HIGH TEMPERATURE CATALYTIC REDUCTION OF STEROIDS AND STEROLS

by

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GENERAL SUMMARY

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"Carbon skeleton" chromatography has been extended to the steroids and sterols and applied to biochemical problems. A method for the reduction of microgram amounts of steroids and sterols to the parent steranes has been developed. The apparatus for the reduction consists of a siliconised glass tube containing 1-3% w/w of platinum catalyst coated on siliconised glass beads. The glass tube is enclosed in an aluminium cylinder and kept at a constant temperature. About 5-10 micrograms of steroids and sterols are injected into the catalyst tube with a stream of hydrogen. The reduction products are trapped and analysed using gas-liquid chromatography. By using this method, a number of steroids and sterols were recovered as the parent hydrocarbons at a catalyst temperature of 170-190°C. The specificity of the method was examined and was found satisfactory. The yields of hydrocarbons were generally related to the number of substituents on the starting materials; about 50% of mono-oxygenated steroids was recovered as sterane, whereas from trioxygenated steroids the yields were 5-10%. A number of anabolic and progestational steroid drugs which are based on a limited number of parent hydrocarbons also gave the expected products; these were generally separable from the hydrocarbons from natural products.

The method was used to characterise the hydrocarbon skeleton of the 16 β-hydroxydehydroepiandrosterone from infant urine and to confirm the specificity/

specificity of a method for measuring pregnanetriol from pregnancy urine extracts. Since the carbon skeleton of a steroid is left largely intact during metabolism in man, the metabolism of 19-nortestosterone, methandrostenolone, oxymetholone and norethandrolone administered in therapeutic doses was studied in man, using the present method for the detection of drug metabolites in the urine extracts. The results from 19-nortestosterone and methandrostenolone have been consistent from the known metabolic fate of these drugs. Two major metabolites of 19-nortestosterone were found in the "conjugated" fraction behaving like 19-noraetiocholanolone and 19-norandrosterone. From methandrostenolone, two metabolites were found in the "free" fraction behaving like 6β-hydroxy-methandrostenolone and an epimer of methandrostenolone. The metabolites of methandrostenolone could also be detected in the freely extractable fraction from urine before any chromatographic separations. The sensitivity of the present method was found satisfactory and the results obtained were similar to those using 4-^{14}C-19-nortestosterone and conventional methods of detection of methandrostenolone metabolites. It, therefore, seemed justifiable to use "carbon skeleton" chromatography in other studies of steroid drugs like oxymetholone and norethandrolone whose metabolic fate was not known.

Oxymetholone was converted to two metabolites which appeared in the "conjugated" fraction. The polar nature of the metabolites suggested that an alteration of the C-2 hydroxymethylene group was possible during metabolism/

metabolism. These metabolites were unstable on gas-liquid chromatography but were stabilised by acetylation. The two major metabolites of norethandrolone were detected in the "conjugated" fraction. Evidence was obtained which suggested that the reduction of the Δ^4-3-ketone group of norethandrolone occurred during metabolism. In all the studies on anabolic steroid drugs, little or no unaltered drug was detectable in urine extracts.

In conclusion, "carbon skeleton" chromatography has been shown to provide a possible solution to the problems of studying the metabolism of normal therapeutic doses of many steroid drugs without using radioactivity. In addition, this technique should solve the problems of detection of the administration of some anabolic steroid drugs.

GENERAL INTRODUCTION

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In the study of biological products, it is often necessary to characterise microgram, µg.+ quantities of organic compounds. Many micro techniques have been developed for this purpose, especially in chromatography. The development of gas-liquid chromatography, g.l.c. with its sensitive detection systems has opened new avenues of research because of its capabilities for the separation and measurement of closely related compounds. The application of g.l.c. to steroids and sterols is noteworthy.

In order to increase the value of g.l.c., other chemical and physical techniques have been combined with it. Examples of such techniques are methods involving chemical reactions and g.l.c. as well as separations by g.l.c. combined with the powerful method of mass spectrometry for final detection and examination. Unlike mass spectrometry, which depends on fragmentation of the molecules, the method of high temperature catalytic reduction described in this thesis yields a simplified pattern of products. These products generally consist of the basic structures of organic compounds, the stable saturated hydrocarbons. In studies on steroids and sterols, proof of the hydrocarbon skeletons have often been sought and the classical Clemmensen and Wolff-Kishner methods of reduction have been used in these problems.

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* The relevant references for this general introduction are cited elsewhere in the thesis.

\+ In this thesis, abbreviations when first mentioned are defined in full; thereafter, abbreviations have been generally used.

These methods have limitations because they require milligram amounts of samples and are applicable only to ketones.

The work presented in this thesis describes, evaluates and applies a more generally useful method for reduction of µg. amounts of steroids and sterols to the parent steranes and their tentative identification by g.l.c. The thesis consists of four chapters. In Chapter I, the development of the method of high temperature catalytic reduction as applicable to various pure naturally occurring steroids and sterols is described. Chapter II is an extension of this approach to steroid drugs and related compounds. In Chapters III and IV, the application of the method to biological problems is presented. Chapter III consists of two sections. In section I, the characterisation of the hydrocarbon skeleton of an unknown compound from infant urine is presented. In section II, this approach has been used to confirm the reliability of a method for measuring pregnanetriol in pregnancy urine. Chapter IV consists of studies on the metabolism of four anabolic steroids - 19-nortestosterone, methandrostenolone, oxymetholone and norethandrolone in man. The metabolism of the last two compounds has not previously been studied. Since the hydrocarbon skeleton of a steroid is not altered during metabolism, the method has been useful in the detection of metabolites by their common denominator, the hydrocarbon skeleton.

CHAPTER I

METHOD FOR THE HIGH TEMPERATURE CATALYTIC REDUCTION OF STEROIDS AND STEROLS

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METHOD FOR THE HIGH TEMPERATURE CATALYTIC REDUCTION OF STEROIDS AND STEROLS

A. INTRODUCTION

Sabatier's classical discoveries in vapour-phase hydrogenation described in 1944 were first applied to micro samples by Thompson, Coleman, Ward & Rall (1960). These workers showed that the identification of the many sulphur containing isomers in petroleum products could be achieved by microhydrogenation and gas chromatographic characterisation of the hydrocarbons produced (Hopkins, Kendall, Thompson & Coleman, 1969). Such identification of the parent hydrocarbons either identified or contributed to the identification of the precursors. This technique was further extended to compounds containing halogen, oxygen and nitrogen (Thompson, Coleman, Hopkins & Rall, 1967). Beroza and other workers further applied this method to many organic compounds including some natural products, for example insect attractants (Beroza & Acree, 1964; Brownlee & Silverstein, 1968) and alkaloids (Beroza, 1963). A similar approach has also been used in the investigation of the queen bee substance (Callow & Johnston, 1960) and in the study of fatty acids from wool wax (Downing, Kranz & Murray, 1960).

Amongst the natural products, steroids and sterols represent an important class of compounds; they occur widely in plants and in animals. Identification/

Identification of an individual steroid or sterol is complicated because they occur only in small quantities in the presence of many closely related substances. The development of chromatographic techniques, especially of gas-liquid chromatography, g.l.c. has been very useful for the identification as well as separation and quantitation of minute quantities of steroids and sterols (Horning, VandenHeuvel & Creech, 1963a; Kuksis, 1966; Horning, Brooks & VandenHeuvel, 1968). Although chromatographic and microchemical techniques for the identification of functional groups are highly developed (Bush, 1961a), the nature of the hydrocarbon skeleton has often been guessed. The complete reduction of a steroid to the parent hydrocarbon is a fundamental method for proving the nature of its carbon skeleton (Fieser & Fieser, 1959a; Klyne, 1965). However, little use had been made of this fundamental approach because techniques were cumbersome and insensitive (Steiger & Reichstein, 1938). Previously it has not been possible to apply this procedure to microgram, µg., quantities of steroids and sterols but Bush (1961b) recognised that the development of g.l.c. now allowed the separation and measurement of the parent hydrocarbons.

In the present chapter a description of the apparatus and the method for high temperature catalytic reduction of steroids and sterols is presented. The factors affecting the method and an examination of the reliability of the method are described. The present apparatus and method have been used successfully in the complete reduction of about 5 to 10 µg. of steroids and sterols to the parent hydrocarbons.

The/

The yields and ratios of the different hydrocarbon products from a number of different types of compounds have been studied at various temperatures. Different types of catalyst 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 skeletons of the starting materials. However, the yield of the parent hydrocarbons depended on the number of substituents in the original compounds.

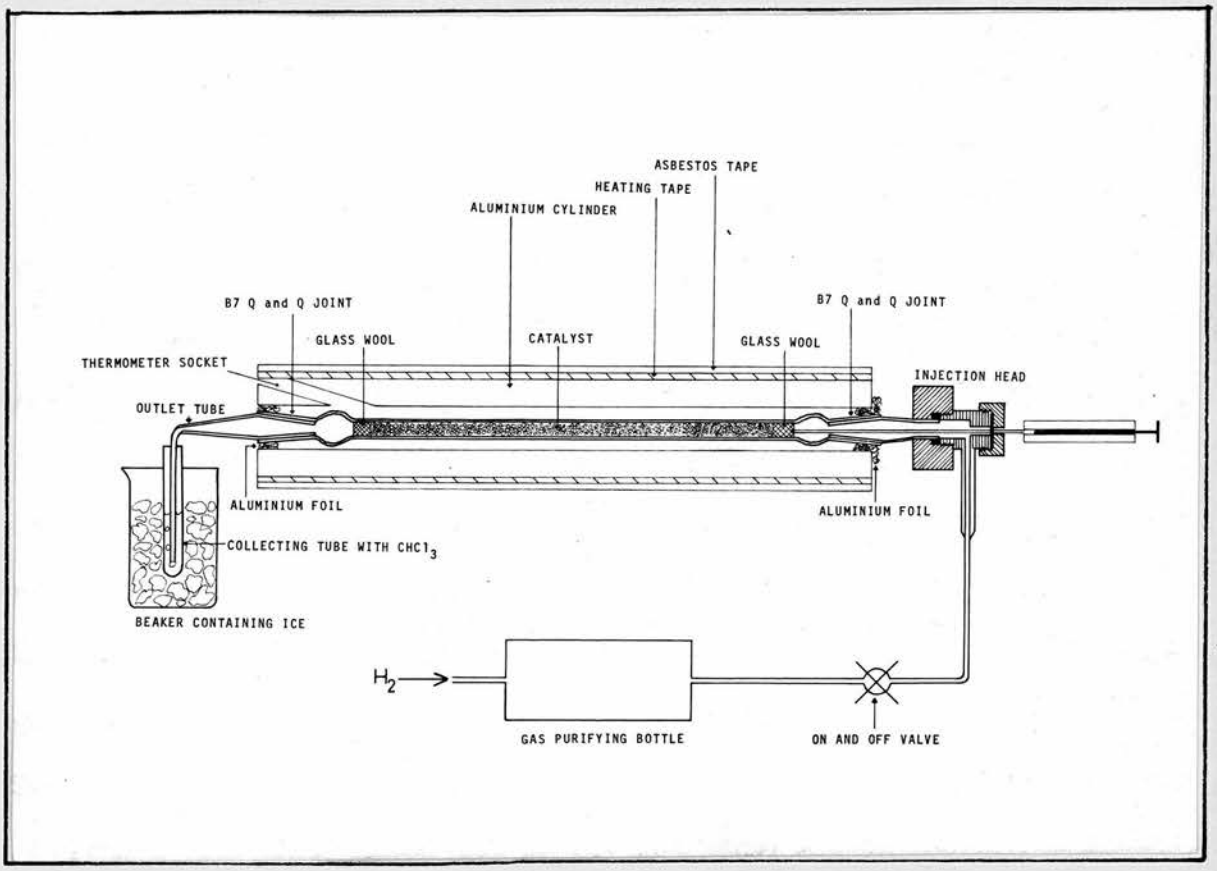

Fig. 1. Diagramatic cross section of apparatus for high temperature catalytic reduction; for details, see text.

B. APPARATUS AND MATERIALS

1. Hydrogenator.

The basic principle of the apparatus is similar to that of the "Carbon skeleton determinator" (National Instrument Laboratories, Inc., Maryland) described by Beroza & Acree (1964) and to the micro-hydrogenation apparatus of Thompson et al. (1967) in which samples were reduced by injection into a stream of hydrogen passing through a heated bed of catalyst. Modifications introduced consist of a siliconised glass tube (21.7 x 0.7 cm. outer diameter, 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 aluminium block (21.7 x 3.2cm. o.d.) drilled approximately to the size of the socket (1.27 cm. diam.), the space between the ends of the glass tube and the aluminium block was sealed with aluminium foil. A metal injection head with a rubber septum (Cat. No. A2994, Pye Unicam 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 (Fig. 1).

The shape of the 'trap tube' is similar to that described by Beroza & Sarmiento (1964). The aluminium 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.)/

(6 mm. diam.) at one end of the aluminium block was drilled to hold a thermometer. The temperature of the system could be maintained ± 0.5°C. A gas purifying bottle (Pye Unicam Ltd.) containing a 13 X type molecular sieve (crystalline sodium aluminosilicate, 1/16" pellets, 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.

2. Catalysts and support.

Platinum catalyst coated on a support of about 40 mesh siliconised glass beads (British Drug Houses Ltd. BDH, Poole, England) was used. Preliminary cleaning and deactivation of the beads was similar to the method used by Horning et al. (1963a) 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. There was less isomerisation of the A/B rings and the yield of the minor products was reduced. The catalyst tube was treated similarly. The cleaned and siliconised beads were then coated with platinum catalyst by the following procedure. The beads (50 gm.) were placed in a beaker with 100 ml. of 1% w/v ethanolic solution of chloroplatinic acid (containing 40% platinum, 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 was 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 when a reference steroid, 3α-hydroxy-5α-androstan-17-one, androsterone * was reduced. About 1-3% w/w Pt. coated on the glass beads was produced by this method. The catalyst was stored in a desiccator and/

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* In this thesis the correct descriptions of the compounds are followed by their simpler trivial names; thereafter, the trivial names are generally used.

and remained active for at least 8 months.

Platinum catalyst was also prepared by the method similar to that used by Beroza and Sarmiento (1964) for the preparation of neutral palladium catalyst. The platinum chloride solution in this case was neutralised by adding 5N alcoholic potassium hydroxide. The preparation of the palladium catalyst was similar to that for platinum. Raney nickel catalyst was prepared according to Vogel (1964).

3. Gas chromatograph.

A Pye Unicam Series 104, model 14 gas chromatograph with a flame ionisation detector and a Honeywell 1mv recorder (Honeywell Ltd. Lanarkshire, Scotland) was used. The recorder chart speed used was 12.5 cm/hr. Nitrogen (40 ml./min.) was used as a carrier gas. The flame ionisation detector was operated at the optimum conditions described by Fowlis, Maggs & Scott (1964). The injection head was modified according to Menini & Norymberski (1965). The flash heater was kept at least 40°C higher than the column temperature. Losses of C_{19} and C_{21} hydrocarbons were found to occur due to radiation from the flash heater. The recoveries obtained from these hydrocarbons were variable. These losses were avoided either by placing a board wrapped on both sides with aluminium foil between the sample holder tube and the flash heater or by using in series two Rotaflo stopcocks, Cat. No. TF2/18 (Quickfit and Quartz Ltd., England) sealed at one end to the gauze holder tube. The latter modification allows injection of single solid samples from outside thus avoiding any risk of losses of more volatile compounds from the sample holder tube.

The stainless steel gauze rings used for deposition of the sample (Dixon Gauze Rings, $\frac{1}{8}$" x $\frac{1}{8}$", Griffin & George Ltd., Wembley, England) were washed 6 times each with chloroform (A.R.), methanol (A.R.), and chloroform (A.R., distilled) in a 50 ml. round bottom flask. Traces of solvent were evaporated using reduced pressure.

The surface of the polytetrafluoroethylene, PTFE dimpled plate used for sample deposition was smoothed by the method of Menini & Norymberski (1965).

Glass wool was cleaned and siliconised by a method similar to that used for the glass beads.

4. Gas chromatograph columns.

Coiled glass columns 152.4 cm. and 121.9 cm. long and 0.4 cm. internal diameter were packed by the method of Horning et al. (1963a). The supports, 100-120 mesh Gas Chrom P (Applied Science Laboratories, State College, Pa.) and acid washed and siliconised 100-120 mesh Supasorb (BDH) were siliconised 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 separations of the hydrocarbon produced by the non-selective phases, SE-30 and JXR, were generally confirmed by the selective phase NGA which retains compounds with carbon-carbon unsaturated groups (Horning, Luukkainen, Haahti, Creech & VandenHeuvel, 1963b). Tentative identification of the products was on the basis of retention times using these columns. The number of theoretical plates on the SE-30, JXR and NGA coated columns at 210°C using 5α-cholestane were 1729, 2152 and 1850 respectively. The separation factors and the relative retention times of the reference hydrocarbons on SE-30 and on NGA columns are presented in Tables 1 and 2. The values obtained on the SE-30 coated columns are similar to those of Brooks & Hanaineh (1963).

For quantitation of the hydrocarbons produced peak heights were measured and compared with those from known quantities of similar external reference hydrocarbons.

TABLE 1

SEPARATION FACTORS OF THE REFERENCE HYDROCARBONS

Columns	Column temperatures	Hydrocarbons	Separation Factors
SE-30	210°C	5β:5α-Cholestane	1.11
SE-30	150°C	5β:5α-Pregnane	1.12
SE-30	150°C	5β:5α-Androstane	1.12
NGA	210°C	5β:5α-Cholestane	1.13
NGA	150°C	5β:5α-Pregnane	1.14
NGA	150°C	5β:5α-Androstane	1.13

TABLE 2

MEAN RETENTION TIMES ± S.D. OF THE REFERENCE HYDROCARBONS

RELATIVE TO 5α-ANDROSTANE (AS 1).

Columns	Column temperature	Hydrocarbons	Relative retention times
SE-30	150°C	5β-Androstane	0.88 ± 0.01 (6)
SE-30	150°C	5β-Pregnane	2.01 ± 0.02 (6)
SE-30	150°C	5α-Pregnane	2.27 ± 0.02 (6)
NGA	150°C	5β-Androstane	0.88 ± 0.01 (6)
NGA	150°C	5β-Pregnane	1.86 ± 0.01 (6)
NGA	150°C	5α-Pregnane	2.13 ± 0.02 (6)

Number of estimations in parentheses.

5. Spectroscopy.

Infrared, i.r., spectroscopy on a micro scale was performed on a Unicam SP200 spectrophotometer. The samples were deposited as a small spot on a polished NaCl plate the spot was then placed at the point of focus of a beam condenser (Sykes & Kelly, 1966). Unicam SP600 spectrophotometer was used for visible regions.

6. Reference standards, reagents and solvents.

All chemicals used were analytical grade where possible. Absolute ethanol (A.R.) was refluxed with solid potassium hydroxide for 12 hours and followed by fractional distillation. Chloroform (A.R.) was redistilled. These solvents were checked for high boiling impurities by depositing about 0.5 ml. on gauze rings and running in g.l.c. for at least 30 minutes using the SE-30 column at 210°C. No rubber or plastic implements were permitted to come into contact with the solvents or the solutions.

The steroids and sterols used were either purchased from the chemical manufacturers or procured as gifts from various sources (see acknowledgments). They were pure when checked by thin-layer chromatography, t.l.c. and in some cases also by g.l.c. except where otherwise noted.

7. Thin-layer chromatography.

Glass plates (20 x 20 cm . and 10 x 20cm .) were coated with silica gel G and silica gel GF_{254} (E. Merck A.G., Darmstadt) in 250 μ thickness using a Quickfit t.l.c. set. The plates were dried in an oven at 100-105°C for/

for 1 hour and stored in a glass tank containing anhydrous silica gel. For t.l.c. the rectangular glass tanks with ground glass lids were kept in a room at 25° ± 1°C. The solvents were saturated and equilibrated overnight by lining one side of the glass tanks with Whatman No. 42 filter papers. The samples in chloroform or in absolute ethanol were dispensed using a 10 µl. Hamilton micro-syringe (Hamilton Company, Whittier, California.) The solvent systems used were chloroform; chloroform - acetone (9:1v/v); chloroform-methanol (19:1, 9:1 and 4:1 v/v) and chloroform-methanol-water (95:35:4 v/v). The last two solvent systems were used only for more polar compounds with 3 or 4 hydroxyl groups.

The steroids and sterols were detected either by their absorption of ultraviolet light, 254 mµ, max. (Hanovia Lamps, Slough, England) on fluorescein impregnated plates or with 3% w/v. phosphomolybdic acid in methanol. This reagent was acidified with a few drops of conc. HCl before use. For detection of steroids with a 17-ketone group on thin-layer plates Zimmermann reagent (Corker, Norymberski & Thow, 1962) was used. The plates were sprayed with a mixture of 1:1 v/v ethanolic solution of m-dinitrobenzene (0.5% w/v) and alcoholic potassium hydroxide (5N). Potassium hydroxide was first dissolved in 1 vol. of distilled water and 2 vol. of ethanol added. The plates were then heated in an oven at 60°C with frequent inspections.

For chromatography of the hydrocarbons on thin-layer plates chloroform or cyclohexane was used. For detection, 5% (w/v) iodine solution in chloroform/

chloroform or iodine vapour was employed. 8% (w/v) phosphomolybdic acid in methanol can also be used for detection; about 2-5 µg. could be detected in about 0.7 cm. diameter spot. These solvent systems on silica gel plates, however, do not separate the androstanes, pregnanes and cholestanes as these hydrocarbons run with the same mobility. The R_F values with the solvents cyclohexane and chloroform were 0.6 and 0.7 respectively. However, this thin-layer separation provided evidence for the presence of any unreduced or partially reduced starting materials.

8. Colour reactions.

When the Zimmermann reaction was used for quantitative estimations in solution the reagents were prepared by dissolving 2 vol. of 0.5% (w/v) m-dinitrobenzene in ethanol with 1 vol. 40% w/w benzyltrimethylammonium-hydroxide. The steroid residues, two known quantities of the standard androsterone (10 µg. each) and two reagent blanks were added with o.1 ml. of m-dinitrobenzene and 0.5 ml of benzyltrimethylammoniumhydroxide. The mixtures were incubated at 25°C in a waterbath for 1 hr. and diluted with 3 ml. of ethanol. Extinctions were measured at 440, 520 and 600 mµ and the results were corrected by the method of Allen (1950).

C. PROCEDURE FOR HIGH TEMPERATURE CATALYTIC REDUCTION.

The catalyst was packed into the glass tube after plugging one end with some siliconised glass wool (Fig. 1). The total weight of support and catalyst used to prepare one tube was approx. 2.25 gm. The tube containing catalyst was then put inside the aluminium block and charged for 3 hrs. 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. Since 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 indicators' solution (BDH). At the end of the charging period, when the effluent was neutral, three injections of 10 µl. absolute ethanol were given at 5 minute intervals. This virtually eliminated the production of compounds which did not correspond to steranes on g.l.c. 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 x 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/

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 an interval of 5 minutes ensured complete elution of any removable compounds which had been retained in the catalyst bed (See Chapter I, DIII 6). In order to increase the recoveries the first blank injection of 10 µl. of absolute ethanol was made at the end of each trapping period as the products were being collected. The whole operation was carried out on a bench fitted with an exhaust fan or inside a fume cupboard. No interruption of gas flow was made throughout the procedure. At the end of procedure as soon as the heater and gas supply were turned off the catalyst tube was closed by a suitable glass stopper. The reduction products which have been trapped in the chloroform were transferred on to a PTFE plate for deposition onto stainless steel gauzes for injection into the g.l.c. 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.

The molecular sieve for drying the hydrogen gas was used as a precautionary/

precautionary measure. In many instances, however, activity of the catalyst was not altered when the molecular sieve was not connected to the gas cylinder.

Spent catalysts were stored under water in a labelled bottle and were discarded into a water carriage drainage system.

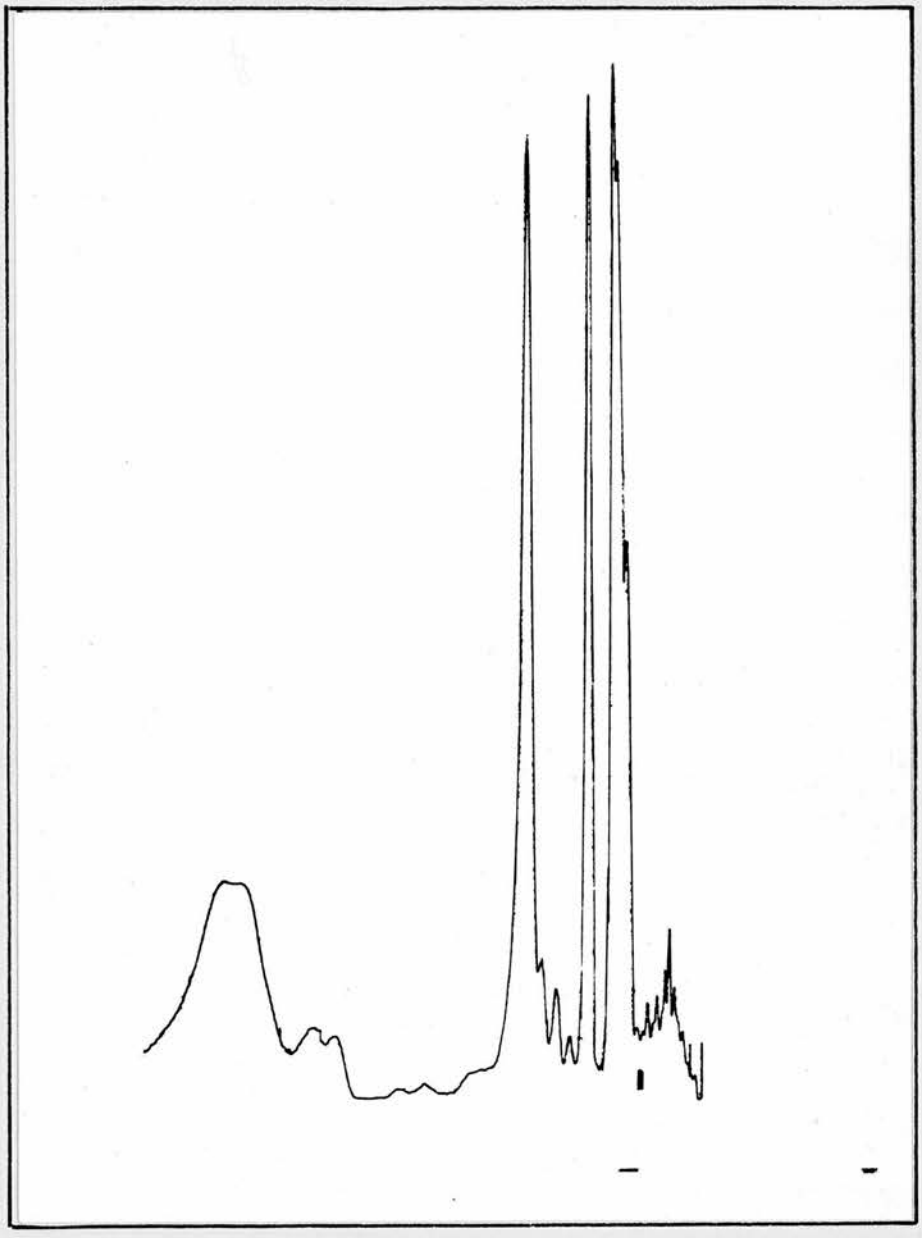


Fig. 2. Reduction products from 10 µg. of androsterone using the "carbon skeleton determinator" at catalyst temperature of 200°C. Chromatographed on an SE-30 coated column at 160°C, attenuation x 500. The retention time of 5α-androstane is indicated by a vertical line. The start of the chromatogram is from the right side.

D. RESULTS - DEVELOPMENT AND EVALUATION OF THE METHOD.

I. Experiments using the previous hydrogenator.

In the present investigation, the "Carbon-skeleton determinator" apparatus previously described (Beroza & Acree, 1964) was found unsatisfactory 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. The products obtained from androsterone are presented in Fig. 2. No products corresponding with the parent steranes were present. A product with the retention time of androsterone was present. Similar results were obtained from 3α-hydroxy-5β-androstan-17-one, aetiocholanolone and 17β-hydroxyandrost-4-en-3-one, testosterone. The results were not improved after siliconisation 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.

II. Development and use of the present hydrogenator and method.

With the present siliconised glass apparatus, unchanged steroid could be recovered quantitatively from the system without catalyst and its support. A steroid, androsterone, was injected into a hydrogen gas stream flowing through the tube and trapped at the outlet of the system. The trapped steroid was estimated by the Zimmermann colour reaction and by g.l.c./

TABLE 3

EFFECT OF TEMPERATURE ON YIELD OF HYDROCARBONS FROM C_{19} STEROIDS

Compounds	Temperature range	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°C	5.26 ± 1.73 (5)	0.94 ± 0.08 (5)	1 : 0.17
	160 - 170°C	9.84 ± 0.75 (5)	2.38 ± 0.10 (5)	1 : 0.24
	180 - 190°C	14.30 ± 2.50 (5)	4.18 ± 0.15 (5)	1 : 0.29
	200 - 210°C	20.48 ± 2.68 (5)	4.10 ± 0.51 (5)	1 : 0.20
	220 - 240°C	23.04 ± 7.11 (6)	3.12 ± 1.23 (6)	1 : 0.13
3α-Hydroxy-5β-androstan-17-one (Aetiocholanolone)	140 - 150°C	1.96 ± 0.97 (5)	4.18 ± 0.76 (5)	1 : 2.13
	160 - 170°C	2.76 ± 0.64 (6)	6.68 ± 0.65 (6)	1 : 2.42
	180 - 190°C	4.38 ± 0.67 (5)	9.16 ± 1.07 (5)	1 : 2.09
	200 - 210°C	9.38 ± 1.16 (5)	4.78 ± 1.66 (5)	1 : 0.50
	220 - 240°C	14.66 ± 4.24 (5)	3.50 ± 0.64 (5)	1 : 0.23
17β-Hydroxyandrost-4-en-3-one (Testosterone)	140 - 150°C	1.14 ± 0.22 (5)	1.31 ± 0.16 (5)	1 : 1.14
	160 - 170°C	4.88 ± 0.90 (6)	5.32 ± 0.98 (6)	1 : 1.09
	180 - 190°C	6.84 ± 2.05 (6)	4.20 ± 1.11 (6)	1 : 0.61
	200 - 210°C	14.30 ± 2.25 (5)	5.48 ± 1.15 (5)	1 : 0.38
	220 - 240°C	15.64 ± 0.93 (5)	4.44 ± 0.98 (5)	1 : 0.28

TABLE 3 (Contd.)

