come from the air absorbed on the surface of the beryllium oxide pellet.

In the irradiated samples, the ratio of oxygen to nitrogen was significantly higher than the same ratio for air, which suggests that some of the oxygen in the irradiated beryllium oxide was released in molecular form on dissolution. In an unirradiated sample, the oxygen to nitrogen ratio was similar to that of air.

Many samples were analyzed for helium content only, because the Materials Division of the Research Establishment was mainly interested in helium content produced in the irradiated beryllium oxide. Table II shows the comparison of the helium content, as determined by gas chromatography and mass spectrometry, for a wide variety of irradiated samples.

The helium content is proportional to dose at 100 °C but considerable losses are apparent above 600 °C. The lowest detection limit for tritium was 6 μCi, but this was quite adequate for the levels of activity encountered. The highest tritium content was about 0.18 Ci per ml of gas extracted.

Most of the tritium formed (~90%) remained in the acid as tritiated water and this is at variance with the results of diffusion experiments (*2*) in which most of the tritium can be released in elemental form when BeO is strongly heated. The results suggest that tritium atoms are trapped in the lattice in a form which is readily oxidized by the hot acid but not by the free oxygen produced during the irradiation of BeO.

The introduction of a scintillation detector in the gas chromatograph has made the method of determining the ratio of free-to-combined tritium simple and fairly rapid and suggests that the method could be used in the study of many other problems concerned with the reactivity of tritons with solids.

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Determination of Carbon Skeletons of Microgram Amounts of Steroids and Sterols by Gas Chromatography after Their High Temperature Catalytic Reduction

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A method is described for the complete reduction of microgram quantities of steroids and sterols to their parent hydrocarbons. The apparatus consists of a siliconized glass tube containing platinum catalyst coated on siliconized glass beads. A slow stream of hydrogen is passed through the heated catalyst containing tube and the samples are injected into the catalyst bed. The reduction products are trapped for analysis by gas–liquid chromatography. The effects of a number of factors involved in the method have been investigated and its reliability has proved satisfactory. Complete reduction of many steroids and sterols could be reproducibly achieved at catalyst temperatures of 170–90 °C, but the yield was related to the structure of the starting material. The method has been used to identify the parent hydrocarbons of many steroids and sterols.

CHROMATOGRAPHIC TECHNIQUES, especially gas–liquid chromatography, GLC, are very useful in the separation and quantitation of minute quantities of compounds of biological origin (*1*). The number of possible structures which natural products may possess is much larger than the number of hydrocarbon skeletons to which their functional groups are attached. Thus complete reduction of these natural products to the "common denominator" of the hydrocarbon skeleton represents a considerable simplification (*2*), and the hydrocarbons produced can be characterized in microgram quantities by GLC. This type of technique has been used to study insect attractants (*3*), the queen bee substance (*4*), alkaloids (*5*), fatty acids from wool wax (*6*), and to convert components of petroleum to the saturated hydrocarbons (*7*).

Steroids and sterols are important classes of the naturally occurring compounds, but their individual identification is complicated because they occur only in small quantities in the presence of many closely related substances. "Carbon skeleton" chromatography has been extended by the present work to the identification of steroids (*8*). This article describes a method for the catalytic reduction of steroids and sterols, studies factors affecting the method, and examines its reliability. It has been used for the complete reduction of about 5–10 μg of steroids and sterols, and the yields of different hydrocarbon products from several types of compounds have been studied at various temperatures. Different types of catalysts and supports for the catalysts have been investigated. The products obtained were consistent at a constant temperature and provided information about the structure of the carbon skeleton of the starting material.

EXPERIMENTAL

Apparatus and Materials. HYDROGENATOR. The basic principle of the apparatus is similar to that of the "Carbon skeleton determinator" described by Beroza and Acree (*3*)

(1) E. C. Horning, W. J. A. VandenHeuvel, and B. G. Creech, in "Methods of Biochemical Analysis," Vol. XI, D. Glick, Ed., Interscience New York, 1963, pp 69–147.
(2) I. E. Bush, "The Chromatography of Steroids," Pergamon Press, London, 1961, p 300.
(3) M. Beroza and F. Acree, Jr., *J. Assoc. Offic. Agr. Chemists*, **47**, 1 (1964).
(4) R. K. Callow and N. C. Johnston, *Bee World*, **41**, 152 (1960).
(5) M. Beroza, *J. Org. Chem.*, **28**, 3562 (1963).
(6) D. T. Downing, Z. H. Kranz, and K. E. Murray, *Aust. J. Chem.*, **13**, 80 (1960).
(7) C. J. Thompson, H. J. Coleman, R. L. Hopkins, and H. T. Rall, *J. Gas Chromatogr.*, **5**, 1 (1967).
(8) P. M. Adhikary and R. A. Harkness, *Biochem. J.*, **105**, 40P (1967).

