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Steroid Hormone Metabolism in Fetal Sheep Kidneys

by

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PREFACE

The abbreviations used in this thesis are defined in "Instructions to Authors" in Biochem.J. (1975) 145:1-20 Non-standard abbreviations are defined in the text.

The following steroids are quoted in the text.

TRIVIAL NAME	SYSTEMATIC NAME
oestrone	3-hydroxy-oestra-1,3,5, (10)-triene-17-one
oestradiol-17β	oestra-1,3,5 (10)-triene-3, 17β-diol
oestradiol-17α	oestra-1,3,5 (10)-triene-3, 17α-diol
DHEA	3β-hydroxy-androst-5-ene-17-one
androstenedione	androst-4-ene-3, 17-dione
testosterone	17β-hydroxy-androst-4-ene-3-one
progesterone	pregn-4-ene-3, 20-dione
pregnenolone	3β-hydroxy-pregn-5-ene-20-one
5α-pregnanedione	5α-pregnan-3, 20-dione
5α-androstanedione	5α-androstan-3, 17-dione
cholesterol	Cholest-5-ene-3β-ol
5α-DHT	17β-hydroxy-5α-androstan-17-one

All other steroids quoted in the text are given systematic names.

Enzyme abbreviations used in the text are:

3β-HSD	(3β-hydroxysteroid:NAD(P)$^+$ oxidoreductase; EC 1.1.1.50)
3α-HSD	(3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase; EC 1.1.1.51)
17β-HSD	(17β-hydroxysteroid:NAD(P)$^+$ 17-oxidoreductase; EC 1.1.1.63-64)
20α-HSD	(20α-hydroxysteroid:NAD(P)$^+$ 20-oxidoreductase; EC 1.1.1.149)
20β-HSD	(20β-hydroxysteroid:NAD(P)$^+$ 20-oxidoreductase; EC 1.1.1.-)
5α-reductase	(3 Ketosteroid:(acceptor) Δ^4-oxidoreductase; EC 1.3.99.-)
Sulphatase	(Aryl-or sterol-sulphate sulphohydrolase EC 3.1.1.6.1 and EC 3.1.6.2)
5β-reductase	(3 Ketosteroid:(acceptor) Δ^4-oxidoreductase; EC 1.3.99.-)

DECLARATION

I declare that the experiments reported in this thesis were carried out by myself except where due acknowledgement has been made. This thesis contains no material previously accepted for the award of any other degree, and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

MARK DOLLING

A preliminary report of some aspects of this work has been published.

Dolling, M; Seamark, R.F.:
Steroid Hormone Metabolism in
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I offer sincere thanks to all who have assisted me during the preparation of this thesis, in particular Miss W. Jones, Miss D. Wilson and Mrs. K. Bliss.

This thesis is dedicated to my parents, whose unfailing support has been of inestimable value.

SUMMARY

1. The in vitro capacity of the fetal sheep kidney to metabolise a number of steroids from all three of the major classes of steroid hormones (C18, C19, C21) has been examined.

2. Homogenates were prepared from fetal kidneys and incubated with C^{14} labelled radioactive tracer steroids. The metabolites formed during these incubations were isolated by thin layer chromatography. Further evidence of the identity of some metabolites was obtained by using sufficient quantities of substrate to allow the metabolites to be analysed using GC and GC-MS. Final proof of the identity of the metabolites was obtained by recrystallisation to constant specific activity. Oestrone was metabolised to oestradiol-17β and oestrone sulphate was hydrolysed, liberating free oestrogen, indicating the presence of 17β-HSD and sulphatase activity. Androstenedione was reduced at several positions indicating the presence of 5α-reductase and 3α/3β-HSD activity, but there was no evidence of any reduction at the 17 position. Progesterone was also reduced at several positions indicating the presence of 5α-reductase, 3α/3β-HSD and 20α-HSD activity. No metabolism of either DHEA or pregnenolone was detected. Of the enzymes present in fetal sheep kidney homogenates, the 5α-reductase was the most active with between 64-75 percent of all the metabolites of androstenedione and progesterone being 5α-reduced.

3. Characteristics of the 5α-reductase were examined. The 5α-reductase has a co-factor specificity for NADPH; NADH at similar concentration being ineffective in raising enzyme activity above control values. A Lineweaver-Burke plot was constructed, from which it was estimated that the 5α-reductase has a Km = 5.9×10^{-6}M, indicating a high apparent binding affinity of 5α-reductase for androstenedione. Examination of the subcellular distribution

of 5α-reductase activity revealed that virtually all the activity was located in the nuclear and cytosol fractions, being about equally distributed between both. The level of activity of 5α-reductase was assayed in the kidneys of both male and female fetuses whose gestational age ranged from about 70 days to term. Most of the values for 5α-reductase activity were between 6-10 p moles/mg of protein/min. No significant difference was observed between the activity in male and female kidneys, and no trend in the level of activity could be demonstrated in relation to gestational age.

4. Data on the body weights and kidney weights were obtained from 52 sets of male-female fetal sheep twins. Analysis of these data revealed that there was a significant difference between the weights of male and female fetal kidneys.

This difference was apparent in fetuses whose gestational age was estimated at 90 days, the numerical value of the difference and level of significance increasing with increasing gestational age. It was also found that the body weight of male fetuses was significantly greater than of female co-twins, however even on a kidney weight per unit body weight basis, the weights of male kidneys for the combined data (n=52) was highly significantly different ($P<0.001$). Thus male fetuses were heavier and had heavier kidneys both by direct comparison of weight, and per unit body weight.

5. Plasma collected from both entire and nephrectomised fetal sheep was analysed, and the pregnanediols in the plasma were identified. Identification was achieved by comparing the retention indicies of derivatised authentic pregnanediols with derivatised plasma extracts on GC using a SCOT column. Evidence of the identity was supported by comparison of GC

retention times after several chemical manipulations and also by comparison of GC-MS characteristics of authentic pregnanediols.

Three pregnanediols were present in fetal plasma, 5β-Pregnane-3β, 20β-diol, 5β-Pregnane-3β, 20α-diol and 5β-Pregnane-3α, 20α-diol. The plasma from both the entire and nephrectomised fetuses contained the same pregnanediols.

6. Fetuses of between 100-120 days gestational age were surgically implanted with arterial and venous cannulae, and either sham operated or bilaterally nephrectomised. Sham operated fetuses were born near term (147 days), whereas nephrectomised fetuses were born almost two weeks premature, at about 135 days of gestation. Plasma samples collected from the cannulae were assayed for pregnanediols. An assay was developed which allowed the level of the three pregnanediols present in the plasma to be assayed simultaneously in each sample. The level of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol in nephrectomised fetuses were from 2-10 times higher than those in sham operated fetuses, whereas the level of 5β-Pregnane-3α, 20α-diol was similar in both. No pattern in pregnanediol levels in nephrectomised fetuses was discernible in relation to gestational age, however in sham operated fetuses, levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol increased from 110 days until 3-4 days prior to parturition, at which time they fell precipitously. In sham operated fetuses, within 12 days of delivery the levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol increase more rapidly and their levels approach each other, 5β-Pregnane-3β, 20α-diol being at highest concentration. This convergence of levels also occurred in the nephrectomised fetuses within a similar time period in relation to delivery.

A concept of the contribution of the steroid hormone metabolising capacity of the fetal sheep kidney to fetal steroid endocrinology is presented.

A. Fetal kidney tissues have little or no de novo steroidogenic capacity, the steroid metabolising enzymes present e.g. 5α-reductase, 3α/3β-HSD, 17β-HSD, 20α-HSD and sulphatase activities probably being concerned with mediating the demonstrable renotrophic activity of steroids such as androgens.

B. No direct causal link between renal tissue steroid metabolism and the steroid endocrine disturbances described for nephrectomised fetuses can be established. The elevated levels of pregnanediols being compatible with the hypothesis that fetal stress associated with the nephrectomised condition leads to a premature activation of the pituitary - adrenal axis. The adrenal being implicated as a source of progesterone in the stressed fetus.

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CHAPTER 1

INTRODUCTION

In adult animals the kidney has a long established role as a secretory organ for body wastes, and as an organ involved in the maintenance of plasma electrolytes. However, recently it has been established that the adult kidney has an important role as an endocrine and para-endocrine organ. Evidence for this includes the kidneys involvement in Vitamin D_3 metabolism (Gray et al, 1972), erythropoietin production (Jacobson, 1957), renin production (Tobian, 1960), the clearance and degredation of insulin and glucagon (Duckworth, 1978), gluconeogenesis (Kaufman, 1971), the peripheral conversion of T_4-T_3 (Chopra, 1977), and the mediation of growth hormone (GH) activity (Wallace et al, 1970).

People maintained on chronic dialysis due to chronic renal disease or kidney loss, exhibit symptoms of a variety of metabolic disturbances which are compatible with the lack of these endocrine functions of the kidney (Ganda et al,1976, and references cited therein). In adults most of these metabolic disturbances can be alleviated by dialysis, which allows these patients to lead otherwise normal lives. However during times of rapid growth and development it is apparent that the endocrine functions of the kidney have greater importance. Young children with severe renal disease and anephric children maintained on chronic renal dialysis become growth retarded unless administered with 1,25-diOH-Vitamin D_3 (Chesney et al, 1978). This vitamin only gains its full biological activity after hydroxylation, which in mammals occurs only in the kidney (Gray et al, 1972). Studies on the anephric human fetus (Potter, 1965) and on the nephrectomised sheep fetus (Thorburn, 1974) demonstrate that in the fetus the lack of kidney function is also associated with growth retardation. Detailed studies on surgically nephrectomised fetal sheep (Thorburn

et al, 1971a,b) have shown that the levels of plasma electrolytes and urea are similar in both nephrectomised and sham operated fetuses. Thus the growth retardation of the nephrectomised fetus is not due to the loss of any waste secretory or plasma electrolyte maintaining function that the fetal kidneys may have, these functions being performed by the placenta. Rather, it seems that the placenta is unable to compensate for the lack of apparently endocrine functions of the fetal kidney.

Of particular interest in these fetal studies was the finding that lack of fetal kidneys was associated with premature delivery in both humans and sheep. Steroid hormones are intimately involved with the maintenance and termination of pregnancy (Csapo, 1969; Liggins et al, 1973); and Nancarrow (1969) and Ainsworth (1972) have shown that the fetal sheep kidney has the capacity to metabolise steroid hormones. These observations raise the question of whether the fetal sheep kidney has a steroid endocrine function, and whether the premature delivery of the nephrectomised fetus is due to an imbalance of steroid hormones caused by the lack of kidney metabolism. The studies of Nancarrow (1969) and Ainsworth (1972) provide insufficient information to give any indication of the role of the steroid metabolising enzymes present in the fetal kidney. Thus the studies reported in this thesis are concerned with the possible role of the fetal kidney in the occurrence and metabolism of steroid hormones; whether this constitutes a steroid endocrine function; and whether this is directly related to the premature parturition of the nephrectomised fetus.

The potential of the fetal kidney for metabolising a number of steroid hormones (C18, C19, C21) has been examined, and the presence of a number of enzymes has been demonstrated, the fetal kidney having a particularly active 5α-reductase.

The effect of fetal nephrectomy on the occurrence of progesterone metabolites in the fetal circulation was also examined and related to the capacity of the fetal kidney to metabolise steroid hormones. The study reveals that nephrectomy causes a gross disturbance in fetal steroid hormone metabolism, but the mechanism by which this occurs remains obscure.

REVIEW

Fetal Factors Involved in Progesterone Production

In many species of mammals progesterone has been established as an essential factor in the maintenance of pregnancy (for review see Bedford et al, 1972b). Progesterone has an anaesthetic action on myometrium, inhibiting the conduction of action potentials through the muscle thus lowering myometrial excitability (Csapo and Weist, 1969). This anaesthetic action of progesterone is a major factor in preventing contraction of the uterine muscle; premature activity of the myometrium resulting in rhythmic waves of contraction either expels the fetus, or has the potential to fatally crush it.

A more recent study has provided compelling evidence to suggest that progesterone has another pregnancy maintaining function; one which is concerned with masking the conceptus from the mothers immune system (Smith et al, 1974; Febres et al, 1978). The conceptus is a foreign body within the mother (having a different genetic complement than the mother), and invades the maternal tissue to establish a contact with the maternal circulation. This invasion of the maternal tissue by the developing trophoblast, and the presence of the conceptus throughout pregnancy has great potential for eliciting a response from the mothers immune system and being rejected by her. Thus progesterone may have two important modes of action in maintaining pregnancy.

The premature parturition of the nephrectomised sheep fetus (Thorburn et al, 1971b) indicates that those factors responsible for the maintenance of pregnancy have been jeopardised. This suggests the possibility that either directly or indirectly the fetal sheep kidney may be involved in the production of progesterone. It is not known whether the fetal sheep kidney is directly involved in producing progesterone and insufficient information is available to provide any indication of whether it is or not. However there is evidence to suggest that fetal factors may be involved in progesterone production and that indirectly the fetal kidney may influence this production.

During pregnancy, progesterone arises from two main sources, the ovaries and the placenta, with the ovaries playing a dominant role early in pregnancy, and the placenta assuming dominance during the latter stages. Ovariectomy of the pregnant ewe before about 50 days of gestation results in abortion (Casida and Warwick, 1945), however after this time abortion does not take place and pregnancy is maintained by placental production of progesterone (Linzell and Heap, 1968). In sheep the essential role that the placenta plays in the production of progesterone for the maintenance of pregnancy is well established, however those mechanisms which are responsible for increasing the production of progesterone by the placenta during gestation are not as clearly defined.

It can be anticipated that the larger the fetus grows, the greater its potential becomes for traumatising the uterus and for eliciting a response from the maternal immune system. Thus as both the fetus and uterus increase in size, increasing amounts of progesterone would be required to maintain the pregnancy. The increase in progesterone production found during gestation is probably the result of the fetus

being involved in directing the progesterone output of the placenta. Data to support this view is provided by the studies of Bassett and Thorburn (1973). These authors found that in normal pregnancies where the birth weight of the sheep fetus is over 3.6Kg there is a linear relationship between the maximal levels of progesterone reached in the maternal plasma and the birth weight of the fetus, the higher the birth weight, the higher the level of progesterone (correlation coefficient = 0.93). A similar relationship exists between placental weight and birth weight ($r > 0.8$) (Alexander, 1964a). Thus it could be rationalised that a large placenta would be capable of producing larger quantities of progesterone and facilitating the growth of a larger fetus. However there is evidence to suggest that during the growth of the placenta, its increasing progesterone producing capacity is not related to its increase in size (i.e. the mass of the placenta is not the only factor which determines its capacity for progesterone production).

Mattner and Thorburn (1971) have measured the progesterone production by the placenta, and found that at 100 days of gestation the production rate of single bearing ewes was about 4mg/day, rising to a maximum of about 33mg/day within 5 days of term. During the same time, from 100 days gestation through to term, the weight of the placenta does not increase, and in some cases actually decreases slightly (Cloete, 1939; Wallace, 1948) (see figure 1A). Thus, although the total mass of the placenta apparently places a limit on the maximum rate of placental progesterone production, weight is not the factor per se which determines the rate, as the total weight remains static while the rate of placental progesterone production increases over 8 fold.

Alexander (1964a) has described morphological changes in the placenta that occur with advancing gestation which are better related to the

production of progesterone. In sheep the placenta exists as discrete entities known as placentomes which are found evenly spaced on the dorsal and ventral surfaces of the pregnant uterine horn. The position of these are determined by some 60 to 150 special endometrial thickenings on the uterus known as caruncles. The cotyledons are composed of both maternal and fetal tissue, and during gestation the amount of fetal tissue in the cotyledons (placenta), changes from a small proportion early in pregnancy to a much greater proportion near the end of gestation. During the period from 100 days to term when placental weight is static, the amount of fetal tissue within the cotyledon increases markedly, as does the vascularity of the placenta (Alexander, 1964a) (see figure 1, B and C). Thus the weight of the placenta is a poor index of its functional capacity for progesterone production, which is apparently directly related to the proportion of fetal tissue within the cotyledon.

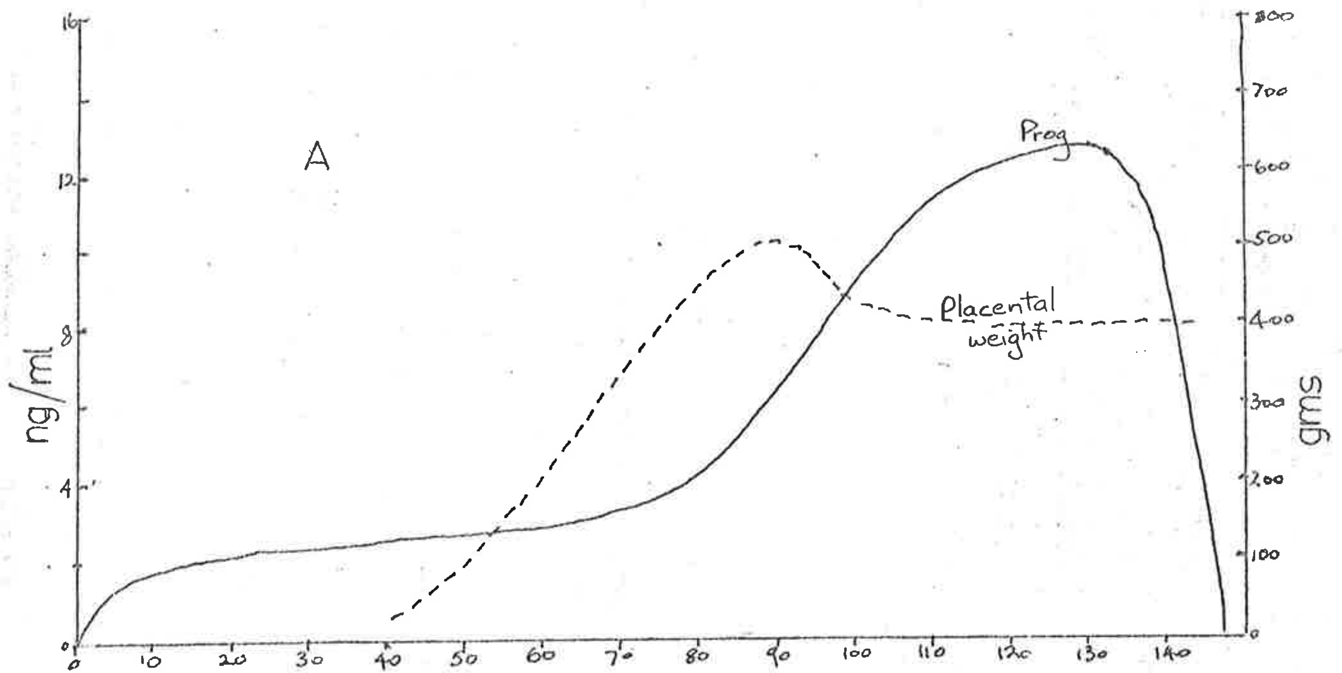
Further observations of Alexander's support this relationship between increased growth of fetal tissue within the cotyledon and increased progesterone production. Alexander (1974) found that on average for single fetuses 70% of the possible uterine sites of cotyledon formation (caruncles) are occupied, 80% in twins, the individual twin thus being severely disadvantaged. However, the total weight of twin placentas is 30% greater than singles, indicating a compensatory increase in growth. This compensatory increase in placental weight does not occur in the maternal portion of the placenta, but is almost entirely due to an increase in the fetal tissue. Thus in fetal twins, where the combined birth weight is high, the large compensatory growth of fetal tissue within the placenta is associated with very high progesterone levels.

FIGURE 1

A. Placental weight and plasma progesterone levels in pregnant sheep as a function of gestational age, progesterone in ng/ml, placenta weight in gm (redrawn from Bedford et al, 1972b).

B. An arbitrary devision of cotyledon cross-sectional appearances as given by Alexander and Williams (1966), who found that types 5,6,7 only occur during the latter stages of normal pregnancies in sheep.

C. Cross sections through cotyledons of the extreme types (taken from Alexander, 1974). The type which appears only during late gestation (7) has cross-sectional appearance similar to that of bovine placenta.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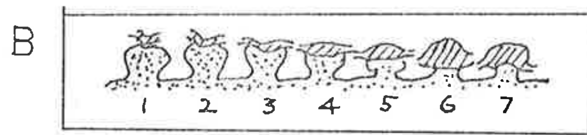
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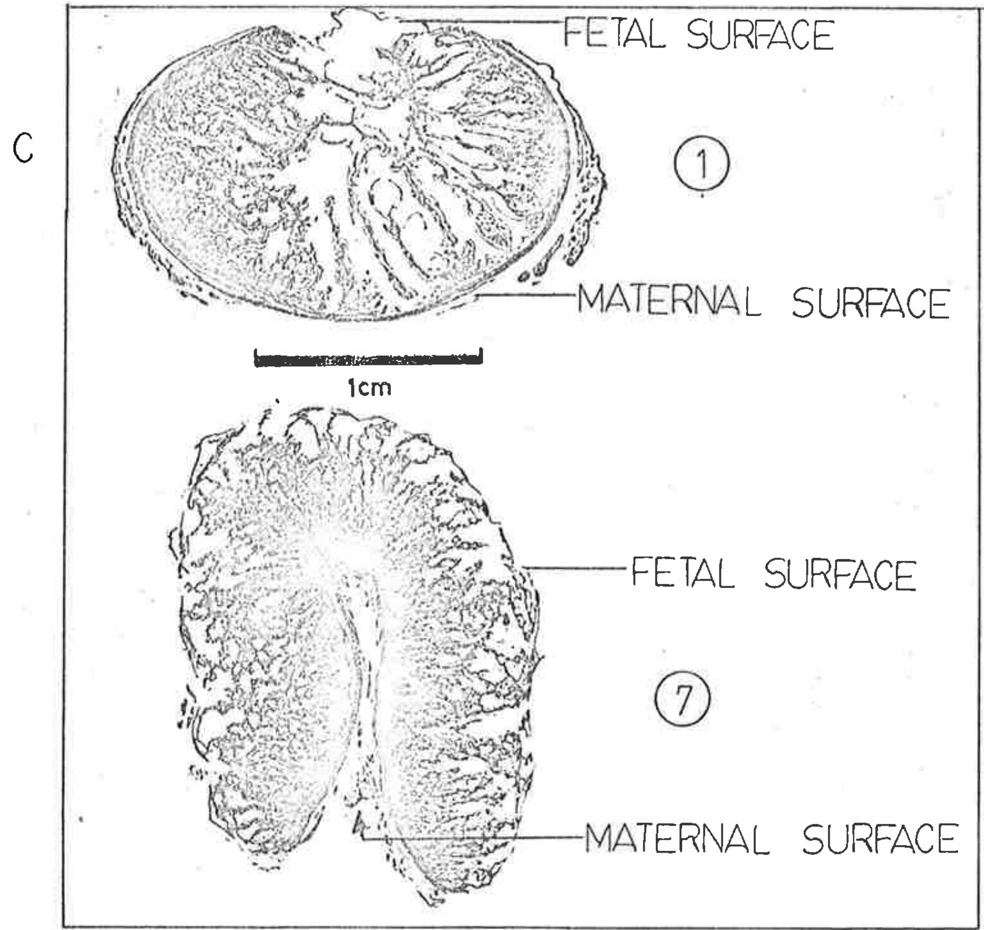
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As this fetal tissue grows, and invades the maternal caruncular tissue within the placentomes, the total surface area of the fetal villi would increase markedly. This would improve the transfer of nutrients to, and wastes from the fetal circulation, thereby facilitating the growth of the fetus, but would also greatly increase the total surface area of fetal tissue that was in close apposition to maternal tissue. If as suggested by Smith et al (1974), progesterone binds to the external villous surface of the placenta to mask it from the maternal immune system, then as the villous surface area increases, greater rates of progesterone production would be required to maintain this effect. Thus the growth of the fetal tissue within the placenta and increased progesterone production should occur concomittantly, in order to maintain the masking action of progesterone. In humans the trophoblast produces progesterone (see Febres et al, 1978), and evidence that has been presented is consistent with this also occuring in sheep (i.e. fetal tissue being responsible for progesterone production).

Bedford et al (1972a) have reported that the almost five fold increase in progesterone production normally seen between 60 and 140 days gestation does not occur in fetuses of below normal weight. This can be interpreted as the result of a lack of efficacy of fetal growth factors which blights the development of fetal tissue within the placenta, which also blights the growth of the fetus, hence low progesterone production in association with low fetal weight. Alternatively it could be interpreted as indicating a lack of maternal factors which normally stimulate the growth of the placenta, and because the placenta does not develop to sufficient size, its progesterone production is limited, and the fetus is deprived of an adequate supply of nutrients and therefore does not reach an optimum size. Basically these two alternatives are (1) that the fetus is to some

degree actively involved in influencing its own growth, or (2) that maternal factors determine the size of the placenta, and that fetal growth is purely passive, merely responding to the amount of nutrients made available to it.

There is no doubt that the maternal environment plays an important role in determining the size of the fetus, with such factors as breed, size, parity, age and nutrition of the mother influencing the birth weight of the fetus (Starke et al, 1958), most probably through influencing the size of the placenta. Similar factors are involved in influencing the size of human fetuses (Butler, 1974). In sheep,growth retarded fetal lambs have also been produced experimentally by surgical reduction of the number of caruncles and hence decreased placental weight (Alexander, 1964b). Thus the growth of the fetus is very much dependent on an adequate supply of nutrients and oxygen by the placenta, particularly during the third trimester, to enable it to sustain its incredibly fast rate of growth. However given a set maternal environment there is evidence to suggest that the fetus is actively involved in influencing its growth by directing the placenta to provide it with optimum nutrition.

Thorburn (1974) reported that in sets of twins, where one twin was bilaterally nephrectomised at 85 days of gestation, that the nephrectomised twin was invariably growth retarded and its placenta was also much smaller than that of the sham operated co-twin. Although care should be taken in using twins for comparisons (Driscoll,1964) it could be validly assumed that the maternal environment the co-twins experience would be similar. Thus the reduced placental mass of the nephrectomised twin provides compelling evidence that fetal factors are involved in the growth of the placenta and possibly the fetus, and that this involves the fetal kidney. How might the fetal kidney be involved?

Wallace et al (1972) found that the MCR of radioiodinated GH in adult ewes was decreased by 58% after nephrectomy, and Thorburn et al (1971a) have reported that GH levels in the nephrectomised twins were higher than in the sham operated cotwins (212ng/ml versus 87ng/ml). This suggests that the fetal kidney is also an important site of metabolism of GH. In the adult sheep this metabolism of GH is not catabolic, as in nephrectomised adult sheep the normal lipolytic action of GH is not seen, and in entire adult sheep ligation of the renal pedicle immediately prior to injection of GH abolishes the lipolytic action of GH (Wallace et al, 1970). Thus the metabolism of GH by the kidney may lead to the formation of an active complex, possibly somatomedin (see Blackard and Andrews page 139, 1974). Somatomedin is generally accepted as the mediator of GH responsive growth (Daughaday, 1971) and the kidney is strongly implicated as its source. Thus the possibility exists that nephrectomised sheep fetuses have high levels of GH due to a lack of GH's major site of metabolism (hence reduced clearance), and that this metabolism is essential for GH to exert an action in promoting growth, mediated by somatomedin. The human placenta has an abundance of high affinity binding sites for somatomedin (Takano et al, 1975) suggesting that somatomedin has a growth promoting action on the human placenta. It is not known whether somatomedin binding sites also exist in the sheep placenta, however this evidence is completely consistent with the view that the sheep placenta (in particular the fetal tissue) is capable of responding to fetal growth factors produced by the kidney.

Thus a model can be envisaged whereby GH produced by the fetus is metabolised by the fetal kidney to an active growth promoting form and that this (among other possible actions e.g. on bone) exerts a growth promoting action on the fetal tissue within the placenta. An action which becomes particularly important in aiding the maintenance of

supply of nutrients to the rapidly growing fetus during the third trimester, and which therefore also indirectly influences the placenta's capacity to produce progesterone.

Thus the above information available in the literature provides a compelling inference that the fetal kidney (via a rather complicated mechanism) is capable of influencing the growth of the placenta, placental progesterone production, and the growth of the fetus.

Progesterone Metabolism by the Fetus

Increased progesterone production whilst essential for the maintenance of pregnancy, results in the fetus being exposed to large amounts of progesterone, which it must metabolise. In humans where placental progesterone production reaches almost 300mg/day, about 75mg/day enters the fetal circulation. In sheep the rate of progesterone production is much lower, the maximum rate being about 56mg/day for a ewe bearing a single fetus. In fetal sheep the plasma level of progesterone has been reported at <2ng/ml (Seamark, 1971; Strott et al, 1974b) which is much less than maternal plasma levels during the latter half of gestation (Bassett et al, 1969), and the MCR of progesterone from the fetal circulation is about five times slower than the maternal circulation (Stupnicki et al, 1970b), indicating that only a portion of the placental progesterone production enters the fetal circulation. However even at about 10mg/day this represents a large influx of progesterone into the fetal circulation which must be metabolised to reduce its biological potenty.

Nancarrow (1969) found that the most striking feature of the metabolism of progesterone by the sheep fetus was its conversion to 20α-OH-preg-4ene-3one by a very active enzyme, 20α-HSD found in fetal red blood cells. This 20α-HSD is able to convert $1.2\mu g$ of prog/ml/min,

effectively reducing the level of progesterone immediately it enters the fetal circulation. The 20α-HSD present in fetal blood catalyses a reversible reaction, but favours reduction of progesterone, (unlike the 20α-HSD present in human placenta) and maintains a ratio of 20α-OH-pregnene-3one: progesterone of about 6:1, the activity of the enzyme being dependent on the rate of metabolism of progesterone by fetal tissues.

Nancarrow (1969) found that the fetal liver, testis, adrenal and kidney are all capable of metabolising progesterone, and that the fetal liver is a major source of progesterone metabolism. It seems that all tissues have 20α-HSD activity, but this may be due to contamination with fetal blood. The fetal sheep liver also has 5β-reductase and 3β-HSD and 20β-HSD (Anderson et al,1970). In the human fetal liver 20α-HSD is the major reducing enzyme for the 20-Ketone group, whereas in sheep fetal liver the 20β enzyme predominates, indicating that a different pattern of 5β-reduced metabolites of progesterone is likely to be found in sheep blood. Reduction of steroids to 5β-reduced metabolites is irreversible and completely abolishes their physiological activity. The 5β-metabolites of progesterone that are formed in the liver, cross the placenta and are removed from the maternal circulation by the kidney.

Nancarrow (1969) has found that fetal sheep kidneys have 5α-reductase, 3β-HSD and 20α-HSD activity, as indicated by the major metabolites formed by in vitro incubation of progesterone. The presence of 20α-HSD may allow for the uptake of progesterone from circulating 20α-OH progesterone. The presence of 5α-reductase is of particular interest, as this irreversible reaction is not usually associated with degradative metabolism, but renders some steroids more effective at the cellular level. This has been shown in the chick oviduct where

5α-pregnane-3, 20-dione is equally as effective as progesterone in stimulating specific protein synthesis (Strott,1974a), and the 5α-reduction of testosterone is thought to be an essential step in its action on the rat ventral prostate (Bruchovsky and Wilson,1968). The physiological role of this 5α-reductase in the fetal kidney is not known, but its presence suggests that progesterone has an action on the fetal kidney. Regardless of the action of progesterone on the kidney, the level of the metabolism of progesterone by the kidney, is most likely to be small in comparison to the major site of metabolism in the fetal liver. The contribution of the kidney would be limited by its very much smaller blood flow in relation to the flow through the fetal liver, which receives blood from the umbilical and portal veins and also from the fetal heart (Abraham et al, 1967a,b).

Steroid Hormones and the Fetal Kidney

The influence of androgen and oestrogens on the development of the fetus or neonate is not limited to reproductive functions, but also affects organs which are not morphologically, but are biochemically differentiated, such as the liver and kidney. These biochemical changes which take place under the influence of sex hormones accentuate the hormonal differences between sexes and are important for normal development of the animal of a particular sex. (Ghraf et al,1975; Kitay et al,1973).

The influence that androgens have on the kidney has been known for a long time. Selye (1939a) found in mice, that androgens enlarge the kidney in both normal and castrate animals of both sexes, and gave a detailed histological description of the changes induced by androgens. These changes take place mainly in Bowman's capsule and the proximal renal tubule, the epithelium in these areas being squamous in females and cuboidal in males, thus accounting for the increase in weight.

These sex differences disappear after castration, and are restored to the male type by the administration of testosterone. Kochakian (1977) has recently reviewed the range of effects that androgens have on the growth and development of the kidney.

In rodents this action of testosterone on the development of the kidney takes place during the neonatal period (see Kochakian, 1977), but rodents are born at a very immature stage of development. In contrast the fetal sheep is born at a far more mature stage of development, (being able to stand, walk and suckle within minutes of birth) and sexual differentiation is far advanced. It is known that this sexual differentiation takes place under the influence of androgens secreted by the fetal testis. The fetal sheep testes actively secrete testosterone at an early stage of pregnancy, (Attal, 1969) and it seems likely that these androgens which cause sexual differentiation of the fetus could also have a trophic effect upon the fetal kidney.

Considering the importance of the fetal kidney to the normal growth of the fetus, the possibility exists that trophic affects of androgens on the fetal kidney may influence the growth of the kidney, and thus indirectly the growth of the fetus. Starke et al (1958) have shown that male lambs at birth tend to be about 0.2Kg heavier than females, and it has been shown that male twins appear to have a competitive advantage in utero over female co-twins (Donald and Purser,1956). Unfortunately no information is available about the relative sizes of male and female fetal kidneys, or whether their enzyme activities are different. These things are examined in this thesis as part of the aim in examining the steroid hormone metabolising capacity of the fetal sheep kidney.

CHAPTER 11

GENERAL MATERIALS

1. Chemicals

All reagents and solvents were of analytical grade. Solvents were redistilled before use and stored in dark bottles. Solvents were purchased from Ajax Chemical Limited Australia (UNIVAR, ANALAR); Merck (Germany); or May and Ba ker (England).

NADH and NADPH as sodium salts were obtained from Boehringer (Manheim), Germany. Tris HCL (7-9) from Sigma; 2-mercaptoethanol from Koch-Light Laboratories Limited, England.
Crystaline iodine (AJAX); Lipidex 5000^R from Packard Instrument Co. U.S.A.; silica gel G Type 60 (Merck), acetic anhydride.

2. Steroids

Unlabelled steroids were obtained from Steraloids, Pawlings USA.

