. Hydroxyl and acetoxyl group retention factors for variously substituted compounds on SE-30

Compound	Group	200° Rel. R_T	200° Factor	225° Rel. R_T	225° Factor
5α-Androstane-3β,17β-diol		0.348		0.395	
5α-Androstane-3β,17β-diol diacetate		-		0.858	
5β-Androstane-3α,17β-diol		0.316		0.358	
5β-Androstane-3α,17β-diol diacetate		-		0.758	
5α-Androstane-3β,7α,17β-triol	7α-OH	0.666	1.91	0.700	1.77
5α-Androstane-3β,7α,17β-triol 3,17-diacetate[a]	7α-OH	-		1.554	1.81
5α-Androstane-3β,7β,17β-triol	7β-OH	0.675	1.93	0.717	1.82
5α-Androstane-3α,7β,17β-triol 3,17-diacetate[a]	7β-OH	-		1.562	1.82
5α-Androstane-3β,11β,17β-triol[a]	11β-OH	0.660	1.89	0.703	1.78
5β-Androstane-3α,11β,17β-triol	11β-OH	0.573	1.82	0.613	1.71
Androst-4-ene-3,17-dione		0.452		-	
11β-Hydroxyandrost-4-ene-3,17 dione	11β-OH	0.822	1.82	-	

Compound	Group	200° Rel. R_T	200° Factor	225° Rel. R_T	225° Factor
5β-Pregnane-3α,20β-diol		0.541		0.561	
5β-Pregnane-3α,20β-diol diacetate		-		1.24	
5β-Pregnane-3α,11β,20β-triol[a]	11β-OH	0.973	1.80	0.99	1.76
3α-Hydroxy-5β-pregnan-20-one		0.527		0.566	
3α-Acetoxy-5β-pregnan-20-one		-		0.825	
3α,6α-Dihydroxy-5β-pregnan-20-one	6α-OH	1.158	2.20	1.168	2.06
Pregn-4-ene-3,20-dione		0.791		0.825	
11β-Hydroxypregn-4-ene-3,20-dione	11β-OH	1.469	1.86	1.45	1.76
11β-Acetoxypregn-4-ene-3,20-dione	11β-OAc	-		1.63	1.98
16α,17α-Epoxypregn-4-ene-3,20-dione		-		0.904	
11β-Hydroxy-16α,17α-epoxypregn-4-ene-3,20-dione	11β-OH	-		1.53	1.69

Table 1 (continued)

Compound	Group	200° Rel. R_T	200° Factor	225° Rel. R_T	225° Factor
5β-Cholane[c]		0.365		0.41	
5β-Cholan-7α-ol[c]	7α-OH	0.672	1.84	0.71	1.
5β-Cholan-7α-ol acetate	7α-OAc	-		0.77	1.89
5β-Cholan-12α-ol	12α-OH	0.618	1.69	0.66	1.63
5β-Cholane-3α,24-diol		-		1.95	
5α-Cholestan-3β-ol		2.14		1.96	1.96
5α-Cholestan-3β-ol acetate		3.41		3.01	3.
5α-Cholestan-7α-ol	7α-OH	-		1.81	1.81
5α-Cholestan-7α-ol acetate	7α-OAc	-		1.79	1.
5α-Cholestane-3β,6α-diol	6α-OH	-		3.90	1.
5α-Cholestane-3β,7α-diol	7α-OH	-		3.58	1.
5α-Cholestane-3β,7α-diol 3-acetate[b]	7α-OH	-		5.49	1.

Table 1 (continued)

Compound	Group	200° Rel. R_T	200° Factor	225° Rel. R_T	225° Factor
5α-Cholestane-3β, 7α-diol diacetate	7α-OAc	-		5.12 *	1.70
5α-Cholestane-3β, 7β-diol	7β-OH	-		3.58	1.83
5α-Cholestane-3β, 7β-diol 3 acetate	7β-OH	-		5.50	1.83
5α-Cholestane-3β, 7β-diol diacetate	7β-OAc	-		5.20 *	1.73

a. Provided by Professor W. Klyne (London)

b. Provided by Professor H. B. Henbest (Belfast)

c. Provided by Dr. M. Martin-Smith (Glasgow)

* Anomalous values

Table 1A. Mean Values for Retention Factors (SE-30, 225°)

Group	No. of examples	Range	Mean
6α-OH	2	1.99 - 2.06	2.03
7α-OH	6	1.75 - 1.83	1.80
7β-OH	4	1.82 - 1.83	1.83
11β-OH	5	1.69 - 1.78	1.74
12α-OH	1	-	1.63
7α-OAc (5α-H)	2	1.70 - 1.79	1.75
7α-OAc (5β-H)	1	-	1.89
7β-OAc	1	-	1.73
11β-OAc	1	-	1.98

quantities of less than a milligram, were acetylated under mild conditions and the products were examined on the SE-30 column at 225°. The retentions of the observed peaks are compared with calculated values using the retention factors of Table 1A, and indicate that the proposed structures were in reasonable agreement with expectation.

The two examples of non-vicinal alcohols included in Table 2 show the close agreement between the observed and calculated retentions of known compounds using 5β-cholane-3α,24-diol as parent compound and the retention factors for the 7α and 12α-positions. Thus they illustrate the applicability of this mode of characterisation to tri- and tetra-substituted steroids.

It must be emphasised however that the characterisation of unknown reaction products from retention data is tentative when based in this way on a single chromatogram, but is very greatly strengthened by comparative runs on two or three columns of different selectivity. The initial use of an almost non-selective phase such as SE-30 is of value in classifying the components essentially according to their molecular weight: only very limited differentiation of the position and type of functional group is possible with this phase. More detailed information is then obtainable from

Table 2. Tentative characterisation of steroids from retention data

Parent Compound	Proposed structure	Relative Retention, 225° Found	Calculated *
Acetylation products			
5α-Androstane-3β,7α,17β-triol	triacetate	1.51	0.858 x 1.75 = 1.50
5α-Androstane-3β,11β,17β-triol	3-acetate		0.395 x 1.54 x 1.74 = 1.06
	or	1.19	
	3,11-diacetate		0.395 x 1.54 x 1.98 = 1.20
	3,17-diacetate	1.48	0.858 x 1.74 = 1.49
	triacetate	1.85	0.858 x 1.98 = 1.70
5β-Androstane-3α,11β,17β-triol	3,17-diacetate	1.25	0.758 x 1.74 = 1.32
	triacetate	1.52	0.758 x 1.98 = 1.50
5β-Pregnane-3α,11β,20β-triol	3,20-diacetate	2.17	1.24 x 1.74 = 2.16
3α,6α-Dihydroxy-5β-pregnan-20-one	3-acetate	1.79	0.825 x 2.03 = 1.68

Table 2 (continued)

Parent Compound	Proposed structure	Relative Retention, 225° Found	Calculated *
Non-vicinal Hydroxy Steroids			
5β-Cholane-3α,12α,24-triol	-	3.22	1.95 x 1.63 = 3.18
5β-Cholane-3α,7α,12α,24-triol	-	5.87	1.95 x 1.80 x 1.63 = 5.72

* From the mean 6α,7α,11β,12α-hydroxyl or acetoxyl factors (Table 1A) and the relative retention of the corresponding disubstituted parent compounds (Table 1).

more selective phases and from examination of suitable derivatives.

(b) Vicinal diols of the cholestane series

The vicinal diols were examined both on SE-30 and on OF-1 columns. In order to assess the interaction between adjacent hydroxyl groups it was necessary first to determine their individual retention factors. Table 3 gives values for a number of 5α- and 5β-cholestanols on SE-30, and includes acetoxyl factors for comparison. With the exception of the strongly hindered 7α and 1α-hydroxyl groups, the secondary alcohols all show factors of the same order, but the tertiary 5α-hydroxyl is sharply distinguished by a lower value. It is noteworthy that acetylation of 5α-cholestan 3α- and 3β-ol leads to a separation factor of 1.12 which permits resolution, as found in practice, but for the 4α- and 4β-alcohols acetylation does not improve the separation factor.

Further hydroxyl factors based on other parent compounds are listed in Table 4 (cf. also Table 1). Some vicinal effects are apparent in the unsaturated sterols. The 3α-hydroxyl factor is 1.85 for cholest-5-ene as against 1.96 for 5α-cholestane. Intramolecular hydrogen bonding, which is known to occur in cholest-5-en-3α-ol (I)(6,7) may account for this effect by reducing the polarity of the diol and weakening its interaction with the liquid phase. Cholest-5-en-4β-ol (II) and ergosta-7, 22-dien-5α-ol (III)(8,10) are other compounds containing a hydroxyl group and double bond suitably placed for hydrogen bonding, and both

Table 3. Retention Factors for the Introduction of Hydroxyl and Acetoxyl Groups into Cholestane

1% SE-30, 225°

	Configuration		Hydroxyl Factor †	Acetoxyl Factor †
5α-Cholestane	1α	(ax)[a]	1.80	(1.86)*
	2β	(ax)	1.92	2.80
	3α	(ax)	1.96	2.68
	3β	(eq)	1.98	3.01
	4α	(eq)	1.97	2.74
	4β	(ax)	1.88	2.61
	5α	(ax)	1.66	-
	6α	(eq)	1.92	2.56
	7α	(ax)	1.81	(1.79)*
5β-Cholestane	3α	(eq)	1.99	2.92
	3β	(ax)	1.97	2.74

† The hydroxyl and acetoxyl factors are relative retentions for the 5α-cholestane derivatives since 5α-cholestane = 1.00

a. Provided by Professor H. B. Henbest (Belfast)

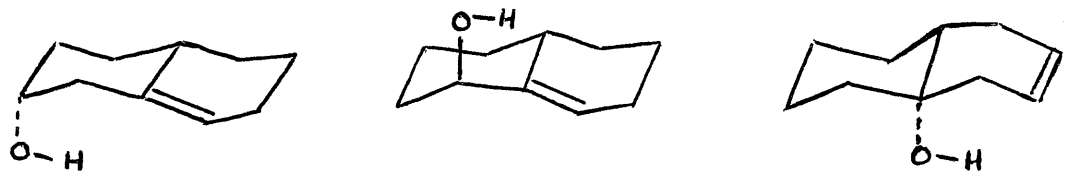
* Anomalous values see Table A under Materials and Methods.

Table 4. Retention factors for hydroxyl groups in various steroids

1% SE-30, 225°

Hydroxyl group position (X)		Parent compound	Relative retention time		Hydroxyl Factor
			X-OH	X-H	
3α	ax	Cholest-5-ene	1.84	0.99	1.85
4β	ax	Cholest-5-ene	1.72	0.99	1.73
5α	ax	5α-Ergosta-7,22-diene	1.87	1.25	1.50
5β	ax/eq	5β-Cholestane	1.53	0.91	1.68
12α	ax	5α-Cholane	0.74	0.44	1.68
12α	ax	5β-Cholane	0.66	0.41	1.63
12β	eq	5β-Cholane	0.67	0.41	1.65
24		Cholest-5-en-3β-ol	3.72	1.95	1.91
24		Cholest-5-en-3β-ol acetate	5.72	3.00	1.90
25		Cholest-5-en-3β-ol	3.28	1.95	1.68

give rise to lower hydroxyl retention factors. Clayton (9) has pointed out similar effects for ergost-7-en-5α-ol and cholest-2-en-5α-ol.



I II III

The data in the foregoing Tables show that the retention factors (on SE-30) for introducing hydroxyl groups at positions 3, 5, 7α and 12α are not markedly affected by a change in nuclear configuration at C_5, as already demonstrated for the 3-position in Section 1.

The purity of the cholestanediols was carefully checked by thin-layer chromatography and Table 5 gives the range of Rf values for some representative compounds. Most of the compounds appeared to be homogeneous, though additional spots were occasionally visible on overloading the chromatoplate. However, some of the apparently pure diols gave several minor peaks on gas-liquid chromatography. This was not unexpected in view of the greater sensitivity and resolving power of the latter technique, and of the possible occurrence of some elimination. For example, 5α-cholestane-1α,2α-diol consistently gave a single spot when examined in the eight solvent systems, but gave rise to three peaks (relative retention on SE-30: 3.1, 2.1, 1.8) on gas-liquid chromatography. It seems probable that the peak at 1.8

Table 5. Range of Rf values of representative Cholestanediols

Compound	Solvent Systems							
	C_6H_6 : $CHCl_3$ (4:6)	$CHCl_3$	Tol : EtOAc (9:1)	C_6H_{12}:EtOAc (7:3)	C_6H_6:EtOAc (6:3)	$CHCl_3$:Me_2CO (9:1)	$CHCl_3$:EtOAc (3:1)	$CHCl_3$:EtOAc (3:2)
5α-Cholestane-1α,2α-diol	0.0-0.13	0.05-0.15	0.05-0.22	0.42-0.52	0.49-0.51	0.39-0.52	0.38-0.66	0.63-0.73
5α-Cholestane-2α,3α-diol	0.0-0.07	0.02-0.15	0.0-0.11	0.17-0.31	0.18-0.40	0.27-0.35	0.19-0.37	0.31-0.50
5α-Cholestane-3α,4α-diol	0.0-0.11	0.0-0.17	0.0-0.11	0.06-0.30	0.18-0.45	0.28-0.42	0.16-0.35	0.23-0.55
Cholestane-4β,5β-diol	0.03-0.21	0.12-0.28	0.02-0.30	0.49-0.50	0.46-0.69	0.48-0.67	0.49-0.60	0.60-0.76
Cholestane-3α,5β-diol	0.0-0.03	0.0-0.05	0.0-0.04	0.01-0.15	0.0-0.21	0.01-0.17	0.0-0.18	0.03-0.28
Cholestane-5α,6α-diol	0.07-0.24	0.10-0.20	0.26-0.40	0.56-0.65	0.66-0.71	0.48-0.73	0.54-0.67	0.66-0.76
5α-Cholestane-6α,7α-diol	0.03-0.26	0.09-0.22	0.04-0.29	0.57-0.65	0.60-0.75	0.38-0.65	0.55-0.72	0.65-0.78
5α-Cholestane-6β,7α-diol	0.06-0.20	0.14-0.28	0.13-0.25	0.47-0.56	0.55-0.74	0.52-0.70	0.44-0.65	0.66-0.78
5α-Cholestane-7α,8α-diol	0.31-0.50	0.36-0.56	0.53-0.64	0.68-0.74	0.83-0.93	0.80-0.93	0.85-0.05	0.88-0.99
5α-Cholestane-7β,8[illegible]-diol	0.02-0.09	0.14-0.20	0.0-0.11	0.24-0.31	0.35-0.53	0.49-0.54	—	0.52-0.72

Tol. = Toluene.

represents a dehydration product such as 5α-cholestan-1-one or 5α-cholestan-2-one. In general the results from thin-layer and gas-chromatography were in agreement.

Table 6 summarises the relative retentions found for a group of cholestanediols on SE-30 at 225°. They are listed in order of decreasing retention time within each set of configurational isomers. In the 5α-cholestane-2,3-diols there is a gradation in relative retention from 3.51 for the diaxial isomer to 3.36 for the diequatorial isomer. The 3,4-diols which have very similar steric environments to the 2,3-diols show an analogous trend: the marginally lower retention times in this group may be ascribed to the effect of the additional 1:3 hydrogen interactions with the C-6 hydrogens.

Most of the remaining diols comprise pairs of epimers. In every case the diaxial isomer shows longer retention than the equatorial-axial type, but even in the former there is evidence of small vicinal effects in that the retentions are mostly lower (3-7%) than would be expected from the individual hydroxyl retention factors. The more marked discrepancies (9-22%) for the equatorial-axial isomers are presumably due to the proximity of the hydroxyl groups, perhaps involving a persistence of hydrogen bonding under the conditions of chromatography.

Table 6. Retention Data for Vicinal Cholestanediols on SE-30 at 225°

Diol	Configuration		H-bonding	Relative Retention Time	
(5α unless stated)	I	II		Found	Calculated
2β, 3α	ax	ax	-	3.51	3.80
2α, 3α	eq	ax	+	3.42	-
2β, 3β	ax	eq	+	3.38	3.76
2α, 3β	eq	eq	+	3.36	-
2β, 3α (5β)	eq	eq	+	3.09	-
3α, 4β	ax	ax	-	3.48	3.72
3α, 4α	ax	eq	+	3.38	3.90
3β, 4β	eq	ax	+	3.30	3.68
1α, 2β	ax	ax	-	3.25	3.45
1α, 2α	ax	eq	+	3.14	-
6β, 7α	ax	ax	-	3.00	-
6α, 7α	eq	ax	+	2.95	3.48
4β, 5α	ax	ax	-	3.02	3.12
4β, 5β	eq	ax	+	2.90	-

Table 6 (continued)

Diol (5α unless stated)	Configuration I	Configuration II	H-bonding	Relative Retention Time Found	Relative Retention Time Calculated
4α, 5α	eq	ax	+	2.55	3.27
5α, 6β	ax	ax	-	2.95	-
5α, 6α	ax	eq	+	2.90	3.18
7α, 8α	ax	eq	+	(1.83)*	-
7β, 8ξ	eq	ax/eq		(1.63)*	-

* Dehydration products.

Table 7 gives hydroxyl factors on QF-1 at 225° computed from 5α- or 5β-cholestane as parent compounds. As already found for SE-30, the 1α- and 7α-hydroxyl factors are markedly lower than the values for other secondary alcohols while the tertiary 5-hydroxyl group is distinguished by even lower factors. The differences in retention between equatorial and axial hydroxy steroids are small on SE-30 (cf. Table 3) but are distinctly greater on QF-1 as already demonstrated in Section 1. The axial 3-alcohols (3α,5α and 3β,5β) gave factors close to 2.8 and the equatorial 3-alcohols (3β,5α and 3α,5β) values near 3.15.

Retention data for cholestanediols on QF-1 are given in Table 8. The sets of configurational isomers are listed in order of decreasing magnitude of the highest retention value in each set. This sequence parallels that found for SE-30 (Table 6), but within each set the order of elution is different. For the QF-1 column the diaxial isomer has a retention intermediate between that of the two equatorial-axial epimers. The one example of a diequatorial diol (2α,3β) shows longer retention than any of its three configurational isomers whereas on SE-30 it shows the shortest of the four values.

Miscellaneous retention data for a few nonvicinal cholestanediols are collected in Table 9. The cholestane-3,5-diols which form a

Table 7. Retention Factors for the Introduction of Hydroxyl groups into Cholestane

1% QF-1, 225°

	Configuration	Hydroxyl Factor
5α-Cholestane	1α (ax)	2.33
	2β (ax)	2.58
	3α (ax)	2.81
	3β (eq)	3.13
	4α (eq)	2.89
	4β (ax)	2.53
	5α (ax)	2.10
	6α (eq)	2.92
	7α (ax)	2.38
5β-Cholestane	3α (eq)	3.16
	3β (ax)	2.75
	5β (ax/eq)	2.22

The hydroxyl factors are relative retentions for the 5α-cholestane derivatives since 5α-cholestane = 1.00

Table 8. Retention Data for Vicinal Cholestanediols on OF-1 at 225°

Diol	Configuration		H-bonding	Relative Retention Time	
(5α unless stated)	I	II		Found	Calculated
2α, 3β	eq	eq	+	6.93	-
2α, 3α	eq	ax	+	6.61	-
2β, 3α	ax	ax	-	6.37	7.25
2β, 3β	ax	eq	+	6.13	8.07
2β, 3α (5β)	eq	eq	+	5.34	-
3α, 4α	ax	eq	+	6.50	8.12
3α, 4β	ax	ax	-	5.72	7.10
3β, 4β	eq	ax	+	5.68	7.92
1α, 2α	ax	eq	+	5.33	-
1α, 2β	ax	ax	-	5.26	6.02
6α, 7α	eq	ax	+	5.00	6.95
6β, 7α	ax	ax	-	4.74	-
4β, 5β	eq	ax	+	4.80	-
4α, 5α	eq	ax	+	4.32	6.07
4β, 5α	ax	ax	-	4.30	5.31

Table 8 (continued)

Diol (5a unless stated)	Configuration I	Configuration II	H-bonding	Relative Retention Time Found	Relative Retention Time Calculated
5a , 6a	ax	eq	+	4.61	6.13
5a , 6β	ax	ax	-	4.25	-
7a , 8a	ax	eq	+	(3.90)*	-
7β , 8ξ	eq	ax/eq		(3.44)*	-

* Dehydration products.

Table 9. Miscellaneous Retention Data for Cholestane Derivatives on SE-30 and QF-1 at 225°

	1% SE-30 Relative Retention Time		1% QF-1 Relative Retention Time	
	Observed	Calculated	Observed	Calculated
Non-vicinal cholestanediols				
Cholestane-3α, 5α-diol	3.20	3.25	6.46	5.90
Cholestane-3β, 5α-diol	3.12	3.28	6.07	6.57
Cholestane-3β, 5β-diol	2.93	3.31	6.23	-
Cholestane-3α, 5β-diol	2.81	3.34	5.82	6.43
5α-Cholestane-3β, 6α-diol	3.90	3.80	8.79	9.14
5α-Cholestane-3β, 7α-diol	3.58	3.58	7.70	7.45
5α-Cholestane-3β, 7β-diol	3.58	-	7.45	-
5β-B-Norcholestane-3α, 6α-diol [c]	2.14	-	4.34	-
5β-B-Norcholestane-3β, 6α-diol [c]	2.05*	-	3.74*	-
5β-B-Norcholestane-3β, 6β-diol [c]	1.94	-	3.89	-
5β-B-Norcholestane-3α, 6β-diol [c]	1.90	-	3.87	-

Table 9 (continued)

	1% SE-30 Relative Retention Time		1% QF-1 Relative Retention Time	
	Observed	Calculated	Observed	Calculated
Ketones				
5α-Cholestan-3-one	2.13	-	5.79	-
5α-Cholestan-6-one	1.94	-	4.69	-
5α-Cholestan-7-one	1.80	-	4.00	-

a. Cholestane = 11.2 min. = 1.00

b. Cholestane = 2.3 min. = 1.00

c. Provided by Dr. J. Joska (Prague)

* Partially decomposed on column giving peaks indicative of dehydration products.

complete set of configurational isomers behave differently on the two phases. On SE-30 the diaxial isomer both in the 5α- and 5β series is retained longer than its equatorial-axial epimer while on QF-1, irrespective of the nuclear configuration at C_5, both equatorial-axial isomers are eluted before the two diaxial epimers. There appears to be no correlation between the agreement of observed and calculated values and the presence of hydrogen bonding. For example cholestane-3α,5α-diol which is known to be intramolecularly hydrogen-bonded in solution (10) has a retention time in agreement with the calculated value on SE-30, and greater by about 8% on the QF-1 phase while the retentions of the non-bonded isomers differ from the calculated values by 5-10% on the two phases. It would appear that the hydrogen bond in cholestane-3α,5α-diol is destroyed at the temperature of the chromatograph. A similar explanation could be extended to 5β-B-norcholestane-3α,6α-diol which is known to be hydrogen-bonded in solution (12) and might be expected to be eluted before its non-bonded isomers.

Among the 5α-cholestane-3β,6- and 3β,7-diols the latter compounds have shorter retentions because of the lower retention factor for the sterically hindered 7-hydroxyl group as already noted for the mono-alcohols. Similarly the cholestane-3,5-diols are

eluted before diols with hydroxyls in the 3,6 and 3,7 positions doubtless because of the tertiary grouping at C_5. The order of elution of the 3,5, 3,6 and 3,7 diols thus follows the order found for the individual cholestan-5,6 and 7-ols.

It may be concluded that the principal factor determining the retention of a vicinal cholestanediol is the nuclear position of the diol grouping, those in ring A conferring longer retentions than those in ring B. Among the non-vicinal cholestane diols, although the order of retentions appears to follow that in the mono-alcohol series, the observed retention times are still not identical with those calculated from the individual hydroxyl factors. It is therefore evident that a composite factor for the diol grouping must be employed in evaluating retention data for such compounds.

1. Clayton, R.B. (1962). Biochemistry, 1, 357.

2. Knights, B.A. & Thomas, G.H. (1962). Analyt. Chem. 34, 1046.

3. Brooks, C.J.W. & Hanaineh, L. (1963). Biochem. J. 87, 151.

4. Knights, B.A. & Thomas, G.H. (1963). Chem. & Ind. p. 43.

5. Stahl, E. (1961). Angew. Chem. 73, 646.

6. Schleyer, P. von R., Trifan, D.S. & Bacskai, R. (1958). J. Amer. chem. Soc. 80, 6691.

7. Ōki, M. & Iwamura, H. (1959). Bull. Chem. Soc. Japan, 32, 306.

8. Dalton, F., Meakins, G.D., Robinson, J.H. & Zaharia, W. (1962). J. chem. Soc. p. 1566.

9. Clayton, R.B. (1961). Nature, 192, 524.

10. Dalton, F., McDougall, J.I. & Meakins, G.D. (1963). J. chem. Soc. p. 4068.

11. Luukkainen, T., VandenHeuvel, W.J.A. & Horning, E.C. (1962). Biochim. biophys. Acta, 62, 153.

12. Pitha, J., Joska, J. & Fajkoš, J. (1963). Coll. Czech. Chem. Comm. 28, 2611.

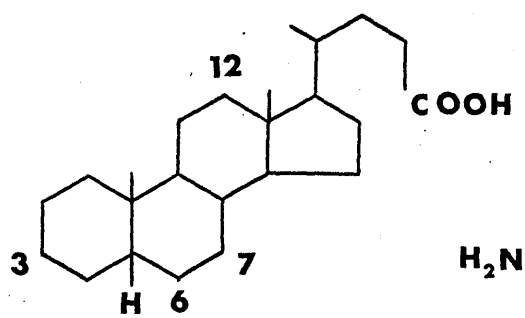
Section 3

Solvent Partition and Gas-Liquid Chromatographic Studies of Bile Acids

The principal bile acids of man are C_{24}-steroids of the 5β-series with a carboxyl group at C_{24} and one, two or three hydroxyl groups in the nucleus (Chart 1). They are degradation products of cholesterol and occur in bile, faeces and serum. In bile they exist mainly as conjugates of glycine and taurine in the form of sodium salts, in faeces the bile acids occur in the unconjugated form while in serum both free and conjugated bile acids are found at low concentrations, totalling about 1 μg./ml.

In most analytical procedures the conjugated bile salts are hydrolysed to the free acids before identification and estimation. The older methods of estimating free bile acids were based upon colour reactions, but owing to lack of specificity they were replaced by procedures employing spectrophotometric (1,2,3,9,10) fluorometric (4,5,6,8) spectroscopic (32) or radioactive (11,12) measurements. Preliminary solvent extraction methods, counter-current distribution, column or paper chromatographic separations were often employed. A procedure for the rapid separation of bile acids on glass fiber paper impregnated with silicic acid or monopotassium phosphate was also devised (13). After VandenHeuvel, Sweeley and

CHART 1



$H_2NCH_2CO_2H$ GLYCINE

$H_2NCH_2CH_2SO_3H$ TAURINE

	Hydroxyl Substitution
Cholic Acid	3α,7α,12α
Deoxycholic Acid	3α,12α
Chenodeoxycholic Acid	3α,7α
Ursodeoxycholic Acid	3α,7β
Hyodeoxycholic Acid	3α,6α
Lithocholic Acid	3α

Horning (14) showed the possibility of separating unconjugated bile acids by gas-liquid chromatography, a number of workers (15-23,48) carried out qualitative or quantitative analyses of bile acids by this method, and by ion exchange followed by gas-chromatography (24). Ion exchange procedures were also applied to the separation of bile acids from bile (50), faeces and serum (49) utilising colorimetric and spectro photometric measurements for determination of the bile acids. In the analysis of bile and serum extracts by the above methods, because of the preliminary hydrolytic step the proportions of bile acids conjugated to glycine and taurine could only be estimated by indirect procedures.

The strongly hydrophilic nature of the conjugated bile acids prevents their ready extraction and separation and has hindered attempts to isolate them without first modifying their properties by hydrolysis. However, in 1952 Ahrens and Craig (27) used counter-current distribution and in 1953 Bergström and Norman (28) employed reversed phase partition chromatography for separating the conjugated bile acids of bile. These methods required large amounts of material and long analysis times, and more convenient paper chromatographic methods were developed by a number of workers (10,26,29-31). Preliminary separations by counter-current distribution or column chromatography were still often employed before paper chromatographic

identification. Sjövall (10) modified his original method so that both conjugated and free bile acids could be separated (30,33), and the method was then applied to bile and duodenal contents. After paper chromatographic separation, the bile acids were eluted and determined by spectrophotometry (34,35). The limit of detection in the above method was 1 mg. of bile acid per 100 ml. of bile. Levin, Johnston and Boyle (36) reported a solvent extraction procedure for the separation of the glycine and taurine conjugates of cholic and deoxycholic acids followed by spectrophotometric determination, and applied their method to the analysis of animal and human bile.

Thin-layer chromatography is yet another technique that has been applied to the study of bile acids. Its main advantage over the older methods of column and paper chromatography is the rapidity with which an analysis can be carried out. After Stahl (39) published a standard procedure for thin-layer chromatography several workers (40,41,42,51,52,53) applied the method to the separation of free and conjugated bile acid standards. Gänshirt, Koss and Morianz (40) worked out a rapid method for the quantitative determination of bile acids by colorimetry after chromatographic separation, while Hofmann (41) described the separation of a few

common bile acids and their conjugates. Eneroth (42) and Hara and Takeuchi (51) carried out a systematic analysis of a large number of bile acids and their derivatives and examined the relationship between structure and adsorptivity (Rf value).

The bile acids in serum are the same as those found in bile. The main acids in order of abundance are cholic, chenodeoxycholic and deoxycholic. The first two are "primary" bile acids formed from cholesterol in the liver, whereas deoxycholic acid is a product of bacterial dehydroxylation of the 7α -hydroxyl group in cholic acid.

Their analysis in serum is complicated by their occurrence mainly in conjugated forms and by their presence in very low concentrations (cf. review by MacIntyre and Wootton (45)). Many attempts have been made to estimate bile acid concentrations in blood, but the results of these studies have varied greatly, the values ranging from 0 to 40 mg./100 ml. plasma (45). During the past few years, preliminary chromatographic separations followed by spectrophotometric (1) or fluorometric (8) measurements have detected no bile acids in normal human serums. Carey, however, examined purified human serum extracts by paper chromatography and observed spots corresponding to bile acids (9). By employing

spectrophotometric methods (3) he found trihydroxy- and dihydroxy cholanic acid concentrations of 0.14 and 0.08 mg./100 ml. serum respectively. Other attempts at estimating blood bile acids have been made by Scott, Grundy and Sjövall (12) using rats and by Portman and Shah (11) using monkeys. Both groups used column chromatography followed by radioactive estimations and arrived at bile acid concentrations similar to those found by Carey for human serum.

More recently, Sjövall et al. (24) extracted bile acids from human serum by passing the serum through an ion exchange column and analysing the eluate (after hydrolysis) by gas-liquid chromatography. They were able to identify cholic, deoxycholic and chenodeoxycholic acids by their retention times and the retention times of their derivatives. Kuron and Tennent (49) have also described an ion exchange procedure applicable to the separation of bile acids from serum in which the bile acids are, after hydrolysis, determined by spectrophotometry.

As previous studies of serum bile acids have involved their hydrolysis to the free acids before estimation, it was the aim of this investigation to examine the gas-liquid chromatography of the conjugated bile acids in the hope that after preliminary solvent extraction, gas chromatographic analysis might ultimately permit

the direct estimation of serum bile acids.

This section deals (i) with studies of the extraction and solvent partition of bile acids from serum and their attempted characterisation by thin-layer chromatography; (ii) with the gas-liquid chromatographic behaviour of glycine-conjugated bile acids.

(i) Solvent Extraction and Thin-layer Chromatography of Serum Bile Acids

Materials and Methods

Most of the bile acids were obtained from L. Light & Co., Ltd., Colnbrook, Bucks, England; Southeastern Biochemicals Inc., Morristown, Tennessee; and Calbiochem, A.G., Lucerne, Switzerland. Authentic samples of glycocholic, glycodeoxycholic and glycolithocholic acids, sodium taurocholate and sodium taurochenodeoxycholate were generously provided by Professor G.A.D. Haslewood (Guy's Hospital Medical School). Solvents were of Analar grade: petroleum ether was of b.p. 60-80°. Solvent mixtures were prepared by volume unless otherwise specified.

In this section, the following abbreviations are used: C = cholic acid, DC = deoxycholic acid, CDC = chenodeoxycholic acid, HDC = hyodeoxycholic acid, UDC = ursodeoxycholic acid, LC = lithocholic acid, Chol = cholesterol, Me = methyl, T = taurine conjugate(s) and G = glycine conjugate(s).

Deoxycholic and chenodeoxycholic acid, as well as their conjugated derivatives, were in most cases unresolved and where only one acid is mentioned reference to the isomer is also implied.

The hydrolysis and extraction procedures applied to serum

were a combination of methods developed by Brooks and Young (37) and Levin, Johnston and Boyle (36) and are shown in Fig. 1. The hydrolysis step was a modification of the procedure of Abell, Levy, Brodie and Kendall (38) as detailed below.

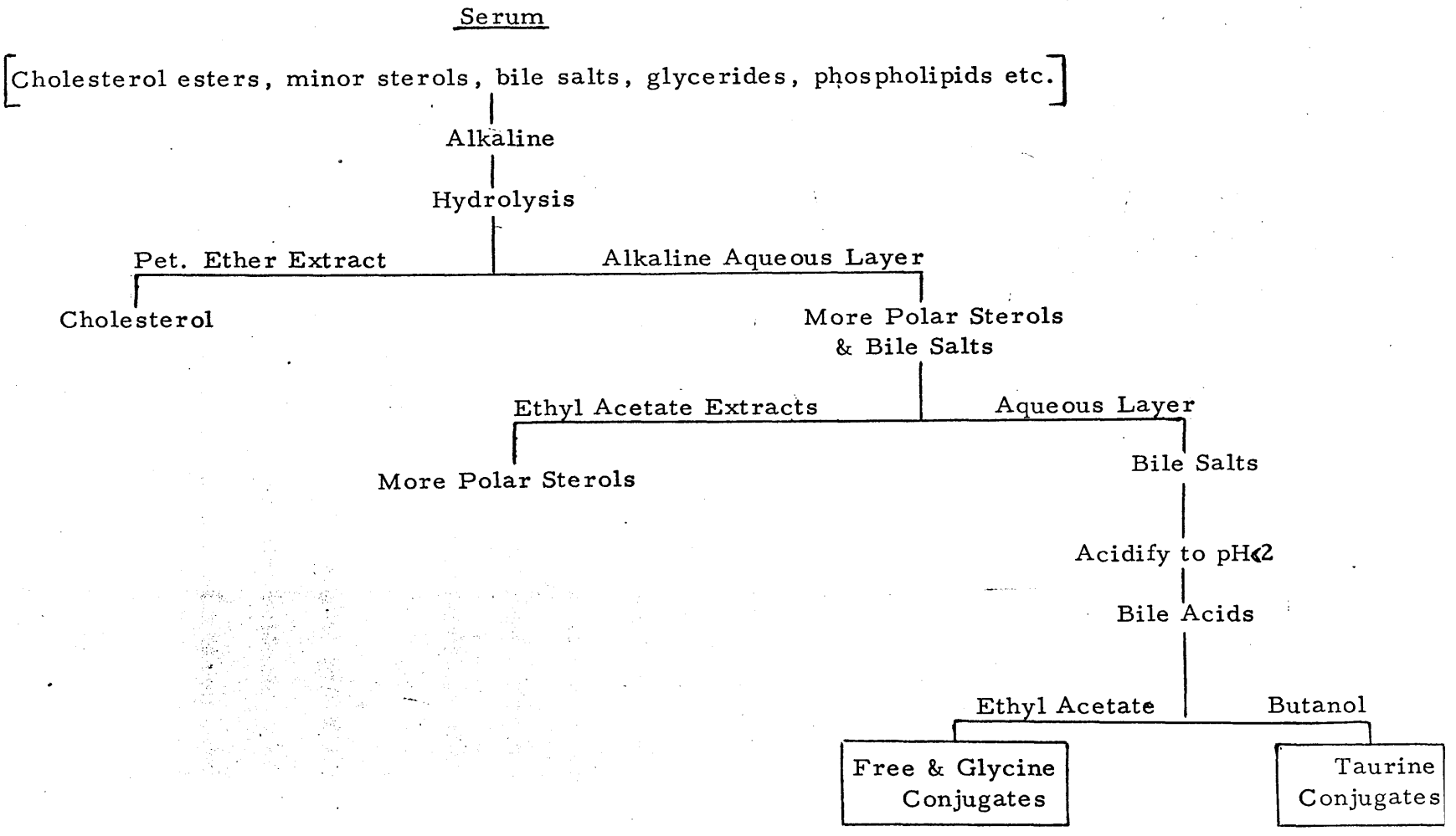
Hydrolysis

A stock 33% (w/v) aqueous solution of potassium hydroxide was diluted to 8% with absolute alcohol, and 5 ml. aliquots were added to 50 ml. polypropylene tubes. Subsequent addition of the serum in 1 ml. aliquots to the alkali ensured good dispersion and consequent complete hydrolysis of the cholesterol esters. A further 5 ml. of absolute alcohol was added to each tube, reducing the concentration of potassium hydroxide to 4%. The tubes were stoppered, shaken well and kept in a water bath at 40°C. for 1 hour. Recovery experiments showed that hydrolysis was at least 95% complete.