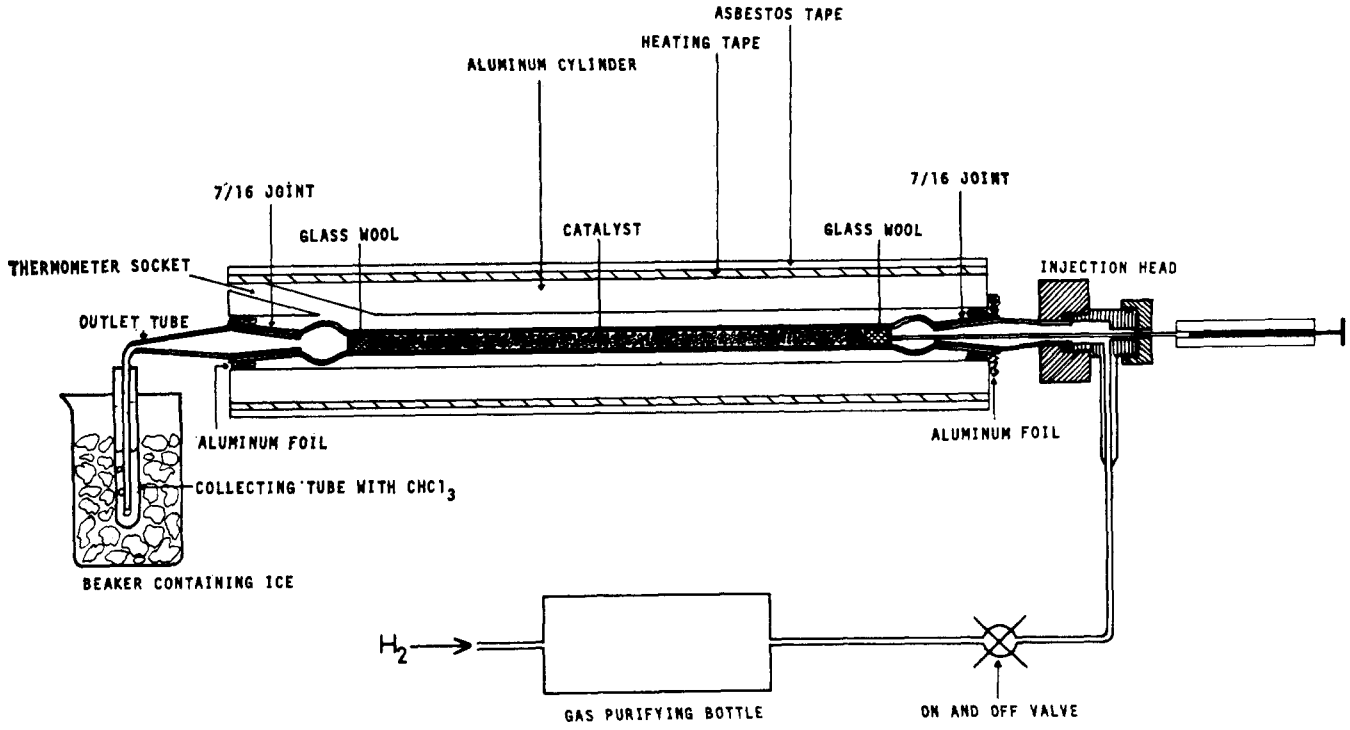


Figure 1. Diagramatic cross section of apparatus for high temperature catalytic reduction; for details, see text

and to the microhydrogenation apparatus of Thompson *et al.* (*7*) in which samples were reduced by injection into a stream of hydrogen passing through a heated bed of catalyst. Modifications introduced consist of a siliconized glass tube (21.7 × 0.7 cm o.d.) with $^7/_{16}$ sockets at its ends as a reaction chamber in place of the metal tube used previously. The tube is placed inside an aluminum block (21.7 × 3.2 cm o.d.) drilled approximately to the size of socket (1.27 cm diam); the space between the ends of the glass tube and the aluminum block was sealed with aluminum foil. A metal injection head with a rubber septum (Cat. No. A2994, W. G. Pye Ltd., Cambridge, England) was fitted to a 3-cm long glass tube ending in a $^7/_{16}$ cone which was connected to the catalyst chamber. The outlet of the catalyst chamber was connected by a $^7/_{16}$ cone to a tapered glass tube for trapping the reaction products (Figure 1).

The shape of the trap tube is similar to that described by Beroza and Sarmiento (*9*). The aluminum block was wrapped with an Electrothermal heating element insulated from the exterior by asbestos tape. The input to the heating tape was controlled using a Variac transformer. A hole (6 mm diam.) at one end of the aluminum block was drilled to hold a thermometer. The temperature of the system could be maintained ±0.5 °C. A gas purifying bottle (W. G. Pye Ltd.) containing a 13 X type molecular sieve (Union Carbide) was connected between the injection port and the hydrogen cylinder to ensure a dry gas supply. An on and off valve between the injection port and the gas purifying bottle prevented the molecular sieve from absorbing moisture from the atmosphere when the hydrogen supply was stopped. This apparatus can be assembled easily in a short time from materials available in most laboratories.

CATALYST AND SUPPORT. Platinum catalyst coated on a support of about 40 mesh siliconized glass beads (British Drug Houses, BDH) was used. Preliminary cleaning and deactivation of the beads was similar to the method used by Horning, VandenHeuvel, and Creech (*1*) for diatomaceous earth supports, but treatment of the beads by overlaying overnight with 5% v/v dichlorodimethylsilane in toluene gave better results than treatment for only 10–15 minutes; the catalyst tube was treated similarly. The cleaned and siliconized beads were then coated with platinum or other catalyst by the following procedure. The beads (50 grams) were placed in a beaker with 100 ml of 1% w/v ethanolic solution of chloroplatinic acid (BDH) and were thoroughly mixed by rotating the beaker during evaporation of the solvent to dryness on a boiling water bath under a stream of compressed air. When the mixture was nearly dry, it was removed and further dried in an oven at 100–105 °C overnight. Alternatively, a rotary evaporator could be used for evaporation of solvent and coating of support on the beads; then final drying was performed in a vacuum desiccator over silica gel. Both procedures gave satisfactory coating of platinum chloride on the support and the activity was similar. About 1–3% Pt w/w coated on the glass beads was produced by this method. The catalyst was stored in a desiccator and remained active for at least 8 months.