Radioactive steroids were obtained from The Radiochemical Centre, Amersham, England, all were (4 - ^{14}C) labelled.
The following radioactively labelled steroids were used

oestrone	53m Ci/mM
oestrone - SO_4	50-60m Ci/mM
DHEA	58m Ci/mM
androstenedione	53m Ci/mM
progesterone	54m Ci/mM
pregnenolone	52m Ci/mM

3. Surgery

Portex polyvinyl tubing (0.63mm ID-1.4mm OD) for cannulae was obtained from Boots Company (Aust) Pty. Ltd. Suture material, Ethicon from Ethnon Pty. Ltd., Sydney, Australia, and Dexon sutures from Davis and Geck, American Cyanamid Company, NY 10965, were used. Chlorhexidine solution

(chlorhexidine, 0.05%, cetrimide, 0.5%, sodium nitrite, 0.4% in 70% ethanol) and hibitane was obtained from ICI Australia. Benyl Penicillin from, Weddel Pharmeceuticals, plastic three way stop cocks, plastic headed luer lock syringe needles from Smith and Nephew Hypodermics Pty. Ltd. Victoria, and syringes from Terumo and Pharmaplast. Astra Chemical Pty. Ltd., N.S.W., Aust., supplied Lignocaine (Xylocaine 2%). Rompun (2% Xylazine) from Bayer Aust. Ltd.; Heparin (mucous) was obtained from Allen and Hanbury, Victoria, Aust.; Fibrinolysin was prepared as a sterile powder by the Pharmacy at the Queen Elizabeth Hospital.

4. Gas Chromatography, Gas Chromatograph-Mass Spectrometry (GC, GC-MS)

t-BDMS tertiary-butyldimethysilyl chloride)
) PIERCE
BSTFA)

Methoxyamine HCL)
) from Eastman, Rochester, USA
Butoxy amine HCL)

Pyridine)
) Merck
Acetonitride)

HMDS hexamethyldisilazane and

N24 n-tetracosane (2400 index units)
and

N32 n-dotriacontane (3200 index units)

from Applied Science Laboratories Inc., State College, Penna 16801

OV101
and
both from Applied Science Laboratories
NGA

5. Buffers

All buffers and aqueous solutions were prepared in glass distilled deionized water.

Buffer A

0.01M TRIS (7-9)

0.001M EDTA
0.05M NaCl
5.0 x 10^{-4}M 2-mercaptoethanol
5.0 x 10^{-3}M $MgCl_2$
pH 7.3

Buffer B

is buffer A made with an additional chemical
0.25 M Sucrose

pH 7.3

Normal Saline NaCl (0.9gm%)

6. Scintillation Fluid

The fluors, 2,5-diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) were purchased from Packard Instrument Co., Inc. USA. Toluene for scintillation fluid only was a commercial grade obtained from Shell Chemicals Aust. Pty. Ltd., and was redistilled before use. The scintillation fluid contained 3g PPO and 0.5g POPOP per litre of toluene. Unless otherwise stated all radioactive samples were counted in 10ml of toluene scintillation fluid and counted in an Nuclear Chicago isocap/3000 for 10 min or 10,000 counts.

CHAPTER III

STEROID METABOLISING ENZYME ACTIVITY IN FETAL SHEEP KIDNEYS

111.a. Introduction

The level of a particular hormone in the circulation is dependent on the relative rates of production and clearance, thus the fetal sheep kidney may influence the level of biologically active steroid hormones by being involved in either their production or metabolism. Nancarrow (1969) has found that fetal sheep kidneys have 5α-reductase, 3β-HSD and 20α-HSD activity, as indicated by the major metabolites formed by the in vitro incubation of progesterone, and Ainsworth (1972) has shown the presence of sulphatase activity. Both these studies were concerned with fetal metabolism of a particular steroid by a number of fetal organs, the kidney being only a small part of the study. Thus these studies while providing evidence of steroid metabolising enzymes within the fetal sheep kidney give little indication of their function, which may be associated with steroid synthesis, degradative metabolism, para-endocrine activity, or with the action of steroids on the fetal kidney.

There are apparently no reports in the literature of the kidneys in any species being capable of de novo steroid synthesis, suggesting that this may be an unlikely function of the fetal sheep kidney. Ghraf et al (1975) have found that the rat kidney has numerous enzymes similar to those of the liver, and suggest that in rats the kidney has an important catabolic function in relation to steroid hormones. The sulphatase activity of the fetal sheep kidney may be associated with a para-endocrine activity, or may be associated with the action of steroids on the kidney. The kidneys of many species of mammals respond to sex steroids, in particular androgens (Kochakian, 1977 review), however insufficient data is available to provide any clear indication of the role of steroid metabolism in the fetal sheep kidney.

The aim of experiments reported in this chapter was to determine the capacity of the fetal sheep kidney to metabolise a range of steroid hormones. It was hoped that by obtaining a more comprehensive assessment of the steroid metabolising enzymes of the fetal sheep kidney that their function would be apparent. The capacity of the fetal kidney to metabolise oestrogens, androgens, and progestagens was examined.

111.b. Methods

(i) Tissue Preparation

Fetal sheep kidneys from freshly slaughtered pregnant ewes were obtained from the local abattoir, SAMCOR. The uterus was excised from the pregnant ewe within 12 min of exsanguination, and the fetus removed from the uterus, at which time its heart was still beating. The crown-rump length, weight and sex of the fetus was recorded, and the gestational age was estimated later using Cloetes' nomogram (1939). The fetal kidneys were removed, placed in a small insulated container, covered with ice and transported to the laboratory. All further steps in the preparation of the homogenate were carried out at 4°C.

The kidneys were weighed, finely chopped and rinsed in ice cold normal saline to remove any clots or excess blood. The chopped pieces were then weighed into 2g portions and added to 10ml of buffer A. The tissue was completely homogenised in a thick walled glass homogeniser with a motor driven teflon pestle. The pestle was driven at about 500r.p.m. and 5-7 strokes were made to obtain an even homogenate. The homogenate was then filtered through 8 layers of medical gauze, collected in a 30ml test tube and kept on ice. The total time taken from the moment the pregnant ewe was killed to the begining of the incubation period was never greater than 2 h.

(ii) Incubation and Extraction Procedure

All steroids were incubated with a 2ml aliquot of freshly prepared homogenate. Radioactively labelled steroid was freshly prepared from a stock solution prior to each incubation, and its purity confirmed by TLC. The stock solution was of benzene: ethanol, 9:1. This solvent was dried under a stream of N_2 at 40^oC. If unlabelled steroid was added to the incubation, this was also dried down with the radioactive material, and both were redissolved in acetone. Only 30μl of acetone was added to each 2ml of homogenate, giving 10^5 c.p.m. per tube with or without unlabelled steroid. After adding the steroid, the homogenate was briefly vortexed and then heated in a thermostatically controlled shaking water bath for one hour at 39°.

The samples were quickly frozen at -70^oC and either stored at -4^oC or thawed and extracted with ethyl acetate. For extraction 0.5ml of saturated NaCl solution was added to the homogenate to reduce emulsions forming, and the homogenate was extracted twice with 2 ml of freshly re-distilled ethyl acetate. The extracts were pooled, blown to dryness under N_2 and redissolved in 100μl of methanol, which was transferred with a fine glass capillary onto the surface of a thin layer chromatographic plate.

(iii) Thin Layer Chromatography (TLC)

Glass plates (5 x 20cms) were coated with a slurry of silica gel G (60g in 110ml water; E. Merck AG Darmstadt) using a Quickfit applicator to give a coating 0.25mm thick. The plates were allowed to dry in air, and were pre-run in system 1 prior to use.

Development was carried out in the following systems at room temperature ($15-25^oC$)

System 1	Chloroform: acetone (9:1)
2	Benzene: ethyl acetate (3:1)
3	Benzene: acetone (4:1)

System 4	Cyclohexane: ethyl acetate (1:1) - Lisboa et al (1962)
5	Chloroform: methanol (9:1)
6	n-hexane: ethyl acetate (3:1) - Lisboa (1964)
7	Cyclohexane: ethyl acetate (3:1)
8	Chloroform: acetone (2:1)

Saturation of the systems was achieved by lining the chambers with Whatmann chromatography paper. The role of solvent vapour in TLC is important (De Zeeuw, 1968) as it has a large bearing on the separation of the compounds. The samples and reference standards were applied to plates 2.5cm from one end of the plate, and a line drawn through the silica gel 2.5cm from the other end of the plate delineated the solvent front. This gave a distance of 15cm over which the plates were developed.

The plates were air dried following development, and all traces of solvent were removed before radioactivity on the plate was detected with a Packard Model 7200 radiochromatogram scanner. Areas of the chromatogram carrying radioactive steroid were eluted with 5-10ml of methanol. After removal of the solvent, the eluted metabolites were either rerun in a different solvent system for further characterisation and/or separation, or used for recrystallisation, or derivatised for analysis by GC-MS.

After the radioactivity had been eluted the position of the reference marker steroids was determined by reaction with iodine vapour or sulphuric acid/vanillan spray.

(iv) Derivatisation to Tertiary - Butyldimethyl silyl ethers (t-BDMS)

Identification of some steroids was aided by analysis of mass-fragmentography patterns of tertiary - butyldimethylsilyl ether derivatives of the unknown

steroids. The metabolites eluted from the silica gel plates, were blown to dryness in screw cap culture tubes. BDMS imidazole complex (50μl) was added to the steroid, the tubes were capped, and heated at 60°C for one hour. The tubes were then cooled and 0.5ml of solvent (heptane: ethyl acetate, 3:1) was added and vortexed. LH-20 columns (2ml volume) were prepared in the same solvent, and the 0.5ml containing the t-BDMS derivative was allowed to run to the top of the column, whilst collecting the effluent. One ml of solvent was added to the reaction tube vortexed and added to the column, and the column allowed to run dry. The total eluent was then puffed to dryness under N_2 and reconstituted in solvent (50-200μl) ready for injection onto the GC-MS. The LH-20 in the column was discarded after a single use.

(v) Gas Chromatography - Mass Spectrometry (GC-MS)

The analytical system consisted of a PYE 104 gas chromatograph (Cambridge, England) interfaced via a single stage membrane separator to an AEI, MS-30 double beam mass spectrometer equipped with an AEI, DS-50 system (AEI, Manchester England). The mass spectrometer was operated under the following conditions: resolution 1000 accelerating voltage 4Kv; ionizing current, 100μA; electron energy 25eV; ion source temperature, 200°; and the molecular separator at 240°.

Several different glass columns were used in the GC. Columns were: glass capillary column, SCOT column (20m x 0.5mm) coated with OV-101 and used with a splitless injection system (all from SGE, Australia); glass coil (1.5m x 2mmID) packed with 1.0% SE-30 on Gas Chrom Q^{R} (100/120 mesh) or with 1.0% OV-101 on the same support, or with 1.1% OV-225: SILAR (10:1) on a SUPELCO^{R} (100/120 mesh) support. The carrier gas flow (N_2) was 30-40mls/min for the packed column, and 16-20 cms/sec was the average linear gas flow for the SCOT column. Oven temperature for the SCOT column was 210°-250° programmed at 1°/min and held at 250 until the

run finished, and for the other columns was 235°C.

A trace of material coming off the GC was visualised by instructing the computer to plot total ion current versus time, and display this on the visual display unit (VDU), from which paper copies were made with a TEKTRONIX hard copier. Peaks were selected from the trace and the relative intensities of ions within the peaks displayed, copied, and compared against standards to determine the most probably identity of the compound.

(vi) Recrystallisation

Recrystallisation to constant specific activity with authentic carrier steroid was taken as final proof of the identity of radioactive metabolites. Each sample to be recrystallised was eluted from the silica gel into a 15ml tapered glass tube (fitted with a ground glass stopper - Quickfit) and the solvent evaporated under N_2. Between 10-30mg of authentic steroid was weighed on an August-Sauter (Germany) torsion balance, to the nearest 0.01mg and then transferred to the tube and dissolved in the solvent or solvents of choice.

A minimal amount of hot solvent was used so that the steroid was only just soluble at the raised temperature, the tube was then stoppered and left undisturbed at room temperature for at least 24 hours, to allow the crystals to form. Once the crystals were formed the mother liquor (ML) was removed and the crystals rinsed with cold solvent, or the solvent in which the steroid was less soluble, if a solvent mixture was used. The ML and rinsings were evaporated and placed in the vacuum dessicator, but only a small portion of the total amount of crystals were used each time.

Once dried the ML and crystals were then weighed on a Beckman Microbalance LM-600 and then assayed for radioactivity following solubilization in

toluene scintillation fluid (10ml). The specific activity was determined and expressed as cpm/mg. Recrystallisation was repeated until the values for the specific activity of the last 3 sets of crystals were within a 5% deviation of their mean value (Axelrod et al., 1965)

111.c. Results

Fetal kidneys were obtained from fetuses whose estimated gestational ages (Cloete,1939) ranged from 97 to 142 days. Homogenates were prepared and incubated separately with one of the following 4 - ^{14}C labelled steroids, oestrone, androstenedione, DHEA, progesterone and pregnenolone. All steroids were incubated with homogenates from at least six fetuses, including different sexes and gestational ages. The identification of a particular metabolite was taken as evidence of the presence of the enzyme capable of its formation e.g. 3β-hydroxy group evidence of 3β-HSD.

111.c. (i) Identification of Oestrone Metabolites from Incubation with Fetal Kidney Homogenates.

Fetal kidneys from a female fetus (2.2kg) of about 121 days gestational age were homogenised and incubated with 10^5c.p.m. (23.2 n g) of ^{14}C oestrone. After TLC in System 1 the scan of radioactivity revealed that oestrone was metabolised to what appeared as a single peak in this chromatographic system. This metabolite peak was eluted and chromatographed in several different systems with oestradiol-17α/β as reference markers (see figure 2). In all the solvent systems used the metabolite ran as a single peak, and its chromatographic mobility (Rf) was the same as oestradiol-17α/β. In all TLC solvent systems oestradiol-17α and 17β did not separate, indicating that TLC would not provide an indication of the spatial arrangement of the hyroxyl groups of the metabolite (assuming it was oestradiol). Thus further identification of this radioactive

FIGURE 2

Oestrone Metabolism

TRACE 1

Scan of radioactivity after TLC of the extract from the incubation of ^{14}C oestrone with fetal sheep kidney homogenate.

TRACE 2,3,4

Repeated chromatography of the polar metabolite.

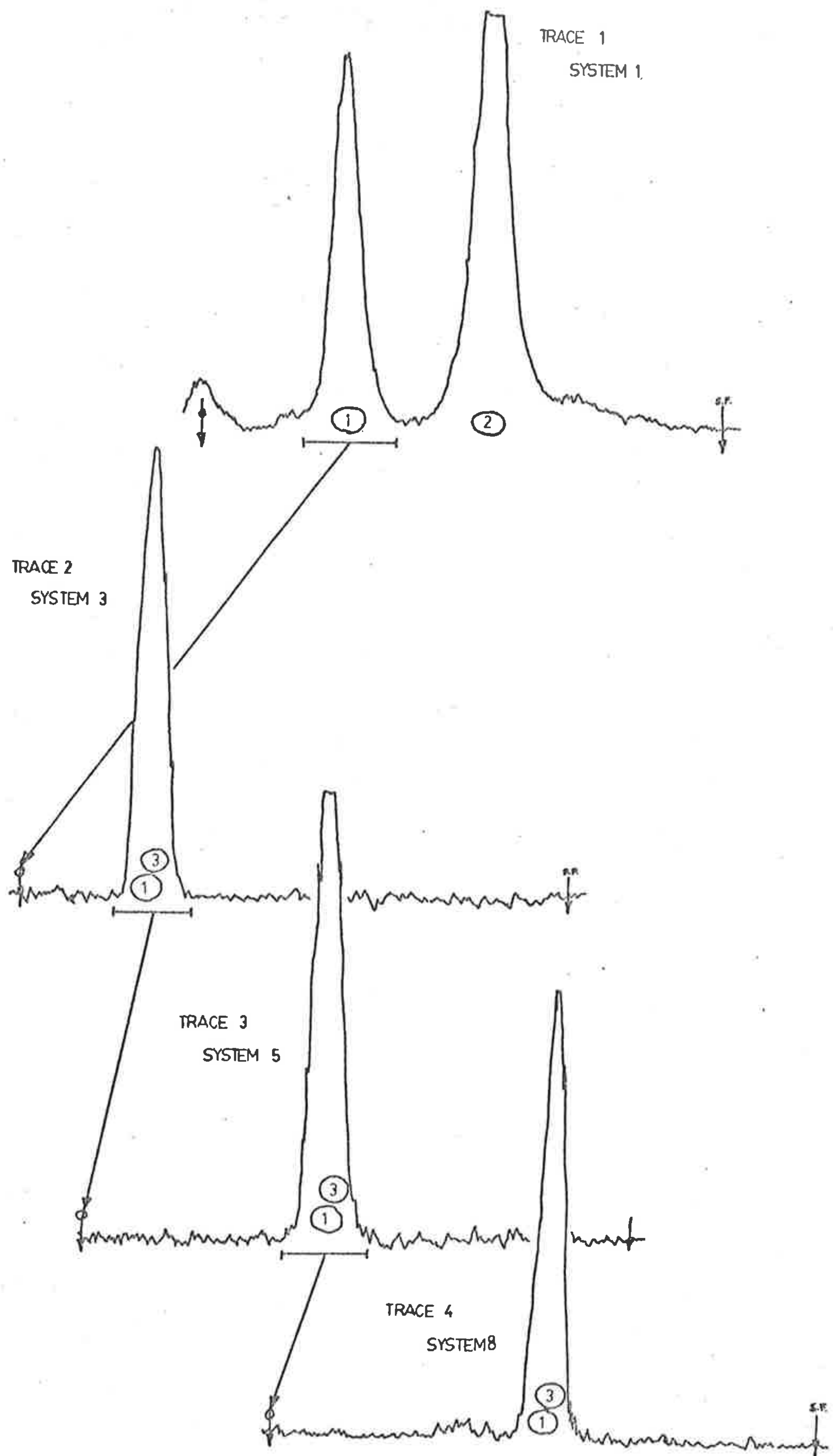
STANDARDS

The circles on the traces indicate the position of reference steroids.

1 = oestradiol-17β

2 = oestrone

3 = oestradiol-17α


TRACE 1
SYSTEM 1
1
2
S.F.
TRACE 2
SYSTEM 3
3
1
TRACE 3
SYSTEM 5
3
1
TRACE 4
SYSTEM 8
3
1
S.F.

metabolite was discontinued in favour of incubating sufficient unlabelled material to allow any metabolites formed to be characterised by GC-MS.

An homogenate was prepared with kidneys from a fetus of about 125 days gestational age. A 20ml incubation volume containing ^{14}C- oestrone, unlabelled oestrone (40μgm), and NADPH (0.2mM), was incubated and extracted as previously described. Five replicates were made, and after the extracts were redissolved in methanol, they were combined, loaded on the origin of a TLC plate (1mm thick), and developed in System 1.

No marker steroids were run, and after scanning the plate for radio-activity, two peaks of a similar Rf to previous incubation were obtained. The peak closest to the origin (the metabolite), was eluted, dried down under N_2, derivatised to a t-BDMS ether and run on GC-MS to obtain its retention time on the GC column and its mass spectrum. This information was compared with authentic oestradiol-17α and -17β which were also derivatised and run on GC-MS by the method previously described.

The computer drawn GC trace of the derivatised metabolite of oestrone showed that only a single major compound was present, any others being estimated at less than 1% of the major peak and indistinguishable from the background. The metabolite had the same retention time as oestradiol-17β, whereas oestradiol-17α came off the GC column approx-imately 1.5 min sooner (see figure 3).

The mass intensity scans from steric isomers such as oestradiol-17α and -17β are very similar, as they both have the same molecular weight, however the spatial arrangement of the t-BDMS derivative at the 17 position influences the relative abundance of various smaller ions due to hinderance or facilitation of the way in which the molecule fragments. Analysis of the scans of ion intensity versus mass, reveal that oestradiol-17α lacks

FIGURE 3

GAS-CHROMATOGRAPHY OF OESTRADIOL

Computer (DS50 system) drawn GC traces from an SE-30 column as constructed from total ion current detected by MS.

A two minute delay precedes the time (minutes) indicated on the top border. The standard for oestradiol-17α-t-BDMS, runs at 34 units and oestradiol-17β-t-BDMS, runs at 45 units (upper trace).

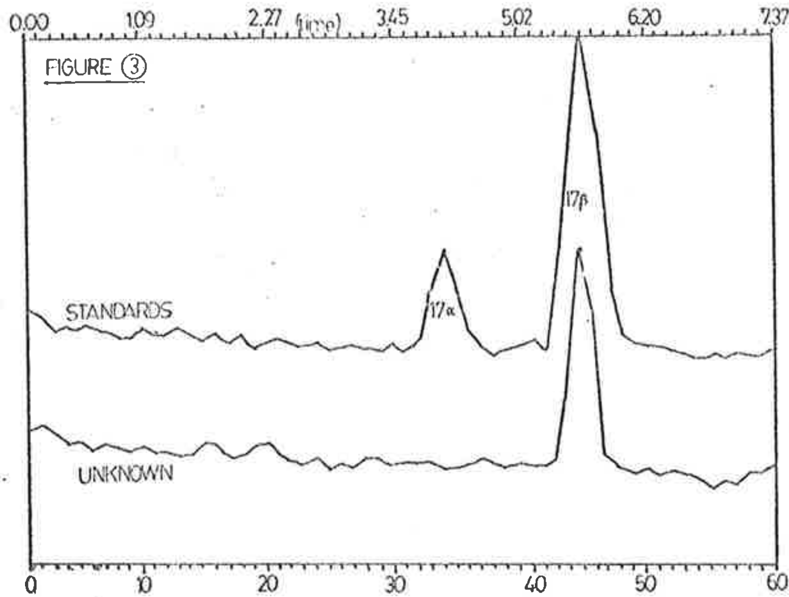
The lower trace is the unknown metabolite which runs at 45 units indicating that only oestradiol-17β is present.

FIGURE 4

OESTRADIOL MASS SPECTRA

Mass spectral traces. Ion intensity versus ion weight.

The spectra for oestradiol-17β and the unknown metabolite are similar, both contain ions at M/e 248 and M/e 328. These ions are absent in the oestradiol-17α spectrum.


0.00
1.09
2.27
3.45
5.02
6.20
7.37
FIGURE (3)
17β
17α
STANDARDS
UNKNOWN
0
10
20
30
40
50
60

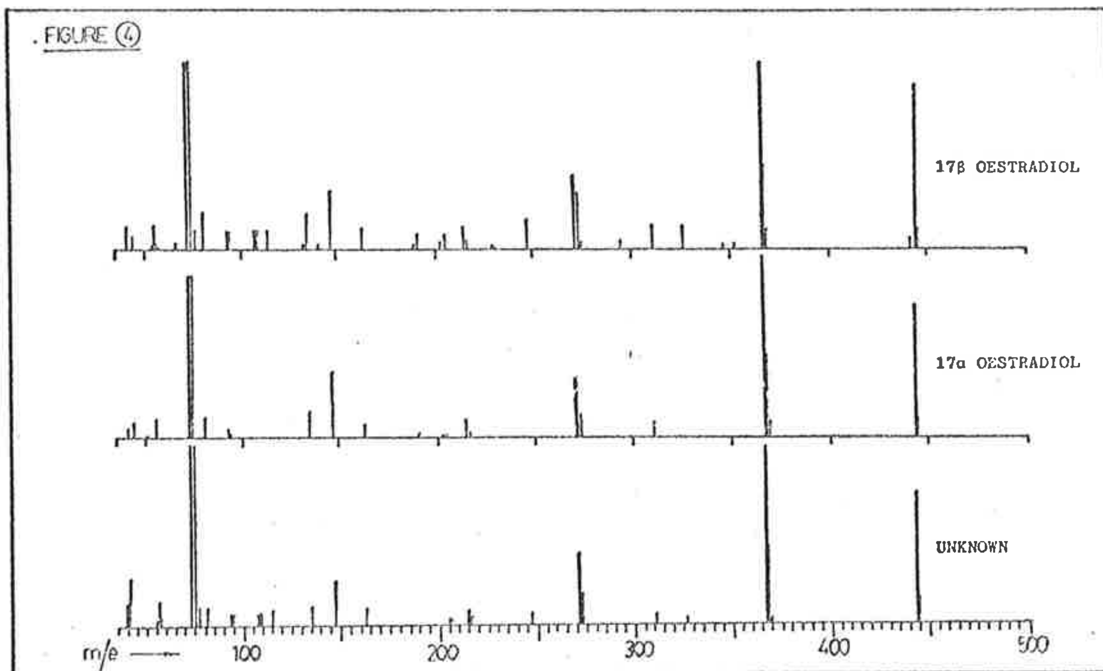


FIGURE (4)
17β OESTRADIOL
17α OESTRADIOL
UNKNOWN
m/e
100
200
300
400
500

two peaks at ion mass values of 248 and 328. Oestradiol-17β and the oestrone metabolite both have these ions, and their scans are essentially identical (see figure 4). Thus the fragment mass spectrum, and the GC retention time both indicate that the metabolite of oestrone is oestradiol-17β.

As final proof of the identity of the metabolite another incubation was performed with radioactive oestrone, and the metabolite recrystallised with authentic oestradiol-17β. Another homogenate was prepared from a male fetus of about 97 days gestational age, and triplicate tubes with only ^{14}C- oestrone were incubated, and extracted as described in the method section. The scans of radioactivity were again similar to previous incubations. The metabolite peak from two tubes was combined and re-crystallised to constant specific activity with oestradiol-17β (see table 1 in the Appendix) and the two peaks from the third tube were assayed for radioactivity.

Peak 1 (oestradiol-17β)	24,732 c.p.m.
Peak 2 (oestrone)	61,779 c.p.m.
TOTAL	86,511 c.p.m.

Indicating that 28% of the added radioactivity, ^{14}C- oestrone is converted to oestradiol-17β. When recrystallised 38,492 c.p.m. were associated with oestradiol-17β from two tubes, hence approximately 19,000 c.p.m. per tube, which allowing for losses in extraction, TLC and elution accounts for virtually all the radioactivity, again indicating the presence of only one major peak.

Thus the chromatographic mobility on TLC and GC, the mass fragmentography spectrum and recrystallisation to constant specific activity provide conclusive evidence that the fetal sheep kidney is capable of metabolising

oestrone to oestradiol-17β indicating the presence of the enzyme 17β-HSD. (17β-hydroxysteroid : NAD(P) + 17- oxidoreductase; EC. 1.1.1.63-64).

111.c. (ii) Metabolism of Oestrone Sulphate

^{14}C- oestrone sulphate was incubated with fetal kidney homogenate for one hour and extracted with benzene. A tube containing only buffer (no homogenate) and ^{14}C- oestrone sulphate was also extracted with benzene, after incubation. The extracts were dried and assayed for radioactivity by liquid scintillation. Of the total radioactivity added to the tubes (10^5c.p.m.) 64% was recovered from the tube containing the homogenate, and only 7% from the control. Oestrone sulphate has limited solubility in organic solvents; the free steroid is infinitely more soluble in benzene. The presence of high levels of radioactivity in the extract from the homogenate was taken as evidence of the liberation of free steroid, indicating the presence of sulphatase activity.

This system was not characterised further, but supports the findings of Ainsworth (1972) that the fetal sheep kidney possesses significant sulphatase activity.

111.c. (iii) Identification of Androst -4-ene-3, 17-dione metabolites from Incubation with Fetal Kidney Homogenates

Androstenedione (10^5c.p.m., 23.2 ng) was incubated with fetal kidney homogenate prepared from kidneys from a fetus of approximately 107 days gestational age (1.5kg). The extract was chromatographed on TLC in solvent system 1, and then scanned for radioactivity. The scan revealed three peaks of radioactivity; a polar metabolite close to the origin, the substrate, and a less polar metabolite (see figure 5).

FIGURE 5

ANDROSTENEDIONE METABOLISM

TRACE 1

Scan of radioactivity after TLC of the extract from the incubation of androst-4-ene-3, 17-dione with fetal sheep kidney homogenate.

TRACE 2,3,4,5

Rechromatography of the polar metabolite in several systems.

TRACE 6

TLC of the polar peak after oxidation with Browns oxidant.

TRACE 7,8,9

Chromatography of the less polar metabolite in several systems.

STANDARDS

1 5α-Androstan -3β-ol-17-one

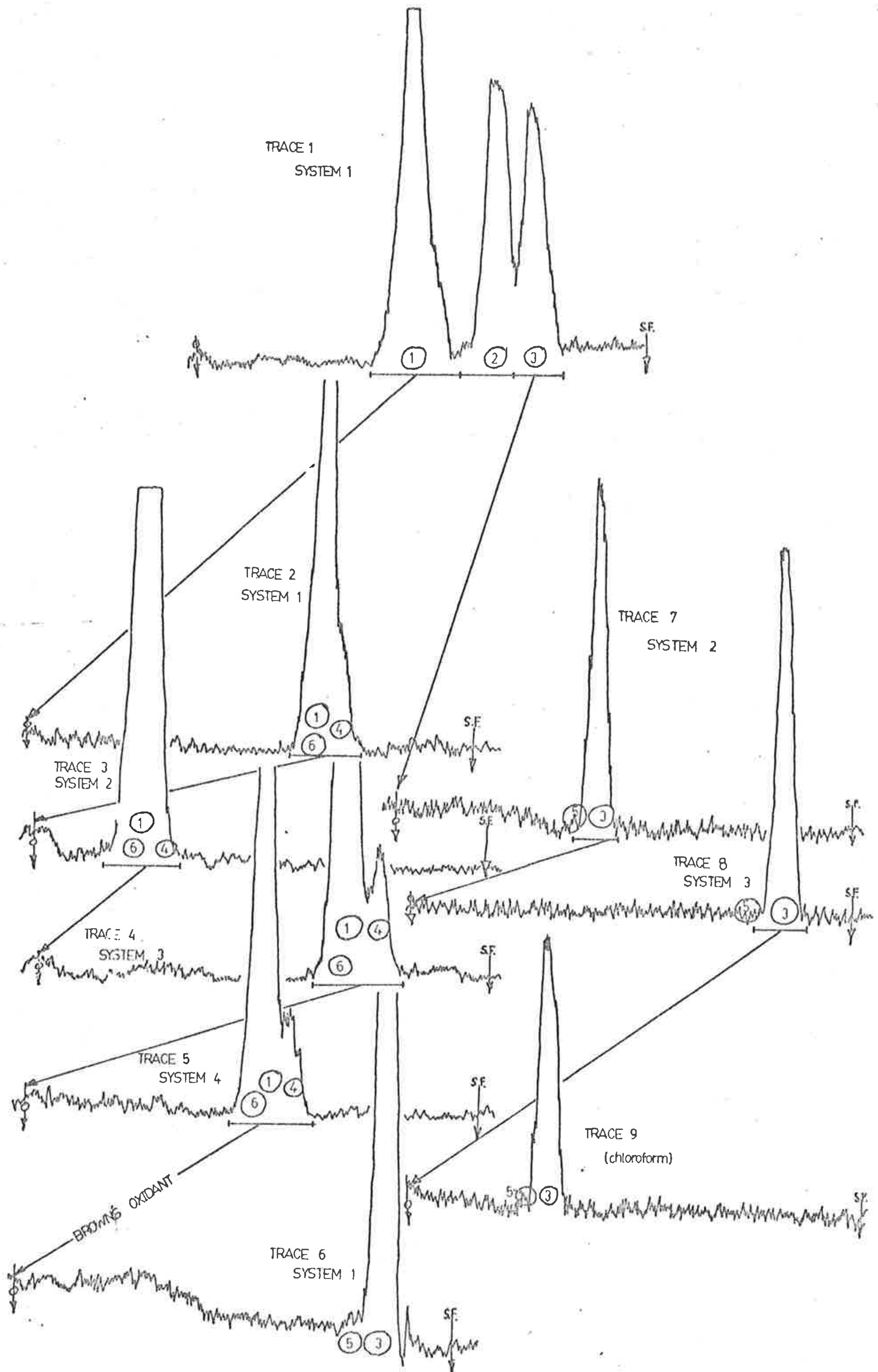
2 Androst-4-ene-3, 17-dione

3 5α-Androstan -3, 17-dione

4 5α-Androstan -3α-ol-17-one

5 5β-Androstan -3, 17-dione

6 5β-Androstan -17β-ol-3-one


TRACE 1
SYSTEM 1
S.F.
1
2
3
TRACE 2
SYSTEM 1
1
4
6
S.F.
TRACE 3
SYSTEM 2
1
6
4
TRACE 4
SYSTEM 3
1
4
6
S.F.
TRACE 5
SYSTEM 4
1
4
6
S.F.
BROWN'S OXIDANT
TRACE 6
SYSTEM 1
5
3
S.F.
TRACE 7
SYSTEM 2
3
S.F.
TRACE 8
SYSTEM 3
3
S.F.
TRACE 9
(chloroform)
3
S.F.

Identification of the Less Polar Metabolite

The radioactivity associated with this less polar metabolite was eluted and using 5α-and 5β-androstanedione as reference markers, was chromatographed on TLC in a number of solvent systems (see figure 5). In all systems the radioactivity remained as a single peak, and its Rf was the same as 5α-androstanedione; 5β-androstanedione ran slower than 5α in all systems, thereby eliminating the possibility of this metabolite being 5β-androstanedione.

Another homogenate was prepared, and radioactive (10^6c.p.m.) and unlabelled androstenedione (200μg) and NADPH (0.2mM) were incubated with 20ml of homogenate, to allow sufficient metabolite to be formed for analysis on GC-MS. The extract was redissolved in 50μl of n-heptane, and 5μl of this was run on TLC in chloroform: acetone, 9:1, and after scanning for radioactivity a pattern similar to that in figure 5 was obtained indicating sufficient amount of metabolite had been formed. Thus the rest of the extract was used for analysis by GC-MS, however the analysis was performed by a slightly different method to that previously described (111.b. (v)). The GC column was a glass coil (1m x 1mm ID) packed with 1.1% OV-225: SILAR (10:1) on a SUPELCO^R (100/120 mesh) support; the column was run at 210^0 with a carrier gas (N_2) flow of approximately 30ml/min; material coming off the column was recorded on a chart recorder. The MS was operated under conditions similar to those previously described, except; resolution 800, and electron energy 70eV.

An aliquot (2μl) of the extract was injected on the GC, and the profile of material coming off the column indicated the presence of five major peaks (see figure 6). The molecular weight (MWt) of the material in the five major peaks as indicated by the parent ion m/e was;peaks (1 and 2) 290,peak (3) 288,peak (4) 386,peak (5) 286. The GC trace for the control incubation, where the homogenate was incubated exactly the same, except that no androstenedione was added, had

only one peak. The material in the peak had the same Rt and MWt as peak (4) from the incubation extract and both are cholesterol. Thus the other peaks in the androstenedione incubation extract, are due to the substrate and its metabolites.

The material in peak (5) had a MWt 286, does not silylate (no hydroxyls), and has the same Rt as androstenedione, indicating that this large peak is unmetabolised substrate.

The material in peak (3) has a MWt 288 which is consistent with this being 5α-androstanedione. An aliquot of the extract was silylated with t-BDMS and the material in peak (3) did not change its Rt indicating that it has no hydroxyl groups, whereas cholesterol and the material in peaks (1) and (2) had shorter Rt's indicating the presence of hydroxyl groups. A clean mass spectral trace was obtained from the silylated material and was again MWt 288.

Authentic 5α and 5β-androstanedione were run several times on GC to establish their retention times (column temperature was raised to 235°). The standards were then run together, and even at the high temperature two peaks were obtained, indicating that the peak in the extract is only a single metabolite. The extract was re-run on GC at the higher temperature to determine the Rt of the material in the peak under examination (MWt 288), and this Rt was compared to those of the standards.