Compounds	Temperature range	Mean percentage yield (± S.D.) 5α-androstane	Mean percentage yield (± S.D.) 5β-androstane	Ratio of products 5α-androstane : 5β-androstane
3β-Hydroxyandrost-5-en-17-one (Dehydroepiandrosterone)	140 - 150°C	1.10 ± 0.01 (5)	0.32 ± 0.05 (5)	1 : 0.29
	160 - 170°C	5.58 ± 0.87 (6)	2.04 ± 0.59 (6)	1 : 0.36
	180 - 190°C	7.32 ± 1.16 (5)	2.48 ± 0.63 (5)	1 : 0.33
	200 - 210°C	13.60 ± 0.92 (5)	2.90 ± 0.55 (5)	1 : 0.21
	220 - 240°C	14.12 ± 2.72 (5)	2.64 ± 0.93 (5)	1 : 0.18

The numbers of estimations are in parentheses.

g.l.c. In two series of 6 experiments using each method of determination, 72.9 ± 5.3% (6) (mean ± S.D.) of the androsterone was recovered unchanged as estimated by g.l.c. and 78.5 ± 9.2% (6) (mean ± S.D.) of the starting material was recovered as estimated by the Zimmermann reaction. From all these results it became obvious that decomposition of the starting material was occurring inside the hot metal surface of the catalyst chamber and that this could be avoided using a glass system.

III. Investigation of the factors altering results from the method.

With the present apparatus factors associated with the optimum operating conditions were studied.

1. 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 3. It is to be noticed that with the rise of temperature there was a linear increase in the yield of hydrocarbons, accompanied by an increase in the relative amount of the more stable 5α isomer. The number and amount of minor products (Fig. 4) was also increased. At the 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-androstane and the ratio of the amounts of these two products were reproducible as shown in Table 4. From the results in Table 4 it can be seen that the selection of a low operating temperature provides more information on the structure of the starting materials. The results in Table 4 show that the yield was partially related/

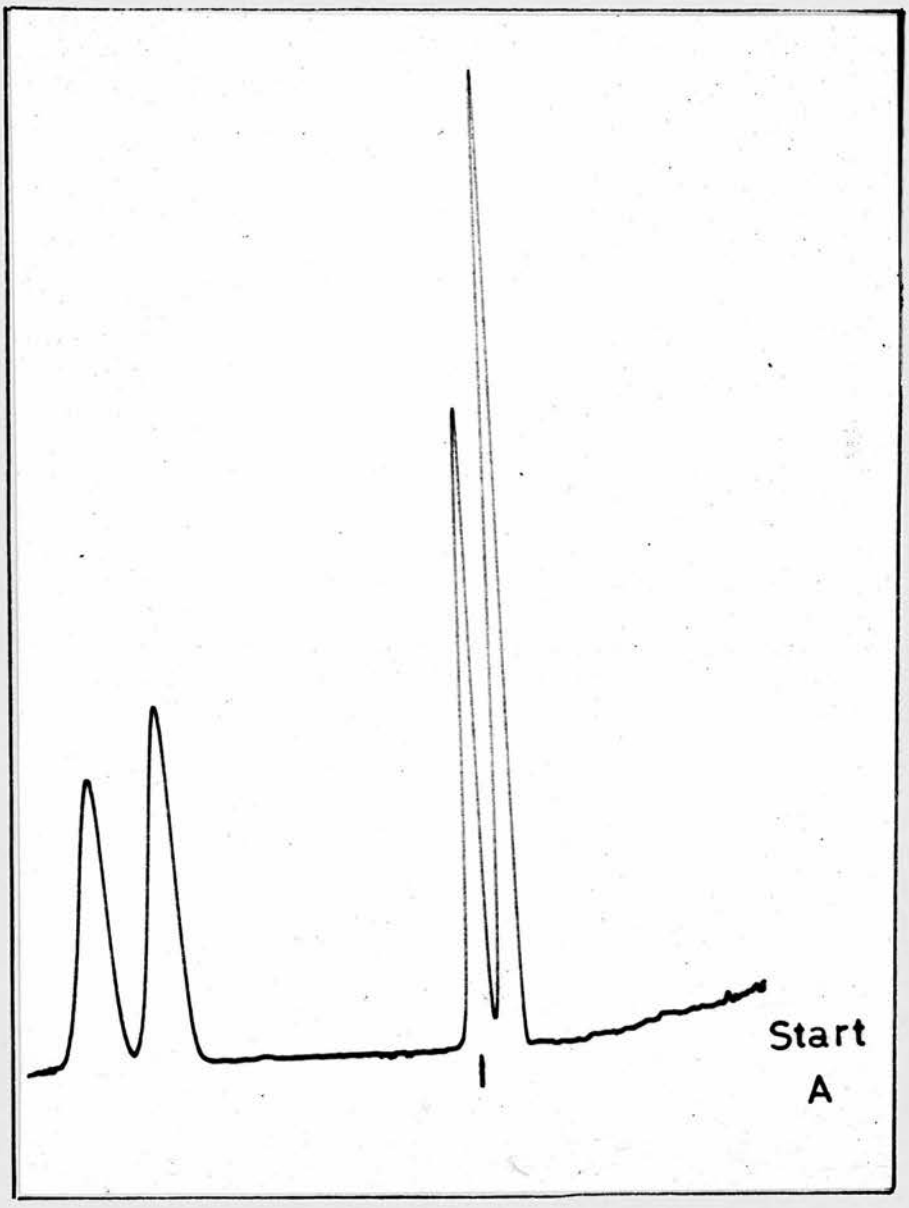
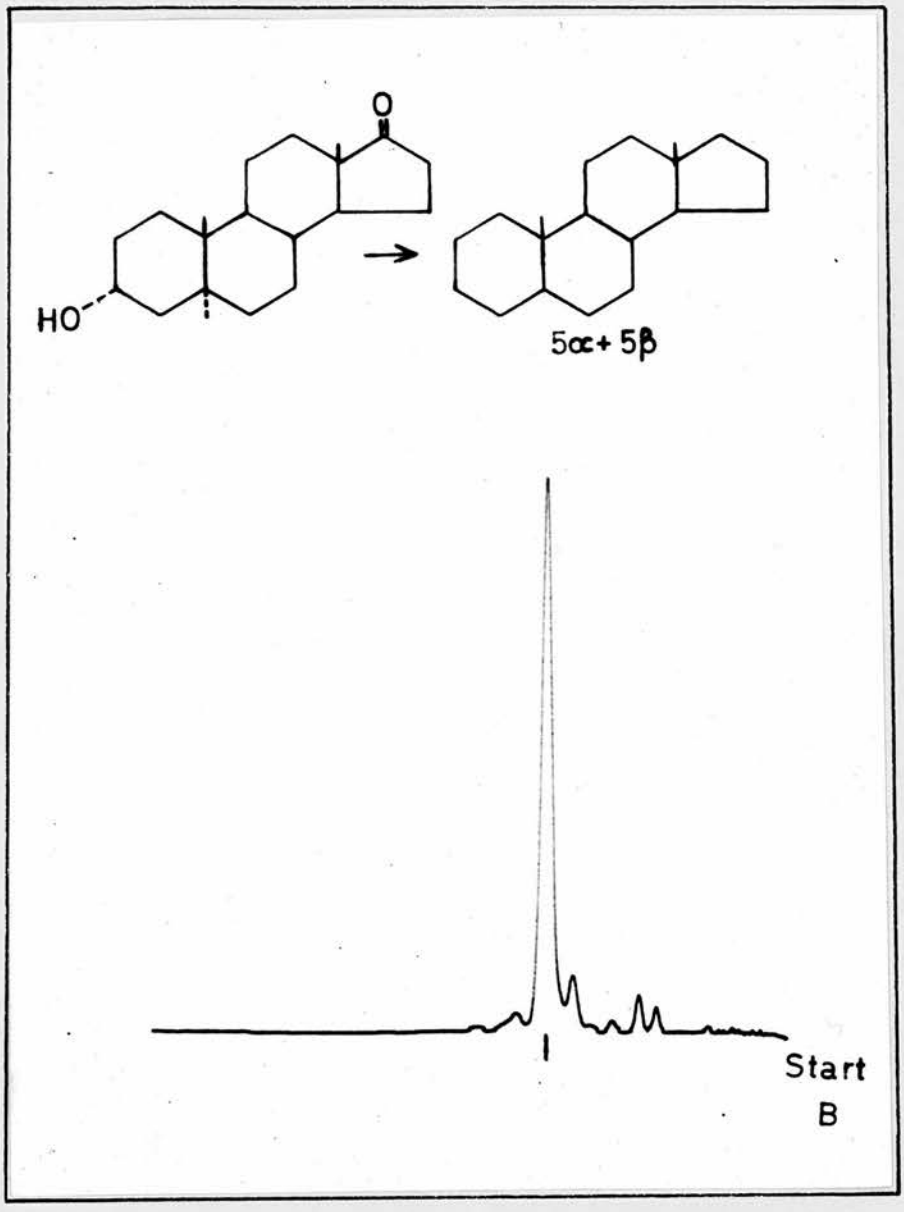


Fig. 3. Gas chromatogram of the standard steranes on a JXR coated column at 140°C, attenuation x 100. In order of increasing retention time, the peaks are 5β-androstane (0.30 µg.), 5α-androstane (0.25 µg., marked by a vertical line), 5β-pregnane (0.30 µg.) and 5α-pregnane (0.25 µg.).



Reduction products from 2.5 µg. of androsterone at catalyst temperature of 170°C chromatographed on a JXR coated column at 140°C, attenuation x 100. The retention time of 5α-androstane is indicated by a vertical line. For the reference steranes, see Fig. 3.

TABLE 4

EFFECT OF CHEMICAL STRUCTURE ON THE YIELD OF HYDROCARBONS FROM DIFFERENT C_{19} STEROIDS AT THE 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)	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 (Aetiocholanolone)	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

contd.

TABLE 4 (Contd.)

Compounds	Mean percentage yield (± S.D.) of 5α + 5β -androstane	Ratio of products 5α-androstane : 5β-androstane
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 ± 0.30 (4)	1 : 0.08

The numbers of estimations are in parentheses.

related to the 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 (Beroza & Sarmiento, 1963).

2. Catalysts and Supports. - Some commonly used catalysts and supports were investigated. Palladium and platinum coated on diatomaceous earth supports as described (Beroza & Acree, 1964; Beroza & Sarmiento, 1965) were not satisfactory. The reduction products contained two major components corresponding to the parent hydrocarbons but their separation was not complete.

With platinum on siliconised 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 non-volatile alkali was added to neutralise the catalyst during preparation as with some steroids the presence of alkali can cause decomposition and isomerisation (Reichstein & Shoppee, 1949). It was also found that an alkaline catalyst was less active than the neutral catalysts. Two steroids aetiocholanolone and androsterone were reduced at 200°C by a neutral platinum catalyst prepared with alcoholic potassium hydroxide (see Chapter I, B2). The yields obtained were about 3% although the reduction patterns were similar.

Beside platinum, palladium and nickel were also tried on a siliconised glass support using androsterone as the starting material. The patterns obtained were similar to those from platinum but the yields of hydrocarbons were/

were comparatively low. The mean yields at the catalyst temperature 210°C of 5α and 5β androstane with an estimate 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). The number of experiments are shown in brackets. So far the platinum catalyst coated on glass beads has given the most satisfactory results and unless otherwise stated all the work reported in this thesis has been carried out using this catalyst and support.

3. Concentration of the platinum catalyst. The effect of an increased concentration of platinum on the reduction of 17β-hydroxy-19-norandrost-4-en-3-one, 19-nortestosterone and 3β, 16α-dihydroxyandrost-5-en-17-one, 16α-hydroxydehydroepiandrosterone was studied. When the amount of platinum chloride was doubled (e.g. 2 gm. on 50 gm. of glass beads) the products with the retention times relative to 5α-androstane of 0.30, 0.32 from 19-nortestosterone (see Fig. 15(A) Chapter IV) and of 0.33, 0.34 from 16α-hydroxydehydroepiandrosterone (see Fig. 11A Table 15 Chapter III, Section I) were obtained in higher yield. From 16α-hydroxydehydroepiandrosterone the parent sterane, 5α-androstane was a minor product. This suggests that the increased concentration of platinum causes extensive hydrogenolysis.

4. 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/

the basis of retention times. The symmetrical and sharp peaks of the products in g.l.c. are characteristic of hydrocarbons (Beroza & Acree, 1964) and because of this property and their high thermal stability these compounds are used as internal standards in many gas chromatographic investigations (VandenHeuvel & Horning, 1962; Brooks & Hanaineh, 1963).

Microchemical treatment of these products from androsterone, aetiocholanolone, testosterone and dehydroepiandrosterone with 3 N sulphuric acid for 30 mins, 3 N sodium hydroxide for 30 mins., oxidation with 10% aqueous chromium trioxide for 15 mins., and acetylation with acetic anhydride and pyridine overnight did not change the retention times.

The i.r. spectra of the products obtained after reduction of androsterone (Table 5), 5α-androstan-3β-ol and 3β-hydroxy-androst-5-ene had identical absorption maxima to that of authentic 5α-androstane. About 100 µg. of the saturated product from each of these compounds was produced by hydrogenation of a number of 5 - 10 µg. samples.

The reduction products from cholesterol (Fig. 6(B)) were analysed using an LKB - 9000-g.l.c. MS combination. The mass spectra showed that the peaks with the retention times of 5α and 5β-cholestanes had the expected molecular weights of 372 (Table 6) and the fragmentation was similar to that of cholestane (Friedland, Lane, Longman, Train & O'Neal, 1959).

The hydrocarbon skeleton from a C_{19} steroid isolated from urine identified by the present method was consistent with evidence obtained using i.r. spectroscopy, g.l.c. with mass spectrometry and direct g.l.c. (Chapter III, Section I C2 & 3). In addition, retention times of the main products of reduction/

TABLE 5.

INFRARED ABSORPTION MAXIMA OF THE REFERENCE 5α-ANDROSTANE AND ANDROSTERONE REDUCTION PRODUCTS

Compounds	Frequency (cm -1)
Reference	2950 s
5α-Androstane	2870 s
	1460 m
	1380 m
	1375 m
Androsterone reduction products	2950 s
	2870 s
	1460 m
	1380 m
	1375 m

s = strong absorption

m = medium absorption

TABLE 6

PARTIAL * MASS SPECTRA OF CHOLESTEROL REDUCTION PRODUCTS.

Compounds	Mass units (m/e)	% abundance
Product corresponding to 5α-cholestane in g.l.c.	41	40
	43	55
	55	64
	57	32
	67	47
	69	31
	81	53
	93	25
	95	51
	109	54
	123	25
	149	54
	217	100
	218	56
	357	29
	372	34
Product corresponding to 5β-cholestane in g.l.c.	41	43
	43	53
	55	61
	57	33
	67	44
	69	30
	81	52
	95	54
	109	52
	149	48
	217	100
	218	60
	357	34
	372	38

* Only mass units showing abundance of above 25% have been shown.

reduction of a number of steroid drugs with common hydrocarbon skeletons were identical (Chapter II, C1 & 2). From this evidence it was concluded that the major products of reduction were the saturated parent hydrocarbons.

5. Trapping. - The efficiency with which the reduction products were trapped was investigated. Table 7 shows the recoveries of C_{19} hydrocarbons after their passage through the complete system at temperatures between 170°C and 200°C. These recoveries are calculated by using as 100% the results obtained by direct estimation of the hydrocarbons by g.l.c. using solid injection and direct liquid injection. The present results agree well with previous work; using liquid nitrogen as a cooling mixture recoveries from 84-88% have been reported for some radioactive steroids and sterols (Brooks & Godefroi, 1964). 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. In order to reduce these large losses a small volume of solvent should be used for transfer to the gauzes. These hydrocarbons could be trapped even without cooling the collecting tubes. In a series of 5 experiments with no cooling arrangements the recoveries were 39.5 ± 10.7 (mean ± S.D.). The results agree closely with those of Brooks & Godefroi (1964) who obtained recoveries at room temperature from 31.2 - 40.3% of injected cholesterol-4-C-14 using a similar type of collecting tube.

A check on the presence of any more volatile products which would otherwise/

TABLE 7.

COMPARISON OF THE RECOVERY OF THE HYDROCARBONS BY DIFFERENT TRAPPING PROCEDURES.

Mean percentage recovery ± S.D.

Compounds	Products trapped with ice and water cooling mixture: estimation by solid transfer to g.l.c.	Products trapped with solid CO_2 and acetone mixture: estimation by direct injection as solution
5β-Androstane	70.5 ± 5.2 (7)	84.0 ± 2.2 (4)
5α-Androstane	77.9 ± 4.2 (7)	87.5 ± 2.1 (4)

Number of estimations in parentheses.

otherwise be lost during trapping and evaporation from PTFE plate was made. The reduction products from androsterone were trapped using a cooling mixture of solid CO_2 and acetone. The products were dissolved in cyclohexane and injected as a solution directly into the g.l.c. column. The chromatograph was temperature programmed from 74° - 146°C at 2°C/min. and then run isothermally. A control experiment using solid injection was also performed under the same conditions. No other products besides those present in the control experiment were seen. However, the injected solvent does prevent the detection of products with short retention times.

6. "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 androsterone was reduced at the catalyst temperature of 200°C the mean percentage recoveries of the retained 5α and 5β-androstane by four serial injection of 10 µl. of absolute ethanol at 5 min. intervals were 9.5, 5.2, 0.9 and 0.06% of the initial sample. These means were derived from the results of 3 sets of experiments.

Two urinary extracts used in routine investigations were studied similarly following injections of 50% ethanol (v/v). The results (Table 8) are the means from two sets of experiments. After the third injection virtually all the retained products were eluted. Though the injections of 50% ethanol inactivate the catalyst more rapidly, it was found necessary to remove polar impurities. When analysing an unknown specimen a longer trapping period should be allowed as the more polar compounds are retained longer by the catalyst (Beroza, 1962a).

TABLE 8.

RECOVERIES OF THE RETAINED SAMPLES FROM THE CATALYST WITH SERIAL 50% ETHANOL INJECTIONS

Steroids from Urinary Extracts	Serial 10 µl. of ethanol injections	Percentage recovery of the retained 5α + 5β-androstane
Testosterone (after paper chromatography)	1	7.5
	2	1.3
	3	0.1
	4	0.0
Aetiocholanolone (after paper chromatography)	1	8.7
	2	2.0
	3	0.3
	4	0.0

7. Flow rates. The effect of different flow rates of hydrogen on the yield of 5α + 5β-androstanes from 5β-androstan-3, 17-dione were studied at the catalyst temperature of 210°C. 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 (Beroza & Acree, 1964). Under similar conditions, a higher flow rate of 60 ml./min. and a longer trapping time of 2 minutes were required for the collection of C_{27} hydrocarbons. The percentage recoveries of 5α + 5β-cholestane from cholest-5-en-3β-ol, cholesterol were 7.5, 12.0 and 20.3 at the flow rates of 20, 40 and 50 ml./min. respectively. These experiments were repeated twice. This may be due to their higher molecular weights.

8. Sample size. With the present length of catalyst bed (17.5cm.) samples from 5 - 10 µg. were generally injected at one time. Even as little as 1 µg. of androsterone, testosterone and 17α-methyl-17β-hydroxyandrost-1, 4-dien-3-one, methandrostenolone have 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 analysed in one working day.

9. Stereoisomerisation. The extent of isomerisation of C_{19} hydrocarbons when passed through the hydrogenator containing catalyst at the temperature of 170°C and 215°C was studied, and it was found that a mean of 4.1% and 5.8% respectively of 5β-androstane isomerised to 5α-androstane. The isomerisation/

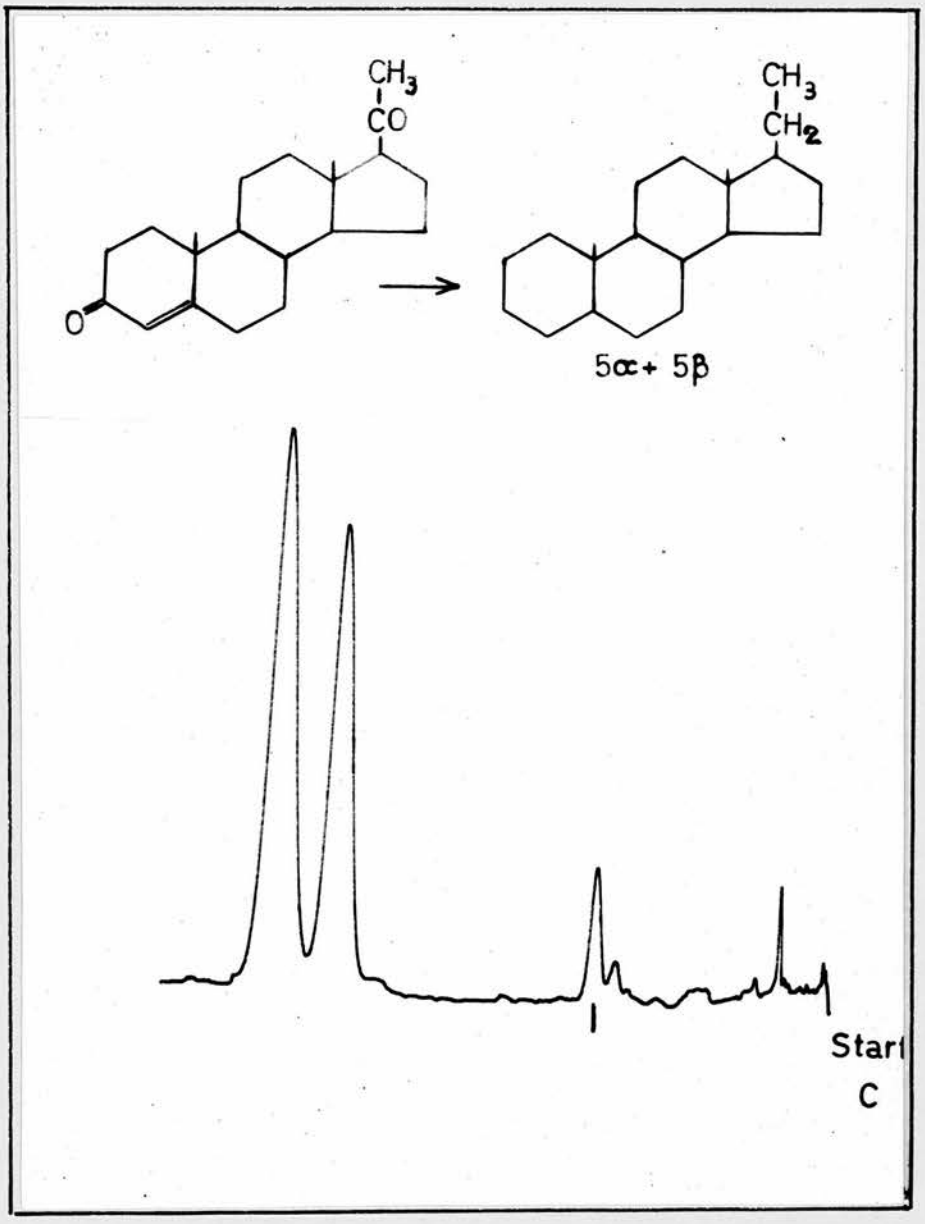
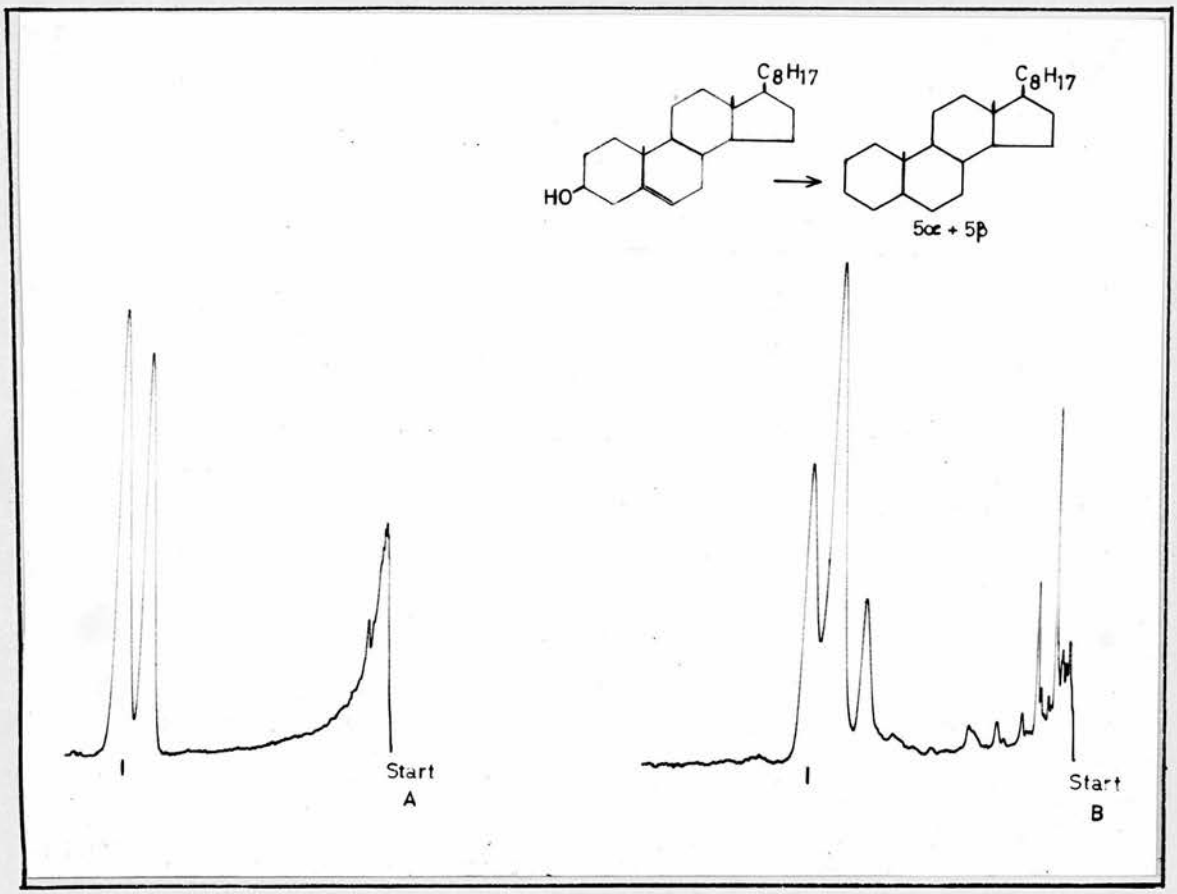


Fig. 5. Reduction products from 5 µg. of progesterone at catalyst temperature of 180°C chromatographed on a JXR coated column at 140°C, attenuation x 100. The retention time of 5α-androstane is indicated by a vertical line. For the reference steranes, see Fig. 3.



6. Gas chromatograms on a JXR coated column at 195°C, attenuation x 200 of

   (A) Standard 5β-cholestane (0.50 µg.) and 5α-cholestane (0.56 µg., indicated by a vertical line).

   (B) Reduction products of 5 µg. of choles-:terol at catalyst temperature of 190°C.

The retention time of 5α-cholestane is indicated by a vertical line.

TABLE 9.

REDUCTION OF C_{27} COMPOUNDS, EFFECT OF DIFFERENT TEMPERATURES AND THE NATURE OF CATALYST ON THE 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)	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)

Number of estimations in parentheses.

isomerisation of 5α-androstane to 5β-androstane when studied under the same conditions were 2.0% and 2.8% respectively. A greater isomerisation of the androstanes was noted before siliconisation of the glass beads used as a support. The amount of the starting material isomerised with the unsiliconised glass beads was 11.2% for 5β-androstane and 10.5% for 5α-androstane at temperatures between 210-215°C. More marked isomerisation was noted with the cholestanes (Table 9). No rearrangement of these hydrocarbons to any other products with longer retention time was found to occur at these temperatures. These experiments were each repeated four times.

The tracings from g.l.c. of the reduction products obtained from some common steroids and a sterol are presented in Figs. 4, 5 & 6. The major reaction products have similar retention times to those of the parent hydrocarbons. The retention time of 5α-androstane has been marked at the base line in the tracings of the products from androsterone and pregn-4-en-3, 20-dione, progesterone. The products from cholesterol, show more 5β-cholestane compared to the 5α-cholestane, the retention time of which is marked on the tracing (Fig. 6(B)).

When cholesterol was reduced at different temperatures the ratios of 5α to 5β-cholestanes were found to change as is shown in Table 9. It is to be noticed that the amount of 5β-cholestane decreased as the temperature increased. When the catalyst was made acid by injections of 1N H_2SO_4 in 50% ethanol (10 µl. x 3) the results obtained with these C_{27} compounds were similar to those obtained with C_{19} and C_{21} steroids, the major product being/

being the stable 5α isomer.

After charging the catalyst three injections of absolute ethanol 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 isomerisation 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 isomerisation 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 g.l.c., similar results were obtained from 19-nortestosterone and aetiocholanolone. Similar results were also obtained when these compounds were introduced in cyclohexane in place of ethanol.

