Department of Veterinary
Clinical Sciences
Massey University
PALMERSTON NORTH

MEASUREMENT OF OVINE PLASMA ANDROGENS BY A COMPETITIVE PROTEIN BINDING METHOD

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science

by

William James TORREY

Massey University

New Zealand

1971

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. R.M. Greenway, for providing this topic and for his help and understanding during the study.

Thanks are due to the Biochemistry Department, Massey University, for allowing me to undertake this topic; to Miss H. Chapman for collection of blood samples; to Dr. A.N. Bruere of the Veterinary Clinical Science Department, Massey University, for providing advice and the experimental animals; to the staff at Palmerston North Public Hospital who provided me with late pregnancy plasma samples; and to Professor G.C. Liggins and his staff, of the Postgraduate School of Obstetrics and Gynaecology, Auckland University, for the helpful advice they have given me.

Special appreciation is extended to Mrs. M. Horsfall for typing and aiding in the printing of this thesis.

Final thanks must go to my parents and those friends dear to me, who have provided emotional and moral support.

ABSTRACT

A method has been developed for measuring plasma testosterone in sheep using competitive protein binding (CPB) techniques. The procedure requires column chromatography on LH-20 Sephadex gel of a methylene chloride extract of plasma, and final determination of plasma testosterone by the CPB technique using salt precipitation of the protein bound fraction. The sensitivity of the assay has allowed application to the measurement of plasma testosterone levels in normal male, normal female, Klinefelter and freemartin sheep. It has also enabled the monitoring of the effect of stimulation - with intravenous pregnant mare serum gonadotrophin and human chorionic gonadotrophin (HCG) - on the plasma testosterone levels of normal male and Klinefelter sheep. A modification to the method, borohydride reduction of the initial methylene chloride extract, has enabled the plasma androstenedione levels to be determined, simultaneously with plasma testosterone, in two of the HCG stimulation studies.

NOMENCLATURE

Trivial names have been used throughout the text for most steroids. These trivial names together with the systematic names according to the International Union of Pure and Applied Chemistry - I.U.P.A.C. - see Biochemistry (Wash.) 8, 2227 (1969) are listed in the section on terminology and abbreviations.

TERMINOLOGY AND ABBREVIATIONS

CPB	competitive protein binding
T-^{3}H	testosterone-^{3}H, tritiated testosterone
TLC	thin layer chromatography
PC	paper chromatography
CC	column chromatography
TPNH	triphosphopyridine nucleotide, reduced form
PMSG	pregnant mare serum gonadotrophin
HCG	human chorionic gonadotrophin
FSH	follicle stimulating hormone
LH	luteinising hormone
17-ketosteroid	steroid with a ketone on carbon-17
CPM (cpm)	counts per minute
IU	international units
ng	nanogram
µg	microgram

Trivial Name	Systematic Name
Androstanediol	5α-Androstan-3β, 17β-diol
Androstenedione	4-Androsten-3, 17-dione
Androsterone	5α-Androstan-3α-ol-17-one
Cholesterol	5-Cholesten-3β-ol
Dehydroepiandrosterone (DHEA)	3β-Hydroxy-5-androsten-17-one
Dihydrotestosterone	17β-Hydroxy-5α-androstane-3-one
Estradiol-17β	1,3,5(10)-Estratrien-3,17β-diol
17α-hydroxypregnenolone	3β,17α-Dihydroxy-5-pregnen-20-one
Lanosterol	5α-Lanosta-8,24-dien-3β-ol
Pregnenolone	3β-Hydroxy-5-pregnen-20-one
Progesterone	4-Pregnene-3,20-dione
Squalene	2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexane
Testosterone	4-Androsten-17β-ol-3-one

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTORY REVIEW

The purpose of this research is threefold:

1. To set up a competitive protein binding technique for assaying plasma androgen levels in sheep.
2. To apply the technique to the study of the circulating plasma testosterone levels in four clinical groups of sheep (normal ewes, Klinefelter rams, freemartin sheep and normal rams).
3. To study the effects of gonadotrophin stimulation on androgen secretion in normal rams and Klinefelter rams using the circulating plasma androgen levels as the index.

It is hoped that this research will provide some insight into the effect the clinical syndromes have on circulating plasma testosterone levels and the response to androgen stimulation by gonadotrophins.

1.1 The Role of Androgens in Sheep

The male and female gonads, the adrenal cortex and the placenta produce steroidal hormones which influence the development and maintenance of the structures directly and indirectly associated with reproduction. Of the three principal types of hormones

involved, the androgens have distinct roles in the reproductive physiology of the male and the estrogens and progestogens comparable roles in the female.

1.1.1 The structure and function of androgens

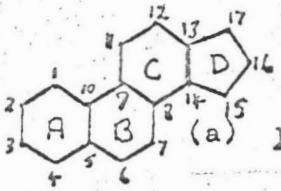
In general naturally occurring steroidal sex hormones are considered derivatives of the saturated tetracyclic hydrocarbon perhydro-13,14- cyclopentanophenanthrene (Fig 1.1a). The structure of the androgens is based on the hydrocarbon androstane (Fig 1.1b) and this structure is common to all androgens. The numbering of the steroid skeleton is outlined in figure 1.1.

The androgens, exemplified by testosterone (Fig 1.1c) are mainly concerned with the development and maintenance of the secondary male sex characters, increasing virility and libido. They also increase nitrogen and water retention and stimulate skeletal growth. Other examples of androgens are androstenedione (Fig 1.1d), androsterone (Fig 1.1e) and dehydroepiandrosterone (Fig. 1.1f).

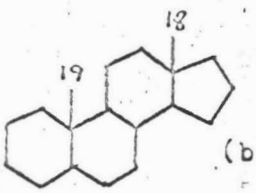
1.1.2 Androgen biosynthesis and secretion

The biosynthetic pathway for androgen synthesis is outlined in figure 1.2. Detailed discussions of the control of androgen biosynthesis Forchielli *et al* (1969) and in vitro synthesis and conversion of androgens Tamaoki *et al* (1969) are documented. In resume, all steroids can arise from cholesterol with pregnenolone as intermediate, cholesterol itself is synthesised from squalene via lanosterol. The biosynthetic steps involved are an initial hydroxylation, a cleavage reaction with the enzyme desmolase, and then a number of hydroxylations by a mechanism involving molecular oxygen directly (Eq. 1.1).

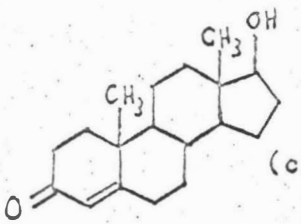
FIG 1.1 Steroid Structure



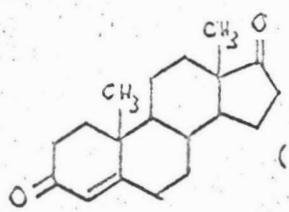
(a) perhydro-13,14-cyclopentanophenanthrene



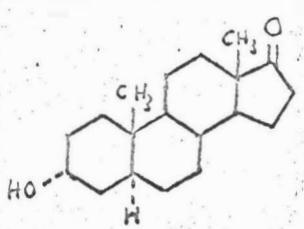
(b) androstane



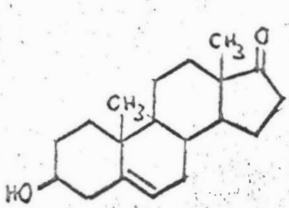
(c) testosterone



(d) androstenedione

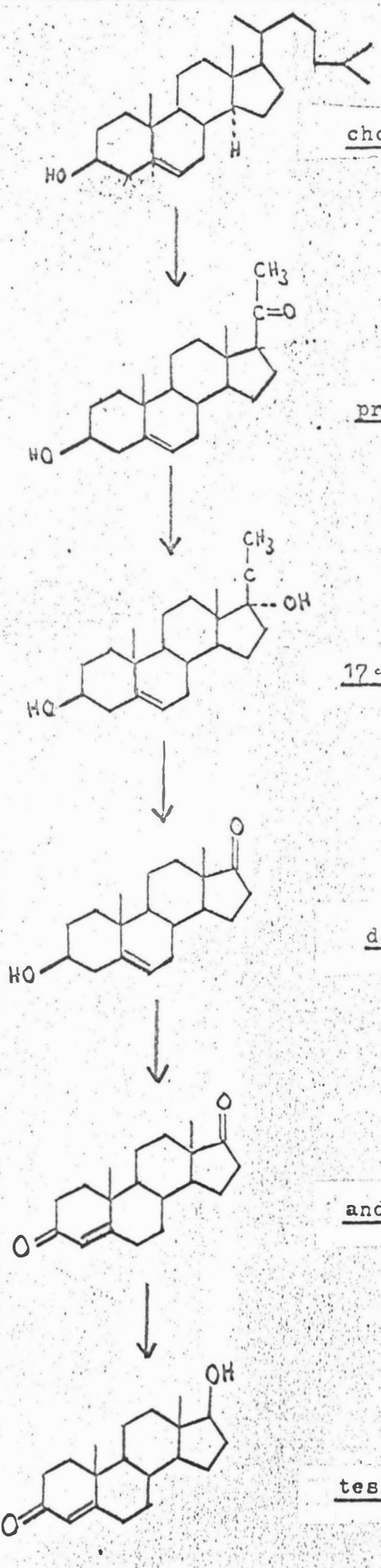


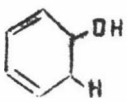
(e) androsterone



(f) dehydroepiandrosterone

FIG 1.2 Testosterone Biosynthesis



Eq. 1.1  + TPNH + H^+ + O_2  + TPN + H_2O

The mechanisms controlling the secretary function of the testis are not very well understood, although three major areas of control have been well defined: Stimuli from the hypothalamus in the form of releasing factors impinge upon the anterior pituitary to release gonadotrophins which stimulate the testis to secrete steroid hormones. The steroid hormones in turn, by virtue of their blood levels, exert control on the hypothalamus and pituitary via negative feedback. Thus there is a homeostatic mechanism which can be used to control production of androgens. A third form of control concerns the direct action of steroid hormones in the various enzyme systems involved in steroidogenesis.

Lindner (1969) has shown that environmental factors such as food intake and season markedly affected testicular androgen secretion in the ram.

1.1.3 Naturally occurring ovine androgenic steroids

Testosterone has been identified by Lindner (1969) in the spermatic vein blood of the ram, accompanied by small amounts of androstenedione. These two androgens are the major naturally occurring androgenic steroids in sheep. The levels for both are documented for HCG treated rams by Lindner (1963) for spermatic vein blood, the respective levels being testosterone 54.1 - 90.1 $\mu g/100\ cm^3$ of plasma, and androstenedione 3.4 - 10.0 $\mu g/100\ cm^3$ of plasma. It is noted that no comparable levels of androstenedione in untreated rams are available, however plasma testosterone levels of spermatic vein blood were 3.6 to 8.7 $\mu g/100\ cm^3$ plasma in the untreated rams.

Levels of the two androgens in peripheral plasma have not been reported, though Lindner does state that plasma testosterone levels in peripheral plasma are less than 1.5 μg/100 cm^3 - these levels being below the measurable sensitivity of his method.

1.1.4 Physiological role of androgens in sheep

The general anabolic action of testosterone (and perhaps also of androstenedione) is bound to be of physiological significance. This action must make the early androgen secretion of the testis an important factor augmenting the growth rate of animals during infancy. In male lambs it could be shown, Lindner (1969), that appreciable amounts of testosterone (0.3 - 0.5 mg/day) were secreted into the spermatic vein blood at the early age of 3½ months (body weight 33-42 kg). Lindner has shown a decreasing proportion of androstenedione in the testicular secretion of the bull with advancing maturation. It remains to be examined whether the shift from androstenedione to testosterone production during pubescence is limited to the bull or whether it is of more general significance. There was no preponderance of androstenedione in the spermatic venous blood of lambs at the age of 3½ months but these animals may have attained a more advanced state of maturity than bull calves at the same age.

For the purpose of this research the shift was assumed to be general, and that the important androgen in mature rams is testosterone rather than androstenedione. As a consequence the majority of the research is directed towards study of testosterone levels.

1.2 Measurement of Androgens by Competitive Protein Binding

Testosterone may be measured by any of a number of methods, including the double isotope derivative technique, gas liquid chromatography, microfluorometry and competitive protein binding (CPB) analysis. Of these techniques, CPB offers the most attractive solution to the problems associated with testosterone determination. It has the advantage of being both faster and easier to use than the other techniques mentioned and in addition is extremely sensitive, allowing measurements in the picogram range. Androstenedione is measured by extension of the testosterone assay.

1.2.1 Theory of competitive protein binding assay

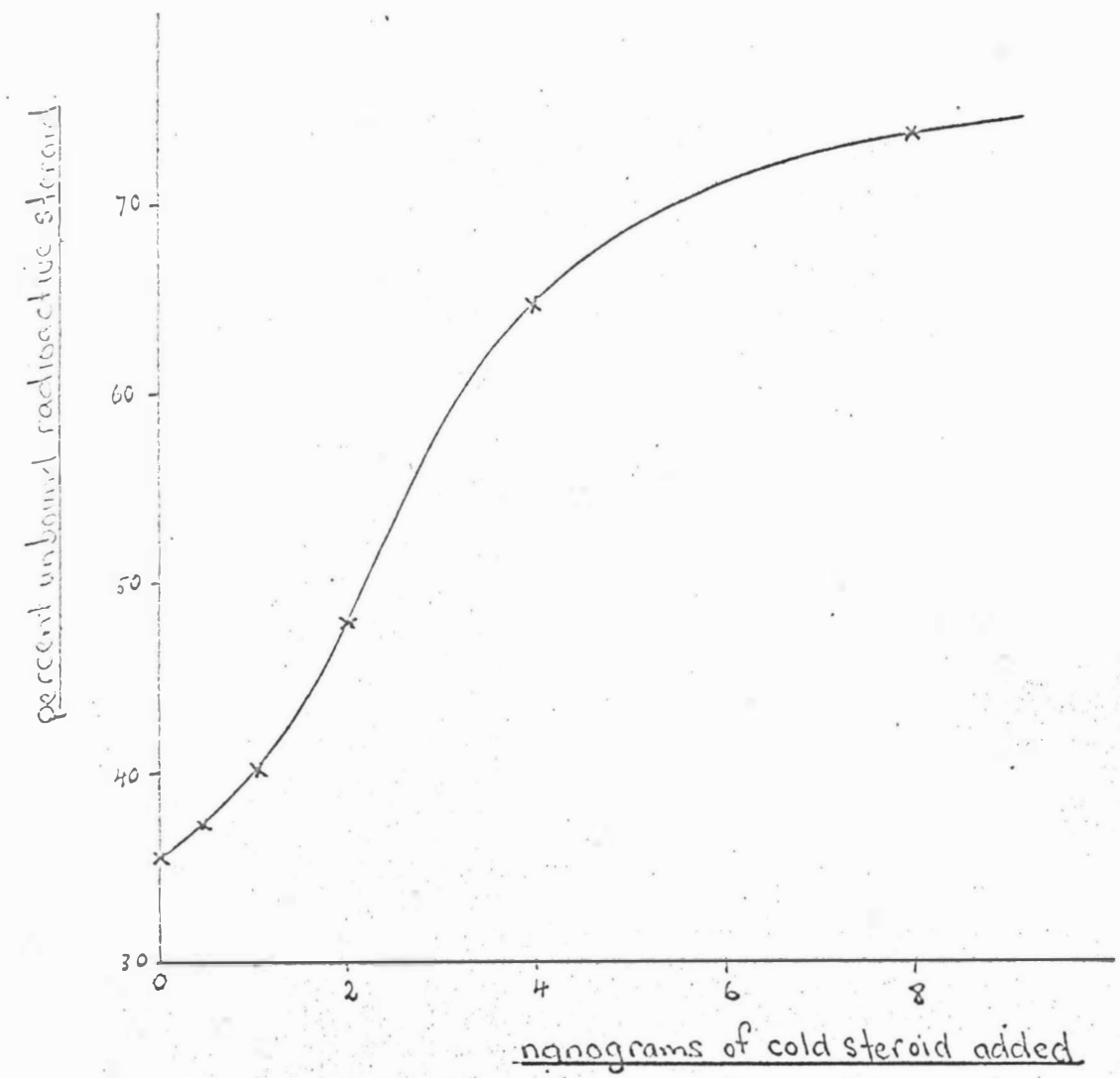
The theory depends on the reversible binding of the steroid (testosterone) to a binding globulin with establishment of equilibrium between free and bound steroid. In practice the binding globulin is just saturated with radioactive steroid, so that all the binding sites are occupied, to give a binding globulin-radioactive steroid complex in solution. If this solution is then equilibrated with non-radioactive steroid, since exchange with bound steroid occurs freely, some of the non-radioactive steroid will replace radioactive steroid already associated with the globulin in the binding complex solution. The amount of displacement is a function of the total amount of steroid present, the relative contribution of the non-radioactive steroid depending on the ratio of non-radioactive steroid to radioactive steroid. Once equilibration has occurred separation of the bound and free steroid must be effected so that an estimation of the degree of displacement of the radioactive steroid from the binding globulin can be made.