TABLE 1

Peak	Retention Times (min-sec)
Unknown metabolite (MWt 288)	2-50, 2-47, 2-49
5α-androstanedione	2-49, 2-46, 2-47
5β-androstanedione	2-33, 2-31, 2-34

FIGURE 6

Identification of Androstenedione Metabolites

EXTRACT

GC trace of the underivatised extract from the incubation of androst-4-ene-3, 17-dione with fetal sheep kidney homogenate.

Peak 1	5α-androstan-3αol-17-one	MWt 290
Peak 2	5α-androstan-3βol-17-one	MWt 290
Peak 3	5α-androstan-3, 17-dione	MWt 288
Peak 4	Cholesterol	MWt 386
Peak 5	androst-4-ene-3, 17-dione	MWt 286

SILYLATED EXTRACT

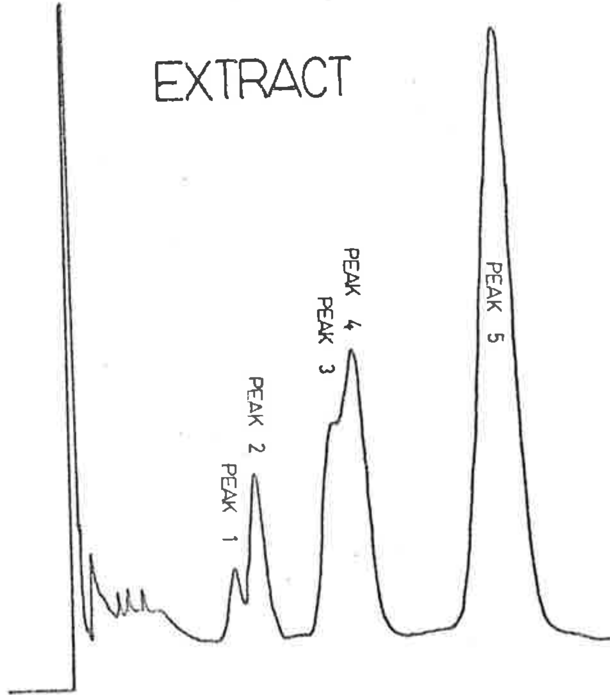
GC trace of the extract after silylation (t-BDMS)

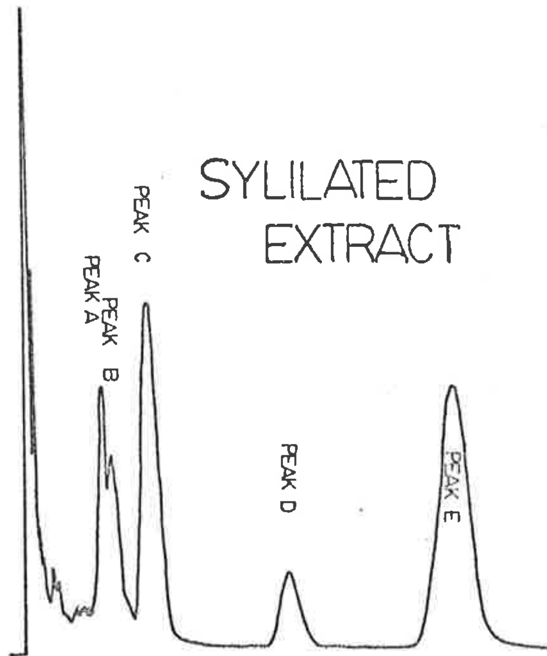
Peak C	Cholesterol t-BDMS ether	
Peak D	5α-androstan-3, 17-dione	MWt 288
Peak E	androst-4-ene-3, 17-dione	MWt 286

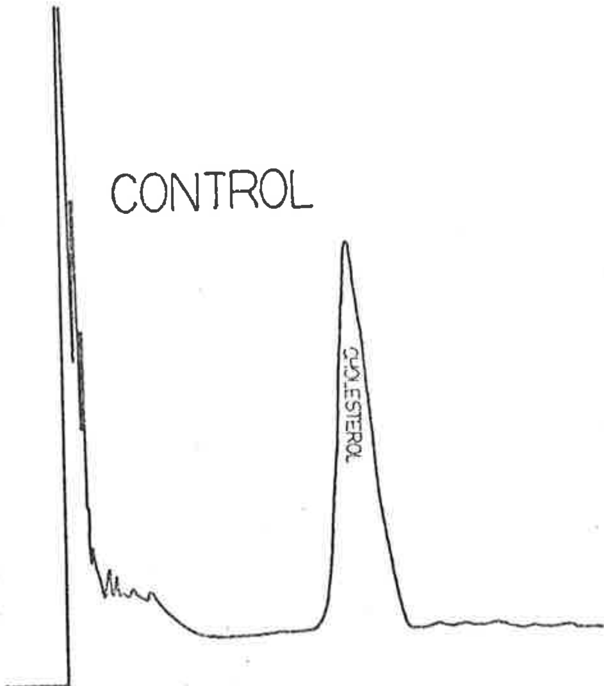
CONTROL

GC trace of the extract from an identical incubation tube as in "extract", except with no added androst-4-ene-3, 17-dione.

The only peak present is cholesterol.


EXTRACT
PEAK 1
PEAK 2
PEAK 3
PEAK 4
PEAK 5


SYLILATED
EXTRACT
PEAK A
PEAK B
PEAK C
PEAK D
PEAK E


CONTROL
CHOLESTEROL

The retention times indicate that the unknown metabolite has the same chromatographic mobility as 5α-androstanedione, confirming that on GC as well as TLC the metabolite behaves identically to 5α-androstanedione.

Thus the TLC of the less polar metabolite, the data from its mass spectrum, its GC characteristics and its lack of hydroxyl groups, together provide compelling evidence that the peak of radioactivity is a single metabolite and is 5α-androstanedione; indicating that the fetal sheep kidney has 5α-reductase activity which is capable of using androstenedione as a substrate.

Identification of the Material in the more Polar Peak (figure 5)

The simplest way in which androstenedione could be metabolised to a more polar metabolite is by reduction of one or both of its two keto groups to hydroxyls. The metabolite could be a androst-4-ene-ol-one, or diol, a 5α-androstan-ol-one or diol, or a 5β-androstan-ol-one or diol, giving at least 24 different possibilities for the identity of the metabolite. However, half of these can be eliminated as peaks (1) and (2) on the GC trace of the extract from the incubation of unlabelled A'dione had a MWt of 290. This leaves 5α/5β-androstan-ol-ones, and androst-4-ene-diols as possibilities.

The radioactive material of the polar peak was repeatedly chromatographed on TLC in a number of different solvent systems (see figure 5) and in all systems the androst-4-ene-diols had lower Rf values than the metabolite. However, a number of 5α-, and 5β-androgens had a similar Rf in all four chromatographic systems. In particular, in some systems a shoulder to the main peak or a small subsidiary peak appeared and matched with 5α-androstan-3α-ol-17-one. 5α-androstan-3β-ol-17-one chromatographed with the radioactive peak in all systems, but so did 5β-androstan-17β-ol-3-one (see figure 5). In an effort to determine if both 5α-, and 5β-metabolites were

present in the peak, the radioactivity was oxidised with Brown's Oxidant (1971) and rechromatographed in chloroform: acetone 95:5 with 5α-, and 5β-androstanedione as reference markers. Only one peak was detected, and this matched 5α-androstanedione, thus eliminating the 5β steroids.

The shoulder to the main peak, on TLC and the two peaks on the GC trace both provide evidence that more than one metabolite is present, but TLC provides insufficient resolution to determine which of the four possible 5α-androstan -ol-ones are present. To further examine this question, an extract from the incubation with unlabelled androstenedione was chromatographed several times on GC (column temperature raised to 235°C). The four 5α-androstan -ol-ones were also run on the column, and their retention times compared with those of the two peaks in the extract.

TABLE 2

GC RETENTION TIMES OF ANDROGEN METABOLITES

Peak	Retention Times (min-sec)
Unknown metabolite (1) (MWt 290)	1-50, 1-48
Unknown metabolite (2) (MWt 290)	2-03, 2-01
5α-Androstan -3α-ol-17-one	1-48, 1-49
5α-Androstan -3β-ol-17-one	2-01, 2-01
5α-Androstan -17α-ol-3-one	2-14, 2-15
5α-Androstan -17β-ol-3-one	2-27, 2-29

The GC retention times indicate that the two metabolites present are 5α-androstan -3β-ol-17-one, and 5α-androstan -3α-ol-17-one.

Another homogenate was prepared and incubated with androstenedione (10^6c.p.m.); the bulk of the extract was run on TLC, scanned for radioactivity, and the polar metabolite which is impure containing two components, was eluted and divided into two equal portions. One half

was recrystallised to constant specific activity with 5α-androstan - 3α-ol-17-one, and the other with 5α-androstan -3β-ol-17-one (see appendix, table 2 and 3). The following radioactivity was associated with these two compounds

5α-Androstan -3β-ol-17-one	214,313 c.p.m.
5α-Androstan -3α-ol-17-one	51,176 c.p.m.

indicating the major metabolite is 3β-hydroxylated by a ratio of about 4.2:1, 3β:3α.

These metabolites show that the fetal kidney has the capacity to metabolise 5α-androstanedione to both 3β, and 3α-hydroxylated compounds, indicating the presence of 3β-hydroxysteroid dehydrogenase (3β-hydroxysteroid: NAD (P)$^+$ oxidoreductase; EC. 1.1.1.51) and 3α-hydroxysteroid dehydrogenase (3α-hydroxysteroid: NAD(P)$^+$ oxidoreductase; EC. 1.1.1.50).

A small portion of the extract from the above incubation was also chromatographed in system 1, scanned for radioactivity and the regions on the plate associated with the radioactivity were scrapped into scintillation vials and assayed for radioactivity.

TABLE 3

PERCENTAGE CONVERSION OF ANDROSTENEDIONE TO 5α METABOLITES

	c.p.m.	% of total	3α	3β
5α-Androstane-3α/3β-ol-17-one	4,333	48=	9	37
Androst-4-ene-3, 17-dione	2,617	29		
5α-Androstan-3, 17-dione	1,952	23		
	8,902			

5α-reductase was the most active enzyme, reducting 71% of the androstenedione, 3β-HSD less active with 37%, and the 3α-HSD the least active with only 9%.

(iv) DHEA and Pregnenolone

Both DHEA and pregnenolone (10^5c.p.m.) were incubated with kidney homogenates from seven fetuses whose estimated gestational ages ranged from 97 to 142 days. The homogenates were extracted, chromatographed, and scanned for radioactivity, however, none of these extracts showed any detectable level of metabolism.

To verify that a metabolite with the same Rf as DHEA and pregnenolone had not been formed in their respective incubations, the single peak of radioactivity from each, was rechromatographed in systems 2,3, and 4 but in all cases only a single peak was present which had the same Rf as the reference steroids (DHEA, pregnenolone). After the final chromatograph, the peak of radioactivity from each plate was eluted, dried under N_2 and recrystallised to constant specific activity.

The radioactivity from the respective incubations was diluted with 38.52mg of authentic DHEA (see appendix table 4) and 41.76mg of authentic pregnenolone (see appendix table 5). The total radioactivity associated with DHEA was approximately 80% (82,792 c.p.m.) of the total radioactivity added to the incubation, which allowing for procedural losses accounts for virtually all the radioactivity. This confirms that there was no detectable metabolism of DHEA. Similarly, the radioactivity associated with pregnenolone (81,254 c.p.m.) was also approximately 80% of the radioactivity, which allowing for procedural losses also confirms that there was no detectable metabolism of pregnenolone.

(v) Identification of the Metabolites formed by the Incubations of Progesterone with Fetal Kidney Homogenates.

Fetal kidneys from a female fetus (2.2kg) of about 121 days gestational age were homogenised and incubated with ^{14}C progesterone (10^5c.p.m.).

The homogenate was extracted, loaded onto a silica gel plate, developed in system 1, and scanned for radioactivity. The scan of radioactivity revealed five major peaks (see figure 7, trace 1). An aliquot of the extract was also run in system 1, and the five peaks were assayed for radioactivity by liquid scintillation. Of the total radioactivity present in the five peaks, peak 1 contained (12.8%), peak 2 (20.2%), peak 3 (19%), peak 4 (20.3%) and peak 5 (27.7%).

Identification of the Metabolites in Area 1 (Figure 7, Trace 1)

In the initial scan of radioactivity this peak had the same Rf as 5α-pregnane-3β,20α-diol. This peak was eluted and chromatographed in systems 2,3,7 and 8, and in all these systems 5α-pregnane-3β,20α-diol, 5α-pregnane-3α,20α-diol, and pregn-4-ene-3β, 20α-diol all had similar Rf values to peak 1. In system 1, peak 1 has an Rf of (0.25). The 5β-pregnanediols all have Rf values less than 0.25, 5β-pregnane-3α,20αdiol being the fastest in system 1 with an Rf 0.20, indicating that no 5β-metabolites are present in peak 1. The 5α-pregnane-3α/β,20β-diols also do not match this peak of radioactivity.

Insufficient radioactivity was present to allow recrystallisation of this peak with the three compounds that did chromatograph with it, so another homogenate was prepared, and ^{14}C- progesterone (10^6c.p.m.) and NADPH (0.2mM) were added to the incubation mixture. After a 30 min incubation, the steroids were extracted and run on TLC in system 1, this time seven peaks of radioactivity were detected and a different pattern of metabolites was formed (see figure 8, c.f. figure 7). Peak 1 (figure 8) still chromatographed with the same Rf as 5α-pregnane-3β,20α-diol and was recrystallised to constant specific activity with (half of the radioactivity in the peak) with 31.96 mg of authentic 5α-pregnane-3β,20αdiol. (see appendix table 5). The other half of the radioactivity of peak 1 was recrystallised with pregn-4-ene—3β,20α-diol (19.48 mg). (see appendix

FIGURE 7

Progesterone Metabolism

TRACE 1

Scan of radioactivity after TLC of the extract from the incubation of ^{14}C- progesterone with fetal sheep kidney homogenate.

TRACE 2,3

Rechromatography of area 1 in two systems.

Contains 5α-Pregnane-3β, 20α-diol, Pregn-4-ene-3β, 20α-diol and others in lesser amounts.

TRACE 4,5

Rechromatography of area 2 from trace 1 contains 5α-Pregnane-3α-ol-20-one and Pregn-4-ene-20α-ol-3-one.

TRACE 6,7,8

Rechromatography of area 3 from trace 1.

Contains 5α-Pregnane-3β-ol-20-one and Pregn-4-ene-3β-ol-20-one.

TRACE 1
System 1

5α Pregnane-3β,20α-diol
Δ^4 Pregnene-20α-ol-3-one
5α Pregnane-3β-ol-20-one
Δ^4 Pregnene-3,20-dione
5α Pregnane-3,20-dione

1 2 3 4 5

S.F.

Trace 2
System 8

Δ^4 Pregnene-3β,20α-diol
5α Pregnane-3β,20α-diol
5α Pregnane-3α,20α-diol

S.F.

TRACE 3
2 × System 7

5β Pregnane-3α,20α-diol
5α Pregnane-3β,20α-diol

S.F.

TRACE 4
System 1

Δ^4 Pregnene-20α-ol-3-one
5α Pregnane-3β-ol-20-one

S.F.

TRACE 5
System 4

Δ^4 Pregnene-20α-ol-3-one
5α Pregnane-3β-ol-20-one

S.F.

TRACE 6
System 4

5α Pregnane-3β-ol-20-one
Δ^4 Pregnene-3β-ol-20-one

S.F.

TRACE 7
System 6

5α Pregnane-3β-ol-20-one
Δ^4 Pregnene-3β-ol-20-one

TRACE 8
System 6

5α Pregnane-3β-ol-20-one
Δ^4 Pregnene-3β-ol-20-one

S.F.

S.F.

table 7). The total radioactivity associated with each compound indicated that within the peak 5α-pregnane-3β,20α-diol accounts for approximately (72%) of the radioactivity present, and pregn-4-ene—3β, 20α-diol (13%). Other diols within this peak were not identified.

Identification of the metabolites in Area 2 (Figure 7, trace 1)

This area was eluted and rechromatographed in system 1. The peak had a shoulder indicating that more than one metabolite was present, and Pregn-4-ene-20α-ol-3-one, and 5α-pregnane-3α-ol-20-one both had the same Rf values as the peak. In system 4 this peak separated into two components which again had the same Rf values as the previously run standards. The peaks were eluted and counted for radioactivity: peak associated with pregn-4-ene-20α-ol-3-one contained 71%, and 5α-pregnane-3α-ol-20-one 29% of the combined radioactivity in the two peaks. The radioactivity in area two (figure 8) was eluted,run in system 4, and the two peaks present were recrystallised to constant specific activity with authentic pregn-4-ene-20α-ol-3-one (27.34 mg) and 5α-pregnane-3α-ol-20-one (35.17 mg) (see appendix tables 8 and 9).

Identification of the Metabolites in Area 3 (Figure 7, Trace 1)

This area was eluted and chromatographed in system 4, and formed two separate peaks. One peak had an Rf (.43) which matched the Rf of the reference steroid 5α-pregnane-3β-ol-20-one, this peak was eluted separately and chromatographed in system 6 and again had the same Rf as the 5α-P-3β-ol-20-one. This peak was eluted and assayed for radioactivity and contained (76,611 c.p.m.).

The other peak had an Rf (.51) which was the same as the reference steroid pregn-4-ene—3β-ol-20-one, this was also eluted and rechromatographed in system 6 and again had the same Rf as pregn-4-ene-3β-ol-20-one. This peak was eluted and assayed for radioactivity, and contained (4,379 c.p.m.).

FIGURE 8

Progesterone Metabolism

TRACE 1

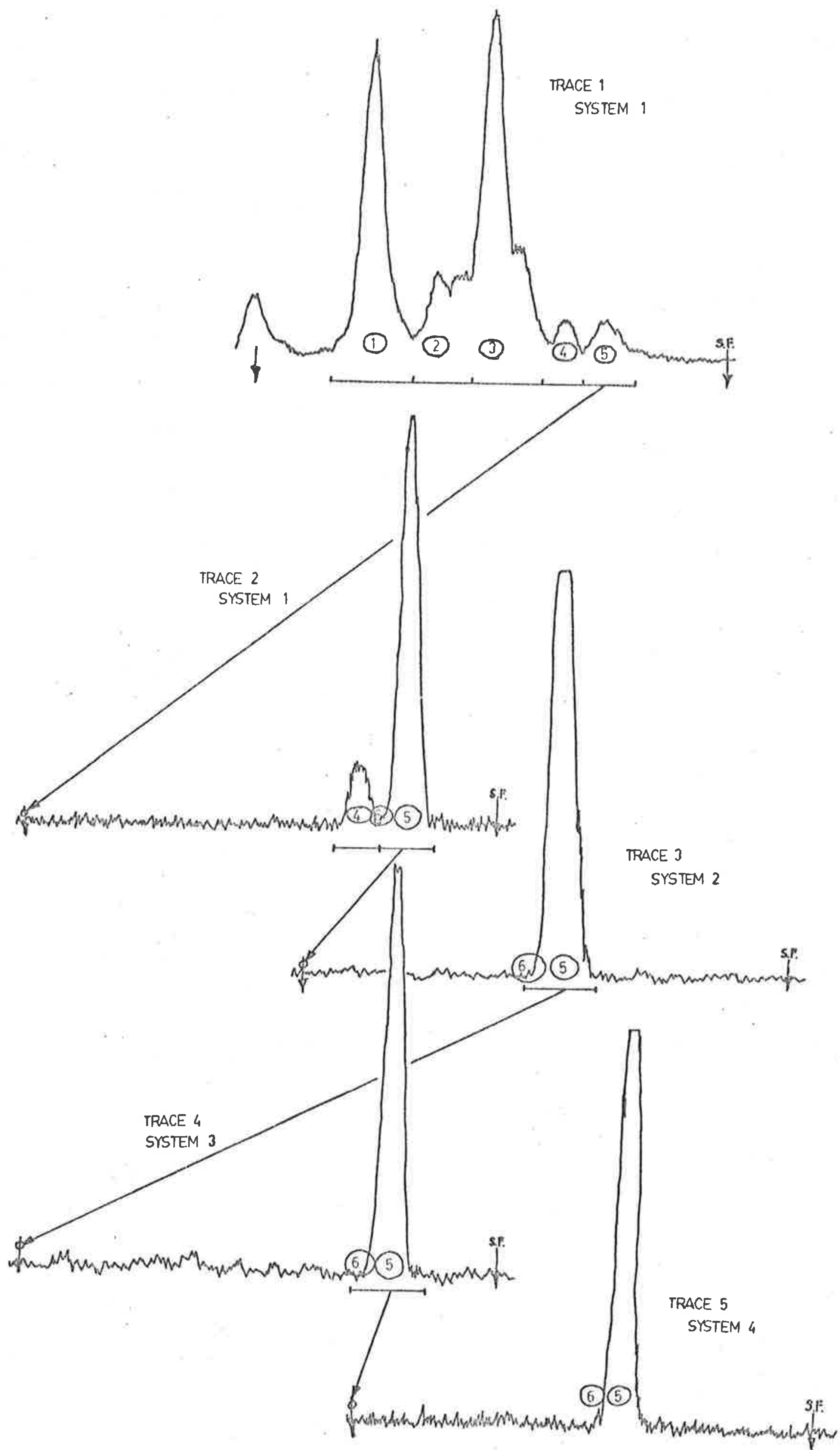
Scan of radioactivity after TLC of the extract from the incubation of ^{14}C- progesterone (+0.2mM, NADPH) with fetal sheep kidney homogenate.

TRACE 2,3,4,5

Rechromatography of area 5 from trace 1 in several different TLC systems. (the radioactivity is actually from figure 8) trace 1, area 5.

STANDARDS

1	5α-Pregnane-3β,20αdiol
2	Pregn-4-ene-20α-ol-3-one
3	5α-Pregnane-3β-ol-20-one
4	Pregn-4-ene-3, 20-dione
5	5α-Pregnane-3, 20-dione
6	5β-Pregnane-3, 20-dione


TRACE 1
SYSTEM 1
1
2
3
4
5
S.F.
TRACE 2
SYSTEM 1
4
5
S.F.
TRACE 3
SYSTEM 2
6
5
S.F.
TRACE 4
SYSTEM 3
6
5
S.F.
TRACE 5
SYSTEM 4
6
5
S.F.

Area 3 from the second incubation (figure 8, trace 1) was eluted chromatographed in system four against the previously matching reference steroids, and then the two separated peaks were eluted and recrystallised to constant specific activity. The peak with the lower Rf was recrystallised with 34.95 mg of authentic 5α-pregnane-3β-ol-20-one (see appendix table 10) and the peak with the higher Rf was recrystallised with 32.76 mg of authentic pregn-4-ene-3β-ol-20-one (see appendix table 11).

The peak which chromatographed against progesterone (figure 8 trace 1) was not characterised, and was assumed to be unmetabolised substrate.

Identification of the metabolite in area 5 (figure 7 trace 1)

The radioactivity in this peak was eluted and chromatographed against 5α-pregnane-3, 20-dione in a number of systems. In every system only a single peak was present which had the same Rf as 5α-pregnane-3, 20-dione; 5β-pregnanedione had a lower Rf value in all systems. The radioactivity was eluted and added to the radioactivity eluted from area 5 (figure 8 trace 1) from the second incubation, and recrystallised to constant specific activity with 26.77 mg of authentic 5α-pregnane-3, 20-dione (see appendix table 12)

The metabolites formed from the incubation of ^{14}C progesterone with fetal kidney homogenate, and the approximate level of radioactivity present as a percentage of the total radioactivity in each of the two incubations is given in the following table.

TABLE 4

PERCENTAGE OF METABOLITES FORMED FROM PROGESTERONE

STEROIDS	PERCENTAGE OF EXTRACTED RADIOACTIVITY FIRST	SECOND INCUBATION
5α-pregnane-3β 20α-diol	9	24
pregn-4-ene-3β 20α-diol	2	4
other polar metabolites	2	4
pregn-4-ene-20α-ol-3-one	14	6
5α-pregnane-3α-ol-20-one	6	7
5α-pregnane-3β-ol-20-one	12	39
pregn-4-ene-3β-ol-20-one	7	7
pregn-4-ene-3, 20-dione	20	4
5α-pregnane-3, 20-dione	28	5
	(see figure 7 trace 1)	(see figure 8 trace 1)

From the above table the relative amount of progesterone metabolised by a particular enzyme is given in the following table.

TABLE 5

RELATIVE ENZYME ACTIVITY FOR PROGESTERONE METABOLISM

	FIRST	SECOND INCUBATION
Δ^4-5α-reductase	64	75
3α-HSD	20	13
3β-HSD	30	74
20α-HSD	25	34

The enzymes 5α-reductase and 3β-HSD are the most active of the enzymes present, which is consistent with the earlier findings of Nancarrow (1969).

A summary of the results presented in this chapter for all five steroids incubated with fetal kidney homogenates is given in the following table.

TABLE 6

STEROID METABOLISING ENZYMES PRESENT IN FETAL SHEEP KIDNEY

STEROID	ENZYMES
C 18 oestrone	17β-HSD
oestrone-SO_4	SULPHATASE* — WHY CAPS?
C 19 Δ^4 androstenedione	(Δ^4-5 - reductase (3β-HSD (3α-HSD
C 19 (Δ^5) DHEA	no metabolism
C 21 (Δ^5) pregnenolone	no metabolism
C 21 (Δ^4) progesterone	(Δ^4-5α-reductase+ (3β-HSD+ (3α-HSD+ (20αHSD+

* previously reported by AINSWORTH 1972
\+ previously reported by NANCARROW 1969

111.d. (1) Discussion

The results show that the fetal sheep kidney has a number of enzymes which are capable of actively metabolising steroids from all three of the major classes of steroids (C18, C19, C21). The results also indicate that the fetal kidney was incapable of metabolising the Δ^5 steroids, pregnenolone, and DHEA, suggesting that Δ^4-Δ^5 isomerase was not present. In organs which are involved in steroid endocrinology such as the ovaries, testes, adrenals, and placenta, the presence of Δ^4- Δ^5 isomerase is crucial for the utilisation of pregnenolone (Ainsworth and Ryan, 1967; Anderson et al, 1970a;Hoppen et al, 1976; Seamark et al, 1977; Attal, 1969). Pregnenolone being a key primary intermediate between cholesterol and the biologically active steroid hormones formed by these endocrine organs. There was also no evidence that the fetal sheep kidney possesses any C17-20 lyase, or aromatase activity, enzymes which are involved in the conversion of C21 to C19 steroids, and C19 to C18

steroids respectively (Steele et al, 1975; Hosoda and Fishman, 1974). Thus even if the fetal sheep kidney were capable of converting pregnenolone to progestagens it would apparently be incapable of metabolising these to androgens, or the androgens to oestrogens. The lack of these enzyme activities also indicates that steroids in the fetal circulation which could be taken up by fetal kidney tissue would also be incapable of acting as precursors for steroid molecules containing less carbon atoms than themselves. Thus unless unique pathways exist in the fetal sheep kidney for production of steroid hormones, of which no evidence was found, the results indicate that the fetal sheep kidney is incapable of de novo synthesis of steroid hormones, and as such cannot be considered as an endocrine organ in relation to steroid hormones.

The fetal kidney does however contain sulphatase activity, which could enable the kidney to cleave the sulphate bonds of steroids such as oestrogen sulphates. Oestrogen sulphates are present in fetal plasma in much higher concentrations (X1000) than free oestrogens (Wong et al, 1972) thus the cleavage of the sulphate bond by the fetal sheep kidney, and subsequent release of oestrogen into the circulation could alter the level of circulating free oestrogens. In this way, although not directly involved with the de novo synthesis of steroids, the cleavage of the sulphate bond would enable the release of biologically active steroid. This type of action has been termed a para-endocrine activity, and the adult kidney in mammals has been shown to possess such activity in relation to Vitamin D_3, whereby the precursor of Vitamin D_3, produced elsewhere in the body, only gains its full biological activity after hydroxylation by an enzyme unique to the kidney (Gray et al, 1972). Although sulphatase activity may provide the fetal sheep kidney with a para-endocrine function through the release of free steroid into the circulation, the free steroids may be released

in order to act on the kidney. This latter alternative is considered later in the discussion.

The results indicate that the fetal kidney is unlikely to be involved in synthesis of steroids, however it may influence the level of physiologically active hormones by metabolising them, thereby effectively removing them from the fetal circulation. The enzymes for steroid metabolism that the fetal kidney does possess also occur in the fetal liver (Ainsworth, 1972; Anderson et al, 1970b; Nancarrow, 1969) suggesting that it may have a similar catabolic function. However the main route of metabolism of steroids in the fetal sheep liver is by their irreversible reduction by 5β-reductase. In the case of progesterone and testosterone, their reduction by 5β-reductase leads to the abolition of their usual biological activity i.e. they become ineffective as progestational or androgenic agents (see Strott et al, 1974a; Robel et al, 1971). (It is interesting however, that they do not become devoid of bilogical activity having a potent ability to induce an enzyme involved in haeme synthesis (Granick and Kappas, 1967), the production of which the kidney is also involved in via erythropoietin production (Jacobson et al, 1957)).

Although the fetal sheep liver contains all the enzymes that were found in the kidney, 5β-reductase was not found in the fetal kidneys, and in contrast to the liver function of deactivativation, the results indicate that the fetal kidneys metabolise steroids to molecules which still have biological activity. Oestrone was metabolised to oestradiol-17β, a more potent oestrogen, and the results indicate that 5α-reductase present in the fetal kidney has the capacity to metabolise both testosterone and progesterone. The 5α-reduction of testosterone is thought to be an essential facet of its action at the cellular level in target organs

such as rat prostate (Bruchovsky & Wilson,1968a) and in some systems 5α-pregnanedione is as efficacious as progesterone (Strott,1974a). Thus rather than having a catabolic activity, the metabolism of steroids by the fetal sheep kidney converts them to a form which is thought to be the active form at the cellular level.

It has been known for a long time that testosterone and progesterone have a trophic effect on the kidney, and that oestrogen has a degenerative effect (Selye 1939a, 1939b). Since then the mass of evidence that has accumulated supports the findings of these initial studies, (this subject has recently been reviewed by Kochakian (1977)), and indicates that sex hormones have an effect on the kidneys of rats, mice, guinea pigs, dogs and man. In investigating the trophic effect of androgens on the kidney Arimasa and Kochakian (1973) found that the rat kidney metabolises testosterone to 5α -androstane-3α,17β-diol. This androgen when administered to rats still has the same renotrophic potential as testosterone, but is markedly less effective in stimulating growth of the seminal vesicle and prostate. The 5α-reductase and 3α/3β-HSD activity in the fetal sheep kidney may have a similar action, emasculating the androgenic potential of testosterone without effecting its renotrophic capacity.

The trophic effect which progesterone has on the kidney may also involve its metabolism by 5α-reductase, similar to that which occurs in the chick oviduct. In this system Strott (1974a) suggests that when progesterone is 5α-reduced to 5α-pregnanedione it becomes physiologically active, and that after acting within the nucleus, its 3-keto group is reduced by 3α/3β-HSD in the cytoplasm. The results indicate that the predominant metabolites of progesterone formed by fetal sheep kidney tissue are 5α-pregnane- 3β-ols which is compatible with the system suggested by Strott (1974a), however, it is not known whether

progesterone or androgens have a renotrophic action on the fetal sheep kidney.

All the studies on the renotrophic action of sex steroids have been performed on neonatal or adult animals, with virtually no reports in the literature of their action on fetuses. Steroid receptor molecules have been studied in order to obtain further information on the mechanism of action of steroid hormones. An oestradiol receptor has been found in the rat kidney (de Vries et al, 1972), an androgen receptor in mouse kidney (Bullock and Bardin, 1974), a progesterone receptor in human kidneys (Concolino et al, 1976) and of particular interest was the presence of an oestradiol receptor in fetal guinea pig kidney (Pasqualini et al, 1974). The existence of this receptor in the fetal guinea pig suggests that sex steroid hormones may be acting on the kidney during fetal life.

The results of this chapter show that there are a limited number of steroid metabolising enzymes present in the fetal sheep kidney. The lack of several key enzymes involved in classical steroid biosynthetic pathways indicate that the kidney is unlikely to be involved in de novo steroid biosynthesis, and the enzymes that are present, although present in the fetal liver, do not equate with the fetal kidney having a catabolic function similar to the fetal liver. The enzymes present are however completely compatible with steroid hormones having an action on the fetal kidney. In this regard, it is interesting to speculate that the sulphatase would allow the release of free steroid, from the large pool of circulating sulphates, to act within the kidney; that 20α-HSD (which is reversible) would allow the kidney to utilize 20αOH-pregnene-3one for progesterone production; that 17β-HSD would metabolise oestrone to oestradiol-17β, a most potent oestrogen; and 5α-reductase and 3α/3β-HSD may be involved

in the action of both testosterone and progesterone on the fetal kidney (by a mechanism similar to that which occurs in PROSTATE and chick oviduct as discussed previously).

Thus the nature, and the limited number of the enzyme activities shown to be present in the fetal kidney are compatible with their being involved in the mechanism of action that steroid hormones may have on the fetal sheep kidney.

CHAPTER IV

5α-REDUCTASE IN FETAL SHEEP KIDNEYS

IV.a.(i) Introduction

The fetal kidneys are essential for the normal growth and development of the fetus as indicated by the malformations, growth retardation, and premature delivery of both the human anephric fetus (Potters Syndrome) and the surgically nephrectomised sheep fetus (Thorburn, 1974). Both these studies indicate that the lack of fetal kidneys is associated with endocrine dysfunction. This study has shown that the fetal sheep kidney has a considerable capacity for metabolising steroid hormones, but does not possess those enzymes usually associated with the de novo synthesis of steroid hormones. It is therefore unlikely that the endocrine disorders associated with nephrectomy are due to a loss of steroid production by the fetal kidney, however the fetal kidney may have important endocrine functions not associated with steroid production, the lack of these other functions may result in the abnormalities of the nephrectomised fetus.

The nature of the enzyme activities that are present in the kidney is compatible with their being involved in mediating the action that steroid hormones such as oestradiol, testosterone or progesterone may have on the kidney. Of the enzymes present, the 5α-reductase was the most active. This enzyme is involved in the action of testosterone in androgen target organs (Bruchovsky and Wilson, 1968). In some species androgens have a renotrophic action (see Kochakian, 1977), thus the presence of 5α-reductase and its high activity, suggest that testosterone may also be acting on the fetal sheep kidney; such an action may have an important influence on fetal kidney functions.

An examination of the characteristics of the 5α-reductase in fetal sheep kidneys would allow a comparison to be made of the 5α-reductases reported in androgen target organs such as the prostate and epididymus. The aim of experiments reported in this chapter was to examine facets of the kinetics of the

5α-reductase, its co-factor requirement, apparent Km value, its subcellular distribution, any sex difference in the level of activity, and change in activity with advancing gestation.

IV.b. Methods

(i) Protein Estimation

Protein estimation was performed using the Folin-Ciocalteu Reagent by the method of Lowry et al (1951). A range of aliquots of the sample (10, 30, 60, 100μl) were used for assay, and were treated with 300μl of alkali to dissolve all protein. Samples were assayed against BSA (Bovine Serum Albumin) as standard, and were read at 750nm.

