ABSTRACT

Sex hormone-binding globulin (SHBG) is a plasma protein which binds testosterone and estradiol *in vitro* with high affinity and specificity. There is conflicting evidence as to the role of SHBG in the transport of estradiol under *in vivo* conditions. Studies describing the distribution of estradiol and testosterone into SHBG-bound, albumin-bound and unbound fractions were carried out using equilibrium dialysis of whole serum at 37°C under isosmotic conditions. The results showed that SHBG was an important binder of estradiol in both pregnant and non-pregnant women, but of minimal significance in men and in newborn infants. Testosterone was significantly bound to SHBG under all circumstances. In order to characterize previously unrecognized substances that may bind to SHBG in pregnancy, maternal serum was fractionated on Sephadex LH-20. A substantial part of the SHBG-bound material eluted as 4 major peaks which did not correspond to any of the steroids thought to bind to SHBG. Comparison of levels in maternal, fetal and placental compartments suggested that all 4 peaks were of placental origin. Three were identified as 5α-pregnane-3,20-dione, progesterone and 2-methoxyestrone on the basis of elution pattern on Sephadex LH-20, binding characteristics, high performance liquid chromatography, and gas chromatography-mass spectrometry. The fourth peak was identified as a 19-nor androgen, i.e. 17β-hydroxy-1,5-estradiene-3-one, on the basis of similar kinds of evidence and in addition, solubility characteristics and ultra-violet absorption spectrum. Since its level fell in association with premature and term labour, its decrease may be involved in the initiation of labour.

RESUME

La globuline liant les hormones sexuelles (Sex Hormone Binding Globulin, SHBG) est une protéine plasmatique qui se lie spécifiquement à la testostérone et à l'estradiol *in vitro*. Cette liaison est de haute affinité. Toutefois, le rôle de la SHBG dans le transport de l'estradiol *in vivo* est mal connu. Nous avons procédé à l'étude des fractions d'estradiol et de testostérone liées à la SHBG ou à l'albumine ou simplement libres en effectuant une dialyse à l'équilibre du sérum à 37°C en conditions isosmotiques. Les résultats ont demontré que la SHBG constitue un facteur important dans la liaison de l'estradiol sérique chez la femme gravide et non gravide. Toutefois, la liaison à l'estradiol s'est avérée minime chez l'homme et chez le nouveau-né. La testostérone était liée à la SHBG en quantité significative dans toutes les conditions que nous avons étudiées. Afin de procéder à la caractérisation de substances liées à la SHBG durant la grossesse et jusqu'à présent non identifiées, le *sérum maternel* a été fractionné sur Sephadex LH-20. Une proportion importante du matériel lié à la SHBG éluait en 4 principaux pics lesquels ne correspondaient à aucune des stéroides que l'on pense se lient à la SHBG. La comparaison des niveaux des quatres pics dans les compartiments maternel, fétal et placentaire suggérait que ces derniers étaient d'origine placentaire. Trois de ces pics représentaient la 5α-pregnane-3,20-dione, la progestérone et la 2-methoxyestrone, ce, sur la base du profil d'élution, des caractéristiques de liaison, et de l'analyse par chromatographie à haute performance, par chromatographie gazeuse et spectrographie de masse. Le quatrième pic representait une 19-nor androgene i.e. 17β-hydroxy-1,5-estradien-3-one, ce, selon le type

d'évidence décrit ci-dessus en plus des caractéristiques de solubilité et d'absorption des rayons UV. Comme les niveaux de cette substance diminuaient avec le travail prématuré et à terme, sa diminution peut être responsable pour l'initiation du travail de l'accouchement.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Beverley E. Pearson Murphy, my research director, for her guidance and support throughout this investigation. Without her criticism, encouragement and friendship, these studies would not have been possible.

I acknowledge with gratitude Dr. P.V. Fennessey, University of Colorado and Dr. C.H. Shackleton, University of California, for the gas chromatography-mass spectrometry (GC-MS) analysis of SHBG-bound substances isolated from pregnancy urine and for the interpretation of the GC-MS data. I thank Dr. O. Mamer, McGill University, and Dr. W. Khalil, University of Western Ontario, for their initial help in the GC-MS analysis of SHBG-bound substances purified from placenta and pregnancy urine.

Thanks are also due to Dr. G. Klein, Montreal General Hospital, Montreal, for the measurement of testosterone levels in serum samples. I am grateful to Dr. G. Just, McGill University, for his interest and sincere attempts in the synthesis of 2,4-dimethoxyestrone. Many thanks go to Mrs. Marigold Hyde, Montreal General Hospital, for carrying out some of the guinea pig progesterone-binding globulin assays.

I wish to thank Ms. Louise Bastien, Department of Obstetrics and Gynecology, Royal Victoria Hospital, Montreal, for the collection of blood samples from patients of various gestational ages.

I am pleased to acknowledge the cooperation of the case room staff of the Jewish General Hospital, Montreal, and the Royal Victoria Hospital, Montreal, for the collection of umbilical cord blood, maternal blood and placentae from patients. I also wish to thank Dr. C. Branchaud of the Montreal Childrens Hospital for the collection of placental samples

of early gestation.

I wish to express my sincere thanks to Dominique Besso for excellent word processing services. I thank Ildico Horvath, Audiovisual department, Montreal General Hospital, for skillful preparation of the graphs and figures. Dr. R. Benvoit is gratefully acknowledged for translating the abstract.

Many thanks to Dr. B. Robaire and Dr. R. Farookhi for helpful discussions. Special thanks are given to Dr. S. Sybulski for proof reading this thesis and to all my colleagues in the laboratory for their encouragement and cooperation. I have enjoyed working with them and their friendship and support are gratefully ackowledged.

I am grateful to the Center for the Study of Reproduction, McGill University, Montreal, for financial support.

Finally, I wish to acknowledge George Philip, my husband, for his support, love, understanding and constant encouragement. Special acknowledgement is also given to my son Justin for providing joy and inspiration.

LIST OF ABBREVIATIONS

alpha feto-protein	AFP
androgen-binding protein	ABP
androstenedione	A-dione
bovine serum albumin	BSA
catechol-o-methyltransferase	COMT
centigrade	C
centimeter	cm
competitive protein binding	CPB
corticosteroid-binding globulin	CBG
cortisol	F
cortisol equivalents	FE
counts per minute	cpm
11-deoxycorticosterone	DOC
5α-dihydro-11-deoxycorticosterone	5α-DHDOC
5α-dihydroprogesterone	5α-DHP
5α-dihydrotestosterone	DHT
estradiol	E_2
estrone	E_1
gas chromatography-mass spectrometry	GC-MS
gram	gm
high performance liquid chromatography	HPLC
hour(s)	hr
human CBG	hCBG
international units	IU
mass/charge	m/z
metabolic clearance rate	MCR
2-methoxyestrone	2-ME_1
4-methoxyestrone	4-ME_1
methylene unit value	MU
microcurie(s)	μCi
microgram(s)	μg (10^{-6} gm)
microliter(s)	μl
micromolar	μM
millicurie	mCi
milliliter(s)	ml
millimeter(s)	mm
millimolar	mM
minutes	min.
molar	M
molecular weight	MW
nanogram(s)	ng (10^{-9} gm)
nanometer(s)	nm
nanomolar	nM
normal (concentration)	N
19-nor-testosterone	19-nor-T
picogram(s)	pg (10^{-12} gm)
progesterone	P
progesterone-binding globulin	PBG
progesterone equivalents	PE
radioimmunoassay	RIA
red blood cells	RBC

revolutions per minute . . . . . rpm
sex hormone-binding globulin . . . . . SHBG
specific activity . . . . . SA
standard deviation . . . . . SD
standard error . . . . . SE
Svedberg unit of sedimentation (10^{-13} cm/sec/dyne/gm) . . . . . S
testosterone . . . . . T
testosterone equivalents . . . . . TE
trimethylsilyl ether . . . . . TMS
ultraviolet . . . . . UV
units . . . . . U
weight . . . . . wt.

GLOSSARY OF TERMS

^{14}C . . . . . Radioactive isotope of carbon, atomic weight 14

^{3}H . . . . . Radioactive isotope of hydrogen, atomic weight 3

^{125}I . . . . . Radioactive isotope of iodine, atomic weight 125

3-Acetoxyestradiol . . . . . 1,3,5(10)-Estratriene-3,17β-diol-3-acetate

17-Acetoxyestradiol . . . . . .1,3,5(10)-Estratriene-3,17β-diol-17-acetate

3-Acetoxyestrone . . . . . 3-Hydroxy-1,3,5(10)-estratriene-17-one-acetate

Androstenedione 4-Androstene-3,17-dione

Androsterone 3α-Hydroxy-5α-androstan-17-one

Corticosterone 11β,21-Dihydroxy-4-pregnene-3,20-dione

Cortisol 11β,17,21-Trihydroxy-4-pregnene-3,20-dione

Dehydroepiandrosterone 3β-Hydroxy-5-androsten-17-one

Dehydroepiandrosterone sulfate 17-Oxo-5-androsten-3β-yl-sulphate

16-Dehydroprogesterone 4,16-Pregnadiene-3,20-dione

11-Deoxycorticosterone 21-Hydroxy-4-pregnene-3,20-dione

3,17-Diacetoxyestradiol . 1,3,5(10)-Estratriene-3,17β-diol-diacetate

5α-Dihydrocorticosterone 11β,21-Dihydroxy-5α-pregnane-3,20-dione

5α-Dihydro-11-deoxycorticosterone . . . 21-Hydroxy-5α-pregnane-3,20-dione

5β-Dihydro-11-deoxycorticosterone . . . 21-Hydroxy-5β-pregnane-3,20-dione

5α-Dihydroprogesterone 5α-Pregnane-3,20-dione

5β-Dihydroprogesterone 5β-Pregnane-3,20-dione

20α-Dihydroprogesterone 20α-Hydroxy-4-pregnen-3-one

20β-Dihydroprogesterone 20β-Hydroxy-4-pregnen-3-one

5α-Dihydrotestosterone 17β-Hydroxy-5α-androstan-3-one

2,4-Dimethoxyestradiol . . . . 1,3,5(10)-Estratriene-2,3,4,17-tetrol-2,4-dimethyl ether

2,4-Dimethoxyestrone	2,3,4-Trihydroxy-1,3,5(10)-estratriene-17-one-2,4-dimethyl ether
Epitestosterone	17α-Hydroxy-4-androsten-3-one
Eticholanolone	3α-Hydroxy-5β-androstan-17-one
Estradiol	1,3,5(10)-Estratriene-3,17β-diol
Estradiol 3-methyl ether	1,3,5(10)-Estratriene-3,17β-diol-3-methyl-ether
Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol
Estrone	3-Hydroxy-1,3,5(10)-estratrien-17-one
Estrone 3-methyl ether	3-Hydroxy-1,3,5,(10)-estratrien-17-one-3-methyl ether
Estrone sulphate	17-Oxo-1,3,5(10)-Estratrien-3-yl-sulphate
2-Hydroxyestrone	2,3-Dihydroxy-1,3,5(10)-estratrien-17-one
6α-Hydroxyprogesterone	6α-Hydroxy-4-pregnene-3,20-dione
6β-Hydroxyprogesterone	6β-Hydroxy-4-pregnene-3,20-dione
11β-Hydroxyprogesterone	11β-Hydroxy-4-pregnene-3,20-dione
15α-Hydroxyprogesterone	15α-Hydroxy-4-pregnene-3,20-dione
16α-Hydroxyprogesterone	16α-Hydroxy-4-pregnene-3,20-dione
2-Methoxyestradiol	1,3,5(10)-Estratriene-2,3,17β-triol-2-methyl ether
4-Methoxyestradiol	1,3,5(10)-Estratriene-3,4,17β-triol-4-methyl ether
2-Methoxyestrone	2,3-Dihydroxy-1,3,5(10)-estratrien-17-one-2-methyl ether
4-Methoxyestrone	3,4-Dihydroxy-1,3,5(10)-estratrien-17-one-4-methyl ether

19-Nor-testosterone	17 β-Hydroxy-4-estren-3-one
Prednisolone	11 β,17 α,21-trihydroxy-1,4-pregnadiene-3,20-dione
Pregnanolones	3 α-Hydroxy-5 α-pregnan-20-one
	3 β-Hydroxy-5 α-pregnan-20-one
	3 α-Hydroxy-5 β-pregnan-20-one
	3 β-Hydroxy-5 β-pregnan-20-one
	20 α-Hydroxy-5 α-pregnan-3-one
	20 β-Hydroxy-5 α-pregnan-3-one
	20 α-Hydroxy-5 β-pregnan-3-one
	20 β-Hydroxy-5 β-pregnan-3-one
Pregnenolone	3 β-Hydroxy-5-pregnen-20-one
Progesterone	4-Pregnene-3,20-dione
Testosterone	17 β-Hydroxy-4-androsten-3-one

TABLE OF CONTENTS

page

LIST OF FIGURES

LIST OF TABLES

PREFACE

The intricate mechanisms whereby hormones regulate the physiological and metabolic activities in the organism are only beginning to be appreciated. Since hormones exert their biological effects away from their site of synthesis, study of their mode of transport and their physiochemical nature in the circulation are of great importance.

Steroid hormones circulate in blood extensively bound to plasma proteins. The binding of steroid hormones to the serum proteins is mediated essentially by hydrophobic forces and hydrogen bonds. The non-covalent complexes that are formed are readily reversible. There are two types of proteins in human plasma that form dissociable complexes with the circulating steroid hormones: (i) albumin, which is present in relatively high concentrations but has only non-selective, low affinity steroid binding properties and (ii) the specific plasma globulins which are present in smaller concentration, but have a high affinity for a limited group of steroids. In the human, two specific steroid-binding plasma globulins have been described: 1. Corticosteroid-binding globulin (CBG) or transcortin which has high affinity for corticosteroids and progesterone; and 2. Sex hormone-binding globulin (SHBG) or testosterone- estradiol-binding globulin (TeBG), the steroid-binding properties of which form the basis of this thesis.

The exact significance of steroid binding to plasma proteins is unclear. While the physiological significance of steroid binding to albumin is controversial it is widely believed that the interaction of steroid hormones with specific binding proteins such as CBG or SHBG is an important determinant of hormonal availability to target tissues. The

generally accepted model of steroid hormone action which is based upon a variety of both experimental and clinical observations suggests that the steady state concentration of unbound steroid resulting from the interaction with plasma binding proteins represents the physiologically active fraction of steroid which can enter the target cell and interact with specific steroid receptors in the cytoplasm. The resulting steroid-receptor complex apparently moves into the nucleus where it modifies the transcriptional activity of chromatin which results in the biological changes characteristic of the hormone. According to this view the steroid hormones bound to CBG or SHBG in plasma are unavailable to target cells (Westphal, 1971). This notion which is still prevalent must now be reexamined since recent evidence suggests that CBG or SHBG of plasma may be directly involved in steroid uptake by target cells. Using immunocytochemical techniques, Siiteri et al (1982) have shown not only the presence of CBG within the target cells of glucocorticoid hormones but also have demonstrated corticosteroid-dependent uptake of CBG by those cells. In addition, direct evidence for the intracellular presence of SHBG in the prostate and other tissues of rhesus monkey and for the uptake of SHBG by human mammary tumor cells (MEF-7) have been obtained by immunofluorescence technique (Bordin and Petra, 1980). Thus it is not clear whether CBG and SHBG are directly involved in cellular uptake and mechanism of action of steroid hormones, or whether their role is only indirect in the regulation of the amount of unbound hormone which is the fraction available for cellular action and metabolism. This has led to the suggestion that the specific steroid-binding plasma proteins may have a dual role (Petra et al, 1983): 1. Direct involvement in the mechanism of steroid hormone action by participating in the uptake across the plasma membrane and the

intracellular action of steroids. 2. Regulation of the metabolic clearance rate of steroid hormones by protecting them from cellular metabolism. The response of any given cell to unbound or protein bound steroid may depend on the individual cell.

Whatever is the true function of specific steroid-binding proteins of plasma, there is little doubt that these proteins have great influence on the destiny of steroid hormones which bind to them. The role of albumin-bound steroid is less well understood. The rapid dissociation of steroids from albumin has led to the suggestion that the albumin-bound steroid may behave essentially as the unbound steroid (Pardridge, 1981).

In any studies on steroid binding to plasma proteins, the basic requirement is to determine the distribution of steroid in plasma into CBG- or SHBG-bound, albumin-bound and unbound fractions. Much of the uncertainty concerning the fate of protein-bound steroid in plasma can be attributed to the lack of convenient, physiologically relevant methods available for the direct estimation of the above binding parameters. Testosterone (T) and estradiol (E_2) are the main circulating biologically active sex steroids. Studies using a variety of techniques have demonstrated the importance of SHBG in the binding of testosterone (Vermeulen and Verdonck, 1968; Mercier-Bodard et al, 1970; Vigersky et al, 1979). Although the studies employed have one or more theoretical or practical limitations, there is general agreement that SHBG is a significant binder of plasma T. However, there is conflicting evidence as to the significance of SHBG in the plasma distribution of E_2. Earlier studies from several laboratories have shown that E_2 can bind to the same binding site as T on SHBG although with a lesser affinity than T itself (Murphy, 1968; Pearlman et al, 1968; Fisher et al, 1974). On the other

hand, the significance of SHBG in the transport of E_2 *in vivo* has recently been questioned by Vigersky and co-workers who used physiological temperature and employed three independent methods (1979). In addition, several clinical conditions in which SHBG levels were altered were not always associated with changes in unbound E_2 or metabolic clearance rate of E_2, although unbound T and metabolic clearance rate of T were altered (Ruder et al, 1971; Galvao-Teles, 1973; Fisher et al, 1974). These observations were interpreted to support the notion that SHBG is not an important binder of E_2 *in vivo*. The discrepancy in the literature regarding the significance of SHBG in the plasma distribution of E_2 is due at least in part to the lower affinity of E_2 for SHBG and thus the increased difficulty in determining its SHBG-bound fraction. None of the studies reported so far have measured E_2 binding to SHBG with the use of undiluted plasma at 37°C under equilibrium conditions (i.e. under physiological conditions). Furthermore, few experimental data are available on the plasma distribution of E_2 and T in the pregnant woman and the newborn infant. The first set of experiments described in this thesis deals with my studies which were directed towards an assessment of the plasma distribution of E_2 and T in nonpregnant women, pregnant women, newborn infants and adult males under conditions which closely approximate those which are physiological.

The significance of the rise (5-8 fold) of SHBG in pregnancy is unknown. The "total sex steroid" level as measured by competitive binding to SHBG in late pregnancy serum is 15-fold higher than in non- pregnant serum (B.E.P. Murphy, unpublished observations). The recognized SHBG-bindable ligands such as T and other androgens, which rise only slightly or not at all, and E_2, which increases greatly, together account for only

approximately half of the SHBG-bindable material present in pregnancy serum. Naturally occurring steroids that bind to SHBG are generally biologically potent sex steroids (e.g.: 5α-hydrotestosterone, T, 5α-androstane-3α{β},17β-diol, 5-androstene-3α{β},17β-diol and E_2). The previously unrecognized ligands of SHBG in pregnancy serum may play a role in the maintenance of pregnancy, in fetal development or in the mechanism of parturition. Alterations in the levels of those substances early in gestation or within a short interval preceding delivery may be of importance in this regard. Furthermore, the presence of high concentrations of competing ligands for SHBG may influence the physiological availability of 'classical' sex steroids such as T and E_2 in the mother and the fetus. The aim of the second set of studies reported in this thesis was to investigate the concentrations, nature and properties of the previously unrecognized substance(s) bound to SHBG during pregnancy.

CHAPTER I: HISTORICAL REVIEW

1.1 Introduction

In the review of literature, emphasis is placed on physiologic aspects of binding of steroids to the human plasma protein, sex hormone binding globulin (SHBG), examining in particular its steroid binding properties. The literature dealing with the binding of steroid hormones to plasma proteins other than SHBG is very extensive, but will be dealt with only briefly. Many excellent and comprehensive reviews on this subject are available (Westphal, 1971; Wagner, 1978; Ballard, 1979). What is known of the general physiochemical properties of SHBG and a comparison of these properties to those of other extracellular "sex steroid" binding proteins will be presented. Studies directed towards an understanding of the physiological role of SHBG and its significance in the plasma distribution of testosterone and estradiol will be described in some detail. Although there is virtually no information available on substances bound to SHBG in pregnancy, the possible physiological significance of these ligands in pregnancy will be discussed.

1.2 General Aspects of Interaction of Steroids with Plasma Proteins

1.2.1 Early studies: Discovery of specific steroid binding proteins of plasma

The association of low molecular weight substances such as lipids, dyes, drugs and steroids to plasma proteins was recognized as early as in 1926 by Bennhold. Brunelli (1935) was one of the first to directly demonstrate the interaction of steroid with protein components of blood. He described the binding of estrone to serum proteins in dialysis

experiments using collodion membranes. However, many studies that followed were inconsistent in their findings. Early studies of Haüssler (1936) supported the findings of Brunelli by showing that serum proteins precipitated with organic solvent was associated with estrogenic activity. However, Mühlbock (1937) was unable to confirm these results. Rakoff et al (1943) employed 3 different methods without the use of organic solvents: salting out with sodium sulfate, precipitation with sulfosalicylic acid and ultrafiltration, and showed that endogenous estrogens are bound to serum proteins in human pregnancy. Using a variety of methods for the precipitation of serum proteins, Szego and Roberts (1946, 1947) also were able to demonstrate that approximately two-thirds of the total estrogen in blood was bound to serum proteins. On the other hand, Bischoff et al (1954) failed to show the association of estrogens with serum proteins in the sera of pregnant women. In addition, no significant association with serum proteins could be demonstrated by Hilmer and Hess (1950) for androgens or by Hooker and Forbes (1949) for progesterone. However, Diczfalusy (1953) obtained results confirming the findings of Szego and Roberts (1946, 1947) that estrogens are associated with proteins in the plasma of pregnant women.

Many of the discrepancies in literature on the binding of steroids to serum proteins during this early period are at least in part due to the use of bioassay methods and colorimetric and spectrophotometric procedures which were inaccurate and tedious. In addition, in many studies serum proteins were precipitated using organic solvents which cause disruption of steroid-protein complexes and denaturation of serum proteins. Furthermore, other experimental conditions such as temperature, pH and ionic strength were not controlled in these studies.

The advent of isotopically labeled steroids with high specific activity during the 1950s greatly facilitated the assessment of the binding of steroid hormones to serum proteins by obviating some of the shortcomings such as lack of sensitivity and accuracy associated with biometric, colorimetric and spectrophotometric methods. In addition, availability of these tools made it possible to study protein binding of the steroid hormones at physiological levels. This led to more systematic investigations for the determination of the specificity and nature of the steroid binding plasma proteins and for an understanding of the consequences of their presence in plasma.

Sandberg et al (1957) and Antoniades et al (1957) administered ^{14}C-labeled steroid hormones to human subjects and studied the various plasma protein fractions obtained by the low temperature ethanol fractionation procedure of Cohn et al (1946). They found that a major portion of most steroid hormones were closely associated with the albumin fraction. The major drawbacks of these studies were the possible dissociation of the steroid-protein complex that can occur with the cold ethanol fractionation method and the possibility that the presence of a steroid in a given protein fraction may not reflect the actual binding of that steroid to the protein in that fraction. However, the extensive studies by various investigators, including that of Sandberg et al (1957), on the binding ability of various Cohn fractions established the very high association capability of albumin to most of the steroids tested.

Although several studies, including those of Brunelli (1935), Szego and Roberts (1946) and Roberts and Szego (1946), have indicated that the globulin fraction was involved in estrogen binding, definite evidence for the presence of a steroid binding plasma globulin was not obtained

until the 1950s. The existence in human plasma of a special protein other than albumin capable of binding corticosteroids with high affinity was first demonstrated by Daughaday in 1956. The low concentration in plasma and the high affinity for corticosteroids clearly distinguished this protein from albumin and it was named by Daughaday (1959) as corticosteroid binding globulin. At about the same time Sandberg et al (1957) and Bush (1957) independently reported steroid binding data describing the presence in human plasma of a specific protein of high binding affinity for corticosteroid hormones which was named transcortin by Slaunwhite and Sandberg (1959). (Both the terms corticosteroid binding globulin (CBG) and transcortin are used in the literature, but the term CBG will be used in this thesis).

The existence of a separate protein which binds testosterone with high affinity and low capacity was first suggested by the experiments of Daughaday (1958). The first clear demonstration of a plasma globulin having high affinity for testosterone and distinct from CBG appeared in 1966 (Mercier et al). Almost simultaneously, results indicating the presence in plasma of a β-globulin capable of interacting with estradiol with high affinity were described (Rosenbaum et al, 1966; Tavernetti et al, 1966). Subsequent reports by various investigators (Murphy, 1968; Steeno et al, 1968; Van Baelen et al, 1968; De Moor et al, 1969; Corvol et al, 1971) showed, on the basis of competition experiments and physiochemical studies, that in both instances the same steroid binding protein was involved. As a result of the initial uncertainty regarding the identity of this protein, various names have appeared in the literature for this protein. Mercier et al (1966) have called this protein testosterone binding globulin initially, but later (Mercier-Bodard et al, 1970) proposed

the term sex steroid binding plasma protein (SBP). Other terms used are steroid binding β-globulin (SBBG; De Moor et al, 1969), testosterone-estradiol binding globulin (TeBG, Vermeulen, 1969) and sex hormone binding globulin (SHBG, Murphy, 1970). The term, sex hormone binding globulin (SHBG) will be used in this thesis.

In the study of steroid hormone-plasma protein interaction, the two major questions to be considered are the physicochemical nature and physiological significance of the interaction.

1.2.2 Molecular aspects of steroid-protein interaction

The physicochemical nature of forces responsible for binding of steroids to plasma proteins is poorly understood. The forces involved in steroid-protein interaction will depend on the stereochemical configuration of the steroid and the three dimensional conformation of the protein. Although the stereochemical structures of a large number of steroids are known, detailed knowledge of the molecular structures of plasma proteins is lacking. Whereas albumin is a protein free of carbohydrate, CBG (an α_1-globulin) and SHBG (a β-globulin) are glycoproteins (Westphal, 1971). The approximate molecular weight of human CBG has been estimated to be 52,000 daltons (Seal and Doe, 1966) and that of human SHBG to be 88,000 daltons (Petra et al, 1983). However, not even the primary structure of these proteins is known.

The binding of steroid hormones to serum proteins is considered to be mediated by three types of non-covalent bonds. They are hydrogen bonds, ionic bonds and hydrophobic bonds such as Van der Waals forces. There are numerous possibilities for the formation of these low energy, short range forces between various sites on the steroid and protein

molecules. Hydrogen bonds are formed between the oxygen functions of the steroid and complementary groups present in the protein. Hydrophobic bonds occur between large areas of the non-polar steroid molecule and lipophilic groups of proteins.

One of the early studies on the nature of forces involved in steroid-protein interactions was that of Eik-Nes et al (1954) who studied the binding of various steroids to bovine serum albumin. He found that the solubility of various steroids in protein solution decreased with an increasing number of hydrophilic groups. These observations led to the formulation of the 'polarity rule' according to which the strength of binding between steroids and protein varies inversely with the number of polar or hydrophilic groups in the steroid. This characteristic of steroid-protein binding has since been confirmed by Westphal and Ashley (1962) who compared the binding strength of a number of Δ4-3-ketosteroids on the basis of a spectral change during their interaction with albumin. Thus the binding affinity of progesterone for albumin is greater than that of testosterone which in turn is greater than that of cortisol. However, the polarity rule alone cannot account for all the experimental observations on protein binding of steroids, even for albumin-steroid interactions.

Conformational changes in protein molecules upon association with steroid have been reported by several investigators. Alfsen (1963) has shown that testosterone binding to bovine serum albumin caused it to become less levorotatory and more acidic. Ryan (1968) and Ryan and Gibbs (1970) found that upon binding to different steroids, human and bovine serum albumin developed a different spectrum in the aromatic absorption region. Corticosteroid dependent conformational changes have been shown to occur for rat (Chader and Westphal, 1968) and rabbit (Chader et al, 1972) CBG.

Removal of steroid caused polymerization of CBG and loss of binding activity while the addition of steroid reversed the aggregation and restored the binding ability. Little is known about the contributions of specific amino acids or carbohydrate residues of SHBG or CBG to steroid binding. Neuraminadase treatment of SHBG (Van Baelen, 1969) and CBG (Muldoon and Westphal, 1967) has indicated that sialic acid residues were not necessary for binding of steroids. Involvement of sulfhydryl groups in the protein-steroid interaction has also been reported. Chader and Westphal (1968) found that treatment of rabbit CBG with paramercuribenzoate caused a 27-fold decrease in its binding affinity for cortisol. Pearlman et al (1969) have shown that treatment of SHBG with various sulfhydryl and disulfide reagents such as N-ethyl maleimide, dithiothreitol or sodium sulfite lowered its testosterone binding activity.

On the basis of their analysis of steroid binding to progesterone binding globulin (PBG) of guinea pig, Stroupe and Westphal (1975) and Westphal et al (1978) reported that steroid-PBG interaction is accompanied by profound alterations in the absorption spectra of both steroids and protein. Their studies on the chemical nature of the steroid binding site showed that chemical modifications of tryptophan, lysine and tyrosine residues of PBG resulted in inactivation of the PBG binding site (Westphal et al, 1978). Their findings led them to conclude that there are strong and specific interactions of the steroid molecule with amino acid residues of the binding site and that almost the entire surface of the steroid is closely associated to the protein.

1.2.3 Quantitative characterization of the interaction

In order to study the degree of interaction, the major parameters

to be estimated are the (i) association constant, (ii) number of binding sites per molecule, and (iii) the concentrations of the reactants. The extent of binding is proportional to the product of binding affinity and the binding capacity. Since the formation of a steroid-protein complex is readily reversible, it is subject to a binding equilibrium which obeys the laws of mass action. If we suppose that a protein P interacts with a steroid S, then $P + S \rightarrow PS$. Applying the laws of mass action and expressing concentration in brackets, the general equation

$$\frac{[PS]}{[P][S]} = \frac{k_1}{k_2} = k$$

is obtained. k_1 and k_2 are the rate constants and k is the equilibrium or association constant. Concentrations of P, S and PS are expressed as moles per liter. k is expressed as liters per mole which is considered as that volume in liters to which a gram-molecule of protein must be diluted in order that the maximal binding of tracer ligand be reduced to 50% (Murphy, 1969). The concentration of a particular binding protein (binding capacity) can be determined directly by radioimmunoassay if a specific antibody to that protein is available or can be estimated according to the binding capacity of the protein for a particular ligand. (The term ligand refers to the smaller molecule in any interaction between protein and another substance when such an interaction is subject to a freely dissociable binding equilibrium). The procedures available for the calculation of the binding affinity, the number of binding sites per molecule of protein and the binding capacity by the analysis binding data obtained by various methods have been reviewed by Westphal (1971).

Marked differences in plasma concentrations and in binding

affinity for steroids are apparent between albumin and the specific binding globulins (Table 1-1). Albumin is present in the highest concentration. In contrast the specific globulins occur in very low concentrations. However, the affinity constants of the steroid complexes with specific globulins are several orders of magnitude higher than those with albumin.

The association constants for CBG and SHBG are strongly temperature dependent (Table 1-1). The binding affinity generally decreases with increasing temperature. CBG and SHBG irreversibly denature and lose binding activity between 60-65°C (Westphal, 1971). However, temperature has only a slight influence on the affinity of albumin for steroids (Table 1-1, Burton and Westphal, 1972).

The association constants of complexes of steroids and specific steroid binding proteins are also pH dependent. The CBG-cortisol complex was found to have maximal affinity beween pH 8 and pH 9 (Westphal, 1969) and for the SHBG-testosterone complex it was between pH 6-8.5 (Forest et al, 1968). Below pH 5 and above pH 10 both proteins were found to have very low binding activity. The binding affinity has also been shown to be influenced by the protein concentration (Brunkhorst and Hess, 1965; Moll and Rosenfield, 1978). However, there are no satisfactory explanations for this phenomenon.

Albumin has been shown to have one binding site per molecule for a particular steroid (Brunkhorst and Hess, 1965). However, there have been reports where albumin was estimated to have up to 20 binding sites per molecule (Alfsen, 1963). This discrepancy in the number of binding sites per molecule can at least in part be attributed to the errors in extrapolations required for the interpretation of the binding data. On the other hand specific steroid binding proteins such as CBG and SHBG are

TABLE 1-1. Concentrations and apparent association constants of steroid binding proteins of human plasma*.

Protein	Concentration	Association Constant M^{-1}		steroid
	μM	4°C	37°C	
Albumin			3.6×10^4**	Testosterone
			6.0×10^4**	Estradiol
	550	11×10^4	6.1×10^4	Progesterone
		0.6×10^4	0.5×10^4	Cortisol
SHBG	0.04	1.9×10^9 @	0.35×10^9 @	Testosterone
		0.6×10^9 @	0.22×10^8 @	Estradiol
CBG	0.72	0.6×10^9	0.3×10^8	Cortisol

* Data summarized from Westphal, unless otherwise indicated.

** Burke and Anderson, 1972

@ Rosner and Smith, 1975

generally agreed to have one binding site per molecule (Muldoon and Westphal, 1967; Seal and Doe, 1966; Rosner and Smith, 1975). This is in contrast to antibodies which may have multiple binding sites of varying affinities for the same ligand. Unlike CBG and SHBG which have high specificity for certain steroid structures, albumin binds with low affinity all natural and synthetic steroids so far examined.

1.2.4 Physiological significance of steroid binding to plasma proteins

The significance of the interaction of steroid hormones with specific intracellular steroid binding proteins, the so called "receptors" for the expression of hormonal activity, is well recognized by most investigators. However, the precise biological importance of the specific steroid binding proteins of plasma in the overall mechanism of steroid hormone action is still open to question.

It has generally been assumed that the association of steroids to proteins provides a means for transporting the steroids in the vascular compartment from their site of synthesis to their target organs. However, Sandberg et al (1966) have suggested that transportation of steroids may not be the major function of plasma proteins, as the solubility of various steroids in saline far exceeds even the pathologically elevated levels of steroid hormones in plasma. Another function which has been proposed for plasma proteins is conservation of steroid hormones by protecting them against nonspecific adsorption to blood and lymph vessels (Westphal, 1961) and against excessive metabolism in liver and increased excretion by the kidney (Sandberg and Slaunwhite, 1963). However, the most important function of plasma proteins is believed to be a regulatory one. It is

widely held that steroid hormones bound to plasma proteins serve as a primarily inert reservoir and only the small amounts of non-protein bound hormone are biologically active. According to this view the biologically inactive steroid-protein complex is capable of rapid dissociation and constitutes a pool of readily available hormone. The plasma proteins function by controlling the concentration of unbound hormone according to the law of mass action. Several clinical observations appear to support this hypothesis. Although the peripheral corticosteroid concentrations are elevated several fold in human pregnancy (Gemzell, 1953) and after estrogen therapy (Wallace et al, 1957), no classical symptoms of hypercorticism can be observed. In 1959, Sandberg and Slaunwhite suggested that in both the above cases CBG levels are greatly increased and that the absence of hypercorticism may be due to the neutralization of the excess amounts of corticosteroids by CBG. Using the same argument it has been generally assumed that, although the plasma androgen levels are increased in pregnancy, the elevated SHBG concentrations prevent virilization.

More compelling evidence of the regulating role of plasma proteins has come from numerous "in vitro" studies which demonstrated the significant effects of SHBG in protecting androgens (Mowszowicz et al, 1970; Lasnitzki and Franklin, 1972) and of CBG in protecting cortisol (Sandberg and Slaunwhite, 1963; Murphy, 1979) from metabolism. Furthermore, several clinical observations suggest that changes in SHBG levels associated with hyperandrogenism and hyperestrogenism (Vermeulen et al, 1969; Southren et al, 1969; Rosenfield, 1975) correlate with changes in metabolic clearance rate of testosterone.

Another line of evidence suggesting the unavailability of protein bound steroids for biological action has been obtained from studies which

demonstrated that the effectiveness of a given amount of streoid is reduced after the addition of plasma proteins to various test systems. Slaunwhite et al (1962) have shown that the cortisol-induced increase in glycogen content in adrenalectomized mice was inhibited by prior injection of CBG. In addition, the presence of CBG has been shown to reverse the inhibitory effect of cortisol on glucose conversion to CO_2 in isolated adipose cells of the rat (Blecher, 1965). Lippman and Thompson (1974) have found that the addition of rat serum increased the amount of cortisol required to induce tyrosine aminotransferase activity in hepatoma tissue culture cells. There are fewer data available on the role of albumin in the regulation of steroid hormone metabolism and action. Matusui and Plager (1966) found that physiological levels of albumin inhibited the effect of cortisol on CO_2 production, suggesting that albumin-bound steroid is physiologically inactive.

Although there is a large body of evidence which supports the concept that the function of steroid binding plasma proteins is indirect by regulation of unbound steroid hormones, a number of new observations indicate that those plasma proteins may play a direct role in the mechanism of steroid hormone action. A possible direct function of SHBG on testosterone uptake and metabolism in the human prostate has been suggested by the studies of Mercier-Bodard et al (1976). In addition, the intracellular presence of CBG in human liver nuclei (Amaral et al, 1974) and that of SHBG in several tissues of adult monkey 'macaca nemestrina' (Bordin and Petra, 1980) have been demonstrated. Furthermore Siiteri et al (1982) have obtained preliminary evidence to suggest that rat pituitary tumor GH_3 cells took up CBG and that this cellular uptake was strongly influenced by temperature and the presence of corticosteroids.

A more detailed analysis of the physiological implications of steroid binding to plasma proteins will be given in section 1.3.5 in connection with the discussion of the function of SHBG.

1.3 Sex Hormone-Binding Globulin

1.3.1 Purification and physicochemical properties of SHBG

1.3.1.1 Early studies of unpurified SHBG. Isolation and purification of SHBG has been difficult because of its very low concentration in plasma and marked instability. The initial fractionation studies of Mercier-Bodard and Baulieu (1968) and Van Baelen et al (1968) through DEAE-cellulose and Sephadex G-200 chromatography and those of Corvol et al (1971) using polyacrylamide gel electrophoresis (PAGE) confirmed the existence of SHBG as a single protein in human plasma, binding both testosterone and estradiol with high affinity and low capacity. Application of protein separation techniques such as mentioned above have demonstrated that SHBG is a β-globulin (Rosenbaum et al, 1966; Steeno et al, 1968; Mercier et al, 1966; Van Baelen et al, 1968). The glycoprotein nature of SHBG was first suggested by the isoelectric focussing experiments of Van Baelen et al (1969). He showed that there was marked microheterogeneity for SHBG when pH gradients over a narrow range were used and that neuraminadase treatment resulted in a higher isoelectric point and simpler elution pattern. Pearlman et al (1969), using sulfhydryl reagents, obtained data to suggest that SHBG may contain a sulfhydryl group and that it may be involved in ligand binding. The thermolability of SHBG has been studied by various groups (Vermeulen and Verdonck, 1968; Steeno et al, 1968) and it has been shown that SHBG gets irreversibly denatured and loses its binding ability at temperatures between 57°-60°C.

Molecular weights (M.W.) and other physicochemical parameters also have been estimated using unpurified or partially purified SHBG. Gel filtration studies of Gueriguian and Pearlman (1968) on Sephadex G-100 showed an approximate M.W. of 110,000 daltons and an apparent Stoke's radius of 46 Å. Using similar methods Rosner et al (1969) calculated a M.W. of 115,000 daltons and Van Baelen et al (1968) obtained a value of 100,000. Corvol et al (1971), using PAGE, suggested that SHBG has a M.W. of 98,000 daltons. Using ultracentrifugation on a sucrose gradient, Hansson et al (1972) showed that it has a sedimentation coefficient of 5.3 S. Isoelectric focussing experiments of Van Baelen et al (1969) and Hansson et al (1972) demonstrated the isoelectric point of SHBG to be between pH 5.2-5.9.

In summary, the early experiments using unpurified or partially purified SHBG suggest that SHBG (i) behaves like a β-globulin (ii) is most probably a glycoprotein (iii) has a M.W. of approximately 98,000- 115,000 daltons and (iv) has an isoelectric point approximately within the range of pH 5.2-5.9.

1.3.1.2 Studies of purified SHBG. The first group to report the preparation of purified SHBG which was homogeneous by physicochemical criteria was that of Mercier et al (1970). They purified SHBG from Cohn fraction IV (Cohn et al, 1946) of human plasma using chromatographies in DEAE cellulose and DEAE Sephadex A-50 and Sephadex G-75. They demonstrated that the final product was homogeneous by PAGE and equilibrium ultracentrifugation. However, the purified preparation had lost most of its binding activity. The M.W. estimated by equilibrium ultracentrifugation was approximately 52,000 daltons. However, Rosner and Smith (1975)

reassessed the molecular weight from the data of Mercier et al (1970) and obtained a value of approximately 100,000.

With the development of affinity chromatography, it has become the method of choice for purification of SHBG. Mickelson and Petra (1975) reported successful purification of SHBG from the ammonium sulphate precipitate of pregnancy plasma using affinity chromatography in combination with preparative PAGE. Although the yield was only 5%, they have obtained a 5,166-fold purification. However, there was a considerable loss in binding activity. Rosner and Smith (1975) took advantage of the protective effect of Ca^{++} on SHBG in its purification by affinity chromatography in combination with other conventional procedures. The pure SHBG had retained most of its binding activity and it was demonstrated that it had one binding site per molecule. The M.W. was estimated to be 94,000 daltons and the isoelectric point was shown to be pH 5.5.

In 1978, Mickelson et al obtained evidence to suggest that SHBG consists of subunits. Under denaturation conditions, in ultracentrifugation in the presence of 6 M guanidine HCl and on PAGE in the presence of sodium dodecyl sulphate (SDS), SHBG had a M.W. of 36,355 daltons and 52,000 respectively whereas on PAGE, in the absence of SDS, the M.W. was 88,000.

In a more recent report, Petra et al (1983) have further characterised SHBG and demonstrated extensive microheterogeneity not only for human SHBG but also for other mammalian preparations. Their studies using improved techniques indicated that SHBG is composed of two polypeptide chains of M.W. of approximately 44,000 daltons which associate noncovalently to form the native structure having a M.W. of 88,000. Further evidence for subunit structure was provided by the studies of Cheng

et al (1983). They have shown microheterogeneity of pure SHBG with regard to size by PAGE and to charge by isoelectric focussing experiments. They have obtained 2 components of M.W. 53,000 and 46,000 on SDS-PAGE and by photolabeling technique. The molecular basis of heterogeneity could not be attributed to differences in carbohydrate content. They concluded that SHBG is a dimer whose monomer exhibits two protomeric forms.