E. DISCUSSION

1. Decomposition of the steroids due to heated metal surfaces and improvements after using a siliconised glass system.

Previously, this technique has been used mainly on more stable compounds than steroids. In steroid gas chromatography problems arising due to decomposition on a heated metal surface have been reduced by inserting a glass tube inside the flash heater (Wotiz & Clark, 1966). An all glass column system and siliconisation of the surfaces have been recommended for working at high temperatures (VandenHeuvel & Horning, 1964). The results obtained in the present investigation using the "Carbon skeleton determinator" suggest that heated metal surfaces should be minimised for reduction of steroids, sterols and possibly other compounds of higher molecular weight. The recommendation of a glass catalyst tube to minimise surface catalytic effects has been made (Komarewsky & Reisz, 1948). This is also consistent with the recommendation of Beroza & Coad (1966) who have previously described alterations to the apparatus for reduction of different classes of compounds. The results of the present investigations show that it was necessary to redesign the entire assembly for the reduction of the relatively high molecular weight steroids and sterols. The present modifications of the apparatus and the catalyst preparations have avoided decomposition of the starting materials; in the present studies the major reaction products have always had the retention times of the expected saturated hydrocarbons.

2./

2. Hydrogenolysis.

The amounts of minor products with shorter retention times than the saturated hydrocarbons are 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 but this has never exceeded the yield of the parent hydrocarbons in studies with C_{19} steroids up to a reduction temperature of 240°C. 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 or hydroxylated steroids (see Chapter II, C3, Table 13) 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 Fig. 5 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 could be due to the removal of the side chain from the C-17 position. These products which may have lost the side chain and the other products possibly due to cleavages at other positions in the side chain are also present in the reduction products from cholesterol (Fig. 6(B)). 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 (Friedland et al, (1959) and in high temperature catalytic reactions of several types of compounds (Beroza, 1962b; Valenta, 1963; Thomson et al, 1967). Beroza & Acree (1964) have compared and contrasted/

contrasted this method with the more complex methods of mass spectrometry and pyrolysis.

3. Reduction at 170-200°C and higher temperatures. Factors affecting the steric course of reduction.

Most of the steroids and sterols so far studied were reduced smoothly at temperatures between 170-200°C and this is found to be the most suitable range for complete reduction. 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 (McQuillin, 1963) and provide information regarding the chemical structure of the starting substance. 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 (Hadler, 1955). However, an increase of temperature changes the steric course of the reaction and favours 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. Increased formation of the stable 5α form at these temperatures was associated with the formation of products with longer retention times than the parent hydrocarbons. This effect was more pronounced with the steroids having an oxygen function at the C-11 position and with sterols at the temperature of 350°C. Such an increased formation of the stable 5α form was also produced by an acid catalyst at low temperatures of about 180°C. Lewis and Shoppee (1955) have used strong acid as a promoter for catalytic reduction of sterols and in the present work an increase in the temperature of reduction also increased/

increased the yield as well as altering the steric course.

4. Reduction of cholesterol and related compounds.

In the cholestane series the structure of the starting material also affected the yield. A difference was noted in the amount of 5α-cholestane produced from the compound 5α-cholestan-3β-ol compared to that from cholesterol (Table 9). The percentage yield of saturated hycrocarbons from this compound was about 2.5 times more than those from other compounds and a comparatively smaller amount of other products with shorter retention times than the parent hydrocarbons were obtained. The amount of the product with a retention time of 0.77 relative to 5α-cholestane running before 5β-cholestane (Fig. 6(B)) was much less with this compound; this product was absent at reduction temperatures above 270°C and also after the use of an acid catalyst with the compounds mentioned in Table 9 both procedures leading to more of the stable 5α-isomer. The concept of catalyst hindrance (Linstead, Doering, Davis, Levine & Whetstone, 1942) the effects of the bulky nature of the angular methyl group (Robinson, 1957) and the nature of the substituents at C-3 position (Lewis & Shoppee, 1955) may be important, at least in part, in explaining these findings.

5. The choice of catalysts and supports.

The choice of the proper catalysts and supports has been found to be important. The present results 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/

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. Thermal decomposition of the pesticide 'Endrin' during gas chromatography was found to be due to surface active sites on the diatomaceous earth supports; glass beads, were free from such active sites (MacDonall & Eaton, 1968). In the present studies it was also found desirable to deactivate the glass surfaces by prolonged treatment with dichlorodimethylsilane. 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 (Beroza, 1962b). Palladium coated on glass beads was found unsatisfactory by previous workers (Thompson et al, 1962).

The diatomaceous earth supports, Chromosorb P and Gas Chrom P, gave products which were not clearly separable. This could be due to 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. The removal of hydroxyl and acetate groups by Chromosorb P (Böttcher & Meijer, 1961) and the catalytic activity of such chemically treated diatomaceous earths (Komarewsky & Reisz, 1948) have been described.

6. "Memory effect" and the separation of the catalytic and chromatographic systems.

The absorption of the reduced products on to the catalyst bed is noticeable/

able especially with the polar compounds. In a continuous catalysis and chromatographic system as described by the previous authors, such a slow release of the reduced products has been overcome by the selection of a proper support for the catalyst (Beroza, 1962b) and by injection of polar liquid and then leaving the system to clear (Walker, 1966). In the present investigations as the catalysis and the chromatographic operations are separate, the removal of the products can be more satisfactorily completed by means of injections of polar solvents after each reduction. In the present system no modifications have to be made to the gas chromatograph and an inert gas can be used as usual for the carrier. Thompson et al. (1967) working with many low molecular weight hydrocarbon products have also found separation of the processes to be useful for the synthesis of reference hydrocarbons from available aromatic compounds.

F. SUMMARY

1. A method has been developed for the complete reduction of microgram quantities of steroids and sterols to their parent hydrocarbons. The modified apparatus consists of a siliconised glass tube containing platinum catalyst coated on siliconised glass beads. The procedures for preparation of the catalyst and deactivation of the surfaces have been modified.

2. A slow stream of hydrogen at 20-30 ml./min. 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.

3. The effect of a number of factors involved in the method have been investigated and its reliability has proved satisfactory.

4. Complete reduction of many steroids and sterols could be reproducibly achieved at catalyst temperatures of 170-190°C. The yields were generally related to the structure of the starting material; about 50% of mono-oxygenated compounds were recovered as hydrocarbons whereas only 5-10% of trioxy compounds were recovered.

CHAPTER II

PRODUCTION OF STERANES FROM STEROID DRUGS AND RELATED COMPOUNDS AFTER HIGH TEMPERATURE CATALYTIC REDUCTION

CHAPTER II

PRODUCTION OF STERANES FROM STEROIDS DRUGS AND RELATED COMPOUNDS AFTER HIGH TEMPERATURE CATALYTIC REDUCTION

A. INTRODUCTION

Many advances in steroid biochemistry have depended on chromatography which has made possible the separation of large numbers of closely related compounds. Chromatographic techniques have also been used for the tentative identification of such biological products in microgram quantities. Paper chromatography has been developed by Bush, (1961a) Edwards & Trafford (1968); gas-liquid chromatography by Knights & Thomas (1962), VandenHeuvel & Horning (1962), Brooks & Hanaineh (1963), Hartman & Wotiz (1964). Thin-layer chromatography has also been useful (Lisboa & Diczfalusy, 1962; Moss & Rylance, 1967). In some steroid drugs the contributions of the functional groups to chromatographic mobility on thin-layer have been evaluated (Hara & Mibe, 1968). Using such techniques, the nature and position of functional groups on the steroid skeleton can generally be determined. However, the identification of the hydrocarbon skeleton to which functional groups are attached has been difficult.

The parent hydrocarbons of a number of steroids and sterols have been obtained using high temperature catalytic reduction as described in the previous chapter. In the present chapter the application of this method to steroid drugs and related compounds on a microgram scale is described. The yields/

yields from these compounds are presented. The reduction products of steroids with the same carbon skeleton have identical retention times. However, the different carbon skeletons produced in the present studies could be separated from each other by g.l.c.

TABLE 10

NAMES AND CARBON SKELETONS OF STEROID DRUGS

Anabolic steroids

Systematic name	Official name	Hydrocarbon skeleton
17α-methyl-17β-hydroxyandrost-1,4-dien-3-one	METHANDROSTENOLONE	17α-methylandrostane
17α-methyl-11β,17β-dihydroxy-9α-fluoroandrost-4-en-3-one	FLUOXYMESTERONE	17α-methylandrostane
17α-methyl-2-hydroxymethylene-17β-hydroxy-5α-androstan-3-one	OXYMETHOLONE	17α-methylandrostane
17α-methyl-4,17β-dihydroxy-androst-4-en-3-one	OXYMESTERONE	17α-methylandrostane
17α-methyl-17β-hydroxyandrost-4-en-3-one	17-METHYLTESTOSTERONE	17α-methylandrostane
1β-methyl-17β-hydroxy-5α-androst-1-en-3-one	METHENOLONE	1β-methylandrostane
17α-ethyl-17β-hydroxyoestra-4-en-3-one	NORETHANDROLONE	17α-ethyl-19-norandrostane
17α-methyl-17β-hydroxy-5α-androstan-3, 2c-pyrazole	STANOZOLOL	17α-methylandrostane-3,2c-pyrazolidine

contd.

TABLE 10 (Contd.)

Progestational and related steroids

Systematic name	Official Name	Hydrocarbon skeleton
17α-ethynyloestr-4-en-3β,17β-diol	ETHYNODIOL	19-norpregnane
17α-ethynyloestr-5(10)-en-17β-ol-3-one	NORETHYNODREL	19-norpregnane
17α-ethynyl-17β-hydroxyoestr-4-en-3-one	NORETHINDRONE	19-norpregnane
17α-ethynyloestra-1,3,5(10)-trien-3, 17β-diol	ETHYNYLOESTRADIOL	19-norpregnane
17α-ethynyl-3-methoxyoestra-1, 3,5(10)-trien-17β-ol	MESTRANOL	19-norpregnane
6α-methyl-17α-acetoxypregn-4-en-3,20-dione	MEDROXYPROGESTERONE ACETATE	6α-methylpregnane
6-methyl-17α-acetoxypregn-4,6-dien-3,20-dione	MEGESTEROL ACETATE	6-methylpregnane
16α-methylpregn-4-en-3,20-dione	16-METHYLPROGESTERONE	16α-methylpregnane
6-chloro-17α-acetoxypregn-4,6-dien-3, 20-dione	CHLORMADINONE ACETATE	pregnane

B. MATERIALS AND METHODS

1. Materials.

The steroid drugs which have been studied are shown in Table 10. This table contains the systematic names, the official names listed in the 8th edition of the Merck Index and the carbon skeleton upon which the drug is based. It should be noted that many drugs possess the same carbon skeleton; for example, 17α-methylandrostane for the anabolic steroids and 19-norpregnane for the progestational agents. It is also of interest that only one of the 17 compounds listed, 'chlormadinone acetate', has the same pregnane carbon skeleton as naturally occurring steroids, in this example, pregnane.

2. Extraction of the drugs from tablets.

Some compounds were obtained by chloroform extraction from tablets. The tablets were made into a suspension in 2 ml. of distilled water and extracted with 5 vol. of chloroform. The extract was washed repeatedly with distilled water until clear, dried by filtration through a small quantity of anhydrous sodium sulphate, and evaporated to dryness. The extract was dissolved in absolute ethanol at a steroid concentration of approximately 1mg./1ml. In such cases no precise quantitative results could be obtained; where relevant, this is noted.

3. High temperature catalytic reduction and gas-liquid chromatography.

The steroids were subjected to high temperature catalytic reduction as described in the previous chapter. The catalyst, 1-3% w/w platinum coated

on/

on glass beads, was used at 170-200°C unless otherwise noted. The products of reduction were studied by g.l.c. on columns coated with 1% w/w SE-30, 1% w/w NGA and 1% w/w JXR unless otherwise stated. The major products, generally two in number, have retention times and yields listed in the tables. Retention time data is given relative to 5α-androstane or 5α-cholestane. The coefficient of variation for a single relative retention time was about 1% (see Chapter I, B3 Table 2). Since the means quoted are generally from four or more determinations, errors would be correspondingly reduced.

4. Measurement of the products.

For quantitation a minimum of three observations was used throughout the work. The amounts of products were estimated by planimetry (Allbrit Planimeter, England) of the peak areas from the g.l.c. tracings and expressed in terms of weight relative to the most closely related available pure sterane. For example, androstane was used as a standard for the "methyl" or "norandrostanes". When external hydrocarbon standards were available estimations were done by comparison of the peak heights as in earlier experiments. The yields given in the tables are the amounts of major products expressed as a percentage of the starting material. The major product is identified in the tables by the letter "M" after its relative retention time; when the peak areas were approximately equal, no comment has been added.

From the results obtained in the previous chapter it can be concluded that/

that the high temperature catalytic reduction produces parent steranes from a number of naturally occurring steroids and sterols. In order to simplify the description and discussion of the results, the reduction products with identical retention times derived from compounds with identical hydrocarbon skeletons are described as parent steranes. The use of the method does not, however, depend upon the correctness of this assumption which seems justifiable from all the available evidence, but only upon the identical retention times.

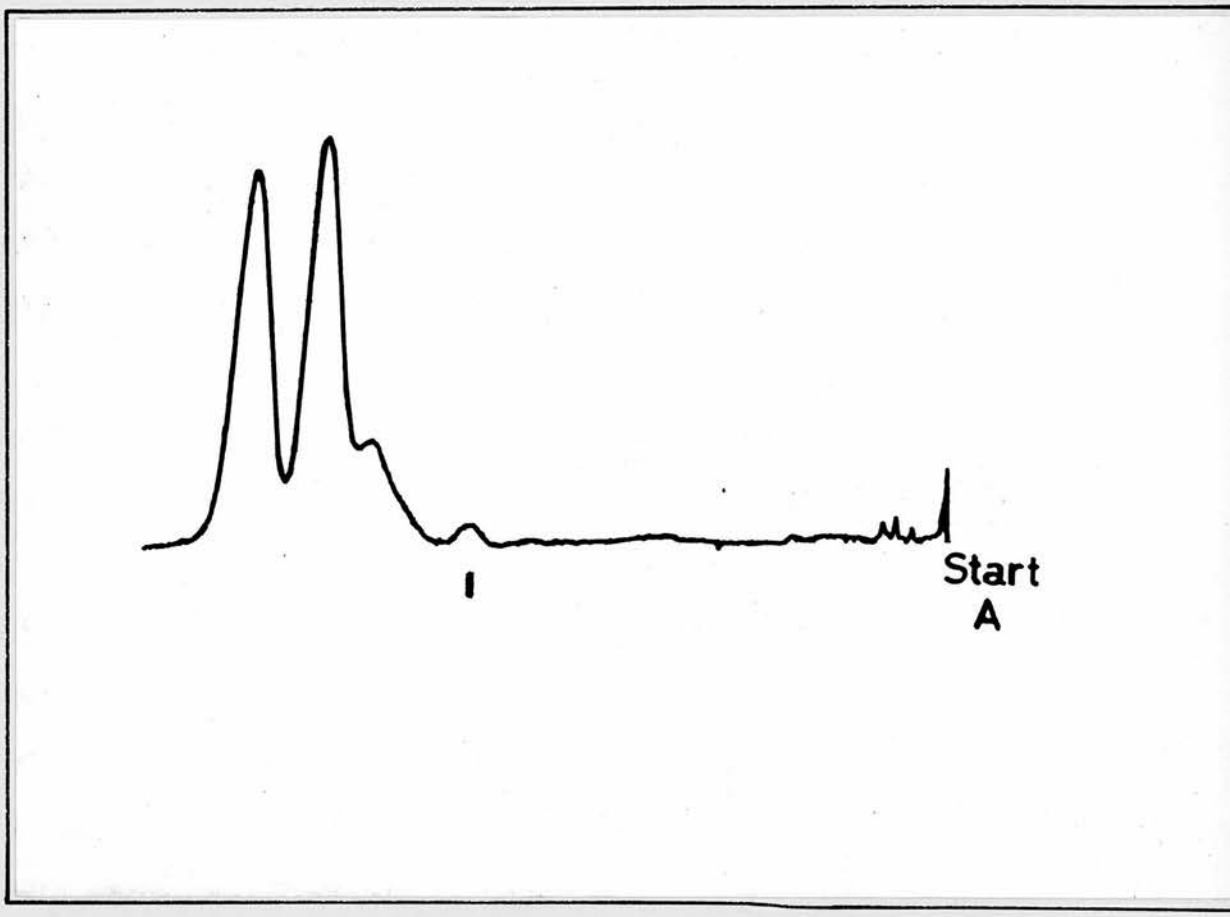


Fig. 7. Reduction products from 5 µg. methyltestosterone, catalyst temperature 180°C, chromatographed on an SE-30 coated column at 150°C; attenuation x 500. The retention time of 5α-androstane is indicated by a vertical line.

C. RESULTS

1. Reduction products of anabolic and related steroids.

Although there are a large number of steroids and sterols, these compounds are based upon a small number of parent hydrocarbons or steranes. This is true for naturally occurring compounds and even for the synthetic steroid drugs as shown in Table 10. Thus reduction to the carbon skeleton is a simplification which has been achieved by the present method. The relative retention times of the reduction products of steroid drugs with the same carbon skeletons are, as predicted, identical. This is shown for the anabolic steroids by the results in Table 11.

Four anabolic steroids with a 17-methylandrostane skeleton, methyltestosterone, methandrostenolone, oxymetholone and oxymesterone, were reduced to products with the same retention times on SE-30 and on NGA coated columns. The other four compounds have similarly given the expected products. The g.l.c. tracing of the reduction products from 17-methyltestosterone is presented in Fig. 7.

The main reduction products of the heterocyclic anabolic steroid stanozolol had a retention time somewhat similar to that of cholestane (Table 11); this product did not correspond to a "methyl androstane". Although Beroza (1962b) found that high temperature catalytic reduction removed amino groups, Thompson et al. (1967) found that high temperatures and a special catalyst were necessary to remove nitrogen from the carbazole ring. It, therefore, seems probable that the pyrazole ring in stanozolol

was/

TABLE 11

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS OF STEROID DRUGS

Anabolic and related steroids

Official name	Mean % yield	Retention times of products relative to 5α-androstane			
		SE-30 column		NGA column	
17-methyltestosterone	20.0	1.28	1.45	1.19	1.31
Methandrostenolone	17.8	1.28	1.45	1.19	1.31
6β-hydroxymethandrostenolone +	ca 15	1.28	1.45	1.19	1.31
Oxymetholone +	12.5	1.28	1.45M	1.19	1.31M
Oxymesterone x	ca 6.5	1.28	1.45M	1.19	1.31M
Norethandrolone	16.8	1.46M	1.60	1.30M	1.46
Methenolone	4.8	1.49			1.48
1α-hydroxy-3β-acetoxyandrost-5-en-17-one +	6.9	1.00M	0.88	1.00M	0.88
3β,15β,17β-trihydroxyandrost-5ene	7.1	1.00M	0.88	1.00M	0.88
Stanozolol	4.7	1.03*			0.81*

* relative to 5α-cholestane at 210°C.

M = Major product

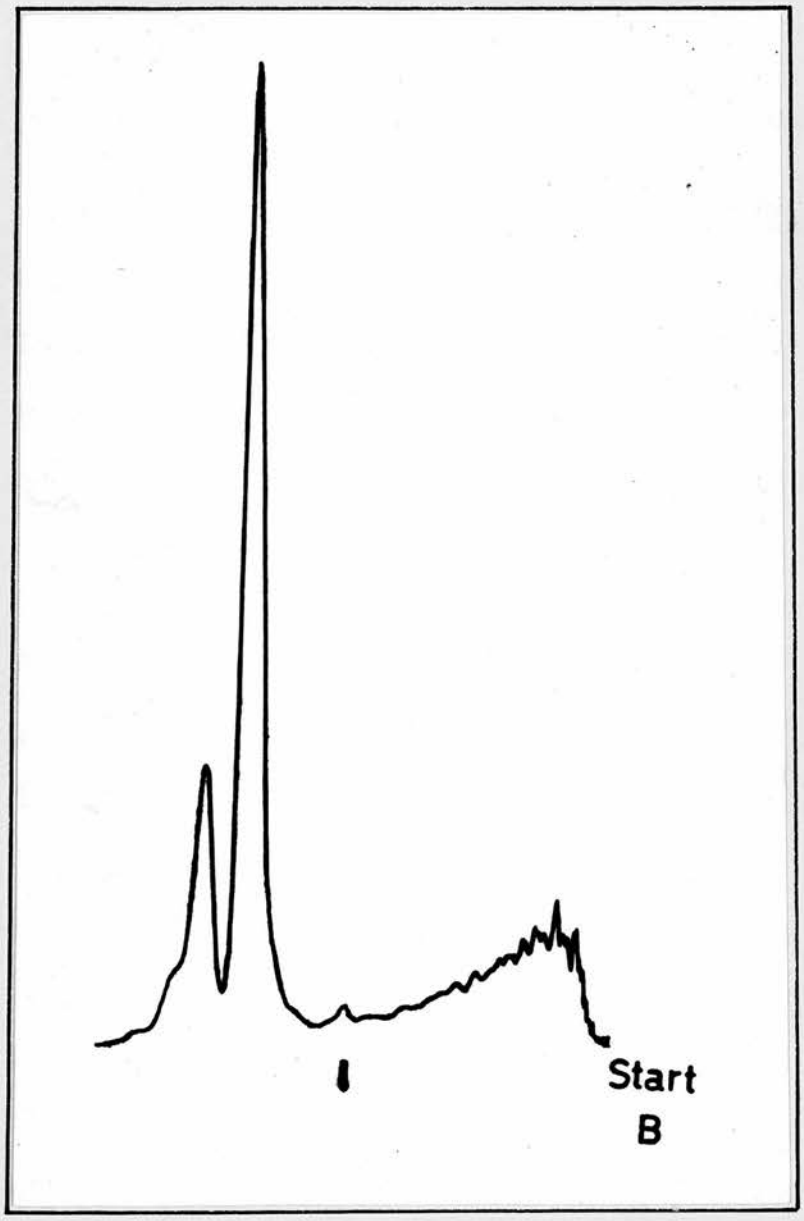


Fig. 8. Reduction products from 5 µg. ethynodiol, catalyst temperature 180°C, chromatographed on an NGA coated column at 150°C; attenuation x 200. The retention time of 5α-androstane is indicated by a vertical line.

was reduced to pyrazolidine and retained as part of the skeleton.

2. Reduction products of Progestational and related steroids.

A number of progestational steroids showed reduction products with identical relative retention times in a similar fashion to the anabolic agents. For example, three commonly used progestational agents with 19-norpregnane skeletons, ethynodiol, norethynodrel and norethisterone, as well as the oestrogens mestranol and ethynyloestradiol, were reduced to products with identical retention times (Table 12). The g.l.c. tracing of the reduction products of ethynodiol is shown in Fig. 8. It should be noted that the product with a relative retention time of 1.60 on a NGA coated column was detectable in all cases, but relatively large amounts were obtained only from mestranol and the impure norethynodrel. The interpretation of these patterns is complicated by the possible rearrangements. Many of the drugs studied possess an 17α-ethynyl side chain which may be rearranged to the more stable 17β-ethynyl side chain during reduction (Fieser & Fieser, 1959b). However, Rapala & Farkas (1958), and Layne, Golab, Arai & Pincus (1963) have shown no alteration of the 17α-ethynyl side chain during reduction. In the present study no drug with 17β-ethynyl side chain was available for comparison, so no conclusions have been made.

3. Reduction products of C-18 and C-19 oxygenated and norsteroids.

The 18- and 19-hydroxy steroids were reduced, as predicted from the work of Beroza (1962b) and that of Thompson et al. (1967) on simpler compounds, largely/

TABLE 12

RELATIVE RETENTION TIMES AND YIELDS OF REDUCTION PRODUCTS OF STEROID DRUGS.

Progestational and related steroids

Official Name	Mean % Yield	Retention times of products relative to 5α-androstane.				
		SE-30 column		NGA column		
Mestranol	8.5	1.41	1.55	1.34	1.53	1.60
Ethynodiol	13.8	1.41M	1.55	1.34M	1.53	
Norethisterone x	ca10	1.41M	1.55	1.34M	1.53	
Norethynodrel +	8.5	1.41M	1.55	1.34M	1.53	1.60
Ethynyloestradiol	13.5	1.41M	1.55	1.34M	1.53	
Megesterol acetate	9.5	2.50M	2.80	2.25M	2.61	
Medroxyprogesterone acetate	11.25	2.50M	2.80	2.25M	2.61	
16-methylprogesterone	8.5	2.48	2.79	2.04	2.34	
Chlormadinone acetate	5.0	2.01	2.27	1.86	2.13	
Progesterone	15.2	2.01	2.27	1.86	2.13	
17α-hydroxypregn-4-en-3,20-dione	3.1	2.01	2.27	1.86	2.13	
Pregnenolone	13.6		2.27		2.13	
5α-pregnane-3,20-dione	17.8		2.27		2.13	
5β-pregnane-3,20-dione	19.2	2.01		1.86		

x tablet
\+ impure

largely to the corresponding "18- or 19-norsteranes"; only about 1.5% of the starting material was recovered as the parent hydrocarbons. The relative retention times of the reduction products from 3-hydroxyoestra-1,3,5 (10) - trien-17-one, oestrone and oestra-1,3,5(10)-triene-3,17-diol, oestradiol are similar to those from 17β -hydroxy-19-norandrost-4-en-3-one and 19-hydroxy androst-4-en-3, 17-dione (Table 13). Similarly, 3β hydroxy-18-norandrost-5-en-17-one, 18-nordehydroepiandrosterone; 3β , 18-dihydroxyandrost-5-en-17-one, 18-hydroxydehydroepiandrosterone; 17β , 18-dihydroxyandrost-4-en-3-one, 18-hydroxytestosterone; 3β, 18-dihydroxypregn-5-en-20-one hemiacetal, 18-hydroxypregnenolone hemiacetal and 11β , 21-dihydroxy-3, 20-dioxypregn-4-en-18-al, aldosterone are reduced to similar products.

Although ethynyloestradiol was reduced completely to the expected products the reduction products from oestradiol and oestrone sometimes included products with a retention times intermediate between androstanes and pregnanes. The retention times of these products relative to 5α -androstane were generally 1.11, 1.17 and 1.54 on SE-30 column and in some cases were the major products. It seems justifiable to conclude from this evidence that the aromatic A ring was more difficult to reduce than the isolated double bonds; this is consistent with studies on some naphthalene derivatives by Beroza (1962b).

3β -acetoxy-26-norcholest-5-en-25-one was also reduced to the expected products. On an NGA column at 210°C the products had retention times relative to 5α -cholestane of 0.77 and 0.85. The yield was 15.1%

4. <u>Reduction products of Corticosteroids</u>.

Reduction/

TABLE 13

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS OF C-18 AND C-19 OXYGENATED AND NORSTEROIDS.

Compound	Mean % yield	Retention times of products relative to 5α-androstane			
		SE-30 column		NGA column	
19-nortestosterone	9.3	0.69		0.60	
19-hydroxyandrost-4-en-3,17-dione	8.7	0.69		0.60	
Oestrone	6.2	0.69		0.60	
Oestradiol-17β	6.6	0.69		0.60	
18-nordehydroepiandrosterone	11.1	0.76	0.83	0.73	0.80
18-hydroxydehydroepiandrosterone	2.4	0.76	0.83	0.73	0.80
18-hydroxytestosterone	2.8	0.76	0.83	0.73	0.80
18-hydroxypregnenolone hemiacetal	2.2	0.76	0.83	0.73	0.80
Aldosterone	0.9	0.76	0.83	0.73	0.80

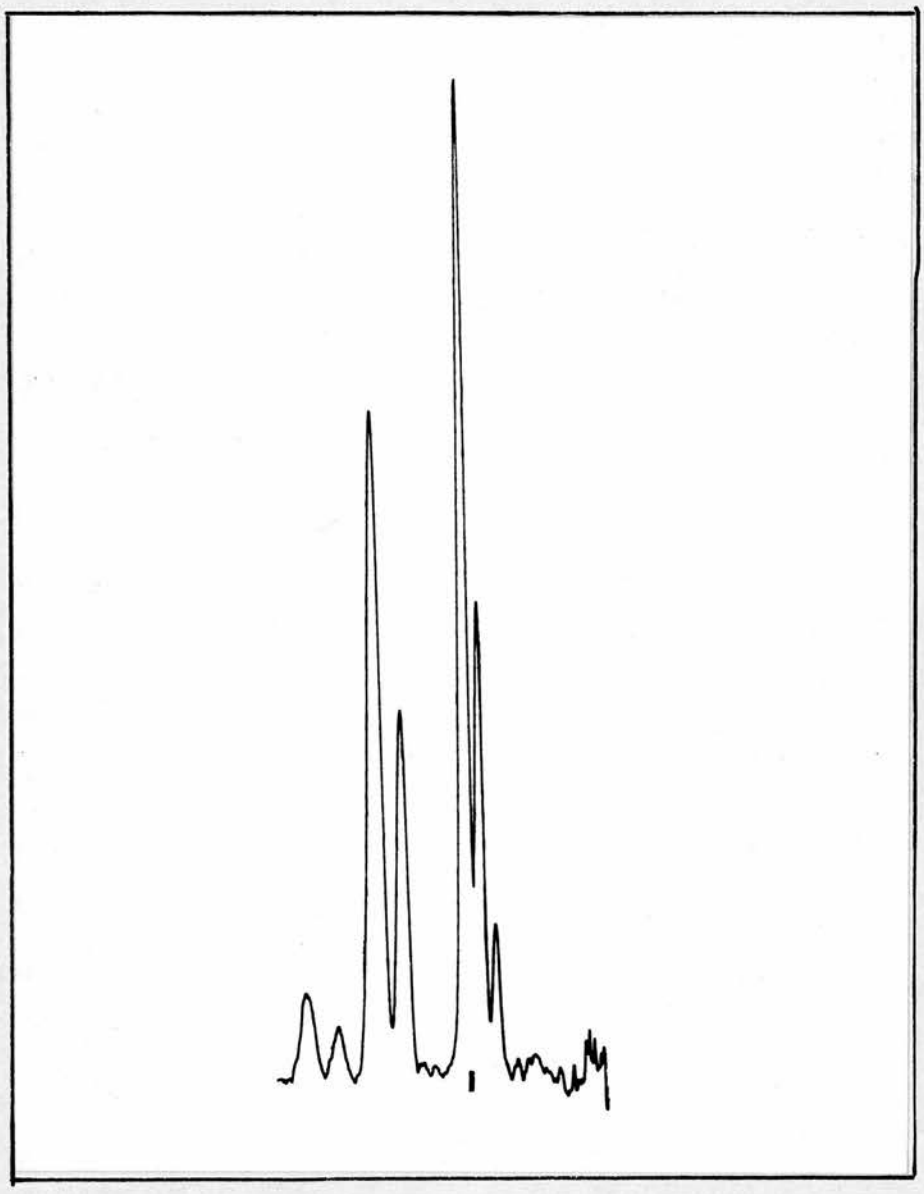


Fig. 9. Reduction products from 10 µg. cortisol, catalyst temperature 190°C, chromatographed on an SE-30 column at 160°C; attenuation x 100. The retention time of 5α-androstane is indicated by a vertical line.