Separation of the bound and free steroid can be effected by several methods including the use of dextran-coated charcoal, Fluorisil and Fuller's earth which adsorb only the free steroid from solution. Alternatively use may be made of salt precipitation to precipitate the globulin-bound steroid out of solution. Using the latter method of protein precipitation estimation of the displacement is obtained by centrifuging the sample and counting the radioactive steroid in the supernatant, which represents the radioactive steroid displaced from the binding globulin. Comparison of this figure with a count of the total radioactive steroid before salt precipitation will give the percentage of unbound radioactive steroid. By allowing a number of different concentrations of non-radioactive steroid to equilibrate with the binding complex solution a standard curve can be obtained. This involves plotting the percent unbound radioactive steroid against the amount of non-radioactive steroid added for equilibration (Fig 1.3).

By equilibrating a sample, with an unknown concentration of steroid, with the binding complex solution, it is possible to determine the percentage of unbound radioactive steroid. Reading this percentage off the standard curve gives the concentration of steroid in the sample. After correcting for losses during the extraction and purification of the sample a final concentration of steroid in the original sample can be obtained.

This provides a broad outline of the theory behind CPB methods: for further reference the Karolinska Symposia on Steroid Assay by Protein Binding (1970) gives a fuller insight into the actual methods used, for assay of various steroids including testosterone.

FIG. 1.3 Standard Curve



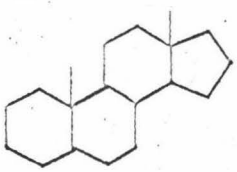
1.2.2 Testosterone binding globulin

Studies by Kato and Horton (1968a) and more recently by Murphy (1970a) have implicated a binding globulin for testosterone and shown that it is not specific for testosterone. These groups have established many features of the steroid configuration necessary for binding to the single globulin which appears to be primarily responsible for both androgen and estrogen binding in human blood. The binding appears to be highly stereo-specific requiring some form of the basic steroid skeleton (Fig 1.4a) and an absolute requirement for an unhindered 17β-hydroxyl group. Dihydrotestosterone (Fig 1.4b) and androstanediol (Fig 1.4c) both have a higher affinity for the binding globulin than testosterone. These steroids are both significantly androgenic and have been implicated as active forms of testosterone. Estradiol 17β (Fig 1.4d) also causes significant displacement of testosterone from the binding globulin, but not as much as testosterone itself. The binding globulin is generally obtained from late pregnancy human plasma or plasma from normal humans undergoing estrogen treatment.

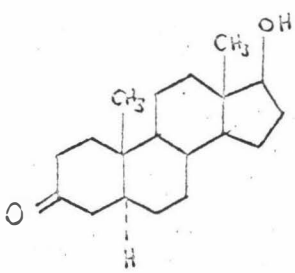
1.2.3 Competitive protein binding methods for plasma testosterone

An excellent review of the current CPB methods for determination of plasma testosterone is provided in a paper by Nugent and Mayes (1970). The techniques are summarised in Table 1 of their paper, which is reproduced, with the addition of the method used in this research, in Table 1.1. It must be noted that these methods are for human plasma, while in this research sheep plasma is studied. As seen in Table 1.1 the basic technique is in most cases very similar, although several modifications are utilised by the different groups. For example, the present method is the only one to utilise column chromatography for purification. The other aspects of the present

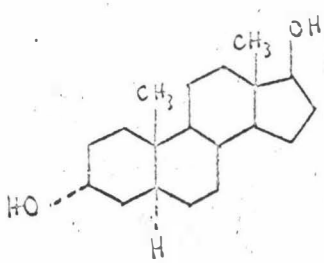
FIG. 1.4 Steroid Structures



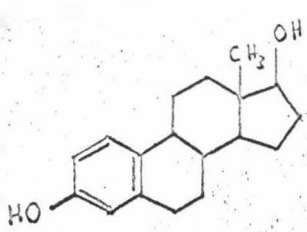
(a) Steroid Skeleton



(b) dihydrotestosterone



(c) androstanediol



(d) estradiol-17β

Table 1.1

Techniques for competitive protein binding assay for plasma testosterone.

Authors	Extraction and Purification*	Protein Binder Source	Bound-free Separation	Plasma (ml) Women	Men
Horton et al. (1967)	Plasma + NaOH, ether extract, water wash	Pregnancy plasma	Dextran coated charcoal		4-5
Frick & Kincl (1969)	Plasma + NaOH, ether extract, TLC X 2	Estrogen treated castrated man	Florisil	2	1
August et al. (1969)	Plasma + NaOH, ether extract, TLC X 2	Pregnancy plasma	Florisil	2-3	0.5
Rosenfield et al. (1969)	Plasma + NaOH, ether extract, H_2O wash X 2, TLC developed X 2	Pregnancy plasma	Charcoal in 20% dextran	2	
Maeda et al. (1969)	Plasma, methylene chloride extract, NaOH and H_2O wash, PC	Estrogen treated women	$(NH_4)_2SO_4$	1-2	0.1-0.2
Kato & Horton (1968)	Plasma + NaOH, NaOH and H_2O wash, PC	$(NH_4)_2SO_4$ precipitate of pregnancy plasma	Sephadex column	5	0.5-1.0
Mayes & Nugent (1968)	Plasma + NaOH, H_2O wash X 2, PC, TLC	Pregnancy plasma	$(NH_4)_2SO_4$	2	0.2
This paper (1971)	Plasma, methylene chloride extract, NaOH and H_2O wash, CC	Late pregnancy plasma	$(NH_4)_2SO_4$	4-5≠	4-5≠

* TLC = thin layer chromatography. PC = paper chromatography.
CC = column chromatography. ≠ sheep plasma.

method are modelled along similar lines to Maeda et al (1969) except for the source of binding plasma. All the methods remove estrogens by the use of NaOH either added to the plasma before extraction or used to wash the plasma extract.

Wide variation in extraction and purification occurs, the extremes being Horton et al (1967) who uses no purification other than a water wash after ether extraction, and Mayes and Nugent (1968) who do not extract the plasma but simply wash and separate the plasma steroids by paper chromatography (PC) and thin layer chromatography (TLC) before treatment with the binding complex solution. The separation of the bound and free steroids is effected by several methods including Sephadex columns, Fluorisil, dextran-charcoal and ammonium sulphate (salt) precipitation of protein.

Concerning the method used in this research, the use of column chromatography with LH-20 Sephadex gel avoids the major problem of high blanks associated with TLC and PC. The ammonium sulphate salt precipitation of bound steroid is effective and simple, and the extraction and washing is as efficient as that in any of the other methods. A fuller discussion of the other methods is contained in Nugent and Mayes (1970) paper.

1.2.4 Androstenedione assay

Since androstenedione does not cause significant displacement of testosterone from the binding globulin (no 17β -hydroxy group) a direct determination by competitive protein binding is not practical. Instead, for CPB analysis, the androstenedione has to be converted to testosterone. This can be done enzymatically, using the 17β -hydroxy-steroid dehydrogenase, Rosenfield (1969b) or chemically, using

borohydride reduction, Moshang et al (1970). The latter method, because of its ease and rapidity has been favoured in this research.

1.3 Action of Gonadotrophic Hormones on Androgen Secretion

Gonadotrophic hormones are the hormones which stimulate the growth and normal functioning of the gonads and the secretion of sex hormones in both men and women. Gonadotrophins are secreted by the anterior lobe of the pituitary gland and are also produced by the placenta and the endometrium during pregnancy. Five different preparations (three of non-pituitary origin) of gonadotrophic hormones are currently in common use. Follicle stimulating hormone (FSH) and luteinising hormone (LH) are directly prepared from pituitary tissue. A gonadotrophin with predominantly luteinising properties is obtained from the urine of pregnant women (human chorionic gonadotrophin, HCG) and one with predominantly follicle-stimulating properties is obtained from the urine of post-menopausal women (human menopausal gonadotrophin, HMG). A gonadotrophin with predominantly follicle-stimulating properties is also obtained from the serum of pregnant mares (pregnant mare serum gonadotrophin, PMSG).

In men, the follicle-stimulating hormone induces the germinal epithelium of the seminiferous tubules to spermatogenesis but does not induce the secretion of androgens. The androgens are formed in the interstitial cells of Leydig under the influence of luteinising hormone. Published work in this field is very limited, particularly in relation to sheep, the species studied in this research. As a consequence where no specific information on sheep is available comparisons with other species, such as the dog, are made.

1.3.1 Pregnant mare serum gonadotrophin

PMSG appears to show both FSH-like and LH-like activity with the emphasis on the former. Eik-Nes and Hall (1965) have shown PMSG to increase the secretion of testosterone and the incorporation of acetate-1-^{14}C into testosterone-^{14}C when administered intravenously to anaesthetized dogs. Lindner (1961a) has shown PMSG injected intravenously at a dose representing 11 1U/kg FSH-like activity and 2 1U/kg LH activity to be effective in stimulating androgen secretion by the calf testis, the rate doubling within forty five minutes of injection. In both cases the research was carried out by monitoring the spermatic vein blood of anaesthetized animals. In the present research peripheral blood was assayed and the animals were not under anaesthetic during the experiments.

1.3.2 Human chorionic gonadotrophin

Intravenous infusion of HCG of total dose 2-5 1U/kg shown by Lindner (1961 a and b) to bring about a prompt increase in the rate of androgen secretion into the spermatic venous blood of all four species examined, that is in the bull, ram, boar and stallion. The early response in mature bulls is not accompanied by an increased blood flow through the testis nor does it depend to a major extent on release of preformed hormone from the gland. It was shown that increased testosterone output was attended by a rise in the testosterone content of the testis, and, in general high rates of testosterone release tend to be associated with a high concentration of testosterone in the testicular tissue, Lindner (1969). The conclusion reached by Lindner is that HCG brings about an immediate increase in androgen synthesis, possibly at first from a steroid precursor in the gland.

This may be followed by an increased synthesis of enzyme protein. Eik-Nes (1967) has observed an even faster response to HCG (within seven minutes) by the dog testis, and this effect of HCG was mimicked by infusion of large amounts of 3',5'-AMP.

Direct reference to sheep is obtained from work by Lindner (1963) in which HCG is used to stimulate the secretion of androgens in rams so that measurements could be made of lympth and plasma levels. Treatment with 1000 IU HCG/24 hours intramuscularly for eight days elevated the plasma testosterone levels in spermatic vein blood into the range 54.1 to 90.1 µg/100ml, from untreated levels in the range 3.6 to 8.7 µg/100ml. In the experiments carried out by Lindner the testosterone levels of peripheral plasma before HCG stimulation were below the limit of sensitivity of the assay method use. After treatment only three of the HCG treated rams had a level of testosterone in arterial blood high enough for precise determination (6.1 to 7.9 µg/100ml). Furthermore, the less sensitive assay methods used by Lindner required the study of spermatic vein, rather than peripheral blood, and the consequent use of anaesthetized animals.

1.4 Clinical Significance of Pathological Entities Studied

Study of androgen levels in intersex sheep and rams with testicular hypoplasia provides information on how these clinical conditions effect androgen levels. The genetic and physical characteristics of these two clinical conditions, as compared to those of normal ewes and rams, enables a study of plasma androgen levels, in particular testosterone, under a wide range of test conditions.

1.4.1 Klinefelter rams

Testicular hypoplasia is not uncommon in the ram. Bruere et al (1969a) considered that the aetiology of testicular hypoplasia in some rams was probably analogous to chromatin-positive Klinefelter's syndrome in man. In Klinefelter's syndrome, the number of chromosomes is increased by the addition of an extra X chromosome, that is, the chromosome number is 47 instead of the normal diploid number of 46.

Klinefelter's syndrome in rams results in an XXY chromosome complex, the extra X chromosome producing the characteristic micro-orchidism (reduced testicular size) and associated testicular pathology (including testicular hypoplasia, azoospermia and apparent aspermatogenesis). The chromatin-positive characteristic of the syndrome is due to the presence of a sex-chromatin body which can be seen in resting somatic cell nuclei, and which represents the extra but 'inactivated' X chromosome of the karyotype.

1.4.2 Freemartin sheep

Freemartin sheep are a form of intersex sheep characterized by permanent white blood cell chimaerism. Two factors are essential for development of freemartin sheep. Firstly, the pregnancy must be of mixed sexes and secondly an early placental vascular anastomosis must develop between the opposed sexes. The male-type freemartin has been reported occassionally in sheep, Bruere et al (1969b). These male-type freemartin sheep have ambiguous external genitalia and testicular gonads, but always have XX (that is, female) sex chromosomes in tissues other than blood and bone marrow, which show XX/XY sex-chromosome mosaicism. The sex-chromosome mosaicism is the result of intrauterine transplacental exchange of blood between opposite sexed foetuses during pregnancy.

Chapter 2

MATERIALS AND EXPERIMENTAL METHODS

2.1 Materials

Androstenedione (Sigma Chemical Company, St. Louis, U.S.A.) was stored without further repurification at a concentration of 0.2 mg/cm^3 in redistilled ethanol.

Testosterone-1 ,2 -^{3}H (T^3H), specific activity 44.6 Ci/mmol (The Radiochemical Centre, Amersham, England) was used after repurification by thin layer chromatography.

Non-radioactive testosterone (Mann Research Laboratories. Inc., New York, U.S.A.) was stored without further repurification at a concentration of 4µg/cm^3 in redistilled ethanol.

Organic solvents: all organic solvents were redistilled before use irrespective of source.

Solution of ammonium sulphate: 50 grams of ammonium sulphate was dissolved in 100 cm^3 of distilled water.

Sephadex-LH-20 (lipophilic) obtained from Sigma and Pharmacia, Uppsala, Sweden. Particle size 25-100µ.

Plasma as a source of testosterone-binding protein was obtained from blood samples from women in late pregnancy at Palmerston North Public Hospital.

Charcoal suspension: charcoal repurified before use by boiling with dilute HCl, after reactivating made up as a 4mg/cm^3 solution in pH 7.8 phosphate buffer.

Liquid scintillation fluid: 6gm PPO (2,5-diphenyloxazol) and 0.5 gm POPOP [p-bis(2-(5-phenyloxazol)benzene)] were added to 1000 cm^3 of redistilled S-free-toluene.

