

STEROID METABOLISM IN UTERO AND IN THE
NEO-NATAL PERIOD

by

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SUMMARY

1. Two steroids were identified for the first time as major steroid components of urine collected from infants and plasma obtained from the umbilical blood vessels. These compounds have the formula $3\beta,16\beta$ -dihydroxyandrost-5-en-17-one and $3\beta,17\alpha$ -dihydroxyandrost-5-ene, but their importance in the metabolism of steroids by the foeto-placental unit is not known.
2. A method was developed for measuring steroids in plasma obtained from the umbilical blood vessels and urine collected from infants. This depends upon the separation of steroids by thin-layer chromatography, and their assay on the thin-layer plates by developing colours with various spray reagents and subsequent densitometric scanning. The accuracy and specificity of the method is discussed.
3. This method was used to establish normal ranges for the excretion of several 3β -hydroxy- Δ^5 steroids found in urine specimens obtained from infants, and to determine the effects upon the excretion of steroids of administering corticotrophin and human chorionic gonadotrophin to newborn infants.
4. The enzyme defects present in three infants with abnormal adrenal glands were investigated by analysis of steroids in urine specimens obtained from these patients.
5. Several 3β -hydroxy- Δ^5 steroids were measured in plasma samples prepared from venous and arterial blood obtained from the umbilical cord. The concentrations present in arterial plasma were higher than in the umbilical vein, indicating a net uptake of these steroids by the placenta, where it is thought that they are converted into 3-oxo- Δ^4 steroids and oestrogens. The relationship between the levels of 3β -hydroxy- Δ^5 steroids in plasma obtained from the umbilical blood vessels and oestrogen excretion by the mother was also investigated.

MATERIALS

Equipment

Thin-layer chromatogram plate spreader	Quickfit and Quartz Ltd., Stone, Staffordshire, England
Thin-layer chromatography tanks	Shandon Scientific Co. Ltd., London, England.
Thin-layer chromatogram scanner "Chromoscan"	Joyce Loebel & Co. Ltd., Gateshead, Durham, England.
Gas chromatograph "Perkin-Elmer 801"	Perkin-Elmer Corp., Norwalk, Conn., U.S.A.
Gas chromatograph-Mass Spectrometer "LKB 9000"	LKB Produkter AB, Stockholm, Sweden.
Liquid-scintillation counter "Packard Tri-Carb"	Packard Corp., Downers Grove, Illinois, U.S.A.
Infra-Red Spectrophotometer "Unicam SP 200"	Unicam Ltd., Cambridge, England.
Infra-Red Internal reflection device	Wilks Scientific Corp., South Norwalk, Conn., U.S.A.
Mechanical agitator "Whirlimixer"	Fisons Scientific Apparatus, Loughborough, Leicestershire, England.

Chemicals

Most of the chemicals (including solvents) were of "analytical reagent" (A.R.) grade and were obtained from British Drug Houses, Poole, Dorset, England. The main exceptions are as follows:-

Silica gel HF 254 Aluminium oxide	Merck AG, Darmstadt, Germany.
Sephadex LH-20	AB Pharmacia, Uppsala, Sweden
Hexamethyldisilazane Trimethyltrichlorosilane	Applied Science Laboratories Inc., P.O. Box 440, State College, PA, U.S.A.
Methoxamine hydrochloride	Kodak Ltd., Kirby, Lancs., England.
Succus Entericus from the snail, Helix pomatia	L'Industrie Biologique Française S.A., Gennevilliers, Seine, France.

Steroids

Reference steroids were obtained from three principal sources:-

- (i) Koch-Light Laboratories,
Colnbrook, Bucks., England.
- (ii) M.R.C. Steroid Reference
Collection.
Professor W. Klyne, Chemistry
Department, Westfield College,
Hampstead, London, N.W.3.
- (iii) Dr. R. W. Kelly and Dr. P. J.
Sykes, Chemistry Department,
Kings Buildings, Edinburgh
University, Edinburgh, Scotland.

STEROID ABBREVIATIONS

<u>Trivial name</u>	<u>Systematic name</u>
Androstenedione	Androst-4-ene-3,17-dione
Testosterone	17 β -Hydroxyandrost-4-en-3-one
Cis-testosterone	17 α -Hydroxyandrost-4-en-3-one
Aetiocholanolone	3 α -Hydroxy-5 β -androstan-17-one
Androsterone	3 α -Hydroxy-5 α -androstan-17-one
DHA (Dehydroepiandrosterone)	3 β -Hydroxyandrost-5-en-17-one
17 α Androstenediol	3 β ,17 α -Dihydroxyandrost-5-ene
17 β Androstenediol	3 β ,17 β -Dihydroxyandrost-5-ene
Androstenetriol	3 β ,16 α ,17 β -Trihydroxyandrost-5-ene
Progesterone	Pregn-4-ene-3,20-dione
Pregnanolone	3 α -Hydroxy-5 β -pregnan-20-one
Pregnanediol	3 α ,20 α -Dihydroxy-5 β -pregnane
Pregnanetriol	3 α ,17 α ,20 α -Trihydroxy-5 β -pregnane
Pregnenolone	3 β -Hydroxypregn-5-en-20-one
Pregnenediol	3 β ,20 α -Dihydroxypregn-5-ene
Pregnenetriol	3 β ,17 α ,20 α -Trihydroxypregn-5-ene
Deoxycorticosterone	21-Hydroxypregn-4-ene-3,20-dione
Dehydrocorticosterone	21-Hydroxypregn-4-ene-3,11,20-trione
Corticosterone	11 β ,21-Dihydroxypregn-4-ene-3,20-dione
Aldosterone	11 β ,21-Dihydroxypregn-4-ene-3,20-dion-18-al
Cortisone	17 α ,21-Dihydroxypregn-4-ene-3,11,20-trione
Cortisol	11 β ,17 α ,21-Trihydroxypregn-4-ene-3,20-dione
Oestrone	3-Hydroxyoestra-1,3,5(10)-trien-17-one
Oestradiol	3,17 β -Dihydroxyoestra-1,3,5(10)-triene
Oestriol	3,16 α ,17 β -Trihydroxyoestra-1,3,5(10)-triene

Notes:

1. Saturated metabolites of corticosteroids are indicated by the prefixes "tetrahydro" (3 α -hydroxy-5 β) and "allotetrahydro" (3 α -hydroxy-5 α).

e.g. Tetrahydrocortisol - 3 α ,11 β ,17 α ,21-Tetrahydroxy-5 β -pregnan-20-one

2. Additional hydroxyl and ketone groups are indicated by the prefixes "-OH-" and "-Oxo-" respectively.

e.g. 16 α -OH-DHA - 3 β ,16 α -Dihydroxyandrost-5-en-17-one
 16-Oxo-Androstenediol - 3 β ,17 β -Dihydroxyandrost-5-en-16-one.

ACKNOWLEDGEMENTS

I should like to thank Dr. F. L. Mitchell for his advice and help and for having guided me as my supervisor throughout the period of this research. I would also like to thank Professor L. G. Whitby for his helpful advice and for providing me with equipment and laboratory space.

I am indebted to Dr. J. R. B. Livingstone who collected the blood samples from umbilical cords, and to Mrs. E. A. Michie who arranged for the collection of urine specimens from infants.

Dr. C. J. W. Brooks of the Chemistry Department, Glasgow University, kindly made available to me the combined gas chromatograph-mass spectrometer and I wish to thank him for this and for his helpful advice on the interpretation of my results.

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PREFACE

PREFACE

One of the earliest signs that there was an alteration in steroid metabolism during pregnancy was the discovery of a large amount of pregnanediol (a metabolite of progesterone) in urine collected from pregnant women. It has been known for several years that this pregnanediol originates from progesterone secreted by the placenta (Diczfalusy and Troen, 1961). The oestrogens are also present in considerable quantity in maternal urine (Doisy et al., 1929; Marrian, 1930; Huffman et al., 1946) and it has been assumed that these compounds are also secreted by the placenta. It is known, however, that the placenta is not able to form from acetate all the steroids it secretes, and it must have available ready-made precursors of, for example, the oestrogens and progesterone. These precursors are produced either by the mother or foetus.

Recently it has been shown that the foetus itself is capable of contributing markedly to the metabolism of steroids in pregnancy, although it had been known for 200 years that the foetal adrenal glands, the most likely site of steroid synthesis, were proportionately much larger than adult adrenals. Winslow, in 1766, describing the foetal adrenal glands noted the problem: "Elles sont chez le foetus extrêmement grosses et diminuent en volume avec l'âge. Ce sont deux problèmes qui méritent attention." The relative weight of the glands reaches a maximum at approximately the 16th week of pregnancy, after which it decreases (Ekholm and Niemineva, 1950). Even at birth, however, the adrenals are still proportionately 20 times as large as adult glands (Eberlein, 1965b). As well as being relatively large, the foetal adrenals are anatomically different from the adult organ as they have a large internal zone (foetal

zone) which regresses after birth (Lanman, 1953, 1957). The significance of this foetal zone is still puzzling endocrinologists, as little difference between the foetal and cortical zones in steroid biosynthetic and metabolic capabilities has yet been found by in vitro isotopic studies (Solomon et al., 1958; Bloch & Benirschke, 1959; Hillman, Stachenko & Giroud, 1962). It seems probable, however, that the foetal zone produces steroids which act as precursors for the synthesis by the placenta of oestrogen and progesterone.

During the later part of pregnancy a cortical zone develops in the foetal adrenal, and it is likely that this zone is responsible for producing the corticosteroids which are essential to the foetus. The evidence available at present suggests that the corticosteroids utilised by the foetus are formed entirely by the foeto-placental unit*, and hormones produced by the maternal adrenal glands play little or no part in meeting the needs of the foetus (Mills, 1967).

The steroid metabolism of the mother, the foetus and the placenta considered individually and collectively will only be fully elucidated by piecing together information obtained from a wide variety of experiments. The first section of the introduction describes many of the techniques which have been used in these experiments ; the subsequent sections describe briefly the evidence which has been obtained so far and which has led to the current understanding of steroid biosynthesis and metabolism by the foeto-placental unit. Mitchell (1967) has conducted a more comprehensive review of the literature up to 1966.

* Explanation of the term "foeto-placental unit":- At the twentieth week of pregnancy, neither the foetus nor the placenta can perform the complete synthesis of a number of steroids. This is because of the lack, or non-

functioning, of certain essential enzymes. However, the enzymatic activities which are lacking in the placenta are present in the foetus, and those not present in the foetus are active in the placenta. Since the foetus and placenta together are capable of elaborating all types of steroid hormones, Diczfalusy (1966) coined the term "foeto-placental unit" when describing the synthesis of steroids by processes which involved both the foetus and the placenta.

INTRODUCTION

INTRODUCTION

1. Methods used in the study of steroid metabolism during pregnancy

The early investigations into the production of steroids during pregnancy depended on the identification and assay of the metabolites of steroids present in maternal urine (Appleby and Norymberski, 1957; Natoli et al., 1957; Fotherby et al., 1965). These studies had only limited value, since it was impossible to delineate the individual parts played by the maternal endocrine glands, the placenta and the foetus in producing the steroids excreted by the mother. To evaluate the contribution of the foetus, the steroid metabolites present in the urine of newborn infants and in blood collected from the umbilical cord have also been examined, on the assumption that many of the compounds present in urine collected from newborn infants would be similar to those produced by the foetus in utero. At birth, blood can easily be obtained from the umbilical cord, and the plasma fraction contains steroid metabolites which have been elaborated by the foetus. Unlike the urine samples, however, plasma derived from the umbilical cord also contains a significant amount of steroids which have come from the placenta: by comparing the concentration of steroid in venous and arterial plasma obtained separately from the umbilical vessels it is possible to define where the individual steroids were formed. There is, for example, a greater concentration of progesterone in plasma derived from blood in the umbilical vein (i.e. from blood going to the foetus) than in corresponding samples obtained from the umbilical arteries (i.e. blood going to the placenta), so it may be assumed that the placenta produces this compound.

Most of the recent work on steroid formation and degradation in utero has involved the use of radioactively labelled steroids. In some

experiments these compounds have been introduced in vivo; for example, by perfusion of a placenta while it is still attached to the uterine wall, or by injection into the foeto-placental circulation. In other experiments the metabolism of the radioactive steroid has been studied in vitro, by using homogenates or slices of foetal or placental tissue. The experiments conducted in vivo approach most closely to the conditions prevailing naturally in utero but, because of the danger to the foetus of harmful radiation, these experiments cannot be carried out during the course of a normal pregnancy, and must be associated with therapeutic abortion. Such terminations of pregnancy are usually carried out before the 24th week of gestation, since the foetus is held to be viable after this period. Normally, the studies in vitro have also been carried out on preparations of foetal tissue obtained as a result of therapeutic abortion, but some investigators have used tissue obtained from infants who died soon after birth (Villey and Loring, 1965).

The principal types of experiment carried out with tissues collected at terminations performed about the 20th week of a pregnancy are as follows:

1. The analysis of steroids in extracts of foetal tissues.
2. The histochemical detection of enzymes involved in steroid biosynthesis and metabolism.
3. Studies using radioactive steroids incubated in vitro with slices, homogenates or minces of foetal or placental tissues.
4. Perfusion of radioactive steroids through:-
 - (a) The foetus alone, after removal from the uterus .
 - (b) The foetus, after operation for the removal of the adrenal glands .
 - (c) The placenta alone, while still attached to the uterine wall

5. Administration of radioactive steroids into the intact foeto-placental unit while the placenta is still attached to the uterus.
6. Administration of steroids into the uterine artery, to investigate the passage of steroids across the placenta.
7. Administration of steroids into the amniotic fluid, before termination of pregnancy. The steroids are then taken up by the foetus through swallowing, or by absorption through the skin, or possibly they are absorbed by the placenta.

The metabolic fate of radioactive steroids is studied by analysing for their metabolites in foetal and placental tissues, in blood collected in the outflow from perfusion experiments and, in some cases, by examining amniotic fluid and urine specimens collected from the mother.

It is possible, by employing the experiments listed, to study the metabolism of steroids by the foetus, by the placenta and by the foeto-placental unit. In addition, by introducing labelled steroids into the mother, it is possible to determine whether steroids produced by the maternal endocrine glands are able to cross the placental barrier, after which they may be further metabolised by the foeto-placental unit.

2. The synthesis of progesterone by the foeto-placental unit

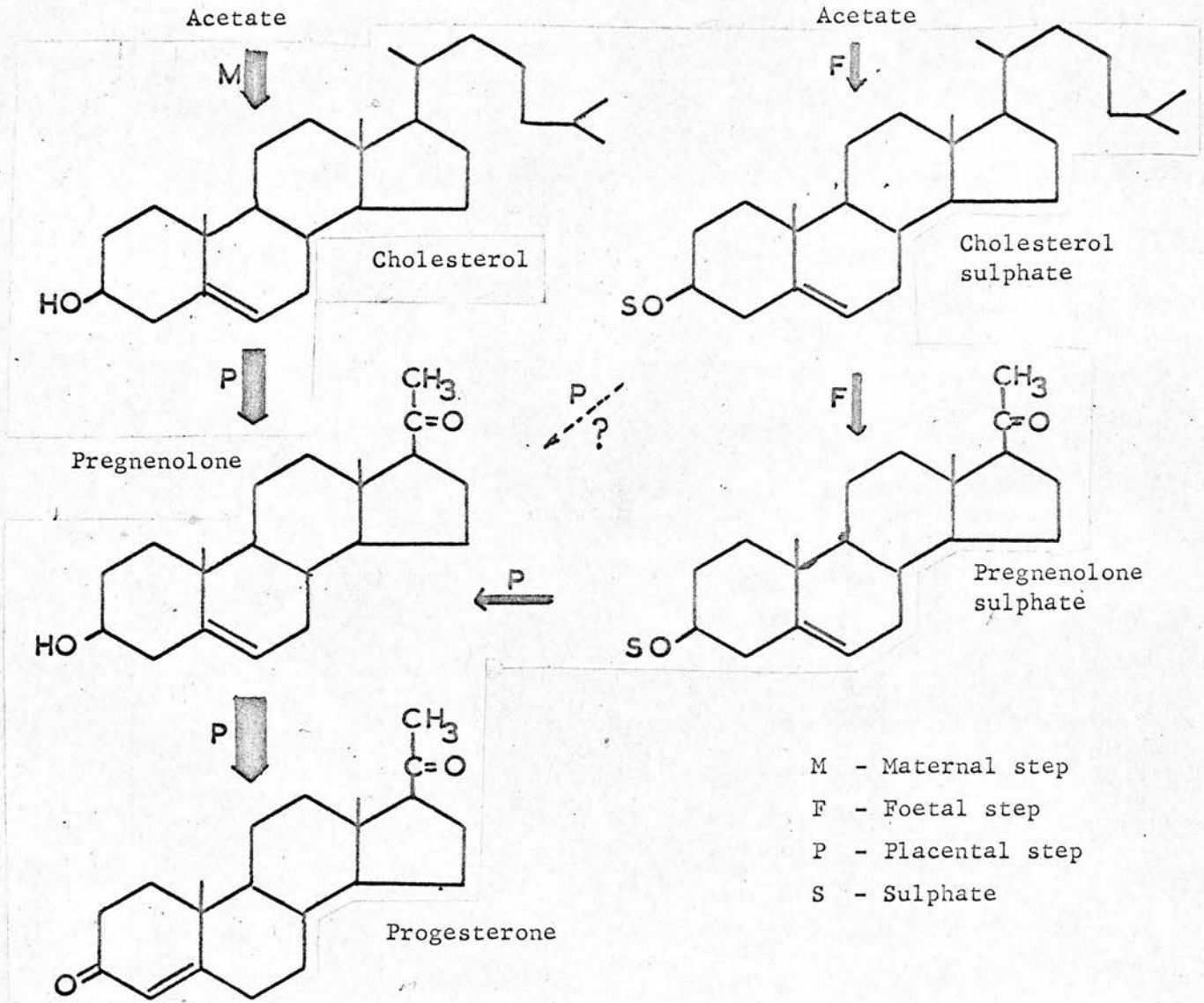
It was considered for many years that progesterone was formed from cholesterol synthesised by the placenta, but it has recently been shown that the placenta is almost unable to synthesise the steroid nucleus de novo. Levitz et al. (1962) found only a very small production of cholesterol by the placenta when perfused with acetate and mevalonate, and Van Leusden and Vilee (1965) reported only a minute conversion (0.002 - 0.005%) by tissue minces of placenta. Since the placenta is unable to form the

steroid nucleus from acetate, it must use preformed steroid precursors for the formation of progesterone.

The most likely blood-borne precursors of progesterone are cholesterol and pregnenolone sulphate, which could be obtained from either the maternal or the foetal circulation. There is a considerable amount of evidence to suggest that the precursor is supplied principally by the mother. Allen (1953) reported two cases of abdominal pregnancy where, after removal of the child, the urinary pregnanediol excretion remained high while the placenta was still in situ. Other workers have shown that urinary pregnanediol excretion remains high after intrauterine death of the foetus (Appleby and Norymberski, 1957; Klopper et al., 1961) and the excretion of pregnanediol by women carrying anencephalic foetuses is little different from normal (Frandsen and Stakeman, 1961), although these monsters have hypoplastic adrenals which would be expected to produce only a small amount of precursor able to be used for progesterone synthesis. The evidence, therefore, suggests that sufficient precursor can be obtained from the mother to provide for the synthesis of progesterone even in the absence of a normal foetus. Insufficient pregnenolone sulphate is supplied to the placenta by the maternal circulation to account for the large amount of progesterone formed each day (Conrad et al., 1967), so it is most likely that the major precursor is cholesterol, a compound which has been shown to be able to be converted to progesterone by placental tissue (Solomon, 1960; Morrison et al., 1965).

The placenta can produce normal amounts of progesterone even though the foetus is not viable or has been removed, but it is nevertheless probable that, during a normal pregnancy, some progesterone is synthesised from precursors produced by the foetus. When labelled progesterone or

Fig. 1. The Biosynthesis of Progesterone by the Foeto-Placental Unit



Progesterone is synthesised in the placenta from precursors supplied by both the foetus and the mother. The major precursor of progesterone is probably cholesterol, which reaches the placenta by the uterine arteries, but it is likely that cholesterol sulphate and pregnenolone sulphate synthesised by the foetus are also important.

pregnenolone sulphate was perfused through placentas at mid-term, approximately 80% of the unconjugated steroid extractable from the tissue, and approximately 50% of the unconjugated steroid isolated from the perfusate was progesterone (Pion et al., 1965a; Palmer et al., 1966), which indicates that the placenta can utilise these steroids as precursors of progesterone.

Eberlein (1965b) first identified pregnenolone sulphate in plasma obtained from the umbilical blood vessels, and more recently it has been shown that this compound is present in greater concentration in samples of arterial plasma than in the venous plasma (Conrad et al., 1967); this suggests that the placenta takes up pregnenolone sulphate from the blood reaching it via the umbilical arteries. It has, therefore, been shown that the foetus can supply pregnenolone sulphate to the placenta, and that this organ contains the enzymes necessary for converting it into progesterone.

The formation of progesterone by the foeto-placental unit is illustrated in diagrammatic form in Fig. 1. Although it has been established that maternal cholesterol and foetal pregnenolone sulphate are efficient precursors of progesterone, it is not yet known whether cholesterol formed by the foetus also contributes to the synthesis of progesterone.

It is unlikely that the foetus itself is capable of the total synthesis of progesterone since most evidence suggests that the 3β -hydroxy-dehydrogenase* which converts pregnenolone to progesterone is

* Two enzymes are required for the formation of a $3\text{-oxo-}\Delta^4$ steroid from a $3\beta\text{-OH-}\Delta^5$ steroid: a 3β -hydroxy-dehydrogenase for converting the $3\beta\text{-OH-}\Delta^5$ steroid into a $3\text{-oxo-}\Delta^5$ steroid, and a steroid $\Delta^{4,5}$ isomerase for converting the $3\text{-oxo-}\Delta^5$ steroid into a $3\text{-oxo-}\Delta^4$ steroid. In this thesis 3β -hydroxy-dehydrogenase refers to both these enzymes,

not active in the foetus in utero. Iwamiya et al. (1966) injected pregnenolone into a mid-term foetus before termination of the pregnancy, and subsequently extracted from the adrenals the sulphates of DHA, pregnenolone and 17α -OH-pregnenolone, and from the liver 20α -dihydro-pregnenolone sulphate. No $3\text{-oxo-}\Delta^4$ steroids were detected in either tissue. Several experiments carried out in vivo by Diczfalusy and co-workers (Diczfalusy et al., 1965; Pion et al., 1965b; Bolté et al., 1966) have also failed to demonstrate any activity of 3β -hydroxydehydrogenase in the foetus at mid-term but $3\text{-oxo-}\Delta^4$ steroids can be formed from pregnenolone by foetal adrenal tissue in vitro (Villem and Loring, 1965). It is thus evident that the enzymes required for the various conversions are present in the adrenal, but they are somehow rendered inactive.

It may be concluded that most of the progesterone is formed in the placenta from cholesterol supplied by the mother, but it is probable that a small portion is also formed from pregnenolone sulphate and from cholesterol produced by the foetus.

3. The synthesis of oestrogens during pregnancy

The formation of the other principal hormones of pregnancy, the oestrogens, is more complex and there have been indications for several years that the foetus is directly involved in their synthesis.

Ten Berge (1958) reported an abnormally low level of oestriol in urine obtained from a woman bearing an anencephalic foetus. This finding was verified by Frandsen and Stakeman (1961), who also found that oestrone and oestradiol were excreted in reduced amount. These workers noted a correlation between the degree of hypoplasia of the foetal adrenal cortex and the amount of oestrogens excreted by the mother, and

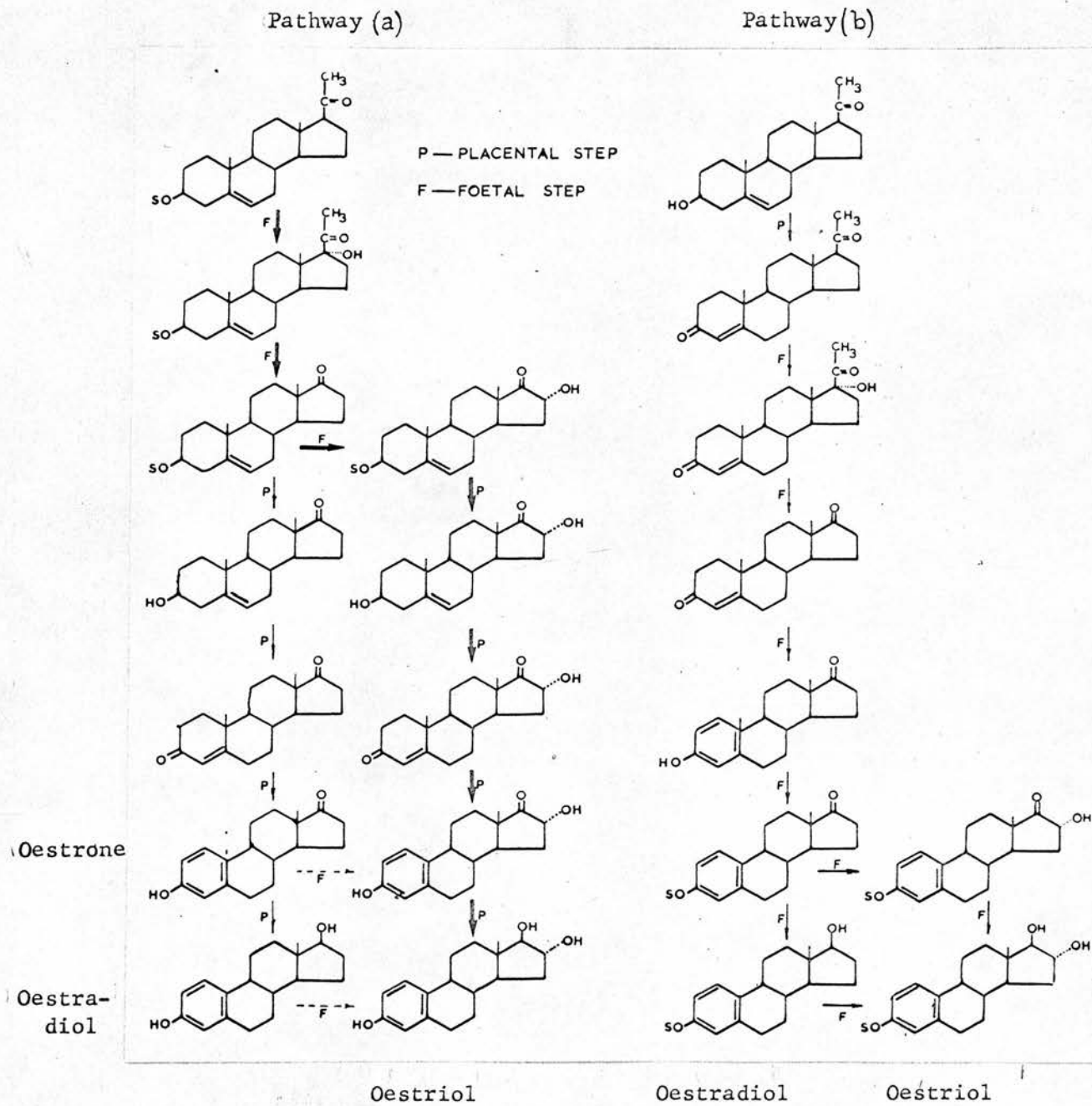
put forward the hypothesis that the foetal adrenal glands were involved in the elaboration of oestrogens, by producing steroid precursors (Frandsen and Stakeman, 1963).

Perfusion of placentas still attached to the uterus with DHA, DHA sulphate, androstenedione or testosterone resulted in the finding of large amounts of oestrone and oestradiol in the placenta and in the perfusate (Bolté et al., 1964). Oestriol, however, was not detected in these experiments, and none was obtained when oestrone and oestradiol were perfused through placentas at mid-term.

These findings from experiments conducted in vivo are in contrast to the findings of Slaunwhite et al. (1965), who found that microsomes prepared from placentas in the third to fifth month of gestation were capable of affecting the 16α -hydroxylation of DHA and oestrone. This represents a further example of the need to interpret the results of in vitro studies with caution.

It has been shown in vivo that the foetal liver can effect the 16α -hydroxylation of oestrone and oestradiol (Schwers et al., 1965a), and of two sulphated steroids, DHA sulphate and oestrone sulphate (Bolté et al., 1966; Schwes et al., 1965b). It is likely, therefore, that the 16α -hydroxy group of oestriol is supplied by the foetus. The foetus is not able to synthesise oestrogens completely, however, due to the inactivity of 3β -hydroxy-dehydrogenase in foetal tissue, so the essential step involving the formation of a $3\text{-oxo-}\Delta^4$ -steroid must take place in the placenta, which has an active 3β -hydroxy-dehydrogenase. Since the foetus can carry out all the other steps in the synthesis, including aromatisation, the oestrogens could arise either by the action of

Fig. 2. The Synthesis of Oestrogen by the Foeto-Placental Unit



placental 3β -hydroxy-dehydrogenase and aromatase on 3β -OH- Δ^5 steroids such as DHA or 16α -OH-DHA (Fig. 2a), or by foetal aromatisation of 3 -oxo- Δ^4 steroids produced by the foetal adrenal from progesterone (Fig. 2b).

Magendantz and Ryan (1964) demonstrated that 16α -OH-DHA could be converted into oestriol by placental microsomes, and this steroid has recently been shown to be an active precursor of oestriol in vivo (Dell'Acqua et al., 1966). Since DHA and 16α -OH-DHA have been shown to be active precursors of oestrogens in vivo, it is significant that the sulphates of these steroids are found in large amount in plasma obtained from the umbilical blood vessels. These compounds have been assayed by Colás et al. (1964), and by Easterling et al. (1966), and were found to be present in greater concentration in the arterial than in the venous plasma obtained from the umbilical vessels, indicating a net uptake of these steroid sulphates by the placenta.

Some oestriol is, however, formed by 16α -hydroxylation of oestrone and oestradiol produced from DHA sulphate synthesised in the maternal adrenal glands. Evidence for this pathway has been supplied by Kirschner et al. (1966), who showed that DHA sulphate administered to the mother was converted to oestriol and that this conversion occurred principally by phenolic intermediates. The DHA sulphate present in the maternal blood is converted by the placenta into oestrone and oestradiol, which are then released into the umbilical vein. Maner et al. (1963) found only 2.8 μ g. oestrone/100 ml. and 0.64 μ g. oestradiol/100 ml. in plasma from the umbilical vein, so it is unlikely that a significant amount of oestriol is formed by 16α -hydroxylation in the foetus of this

oestrone or oestradiol. The concentration of DHA sulphate in plasma obtained from the umbilical blood vessels is several times greater than its concentration in plasma obtained from the mother (Colás et al., 1964); this is further evidence that most of the precursors of oestrogens are synthesised by the foetus.

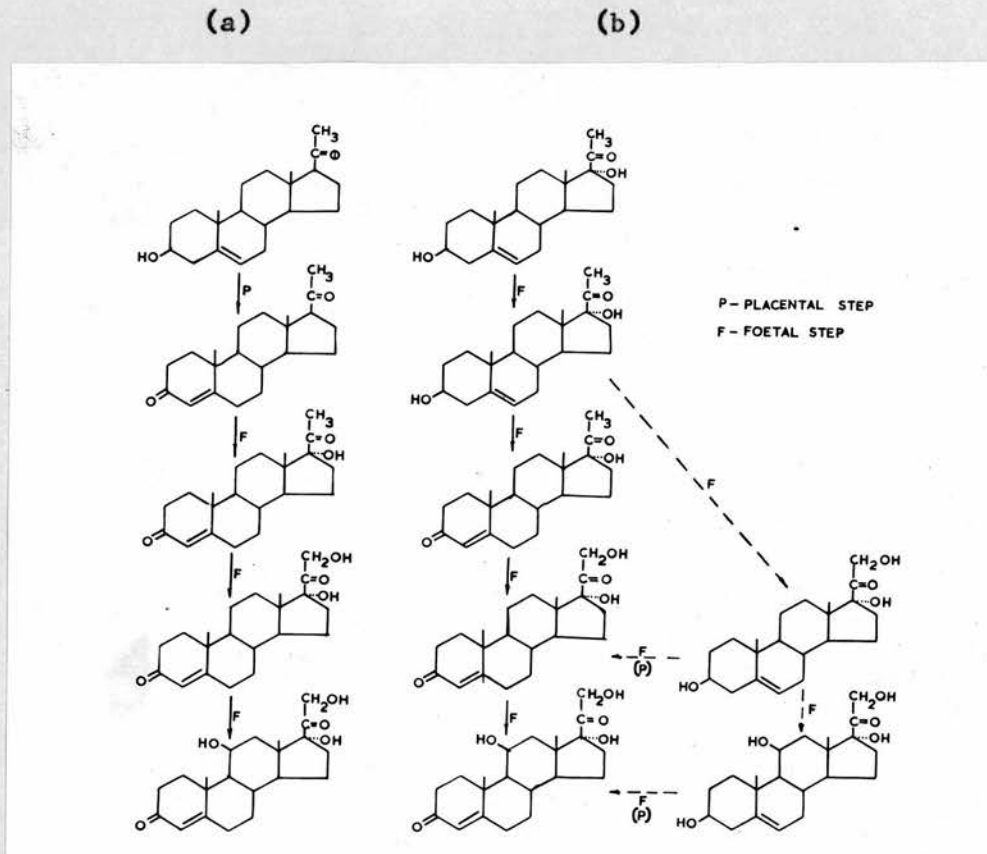
It has recently been shown that foetal liver is able to aromatise 3-oxo- Δ^4 steroids (Mancuso et al., 1965), so it is possible that oestrogens can also be formed from androstenedione and testosterone produced in the foetal adrenals from progesterone supplied by the placenta. It is probable, however, that this pathway (Fig. 2b) is only of minor importance, and from the evidence available it is concluded that most of the oestrogens are formed in the placenta from DHA sulphate and 16 α -OH-DHA sulphate produced by the foetus.

4. The biosynthesis of corticosteroids by the foeto-placental unit

The general metabolic balance of the foetus during pregnancy is not thought to be controlled to any extent by cortisol supplied by the mother, and it is likely that the foeto-placental unit is itself capable of forming sufficient cortisol for foetal use. Migeon et al. (1961) showed that ^{14}C cortisol administered to the mother could cross the placental barrier to the foetus, but by administering cortisol into the amniotic fluid, these workers demonstrated that it could also be transported in the other direction. Mills (1967) considers that because plasma derived from blood collected from the mother has high cortisol-binding power, whereas plasma obtained from the foetus has low cortisol-binding power, it is most likely that throughout much of pregnancy the mother "steals" cortisol produced by the foeto-placental unit.

The newborn infant is evidently able to produce sufficient cortisol to maintain life, but throughout most of pregnancy the complete foeto-placental unit is probably required for the synthesis of this compound, due to the inactivity of the 3β -hydroxy-dehydrogenase in foetal tissue. Because of this enzyme deficiency, the foetus requires a 3-oxo- Δ^4 precursor such as progesterone for the synthesis of cortisol. It has been estimated by Zander (1961) that half of the large amount of progesterone produced in pregnancy (estimated at about 250 mg./day near term; Pearlman, 1957) is secreted by the placenta directly into the foetus. Autoradiographic studies carried out by Bengtsson et al. (1964) on slices of foetal tissue perfused with radioactive progesterone revealed an accumulation of radioactivity in the adrenals, indicating that this was probably the major site of progesterone metabolism. Several investigators have demonstrated in vitro that the foetal adrenals, ovaries and testicles can convert progesterone into a variety of steroids. Of particular interest is the production of cortisol by incubating progesterone with minced adrenal tissue (Villem and Driscoll, 1965), or by organ cultures of foetal adrenals (Bloch et al., 1965). Solomon et al. (1967) perfused foetuses of gestational age 17-21 weeks with ^{14}C progesterone and isolated the following radioactive steroids from the adrenal gland; 16α -OH-progesterone, corticosterone sulphate, 20α -dihydroprogesterone, cortisol, 17α -OH-progesterone and pregnanolone. This important work indicates that the foetal adrenal at mid-term can utilise circulating progesterone for the synthesis of corticosteroids. It has, therefore, been established that the foetus at mid-term can effect the 16α -, 17α -, 21- and 11β -hydroxylation of progesterone,

Fig. 4. The Biosynthesis of Cortisol by the Foeto-Placental Unit



Established pathway
of cortisol formation

Proposed alternative
pathway of cortisol
formation.

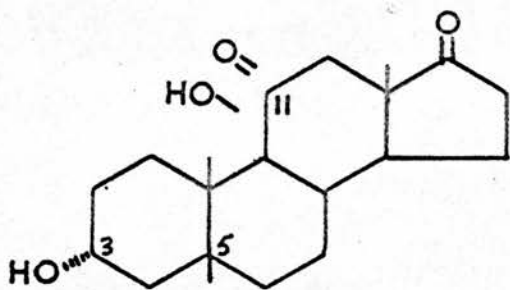
Note: Progesterone is not an intermediate in the pathway which is proposed here as an alternative route.

whereas it has recently been shown that the placenta has little if any ability to carry out these hydroxylations, its metabolism of progesterone being limited to 6β -hydroxylation and 20α -reduction (Diczfalusy, 1966). Experiments with adrenalectomised fetuses (Wilson et al., 1966) have indicated that most of the metabolism of progesterone takes place in the adrenals; only 6β - and 17α -hydroxylation takes place in other tissues. Small amounts of aldosterone have been isolated from the adrenals of fetuses perfused with labelled corticosterone (Pasqualini et al., 1966), so it is apparent that, using placental progesterone as a precursor, the adrenals of fetuses at mid-term are capable of elaborating all the biologically important corticosteroids (Fig. 3a, 4a).

Iwamiya et al. (1966) have shown that the foetus lacks the 3β -hydroxy-dehydrogenase required for converting pregnenolone into progesterone, but there are indications that other 3β -hydroxy-dehydrogenases are active in foetal tissue and that these can convert hydroxylated pregnenolone derivatives to their corresponding $3\text{-oxo-}\Delta^4$ steroids. Recently, Pasqualini and co-workers (1968) obtained cortisol from fetuses perfused with labelled $3\beta,17\alpha,21$ -trihydroxypregn-5-en-20-one, at mid-term, so it is possible that the foetus and the newborn infant may obtain cortisol by biosynthetic pathways not involving progesterone as an intermediate. The possible formation of corticosterone and cortisol by these pathways is illustrated in Figs. 3b and 4b. Further evidence that these routes may be important has been obtained by analysis of the steroid metabolites present in urine collected from infants and in plasma obtained from the umbilical cord. 21-OH-Pregnenolone

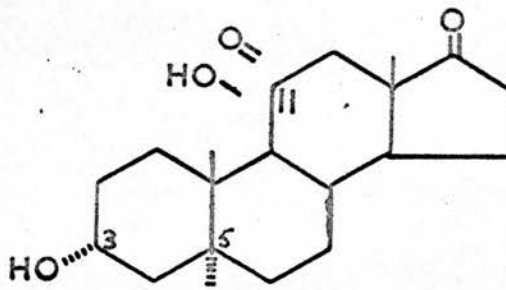
Fig. 5. The Major Metabolites of Steroids Excreted in Urine by Adults

The 17-oxosteroids



Aetiocholanolone
(3 α -OH-5 β)

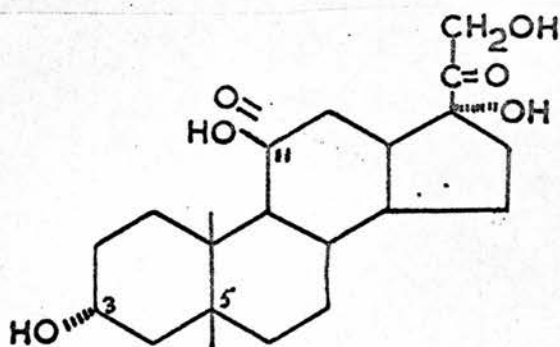
Also 11-oxo-aetiocholanolone
11-OH-aetiocholanolone



Androsterone
(3 α -OH-5 α)

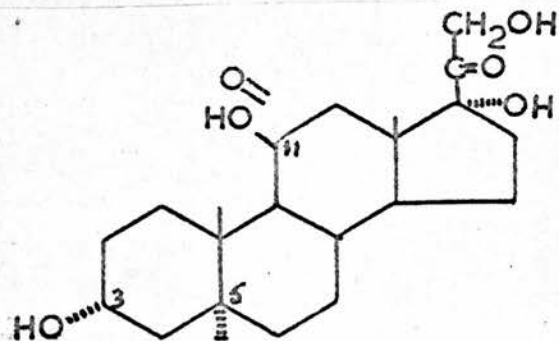
Also 11-oxo-androsterone
11-OH-androsterone

The metabolites of cortisol



Tetrahydrocortisol
(3 α -OH-5 β)

Also tetrahydrocortisone



Allotetrahydrocortisol
(3 α -OH-5 α)

Also allotetrahydrocortisone

is a possible intermediate in the formation of corticosterone (Fig. 3b), and this steroid has been identified in these blood (Eberlein, 1965b) and urine specimens (Birchall and Mitchell, 1965). A 3β -hydroxy-dehydrogenase is possibly present in the foetus and capable of converting 21-OH-pregnenolone to deoxycorticosterone; alternatively, $3\beta,11\beta,21$ -trihydroxypregn-5-en-20-one could be formed from 21-OH-pregnenolone and then dehydrogenated directly to form corticosterone. Eberlein (1965b) has tentatively identified other 3β -OH- Δ^5 steroids in plasma from the umbilical cord, including 17α -OH-pregnenolone, $3\beta,17\alpha,21$ -trihydroxypregn-5-en-20-one, and $3\beta,11\beta,17\alpha,21$ -tetrahydroxypregn-5-en-20-one, all of which could be intermediates in the biosynthetic pathway illustrated in Fig. 4b for the production of cortisol. Further research is necessary to determine which of these alternative pathways are important for the production of corticoids by the foetus.

5. The structure of steroid metabolites in urine collected from infants

Qualitative analyses of steroids in urinary extracts have revealed considerable differences between specimens collected from infants and from adults. The major steroids excreted by adults are the 17-oxosteroids and the 17-hydroxycorticosteroids; most of the compounds falling into these groups are formed by reduction of 3 -oxo- Δ^4 steroids in the liver, with the formation of 3α -OH- 5α steroids and 3α -OH- 5β steroids (Fig. 5). These steroids are almost invariably excreted as their conjugates with glucuronic acid. In urine collected from infants, however, the major steroids present have the 3β -OH- Δ^5 structure. This is due to the relative deficiency of 3β -hydroxy-dehydrogenase in the adrenal glands of newborn children. These 3β -OH- Δ^5 steroids are almost entirely excreted as their

sulphate conjugates, and there is relatively little steroid excreted as the glucuronide conjugate in urine collected from the newborn infants. When group assay methods were used to measure the 17-oxosteroids and the cortisol metabolites in urine samples from infants, results were high but it has since been shown that the "adult-type" individual 17-oxosteroids and cortisol metabolites are only present in small amount in these specimens (Cathro et al., 1963).

Among the cortisol metabolites, tetrahydrocortisone and tetrahydrocortisol are the major reduced metabolites in the urine of adults, but in infants neither is the dominant reducing compound. Only 10% of the radioactivity injected into infants as ^{14}C labelled cortisol was recovered as tetrahydrocortisone, tetrahydrocortisol and allotetrahydrocortisol (Bertrand et al., 1963) compared to approximately 36% when labelled cortisol was injected into adults (Flood et al., 1961). The metabolism of cortisol by infants is therefore very different from its metabolism in adults.

In the newborn the enzymes which catalyse the reduction of the A ring of the steroid nucleus are apparently relatively inactive (Bongiovanni et al., 1958; Migeon, 1959; Reynolds et al., 1962), and this deficiency results in a large proportion of the cortisol being either excreted unchanged or being further metabolised by other routes. An important pathway in newborn infants is 6β -hydroxylation (Ulstrom et al., 1961), the resulting 6β -OH-cortisol being excreted unconjugated.

Cathro and co-workers (1963) determined the individual 17-oxosteroids and reducing compounds by a paper chromatographic method, and found many hitherto unidentified bands which were probably unknown 17-oxosteroids.

and metabolites of corticosteroids. Many of these unidentified compounds were quantitatively very important, and interfered with the attempts by these workers to measure small amounts of the "adult-type" 17-oxosteroids and tetrahydro metabolites of cortisol.

Little is known, therefore, about the major routes for the metabolism of the adrenal steroids in neo-natal life, and in recent years most attention has been paid to the identification of $3\beta\text{-OH-}\Delta^5$ steroids, since these are quantitatively the most important steroids in urine collected from newborn infants. Some of these $3\beta\text{-OH-}\Delta^5$ steroids can act as precursors for steroid synthesis in utero, the most important example being $16\alpha\text{-OH-DHA}$, a compound which has been shown to be a major precursor of oestriol (Dell'Acqua et al., 1966); this steroid was identified in urine from newborn infants by Bongiovanni (1962).

Birchall and Mitchell (1965) identified $21\text{-OH-pregnenolone}$ in extracts from urine collected from infants and it is possible that this compound is a precursor of deoxycorticosterone and of corticosterone in the foetus. Quantitatively the most important steroid in infant urine is $16\alpha\text{-OH-pregnenolone}$ which was identified by Reynolds (1963), but the function of this compound is not known, since it is unlikely to play a part as a precursor in the synthesis of other steroids, and is more probably a metabolite of pregnenolone formed prior to excretion in the urine.

Although DHA is quantitatively the most important $3\beta\text{-OH-}\Delta^5$ steroid in urine collected from adults, little is found in urine obtained from newborn infants (Cathro et al., 1963), presumably due to the high activity of $16\alpha\text{-hydroxylase}$ in the liver, both in the foetus and the newborn.

6. The control of steroid production by the foetus and by the newborn infant

In anencephalic foetuses the adrenal glands are severely hypoplastic (Frandsen and Stakeman, 1961). This may be explained by the gross disturbance of the hypothalamic-pituitary-adrenal axis and may indicate that the foetal adrenal is controlled by foetal adrenocorticotrophic hormone. Further evidence that this is the case has been produced by Simmer et al. (1966), who showed that corticosteroids administered to the mother markedly lowered the levels of DHA and 16α -OH-DHA in blood collected from the umbilical cord. This indicated that these steroids had crossed the placenta and suppressed the production of adrenocorticotrophin by the foetus. It has also been shown that adrenocorticotrophin stimulates the synthesis of steroids by foetal adrenals in the third trimester (Bloch, 1966).

In the neonatal period, blood cortisol is certainly under the control of adrenocorticotrophic hormone, since Bertrand et al. (1962) and Hillman and Giroud (1965) found considerable increases in its concentration in blood after the administration of adrenocorticotrophin to infants.

Recent work by Lauritzen and Lehmann (1965, 1967) has shown that human chorionic gonadotrophin as well as adrenocorticotrophin can affect steroid excretion by the newborn. These workers found that administration of human chorionic gonadotrophin caused a very significant increase in DHA excretion. Since human chorionic gonadotrophin is produced by the placenta in large amounts, particularly near the beginning of pregnancy, this finding could have considerable importance.

It seems probable therefore that the synthesis of steroids by the

foetus is regulated by adrenocorticotrophic hormone and human chorionic gonadotrophin.

7. The purpose of the research undertaken for this thesis

In 1963, Cathro et al. reported the presence of many unknown steroid-like compounds in urine collected from newborn infants. It was considered by these workers that the compounds were formed by biosynthetic systems designed for special functions required for foetal life, and which were still operating after birth. The research reported in this thesis was undertaken in an attempt to identify and assay some of these uncharacterised compounds. It was considered that a more complete knowledge of the excretion of steroids by newborn infants would be useful in understanding the metabolism of steroids in the foetus and the newborn infant. Since plasma obtained from the umbilical vessels also contains steroid metabolites formed in the foetus, a method was developed for the analysis of these compounds.

Since both human chorionic gonadotrophin and adrenocorticotrophin appeared to be involved in the regulation of steroid metabolism in the foetus (Lauritzen and Lehmann, 1965; Bertrand et al., 1962), a study was undertaken of the effect of these trophic hormones on the excretion of a number of steroids in urine collected from infants.

PART I.

DEVELOPMENT OF A METHOD FOR DETERMINING STEROID METABOLITES IN URINE
SAMPLES COLLECTED FROM NEWBORN INFANTS, AND IN PLASMA SAMPLES
OBTAINED FROM THE UMBILICAL BLOOD VESSELS.

I CHOICE OF TECHNIQUES

1. Introduction

In the last two decades many techniques have been developed for the assay of steroids in biological fluids; most of these have been useful aids for the particular topics being studied. Before developing a new method for the determination of steroids in specialised biological material it is, therefore, necessary to decide on the types of method to be investigated. The choice will depend on the concentrations of the steroids to be assayed, the accuracy needed, and the number and type of steroids which are to be determined in any particular estimation. The accuracy of a method designed specifically for measuring a single compound is usually better than the accuracy of a method for the quantitative estimation of many compounds. A balance must, therefore, be kept between the requirements of reliability and practicability.

The methods of steroid assay can be divided into two main types. Firstly, methods which assay groups of steroids containing a common functional moiety, such as the 17-oxosteroids and the 17-hydroxycorticosteroids; secondly, methods for the assay of individual steroids, which have first been separated by chromatographic or other techniques.

It was found early in the study of steroid metabolism in newborn infants that group assay methods suitable for adult work were not satisfactory when applied to the determination of steroids in urine collected from infants. Difficulties mainly arose because non-specific chromogens interfered with the colorimetric assays of the groups (Migeon, 1959; Ulstrom et al., 1959; Hughes et al., 1962), but there was also the more serious limitation that these methods had been devised to measure

TABLE 1

Methods for Measuring Individual Steroids by Chromatographic Techniques

<u>Type of chromatography</u>	<u>Method of final measurement</u>	<u>Publications using these techniques</u>
Column	1. Colour reaction in collection tube	Colás <u>et al.</u> (1964) The measurement of DHA and 16 α -OH-DHA in umbilical plasma.
	2. Measurement by automatic instrument	James <u>et al.</u> (1964) The separation and assay of lipids e.g. cholesterol.
Paper	1. Elution of compound from paper followed by colour reaction in tube	Reynolds (1965a,b, 1966a, b) The measurement of 16 α -OH-DHA, 16 α -OH-pregnenolone and androstenetriol in urine collected from newborn infants.
	2. Direct staining of chromatogram and densitometric scanning	Cathro <u>et al.</u> (1963) The assay of Zimmermann and blue tetrazolium staining compounds in urine obtained from infants.
Thin-layer (TLC)	1. Elution of compound from thin-layer material followed by colour reaction in tube	Hamman and Martin (1964) Determination of the six urinary 17-oxosteroids by two-dimensional TLC.
	2. Direct spraying of colour reagent on to plate followed densitometric scanning	Zöllner <u>et al.</u> (1962) The measurement of cholesterol esters in plasma.
Gas-liquid	Measurement of peak height or area	Vihko (1966) The determination of 3 β -OH- Δ^5 steroids in plasma. Gardiner <u>et al.</u> (1966) The measurement of all types of steroids in urine specimens obtained from adults and infants.

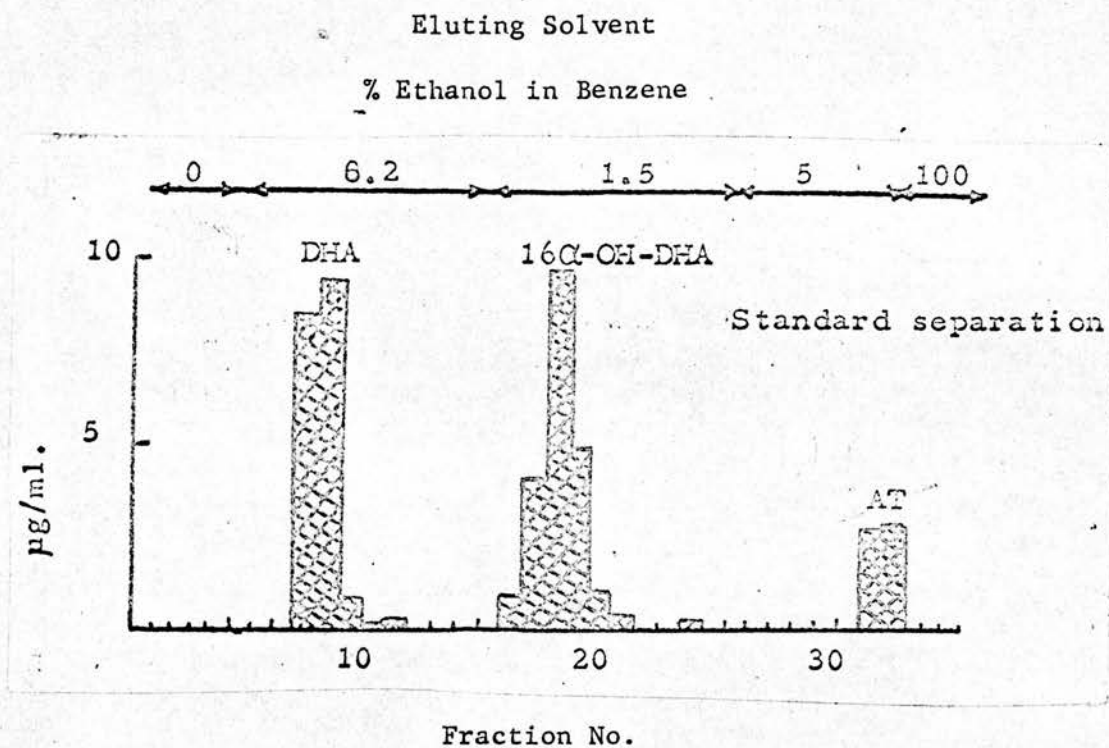
the principal metabolites occurring in the urine of adults.

Group methods for assaying steroids have been found useful in the study of large numbers of clinical cases, and form the basis of many routine methods performed in the clinical laboratory. However, in many cases, measurement of individual steroids can reveal subtle changes not detected by group assays. To elucidate the normal pattern of steroid metabolism by the newborn infant a method was required that would allow the determination of a wide spectrum of steroids. It was necessary to separate and quantitate $3\beta\text{-OH-}\Delta^5$ steroids, 17-oxosteroids and the metabolites of cortisol.

Before individual steroids can be measured, they must first be separated by chromatographic or other techniques. Four chromatographic methods have been widely used: a) column chromatography; b) paper chromatography; c) thin-layer chromatography (TLC), and d) gas-liquid chromatography (GLC). Frequently, more than one of these methods have been used in a particular assay. This section discusses some of the methods used for separation and quantitative estimation using these types of chromatography.

Table 1 shows how the four types of chromatography have been applied to the quantitative measurement of individual compounds. The examples given have been chosen for their applicability to the work to be undertaken. Each type of technique will be described in more detail, with particular reference to the requirements of the present study.

Fig. 6. Method of Colás et al. (1964) for Fractionating 3β -OH- Δ^5 Steroids Present in Plasma Obtained from the Umbilical Blood Vessels



Alumina columns (0.4 x 5.5 cm.) were used for separation. The steroids were determined after separation by using the Pettenkoffer reaction.

AT = Androstenetriol

2. Column Chromatography

2.1 With colour development in the collection tube

Colás and co-workers (1964) determined DHA and 16α -OH-DHA in plasma obtained from the umbilical vessels. They used a method which depended on solvolysis of the steroid conjugates followed by column chromatography. Very small columns were used (internal diameter 0.4 cm., length 5.5 cm.) and a typical separation is shown in Fig. 6. The Pettenkoffer reaction (Fotherby, 1958), which is specific for 3β -OH- Δ^5 steroids, was used for colour development. The practicability of the method is good but specificity is low, since there are many other compounds which develop a colour in the Pettenkoffer reaction and which are present in these plasma samples (Eberlein, 1965b); these other compounds have similar polarities and the resolution of the columns used is not high. 16α -OH-DHA separated in this technique has been found recently to be contaminated with 16-oxo-androstenediol (Colás et al., 1965).

2.2 With automatic determination

This method has only recently been developed, and has not as yet been applied to the measurement of steroids. It has been included since it will probably prove useful for the assay of steroids. A moving wire passing through the eluant from the column collects a thin coating, and any material eluted remains on the wire when the solvent is evaporated. The wire passes to an oven where pyrolysis and vaporisation take place, and any material present is then detected by an argon or flame-ionisation detector of the type used for GLC. Peaks due to the separated compounds are registered on a recorder. This method has been shown to give reproducible quantitative results when used for the separation of lipids, and will certainly be applicable to column chromatography of steroids.

3. Paper chromatography

3.1 Elution method

The major metabolite in urine specimens collected from infants, 16 α -OH-pregnenolone, was isolated by Reynolds (1963) using a method involving paper chromatography. This technique was later developed for the quantitative estimation of this compound and of two other important metabolites, 16 α -OH-DHA and androstenetriol (Reynolds, 1965a, 1966b). In this procedure the sulphate esters were hydrolysed by solvolysis, and the steroids obtained were separated by a single chromatographic run. Part of the chromatogram was stained with phosphomolybdic acid, to determine the position of the various compounds; these were then eluted from the corresponding positions on the remainder of the paper. The material obtained was further purified by using small columns of silica gel, and the final measurement made the Oertel reaction (Oertel and Eik-Nes, 1959).

This method is satisfactory for the measurement of a small number of compounds, but if many steroids are to be determined in a single sample, then a method which makes use of a colour reaction developed directly on the paper chromatogram is preferable.

3.2 Direct staining and quantitation on paper

Birchall and co-workers (1963) developed a paper chromatographic technique for investigating the types of steroid that were excreted by normal newborn infants, but it was found to be impossible to delineate and elute the many compounds detected. Therefore, different groups of steroids were measured directly on the chromatograms by dipping the papers into suitable staining solutions. For example, the blue tetrazolium reagent was used for detecting α -ketolic steroids and the Zimmermann

reagent for 17-oxosteroids. The spots were measured densitometrically using a modified spectrophotometer. A considerable disadvantage of this method was the time taken to complete an estimation on a single sample of urine, since several long paper chromatographic runs were involved with the necessity of eluting steroids from one chromatogram and transferring them to another.

4. Thin-layer chromatography (TLC)

There are two methods involving TLC, one of which relies on elution followed by the development of a colour reaction in a tube, the other depends on direct estimation, by spraying with a suitable reagent followed by densitometric measurement.

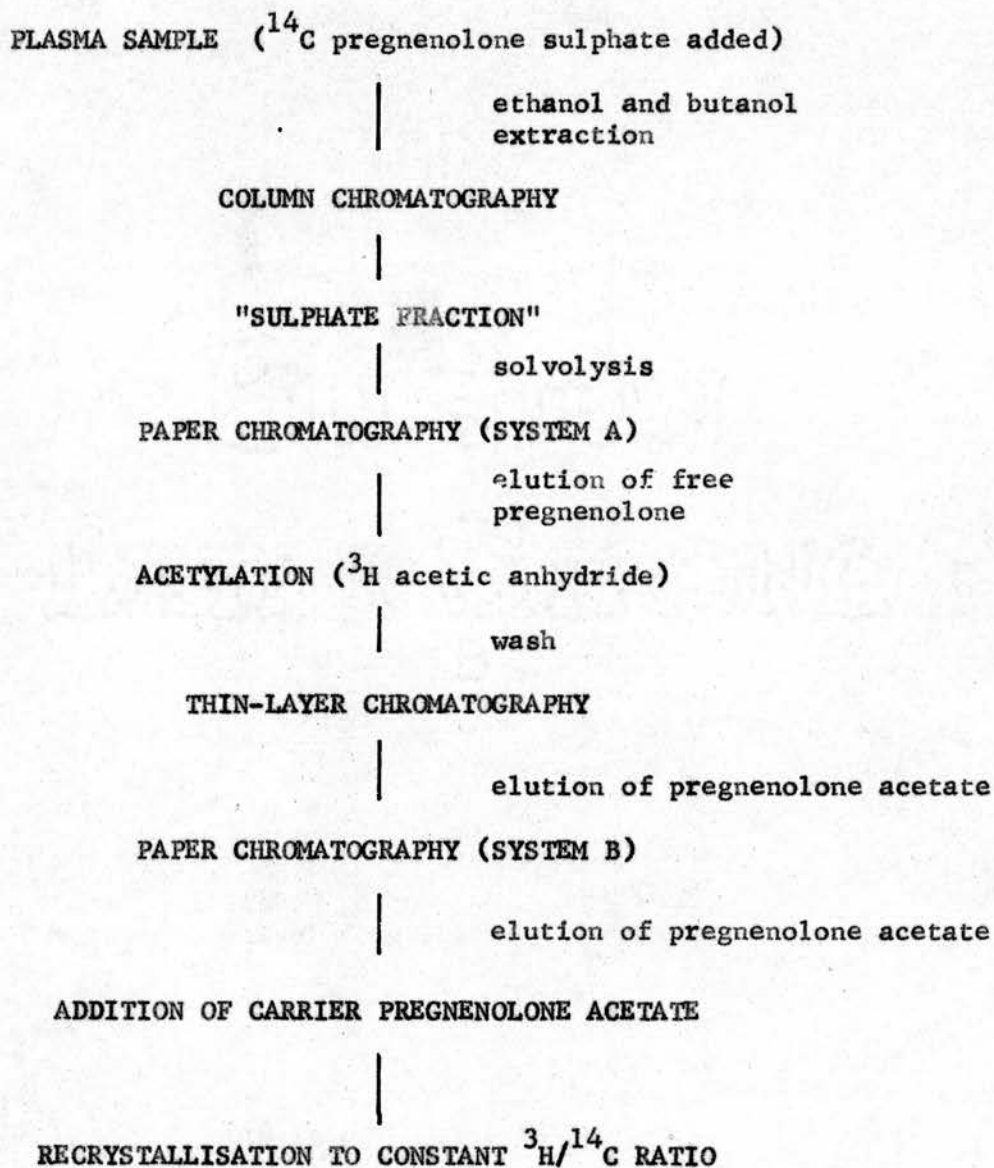
4.1 Elution method

A method was developed by Hamman and Martin (1964) for measuring the six principal 17-oxosteroids in urine. Separation was effected by two-dimensional chromatography on alumina. This was carried out in duplicate for each extract, one plate being used for locating the steroids; the corresponding areas on the other plate were then scraped off and eluted. The final assay was performed using the Zimmermann reagent. This method is open to the same criticism as applied to the comparable method involving elution after paper chromatography. To separate the steroids sufficiently for elution to be reliably performed of spots containing only one compound, two-dimensional chromatography was necessary. This meant that only one sample could be run on each thin-layer plate.

4.2 Quantitative estimation by direct staining of thin-layer chromatography plates

A more practicable approach to quantitative TLC makes use of a

Fig. 7. Double Isotope Dilution Method for the Determination of Pregnenolone Sulphate in Plasma



from Conrad et al. (1967).

system developed by Zöllner et al. (1962) for the measurement of cholesterol esters in plasma. The steroids were separated by TLC and detected by means of spray reagent consisting of antimony trichloride dissolved in chloroform. The coloured areas that developed were scanned with a densitometer, and the peak areas were related to those formed by standard compounds. Such a method is particularly useful since many different spray reagents can be used, including strong acids which would destroy paper chromatograms. Another advantage is that runs are short compared to the time required when using paper chromatography, and sensitivity is considerably increased due to the much smaller diameter of the spots. This last consideration is particularly important when steroids present in small amount are to be determined, for example in blood samples collected from the umbilical cord.

5. Double isotope dilution

The most accurate methods for measuring single steroids when present in small amount often employ double isotope dilution together with one or more of the above techniques. These methods have been used for several years, for measuring such important compounds as aldosterone (Kliman and Peterson, 1960) and testosterone (Riondel et al., 1963). Recently the technique has been developed for the measurement of pregnenolone in blood samples collected from mothers and from the umbilical cords of their infants (Conrad et al., 1967). Radioactive ^3H pregnenolone sulphate is added initially to plasma to monitor recovery. Multiple chromatograms are run (see Fig. 7) before and after acetylation with ^{14}C acetic anhydride, and finally recrystallisation has to be performed until a constant ratio of $^3\text{H}/^{14}\text{C}$ is obtained. This method is accurate and reliable, but it would

be impossible to apply it to more than one or two important compounds; it is not practicable for analysing many steroids in a single sample.

6. Gas chromatography (GLC)

Gas chromatography has been used for the determination of oestrogens, pregnanediol, the 17-oxosteroids and testosterone and several other steroids. It has recently been applied to the measurement of the solvolysable $3\beta\text{-OH-}\Delta^5$ steroids present in plasma (Vihko, 1966; Sjövall and Vihko, 1966). The technique has good reproducibility and high sensitivity, but problems arise with corticosteroids which are unstable to heat; for these thermolabile compounds it is necessary to form derivatives of either the hydroxyl or the ketone groups, or sometimes of both groupings. Horning and co-workers (1967) have successfully separated the complete range of steroid metabolites present in urine specimens collected from adults or from infants by preparing the O-methyloxime derivatives of the ketone groups and the trimethylsilyl ethers of hydroxyl groups. The method was rendered specific by identifying the peaks that were produced using a combination of gas-liquid chromatography and mass-spectrometry. This technique has not yet been made quantitative, but when it has been modified so as to allow the quantitative esterification of all the functional groups, it may prove to be the method of choice for investigating the steroids in urine samples collected from infants. If the specificity afforded by mass-spectrometry is not available, quantitative methods which depend upon specific colour reactions such as the methods described above for column, paper or thin-layer chromatography are more satisfactory than GLC.

7. Conclusions

Two methods were selected because they showed the greatest promise: (a) column chromatography followed by colour development (Colás et al., 1964); and (b) thin-layer chromatography with colour development on the chromatograms (Zöllner, 1962). Both these techniques could be adapted for the measurement of several groups of steroids, by utilising different colour reactions. By using differential colorimetry, it did not appear that it would be necessary to separate every steroid that was to be measured, but only those producing a colour in a particular reaction. For sensitivity with high resolution, it was finally decided to develop a method based on separation by TLC using specific staining reactions followed by densitometric scanning.

II. DEVELOPMENT OF A THIN-LAYER CHROMATOGRAPHIC METHOD
FOR DETERMINING STEROID METABOLITES IN URINE AND IN
PLASMA

1. Preliminary treatment of plasma

Nearly all the known $3\beta\text{-OH-}\Delta^5$ steroids, 17-oxosteroids and corticosteroids are present in cord blood and in urine as sulphates and glucuronides. Before determination, these conjugates are usually hydrolysed by acid, by enzymes, or by solvolysis. The steroids that are released are then extracted into organic solvents. Using enzymatic hydrolysis, the steroids in urine can be released by adding enzymes directly to urine, but plasma contains protein and fat which interfere with the later stages of the method. Protein interferes with the mechanism of extraction into organic solvents, and fat (which is extracted by the solvent) interferes with the subsequent chromatography. It is therefore necessary to remove protein and as much of the fat as possible prior to the hydrolysis step.

1.1 The extraction of steroids by enzymatic hydrolysis and solvolysis of a crude extract of plasma.

Initially, a simple method was investigated for extracting steroids present in plasma samples obtained from the umbilical blood vessels. The details are as follows:-

- (a) Protein was precipitated with 4 vol. ethanol (v/v) and removed by centrifugation. The supernatant was collected.
- (b) The centrifugate was extracted with 4 vol. 80% ethanol (v/v) and the suspension centrifuged again. The supernatant was again collected.

(c) The supernatants were combined and evaporated to dryness on a rotary evaporator.

(d) 30 ml. distilled water were added and the suspension was extracted twice with 30 ml. ether. These extracts were combined; they contained unconjugated steroids and some fatty material.

(e) The steroid conjugates remaining in the aqueous phase were hydrolysed, and the liberated steroids extracted, using the technique which is described later and which was developed for determining steroids in urine samples obtained from newborn infants. This method is described in detail in sections 2.3 (p. 37), 2.4 (p.38) and 2.5 (p.39).

The final extract occasionally contained polar fatty material which interfered with chromatographic separations. Since the polarity of the fatty impurity was similar to the steroids to be measured, the methods commonly used for removing fat, such as precipitation with 70% methanol in the cold or extracting aqueous methanol with non-polar solvents, were not successful. Silica gel column chromatography of the final extract was also attempted, but the fatty impurity invariably remained with the steroid fraction. From a preliminary investigation, it appeared that this fat was being released during the hydrolytic procedure, so it was considered necessary to obtain the steroid conjugates in a purer state prior to hydrolysis. For this reason other methods were investigated.

1.2. Methods based on the extraction of steroid conjugates from plasma

(a) Extraction of steroid sulphates as their pyridinium salts

McKenna and Rippon (1965) described a method for extracting steroid

sulphates from plasma; this method was based on a technique developed by McKenna and Norymberski (1960) for the extraction of steroid conjugates from urine. By the addition of pyridinium sulphate to plasma, sodium salts of steroid sulphates are converted into their corresponding pyridinium salts. Unlike the sodium salts, the pyridinium sulphates of steroids are soluble in organic solvents and may be readily extracted from the plasma into chloroform.

The principle involved has already been applied to the measurement of DHA sulphate and of 16α -OH-DHA sulphate in plasma samples collected from the umbilical blood vessels (Simmer et al., 1964; Easterling et al., 1966; Simmer et al., 1966). The details of this technique are as follows:-

- (a) 5.0 ml. plasma were shaken vigorously with 50 ml. chloroform and the mixture was centrifuged.
- (b) 4.5 ml. of the aqueous layer were transferred to a centrifuge tube containing 4.5 ml. 3M pyridinium sulphate. After mixing, 45 ml. of chloroform were added, the tube was shaken and the two layers were then separated by centrifugation.
- (c) 43 ml. of the chloroform layer were transferred to a flask and evaporated to dryness on a rotary evaporator.
- (d) The steroid conjugates present in the dried extract were hydrolysed, and the liberated steroids were extracted using the technique described in sections 2.3 (p. 37), 2.4 (p. 38) and 2.5 (p. 38).

The technique of McKenna and Rippon (1965) did not include an extraction with chloroform ^{prior to the addition of pyridinium sulphate.} This step was added so as to remove much of the fat that would otherwise have interfered with chromatography at a

TABLE 2.

The Extraction of Steroid Conjugates as Pyridinium Sulphates
from Plasma Obtained from Umbilical Blood Vessels

	Pyridinium sulphate extraction
	% Recovery
Pregnenolone	52
DHA	69
17 α -OH-pregnenolone	62
21-OH-pregnenolone	0
16 α -OH-DHA	30
U ₂ (16 β -OH-DHA)	0
16 α -OH-pregnenolone	62
17 α Androstenediol	10

Legend:

After pyridinium sulphate is added to plasma, the sodium salts of steroid sulphates are converted to pyridinium salts; these are soluble in organic solvents and can be extracted with chloroform.

The suitability of this method for the extraction of steroid sulphates from plasma obtained from the umbilical blood vessels was investigated. This table shows the recovery of 3 β -OH- Δ^5 steroids by pyridinium sulphate extraction as a percentage of the results obtained using the method employing Sephadex chromatography. Enzymatic hydrolysis was used to hydrolyse the purified steroid sulphates, both after pyridinium sulphate extraction and after Sephadex chromatography.

later stage. The extracts obtained were apparently free from all fatty impurities but the results given in Table 2 show that overall losses of steroid were considerable. Using a similar method, Easterling et al. (1966) only recovered about 30% of the 16α -OH-DHA sulphate present in plasma samples obtained from the umbilical blood vessels.

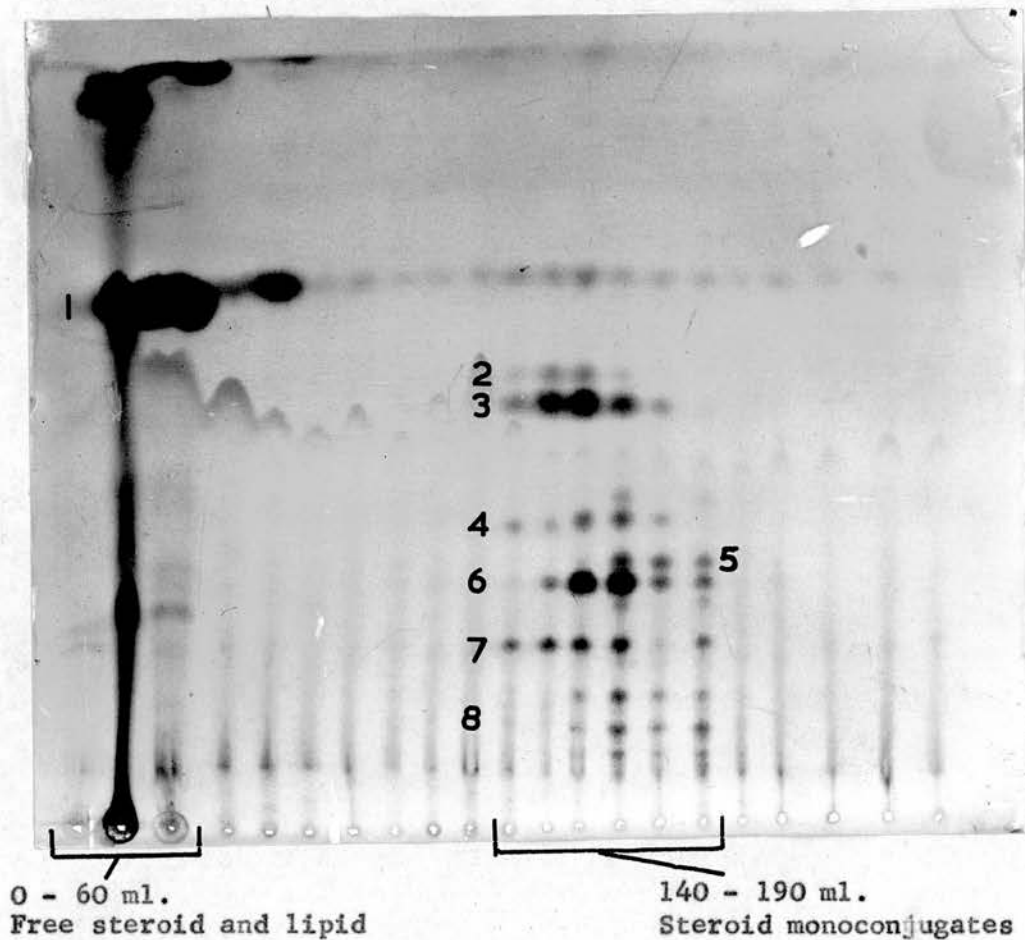
(b) The conversion of steroid sulphates to steroid acetates

Another technique has recently been applied to the extraction of steroid conjugates from plasma (Eberlein, 1962). In this method, the steroid sulphates are converted to steroid acetates, which can be readily extracted into organic solvents. Eberlein (1965b) characterised many of the steroids present in cord blood using this technique, but some of the recoveries that he obtained appeared to be very low. For instance, 16α -OH-DHA, which is quantitatively the most important steroid in plasma obtained from the umbilical cord, was found by Eberlein (1965b) to be present at a concentration of only 2 $\mu\text{g.}/100$ ml. plasma, whereas the concentration found in the present study, using the method finally adopted, ranged between 190 and 560 $\mu\text{g.}/100$ ml. plasma. Due to the low recoveries obtained by Eberlein (1965b), this method was not investigated in the present study.