Reduction of the corticosteroids, 11β, 17α, 21-trihydroxy-pregn-4-en-3, 20-dione, cortisol; 11β, 21-dihydroxypregn-4-ene-3, 20-dione, corticosterone and 21-hydroxypregn-4-ene-3, 20-dione, deoxycorticosterone, with highly oxygenated side chains produced about 1-3% of the parent pregnanes and about 5-10% as androstanes (Table 14). However, peak areas of about 34-95% of the major peak area appeared as products with retention times relative to 5α-androstane of 1.40 and 1.56 on an NGA coated column. In contrast to the products from the majority of steroids and sterols, there was a complex pattern of reduction products from corticosteroids (Fig. 9).

The yield of reduction products from the corticosteroids was more variable than from compounds like progesterone and the 17-oxosteroids; the coefficient of variation for a single observation was about 5-10% for 17-oxosteroids in contrast to 30-50% for corticosteroids. However, the major reduction products were generally the androstanes and the parent hydrocarbon could always be detected.

5. Reduction products of Bile acids.

The carboxyl group in the bile acids was reduced in low yield. Reduction products with relative retention times identical with those of 5α-cholane were always detected after reduction of bile acids. The compounds reduced were, 3α, 12α-hydroxy-5β-cholenic acid methyl ester, methyldeoxycholic acid; 3α, 7α-hydroxy-5β-cholenic acid, chenodeoxycholic acid; 3α, 7α, 12α-hydroxy-5β-cholenic acid methyl ester, methyl cholate; 3β-hydroxy-5-cholenic acid; 3α-hydroxy-5β-cholenic acid, lithocholic acid; 5β-cholane-7-one at catalyst temperature of 200°C and 210°C. From all these compounds except/

TABLE 14

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS OF CORTICOSTEROIDS.

Compounds	Mean % yield of 5α & 5β -pregnanes	Retention times of products relative to 5α-androstane			
		SE-30 column		NGA column	
Cortisol	1.9	1.00M	0.88	1.00M	0.88
		2.01	2.27	1.86	2.13
Corticosterone	2.6	1.00M	0.88	1.00M	0.88
		2.01	2.27	1.86	2.13
Deoxycorticosterone	2.0	1.00M	0.88	1.00M	0.88
		2.01	2.27	1.86	2.13

except 5β-cholane-7-one, the parent hydrocarbons were the minor products representing less than 1% of the starting material; the major products had retention times relative to 5α-cholane of 0.66 and 0.75 on a JXR coated column (195°C) and on an NGA coated column (185°C) respectively. 5β-cholane-7-one under similar conditions gave about 10% of "5α-cholane" with a small quantity of other products. The retention time of 5α-cholane relative to 5α-cholestane was 0.45 on a JXR column. From the work of Beroza (1962b) it was expected that the major products would have lost the C-24 carbon atom.

6. Reduction products of Lanosterol.

Two products which behaved like the parent hydrocarbons were obtained from a C_{30} compound, 4α, 4β, 14α-trimethyl-5α-cholesta-8, 24-dien-3β-ol, lanosterol. The retention times relative to 5α-cholestane of the major reduction product on JXR (190°C), NGA (210°C) and SE-30 (230°C) columns were 1.63, 1.47 and 1.53 respectively and the yield was 8.2%. The reduction products when chromatographed on thin-layer with the solvent system chloroform-acetone (9:1 v/v) had the R_F values of 0.65 (major component) and 0.48 and did not absorb ultraviolet light (254 mμ max.). Lanosterol under similar conditions had an R_F of 0.41 and showed weak u.v. absorption. In g.l.c. using an SE-30 column (230°C) standard lanosterol contained two peaks with retention times relative to 5α-cholestane of 2.87 and 3.17 (Major). The two reduction products with relative retention times of 1.67 and 1.87 could have arisen from the impurity present in the standard. When the log relative/

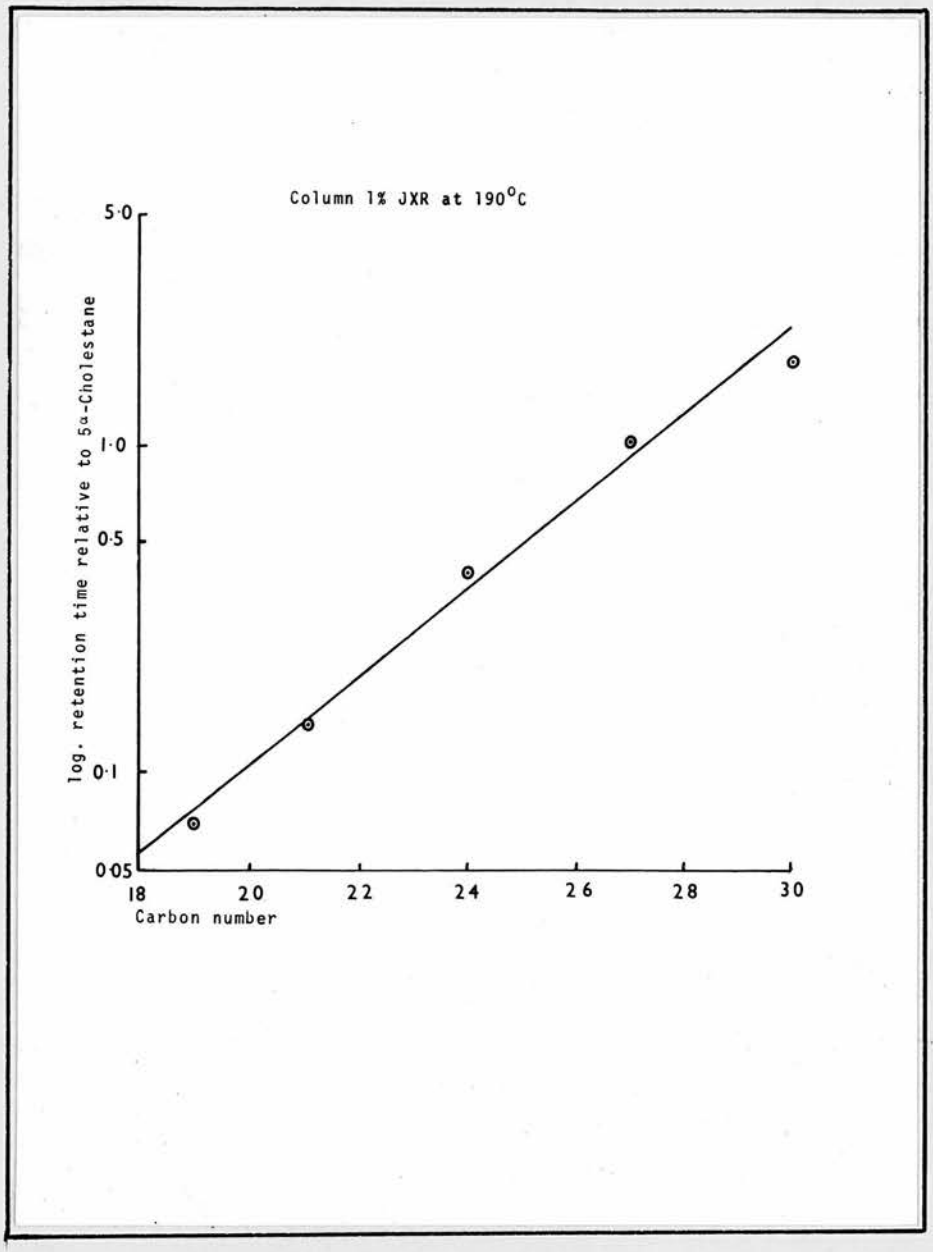


Fig. 10. Relationship between carbon number of steranes and their relative retention time using iso-:thermal conditions for gas chromatography.

relative retention time of the major product was plotted against carbon number it lay near the same straight line as the other steranes (Fig. 10). Further confirmation for the reduction of the inert Δ 8(9) double bond (Doree, McGhie & Kurzer, 1948) would be possible from the mass of the parent ion which could be obtained by mass spectrometry.

D. GENERAL STATEMENT OF RESULTS

Using the present method, products with the retention times of the parent steranes were always detectable and were generally the major products. The different steranes from the compounds C_{18} to C_{30} were separated by g.l.c. and these separations were in most cases good. Products which had different numbers of carbon atoms were clearly distinguished; as expected, the log retention times were approximately linearly related to the carbon number as shown in the Fig. 10. The result agrees with the findings of VandenHeuvel & Horning (1962). It should also be noted that different structures with the same number of carbon atoms were also separable. The "18-norandrostanes" were separated from the "19-norandrostanes" (Table 13). "17-methylandrostanes" were separable from "1-methylandrostanes" and "6-methylpregnanes" from "16-methylpregnanes" (Tables 11 & 12).

The amount recovered as steranes varied with the structure of the starting material. Recoveries were reduced by extra substituents, for example, hydroxyl groups. This is illustrated by the yield of 20% from 17-methyltestosterone compared to 6.5% from oxymesterone with an extra hydroxyl group at position 4.

The position of a methyl group could have an important effect on the yield. The recovery of steranes from 17-methyltestosterone was similar to that from testosterone whereas from methenolone with a 1-methyl group only 4.8% was recovered. In this compound the 1-methyl group may hinder the reduction of the double bond at C-1 (Djerassi, Riniker & Riniker, 1956).

This/

This double bond was readily reduced when relatively unhindered as in methandrostenolone, ethynyloestradiol and mestranol (Tables 11 & 12).

Using the present method, various groups could be reduced or removed from the carbon skeleton. Hydroxyl or ketone groups were readily reduced; fluoro and chloro substituents, at C-9 and C-6 respectively, could be removed, although fluoxymesterone only yielded small amounts of "17-methylandrostanes", about 3% of the starting material, with significant quantities of other products with shorter retention times which were the major products. The retention times relative to 5α-androstane were 0.98, 0.58 and 0.42. These products in contrast to the hydrocarbons had not sharp peaks. However, it must be emphasised that "17-methylandrostanes" were produced from fluoxymesterone as from the other anabolic steroids with a "17-methylandrostane" skeleton (Table 10).

Ester and ether linkages were readily split. Double bonds at various positions, 1, 3, 5 and 5 (10) were readily reduced. After reduction of hydroxyl groups at various positions, the parent hydrocarbon was always detectable; steroids with hydroxyl groups at positions 1, 2, 3, 4, 6, 7, 11, 12, 15, 16, 17, 18, 19, and 21 were successfully reduced. The position of the hydroxyl groups did not generally affect the production of a single major product corresponding to the parent hydrocarbon except where the hydroxyl group was in a terminal position, for example, C-18, C-19 or C-21. The 2-hydroxymethylene group was removed from oxymetholone.

E. DISCUSSION

Using the present technique crude fractions can be shown to contain steroids. Thus at a very early stage in work on the isolation and identification of unknown natural products evidence of a sterane hydrocarbon skeleton can be obtained (Chapter III, Section I). It is difficult to assess the importance of this from the literature because mistakes are rarely acknowledged; although caffeine has been recognised as a source of confusion (Nishizawa & Eik Nes, 1963).

The results of the relative retention times in the present study show that the steranes are not difficult to separate, and even better results have been obtained on columns coated with 1% w/w OV-17 (phenyl methyl silicone - 50% mole phenyl). The separation factors of 5β : 5α-androstane and 5β: 5α-pregnane at the column temperatures of 150°C and 180°C were 1.15 and 1.22 respectively. Thus many steroid drugs and their metabolites with carbon skeletons which do not occur in nature, should be detectable. Although O-methylation is a significant pathway of drug catabolism (Parke, 1968a) it is quantitatively unimportant for steroids. In addition, methoxy groups are removed by the present method as are additional hydroxyl groups.

The stability of the carbon skeleton will be important in determining the sensitivity of the method. However, even the highly unstable corticosteroids yield about 1% as parent pregnanes. Since the flame ionisation detector is highly sensitive and losses of hydrocarbons even on present columns are small, corticosteroids should be detected in quantities down to 10 µg;/

10 µg; correspondingly smaller amounts of more stable steroids should be detectable. Thus normal therapeutic quantities of some steroid drugs can be administered and the metabolites detected, in contrast to the large doses previously necessary (Engel, Alexander & Wheeler, 1958; Rongone, Segaloff, Fried & Sabo, 1961). The metabolism of normal therapeutic quantities of some steroid drugs can be studied without the difficulties of obtaining pure radioactively labelled compounds and the potential dangers of their use in man (Chapter IV).

F. SUMMARY

1. Carbon skeleton chromatography of steroids and sterols from C_{18} to C_{30} was carried out following high temperature catalytic reduction on a microgram scale. Steroids with the same carbon skeleton were reduced to products with the same relative retention times on gas chromatography. The 'parent steranes' from many steroid drugs could generally be distinguished from the carbon skeletons of the naturally occurring steroids. About 5-20% of the starting material could be recovered as steranes. Although the yield from any one compound was reproducible, the recoveries were generally dependent on the position, nature and number of substituents in the starting material.

2. Expected products were obtained from anabolic and progestational steroid drugs as well as related compounds for example the C-18 and C-19 hydroxylated and norsteroids. Lanosterol was also studied. Corticosteroids and bile acids gave the parent hydrocarbons as the minor reduction products.

CHAPTER III

CHARACTERISATION OF THE HYDROCARBON SKELETONS OF COMPOUNDS FROM URINE EXTRACTS

SECTION I - THE IDENTIFICATION OF 16β-HYDROXYDEHYDROEPIANDROSTERONE FROM INFANT URINE.

SECTION II - THE MEASUREMENT AND IDENTIFICATION OF PREGNANETRIOL IN PREGNANCY URINE

CHAPTER III

SECTION I

THE IDENTIFICATION OF 16β-HYDROXYDEHYDROEPIANDROSTERONE FROM INFANT URINE

A. INTRODUCTION

In urine from infants of 1-3 days old, a large number of steroid and steroid-like compounds are present which become undetectable after the first 6 months of life (Mitchell, 1967). One such compound was isolated and the fraction in which it was the major component was initially called U2. In its structural identification as 16β -hydroxydehydroepiandrosterone (Shackleton, Kelly, Adhikary, Brooks, Harkness, Sykes & Mitchell, 1968), the recognition of the steroid skeleton and the pattern of reduction products were useful.

The present section describes the application of the method of high temperature catalytic reduction to the characterisation of U2. The uses and limitations of the present method in the identification of compounds of biological origin are discussed.

B. MATERIALS AND METHODS

The method described in Chapter I was used. The reduction temperatures used were 190°C, 200°C, 210°C and 220°C. The samples were dissolved in 0.1 ml. of absolute ethanol. 10 µl of this solution was generally used for reduction at one time.

1. Nature of the sample.

About 100 µg. of U2 obtained by the method of Shackleton et al. (1968) was used for the present study. At the start of this investigation, the microchemical and chromatographic evidence for the structure of the major component of the fraction were: (a) it was unstable in g.l.c. (b) it produced colours in the Zimmermann and blue tetrazolium reactions (c) on t.l.c. it was more polar than 3β, 21-dihydroxypregn-5-en-20-one and less polar than 3β, 17α, 21-trihydroxypregn-5-en-20-one (d) it probably contained 3β , 16α -dihydroxyandrost-5-en-17-one, 16α-hydroxydehydroepiandrosterone or 3β , 17β -dihydroxyandrost-5-en-16-one, 16-ketoandrostenediol as a contaminant.

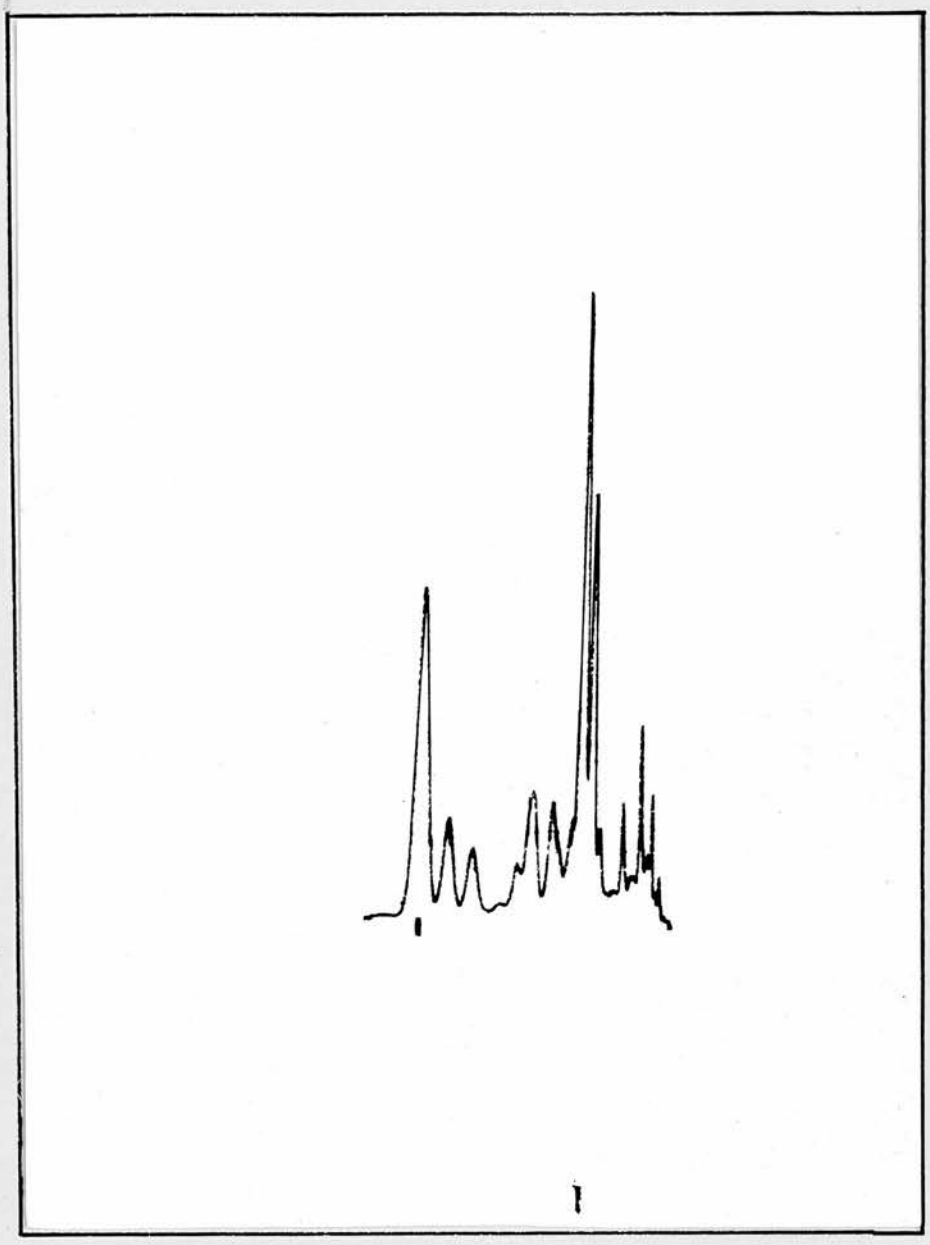


Fig. 11A. Reduction products from 5 µg. 16α-dehydro-:epiandrosterone, catalyst temperature 200°C, chromatographed on an NGA coated column at 150°C; attenuation x 100. The retention time of 5α-androstane is indicated by a vertical line. The start of the chromatogram is from the right side.

C. RESULTS

1. Reduction products of the unknown U2.

The reduction products of U2 at 200°C consisted of 5α-androstane and 5α-pregnane with other products having retention times relative to 5α-androstane of 0.33, 0.34, 0.73 and 0.80. The products with the relative retention times of 0.33 and 0.34 were obtained regularly from the 16, 17-dioxyandrostane derivatives (Table 15, Fig. 11A). Similarly, the products with the relative retention times of 0.73 and 0.80 were obtained consistently from 18-hydroxylated or 18-norsteroids (See Chapter II, C3, Table 13). The reduction patterns from U2 were similar to 16, 17-dioxyandrostane derivatives plus 18-hydroxy or 18-norsteroids. The presence of the two steranes, androstane and pregnane suggested that U2 was similar in structure to the thermally unstable corticoids like 3β, 21 -dihydroxypregn-5-en-20-one and aldosterone; in addition it was already known that U2 probably contained a "contaminant" 16α-hydroxydehydroepiandrosterone or 16-ketoandrostenediol.

2. Reduction products of the purified unknown compound U2.

After repeated separation by t.l.c. about 50 µg. of a purified sample of U2 was obtained. The reduction patterns at a catalyst temperature of 200°C are presented in Fig. 11B. 16β-hydroxydehydroepiandrosterone containing a small amount of 16 -ketoandrostenediol was also obtained; the reduction patterns are presented in Fig. 11C. The patterns are similar but not identical. Two minor products with retention times relative to 5α-androstane of 0.73 and 0.80 were still present. These products were similar to those obtained from 18-hydroxy or 18- norsteroids. From these results/

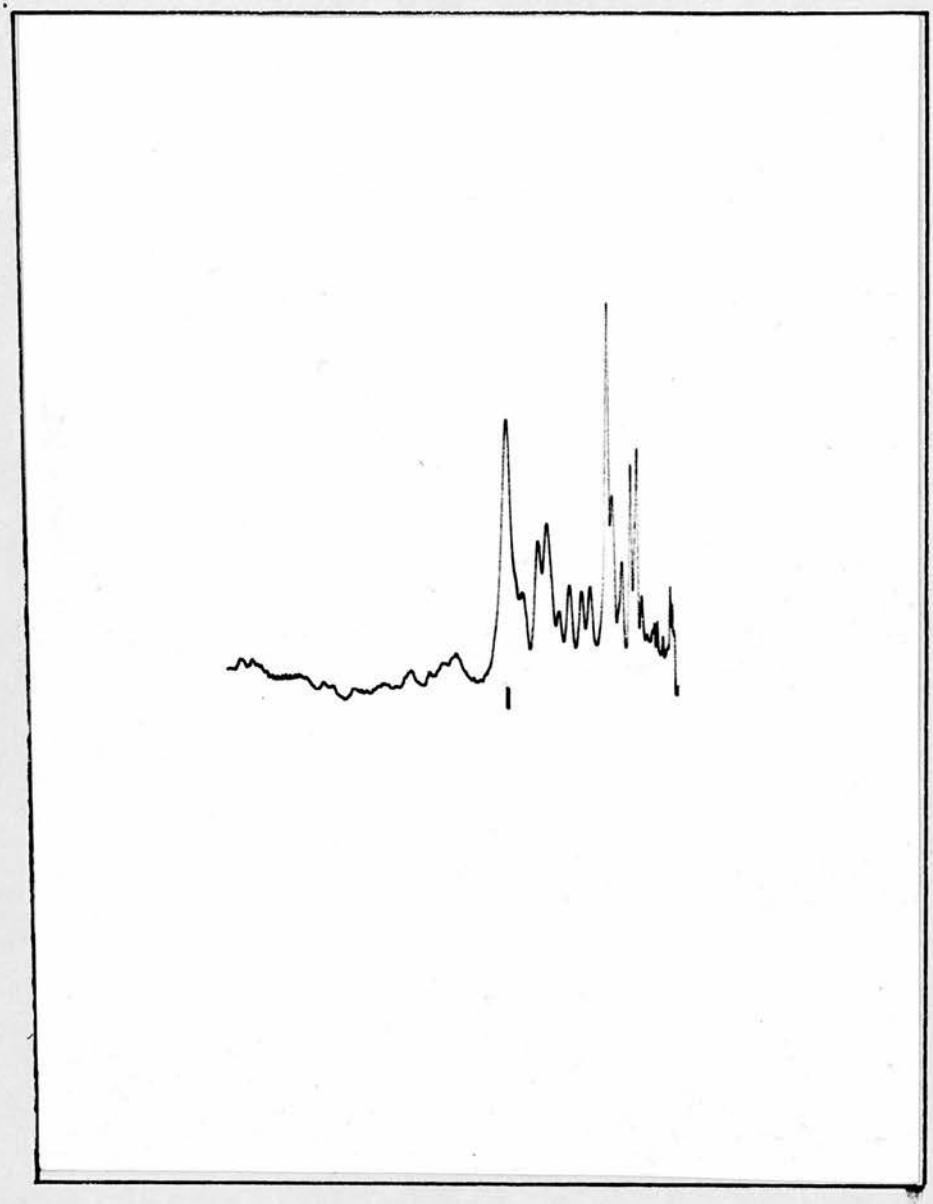


Fig. 11B. Reduction products from about 5 µg. of purified compound U2, catalyst temperature 200°C, chromatographed on an SE-30 coated column at 150°C, attenuation x 100. The retention time of 5α-androstane is indicated by a vertical line. The start of the chromatogram is from the right side.

TABLE 15

RELATIVE RETENTION TIMES AND YIELDS OF THE REDUCTION PRODUCTS FROM 16, 17-DIOXYANDROSTANE DERIVATIVES.

Compound	Mean % yield of 5α & 5β-androstanes.	Retention times of products relative to 5α-androstane. NGA column	
16α-hydroxydehydroepiandrosterone	5.8	0.88	1.00M
		0.33	0.34
16β-hydroxydehydroepiandrosterone +	6.5	0.88	1.00M
		0.33	0.34M
3β,16α,17β-trihydroxyandrost-5-ene	5.7	0.88	1.00M
		0.33	0.34
3β,17β-dihydroxyandrost-5-en-16-one	6.0	0.88	1.00M
		0.33	0.34

+ impure

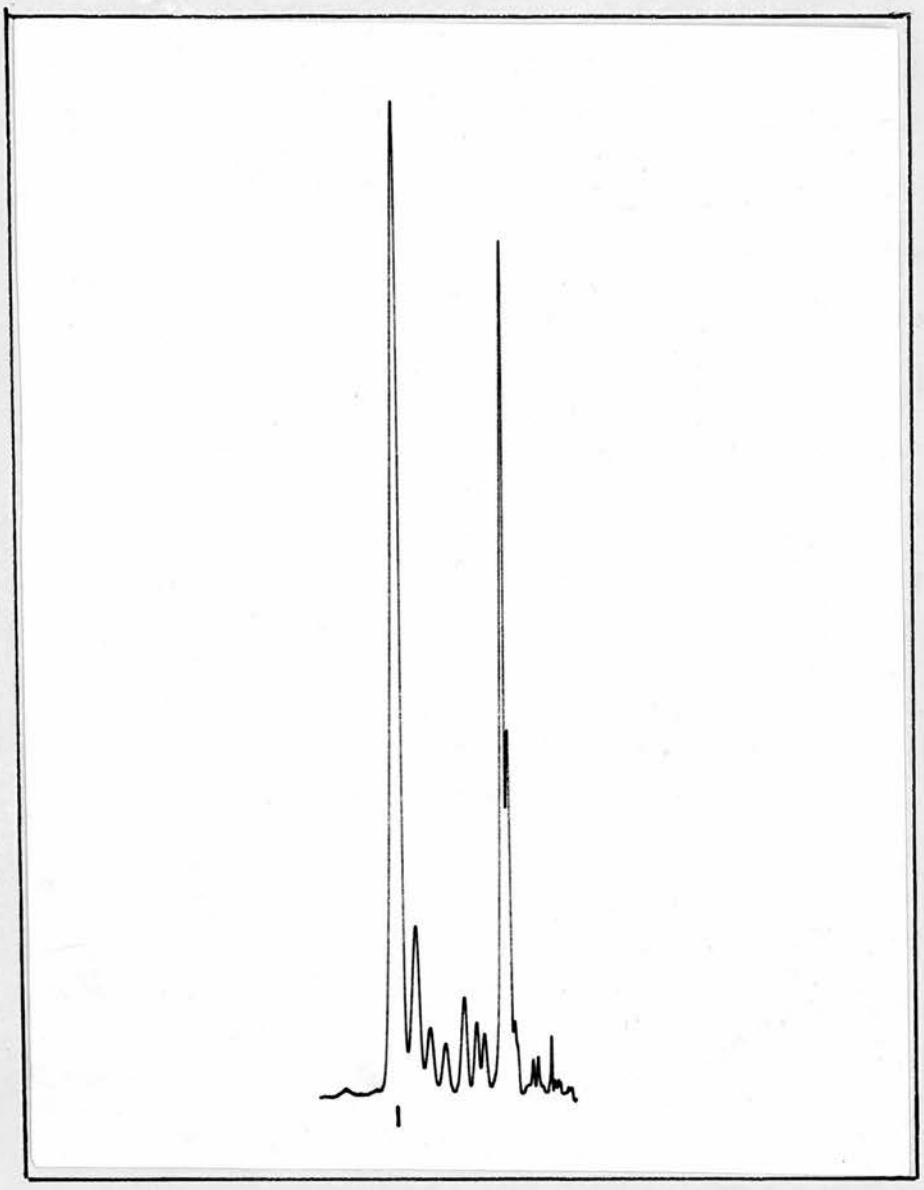


Fig. 11C. Reduction products from about 10 µg. 16β-dehydro-:epiandrosterone, catalyst temperature 200°C, chromatographed on an SE-30 column at 150°C; attenuation x 1000. The retention time of 5α-androstane is indicated by a vertical line. The start of the chromatogram is from the right side.

results it was concluded that the hydrocarbon skeleton of the major component of the purified sample of U2 was androstane and that this component had similar properties to 16, 17-dioxygenated androstane derivatives. The minor component could be a 18-hydroxy compound possibly of the C_{21} series. The characterisation of the major component in U2 as a 16, 17-dioxygenated androstane was consistent with other findings (Shackleton et al, 1968). The minor component of the purer fraction probably with an 18-hydroxyl group has not been detected by other techniques possibly due to its instability. No further study has been made of this component.