The carbohydrate content of pure SHBG estimated by various investigators was as follows: Rosner and Smith (1975) - 32.1%, Mickelson et al (1979) - 18%, Iqbal and Johnson (1979) - 34.4%. In addition, these studies have suggested that almost one-third of the carbohydrate was sialic acid. Three laboratories have so far reported the amino acid composition of SHBG. While there is some agreement between two reports (Rosner and Smith, 1975; Iqbal and Johnson, 1979), the amino acid composition reported by the third group (Mickelson et al, 1978) is entirely different from the other two.

In summary, although successful purification of SHBG has been reported by many laboratories, the exact M.W. is still a controversial issue. Available evidence suggest that the M.W. is in the range of 80,000-100,000 daltons. Most of the recent studies with improved techniques suggest a subunit structure for SHBG; according to the current consensus, there are two subunits per molecule of native SHBG. Although most investigators agree that SHBG is a glycoprotein having one binding site per native molecule, there is no uniform agreement on the amino acid composition of SHBG. Preparations of monospecific antibodies to SHBG have been reported by many laboratories (Mercier-Bodard et al, 1979; Khan et al, 1982; Cheng et al, 1983). An understanding of the molecular structure of SHBG and the availability of specific antibodies to SHBG would be

instrumental in the elucidation of its physiological role.

1.3.2 Steroid binding parameters of SHBG

1.3.2.1 Specificity of SHBG. In general, stereospecific binding proteins such as antibodies, hormone receptors, enzymes and steroid binding plasma proteins bind more than one ligand with high affinity. The apparent specificity of SHBG has been studied in many laboratories and some of the structural requirements of steroid molecules for SHBG binding have been established. The apparent binding ability of a compound to SHBG can be estimated indirectly by means of competition experiments in which the compound is allowed to compete for binding sites with ^{3}H-testosterone and by measuring its ^{3}H-testosterone-displacing ability compared with that of unlabeled testosterone. Table 1-2 lists a selection of the steroids that have been tested. The relative binding is expressed in terms of testosterone which was assigned an arbitrary value of 100. It is assumed that SHBG is the only high affinity binding site for testosterone in human plasma. Evidence for this assumption will be presented in section 1.4.

The values shown in Table 1-2 do not represent the true binding affinity. They are only approximations as the figures vary according to the assay conditions employed. However, it is reasonable to assume that the ^{3}H-testosterone displacement ability is proportional to the binding affinity of the steroid to SHBG. The nonspecific binding by the high affinity, low capacity sites of albumin was minimized by dilution of plasma. For example, Murphy (1968,1969) used 1:200 and Kato and Horton (1968) used 1:6 diluted pregnancy plasma. Vermuelen and Verdonck (1968) used undiluted pooled plasma; however, the Sephadex G-25 filtration technique they used to separate SHBG bound and unbound steroid ensured

TABLE 1-2. Relative ability of various steroids to displace ^{3}H-testosterone from binding sites in human plasma

Trivial Name	Vermeulen & Verdonck, 1968	Kato and Horton, 1968	Murphy, 1968, 1969
Testosterone	100	100	100
Dihydrotestosterone	300	240	340
5α-Androstane-3β,17β-diol	160	141	250
5α-Androstane-3α,17β-diol	160	98	200
4-Androstene-3β,17β-diol	93	--	200
5-Androstene-3β,17β-diol	50	--	130
5-Androstene-3α,17β-diol	--	83	--
Androsterone	3	< 5	1
5α-Androstanedione	--	< 5	2.3
Androstenedione	5	< 5	1.4
Dehydroepiandrosterone	4.5	< 5	2.5
5β-Androstane-3α,17β-diol	24	--	0.6
5β-Androstane-3α,17α-diol	--	--	1.4
Etiocholanolone	--	< 5	0.1
5β-Androstanedione	--	--	0.1
Epitestosterone	4	--	1
19-Nortestosterone	22	18	8
Estradiol	62	28	60
Estrone	4	< 5	0.6
Estriol	3.5	< 5	0.1
Estrone sulfate	--	--	0.03
Dehydroepiandrosterone sulfate	--	--	0.02
Progesterone	2.5	< 5	0.2
Cortisol	2	< 5	0.1

complete dissociation of albumin bound steroid during the separation.

It seems clear from the figures shown in Table 1-2 that SHBG is highly stereospecific for certain steroid structures. These data suggest that the presence of a 17β-hydroxyl group on ring D of the steroid is the most important structural requirement for SHBG binding. Epitestosterone which differs from testosterone in having a 17α-OH group rather than a 17β-OH group has little or no binding activity. 5α-dihydrotestosterone which differs from testosterone only by the saturation of the double bond between carbon 4 and 5 is the most potent of all steroids so far tested, being approximately 3 times as effective as testosterone itself. On the other hand, 5β-androstane steroids show almost no competition. Another structural feature that is indicated to be important in SHBG binding is an oxygen function (3-ketone or 3-hydroxyl) in ring A. The presence of a 16α-hyroxyl group (estriol) appears to inhibit the binding of the 17β-hydroxy group. Elimination of the 19-methyl group (19-nortestosterone) results in a drastic decrease in binding. Estradiol, however, with an aromatic A ring, binds to SHBG almost half as strongly as testosterone. C21-pregnane steroids such as progesterone and cortisol, even at high concentrations, are less effective in causing the displacement of ^{3}H-testosterone.

Further studies of the structural requirements of steroids for SHBG binding by Murphy (1970) and Cunningham et al (1981) have suggested that while the addition of a methyl group at carbon 4 enhanced binding, affinity was reduced by a 7α-methyl or a 17α-methyl group. Most modifications at carbons 2, 6, 9 or 11 also impaired affinity.

Interestingly, comparison of the relative binding activity with the biological potency of the various steroids suggests that there is a

strong positive correlation between the binding ability and the biological effectiveness of these steroids (Murphy, 1969). Thus the highly androgenic steroids such as dihydrotestosterone (Dorfman and Dorfman, 1963), testosterone (Hilgar and Hummel, 1964) and androstanediols (Baulieu et al, 1969) bind SHBG strongly while steroids such as androstenedione and dehydroepiandrosterone, which have low androgenic potency, bind poorly. Similarly, the binding ability of highly estrogenic estradiol-17β is higher than that of less estrogenic estrone or estriol.

1.3.2.2 Equilibrium constants of major ligands of SHBG.

The major circulating steroids which bind to SHBG are testosterone (T), 5α-dihydro-testosterone (DHT) and estradiol (E_2). The equilibrium association constants of these steroids have been determined in various laboratories using different procedures both for unpurified or partially purified SHBG and for purified SHBG. Some selected values are given in Table 1-3. Although there are wide variations in the values obtained from different laboratories, there is uniform agreement that the affinity of DHT is higher than that of T which in turn is greater than that of E_2. In addition, the figures in Table 1-3 show that there is a marked effect of temperature on the equilibrium constant of association. The equilibrium constant is higher at 4°C than at 37°C for all 3 steroids. These binding properties are more obvious when all the values were obtained in the same laboratory using a purified preparation of SHBG (Rosner and Smith, 1975).

The kinetics of dissociation of various tritium-labeled 17β-hydroxysteroids from SHBG has been studied by Heyns and De Moor (1971)

TABLE 1-3. Equilibrium constants of association of steroid-SHBG complexes (10^9 M^{-1})

Reference	Dihydrostestosterone		Testosterone		Estradiol	
	4°C	37°C	4°C	37°C	4°C	37°C
*Mercier-Bodard and Baulieu, 1968			1.7		0.6	
*Lebeau et al., 1969			1.2		0.46	
*Vermeulen et al., 1971				0.75		
*Burke and Anderson, 1972				1.6		0.6
*Forest and Bertrand, 1972		1.3				
*Shanbhag et al., 1973	2.2		0.9			
*Södergard et al., 1982		1.07		0.60		0.31
#Rosner and Smith, 1975	2.4	0.99	1.1	0.35	0.6	0.22

* using unpurified or partially purified SHBG
# using purified SHBG

by ammonium sulphate precipitation of the steroid-SHBG complex at different intervals after the addition of an excess of unlabeled steroid (Table 1-4). Marked differences in the dissociation rates between DHT, T and E_2 were observed. The relative value of the dissociation rate constant ($k\ min^{-1}$) was lowest for DHT and highest for E_2, testosterone having an intermediate value. The dissociation rate constant was not affected by the concentration of SHBG in the sample nor by endogenous steroid concentrations. In addition to showing the difference in the strength of interaction between the three steroids and SHBG, the values presented in Table 1-4 also demonstrate that temperature has a marked effect on the rate constants of dissociation of steroid-SHBG complexes.

Thus the data on the association constant and dissociation rate constant of steroid-SHBG interaction suggest that the relative binding activities of steroids obtained in dilute serum generally reflect their true binding affinities.

1.3.2.3 Factors affecting steroid binding to SHBG. As already mentioned, the binding of steroids to SHBG is temperature dependent. An increase in temperature from 4°C to 37°C resulted in decreased binding of steroids. Whereas the diminution in binding up to 37°C was largely reversible, at temperatures above 50-60°C, irreversible denaturation of SHBG binding sites occurred (Steeno et al, 1968; Kato and Horton, 1968; Vermeulen and Verdonck, 1968). Changes in pH have also been shown to affect steroid binding to SHBG. Kato and Horton (1968) have shown that while steroid binding was relatively unaffected by alkaline pH for one hour up to pH 11, irreversible loss of binding activity took place below pH 5. Forest et al (1968) have reported that maximal binding of steroids to

TABLE 1-4. Rate constants of dissociation of steroid-SHBG complexes.

Temperature	Rate constants of dissociation* k min^{-1}		
	DHT	T	E_2
0°C	.0091	0.054	0.21
37°C	0.395	1.89	--

* using unpurified SHBG; Heyns and DeMoor, 1971

SHBG occurred at pH between 6-8.5. The binding is also affected by the concentration of the binding protein, and by the presence of other binding proteins and competing ligands (Murphy, 1969). The protective effect of calcium ions on the binding activity of SHBG has been well documented (Rosner et al, 1974; Mickelson and Petra, 1975). Other agents which have been shown to protect the binding sites of SHBG are glycerol and saturating amounts of DHT (Rosner et al, 1974). Ions such as cadmium, cobalt and manganese (Rosner et al, 1974) and mercury, silver and copper (Dessypris, 1970) have been reported to diminish SHBG binding activity. This effect of the ions on SHBG binding might be a consequence of their reaction with sulfhydryl groups on the protein. Pearlman et al (1969) have demonstrated that SHBG was sensitive to sulfhydryl and disulfide reagents and that the binding was reduced by treatment with those agents. Studies by Van Baelen et al (1969) have showed that an intact carbohydrate moiety was not required for steroid binding because neuraminadase treatment of SHBG affected the isoelectric point but not the binding activity.

1.3.2.4 Application of steroid-SHBG interaction for the quantification of steroids and SHBG binding sites. (a) Determination of steroid levels - The remarkable specificity of SHBG for certain steroid hormones enables the hormone-SHBG interaction to be used for the assay of those hormones. The general method is commonly known as competitive protein binding (CPB) analysis, or more specifically as radiotransinassay, and was first described by Murphy et al in 1963 for the determination of corticoids. The term 'transin' refers to an indigenous binding protein such as SHBG or CBG as defined by Murphy. 1975b. The principle is similar to that of radioimmunoassay. Murphy (1969) has defined the CPB assay as "one in which a compound is measured according to its

its ability to compete for a limited number of binding sites with a tracer possessing similar binding characteristics."

Since the binding affinity of the ligand for SHBG is greater at low temperature, the equilibration is usually done below 10°C. Late pregnancy plasma which is a rich source of SHBG is often used as the assay protein. High sensitivity can be achieved by reducing the amount of assay protein by dilution of plasma (Murphy, 1969). In addition, the dilution of assay protein preparation is important in minimizing the binding due to other proteins of higher capacity but of lower affinity (e.g.: albumin) which may compete for the same ligand. The endogenous ligand is usually removed by "stripping" the sample with dextran coated charcoal.

A critical evaluation of the CPB method has been made by Mayes and Nugent (1968) for the determination of testosterone in plasma. They found that the testosterone values obtained were statistically indistinguishable from those given by the double isotope derivative method. Several laboratories have used the CPB assay using SHBG as the binding protein to measure small quantities of testosterone and estradiol (Murphy, 1967; August et al, 1969; Frick and Kincl, 1969). Horton et al (1967) have reported details on specificity, precision and accuracy of the CPB method in the determination of testosterone concentrations. In the above studies, late pregnancy plasma was used as the source of SHBG and separation of bound from unbound steroid was accomplished by ammonium sulphate, Florisil or dextran-coated charcoal. A detailed account of the application of the CPB method using SHBG as the binding protein for the determination of steroid levels has been published by Murphy (1969).

(b) Determination of SHBG concentration - The principle of the CPB technique described above for the assay of steroids can be used in reverse

to measure the concentration of binding sites. The concentration of SHBG binding sites has been estimated, using this method, by several investigators (Vermeulen and Verdonck, 1968; Rosenfield, 1971; Nisula et al, 1978; Södergard et al, 1982; Hertz and Johnsen, 1983). In these studies, the concentration of SHBG is estimated indirectly from the measurements of bound and unbound steroid at various steroid concentrations and analysing the data using a Scatchard (1949) type plot. In Scatchard plots the ratio of bound to unbound ligand is plotted against the concentration of bound ligand. The intercept on the abscissa of the line describing the ligand-SHBG interaction is equal to the binding capacity of SHBG and the slope of the line represents the affinity of the ligand for SHBG. A detailed account of the theoretical basis of Scatchard plots has been described by Westphal (1971, p. 66).

Analysis of the binding data by the Scatchard plot is the most popular method for the determination of SHBG concentration since both the precise molecular weight of SHBG and the accurate number of binding sites per molecule are not known. The concentration of SHBG binding sites is expressed as the maximal concentration of steroid binding to SHBG at saturation. Recently, immunological methods for the direct estimation of SHBG concentration using specific antibodies to SHBG have been reported by various laboratories (Mercier-Bodard et al, 1979; Khan et al, 1982; Cheng et al, 1983). However, since neither pure SHBG nor the antibodies are yet commercially available, the determination of the concentration of SHBG based on its binding capacity for a ligand will continue to be used for some time to come.

1.3.3 Species distribution of SHBG and comparison of its properties to those of other extracellular sex steroid binding proteins

1.3.3.1 Species distribution. Unlike CBG which has been shown to be present in all 131 vertebrate species so far examined (Seal and Doe, 1965), SHBG appears to have a more limited species distribution. Early data obtained by Murphy (1968) have shown that human, bovine and frog plasma contained protein(s) with high affinity binding to estradiol while specific estradiol binding was absent in the rat, rabbit, dog and duck plasma. In these studies, high affinity binding for testosterone was present in all seven species. Idler and Freeman (1968) have found high affinity testosterone binding in thorny skate and Atlantic salmon. In contrast to human SHBG which loses most of its activity by heating at 60°C, only slight loss of testosterone-binding activity was observed on heating of diluted skate sera at 60°C for 45 min. SHBG having high affinity for DHT, T and E_2 has been characterized in two species of monkeys: the stumptailed macaque (Gauthier-Wright et al, 1973) and the chimpanzee (McCormack, 1971).

Corvol and Bardin (1973) have identified testosterone binding activity in the plasma of 13 of 21 species examined using polyacrylamide gel electrophoresis; high affinity binding was observed in primates, goats, rabbits, cows, sheep, reptiles and amphibians. It was missing in birds, donkeys, cats, dogs and rats. Estradiol binding could be demonstrated in 5 of the above species namely, man, monkey, rabbit, salamander and skate. Studies using improved detection techniques have more recently permitted the identification of a SHBG having low affinity for testosterone in dog (Tabei et al, 1978) and cat (Bordin et al, 1978). Wenn et al (1977), using

an ammonium sulfate precipitation technique demonstrated the presence of specific testosterone binding for the first time in a marsupial, the kangaroo. They have also found trace amounts of high affinity testosterone binding in certain rodents such as hamster and in birds such as turkey, pigeon. These observations have led them to suggest that the apparent absence of SHBG in certain eutherian mammal orders (e.g. horse, donkey) might be due to a secondary loss during evolutionary development.

Although several studies (Raynaud et al, 1971; Wenn et al, 1977; Renoir et al, 1980) have since confirmed the early observation of Murphy (1968) that rat plasma lacked a specific testosterone binding protein, a recent report by Suzuki and Sinohara (1981) has shown the existence of low levels of a testosterone binding protein, having the same physicochemical properties as SHBG in male rats aged 3 weeks. This activity reached a peak at about 20 days of age, then decreased to lower levels and remained relatively unchanged until the end of the experimental period. The activity was not present in the serum of female rats and was not due to α-fetoprotein (see below) as the T binding activity did not parallel the estradiol binding activity. However, it may be due to the androgen binding protein (see below) since Gunsalus et al (1978) have identified the androgen binding protein of testicular origin in the male rat plasma by radioimmunoassay. A specific binding protein having high affinity for DHT, T and E_2 and similar electrophoretic mobility as other mammalian SHBG has been demonstrated by Damassa et al (1982) in the plasma of the male little brown bat, Myotis lucifugus lucifugus (chiroptera: vespertilionidea). Thus SHBG has been found in an increasing number of mammalian species and its species distribution may not be as limited as had previously been thought.

SHBG has been purified to homogeneity from the plasma of several

species other than human. They include thorny skate (Freeman and Idler, 1969), rabbit (Mickelson and Petra, 1978), dog (Tabei et al, 1978), bovine (Suzuki et al, 1977), rat (Suzuki et al, 1981), monkey and baboon (Petra, 1983). Basic similarity in structure between SHBG from various species has been supported by earlier observations of Corvol and Bardin (1973) who found that SHBG of 13 species had similar electrophoretic mobility under different experimental conditions. SHBGs from all species so far characterized have been shown to be large (≃ 80-100,000 daltons) glycoproteins. Close similarities in the electrophoretic mobilities, sedimentation coefficients and subunit structures have also been described for purified SHBG of rabbit, bovine and canine plasma (Suzuki et al, 1981) and for purified SHBG of human, monkey and baboon plasma (Petra, 1983).

Steroid binding and immunological studies of SHBG from various species have revealed certain interesting differences. SHBG of primates (man and monkey) bind both androgens and estrogens. However, SHBG from non-primate species bind androgens with the same range of affinity, but do not bind estradiol to any significant extent (Renoir et al, 1980). The major exception is the SHBG in the plasma of male little brown bat, Myotis lucifugus licifugus, which binds DHT, T and E_2 (Damassa et al, 1982). The binding of E_2 by SHBG in this species was in fact higher than that of testosterone. SHBG from the plasma of the fish which binds androgen, estrogen and progesterone with weak affinity (Freeman and Idler, 1969; Fostier and Breton, 1975) is another steroid binding plasma protein which does not conform to the primate and non-primate classification of SHBG, but may belong to another category of steroid binding proteins. Among the non-primate mammals, dog is unusual in that its SHBG binds DHT and T with a lower affinity, but binds E_2 with higher affinity than the SHBG of other

non-primate mammals (Renoir et al, 1980).

Detailed studies (Renoir et al, 1980) on the steroid binding specificity of SHBG in monkeys showed that, in addition to DHT, T and E_2 primate SHBGs bound 5α-androstanediols with high affinity, but had no affinity for estriol, diethylstilbestrol, progesterone or cortisol. Interestingly, estrone was a good competitor of ^{3}H-DHT binding in baboon, rhesus monkey, squirrel monkey, marmoset and gorilla, but not in chimpanzee and man. Systematic studies using monospecific antibodies against human SHBG (Bordin et al, 1978; Renoir et al, 1980) and rabbit SHBG (Bordin et al, 1978) have shown that the differences in the steroid binding of SHBGs from different species can be correlated with their respective immunocross-reactivities. Thus, human SHBG cross-reacted with sera from primates but not with sera from different non-primate species. The cross-reaction was complete in the chimpanzee and gorilla while it was partial with baboon and Rhesus monkey and weak in Prosimii (Microcebus murinus). Rabbit SHBG did not cross-react with the SHBGs of human, monkey, baboon, cat, dog, sheep, goat, cow or calf. Thus, it appears that there has been less evolutionary pressure for the conservation of specific steroid binding plasma proteins than for intracellular steroid receptors. For example, antibodies directed against estradiol receptors of one species have been shown to cross-react with those of another species (Radanyi et al, 1979).

The concentration of SHBG has been measured in various species of monkeys. Anderson et al (1976) found that, in the adult female of the bonnet and rhesus monkey, SHBG levels were 2.5-fold higher than in the human adult female. Data obtained by Renoir et al (1980) have shown that the levels of SHBG in the female chimpanzee was similar to that of human

female, while the SHBG levels of gorilla, baboon and Rhesus monkeys were 2-5 times higher. As in human, a sex difference in the levels of SHBG, females having higher levels, has been demonstrated in several species of monkeys (Burry et al, 1980). The levels of SHBG have been determined in monkeys also during pregnancy. Schiller et al (1978) have shown that changes in SHBG concentration during pregnancy in Macaca nemestrina were markedly different from those in women. In the Macaca nemestrina, SHBG levels rose significantly in early pregnancy, remained elevated through 56 days, then fell to very low values by 130-150 days post-conception. In contrast, SHBG levels in the human have been shown to increase 5-fold during pregnancy and to remain high at term (Anderson et al, 1976). Data published by Anderson et al (1976) showed that, in the bonnet monkey, maternal SHBG levels remained unchanged during pregnancy while in the rhesus monkey the levels fell markedly. Concentration of SHBG measured in infants of the bonnet and rhesus monkeys were much lower than their maternal levels and comparable to those of the human infant which was 20-fold lower than that of the pregnant woman. Evidently, the regulation of SHBG levels in the human mother and fetus and in mothers of various species are different. Physiological alterations in SHBG levels have also been shown in various species during reproductively important periods such as puberty (Berger et al, 1980; Blank et al, 1978) and renewal of reproductive activity in seasonal breeders (Maurel et al, 1980).

1.3.3.2 Androgen binding protein. Androgen binding protein (ABP) has been extensively studied in the rat and other mammalian species (French and Ritzen, 1973, Hansson et al, 1974; Ritzen and French, 1974; Vigersky et al, 1976). In the rat and rabbit it has been shown to be produced by the Sertoli cells of the testis, secreted into the lumen of the

seminiferous tubule and transported to the epididymis (French and Ritzen, 1973; Weddington et al, 1974). Direct evidence showing the site of synthesis as Sertoli cells has been obtained using culture studies (Louis and Fritz, 1979). There is no evidence to indicate that ABP is produced in the female rat. ABP has been shown to be a heat stable protein with high affinity for androgens and estrogens but not anti-androgens (Vernon et al, 1974). Data on the physicochemical characteristics of ABP has indicated great similarity to SHBG (Ritzen et al, 1973; Weddington et al, 1974).

Rat ABP has been purified to homogeneity employing affinity chromatography (Musto et al, 1980). ABP purified from rat testis is considered to be unambiguous and free of contamination by plasma SHBG since the levels of SHBG is undetectable (Corvol and Bardin, 1973; Renoir et al, 1980) or very low (Suzuki et al, 1981) in the rat plasma. SDS-PAGE analysis of rat ABP has suggested a subunit structure for ABP (Musto et al, 1978). Two species of monomers of apparent molecular weights 41,000 and 45,000 have been demonstrated. In addition, micro-heterogeneity due to differences in charge has been demonstrated for rat ABP. Furthermore, the heterogeneity has been shown to vary in different parts of the reproductive tract (Lee et al, 1980).

A highly purified ABP has also been prepared from the rabbit epididymis (Weddington et al, 1974). The purified rabbit ABP was similar or identical to rabbit SHBG with respect to steroid specificity, molecular weight and heat stability, but differed in concanavalin-A binding (Cheng et al, 1980).

ABP has also been described in the testis of man (Vigersky et al, 1976; Hsu and Toren, 1978) and monkey (Vigersky et al, 1976). Vigersky et al have reported that they were unable to distinguish human ABP from human

SHBG on the basis of physicochemical parameters such as isoelectric point, steroid specificity or dissociation constant. They have reported that although testicular and epididymal cytosols of man and monkey, on analysis by PAGE, were found to contain multiple species of DHT binding proteins they were indistinguishable from SHBG or could be related to SHBG as size or charge isomers. Hsu and Toren (1978), however, found that human testicular ABP, prepared without plasma contamination, could be distinguished from human plasma SHBG on the basis of charge, binding affinity for DHT, and pH optimum for binding. The molecular weight, ligand specificity and heat sensitivity of the two proteins were similar. It may be that human SHBG and human ABP have the same primary structure but differ in their carbohydrate content.

Studies in animals have suggested that the synthesis and secretion of ABP are under the control of gonadotropin and androgens. Several studies have shown a decrease in testicular and epididymal ABP levels after hypophysectomy and an increase in ABP levels after the administration of follicle stimulating hormone (FSH; Sanborn et al, 1975; Tindall et al, 1978) and testosterone (Elkington et al, 1975; Louis and Fritz, 1977). The data obtained by Louis and Fritz (1979) using Sertoli cells in culture have suggested that the actions of FSH and testosterone to increase ABP production were independent of each other. In addition, the concentration of ABP has been found to vary directly as a function of testicular testosterone in the human (Lee et al, 1980). The hormonal control of ABP appears to be quite different from that of SHBG.

The precise function of ABP is not understood. It has been suggested that ABP may participate in the transport of androgens within seminiferous tubules as well as to androgen binding epithelial cells in the

epididymis (Weddington et al, 1975; Tindall and Means, 1976). ABP has been shown to be present in caput epididymis in high concentrations. Immunocytochemical studies have provided evidence to show that ABP is selectively internalized by the epithelial cells of caput but not of other portions of the epididymis (Pelliniemi et al, 1980). These observations have led to the suggestion that ABP might function as a transmembrane carrier for androgens (Pelliniemi et al, 1980). Studies of Hsu et al (1977) have indicated that the number of binding sites of human ABP decreased in the aging testis.

Since ABP has been thought to be secreted behind the blood testis barrier, it was not believed to enter the blood and in fact no ABP could be detected in the plasma of rat, a species which has no detectable or low levels of SHBG. However, Gunsalus et al (1978) have recently reported that they have detected ABP in male rat plasma using an antibody raised against purified rat ABP. However, the plasma levels of immunoreactive ABP were very low in the male (1.5 percent of epididymal tissue concentration) and absent in the female. Testicular origin of plasma ABP was shown by an exponential decrease in its plasma levels after orchiectomy. It is not known whether testicular ABP is released into the blood circulation in man. Belgorosky et al (1983) have found significantly lower levels of SHBG in the spermatic vein than in the arterial blood. Since their method could not differentiate SHBG from ABP, they suggested that ABP is not secreted into blood in humans. In summary, available evidence suggests that an androgen binding protein is synthesized by the testis, is present in high concentrations in the epididymis and its levels are regulated by FSH and T. However, at present the data are insufficient to ascertain whether ABP and SHBG in the same species are similar but distinct proteins or the same

protein made in different tissues or partially metabolized glycoproteins.

1.3.3.3 Alpha-fetoprotein. Fetal and newborn rat sera have been shown to contain a unique estrogen binding α_1-globulin which was demonstrated to be the rat serum alpha$_1$-fetoprotein (AFP; Nunez et al, 1971a, 1971b). The AFP in the rat fetal serum has been shown to reach maximum concentration before birth and to decline after birth, almost completely disappearing by the 28th day of postnatal life (Stanislawsky-Birencwajg, 1967). The rat AFP has been demonstrated to bind estrone and estradiol, but not testosterone, with high affinity (Nunez et al, 1971b). It has been shown that adult rat serum has neither estrogen nor testosterone binding activity (Nunez et al, 1971a).

The estrogen binding property of serum AFP is species specific. Only rat and mouse AFP have been shown to exhibit high binding affinity for estrogens. In the human, AFP has been shown to be present in the fetal serum throughout gestation, disappearing almost completely after birth (Masseyeff, 1972); however, human AFP has been shown to have no estrogen binding activity (Nunez et al, 1974).

The steroid binding parameters of rat AFP have been studied in many laboratories (Raynaud et al, 1971; Savu et al, 1972; Aussel et al, 1973). The data from these studies has suggested that the binding affinity of rat AFP for E_2 was 2-3 orders of magnitude higher than that of albumin and 1-2 orders of magnitude lower than that of human SHBG. Purification and characterization of rat AFP has been reported (Benassayag et al, 1975; Valette et al, 1977). Separation of purified AFP has revealed molecular microheterogeneity as in the case of SHBG. However, in addition to molecular heterogeneity, rat AFP has exhibited functional heterogeneity, the various isomers showing different estrogen-binding characteristics.

Antibodies raised against human and rat AFP did not cross-react with human SHBG and, conversely, antibodies against human SHBG showed no cross-reactivity to human AFP (Renoir et al, 1980).

1.3.4 Concentration of SHBG in plasma

1.3.4.1 Synthesis and metabolism. There is no definitive evidence available for the site of synthesis of SHBG. It is generally believed to be synthesized by the liver although no direct experimental evidence has been published to support this hypothesis. However, several indirect lines of evidence are available. Liver has been shown to be the site of synthesis of several serum protein components including glycoproteins (Miller and Bale, 1954; Molnar et al, 1964; Robinson et al, 1964; Athineos et al, 1964). Data obtained by Gala and Westphal (1966) have suggested that subtotal hepatectomy resulted in a marked decrease in CBG activity. In addition, direct evidence for the synthesis of CBG in the liver has recently been obtained in the rat (Weiser et al, 1979; Wolf et al, 1981) and in the guinea pig (Perrot-Applalanat, 1979). Natural and synthetic estrogens which have been shown to alter the synthesis of plasma proteins of hepatic origin also influence levels of SHBG (Song et al, 1970; Wynn, 1977; Laurell and Rannevik, 1979). In addition, the only major nonendocrine illness in which SHBG is altered is cirrhosis of the liver (Anderson, 1974).

Little information is available on the metabolism of SHBG. It is generally assumed that it is metabolized in the liver as are many other glycoproteins. Suzuki and Sinohara (1979) have recently shown that ^{125}I-labeled bovine SHBG injected into rat tail vein was taken up by liver. When ^{125}I-labeled asialo-SHBG was injected, a much faster uptake by liver was observed. Rapid clearance from blood and concomitant accumulation in

liver as a result of the removal of sialic acid have been shown for a number of glycoproteins (Ashwell and Morell, 1974).

Metabolism of SHBG might also occur by uptake into target cells. Using immunofluorescence with monospecific antibodies to human SHBG, Bordin and Petra (1980) have shown intracellular presence of SHBG not only in liver but also in several tissues involved in reproduction in the monkey. They also demonstrated, in experiments performed with cultured MCF-7 cells, that SHBG can apparently cross the plasma membrane and enter cytoplasm. Data obtained by Belgorosky et al (1983) suggest that SHBG is cleared at least partially in extrahepatic tissue in children and that it is taken up by sexually mature testis of adults.

1.3.4.2 Concentration of SHBG in normal subjects. A summary of the data obtained in various laboratories on the normal concentration of SHBG in adult men, non-pregnant adult women and pregnant women is given in Table 1-5. The different methods used in these studies for the determination of SHBG are based on the binding of tritiated T or DHT to SHBG followed by separation of SHBG-bound steroid from that which is not bound to SHBG. Although there are discrepancies in the values of SHBG concentrations obtained in different laboratories, the relative differences between different groups of subjects are in reasonable agreement. All studies clearly show that there are significant differences in SHBG binding capacity between men, non-pregnant women and pregnant women.

1.3.4.3 Physiological changes in SHBG concentration. Concentrations of SHBG in the fetal and neonatal plasma have been shown to be much lower than in maternal plasma during late pregnancy or at term (Rivarola et al, 1968; Abramovich et al, 1978). Forest et al (1971) have found a 20-fold gradient and Anderson et al (1976) a 21-fold gradient in

TABLE 1-5. Concentration of SHBG expressed as concentration of steroid binding sites for T or DHT (mean ± SEM, nM).

Reference	Method of separation of bound from unbound fraction*	adult men	adult women	pregnant women
Vermeulen et al., 1969	GF	46±4 (12)†	74±8 (12)	324±52 (16)
Heyns and De Moor, 1971b	ASP	37±3 (24)	61±5 (18)	434±18 (11)
Corvol et al., 1971	PAGE	17±1 (10)	49±8 (14)	376±26 (6)
Rosner, 1972	ASP	32±2 (27)	64±4 (16)	410±23(19)
Shanbhag et al., 1973	AQP	61 (2)	103 (2)	438 (3)
Rudd et al., 1974	ASP	50±4 (10)	78±12 (12)	290±38(10)
Ritzen et al., 1974	SS-PAGE	59±7 (5)	103±9 (5)	--
Anderson et al., 1976	ASP	35±2 (23)	74±9 (40)	367±21 (24)
Dennis et al., 1977	DCC	29±10 (13)	64±28 (8)	168 (5)
Nisula et al., 1978	Con A	27±2 (32)	37±4 (10)	399±107 (5)

* GF - gel filtration; ASP - ammonium sulfate precipitation; PAGE - polyacrylamide gel electrophoresis; AQP - aqueous partition; SS-PAGE - steady state polyacrylamide gel electrophoresis; DCC - dextran coated charcoal; con A - concanavalin A

† Number of subjects

SHBG concentration from mother to fetus which was independent of the sex of the fetus. The SHBG concentration (mean ± S.E.M) in the cord blood of 23 newborn infants was 1.7 ± 1.3 nmoles/liter (Anderson et al, 1976). During the first few weeks of life SHBG activity has been shown to rise in both boys and girls (Forest et al, 1973). Tulchinsky and Chopra (1973) measured, in 3 day old infants, a 1.7-fold increase in the SHBG concentration over that measured at birth.

Vermeulen et al (1969) have found that SHBG levels in prepubertal children were in the adult non-pregnant female range (Table 1-5) which was higher than the adult male values. Similar findings have been reported by August et al (1969) and Forest and Bertrand (1972). Wagner (1978) has shown that increased SHBG levels in boys and girls were maintained until puberty.

During puberty, plasma SHBG levels have been shown to fall markedly in boys (Horst et al, 1977; Wagner, 1978) and to a lesser extent in girls (Bartseh et al, 1980; Wagner, 1978). The differential decline in SHBG levels in males and females during puberty result in a marked sex difference in adults which is maintained until menopause. The SHBG levels in adult men are only half of those found in women of comparable age. The SHBG levels in women during the menstrual cycle have been studied by many groups (Pearlman et al, 1967; De Moor et al, 1969; Wagner, 1978; Odlind et al, 1982). No significant variation in plasma SHBG levels were found during any phase of the menstrual cycle. However, Plymate et al (1985) who have measured SHBG levels using a more sensitive binding assay and an RIA method have demonstrated that SHBG changed in association with E_2 during the menstrual cycle.

During pregnancy, SHBG levels increase markedly to some 5-10

times those found in the non-pregnant female (Table 1-5). A significant increase over non-pregnant levels could be detected as early as 10 weeks gestation (De Hertogh et al, 1976; Uriel et al, 1981). Although maximal increase in SHBG occurred in the first half of gestation, SHBG concentration continued to increase gradually during the second half of pregnancy and levelled off between 25-30 weeks of gestation (De Hertogh et al, 1976; Uriel et al, 1981). A decline in SHBG levels prior to delivery was observed by Vermeulen et al (1969) and Uriel et al (1981) but not by Anderson et al (1976) and De Hertogh et al (1976). Data obtained by Uriel et al (1981) have shown that there was considerable variation in individual serum patterns throughout gestation. No significant difference in SHBG concentration could be associated with the sex of the fetus (Anderson et al, 1976; Uriel et al, 1981). SHBG levels have been shown to decrease after delivery. Anderson et al (1976) found that SHBG Levels fall immediately post-partum with a half life of 7.1 days.

SHBG activity was first detected in amniotic fluid by Caputo and Hosty (1972). Hammond et al (1983) have recently confirmed the presence of SHBG in amniotic fluid and measured its levels by a competitive saturation analysis method using ^{3}H-DHT. They have obtained similar SHBG concentrations in early (13-20 weeks, 8.5 nmole/liter) and late (36-37 weeks, 8.7 nmole/liter) gestation which were lower than in pregnancy serum (390 nmole/liter). No difference in amniotic fluid levels with respect to fetal sex was observed.

A definite increase in the levels of SHBG in adult men after the age of 50 years has been demonstrated (Vermeulen et al, 1972; Anderson, 1974). In contrast, a sharp decrease in SHBG concentration has been observed in women during menopause (Vermeulen et al, 1969; Wagner, 1978).

From the above discussion it is clear that SHBG concentrations are influenced by changes in hormonal levels such as those occurring during pregnancy.

1.3.4.4 Hormonal influences on SHBG concentration. As suggested by the studies of various physiological and pathological conditions in which SHBG levels are altered, plasma SHBG is under the control of various hormonal factors. It is well documented that the levels of SHBG are markedly increased when plasma estrogen concentrations are greatly elevated such as in pregnancy (Vermeulen et al, 1969; Rosner, 1972; Heyns and De Moor, 1971b) and after estrogen administration (Vermeulen and Verdonck, 1968; Murray et al, 1973; Van Kammen et al, 1975). Androgens, conversely, have been shown to depress the levels of SHBG in men (De Moor et al, 1969; Mauvais-Jarvis et al, 1971) and in women (Vermeulen et al, 1969; Tochimoto et al, 1970). The remarkable sensitivity of SHBG to changes in sex steroid concentration can be seen from several studies in which exogenous androgens or estrogens were adminstered. Wang et al (1975) have studied SHBG levels in Klinefelter's syndrome and other hypogonadal states and demonstrated that SHBG levels can be effectively controlled by small amounts of androgens. Administration of 20 μg daily of ethinylestradiol for 5 weeks to male criminal sex offenders, resulted in a 150% increase in SHBG levels (Murray et al, 1973). Medroxyprogesterone acetate, a progestational agent, when administered, appeared to decrease the SHBG concentration in both sexes before and after puberty (Forest et al, 1968; Forest and Bertrand, 1972). The synthetic anti-estrogen, clomiphene citrate, produced a moderate increase in SHBG concentration (Dray et al, 1970; Marshall et al, 1972). Van Look et al (1981) have recently studied SHBG activity in normal men treated with oral

ethinylestradiol for 5 days and reported that the estrogen-induced rise in SHBG occurred very rapidly. Significant elevations of SHBG could be detected as early as 72 hours after estrogen treatment.

It is generally believed that the greatly elevated concentration of plasma estradiol is responsible for the markedly increased SHBG levels in maternal plasma. However, despite the relatively high estradiol levels in fetal serum (Tulchinsky and Chopra, 1973), the SHBG concentration is very low in both the cord blood and peripheral circulation of the newborn, as previously mentioned. In the fetus, evidently, estrogen does not appear to induce SHBG synthesis. Furthermore, the SHBG levels rise in the early neonatal period when the circulating levels of estrogens have fallen to very low levels (Tulchinsky and Chopra, 1973). It has been suggested that the postnatal increase in thyroxine and triiodothyronine production may be responsible for the increase in SHBG production during this time (Anderson et al, 1976).

The relatively high levels of SHBG before puberty are also difficult to interpret since sex steroid production is minimal in both sexes (Faiman and Winter, 1974). In addition, Horst et al (1977) have found that, in boys younger than 12 years of age, SHBG binding capacity did not correlate with total testosterone concentration. Furthermore, Bartsch et al (1980) have observed no significant alterations in SHBG levels despite significant increase in T and DHT in girls aged 1-8 years.

The pronounced fall in SHBG during puberty in males is often used as evidence to support the concept that androgens lower the concentration of SHBG in plasma. Indeed, a significant negative correlation between SHBG and androgens has been found in both boys (Horst et al, 1977) and girls (Bartsch et al, 1980) as they pass through puberty. However, based on

their studies in boys with isolated gonadotropin deficiency and in their siblings with complete androgen insensitivity, Cunningham et al (1984) have suggested that factors other than elevated androgen levels are involved in pubertal fall in SHBG activity. They observed that decrease in SHBG levels occurred in boys during the second decade of life regardless of androgen activity.

The significant difference in SHBG levels between normal men and women is thought to be due to higher estrogen levels in women and elevated androgen levels in men. The higher SHBG binding capacity in women taking oral contraceptives is most likely to be caused by the high doses of estrogen in the contraceptive preparations. The increase in SHBG concentration observed in men after 50 years of age is most likely due to the associated decrease in the concentration of testosterone (Baker et al, 1976). Similarly, the decrease in the levels of SHBG in women after menopause may reflect the parallel decrease in estrogen levels caused by the termination of ovarian function (Vermeulen et al, 1969).

Among the non-steroidal hormones that influence SHBG levels, thyroid hormones have the most marked effect. These hormones produce a dramatic rise of SHBG levels in both sexes and hirsute women when administered slightly above the replacement doses (Dray et al, 1969; Ruder et al, 1971; Anderson et al, 1972). Crepy et al (1967) first observed that patients with spontaneous thyrotoxicosis had markedly elevated SHBG levels and this observation has since been confirmed in several laboratories (Dray et al, 1969; Vermeulen et al, 1969; Olivo et al, 1970; Ruder et al, 1971; Tulchinsky and Chopra, 1973). On treatment with anti-thyroid drugs, SHBG levels usually return to normal (Crepy et al, 1967; Olivo et al, 1970; Anderson, 1974). The increased levels of SHBG found in hyperthyroid

patients may be due to the direct effect of thyroid hormones in these patients. However, it is possible that the effect of thyroid hormones is indirect because hyperthyroidism is accompanied by elevated estrogen and LH levels (Chopra and Tulchinsky, 1974; Akande and Anderson, 1975). Thyrotoxic women often have amenorrhoea (Akande and Anderson, 1975), and men with thyrotoxicosis often suffer from gynaecomastia and reduced libido (Chopra et al, 1972).

Low levels of SHBG have been reported in acromegaly (De Moor et al, 1972; Anderson, 1974) and in Cushing's syndrome (De Moor et al, 1969; Anderson, 1974). Growth hormone has been found to lower SHBG levels in children (De Moor et al, 1972) and may be responsible for the decreased SHBG levels in acromegaly. Administration of high doses of glucocorticoids to patients with chronic asthmatic bronchitis has been shown to decrease SHBG levels (Vermeulen et al, 1969) which may be relevant to the depressed SHBG levels observed in Cushing's syndrome.