1.3 The purification of steroid conjugates present in plasma samples using chromatography on Sephadex LH 20

Vihko (1966) used Sephadex LH 20 to separate unconjugated steroids from their monosulphate and disulphate conjugates. Sephadex LH 20 is prepared from Sephadex by introducing 2-hydroxyethyl groups bound in ether linkage to the free hydroxyl groups. This enables the Sephadex to be used with organic solvents.

Fig. 8. Sephadex LH20 Chromatography of Steroid Conjugates Present in Plasma
Obtained from the Umbilical Blood Vessels



- Steroids Indicated:
1. Cholesterol
 2. Pregnenolone
 3. DHA
 4. 17α -OH-Pregnenolone, Pregnenediol
 5. 16 -oxo-androstenediol
 6. 16α -OH-DHA
 7. 16α -OH-Pregnenolone
 8. Androstenediol

This figure illustrates the separation by Sephadex LH20 chromatography of steroid conjugates from lipid material present in the plasma. Methanol: chloroform (1:1, v/v) was the eluting solvent used and 10 ml. fractions were collected. Each fraction was hydrolysed by *Helix pomatia* sulphatase enzymes and, following extraction, the steroids were separated on this chromatogram. It can be seen from this figure that the fractions containing steroids are well separated from those containing unwanted lipid material. Since diconjugated steroids require a large volume of solvent to pass through the column before they are eluted, the fractions containing these steroids are not illustrated in this figure.

Figure 8 shows the separation of unconjugated steroids and fatty impurities from steroid monosulphates by chromatography on Sephadex LH20. A crude extract of the plasma steroid conjugates was obtained using stages one to three of the method described on page 28. This crude extract was applied to the column of Sephadex LH20 (17g.) using a small volume of methanol:chloroform (1:1 v/v), which was also the solvent mixture used for elution. Ten ml. fractions of the eluant were collected, apart from the first and second fractions which were 30 ml. and 20 ml. in volume respectively .

The steroid conjugates present in the fractions of the eluate were hydrolysed both by enzymatic means and by solvolysis. The details of the methods used for enzymatic hydrolysis and for solvolysis, as well as for subsequent extraction into organic solvents, are given in sections 2.3 (p. 37), 2.4 (p.38) and 2.5 (p. 38). The compounds present in the individual fractions were separated on thin-layer plates as illustrated in Fig. 8.

The free steroids and fat began to appear in the eluant after 30 ml. of the eluting solvent had passed through the column; after 60 ml. had been collected, almost all the free steroid and fat had been eluted. The steroid monosulphates were eluted after between 140 ml. and 190 ml. solvent had passed through the column, and the disulphated steroids were eluted after between 350 ml. and 400 ml. had passed through. Only the fractions comprising the first 240 ml. of eluant are represented on the thin-layer plate shown in Fig. 8. This method was not reproducible when the same columns were used repeatedly, and occasionally separation between less polar material and steroid monosulphates was poor. The separations

were improved if the solvent was saturated with sodium chloride, but this meant that much greater volumes were required for elution of steroid disulphates (Vihko, 1966). Steroid monosulphates were eluted between 150 and 220 ml. but disulphates were in fractions between 550 and 650 ml., the elution of non-polar material being unchanged.

Although time-consuming, the method which includes chromatography on Sephadex LH20 was finally adopted for assaying steroids present in plasma samples obtained from the umbilical blood vessels. The technique was simplified, however, when used as a routine procedure. The free steroid and fat were collected in one fraction comprising the first 100 ml. of the eluting solvent (methanol:chloroform, 1:1, v/v; saturated with sodium chloride). All the steroid conjugates were then obtained in a second fraction by eluting off the column with 300 ml. methanol. This polar solvent removed both steroid monosulphates and disulphates from the column, and also served to clean the column before it was used again. After collection, the fraction containing the conjugates was hydrolysed, and the free steroids were extracted into organic solvents using the methods described in sections 2.3 (p. 37), 2.4 (p. 38) and 2.5 (p. 38).

The unconjugated plasma steroids present in the first 100 ml. of eluate could be separated from most of the fat by preparative TLC; they could then be examined by quantitative chromatographic methods. Since only small amounts of unconjugated $3\beta\text{-OH-}\Delta^5$ steroid were present in plasma, this procedure was not usually necessary.

2. The hydrolysis of steroid conjugates and the extraction of the liberated steroids

2.1. Enzymatic methods of hydrolysis: selection of a method

It was necessary to measure both sulphate and glucuronide conjugates of steroids, present in urine and in plasma samples. Enzymatic hydrolysis was therefore chosen, since acid hydrolysis results in the destruction of some steroids, and solvolysis only hydrolyses steroid sulphates.

The two commonly used sources of enzymes for hydrolysing steroid conjugates are the visceral hump of the limpet (*Patella vulgata*), and the crop fluid of the Roman snail (*Helix pomatia*). The enzymes in these materials are not usually purified for this purpose. Leon et al. (1960) showed that the limpet preparation was more efficient than the snail preparation in hydrolysing glucuronides, but that the reverse was true for the hydrolysis of sulphates.

Only the enzymes obtained from *Helix pomatia* can hydrolyse sulphate esters of 3α -OH- 5β steroids, but neither enzyme preparation will hydrolyse the sulphate esters of 3α -OH- 5α steroids. In adults, practically all the 3α -OH- 5α steroids are excreted as glucuronides, so the hydrolysis of sulphate esters of this group of steroids does not present a problem. In infants, however, steroid sulphates form the largest group of steroid metabolites present in urine, and it is likely therefore that sulphate esters of 3α -OH- 5α steroids will be present; if this is the case, they must be hydrolysed by solvolysis. During the present study, it was found that 17α sulphate esters and 20α sulphate esters of steroids were present in urine samples collected from infants. These esters are not hydrolysed by the enzymes present in the preparation from *Helix pomatia*, so they

TABLE 3.

Evaluation of the Procedure Developed for the
Hydrolysis of Steroid Conjugates

Preliminary Treatment	1	2	3	4	5
1. Adjust to pH 11.5	+	-	-	-	Pyridinium sulphate
2. Addition of Barium chloride	+	+	+	-	extraction and
3. Washing extract with sodium hydroxide	+	+	-	-	Helix pomatia hydrolysis
Steroid					
DHA (added)	100	72	64	63	117
Compound U ₁	100	78	62	53	85
21-OH-pregnenolone					
16-oxo-androstenediol	100	45	36	21	58
16 α -OH-DHA	100	96	79	44	90
Compound U ₂ (16 β -OH-DHA)	100	57	53	35	0
16 α -OH-pregnenolone	100	103	87	65	105
Androstenetriol	100	105	94	46	0

Legend:

These experiments were carried out to determine the best method for the hydrolysis and extraction of urinary steroids. Five parallel experiments were carried out in duplicate on the same sample of urine, using various modifications of the standard technique. The percentage recovery of steroids by the various modified techniques are based on a recovery of 100% for the standard method (1).

The results show that it is necessary to remove enzymic inhibitors by precipitation with barium chloride at pH 11.5. It is also necessary to wash the extracts with sodium hydroxide, since a very high background colour develops when the thin-layer chromatograms are sprayed with reagents if this is omitted. This background colour masks the colours given by the steroids. The recovery of polar steroids (e.g. androstenetriol) and of steroid disulphates (16 β -OH-DHA) is zero when the pyridinium sulphate method is used.

must also be hydrolysed by solvolysis.

A two stage hydrolytic procedure was finally adopted for the hydrolysis of the steroid conjugates present in urine samples collected from infants, and in plasma obtained from the umbilical blood vessels. The urine (or plasma extract) was first treated with the enzyme preparation from *Helix pomatia*, and the steroid sulphates, which resisted this treatment, were then hydrolysed by solvolysis.

2.2 The removal of enzyme inhibitors from urine

Certain inhibitors have to be removed from urine before the steroid conjugates can be hydrolysed by enzymes. The sulphatase activity of *Helix pomatia* preparations is strongly inhibited by phosphate ions (Henry and Thevenet, 1952). Similarly, Roy (1956) and Stitch et al. (1956) reported that the activity of the enzymes obtained from *Patella vulgata* was inhibited both by sulphate and by phosphate ions.

Jayle and Baulieu (1954) showed that the sulphatase activity of *Helix pomatia* was increased considerably when sulphate and phosphate ions were removed by precipitation with barium chloride at pH 11.5; this procedure also removed saccharo-1:4-lactone, a specific inhibitor of β -glucuronidase (Marsh, 1962). The importance of this preliminary treatment with barium chloride is shown by the results of the experiment described in Table 3. However, some artifacts were formed, for instance, 16 β -OH-DHA was completely converted into 16-oxo-androstenediol under these alkaline conditions. Since 16 β -OH-DHA is present in urine samples collected from infants mostly as its 3 β ,16 β -diconjugate, which does not isomerise, a considerable proportion of this steroid could still be determined. It appears from this experiment that much of the 16-oxo-

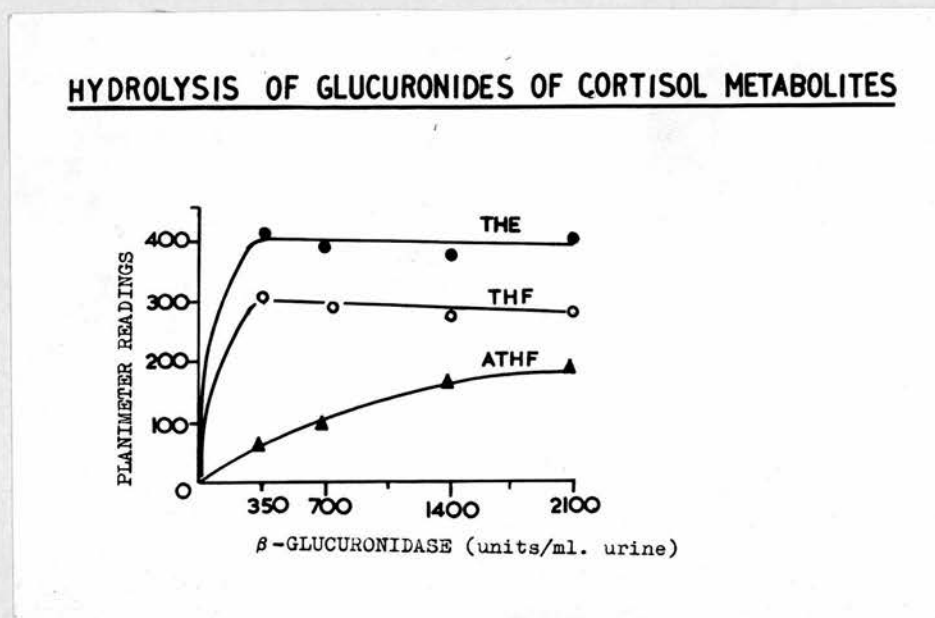
androstenediol assayed in urine arises as an artifact from 16β -OH-DHA.

Another method for avoiding the effects of enzyme inhibitors present in urine is to extract the steroid conjugates before subjecting them to enzymatic hydrolysis. This principle was adopted by McKenna and Norymberski (1960), who devised a simple method for the extraction of steroid sulphates from urine. By adding pyridinium sulphate to give a final concentration of 0.3 M, pyridinium sulphates of steroids are formed and these can then be extracted into chloroform. Menini (1966) modified this technique by increasing the concentration of pyridinium sulphate to 1 M, and found that the steroid glucuronides were also extracted. After extraction, the conjugates were hydrolysed either by enzymatic means or by solvolysis. Since steroid extracts of urine obtained using this method contain little material other than steroids, this method was attempted for the extraction of steroid conjugates present in urine samples collected from infants. The details of the method are as follows:-

- (a) 10 ml. of 3M pyridinium sulphate was added to 20 ml. of urine, and the mixture was then extracted with 30 ml. of chloroform.
- (b) 25 ml. of the chloroform layer was removed and dried.
- (c) The extract after drying contained the steroid conjugates. These conjugates were hydrolysed by enzymatic hydrolysis and solvolysis in the manner described for plasma extracts in sections 2.3, 2.4 and 2.5.

The results of this experiment are compared in Table 3 (experiment 5) with the results obtained by the method which involves direct

Fig. 9.



THE - Tetrahydrocortisone
THF - Tetrahydrocortisol
ATHF - Allotetrahydrocortisol

It was found that only a very small amount of *Helix pomatia* enzyme was required for the complete hydrolysis of the conjugates of urinary tetrahydrocortisone and tetrahydrocortisol. However, the hydrolysis of allotetrahydrocortisol glucuronide increased considerably with increasing concentration of enzyme.

enzymatic hydrolysis of urine samples. Satisfactory recoveries were obtained for DHA, 16 α -OH-DHA and 16 α -OH-pregnenolone, but the recoveries of polar compounds and of steroid disulphates were poor.

2.3 Conditions for hydrolysis

Urine:

After precipitation with barium chloride, the pH was adjusted to 4.5 - 5.0 with 10% (v/v) 5M acetate buffer (5M acetic acid: 5M sodium acetate, 2:3). The sulphatases of both the limpet, and the snail have maximum activity at pH 5, and the glucuronidases are most active at pH 4.5 (Henry and Thevenet, 1952; Jarrige and Henry, 1952). The steroid conjugates were hydrolysed at 37 $^{\circ}$ for 48 hours, using 700 units/ml. β -glucuronidase and 1000 units/ml. sulphatase. These same amounts of the enzymes were added again after 24 hours, to replace inactivated enzyme. The amount of *Helix pomatia* glucuronidase used is equivalent to the amount used by Birchall and co-workers (1963), but is greater than that advocated by Hobkirk and Cohen (1960) for the maximum release of 17-oxosteroids and corticosteroids from their conjugates. The amounts used were also satisfactory for the hydrolysis of the glucuronides of tetrahydrocortisone and tetrahydrocortisol, but were not sufficient to hydrolyse the conjugate of allotetrahydrocortisol completely; the hydrolysis of conjugates of allotetrahydrocortisol increased with increasing concentration of enzyme (Fig. 9), a finding also reported by Nielsen (1966). It is possible that the glucuronide conjugate of allotetrahydrocortisol may require a specific glucuronidase; if so, there would appear to be only a small amount of this enzyme in the extract obtained from *Helix pomatia*.

Plasma:

The extract of steroid conjugates present in plasma was dissolved in 5 ml. 0.5M acetate buffer (0.5 M acetic acid:0.5 M sodium acetate, 2:3, pH 4.5). The steroid conjugates were then hydrolysed by the enzymes present in the *Helix pomatia* preparation (700 units β -glucuronidase, 1000 units sulphatase) for 48 hours at 37°. A second amount of the enzyme preparation, equal to the first addition, was added after 24 hours.

2.4 Extraction of the steroids after their release from conjugation

The steroids were extracted twice into two volumes of ether;ethyl acetate (2:1, v/v), the solvent mixture used by Birchall and co-workers (1963). Good recoveries of polar and non-polar steroids were obtained. The extracts were washed repeatedly with N sodium hydroxide (one-tenth the volume of the organic phase), to remove phenolic compounds and other impurities. Washing with sodium hydroxide was judged to be sufficient when no more coloured material was extracted, and a final wash with water (one-tenth volume) was then performed. After these washings, the organic extracts were evaporated to dryness on a rotary evaporator at 45-50°. The solid residue was then dissolved in a small volume of chloroform and transferred to a test-tube, after which the solvent was again removed by evaporation under a stream of nitrogen.

2.5 Solvolysis of the remaining steroid sulphates

Some of the steroid sulphates present in urine collected from newborn infants, and in plasma obtained from the umbilical cord, are not hydrolysed by the enzymes obtained from *Helix pomatia*; instead they have to be hydrolysed by solvolysis. The method described by Burstein and

Liebermann (1958) was adopted; this uses organic solvents such as ethyl acetate. The details of the technique are as follows:-

The urine (after enzyme hydrolysis and extraction) was saturated with ammonium sulphate, and the pH adjusted to pH 1 by addition of sulphuric acid (50%, v/v). Following these steps, the urine was extracted with an equal volume of ethyl acetate, and the ethyl acetate extract was incubated at 38° for 24 hours. This extract was then washed repeatedly with N sodium hydroxide (one-tenth the volume of the ethyl acetate) until the washings were alkaline, and finally the extract was washed with water.

This method gave poor recoveries of those steroid sulphates which were also susceptible to hydrolysis by enzymatic methods. It is likely, therefore, that the results obtained for the concentration in urine and in plasma of steroid conjugates resistant to hydrolysis by sulphatase are unduly low.

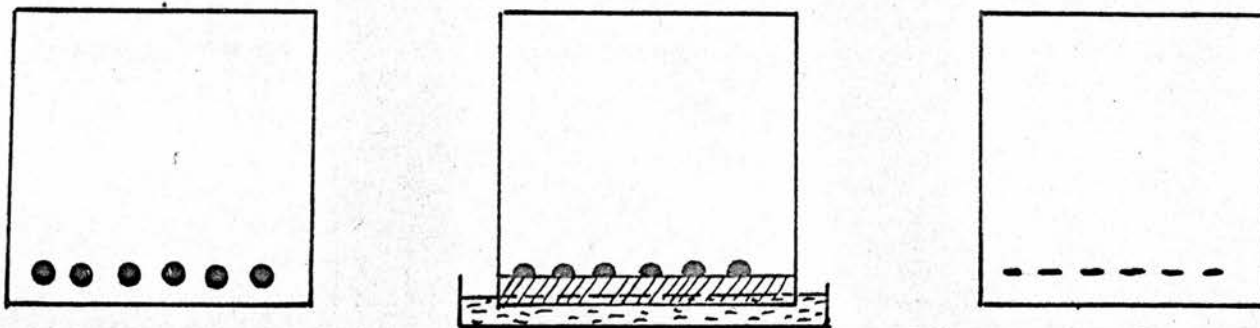
The steroid extracts obtained after hydrolysis by enzymes and after solvolysis can either be combined or be kept separate for subsequent chromatographic separations and quantitative examination.

3. Thin-layer chromatography

All chromatographic separations for the quantitative method have been carried out using 0.25 mm. thickness of silica gel HF 254 spread on 20 cm. by 20 cm. plates. No activation of plates was necessary prior to use, but they were allowed to dry for 24 hours after preparation. The plates were developed at room temperature in tanks lined with a double layer of chromatography paper. After the addition of solvent, tanks were allowed to equilibrate for one hour before use. Fresh solvent

Fig. 10. "Running Up" of Thin-Layer Chromatograms

After the application of the spots, the plate is dipped into ethylacetate:methanol (3:1, v/v) contained in a trough. When the solvent has reached a level just above the spots, the plate is removed and dried. The dipping and drying are repeated twice more; in this way the steroids are concentrated into narrow bands, which helps in the subsequent chromatographic separations.



systems were prepared daily for chromatography.

3.1 Application of material to thin-layer plates

Extracts and standards were applied in ethanolic solution, using a series of micropipettes delivering between 5 μ l. and 100 μ l. The spots were dried in a stream of cold air delivered from a domestic hair-dryer. Care was taken to keep the spots to the same size, since the final response given by the densitometer depended on the size of the spot as well as upon its optical density. It was not difficult to keep the spots of equal size, but when spots were produced that were too large they were reduced in size by "running in" with ethanol before development. To "run in" a large spot, drops of ethanol from a micropipette are placed around the spot; ethanol carried the extract more into the centre of the area. Using a polar solvent (ethyl acetate:methanol, 3:1) the round spots produced were "run up" to form narrow bands at a new origin. This concentrating technique considerably enhances separation and is illustrated in diagrammatic form in Fig. 10.

Normally, up to six extracts were applied in duplicate to each plate, spaced between four spots containing standard steroids in varying amounts. For most of the determinations carried out during this study, several compounds were present in each of the standard spots. Ideally, each run of standards should contain as many as possible of the steroids to be assayed but, in order to conserve valuable reference compounds, an alternative method was occasionally used. For three of the positions allocated to standards on the chromatograms, a single steroid was applied, and the remaining steroids were present only in the fourth spot. Using this method, the linearity of the standard curve was established for one

TABLE 4.

Major Metabolites Measured Using the Quantitative Method
(Compounds listed in order of increasing polarity)

<u>3β-OH-Δ^5 steroids</u>		<u>17-oxosteroids</u>		<u>Reducing compounds</u>
<u>Urine from infants</u>	<u>Plasma from umbilical blood vessels</u>	<u>Urine from adults</u>	<u>Urine from adults</u>	
DHA	Pregnenolone	DHA	Androsterone	3 α ,17 α ,21-trihydroxy-5 β -pregnan-20-one (Tetrahydro substance S)
Compound U ₁	DHA	Androstenediol	DHA	Cortisol
21-OH-pregnenolone	17 α -OH-pregnenolone	16 α -OH-DHA	Aetiocholanolone	Tetrahydrocortisone
Androstenediol	21-OH-pregnenolone	Pregnenetriol	11-oxo-androsterone	Allotetrahydrocortisol
16-oxo-androstenediol	Androstenediol*	Pregnanetriol**	11-oxo-aetiocholanolone	Tetrahydrocortisol
16 α -OH-DHA	16-oxo-androstenediol	Androstenediol	11-OH-androsterone	
Compound U ₂ (16 β -OH-DHA)	16 α -OH-DHA		11-OH-aetiocholanolone	
16 α -OH-pregnenolone	Compound U ₂ (16 β -OH-DHA)			
Androstetriol	16 α -OH-pregnenolone			
[Pregnanetriol]**	Androstetriol			

* This is extracted only after solvolysis so it can be determined separately.

** Although not a 3 β -OH- Δ^5 steroid this compound can be determined using the antimony trichloride reaction.

[] Only quantitatively important in adrenal disorders.

compound, and the standard curves for the others were drawn in using relative values for the intensity of the colours which these other compounds developed, as revealed by their behaviour following the separation of the mixed standards.

3.2 Separation and detection of steroids by thin-layer chromatography

This section describes reactions used for detecting the 17-oxosteroids, the cortisol metabolites and the $3\beta\text{-OH-}\Delta^5$ steroids, and the solvent systems that were developed for their separation. The compounds listed in Table 4 include many of the major metabolites present in the biological fluids studied, and solvent systems were chosen which would separate as many compounds as possible on a single thin-layer plate. The individual systems used are listed in Table 5, but all separations were achieved using more than one development, and often more than one solvent system. Multiple running with solvent systems giving low Rf values gave better separations than the more normally used procedure, which attempts to produce a separation in a single stage. At each stage of chromatographic development, there is a concentrating effect since the lower part of the band moves while the upper edge is stationary (Dohmann, 1965).

As the method relies for quantitative assessment upon the direct densitometric scanning of stained spots, investigations of specificity were most important. Each coloured area which develops after spraying a plate should contain a single compound responsible for the colour reaction. For this reason, after describing the systems used for separation, there is a paragraph on the evaluation of specificity.

TABLE 5

Thin Layer Chromatography Systems

<u>Solvent</u>	<u>Relative Proportions</u>
<u>Adsorbent: Silica Gel</u>	
A Benzene : ethanol	99 : 1
B Benzene : ethanol	96 : 4
C Benzene : ethanol	95 : 5
D Benzene : ethanol	80 : 20
E CHCl ₃ : ethanol	99 : 1
F CHCl ₃ : ethanol	95 : 5
G CHCl ₃ : ethanol	90 : 10
H Cyclohexane : ethyl acetate	50 : 50
I Cyclohexane : ethyl acetate : ethanol	45 : 45 : 10
J Ethyl acetate : water saturated n-hexane : glacial acetic acid : ethanol	72 : 13.5 : 10 : 4.5
K Benzene : acetone : ethanol : water	30 : 30 : 30 : 10
<u>Adsorbent: Alumina</u>	
L Dichloromethane : ether	80 : 20

Colours produced by the antimony trichloride reaction with various steroids on silica-gel thin-layer chromatograms

3 β -Hydroxy- Δ^5 steroids

Androstenes

3 β -Hydroxyandrost-5:16-dien	red
3 β -Hydroxyandrost-5-en-17-one	red
3 β -Hydroxyandrost-5,15-dien-17-one	red
3 β -Hydroxyandrost-5-en-11:17-dione	red
3 β -16 α -Dihydroxyandrost-5-en-17-one	red
3 β -16 β -Dihydroxyandrost-5-en-17-one	red
3 β -17 β -Dihydroxyandrost-5-en-17-one	red
3 β -17 α -Dihydroxyandrost-5-en-16-one	red
3 β -15 α ,17 β -Trihydroxyandrost-5-en	red
3 β -16 α ,17 α -Trihydroxyandrost-5-en	red
3 β -16 α ,17 β -Trihydroxyandrost-5-en	red
3 β -17 α ,17 β -Trihydroxyandrost-5-en	red
3 β -17 α -Dihydroxyandrost-5-en	pale blue-grey
3 β -17 β -Dihydroxyandrost-5-en	pale blue-grey
3 β -17 α -Dihydroxyandrost-5-en-17-one	dark blue-grey
3 β -17 β -Dihydroxyandrost-5-en-17-one	dark blue-grey
3 β -17 α ,17 β -Trihydroxyandrost-5-en	dark blue-grey
3 β -15 α ,17 β -Trihydroxyandrost-5-en	dark blue-grey
3 β -7 α -Dihydroxyandrost-5-en-17-one	turquoise*
3 β -7 β -Dihydroxyandrost-5-en-17-one	turquoise*
3 β -Hydroxyandrost-5-en-7,17-dione	no colour

Other steroids

<u>3-Oxo-Δ^4 steroids</u>		
Androst-4-en-3,17-dione	no colour
Androst-4-en-3,11,17-trione	no colour
17 β -Hydroxyandrost-4-en-3-one	no colour
17 α -Hydroxyandrost-4-en-3-one	blue
Pregn-4-en-3,20-dione	no colour
16 α -Hydroxypregn-4-en-3,20-dione	no colour
17 α -Hydroxypregn-4-en-3,20-dione	no colour
21-Hydroxypregn-4-en-3,20-dione	no colour
16 α ,21-Dihydroxypregn-4-en-3,20-dione	no colour
21-Hydroxypregn-4-en-3,11,20-trione	no colour
11 β ,21-Dihydroxypregn-4-en-3,20-dione	no colour
17 α ,21-Dihydroxypregn-4-en-3,20-dione	no colour
17 α ,21-Dihydroxypregn-4-en-3,11,20-trione	no colour
11 β ,17 α ,21-Trihydroxypregn-4-en-3,20-dione	no colour
6 β ,11 β ,17 α ,21-Tetrahydroxypregn-4-en-3,20-dione	no colour
Androst-1,4,dien-3,17-dione	no colour
17 β -Hydroxy-1,4,dien-3-one	yellow

Miscellaneous

Androst-5-en-3,11,17-trione	no colour
3 β -Hydroxycholest-4-en	red*
3 β -Hydroxycholest-1-en	red*
Cholest-5-en-3-one	no colour

Pregnenes

3 β -Hydroxypregn-5-en-20-one	red
3 β -Hydroxypregn-5,16-dien-20-one	red
3 β ,16 α -Dihydroxypregn-5-en-20-one	red
3 β ,17 α -Dihydroxypregn-5-en-20-one	red
3 β ,20 β -Dihydroxypregn-5-en	red
3 β ,21-Dihydroxypregn-5-en-20-one	red
3 β ,17 α -Dihydroxypregn-5-en-11,20-dione	red
3 β ,17 α ,20 β -Trihydroxypregn-5-en-20-one	red
3 β ,20 β ,21-Trihydroxypregn-5-en	red
3 β ,20 α -Dihydroxypregn-5-en	blue-grey
3 β ,17 α ,20 α -Trihydroxypregn-5-en	blue-grey
3 β ,17 α ,20 β -Trihydroxypregn-5-en	blue-grey
3 β ,17 α ,20 α ,21-Tetrahydroxypregn-5-en	blue-grey

Cholestenes

Cholesterol	violet
7 α -Hydroxy cholesterol	turquoise*
7 β -Hydroxy cholesterol	turquoise*

Saturated steroids

3 α ,17 α ,21-Trihydroxy-5 β -pregnan-20-one	no colour
3 α ,17 α ,21-Trihydroxy-5 β -pregnan-11-20-dione	no colour
3 α ,11 β ,21-Trihydroxy-5 α -pregnan-11-20-dione	no colour
3 α ,11 β ,17 α ,21-Tetrahydroxy-5 β -pregnan-20-one	no colour
3 α ,11 β ,17 α ,21-Tetrahydroxy-5 α -pregnan-20-one	no colour
3 α -Hydroxy-5 β -androst-17-one	no colour
3 α -Hydroxy-5 β -androst-11,17-dione	no colour
3 α ,11 β -Dihydroxy-5 β -androst-17-one	no colour
3 α -Hydroxy-5 α -androst-17-one	no colour
3 α -Hydroxy-5 α -androst-11,17-dione	no colour
3 α ,11 β -Dihydroxy-5 α -androst-17-one	no colour
3 α -Hydroxy-5 α -androst-11,17-dione	yellow
3 α ,11 β -Dihydroxy-5 α -androst-11,17-dione	yellow
3 α ,11 β -Dihydroxy-5 β -androst-17-one	yellow
3 α ,11 β -Dihydroxy-5 β -androst-11,17-dione	yellow
3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-11-one	yellow
3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-20-one	orange

Chromatograms were developed with 10 μ g. of steroid in each case.

* Immediate colour produced without heating.

(i) Separation and detection of 3β -OH- Δ^5 steroids

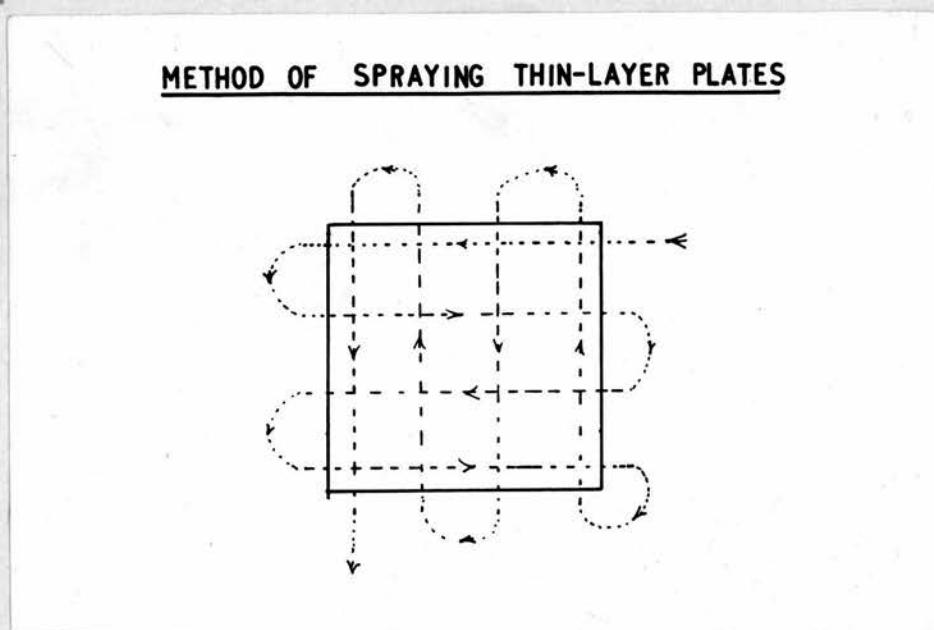
Detecting reagent

Two spray reagents have been used recently for the determination of 3β -OH- Δ^5 steroids on thin-layer plates. Eberlein developed a reagent for the determination of steroids in plasma obtained from the umbilical blood vessels (Eberlein, 1965a, 1965b). The composition of this reagent was 36 ml. acetic acid containing 100 mg. picric acid, and 6 ml. 70% (v/v) perchloric acid. 3β -OH- Δ^5 steroids gave red spots with this reagent but no colour developed with 3-oxo- Δ^4 steroids or with saturated compounds, even at a concentration of 100-250 $\mu\text{g}/\text{cm}^2$. The other reagent which has been used is a saturated solution of antimony trichloride in chloroform. Although this latter reagent is relatively specific for 3β -OH- Δ^5 steroids when the chromatogram is viewed in daylight (Neher, 1964; Lisboa, 1965), all types of steroid give a fluorescent reaction when the sprayed chromatogram is viewed under ultra-violet light of wavelength 365 m μ (Neher, 1964). The reagent has also been used in a thin-layer densitometric method for measuring cholesteryl esters (Zöllner, 1962).

The antimony trichloride has the advantage of being a "dry reaction". The chloroform evaporates soon after it is sprayed on to the plate and less background colour develops and less diffusion of spots occurs than with Eberlein's reagent.

An extensive investigation was carried out to determine the specificity of the antimony trichloride reagent since it was required in the method being developed to measure 3β -OH- Δ^5 steroids in the presence of 3α -hydroxy steroids and 3-oxo- Δ^4 steroids. The colours given by 10 μg . of a variety of steroids are shown in Table 6. All the 3β -OH- Δ^5 steroids

Fig. 11.



In order to ensure that the thin-layer chromatography plates are evenly covered with detecting reagent, a definite "pattern" of spraying is followed. Normally the plates are sprayed for a total of 30 seconds.

determined gave a strong colour except when a 7-oxo group was present. Most gave a red to blue colour after heating for five min. at 100°, but those containing a 7-hydroxy group gave an immediate turquoise reaction at room temperature. The only other compounds which gave a similar colour to the $3\beta\text{-OH-}\Delta^5$ steroids were $3\beta\text{-OH-}\Delta^4$ steroids but these could be distinguished since they gave an immediate colour without heating. Pregnanediol and pregnanetriol gave yellow spots, a reaction which can be used for the specific assay of these compounds.

Particular care must be taken to ensure that plates are evenly covered with the reagent, as uneven spraying leads to great inaccuracies when the method is used quantitatively. The plates were therefore sprayed in a definite pattern (Fig. 11) for 30 seconds.

The separation of the $3\beta\text{-OH-}\Delta^5$ steroids present in urine obtained from newborn infants

The Rf values of many reference $3\beta\text{-OH-}\Delta^5$ steroids for single runs in the most important thin-layer systems are given in Table 20 (Appendix). Usually the separation of steroids present in urine and plasma required more than one development in a thin-layer system. If a thin-layer plate has been developed three times in system C, for example, then the system used is often reported as system C (x 3).

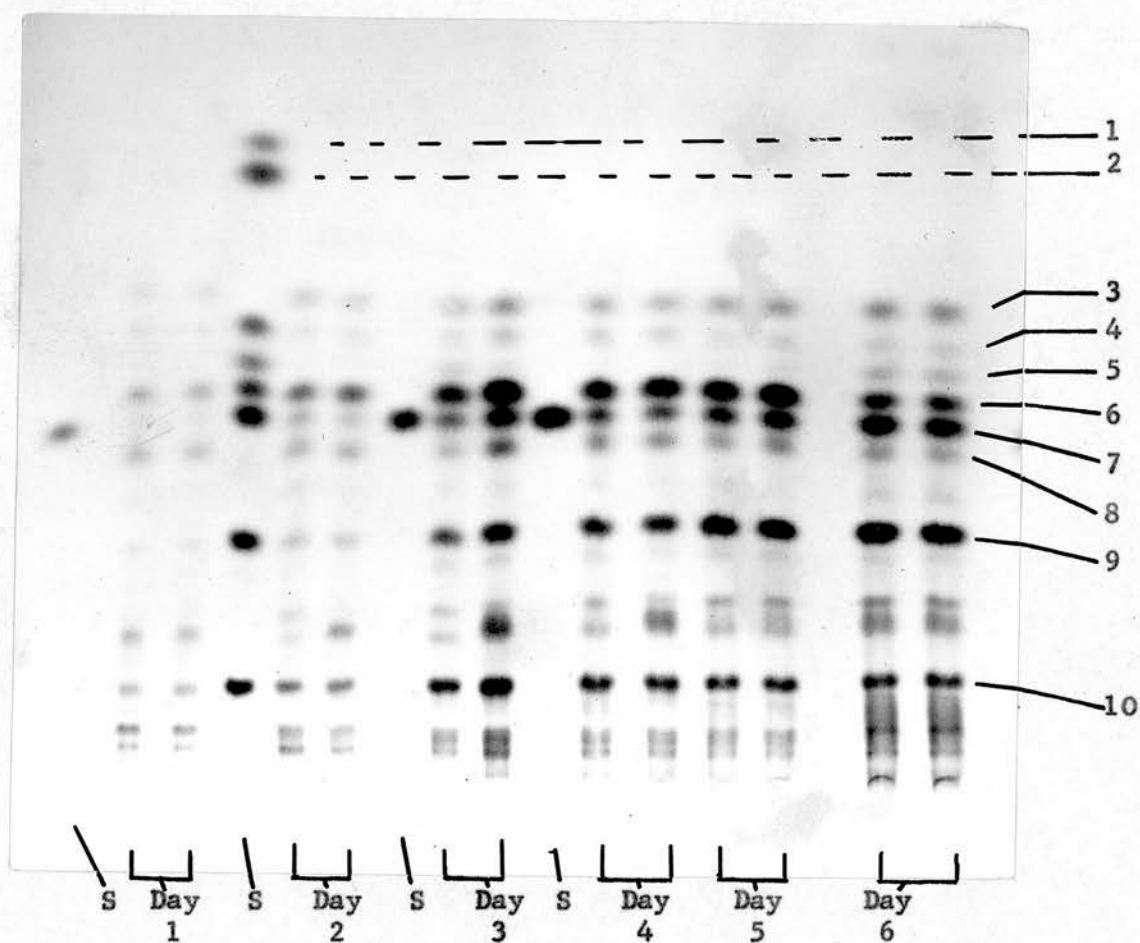
The separation of the quantitatively important $3\beta\text{-OH-}\Delta^5$ steroids present in urine collected from infants (Table 4) can be achieved by running a plate three times in system E (to separate compound U_1 from 21-OH-pregnenolone), and three times in system B (to separate 16-oxo-androstenediol from 16 α -OH-DHA). This separation is illustrated in Fig. 12. It was occasionally necessary to vary slightly the percentage of ethanol in each of these systems when using different batches of silica

Fig. 12. The Separation of Medium Polarity 3β -OH- Δ^5 Steroids Present in Urine Collected from Infants

Solvent system - E (x3), B (x3).

Detecting reagent - Antimony trichloride

This figure illustrates the assay of steroids in the urine of a single infant during the first six days of life. This infant showed a relatively low excretion of the major steroids on the first two days of life. The absence of DHA from the urine of newborn infants is also apparent from this figure.



S - Standard steroids

Steroids illustrated: Pregnenolone (1); DHA (2); Unknown 1 (3);
 21-OH-pregnenolone (4); androstenediol (5); 16-oxo-androstenediol (6);
 16 α -OH-DHA (7); Unknown 2 (16 β -OH-DHA) (8); 16 α -OH-pregnenolone (9);
 androstenetriol (10).

gel. The 16-oxygenated compounds were often present in much greater concentration in urine than 21-OH-pregnenolone and compound U₁, and it was not always possible to determine all these steroids on the same chromatogram. In these cases it was necessary to repeat the chromatographic separation using a larger amount of extract.

Towards the end of this study, two plates were almost invariably used for determining the urinary 3 β -OH- Δ^5 steroids that had medium polarity. One plate was used for the assay of compound U₁ and 21-OH-pregnenolone, and was developed twice in system F. The other plate was used for separating the 16-oxygenated 3 β -OH- Δ^5 steroids, and was run three times in system C.

Since androstenetriol is much more polar than the other compounds to be measured, when adequate distribution of the main group was obtained it moved only a short distance from the origin and was often poorly separated from neighbouring substances. For this reason another chromatogram was normally prepared and run twice in the more polar system G (Fig. 13). In patients with congenital adrenal hyperplasia, pregnanetriol is of diagnostic importance and can also be measured using this system (Fig. 32).

17 α Androstenediol and pregnanetriol are both present in the solvolysis extract; these compounds are separated by almost all systems, and two developments in system H were found to give a satisfactory separation.

Specificity and identification:

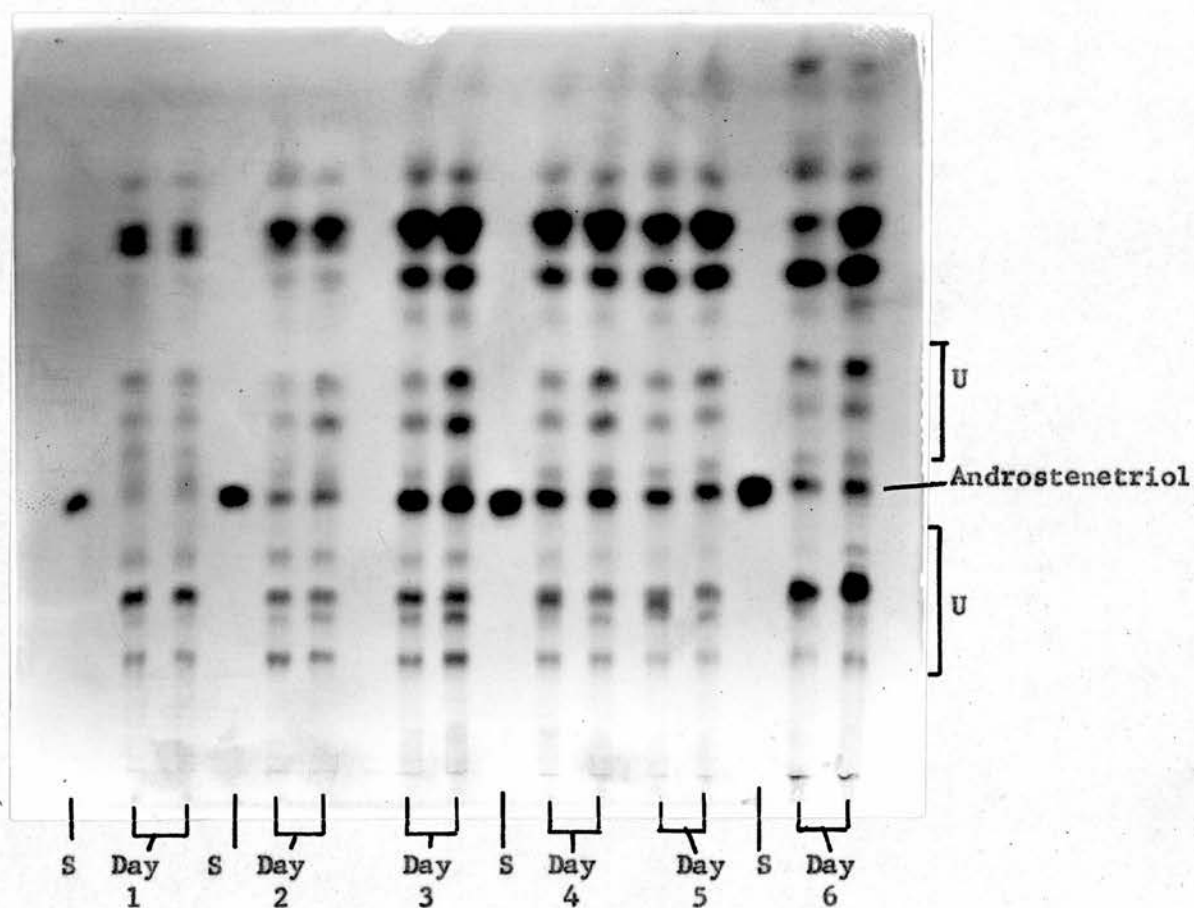
Extracts of urine were run first in one direction with the systems normally used for quantitation, and a second development was then carried

Fig. 13. The Separation of Polar $3\beta\text{-OH-}\Delta^5$ Steroids Present in Urine
Collected from Infants

Solvent system - G (x2)

Detecting reagent - Antimony trichloride

This figure illustrates the assay of androstenetriol in the urine of a single infant during the first six days of life. The presence of several $3\beta\text{-OH-}\Delta^5$ steroids of unknown structure in the urine of infants is also evident from this figure.



U - Unknown compounds
S - Standard steroids

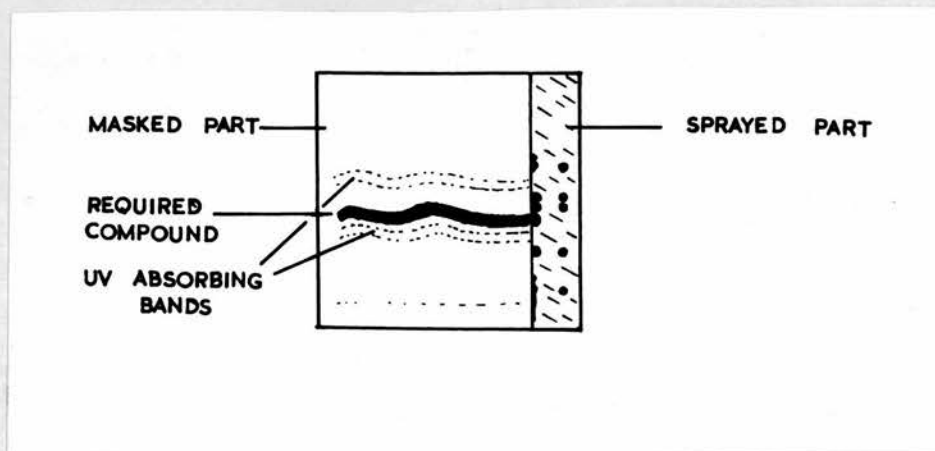
out at right angles to the first, using solvent systems C, F, H and J. In no case could an interfering chromogen be separated from a spot normally assayed. In addition, another form of two-dimensional chromatography was carried out, but in this case the steroids were acetylated in situ after completing the run in the first direction. Acetylation was achieved by spraying the edge of the plate containing the separated extract with acetic anhydride:pyridine (1:1 v/v). After standing for two hours, the plates were "run up" at right angles to the line of the first development, thereby forming a new origin for a second chromatographic run, which was then carried out using system A. For the compounds normally assayed, no other spots staining with antimony trichloride could be separated.

Using an extract prepared from urine collected from several infants, 50 to 100 μg of DHA*, androstenediol, 16-oxo-androstenediol, 16 α -OH-DHA, 16 α -OH-pregnenolone and androstenetriol were isolated by the following method:-

The extract was streaked along the origin of a thin-layer plate, and this was developed in the chromatographic system used for quantitative assay of steroids present in urine samples collected from infants. The plate was "masked" using a clean glass plate, except for one edge which was sprayed with the antimony trichloride reagent. The colours were allowed to develop at room temperature, to protect the steroids on the rest of the plate from possible destruction by heat. The reaction was much slower at

* DHA was only identified in urine samples collected from infants treated with human chorionic gonadotrophin during the first few days of life.

Fig. 14. Preparative Thin-Layer Chromatography



The steroid extract is applied as a band across the chromatogram. After development most of the layer is masked with a glass plate. The edge of the separated band is left unmasked and this part is sprayed with detecting reagent in order to display the position of the required compounds. The plate is then viewed under ultra-violet light ($254\text{ m}\mu$) as it can usually be seen from the position of the absorbing bands whether the band has run regularly along its length. Allowing for any irregularity, the required zone is scraped off the plate and is eluted with solvent.

room temperature and it normally required about 15 minutes for the colour to become reasonably intense.

Although the positions of each compound on the preparative plate could be determined by spraying the edge of the band with antimony trichloride reagent, it could not be assumed that the compounds had each run evenly right across the chromatogram. Bands of material do not usually run regularly, and it was important to determine the degree of irregularity before the band containing the desired steroid was removed from the plate. Detection was achieved by viewing the chromatogram under ultra-violet light of wavelength 254 m μ ., when compounds capable of absorbing ultra-violet light and present in the urinary extract gave a clear indication of the extent of irregularity in the running of the chromatogram. The bands could then be scraped off the plate by following lines parallel to the ultra-violet absorbing bands. Figure 14 illustrates this technique in diagrammatic form.

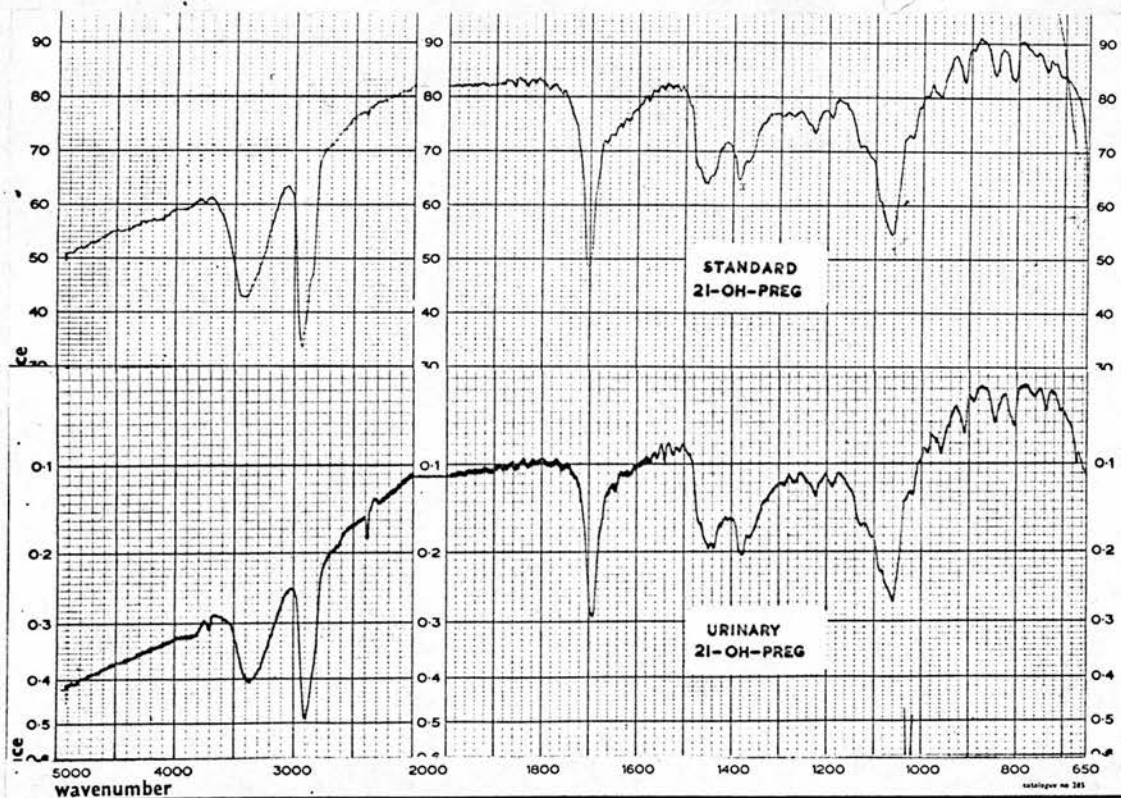
For eluting the steroids, silica gel was placed in 15 ml. centrifuge tubes, a drop of water was added and the steroids were eluted with ether. The "whirlimixer" was used for agitation; this instrument mixes by producing a vortex in the tubes. The tubes were centrifuged, the solvent decanted, and the elution with ether was repeated twice more. The compounds obtained in this way were identified by combined gas chromatography-mass spectrometry. The details of the methods used in this identification are given in Part 3 (p. 85), in the section which describes the identification of urinary steroids.

Gas chromatographic-mass spectrometric analysis of the steroid trimethylsilyl ethers indicated that the major peaks had the same

retention times as the reference compounds, and gave identical mass-spectra. There were no significant peaks on the GLC record apart from the compounds which had been identified. Since the compounds analysed were originally separated by preparative methods using the same thin layer chromatographic systems as had been developed for quantitative determinations of the steroids, this in itself provided a good test of specificity as well as a means of identification. Figure 37, for example, illustrates the gas chromatographic record of 16 α -OH-DHA prepared from urinary extracts. The only other large peak present corresponds to 16-oxo-androstenediol, a contaminant to be expected because of the nature of the thin-layer system used for the separation (Fig. 12).

21-OH-Pregnenolone was identified by means of infra-red spectroscopy in urine samples collected from infants. For compounds to be analysed by infra-red spectroscopy, they must be obtained in a completely pure state; for this reason 21-OH-pregnenolone was obtained from the steroid disulphate fraction after separating the urinary conjugates on Sephadex LH 20. By this means, most of the other steroids were removed before hydrolysis, and it was then easier to remove the remaining unwanted steroids by TLC. Systems C (x3), F (x2) and H (x2) were used for the preparative separation. The compound was eluted with ether from the first two plates in the same manner as for other steroids but, after the final preparative chromatography, Analar acetone (2 x 5 ml.) was used for eluting 21-OH-pregnenolone from the silica gel. This solvent proved suitable for infra-red spectroscopy without distillation. The acetone extract was dried and 21-OH-pregnenolone was transferred to an

Fig. 15. Infra-Red Spectra of Authentic 21-OH-Pregnenolone and of the Compound Prepared from Urine Excreted by Infants



21-OH-PREG - 21-OH-pregnenolone

internal-reflectance plate (p. 92) with about 200 μ l. of acetone. The infra-red spectrum produced was essentially identical with the spectrum of a standard specimen of 21-OH-pregnenolone (Fig. 15).

The separation of the 3β -OH- Δ^5 steroids present in plasma samples obtained from the umbilical blood vessels

Steroids present in plasma samples collected from the umbilical blood vessels cannot be separated by the same thin-layer system as the one used for the separation of steroids in urine samples collected from infants. This is because 17α -OH-pregnenolone and pregnenediol are present in plasma obtained from the umbilical artery and vein, but were not detected in the urine samples. 17α -OH-Pregnenolone is only separated from 21-OH-pregnenolone in system H, and the most satisfactory separation of the steroids present in plasma samples has been obtained by developing the chromatograms once in system C, followed by two runs in system H (Fig. 16).

Androstenediol and pregnenetriol, present in the extract obtained after solvolysis, were separated by two runs in system H.

Specificity and identification

The extracted compounds gave the same colour reactions and had the same Rf values as standard steroids in several different solvent systems. After two-dimensional chromatography (first direction system G, second direction, systems C, E, H and finally J), it was not possible to detect other 3β -OH- Δ^5 steroids - these would have interfered with the quantitative determinations.

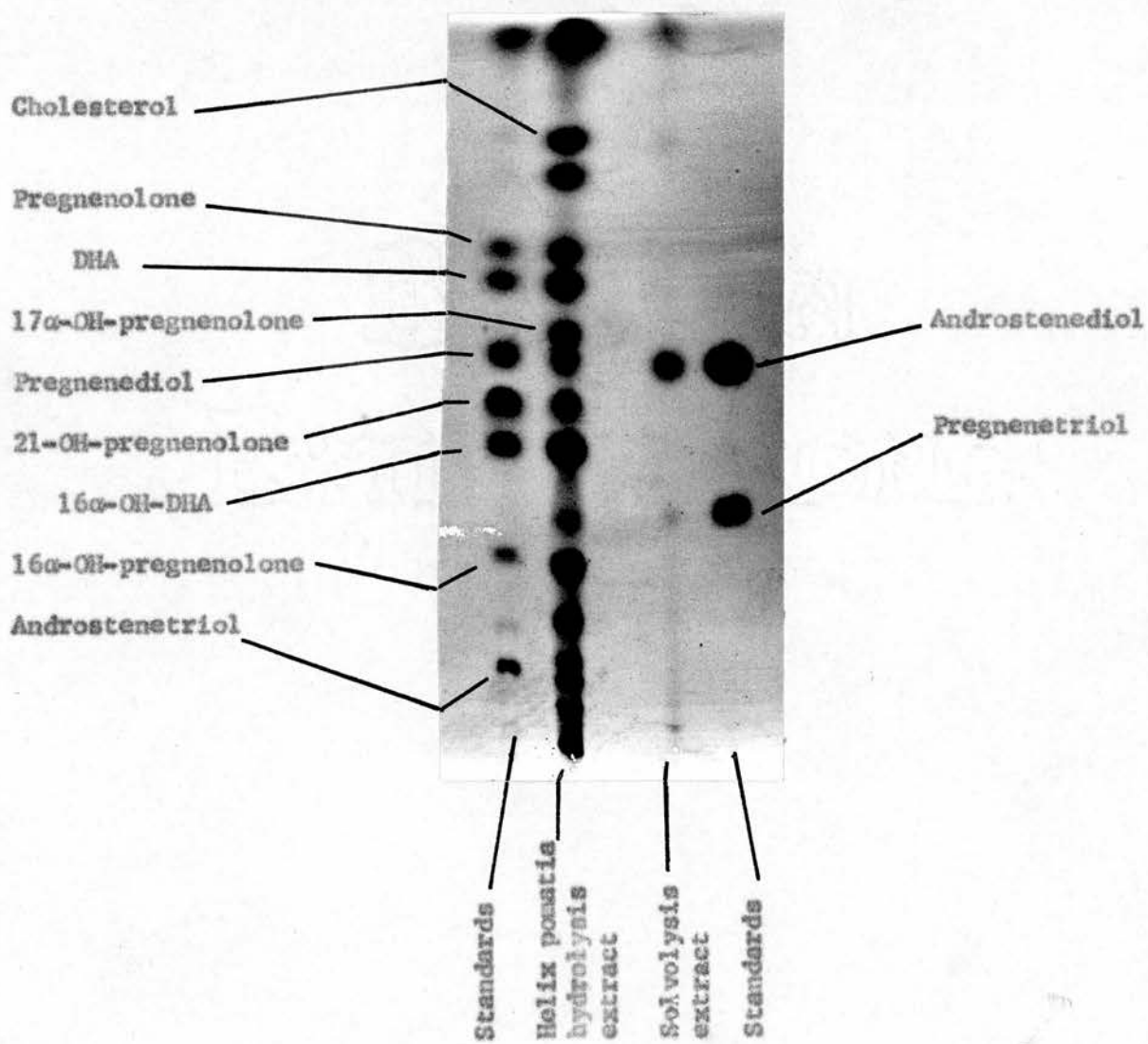
The separation of 3β -OH- Δ^5 steroids present in urine samples collected from adults

Urine samples collected from adults contain less 3β -OH- Δ^5 steroid

Fig. 16. The Separation of 3 β -OH- Δ^5 Steroids in Plasma Obtained from the Umbilical Vein

Solvent system - C, H (x2)

Detecting reagent - Antimony trichloride



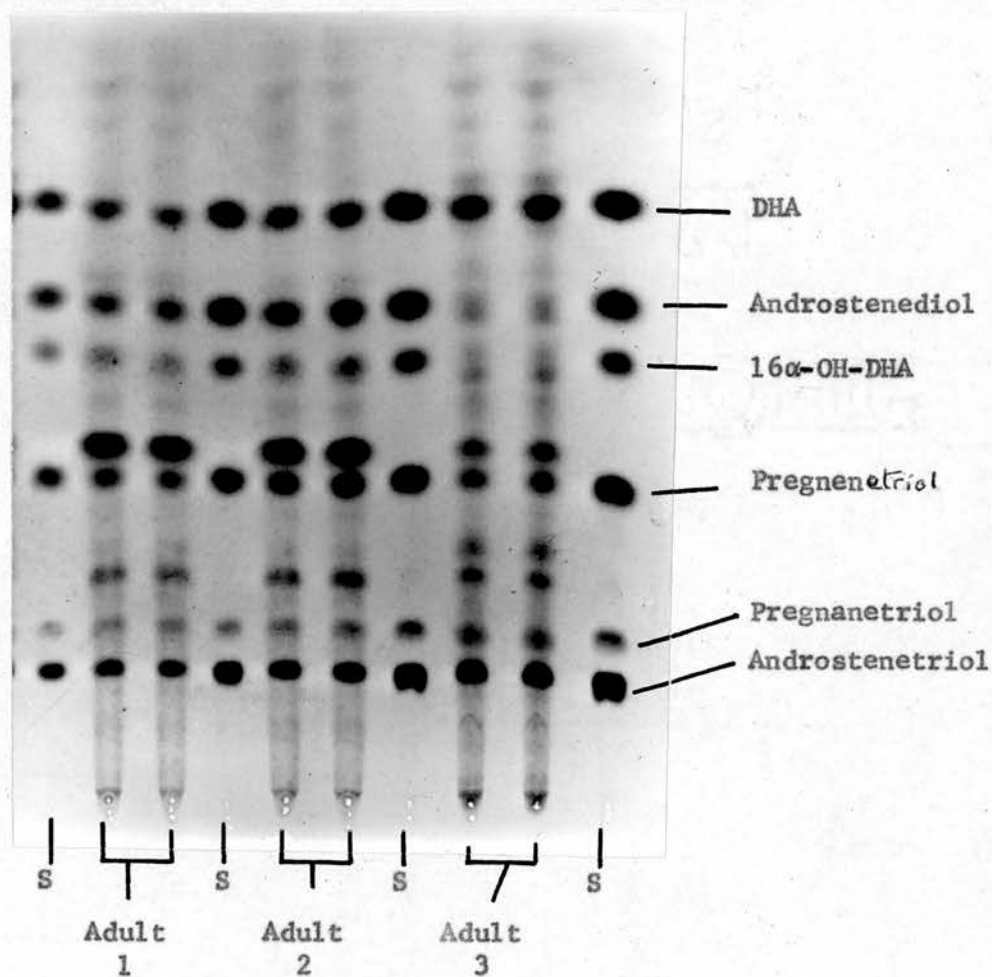
* Standard 17 α -OH-pregnenolone omitted in error.

Fig. 17. The Assay of $3\beta\text{-OH-}\Delta^5$ Steroids Present in Urine from Adults

Solvent system - H, F (x2)

Detecting reagent - Antimony trichloride

This figure illustrates the separation of urinary $3\beta\text{-OH-}\Delta^5$ steroids. Samples collected from three adults.



S = standard steroids

than urine samples collected from newborn infants. Pregnenetriol is quantitatively important, and is present in the extract containing steroids liberated by the enzymes present in the *Helix pomatia* preparation. In urine samples obtained from infants, the conjugate of pregnenetriol is only hydrolysed by solvolysis. For adults, therefore, the problems of separation are different but less complex, and a procedure utilising a single chromatographic separation has been chosen.

The plates are run once in system H, then twice in system F, and it can be seen from Fig. 17 that androstenediol and pregnanetriol have moved sufficiently far from the origin to be separated and measured. 16α -OH-DHA and 16-oxo-androstenediol are not separated, but there is in fact little (if any) 16-oxo-androstenediol in urine samples obtained from adults.

Specificity and identification

The specificity of the separation of 3β -OH- Δ^5 steroids was determined for adults in the same way as with steroids obtained from infants, using two-dimensional chromatography with and without acetylation. The steroids were identified by their ability to develop specific colours with antimony trichloride, and by comparing their Rf values with those of standards in the thin-layer systems C, D, G, H, I and J.

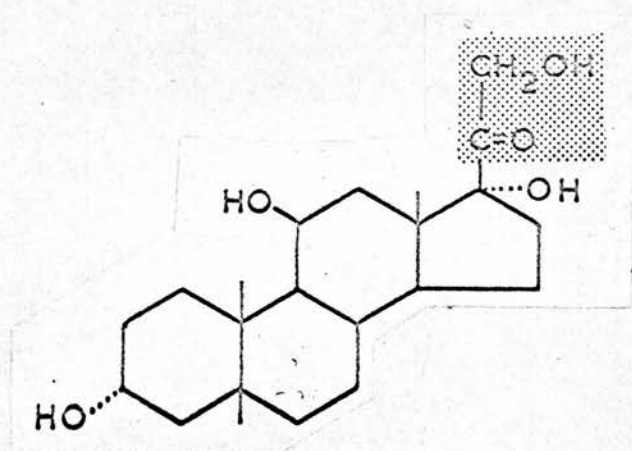
(ii) Separation and detection of reducing compounds

Detecting reagent

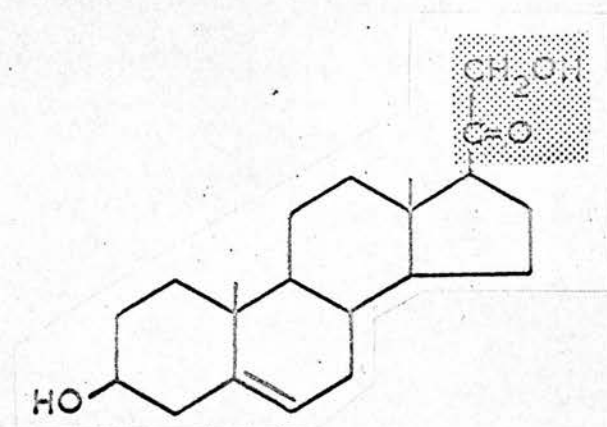
The blue tetrazolium reaction was used for detecting all compounds with an α -ketol group, i.e. steroids containing a hydroxyl and a ketone group on adjacent carbon atoms (the hydroxyl group is in the " α " position with respect to the carbonyl group). The major compounds of this type in

Fig. 18. The Blue Tetrazolium Reaction.

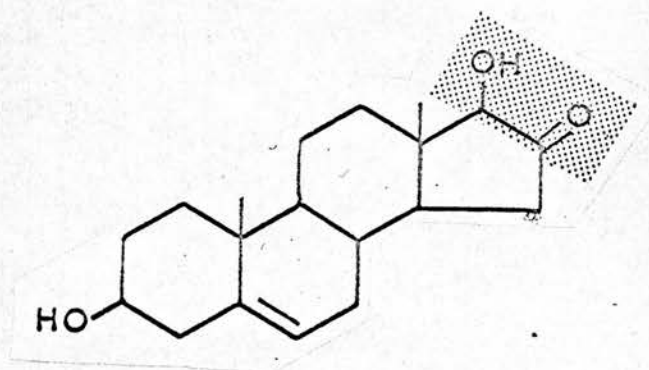
C₁₉ and C₂₁ steroids containing an α -ketol group develop blue colours with blue tetrazolium. The four steroids illustrated all give the blue tetrazolium reaction. The shaded areas indicate the α -ketol group that reacts with this reagent. .



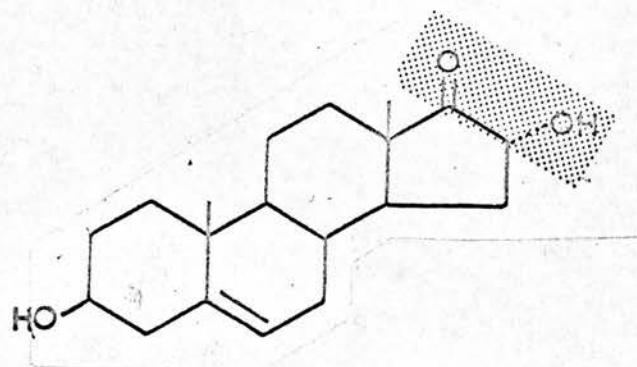
Tetrahydrocortisol



21-OH-Pregnenolone



16-Oxo-androstenediol



16 α -OH-DHA

* An α -ketol group consists of a hydroxyl group attached to the carbon atom adjacent (α) to a carbonyl group.

urine samples collected from adults are the 20,21 ketols, tetrahydrocortisone, tetrahydrocortisol and allotetrahydrocortisol. In urine obtained from newborn infants the 16,17 ketols, 16 α -OH-DHA, 16 β -OH-DHA and 16-oxo-androstenediol are quantitatively more important (Fig. 18).

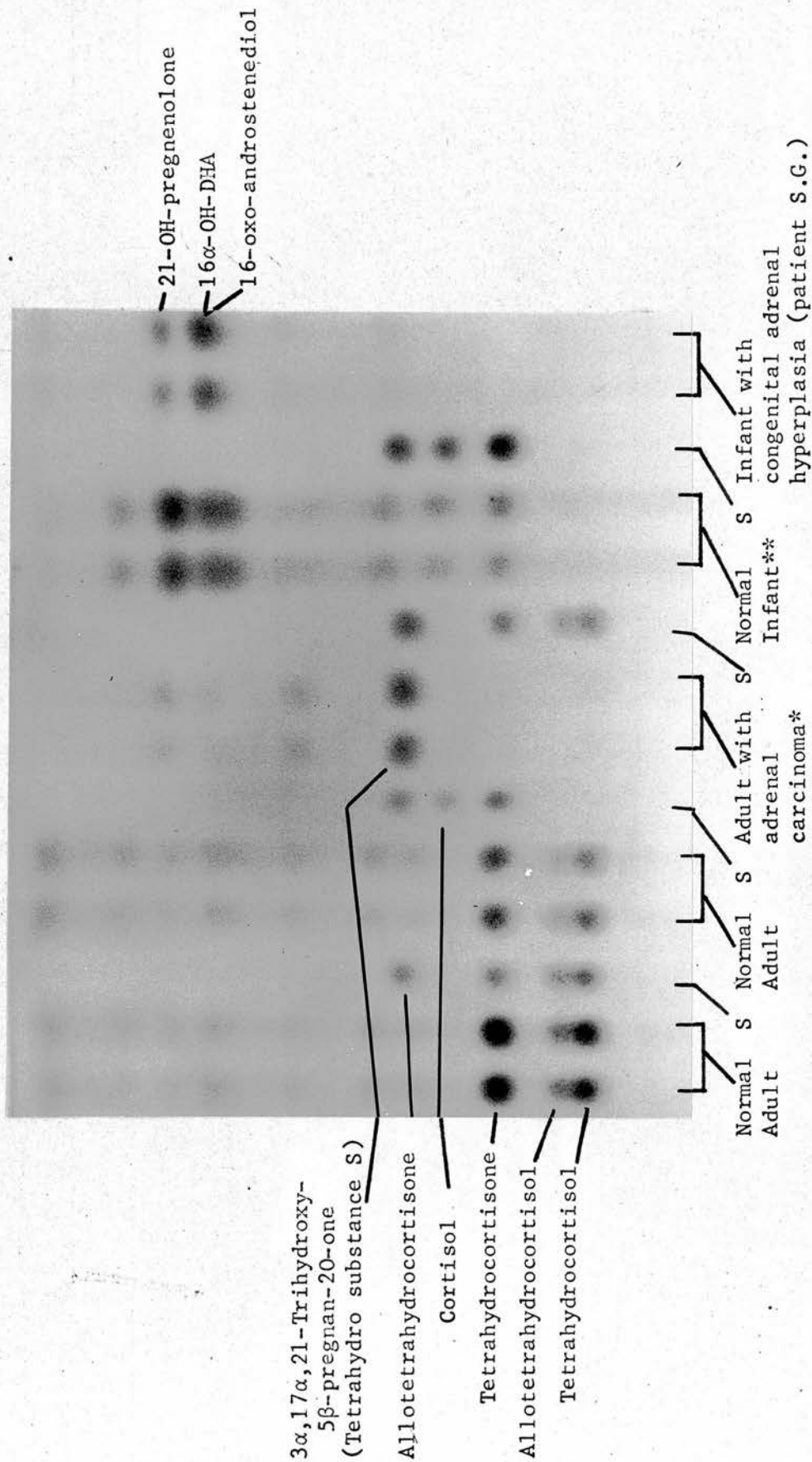
The blue tetrazolium reagent was made up just before use from the following constituents: 1% (w/v) blue tetrazolium in ethanol (2 parts); 3.5 N potassium hydroxide in ethanol (5 parts); and water (3 parts). This reagent contains less water than the similar reagent used for staining paper chromatograms (Birchall *et al.*, 1963), since it was found that excessive amounts of water caused the spots to diffuse and the silica gel layer to break up. The plates were sprayed until uniformly soaked, and colour development took place while the plates were being dried by a draught of hot air from a domestic hair-dryer. Good colour reactions have also been obtained by heating the plates at 50^o prior to spraying. In this method the spots show up immediately after spraying, and it is not necessary to heat with a hair-dryer. Both these methods give a light background, and in this respect are superior to the heating of plates in an oven after spraying.

The intensity of colour developed by different steroids varied considerably from plate to plate and it was necessary, therefore, to have standards present for every compound to be measured. This was particularly noticeable with the three α -ketolic androstenes, 16-oxo-androstenediol, 16 α -OH-DHA and 16 β -OH-DHA. 16 α -OH-DHA initially gave a very weak reaction compared to the other two compounds, but if left for several hours the intensity of its colour increased.

Cortisol was present in urine samples collected from those infants

Fig. 19. The Quantitative Estimation of Polar Blue Tetrazolium reacting Steroids

This figure illustrates the urinary excretion of reducing compounds by normal adults, by an adult with adrenal carcinoma, by a normal infant and by an infant with congenital adrenal hyperplasia.



Solvent system - G (x2)

Detecting reagent - Blue tetrazolium

S - Standard steroids

* Only 3 α ,17 α ,21-trihydroxy-5 β -pregnan-20-one (tetrahydro substance S) visible.

** The spots present are not necessarily equivalent to any of the reference compounds of similar polarity as the polar blue tetrazolium reacting steroids present in the urine of infants have not been identified.

that had been treated with adrenocorticotrophic hormone (ACTH). When the thin-layer plates used for the quantitative measurements were viewed under an ultra-violet lamp, cortisol showed up as a dark area due to the presence of an additive in the silica gel which fluoresces at 254 m μ . In urine collected from these infants, cortisol was assayed by comparing visually the absorbance given by the cortisol spot in the urinary extract with the absorbance of varying amounts of cortisol that had been applied to the thin-layer plate as reference compound.

Thin-layer system

Tetrahydrocortisone, tetrahydrocortisol and allotetrahydrocortisol were separated by two runs in system G (Fig. 19); this also separates these compounds from tetrahydrodehydrocorticosterone, tetrahydrocorticosterone and 3 α , 17 α , 21-trihydroxy-5 β -pregnan-20-one (tetrahydro substance S). These latter steroids are sometimes present as a result of malfunctioning of the adrenal glands.

In system G (x 2) separation of the polar steroids that react with blue tetrazolium and which can be extracted from urine collected from infants demonstrated that the tetrahydro metabolites of cortisol were quantitatively of minor importance (Fig. 19), and these steroids could not be measured quantitatively. It was possible, however, to estimate steroids of polarity similar to tetrahydrocortisol and allotetrahydrocortisol in urine samples obtained from infants treated with adrenocorticotrophin.