3. Reduction products of 16, 17-dioxyandrostane derivatives.

In the compounds with adjacent hydroxyl and ketone groups at C-16 and C-17, the parent hydrocarbons were the major products; in addition, two other products with short retention times were regularly produced, (Fig. 11A, 11C & Table 15) possibly by hydrogenolysis (Thompson et al, 1967). These two products could be more easily lost during solvent evaporation than the parent steranes.

D. DISCUSSION

After purification of the extract the hydrocarbon skeleton of the major component was androstane; pregnane was not detectable but two products similar to those obtained from 18-hydroxylated compounds were present as minor components (Fig. 11B). Since, many of the unstable corticoids give about 1-3% pregnane after reduction (Chapter II, C4) small quantities of corticoids might only be detectable as androstane. The losses on g.l.c. and on t.l.c. in the further purification also suggested instability of the other component or components of the initial extract. 18-hydroxyandrosterone and 18-hydroxyaetiocholanolone have been isolated and identified in the urine of adults (Fukushima, Bradlow, Hellman & Gallagher, 1962); these compounds were probably derived from hepatic metabolism. The presence of such compounds in infants' urine has not been reported. The isolation and identification of 18-oxygenated compounds have been difficult (Simpson, Tait, Wettstein, von Euw & Reichstein, 1953; Loke, Marrian & Watson, 1959). It therefore seems justifiable to suggest that the initial extract may have contained an unstable 18-hydroxylated pregnane derivative.

E. SUMMARY

1. The hydrocarbon skeleton of an unknown compound, U2, from 1 to 3 day-old infant urine was characterised using the method of high temperature catalytic reduction. On reduction the sample before repeated t.l.c. gave androstanes and a small quantity of pregnanes. The reduction patterns of U2 were similar to those from 16-17-dioxygenated androstane and 18-oxygenated steroids.

2. The purified fraction was reduced mainly to androstane without pregnane . The characteristic products from 16, 17-dioxy-androstane compounds were prominent. The products from 18-hydroxylated steroids were just detectable. This suggested that the major component of U2 consisted of a 16-17-dioxyandrostane derivative. This evidence was consistent with its final identification as 16β-hydroxydehydropiandrosterone.

CHAPTER III

SECTION II

THE MEASUREMENT AND IDENTIFICATION OF PREGNANETRIOL IN PREGNANCY URINE

A. INTRODUCTION

The excretion of 5β-pregnane-3α, 17α, 20α-triol, pregnanetriol by women is increased during pregnancy (see Harkness & Love, 1966a); this increase is not observed in mothers with anencephalic foetuses in whom the foetal adrenals are atrophic (Harkness & Love, 1966b). This evidence suggested that the rise was connected with the foetal adrenal cortex. Pregnanetriol excretion, in a mother with a foetus later shown to have a steroid 21-hydroxylation defect was therefore studied.

In the present investigation the structure of the "aetiocholanolone" obtained in the final extract from this method was examined in detail to prove the specificity of the method for urinary pregnanetriol when used on the complex mixture of steroids that are present in pregnancy urine. The method includes vicinal glycol oxidation; the aetiocholanolone finally formed from pregnanetriol is measured. Evidence of a 5β-androstane carbon skeleton with a 17-ketone and an unhindered hydroxyl group were obtained; the quantities of 5β-androstane precursor approximated to those from g.l.c. of the unmodified aetiocholanolone.

B. MATERIALS AND METHODS

The extracts containing aetiocholanolone derived from pregnanetriol in pregnancy urine were obtained by the method of Harkness & Love (1966a).

Zimmermann colour reaction was performed as described in Chapter I, B3.

High temperature catalytic reduction was done at 170°C. The extracts were dissolved in 0.1 ml. of absolute alcohol; 30 µl. each of this solution was used for reduction. The reduction products were analysed by g.l.c. using SE-30 and NGA columns at 150°C. The parent steroid of the neutral extracts were chromatographed on a 1% w/w QF-1 (trifluoro propyl methyl silicone) column and the SE-30 column. Serial "quantitative" estimates of the 5β-androstane precursor were obtained from reduction of known amounts of aetiocholanolone in the same batch as the extracts, that is an 'internal standard'.

Acetylation. The steroid residues were treated with 0.3 ml. of acetic anhydride and 0.2 ml. of pyridine. The reaction mixtures were left overnight at room temperature. The reagents were removed by a stream of nitrogen; the steroid residues diluted with 5 ml. of distilled water and extracted twice with 1 vol. of distilled chloroform. The chloroform extracts were washed with 0.1 vol. of distilled water until the washings were neutral; filtered through anhydrous sodium sulphate and evaporated to dryness at the waterbath temperature of about 40°C using a blast of filtered air.

C. RESULTS.

The yield of hydrocarbons from the standard aetiocholanolone was 11.5%. The ratio of 5α to 5β -androstane from the reference aetiocholanolone and those of the extracts was 1:2.8. No products corresponding to 5β and 5α -pregnane were detected in the extracts after high temperature catalytic reduction.

The results of the hydrocarbon estimations from the urine extracts are presented in Table 16. For the sake of comparison the values obtained by Zimmermann colour reaction and g.l.c. of the unmodified aetiocholanolone are also presented. The results obtained by reduction although only semi-quantitative, in general agree with the estimation of unmodified aetiocholanolone by g.l.c. The values obtained by Zimmermann colour reaction are slightly higher than from these two methods.

The urine extracts and reference aetiocholanolone were acetylated and the products chromatographed using SE-30 (200°C) and QF-1 (210°C) columns. The retention times of the acetylated aetiocholanolone and those from the extracts were identical in these columns. The estimations by g.l.c. as aetiocholanolone acetate are similar to those of aetiocholanolone alone (Table 16).

The estimates obtained by Zimmermann colour reaction and by g.l.c. as aetiocholanolone and aetiocholanolone acetate showed no marked fall in levels at the 38th 39th weeks of pregnancy. Levels, however, were not unduly high.

TABLE 16

ESTIMATION OF PREGNANETRIOL (mg./24hr.) IN PREGNANCY URINE.

Week of pregnancy	By Zimmermann colour reaction	By Gas-Liquid Chromatography		
		as aetiocholanolone.	as aetiocholanolone acetate.	as 5β& 5α-androstane*
31	1.27	0.64	0.63	0.40
32	1.18	0.90	0.98	0.93
33	1.43	1.35	1.53	1.02
34	1.71	1.22	1.32	0.98
35	1.34	1.40	1.30	1.71
36	0.90	0.97	1.16	2.12
37	1.63	1.30	1.35	1.64
38	1.18	0.97	1.00	1.77
39	1.87	1.31	1.36	0.87
5 week after delivery	0.45	0.30	0.32	0.33

* semiquantitative estimation.

D. DISCUSSION

Evidence has been obtained for aetiocholanolone being the major component of the final extract from pregnancy urine obtained by the method of Harkness & Love (1966a). The presence of colour due to the Zimmermann reaction and of an acylable group suggested that the major component of the extracts possessed a 17-ketone and an unhindered hydroxyl group as in aetiocholanolone. The major hydrocarbon product from the fraction was 5β-androstane. The ratio of 5α to 5β-androstane and the yields suggested that the starting material was aetiocholanolone (Chapter I, D1, Table 4). It therefore seemed justifiable to conclude that the method was largely measuring pregnanetriol.

The quantitative methods used for estimating pregnanetriol showed a similar trend in levels. The less accurate estimates by high temperature catalytic reduction also showed increased levels during pregnancy.

E. SUMMARY

1. The excretion of pregnanetriol during pregnancy was estimated by gas-liquid chromatography as aetiocholanolone; as the hydrocarbons 5β and 5α - androstane and by Zimmermann colour reaction. An increase in the excretion of pregnanetriol was observed by these methods; slightly higher levels were obtained with the colour reaction. The semiquantitative estimates from the hydrocarbons were in general agreement with the estimates by gas-liquid chromatography.

2. The use of the high temperature catalytic reduction thus provided additional evidence for the specificity of an analytical method for estimating pregnanetriol in pregnancy urine.

CHAPTER IV

STUDIES ON THE METABOLISM OF ANABOLIC STEROIDS

CHAPTER IV

STUDIES ON THE METABOLISM OF ANABOLIC STEROIDS

A. INTRODUCTION

The metabolism of the natural androgens has been studied in detail (Dorfman & Shipley, 1956; Stylianou, Forchielli, Tummillo & Dorfman, 1961; Pearlman & Pearlman, 1961 and others). The catabolism of many hormones, drugs and vitamins consists generally of reduction, hydroxylation and conjugation. The intermediary metabolism of one natural androgen, testosterone, consists essentially of three main reactions, the oxidation of 17β-hydroxyl group to the 17-keto group by the 17β-hydroxysteroid dehydrogenase; the reduction of the 4, 5-double bond in ring A by the Δ^4-5α or the Δ^4 5β-reductases and the reduction of the 3-ketone group by 3 - hydroxysteroid dehydrogenases (Dorfman & Shipley, 1956; Krüskemper, 1968). Conjugation of hydroxyl groups at C-3 and C-17 proceeds rapidly but hydroxylation is not a major pathway for testosterone.

In recent years, a number of synthetic analogues of the natural androgens have been used for their stimulation of the biosynthesis of proteins. The metabolism of these anabolic steroids has been little studied (Krüskemper, 1968). In many biological investigations a knowledge of the fate of a drug in the body and also an understanding of the nature of metabolites are necessary. Such investigations may be valuable in understanding the causes of drug toxicity (Fingl & Woodbury, 1966a; Parke, 1968b) and are an essential basis for methods of detecting drug administration.

The/

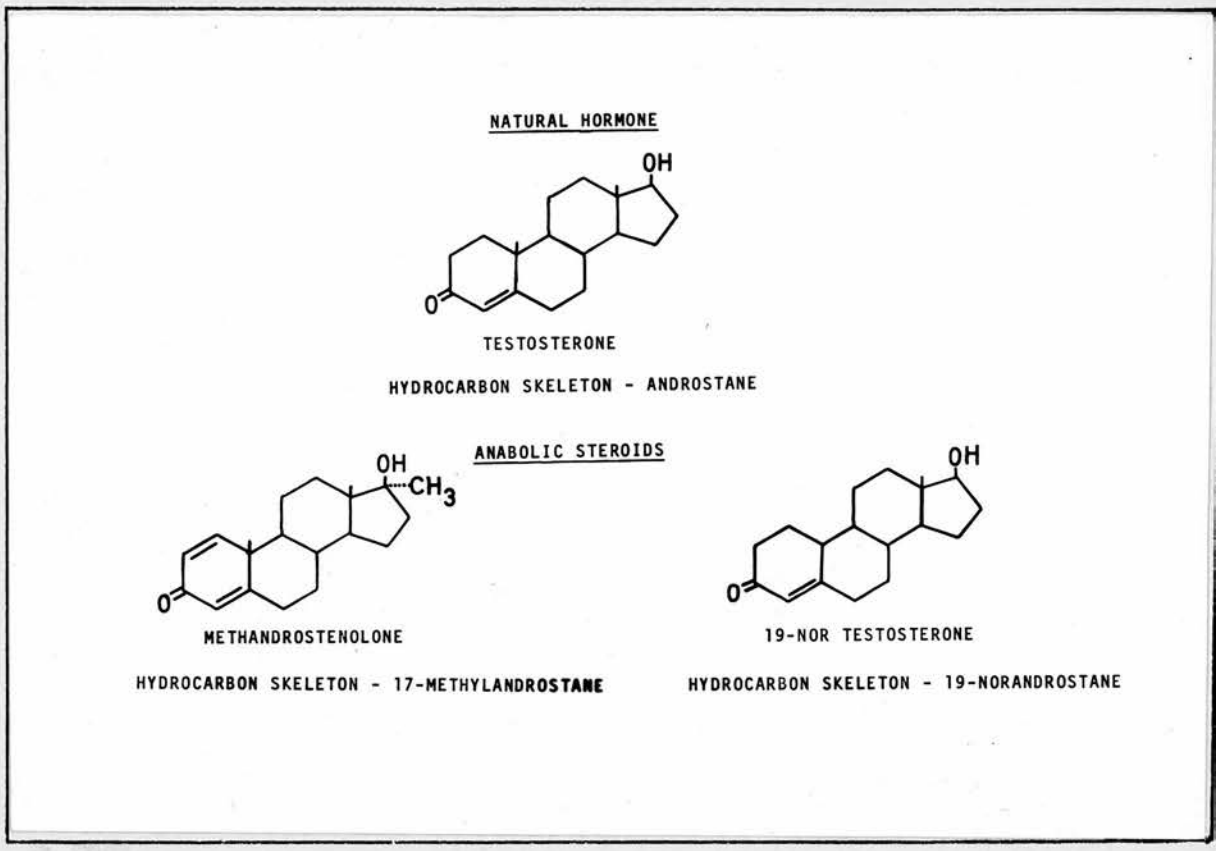


Fig. 12. Examples of different hydrocarbon skeletons of the natural and synthetic androgens which are separable by using gas-liquid chromatography.

The study of the metabolism of a steroid drug has initially depended on the techniques used for detection of drugs or its metabolites. The sensitive techniques for the detection of radioactivity have been extremely useful in the study of the drug metabolism using small doses of radioactively labelled drugs (Layne et al, 1963; Kamyab, Littleton & Fotherby, 1967; Cooper & Kellie, 1968). Such labelled compounds are generally difficult to obtain and are potentially dangerous due to radiation. The administration of labelled compounds is rarely justified in children and in pregnant women.

The work presented in this chapter is an attempt to study the metabolism of normal therapeutic doses of steroid drugs without using compounds labelled with radioactive isotopes. Since the carbon skeleton of the steroids remains intact during metabolism (Turfitt, 1948; Talalay, 1957; Cooper & Kellie, 1968) this 'label' is retained by the metabolites of the steroids. The present technique thus offers the possibility of a simple method for detection of those steroids and their metabolites which have different carbon skeletons from the natural products. This is illustrated in Fig. 12. The metabolism of four common anabolic steroids, 19-nortestosterone, methandrostenolone, oxymetholone and norethandrolone whose hydrocarbon skeletons are different from the natural hormones were studied in man using "carbon skeleton" chromatography for detection. Normal therapeutic doses of the drugs were administered.

The results obtained from 19-nortestosterone and methandrostenolone have been consistent with the results of Engel, Alexander & Wheeler (1958) and Rongone & Segaloff (1963) respectively. It therefore appeared justifiable

to use "carbon skeleton" chromatography to study drugs whose metabolism was not known. The results from oxymetholone and norethandrolone also suggested that the predicted metabolic transformations like reduction and conjugation with glucuronic acid had occured. Surprisingly, it should also be noted that little or no unaltered drug was detectable in urine.

B. ADMINISTRATION OF DRUGS AND URINE COLLECTIONS

The subjects for the study were normal adult males, P, R, A and T except a boy, 7 year-old, M who was under treatment for hypoplastic anaemia. The anabolic steroids studied and the doses administered were as follows:-

1. 19-nortestosterone (Searle) - 20 mg. powder.
2. 4-14C-19-nortestosterone (The Radiochemical Centre), Sp. activity 184μc/mg. (1μc = 5.4 μg.).
3. Methandrostenolone (Ciba) - 5 mg. tablet.
4. Oxymetholone (Syntex) - 5 mg. x 2 tablets.
5. Norethandrolone (Searle) - 10mg. x 2 tablets.

A single dose of the drugs was given by mouth. 1 μc of 4-14C-19-nortestosterone was dissolved in 20 ml. of 10% v/v ethanol. This solution was taken followed by drinking of a small volume of water. Complete 24 hr. urine collections were obtained before the start of the drugs administration as control specimens. Two complete 24 hr. urine collections were obtained following administration of the drugs, unless otherwise noted. In order to simplify calculations specimens of less than 1200 ml. were made up to that volume with water. For larger volumes the dilutions were up to the nearest even volume i.e. 1250-1400 ml. Aliquots of urine specimens were extracted immediately and the remaining volumes were stored at $-20^{\circ}C$.

C. MATERIALS AND METHODS

1. Reference hydrocarbons.

40 µg. of the drugs were reduced at catalyst temperatures of 180 to 190°C. The products were dissolved in 0.1 ml. of absolute ethanol. 10 µl. of this solution was generally used for reference purposes. The mean percentage yield of the hydrocarbons from 19-nortestosterone, methandrostenolone, oxymetholone and norethandrolone were 9.3, 17.8, 12.5 and 16.8 respectively (Chapter II, C1, Tables 11 & 13).

2. Paper chromatography.

In order to achieve a better separation of the closely related metabolites paper chromatography, p.c. was used. It was performed by methods similar to those described by Bush (1961c) on Whatman No. 42 paper in all glass tanks at 25° ± 1°C. The solvent systems used are shown in Table 17. In order to simplify reference to these systems the solvent systems have been assigned a code indicating the mobile phase.

The paper chromatographs were run automatically overnight after equilibration for 10 to 12 hr. An alarm clock was modified to turn a tap which filled the trough with 100 ml. of solvent for descending development. The papers which were "overrun" were "saw toothed" to avoid accumulation of solvent at this site (Jermyn & Isherwood, 1949).

For the detection of drugs on papers the reagent used were as described in Chapter I, B7. All these drugs except oxymetholone have strong u.v. absorption/

TABLE 17

THE SOLVENT SYSTEMS USED IN PAPER CHROMATOGRAPHY

Solvent systems by volume	Code
1. Petroleum ether * 5: Methanol 4: Water 1	PE
2. Petroleum ether 33: Toluene 66: Methanol 80: Water 20	PT1
3. Petroleum ether 1: Toluene 9: Methanol 8: Water 2	PT2

* b.p. 80 - 100°C

absorption (254 mμ. max.); this method of detection was generally used to mark the position of the standards. About 10 μg. of Oxymetholone also shows a weak u.v. avsorption on t.l.c. but not on paper. For detection of drug metabolites 8% w/v phosphomolybdic acid was generally used.

For the separation of metabolites on t.l.c. the methods used were as described in Chapter I, B7.

3. Elution of paper and thin-layer chromatograms.

The pencil marked paper was cut into pieces about 7 mm. square; placed in 19/26 tubes to which 5 ml. of methanol was added. The tubes were stoppered and shaken for 20 minutes in a mechanical shaker. The methanol solution was filtered through the sintered glass disc (porosity G3). The tubes were rinsed twice with 1 ml. of methanol which was similarly filtered.

Marked strips of silica gel from t.l.c. were scraped, using two scalpel blades, into 19/26 tubes containing 5 ml. of water saturated chloroform. The tubes were shaken and the suspension filtered as described for paper chromatograms using water saturated chloroform for rinsing the tubes.

The solvents were evaporated to dryness in a waterbath at about 70°C using a stream of filtered air.

TABLE 18

PARTITION COEFFICIENTS OF THE ANABOLIC STEROIDS.

Compounds	Percentage of the drug extracted		
	In petroleum ether from 80% ethanol (v/v)	In benzene from 50% ethanol (v/v)	In chloroform from 20% ethanol (v/v)
Methandrostenolone	2.7	91.8	-
6β-hydroxymethandrostenolone	-	56.0	21.0
Oxymetholone	3.8	54.0	3.8
Norethandrolone	5.0	88.7	-

4. Solvent systems for the partition of drugs.

For preliminary purification of the drugs and their metabolites a partition system was designed. The dry steroid residues were dissolved in 4 ml. of 80% aqueous ethanol (v/v) and extracted twice with 1 vol. of petroleum ether (40-60°C, b.p.). The petroleum ether layer was separated; washed twice with 0.1 vol. of distilled water and evaporated to dryness. The ethanol layer was diluted by water to 50% (v/v) and extracted twice with 1 vol. of distilled benzene. The benzene layer was separated; washed twice with 0.1. vol. of distilled water and evaporated to dryness. The 50% ethanol layer was further diluted to 20% (v/v) and extracted once with 1 vol. of distilled chloroform. The chloroform layer washed twice with 0.1 vol. of distilled water and evaporated to dryness.

The recoveries of the reference anabolic steroids using these partition systems are shown in Table 18. These values were estimated by g.l.c. using SE-30 (210°C) and JXR (195°C) coated columns.

3α, 17α, 21-trihydroxy -5β -pregnane-11, 20-dione, tetrahydrocortisone was also partitioned by the above system in order to determine the recoveries of such polar compounds. When estimated as the product 5β -androstan-3, 11, 17-trione by g.l.c., the recoveries in the benzene layer after extraction from the 50% ethanol were about 11.4% and in the chloroform layer after extraction from the 20% ethanol were about 42.7%.

5. Determination of radioactivity.

Measurement of radioactivity due to ^{14}C was by the method of Harkness & Fotherby (1963). The residues were dissolved in 3 drops of ethanol and added to 4 ml. of liquid scintillator, NE 213 (Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh). The solutions were then transferred into the counting vials and were counted in a Packard liquid scintillation spectrometer, model 2002 (Packard Instrument Company, Inc., Ill.) for 1000 sec. The instrument settings were, gain 8%, 20:1 window ratio of upper discriminator to baseline discriminator which was set at 50. The efficiency for counting ^{14}C was 83% with these settings. The quenching correction was estimated automatically from an external standard.

6. Enzyme hydrolysis and extraction of urine.

The β-glucuronidase preparation was obtained from the common limpet (Patella vulgata) by the method of Fotherby & Love (1960). This preparation had an activity of 2 million Fishman units /g. Urine was adjusted to pH 4.7 and 0.1 vol. of 5M-acetate buffer, pH 4.7 added. The enzyme powder was then added to give a concentration of 1500 units/ml. of urine. The mixture was well shaken and incubated at 37°C in a waterbath for 48 hr.

The urine was extracted twice with 1 vol. of chloroform or benzene; the chloroform or the benzene extract was washed twice with o.1 vol. of N NaOH and then with 0.1 vol of distilled water until the washings were neutral. Traces of water were removed with anhydrous sodium sulphate and the solvent was distilled off using a boiling waterbath. The dry residue was dissolved in/

in 5ml. of absolute ethanol; a 4.5ml. aliquot of this was then transferred to a conical centrifuge tube and evaporated to dryness at about 70°C. This extract represented the "conjugated" fraction.

The urine before enzyme hydrolysis was extracted similarly. This extract represented the unconjugated or the "free" fraction.

7. Microchemical treatments.

Potassium borohydride reduction.

Potassium borohydride (BDH) was dissolved in methanol at a concentration of 1 mg./ml. The dry steroid residue was dissolved in 0.1 ml. of methanol and treated with 1ml. of potassium borohydride solution. The reaction mixture was left overnight at room temperature. The methanol was evaporated to dryness in a waterbath at about 70°C using a blast of filtered air. The residue was extracted into chloroform from distilled water in a similar way to that described for acetylation (see Chapter III, Section II, B).

The Girard separation was performed by the method of Girard & Sandulesco (1936).

SCHEME 1

A GENERAL PROCEDURE FOR THE DETECTION OF ANABOLIC STEROIDS AND THEIR METABOLITES IN URINE

200 ml urine

Extraction with $CHCl_3$ or with C_6H_6

Urine	"Free" Fraction	
	Partition	T.l.c.
Enzyme hydrolysis	*	
"Conjugated" fraction	H.t.c.r. & G.l.c.	elution of fractions
T.l.c. or P.c.	(total metabolites)	H.t.c.r. & G.l.c.
Elution of fractions		
H.t.c.r. & G.l.c. (First scan)		
Partition of the positive fractions		
H.t.c.r. & G.l.c. (Final scan)		

* High temperature catalytic reduction

D. GENERAL PROCEDURE

An outline of the general procedure used for the detection of anabolic steroids and their metabolites is presented in Scheme I. 200 ml. of the first 24 hr. urine collection after taking the drug was extracted by chloroform or benzene. The urine after extraction of the "free fraction was hydrolysed to obtain the "conjugated fraction. The extracts were halved. One part was used for t.l.c. or p.c. The chromatographic fractions were collected as elutes from 1 to 2.5 cm. strips. The dry fractions were then dissolved in 0.1 ml. of absolute ethanol. 10 µl. of this solution from each fraction was used for the high temperature catalytic reduction at 180°-190°C using 1-3% w/w platinum catalyst. The reduction products were run on g.l.c. using the SE-30 and the NGA columns with hydrocarbon standard from the drug.

From g.l.c. results chromatographic fractions were selected which contained compounds with the same carbon skeleton as the drug (positive fractions). These fractions were then purified by the partition systems described in Chapter IV, C4. Further confirmation of the carbon skeleton of the compound or compounds in these fractions (final scan) was then obtained by g.l.c. after high temperature catalytic reduction. The remaining halves of the urine extracts were also chromatographed and the fractions containing drug metabolites were pooled. These chromatographic fractions were then tentatively identified by their chromatographic mobilities before and/

and after microchemical treatments like acetylation with acetic anhydride and reduction by potassium borohydride.

The "free" urine fraction was similarly treated except that to obtain an estimate of the total metabolites present the urine was extracted in benzene and the dry residue obtained after evaporation of benzene was partitioned in the system described in Chapter IV, C4 prior to t.l.c. or p.c. The benzene layer was collected and evaporated to dryness. The dry residue was dissolved in 0.2 ml. of absolute ethanol; 20 µl. of this solution was then injected for high temperature catalytic reduction. As expected the extracts obtained from chloroform were much bulkier than those from benzene.

The control urine specimens were similarly treated up to the "first" scan. The presence of non-specific products from them were noted for the interpretation of the results.

The studies of the metabolism of individual anabolic steroids are presented under the separate headings on the following pages. The estimates of the metabolites present in chromatographic fractions in µg. of steranes (Figs. 15, 17, 19 and 21) represent the amounts present in the aliquots of the samples used for high temperature catalytic reduction. The estimates of the quantities of drug metabolites excreted have been calculated from the yields of the hydrocarbons obtained from the parent drugs, and from the total 24 hr. urine volumes except where otherwise noted. The accuracy of this correction is dependent on the nature of the metabolites. The results,/

results, however, have been surprisingly reproducible.

In order to avoid repetitions only the changes in the procedure from that described above have been specified under individual headings.

E. METABOLISM OF 19-NORTESTOSTERONE

The metabolism of 19-nortestosterone has been studied by Engel, Alexander & Wheeler (1958) in a woman after administration of 550 mg. of the drug. These workers have shown that the two urinary metabolites of this drug were 19-noraetiocholanolone and 19-norandrosterone. The present study using high temperature catalytic reduction and radioactivity confirms their results using 20 mg. of the drug. The sensitivity of high temperature catalytic reduction for detection of metabolites has been satisfactory.

a. EXPERIMENTAL

400 ml. of the 48 hr. pooled urine collection from the subject P, was used to obtain "free" and "conjugated" urine extracts which were separated by p.c. using the solvent system PE as described on Chapter IV, C2. The chromatogram was run for 6 hr. allowing the solvent to overrun the paper. The marker standards, androsterone, aetiocholanolone and 19-nortestosterone were also run alongside the extract. The reference 4-^{14}C-19-nortestosterone used was homogeneous on p.c. and on t.l.c. in the relevent solvent systems (Chapter I, B7 and Chapter IV, C2).

High temperature catalytic reduction was performed at 180°C. The yield of the parent hydrocarbon from 19-nortestosterone was less than those from methandrostenolone and norethandrolone. By using an increased concentration of platinum, two split products with shorter retention times than "19-nor-androstane" were obtained probably due to splitting off the 18-methyl group. Two other products of much longer retention times probably due to dehydrogenation were also obtained after using such a catalyst. It was therefore essential to keep the concentration of the platinum to near 1% as obtained by the procedure described in Chapter I, B2.

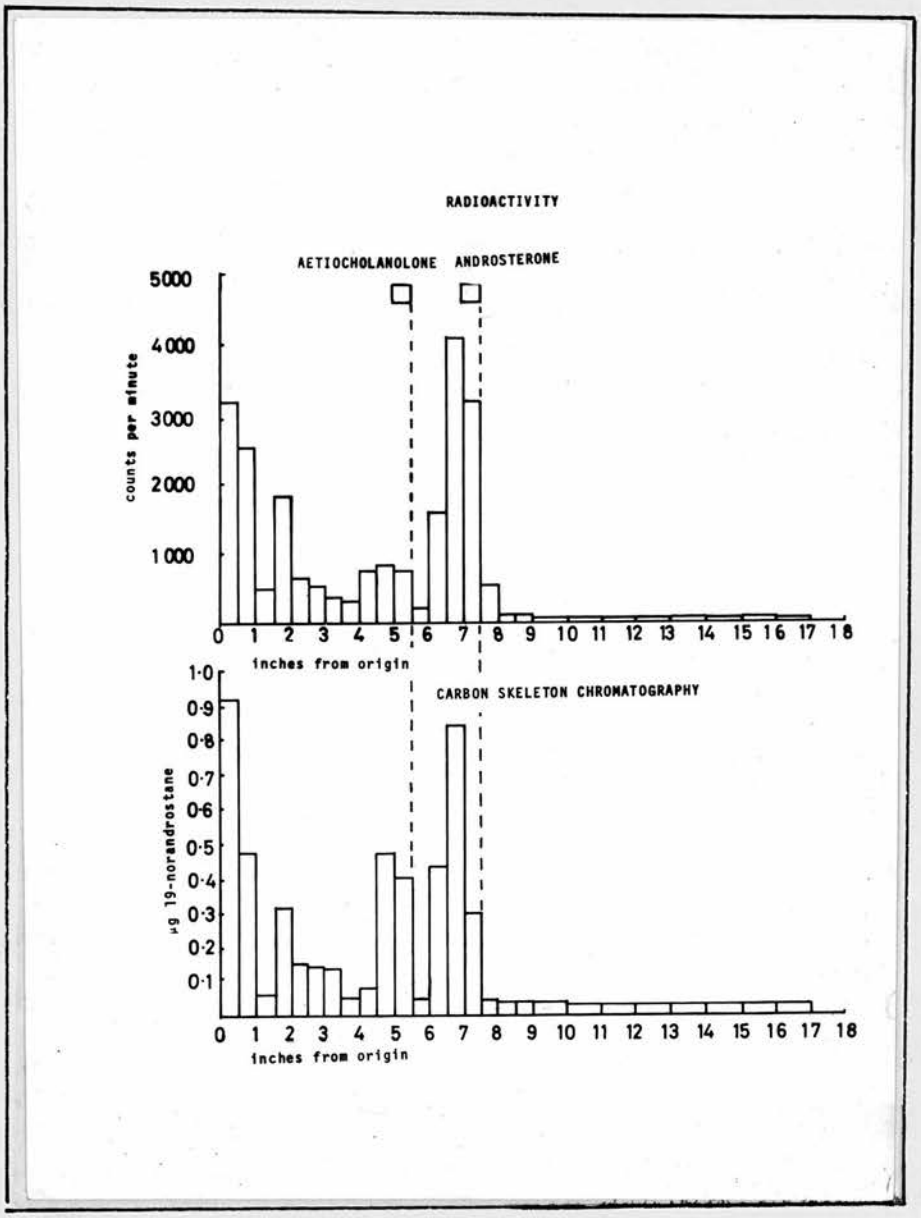


Fig. 13. The pattern of 19-nortestosterone metabolites on paper chromatograms. Detection of metabolites by the methods of liquid scin-:tillation counting and "carbon skeleton chromatography".

b. RESULTS

The reduction of 19-nortestosterone gave a major product probably a "19-norandrostane" (Fig. 15) having a retention time relative to 5α -androstane of 0.6 on an NGA column. This product was clearly separable from the androstanes and was also obtained from 19-hydroxylated androstane derivatives and oestrogens (Chapter II, C3.).