Pregnant mare serum gonadotrophin (Sigma) the sample of PMSG containing 10,000 1U made up in a 0.9% saline solution to give a concentration of 50 1U/cm^3. After sterilization with a millipore filter the stock solution was stored at 5°C.

Human chorionic gonadotrophin (Park Davis and Company, Detroit, U.S.A.) Antuitrin "S", chorionic gonadotrophin of human origin was obtained in a sterile kit. When the kit was made up it consisted of a 10 cm^3 solution containing 500 1U/cm^3. This was stored at 5°C and used within ninety days.

Sodium borohydride (B.D.H. Chemical Division, Poole, England), 0.005 gm made up to 25 cm^3 with redistilled methanol to give an 0.02% solution of $NaBH_4$ in methanol.

Thin layer chromatography plates: these were pre-prepared silica gel plates (Riedel-De Haen, Hannover). After activation by heating at 110°C for one hour they were washed for 24 hours in benzene:ethylacetate (2 : 1) before use.

2.2 Experimental Sheep

All experimental animals were obtained through the department of Veterinary Clinical Sciences, Massey University. The work with these animals was supervised by Dr. A.N. Bruere.

2.2.1 Normal sheep

The normal rams and ewes in this study were obtained from flocks maintained for the Veterinary Clinical Sciences; in particular experimental animals used by Mr. A. Rhodes and Miss H. Chapman.

2.2.2 Klinefelter rams

These rams were a flock of six maintained by Dr. Bruere, and were obtained from genetic screening of rams with micro-orchidism, sent in from veterinary practices. All six have an XXY sex chromosome and exhibit the characteristics of the Klinefelter syndrome.

2.2.3 Freemartin sheep

These are also a flock maintained by Dr. Bruere, being confirmed freemartins from suspected animals sent in from veterinary practices. They have normal XX female sex chromosome in all tissues except blood and bone marrow which have XX/XY sex chromosome mosaicism.

2.3 Collection and Preservation of Samples

2.3.1 Collection

Samples were collected from either jugular vein into 10 cm^3 vacutainer tubes using a No 20 needle. The vacutainer tubes contained heparin as an anticoagulant.

2.3.2 Treatment and storage

The samples were retained in the vacutainer tubes for varying periods at $5^{o}C$ and then centrifuged. The resultant plasma samples were stored at $5^{o}C$ until ready for assay. The storage period was not longer than 2 to 3 days, usually less.

2.4 Purification of Binding Plasma

Late pregnancy human plasma was stripped of endogenous steroid by heating to 45°C for two minutes with an equal volume of charcoal suspension. After centrifugation and filtration (twice) through Whatman No 42 filter paper, the 50% plasma solution was stored at 5°C (for immediate use) or at -20°C (for later use).

2.5 Preparation of Binding Complex Solution

The binding complex solution consists of the binding globulin (of the binding plasma solution) and the T-^{3}H bound to it, in solution. A known amount of the T-^{3}H in ethanolic solution plus a known amount of cold testosterone in ethanolic solution was evaporated down in a 50 cm^3 conical flask. The purified binding plasma was diluted with distilled water to the required concentration, then added and gently mixed. The mixture was freshly prepared for each experiment. For routine study the mixture was made up to give a concentration of approximately 50,000 cpm/cm^3 of T-^{3}H (less than 0.01ng testosterone) and 0.002 $\mu g/cm^3$ of cold testosterone, so that approximately 5,000 cpm in the final counting vial. The solution was stored at 5°C and used within two days.

2.6 Standard Curve

Duplicate tubes set up containing a range of standards and a control. For the later experiments the standards used were 0, 0.5, 1, 2, 4 and 8 ng of cold testosterone along with a control containing no added cold testosterone. Each sample was evaporated to dryness under dry nitrogen, then 0.5 cm^3 of the binding complex solution

added to each tube and gently mixed with a vortex mixer. The tubes were equilibrated for 20 minutes at room temperature and then 1.5 cm^3 of ammonium sulphate solution added to all tubes except the control. 1.5 cm^3 of distilled water was added to the control. After gentle mixing the tubes were left for 10 minutes then centrifuged for 20 minutes (at full speed on bench centrifuges). A 0.5 cm^3 aliquot of the supernatant was transferred to a counting vial containing 8 cm^3 of scintillation fluid, and then counted for tritium in a Packard 3375 (Tri-carb Liquid Scintillation Spectrometer). The unbound fraction of T-^{3}H was expressed as a percentage of the total radioactivity added (control) to each tube.

2.7 Extraction of Plasma Neutral Steroids

5 cm^3 samples of plasma were placed in 50 cm^3 stoppered separating funnels and approximately 5,000 cpm of T-^{3}H added to each sample (and a standard counting vial) for recovery purposes. A short time was allowed for equilibration of the T-^{3}H with the plasma and then the samples were extracted by vigourous shaking with 5 cm^3 of methylene chloride. After settling, the methylene chloride phase was separated off and washed once with 1 cm^3 of 0.1N NaOH and once with 1 cm^3 of distilled water. The methylene chloride phase was then centrifuged and transferred to clean tubes for evaporation to dryness.

2.8 Chromatographic Purification of Testosterone

Three gram quantities of LH-20 Sephadex gel were allowed to swell in the column eluant, benzene:methanol (85:15), then packed by gravity into 8 mm internal diameter columns in the eluant. The prepared columns had an approximate height of Sephadex of 20 cm. The methylene

chloride extract was resuspended in 1 cm^3 of the eluant and put onto a column. On elution with 3 X 6 cm^3 aliquots of the same eluant, fraction two (7-12 cm^3) was collected as the testosterone containing fraction.

2.9 Recovery of Added Tracer

The benzene:methanol fraction collected from the column was blown down under nitrogen, then resuspended in 1 cm^3 of methanol. Two-tenths of the sample (0.2 cm^3) was placed in a counting vial, dried, scintillant added and counted for radioactivity.

2.10 Aliquots for the Standard Curve

Duplicate 0.2 cm^3 aliquots of the resuspended sample accurately pipetted into the assay tubes and processed in the manner described for the standard curve.

2.11 Displacement due to the Non-specific "Blank"

This was prepared by washing 5 cm^3 of methylene chloride with 1 cm^3 of 0.1N NaOH, 1 cm^3 of distilled water, then treating in an identical manner to the samples with the exception of the recovery step. This provides a "method" blank due to non-specific displacement of a small amount of tritiated testosterone. This blank value is subtracted from the sample values, determined from the standard curve, before calculating the levels of testosterone.

2.12 Calculation

On each sample the ratio A/(B+C) was determined where A represents the counts in the supernatant; B+C the total count in the assay tube

which is made up of B the counts added as testosterone-binding protein complex; and C the counts of tracer testosterone recovered in the estimation for correction of losses. From the value of this ratio, which gives a measure of the amount of unbound testosterone-^{3}H in solution (multiplying the ratio by $^{100}/_{1}$ gives the % unbound T-^{3}H), the unknown testosterone in the total extract is estimated from the standard curve. After allowing for the blank, the amount of testosterone is corrected for recovery and the final plasma testosterone is expressed in $\mu g/100\ cm^3$. A sample calculation is provided in appendix I.

2.13 Androstenedione Assay

Duplicate 5 cm^3 samples of plasma, with tracer added for recovery purposes, were extracted with 5 cm^3 of methylene chloride. One extract was purified and assayed for plasma testosterone, the other extract was evaporated down under dry nitrogen and treated as follows: The dried extract was cooled in iced water and 0.15 cm^3 of 0.02% $NaBH_4$ (sodium borohydride) in methanol added and mixed. After an interval of fifteen minutes ten drops of glacial acetic were added and mixed to destroy the reagent and stop the reaction. A further addition of 1.5 cm^3 of distilled water was followed by extraction with 5 cm^3 of methylene chloride. This second methylene chloride extract was purified and assayed for plasma testosterone.

2.14 Gonadotrophin Stimulation Studies

2.14.1 Pregnant mare serum gonadotrophin stimulation of androgen secretion

The experimental animals were shaved in the region of the jugular

vein (in the neck) to facilitate blood sampling. After taking an initial (zero time) blood sample of approximately 20 cm^3 (two vacutainer tubes), 10 cm^3 of PMSG solution was injected intravenously into the jugular vein. Further blood samples were collected at given time intervals after injection of the PMSG solution, that is after 1, 2, 3 and 4 hours. All blood samples were centrifuged immediately and the plasma assayed for testosterone within 24 hours.

2.14.2 Human chorionic gonadotrophin stimulation of androgen secretion

The experimental sheep were prepared in the same way as in section 2.14.1. The HCG was administered as an 0.3 cm^3 intravenous injection into the jugular vein after the initial 20 cm^3 blood sample had been collected. Further blood samples were collected at intervals of 0.5, 1, 2 and 4 hours after administration of the HCG. All the blood samples were centrifuged immediately after collection and the plasma assayed for testosterone within 24 hours (in most cases).

Chapter 3

DISCUSSION OF METHOD DEVELOPMENT

Though the CPB analysis is a faster and easier technique than others available, it requires a great deal of trial and error in setting the method up. Many problems can arise in developing the method and these are discussed under the relevant sections of the method. However, once the technique has been standardised the operation becomes routine and very rapid, samples being taken through the method in about 10 - 12 hours. The major section of this thesis with respect to the time spent on it has been the setting up of the method. Once this had been achieved it was simply a matter of application to the systems studied.

3.1 Sample Treatment

Every effort was made to centrifuge the blood samples as soon as possible after collection to avoid prolonged contact with the erythrocytes. Consequently the samples were stored as plasma rather than as whole blood. The reason that contact with erythrocytes is avoided is because of the erythrocytic enzyme peculiar to ruminants, 17α-hydroxy-C_{19} - steroid dehydrogenase, Lindner (1965). Immediate centrifugation was technically possible only with the gonadotrophin studies, and other samples were often

left for a period of several hours as whole blood before centrifugation. Since the samples were heparinised no clotting occurred but enzyme action may have had some effect.

Plasma samples were normally assayed within 24 hours of preparation, although occassionally this time had to be extended in some of the larger experiments which were limited by the amount of equipment available.

3.2 Reasons for Purification of Binding Plasma

This step ensures that the maximum number of binding sites are available for the binding and displacement of testosterone. The source of binding globulin (plasma), late pregnancy human blood, is high in endogenous steroids (in particular estrogens) as well as binding protein. Since the binding globulin is not specific for testosterone but also binds other steroids with the 17-β hydroxyl group, for example estradiol 17β, these steroids have to be removed before the binding plasma can be used. The most practical method available is the use of charcoal adsorption. This method is simple and efficient and is based on preferential adsorption of the steroids to charcoal which is then removed from the system by centrifugation and filtration. The adsorption is hastened by transient heating of the system to 45^{o}C for two minutes, which also speeds the dissociation of the steroid-protein complex. Use of equal volumes of binding plasma and charcoal solution provides a stock solution of purified, 50% binding plasma solution. The most suitable concentration of charcoal was found to be 4 mg/cm^3 in pH 7.8 phosphate buffer, (personal communication - staff of the Postgraduate School of Obstetrics and Gynaecology, National Womens Hospital, Auckland).

3.3 Dependence of Range of Standard Curve on Preparation of Binding Complex Solution

The method of preparation is simple. A known amount of high-specific-activity T-^{3}H is equilibrated with a given dilution of the purified binding plasma solution. The relative proportion of these govern the shape and range of the standard curve. The shape will be considered more fully in the next section. A suitable range is required to fit the experimental measurements to be made. The range of the standard curve is directly dependent on the dilution of binding plasma. With reference to Maeda et al (1969) a 25% solution of the original binding plasma solution gives a range of 0 to 40 ng, while an 0.5% solution gives a range of 0 to 0.6 ng. For the purpose of experimental results it was found that a 3% solution of the original binding plasma solution gave a good working curve in the range 0 to 4 ng, which was ideally suited to the range in which measurements were made.

3.4 Factors Affecting the Shape of the Standard Curve

The first step in developing the standard curve was to select a method for separating bound and free testosterone. After initial literature surveys it was decided to follow the procedure of Maeda et al (1969) and use ammonium sulphate precipitation. This method precipitates the protein bound steroid, leaving the free steroid in solution. It has many advantages in particular the ease and short time required and the ability to operate at room temperature.

The second step was to control the shape of the standard curve over the working range required. The shape of the standard curve is dependent on the degree of saturation of the binding sites. Early standard curves had a flat initial section, where added testosterone was occupying the still-available binding sites.

No effective displacement was occurring until these sites were fully saturated. Full saturation provides better displacement at low levels of added testosterone and can be achieved by either diluting the binding plasma, or increasing the amount of radioactive testosterone (and the amount of testosterone), or by simply adding cold testosterone (to lower the effective specific-activity of T-^{3}H). Since the range of the standard curve is directly dependent on the dilution of plasma, dilution of plasma cannot be used as a variable. The next alternative was to increase the number of counts, however the specific activity of the T-^{3}H was such that even a 5-fold increase in the number of counts did not increase the testosterone level enough to fully saturate the binding sites.

The problem was finally solved by adding a known amount of cold testosterone to the T-^{3}H before equilibration with the binding plasma. The effect of this was to more fully saturate the binding sites and move the curve in Fig 3.1 to the corresponding position in Fig 3.2, successfully removing the flat initial section of the curve. It was found that with a 3% binding plasma solution, approximately 50,000 cpm and 4 ng cold testosterone per cm^{3} of binding plasma solution, gave a working curve in the range of 0 to 4 ng of added testosterone.