Specificity and identification:

The R_f values of tetrahydrocortisone, tetrahydrocortisol and allotetrahydrocortisol obtained from urine were the same as the corresponding



standards in solvent systems D, I and J, and no other blue tetrazolium staining compounds could be separated from them when two-dimensional chromatography was carried out on urinary extracts.

Cortisol was prepared from the urine of infants treated with adrenocorticotrophin, and was identified by infra-red spectroscopy. Two plates were used for purification; the cortisol was first separated as a band in system G (x 2); after elution, it was further purified by two-dimensional chromatography in systems J and I (x 2). The purification of 3-oxo- Δ^4 steroids is simple, since the band, or spot, to be eluted can be detected under ultra-violet light of wavelength 254 m μ , if silica gel containing a fluorescent additive is used.

(iii) The separation and detection of 17-oxosteroids

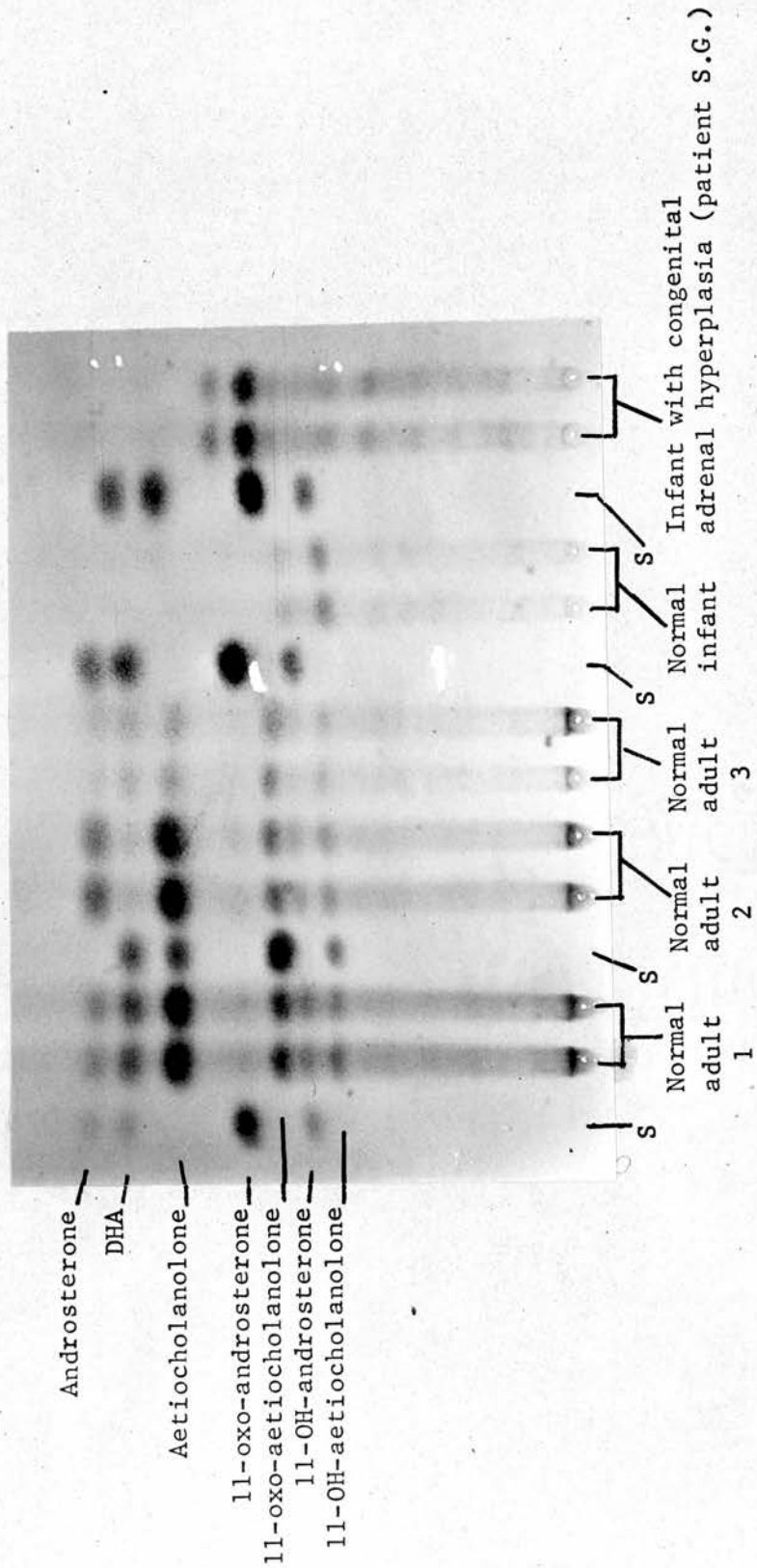
Detecting reagent

The 17-oxosteroids were detected using the Zimmermann reaction, which gives blue to violet colours with unsubstituted methylene groups adjacent to a carbon atom containing a ketone group. This reaction was used to detect steroids present in urine excreted by adults, where the amount of steroids containing carbonyl groups at the 3 and 20 positions of the steroid molecule is relatively small compared to the 17-oxosteroids. In urine collected from infants, however, there are too many Zimmermann chromogens present, apart from the 17-oxosteroids, for the reaction to be useful for the quantitative estimation of steroids by densitometry of the thin-layer chromatograms.

The reagent consisted of 2% (w/v) metadinitrobenzene in ethanol (2 parts), and 3.5 N potassium hydroxide in ethanol (1 part). It was made up before use from solutions kept in a deep-freeze. The plates were

Fig. 20. The Quantitative Estimation of Individual 17-oxosteroids

Solvent system - C (x3) Detecting reagent - Zimmermann reagent



S - standard steroids

The photograph shows the separation of individual 17-oxosteroids from the urine of three normal adults, of a normal infant and of an infant with congenital adrenal hyperplasia (patient S.G.). The normal infant shows little or no excretion of the classical oxosteroids; the infant with congenital adrenal hyperplasia, however, shows a large excretion of 11-oxo-androsterone and 11-oxo-aetiocholanolone.

sprayed by the method described previously for the blue tetrazolium reaction (p. 50).

The seven principal 17-oxosteroids gave violet spots; the intensity of colour developed by the 11-oxo-17-oxosteroids was about twice as great as the colour developed by the 11-deoxy-17-oxosteroids and the 11-hydroxy-17-oxosteroids.

Thin-layer system

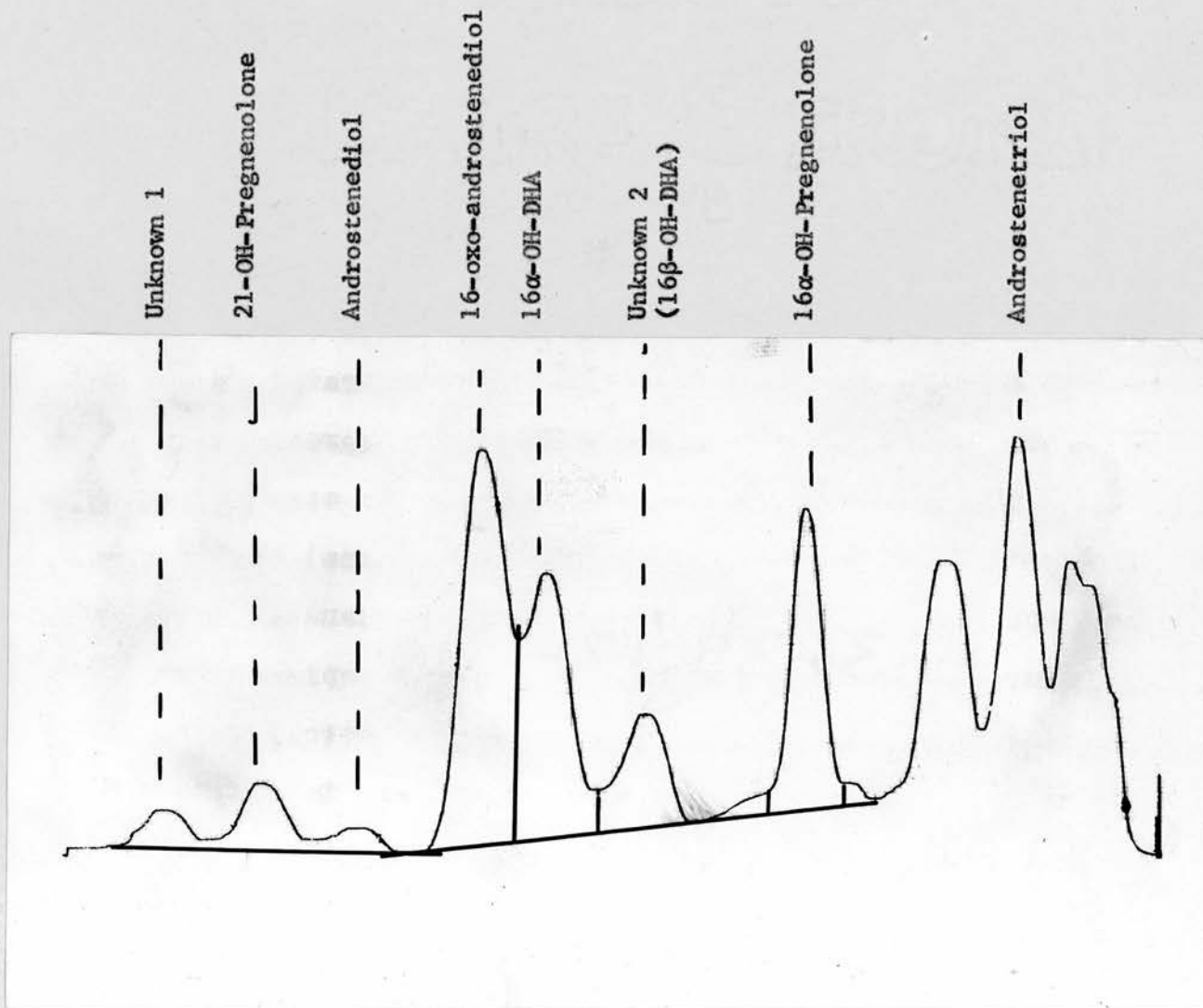
The principal 17-oxosteroids present in urine excreted by adults (Table 4) were separated by running the chromatogram three times in system C; the results are illustrated in Fig. 20. There are only very small amounts of the classical 17-oxosteroids present in urine samples collected from newborn infants; when these are measured by the Zimmermann reaction, the 17-oxosteroids are often interfered with by other compounds giving an unspecific reaction. Measurement of 17-oxosteroids is of value, however, when infants are suspected of having congenital adrenal hyperplasia, as this disorder is frequently associated with an abnormally high excretion of these compounds.

Specificity and identification

Two dimensional chromatograms, with and without acetylation after development in the first direction, were prepared in the manner described for the $3\beta\text{-OH-}\Delta^5$ steroids. The spot which was identified as DHA was shown to contain a second chromogen when subjected to chromatography in system H (x 2); the colour formed by the contaminant indicated that it was present at one-tenth of the concentration of DHA. This substance was shown to have the same Rf values as epiandrosterone ($3\beta\text{-hydroxy-5}\alpha\text{-androstan-17-one}$) when compared with standards in three solvent systems.

Fig. 21. Densitometer Tracing of the 3β -OH- Δ^5 Steroids Present in Urine
Collected from Infants after Separation by Thin-Layer Chromatography

Following separation by thin-layer chromatography, steroids present in urine collected from infants are detected by the antimony trichloride reaction and assayed by reflectance densitometry. This figure illustrates the tracing drawn by the densitometer recorder of the medium polarity 3β -OH- Δ^5 steroids; the separation is illustrated in Fig. 12. This figure also illustrates the method of delineating overlapping peaks and drawing the baseline.



The 11-oxygenated metabolites also showed the presence of small amounts of impurities (probably due to epimers) when the chromatograms were developed further by running again at right angles in system H; quantitatively these impurities also amounted to about one-tenth of the major constituents in their spots, and these amounts were not considered to be sufficient to affect the value of the method seriously.

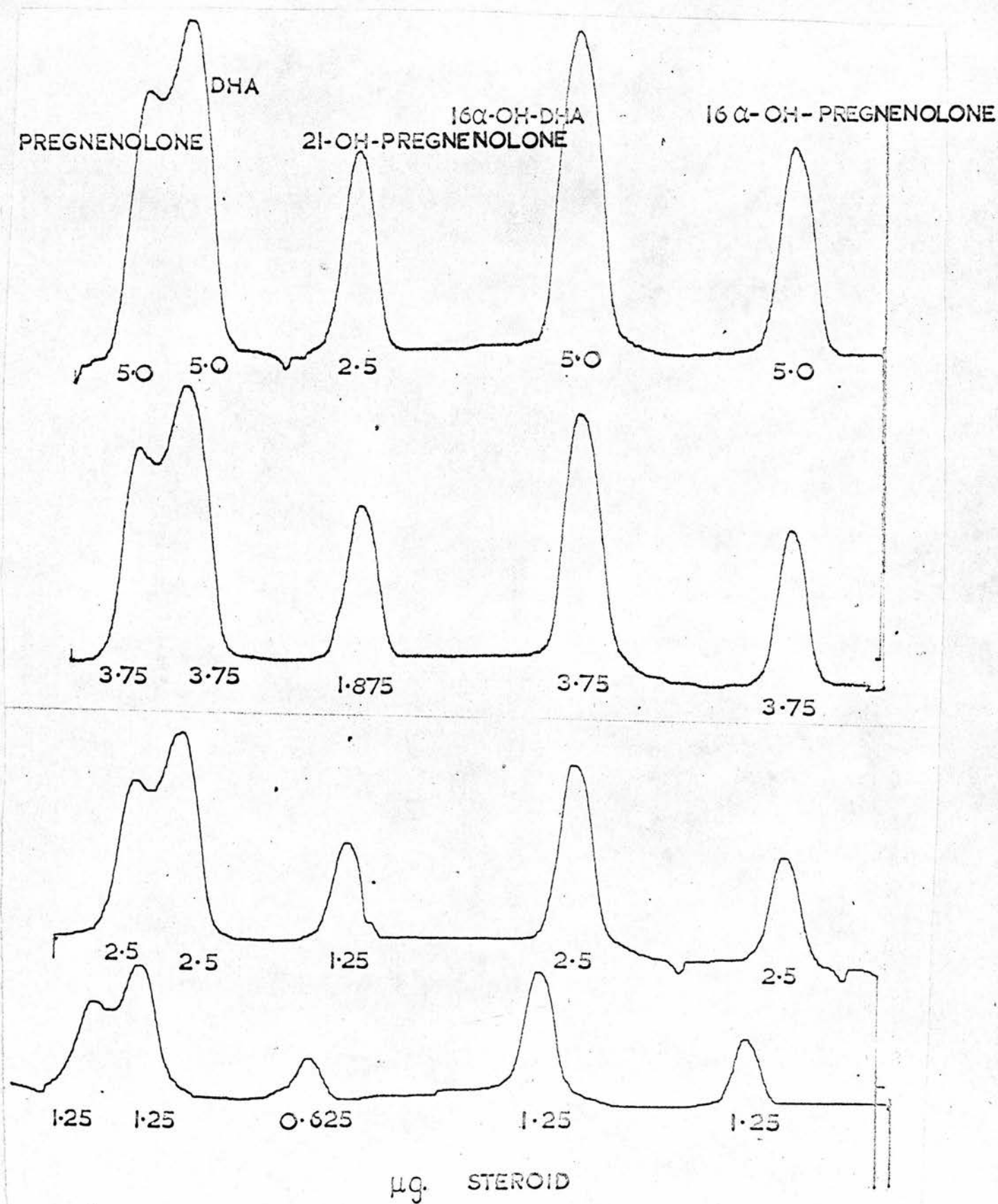
3.3 Quantitation

Following development of the colour reaction the steroids were determined by scanning the thin-layer plates by reflectance densitometry. A "Chromoscan" was used for this purpose. The light beam was produced by a slit of dimensions 1 x 7 mm. and green filters were used for all colour reactions (wavelength 490 m μ for the antimony trichloride reaction, 520^{m μ} for the Zimmermann reaction and 550 m μ for the blue tetrazolium reaction). A recording made by the densitometer of steroids in urine obtained from infants is illustrated in Fig. 21. The steroids were determined by comparing peak areas produced (measured by planimeter) to peak areas given by known amounts of standard compounds. Although the Chromoscan instrument possessed an integrator, this could not be used since the background colouration was not constant, resulting in an uneven baseline. For this reason the baseline was drawn by hand and overlapping peaks were delineated by dropping perpendicular lines to this baseline (Fig. 21). After measuring the areas of peaks produced by reference compounds (Fig. 22), standard curves were drawn (Fig. 23), from which the concentration of steroids in the biological sample could be determined.

4. Summary of routine method

Since the description of the method in the previous sections has included results of a considerable amount of preliminary work, this section will describe briefly the routine method that was in use in the later stages of this study.

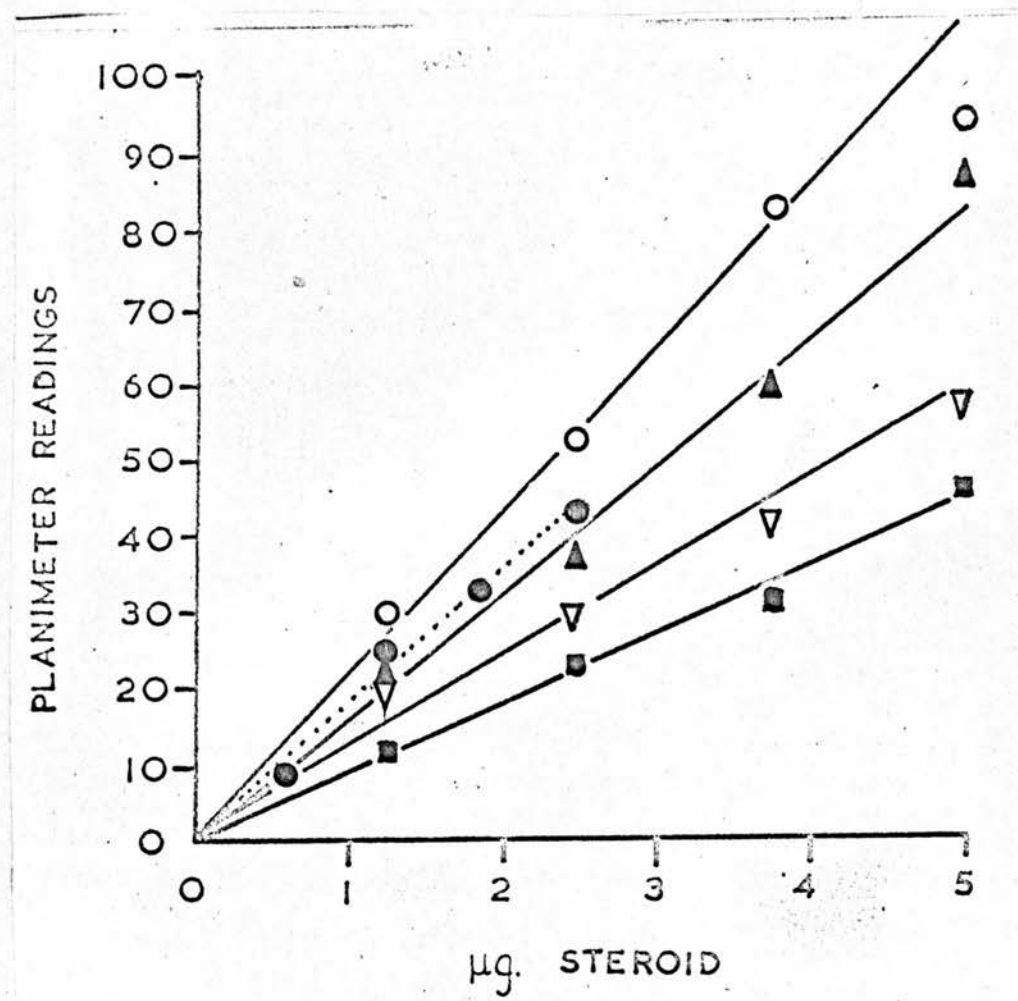
Fig. 22. Densitometer Tracing of Standard Steroids



Legend:

Normally four spots are applied to each plate of standards, each spot containing several steroids. These standard spots, as a rule, contain the same steroids but in increasing amount. After separation and spraying with reagent for their detection, the coloured spots are measured quantitatively using a densitometer. This figure shows densitometer tracings of the four standard spots used for the assay of steroids in plasma obtained from the umbilical blood vessels. The quantity of each reference compound in each standard spot is given. A planimeter is used to determine the areas under the peaks and standard curves are then plotted for each compound. Figure 23 illustrates the standard curves obtained for these steroids.

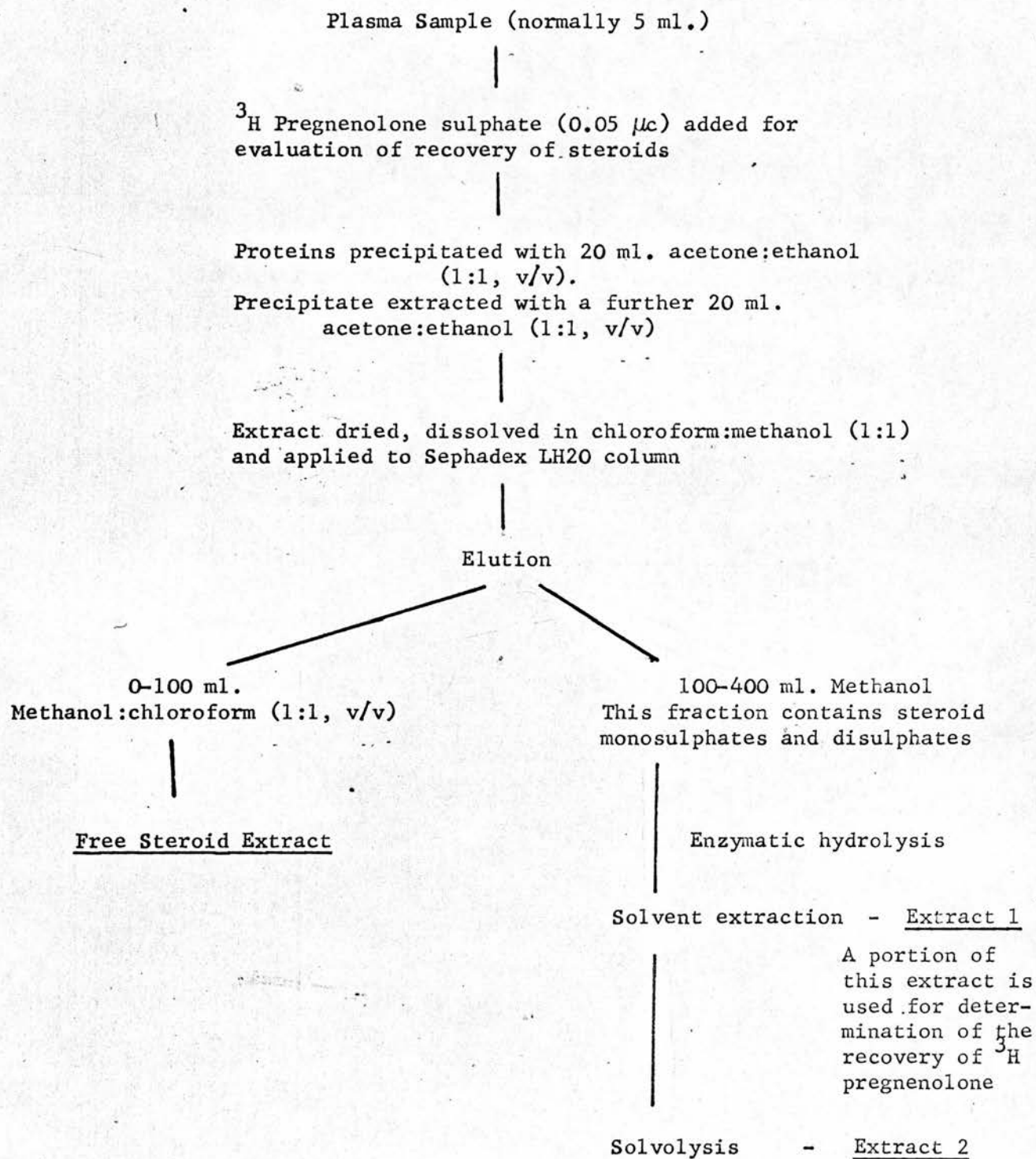
Fig. 23. Calibration Graphs for the Estimation of Steroids by Densitometry



- O - DHA
- ● - 21-OH-Pregnenolone
- ▲ - 16 α -OH-DHA
- ▽ - Pregnenolone
- ■ - 16 α -OH-Pregnenolone

A standard curve is drawn for each of the reference compounds determined by densitometric scanning (Fig. 22). The concentration of steroid in extracts of urine and plasma can then be measured by relating the areas of the peaks produced to the areas given by standard steroids.

Fig. 24. Flow-Sheet of Method Used for Extraction of Steroids from Plasma



4.1 Preliminary treatment and hydrolysis

Urine:

Normally 1/16 of a 24 hour collection of urine from infants and 1/60 of a 24 hour collection from adults was used for analysis.

The urine was treated as follows:-

1. ^3H Pregnenolone sulphate (0.05 μc) was added to the urine to monitor the recovery of steroid sulphates through the whole procedure.
2. The urine was brought to pH 11.5 with 4 N sodium hydroxide. At this pH saccharo-1:4-lactone is precipitated (this compound inhibits β -glucuronidase activity).
3. Barium chloride (10%, w/v) was added until no more barium sulphate was precipitated. (Sulphate ions inhibit sulphatase activity and must be removed before enzymatic hydrolysis).
4. The urine sample was adjusted to pH 4.5 by adding 5 N acetate buffer (5 N acetic acid:5 N sodium acetate, 2:3, v/v). The volume of this buffer that was added was equivalent to one-tenth the volume of the urine.
5. 0.1 ml. of the succus entericus of *Helix pomatia* was added per 20 ml. urine. The urine was then incubated at 37-38 $^{\circ}$ for 48 hours, a further 0.1 ml. of the enzyme preparation being added after 24 hours.

Plasma:

A summary of the method used for the extraction of steroids from plasma is illustrated in Fig. 24. Duplicate assays were normally carried out on arterial and venous plasma. Five ml. of plasma was generally used for each analysis.

The plasma samples were treated as follows:-

1. ^3H Pregnenolone sulphate (0.05 μc) was added to 5 ml. plasma.
2. The plasma proteins were precipitated with 20 ml. acetone:ethanol

(1:1, v/v) and were then removed by centrifugation. The precipitate was washed by suspending in a further 20 ml. acetone:ethanol and the centrifugation repeated. The acetone:ethanol extracts were combined and dried on a rotary evaporator. The protein precipitate was discarded.

3. The dried extract was applied to the Sephadex column (17 g. Sephadex LH 20 in columns 2 cm. in diameter) with 2 x 5 ml. methanol:chloroform, 1:1 (saturated with sodium chloride). The flask was washed with a further 90 ml. of the eluting solvent; this was then put in the column reservoir.

4. The first 100 ml. of solvent which passed through the column were collected and dried; this fraction contained free steroids, cholesterol and fat.

5. Monosulphates and disulphates were washed off the column with 300 ml. methanol. No attempt was made to separate the monosulphates from the disulphates. The eluate was dried.

6. The dried extract, containing the steroid monosulphates and disulphates, was dissolved in 10 ml. 0.5 N acetate buffer (0.5 N acetic acid: 0.5 N sodium acetate, 2:3). Incubation was then carried out for 48 hours at 37-38°, with 0.05 ml. of the *Helix pomatia* enzyme preparation. A further 0.05 ml. of the *Helix pomatia* enzyme preparation was added after 24 hours.

4.2 Extraction Procedure

Urine and plasma

1. After enzymatic hydrolysis, the steroids were extracted twice with two volumes of ether:ethyl acetate (2:1, v/v).

2. The solvent extract was washed repeatedly with N sodium hydroxide (an amount equivalent to one-tenth the volume of the extract), until the washings were colourless.

3. Further washings were carried out once with water containing a few drops of acetic acid, and once with water alone.

4. The extract was dried on a rotary evaporator at 45°.
5. The dried extract was transferred with chloroform to a small tube, and the chloroform was removed under a stream of nitrogen. This extract contained the steroids which were released by enzymatic hydrolysis.
6. Sulphuric acid (50%, v/v) was added to the aqueous phase (from stage 1) to bring it to pH 1. The aqueous phase was then saturated with ammonium sulphate.
7. The steroid sulphates remaining in the aqueous phase were extracted into an equal volume of ethyl acetate. One half of the ethyl acetate extract was incubated for 24 hours at 38°, the other half was discarded with the urine.
8. Stages 2 - 5 were repeated. The extract obtained contained the steroids released from conjugation by solvolysis.

4.3 Use of extracts

(a) Urine from infants

Extract following enzymatic hydrolysis

The steroid residue was normally dissolved in 0.5 ml. ethanol. The tubes containing extracts were kept stoppered at -20° when not in use.

For the determination of overall losses, 25 μ l. of the extract solution were taken for measurement of the radioactivity by liquid scintillation counting.

Chromatography:

The following portions of each extract, with standards, were applied to and run on three thin-layer plates. Due to the wide variation in the urinary excretion of steroids by infants, it was often necessary to repeat assays using either more or less of the extract.

- (i) 50 μ l. was used for the determination of compound U₁ and 21-OH-

TABLE 7

Standards

		<u>Steroid ($\mu\text{g. per spot}$)</u>	<u>Spot</u>			
			1	2	3	4
<u>STANDARD 1</u>						
Medium polarity $3\beta\text{-OH-}\Delta^5$ steroids	Pregnenolone	1.25	2.50	3.75	5.00	
	DHA	1.25	2.50	3.75	5.00	
	21-OH-pregnenolone	0.625	1.25	1.875	2.50	
	16 α -OH-DHA	1.25	2.50	3.75	5.00	
	16 α -OH-pregnenolone	1.25	2.50	3.75	5.00	
	Androstenetriol	0.625	1.25	1.875	2.50	
<u>STANDARD 1a</u>	17 α -OH-pregnenolone	0.625	1.25	1.875	2.50	
<u>STANDARD 2</u>						
Solvolysis Steroids	Androstenediol	1.25	2.50	3.75	5.00	
	Pregnenetriol	0.625	1.25	1.875	2.50	
<u>STANDARD 3</u>						
Polar steroids	Androstenetriol	1.25	2.50	3.75	5.00	
<u>STANDARD 4</u>						
Adult $3\beta\text{-OH-}\Delta^5$ steroids	DHA	1.25	2.50	3.75	5.00	
	Androstenediol	1.25	2.50	3.75	5.00	
	16 α -OH-DHA	1.25	2.50	3.75	5.00	
	Pregnenetriol	1.25	2.50	3.75	5.00	
	Pregnanetriol	1.25	2.50	3.75	5.00	
	Androstenetriol	1.25	2.50	3.75	5.00	
<u>STANDARD 5</u>						
17-oxosteroids	Androsterone	2.50	-	5.00	-	
	DHA	1.25	2.50	3.75	5.00	
	Aetiocholanolone	-	2.50	-	5.00	
	11-oxo-androsterone	2.50	-	5.00	-	
	11-oxo-aetiocholanolone	-	2.50	-	5.00	
	11-OH-androsterone	2.50	-	5.00	-	
	11-OH-aetiocholanolone	-	2.50	-	5.00	
<u>STANDARD 6</u>						
Reducing steroids	Tetrahydrocortexolone	-	2.50	-	5.00	
	Allotetrahydrocortisone	2.50	-	5.00	-	
	Cortisol	-	2.50	-	5.00	
	Tetrahydrocortisone	2.50	3.75	5.00	7.50	
	Allotetrahydrocortisol	2.50	-	5.00	-	
	Tetrahydrocortisol	2.50	-	5.00	-	

pregnenolone.

Standard 1 (Table 7) was applied to the thin-layer plate, which was then developed in system F (x 2).

(ii) 20 μ l. was used for the determination of the 16-oxygenated steroids.

Standard 1 (Table 7) was applied to the thin-layer plate; this was then developed in system C (x 3).

(iii) 25 μ l. of the extract was used for the determination of androstenediol.

Standard 3 (Table 7) was applied to the plate, which was then developed in system G (x 2).

Initially, 21-OH-pregnenolone, compound U₁ and the 16-oxygenated steroids were determined on the same chromatogram after six developments in chromatographic systems [system E (x 3) and system B (x 3)].

Solvolysis extract

One quarter of the solvolysis extract was used for the assay of androstenediol.

Standard 2 (Table 7) was used for quantitation, and system H (x 2) for development.

(b) Urine from adults

One sixth of the extract was normally taken for the determination of the 3 β -OH- Δ^5 steroids, the 17-oxosteroids and the cortisol metabolites.

For chromatography, the following standards, development systems and staining reagents were used:-

3 β -OH- Δ^5 steroids	Standard 4 (Table 7), development in system H followed by system F (x 2). Detection by the antimony trichloride reagent.
17-oxosteroids	Standard 5 (Table 7), development in system C (x 3).

TABLE 8

Precision of Determinations as Shown by the Concordance of
Areas Under the Peaks of Chromatogram Scans

Compound assayed	Number of duplicate assays	Standard deviation of the area (%)*
Compound U ₁	22	7.3
21-OH-pregnenolone	22	8.9
Androstenediol	11	8.5
16-oxo-androstenediol	22	4.5
16 α -OH-DHA	22	7.6
Compound U ₂ (16 β -OH-DHA)	17	7.0
16-OH-pregnenolone	22	4.4
Androstenetriol	22	5.7
Androsterone	10	10.8
DHA	12	8.6
Aetiocholanolone	12	9.3
11-oxo-aetiocholanolone	12	10.4
11-OH-aetiocholanolone	9	10.0
11-OH-androsterone	9	9.2
Tetrahydrocortisone	11	7.4
Allotetrahydrocortisol	6	8.6
Tetrahydrocortisol	11	9.8

* The standard deviation has been calculated from the formula

$$\sqrt{\frac{d^2}{2N}}$$

where d = % difference between duplicate areas and

N = the number of duplicate assays.

Cortisol metabolites Detection by the Zimmermann reagent.
Standard 6 (Table 7), development in
system G (x 2).
Detection by the blue tetrazolium reaction.

(c) Plasma

Free steroid extract:- This was not normally used.

Extract following enzymatic hydrolysis

For the determination of overall losses, two portions equivalent to 1/40 of the total extract were taken for liquid scintillation counting.

Chromatography:-

16 α -OH-DHA was present in much larger quantity than the other 3 β -OH- Δ^5 steroids and for this reason two portions of different amount were applied to the same plate. One quarter of the total extract was used for the determination of 16 α -OH-DHA, and one half for the determination of the remaining 3 β -OH- Δ^5 compounds.

Standards 1 and 1a (Table 7) were used together.

The plates were developed once in system C and twice in system H.

Solvolysis extract

One half of the extract was used for chromatography. The extract itself usually represents only one half of the solvolysable steroids as the remainder was discarded at stage 7 of the extraction procedure.

5. Precision of the method

The precision, defined as the concordance of measurements of the same quantity, has been determined by calculating the standard deviation from the differences between the chromatogram peak areas for duplicate runs; all the steps of the procedure were performed in duplicate. Table 8 shows the results obtained with the 3 β -OH- Δ^5 steroids, the blue tetrazolium reacting steroids

and the Zimmermann staining compounds. In investigating the precision, widely differing values were occasionally found in the duplicate determinations, but these have not been included since such clearly unacceptable determinations were always repeated.

6. Accuracy of the method

Accuracy, defined as the concordance between the determination and the true value of the quantity measured, was assessed by measuring the recovery of DHA from DHA sulphate, and aetiocholanolone and tetrahydrocortisone from their glucuronides.

Dehydroepiandrosterone sulphate (250 µg.) was added to six 10 ml. portions of urine collected from infants; after hydrolysis and extraction 1/50 of each extract was separated by TLC, and the DHA assayed. The recoveries of DHA for the six determinations ranged between 73 and 90% with a mean of 80% ± 6.2 (S.D.*).

To estimate the recovery of steroids from steroid glucuronides, known amounts of aetiocholanolone glucuronide and tetrahydrocortisone glucuronide were added to ten equal portions of urine. Aetiocholanolone and tetrahydrocortisone were then assayed after hydrolysis of the urine samples. The results obtained were compared with the results for the analysis of these steroids in ten portions of the same urine to which no steroid glucuronide had been added. The recovery of tetrahydrocortisone from its glucuronide was 72% ± 5.4 (S.D.), and the recovery of aetiocholanolone from aetiocholanolone glucuronide was 91% ± 6.9 (S.D.).

* The standard deviations have been determined using the

formula

$$\text{S.D.} = \sqrt{\frac{\sum d^2}{N} - \left(\frac{\sum d}{N}\right)^2}$$

The accuracy of the method for steroids in plasma was determined by adding 0.5 μ c. ^3H pregnenolone sulphate to the plasma samples. The ^3H pregnenolone present after hydrolysis was determined by counting a portion of the steroid extract in a Packard liquid scintillation spectrometer. For the plasma samples, the recovery of ^3H pregnenolone from ^3H pregnenolone sulphate varied between 76 and 99% with a mean of $89\% \pm 7.9$ (S.D.). Since this method depends on the radiochemical purity of the ^3H pregnenolone sulphate, experiments were carried out to check whether all the radioactivity in the standard solution was associated with the ^3H pregnenolone sulphate. When ^3H pregnenolone sulphate was developed on a thin-layer chromatogram using system K only one peak, corresponding to pregnenolone sulphate, was detected when the plate was scanned for radioactivity using a Nuclear Chicago radiochromatogram scanner. Similarly, after enzymatic hydrolysis of the ^3H pregnenolone sulphate, all the radioactivity was found to be associated with ^3H pregnenolone. It was therefore concluded that the ^3H pregnenolone sulphate was radiochemically pure.

III DISCUSSION OF THE METHOD

The method that has been developed is particularly useful for the determination of $3\beta\text{-OH-}\Delta^5$ steroid metabolites present in urine obtained from infants. It is preferable to other methods developed in recent years since it permits the determination of a wider range of steroids.

Reynolds (1965 a, b; 1966 a, b) described a technique for the assay of $16\alpha\text{-OH-DHA}$, $16\alpha\text{-OH-pregnenolone}$ and androstenetriol in urine collected from infants. The method described in this thesis, however, permits the measurement of several other steroids as well, including $21\text{-OH-pregnenolone}$ and pregnanetriol, the measurement of which is particularly useful in the diagnosis of congenital adrenal hyperplasia. The presence of any abnormal steroid which reacts with one of the three spray reagents is also immediately evident.

Two methods have recently been described for the measurement of DHA sulphate and $16\alpha\text{-OH-DHA}$ sulphate in plasma obtained from the umbilical blood vessels; a method based on column chromatography (Colás et al., 1964) and the paper chromatographic technique developed by Simmer and co-workers (Simmer et al., 1964, 1966; Easterling et al., 1966). Eberlein (1965b) showed that plasma obtained from the umbilical blood vessels contains other important $3\beta\text{-OH-}\Delta^5$ steroids and the technique described here makes possible the determination of several of these, including pregnenolone, $16\alpha\text{-OH-pregnenolone}$, $17\alpha\text{-OH-pregnenolone}$ and $21\text{-OH-pregnenolone}$, as well as DHA and $16\alpha\text{-OH-DHA}$.

The sensitivity is adequate for the measurement of the steroids so far determined, as there is generally sufficient material in about 1/400 of a 24 hour sample of urine collected from an infant to permit measurement

of the principal $3\beta\text{-OH-}\Delta^5$ steroids. In order to assay minor, but important, components such as DHA and pregnenolone accurately, it will be necessary to develop the method further by including a step for removing many of the steroids present in large amount.

To measure the metabolites of pregnenolone and DHA in plasma obtained from the umbilical vein and artery, an amount of extract equivalent to 2.5 ml. of plasma is applied to the thin-layer plates. It is usually possible to obtain at least 5 ml., and often 10 or 20 ml. of arterial and venous plasma from the umbilical blood vessels, so the method is sufficiently sensitive for the material available.

The precision obtainable is adequate for the purposes for which the technique is designed. The standard deviations shown in Table 8 refer to single assays, but in practice the average of duplicate measurements is used and the precision is thereby considerably increased. The accuracy, judged by the recovery of added steroid conjugates, is satisfactory. Since no more $3\beta\text{-OH-}\Delta^5$ steroids are released by solvolysis after enzymatic hydrolysis, apart from those steroids which are only released from conjugation by solvolysis, it may be assumed that the hydrolytic procedure releases almost all steroids. The specificity has been thoroughly determined for the $3\beta\text{-OH-}\Delta^5$ steroids present in urine collected from infants since most of the compounds have been identified by combined gas chromatography-mass spectrometry. The steroids in plasma obtained from the umbilical cord, and in urine collected from adults, have not been so thoroughly identified. These steroids, however, gave the same colour reactions as standards, and their mobility on TLC was identical to the standard compounds.

By using the Zimmermann and blue tetrazolium reactions, the method was extended for the assay of the major group of steroid metabolites excreted by adults, namely the 17-oxosteroids and the metabolites of cortisol. A particular advantage of this technique is that it can be used without any elaborate equipment since it is possible to assess visually, with satisfactory accuracy, the quantity of steroid present in any chromatographic spot, providing a suitable number of standards have been run simultaneously. The method can therefore be used routinely for the investigation of adrenocortical function. It has not yet proved possible to determine accurately the individual 17-oxosteroids and cortisol metabolites present in urine collected from infants, but these groups of steroids are quantitatively of minor importance in these samples and it is difficult to determine them in the presence of large amounts of the $3\beta\text{-OH-}\Delta^5$ steroids.

EXTRACTS FROM
STRENGTH

PART 2

QUANTITATIVE RESULTS OBTAINED FROM THE ANALYSIS OF STEROIDS
PRESENT IN URINE AND PLASMA.

TABLE 9

Mean Urinary Excretion of Individual 3β -OH- Δ^5 Steroids by Newborn Infants ($\mu\text{g}/24 \text{ hr}$)

	Number of determinations	Mean Excretion	Range	Ratios
Pregnenolone	3	33	10-50	$\frac{16\alpha\text{-OH-pregnenolone}}{16\alpha\text{-OH-DHA}} = 0.98$
DHA	4	29	10-40	
Compound U ₁	14	534	20-1360	$\frac{16\alpha\text{-OH-pregnenolone}^{**}}{21\text{-OH-pregnenolone}^{**}} = 4.76$
21-OH-pregnenolone	14	201	15-471	
Androsterediol	32	140	10-840	$\frac{16\alpha\text{-OH-DHA}}{16\beta\text{-OH-DHA}} = 1.42$
16-oxo-androsterediol	32	680	30-2430	
16 α -OH-DHA	32	750	32-2240	$\frac{16\alpha\text{-OH-DHA}}{\text{Androstenetriol}} = 1.53$
Compound U ₂ (16 β -OH-DHA)	32	528	20-1560	
16 α -OH-pregnenolone	32	737	92-2100	
Androstenetriol	32	495	20-1620	
Total*		3699	263-11,988	

* Not including DHA and pregnenolone

** The values of 16 α -OH-pregnenolone and 21-OH-pregnenolone used for this ratio were only obtained from the 14 German infants since earlier in the study 21-OH-pregnenolone had not been assayed.

I. THE MEASUREMENT OF 3β -OH- Δ^5 STEROIDS IN URINE COLLECTED FROM NORMAL NEWBORN INFANTS

The earlier analyses in this study were on urine obtained from normal infants born in the Simpson Memorial Maternity Pavilion, Edinburgh. The urine was collected in plastic bags, refrigerated soon after collection, and stored at -20° until assayed. Most of the later steroid determinations have been carried out in conjunction with Professor Ch. Lauritzen of Kiel, Germany, to study the effect of adrenocorticotrophin and of human chorionic gonadotrophin on steroid excretion by the newborn infant. In these experiments, six day urine collections were made from four normal untreated infants as controls, and ten infants treated with human chorionic gonadotrophin or adrenocorticotrophin during the fourth, fifth and sixth days of life.

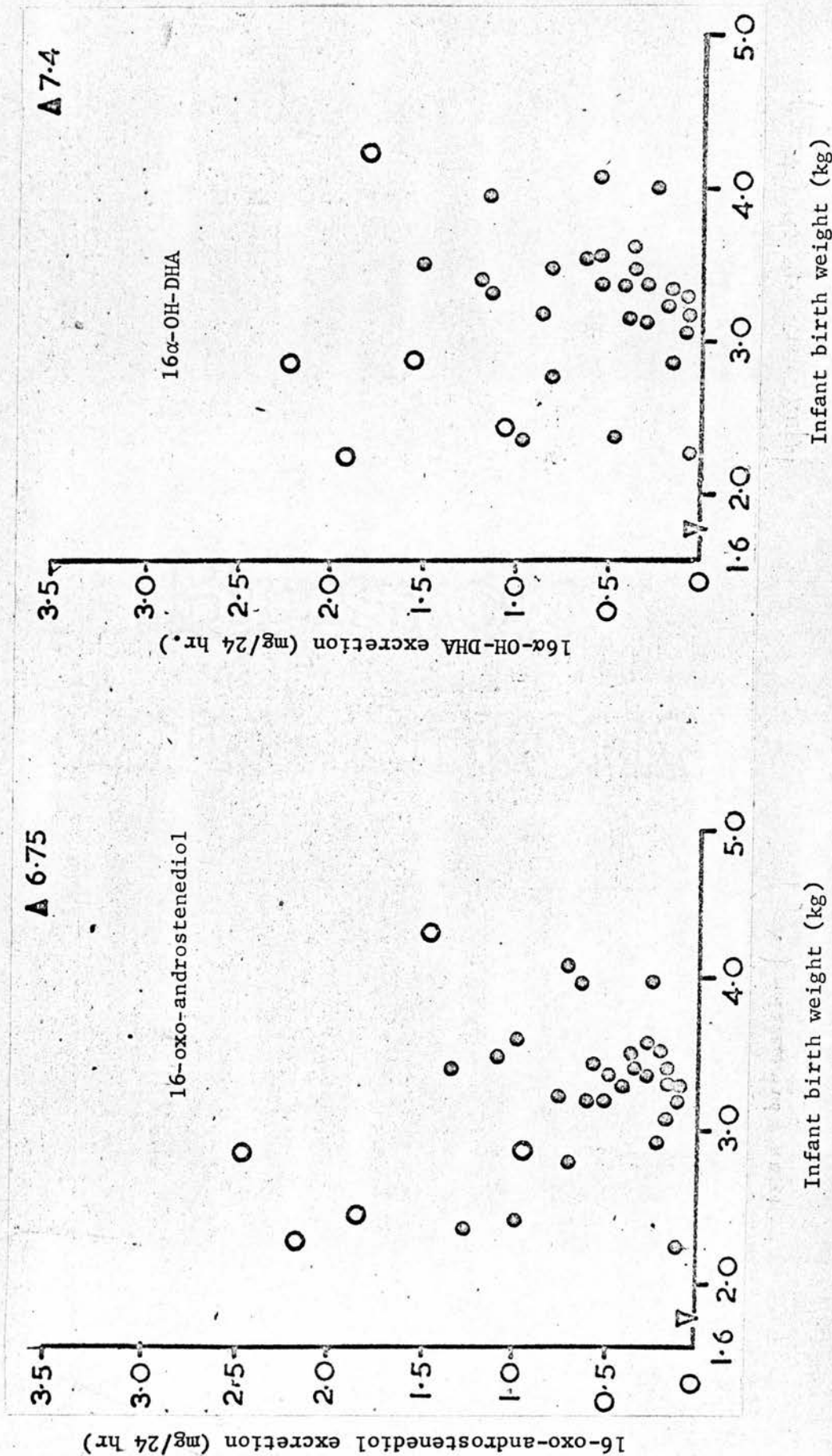
Table 22 (Appendix) gives the quantitative results for the excretion of all the 3β -OH- Δ^5 steroids, together with the weight of the infants at birth and the length of gestation. In an attempt to detect possible changes of steroid excretion with foetal maturity, ratios have been calculated of 16α -OH-pregnenolone/ 16α -OH-DHA, 16α -OH-pregnenolone/ 21 -OH-pregnenolone, 16α -OH-DHA/ 16β -OH-DHA, and 16α -OH-DHA/androstenetriol. It was envisaged that any correlation in desmolase, 17β -reductase and 16α - and 21 -hydroxylase with infant weight, would be apparent from these ratios.

Table 9 gives the range, and mean for the excretion of 3β -OH- Δ^5 steroids; this table also shows the mean steroid ratios and their range.

In the earlier part of this work 21 -OH-pregnenolone and compound U_1 were not separated by TLC, so these steroids were determined together.

Fig. 25. 16-Oxo-androstenediol and 16 α -OH-DHA in Urine Samples Collected from Infants, Plotted in Relation to Birth Weight

- Normal infants
- Infants that had been subject to stress
- ▲ An infant with congenital adrenal hyperplasia (S.G.)
- ▽ An infant with congenital adrenal hypoplasia (B.O.)



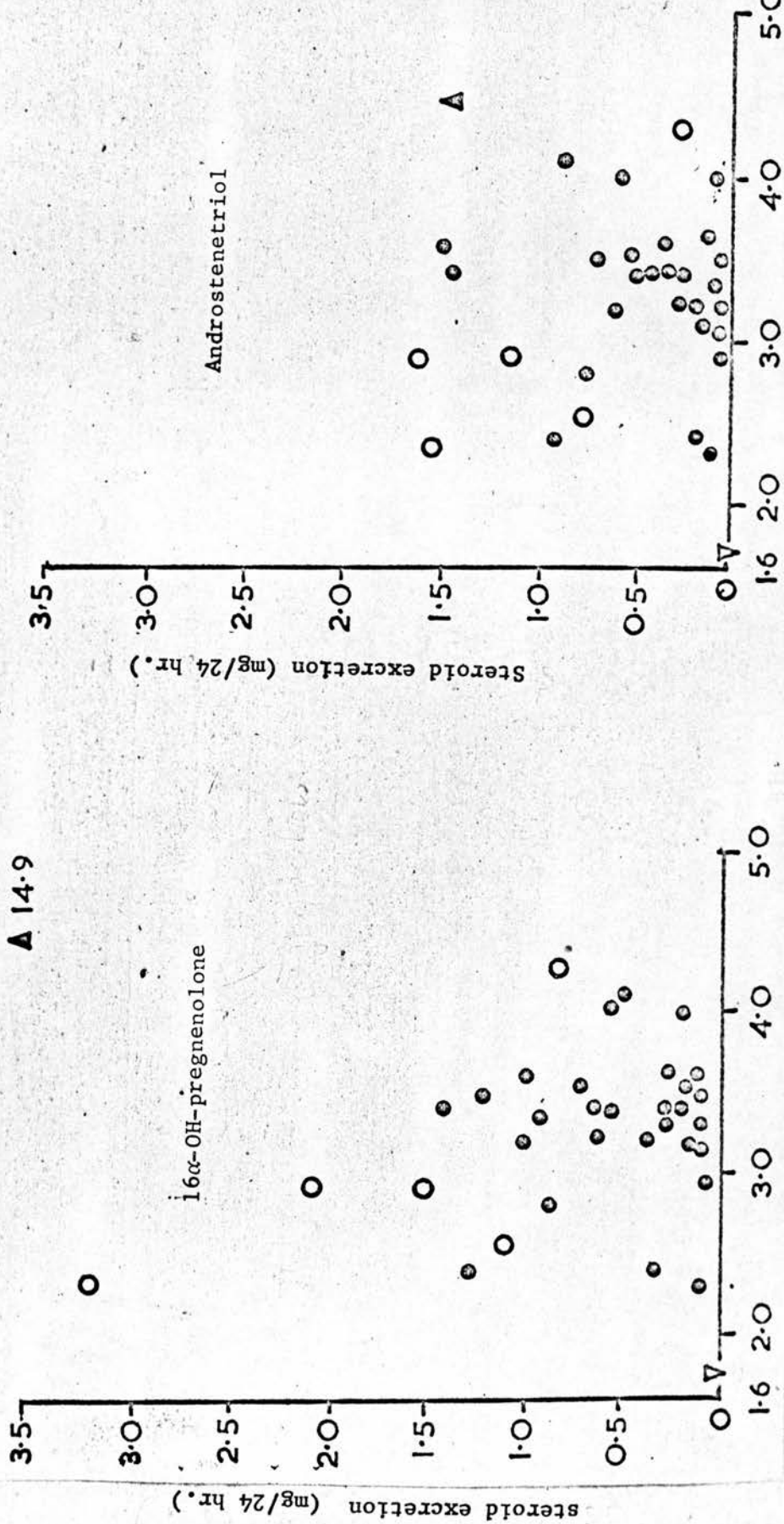
The mean values for the excretion of 21-OH-pregnenolone and compound U_1 were obtained only from the first 24 hour collection of the fourteen German infants.

Scatter diagrams have been drawn for the excretion of four of the principal 3β -OH- Δ^5 steroids; these are illustrated in Fig. 25 and 26. The steroid excretion has been related to infant birth-weight. Five of the German infants were possibly under stress as they had to be treated with adrenocorticotrophic hormone for therapeutic reasons (see p. 68). It was noticed that these infants tended to give a rather high excretion of steroids in the control period (first three days of life) so they have been illustrated differently (with open circles) on the diagram. Two infants with adrenal disorders have also been studied; one with adrenal hyperplasia and the other with adrenal hypoplasia. The steroid excretion of these infants is described in more detail later (see p. 73) but to illustrate their excretion of the 16-oxygenated 3β -OH- Δ^5 steroids relative to the excretion of these steroids by normal newborn infants, they have also been included on the diagrams.

Table 22 in the Appendix gives the daily excretion for the four infants studied for six consecutive days as controls for the infants treated with adrenocorticotrophin and human chorionic gonadotrophin. Fig. 27 illustrates the excretion of the principal compounds by these infants in the form of a histogram. From the mean values, a slight increase in excretion towards the middle of the period is apparent, but individual infants show widely variable results. Infant CON 1, for example, showed a fivefold increase in the excretion of 16α -OH-pregnenolone throughout the six days, whilst infant CON 2 showed a fall

Fig. 26. 16 α -OH-Pregnenolone and Androstenediol in Urine Samples Collected from Infants, Plotted in Relation to Birth Weight

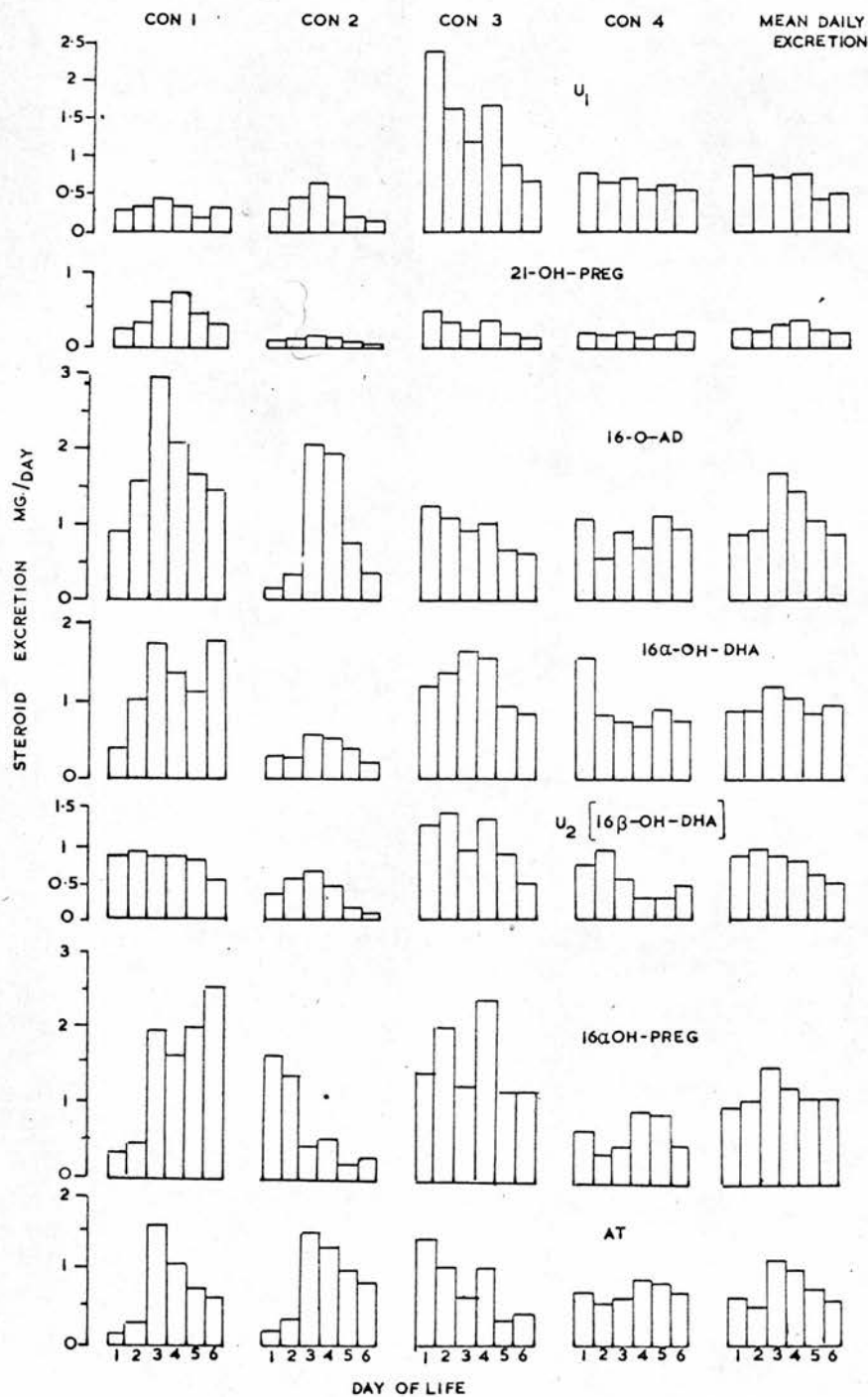
- Normal infants
- Infants that had been subject to stress
- ▲ An infant with congenital adrenal hyperplasia (S.G.)
- ▽ An infant with congenital adrenal hypoplasia (B.O.)



Infant weight (kg)

Infant weight (kg)

Fig. 27. $3\beta\text{-OH-}\Delta^5$ Steroid Excretion by Normal Infants During the First Six Days of Life



21-OH-PREG - 21-OH-pregnenolone
 16-O-AD - 16-oxo-androstenediol
 $16\alpha\text{-OH-PREG}$ - $16\alpha\text{-OH-pregnenolone}$
 AT - Androstenediol

in the excretion of this compound during the same period. In both these infants there is a gradual change apparent, but all four infants showed a considerable fluctuation in steroid excretion from day to day.

II. THE EFFECT OF ADRENOCORTICOTROPHIN ADMINISTRATION
ON STEROID EXCRETION BY NEWBORN INFANTS

The infants used for the study were all male and were being treated with adrenocorticotrophic hormone for prematurity, hyperbilirubinemia, anorexia with dyspepsia, or laryngitis subglottica. It was considered that in all cases the disorders were of non-endocrine origin and that the results found should not differ markedly from those to be expected in normal individuals. Adrenocorticotrophin was administered intramuscularly, twice daily, at a dose of eight units per day for three successive days. Urine was collected from these infants during treatment, and for the three days prior to treatment.

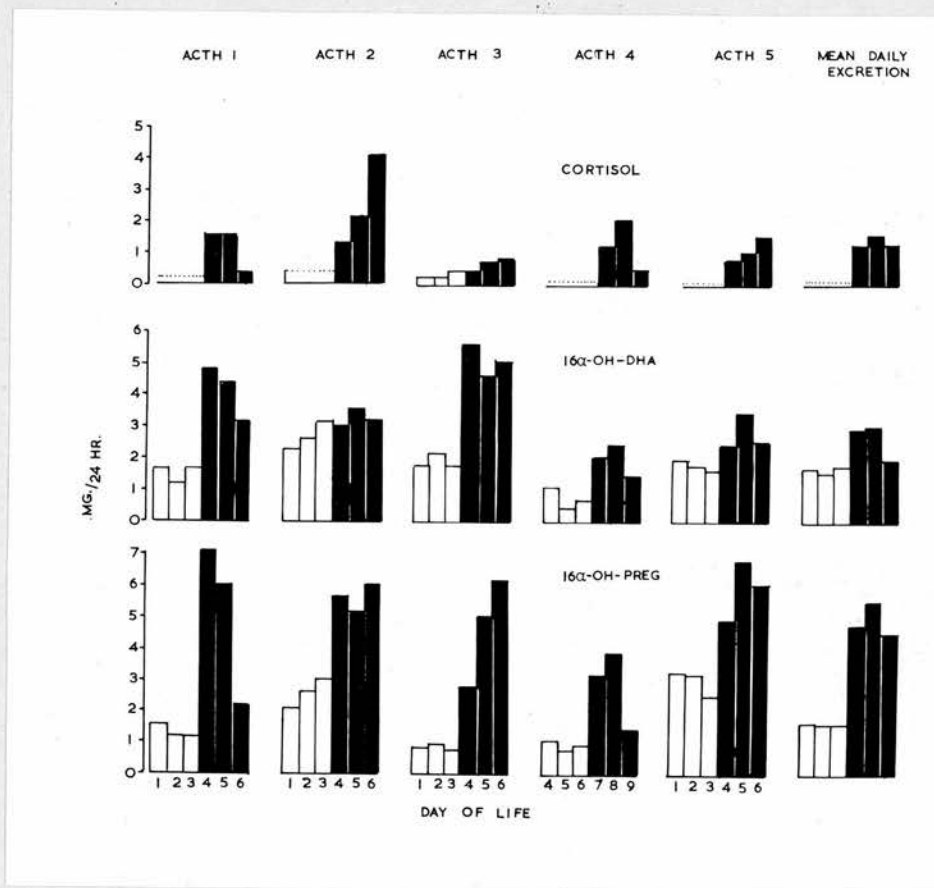
Table 23 (Appendix) gives the particulars of the infants studied and the results of the individual steroid assays for each infant. It was noted that the excretion of $3\beta\text{-OH-}\Delta^5$ steroid during the three control days was higher than for normal untreated control infants (Fig. 25). These infants may be producing a greater than normal amount of adrenocorticotrophin because of the "stress" of the clinical symptoms for which they were being treated. If there was an increase in endogenous adrenocorticotrophic hormone due to stress, this would result in stimulation of the adrenal glands to produce more steroid.

In nearly every case the excretion of all the $3\beta\text{-OH-}\Delta^5$ steroids was elevated after adrenal stimulation by administered adrenocorticotrophin. The average daily excretion of steroids for each infant for the three days prior to treatment, and the three days during treatment, was determined and the percentage increase calculated. The percentage increase for each infant for all the compounds assayed, and the average

Fig. 28. The Excretion of Steroids by Infants Treated with Adrenocorticotrophic Hormone (ACTH)

Histograms illustrating the increased excretion of cortisol, 16α -OH-DHA and 16α -OH-pregnenolone by infants treated with adrenocorticotrophin.

Infants



□ - Control period

■ - Days on which adrenocorticotrophin was administered

16α -OH-PREG - 16α -OH-pregnenolone

percentage increase of all the infants is given in Table 10. By comparing these results with the results given previously for four untreated infants whose urine was collected for six consecutive days (Table 22 and Fig. 27), it was apparent that adrenocorticotrophin administration gave rise to a very significant stimulation of $3\beta\text{-OH-}\Delta^5$ steroid production. This was particularly noticeable for $16\alpha\text{-OH-DHA}$ and $16\alpha\text{-OH-pregnenolone}$. Figure 28 shows the increased production of these two compounds for the five infants studied. It may also be significant that the $16\alpha\text{-OH-pregnenolone}/16\alpha\text{-OH-DHA}$ ratio increased from about 1.0 to 1.5 on treatment with adrenocorticotrophin. A higher ratio than normal was also found in cases of congenital adrenal hyperplasia where it is likely that adrenocorticotrophin is being produced in abnormally large amount (see Table 11).

Since DHA and pregnenolone are difficult to measure accurately due to their low concentration in urine, the assay of these steroids was only attempted for two of the infants studied. Both showed a considerable increase in the production of DHA and pregnenolone during treatment with adrenocorticotrophin; the absolute values for DHA excretion, however, remained very small in comparison to stimulation by human chorionic gonadotrophin (Table 24, Fig. 29).

The increased production of cortisol and its metabolites was even more significant. In the control period these compounds were generally not determined due to their low concentration in the urine, but an estimate was made of the smallest quantity that could be determined on each of the thin-layer plates, and the control values were reported as "less than" this figure. It was apparent that the increased excretion

TABLE 10

The Increase in Excretion of Steroids by Infants

Calculated from the total excretion of each steroid by five infants in the three
As a control, the average increase in excretion of steroids between the 4th, 5th
normal untreated infants is also given.

Infant	Treatment	Average urine vol. ml/24 hr.	Steroid excretion µg/24 hr.					
			Pregnenolone	DHA	Compound U ₁	21-OH-pregnenolone	Androstenediol	16-oxo-androstenediol
ACTH 1	None	290	<50	<50	320	190	98	1087
	ACTH	350	<50	<50	336	495	88	4052
	% increase				5	160	-10	200
ACTH 2	None	205	31	37	1140	352	713	2213
	ACTH	274	91	110	1024	285	527	1787
	% increase		300	198	-10	-19	-26	-19
ACTH 3	None	31	12	12	382	436	713	2573
	ACTH	265	18	19	462	711	1027	5130
	% increase		50	58	21	63	44	100
ACTH 4	None	127	<50	<50	260	213	98	1320
	ACTH	198	<50	<50	413	407	167	2253
	% increase				91	90	70	71
ACTH 5	None	290	<50	<50	633	207	70	2040
	ACTH	323	<50	<50	1173	647	75	2240
	% increase				85	213	7	10
Grand Average 1-5	None				547	275	338	1827
	ACTH				681	509	377	2935
	% increase				25	82	11	59
Grand Average of 4 un- treated infants	1st 3 days				820	243	69	1162
	2nd 3 days				585	226	65	1106
	% change				-29	7	6	5

Treated with Adrenocorticotrophin (A.C.T.H.)

days prior to treatment and the three days during treatment.

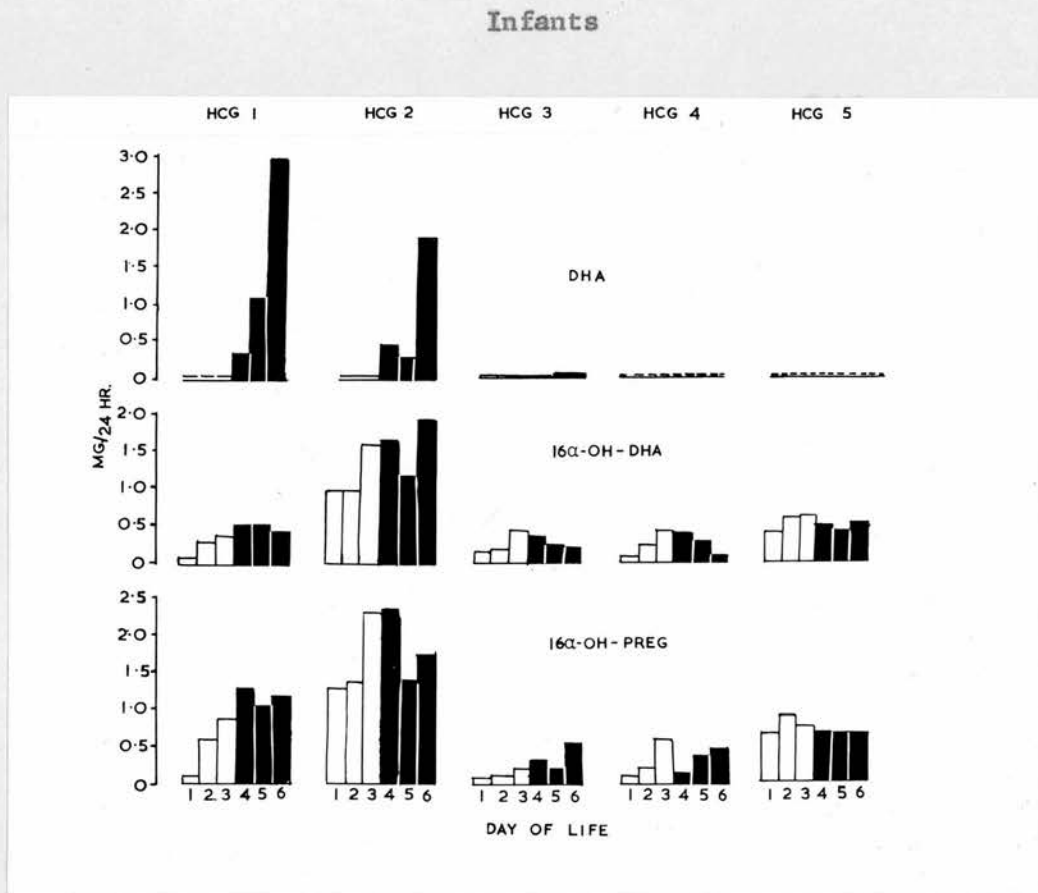
and 6th days and 1st, 2nd and 3rd days for four

16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenetriol	Cortisol	Tetrahydro Group
1460	220	1300	1327	<80	<40
4052	932	5088	1769	1000	1038
178	324	289	33	1100	2600
1660	1246	2460	1173	<400	<240
3240	886	5813	1153	2400	2733
95	-29	136	-2	500	1000
1887	1736	850	680	200	120
5080	1480	4653	1707	550	816
170	16	441	151	161	580
740	226	920	673	<100	<70
1967	586	2827	1007	1200	632
166	159	207	50	1100	802
1107	1533	2993	1467	<100	110
2815	2193	5933	1393	1080	737
156	41	98	-5	983	570
1371	1002	1707	1064	170	116
3430	1215	4863	1406	1250	1200
150	21	185	32	>600	>1000
975	853	1039	767		
932	604	1211	816		
4	-29	16	6		

of cortisol and cortisol metabolites during stimulation by adrenocorticotrophin was much greater than the increase in the excretion of $3\beta\text{-OH-}\Delta^5$ steroids.

Determination of the individual 17-oxosteroids present in the urine of these infants before, and during, treatment was also attempted but there was no apparent increase in any Zimmermann reacting compounds.

Fig. 29. The Excretion of Steroids by Infants Treated with Human Chorionic Gonadotrophin (HCG)



□ - Control period

■ - Days on which human chorionic gonadotrophin was administered

16α-OH-PREG - 16α-OH-pregnenolone.

This figure illustrates the increased excretion of DHA by two infants treated with human chorionic gonadotrophin: no increase was found in the other three infants. The excretion of the most important metabolite of DHA, 16α-OH-DHA, was not significantly increased even in those infants who showed a marked elevation of DHA production.

III. THE EFFECT OF HUMAN CHORIONIC GONADOTROPHIN
ON STEROID EXCRETION

The five infants chosen for this study were being treated with human chorionic gonadotrophin for the therapeutic effect of this hormone on undescended testicles. There was no reason to consider that any of these infants was endocrinologically abnormal.

After a three day control period, during which urine was collected to determine the normal values of steroid excretion, 5000 i.u. of human chorionic gonadotrophin was administered intramuscularly on the fourth, fifth and sixth days of life. The quantitative results for the excretion of $3\beta\text{-OH-}\Delta^5$ steroids by these infants is given in Table 24.

From the work of Lauritzen and Lehmann (1965, 1967), it was expected that the infants studied would show a greatly increased excretion of DHA during treatment with human chorionic gonadotrophin; two of the infants (HCG 1 and HCG 2) showed this effect (Fig. 29), but no significant increase in the production of DHA was apparent in the other three infants (HCG 3, HCG 4 and HCG 5).

The daily excretion of the major metabolite of DHA, $16\alpha\text{-OH-DHA}$, was only slightly increased (average increase 26%) during treatment with human chorionic gonadotrophin, even in the two infants who showed a large increase in DHA excretion. No significant elevation in the production of the other $3\beta\text{-OH-}\Delta^5$ steroids was apparent.

In order to investigate whether any increase in the production of cortisol occurred, the polar blue tetrazolium reacting steroids were separated and detected. In contrast with the effect of adrenocorticotrophin administration to newborn infants, the excretion of cortisol

and its metabolites was not affected by human chorionic gonadotrophin.

It is possible that the hormone preparation administered to the three infants who showed no response was inactive, since the infants who showed a response in the form of increased DHA excretion were treated with a different batch of human chorionic gonadotrophin.

IV. STEROID EXCRETION BY INFANTS WITH CONGENITAL
ADRENAL ABNORMALITY

The steroid excretion of three abnormal infants has been determined during the course of this study. Congenital adrenal hyperplasia was the diagnosis in two cases, and congenital adrenal hypoplasia in the third.

Congenital adrenal hyperplasia. Infant S.G.

This infant had a normal birth after 40 weeks gestation. His weight was above average, at 4432 g., and he appeared healthy at birth. On the fourth day of life he started vomiting; this was accompanied by loss in weight, and these symptoms continued for several days. Between the fifth and twelfth days of life there was a fall in the concentration of plasma sodium associated with an increase in the plasma potassium concentration. This electrolyte imbalance is often associated with congenital adrenal hyperplasia due to a defect in the production of cortisol. On the twelfth day of life the condition of the baby deteriorated rapidly and because of the provisional diagnosis of adrenal hyperplasia, he was given intravenous injections of cortisol. Twenty-four hour urine collections were taken on the seventh, eighth, twelfth, thirteenth and fifteenth days of life so that individual steroid assays could be carried out to determine the type of enzyme deficiency, if any, that was present in this patient.

The quantitative results for all the steroids determined are listed in Table 11, but the individual findings using the three staining reagents will be described in more detail for each day of collection. Fig. 30 illustrates in diagrammatic form the daily excretion of the principal steroids.

TABLE 11

The Excretion of Steroids by Infants with Congenital

(μg/24 hr.)