GAS CHROMATOGRAPH. A Pye model 104 with a flame ionization detector was used with the injection head modified according to Menini and Norymberski (*10*). The flash heater was kept at least 40 °C higher than the column temperature. Nitrogen (40 ml/min) was used as a carrier gas. The flame ionization detector was operated at the optimum conditions described by Fowlis, Maggs, and Scott (*11*). Coiled glass columns, 152.4 cm and 121.9 cm long and 0.4 cm i.d., were packed by the method of Horning *et al.* (*1*). The supports, 100–120 mesh Gas Chrom P (Applied Science Laboratories) and acid washed and siliconized 100–120 mesh Supasorb (BDH), were siliconized with 5% v/v dichlorodimethylsilane in toluene. These supporting materials were coated with 1% w/w neopentyl glycol adipate polyester, NGA, and with methyl silicone polymers, 1% w/w JXR (Applied Science Laboratories) and 1% w/w SE-30 (General Electric Co.). The coating material NGA was dissolved in acetone; SE-30 and JXR were dissolved in toluene.

The hydrocarbons produced were tentatively identified on

(9) M. Beroza and R. Sarmiento, ANAL. CHEM., **36**, 1744 (1964).

(10) E. Menini and J. K. Norymberski, *Biochem. J.*, **95**, 1 (1965).

(11) A. Fowlis, R. J. Maggs, and R. P. W. Scott, *J. Chromatogr.*, **15**, 471 (1964).

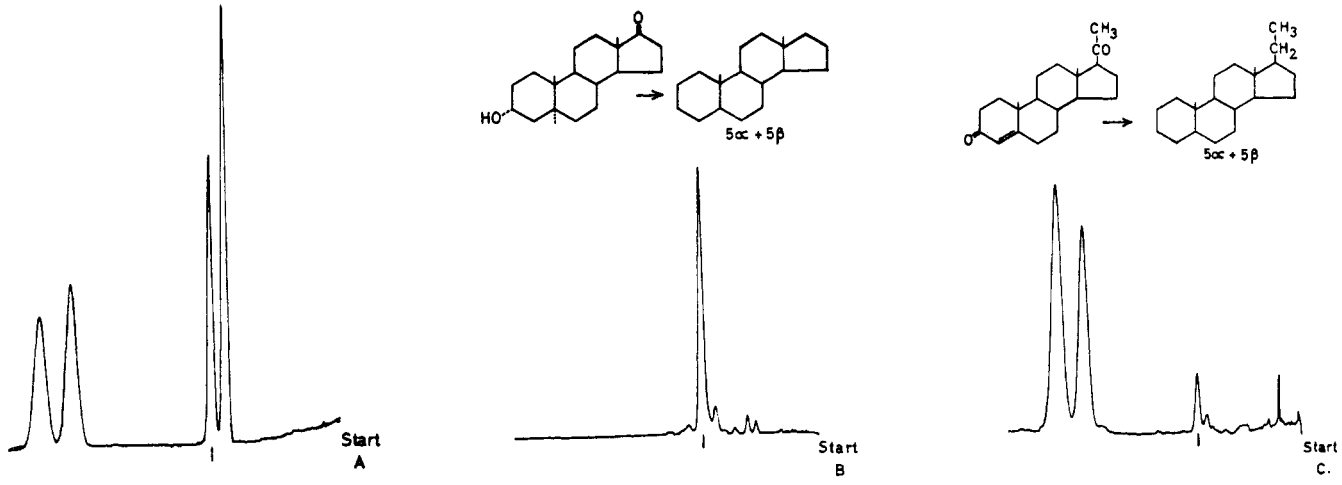


Figure 2. Gas chromatograms of standard steranes and reduction products from steroids on 1% JXR coated columns at 140 °C

A. **Gas chromatogram of 0.25–0.30 μg of steranes; in order of increasing retention time the peaks are 5β-androstane, 5α-androstane (marked by a vertical line), 5β-pregnane and 5α-pregnane**

B. **Gas chromatogram of reduction products from 2.5 μg of androsterone; retention time of 5α-androstane is indicated by a vertical line**

C. **Gas chromatogram of reduction products from 5 μg of progesterone; the retention time of 5α-androstane is indicated by a vertical line**

the basis of retention times derived by using these columns. Relative retention times and separation factors for the hydrocarbons on SE-30 coated columns were similar to those of Brooks and Hanaineh (*12*).

SPECTROMETRY. Infrared, IR, spectrometry on a micro scale was performed on a Unicam SP200 spectrophotometer using solid samples in a beam condenser.

REFERENCE STANDARDS, REAGENTS, AND SOLVENTS. All chemicals and solvents were analytical grade where possible. The steroids and sterols were pure.