3.5 Background to the Final Extraction Method

The extraction presented few problems in the method development. Any problems arising generally came as artifacts of the extraction, and were not apparent until later in the method. The procedure used initially was a methylene chloride extraction, a water wash of the extract, then purification by TLC. When TLC was implicated as the major problem with the blank (see section 3.8) the method was

FIG 3.1 Inferior Standard Curve

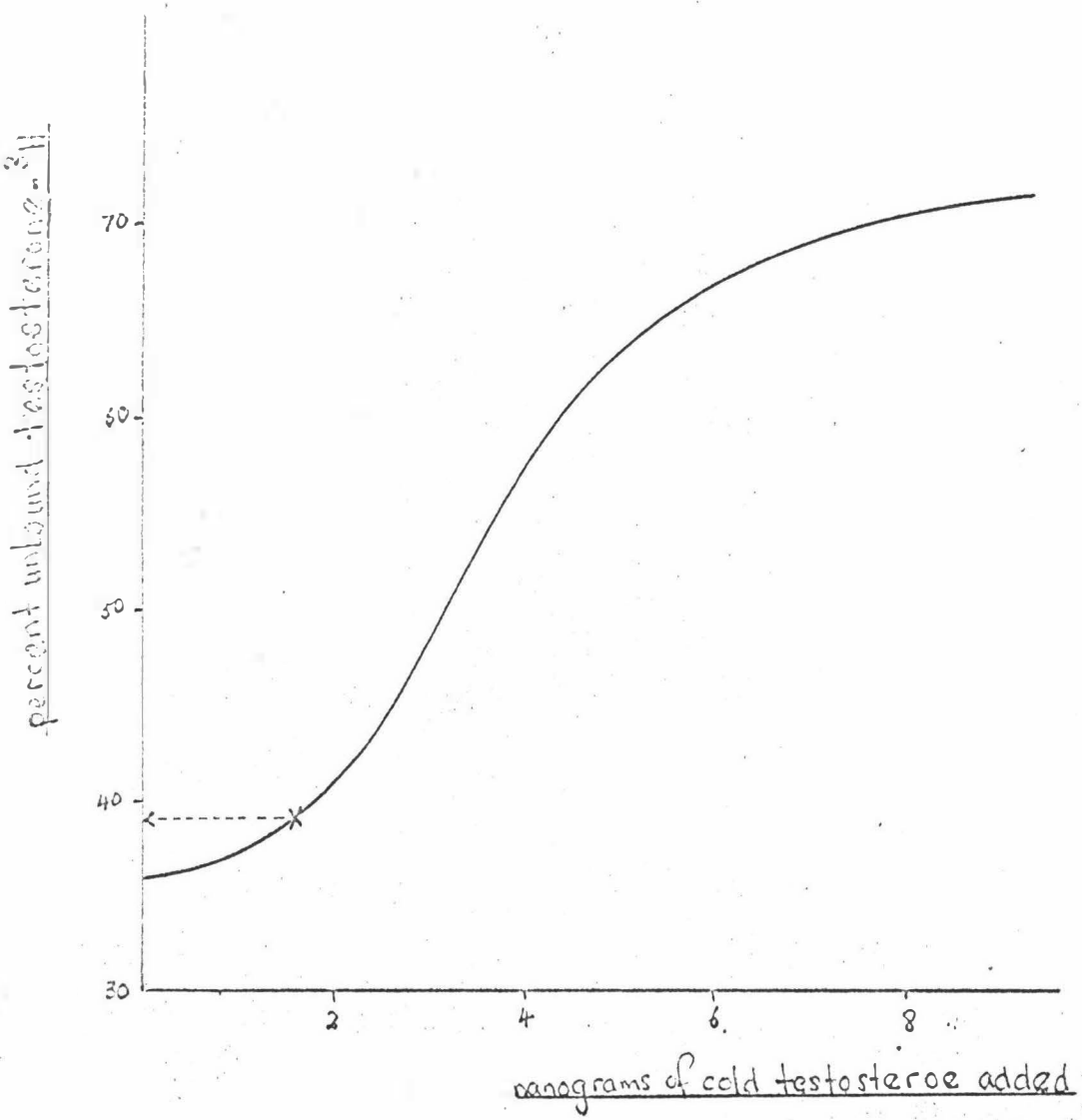
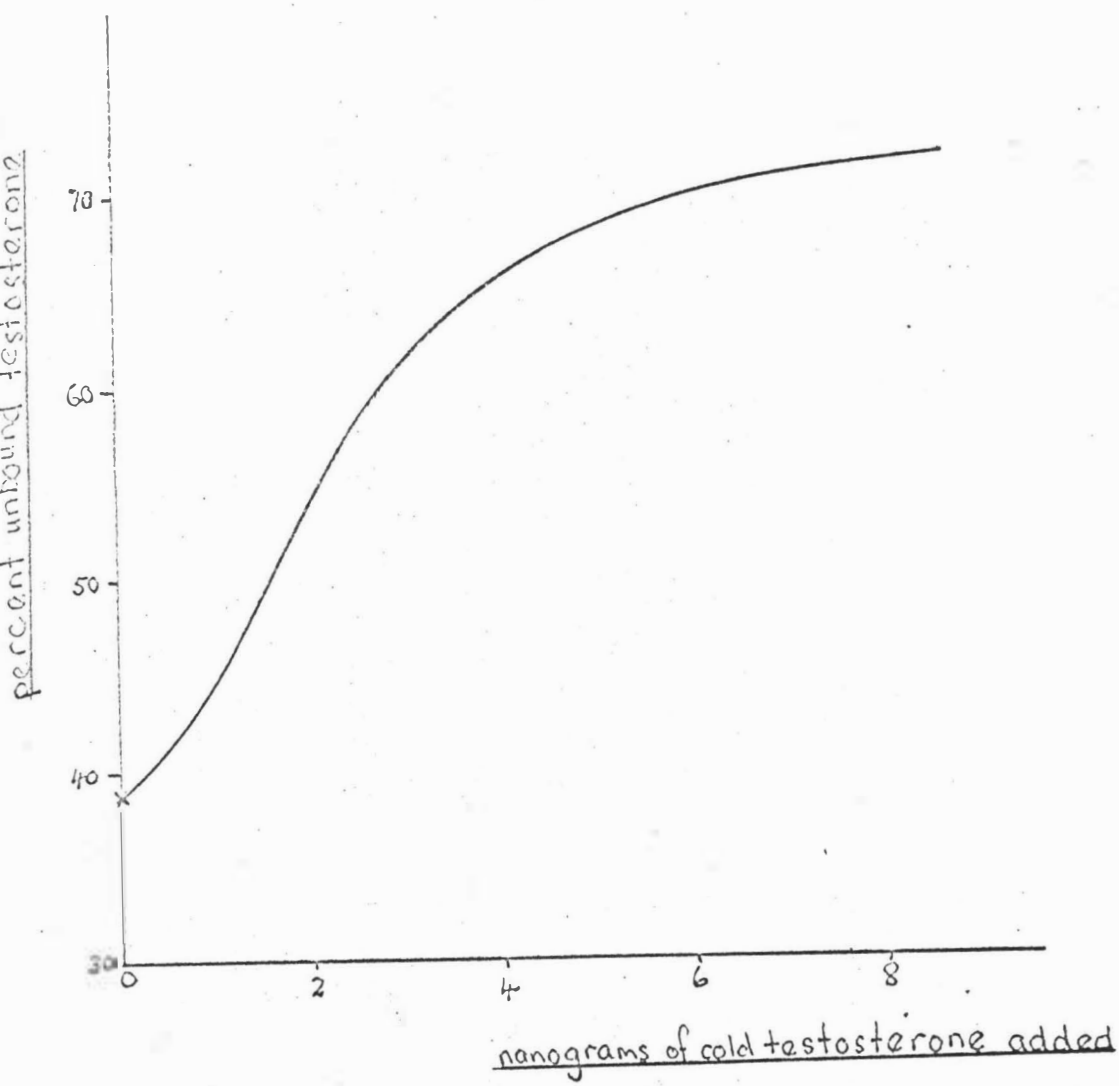


FIG 3.2 Improved Standard Curve



modified to include a NaOH wash before the water wash. The aim of the NaOH wash was to remove most of the estrogens before the water wash and column chromatography. In an effort to keep the reagent contribution to the blank as low as possible, methylene chloride was used for the extraction instead of ether as in many other methods, and the volume was kept low (5cm^3). In addition the washes were limited to 1 cm^3 volumes.

3.6 Steroid Separation by Chromatography

3.6.1 Column chromatography

The use of a lipophilic gel, LH-20 Sephadex, for separating steroids by column chromatography is a recent innovation, some work being published by Murphy (1970b). The use of an 8 mm internal diameter column with 3g of LH-20 Sephadex provides, after swelling, a column approximately 20 cms in length. The gel was allowed to swell for several hours in the eluant (benzene:methanol 85:15) before the columns were packed. The elution profile obtained with these specifications (Figs 3.3 and 3.4) places the majority of the testosterone coming off in the 7th to 12th cm^3 of eluant through the column, after the sample had been put on. The same pattern was obtained whether testosterone was assayed by its absorbance at 250 nm (Fig 3.3) or by counting of T-^{3}H (Fig 3.4). The sharper elution profile with the tracer studies is expected as the high specific activity T-^{3}H does not overload the column.

The elution profile obtained permitted separation of more polar or less polar steroids from testosterone. For example, progesterone is eluted off by the first six cm^3 of eluant. The only other steroids which elute off with testosterone in the second six cm^3 of

FIG 3.3 LH-20 Elution Profile -OD

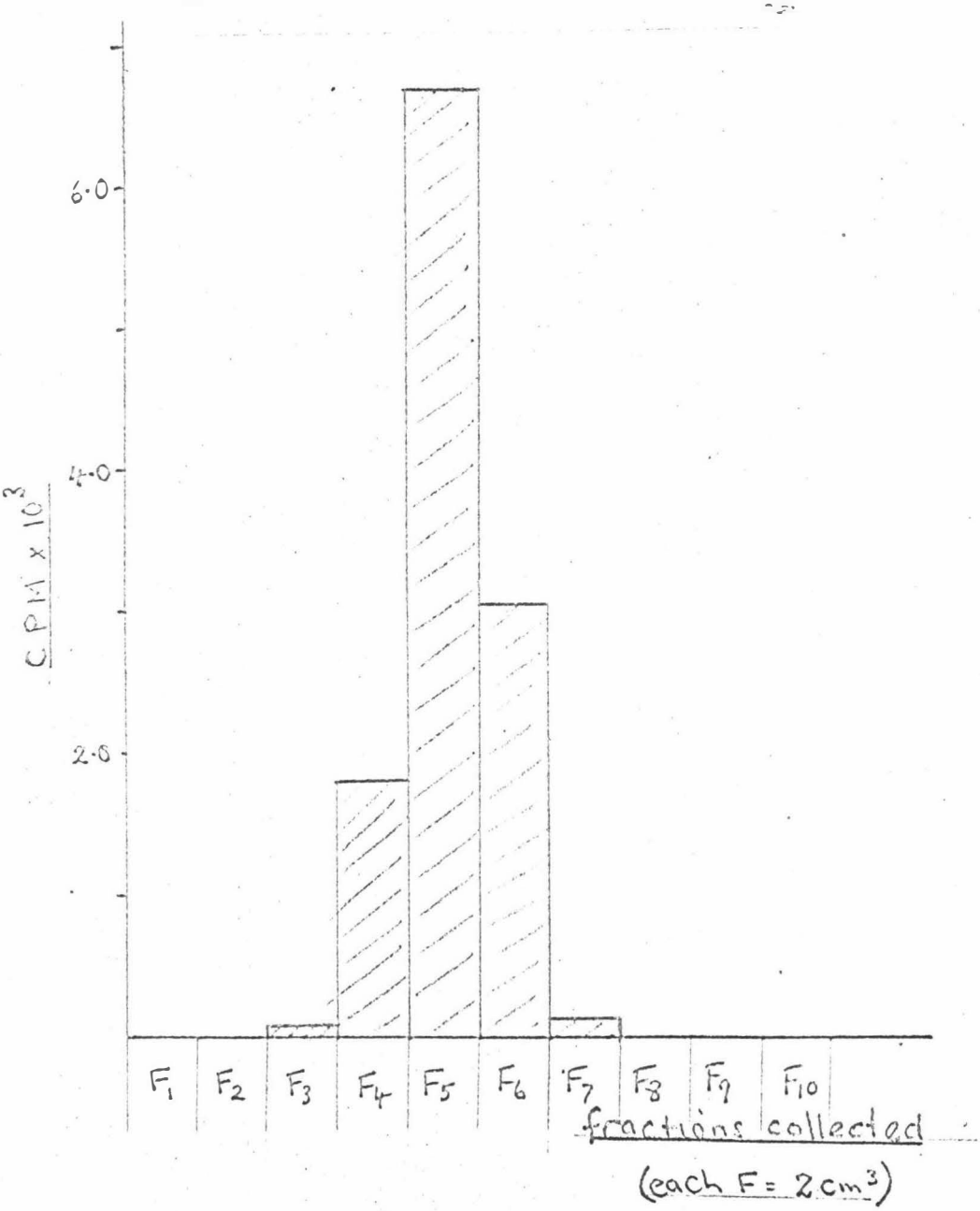
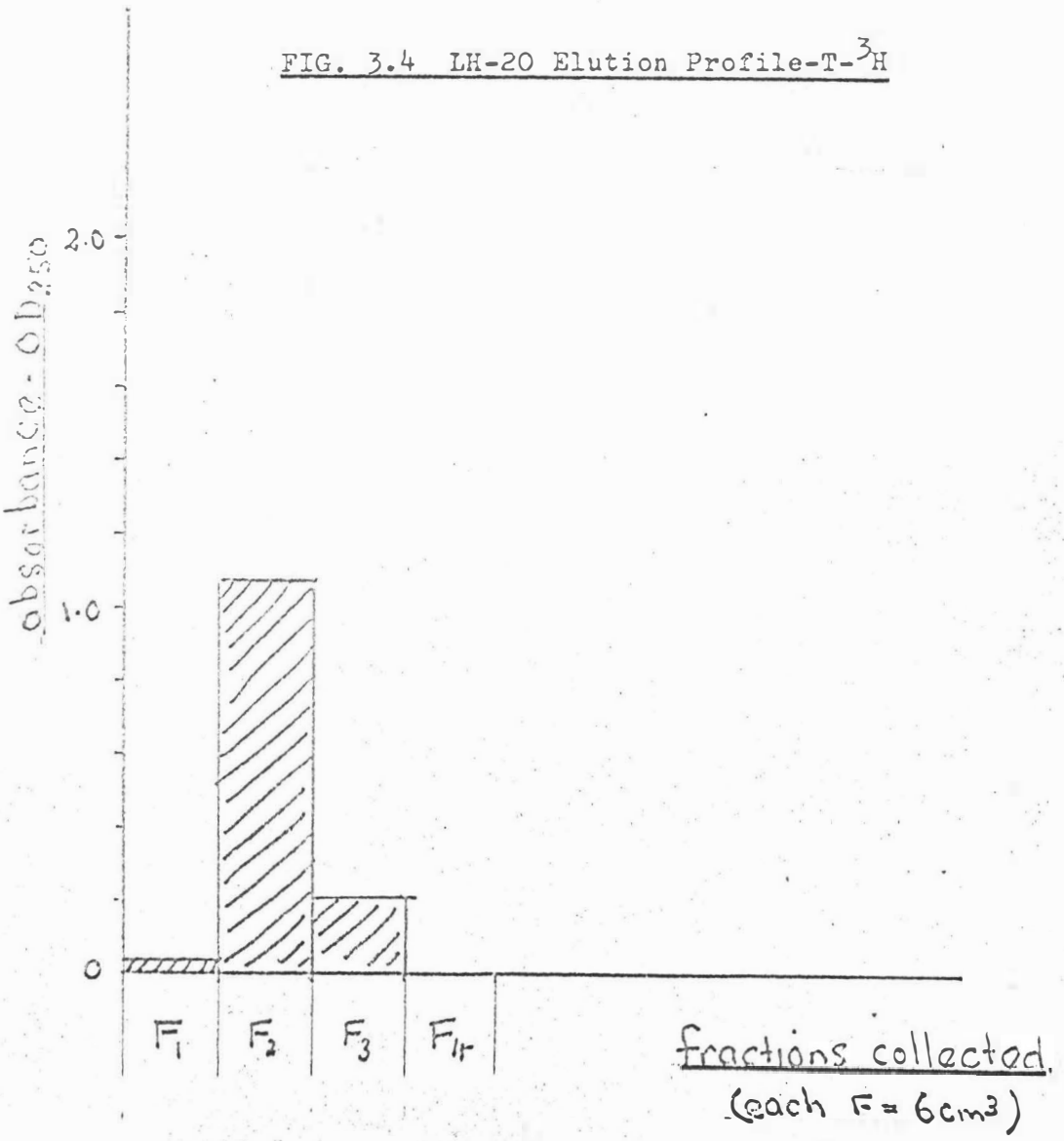


FIG. 3.4 LH-20 Elution Profile-T-^{3}H



eluant are androstenedione and DHEA, neither of which can cause significant displacement of testosterone from binding protein, particularly at levels likely to be present. The steroids likely to cause displacement, estradiol-17β and 5α-Androstane-3α,17β diol, because of their 17β hydroxyl group are well separated from testosterone because of their greater polarity (both diols), or by NaOH wash (estradiol)

3.6.2 Thin layer chromatography

Thin layer chromatography provides an efficient method of separating steroids, however, due to the necessity for mixtures of organic solvents and residues from the TLC plates themselves, a TLC step can affect the non-specific blank in CPB analysis, Vermeulen et al (1970). This is discussed more fully in section 3.8 which deals specifically with the problems arising with the blank. Extensive prepurification of the TLC plates is required before they can be used in CPB analysis. In this research the TLC plates were washed for 24 hours in benzene:ethylacetate (2:1) before use, however, this did not lower the blank sufficiently and the TLC step was accordingly omitted from the method.