Birth weight gestation	Diagnosis	Day of life	Urine volume	Compound U ₁	21-OH-pregnenolone	Androstenediol	16-oxo-androstenediol	16α-OH-DHA	Compound U ₂ (16β-OH-DHA)	16α-OH-pregnenolone	Androstetriol	Unknown BT
S.G.	Congenital adrenal hyperplasia	7	150	1350	1350	638	6750	7425	995	14,925	1460	<50
4432g.		8	90	1040	220	165	640	2400	160	6900	1600	1000
40 wk.		12	480	780	30	<20	144	320	23	1470	780	2480
		13	230	540	<20	<20	60	200	12	1280	520	1280
		15	165	80	<20	<20	20	52	<10	408	80	1280
B.J.	Congenital adrenal hyperplasia	4	960	150	<50	150	100	100	20	1550	240	<50
*												
B.O.	Congenital adrenal hypoplasia	2	56	<10	<10	<10	45	98	<10	40	<10	850
1710g.												
38 wk.												

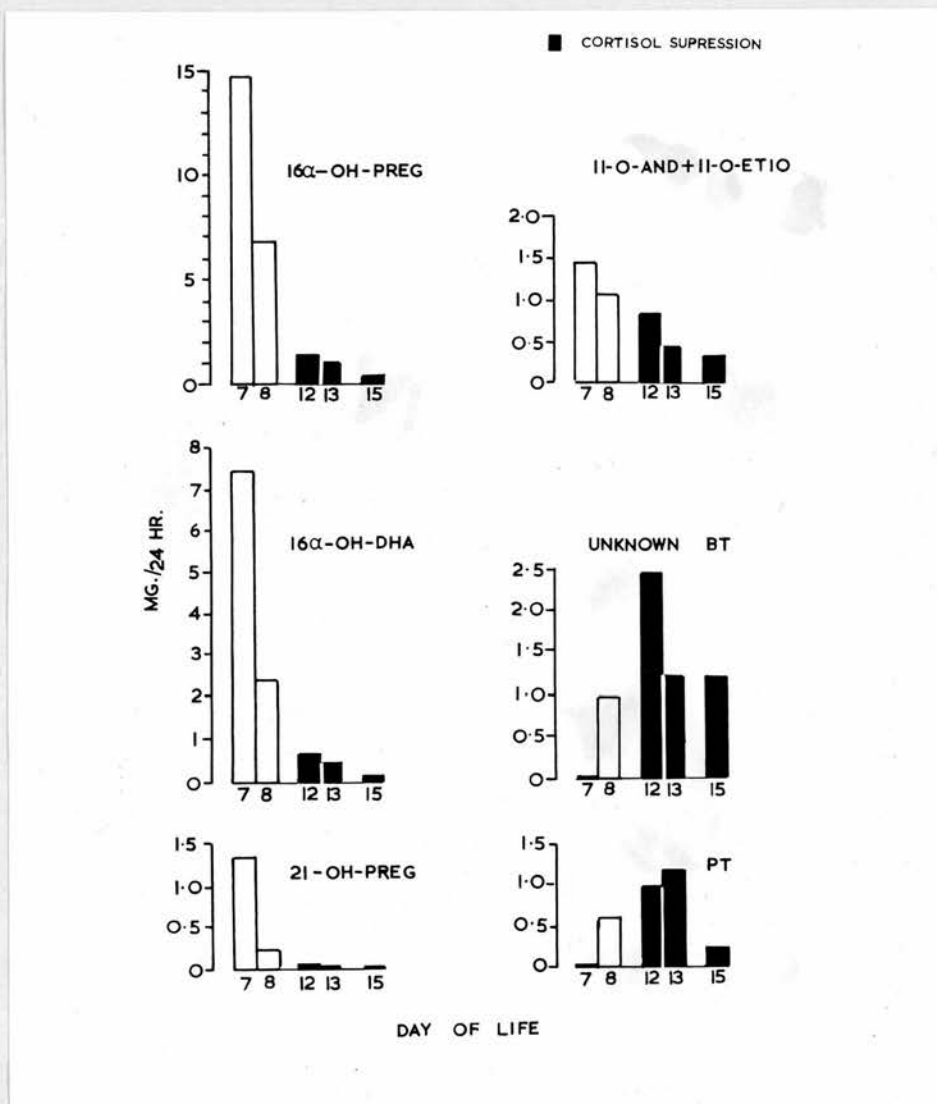
* Birth weight and gestation period not known

** The 17-oxosteroids were not assayed

Adrenal Hyperplasia or Hypoplasia

Pregnanetriol	11-oxo-androsterone	11-oxo-aetiocholanolone	Total 3 β -OH- Δ^5 steroid	$\frac{16\alpha\text{-OH-pregnenolone}}{16\alpha\text{-OH-DHA}}$	$\frac{16\alpha\text{-OH-pregnenolone}}{21\text{-OH-pregnenolone}}$	$\frac{16\alpha\text{-OH-DHA}}{U_2 (16\beta\text{-OH-DHA})}$	$\frac{16\alpha\text{-OH-DHA}}{\text{Androstenetriol}}$	Treatment (During day of collection)
<100	665	775	34,893	2.00	11.05	7.46	5.08	No treatment
600	500	553	13,125	2.89	31.36	15.00	1.50	No treatment
985	408	416	3547	4.59	49.00	13.91	0.41	Cortisol 150 mg.
1170	220	235	2612	6.40	>64.00	16.66	0.38	Cortisone acetate 75 mg.
208	155	160	640	7.84	>20.40	>5.20	0.65	Cortisone acetate 50 mg.
<50	<50		2320	15.50	31.00	5.00	0.42	Adrenocorticotrophin 20 units. Dexamethasone 1 mg. Deoxycorticosterone acetate 1 mg.
<10	-	-**	183	0.40	>4.0	>9.8	>9.8	No treatment

Fig. 30. The Excretion of Steroids by an Infant with Congenital Adrenal Hyperplasia (Patient S.G.)



Steroid abbreviations:

- 11-O-AND = 11-oxo-androsterone
- 11-O-ETIO = 11-oxo-aetiocholanolone
- 21-OH-PREG = 21-OH-pregnenolone
- 16α-OH-PREG = 16α-OH-pregnenolone
- PT = pregnanetriol
- UNKNOWN BT = Unidentified steroid

Legend:

This diagram illustrates the excretion of some of the principal steroids by patient S.G., an infant with congenital adrenal hyperplasia. Steroid assays carried out on the seventh day of life gave little indication of the enzyme deficiency since the urine contained a large amount of 21-OH-pregnenolone, and pregnanetriol could not be detected. However, the excretion of pregnanetriol had increased by the eighth day of life.

Steroid excretion on the seventh day of life

Antimony trichloride reaction:

All the $3\beta\text{-OH-}\Delta^5$ steroids measured were excreted in increased amount, generally between five and ten times the amount present in urine from normal newborn infants; this indicated a probable deficiency of 3β -hydroxy-dehydrogenase in this infant. This diagnosis was also considered probable due to the high excretion of 21-OH-pregnenolone, a compound which would be expected to be present in urine in small amounts if the adrenal hyperplasia was due to a deficiency of 21-hydroxylase. The ratio of $16\alpha\text{-OH-pregnenolone}$ to 21-OH-pregnenolone was slightly more than 11/1 which was greater than the average ratio found in normal infants (Table 9), but within the normal range. No pregnanetriol was detected, which also suggested that a deficiency at 21-hydroxylase was unlikely.

Blue tetrazolium reaction:

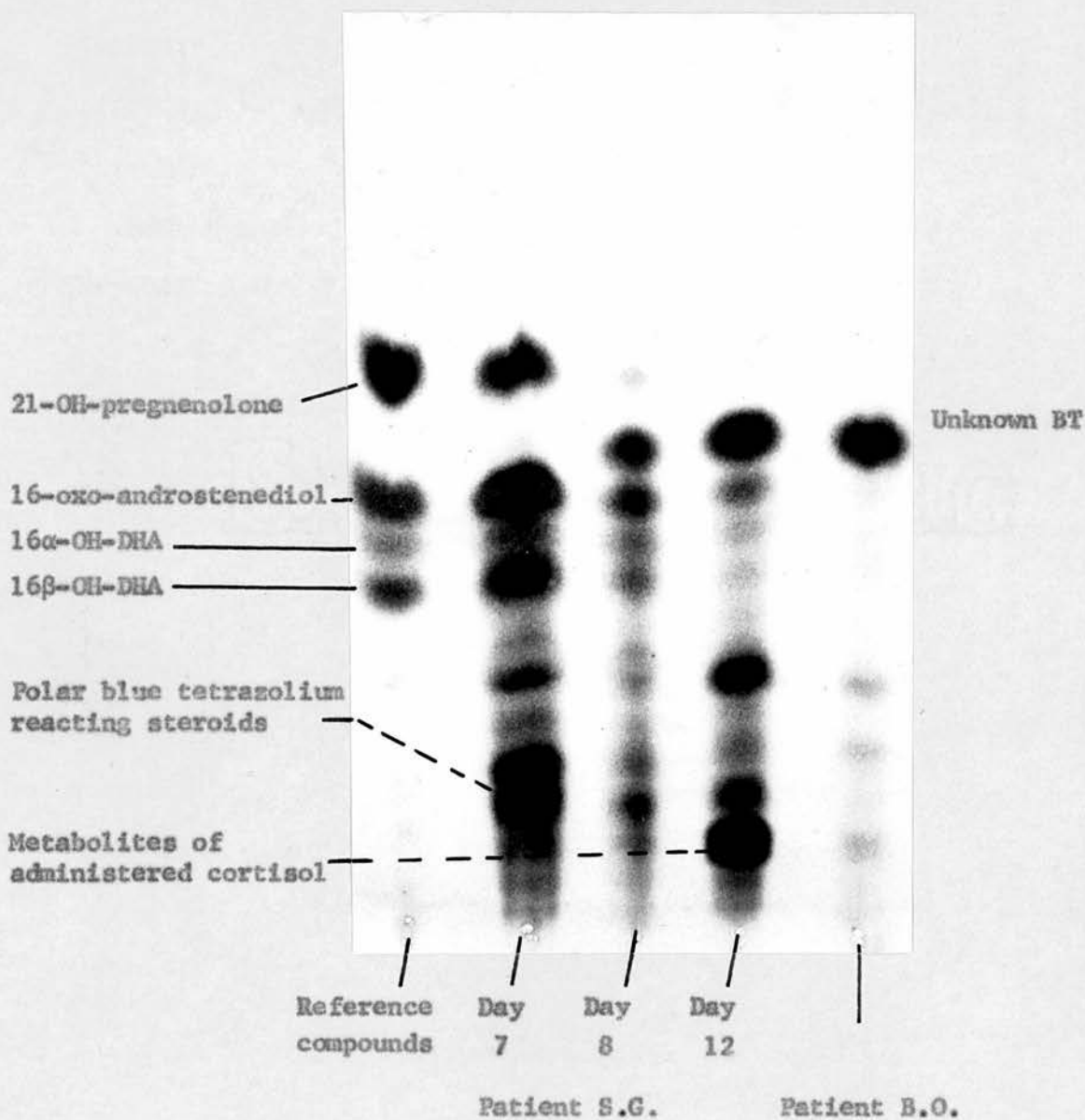
After separation of the medium polarity steroids in thin-layer system C (x 3), 21-OH-pregnenolone was again measured, this time using the blue tetrazolium reaction to check the identity of this compound.* The separation of the medium polarity blue tetrazolium staining compounds is illustrated in Fig. 31. The polar blue tetrazolium reacting steroids were separated using system G (x 2) and these appeared to be present in normal amount: this may not be significant as the identity of the major blue tetrazolium reacting steroids in urine obtained from normal infants is not known, and some are probably not metabolites of the corticosteroids. No specific measurement of cortisol metabolites was possible.

* As an additional precaution, 21-OH-pregnenolone was identified by combined gas chromatography-mass spectrometry.

Fig. 31. The Medium Polarity α -Ketolic Compounds Excreted by Patients S.G. and B.O.

Solvent system - C (x3)

Detecting reagent - Blue tetrazolium



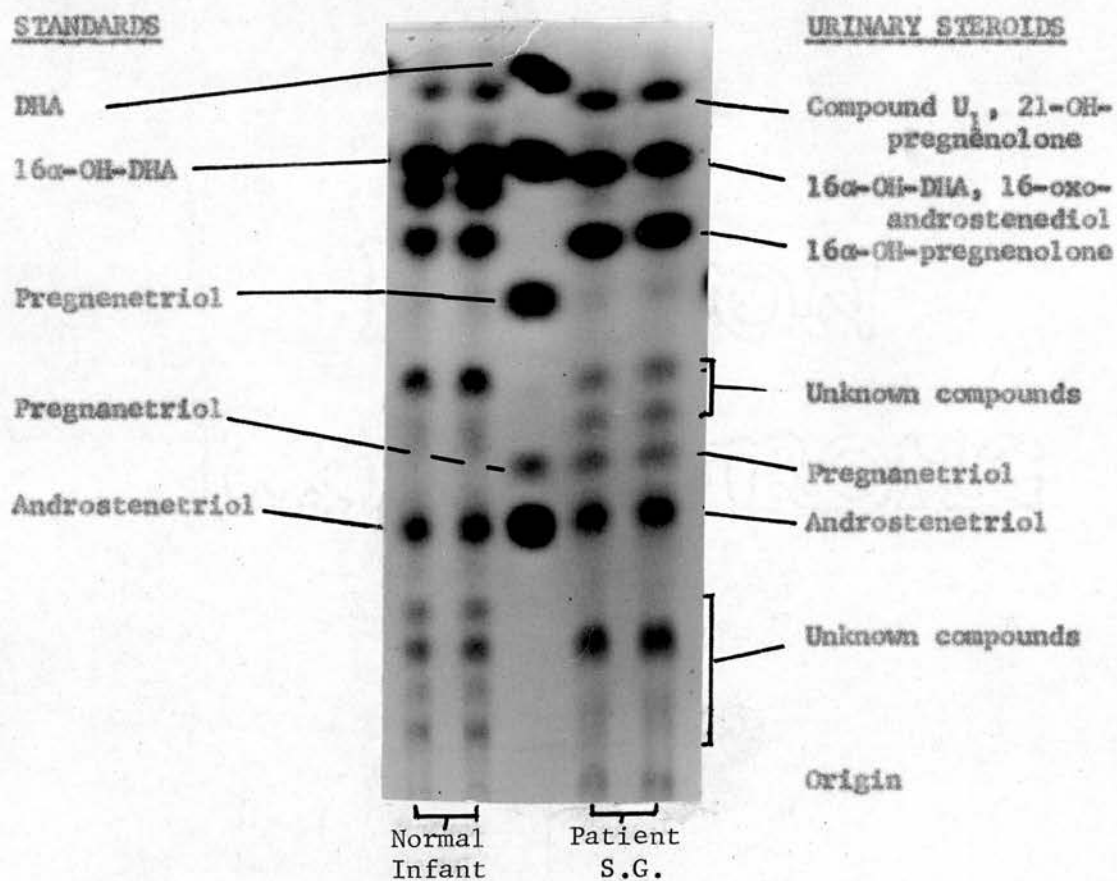
Legend:

This figure illustrates the large amounts of 16α -OH-DHA, 16-oxo-androstenediol, 16β -OH-DHA and 21-OH-pregnenolone present in the urine of patient S.G. on the seventh day of life. The levels of these compounds had fallen considerably by the eighth day (particularly 21-OH-pregnenolone), but a new blue tetrazolium-reacting steroid had appeared (Unknown BT); steroid assays on urine collected on the twelfth day of life indicated that the excretion of this compound had increased. A similar compound was detected in urine from an infant with congenital adrenal hypoplasia (Patient B.O.).

Fig. 32. Separation of the Antimony Trichloride Reacting Steroids Excreted by a Normal Infant and by Patient S.G.

Solvent system - G (x2)

Detecting reagent - Antimony trichloride



This figure illustrates the presence of pregnanetriol in the urine of patient S.G. (8th day of life) and the absence of this compound from urine obtained from normal infants. Other qualitative differences are also illustrated by the chromatographic separation.

Zimmermann reacting steroids

The separation of these compounds in system C (x 3) is illustrated in Fig. 20, together with the pattern shown by an extract from urine collected from normal infants. It was not possible to measure accurately the excretion of the 11-deoxy-17-oxosteroids nor the 11-hydroxy-17-oxosteroids, due to the presence of many unidentified Zimmermann chromogens, but 11-oxo-androsterone and 11-oxo-aetiocholanolone gave clear spots and were present in abnormal amount.

Steroid excretion on the eighth day of life

Antimony trichloride reaction:

The 3β -OH- Δ^5 steroids were present in lower concentration than the previous day, but were still in greater amount than in urine from normal infants. A particularly interesting trend was seen in the relative amounts of 21-OH-pregnenolone and the other 3β -OH- Δ^5 steroids. The ratio of 16α -OH-pregnenolone to 21-OH-pregnenolone increased to 31/1 on the eighth day of life. This was greater than the figure for the seventh day (11/1) and much greater than the ratio obtained for normal infants (Table 9). In contrast to the fall in excretion of 21-OH-pregnenolone, there was a marked increase in the excretion of pregnanetriol*. Taken together, these measurements indicated a probable deficiency of 21-hydroxylase, a deficiency that was not apparent from steroid determinations carried out on urine collected on the previous day.

Blue tetrazolium reaction:

The assay of the medium polarity blue tetrazolium reacting steroids also showed a decrease in 21-OH-pregnenolone, and this was associated with

* The presence of pregnanetriol in urine is easily detected by thin-layer chromatography (Fig. 32). This compound was finally identified in urine obtained from patient S.G. by combined gas chromatography-mass spectrometry.

the appearance of an abnormal steroid (see Fig. 31). This blue tetrazolium reacting compound (hereafter referred to as "Unknown BT") was intermediate in polarity between 21-OH-pregnenolone and 16-oxo-androstenediol when stained after developing the thin-layer plate three times in system C; it was present in greater amount than 21-OH-pregnenolone. A blue tetrazolium reacting compound of this polarity has never been detected in urine obtained from normal infants, although a similar compound was the principal "steroid" excreted by an infant with adrenal hypoplasia (Infant B.O.). There is, however, little evidence to prove that this compound is a steroid.

The polar steroids (possibly corticosteroid metabolites) assayed by this reaction were not present in such large amount on the eighth day of life (Fig. 31). When considered with the finding of a reduced amount of 21-OH-pregnenolone and an increased excretion of pregnanetriol, this probably represents a progressive decline in the ability of the infant to form 21-hydroxylated corticosteroids.

Zimmermann reacting steroids:

11-oxo-androsterone and 11-oxo-aetiocholanolone were again present in much greater amount than in urine samples from normal infants, and the levels of these compounds were similar to those found for Day 7.

Steroid excretion on the twelfth, thirteenth and fifteenth days of life

During this period cortisol was administered, because of the rapid deterioration in the condition of the baby. Consequently, there was suppression of steroid production and the appearance of cortisol metabolites in the urine.

The excretion of $3\beta\text{-OH-}\Delta^5$ steroids decreased and, by the fifteenth

day, the excretion of the 16-oxygenated compounds was below the average value for normal newborn infants. However, the urinary excretion of pregnanetriol and Unknown BT increased considerably during the first two days of cortisol administration. The excretion of the 11-oxo-17-oxosteroids decreased with cortisol treatment.

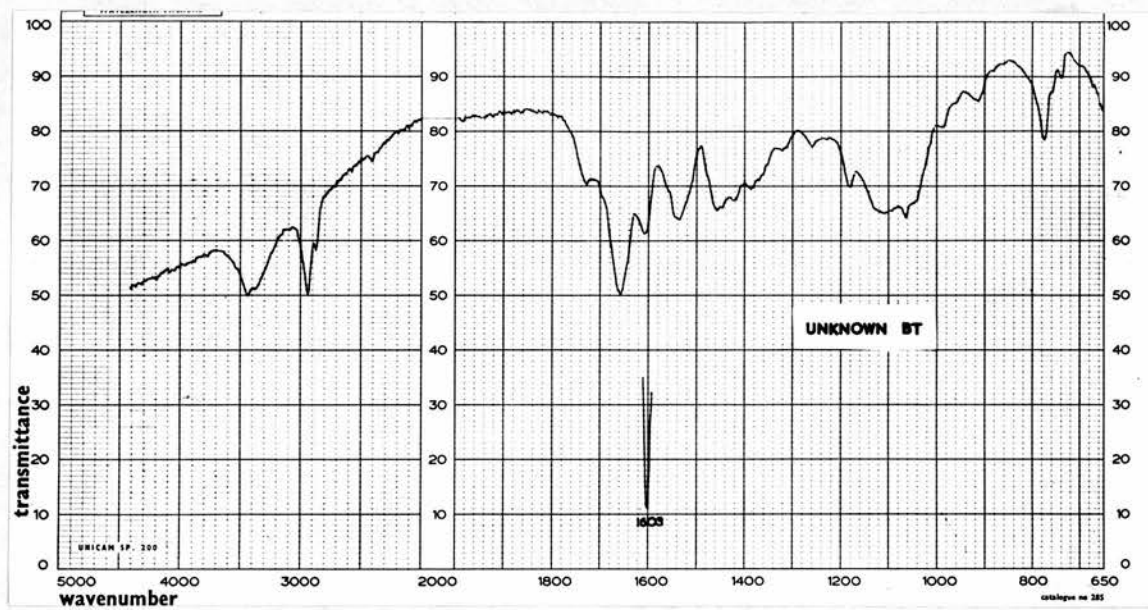
Congenital adrenal hyperplasia. Infant B.J.

The steroid excretion of this infant was determined early in this study, before suitable techniques had been developed for a full investigation of the steroid production by newborn infants. The baby had abnormal male external genitalia, and showed evidence of salt imbalance. Urine was collected on the third and fourth days of life, but since birth the infant had been treated with dexamethasone, deoxycorticosterone acetate and adrenocorticotrophin so the steroid assays could not give an accurate picture of the untreated disorder. The results of the assay of $3\beta\text{-OH-}\Delta^5$ steroids are given in Table 11. $16\alpha\text{-OH-pregnenolone}$ was present in relatively large amount compared to the other steroids. Although they were not accurately assayed, the 17-oxosteroids did not appear to be present in abnormal amount. Pregnanetriol was detected but not measured quantitatively; by visual assessment, it was estimated that the excretion of pregnanetriol was about 200 $\mu\text{g}/24$ hr. The disorder therefore appeared to be congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

Congenital adrenal hypoplasia. Infant B.O.

Infant B.O. was born after 38 weeks gestation and weighed 1710 g. The baby was male but had maldeveloped gonads. He died from respiratory failure after 30 hours of life. An abnormally low level of oestriol was

Fig. 33. Infrared Spectrum of the Unknown Blue Tetrazolium Reacting Steroid (Unknown BT) Extracted from the Urine of Infant B.O.



found in plasma obtained from the mother before the baby was born; this suggested hypoplasia of the foetal adrenals. Post-mortem examination of the infant revealed the presence of small adrenal glands, thereby confirming the diagnosis.

A complete 24 hour collection of urine was taken for steroid analysis. The three major 16-oxygenated 3β -OH- Δ^5 steroids were present in sufficient quantity to be assayed and the results are given in Table 11. These results are compared with the findings for normal infants in Fig. 25. Although the amounts excreted were extremely low, they were within the range found for normal infants as illustrated in Table 21 (in the Appendix).

The blue tetrazolium reaction demonstrated the presence of a relatively large amount of a compound of polarity intermediate between 16-oxo-androstenediol and 21-OH-pregnenolone (Fig. 31). This compound was designated "Unknown BT" and its polarity was identical to a similar compound found in urine collected from patient S.G., an infant with congenital adrenal hyperplasia. Fig. 31 also illustrates the very small amount of 16-oxo-androstenediol and 16α -OH-DHA that is present, since they can hardly be seen when an amount of extract sufficient for the assay of Unknown BT is separated on the plate. Unknown BT does not react with either the antimony trichloride or the Zimmermann reagents. It is not extracted by solvent, prior to hydrolysis of the urinary steroid conjugates, so it is likely that it is present in urine as an enzyme hydrolysable conjugate. An infra-red spectrum of the compound isolated from the urine of infant B.O. is illustrated in Fig. 33. Unfortunately there is insufficient evidence available for a structure to be assigned to this compound.

V. THE QUANTITATIVE ESTIMATION OF $3\beta\text{-OH-}\Delta^5$
STEROIDS IN PLASMA OBTAINED FROM THE UMBILICAL BLOOD
VESSELS.

Blood was collected from the umbilical blood vessels of infants delivered by Caesarian section. It was considered that the quantity of steroid present under these circumstances would indicate the normal foetal production of steroids more closely than if the collection had been made after the considerable stress of a normal birth. The venous and arterial blood obtained from the umbilical blood vessels was independently withdrawn through catheters by gently squeezing the umbilical cord along its length. The blood was collected into heparinised receptacles, and the plasma was separated as soon as possible. In most of the cases studied it was possible to obtain sufficient plasma for duplicate assays of venous and arterial plasma, but for some it was not possible to obtain any arterial plasma, and in others amounts sufficient for only a single assay were obtained.

Both DHA sulphate and $16\alpha\text{-OH-DHA}$ sulphate are synthesised by the foetus and are probably precursors of oestrogens. The oestrone, oestradiol and oestriol were therefore determined in urine collected from the mother during the week prior to delivery. These assays were performed by Mrs. E. A. Michie who used the method of Coyle and Brown (1963).

The results of all the assays are given in Table 25 in the Appendix. This table also gives the arterio-venous gradient (arterial value in $\mu\text{g}/100\text{ ml.}$ minus the venous value in $\mu\text{g}/100\text{ ml.}$) for each of the steroids assayed, and this figure expressed as a percentage of the arterial assay. Eleven of the fourteen cases studied (Table 25, Nos. 1 - 11) were

TABLE 12

The Mean Values and Ranges of 3β -OH- Δ^5 Steroids in Plasma Obtained from the Blood Vessels in the Umbilical

Cord and the Corresponding Figures for Oestrogens in Urine Collected from the Mother

		3 β -OH- Δ^5 Steroids in Plasma Samples (μ g/100 ml.)						Oestrogens in Urine (mg/24 hr.)					
		DHA	17 α -OH-preg- nenolone	21-OH- pregnenolone	16 α -OH-DHA	U ₂ (16 β -OH-DHA)*	16 α -OH- pregnenolone	Androstenediol	Oestrone	Oestradiol	Oestriol		
ARTERIAL													
	Mean	91.4	80.2	56.6	62.4	342.7	27.1	178.1	122.6	Mean	0.87	0.258	19.9
	Range	56-157	47-107	25-103	30-115	114-560	10-73	35-309	10-240	Range	0.36-2.26	0.031-0.52	5.3-34.6
VENOUS													
	Mean	64.0	63.3	46.3	55.6	276.5	20.6	141.7	111.4				
	Range	27-147	24-100	14-94	18-115	93-525	10-52	34-305	24-220				
	Average A-V	27.4	16.9	10.3	6.8	66.2	6.5	36.4	11.2				
	$\frac{A-V}{A}$ x 100%	30.0	21.1	18.2	12.5	19.4	24.0	20.2	9.25				

* This compound was only determined in six cases.

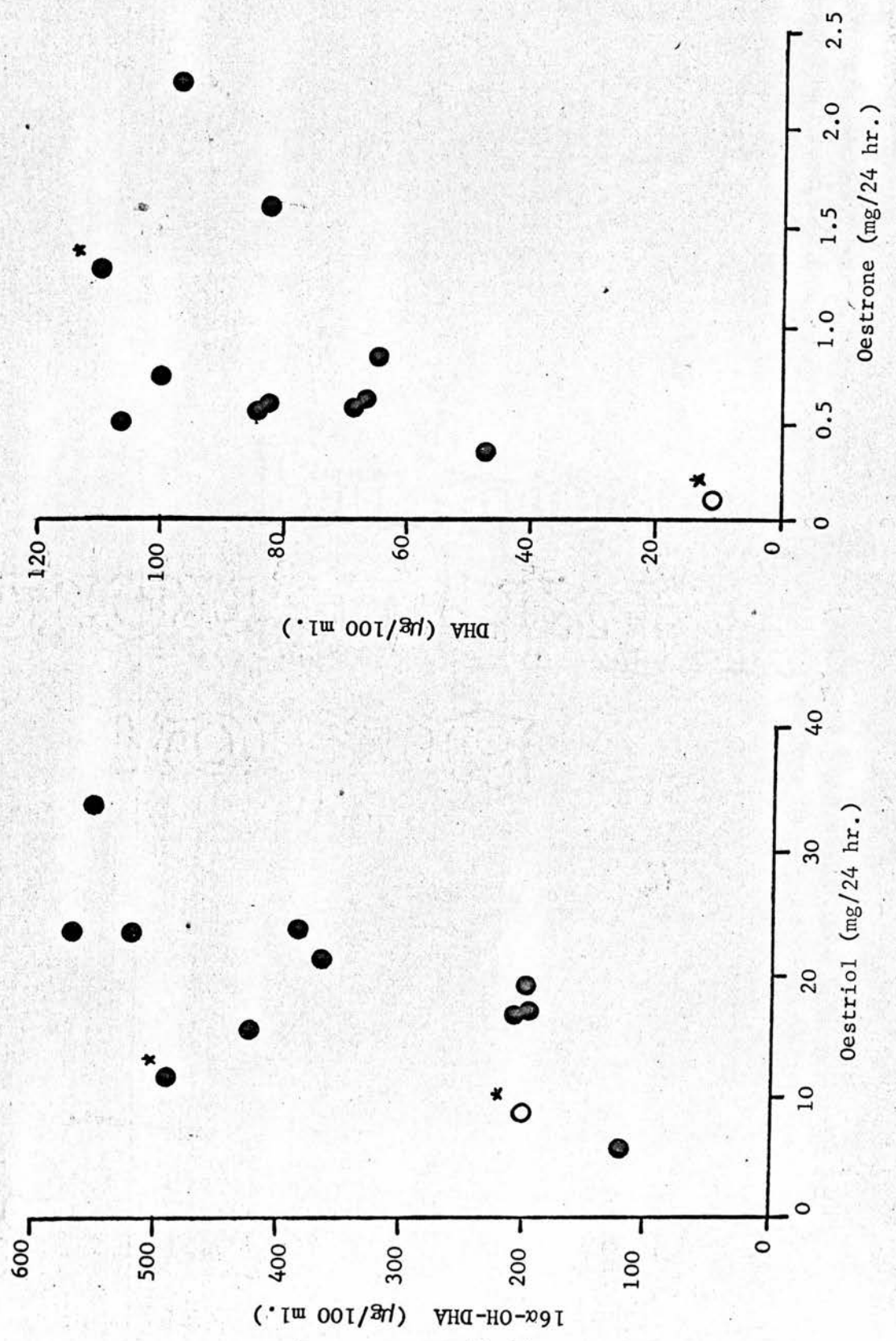
considered normal; two of the other infants were in a weak state immediately after birth (Table 25, Infants 12 and 13) and one of these infants died on the second day of life (Infant 12). Infant 14 was delivered from a woman who had been taking prednisolone during pregnancy.

The average values and the range for each of the compounds measured in the eleven "normals" are given in Table 12. Although 16-oxo-androstenediol and 16 α -OH-DHA were determined independently in extracts of urine collected from infants, only in the preliminary studies on plasma was a thin-layer system used that was capable of separating the isomers. It was found in these early experiments that 16-oxo-androstenediol was present in the plasma at a level only about one-tenth that of 16 α -OH-DHA, and it was not considered necessary to assay this compound separately.

All the compounds determined were usually present in greater concentration in plasma obtained from the arteries than in plasma obtained from the vein; this indicates a net uptake of these steroids by the placenta. The plasma levels of 16 α -OH-DHA and DHA have been compared individually with the corresponding results for oestrone and oestriol obtained by assaying these compounds in urine collected from the mother (Fig. 34). From the limited number of results available, there seemed to be a slight correlation between these findings. Little correlation was apparent between the birth weight of the infants and the concentrations of 16 α -OH-DHA in the plasma samples (Fig. 35).

The infant born of the mother taking prednisolone (Table 25, Infant 14) gave lower than normal results for all the 3 β -OH- Δ^5 steroids; the concentration of DHA and pregnenolone was particularly low, the remaining steroids being within the normal range. These low results

Fig. 34. DHA and 16 α -OH-DHA in Plasma Samples Obtained from the Umbilical Blood Vessels Plotted in Relation to the Oestrogen Excretion of the Mother



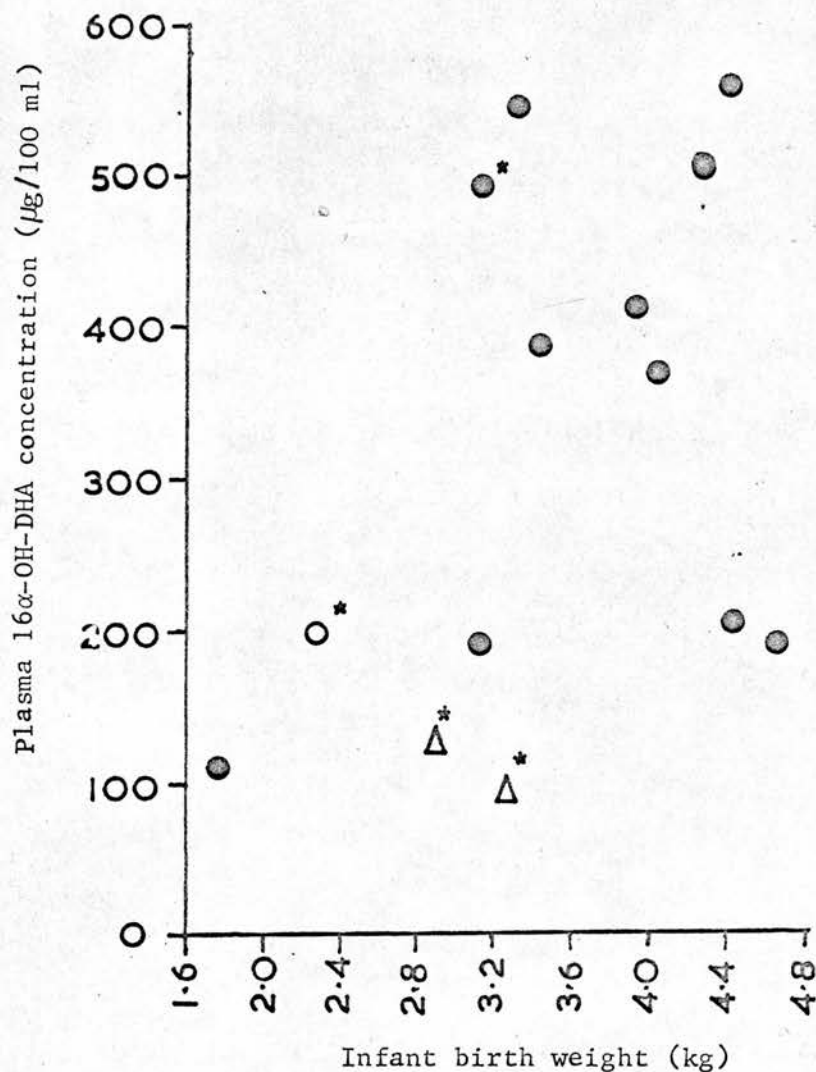
● Normal arterial plasma

○ This analysis was carried out on plasma obtained from an infant whose mother had been taking prednisolone during pregnancy.

Prednisolone would be expected to suppress the production of adrenal steroids by the foetus.

* These assays were carried out on venous plasma; arterial plasma was not available.

Fig. 35. 16 α -OH-DHA in Plasma Obtained from the Umbilical Blood Vessels,
Plotted in Relation to Infant Birth Weight



● Normal infants (arterial blood)

○ This analysis was carried out on plasma obtained from an infant whose mother had been taking prednisolone during pregnancy. Prednisolone would be expected to suppress the production of adrenal steroids by the foetus.

△ These infants were extremely weak at birth and one died on the second day of life.

* These assays were carried out on venous plasma; arterial plasma was not available.

indicated suppression of the "feed-back" mechanism in the foetus.

Insufficient plasma was obtained from the umbilical blood vessels from infants 12 and 13 for accurate assay, but both showed a relatively small concentration of 16α -OH-DHA compared to the normals.

In the method for the preliminary purification of steroid conjugates by Sephadex column chromatography, unconjugated steroids present in the first 100 ml. of the column eluant were normally discarded. However, an experiment was carried out to determine how much, if any, of the 3β -OH- Δ^5 steroids assayed in the conjugated fraction were also present as free steroids in plasma obtained from the umbilical vein.

It was not possible to measure directly the 3β -OH- Δ^5 steroids present in the free steroid fraction, due to the presence of a large amount of lipid material which interfered with the chromatography. It was therefore necessary to remove as much of this material as possible before the final TLC separation and quantitative measurement. This purification was achieved by preparative TLC using system C (x 2). Since all the known 3β -OH- Δ^5 steroids present in plasma obtained from the umbilical blood vessels have mobilities between pregnenolone and androstenetriol, all the silica gel between the positions of these two steroids on the plate was removed and eluted with ether. This effectively removed most of the non-polar and highly polar lipid, retaining only compounds of polarity between pregnenolone and androstenetriol. The eluate was dried and the residue was applied as a spot to a thin-layer plate, which was developed three times in system C. After development the plate was sprayed with antimony trichloride to detect the 3β -OH- Δ^5 compounds, if they were present. No spots corresponding to any 3β -OH- Δ^5

steroids could be detected, and it was estimated therefore that none of these compounds could be present at a concentration greater than 5 $\mu\text{g}/100$ ml. plasma. The concentration of free $3\beta\text{-OH-}\Delta^5$ steroids in plasma obtained from the umbilical blood vessels is therefore very low.

Apart from the $3\beta\text{-OH-}\Delta^5$ steroids determined quantitatively in plasma obtained from the umbilical blood vessels, three other compounds were detected but not accurately measured. These were pregnenediol, pregnenetriol and androstenetriol, Although they were assayed in a pool of venous plasma obtained from several umbilical cords. The results obtained were:-

Pregnediol	50 $\mu\text{g.}/100$ ml.
Pregnenetriol	65 $\mu\text{g.}/100$ ml.
Androstenetriol	68 $\mu\text{g.}/100$ ml.

VI. THE CONJUGATION OF 3β -OH- Δ^5 STEROIDS PRESENT IN URINE OBTAINED FROM INFANTS, AND IN PLASMA COLLECTED FROM THE UMBILICAL BLOOD VESSELS.

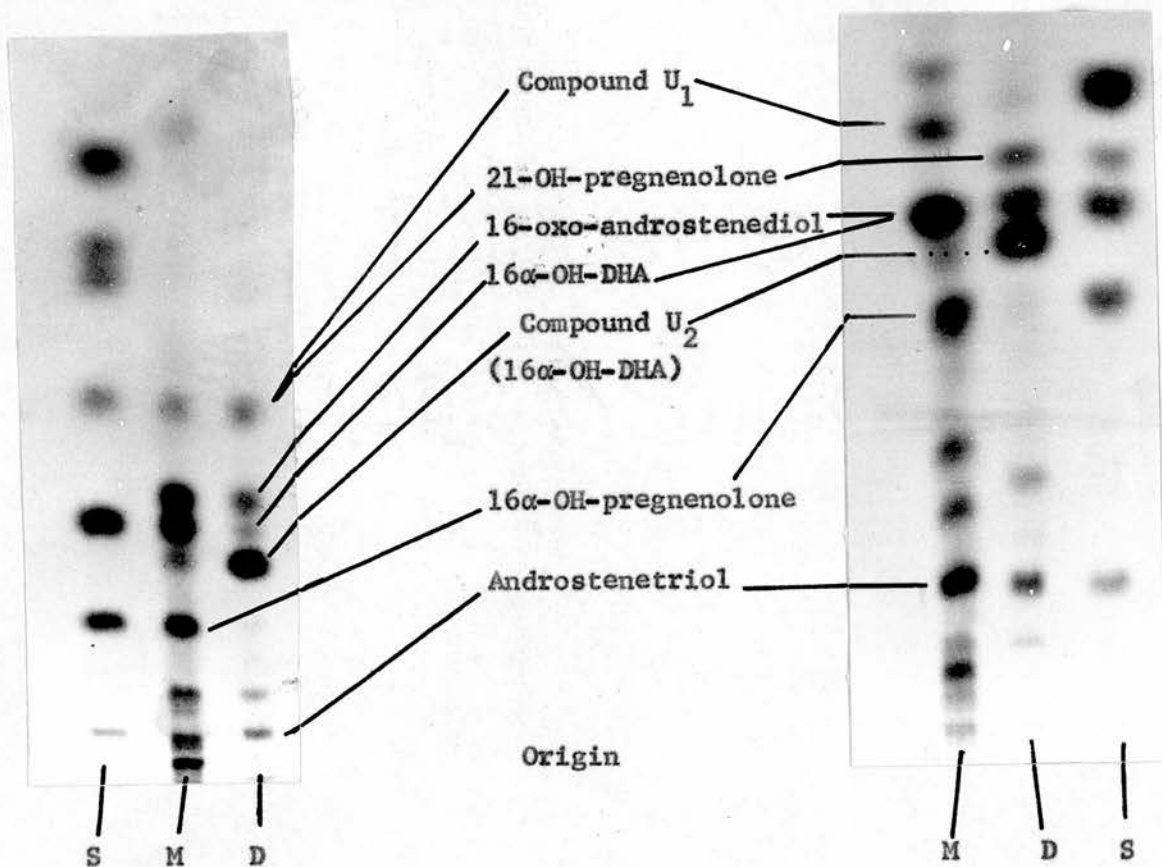
The method used for the extraction and fractionation of plasma steroid conjugates has been given in detail in Part I (p. 31). An identical method was used for the preparation of the crude extract of steroid conjugates obtained from urine collected from infants. After applying this extract to the column of Sephadex LH 20, sequential 25 ml. fractions of the eluting solvent (methanol:chloroform, 1:1; saturated with sodium chloride) were collected, dried, dissolved in acetate buffer, and were hydrolysed with *Helix pomatia* sulphatase and glucuronidase (Part I, sections 2.3, 2.4 and 2.5). After extraction with solvent, a portion of each fraction was separated by TLC to determine which contained steroids. The fractions containing steroids were then pooled to form two fractions, one containing the steroid monoconjugates, the other the steroid diconjugates. For some unexplained reason the conjugates obtained from urine were eluted from the column with a much smaller volume of eluting solvent than was required for the plasma conjugates; a satisfactory separation of steroid monoconjugates from free steroids and lipid material was not obtained. The monoconjugates appeared in the eluant after 50 ml. of solvent had passed through the column; no more monoconjugates were obtained after 150 ml. had been collected. The diconjugates were eluted between 250 ml. and 400 ml.

The steroids present in the monoconjugated and diconjugated fractions of plasma and urine were assayed by TLC, and the percentage of

Fig. 36. The 3β -OH- Δ^5 Steroids Present in the Monoconjugated and Diconjugated Fractions Prepared from Urine Collected from Infants and Separated by Sephadex Chromatography

Solvent system C (x3)
(separates 16-oxo-androstenediol
from 16 α -OH-DHA)

Solvent system F (x2)
(separated compound U₁
from 21-OH-pregnenolone)



- S - Standard compounds
- M - Monoconjugated derivatives
- D - Diconjugated derivatives - the amounts applied to the chromatograms was equivalent to twice the quantity of extract used for the separation of the monoconjugated derivatives.

each steroid in each fraction was determined. The results of these assays are given in Tables 13 and 14. Figure 36 illustrates the thin-layer separation of the $3\beta\text{-OH-}\Delta^5$ steroids obtained from urine collected from infants. Two different development systems were used; system C (x 3) for resolving 16-oxo-androstenediol and 16 α -OH-DHA, and system F (x 2) for the separation of compound U_1 and 21-OH-pregnenolone. The figure illustrates that most of the 16 β -OH-DHA (U_2), and all the 21-OH-pregnenolone, are present in the diconjugate fraction, the other $3\beta\text{-OH-}\Delta^5$ steroids being mainly in the monoconjugate fraction. It can be seen that almost all the uncharacterised polar compounds are excreted as monoconjugates. It is likely that most of the $3\beta\text{-OH-}\Delta^5$ steroids are sulphated and that some, particularly 16 β -OH-DHA and 21-OH-pregnenolone, are predominantly disulphated. This study has demonstrated that the *Helix pomatia* enzyme preparation has enzymes capable of hydrolysing the 16 α -, 16 β - and 21- esters of $3\beta\text{-OH-}\Delta^5$ steroids. Androstenediol and pregnenetriol are present in the diconjugated fraction that can be hydrolysed by solvolysis following enzymatic hydrolysis; it is probable that these conjugates are disulphates, but that the 17 α -sulphate and the 20 α -sulphate are not hydrolysed by the enzymes present in the digestive juices of *Helix pomatia*.

TABLE 13

The State of Conjugation of 3 β -OH- Δ^5 Steroids Present in UrineCollected from Infants

The steroid sulphates in urine were separated into a monosulphate and a disulphate fraction by Sephadex LH 20 chromatography. After hydrolysis of the steroid sulphates by *Helix pomatia* sulphatase, the steroids in the monosulphate and disulphate fractions were separated by TLC and assayed.

The percentage of each steroid present, as its monoconjugated and diconjugated derivatives, is listed in the table.

	Monoconjugates (%)		Diconjugates (%)	
	Pool 1*	Pool 2	Pool 1	Pool 2
Compound U ₁	100	100	0	0
21-OH-pregnenolone	0	0	100	100
Androstenediol	0	0	100	100
16-oxo-androstenediol	78	80	22	20
16 α -OH-DHA	89	77	11	23
Compound U ₂ (16 β -OH-DHA)	34	42	66	58
16 α -OH-pregnenolone	100	94	0	6
Androstenetriol	81	90	19	10

* Two samples of pooled urine were used for these analyses.

TABLE 14

The State of Conjugation of 3 β -OH- Δ^5 Steroids Present in Plasma Obtained
from the Umbilical Blood Vessels

The steroid sulphates in plasma samples were separated into a monosulphate and a disulphate fraction by Sephadex LH 20 chromatography. After hydrolysis of the steroid sulphates by *Helix pomatia* sulphatase, the steroids in the monosulphate and disulphate fractions were separated by TLC and assayed.

The percentage of each steroid, as monoconjugated and diconjugated derivative, is listed in the table.

	Monoconjugate (%)	Diconjugate (%)
Pregnenolone	100	0
DHA	100	0
17 α -OH-pregnenolone	100	0
Pregnenediol	100	0
21-OH-pregnenolone	0	100
16-oxo-androstenediol	50	50
16 α -OH-DHA	90	10
U ₂ (16 β -OH-DHA)	0	100
16 α -OH-pregnenolone	100	0
Androstenetriol	60	40

PART 3

THE IDENTIFICATION OF STEROIDS

I. METHODS USED IN THE IDENTIFICATION OF STEROIDS

1. Chemical methods for the formation of steroid derivatives

1.1 The separation of ketonic and non-ketonic steroids

Steroids containing carbonyl groups (ketonic steroids) form water soluble hydrazones when reacted with Girard's reagent T; this enables them to be separated from steroids without carbonyl groups. After formation of the hydrazones, the unreacted steroids (i.e. steroids without any ketone groups) can be extracted from the aqueous phase with organic solvents. Since steroid hydrazones are hydrolysed by acid, the ketonic steroids may be recovered by acidification to pH 1 followed by extraction into organic solvent.

Most steroid carbonyl groups form Girard hydrazones. An 11-ketone group, however, will not form a derivative due to steric hindrance of this position by the C-18 and C-19 methyl groups of the steroid molecule.

The formation of Girard hydrazones is often used to simplify the purification of steroids from crude extracts of biological materials. This reaction is also carried out to determine whether or not an unknown steroid contains a carbonyl group.

Formation of Girard hydrazones (Bush, 1961)

(i) The steroid (or steroid mixture) was dissolved in 0.1 ml of glacial acetic acid.

(ii) Girard reagent T (10 mg) was added, and the mixture either left overnight at room temperature or heated in a water bath at 90° for 20 minutes.

(iii) Three ml. water and 0.7 ml. 10% sodium hydroxide (w/v) was added. This mixture was extracted with three 10 ml. portions of ether to obtain the non-ketonic steroids.

(iv) Hydrochloric acid (50%, v/v) was added to the aqueous phase to bring the solution to pH 1. This mixture was continuously extracted for two hours with 10 ml. ether, and the extraction was repeated twice with further portions of ether (10 ml.). The ether extracts (containing the ketonic steroids) were combined and dried.

1.2 The reduction of carbonyl groups

Steroid carbonyl groups can be reduced to hydroxyl groups by sodium borohydride. Ketone groups on almost every position of the steroid nucleus are reduced completely in two hours, but ^{the} carbonyl group at position 11 requires about 16 hours before it is completely converted to an 11 β hydroxyl group. A mixture of the two possible epimeric hydroxyl groups is normally produced, but one of these epimers usually predominates.

Reduction with sodium borohydride (Bush, 1961):

(i) The steroid to be reduced was dissolved in 0.2 ml. of methanol.

(ii) Sodium borohydride (10 mg.) was added: this mixture was left at room temperature for two hours.

(iii) Excess sodium borohydride was destroyed by the addition of glacial acetic acid.

(iv) Distilled water (5 ml.) was added. This mixture was extracted three times with 10 ml. ether. The ether extracts (containing the reduced steroid) were combined and dried.

1.3 The formation of O-methyloxime derivatives

Steroid O-methyloxime derivatives are usually prepared in order to

protect the C-20 carbonyl group, in steroids containing a dihydroxyacetone side-chain, from destruction during gas chromatography. It is also useful to prepare these compounds for thin-layer chromatographic analysis, since steroid O-methyloxime derivatives are less polar than free steroids and it is therefore simple to deduce whether or not a steroid contains a carbonyl group.

Preparation of O-methyloxime derivatives (Brooks, 1966):

The steroid was dissolved in 0.2 M methoxamine hydrochloride dissolved in dry pyridine, using a three to five-fold excess of reagent per reactive carbonyl group. After four hours, portions of the mixture were taken for analysis by thin-layer chromatography.

1.4 The preparation and purification of trimethylsilyl ethers

Steroids are often polar, non-volatile compounds and before they are analysed by gas chromatography it is usual to make them more volatile by preparing derivatives of the hydroxyl groups. The derivatives most often prepared are the trimethylsilyl ethers: under certain conditions, these can be formed with all the steroid hydroxyl groups.

Formation of trimethylsilyl ethers

The method described by Vihko (1966) was used. This does not normally effect the formation of trimethylsilyl ether derivatives of tertiary hydroxyl groups.

A solution of hexamethyldisilazane:trimethylchlorosilane:pyridine (3:1:4) was prepared. 50 μ l. of this solution was added to a vial containing about 20 μ g of steroid; this was then closed and left overnight. The steroid trimethylsilyl ether was purified for gas chromatography-mass spectrometry by preparative gas chromatography using a Perkin-Elmer 801

gas chromatograph with an E 301 column. Either single peaks, or alternatively the whole of the run, were collected into glass collection tubes placed on the outlet pipe of the chromatograph (Sparagana, 1966). The steroid trimethylsilyl ethers were then transferred with chloroform into vials and dried under a stream of nitrogen. The steroid trimethylsilyl ethers were then sufficiently pure to be studied by combined gas chromatography-mass spectrometry.

2. Mass spectrometry

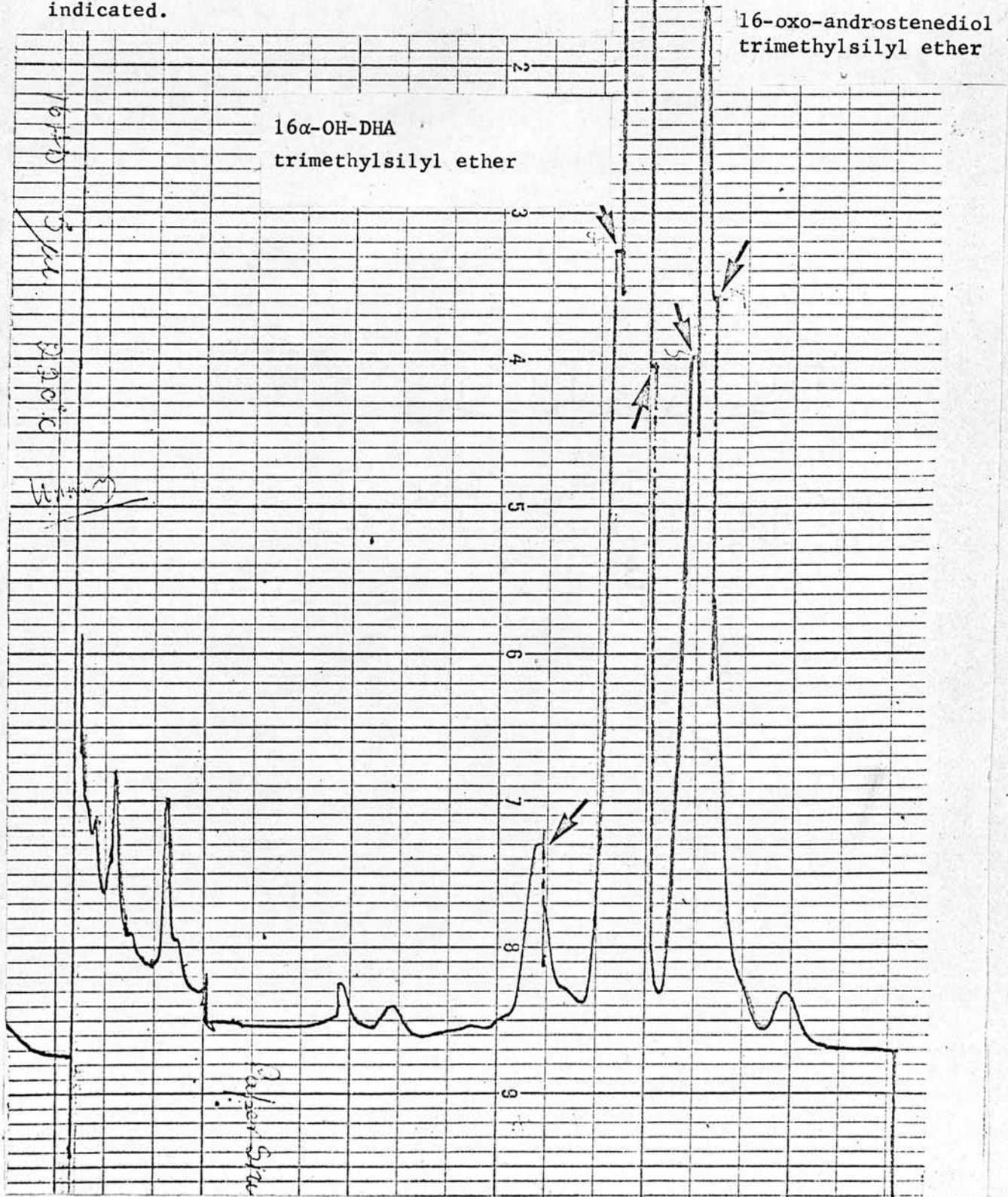
Introduction:

The advent of combined gas chromatography-mass spectrometry has greatly simplified the problems involved in identifying small quantities of steroid metabolites in urine and plasma since a mass-spectrum can be obtained with as little as 0.06 μg . of the trimethylsilyl derivative (Sjövall and Vihko, 1966). One of the major difficulties which hindered the development of gas chromatography of steroids was the protection of heat-labile compounds from destruction at the high temperatures required for volatilisation. The formation of trimethylsilyl ethers is suitable for the protection of hydroxyl groups in androstane and in polyhydroxypregnane compounds lacking a C-20 carbonyl group (Rosenfeld, 1964), but for the chromatography of steroids containing an α -ketolic side chain it is necessary to form a derivative of the C-20 carbonyl group. To protect carbonyl groups, the formation of O-methyloxime or O-trimethylsilyloxime derivatives is suitable (Gardiner and Horning, 1966). When mass-spectrometry is combined with gas chromatography, these derivatives have the added advantage of directing fragmentation in a recognisable manner (Brooks et al., 1966). O-Methyloxime trimethylsilyl ethers have been used

Fig. 37. The Identification of 16 α -OH-DHA
by Combined Gas Chromatography - Mass
Spectrometry.

Stationary phase 1% SE 30
Temperature 220^o

Mass spectra were taken at the positions
indicated.



by Brooks and co-workers (1966) in the study of the excretion of $3\beta\text{-OH-}\Delta^5$ steroids by newborn infants and trimethylsilyl ethers were used by Sjövall and Vihko (1966, 1968) in their quantitative study of the solvolysable steroids in peripheral blood.

The mass-spectrum of a steroid trimethylsilyl ether usually gives the molecular weight $(M)_{\Lambda}$ ^{and} the number of trimethylsilyl groups in the molecule; other detailed information about the structure can be obtained from the fragmentation pattern (Budzikiewicz et al., 1964). Steroids containing a $3\beta\text{-OH-}\Delta^5$ group, for example, normally give an intense fragment at m/e 129 and m/e ^M _{Λ} -129; this fragmentation has been used by Eneroth et al. (1964, 1965) to identify this type of steroid.

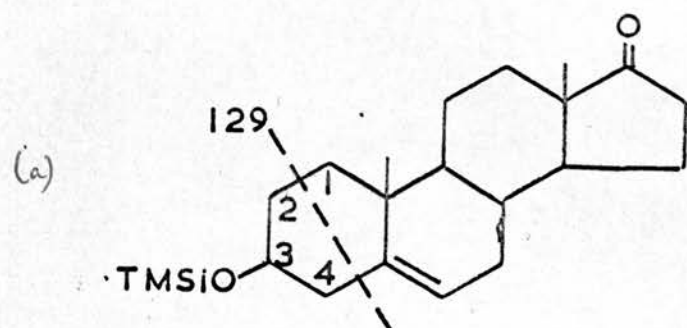
In this study combined gas chromatography-mass spectrometry has been used to identify most of the compounds measured in the quantitative method in order to evaluate its specificity. The mass-spectrum fragmentation patterns of several other reference compounds have also been determined, even though these compounds have not been identified either in urine collected from infants or plasma obtained from the umbilical blood vessels.

Method

The sample of trimethylsilyl ether was dissolved in chloroform and a suitable amount was injected into the column of the LKB 9000 gas chromatograph-mass spectrometer. For reference compounds, the mass-spectrum was normally recorded at the centre of the peak, but for the identification of urinary compounds a "scan" was taken on the ascending side of the peak and also on the descending side (see Fig. 37) to determine whether the peak was homogeneous or composed of more than one compound. If two or more compounds were present it was considered that these would probably

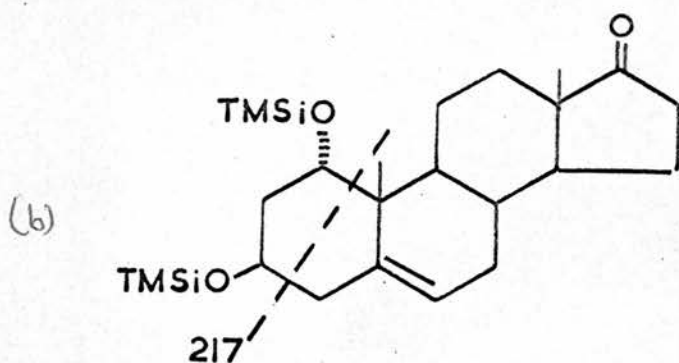
Fig. 38. The "A" Ring Fragmentation of Trimethylsilyl Ether Derivatives of $3\beta\text{-OH-}\Delta^5$ Steroids

TMSiO — Trimethylsilanol group.



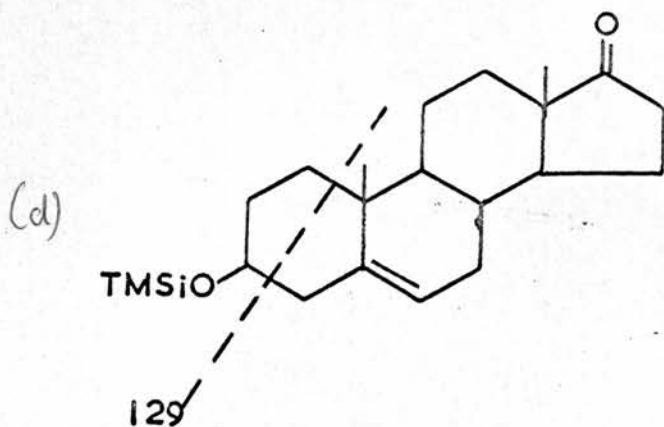
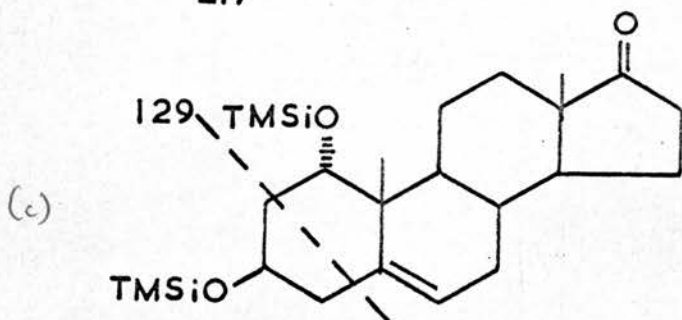
DHA trimethylsilyl ether

This method of fragmentation was suggested by Eneroth *et al.* (1964). It has since been shown to be incorrect.



1 α -OH-DHA trimethylsilyl ether

The mass-spectrum of this compound (Fig. 58) shows a strong peak at m/e 217 but no peak at m/e 129. This indicates that the fragment must contain carbon atoms 1, 2 and 3. If the fragment formed from the A ring had contained carbon atoms 2, 3 and 4 (as suggested by Eneroth *et al.*, 1964), a strong m/e 129 peak would have been present in the mass-spectrum.



DHA trimethylsilyl ether

The fragmentation of the A ring of $3\beta\text{-OH-}\Delta^5$ steroids occurs as shown in this figure.

have slightly differing retention times, so differences might be detected in the mass-spectra taken at each side of the peak. Occasionally three scans were taken, the third being taken at the top of the peak.

Figure 37 shows the gas chromatograph record of 16α -OH-DHA (as its trimethylsilyl ether) obtained after preparative TLC carried out on the extract of urine collected from infants. There is one other major peak which is due to 16-oxo-androstenediol, a compound which is extremely difficult to remove by the systems used for quantitative TLC.

After calculating the relative intensities of all the peaks in the mass-spectrum, line-diagrams have been drawn for several of the compounds analysed during this study. These line-diagrams are illustrated in Figs. 55-68. Table 15 gives the relative retention times on gas chromatography of all the steroids analysed.

Fragmentation of 3β -OH- Δ^5 steroids

(a) Fragmentation peaks at m/e 129 and m/e M-129

The tendency of 3β -trimethylsilyloxy- Δ^5 steroids to yield intense fragments of m/e 129 and m/e M-129 has been used in the identification of 3β -OH- Δ^5 steroids (Eneroth et al., 1964, 1965). These workers originally considered that this fragmentation was due to the splitting off of carbon atoms 2, 3 and 4 of the steroid molecule (as illustrated in Fig. 38a) but it now seems more probable that the fragment contains carbon atoms 1, 2 and 3 (Fig. 38d). Evidence to support this theory comes from the fragmentation of 1α -OH-DHA trimethylsilyl ether (Fig. 58). This compound does not have a major peak at m/e 129, although such a peak would have been expected if the fragment had contained carbon atoms 2, 3 and 4 (Fig. 38c). Instead, it has a base peak at m/e 217, which fits with a fragment containing carbon atoms

TABLE 15

Gas Chromatographic Retention Times of Steroid Trimethyl Silyl Ethers

(Relative to DHA trimethyl silyl ether)

Stationary phase 1% SE₃₀

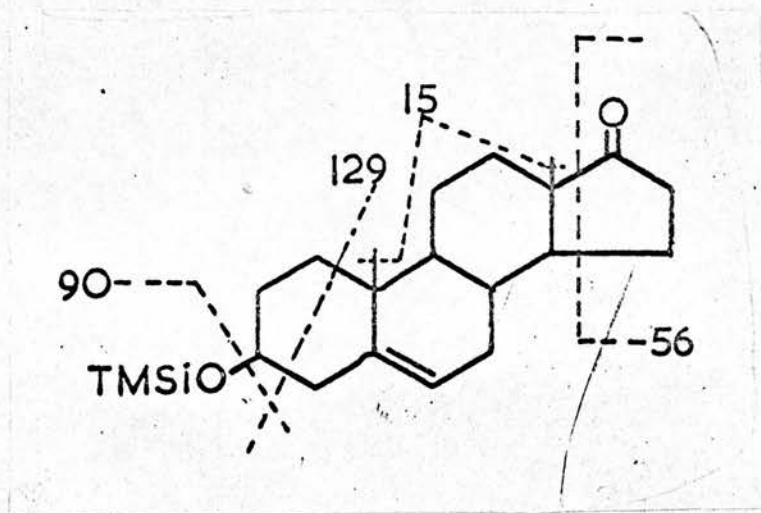
Column temperature 220°

Steroid	Number of trimethyl silyl ether groups	Relative retention time
Androst-5,16-dien-3 β -ol	1	0.56
DHA	1	1.00
7-oxo-DHA	1	1.55
11-oxo-DHA	1	1.15
1 α -OH-DHA	2	1.13
7 α -OH-DHA	2	1.20
7 β -OH-DHA	2	1.42
11 β -OH-DHA	2	1.72
14 α -OH-DHA	(2)*	1.51
16 α -OH-DHA	2	1.63
16 β -OH-DHA	2	1.80
17 α androstenediol	2	1.04
17 β androstenediol	2	1.17
11-oxo-androstenediol	2	1.75
16-oxo-androstenediol	2	1.80
Androstenetriol	3	2.18
Androst-5-ene-3 β ,15 α ,17 β -triol	3	1.41
Androst-5-ene-3 β ,15 β ,17 β -triol (i) **	3	1.69
Androst-5-ene-3 β ,15 β ,17 β -triol (ii) *	3	1.83
Androst-5-ene-3 β ,11 β ,17 β -triol	3	1.92
Compound U ₁	1	1.98

* The mass-spectrum did not give a peak corresponding to the MW of the parent ion.

** Androst-5-ene-3 β ,15 β ,17 β -triol gave two peaks which had similar mass spectra.

Fig. 39. Fragmentation of DHA Trimethylsilyl Ether
(Mass-spectrum illustrated in Fig. 56)



TMSiO — Trimethylsilyl group

Characteristic Peaks (m/e)

M-15	129
M-90	M-129
M-90+15	
M-56	

1, 2 and 3 (Fig. 38b); this would also explain the results given for the fragmentation of 4-methyl- and 4,4-dimethyl-cholesteryl trimethylsilyl ether (Brooks *et al.*, 1966).

The peak at m/e 129 is frequently the base peak for trimethylsilyl ethers of 3β -OH- Δ^5 steroids, but it has been shown that androstanes with a 17-hydroxy group (but no 3β -OH- Δ^5 group) also give intensive peaks at m/e 129; for example, the epimeric androstane-3,17-diols (Vihko, 1966) give such a fragment.

(b) Fragmentation peak at m/e M-15.

This fragment represents the loss of an angular methyl group (Fig. 39).

(c) Fragmentation peak at m/e M-90.

This fragment is due to the loss of trimethylsilanol, and indicates the number of esterifiable hydroxyl groups. Dehydroepiandrosterone, for example, gives only an M-90 peak, while in the spectrum of androstenediol there are predominant peaks at m/e M-90 and m/e M-(2x90). Each of these fragments can also be associated with the loss of an angular methyl group. For each m/e M-(x 90) peak (x = number of esterifiable hydroxyl groups), therefore, there is a peak at M-(x x 90) + 15. This becomes clearer from an examination of the fragmentation of mono-, di- and trihydroxy androstenes (Figs. 40, 55, 63 and 67).

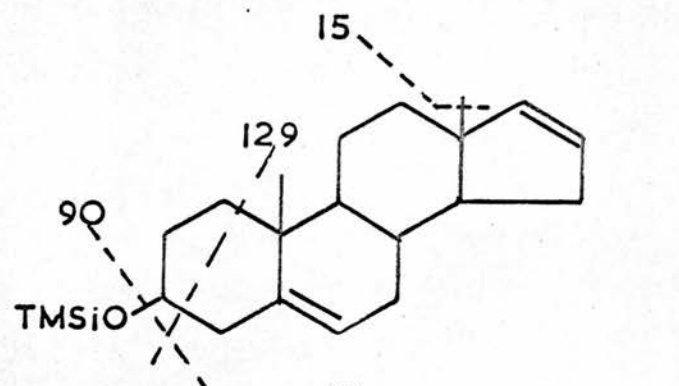
(d) Fragmentation peak at m/e M-56.

Budzikiewicz and Djerassi (1962) showed that a peak at m/e M-56 represented the splitting of the D ring of steroids containing $\overset{\alpha}{\text{C}}$ carbonyl group at positions 15, 16 or 17. This fragmentation is illustrated in Fig. 39 for DHA trimethylsilyl ether (mass-spectra shown in Fig. 56).

Fig. 40. Fragmentation Peaks Expected for Mono-, Di-, and Tri-Hydroxy Androstenes.

(See Figures 55, 63 and 67).

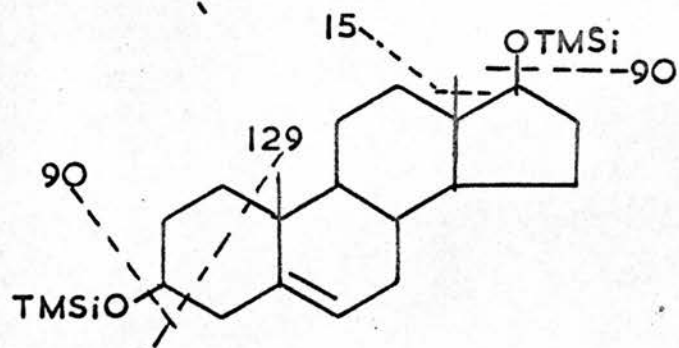
(TMSiO - Trimethylsilanol group)



Characteristic peaks (m/e)

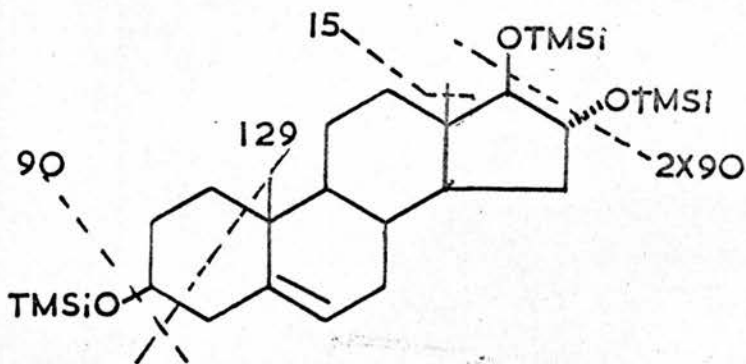
3β-Hydroxyandrost-5,16-ene

M-15	129
M-90	M-129
M-90+15	



17β Androstenediol trimethylsilyl ether

M-15	129
M-90	M-129
M-(2x90)+15	



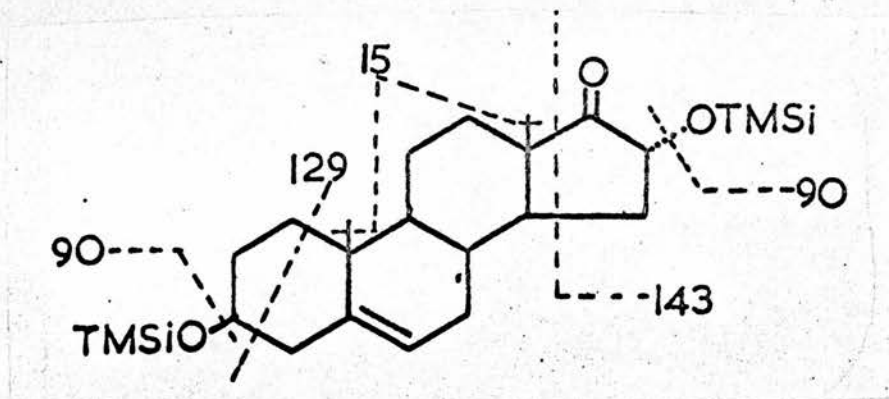
Androstenetriol

M-15	129
M-90	M-129
M-90+15	
M-(2x90)+15	
M-(3x90)	
M-(3x90)+15	

Fig. 41. Fragmentation of 16 α -OH-DHA Trimethylsilyl Ether

(Mass-spectrum illustrated in Fig. 47)

(TMSiO = trimethylsilanol group)



Characteristic peaks (m/e)

M-15	129
M-90	M-129
M-90+15	
M-143	
M-(2x90)	
M-(2x90)+15	

The strong m/e M-144 (m/e 304) peak present in the spectrum of 16 α -OH-DHA trimethylsilyl ether (Fig. 47) may also be related to the loss of the D ring (Fig. 41).

(e) Unique features in the fragmentation of some of the compounds analysed

The trimethylsilyl ethers of 3 β , 15 α , 17 β -trihydroxyandrost-5-ene (Fig. 66) and 3 β , 15 β , 17 β -trihydroxyandrost-5-ene did not give peaks representing the parent ions (M^+), but gave peaks at m/e M-15. However, 14 α -OH-DHA trimethylsilyl ether (Fig. 61) did not give peaks at M, M-15, M-90 or M-90+15, and this compound therefore appears to disintegrate rapidly, giving little structural information apart from the presence of a base peak at m/e 129 indicating that it is a 3 β -OH- Δ^5 steroid.

With the exception of the androstenediol epimers, and 1 α -OH-DHA, the only steroid trimethylsilyl ether studied that failed to give a base peak at m/e 129 was 7 α -OH-DHA (Fig. 59). This compound has a base peak at m/e M-90 (m/e 358) indicating the rapid removal of trimethylsilanol at position 7.