Petroleum ether extraction

Water (10 ml.) and petroleum ether (20 ml.) were added to the cool hydrolysed mixture. The tubes were shaken vigorously for 1 minute, centrifuged for 5 minutes and the layers separated. The process was repeated with a further 2 x 20 ml. petroleum ether and the combined petroleum ether layers, containing principally cholesterol, were discarded.

FIG. I. Simplified Scheme of Extraction of Bile Acids from Serum.



Ethyl acetate extraction

The alkaline aqueous layer was further extracted with ethyl acetate (3 x 10 ml.) with the aim of removing more polar sterols and any cholesterol not removed by the previous extraction. The remaining aqueous layer was expected to contain the bile salts in addition to bile pigments, phospholipids, glycerol, salts of fatty acids etc. The procedure up to this point was that developed by Brooks and Young, but the following separation of bile acids into glycine and taurine conjugates was that of Levin et al.

Extraction with ethyl acetate and n-butanol

The aqueous layer was first acidified with 6N-hydrochloric acid to pH<2 in order to convert the bile salts to the free acids. This was followed by extraction with ethyl acetate (3 x 10 ml.) to remove the free and glycine conjugated bile acids and with n-butanol to remove the taurine conjugates.

In the method of Levin et al. (36) the reagent grade ethyl acetate and n-butanol were washed with hydrochloric acid and ammonium hydroxide before use. In our work these solvents were instead twice distilled. These authors' use of concentrated hydrochloric acid and ammonium hydroxide in the final extraction stage was eliminated as it caused the precipitation of salts. The use of separating funnels was avoided because of a tendency for

emulsification to occur, and solvent extraction was carried out in polypropylene tubes permitting centrifugal separation of the layers.

Other components of serum

Thin-layer chromatography of the ethyl acetate fraction of serum showed a number of extraneous spots near the solvent front which overlapped with some of the faster-running bile acids.

Two solvent systems were examined, in conjunction with selective spray reagents, with the aim of distinguishing bile acids from contaminants. The systems (a) chloroform:acetic acid = 48:2, and (b) toluene:acetic acid:water = 5:1:1, did not achieve good separations of the bile acids from the faster-running components. However, with the aid of 0.1% dichloro-R-fluorescein in ethanol as spray reagent, three of the extraneous spots in the ethyl acetate fraction were shown to correspond to fatty acids, cholesterol and cholesterol esters (e.g. cholesterol stearate). The fatty acids from C_8 (n-caprylic) to C_{18} (stearic, oleic etc.) were unresolved in both solvent systems examined. The presence of fatty acids in the extract is expected, and it is conceivable that traces of cholesterol esters remain after hydrolysis and solvent extraction because of the relatively large amounts originally present. Since the non-bile acid components of serum were not

of primary interest in this study no further attempt at characterisation was made.

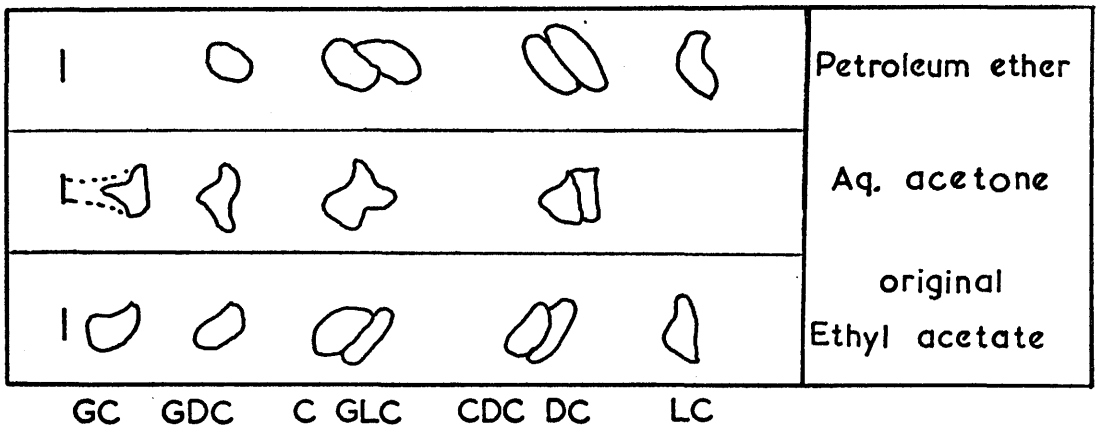
The residues obtained by evaporation of the ethyl acetate extracts were partitioned between hydrocarbon and hydroxylic solvents with the aim of eliminating contaminants. Preliminary investigations were carried out on processed reference compounds with the following combinations of solvents:

(a) Petroleum ether and aqueous acetone :— Petroleum ether (3 ml.) and 75% acetone (4 ml.) were added to the ethyl acetate residue. After mixing and centrifuging, the petroleum ether layer was removed. The aqueous acetone layer was extracted with a further 3 ml. of petroleum ether and the combined extracts were evaporated to dryness. The remaining aqueous acetone layer was also evaporated to dryness and both fractions were redissolved in small volumes of solvent for thin-layer chromatography. Partitioning was not very effective, as with the exception of lithocholic and glycocholic acid, the bile acids were found in both fractions (Fig. 2 (a)).

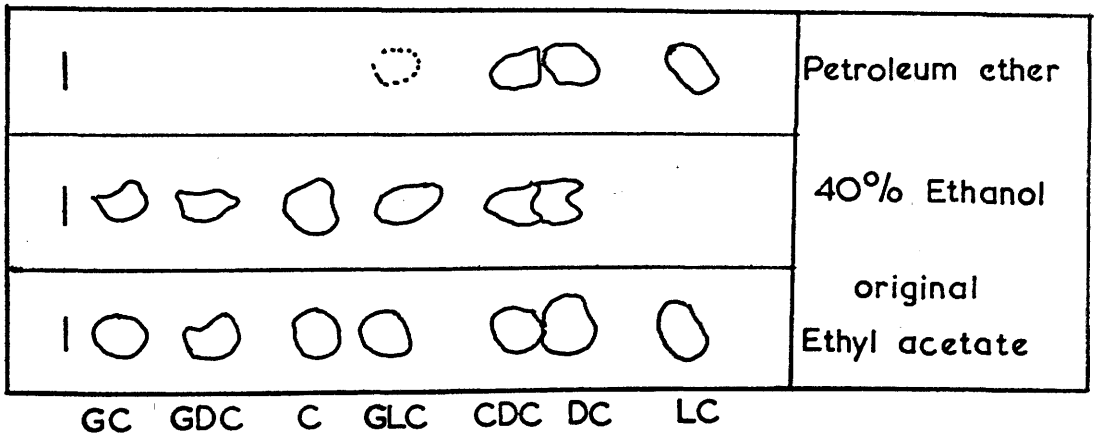
(b) Petroleum ether and 40% ethanol :— The procedure described in (a) was used, 40% ethanol being substituted for 75% acetone. Fig. 2(b) shows a chromatogram of both fractions as

FIGURE 2

a)



b)



SOLVENT Modified Ga I

SPRAY REAGENT Ceric sulphate

compared with the original unpartitioned ethyl acetate. Better separation was achieved in this case, as the petroleum ether fraction contained only the unconjugated mono- and dihydroxy bile acids.

(c) Water/petroleum ether and water/ethyl acetate :— The ethyl acetate residue was dissolved in water, then extracted with petroleum ether followed by ethyl acetate. Petroleum ether was found to extract lithocholic acid, also cholesterol and other non-bile acids, while the ethyl acetate layer contained the remaining lithocholic acid and cholesterol in addition to the other bile acids. Procedures (b) and (c) were used in the partitioning of serum extracts.

Thin-layer chromatography

The preparation of thin-layer "chromatoplates" was according to the method of Stahl (39). Experimental details are given in Part A, section 2. At first the chromatograms were developed over 10 cm. but later a 15 cm. run was found to give better separations. The unconjugated bile acids were dissolved in acetone and the conjugated acids in methanol for application to the chromatoplate.

Solvent systems

The solvent systems used for thin-layer chromatography were

those introduced by Gänshirt, Koss and Morianz (40), Hofmann (41) and Eneroth (42)*.

(a) Gänshirt No. I - "Ga I"
toluene:acetic acid:water = 5:5:1 (upper phase)

(b) Modified Gänshirt No. I - "modified Ga I"
benzene:acetic acid:water = 5:5:1 (upper phase)

"Ga I" and "modified Ga I" were suitable for separating the glycine conjugated and unconjugated bile acids.

(c) Gänshirt No. II - "Ga II"
n-butanol:acetic acid:water = 10:1:1 (single phase)

"Ga II" was suitable for separating the various glycine and taurine conjugated bile acids. A disadvantage of this system is the long development times (3-3½ hours) required for a 15 cm. chromatoplate.

(d) Hofmann No. I - "Ho I"
acetic acid:carbon tetrachloride:di-isopropyl ether:isoamyl acetate:n-propanol:benzene = 5:20:30:40:10:10 (single phase)

(e) Eneroth S_{11} - "En S_{11}"
trimethylpentane:ethyl acetate:acetic acid = 10:10:2 (single phase)

Rf values relative to deoxycholic acid were of the same order as those quoted by Eneroth (42). "Ho I" and "En S_{11}" were suitable for separating the free and glycine conjugated bile acids.

* Publication of Eneroth's system appeared only towards the end of this investigation.

Table 1 gives typical Rf values of reference compounds in the five solvent systems. The generally poor reproducibility of Rf values (30,31) made it necessary to include reference compounds in all chromatograms of unknowns. The Rf values are expressed in the usual way as the ratios of the distance covered by the spots and by the solvent front.

To improve the resolution of certain pairs of bile acids (C/GLC and DC/CDC) development of a chromatoplate in two successive solvent systems was tried. Reference bile acids on a 15 cm. neutral chromatoplate were run first in methanol:chloroform:water = 25:65:4 ("MCW1") then in En S_{11}. Excellent separation of the acids GC, GDC, GLC and C was achieved but the acids GLC/HDC and DC/UDC/CDC were not resolved.

To confirm that the spots observed on thin-layer chromatography were due to bile acids, other possible constituents of the serum extracts were also examined. Of the following compounds: urea, lecithin, glucose; lactic, citric and pyruvic acids, only citric acid gave rise to a spot which could be mistaken for glycocholic acid in the system Ga I. However, it was readily distinguishable from glycocholic acid in other solvent systems.

The spray reagents used to detect the bile acids were 1% ceric sulphate in 10% H_2SO_4, 10% phosphomolybdic acid in ethanol ("PMoA")

Standard Bile Acid Rf Values.

Bile Acids	Modified Ga I	Ga I	Ga II	Ho I	En S_{11}
Taurocholic	0.00	0.00	0.22-0.27	0.00	0.00
Taurodeoxycholic	0.00	0.00	0.35-0.43	0.00	0.00
Glycocholic	0.10-0.16	0.01-0.03	0.49-0.57	0.00	0.0-0.04
Glycodeoxycholic	0.28-0.34	0.10-0.12	0.64-0.72	0.03-0.05	0.05-0.11
Glycolithocholic	0.47-0.57	0.26-0.28	0.71-0.77	0.25-0.35	0.25-0.31
Cholic	0.47-0.50	0.25-0.30	0.78-0.79	0.15-0.18	0.16-0.21
Hyodeoxycholic	-	-	-	-	0.31-0.37
Ursodeoxycholic	-	-	-	-	0.36-0.43
Chenodeoxycholic	0.65-0.77	0.41-0.45	0.80-0.85	0.42-0.48	0.42-0.48
Deoxycholic		0.43-0.48	0.81-0.85		0.43-0.48
Lithocholic	0.85-0.91	0.56-0.60	0.81-0.85	0.70-0.77	0.54-0.58

The range of Rf values given is the result of numerous chromatograms run in each solvent system.

(7) and a saturated solution of antimony trichloride in chloroform. After spraying, the plates were dried in an oven at 100° for 10 minutes. Ceric sulphate gave rise to fluorescent yellow spots when the plate was viewed under ultraviolet light: under these conditions the limit of detection was about 0.1 µg. Phosphomolybdic acid gave dark blue spots on a yellow background, and antimony trichloride orange-yellow spots on a purple background when viewed in ordinary light.

At a late stage in the work, Kritchevsky, Martak and Rothblat (44) reported a reagent which gave rise to distinctive colours with different bile acids. It consisted of anisaldehyde (0.5 ml.), glacial acetic acid (50 ml.) and concentrated sulphuric acid (1 ml.), mixed just before use. Although distinctive colours were indeed observed with our reference samples, the reagent was found unsatisfactory for serum extracts as the colours were masked by background impurities. Table 2 summarizes the colours observed with the various reagents.

Elution of bile acids

When a plate was to be eluted the chromatogram was run for the usual 10 or 15 cm. From a knowledge of the Rf values of the compounds, zones incorporating the required spots were scraped

Table 2.

Bile acid colours observed with different spray reagents.

Bile Acids	Anisaldehyde	Salicylaldehyde	Antimony trichloride	Ceric Sulphate
Glycocholic	brown-purple	-	yellow	bright yellow
Glycodeoxycholic	pale-purple	-	yellow	white-yellow
Glycolithocholic	turquoise	-	orange	pale-yellow
Cholic	brown purple	pink	blue-yellow	white-yellow
Hyodeoxycholic	dark blue	olive-green	pink	white-yellow
Ursodeoxycholic	pale blue	green-grey	-	white-yellow
Chenodeoxycholic	pale blue	green-grey	orange-yellow	pale yellow
Deoxycholic	brown	orange	yellow	white-yellow
Lithocholic	green	green-grey	pale purple	pale yellow
3,6-Dioxo-5β-cholanic	orange	-	-	-

off into small centrifuge tubes. (For checking purposes a margin of silica gel with the reference bile acids was left for spraying.) The silica gel was extracted three or four times with an appropriate solvent, centrifuging and removing the supernatant each time. The combined extracts were then evaporated in a stream of nitrogen and the residue examined by thin-layer chromatography.

Chloroform and ethyl acetate were initially used for elution but proved unsuitable, giving low recoveries of bile acids heavily contaminated with extraneous material. After many trials, methanol was found to be the most effective solvent for elution. The results showed that whereas the unconjugated acids were recovered to the extent of 90%, the recovery of glycine conjugates was much lower. The possibility of improved elution of bile acids from chromatoplates prepared with 0.05N oxalic acid solution ("acidic" plates) was briefly examined. It was found that such "acidic" plates could not be run in the solvent systems so far employed and the neutral system methanol:chloroform:water = 10:60:0.5 ("MCW2") was adopted. Since ceric sulphate detected only the trihydroxy acids (cholic and glycocholic) on "acidic" plates, other spray reagents were examined (distilled water, concentrated sulphuric acid, iodine vapour and phosphomolybdic acid) of which the last was the most suitable. The elution of bile acids from "acidic" chromatoplates

gave no improvement over the results obtained with neutral plates.

Bile acid methyl esters

Reference samples of unconjugated and glycine-conjugated bile acids were methylated as described in the following section (p.188). For the serum extracts methylation was effected as follows: the extract was evaporated to dryness and dissolved in a small volume of warm methanol. Excess of an ethereal solution of diazomethane was added to the extract and the mixture allowed to stand for 15-30 minutes. The solution was evaporated to dryness and the residue taken up in acetone for chromatography.

Gas-liquid chromatography

Details pertaining to the apparatus and the preparation of columns are given in Section 1 under Materials and Methods. The Chromatograph was operated under the following standard conditions: column temperature, 245° for SE-30 and 235° for QF-1; injection block temperature, 280 ± 5° (245 ± 5° for trifluoro acetates); argon flow rate, 40 ml./min. at outlet (for SE-30) and 80 ml./min. (for QF-1); nominal detector voltage, 1250V; sensitivity setting,10.

Trifluoroacetates

The trifluoroacetates of the free and conjugated bile acid methyl esters were prepared on a microgram scale according to the method of Sjövall (20) as follows: the methyl esters (200-400 µg.) were

dissolved in trifluoroacetic anhydride (200 µl.) and heated in an aluminium block at 30°C for 20 minutes. The reaction mixture was evaporated to dryness in a stream of nitrogen, and the residue taken up in acetone for chromatography. Completion of the reaction and purity of the samples were checked by thin-layer chromatography, in benzene for the unconjugated derivatives and in ethyl acetate for the glycine-conjugated trifluoroacetates. The unconjugated derivatives gave satisfactory results whereas the conjugated derivatives each showed several additional spots suggesting decomposition.

Results and Discussion

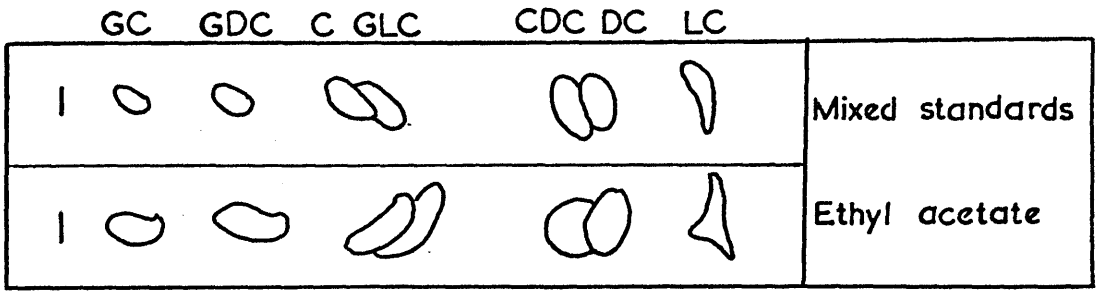
The solvent extraction procedure finally developed proved satisfactory for model mixtures of bile acids, but was found unsuitable for application to serum extracts, owing to interference by extraneous materials. The observations on serum extracts recorded below are therefore of an inconclusive nature, but are of possible value as a guide to future work. The solvent extraction method was applied on a semiquantitative basis to a synthetic mixture of bile acids before it was used for serum.

Extraction of bile acids from a solution of mixed standards

Solutions of one mg. of each of the reference samples (TC, TDC, GC, GDC, GLC, C, DC, CDC and LC) in 10 ml. 0.012N hydrochloric acid were extracted in the manner already described (p.145). Thin-layer chromatograms of the ethyl acetate and n-butanol fractions are shown in Figs. 3(a) and 3(b) respectively. Known amounts of reference samples were included, to permit semiquantitative evaluation of the extracted bile acids. Comparison of areas and intensities (Fig. 3(a)) indicated 80-90% extraction of free and glycine-conjugated bile acids in the ethyl acetate fraction. The butanol fraction (Fig. 3(b)) contained about 90% of the taurine conjugates, about 20% of glycocholic acid and 4-8% of the other

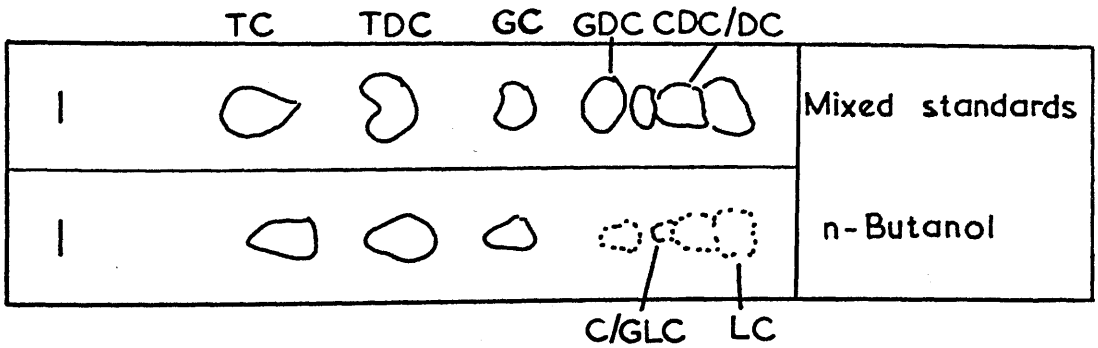
FIGURE 3

a)



SOLVENT Modified Ga I

b)



SOLVENT Ga II

SPRAY REAGENT Ceric sulphate

bile acids.

Re-extraction of the aqueous layer with n-butanol (Table 3, "n-Butanol 2") confirmed that the taurine conjugates had not been completely extracted. Detailed examination of the ethyl acetate and n-butanol fractions established that the former solvent extracted only the free and glycine-conjugated bile acids while n-butanol extracted predominantly the taurine conjugates. The approximate percentages of bile acids extracted in each layer are given in Table 3: there is considerable inaccuracy in the estimates of spots exceeding about 10 μg. The results show that with model mixtures this method is capable of yielding an extract free from taurine conjugates, but that in the isolation of the latter the presence of some glycine conjugates (largely glycocholic acid) cannot be avoided.

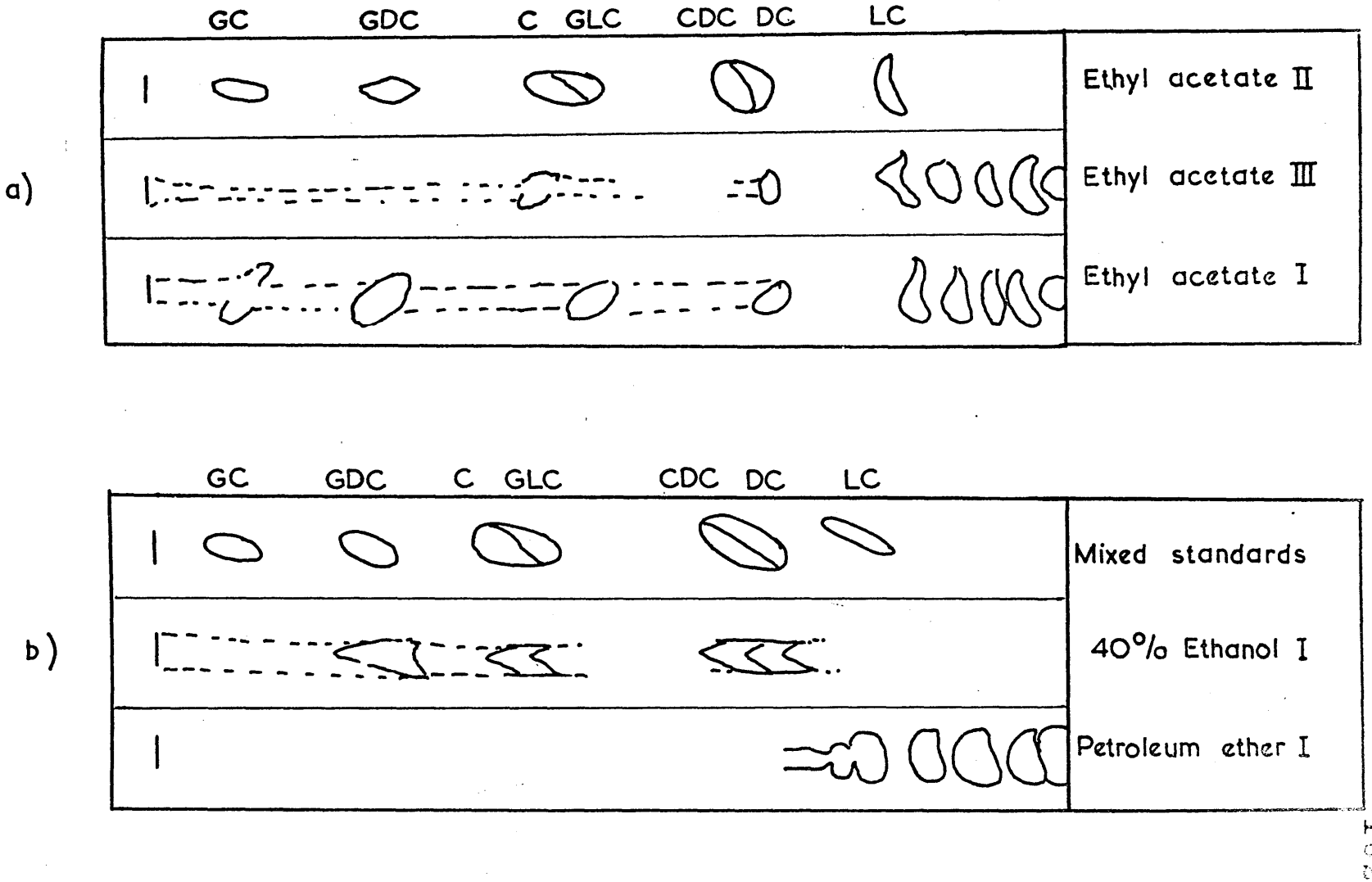
Extraction of bile acids from serum (I), mixed standards (II) and serum + mixed standards (III).

Qualitative application of the above method to normal serum wasattempted. Parallel experiments were carried out on serum, on a mixture of standard compounds (LC, DC, CDC, C, GLC, GDC, GC) and on serum to which the standard mixture had been added. The hydrolysed samples (solutions I, II & III) were first subjected to solvent extraction as outlined in Fig. 1. The ethyl acetate fractions from serum (I), mixed standards (II) and serum + mixed standards (III) were examined and the results are shown in Fig. 4(a).

Estimated Percentage of Bile Acids Extracted in Each Layer.

	Ethyl acetate				n-Butanol 1						n-Butanol 2	
	µg.		%		µg.			%			µg.	%
	A*	B *	A	B	A	B	C*	A	B	C		
TC	-	-	-	-	10	9.0	6.0	100	90	100	1.8	9
TDC	-	-	-	-	10	9.0	5.5	100	90	90	1.9	9.5
GC	⩾ 10	0.8	⩾ 50	80	5	2.5	1.2	50	25	20	0.4	2
GDC	⩾ 10	0.9	⩾ 50	90	< 1	0.5	0.2	< 10	5	3	0.5	2.5
GLC	⩾ 10	0.9	⩾ 50	90	< 1	0.5	0.2	< 10	5	3	0.4	2
C	⩾ 10	0.9	⩾ 50	90	< 1	0.5	0.2	< 10	5	3	0.8	4
DC	⩾ 10	0.9	⩾ 50	90	≪ 1	< 0.5		< 5	< 5			
CDC	⩾ 10	0.9	⩾ 50	90	≪ 1	< 0.5	0.2	< 5	< 5	3	0.8	4
LC	⩾ 10	0.9	⩾ 50	90	≪ 1	< 0.5		< 5	< 5			

A, B & C are separate experiments all starting with 1 mg. of each sample.
In A, aliquot applied to plate = 20 µg. of each acid if fully extracted.
In B, aliquot applied to plate = 10 µg. of each acid.
In C, aliquot applied to plate = 6 µg. of each acid.



SOLVENT Modified Ga I

SPRAY REAGENT Ceric sulphate

The serum extract "Ethyl acetate I" showed four well-defined spots corresponding in Rf values to the following bile acids: glycodeoxycholic, glycolithocholic, deoxycholic and (tentatively) lithocholic. The spot corresponding to lithocholic acid was unusually dark and intense, and could have been due to cholesterol (see further, p. 164). The numerous spots beyond lithocholic acid in the ethyl acetate extracts I (serum) and III (serum + mixed standards) are not due to bile acids.

Partition between petroleum ether and 40% ethanol

(Procedure (b) p. 147):- The ethyl acetate extracts (I, II & III) were next partitioned between petroleum ether and 40% ethanol. Fig. 4(b) shows a chromatogram of both fractions derived from "Ethyl acetate I". The bile acid spots occurred chiefly in the 40% ethanol fraction but were too diffuse for clear identification. Chromatograms of the petroleum ether and aqueous ethanol fractions derived from "Ethyl acetate II" and "III" in Ganshirt's and other solvent systems showed similar poor results. In some experiments the ethanolic fraction showed only broad fluorescent streaks with darker areas corresponding to the bile acid spots.

Bile acids in the serum of normal and jaundiced patients

Serum from a normal patient was hydrolysed, extracted and

partitioned as in Fig. 1. The ethyl acetate fraction containing the unconjugated and glycine-conjugated bile acids was examined in three solvent systems. Table 4 gives the bile acids tentatively detected. Spots corresponding to glycocholic and glycodeoxycholic acid were consistently observed in the normal serum extracts. Lithocholic acid and cholesterol had very similar Rf values but were distinguishable by initial spraying with $SbCl_3$ to detect cholesterol (pink to blue) followed by $Ce(SO_4)_2$ to detect lithocholic acid. In the cases where $SbCl_3$ was not used both possibilities are listed. Glycolithocholic and cholic acid were unresolved in the majority of solvent systems and occasionally appeared in trace amounts. Spots running ahead of cholesterol are omitted from Table 4 as their presence did not interfere with the identification of the bile acids.

The ten to twenty-fold higher concentration of bile acids in jaundiced serum (43) led to its use in subsequent investigations. Serum from a hypercholesterolaemic and jaundiced patient was hydrolysed and extracted as in Fig. 1. The final ethyl acetate fraction was examined in four solvent systems as shown in Table 4. Spots corresponding to glycocholic and glycodeoxycholic acid were again consistently observed, indicating their presence in normal and jaundiced serum. The other acids were tentatively detected only in certain solvent systems.

Table 4.

Tentative Identification of Bile Acids in Normal and Jaundiced Serum.

Index	Solvent System	Spray reagent	Bile Acids Present							
			GC	GDC	GLC	C	DC	LC	Chol.	UDC
				GCDC			CDC			
	Normal Serum									
a	Modified Ga I	$Ce(SO_4)_2$	+	+	(+)	(+)	(+)	+ &/or +		*
b	Ga I	$Ce(SO_4)_2$	+	+	(+)	(+)	(+)	+ &/or +		*
c	Ho I	$Ce(SO_4)_2$	+	+	-	-	-	+ &/or +		*
	Jaundiced Serum									
a	Modified Ga I	$Ce(SO_4)_2$	+	+	-	-	+	+ &/or +		-
			+	+	-	-	+	+ &/or +		-
b	Ga I	$Ce(SO_4)_2$	+	+	(+) &/or (+)		-	+	(+)	-
			+	+	(+) &/or (+)		-	+ &/or +		+
			+	+	(+) &/or (+)		-	(+) &/or (+)		-
		$SbCl_3$	+	(+)	-	-	-	-	+	-
		$Ce(SO_4)_2$	+	+	(+) &/or (+)		+	+	+	-

continued on next page.

Table 4 (continued).

Index	Solvent System	Spray reagent	Bile Acids Present							
			GC	GDC	GLC	C	DC	LC	Chol.	UDC
c	Ho I	$Ce(SO_4)_2$	+	+	+ &/or +		-	+ &/or +		-
d	"MCW2" ("acidic" plates)	"PMoA"	+	(+)	(+) &/or (+)		-	-	-	-

+ compound present

- compound not detected

(+) compound present in trace amounts

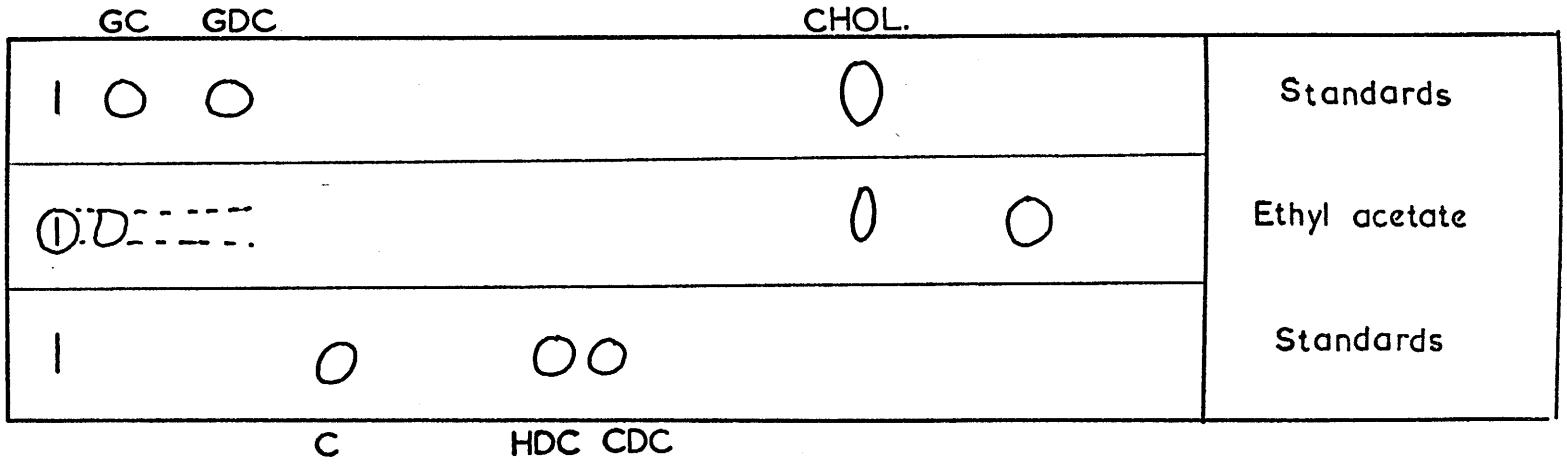
* standard not included in chromatogram.

The presence of both lithocholic acid and cholesterol was shown as described above. Fig. 5(a) shows the chromatograms of the ethyl acetate fraction after spraying with antimony trichloride, and Fig. 5(b) the results observed on overspraying with ceric sulphate.

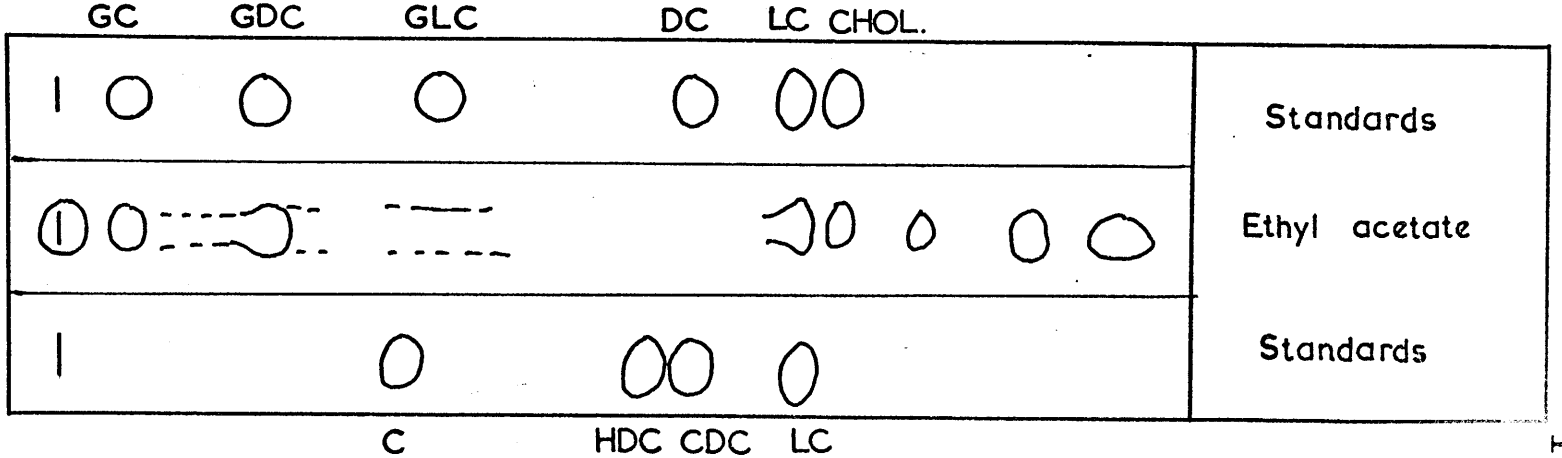
Figs. 5(a) and (b) show a spot at the origin which could be attributed to taurine conjugates not normally expected in the ethyl acetate fraction. Accordingly, the extract was examined in a system more suitable for the identification of glycine and taurine conjugates: six replicate chromatograms were run in system Ga II, yielding the results shown in Table 5. The presence of glycocholic acid, as found in the other solvent systems, was confirmed. However, glycodeoxycholic acid which was indicated in all the previous systems (Table 4) appeared only in trace amounts. On the other hand, the unresolved glycolithocholic and cholic acid which were previously only detected in trace amounts appeared as definite spots in this system. The presence of taurine conjugates in the extract was not established as they were indicated only in certain chromatograms e.g. Fig. 5(c) shows the last chromatogram listed in Table 5. The spot corresponding to lithocholic acid in Rf value is probably a mixture of unconjugated

FIGURE 5 (a) & (b)

a)



b)



SOLVENT Ga I

SPRAY REAGENTS a) $SbCl_3$ b) $Ce(SO_4)_2$

Table 5.