(ii) Tissue Preparation

Tissue was collected, prepared and homogenised as previously described (111.b.i). Both 10 and 20% w/v homogenates were used.

(iii) Assay of 5α-Reductase Activity

5α-reductase activity was assayed using ^{14}C androstenedione (10^5c.p.m., approximately 25ng) with 2μg of unlabelled androstenedione. The homogenate (1 or 2ml) was incubated and extracted as previously described (111.b.iii), and the dried extract was chromatographed on TLC in chloroform: acetone (95:5) (Androstenedione Rf 0.5). The products from the reaction have been identified (111.c.ii) and thus 50μl of a 1mg/ml solution of standards (5α-androstan-3β-ol-17-one, androst-4-ene-3, 17-dione, 5α-androstan-3, 17-dione) was added to the dried down steroid prior to TLC, and thus was run as an internal standard. The position of these standards was visualised with iodine vapour, the plate scanned for radioactivity, and the areas associated with the standards were scraped into scintillation vials, 1ml of water added,+10ml of toluene scintillator, and the solubilised radioactivity assayed. The activity of 5α-reductase was then calculated from the percentage of the total radioactivity recovered

that was converted to 5α-reduced metabolites.

From the percent conversion the velocity of the reaction (pM/mg of protein/min) was calculated using the known amount of unlabelled androstenedione added to the incubation mixture. When comparing the activity between fetuses, replicate tubes were run at, 0, 5, 10 and 15 minutes to ascertain linearity of the reaction over the incubation time, and the value for activity was obtained from the initial linear portion of the curve. The maximum percentage conversion of substrate was kept below 30% to reduce any error in estimation of activity due to an increasing velocity of the reverse reaction at high product concentration. It should be noted however, that Roy (1971) in investigating the kinetics of 5α-reductase with testosterone in rat prostate, found no evidence of the reverse reaction of 5α-DHT to testosterone.

(iv) Preparation of Subcellular Fractions

An homogenate of fetal sheep kidney was prepared with Buffer B containing 0.25 M Sucrose using the previously described method (III.b.ii). Subcellular fractions were prepared essentially as described in "Methods in Enzymology Volume 10" and de Duve (1963). All procedures were carried out at 4°C.

Nuclear Fraction

10ml of homogenate layered carefully onto 10ml of 0.34M sucrose in 30ml centrifuge tube. Centrifuge at 850g for 10 minutes. Completely remove supernatant, and resuspend the pellet in Buffer B and repeat procedure. Resuspend second pellet in Buffer B (10ml) and use this as nuclear fraction.

Mitochondrial Fraction

Centrifuge homogenate (Buffer B) at 850g for 20 minutes and remove supernatant. Centrifuge this first supernatant at 15,000 g for 15 minutes and remove supernatant. This supernatant is used for preparing microsomal and cytosal fractions.

The pellet was resuspended in Buffer B (½ volume of homogenate) and used as the mitochondrial fraction.

Microsomal Fraction

The supernatant from the 15,000 g spin in the mitochondrial preparation was centrifuged at 100,000 g for 60 minutes in a Beckman preparative ultra centrifuge (30,000 r.p.m.). The pellet was resuspended in (½ volume) of Buffer B, and the supernatant was used as the Cytosol fraction. All fractions were kept on ice until used.

Results

IV.c.(i) Co-Factor Specificity of 5α-Reductase

A male and female twin pair of approximately 135 days gestational age with kidney weights of 18.3 gm and 13.8 gm respectively was obtained from the abattoirs. Homogenates (20%) were prepared separately for the kidneys, and the activity of 5α-reductase with either NADH or NADPH added to the incubation mixture was compared with the activity of the control containing no co-factor. Incubation mixture contained 1ml of 20% homogenate, ^{14}C-androstenedione (10^5 c.p.m. (25ng) added in 10μl of acetone), unlabelled androstenedione (2μgm added in 10μl of acetone), and either 100μl of buffer (control) or buffer containing (0.1m M) NADH or (0.1m M) NADPH.

Tubes were preincubated for 15 minutes prior to addition of co-factor, then unlabelled androstenedione was added followed by ^{14}C androstenedione. At each time interval the respective tubes were removed from the shaking 37°C water bath and quickly frozen at -70°C and stored at -4°C until processed. Tubes were extracted, the dried extracts run on TLC and the radioactivity associated with 5α-reduced metabolites was assayed.

TABLE 7

Percentage Conversion to 5α-Reduced Metabolites

Female Twin

Time (min)	Control	NADH	NADPH
0	5.0	4.6	5.1
5	4.7	4.4	10.9
10	5.6	6.9	14.4
20	5.9	7.8	25.6
30	6.4	8.0	27.6

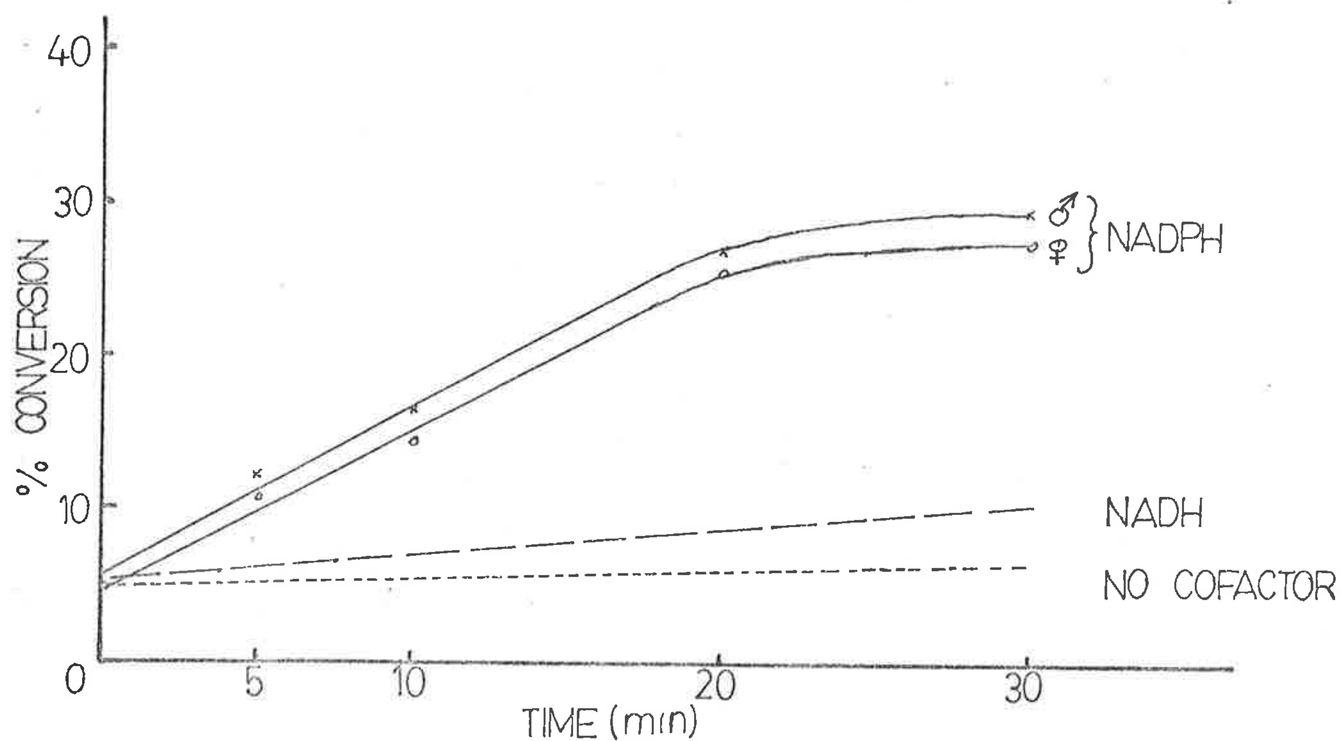
Male Twin

Time (min)	Control	NADH	NADPH
0	4.9	5.1	5.0
5	5.2	6.4	12.0
10	5.6	7.3	16.5
20	5.4	8.5	26.9
30	6.0	9.7	28.9

FIGURE 9

Co-Factor Specificity

Percentage conversion to 5α-reduced metabolites versus time.



The results indicate that NADH is ineffective in raising the level of activity above that of the control, whereas NADPH causes a marked increase (over ten fold increase above control) in the level of activity of 5α reductase, indicating that this enzyme is highly specific in its co-factor requirement for NADPH. The level of activity of the 5α-reductase is very similar in both male and female fetal kidneys of the twins used in this experiment. (see table 7, and figure 9).

IV.C.(ii) 5α-Reductase: Estimation of Km for Androst-4-ene-3,17-dione

A 10% homogenate was prepared from the kidneys (16.6 gm, 15.3 gm) of two male fetuses of approximately 123 days gestational age; sufficient homogenate was prepared (and pooled) for 15 incubation tubes each containing 10ml of homogenate. The 15 tubes contained the following array of NADPH and androstenedione concentrations.

NADPH	0.1, 0.2, 0.4 mM
Androstenedione	1.0, 2.0, 3.33, 5, 10mM

All tubes were preincubated for 15 minutes before addition of substrate and co-factor; after these were added the tubes were vortexed and placed in a shaking water bath at 37°C. Immediately after vortexing, and at 4, 7, 11, 20, 25 and 30 minutes 100μl samples were taken from each tube and rapidly frozen. Each sample was extracted (0.5 ethyl acetate), the extract dried, chromatographed on TLC, the products and substrate eluted and the radioactivity assayed. The percentaged conversion to 5α-reduced metabolites plotted against time was used to give an estimate of the initial velocity of the reaction. A 10μl sample of the homogenate in each tube was taken for protein estimation (Lowry, 1951) and gave a mean of 5.6mg/ml (n=15), range 5.2 - 5.8mg/ml. All velocities were calculated using the mean value for protein concentration. The initial relative velocity for each incubation

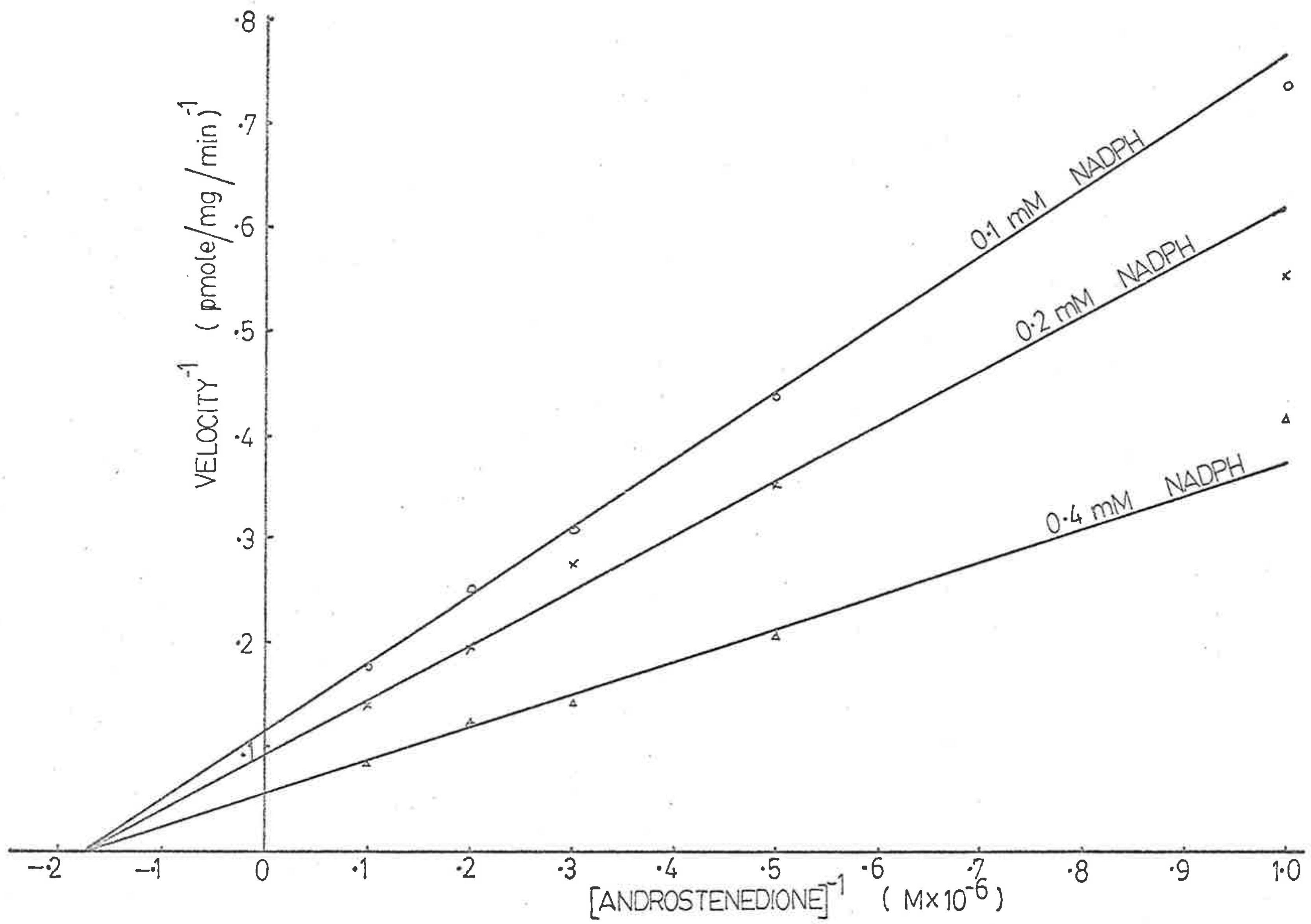
FIGURE 10

LINEWEAVER - BURKE PLOT OF 5α-REDUCTASE ACTIVITY

The graph is a representation of initial velocities of fetal sheep kidney 5α-reductase activity on androstenedione, measured at pH 7.3 at 37°C.

The reciprocal velocity is plotted against the reciprocal androstenedione concentration.

NADPH at	0.1 mM, 0.2 mM, 0.4 mM
Androstenedione	1.0, 2.10 3.33, 5, 10 mM



VELOCITY^{-1} (pmole/mg/min)$^{-1}$
·8
·7
·6
·5
·4
·3
·2
·1
0·1 mM NADPH
0·2 mM NADPH
0·4 mM NADPH
−·2
−·1
0
·1
·2
·3
·4
·5
·6
·7
·8
·9
1·0
[ANDROSTENEDIONE]$^{-1}$ (M x 10^{-6})

tube was plotted against substrate concentration using a Lineweaver-Burke plot to gain an estimate of Km and V max (see figure 10).

$Km = 5.9 \times 10^{-6}$

NADPH (mM)	V max (pmoles/mg/min)
0.1	9.1
0.2	11.1
0.4	16.7

IV.C.(iii) <u>5α-Reductase Activity in Subcellular Fractions of the Fetal Kidney</u>

The kidneys from a male fetus of approximately 110 days gestational age (kidney weight 13.2 gms) were homogenised in Buffer B (10% w/v). Subcellular fractions; nuclear, mitochondrial, microsomal and cytosol were prepared as described in the method section (IV.b.iii). The preparation for each fraction (1ml) and the homogenate (1ml) were incubated with NADPH (0.2mM, added in 0.1ml Buffer B), ^{14}C androstenedione and unlabelled androstenedione (2ng 6.7nM) giving a final volume of 1.1 ml. Replicate tubes were prepared for each subcellular fraction and run as 0, 10, 20, 30, minute incubations, frozen at -70°C, extracted with 2 x 2 volumes of ethyl acetate, chromatographed, areas of radioactivity assayed, and the percentage conversion to 5α-reduced metabolite calculated. The straight line drawn through the plot of percentage conversion versus time was used to calculate the velocity of the reaction. Usually after 20 min , the velocity of the reaction decreases, thus only the initial velocity was used and expressed as pM/mg of protein/min.

TABLE 8

<u>Subcellular Distribution of 5α-Reductase Activity</u>

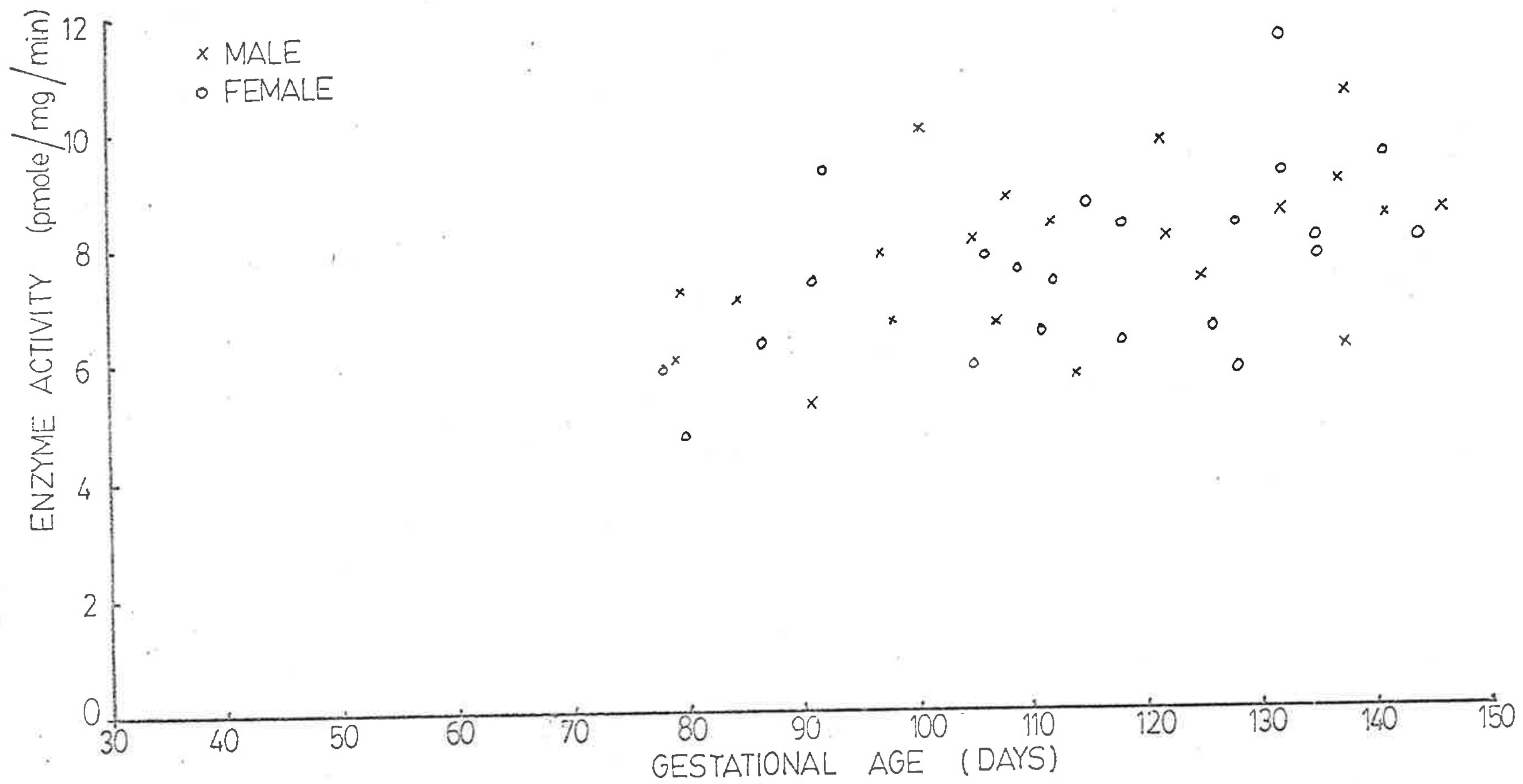
Subcellular Fraction	Activity (p mole/mg/min)	Protein Concentration (mg/ml)
Homogenate	3.2	5.2
Nuclear	9.9	2.5
Mitochondrial	3.2	3.5
Microsomal	7.0	1.2
Cytosol	1.9	2.5

FIGURE 11

5α-REDUCTASE ACTIVITY versus GESTATIONAL AGE

Enzyme activity was measured using the assay described in the text. Kidneys were obtained from fetuses whose gestational age was estimated from measurements of crown-rump length and body weight with reference to Cloete (1939). Enzyme activity is expressed in pmole/mg of protein/min.

The graph shows that no sex difference is apparent, nor any change with gestational age.



ENZYME ACTIVITY (pmole/mg/min)
12
10
8
6
4
2
0
× MALE
o FEMALE
30
40
50
60
70
80
90
100
110
120
130
140
150
GESTATIONAL AGE (DAYS)

The results indicate that the nuclear and microsomal fractions contain the greatest activity; the activity in the nuclear fraction being equivalent to the total combined activity of the other fractions. The same pattern of activity in the fractions was found when this experiment was repeated. Marker enzymes which are known to be present exclusively in individual fractions (de Duve,1963) were not assayed to determine the level of contamination between fractions. It may be that activity in the mitochondrial and cytosol fractions is due to contamination, and that all the activity resides in the microsomes and the nucleus, which clearly possess the greatest activity.

IV.C.(iv) Kidney 5α-Reductase Activity with Advancing Gestational Age

Kidneys from fetuses of estimated gestational ages (Cloete, 1939) ranging from 78 days until near term was assayed for 5α-reductase activity. Homogenates were prepared (10%, w/v), 1ml of homogenate was incubated with NADPH (0.2mM, in 100μl Buffer A), 4-^{14}C androstenedione and unlabelled androstenedione (2μg, 6.7nM). Replicate tubes were run at 0, 10, 20 minutes and the straight line through the plot of percentage conversion was again used to calculate the activity present. The results are shown in figure 11. The results indicate that the level of activity does not change with increasing gestational age, with virtually all values from 80 days to term being between 6-10 p mole/mg/min. The results did not indicate that there was any difference in the level of activity between male and female fetal kidneys.

Discussion

The results indicate the similarity of the 5α-reductase activity present in the fetal sheep kidney with those reported for rat prostate (Roy, 1971) and rat epididymus (Robaire et al, 1977) both of which are target organs for androgens. The 5α-reductase present in these tissues and others,

requires NADPH as co-factor, NADH being ineffective (Kitay et al, 1973; Maynard and Cameron, 1973); the 5α-reductase present in fetal sheep kidneys also requires NADPH as co-factor.

The values reported for Km and V for the 5α-reductase activity on testosterone in rat prostate and epididymus are also similar to the values obtained for androstenedione 5α-reduction in the fetal sheep kidney.

Apparent Kinetic Constants for 5α-Reductase

	V(p mole/mg/min)	Km (M)
Rat Prostate [1]	0.35	1.15×10^{-6}
Rat Liver	750	-
Rat Epididymus [2]	30	3.6×10^{-7}
Fetal Sheep Kidney	9	5.9×10^{-6}

all the velocities were with 0.1mM NADPH

1 = Roy, 1971; 2 = Robaire et al, 1977.

However, Roy in investigating the properties of the 5α-reductase activity of rat prostate activity found as in this thesis that there was no evidence of the reverse reaction of 5α going to Δ^4-steroid. The irreversible nature of this reaction is not consistent with the direct utilisation of NADPH by the 5α-reductase enzyme; in contrast to this is the reversible 5α-reduction of cortisone (Tompkins and McGuire,1960). Using a range of inbibitors Roy obtained results which indicated that a system with similar properties to cytochrome P450 was involved in the 5α-reduction by prostate. Thus the significance of the Km value is obscure, probably being related to a combination of enzyme activities which ultimately provide the donor hydrogen. The presence of a system such as this would account for the lack of reversibility of the 5α-reductase in fetal sheep kidneys, and suggests that the values for the kinetic constants

may also be related to a combination of enzyme activities. It may be that both the lack of reversibility and the similar values in apparent kinetic constants to those in the rat prostate and epididymus are indicative of a similar mechanism of 5α-reduction; one which is involved in hormone action. In contrast, in the liver, which has a catabolic function in relation to steroid hormones, not only is the 5α-reductase reversible but the level of enzyme activity is several orders of magnitude higher (100 fold higher).

Another similarity between the 5α-reductase of fetal sheep kidneys and rat prostate is the similar distribution of activity within the subcellular fractions. Roy (1971) found that the nuclear and microsomal fractions were the most active, containing approximately equal activity and accounting for 76% of the 5α-reductase activity present in all the fractions. In this thesis the nuclear and microsomal fractions were also approximately equally active and contained between 77% - 83% of the combined activity in the fractions. This equal distribution of activity between the nucleus and cytoplasm is believed to be associated with the way in which androgens act in target organs (Bruchovsky and Wilson, 1968 a,b). In contrast, the liver which also contains 5α-reductase activity, has most of its activity located in microsomes, the cytoplasm containing 96% of the activity and the nucleus less than 4%. The total activity of the liver is much higher than that of the prostate, and thus even at 4%, the activity in the liver nucleus is comparable to that of the prostate nucleus. A major function of the liver is catabolism, part of which involves numerous enzymes which catabolise steroid hormones. There are distinct sex difference in the levels of these steroid metabolising enzymes, and 5α-reductase in the nucleus may be involved in the action that androgens have in altering the pattern of catabolic enzyme activity in the liver (Denef, 1973). Thus the much higher total level of activity in the liver may be due to the combination of

activity associated with the major catabolic function of the liver, and a much smaller level of activity associated with the livers response to androgens.

The results show that the 5α-reductase activity in fetal sheep kidneys is much less than in the liver, is not reversible, and its distribution of activity between subcellular fractions is also different from that in the liver. The blood flow to the fetal sheep kidney is much smaller than to the fetal liver (Abraham, 1967), thus even if the fetal kidney 5α - reductase has a catabolic function (and this seems unlikely) its contribution to total steroid catabolism within the fetus would be greatly limited. Thus the results for the kinetic data on fetal sheep kidney 5α-reductase demonstrate that it is different from that found in liver, but is very similar to that found in a range of mammalian tissues known to be target tissues for androgens. The 5α-reductase in these tissues has similar co-factor requirements, apparent binding affinity and reaction rate, and similar subcellular distribution to that in the fetal sheep kidney. This evidence provides support for the postulate that the role of steroid metabolism in the fetal sheep kidney, involves the action that steroid hormones have on the kidney.

The results did not show any sex difference in the fetal kidney 5α-reductase activity, with the values for both males and females being apparently evenly scattered. Testosterone has been found in the plasma of both male and female sheep, and although the levels are higher in males than females, the levels are highly variable between individuals (Pomerantz and Nalbandov, 1975). The variability may account for the lack of any demonstrable sex difference in 5α-reductase activity. The presence of testosterone in fetal sheep plasma and active 5α-reductase in the kidneys of both sexes suggests that testosterone could have an action on the kidneys in both sexes.

Thus the results presented in this chapter indicate the similarity of the 5α-reductase present in both fetal sheep kidneys and androgen target organs; and are compatible with androgens having an action in both male and female fetal sheep kidneys.

CHAPTER V

SEX DIFFERENCE IN FETAL SHEEP KIDNEY WEIGHT

V.a. Introduction

In several species of mammals it has been established that testosterone has an action on the kidney, which among other changes results in an increase in kidney weight (see Kochakian, 1977). In these mammalian kidneys several steroid metabolising enzymes are present which are thought to be directly involved in the mediation of testosterones action (Ohno et al, 1971). One of these enzymes, 5α-reductase, is found in androgen target organs, and is thought to be essential for testosterone to exert its effect (Bruchovsky and Wilson, 1968a). The results of the previous chapters demonstrate that the fetal sheep kidney also possesses an active 5α-reductase, and that the nature of the other steroid metabolising enzymes present are very similar to those present in androgen responsive kidneys (Ohno et al, 1971). This suggests that the fetal sheep kidney may also be responsive to androgens.

The increase in kidney weight in response to testosterone in other species, results in a marked sex difference in kidney weight. It is not known whether a similar sex difference in kidney weight exists in fetal sheep. The existence of such a difference is dependent on higher levels of testosterone in the circulation of the male fetus. Attal (1969) reported that testosterone was present in the testes of male sheep fetuses from day 30 of gestation, and that sufficient of it is released into the circulation for differentiation of the external genitalia to be observable by day 45 of gestation. Pomerantz and Nalbandov (1975) have reported that the levels of testosterone in male fetal sheep plasma are consistently higher than in the female throughout gestation. Thus the male fetal sheep kidney may also respond to this higher level of circulating testosterone, resulting in a sex difference in fetal kidney weight.

The aim of experiments reported in this chapter was to establish whether a difference exists between the weights of male and female fetal sheep kidneys.

V.b. Methods

Fetuses were obtained from pregnant ewes slaughtered at the local abattoirs (SAMCOR). Fetal body weight, kidney weight and sex was recorded, and the data analysed statistically.

V.c. Results

Fetal body weight, kidney weight and sex of 63 singleton fetuses were recorded (see appendix I table 13). Examination of the data revealed that within a given body weight range (e.g. 2.0-2.5 kg) the variability in fetal kidney weight was large (range; female, 13.6-16.5 gm; male, 12.6-23.3 gm), and although the mean was slightly higher in males (17.1 gm) the variance (σ) was high (6.3), thus the means were not significantly different. Using the approach of Clarke (1969) an estimate was made of the number of replicates needed to establish a significant difference ($P < 0.05$) in the means. Using this method two means may be declared significantly different if the numerical value of their difference exceeds $t_f\sqrt{(2\sigma^2/r)}$. Where t_f is Students t distribution with f degrees of freedom , and r is the number of replicates. It was estimated that at least 30 replicates of each sex at every given range of body weight would be required if the experiment was to provide a significant result, i.e. a total of approximately 600 fetuses. From practical consideration this was viewed as unsuitable.

Rather than compare kidney weights in groups of male and female singleton fetuses it was thought that a direct comparison between male-female fetal co-twins would yield a significant result with less replicates. 52 male-female fetal twin pairs were obtained, and the data were tabulated as in

TABLE 9

FETAL TWINS (DATA FOR 52 MALE-FEMALE TWIN PAIRS)

Est. Age (days)	Body Wght. (Kg)	Kid. Wght. (gms)	Kid. Wght. / Body Wght.	Estim. Age	Body Wght. (Kg)	Kid. Wght. (gms)	Kid. Wght. / Body Wght.
72	0.18	1.5	8.33	123	1.9	11.4	6.0
	0.195	1.75	8.97		2.2	11.8	5.36
94	0.625	5.85	9.36	124	2.3	20.3	8.82
	0.567	4.95	6.97		1.9	14.4	7.57
96	0.68	7.5	11.10	126	2.4	16.7	6.96
	0.59	5.5	9.32		2.2	13.9	6.32
101	0.8	8.0	10	127	2.5	17.4	6.96
	0.85	8.7	10.23		2.2	14.4	6.54
101	0.875	7.3	8.34	128	2.56	16.2	6.34
	0.660	6.1	9.24		2.05	11.3	5.51
103	0.97	10.2	10.52	129	2.61	17	6.51
	0.90	8.7	9.67		2.58	15.9	6.16
104	1.02	10.8	10.58	129	2.62	16.3	6.22
	.96	7.5	7.81		2.09	11.3	5.41
107	1.10	13.1	11.9	130	2.3	15	6.52
	1.11	13.5	12.16		2.7	14.3	5.3
107	1.15	8.4	7.3	130	2.65	19.6	7.4
	1.15	8.4	7.3		2.38	16.4	6.89
110	1.22	8.8	7.21	130	2.73	18	6.59
	1.31	9.4	7.17		2.47	14.2	5.75
110	1.30	11.4	8.77	130	2.7	13.7	5.07
	1.20	11.1	9.25		2.8	15.4	5.5
111	1.35	13	9.6	131	2.8	18.7	6.7
	1.35	11.4	8.4		2.8	14.5	5.2
112	1.4	12.1	8.6	132	2.9	17.2	5.9
	1.25	11.5	9.2		2.8	15.3	5.5
113	1.48	15.9	10.74	133	3.13	16.9	5.39
	1.04	9.1	8.75		3.05	15.5	5.08
114	1.54	13.1	8.51	134	3.0	19.7	6.57
	1.53	9.9	6.47		2.8	17.0	6.07
114	1.53	13.7	8.95	134	3.1	21.1	6.8
	1.17	9.6	8.21		2.8	20.0	7.1
115	1.6	15.3	9.56	134	3.0	18.3	6.1
	1.43	13.4	9.37		2.75	13.8	5.02
117	1.72	14.2	8.25	135	3.1	15.5	5.0
	1.67	13.1	7.84		3.05	17	5.57
118	1.77	16.5	9.32	136	3.2	19.2	6.00
	1.48	13.7	9.26		3.0	14.6	4.87
118	1.82	13.6	7.47	137	3	13.6	4.53
	1.72	12.9	7.49		3.3	14.6	4.42
119	1.91	15.0	7.85	137	3.2	16.1	5.03
	1.60	13.4	8.35		3.3	17.0	5.15
120	2.03	17.0	8.37	138	3.4	19.2	5.6
	1.80	14.6	8.11		3.0	14.9	4.9
121	2.05	16.5	8.05	139	3.5	22.5	6.43
	1.73	12.1	6.99		3.0	13	4.33
122	2.1	12.6	6.0	139	3.5	22.8	6.51
	1.9	12.3	6.47		2.7	18.3	6.77
122	2.12	14.5	6.84	139	3.5	23.1	6.6
	2.06	13.6	6.65		3.1	18.0	5.8
122	2.18	18.0	8.26	148	4.3	25.5	5.93
	1.85	17.5	9.46		3.5	20.1	5.74

table 9, where the top line in each pair is the data for the male fetus.

The gestational age is only approximate, being taken from the weight of the heaviest twin, with reference to Cloete (1939). At birth singleton fetuses are on average 0.5Kg heavier than each twin (Starke et al,1958), thus the twins are most likely to be older than their estimated age.

The fetal twins were divided into four groups according to the weight of the heaviest twin in each pair, and kidney weight was compared using a paired t-test. The analysed data are summarised in table 10.

TABLE 10

Analysis of Paired Kidney Data

Estimated Age	Body Weight (Kg)	Mean Kidney Weight Male Twins	Female Twins	Paired t-test	d.f.	
100-109	0.9-1.19	9.63	8.81	1.33	5	(NS)
110-119	1.2-1.99	13.55	11.54	3.47	11	P<0.01
120-129	2.0-2.69	16.15	13.5	4.173	11	P<0.01
130-139	2.7-3.5	18.3	15.7	3.802	17	P<0.002

These data are presented in figure 12.A., and show that in fetal twins the kidneys of male fetuses are significantly heavier than those of their female co-twins. However, of the 52 twins the males are heavier in 42 pairs (80.8%), thus although the male kidneys are heavier, the body weight of male fetuses is also heavier.

Fetal kidney weight was plotted against body weight for each individual fetus (figure 12.B) rather than against the body weight of the heaviest twin. From figure 12.B., it can be seen that kidney weight increases rapidly before the fetus reaches 1Kg, after which it increases at a slower rate. This figure also shows that even though considerable variation exists in the values for both

FIGURE 12

KIDNEY WEIGHTS OF FETAL TWINS

All the data presented in this figure was obtained from 52 sets of male - female fetal twins, shown in table 9.

A. Grouped Twins; Kidney Weight vs Body Weight

The kidney weights of four sets of twins are shown at 0.2, 0.625, 0.68 and 4.3kg these being the weights of the heaviest fetus in each pair.