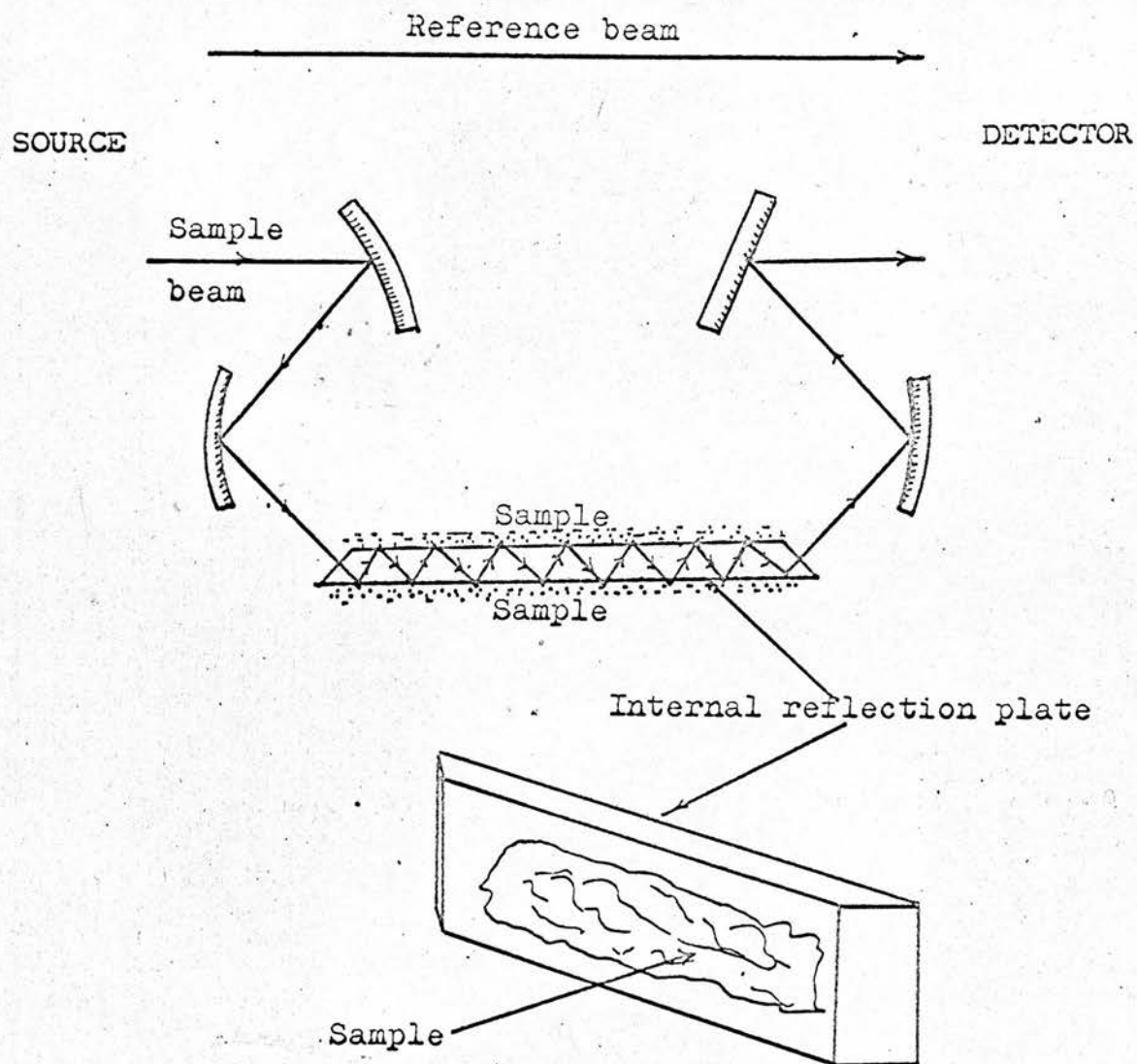
Epimers frequently give almost identical mass-spectra. An example of this is given for 17 α and 17 β androstenediol; the mass-spectra of these compounds are shown in Figs. 62 and 63.

3. Infra-red spectroscopy

A Unicam S.P. 200 spectrophotometer was used for analysis of steroids by infra-red spectroscopy. This was a double beam instrument, one beam being used for reference and one for the sample. By utilising a system of mirrors, the sample beam was made to pass through a KRS 5 internal reflectance plate (Fig. 42). The plate was made of a synthetic mixed crystal of thallos bromide and thallos iodide, a strong resistant

Fig. 42. Internal Reflection Infra-Red Spectroscopy

By employing a reflection plate, the sample beam can be made to reflect off the steroid many times as it travels to the detector; this greatly increases the sensitivity.



material which does not absorb in the infra-red region. The sample to be analysed was dissolved in a small volume (200 μ l.) of a suitable volatile solvent (acetone was used in this study) and the solution was streaked on both sides of the plate, using a micropipette. Radiation from the source is directed by the mirrors into the plate, where it reflects internally many times as it travels the length of the plate, penetrating into the sample with each reflection. The additive effect of a large number of internal reflections provides a reasonable spectrum on a small amount of material. About 25 μ g. of steroid was found to be the smallest amount that gave a satisfactory spectrum.

II THE IDENTIFICATION (OR PARTIAL IDENTIFICATION) OF FOUR
STERIODS PRESENT IN URINE COLLECTED FROM INFANTS

1. Partial identification of compound U₁

This steroid was excreted by infants as a conjugate which could be hydrolysed by the enzymes present in the crop fluid of *Helix pomatia*. Compound U₁ was not detected in extracts of urine collected from adults, nor in plasma obtained from the umbilical blood vessels.

(a) Polarity:

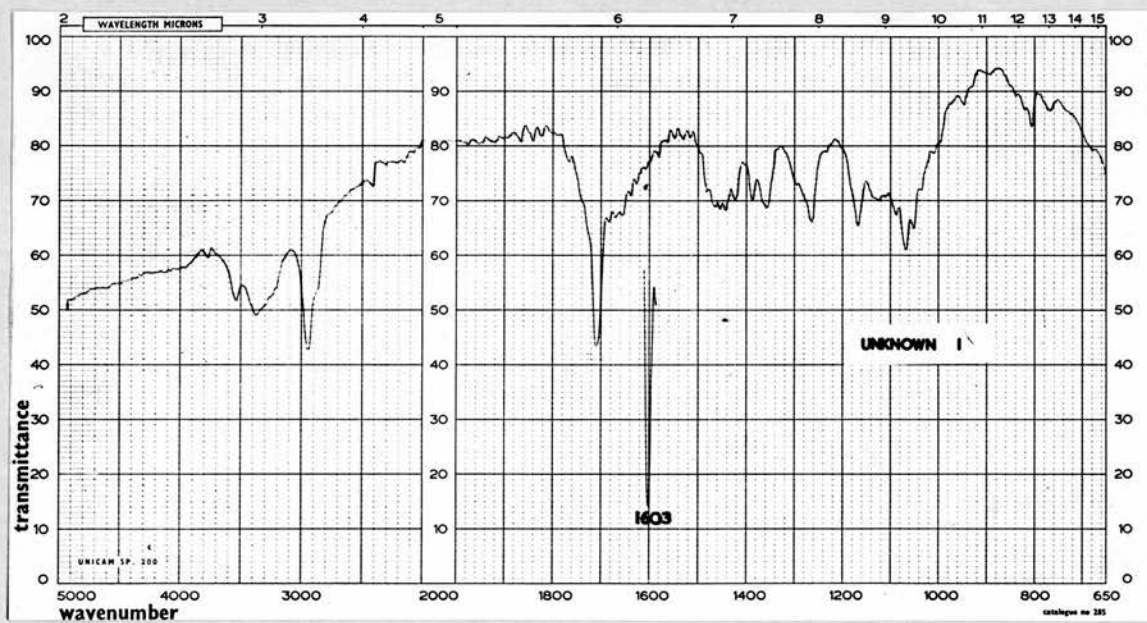
Compound U₁ had a similar mobility to 21-OH-pregnenolone in all the thin-layer solvent systems used (Table 20), but these two compounds could be separated satisfactorily in systems based on chloroform, as compound U₁ was slightly less polar. Its mobility with respect to a 3 β -OH- Δ^5 androstene was similar to 11-oxo-DHA; it was less polar than androstenediol in systems C and F, but more polar in system H.

If compound U₁ is a 3 β -OH- Δ^5 pregnene, it is probable that it either has two hydroxyl groups, two hydroxyl groups and one ketone group or one hydroxyl group and two ketone groups. If it is a 3 β -OH- Δ^5 androstene, it is most likely to contain two hydroxyl groups, or one hydroxyl and two ketone groups, as it was found to be less polar than all the androstene dihydroxy-monoketone standards available.

(b) Colour reactions:

Compound U₁ was detected on thin-layer chromatograms by the antimony trichloride reaction, in which it produced the typical red colour given by 3 β -OH- Δ^5 steroids. The Zimmermann reaction was negative, indicating the absence of a carbonyl group adjacent to an unsubstituted

Fig. 43. Infra-red Spectrum of Compound Unknown 1



methylene group. Compound U_1 did not react with blue tetrazolium, which indicated that it did not contain an α -ketol group.

(c) Derivative formation

Compound U_1 did not react with Girard's reagent T since it was extracted with the non-ketonic fraction. No derivative was formed with methoxamine hydrochloride, since the R_f value on thin-layer chromatography remained unchanged; steroid O-methyloxime derivatives are less polar than the free steroids. These findings are consistent with the steroid either having no ketone group, or else a hindered one which does not readily form derivatives, for example at position 11 of the steroid molecule.

Gas chromatography-mass spectrometry showed that the compound formed a mono-trimethylsilyl ether, indicating the presence of at least one hydroxyl group.

(d) Reduction

Two products were formed when compound U_1 was reduced with sodium borohydride in methanol.

(e) Infra-red analysis

The infra-red spectrum of compound U_1 (Fig. 43) contained a large peak at 1710 cm^{-1} . This is probably due to a ketone group, but the wavelength is too low for the ketone to be in ring D (about $1720 - 1740\text{ cm}^{-1}$), and too high for the carbonyl group of 3-oxo- Δ^4 steroids ($1660 - 1680\text{ cm}^{-1}$). Carbonyl groups at positions 11 and 20, however, absorb at this frequency. Since there was only one peak in the part of the spectrum characteristic for ketone groups, it is probable that only a single carbonyl group is present in the molecule.

(f) Ultra-violet analysis

Compound U_1 did not absorb light of wavelength 240 $m\mu$, which indicates that there is no α, β unsaturation.

(g) Mass-spectrometric analysis

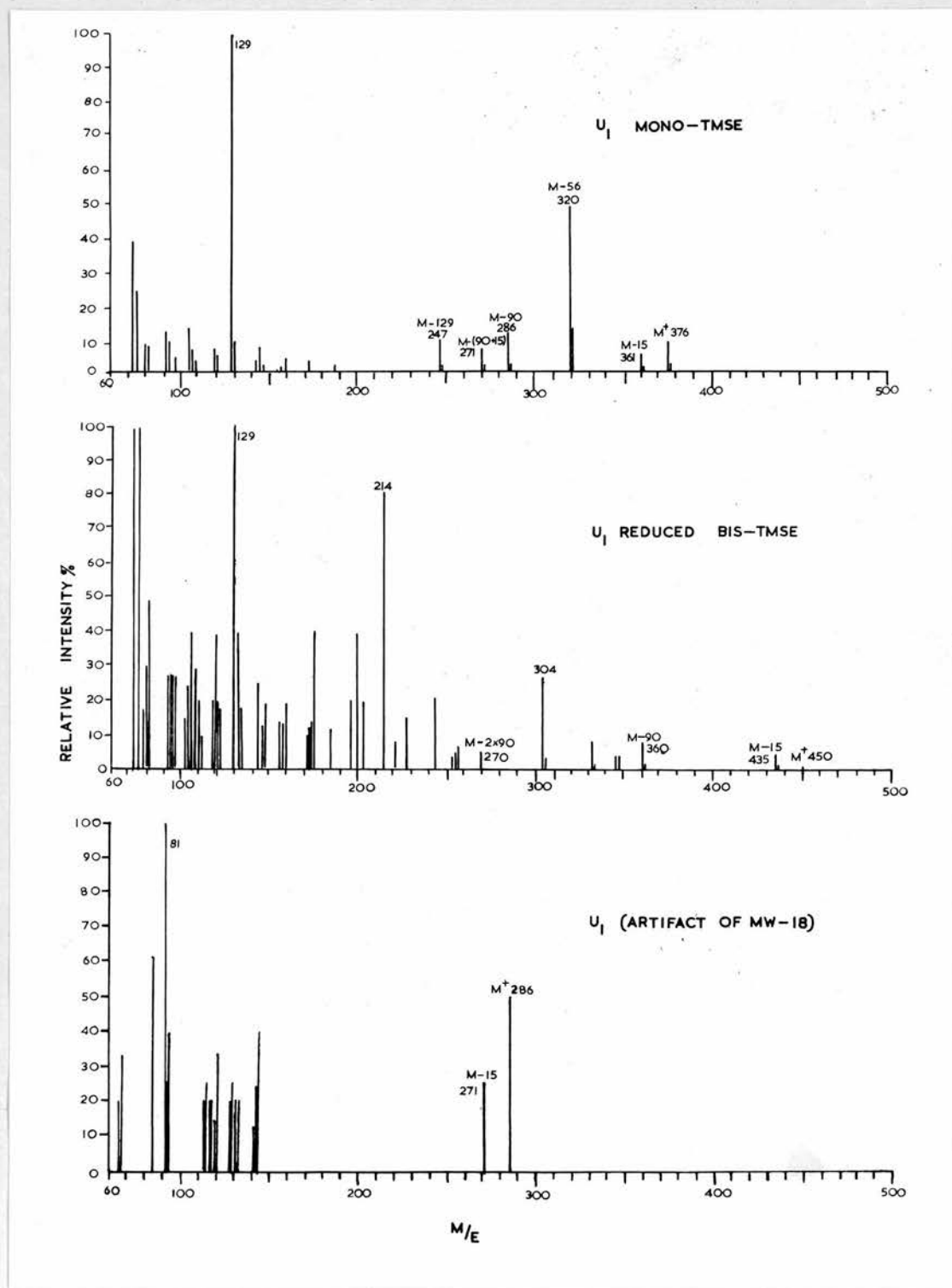
The mass-spectrum of the steroid trimethylsilyl ether (Fig. 44) showed that the base peak was at m/e 129, indicating the probable presence of a $3\beta\text{-OH-}\Delta^5$ group, although 17-hydroxy androstanes without a $3\beta\text{-OH-}\Delta^5$ group also give base peaks at m/e 129. The mass-spectrum indicated that the molecular weight was 376, and the fragmentation pattern showed the presence of one trimethylsilyl group, since there were peaks at m/e $M-90$ and m/e $M-90+15$, but none at m/e $M-(2 \times 90)$ nor at m/e $M-(2 \times 90) +15$. A mono-trimethylsilyl ether of molecular weight 376 indicated that the free compound has a molecular weight of 304 since the formation of a trimethylsilyl ether adds a mass of 72. These findings would be consistent with a $3\beta\text{-OH-}\Delta^5$ androstene containing an additional hydroxyl group (which does not form a trimethylsilyl ether) and a ketone group. There was a relatively strong peak at m/e $M-56$, a fragment which often indicates the presence of a carbonyl group in the D ring (Fig. 39). However a ring D ketone is inconsistent with the ketone frequency observed by infra-red analysis, and with the lack of formation of O-methyloxime and Girard derivatives.

Analysis of the "free" compound by combined gas chromatography-mass spectrometry resulted in the formation of two peaks, one of which was compound U_1 and the other of which had a molecular weight of 286 (mass-spectrum Fig. 44). It is probable that compound U_1 is heat labile, and partially dehydrates immediately after injection into the column, due to the removal of one of its hydroxyl groups. One of the reduction products

Fig. 44. Mass Spectra of Derivatives of Unknown₁ (U₁)

MONO-TMSE - Mono-trimethylsilyl ether

BIS-TMSE - Di-trimethylsilyl ether



of compound U_1 was also examined by gas chromatography-mass spectrometry (Fig. 44). The molecular weight of this steroid was 450, and the fragmentation pattern indicated the presence of two trimethylsilyl ether groups. This mass-spectrum again indicated the probable presence of an unsubstituted hydroxyl group.

If this compound is a 3β -OH- Δ^5 androstene, then it is likely that the additional hydroxyl is at position 8, 9 or 14 since these are the only tertiary carbon atoms that could support an additional group. It is also most probable that only a tertiary hydroxyl group is unable to form a trimethylsilyl ether. The carbonyl group must be unreactive and cannot be in the D ring; it may therefore be at position 11.

More work will have to be carried out on this unknown steroid before any definite structure can be assigned, since the available evidence is so conflicting.

2. The identification of compound U_2 (16β -OH-DHA)

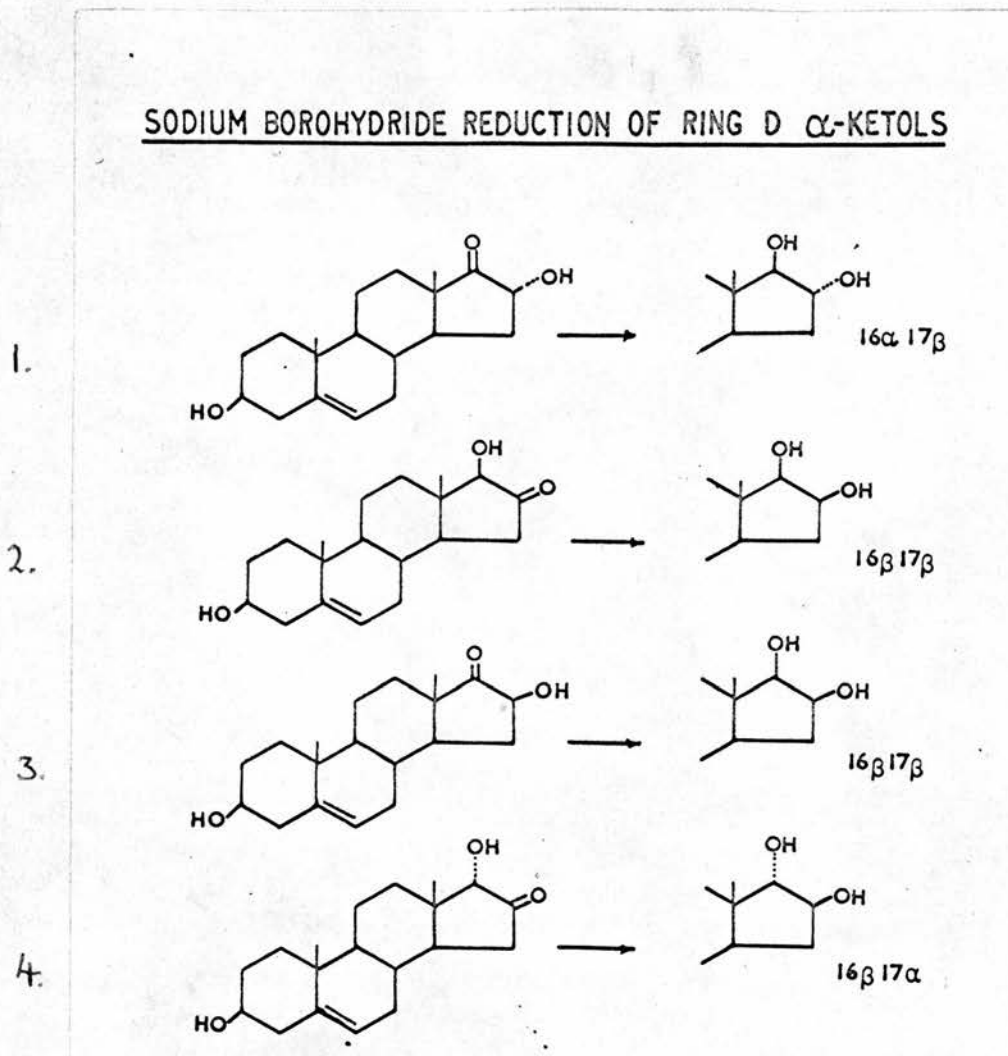
Compound U_2 was present in extracts of urine obtained after hydrolysis with the *Helix pomatia* enzyme preparation and a similar compound was detected in plasma obtained from the umbilical blood vessels. However compound U_2 was not detected in extracts of urine collected from adults. Compound U_2 gave the antimony trichloride reaction characteristic of 3β -OH- Δ^5 steroids, and was slightly more polar than 16α -OH-DHA in all the TLC solvent systems used (see Fig. 12). A strong reaction was given with the blue tetrazolium reagent, similar to that given by 16-oxo-androstenediol; the reaction given by 16α -OH-DHA is weak and slow to develop. Compound U_2 also gave a weak reaction with the Zimmermann reagent. Because of the similar polarities of compound U_2 , 16-oxo-androstenediol and 16α -OH-DHA, and their similar

reactions with the blue tetrazolium and the Zimmermann reagents, it was considered probable that compound U_2 was another steroid with an α -ketolic group in ring D.

Compound U_2 was purified by preparative thin-layer chromatography (p. 45) in three solvent systems [system C (x3), H (x2) and F (x1)]. All the solvent systems used were able to separate compound U_2 from 16α -OH-DHA and 16-oxo-androstenediol, although all three compounds had similar mobility. However, it proved impossible to remove all traces of a compound with the same mobility as 16-oxo-androstenediol, although it was possible to remove all 16α -OH-DHA. This finding was surprising since, in one of the systems used for preparative chromatography (system C), compound U_2 and 16-oxo-androstenediol were separated by a considerable distance. In this system 16α -OH-DHA would have been the expected impurity, since it was of polarity intermediate between 16-oxo-androstenediol and compound U_2 (Fig. 12). It was apparent, therefore, that 16-oxo-androstenediol was being formed as an artifact during the preparative procedure. The formation of 16-oxo-androstenediol from compound U_2 suggested that this unknown steroid was in fact 16β -OH-DHA. 16β -Hydroxydehydroepiandrosterone is the least stable of the isomeric α -ketols, and would be expected to isomerise readily to the α -ketol with the most stable configuration, namely 16-oxo-androstenediol. The same difficulty with isomerisation was experienced by Layne and Marrian (1958) in the identification of 16β -OH-oestrone in urine.

The principal reduction product of compound U_2 had the mobility of either $3\beta,16\alpha,17\alpha$ -trihydroxyandrost-5-ene or $3\beta,16\beta,17\beta$ -trihydroxyandrost-5-ene in solvent system G; these "cis" epimers could not be resolved in this system, but were much less polar than the "trans" epimers,

Fig. 45.



An androstenetriol with 16α and 17α hydroxyl groups cannot be formed by reduction of an α -ketol, since both 16 and 17 carbonyl groups reduce to a " β " hydroxyl.

3 β ,16 α ,17 β -trihydroxyandrost-5-ene and 3 β ,16 β ,17 α -trihydroxyandrost-5-ene. Since carbonyl groups at positions 16 and 17 of the steroid molecule reduce to " β " hydroxyl groups with sodium borohydride (Fig. 45), the product formed by reduction of compound U₂ cannot be 3 β ,16 α ,17 α -trihydroxyandrost-5-ene; the structure of the product must therefore be 3 β ,16 β ,17 β -trihydroxyandrost-5-ene. Only 16-oxo-androstenediol (Fig. 45, compound 2) or 16 β -OH-DHA (Fig. 45, compound 3) could give this reduction product, so reduction with sodium borohydride provides further evidence for the structure of compound U₂ being 16 β -OH-DHA.

On the basis of the evidence obtained, reference 16 β -OH-DHA was synthesised (this compound was prepared by Dr. R. W. Kelly). Synthetic 16 β -OH-DHA was shown to have the same mobility as compound U₂ in all the thin-layer solvent systems used, and it gave the same reactions as compound U₂ with the antimony trichloride reagent, the blue tetrazolium reagent and the Zimmermann reagent. Gas chromatography-mass spectrometry also indicated that compound U₂ and 16 β -OH-DHA were identical. Both compounds had the same retention times on SE 30 stationary phase at 225° (Table 15), and gave similar mass-spectra (Fig. 47). It was not possible to separate 16 β -OH-DHA from 16-oxo-androstenediol under the conditions used, and the major peak in the gas chromatographic tracing illustrated in Fig. 46 is a mixture of these two compounds. This is particularly noticeable from an examination of the fragmentation patterns (Fig. 47), since compound U₂ isolated from urine gives large peaks at M, M-15 and m/e 129; these peaks are more important with 16-oxo-androstenediol than with 16 β -OH-DHA.

When the steroid conjugates present in urine collected from infants were separated into monosulphates and disulphates by Sephadex chromatography,

Fig. 46 The Identification of Compound U₂ (16 β -OH-DHA) by Combined Gas Chromatography-Mass Spectrometry

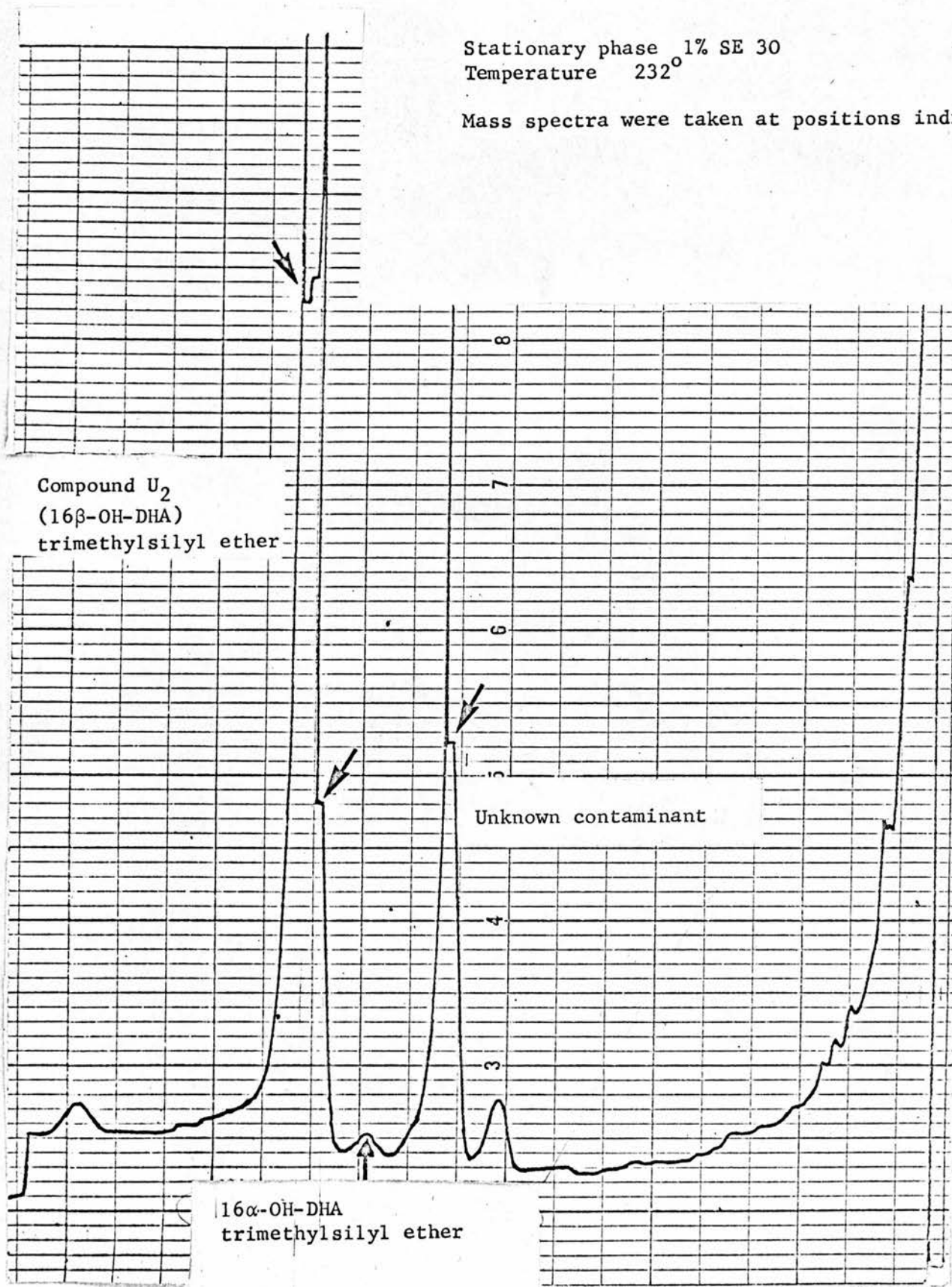


Fig. 47. Mass Spectrometric Identification of Compound U₂

This figure illustrates the mass-spectra of the trimethylsilyl ethers of three ring D α -ketols and of compound U₂

TMSiO - Trimethylsilanol group

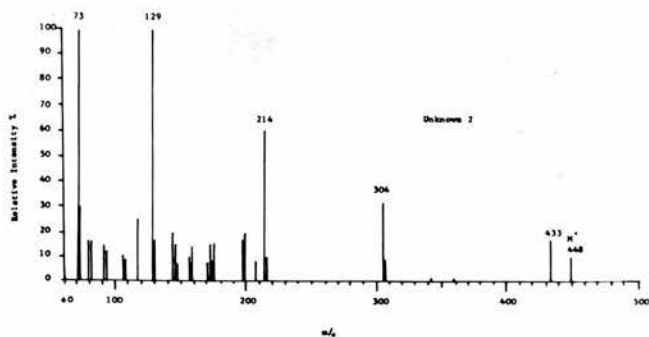
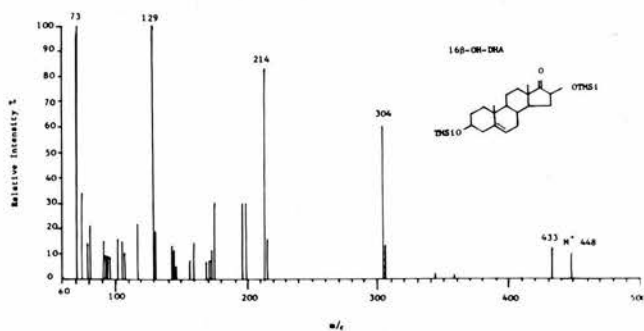
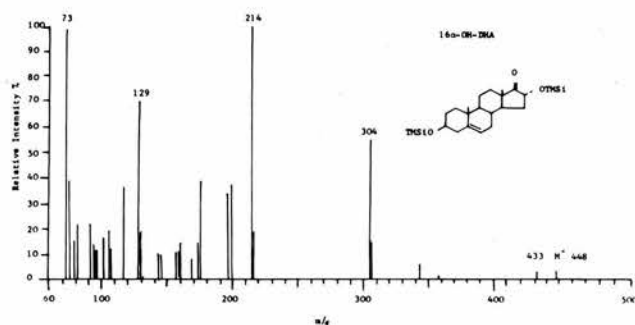
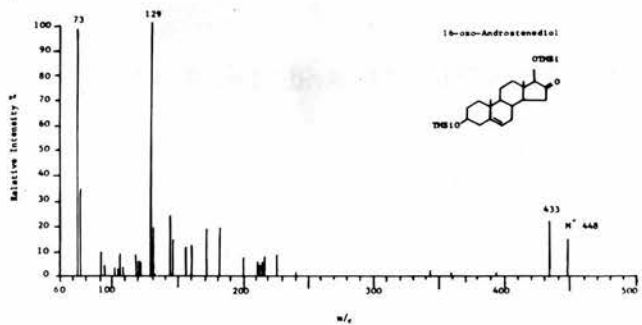
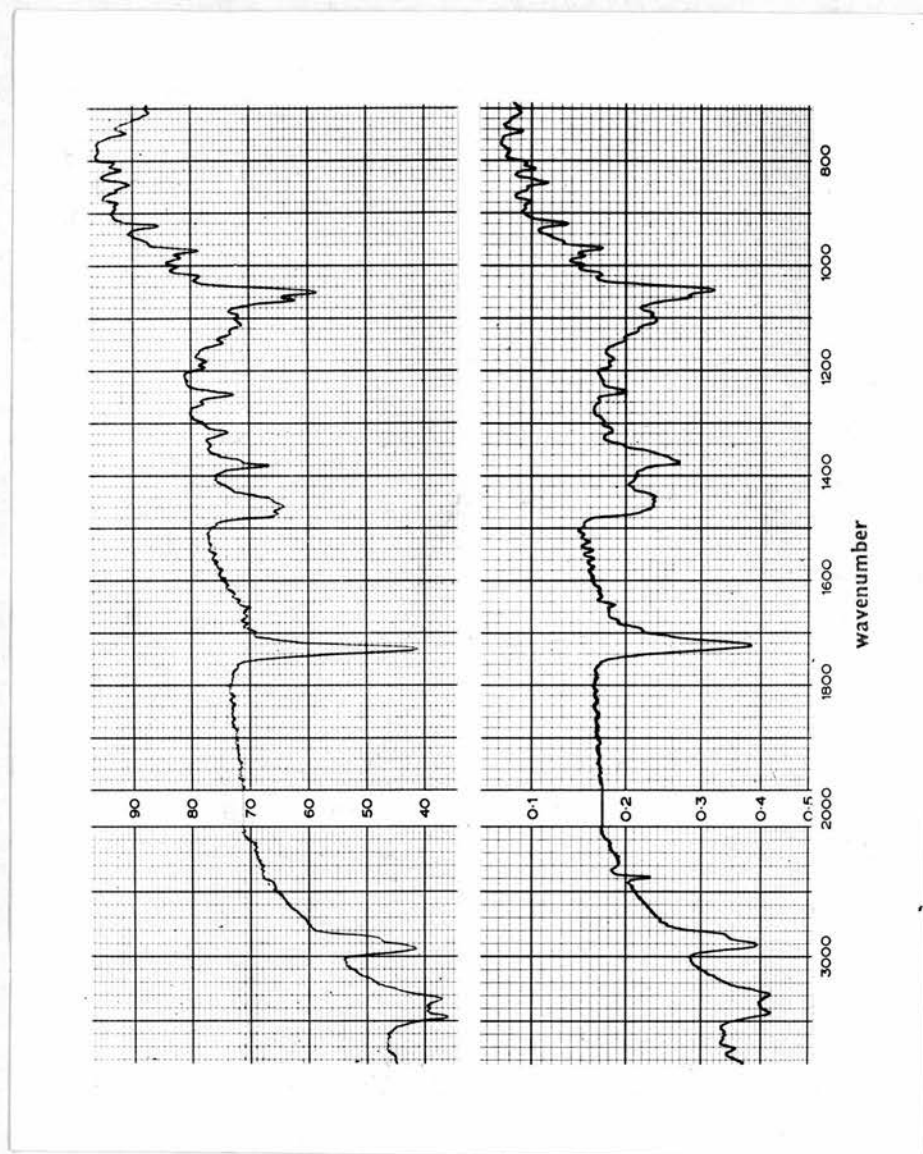


Fig. 48. Infrared Spectrum of Compound U₂ (16 β -OH-DHA)



Standard 16 β -OH-DHA

Compound U₂ prepared
from urine collected
from infants

it was apparent that most of the compound U_2 was present in the disulphate fraction. It was also clear that this conjugate could be hydrolysed by the enzymes present in the *Helix pomatia* preparation. In the diconjugated fraction, compound U_2 was present in large amount relative to 16α -OH-DHA and 16 -oxo-androstenediol (Fig. 36): by preparing compound U_2 from the disulphate fraction, therefore, it was obtained in sufficiently pure form for infra-red spectroscopy. Earlier attempts to obtain compound U_2 sufficiently pure for infra-red analysis had been unsuccessful. Figure 48 illustrates the infra-red spectra of compound U_2 and of synthetic 16β -OH-DHA. The two spectra are very similar, although there is some distortion of the spectrum of compound U_2 due to contaminants.

The evidence for the presence of 16β -OH-DHA in the diconjugated fraction of steroids obtained from urine excreted by infants is therefore conclusive. It is not yet known, however, whether this compound is also excreted as a monoconjugate.

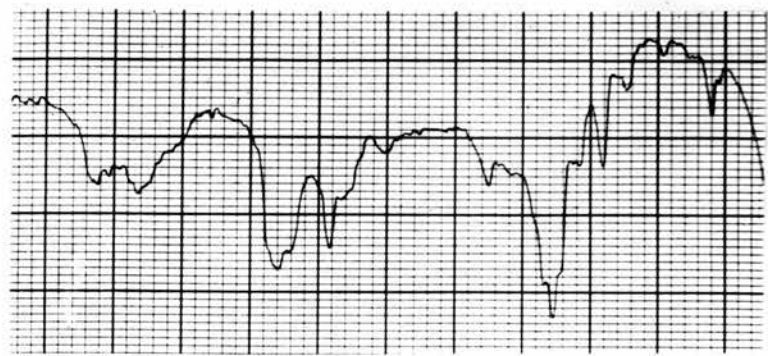
3. The identification of 17α androstenediol

After removing the steroids hydrolysed by the *Helix pomatia* enzyme preparation, solvolysis of urine and ^{of} plasma obtained from newborn infants resulted in the extraction of more steroids. The principal steroid in this solvolysis extract has been identified as 17α androstenediol ($3\beta,17\alpha$ -dihydroxyandrost-5-ene). This finding is of interest since the major epimer of androstenediol produced by adults has a 17β hydroxyl group. Since 17α androstenediol is not released from conjugation by the enzymes present in the *Helix pomatia* preparation, it is probable that this steroid is present in urine and in plasma as a diconjugate (probably disulphate).

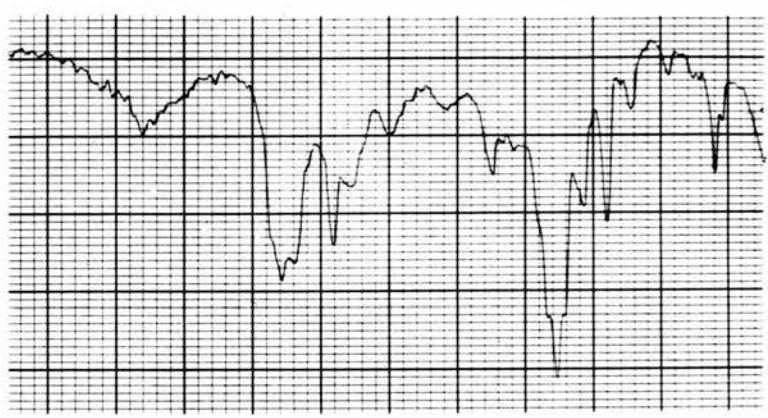
17α Androstenediol obtained from urine was purified by two

Fig. 49. Identification of 17 α Androstenediol by Infrared Spectroscopy
in a Specimen Prepared from Urine Collected from Infants

17 α Androstenediol
In urine collected
from infants



17 α Androstenediol
Standard



17 β Androstenediol
Standard



1800 1600 1400 1200 1000 800
wavenumber

preparative TLC separations. The androstenediol epimers were satisfactorily separated from all other $3\beta\text{-OH-}\Delta^5$ steroids by two developments in solvent system G. For the separation of 17α and 17β androstenediol, however, it was necessary to use alumina (solvent system L), rather than silica gel as the support: none of the solvent systems used for silica gel chromatography was capable of resolving these epimers.

Following purification, part of the urinary 17α androstenediol was transferred to an internal reflection plate for infra-red analysis. It was found that the 17α androstenediol isolated from urine gave a similar spectrum to reference 17α androstenediol (Fig. 49). There was, however, a small peak in the carbonyl region of the spectrum due to an impurity still present in the isolated compound.

A further portion of the purified steroid was analysed by gas chromatography-mass spectrometry after formation of the trimethylsilyl ether. The mass-spectrum recorded was identical to the spectrum of standard 17α or 17β androstenediol trimethylsilyl ether (Figs. 62, 63), since the fragmentation patterns of the two epimers could not be distinguished. Gas chromatography on SE 30 stationary phase, however, resulted in the separation of the two epimers, so a combination of the two techniques in combined gas chromatography-mass spectrometry gave absolute identification of both epimers. The retention times of 17α and 17β androstenediol relative to DHA are given in Table 15.

17α Androstenediol extracted from urine collected from infants, and from plasma obtained from the umbilical blood vessels, had Rf values identical with standard 17α androstenediol in all the thin-layer solvent systems used in this study. The isolated compound gave the same reaction

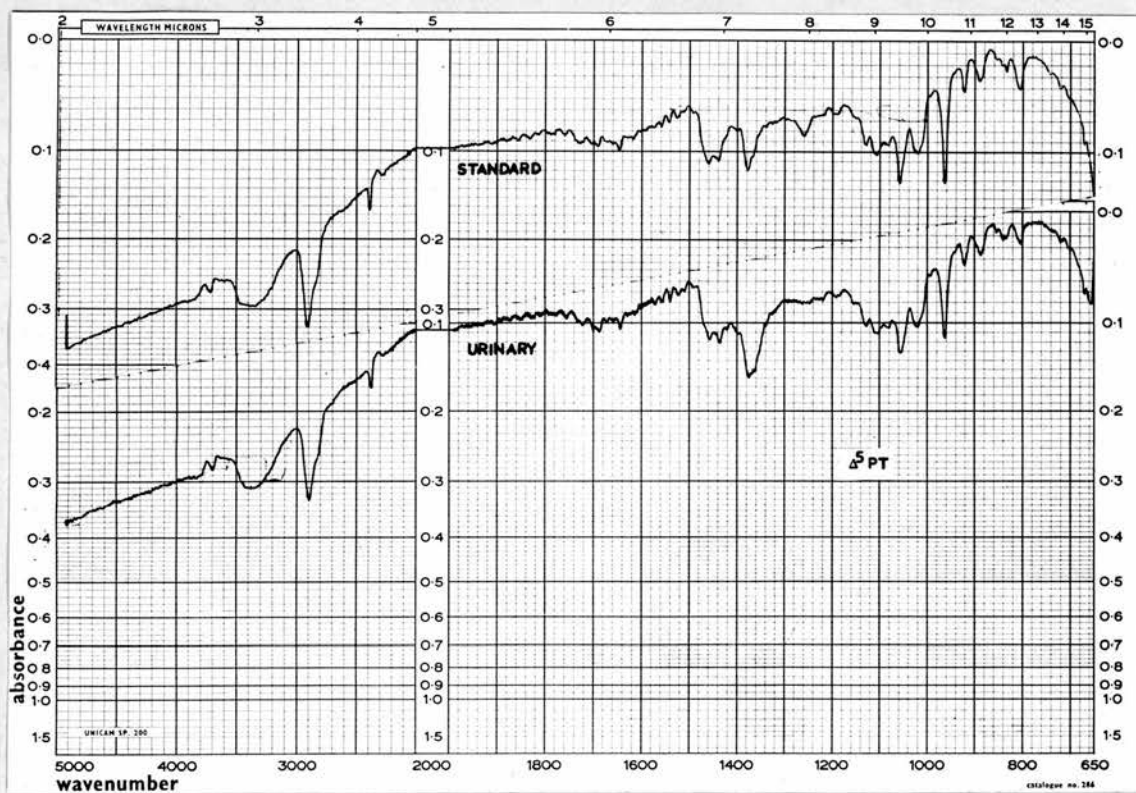
with antimony trichloride as 17α androstenediol. 17β Androstenediol gave a red-blue reaction with this reagent whereas 17α androstenediol produced a grey-blue colour.

It has been suggested that the solvolysis of 17β androstenediol disulphate may result in epimerisation of the 17β hydroxyl group to a 17α hydroxyl group (Sjövall and Vihko, 1966). In order to show that 17α androstenediol was not being formed as an artifact from 17β androstenediol disulphate, a sample of the latter conjugate was prepared using the technique of steroid sulphate formation described by Levitz (1963). The product was purified by TLC using solvent system K. The formation of the disulphate was indicated by the compound formed having an Rf value of 0.11, compared with values of 0.79 for androstenediol and 0.42 for DHA sulphate. In system K, free steroids, monosulphates and disulphates tend to run as three groups with Rf. values similar to those indicated. After removal from the plate, the silica gel containing the disulphate was eluted with ethanol:water (50:50 v/v), the solvent was evaporated, and the residue was hydrolysed by solvolysis for 24 hours. Subsequent chromatography on alumina showed that only the 17β epimer of androstenediol was present so it can be assumed that no epimerisation had taken place during solvolysis.

4. Identification of pregnenetriol

A substance of polarity similar to pregnenetriol was detected in the solvolysis extract of urine obtained from infants. This steroid was purified on a preparative scale by thin-layer chromatography in system C (x3) and system H (x2). In these and other solvent systems, the compound extracted from urine and standard pregnenetriol had identical Rf values. Infra-red analysis of the purified steroid confirmed the identification,

Fig. 50. Infra-red Spectrum of Standard Pregnenetriol and of the Compound Prepared from Urine Collected from Infants



(Δ^5 PT = Pregnenetriol)

since the spectra of reference pregnenetriol and the steroid extracted from urine were almost identical (Fig. 50). There was, however, a slight impurity in the urinary steroid which absorbed at 1500 cm^{-1} .

Pregnenetriol was also detected in urine collected from adults, although here it is probably present as a monoconjugate since it was released from conjugation by the enzymes present in the *Helix pomatia* preparation.

TABLE 16

The Identification of 3β -OH- Δ^5 Steroids in Urine SamplesObtained from Infants

The following 3β -OH- Δ^5 steroids have been sought, but have not been found in an extract of pooled urine.

 3β -OH- Δ^5 AndrostenesAndrost-5,16-dien-3 β -ol

11-oxo-DHA

1 α -OH-DHA7 α -OH-DHA7 β -OH-DHA11 β -OH-DHA14 α -OH-DHA14 β -OH-DHA15 α -OH-DHA*15 β -OH-DHA*

18-OH-DHA

11-oxo-androstenediol

3 β ,11 β ,16 α -trihydroxyandrost-5-en-17-one3 β ,16 α -dihydroxyandrost-5-en-11,17-dioneAndrost-5-ene-3 β ,11 β ,17 β -triolAndrost-5-ene-3 β ,15 α ,17 β -triolAndrost-5-ene-3 β ,15 β ,17 β -triolAndrost-5-ene-3 β ,16 β ,17 β -triolAndrost-5-ene-3 β ,17 β ,18-triol 3β -OH- Δ^5 Pregnenes3 β ,16 α -dihydroxypregn-5-ene-11,20-dione3 β ,17 α -dihydroxypregn-5-ene-11,20-dione3 β ,16 α ,17 α -trihydroxypregn-5-en-20-one3 β ,17 α ,21-trihydroxypregn-5-en-20-one3 β ,11 β ,17 α ,21-tetrahydroxypregn-5-en-20-onePregn-5-ene-3 β ,11 β ,17 α ,20 α ,21-pentol

- * Reference compounds for 15 α -OH-DHA and 15 β -OH-DHA were not available. Since neither androst-5-ene-3 β ,15 α ,17 β -triol nor androst-5-ene-3 β ,15 β ,17 β -triol could be detected in an extract of urine which had been treated with sodium borohydride, it may be assumed that 15 α -OH-DHA and 15 β -OH-DHA are not important urinary steroids.

III. THE ABSENCE OF CERTAIN 3β -OH- Δ^5 STEROIDS FROM URINE
COLLECTED FROM INFANTS

Another approach to the characterisation of unknown steroids in urine obtained from newborn infants has also been attempted. All the 3β -OH- Δ^5 steroids listed in Table 16 have been sought in a pooled extract of 200 ml. of urine collected from several infants. Although very many unidentified steroids were detected when the extract was fractionated by thin-layer chromatography, none of these unknown compounds had the same mobility or gave quite the same colour with antimony trichloride as these reference steroids. Although neither $3\beta,15\alpha,17\beta$ -trihydroxyandrost-5-ene nor $3\beta,15\beta,17\beta$ -trihydroxyandrost-5-ene could be detected, it was considered that 15α -OH-DHA or 15β -OH-DHA might be present in the urinary extract; however, no standards were available for these compounds. For this reason a portion of the urinary extract was reduced with sodium borohydride so that 15α -OH-DHA or 15β -OH-DHA, if present, would be reduced to $3\beta,15\alpha,17\beta$ -trihydroxyandrost-5-ene, and to $3\beta,15\beta,17\beta$ -trihydroxyandrost-5-ene. Neither of these products could be detected.

The concentration of these 3β -OH- Δ^5 steroids that would have been detected in this analysis was of the order of 1/50 of the concentration of the major 16-hydroxylated 3β -OH- Δ^5 metabolites. The presence of these compounds in urine collected from infants has therefore not been completely excluded; if present, however, they are only present in small amount.

DISCUSSION

1. The analysis of $3\beta\text{-OH-}\Delta^5$ steroids in urine obtained from infants

Metabolites of DHA and pregnenolone with a carbonyl or hydroxyl group at position 16 of the steroid nucleus were found to be quantitatively the most important steroids in the urine samples collected from infants. The presence in urine of a relatively large amount of these compounds, together with the barely detectable amounts of DHA and pregnenolone can be related to a high activity of 16α -hydroxylase and a low activity of 3β -hydroxy-dehydrogenase in foetal and infant tissues.

The major 16α -hydroxylated compound excreted by infants was characterised as $16\alpha\text{-OH-pregnenolone}$, a steroid first identified by Reynolds (1963). Other quantitatively important steroids that were identified and measured included the three isomeric α -ketols, $16\alpha\text{-OH-DHA}$, $16\text{-oxo-androstenediol}$ and $16\beta\text{-OH-DHA}$. A reduction product of $16\alpha\text{-OH-DHA}$, androstenediol , was characterised during this study (Shackleton and Mitchell, 1966) in extracts of urine obtained from infants and this compound was simultaneously identified by other workers (Brooks et al., 1966; Reynolds, 1966a). Although these $3\beta\text{-OH-}\Delta^5$ steroids are so important, only three quantitative studies have been made of their excretion by newborn infants. For this reason Table 17 gives the mean results for the daily excretion of the 16-oxygenated $3\beta\text{-OH-}\Delta^5$ steroids observed during the present study, and in the studies of Reynolds (1965a,b, 1966a,b) and of Cleary and Pion (1968). Considering the different methods used for the analysis of these steroids — Reynolds used a method based on paper chromatography, whereas Cleary and Pion used gas-chromatography — and the great variation in the excretion of $3\beta\text{-OH-}\Delta^5$ steroids by individual infants, the agreement

TABLE 17

Comparison of the Results of Three Studies on the Quantitative Estimation of 16-oxygenated 3 β -OH- Δ^5

Steroids Present in Urine Collected from Newborn Infants

($\mu\text{g.}/24 \text{ hr.}$)

The results (average values) are corrected for losses incurred during the estimation

	Present Study	Reynolds (1965a) (Full-term infants)	Reynolds (1966b) (Premature infants)	Cleary & Pion (1968)
Number of infants	32	11	19	8
16-oxo-androstenediol	850	-	-	1630
16 α -OH-DHA	925	290	960	1080
16 α -OH-pregnenolone	921	980	1000	-
Androstenetriol	619	-	390	-

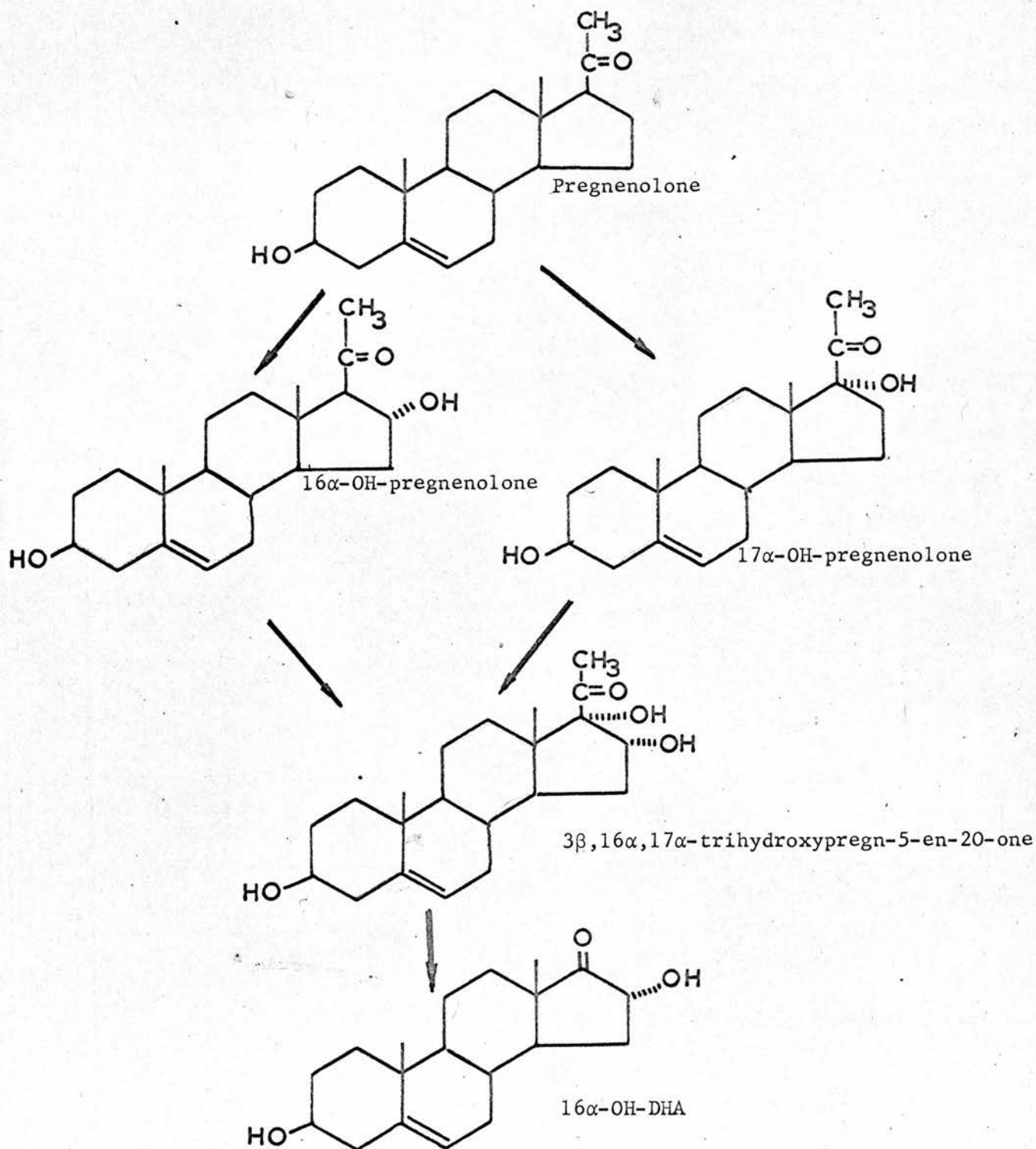
between the results obtained in these studies is considered reasonable.

No relationship could be established between the weight of the babies and the excretion of the 16-oxygenated steroids; there was, however, a greater excretion of these compounds in those infants studied who were under a stress for which treatment with adrenocorticotrophin was to be given. In the infants whose steroid excretion was determined for the first six days of life, no constant pattern of daily excretion of steroids was observed; there was considerable day to day fluctuation, an observation also reported by Cleary and Pion (1968), who determined the excretion of 16 α -OH-DHA and 16-oxo-androstenediol by individual infants during the first three days of life.

The urinary 16-hydroxylated steroids arise principally by hydroxylation of DHA and pregnenolone in the adrenal glands and liver of the foetus and the newborn infant. The 16 α -hydroxylase enzymes present in each of these organs have different substrate specificity. From perfusion experiments carried out with foetuses under conditions obtaining in vivo, it has been shown that 3-oxo- Δ^4 steroids cannot be hydroxylated at position 16 by foetal liver, and that 3 β -OH- Δ^5 steroids do not act as substrates for adrenal 16 α -hydroxylase (Solomon, 1966; Dell'Acqua et al., 1966). The foetal liver will, therefore, only hydroxylate 3 β -OH- Δ^5 steroids in the 16 α -position, and the adrenal glands will only act similarly on 3-oxo- Δ^4 steroids. The conversion of labelled pregnenolone to 16 α -OH-pregnenolone by foetal liver in vivo has been shown to occur at mid-pregnancy; it is, therefore, probable that 16 α -OH-DHA and 16 α -OH-pregnenolone are formed by foetal liver from DHA and pregnenolone supplied by the adrenal glands. Although the formation of 16 α -hydroxylated 3 β -OH- Δ^5 steroids by foetal

Fig. 51. Possible Routes for the Synthesis of 16 α -OH-DHA from Pregnenolone by Pathways in which 16 α -Hydroxylation Precedes Cleavage of the Side-Chain.

These reactions may take place with the steroids either in the free form, or alternatively as monosulphates.



adrenal tissue has not been demonstrated under in vivo conditions, they can be formed by foetal adrenal glands and newborn anencephalic adrenal tissue in vitro (Villem and Loring, 1965; Shahwan et al., 1968).

Although 16α -OH-pregnenolone is produced in large amounts by the foetus and by the newborn infant, this compound has no known function in utero and it is generally accepted that 16α -OH-pregnenolone is merely formed due to the high activity of 16α -hydroxylase in foetal liver. It is possible, however, that 16α -OH-pregnenolone could be an intermediate in the conversion of pregnenolone to 16α -OH-DHA by a pathway not involving DHA as an intermediate (Fig. 51). Shahwan et al. (1968) have demonstrated the conversion of ^{14}C pregnenolone to 16α -OH-DHA by adrenal cortex tissue from anencephalic fetuses. These workers did not, however, find any conversion of pregnenolone to $3\beta,16\alpha,17\alpha$ -trihydroxypregn-5-en-20-one, although this is presumably an essential intermediate in the process leading to 16α -OH-DHA. There is evidence that such a pathway may be important since Villem et al. (1967) have demonstrated by studies conducted in vitro with foetal adrenal glands that pregnenolone is a better precursor of 16α -OH-DHA than 17α -OH-pregnenolone, the classical precursor of DHA. Slaunwhite and co-workers (1966) studied the conversion in vitro of ^3H 17α -OH-pregnenolone to 16α -OH-DHA by liver tissue obtained from adults, and found no tritium associated with either DHA or androstenediol. It was therefore concluded that 16α -hydroxylation must precede cleavage of the side-chain, and that an intermediate such as $3\beta,16\alpha,17\alpha$ -trihydroxypregn-5-en-20-one must be involved. Villem and Villem (1964) have demonstrated the formation of $16\alpha,17\alpha$ -dihydroxypregn-4-ene-3,20-dione from progesterone during incubation studies with foetal

adrenal tissue. It is therefore possible that the analogous $3\beta\text{-OH-}\Delta^5$ steroid, $3\beta,16\alpha,17\alpha\text{-trihydroxypregn-5-en-20-one}$ is formed during synthesis of $16\alpha\text{-OH-DHA}$, but is an exceptionally short-lived intermediate. This compound was not detected in urine obtained from newborn infants.

Slaunwhite et al. (1965) demonstrated that foetal liver (in vitro) could hydroxylate DHA both in the 16α and the 16β positions, but the identification in the present study of $16\beta\text{-OH-DHA}$ in urine obtained from infants provides the first evidence that $16\beta\text{-hydroxylation}$ does occur under in vivo conditions. $16\beta\text{-OH-DHA}$ was found to be one of the major steroids present in urine collected from infants, and this compound was often excreted in larger quantity than $16\alpha\text{-OH-DHA}$. It is surprising, therefore, that $16\beta\text{-OH-DHA}$ had not been detected previously by other workers. Two isomers of $16\beta\text{-OH-DHA}$, $16\text{-oxo-androstenediol}$ and $16\alpha\text{-OH-DHA}$, have been assayed in urine obtained from infants (Reynolds, 1965a; Cleary and Pion, 1968) and it is particularly difficult to separate $16\beta\text{-OH-DHA}$ from these compounds by chromatographic techniques. It seems likely that the method used by Reynolds (1965a) and by Cleary and Pion (1968) for extracting steroid conjugates prior to solvolysis is unable to extract disulphated steroids efficiently. Hydrolysis of the steroid conjugates by enzymes is probably necessary for the analysis of $16\beta\text{-OH-DHA}$ in plasma and urine. Other $16\beta\text{-hydroxylated}$ steroids have been isolated from several sources. A reduced metabolite of $16\beta\text{-OH-DHA}$, $3\beta,16\beta,17\beta\text{-trihydroxyandrost-5-ene}$, was identified in urine obtained from adults by Fotherby (1957) and it is significant that none of this androstenetriol was detected in urine collected from infants, even though some might be expected to be present as a reduction product of $16\beta\text{-OH-DHA}$ or $16\text{-oxo-androstenediol}$. It is possible that the 16-carbonyl

group of 16-oxo-androstenediol cannot be reduced enzymatically, or that rapid disulphation of 16 β -OH-DHA by the foetus prevents any further metabolism by reduction of the ketone group at position 17. It is well established that 16 α -OH-DHA sulphate, elaborated by the foetal adrenal glands and by foetal liver, is an important precursor of the oestriol produced during pregnancy (see p. 11) (Magendantz and Ryan, 1964; Colás et al., 1964; Dell'Acqua et al., 1966), but it is not known whether 16 β -OH-DHA is a precursor of the two 16 β -hydroxylated oestrogens, 16-epioestriol and 16 β -OH-oestrone, excreted by pregnant women. These oestrogens have been assayed by Breuer (1964) in urine obtained from pregnant women, and it is likely that these steroids are formed by pathways similar to those utilised in the synthesis of oestriol, i.e. both by aromatisation of 16 β -OH-DHA and by 16 β -hydroxylation of oestrone and oestradiol.

16-Oxo-androstenediol is another steroid which has no known function. Reynolds (1964) considered that it was formed in the adrenal glands, but it is possible that much of this 16-oxo-androstenediol assayed in urine obtained from infants arises by isomerisation of 16 β -OH-DHA during the procedures of hydrolysis and extraction. It is therefore difficult to determine how much has been formed enzymatically in foetal and newborn infants tissues. Recently it has been shown that 16-oxo-testosterone can be formed by perfusion of placentas with 16-oxo-androstenediol (Reynolds et al., 1968), but it was not stated in this report whether there had been any conversion to 16-oxo-oestradiol, a steroid found in urine obtained from pregnant women (Breuer, 1964).

Dehydroepiandrosterone is the principal 3 β -OH- Δ^5 steroid excreted by adults, but the amount of this compound excreted by infants is very low.

Nevertheless, an attempt was made to measure DHA in some of the samples of urine collected from infants. The levels found during this study were much lower than those reported by Lauritzen and Lehmann (1965, 1967), who used the technique of Fotherby (1959). No explanation can be given for the high results of these workers, but it is thought that their methods were unreliable. Urine samples collected from five infants (four samples supplied by Dr. Lauritzen, and one provided from this study) were assayed for DHA by Dr. Fotherby, and were found to contain between 2.5 and 7.5 $\mu\text{g}/20$ ml. urine, indicating a total daily excretion of about 6-15 μg . It is difficult to reconcile these results with those obtained by Dr. Lauritzen who found normal values for daily excretion ranging between 70 and 250 $\mu\text{g}/24$ hours. The finding in this study of only a small amount of DHA in the urine collected from newborn infants is in agreement with the results reported by Birchall et al. (1961), Cathro et al. (1963) and Paulsen et al. (1966).

2. Control of the production of steroids by the foetus and by the newborn infant.

Many of the $3\beta\text{-OH-}\Delta^5$ steroids which form such a quantitatively important group of steroids produced during pregnancy are probably precursors of the oestrogens, progesterone and the corticosteroids. Although these $3\beta\text{-OH-}\Delta^5$ steroids are so important, relatively little is known of the factors which control their production. If it is assumed that the level of excretion of $3\beta\text{-OH-}\Delta^5$ steroids by infants during the first few days of life is proportional to their production, then a study of the effect of adrenocorticotrophic and gonadotrophic hormones upon their rates of excretion should yield useful information, providing that it can be assumed that the

production of these steroids still proceeds by the same mechanisms as were used during pregnancy. As in later life, adrenocorticotrophin produced by the foetal pituitary gland is of major importance in the regulation of foetal corticosteroid synthesis. It is, however, likely that human chorionic gonadotrophin secreted by the placenta also exerts some control over steroid formation by the foetus.

Lauritzen and Lehmann (1965, 1967) showed that the administration of adrenocorticotrophin to infants during the first week of life caused a significant increase in the excretion of DHA, a precursor of oestrogen, but the effect of stimulation by adrenocorticotrophin on the production of other $3\beta\text{-OH-}\Delta^5$ steroids by the foetus and newborn infant has not previously been determined. In view of the relative unimportance of DHA in urine obtained from newborn infants, measurement of this steroid was only attempted for two of the infants studied, and the quantities found were very low even during stimulation of the adrenals by adrenocorticotrophic hormone. Since adrenocorticotrophin did have an effect on the excretion of all the $3\beta\text{-OH-}\Delta^5$ steroids measured, it is probable that adrenocorticotrophic hormone in foetal blood has some influence on the production of oestrogens and $3\text{-oxo-}\Delta^4$ pregnenes (e.g. progesterone), since the $3\beta\text{-OH-}\Delta^5$ steroids are almost certainly precursors of these two groups of steroids. Further evidence supporting this theory has been provided by Dässler (1966), who reported that excretion of oestriol by women in late pregnancy was increased following the infusion of adrenocorticotrophin; no such increase was obtained when intra-uterine foetal death had occurred. These findings suggest that the administration of adrenocorticotrophin stimulated the foetal adrenal glands to produce more DHA; after 16α -hydroxylation in the foetal liver, the

16 α -OH-DHA was then converted by the placenta to oestriol.

The great increase found in the excretion of cortisol and its metabolites by infants during treatment with adrenocorticotrophin agreed with the increases in blood cortisol in infants reported by Bertrand et al. (1962) and Hillman and Giroud (1965). It is difficult to find an explanation for the greater stimulation of cortisol production, as compared with 3 β -OH- Δ^5 steroid production, but the following alternatives exist:-

1. It is possible that adrenocorticotrophin specifically stimulates steroid production by the cells of the adrenal cortex; these cells are designed primarily to produce corticosteroids. This hormone may have little effect on the synthesis of 3 β -OH- Δ^5 steroids by the foetal zone of the foetal adrenal glands. If this is the case, there must be another hormone which controls the formation of the 3 β -OH- Δ^5 steroids, the precursors of oestrogen.
2. Adrenocorticotrophin may stimulate steroid synthesis by the whole adrenal gland. However, the potential of the cortical zone (a developing tissue) for steroid synthesis may be greater than that of the foetal zone (a degenerating tissue). Adrenocorticotrophin may therefore be able to stimulate cortisol synthesis to a greater extent.

The effect of human chorionic gonadotrophin on the production of steroids by the newborn infant is even more complex than the effect of adrenocorticotrophin. Lauritzen and Lehmann (1965, 1967) have shown that DHA excretion by newborn infants is very significantly increased following intramuscular administration of human chorionic gonadotrophin. These workers were not able to measure the excretion of other 3 β -OH- Δ^5 steroids,

TABLE 18

The Specificity of Action of Human Chorionic Gonadotrophin and
of Adrenocorticotrophin on Steroid Excretion by Newborn Infants

This table gives the mean factor by which the excretion of individual steroids is increased as a result of administration of human chorionic gonadotrophin or of adrenocorticotrophin to newborn infants. These factors have been obtained by comparing the average excretion for the three days when no treatment was given with the amounts excreted for the three days when treatment was being given. The figures for the infants treated with human chorionic gonadotrophin represent only the average change in steroid production for the two infants who showed a considerable increase in excretion of DHA.

<u>Steroid</u>	<u>Human Chorionic Gonadotrophin</u>	<u>Adrenocorticotrophin</u>
DHA	> x 34	x 2
16 α -OH-DHA	x 1.04	x 2
Cortisol	No increase	> x 7

but they did report that, in contrast to the action of adrenocorticotrophin, the 17-hydroxycorticosteroid excretion was not increased following administration of human chorionic gonadotrophin.

In this study the effect of human chorionic gonadotrophin on the production of all the major $3\beta\text{-OH-}\Delta^5$ steroids was examined. Of the five infants studied, none showed a significant increase in the excretion of the 16-hydroxylated metabolites of DHA, but two of the infants showed a considerable increase in the excretion of DHA. Findings for the excretion of 17-hydroxycorticosteroids were in agreement with those of Lauritzen and Lehmann (1967), as no increased excretion of cortisol or of its reduced metabolites was found. It appeared, therefore, that human chorionic gonadotrophin stimulated solely the excretion of DHA.

It may be significant that the two infants that showed an increased excretion of DHA were premature (35 and 36 weeks gestation), as Lauritzen and Lehmann (1967) demonstrated that premature infants responded to human chorionic gonadotrophin to a greater extent than did infants born at term. The extent to which the excretion of the three important steroids (DHA, $16\alpha\text{-OH-DHA}$ and cortisol) are increased by human chorionic gonadotrophin and by adrenocorticotrophin are given in Table 18. Average values have been calculated for the increase in steroid production by adrenocorticotrophin, but for stimulation by human chorionic gonadotrophin only the figures representing the two infants who showed an increased DHA excretion have been included.

The origin of the large amounts of DHA produced during stimulation with human chorionic gonadotrophin is unknown. It is likely that it is formed in the adrenal glands, since this tissue normally produces large

amounts of DHA; most of the DHA is further metabolised before being excreted, for example, by 16-hydroxylation. Human chorionic gonadotrophin may have more effect on the metabolism of DHA prior to excretion, than on the synthesis of DHA by the adrenals. There is no direct evidence, that human chorionic gonadotrophin can control steroid biosynthesis in the foetal adrenal glands, but it has been shown that administration of this hormone produces marked histological changes in the foetal adrenal glands (Johannisson, 1968). Possibly the DHA formed during stimulation by human chorionic gonadotrophin is synthesised in other foetal tissues, for example by the foetal testes. However, for the purpose of this discussion, it will be assumed that the excess DHA produced is synthesised in the adrenal glands.

Since stimulation of steroid synthesis by human chorionic gonadotrophin gives rise to an increase in the ratio of DHA to 16 α -OH-DHA, it could be argued that the 16-hydroxylating enzymes are saturated with the excess of DHA produced. This seems unlikely, however, since the production of 16-hydroxylated compounds is increased considerably by adrenocorticotrophin to levels about twice as high as those found in infants treated with human chorionic gonadotrophin, and it is also unlikely therefore that the 16-hydroxylating enzymes are working at full capacity. It is also possible that human chorionic gonadotrophin increases the excretion of DHA by inhibiting the 16-hydroxylating systems in foetal tissue and tissues from newborn infants. Thus human chorionic gonadotrophin may be concerned with regulating the supply of DHA and 16 α -OH-DHA to the placenta, where these steroids serve as precursors for placental oestrone, oestradiol and oestriol. If, as is suggested, human chorionic gonadotrophin acts in the foetus by

increasing the ratio of DHA to 16α -OH-DHA, an increase in the ratio of oestrone and oestradiol to oestriol (i.e. 16-deoxy-oestrogen to 16-hydroxy-oestrogen) would be the result of the action of human chorionic gonadotrophin. This hormone would then directly control the ratio of "active" oestrogen (oestradiol) to "inactive" oestrogen (oestriol) produced during pregnancy. Adrenocorticotrophin may act to a limited extent as an antagonist of human chorionic gonadotrophin, since it gives rise to a considerable increase in the production of 16α -OH-DHA which would in turn lead to an increased synthesis of oestriol. This scheme is supported by the work of Dässler (1966), who has shown that excretion of oestriol by pregnant women is increased considerably following administration of adrenocorticotrophin.

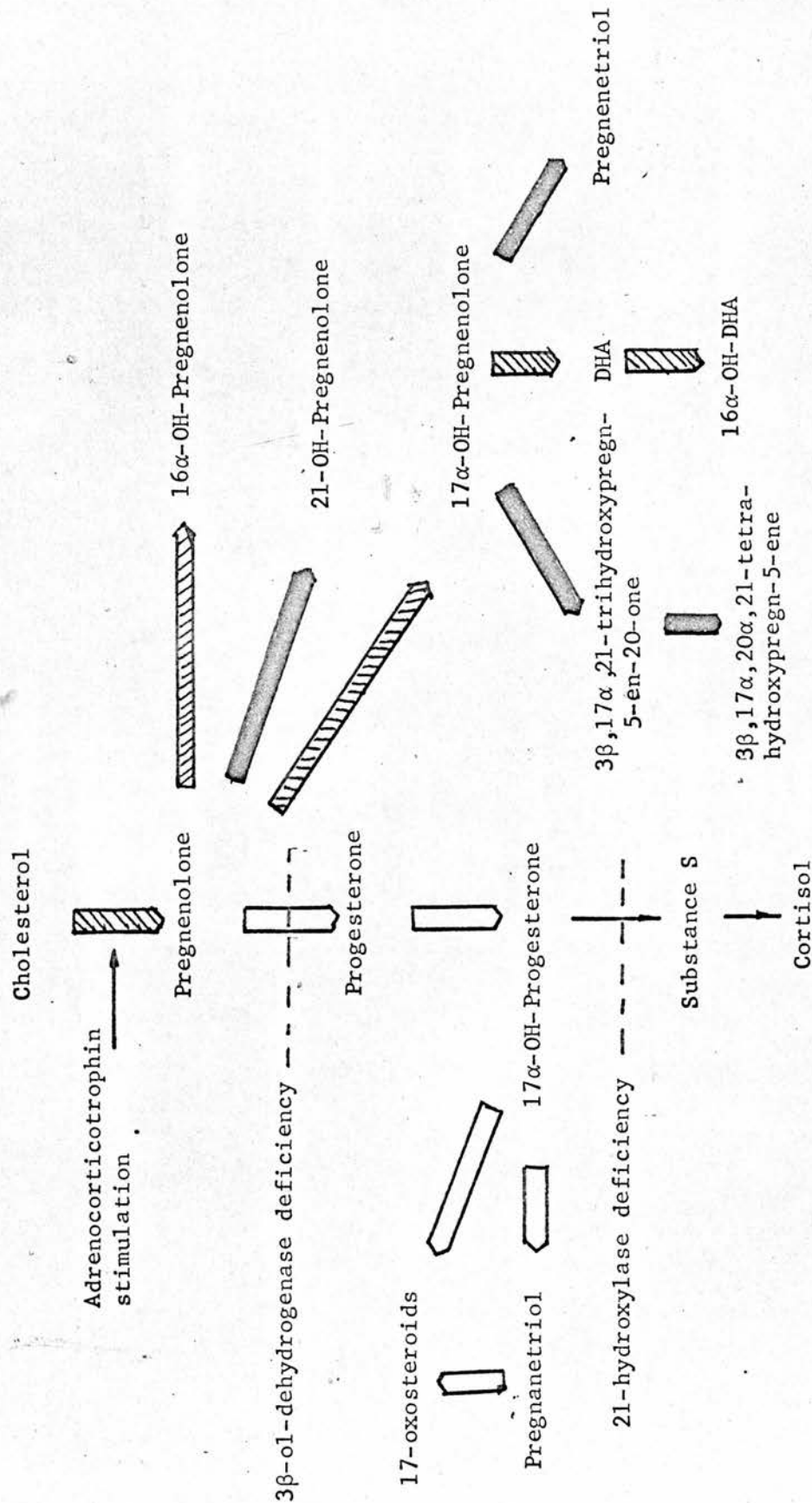
In summary, it seems probable that both adrenocorticotrophin and human chorionic gonadotrophin are important for controlling steroid synthesis by the foeto-placental unit. Adrenocorticotrophin has been shown to be primarily involved in the control of cortisol production, and to a more limited extent in the control of oestrogen synthesis. The true role of human chorionic gonadotrophin is still obscure, but it has been suggested above that human chorionic gonadotrophin is probably concerned with regulating the formation of oestrogens by the placenta, by controlling the synthesis and metabolism of DHA in the foetus.