Index	Solvent System	Spray Reagent	Bile Acids Present					
			TC	TDC	TCDC	GC	GDC	GLC [a]
	Jaundiced Serum							
a	Ga II	$Ce(SO_4)_2$	-	(+)	*	+	-	+
			+	Rf not identical	*	+	(+)	+
			(+)	area	*	+	(+)	+
			-	*	-	+	*	+
			(+)	*	+	+	*	Rf not identical
			+	*	+	+	(+)	+

+ compound present

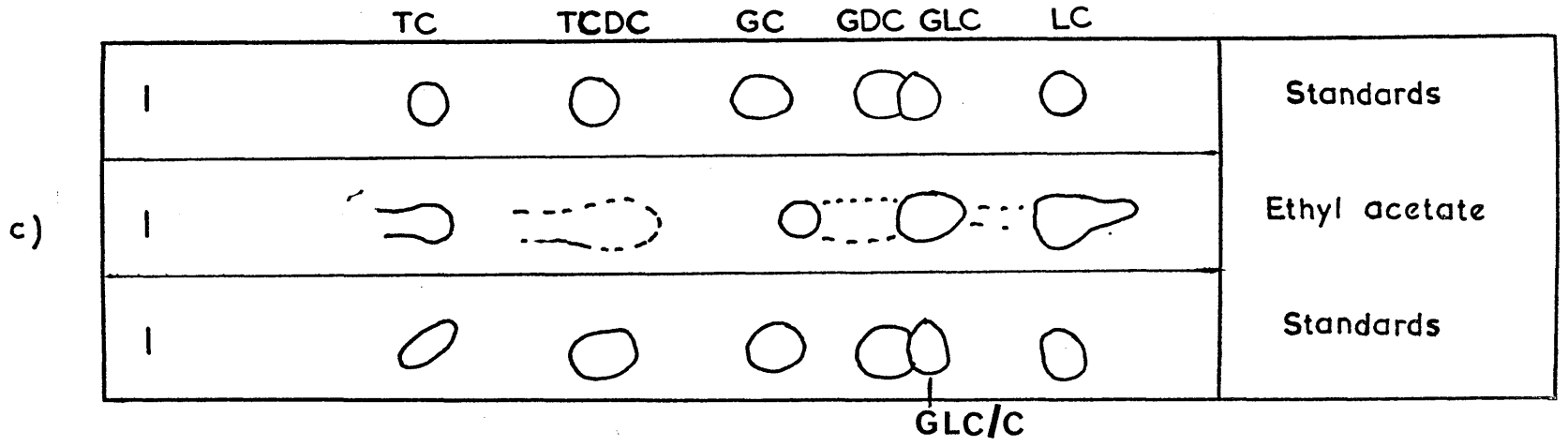
- compound not detected

(+) compound present in trace amounts

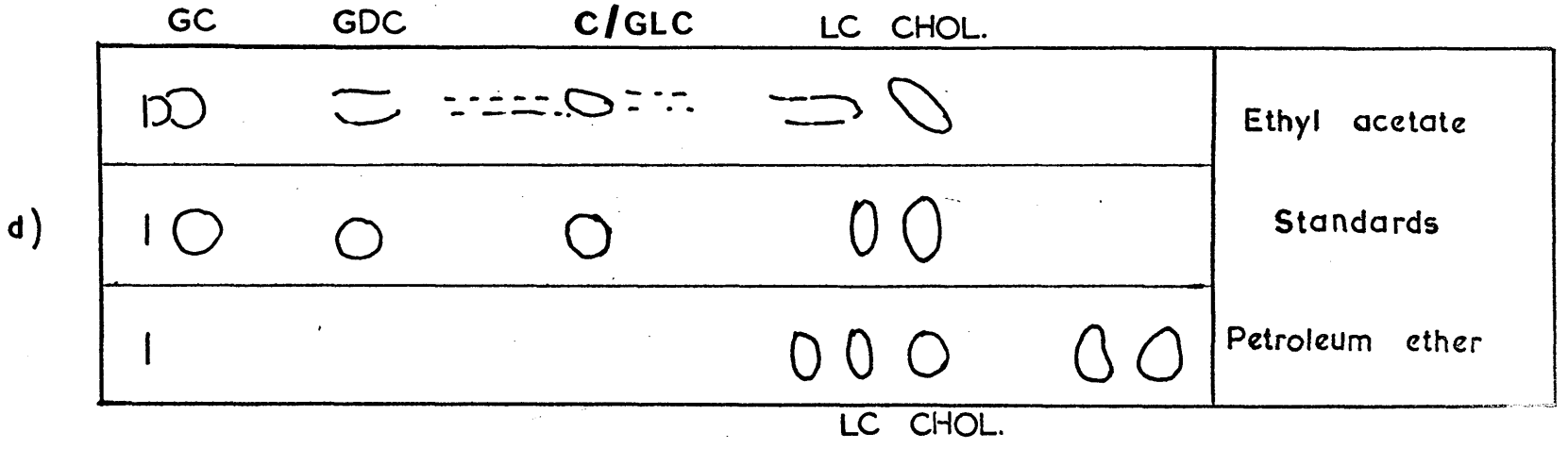
* standard not included in chromatogram.

a the presence of cholic acid which has similar Rf value is also implied.

FIGURE 5 (c) & (d)



c)



d)

SOLVENT c) Ga II d) Ga I SPRAY REAGENT $Ce(SO_4)_2$

bile acids and non-bile acid components of serum.

Partition between water/petroleum ether and water/ethyl acetate

(Procedure (c) p.149):- Further partition of the ethyl acetate fraction between water and petroleum ether followed by water and ethyl acetate resulted in elimination of the non-steroid constituents (Fig. 5(d): cf. Fig. 5(b)). The petroleum ether fraction contained lithocholic acid, cholesterol (detected by $SbCl_3$)and other non-bile acids while the new ethyl acetate fraction contained all the bile acids and some cholesterol. From the chromatograms it was now possible to identify with more confidence glycocholic acid, glycodeoxycholic acid, glycolithocholic (or cholic) acid, lithocholic acid and cholesterol. The probable presence of all these compounds in the unpartitioned ethyl acetate fraction has already been shown (Tables 4 and 5). Attempts to elute the components of an ethyl acetate fraction from a jaundiced serum extract proved unsuccessful.

In a further experiment, bile acids from the fasting and non-fasting sera of a jaundiced patient were examined on acidic and neutral chromatoplates (p.155). The sera were processed as in Fig. 1 and the final ethyl acetate fraction was examined for the presence of bile acids (Table 6). The presence of glycocholic and glycodeoxycholic acid and possibly some unresolved glycolithocholic

Table 6.

Index	Solvent System	Spray reagent	Bile Acids Present								
			TC	TDC	GC	GDC	GLC	C	DC	HDC	LC
				TCDC		GCDC			CDC		
	Jaundiced Serum Unpartitioned ethyl acetate †										
a	"MCW2" ("acidic" plates)	"PMoA"	*	*	+	+	+ &/or +		-	*	-
b	En S_{11}	Ce $(SO_4)_2$	*	*	+	(+)	+ &/or +		+	*	-
			*	*	+	+	(+) &/or (+)		-	*	-
c	Ga II	Ce $(SO_4)_2$	(+)	(+)	+	(+)	-	*	*	*	*
			(+)	(+)	+	(+)	-	*	*	*	*
	Ethyl acetate Fraction ‡										
a	En S_{11}	Ce $(SO_4)_2$	*	*	+	+	+ &/or +		(+)	(+)	-
b	"MCW1" followed by En S_{11}	Ce $(SO_4)_2$	*	*	+	+	+ &/or +		-	*	(+)
c	Ga II	Ce $(SO_4)_2$	-	(+)	+	+	+ &/or +		-	—	-

Footnotes to Table 6 overleaf.

Table 6. (continued).

† This is the original ethyl acetate fraction resulting from extraction of the aqueous serum layer with ethyl acetate.

‡ This is the fraction resulting from partitioning of the original ethyl acetate extract between water/petroleum ether and water/ethyl acetate.

\+ compound present

\- compound not detected

(+) compound present in trace amounts

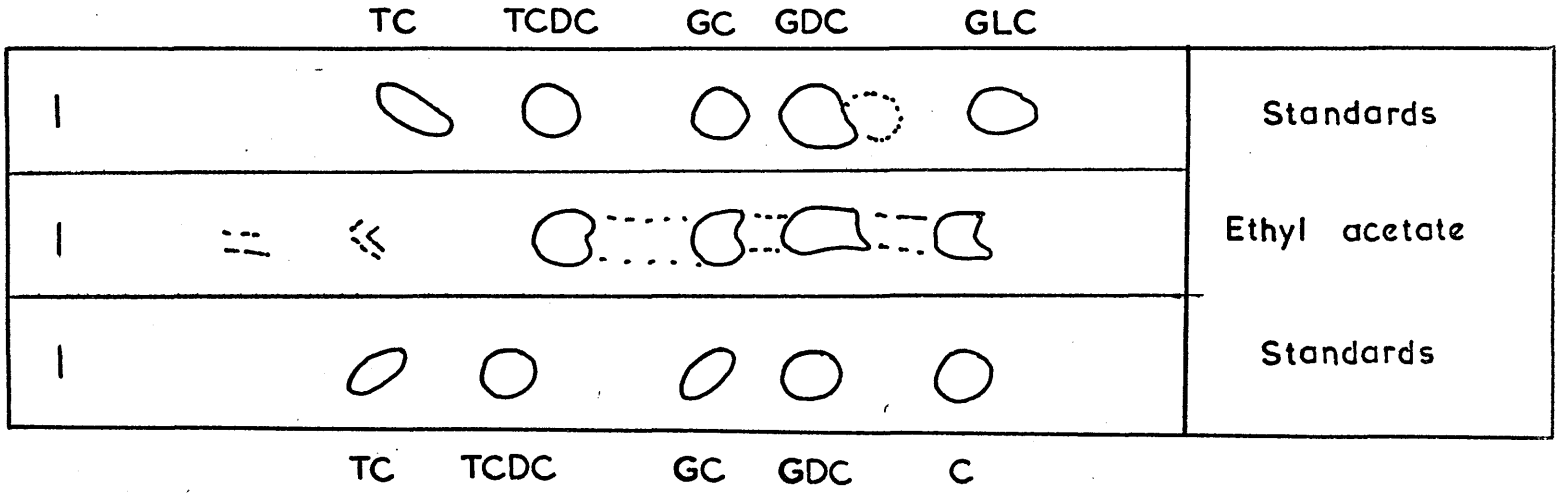
* standard not included in chromatogram.

and cholic acid was again demonstrated. As the bile acid patterns for fasting and non-fasting sera were the same, the extracts were combined and partitioned between water and petroleum ether followed by water and ethyl acetate (procedure (c), p.149). The petroleum ether fraction showed the presence of the usual contaminants as well as traces of lithocholic acid. The new ethyl acetate fraction (Table 6 and Fig. 6) confirmed the presence of the bile acids found in the unpartitioned ethyl acetate fraction mentioned above. The results of Table 6 thus show that jaundiced serum contains glycocholic acid, glycodeoxycholic acid and glycolithocholic (or cholic) acid. The presence of traces of other bile acids is suggested.

Bile acid methyl esters

As the identification of bile acids by thin-layer chromatography was generally inconclusive, conversion to their methyl esters and examination by thin-layer and gas-liquid chromatography was attempted. The ethyl acetate fraction derived from jaundiced serum by procedure (c) was examined by thin-layer chromatography before and after methylation and the results are given in Table 7 and in Figs. 7(a) and 7(b). Identification of the unmethylated bile acids (Fig. 7(a)) was still tentative, but the results for the methylated extract (Fig. 7(b)) appeared to confirm the presence of glycocholic acid,

FIGURE 6



SOLVENT Ga II SPRAY REAGENT $Ce(SO_4)_2$

Table 7.

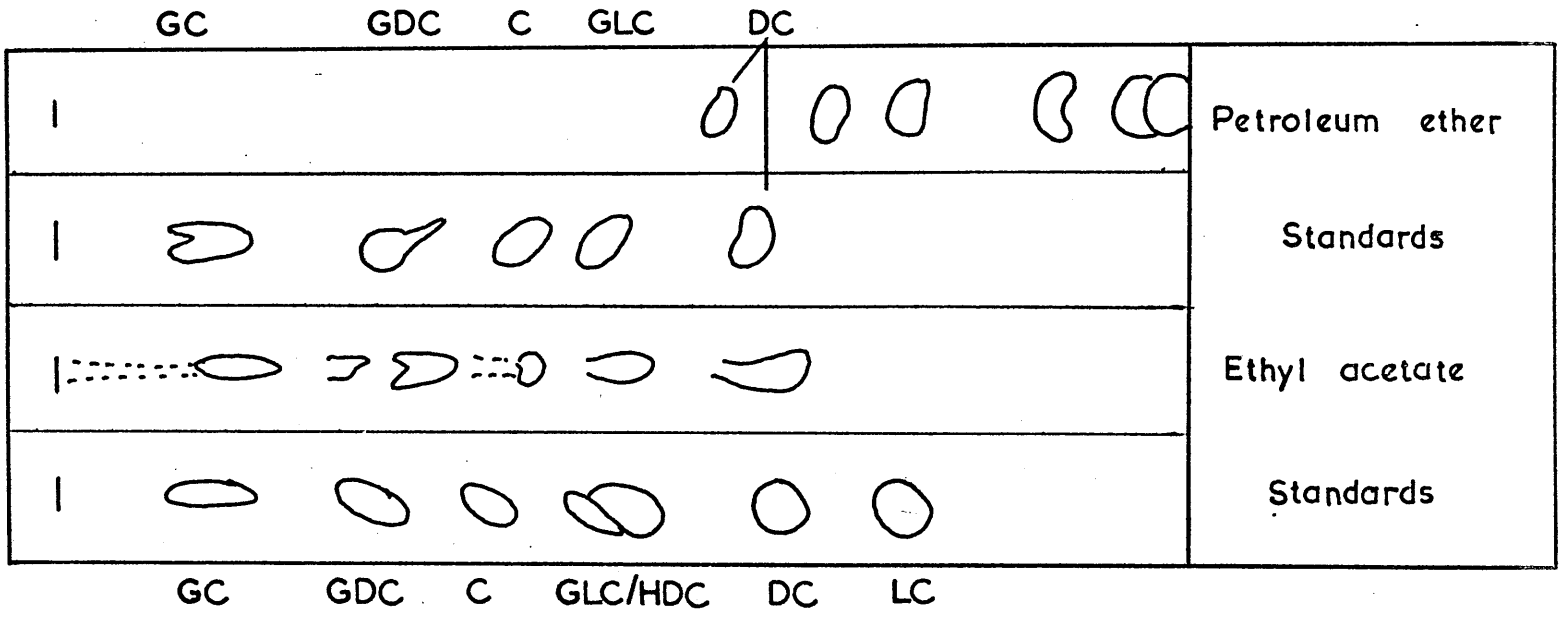
	Solvent system or Liquid phase	Spray reagent or Temperature & flow	Bile acids present								
			GC	GDC	GLC	HDC	C	DC	UDC	CDC	LC
	Thin-layer chromatography										
	Unmethylated ethyl acetate										
a	En S_{II}	$Ce(SO_4)_2$	+	+?	+	&/or +	+?	+	*	*	-
	Methylated ethyl acetate										
a	EtOAc	$Ce(SO_4)_2$	+	-	*	+	-	+ &/or +		*	+
	Gas-liquid chromatography										
	Methylated ethyl acetate										
a (I)	1% SE-30	245°; 40 ml./min.	*	*	*	+	+	+	+ &/or +		+
(II)			*	*	*	+	+	+	+ &/or +		+
(III)			*	*	*	+?	+	+	+ &/or +		+
	Methylated ethyl acetate (TFAs)										
a	1% SE-30	245°; 40ml./min.	*	*	*	*	+	+	+ &/or +	+	+
b	1% OF-1	235°; 80ml./min	*	*	*	-	-	-	+	-	-

(over)

Table 7 (continued)

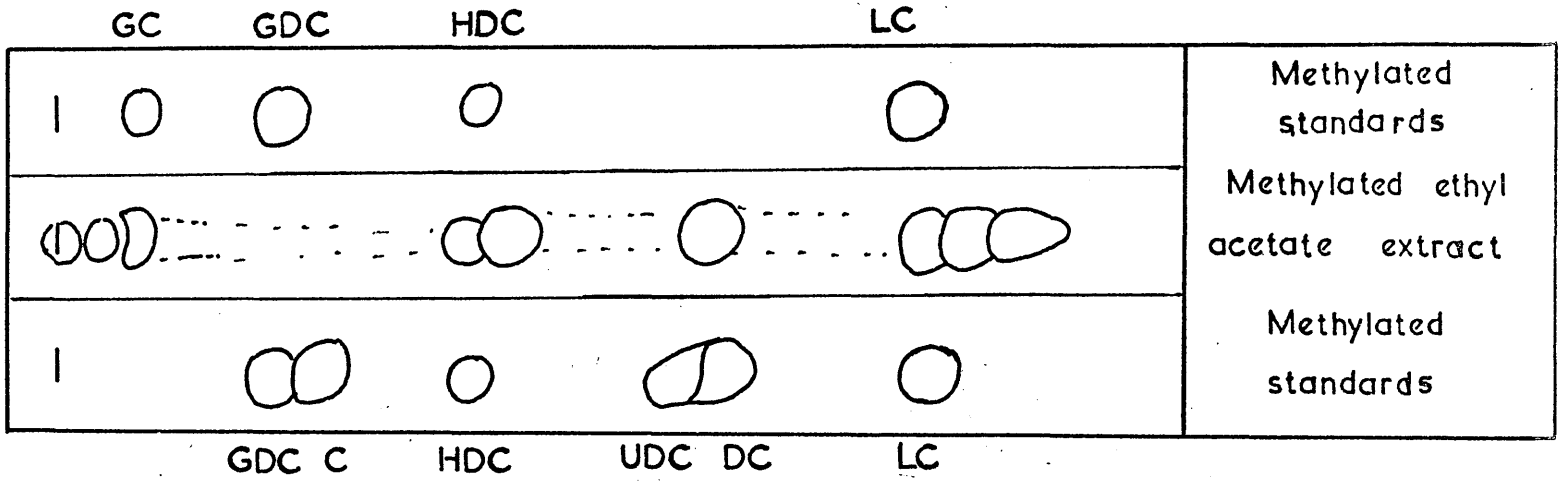
+	compound present
+?	presence of compound not definite
-	compound not detected
*	standard not included in chromatogram.

FIGURE 7(a)



SOLVENT En S_{II} SPRAY REAGENT $Ce(SO_4)_2$

FIGURE 7 (b)



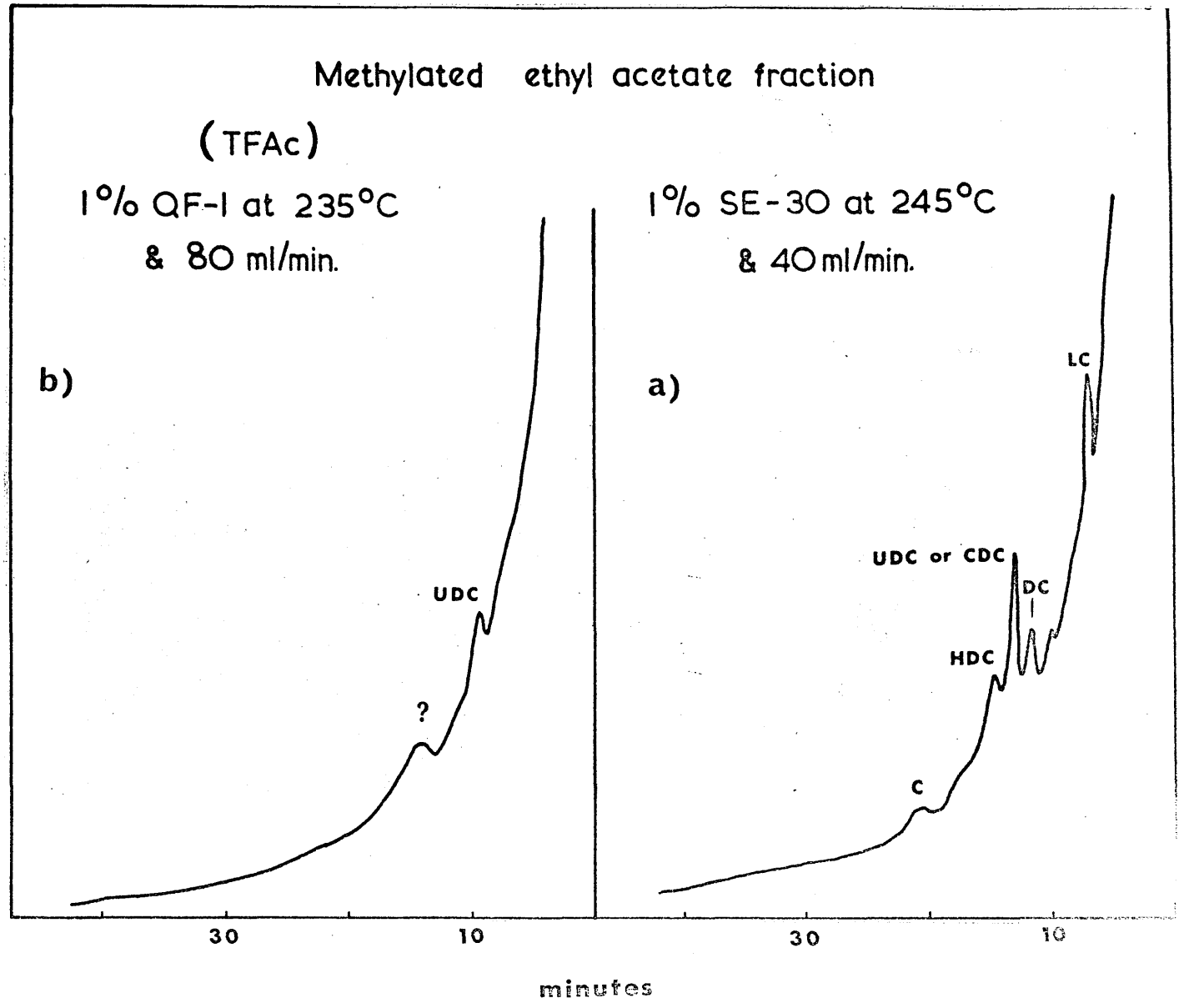
SOLVENT Ethyl acetate SPRAY REAGENT $Ce(SO_4)_2$

hyodeoxycholic acid and deoxycholic (or ursodeoxycholic) acid in the ethyl acetate fraction. The unmethylated petroleum ether fraction indicated the presence of deoxycholic (or chenodeoxycholic) acid and possibly lithocholic acid (Fig. 7(a)).

The methylated ethyl acetate and petroleum ether fractions were further examined by gas-liquid chromatography using SE-30 and QF-1 columns. The results observed for the ethyl acetate fraction are summarised in Table 7 and representative chromatograms are shown in Figs. 8 and 9. The results for the methylated extract on an SE-30 column are shown in Fig. 8(a) where tentative assignments to the peaks have been made. Further evidence for the occurrence of these bile acids was obtained by conversion to their trifluoroacetates (TFAc) and re-examination on the gas chromatograph. On QF-1 (Fig. 8(b)) only methyl ursodeoxycholate trifluoroacetate could be detected whereas on SE-30 (Fig. 9) the majority of bile acids observed in Fig. 8(a) were also detected as their trifluoroacetates. In addition, two large unidentified peaks were observed at about 29 and 37 minutes. The retention data for various compounds on the two columns are tabulated in Table 7 of the following section, (p.206)

Gas-liquid chromatography of the methylated petroleum ether fraction on QF-1 showed the presence of a peak corresponding to

FIGURE 8


Methylated ethyl acetate fraction
(TFAc)
1% QF-1 at 235°C
& 80 ml/min.
1% SE-30 at 245°C
& 40 ml/min.
b)
a)
UDC
?
LC
UDC or CDC
DC
HDC
C
30
10
30
10
minutes

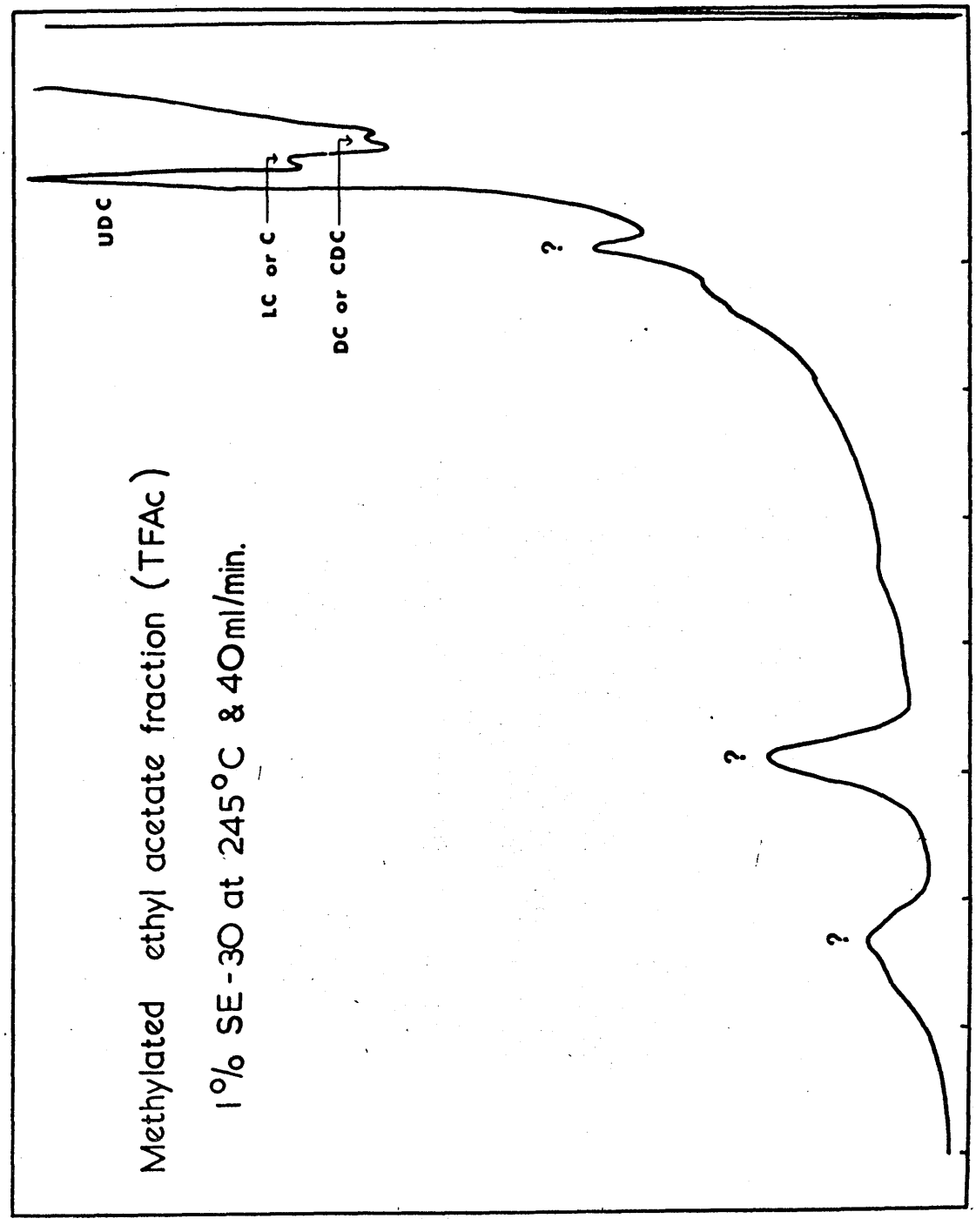

Methylated ethyl acetate fraction (TFAc)
1% SE-30 at 245°C & 40ml/min.
UDC
LC or C
DC or CDC
?
?
?
10
20
30
40

FIGURE 9

chenodeoxycholic acid (cf. thin-layer results Fig. 7(a)).

Comparison of the results obtained with thin-layer and gas-liquid chromatography (Table 7) suggests the presence of compounds corresponding to hyodeoxycholic, cholic, deoxycholic (or chenodeoxycholic or ursodeoxycholic) and lithocholic acid. From thin-layer chromatography data, the presence of glycocholic acid is shown.

More effective studies of extracts of this type might have been possible following the development of gas-chromatographic conditions for the detection of the glycine-conjugated acids, as described below. However, lack of time precluded further investigations along these lines.

(ii) Gas-Liquid Chromatography of Glycine-Conjugated Bile Acids

In the last few years a number of workers have investigated the gas chromatographic behaviour of unconjugated bile acids using a variety of silicone polymer phases of the type introduced by Horning and co-workers (46). The separation of bile acid mixtures on non-polar phases was often inadequate, and since the use of polar phases led to unduly long retention times, it became necessary to convert these polyhydroxy compounds to less polar derivatives. The derivatives most frequently employed in gas-chromatographic studies of unconjugated bile acids (as methyl esters) have been the trifluoroacetates (17) the trimethylsilyl ethers (21) and the acetates (16). A synopsis of previous gas chromatographic studies of bile acids is given in Table 1.

Materials and Methods

For the bile acids see previous section (p.142). Bile alcohols were obtained from Southeastern Biochemicals Inc., except 5β-cholane, 3α,12α,24-triol which was prepared by reduction of methyl deoxycholate (98 mg.) with excess lithium aluminium hydride in refluxing ether. After working up in the usual way, the crude product was recrystallised from ethyl acetate giving 67 mg. m.p. 115-120° and

Table 1. Previous gas chromatographic studies on unconjugated bile acids

Authors	Derivatives investigated
VandenHeuvel, Sweeley & Horning (1960)	Methyl esters
Blomstrand (1961)	Methyl esters
Sjövall, Meloni & Turner (1961)	Methyl esters & acetates
VandenHeuvel, Sjövall & Horning (1961)	Methyl esters & trifluoroacetates
Holmes & Stack (1962)	Methyl esters
Bloomfield* (1962)	Methyl esters
Ellin, Mendeloff & Turner (1962)	3,7,12-triketo cholanic acid methyl ester
Sjövall (1962)	Methyl esters & trifluoroacetates
Makita & Wells* (1963)	Methyl esters & trimethylsilyl ethers
Kuksis & Gordon* (1963)	Methyl esters, acetates & trifluoroacetates
Danielsson, Eneroth, Hellstrom, Lindstedt & Sjövall (1963)	Methyl esters
Sjövall, Sandberg, Sjövall & Turner (1963)	Methyl esters, trifluoroacetates & trimethylsilyl ethers
Lin, Rubinstein & Holmes (1963)	Methyl esters

* Quantitative gas-liquid chromatography of bile acids

Liquid phase & temperature	Ref. No.
0.75% SE-30; 222°	14
12% SE-301; 245°	15
SE-52 with 20 & 30 moles% phenyl group; 215°-220°	16
0.75% SE-30; 222° and 0.75% NGS; 210°	17
0.56% SE-52; 250°	18
0.75 to 1% SE-30; 235°-256°	19
0.5% nitrile polysiloxane; 228°	25
0.5% QF-1 and a mixture of 0.5% QF-1 & 0.05% EGIP	20
0.5% Hi Eff-8B; 245° & 280°	21
1% QF-1, 200°; 2.25% SE-30, 195°; 2% XE-60, 220° & 200°	22
0.5% QF-1; 219°	23
QF-1 and cyanoethyl polysiloxane	24
0.72% SE-52; 250°	48

characterised as the triacetate m.p. 79-80 (lit. 79.5-80.5 (54)).

Thin-layer chromatography was applied to check the purity of the bile acids and their derivatives. Recrystallisation of the glycine conjugates was not found to provide an effective means of purification, but the samples were adequately pure for the present investigation. Solutions of unconjugated bile acids were made in acetone, and of glycine conjugates in methanol. Redistilled acetic anhydride and pyridine (distilled over potassium hydroxide) were used in the preparation of the acetates. For the trimethylsilyl ethers hexamethyldisilazine and trimethylchlorosilane were used without purification.

Apparatus

Details of the Pye Argon Chromatograph have been given in Section 1 (Materials and Methods, p 24). In the work described in the present section, two types of glass column were used:
(i) the standard 3.5-4 mm. bore 4 ft. columns (ii) modified 4 ft. columns comprising lengths of standard bore tubing joined to capillary tubing (1 mm. bore). Modified columns having 1, 2 and 3 ft. capillary sections were employed: the capillary formed the lower portion of the column, terminating in a B7 cone fitted with a sintered disc.

The chromatographic conditions varied for the different columns and according to the type of compound being examined. They are given individually with the Tables and Figures. The column temperature ranged from 235° to 250° and the injection block temperature was kept at 250 ± 5° or 280 ± 5° depending on which led to the better peak shapes. Nominal detector voltages 1250 or 1500V were used and the sensitivity settings were 10, 3 or 1.

Preparation of columns

The methyl siloxane polymer SE-30 and the methyl phenyl siloxane polymer SE-52 (General Electric Co., Schenectady, N.Y., U.S.A.), the fluoroalkyl siloxane polymer QF-1-0065 (viscosity 10,000 centistokes) and the methyl p-chlorophenyl siloxane polymer F-60 (Dow-Corning Corp., Inc.; Midland Silicones Ltd., London) were kindly provided by Dr. E.C. Horning.

Column packings were prepared on the support Gas-Chrom P, 100-120 mesh (Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A.) which had been acid washed, "silanized" and coated according to Horning's technique as already described. Column packings so prepared contained approximately 1% (w/v)

SE-30 and QF-1, 0.5% SE-52 and 1.25% F-60. The capillary sections of the glass columns were coated internally by keeping a solution of the appropriate liquid phase in them overnight. The solution was then removed and the capillary tube dried. A small plug of "silanized" glass wool was placed at the junction of capillary and ordinary tubing to prevent any packing from falling into the capillary, and the section of standard bore was then packed with the coated support as already described. It was found that filling the small cone at the bottom of the column with coated support improved the column efficiency presumably as a result of reducing the dead volume.

Before any new column was used in the Chromatograph it was heated in a slow stream of argon for 24-48 hours to remove volatile products. SE-30, SE-52 and F-60 columns were preheated to 300° and QF-1 to 250°.

Bile acid methyl esters

Reference samples of unconjugated and glycine-conjugated bile acids were methylated with diazomethane prior to gas-liquid chromatography. Diazomethane was prepared in the usual way from N-methyl-N-nitrosourea (47). Initially the diazomethane was standardised by methylating a known weight of benzoic acid with a

known volume of diazomethane and titrating the excess benzoic acid with 0.1N sodium hydroxide. However, it was found that the use of a large excess of diazomethane and short reaction times gave satisfactory results. For methylation of the unconjugated bile acids, 150 mg. portions each dissolved in a small volume of warm methanol, were treated with a large excess of ethereal diazomethane. After standing for 15-30 minutes, the solutions were evaporated to dryness and the residues redissolved in ether. The filtered ether solutions were washed with 5% sodium hydrogen carbonate to remove any unmethylated acids, then with water, dried over sodium sulphate and evaporated to dryness. The crude products were recrystallised from ether/petroleum ether (b. p. 40-60°), and their purity was checked by thin-layer chromatography in ethyl acetate.

Methyl esters of the conjugated bile acids were prepared in 15 mg. quantities and were not isolated in crystalline form. The reaction mixtures were evaporated to dryness under nitrogen and the residues each dissolved in 1.5 ml. methanol giving a concentration of ca. 10 µg./µl. Sample purity was again checked by thin-layer chromatography before and after methylation. For the unmethylated acids chromatography was effected in Ganshirt's

solvent systems mentioned previously (p.150) while the methyl esters were examined in ethyl acetate or methanol.

Trimethylsilyl ethers

The trimethylsilyl ethers of the free and conjugated bile acid methyl esters were prepared on a microgram scale according to the method of Makita and Wells (21) as follows: the methyl esters (100-200 μg.) were dissolved in a mixture of dry pyridine (10 μl.) and hexamethyldisilazine (100 μl.). Trimethylchlorosilane (20 μl.) was added and the mixture allowed to stand at room temperature for 15-30 minutes. (If trimethylchlorosilane was omitted the reactions appeared to be incomplete.) The reaction mixture was centrifuged and the supernatant evaporated to dryness in a stream of nitrogen. The residue was taken up in a suitable volume of acetone for gas-liquid chromatography. The completion of the reaction and the purity of the derivatives were checked by thin-layer chromatography in ethyl acetate or methanol. Rf values in ethyl acetate were as follows: methyl glycolithocholate 0.39; trimethylsilyl ether 0.63; methyl glycodeoxycholate 0.11; trimethylsilyl ether 0.69; methyl glycocholate 0.00. Rf values in methanol were: methyl glycocholate 0.64; trimethylsilyl ether 0.68.

Acetates

Acetylation was carried out by treating a solution of the bile acid methyl ester (100-300 µg.) in dry pyridine (10 µl.) with acetic anhydride (50-100 µl.) and leaving at room temperature overnight. The reagents were evaporated to dryness and the residue was taken up in acetone for gas-liquid chromatography. Rf values for thin-layer chromatography of the methyl ester acetates in ethyl acetate were: glycolithocholate 0.58; glycodeoxycholate 0.51; glycocholate 0.44.

Trifluoroacetates

Trifluoroacetylation of the free and glycine-conjugated bile acid methyl esters was effected as described in the previous section (p.156). Gas-liquid chromatography of the unconjugated trifluoroacetates was satisfactory but the glycine-conjugated esters gave rise to multiple peaks although from retention considerations the main peak could be recognised. Thin-layer chromatography of the glycine derivatives also showed numerous extraneous spots suggesting that decomposition was occurring during trifluoroacetylation as well as on the chromatographic column.

Results and Discussion

The conjugated bile acid methyl esters and their derivatives

were studied on a number of silicone polymer phases (SE-30, F-60, SE-52 and QF-1) at temperatures ranging from 235° to 250°C. The study is limited to derivatives of the glycine conjugates of lithocholic, deoxycholic and cholic acid which are designated as A, B and C in the Figures. Successful chromatography of the taurine conjugates was not achieved. In a preliminary study of derivatives for the glycine-conjugated bile acid methyl esters, the trimethylsilyl ethers and the acetates proved the most suitable. Although acetates are less polar and more thermostable than the parent hydroxy compounds, they have not been widely applied to bile acids, perhaps because with other steroids they normally lead to longer retention times. In comparison, formation of trifluoroacetates (17) or trimethylsilyl ethers (21) tends to reduce the retention time; however, the trifluoroacetates were unsatisfactory for the conjugated derivatives under the present chromatographic conditions.