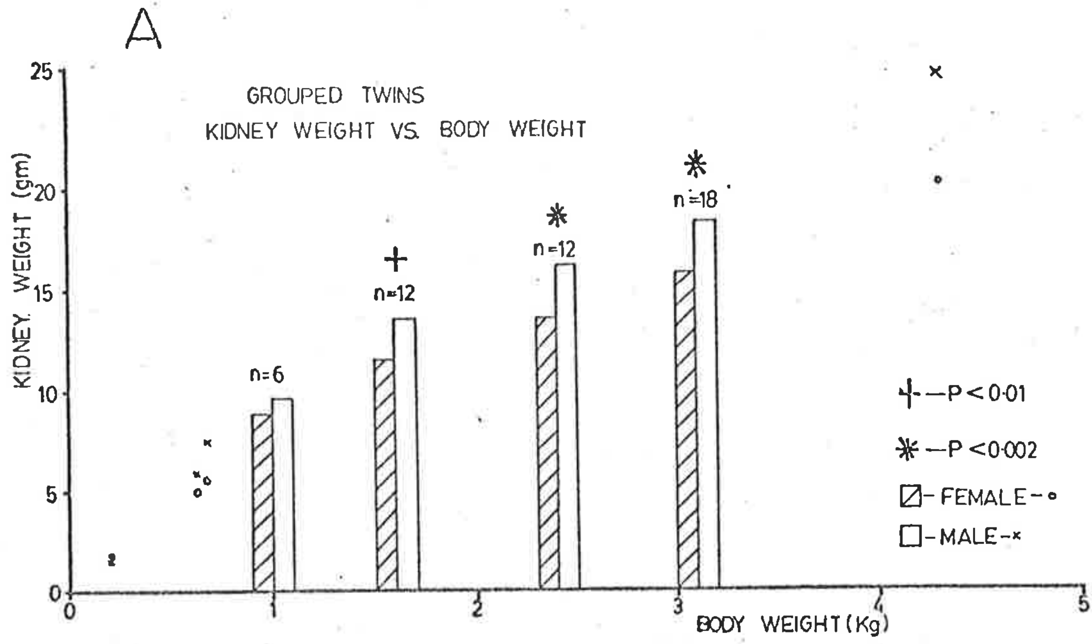
The paired barographs represent the mean kidney weights of male and female fetuses within given weight ranges (0.8 - 1.19, 1.2 - 1.99, 2.0 - 2.69, 2.7 - 3.5 kg) and n is the number of twins in each group. Using a paired t-test the weights of male and female kidneys were significantly different in three groups.

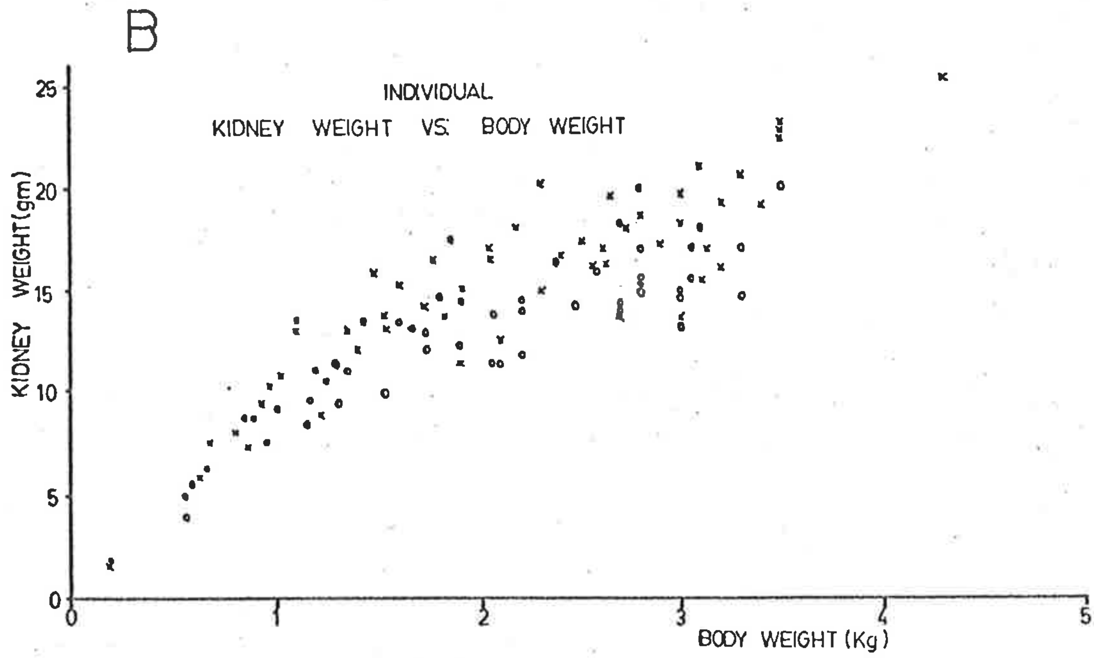
B. Individual Kidney Weight vs Body Weight

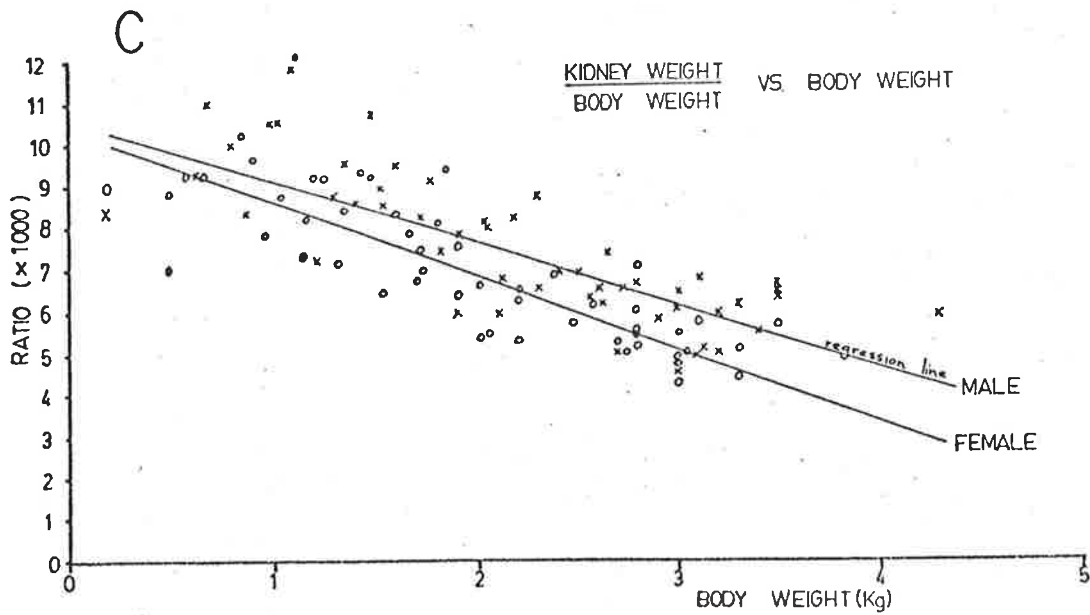
The data from table 9 plotted as 104 individual fetuses rather than as twin pairs.

C. The Ratio of Kidney Weight per Body Weight as 104 Individual Fetuses (52 twins)

The regression lines are not significantly different in slope, but show that kidney weight per body weight decreases with increasing gestational age.


A
GROUPED TWINS
KIDNEY WEIGHT VS. BODY WEIGHT
KIDNEY WEIGHT (gm)
BODY WEIGHT (Kg)
n=6
n=12
n=12
n=18
—P<0·01
—P<0·002
- FEMALE - o
- MALE - x


B
INDIVIDUAL
KIDNEY WEIGHT VS. BODY WEIGHT
KIDNEY WEIGHT (gm)
BODY WEIGHT (Kg)


C
KIDNEY WEIGHT / BODY WEIGHT VS. BODY WEIGHT
RATIO (×1000)
regression line
MALE
FEMALE
BODY WEIGHT (Kg)

male and female kidney weights, at any particular body weight there are more female kidneys below the mean kidney weight than males. This trend was shown more clearly in figure 12.C. where the ratio of kidney weight per unit body weight was plotted against body weight. A linear regression analysis performed separately on all male and all female fetuses in the 52 twins gave the regression lines as shown in figure 12.C. Although the slopes of the regression lines are not significantly different, the position of the lines indicates the trend that even on a weight per unit body weight basis, the male kidneys are heavier than females. From the regression lines it was estimated that the difference in kidney weight between male and female fetuses of the same weight was about 6% when fetal body weight was 1Kg, increasing with increased body weight to about 17% at 3Kg body weight.

A comparison was also made of the kidney weight per unit body weight for male and female co-twins using a paired t-test. This gave a value of $t=3.8$, which with 51 degrees of freedom is significant at $P<0.001$.

DISCUSSION

The results indicate that a sex difference exists in the kidney weight of fetal sheep, with the kidney weight per unit body weight in males being heavier than in females. From the regression analysis depicted in figure 12C it was estimated that the sex difference in kidney weight between fetuses of the same body weight was about 6% at 1Kg body weight and 17% at 3Kg. These percentage differences in kidney weight between sexes are comparable to those reported for other species, such as the rat, guinea-pig, dog and man, where there is approximately a 10% difference (for review see Kochakian,1977). In mice the difference is even greater, being approximately 40%. It has been established via experiments involving castration and androgen administration that this sex difference in kidney weight is due to the protein anabolic action of androgens on the kidney (review Kochakian,1977). The sex difference in kidney weight seen in fetal sheep may also be due to the action of androgens. Testosterone is present in fetal sheep testes at 30 days of gestation (Attal, 1969) and testicular secretion of testosterone results in a higher level of plasma testosterone in males than females at about 70 days of gestation (Pomerantz and Nalbandov (1975)). This early appearance of higher testosterone levels in the male fetus is consistent with the results, which indicate that a sex difference in kidney weight is apparent at 95 days of gestation.

In mammalian development there is an intimate association between the ontogeny of the renal and reproductive system; the gonads developing from a fold on the surface of the mesonophros, and the Wolffian duct being of mesonophric origin; and the Mullerian duct is said to be of pronephric origin (Witschi, 1959). In the male, differentiation of the external genitalia, under the influence of testicular testosterone secretion, begins at about day 45 of gestation (Attal,1969). Clarke et al,(1976)suggest that the morphological differentiation of the external genitalia of both sexes is virtually complete by about 70 days of gestation, and thus the sexual dimorphism of the urethra

must also be established by this stage of gestation. The kidney is also well developed by this stage, having the functional capacity to produce urine at 60 days of gestation (Alexander et al, 1958). Thus both the renal and reproductive systems develop relatively early in gestation, and the androgens which cause differentiation of the male genitalia, may also begin to influence the growth of the fetal sheep kidney as early as 45 days of gestation. However, due to large variability in kidney weights, and the smaller percentage difference in weight at younger gestational ages, a very large number of younger fetuses would be required to ascertain any sex difference in kidney weight prior to 95 days gestational age.

Pomerantz and Nalbandov (1975) found that testosterone levels in male and female fetuses change during gestation. The plasma levels of testosterone in males was assayed at 658$\pm$145 pg/ml at about 70 days of gestation falling to 279$\pm$51 pg/ml at about 100 days of gestation after which it rose to 866$\pm$141 pg/ml near term. In females the levels were consistently lower than in males, and were reported to be 112$\pm$20 pg/ml at about 70 days of gestation, rising steadily to 459$\pm$223 pg/ml near term. These authors found that the female gonads contained no testosterone, and stated that the increasing levels of testosterone were most likely to be of adrenal and/or placental origin. They also found that testicular concentration of testosterone decreased continually from 70 days till term, however, due to increasing testicular weight, sufficient testosterone was still secreted throughout gestation for the levels in male plasma to remain consistently above those in females. (It should be noted that fetal plasma testosterone levels never reach the 1-20 ng/ml observed in adult rams; Katongole et al, 1974). The results indicate that the sex difference in kidney weight increases with increasing gestational age. In view of the higher testosterone levels in the male fetus throughout gestation, this result is consistent with the male fetal kidney responding to androgens with an increased rate of growth. Thus even though

the female fetus has increasing levels of testosterone which may influence kidney growth in the female, it may be that the rate of increase in the male kidney is slightly larger than the female due to the higher circulating levels of testosterone in the male.

Another characteristic of fetal sheep kidney weight is the high degree of variability in the weight of both male and female kidneys. It seems reasonable to assume that genetic variation would result in a variation in kidney weight between individual fetuses similar to that which occurs in body weight, or crown rump lenght, or adrenal weight, or heart size, all of which exhibit variability (Cloete,1939). However, in a normal population the variability in these parameters is not as great as that which is apparent in fetal kidney weight, suggesting that an additional source of variability may be influencing fetal kidney weight. In this regard it is of particular interest that fetal testicular and plasma testosterone levels also exhibit a high degree of variability (Attal,1969; Strott et al, 1974b;Pomerantz et al, 1975). Thus knowing that testosterone is present in the circulation of both male and female fetuses and that its level is highly variable in both sexes, it may be that the large variation in fetal sheep kidney weight is the result of the combination of both genetic and androgenic influences.

From figure 12.C it is apparent that fetal kidney weight per unit body weight decreases as body weight increases. This arises due to the approximately linear increase in kidney weight in relation to gestational age (c.f. crown-rump length) whereas fetal body weight increases more rapidly at a non-linear rate, especially during the third trimester.

CHAPTER VI

IDENTIFICATION OF PROGESTERONE METABOLITES IN FETAL SHEEP PLASMA

VI.a. Introduction

The anephric human fetus (Potter, 1965) and the surgically nephrectomised sheep fetus (Thorburn, 1971a) are both born prematurely indicating that those factors which are responsible for the maintenance of pregnancy have been jeopardised. Progesterone production in increasing amounts is essential for the maintenance of pregnancy in both women and sheep (Bedford et al, 1972b) and in those fetuses without kidneys the site of progesterone production, the placenta, is markedly reduced in size, suggesting that progesterone production may also be altered.

The fetal kidney metabolises progesterone having a particularly active 5α-reductase (111.c.v.) which as well as acting on androgens also converts progesterone to 5α-pregnanedione. The level of progesterone within the fetus is a complex function of production, release into the circulation and metabolism by a variety of enzymes both in the blood and in tissues. In the fetal sheep liver the predominant catabolic pathway for most steroids involves reduction by 5β-reductase and 3β-HSD; progesterone being converted to pregnanediols. Pregnanediol is formed exclusively from progesterone and DOC, and the high level of placental progesterone production would completely obscure the miniscule contribution that DOC makes to the level of pregnanediols in the fetal circulation.

To ascertain the level of progesterone metabolism performed by the fetal kidney, the pregnanediols in fetal plasma have been identified to see if 5α-reduced pregnanediols form a significant portion of those present. The plasma pregnanediols in entire and nephrectomised fetuses have also been examined for changes in the level of isomers present.

VI.b. Methods

VI.b.(i) Animals

Merino crossbred ewes of known mating date were obtained from the University of Adelaide's Experimental Farm at Mintaro, South Australia. The pregnant ewes were brought to the Research Laboratories Animal Housing facility at the Queen Elizabeth Hospital at least seven days prior to surgery. The ewes were housed under conditions of 12 hour light and 12 hour dark at constant temperature (25°C), and maintained on 800 g of lucerne chaff daily with water ad libitum. Food, but not water was withheld for 24 hours prior to surgery. Pregnancy was usually confirmed by palpation of the abdomen and appearance of the mammary glands, and in certain instances by laparoscopy (3cm incision).

VI.b.(ii) Surgical Procedures

(a) Preparation of the Ewe

Starved ewes 90-120 days pregnant were sedated with Rompun (0.4 - 0.8ml, 0.008ml/kg) injected intra muscularly. The wool covering an area over the 3rd and 4th secral vertebra was closely clipped and then swabbed with chlorhexidine/cetrimide solution and lumbosacral anaesthesia was then induced with 10ml Lignocaine (2% Xylocaine) using a technique similar to that described by Hopcroft (1967).

As soon as the ewes lost sensory response in the hind legs all the wool covering the abdomen and area around the groin was clipped with both coarse and very fine clippers. The clipped area was then thoroughly cleaned with an antiseptic detergent solution (SAPODERM), dried with a clean towel and then liberally soaked with an alcoholic chlorhexidine/cetrimide solution. The ewe was carried from the preparation area, and placed on the operating table in the supine position on a curved slatted wooden stretcher. Once secured on the operating table the abdomen was again carefully soaked with

chlorhexidine/cetrimide solution and excess solution from the edges of the clipped area was swabbed with sterile swabs.

(b) Cannulation of Fetal Vessels

Strict aseptic techniques were maintained during all procedures. Sterile gloves and gowns were worn with hats and masks, all instruments were steam sterilized immediately prior to commencing the operation. All cannulae were sterilized (in plastic bags) with ethylene oxide and prepared prior to commencing surgery. Preparation involved placing disposable three way stop cocks on square tipped needles inserted into the catheters. The needles were tied in place with 2/0 black braided silk and labelled to identify arterial and venous. The tip of the catheter was cut at 45^0 to give a relatively blunt point to allow easier insertion into the fetal vessels. The catheters were filled with normal saline. Sterile Drapes were placed over the ewe leaving only a small area over the site of incision. The ewes head was also draped, as it was found that ewes remained more docile if not visually aware of their surroundings.

After covering the ewe with sterile cloth drapes, laparotomy was performed with an infraumbilicular longitudinal incision (11-12cms) approximately 3cms lateral to the mid-line. The veins draining the mammary glands traverse the mid-line in this region making the mid-line an unsuitable site for incision. The scapel blade (No 12) was wiped with a sterile gauze swab to remove any blood. The fetus was palpated and the hind legs grasped and the uterus and fetus were carefully positioned so that an area of the crest of the pregnant horn (least vascular) was brought adjacent to the abdominal incision by carefully pulling on the uterus with sterile swabs and pushing the lower half of the fetus' hind leg against the inside surface of the uterus. Careful choice of an intercotyledonary area of the uterus and correct positioning of the fetus were considered an essential aspect of obtaining a good prepar-

ation. A small (1cm) incision was made in the uterus and Allis clamps carefully positioned either side. The incision was then enlarged (with scissors), whilst viewing through the incision any small vessels on the inside of the uterus, making sure not to cut any vessels. The fetal hind quarters were brought through the incision, the pulse in the femoral artery palpated and using a clean scapel blade a 3-4cm incision was made on the inside surface of the leg toward the groin. Connective tissue surrounding the underlying muscle was removed and by blunt dissection the connective tissue between two larger bundles of sketetal muscle fibres were parted to reveal the femoral artery, vein and nerve. Both the artery and vein were cannulated using Portex vinyl 1 tied in place with 2/0 black braided silk, after tying the cannulae in place their patency was checked by infusing saline. The incision was closed with a purse string suture using 4/0 chromic (Ethinon) or 5/0 Dexon. The cannulae were then tied (4-6 times) to the fetal skin with 2/0 black braided silk to reduce the risk of the catheters being accidently pulled out by fetal movements. The fetal hindquarters were replaced in the uterus and the uterine incision closed with a purse string suture of 2/0 dexon and oversewn after introducing about 20cms of cannulae into the amniotic fluid to allow for fetal movement. The uterus was placed back in the abdominal cavity and the fetus positioned the same as it was when found. The peritoneum and muscle was closed with 0-DEXON and oversewn, the wound was then swabbed if any blood was present, and then sprayed with Neotracin (an antibiotic aerosol) and then the skin was closed with a continuous purse string suture using 0-DEXON. The cannulae were brought out through the abdominal wound and held in place by suturing to the abdominal skin and to the flank of the ewe. A canvas sash was placed around the ewe and the cannulae led under this and into a small canvas pouch attached to the sash. The ends of the cannulae were plugged with plastic stoppers in the 3 way stop cocks all of which were sprayed with a dilute iodine solution (70%V/V,ethanol/water) and placed in a sterile plastic bag.

(c) Fetal Nephrectomy

Some fetuses were nephrectomised and cannulated. When fetuses were nephrectomised and cannulated, nephrectomy was performed first. The procedure is similar to that reported briefly by Thorburn (1974) and uses an approach similar to that reported by Drost (1968) for bilateral adrenalectomy.

All procedures leading to the delivery of the fetal hind legs were the same as for cannulation. Both hind legs were brought through the uterine incision, which means that extreme care must be taken to prevent the umbilical cord from being traumatised. The position of the kidney was determined by palpation and an incision (3cm) made in the skin directly over it; overlaying fetal abdominal muscle was parted by blunt dissection, the peritoneum cut and connective tissue and perirenal fat was cleared from around the kidney. A ligature (2/0 black braided silk) was passed around the hilus of the kidney, the kidney exteriorised, the ligature tied, and the kidney excised. The ligature was checked to confirm no bleeding occurred, and the fetal abdominal incision closed with a single continuous purse string suture (5/0 DEXON) through the muscle and peritoneum. The skin was similarly sutured. During this entire procedure the fetus was kept moist and warm by intermittently changing swabs soaked in sterile saline kept at 45°C, and wrapping these around exposed areas of the fetus. After both kidneys were removed, the fetus was cannulated. Those fetuses which were to act as controls were sham operated; fetal laparotomy was performed, but the fetal kidneys were not removed.

(d) Post-Operative Care

Immediately after surgery the ewe was returned to her pen (slatted wooden floor) and given food and water. Ewes were not kept in tight confinement in metabolism cages as it was invariably found that they lost condition

rapidly with subsequent loss of the preparation. The use of a canvas sash with a small pouch (containing the cannula ends) allowed the ewe free movement, without unduly risking the preparation due to damage to the catheters. As the main cause of fetal death was due to infection by Streptococous faecalis and Pseudomonas aeruginosa, the fetus was given benzyl pennicillin (10^5IU) by arterial infusion in heparinised saline, and the ewe benzyl penicillin (10^6IU) by intra muscular injection, immediately after surgery.

Both arterial and venous cannulae were flushed morning and night with 5ml of heparinized saline (100IU/ml). Strict aseptic procedures were adhered to when handling the cannulae to minimise the risk of post operative infection. Sterile syringes and solutions were used, hands were rinsed in dilute ethanolic iodine solution, and after sealing the cannulae ends, these and the plastic bag were also sprayed lightly with the iodine solution, excess solution was drained.

(e) Blood Samples

Blood samples were not taken from fetal cannulae until at least five days after surgery to allow for recovery of the ewe and fetus from the stress of the operation. There are many reports of gross changes in physiology of the fetus due to surgical intervention, and in some cases (fetuses over 120 days gestational age) the fetuses do not fully recover and go into premature labour (Bassett and Thorburn, 1969); Mellor, Slater, and Cockburn (1971); Comline and Silver (1970); Mellor and Slater, (1971, 1972); Gresham et al, (1972)).

Samples were collected on alternative days between 9-10 a.m. Blood (10ml) was withdrawn slowly and collected into heparinised tubes, placed on ice, and centrifuged within 5 min of collection. The plasma was aspirated

and stored at $-4^{o}C$ until assayed for pregnanediols.

VI.b.(iii) Extraction of Pregnanediols

Steroids present in fetal plasma exist predominantly as sulphoconjugates (Wong et al. 1972), Seamark (1973) also reported that pregnanediols exist as conjugates. Sulphoconjugated pregnanediols in fetal plasma were released as free steroids by acid hydrolysis (Klopper, 1962). 1 ml of fetal plasma was diluted with 1 ml of saturated NaCl solution, 2 ml of ethyl acetate was added and 100μl of 55% H_2SO_4 (giving pH1.0-1.1). This was immediately vortexed, and heated in a shaking water bath at $37^{o}C$ for 18 h , centrifuged to break emulsions, and the organic phase aspirated. The aqueous phase was re-extracted with 2 ml of ethyl acetate and the organic phases combined, 2 ml of 8% $NaHCO_3$ was added and vortexed to neutralize acid in the ethyl acetate. The ethyl acetate was finally washed with 2 ml of water and blown to dryness (N_2). at 37°.

VI.b.(iv) Derivatisation for GC and GC-MS

Methoxyamine-trimethylsilyl derivatisation by the method of Thenot et al (1972) and Axelson et al (1974). To the dried down extract 50μl of methoxyamine -HCL (100mg/ml in pyridine) was added and heated at $60^{o}C$ for 30 minutes. The pyridine was blown to dryness under N_2 at 45° and 50μl of imidazol-TMS was added and heated for 120 min at $100^{o}C$ after vortexing vigorously. This was allowed to cool and then 0.5ml of solvent (cyclohexane:pyridine:HMDS; 98:1:1;v/v) was added and vortexed with the derivatised samples. This was run of Lipidex 5000 (1.5 ml in 2ml column (12cm long)). This removes the reagents and the derivatised sample is eluted with a further 1.5 ml of solvent. The derivatised sample is blown to dryness under N_2 and redissolved in 70μl of n-hexane ready for injection into the GC.

GC-MS was performed as previously described using a SCOT column OV-101 (18m x 0.5mm) for GC, and a DS-50 system. GC involved the use of a 0.1% NGA on

Gas chrom Q^R (100/120 mesh) operated at 216°C and 1% OV101 on the same support at 230°C, carrier gas N_2 at 30-40ml/min.

VI.c. Results

VI.c.(i) Underivatised Pregnanediols; GC on packed Column (1.0m x2mm ID, 0.1% NGA)

Previous methods for measuring pregnanediols in fetal plasma (Seamark 1973) have involved the use of GC with an NGA column, as the pregnanediols are present at several μg /ml which allows their detection by flame ionisation. Underivatised pregnanediol standards were run on a 0.1% NGA column (216°C, 1.5mm x 2mm ID, carrier gas 30-40ml/min N_2).

TABLE 11

Retention Distance of Pregnanediols on NGA

Pregnanediol	Distance (in cms)	
5β P 3α 20α	2.80	2.80
5β P 3β 20α	2.49	2.46
5β P 3α 20β	2.33	2.40
5β P 3β 20β	2.15	2.16
5α P 3β 20α	3.41	3.45
5α P 3β 20β	2.94	2.94
5α P 3α 20α	2.80	2.80

The separation achieved on this column (peaks did not tail) was insufficient for use in identifying pregnanediols, as even with lower temperatures and programmed increases in temperature, when all seven standards were run together there was incomplete separation of any peak. Identification using this previously used method was not persevered with further.

(ii) Pregnanediol - TMS; GC on Packed Column (1%, OV-101)

Results from the NGA column indicated that underivatised material would not separate on packed columns. Pregnanediol standards were derivatised with TMS to effectively exaggerate the spatial arrangement of the hydroxy groups, which

also increases the size of the molecule and changes its polarity. Thus a different liquid phase such as OV-101 was required for optimal separation. OV-101 is a relatively non polar liquid phase which interacts with the derivatised steroids in relation to their size. The results are shown in Table 12.

TABLE 12

Retention Times (min) on Packed Column OV-101

Pregnanediol		Rt.				Lower Temperature	
5β P	3β 20β	5.26	5.90	5.93	5.97	6.15	6.0
	3β 20α	6.37	6.35	6.34		6.53	
	3α 20β	6.15	6.11	6.11	6.15	6.41	
	3α 20α	6.53	6.55			6.61	6.61
5α P	3α 20α	6.41	6.43	6.42		6.85	
	3α 20β	5.57					
	3β 20α	7.94	7.88	7.93	7.91	8.12	
	3β 20β	7.48	7.48	7.47		7.97	7.79

This column gave better separation than underivatised material on NGA, however once again when all the standards were combined in a single injection all but the 5α-Pregnane-3β , 20α/β-diols were merged.

(iii) Pregnanediol - TMS, GC on a SCOT Column (OV-101)

The packed column provided insufficient resolving power to allow unequivocal identification of the pregnanediols present in fetal plasma. Use of a SCOT column which has a far greater resolving capacity was indicated. Use of a column with the same stationary phase meant that the pregnanediols would have similar relative retention properties on the SCOT column as on the packed OV-101 column.

A comparison of gas chromatographic characteristics of the various steroids run on the SCOT column was made, by use of retention indices, by a method described by Kovats (1965). The retention index was calculated as methyl

equivalents by interpolation between two alkane series compounds, η-tetracosane (2400 units) and η-dotriacontane (3200 units). The values determined by this method were highly reproducible for a particular column, and were largely unaffected by minor changes in column temperature, gas flow etc. (see Table 13).

TABLE 13

Retention Indices for Pregnanediols on SCOT Column (OV-101)

Pregnanediol		Ri	(Column 1)			(Column 2)	
5β P	3β 20β	2725	2727			2665	2664
	3β 20α	2760	2758	2758			
	3α 20β	2745	2747			2682	2681
	3α 20β	2781	2783	2784	2784	2711	2710
5α P	3α 20α	2766	2764			2698	2696
	3α 20β	2743	2743				
	3β 20α	2873				2784	2783
	3β 20β	2845				2760	2760

The SCOT column gave far better resolution than either of the packed columns, giving complete baseline separation of almost all the standards when run together, (see figure 13, trace 1). Four of the pregnanediols formed two peaks with distinct shoulders, and virtual baseline separation, with 5α/β-Pregnane-3α, 20β-diols running close together but separating from 5α-Pregnane-3α, 20α-diol and 5β-Pregnane-3β, 20α-diol which also ran together. These pregnanediols were chromatographed repeatedly and were spiked with one of the closely running pregnanediols in both combined peaks to provide a definite indication of the relative retention times. Both sets of peaks had distinct shoulders and gave highly repeatable retention indicies indicating that unknown pregnanediols could be identified with a high degree of confidence.

(IV) Retention Indicies of Plasma Peaks

Plasma was collected from a fetus of approximately 122 days gestational age, obtained from the abattoirs. The plasma was solvolysed, extracted,

FIGURE 13

GAS CHROMATOGRAPHY OF DERIVATISED PREGNANEDIOLS ON A SCOT COLUMN (OV-101)

Trace 1 5α/-5β-Pregnanediols (5α-Pregnane-3α, 20β-diol not run).

Trace 2 - The same pregnanediols as run in Trace 1, however some of the peaks have been spiked with more authentic steroid (-TMS).

Retention Index	Pregnanediol
2664	5β-Pregnane-3β, 20β-diol
2681	5β-Pregnane-3α, 20β-diol spiked
shoulder to 2696 is	5β-Pregnane-3β, 20α-diol
2696	5α-Pregnane-3α, 20α-diol
2710	5β-Pregnane-3α, 20α-diol
2760	5α-Pregnane-3β, 20β-diol spiked
2784	5α-Pregnane-3β, 20α-diol

Trace 3 - Fetal (DAY 122) plasma pregnanediols (plasma obtained by cadiac puncture of fetuses obtained from local abattoirs).

2664 = 5β-Pregnane-3β, 20β-diol, 2691 = 5β-Pregnane-3β, 20α-diol

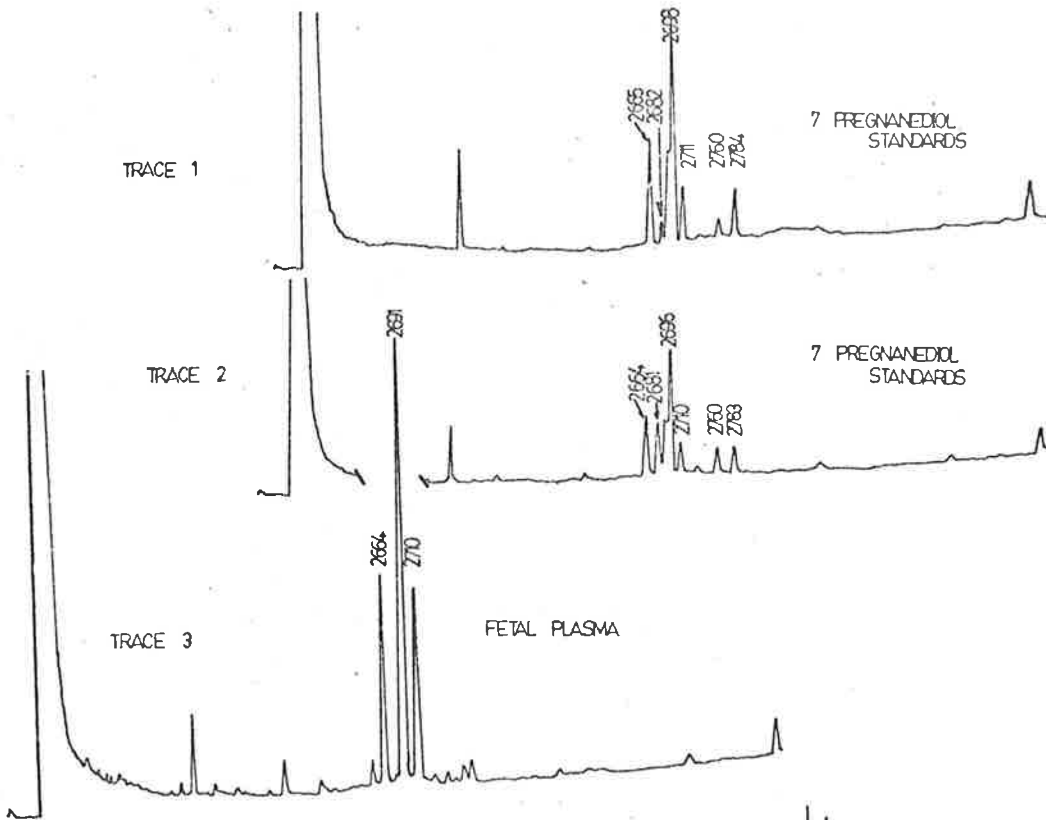
2710 = 5β-Pregnane-3α, 20α-diol

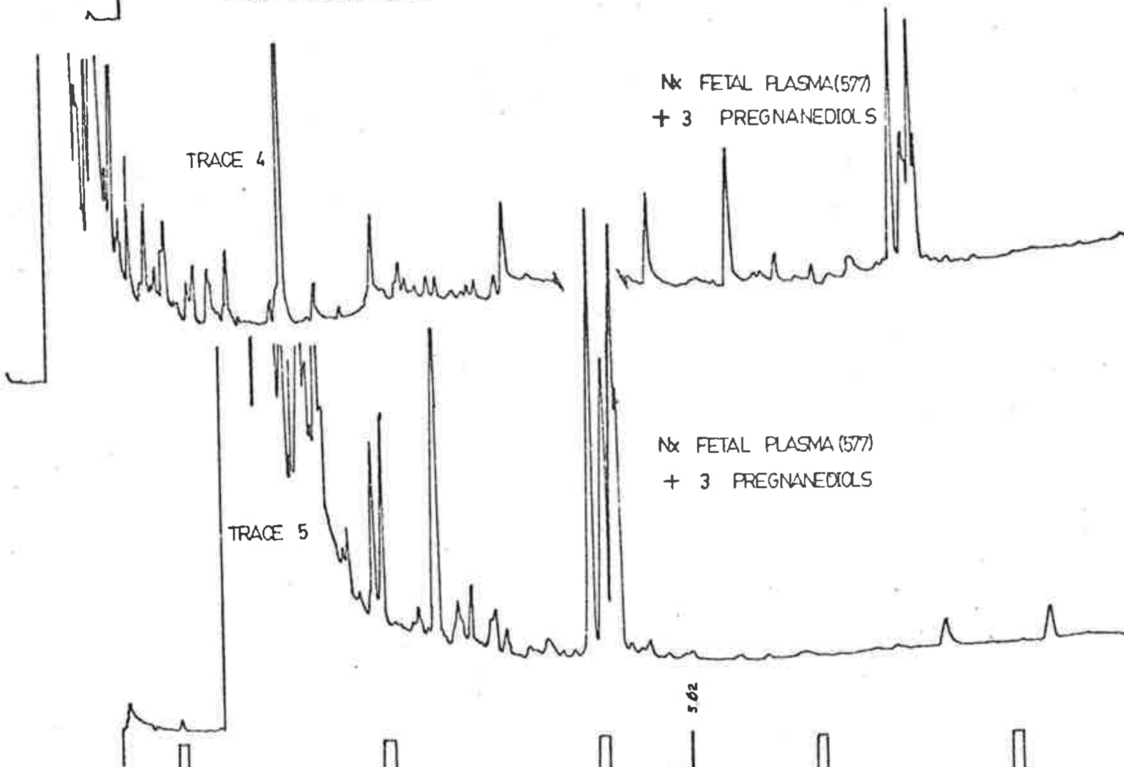
Trace 4 and 5 - Nephrectomised fetal (DAY 110) plasma pregnanediols (see text). The two major peaks are from the fetal plasma extract, and the lower peaks are 5β-Pregnane-3α, 20β-diol, 5α-Pregnane-3α, 20α-diol and 5α-Pregnane-3α, 20β-diol; 5β-Pregnane-3α, 20β-diol runs just in front of 5β-Pregnane-3β, 20α-diol.