Hirsute or virilized women, regardless of their menstrual history, frequently have reduced plasma levels of SHBG (Dray et al, 1969; Vermeulen et al, 1969; Rosenfeld, 1971; Anderson, 1974). The levels of SHBG in hirsute women often approach those found in normal men with some overlapping with the levels in normal women. Depressed levels of SHBG have also been observed in obese subjects (De Moor and Joossens, 1970; Siiteri et al, 1982). A significant negative correlation between SHBG and body weight was found by both groups. The low levels of SHBG found in hirsutism and obesity may be due to excessive androgen action which may be caused by either elevated androgen levels or increased androgen sensitivity (Cunningham et al, 1983; Kirschner et al, 1983).

In men with hypogonadism, SHBG levels have been found to be above

the normal male range and may approach female levels (Kato and Horton, 1968; Vermeulen et al, 1969). Decreased SHBG levels have also been observed in men with testicular feminization where there was end organ resistance (Mauvais-Jarvis, 1970; Rosenfield et al, 1971). Although it has been suggested that SHBG structure may be abnormal in testicular feminization, Anderson (1974) has found that plasma binding of testosterone and estradiol were normal in this syndrome. Thus the decreased levels of androgen in hypogonadal men and the impaired action of androgens in testicular feminization syndrome may be responsible for the high SHBG levels in those two conditions. The increased levels of SHBG found in gynaecomastia may be explained by the elevated concentration of estrogen associated with that state.

Finally, the only non-endocrine illness in which SHBG is altered is cirrhosis of the liver (the liver is believed to be the site of synthesis of SHBG). Increased SHBG levels have been found in men with cirrhosis of the liver (Tavernetti et al, 1967; Rosner, 1972) or chronic liver disease (Galvao-Teles et al, 1973). Interestingly, in two hirsute women with cirrhosis of the liver, Anderson (1974) reported SHBG levels within the normal female range. In summary, the concentration of SHBG is under complex multi-hormonal control, the major regulatory factors being the steroid hormones which are strongly bound by the SHBG binding site.

1.3.5 Physiological function of SHBG

The presence of SHBG or similar proteins capable of binding sex steroids in the plasma of a wide variety of species suggests an important function for such proteins in the biological action of sex steroids. The physiological function of SHBG appears to be complex as its plasma concentration is under the regulation of steroids which are themselves

strongly bound to its binding site. As already mentioned in section 1.2.4, the most widely held theory on the function of SHBG is the 'free hormone hypothesis' according to which the level of SHBG regulates the concentration of unbound hormone and despite its small size it is the unbound hormone which is physiologically active. Although the precise mechanism by which SHBG regulates sex hormone action is still a controversial issue, most of the proposed roles of SHBG are discussed below. Some of those roles are consistent with the 'free' hormone hypothesis while others are not.

1.3.5.1 Proposed roles of SHBG which are consistent with the 'free' hormone hypothesis.

(i) Regulation of metabolism and biological action of sex steroids - Several in vivo and in vitro studies support the contention that SHBG retards the metabolism of steroids that are bound to it. In vivo it was noted that in the human the metabolic clearance rate (MCR) of testosterone is inversely related to SHBG levels in blood (Vermeulen et al, 1969; Southren et al, 1969). The MCR of T in men is about twice that in women, presumably due to the lower SHBG levels in men (Vermeulen et al, 1969). In addition, androgen administration in women results in increased MCR of T and decreased levels of SHBG. In several clinical conditions such as hirsutism or hyperthyroidism, an inverse relationship between MCR and T and SHBG concentrations was observed (Vermeulen et al, 1969; Mahoudeau et al, 1971). Further, androstenedione which is poorly bound to SHBG is cleared twice as fast as testosterone (Bardin and Lipsett, 1967). In contrast, in species such as donkey and dog which lack or have only low levels of SHBG, androstenedione and T are cleared at the same rate (Corvol and Bardin, 1973). Indeed, an inverse correlation between the MCR of an androgen and

the strength of its binding to SHBG has been demonstrated (Rosenfield, 1975). However, the MCR of sex steroids is not uniquely determined by their affinity for SHBG and the plasma concentration of SHBG. The contribution of local blood flow and intracellular steroid metabolizing enzymes also are of importance (Vermeulen et al, 1969). Further, while certain metabolic events lead to inactivation of steroids, others may result in activation. For example, intracellular metabolism of T to DHT has been demonstrated in many target tissues.

One of the most important pieces of evidence often used to support the concept that SHBG regulates the biological activity of steroids bound to it comes from the clinical finding that the increased plasma testosterone concentrations found in association with elevated SHBG levels in conditions such as pregnancy and hyperthyroidism do not cause virilization. This observation suggests that the unbound rather than the SHBG-bound fraction is biologically active. In hirsute women, the symptoms of hyperandrogenicity correlate better with unbound androgen levels than with total androgen concentration (Vermeulen et al, 1971; Rosenfield, 1971). A number of <u>in vitro</u> studies also have suggested that SHBG-bound steroid is less available for metabolism or expression of biological activity. Mowszowicz et al (1970) have reported that aromatization of testosterone by human placental microsomes was significantly decreased by the presence of SHBG or high concentrations of albumin in the incubation medium. Similar findings were obtained by Hampl and Starka (1973). In addition, decrease in the yields of testosterone glucuronide when testosterone was incubated with rat liver microsomes in the presence of SHBG has been shown by Hampl et al (1975). Lasnitzki and Franklin (1972), using explant cultures of rat ventral prostate, demonstrated that

testosterone uptake by tissues decreased with rising serum content in the media. The uptake and metabolism of testosterone were significantly lower in explants incubated with pregnancy serum than in those incubated with male serum. Furthermore, there was good agreement between uptake and metabolism of testosterone and the degree of maintenance of the prostatic epithelium. These experiments suggested that the concentration of SHBG regulated the biological action of testosterone by determining the levels of unbound hormone. Mercier-Bodard et al (1976), using human prostate explants in constant flow organ culture, have obtained similar results. The presence of SHBG and albumin in the superfusion medium decreased the uptake of radioactive T in direct proportion to the unbound T fraction in the medium.

(ii) Regulation of androgen-estrogen balance - Burke and Anderson (1972) have proposed that SHBG may control the androgen-estrogen balance by regulating the ratio of unbound testosterone to unbound estradiol. They observed that alterations in SHBG concentration produced much more pronounced changes in the percentage unbound T levels than in unbound E_2 levels. On the other hand, increasing the concentration of T over the physiological range did not significantly alter unbound E_2. These observations can be explained by their finding that at 37°C T bound twice as strongly to SHBG and only half as strongly to albumin as did E_2. Now, considering that both T and E_2 bind to the same binding site on SHBG and that they have opposite effects on the synthesis of SHBG, this protein may regulate the androgen- estrogen balance in the following manner: an estradiol-induced rise in SHBG might tip the androgen-estrogen balance in favour of estradiol by increasing the ratio of unbound E_2/unbound T. Conversely, a T-induced fall in SHBG might promote T action by an increase

in the ratio of unbound T/unbound E_2. Thus, SHBG may function as a sex hormone amplifier.

Findings in a number of endocrine disorders appear to support this hypothesis. The symptoms of androgenicity often found in hirsute women in association with low SHBG levels can be explained by the relative increase in unbound androgen concentration. Similarly, the estrogen-induced increase in SHBG levels in pregnancy may prevent virilization by high androgen levels by checking the relative increase in unbound androgen concentration.

(iii) Selective concentration of active steroid in the target cell - Robel (1971) has proposed the following possible role for SHBG in the biological action of T in the prostate. In plasma the only high affinity binder for testosterone is SHBG (section 1.4.1). T entering the cell can bind to the cytoplasmic androgen receptor or to the 5α-reductase enzyme. T bound to 5α-reductase gets converted to DHT which in turn can bind to the high affinity cytoplasmic receptor or to the 3α/3β ketoreductase. The 3α or 3β androstenediols formed are not bound by the cytoplasmic receptor and diffuse out of the cell. In plasma they can displace T from SHBG binding sites since they have a higher affinity to SHBG than T (Table 1-2), thus favouring the entry of T into the cell. This series of events results in a one-way circle whereby selective concentration of T in the target cell is achieved.

(iv) Facilitation of the transport of steroid hormones from the site of biosynthesis into the blood stream - This function for steroid binding plasma proteins was first proposed by Westphal (1970). Further studies in his laboratory showed that the rate of removal of corticosterone from

diluted rat serum by dialysis was increased by the presence of albumin, charcoal suspension and CBG in the outside fluid (Westphal, 1975). Data obtained by Ewing et al (1976) from in vitro perfusion studies of rabbit testis appear to support this hypothesis. They have obtained more than 4-times higher testosterone secretion when the perfusion medium contained 3% bovine serum albumin (BSA) than when it contained 3% dextran in place of BSA. These findings suggest that the steroid binding plasma proteins may enhance the secretion of steroids by rapidly removing the steroid from its site of synthesis into the blood stream.

Recently, Egloff et al (1981) have provided evidence to show that SHBG indeed may function in this manner. They demonstrated that the conversion of androstenedione to testosterone by human erythrocytes was markedly increased by the presence of SHBG while the presence of total plasma and albumin decreased the conversion. The effect of SHBG was largely abolished by heating to 60°C for 1 hr or by saturating the binding sites with DHT. These findings taken with the known fact that SHBG is a poor binder of androstenedione and a strong binder of T led the authors to propose that SHBG stimulates T production in erythrocytes by facilitating the diffusion of T out of the cell and thereby displacing the chemical equilibrium within the cell. Similarly albumin, which is a low affinity high capacity binder of both androstenedione and T, may exert its inhibitory effect on the conversion of androstenedione by preventing that steroid from entering the cell.

(v) <u>Regulation of unbound hormone concentrations in vivo: Pardridge's hypothesis (Pardridge, 1981)</u> - This theory is consistent with the 'free hormone hypothesis' in that according to this theory the unbound hormone is

a necessary intermediate in the steroid hormone action and metabolism *in vivo*. However, it advances the concept that the large protein-bound moiety is available for transport into tissues *in vivo* depending on the capillary transit time of the protein, the unidirectional dissociation rate of the steroid from the protein and the membrane permeability (Pardridge, 1981). This notion is not compatible with the classical free hormone hypothesis according to which the unbound (dialyzable) fraction *in vitro* represents the fraction available for transport into tissues *in vivo*.

The unbound hormone fraction *in vivo* was estimated by Pardridge (1981) using the Oldendorf (1970) technique. The *in vivo* uptake index (first pass extraction) by rat brain or liver of T and E_2 were determined after rapid injection of radioactive T or E_2 mixed with various concentrations of SHBG (different types of serum) or albumin into the carotid artery (brain) or portal vein (liver) of the anesthetized rat.

The results they have obtained from the above studies suggested that (i) albumin-bound T and E_2 were available for transport into brain and liver tissues, (ii) SHBG-bound T was not available for transport into brain or liver, (iii) SHBG-bound E_2 was not available for transport into brain tissue, but was available for clearance by liver tissue, (iv) brain uptake of T and E_2 were tightly regulated by changes in SHBG concentration, and (v) changes in SHBG concentration had greater influence on the uptake of T than on E_2 uptake. Based on the above findings, the authors proposed that since changes in SHBG concentration will cause more pronounced changes in T clearance relative to changes in E_2 clearance, the E_2/T clearance ratio will rise linearly with increases in SHBG. Thus, increases in SHBG concentration will amplify the amount of estradiol taken up by tissues

relative to the amount of testosterone cleared by tissues and SHBG may function as an estradiol amplifier as suggested by Burke and Anderson (1972). In addition, they speculated that since increases in SHBG may cause the ratio of percent unbound E_2/percent unbound T to rise faster in liver relative to brain, SHBG may also function as a selective amplifier of hormone entry into hepatic tissues vs peripheral tissues.

These studies have suggested that the unbound hormone *in vivo* is not equivalent to unbound hormone measured *in vitro*. However, determination of unbound hormone *in vitro* is still useful, as it will be proportional to the *in vivo* unbound concentration under most circumstances. The only situations where this assumption may not be strictly valid are when the primary change is in the albumin binding parameter or when the SHBG-bound hormone is available for uptake or clearance.

1.3.5.2. Proposed roles of SHBG which are inconsistent with the 'free' hormone hypothesis.

(i) SHBG functions as a transmembrane carrier for sex steroids - Several authors have suggested that SHBG may function as a transmembrane carrier of steroid hormones (Bordin and Petra, 1980; Lobl, 1981; Siiteri et al, 1982). Bordin and Petra (1980) have elegantly demonstrated the presence of SHBG in the cells of several tissues of the monkey, Macaca nemestrina. The plasma of this monkey has been shown to contain SHBG very similar in physicochemical properties to human SHBG. Using immunofluorescence technique with a specific antibody against human SHBG, they have detected SHBG within the cells of prostate, epididymis and the liver tissues of the monkey. Furthermore, their experiments with cultured MCF-7 cells indicated that SHBG can cross the plasma membrane and enter the cytoplasm. In both

cases SHBG was localized in the cytoplasm and did not show any evidence of entry into the nucleus. The authors speculated that SHBG may serve as a specific carrier of sex steroids from plasma into target cells and from steroid synthesizing cells into plasma. Intracellular presence and steroid dependent uptake of CBG have also been shown in the rat by Siiteri et al (1982, see below).

It is generally assumed that steroids are absorbed into and secreted from cells by simple diffusion. However, some evidence for carrier-mediated transport has been presented in the literature. Gurpide et al (1971) have reported that the uptake of E_2 by endometrial cells was decreased by the presence of albumin but was unchanged or increased by the presence of SHBG in the perfusion medium. Milgrom et al (1973), based on their studies of E_2 and DES uptake by isolated endometrial cells in the presence or absence of various inhibitors, have demonstrated that the cytoplasmic high affinity receptor binding alone could not acount for the rate of uptake of steroids into these cells. Data obtained by Rao et al (1977) have suggested that sex steroid uptake by liver cells in culture is a saturable, energy dependent process. In addition, Szego and Pietras (1981) have described the presence of E_2 binding sites on the plasma membranes of hepatic and uterine cells of the rat. Although none of the above studies have considered the possible involvement of SHBG in the carrier-mediated transport of sex steroids, it is possible that SHBG may directly participate in the transmembrane transport of sex steroids. Specific recognition sites for SHBG on the target cell membranes will allow specificity of steroid uptake as contrasted with simple diffusion. Indeed, the presence of a recognition system for SHBG-E_2 complex on the plasma

membranes of human decidual endometrium has recently been demonstrated (Strel'Chyonok et al, 1984).

(ii) Direct participation in the uptake and mechanism of action of steroids - Siiteri et al (1982), based on the data obtained in their own laboratory and which have been recently reported in the literature, have proposed an alternative model for the role of SHBG in steroid hormone action. This model and the hypothesis of SHBG acting as a transmembrane carrier (discussed above) are not mutually exclusive. According to the theory proposed by Siiteri et al (1982), steroid hormones complexed with specific steroid binding plasma proteins such as SHBG are recognized and bound by specific binding sites on the outer surface of the plasma membranes of their target cells and are subsequently internalized. The internalized steroid-SHBG complex either supplies the hormone to the classical intracellular steroid receptors or is converted by an unknown mechanism into such receptors. The steroid- receptor complex is then translocated into the nucleus and interacts with chromatin to modify the transcriptional activity which results in altered cell function. Alternatively, the steroid-SHBG complex may be distributed to various subcellular compartments to modify various activities in those compartments. The data supporting this hypothesis, obtained in the laboratory of Siiteri et al (1982), are summarized below. Using immunocytochemical detection technique with antiserum raised against pure rat CBG it was found, in the rat, that several tissues contained cells which stained positively for CBG. These tissues included liver, kidney, pituitary gland, spleen and uterus. The intracellular CBG-like binding sites were found to be similar to CBG in antigenic and steroid-binding

properties to CBG and could be distinguished from the classical dexamethasone binding sites. When adrenalectomized, ovariectomized rats were injected with ^{125}I-CBG alone or together with 100 µg corticosterone, it was found that certain tissues such as the uterus and kidney showed steroid-dependent uptake of CBG. In addition, they demonstrated that rat pituitary tumour GH_3-cells internalized fluorscein-labeled CBG and that the rate of uptake was strongly influenced by temperature and the presence of steroid. Furthermore, the cells were found to display marked nuclear concentration of CBG. This is in contrast to the finding of Bordin and Petra (1980) who observed cytoplasmic presence of SHBG but no nuclear manifestation in several tissues involved in reproduction in the monkey.

Siiteri et al (1982) have also provided a further piece of circumstantial evidence in favour of a direct role of SHBG in T action based on their comparative studies of steroid binding in primates. Although the binding affinity of SHBG for T was about 8-fold higher in the rhesus than in the squirrel monkey, the T levels were similar in both species during the nonbreeding season. However, the testosterone levels in squirrel monkey are 5-10-fold higher than in the rhesus monkey during the breeding season. This observation is opposite to what one would expect if the free hormone hypothesis were valid. The authors have speculated that the squirrel monkey adapted to the presence of the low affinity SHBG by increasing its capacity to produce high levels of T and thus achieving effective levels of SHBG-testosterone complexes. Siiteri et al (1982) further argue that, in women, even if both free and albumin bound fractions of plasma E_2 were available to the cytoplasmic E_2 receptors as suggested by Pardridge (1981), they would saturate only a fraction of the E_2 receptors.

Thus, the authors speculated that the steroid-binding plasma proteins may be involved in increasing the intracellular steroid concentration which is required for a full biological response.

Data obtained by Mercier-Bodard et al (1976) using human prostate explant in constant flow organ culture appear to support the hypothesis of Siiteri. The pattern of T metabolism was found to be different when the superfusion medium contained SHBG than when it contained albumin or buffer alone. It was observed that although the uptake of T was decreased in the presence of SHBG, the DHT/T ratio in the prostate explant tissues was critically dependent on the SHBG concentration in the superfusion medium. This suggested that SHBG has a direct effect on T uptake and metabolism in the human prostate.

Available evidence suggests that there is no physicochemical relationship between SHBG and intracellular E_2 or T receptors. Their binding characteristics also are known to be different. However, it is conceivable that changes in structural or binding properties of SHBG may occur during its internalization by mechanisms e.g. as the removal of the carbohydrate moiety. It is of interest to note in this regard that a statistically significant ($P < 0.001$) inverse relationship between the levels of plasma SHBG and E_2 receptors in breast tissue has recently been reported in breast cancer patients (Namkung and Petra, 1982).

The model proposed by Siiteri et al (1982) may provide an explanation for certain clinical conditions such as resistance to estrogens that develops following prolonged estrogen therapy, hirsutism and polycystic ovarian disease. For example, the presence of surface receptors for SHBG may provide a mechanism for their down-regulation by estrogens

leading to estrogen resistance. The virilization associated with hirsutism, a condition in which androgen levels are high and SHBG levels low, may be explained by an increased uptake of SHBG-androgen complexes. The model, however, fails to explain the vast majority of data supporting the free hormone hypothesis accumulated over the past 20 years. Pregnant women, despite having high SHBG, CBG and androgen levels, are neither virilized nor Cushingoid. Synthetic compounds such as dexamethasone and diethylstilbesterol, although having low affinity for CBG and SHBG, are biologically very potent. Thus it may be that the presence of SHBG in various tissues may simply represent the site of synthesis or catabolism. Critical experiments using labeled SHBG and steroid to demonstrate the intracellular presence of intact SHBG-steroid complex have not yet been attempted. In conclusion, although no convincing evidence has been presented to authenticate any of the proposed models, they provide a framework for designing studies to delineate the role of SHBG in the overall mechanism of sex hormone action.

1.4 Significance of SHBG in the Plasma Distribution of Testosterone and Estradiol

Over the past two decades convincing evidence has accumulated to show that T and E_2 exist in plasma in mainly three forms, namely, SHBG-bound, albumin-bound and unbound (Vermeulen and Verdonck, 1968; Anderson, 1974; Abramovich et al, 1978). The unbound concentrations of T and E_2 are believed to represent only 1-4% of the total concentration in most studies (Vermeulen and Verdonck, 1968; Burke and Anderson, 1972; Fisher et al, 1974; Moll et al, 1981).

1.4.1 SHBG: The only high affinity binder for both testosterone and estradiol

Studies in several laboratories using a variety of techniques have shown that SHBG is the only significant high affinity binding protein for both T and E_2 in human plasma. Murphy (1968) obtained direct evidence to demonstrate that T and E_2 are bound to the same binding site on SHBG by competition experiments using tritiated T and E_2 in the presence of non-radioactive steroids. De Moor et al (1969) have determined the correlation coefficients for T and E_2 binding in 59 plasma samples of human subjects and showed that the high affinity binding of T and that of E_2 in plasma are due to actually one and the same protein. A summary of these findings has been presented by Murphy (1969) in her treatise on protein binding and the assay of non-antigenic hormones. Competition of T and E_2 for binding sites of purified SHBG has been shown by Rosner (1975). Other major reports that have provided compelling evidence to show SHBG as the only high affinity binder and albumin as the only significant low affinity binder for T and E_2 include those of Mercier-Bodard et al (1970) and Burke and Anderson (1972). In addition, Abramovich et al (1978) who have done a systematic study of the sex steroid binding proteins in maternal and fetal serum, using steady state gel electrophoresis, have failed to demonstrate any additional estrogen or androgen binding protein other than SHBG and albumin. Although T has been shown to bind to CBG at high T concentrations (Shanbhag et al, 1973) and at low temperatures (Ritzen et al, 1974), data obtained by several investigators (Burke and Anderson, 1972; Ritzen et al, 1974; Vermeulen, 1977; Nisula and Dunn, 1979) show that T binding by CBG is insignificant at physiological concentrations in undiluted serum at 37°C.

1.4.2 Methods used for the determination of plasma distribution of T and E_2

Direct quantitation of protein-bound hormones has not been possible due to theoretical and practical limitations. Available methods for the determination of protein-bound and unbound forms depend on their ability to distinguish the bound form of the ligand from the unbound form. The procedure usually involves addition of a known amount of the radioactive steroid to plasma samples followed by determination of the percentage of total steroid that is protein-unbound using one of the methods described below. The percentage distribution of the steroid into SHBG-bound and albumin-bound forms is usually determined indirectly using the values of total concentration of the ligand, equilibrium constant of association and the binding capacity. The total plasma concentration of the ligand is usually determined by radioimmunoassay or a radiotransin-assay. (The term 'transin' refers to an indigenous binding protein such as SHBG or CBG, as defined by Murphy, 1975b). The equilibrium constant of association and the binding capacity of SHBG can be determined by analysing the binding data obtained in the presence of increasing concentrations of non-radioactive ligand. The analysis of the data is usually done by Scatchard plot already mentioned (section 1.3.2.4) or by solving the equations describing the complex equilibrium reactions between steroid and proteins. A brief description of all the widely used methods that have been described as well as most of the recently described ones for the determination of T and E_2 binding to plasma proteins is given below. The disadvantages of these methods will be high-lighted since one of the objectives of the first part of the work presented in this thesis was to develop a method which is free of these limitations.

1.4.2.1 Equilibrium dialysis. In equilibrium dialysis, two compartments are separated by a semipermeable membrane which is impermeable to high molecular weight substances such as proteins while allowing the passage of smaller molecular weight substances such as steroids. In one compartment the plasma sample is placed and in the other one a buffer solution is placed. A small (known) quantity of radiolabeled ligand is then introduced into one of the two compartments. Dialysis is continued until equilibrium is reached. At equilibrium the concentration of unbound ligand will be uniform throughout the system. The concentration of ligand in the compartment containing the protein (sample compartment) will represent the total concentration of protein-bound and unbound ligand while that in the protein-free (dialysate) compartment will represent the unbound ligand concentration.

The major advantage of equilibrium dialysis over other methods is its thermodynamic validity (Westphal, 1971). Accurate, reproducible values can be obtained if the experiment is conducted under in vivo conditions using undiluted plasma. However, the equilibrium dialysis procedures described by various investigators have several limitations. An inherent drawback is the change in equilibrium during dialysis. In other words the ligand concentration changes during the course of dialysis and the post-dialysis equilibrium is not identical to pre- dialysis equilibrium. The magnitude of this change may be considerable if a high dialysate/sample ratio is used (Smith and Jubiz, 1980). A second drawback is the volume shift that may occur across the semipermeable membrane when the solutions on both sides of the membrane are not isosmotic. In order to minimize this problem, most investigators have used diluted serum. However, the

alterations in serum protein concentration have been shown to change the binding site characteristics (Moll and Rosenfield, 1978). Another potential problem is the adsorption of ligand to surfaces. However, this can easily be tested by checking the recovery of the ligand.

1.4.2.2 Ultrafiltration. The principle of this method is the same as that of dialysis. The procedure has been described in detail by several investigators (Chen et al, 1961; O'Connell and Welsh, 1969). The separation of unbound ligand from the sample is achieved by the application of centrifugal force on the sample whereby the solvent and low molecular weight substances are forced through a semipermeable membrane. An advantage of ultrafiltration technique is that it can be carried out in a relatively short time. In addition, undiluted plasma can be used without the danger of volume shift across the membrane. However, there is one major drawback to the ultrafiltration procedure. During ultrafiltration the protein concentration in the sample being filtered increases resulting in continuous change in the binding equilibrium.

1.4.2.3 Gel filtration. In this method the separation of the bound and unbound portion of the ligand is accomplished by chromatography. The unbound ligand is separated from protein-ligand complex by molecular exclusion based on the difference in their molecular size. The original gel filtration method (zonal analysis) which has been used for years (De Moor et al, 1962; Westphal, 1969) is not an equilibrium procedure. This is mainly due to the fact that the fraction bound to specific proteins also undergoes partial dissociation during the filtration procedure. In addition, when small volumes of plasma samples are used, the concentration of plasma protein is not maintained during gel filtration

because of zone spreading. Burke (1969) has corrected these drawbacks by using frontal analysis, a method by which a steady state is maintained. by applying a larger volume of sample (e.g.: 25 ml serum), thus largely eliminating the problem of dissociation. The obvious limitation of this procedure is the large sample volume required.

1.4.2.4 Gel equilibration. This method combines the principle of gel filtration method with that of equilibrium dialysis. The essential features of this procedure have been summarized by Pearlman (1970). It involves mixing the sample and a small amount of radioactive ligand with Sephadex G-25 in phosphate buffer. Whereas the unbound ligand can freely enter the internal volume of Sephadex beads, the protein-ligand complex is excluded. The major advantages of this procedure are the small amount of sample required and the rapid equilibration time. The major limitation is that the internal volume cannot be sampled directly. In addition, reversible adsorption of ligand to Sephadex beads presents a potential problem which will lead to an altered partition coefficient.

1.4.2.5 Electrophoretic technique. Although steroids are uncharged molecules and do not show electrophoretic mobility, they will migrate in an electric field when associated to plasma proteins. However, this method has the disadvantage of rapid dissociation of the steroid-protein complex that occurs during the electrophoresis. In order to minimize this problem, electrophoresis under steady state have been developed more recently (Ritzen et al, 1974). The method combines the desirable features of high resolution gel electrophoresis with those of steady state condition. Using this method, the binding of SHBG and albumin can be studied simultaneously in one steady state electrophoresis under

identical experimental conditions. In addition, only small amounts of sample are required. However, in this method, the plasma is distributed on a gel and the state differs from that of whole plasma. In addition, the estimation of the ratio of bound/free ligand may be difficult due to the high background of free radioactivity.

1.4.2.6 Partition methods. A partition method in which the protein stays in an aqueous phase, while the unbound ligand partitions between an organic and aqueous phase has been used by Schellman et al (1954) for the determination of albumin-testosterone interactions. However, the organic solvent chosen must not interact with the protein molecule and influence its intact structure or its binding affinity. On the other hand, the solvent has to be polar enough to allow the extraction of sufficient amounts of ligand.

More recently, Shanbhag et al (1973) have introduced a new partition method to study testosterone binding to SHBG. The method was based on equilibrium partition in an aqueous, 2 phase system containing 1% dextran, 7% polyethyleneglycol and 0.1 M KSCN in 0.005 M phosphate buffer. In this system, 99% of the total plasma protein is confined to the lower phase while the unbound ligand equilibrates between the two phases. This method is rapid, simple and requires only small amounts of sample. However, it is not known whether the system is applicable to study the binding of more polar steroids such as estradiol.

1.4.2.7 Ammonium sulphate precipitation. This is a non-equilibrium method and is usually done under non-physiological conditions. The procedure, however, is simple and rapid. Van Baelen et al (1968) first demonstrated that SHBG and SHBG-steroid complex can be

precipitated by ammonium sulphate. Heyns and De Moor (1971) used this method to study the kinetics of dissociation of various steroids from SHBG. Rosner (1972) has shown that 90% of DHT is precipitated by 50% ammonium sulphate while albumin bound and CBG bound steroids stayed in the supernatant. However, it is necessary that all the SHBG is precipitated and that other proteins that bind to the radioactive ligand not be precipitated. In addition, the bound steroid should not dissociate from SHBG after the addition of ammonium sulphate. In this method, the precipitation is usually performed at 0°C in order to avoid dissociation. Further, it is often necessary to use diluted plasma to avoid a high percentage of unbound (albumin bound) steroid in the supernatant.

1.4.2.8 Adsorption methods. Adsorption of the unbound moiety is a non-equilibrium method for the separation of bound ligand from unbound ligand which has found wide application. The method is inexpensive, rapid and requires only limited space and equipment. The procedure involves addition of a tracer amount of radioactive ligand to a small volume of sample (usually diluted serum). The adsorbent is added, allowed to react and then removed, usually by centrifugation. The adsorbent should remove only the unbound ligand and the concentration of bound ligand is determined by analyzing the supernatant. The most commonly used adsorbents are dextran-coated charcoal, Florisil and Fuller's earth. The major pitfall of this procedure is that it is a non-equilibrium, non-physiological method. The adsorption of unbound steroid varies with time since the dissociation of steroid from the adsorbent is relatively non-reversible while the dissociation from protein is readily reversible. In addition, the adsorbent may adsorb protein while removing the unbound ligand.

1.4.2.9 Solid phase method. The solid phase method was first described by Nisula and Dunn (1979). It is based on the ability of concanavalin A (con A) to adsorb SHBG. The procedure involves incubation of plasma sample with 50% (v/v) slurry of con A sepharose in phosphate-buffered saline. The gel sediment is then washed for complete removal of albumin and other non-glycoproteins and incubated with the radioactive ligand at 37 C. The radioactivity in the aqueous phase and the gel sediment is then determined to obtain the unbound and bound ligand concentrations, respectively. Although the incubation can be done at 37°C, the method has most of the disadvantages of the non-equilibrium adsorption methods. The concentrations of proteins and ligands are not the same as those of whole plasma.

1.4.2.10 Flow dialysis. Moll and Rosenfield (1977, 1979) have described a flow dialysis technique for the determination of testosterone binding in undiluted plasma at 37°C under conditions which closely approximated physiological. In this method, Ringers lactate solution is perfused through the lower chamber of a Colowick-Womack cell (Colowick and Womack, 1969), the upper chamber of which contains 10 ml of plasma and tracer amounts of ligand. The two chambers are separated by a semipermeable membrane. The rate of diffusion of the radioactive ligand into the outflow chamber will be proportional to the concentration of unbound ligand in the plasma chamber. The major advantage of this technique is that it can be performed at 37°C using undiluted plasma under near physiological conditions. However, large volumes of plasma (10 ml) are required. In addition, the procedure is tedious and involves standardization and collection of fractions.

1.4.2.11 Ultrafiltration dialysis. This new method reported by Hammond et al (1980) combines some of the desirable features of equilibrium dialysis with those of ultrafiltration. Plasma samples are pre-incubated with ^{3}H-ligand and an internal standard such as ^{14}C-glucose in a small tube which is closed at one end with a dialysis membrane. These tubes are placed in minivials which have 3 pads of filter paper at the bottom and the vials are centrifuged at 3000 rpm for 1 hr at 37°C. The percentage of unbound steroid is determined as:
($^{3}H/^{14}C$ ratio in the minivial) / ($^{3}H/^{14}C$ ratio in the inner tube).

This method requires only small volumes of sample, utilizes undiluted plasma and is faster than equilibrium dialysis. However, for an accurate determination of $^{3}H/^{14}C$ ratio, relatively high concentrations of ^{3}H-ligand are required, which will significantly alter the endogenous ligand concentration. In addition, during centrifugation, there is a concentration gradient of plasma components in a 3000 rpm centrifugal field and a certain amount of dialysate volume is lost, thus concentrating the protein in the sample. Further, temperature control during centrifugation may present a problem.

1.4.3 Apparent binding of testosterone to SHBG

Numerous in vitro studies by a variety of methods have demonstrated that T binds to SHBG with high affinity (Mercier-Bodard et al, 1970; Murphy, 1968; Kato and Horton, 1968; Vermeulen, 1969; Ritzen et al, 1974). Nevertheless, the present estimates of the fraction of plasma testosterone bound to SHBG are open to question. This is mainly because of the methodological difficulties in making those determinations under physiological conditions. Early studies which have established the high

affinity binding of T to SHBG were done at low temperatures and usually utilized diluted plasma to eliminate the albumin effect. The binding parameters obtained at 4°C may be quite different from those at 37°C. Although results obtained under non-physiological conditions may give an index of binding, such an index may not reflect the true binding of a steroid to different plasma proteins in vivo. It should also be borne in mind that, even when obtained under "physiological" conditions, in vitro results only approximate the in vivo situation.

Whereas much attention has been directed towards developing experimental methods for measuring the unbound fraction of T because of its presumed physiological significance, only a limited number of studies (Vermeulen, 1977; Moll et al, 1981; Dunn et al, 1981; Södergard et al, 1982) have described the distribution of T into SHBG-bound and albumin-bound fractions. Each of these studies has one or more drawbacks which will be discussed in detail in section 4.1.4. Determination of the individual values for the fraction that is bound to SHBG and the fraction that is bound to albumin is especially important in the light of recent findings which indicate that albumin-bound steroid is available for tissue metabolism in vivo (Pardridge, 1981) and that the SHBG-bound steroid may directly participate in the intracellular steroid hormone action (Siiteri, 1982).

Although the values of percentage T bound to SHBG reported by various investigators (Vermeulen, 1977; Moll et al, 1981; Dunn et al, 1981; Södergard et al, 1982) vary widely, there is general agreement that SHBG is a significant binder of plasma T in vivo. However, a direct experimental approach for the determination of percentage of T bound to SHBG under

physiological conditions has not so far been described. All the methods discussed above depend on indirect estimations of SHBG-bound fraction from values of the binding affinity, binding capacity and plasma concentration of ligands. Early studies of Vermeulen and Verdonck (1968) and those of Forest et al (1971) have shown that T binding to SHBG in pregnancy was significantly increased over non-pregnant state. Although Forest et al (1971) have estimated the partition of T into SHBG-bound, albumin-bound and unbound fractions in maternal and cord plasma, they have used 1:5 diluted plasma and a sample:dialysate ratio of 1:10. No studies other than that of Dunn et al (1981) who used a computer simulation technique have described the plasma distribution of T into SHBG-bound, albumin-bound and unbound fractions in whole serum in pregnant women and newborn infants. In order to study the biological role of the different fractions of T in plasma it is important to determine the distribution of T into SHBG bound, albumin bound and unbound fractions under in vivo conditions.

1.4.4 Apparent binding of estradiol to SHBG

Several in vitro studies employing methods such as competition technique (Murphy, 1968), polyacrylamide gel electropheresis (Mercier-Bodard et al, 1970), steady state gel filtration (Fisher et al, 1974) and steady state polyacrylamide gel electrophoresis (Ritzer et al, 1974) have shown that estradiol can bind to the same binding site as testosterone on sex hormone-binding globulin. In these studies estradiol was shown to bind to SHBG to the extent of 10% to 65% of that of testosterone. On the other hand, the significance of SHBG in the binding of estradiol has recently been questioned. Vigersky et al (1979) have studied E_2 binding to SHBG at 37°C using three independent methods namely equilibrium dialysis, steady

state polyacrylamide gel electrophoresis and dissociation rate analysis. They reported that SHBG does not bind plasma E_2 at physiological temperature. In addition, several clinical conditions such as hyperthyroidism (Ruder et al, 1971), liver disease (Galvo-Teles et al, 1973) and hirsutism (Fisher et al, 1974) in which SHBG levels were altered were not always associated with changes in unbound estradiol or metabolic clearance rate of estradiol although unbound T and metabolic clearance rate of T were altered. These observations were interpreted to support the notion that SHBG is not an important binder of plasma estradiol (Vigersky et al, 1979; Wu, 1979).

None of the reports mentioned above which studied the binding of E_2 to SHBG were done under physiological conditions. The limitations of these studies included non-equilibrium conditions, low incubation temperatures, dilution of plasma or a high buffer/sample ratio in dialysis systems. In addition, there is very little information available on the plasma distribution of E_2 into SHBG-bound, albumin-bound and unbound fractions. Wu et al (1976) have studied the plasma distribution of E_2 in women during the menstrual cycle. Although they have used undiluted plasma for dialysis, the dialysate compartment contained only buffer and the buffer:sample ratio was 1:11.7. Moll et al (1981) have described the plasma distribution of E_2 at 37°C. However, the SHBG-bound and albumin-bound fractions of E_2 were calculated indirectly using the data on T binding and required large amounts of sample. Dunn et al (1981), using a computer simulation technique, and Södergard et al (1982), using a mathematical model, have also determined the plasma distribution of E_2. However, mathematical modeling and computer simulation will depend

critically on the number and nature of ligands included in the calculations and may not accurately reflect the plasma distribution in vivo.

In summary, data on binding of E_2 to SHBG in male and female plasma under physiological conditions are limited. In addition, with the single exception of the report of Dunn and coworkers (1981) there are virtually no studies of the plasma distribution of E_2 in the pregnant woman and the fetus. Since, in the pregnancy serum, the nature of a major part of the substances bound to SHBG is unknown and therefore cannot be included in the computer simulation program of Dunn et al (1981), this method is inadequate to describe the plasma distribution of E_2 in pregnancy. The ability to determine the distribution of E_2 in plasma into SHBG-bound, albumin-bound and unbound fractions would allow a more meaningful analysis of plasma E_2 concentrations in various physiological states.

The first series of experiments described in this thesis deals with studies on the significance of SHBG in the serum transport of T and E_2 in adult men, non-pregnant women, pregnant women and newborn infants using direct experimental approaches under conditions which closely approximate physiological ones.

1.5 Substances Bound to SHBG in Pregnancy Serum

The concentrations of SHBG are elevated during pregnancy. Rivarola et al (1968) were the first to report that SHBG levels are high in the pregnant woman. Since then it has been confirmed that the concentration of SHBG is increased more than 5-fold in pregnancy compared to the levels in the non-pregnant state (Forest et al, 1971; Anderson et al, 1976). A significant increase, during pregnancy, in the fraction of T and E_2 bound to SHBG with corresponding decrease in the albumin-bound

fraction and unbound fraction has been reported by Dunn et al (1981). A marked decrease in the percentage of unbound T during pregnancy has also been shown by Rivarola et al (1968), Forest et al (1971) and Bamman et al (1980). The significance of the increase in the concentration and the increased binding capacity of SHBG during pregnancy is poorly understood.

Quantitative and qualitative changes in the synthesis and metabolism of steroids are major characteristics of human pregnancy. Although the levels of estradiol show dramatic increases, concentrations of other more potent ligands of SHBG such as T, DHT, 5α-androstane-3β,17β-diol and androst-5-ene-3β,17β-diol rise only moderately or not at all during pregnancy. Early studies of Murphy (unpublished observations, 1971) have shown that the total concentration of substances bound to SHBG in pregnancy serum as determined by competitive binding to SHBG was much higher than could be accounted for by the levels of known sex steroids. This suggested that unknown substances may be bound to SHBG in pregnancy.

The data presented in Table 1-6 illustrate the above concept. In this table, a comparison of SHBG-bound material of non-pregnant and pregnant serum is shown. The plasma concentrations of the recognized ligands of SHBG and their ability to bind SHBG relative to that of T were obtained from the literature. Using these two parameters, the plasma concentration of each steroid in T equivalents (the amount of T required to cause the same displacement of radioactive tracer as the plasma concentration of steroid) was then calculated. The sum of these values will represent the total calculated SHBG-bindable activity in T equivalents. At the bottom of the table, the total measured SHBG-bound material in T equivalents is shown as determined by assaying the organic solvent extract of the serum in a competitive binding assay using SHBG as

TABLE 1-6. Comparison of SHBG bindable activity in the plasma of non-pregnant and pregnant women.

Steroids in plasma known to bind to SHBG	Relative binding (Murphy, 1968)	Plasma Concentration - ng/ml			
		Non-pregnant women		Pregnant women	
		Reported levels	T equivalents	Reported levels	T equivalents
Testosterone	100	0.43 (1)	0.43	1.2 (1)	1.2
Androstenedione	1.4	1.8 (1)	0.025	2.49 (1)	0.035
Dehydroepiandrosterone	2.5	5.0 (1)	0.125	3.6 (1)	0.09
Dihydrotestosterone	300	0.15 (2)	0.45	0.18 (3)	0.54
5-Androstene3β,17β-diol	130	0.68 (4)	0.85	0.65 (3)	0.85
5α-Androstane-3α,17β-diol	200	0.114 (5)	0.228	0.199 (5)	0.398
5α-Androstane-3β,17β-diol	250	0.515 (5)	1.28	0.704 (5)	1.76
Estradiol	60	0.08 (6)	0.05	16 (7)	9.6
Total calculated			3.4		14.5
Total measured			2.0 (8)		32.5 (8)

(1) Gurpide and Holinka, 1980
(2) Meikle et al., 1979
(3) Buster et al., 1979
(4) Rosenfield and Otto, 1972
(5) Habrioux et al., 1978
(6) Moll et al., 1981
(7) Tulchinsky et al., 1972
(8) Philip and Murphy, unpublished observations

the binding protein and testosterone as the standard. The data clearly show that while the total SHBG-bound material in the non-pregnant serum can be accounted for by the individual concentrations of recognized ligands of SHBG, these steroids account for only about half of the total SHBG-bound material measured in pregnancy serum.

There has been no systematic investigations of steroid binding to SHBG in pregnant women and there is virtually no information available as to the nature and properties of the unknown substances bound to SHBG in pregnancy. As mentioned in section 1.3.2.1, available evidence suggests that there is a positive relationship between the binding affinity of estrogenic and androgenic steroids to SHBG and their biological potency (Murphy, 1969). Thus, the "unknown" SHBG-bindable material in pregnancy serum, if bound to SHBG under in vivo conditions, may be biologically potent compounds. These compounds may be involved in fetal growth and differentiation or maintenance of pregnancy and/or parturition. In addition, the presence of these competing steroids may influence the unbound levels of classical sex steroids such as T and E_2. This aspect may be especially important in the fetal serum where SHBG levels have been shown to be only 1/20 of those of the mother (see section 1.3.4.3).