3.7 Reasons for the Recovery Determinations

In order to compensate for losses of testosterone in the early stages of sample processing a known amount of T-^{3}H was added to each sample before extraction. One source of loss is not compensated for in this way. If the samples are left dry for too long then some breakdown of testosterone can occur, and since the breakdown products remain in the system no difference in recovery is seen. The net effect is to give a lower testosterone

level than expected because the breakdown of the testosterone is not allowed for in the recovery. This problem arises from the need to evaporate down the samples at several stages in the method, particularly in the last step before addition of the binding complex solution (binding plasma-T-^{3}H). It became apparent when samples were left to evaporate down in air, when they may be dry for several hours before addition of the binding complex solution. The effect was not noticed when evaporation under dry nitrogen was used as the process was more rapid and meant only a short period of dryness before equilibration with the binding complex solution.

There was considerable variation in the percentage of tracer recovered (30-80%). While dependent on several steps in the processing of the sample this appears mainly due to the initial step of the extraction. Because of the likelihood of emulsions forming care must be taken when extracting the plasma sample with methylene chloride. It was found that a fairly vigourous shaking for 2 - 3 minutes (with regular release of pressure) was sufficient to give final recoveries of the order 60 - 70%. However, small variation in the amount of shaking gave markedly different recoveries in the range 30 - 80%. One method of avoiding this problem would be the use of automatic shakers, but these would be rather time consuming and could still lead to emulsions.

3.8 Contributing Factors in the Non-specific "Blank"

Many of the problems arising in CPB methods are related to the blank. For the purpose of these experiments a "method" blank was used. This provides an estimation of the amount of displacement caused by a blank prepared by treating a pure methylene chloride

control identically to the methylene chloride extract of the samples. Several factors can contribute to the blank, such as solvent, reagents and preparation of glassware and adsorbents. The contribution from TLC was such that this step in the purification had to be omitted. For example, when two equivalent samples of plasma were extracted and the first purified with TLC and CC, the second only with CC the resulting blanks were: with TLC 5.925ng, without TLC 0.32ng. The contribution of TLC to the blank is obvious and though a similar contribution to the sample might be expected, using TLC must lead to inaccuracies. TLC was originally intended to separate androstenedione and DHEA from testosterone, and after considering the implications of their levels in plasma and effects on displacement of testosterone, the TLC step was omitted, providing a much lower blank.

Another early problem which arose was the small differences between the blank and the samples. This gave rise to a great deal of inaccuracy and was overcome by increasing the sample size. In the early experiments a sample volume of 0.5 cm^3 plasma was used, but after several experiments it was found that a much higher sample volume was required to get a significant difference between the blank and the samples. The sample volume found to be most satisfactory, both in terms of collection and handling, was a 5 cm^3 volume. However, this could be varied slightly depending on the volume available. Normally a sample volume of 4 or 5 cm^3 used.

The relative contribution of solvent residues (redistilled) to the blank is summarized by Vermeulen et al (1970). Of the solvents tested methylene chloride had one of the lowest values and was accordingly used for the extraction. A fuller discussion of some aspects of the blank problem is provided by Vermeulen.

3.9 Development of the Androstenedione Assay

A method for determination of plasma androstenedione was devised from the testosterone assay by utilising the conversion of androstenedione to testosterone. With reference to Moshang et al (1970) the conversion was carried out by borohydride reduction of the dried methylene chloride extract before purification and assay. The elevation of testosterone level following the conversion was measured by determining the plasma testosterone levels before and after reduction, the increase being due to the androstenedione converted to testosterone.

The specificity of the borohydride reduction in hydroxylating the 17-keto group provides the basis for an accurate measurement of androstenedione, any other compounds likely to interfere with the competitive protein binding displacement being removed in the purification steps following the reduction.

3.10 Determination of Dose Level and Sampling Times for Gonadotrophin Studies

3.10.1 Action of pregnant mare serum gonadotrophin

The dose rate was determined by following Lindner (1969). Lindner uses a dose of PMSG representing 11 lU/kg FSH-like activity and 2 lU/kg LH activity, administered in a 10 cm^3 intravenous injection to an immature calf. It was assumed that the activity expressed by Sigma as "10,000 lU" is the combined FSH-like and LH-like activity, and that the proportions of the two components are of the order expressed by Lindner, that is 2 lU LH activity and 11 lU FSH-like activity in every 13 lU.

Using this assumption and the evidence of Lindner the PMSG sample of "10,000 IU" made up in the saline solution to give 50 IU/cm^3, that is, so a 10 cm^3 solution contained 500 IU. Administration of a 10 cm^3 intravenous injection to a 50 kg ram would then be equivalent to a dose of 10 IU/kg.

The sampling times were modelled on the work by Lindner (1969) on the immature calf. Since these experiments performed on mature rams the response is expected to be more pronounced and Lindner's sampling times, of zero time and 45 minutes after injection, extended to hourly sampling for a four hour period after the initial sample and injection of PMSG.

3.10.2 Action of human chorionic gonadotrophin

Again the dose rate determined by following Lindner (1969). In this case the dose rate used in the four species studied (including rams) was 2 - 5 IU/kg. Since the prepared HCG solution contains 500 IU/cm^3 it was decided to administer a dose of 150 IU as an 0.3 cm^3 intravenous injection, so that a 50 kg ram would receive a dose of 3 IU/kg.

The sampling times were modelled on Lindner (1969), who studied the action of HCG on the mature bull. The response with HCG is expected to be more rapid than with PMSG (because of its more specific action), hence sampling times of 0.5, 1, 2 and 4 hours after initial sample and HCG injection used. These times were based on the PMSG studies and the 0, 25, 45 and 90 minute sampling times of Lindner.

Chapter 4

EXPERIMENTAL AND RESULTS

4.1 Characterization of Competitive Protein Binding Method

Before any experimental method can be used it must first be tested for accuracy and reproduceability. This involves estimating the recovery of added steroid, checking the effects of aliquot sizes for the standard curve and investigating the statistical significance of measurements made with the method.

4.1.1 Recovery of added testosterone

Two duplicate 0.5 cm^3 plasma samples were placed in a 50 cm^3 separating funnel and T-^3H added to both in tracer amounts for recovery purposes. Sample A was extracted without further addition, while sample B had 8 ng of cold testosterone added to it before extraction. Both samples were then processed and assayed for testosterone. The levels of plasma testosterone obtained were:

$$A = 0.425\ \mu g/100\ cm^3, \qquad B = 1.79\ \mu g/100\ cm^3$$

This means that an increase of 1.265 $\mu g/100\ cm^3$ of plasma testosterone. Now 8 ng of cold testosterone added to 0.5 cm^3 of plasma represents 1.6 $\mu g/100\ cm^3$. Since the experiment has detected 1.265 $\mu g/100\ cm^3$ of added testosterone, then 79% of testosterone added has been recovered.

It must be noted that for the main body of the experimental work sample sizes of 5 cm^3 rather than 0.5 cm^3 were used, and the standard curve was better developed (the initial flat region removed). Since this recovery experiment was carried out before these modifications were incorporated, the actual recovery may probably have been better than that shown in the experiment (see section 4.1.4).

4.1.2 Check on aliquot size for the standard curve

This is necessary to confirm that the aliquot size for the standard curve is not affecting the measurement of plasma testosterone. It is related to the fact that the assay "blank" effect may vary with different aliquot sizes. Duplicate 0.2 cm^3 and 0.4 cm^3 aliquots of a 2.0 cm^3 solution of processed ram plasma (Br20) were assayed for testosterone along with 0.2 cm^3 and 0.4 cm^3 blanks. The plasma testosterone levels obtained were: For the 0.2 cm^3 sample 0.079 $\mu g/100\ cm^3$, and for the 0.4 cm^3 sample 0.087 $\mu g/100\ cm^3$. These results give a mean of 0.083 $\mu g/100\ cm^3$ and a standard deviation of 0.004 $\mu g/100\ cm^3$. The difference between samples is very small, so it can be assumed that the aliquot size is not affecting the overall plasma testosterone level. However, for the sake of uniformity, a 0.2 cm^3 aliquot size from a 1.0 cm^3 solution was used in all assays.

4.1.3 Reproduceability of samples

The variation in plasma testosterone levels between assays of samples from the same animal was determined. Using the values for ram Br20 obtained in different assays, the following data was obtained: Experiment A (20/7/71) 0.083 $\mu g/100\ cm^3$; experiment B (28/7/71) 0.085 $\mu g/100\ cm^3$; and experiment C (10/9/71) 0.091 $\mu g/100\ cm^3$.

These three independent determinations of plasma testosterone for ram Br20 give a mean 0.0863 $\mu g/100\ cm^3$ and a standard deviation of 0.00346 $\mu g/100\ cm^3$. These results are in good agreement, and taking into consideration the closeness of the two determinations that were only a week apart, that is 0.083 and 0.085 $\mu g/100\ cm^3$, the reproduceability is shown to be fairly accurate. The higher level obtained after an interval of six weeks (0.091 $\mu g/100\ cm^3$) could easily be explained by variation in diet and behaviour.

4.1.4 Recovery of added androstenedione

Two duplicate 5 cm^3 plasma samples were placed in 50 cm^3 separating funnels. To the first sample tracer T-^{3}H added for recovery, while to the second the recovery tracer T-^{3}H and 3 ng of cold androstenedione added. Both samples were subjected to borohydride reduction and subsequent assay for plasma testosterone. The resulting values for plasma testosterone give A, the plasma testosterone plus androstenedione before addition of cold androstenedione and B, the plasma testosterone plus androstenedione after addition of cold androstenedione. The values obtained: A = 0.066 $\mu g/100\ cm^3$ and B = 0.123 $\mu g/100\ cm^3$ give an increase of 0.057 $\mu g/100\ cm^3$, which is due to the added cold androstenedione. This increase is equivalent to 2.85 ng/5 cm^3 plasma, that is, since 3 ng/5 cm^3 of cold androstenedione was actually added, then 95% of added androstenedione had been recovered. A further test gave an average recovery of 2.80 ng, that is 93%.

Comparison of this recovery with that for added testosterone (section 4.1.1) shows a marked improvement, probably due to the increased accuracy with the larger sample size and improved standard curve.

4.2 Determination of Plasma Testosterone

The plasma testosterone levels were determined in four different groups of sheep, distinguished both genetically and physiologically. The four groups (normal ewes, Klinefelter rams, freemartin sheep and normal rams) provide a spectrum of clinical conditions for studying plasma testosterone.

4.2.1 Normal ewes

The plasma testosterone levels for six normal ewes were determined. 5 cm^3 samples from each ewe were processed and resuspended in 1.0 cm^3 of methanol. 0.2 cm^3 duplicates were then assayed for plasma testosterone. The results obtained from the individual ewes were:

Ewe	I	0.049 $\mu g/100\ cm^3$
	II	0.039 $\mu g/100\ cm^3$
	III	0.115 $\mu g/100\ cm^3$
	IV	0.042 $\mu g/100\ cm^3$
	V	0.059 $\mu g/100\ cm^3$
	VI	0.058 $\mu g/100\ cm^3$

These results give a mean of 0.060 $\mu g/100\ cm^3$, a standard deviation of 0.025 $\mu g/100\ cm^3$ and a range of 0.039 to 0.115 $\mu g/100\ cm^3$, for a test population of six.

4.2.2 Klinefelter rams

The plasma testosterone levels for five of the six Klinefelter rams were determined. 4 cm^3 plasma samples from each ram were

processed and assayed for testosterone. Resulting levels were:

Ram	Br 18	0.076 $\mu g/100\ cm^3$
	Br 20	0.085 $\mu g/100\ cm^3$
	Br A114	0.075 $\mu g/100\ cm^3$
	Br 130	0.109 $\mu g/100\ cm^3$
	Br 182	0.074 $\mu g/100\ cm^3$

These results give a mean of 0.084 $\mu g/100\ cm^3$, a standard deviation of 0.0136 $\mu g/100\ cm^3$ and a range of 0.074 to 0.109 $\mu g/100\ cm^3$.

Note: The level of the sixth Klinefelter ram, Br 146, obtained from the preinfusion level in the HCG studies was found to be 0.072 $\mu g/100\ cm^3$. This value is in close agreement (within one standard deviation) with the mean obtained for the group of five. It is only left out of the group to show that an independent measurement of a Klinefelter ram fits in with the mean obtained for the group. If the value for Br 146 is included in the group the mean and standard deviation change slightly to 0.083 $\mu g/100\ cm^3$ and 0.013 $\mu g/100\ cm^3$ respectively, for a test population of six.

4.2.3 Freemartin sheep

The plasma testosterone levels for five freemartin sheep were obtained by processing and assaying 4 cm^3 samples of plasma. The resulting levels were:

Sheep No.	14	0.064 $\mu g/100\ cm^3$
	144	0.107 $\mu g/100\ cm^3$
	197	0.056 $\mu g/100\ cm^3$
	198	0.200 $\mu g/100\ cm^3$
	196	0.067 $\mu g/100\ cm^3$

These results give a mean of 0.098 µg/100 cm^3, a standard deviation of 0.053 µg/100 cm^3, and a range of 0.056 to 0.200 µg/100 cm^3, for a test population of five.

4.2.4 Normal rams

The plasma testosterone levels of six normal rams were determined by processing and assaying 5 cm^3 samples of plasma. The resulting levels were:

Ram	No. 12	0.110	µg/100 cm^3
	24	0.170	µg/100 cm^3
	53	0.250	µg/100 cm^3
	74	0.200	µg/100 cm^3
	76	0.290	µg/100 cm^3
	77	0.211	µg/100 cm^3

These figures give a mean of 0.205 µg/100 cm^3, a standard deviation of 0.057 µg/100 cm^3 and a range of 0.110 to 0.290 µg/100 cm^3, for a test population of six.

Another normal ram (R253) assayed independently gave a value of 0.24 µg/100 cm^3 which is within one standard deviation of the mean obtained for the group of six.

4.3 Action of Pregnant Mare Serum Gonadotrophin

The action of PMSG in stimulating androgen secretion was studied in one normal ram (R285) and one Klinefelter ram (Br146). The timed samples were processed and assayed for plasma testosterone, the results being expressed in table 4.1.

Table 4.1

Response to PMSG Stimulation

Time (hours)		0	1	2	3	4
Plasma testosterone ($\mu g/100\ cm^3$)	Normal (R285)	0.080	0.054	0.047	0.096	0.121
	Klinefelter (Br146)	0.060	0.051	0.047	0.076	0.080

Only one trial was performed with PMSG since it is not as specific as HCG in stimulating androgen production. The resultant stimulations are plotted against time in figure 4.1.

4.4 Action of Human Chorionic Gonadotrophin

The effect of HCG was studied with respect to both the naturally occurring androgens, (testosterone and androstenedione).

4.4.1 Effect of human chorionic gonadotrophin on plasma testosterone

The action of HCG was studied in two normal rams: R253 and R195/69; and in three Klinefelter rams: Br20, Br146 and Br182 (twice). Samples taken at 0.5, 1, 2 and 4 hour intervals after infusion of HCG were processed and assayed for plasma testosterone, along with a preinfusion control. The results are summarized in table 4.2.