PROCEDURE. The catalyst was packed into the glass tube after plugging one end with some siliconized glass wool (Figure 1). The total weight of support and catalyst used to prepare one tube was approx. 2.25 grams. The tube containing catalyst was then put inside the aluminum block and charged for 3 hr by passing hydrogen (20–30 ml/min) through the catalyst bed at temperatures of 180–200 °C. As the rise of temperature with this apparatus is slow, overheating of the catalyst can be avoided. Because HCl is split off the chloroplatinic acid, a check on the pH of the effluent was made during the process by testing it with a few drops of universal indicator solution (BDH). At the end of the charging period, when the effluent was neutral, three injections of 10 μl of absolute ethanol were given at 5-minute intervals. This virtually eliminated the production of compounds which did not correspond to steranes on GLC. The activity of the charged catalyst was not found to alter even after three months' storage.

Samples were dissolved in absolute ethanol (approx. 1 mg/ml) and 5–10 μl were injected with a Hamilton micro-syringe. The products were collected for about 1 minute in a tube (7.5 × 0.6 cm) containing about 0.2 ml of chloroform which was kept cool in ice. Finally, the outlet was removed and replaced immediately by another. The outlet was rinsed with a few drops of chloroform which were then transferred into the collecting tube. A U-shaped outlet cooled in ice water can also be used for trapping the reduced products.

Injections of 10 μl of absolute ethanol or 50% ethanol three times at intervals of 5 minutes ensured complete elution of any removable compounds which had been retained in the catalyst bed. The operation was carried out in a hood with no interruption of gas flow. Then the heater and gas supply were turned off and the catalyst tube was closed by a suitable glass stopper to avoid condensing water inside the catalyst bed. The reduction products in chloroform were transferred on to a polytetrafluoroethylene (PTFE) plate for deposition on to stainless steel gauzes which were then injected into the GLC. The chromatographic columns' temperatures were 140–170 °C for C_{19}-androstanes and C_{21}-pregnanes. For C_{27}-cholestanes and C_{24}-cholanes, temperatures of 190–210 °C and 170–180 °C, respectively, were required with NGA, JXR, and SE-30 coated columns.

As the yield of saturated hydrocarbons depends upon the activity of the catalyst, this should be checked frequently by reduction of a known quantity of a reference compound. In our experience, a new batch of charged catalyst gives a reproducible yield for at least 4–6 days.

RESULTS

The "Carbon skeleton determinator" (*3*) was tried for reduction of steroids. The products were a complex mixture with a large range of retention times, some of which were considerably longer than that of the parent hydrocarbon. No product corresponding with the parent sterane was present. The results were not improved after siliconization of the metal surface or by putting a glass liner inside; there still remained a small part of the metal tube which could not be glass lined. These complex changes occurred with and without catalyst and support in the tube. The new apparatus overcame these difficulties; unchanged 3α-hydroxy-5α-androstan-17-one, androsterone, was recovered from the system in a mean yield ± S.D. of 72.9 ± 5.3% [6]. The number of experiments is shown in brackets.

A number of factors associated with the optimum operating conditions were studied using the present apparatus.

Effect of Temperature. The effects of temperature on the yield of saturated products from some typical C_{19} steroids were studied from 140–240 °C. The results are presented in Table I. It is to be noticed that with the rise of temperature there was an associated increase in the yield hydrocarbons, accompanied by an increase in the relative amount of the more stable 5α isomer. The number and amount of minor products (see Figure 2) were also increased; higher temperatures have been shown to favor hydrogenolysis (*9*). At the

(12) C. J. W. Brooks and Leila Hanaineh, *Biochem. J.*, **87**, 151 (1963).

Table I. Effect of Temperature on Yield of Hydrocarbons from C_{19} Steroids

Compounds	Temperature range, °C	Mean percentage yield (± S.D.) 5α-Androstane	Mean percentage yield (± S.D.) 5β-Androstane	Ratio of products 5α-Androstane : 5β-Androstane
3α-Hydroxy-5α-androstan-17-one (Androsterone)	140–150	5.26 ± 1.73 (5)[a]	0.94 ± 0.08 (5)	1 : 0.17
	160–170	9.84 ± 0.75 (5)	2.38 ± 0.10 (5)	1 : 0.24
	180–190	14.30 ± 2.50 (5)	4.18 ± 0.15 (5)	1 : 0.29
	200–210	20.48 ± 2.68 (5)	4.10 ± 0.51 (5)	1 : 0.20
	220–240	23.04 ± 7.11 (6)	3.12 ± 1.23 (6)	1 : 0.13
3α-Hydroxy-5β-androstan-17-one (Etiocholanolone)	140–150	1.96 ± 0.97 (5)	4.18 ± 0.76 (5)	1 : 2.13
	160–170	2.76 ± 0.64 (6)	6.68 ± 0.65 (6)	1 : 2.42
	180–190	4.38 ± 0.67 (5)	9.16 ± 1.07 (5)	1 : 2.09
	200–210	9.38 ± 1.16 (5)	4.78 ± 1.66 (5)	1 : 0.50
	220–240	14.66 ± 4.24 (5)	3.50 ± 0.64 (5)	1 : 0.23
17β-Hydroxyandrost-4-en-3-one (Testosterone)	140–150	1.14 ± 0.22 (5)	1.31 ± 0.16 (5)	1 : 1.14
	160–170	4.88 ± 0.90 (6)	5.32 ± 0.98 (6)	1 : 1.09
	180–190	6.84 ± 2.05 (6)	4.20 ± 1.11 (6)	1 : 0.61
	200–210	14.30 ± 2.25 (5)	5.48 ± 1.15 (5)	1 : 0.38
	220–240	15.64 ± 0.93 (5)	4.44 ± 0.98 (5)	1 : 0.28
3β-Hydroxyandrost-5-en-17-one (Dehydroepiandrosterone)	140–150	1.10 ± 0.01 (5)	0.32 ± 0.05 (5)	1 : 0.29
	160–170	5.58 ± 0.87 (6)	2.04 ± 0.59 (6)	1 : 0.36
	180–190	7.32 ± 1.16 (5)	2.48 ± 0.63 (5)	1 : 0.33
	200–210	13.60 ± 0.92 (5)	2.90 ± 0.55 (5)	1 : 0.21
	220–240	14.12 ± 2.72 (5)	2.64 ± 0.93 (5)	1 : 0.18