The objective of the second set of studies described in this thesis was to investigate the nature and properties of previously unrecognized ligands of SHBG in pregnancy.

CHAPTER II: MATERIALS AND METHODS

2.1 Subjects

Blood specimens were obtained from the following categories of subjects. All subjects included in the study were healthy volunteers.

(i) Adult men, 22-40 years of age who were students and employees of the Montreal General Hospital, Montreal.

(ii) Non-pregnant normally menstruating women aged 23-35 years who were also students and employees of the Montreal General Hospital, Montreal. None of the subjects selected were on oral contraceptive pills.

(iii) Pregnant women of 12-42 weeks gestation. All pregnant patients were healthy women attending the prenatal clinic of the Royal Victoria Hospital, Montreal or were admitted to the obstetrical unit of the Jewish General Hospital or Royal Victoria Hospital, Montreal.

(iv) Newborn infants delivered either vaginally or by Cesarean section at the Jewish General Hospital or the Montreal General Hospital. Placental cord blood of these infants was obtained at birth.

Placental samples of various gestational ages and liver tissue were obtained at therapeutic abortion, hysterotomy or at delivery from the Montreal General Hospital, the Notre Dame Hospital or the Jewish General Hospital. The samples were transported to the laboratory on ice and were analysed immediately or were stored at -20°C until analysis.

24 Hour urine samples were collected from pregnant women of 28-38 weeks gestation and were stored at -20°C until analysis.

Gestational age was estimated by the date of the last menstrual period or by ultrasonic determination. When there was a discrepancy as to the gestational age, the assessment by ultrasonic technique was relied

upon. The blood specimens, one from each subject, were refrigerated and centrifuged at 4°C. The sera were stored at -20°C until analysis. Samples which showed any hemolysis were excluded from the study since the percentage binding of steroids to SHBG in such samples was lower, presumably due to some degree of denaturation of SHBG. For maternal blood, the presence of any labour and, if present, the type of labour (spontaneous or induced) at the time of sample collection was carefully noted. For cord blood and placental samples, the type of delivery (vaginal with spontaneous labour or induced labour and Cesarean section with or without labour) was determined from the case room records or the case room staff.

2.2 Materials

2.2.1 Solvents, reagents, and chemicals

The following all glass-redistilled organic solvents (analytical grade) were obtained from Fisher Scientific Co., Montreal: ethyl acetate, methylene chloride, benzene, methanol, heptane, and diethyl ether.

Sodium monophosphate ($NaH_2PO_4 \cdot 2H_2O$) and sodium diphosphate ($Na_2HPO_4 \cdot 7H_2O$) were also obtained from Fisher Scientific Co.

Solvents and reagents purchased from other sources included: absolute alcohol (pharmacy, Montreal General Hospital); hexane (American Chemical Ltd., Montreal); Econofluor (New England Nuclear Corp., Boston, Mass).

Sephadex LH-20 and Dextran-T40 (Rheomacrodex) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Charcoal powder and Florisil were obtained from Fisher Scientific Co. Florisil was washed with 95% ethanol several times, the fines discarded and the remainder dried at room temperature for 24 hours. Gelatin was purchased from a local grocery store.

The cellulose casing used as dialysis membrane (Dialyzer tubing, 3.1 cm width) was obtained from Fisher Scientific Co., Montreal. The seamless cellulose tubing had a molecular weight cut-off point of 12,000 daltons and contained glycerin, water and 1% sulfur. The pore diameter of the membrane was 4.8 μm.

Enzyme preparations included: (1) Glucurase (β-D-glucuronide glucoronohydrolase, E.C. No. 3.2.1.31, prepared from bovine liver, 5000 Sigma units/ml of β-glucuronidase activity, devoid of sulfatase activity, Sigma Chemical Co., St. Louis ,Mo.; (2) Glusulase (a preparation of the intestinal juice of the snail Helix pomatia, approx. 10,000 units sulfatase and 90,000 units of β-D-glucuronidase activity per ml, Dupont Pharmaceuticals, Wilmington, Delaware).

2.2.2 Radioactive steroids

Radioactive steroids were obtained from New England Nuclear Corp., Boston, Mass. They were diluted with redistilled ethanol to a concentration of 50-200 μCi/ml and stored at -10°C.

compound	specific activity
Estrone, [2,4,6,7-^{3}H(N)]	87.8 Ci/mMol
Estradiol, [2,4,6,7-^{3}H(N)]	115.0 Ci/mMol
Testosterone, [1,2,6,7-^{3}H(N)]	93.9 Ci/mMol
4-Androstene-3,17-dione, [1,2,6,7-^{3}H(N)]	108.0 Ci/mMol
Dihydrotestosterone, [1,2-^{3}H(N)]	52 Ci/mMol
Progesterone, [1,2,6,7-^{3}H(N)]	112.0 Ci/mMol
5α-Dihydroprogesterone, [1,2-^{3}H(N)]	57.0 Ci/mMol
Corticosterone, [1,2,6,7-^{3}H(N)]	105.0 Ci/mMol
Cortisol, [1,2,6,7-^{3}H(N)]	114.5 Ci/mMol
Cholesterol, [1,2,6,7-^{3}H(N)]	82.7 Ci/mMol

4-Androstene-3,17-dione, [4-^{14}C]	52.0 mCi/mMol
Progesterone, [4-^{14}C]	57.2 mCi/mMol

The radiochemical purity of the radioactive steroids was checked by Sephadex LH-20 column chromatography using a suitable solvent system. The purity of radioactive testosterone and estradiol used in dialysis experiments was better than 98%. In all other experiments the purity of isotopes used was greater than 95%.

2.2.3 Unlabeled steroids

Steroid standards were obtained from either Sigma Chemical Co., St. Louis, Mo. or from Steraloids Inc., Wilton, N.H. All steroids were dissolved in redistilled ethanol and stored at -10°C. Unlabeled steroids found impure on Sephadex LH-20 chromatography were purified by repeated crystallizations in solvent systems benzene:hexane or acetone:water.

2.3 General Methods

2.3.1 Assay of radioactivity

Radioactivity was determined in a Philips liquid scintillation analyser (Philips Electronic Instruments, Montreal). The sample was placed in a 4 ml plastic vial. Econofluor, 2 ml, was added to dry samples or to 0.5 ml aqueous buffer (competitive binding assays). Although the latter gave a 2-phase system, the level of the aqueous phase was low and did not interfere with the counting. Since both standards and samples contained the same volume of aqueous buffer, quenching was similar for both. All samples were shaken thoroughly for complete extraction and mixing of the steroids into the Econofluor solution.

All samples were counted for a minimum of 10 minutes or till 10,000 counts were accumulated to minimize the counting error. The

background activity was determined by counting 1 mg of non-radioactive steroid dissolved in 2 ml of Econofluor under identical conditions.

In order to count two isotopes (tritium and carbon-14) present in the same vial, a dual label counting technique was employed. For dual label counting, carbon-14 cpm were present in sufficiently low amounts to avoid excess carbon-14 spill over to the tritium channel.

2.3.2 Extraction of samples

To a suitable aliquot of serum, urine or minced tissue, a small amount (4-8,000 cpm each) of ^{3}H-androstenedione and ^{3}H-testosterone were added as radioactive markers for the determination of the elution pattern and to calculate recoveries. The sample was then extracted 3 times with 4 volumes of ethylacetate and the combined extract was evaporated to dryness.

2.3.3 General procedure used for column chromatography

Sephadex LH-20 has been used almost exclusively for separation procedures in this thesis as it does not contribute any significant blank value to the competitive protein-binding assays (radiotransin and radioimmunoassays) which were used for the detection and quantitation of steroids. Although thin layer and paper chromatography can be used for effective separation of steroids, these procedures give rise to high blank values in assays employing binding proteins (Murphy, 1971). HPLC became available only at the end of the study.

Sephadex LH-20 is a dextran gel prepared by the hydroxypropylation of Sephadex G-25. It has both hydrophilic and lipophilic properties. The separation depends on molecular size, adsorption, partition, ion exclusion, retardation and other unknown

mechanisms (Information Booklet, Pharmacia Fine Chemical Co.).

2.4 Methods Used in the Study of the Significance of SHBG in the Transport of Estradiol and Testosterone

2.4.1 Equipment used for equilibrium dialysis

The microanalysis chambers (Model A, capacity 0.1 ml, No. 9035/408) were purchased from Chemical Rubber Co. (now known as Lab Apparatus Co.), Cleveland, Ohio or were made (capacity 0.1 or 2 ml) with identical material by the Medical Physics Department, Montreal General Hospital. Hamilton syringes with and without fixed needle of a capacity of 100 microliters (µl) were purchased from Canadian Laboratory Supplies Ltd., Montreal.

2.4.2 General procedure used for equilibrium dialysis

In equilibrium dialysis, two compartments are separated by a semipermeable membrane that retains the protein while allowing the passage of steroid. Dialysis is continued until equilibrium is achieved, i.e., until the concentration of free ligand is uniform throughout the system. The procedure involved adding a tracer amount of ^{3}H-E_2 or ^{3}H-T to either the sample or the dialysate side and allowing it to equilibrate between the two chambers. The recovery of the steroid was tested for each experiment by comparing the cpm added and the cpm recovered from both the chambers at the end of dialysis. The percentage of E_2 and T bound to SHBG and the percentage of E_2 and T unbound in whole serum were determined using equilibrium dialysis procedures at 37°C.

2.4.3 Determination of the percentage of estradiol and testosterone specifically bound to SHBG.

The 0.1 ml capacity microdialysis cells were prepared by inserting a piece of cellulose membrane (soaked in distilled water for

1 min and drained on a piece of gauze) between the two halves of the dialysis cell and tightening the bolts. Using a 100 µl Hamilton syringe, 0.1 ml of undiluted serum (sample) containing ^{3}H-E_2 or ^{3}H-T (60-70 pg or 30,000 cpm per ml of serum) was pipetted into one chamber. Into the other chamber, 0.1 ml of the same serum heated at 60°C for 1 hour (dialysate) was pipetted. So as not to break the cellulose membrane, a blunted needle was used. The chambers were then closed and the cell was ready for incubation. [The heating of serum at 60°C for 1 hour has been shown to inactivate SHBG while albumin binding remains unaltered (Westphal, 1971, p. 361). The completion of such heat inactivation was tested by comparing the percentage of ^{3}H-T specifically bound in the absence and presence of excess non-radioactive T in native and heated sera.]

The microdialysis cells prepared for incubation were placed inside two layers of plastic bags and were immersed in a gently shaking Dubnoff water bath at 37°C for 20 hours. [The time required for equilibration between the sample and the dialysate sides was determined by measuring the percentage of T bound to SHBG at different time intervals (½ hr to 72 hrs)]. The same speed of shaking was used for all dialysis experiments and a thermostat insured a constant temperature during incubations.

Dialyses were done in duplicates or triplicates in all cases. At the end of dialysis, the material from each side was transferred individually into narrow 2 ml tubes using a Hamilton syringe fitted with a flexible teflon needle. The small tubes were closed with parafilm in order to prevent evaporation. Equal aliquots (to insure same amount of quenching) from the sample and dialysate sides were taken in duplicates (e.g.: 25 µl each) for the determination of radioactivity. Aliquots were

assayed for radioactivity directly after the addition of 2 ml of Econofluor.

For calculations, it was assumed that SHBG and albumin are the only binding proteins present in the serum for E_2 and T. [T binding by CBG has been shown to be insignificant at physiological concentrations at 37°C (Burke and Anderson, 1972; Ritzen et al, 1974; Vermeulen, 1977)]. Since SHBG is present only on the side containing native serum and since the concentration of albumin was the same on both the native side and the heated side (Fig. 2-1a), the percentage of E_2 or percentage of T not bound to SHBG could be directly calculated as,

% SHBG unbound or (% albumin bound + % unbound)

$$= \frac{\text{cpm/ml in the heated serum}}{\text{cpm/ml in the native serum}} \times 100$$

% SHBG bound = 100 - % SHBG unbound

2.4.4 Determination of the percentage of unbound estradiol and testosterone

The procedure followed was essentially the same as that described above. One ml of undiluted native serum that contained ^3H-E_2 or ^3H-T (30,000 cpm or 60-70 pg per ml serum) was dialyzed against 1.0 ml of 2.8% (for late pregnancy serum) or 3% (for all other types of serum) dextran. [The concentration of dextran that is isosmotic with serum was determined by examining fluid shift across the membrane when serum was dialysed against a series of dextran concentrations. The lack of binding of dextran to T and E_2 was established by dialysing dextran solution containing ^3H-T or ^3H-E_2 against a buffer solution and determining the ratio of radioactivity on the dextran side to that on the buffer side. To study the effect of CBG on the value of % unbound T, dialyses were done in the

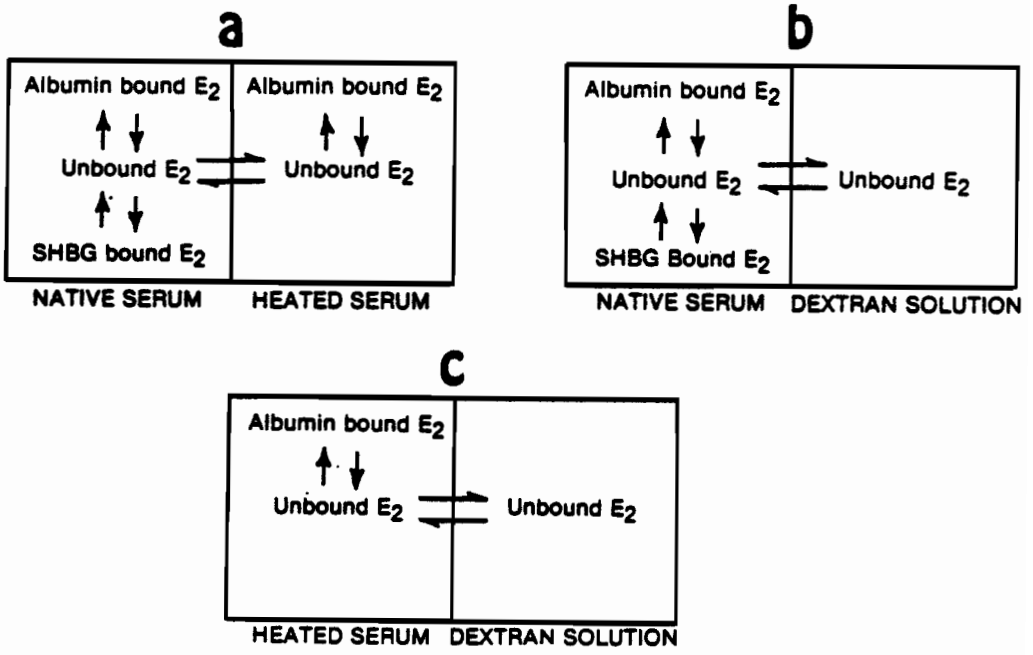


Figure 2-1. Schematic representation of the equilibrium dialysis procedures used for the determination of percentage estradiol bound to (A) SHBG, (B) whole serum and (C) albumin in the absence of SHBG. The same procedures were used to study the distribution of testosterone.

presence of 8 µg% cortisol in some cases.] The percentage of unbound E_2 or T unbound in whole serum was calculated as, (Fig. 2-1b)

$$\% \text{ unbound} = \frac{\text{cpm/ml in the dextran solution}}{\text{cpm/ml in the native serum}} \times 100$$

% protein bound = (% SHBG bound + % albumin bound)

= 100 - % unbound

2.4.5 Determination of the percentage of estradiol and testosterone bound to albumin

The percentage of E_2 and T bound to albumin in serum was calculated as follows:

% albumin bound = 100 - (% SHBG bound + % unbound)

2.4.6 Determination of the percentage of estradiol and testosterone bound to albumin in the absence of influence of SHBG

Percentage albumin bound in the absence of native SHBG was determined by dialyzing 0.1 ml of undiluted serum heated at 60°C for 1 hour, containing ^3H-E_2 or ^3H-T (30,000 cpm or 60-70 per ml serum) against 0.1 ml of isosmotic dextran solution (Fig. 2-1c).

Percentage albumin bound in the absence of unoccupied SHBG binding sites was determined by dialyzing 0.1 ml of undiluted native serum containing ^3H-E_2 or ^3H-T (30,000 cpm or 60-70 pg per ml serum) against 0.1 ml of isosmotic dextran solution in the presence of excess amounts of nonradioactive T.

2.4.7 Radioimmunoassay of estradiol

The preparation of serum samples for the assay was done as follows: aliquots of serum (0.5 ml in duplicate) were extracted twice with 5 volumes of diethyl ether. For sera of pregnant women in the third

trimester and of newborn infants, fractionation of the extract was carried out using Sephadex LH-20 chromatography. Five ml disposable pipettes were used as columns (18 × 0.6 cm). Serum extracts, to which known amounts (≃ 2000 cpm each) of ^{3}H-estrone (^{3}H-E_1) and ^{3}H-E_2 had been added, were applied to the column. The steroids were eluted using a solvent system of methylene chloride:methanol (98:2). One ml fractions were collected. An aliquot was counted from each fraction in order to determine the elution pattern of ^{3}H-E_1 and ^{3}H-E_2. ^{3}H-E_1 was eluted at 7-10 ml and ^{3}H-E_2 at 16-20 ml. The procedural loss during chromatography was accounted for on the basis of ^{3}H-E_2 lost during the procedure. The eluate fractions under ^{3}H-E_2 peak were combined and evaporated to dryness, redissolved in 1 ml of ethanol and duplicate aliquots (20 µl each) were assayed for E_2. The serum extracts of nonpregnant women and adult men were assayed directly after extraction.

The procedure used for the assay of E_2 was that used routinely in the laboratory, and monitored using World Health Organization standards for estradiol. To 10 ml polypropylene tubes (Fisher Scientific Co.) containing nonradioactive E_2 standard (20, 40, 80, 160, 320 pg) or unknown sample, 0.1 ml of 0.1M phosphate buffer containing ^{3}H-E_2 (22,500 cpm or 0.02 µci) and one ml of the antibody solution(diluted 1:250,000 in buffer) were added. The cross-reactivity of the antiserum (prepared according to Ferin et al, 1968) relative to that of estradiol was 77% for estrone and 2.6% for estriol; all other steroids tested were less than 0.1%. The tubes were vortexed well and were pre-incubated at 45°C for 5 min, followed by incubation at 4°C for 18 hrs or more. At the end of incubation, 1.0 ml of dextran-charcoal suspension was added. Tubes were allowed to stand for 5 min, centrifuged at 2000 rpm. and 0.5 ml of the supernatant was counted.

2.4.8 Radioimmunoassay of testosterone

Serum T levels were measured by the Endocrine Laboratory, Montreal General Hospital (courtesy, Dr. G. Klein) using an RIA method according to the instructions of the manufacturer of the kit (Pantex, Santa Monica, California). The cross-reactivity of the testosterone antibody was: T - 100%; dihydrotestosterone - 6.9%, androsterone - 0.5% and all other steroids tested - less than 0.1%. The radioactive tracer used was ^{125}I-T and the antiserum was prepared in the rabbit.

2.5 Methods Used in the Study of the Characterization of Previously Unrecognized Ligands of SHBG in Pregnancy

2.5.1 Preliminary studies

The initial fractionation studies were done to reproduce the previous observations (Murphy, unpublished observations) in our laboratory which showed that pregnancy serum contained large amounts of unidentified nonpolar material capable of binding to SHBG. A similar procedure (shown in Figure 2-2) which involved Sephadex LH-20 chromatography and SHBG assay was therefore used in the initial studies for the detection of the unknown SHBG-bound material. Details of the SHBG assay are described later in this chapter.

2.5.2 Detailed chromatographic analysis

The general scheme used is shown in Fig. 2-3.

2.5.2.1 Preparation and initial purification of the sample. The samples were prepared for detailed chromatography by extraction and an initial purification on a short Sephadex LH-20 column (10 × 0.9 cm) using a solvent system of methylene chloride:heptane:methanol (50:50:1). A maximum of 7 samples were processed in each batch. Four ml fractions were collected at a flow rate of 1 ml/3 min. Ten fractions or 40 ml (or until

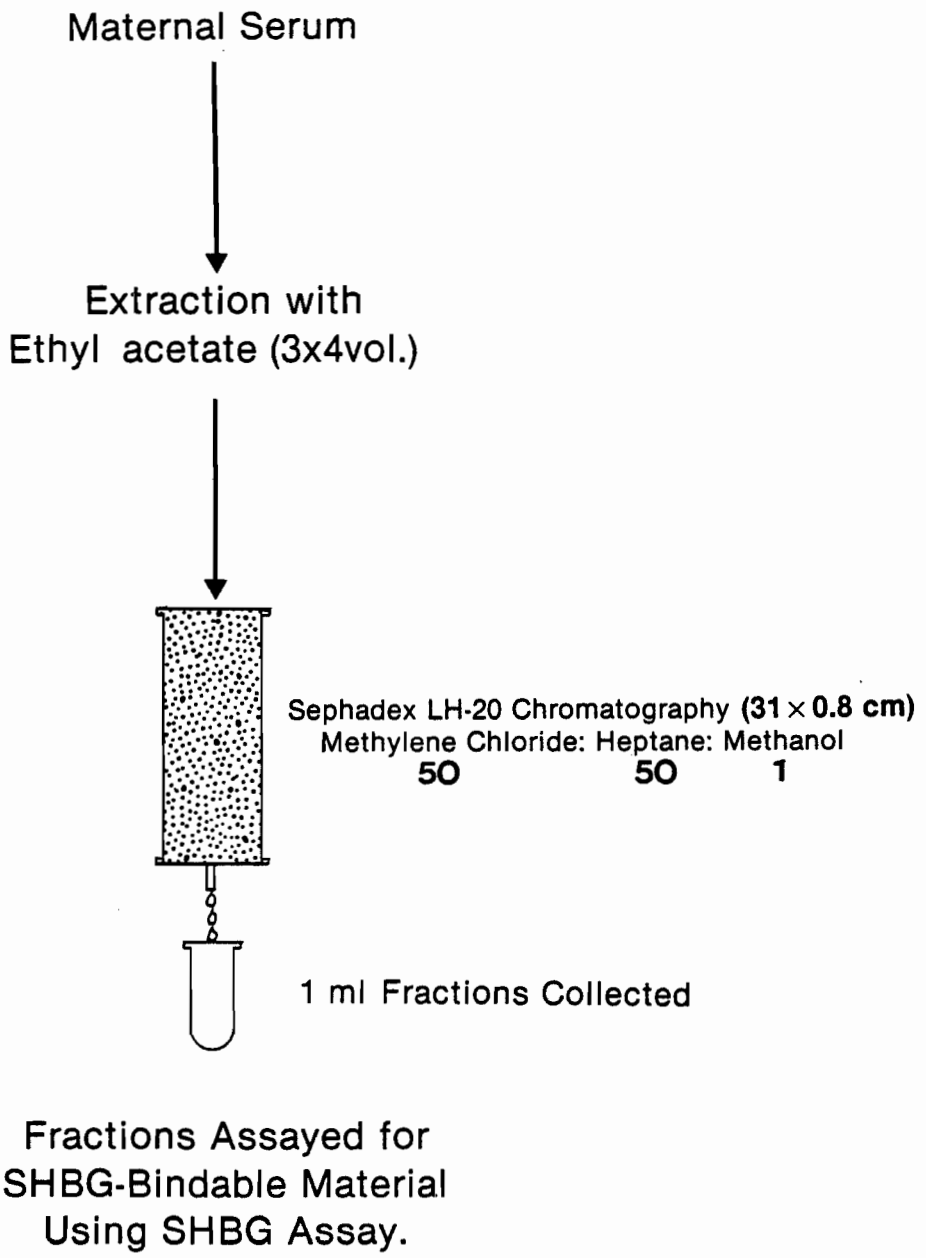


Figure 2-2. Procedure used for preliminary studies of SHBG-bindable material in maternal serum.

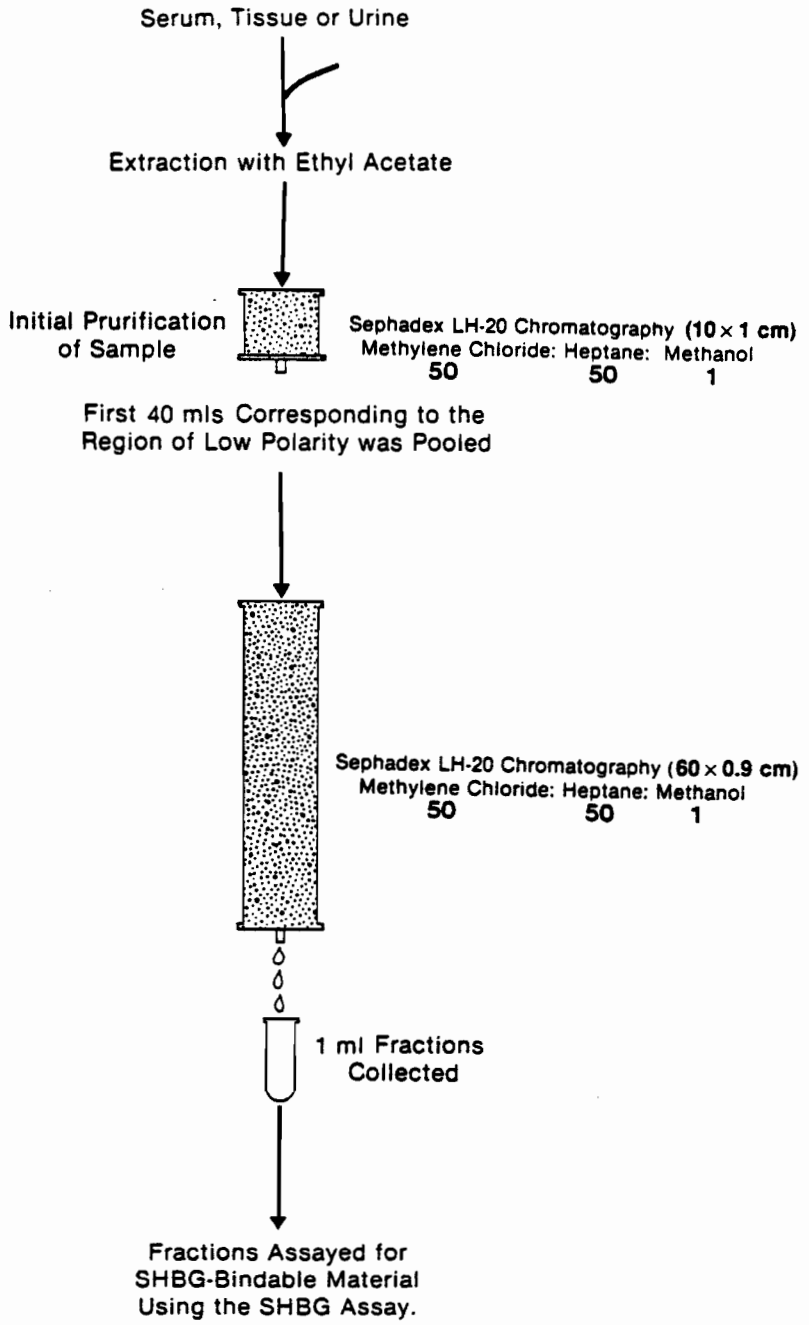


Figure 2-3. Procedure used for more detailed chromatography to study the nature of unknown SHBG-bindable material.

the ^{3}H-testosterone peak was eluted) were collected. More polar steroids were retained in the column and were not eluted at the above elution volume. An aliquot from each fraction was counted for radioactivity to locate the tracer peaks. The fractions were pooled, evaporated to dryness and redissolved in a known volume of the column solvent. The recovery of the radioactive markers up to this stage was determined by counting an aliquot. The pooled fractions were dissolved in a minimum volume in a centrifuge tube and were then ready for more detailed chromatography.

2.5.2.2 Procedure used for detailed chromatography. More detailed chromatography of samples was done on a series of seven Sephadex LH-20 columns of 60 × 0.9 cm (Glenco Scientific, Inc., Houston, Texas) using a solvent system of methylene chloride: heptane: ethanol (50:50:1). The solvent was picked up by a Teflon capillary tubing using a polystaltic infusion pump (Buchler Instruments Inc., Fort Lea, N.J.) on to the column and the packing of the column was achieved by an upward flow of the solvent.

The sample was applied to the column and the pump was run at a constant rate of 1 ml/5 min. The first 10 ml was collected as one fraction, evaporated and checked for blank value. One ml fractions were collected up to 50 ml. The columns were washed with an additional 100 ml column solvent and were ready to be used again. Suitable aliquots were taken from each fraction for counting to locate the radioactive steroid peaks and for the detection and quantitation of substances bound to SHBG using a radiotransinassay for testosterone (SHBG assay) which is described below.

2.5.3 Radiotransinassay for testosterone: SHBG assay

The term transin is used here to denote indigenous extracellular

binding proteins such as SHBG and CBG as defined by Murphy (1975b).

For the SHBG assay, late pregnancy serum SHBG was used as the binding protein and non-radioactive testosterone was used as the standard. The SHBG bindable activity in the sample was read off from the standard curve in terms of testosterone equivalents.

2.5.3.1 Reagents. A phosphate-gelatin buffer was prepared by dissolving 0.5 mg/ml of gelatin in 0.2 M phosphate buffer (pH 6.5). The dextran-charcoal suspension was prepared by mixing 0.125 g Norit A charcoal and 12.5 mg dextran in 100 ml of 0.2M phosphate buffer (pH 6.5).

For the preparation of protein-tracer solution, 0.1 ml of late pregnancy serum was "stripped" as follows: the serum was diluted with 0.5 ml of distilled water, 10 mg of charcoal was added and vortexed for 1 min. The tube was centrifuged twice and the supernatant was transferred into a flask containing ^{3}H-testosterone (12,000 cpm or 3 µci). The volume was made up to 10 ml with 0.2 M phosphate buffer. This solution was freshly made up immediately before each assay. In the protein-tracer solution, albumin binding was minimal due to the high dilution of the late pregnancy serum.

2.5.3.2 Procedure. To tubes (disposable borosilicate culture tubes, 16 × 100 mm; Fisher Scientific Co.) containing increasing amounts of nonradioactive T standard (50, 100, 200, 400, 800 and 1200 pg) or the unknown samples, 0.1 ml of the protein-tracer solution was added. The tubes were vortexed and pre-incubated at 45°C for 5 min. They were then incubated in a refrigerated water bath at 4°C for 30 min. At the end of incubation, 1.0 ml of the dextran-charcoal suspension was added. The tubes were allowed to stand for 5 min, mixed on an automatic shaker for 1 min and centrifuged at 2000 rpm for 5 min. The tubes were placed back in

the water bath at 4°C and 0.5 ml of the supernatant was pipetted into 5 ml counting vials. 2 ml of Econofluor was added, mixed well and counted for 10 min or a preset count of 10,000. All incubations were done in duplicates. The total counts incubated were determined by adding 0.1 ml of the protein-tracer solution to 1.0 ml of 0.2 M phosphate buffer and counting 0.5 ml from it as usual.

Since the time of contact with the adsorbent was critical, care was taken to keep this as uniform as possible throughout each assay. One set of standards (in duplicates) were done for every 24 sample tubes.

2.5.4 Radiotransinassay for progesterone: PBG assay

The procedure for this assay has been described by Tan and Murphy (1974). In this assay, pregnant guinea pig serum (obtained from the animal colony of Mc Intyre Medical Sciences building, McGill University) was used as the binding protein and ^{3}H-progesterone as the radioactive tracer. The pregnant guinea pig serum has been shown to contain a distinct protein in very high concentrations which has a high binding affinity for progesterone. The specificity of this protein has been shown to be: progesterone - 100, cortisol - 0.5, testosterone - 0.5, estradiol - 0.5 (Tan and Murphy, 1974). At the very high dilution of guinea pig serum used in the assay, there was no detectable binding of cortisol and it was assumed that the effect due to CBG and albumin were negligible (Tan and Murphy, 1974).

Briefly, the procedure involved the following. One ml of the protein-tracer solution (6.5 µCi ^{3}H-progesterone, 10 µl of serum from a pregnant guinea pig of 50-55 days gestation, 10 ml of 0.75 M phosphate buffer of pH 7.5, 45 mg gelatin and water to 100 ml) was added to tubes containing increasing concentrations of non-radioactive progesterone

standard (0, 100, 200, 400, 800, 1200 and 2000 pg) or the test substance. It was then pre-incubated at 45 °C for 5 min. and the incubation was continued at 4°C for 80 min. At the end of incubation, 60 mg of Florisil was added and shaken for 1 min on an automatic shaker. It was then centrifuged at 4°C for 5 min at 2000 rpm. The supernatant, 0.5 ml, was counted for radioactivity.

2.5.5 Radiotransinassay for cortisol: human CBG assay

In this assay, human late pregnancy serum CBG was used as the binding protein. The specificity of human CBG in this assay has been shown (Murphy, 1967) to be: cortisol: 100, corticosterone: 61, progesterone: 20 and testosterone: 4.

Briefly the procedure was as follows: one ml of protein-tracer solution (5 μCi of ^{3}H-cortisol, 10 ml of 0.75 M phosphate buffer pH 7.5, 1 ml of human late pregnancy serum, 45 mg gelatin and water to 100 ml), was added to tubes containing increasing concentrations of non-radioactive cortisol standard (0, 100, 200, 400, 800, 1200 and 2000 pg) or the test substance. The tubes were incubated and processed in the same manner as for the PBG assay.

2.5.6 Radiotransinassay for 2-methoxyestrone: 2-ME1 assay

The procedure used for the assay was the same as that for SHBG assay except that 2-methoxyestrone rather than tesosterone was used as the nonradioactive standard. The standard curve employed 25, 50, 100, 200, 400, and 800 pg of nonradioactive 2-methoxyestrone dissolved in ethanol containing 1mg% ascorbic acid.

2.5.7 Radioimmunoassay for progesterone: RIA for progesterone

In this assay an antibody to progesterone raised in the rabbit in our laboratory (courtesy, Dr. B.E.P. Murphy) was used. The specificity of

this antibody has been shown in our laboratory to be: progesterone: 100, 5α-dihydroprogesterone: 7, androstenedione: 0.1, corticosterone: 4.6 and deoxycorticosterone: 6. To tubes containing increasing concentrations of non-radioactive progesterone (0, 100, 200, 400, 800, and 1600 pg) or test substance, 0.1 ml of protein-tracer solution was added. The protein-tracer solution consisted of 2 μCi ^{3}H-progesterone, 1 ml 0.75 M phosphate buffer pH 7.5, 10 μl antiserum diluted 1:5, 4.5 mg gelatin and water to 10 ml. The tubes were mixed well and incubated at 37°C for 1 hr. They were then transferred to a waterbath at 4°C and the incubation was continued for another 30 min. At the end of incubation, 1 ml of dextran coated charcoal solution (125 mg Norit A charcoal and 12.5 mg dextran in 100 ml of phosphate buffer) was added. The tubes were left to stand in the waterbath at 4°C for 4 min. They were shaken on an automatic shaker for 1 min and centrifuged immediately at 4°C for 5 min at 2000 rpm. The supernatant, 0.5 ml, was counted.

2.5.8 Comparison of the elution patterns and the relative binding affinities of unknown peaks with those of authentic steroid standards

Tentative identification of the unknown peaks eluted in the nonpolar region was attempted based on studies of the elution pattern of authentic steroid standards, the cross-reactivity of these standards in various binding (radiotransin and radioimmuno) assays and the plasma levels of these steroids when known. (As mentioned earlier the term transin is used here to denote indigenous extracellular binding proteins such as CBG and SHBG as defined by Murphy, 1975b).

2.5.8.1 Elution pattern. If the elution pattern of an authentic steroid standard on a detailed Sephadex LH-20 chromatogram was

the same as that of one of the unknown peaks, then that steroid was considered as a possible candidate for that unknown peak. The binding properties of that steroid to various proteins were then examined and compared to those of the unknown peak.

2.5.8.2 Relative binding affinities. The principle involved in the method of comparing the relative binding affinities to various binding proteins as a means of verifying the identity of ligands has been described by Murphy (1973).

The cross-reactivities of authentic steroid standards were determined in one or more radiotransin (immuno) assays described above and compared with the values (equivalents) obtained for the unknown peak in those assays.

The relative binding or cross-reactivity of each of the steroid standards was estimated from the standard curve obtained by plotting 'cpm of bound radioactive tracer' vs 'concentration of the nonradioactive standard'. The values were expressed in terms of the nonradioactive standard used. The percentage cross-reactivity was calculated as:

$$\frac{\text{mass of nonradioactive standard required to displace 50\% of bound } {}^{3}\text{H-tracer}}{\text{mass of test steroid required to displace 50\% of bound } {}^{3}\text{H-tracer}} \times 100$$

2.5.9 Procedure used to test the binding of unknown SHBG-bindable material under physiological conditions

Since the detection of SHBG-bindable material using the SHBG assay is done under nonphysiological conditions (using a highly diluted

serum at 4°C), it was important to test whether the unknown SHBG bindable material was bound to SHBG under physiological conditions (i.e. using undiluted serum at 37°C). The following dialysis procedure was used for this purpose. (The scheme of procedure is shown in Fig. 2-4).

Four ml of late pregnancy serum was dialyzed at 37 C for 20 hours against the same serum heated at 60 C for 1 hour. The dialysis tubing used was 14 cm long and 3.1 cm diameter (Fisher Scientific Co.). The heated serum was placed inside the dialysis tubing. The tubing was tied twice on both ends before placing it in a 10 ml glass vial containing the native serum. At the end of dialysis, the vial was taken out and the serum was withdrawn from the tubing and the vial individually into centrifuge tubes. Serum from each side was extracted with ethyl acetate and the extracts were analysed by detailed chromatography and each fraction was assayed for SHBG bindable activity using the SHBG assay.

2.5.10 Saponification studies

In order to test whether the unknown SHBG bindable peak eluting as peak 2 in the region of low polarity on the Sephadex LH-20 column could be accounted for by fatty acid esters of more polar steroids, saponification of the unknown SHBG-bindable material was attempted using two different procedures, namely digestion with saturated potassium carbonate solution (Janocko and Hochberg, 1983) and digestion with ethanolic potassium hydroxide (Ichikawa et al., 1971).

To an aliquot of the ethyl acetate extract of pooled late pregnancy serum, 1 ml of saturated potassium carbonate solution was added and the tube was mixed thoroughly and incubated at room temperature for 18 hrs. To another aliquot of the same extract, 1 ml of 5% KOH in 95% ethanol was added and the tube was mixed thoroughly and incubated at 60°C for 1 hr.

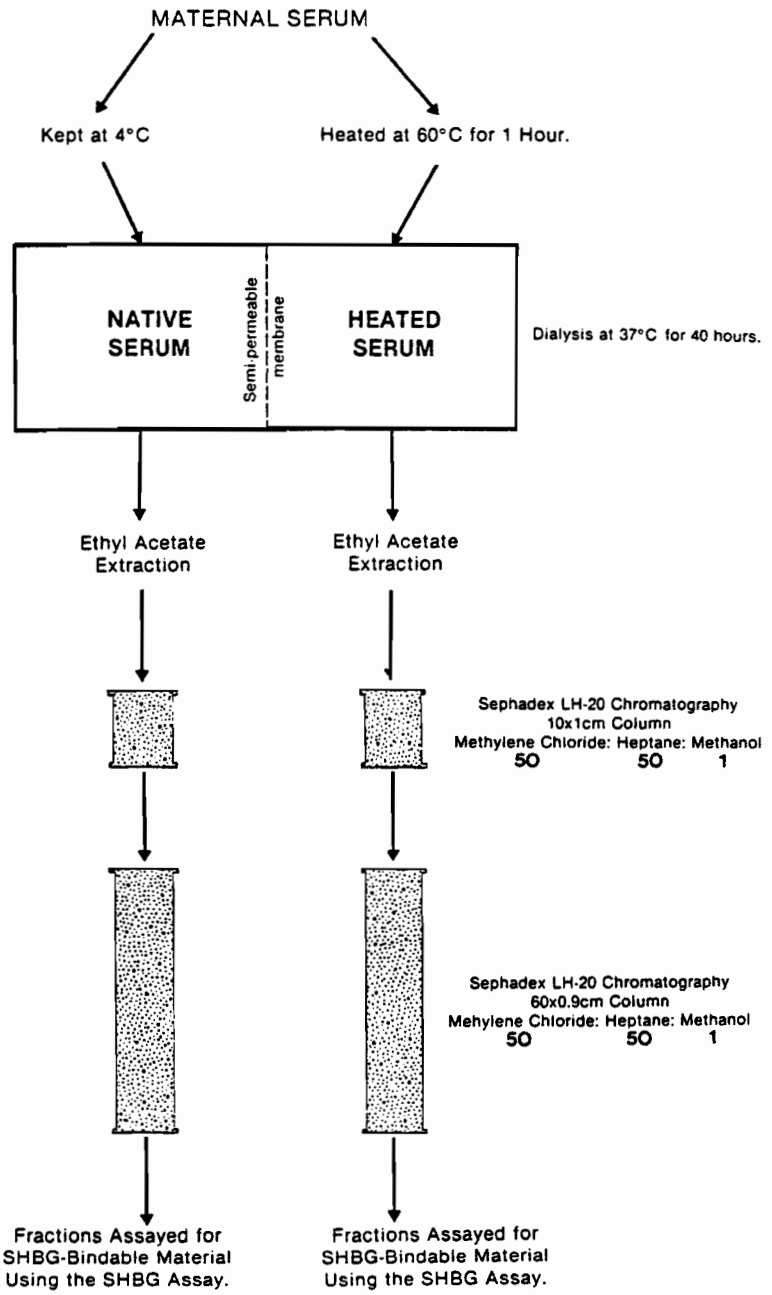


Figure 2-4. Procedure used to determine whether the unknown SHBG-bindable material is bound to SHBG under physiological conditions. Undiluted maternal serum was dialyzed at 37°C for 40 hours against the same serum heated at 60°C for 1 hour. At the end of dialysis, both sides were extracted, fractionated (1ml/fraction) and each fraction was measured using SHBG assay.

At the end of incubation, the pH was brought back to 7.0 and 8000 cpm of ^{3}H-androstenedione was added. The neutral solutions were extracted with ethyl acetate and the extracts were evaporated to dryness. An aliquot of the original extract not subjected to any digestion procedure served as the control. All were then analysed by detailed chromatography and each fraction was assayed for SHBG- bindable activity using the SHBG assay.