Table 4.2

Response to HCG Stimulation (testosterone)

Time (hours)		0	0.5	1	2	4
Plasma testosterone ($\mu g/100\ cm^3$)	Normal (R253)*	—	0.135	0.21	0.312	0.227
	Normal (R195/69)≠	0.120	0.117	0.112	0.215	0.614
	Klinefelter (Br146)	0.072	0.077	0.141	0.196	0.134
	Klinefelter (Br182a)	0.056	0.070	0.081	0.083	0.084
	Klinefelter (Br182b)	0.060	0.090	0.099	0.078	0.089
	Klinefelter (Br20)	0.091	0.143	0.204	0.200	0.226

* the zero time (preinfusion) sample was lost.

≠ the HCG was not injected intravenously (see section 5.5.2).

These results are graphed in figure 4.2.

4.4.2 Effect of human chorionic gonadotrophin on plasma androstenedione

The action of HCG on plasma androstenedione was studied simultaneously with plasma testosterone in one normal ram (R195/69) and one Klinefelter ram (Br182b). With reference to section 2.1.3 the androstenedione was assayed by conversion to testosterone by borohydride reduction and subsequent determination of the increase in testosterone. The results are summarized in table 4.3.

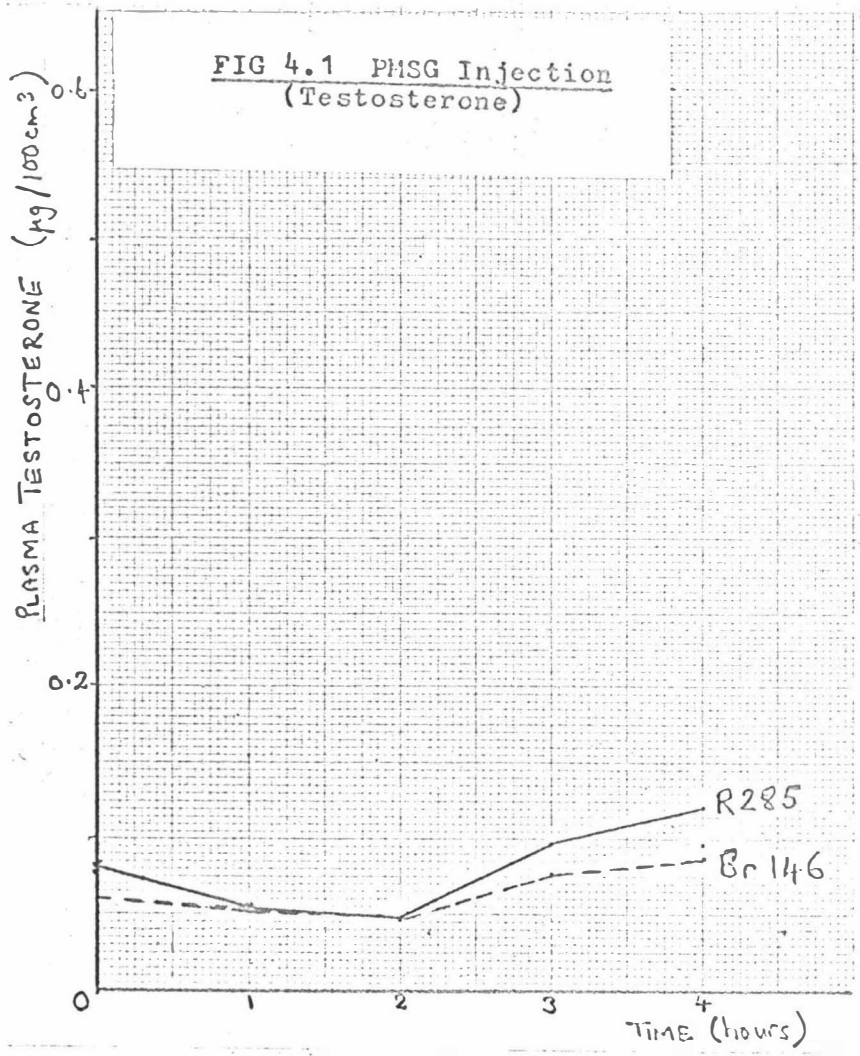


FIG 4.1 PMSG Injection
(Testosterone)
PLASMA TESTOSTERONE (µg/100cm³)
0·6
0·4
0·2
0
1
2
3
4
TIME (hours)
R285
Br 146

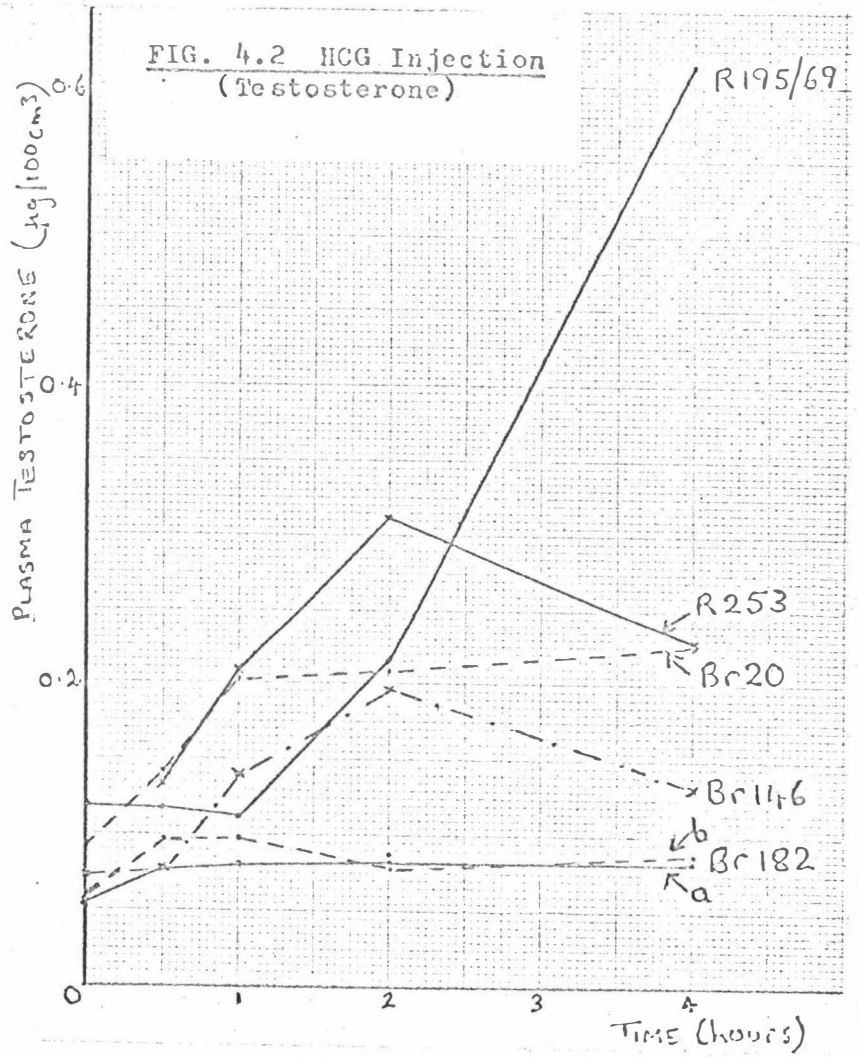


FIG. 4.2 HCG Injection
(Testosterone)
PLASMA TESTOSTERONE (µg/100cm³)
0·6
0·4
0·2
0
1
2
3
4
TIME (hours)
R195/69
R253
Br20
Br146
b
Br182
a

Table 4.3

Response to HCG Stimulation (androstenedione)

Time (hours)		0	0.5	1	2	4
Plasma androstenedione ($\mu g/100\ cm^3$)	Normal (R195/69)	0.060	0.067	0.048	0.055	—
	Klinefelter (Br182b)	0.029	0.031	0.022	0.004	—

These results are graphed in figure 4.3

Note: No detectable level of androstenedione after four hours, probably due to the levels falling below the sensitivity of the method.

FIG. 4.2 HCG Injection
(Testosterone)

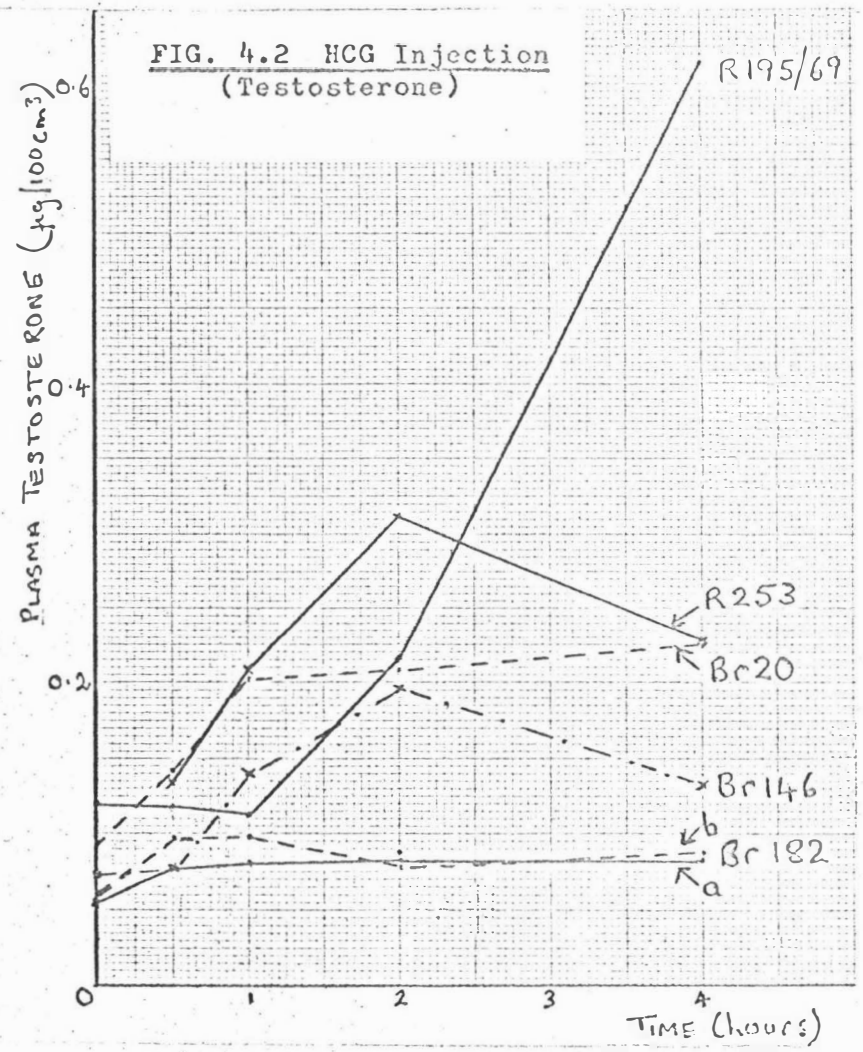
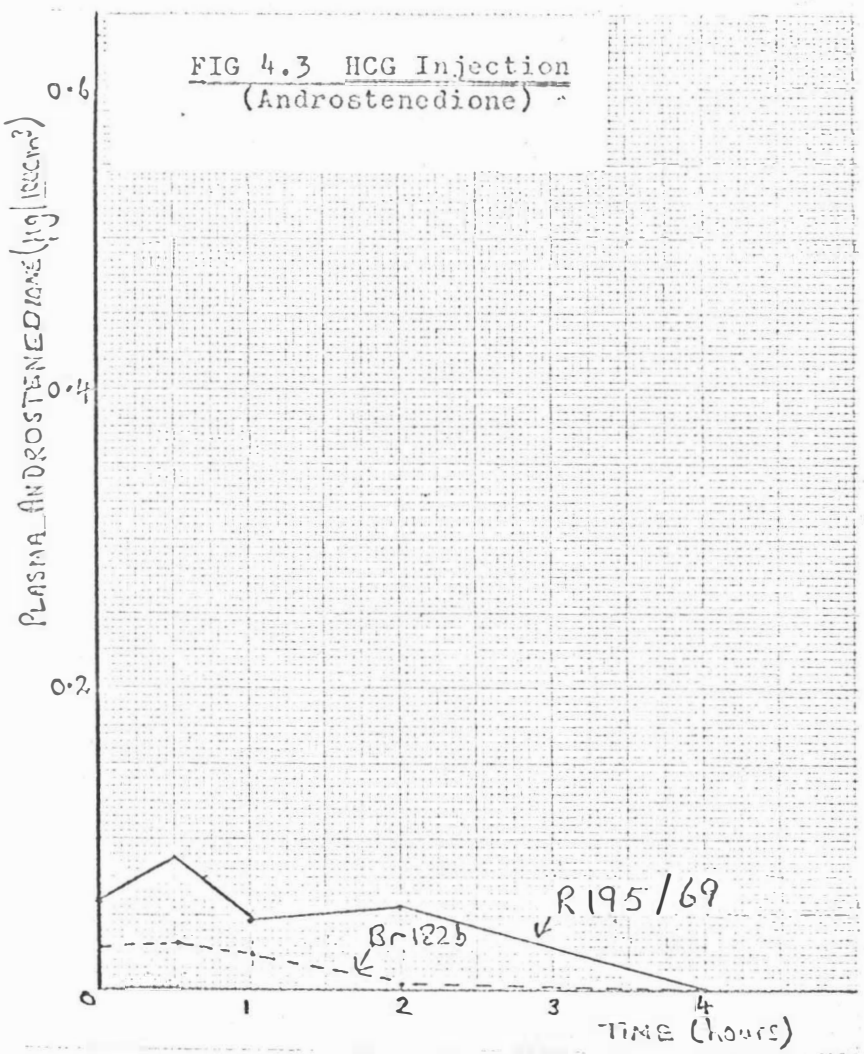


FIG 4.3 HCG Injection
(Androstenedione)



Chapter 5

DISCUSSION

5.1 Assay Method

The development of the method has been discussed fully in Chapter 3. However, a brief description of blank levels, precision, accuracy and sensitivity, as compared to other methods, gives further insight into the method used in this research.

5.1.1 Blank

The highest blanks documented in CPB techniques in use for plasma testosterone assays are those by August et al (1969) (0.18 ± 0.08 ng/cm^3) and Kato and Horton (1968) (0.21 ± 0.16 ng/cm^3). Many of the methods tend to regard blanks rather vaguely, Mayes and Nugent (1968) while qualifying that their water blank is very low do not subtract it from their samples, and Maeda et al (1969) does not even mention a blank. The blank equivalent in the present experiments was of the order 0.10 ± 0.04 ng/cm^3, which though an improvement on those previously mentioned, is still rather high. However, the assay was developed within a limited time, and the blank could possibly have been reduced if more time had been available.

It has been noted by Barden and Lipsett (1967) that the blank is related to plasma volume and recovery. The blank is lowered,

although not linearly, with larger plasma volumes and increased recoveries. This effect was noted when the sample volume was increased from 0.5 cm^3 to 5 cm^3. Another factor which could have been contributing to the variation in the blank was the distilled water used to wash the methylene chloride extract. This was one of the few variables in the assay and could also have been contributing to the high blanks obtained, as a similar effect of distilled water on CPB blanks has been observed (personal communication - staff of the Postgraduate School of Obstetrics and Gynaecology, National Womens Hospital, Auckland). The distilled water was not glass distilled, and was stored and piped to the laboratory. Perhaps any contribution to the blank from the distilled water could be reduced by using glass distilled, deionized water.

Since the main interest of the experiments lay in the comparison between the plasma testosterone levels of the clinical groups, rather than the exact levels, the blank did not produce a major problem, though it must be noted that it may have led to slight discrepancies in the results.