3. The excretion of steroids by infants with congenital adrenal hyperplasia

During the course of this study an extensive analysis was made of the steroids excreted by an infant with congenital adrenal hyperplasia. This disorder is normally related to a reduced production of cortisol by the adrenal glands since deficiency of cortisol leads to an excessive secretion of adrenocorticotrophin by the pituitary gland, and this in turn causes

Fig. 52. The Major Metabolic Blocks in Congenital Adrenal Hyperplasia

Alternative pathways of metabolism which result from deficiencies in the enzyme 3 β -hydroxydehydrogenase and 21-hydroxylase.



(Insufficient cortisol is produced to suppress the formation of adrenocorticotrophin by the pituitary)

Legend:



Major alternative pathways followed when there is a deficiency of either enzyme



Major alternative pathways followed when there is a deficiency of 3 β -hydroxydehydrogenase



Major alternative pathways followed when there is a deficiency of 21-hydroxylase

continuous adrenal stimulation. The abnormally low production of cortisol is due to a defect in one of the enzyme systems required for the synthesis of this compound; this defect leads to an increased production of those steroids whose synthesis is not affected by the enzyme block. Since the $3\beta\text{-OH-}\Delta^5$ steroids are precursors of all other types of steroid, a defect in most of the enzyme systems required for cortisol synthesis leads to an increased production and excretion of compounds with this structure.

Three forms of congenital adrenal hyperplasia are associated with an increased production of $3\beta\text{-OH-}\Delta^5$ steroids due to inadequate adrenal suppression. These are (a) a deficiency (or inactivity) of 21-hydroxylase (the most common form of the disorder); (b) a deficiency (or inactivity) of 3β -hydroxydehydrogenase; and (c) a deficiency of 11β -hydroxylase. Since the infant studied (patient S.G.) excreted large amounts of the $11\text{-oxo-}17\text{-oxosteroids}$, inactivity of one of the enzymes required for 11β -hydroxylation is unlikely. This discussion of the results will therefore be limited to a consideration of the means of distinguishing a defect in 21-hydroxylase from a deficiency of 3β -hydroxy-dehydrogenase. Figure 52 illustrates the enzymic defects present in these forms of the disorder, and the major metabolic pathways for steroids utilised as a result of these defects.

Eberlein (1966) and Reynolds (1965b) showed that congenital adrenal hyperplasia due to a 21-hydroxylase defect was associated with a greatly increased excretion of the $3\beta\text{-OH-}\Delta^5$ steroids normally excreted by newborn infants. Bongiovanni (1962) reported two cases of congenital adrenal hyperplasia due to a deficiency of 3β -hydroxy-dehydrogenase, the diagnosis being based on the large amounts of $3\beta\text{-OH-}\Delta^5$ steroids which were

excreted. Since both a lack of 21-hydroxylase and of 3 β -hydroxy-dehydrogenase results in increased production of 3 β -OH- Δ^5 steroids, the position of the metabolic block is often difficult to ascertain.

The most marked abnormality of steroid metabolism in patient S.G. on the seventh day of life (the first day when urine was collected) was the excretion of a much larger amount of 3 β -OH- Δ^5 steroids than is normally excreted by infants. There appeared to be no abnormality of a qualitative nature; all the usual 3 β -OH- Δ^5 steroids were present in the urine in approximately the normal proportions, and there was no evidence of an increased amount of any unusual 3 β -OH- Δ^5 steroids (e.g. DHA or pregnetriol) or pregnetriol. However, 21-hydroxylase deficiency in the first few days of life is not always associated with an increased excretion of pregnetriol (Eberlein, 1966). Because of the difficulty of measuring urinary pregnetriol during the first few days of life (Eberlein, 1966), and the abnormally high urinary levels of 3 β -OH- Δ^5 steroids present both in the 3 β -hydroxy-dehydrogenase and the 21-hydroxylase types of the disorder, it is useful to study the excretion of a 21-hydroxylated steroid, e.g. 21-OH-pregnenolone.

Cathro et al. (1965) described a three day old infant suspected of having a deficiency of 3 β -hydroxy-dehydrogenase and who excreted an excessive amount of 21-OH-pregnenolone; these workers used this finding as a means of diagnosing 3 β -hydroxy-dehydrogenase deficiency. Reynolds (1965b) found that the ratio of 16 α -OH-pregnenolone excretion to 16 α -OH-DHA excretion was greater for infants with 21-hydroxylase deficiency than for normal infants. However, for the diagnosis of a 21-hydroxylase block, it would be more valuable to measure the ratio of 16 α -OH-pregnenolone to

21-OH-pregnenolone since this ratio would presumably be very much higher than normal when deficiency of this enzyme existed. This is only true providing it is assumed that a single hydroxylating system catalyses the 21-hydroxylation of all steroids. A large amount of 21-OH-pregnenolone was detected in the urine of patient S.G. on the seventh day of life, but the ratio of 16 α -OH-pregnenolone to 21-OH-pregnenolone was within the normal range, although it was slightly low compared to the average values for normal infants. Initially, it was concluded that the patient did not have 21-hydroxylase deficiency, but that there was a congenital deficiency of 3 β -hydroxy-dehydrogenase. There were several important differences, however, when the steroid excretion of patient S.G. was compared with earlier cases studied by other workers. Bongiovanni (1962) found that the steroids mainly excreted by patients with this disorder were DHA, pregnenetriol, and 3 β ,17 α ,20 α ,21-tetrahydroxypregn-5-ene; these steroids were only excreted in small quantity by patient S.G.. According to Bongiovanni (1966), DHA is the major 17-oxosteroid excreted in this form of adrenal hyperplasia, whereas a high excretion of 11-oxygenated 17-oxosteroids is more usual for 21-hydroxylase deficiency. Therefore, from a study of the steroids excreted by patient S.G. on the seventh day of life, the results obtained were ambiguous and no definite metabolic defect could be ascertained.

A more typical pattern emerged with the second collection, when a fall in the excretion of 21-OH-pregnenolone was associated with the appearance of relatively large quantities of pregnanetriol in the urine. The ratio of 16 α -OH-pregnenolone to 21-OH-pregnenolone (30/1) was now above normal for an infant of this age; it therefore appeared that the patient had a partial deficiency of 21-hydroxylase.

Cathro et al. (1965) give as evidence for 3β -hydroxy-dehydrogenase deficiency the large amount of 21-OH-pregnenolone present in urine obtained from an infant with congenital adrenal hyperplasia, but the assay of this steroid alone amongst the 3β -OH- Δ^5 steroids is inadequate for diagnosis since this finding does not exclude a defect in 21-hydroxylase. The excretion of 21-OH-pregnenolone by patient S.G., who had 21-hydroxylase deficiency, was seven times greater than the average value for newborn infants. This study demonstrated, therefore, the difficulty in identifying positively the enzyme deficient in newborn infants with congenital adrenal hyperplasia solely by the assay of the major urinary steroids. The investigation of the urinary steroids of patient S.G. also emphasised the need to study the excretion of steroids on more than one day, since a very significant change (both qualitative and quantitative) occurred between the seventh and eighth days of life.

4. The assay of steroids in plasma obtained from the umbilical cord.

The assay of steroids present in venous and arterial plasma obtained from the umbilical blood vessels showed that the 3β -OH- Δ^5 steroids were almost invariably present in greater concentration in arterial plasma than in venous plasma. This finding indicates that they are synthesised by the foetus and agree with the recent findings of other workers in respect of pregnenolone, DHA and 16α -OH-DHA (Colás et al., 1964; Simmer et al., 1966; Conrad et al., 1967). It is well established that DHA sulphate and 16α -OH-DHA sulphate are precursors of oestrogens, and two infants studied who were born to mothers having low urinary oestriol excretion (one due to corticosteroid therapy) gave low values for DHA and 16α -OH-DHA in plasma. This agrees with the findings of Simmer and Easterling and their co-workers

TABLE 19

Comparison of the Results of Four Studies on the Quantitative Estimation of 3β -OH- Δ^5 Steroid Sulphates

in Arterial and Venous Plasma Obtained from the Umbilical Vessels

The results are corrected for losses incurred during the estimation.

All figures are expressed in $\mu\text{g}/100 \text{ ml.}$

	Present Study		Colás et al. (1964)		Simmer et al. (1964, 1966)		Easterling et al. (1966)		Conrad et al. (1967)	
	Arterial	Venous	Arterial	Venous	Arterial	Venous	Arterial	Venous	Arterial	Venous
Pregnenolone sulphate	133	95	-	-	-	-	210	135	-	-
DHA sulphate	118	100	116	97	162	130	-	-	-	-
16 α -OH-DHA sulphate	566	438	206	162	370	292	-	-	-	-

(Simmer et al., 1966; Easterling et al., 1966).

There was only a slight degree of correlation between normal levels of the oestrogens excreted by the mother, and DHA and 16α -OH-DHA assayed in plasma obtained from the umbilical blood vessels. This is partly due to the fact that DHA sulphate produced by the mother is also an important precursor of oestrogen (Kirschner et al., 1966). The results given for the plasma levels of pregnenolone, DHA and 16α -OH-DHA are similar to those reported by other workers; the comparative results of four studies are presented in Table 19.

The level of androstenetriol sulphate in plasma obtained from the umbilical cord, although only accurately determined in a sample of pooled venous plasma, was very small compared to the concentration of 16α -OH-DHA sulphate (about 1/20). It is therefore unlikely that this steroid is a significant precursor of oestriol even though it is more similar in structure to oestriol than 16α -OH-DHA. Thus, it is almost certain that reduction of the 17-carbonyl group occurs after aromatisation of the neutral steroid by the placenta.

Dell'Acqua et al. (1966) have demonstrated by perfusion of placentas in situ that 16α -OH-DHA is a better precursor of oestriol than is androstenetriol. By employing similar techniques it was shown that 16α -OH-androstenedione was a better precursor of oestriol than is 16α -OH-testosterone. These workers suggested that the vicinyl $16\alpha,17\beta$ -hydroxyl groups of androstenetriol and of 16α -OH-testosterone interfered with the removal of the angular methyl group at position C-10 of the steroid molecule.

Pregnenolone sulphate was first detected in plasma obtained from

the umbilical blood vessels by Eberlein (1965b) and it has been determined in arterial and venous plasma obtained from the umbilical cord by Conrad et al. (1967) who found it to be in considerably greater concentration in the arterial samples. The determinations reported in this thesis agree with this finding; it therefore seems likely that a considerable amount of pregnenolone sulphate is synthesised by the foetus and taken up by the placenta. Solomon (1966), however, considers that the foetus is not an important producer of pregnenolone because acetate and cholesterol are poor precursors of neutral steroids in the adrenal glands of the foetus, although acetate and cholesterol are active precursors of pregnenolone in the placenta. From the results reported here, and from the work of Conrad et al. (1967), it is clear that relatively large amounts of pregnenolone sulphate must be synthesised by the foetus. The pregnenolone sulphate present in the umbilical circulation must arise solely from the foetus, since the level of this compound is higher in the umbilical arteries than in the vein. Pregnenolone may be produced in greater amount by the placenta than by the foetus but little, if any, escapes into the umbilical circulation. Pion et al. (1966) demonstrated that pregnenolone was an extremely efficient precursor of progesterone in the placenta and it is likely that some of the progesterone elaborated during pregnancy is formed from pregnenolone sulphate synthesised by the foetus. Most however is, or can be, synthesised from pregnenolone produced in the placenta from maternal cholesterol since death of the foetus does not result in a marked reduction of pregnanediol excretion by the mother.

The steroid assays carried out on plasma obtained from the umbilical cord have also indicated that pregnenolone or pregnenolone sulphate is

metabolised by the foetus into 17α -OH-pregnenolone sulphate, 21-OH-pregnenolone disulphate, pregnenediol sulphate and pregnenetriol disulphate. Diczfalusy (1966) considers that pregnenolone metabolites such as these produced by the foetus could be converted into 3-oxo- Δ^4 steroids on reaching the placenta, these steroids then being recirculated to the foetus. If this scheme is at all important in corticosteroid synthesis, then it will only be possible to form corticosterone sulphate (and not free corticosterone) since 21-OH-pregnenolone is present as a disulphate, and the placenta has little or no 21-sulphatase activity (Pasqualini and Diczfalusy, 1966). It is more likely that 17α -OH-pregnenolone are intermediates in the synthesis of corticosteroids in the foetus and only "escape" into the foeto-placental circulation.

Pasqualini et al. (1968) have demonstrated the formation of labelled cortisol by foetuses perfused with blood containing labelled $3\beta,17\alpha,21$ -trihydroxypregn-5-en-20-one. This study indicated that the foetus has a 3β -hydroxy-dehydrogenase capable of performing this conversion even in the absence of an enzyme for the conversion of pregnenolone to progesterone. It is therefore probable that the foetus has 3β -hydroxy-dehydrogenase enzymes capable of converting 17α -OH-pregnenolone and 21-OH-pregnenolone into the analogous 3-oxo- Δ^4 steroids.

The hydroxylated pregnenolone derivatives detected and assayed in this study have all been previously characterised in plasma obtained from the umbilical blood vessels (Eberlein, 1965b), but none have been previously measured quantitatively.

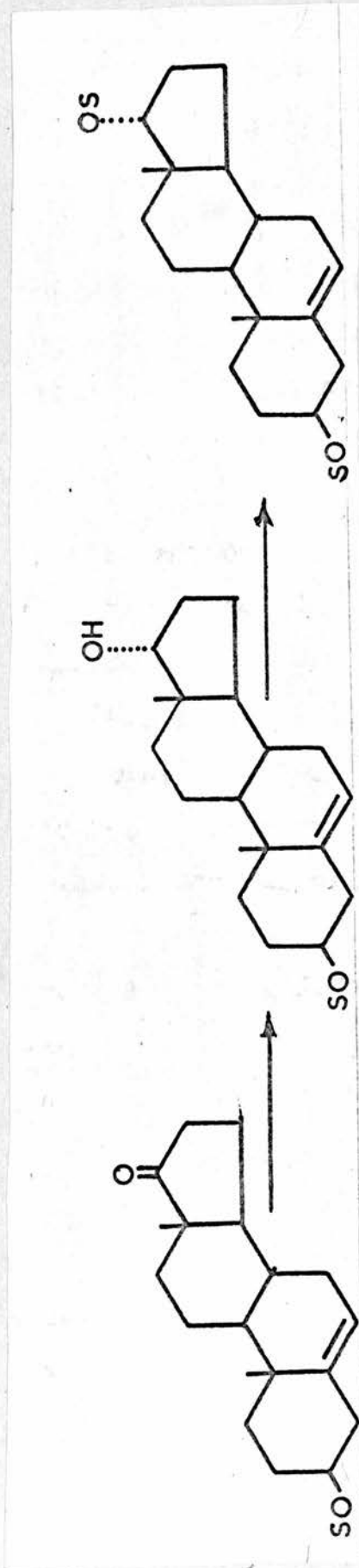
Plasma obtained from the umbilical cord and urine collected from infants have also been shown in this study to contain a relatively large

amount of 17α androstenediol disulphate, a steroid conjugate which has recently been detected in small quantity in peripheral plasma obtained from adults by Sjövall and Vihko (1966, 1968). These workers demonstrated that the major conjugate of androstenediol present in plasma obtained from adults was 17β androstenediol monosulphate. Eberlein (1965b) detected 17β androstenediol in the sulphate fraction prepared from blood samples collected from the umbilical cord at a concentration of about $5\mu\text{g./100 ml.}$, although this compound was possibly not distinguished from its 17α epimer. It is surprising, however, that Eberlein (1965b) did not detect the large quantity of androstenediol reported in the present study; this may have been due to an inadequate recovery of disulphated steroids by the method used in his analyses.

Bolté et al. (1966) isolated 17β androstenediol monosulphate after perfusion of the foeto-placental unit (in vivo) with DHA and DHA sulphate. The monosulphated steroids from this experiment were separated by paper chromatography and were identified after hydrolysis. No attempt was made to isolate any disulphated steroids, and this may be a reason why Bolté et al. (1966) did not find any conversion to 17α androstenediol monosulphate as it was possibly formed but immediately converted to the disulphate. Since these experiments were carried out at mid-pregnancy, the development of 17α -dehydrogenase activity later in pregnancy cannot be excluded. These workers found that 17β -dehydrogenase activity was located principally in the adrenal glands and liver and it seems probable that the 17α -dehydrogenase will also be located in these tissues. Foetal tissue is capable of forming 17β -sulphate esters of steroids at mid-pregnancy, since Mancuso et al. (1967) have reported the formation of testosterone sulphate by foetal

Fig. 53. The Probable Pathway for the Biosynthesis of 17 α Androstenediol Disulphate by the Foetus

This biosynthesis probably takes place with the 3 β -hydroxy group sulphates. It is possible, however, that some 17 α -androstenediol disulphate arises by conjugation of free 17 α androstenediol.



DHA sulphate

17 α Androstenediol
monosulphate

17 α Androstenediol
disulphate

S = Sulphate group

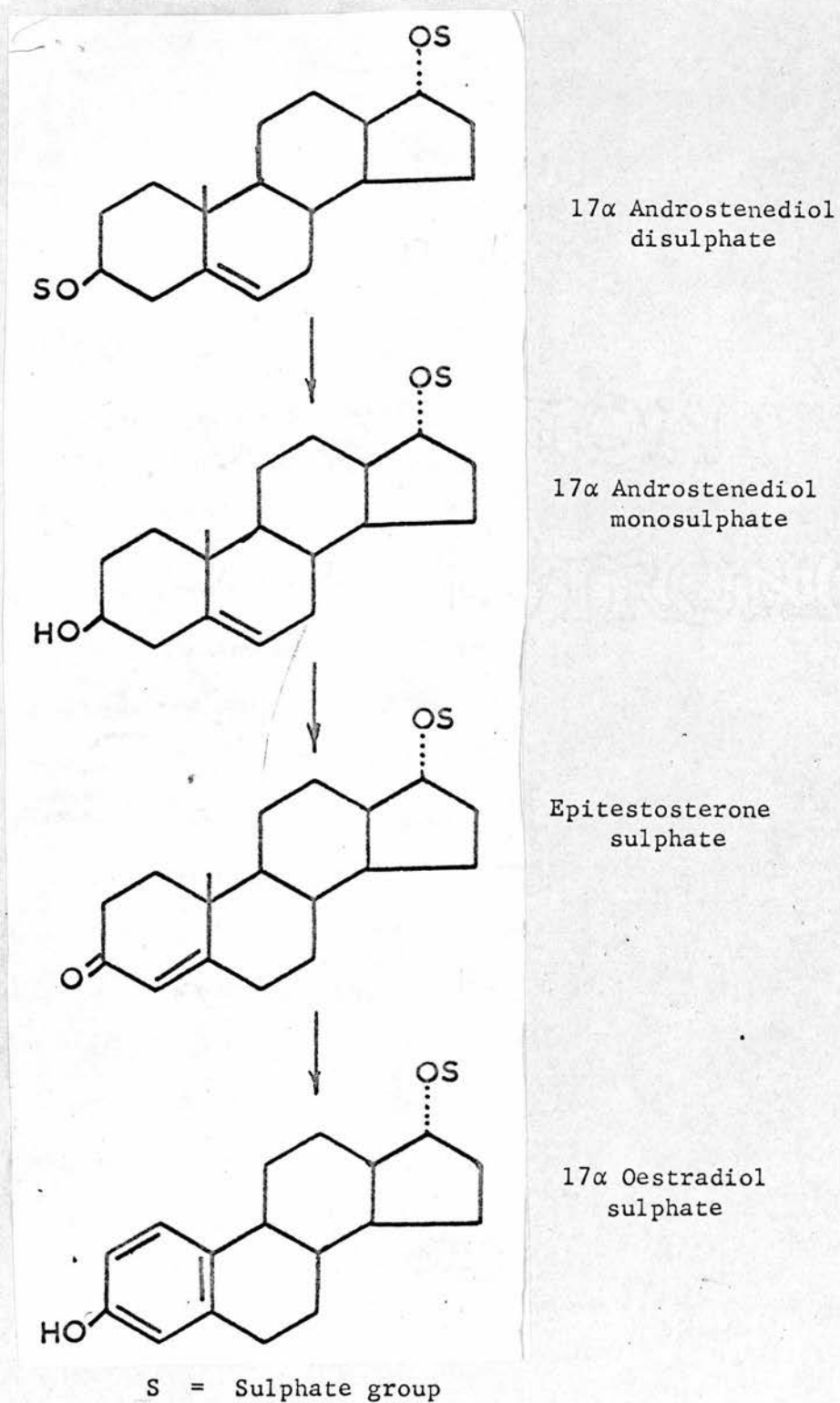
adrenal glands.

The probable biosynthetic pathway for the formation of 17α androstenediol disulphate is illustrated in Fig. 53. Baulieu and Dray (1963) have demonstrated the formation of 17β androstenediol monosulphate from DHA sulphate under in vitro conditions by experiments employing doubly labelled steroid sulphates (^{14}C and ^{35}S). These experiments proved that 17β androstenediol monosulphate could be formed from DHA sulphate without hydrolysis of the sulphate esters. The reverse reaction has been demonstrated by Baulieu et al. (1963), a finding also reported by Roberts et al. (1964). Assuming that 17α androstenediol disulphate is formed from DHA sulphate, the difference between the reduction products of DHA produced by infants and by adults is particularly interesting, since the major metabolite in infants and in the foetus is 17α androstenediol disulphate whereas the major metabolite formed by adults is 17β androstenediol monosulphate. The reason for this difference in metabolism is not known. 17α -Hydroxy-C19-steroids are hormonally inactive so the preferential formation of this epimer by the foetus and by the newborn infant is unusual.

Not all 17-oxo-C19 steroids produced by the foetus are preferentially reduced to 17α -hydroxy steroids. $3\beta,16\alpha,17\beta$ -trihydroxyandrost-5-ene (androstetriol) is the major metabolite of 16α -OH-DHA; no $3\beta,16\alpha,17\alpha$ -trihydroxyandrost-5-ene has yet been detected in extracts of plasma or urine. It is possible that only a small amount of 17α androstenediol is actually formed compared to 17β androstenediol, but the 17α epimer may not be so extensively metabolised as the 17β epimer (e.g. by 16α -hydroxylation).

The metabolism of 17α androstenediol disulphate by the foeto-placental unit is not known but it is possible that this compound could be metabolised to cis-testosterone sulphate by the placenta (Fig. 54); cis-testosterone

Fig. 54. Possible Pathway for the Formation of 17 α Oestradiol Sulphate from 17 α Androstenediol Disulphate



sulphate has recently been identified in adult peripheral blood by Dray et al. (1967). Cis-testosterone sulphate could in turn be aromatised to 17α oestradiol sulphate, a compound which has been identified by Schott and Katzmann (1964) in urine obtained from pregnant women. However, this is unlikely since testosterone sulphate cannot be aromatised by the placenta under in vitro conditions (Baulieu et al., 1965) and this would indicate that hydrolysis of the sulphate group must occur before aromatisation can take place. It is not yet known whether the placenta has the necessary sulphatase for the removal of a 17α -sulphate group, but French and Warren (1966) have shown that this tissue has no 17β -sulphatase activity. Mancuso et al. (1967) demonstrated that testosterone sulphate was formed by fetuses perfused with testosterone, and suggested that this compound accumulated in the foeto-placental unit due to the relative lack of any further metabolism, and to the difficulty that steroid conjugates have in crossing the placental barrier into the mother. If accumulation of this type is possible, 17α -androstenediol disulphate could also share the same fate, and the level of this compound in the foeto-placental circulation may not be directly related to the actual production by the foetus. In this scheme, 17α -androstenediol disulphate has no role to play in the biosynthesis of steroids by the foeto-placental unit, but is merely an end product of the metabolism of DHA sulphate. It will be necessary to study further the biosynthesis of this compound by the foetus and its metabolism by the foeto-placental unit before its importance can be properly assessed.

5. The characterisation of unknown steroid metabolites present in urine obtained from newborn infants

It is evident that many of the unknown steroids present in urine collected from infants have hydroxyl or ketone groups at unusual positions in the steroid molecule. For this reason, several steroids were synthesised during the course of this study (Table 16) for reference purposes, but none of these has been detected yet in extracts of urine.

Several workers in recent years have demonstrated that steroids can be hydroxylated by mammalian enzyme systems in positions other than the classical ones. Knuppen et al. (1966) demonstrated the formation by foetal tissue in vivo and in vitro of oestrogens hydroxylated at the 2, 6 α , 15 α , 16 α and 16 β positions. In addition, these workers identified 15 β -OH-oestrone and 15 β -OH-oestradiol in urine obtained from pregnant women, and Lisboa et al. (1967) identified 15 α -OH-oestradiol from the same source. Zucconi et al. (1967) identified 15 α -OH-oestriol in urine obtained from newborn infants and pregnant women; this was the first report of a naturally occurring human steroid with three functional groups in the D ring.

Studies on the aromatisation of androstenedione and of testosterone by fetuses at midpregnancy showed that 15 α -hydroxylated phenolic metabolites were formed by the foetal liver (Mancuso et al., 1968). However, these workers could not isolate any 15 α (or 16 α)-OH-androstenedione or 15 α (or 16 α)-OH-testosterone from the foetal livers. This suggested that 15 α -hydroxylation only occurred with oestrogens. Since 16 α -hydroxylation by liver enzymes occurs both with oestrogens and with 3 β -OH- Δ^5 steroids, it was considered that the foetal liver might also be capable of effecting the 15 α -hydroxylation of 3 β -OH- Δ^5 steroids because it could hydroxylate oestrogens

in the 15 position. In this study, standard $3\beta,15\alpha,17\beta$ -trihydroxyandrost-5-ene and $3\beta,15\beta,17\beta$ -trihydroxyandrost-5-ene were synthesised, but no evidence was found for the presence of either of these two compounds (or of 15α -OH-DHA) in urine collected from newborn infants. It is therefore likely that 15α -hydroxylated oestrogens are formed by direct hydroxylation, in contrast to the formation of 16α -hydroxylated oestrogens (e.g. oestriol) which are principally formed from neutral 16α -hydroxylated compounds (e.g. 16α -OH-DHA).

No evidence has been found for the presence of 18 -OH-DHA or of $3\beta,17\beta,18$ -trihydroxyandrost-5-ene, although 18 -OH-oestrone has been detected by Loke et al. (1957) in urine collected from pregnant women, and 18 -OH-androsterone and 18 -OH-aetiocholanolone have been identified in urine collected from adults (Fukushima et al., 1962).

11 -Oxygenated 3β -OH- Δ^5 steroids were not detected in urine or plasma, although two compounds ($3\beta,17\alpha,21$ -trihydroxypregn-5-ene- $11,20$ -dione and $3\beta,11\beta,17\alpha,21$ -tetrahydroxypregn-5-en- 20 -one) have been provisionally identified by Eberlein (1965b) in plasma obtained from the umbilical blood vessels. Another of the steroids sought, $3\beta,11\beta,16\alpha$ -trihydroxyandrost-5-en- 17 -one, was a steroid which had previously been tentatively identified by Brooks et al. (1966) in urine obtained from infants. However, thin-layer chromatography did not reveal the presence in urinary extracts of any steroid with mobility similar to this compound.

14α -OH-oestrone has recently been shown to be a major metabolite of oestrone in bovine adrenal tissue (Knuppen et al., 1967) but it was not found by these workers in urine obtained from pregnant women. No 14α -OH-DHA was detected in urine collected from infants.

Evidence has been presented by O'Kelly and Grant (1967) and by Fahmy et al. (1967) for the formation of 19-OH-DHA as an intermediate in the synthesis of oestrogens. It will be of interest to examine extracts of urine for the presence of this compound and its 16 α -hydroxy metabolite (3 β ,16 α ,19-trihydroxyandrost-5-en-17-one).

6. Concluding remarks

Analysis of the steroids excreted in urine by infants has revealed the presence of many compounds which are absent from urine collected from adults. Several of the quantitatively important steroids have been characterised, but there are many more of which the structures are still unknown.

Three populations of steroids may be considered to be formed and/or metabolised by the foetus and newborn infant. These steroids may be expected to be constituents of urine collected from infants and of plasma obtained from the umbilical blood vessels.

1. Steroids arising from the metabolism of the large quantities of oestrogen and of progesterone received from the placenta.
2. Metabolites of 3 β -OH- Δ^5 steroids produced by the foetus for conversion by the placenta into oestrogens and progesterone (e.g. 16 α -OH-DHA and 16 α -OH-pregnenolone). Steroids used by the foetus in the biosynthesis of corticosteroids may also be considered in this group (e.g. 21-OH-pregnenolone and 17 α -OH-pregnenolone).
3. Metabolites of androgens, cortisol and other corticosteroids (corticosteroid and androstane derivatives containing a 3 α -OH-5 α (or 5 β) group).

Steroids with a 3 β -OH- Δ^5 group form quantitatively the most important

group of steroids found in urine excreted by infants. This indicates that the foetus produces much more of these "precursor steroids" than is ever converted into active hormones. The situation in adults is very different since the steroids mainly present in urine are 3α -OH- 5β (or 5α) metabolites of the androgens or of the corticosteroids. The cause of this difference in the pattern of excretion is a continuation into extrauterine life of synthetic systems for steroids developed for use in utero. The relatively small proportion of cortisol metabolites in urine obtained from infants renders their investigation, and therefore the investigation of cortisol production, difficult. It was found impossible in the present study to determine the production of cortisol by an infant with congenital adrenal hyperplasia. For this reason diagnosis of a 21-hydroxylase defect was based on the excessive excretion of 3β -OH- Δ^5 steroids, the high ratio of 16α -OH-pregnenolone to 21-OH-pregnenolone and the presence of pregnanetriol in the urine.

The results reported in this study suggest that human chorionic gonadotrophin and adrenocorticotrophin play an important part in the control of steroid formation during pregnancy. Both hormones are available to the foetus (human chorionic gonadotrophin from the placenta, adrenocorticotrophin from the foetal pituitary) and it is suggested that adrenocorticotrophin controls cortisol synthesis whereas human chorionic gonadotrophin controls the synthesis of foetal precursors of oestrogen. It is possible that human chorionic gonadotrophin increases DHA (and oestrone) synthesis by inhibiting foetal 16α -hydroxylase.

Many steroid-like compounds present in urine obtained from infants have still to be identified, and it may be several years before the metabolism

of steroids by the foetus and newborn infant has been fully elucidated. A complete understanding of the metabolism of steroids will only be obtained by gathering together information obtained by perfusion studies, incubation experiments, and the analysis of steroids in urine, amniotic fluid, plasma and foetal tissue.

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APPENDIX

TABLE 20

Rf Values (x 100) of 3β -OH- Δ^5 Steroids on Thin-Layer Chromatography

These results represent the mobilities of steroids on chromatograms developed once in some of the solvent systems listed in Table 5. The figures represent the arithmetic mean of four determinations.

Medium polarity steroids

	System:-	H	C	F
Androst-5,16-dien-3 β -ol		54	44	55
DHA		40	34	51
11-OXO-DHA		24	27	45
1 α -OH-DHA		4,5	7.0	14
7 α -OH-DHA		3.5	6.5	14
7 β -OH-DHA		5.5	7.0	15
11 β -OH-DHA		24	17	29
14 α -OH-DHA		20	14	27
14 β -OH-DHA		17	13	25
16 α -OH-DHA		22	16	34
16 β -OH-DHA		20	15	30
18-OH-DHA		12	11	24
Androstenediol		33	19	37
11-oxo-androstenediol		14	19	21
16-oxo-androstenediol		22	11	34
3 β ,16 α -dihydroxyandrost-5-en-11,17-dione		11	13	24
3 β ,11 β ,16 α -trihydroxyandrost-5-en-17-one		12	10	16
[Compound U ₁		23	26	45]
Pregnenolone		44	36	51
16 α -OH-pregnenolone		16	13	26
17 α -OH-pregnenolone		36	27	42
18-OH-pregnenolone		38	34	48
21-OH-pregnenolone		24	27	43
Pregnenediol		32	19	37
Pregnenetriol		17	13	22
3 β ,17 α ,21-trihydroxypregn-5-en-20-one		17	13	24

Polar steroids

	System:-	I	G
Androst-5-ene-3 β ,11 β ,17 β -triol		25	17
Androst-5-ene-3 β ,15 α ,17 β -triol		14	11
Androst-5-ene-3 β ,15 β ,17 β -triol		21	17
Androst-5-ene-3 β ,16 α ,17 α -triol		26	24
Androst-5-ene-3 β ,16 α ,17 β -triol		18	15
Androst-5-ene-3 β ,16 β ,17 β -triol		27	26

TABLE 21

The Excretion of 3β -OH- Δ^5 Steroids by Newborn Infants

Number	Day of life	Birth weight	Weeks gestation	Urine vol.	Compound U ₁	21-OH-pregnenolone	Androstenediol	16-oxo-androstenediol	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androsteneetriol	Total	16 α -OH-pregnenolone	16 α -OH-DHA	16 β -OH-DHA	16 α -OH-DHA	21-OH-pregnenolone	16 α -OH-DHA	Androsteneetriol
1	1	3120	39	62	36	15	40	87	72	124	67	108	549	0.93	0.58	0.46	0.66	4.46	0.66	108
2	1	2920	37	42	151	43	40	131	115	94	52	84	710	0.45	1.22	1.21	1.36	1.21	1.36	84
3	1	2450	35½	30	260	210	220	960	420	900	320	140	3430	0.76	0.53	1.53	1.51	1.53	1.51	140
4	1	3450	42	21	335	74	620	136	273	399	168	280	228	0.62	1.62	2.28	0.95	2.28	0.95	280
5	1	3450	41	105	2357	471	111	1203	1161	1262	1434	1434	943	1.2	0.92	3.05	0.81	3.05	0.81	1434
6	1	3500	41	34	793	177	20	1050	1570	730	710	700	5750	0.45	2.15	4.00	0.22	4.00	0.22	700
7	1	2900	38	270	200	200	100	960	1600	260	1520	1620	6460	0.95	6.15	7.60	0.99	7.60	0.99	1620
8	1	2900	-	148	1360	328	840	2420	2240	1520	2100	1180	11988	0.94	1.47	6.40	1.89	6.40	1.89	1180
9	1	4300	41	15	312	420	460	1460	1760	1480	840	240	6972	0.48	1.18	2.00	7.33	2.00	7.33	240
10	1	2480	36	230	345	300	120	1260	990	370	1280	862	5527	1.29	2.67	4.27	1.15	4.27	1.15	862
11	1	2320	35	47	20	20	10	38	32	51	96	100	367	3.00	0.63	5.00	0.32	5.00	0.32	100
12	1	2320	37	220	620	280	7	2160	1920	1560	3280	1520	11347	1.71	1.23	11.70	1.26	11.70	1.26	1520

13	1	3460	40	25	156	75	41	275	387	187	612	390	2123	1.01	8.16	2.10	0.99
14	4	2420	38	150	560	320	160	1880	1680	500	1100	780	6980	1.02	3.12	2.16	1.40
15	1	3348	39	37	157 ^a		171	347	1180	444	880	370	3549	0.745		2.65	3.2
16	1	3272	42	41	158		51	477	354	369	610	200	2219	1.72		0.96	1.77
17	1	3272	42	73	219		91	577	839	912	1003	591	4232	1.19		0.92	1.42
18	1	3214	42	103	20		20	30	50	20	103	20	263	2.06		2.50	2.5
19	1	3424	39	40	150		172	460	517	150	540	483	2472	1.04		3.44	1.07
20	1	3419	39	47	289		289	258	176	247	188	295	1743	1.07		0.71	0.59
21	1	3348	-	30	109		10	164	138	109	100	88	718	0.724		1.26	1.57
22	1	3571	40	45	219		180	325	349	219	95	76	1463	2.72		1.59	4.54
23	1	3600	-	14	70		133	182	367	214	233	129	1328	0.63		1.71	2.84
24	2	2616	40	72	222		277	222	555	351	92	306	2025	1.67		1.58	1.79
25	2	4071	41	33	41		124	214	255	66	148	93	941	3.65		3.86	2.74
26	2	4071	39	30	375		353	615	1116	372	540	560	3931	0.504		2.98	1.95
27	3	4100	42	53	344		371	715	556	477	477	848	3788	1.77		1.16	0.655
28	4	3272	39	59	103		221	486	324	531	354	250	2269	1.09		0.61	1.291
29	5	3526	41	80	220		270	560	820	60	1220	520	3670	1.48		13.66	1.58
30	8	3616	40	312	434		633	908	592	237	948	1465	5217	1.75		2.50	0.40
31	8	2803	38	75	435		452	696	737	285	820	743	4168	1.11		2.58	0.99
32	9	3325	-	132	84		17	34	51	20	238	99	459	1.94		5.0	0.51

(a) The analyses of urine specimens collected from infants 15-32 were carried out early in the study, before a technique had been developed for the separation of compound U₁ from 21-OH-pregnenolone. The figure given represents compound U₁ and 21-OH-pregnenolone assayed together.

TABLE 22

The Daily Excretion of 3β -OH- Δ^5 Steroids by Four Normal Infants During the First Six Days of Life

Infant birth weight and gestation	Day of life	Volume	Pregnenolone	DHA	Compound U ₁	21-OH-pregnenolone	Androstenediol	16-oxo-androstenediol	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol
CON. 1 2450 g 35 wk.	1	30	<50 ^b	<50	260	210	<20	960	420	900	320	140
	2	48	<50	<50	320	300	<20	1600	1020	940	480	340
	3	126	<50	<50	410	580	95	2960	1780	900	2000	1600
	AV ^a	68	<50	<50	330	363	45	1840	1073	913	933	693
	4	152	<50	<50	320	699	20	2080	1400	900	1660	1070
	5	198	<50	<50	185	435	20	1680	1140	820	2080	760
	6	228	<50	<50	320	320	20	1480	1800	560	2680	660
AV	192	<50	<50	275	484	20	1746	1446	760	2140	830	
CON. 2 3450 g 42 wk.	1	21	17	<5	335	74	20	1136	273	399	1680	280
	2	25	20	7	481	96	42	346	262	588	1365	340
	3	76	12	33	641	139	50	2020	590	710	330	1520
	AV	41	16	15	485	103	27	834	375	565	1125	713
	4	115	12	<5	490	104	168	1940	539	455	455	1300
	5	150	9	<5	212	42	39	715	392	176	264	1000
	6	152	10	<5	150	28	30	340	240	100	280	820
AV	139	10	<5	284	58	79	992	350	243	313	1040	

CON. 3	1	105	12	< 5	2357	471	111	1203	1161	1262	1434	1434
3450	2	110	10	< 5	1608	311	202	1070	1393	1444	2040	1030
41 wk.	3	105	10	< 5	1221	253	212	969	1680	919	1282	606
	AV	107	10	< 5	1728	345	175	1080	1411	1208	1585	1023
	4	200	8	< 5	1690	330	171	1030	1565	1393	2424	1010
	5	240	10	< 5	895	185	20	686	939	878	1201	383
	6	242	8	< 5	675	143	20	646	828	800	1200	404
	AV	227	8	< 5	1086	219	70	787	1110	1023	1608	599

CON. 4	1	34	40	13	793	177	< 20	1050	1570	730	710	700
3500	2	60	18	< 5	682	148	< 20	760	810	930	370	580
	3	132	25	< 5	740	160	< 20	880	750	520	470	648
	AV	75	27	7	738	161	< 20	896	1043	726	516	642
	4	175	23	< 5	555	115	220	660	700	350	910	820
	5	210	27	< 5	655	145	40	1100	900	350	900	832
	6	186	37	7	884	176	20	930	750	470	490	740
	AV	190	29	< 5	698	145	93	896	783	390	766	797

(a) AV - Average daily excretion for the three day period

(b) A "less-than" symbol (<) indicates that the steroid was not accurately assayed, but was present at a concentration less than the figure given.

TABLE 23

The Daily Excretion of 3β -OH- Δ^5 Steroids, and of Cortisol and its Metabolites, by Five Infants Treated with

Adrenocorticotrophic hormone (ACTH)

Infant birth weight and gestation	Day of life	Volume	Pregnenolone	DHA	Compound U ₁	21-OH-pregnenolone	Androstenediol	16-oxo-androstenediol	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol	Cortisol	Tetrahydrocortisol	Allosterahydrocortisol	Treatment with adrenocorticotrophic hormone (ACTH)
ACTH 1 2900 g 38 wk	1	270	<50 ^b	<50	200	200	100	960	1600	260	1520	1620	<80	<40	<40	-
	2	230	<50	<50	285	140	90	840	1140	200	1200	1220	<80	<40	<40	-
	3	360	<50	<50	475	220	104	1460	1640	200	1180	1140	<80	<40	<40	-
	Av ^a	290	<50	<50	320	186	98	1086	1460	220	1300	1326	<80	<40	<40	-
	4	310	<50	<50	270	505	86	3240	4800	960	7080	1600	1400	900	900	8 i.u. ACTH
	5	280	<50	<50	290	500	75	3640	4240	1000	6000	1800	1400	1780	1780	8 i.u. ACTH
ACTH 2 2900 g	1	148	12	25	1360	328	840	2420	2240	1520	2100	1180	<400	<240	<240	-
	2	215	12	36	1140	416	680	2180	2560	1160	2260	1120	<400	<240	<240	-
	3	252	40	50	920	312	620	2040	3180	1060	3020	1220	<400	<240	<240	-
	Av	205	21	37	1140	352	713	2213	2660	1246	2460	1173	<400	<240	<240	-
	4	230	100	120	776	176	420	740	3000	400	5680	1180	1200	1380	1380	8 i.u. ACTH
	5	250	72	50	888	264	600	2180	3520	1080	5160	1180	2000	2920	2920	8 i.u. ACTH
-	6	344	100	160	1408	416	560	2440	3200	1180	6000	1100	4000	3900	3900	8 i.u. ACTH
	Av	274	90	110	1024	285	526	1786	3240	886	5813	1153	2400	2733	2733	8 i.u. ACTH

ACTH 3	1	15	<12	<12	312	420	460	1460	1760	1480	840	240	160	<120	-
4300 g	2	38	<12	<12	420	492	800	3000	2160	2080	980	680	160	<120	-
41 wk	3	40	<12	<12	416	396	880	3260	1740	1740	760	1120	320	<120	-
Av	4	31	<12	<12	382	436	713	2573	1886	1766	860	680	213	<120	-
	5	125	15	17	196	452	680	5480	5600	1480	2800	1200	320	352	8 i.u. ACTH
	6	320	20	25	392	864	1140	4320	4600	1080	5000	1980	640	1008	8 i.u. ACTH
Av	6	350	20	15	800	816	1260	5600	5040	1880	6160	1940	700	1088	8 i.u. ACTH
	Av	265	18	19	462	710	1026	5133	5080	1480	4653	1706	553	816	-

ACTH 4	4	150	<50	<50	360	320	160	1880	1080	500	1100	780	<100	<70	-
2420 g	5	120	<50	<50	200	160	120	980	480	80	740	720	<100	<70	-
38 wk	6	112	<50	<50	220	160	140	1100	660	100	920	520	<100	<70	-
Av	7	127	<50	<50	260	213	140	1320	740	226	920	673	<100	<70	-
	8	130	<50	<50	400	360	160	2040	2040	400	3200	960	1200	400	8 i.u. ACTH
	9	216	<50	<50	640	600	160	3160	2400	720	3840	1200	2000	1310	8 i.u. ACTH
Av	9	250	<50	<50	200	260	180	1560	1460	640	1440	860	400	185	8 i.u. ACTH
	Av	198	<50	<50	413	406	166	2253	1966	586	2826	1006	1200	631	-

ACTH 5	1	220	<50	<50	620	280	<7	2160	1920	1560	3280	1520	<100	110	-
2320 g	2	310	<50	<50	640	180	<7	2160	1800	1680	3200	1280	<100	100	-
37 wk	3	340	<50	<50	640	160	<7	1800	1660	1420	2500	1600	<100	120	-
Av	4	290	<50	<50	633	206	<7	2040	1793	1553	2993	1466	<100	110	-
	5	280	<50	<50	780	240	<7	2360	2480	2040	4920	1580	750	590	8 i.u. ACTH
	6	320	<50	<50	1440	860	109	2160	3424	2320	6800	1360	1000	840	8 i.u. ACTH
Av	6	370	<50	<50	1300	840	109	2200	2540	2220	6080	1240	1500	730	8 i.u. ACTH
	Av	323	<50	<50	1173	646	675	2240	2814	2195	5933	1393	1083	736	-

(a) Av - average daily excretion for the three day period.

(b) A "less than" symbol (<) indicates that the steroid was not accurately assayed, but was present at a concentration less than the figure given.

(c) Tetrahydrocortisol and allotetrahydrocortisol were not individually determined.

TABLE 24

The Daily Excretion of 3β -OH- Δ^5 Steroids by Five Infants Treated with Human Chorionic Gonadotrophin (HCG)

Infant birth weight and gestation	HCG	Day of life	Volume	Pregnenolone	DHA	Compound U ₁	21-OH-pregnenolone	Androstenediol	16-oxo-androstenediol	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol	Treatment with HCG
HCG 1	232 g	1	47	<20 ^b	<20	220	20	10	38	32	51	96	100	
		2	100	<20	<20	290	26	12	243	256	205	600	660	
		3	100	<20	<20	198	90	38	224	314	230	875	590	
		AV ^a	82	<20	<20	169	45	20	168	200	162	523	450	
		4	170	<20	350	205	109	77	410	475	435	1280	790	5000 i.u. HCG
		5	150	<20	1120	109	166	64	314	475	268	1025	500	5000 i.u. HCG
		6	190	<20	3000	109	51	64	364	400	230	1190	632	5000 i.u. HCG
		AV	170	<20	1490	141	108	68	362	450	311	1165	640	
HCG 2	2480 g	1	230	<50	<50	345	300	120	1260	990	370	1280	862	
		2	140	<50	<50	465	460	210	1080	950	360	1320	400	
		3	260	<50	<50	615	590	205	1570	1570	660	2300	590	
		AV	210	<50	<50	475	450	178	1303	1170	463	1633	617	
		4	200	<50	450	475	616	220	1970	1640	330	2380	330	5000 i.u. HCG
		5	300	<50	320	190	315	245	980	1140	230	1400	230	5000 i.u. HCG
		6	310	<50	1940	235	315	175	1770	1930	129	1730	215	5000 i.u. HCG
		AV	270	<50	903	300	413	213	1573	1570	229	1836	258	

HCG 3	1	42	36	18	151	43	20	131	115	94	52	84
2920 g	2	72	22	6	185	65	20	80	170	285	80	112
37 wk	3	80	37	7	460	165	110	285	410	410	200	250
AV	AV	64	31	10	265	91	50	165	231	263	110	148
4	4	155	7	7	389	131	236	393	314	411	288	481
5	5	140	8	3	281	96	181	342	257	237	189	251
6	6	150	10	4	219	98	243	262	192	191	523	147
AV	AV	158	9	4	296	108	220	332	254	279	333	293

5000 i.u. HCG
5000 i.u. HCG
5000 i.u. HCG

HCG 4	1	62	10.3	32	36	15	10	87	72	124	67	108
3120 g	2	68	9.0	53	130	40	10	195	245	300	170	395
39 wk	3	67	2.9	8.8	374	128	39	532	418	807	591	985
AV	AV	65	7.4	31	180	61	19	271	245	410	276	496
4	4	120	3.0	9.0	235	105	10	355	400	200	130	660
5	5	160	8.3	72	124	37	10	240	277	88	383	365
6	6	185	18.1	43	74	37	10	260	74	64	454	362
AV	AV	155	9.8	41	144	59	10	285	250	117	322	462

5000 i.u. HCG
5000 i.u. HCG
5000 i.u. HCG

HCG 5	1	25	53	40	156	75	40	275	387	187	612	390
3460 g	2	50	105	32	388	137	83	571	576	601	868	569
40 wk	3	84	18	14	504	126	68	546	588	656	703	976
AV	AV	53	58	28	349	112	63	464	517	481	727	645
4	4	140	28	22	230	155	23	415	470	270	690	790
5	5	130	19	20	249	111	23	356	425	175	601	883
6	6	180	11	18	264	113	76	345	508	145	621	836
AV	AV	150	19	20	247	126	40	372	467	196	637	836

5000 i.u. HCG
5000 i.u. HCG
5000 i.u. HCG

(a) AV - Average daily excretion for the three day period.

(b) A "less than" symbol (<) indicates that the steroid was not accurately assayed, but was present at a concentration less than the figure given.

TABLE 25

Quantitative Results for the Determination of 3β -OH- Δ^5 Steroids in Plasma
Obtained from the Umbilical Blood Vessels and the Corresponding Figures
for Oestrogens in Urine Collected from the Mother

- A - Arterial result
 V - venous result
 A-V - arterio-venous difference
 % - arterio-venous difference as percentage of arterial result.

Infant	Weight (g)	Gestation (wk)	Plasma 3β -OH- Δ^5 steroids ($\mu\text{g}/100\text{ ml.}$)								Urinary oestrogen (mg/24 hr.)			
			Pregnenolone	DHA	17 α -OH-pregnenolone	21-OH-pregnenolone	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol	Oestrone	Oestradiol	Oestriol	
1.	3465	39	A	108	83	47	53	386	17	142	107	0.62	0.12	24.3
			V	41	74	37	46	327	16	125	96			
			A-V	67	9	10	7	59	1	17	11			
			%	62	11	21	13	15	6	12	10			
2.	4402	39	A	56	65	30	45	200	20	100	160	0.85	0.57	16.9
			V	33	42	16	30	180	16	80	76			
			A-V	23	23	14	15	20	4	20	84			
			%	41	35	47	33	10	20	20	52			
3.	4100	42	A	101	82	82	103	363	73	200	110	1.65	0.28	20.6
			V	62	62	71	94	238	52	168	110			
			A-V	39	20	11	9	125	21	32	0			
			%	39	24	13	9	34	29	16	0			

TABLE 25 (cont.)

Infant	Weight (g.)	Gestation (wk.)		Pregnenolone	DHA	17 α -OH-pregnenolone	21-OH-pregnenolone	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol	Oestrone	Oestradiol	Oestriol
4.	3124	39	A	87	100	54	60	192	16	94	240	0.69	0.031	17.2
			V	55	60	28	37	148	12	34	220			
			A-V	32	40	26	23	44	4	60	20			
			%	37	40	48	38	23	25	64	8			
5.	4487	40	A	68	98	83	115	560	-	294	210	2.26	0.52	23.0
			V	68	80	44	77	420	-	164	200			
			A-V	0	18	39	38	140	-	130	10			
			%	0	18	47	33	25	-	44	5			
6.	1789	39	A	83	47	25	30	114	18	35	64	0.36	0.16	5.3
			V	27	24	14	18	93	13	30	48			
			A-V	56	23	11	12	21	5	5	16			
			%	67	49	44	40	18	29	14	25			
7.	3181	40	A	-	-	-	-	-	-	-	-	1.30	0.039	11.8
			V	60	110	38	90	490	38	260	104			
			A-V	-	-	-	-	-	-	-	-			
			%	-	-	-	-	-	-	-	-			

TABLE 25 (cont.)

Infant	Weight (g.)	Gestation (wk.)		Pregnenolone	DHA	17 α -OH-pregnenolone	21-OH-pregnenolone	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol	Oestrone	Oestradiol	Oestriol
8.	4629	39	A	57	68	42	36	192	-	154	110	0.61	0.25	19.3
			V	67	73	35	30	152	-	120	112			
			A-V	-10	-5	7	6	40	-	34	-2			
			%	-18	-7	17	17	21	-	22	-2			
9.	3380	39	A	157	107	103	109	546	19	309	65	0.54	0.20	34.6
			V	147	99	94	115	525	15	305	80			
			A-V	11	8	8	-6	21	4	4	-15			
			%	6	8	8	-5	4	22	1	-23			
10.	3860	-	A	105	84	44	31	412	-	218	150	0.56	0.17	15.7
			V	54	55	37	46	319	-	181	148			
			A-V	50	29	7	-15	92	-	31	2			
			%	48	35	17	-49	22	-	14	1			
11.	4270	40	A	92	68	56	42	512	-	235	10	0.63	0.28	22.6
			V	86	64	47	63	363	-	210	24			
			A-V	6	4	9	-21	149	-	25	-14			
			%	7	6	15	-50	29	-	11	-140			

TABLE 25 (cont.)

Infant	Weight (g.)	Gestation (wk.)		Pregnenolone	DHA-OH-pregnenolone	17 α -OH-pregnenolone	21-OH-pregnenolone	16 α -OH-DHA	Compound U ₂ (16 β -OH-DHA)	16 α -OH-pregnenolone	Androstenediol	Oestrone	Oestradiol	Oestriol
12.	2850	38	A	-	-	-	-	-	-	-	-	-	-	-
(see note 1)			V	117	105	92	42	131	-	202	100			
			A-V	-	-	-	-	-	-	-	-			
			%	-	-	-	-	-	-	-	-			
13.	3250	40	A*	14	14	-	-	90	-	40	111	-	-	-
(see note 1)			V	-	-	-	-	-	-	-	-			
			A-V	-	-	-	-	-	-	-	-			
			%	-	-	-	-	-	-	-	-			
14.	2300	39	A	-	-	-	-	-	-	-	-	0.163	0.074	8.4
(see note 2)			V	11	11	20	53	200	10	90	80			
			A-V	-	-	-	-	-	-	-	-			
			%	-	-	-	-	-	-	-	-			

* Assay on a specimen (total volume 2.8 ml.) consisting of plasma obtained from both arterial and venous sources (0.8 ml. and 1.5 ml. volumes respectively).

Notes:

1. Infants 12 and 13 were in a distressed condition at birth. Infant 13 died the following day.
2. The mother of this infant was taking prednisolone during pregnancy. This steroid may be expected to suppress indirectly the production of steroids by the adrenal glands of the fetus.

Figures 55 - 68.

Mass-Spectra of 3β -OH- Δ^5 Steroids

These figures illustrate the mass-spectra obtained for several reference 3β -OH- Δ^5 steroids. Some of these steroids were identified in urine obtained from infants, i.e. the mass-spectrum of a reference compound and a steroid isolated from urine were virtually identical.

The heights of each peak (relative intensity) were measured relative to the "base-peak", i.e. the highest peak in the spectrum. Several of the spectra contain very large peaks at m/e 73 and 75 but these are due to impurities and should not be confused with the base-peak.

M^+ represents the molecular ion

$M - x$ represents a loss of x mass units from the molecular ion

TMS10 - Trimethylsilyl ether.