Table 2 gives the retention times of the glycine-conjugated methyl esters and their derivatives on the four liquid phases examined. The voltages and sensitivities tabulated are these which were found experimentally to be the most satisfactory. Among the methyl esters themselves, only methyl glycolithocholate regularly gave a well defined peak. Methyl glycodeoxycholate

Table 2. Retention times (minutes) of glycine-conjugated bile acid esters and their derivatives

Chromatographic Conditions					Me Glycolithocholate		Me Glycodeoxycholate			Me Glycocholate	
Length*	Temperature	Flow† ml./min.	Voltage§	Sensitivity※	OH	TMSi	OAc	OH	TMSi	OAc	TMSi	OAc
1% SE-30												
4ft/0ft*	**245°**	40	1250	10	34.5	-	-	52.7	-	-	-	-
	245°	70	1250	1	30.4	33.0	41.8	48.8	38.1	54.6	40.6	(69.8)
3ft/1ft.	**240°**	50	1250	3	-	31.5	-	-	37.0	-	38.6	-
2ft/2ft.	**245°**	50	1500	3	14.5	14.3	18.3	-	15.3	22.8	16.3	29.3
	240°	30	1500	1	-	25.2	-	-	28.1	-	31.2	-
	240°	76	1500	3	13.0	-	17.7	(19)	-	22.1	-	30.1
1ft/3ft.	**245°**	30	1500	10	12.5	11.8	16.0	-	12.7	19.5	13.4	24.3
	240°	40	1500	10	13.0	-	17.5	-	-	21.8	-	28.0
0.5% SE-52												
4ft/0ft.	**245°**	70	1250	10	14.5	11.7	-	-	14.0	-	14.7	-
1% OF-1												
4ft/0ft.	**235°**	80	1500	3	54.9	27.0	-	(100)	27.7	-	28.8	-
2ft/2ft.	**235°**	40	1500	1	-	13.0	45.5	-	15.0	-	15.7	-
1.25% F-60												
4ft/0ft.	**245°**	60	1500	3	25.5	24	37.8	(34)	27.1	-	29.3	-
	245°	74	1500	3	22	-	31.3	-	-	42.5	-	68.5
	250°	74	1500	3	18.6	-	25.2	(29)	-	36.5	-	51.5

Table 2 (continued).

	Chromatographic Conditions				Me Glyco		Ethocholate	Me Glycodeoxycholate			Me Glycocholate	
Length*	Temperature	Flow† ml./min.	Voltage§	Sensitivity*	OH	TMSi	OAc	OH	TMSi	OAc	TMSi	OAc
F-60												
3ft/1ft.	245°	70	1500	3	27	28.0	44.5	-	31.8	-	34.4	-
	250°	70	1500	3	21.5	22.1	30.6	(37)	24.8	46.9	27.6	62

* 4ft/0ft = the first figure refers to the length of standard bore (3.5-4 mm.) tubing, the second figure to the capillary tubing.

† Argon flow at the outlet

§ Voltage refers to detector voltage

* The figures refer to instrument settings: 1 represents approximately full scale deflection for a signal of 1×10^{-8} ma., 3 for 3×10^{-8} ma. and 10 for 1×10^{-7} ma.

\- Compound not examined

Values in parenthesis are very approximate.

TMSi = trimethylsilyl ether

OAc = acetates

OH = parent compound

generally decomposed except on a 4 ft. SE-30 column where reproducible peaks were obtained (Fig. 1). In spite of the low concentrations of liquid phase employed, losses of the conjugated methyl esters were evidently considerable as relatively large samples were required. Typical amounts for 4 ft. columns were: 10-20 μg. for an SE-30 phase and 20-30 μg. for F-60, QF-1 and SE-52 phases. Consequently conversion to the trimethylsilyl ethers and acetates was carried out and it was found that 1-5 μg. of each derivative was sufficient for identification. Methyl glycolithocholate, methyl glycodeoxycholate and the corresponding trimethylsilyl and acetyl derivatives are shown in Figs. 2 and 3 for a 4 ft. column of F-60.

The peaks observed early in the chromatograms were due in part to derivatives of unconjugated bile acids, present as impurities, and in part to decomposition on the column, which was more marked in the deoxycholate series than in the lithocholate series.

As can be seen from Table 2, trimethylsilylation of methyl glycolithocholate caused only minor changes in retention time except on QF-1 where a considerable reduction was observed. For all three conjugates, the trimethylsilyl ethers had shorter

FIGURE 1

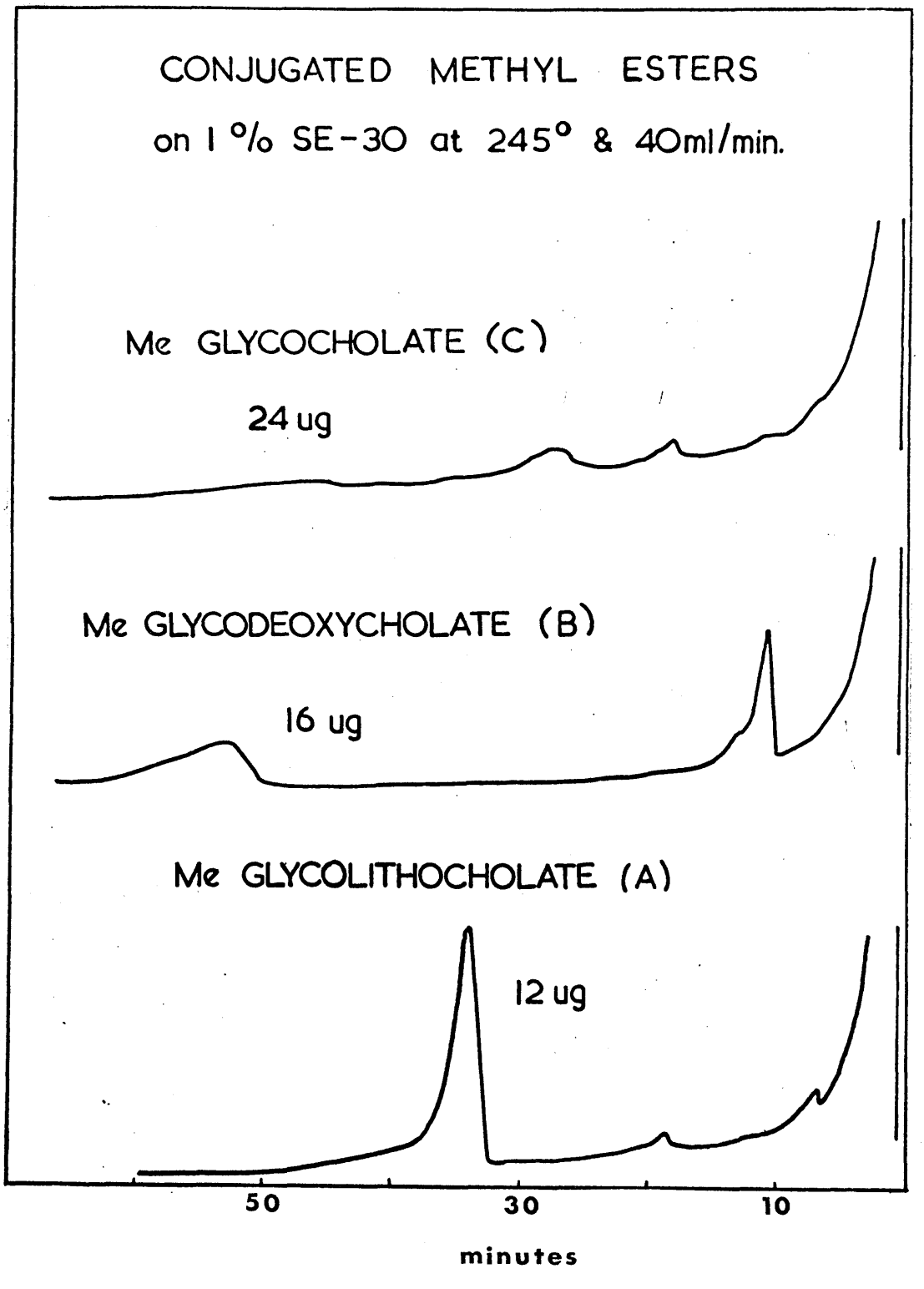

CONJUGATED METHYL ESTERS
on 1 % SE-30 at 245° & 40ml/min.
Me GLYCOCHOLATE (C)
24 ug
Me GLYCODEOXYCHOLATE (B)
16 ug
Me GLYCOLITHOCHOLATE (A)
12 ug
50
30
10
minutes

FIGURE 2

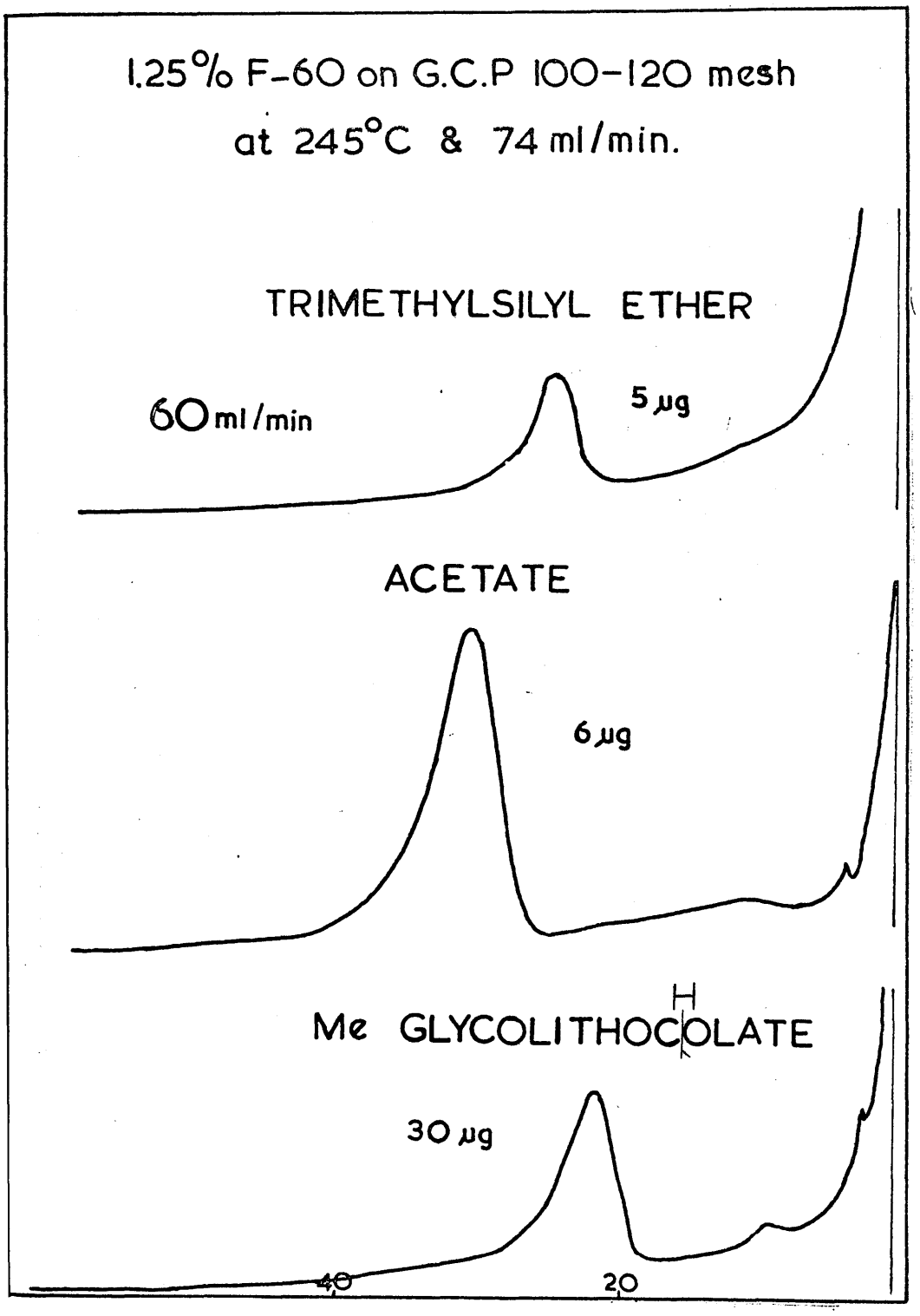
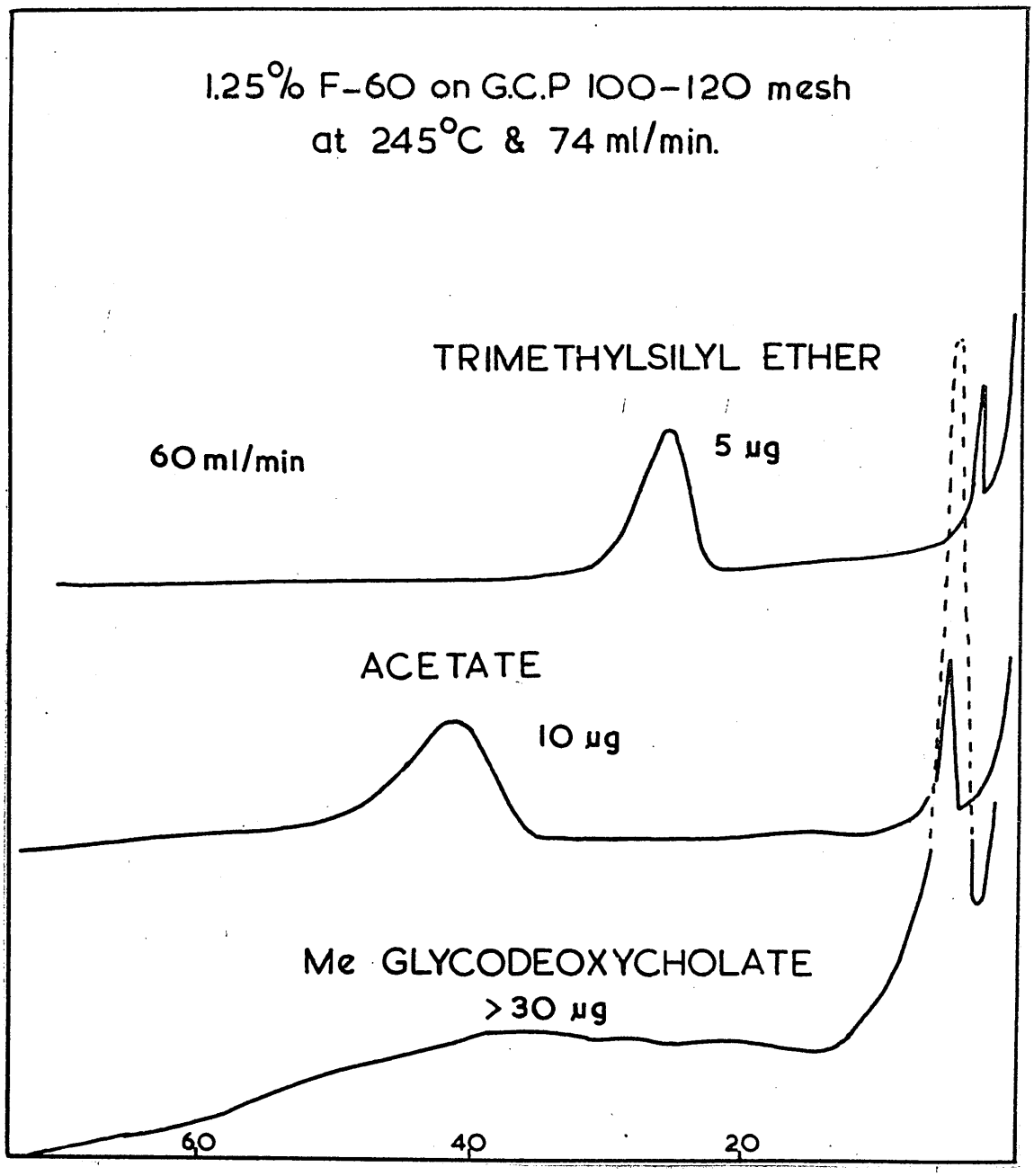

1.25% F-60 on G.C.P 100-120 mesh
at 245°C & 74 ml/min.
TRIMETHYLSILYL ETHER
60 ml/min
5 µg
ACETATE
6 µg
Me GLYCOLITHOCOLATE
H
30 µg
40
20
minutes

FIGURE 3


1.25% F-60 on G.C.P 100-120 mesh
at 245°C & 74 ml/min.
TRIMETHYLSILYL ETHER
60 ml/min
5 µg
ACETATE
10 µg
Me GLYCODEOXYCHOLATE
> 30 µg
60
40
20
minutes

retention times than the acetates, the differences becoming more marked with increasing substitution. On QF-1 the acetates had unduly long retention times resulting in very broad peaks and substantial decomposition. For example, on a 2 ft. standard bore/2 ft. capillary column of QF-1 only methyl glycolithocholate acetate gave a well defined peak, with a retention time more than three times that of the corresponding trimethylsilyl ether.

In general shortening of the packed columns from 4 ft. to 2 ft. achieved (in the case of SE-30 and QF-1 phases) an appreciable reduction in retention times, but no further useful effect was noted on proceeding to 1 ft. packed columns. For the F-60 columns examined reduction in column length had little effect, but the retention times appeared to be markedly affected by a change in temperature from 245 to 250°, in contrast with the results for SE-30. The reason for this difference in behaviour is obscure.

Data for the trimethylsilylation of unconjugated and conjugated bile acids on SE-30 are given in Table 3, which shows the successively lower gross factors (defined as the retention ratios of the trimethylsilylated and parent compounds) observed with increasing number of hydroxyls. The similarity of the factors for corresponding unconjugated and conjugated compounds suggests

Table 3.

Gross Factors for Trimethylsilylation of Bile Acids.

1% SE-30 (4ft. column) at 245° and 70 ml./min.

Bile Acid Methyl Ester	Retention Times * Parent Compound	TMSi Derivative	Gross TMSi Factor
Me Lithocholate	5.70	6.17	1.08
Me Deoxycholate	8.90	7.45	0.84
Me Cholate	15.70	8.50	0.54
Me Glycolithocholate	30.4	33.0	1.09
Me Glycodeoxycholate	48.8	38.1	0.78
Me Glycocholate	-	40.6	-

* in minutes.

that they are behaving in a similar manner on the column. Analogous results are observed for the same compounds on a 4 ft. column of F-60 (Table 4).

A similar correlation can be made in terms of gross acetate factors, defined as the retention ratios of the acetylated and unacetylated bile acid methyl esters. Factors of this type, derived from data found with SE-30 and F-60 columns, are given in Table 5. The factor diminishes with each additional substituent, and it has not yet been established* whether this effect (which contrasts with the regular increments found for acetylation of steroid alcohols) is due to loss of substituents by elimination. The striking agreement between the factors for unconjugated and conjugated analogues strongly suggests that any such transformation is a highly regular one. The data of Kuksis and Gordon for SE-30 (22) and of Sjövall, Meloni and Turner for SE-52 (16) show almost identical factors (Table 6). On QF-1 the steady increase in the factor from lithocholate through deoxycholate to cholate provides further, though not compelling, evidence against the occurrence of elimination during chromatography.

* For further details see Appendix 2.

Table 4.

Gross Factors for Trimethylsilylation of Bile Acids.

1.25% F-60 (4 ft. column) at 245°C and 60 ml./min.

thylated Bile Acid	Retention Times * Parent Compound	TMSi Derivative	Gross TMSi Factor
Lithocholate	3.47	3.55	1.02
Deoxycholate	6.07	4.40	0.72
Cholate	11.10	4.60	0.40
Glycolithocholate	25.5	24.0	0.94
Glycodeoxycholate	(34)	27.1	0.80
Glycocholate	-	29.3	-

* in minutes.

Table 5. **Factors for Acetylation of Bile Acids.**

1% SE-30 at 245°C and 70 ml./min.

Methylated Bile Acids	Retention Times* Parent Compound	Retention Times* OAc Derivative	Gross Acetate Factor
Me Lithocholate	5.70	8.0	1.40
Me Deoxycholate	8.90	10.2	1.14
Me Cholate	13.7	13.5	0.86
Me Glycolithocholate	30.4	41.8	1.37
Me Glycodeoxycholate	48.8	54.6	1.12
Me Glycocholate	-	69.8	-

* in minutes.

1.25% F-60 at 250°C and 74 ml./min.

Methylated Bile Acids	Retention Times* Parent Compound	Retention Times* OAc Derivative	Gross Acetate Factor
Me Lithocholate	2.66	3.69	1.38
Me Deoxycholate	4.43	5.30	1.19
Me Cholate	8.62	7.50	0.87
Me Glycolithocholate	18.65	25.2	1.35
Me Glycodeoxycholate	(29)	36.5	(1.2)
Me Glycocholate	-	51.5	-

* in minutes.

Table 6. Comparative Data for Gross Acetate Factors.

Methylated Bile Acids	I 1% SE-30 245°	II* 2.25% SE-30 195°	I 1.25% F-60 250°	III* SE-52 215°-220°	II* 1% QF-1 200°
Me Lithocholate	1.40	1.38	1.38	1.46	1.28
Me Deoxycholate	1.14	1.12	1.19	1.15	1.47
Me Cholate	0.86	0.86	0.87	0.86	1.63
Me Glycolithocholate	1.37	-	1.35	-	-
Me Glycodeoxycholate	1.12	-	(1.2)	-	-
Me Glycocholate	-	-	-	-	-

I Present work: Pye Argon chromatograph, 4 ft. columns
II Kuksis & Gordon (22): Aerograph chromatograph, 3-5 ft. columns
III Sjovall, Meloni & Turner (16): Barber-Coleman chromatograph, 6 ft. column

* The gross acetate factors were computed from the data of the above authors.

The effect of converting the unconjugated methyl esters to the glyco-derivatives can be computed from Tables 3,4 and 5. The introduction of a glycine group increases the retention time by an average factor of 5.3 (range 4.78-5.48) on SE-30 and 6.8 (range 6.16-7.35) on F-60.

Retention data for trifluoroacetates of the bile acid methyl esters are collected in Table 7. As already mentioned, the derivatives of the conjugated acids gave multiple peaks, and the tabulated values refer to the major peak in each case. On all three phases the gross trifluoroacetylation factors diminish with increasing substitution, and even for methyl lithocholate, trifluoroacetylation reduces the retention time, in agreement with the effect observed in the sterol series (17).

Factors derived from Table 7 for conversion of the unconjugated esters to their glyco-analogues differ slightly from those quoted above, the mean values being 4.4 (range 4.0-4.7) on SE-30 and 6.1 (range 5.5-6.6) on F-60. The discrepancies reflect the unreliability of the data for the trifluoroacetates of the conjugated bile acid esters.

Data for the introduction of 12α-substituents into the lithocholate and glycolithocholate series, and 7α-substituents

Table 7. Gross Factors for Trifluoroacetylation of Bile Acids

Methylated Bile Acids	1% SE-30 at 245°C and 70ml./min.			1% OF-1 at 235°C and 80ml./min.			1.25% F-60 at 245° and 60ml./min.		
	Retention Times *			Retention Times *			Retention Times *		
	Parent Compound	TFAc Derivative	Gross TFAc Factor	Parent Compound	TFAc Derivative	Gross TFAc Factor	Parent Compound	TFAc Derivative	Gross TFAc Factor
Me lithocholate	7.4	5.8	0.78	5.3	4.3	0.81	4.0	2.4	0.60
Me deoxycholate	11.8	5.3	0.45	10.1	6.3	0.62	6.7	2.1	0.31
Me chenodeoxycholate	11.5	5.5	0.48	11.9	8.2	0.69	7.5	2.5	0.33
Me ursodeoxycholate	12.5	6.6	0.53	12.7	9.4	0.74	7.4	2.7	0.37
Me hyodeoxycholate	14.0	-	-	14.6	-	-	8.9	-	-
Me cholate	20.6	5.6	0.27	23.4	12.9	0.55	13.0	2.4	0.19
Me glycolithocholate	34.4	27.3	0.80	54.8	43.5	0.79	25.0	15.8	0.63
Me glycodeoxycholate	53.0	22.1	0.42	100.8	61.3	0.61	-	12.8	-
Me glycocholate	-	22.4	-	-	51.3	-	-	13.2	-

* in minutes.

into the deoxycholate and glycodeoxycholate series, are given in Table 8. Hydroxyl, trimethylsilyloxy and acetoxyl factors are derived from the corresponding derivatives of the parent compounds. The following comments can be made :

(1) On a given phase remarkably constant factors are observed with various column dimensions, temperatures and flow rates. It should also be noted that this degree of consistency is found from actual retention times, without the use of internal standards.

(2) Factors found for substitution at the 7α and 12α positions mostly have similar values; only for the hydroxyl groups on F-60 is there a distinct difference.

(3) Factors for acetoxylation exceed those for trimethylsilyloxy substitution by about 20% on SE-30 and about 30% on F-60.

(4) Hydroxyl and acetoxyl factors, ~~factors~~ computed from the data of Sjövall, et al. for SE-52, show the same relationships as those observed for SE-30 and F-60.

The trimethylsilyl derivatives of the conjugated esters were examined on the four phases previously mentioned to determine the best phase for the resolution of mixtures. In Fig. 4 the retention times of the three derivatives (A, B & C) are compared

Table 5

Factors for Introduction of 12a-substituents

Parent Compound		1% SE-30			1.25% F-60				SE-52 (16)	
		12a-OH	12a-TMSi*	12a-OAc†	12a-OH	12a-TMSi*	12a-OAc†		12a-OH	12a-OAc†
Methyl Lithocholate	**a**	**1.56**	**1.21**	**1.27**						
	b				1.75	1.24	1.42	h	1.85	1.45
	c				1.68	-	1.43	i	2.04	1.70
	d				1.72	-	1.41			
	e				1.78	-	1.42			
Methyl glycolitho-cholate	a	1.60	1.15	1.36						
	b				-	1.13	-			
	f	-	1.07	1.24	(1.56)	-	1.45			
	d				-	1.13	-			
	g	-	1.08	1.22	(1.72)	1.12	1.53			

Factors for Introduction of 7a-substituents

		7a-OH	7a-TMSi*	7a-OAc†	7a-OH	7a-TMSi*	7a-OAc†		7a-OH	7a-OAc†
Methyl deoxycholate	**a**	**1.76**	**1.14**	**1.32**						
	b				1.98	1.05	1.46	h	2.20	1.63
	c				1.94	-	1.42	i	2.32	1.66
	d				2.07	-	-			
	e				2.04	-	1.46			
Methyl glycodeoxy-cholate	a	-	1.08	1.28						
	b				-	1.07	-			
	f	-	1.07	1.29	-	-	1.42			
	d				-	1.13	-			
	g	-	1.06	1.25	-	1.11	1.32			

Footnotes to Table 5 overleaf.

Table 8. (continued).

a. 1% SE-30 at 245° & 70 ml./min (4 ft. column)

b. 1.25% F-60 at 245° & 60 ml./min (4 ft. column)

c. 1.25% F-60 at 250° & 74 ml./min (4 ft. column)

d. 3 ft./1 ft. F-60 at 245° & 70 ml./min

e. 3 ft./1 ft. F-60 at 250° & 70ml./min

f. 2 ft./2 ft. SE-30 at 245° & 50 ml./min

g. 1 ft./3 ft. SE-30 at 245° & 30 ml./min

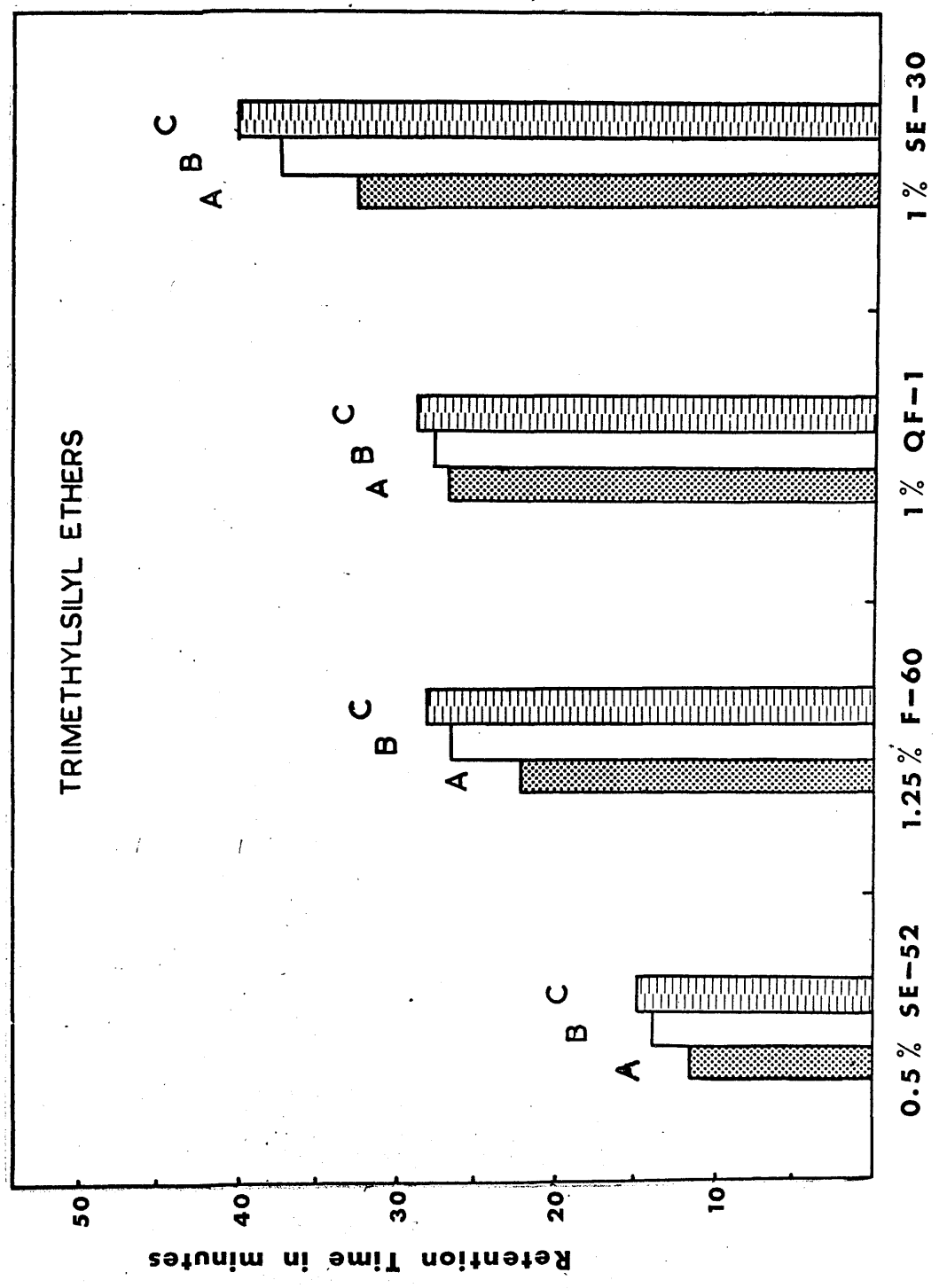
h. 6 ft. SE-52 with 20 mole per cent phenyl groups at 215-220° & 40-70 ml./min

i. 6 ft. SE-52 with 35 mole per cent phenyl groups at 215-220° & 40-70 ml./min.

* Computed using trimethylsilyl ether of parent compound.

† Computed using acetate of parent compound.

FIGURE 4


TRIMETHYLSILYL ETHERS
Retention Time in minutes
50
40
30
20
10
A
B
C
0.5% SE-52
1.25% F-60
1% QF-1
1% SE-30
Stationary Phases

as heights; the largest differences were observed on 4 ft. columns of SE-30 and F-60 and most of the work was confined to these phases. Fig. 5 shows nearly complete resolution of a mixture of trimethylsilyl ethers and complete resolution of acetates on a 4 ft. column of SE-30. For the acetates, however, the retention times were unduly long (cf. Table 2).

In the hope of reducing the retention times and the amounts of sample necessary, the bile acid methyl esters were examined on columns modified by inclusion of a capillary portion as described above (p. 186). Some results are are shown in Figs. 6-8.

In Fig. 6 the trimethylsilyl ethers are shown individually and in admixture on a 2 ft. packed/2 ft. capillary SE-30 column. Samples of about 1 μg. were detectable but the resolution of the mixture was less satisfactory than for the 4 ft. column: cholate and deoxycholate were not separated.