2.5.11 Urinary excretion pattern of SHBG-bound material

2.5.11.1 Unconjugated fraction of urine. A twentyfour hour urine sample collected from a pregnant woman of 33 weeks gestation with twin pregnancy (both fetuses female) was used. An aliquot of 25 ml from a total volume of 1400 ml was taken and extracted with ethyl acetate after the addition of 12,000 cpm of ^{3}H-androstenedione. The aqueous fraction containing the conjugated steroids was kept frozen at -20 C until analyzed. The ethyl acetate extract containing the unconjugated steroids was evaporated to dryness. The residue was fractionated on Sephadex LH-20 and each fraction was assayed for material bound to SHBG using the SHBG assay.

2.5.11.2 Glucuronide fraction of urine. The aqueous fraction of the urine containing the conjugated steroids was hydrolyzed as follows: The pH of the urine was adjusted to 5.0 with acetic acid. Acetate buffer, 1.0 M, was added in amounts of 0.2 ml/ml urine. The Glucurase (0.2 ml/ml urine) was then added, mixed, and incubated in a shaking bath at 37°C for 24 hr. The incubate was extracted with ethyl acetate after the addition of ^{3}H-androstenedione as marker. The aqueous fraction was kept frozen at -20° C until analysed. The ethyl acetate extract was fractionated on Sephadex LH-20 and each fraction was assayed for SHBG-bound material as usual.

2.5.11.3 Sulfate fraction of urine. The aqueous fraction frozen after the removal of the unconjugated steroids and the glucuronide conjugates of steroids was hydrolyzed for 24 hr at 37°C using Glusulase at pH 5.2 and a final concentration of 0.1M acetate buffer. The incubate was extracted with ethyl acetate after adding ^{3}H-androstenedione as marker, fractionated on Sephadex LH-20 and each fraction was assayed for SHBG-bound material as usual.

2.5.12 Analysis of the unknown SHBG-bound material by gas chromatography-mass spectrometry(GC-MS)

Preliminary experiments showed that the levels of peak 2 and peak 3 in the conjugated fraction of urine were sufficiently high to use urine as a source for further purification of those peaks for analysis by GC-MS. In addition it was assumed that since urine contains lesser amounts of fatty material than the maternal serum or placenta the purification procedure might be simpler. Attempts to purify peak 2 prepared from serum and placenta were not successful due to heavy contamination with lipid.

2.5.12.1 Sample collection, digestion and extraction. Twenty four hour urine samples were collected from 4 different pregnant women of 28-38 weeks gestation. The urine was kept frozen until digestion. One liter of urine was taken from each of the four collections and the pH was adjusted to 5.2 with 25% acetic acid. To each liter of urine 45 ml of 2 M acetate buffer of pH 5.2, 40 ml of Glusulase (see section 2.5.12.3) and a few drops of chloroform were added. The flasks were mixed well and incubated at 38°C for 50 hours. To the resulting digested urine samples containing the unconjugated, glucuronide and sulfated fractions, 2×10^{6} cpm of ^{3}H-estrone was added. The urine was then allowed to settle and extracted as follows: the clear urine was extracted using reversed phase

C_{18} cartridges (9 mm long x 10 mm internal diameter) (Sep-pak, Waters Associates Inc., Mississauga, Ontario) as described by Heikkinen et al (1981). The urine was buffered with 100 ml of 1.5 M acetate buffer, pH 3.0. The Sep-pak columns were primed by washing the columns with 10 ml water, 5 ml methanol and then 10 ml water. Ten such columns were set up. Glass syringes of 50 ml capacity served as reservoirs. Seventy ml of urine was passed through each column. The column was then washed with 25-30 ml of distilled water and the steroids eluted with 25 ml of methanol at a flow rate of 1 ml/min. An aliquot from the urine wash, water wash and methanol eluate was counted to check the ^{3}H-estrone recovery. The methanol eluates containing more than 90% of the added ^{3}H-estrone counts were kept frozen.

The cloudy urine was extracted with two volumes of ethyl acetate. An aliquot from the ethyl acetate extract was counted for ^{3}H-estrone recovery. The methanol eluates and the ethyl acetate extracts were pooled and evaporated to dryness using a rotor evaporator.

2.5.12.2 Detailed chromatography on Sephadex LH-20 column.

To the pooled urine extract, 1×10^{6} cpm of ^{3}H-androstene-3,17-dione was added and the extract was purified on Sephadex LH-20 columns as described before (section 2.5.2). Fractions corresponding to each of the peaks (peak 1a, peak 1b, peak 2 and peak 3) were then subjected to high pressure liquid chromatography.

2.5.12.3 High performance liquid chromatography (HPLC). The column used (Econosphere silica, 5 micron particle size, 25 cm length and 4.6 mm internal diameter) was purchased from Alltech Associates Inc., Deerfield, IL. The remaining components of the HPLC system were designed by Gilson Medical Electronics (purchased from Mandel Scientific Co.,

Rockwood, Ontario). The set-up consisted of two model 302 single piston pumps (pump A and pump B), model 811 dynamic mixer, model 7125 Rheodyne injection valve, model 111-HPLC detector (UV detector with two selectable wavelengths 254/280 nm), a model 202 fraction collector and an Apple II plus gradient controller. The chart recorder (Fisher Recordall, series 5000) was obtained from Fisher Scientific Co.

The solvents were HPLC grade and were filtered and de-aerated before use. All analyses were performed at room temperature with a flow rate of 1.0 ml/min and at a detection wavelength of 280 nanometers. The column pressure varied between 1000 to 1800 psi. For injection, standards or samples were dissolved in 35 µl of methylene chloride. 25 µl from this was used for injection using a 25 µl capacity Hamilton syringe (Fisher Scientific Co.). To the samples, approximately 6000 cpm each of ^{3}H-5α pregnane-3,20-dione and ^{3}H-progesterone were added before injection as radioactive markers.

For the separation of steroids, a gradient elution technique was used. The solvent system consisted of A = methylene chloride which was pumped by pump 1 and B = hexane:ethanol (90:10) which was pumped by pump 2. The system was set up to use 98% A and 2% B during the first 10 minutes. During the next 50 minutes, a solvent gradient of 2% B to 30% B (98% A to 70% A) was employed. 1.0 ml (1 minute) fractions were collected and an aliquot from each fraction was assayed to detect the SHBG-bound material. Fractions corresponding to each SHBG-bindable peak obtained by the HPLC of peak 1a, peak 1b, peak 2 and peak 3 were subjected to gas chromatography-mass spectrometry.

2.5.12.4 Gas chromatography-mass spectrometry The GC-MS analysis of all the peaks obtained by the HPLC of peak 1a, peak 1b, peak 2

and peak 3 of pregnancy urine was done by Dr. P.V. Fennessey, University of Colorado Health Sciences Center, Colorado. The procedure used was that described by Pike et al (1984). The samples were converted to their methyloxime, trimethylsilyl (TMS) ether derivatives as follows: to the dry sample extract, 100 µl of 2% methoxyamine-HCl in pyridine were added and incubated at 60°C overnight. After the removal of pyridine and the residual moisture, TMS derivatives were formed by the addition of 100 µl of Tri-Sil/TBT reagent (Applied Sciences Labs) and allowing the reaction to continue for 2 hours at 100°C. The derivatized sample, purified on a Lipidex-5000 column, was diluted to 100 µl and 1 to 1.5 µl was injected. Four hydrocarbon standards (C-24, 28, 32 and 36) were added to each sample to help determine the methylene unit value of each peak.

The analyses were performed on a Hewlett-Packard 7620A gas chromatograph fitted with a modified Van den Berg injector. The GC column consisted of 30 m × 0.2 mm, film thickness 0.25 µm, DBI fused silica open tubular column (J and W Scientific, Cordova, CA). The velocity of the carrier gas (helium) was 40 cm/sec. The injection block and transfer line temperature were set at 300°C. This was interfaced to a vacuum generator MM-16 low resolution magnetic sector instrument. The ionizing energy was 70 eV and the ion source temperature was 200°C for 4 min, then increased to a final temperature of 300°C at a rate of 4°C/min.

CHAPTER III: RESULTS

3.1 Study of the Significance of SHBG in the Transport of Testosterone and Estradiol

3.1.1 Introduction

Since the study was originally undertaken to determine the significance of steroid binding to SHBG during pregnancy, most of the initial experiments to test the validity of the dialysis method were done using pregnancy serum.

3.1.2 Recovery of radioactivity

To test whether ^{3}H-testosterone or ^{3}H-estradiol was adsorbed to the dialysis membrane or to the chambers during dialysis, aliquots from both the sample and the dialysate sides were counted at the end of dialysis and the recovery of ^{3}H-T and ^{3}H-E_2 calculated. The results obtained in individual dialysis experiments using the same pool of pregnancy serum to which a known amount of ^{3}H-T or ^{3}H-E_2 was added are given in Table 3-1. In these experiments 0.1 ml of native serum was dialysed at 37°C for 20 hrs against 0.1 ml of the same serum heated at 60°C for 1 hr. The data obtained suggest that the recovery value at the end of dialysis closely approximated 100% and that the loss due to adsorption was insignificant.

3.1.3 Evidence for heat denaturation of SHBG

The heating of serum at 60°C for 1 hour has been shown to inactivate SHBG while albumin remains unaltered (Steeno et al, 1968; Westphal, 1971). Completion of such heat inactivation of SHBG was checked by the failure to decrease the percentage of ^{3}H-T specifically bound to serum by the addition of excess non-radioactive testosterone. These

TABLE 3-1. Recovery of the tracer amounts of ^{3}H-testosterone and ^{3}H-estradiol added to dialysis cells when 0.1 ml of native serum was dialyzed against 0.1 ml of the same serum heated at 60°C.

Testosterone			Estradiol		
Total counts added to the cell, cpm	Recovery in individual dialysis experiments		Total counts added to the cell, cpm	Recovery in individual dialysis experiments	
	cpm	%		cpm	%
	6208	104.6		5400	101.9
	6332	106.8		5148	97.1
5930	5796	97.7	5300	5320	100.4
	5592	94.3		5440	102.6
				5260	99.2
Mean ± SD		100.9 ± 5.8			100.2 ± 2.2

results are shown in Figure 3-1. Addition of 200 ng (2500 times the concentration of ^{3}H-T) of non-radioactive T resulted in the displacement of ^{3}H-T bound in unaltered late pregnancy serum but not in the same serum heated at 60°C for 1 hour. When 1 mg of non-radioactive T was added, a slight displacement of ^{3}H-T bound in heated serum was observed which was probably due to displacement from albumin binding sites. These results suggest that heating the serum at 60°C for 1 hour denatured SHBG (high specificity, low capacity binding sites) while albumin (low specificity, high capacity) remained unaltered.

3.1.4 Time course of dialysis

Time required for equilibration between the sample and the dialysate sides was estimated by measuring the percentage of T bound to SHBG in late pregnancy serum at different time intervals (i.e. ½ hour to 72 hours). Results shown in Figure 3-2 suggest that equilibrium was reached by 20 hours and that no further change in percentage T bound to SHBG occurred up to 72 hours. The data further indicate that the SHBG binding sites remained intact during the course of dialysis.

3.1.5 Effect of changing sample:dialysate ratio from 1:1 to 9:1

During dialysis, the steroid concentration in the sample compartment changes as the unbound steroid passes across the membrane. In many previous studies, the ratio of sample:dialysate was 1:10. This must have caused the steroid concentration in the sample compartment at the end of dialysis to be quite different from that of the original sample. Although a ratio of sample:dialysate of 1:1 was used in the present study, it was of interest to see whether further decrease in the proportion of

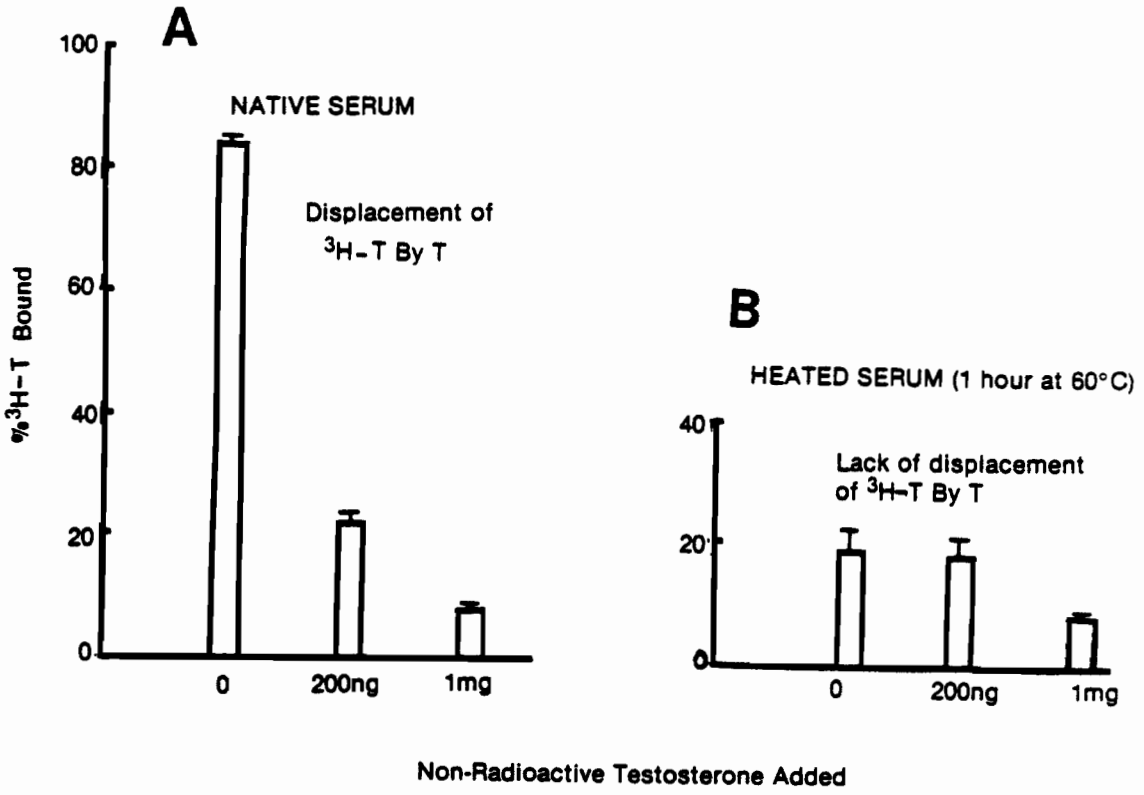


Figure 3-1. Evidence for heat denaturation of SHBG. Native serum (A) and the same serum heated at 60°C for 1 hour (B) were incubated with ^{3}H-testosterone (^{3}H-T) alone or with ^{3}H-T in the presence of excess non-radioactive testosterone (200 ng or 1 mg) at 4°C for 30 min. Percentage of ^{3}H-T bound was determined and plotted against the concentration of non-radioactive testosterone added. Each value is the mean±SD of 4 determinations.

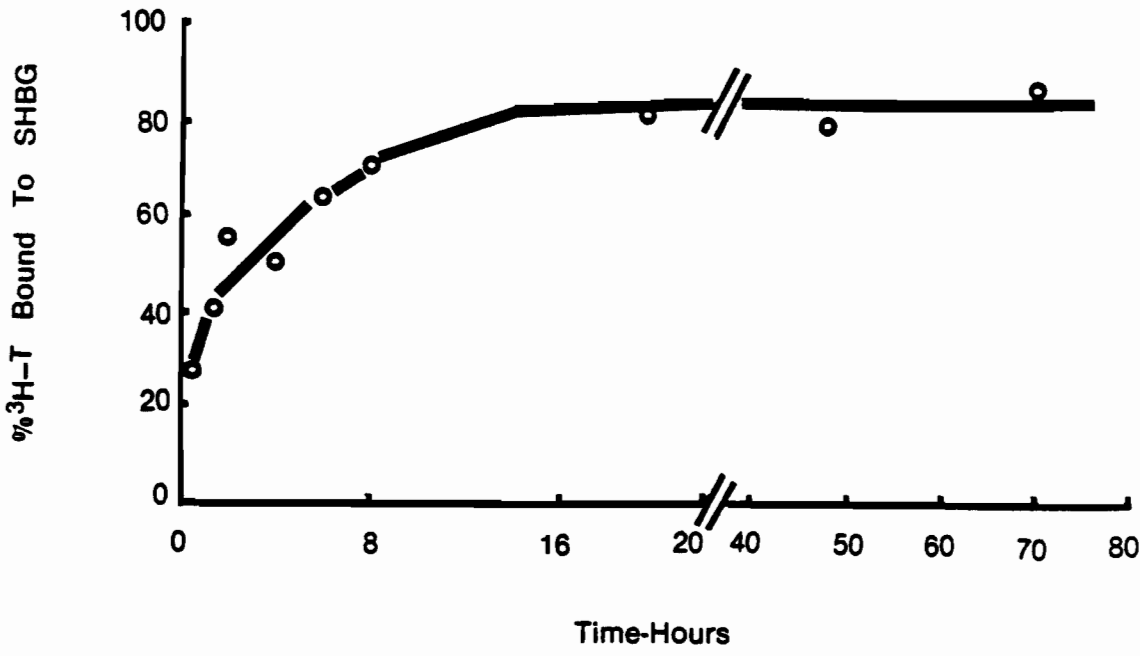


Figure 3-2. Time course of dialysis. Sera (0.1 ml, undiluted) of pregnant women in third trimester was dialyzed against the same sera (0.1 ml, undiluted) heated at 60°C for 1 hour for different periods of time using ^{3}H-testosterone as the tracer. Percentage of ^{3}H-testosterone bound to SHBG was calculated as described in text. Each point represents the mean of duplicate or triplicate determinations.

dialysate volume would alter the values for percentage of steroid bound to SHBG. When percentage of T specifically bound to SHBG in late pregnancy serum was determined using a ratio of sample:dialysate of 9:1, the values obtained were not significantly different from those obtained using a 1:1 ratio for the same pool of late pregnancy serum. These data, depicted in Table 3-2, suggest that a further decrease in the proportion of the volume of dialysate does not significantly alter the percentage of T specifically bound to SHBG at least in late pregnancy serum.

3.1.6 Determination of the concentration of dextran that is isosmotic with serum and evidence for the lack of binding of testosterone and estradiol to dextran

The concentration of dextran that is isosmotic with serum was determined by examining fluid shift across the membrane when serum was dialyzed against different concentrations of dextran solutions prepared by diluting a 10% dextran T-40 solution with 0.2 M phosphate buffer, pH 6.0. After equilibration, fluid shift across the membrane was estimated by measuring the change in the level of the fluid in the transparent, long, narrow chambers of the dialysis cell. It was found that 3% dextran solution was isosmotic to the sera of adult men, non-pregnant women and newborn infants while 2.8% dextran solution was isosmotic to the sera of pregnant women in the third trimester as shown by the absence of any fluid shift across the membrane at those dextran concentrations.

The inert nature (i.e. its lack of binding) of dextran was established by studying its binding to ^{3}H-T and ^{3}H-E_2 at 37°C. One ml of 3% dextran solution was dialysed against 1 ml of 0.2 M phosphate buffer after adding 25,000 cpm per ml of ^{3}H-E_2 or ^{3}H-T to either the dextran or the buffer solution. At the end of dialysis when the ratio of the

TABLE 3-2. Effect of changing the ratio of sample : dialysate volume from 1:1 to 9:1 on the percentage of testosterone bound to SHBG.

	% SHBG bound to testosterone	
Number of determinations	sample : dialysate 9 : 1	sample : dialysate 1 : 1
1	77.7	76.5
2	76.5	79.7
3	79.6	81.4
4	78.7	75.0
5	77.8	80.5
6	79.2	81.2
7	83.1	80.2
8	77.7	78.5
*Mean ± SD	78.8 ± 1.9	79.1 ± 2.2
Coefficient of variation	2.4%	2.8%

* Not significantly different

radioactivity on the dextran side to that on the buffer side was determined, it was found to closely approximate 1.0.

3.1.7 Precision of the dialysis method

The coefficients of variation of 8 determinations of percentage of T bound to SHBG in the same pool of pregnancy serum in two different dialysis experiments were 2.4% and 2.8% (data recorded in Table 3-2).

The precision of the dialysis methods determined from duplicate measurements of 8 different samples of pregnancy serum is shown in Table 3-3. The coefficient of variation for the estimation of percentage of T bound to SHBG was 2% and that for percentage unbound T in whole serum was 3.6%. These data indicate that the dialysis method used was reproducible.

3.1.8 Influence of CBG on percentage of unbound testosterone in serum

In order to test whether T is bound to CBG at 37°C in undiluted serum, percentage of T unbound in whole serum was determined in the absence and presence of excess cortisol. If T is bound to CBG, excess cortisol will eliminate any T binding by CBG, leading to an altered percentage unbound value for T. Values for percentage of unbound T when undiluted pregnancy or cord serum was dialyzed against isosmotic dextran solution in the absence or presence (8 µg/100 ml serum) of cortisol are shown in Table 3-4. Four determinations each were made with a pregnancy serum pool and cord serum pool. The data suggest that T binding by CBG in undiluted serum at 37°C is insignificant, at least for pregnant women in third trimester and for newborn infants.

TABLE 3-3. Precision of dialysis method.

Late pregnancy serum Sample No.	% T bound to SHBG* duplicate determinations		% T unbound† duplicate determinations	
	1	2	1	2
1	77.7	76.4	2.50	2.47
2	79.2	77.6	2.82	3.10
3	79.6	83.0	2.51	2.48
4	78.7	77.7	2.95	2.78
5	81.3	79.7	2.56	2.70
6	81.6	80.4	2.49	2.37
7	75.2	74.1	2.78	2.76
8	80.2	75.9	2.47	2.48
Mean	78.6		2.64	
SD** $= \frac{\Sigma\Delta^2}{2N}$	1.56		0.09	
Coefficient of variation $= \frac{SD \times 100}{mean}$	2.0%		3.6%	

* % T bound to SHBG was determined by dialysing 0.1 ml of undiluted late pregnancy serum against 0.1 ml of the same serum heated at 60°C for 1 hour

† % T unbound in whole serum was determined by dialysing undiluted late pregnancy serum against 2.8% dextran solution.

** SD = standard deviation; Δ = difference between duplicates

TABLE 3-4. Percentage of testosterone (T) bound when native serum was dialysed against isosmotic dextran solution in the absence or presence of non-radioactive cortisol (8 μg/100 ml serum).

Sample	Experiment #	% T bound in the absence of added cortisol	% T bound in the presence of added cortisol
Pooled Pregnancy Serum	1	97.1	97.6
	2	97.2	96.8
	3	97.4	97.5
	4	97.3	97.6
Mean ± SD		97.3 ± 0.16	97.4 ± 0.39*
Pooled Cord Serum	1	93.1	93.4
	2	93.5	92.9
	3	90.8	91.6
	4	89.8	90.2
Mean ± SD		91.8 ± 1.8	92.0 ± 1.4*

* Not significantly different

3.1.9 Percentage of testosterone and estradiol specifically bound to SHBG

The percentage (mean ± SD) of T and E_2 specifically bound to SHBG in whole serum of women with normal menstrual cycles, pregnant women in third trimester, newborn infants and adult men are given in Table 3-5(top). Percentages of T and E_2 bound to SHBG in nonpregnant women and pregnant women were significantly ($P < 0.001$) higher than in adult men and newborn infants. There was significant binding of E_2 by SHBG in women, being greater ($P < 0.001$) in pregnant (66.2 ± 1.4%) than in non-pregnant (41.7 ± 2.8%) women. E_2 binding by SHBG was minimal in adult men (2.7 ± 1.5%) and undetectable in newborn infants. T was significantly bound to SHBG in all types of sera studied.

3.1.10 Percentage of unbound testosterone and unbound estradiol in serum

The mean ± SD percentages of T and E_2 unbound in the same sera are shown in Table 3-5(middle). Percentages of unbound T and E_2 were highest in the newborn infants, lowest in pregnant women in third trimester and intermediate in nonpregnant women and adult men, men having higher values ($P < 0.001$ for T and $P < 0.02$ for E_2) than women. The difference in the values of percentage unbound between any one group and another was statistically significant for both T and E_2.

3.1.11 Percentage of testosterone and estradiol bound to albumin in the presence of SHBG

The mean ± SD percentages of T and E_2 bound to albumin in the same sera, estimated indirectly from the values of percentage specifically bound to SHBG and the percentage unbound values in whole serum, are presented in Table 3-5 (bottom). They were particularly high for E2 in

TABLE 3-5. The percentage SHBG-bound (top), unbound (middle) and albumin-bound (bottom) fractions of testosterone and estradiol in serum - mean ± S.D.

Subject	% T bound to SHBG	% E_2 bound to SHBG
Nonpregnant women	77.7 ± 3.5 (n = 7)	41.7 ± 2.8 (n = 4)
Pregnant women	79.3 ± 2.7 (n = 12)	66.2 ± 1.4 (n = 4)
Newborn infants	28.8 ± 4.9 (n = 12)	Undetectable (n = 5)
Adult men	30.3 ± 5.0 (n = 10)	2.7 ± 1.5 (n = 4)

Subject	% T unbound	% E_2 unbound
Nonpregnant women	3.63 ± 0.11 (n = 8)	1.98 ± 0.13 (n = 4)
Pregnant women	2.64 ± 0.21 (n = 8)	1.29 ± 0.09 (n = 4)
Newborn infants	6.09 ± 1.06 (n = 8)	4.38 ± 0.17 (n = 4)
Adult men	4.41 ± 0.43 (n = 4)	2.50 ± 0.27 (n = 4)

Subject	% T bound to albumin	% E_2 bound to albumin
Nonpregnant women	18.7 ± 3.5	56.3 ± 2.8
Pregnant women	18.1 ± 2.7	32.5 ± 1.4
Newborn infants	65.1 ± 5.0	95.6 ± 0.2
Adult men	65.3 ± 5.0	94.8 ± 1.5

* $P < 0.02$ ** $P < 0.001$

newborn infants and men, reflecting the low % bound to SHBG.

3.1.12 Serum distribution of testosterone and estradiol

The percentage distributions (mean ± SD) of estradiol and testosterone in undiluted serum at 37°C into SHBG-bound, albumin-bound and unbound fractions in newborn infants, adult men, nonpregnant women and pregnant women are given in Figure 3-3. The data are taken from Table 3-5 and are plotted in graphical form for easy comparison.

3.1.13 Percentage of testosterone and estradiol bound to albumin in the absence of SHBG

Data obtained when the percentage of T and E2 bound to albumin in the absence of SHBG was determined by dialysing serum heated at 60°C for 1 hr against an isosmotic dextran solution are shown in Fig. 3-4. Values for the percentage of T and E2 bound to albumin in the presence of SHBG are also shown for comparison. It is clear that in the absence of SHBG, albumin has the ability to bind greater than 96% of serum E_2 and greater than 90% of serum T in pregnant women and newborn infants. Data corroborating this were obtained when the percentage of unbound testosterone was determined in the absence and presence of increasing concentrations of non-radioactive testosterone in the serum of a pregnant woman in the third trimester of pregnancy (Table 3-6). Addition of increasing concentrations of non-radioactive testosterone progressively increased the percentage of unbound testosterone in whole serum and the unbound level reached a plateau when larger amounts (0.56 µg - 1.4 µg) of testosterone were added. At very high concentrations of testosterone, all the specific testosterone binding sites in the serum are saturated and the binding activity (100% - unbound) represents albumin binding in the absence of the influence of SHBG.

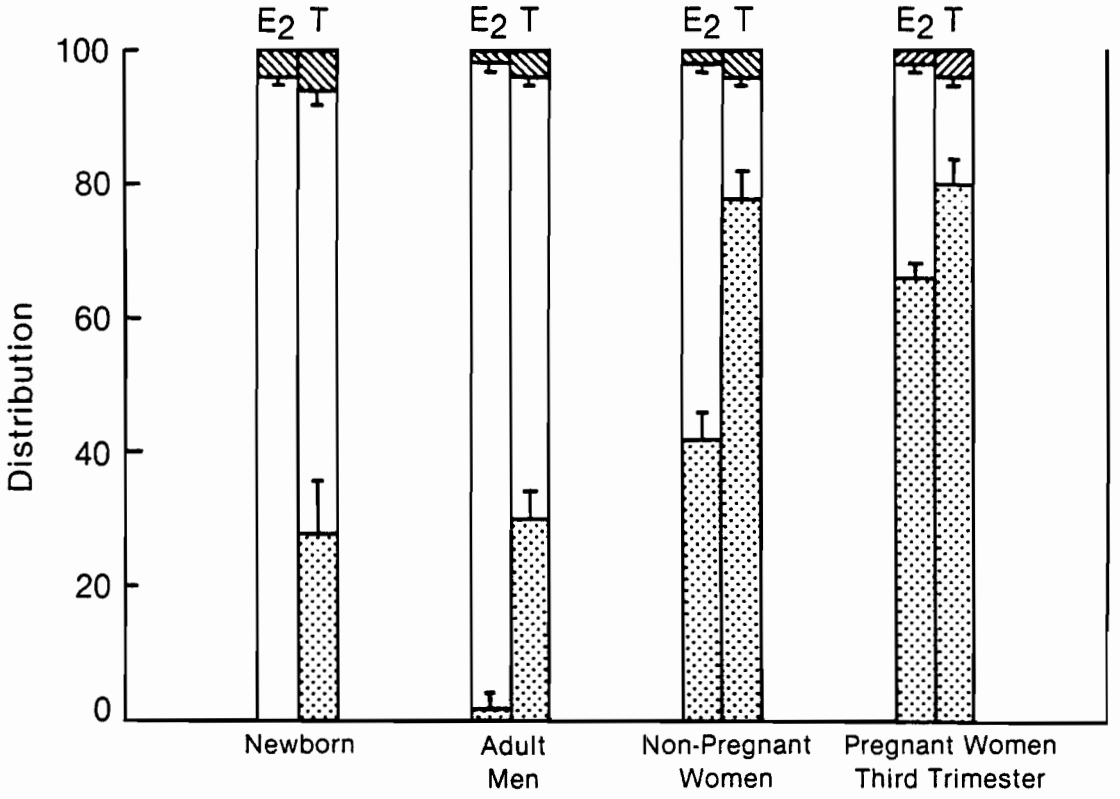


Figure 3-3. Percentage destribution (mean ± SD) of serum estradiol (E_2) and testosterone (T) into SHBG bound (stippled bars), albumin bound (open bars) and unbound (diagonally striped bars) fractions in newborn infants, adult men, non-pregnant women with normal menstrual cycles and pregnant women in third trimester.

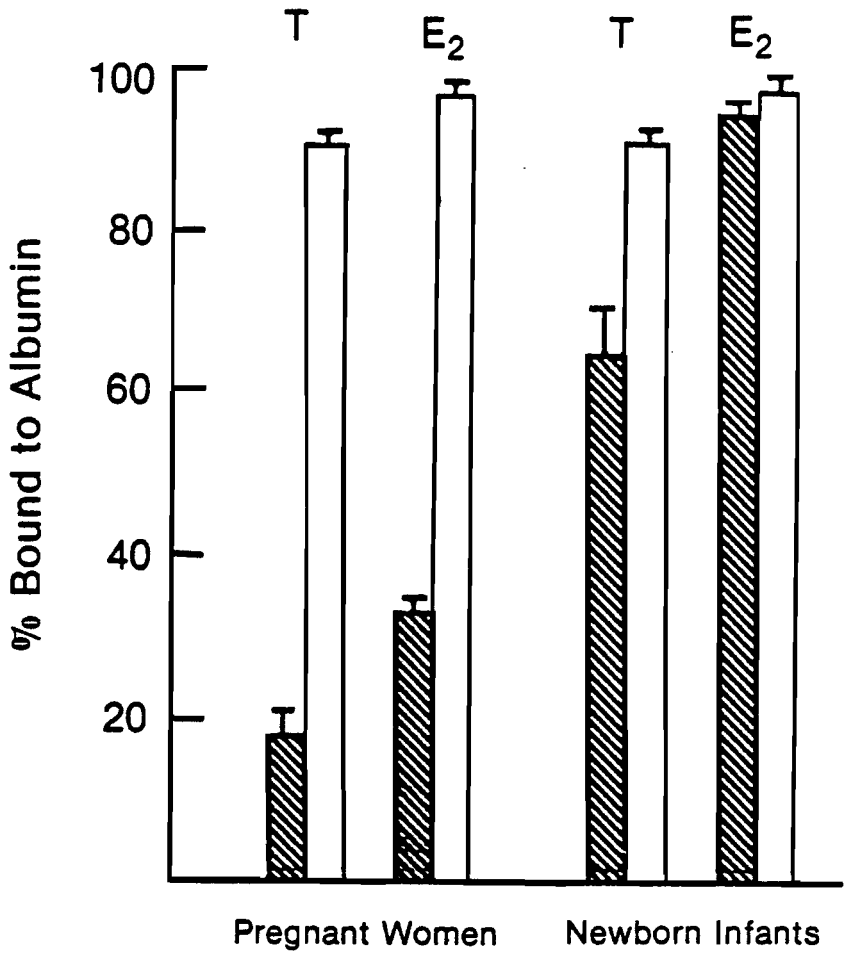


Figure 3-4. The percentage of testosterone (T) and estradiol (E_2) bound to albumin in the presence (▨) and absence (▭) of influence of SHBG in the sera of pregnant women in third trimester and in the sera of newborn infants.

TABLE 3-6. Percentage of unbound ^{3}H-testosterone in the serum of a pregnant woman in the third trimester in the presence of increasing concentrations of non-radioactive testosterone.

Non-radioactive testosterone added ng	% T unbound in whole serum
0	2.2
70	3.6
140	4.4
560	7.0
700	6.8
1120	7.1
1400	6.5

3.1.14 Serum levels of testosterone and estradiol

Total serum concentrations of T and E_2 were determined by radioimmunoassays. Concentrations of T and E_2 that are SHBG-bound, albumin-bound and unbound were then estimated as the total concentration × corresponding percentage distribution indicated in Table 3-5. The total concentration and the calculated amounts that are SHBG-bound, albumin-bound and unbound are shown in Table 3-7 for T and E_2.

The concentration of unbound E2 in serum calculated as the total concentration of E2 x % unbound E2 was 1.8 times higher in newborn infants than in pregnant women. The unbound E2 level was at least 70 times higher in pregnant women (215 ± 79 pg/ml) than in non-pregnant women (3.1 ± 2.0 pg/ml). The level of unbound E2 was the lowest (1.1 ± 0.3 pg/ml) in men. In fact the concentration of unbound E2 in non-pregnant women and adult men can be up to 40 % lower than shown in Table 3-7 since total E2 levels were measured without prior chromatography and included 77 % of estrone in these groups.

The concentration of unbound T in prgnant women (46 ± 14 pg/ml), calculated as the total serum concentration of T x % unbound T, was double that in non-pregnant women (21 ± 7 pg/ml). In newborn infants, the unbound T levels (88 ± 29 pg/ml) in turn were double those in pregnant women. The concentration of unbound T was highest (334 ± 124 pg/ml) in adult men.

If one assumes that only the unbound hormone is biologically active, it would then follow that the fetus and mother are exposed to high levels of physiologically active estrogen and androgen.

TABLE 3-7. Concentrations (mean ± SD) of total, SHBG-bound, albumin-bound and unbound hormone

Subjects	Total Concentration ng/ml	SHBG bound ng/ml	Albumin bound ng/ml	Unbound pg/ml
		E S T R A D I O L		
Non-pregnant women*	0.155 ± 0.102 (n = 6)	0.065 ± 0.042	0.087 ± 0.057	3.1 ± 2.0
Pregnant women	16.5 ± 4.8 (n = 6)	10.9 ± 3.2	5.36 ± 1.57	213 ± 64
Newborn infants	8.7 ± 3.0 (n = 6)	undetectable	8.34 ± 2.87	382 ± 132
Adult men*	0.042 ± 0.008 (n = 6)	0.001 ± 0.0006	0.040 ± 0.008	1.1 ± 0.2
		T E S T O S T E R O N E		
Non-pregnant women	0.57 ± 0.19 (n = 5)	0.44 ± 0.15	0.11 ± 0.04	20.5 ± 6.9
Pregnant women	1.78 ± 0.34 (n = 6)	1.41 ± 0.27	0.32 ± 0.08	46.3 ± 9.7
Newborn infants	1.44 ± 0.22 (n = 6)	0.42 ± 0.09	0.94 ± 0.16	87.8 ± 20.3
Adult men	7.58 ± 2.12 (n = 6)	2.30 ± 0.74	4.95 ± 1.43	334 ± 99

Note: Concentrations of steroid bound to SHBG, albumin and the amount that is unbound were calculated as total concentration × corresponding percentage distribution value given in Tables 3-5.

* The serum extract was assayed without prior chromatography and the value included 77% of estrone present in the serum. Thus the actual values of total, SHBG-bound, albumin-bound and unbound E2 can be up to 40% lower than shown.

3.2 Characterization of Certain Previously Unrecognized Ligands of SHBG in Pregnancy

3.2.1 Introduction

Early observations of Dr. Murphy suggested that a large portion of the SHBG-bound material eluted in the non-polar region when maternal serum was fractionated on a Sephadex LH-20 column and that this material was not detectable in the serum of nonpregnant women (Fig.3-5). The initial attempt in the present study was to confirm these results. Thus,the elution profile of SHBG-bindable activity on a Sephadex LH-20 column (31 × 0.8 cm) was studied in the sera of pregnant and non-pregnant women. The data presented in Figure 3-6 show that in maternal serum a large amount of the activity was eluted near androstenedione as a single peak. However, androstenedione cannot account for this material, as the levels of androstenedione during pregnancy (2.49 ng/ml) and its cross-reactivity in the SHBG assay (1.4%) are too low.

3.2.2 Detection and quantitation of unknown SHBG-bound material

An example of the (SHBG assay) standard curve used for the detection and quantitation of the unknown material is shown in Figure 3-7. The intraassay coefficient of variation of replicate measurements (n = 10) of a pregnancy serum extract was 6%. The interassay variation determined from duplicate measurements of Sephadex LH-20 fractions of pregnancy serum was 13% for the range of 200-800 pg (n = 20) and 18% for the range of 50-200 pg (n = 20). The sensitivity of the assay defined as 2 standard deviations of the reagent blank value was 20 pg. The specificity of the assay would be the same as that of SHBG (Table 1-2; Table 3-16).

In order to determine the nature of the unknown material eluting in the region of androstenedione, detailed analysis of maternal serum on a

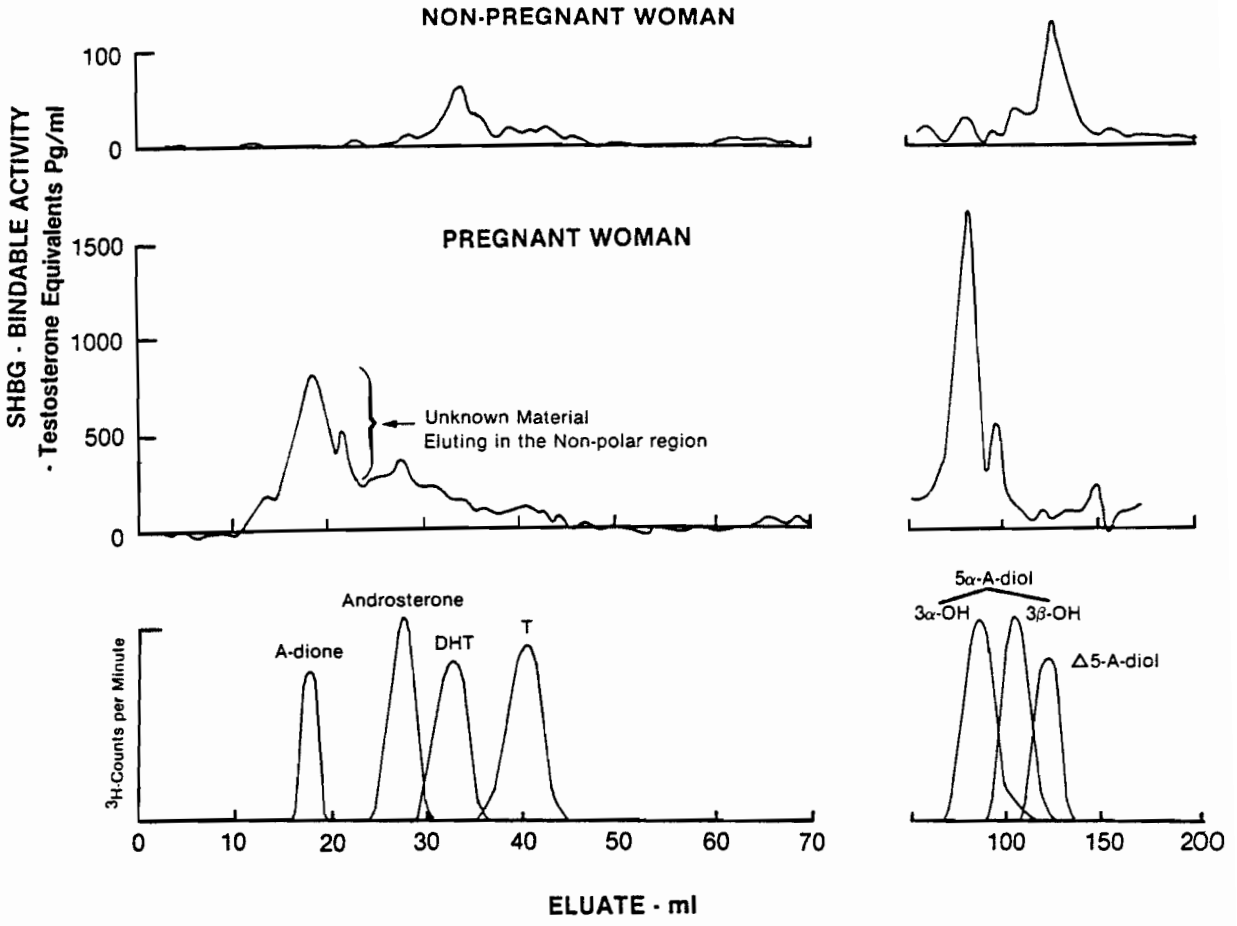


Figure 3-5. Preliminary data (Murphy, B.E.P., unpublished observations) showing the elution pattern on Sephadex LH-20 column (31×0.8 cm) of SHBG-bindable activity in the sera of non-pregnant and pregnant women. The bottom panel shows the fractionation pattern of radioactive steroid standards. The solvent system consisted of methylene chloride:heptane: methanol (50:50:1). 5α-A-diol-3α-OH : 5α-androstane-3α,17β-diol; 5α-A-diol-3β-OH : 5α-androstane-3β,17β-diol; Δ5-A-diol : 5-androstene-3α,17β-diol

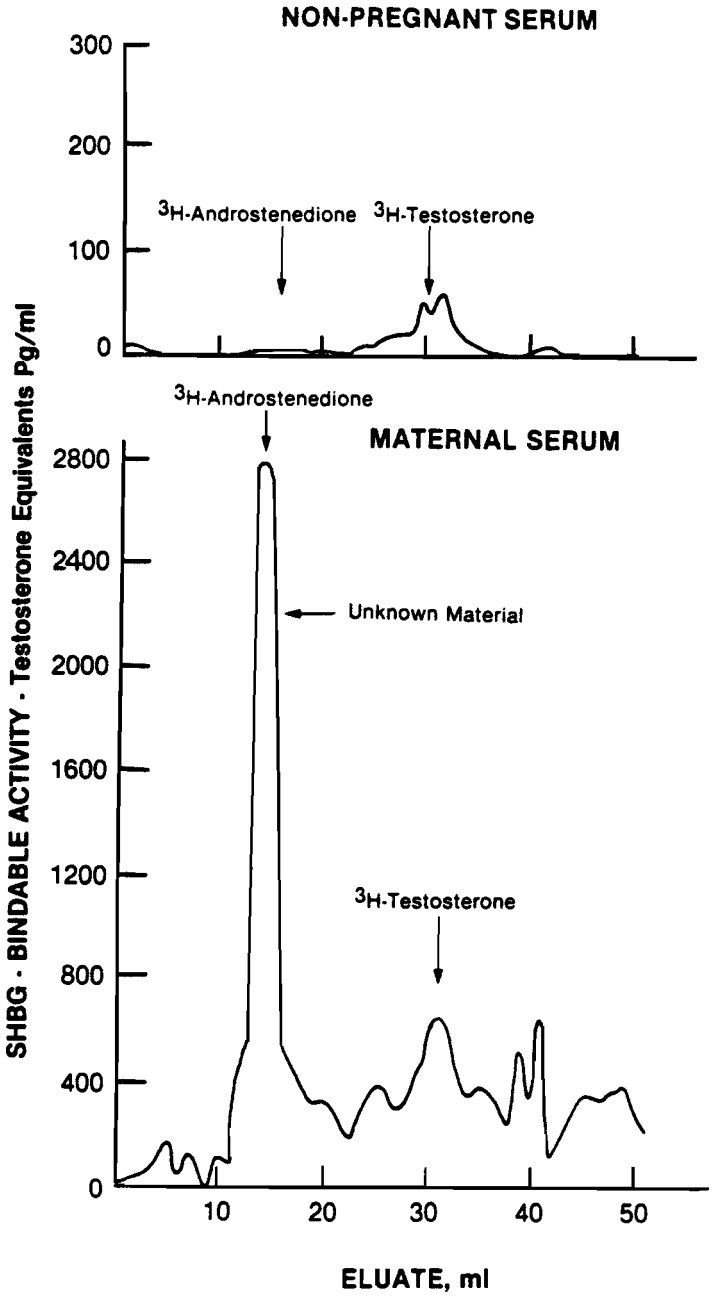


Figure 3-6. Data corroborating the results shown in Figure 3-5. Elution pattern of SHBG-bindable activity in the region of low polarity when maternal serum was fractionated on a Sephadex LH-20 column (31×0.8 cm). The arrows denote the elution volumes of ^{3}H-androstenedione and ^{3}H-testosterone. The solvent system consisted of methylene chloride: heptane:methanol (50:50:1).