No product corresponding to the hydrocarbon "19-norandrostane" was detected in the "free" fraction and no radioactivity was detectable in the "free" fraction. This fraction was therefore not investigated further.

1. Detection of the metabolites in the "conjugated" fraction.

The scans of the chromatograms using radioactivity and carbon skeleton chromatography as methods of detection are presented in Fig. 13. The positions of the markers aetiocholanolone and androsterone in the chromatograms have been indicated. The marker 19-nortestosterone in the system ran to 4.5 cm from the origin. The patterns of metabolites are almost identical using both methods of detection and show the presence of two major metabolites with similar R_{F}s to those of the reference aetiocholanolone and androsterone.

In addition to the two major metabolites at least three more polar metabolites were separated by p.c. using the solvent system PT2. In this system androsterone and aetiocholanolone were run off the paper and 19-nortestosterone was run to the end of the paper. The patterns of metabolites obtained/

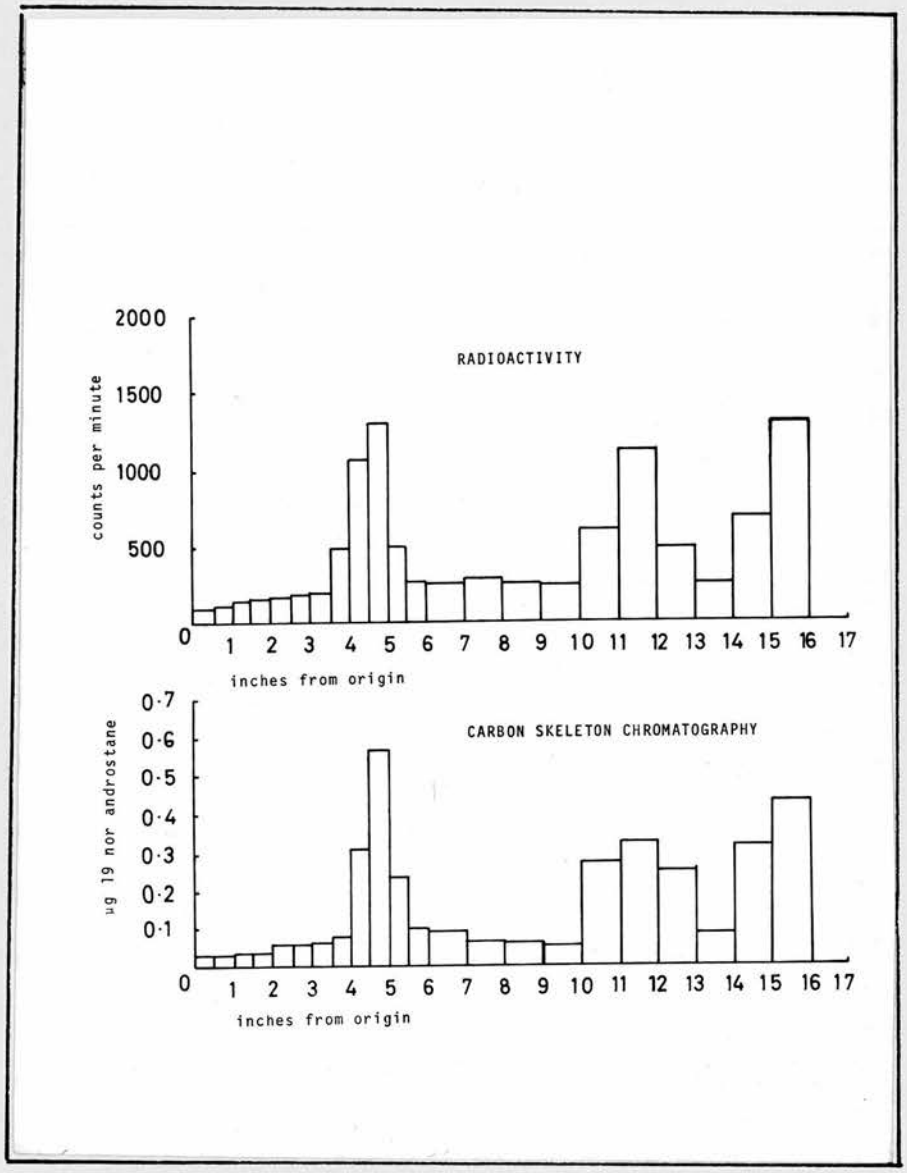


Fig. 14. The pattern of polar metabolites of 19-nortestosterone on paper chromatograms. Detection of metabolites by the methods of liquid scintillation counting and "carbon skeleton chromatography".

obtained using radioactivity and high temperature catalytic reduction are presented in Fig. 14 and are almost identical.

The total amount of the "conjugated" metabolites represented approximately 31% of the administered dose in the 48 hr. urine volume.

2. Tentative identification of the metabolites of 19-nortestosterone.

Four fractions running like 19-noraetiocholanolone (E1), aetiocholanolone (E2), 19-norandrosterone (A1) and androsterone (A2) were collected by using preparative g.l.c. on a column coated with 3% SE-52, phenyl methyl silicone at 230°C. No satisfactory separation of these fractions was possible using p.c. or t.l.c. The half portions of the collected fractions were counted for radioactivity which was detected only in E1 and A1. These radioactive fractions after high temperature catalytic reduction gave a major product with an identical retention time to the product from similarly reduced 19-nortestosterone (Fig. 15).

E1 and A1 did not absorb u.v. confirming the reduction of the Δ^4-3-ketone group of 19-nortestosterone during metabolism. After reduction by potassium borohydride and after acetylation by acetic anhydride the R_{Fs} of E1 and A1 were altered and were like similarly treated aetiocholanolone and androsterone on t.l.c. in the solvent system chloroform-methanol (19:1 v/v). These results indicated the presence of a reducible ketone and an acylable hydroxyl group in E1 and A1.

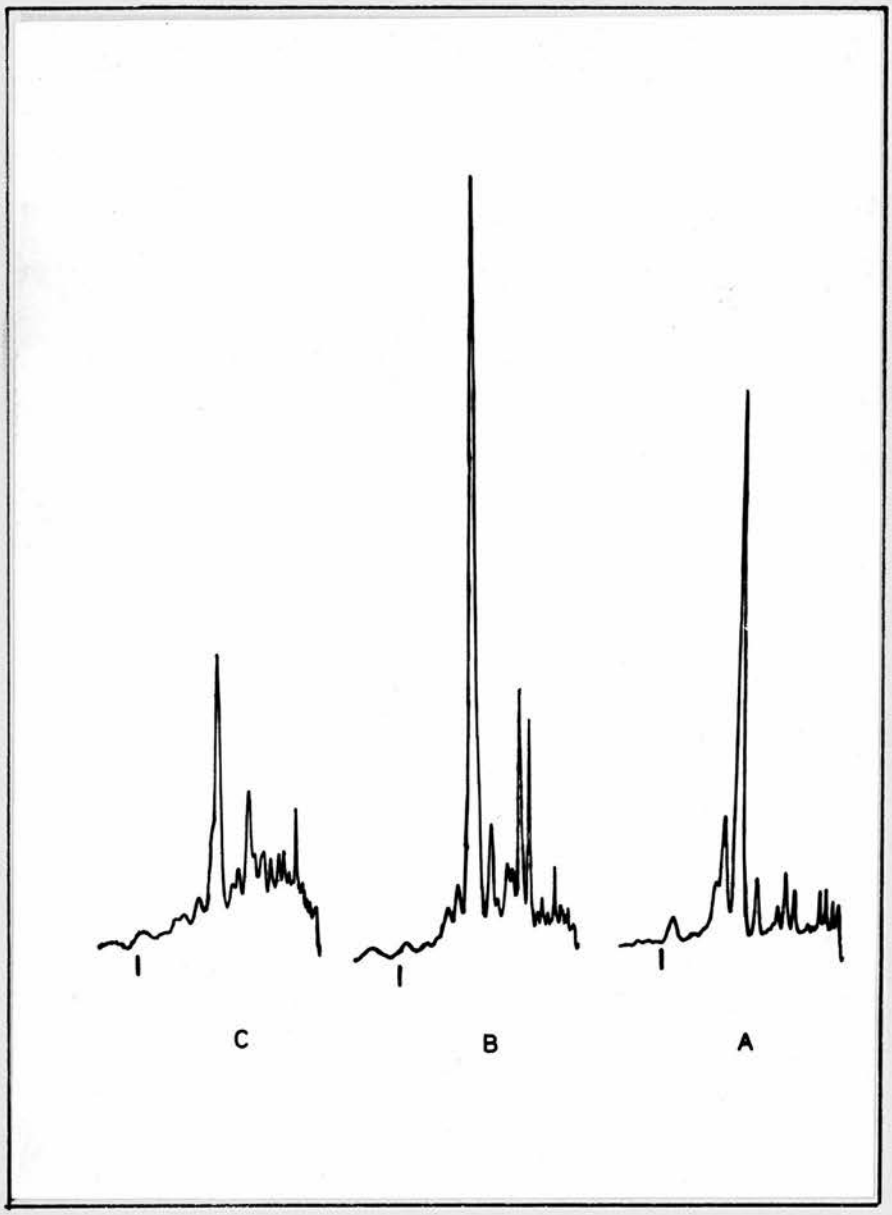


Fig. 15. Gas chromatograms on an NGA coated column at 150°C, attenuation x 200 of

(A) Reduction products of 4 µg. 19-nortestosterone.

(B) Reduction products of the metabolite like 19-norandrosterone (A1).

(C) Reduction products of the metabolite like 19-noraetiocholanolone (E1).

The catalyst temperature used in these experiments was 180°C; the retention time of 5α-androstane is indicated by a vertical line on the tracings.

3. Conclusion.

The above results suggest that the two major urinary metabolites of 19-nortestosterone, E1 and A1 are 19-noraetiocholanolone and 19-norandrosterone as described by Engel et al. (1958). The patterns of the metabolites obtained using high temperature catalytic reduction and radioactivity as detection methods have been similar.

F. METABOLISM OF METHANDROSTENOLONE

The metabolism of methandrosterolone was studied by Rongone & Segaloff (1963) in a woman with adenocarcinoma of the lung. She was given methandrostenolone, 1 g. daily for 4 days and from the urine two metabolites were isolated. The major metabolite was identified as 6β-hydroxymethandrostenolone; it was suggested that the other metabolite was an epimer of methandrostenolone. Sandor & Lanthier (1963) studied the metabolism of methandrostenolone-17α-C^{14} in a dog and also showed the presence of two major metabolites. From all these results it was seen that the metabolism of methandrostenolone was virtually complete and no unaltered drug was excreted.

The present study confirms the findings described above. The application of high temperature catalytic reduction and g.l.c. is especially useful in studies of drugs like methandrostenolone which are apparently completely metabolised in the body. Such drug metabolites though possessing different properties should be detectable by their common hydrocarbon skeleton.

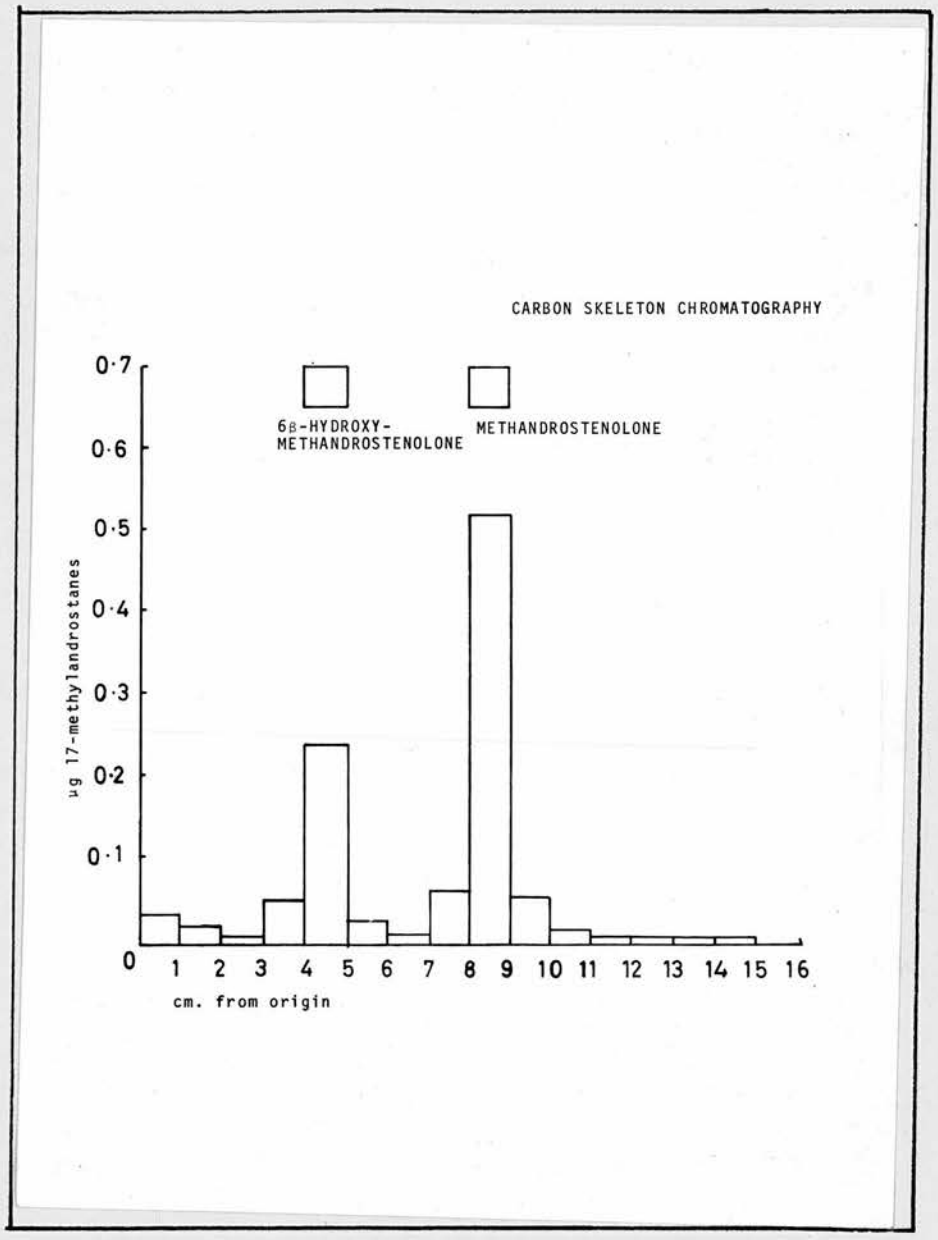


Fig. 16. The pattern of methandrostenolone metabolites on thin-layer chromatograms. Detection of metabolites by "carbon skeleton chromatography".

a. EXPERIMENTAL

Urine aliquots from the subjects P, A and R were extracted by chloroform. The "free" and the "conjugated" fractions were chromatographed on thin-layer in the solvent system chloroform-methanol (19:1 v/v). The marker standards 6β-hydroxymethandrostenolone and methandrostenolone were also run alongside. High temperature catalytic reduction was done at a catalyst temperature of 190°C.

b. RESULTS

1. Detection of the metabolites in "free" fractions.

The scan of the carbon skeletons of methandrostenolone metabolites in chromatographic fractions from subject A is presented in Fig. 16. The two fractions showing the carbon skeleton of methandrostenolone corresponded to the positions of the markers 6β-hydroxymethandrostenolone and methandrostenolone in t.l.c. and were just detectable by their u.v. absorption and by staining with 8% w/v phosphomolybdic acid. The fractions remaining at about the origin of the chromatograph have also shown similar carbon skeletons but the quantities of these minor metabolites were too small for further studies.

The two fractions hereafter called M1, corresponding to 6β-hydroxymethandrostenolone and M2, corresponding to methandrostenolone were partitioned in the system described in Chapter IV, C4; the benzene portion was collected and evaporated to dryness. The reduction patterns from the reference/

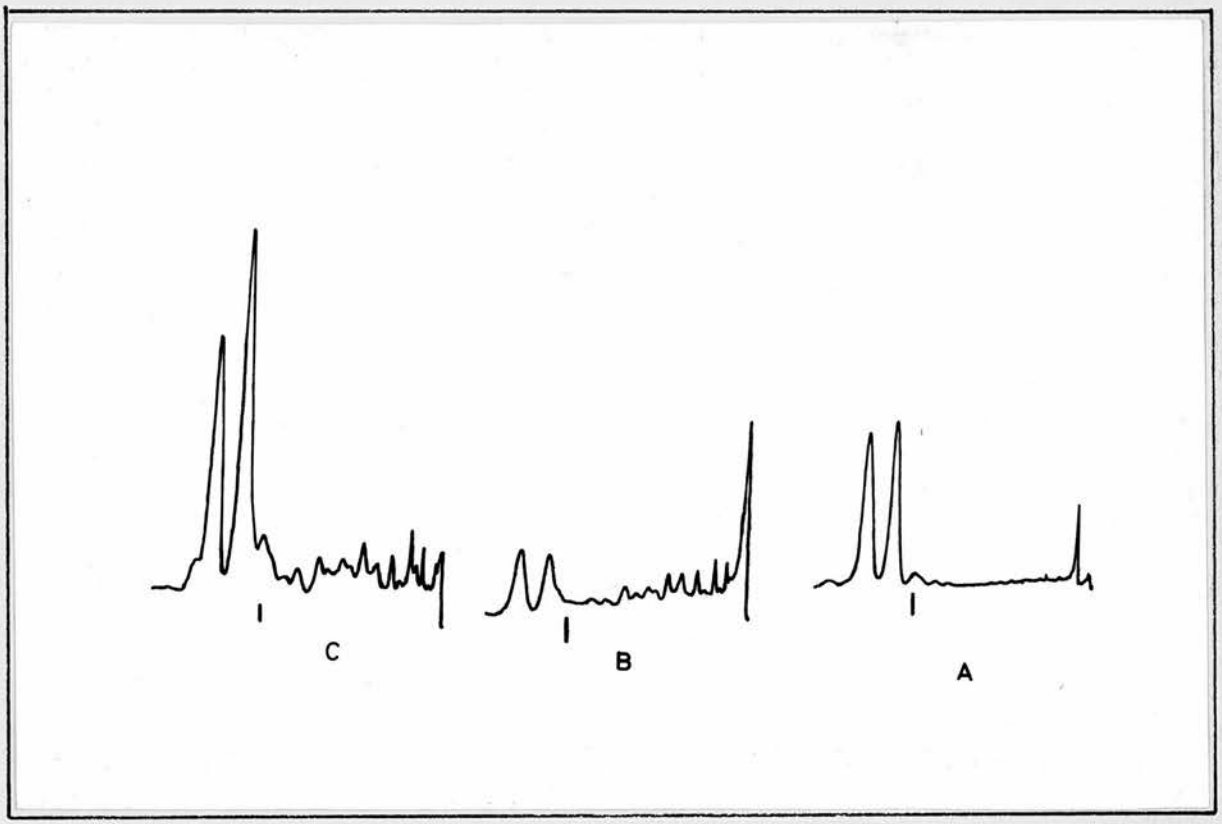


Fig. 17. Gas chromatograms on an NGA column at 150°C of the reduction products of methandrostenolone and its metabolites reduced at the catalyst temperature of 190°C:

(A) 2.0 µg. of standard methandrostenolone; attenuation x 500.

(B) The compound behaving like 6β-hydroxy-:methandrostenolone (M1); attenuation x 500.

(C) The compound behaving like an epimer of methandrostenolone (M2); attenua-:tion x 200.

The retention time of 5α-androstane is indicated by a vertical line on the tracings.

reference methandrostenolone and those from M1 and M2 are shown in Fig. 17. The patterns of reduction from these compounds are similar and the major products have identical retention times. The two products with retention times relative to 5α-androstane of 1.19 and 1.31 (Fig. 17)., are probably the 5β and 5α-isomers "17-methylandrostane". These products were also obtained from 17α-methyltestosterone (see Chapter II, C1). Similar results were obtained from subjects P and R. The total quantities of the metabolites recovered from the urine of subjects P, A and R were 4.1%, 5.7% and 5.3% respectively of the administered drug. No metabolites were detected by any method in the comparable control urine samples.

2. Detection of Metabolites in the total "free" extracts".

In order to study the duration and the maximum excretion of metabolites a serial urine collection of one week was obtained from the subject P after administration of methandrostenolone, 5 mg. daily for 2 days; the tablet being taken at the start of the urine collections. The residues obtained after extraction of the urine by benzene were partitioned in the system described on Chapter IV C4; the benzene portion was collected and then evaporated to dryness. The fractions were then reduced. The amounts of the hydrocarbons in these fractions from the first day to the fourth day were 0.54 µg., 0.75 µg., 0.11 µg. and 0.00 µg. respectively. The maximum amount of metabolites was, as expected on the second day and a small amount was still/

still detectable 48 hr. after drug administration. These urine residues were also reduced without purification by partition. The "17α-methylandrostane" hydrocarbon peaks were detectable but the bulky nature of the total extracts was found to deactivate the catalyst rapidly. The "conjugated" fractions from the subjects P, A and R were chromatographed on thin-layer and then reduced. The scans from the chromatograms showed no products corresponding to the "17α-methylandrostane" on g.l.c. No further study on this fraction was therefore done.

3. Tentative identification of the metabolites of methandrostenolone.

The fractions M1 and M2 absorbed u.v. suggesting that at least one of the double bonds in ring A and the ketone at C-3 in methandrostenolone remained intact during metabolism. M1 and M2 had identical R_{F}s to 6β-hydroxymethandrostenolone and methandrostenolone respectively on t.l.c. in the solvent systems, chloroform-methanol (19:1 v/v); chloroform-acetone (19:1v/v) and on p.c. in the solvent system PT2.

On g.l.c. using an SE-30 and QF-1 columns at 210^{o}C, M1 had identical retention times to 6β-hydroxymethandrostenolone. The retention times of M2 and methandrostenolone on the SE-30 column were 22.0 and 23.0 min. respectively; on the QF-1 column the retention times were 23.9 and 25.3 min. respectively. The g.l.c. results thus suggested M2 was a metabolite of methandrostenolone.

After acetylation the R_{F} of M1 and 6β-hydroxymethandrostenolone on t.l.c. /

t.l.c. in the solvent system chloroform-methanol (19:1 v/v) was found to change and was again identical. Under the same condition the R_F of M2 and methandrostenolone remained unchanged indicating the unreactivity of the C-17 tertiary hydroxyl group to acetylation (Bush, 1961d). These results suggested the presence of an acylable hydroxyl group in M1 similar to that in 6β-hydroxymethandrostenolone.

After potassium borohydride reduction the R_F of M1 and M2 remained unchanged on t.l.c. in the solvent system (19:1 v/v) suggesting the absence of a reducible ketone group. Methandrostenolone did not appear in the ketonic fraction after the Girard separation possibly due to its hemiquinone structure.

4. Conclusion.

The above results show that in its chemical properties and chromatographic mobilities the fraction M1 behaved like 6β-hydroxymethandrostenolone; this is consistent with the metabolite of methandrostenolone identified by Rongone & Segaloff (1963). For M2, the slight difference in its retention times on g.l.c. to that of methandrostenolone and also its lack of chemical reactivity, like methandrostenolone, suggest that it was an epimer (17β-CH_3) of methandrostenolone rather than an oxygenated metabolite like M1. It therefore seems likely that M2 is similar to the unidentified metabolite of methandrostenolone described by Rongone & Segaloff (1963). These metabolites which were easily detected by using high temperature catalytic reduction were thus similar to those just detectable by conventional methods such/

such as u.v. absorption and 8% w/v phosphomolybdic acid. No unaltered methandrostenolone was detected.

G. METABOLISM OF OXYMETHOLONE.

The synthesis of oxymetholone was described by Ringold, Batres, Halpern & Necoechea (1959). The anabolic and therapeutic properties of this compound have been evaluated (Myerson, 1961; Sanchez-Medal, Pizzuto, Torre-Lopez & Derbez, 1964). The metabolism of oxymetholone however has not been reported. In the present study two fractions with the carbon skeleton of "17-methylandrostane" were detected in urine after the administration of oxymetholone. The polar nature and instability of the metabolites on g.l.c. suggest an alteration of the hydroxymethylene group during metabolism.

a. EXPERIMENTAL

The subjects of the study were P, R and T. The urine aliquots were extracted by benzene. For t.l.c. separation of the extracts the solvent system chloroform-methanol (9:1 v/v) was used. The high temperature catalytic reduction was performed at 190°C.

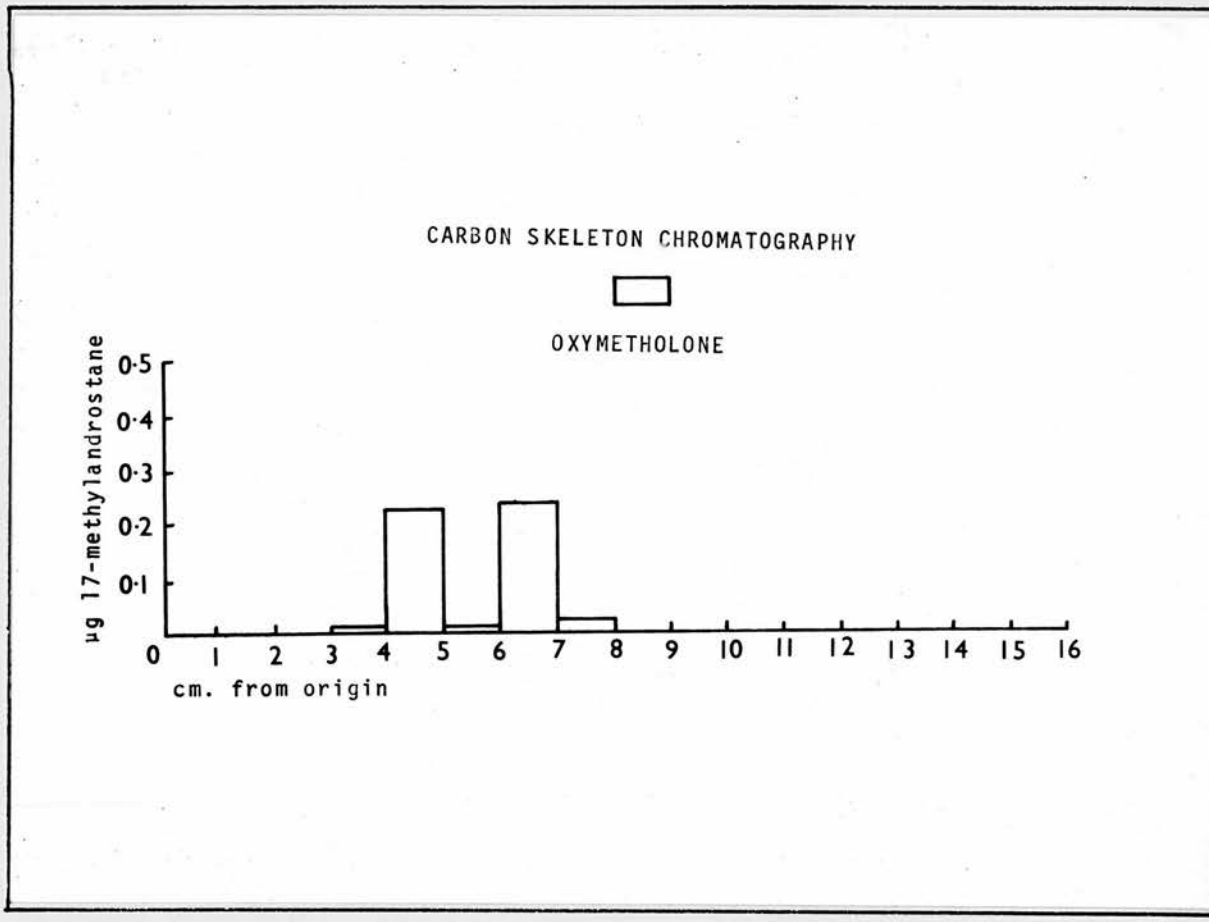


Fig. 18. The pattern of oxymetholone metabolites on thin-layer chromatograms. Detection of metabolites by "carbon skeleton chromatography".

b. RESULTS

No products with the retention times of the hydrocarbons obtained from oxymetholone were detected in the "free" fractions.