The acetylated conjugates on the same column but under different conditions are shown in Fig. 7. The analysis time was about 30 minutes compared to 70 minutes on the 4 ft. column (cf. Fig. 5) and the mixture of acetates was sufficiently well resolved for identification. The resolution of trimethylsilyl

FIGURE 5

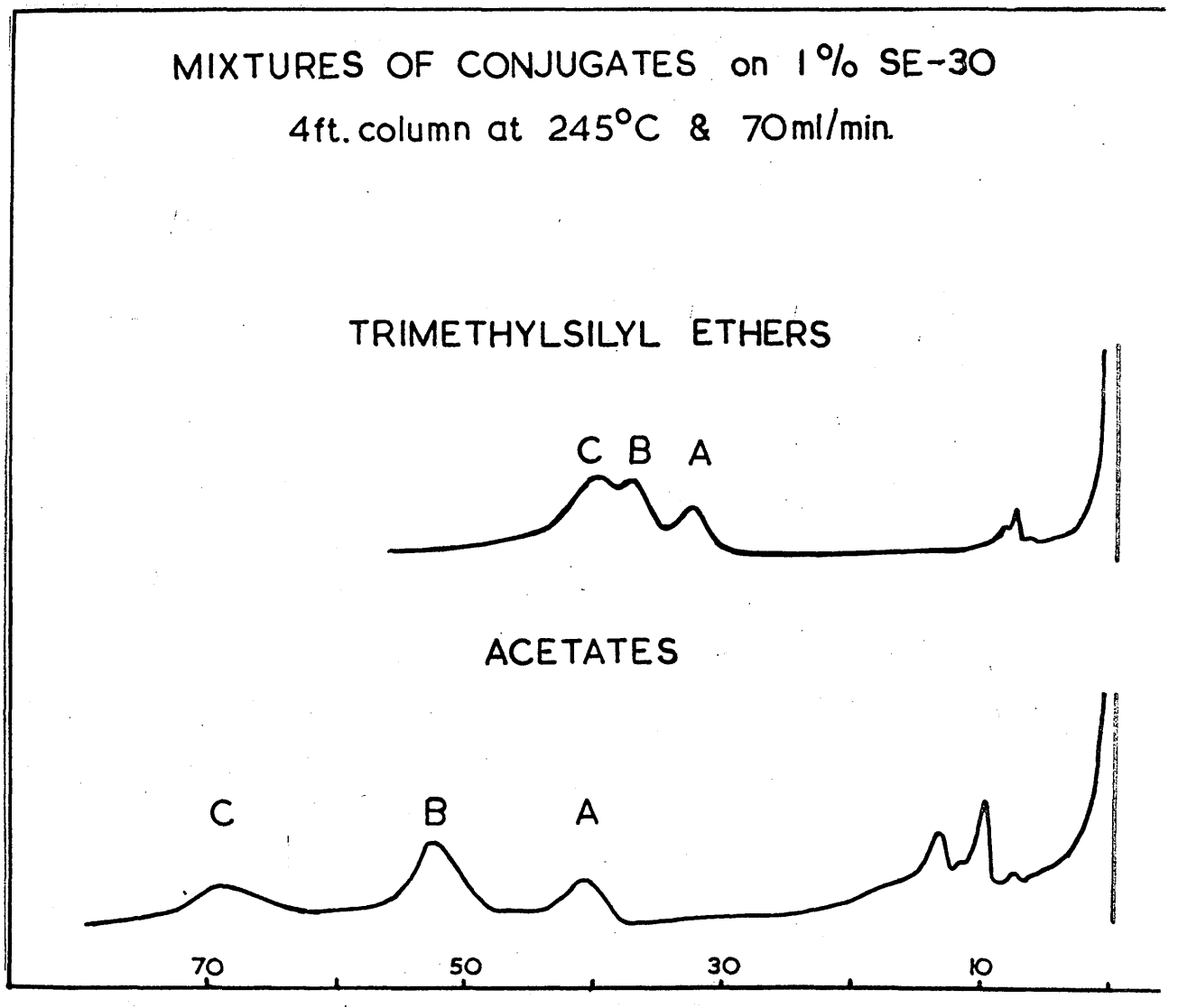

MIXTURES OF CONJUGATES on 1% SE-30
4ft. column at 245°C & 70ml/min.
TRIMETHYLSILYL ETHERS
C B A
ACETATES
C
B
A
70
50
30
10
minutes

FIGURE 6

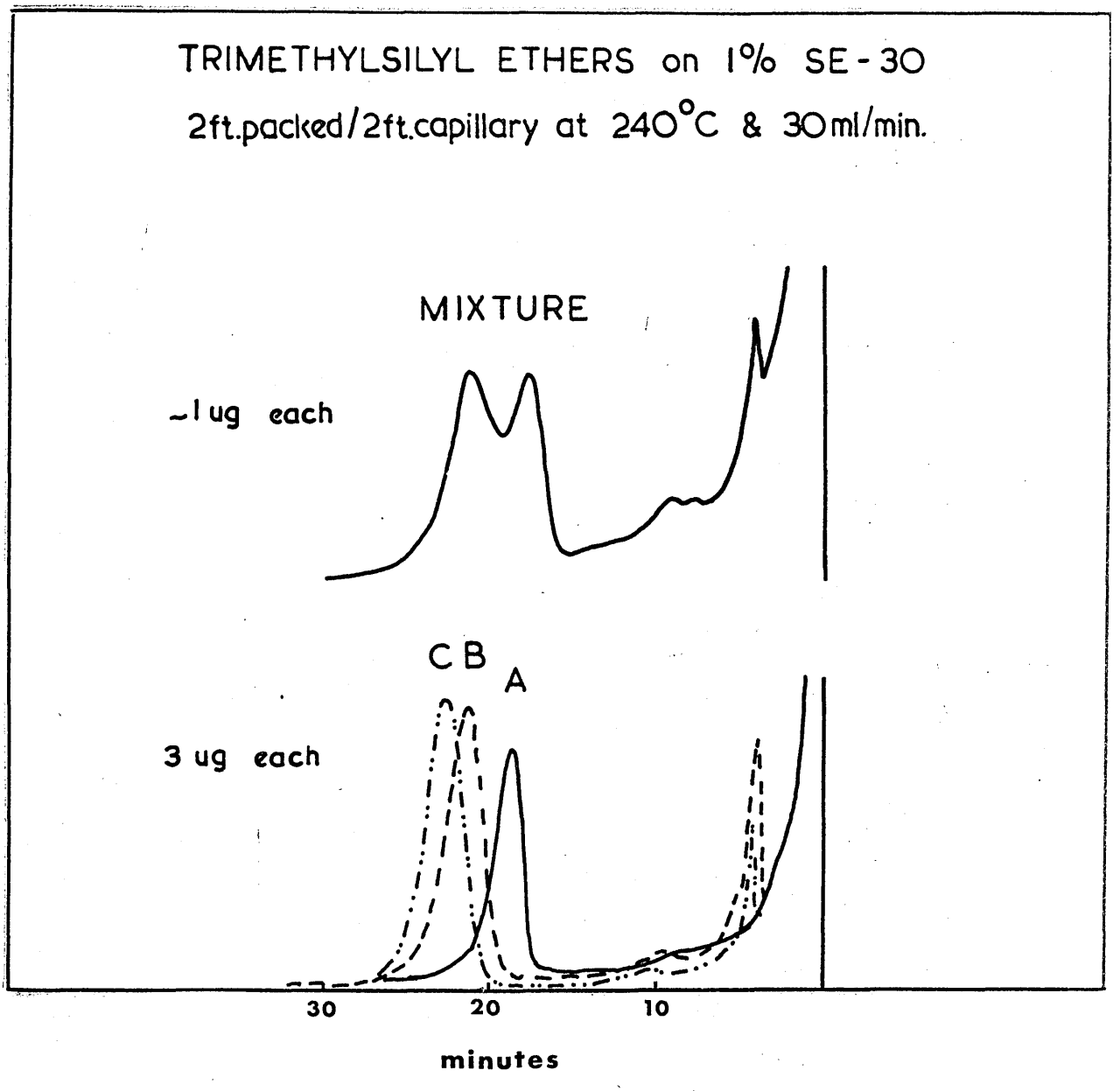
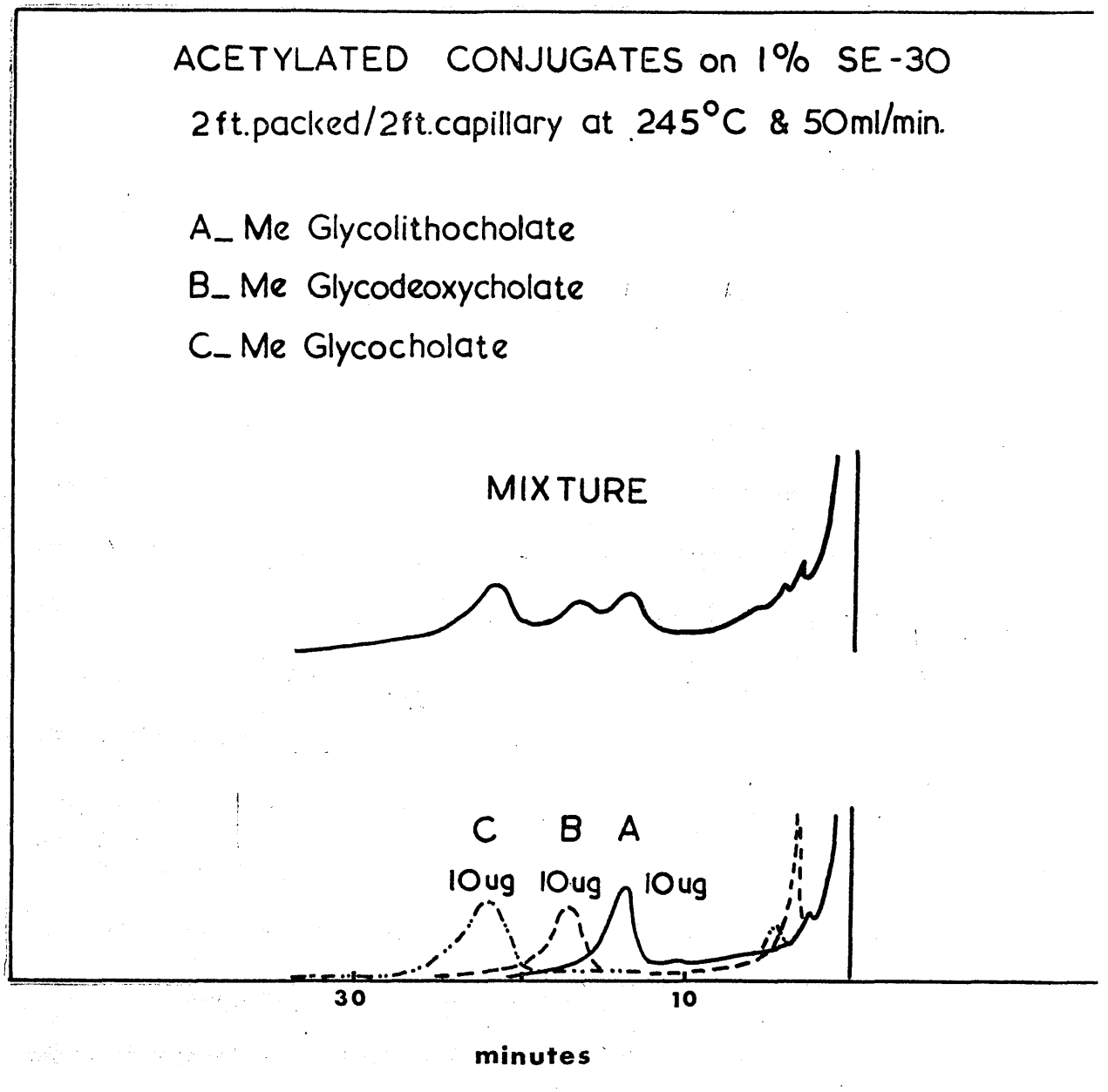

TRIMETHYLSILYL ETHERS on 1% SE-30
2ft.packed/2ft.capillary at 240°C & 30ml/min.
MIXTURE
~1 ug each
C B A
3 ug each
30
20
10
minutes

FIGURE 7


ACETYLATED CONJUGATES on 1% SE-30
2ft.packed/2ft.capillary at 245°C & 50ml/min.
A_ Me Glycolithocholate
B_ Me Glycodeoxycholate
C_ Me Glycocholate
MIXTURE
C
B
A
10ug
10ug
10ug
30
10
minutes

ethers and of acetates under different conditions is shown in Fig. 8. Both for trimethylsilyl ethers and acetates the separations were sensitive to minor changes in temperature and to the flow rate.

The behaviour of the trimethylsilyl ethers on a 4 ft. and a 2 ft. column of SE-30 is compared in Fig. 9. For these derivatives, the standard 4 ft. column gave satisfactory resolution while the modified column was inferior. Chromatograms of the acetylated conjugates on SE-30 columns of different effective lengths are shown in Fig. 10. All three columns were operated at 245^{o} but at different flow rates. The 1 ft. column failed to resolve the mixture while the 4 ft. column gave complete separation but at the expense of long retention times. The best results were observed with the 2 ft. column where nearly complete resolution was achieved within 35 minutes, and where the proportions of decomposition products were smaller than with the 4 ft. column.

On the basis of the above results, the most satisfactory derivatives for the chromatography of glycine-conjugated bile acid methyl esters are their trimethylsilyl ethers. The disadvantage of incomplete resolution (except on a 4 ft. SE-30

FIGURE 8

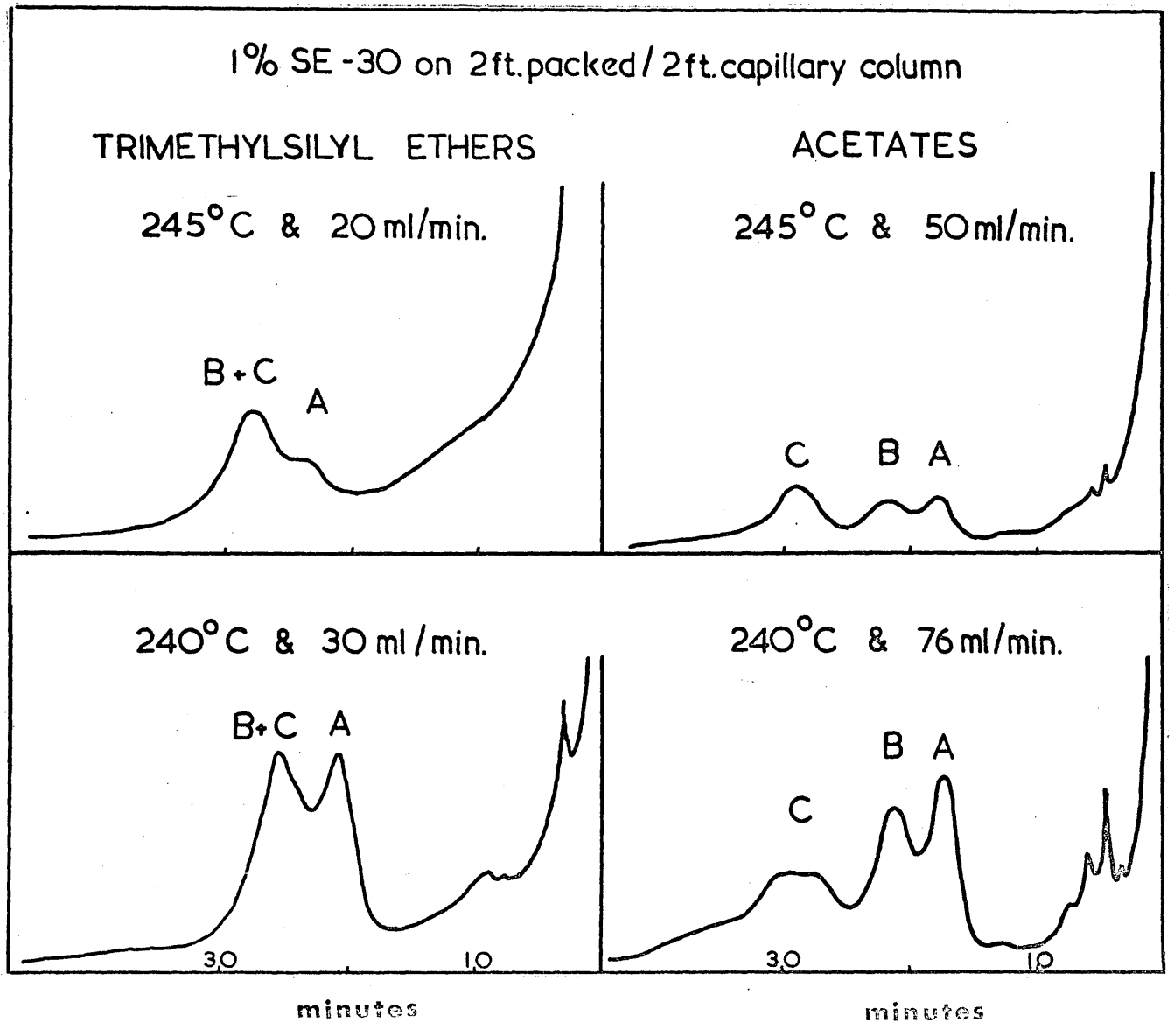

1% SE-30 on 2ft. packed / 2ft. capillary column
TRIMETHYLSILYL ETHERS
ACETATES
245°C & 20 ml/min.
245°C & 50 ml/min.
B+C
A
C
B
A
240°C & 30 ml/min.
240°C & 76 ml/min.
B+C
A
B
A
C
3.0
1.0
3.0
1.0
minutes
minutes

FIGURE 9

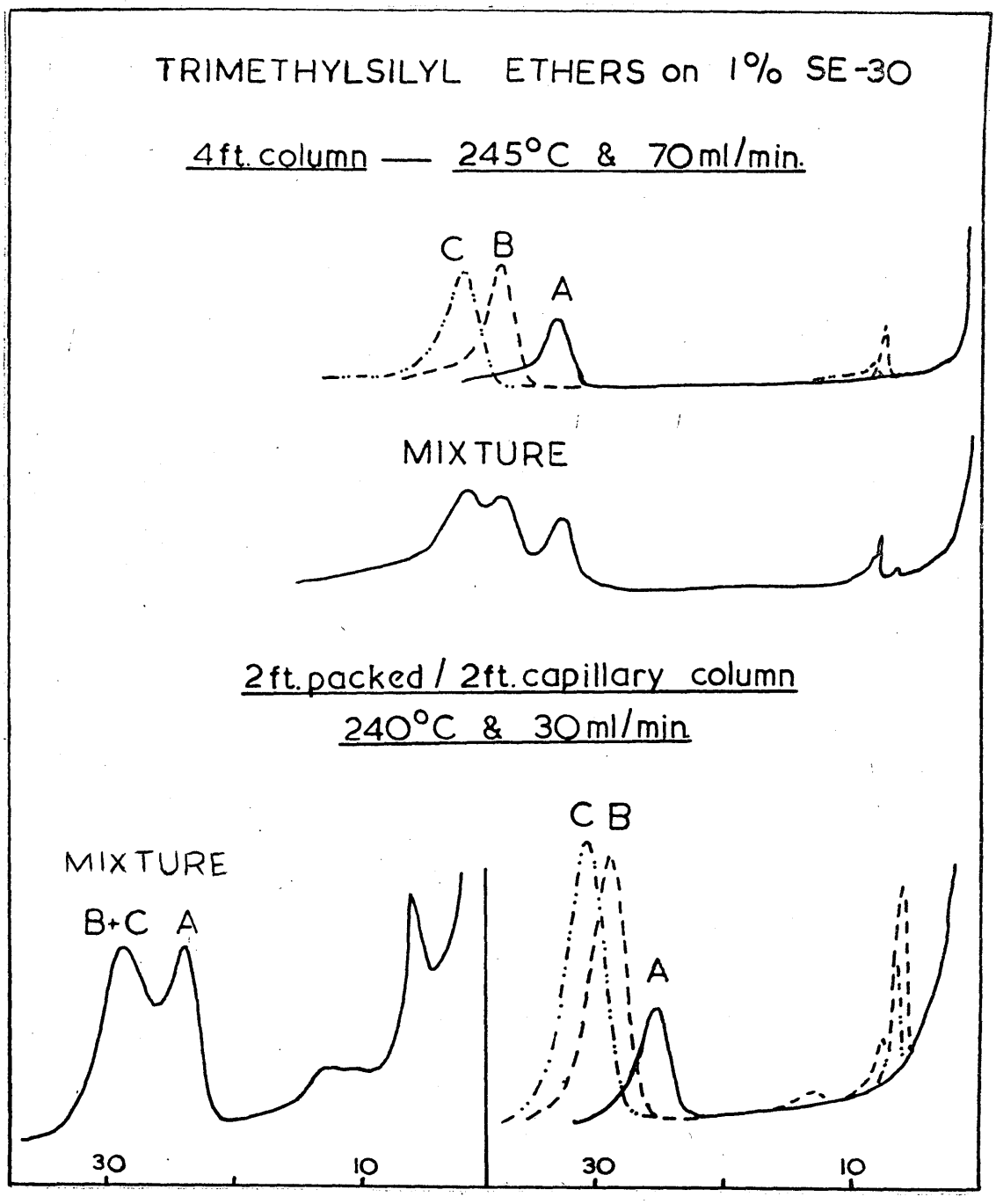
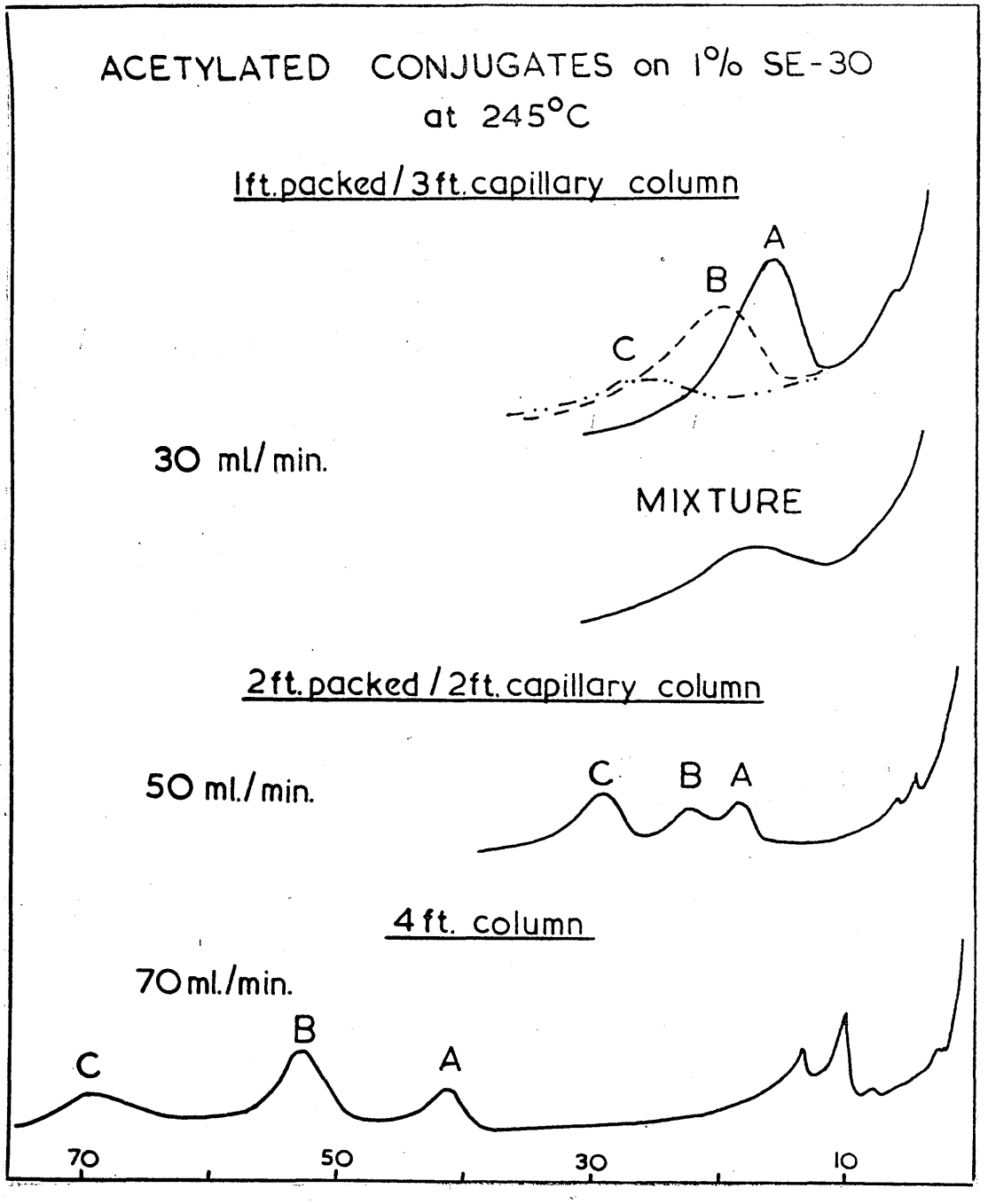

TRIMETHYLSILYL ETHERS on 1% SE-30
4ft. column — 245°C & 70ml/min.
C B
A
MIXTURE
2ft. packed / 2ft. capillary column
240°C & 30ml/min.
MIXTURE
B+C A
C B
A
30
10
30
10
minutes

FIGURE 10


ACETYLATED CONJUGATES on 1% SE-30
at 245°C
1ft. packed / 3ft. capillary column
A
B
C
30 ml/min.
MIXTURE
2ft. packed / 2ft. capillary column
50 ml./min.
C B A
4ft. column
70 ml./min.
B
C
A
70
50
30
10
minutes

column) is offset by the following advantages:

(1) All three conjugates yield well-defined peaks with short retention times. For the acetates, the long retention times for glycodeoxycholate and glycocholate tend to produce ill-defined peaks.

(2) About 1 μg. suffices to give a clear peak.

(3) The derivatives are readily prepared and are stable to hydrolysis if kept at 5° and protected from moisture.

(4) Judging from retention data they appear to be stable on the columns. In the case of the acetates it is not yet clear whether they are unchanged or undergo elimination.*

Nevertheless, the complete separation of the acetate mixture on SE-30 and F-60 makes these derivatives convenient, and in combination with the trimethylsilyl ethers they should permit the tentative identification of glycine conjugates in admixture.

It should be emphasised that the results described above were obtained with an instrument designed for use with 4 ft. columns, and that the modifications made to secure shorter effective column lengths were necessarily rather crude. Use of a chromatograph suitable for operation with a simple 1 ft. or 2 ft. column, with a minimum of dead volume, would be expected to afford better results.

* For further details see Appendix 2.

A further improvement would be likely to result from temperature programming beyond 250° (the limit in this work), especially in the case of the acetylated derivatives.

1. Rudman, D., & Kendall, F.E. (1957). J. Clin. Invest. 36, 530.

2. Carey, J.B. (1956). J. Clin. Invest., 35, 695.

3. Carey, J.B. (1958). J. Clin. Invest., 37, 1494.

4. Levin, S.T., Irvin, J.L., & Johnston, C.G. (1961). Analyt. Chem., 33,856.

5. Levin, S.T. & Johnston, C.G. (1962). J. Lab. Clin. Med., 59, 681.

6. Turner, M.D. Osborn, E.C. & Wootton, I.D.P. (1957). Biochem. J., 67, 31P.

7. Kritchevsky, D. & Kirk, M.R. (1952). Arch. Biochem. Biophys., 35, 346.

8. Osborn, E.C., Wootton, I.D.P., Da Silva, L.C., & Sherlock, S. (1959). Lancet, 2, 1049.

9. Carey, J.B. (1956). Science, 123, 892.

10. Sjövall, J. (1952). Acta. chem. scand. 6, 1552.

11. Portman, O.W. & Shah, S. (1962). Arch. Biochem. Biophys., 96, 516.

12. Scott, M., Grundy, S.M., & Sjövall, J. (1961). Proc. Soc. exp. Biol. Med., 107, 306.

13. Hamilton, J.G., & Dieckert, J.W. (1959). Arch. Biochem. Biophys., 82, 203.

14. VandenHeuvel, W.J.A., Sweeley, C.C. & Horning, E.C. (1960). Biochem. Biophys. Res. Comm., 3, 33.

15. Blomstrand, R. (1961). Proc. Soc. exp. Biol. Med., 107, 126.

16. Sjövall, J., Meloni, C.R. & Turner, D.A. (1961). J. Lipid Res., 2, 317.

17. VandenHeuvel, W.J.A., Sjövall, J. & Horning, E.C. (1961). Biochim. biophys. Acta, 48, 596.

18. Holmes, W.L. & Stack, E. (1962). Biochim. biophys. Acta, 56, 163.

19. Bloomfield, D.K. (1962). Analyt. Chem., 34, 737.

20. Sjövall, J. (1962). Acta chem. scand. 16, 1761.

21. Makita, M., & Wells, W.W. (1963). Analyt. Biochem., 5, 523.

22. Kuksis, A. & Gordon, B.A. (1963). Can. J. Biochem. & Physiol., 41, 1355.

23. Danielsson, H., Eneroth, P., Hellström, K. Lindstedt, S. & Sjövall, J. (1963). J. biol. Chem., 238, 2299.

24. Sjövall, J., Sandberg, D., Sjövall, K. & Turner, D.A. (1963). Fed. Proc. 22, 198.

25. Ellin, R.I., Mendeloff, A.I. & Turner, D.A. (1962). Analyt. Biochem. 4, 198.

26. Bridgwater, R.J., Haslewood, G.A.D. & Tammar, A.R. (1962). Biochem. J. 85, 413.

27. Ahrens, E.H. & Craig, L.C. (1952). J. biol. Chem. 195, 763.

28. Bergström, S. & Norman, A. (1953). Proc. Soc. exp. Biol. Med. 83, 71.

29. Kritchevsky, D. & Kirk, M.R. (1952). J. Amer. chem. Soc. 74, 4713.

30. Sjövall, J. (1955). Ark. Kemi, 8, 299.

31. Haslewood, G.A.D. & Sjövall, J. (1954). Biochem. J., 57, 126.

32. Wiggins, H. & Wootton, I.D.P. (1958). Biochem. J., 70, 349.

33. Sjövall, J. (1954). Acta chem. scand. 8, 339.

34. Sjövall, J. (1959). Clin. Chim. Acta, 4, 652.

35. Sjövall, J. (1960). Clin. Chim. Acta, 5, 33.

36. Levin, S.J., Johnston, C.G. & Boyle, A.J. (1961). Analyt. Chem. 33, 1407.

37. Brooks, C.J.W. & Young, J.S. (1962). Biochem. J. 84, 53P.

38. Abell, L.L., Levy, B.B. Brodie, B.B. & Kendall, F.E. (1952). J. biol. Chem. 195, 357.

39. Stahl, E. (1961). Angew. Chem. 73, 646.

40. Gänshirt, H. Koss, F.H. & Morianz, K. (1960). Arzneimittel Forsch. 10, 943.

41. Hofmann, A.F. (1962). J. Lipid Res. 3, 127.

42. Eneroth, P. (1963). J. Lipid Res. 4, 11.

43. Carey, J. (1961). Gastroenterology, 41, 285.

44. Kritchevsky, D., Martak, D.S. & Rothblat, G.H. (1963). Analyt. Biochem. 5, 388.

45. MacIntyre, I. & Wootton, I.D.P. (1960). Annu. Rev. Biochem. 29, 635.

46. Horning, E.C., VandenHeuvel, W.J.A. & Creech, B.G. (1963). In Methods of Biochemical Analysis, vol. 11, Ed. by Glick, D. New York. John Wiley and Sons Inc.

47. Vogel, A.I. (1948). "A Textbook of Practical Organic Chemistry". Longmans, Green and Co., London.

48. Lin, T.H., Rubinstein, R. & Holmes, W.L. (1963). J. Lipid Res., 4, 63.

49. Kuron, G.W. & Tennent, D.M. (1961). Fed. Proc. 20, 268.

50. Gordon, B.A., Kuksis, A., & Beveridge, J.M.R. (1963). Can. J. Biochem. & Physiol., 41, 77.

51. Hara, S. & Takeuchi, M. (1963). J. Chromat., 11, 565.

52. Hanton, J.C. (1963). Bull. Soc. Chim. biol., Paris, 45, 819.

53. Usui, T. (1963). J. Biochem., Japan, 54, 283.

54. Spero, G.B., McIntosh, A.V. & Levin, R.H. (1948). J. Amer. chem. Soc. 70, 1907.

Appendix 2

Gas Chromatographic Behaviour of Bile Alcohols and their Acetylated Derivatives

As discussed above, the gas chromatographic behaviour of acetylated bile acid methyl esters did not provide clear evidence for their stability on the column, because acetylation at $C_{(7)}$ and $C_{(12)}$ led to markedly dissimilar retention changes to those observed in the 5α-cholestane series. The differences could have been due to the 5β-configuration, to interactions arising in tri- and tetrafunctional derivatives, and/or to the presence of the carbomethoxyl group in the bile acid series. To assess the effect of a carbomethoxyl group at $C_{(24)}$, the corresponding bile alcohols and their acetates were examined.

The alcohols (5β-cholan-24-ol and 5β-cholane-3α,24-diol, 3α, 12α, 24-triol and 3α,7α,12α,24-tetrol) were acetylated by three procedures: (i) with acetic anhydride and pyridine at room temperature overnight; (ii) with the same reagents at reflux temperature for 1-2 hours; (iii) with acetic anhydride in the presence of p-toluene sulphonic acid. Procedure (ii) was found to be most suitable for effecting complete acetylation. The products were characterised by their mode of preparation and by thin-layer chromatographic

examination. Rf values are given in Table 9: the decrease in mobility with increase in the number of acetoxyl substituents parallels the effect reported for corresponding derivatives of methyl cholanate (53).

Gas chromatography was confined to the SE-30 column operated at 240°. Table 10 gives the retention data, together with gross acetate factors, which may be compared with the results for the bile acid series (at 245°: Table 5). The results are self-consistent and the gross acetate factors for the fully acetylated diol, triol and tetrol are in the ratio 100: 83: 72 compared with 100: 82: 75 for the corresponding bile acid derivatives. Similarly the derived acetoxyl factors for the 7α and 12α positions are 1.33 comparable with 1.32 and 1.27 respectively observed for the bile acid series (Table 8). Characterisation of the partially acetylated alcohols was strengthened by the agreement between the derived hydroxyl factors (Table 10, column 6) and those computed from the parent alcohols as shown in Table 10 (column 2).

It is therefore clear that the carbomethoxyl group has no unusual effect on the retention behaviour of the acetylated bile acid methyl esters. It seems probable that the decrease in gross acetate factors for the tri- and tetrasubstituted compounds is a

Table 9. Rf Values for Thin-layer Chromatography of Bile Alcohols and their Acetates in Benzene-Diethyl Ether (80:20)

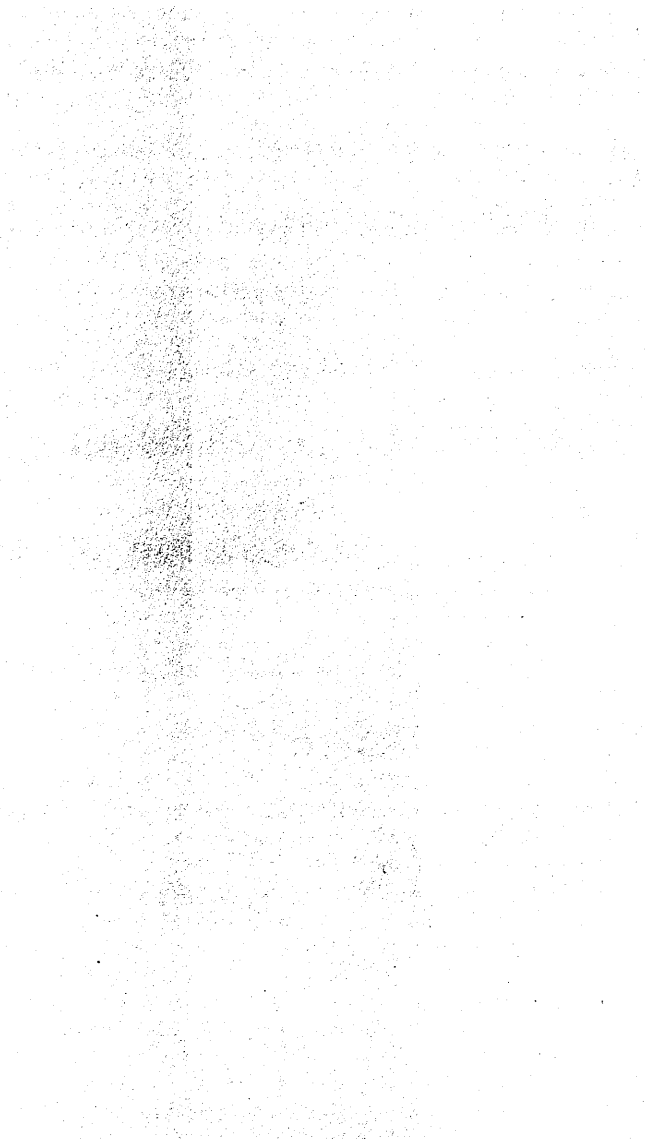
Alcohol (5β-Cholane derivative)	Rf	Acetate	Rf
24-ol	0.47	24-acetate	0.92
3α,24-diol	0.08	24-monoacetate	0.35
		3,24-diacetate	0.87
3α,12α,24-triol	0.00	3,24-diacetate	0.42
		3,12,24-triacetate	0.69
3α,7α,12α,24-triol	0.00	3,24-diacetate	0.05
		3,12,24-triacetate	0.22
		3,7,12,24-tetraacetate	0.50

Table[10]. Relative Retentions and Gross Acetylation Factors for Bile Alcohols

1% SE-30 at 240° and 60 ml./min.

Alcohol (5β-Cholane Derivatives)	Relative Retention	Derived Hydroxyl Factor	Acetyl derivatives: Relative Retention	Assignment	Derived Factors: Hydroxyl Factor	Acetoxyl Factor	Gross acetate factor
5β-Cholane	0.425	2.32	-	-	-	-	-
24-ol	0.987		1.38	24-OAc	-	3.24	1.40
3α,24-diol	1.87	1.89	2.65	24-OAc	1.92	-	1.42
			3.70	3,24-diOAc	-	2.68	1.98
3α,12α,24-triol	3.00	1.61	5.95	3,24-diOAc	1.61	-	1.98
			4.91	3,12,24-triOAc	-	1.33	1.64
3α,7α,12α-24-tetrol	5.50	1.83	10.60	3,24-diOAc	1.78	-	1.93
			8.70	3,12,24-triOAc	1.77	-	1.58
			6.54	3,7,12,24-tetraOAc	-	1.33	1.18

feature of the substitution pattern, i.e. that it arises from vicinal interactions, and is not due to elimination. Such "vicinal effects" must be confined to the acetylated derivatives since the hydroxyl factors for the 3α, 7α and 12α positions derived from the tri- and tetrasubstituted bile alcohols are in agreement with the factors computed from 5α-cholestane and 5β-cholane. Conclusive evidence against the occurrence of elimination must await isolation of the acetylated products in the chromatographic effluent.



Section B

INFRARED SPECTROSCOPY

D. INFRARED SPECTROSCOPY

Infrared Studies of Solvent Effects on Carbonyl and Hydroxyl Stretching Frequencies

Introduction

Infrared absorption spectroscopy has been widely applied to the structural determination of organic molecules. One important aspect of the method is based on the association of particular absorption bands with the presence of specific groups in the molecule. For example, characteristic vibration frequencies are associated with the presence of C-H, C=O, O-H bonds in the compound. With the advent of high resolution spectrometers in the last few years, infrared spectroscopy has become a powerful analytical tool for the study of conformational and configurational details. This is made possible by the sensitivity of the characteristic group vibrations, referred to above, to small changes in environment, resulting in characteristic changes in the absorption frequency, half-band width and peak intensity. The present work is concerned with the study of such effects in the carbonyl and hydroxyl regions.

Considerable attention has been focused in the past few years on the variation of carbonyl group stretching frequencies with solvent (1-10). Several authors (1-4,11,12) have considered solvent effects from the standpoint of simple dielectric theory, while BELLAMY and

WILLIAMS (10,13) have explained frequency shifts in terms of specific solute-solvent interactions in which the dielectric constant of the medium plays a minor part. Others maintain that the relative significance of these factors varies from case to case (5,6). The problem has also been examined in terms of solute-solution interaction energies (8), polarisability of the functional group (7), and inductive and mesomeric influences (9).

Much work in this field has been concerned with series of compounds studied in a large number of solvents of diverse polarity. BELLAMY and HALLAM (14), ITO et al. (4) and HALLAM and RAY (6) have employed mixtures of solvents to assess the importance of the dielectric constant in the solvent-induced frequency shifts of N-H, C=0 and C-halogen chromophores respectively. Recently WHETSEL and KAGARISE (16,17) have made detailed studies of the carbonyl absorptions of certain ketones and esters in mixed solvents. They examined, among other compounds, cyclohexanone in chloroform-cyclohexane and in carbon tetrachloride-cyclohexane solvent systems, in which they demonstrated specific association between chloroform or carbon tetrachloride and cyclohexanone. Their results supported BELLAMY'S proposal that frequency shifts are determined predominantly by specific interactions between the solute and the solvents (10,13,14).

In spite of the many investigations of solvent effects on carbonyl frequencies, few studies have been made in relation to steric factors (18,19,33). MARONI (18) has carried out a systematic study on the influence of steric factors on the carbonyl frequency of saturated aliphatic ketones studied in the liquid state. He found that the carbonyl frequency decreased with increasing α - and β-substitution, though a very marked decrease occurred only upon full α-substitution as in di-t-butylketone. In the steroid and triterpenoid fields the principal works are those of JONES et al. (20-24) and COLE and co-workers (25,26) who correlated carbonyl frequencies with molecular structure. In the present work, infrared spectroscopy has been used to study steric influences on the interaction of carbonyl groups with solvents (Section 1) and on the hydrogen bond association between carbonyl and phenol groups (Section 2).

Solvent effects on hydroxyl stretching frequencies have been extensively investigated and in particular, attention has been focused on the association of phenols with ketones and ethers (15, 27-31). In the majority of cases the magnitude of the shift in hydroxyl frequency has been related to the strength of the hydrogen bond, but a few investigators (15,27,28,30) have supplemented their absorption data by evaluating formation of dissociation constants.

In contrast to the limited investigations of steric factors in the carbonyl region, their influence on hydroxyl absorption has been extensively reported (e.g. refs. 30-34) both for single and mixed solvent systems. In Section 2 of this work, dealing with the association of ortho-substituted phenols with alkyl cyclohexanones, the hydroxyl absorptions were examined in parallel with studies of the carbonyl region.