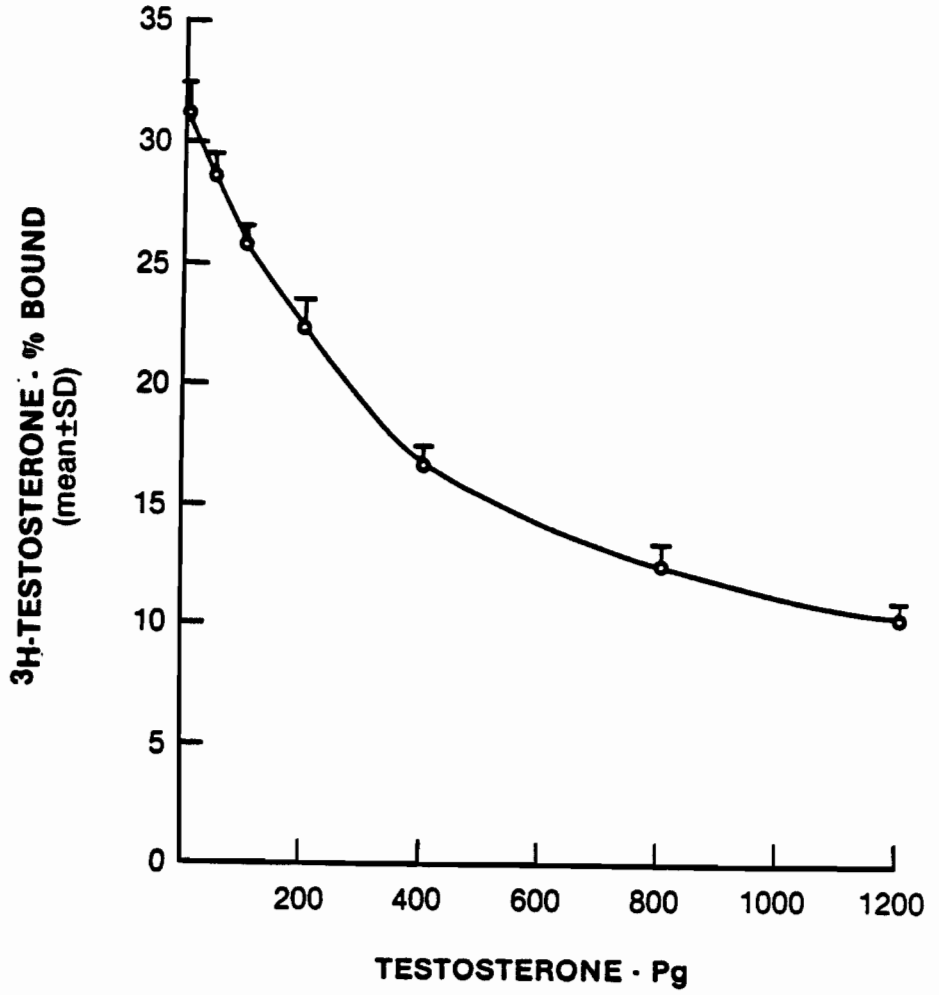


Figure 3-7. Example of a standard curve used for the measurement of SHBG-bindable activty (SHBG-assay). Displacement of ^{3}H-testosterone by non-radioactive testosterone from specific binding sites in pregnancy serum. Values for the unknown were read from the standard curve and expressed as testosterone equivalents.

longer Sephadex LH-20 column (60 × 0.9 cm) was carried out. The bulk of the unidentified material eluting near androstenedione resolved into 4 major peaks. These peaks were arbitrarily named as peaks 1a, 1b, 2 and 3. While it is possible that each of these peaks may represent more than one compound, it was hypothesized that one major compound may account for most of the activity eluting under each curve. Typical elution profiles of SHBG-bindable activity indicating these peaks, obtained in the serum of 5 pregnant women of different gestational ages, are shown in Figure 3-8. A large number of pregnancy serum samples of various gestational ages were analysed and the levels of peak (1a + 1b), peak 2 and peak 3 were calculated. Elution patterns of the SHBG bindable activity defined in maternal serum were also studied at various gestational ages in cord serum, placenta and in a sample (24-hr collection) of maternal urine and are shown in Figures 3-9, 3-10 and 3-11,respectively. The elution profiles obtained in cord serum and placenta and those obtained in preliminary studies in maternal urine resembled those of maternal serum and the peaks (1a + 1b), 2 and 3 of cord serum, maternal urine and placenta were assumed to be the same as those of maternal serum. While it is possible that different substances may have similar retention times even on a long (60 × 0.9 cm) Sephadex LH-20 column, the chances of these substances having the same binding properties are slight.

The levels of peak (1a + 1b), 2 and 3 shown in Fig. 3-9, 3-10 and 3-11 for individual samples were not corrected for procedural losses. The actual levels were estimated by summing the total activity under the curve after correction for procedural losses. These values will be given in later sections. However, it can be seen that levels of these peaks vary with gestational age. For example, the levels of peak 2 in maternal serum and

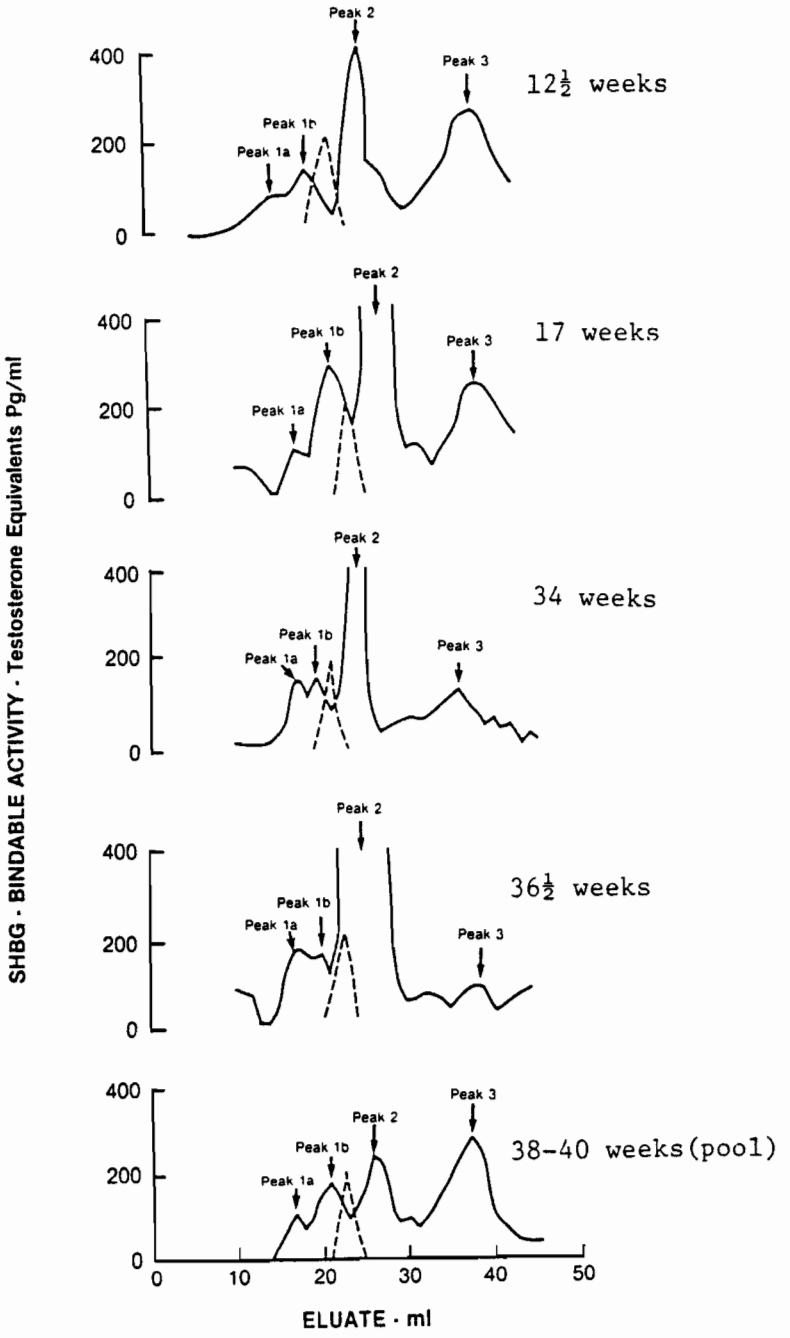


Figure 3-8. The elution patterns of the unknown SHBG-bindable activity in the region of low polarity in the serum of 5 pregnant women using detailed chromatography on Sephadex LH-20 (column 60×0.9 cm; solvent system - methylene chloride:heptane:methanol, 50:50:1). One ml fractions were collected and an aliquot from each fraction was assayed. None of the women were in labour when the samples were collected. The data shown are not corrected for procedural losses. Dotted lines represent the elution of ^{3}H- androstenedione marker added to each serum before extraction and chromatography.

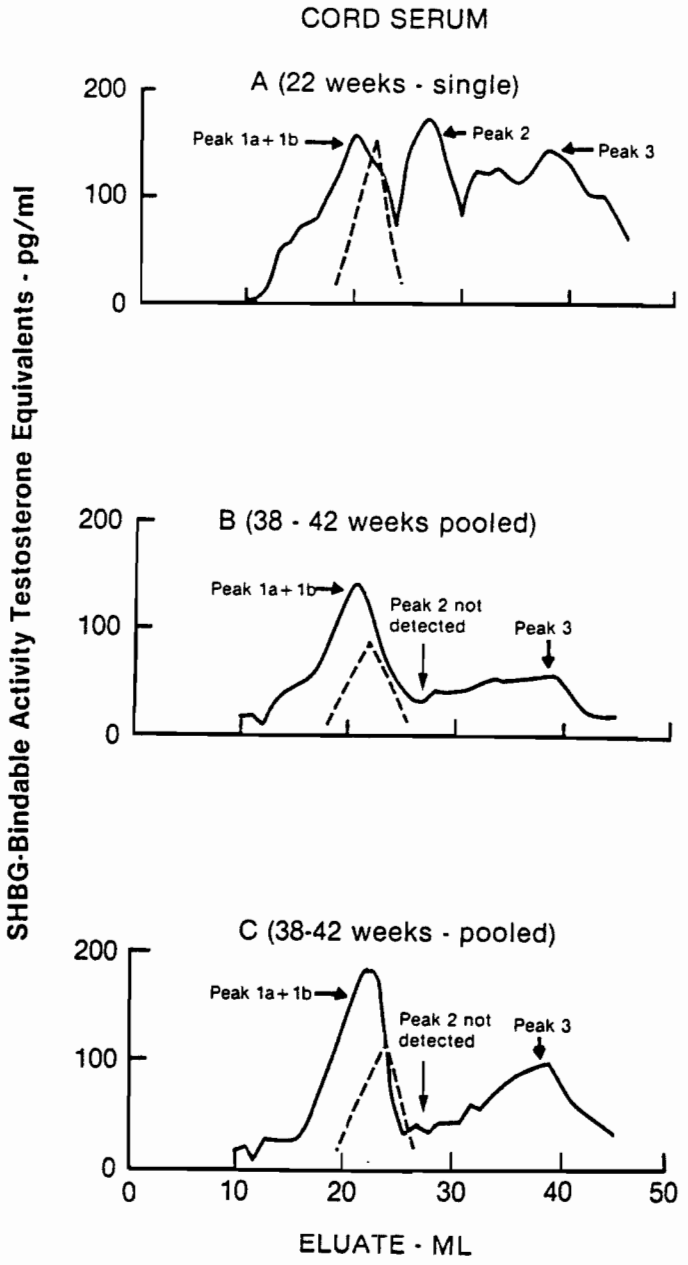


Figure 3-9. Elution profile of the unknown SHBG-bindable activity in cord serum using detailed chromatography on Sephadex LH-20 (column 60 x 0.9 cm; solvent system - methylene chloride:heptane:methanol 50:50:1). One ml fractions were collected and an aliquot from each fraction was assayed. The dotted lines show the elution of ^{3}H-androstenedione marker. Values are not corrected for procedural losses. (A) 22 weeks gestation obtained at hysterotomy in the absence of labour, (B) 38-42 weeks gestation (pooled serum) obtained at cesarean section in the absence of labour and (C) 38-42 weeks gestation (pooled serum) obtained at vaginal delivery with spontaneous labour.

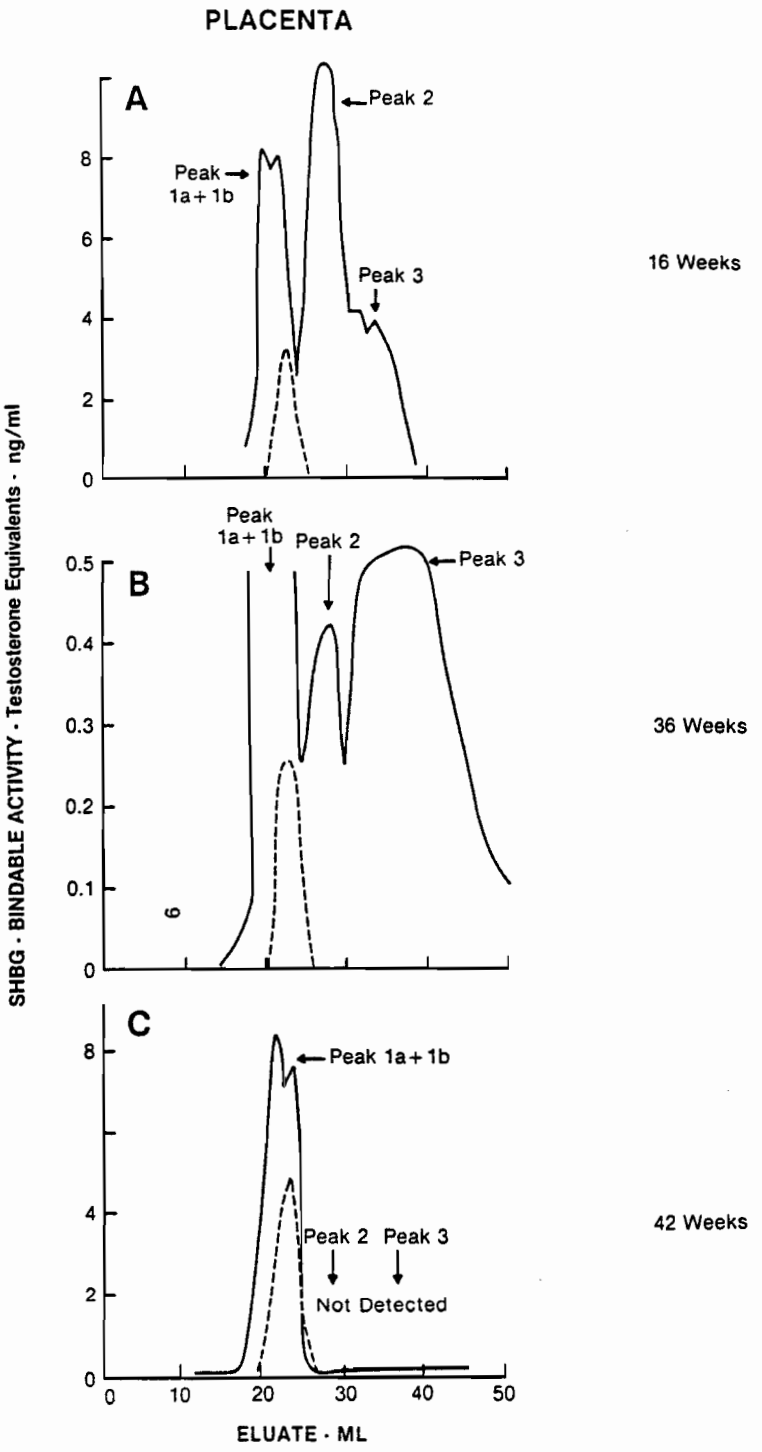


Figure 3-10. Elution pattern of the unknown SHBG-bindable activity in placentae using detailed chromatography on Sephadex LH-20 (column 60 x 0.9 cm; solvent system - methylene chloride;heptane:methanol 50:50:1). One ml fractions were collected and an aliquot from each fraction was assayed. The dotted lines represent the elution of the ^{3}H-androstenedione marker. Values are not corrected for procedural losses. (A) 16 weeks gestation obtained at hysterotomy (B) 36 weeks gestation obtained at Cesarean section (placenta previa) and (C) 42 weeks gestation obtained at Cesarean section. None of the mothers were in labour.

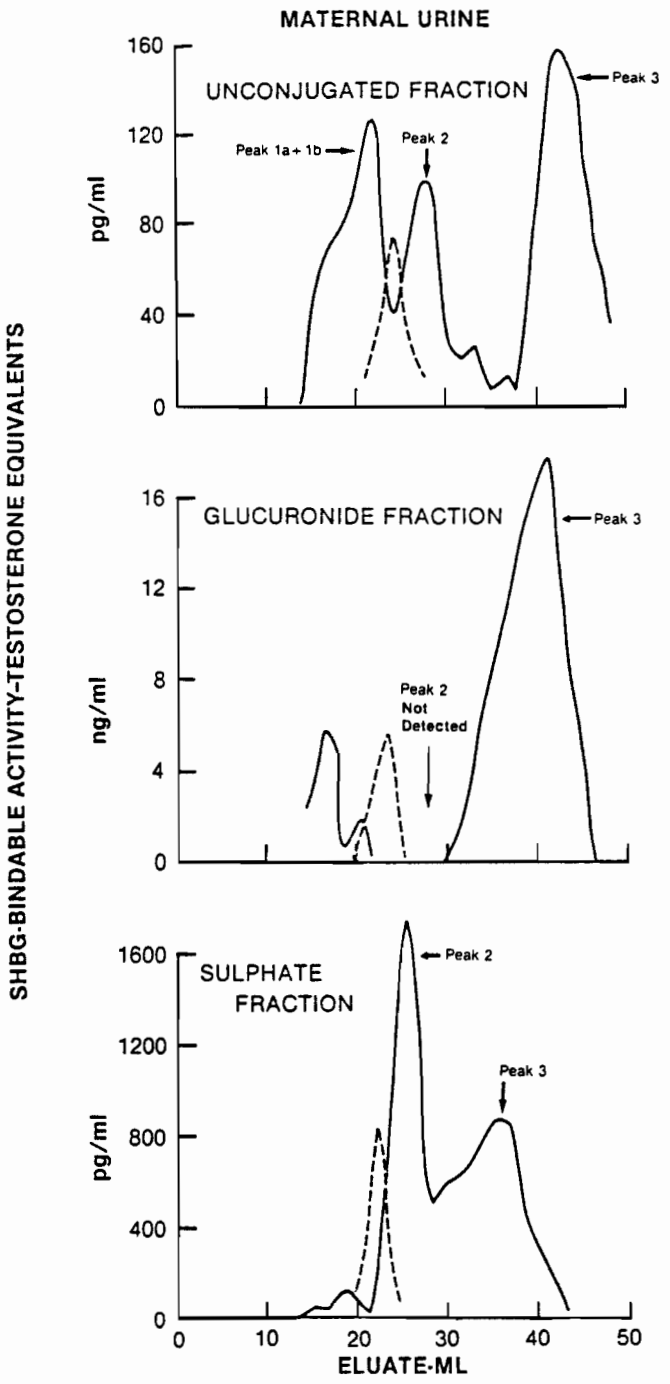


Figure 3-11. Preliminary data showing the elution pattern of the unknown SHBG-bindable activity in the unconjugated, glucuronide and sulfate fractions in the urine of a woman of 33 weeks gestation (not in labour; twin pregnancy) using Sephadex LH-20 (60 x 0.9 cm; solvent system - methylene chloride:heptane:methanol 50:50:1). One ml fractions were collected and an aliquot from each fraction was assayed. The dotted lines represent the elution of ^{3}H-androstenedione. Values are not corrected for procedural losses.

Note: The SHBG-bindable material detected in the region of peak 1a + 1b in the conjugated fractions is most likely an artifact (see section 3.2.2 and 4.2.4).

placenta were low or undetectable close to term while they were high in mid-pregnancy. In addition, the levels of peak 2 and peak 3 usually were much higher than those of peak 1a and 1b. The activity observed in the region of peak (1a + 1b) in the conjugated fractions in the preliminary study of a maternal urine sample (Fig. 3-11) is most likely an artifact since these peaks were later identified as 5α-DHP and P, respectively, by GC-MS analysis of peaks purified from hydrolysed pregnancy urine (see Section 4.2.4).

The nature of the unknown SHBG-bound peaks was further investigated by comparison of their relative elution volumes to those of reference steroids, by studying their binding affinities to various proteins, by comparison of measured and predicted serum concentrations, by HPLC, and by GC-MS analysis.

3.2.3 Concentration of peak (1a + 1b) during pregnancy

Since the resolution between peak 1a and peak 1b was often incomplete when one ml fractions were collected, the levels of peak (1a + 1b) were estimated together for all samples. (Peaks 1a and 1b, however, were readily separable when 0.5 ml fractions were collected as shown in Fig. 3-12; p. 137). The concentration of peak (1a + 1b) measured in T equivalents in maternal serum, placenta and cord serum is shown in Table 3-8. The levels of peak (1a + 1b) in maternal serum were significantly higher at 30-38 weeks gestation ($P < 0.001$) and 39-42 weeks gestation ($P < 0.001$) than at 12-24 weeks gestation. The concentrations at 30-38 weeks gestation were not significantly different from those at 39-42 weeks gestation for the number of samples measured. In the cord serum of 38-42 weeks gestation, although the levels were slightly higher than maternal levels at 30-38 and 39-42 weeks gestation, the difference

TABLE 3-8. Levels of peak (1a + 1b) during pregnancy.

Compartment	Gestational age weeks	Number analysed	Peak (1a + 1b) level* testosterone equivalents ng/ml or ng/gm
Maternal serum:			
	12-24	11	1.1 ± 0.3#
	30-38	14	2.6 ± 1.2#
	39-42	9	2.1 ± 1.2#
Cord serum:			
	22	1	1.7
	38-42	6 (pools)**	2.7 ± 1.2#
Placenta:			
	14	1	62
	16	2	55
	36	1	58
	39-42	3	45 ± 5.2#

* values are corrected for procedural losses
\# mean ± SD
** 4-5 samples were pooled in each case

was not significant for the number of samples studied.

Preliminary studies of urine at 33 weeks gestation (Fig. 3-11) showed that the level of material eluting as peak (1a + 1b) in the unconjugated fraction was low. Although some SHBG-bindable activity was detectable in the peak (1a+1b) area in the glucuronide and sulfate fractions of that sample, it is most likely an artifact as mentioned above.

The concentration of material eluting as peak (1a + 1b) was highest in the placenta. The levels varied from 45 to 62 ng/gm tissue wet weight. The fact that the placental levels were several fold higher than maternal and cord serum levels suggests that this material, if the same in all 3 types of samples, originates in the placenta.

3.2.4 Tentative identification of peak 1a as 5 α-pregnane-3,20-dione (5 α-dihydroprogesterone or 5 α-DHP) and peak 1b as progesterone

The first piece of evidence came from the elution pattern of authentic 5 α-DHP and progesterone on Sephadex LH-20 column. ^{3}H-5 α-DHP and ^{3}H-progesterone co-eluted with peaks 1a and 1b respectively (Figure 3-12 A). None of the 45 other possible metabolites of androgen, progesterone or estrogen tested had the same relative elution volume as peak 1a or peak 1b on detailed chromatography (Table 3-16; p. 168); 5 β-pregnane-3,20-dione was an exception which had the same relative elution volume as 5 α-DHP (however, this compound was ruled out on the basis of its cross-reactivity and expected serum levels - see Chapter IV).

Further evidence for the identity of peak 1a and 1b was obtained from cross-reactivity studies and comparison of known and predicted serum levels. The principle described by Murphy (1973) for the verification of identity of a ligand on the basis of its relative binding affinities to

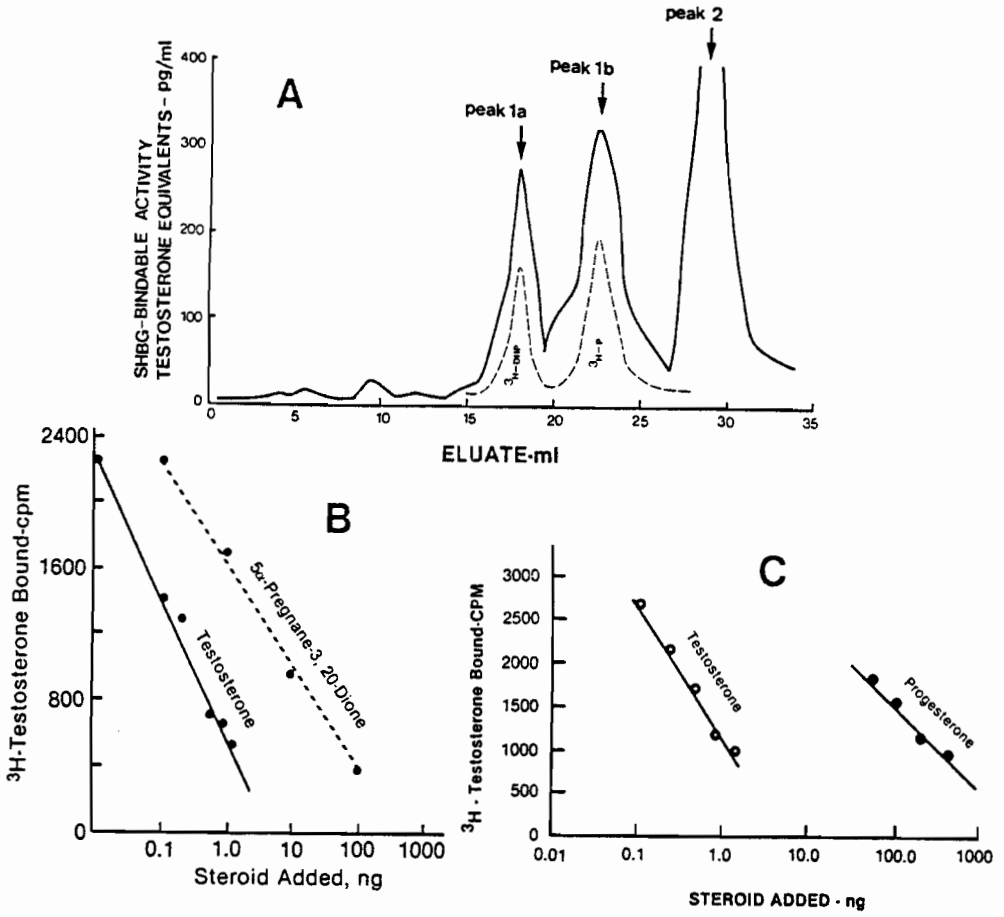


Figure 3-12. (A) Co-elution of ^{3}H-5α-pregnane-3,20-dione and ^{3}H-progesterone with peak 1a and 1b, respectively when maternal serum was fractionated on Sephadex LH-20 column (60 x 0.9 cm; solvent system methylene chloride:heptane:methanol 50:50:1). The figure also shows almost complete resolution of peak 1a and 1b when 0.5 ml fractions were collected and assayed. (B) and (C) Cross-reactivity data showing the competitive displacement of ^{3}H-testosterone from SHBG by 5α-pregnane-3,20-dione and ^{3}H-progesterone, respectively.

various binding proteins was used for this purpose. When an aliquot from peak 1a fraction measuring 0.11 ng TE in the SHBG assay, was assayed using guinea pig PBG assay, it measured 2.2 ng PE (progesterone equivalents) in the PBG assay. Since 5α-DHP cross-reacted 3.5% as well as testosterone in the SHBG assay (Fig. 3-12 B) and 100% as well as progesterone in the PBG assay, if peak 1a is indeed 5α-DHP, the actual amount of 5α-DHP in the aliquot tested will be 3.1 ng (i.e. 0.11 ng at 3.5% cross-reactivity) according to the SHBG assay and 2.2 ng (i.e. 2.2 ng PE at 100 % cross-reactivity) according to the PBG assay. Since these two values are the same within experimental error, it suggests that peak 1a is 5α-DHP.

Similarly, an aliquot of peak 1b measuring 0.20 ng TE in the SHBG assay measured 73 ng PE in the RIA for progesterone. Studies on the binding properties of progesterone showed that it cross-reacted 0.3% in the SHBG assay, (Fig. 3-12 C) and 100% in the RIA for progesterone. If peak 1b is in fact progesterone, its level in the aliquot measured in the SHBG assay (0.2 ng TE at 0.3% cross-reactiviy) will be 66 ng and in the RIA for progesterone (73 ng PE at 100 % cross-reactivity) will be 73 ng. These two values are the same within experimental error which is in accord with the hypothesis that peak 1b is progesterone.

Further, the reported plasma concentrations of 5α-DHP and progesterone support the above hypothesis. The measured serum levels of peak(1a + 1b) could be accounted for by the reported plasma concentrations of 5α-DHP and progesterone during pregnancy. Stoa and Bessessen (1975) have reported that mean ± SD concentration of 5α-DHP was 19.6 ± 11.0 ng/ml for 27-29 weeks gestation and 56.9 ± 29.3 ng/ml for 40-42 weeks gestation. Since the cross-reactivity of 5α-DHP in the SHBG assay is 3.5%, the above DHP concentrations will be expected to measure as (mean ± SD)

0.7 ± 0.4 ng/ml and 2.0 ± 1.0 ng/ml in TE at 27-29 and 40-42 weeks respectively. The plasma concentration (mean ± SD) of progesterone reported by Stoa and Bessessen (1975) was 62.4 ± 20.4 ng/ml for 27-29 weeks gestation and 167.9 ± 11.2 ng/ml for 40-42 weeks gestation. Since the cross-reactivity of progesterone is 0.3% in SHBG assay, the above progesterone levels will be expected to measure as 0.2 ± 0.1 ng/ml and 0.5 ± 0.0 ng/ml in TE for 27-29 weeks and 40-42 weeks gestation respectively. Thus the total expected range of plasma levels for(5 α-DHP + progesterone) at 27-29 and 40-42 weeks will be 0.9 ± 0.4 and 2.5 ± 1.0 ng/ml TE, respectively. The actual levels of peak (1a + 1b) measured for gestational ages of 12-24 weeks and 39-42 weeks gestation were 1.1 ± 0.3 and 2.1 ± 1.2 ng/ml TE respectively. Thus the measured levels of peak (1a + 1b) in maternal serum could be accounted for by total expected serum levels (TE) of 5 α-DHP and progesterone during pregnancy.

3.2.5 Concentration of peak 3 during pregnancy

The concentration (TE) of material eluting as peak 3 in various compartments during pregnancy is shown in Table 3-9. There was a significant rise ($P < 0.05$) in peak 3 concentration from 12-24 weeks gestation (1.6 ± 1.0 ng/ml) to 30-38 weeks gestation (3.3 ± 2.2 ng/ml). The level at 39-42 weeks gestation (3.2 ± 2.0 ng/ml) was not significantly different from that at 30-38 weeks gestation. There was considerable individual variation in all 3 gestational age groups.

The levels of peak 3 in cord serum at 38-42 weeks gestation (1.3 ± 0.7 ng/ml) was significantly lower ($P < 0.05$) than maternal levels at 30-38 and 39-42 weeks gestation. The level of peak 3 in a single cord serum sample of 22 weeks gestation was also low (1.8 ng/ml).

Levels of peak 3 in the unconjugated fraction (1.5 ng/ml)

TABLE 3-9. Levels of peak 3 during pregnancy

Compartment	Gestational age weeks	Number analysed	peak 3 level* testosterone equivalents ng/ml or ng/gm
Maternal serum			
	12-24	11	1.6 ± 1.0†
	30-38	9	3.3 ± 2.2†
	39-42	9	3.2 ± 2.0†
Cord serum			
	22	1	1.8
	38-42	6 (pools)**	1.3 ± 0.7†
Maternal urine	33	1	
- unconjugated fraction			1.5
- glucuronide fraction			203
- sulfate fraction			11.7
Placenta			
	14	1	54
	16	2	41
	36	1	13
	39-42	3	UD††

* values are corrected for procedural losses
† mean ± SD
** 4-5 samples were pooled in each case
†† undetectable

observed in the preliminary study of a urine sample at 33 weeks gestation was lower than maternal serum levels at 30-38 and 39-42 weeks gestation. However the levels of peak 3 in the glucuronide fraction (203 ng/ml) and in the sulfate fraction (11.7 ng/ml) were much higher.

The concentration of peak 3 was high in a placenta of 14 weeks (54 ng/gm TE) and in two placentae of 16 weeks (41 ng/gm TE) gestation. However, the level was considerably lower (13 ng/gm TE) in the placenta of a woman of 36 weeks gestation with placenta previa. Furthermore, in 3 normal placentae of term (39-42 weeks) gestation, peak 3 was undetectable when the same amount of placental tissue was analysed. On the other hand, the levels of peak (1a + 1b) in the above four late gestation placentae of 36, 39, 40 and 42 weeks were comparable to those of mid-gestation placentae of 14 and 16 weeks. Since it is reasonable to assume that the maternal, cord and placental peak 3 are the same, the high levels of peak 3 in mid-gestation placenta suggest that placenta may be a source of peak 3 in pregnancy. However, since peak 3 was undetectable in term placenta, the ability of placenta to synthesize the material eluting as peak 3 may be decreased or lost towards term.

3.2.6 Tentative identification of peak 3 as 2-methoxyestrone (2-MEI)

As for peak (1a + 1b), the first line of evidence for the identity of peak 3 came from the elution profile. Standard 2-MEI eluted in the same position as peak 3 on Sephadex LH-20 (Fig. 3-13A). None of the 45 other possible steroids tested had the same relative elution volume as peak 3 on detailed chromatography.

Further evidence for its identity was obtained by studying its binding affinity (cross-reactivity) and by comparison of known and predicted serum levels. The ability of 2-MEI to displace ^{3}H-testosterone

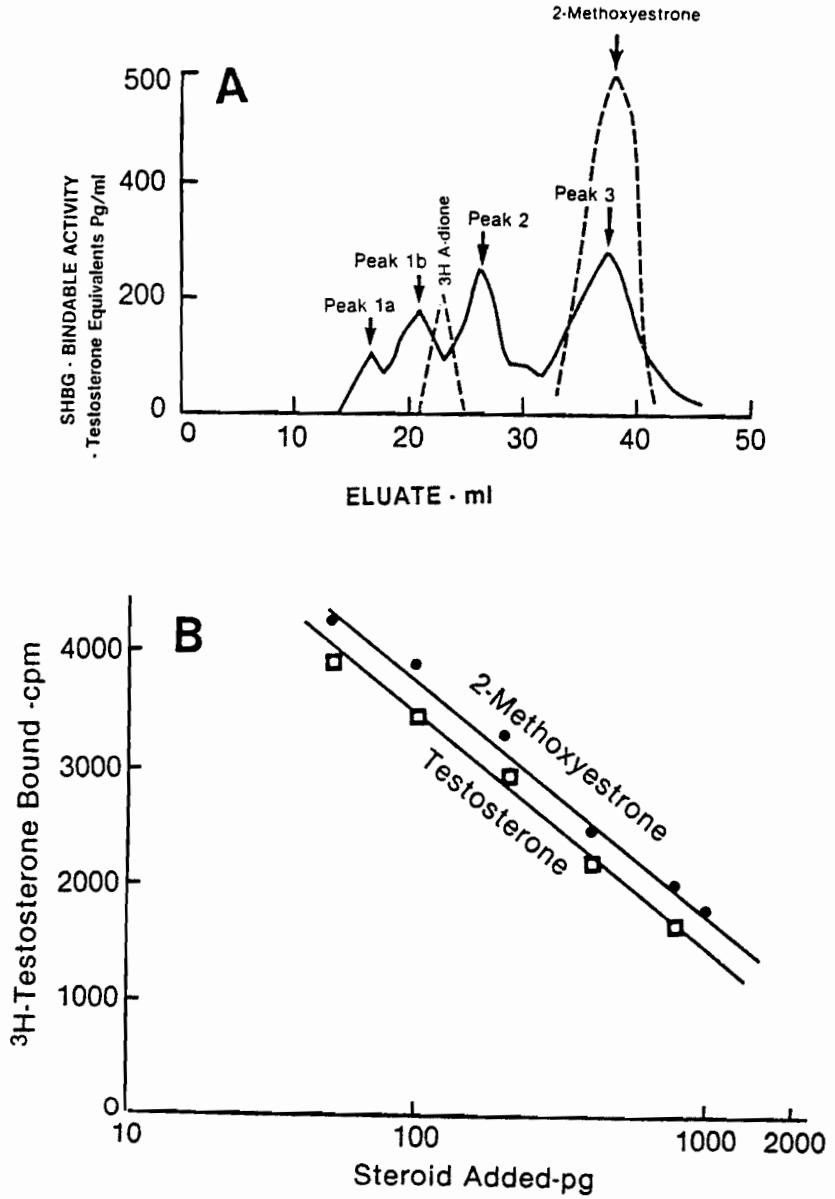


Figure 3-13. (A) The elution pattern of authentic 2-methoxyestrone showing that its relative elution volume is the same as that of peak3 in maternal serum (Sephadex LH-20 column 60 x 0.9 cm; solvent system methylene chloride:heptane:methanol 50:50:1). One ml fractions were collected and assayed. (B) Cross reactivity data showing competitive displacement of ^{3}H-testosterone from SHBG by 2-methoxyestrone.

from SHBG is shown in Figure 3-13B. 2-MEI cross-reacted 80% as well as testosterone in the SHBG assay. Plasma levels of 2-MEI in maternal serum have recently been measured by Berg et al (1983) using a radioimmunoassay at 11-16 weeks and 37-40 weeks and during labour and are shown in Table 3-10. These values, expressed in T equivalents, will be the expected levels of peak 3 if it were 2-MEI. The maternal serum concentration of peak 3 measured in the present study is also shown in Table 3-10. Comparison of the expected concentrations of 2-MEI in T equivalents and the levels of peak 3 actually measured suggests that peak 3 in maternal serum can be accounted for by the amounts of 2-MEI present in maternal serum during pregnancy.

3.2.7 A radiotransinassay for the determination of 2-MEI levels

In the present study a radiotransinassay for the determination of 2-MEI levels was developed by taking advantage of the capacity of 2-MEI to bind strongly (80% as well as T) to SHBG.

An example of the standard curve is shown in Figure 3-14. Binding curves for testosterone and estrone are also shown for comparison.