[a] The numbers of estimations are in parentheses.

temperature 170 °C smaller amounts of the minor products were found, and there was less conversion to the stable 5α, trans, isomer. The amount of starting materials recovered as 5α and 5β-androstanes and the ratio of the amounts of these two products were reproducible as shown in Table I.

From the results in Table I and Table II, it can be seen that the selection of a low operating temperature minimizes isomerization to the 5α form and thus provides more information on the structure of the starting materials. The results in Table II show that the yield was partially related to number of oxygen functions in the starting material; about 50% of the mono-oxygenated androstanes were converted to androstanes, whereas only about 5–10% of the trioxy compounds were recovered as steranes. Low yields have previously been noted from compounds with two or more polar groups (*13*).

Catalysts and Supports. Some commonly used catalysts and supports were investigated. Palladium and platinum coated on diatomaceous earth supports as described in (*3*, *14*) were not satisfactory. The reduction products contained two major components corresponding to the parent hydrocarbons but their separation was not complete.

With platinum on siliconized glass beads, the compounds obtained were clearly separable and consisted mainly of two peaks with the retention times of the parent hydrocarbons. In the present investigation, no nonvolatile alkali was added to neutralize the catalyst during preparation as with some steroids the presence of alkali can cause decomposition and isomerization (*15*). It was also found that an alkaline catalyst was less active than the neutral catalysts.

Beside platinum, palladium and nickel were tried on a siliconized glass support using androsterone as the starting material. The patterns obtained were similar to those from platinum but the yields of hydrocarbons were comparatively low. The mean yield of 5α and 5β-androstane with estimates of the standard deviations were for platinum, 24.7 ± 4.6% [4]; for nickel, 8.1 ± 1.3% [4]; and for palladium, 2.9 ± 0.68% [6] at a catalyst temperature of 210 °C. So far the platinum catalyst coated on glass beads has given the most

Table II. Effect of Chemical Structure on Yield of Hydrocarbons from Different C_{19} Steroids at Constant Reduction Temperature of 170 °C

Compounds	Mean percentage yield (± S.D.) of 5α + 5β-Androstane	Ratio of products 5α-Androstane : 5β-androstane
3β-Hydroxyandrost-5-ene	59.30 ± 6.67 (4)[a]	1 : 0.28
5α-Androstan-3β-ol	53.37 ± 6.93 (4)	1 : 0.19
3α-Hydroxy-5α-androstan-17-one) (Androsterone	11.70 ± 1.45 (5)	1 : 0.22
3α-Hydroxy-5β-androstan-17-one (Etiocholanolone)	11.01 ± 0.86 (4)	1 : 2.65
17β-Hydroxyandrost-4-en-3-one (Testosterone)	10.73 ± 1.21 (5)	1 : 1.18
Androst-4-ene-3, 17-dione	9.46 ± 0.42 (4)	1 : 1.18
3β-Hydroxyandrost-5-en-17-one (Dehydroepiandrosterone)	7.23 ± 1.89 (5)	1 : 0.42
5α-Androstan-3,17-dione	14.22 ± 1.01 (4)	1 : 0.11
5β-Androstan-3,17-dione	17.81 ± 1.32 (5)	1 : 3.24
5α-Androstan-3β,17β-diol	7.55 ± 1.40 (4)	1 : 0.16
16α-Hydroxyandrost-5-en-17-one	4.66 ± 0.30 (4)	1 : 0.15
3β-Methyl-3α,3′-epoxy-17β-hydroxy-5α-androstane	4.96 ± 0.46 (4)	1 : 0.20
Androst-1,4-diene-3,11,17-trione	6.00 ± 1.30 (4)	1 : 0.43
Androst-4-ene-3,11,17-trione	8.20 ± 1.42 (5)	1 : 0.24
5α-Androstan-3,11,17-trione	7.12 ± 0.80 (4)	1 : 0.08

[a] The numbers of estimations are in parentheses.

(13) M. Beroza and R. Sarmiento, ANAL. CHEM., **35**, 1353 (1963).
(14) *Ibid.*, **37**, 1040 (1965).
(15) T. Reichstein and C. W. Shoppee, *Discuss. Faraday Soc.*, **7**, 305 (1949).