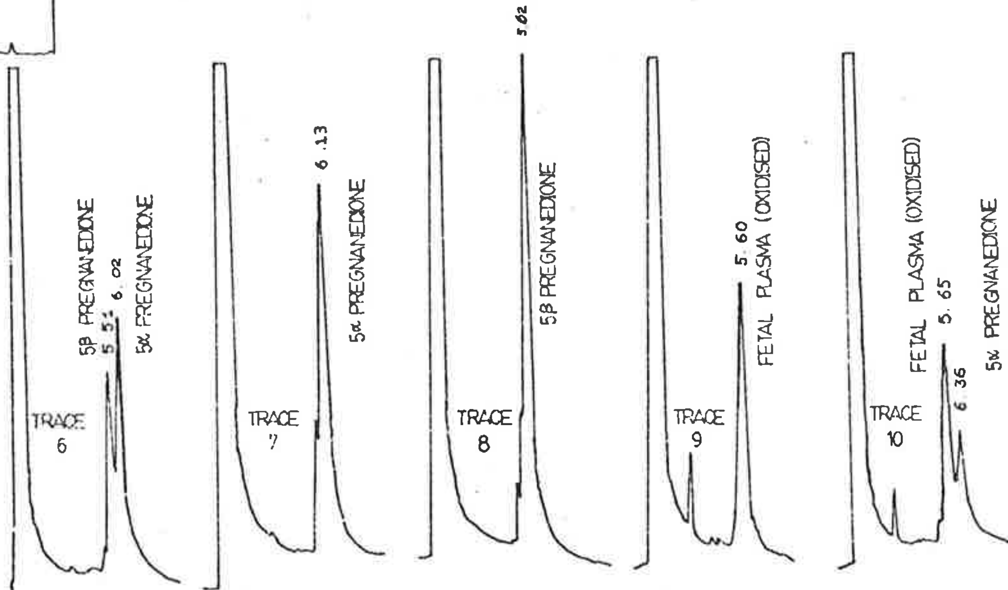
Trace 5 is the material in trace 4 rerun with addional 5β-Pregnane -3α, 20β-diol and 5α-Pregnane-3α, 20α-diol which are both separate from the plasma peak, indicating its identity is 5β-Pregnane-3β, 20α-diol.

Trace 6, 7, 8, 9 and 10 are on a packed column (OV-101).

Trace 9 shows that oxidized fetal plasma contains only one peak; and the other traces indicate that this corresponds to 5β-Pregnanedione.


TRACE 1
7 PREGNANEDIOL STANDARDS
2655
2682
2698
2711
2750
2784
TRACE 2
7 PREGNANEDIOL STANDARDS
2691
2654
2681
2696
2710
2750
2783


2664
2691
2710
TRACE 3
FETAL PLASMA
TRACE 4
Nx FETAL PLASMA (577)
+ 3 PREGNANEDIOLS
TRACE 5
Nx FETAL PLASMA (577)
+ 3 PREGNANEDIOLS


TRACE 6
5β PREGNANEDIONE
5α PREGNANEDIONE
6.02
TRACE 7
6.13
5α PREGNANEDIONE
TRACE 8
5.82
5β PREGNANEDIONE
TRACE 9
5.60
FETAL PLASMA (OXIDISED)
TRACE 10
FETAL PLASMA (OXIDISED)
5.65
6.36
5α PREGNANEDIONE

derivatised and run on the SCOT column (figure 13, trace 3). Only three major peaks were present in the area corresponding to pregnanediols; all gave clean baseline separation with no shoulders to the peaks, all appeared as 5β-isomers, and had the same retention indicies as 5β-Pregnane-3β, 20β-diol, 5β-Pregnane-3β,20α diol, and 5β-Pregnane-3α, 20α-diol.

Plasma from a nephrectomised fetus (577, 110 days gestational age) was similarly prepared and chromatographed indicating that two major peaks were present which had retention indicies the same as 5β-Pregnane-3β, 20β-diol, and 5β-Pregnane-3β, 20α-diol. This derivatised extract was repeatedly chromatographed after the addition of the 3 standards which had retention indicies similar to 5β-Pregnane-3β, 20α-diol. The 5α-Pregnane-3α, 20β-diol and 5β-Pregnane-3α, 20β-diol clearly separated from the peak in the nephrectomised plasma, as did 5α-Pregnane-3α, 20α-diol which although not clearly separating from the plasma peak formed a distinct slower running shoulder (see figure 13, trace 4,5). Plasma from other nephrectomised fetuses was also prepared and chromatographed and gave a pattern similar to that of the normal fetus.

(V) Comparison of Oxidised Plasma Peaks with Pregnanediones

In both the nephrectomised and control fetuses the data from retention indicies indicated that only three pregnanediols were present (see above) and that these were all 5β-isomers. A portion of the extract of solvolysed material from the plasma used in figure 13, trace 3 was oxidised using Brown's oxidant (Brown et al, 1971).

This oxidised plasma extract and 5β/5α-pregnane-3, 20-dione were chromatographed repeatedly on the packed column (OV-101) (see figure 13, traces 6, 7, 8, 9, and 10). Only a single major peak was present in the oxidised

FIGURE 14

GAS CHROMATOGRAPHY OF METHOXY AND BUTOXY DERIVATIVES

All the traces are of material run on a SCOT column (OV-101).

Trace 1 - Pregnenolone - methoxy derivative.

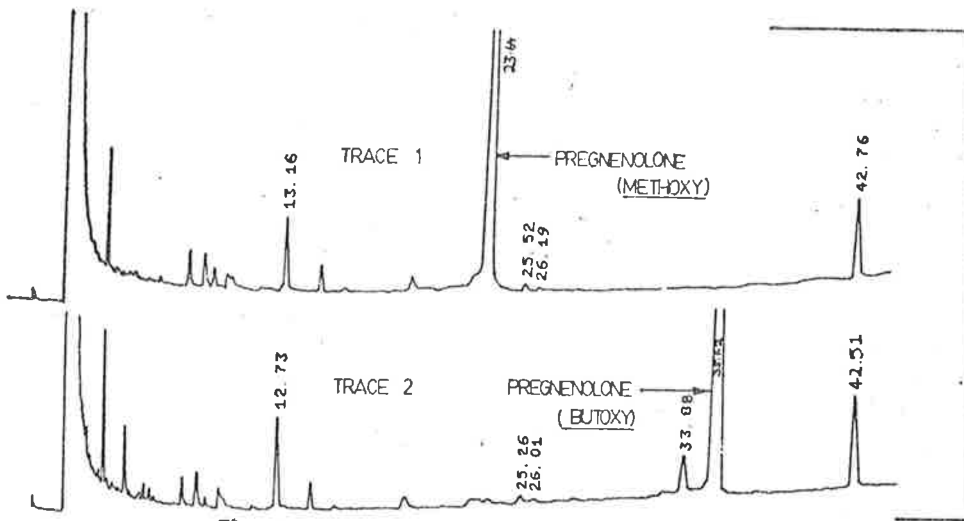
Trace 2 - Pregnenolone - butoxy derivative (much slower running due to the presence of the larger butoxy group).

Trace 3 - Plasma from a nephrectomised fetus (577), treated with methoxy amine HCl.

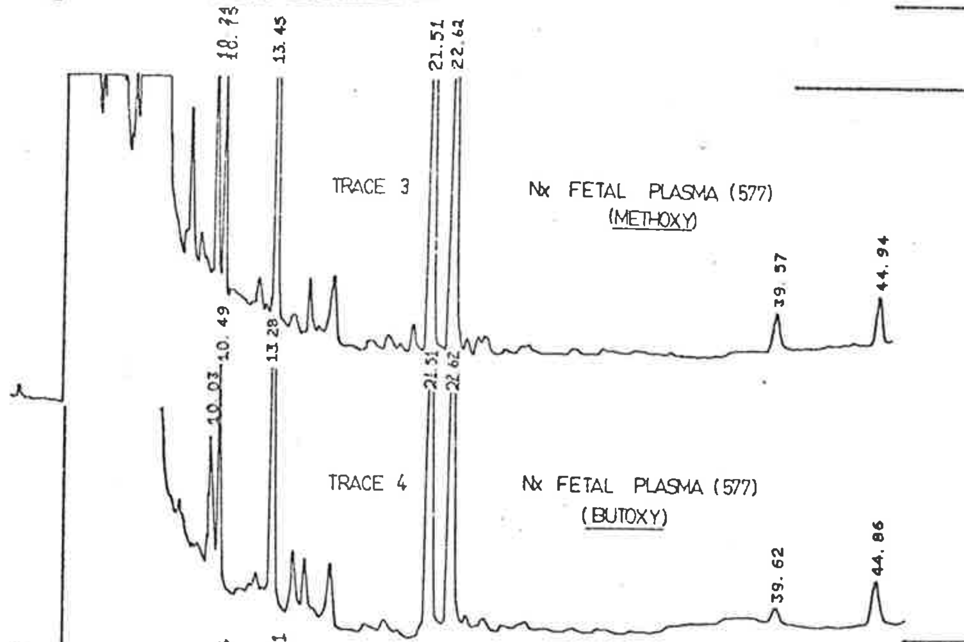
Trace 4 - Plasma from a nephrectomised fetus (577), treated with butoxy amine HCl. There is no marked increase in the retention time indicating a lack of any keto groups on the material which forms the peaks in the trace.

Trace 5 - Normal fetal plasma treated with methoxy amine HCl.

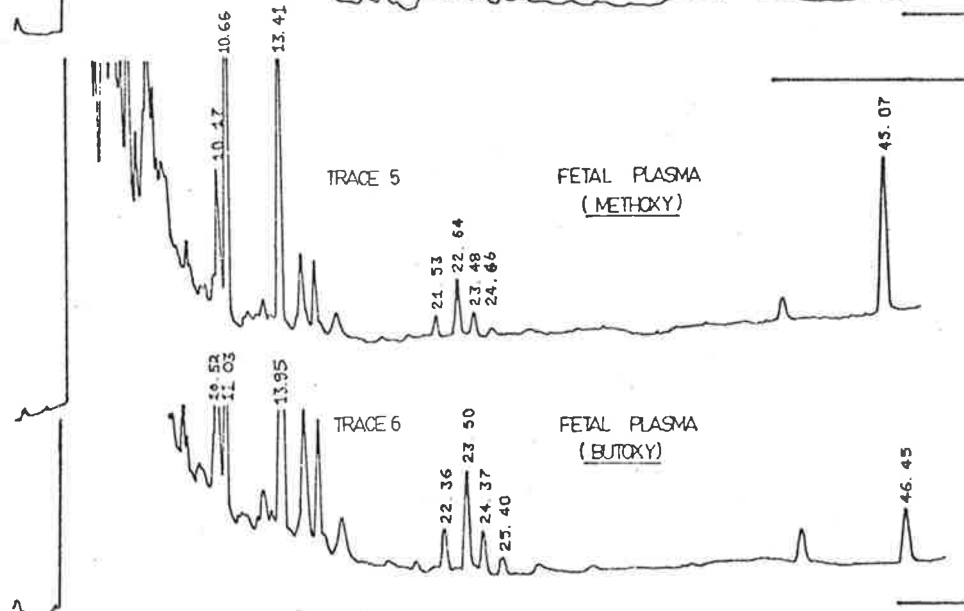
Trace 6 - Normal fetal plasma treated with butoxy amine HCl. no marked increase in retention time, hence in keto groups.



TRACE 1
PREGNENOLONE
(METHOXY)
13.16
25.52
26.19
42.76
TRACE 2
PREGNENOLONE
(BUTOXY)
12.73
25.26
26.01
33.88
42.51



13.45
21.51
22.62
TRACE 3
Nx FETAL PLASMA (577)
(METHOXY)
39.57
44.94
10.49
13.28
10.03
21.51
22.62
TRACE 4
Nx FETAL PLASMA (577)
(BUTOXY)
39.62
44.86



10.66
13.41
10.17
TRACE 5
FETAL PLASMA
(METHOXY)
45.07
21.53
22.64
23.48
24.66
13.95
TRACE 6
FETAL PLASMA
(BUTOXY)
22.36
23.50
24.37
25.40
46.45

cf legend for figure 13.

plasma extract, and this had the same retention time as 5β-pregnanedione, and clearly separated from 5α-pregnanedione; supporting the previous data that the major peaks present are all 5β-pregnanediols.

(VI) Methoxy Versus Butoxy Derivatives

It is possible that the peaks may be 5β-pregnane-ol-ones, some of which have similar retention indicies to the pregnanediols. To ascertain whether the major peaks in both the nephrectomised and normal plasma contain any keto groups, the solvolysed extracts were halved. One half was reacted with methoxyamine -HCl, and the other half with butoxyamine -HCl, and then both were reacted with imidazol-TMS. The butoxy derivative is much larger than the methoxy, thus if a keto group is present on the molecule the retention index of the butoxy derivative will be much greater, as per pregnenolone (figure 14, trace 1 and 2). The butoxy and methoxy treated extracts from the nephrectomised and normal plasma were chromatographed on the SCOT column, and there was no chage in the retention indicies of the major peaks indicating that they do not contain any keto groups (figure 14, traces 3, 4, 5 and 6). This is consistent with the peaks being 5β-pregnanediols.

(VII) GC-MS of Plasma Peaks

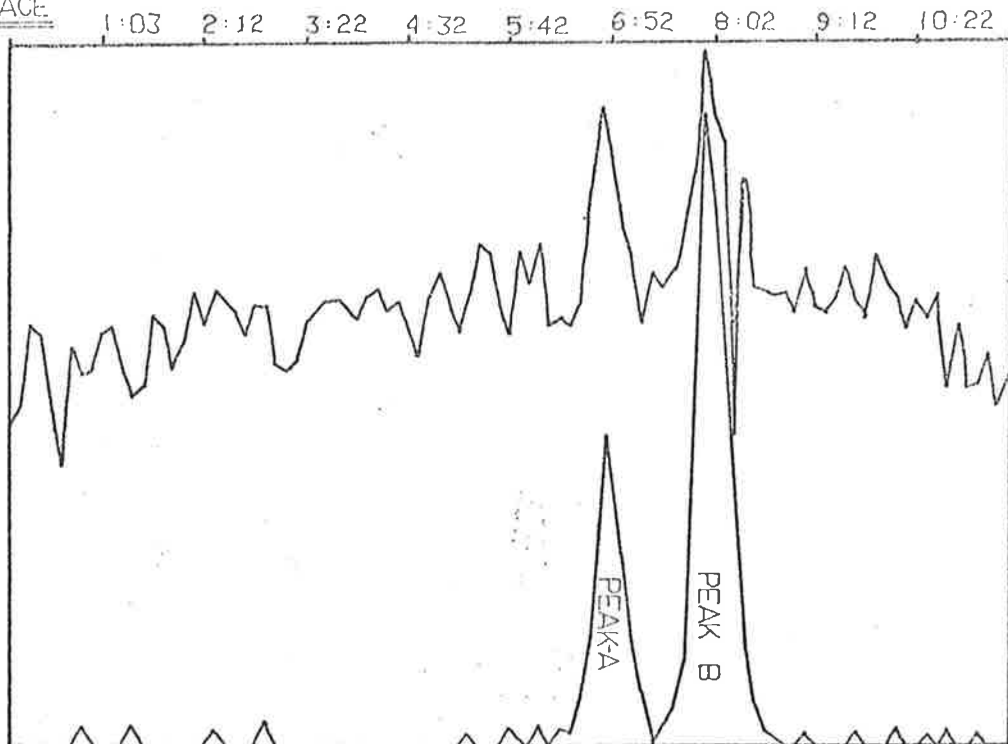
Plasma (10ml) was pooled from all the samples collected from the nephrectomised fetus 577. The plasma steroids were solvolysed, extracted, and derivatised (MO-TMS) and then run on GC-MS using a SCOT column interfaced to the MS, and the MS operated with a DS-50 system. The plasma from fetus 577 was used, as the level of the major peaks in this plasma appeared much higher than those of other plasma and would therefore give greater ion intensities. After injecting the derivatised extract, a 15 minute delay was allowed before starting the DS-50 scans. Only two major peaks were present, and these had similar retention times to the pregnanediols. The

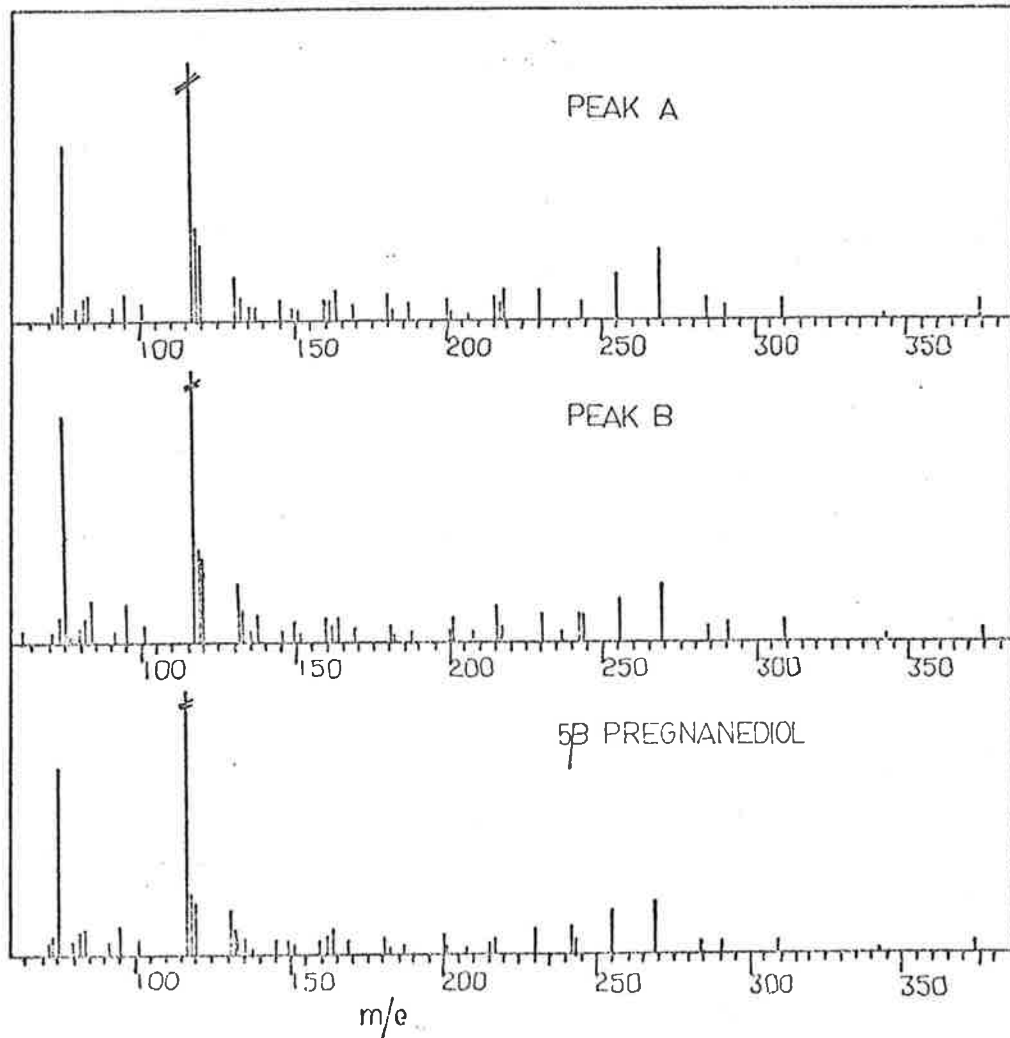
FIGURE 15

GC-MS OF FETAL PLASMA PREGNANEDIOLS

All the data is from the DS-50 system.
A 15 min delay preceeds start of the GC trace. The material in this sample was obtained from pooled plasma taken from the nephrectomised Fetus 577. Two major peaks are present (compares well with figures 13 and 14).

The mass spectra indicate peaks at m/e 374 and 284 in the material that forms both peaks, these peaks are characteristic of pregnanediols. The spectra are similar to 5β-Pregnane-3α, 20α-diol providing evidence that the material in both peaks in nephrectomised plasma is also pregnanediol.


GC - TRACE
1:03
2:12
3:22
4:32
5:42
6:52
8:02
9:12
10:22
PEAK-A
PEAK B


MASS SPECTRA
PEAK A
PEAK B
5β PREGNANEDIOL
100
150
200
250
300
350
m/e

mass spectral traces of both these peaks revealed peaks at 284, and 374. The MWt of pregnanediol with two+TMS groups is 464, thus 374 = M^+ -TMS-OH and 284 = M^+ -2(TMS-OH). These peaks occur in the pregnanediol standard and are characteristic of pregnanediols. Thus the mass spectral data confirms that the two major peaks in the nephrectomised plasma are pregnanediols (see figure 15).

DISCUSSION

The combined evidence of GC retention indicies, chromatographic properties of the oxidation products, lack of derivatisation with butoxyamine-HCl, and the mass spectral data provide compelling evidence of the presence of three pregnanediols in fetal sheep plasma, which are 5β-Pregnane-3β, 20β-diol, 5β-Pregnane-3β, 20α-diol, 5β-Pregnane-3α, 20α-diol.

In fetal sheep the presence of 5β-Pregnane-3α, 20α-diol (at about 1ng/ml) has been previously reported by Seamark (1973); he also found that 5β-Pregnane-3α, 20β-diol was present, but at lower levels than 5β-Pregnane-3α, 20α-diol. Seamark et al (1973) did not report the presence of 5β-Pregnane-3β, 20β-diol or 5β-Pregnane-3β, 20α-diol which is unusual considering that the results in this chapter indicate that 5β-Pregnane-3β, 20β-diol is present at a similar level to 5β-Pregnane-3α, 20α-diol and 5β-Pregnane-3β, 20α-diol at levels almost twice as much as 5β-Pregnane-3α, 20α-diol. No 5β-Pregnane-3α, 20β-diol was detected, however at the levels (<100ng/ml) indicated by Seamark et al (1973) this would be close to the limit of detection by the method used in this chapter.

Seamark et al (1973) used packed columns (NGA) for gas chromatography in identifying the pregnanediols in fetal sheep plasma. On NGA, 5β-Pregnane-3α, 20β-diol has a retention value similar to 5β-Pregnane-3β, 20β-diol; and 5β-Pregnane-3β, 20α-diol and 5β-Pregnane-3α, 20α-diol have peaks which are completely merged.

The experiments reported in this chapter indicate that the packed columns have insufficient resolving capacity to allow an accurate identification of the pregnanediols when these are mixed together as in a sample of fetal plasma. The high degree of structural, chemical and chromatographic similarity between all the 5α/5β-Pregnane-3, 20-diols highlights the necessity for sophisticated and powerful analytical techniques to be assured of correctly identifying these isomers in a biological sample.

The relative peak heights of the pregnanediols indicates that 5β-Pregnane-3β, 20β-diol, 5β-Pregnane-3β, 20α-diol and 5β-Pregnane-3α, 20α-diol are present respectively in the ratio of 1:2:1. 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol have both 3β-hydroxy groups, and 5β-Pregnane-3β, 20α-diol and 5β-Pregnane-3α, 20α-diol both have 20α-hydroxy groups, indicating that approximately 75% of all the pregnanediols contain a 3β-hydroxy group and 75% contain 20α, and the major metabolite is 5β-Pregnane-3β, 20α-diol. All the pregnanediols are 5β-reduced. The results strongly indicate that the major site of progesterone metabolism within the fetus is the liver. The pregnanediols that are formed mirror the most active enzymes found in the liver (Anderson et al, 1970b), where the major metabolites are 5β, 3β, reduced; and in the blood where 20αHSD is extremely active. Thus the major metabolite 5β-Pregnane-3β, 20α-diol is consistent with the most active enzymes known to be present in both the blood and liver.

5β-Pregnane-3β, 20α-diol as the major metabolite in fetal plasma contrasts with the results from maternal metabolism of progesterone, where 5β-Pregnane-3α, 20α-diol is the major urinary metabolite of progesterone infused into the jugular vein of the ewe (Stupnicki et al, 1968). No information is available about the relative distribution of placental progesterone between ewe and fetus, or of the relative importance each plays in the total metabolism of progesterone during gestation.

No 5α-Pregnanediols were identified in fetal plasma; if any were present they were estimated at less than 1% of the 5β-Pregnanediols present in "normal" fetal plasma. The fetal kidney has an active 5α-reductase (see Chapter IV), but the presence of a 5β-reductase has not been demonstrated. The lack of any detectable amounts of 5α-Pregnanediols in fetal plasma indicates that progesterone metabolism by the kidney is small in relation to the total progesterone metabolism by the fetus. This is supported by the result that the same pregnanediols were present in the plasma of both the nephrectomised and sham operated fetuses. If the kidney was responsible for the production of one of the pregnanediols, then it would have been absent in the nephrectomised fetal plasma.

Although the same pregnanediols were present, the results indicate that their level may be much higher in the nephrectomised fetus; indicating that the fetal kidney may be indirectly involved in progesterone metabolism. The GC traces shown in figure 14, of material extracted and prepared identically from both a sham operated and nephrectomised fetus, shows the height of the peaks associated with 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol is far greater in the nephrectomised fetus. Internal recovery standards for estimating procedural losses were not used, but even allowing for a large difference in losses, which is unlikely, the level of pregnanediol in fetus 577 appears to be about 10 fold higher than in the sham operated fetus.

Thus the results indicate that although progesterone metabolism by the fetal kidney is insignificant in its contribution of pregnanediols to the fetal circulation, nephrectomy causes a marked disturbance in the metabolism of progesterone, however the mechanism by which this occurs is unknown.

CHAPTER VII

PREGNANEDIOL LEVELS IN FETAL SHEEP PLASMA

VII.a. Introduction

The progesterone which is produced in large quantities (300mg/day, see Little and Billiar, 1968) by the human placenta may enter either the fetal or maternal circulation, and it has been estimated that up to 75mg/day enters the fetal circulation. In sheep it has been established that placental progesterone production is much lower than in humans, reaching a maximum of about 33mg/day (Mattner and Thorburn 1971), however no information exists on what proportion of this enters the fetal circulation. Measurement of this is greatly complicated by tissue metabolism within the fetus, reversible 20α-HSD activity in fetal red blood cells (Nancarrow 1969), and metabolism of progesterone by the sheep placenta (Pierrepoint et al, 1973). Thus measurement of fetal plasma progesterone levels provide little information about the fate of progesterone within the fetus.

The reduction of progesterone to 5β-pregnanediol by 5β-reductase within the fetal sheep liver (Anderson et al, 1970b)is irreversible, thus the level of pregnanediols in fetal plasma may give a better indication of changes in progesterone availability within the fetus. Three pregnanediols have been identified in fetal plasma (see previous chapter). Their steric configuration indicates that they are not progesterone metabolites formed by the fetal kidney, however the results of Chapter VI give a preliminary indication of a change in their level, and relative proportion in the plasma of nephrectomised fetuses. Nephrectomy appears to cause a change in the normal metabolism of progesterone, which may be associated with the premature birth characteristically seen in the nephrectomised fetus (Thorburn et al, 1971b).

The aim of experiments reported in this chapter was to measure the level of the three pregnanediols in sequential samples of fetal plasma taken from both nephrectomised and sham operated fetuses. It was hoped to confirm the preliminary observation on pregnanediol levels of the previous chapter, and by comparison with the control group of fetuses provide some insight into changes in fetal progesterone metabolism associated with the premature birth of the nephrectomised fetus.

VII.b. Methods

Animals, surgical procedures, post operative care, and collection of samples from both sham operated and nephrectomised fetuses were all performed as described in the method section for Chapter VI.

VII.b. (i) Assay for Plasma Pregnanediols

The method for assay of pregnanediols in fetal sheep plasma was adapted from that reported by Phillipou et al, (1978) for the analysis of neutral urinary steroids. A 1ml fraction of the plasma sample was used for assaying the pregnanediols. The first step involved the addition of the internal recovery standard (IRS, 1ug of 5β-Pregnane-3α, 17α, 20α-triol). Solvolysis and extraction with ethyl acetate was performed as described in Section VI b. (iii). 4-C^{14}-DHEA-SO_4 and 4-C^{14}-oestrone-SO_4 added to fetal plasma were used to assess the efficiency of the solvolysis procedure, and gave quantitative yield (>98%) as determined by the amount of radioactivity in the organic phase as a percentage of that initially added. It was assumed that pregnanediol sulphates would also be quantitatively hydrolysed in this process. In using 5β-pregnanetriol as IRS,no correction was made for any possible difference in the relative proportion of it extracted in relation to the amount of pregnanediols extracted. After being blown to dryness under N_2 the dried extract was purified on liquid - gel chromatography on Lipidex 5000.

(a) Chromatography on Lipidex 5000

The use of Lipidex 5000 for the separation of steroids has been reported previously (Apter et al, 1976; Anderson et al, 1974), this material having a potentially high resolving capacity. Optimal resolving characteristics were not required as chromatography was used to partially purify the extract by removing material less polar than the pregnanediols. Solvents of increasing polarity were used to give smaller elution volumes than previously reported.

Each column consisted of a 2ml serological pipette joined to a 7ml reservoir, and contained 1.7ml of Lipidex pre-swollen in PETROLEUM spirit (60^0-80^0): ethyl acetate (95:5) (solvent 1). The extracted sample obtained after solvolysis was blown to dryness and redissolved in 0.5ml of solvent 1, vortexed and placed on the column, another 0.5ml was vortexed in the same tube and also loaded on the column after the first 0.5ml had reached the top of the column. A further 1ml of solvent 1 was added to the tube containing the remains of the extract, vortexed and added to the reservoir. When the solvent surface reached the top of the column 6ml of solvent 1 was added to the reservoir, giving a total of 7ml of eluting solvent to form fraction 1. This fraction removed cholesterol, and lipid soluble material of low polarity and was discarded. ETHYL ACETATE (7MLS) was used to elute fraction 2 which contained the pregnanediols and the IRS. This fraction was collected for derivatisation. The columns were washed with 7ml of methanol and then re-equilibrated with solvent 1 before reuse.

24 columns were used in the assay, the elution profile using, cholesterol, 3 pregnanediols and IRS standards was highly repeatable. When 50μg of standards of the above steroids were chromatographed there was no detectable overlap of steroids between fractions, fraction 1 contained only cholesterol,

and fraction 2, the three pregnanediols and IRS; no IRS remained on the column. A pooled fetal plasma standard also gave highly repeatable elution profiles, however due to the very high levels of cholesterol in fetal plasma, some cholesterol appears in fraction 2. Fresh columns were prepared each month, and elution profiles checked. All solvent volumes were measured using 10ml Oxford Pipettors. (Oxford Laboratories, Foster City, Ca 94404 USA).

(b) Derivatisation to TMS Ethers

Fraction 2 was collected and blown to dryness under N_2 at 45°C in a stoppered test tube with a conical shaped bottom.

To the dried fraction 2, acetonitrile (100μl) BSTFA (50μl) and TMCS (50μl) were added, vortexed, and heated at 60°C for 2 hours. This was either directly used for GC or blown to dryness under N_2 and redissolved in 25-75μl of n-heptane.

MO-TMS derivatives (see section VI b. iv) were not used, there being no difference in the GC traces of column chromatographed extract from the same sample of plasma when derivatised by either procedure. Also the three pregnanediols to be measured contained no hindered hydroxyl groups, and even though the 20β-hydroxyl group reacts slower than the 3α/β or 20α-hydroxyls,it is completely reacted after 2 h at 60°C. The above procedure has the added advantage of being simpler and quicker not requiring two reactions or any further chromatographic steps, hence an increase in recovery.

(c) GC on SCOT Column (OV-101)

A Pye 104A instrument was used fitted with a splitless injection system (SGE, Melbourne, Aust.) and a 20m x 0.5mm, OV-101, glass SCOT column

(SGE, Aust.). Sample injection volumes were always less than 1.5µl. The GC conditions were as follows: injector temp. 250°; column temp.: $215-265^{o}$ programmed at 1° per minute; average linear gas flow (N_2): 16-20cms/sec.

(d) Steroid Identification

The three pregnanediols, 5β-pregnane-3β, 20β-diol, 5β-pregnane-3β, 20α -diol and 5β-pregnane-3α, 20α-diol have already been identified (see Chapter VI). Rountine identification of these pregnanediols in the fetal plasma samples was achieved by comparison of retention indices calculated as methyl equivalents relative to two alkane series standards (N_{24}, N_{32}) as previously reported by Kovats (1965). These values were compared against standards run prior to plasma samples, and were found to be highly repeatable.

(e) Steroid Quantitation

The level of all three pregnanediols in fetal plasma was determined using the ratio of peak area of the individual pregnanediols to the peak area of the IRS. Four standards were run prior to running samples, each standard contained all three of the identified pregnanediols at one of the following amounts, 0.5µgm, 1µgm, 2µgm and 3µgm, and all contained 1µg of the IRS. Peak areas were measured using a Hewlett-Packard 3380A integrator; the program used to calculate peak area provides a more accurate estimate, when a steady baseline has been reached, when peaks are not merged, and when the peak starts at, and returns to the baseline. Every effort was made to ensure these conditions prevailed. Samples were also repeated if the value of the peak area, for the pregnanediol present in least amount, was below 10,000 units. The ratio of peak areas was plotted against µgms of pregnanediol, and the standard plot was found to be linear to at least 10µgms of pregnanediol.

Allowing for the small variation (CV = 2.7-3.4%) all three pregnanediols gave equal responses, thus the standard curve was drawn using the mean ratio of the three pregnanediols at each concentration, and the level of all three pregnanediols in samples was extrapolated from the one standard curve.