1. Detection of metabolites in "conjugated" fractions.

The scan for the carbon skeleton "17-methylandrostane" in chromatographic fractions from subject R is presented in Fig. 18. The two fractions hereafter respectively called O1 and O2 for the polar and the less polar components were partitioned in the system described in Chapter IV, C4 except that after separation of the petroleum ether the 80% ethanol was diluted to 20% ethanol by distilled water which was then extracted twice with 1 vol. of chloroform. Due to the polar nature of these metabolites it was found difficult to obtain clean fractions by the simple partition methods used. The reduction products of O1 and O2 and those from standard oxymetholone are presented in Fig. 19. It is to be noted that the products from oxymetholone have identical retention times to the reduction products of other compounds with a 17-methylandrostane skeleton (Chapter II, C1); this is probably due to the splitting of the hydroxymethylene group of oxymetholone.

The total amounts of the metabolites in urine from subjects P, R and T were 5.1%, 6.0% and 4.9% respectively of the administered drug. O1 and O2 were present in about the ratio of 1:1.

2. Tentative identification of the metabolites of oxymetholone.

In the solvent system chloroform-methanol (9:1 v/v) on t.l.c., the R_F of/

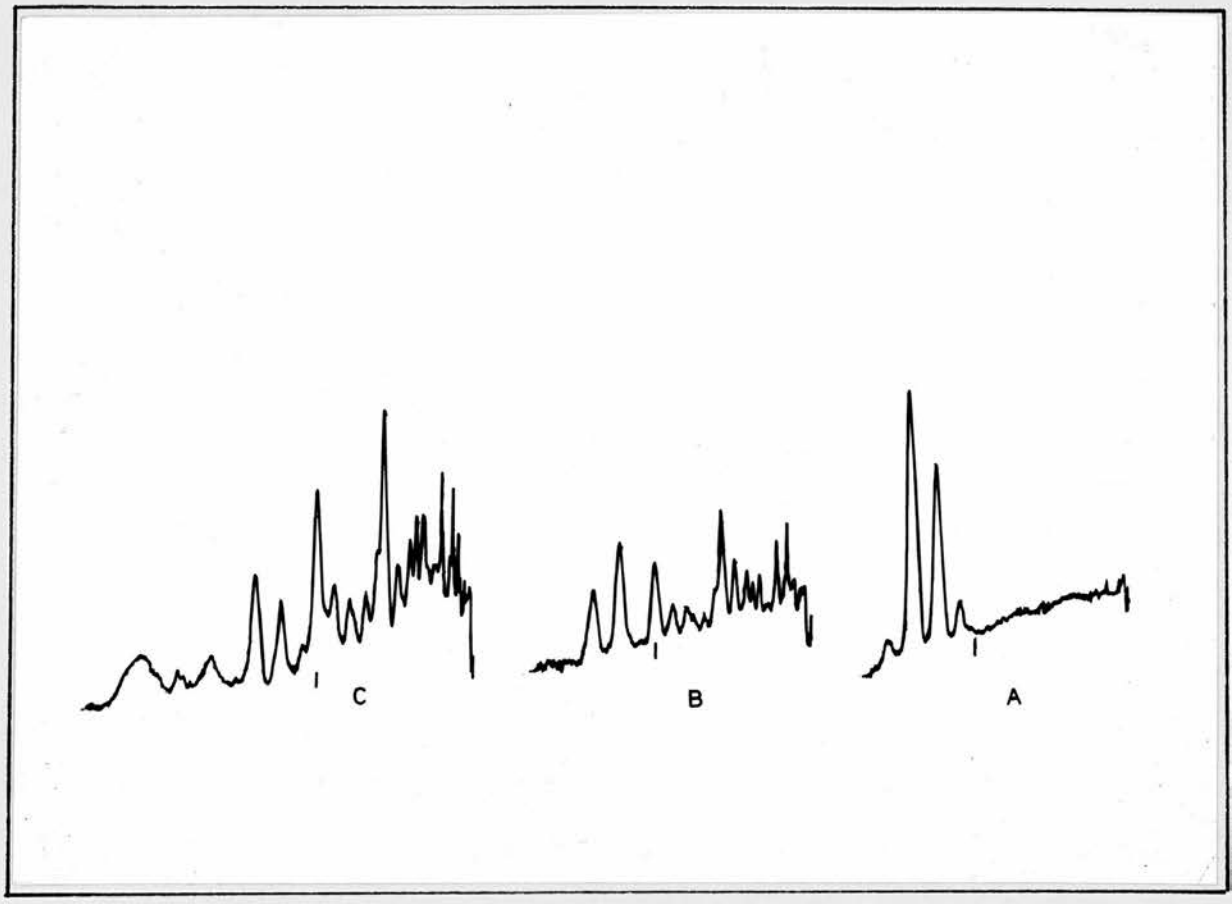


Fig. 19. Gas chromatograms on an NGA column at 150°C of the reduction products of oxymetholone and its metabolites reduced at the catalyst temperature of 190°C; attenuation x 200:

(A) 4 µg. of standard oxymetholone.

(B) The most polar metabolite (01).

(C) The polar metabolite (02).

The retention time of 5α-androstane is indicated by a vertical line on the tracings.

of O1, O2 and the standard oxymetholone respectively were 0.36, 0.50 and 0.78. After potassium borohydride reduction of these compounds, in the above t.l.c. system, the R_F of O1 remained unchanged whereas the R_F of O2 was changed and was identical to that of O1. Oxymetholone after potassium borohydride reduction had a different R_F (0.46).

These results suggested the presence of a reducible ketone group in O2 which was absent in O1.

The acetylated products of O1 and O2 had R_{Fs} of 0.42 and 0.40 respectively on t.l.c. in the solvent system chloroform-acetone (9:1 v/v). The similarly treated oxymetholone ran with the R_F of 0.39.

After potassium borohydride reduction and acetylation O1 and O2 had identical R_{Fs} of 0.78 on t.l.c. in the solvent system chloroform-methanol (19:1 v/v) and also in the less polar solvent system of chloroform alone (R_F = 0.20). All these results suggested that O2 after reduction by potassium borohydride was similar to O1.

On g.l.c. in an SE-30 column at 215°C and in a QF-1 column at 220°C both O1 and O2 gave at least three products with shorter retention times than that of standard oxymetholone showing their unstable nature. Oxymetholone showed a poor response and 'peak tailing'. The products of potassium borohydride reduction of oxymetholone, O1 and O2 were all unstable and gave similar split products on g.l.c. However, acetylation of oxymetholone, O1 and O2 allowed the elution of a single peak from these compounds with different retention times which are shown in Table 19.

TABLE 19

RETENTION TIMES OF OXYMETHOLONE AND ITS METABOLITES AFTER ACETYLATION

Compounds	Retention times in minutes	
	On SE-30 column at 215°C	On QF-1 column at 220°C
Oxymetholone	30.0	22.5
O 1	55.9	25.3
O 2	24.4	18.8
Oxymetholone (before acetylation)	26.3	14.5
Oxymetholone (KBH_4 reduction and acetylation)	51.7	

3. Conclusion.

The g.l.c. results from O1 after acetate formation confirm those of t.l.c. and suggest the reduction of the C-3 ketone group of oxymetholone during metabolism. On the other hand, the similar mobilities of O2 to that of O1 on t.l.c. after the potassium borohydride reduction suggest that the C-3 ketone has remained intace in O2.

The instability on g.l.c. of the metabolites O1, O2 and of the oxymetholone after potassium borohydride reduction and the "17-methylandrostane" hydrocarbon obtained after high temperature catalytic reduction, indicate the labile C-2 substitution of these compounds. This group therefore could have been altered during metabolism.

H. METABOLISM OF NORETHANDROLONE

The preparation of norethandrolone has been described by Colton, Nysted, Riegel & Raymond (1957). It is a widely used anabolic steroid (Brooks & Prunty, 1957; Brendler & Wrinkler, 1959). The metabolic fate of this compound however is not known. The present study shows two major urinary metabolites of norethandrolone which are probably the predicted reduction products. From its structure, the metabolism of this compound was likely to be comparable to that of 17-methyltestosterone (Rongone & Segaloff, 1962) and 19-nortestosterone (Engel et al. 1958).

a. EXPERIMENTAL

The subjects of the study were P, R, T and M. The urine aliquots were extracted by benzene. The "free" and "conjugated" fractions were chromatographed on thin-layer in the solvent system chloroform-methanol (9:1 v/v). The chromatographic fractions were eluted and processed as described under the general procedure (Chapter IV, D).

The subject M, a child, was given 10 mg. of norethandrolone. Half of the 24 hr. urine collection was similarly extracted by benzene and separated on thin-layer. Each chromatographic fraction was dissolved in 0.4 ml. of absolute ethanol; 10 µl. of this solution was used for reduction as usual. The high temperature catalytic reduction was done at 190°C.

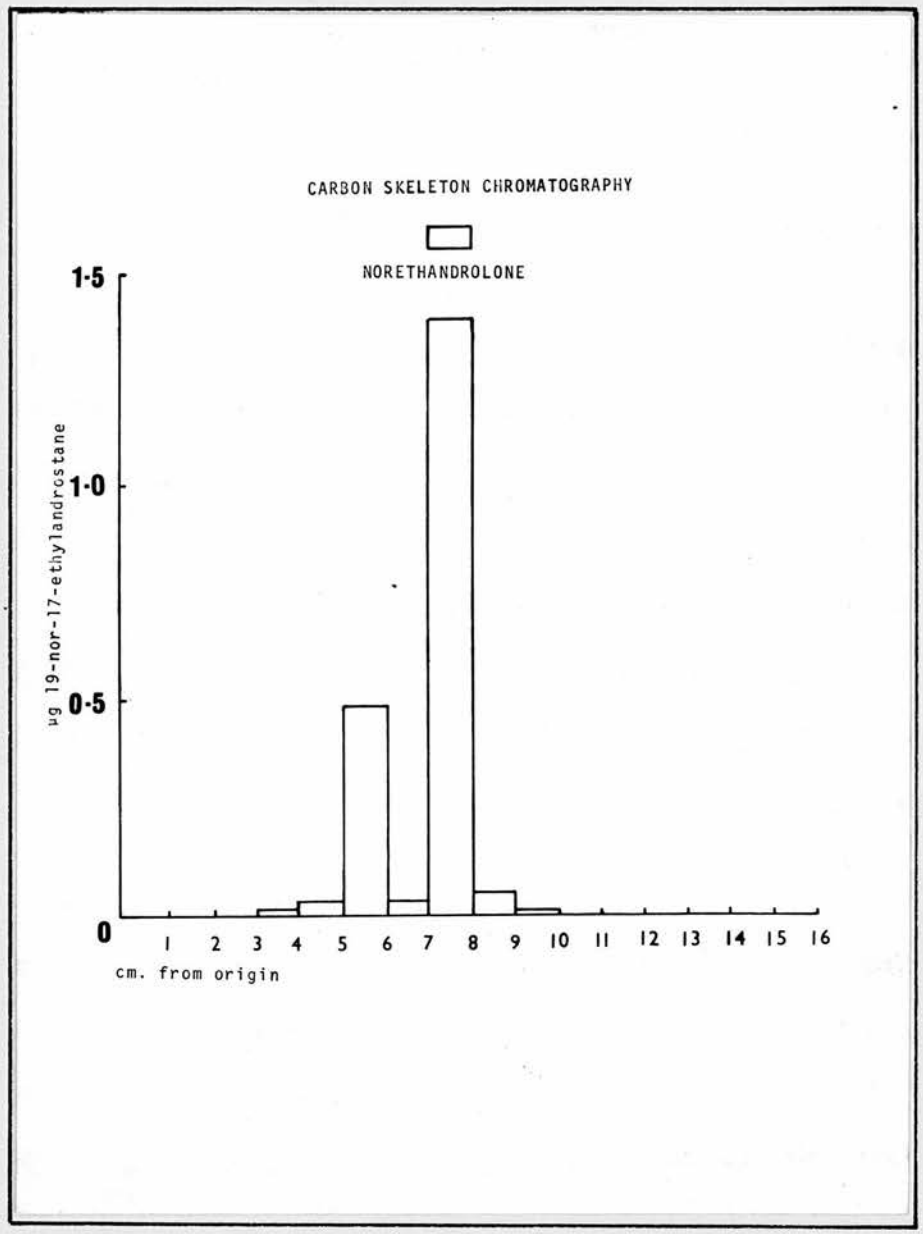


Fig. 20. The pattern of norethandrolone metabolites on thin-layer chromatograms. Detection of metabolites by "carbon skeleton chromatography".

b. RESULTS

1. Detection of the metabolite in "free" fractions.

No product corresponding to the parent hydrocarbon of norethandrolone was detected in the "free" fractions from P, R and T. In subject M, the fraction corresponding to norethandrolone on t.l.c. gave a product with an identical retention time to that of the hydrocarbon standard. This fraction amounted to 0.02% of the administered drug. On g.l.c. this component had the same retention time as norethandrolone in columns coated with SE-30 at 205°C and with QF-1 at 210°C. This fraction corresponding to norethandrolone was not detectable by u.v. absorption on t.l.c. probably due to the minute amount present.

2. Detection of the metabolites in "conjugated" fractions.

The scan for the carbon skeleton of norethandrolone in the chromatographic fractions from subject R is presented in Fig. 20. Two fractions, one slightly more polar than norethandrolone (N1) and the other corresponding to norethandrolone (N2) on t.l.c. were partitioned in the system described in Chapter IV, C4. The benzene portion was collected and evaporated to dryness. The reduction patterns from N1 and N2 and the reference norethandrolone were similar and the components had identical retention times (Fig. 21). Similar results were also obtained from subjects P, T and M. The total amounts of the conjugated metabolites in the urines of P, R, T and M were 11.2%, 11.2%, 9.1% and 10.9% respectively of the administered drug. The ratio of N1 to N2 was about 1:2.8 in these subjects.

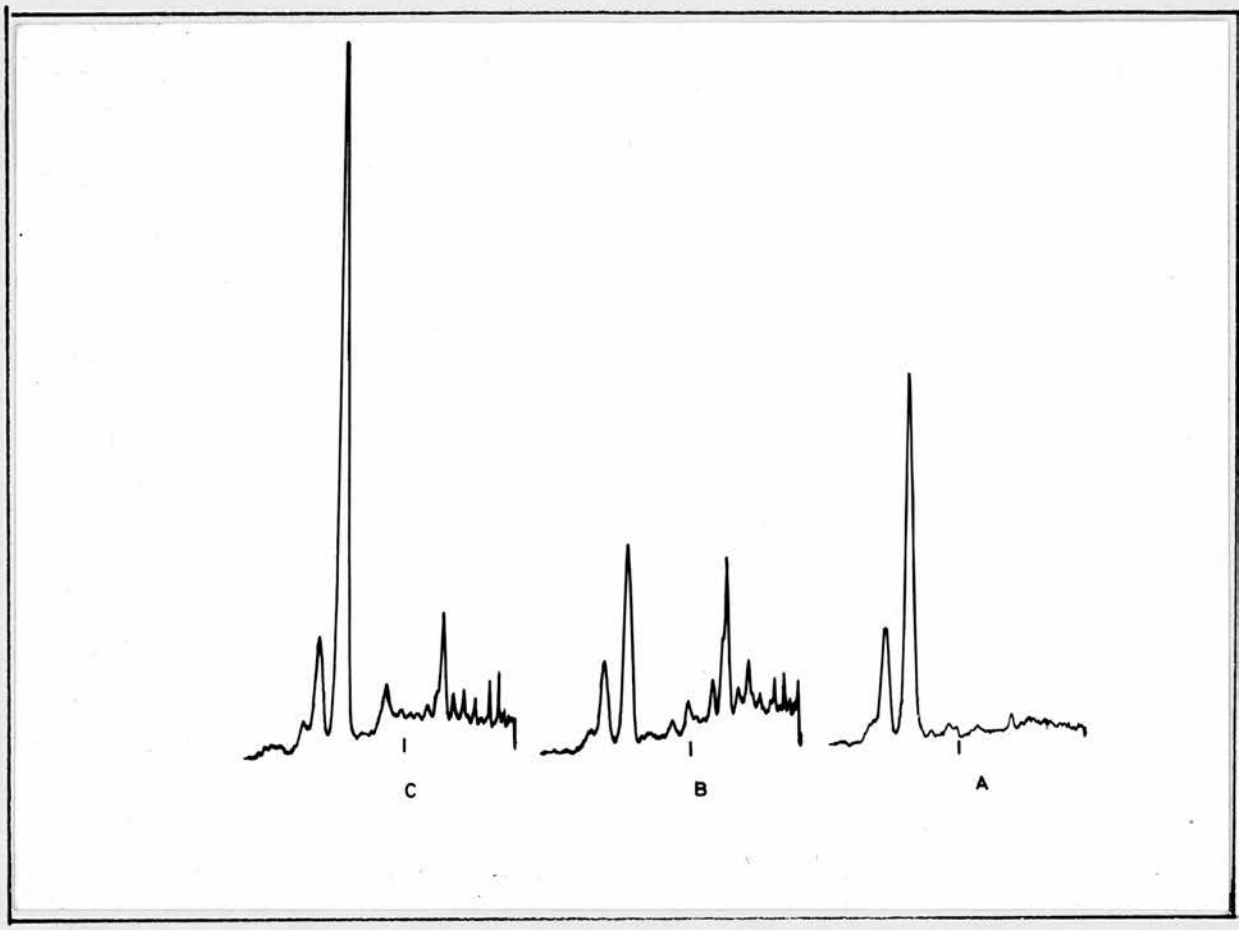


Fig. 21. Gas chromatograms on an NGA column at 150°C of the reduction products of norethandrolone and its metabolites reduced at the catalyst temperature of 190°C; attenuation x 200:

(A) 4 µg. of standard norethandrolone.

(B) The compound more polar than norethandrolone (N1).

(C) The compound with R_F similar to norethandrolone on t.l.c.

The retention time of 5α-androstane is indicated by a vertical line on the tracings.

3. Tentative identification of the metabolites of norethandrolone.

The two chromatographic fractions N1 and N2 did not absorb u.v. which suggested the reduction of the Δ^4-3-ketone group of norethandrolone during metabolism. These fractions were readily detectable by using 8% w/v phosphomolybdic acid.

On t.l.c. in the solvent system chloroform-acetone (9:1 v/v) N1 had R_F of 0.34. N2 and reference norethandrolone in the same t.l.c. had identical R_{FS} of 0.46.

On p.c. in the solvent system PT1, the R_F of N2 and the reference norethandrolone were different (R_F of N2 = 0.78; R_F of the reference norethandrolone = 0.73). In the same system N1 ran with the R_F of 0.74.

On g.l.c. N2 and reference norethandrolone were further separable. The retention times of norethandrolone, N1 and N2 are shown in Table 20.

In fraction N2 a very small quantity of norethandrolone was probably present. This product had identical retention times to those of norethandrolone in the SE-30 and QF-1 columns. The percentage of this norethandrolone like component in the subject P was 0.21% of the administered drug; it was not detectable on t.l.c. or on p.c. by the conventional methods of detection like u.v. absorption and 8% w/v phosphomolybdic acid.

After acetylation N1 and N2 had R_{FS} of 0.56 and 0.66 respectively on t.l.c. in the solvent system chloroform-acetone (9:1 v/v). The R_F of similarly treated norethandrolone remained unchanged (R_F 0.41). These results suggested the presence of an acylable hydroxyl group in N1 and N2.

After/

TABLE 20

RETENTION TIMES OF NORETHANDROLONE AND ITS METABOLITES

Compounds	Retention times in minutes	
	On SE-30 column at 205°C	On QF-1 column at 210°C
Norethandrolone	30.0	22.5
N 1	15.9	10.1
N2	16.9	10.3

After potassium borohydride reduction the R_{F_S} of N1 and N2 remained unchanged on t.l.c. in the solvent system chloroform-acetone (9:1 v/v) which suggested the absence of a reducible ketone group in N1 and N2.

4. Conclusion.

From the similarity of their chromatographic mobilities and chemical properties the metabolites of norethandrolone, N1 and N2 are probably somewhat similar. Since, N1 had a shorter retention time than N2, N1 may be a 5β-isomer of N2. This evidence suggests a similar metabolic fate for norethandrolone to that of 17α-methyltestosterone which is reduced to two major urinary metabolites 17α-methyl-5β-androstan-3α, 17β-diol and 17α-methyl-5α-androstan-3α, 17β-diol (Rongone & Segaloff, 1962).

I. DISCUSSION.

a. General consideration of the metabolism of anabolic steroids.

In general, the foregoing results have shown that the anabolic steroids studied were almost entirely metabolised. Their excretion in urine, however, represented approximately 5 to 31% of the dose and occurred relatively rapidly. The excretion was generally less with C-17 alkylated compounds, especially when compared to the natural C-19, 17-oxosteroids. This may be due to the hindrance of metabolic changes at C-17 such as glucuronyl transfer (Hsia, Riabov & Dowben, 1963). The known metabolism of C_{19} steroids suggests that biliary excretion of anabolic steroids metabolites is likely to be small. Urine is probably the main route of excretion. The conversion of these compounds to oestrogens is also a likely but quantitatively unimportant pathway (Engel et al. 1958; Breuer, 1962). The rapid and complete metabolism of anabolic steroid drugs is surprising since androgens given in smaller doses over a long period act mainly as anabolic agents (van der Vies, personal communication). Esterification at C-17 with higher fatty acids has been shown to increase the anabolic property of 19-nortestosterone by means of slowing its release in the body (Callow, Callow & Emmens, 1939; van der Vies, 1969). Prolonged action is therefore a feature of anabolic agents.

The characterstic features of their metabolism will be discussed under individual headings.

1. 19-nortestosterone.

In order to ascertain whether "carbon skeleton" chromatography can be used in studies of steroid drug metabolism, the metabolism of 19-nortestosterone was restudied in the present work. This study also made it possible to examine the results obtained by using the present method to that obtained by detection of radioactive metabolites as the starting drug 4-^{14}C-19-nortestosterone was readily available for this purpose; availability is a major factor limiting studies of radioactive drug metabolism. The results obtained have been similar to those obtained previously by Engel et al. (1958). The two known metabolites 19-noraetiocholanolone and 19-norandrosterone are probably identical to E1 and A1 of the present study. The quantitative aspects differ; the ratio of 19-noraetiocholanolone to 19-norandrosterone was found to be 1:1 by Engel et al. (1958) in the present study the ratio of E1 to A1 was about 1:5. Sex differences in the response to drugs are known in animals and after administration of androgens, the activities of the microsomal enzymes have been found to increase in female rats (Parke, 1968c). These factors may be responsible for the differences in the two studies because Engel et al. (1958) studied a woman whereas the present work was performed on men.

Besides the major metabolites E1 and A1, at least three more polar metabolites were also detected in the "conjugated" fraction by their carbon skeleton and also by their radioactivity. The chromatographic behaviour of these metabolites suggests further hydroxylation of 19-nortestosterone.

These/

These are, however, minor metabolites.

In the detection of metabolites by using "carbon skeleton" chromatography slightly higher values were sometimes obtained from chromatographic fractions when the carbon skeleton of the compound studied was smaller than that from the related natural products. This is seen in Fig. 13, especially in the quantitation of the fraction corresponding to 19-noraetiocholanolone. This is probably due to the fact that many of the C_{19} steroids by the present method produce a small amount of a split product corresponding to "19-norandrostane" (Chapter I, DI). Such an increased yield of "19-norandrostane" therefore could have been derived from the naturally occurring androgens like aetiocholanolone and androsterone which are likely to be present in the chromatographic fractions due to their poor separation from the drug metabolites. It should also be noted that the natural oestrogens which can be reduced to "19-norandrostane" appear in the phenolic not the neutral fraction.

The sensitivity of the detection of metabolites by "carbon skeleton" chromatography was found to be satisfactory for metabolic studies using milligram doses. The sensitivity of "carbon skeleton" chromatography however depends on the number of substituents in the starting sample (Chapter I, DI). This does not affect the sensitivity of detection using ^{14}C ring labelled compounds. In this way "carbon skeleton" chromatography will prove less sensitive than studies using high specific activity material.

2. Methandrostenolone.

The two metabolites of methandrostenolone, M1 and M2 detected in the present study are similar to 6β-hydroxymethandrostenolone and an epimer of methandrostenolone respectively as described by Rongone & Segaloff (1963). The ratio of the hydroxylated metabolite M1 to M2 was 1:2 in the present study; this result is different to that of Rongone & Segaloff (1963) who found a ratio of 2:1. The presence of more of the 6β-hydroxy metabolite in their sick woman raises the question of regulation of the detoxication mechanism in the body. Hydroxylation is one of the major routes for deactivation of a large number of foreign compounds (Parke, 1968d) and in steroids C-6 oxygenation has been found to be important (Nadel, Burstein & Dorfman, 1956; Florini, Smith & Buyske, 1961). The extent of hydroxylation is dependent on the state of a person receiving the drug (Fingl & Woodbury, 1966b). Many drugs are known to stimulate or inhibit the hepatic microsomal enzyme activities (Conney, Jacobson, Schniedman & Kuntzman, 1965; Tephly & Mannering, 1963); thus drug administration modifies drug metabolism. The study of Rongone & Segaloff (1963) was done on a patient suffering from adenocarcinoma of the lung, as such, she might have been treated simultaneously with other drugs. This could be responsible for the active hydroxylation mechanism in their patient, especially of 6β-hydroxylation (Kuntzman, Jacobson & Conney, 1966; Kuntzman, Jacobson, Levin & Conney, 1963).

It was interesting to find that methandrostenolone was deactivated in the body apparently without conjugation. The stability of this compound was shown/

shown by its unreactive nature in many chemical reactions. Such a drug is likely to be excreted largely "unchanged" or after hydroxylation.

Methandrostenolone is a popular 'muscle building' drug and it has been suggested that athletes use it to increase their performance. The detection of the total metabolites of methandrostenolone without the chromatographic separation was therefore investigated in serial 1 to 4 day urine collections after a single dose of the drug (see Chapter IV, b2). It was interesting to find that though the major amount of the metabolites was excreted within the first 24 hr., a minute amount was still detectable up to 48 hrs. after the drug intake. Since, the yield of hydrocarbons from methandrostenolone is satisfactory and, since the metabolites are extractable in the fairly clean "free" fraction of the urine, detection of this drug should be possible within about 48 hr. of the drug intake.

3. Oxymetholone.

In the present study two metabolites of oxymetholone, O1 and O2, were detected by "carbon skeleton" chromatography. As these metabolites were found in the "conjugated" fraction the position of the glucuronide residue is important. This could be attached by way of the 17β-hydroxyl group although this group is highly hindered (Reifenstein, Forbes, Albright, Donaldson & Carroll, 1945; Leach, Maddock, Tokuyoma & Paulsen, 1956). In addition, the available evidence suggests that C-3 hydroxyl group might be available in O1 for conjugation. Both O1 and O2 may possess a C-2 hydroxyl group. However, it must be emphasised that these assignments are tentative.

Since, O1 was more polar than oxymetholone after potassium borohydride reduction, either further hydroxylation or an alteration of the hydroxymethylene group is also indicated. But, hydroxylation seems unlikely as such a tetrahydroxy compound would probably be very much more polar on t.l.c. Many foreign compounds are detoxicated by a dealkylation mechanism which results in the introduction of a hydroxyl group after oxidative scission of the alkyl group (Brown, 1962; Parke, 1968e). Evidence for the dealkylation of 2-methoxyoestrone with unmasking of a C-2 hydroxy metabolite suggests that oxymetholone, although it possesses a different A ring, may undergo a similar process. 2-Hydroxyoestrone, isolated by Fishman, Cox & Gallagher (1960), has been assumed to be a demethylation product of 2-methoxyoestrone (Brown, 1962). By high temperature catalytic reduction the hydroxymethylene group was completely removed from oxymetholone as the products had the retention times of "17α-methylandrostane". This indicates the instability of this group.

From/

From the limited available data, it is assumed that the metabolites O1 and O2 are without the hydroxymethylene group. O1 has the C-3 ketone group reduced but this ketone group is intact on O2.

4. Norethandrolone.

The metabolism of norethandrolone seems to show similar features to those of 17α-methyltestosterone (Rongone & Segaloff, 1962). The isolation of the ring A reduced 5β and 5α-isomers of 17α-methyltestosterone by these workers and the results of the present investigations suggest that the metabolites N1 and N2 of norethandrolone could be similar products. The presence of a 17-alkyl group in norethandrolone should have hindered the oxidation of the 17β-hydroxyl group to a 17-ketone group (Levedahl & Samuels, 1950; Partridge, Boling, De Wind, Margen & Kinsell, 1953).

The presence of a small amount of a product like norethandrolone in the "conjugated" fraction suggests the possibility of conjugation at C-17 without reduction of ring A. This is quantitatively a very minor metabolic in the present study. However, it is interesting that the hindered 17β-hydroxyl group (Reifenstein et al. 1945) may be conjugated in this compound.

In subject M, a small amount of the product corresponding to norethandrolone was found in the "free" fraction. This may be due to the dose of norethandrolone relative to the body weight being much larger in this subject compared to the other subjects P, R and T.

J. SUMMARY

The metabolic fates of four anabolic steroids, 19-nortestosterone, methandrostenolone, oxymetholone and norethandrolone administered in therapeutic doses were studied in man. The detection of the drugs and their metabolites in urine extracts was by the method of high temperature catalytic reduction following chromatographic separations. The results showed an extensive metabolism of these drugs. Little or no unaltered drug was detected.

1. 19-nortestosterone:- The metabolism of 19-nortestosterone showed two major metabolites behaving like 19-noraetiocholanolone and 19-norandrosterone in the ratio of 1:5. Three more polar metabolites were also detected. The total amount of the metabolites in the "conjugated" fraction was 31% of the administered drug.

2. The results of high temperature catalytic reduction used as a detection method were checked by simultaneous detection of radioactive metabolites by liquid scintillation counting. Detection by both methods gave similar patterns of metabolites.

3. Methandrostenolone:- Two major metabolites behaving like 6β-hydroxymethandrostenolone (M1) and like an epimer of methandrostenolone (M2) were freely extractable from the urine of three adult males. These metabolites were just detectable using standard chemical methods and reference compounds. The ratio of M1 to M2 was about 1:2 and amounted to about 5% of the total administered drug in the 24 hr. urine collection. The total metabolites were detectable/

detectable up to 48 hr., after administration of methandrostenolone, from the urine extracts after a simple partition.