Table III. Reduction of C_{27} Compounds, Effect of Different Temperatures and Nature of Catalyst on Amount of 5β-Cholestane Produced Relative to 5α-Cholestane (the Yield of 5α-Cholestane as 1.0)

Compounds	180 °C	210 °C	270 °C	300 °C	350 °C	Acid catalyst at 180 °C
Cholesterol	1.60 (6)[a]	1.68 (3)	1.29 (2)	1.25 (2)	0.40 (2)	0.74 (3)
5α-Cholestan-3β-ol	0.69 (4)	1.13 (4)	1.09 (3)	1.03 (2)	0.58 (2)	0.03 (3)
5β-Cholestan-3-one	1.96 (3)	2.12 (4)	2.00 (2)	1.18 (2)	0.81 (2)	1.57 (3)
Cholest-4-en-3-one	1.81 (3)	2.05 (3)	2.00 (2)	1.57 (2)	0.51 (2)	2.00 (3)
5α-Cholestane	2.22 (4)	1.50 (3)			0.23 (2)	0.02 (3)
5β-Cholestane	2.87 (3)					0.29 (3)

[a] Number of estimations in parentheses.

satisfactory results and unless otherwise stated all the work reported has been carried out using this catalyst and support.

Specificity. The identities of the major reaction products obtained by using the present method were examined in different ways.

In a series of experiments, the products have been run on SE-30, JXR, and NGA coated columns and then tentatively identified as parent hydrocarbons on the basis of retention times.

The IR spectra of the products obtained after reduction of androsterone, 5α-androstan-3β-ol, and 3β-hydroxyandrost-5-ene had identical absorption maxima to those of authentic 5α-androstane.

The reduction products from cholesterol (see Figure 3) were analyzed using an LKB-9000 GLC-MS combination. The mass spectra showed that the peaks with the retention times of 5α and 5β-cholestanes had the expected molecular weight of 372.

The hydrocarbon skeleton from a C_{19} steroid isolated from urine and identified by the present method was consistent with evidence obtained using IR spectroscopy, GLC with mass spectrometry and direct GLC (*16*). In addition, retention times of the main products of reduction of a number of steroid drugs with a common hydrocarbon skeleton were identical. From this evidence it was concluded that the major products of reduction were the saturated parent hydrocarbons.

Trapping. The efficiency with which the reduction products were trapped was investigated and found, in agreement with the results of Brooks and Godefroi (*17*), to be satisfactory. 5α and 5β-androstane were passed through the system at 170–200 °C and trapped with ice and water as a coolant. The samples were transferred on gauzes to GLC. The mean recoveries ± S.D. were 77.9 ± 4.2% [7] and 70.5 ± 5.2% [7] for 5α and 5β-androstane, respectively. When solid CO_2 and acetone was used as a coolant and the trapped hydrocarbons injected in solution directly into the GLC, the mean recoveries ± S.D. were 87.5 ± 2.1% [4] and 84.0 ± 2.2% [4] for 5α and 5β-androstane, respectively. These recoveries are calculated by using as 100% the results obtained by direct estimation of the hydrocarbons by GLC using solid injection and direct liquid injection. The losses experienced in the solid transfer procedure may be occurring during trapping from the effluent, in transfer to the gauzes, and with the evaporation of the solvent from the gauzes. When 5α-androstane was left overnight on a stainless steel gauze on a PTFE plate, more than 90% of it was lost. It is, therefore, suggested that this loss should be reduced by using a small volume of solvent for transfer to the gauzes. When liquid injection was used, there was no evidence of any low molecular weight reduction products which might have been lost from the PTFE plate.

"Memory" Effect. It was noted that some compounds were retained on the catalyst and could be subsequently eluted with injection of solvent; this memory effect could be avoided by injecting absolute ethanol after the reduction was completed when pure reference compounds were used, like androsterone. The mean percentage recoveries of retained samples from the catalyst by four serial injections of 10 μl of ethanol were 9.5, 5.2, 0.9, and 0.06% of the initial sample. These means were derived from the results of three sets of experiments. Similar results were obtained after the injection of crude extracts by following the same procedure with 50% aqueous ethanol (v/v). Though this operation inactivates the catalyst more rapidly, it was found necessary to remove polar impurities. When analyzing an unknown specimen, a longer trapping period should be allowed as the more polar compounds are retained longer by the catalyst (*18*). The support for the catalyst (*19*) and the polarity of the liquid injected (*20*) markedly affect this retention of reduction products. It should be noted that this memory effect can be more satisfactorily overcome when catalysis and chromatography are separate.

Flow Rates. The effect of different flow rates of hydrogen on the yield of 5α + 5β-androstanes from 5β-androstan-3,17-dione was studied. At flow rates of 5, 20, 40, and 80 ml/min, the mean percentage yields were 10.0, 55.2, 45.0, and 36.8%. The means were derived from the results of three sets of experiments. An optimum flow rate of 20–40 ml/min is similar to previous results (*3*). In the present work, a higher flow rate of 60 ml/min and a longer trapping time of two minutes were required for the collection of C_{27} hydrocarbons; this may possibly be caused by their higher molecular weights.

Sample Size. With the present length of catalyst bed (17.5 cm) samples from 5–10 μg were generally injected at one time. Even as little as 1 μg of a steroid has given the expected products. Depending upon their purity 30–40 samples could be reduced once the catalyst was charged. About 10–12 samples could be analyzed in one working day.