The same amount of IRS (1μg) was added to the plasma samples (1ml) as was added to each standard, thus the sample value interpolated from the standard curve gave the level of pregnanediols in μgm/ml of plasma. The lower limit of detection of plasma pregnanediol was estimated at 10ng per injected sample, or approximately 80ng/ml of plasma. Plasma from near term fetuses obtained from the local abattoirs was pooled and used as a quality control (QC). Repeated determinations of the level of the three pregnanediols in the QC (assayed independently) gave a coefficient of variation (CV) of 10-13.3%, indicating an acceptable degree of repeatability. (see table 14).

TABLE 14

Repeated Independent Analysis of Pregnanediols in QC (level in μg/ml)

5β Pregnane-3β, 20β-diol	1.334	1.131	1.216	1.169	0.986	1.047	1.245	1.103	1.266	1.076	1.361	1.158	1.286	1.013	1.116	0.906	1.209	1.316	CV10.9%	$\bar{X}$ = 1.163
5β Pregnane-3β, 20α-diol	2.265	1.872	1.970	1.978	1.675	1.835	2.013	1.745	1.982	1.699	2.318	1.906	2.072	1.868	1.897	1.581	1.964	2.145	CV10.0%	$\bar{X}$ = 1.932
5β Pregnane-3α, 20α-diol	0.501	0.389	0.464	0.423	0.358	0.416	0.486	0.399	0.451	0.382	0.541	0.417	0.457	0.396	0.386	0.337	0.447	0.537	CV13.3%	$\bar{X}$ = 0.433

VII c. Results

Over eighty fetuses were operated on, however due to a variety of reasons success rate was only about 25%; success being judged as the birth of an uninfected fetus, after the collection of serial blood samples from its

implanted cannulae.

While developing the surgical techniques, fetuses were nephrectomised but not cannulated. Of these, 9 fetuses were uninfected and were born approximately two weeks prematurely at 133 (median) (range 126-139) days gestational age; all were still-born. These results confirm the studies reported by Thorburn et al (1971b).

All fetuses were between 100 and 106 days gestational age at the time of surgery; this age range was found to be the most suitable due to a number of reasons. The smaller the cannulae the more easily they become occluded, hence the use of cannulae smaller than polyvinyl 1 (Boots, Aust.) although allowing cannulation of smaller fetuses was unlikely to allow samples to be collected over an extended period of time. The intercotyledonary distance is smaller, fetal tissues more delicate, and the vessels smaller in younger fetuses, making surgery on fetuses younger than 100 days more time consuming, which also decreases the probability of success of the preparation. Thus fetuses between 100-110 days gestational age gave the best degree of success at the earliest age.

The values for plasma pregnanediol levels are presented in three sections, (i) sham operated (controls), (ii) nephrectomised, (iii) sham operated (aborted).

VII.c.(i) Sham Operated Fetuses (Controls)

Four sham operated fetuses implanted with arterial and venous cannulae were born normally at or near term (147 days). The live birth of these fetuses at term was used as the criterion for a successful preparation and added to the validity of the results.

Fetus 137, a single female fetus was operated on day 103 of gestation and born

on day 145. Samples were collected from the venous cannula from day 108 to day 133 after which the cannula blocked. The arterial cannula blocked after day 116.

Fetus 111, a single male fetus was operated on day 102 of gestation and was born on day 146. Samples were collected from the venous cannula from day 108 to 143, the arterial cannula blocked on the fourth day after surgery and could not be cleared. Both catheters were still implanted in the fetus at the time of birth.

Fetus 305, a single female fetus was operated on day 101 and born on day 146. The arterial cannula blocked before the first samples were taken on day 108; the venous cannula blocked on day 111.

Fetus 266, a single male fetus was operated on day 106 and the ewe went into labour on day 145. Samples were collected from both cannulae from day 111 until day 129 when the arterial cannula blocked, and venous samples were collected until day 145.

The results for all four fetuses are shown in figure 16.

In all fetuses the arterial cannula became blocked first, and as no difference in the level was found between arterial and venous plasma pregnanediols, only the results for venous plasma are shown. The results show that in all fetuses 5β-Pregnane-3α, 20α-diol remains between 0.2-06ug/ml over the time from 108 days to term. The levels of 5β-Pregnane-3β, 20β-diol and 5βPregnane-3β, 20α-diol are similar to 5β-Pregnane-3α, 20α-diol at about day 108 after which 5β-Pregnane-3β, 20α-diol begins to slowly increase, but at a rate faster than 5β-Pregnane-3β, 20β-diol which also increases. About twelve days before birth both 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol increase more rapidly, especially 5β-Pregnane-3β, 20β-diol, such that their levels are very close within a week of term. Approximately 4 days before term 5β-Pregnane-3β,

FIGURE 16

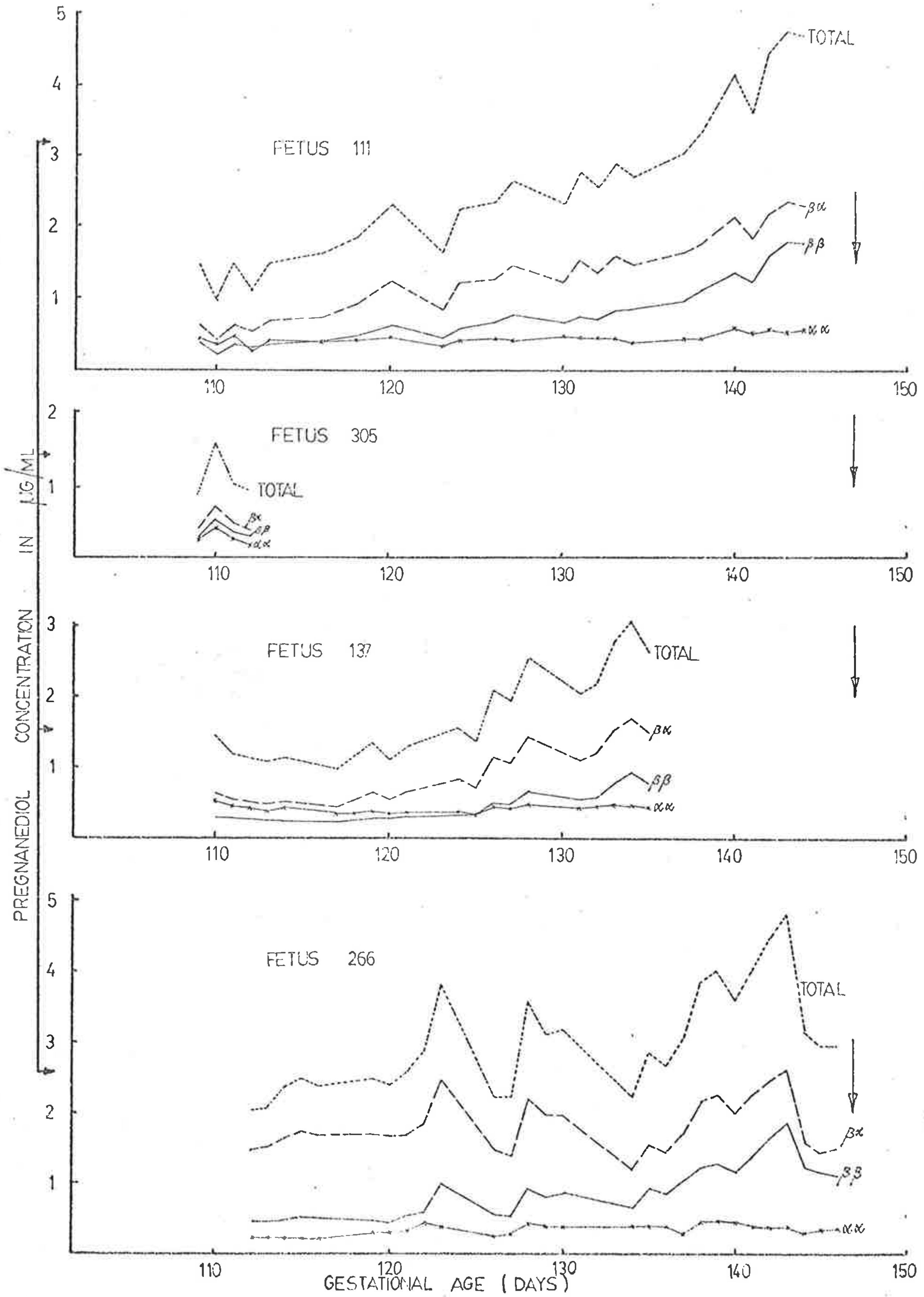
FETAL PLASMA PREGNANEDIOLS (SHAM OPERATED (CONTROLS))

All the graphs indicate the level of pregnanediols in venous plasma samples. Gestational age in days is plotted on the x-axis and pregnanediol concentration in µg/ml on the y - axis. βα, ββ and αα refer to the spatial arrangement of the hydroxyl groups at positions 3 and 20 respectively on the 5β-pregnanediol molecule.

The gestational ages by mating date have been adjusted to give all fetuses a birth date of 147 days (see text for actual date details of fetuses).

The total pregnanediol is the arithmetic sum of the values for each individual pregnanediol in each plasma sample.

FETAL PLASMA PREGNANEDIOLS



20β-diol and 5β-Pregnane-3β, 20α-diol reach their maximum levels, after which they decrease toward term.

VII c. (ii) Nephrectomised Fetuses

Five fetuses of between 102-106 days gestational age were nephrectomised and cannulated; three were born prematurely at 134, 135 and 136 days gestational age, one at 146 days, and one which was a twin died in utero at 137 days. All were uninfected at the time of birth.

Fetus 577 was male, single, was nephrectomised and cannulated on day 105 of gestation and born prematurely on day 134. Plasma samples were taken from day 110 onwards, however the plasma was unable to be sampled from day 117 onwards although saline could be infused. On day 131 a final venous sample was collected. ADRENAL WEIGHT 302 mg.

Fetus 127 was a male of a male-female twin pair; was nephrecomised and cannulated on day 105 of gestation and died in utero after 137 days of gestation, as no further samples were obtained. The twins were born on day 151 and the female twin was healthy, however the nephrectomised twin still had the catheters implanted and although uninfected was in the process of being resorbed. NO ADRENAL WEIGHT WAS MEASURED.

Fetus 253 was a female, single; nephrectomised and cannulated on day 106 of gestation and born prematurely on day 135. Plasma samples were collected from day 113 to day 125, both arterial and venous samples were collected. ADRENAL WEIGHT 188 mg.

Fetus 18 was a female, single; nephrectomised and cannulated on day 104 of gestation and born prematurely on day 136. Plasma samples were collected from day 111 until day 134, both arterial and venous samples were collected. ADRENAL WEIGHT 382 mg.

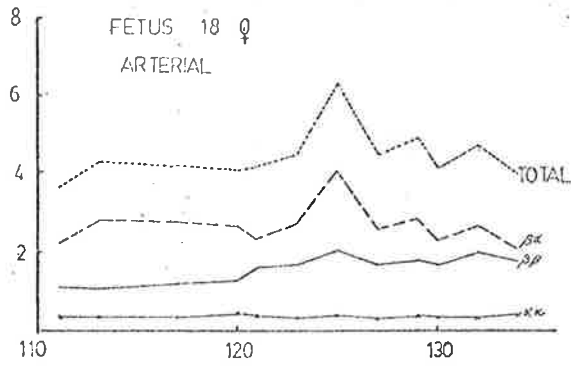
FIGURE 17

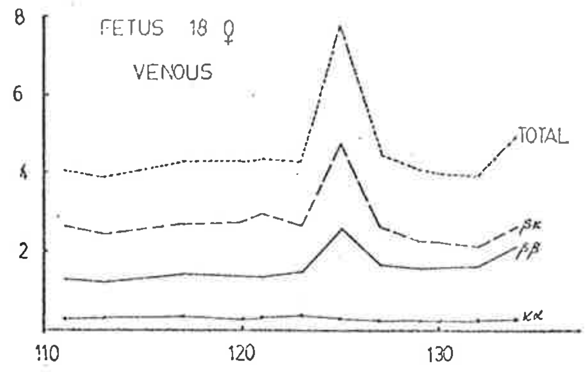
FETAL PLASMA PREGNANEDIOLS (NEPHRECTOMISED)

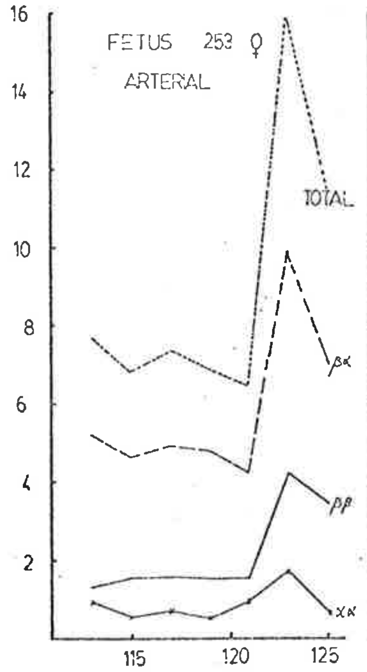
The levels of pregnanediols are shown for both arterial and venous plasma samples. Gestational age in days is on the x-axis and pregnanediol levels in μg/ml on the y-axis.

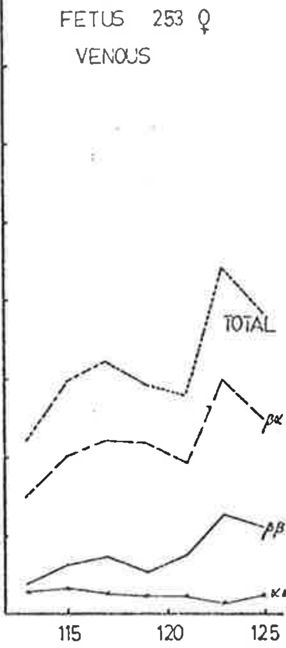
Fetus 18 was a female singleton born on day 136.
Fetus 253 was a female singleton born on day 135.
Fetus 127 was a male of a male-female twin pair.
After day 137 the plasma samples obtained from this fetus were haemolysed indicated the fetus was dead.

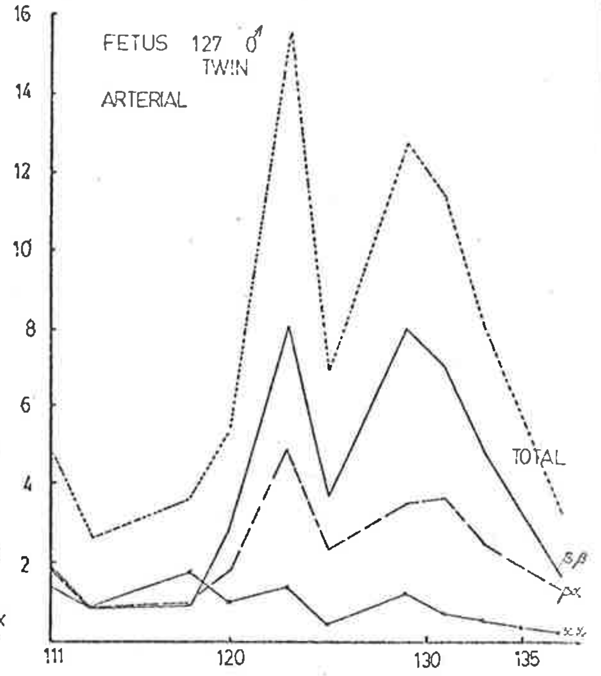
Fetus 577 was a male singleton born on day 134.
Fetus 104 was a female singleton born on day 146.
All but fetus 104 were still born (see text).

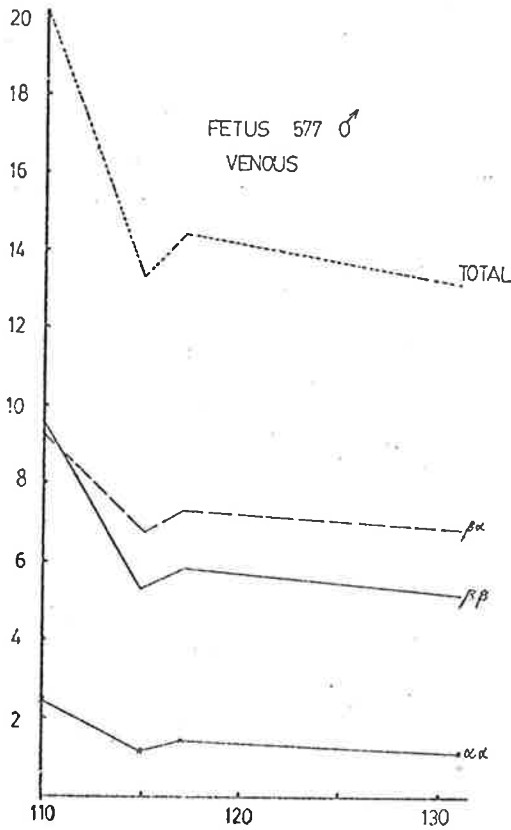

FETUS 18 ♀
ARTERIAL
TOTAL
βα
ββ
αα
8
6
4
2
110
120
130

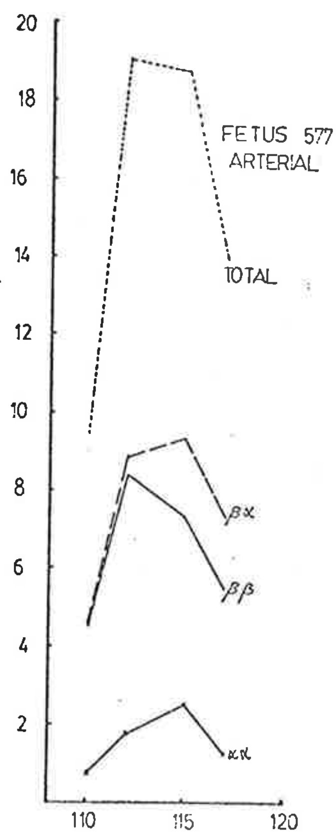

FETUS 18 ♀
VENOUS
TOTAL
βα
ββ
αα
8
6
4
2
110
120
130

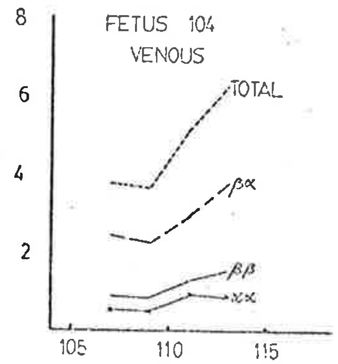

FETUS 253 ♀
ARTERIAL
TOTAL
βα
ββ
αα
16
14
12
10
8
6
4
2
115
120
125


FETUS 253 ♀
VENOUS
TOTAL
βα
ββ
αα
115
120
125


FETUS 127 ♂
TWIN
ARTERIAL
TOTAL
ββ
βα
αα
16
14
12
10
8
6
4
2
111
120
130
135


FETUS 577 ♂
VENOUS
TOTAL
βα
ββ
αα
20
18
16
14
12
10
8
6
4
2
110
120
130


FETUS 577
ARTERIAL
TOTAL
βα
ββ
αα
20
18
16
14
12
10
8
6
4
2
110
115
120


FETUS 104
VENOUS
TOTAL
βα
ββ
αα
8
6
4
2
105
110
115

Fetus 104 was a female, single; nephrectomised and cannulated on day 102 of gestation and born alive on day 146 but died within minutes of birth. The cannulae were still implanted at the time of birth however samples were only able to be collected on days 107-113 and by cardiac puncture on day 146. ADRENAL WEIGHT 347 mg.

The plasma levels of pregnanediols are shown in figure 17. In the nephrectomised fetuses the total plasma pregnanediols are 2-10 times higher than the levels in the sham operated fetuses however, the level of 5β-Pregnane-3α, 20α-diol is similar to that of the sham operated fetuses. The level of 5β-Pregnane-3α, 20α-diol also remains at a steady level near 0.5ug/ml and in most cases actually decreases with advancing gestation. The levels of both 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol are much higher in the nephrectomised fetuses than their levels in sham operated fetuses, being at least double at all gestational ages. In the nephrectomised fetuses the levels of 5β-Pregnane-3β,20β-diol and 5β-Pregnane -3β, 20α-diol are also much higher than the level of 5β-Pregnane-3α, 20α-diol, especially at a time close to premature parturition. At this time the levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol approach each other, this trend is clearly seen in fetus 18, and is similar to the changes in pregnanediol levels seen near term in the sham operated fetuses.

A comparison of the total level of pregnanediols in both sham operated and nephrectomised fetuses is shown in figure 18, which clearly demonstrates the much higher levels of pregnanediols in the nephrectomised fetuses.

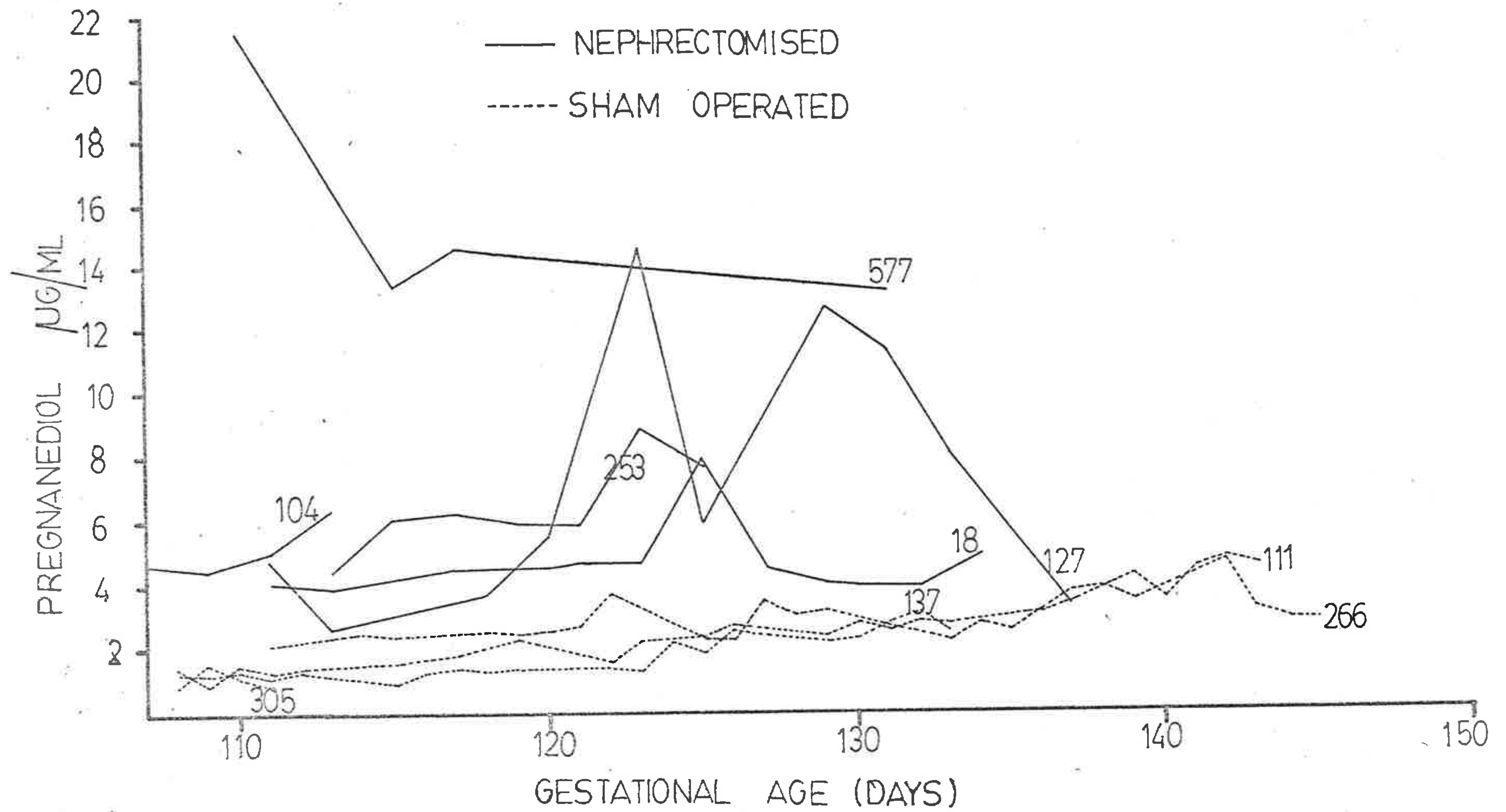
VII c. (iii) Sham Operated Fetuses (Aborted)

The fetuses included in this group are sham operated fetuses from which serial samples have been collected, but which aborted due to post-operative infection.

FIGURE 18

<u>COMPARISON OF THE PLASMA PREGNANEDIOL LEVELS IN NEPHRECTOMISED AND SHAM OPERATED FETUSES</u>

The graph shows clearly that the levels of total pregnanediols in nephrectomised fetuses are much higher than those in sham operated fetuses.


NEPHRECTOMISED
SHAM OPERATED
PREGNANEDIOL μG/ML
22
20
18
16
14
12
10
8
6
4
2
577
253
104
18
127
111
137
266
305
110
120
130
140
150
GESTATIONAL AGE (DAYS)

(see figure 19 for plasma pregnanediol levels).

Fetus 145 a female, single, was of 106 days gestational age at the time of operation (cannulation). Samples were collected from both arterial and venous cannulae until day 129, and heamolysed blood was obtained on day 132 indicating that the fetus had died. The fetus was aborted on day 141 and was infected. The plasma level of pregnanediols as indicated in figure 19, show a pattern similar to that seen in the control group. After day 118 the levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol were elevated about 3 fold higher than the levels of these pregnanediols in the control group, and were similar to those in the plasma of nephrectomised fetuses.

Fetus 149 and fetus 143 were both aborted on day 128 of gestation, both were infected, and both had plasma pregnanediol levels that were elevated to a level similar to the nephrectomised fetuses.

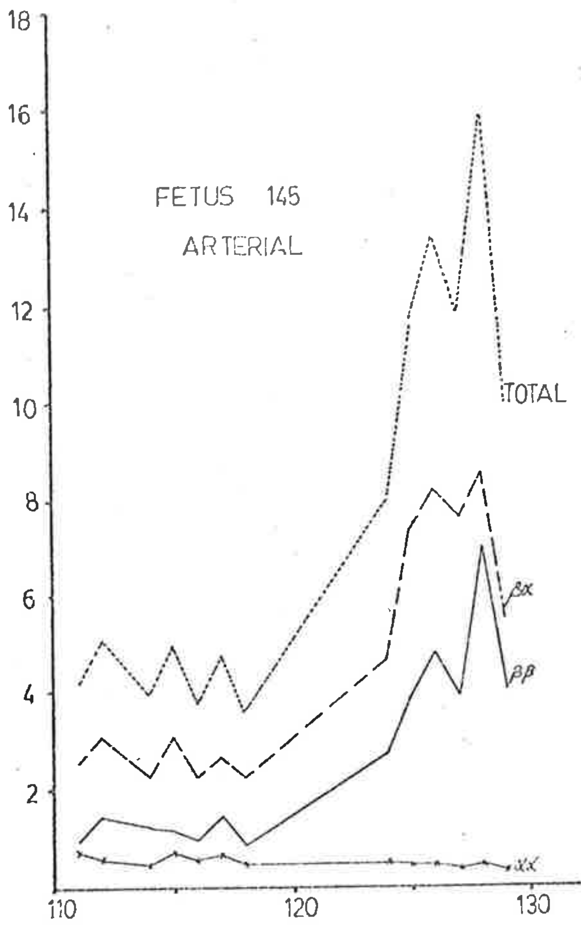
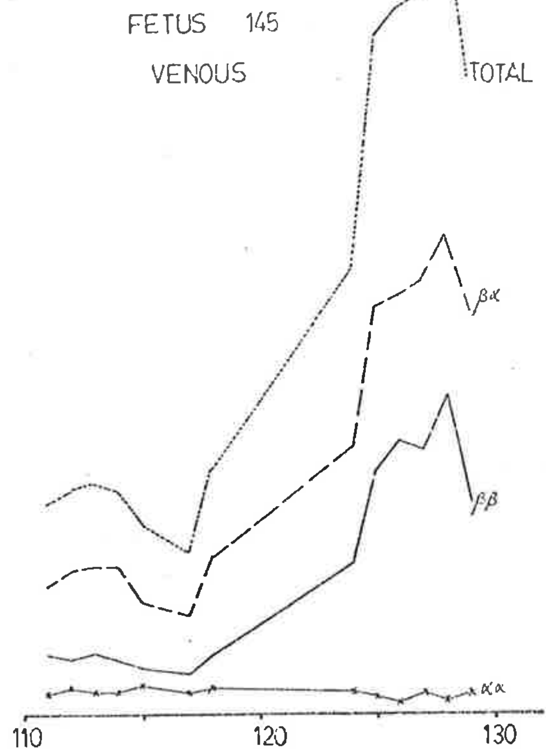
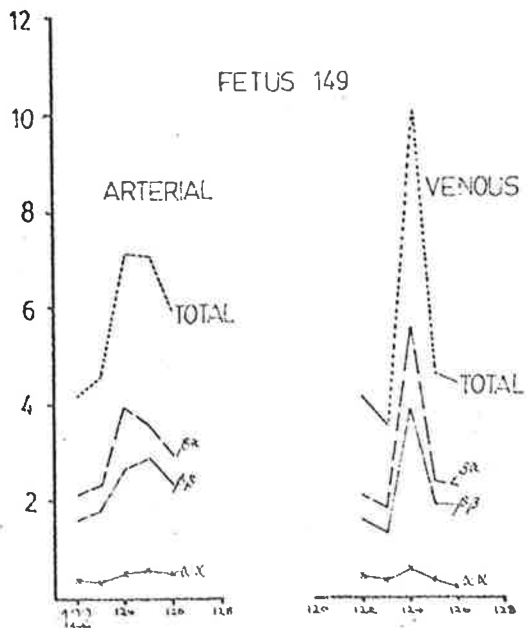
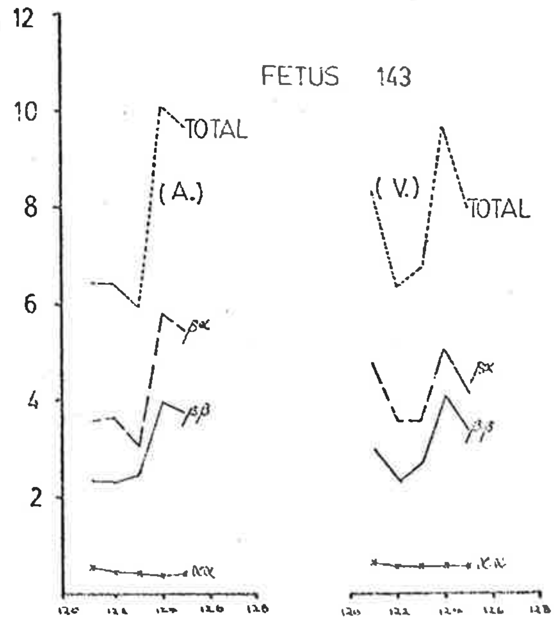
The results (see figure 19) show that in all three fetuses there is little difference between arterial and venous plasma levels of pregnanediols, and that although the total pregnanediol level is grossly elevated, the level of 5β-Pregnane-3α, 20α-diol remains at values close to 0.5ug/ml.

FIGURE 19

FETAL PLASMA PREGNANEDIOLLEVELS IN INFECTED FETUSES

The levels of pregnanediols are shown for both arterial and venous plasma samples. Gestational age in days on the x-axis and pregnanediol level in µg/ml on the y-axis.

All three fetuses were aborted spontaneously, and were found to be infected. The pregnanediol levels are elevated, being of similar magnitude to those in nephrectomised fetuses.


FETUS 145
ARTERIAL
TOTAL
βα
ββ
αα
18
16
14
12
10
8
6
4
2
110
120
130

FETUS 145
VENOUS
TOTAL
βα
ββ
αα
110
120
130

FETUS 149
ARTERIAL
VENOUS
TOTAL
TOTAL
12
10
8
6
4
2

FETUS 143
TOTAL
(A.)
(V.)
TOTAL
βα
ββ
αα
12
10
8
6
4
2

DISCUSSION

The results confirm those of the previous chapter, with plasma levels of pregnanediols being higher in the nephrectomised fetuses than the levels in sham operated fetuses. The total level of the three pregnanediols in the sham operated fetuses is close to 1μg/ml at 110 days of gestation, increases slowly during the next 25 days, doubling to about 2μg/ml at 135 days of gestation and then increases more rapidly, doubling in about seven days to 4μg/ml at 142 days. Approximately 3-4 days prior to term the total pregnanediol levels drop rapidly toward 1ug/ml. The nephrectomised fetuses show a much greater variability in levels, thus because of the small number of fetuses present in this group, no clear trend with advancing gestation is apparent. No samples were collected from nephrectomised fetuses in the 3 days prior to term thus it is not known whether the total levels show a similar drop, near term, to that seen in the sham operated fetuses. Although no trend is apparent, the results show clearly that the total pregnanediol levels are from 2-10 times higher in the nephrectomised fetuses (see figure 18).

The levels of pregnanediols in sham operated fetuses which become infected are also grossly elevated and show a high degree of variability. These levels are of similar magnitude to those in nephrectomised fetuses, indicating that high fetal plasma pregnanediol levels are not a unique occurrence associated with fetal nephrectomy. Nephrectomised fetuses have a number of abnormalities, which collectively suggest a gross disturbance in metabolism, indicating that nephrectomy may stress the fetus in trying to compensate for the loss of kidney functions. Thorburn et al, (1971a) found that fetal GH levels were elevated in nephrectomised fetuses, which is consistent with the fetus being under stress; fetal GH is released in response to stress, such as hypoxia and haemorrhage (Alexander et al, 1973). Infection places the fetus under great stress as it tries unsuccessfully to combat the invading bacteria and toxins

with its poorly developed immune system. It maybe that nephrectomy also causes fetal stress, and that this is the common factor between infected and nephrectomised fetuses which results in high pregnanediol levels.

An increase in pregnanediol levels is indicative of either a decrease in progesterone clearance or an increase in production. If the fetal kidney were an important site of progesterone clearance then its removal could lead to increased levels of pregnanediol. Studies on the flux of deuterated progesterone into amniotic fluid, and also the low levels of progesterone and pregnanediols in amniotic fluid (Seamark et al, unpublished data) indicate that the fetal kidney is not a major site of clearance of progesterone. The infected fetuses still have kidneys, thus if a common mechanism accounts for the increase in pregnanediol levels in both the nephrectomised and infected fetuses, this involves the kidney only indirectly, and is probably due to an increased progesterone production.

The production of progesterone by the pregnant ewe has been the subject of extensive investigation, which has established the importance of the ovary and placenta in progesterone production, and to a lesser extent the adrenal during late gestation (Thompson and Wagner,1974). Total progesterone production as measured in the peripheral plasma of the ewe reaches a maximum at about 135 days of gestation (Bedford et al, 1972a) after which it declines slowly until approximately 5 days prior to term when it drops rapidly. This maximum level near 135 days is the sum of ovarian and placental production (Mattner and Thorburn,1971) with 80% of the production being accounted for by the placenta at 119-126 days of gestation (Linzell and Heap,1968), after which the ovarian contribution declines being insignificant within 5 days of term (Thompson and Wagner,1974). Even though total production decreases, placental production continues to rise steadily, increasing by 10% between 120-140 days of gestation and reaching a maximum level of about 33mg/day

approximately 5 days prior to term (Mattner and Thorburn 1971). This measurement of placental progesterone production was made from plasma taken from the utero-ovarian vein of a dozen ovariectomised ewes, and hence actually indicates the level of production by the conceptus (placenta + fetus) rather than just the placenta.