5.1.2 Precision and accuracy

Precision and accuracy are defined by Diczfalusy (1957) as variation within replicates and closeness of measurements to the true values respectively. In these experiments the precision for three samples from one ram (Br20) gave a variation of $\pm$ 0.00346 $\mu g/100\ cm^3$ from the mean of 0.0863 $\mu g/100\ cm^3$, that is a variation of $\pm$ 4%. With respect to the accuracy, assay of added testosterone gave a 79% recovery, while two assays of added androstenedione gave

93% and 95% recoveries. It must be noted that the testosterone experiment was carried out in the early stages of method development, when a small sample size (0.5 cm^3) and inferior standard curve (an initial flat region) were being used. The recoveries with androstenedione are with a sample size of 5 cm^3 and the improved standard curve (see section 3.4). While this accuracy is not exceptional it is sufficient for these experiments.

5.1.3 Sensitivity

Sensitivity is defined as the smallest amount which differs significantly from zero. In these experiments the blank ranged from 0.3 to 0.6 ng displacement equivalent on the standard curve. The lowest difference between blank and sample used was 0.3 ng, that is, a 50 - 100% increase over the blank. This gives a sensitivity of 0.3 ng and consequently no values were determined for samples showing less than a 50% increase over the blank.

5.1.4 Aliquot size for the standard curve

Because of the effect of the non-specific blank, the increase in blank caused by increasing the aliquot size may not be linear. In these experiments the determination of the same sample, using different aliquot sizes, gave a mean of 0.083 $\mu g/100\ cm^3$ and a standard deviation of 0.004 $\mu g/100\ cm^3$. The values obtained are sufficiently close to allow the assumption that the relationship is linear to hold for these experiments. To alleviate the importance of this assumption the same aliquot size was used for all the samples and blanks determined.

5.2 Plasma Testosterone Levels

Four clinical groups of sheep were studied - normal ewes, Klinefelter rams, freemartin sheep and normal rams. The means and standard deviations for the groups plasma testosterone levels were:

Normal ewes	:	0.060 ± 0.025	$\mu g/100\ cm^3$	(N = 6)
Klinefelter rams	:	0.083 ± 0.013	$\mu g/100\ cm^3$	(N = 5)
Freemartin sheep	:	0.098 ± 0.053	$\mu g/100\ cm^3$	(N = 5)
Normal rams	:	0.205 ± 0.057	$\mu g/100\ cm^3$	(N = 6)

The distribution for each group is plotted in figure 5.1.

It can be seen from these results that the normal ewes and Klinefelter rams both provide fairly compact groups (low standard deviations) while the freemartins and normal rams show more diversity (higher standard deviations). The normal ewes showed little variation in levels, in fact, apart from one ewe with a plasma testosterone level of 0.115 $\mu g/100\ cm^3$ they lie in a range of 0.02 $\mu g/100\ cm^3$ (0.039 to 0.059 $\mu g/100\ cm^3$). The Klinefelters are even more uniform, apart from one ram with a level of 0.109 $\mu g/100\ cm^3$, they lie in a range of 0.013 $\mu g/100\ cm^3$ (0.072 to 0.085 $\mu g/100\ cm^3$).

More variation is apparent with the freemartins, as seen by the high standard deviation (0.053 $\mu g/100\ cm^3$), but this is probably expected (see section 5.3.1). The normal rams show a very wide spread with their high standard deviation (0.057 $\mu g/100\ cm^3$). The normal rams show an almost patterned spread from the calculated mean, occurring at fairly evenly spaced intervals from 0.110 to 0.290 $\mu g/100\ cm^3$. The reason for the wide spread with the normal ram plasma testosterone levels is uncertain, since they were all kept under the same conditions it cannot be attributed to the diet.

FIG 5.1 Plasma Testosterone Baseline Levels

Unfortunately no real comparison with other work is possible as the only documented levels of testosterone in sheep have been for spermatic vein blood (Lindner, 1963). The levels in peripheral blood were shown to be below the limits of sensitivity of Lindner's assay method and his only mention of peripheral plasma levels was that they are less than 1.5 μg/100 cm^3. However, it is significant that the ratio of the means for normal males to normal females of 3 to 4 for sheep, found in these experiments, is similar to that found in humans.

5.3 Relation of Plasma Testosterone to the Clinical Syndrome

In addition to normal males and females two other clinical entities have been studied, the freemartin type sheep and the Klinefelter sheep. It is more than coincidence that the more strictly controlled group, the Klinefelters, had the least variation in plasma testosterone levels within the group, while the more random grouping of the freemartins showed wider variation.

5.3.1 Freemartin sheep

The freemartins as a group were selected from sheep suspected of being freemartins sent in from veterinary practices. By definition a freemartin is an intersex sheep with the female (XX) sex chromosome in all tissues except blood and bone marrow which have XX/XY sex chromosome mosaicism. The development of the freemartin is dependent on an early placental vascular anastomosis between opposed sexes and can exhibit varying degrees of masculinization.

Lillie (1917) and Chapin (1917) showed that the degree of masculinization of the potentially female gonad was dependent on the stage at which anastomosis occurred between the placentae. Thus, the earlier the anastomosis, the more marked was the masculinization of the female gonad. Willier (1921) showed that in addition to the freemartin gonad exhibiting a series of gradations between "near female" and "near male" the degree of transformation of the genital organs (vas deferens, seminal vesicles and uterus) was correlated with the same range form in bovine species.

This introductory background could provide a basis for speculation, with respect to interpretation of the results for the freemartins. However, the variation between samples, and the small number of animals tested, rule out any possible correlations. The one fact emerging from the experiments is the lack of correlation between the level of circulating plasma testosterone and the degree of masculinization, seeming to rule out any connection between these two factors. Present lines of thought tend towards a humoral substance (inductor), transferred in the blood during the anastomosis, acting on the "indifferent" gonad of the female foetus to masculinize it, Witschi (1934). Short et al (1969) also supports this idea. The now masculinized gonad, produces its own androgen and is responsible for the development of the secondary male sex characteristics in the female freemartin. The time of anastomosis is still possibly important in the degree of masculinization developed, but over a very short time (a matter of minutes). The main contributing factor now being the amount of humoral substance transferred rather than the time at which the transfer occurs. The degree of masculinization will also depend on the response of the individual gonad.

The results show a wide range of plasma testosterone values for the freemartin. Three of the five freemartins tested show levels (0.064, 0.056 and 0.067 μg/100 cm^3) close to the mean for normal ewes (0.060 μg/100 cm^3). The fourth freemartin has an intermediate level (0.107 μg/100 cm^3) while the fifth has a level (0.200 μg/100 cm^3) which is very close to the mean for normal males (0.205 μg/100 cm^3). A cursory physical examination of the external genitalia of the freemartins has shown that the level of circulating plasma testosterone is not related to the degree of masculinization. However, a more thorough examination when the animals are killed could provide more information.

5.3.2 Klinefelter rams

The Klinefelter rams were selected from clinical referral from veterinary practices. The subjects were effectively screened so that the final group of six all had the XXY sex chromosome and as a group showed uniform bilateral testicular hypoplasia. As a consequence, they would be expected to have fairly uniform plasma testosterone levels. This is shown to be so as the variation within the group is only ± 0.013 μg/100 cm^3 from the mean of 0.083 μg/100 cm^3. Furthermore, five of the group lie within 0.013 μg of each other, in the range 0.072 to 0.085 μg/100 cm^3.

From the results it is apparent that the extra X chromosome has markedly feminized the Klinefelter rams and by some mechanism has lowered the plasma testosterone levels to a level closer to normal females than to normal males. The respective levels for normal ewes (0.060 μg/100 cm^3), Klinefelter rams (0.083 g/100 cm^3) and normal rams (0.205 μg/100 cm^3) compare favourably with the relative

concentrations in similar human groups studied - female 0.08 to 0.10 μg/100 cm^3, Klinefelter 0.28 μg/100 cm^3, normal male 0.67 μg/100 cm^3. A more thorough discussion of the comparison with humans is provided in section 5.4.1.

5.4 Comparison of the Klinefelter Syndrome in Human and Ovine Species

The Klinefelter syndrome was studied in sheep both with respect to circulating plasma testosterone levels and response to gonadotrophin stimulation. Since there are no documented results for sheep, the results were compared with those found in humans by Paulsen *et al* (1968).

5.4.1 Circulating plasma testosterone

The mean level of plasma testosterone in normal rams was found to be 0.205 μg/100 cm^3 and the range of values from 0.110 to 0.290 μg/100 cm^3. The corresponding level in normal human males was 0.67 μg/100 cm^3 and the range of values from 0.28 to 1.44 μg/100 cm^3. In the Klinefelter rams the mean value for plasma testosterone was 0.084 μg/100 cm^3 and the range from 0.072 to 0.109 μg/100 cm^3 while in the human Klinefelter males the mean was 0.28 μg/100 cm^3 and the range from 0.03 to 0.88 μg/100 cm^3. The mean values are listed for comparison in table 5.1.

Table 5.1

Mean plasma testosterone ($\mu g/100\ cm^3$) in arterial blood

Species / Clinical Condition	Human	Ovine	Ratio Human/Ovine
Normal	0.67	0.205	3.2
Klinefelter	0.28	0.088	3.2
Ratio Normal/Klinefelter	2.4	2.4	

The ratio of the means of normal to Klinefelter is 2.4 for both ovine and human species, in addition the ratio of the means of human to ovine plasma testosterone in normal males of 3.2 is also applicable to the Klinefelter condition. The conclusion reached from these figures is that the Klinefelter syndrome has a similar effect on plasma testosterone in both human and ovine species.

5.4.2 Gonadotrophic stimulation

The reports of the effect of HCG stimulation on plasma testosterone levels in human Klinefelter males are rather conflicting. Hudson et al (1965), Briefer et al (1965) and Lipsett et al (1965) have concluded that plasma testosterone levels do not significantly increase when HCG is administered to patients with Klinefelter's syndrome

suggesting that the Leydig cells of these patients may already be maximally stimulated by endogenous gonadotrophin. These observations however, are at variance with those of Leach et al (1956), Leon et al (1959) and Paulsen (1965) who demonstrated either increased sexual development, increased 17-keto-steroid excretion, increased urinary estrogen excretion or mitotic activity of Leydig cells after long term HCG administration. Thus Paulsen et al (1968) has been led to conclude that the Leydig cells in patients with Klinefelter's syndrome can respond to HCG but have a limited reserve.

The situation with ovine species is no less complicated and it would appear that the data obtained supports both schools of thought with human Klinefelters. Of the three Klinefelter rams studied with HCG stimulation, two showed a mild response (as compared to normal rams) and one showed a negligible response. The latter animal was re-examined with further HCG stimulation and was still found to have a negligible response. As a consequence, the conclusion reached by Paulsen with human Klinefelters seems to be the most plausible, that is, in the Klinefelter ram the Leydig cells can respond to HCG but have a limited reserve. In some cases, as with the ram with negligible response, the reserves may be extremely limited, that is, almost saturated by endogenous gonadotrophin.

5.5 Response to Gonadotrophins

Three aspects of gonadotrophin stimulation were studied in the present research: The action of PMSG on plasma testosterone levels in one normal and one Klinefelter ram. The action of HCG on plasma testosterone levels in two normal and three Klinefelter rams; and the action of HCG on the plasma androstenedione levels in one normal

and one Klinefelter ram. Each aspect provides insight into the action of the gonadotrophins in stimulating androgen secretion.

5.5.1 Action of pregnant mare serum gonadotrophin

Little is known about the effect PMSG has on steroid production by the testis. PMSG appears to show both FSH-like and LH-like activity with the greater emphasis on the former. The only reports of the action of PMSG upon the production of testosterone by the testis concern a single observation by Lindner (1961a) in a "poorly developed three month old Friesian calf" where the administration of PMSG resulted in increased secretion of testosterone; and a study by Eik-Nes and Hall (1965) which showed PMSG to increase the secretion of testosterone and the incorporation of acetate-1-^{14}C into testosterone-^{14}C in dogs invivo and in slices of rabbit testis invitro.

The present research has studied the effect of PMSG stimulation of testosterone secretion in a normal and a Klinefelter ram. The results support the stimulatory action of PMSG, an overall increase of about 50% occurring in the plasma testosterone of both animals studied (normal 0.080 to 0.121 $\mu g/100\ cm^3$, Klinefelter 0.060 to 0.088 $\mu g/100\ cm^3$). It was notable that in both cases an initial drop in the plasma testosterone levels occurred in the first two hours after injection of PMSG, to a level of 0.047 $\mu g/100\ cm^3$. The response after this drop was more marked in the normal (an increase of 0.074 $\mu g/100\ cm^3$ in the next two hours) than in the Klinefelter (0.041 $\mu g/100\ cm^3$/2 hours). However, the overall picture seems to be of a similar relative response in the normal and the Klinefelter rams, that is, a similar overall percentage

increase, even though the actual increment is higher in the normal (+0.041 µg/100 cm^3/4 hours) than in the Klinefelter (+0.028 µg/100 cm^3/ 4 hours).

A comparison with the results of Lindner (100% increase in 45 minutes) and Eik-Nes and Hall (600% increase in 60 minutes) is not completely valid since their measurements were on spermatic venous blood. Since their experiments, and those in this research, were carried out on the intact animal, participation of the animals own pituitary in the response cannot be excluded. In addition both the groups mentioned have their results further complicated by the possibility of interaction with anaesthetic affecting the response, since the animals being experimented on are under anaesthesia during their experiments. The work in this research did not entail the use of anaesthetic and hence this factor does not need to be considered.

The comparisons obtained, as expected, show that the response in the spermatic vein blood is much more rapid and marked than that in the peripheral blood as measured in these experiments. This would be expected as the main route of release of testosterone to the peripheral blood is via the spermatic venous blood.

5.5.2 Action of human chorionic gonadotrophin on plasma testosterone levels

More evidence is available for HCG studies than there is for PMSG studies. In particular Lindner (1969) has reported the following result for work in sheep: That intravenous infusion of HCG (total dose 2 - 5 IU/kg) brought about a prompt increase in the androgen secretion into the spermatic venous blood of rams (and bulls, stallions and boars). In addition Lindner (1963)

also documents the levels of plasma testosterone in rams in response to prolonged HCG treatment (1000 IU HCG/24 hours, intramuscularly for 8 days). In untreated rams the plasma testosterone levels in spermatic venous blood range from 3.6 to 8.7 $\mu g/100\ cm^3$. In samples taken two hours after the last injection of HCG in treated rams, the levels had risen to - 54.1 to 90.1 $\mu g/100\ cm^3$. Though no levels are given for peripheral plasma in untreated rams (less than 1.5 $\mu g/100\ cm^3$) the levels in HCG treated rams was 6.7 to 7.9 $\mu g/100\ cm^3$ (the level only rose to measurable levels in 3 out of the 5 rams treated with HCG).

In these experiments the actual levels of stimulation were secondary to the comparison of the effect of stimulation in normal and Klinefelter rams. However, the two normal rams, after treatment with 150 IU of HCG showed a 200% and 500% increase in the level of circulating plasma testosterone in four hours. Though the final levels are nowhere near those obtained by Lindner, it must be remembered that he administered 1000 IU HCG/24 hours, intramuscularly for eight days before testing the peripheral plasma testosterone levels. In this research a single 150 IU dose of HCG is injected intravenously into the first ram and subcutaneously into the second ram (it was injected around, rather than into, the vein by mistake). It is evident from these results that HCG has a marked effect on the levels of circulating plasma testosterone levels in normal rams.