Stereoisomerization. The extent of isomerization of C_{19} hydrocarbons when passed through the hydrogenator containing catalyst at the temperatures of 170 and 215 °C was

(16) C. H. L. Shackleton, R. W. Kelly, P. M. Adhikary, C. J. W. Brooks, R. A. Harkness, P. J. Sykes, and F. L. Mitchell, *Steroids*, in press.
(17) S. C. Brooks and V. C. Godefroi, *Anal. Biochem.*, **7**, 135 (1964).
(18) M. Beroza, *Nature*, **196**, 768 (1962).
(19) M. Beroza, ANAL. CHEM., **34**, 1801 (1962).
(20) E. A. Walker, in "4th Wilkins Gas Chromatography Symposium," University of Manchester, England, 1966, p 15.

studied, and it was shown that a mean of 4.1 and 5.8%, respectively, of 5β-androstane isomerized to 5α-androstane. The isomerization of 5α-androstane to 5β-androstane, when studied under the same conditions, were 2.0 and 2.8%, respectively. More marked isomerization was noted with the cholestanes (Table III). Isomerization of the androstanes was 2–3 times greater with unsiliconized glass beads. No rearrangement of these hydrocarbons to any other products with longer retention times was found to occur at these temperatures.

The tracings from GLC of the reduction products obtained from some common steroids and a sterol are presented in Figures 2 and 3. The major reaction products have the same retention times as the parent hydrocarbons. The retention time of 5α-androstane has been marked at the base line on the tracings of the products from androsterone and pregn-4-en-3,20-dione, progesterone. The products from cholest-5-en-3β-ol, cholesterol, show more 5β-cholestane compared to the 5α-cholestane, the retention time of which is marked on the tracing (Figure 3). However, when cholesterol was reduced at different temperatures, the ratios of 5α to 5β-cholestanes were found to change as is shown in Table III. It is to be noticed that the amount of 5β-cholestane decreased as the temperature increased. When the catalyst was made acid by injection of 1*N* H_2SO_4 (10 μl × 3), the results obtained with these C_{27} compounds were similar to those obtained with C_{19} and C_{21} steroids, the major product generally being the stable 5α isomer. However, the structure of the starting material still altered the ratio of the 5α to 5β-cholestane. After charging the catalyst, three injections of absolute alcohol and subsequent check on the pH of the effluent probably ensured the removal of acid sites. When this operation was not carried out, an increased isomerization to the stable trans form was noted even at low temperatures with C_{19} steroids. A similar result was also obtained when platinum chloride was dissolved in acetic acid during preparation. This suggests that isomerization of the products was associated with the presence of acid.

On some occasions, products with longer retention times than the parent hydrocarbon were produced from C_{19} steroids. The reasons for this were either an inactive catalyst or an insufficient quantity of the catalyst in the tube; for example, when using only a quarter or a half of the usual length of the catalyst bed.

In the present investigations, the ethanol used for injection of samples had no effect on the absolute or the relative yields of hydrocarbons because when the hydrogenator was modified for the solid injection of the sample in a manner similar to that used in GLC the same results were obtained. Similar results were also obtained when samples were introduced in cyclohexane in place of ethanol.

DISCUSSION

In the present method, a siliconized all glass system which has been recommended for use at high temperatures (*21*) and to minimize surface catalytic effects (*22*) has avoided decomposition of steroids. The factors affecting the design of apparatus for reaction gas chromatography have already been reviewed (*23*).

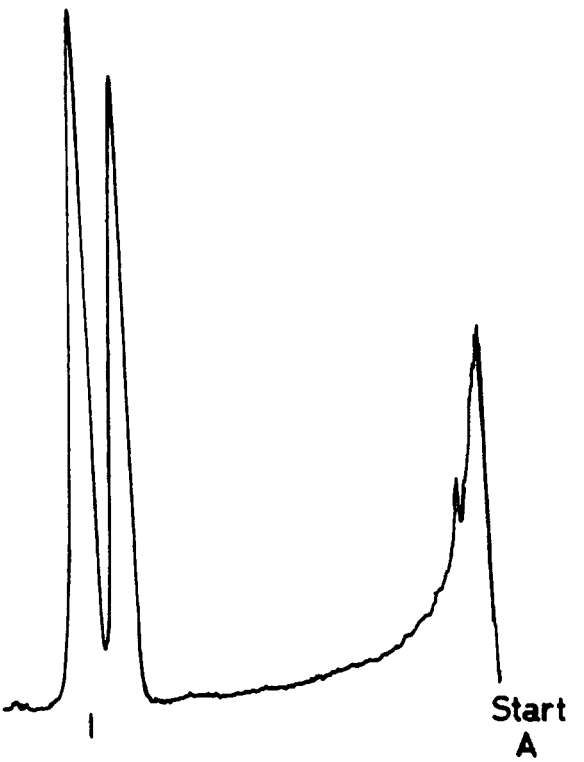
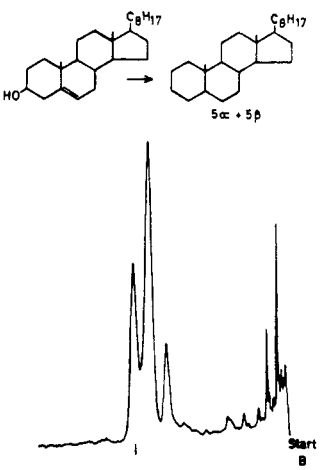


Figure 3. Gas chromatograms on 1% JXR coated columns at 195 °C of

A. **Standard 5β and 5-αcholestane (marked by a vertical line)**



B. **Reduction products from 5 μg cholesterol; retention time of 5α-cholestane is indicated by a vertical line**

The major reaction products from the present method have always had the retention times of the expected saturated parent hydrocarbons and the yields have been reproducible.