Fig. 55. Mass-Spectrum of Androst-5,16-dien -3 β -ol Trimethylsilylyl Ether

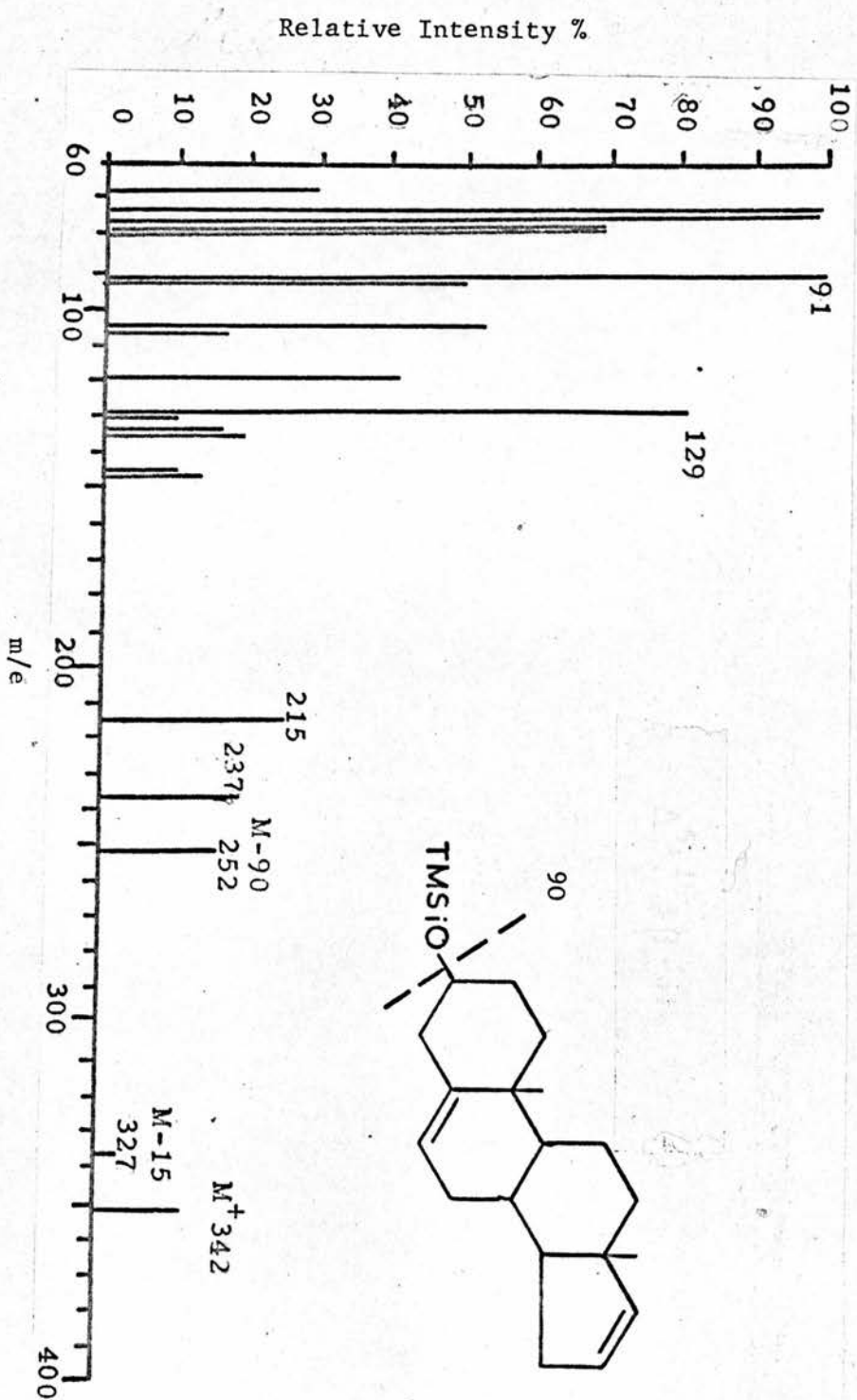


Fig. 56. Mass-Spectrum of Dehydroepiandrosterone (DHA) Trimethylsilyl Ether

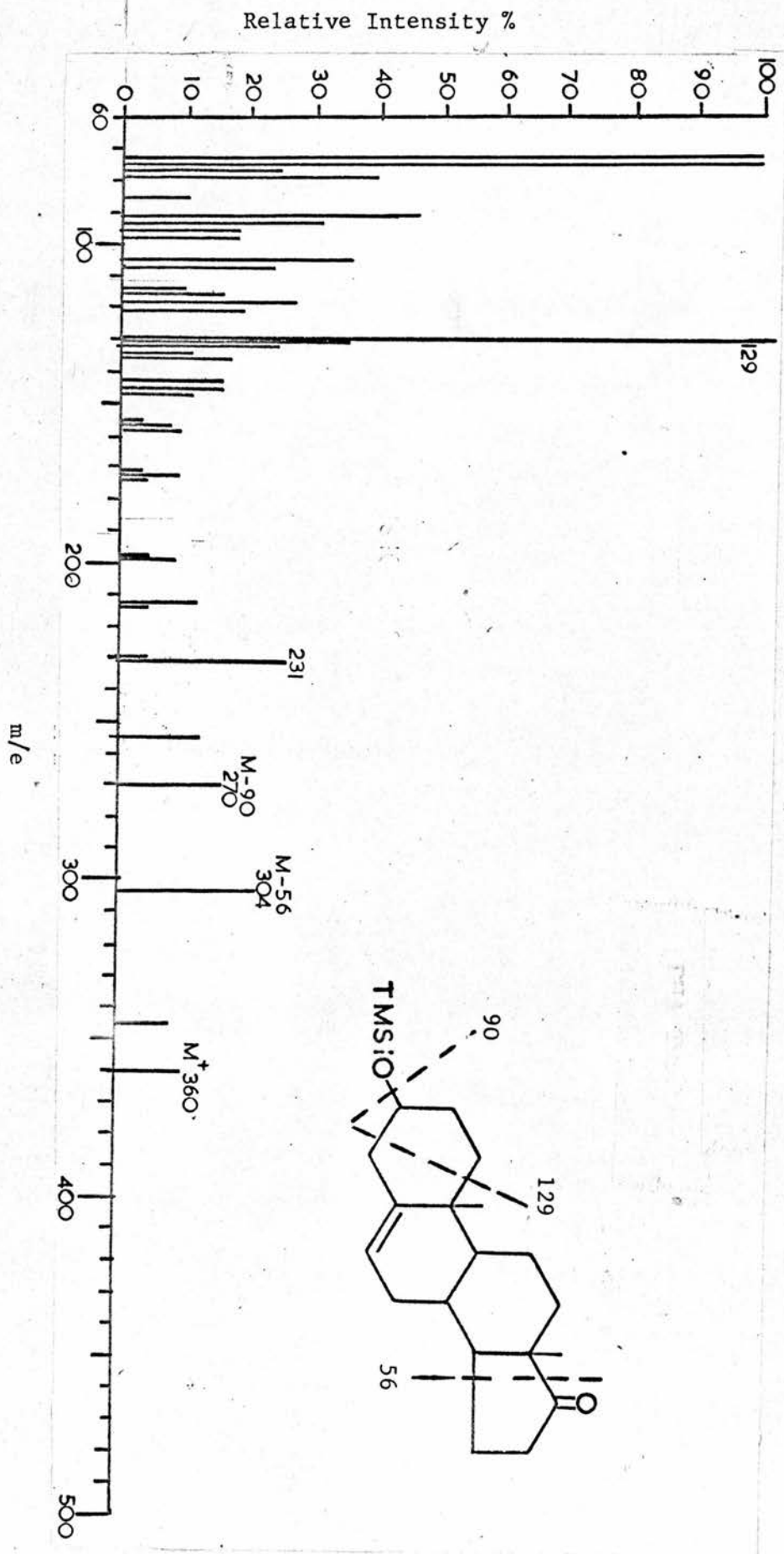


Fig. 57. Mass-Spectrum of 11-oxo-DHA Trimethylsilyl Ether

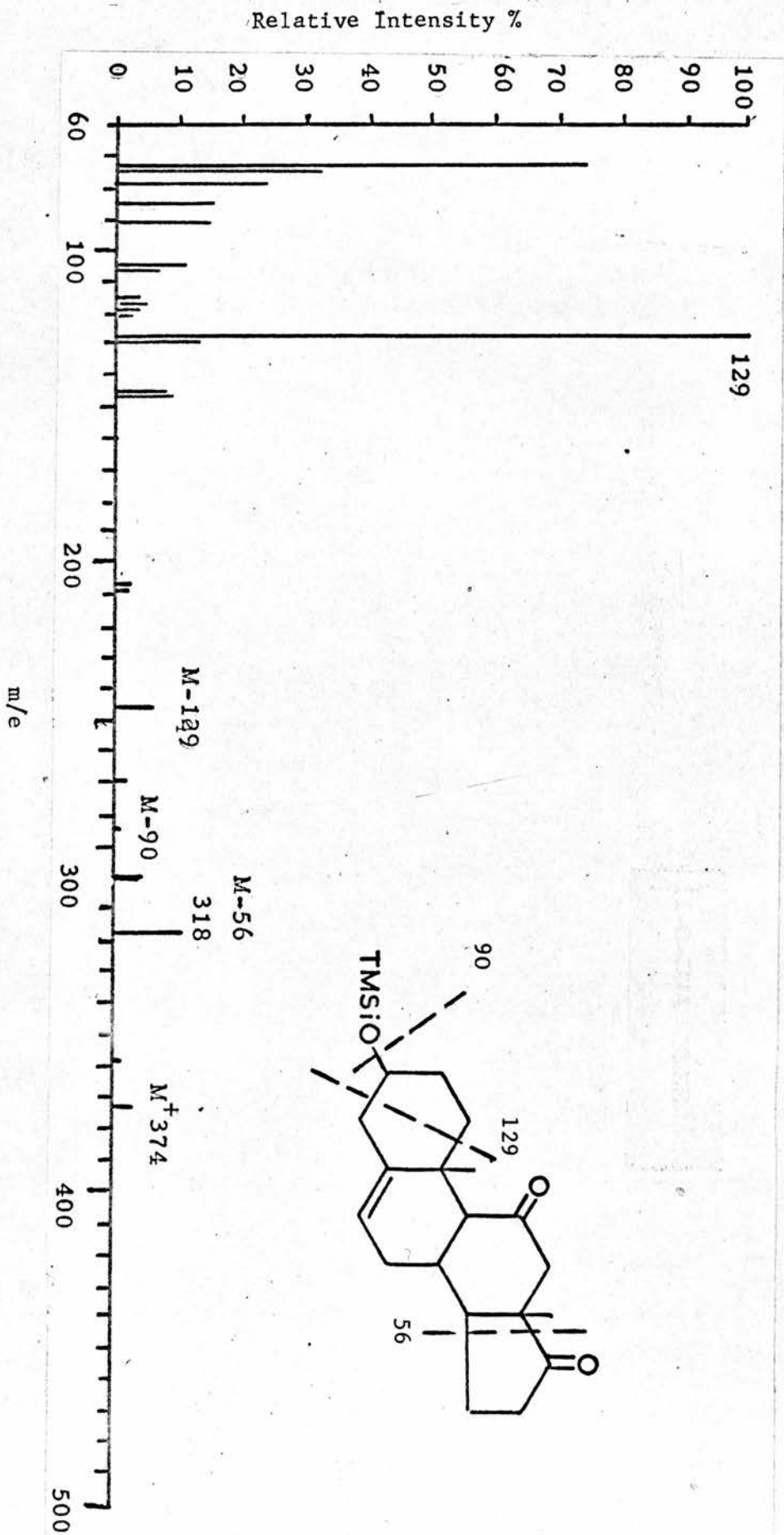


Fig. 58. Mass-Spectrum of α -OH-DHA Trimethylsilyl Ether

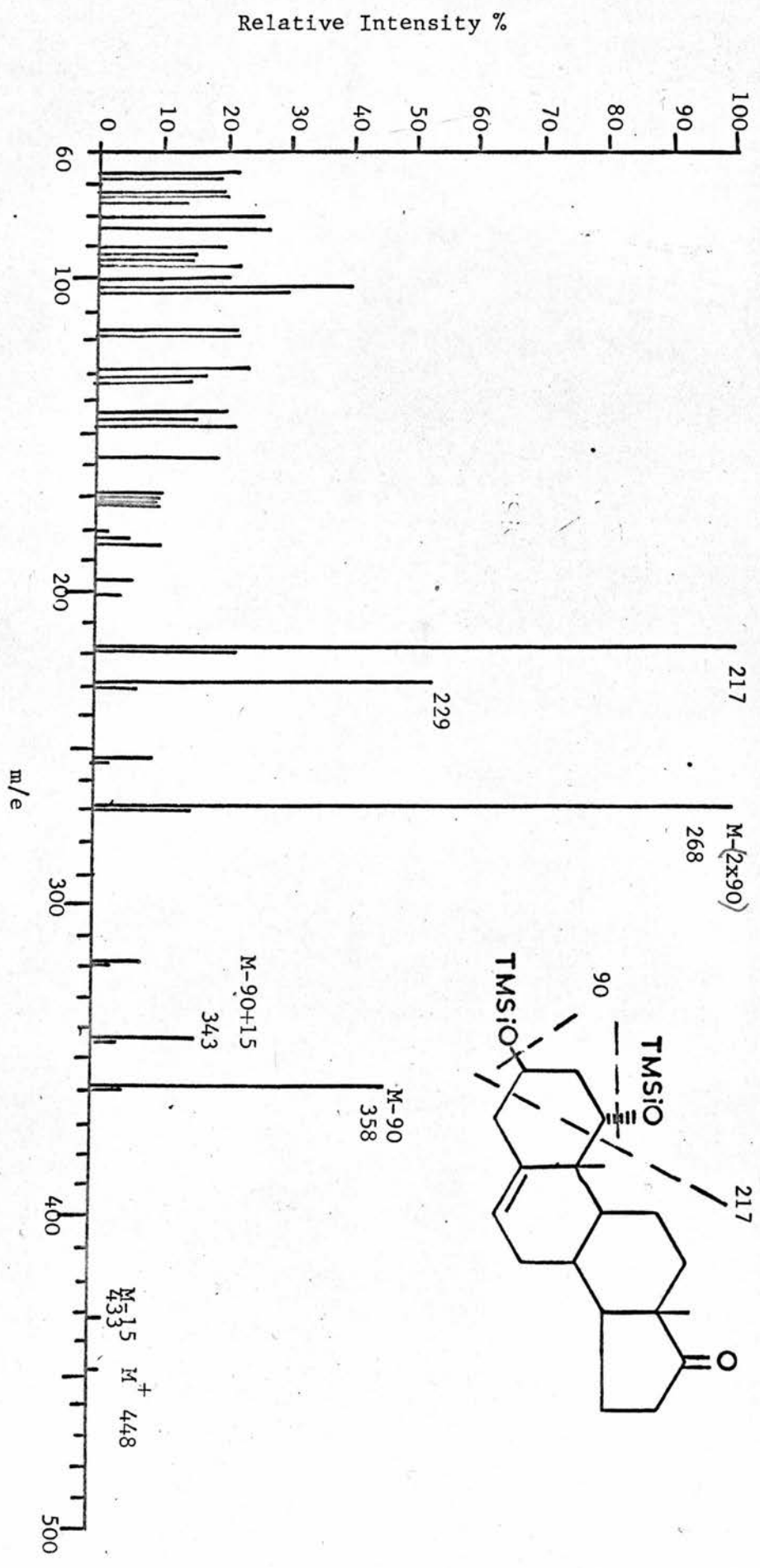


Fig. 59. Mass-Spectrum of 7 α -OH-DHA Trimethylsilyl Ether

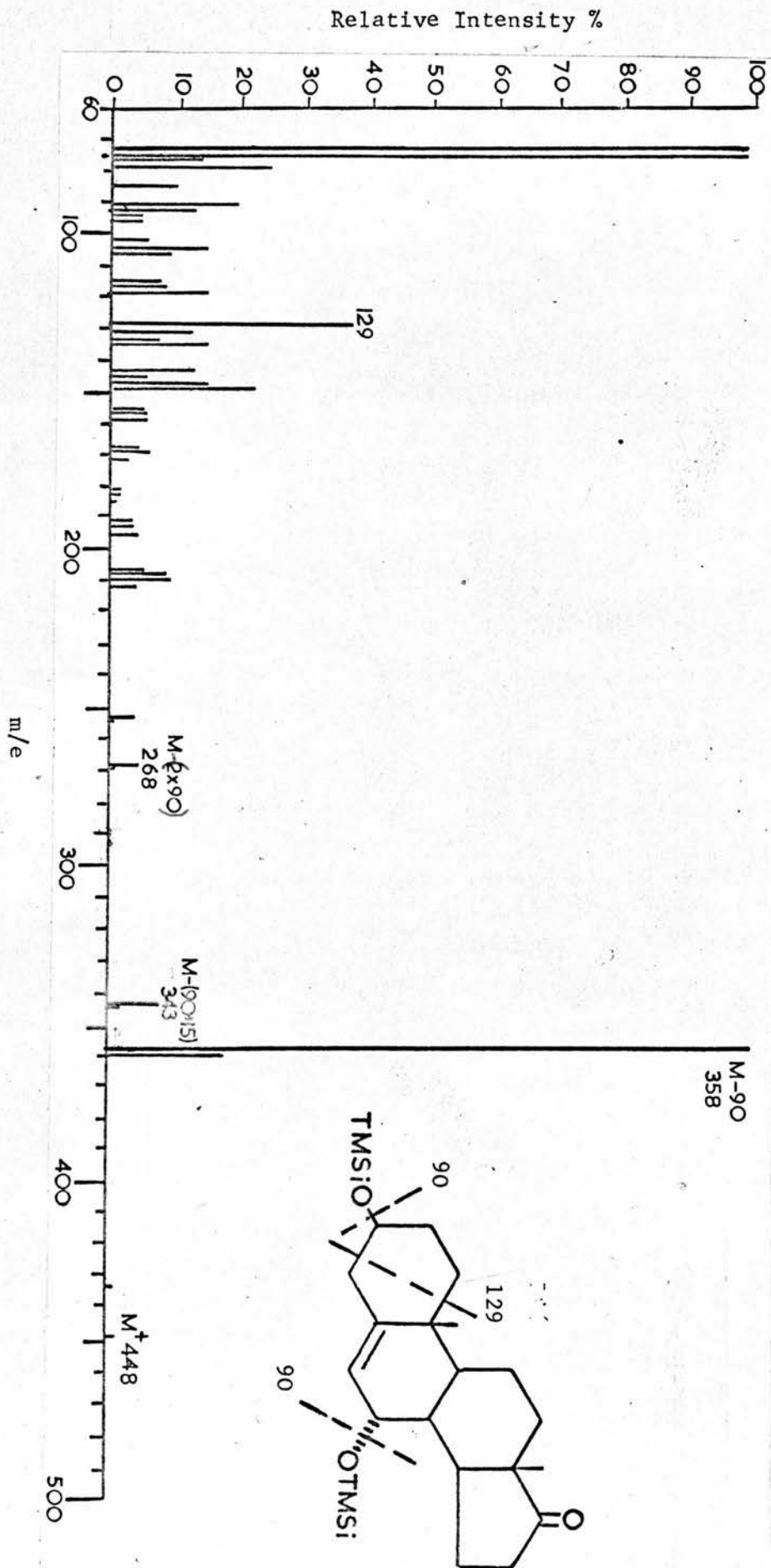


Fig. 60. Mass-Spectrum of 11 β -OH-DHA Trimethylsilyl Ether

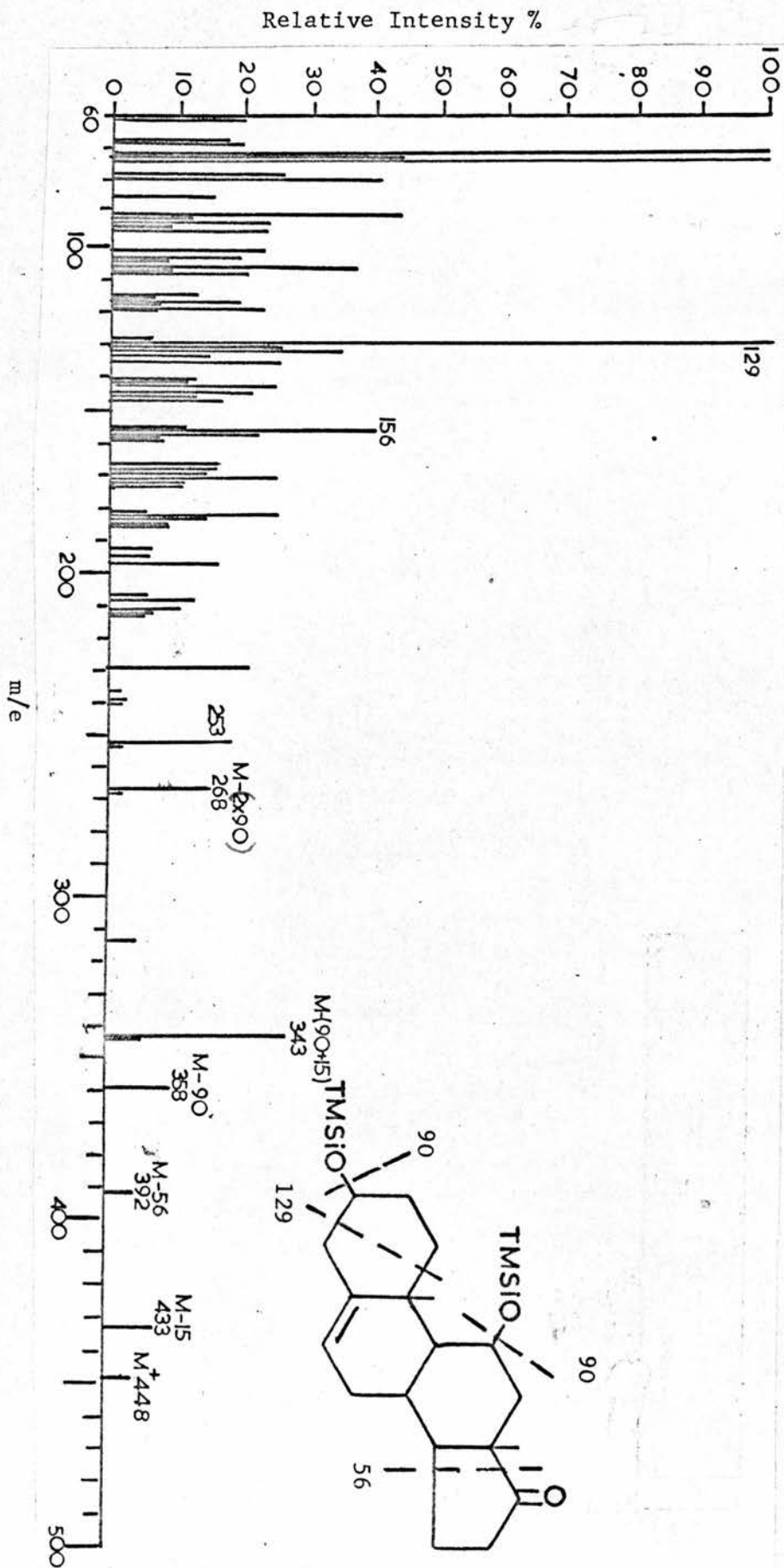


Fig. 61. Mass-Spectrum of 14 α -OH-DHA Trimethylsilyl Ether

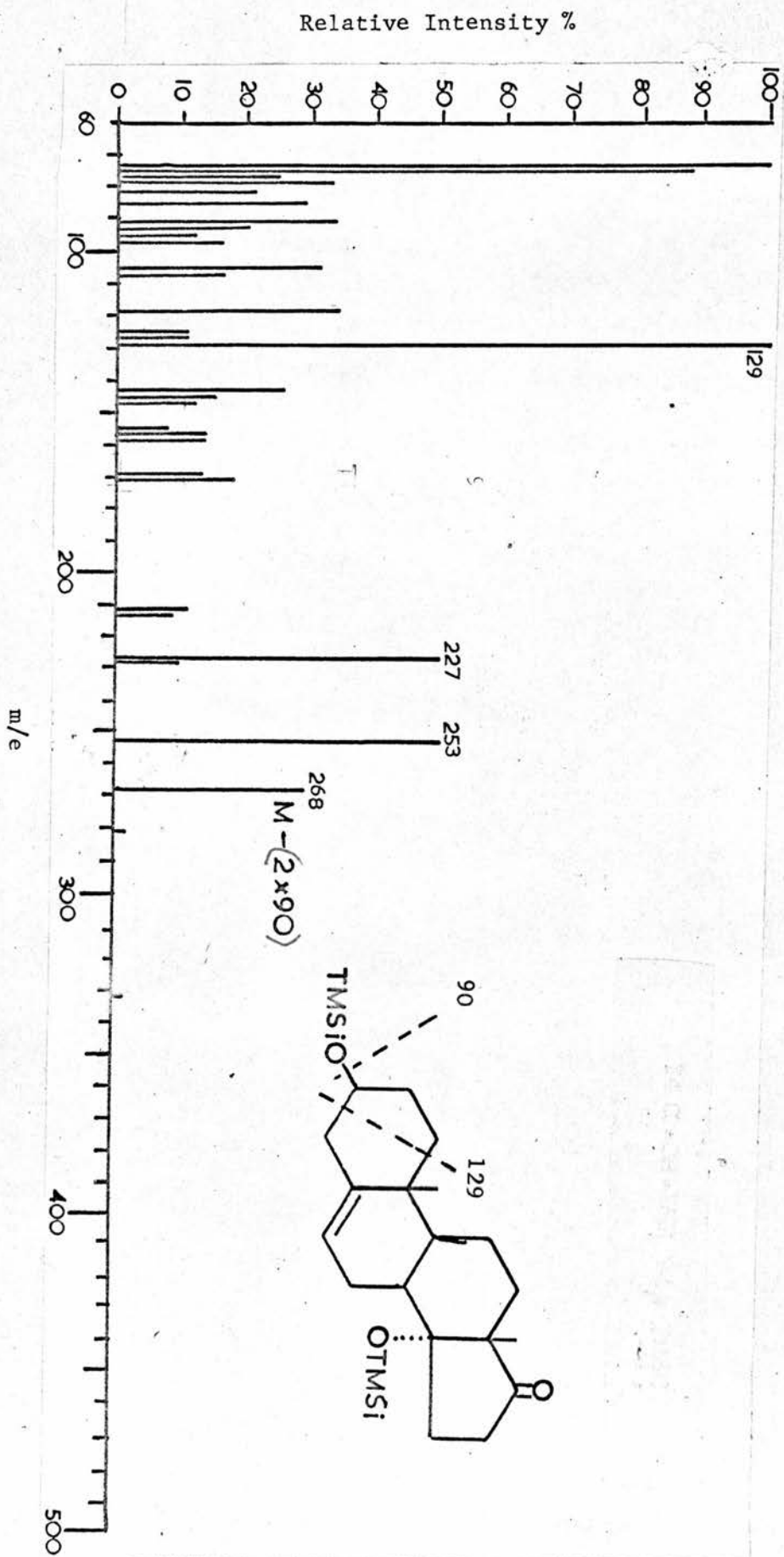


Fig. 62. Mass-Spectrum of 17 α Androstenediol Trimethylsilyl Ether

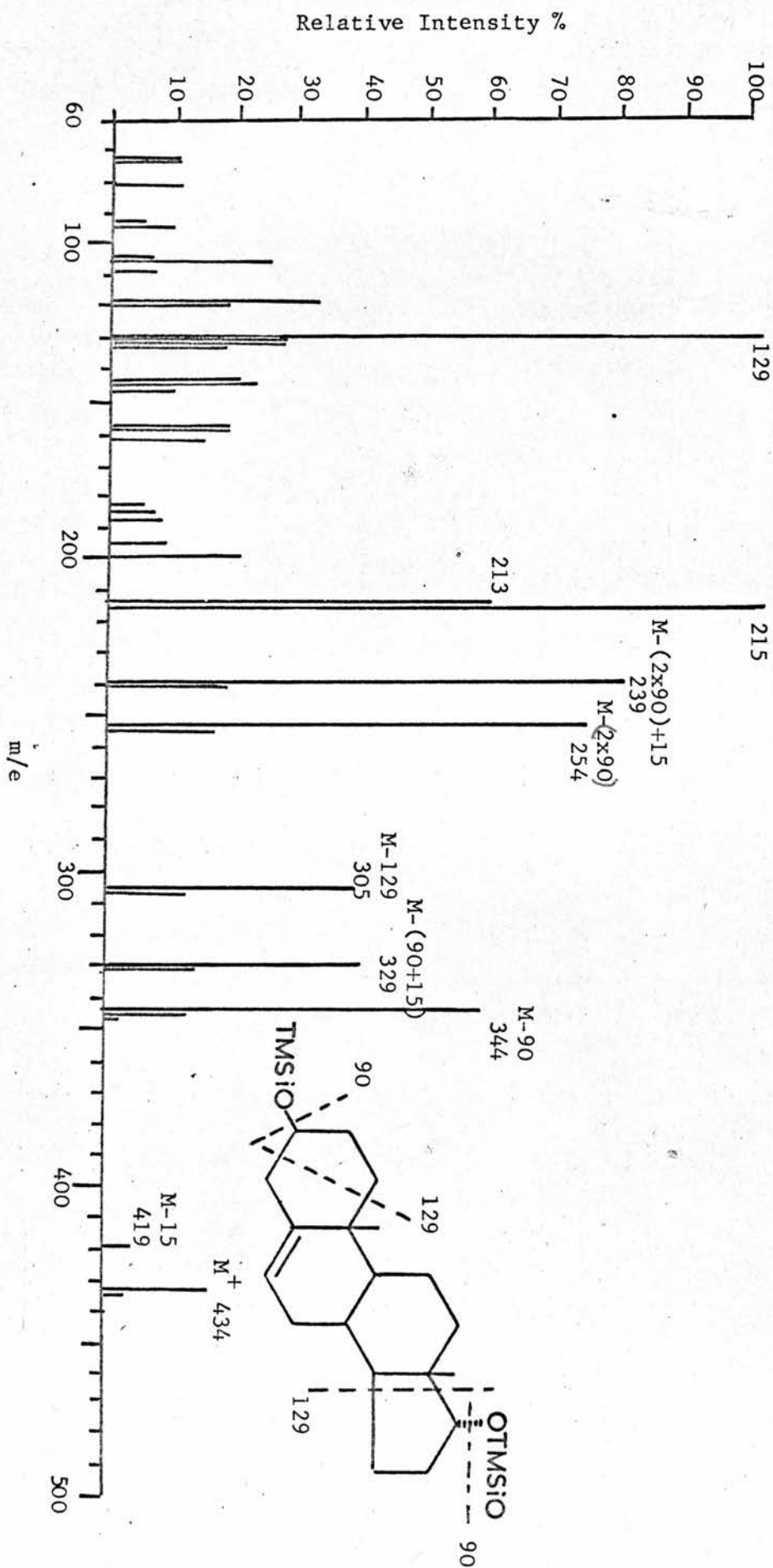


Fig. 63. Mass-Spectrum of 17 β -Androstenediol Trimethylsilyl Ether

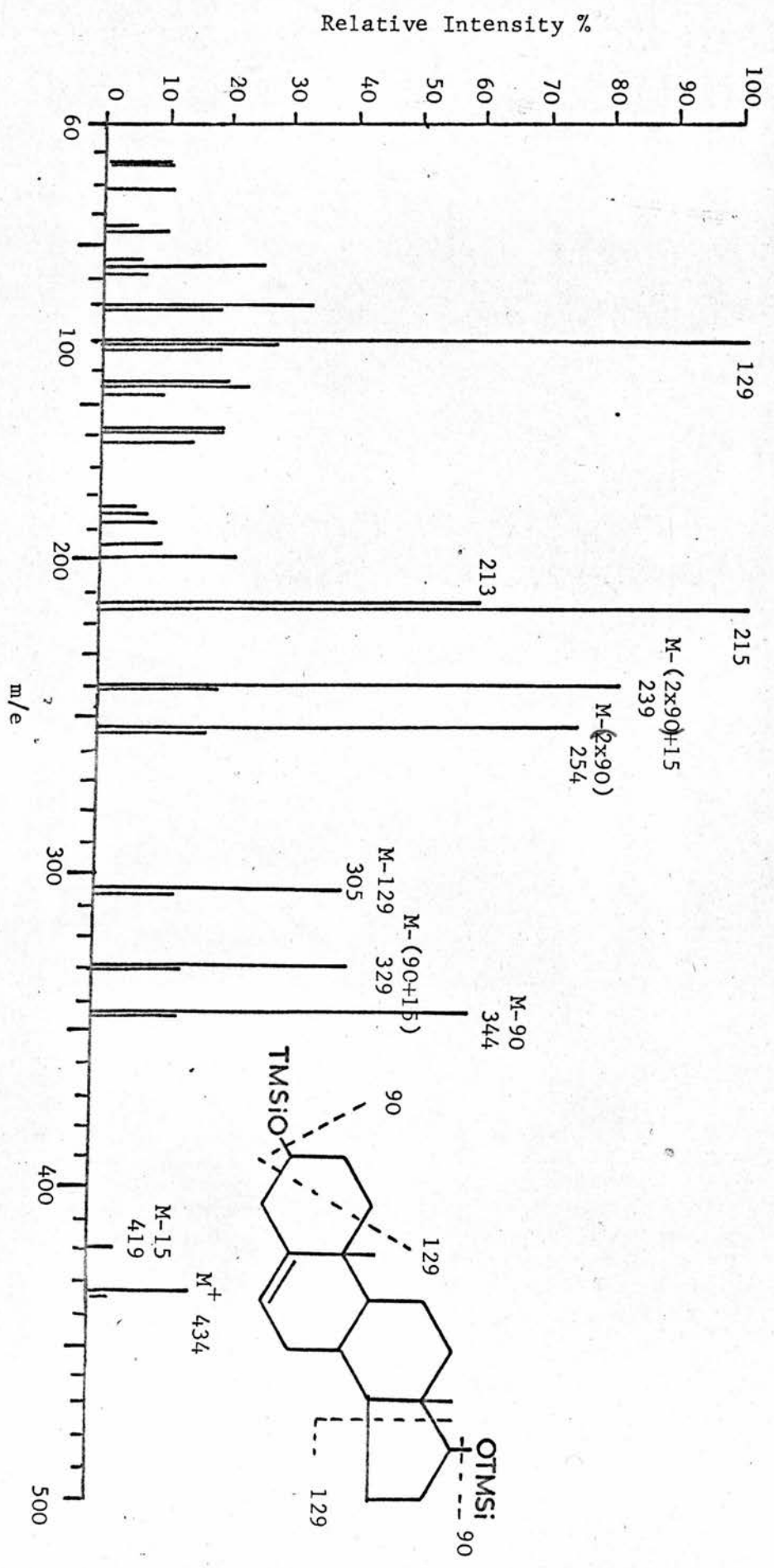


Fig. 64. Mass-Spectrum of 11-oxo-androstenediol Trimethylsilyl Ether

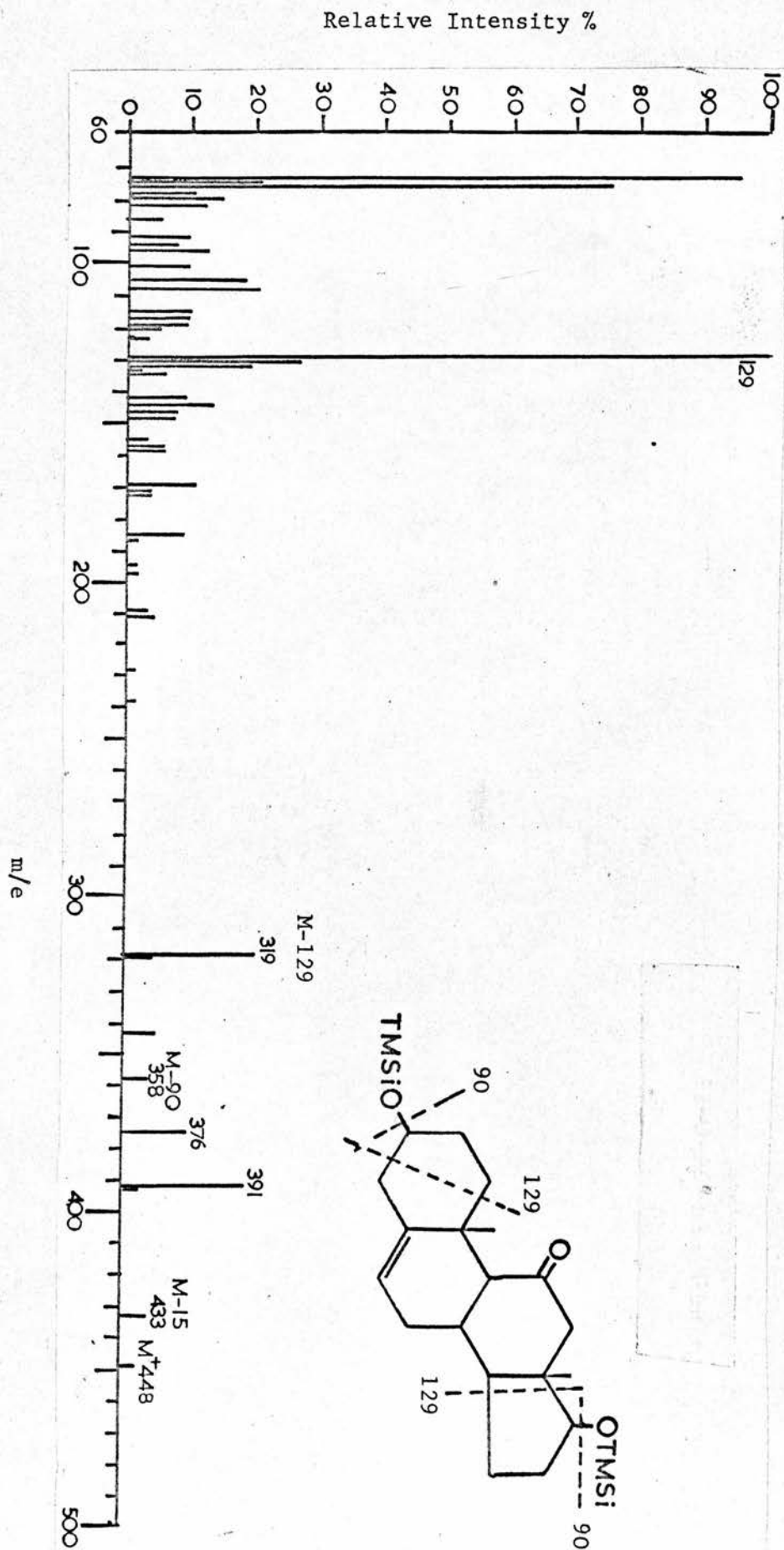


Fig. 65. Mass-Spectrum of Androst-5-ene-3 β ,11 β ,17 β -triol Trimethylsilyl Ether

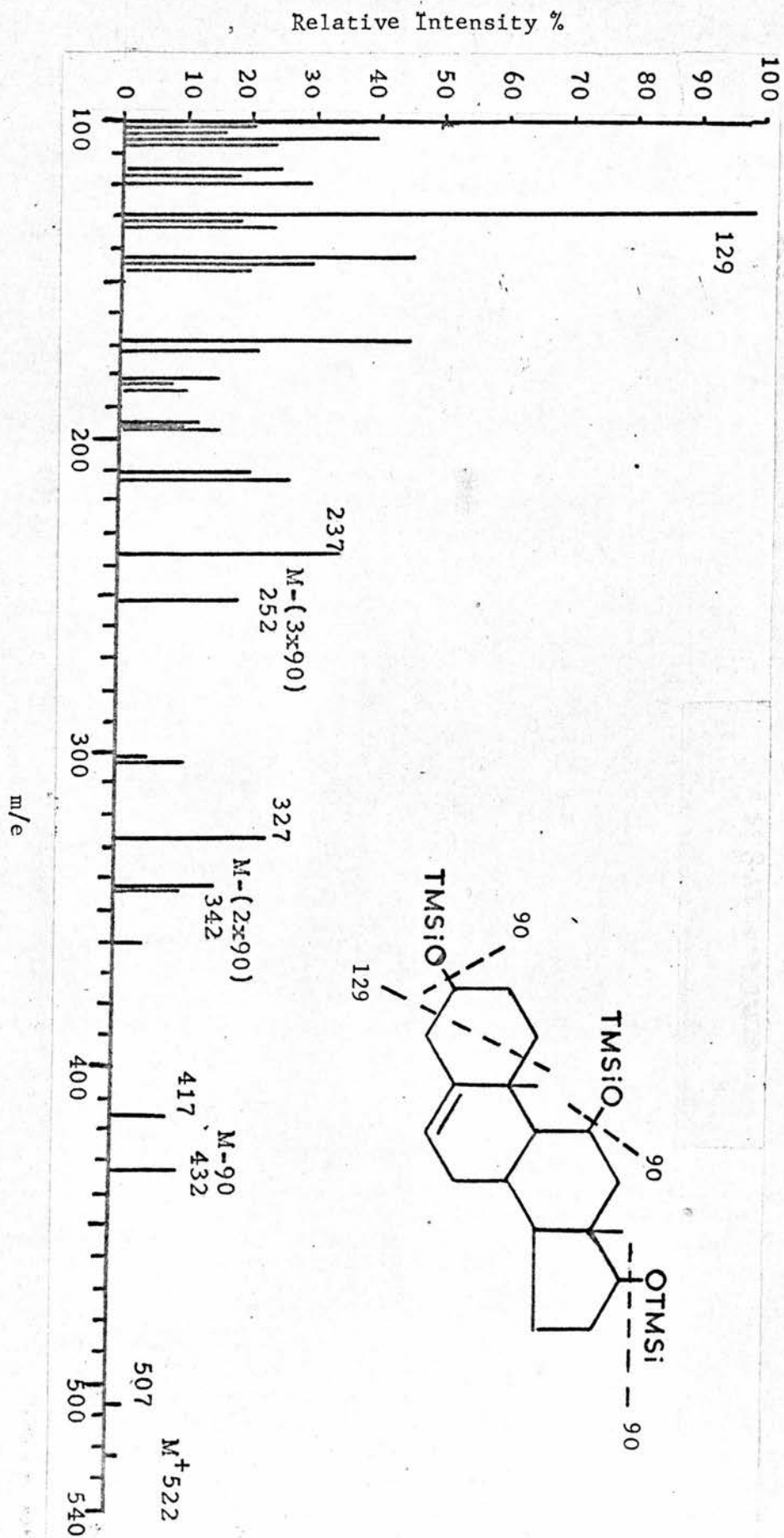


Fig. 66. Mass-Spectrum of Androst-5-ene-3 β ,15 α ,17 β -triol Trimethylsilyl Ether

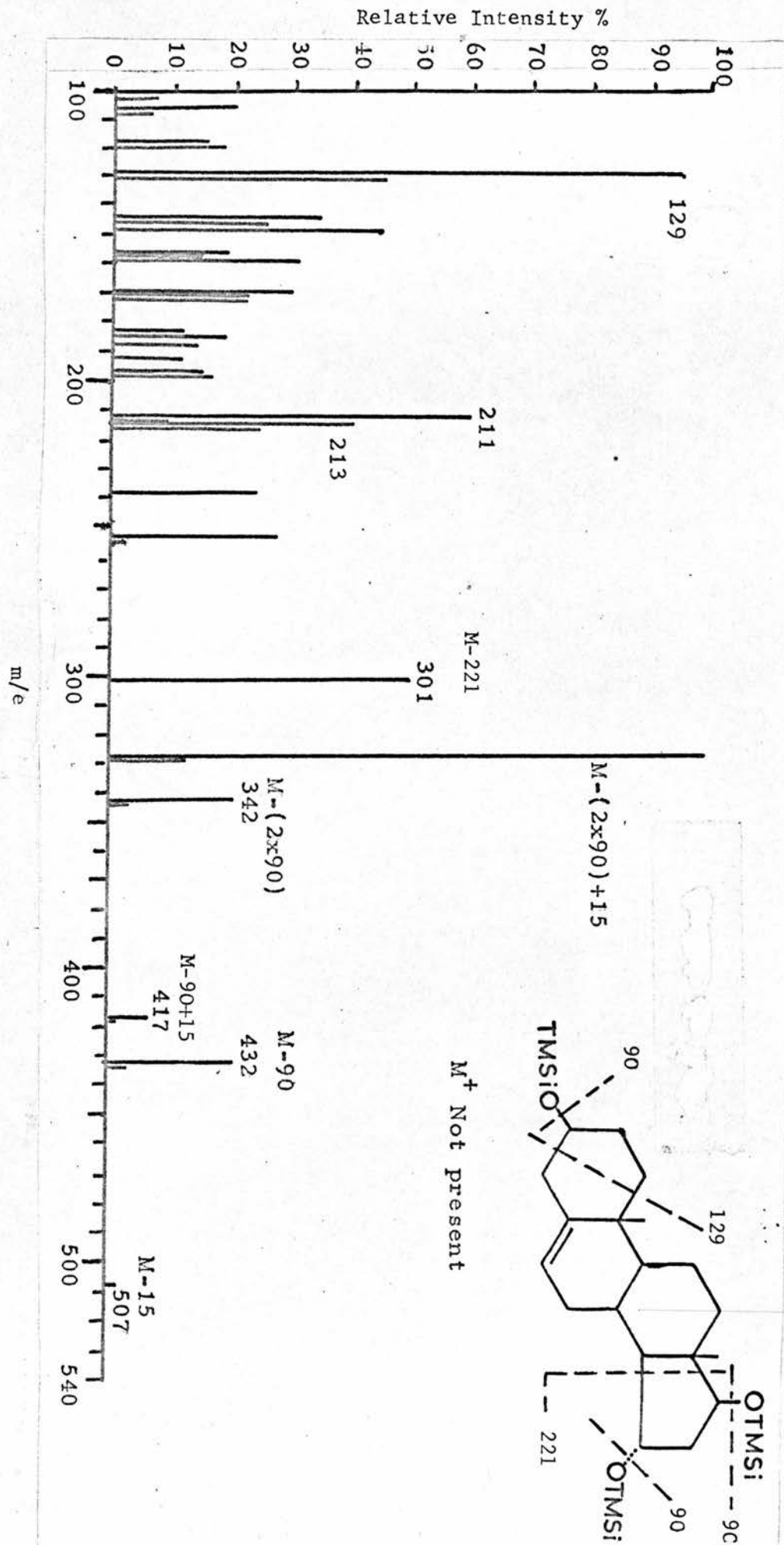


Fig. 67. Mass-Spectrum of Androstenediol Trimethylsilyl Ether

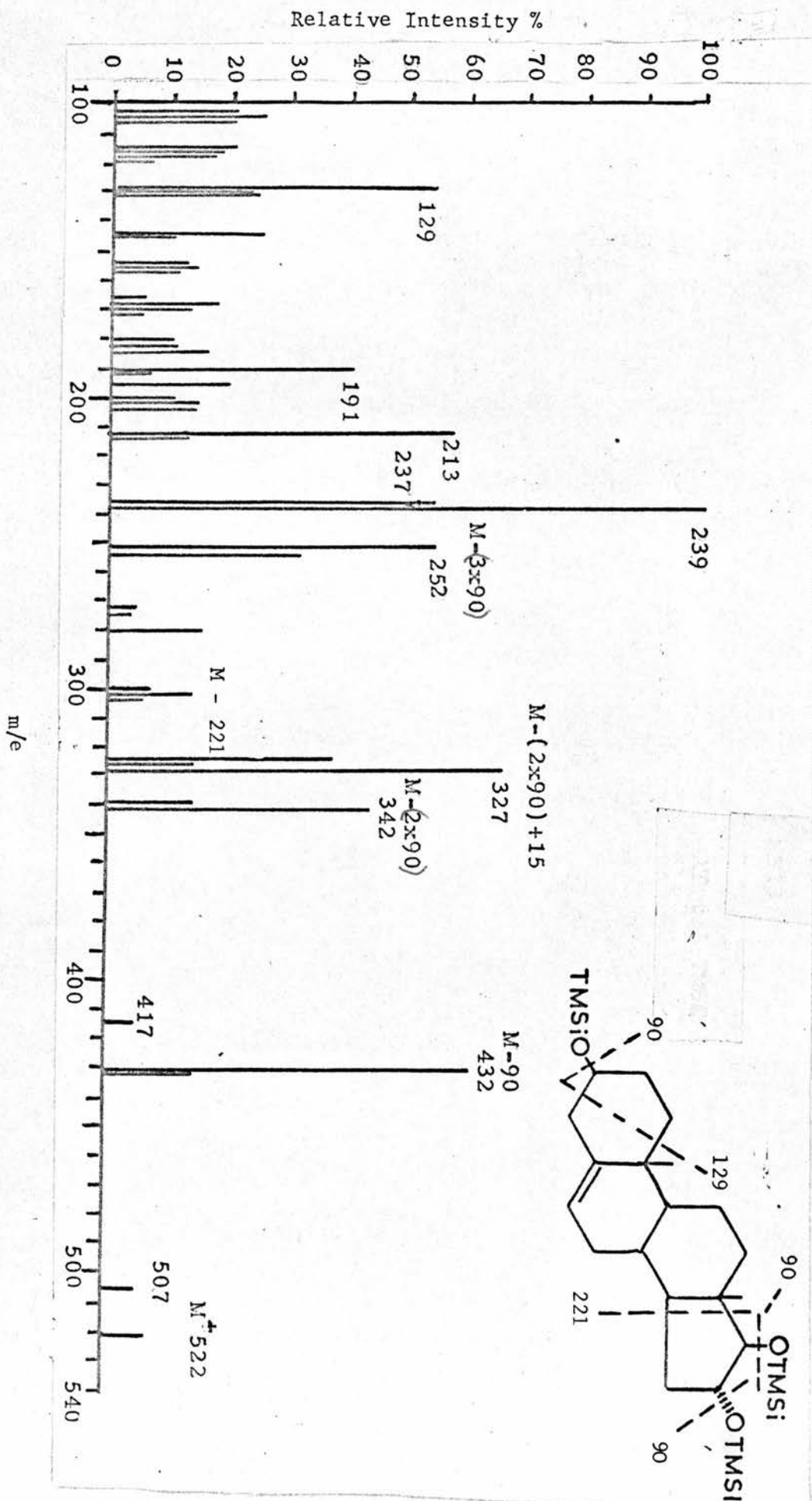
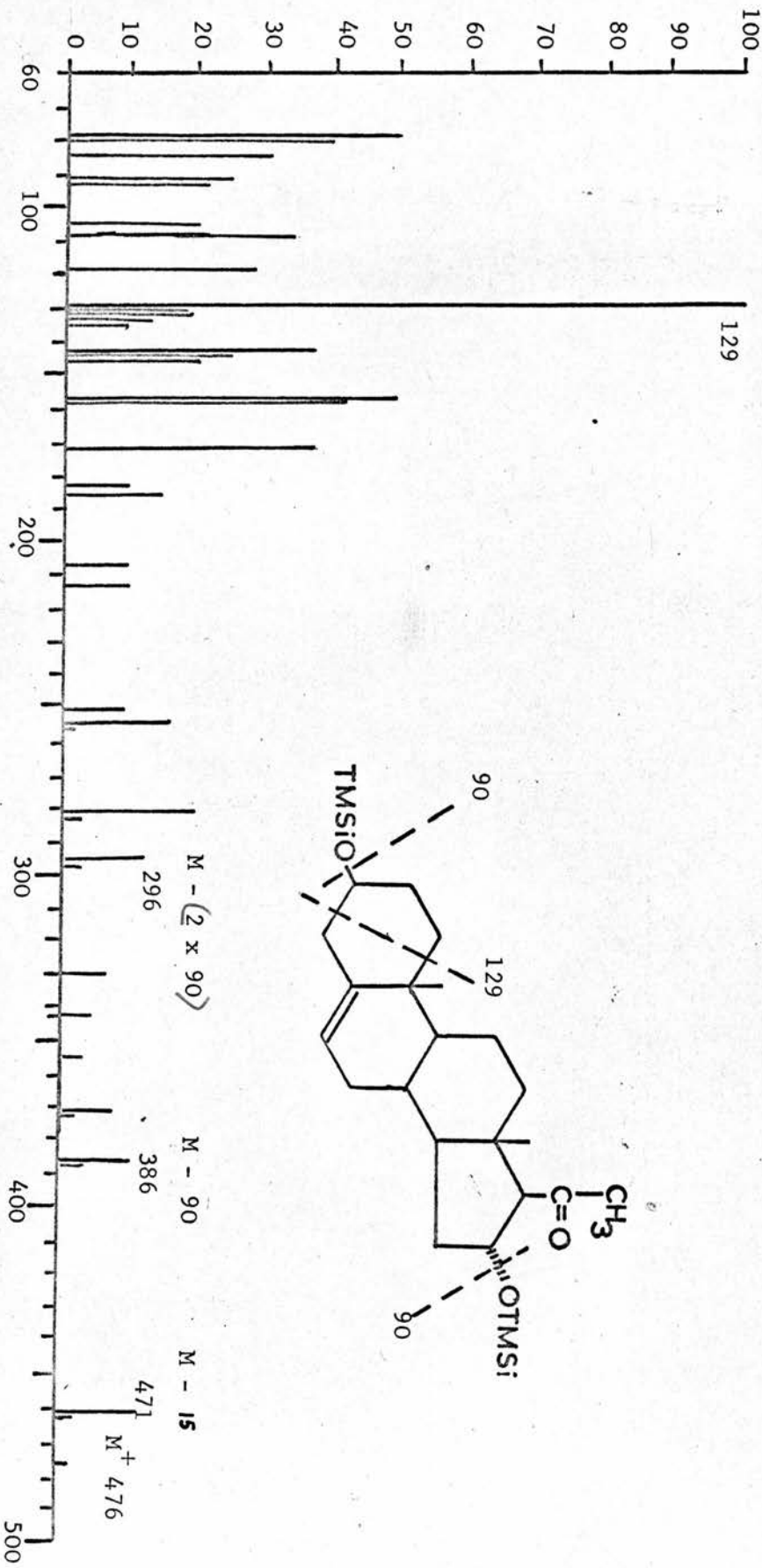


Fig. 68. Mass-Spectrum of 16 α -OH-Pregnenolone Trimethylsilyl Ether



PROCEEDINGS
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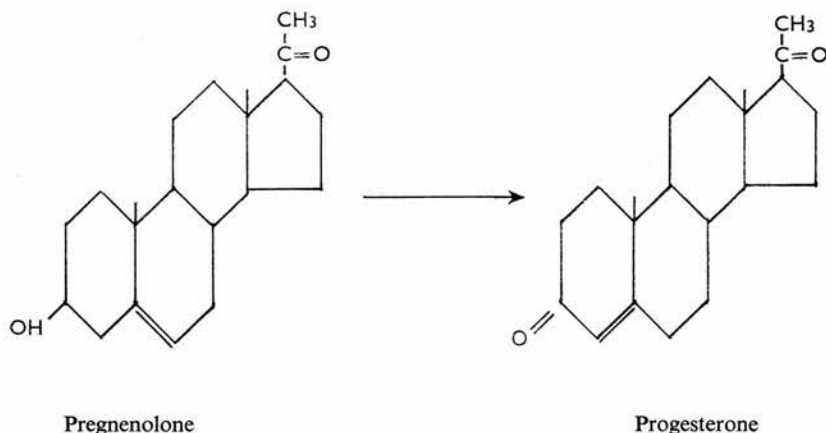
C. H. L. SHACKLETON AND F. L. MITCHELL

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Steroid metabolism *in utero* and in the newborn period has recently been shown to be different in many ways from that found in the adult (Cathro, Birchall, Mitchell and Forsyth, 1963), and up to two years may elapse before the infant's steroid metabolism is completely replaced by the adult mechanism (Barr, Diczfalusy and Tilligen, 1961). One of the main differences appears to be due to a relative deficiency or inactivity in the infant of the steroid enzyme 3 β -hydroxydehydr-

ogenase which catalyses the dehydrogenation and isomerisation of a 3 β -OH- Δ^5 -steroid to a 3-oxo- Δ^4 -steroid: e.g. pregnenolone converted to progesterone and dehydroepiandrosterone (DHA) to androstenedione.

Inability to carry out this conversion completely, results in a build up of pregnenolone which then appears to be acted upon by enzymes which would normally act upon progesterone to



produce corticoids. For instance, instead of forming deoxycorticosterone from progesterone, 21-hydroxylase would produce 21-hydroxy-pregnenolone, a steroid found in significant quantity in urine from infants but not normally detectable in adults (Cathro, Birchall, Mitchell and Forsyth, 1965). A large number of compounds showing many of the chemical properties of Δ^5 -steroids are present in infant urine, cord blood, amniotic fluid, *etc.* and have not yet been identified but could be produced by enzymes such as 11 β or 17 α -hydroxylase, and 17 β (acting on 17 oxosteroids) or 20 α -reductase, acting upon Δ^5 instead of Δ^4 -steroids.

A further major difference in metabolism results from the fetus and infant having a high activity of the enzyme 16 α -hydroxylase, and where one would expect from the above a relatively high excretion of Δ^5 -pregnenolone and DHA, this does not in fact occur, but further metabolism to the 16-hydroxy compounds causes the excretion of a relatively large quantity of 16 OH-pregnenolone and 16 OH-DHA (Reynolds, 1965). These compounds are undetectable in normal adult urine. The reason for this high activity of 16 α -hydroxylase is not known.

Many of the metabolites of pregnenolone and DHA so far remain uncharacterised, but it is thought that they may be hydroxylated in any of the following positions: 11, 16, 17, 20 or 21. Little is known about the importance of these compounds in the overall metabolism of the foeto-placental unit and, to make investigation possible, a method has had to be devised for their separation and quantitation.

After hydrolysis of urine, plasma or amniotic

fluid by β -glucuronidase and steroid sulphatases, the free compounds are extracted with ether: ethyl acetate 2:1 (v/v) and then separated into ketonic and non-ketonic fractions by Girard Reagent T. Thin-layer chromatography is then carried out on each fraction, using the following systems.

System C (Lisboa, 1965)

ethyl acetate: cyclohexane 50:50 (v/v).

System L (Lisboa, 1965)

chloroform: ethanol 95:5 (v/v).

System D (Lisboa, 1965)

chloroform: ethanol 90:10 (v/v).

System C is suitable for relatively non-polar steroids, system L for steroids of all polarities and system D for relatively polar steroids. Heating, after spraying with a saturated solution of antimony-trichloride in chloroform gives a reasonably specific red colour with 3 β -OH Δ^5 -steroids. By running suitable standards on the same plate an approximate quantitative assay may be obtained by the visual comparison of spot intensity. The use of the thin-layer attachment to a Chromoscan (Joyce, Loebel & Co., Princesway, Gateshead-on-Tyne) gives considerable improvement in quantitative accuracy.

It has been possible, by using this technique, to demonstrate in addition to those already specifically mentioned by name, at least 8 Δ^5 -steroid-like compounds which are present in relatively large quantity in the infant but not demonstrable in the normal adult. It will be interesting to discover whether these compounds are produced by processes present in the infant purely for detoxication purposes, or whether their production and subsequent use is an essential part of foetal life and growth. Whatever

the reason, the fact that they form a large proportion of the steroid excretion of the newborn must be borne in mind in the interpretation of any conventional steroid assay which may be carried out on infant urine.

Acknowledgment

We are grateful to the Medical Research Council for financial support.

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THE MEASUREMENT OF 3β -HYDROXY- Δ^5 STEROIDS IN HUMAN FETAL BLOOD, AMNIOTIC FLUID, INFANT URINE AND ADULT URINE

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ABSTRACT

A relatively simple new technique is reported for the separation and measurement of the following Δ^5 steroids in human fetal blood, amniotic fluid and infant and adult urine: 3β -hydroxypregn-5-en-20-one, 3β -hydroxyandrost-5-en-17-one, $3\beta, 21$ -dihydroxypregn-5-en-20-one, $3\beta, 17\alpha$ (and 17β)-dihydroxyandrost-5-ene, $3\beta, 17\beta$ -dihydroxyandrost-5-en-16-one, $3\beta, 16\alpha$ -dihydroxyandrost-5-en-17-one, $3\beta, 16\alpha$ -dihydroxypregn-5-en-20-one, $3\beta, 16\alpha, 17\beta$ -trihydroxyandrost-5-ene and two unidentified compounds. After enzyme hydrolysis and solvolysis the steroids are extracted from the biological material and separated on thin-layer silica-gel chromatograms. Assay is by direct photo-electric scanning of the coloured bands developed on the chromatograms by an antimony trichloride reagent. The identity of the compounds has been checked by gas chromatography-mass spectroscopy and further thin-layer chromatography. The accuracy, specificity and precision of the method have been evaluated. The total urinary excretion of the compounds when expressed in $\mu\text{g}/24 \text{ hr}/\text{m}^2$ body area is approximately 10 times that of the adult. The 2 unknown compounds, 21-hydroxypregnenolone and 16-hydroxypregnenolone are undetectable in adult urine. In cases of the adrenogenital syndrome the excretion of 16-hydroxypregnenolone was markedly increased.

INTRODUCTION

Steroids with the Δ^5 - 3β -hydroxy configuration predominate quantitatively and qualitatively over all other steroids (cholesterol apart) in fetal blood and infant urine. In adults the quantitative importance of such steroids is largely limited to dehydroepiandrosterone (DHA)¹, but in the fetus and in infants a large number of other Δ^5 steroids and Δ^5 steroid-like compounds are present, some in considerable quantity (1 - 3).

Easterling et al. (4) have shown that the concentration of DHA-sulfate and 16 α -hydroxy-DHA-sulfate in fetal blood totals 200 - 300 μ g/100 ml. and Reynolds (5) has recorded a total urinary excretion of 16 α -hydroxypregnenolone (16-OH-Preg.), 16 α -hydroxy-DHA (16-OH-DHA) and androstetriol (AT) of 14 mg./24 hr. in a 3 week old premature infant and of over 8 mg./24 hr. in 6 similar infants aged up to 5 weeks.

It is only comparatively recently that the importance of the Δ^5 steroids in early life has been appreciated and the techniques of assay so far used have been tedious, most of them having been designed more to achieve the isolation of pure compounds rather than for assay purposes (1,3-8). It is apparent that much is to be gained from a quantitative study of the individual Δ^5 steroids circulating in the feto-placental unit and excreted by the infant during the first few months of independent life (9); also there is an immediate clinical application in that an early diagnosis of any dysfunction in adrenocortical steroid synthesis may depend upon an ability to measure

¹ Abbreviations for compounds used throughout the text are shown in Table 1.

accurately the Δ^5 steroids in day-old infant urine (10). In adults it is not usually considered worthwhile to investigate the 3 β -hydroxy- Δ^5 steroids as a group but as they constitute by far the most abundant group in infants, it is of considerable importance that more should be known of their excretion in the normal and abnormal infant.

During the development of a suitable technique, an attempt has been made to balance specificity, sensitivity and reliability against

ease of operation and the possibility of measuring the individual steroids in a urine sample from a day-old infant, and in the small samples of arterial and venous cord blood which can be obtained from single umbilical cords. The compounds chosen for assay are shown in Table 1 and include two chromatographic bands of material staining with antimony trichloride which have not been identified, but they are probably steroidal and if so, are of considerable quantitative significance.

The technique has been used to measure the excretion of urinary Δ^5 steroids during the first few days of infant life, and the rates of excretion are compared with those found in adults. Findings in 2 cases of the adrenogenital syndrome are also reported.

MATERIALS

All the reagents used were of analytical grade unless otherwise specified. Other material used was obtained as follows:-

Succus entericus from the snail *Helix pomatia* - L'Industrie Biologique S.A., Gennevilliers, France.

Silica-gel, grade Hf 254 - Merck A.G., Darmstadt, Germany.

Thin-layer chromatography spreader - Quickfit and Quartz Ltd., Stone, England.

Thin-layer chromatography tanks - Shandon Scientific Co. Ltd., London, England.

Thin-layer chromatogram scanner - Joyce, Loebel and Co. Ltd., Gateshead 11, England.

Gas chromatograph-mass spectrograph - LKB 9000. LKB Produkter AB, Stockholm, Sweden.

The source of steroids used as authentic standards is indicated in Table 1.

Complete 24 hr. specimens of urine were collected from different normal infants on each day for the first 6 days of life, and also from normal adult males. Preservation was by storage at 4° followed by freezing at -24° as soon as possible. Umbilical cord blood was heparinized and the plasma separated as soon as possible. Plasma and amniotic fluid were stored at -24°.

METHOD

Choice of Technique

To ensure the extraction and measurement of as much steroid material as possible a comprehensive hydrolysis procedure was chosen, and for separating the steroids in the extract, thin-layer was decided upon in preference to column, paper or gas-liquid chromatography. The quantities of steroid being dealt with, particularly in blood, were small and the assay of bands of material on paper or thin-layer chromatograms was preferred to the use of column chromatography involving the collection of many eluate fractions. Paper chromatography was found to necessitate a complex preliminary purification stage (6), long development periods were required and the spots or bands of steroid formed tended to be more diffuse than those obtained using thin-layer chromatography. Many of the compounds were found to be unstable when subjected to gas-liquid chromatography.

Elution of the bands of material from the thin-layer plates, with subsequent colorimetric assay did not prove practicable because many were too close together for accurate detection and delineation using a parallel run of standards. Assay was therefore achieved by direct staining and quantitative scanning.

Preliminary studies

Hydrolysis. Leon et al. (11) have shown that the crop fluid of the snail *Helix pomatia* contains both β -glucuronidase and sulfatases. Eberlein (1) has demonstrated that the Δ^5 - 3β -OH steroids are present in cord blood mainly in sulfated form, and it was found that the use of the *Helix pomatia* enzyme alone did not achieve complete hydrolysis of the Δ^5 steroids present in the biological fluids studied. A second stage involving a solvolytic procedure (12) was essential.

To test the completeness of hydrolysis by the two-stage process, the residual hydrolysate after extraction with ether:ethyl-acetate was further extracted with butanol to remove any material still conjugated. The butanol extract was evaporated to dryness, taken up in water and the two stage hydrolysis repeated. This procedure was carried out 6 times on infant urine and no detectable spots were produced after spraying the chromatograms finally produced from the extracts.

TABLE 1

Compounds assayed, together with their abbreviations, trivial names and the source of origin of the standards used. (Given in order of increasing polarity).

Pregnenolone (Preg)	3 β -hydroxypregn-5-en-20-one. ^(a)
Dehydroepiandrosterone (DHA)	3 β -hydroxyandrost-5-en-17-one. ^(a)
U ₁	Unknown SbCl ₃ staining compound.
21-Hydroxypregnenolone (21-OH-Preg)	3 β ,21-dihydroxypregn-5-en-20-one ^(b)
Androstenediol (AD)	3 β ,17 α (and 17 β)-dihydroxyandrost-5-en ^(b)
16-oxoandrostenediol (16-O-AD)	3 β ,17 β -dihydroxyandrost-5-16-one ^(c)
16 α -Hydroxydehydroepiandrosterone (16-OH-DHA)	3 β ,16 α -dihydroxyandrost-5-en-17-one ^(d)
U ₂	Unknown SbCl ₃ and blue tetrazolium staining compound
16 α -Hydroxypregnenolone (16-OH-Preg)	3 β ,16 α -dihydroxypregn-5-en-20-one ^(b)
Pregnenetriol	3 β ,17 α ,20 α -trihydroxypregn-5-en ^(b)
Pregnanetriol	3 α ,17 α ,20 α -trihydroxy-5 β -pregnan ^(b)
Androstenetriol (AT)	3 β ,16 α ,17 β -trihydroxyandrost-5-en ^(c)

(a) Koch-Light Laboratories Ltd., Colnbrook, England.

(b) Medical Research Council Reference Collection.

(c) Synthesized by P.J. Sykes and R.W. Kelly.

(d) Drs. D. Fukushima, P.J. Sykes and R.W. Kelly.

Preliminary purification. With the thin-layer chromatography systems used, it was found that the ether:ethyl acetate extract of urine and the ethanol:ether (see later) extracts of amniotic fluid and blood, needed only alkali, mild acid and water washes.

Thin-layer Chromatography. In order to achieve adequate separation of the antimony trichloride (see later) staining compounds which appear to be important in the biological materials studied, a

procedure of chromatogram development using multiple solvent runs was designed using the same or different solvent systems. Multiple running with systems giving low Rf values was found to give better separations than the more normally used single stage development procedure. In all cases plates were prepared using silica-gel and the following solvent systems were selected for use:-

- System A - Chloroform
- System B - Chloroform : absolute ethanol (95:5 v/v)
- System C - Chloroform : absolute ethanol (90:10 v/v)
- System D - Benzene : absolute ethanol (96:4 v/v)
- System E - Cyclohexane : ethyl acetate (50:50 v/v)

Compounds which proved difficult to separate either from each other or from interfering chromatogenic material were:-

1. DHA from Preg
2. U₁ from 21-OH-Preg
3. 16-O-AD from 16-OH-DHA
4. AT

The use of System D will satisfactorily separate the substances listed under 1 and 3 but not those under 2, separation of these may however be achieved by using System A. The desired properties of the two systems may be combined by running the plates three times in System A then three times in System D; the resulting separation then produced for infant urine is shown in Fig. 1.

Since AT is considerable more polar than the other compounds to be assayed, when adequate distribution of the main group is obtained AT moves only a short distance from the origin and is poorly separated from neighbouring substances. A second chromatogram must therefore be prepared and run in the more polar solvent system C.

Fig. 2 shows the separation achieved for infant urine after two runs in this system. It will be seen that pregnanetriol is also separated, staining as a yellow band. Quantitative information on the content of this steroid in infant urine can be of considerable diagnostic importance in addition to the measurement of the Δ^5 steroids, in cases of the adrenogenital syndrome.

In adult urine there is qualitatively and quantitatively less Δ^5 steroid present, and unlike infant urine, pregnenetriol assumes quantitative importance, but in the systems designed for infants an unidentified band staining with antimony trichloride is not separated from pregnenetriol. For adults therefore, the problems of separation are different but less complex and a procedure utilizing a single chromatogram has been chosen.

The plates are run once in System E, then twice in System B and it will be seen in Fig. 3 that AT and pregnanetriol move sufficiently far from the origin to be separated and measured, but 16-OH-DHA and 16-O-AD are not separated. This disadvantage may easily be overcome if necessary, by carrying out additional runs in the systems designed for infants.

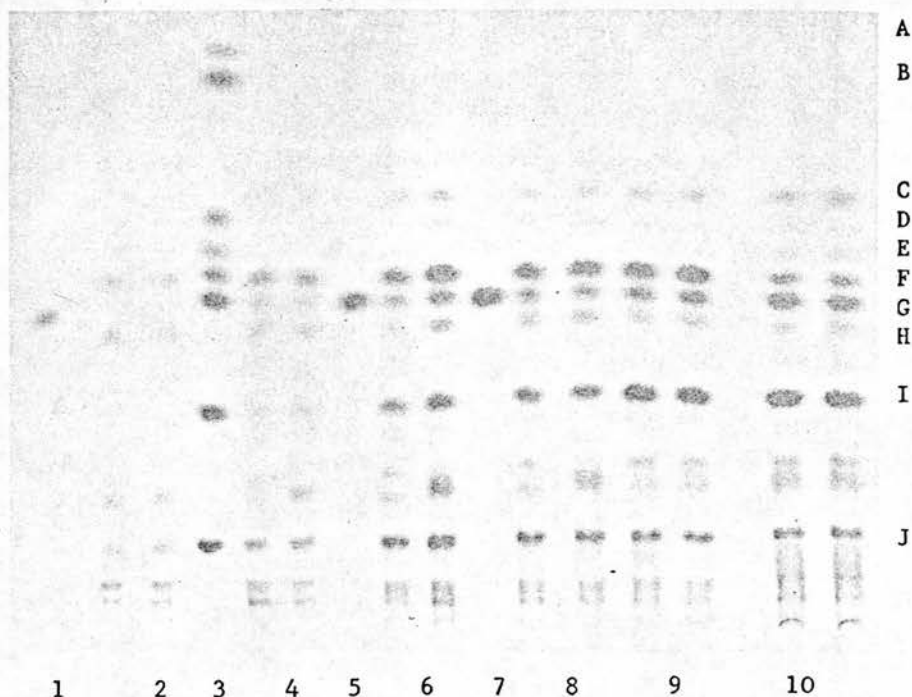


Figure 1. A typical 20 x 20 cm. thin-layer chromatography plate for the assay of the following compounds in infant urine: A, pregnenolone; B, DHA; C, unknown 1; D, 21-OH-pregnenolone; E, androstenediol; F, 16-oxo-androstenediol; G, 16-OH-DHA; H, unknown 2; I, 16-OH-pregnenolone. The position of androstenetriol (not assayed) is shown at J. Samples of 16-OH-DHA were run in positions 1 (1.25 μg), 5 (3.75 μg) and 7 (5.0 μg). Samples (2.5 μg) of all the steroids measured (unknown compounds apart) were run in position 3. Duplicate extracts of urines collected from the same baby on days 1, 2, 3, 4, 5 and 6 of life were run in positions 2, 4, 6, 8, 9 and 10. The plate was sprayed with antimony trichloride followed by heating.

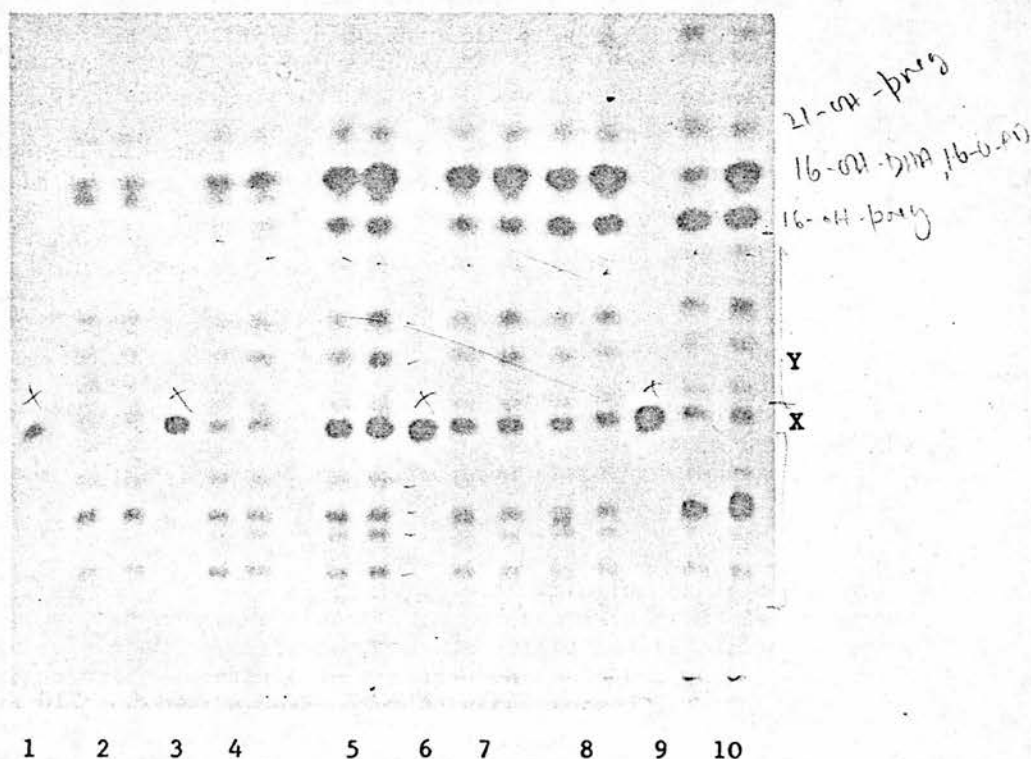


Figure 2. A typical 20 x 20 cm. thin-layer chromatography plate for the assay of androstenediol (X) in infant urine. The position of pregnanetriol, if present, is shown at Y. Standard samples of androstenediol were run in positions 1 (1.25 μg), 3 (2.50 μg), 6 (3.75 μg) and 9 (5.0 μg). Urine extracts run in duplicate in positions 2, 4, 5, 7, 8 and 10 were the same as shown in Fig. 1. The plate was sprayed with antimony trichloride followed by heating.

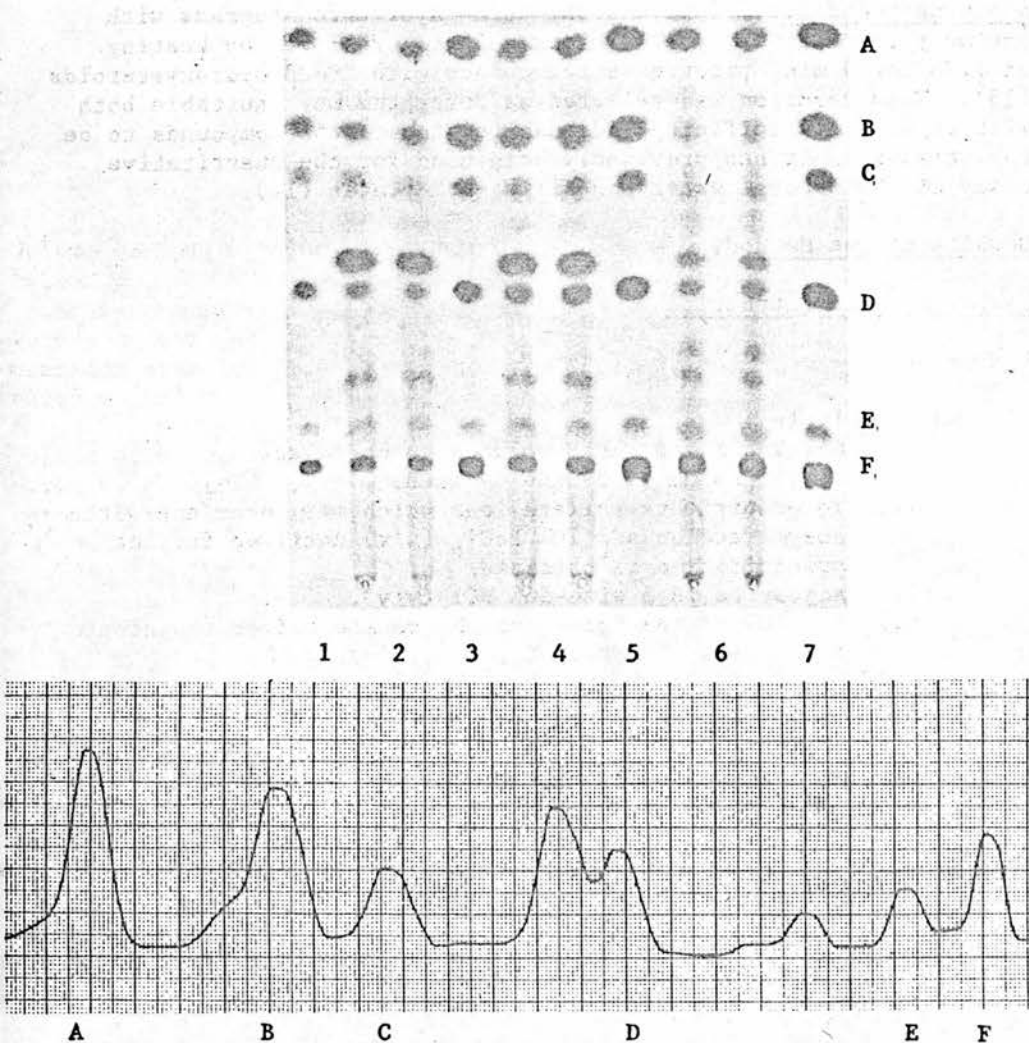


Figure 3. (Above). Part of a typical thin-layer chromatography plate for the assay of the following compounds in adult urine: A, DHA; B, androstenediol; C, 16-OH-DHA and 16-oxo-androstenediol; D, pregnenetriol; E, pregnanetriol; F, androstenetriol. Standard steroids were run in positions 1 (1.25 μg), 3 (2.50 μg), 5 (3.75 μg) and 7 (5.0 μg). Duplicate extracts of urines from 3 different adults were run in positions 2, 4 and 6. The plate was sprayed with antimony trichloride followed by heating.

(Below). The recording produced by run 4 when scanned in a photoelectric reflectance densitometer.

Staining technique. Spraying the thin-layer chromatograms with antimony trichloride in chloroform solution followed by heating at 110° for 5 min. produces a red colour with Δ^5 - 3β -hydroxysteroids (13). This reaction was selected as being the most suitable both with regard to specificity and quantitation of the compounds to be investigated. It has previously been used for the quantitative assay of cholesteryl esters on silica-gel plates (14).

Details of the Method.

Hydrolysis and Extraction

1. Urine

(a) Hydrolysis

- (i) See Table 2 for the volumes to be taken.
- (ii) Adjust to pH 11.5 with 4N NaOH.
- (iii) To precipitate sulfate ions which may interfere with enzyme action add 10% BaCl_2 (w/v) until no further precipitation is obtained.
- (iv) Adjust to pH 5 with 50% HCl (v/v).
- (v) Add 10% of the volume of 5N acetate buffer (5N acetic acid, 5N sodium acetate, 2:3, v/v).
- (vi) Add 0.1 ml. of the succus entericus of *Helix pomatia* per 20 ml. urine. Incubate at 37° for 24 hr.
- (vii) Repeat (vi).

(b) Extraction

- (i) Extract twice with 2 volumes of ether:ethyl acetate (2:1, v/v).
- (ii) Wash the extract repeatedly with 10% of the volume of N NaOH until the washings are clear.
- (iii) Wash once with 10% of the volume of water with 2-3 drops of acetic acid added.
- (iv) Wash once with 10% of the volume of water.
- (v) Evaporate to dryness on a rotary evaporator at 45° .
- (vi) Transfer the extract with chloroform into a small tube, dry down under nitrogen.

(c) Solvolysis

- (i) Bring the aqueous phase from b (i) to pH 1 with 50% H_2SO_4 (v/v).
- (ii) Saturate with ammonium sulfate.
- (iii) Extract twice with 1 volume of ethyl acetate, incubate the extract at 38° for 24 hr.
- (iv) Repeat b (ii to vi). The solvolysis extract is combined with the enzyme freed extract in the same tube.

TABLE 2

Volumes of urine, extract etc. required
at various stages in the technique.

	Infant urine	Adult urine	Amniotic fluid	Umbilical cord plasma
Volume normally taken	1/16 24 hr. spec.	1/60 24 hr. spec.	100 ml	10 ml
Volume of ethanol used for dissolving extract (μ l)	500	160	140	100
Volume of ethanol sol. normally required per chromatogram spot (μ l)				
Preg	100 - 200	-	50	50
DHA	100 - 200	20	50	50
U ₁	10 - 40	-	20	50
21-OH-Preg	10 - 40	-	20	50
AD	10 - 40	20	20	50
16-O-AD	10 - 20	-	20	50
16-OH-DHA	10 - 20	20	20	50
U ₂	10 - 20	-	20	50
16-OH-Preg	10 - 20	-	20	50
Pregnenetriol	-	20	-	-
Pregnanetriol	-	20	-	-
AT	10 - 20	20	40	50

1. Amniotic Fluid and Umbilical Cord Plasma

- (i) See Table 2 for the volumes to be taken
- (ii) Precipitate proteins with 4 volumes of ethanol, centrifuge.
- (iii) Extract the precipitate with 100 ml. of 80% ethanol (v/v), centrifuge.
- (iv) Combine the ethanolic solutions and evaporate to dryness on a rotary evaporator at 45°.
- (v) Add 30 ml. of distilled water and extract twice with 30 ml. ether.

- (vi) The extract contains the free steroids and because of fatty impurities it is not suitable for thin-layer chromatography and is normally discarded. If required for other purposes it may be further treated as under lb (ii-vi).
- (vii) Hydrolyse and extract the aqueous fraction from (v) as for urine. The final extract will contain the conjugated steroids.

Thin-layer Chromatography. Silica-gel is spread onto 20 x 20 cm plates to a thickness of 0.25 mm. No activation is required but the plates are allowed to stand for at least 24 hr. at room temperature before use. Extracts are "spotted" onto two prepared plates in the amounts shown in Table 2. Duplicate extracts of up to 6 urines may be run on each plate (see Figs. 1 - 3). Care must be taken to ensure that all the spots are as nearly as possible 0.5 cm diameter. The spot pairs are interspersed with spots containing 1.25, 2.50, 3.75 and 5.0 μg of 16-OH-DHA (for infant medium polarity compounds), AT (for infant high polarity compounds) and DHA and 16-OH-DHA (for adult Δ^5 compounds), to be used for quantitative calibration. One spot containing 2.5 μg of each steroid to be assayed is also prepared to check the quantitation of each steroid.

The plates thus prepared are placed momentarily in a highly polar development system (ethyl acetate:methanol, 3:1 v/v) to "run up" the spots approximately 0.5 cm, so that bands are produced instead of circles. This procedure is repeated a second time, allowing the plate to dry in between. Very narrow bands of material are thus produced, making it possible to achieve maximal chromatographic separation.

The solvent systems used for development have been described in a previous section. Multiple runs are carried out by drying the plates in air after development in one system, then immediately placing them for a further run either in the same or in another system. Details of development are as follows:

infant urine)	for medium polarity compounds	(3 x System A
cord plasma)		(3 x System D
amniotic fluid)	for AT	2 x System C
adult urine			(1 x System E
			(2 x System B

Colour Development. The fully developed plates are sprayed evenly with a saturated solution of antimony trichloride in chloroform and heated for 5 min. in an oven with an even temperature of 110°. The colours formed will fade after several hours in the open air but if the chromatograms are covered with a glass plate they may be kept successfully in the refrigerator for several days. It is however recommended that they be scanned as soon as possible.

Quantitation. Each developed chromatogram spot is scanned in succession in the thin-layer scanner using reflectance, a slit 1 x 7 mm and the no. 490 filter (max. transmission 490 m μ). The base-line is drawn in

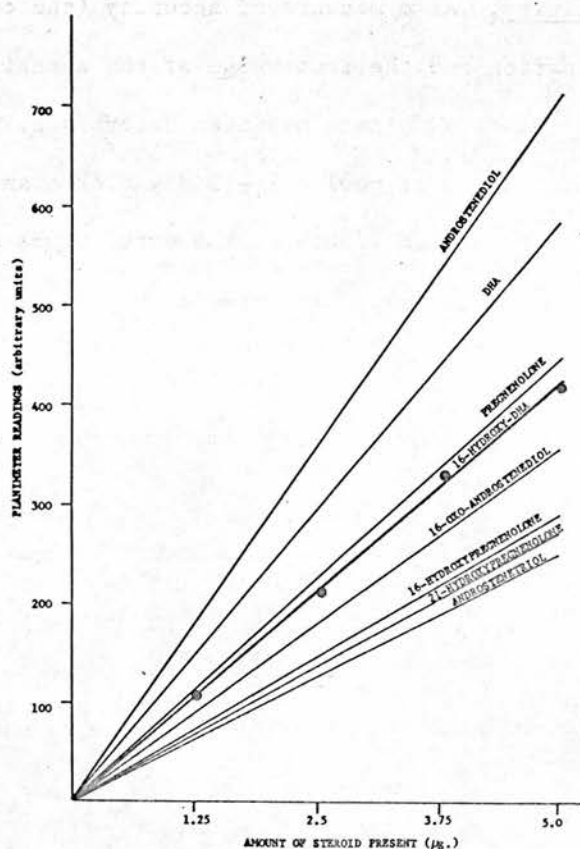


Figure 4. Calibration graphs for standard steroids run on thin-layer chromatograms, stained with antimony trichloride, scanned by a reflecting densitometer and the areas under the peaks measured by planimetry.

on the resulting recorder trace (Fig. 3) and perpendicular lines are inserted to divide each peak, areas are measured by planimetry. The integrator on the scanner is not used because no correction can then be made for a base-line which is often slightly sloping. A standard curve is constructed from the areas under the standard 16-OH-DHA peaks and similar graphs based on the single multistandard run are drawn for the other compounds assayed (Fig. 4). The graphs are then used for quantitation of the compounds in the extracts. (Greater precision may be obtained by using 4 multistandard runs but the above scheme is suggested as it is usually necessary to conserve valuable steroid standards).

RESULTS

Method Evaluation

Accuracy and Specificity. As a measure of accuracy (the concordance between the determination and the true value of the quantity measured), the recovery of DHA from DHA sulfate has been determined. DHA sulfate (250 μg) was added to 10 ml. of pooled 1 - 3 day old infant urine and after hydrolysis and extraction 1/50th of the extract was chromatographed and assayed. The average recovery for 6 experiments was $80\% \pm 6.2$ (S.D.).

The antimony trichloride reaction used for colour development was thoroughly investigated to determine its specificity (the ability of a technique to determine solely the compound it purports to measure). The preliminary purification and separation stages of the method were chosen purposely for their simplicity and ease of operation, and consequently the substances to be assayed are not obtained on the chromatograms in pure form; the specificity of the colour reaction is therefore important.

The fluorescence producing reactions of antimony trichloride are very unspecific (15) but the reaction considered here produces in normal light, specific colours for certain groups of steroids when the amount of each steroid on the chromatogram is less than 10 μg .

Table 3 indicates the various colours which were found to be produced; some others have been reported previously (13, 16, 17). With the exception of the estrogens (removed in the preliminary alkali wash) most of the major biologically common groups of steroids are represented and it will be seen that all the $3\beta\text{-OH-}\Delta^5$ steroids

TABLE 3 Colours produced by the antimony trichloride reaction with various steroids on silica-gel thin-layer chromatograms

3 β -Hydroxy- Δ^5 steroids

Androstanes

3 β -Hydroxyandrost-5:16-dien red
 3 β -Hydroxyandrost-5-en-17-one red
 3 β -Hydroxyandrost-5,15-dien-17-one red
 3 β -Hydroxyandrost-5-en-11:17-dione red
 3 β ,16 α -Dihydroxyandrost-5-en-17-one red
 3 β ,16 α -Dihydroxyandrost-5-en-17-one red
 3 β ,17 α -Dihydroxyandrost-5-en-17-one red
 3 β ,17 α ,20 α -Trihydroxyandrost-5-en-16-one red
 3 β ,15 α ,17 α -Trihydroxyandrost-5-en red
 3 β ,16 α ,17 α -Trihydroxyandrost-5-en red
 3 β ,16 α ,17 β -Trihydroxyandrost-5-en red
 3 β ,17 α -Dihydroxyandrost-5-en pale blue-grey
 3 β ,17 α -Dihydroxyandrost-5-en pale blue-grey
 3 β ,17 α -Dihydroxyandrost-5-en-17-one dark blue-grey
 3 β ,16 α -Dihydroxyandrost-5-en-17-one dark blue-grey
 3 β ,11 β ,17 α -Trihydroxyandrost-5-en dark blue-grey
 3 β ,15 β ,17 β -Trihydroxyandrost-5-en dark blue-grey
 3 β ,7 β -Dihydroxyandrost-5-en-17-one turquoise*
 3 β ,7 β -Dihydroxyandrost-5-en-17-one turquoise*
 3 β -Hydroxyandrost-5-en-7,17-dione no colour

Other steroids

2-Oxo- Δ^4 steroids

Androst-4-en-3,17-dione no colour
 Androst-4-en-3,11,17-trione no colour
 17 β -Hydroxyandrost-4-en-3-one no colour
 17 α -Hydroxyandrost-4-en-3-one blue
 Pregn-4-en-3,20-dione no colour
 16 α -Hydroxypregn-4-en-3,20-dione no colour
 17 α -Hydroxypregn-4-en-3,20-dione no colour
 21-Hydroxypregn-4-en-3,20-dione no colour
 16 α ,21-Dihydroxypregn-4-en-3,20-dione no colour
 21-Hydroxypregn-4-en-3,11,20-trione no colour
 11 β ,21-Dihydroxypregn-4-en-3,20-dione no colour
 17 α ,21-Dihydroxypregn-4-en-3,20-dione no colour
 17 α ,21-Dihydroxypregn-4-en-3,11,20-trione no colour
 11 β ,17 α ,21-Trihydroxypregn-4-en-3,20-dione no colour
 6 β ,11 β ,17 α ,21-Tetrahydroxypregn-4-en-3,20-dione no colour
 Androst-1,4,dien-3,17-dione no colour
 17 β -Hydroxy-1,4,dien-3-one yellow

Miscellaneous

Androst-5-en-3,11,17-trione no colour
 3 β -Hydroxycholest-4-en red*
 3 β -Hydroxycholest-1-en red*
 Cholest-3-en-3-one no colour

Pregnenes

3 β -Hydroxypregn-5-en-20-one red
 3 β ,16 α -Dihydroxypregn-5-en red
 3 β ,17 α -Dihydroxypregn-5-en red
 3 β ,20 α -Dihydroxypregn-5-en red
 3 β ,21-Dihydroxypregn-5-en red
 3 β ,17 α -Dihydroxypregn-5-en-11,20-dione red
 3 β ,17 α ,21-Trihydroxypregn-5-en-20-one red
 3 β ,20 α ,21-Trihydroxypregn-5-en blue-grey
 3 β ,20 α -Dihydroxypregn-5-en blue-grey
 3 β ,17 α ,20 α -Trihydroxypregn-5-en blue-grey
 3 β ,17 α ,20 α ,21-Tetrahydroxypregn-5-en blue-grey

Cholestenes

Cholesterol violet
 7 α -Hydroxy cholesterol turquoise*
 7 β -Hydroxy cholesterol turquoise*

Saturated steroids

3 α ,17 α ,21-Trihydroxy-5 β -pregnan-20-one no colour
 3 α ,17 α ,21-Trihydroxy-5 β -pregnan-11-20-dione no colour
 3 α ,17 α ,21-Trihydroxy-5 α -pregnan-11-20-dione no colour
 3 α ,11 β ,17 α ,21-Tetrahydroxy-5 β -pregnan-20-one, no colour
 3 α ,11 β ,17 α ,21-Tetrahydroxy-5 α -pregnan-20-one, no colour
 3 α -Hydroxy-5 β -androst-17-one no colour
 3 α -Hydroxy-5 α -androst-11,17-dione no colour
 3 α ,11 β -Dihydroxy-5 β -androst-17-one no colour
 3 α -Hydroxy-5 α -androst-11,17-dione no colour
 3 α ,11 β -Dihydroxy-5 α -androst-17-one no colour
 3 α ,11 β -Dihydroxy-5 α -androst-11,17-dione no colour
 3 α ,11 β -Dihydroxy-5 α -androst-11,17-dione no colour
 3 α ,11 β -Dihydroxy-5 α -androst-17-one no colour
 3 α ,11 β -Dihydroxy-5 α -androst-11,17-dione no colour
 3 α ,11 β -Dihydroxy-5 α -androst-17-one no colour
 3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-11-one yellow
 3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-11-one yellow
 3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-11-one yellow
 3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-11-one yellow
 3 α ,17 α ,20 α -Trihydroxy-5 β -pregnan-11-one yellow
 3 α ,6 α -Dihydroxy-5 β -pregnan-20-one orange

Chromatograms were developed with 10 μ g. of steroid in each case.