4. Oxymetholone:- The metabolism of oxymetholone in 3 adult males showed two urinary metabolites named O1 and O2 in the conjugated fraction. It is suggested that dealkylation of this compound occurs to produce 2-hydroxyl group in both O1 and O2. The metabolite O1 probably has a reduced C-3 oxo group.

5. The total metabolites accounted for about 5.3% of the administered drug. The ratio of the metabolites was about 1:1.

6. Norethandrolone:- The metabolism of norethandrolone was studied in three adult males and a 7 year-old boy. Two major urinary metabolites named N1 and N2 were detected in the "conjugated" fraction. These compounds were probably ring A reduction products. The ratio of N1 to N2 was about 1:2.8. The total metabolites accounted for about 10.4% of the administered drug in a 24 hr. urine specimen. On gas-liquid chromatography of the unmodified fraction N2, a product corresponding to norethandrolone was present and accounted for about 0.2% of the administered drug.

7. In the freely extractable fraction from the urine of the boy, a product corresponding to norethandrolone was also present. This amounted to about 0.02% of the administered drug.

TABLE 21

A GENERAL SUMMARY OF THE METABOLISM OF ANABOLIC STEROIDS IN MAN

Anabolic Steroids	Dose	Number of subjects studied	Number of metabolites detected in urine.		Mean ratio of the major metabolites	Mean % of the total dose in urine	Nature of the metabolites.
			In "free" fraction	In "conjugated" fraction			
19-Nortestosterone	20mg, 1μc(4-^{14}C)	1	none	2 major-E1&A1; 3 minor	E1:A1 = 1:5	31*	E1 & A1 like 5β & 5α reduction products
Methandrostneolone	5mg.	3	2 - M1 & M2	none	M1:M2 = 1:2	5	M1 like hydroxylation product; M2 like an epimer of the drug.
Oxymetholone	10mg.	3	none	2 - O1 & O2	O1:O2 = 1:1	5.3	O1 & O2 both more polar than oxymetholone.
Norethandrolone	20mg. & 10mg.	4	very small quantities of unchanged drug in one case only	2 - major- N1 & N2 1 - minor	N1:N2 =1:2.8	10.4	N1 & N2 like 5β & 5α reduction products.

* in 48 hr. urine specimen.

GENERAL DISCUSSION

GENERAL DISCUSSION

1. "Carbon skeleton" chromatography - its applications and limitations.

By utilising the method of high temperature catalytic reduction, a large number of steroids and sterols in µg. amounts were converted to the expected parent hydrocarbons in quantities generally satisfactory for analyses using g.l.c. The method should, therefore, be applicable to many other steroids and sterols isolated in µg. quantities from biological sources.

Metabolic studies have been performed on anabolic steroid drugs. It has been shown that "carbon skeleton" chromatography can be used to detect metabolites. The metabolism has been described of two drugs which have not been previously studied. Among the many such drugs reduced and separated by g.l.c. (Chapter II, B, Table 10) it has only been possible to study the metabolism of a few. The possibility for similar studies exists for many of these compounds.

"Carbon skeleton" chromatography may be helpful in forensic and toxicological problems in which a drug is largely or completely metabolised. Although unchanged drugs can satisfactorily be detected by chemical or other chromatographic methods, the present method in combination with g.l.c. can be used in detection of metabolites as well as unchanged drugs.

In structural identification, the method can be a useful companion to mass spectrometry as no other method applicable to µg. amounts of steroids and sterols is yet known to give much information on the identity of the parent hydrocarbon. In the biological field, a direct comparison of the physical/

physical and chemical properties of unknown natural products with the compounds of known structure is difficult, as this involves a large number of possible reference compounds. The present method for the tentative identification of the hydrocarbon skeleton supplemented by existing micro-chemical identification of the functional groups could be useful in suggesting a limited number of possible structures for an unknown steroid or sterol, and ease the selection and supply of relevant compounds for comparison. These possibilities can then be investigated by other methods.

The sensitivity of the present method, however, depends on the nature of the starting compounds because the steroids with more than two oxygenated functional groups were recovered as hydrocarbons in smaller amounts than those containing only one or two groups (Chapter I, D III 1, Table 4; Chapter II, C 1, Table 11). These problems of chemical reactivity of the molecules due to the presence of polar groups (Beroza & Sarmiento, 1963), hindered groups like the C-11 oxygenated group (Harkness & Fotherby, 1961), and steric interference by certain substituents like the C-1 β -methyl group (Djerassi et al. 1956), limit the sensitivity of the method. These limitations are important if the compounds are present in small amounts, i.e. in less than 5 µg.

The amount of contaminants is important. The sulphate conjugated metabolites are generally present in small quantities with large amounts of contaminating material. The glucuronide conjugated metabolites are present in larger quantities in a 'cleaner' fraction (Layne et al. 1963; Cooper & Kellie, 1968; Kamyab, Fotherby & Klopper, 1968; Harkness, Davidson & Strong, 1969);/

1969); these studies using radioactively labelled compounds have shown the difficulty of isolation and characterisation of metabolites in the sulphate fraction, mainly due to the minute amounts present. In the present study, the sulphate fractions of methandrostenolone were reduced. Due to the bulky extracts obtained after solvolysis (Burstein & Lieberman, 1958), and possibly due to the minute amounts of such metabolites present, metabolites could not be detected using the present method. Similar limitations have been encountered by the workers listed above using other methods including radioactivity.

2. Steranes and other hydrocarbons as markers of biological evolution.

In the search for evidence of the origin of life, interest has recently been directed towards the organic compounds in ancient shales, oils and carbonaceous chondrites ranging from several million to over 2.7 billion years old. The existence of the hydrocarbons phytane and pristane in the isoprenoid series (Johns, Belsky, McCarthy, Burlingame, Haug, Schnoes, Richter & Calvin, 1966) and steranes (Burlingame, Haug, Belsky & Calvin, 1965) in those ancient samples have been proved by g.l.c. and mass spectrometry. The presence of these complex structures has been interpreted as suggesting the existence of "biological" systems. Purely "physical" systems seem less likely to produce such structures. However, lower isoprenoid hydrocarbons from C_9 to C_{14} have been prepared by abiogenic methods (McCarthy & Calvin, 1967); emphasis has therefore shifted to the steranes. McCarthy & Calvin (1967) emphasise that the development of abiogenic methods and the understanding of products obtained from them could in future define "biological markers"; that is, the compounds produced solely by biological systems but not obtained by abiogenic processes.

After the brief communication on the development of the present method to the Biochemical Society (Adhikary & Harkness, 1967), an enquiry was made by Sir Robert Robinson regarding any similarity in the patterns on g.l.c. of reduction products from cholesterol and the steranes identified in Colorado Green River shale by Burlingame et al. (1965). It was interesting to note that the reduction products of cholesterol by the present method show a characteristic pattern of hydrocarbons similar to that present in the Colorado Green River shale (see Burlingame et al. 1965, Fig. 1, and Chapter I, D III 9,

9, Fig. 6B). The present work has thus shown that the cholestane present in an ancient shale (52 x 10^6 years old) could have come from cholesterol - (Sir Robert Robinson, personal communication). It should also be noted that a C_{30} compound which may have been a sterane was detected by Burlingame et al. (1965) in larger quantities than the C_{27} sterane. Since in the present study lanosterol was reduced probably to a C_{30} hydrocarbon (Chapter II, C6), it seems justifiable, as pure speculation, to suggest that the biosynthetic demethylation of lanosterol was beginning to appear at about the time that this shale was laid down.

LIST OF PUBLICATIONS FROM THE CONTENTS OF THIS THESIS.

High-temperature catalytic reduction of steroids - P.M. Adhikary & R.A. Harkness (1967). Biochem J. 105, 40P.

The identification and measurement of a new steroid 16β-hydroxydehydroepiandrosterone in infant urine - C.H.L. Shackleton, R.W. Kelly, P.M. Adhikary, C.J.W. Brooks, R.A. Harkness, P.J. Sykes & F.L. Mitchell. (1968). Steroids, 12, 705.

Determination of the carbon skeleton of microgram amounts of steroids and sterols by gas chromatography after their high temperature catalytic reduction - P.M. Adhikary & R.A. Harkness (1969). Analyt. Chem. 41, 470.

Production of the parent hydrocarbons from steroid drugs and their separation by gas chromatography - P.M. Adhikary & R.A. Harkness. (1969). J. Chromatog. 42, 29.

The detection of steroid drug metabolites by gas-liquid chromatography of their carbon skeletons - P.M. Adhikary & R.A. Harkness. (1969). Biochem. J. 112, 30P.

THE IDENTIFICATION AND MEASUREMENT OF A NEW STEROID 16β-HYDROXYDEHYDROEPIANDROSTERONE IN INFANT URINE

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ABSTRACT

Extracts from 1-3 day-old infant urine which had been hydrolysed by Helix pomatia enzyme followed by solvolysis, were subjected to thin-layer chromatography. A band of material giving the 3β-hydroxy-Δ^5 steroid colour reaction with antimony trichloride staining reagent and previously named "U_2", was purified by further thin-layer chromatography and identified as 16β-hydroxydehydroepiandrosterone. Proof of identity was obtained by comparing the chemical and physical characteristics of the chemically synthesized compound, with those of the unknown. Techniques used were: thin-layer and paper chromatography in 5 systems, gas chromatography, mass spectroscopy, infrared spectroscopy and high temperature catalytic reduction. It is excreted in infant urine mainly as a di-conjugate.

INTRODUCTION

Infant urine has been shown to contain a large number of steroids and steroid-like compounds which are undetectable in later life, many of these being present in considerable quantity (1). [for review see Mitchell (2)]. Due to a relative lack of the enzyme 3β-hydroxysteroid dehydrogenase in the adrenal in utero and in early infancy, many of these compounds have the Δ^5 configuration. The separation and

measurement of 8 such compounds by thin-layer chromatography followed by antimony trichloride staining has been reported previously (3) and two compounds, then unidentified, were labelled U_1 and U_2. The more polar of these (U_2) has now been identified as 3β,16β-dihydroxyandrost-5-en-17-one (16β-hydroxy-DHA). The 16α epimer has already been reported as being a major component of fetal blood (4,5) and infant urine (3,6,7). The newly identified compound is excreted in infant urine at an average rate of approximately 600 μg/24 hr. during the first 6 days of life (3).

ISOLATION AND PURIFICATION

Hydrolysis (by Helix pomatia enzyme and solvolysis), followed by extraction and thin-layer chromatography, was carried out as previously described (3). For preparation purposes extracts were streaked along the origin of the chromatogram plate and the positions of the bands after chromatography determined by carrying out the antimony trichloride reaction only on the two outer edges of the plates. The band of material in the centre, thus delineated, was eluted from the silica-gel with ether; the silica-gel having first been deactivated with a drop of water.

After separation in the thin-layer systems used for the normal assay technique (3), the band of material previously referred to as U_2 was further purified by re-chromatography on silica-gel once in the system; chloroform:absolute ethanol (95:5 v/v) and twice in cyclohexane:ethyl acetate (50:50 v/v).

PREPARATION OF AUTHENTIC 16β-HYDROXY-DHA

The preparation of 3β,16β-diacetoxyandrost-5-en-17-one from 3β-acetoxy-5α,6β-dichloroandrostan-17-one has been described by Aoki et al. (8) but the preparation of the free ketol has not so far been reported. In the method of Aoki et al. (8) the 17-enol acetate is prepared and treated with lead tetraacetate to give 3β,16β-diacetoxy-5α,6β-dichloroandrostan-17-one which is then dechlorinated with zinc in acetic acid.

Hydrolysis of the 16β-acetate is difficult since only mild conditions must be used if isomerisation of the sensitive ketol is to be minimised. The best yields were obtained by the use of aqueous acidic methanol at 30° for 60 hr. The product was purified by fractional crystallization from two solvent systems. Identification was by infrared and nuclear magnetic resonance spectroscopy. The n.m.r. spectrum (in pyridine) showed that the compound was free from the 17β-hydroxy-16-oxo epimer (C-18 methyl of 3β,17β-dihydroxyandrost-5-en-16-one resonates at τ 9.08 in pyridine).

Experimental

3β,16β-Diacetoxyandrost-5-en-17-one (330 mg) was dissolved in methanol (180 ml), water (20 ml) and concentrated HCl (5 ml) and left at 30° for 60 hr. A further 30 ml. of water was added and the solution evaporated to 100 ml in vacuo at 30°. The crystalline product which formed during this evaporation was filtered off, washed well with water and dried. The material was then dissolved in excess boiling methanol and the solution allowed to cool, the precipitate was discarded and the solvent evaporated to dryness. This treatment was repeated twice using aqueous methanol as the solvent; the total weight of discarded material from the three operations was 120 mg. The final residue was recrystallized twice from acetone-hexane to give 53 mg of pure material; m.p. 187° - 192° $[\alpha]_D$ - 19° (c 0.1, dioxan); infrared (see Fig. 1); n.m.r. (pyridine) τ 8.96 (C-19 methyl), τ 9.00 (C-18 methyl).

Instruments used were as follows: melting points - Kofler block, optical rotation - Perkin Elmer polarimeter 141, infrared spectrum - Unicam S.P. 200 spectrophotometer with thallous-bromide-iodide internal reflection plate, n.m.r. spectrum - Perkin Elmer R10 (60 mc/s).

IDENTIFICATION

Considerable difficulty was experienced during the purification of compound U_2 since, as found by Layne and Marrian (9) during the isolation of 16β-hydroxyestrone, many of the procedures used resulted in its partial isomerization to the more stable α-ketol, in this case 3β,17β-dihydroxyandrost-5-en-16-one (16-oxoandrostenediol). This spontaneous conversion was proved by separating 16-oxoandrostenediol from compound U_2 and recording its infrared spectrum. The isomerization proceeds especially rapidly in alkaline conditions and these were avoided as much as possible. It was not however possible to avoid bringing the urine to pH 11.5 before hydrolysis and extraction since this is an important step in the

essential removal of phosphate and sulfate ions (10) which would otherwise inhibit the enzyme hydrolysis. In an experiment in which water containing pure 16β-hydroxy-DHA was treated identically to urine, only 16-oxoandrostenediol could be isolated from the final extracts. It is assumed that when natural 16β-hydroxy-DHA is present as the diconjugate it is immune to isomerization before hydrolysis.

Gas chromatography could not be used for purification because the compound was destroyed on the column when chromatographed in the free form.

Polarity on thin-layer chromatography

The compound has identical Rf values to 16β-hydroxy-DHA on silica-gel in the following thin-layer systems:-

chloroform:absolute ethanol (95:5 v/v)	Rf	=	0.30
benzene:absolute ethanol (95:5 v/v)	Rf	=	0.15
cyclohexane:ethyl acetate (50:50 v/v)	Rf	=	0.20

and on paper in the systems:-

toluene:methanol:water (100:75:25 v/v)	Rf	=	0.450
light petroleum (b.p. 100-120°):benzene:methanol:water (66:33:80:20 v/v)	Rf	=	0.068

In all the systems mentioned it is slightly more polar than 16α-hydroxy-DHA and 3β,17β-dihydroxy-5-en-16-one. On thin-layer alumina it isomerizes to 16-oxoandrostenediol.

Chemical reactivity

Blue tetrazolium was reduced, indicating the presence of an α-ketolic group. On reduction with potassium borohydride a compound was produced with the polarity on thin-layer chromatography of 3β,16α,17α (or 16β,17β)-trihydroxyandrost-5-ene (the two epimers were inseparable in the systems used). Since the original compound was an α-ketol and both 16 and 17-oxo groups are reduced to 16β- and 17β-hydroxy groups by borohydride it may be presumed that the 16β,17β-epimer was formed.

A blue colour typical for a 17-oxosteroid was produced with the Zimmermann reagent and a red colour typical for a 3β-hydroxy Δ^5 steroid was produced with the antimony trichloride reaction on a thin-layer chromatogram (3).

Infrared spectroscopy

Samples of U_2 and authentic 16β-hydroxy-DHA were dissolved in acetone and smeared on both sides of a thallous bromide-iodide internal reflection plate. The infrared spectrum was then recorded in a Unicam SP 200 spectrophotometer. Owing to the difficulty, mentioned previously, of isomerism taking place during purification it has proved impossible to record a perfect infrared spectrum of U_2. The spectrum shown in Fig. 1 was produced from a purified sample specially prepared by enzyme hydrolysis of the conjugate obtained from infant urine by Sephadex chromatography.

Gas chromatography

The free compound was unstable on the column but the trimethylsilyl ether (TMSE) derivative of U_2 gave the following retention time on a 1% SE-30 column. Derivatives of related compounds are shown for comparison:-

DHA (3β-hydroxyandrost-5-en-17-one) TMSE	- 4.20 min.
16α-hydroxy-DHA bis-TMSE	- 8.66 min.
16-oxo-androstenediol bis-TMSE	- 9.33 min.
16β-hydroxy-DHA bis-TMSE	- 9.33 min.
U_2 TMSE	- 9.33 min.

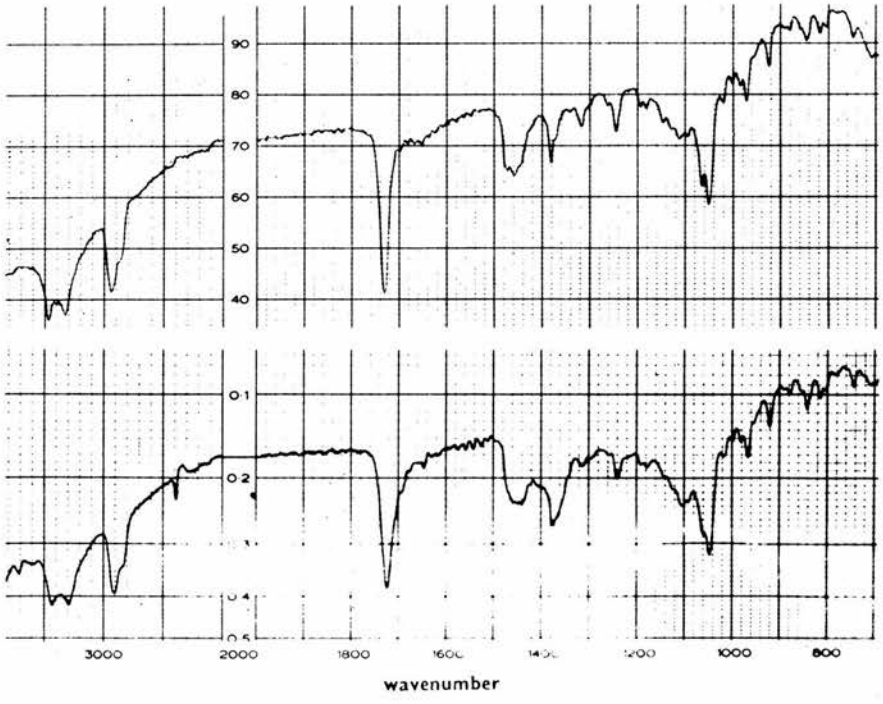


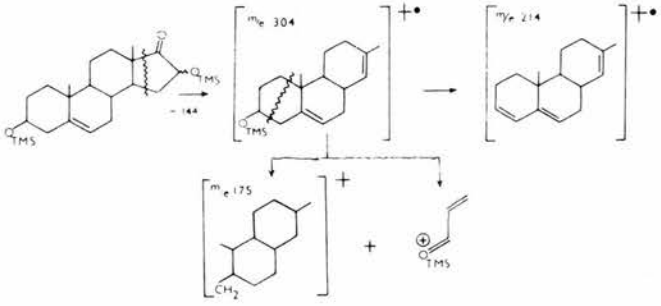
Figure 1. Infrared spectra of (top) authentic 16β-hydroxy-DHA and (lower) compound U_2. The spectra were obtained by the use of a thallous bromide-iodide internal reflection plate.

These data show clearly that U_2 is not 16α-hydroxy-DHA: they do not distinguish it from 16-oxo-androstenediol, but the mass spectrometric data (see below) rule out this structure.

Gas chromatography-mass spectrometry

The TMSE derivatives of 16-oxo-androstenediol, 16α-hydroxy-DHA, 16β-hydroxy-DHA and U_2 were subjected to gas chromatography-mass spectrometry using an LKB 9000 instrument (LKB Produkter AB, Stockholm, Sweden) operated at electron energy 70 E.V. The column was packed with 1% SE-30 on silanized Gas Chrom P and operated at 232°. As with infrared spectrometry, it proved impossible to record a spectrum of compound U_2 completely free from 16-oxo-androstenediol. It will be seen from the results shown in Fig. 2 that the peak at m/e 129 in the spectrum produced by compound U_2 is slightly larger than in the authentic spectrum - an effect readily ascribed to the small proportion of 16-oxo-androstenediol derivative present as contaminant.

The trimethylsilyl ethers of 16α- and 16β-hydroxy-DHA give closely similar mass spectra, which are however quite different from that of the 16-oxo-androstenediol derivative. The latter spectrum is dominated by the peak at m/e 129 characteristic of 3-trimethylsilyloxy-Δ^5-steroids (11, 12): there are no abundant ions above m/e 200 except at m/e 448 (M) and m/e 433 (M-15). In contrast, the 16-trimethylsilyloxy-17-ketones give (in addition to the peak at m/e 129) strong, characteristic peaks at m/e 214 and 304. The exact origin of these has not been established: a formal possibility, which accounts also for the ion at m/e 175, is indicated below.



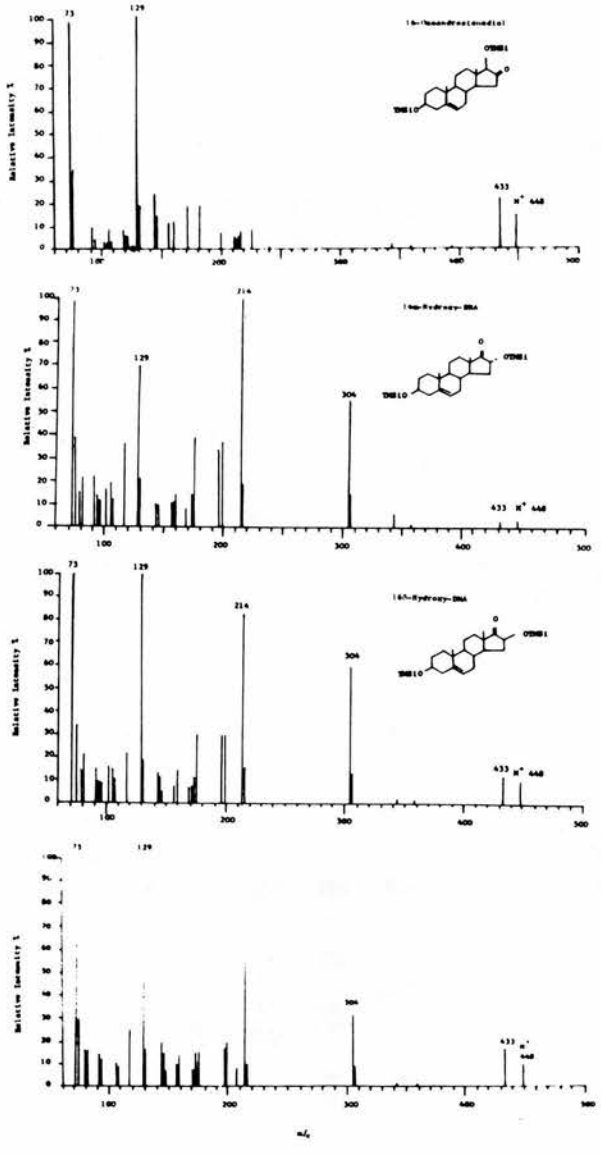


Figure 2. Mass spectra diagrams for trimethyl silyl ethers of authentic 16-oxoandrostenediol, 16α-hydroxy-DHA, 16β-hydroxy-DHA and compound U_2. The peaks at m/e 73 were off scale.

High temperature catalytic reduction

The compound was subjected to high temperature catalytic reduction by passing it with hydrogen, in a manner similar to that used for gas chromatography, through a siliconized glass tube containing 1-3% (w/w) platinum on siliconized glass beads at a temperature of 200°. The trapped products were then examined by gas chromatography on both 1% NGA and 1% SE-30 columns (13). Reduction products were produced similar to those from 16α- and 16β-hydroxy-DHA and 16-oxo-androstenediol; the main products having retention times, relative to 5α-androstane, of 1.0 and 0.34 on both columns. This evidence is thus consistent with the original compound being 16β-hydroxy-DHA. The specific pattern of reduction products was unique for 16 oxygenated steroids amongst many similar steroids studied.

CONJUGATION

It has been shown by Sjovall and Vihko (14) that free and mono- and di-conjugated steroids may be clearly and reliably separated on Sephadex LH 20 (Pharmacia, Uppsala, Sweden). Elution volumes using this technique were checked with a range of authentic free and conjugated steroids and the method was subsequently used to fractionate the free steroids and conjugates in infant urine. Separation of the steroid components of each fraction was achieved by enzyme hydrolysis followed by thin-layer chromatography.

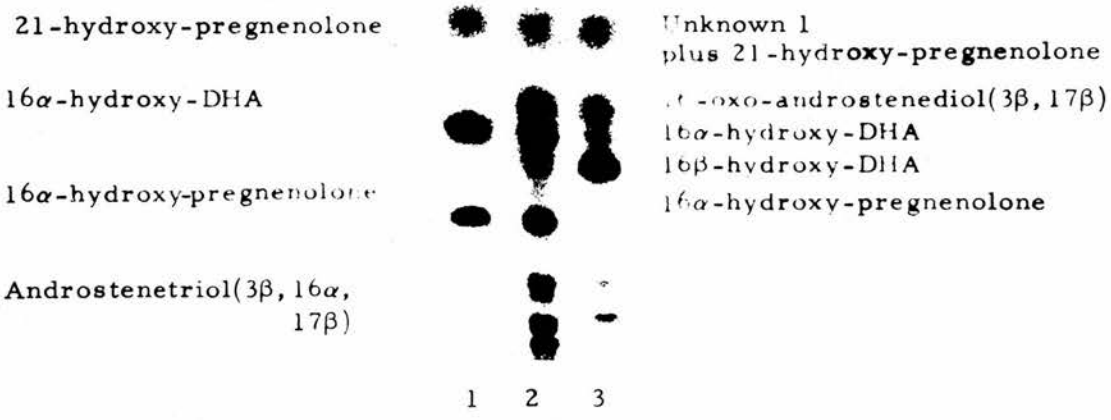


Figure 3. Thin-layer silica-gel chromatography of 1, the standard compounds indicated; 2, the mono-conjugated steroid fraction from infant urine; and 3, the di-conjugated fraction. Colour development was by $SbCl_3$ reagent.

To separate the free, mono- and di-conjugated steroids, 10 ml from a pool of urine from 1-3 day-old infants was extracted twice with 2 volumes of ether, and the extract of free steroids was evaporated to dryness. The aqueous phase was added to 4 volumes of ethanol:acetone (50:50 v/v) to precipitate protein, the precipitate was washed with one volume of ethanol:acetone and the combined extracts were evaporated to dryness on a rotary evaporator at 45°. The dried extract was dissolved in 10 ml of methanol:chloroform (50:50 v/v) saturated with NaCl, and the whole applied to a Sephadex column [prepared by standing 17 g of Sephadex LH 20 (Pharmacia Uppsala, Sweden) in methanol:chloroform (50:50 v/v) saturated with NaCl for 1 hour, pouring into a 2 cm diameter column and passing further solvent through for 6 hr before use]. Elution was carried out by adding more NaCl saturated methanol: chloroform. Mono-conjugates were found to be contained in the 50-150 ml fraction of eluate and di-conjugates in the fraction 250-400 ml. After evaporation to dryness, hydrolysis of the conjugates by Helix pomatia enzyme was carried out as previously described (3). In neither fraction could further free 16β-hydroxy-DHA be obtained by solvolysis.

Extracts containing the free fraction and the hydrolysed conjugates were chromatographed by running 3 times in the system, ethanol: benzene (5:95 v/v). No 16β-hydroxy-DHA could be detected in the free fraction and it will be seen from Fig. 3 that the major proportion is di-conjugated. In the urine specimens studied 34% was mono and 66% di-conjugated. Fig. 3 also shows that of the 3β-hydroxy-Δ^5 steroids staining with $SbCl_3$ and hydrolysed by Helix pomatia enzyme, 16β-hydroxy-DHA is the major compound present as the di-conjugate. Another major unknown compound (unknown 1) is also indicated.

DISCUSSION

The proven presence of 16β-hydroxy-DHA in infant urine plus the known fact that under certain conditions it spontaneously isomerizes to the more stable 16-oxoandrostenediol, now raises the question as to what extent 16-oxo-androstenediol, which has been reported as one of the major components of infant urine (3, 15) and tentatively identified in umbilical cord blood (16), has been produced during extraction and purification as an artefact.

An explanation for the presence of 16β-hydroxy-DHA in quantity in infant urine and its use by the fetus is not immediately apparent. 16α-hydroxy-DHA in quantity is readily aromatized by placentas when perfused in situ, to form estriol (3β, 16α, 17β-trihydroxyestra-1, 3, 5 (10)-triene (17) and 16β-hydroxy-DHA might similarly form 16-epiestriol, 16β-hydroxyestrone (3, 16β-dihydroxyestra-1, 3, 5(10-trien-17-one) or its isomer 16-oxoestradiol (3, 17β-dihydroxyestra-1, 3, 5 (10)-trien-16-one), though the urinary output of these compounds in pregnancy is not great, being respectively 0.8, 0.7 and 1.1 mg/24 hr (18).

The presence of both 16α- and 16β-hydroxylase for DHA has been demonstrated in fetal liver (19), though it is not known in what ratio the two epimers are produced. The formation of 16β- from 16α-hydroxy-DHA must not be discounted since a high activity of a 16α to a 16β epimerizing system has been demonstrated for estriol in avian liver (20), and for 16α-hydroxyestrone in human placenta and other tissues(21).

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DECLARATION

I hereby declare that this thesis has been composed by me and the work presented has been done by myself. The methods followed in the investigation were generally based on those of the previous workers which have been specifically acknowledged in the text by means of references.

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