Section 1

Solvent Effects on Carbonyl Stretching Frequencies in Relation to Steric Hindrance

The present section is concerned with the influence of steric and solvent effects on the carbonyl absorption of a number of cyclohexanones, and of steroid and triterpenoid ketones and acetoxy-ketones. These are investigated in three solvents and the frequency shifts, half-band widths and intensities are discussed with reference to stereochemical features. Chart 2 gives formulae of the compounds studied.

Materials and Methods

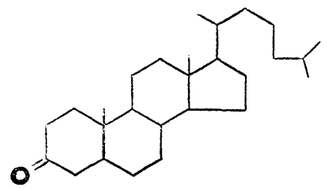
Cyclohexanones

See Section 2 for experimental details.

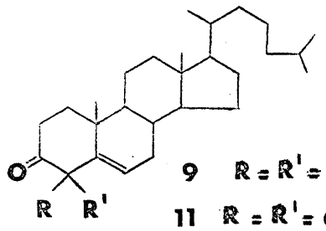
Steroid and Triterpenoid Ketones and Acetoxy-ketones

The compounds 21,23,25-28,31 and 33 were generously provided by Dr. P. Bladon; the compounds 29,37,41,44,45,50 and 51 by Dr. J. McLean; compounds 11,12,15,22,35 and 57 by Dr. G. Eglinton; compounds 8,34 and 36 by Dr. C. J. W. Brooks and the compounds 19 and 30 by Dr. M. Martin-Smith. Compounds 24,47 and 52 were prepared by acetylation of the corresponding hydroxy-derivatives, while compounds 31,40 and 49 were prepared by alkaline hydrolysis of compounds 32,41 and 50 respectively. The remaining compounds were obtained from commercial sources, mainly from L. Light & Co., Ltd., Colnbrook,

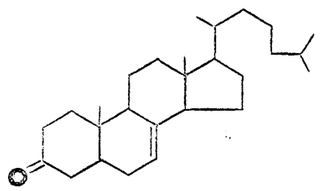
CHART 2 COMPOUNDS EXAMINED BY INFRARED SPECTROSCOPY



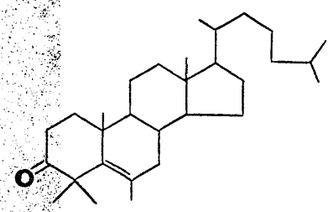
8



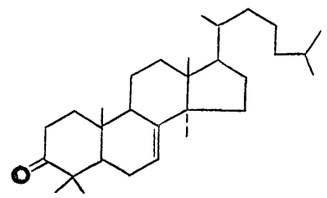
9 R = R' = H
11 R = R' = CH_3



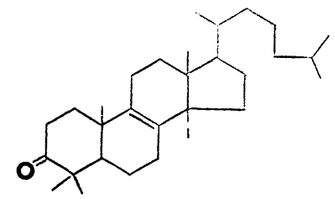
10



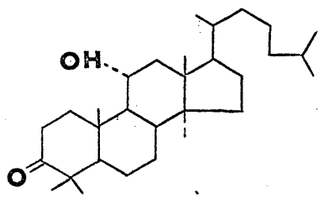
12



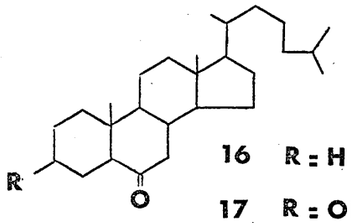
13



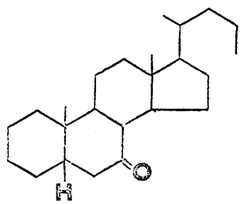
14



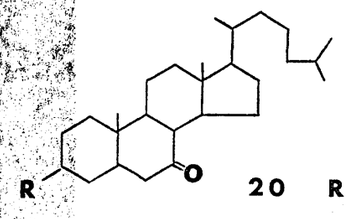
15



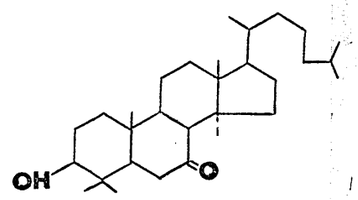
16 R = H
17 R = OH
18 R = OAc



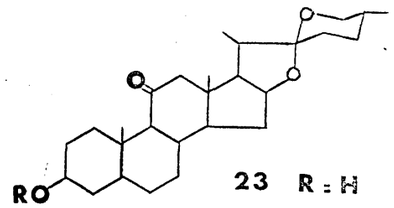
19



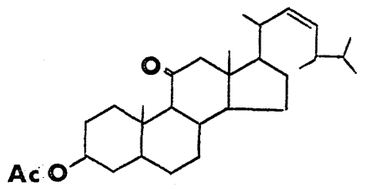
20 R = H
21 R = OAc



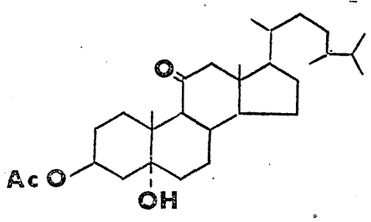
22



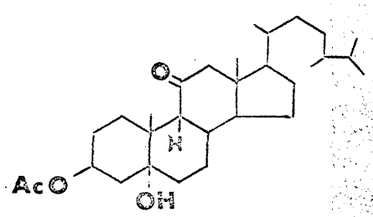
23 R = H
24 R = Ac



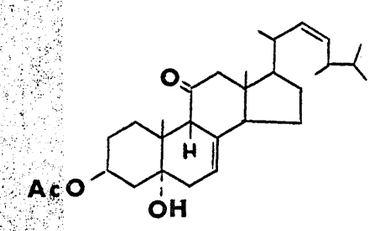
25



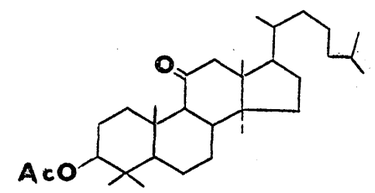
26



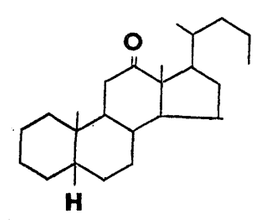
27



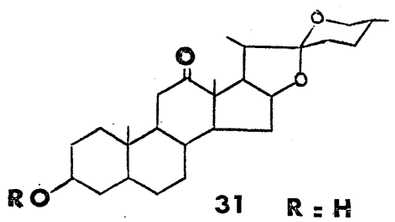
28



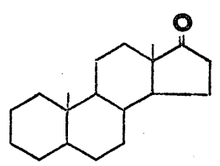
29



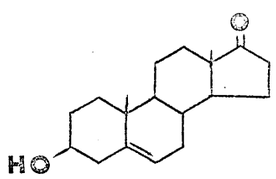
30



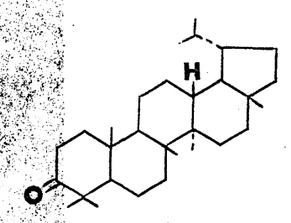
31 R = H
32 R = Ac



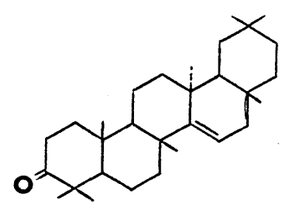
33



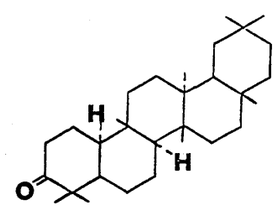
34



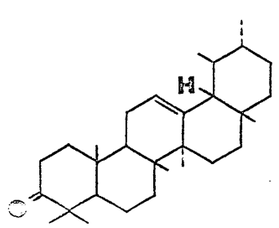
35



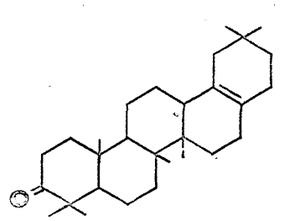
36



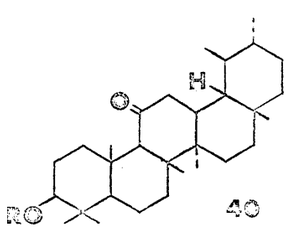
37



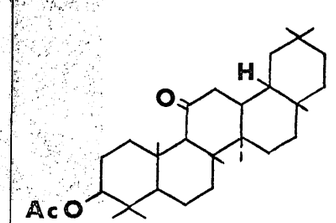
38



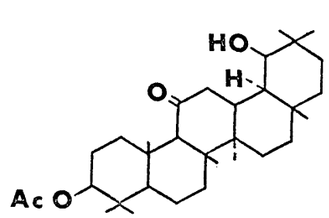
39



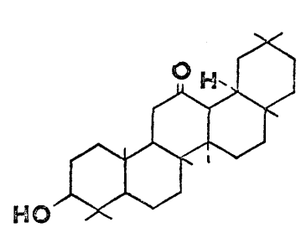
40 R = H
41 R = Ac



42

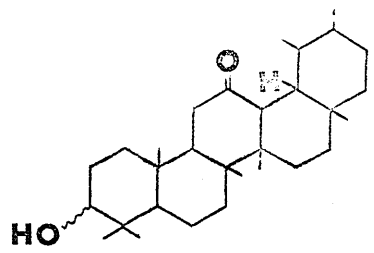


43

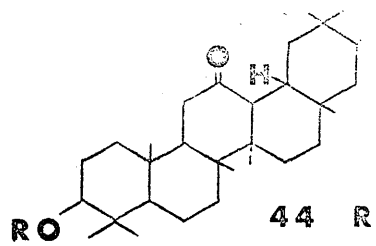


45

(continued)

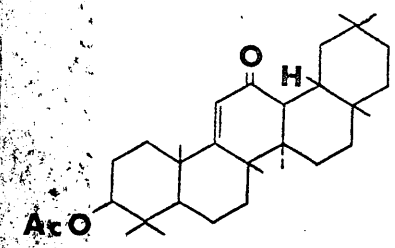


46

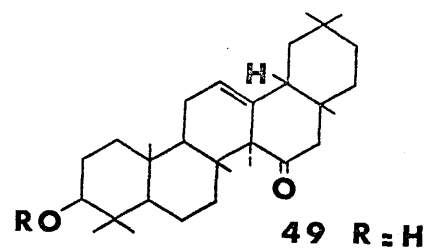


44 R = H

47 R = Ac

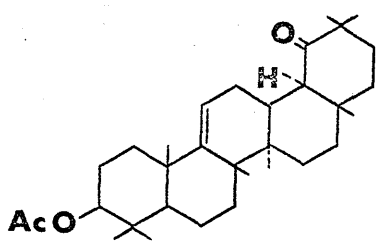


48

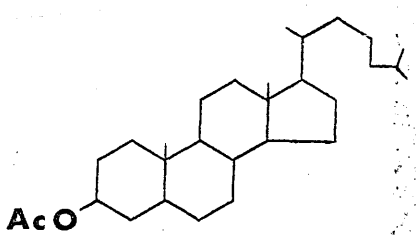


49 R = H

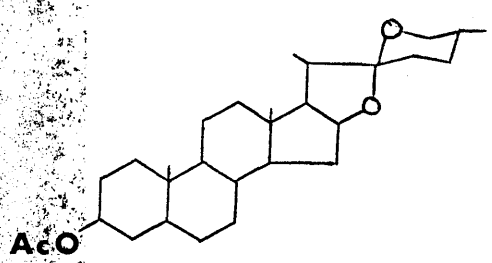
50 R = Ac



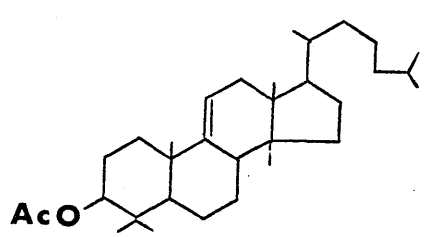
51



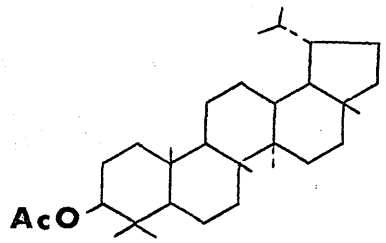
52



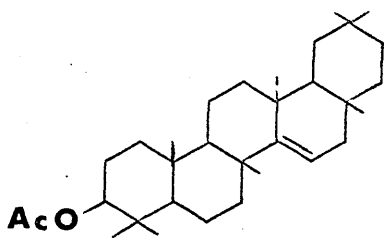
53



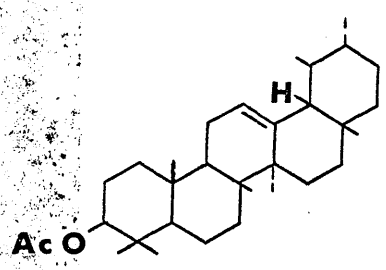
54



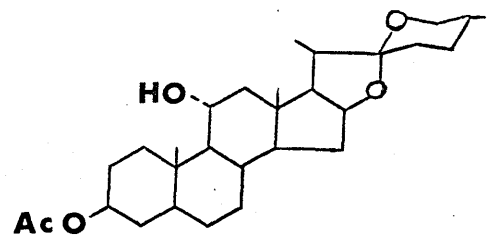
55



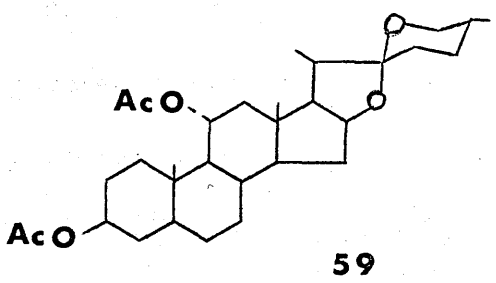
56



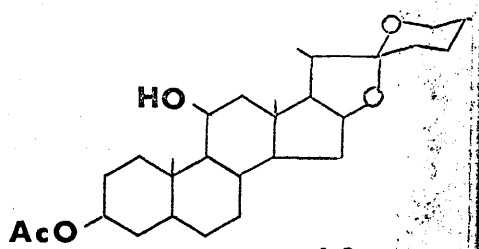
57



58



59



60

Bucks and Southeastern Biochemicals, Morristown, Tennessee. Compounds of doubtful purity were recrystallised and their purity checked by melting-point, thin-layer and/or gas-liquid chromatography while those of known purity were used directly.

Solvents

n-Hexane (AnalaR) and carbon tetrachloride (AnalaR) were used without purification. Chloroform (AnalaR) was freed from ethanol by two successive passages through blue silica gel immediately before use.

Measurements

All 1 ml. and 2 ml. solutions were measured by weight, and 5 ml. and 10 ml. solutions by volume. Spectra were recorded linearly in $cm.^{-1}$ as percentage transmission with a Unicam S. P. 100 double beam infrared spectrophotometer operated under vacuum. An S. P. 130 sodium chloride prism-grating double monochromator (1,500 lines/inch (650-1,800 $cm.^{-1}$)) and 3,000 lines/inch (3,200-3,650 $cm.^{-1}$) was used. The cell-well temperature was $30 \pm 2^{\circ}$. Frequency calibration (52) was based on the spectrum of water vapour, a calibration being run after each uninterrupted series of measurements. The measurements are considered accurate to within $\pm$ 1 $cm.^{-1}$ at 1,700 $cm.^{-1}$ and to within $\pm$ 5 $cm.^{-1}$ at 3,000 $cm.^{-1}$. In examples where split bands were present

the mean value of the frequencies was taken for the calculation of frequency shifts. The theoretical spectral slit widths, computed from tables supplied by Messrs. Unicam Instruments Ltd., were approximately 4 cm.$^{-1}$ for the carbonyl region (1,700 cm.$^{-1}$) and 5 cm.$^{-1}$ for the hydroxyl region (3,000 cm.$^{-1}$). The carbonyl region was scanned at 14 cm.$^{-1}$ per minute and the hydroxyl region at 80 cm.$^{-1}$ per minute.

Unless otherwise specified the peaks were symmetrical and the apparent half-band widths ($\Delta\nu$ a/2) are quoted to the nearest whole number. Where necessary half-band widths were determined by reflection of the undisturbed sides of the asymmetric bands. All intensity measurements were made on bands of at least 10% transmission. Peak intensities are given as apparent extinction coefficients ε^{a} (l. mole^{-1} cm.$^{-1}$) rounded to the nearest 10 units and measured from a solvent-solvent baseline superimposed on the absorption of the solution recorded with solvent in the reference cell.

Results and Discussion

Cyclohexanones

It is well known that a carbonyl compound normally exhibits its highest frequency in the vapour phase, while in solution the highest values are observed in solvents of low polarity. Table 1 shows that for variously substituted cyclohexanones a general decrease in frequency

Table 1. **Cyclohexanones**

No.	Compound	ν_1 n-Hexane $\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	ν_2 CCl_4 $\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	ν_3 $CHCl_3$ $\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	Shifts $\Delta\nu_{1-2}$
1.	Cyclohexanone	1725	12	450	1717 †	16	480	1705 †	17	460	8
2	2-Methylcyclohexanone	1722	9	570	1716	12	550	1706	19	430	6
3	2,6-Dimethylcyclohexanone	1720 †	10	530	1715 †	14	430	1705 †	20	370	5
4	2,2,6-Trimethylcyclohexanone	1714 †	10	440	1709	11	490	1703 †	19	360	5
5	2,2,6,6-Tetramethylcyclohexanone	1701 † (1707sh)	9	450	1698 † (1713sh)	9	470	1692 † (1707sh)	17	350	3
6	4-Methylcyclohexanone	1725	7	650	1719	9	640	1711	18	440	6
7	4-t-Butylcyclohexanone	1726	7	710	1720	9	670	1711	16	480	6

Solutions were ca. 0.02M examined in 0.51 mm. cells.

† Asymmetric peak

sh Shoulder

Values in parenthesis are approximate

$\Delta\nu_{1-2} = \nu_1 - \nu_2$

$\Delta\nu_{2-3} = \nu_2 - \nu_3$

on passing from n-hexane through carbon tetrachloride to chloroform is attended by an increase in half-band width and a fall in apparent molecular extinction coefficient. Although apparent molecular extinction coefficients [a] may be as much as 20% below the true coefficients (35), they are of value in comparing absorption intensities provided that variations in half-band width are taken into account.

WHETSEL and KAGARISE (16) have demonstrated specific, though weak, interaction between cyclohexanone and carbon tetrachloride on the basis of changes in the shape of the asymmetric carbonyl band in solvent mixtures of varying composition. The effects observed with cyclohexanone and chloroform were more distinct, indicating the presence of 1:1 and 1:2 cyclohexanone-chloroform complexes. Comparison with our results for cyclohexanone in n-hexane, carbon tetrachloride and chloroform (Fig. 1A) shows them to be in close agreement.

Introduction of α-substituents into cyclohexanone tends to shield the carbonyl group from solvent molecules. Table 1 shows that the frequency shift $\Delta\nu(C=O)$, which is a measure of the strength of the association, diminishes with increasing α-methyl substitution. The magnitudes of the carbonyl frequencies ($\nu C=O$) also show a decreasing trend as the number of α-methyl substituents is increased; markedly lower frequencies are found for 2,2,6-trimethyl and 2,2,6,6-tetra-

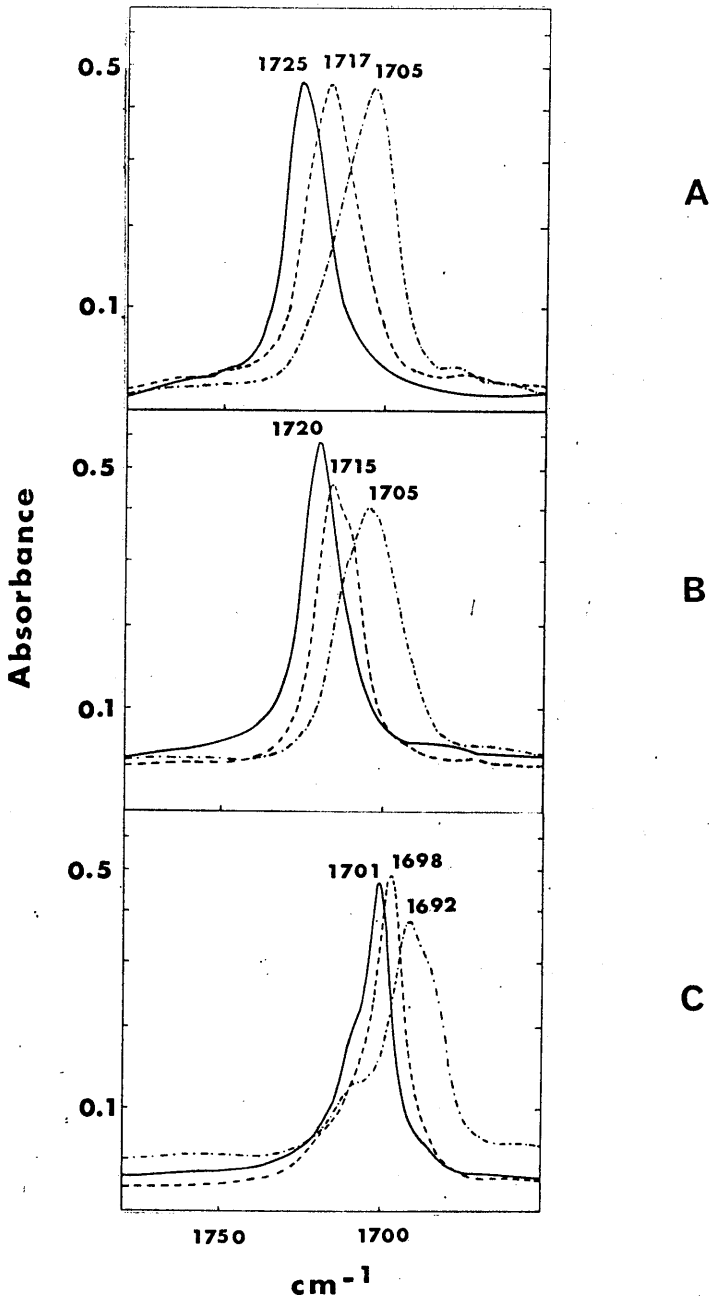


Fig. 1. Carbonyl absorptions: (A) cyclohexanone; (B) 2,6-dimethylcyclohexanone; (C) 2,2,6,6-tetramethylcyclohexanone. All measurements on 0.02M solutions in n-hexane (——), carbon tetrachloride (– – –) and chloroform (–·–·) in 0.51 mm. cells.

methylcyclohexanone, paralleling the effects reported for analogous acyclic ketones (e.g. di-t-butylketone 1,686 cm.$^{-1}$ in the liquid state and in carbon tetrachloride)(18,19). Examples are depicted in Fig. 1.

In comparison introduction of a 4-methyl or a 4-t-butyl group into cyclohexanone causes little change in carbonyl frequency but leads to much sharper bands in n-hexane and carbon tetrachloride. This effect may be ascribed to the existence of the 4-alkyl cyclohexanones in a single preferred conformation in which the substituent adopts an equatorial position, as discussed by ALLINGER and BLATTER (36) and HÜCKEL (37), among others. In contrast, cyclohexanone exists as an equilibrium mixture of conformational isomers, leading to broader absorption bands having Gaussian character as described by SESHADRI and JONES (38). The large values of $\Delta\nu_{\frac{1}{2}}$ in chloroform reflect the composite nature of the observed carbonyl bands.

The apparent extinction coefficients for compounds 1,2,6 and 7 in carbon tetrachloride are about 40% higher than those reported by CETINA and MATEOS (39), indicating the improved resolution obtained with the prism/grating spectrophotometer used here, compared with the prism instrument employed by these authors.

Steroid Ketones

In contrast to the cyclohexanones, the steroids possess relatively

rigid conformations, permitting the study of carbonyl groups in a variety of defined environments, and the comparison of the influence of different substituents, more or less remote from the carbonyl group. Table 2 illustrates data for keto steroids (including lanostane derivatives) showing the variation in the frequency of the carbonyl stretching vibration ($\nu C=O$) in differently constituted compounds: mean values are given in Table 2A. The sequence of carbonyl stretching frequencies in n-hexane and carbon tetrachloride (17-one > 3-one $\geqslant$ 6-one > 4,4,-dimethyl-3-one ~ 7-one $\geqslant$ 12-one > 11-one) is in general agreement with the data of R. N. JONES (23) and (for the six-membered ketones) parallels increasing steric congestion round the carbonyl group.

Steric hindrance of a carbonyl group is also reflected in the frequency shift values observed on proceeding from n-hexane to carbon tetrachloride and from carbon tetrachloride to chloroform (cf. cyclohexanone and 2,2,6,6-tetramethylcyclohexanone in Table 1). The largest solvent-induced frequency shifts (Table 2A) are observed for the relatively unhindered 17-, 3-, 6- and 7-ketones, the mean values being 7 $cm.^{-1}$ (n-hexane - CCl_4) and 9 $cm.^{-1}$ (CCl_4 - $CHCl_3$) for most steroids. (Data for 3β-Acetoxy-5α-cholestan-6-one and 7-one (Nos. 18 and 21) have been omitted in computing mean values as these compounds show anomalous absorptions in chloroform attributable to the proximity of the acetate and carbonyl groups.) The more hindered 11- and 12-ketones show

Table 2. **Steroid Ketones**

No.	Compound	ν_1 n-Hexane			ν_2 CCl_4			ν_3 $CHCl_3$			Shifts ($cm.^{-1}$)	
		$\nu C{=}O$	$\Delta\nu a/2$	ε^a	$\nu C{=}O$	$\Delta\nu a/2$	ε^a	$\nu C{=}O$	$\Delta\nu a/2$	ε^a	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	3-Ketones											
8	5α-Cholestan-3-one	1724	12	640	1716	14	560	1706	20	500	8	10
9	Cholest-5-en-3-one	1729	16	460	1722	16	490	1713	20	470	7	9
10	5α-Cholest-7-en-3-one	-	-	-	1719	17	510	1706‡	21	480	-	13
11	4,4,-Dimethylcholest-5-en-3-one	-	-	-	1712	12	610	1703	19	400	-	9
12	4,4,6-Trimethylcholest-5-en-3-one	1716	7	750	1710	12	550	1702	22	360	6	8
13	Lanost-7-en-3-one	-	-	-	1710	11	580	1701	21	410	-	9
14	Lanost-8(9)-en-3-one	-	-	-	1709	13	500	1699	22	410	-	
15	11α-Hydroxylanostan-3-one	-	-	-	1708	13	480	1699	22	410	-	9
	6-Ketones											
16	5α-Cholestan-6-one	-	-	-	1712	13	550	1702	20	420	-	10
17	3β-Hydroxy-5α-cholestan-6-one	-	-	-	1716	13	440	-	-	-	-	-
18	3β-Acetoxy-5α-cholestan-6-one	1722	10	720	1716	14†	650	1710	27†	610	6	6
	7-Ketones											
19	5β-Cholan-7-one	1718‡	12	880	1711	13	570	1701‡	19	360	7	10
20	5α-Cholestan-7-one	1716	9	550	1710	12	500	1700	19	400	6	10

Table 2 (continued)

No.	Compound	ν_1 n-Hexane $\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	ν_2 CCl_4 $\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	ν_3 $CHCl_3$ $\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	Shifts ($cm.^{-1}$) $\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	7-Ketones (continued)											
21	3β-Acetoxy-5α-cholestan-7-one	1718	11	490	1712	13	470	(1707)	23 †	450	6	(5)
22	3β-Hydroxylanostan-7-one	-	-	-	1708	13	450	1696	19	380	-	12
	11-Ketones											
23	11-Oxotigogenin	-	-	-	1709	14	420	1703	18	340	-	6
24	11-Oxotigogenin acetate	1714	11	*	1709	15	430	1703	19 †	380	5	6
25	3β-Acetoxyergost-22-en-11-one	1711	10	570	1707	13	530	1702	20 †	500	4	5
26	3β-Acetoxy-5α-hydroxyergostan-11-one	1710	15	580	1705	14	410	1699	19 †	480	5	6
27	3β-Acetoxy-5α-hydroxy-9β-ergostan-11-one	1705	11	540	1700	14	540	1692	21	420	5	8
28	3β-Acetoxy-5α-hydroxy-9β-ergosta-7,22-dien-11-one	-	-	-	1710	15 †	340	1704	22 †	370	-	6
29	3β-Acetoxylanostan-11-one	1705	10	450	1700	12	500	1695 ‡	19 †	430	5	5
	12-Ketones											
30	5β-Cholan-12-one	1712	9	660	1706	12	670	1699 ‡	19	470	6	7
31	Hecogenin	-	-	-	1710	11	460	-	-	-	-	-
32	Hecogenin acetate	1716	12	*	1711	13	630	(1707)	23 †	570	5	4
	17-Ketones											
33	5α-Androstan-17-one	1749	6	900	1743	11	780	1731 ‡	18	450	6	12
34	3β-Hydroxyandrost-5-en-17-one	-	-	-	1743	10	730	-	-	-	-	-

Footnotes to Table 2

‡ Asymmetric peak

† Estimated by band reflection

\- Not measured

sh Shoulder

Values in parenthesis are approximate

* Saturated solution in n-hexane

Solutions in n-hexane and chloroform were 0.15M ± 0.0005M (0.51 mm. cells) with the following exceptions: in n-hexane compound Nos. 26 & 27 (0.006M).

Solutions in carbon tetrachloride were 0.0015M ± 0.0003M (5 mm. cells) with the following exception: compound No. 26 (0.00212M).

Table 2A. **Mean carbonyl frequencies and solvent shifts for steroid ketones**

Type of ketone	Mean ν C=0			Mean shifts (cm.$^{-1}$)	
	ν_1 n-hexane	ν_2 CCl_4	ν_3 $CHCl_3$	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
17-one	1749	1743	1731		
3-one	1726 ± 3	1719 ± 3	1708 ± 5		
6-one	1722	1714 ± 2	1702	7 ± 1	10 ± 3
4,4-dimethyl-3-one	1716	1710 ± 2	1701 ± 2		
7-one	1717 ± 1	1710 ± 2	1699 ± 3		
12-one	1714 ± 2	1709 ± 3	1703 ± 4	5 ± 1	6 ± 2
11-one	1709 ± 5	1705 ± 5	1700 ± 5		

distinctly smaller frequency shifts averaging 5 cm.$^{-1}$ from n-hexane to carbon tetrachloride and 6 cm.$^{-1}$ from carbon tetrachloride to chloroform. Figs. 2A, 2B and 2C are representative diagrams for compounds with a carbonyl group at positions 3, 7 and 12 (in a triterpenoid example) respectively. The considerable broadening of the bands in chloroform, similar to that observed for the alkyl cyclohexanones (Table 1) suggests the participation of more than one hydrogen-bonded species.

Introduction of unsaturation at Δ^5 or Δ^7 in 5α-cholestan-3-one raises the carbonyl frequency by 6 and 3 cm.$^{-1}$ respectively whereas introduction of a gem-dimethyl group at $C_{(4)}$ in three examples of 3-ketones lowers the frequency by 8-10 cm.$^{-1}$ (40, 41). In such compounds as 4,4,-dimethylcholest-5-en-3-one and lanost-7-en-3-one the frequencies observed represent an approximate summation of these effects. The 4,4,-dimethyl group also seems to lower the frequency of the 11-keto group (cf. Nos. 25 & 29) and possibly also of the 7-keto group (cf. Nos. 20 & 22). The shifts may be due to the steric effect of the 4β (axial) methyl group acting through the 10β-methyl and 6β-hydrogen substituents respectively.

In the 11-oxoergostane series (Nos. 26-28) the observed carbonyl frequencies may be rationalised as follows. In No. 26 the 5α-hydroxyl group is too distant from the 11-ketone to interact with it and the carbonyl

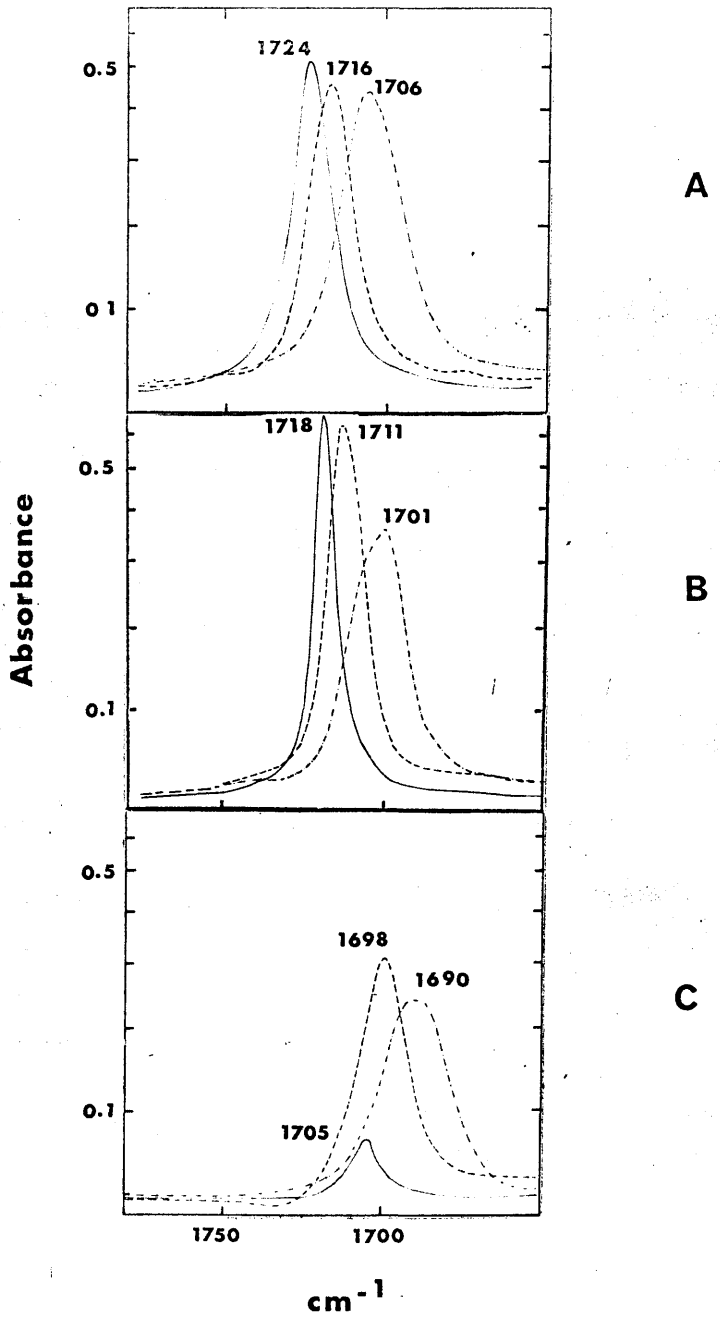


Fig. 2. Carbonyl absorptions: (A) 5α-cholestan-3-one; (B) 5β-cholan-7-one; (C) 3β-hydroxyoleanan-12-one. Measurements on ca. 0.015M solutions in n-hexane (——) (except C, saturated solution) and chloroform (-·-·) in 0.51 mm. cells, and ca. 0.0015M solutions in carbon tetrachloride (– – –) in 5 mm. cells.

frequency is essentially normal. However on changing the configuration at $C_{(9)}$ from α to β the two groups are brought into close proximity (1.8 Å) and the lower carbonyl frequency may be due to hydrogen-bonding. In the presence of a double bond at $C_{(7)}$ (No. 28) the hydroxyl and keto groups are too far apart for interaction: the higher frequency for this compound may partly ensue from the "homoconjugation" of the Δ^7 and 11-keto groups, analogous to the elevating effect of the Δ^5 bond on the 3-ketone frequency.

Triterpenoid Ketones

Table 3 shows the carbonyl frequencies for pentacyclic triterpenoid ketones at positions 3,11,12,15 and 19. Consideration of the carbonyl frequencies for the 3-position in steroid and triterpenoid systems (Tables 2 and 3) shows that all the 3-ketones with a 4,4-dimethyl group have frequencies 8-10 cm.$^{-1}$ lower in carbon tetrachloride than comparable 3-ketones unsubstituted at $C_{(4)}$. Such decreases in carbonyl frequency have been noted by other workers (40, 41) and related to the changes in conformation caused by the dimethyl group. ALLINGER and DAROOGE (42), using HALFORD'S relationship between bond angle expansion and depression of the carbonyl frequency (43), have suggested a flattened chair conformation for ring A in 4,4-dimethyl-3-keto steroids where the angle between $C_{(2)}$ and $C_{(4)}$ is slightly expanded. LEHN et al. (44,45) have concluded that ring A in

Table 3. Pentacyclic triterpenoid ketones

No.	Compound	ν_1 n-hexane			ν_2 CCl_4			ν_3 $CHCl_3$			Shifts ($cm.^{-1}$)	
		$\nu_{C=0}$	$\Delta\nu_{a/2}$	ε	$\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	$\nu_{C=0}$	$\Delta\nu_{a/2}$	ε^a	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	3-ketones											
35	Lupanone	1713	10	560	1707	13	500	1697	21	420	6	10
36	Taraxerone	1712	9	*	1707	12	530	1698	20	410	5	9
37	Almusanone	1717	6	890	1711	10	700	1702	19	420	6	9
38	Urs-12-en-3-one	-	-	-	1707	13	490	1698	21	400	-	9
39	28-Norolean-18(17)-en-3-one (oleanone)	-	-	*	1708	14	510	1699	21	430	-	9
	11-ketones											
40	3β-Hydroxyursan-11-one	1710	10	*	1706	15	420	(1699)	25 †	340	4	(7)
41	3β-Acetoxyursan-11-one	-	-	-	1706	14	420	-	-	-	-	-
42	3β-Acetoxyoleanan-11-one	-	-	*	1704	15	450	1699 ‡	26 †	440	-	5
43	3β-Acetoxy-19β-hydroxy-18α-oleanan-11-one	(1707)	*	*	1704	16	520	1697	22 †	490	(3)	7
	12-ketones											
44	3β-Hydroxyoleanan-12-one	1705	13	*	1698	15	400	1690	22	310	7	8

Table 3 (continued)

No.	Compound	ν_1 n-hexane			ν_2 CCl_4			ν_3 $CHCl_3$			Shifts ($cm.^{-1}$)	
		$\nu C=0$	$\Delta\nu a/2$	ε^a	$\nu C=0$	$\Delta\nu a/2$	ε^a	$\nu C=0$	$\Delta\nu a/2$	ε^a	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	12-ketones (continued)											
45	3β-Hydroxy-18α-oleanan-12-one	(1711)	*	*	1706	13	540	1700	18	450	(5)	6
46	3ξ-Hydroxyursan-12-one	-	-	-	-	-	-	1680	30	220	-	-
47	3β-Acetoxyoleanan-12-one	1706	13	*	1700	17	410	1692	22†	380	6	8
48	3β-Acetoxyolean-9(11)-en-12-one	-	-	-	-	-	-	1648‡	22	450	-	-
	15-ketones											
49	3β-Hydroxyolean-12-en-15-one	-	-	-	1706	15	430	-	-	-	-	-
50	3β-Acetoxyolean-12-en-15-one	1709	13	*	1705	17	360	(1702)	26†	410	4	(3)
	19-ketones											
51	3β-Acetoxy-18α-olean-9(11)-en-19-one	1711	6	640	1707	9	640	1702	18†	600	4	5

Symbols have the same meaning as those given in the footnotes to Table 2.