* Immediate colour produced without heating.

give a strong colour except when a 7-oxo group is present. Compounds with a 7-OH group are readily distinguished by their producing an immediate turquoise colour without heating. None of the 3-oxo- Δ^4 steroids listed gives a colour except epitestosterone (17 α -hydroxyandrost-4-en-3-one) which produces a blue colour, and a yellow colour is produced by a $\Delta^{1,4}$ steroid. Amongst the saturated steroids, only a 6 α -OH steroid gave an orange colour, all others tested produce either a weak yellow colour or no colour at all. Pregnanetriol gives an intense yellow colour by which it may be assayed. If careful consideration is given to the data in Table 3 and chromatogram development systems are carefully chosen, the antimony trichloride reaction on thin-layer chromatograms can in practice be very specific.

Extracts from the biological materials under investigation were run firstly in one direction using the systems normally used, and then a second development was carried out at 90° to the first using the following thin-layer chromatography solvent systems:

chloroform: absolute ethanol (95:5, v/v)

ethyl acetate: water saturated with n-hexane:

glacial acetic acid: absolute ethanol (72:13.5:10:4.5, by vol.)

cyclohexane: ethyl acetate (50:50, v/v)

cyclohexane: ethyl acetate: absolute ethanol (45:45:10, by vol.)

In no case could an interfering chromogen be separated from a spot normally assayed. In addition, after completing "standard" runs of extracts, the separated spots were acetylated by spraying the plates with acetic anhydride: pyridine (50:50, v/v); after standing for 2 hr. they were then "run up" at 90° to the line of the first development,

thus forming new origins for a second development carried out using ethanol: benzene (2:98, v/v). For the compounds normally assayed no additional antimony trichloride staining spots could be separated.

Identification of Compounds. From extracts of infant urine, 50 to 100 $\mu\text{g.}$ of AD, 16-O-AD, 16-OH-DHA, 16-OH-Preg and AT were prepared as follows: the extract was streaked along the origin of thin-layer chromatography plates which were then developed in the normal systems; the antimony trichloride reaction was carried out only on the two outer edges of the plates and the bands of material in the centre, thus delineated, were eluted from the silica-gel with ether, the silica-gel having first been deactivated with a drop of water. The compounds were converted to their trimethylsilyl ethers and subjected to gas-liquid chromatography using an E30L column. In each case they had the same retention times as the corresponding authentic standards treated in the same way.

The trimethylsilyl ether of each eluate was then subjected to gas chromatography-mass spectroscopy, and mass spectra were produced on the beginning, centre and end of each gas chromatography peak. In each case all three spectra were identical with each other and with similar spectra produced by the authentic standard steroids (the identification of Δ^5 steroids using gas chromatography-mass spectroscopy will be the subject to a later publication with Dr. C. J. W. Brooks).

Precision. The precision of the method (the concordance of measurements of the same quantity) has been determined by calculating the standard deviation from the differences between the chromatogram peak areas of duplicate extracts (duplicated through the whole procedure) of the

urinary assays reported in this paper. Table 4 shows the results obtained.

TABLE 4

Precision, as shown by the concordance of areas under the peaks of chromatogram scans.

Compound assayed	No. of duplicate assays	Average area (planimeter readings)	Standard deviation of the area (%)*
U ₁	22	81	7.3
21-OH-Preg	22	43	8.9
AD	11	95	8.5
16-O-AD	22	252	4.5
16-OH-DHA	22	211	7.6
U ₂	17	85	7.0
16-OH-Preg	22	245	4.4
AT	22	162	5.7

* The standard deviation has been calculated from the formula $\sqrt{\frac{\sum d^2}{2N}}$ where d = % difference between duplicate areas and N = no. of duplicate assays.

Labour involved. Working a normal 8 hr. day, it is possible for one reasonably skilled technician to assay 6 specimens each week.

Findings in normal individuals

Table 5 compares the excretion (in $\mu\text{g}/24 \text{ hr}/\text{m}^2$) of the major identified Δ^5 steroids and two unknown Δ^5 steroid-like compounds (measured in terms of the chromogenicity of the Δ^5 steroid nearest in polarity, 21-OH-Preg and 16-O-AD) in the day-old infant with that found in normal adult males. AD, 16-O-AD, 16-OH-DHA, 16-OH-Preg and AT have

TABLE 5
URINARY EXCRETION OF Δ^5 STEROIDS
 $\mu\text{g}/24 \text{ hr}/\text{m}^2$

STEROID	DAY OLD INFANT		ADULT MALE	
	Average	Range	Average	Range
Dehydroepiandrosterone (DHA)	-	-	449	120 - 890 (10)
UNKNOWN 1	2,415	180 - 11,785 (11)	-	-
21-Hydroxypregnenolone (21-OH-Preg)	1,050	<100 - 2,355 (11)	-	-
Androstenediol (17 α + 17 β) (AD)	860	<100 - 4,200 (11)	370	73 - 417 (4)
16-oxoandrostenediol (16-O-AD)	4,515	435 - 12,100 (11)	99	<20 - 164 (4)
16-Hydroxydehydroepiandrosterone (16-OH-DHA)	4,810	300 - 11,200 (11)	745	201 - 871 (4)
UNKNOWN 2	3,330	470 - 7,600 (11)	-	-
16-Hydroxypregnenolone (16-OH-Preg)	4,135	260 - 7,600 (11)	-	-
Androstenetriol (AT)	3,200	420 - 8,100 (11)	341	200 - 555 (10)
Pregnenetriol	-	-	298	120 - 485 (10)
Total of averages	24,315		2,302	

- Denotes presence only in barely detectable or undetectable quantity.

The figures in parentheses give the number of subjects studied.

TABLE 6. URINARY EXCRETION OF Δ^5 STEROIDS IN EARLY INFANCY ($\mu\text{g}/24$ hr.)

STEROID	AGE IN DAYS () = no. of infants.					
	1 (11)	2 (11)	3 (11)	4 (5)	5 (5)	6 (5)
UNKNOWN 1	483	555	616	683	429	450
	36 - 2,357	285 - 1,608	374 - 1,221	320 - 1,690	212 - 895	150 - 675
21-Hydroxypregnenolone (21-OH-Preg)	210	236	279	311	193	165
	15 - 471	40 - 492	128 - 590	104 - 690	42 - 435	28 - 320
Androstenediol (17 α + 17 β) (AD)	172	191	208	144	47	37
	<20 - 840	<20 - 800	<20 - 880	<20 - 220	<20 - 120	<20 - 140
16-Oxoandrostenediol (16-O-AD)	903	1,060	1,502	1,518	1,031	899
	87 - 2,420	80 - 3,000	285 - 3,260	660 - 2,080	680 - 1,680	340 - 1,480
16-Hydroxydehydroepiandrosterone (16-OH-DHA)	962	1,026	1,304	1,057	770	855
	72 - 2,240	170 - 2,560	410 - 3,180	539 - 1,565	900 - 1,140	240 - 1,800
UNKNOWN 2	666	808	780	719	460	406
	94 - 1,520	200 - 1,444	200 - 1,740	350 - 1,393	176 - 878	100 - 800
16-Hydroxypregnenolone (16-OH-Preg)	827	900	1,167	1,298	1,037	1,114
	52 - 1,520	80 - 2,260	200 - 3,020	910 - 2,424	264 - 2,080	280 - 2,680
Androstetriol (AT)	640	626	968	996	759	621
	84 - 1,620	112 - 1,220	250 - 1,600	820 - 1,300	383 - 1,100	404 - 820
Total of averages	4,863	5,402	6,824	6,726	4,726	4,547

been identified by comparison with authentic standards during thin-layer chromatography in several systems and also by gas-liquid chromatography of their trimethylsilyl ethers followed by mass spectroscopy. DHA, 21-OH-Preg and pregnenetriol have only been partially identified from their Rf values in several thin-layer chromatography systems and the specificity of the antimony trichloride staining bands.

Table 6 shows the excretion during the first few days of life of the 8 compounds shown in Table 5 to be prominent in infant urine. There was a considerable variation in the excretion of each compound by separate individuals.

The excretion in 2 cases of the adrenogenital syndrome is shown in Table 7.

TABLE 7
URINARY EXCRETION OF Δ^5 STEROIDS IN TWO CASES OF
THE ADRENOGENITAL SYNDROME
($\mu\text{g}/24 \text{ hr}$)

STEROID	CASE 1 aged 2 days	CASE 2 aged 7 days
UNKNOWN 1	150	1,350
21-Hydroxypregnenolone (21-OH-Preg)	not assayed	1,350
Androstenediol (17α and 17β) (AD)	150	638
16-oxoandrostenediol (16-O-AD)	100	6,750
16-Hydroxydehydroepiandrosterone (16-OH-DHA)	100	7,425
UNKNOWN 2	<20	995
16-Hydroxypregnenolone (16-OH-Preg)	1,550	14,925
Androstenetriol (AT)	200	1,460
Total	2,250	34,893

DISCUSSION

The relatively large number of Δ^5 steroids present in the biological material being assayed and the small quantity of original

material available, led to the choice of a single technique which would maintain a reasonable degree of reproducibility while measuring all the compounds required, with a fairly high degree of specificity. The chromatograms produced also give a comprehensive indication of the amounts of other Δ^5 steroid-like compounds present, and this feature is important in view of the large number of these compounds and the present lack of knowledge as to their identity and importance.

All the steroids on the chromatograms which have been referred to by name have been identified before either in infant urine or fetal blood or tissue:

Preg (1,18), DHA (3,7), 21-OH-Preg (1,19), AD (1,2,9,20).

16-O-AD (9,21,22), 16-OH-DHA (2,5,7,8,9,23,24,25),

16-OH-Preg (1,2,5,8,9,24), AT (2,5,9,20,25,26). The bands labelled U_1 and U_2 together with the other unlabelled bands shown in Figs. 1 and 2 remain to be identified. In the present investigation only the last five compounds mentioned by name above (the major identified Δ^5 steroids in infant urine) have been fully characterized. AD includes both 17α and 17β androstenediol, separation of the epimers only being possible after further chromatography.

The precision obtainable is adequate for the purposes for which the technique is designed. The standard deviations shown in Table 4 are true for single assays, when in practice the average of duplicate measurements is used the precision is considerably increased.

The excretion of such a large quantity and variety of Δ^5 steroids in early life is undoubtedly related to the low activity of 3β -hydroxy steroid dehydrogenase which is known to obtain in the fetus and which must persist during early childhood. Similarly the high rate of

excretion of 16-OH-DHA in the absence of barely detectable amounts of DHA must be related to the known high activity of 16 α -hydroxylase in the fetus and infant and not in the adult.

The results shown in Table 6 indicate that on the whole the compounds measured are being synthesized by the infant in fairly constant amount during the first 6 days of life. The normal newborn infant may be considered, because of its deficiency in 3 β -hydroxy dehydrogenase, to possess a mild form of the congenital adrenal hyperplasia due to severe congenital deficiency of 3 β -hydroxy dehydrogenase first described by Bongiovanni (27). In babies with this disorder, large quantities of Δ^5 steroids are produced (10, 27), presumably by overactivity of an adrenal under the stimulus of excessive corticotrophin from a pituitary endeavouring to maintain an adequate level of blood cortisol.

It is probable that the normal fetus and infant synthesize cortisol and corticosterone differently from the major route used by adults, the limited activity of 3 β -hydroxy dehydrogenase acting subsequent to 17 α , 21- and 11 β -hydroxylase (9,10). This would account for the presence of 3 β ,21-dihydroxypregn-5-en-20-one in infant urine (19) and cord blood (1) and 3 β ,17 α ,21-trihydroxypregn-5-en-20-one and 3 β ,11 β ,17 α ,21-tetrahydroxypregn-5-en-20-one in cord blood (1). If, in the normal infant, excess corticotrophin is being produced to stimulate normal cortisol production in the face of a lack of 3 β -hydroxy dehydrogenase, the C-19 Δ^5 steroids (DHA, 16-OH-DHA etc) would be incidentally increased in the same way that 17 oxosteroids are over-produced in the...

more common forms of congenital adrenal hyperplasia due to deficiency of 11 β - and 21-hydroxylase. As with increasing age the deficiency of 17 β -hydroxy dehydrogenase is rectified, cortisol production becomes more efficient and the "by-products" are no longer formed in quantity.

Of the compounds measured, AD, 16-O-AD, 16-OH-DHA and AT may be considered to be estrogen precursors (or their metabolites) produced by the fetus for aromatization in the placenta. 16-OH-Preg however is not an immediate precursor of estrogen but is possibly derived, as is 16-OH-DHA, by 16 hydroxylation in the liver of the parent compound produced by the adrenal (20). The reported levels of pregnenolone in fetal blood vary considerably (23 μ g/100 ml., (1); 45 μ g/100 ml., (18); 100 μ g/100 ml., (28)). It can be utilized by the placenta for progesterone production (29) and by the fetal adrenal for DHA production (30). Further work is required to elucidate the reason for its considerable quantitative significance as indicated by the large amount of 16-OH-Preg excreted in infant urine.

In 2 cases of the adrenogenital syndrome whose results are shown in Table 7, there is a selective increase in the excretion of 16-OH-Preg. Case 1 shows a reduction in the excretion of the other compounds measured, but Case 2 shows a considerable increase in all compounds. Reynolds (31) has measured 16-OH-Preg and 16-OH-DHA excretion in cases with 21-hydroxylase deficiency and has shown that during the first few months of life the ratio of 16-OH-Preg/16-OH-DHA is increased from a normal average of 3:2 to 10:1 in the adrenogenital group. As for normal infants he found that both steroids were almost undetectable in the urine of patients aged over 5 months. The marked increase in 16-OH-Preg excretion in the cases reported here, together with the findings of Reynolds would seem to indicate that in the patients studied there was for some reason a relative inactivity of 17-20 desmolase. It is

difficult to suggest a reason for this and further study is required. The considerable difference from normal in the excretion of Δ^5 steroids by cases of the adrenogenital syndrome indicates the importance of their assay in the investigation of such disorders.

Work carried out in recent years has uncovered the existence of complex interrelationships in the steroid metabolism of the mother, the fetus and the placenta (9), but much of this work has so far only been qualitative. The Δ^5 steroids certainly play a major role and the use of the technique described, should expedite work on the quantitative relationships of these steroids in the feto-placental unit and in the newborn, and facilitate the isolation and identification of more compounds. As thin-layer chromatography forms the basis of the separations, the chances of transformation, destruction or artifact formation taking place are considerably less than would be likely with the alternative gas-liquid chromatography.

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THE CONJUGATED 17-HYDROXY EPIMERS OF Δ^5 -ANDROSTENE-3 β -17-DIOL
IN INFANT AND ADULT URINE AND UMBILICAL CORD PLASMA

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ABSTRACT

The 17 α and 17 β epimers of androstenediol (3 β ,17-dihydroxy-androst-5-ene) have been separated in umbilical cord venous plasma and infant and adult urine and the 17 α epimer isolated and identified in infant urine. The ratio of 17 α - to 17 β -androstenediol is at least 9 to 1 in both media, and is the opposite ratio to that found in adult urine. It is suggested that the predominance of the 17 α epimer in the fetus and in infants is due to a preferential 16 α hydroxylation of 17 β -androstenediol by the high activity of 16 α hydroxylase in early life, coupled in the fetus by possible preferential placental aromatization of the 17 β epimer to form estradiol. Androstenediol is one of the major components of umbilical cord plasma, the approximate levels are: arterial plasma 126 $\mu\text{g}/100\text{ ml.}$, venous plasma 94 $\mu\text{g}/100\text{ ml.}$ The 17 α -epimer is present in infant urine as the disulfate.

INTRODUCTION

The 17 β -hydroxy epimer of androstenediol was first isolated from male urine by Fotherby (1) and identified in adrenal vein blood by Hirschmann et al. (2) and Wieland et al. (3). It has been detected (although possibly not distinguished from the 17 α epimer) and measured (5 $\mu\text{g}/100\text{ ml}$) in the sulfate fraction from umbilical cord plasma (4) and isolated from fetal tissue after perfusion with DHA and DHA sulfate (5). It has been identified and measured in adult human peripheral blood by Vihko (6) who reported levels of between 0 and 90 $\mu\text{g}/100\text{ ml}$ (with one of 160 $\mu\text{g}/100\text{ ml}$) in the solvolysable steroid fraction. Sjoval and Vihko (7) have recently shown that some at least of the 17 β epimer is present in the disulfate. 17 α -androstenediol has been

measured in human biological fluids but has been demonstrated in the urine of a boy with adrenocortical carcinoma (8) and small quantities have been reported in the di-sulfate fraction of adult peripheral blood (7).

Steroids with the 3β -hydroxy- Δ^5 configuration predominate quantitatively in infant urine and androstenediol is one of the major components of the group. When a method was being developed for their assay (9), it became evident that the majority of the androstenediol present had the 17α configuration, furthermore, unlike the other 3β -hydroxy- Δ^5 steroids measured, it was not hydrolysed by the *Helix pomatia* enzyme but only by solvolysis. It was decided to identify the 17α epimer fully and to investigate its relative importance and mode of conjugation in infant urine and umbilical cord plasma.

EXPERIMENTAL

Abbreviations

17α (or 17β) Androstenediol: $3\beta,17\alpha$ (or 17β) dihydroxy-androst-5-ene.
16-Oxo-androstenediol: $3\beta,17\beta$ -dihydroxy-androst-5-en-16-one.
Androstenetriol: $3\beta,16\alpha,17\beta$ -trihydroxy-androst-5-ene.
DHA: 3β -hydroxy-androst-5-en-17-one.
 16α -OH-DHA: $3\beta,16\alpha$ -dihydroxy-androst-5-en-17-one.
Estradiol: $3,17\beta$ -dihydroxy-estra-1,3,5(10)-triene.
 16α -OH-Pregnenolone: $3\beta,16\alpha$ -dihydroxy-pregn-5-en-20-one.
21-OH-Pregnenolone: $3\beta,21$ -dihydroxy-pregn-5-en-20-one.
Testosterone: 17β -hydroxy-androst-4-en-3-one.
epi-Testosterone: 17α -hydroxy-androst-4-en-3-one.

Materials

Complete 24 hr. specimens of urine were collected from normal male infants during the first 6 days of life and also from adult males and females. Preservation was by storing at 4° followed by freezing at -24° as soon as possible. Umbilical cord venous plasma was obtained by gently withdrawing the venous blood in cords from normal babies into receptacles containing sufficient heparin to prevent clotting, separating the plasma as soon as possible and storing at -24° .

Hydrolysis and extraction

The basic quantitative technique used has been described previously (9). For urine, this was unaltered but recent experience has shown that for plasma the extract occasionally contains sufficient fatty material to prevent chromatographic separation. This fatty impurity proved to be difficult to remove since its polarity was very similar to that of the group of steroids being separated. Removal was finally achieved by performing the separation while the steroids were still in conjugated form, and to this end the following additional procedure has been included for plasma specimens only. Plasma (5 ml.) was dripped into 4 vol. ethanol:acetone (50:50 v/v) to precipitate protein, the precipitate was washed with one vol. ethanol:acetone and the combined extracts were evaporated to dryness on a rotary evaporator at 45°. The dried extract was dissolved in 10 ml 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 hr, pouring into a 2 cm column and passing further solvent through for 6 hr before use). Fatty impurities and the free steroids were eluted with a further 100 ml of NaCl saturated methanol-chloroform and finally the conjugated steroids were eluted with 200 ml methanol. This was evaporated to dryness and the residue dissolved in 5 ml acetate buffer (0.5 N acetic acid: 0.5 N sodium acetate 2:3 v/v); after hydrolysis with a *Helix pomatia* enzyme preparation and extraction (to free other plasma steroids), the androstenediol was obtained by solvolytic extraction (9) with ethyl acetate. Separation and assay was as previously described (9). The technique for urine briefly consisted of an initial hydrolysis with *Helix pomatia* enzyme followed by extraction with ether:ethyl acetate (2:1 v/v). The aqueous phase was further subjected to solvolysis and the extracts combined.

Thin-layer chromatography and quantitation

Plates (20 x 20 cm) were spread with silica-gel or alumina (both from Merck A.G., Darmstadt, Germany) to a thickness of 0.25 mm. The silica-gel plates were left to stand at least 24 hr. before use but those prepared with alumina were used immediately they were dry. The solvent systems referred to later were as follows:-

Silica-gel

System A - Cyclohexane:ethyl acetate (50:50 v/v)

System B - Benzene:acetone:ethanol:water (30:30:30:10 v/v)

Alumina

System C - Methylene dichloride:ether (90:10 v/v)

Colour development was by spraying with a saturated solution of antimony trichloride in chloroform followed by heating for 5 min at 110°. The bands produced were quantitated by densitometric scanning (9).

RESULTS

Identification of androstenediol-17 α in infant urine

The 17-hydroxy epimers of androstenediol are not separated in the thin-layer silica-gel chromatographic systems previously described (9) and to achieve an adequate separation it was found necessary to use alumina. A sample of the major epimer in infant urine was prepared for identification as follows:- 500 ml. 1-6 day old infant urine were treated as described, and the extracts streaked onto almost the full width of a 20 x 20 cm silica-gel thin-layer chromatography plate which was developed by 2 runs in System A. With the rest of the chromatogram masked, the edges were sprayed with antimony trichloride and the band of silica-gel containing the androstenediol thus located in the unsprayed portion was removed from the glass and eluted with absolute ethanol. The material obtained was streaked onto an alumina thin-layer plate later developed by 2 runs in System C (Fig. 1 shows the separation of the epimers obtained in this system). The band containing the 17 α epimer was located and eluted as before and the material obtained after



Figure 1. Thin layer alumina chromatography of 1, androstenediol (17 α); 2, the androstenediol (17 α and β) fraction from infant urine; and 3, androstenediol 17 β . The separation was achieved by 2 runs in System C, and colour development was by antimony trichloride. A part only, of the chromatography plate is shown.

evaporation of the ethanol transferred to a 1 mm germanium internal reflector plate with acetone. The infrared spectrum produced with a Unicam SP 200 spectrophotometer is shown in Fig. 2. It is practically identical to the spectrum for authentic 17α androstenediol produced under similar conditions and also shown in Fig. 2. The spectrum of 17β -androstenediol is also shown for comparison.

Gas chromatography-mass spectroscopy was carried out on the unseparated epimers from the silica-gel chromatography of infant urine (9) and the spectrum was, for practical purposes, identical to that of either epimer. The fragmentation patterns of the epimers cannot be distinguished (6).

The mode of conjugation of androstenediol in infant urine and cord plasma

When the 3β -hydroxy- Δ^5 steroids freed by the *Helix pomatia* enzyme hydrolysis of infant urine are compared with those freed by the further action of solvolysis, it is evident (Fig. 3) that androstenediol in infant urine is unique in being freed practically entirely by solvolysis only. The bulk of the known steroid material shown in Fig. 3 to be freed by the enzyme is made up of 16-oxo-androstenediol, 16α -OH-DHA, 21-OH-pregnenolone, 16α -OH-pregnenolone and androstenetriol. Very little material other than androstenediol is freed by the solvolysis.

Androstenediol also behaves uniquely together with 21-OH-pregnenolone when the steroid conjugate extract of cord plasma is subjected to Sephadex chromatography. Chromatography was carried out as described except that the eluting solvent was not saturated with NaCl

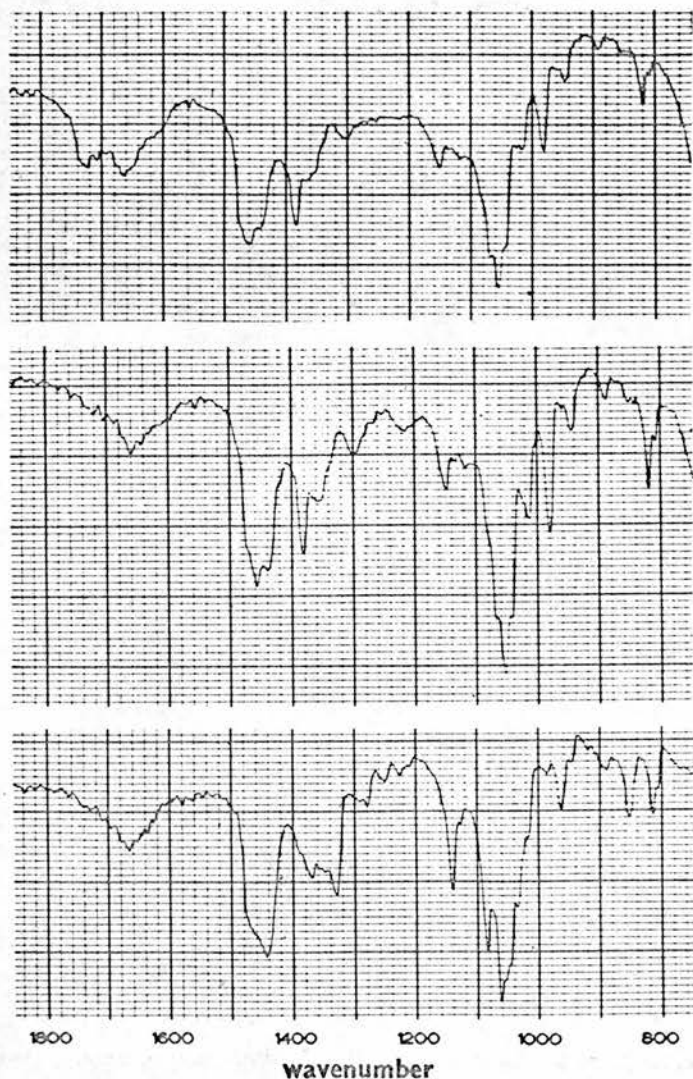


Figure 2. Infrared spectra of (top) androstenediol (17α) fraction from infant urine, (centre) authentic androstenediol (17α), and (bottom) authentic androstenediol (17β).

and the eluate was collected in 10 ml. fractions. All fractions were then subjected to hydrolysis with *Helix pomatia* and the extracts of freed steroids were separated by thin-layer chromatography in System A and shown up by antimony trichloride staining. The steroids listed

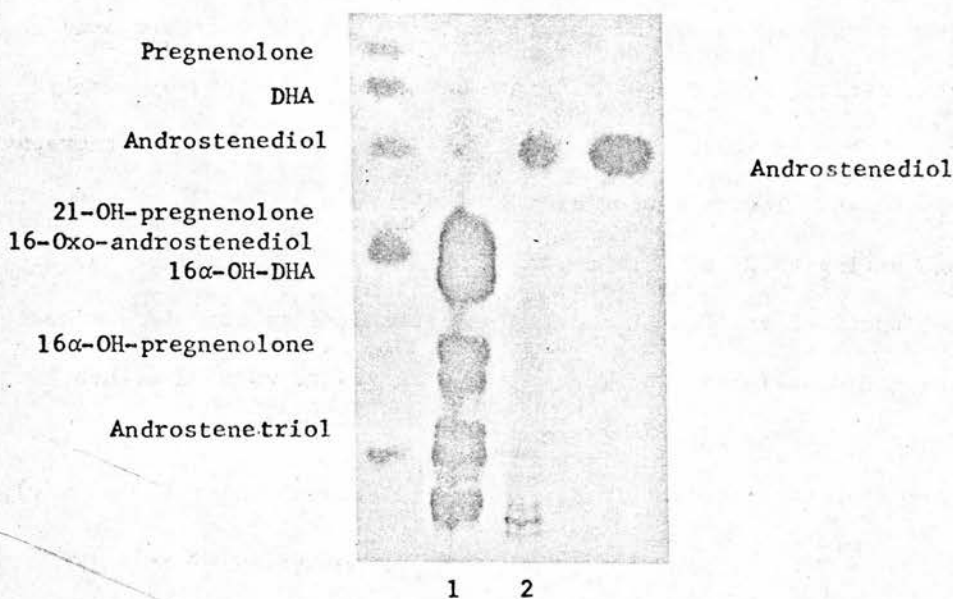


Figure 3. Thin-layer silica-gel chromatography of 1, 3β -hydroxy- Δ^5 steroids freed from infant urine by *Helix pomatia* enzyme hydrolysis and 2, those freed by subsequent solvolysis. Standards have been run as shown. Separation was by 2 runs in System A and colour development was by antimony trichloride. Run 1 has purposely been overloaded to allow a demonstrable amount of androstenediol in run 2. A part only of the chromatography plate is shown.

above together with other unidentified material were present in the 140 to 190 ml of eluate, with the exception of 21-hydroxypregnenolone which was shown to be in the fraction 350-380 ml. The eluate fractions were further subjected to solvolysis and the extracts again separated by thin-layer chromatography. Androstenediol now appeared in the extracts from 350-380 ml. and was the only detectable spot on all the chromatograms. The relative elution volume of androstenediol and 21-hydroxypregnenolone to the other 3β -hydroxy- Δ^5 steroids ($\frac{375}{165} = 2.27$) is consistent with their being disulfated compared with the more usual monoconjugation of the other 3β -hydroxy- Δ^5 steroids (10).

It was thought possible that 17α -androstenediol was being formed as an artifact during the hydrolysis of 17β -androstenediol disulfate.

To discount this, a sample of the 17β -epimer disulfate was prepared using the technique of steroid sulfate formation described by Levitz (11). The product was purified by thin-layer silica-gel chromatography using System B. The formation of the disulfate was indicated by the compound having an Rf of 0.11 compared with values of 0.79 for androstenediol and 0.42 for DHA sulfate. In System B, free steroids, mono-sulfates and disulfates tend to run as three groups with Rf values similar to those indicated. After removal from the plate the silica-gel containing the disulfate was eluted with ethanol:water (50:50 v/v), the solvent evaporated and the residue hydrolysed by solvolysis for 24 hr. Subsequent chromatography in System C showed that only 17β -androstenediol was present.

Quantitative assay

Infant urine. The levels of 17β -androstenediol in infant urine are too small for accurate assay by the techniques used but it can be seen from Fig. 1 that the ratio of the 17β to the 17α epimer is low (allowing for the decreased chromogenicity of 17α -androstenediol, see Fig. 4). It has been estimated from many similar separations on different urines to be less than 1 to 9. The urinary excretion of both epimers measured together during the first 6 days of life has been previously reported (11) and was found to vary from less than 20 to 880 $\mu\text{g}/24$ hr.

Adult urine. The 24 hr. urine specimens from 4 male and 4 female adults were pooled and portions subjected to Helix pomatia hydrolysis with extraction followed by solvolysis, and to solvolysis alone. The two epimers of androstenediol were then separated and measured in each

extract. Fig. 4 shows the resulting chromatograms. The following percentages of each isomer in each extract were obtained:

	17 α	17 β
enzyme hydrolysis (1)	38	62
solvolysis after enzyme hydrolysis (2)	25	75
[(1) + (2)]	34	66]
solvolysis alone	50	50



Androstenediol (17 β)

Androstenediol (17 α)

1 2 3 4 5

Figure 4. Thin-layer alumina chromatography of 1, 2 μ g each of androstenediol (17 α) and (17 β); 2, the enzyme hydrolysable androstenediol fraction from adult urine, previously purified on thin-layer silica-gel; 3, the solvolysable fraction remaining after enzyme hydrolysis; 4, the fraction obtained from adult urine after solvolysis alone; 5, 4 μ g each of androstenediol (17 α) and (17 β). The separation was achieved by 2 runs in System C, and colour development was by antimony trichloride. Each extract was obtained from approximately the same quantity of urine. A part only of the chromatography plate is shown.

The average total excretion of both epimers of androstenediol in 4 adult males was 560 (range 150 - 830) μ g/24 hr.

From Fig. 4 it is evident that adults differ from infants in having a considerable proportion of both 17 α and 17 β androstenediol which is hydrolysable by *Helix pomatia* (17 α , 83%; 17 β , 89%; total 87%) and a relatively small portion (17 α , 17%; 17 β , 33%; total 27%) which can

freed by solvolysis. This could be accounted for by a major quantity of the androstenediol being present as glucosiduronate.

Amnion cord plasma. The ratio of the 17 β to the 17 α epimer was approximately the same as in infant urine. The concentration of both epimers in a 70 ml pool of venous cord plasma was 190 $\mu\text{g}/100\text{ ml}$.

Measurements in arterial and venous cord plasma ($\mu\text{g}/100\text{ ml}$) from 3 infants were as follows:

	Arterial plasma	Venous plasma
Infant 1	160	76
" 2	107	96
" 3	110	110

DISCUSSION

In infants and in utero the 17 α epimer of androstenediol predominates approximately 9 times over its 17 β counterpart whereas in adults the 17 β epimer is dominant. Furthermore the 17 α -androstenediol in infants would appear to be in disulfate form with at least one of the ester sulfate groups not hydrolysable by the *Helix pomatia* enzyme preparation (the title of "sulfate" has been given to solvolysable compounds with the retention volumes of sulfates on Sephadex LH-20). The difficulty with enzyme hydrolysis may account for Bolté et al. (5) isolating only the 17 β epimer after perfusion of fetus with DHA and DHA sulfate. 17 α Androstenediol appears to be the only 3 β -hydroxy- Δ^5 steroid present in any quantity in cord blood and not completely hydrolysed by the *Helix pomatia* enzyme. It is the only 17 α hydroxy C₁₉ steroid detected in cord blood and is apparently the sole 3 β -hydroxy- Δ^5 C₁₉ steroid excreted by infants in any quantity as the di-sulfate. 21-Hydroxypregnenolone, the only other 3 β -hydroxy-

Δ^5 steroid present as the disulfate, has been shown by Pasqualini (12) to be present exclusively as such in adults. It is hydrolysed by enzyme hydrolysis.

The reason why the 17α epimer predominates in the fetus and in infancy is not readily apparent. One possibility is that both epimers are produced (perhaps 17β -androstenediol in greater quantity as found in adult urine) and 16α hydroxylase [abundant in the fetus and infant and not in adults (13)], selectively acts on 17β -androstenediol producing androstenetriol. This compound is present in considerable amount in infant urine (9) and $16\alpha,17\beta$ -dihydroxy-androst-4-en-3-one has been identified as a major steroid component of placental tissue (14). 17α Androstenetriol is undetectable in infant urine by the techniques used.

This selective removal of the 17β epimer would not operate in the adult because of the low level of activity of 16α -hydroxylase. The possibility of an enhancement of the effect in utero by the placenta selectively aromatizing the 17β epimer to form estradiol, cannot be discounted.

It is possible that an equilibrium exists in vivo between DHA and androstenediol since Baulieu et al. (15) has shown that 17β androstenediol sulfate can be converted in humans to DHA sulfate and the reverse conversion has been indicated in adults by Roberts et al. (16) and in the fetus by Bolté et al. (5). It is not known what would be the ratio of 17α - to 17β - androstenediol in such an equilibrium, or how much, if any, androstenediol is produced as such by the adrenal.

An important aspect of the relative amounts of circulating 17α and 17β epimers arises if androstenediol is an important intermediate

in the synthesis of testosterone as has been suggested may be the case (17,18). An excessive amount of 17α -androstenediol may conceivably lead to an excessive production of epitestosterone relative to testosterone.

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INTERNATIONAL SYMPOSIUM ON FOETO-PLACENTAL UNIT

Milan, September 4-6, 1968

62. Arterio-venous blood and amniotic fluid levels of individual 3β -hydroxy- Δ^5 -steroids

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The concentration of many of the 3β -hydroxy- Δ^5 steroids in umbilical cord blood is higher than that of any other known steroid (cholesterol apart) in human blood at any time, and the measurement of the differences in concentration between the arterial and venous blood in the cord can give an indication of their production or utilization by the foetus or placenta. The following known steroids have been measured in cord arterial and venous blood: dehydroepiandrosterone, pregnenolone, 17α -hydroxypregnenolone, 16α -hydroxypregnenolone, 21 -hydroxypregnenolone, 16α -hydroxydehydroepiandrosterone, and androstenediol. In addition, two new steroids, 16β -hydroxydehydroepiandrosterone and androstenediol (17α) have been identified and measured. In general the concentration of all compounds is greater in arterial than in venous blood. By the methods used, none of the steroids were detectable in the free form and all were present as mono-conjugates except for the di-conjugation of androstenediol (17α), 16β -hydroxydehydroepiandrosterone and some of the 16α epimer, 21 -hydroxypregnenolone, and some of the androstenediol. The di-sulphate of androstenediol (17α) could not be completely hydrolysed by sulphatases present in placental tissue and as they are absent in foetal tissue this may be one reason why such di-conjugates build up in the circulation, since if they cannot be hydrolysed they may not readily be removed.

The steroids mentioned have also been assayed in amniotic fluid where their profile resembles that in neonatal urine more than in cord blood. Negligible quantities were found in cases with anencephalic fetuses presumably due to their low synthesis and excretion by these infants.

The identification and importance of new steroids in the foeto-placental unit.

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Until recently it was believed that the placenta, acting as a single endocrine organ, was responsible for the production of the large quantity of the steroids associated with pregnancy, but it is now known that the foetus itself plays a major role in their synthesis and probably also in the control of their production. Diczfalusy has suggested the term foeto-placental unit to describe the complete endocrine organ responsible.

If it is accepted that a high level of oestrogen and progesterone is necessary for a successful pregnancy, then the involvement of the foetus in their production means that in some respects it is responsible for its own future and any fault which develops in its steroid synthesis may have serious repercussions for its well-being. Study of steroid metabolism in the intact foetus is not easy, but the mechanisms of steroid production by the foetus are still functional at birth, and it may be assumed that the steroids in newborn urine are indicative of production *in utero*. Many new steroids have recently been discovered both in foetal blood and in infant urine and undoubtedly many more remain to be identified; other already known steroids have been shown to be present in considerable quantity. It is not known what role many of these compounds play with regard to the overall requirement for steroids in pregnancy and certainly the control of their production is not understood.

The majority of the compounds involved have the 3β -hydroxy- Δ^5 configuration and the chemical synthesis of model compounds for identification purposes presents problems in introducing functional groups, sensitive and otherwise, in the presence of the 3β -hydroxy- Δ^5 structure.

The excretion of variously hydroxylated oestrogens in pregnancy urine has raised the question as to whether the correspondingly hydroxylated dehydroepiandrosterone derivatives are precursors. In an endeavour to answer this, model compounds oxygenated at positions 14, 15, 16 and 18 have had to be synthesized. Most of these have not been found in infant urine, but two have been identified which are excreted by the newborn infant in quantity and are present in high concentration in cord blood, $3\beta,17\alpha$ -dihydroxyandrost-5-ene and $3\beta,16\beta$ -dihydroxyandrost-5-en-17-one. Both these compounds are the epimers (at C-17 and C-16 respectively) of steroids previously identified in adult urine and, in greater quantity, in infant urine. If they are aromatized in the placenta they should form 17α -oestradiol and 16β -hydroxy-oestrone (leading to 16β -epioestrinol) respectively. These oestrogens are only excreted by the mother in very small quantity. Possible factors limiting their aromatization will be discussed.

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THE EFFECT OF EXOGENOUS CORTICOTROPHIN ON STEROID EXCRETION IN THE NEWBORN

By

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and F. L. Mitchell

ABSTRACT

The urinary excretion of the individual 3β -hydroxy- Δ^5 steroids together with cortisol and the chromatographic bands of material containing its tetrahydrometabolites, have been studied in early infancy before and after stimulation by exogenous corticotrophin (ACTH). The average increase in the excretion of the Δ^5 steroids after stimulation is relatively small (less than 185%) compared with the increase in cortisol and its metabolites (greater than 600%) and it is suggested that ACTH stimulation cannot be solely responsible for the formation of the considerable quantities of Δ^5 steroids in early infancy and the high Δ^5 steroid/cortisol production ratio *in utero*.

Steroid metabolism in early infancy differs markedly from that in adults (Mitchell 1967). Factors are still in operation which presumably were designed to meet circumstances prevailing *in utero*. These include the production of oestrogen and possibly progesterone precursors, 3β -hydroxy- Δ^5 steroids, which *in utero* would be converted by the placenta into the active hormones but in early independent life are excreted in the urine either unchanged, 16α hydroxylated or otherwise metabolized. They make up by far the largest fraction of the total steroid content in infant urine during the few weeks immediately following birth.

The control of oestrogen and progesterone production in pregnancy is not understood, but if it is assumed that the production of their precursors in early infancy proceeds by the same mechanisms as *in utero*, a study of the effects of

certain agents upon their rates of production should yield useful information. *Lauritzen & Lehmann* (1965) and *Lauritzen* (1966) have shown that the administration of corticotrophin (ACTH) and human chorionic gonadotrophin (HCG) to infants during the first week of life, causes a statistically significant increase in the excretion of the oestrogen precursor dehydroepiandrosterone (DHA). A study has now been made of the effect of ACTH on the urinary excretion of the major 3β -hydroxy- Δ^5 steroids found in infant urine and this is compared to the effect on urinary cortisol and its tetrahydro metabolites.

MATERIAL AND METHODS

Abbreviations

PREG: pregnenolone (3β -hydroxy-pregn-5-en-20-one); DHA: dehydroepiandrosterone (3β -hydroxy-androst-5-en-17-one); 21-OH-PREG: 21-hydroxypregnenolone (3β , 21-dihydroxy-pregn-5-en-20-one); AD: androstenediol (3β , 17 α (and 17 β)-dihydroxy-androst-5-en); 16-O-AD: 16-oxoandrostenediol (3β , 17 β -dihydroxy-androst-5-en-16-one); 16-OH-DHA: 16 α -hydroxydehydroepiandrosterone (3β , 16 α -dihydroxy-androst-5-en-17-one); 16-OH-PREG: 16 α -hydroxypregnenolone (3β , 16 α -dihydroxy-pregn-5-en-20-one); AT: androstenetriol (3β , 16 α , 17 β -trihydroxy-androst-5-en); cortisol: 11 β , 17 α , 21-trihydroxy-pregn-4-ene-3,20-dione; THE: tetrahydrocortisone (3 α , 17 α , 21-trihydroxy-5 β -pregnane-11,20-dione); THF: tetrahydrocortisol (3 α , 11 β , 17 α , 21-tetrahydroxy-5 β -pregnan-20-one).

Subjects

Infants used for the study were all male and were being treated with ACTH for either prematurity, hyperbilirubinaemia, anorexia with dyspepsia leading to anhydraemic shock or laryngitis subglottica (pseudokrup). It was considered that in all cases the disorders were of non-endocrine origin and the results found should not differ markedly from those to be expected in normal individuals. ACTH (Corticotrophine-Z, Organon, Oss) was administered intramuscularly twice daily at the rate of 8 units per day for 3 successive days. Urine was collected from these infants during treatment and for the 3 days previously. Collections of 24 h urines were also made from 4 normal infants during the first 6 days of life.

Urine was collected in plastic bags attached to the infant, refrigerated as soon as possible and stored at -20°C until processed.

Extraction, chromatography and assay

The basic method was that reported previously for the 3β -hydroxy- Δ^5 steroids (*Shackleton & Mitchell* 1967), modified to include cortisol and its tetrahydro metabolites. These required a longer period of enzyme hydrolysis, and to achieve this, penicillin (500 IU/ml urine) and streptomycin calcium chloride B. P. (0.625 mg/ml urine) were added to the urine before the addition of 750 units per ml of β -glucuronidase from *Helix pomatia*. Hydrolysis was allowed to proceed for 48 h at 37°C , a further 750 units of enzyme being added after 24 h. Extraction by ethyl acetate, solvolysis, chromatography on thin-layer silica-gel and quantitation by direct scanning of the chromatogram after colour development by antimony trichloride reagent was then done for 3β -hydroxy- Δ^5 steroids as previously described.

For cortisol and its metabolites, thin-layer chromatography was done by running twice in the system, chloroform: absolute ethanol (90:10 v/v) and colour development for the tetrahydro compounds was by spraying the plates, until they were uniformly soaked, with a blue tetrazolium reagent (1% blue tetrazolium chloride in absolute ethanol w/v: 3.5 N KOH in absolute ethanol:water, 2.5:3) and drying with a domestic hair dryer held at a distance of approximately 30 cm. Only the bands of material containing THE and THF were quantitatively measured; this was done by densitometric comparison with standard runs of cortisol. Cortisol was assayed by visually comparing the absorption of its chromatographic band in ultraviolet light of wavelength 254 μ with standards, using the precautions for visual assessment described for fluorescent spots by *Bush & Mahesh* (1959).

Identification

The 3β -hydroxy- Δ^5 steroids AD, 16-O-AD, 16-OH-DHA, 16-OH-PREG and AT were identified by methods including gas chromatography-mass spectroscopy as previously described (*Shackleton & Mitchell* 1967). The cortisol band was eluted from the silica-gel with absolute ethanol and subjected to two dimensional thin-layer chromatography in the systems: a) two runs in ethyl acetate:cyclohexane: absolute ethanol (45:45:10), b) one run in ethyl acetate:water saturated n-hexane:acetic acid: absolute ethanol (72:13.5:10:4.5). No other ultraviolet light absorbing spot could be separated. The cortisol spot was eluted with absolute ethanol and the residue after evaporation to dryness dissolved in acetone and transferred to a 2 mm germanium internal reflector plate for infra-red spectroscopy. The spectrum showed major peaks at 1060, 1135, 1240, 1280, 1460, 1660 and 1710 wavenumbers and agreed well with the spectrum of authentic cortisol prepared in a similar way.

The pattern of reducing steroids in infant urine is complex (*Cathro et al.* 1963) and the chromatographic bands of material containing THE and THF undoubtedly contain other compounds which have not been identified. A full investigation of the material in these bands has not been attempted.

RESULTS

In Table 1 the results obtained in μ g/24 h have been averaged for the 3 days prior to treatment and for the 3 days during the administration of ACTH. Table 2 shows findings over a similar period for 4 normal untreated individuals and Table 3 gives the average excretion for the 5 ACTH treated infants on the first, second and third days of treatment.

DISCUSSION

Of the major 3β -hydroxy- Δ^5 steroids studied, DHA (*Ryan* 1959), 16α -OH-DHA (*Colás et al.* 1964) and androstetriol (*Ryan* 1958) have been shown to act in the placenta as precursors of oestrogens, and 16-oxoandrostenediol is possibly implicated also (*Dell'Acqua et al.* 1967). Pregnenolone has been found to be converted to progesterone in high yield when perfused through placentas *in situ* (*Palmer et al.* 1966), though it is not known how this pathway of synthesis

Table 1.

Urinary steroid excretion in the newborn, averaged for the first three days of life with no treatment and for days 4, 5 and 6 during which ACTH was administered ($\mu\text{g}/24\text{ h}$)

Infant	Treatment	Average urine volume ml/24 h	PREG	DHA	21-OH-PREG	AD	16-O-AD	16-OH-DHA	16-OH-PREG	AT	Cortisol	Tetrahydro group
1	None	290	<50	<50	190	98	1087	1460	1300	1327	<80	<40
	ACTH	350	<50	<50	495	88	3262	4052	5088	1769	1000	1088
2	% increase				160	-10	200	178	289	33	>1100	>2600
	None	205	<31	37	352	713	2213	1660	2460	1173	<400	<240
3	ACTH	274	91	110	285	527	1787	3240	5813	1153	2400	2733
	% increase		>300	198	-19	-26	-19	95	136	-2	>500	>1000
4 ^{a)}	None	31	<12	<12	436	713	2573	1887	860	680	200	<120
	ACTH	265	18	19	711	1027	5180	5080	4653	1707	550	816
5	% increase		>50	>58	63	44	100	170	441	151	161	>580
	None	127	<50	<50	213	98	1320	740	920	673	<100	<70
6	ACTH	198	<50	<50	407	167	2253	1967	2827	1007	1200	632
	% increase				90	70	71	166	207	50	1100	802
7	None	290	<50	<50	207	<70	2040	1107	2993	1467	<100	110
	ACTH	323	<50	<50	647	75	2240	2815	5933	1393	1080	737
8	% increase				213	<7	10	156	98	-5	983	570
	None				275	338	1847	1371	1707	1064	<170	<116
9	(Grand average)				509	377	2935	3430	4863	1406	>1250	>1200
	% increase ^{b)}				82	>11	59	150	185	32	>600	>1000

a) For this infant, control days were 4, 5 and 6 with ACTH given on days 7, 8 and 9.

b) Calculated from grand totals before and after treatment.

Table 2.
Average urinary steroid excretion in 4 normal infants during days 1, 2, 3 and 4, 5, 6 of life ($\mu\text{g}/24\text{ h}$).

Days of life	Average urine volume ml/24 h	PREG	DHA	21-OH-PREG	AD	16-O-AD	16-OH-DHA	16-OH-PREG	AT	Cortisol	Tetrahydro group
1, 2, 3	73	18	<9	243	<70	1163	976	1040	768	<100	<100
4, 5, 6	187	16	<5	226	<70	1107	932	1212	817	<100	<100

Table 3.
 Urinary steroid excretion during the 3 days on ACTH, averaged for 5 infants ($\mu\text{g}/24 \text{ h}$).

Days on ACTH	PREG ^{a)}	DHA ^{c)}	21-OH-PREG	AD	16-O-AD	16-OH-DHA	16-OH-PREG	AT	Cortisol	Tetrahydro group
1	58	69	347	270	2772	3584	4736	1304	974	724
2	46	38	617	417	3092	3637	5480	1504	1480	1571
3	60	88	562	443	2941	3071	4373	1409	1360	1308

a) Assayed for 2 infants only.

using circulating pregnenolone compares quantitatively with the use of circulating cholesterol which can also act as a precursor (*Solomon et al.* 1960). Both DHA and PREG are present in considerable quantity in foetal blood (*Simmer et al.* 1964; *Conrad et al.* 1967) and their low rate of excretion in infant urine together with the high excretion of their 16α -hydroxylated derivatives may be explained by the considerable activity of 16α -hydroxylase in the liver at this time (*Reynolds* 1966). On the whole the 3β -hydroxy- Δ^5 steroids measured are being excreted by the infant in fairly constant amount during the first 6 days of life (Table 2 and *Shackleton & Mitchell* 1967) and without ACTH stimulation no significant increase would be expected.

In view of the relative quantitative unimportance of DHA and PREG in infant urine, measurement has only been attempted for 2 of the infants studied. The use of different methodology would account for the somewhat higher values for DHA excretion found by *Lauritzen & Lehmann* (1965). The undetectable or very small amounts indicated by the present technique agree with the findings of *Birchall et al.* (1961) and *Paulsen et al.* (1966). The increase in urinary cortisol after ACTH may be compared with the increases found by *Bertrand et al.* (1962) and *Hillman & Giroud* (1965) in blood cortisol in infants (average increases 170 % and 340 %) after ACTH stimulation.

It is of interest to compare the increases in the major 3β -hydroxy- Δ^5 steroids (16-OH-DHA and 16-OH-PREG) under ACTH stimulation with those found by other workers for the major group of urinary steroids not emanating to any extent from cortisol, i. e. the 17-oxosteroids. For androsterone, aetiocholanolone and DHA in adults, *Jungmann et al.* (1967) have listed the findings of 7 laboratories and though the results for individuals vary considerably, the average overall increase is 120 %. For 12 children between the ages of 3 and 14 years, *Paulsen et al.* (1966) showed an average increase in total 17-oxosteroids of 35 % and the average increase in 30 children aged from 5 months to 14 years, with 3 days stimulation, investigated by *Clayton et al.* (1963) was 350 %. A direct comparison in infants, of the effects of ACTH on the 3β -hydroxy- Δ^5 steroids and upon the adult type 17-oxosteroids, cannot be made because of the virtual absence of the latter from infant urine (*Birchall et al.* 1961).

If the rate of excretion can be taken as a measure of production, it appears that the 3β -hydroxy- Δ^5 steroids in infancy are about as sensitive to ACTH stimulation as are the 17-oxosteroids later in life, and it is thus possible that ACTH in foetal blood has some influence on the production of oestrogens and possibly progesterone in pregnancy. *Dässler* (1966) has reported a considerable increase in the urinary excretion of oestriol by women in late pregnancy after the infusion of ACTH; no such increase was obtained in cases of intrauterine foetal death.

The relatively small increase (occasionally a decrease) in the 3β -hydroxy- Δ^5

steroids, however, has been produced together with a very considerable increase in cortisol and its metabolites, and if urinary excretion is taken as a measure of production it is evident that to produce the opposite effect *in utero* of a considerable production of 3β -hydroxy steroids with an apparently fairly normal, compared proportionately with adults (Mitchell 1967), production of cortisol, a different or more complex mechanism of control must obtain. The qualitative difference in steroid production is so great that it cannot be explained by peculiarities in the catabolism of cortisol which undoubtedly obtain in the newborn (Mitchell 1967). It has been suggested (Shackleton & Mitchell 1967) that the explanation for the large quantity of 3β -hydroxy- Δ^5 steroids produced after birth lies in their incidental formation by ACTH stimulation, during a somewhat inefficient synthesis of cortisol via hydroxylated Δ^5 intermediates. It now appears that some other reason for their presence must be found since under exogenous ACTH stimulation the efficiency of cortisol, relative to Δ^5 steroid production, is apparently considerably improved.

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SEPARATUM

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THE EFFECT OF EXOGENOUS HUMAN
CHORIONIC GONADOTROPHIN ON STEROID EXCRETION
IN THE NEWBORN

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C. Lauritzen, C. H. L. Shackleton and F. L. Mitchell**

ABSTRACT

The urinary excretion of a range of steroids and steroid-like compounds, including the major compounds with the 3β -hydroxy- Δ^5 configuration, have been studied in early infancy before and after the administration of human chorionic gonadotrophin. The only compound which showed any increase in excretion was dehydroepiandrosterone which was increased in four out of seven cases studied.

In a previous communication (*Lauritzen et al.* 1968) we described the effect of corticotrophin on the urinary excretion, in the first few days of life, of the individual 3β -hydroxy- Δ^5 steroids together with cortisol and the chromatographic bands of material containing its tetrahydro metabolites. It was then found that though cortisol and its metabolites were increased over 600 per cent, the increase in the 3β -hydroxy- Δ^5 compounds was very variable but averaged approximately 60 per cent, indicating that other factors must normally be operating *in utero* and after birth, to produce the opposite effect of a considerable production of 3β -hydroxy- Δ^5 steroids with a very much smaller amount of cortisol and its metabolites.

It has been shown that human chorionic gonadotrophin (HCG) can stimulate the excretion of dehydroepiandrosterone in newborn infants (*Lauritzen & Lehman* 1965, 1967). In order to compare fully the effect of the two stimulatory

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hormones, the individual urinary steroids have been investigated after the administration of HCG.

MATERIALS AND METHODS

Abbreviations

PREG: pregnenolone (3β -hydroxy-pregn-5-en-20-one);
DHA: dehydroepiandrosterone (3β -hydroxy-androst-5-en-17-one);
21-OH-PREG: 21-hydroxypregnenolone ($3\beta,21$ -dihydroxy-pregn-5-en-20-one);
AD: androstenediol ($3\beta,17\alpha$ (and 17β)-dihydroxy-androst-5-en);
16-O-AD: 16-oxoandrostenediol ($3\beta,17\beta$ -dihydroxy-androst-5-en-16-one);
16 α or β -OH-DHA: 16 α -hydroxydehydroepiandrosterone (3β -16 α or β -dihydroxy-androst-5-en-17-one);
16 α -OH-PREG: 16 α -hydroxypregnenolone ($3\beta,16\alpha$ -dihydroxy-pregn-5-en-20-one);
AT: androstenetriol ($3\beta,16\alpha,17\beta$ -trihydroxy-androst-5-en);
Cortisol: 11 $\beta,17,21$ -trihydroxy-pregn-4-ene-3,20-dione.

Subjects

Only male infants were used in the study and all were being treated with HCG for undescended testicles. It was considered that this disorder would not markedly alter the results from those to be expected in normal infants. HCG (Predalon®, Organon, Oss) was administered intramuscularly twice daily at the rate of 5000 IU per day on the 4th, 5th and 6th day of life. Urine was collected for the first three days of life and also on the three days during treatment, by attaching plastic bags to the infant. It was refrigerated as soon as possible and stored at -20°C until processed.

Assay technique

The method was the same as that used for the study with corticotrophin stimulation (Lauritzen *et al.* 1968) and was as previously described (Shackleton & Mitchell 1967; Shackleton *et al.* 1968). Briefly, the urine was extracted with ether-ethyl acetate after being subjected to hydrolysis by *Helix pomatia* enzyme followed by ethyl acetate solvolysis. Steroid separation was done by thin-layer chromatography and assay was by direct reflectance densitometry of the coloured bands produced by spraying with antimony trichloride and blue tetrazolium reagents. Cortisol was assayed by comparing visually with standards, the absorption of its chromatographic band in ultra-violet light of wavelength 254 $m\mu$.

Identification

Since the only investigated chromatographic band of material showing an increase after stimulation was that containing DHA it was considered important to identify fully the material present in this band after stimulation. The band was eluted from the silica gel with ether, the silica gel having first been deactivated with a drop of water. The trimethylsilyl ether was prepared and subjected to gas-liquid chromatography using an E 301 column. Only one major peak was present (the total area of other minor peaks was less than 5 per cent of the major peak) and this had the retention time of DHA trimethylsilyl ether. It was collected and gas chromatography-mass spectroscopy was carried out 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° C. The spectrum produced was identical with that of authentic DHA.

RESULTS

In Table 1 the results obtained for the 3 β -hydroxy- Δ^5 steroids assayed have been averaged for the three days prior to treatment and for the three days

Table 1.

Urinary steroid excretion in the newborn, averaged for the first three days of life with no treatment and for days 4, 5 and 6 during which HCG was administered ($\mu\text{g}/24 \text{ h}$).

Infant	Treatment	Average urine volume (ml/24 h)	PREG.	DHA	21-OH-PREG	AD	16-O-AD	16 α -OH-DHA	16 β -OH-DHA*	16 α -OH-PREG	AT
1	None	64	30	10	90	50	170	230	260	110	150
	HCG	148	8	4	110	220	330	250	280	330	290
	% Increase	131	-73	-60	22	340	94	9	8	200	93
2	None	70	7	30	60	20	270	250	410	280	500
	HCG	160	10	40	60	10	290	250	120	220	460
	% Increase	129	43	33	0	-50	7	0	-71	14	-8
3	None	50	60	28	110	60	460	520	480	730	650
	HCG	150	19	20	130	40	370	470	200	640	840
	% Increase	200	-68	-29	18	-33	-20	-10	-58	-12	29
4	None	80	<20	<20	45	20	170	200	160	520	450
	HCG	170	<20	1490	108	68	360	450	310	1170	640
	% Increase	113	0	>7350	140	240	112	125	94	125	42
5	None	210	<50	<50	450	180	1300	1170	460	1630	620
	HCG	270	<50	903	410	210	1570	1570	230	1840	260
	% Increase	29	0	>1706	-9	17	21	34	-50	13	-58
6	None	90	<12	60	80	90	390	660	560	1130	290
	HCC	120	<12	460	60	20	290	510	410	670	220
	% Increase	33	0	667	-25	-78	-26	-23	-27	-41	-24
7	None	100	<12	20	50	90	320	550	340	530	150
	HCG	100	<12	110	30	120	180	390	240	430	180
	% Increase	0	0	450	-40	33	-44	-29	-29	-19	20

* Recently identified (*Shackleton et al.* 1968).

during the administration of HCG. Neither cortisol nor any of the bands of material shown up with either blue tetrazolium or Zimmermann reagent showed any increase, and the values found are not reported.

The individual results for DHA in the four infants displaying an increase are shown in histogram form in Fig. 1. Since 16α -OH-DHA is normally the major metabolite of DHA in infant urine, its excretion rate is also shown.

DISCUSSION

Of the steroids investigated, only DHA shows on average an increased rate of excretion after stimulation by HCG. The effect is not consistent, however; no response was obtained in the first three infants studied, and the increase in excretion for the remaining four was variable. A similar variation in response was found by *Lauritzen & Lehmann* (1965, 1967) measuring DHA only, but on

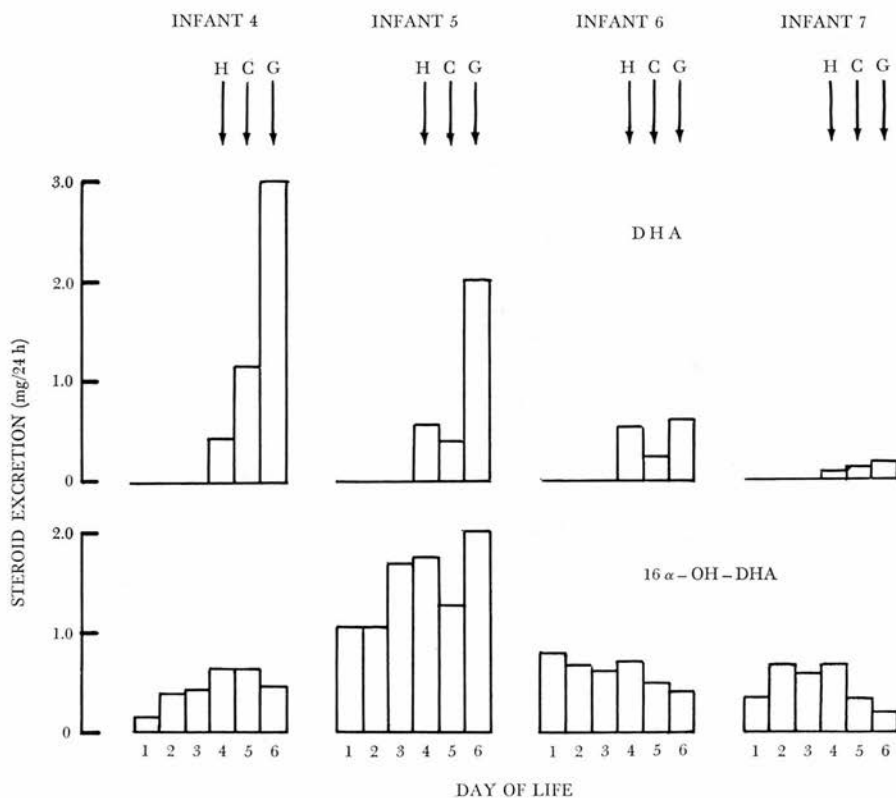


Fig. 1.

Daily excretion of DHA and 16α -OH-DHA by infants treated with HCG. Only those infants showing an increase in DHA excretion have been included.

no occasion did they obtain no response. The somewhat higher values found by them for DHA may be accounted for by the different methodology used.

In the previous investigation, where stimulation was by corticotrophin (Lauritzen *et al.* 1968), there was a considerable increase in the excretion of cortisol and its metabolites, but the increase in the excretion of the 3β -hydroxy- Δ^5 steroids was only comparable with that obtained for the 17-oxosteroids in older individuals. It was suggested then that in order to produce large quantities of 3β -hydroxy- Δ^5 steroid *in utero* and in early infancy a different or more complex control mechanism must exist.

The function of the large quantities of HCG produced in pregnancy is not known but the opinion has been held that it is not intended for use by the foetus because although it is produced in the trophoblast it tends to be secreted unidirectionally into the maternal circulation (Lanman & Dinerstein 1959; Bruner 1951). Lauritzen & Lehmann (1967) have shown, however, that the concentration of HCG in umbilical cord blood is higher in the arterial than in the venous, suggesting a net uptake by the foetus. Rotter (1949) and Jones (1955) have suggested that HCG is the stimulant for the foetal adrenal, and Niemi *et al.* (1967) have pointed out that since the maximal output of urinary chorionic gonadotrophin during pregnancy just precedes maximal enzyme activity, and the maximal volume of interstitial cell tissue in the testes in male foetuses (maximal activity between the 12th and 16th week), it may provide the stimulus for this activity and thus be responsible for the masculinisation of the male foetus. In support of this Brody & Carlström (1965) have shown a connection between HCG and the sex of the foetus; mothers with male foetuses have a significantly lower level of serum HCG in the last trimester of pregnancy than those producing female infants.

The possible implication of HCG in the stimulation of the foetal adrenal has been discussed previously (Lauritzen & Lehmann 1967) and reasons were then given in favour of the view that the increase of DHA noted following HCG injection stems from the adrenals and not from the gonads. The finding that the stimulation is highly selective for DHA now complicates the question since it has been generally assumed that the low level of excretion of DHA by the infant, subsequent to there being a high concentration in umbilical cord blood, is due to the high activity of liver 16-hydroxylase (Mitchell 1967). It is not impossible for HCG to suppress 16-hydroxylation, but no concomitant fall in the excretion of 16α or β -hydroxy DHA is evident.

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THE IDENTIFICATION AND MEASUREMENT OF A NEW STEROID
16 β -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 3 β -hydroxy- Δ^5 steroid colour reaction with antimony trichloride staining reagent and previously named "U₂", was purified by further thin-layer chromatography and identified as 16 β -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 3 β -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 $3\beta, 16\beta$ -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 $600 \mu\text{g}/24 \text{ hr.}$ 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 $3\beta, 16\beta$ -diacetoxyandrost-5-en-17-one from 3β -acetoxy- $5\alpha, 6\beta$ -dichloroandrost-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 $3\beta, 16\beta$ -diacetoxy- $5\alpha, 6\beta$ -dichloroandrost-17-one which is then dechlorinated with zinc in acetic acid.

Hydrolysis of the 16 β -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 $^{\circ}$ 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 17 β -hydroxy-16-oxo epimer (C-18 methyl of 3 β ,17 β -dihydroxyandrost-5-en-16-one resonates at τ 9.08 in pyridine).

Experimental

3 β ,16 β -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 $^{\circ}$ for 60 hr. A further 30 ml. of water was added and the solution evaporated to 100 ml in vacuo at 30 $^{\circ}$. 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 $^{\circ}$ - 192 $^{\circ}$ [α]_D - 19 $^{\circ}$ (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 U₂ since, as found by Layne and Marrian (9) during the isolation of 16 β -hydroxyestrone, many of the procedures used resulted in its partial isomerization to the more stable α -ketol, in this case 3 β ,17 β -dihydroxyandrost-5-en-16-one (16-oxoandrostenediol). This spontaneous conversion was proved by separating 16-oxoandrostenediol from compound U₂ 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 R_f values to 16β -hydroxy-DHA on silica-gel in the following thin-layer systems:-

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

and on paper in the systems:-

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

In all the systems mentioned it is slightly more polar than 16α -hydroxy-DHA and $3\beta, 17\beta$ -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 $3\beta, 16\alpha, 17\alpha$ (or $16\beta, 17\beta$)-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 $16\beta, 17\beta$ -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 thallos 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 U_2 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.

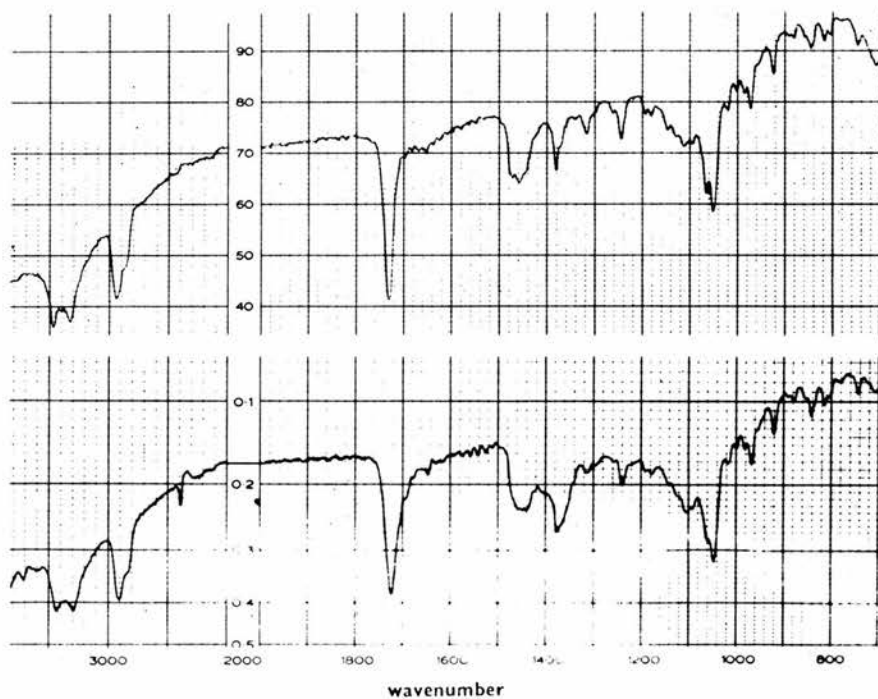


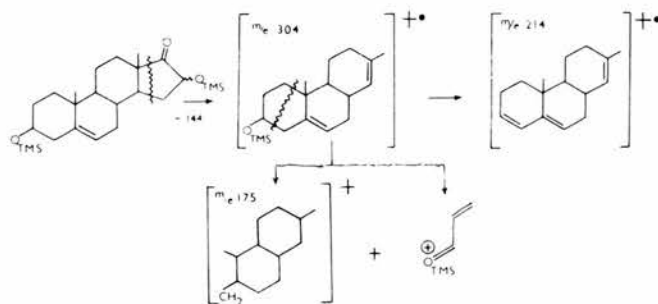
Figure 1. Infrared spectra of (top) authentic 16β -hydroxy-DHA and (lower) compound U_2 . The spectra were obtained by the use of a thallos bromide-iodide internal reflection plate.

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.



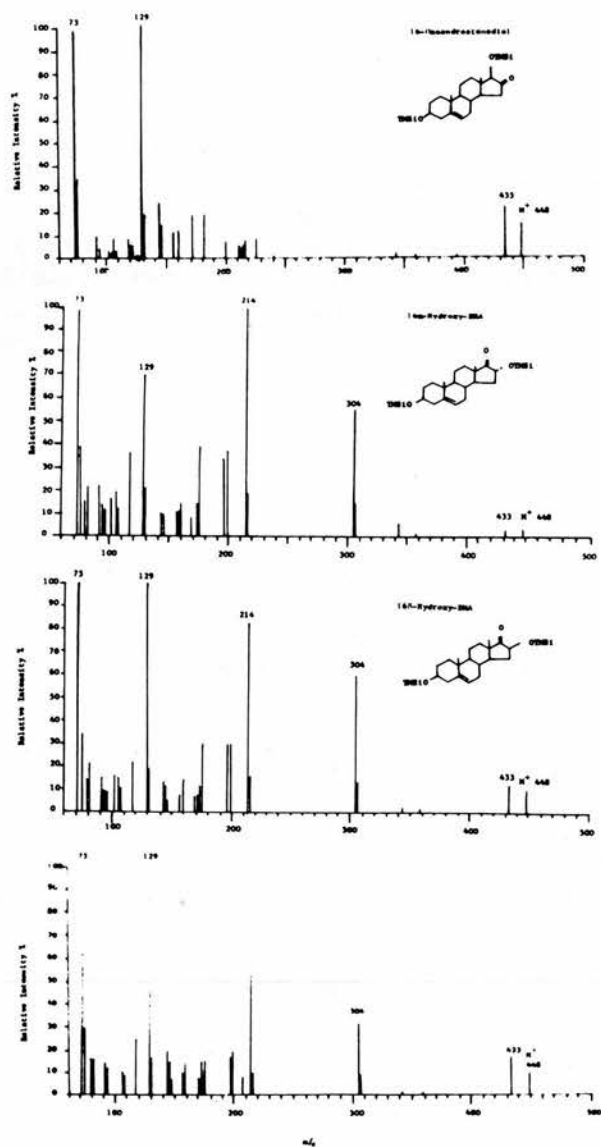


Figure 2. Mass spectra diagrams for trimethyl silyl ethers of authentic 16-oxoandrostenediol, 16 α -hydroxy-DHA, 16 β -hydroxy-DHA and compound U₂. The peaks at m/e 73 were off scale.

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 16 α - and 16 β -hydroxy-DHA and 16-oxo-androstenediol; the main products having retention times, relative to 5 α -androstane, of 1.0 and 0.34 on both columns. This evidence is thus consistent with the original compound being 16 β -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 Sjovald 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.

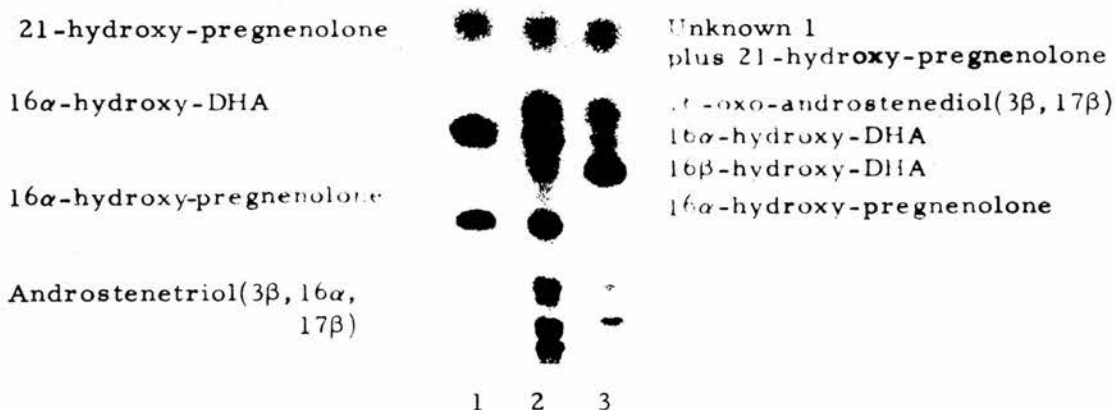


Figure 3. Thin-layer silica-gel chromatography of 1, the standard compounds indicated; 2, the mono-conjugated steroid fraction from infant urine; and 3, the di-conjugated fraction. Colour development was by SbCl₃ reagent.

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 16 β -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 16 β -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 3 β -hydroxy- Δ^5 steroids staining with SbCl₃ and hydrolysed by *Helix pomatia* enzyme, 16 β -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 16 β -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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