Data for the precision and accuracy of the assay are shown in Table 3-11. The sensitivity of the assay, determined as the lowest amount of 2-MEI which could be distinguished from zero with 95% confidence, was 20 pg. The intraassay coefficient of variation determined for two sets of samples was 11% and 4%. The interassay coefficient of variation was not investigated thoroughly. It was 18% in 3 different assays. Recovery of 50 ng of 2-MEI added to a pregnancy serum sample having 17 ng SHBG-bindable activity was approximately 78% after fractionation on Sephadex LH-20 column. Recovery of 50 ng of authentic 2-MEI after Sephadex LH-20 chromatography with and without added ascorbic acid was 104% and 73%

TABLE 3-10. Comparison of the levels of 2-methoxyestrone and peak 3 during pregnancy

LEVELS OF 2-METHOXYESTRONE (Berg et al, 1983): -

Gestational age (weeks)	Reported plasma level as measured by a radioimmuno-assay - ng/ml		Expected plasma levels using SHBG assay at 80% crossreactivity ng/ml, T.E.*	
	median	range	median	range
11-16 (46)**	0.67	0.22 - 1.68	0.5	0.2 - 1.3
37-40 (34)	3.77	2.04 - 10.6	3.0	1.6 - 8.6
In labour (41)	3.58	1.35 - 9.97	2.9	1.1 - 8.0

LEVELS OF PEAK 3 (present study): -

Gestational age (weeks)	Peak 3 levels in maternal serum: ng/ml, T.E. (mean ±S.D.)
12 - 24 (11)**	1.6 ± 1.0
30 - 38 (9)	3.3 ± 2.2
39 - 42 (9)	3.2 ± 2.0

Thus, the measured levels (ng/ml T.E.) of peak 3 can be accounted for by the expected amounts (ng/ml T.E.) of 2-methoxyestrone in maternal serum.

* testosterone equivalents
** numbers in parentheses denote number of samples analysed.

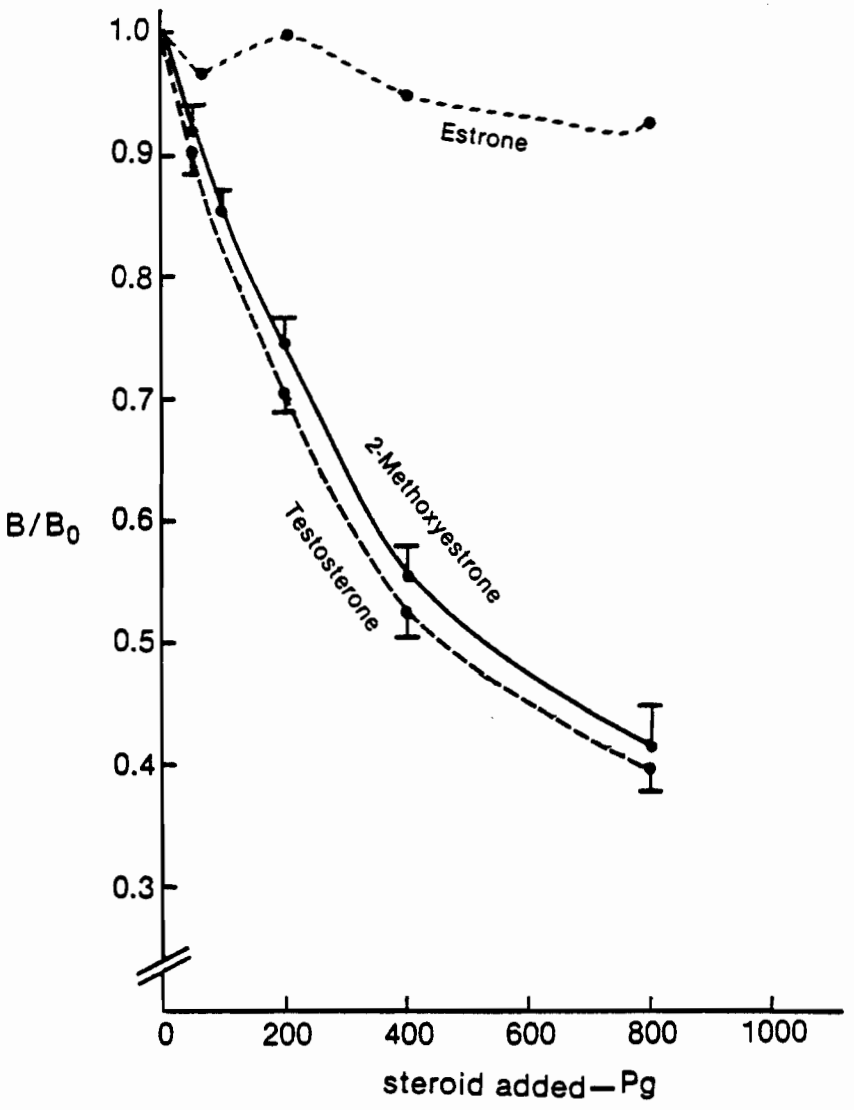


Figure 3-14. Example of a standard curve developed for 2-methoxyestrone. Binding curves obtained for testosterone and estrone are also shown for comparison. B/B_0 = ^{3}H-testosterone bound in the presence of competitor ÷ ^{3}H- testosterone specifically bound in the absence of competitor.

TABLE 3-11. A radiotransinassay for the measurement of 2-methoxyestrone (2-ME_1) levels: sensitivity, precision and accuracy.

A. Sensitivity:

^{3}H-testosterone bound in the absence of non-radioactive steroid mean ± SD	2-ME_1 measured at 4750 cpm (i.e. 2 × SD at zero concentration)
5062 ± 156 cpm n = 9	20 pg

B. Precision and accuracy:

Experiment #	Serum extract pg	Serum extract + 50 pg 2-ME_1 pg
1	310	340
2	240	370
3	290	340
4	330	330
5	310	350
Mean ± S.D.	296 ± 34	346 ± 15
Coefficient of variation	11%	4%

C. Recovery after chromatography on Sephadex LH-20:

Sample	2-ME_1 measured after chromatography* ng	% Recovery
1. Pregnancy serum extract	17	—
2. Pregnancy serum extract + 50 ng 2-ME_1	56	78
3. 50 ng 2-ME_1	37	73
4. 50 ng 2-ME_1 + ascorbic acid	52	104

* values are corrected for procedural losses

respectively (the recovery values were corrected for procedural losses). The data on recovery studies suggest that addition of ascorbic acid is important for complete recovery of 2-MEI.

Specificity of the assay for 2-MEI depends entirely on the separation procedure and on the specificity of SHBG. The relative binding to SHBG of a number of steroids obtained in the present study is shown in Table 3-16; (p. 168). Such values reported by other investigators have been summarized in Table 1-2 (p. 24).

3.2.8 Concentration of peak 2 during pregnancy

Levels of peak 2 in T equivalents in various compartments at different gestational ages are shown in Table 3-12. The mean (± SD) concentration of peak 2 in maternal serum at 12-24 weeks gestation (3.5 ± 4.4 ng/ml) was not significantly different from that at 30-38 weeks of gestation (3.6 ± 1.5 ng/ml). However, there was a significant decrease ($P < 0.05$) in the concentration of material eluting as peak 2 at 39-41 weeks gestation (1.8 ± 0.7 ng/ml) from that at 30-38 weeks gestation. Although there was considerable individual variation in peak 2 values at 12-24 weeks gestation (0.4-14 ng/ml), the variation was lower at 30-38 weeks gestation (1.4-8.8 ng/ml)and lowest at 39-40 weeks gestation (1.0 -2.7 ng/ml). While the reason for the high variation at 12-24 weeks is not clear, possible explanations include a temporal difference in the ontogeny of peak 2 synthesis among individuals and differences in factors affecting the synthesis or metabolism of peak 2.

In six samples of pooled cord serum obtained at term gestation (38-42 weeks), peak 2 was undetectable. In one cord serum sample of 22 weeks gestation, peak 2 measured as 1.5 ng/ml.

Preliminary studies on the excretion pattern of peak 2 in the

TABLE 3-12. Levels of peak 2 during pregnancy

Compartment	Gestational age weeks	Number analysed	peak 2 level* testosterone equivalents ng/ml or ng/gm
Maternal serum			
	12-24	12	3.5 ± 4.4†
	30-38	12	3.6 ± 1.5†
	39-41	4	1.8 ± 0.7†
Cord serum			
	22	1	1.5
	38-42	6 (pools)**	UD††
Maternal urine	33	1	
- unconjugated fraction			0.7
- glucuronide fraction			UD
- sulfate fraction			10.7
Placenta			
	14	1	28
	16	2	82
	36	1	3.6
	39-42	3	UD
Fetal tissues			
- liver	16	1	UD
- adrenal***	14	1	UD
- testis***	13	1	UD
- ovary***	14	1	UD

* values are corrected for procedural losses
† mean ± SD; all maternal serum samples included in this table were obtained from mothers not in labour
** 4-5 samples were pooled in each case
†† undetectable
*** Murphy, unpublished observations; Peak 2 was not studied separately. However, no detectable SHBG-bindable peak was observed in the region of low polarity

urine at 33 weeks gestation showed that the level of peak 2 in the unconjugated fraction was low (0.7 ng/ml). In the glucuronide fraction, peak 2 was undetectable and in the sulfate fraction, the level of material eluting as peak 2 was higher (10 ng/ml) than in maternal serum. Since it is reasonable to assume that serum peak 2 is the same as urinary peak 2, these results suggest that peak 2 is mainly excreted in the sulfated form.

The concentration of peak 2 in a placenta of 14 weeks gestation was 28 ng/gm and in two placentae of 16 weeks gestation was 82 ng/gm. However, in a placenta of 36 weeks gestation, the level was only 3.6 ng/gm. Interestingly, in three placentae of 38-42 weeks gestation, peak 2 was undetectable. This decrease in the levels of peak 2 towards term was also found in maternal serum.

Peak 2 was undetectable in the four main fetal tissues involved in the synthesis and metabolism of steroids (i.e. liver, adrenal, testis and ovary) obtained at 16-18 weeks. The low level of peak 2 in the cord serum sample of 22 weeks gestation and the absence of any detectable levels in cord sera of 38-42 weeks gestation and in the four fetal tissues capable of steroidogenesis suggest that peak 2 is not of fetal origin.

3.2.9 Analysis of peak 2 concentration according to labour and gestational age

The levels of peak 2 in maternal serum, cord serum and placenta analysed according to gestational age and the presence or absence of labour at the time of collection of the sample are given in Table 3-13. Peak (1a + 1b) and peak 3 levels are also given for comparison.

When the levels of the SHBG-bound peaks were analysed according to gestational age in the serum of mothers not in labour, it was found that

TABLE 3-13. Analysis of peak 2 levels according to labour and gestational age. Peak (1a + 1b) and peak 3 levels are also given for comparison - mean ± SD

Compartment	Gestational age weeks	Labour	Concentration - ng/ml or ng/gm, Testosterone Equivalents		
			peak 2	peak (1a + 1b)	peak 3
Maternal serum	12 - 24	None	3.5 ± 4.4 (12) ‡	1.1 ± 0.3 (11)	1.6 ± 1.0 (11)
	30 - 38	All@	2.9 ± 2.2 (17)	2.5 ± 1.2 (14)	3.3 ± 2.2 (9)
		None	3.6 ± 1.5 (12)	2.6 ± 1.0 (10)	3.3 ± 1.9 (6)
		Spont. labour	0.8 ± 0.5 (5)	2.4 ± 1.8 (4)	3.3 ± 2.0 (3)
	39 - 42	All	1.8 ± 0.6 (12)	2.1 ± 1.2 (9)	3.2 ± 2.0 (9)
		None	1.8 ± 0.7 (4)	NA ‡‡	NA
		Spont. labour	1.1 ± 0.1 (3)	NA	NA
Cord Serum	22	None	1.5 (1)	1.7 (1)	1.8 (1)
	38 - 42●●	All	UD● (6)	2.7 ± 1.2 (6)	1.3 ± 1.0 (1)
		None	UD (1)	1.6 (1)	0.8 (1)
		Spont. or ind. labour	UD (5)	3.0 ± 1.2 (5)	1.4 ± 0.7 (5)
Placenta	14	None	29 (1)	63 (1)	54 (1)
	16	None	82 (2)	55 (2)	41 (2)
	36	None	3.6 (1)	58 (1)	13 (1)
	39 - 42	All	UD (3)	45 ± 5.2 (3)	UD (3)
		None	UD (1)	44 (1)	UD (1)
		Spont. labour	UD (1)	41 (1)	UD (1)
		Ind. labour	UD (1)	51 (1)	UD (1)

* $p < 0.05$
** $p < 0.01$
‡ Numbers in parentheses denote number of samples analysed
@ Includes all samples i.e. no labour + spontaneous or induced labour
‡‡ Not analysed due to insufficient number of samples
● Undetectable
●● Pooled serum samples were used

while there was a significant increase in the mean serum concentration of peak (1a + 1b) and peak 3 ($P < 0.05$ in both instances) from 12-24 weeks gestation to 30-38 weeks gestation, there was no significant increase in the mean level of peak 2. This may be due to the large variations in peak 2 levels in individual serum samples of 12-24 weeks gestation. Interestingly however, there was a significant decrease ($P < 0.05$) in the serum levels of peak 2 (but not of peak (1a + 1b) and peak 3) from 30-38 weeks gestation to 39-41 weeks gestation.

When the levels of the peaks were analysed according to labour, at 30-38 weeks gestation, peak 2 levels, but not peak (1a + 1b) and peak 3 levels, were significantly lower ($P < 0.01$) in the serum of mothers in spontaneous labour (0.8 ± 0.5 ng/ml) than in the serum of mothers not in labour (3.6 ± 1.5 ng/ml) (Table 3-13). The data presented in Figure 3-15 show the markedly decreased serum levels of peak 2 in a pregnant woman of 34 weeks gestation after spontaneous onset of labour compared to that in a woman of the same gestation, but not in labour. Analysis according to labour was not possible for maternal serum samples at 12 to 24 weeks gestation since all the samples were collected from mothers not in labour. At 39-42 weeks gestation, the number of maternal serum samples measured was insufficient to give a significant difference with respect to labour although the mean value for women in labour was lower than that for women not in labour. (Five of the values were for mixed pools and could not be included).

The concentrations of peak 2 in maternal serum at different stages of gestation in the presence and absence of labour (taken from Table 3-13) are represented in a graphical form in Figure 3-16.

In cord serum, although peak 2 was detectable in the single

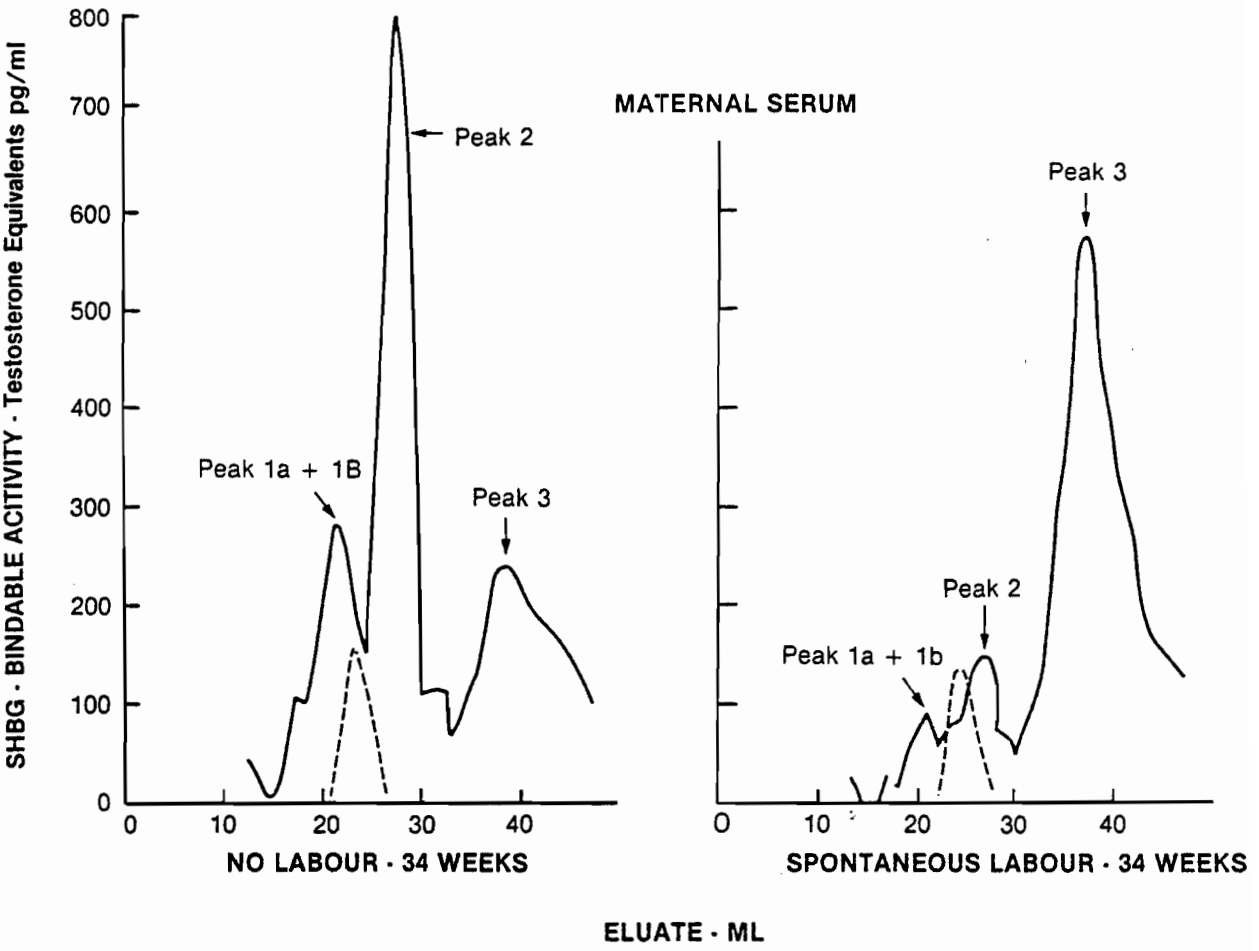


Figure 3-15. Elution pattern of the unknown SHBG-bindable activity in the serum of a pregnant woman of 34 weeks gestation not in labour compared to that in the serum of a woman of same gestation in spontaneous labour using Sephadex LH-20 (column 60 x 0.9 cm; solvent system - methylene chloride:heptane:methanol 50:50:1). One ml fractions were collected and assayed. Dotted lines represent elution pattern of the androstenedione marker.

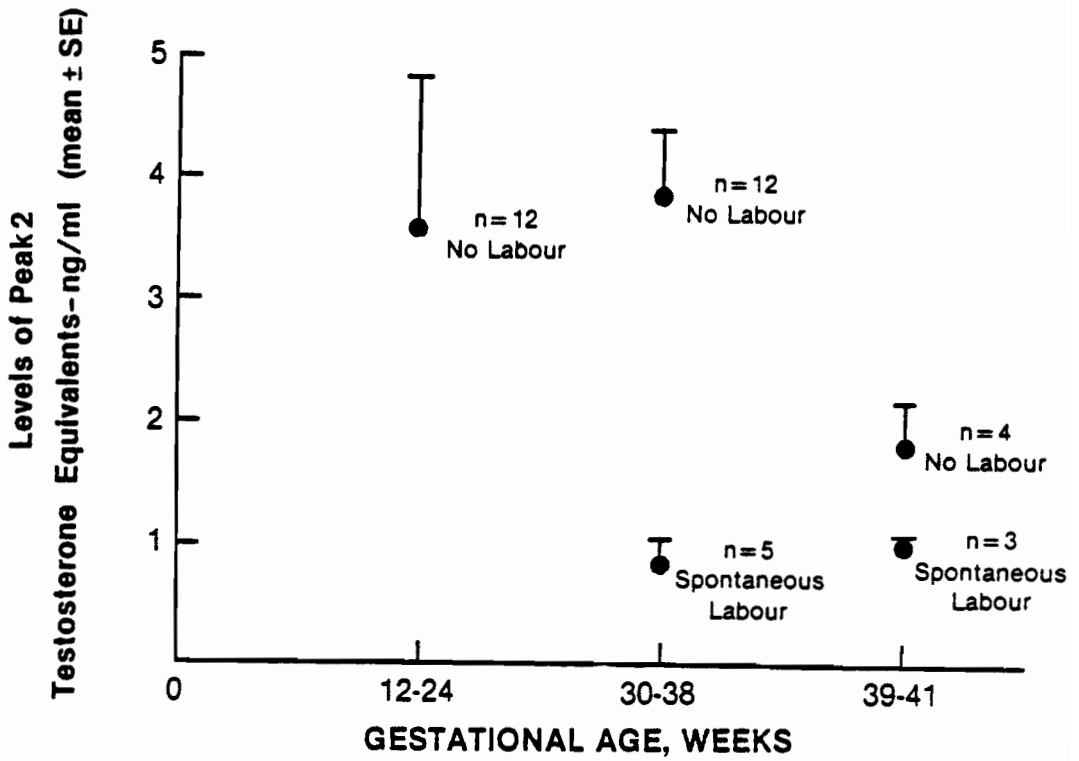


Figure 3-16. Concentration of peak 2 in maternal serum at gestational ages 12-24 weeks, 30-38 weeks and 39-41 weeks when no labour was present and at 30-38 weeks and 39-41 weeks after spontaneous onset of labour. Values are corrected for procedural losses.

sample of 22 weeks gestation (obtained at hysterotomy in the absence of labour), it was undetectable in 6 pools of 38-42 weeks gestation which included 1 pool obtained at caesarian section in the absence of labour and 5 pools obtained at spontaneous or induced vaginal delivery. Fetal sex made no difference. Peak 2 was undetectable in pooled male and pooled female cord sera of 38-42 weeks gestation collected at spontaneous or induced delivery. These data are shown in Figure 3-17. Peak (1a + 1b) and peak 3 were detectable in all cord serum samples studied. The elution patterns of peak (1a + 1b), peak 2 and peak 3 in typical cord serum samples have already been shown in Figure 3-9.

Levels of peak 2 were high at 14 weeks (28 ng/gm) and 16 weeks (82 ng/gm) gestation in placentae which were obtained at hysterotomy in the absence of labour. In one placenta of 36 weeks gestation obtained at caesarian section in the absence of labour (placenta previa), peak 2 level was low (3.6 ng/gm). Peak 2 was undetectable in 3 placentae of term gestation obtained at caesarian section in the absence of labour (42 weeks), at spontaneous delivery (39 weeks) and at induced delivery (40 weeks). Peak (1a + 1b) levels were high in placentae of all gestational ages studied. The pattern of peak 3 levels in placentae of different gestational ages was similar to that of peak 2, i.e. high levels at 14 and 16 weeks (54 and 41 ng/gm) and low levels at 36 weeks (12 ng/gm) and undetectable at 39-42 weeks in the absence or presence of labour. Elution patterns of peak 2, peak (1a + 1b) and peak 3 in typical placental samples have already been shown in Figure 3-10.

3.2.10 Source of peak 2

Concentration of peak 2 in different compartments in pregnancy are depicted in Figure 3-18. Since it is reasonable to assume that the

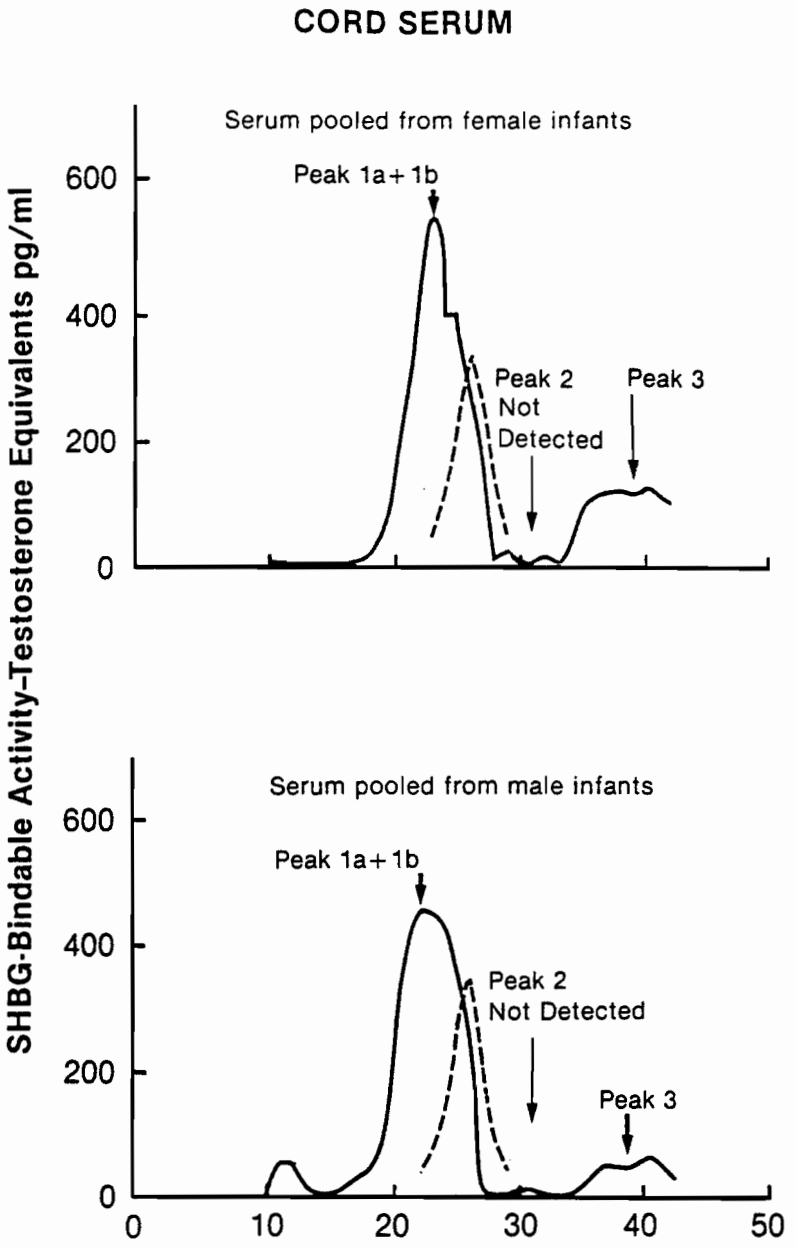


Figure 3-17. Elution profile of the unknown SHBG-bindable activity in pooled male and female cord sera of 38-42 weeks gestation using Sephadex LH-20 (column 60 x 0.9 cm; solvent system - methylene chloride:heptane: methanol 50:50:1). One ml fractions were collected and assayed. The dotted lines show the elution pattern of the ^{3}H-androstenedione marker. The serum samples were collected at vaginal delivery after induced or spontaneous labour.

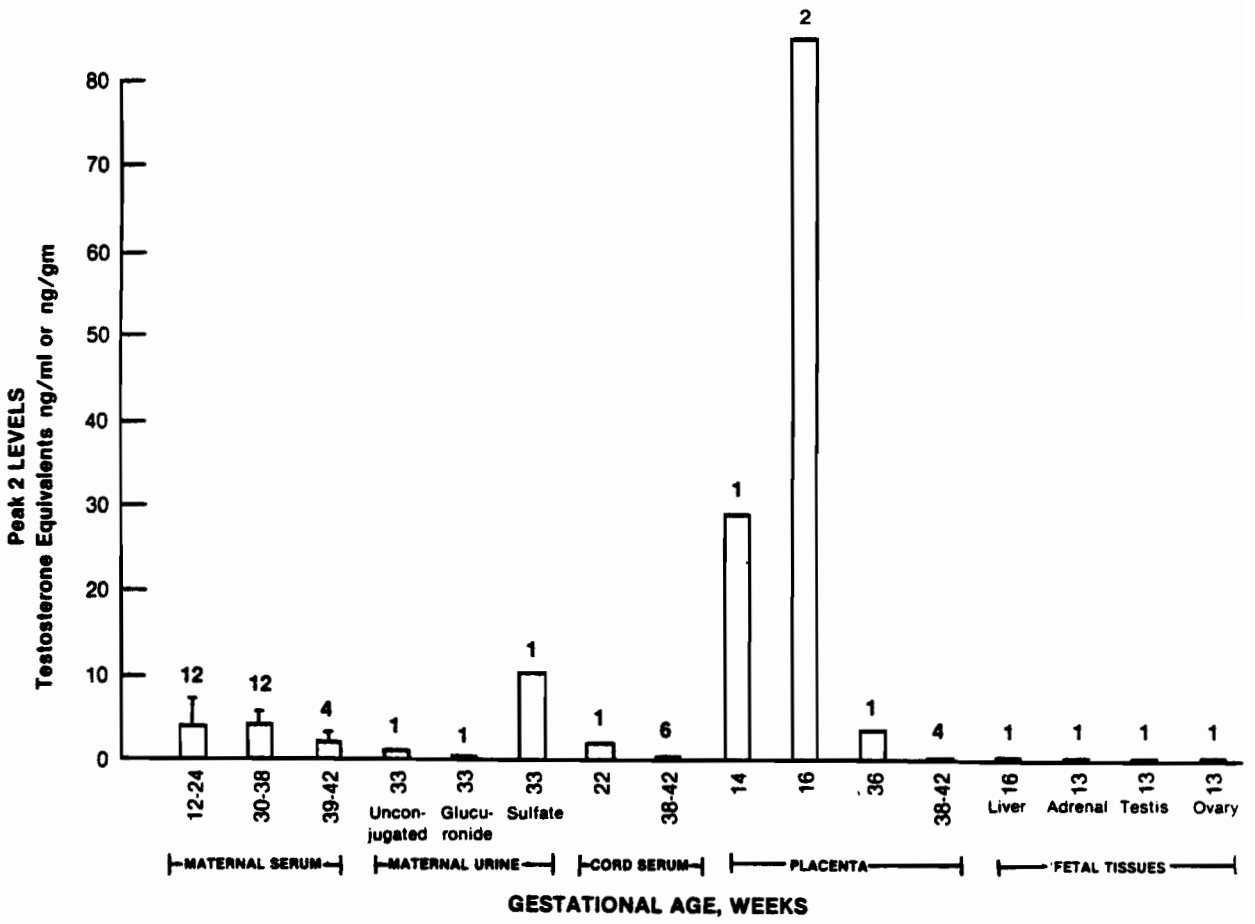


Figure 3-18. Concentration of peak 2 in different compartments during pregnancy. Since the levels of peak 2 in placenta are several fold higher than that of maternal serum, cord serum and fetal tissues, it was tentatively concluded that placenta is the source of peak 2 in pregnant women. Values are corrected for procedural losses. Figures above bars indicate number of samples.

material eluting as peak 2 is the same in all compartments, the several fold higher levels of peak 2 in mid-gestation placentae as compared to those of maternal serum, cord serum and fetal tissues, suggest that placenta is the source of peak 2. (Although less likely, another possibility is that the higher levels of peak 2 in the placenta are due to some mechanism present in that tissue for concentrating this material). Failure to detect peak 2 in placentae of 39-42 weeks gestation suggests that placenta may lose (or become deficient in) its ability to synthesize peak 2 towards term. This decrease or absence of peak 2 levels in placenta towards terms was also reflected in maternal serum.

3.2.11 Relative binding of peak 2 to SHBG

Since the identity of peak 2 was not known, it was not possible to calculate the cross-reactivity of peak 2 in the SHBG assay. The binding curves obtained by the competitive displacement of ^{3}H-testosterone by peak 2 and nonradioactive testosterone are shown in Figure 3-19. Comparison of the peak 2 curve with the T-curve suggests that peak 2 behaves similarly to testosterone at 4°C in a highly diluted system (i.e. SHBG assay).

When the binding of peak 2 to SHBG under 'physiological' conditions, (i.e. undiluted serum at 37°C) was measured by dialyzing native serum against the same serum heated at 60°C for 1 hour, it was found that peak 2 concentration in the unaltered side which contained intact SHBG was 3 to 6 times higher than in the heated side containing denatured SHBG. The elution pattern of peak 2 in the native side and heated side is shown in Figure 3-20. From these data, the strength of binding of peak 2 to SHBG in comparison to that of testosterone was calculated and is recorded in

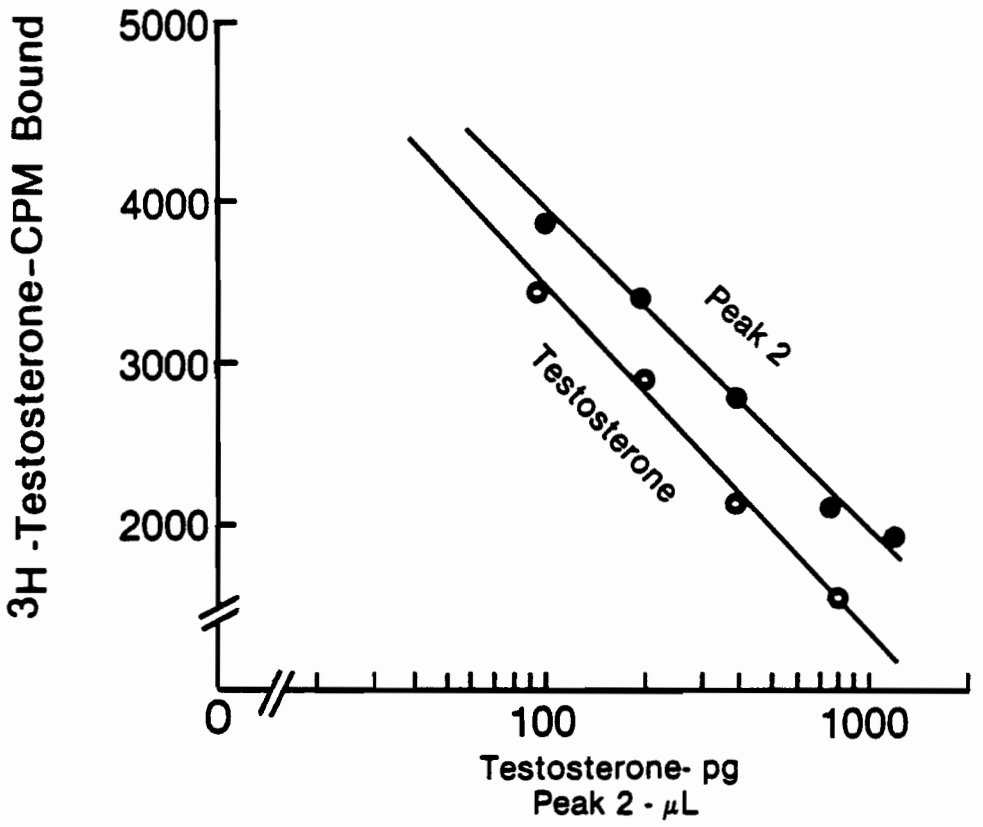


Figure 3-19. Competitive displacement of ^{3}H-testosterone from SHBG by non-radioactive testosterone and peak 2. Peak 2 behaves similarly to testosterone at 4°C in a highly diluted system, i.e. SHBG assay.

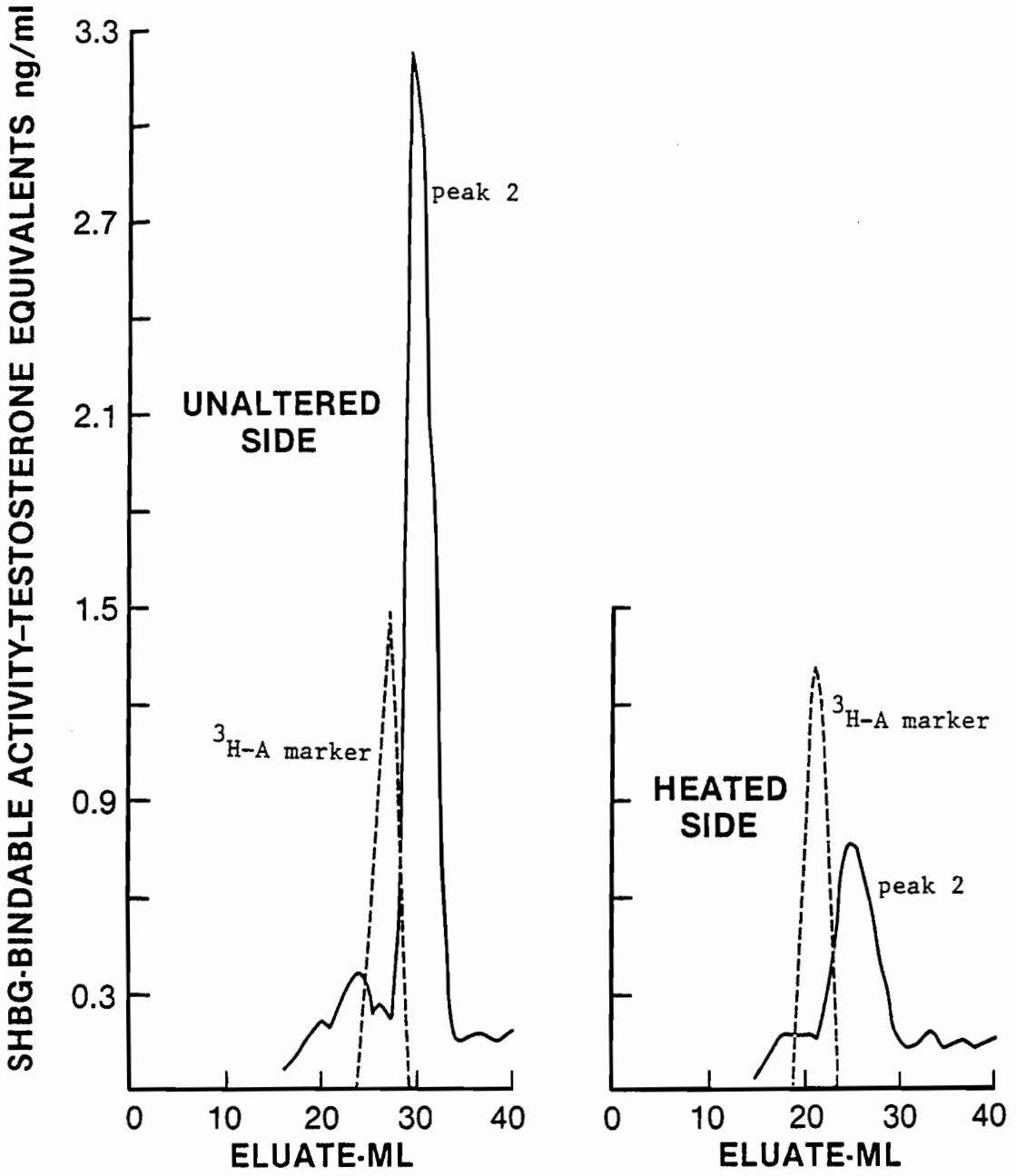


Figure 3-20. Binding of peak 2 to SHBG under physiological conditions. Undiluted maternal serum was dialyzed at 37°C for 20 hours against the same serum heated at 60°C for 1 hour. At the end of dialysis both sides were extracted, fractionated (1 ml/fraction) on Sephadex LH-20 (column 60 x 0.9 cm; solvent system - methylene chloride:heptane:methanol 50:50;1) and assayed.

Table 3-14. Percentage of peak 2 bound to SHBG when native serum was dialysed against heated serum at 37°C was calculated as:

$$100 - \left(\frac{\text{ng/ml peak 2 in the heated side}}{\text{(ng/ml peak 2 in the native side)}}\right) \times 100$$

These results were compared to the percentage of testosterone bound to SHBG under the same conditions after the addition of tracer amounts of ^{3}H-testosterone to late pregnancy serum. The percentage of ^{3}H-testosterone bound to SHBG was calculated as:

$$100 - \left(\frac{\text{cpm/ml in the heated side}}{\text{(cpm/ml in the native side)}}\right) \times 100$$

The principle behind this dialysis procedure for direct estimation of percentage bound to SHBG has already been presented in section 2 (Figure 2-1a). The dialysis data suggest that approximately 75% of peak 2 and 79% of ^{3}H-testosterone was bound to SHBG under the same experimental conditions. Thus, peak 2 appears to bind approximately 95% as strongly to SHBG as does testosterone under physiological conditions (i.e. in undiluted serum at 37°C).

3.2.12 Is peak 2 saponifiable?

It was of interest to test whether peak 2, which eluted in the region of low polarity on fractionation on Sephadex LH-20 column, is a lipoidal derivative. In order to test this, the elution profile of SHBG-bindable activity of late pregnancy serum was studied before and after saponification. Two procedures were used for saponification, namely hydrolysis with saturated potassium carbonate solution and hydrolysis with 5% KOH in 95% ethanol. The fatty acid esters of steroid hormones have been shown to readily undergo hydrolysis by treatment with a mild alkali such as K_2CO_3 (Janocko and Hochberg, 1983). The amount of material eluted as

TABLE 3-14. The affinity of peak 2 to SHBG compared to that of testosterone in undiluted serum at 37°C.

Sample	Amount of peak 2 after dialysis* ng			Sample	Amount of ^{3}H-testosterone after dialysis* cpm		
	Native side	Heated side	% bound		Native side	Heated side	% bound
1	12.4	4.1	67	1	1436	292	79.7
2	6.6	1.1	84	2	1256	235	81.3
				3	878	183	79.2
				4	885	197	77.8
Mean			75.1				79.5

* Late pregnancy serum was dialysed at 37°C against the same serum heated at 60°C for 1 hour until equilibrium was reached. Details of dialysis procedures used for the determination of percentage of peak 2 and ^{3}H-testosterone bound to SHBG are given in section 2.5.9 and section 2.4.3 respectively.

peak 2 after saponification by both the procedures was closely similar to that eluted in control (untreated) for the same pool of late pregnancy serum. These results shown in Figure 3-21 suggest that peak 2 is not saponifiable and that it is unlikely to be a fatty acid ester (non-polar conjugate) of a more polar steroid hormone.

3.2.13 Evidence to show that peak 2 is not strongly bound to human CBG, and guinea pig PBG

A purified placental preparation was used to study the binding of peak 2 to human CBG and guinea pig PBG. Purification was achieved by repeated detailed chromatography on Sephadex LH-20 using two different solvent systems (methylene chloride: heptane: ethanol - 50:50:1 and hexane:benzene:methanol - 85:10:5) followed by a reverse phase chromatography on a C18 silica gel column (10 x 0.8 cm; 20-40 μ particle size) using methanol:water - 80:20.

Aliquots of purified peak 2 preparation that measured 1.75 ng and 3.25 ng testosterone equivalents in SHBG assay measured zero cortisol equivalents (i.e. undetectable) in the CBG assay (Table 3-15a). All measurements were made in duplicates or triplicates. The lack of displacement of ^{3}H-cortisol by 1.75 ng and 3.25 ng TE of placental peak 2 while there was a significant displacement of ^{3}H-cortisol by 0.50 ng of non-radioactive cortisol suggest that placental peak 2 does not bind to CBG strongly.