Solutions in n-hexane and chloroform were 0.015M ± 0.0005M (0.51 mm. cells) with the following exceptions: in n-hexane compound No. 35 (0.0099M); in $CHCl_3$ Nos. 43 (0.01196M), 47 (0.00935M), 50 (0.00615M) and 51 (0.00659M)

Solutions in carbon tetrachloride were 0.0015M ± 0.00002M (5 mm. cells).

3-keto triterpenoids having an 8-methyl group also exists in a flattened chair form and that ring B is also distorted in this case.

From Table 3 it appears that the dependence of the frequency of a carbonyl group on its position in the nucleus is even less marked than in the steroid system (25). Ketones at positions 3,11,12,15 and 19 in the triterpenoid skeleton absorb between 1698 and 1711 cm.$^{-1}$ in carbon tetrachloride and 1690 and 1702 cm.$^{-1}$ in chloroform, and probably only the 12-ketones could be distinguished from the others as already suggested by COLE and THORNTON (25). By virtue of the large number of angular methyl groups the steric environments of the carbonyl groups at positions 11,12,15 and 19 are similar and this is further reflected in their $\Delta\nu$ (C=0) values which alter only slightly from one position to another. The $\Delta\nu$(C=0) values for the 3-position are largest and comparable to the values found for the corresponding 3-ketosteroids.

Alnusanone, which has an inverted A/B ring junction, shows the highest frequencies of the group. For the epimeric 12-ketones (Nos. 44 and 45) changing the configuration at $C_{(18)}$ from β to α raises the carbonyl frequency by 6-10 cm.$^{-1}$. From molecular models it is apparent that this change is attended by a decrease in carbonyl-$C_{(19)}$-hydrogen distance from ca. 2.3 Å to ca. 1.9 Å (1:4 interaction). A similar situation should arise in the 19-keto

compounds on changing the configuration at $C_{(18)}$ but the effect could not be examined as only the 18α-epimer (No. 51) was available.

Although the data of Tables 2 and 3 are somewhat heterogenous, and are also limited by the insolubility of many of the compounds in n-hexane, some trends can be discerned in half-band widths and extinction coefficients. The most complete data are for carbon tetrachloride. Among the steroids, the 17-ketones ($\Delta\nu_{\frac{1}{2}} \sim 10$ cm. $^{-1}$) show the narrowest, and the 3-ketones ($\Delta\nu_{\frac{1}{2}} \sim 16$ cm. $^{-1}$) the broadest bands. Among the triterpenoids the one 19-ketone examined has $\Delta\nu_{\frac{1}{2}}$ 9 cm. $^{-1}$, markedly less than the values for the 3-ketones (~12 cm. $^{-1}$, also found for 4,4-dimethyl-3-ketosteroids) and for carbonyls at other positions (11-, 12- and 15-, ~15 cm. $^{-1}$). A similar pattern is seen in the more fragmentary data for n-hexane, in which all the half-band widths are 3-5 cm. $^{-1}$ less than in carbon tetrachloride. It is of interest to note that in chloroform all the steroid ketones show values of $\Delta\nu_{\frac{1}{2}}$ in the range 17-21 cm. $^{-1}$ and no structural differentiation is discernible.

The apparent extinction coefficients lie within the following ranges (the anomalous value for No. 46 is neglected): in n-hexane, 450 to 900; in carbon tetrachloride, 340 to 780; in chloroform, 310 to 610. The data thus support the rule, referred to by JONES

and SANDORFY (53), that a value of $\mathcal{E}^a$ exceeding 300 units may be regarded as indicative of a carbonyl function as distinct from an ethylenic or aromatic group. No correlation of $\mathcal{E}^a$ values with the structures of the ketones is evident from the data.

Steroid and Triterpenoid Acetates

Table 4 gives the 3-acetate carbonyl absorption data for nine steroids and seven triterpenoids of Tables 2 and 3 as well as nine examples of simple acetates. The values for all the 3β-acetates range from 1732-1738 cm.$^{-1}$ in carbon tetrachloride and from 1718-1726 cm.$^{-1}$ in chloroform, in agreement with the date of COLE and THORNTON (25) for a group of eleven triterpenoid 3β-acetates. This constancy of 3-acetate frequency would suggest that the presence of a ketonic carbonyl, with the exception of 6- and 7-keto groups discussed below, has little effect on the position of the acetate band. JONES et al. (21) studying the C-O stretching vibrations of 3-acetoxy steroids in the region 1200 to 1250 cm.$^{-1}$ have noted a similar constancy of the characteristic spectrum in the presence of a ketonic carbonyl, with the possible exception of a 6-ketone group. Similarly introduction of a gem-dimethyl group at $C_{(4)}$ has no apparent effect on the frequency or symmetry of a 3β-acetoxyl group in contrast to the lower frequencies observed for the 3-ketones.

Table 4. **Steroid and triterpenoid acetates**

No.	Compound	ν_1 n-hexane			ν_2 CCl_4			ν_3 $CHCl_3$			Shifts (cm.$^{-1}$)	
		ν_{OAc}	$\Delta\nu_{a/2}$	ε^a	ν_{OAc}	$\Delta\nu_{a/2}$	ε^a	ν_{OAc}	$\Delta\nu_{a/2}$	ε^a	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	Simple Acetates											
52	3β-Acetoxycholestane	1744	14	590	1733‡	14	590	1724‡	25	440	11	9
53	Tigogenin acetate	1745	11	750	1734‡	17	610	1724‡	25	460	11	10
54	3β-Acetoxylanost-9(11)-ene	1744	9	810	1736‡	17	560	1724‡	25	470	8	12
55	3β-Acetoxylupane	1745	9	820	1738‡ (1740 sh.)	19[c]	550	1723‡	25	460	(9)[b]	(13)[b]
56	Taraxeryl acetate	(1745)	*	*	1733	14	630	1723‡	26	470	(12)	10
57	3β-Acetoxyurs-12-ene (α-amyrin acetate)	1743	12	600	1735	15	550	1720	26	470	8	15
58	11α-Hydroxytigogenin 3-acetate	-	-	-	1733	18	770	1722	25	680	-	11
59	11α-Hydroxytigogenin diacetate	-	-	-	1733	18	1170	1722	25	960	-	11
60	11β-Hydroxytigogenin 3-acetate	-	-	-	1733	16	580	1722	20	570	-	11
	Keto steroids											
	6-Ketones											
17	3β-Acetoxy-5α-cholestan-6-one	1742	10	740	1735	14	730	1726	19†	530	7	9
	7-Ketones											
21	3β-Acetoxy-5α-cholestan-7-one	1745	9	860	1737	16	720	1725‡	25†	520	8	12

Table 4 (continued)

No.	Compound	ν_1 n-hexane			ν_2 CCl_4			ν_3 $CHCl_3$			Shifts (cm.$^{-1}$)	
		ν_{OAc}	$\Delta\nu_{a/2}$	ε^a	ν_{OAc}	$\Delta\nu_{a/2}$	ε^a	ν_{OAc}	$\Delta\nu_{a/2}$	ε^a	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	11-Ketones											
24	11-Oxotigogenin acetate	1743	15	*	1733	19	380	(1721)	-	-	10	(12)
25	3β-Acetoxyergost-22-en-11-one	1742 ‡	16	660	1733	16	660	1723 ‡	24 †	490	9	10
26	3β-Acetoxy-5α-hydroxyergostan-11-one	1741 ‡	15	400	1733	17	460	1723 ‡	24 †	500	8	10
27	3β-Acetoxy-5α-hydroxy-9β-ergostan-11-one	1746	15	580	1736 ‡	17	650	(1725)	29	450	10	11
28	3β-Acetoxy-5α-hydroxy-9β-ergost-7,22-dien-11-one	-	-	-	1736 ‡	21	520	1726 ‡	21 †	530	-	10
29	3β-Acetoxylanostan-11-one	1742 ‡	15	520	1732 ‡	13	680	(1720)	26 †	460	10	(12)
	12-Ketones											
32	Hecogenin acetate	1746	10	*	1736 ‡	20	640	(1722)	26 †	540	10	(14)
	Ketotriterpenoids											
	11-Ketones											
41	3β-Acetoxyursan-11-one	1745	7	*	1738 (1729 sh.)	23[c]	440	1720 ‡	24 †	380	(11)[b]	(14)[b]
42	3β-Acetoxyoleanan-11-one	-	-	-	1737 (1729 sh.)	23[c]	480	1720 ‡	25 †	480	-	(13)[b]
43	3β-Acetoxy-19β-hydroxy-18α-oleanan-11-one	1744	*	*	1736 ‡	20	580	1720	26 †	510	8	16

Table 4 (continued)

No.	Compound	ν_1 n-hexane			ν_2 CCl_4			ν_3 $CHCl_3$			Shifts ($cm.^{-1}$)	
		νOAc	$\Delta\nu$ a/2	ε^a	νOAc	$\Delta\nu$ a/2	ε^a	νOAc	$\Delta\nu$ a/2	ε^a	$\Delta\nu_{1-2}$	$\Delta\nu_{2-3}$
	12-Ketones											
47	3β-Acetoxyoleanan-12-one	1746	9	*	1732 (1740 sh.)	21[c]	540	1722	29	520	(10)[b]	(14)[b]
48	3β-Acetoxyolean-9(11)-en-12-one	-	-	-	-	-	-	1725	29	450	-	-
	15-Ketones											
50	3β-Acetoxyolean-12-en-15-one	1745	9	*	1732	19	550	(1720)	26 †	550	13	(12)
	19-Ketones											
51	3β-Acetoxy-18α-olean-9(11)-en-19-one	1743	10	660	1735	14	650	(1718)	32 †	480	8	(18)

Symbols are the same as those given in Table 2.

b The mean value of the frequencies is taken in cases of split bands for calculating frequency shifts.

c Composite band

Solutions in n-hexane and chloroform were 0.015M ± 0.0005M (0.51 mm. cells) with the following exceptions: in n-hexane compound Nos. 26 and 27 (0.0060M); in $CHCl_3$ Nos. 43 (0.01196M), 47 (0.00935M), 50 (0.00615M) 51 (0.00659M) and 59 (0.00707M).

Solutions in carbon tetrachloride were 0.0015M ± 0.00002M (5 mm. cells) with the following exceptions: compound Nos. 26 (0.00212M) and 59 (0.00076M).

Examination of the half-band widths and apparent extinction coefficients shows that in general these are higher for the acetate carbonyl than for the ketone carbonyl bands. The mean apparent extinction coefficients for the ketone carbonyl and acetate carbonyl bands are 640 and 670 in n-hexane; 520 and 590 in carbon tetrachloride and 430 and 500 in chloroform. This agrees with the results of JONES et al. (35) who showed that the integrated intensities of steroid acetate carbonyls were larger than those of analogous ketones. No clear correlations of structure with half-band width or intensity are detectable. In chloroform solution the simple acetates show half-band widths close to 25 $cm.^{-1}$.

In spite of the relative independence of the 3-acetate carbonyl group frequency, splitting of the acetate band and variations in symmetry have been observed in some instances. Examples of compounds giving rise to complex carbonyl absorptions have often been quoted (46-51) and the effects have been ascribed (in the absence of intramolecular hydrogen bonding) to one of the following causes: (i) the overlap of independent absorption bands due to more than one stable conformation, (ii) Fermi resonance or (iii) solute-solvent association. In the present study association with the solvent is considered unlikely, as splitting of the acetate band has only been observed for a few compounds and only in carbon tetrachloride

solutions. (In chloroform solutions such features of symmetry may be obscured because of the width of the absorption bands.). Explanations on the basis of conformational isomers or Fermi resonance therefore remain to be considered.

Fig. 3 shows the carbonyl absorption bands of representative acetoxy-ketones illustrating the variations in the shape of the acetate band. Fig. 3A refers to 3β-acetoxylanostan-11-one, which shows simple ketone and acetate bands separated by 25-37 cm.$^{-1}$. In 3β-acetoxyoleanan-11- and 12-one (Figs. 3B and 3C) the acetate carbonyl band in carbon tetrachloride shows a definite shoulder on the low and high frequency sides respectively. Both equilibria between conformational isomers and Fermi resonance can give rise to modifications of the carbonyl stretching bands with changes in solvent and temperature (49)*. In the case of the 3β-acetoxy-oleananones (Fig. 3B and 3C) rings A and E are the flexible parts of the molecule. Ring A, as already mentioned, probably exists in a flattened chair conformation (44,45) and from models the chair conformation for ring E seems the least sterically hindered. In order that two conformational isomers may coexist in solution the energy difference between them must be small to permit the existence of an appreciable proportion of the less stable isomer

* The two effects can sometimes be distinguished by selective deuteration which may alter the proportion of conformational

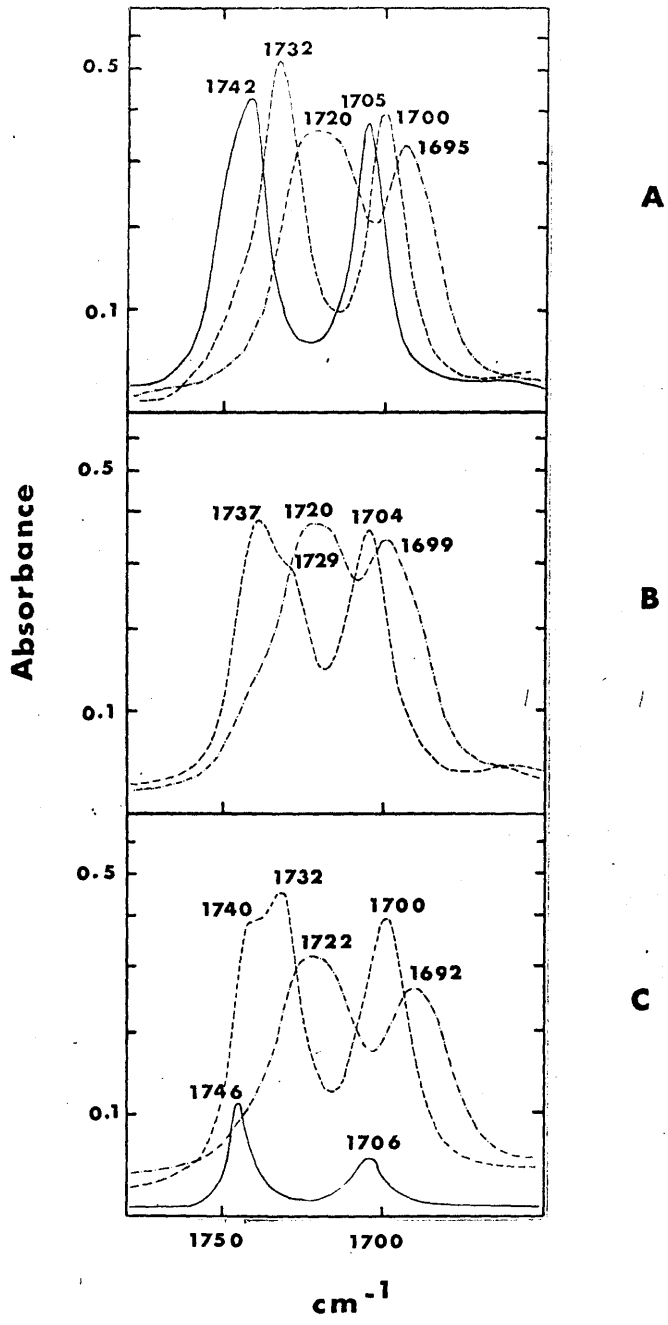


Fig. 3. Carbonyl absorptions: (A) 3β-acetoxylanostan-11-one; (B) 3β-acetoxyoleanan-11-one; (C) 3β-acetoxyoleanan-12-one. Measurements on ca. 0.015M solutions in n-hexane (——) (except C, saturated solution) and chloroform (-·-·-) in 0.51 mm. cells, and ca. 0.0015M in carbon tetrachloride (- - - -) in 5 mm. cells.

at room temperature. Furthermore, the environment of the carbonyl group in the two isomers must be sufficiently different to give rise to two distinct carbonyl absorptions. Since the near environment of the 3-acetoxyl group is essentially the same and variations mostly occur in the more distant rings C, D and E, an explanation based on an equilibrium of conformational isomers seems improbable. Besides, the occurrence of the split acetate band in only four out of twenty steroid and triterpenoid acetates, would suggest a coincidental overlap of the fundamental carbonyl band with an overtone or combination band (46). It is to be noted that the four compounds showing the split acetate band belong to the triterpenoid series although this may be fortuitous.

Fig. 4 compares the carbonyl regions of some 3β-acetoxy ketones and corresponding acetates and ketones. Figs. 4A and 4D depict tigogenin derivatives in carbon tetrachloride and chloroform solutions. The 11-carbonyl absorption is strikingly unaffected by the presence of the 3β-acetate group although the intensity of the latter peak is much reduced in the acetoxy ketone. Overlap of the ketone and acetate bands occurs in chloroform solution because of the small frequency separation and substantial broadening of the carbonyl bands in this solvent. 3β-Acetoxyursan-11-one (Fig. 4B) is an example of complex acetate carbonyl absorption, which has

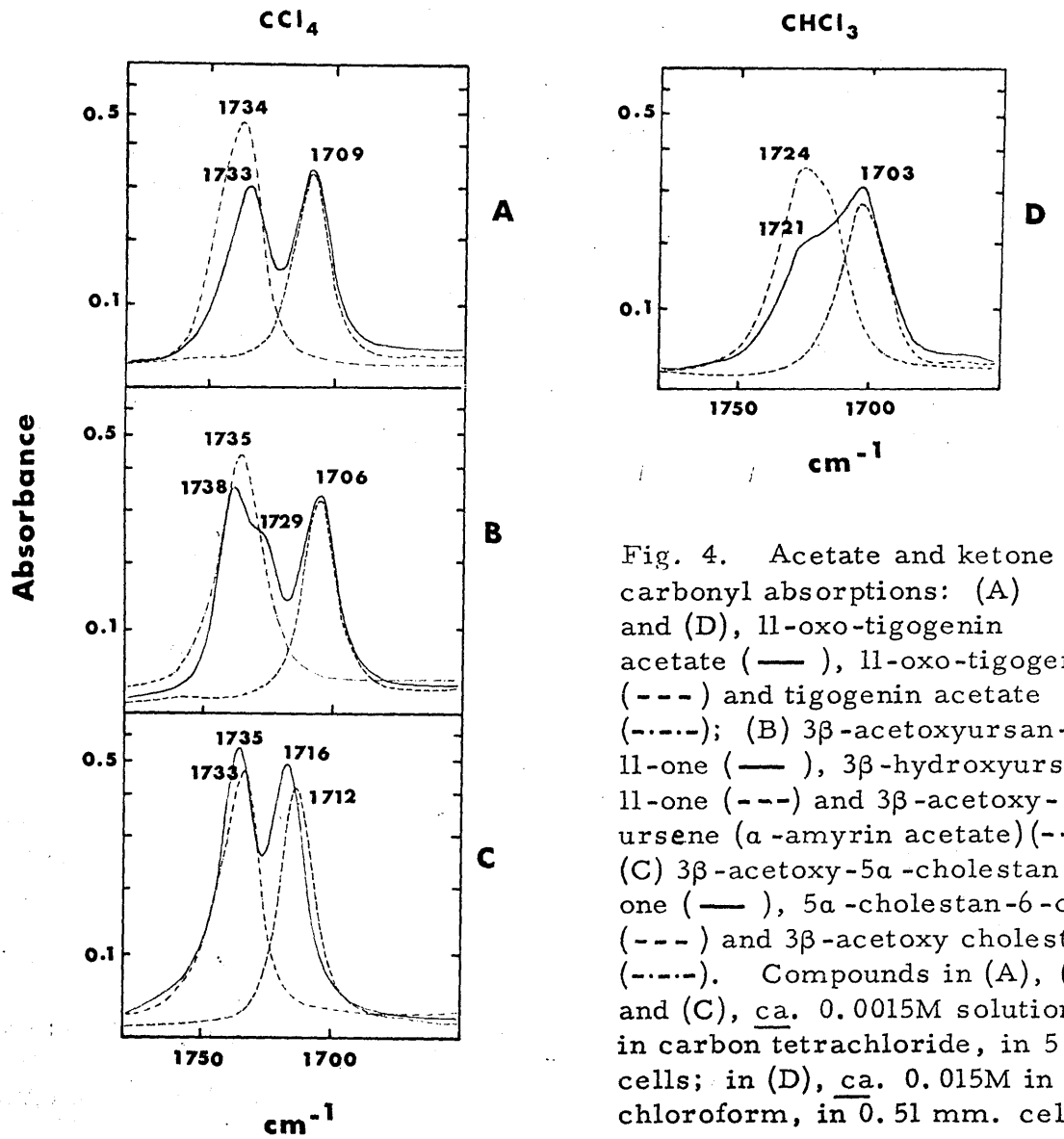


Fig. 4. Acetate and ketone carbonyl absorptions: (A) and (D), 11-oxo-tigogenin acetate (——), 11-oxo-tigogenin (- - -) and tigogenin acetate (-·-·-); (B) 3β-acetoxyursan-11-one (——), 3β-hydroxyursan-11-one (- - -) and 3β-acetoxy-ursene (α-amyrin acetate) (-·-·); (C) 3β-acetoxy-5α-cholestan-6-one (——), 5α-cholestan-6-one (- - -) and 3β-acetoxy cholestane (-·-·-). Compounds in (A), (B) and (C), ca. 0.0015M solutions in carbon tetrachloride, in 5 mm. cells; in (D), ca. 0.015M in chloroform, in 0.51 mm. cells.

also been observed in the oleanane series (Figs. 3β and 3C). Fig. 4B shows that the 11-keto band is the same in the acetoxy ketone as in 3β-hydroxyursan-11-one although again the acetate band in the acetoxy ketone is of reduced intensity. In 3β-acetoxy-5α-cholestan-6-one, where the two substituents are in closer proximity, the frequencies of both carbonyls are slightly raised in comparison with those observed for the individual groups. A similar effect is noted for 3β-acetoxy-5α-cholestan-7-one (No. 21, Tables 2 and 4).

In conclusion it may be said that where "vicinal effects" are not operating, frequencies and frequency shifts roughly parallel the steric environment of the carbonyl group. In the simple alkyl cyclohexanones, the carbonyl frequency decreases with increasing substitution in the α-positions as already found by MARONI (18) for aliphatic ketones in the liquid state. Among the more complex ketones of the steroid and triterpenoid series, the trends observed in absorption data can only be broadly correlated with the situations of the carbonyl groups. The variations in angular methyl groups in the triterpenoid series introduce additional stereochemical features so that the results are not readily interpreted in terms of a single factor. Moreover, the complex acetate carbonyl bands observed in four triterpenoid acetoxy-ketones are a further complication, probably due to

vibrational interactions. Further examples and more detailed studies of acetoxy-ketones having carbonyl groups in rings A and B would be necessary to assess the magnitude of "vicinal effects" on the frequencies and intensities of carbonyl bands.

Section 2

Hydrogen-bond association of alkyl cyclohexanones with ortho-substituted phenols

Solute-solvent association can be readily observed in hydrogen bonding systems where both free and associated species give rise to distinct absorption bands. In this work substituted cyclohexanones have been studied in phenol-n-hexane solvent systems where the concentration and the alkyl substitution of the phenol have been varied. The extent to which the carbonyl and hydroxyl stretching absorptions are perturbed by the formation of the hydrogen bond has been investigated. Previous studies of the interaction between ketones and phenols in dilute solutions have been made, notably by WIDOM, PHILIPPE and HOBBS (27), WHETSEL and KAGARISE (15), GRAMSTAD (28) and LASCOMBE (29). In our studies emphasis has been placed on the steric environments of the hydroxyl and carbonyl groups and the effects of alkyl substitution on the strength and degree of hydrogen-bond association.

Materials and Methods

Cyclohexanones

Compounds 1-6 were obtained from commercial sources and purified by distillation under reduced pressure (0.1-1 mm.). 4-t-Butylcyclohexanone was prepared by oxidation of 4-t-butylcyclohexanol (L. Light & Co., Ltd.) using chromium trioxide in a two-phase system (benzene

and aqueous acetic acid): the product was sublimed at reduced pressure, m.p. 46-48° (lit. 49-50° (54)).

The purity of the ketones was checked by gas-liquid chromatography using a column of 7% F-60 (Dow-Corning Corp.) - 1% EGPS-Z (Applied Science Laboratories, Inc.) supported on Gas-Chrom P at 75° with the argon flow rate 52 ml./min. The following retention times were observed: cyclohexanone 4.25 min.; 2-methylcyclohexanone 6.0 min.; 2,6-dimethylcyclohexanone 8.6 min.; 2,2,6-trimethylcyclohexanone, 11.0 min.; 2,2,6,6-tetramethylcyclohexanone, 13.1 min. No significant impurities were observed except in 2,6-dimethylcyclohexanone which contained 2-methylcyclohexanone (≯5%), and in 2,2,6-trimethylcyclohexanone which contained 2,2,6,6-tetramethylcyclohexanone (≯7%).

Phenols

p-Cresol, 2,6-dimethylphenol and 2,6-di-t-butyl-4-methylphenol were purified by sublimation under reduced pressure.

Solvents

n-Hexane ('AnalaR') was used without purification. Mixed solvents comprising various concentrations of a particular phenol in n-hexane were prepared by weighing the required amount of phenol into a volumetric flask and making up to the appropriate volume with n-hexane.

Measurements

General details are given in Section 1. All measurements were made in 0.51 mm. cells with n-hexane in the reference cells.

Results and Discussion

Association of cyclohexanone and alkyl-substituted cyclohexanones with p-cresol

Data for cyclohexanones in 0.03M p-cresol-n-hexane solution are given in Table 1, and typical carbonyl and hydroxyl absorptions in Fig. 1. By keeping the p-cresol at constant molarity while varying the substituents in cyclohexanone the effects of alkyl substitution on hydrogen bonding could be compared. The steroid 5α-cholestan-3-one is included for comparison.

In the carbonyl region, bands due to free and associated carbonyl groups were observed in each case. The free carbonyl frequencies were virtually identical with those observed in the absence of p-cresol (cf. Section 1, Tables 1 and 2): thus the "associated" bands clearly result from specific interactions. As already shown for single solvents, introduction of α-methyl groups into cyclohexanone results in some lowering of the carbonyl frequency. Similar behaviour is noted (Table 1 Fig. 1) for both free and associated carbonyl frequencies of 2,6-dimethyl- and 2,2,6,6-tetramethyl-cyclohexanone in p-cresol-n-hexane. Introduction of 4-alkyl

Table 1. Carbonyl stretching frequencies of alkyl-substituted cyclohexanones[a] in 0.03M p-cresol-n-hexane solution

Compound	Free			Associated				
	ν	$\Delta\nu a/2$	ε^{a*}	ν	$\Delta\nu a/2$	ε^{a*}	$\Delta\nu$	$\varepsilon f/\varepsilon a$	% free carbonyl
Cyclohexanone	1725	11 †	330	1708	14 †	250	17	1.32	75
2,6-dimethylcyclohexanone	1721 ‡	11	400	1707	16 †	210	14	1.91	76
2,2,6,6-tetramethylcyclohexanone	1703 ‡ 1710 sh	10	370	1690	14 †	150	13	2.47	88
4-methylcyclohexanone	1726	8	480	1714	20 †	240	12	2.00	71
4-t-butylcyclohexanone	1727	8	490	1714	18 †	260	13	1.89	69
5α-cholestan-3-one	1724	11 †	390	1709	11 †	250	15	1.56	61

a Ketones were 0.0149 ± 0.00006M examined in 0.51 mm. cells.

* Apparent extinction coefficients are approximated to the nearest 10 units.

$\varepsilon f/\varepsilon a$ - Ratio of extinction coefficients of free (εf) to associated (εa) carbonyl bands

$$\% \text{ free carbonyl} = \frac{\varepsilon^a \text{ in p-cresol-n-hexane X100}}{\varepsilon^a \text{ in n-hexane}}$$

‡ Asymmetric peak

† Estimated by band reflection

sh Shoulder

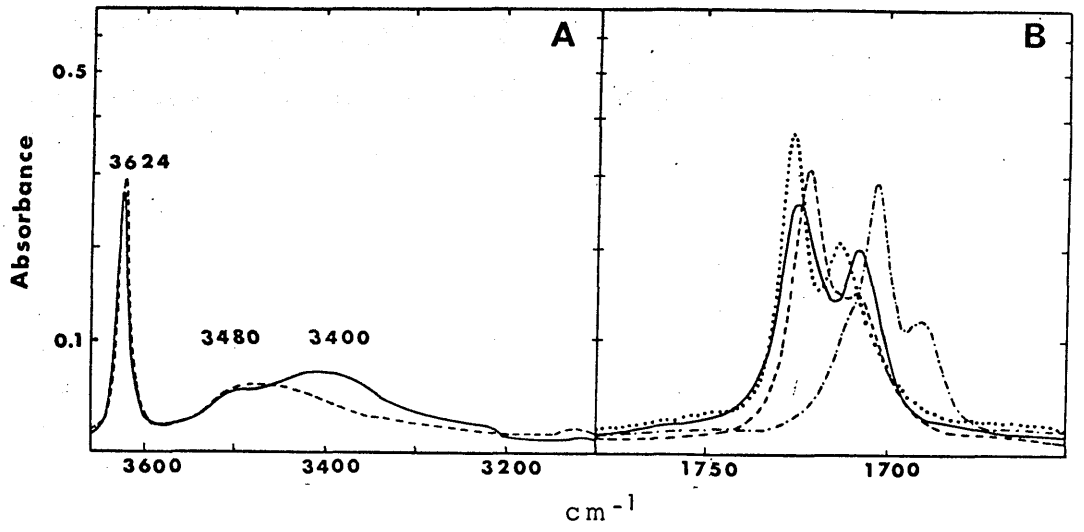


Fig. 1. Hydroxyl and carbonyl absorption of solutions of various ketones (0.015M) with p-cresol (0.03M) in n-hexane (0.51 mm. cells).

A. Hydroxyl absorption: combined spectra of solutions containing cyclohexanone, 4-methyl- and 4-t-butylcyclohexanone (——); 2,6-dimethyl- and 2,2,6,6-tetramethylcyclohexanone (- - -).

B. Carbonyl absorption: cyclohexanone (——); 2,6-dimethylcyclohexanone (- - -); 2,2,6,6-tetramethylcyclohexanone (- · - · -); 4-t-butylcyclohexanone (·····).

substituents hardly alters the free carbonyl frequency (though giving sharper bands, as in single solvents) but raises the associated carbonyl frequency. The free and associated carbonyl frequencies of 5α-cholestan-3-one are very similar to those observed for cyclohexanone. Thus within the whole group of compounds, no marked variations are observed in the frequency separations between free and associated carbonyl bands, and it would appear that the alkyl substitution does not greatly affect the strength of the association.

Some effect is noted on the equilibrium between free and hydrogen-bonded species: for equimolar solutions of alkyl-substituted cyclohexanones the ratios of absorbances of "free" and "associated" bands suggest that cyclohexanone (1.32) shows the greatest degree of association and 2,2,6,6-tetramethylcyclo-hexanone (2.47) the least. The more reliable data of the percentages of free carbonyl species indicate that whereas 2,2,6,6-tetramethyl-cyclohexanone indeed shows the least degree of association, the ketones substituted in the 4 or 3 and 4 (5α-cholestan-3-one) positions show greater association than cyclohexanone (cf. Table 5). The results of BELLAMY EGLINTON and MORMAN (30) for the association of phenols with ethers showed that, except in extremely hindered examples, variations in alkyl groups on the donor or acceptor molecules did not prevent formation of hydrogen bonds of

normal strength but affected the likelihood of their formation.

In the hydroxyl region, cyclohexanone and its 4-methyl and 4-t-butyl derivatives (Fig. 1A, continuous line) each form a hydrogen bonded complex with p-cresol, giving rise to a broad band at about 3400 cm.$^{-1}$. The more hindered dimethyl and tetramethyl derivatives (Fig. 1A, broken line) give a weaker band with a peak at about 3480 cm.$^{-1}$. This higher frequency suggests a weaker hydrogen bond in these α-alkyl cyclohexanones: the result is somewhat unexpected for the dimethyl cyclohexanone, for which the carbonyl data do not indicate marked steric hindrance to association.

At p-cresol and ketone concentrations of 0.03M and 0.015M respectively the percentages of bonded ketone range from 24 to 39%, no striking steric effects being observed except in the case of 2,2,6,6-tetramethylcyclohexanone (12% bonded). At this concentration, self-association of p-cresol in the presence of ketone is small (see Table 5). A broad shoulder consistently observed near 3500 cm.$^{-1}$ has been noted by others (30,31) and attributed to a water-solvent complex of the type R-O-H ....OH_2. Fig. 2C shows this shoulder as a definite peak at a p-cresol concentration of 0.12M and since it is present to a marked degree in

the solvent mixture it must arise from association of molecules other than the ketone.

Table 2 gives the relevant data for free and associated carbonyl bands of cyclohexanone in progressively higher concentrations of p-cresol in n-hexane. The frequencies of both "free" and "associated" bands show only very small downward shifts as the p-cresol concentration increases from 0.03M to 0.12M. Over this range, the band separation ($\Delta\upsilon$, 17 cm. $^{-1}$) and half-band widths remain almost unchanged. The relative intensities of the free and associated carbonyl bands are illustrated in Fig. 2B, while Fig. 2D shows the corresponding band of cyclohexanone in n-hexane.

For 0.030M cyclohexanone in p-cresol concentrations of 0.03M, 0.045M and 0.12M the percentages of free carbonyl present are approximately 75, 66 and 46% respectively. These values are of the same order as those reported by WHETSEL and KAGARISE (15) for 0.039M cyclohexanone in corresponding p-cresol-cyclohexane mixtures.

Figs. 2A and 2C show the hydroxyl absorptions in the presence and absence of cyclohexanone. In the former, the broad absorption at about 3400 cm. $^{-1}$ is due to ketone-phenol association, while increased self-association of p-cresol at the highest concentration

Table 2. Carbonyl stretching frequencies of cyclohexanone[a] in solutions of p-cresol in n-hexane

p-cresol	Free			Associated				
in n-hexane	ν	$\Delta\nu$ a/2	ε^a	ν	$\Delta\nu$ a/2	ε^a	$\Delta\nu$	$\varepsilon_f/\varepsilon_a$	% free carbonyl
-	1725	12	440	-	-	-	-	-	100
0.0301M	1726	14	330	1709	13 †	200	17	1.65	75
0.0448M	1724	12 †	290	1707	14 †	250	17	1.21	66
0.119M	1724	12 †	200	1707	16	330	17	0.61	46

a Cyclohexanone was 0.0295 ± 0.0005M in p-cresol-n-hexane solutions examined in 0.51 mm. cells.

- No absorption.

For other details see footnotes to Table 1.