The results obtained with steroids at temperatures of 170–200 °C are generally consistent with the products obtained by other types of catalytic hydrogenation using a platinum catalyst (*24*) and provide information regarding the structure of the starting substances. For example, the reduction of a double bond at C-5 produced mainly the 5α isomer, whereas reduction of a double bond at C-4 yielded appreciable amounts of the 5β isomer (*25*). The ratio of 5α to 5β steranes could

(21) W. J. A. VandenHeuvel and E. C. Horning, in "Biomedical Applications of Gas Chromatography," H. A. Szymanski, Ed., Plenum Press, New York, 1964, p 92.
(22) V. I. Komarewsky and C. H. Riesz, in "Technique of Organic Chemistry," Vol. II, A. Weissberger, Ed., Interscience, New York, 1948, pp 1–78.
(23) M. Beroza and R. A. Coad, *J. Gas Chromatogr.*, **4**, 199 (1966).
(24) F. J. McQuillin, in "Technique of Organic Chemistry," Vol. XI, Part 1, A. Weissberger and K. W. Bentley, Eds., Interscience, New York, 1963, p 528.
(25) H. I. Hadler, *Experientia*, **11**, 175 (1955).

thus be used to provide evidence as to whether the hydrocarbon skeleton of the starting material has a 5α, 5β, Δ^4, or Δ^5 structure.

However, an increase in temperature changes the steric course of the reaction and favors more of the stable 5α product. This change occurred at about 200 °C in the C_{19} and C_{21} steroids and at about 350 °C in the C_{27} series. The amounts of minor products with shorter retention times than the saturated hydrocarbons are also affected by the temperature of the catalyst. At a catalyst temperature between 160–180 °C, these are present in negligible amounts. As the catalyst temperature is increased, the yield of these products increases linearly with the increase in the yield of the parent saturated hydrocarbons. In a search for the identity of these products with short retention times, we have noted that the major products obtained from angular C-18 or C-19 demethylated steroids have the same retention times. Thus the minor products with shorter retention times from compounds like androsterone are probably 18 or 19 nor steroids. In Figure 3 it is to be noted that in the reduction products from progesterone there are minor products with the retention times of the 5α and 5β-androstanes. This is possibly because of the removal of the side chain from the C-17 position. These products which have lost the side chain and other products possibly because of cleavage at other positions in the side chain are also present in the reduction products from cholesterol (Figure 3). As the temperature is increased, this effect is more pronounced. Such a loss of methyl groups and the side chain from steroids is a significant process in mass spectrometry (*26*) and in high temperature catalytic reactions of several types of compounds (*7*, *19*, *27*).

Reduction at high temperatures sometimes produced small amounts of compounds with longer retention times than the parent hydrocarbons from which they could readily be separated. This effect was more pronounced with the compounds having an oxygen function at the C-11 position in C_{19} steroids and with sterols at the temperature of 350 °C. No products with the retention times of the pregnanes were formed from androstane derivatives.

The choice of catalysts and supports has been found to be important. The results of the present investigations suggest that a highly active catalyst on an inert support is the best combination. It was interesting to find that platinum metal coated on Chromosorb P did not give better results than those obtained from palladium or nickel on the same support, but when glass beads were substituted as a support for platinum, the results obtained were satisfactory. The use of platinum coated on porous glass at lower temperatures has also been found superior to palladium for reducing ketones, alcohols, ethers, acids, and anhydrides (*19*). Palladium coated on glass beads was found unsatisfactory by previous workers (*28*). The diatomaceous earth supports, Chromosorb P and Gas Chrom P, gave products which were not clearly separable; this could be because of the production of both saturated and unsaturated products by these agents in association with the metal catalysts. When acid-washed Chromosorb P alone was used, products corresponding to the saturated hydrocarbons were seen along with other products with longer retention times. Removal of hydroxyl and acetate groups by Chromosorb P has been described (*29*) and the catalytic activity of such chemically treated diatomaceous earths has been found useful in cracking hydrocarbons (*22*).

Application of this relatively simple technique to microgram quantities of compounds with lower molecular weights than steroids has already been described (*3*, *7*). The method may be a useful companion to mass spectrometry. In the biological field a direct comparison of the physical and chemical properties of unknown natural products with compounds of known structure is difficult, as this usually involves a large number of possible reference compounds. The present method for the tentative identification of the hydrocarbon skeleton supplemented by existing microchemical identification of the functional groups can be used to suggest a limited number of possible structures, thus easing the selection and supply of relevant compounds for comparison. These possibilities can then be investigated by other methods.

Since the hydrocarbon skeletons of many steroids are not broken down in man (*30*, *31*), the presence of the metabolites of such compounds can be detected in body fluids using carbon skeleton chromatography. Thus high temperature catalytic reduction has proved useful in the detection of the metabolites of those steroid drugs which have different hydrocarbon skeletons from the natural products. The metabolism of normal therapeutic quantities of drugs can be studied without the difficulties of obtaining pure radioactively labeled compounds and the potential dangers of their use in man (*32*).

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