5.5.3 Effect of human chorionic gonadotrophin on plasma androstenedione levels

Only one study was carried out, comparing the response in a Klinefelter and a normal ram. Lindner (1963) has shown that HCG administration increased plasma androstenedione to the measurable

level of 3.4 to 10.0 μg/100 cm^3 in spermatic venous blood (as compared to elevation of plasma testosterone levels from 3.6 - 8.7 μg/100 cm^3 to 54.1 - 90.1 μg/100 cm^3). This research has shown that although a slight increase occurred in the plasma androstenedione level in peripheral blood of the normal ram, it was not really significant and the response in both the Klinefelter and the normal ram was deemed to be negligible. Furthermore, the level of androstenedione actually seemed to decrease, the effect being more marked in the Klinefelter ram studied. This supports the original idea that in the mature ram testosterone rather than androstenedione is the important androgen (see section 1.1.4). The decrease is possibly related to conversion of androstenedione to testosterone, to increase the testosterone levels, under the stress of gonadotrophin stimulation. More extensive study would be required to substantiate this possibility.

5.5.4 Comparison of PMSG and HCG action

Eik-Nes (1962) has shown that 1-2 IU PMSG/kg body weight administered intravenously to a dog prevented the decline in the secretion of testosterone usually seen in the anaesthetized dog. These responses are comparable to those reported for 1-2 IU HCG/kg body weight (Brinck-Johnsen and Eik-Nes, 1957) suggesting that in the dog the two hormones are of approximately equal activity, unit for unit. Also the time course of the response to PMSG invivo and the dose (expressed in IU) required to produce maximal stimulation of testosterone secretion are in general similar to those previously reported for HCG. Lindner (1969) though not making direct reference to the relative activities uses 2-5 IU HCG/kg and (11 IU FSH, 2 IU LH) PMSG/kg, suggesting that the relative activities of PMSG

and HCG are dependent on the LH activity, a logical assumption since it is the LH which affects the Leydig cells, which are the site of testicular testosterone production.

In the present research 3 IU HCG/kg and 10 IU PMSG/kg are used respectively. The responses obtained in normal rams were far greater with HCG (200 - 500% increases in four hours) than with PMSG (50% increase in four hours). This would tend to support Lindner, since it is assumed that the activity of PMSG (10 IU) is the combined FSH-like and LH-like activity. From Lindner's ratio of FSH : LH of 11 : 2, the 10 IU of PMSG administered would have approximately 1.5 IU LH/kg. Thus the relative responses of 200 - 500% for 3 IU HCG/kg and 50% for 1.5 IU LH/kg, do have some degree of correlation, since the effect of an increasing dose of gonadotrophin on testosterone secretion is not necessarily linear.

SUMMARY

The research carried out has provided information on plasma androgens in sheep, which at the time of writing, could not be found documented elsewhere. The levels of circulating plasma testosterone provide comparisons between the clinical syndromes, but because of the small test populations the overall picture is by no means clear. However, in part, the results obtained can lead to further understanding in conjunction with results of other groups, when and if these are documented. With respect to circulating plasma testosterone levels the normal rams and normal ewes show levels which approximate those expected, and the Klinefelter rams showed the expected effect of feminization on the male sex hormone levels. The levels of circulating plasma testosterone in the freemartin sheep do not provide any outstanding information apart from the randomness of the levels. More information on the syndromes themselves and the associated plasma testosterone levels is required to substantiate the present results.

With regard to the gonadotrophin studies a similar situation arises, in that studies on a small number of experimental animals are far from conclusive. Even where results are well documented, for example, HCG studies in normal and Klinefelter human males, the evidence from different groups is conflicting. Consequently the value of the present results lies in limited indications requiring study of more cases.

In conclusion, the method set up has provided a technique for assaying plasma testosterone and plasma androstenedione at low levels. Further refinements, such as lowering the blank even more, could lead to an extremely useful tool for studying androgens, applicable to many different species.

LITERATURE CITED

August, G.P., Tkachuk, M., and Grumbach, M.M. : J. Clin. Endocrin. 29, 495, (1969).

Barden, C.W., and Lipsett, M.D. : Steroids 9, 71, (1967)

Briefer, C.Jr., Rotner, H.E., Forbes, A.P., and Kliman, B. : Clin. Res. 13, 240, (1965)

Brinck-Johnsen, T., and Eik-Nes, K. : Endocrinology 61, 676, (1957)

Bruere, A.N., and Macnab, J. : Res. Vet. Sci. 9, 170, (1968)

Bruere, A.N., Marshall, R.B., and Ward, D.P.J. : J. Reprod. Fert. 19, 103, (1969a)

Bruere, A.N., McDonald, M.F., and Marshall, R.B. : Cytogenetics 8, 148, (1969b)

Chapin, C.L. : J. exp. Zool. 23, 453, (1917)

Diczfalusy, E. : Acta endocr. (Kbh) Suppl. 31, 11 (1957)

Eik-Nes, K. : Endocrinology, 71, 101, (1962)

Eik-Nes, K.B., and Hall, P.F. : J. Reprod. Fert. 9, 233, (1965)

Eik-Nes, K.B. : In "Endocrinology of the Testis", Wolstenholme, G.E.W., O'Connor, M., eds Ciba Foundation Colloquia on Endocrinology 16, 120 (1967)

Forchielli, E., Menon, K.M.J., and Dorfman, R.I. : In "The Gonads", pp 519-545, McKerns, K.W., eds Meredith Corporation, New York, (1969)

Frick, J., and Kincl, F.A. : Steroids 13, 495, (1969)

Horton, R., Kato, T., and Sherins, R. : Steroids 10, 245, (1967)

Hudson, B., Coghlan, J.P., Dulmanis, A., and Wintour, M. : 2nd Intern. Congr. Endocrinol. pp 1127-1133. Excerpta Med. Found. Intern. Congr. Ser. No. 83, (1965), Amsterdam.

Karolinska Symposia on Research Methods in Reproductive Endocrinology, 2nd Symposium. "Steroid Assay by Protein Binding". Acta endocrinologica, Suppl. 147, (1970)

Kato, T., and Horton, R. : J. Clin. Endocrin. 28, 1160, (1968a)

Kato, T., and Horton, R. : Steroids 12, 631, (1968b)

Leach, R.B., Maddock, W.O., Tokuyama, I., Paulsen, C.A., and Nelson, W.O. : Recent Progr. Hormone Res. 12, 377, (1956).

Leon, M., Wajchenberg, B.L., Montenegro, M.R., and Cintra, A.B.U. : J. Clin. Endocrin. 19, 1667, (1959)

Lillie, F.R. : J. exp. Zool. 23, 371, (1917)

Lindner, H.R. : J. Endocrin. 23, 139, (1961a)

Lindner, H.R. : J. Endocrin. 23, 171, (1961b)

Lindner, H.R. : J. Endocrin. 25, 483, (1963)

Lindner, H.R. : Steroids, Suppl. 2, 133, (1965)

Lindner, H.R. : In "The Gonads", pp 615-640, McKerns, K.W., eds Meredith Corporation, New York, (1969)

Lipsett, M.B., Davis, T.E., Wilson, H., and Canfield, C.J. : J. Clin. Endocrin. 25, 1027, (1965)

Maeda, R., Okamoto, M., Wojienka, L.C., and Forsham, P.H. : Steroids 13, 83, (1969)

Mayes, D., and Nugent, C.A. : J. Clin. Endocrin. 28, 1169, (1968)

Moshang, T., Rudd, B.T., Eberlein, W.R., and Bongiovanni, A.M. : Steroids 15, 195, (1970)

Murphy, B.E.P. : Steroids 16, 791, (1970a)

Murphy, B.E.P. : Acta Endocrinologica Suppl. 147, 37 (1970b)

Nugent, C.A., and Mayes, D. : Acta Endocrin. Suppl. 147, 257, (1970)

Paulsen, C.A. : In "Estrogen Assays in Clinical Medicine", pp 279-282, University of Washington Press, Seattle, Washington, (1965)

Paulsen, C.A., Gordon, D.L., Carpenter, R.W., Gandy, M.H., and Drucker, W.D. : Recent Progress in Hormone Research 24, 321, (1968)

Rosenfield, R.L., Eberlein, W.R., and Bongiovanni, A.M. : J. Clin. Endocrin. 29, 854, (1969a)

Rosenfield, R.L. : Steroids 14, 251 (1969b)

Short, R.V., Smith, J., Mann, T., Evans, E.P., Hallet, J., Fryer, A., and Hamerton, J.L. : Cytogenetics 8, 369, (1969)

Tamacki, E., Inano, H., and Nakano, H. : In "The Gonads", pp 547-613, McKerns, K.W., eds Meredith Corporation, New York (1969)

Vermeulen, A., and Verdonck, L. : Acta Endocrin. Suppl. 147, 239, (1970)

Willier, B.H. : J. exp. Zool. 33, 63, (1921)

Witschi, E. : Biol. Rev. 9, 60, (1934)

Appendix I

CALCULATION

Several calculations are required in determining the final plasma testosterone concentration in $\mu g/100\ cm^3$ of the initial plasma sample.

a) Recovery - by adding labelled testosterone and counting the re-isolated steroid a determination of how much of the original sample testosterone is left can be made. Each sample is corrected for background, and in addition the sample is corrected for the aliquot counted.

for example:			corrected value
	Background CPM	= 15.7	
	Counts added	= 5712.8	5697.1
	Recovered sample ($^1/_5$ aliquot)	= 337.9	322.2(x5)

then the percentage recovery is:

$$\frac{322.2 \times 5}{5697.1} \times \frac{100}{1} = 28.27\%$$

b) Standard curve and blank - the CPM in the supernatant after addition of the binding complex solution to the standard is corrected for background (total CPM). The CPM in the supernatant of each

sample is corrected for background, then divided by the total CPM and multiplied by $^{100}/_{1}$ to give the percentage of unbound T-^{3}H.

			corrected values	% unbound T-^{3}H
Background CPM	=	15.7		
Standard (no pptn)	=	6211.1	6195.4	
nanograms of cold testosterone added to each assay tube: 0	=	2413.3	2397.6	38.70
½	=	2516.85	2501.15	40.37
1	=	2768.65	2752.95	44.43
2	=	3689.8	3674.1	59.30
4	=	4158.9	4143.2	66.87
8	=	4601.8	4586.1	74.02
Blank*	=	2359.7	2524.1	40.74

* the blank is the nonspecific displacement caused by solvent residues.

The points are plotted in figure I, and the blank value read off.

c) Sample - the percentage unbound T-^{3}H in the sample must make allowance for the added labelled testosterone. This is done by using the ratio A/(B+C) where A represents the corrected counts in the supernatant; B+C the total counts in the assay tube which is made up of B the corrected counts added in the binding protein complex solution; and C the corrected counts of the tracer (added labelled testosterone) recovered. Multiplying this ratio by $^{100}/_{1}$ gives the percentage of unbound T-^{3}H in the sample.

For ram No. 74 A = 3033.35

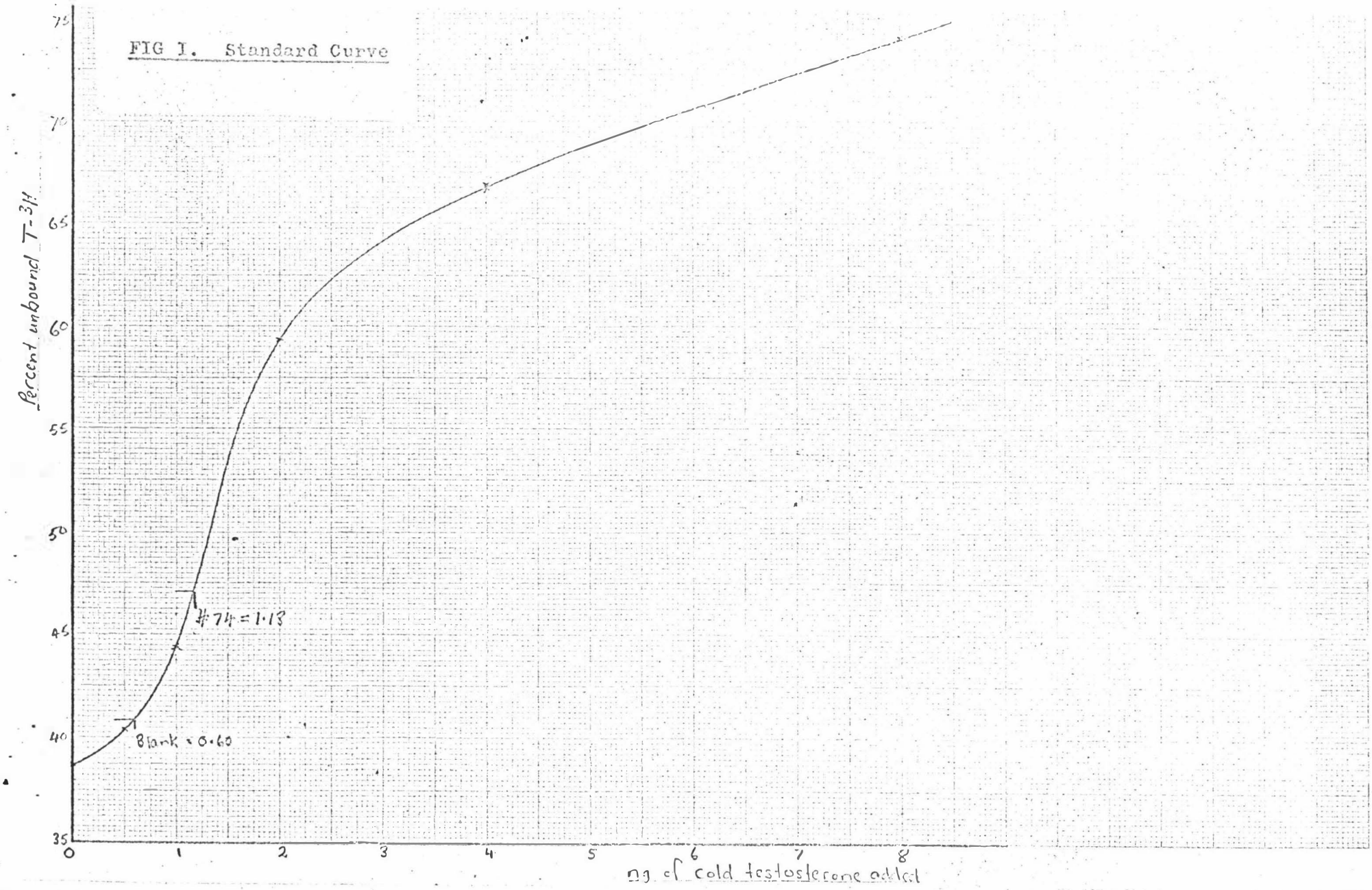
B = 6195.4

C = 322.2

$$\text{then \% unbound T-}^{3}\text{H for No. 74} = \frac{3033.35}{(6195.4 + 322.2)} \times \frac{100}{1}$$

$$= 46.54\%$$

FIG I. Standard Curve



d) Testosterone in sample - from the standard curve the blank has the equivalent of 0.60 ng testosterone and sample No. 74 has the equivalent of 1.18 ng testosterone, that is, sample No. 74 actually contains 0.58 ng testosterone.

e) Conversion to $\mu g/100\ cm^3$ - the following factors must be allowed for:

(i) Allowance for aliquot of extract assayed, i.e. 0.2 cm^3 from a total extract volume of 1.0 cm^3 (i.e. x 5)

(ii) Correction for incomplete recovery (i.e. x 100/28.27)

(iii) Scaling of original plasma sample volume of 5 cm^3 to 100 cm^3 (i.e. x 20)

(iv) Conversion of ng to μg (i.e. x 0.001)

then, the amount of testosterone in the initial sample of plasma is:

$$\text{No. 74} = 0.58 \times 5 \times 100/28.27 \times 20 \times 0.001$$

$$= \underline{0.20\ \mu g/100\ cm^3}$$