While progesterone production by the placenta increases steadily after 135 days, fetal pregnanediol levels (see figure 20, section B) show a 2 fold increase indicating a rapid increase in the amount of progesterone available to the fetal liver. It is not known what proportion of placental progesterone production enters the fetal circulation. However progesterone levels in the fetus are lower than those in the mother (1ng/1ml vs 2-15ng/ml) (Bedford et al,1972a)(Strott et al,1974b),while clearance is approximately five times slower (Stupnicki and Stupnicki,1970a),suggesting that only a small portion of the total placental progesterone production enters the fetus. If placental progesterone is responsible for the increased progesterone within the fetus, then either an increased percentage would have to enter the fetal circulation which would lead to a decrease in utero-ovarian vein progesterone concentration; or, if the same percentage entered the fetus, then placental progesterone production would have to increase.

Neither of these occur, which indicates that the increased progesterone is most likely to be of fetal origin.

The study of Linzell and Heap (1968) provides supporting evidence for this contention. They found that umbilical arterial progesterone levels were higher than umbilical vein levels, suggesting a greater flow of progesterone from the fetus to the placenta than vice versa. The method used by these authors to collect their samples would have placed the fetus under stress

(see references in surgical methods IV b. ii. (e)), suggesting that this increased fetal progesterone may only be significant when the fetus is stressed, and hence may be of adrenal origin. The fetuses in this study were aged between 119-126 days of gestation, and it has been shown that after about 90 days of gestation the fetus is able to respond to stress with a very large increase of ACTH, but does not produce increased quantities of cortisol, suggesting that the adrenal of the fetus responds differently to ACTH, or is less responsive than the adult adrenal (Alexander et al, 1973). Anderson et al (1972) found that in the fetal sheep adrenal the metabolic route from pregnenolone to cortisol through progesterone (rather than 17αOH-pregnenolone) is of greater importance than it is in the adult sheep adrenal. They also found that although this pathway is more prominent, the progesterone that is formed is only slowly metabolised until the fetus reaches approximately 140 days gestational age, after which progesterone is rapidly converted through to cortisol. Thus from the available evidence we may speculate that if the fetus becomes stressed it releases ACTH which in pre-term fetuses leads to an increase in progesterone production by the adrenal. It seems that the developing lamb is equipped to increase its chances of survival. In the uterus it can produce progesterone in response to stress, where an increase in cortisol could be deleterious to its survival, and once outside the uterus it can immediately produce cortisol which then aids in its survival (where progesterone would be of little use); this can be achieved by a subtle change in enzyme activity which is an essential facet of the birth of the lamb.

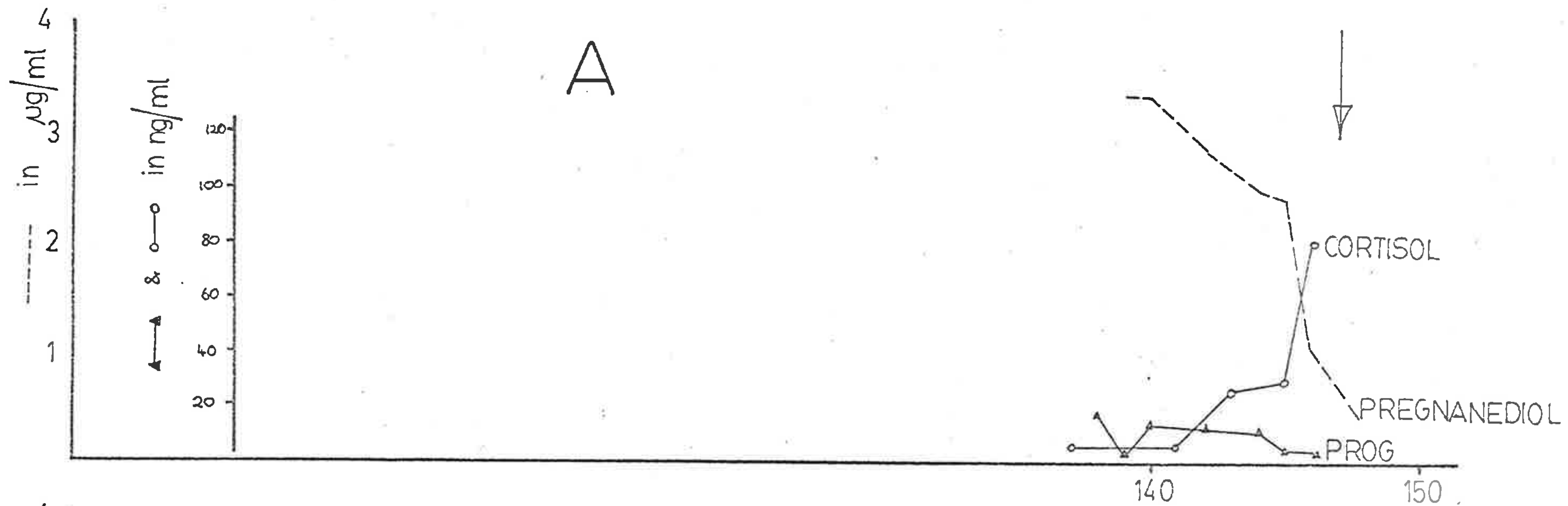
The rise in fetal pregnanediol levels in sham operated fetuses (figure 20, section B) reflect the steady rise in placental production during the period from 110 to 135 days of gestation; although placental production continues to rise steadily till about 5 days prior to term, fetal pregnanediol levels increase abruptly after day 135 at a time when the fetal adrenal also shows

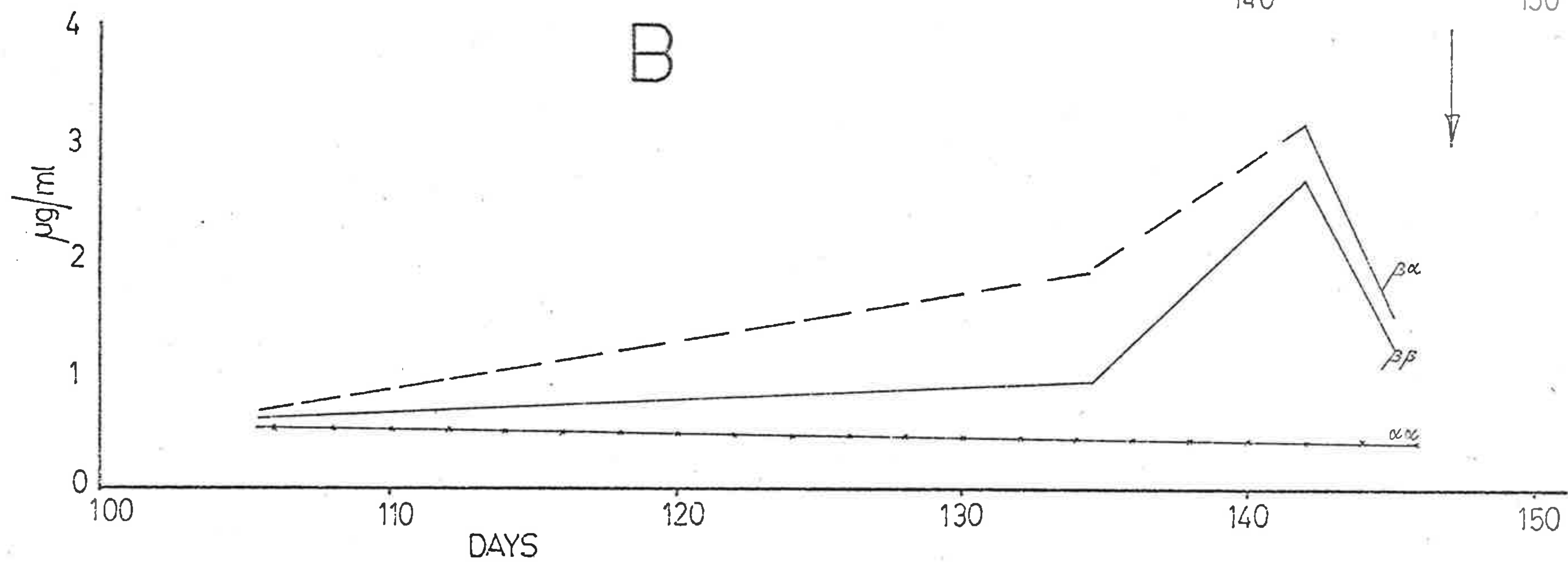
FIGURE 20

CHANGES IN FETAL PLASMA PREGNANEDIOL LEVELS WITH ADVANCING GESTATION AGE AND THEIR RELATIONSHIP TO FETAL CORTISOL LEVELS

A. The levels of pregnanediol (5β-Pregnane-3β,20 α -diol) progesterone, and cortisol in fetal plasma in serial samples collected near term (the arrow represents the time of birth). The samples were collected from a cannula implanted in the femoral artery of the fetus, and were analysed by GC-MS using multiple ion monitoring (unpublished data of Seamark).

B. Changes in fetal plasma pregnanediol levels (as indicated by the data presented in figure 16) in sham operated fetuses.


A
CORTISOL
PREGNANEDIOL
PROG
in μg/ml
in ng/ml
140
150


B
μg/ml
βα
ββ
αα
DAYS
100
110
120
130
140
150

an abrupt increase in weight (Comline and Silver,1961; Liggins,1969a). A comparison of the traces in figure 20, section A, and B, indicates that pregnanediols increase up until the time adrenal cortisol begins to rise, then as cortisol levels increase adrenal progesterone release would decline as progesterone is converted to cortisol in increasing rate (Anderson et al,1972). This fetal cortisol also decreases placental progesterone production (Liggins et al,1973) so that as cortisol levels increase, and increase more rapidly, both adrenal and placental sources of progesterone decline and pregnanediol levels plummet.

The five fold slower metabolic clearance rate of progesterone from the fetus and smaller size of the fetus mean that even a production rate of 1mg/day within the fetus would increase pregnanediol levels. The fetal adrenal within a few days of term is capable of producing 10mg/day of cortisol (Nathanielsz, Comline, Silver and Paisey,1972) thus it is certainly capable of producing a few mg/day of progesterone (Glickman, 1979). Thus the fetal adrenal would be capable of producing sufficient progesterone to cause the rapid increase in pregnanediols seen near term.

The above evidence also indicates that the high pregnanediol levels in the sham operated and nephrectomised fetus may also be due to adrenal production of progesterone. Fetal lambs infused with *Clostridium perfringes* toxin respond with accelerated adrenocortical growth, the extent of the response increasing with advancing gestational age (Liggins,1969b). The growth of the adrenal is due to release of ACTH from the pituitary, but the adrenal cortisol production is depressed between 90-130 days of gestation (Wintour et al, 1975), and it seems likely that instead of cortisol this increase in ACTH may result in increased progesterone production.

Thorburn et al (1971b) have postulated that premature delivery of nephrectomised fetuses is due to a premature activation of the hypothalamic-pituitary-

adrenal axis, having found elevated cortisol levels in nephrectomised fetuses after day 130 of gestation (birth at approximately day 134). In the present study, nephrectomised fetuses had adrenal weights (at the time of delivery) which were significantly elevated in comparison with the weights of normal fetal adrenals of the same gestational age as reported by Comline and Silver (1961). Nephrectomised (188-383mg) versus normals (approximately 130mg) at day 134 of gestation. Thorburn (1974) also found that placental weight was significantly reduced in nephrectomised fetuses, and considering the direct relationship between placental weight and progesterone production it would be expected that in nephrectomised fetuses placental progesterone production would be lower than normal. If fetal pregnanediols were produced entirely from placental progesterone, then it would have been expected that pregnanediol levels would be depressed, but this is contrary to the results and is taken as further evidence that high fetal pregnanediol levels are due to increased secretion of progesterone within the fetus, most probably by the fetal adrenal.

The total pregnanediol levels are the sum of the three individual pregnanediols 5β-Pregnane-3β, 20β-diol, 5β-Pregnane-3β, 20α-diol, and 5β-Pregnane-3α, 20α-diol and as total pregnanediol levels change, the relative level of the individual pregnanediols also change, as indicated in figures 16, 17, and 19. When total pregnanediols are elevated, 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α -diol are elevated, but 5β-Pregnane-3α, 20α-diol remains at approximately the same level with advancing gestation in virtually all fetuses, regardless of the total level. Fetal progesterone concentrations remain at about the same level throughout gestation and are low, rarely rising above 1ng/ml (Strott et al 1974b) which suggests that 5β-Pregnane-3α, 20α-diol may be related to direct fetal liver metabolism of the free progesterone in the fetal circulation, whereas 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol may be

related to metabolism of progesterone metabolites in the fetal circulation, such as 20αOH-preg-4-ene-3one.

The relative proportion of the three pregnanediols in sham operated fetuses with advancing gestation is shown in figure 20, section B, which shows that when pregnanediols rise quickly, the levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol move closer together. This is also seen in the nephrectomised fetuses, and is shown clearly in fetus 18 where within about 12 days of term (136) the level of 5β-Pregnane-3β, 20β-diol increases toward the level of 5β-Pregnane-3β, 20α-diol. The increase in the sham operated fetus also occurs within approximately 12 days of birth, and occurs at a time when the adrenal first starts to secrete slightly increased levels of cortisol. This cortisol has many extremely important functions which in general cause maturation of particular organ systems e.g. the production of surfactant in the fetal lung, which is essential if the fetus is to survive after birth, occurs under the stimulus of cortisol. The change in relative levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol during this time suggests that the fetal liver may also be undergoing maturational changes. Regardless of the mechanism by which these changes occur, the similarity in the change in relative levels of 5β-Pregnane-3β, 20β-diol and 5β-Pregnane-3β, 20α-diol in relation to the date of birth in both sham operated and nephrectomised fetuses, lends support to Thorburn's (1974) hypothesis that the premature parturition seen in nephrectomised fetuses is due to a premature activation of the hypothalamic-pituitary-adrenal axis.

CHAPTER VIII

GENERAL DISCUSSION

The importance of the endocrine functions of the kidney has only recently been recognised, and is highlighted by the numerous metabolic disturbances which are apparent in people with chronic renal disease or kidney loss (Ganda et al, 1976 and reference cited therein). These people are maintained on chronic renal dialysis, and their metabolic disturbances are compatible with the lack of the endocrine functions of the kidney. There is evidence that these functions become more critical during times of rapid growth and development. Young children with severe renal disease and anephric children maintained on chronic renal dialysis become growth retarded unless administered with 1,25-diOH-Vitamin D_3 (Chesney et al, 1978), which only gains its full biological activity within the kidney. Studies on the anephric human fetus (Potter, 1965) and on the nephrectomised sheep fetus (Thorburn, 1974) demonstrate that in the fetus the lack of kidney function is also associated with growth retardation. These fetal studies also demonstrate that the lack of fetal kidneys is associated with premature delivery in both humans and sheep.

Liggins et al, (1973) have demonstrated the essential role that steroids, in particular, progesterone, cortisol, and oestradiol-17β play in the maintenance and termination of pregnancy in sheep, and Nancarrow (1969) and Ainsworth (1972) demonstrated that the fetal sheep kidney was capable of metabolising steroid hormones. These observations led to the studies reported in this thesis, which were aimed at elucidating whether the steroid metabolising enzymes of the fetal sheep kidney were associated with steroid endocrine function and whether they were associated with the premature delivery of the nephrectomised fetus.

The results demonstrate that the fetal sheep kidney possesses a number of steroid metabolising enzymes as indicated in Table 6. However, the fetal

sheep kidney was not capable of utilizing the precursors, pregnenolone or DHEA indicating a lack of Δ^4-Δ^5 isomerase. Pregnenolone and DHEA are important precursors known to be involved in the biosynthetic pathways of progesterone, testosterone and oestradiol in sheep endocrine organs. There was also no evidence of C17-20 lyase or aromatase activity suggesting that the fetal kidney was incapable of converting C21 to C19 steroids or C19 to C18 steroids, which combined with the lack of Δ^4-Δ^5 isomerase activity suggests that the fetal kidney is unlikely to contribute to steroid production within the fetus. The sulphastase activity may provide the fetal kidney with a para-endocrine function via the release of biologically active free steroids from their sulphates. These free steroids may either act within the kidney or be released into the circulation, but considering that in other species the kidney is a target organ for sex steroids, it seems likely that they act within the kidney.

Only a limited number of steroid metabolising enzymes are present in the fetal sheep kidney and all may be involved in the action of steroids on the kidney. The results provide evidence which is compatible with the fetal kidney being responsive to sex steroid hormones, and in particular to androgens. 5α-reductase is thought to be involved in the action of androgens in target organs (Bruchovsky and Wilson,1968a), and the nature of the 5α-reductase in fetal sheep kidneys is similar to that in the prostate and epididymus but dissimilar to that in the liver. The mouse kidney responds to androgens, and there is a striking similarity between the enzymes involved in the action of testosterone on the mouse kidney, and the enzymes present in the fetal sheep kidney. Arimasa and Kochakian (1973) found that the mouse kidney contains 5α-reductase and 3α/3β-HSD activity, with the major product of testosterone being 5α androstane-3α, 17β-diol. These enzymes were also found in the fetal sheep kidney, however 5α-, 3β-reduced products were predominant, which may merely reflect a species difference in preferred steric configuration.

Ohno et al (1971) found that 5α-androstane-3α, 17β-diol induces β-glucuronidase in the mouse kidney and that this is a prerequisite for subsequent hypertrophy. This induction only occurs in the cells of the proximal convoluted tubule; cells which are involved in reabsorption of material from the glomerular filtrate by pinocytosis, (Baxter and Yoffey, 1948), and are the specific cells within the kidney which undergo hypertrophy (Selye, 1939a). Due to the lack of cellular receptors (permeases) for androstanediols, 5α-androstane-3α, 17β-diol does not enter these kidney cells from the blood, and Ohno et al suggest that it enters the cells by pinocytotic reabsorption from the glomerular filtrate. Most androgenic steroids normally excreted through the glomeruli are in conjugated form, thus the induction of β-glucuronidase as an initial step, is logical, allowing the larger quantities of conjugated steroids to exert their action on the kidney. Ohno et al (1971) propose a two step mechanism for the action of androgens on the kidney, with androstanediol causing an initial induction of specific proteins such as β-glucuronidase, and possibly an androgen receptor protein, followed by non specific stimulation of transcription under the influence of 5αDHT, resulting in hypertrophy.

A difference was found between the weights of male and female fetal sheep kidneys, which in view of the data reported in the literature, strongly suggests that this is the result of androgen action. When the enzymes activities of the fetal sheep kidney are viewed from this perspective they fit exceptionally neatly into a scheme similar to that proposed by Ohno et al (1971). The only readily apparent differences being that in fetal sheep plasma, sulphated steroids are the predominant form of conjugate, hence sulphatase is present rather than β-glucuronidase,and that reduction by 3βHSD is favoured over 3αHSD. It is not known whether the sulphatase in fetal sheep kidneys is inducible, but although no inference should be made, it is interesting to note that there is an almost two fold difference in

the level of kidney sulphatase activity between the male and female fetuses reported by Ainsworth (1972). Conclusive evidence that androgens influence kidney growth in the sheep fetus will require extensive studies on castrated and normal fetuses, both with or without testosterone implants, however the nature of the enzymes in the fetal kidney indicates that they are almost certainly involved in the action of steroids on the fetal sheep kidney.

Thus the results of chapters 3, 4 and 5 indicate that the fetal sheep kidney is most unlikely to have a steroid endocrine function, nor is it likely to influence the level of circulating steroids by being an important site of catabolism. Hence it is most unlikely that the premature parturition of the nephrectomised fetus would result directly from the loss of the steroid metabolising capacity of the fetal sheep kidney. This activity apparently being concerned with the actions of steroids within the kidney, rather than influencing the steroid milieu outside it. To provide support for this contention, the pregnanediols were examined in the plasma from both normal and nephrectomised fetuses.

Removal of the kidneys results in premature parturition, indicating that if the kidneys are directly involved then they are involved with those factors concerned with the maintenance of pregnancy. Progesterone is the steroid hormone which is essential for the maintenance of pregnancy, and pregnanediols are progesterone metabolites, hence an examination of plasma pregnanediols would give an indication of changes in progesterone metabolism. The results indicate that the pregnanediols present in fetal plasma in greatest quantity (>99%) are all 5β-reduced and thus are not metabolites formed by the fetal kidney; 5β-reductase is not present in the fetal kidney. The same pregnanediols were found in both nephrectomised and entire fetuses, also indicating that kidney metabolism of progesterone is insignificant. However, the results

showed that the level of pregnanediol was much higher in the nephrectomised fetuses than in the sham operated controls. This was unexpected, as the fetal kidney is not an important site of pregnanediol or progesterone excretion (Seamark et al, unpublished data) and its metabolism of progesterone is insignificant, hence clearance is unlikely to decrease significantly due to kidney loss. The placenta is decreased in size in nephrectomised fetuses (Thorburn, 1974) suggesting decreased placental progesterone production, thus without a change in clearance it was expected that pregnanediol levels would be lower in the nephrectomised fetus. This was not found, and suggested that although placental progesterone production was probably depressed, the production within the fetus was higher than normal.

The levels of pregnanediols in infected fetuses were also elevated indicating that the phenomena is not uniquely associated with the loss of kidneys. Data available on infected fetuses (Liggins,1969b) and on nephrectomised fetuses (Thorburn et al, 1971a) are both compatible with the fetus experiencing stress, with an associated premature activation of the pituitary - adrenal axis. In the sheep adrenal the Δ^4 pathway is favoured for the production of cortisol, implicating the fetal adrenal as the most likely source of increased progesterone production within the stressed fetus. In this regard an observation of Bedford et al (1972a) is pertinent. In experiments designed to measure progesterone production in pregnant and non pregnant ewes, they excluded the data from a particular ewe from the non pregnant group because the ewe was unusually agitated (stressed) and its progesterone production rate was equivalent to that seen in pregnant sheep at between 60-100 days of gestation (much higher than any other in the non pregnant group). At the very least this does provide an example of a stressed adult sheep being associated with high progesterone levels. Finally the rapid increase in pregnanediol levels in the fetal sheep plasma during

the last twelve days of gestation, at a time when maternal ovarian production of progesterone has ceased, and placental production increases only slightly (Mattner and Thorburn, 1971), provides further evidence which is compatible with the fetal adrenal being the source of the increased progesterone.

In conclusion the steroid metabolising enzymes of the fetal sheep kidney are apparently associated with the action of steroids on the fetal kidney, and are not involved in steroidogenesis, and are unlikely to significantly alter circulating levels of steroids. In providing confirmatary evidence for these assertions, an examination of fetal pregnanediol levels also provided evidence which strongly implicates the fetal adrenal as a significant source of fetal progesterone in stressed fetuses of abnormal pregnancies and during the final stages of normal pregnancy.

APPENDIX I

RECRYSTALLISATION DATA

TABLE 1

Recrystallisation of the single metabolite from the incubation of ^{14}C-oestrone with fetal kidney homogenate.

32.51 mg of authentic carrier steroid added.

17β Oestradiol

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
benzene/acetone	Xtal	0.6309	926	1468
	ML	3.256	7723	2372
benzene/acetone	Xtal	0.7875	1027	1304
	ML	1.480	2602	1758
benzene/acetone	Xtal	0.7658	950	1241
	ML	1.810	2684	1483
benzene/methanol	Xtal	0.4907	517	1054
	ML	2.203	2796	1269
benzene/methanol	Xtal	0.6845	778	1137
	ML	1.957	1939	991

$\bar{x}$ = 1184

Thus the total radioactivity associated with 17β oestradiol is 38,492

TABLE 2

Recrystallisation of the polar metabolite from the incubation of ^{14}C-Δ^4- androstene-3, 17-dione with fetal sheep kidney homogenate.

31.25 mg of authentic 5α-androstane-3βol-17-one was added as carrier steroid.

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
acetone/heptane	Xtal	-	8,931	-
	ML	-	23,362	-
acetone/heptane	Xtal	0.7500	5,039	6718
	ML	2.2021	18,414	8331
acetone/heptane	Xtal	0.7158	5,001	6986
	ML	1.847	14,701	7959
acetone/heptane	Xtal	0.7575	5,205	6871
	ML	3.380	24,600	7278

$\bar{x}$ = 6858

Total radioactivity associated with 5α-androstane-3β-ol-17-one is 214,313 c.p.m.

TABLE 3

Recrystallisation of the polar metabolite from the incubation of ^{14}C androst-4-ene-3, 17-dione with fetal sheep kidney homogenate.

This recrystallisation was performed on a single peak of radioactivity containing two components which do not separate chromatographically. The data presented in this table is of material present at lower concentration.

27.44 mg of authentic 5α-androstane-3α-ol-17-one was added as carrier steroid

SOLVENT	WEIGHT		ACTIVITY	SPECIFIC ACTIVITY (c.p.m.)/(mg)
acetone/heptane	Xtal	0.7314	3,873	5,296
	ML	3.247	143,319	44,139
acetone/heptane	Xtal	0.7091	2,019	2,847
	ML	2.386	65,586	27,488
acetone/heptane	Xtal	0.7613	1,487	1,953
	ML	2.754	11,454	4,159
acetone/heptane	Xtal	0.7250	1,303	1,797
	ML	1.918	4,526	2,360
acetone/heptane	Xtal	0.8033	1,482	1,845
	ML	2.241	4,849	2,164

$\bar{x}$ = 1,865

Total radioactivity associated with 5α-androstane-3α-ol-17-one is 51,176 c.p.m.

TABLE 4

Recrystallisation of the single radioactive peak from the incubation of DHEA with fetal sheep kidney homogenate.

38.52 mg of androst-5-ene-3β-ol-17 one was added as carrier steroid.

SOLVENT	WEIGHT		ACTIVITY	SPECIFIC ACTIVITY (c.p.m.)/(mg)
methanol/water	Xtal	.9181	2,011	2,190
	ML	2.433	6,146	2,526
methanol/water	Xtal	.7703	1,903	2,471
	ML	3.190	9,079	2,846
methanol/water	Xtal	.7197	1,552	2,156
	ML	2.906	6,501	2,237
methanol/water	Xtal	.6583	1,305	1,983
	ML	2.265	5,248	2,317
methanol/water	Xtal	.8159	1,884	2,309
	ML	2.873	7,349	2,558

$\bar{x}$ = 2,149

Total radioactivity associated with DHEA was 82,792 c.p.m.

TABLE 5

Recrystallisation of the single radioactive peak from the incubation of pregnenolone with fetal sheep kidney homogenate.

41.76 mgs preg-5-ene-3β-ol-20-one was added as carrier steroid.

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
benzene/n-hexane	Xtal	.5628	1,318	2,342
	ML	3.719	9,725	2,615
benzene/n-hexane	Xtal	.8831	1,761	1,994
	ML	2.406	5,430	2,257
benzene/n-hexane	Xtal	.7149	1,375	1,923
	ML	3.048	5,739	1,883
methanol/toluene	Xtal	.7322	1,477	2,017
	ML	2.513	5,423	2,158
methanol/toluene	Xtal	.7946	1,451	1,849
	ML	2.067	4,312	2,086

$\bar{x}$ = 1,946

Total radioactivity associated with pregnenolone was 81,254 c.p.m.

TABLE 6

Recrystallisation of half of the radioactivity present in peak 1 (figure 10) of the metabolites formed from the incubation of Δ^4-pregnene-3, 20-dione with fetal sheep kidney homogenate.

31.96 mg of authentic 5α-pregnane-3β, 20-diol was added as carrier steroid.

SOLVENT	WEIGHT		ACTIVITY	SPECIFIC ACTIVITY (c.p.m.)/(mg)
chloroform/n-hexane	Xtal	0.445	1,876	4,213
	ML	2.314	14,586	6,303
chloroform/n-hexane	Xtal	0.7459	2,653	3,557
	ML	3.068	14,632	4,769
chloroform/n-hexane	Xtal	.6232	2,184	3,504
	ML	2.745	11,417	4,159
chloroform/n-hexane	Xtal	.8791	3,056	3,476
	ML	2.667	9,859	3,697
chloroform/n-hexane	Xtal	.7349	2,639	3,591
	ML	3.165	11,403	3,603

$\bar{x}$ = 3,523

Total radioactivity with 5α-pregnane-3β,20α-diol is 112,616 c.p.m.

TABLE 7

Recrystallisation of half of the radioactivity present in peak 1 (figure 10) of the metabolite formed from the incubation of pregesterone with fetal sheep kidney homogenate.

This recrystallisation was performed on a single peak of radioactivity containing two components which do not separate chromatographically. The data presented in this table is of material present at lower concentration.

18.35 mgs of preg-4-ene-3β, 20α-diol was added as carrier steroid.

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
chloroform/n-hexane	Xtal	0.5795	1,348	2,326
	ML	1.436	93,275	64,955
chloroform/n-hexane	Xtal	0.4275	506	1,183
	ML	1,283	20,786	16,201
chloroform/n-hexane	Xtal	0.6044	692	1,145
	ML	1.643	2,277	1,386
chloroform/n-hexane	Xtal	0.4576	583	1,274
	ML	1.536	1,748	1,138
chloroform/n-hexane	Xtal	0.4839	531	1,097
	ML	1,265	1,255	995

$\bar{x}$ = 1,172

Total radioactivity associated with Δ^4-pregnene-3β, 20α-diol 21,506 c.p.m.

TABLE 8

Recrystallisation of Δ^4 pregnene-20α ol-3 one formed from the metabolism of pregesterone by fetal sheep kidney homogenate.

27.34 mg of Δ^4-pregnene-20α-ol-3-one was added as carrier steroid.

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
chloroform/n-hexane	Xtal	0.6895	1,067	1,548
	ML	2.711	4,481	1,653
chloroform/n-hexane	Xtal	0.7136	961	1,347
	ML	2.407	3,122	1,297
ethyl acetate/n-hexane	Xtal	0.7225	1,038	1,435
	ML	2.986	4,138	1,386
acetone/n-hexane	Xtal	0.7307	1,016	1,391
	ML	2.293	2,871	1,252

$\bar{x}$ = 1,391

Total radioactivity associated with Δ^4-pregnene-20α-ol-3-one is 38,030 c.p.m.

TABLE 9

Recrystallisation of 5α-Pregnane-3α-ol-20-one formed from the metabolism of progesterone by fetal sheep kidney homogenate.

35.17 mg of authentic 5α-Pregnane-3α-ol-20 one was added as carrier steroid.

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
acetone/n-heptane	Xtal	.8427	1,235	1,465
	ML	3.071	4,616	1,503
acetone/n-heptane	Xtal	.6895	944	1,369
	ML	2.9138	4,076	1,399
acetone/n-heptane	Xtal	.7872	1,045	1,328
	ML	3.193	4,394	1,376
chloroform/n-hexane	Xtal	.8104	988	1,219
	ML	2.647	3,401	1,285
chloroform/n-hexane	Xtal	.7155	917	1,282
	ML	3,143	4,010	1,276

$\bar{x}$ = 1,276

Total radioactivity associated with 5α-pregnane-3α-ol-20-one was 44,877 c.p.m.

TABLE 10

Recrystallisation of 5α-pregnane-3β-ol-20-one formed from the incubation of ^{14}C progesterone with fetal sheep kidney homogenate.

34.95 mg of authentic 5α-pregnane-3β-ol-20-one was added as carrier steroid

SOLVENT		WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
acetone/n-heptane	Xtal	0.6570	4,742	7,218
	ML	3.217	24,021	7,467
acetone/n-heptane	Xtal	0.8045	5,545	6,893
	ML	2.962	21,566	7,281
acetone/n-heptane	Xtal	0.7381	5,272	7,142
	ML	3.028	22,150	7,315
acetone/n-heptane	Xtal	0.6499	4,593	7,068
	ML	2.631	19,057	7,293

$\bar{x}$ = 7,034

Total radioactivity associated with 5α-pregnane-3β-ol-20-one is 245,838 c.p.m.

TABLE 11

Recrystallisation of Δ^4-pregnene-3β-ol-20-one formed from the incubation of ^{14}C progesterone with fetal sheep kidney homogenate.

32.76 mg of authentic Δ^4 pregnene-3β ol 20 one.

SOLVENT	WEIGHT (mg)	ACTIVITY (c.p.m.)	SPECIFIC ACTIVITY (c.p.m.)/(mg)
chloroform/n-hexane	-	1,137	-
	-	-	-
chloroform/n-hexane	0.8629	1,291	1,496
	2.845	3,946	1,387
acetone/n-heptane	0.7138	970	1,359
	3.029	3,992	1,318
acetone/n-heptane	0.7461	3,898	1,287
	3.485	4,408	1,265
acetone/n-heptane	0.6953	1,000	1,438
	2.721	3,662	1,346

$\bar{x}$ = 1,361

Total radioactivity associated with Δ^4-pregnene-3β-ol-20-one is 44,586 c.p.m.

TABLE 12

Recrystallisation of 5α-Pregnane-3, 20-dione formed from the incubation of ^{14}C progestrone with fetal sheep kidney homogenate.

26.77 mg of authentic 5α-Pregnane-3, 20-dione was added as carrier steroid.

SOLVENT	WEIGHT	ACTIVITY	SPECIFIC ACTIVITY (c.p.m.)/(mg)
benzene/n-hexane	0.7879	1,180	1,498
	2.637	4,330	1,642
benzene/n-hexane	-	962	-
	-	4,158	-
acetone/n-hexane	0.7093	826	1,164
	3.117	3,971	1,246
benzene/n-hexane	0.7832	1,005	1,283
	2.900	3,950	1,362
benzene/n-hexane	0.6843	736	1,075
	2.597	2,584	995

$\bar{x}$ = 1,174

Total radioactivity associated with 5α-Pregnane-3, 20-dione is 31,428 c.p.m.

TABLE 13

SINGLETON MALE AND FEMALE FETUSES

MALE FETUSES

Weight (Kg)	Kidney Weight (gm)
1.1	11.6
1.4	10
1.75	16.35
1.95	16.2
2.05	16.2
2.1	12.6
2.17	16.7
2.18	23.3
2.2	20.8
2.45	19.4
2.7	21.1
2.75	18.8
2.8	18.4
2.85	17.9
2.9	21.3
3.0	16.5
3.0	17.8
3.25	19.3
3.65	23.1
3.7	24.7
3.75	19.9
3.8	19.2
3.85	22.8
3.9	19.7
4.0	22.8
4.0	22
4.4	21.25
4.95	19.9
5.1	24.6
5.5	22.3
5.6	21.1

FEMALE FETUSES

Weight (Kg)	Kidney Weight (gm)
0.6	6.75
1.0	7.8
1.3	12.6
1.45	12
1.5	14.1
1.9	15.3
1.95	18.3
2.14	14.0
2.2	13.6
2.2	14.2
2.3	15.3
2.4	13.9
2.4	16.5
2.6	14.9
2.6	16.7
2.6	18.35
2.65	23
2.9	19.7
3.1	13.3
3.1	19.4
3.2	15.9
3.3	20
3.4	20.8
3.75	17.1
3.9	17.7
3.9	21.6
4.2	19.7
4.2	22.5
4.7	22.1
5.4	21.9

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