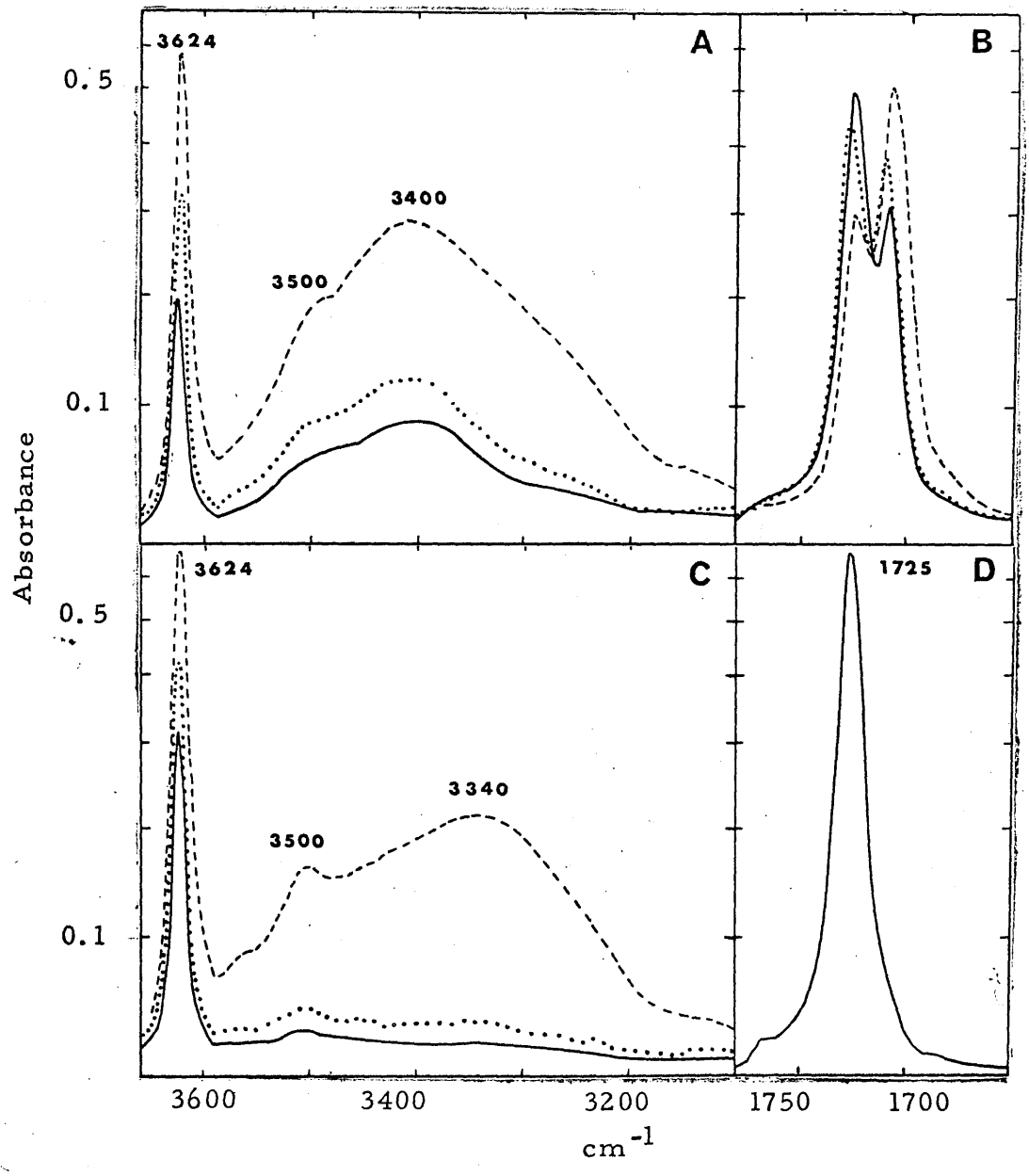


Fig. 2. A and B: Hydroxyl and carbonyl absorptions of mixtures of cyclohexanone (0.03M) with p-cresol, 0.03M (——), 0.05M (·····) and 0.12M (- - - -) in n-hexane (0.51 mm. cells).

C and D: Absorptions of corresponding solutions of p-cresol in n-hexane and cyclohexanone in n-hexane respectively.

is evident from the broadening of the band on the low frequency side, near 3340 cm.$^{-1}$.

The free hydroxyl band occurs at 3624 cm.$^{-1}$ and the fall in intensity of this band in the presence of ketone is a measure of the p-cresol involved in complex formation. Table 5 (see below) gives approximate data for the distribution of free, carbonyl-bonded and self-associated p-cresol in these solutions.

Association of cyclohexanone with ortho-substituted phenols

Table 3 and Fig. 3 give the relevant data for the association of 0.015M solutions of cyclohexanone with a range of ortho-substituted phenols in n-hexane. The frequencies attributable to the unbonded carbonyl group are almost unchanged up to phenol concentrations of 0.5M. With an approximate 2:1 ratio of p-cresol to cyclohexanone two well-separated carbonyl bands are observed corresponding to free and associated cyclohexanone. The small value for the ratio of absorbances shows that the free carbonyl band is only slightly more intense than the associated band (Fig. 3B). With 2:6-dimethylphenol (about 3:1 mole ratio) there is some reduction in phenol-ketone association as a result of introducing two ortho-methyl groups. The band separation, the ratio of absorbances and particularly the shapes of the carbonyl bands are quite close to those of 2,6-dimethylcyclohexanone in p-cresol-n-hexane (Table 1 &

Table 3. Carbonyl stretching frequencies of cyclohexanone[a] in solutions of phenols in n-hexane

Phenol in n-hexane	Free			Associated					
	ν	$\Delta\nu_{a/2}$	ε^a	ν	$\Delta\nu_{a/2}$	ε^a	$\Delta\nu$	$\varepsilon_f/\varepsilon_a$	% free carbonyl
-	1725	12	440	-	-	-	-	-	100
0.0301M p-cresol in n-hexane	1725	11†	330	1708	14†	250	17	1.32	75
0.0503M 2,6-dimethylphenol in n-hexane	1726‡	15‡ (10†)	370	1714	16†	(200)	12	1.85	84
0.1504M 2,6-dimethylphenol in n-hexane	1725	11†	280	1711	14†	280	14	1.00	64
0.501M 2,6-di-t-butyl-4-methyl phenol in n-hexane	1725‡	17‡ (11†)	340	-	-	-	<5	-	77
2.0M 2,6-di-t-butyl-4-methyl phenol in n-hexane	1720‡	21‡ (17†)	300	-	-	-	<5	-	68

a Cyclohexanone was 0.0152 ± 0.0002M in phenol-n-hexane solution examined in 0.51 mm. cells

Values in parenthesis are approximate

Symbols are as given in the footnotes to Tables 1 and 2.

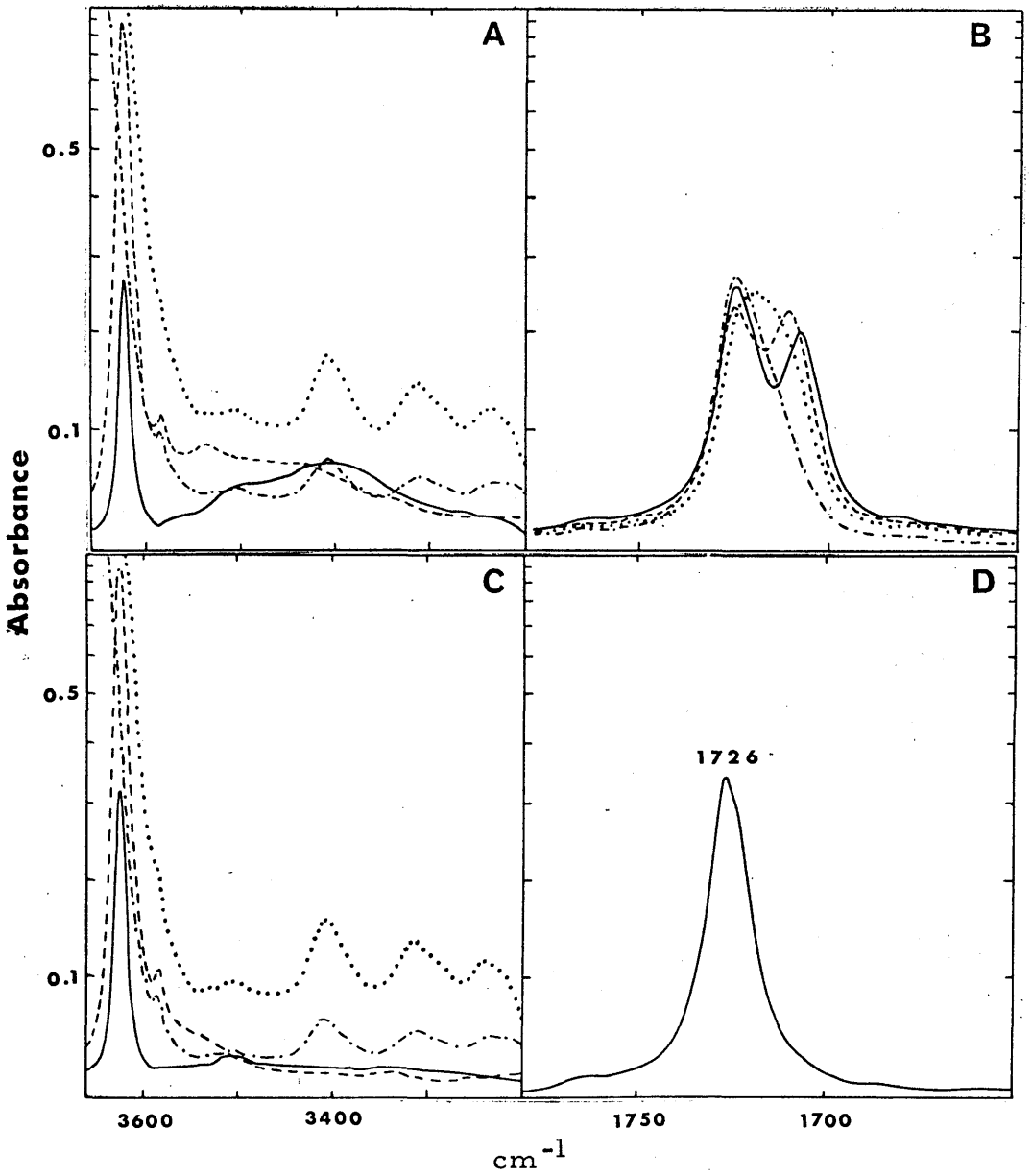


Fig. 3. A and B: Hydroxyl and carbonyl absorptions of mixtures of cyclohexanone (0.015M) with various phenols in n-hexane (0.51 mm. cells): p-cresol (—— , 0.03M); 2,6-dimethylphenol (– – –, 0.15M); 2,6-di-t-butyl-4-methylphenol (–·–·, 0.50M; ····· 2.0M).

C: Hydroxyl absorptions of the corresponding phenols in n-hexane.

D: Cyclohexanone (0.015M) in n-hexane.

Fig. 1). The lower ratio (about 2:1) of p-cresol to 2,6-dimethylcyclohexanone necessary to give analogous results suggests that α-methyl substitution in cyclohexanone is more effective than ortho-methyl substitution in phenol in hindering hydrogen-bond formation. This is not unexpected in view of the rigid nature of the carbonyl group and its short bond length. Raising the concentration of 2,6-dimethylphenol to 0.15M (i.e. a 10:1 ratio of phenol to ketone) produces two well resolved bands of approximately equal intensity.

When two t-butyl groups are present in ortho-positions not even a 2M-concentration of 2,6-di-t-butyl-4-methylphenol produces a resolved carbonyl band. The only effects of increasing the molarity of this phenol from 0.5M to 2M are a small shift in frequency to lower values, an increase in half-band width and a small fall in intensity. These changes, in addition to development of marked asymmetry, are indicative of the presence of unresolved bands.

The results of Table 3 show the interplay of two opposing factors - higher concentrations of phenol leading to an increased occurrence of hydrogen bonding and steric congestion affecting both the strength and degree of hydrogen bonding. Whereas for

2,6-dimethylphenol a 10:1 ratio of phenol to ketone gives two distinct bands, for 2,6-di-t-butyl-4-methylphenol even a 133:1 ratio of phenol to ketone still gives a broad, unresolved carbonyl band. These results can be understood from an examination of Dreiding models. For phenolic hydroxyls to form moderately strong hydrogen bonds certain stereochemical requirements are necessary: approximate coplanarity of the C-O-H bond with the benzene ring (30,55) and an O ....O distance of approximately 2.7 Å with the hydrogen atom almost collinear with the two oxygen atoms (55).

Cyclohexanone would encounter repulsion from the methyl groups of 2,6-dimethylphenol in conforming to the above requirements since from models the hydrogen atom at C-2 of cyclohexanone and one of the hydrogens of the ortho-methyl groups of the phenol appear to be only ca. 1.6 Å apart. Hence the necessity for a high concentration of phenol before distinct carbonyl absorptions due to free and associated species are observed. In addition to steric effects the slight decrease in the acidity of the phenolic hydroxyl group on ortho-alkyl substitution (30,31) may be a cause of reduced hydrogen bonding (pKa's for aqueous solutions:- p-cresol, 10.2; 2,6-dimethylphenol 10.6). (56)

It has already been shown that 2,6-di-t-butylphenol is subject to

considerable steric hindrance to solvent association (30, 57). (A para-methyl group reduces the acidity of the phenolic hydroxyl group even further (32, 58, 59)). Only a considerable displacement of the hydroxyl group out of the plane of the ring would enable a cyclohexanone molecule to approach with reduced steric interference from the t-butyl groups. However, there is good evidence that the hydroxyl group in phenols possessing bulky ortho-substituents lies in the plane of the phenolic ring (7, 34, 60, 61), probably with a slight opening of the C-O-H angle (30). This conformation for 2,6-di-t-butyl-4-methylphenol is supported by the high intensity of the free hydroxyl band (Figs. 3A and 3C). Accordingly, the very broad carbonyl band of cyclohexanone in the 2M solution of 2,6-di-t-butyl-4-methylphenol could be interpreted as being due to the superposition of bands due to free and very weakly associated carbonyls.

In Table 3 the $\Delta\nu$ values for cyclohexanone in p-cresol, 2,6-dimethylphenol and 2,6-di-t-butyl-4-methylphenol are 17, 13 and < 5 respectively: thus the bond strength is considerably reduced only when very strong steric hindrance to association occurs.

Examination of the hydroxyl regions (Fig. 3A and 3C) shows some complex features. For the unhindered p-cresol the broad band at 3400 cm.$^{-1}$ is due to hydrogen bonding with cyclohexanone, and the shoulder at 3500 cm.$^{-1}$ has been ascribed to phenol-water association.

For 0.15M 2,6-dimethylphenol in n-hexane (Fig. 3C) the distinctive features of the hydroxyl region are weak absorptions at 3340 cm.$^{-1}$, 3540 cm.$^{-1}$ and 3585 cm.$^{-1}$ and an intense free hydroxyl band at 3624 cm.$^{-1}$. The absorption at 3340 cm.$^{-1}$ has been attributed to polymeric self-association (cf. Fig. 2C), which is here much reduced by the presence of two ortho-methyl groups. The broad absorption at 3540 cm.$^{-1}$ may be due to dimeric association, which tends to be favoured in ortho-substituted phenols (62). In the presence of cyclohexanone (Fig. 3A) 2,6-dimethylphenol exhibits a broad band at a higher frequency than that observed with p-cresol. The band centre (~3480 cm.$^{-1}$) is close to that found for mixtures of 2,6-dimethyl or 2,2,6,6-tetramethylcyclohexanone with p-cresol (Fig. 1A). This absorption may be attributed to a weaker association, since the smaller frequency shift from the free hydroxyl band at 3624 cm.$^{-1}$ is an indication of weaker hydrogen bonding. In the case of 2,6-di-t-butyl-4-methylphenol, the hydroxyl absorption remains almost unaltered in the presence of cyclohexanone, in conformity with the very weak association indicated in the carbonyl region (Fig. 3B).

Association of 2,2,6,6-tetramethylcyclohexanone with substituted phenols

Finally, a study was made of the association of ortho-substituted phenols with 2,2,6,6-tetramethylcyclohexanone (Table 4, Fig. 4). In n-hexane, this ketone shows a characteristic sharp band with a

Table 4. Carbonyl stretching frequencies of 2,2,6,6-tetramethylcyclohexanone[a] in solutions of phenols in n-hexane

Phenol in n-hexane	Free			Associated					
	ν	$\Delta\nu_{a/2}$	ε^a	ν	$\Delta\nu_{a/2}$	ε^a	$\Delta\nu$	$\varepsilon_f/\varepsilon_a$	% free carbonyl
-	1703 1710 sh.	9	420	-	-	-	-	-	100
0.0301M p-cresol in n-hexane	1703 ‡ 1710 sh.	10	370	1690	14 †	150	13	2.47	88
0.1504M 2,6-dimethylphenol in n-hexane	1702 1710 sh.	9 †	350	1694	14 †	190	8	1.84	84
0.501M 2,6-di-t-butyl-4-methyl phenol in n-hexane[b]	1702 ‡ 1710 sh.	11	380	-	-	-	-	-	91

a 2,2,6,6-tetramethylcyclohexanone is 0.0150 ± 0.0001M examined in 0.51 mm. cells

b 2,2,6,6-tetramethylcyclohexanone is 0.0141M

Symbols are as given in the footnotes to Tables 1 and 2.

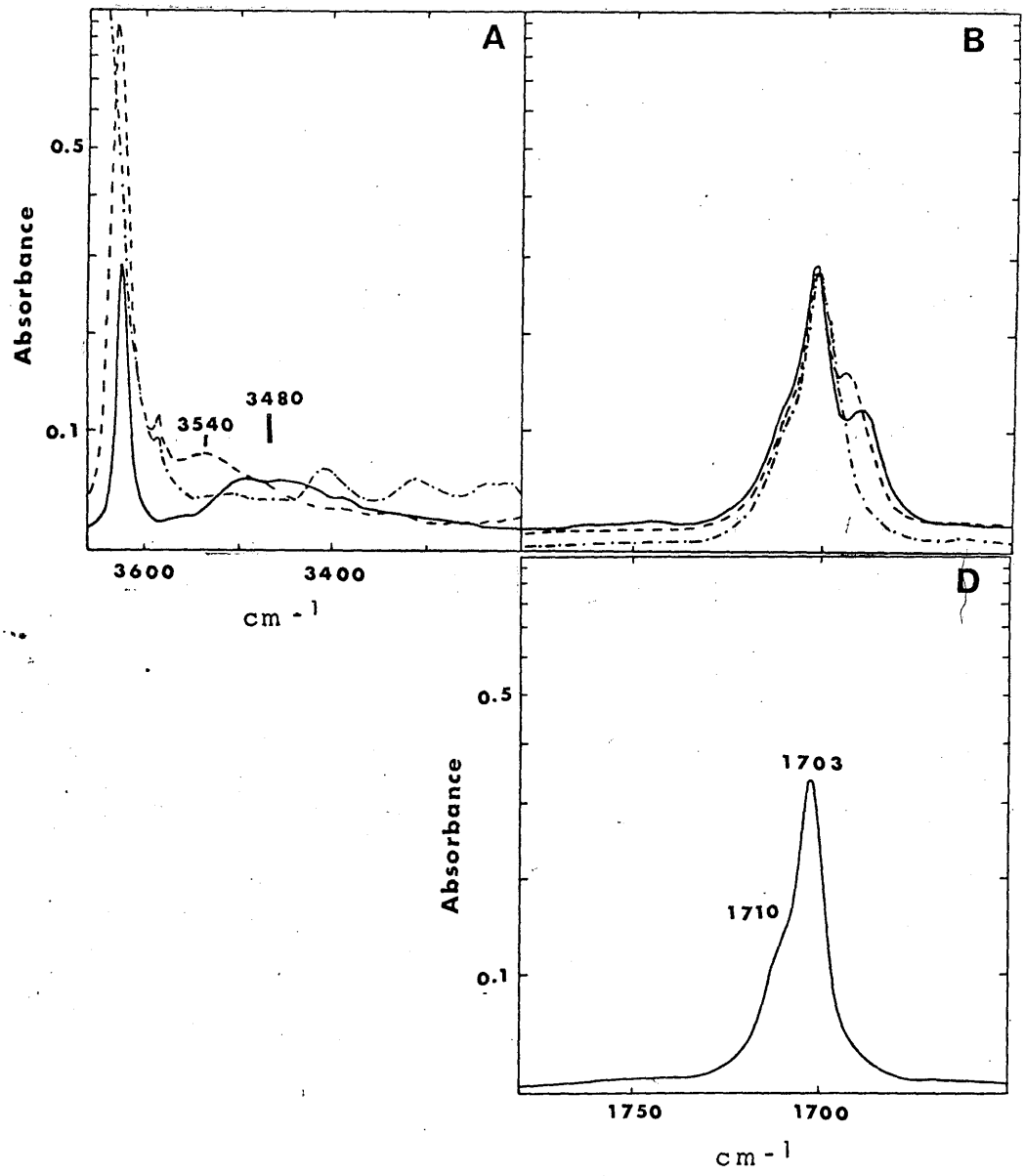


Fig. 4. A and B. Hydroxyl and carbonyl absorptions of solutions containing 2,2,6,6-tetramethylcyclohexanone (0.015M) in n-hexane in the presence of various phenols: p-cresol (——, 0.03M); 2,6-dimethylphenol (– – –, 0.15M); 2,6-di-t-butyl-4-methylphenol (– · – ·, 0.50M).

D. 2,2,6,6-Tetramethylcyclohexanone (0.015M) in n-hexane.

shoulder at 1710 cm.$^{-1}$ (Fig. 4D). With 0.33M p-cresol, where the ratio of concentrations (phenol:ketone) is 2:1, the separation between free and associated carbonyl bands is 13 cm.$^{-1}$ as compared with 17 cm.$^{-1}$ for cyclohexanone. The ratio of absorbances is 2.47 (ca. 88% free carbonyl) compared with 1.32 (ca. 75% free carbonyl) for cyclohexanone. Thus both the hydrogen bond strength and the degree of association of p-cresol with the tetramethyl ketone appear to be somewhat reduced by the steric effects of the α-methyl groups.

In the presence of 0.15M 2,6-dimethylphenol, equimolar solutions of tetramethylcyclohexanone and cyclohexanone contain respectively about 84% and 64% free carbonyl (Tables 4 and 3). Examination of models shows that a frontal approach by 2,6-dimethylphenol in the plane of the carbonyl group leads to overlap of the α- and ortho-methyl groups, hence the higher percentage of unbonded carbonyl in the tetramethyl ketone. The band separation is also much less than for cyclohexanone (Table 3) indicating that the association is weaker, as well as of reduced extent.

The frequency of the free carbonyl band hardly alters on proceeding from n-hexane through the sequence of phenol solutions to 0.5M 2,6-di-t-butyl-4-methylphenol in n-hexane (Figs. 4D and 4B)

and there are only small changes in half-band width. The association between 2,2,6,6-tetramethylcyclohexanone and 2,6-di-t-butyl-4-methylphenol is extremely weak as the only sign of interaction is a slight broadening of the free carbonyl band. The existence of 91% of the ketone in an unbonded form in the presence of a large excess of phenol (0.5M) tends to confirm the weak nature of the association. These observations of the inhibitory effect of bulky ortho-substituents on hydrogen bond formation by phenols are comparable with the results of BELLAMY, EGLINTON and MORMAN (30) for the association of 2,6-di-t-butyl-phenol with ether, and those of BROWN, EGLINTON and MARTIN-SMITH (31,32) for alkyl substituted ortho-bromo phenols in ether - carbon tetrachloride mixtures.

The hydroxyl region (Fig. 4A) shows a broad band with maximal absorption about 3480 cm.$^{-1}$ for a 0.03M solution of p-cresol in n-hexane. This band is at the same frequency as that found for 2,6-dimethyl and 2,2,6,6-tetramethylcyclohexanone in 0.03M p-cresol (Fig. 1A). In 2,6-dimethylphenol this band seems to have shifted to higher frequencies (3540 cm.$^{-1}$) which probably signifies an even weaker association. That this band is due in this particular case to a ketone-phenol complex and not to a phenol dimer is suggested by the absence of any other bonded hydroxyl absorption, and is consistent with the evidence for the associated species in the carbonyl region (Fig. 4B).

The hydroxyl absorption of 2,6-di-t-butyl-4-methylphenol in n-hexane (Fig. 3C) is virtually unchanged in the presence of tetramethylcyclohexanone (Fig. 4A), confirming that very little association occurs.

Representative quantitative measurements were made in the hydroxyl region for mixtures of p-cresol with cyclohexanone, 2,2,6,6-tetramethylcyclohexanone and 5a-cholestan-3-one respectively. The data permitted the calculation of the approximate distribution of p-cresol between various species, as given in Table 5. The apparent extinction coefficient of the free hydroxyl band was found by extrapolation to infinite dilution (Fig. 5A) of the values observed for several concentrations of p-cresol in n-hexane. A calibration curve (Fig. 5B) was also drawn of the absorbance of this band against the total concentration of p-cresol. In the presence of a ketone, the absorbance of the free hydroxyl band would then be used to determine the total concentration of free and self-associated p-cresol in the equilibrium mixture. The range of measurements was inadequate to allow computation of formation constants from the hydroxyl absorption data alone, as was done by WHETSEL and KAGARISE (15). However, by using the absorbance of the free carbonyl band to calculate both the free ketone concentration and

Table 5. Distribution of various species in ketone-p-cresol solutions

Ketone	Molarity		Free p-cresol			Free + self-associated p-cresol			Self-associated p-cresol		Carbonyl molarity		Formation* constant
	Ketone	p-Cresol	ε^a	Molarity [B]	%	Absorbance	Molarity	%	Molarity	%	Free [A]	Bound [C]	
Cyclohexanone	0.015	0.030	165	0.021	69	0.252	0.024	80	0.003	10	0.0112	0.0038	16
	0.030	0.030	124	0.016	52	0.190	0.017	57	0.001	3	0.0225	0.0075	21
	0.030	0.045	131	0.025	55	0.300	0.030	67	0.005	11	0.0198	0.0102	21
	0.030	0.120	96	0.045	38	0.560	0.079	66	0.034	28	0.0138	0.0162	26
2,2,6,6-Tetramethyl cyclohexanone	0.015	0.030	183	0.023	76	0.280	0.0275	92	0.0045	15	0.0132	0.0018	5.9
	0.030	0.030	146	0.018	61	0.224	0.0215	72	0.0035	12	0.0240	0.0060	13.9
5α-Cholestan-3-one	0.015	0.030	150	0.019	63	0.230	0.022	73	0.003	10	0.0092	0.0058	33

* Computed as $\frac{[C]}{[A]\ [B]}$: units 1/mole

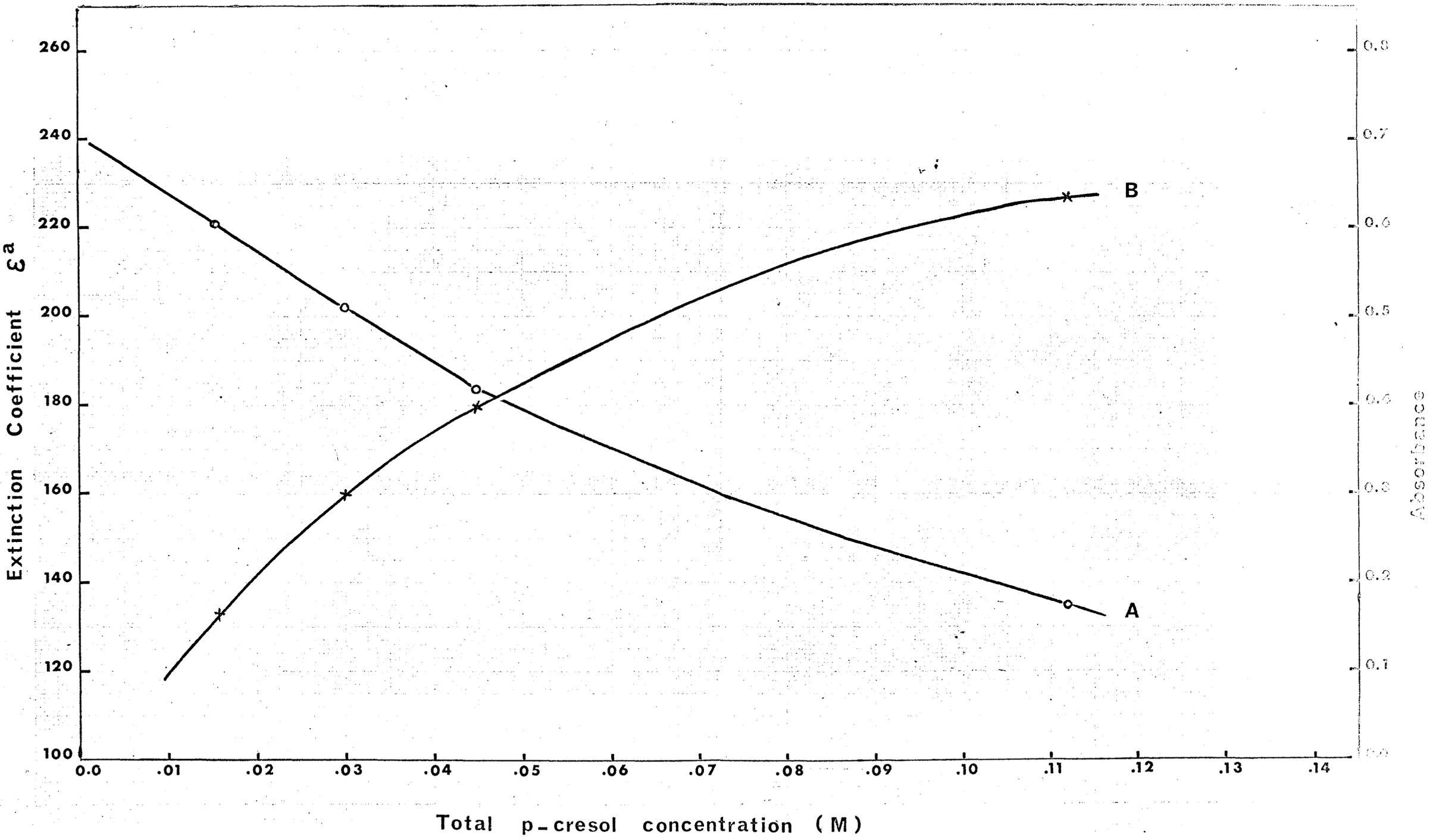


Fig. 5. Measurements on the free hydroxyl band of p-cresol at 3624 cm.$^{-1}$ in p-cresol-n-hexane solutions. A Apparent extinction coefficient against total phenol concentration. B. Absorbance against total phenol concentration.

(by difference from the total ketone concentration) that of the 1:1 complex (higher complexes being neglected), formation constants were evaluated as in Table 5. For the more dilute solutions, in which the proportions of higher complexes are small, the constant of about 19 l/mole for p-cresol-cyclohexanone is in good agreement with the value of 18.5 obtained by WHETSEL and KAGARISE for the same complex in cyclohexane at 30°C. As expected from the more qualitative observations, a markedly lower formation constant was found for the p-cresol-tetramethyl-cyclohexanone complex, whereas for cholestanone the value was significantly greater than that for cyclohexanone.

(1) M. L. JOSIEN and J. LASCOMBE, J. Chim. Phys. 52, 162 (1955).

(2) N. W. BAYLISS, A. R. H. COLE and L. H. LITTLE, Australian J. Chem. 8, 26 (1955).

(3) A. D. BUCKINGHAM, Proc. Roy. Soc. (London) 248A, 169 (1958).

(4) M. ITO, K. INUZUKA and C. IMANISHI, Bull. Chem. Soc. Japan 34, 467 (1961).

(5) G. L. CALDOW and H. W. THOMPSON, Proc. Roy. Soc. (London) 254A, 1 (1960).

(6) (a) H. HALLAM and T. C. RAY, Nature (London) 189, 915 (1961).
(b) H. HALLAM and T. C. RAY, Trans. Faraday Soc. 58, 1299 (1962).

(7) R. J. W. LE FEVRE Australian J. Chem. 14, 312 (1961).

(8) R. S. NORRISH, Nature 187, 142 (1960).

(9) H. W. THOMPSON and D. J. JEWELL, Spectrochim. Acta 13, 254 (1958).

(10) L. J. BELLAMY and R. L. WILLIAMS, Trans. Faraday Soc. 55, 14 (1959).

(11) W. WEST and R. T. EDWARDS, J. Chem. Phys. 5, 14 (1937).

(12) E. BAUER and M. MAGAT, J. Phys. radium 9, 319 (1938).

(13) L. J. BELLAMY and R. L. WILLIAMS, Proc. Roy. Soc. 255A, 22 (1960).

(14) L. J. BELLAMY and H. E. HALLAM, Trans. Faraday Soc. 55, 220 (1959).

(15) K. B. WHETSEL and R. E. KAGARISE, Spectrochim. Acta 18, 315 (1962).

(16) K. B. WHETSEL and R. E. KAGARISE, Spectrochim. Acta 18, 329 (1962).

(17) K.B. WHETSEL and R.E. KAGARISE, Spectrochim. Acta 18, 341 (1962).

(18) P. MARONI, Ann. Chim. (Paris) 2, 757 (1957).

(19) R. MECKE and K. NOACK, Chem. Ber. 93, 210 (1960).

(20) R.N. JONES, P. HUMPHRIES and K. DOBRINER, J. Amer. Chem. Soc. 71, 241 (1949).

(21) R.N. JONES, P. HUMPHRIES, F. HERLING and K. DOBRINER, J. Amer. Chem. Soc. 73, 3215 (1951).

(22) A.R.H. COLE, R.N. JONES and K. DOBRINER, J. Amer. Chem. Soc. 74, 5571 (1952).

(23) R.N. JONES and F. HERLING, J. Org. Chem. 19, 1252 (1954).

(24) R.N. JONES and G. ROBERTS, Chem. & Ind. p. 1269 (1957).

(25) A.R.H. COLE and D.W. THORNTON J. Chem. Soc. 1, 1007 (1956).

(26) A.R.H. COLE and R.L.S. WILLIX, J. Chem. Soc. 1, 1212 (1959).

(27) J.M. WIDOM, R.J. PHILIPPE and M.E. HOBBS, J. Amer. Chem. Soc. 79, 1383 (1957).

(28) T. GRAMSTAD, Spectrochim. Acta 19, 497 (1963).

(29) J. LASCOMBE, Thesis, University of Bordeaux (1960).

(30) L.J. BELLAMY, G. EGLINTON and F. MORMAN, J. Chem. Soc. 4762 (1961).

(31) I. BROWN, G. EGLINTON and M. MARTIN-SMITH, Spectrochim. Acta 19, 463 (1963).

(32) I. BROWN, G. EGLINTON and M. MARTIN-SMITH, Spectrochim. Acta 18, 1593 (1963).

(33) C.J.W. BROOKS, G. EGLINTON and J.F. MORMAN, J. Chem. Soc. 661 (1961).

(34) N.A. PUTTNAM, J. Chem. Soc. 486 (1960).

(35) R.N. JONES, D.A. RAMSAY, D.S. KEIR and K. DOBRINER, J. Amer. Chem. Soc. 74, 80 (1952).

(36) N.L. ALLINGER and H.M. BLATTER, J. Amer. Chem. Soc. 83, 994 (1961).

(37) W. HÜCKEL, Bull. Soc. Chim. France 1 (1963).

(38) K.S. SESHADRI and R.N. JONES, Spectrochim. Acta 19, 1013 (1963).

(39) R. CETINA and J.L. MATEOS, J. Org. Chem. 25, 704 (1960).

(40) Y. MAZUR and F. SONDHEIMER, J. Amer. Chem. Soc. 80, 5220 (1958).

(41) E.G. CUMMINS and J.E. PAGE, J. Amer. Chem. Soc. 79, 3847 (1957).

(42) N.L. ALLINGER and M.A. DAROOGE, J. Amer. Chem. Soc. 84, 4561 (1962).

(43) J.O. HALFORD, J. Chem. Phys. 24, 830 (1956).

(44) J.M. LEHN, J. LEVISALLES and G. OURISSON, Bull. Soc. Chim. France 1096 (1963).

(45) P. WITZ, H. HERMANN, J.M. LEHN and G. OURISSON, Bull. Soc. Chim. France 1101 (1963).

(46) H. MINATO, Bull. Chem. Soc. Japan 36, 1020 (1963).

(47) P. YATES, N. YODA, W. BROWN and B. MANN J. Amer. Chem. Soc. 80, 202 (1958).

(48) P. YATES and L.L. WILLIAMS, J. Amer. Chem. Soc. 80, 5896 (1958).

(49) C.L. ANGELL, P.J. KRUEGER, R. LAUZON L.C. LEITCH, K. NOACK, R.J.D. SMITH and R.N. JONES, Spectrochim. Acta 11, 926 (1959).

(50) R.N. JONES, C.L. ANGELL, T. ITO and R.J.D. SMITH, Canad. J. Chem. 37, 2007 (1959).

(51) R.N. JONES and B.S. GALLAGHER, J. Amer. Chem. Soc. 81, 5242 (1959).

(52) Table of Wavenumbers for the Calibration of Infra-Red Spectrometers. p. 652-655. Butterworths (1961).

(53) R.N. JONES and C. SANDORFY in "Chemical Applications of Spectroscopy" p. 466. Interscience, New York (1956).

(54) L. SCHMERLING, J. Amer. Chem. Soc. 69, 1121 (1947).

(55) T. CAIRNS and G. EGLINTON, Nature 96, 535 (1962).

(56) H.C. BROWN, D.H. McDANIEL and O. HAFLIGER in "Determination of Organic Structures by Physical Methods" (1955) p. 603, Ed. by E.A. BRAUDE & F.C. NACHOD, New York, N.Y. Academic Press Inc.

(57) L.J. BELLAMY and R.L. WILLIAMS, Proc. Roy. Soc. 254A, 119 (1960).

(58) M. ST.C. FLETT, Spectrochim. Acta 10, 21 (1957).

(59) P.J. STONE and H.W. THOMPSON, Spectrochim. Acta 10, 17 (1957).

(60) R.F. GODDU, J. Amer. Chem. Soc. 82, 4533 (1960).

(61) I. BROWN, Thesis, University of Glasgow (1962).

(62) L.J. BELLAMY, "The Infra-red Spectra of Complex Molecules" (1958). p. 101. London: Methuen & Co., Ltd.