Cross-reactivity studies have also shown that aliquots of purified placental peak 2 measured 6-7-fold lower in the guinea pig PBG assay than in the SHBG assay (see data presented in Table 3-17). Similarly, aliquots of peak 2 preparation from maternal serum which measured 1-3 ng TE in SHBG assay, measured several-fold lower ($\leq$ 0.1 ng)

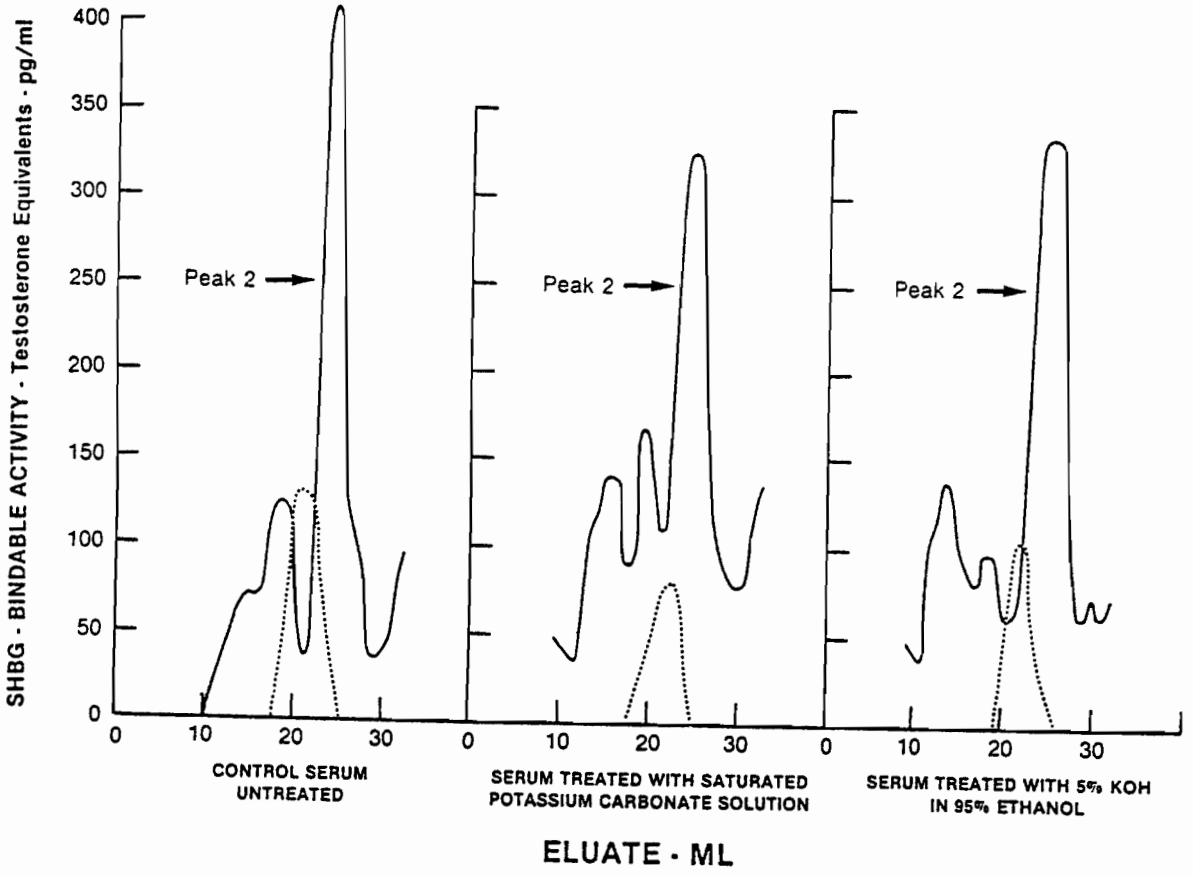


Figure 3-21. Saponification of peak 2. Elution pattern of peak 2 was determined in the same pool of late pregnancy serum using Sephadex LH-20 (column 60 x 0.9; solvent system - methylene chloride:heptane: methanol 50:50:1). One ml fractions were collected and an aliquot from each fraction was assayed. The dotted lines depict the elution pattern of the ^{3}H-androstenedione marker. (A) when untreated, (B) when treated with saturated potassium carbonate solution and (C) when treated with 5% KOH in 95% ethanol.

TABLE 3-15a. Evidence to show that peak 2 is not bound to CBG strongly.

Purified peak 2 aliquot*	SHBG assay @ Testosterone equivalents	Human CBG assay @ Cortisol equivalents
50 µl	1.75 ng	undetectable
100 µl	3.25 ng	undetectable

* Aliquots were taken in duplicates from the same placental peak 2 preparation obtained after several purification procedures.
@ Sensitivity was 20 pg for both SHBG and CBG assays.

TABLE 3-15b. Evidence to show that peak 2 is extractable by 0.1N NaOH*

Sample	SHBG-bound actiity MeCl2 phase pg	NaOH phase pg	Extracted by 0.1N NaOH %
Purified placental peak 2	200	3000	94
	320	4500	93
Crude placental peak 2	646	653	50
Crude serum peak 2	303	208	40
Crude urine peak 2 **	1660	575	25

* Peak 2 was partitioned between 0.1 N NaOH and methylene chloride (MeCl2).

** obtained from a single chromatography of 2 litres of hydrolysed pregnancy urine on Sephadex LH-20.

in the RIA for progesterone and was barely detectable (4-8 pg) in the RIA for estradiol (data not shown).

3.2.14. Evidence to show that peak 2 is extractable by 0.1 N NaOH

Data given in Table 3-15b show that when a purified preparation of placental peak 2 (same as that used to study binding to human CBG) was partitioned between methylene chloride and 0.1N NaOH, more than 90% of the SHBG-bound activity was extracted into the alkaline phase. This extraction into 0.1 N NaOH was also demonstrable for crude preparations of placenta, serum and urine peak 2. Crude placental and serum peak 2 were obtained after detailed chromatography of 5 gm placental tissue and 4 ml maternal serum, respectively, on Sephadex LH-20. The urinary peak 2 preparation was the least pure since it was obtained by only one Sephadex LH-20 chromatography of 2 litres of hydrolysed pregnancy urine (the purified peak 2 having been used up for GC-MS analysis). The solubility of peak 2 in 0.1 N NaoH suggests that it behaves like an estrogen.

Further, preliminary studies showed that the elution volume of crude serum and urine peak 2 extracted into 0.1 N NaOH phase is shifted to the position of estradiol on Sephadex LH-20 chromatography using a solvent system of methylene chloride:methanol (95:5).

3.2.15 Ultraviolet (UV) absorption spectrum of peak 2

The UV absorption spectrum of purified placental peak 2 (the same preparation as that used to study binding to CBG and solubility in NaOH), dissolved in absolute ethanol, was determined using a spctrophotometer (Cary 219, Varian Instruments). The characteristic absorption maximum obtained was at 232 nm (Fig. 3-22). This suggests the presence of a 1-en-3-one structure (Fieser and Fieser, 1959; Klyne, 1957).

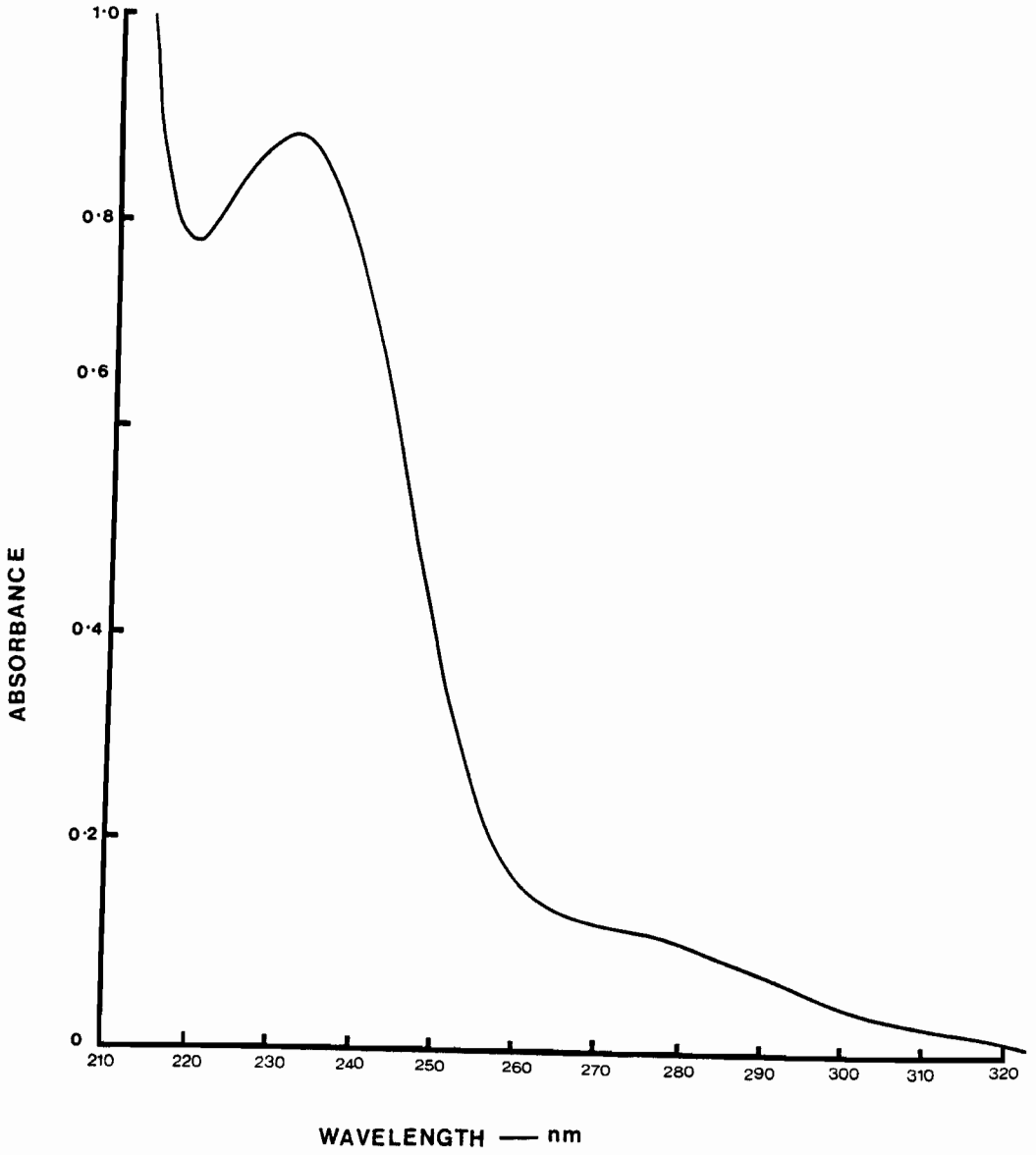


Figure 3-22. The ultraviolet absorption spectrum in ethanol of purified (placental) peak 2.

3.2.16 Possible compounds tested to date to account for peak 2

A large number of compounds was tested for their elution profiles on Sephadex LH-20 and for their cross-reactivity in the SHBG assay in order to account for peak 2 in maternal serum. When the serum levels of these compounds were known, the expected levels in TE were calculated from their cross-reactivity and were matched with the measured levels. The compounds tested included all known androgens of low polarity, all known progesterone metabolites of low polarity and all known estrogen metabolites of low polarity. A list which includes all the compounds tested is shown in Table 3-16. Each of the compounds listed was ruled out on the basis of its elution pattern on Sephadex LH-20 column, cross-reactivity in SHBG assay or its serum levels during pregnancy.

3.2.17 Compounds considered but not tested to account for peak 2

Possible compounds not tested to account for peak 2 included 2,4-dimethoxyestrone and 2,3-dimethoxyestrone. These compounds were not available commercially. Although the synthesis of the dimethoxyestrogens were attempted (courtesy, Dr. G. Just, McGill University) using the method of Zheng et al (1982), those attempts were not sucessful.

3.2.18 Evidence to show that peak 2 is not 5α-dihydro-11-deoxycorticosterone

Of all the compounds listed in Table 3-16, only 5α-DHDOC had the same relative elution volume as peak 2. However, further studies on the binding properties of 5α-DHDOC using various radiotransinassays provided evidence to show that peak 2 cannot be accounted for as 5α-DHDOC (Table 3-17). Aliquots in duplicates of a purified preparation of placental peak 2 (the same as that employed to study binding to CBG) were used. A 100 µl aliquot of peak 2 measuring 350 pg TE in SHBG assay, measured as

TABLE 3-16. Possible compounds tested to date to account for peak 2*.

	Relative elution volume**	Cross-reactivity % ***	Serum concentration required to account for peak 2 in late pregnancy (ng/ml)
4-Androstene-3,17-dione	1	1.4	214
Testosterone	2.85	100	3
Peak 2	1.24	95	3
A. Known Androgen (C_{19}) metabolites of low polarity:			
5α-Androstane-3,17-dione	0.76	1.6	188
5β-Androstane-3,17-dione	0.76	0.3	1000
Androsterone	1.90	1.0	300
Dihydrotestosterone	2.38	300	1
B. Known Progesterone (C_{21}) metabolites of low polarity:			
5α-Pregnane-3,20-dione	0.81	3.5	86
5β-Pregnane-3,20-dione	0.81	1.0	300
Progesterone	0.86	0.3	1000
20α-Dihydroprogesterone	1.90	0.3	1000
20β-Dihydroprogesterone	2.00	< 0.3	> 1000
3α-Hydroxy-5α-pregnan-20-one	----	1.7	176
3β-Hydroxy-5α-pregnan-20-one	2.05	2.0	150
3α-Hydroxy-5β-pregnan-20-one	----	0.9	333
3β-Hydroxy-5β-pregnan-20-one	2.14	5.8	52
20α-Hydroxy-5α-pregnan-3-one	2.24	4.0	75
20β-Hydroxy-5α-pregnan-3-one	----	0.4	750
20α-Hydroxy-5β-pregnan-3-one	----	2.5	120
20β-Hydroxy-5β-pregnan-3-one	----	< 0.3	> 1000
16α-Hydroxyprogesterone	----	< 0.3	> 1000
6α-Hydroxyprogesterone	----	< 0.3	> 1000
6β-Hydroxyprogesterone	----	< 0.3	> 1000
15α-Hydroxyprogesterone	----	< 0.3	> 1000
11β-Hydroxyprogesterone	----	< 0.3	> 1000
16-Dehydroprogesterone	----	0.5	600
5α-Pregnane-3α,20α-diol	> 2	---	---
Pregnenolone	> 2	0.3	1000
11-Deoxycorticosterone	1.52	4.0	75
5α-Dihydro-11-deoxycorticoste	1.24	4.5	67
5β-Dihydro-11-deoxycorticostee	----	0.5	600
Corticosterone	> 2	1.0	300
5α-Dihydrocorticosterone	> 2	0.8	375

TABLE 3-16 (continued)

	Relative elution volume**	Cross-reactivity % ***	Serum concentration required to account for peak 2 in late pregnancy (ng/ml)
C. Known Estrogen (C_{18}) metabolites of low polarity:			
Estradiol	> 4	50	6
Estrone	3.10	3	100
2-Methoxyestrone	1.76	81	3.7
2-Methoxyestradiol	> 3	120	2.5
4-Methoxyestrone	2.4	4	75
4-Methoxyestradiol	> 4	6	50
Estrone-3-methyl ether	----	0.2	1500
Estradiol-3-methyl ether	----	0.1	3000
2,4-Dimethoxyestradiol @	1.86	----	----
3-Acetoxyestrone	----	0.5	600
3-Acetoxyestradiol	----	16	19
17-Acetoxyestradiol	----	0.04	7500
3,17-Diacetoxyestradiol	----	0.02	15000

* Each compound listed was ruled out on the basis of its elution pattern on Sephadex LH-20 column, cross-reactivity in SHBG assay or serum levels during pregnancy.

** Relative elution volume = $\frac{\text{Elution volume of test steroid}}{\text{Elution volume of } {}^{3}\text{H-androstenedione}}$

***cross-reactivity, % = $\frac{\text{mass of T}}{\text{mass of test steroid required to displace 50\% } {}^{3}\text{H-T}} \times 100$

@ donated by Dr. Zheng, Sichuan University, People's Republic of China.

TABLE 3-17. Evidence to show that peak 2 is not 5α-dihydro-11-deoxycorticosterone (5α-DHDOC)

(i) Binding Data of Peak 2 and 5α-DHDOC

	SHBG Assay @ TE*	PBG Assay @ PE*	CBG Assay @ FE*
Peak 2 aliquot**			
100 µl	350 pg	50 pg	not detectable
200 µl	650 pg	110 pg	not detectable
500 µl	Above range	Not done	not detectable
1000 µl	Above range	Not done	not detectable
5α-DHDOC Cross-reactivity	6%	60%	2%

* TE - testosterone equivalents; PE - progesterone equivalents; FE - cortisol equivalents.
** Aliquots were taken from the same purified preparation of placental peak 2.
@ Sensitivity of all 3 assays was approximately 20 pg.

(ii) Explanation of Binding Data

If peak 2 is 5α-DHDOC:
200 µl or 650 pg TE of peak 2 = 10.8 ng 5α-DHDOC
and 1000 µl or 3250 pg TE of peak 2 = 54.2 ng 5α-DHDOC

Then, expected amounts will be:
200 µl peak 2 or 10.8 ng 5α-DHDOC = 6.5 ng PE
and 1000 µl peak 2 or 54.2 ng 5α-DHDOC = 1.1 ng FE

However, actually measured amounts are:
200 µl peak 2 = 110 pg PE
and 1000 µl peak 2 = not detectable, FE

Thus, the expected and measured amounts of peak 2 in two different assays are widely different and peak 2 cannot be accounted for as 5α-DHDOC.

50 pg PE in PBG assay and was undetectable in human CBG assay. Similarly, a 200 µl aliquot of peak 2 measured 650 pg TE and 110 pg PE in the SHBG and PBG assays respectively and was undetectable in the hCBG assay. When 500 µl and 1000 µl aliquots of peak 2 were tested, the values were above the range of standard curve in the SHBG assay, but again no displacement of ^{3}H-cortisol was detectable in the hCBG assay. When the cross-reactivity of authentic 5α-DHDOC was studied in various radiotransin assays, it was 6% in SHBG assay, 60% in PBG assay and 2% in CBG assay.

If peak 2 is in fact 5α-DHDOC, 200 µl of peak 2 preparation which measured 650 pg TE in SHBG assay will contain 10.8 ng 5α-DHDOC. Similarly, 1000 µl of peak 2 preparation measuring 3.25 ng TE in SHBG assay will contain 54.2 ng 5α-DHDOC. In the PBG assay, 200 µl of peak 2 preparation containing 10.8 ng 5α-DHDOC is expected to measure as 6.5 ng PE. Similarly, 1000 µl of peak 2 preparation containing 54.2 ng 5α-DHDOC is expected to measure 1.1 ng FE in the CBG assay. However, the actually measured value for 200 µl of peak 2 preparation was 110 pg PE in the PBG assay, and for 1000 µl of peak 2 preparation, the actually measured value was 0 pg FE (i.e. undetectable) in the CBG assay. Thus, peak 2 cannot be accounted for as 5α-DHDOC.

3.2.19 Identification of peaks 1a, 1b, 2 and 3 by gas chromatography-mass spectrometry (GC-MS)

Although further purification of the unknown SHBG-bindable material was attempted from maternal serum and placenta, GC-MS analysis showed that they were heavily contaminated with lipids. Therefore the unknown SHBG-bindable material purified from hydrolysed pregnancy urine was used for GC-MS analysis. It was assumed that the SHBG-bindable peaks of maternal urine were the same as those of maternal serum (see section 4.2.2)

Peaks 1a, peak 1b, peak 2 and peak 3 prepared from hydrolysed pregnancy urine by detailed chromatography on Sephadex LH-20 column were individually purified by HPLC using a gradient elution technique. The elution profile on HPLC of steroid standards ^{3}H-5α-DHP, ^{3}H-P and non-radioactive 2-ME$_{1}$ and that of peak 2 preparation are shown in Fig. 3-23 A and B respectively. HPLC profiles of peaks 1a, 1b and 3 preparations are not shown. However, the elution volumes of the largest SHBG-bindable peak obtained on HPLC of the above peak preparations are indicated by arrows in Fig. 3-23B. The SHBG-bindable peaks obtained by HPLC were converted to their corresponding methyloxime trimethylsilyl derivatives and analysed by GC-MS. Thus, the components of peak 2 were identified as follows (Fig. 3-23B): P: phthalate and hydrocarbon (Parafilm); Q: 3β-hydroxy-5β-pregnan-20-one; R: 20α-hydroxy-5α-pregnan-3-one; X: 17ε-hydroxy-estradiene-3-one; ε-hydroxy-5α-pregnan-3,20-dione; 21-hydroxy-5α-pregnan-3,20-dione, (5α-DHDOC) and 3ε-hydroxy-5ε-pregnan-11,20-dione; Y: not analysed - elution volume same as that of 2-methoxyestrone.

A summary of the results obtained when the SHBG-bindable peaks obtained by the HPLC of peaks 1a, 1b, 2 and 3 were subjected to GC-MS after derivatization to their corresponding methyloxime-trimethylsilyl ethers is given in Table 3-18. [The GC-MS analysis and the interpretation of mass spectral data were kindly done by Dr. Fennessey, University of Colorado.]

From the GC-MS data presented in Table 3-18 on the identification of compounds detected in the peak 1a, 1b, 2 and 3 preparations of urine, and from the data on the binding characteristics and chromatographic properties of these compounds on Sephadex LH-20, and maternal serum levels when known, the identities of the substances responsible for peaks 1a, 1b, 2 and 3 in pregnancy serum were deduced. Thus, peak 1a was identified as

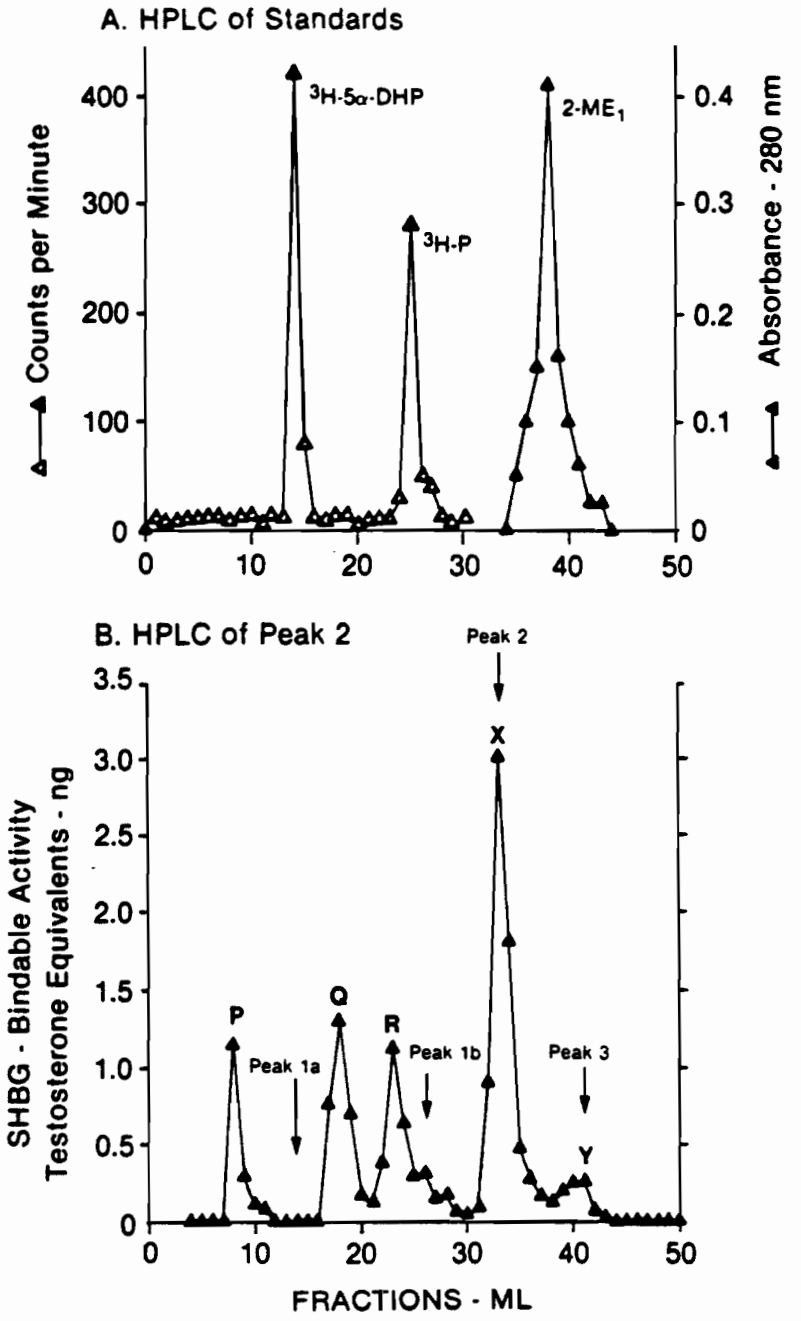


Figure 3-23. A. Elution profile of ^{3}H-5α-dihydroprogesterone (5α-DHP or 5α-pregnane-3,20-dione), ^{3}H-progesterone (P) and non-radioactive 2-methoxyestrone (2-ME$_1$) on HPLC. B. Elution pttern on HPLC of peak 2 obtained from Sephadex LH-20 chromatography. The elution volumes of the largest peaks obtained on HPLC of peaks 1a, 1b, 2 and 3 are indicated by arrows. See text for the data on the GC-MS analysis of peaks P, Q, R, X and Y. An Econosphere silica column of 5 micron particle size, 25 cm length and 4.6 mm internal diameter was used. The steroids were eluted using a gradient elution technique. The solvent system consisted of (A) methylene ch loride and (B) hexane:ethanol (90:10). For the first 10 minutes after the injection, 98% A and 2% B was run and for the next 50 minutes a gradient of 2-30% B (98-70% A) was applied. 1 ml fractions at a flow rate of 1 ml/min were collected.

Table 3-18: Mass spectrometric identification and methylene unit values (MU) of peaks obtained by GC:*

		MU	Approx. Amount ‡
Peak 1a:	5-cholestene-3β-ol (cholesterol)	31.37	-
	5α-pregnan-3,20-dione (5α-DHP)	28.56	20%
	5β-pregnan-3,20-dione (5β-DHP)	28.18	-
	20α-hydroxy-5α-pregnan-3-one (a pregnanolone)	29.27, 29.32	-
Peak 1b:	4-Pregnene-3,20-dione (progesterone)	28.99	80%
	20α-hydroxy-5α-pregnan-3-one (a pregnanolone)	29.27, 29.32	-
Peak 2:	17ε-hydroxyestradiene-3-one	27.03, 27.11	40%
	3β-hydroxy 5β-pregnan-20-one (a pregnanolone)	27.37	-
	20α-hydroxy-5α-pregnan-3-one (a pregnanolone)	29.27, 29.32	-
	ε-hydroxy-5α-pregnan-3,20-dione (a pregnanedionol)	29.24	-
	21-hydroxy-5α-pregnan-3,20-dione (5α-DHDOC)	30.62	-
	3ε-hydroxy-5ε-pregnan-11,20-dione (11-ketopregnanolone)	28.70	-
Peak 3:	2,3-dihydroxy-1,3,5(10)-estratriene-17-one-2-methylether (2-methoxyestrone, 2-ME$_1$)	28.24	**
	3α-hydroxy-5α-androstan-17-one (androsterone)	25.55	-
	5α-pregnane-3α,20α-diol (pregnanediol)	27.79	-
	3ε-hydroxy-5ε-pregnan-20-one (a pregnanolone)	25.68	-

* Data obtained by Dr. P.V. Fennessey or by Dr. C. Shackleton.
**Could not be estimated since the standard was impure.
‡ Approximate proportion in the sample used for GC-MS.

5α-DHP, peak 1b as progesterone, peak 2 as a 19-nor-compound (17β-hydroxy-1,5-estradiene-3-one) and peak 3 as 2-ME_1. Major factors which support these deductions will be discussed in chapter 4.

The chemical structures of the derivatives of 5α-DHP, P, the 19-nor-compound and 2-ME_1 which were used for MS and the origin of some major ions which will help explain the mass spectra are shown in Fig. 3-24.

The mass spectra obtained for methyloxime derivatives of standard 5α-pregnane-3,20-dione and the steroid isolated from the peak 1a preparation are shown in Fig. 3-25. The mass spectrum of the sample shows some background ions, most probably originating from silicone grease which was a contaminant of the extract (Dr. Fennessey assured me that silicone grease is a common, readily recognizable contaminant and that its presence did not interfere with the interpretation of the data).

The mass spectra obtained for the methyloxime derivative of standard progesterone and of the steroid isolated from peak 1b preparation are shown in Fig. 3-26. In the mass spectrum of the sample, although the major component was identified as progesterone, there were some low mass ions present which suggest the presence in low concentrations of a 17-hydroxy C21-steroid.

The mass spectra for the methyloxime-trimethylsilyl ether of standard 19-nor-T and of the 19-nor-compound isolated from peak 2 are shown in Fig. 3-27. The mass spectrum of the standard 19-nor-T, although it showed some similarity, was not identical to that of the isolated 19-nor-compound. However, no other standard having a more similar mass spectrum was found in a large collection of steroid reference spectra.

In Fig. 3-28, the mass spectra of methyloxime-trimethylsilyl ether of standard 2-ME_1 and of the steroid isolated from peak 3 preparation

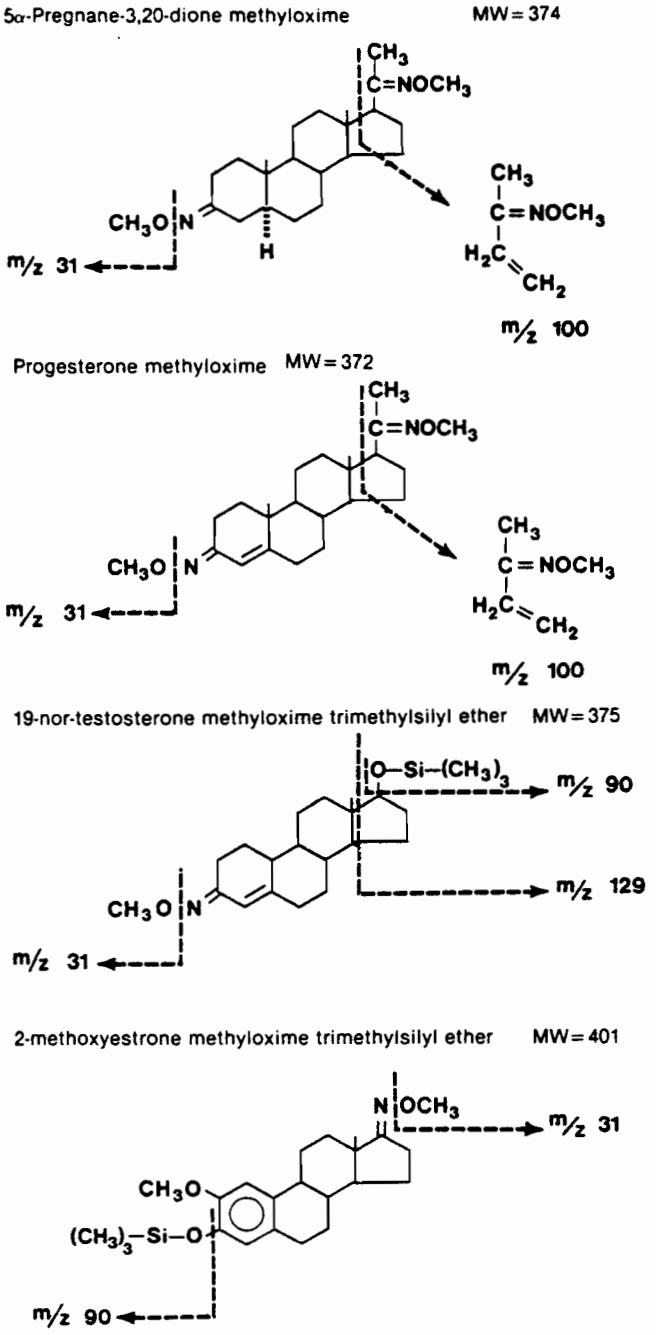


Figure 3-24. The chemical structures of 5α-pregnane-3,20-dione methyloxime, progesterone methyloxime, 19-nor-testosterone methyloxime trimethylsilyl ether and 2-methoxyestrone methyloxime trimethylsilyl ether. The origins of some major ions in the mass spectra of the above compounds are also shown.

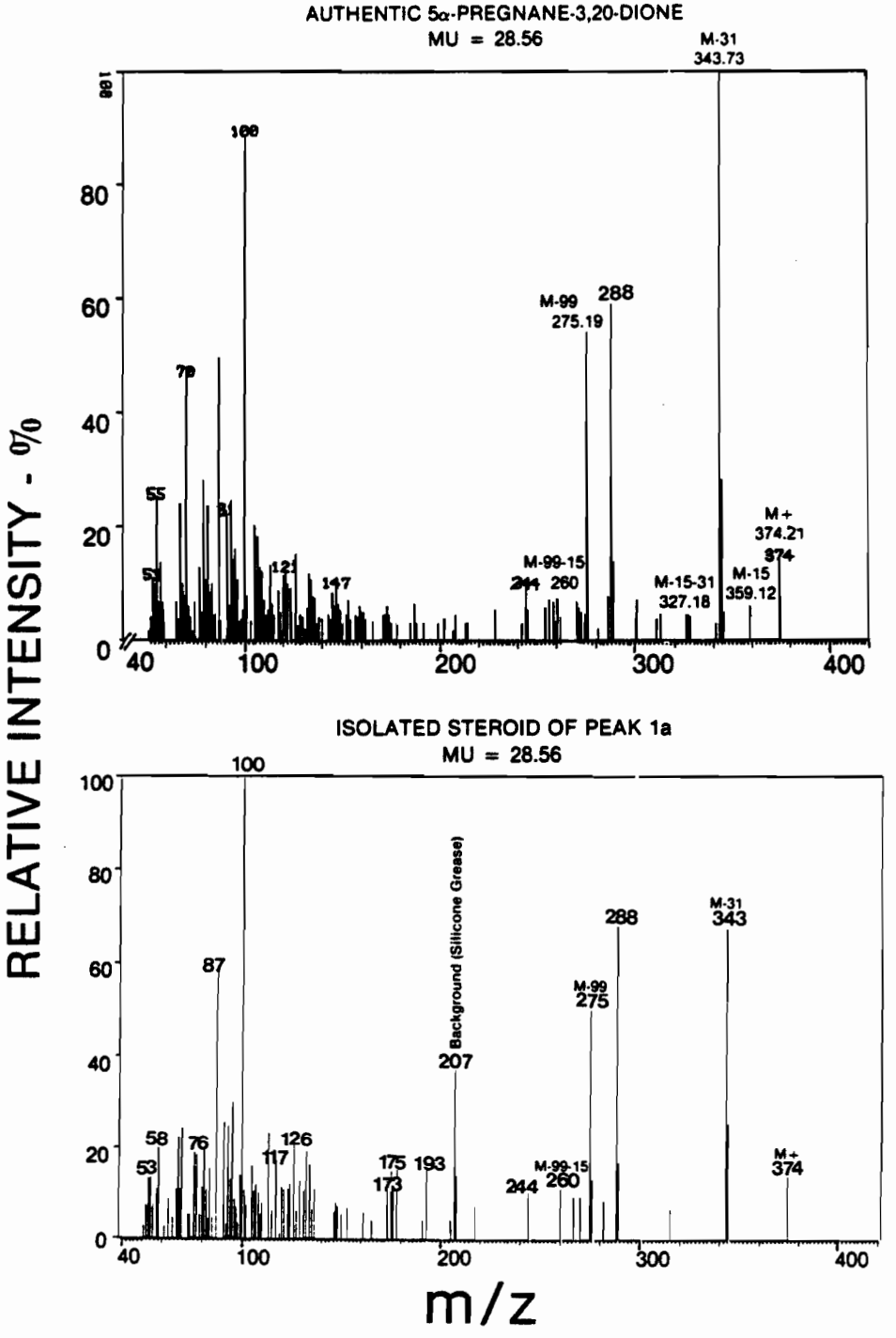


Figure 3-25. Mass spectra of authentic 5α-pregnane-3,20-dione methyloxime and the methyloxime derivative of the isolated steroid of peak 1a. The methylene unit value (MU) obtained on gas chromatogram is indicated.

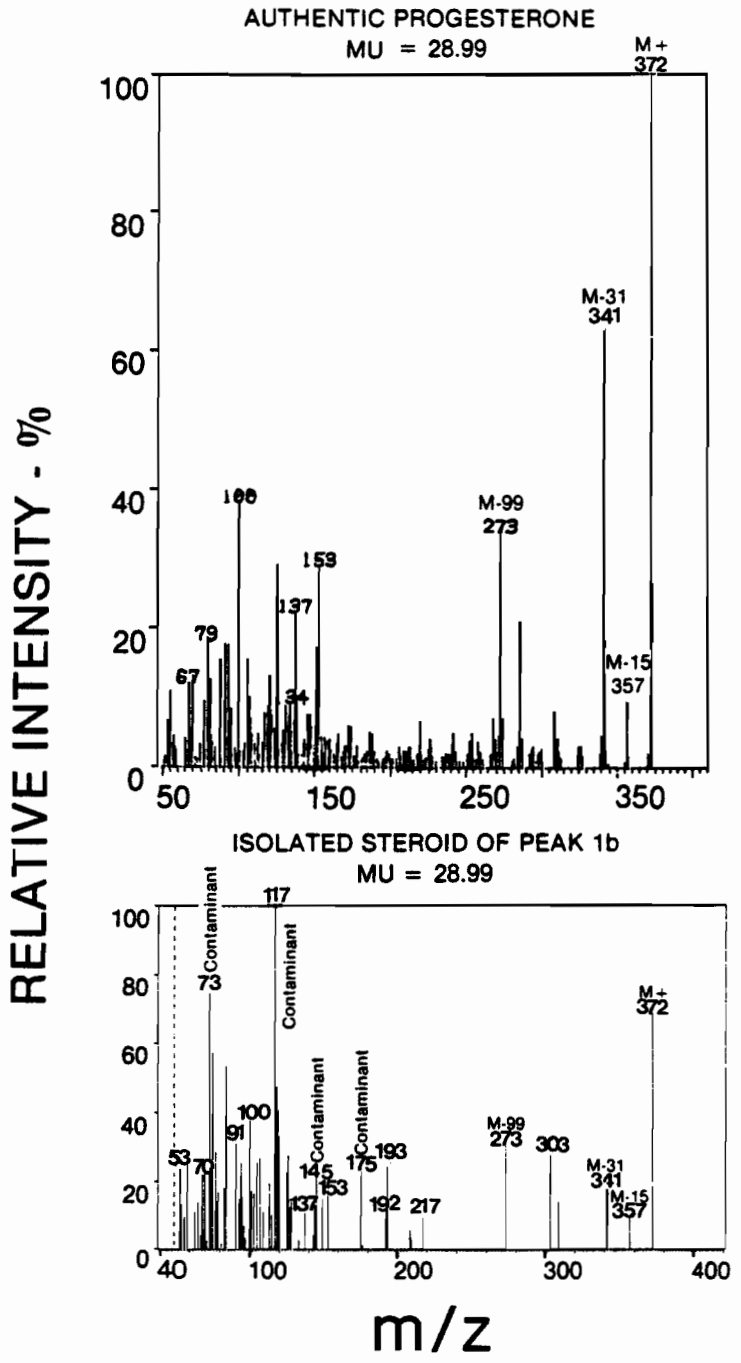


Figure 3-26. Mass spectra of authentic progesterone methyloxime and the methyloxime derivative of the isolated steroid of peak 1b. The methylene unit value (MU) obtained on gas chromatogram is indicated.

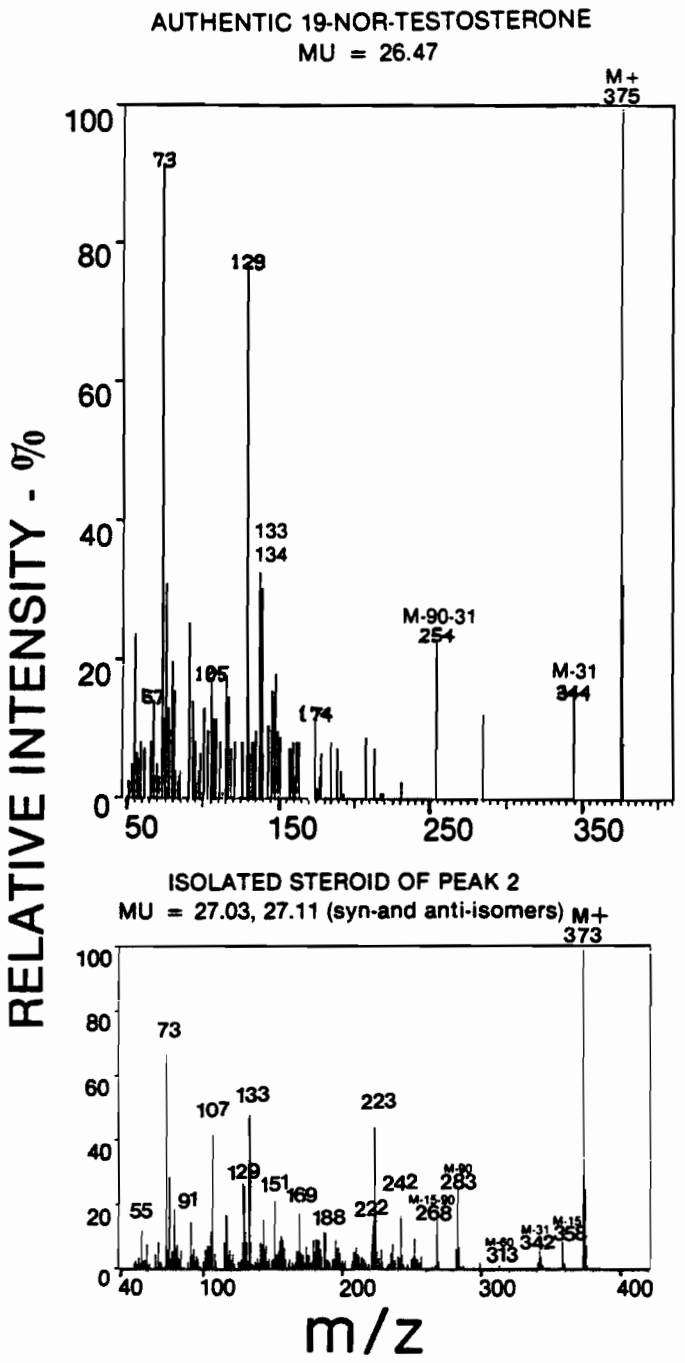


Figure 3-27. Mass spectra of authentic 19-nor-testosterone methyloxime trimethylsilyl ether and the methyloxime trimethylsilyl ether derivative of the isolated steroid of peak 2. The methylene unit value (MU) obtained on gas chromatogram is indicated.

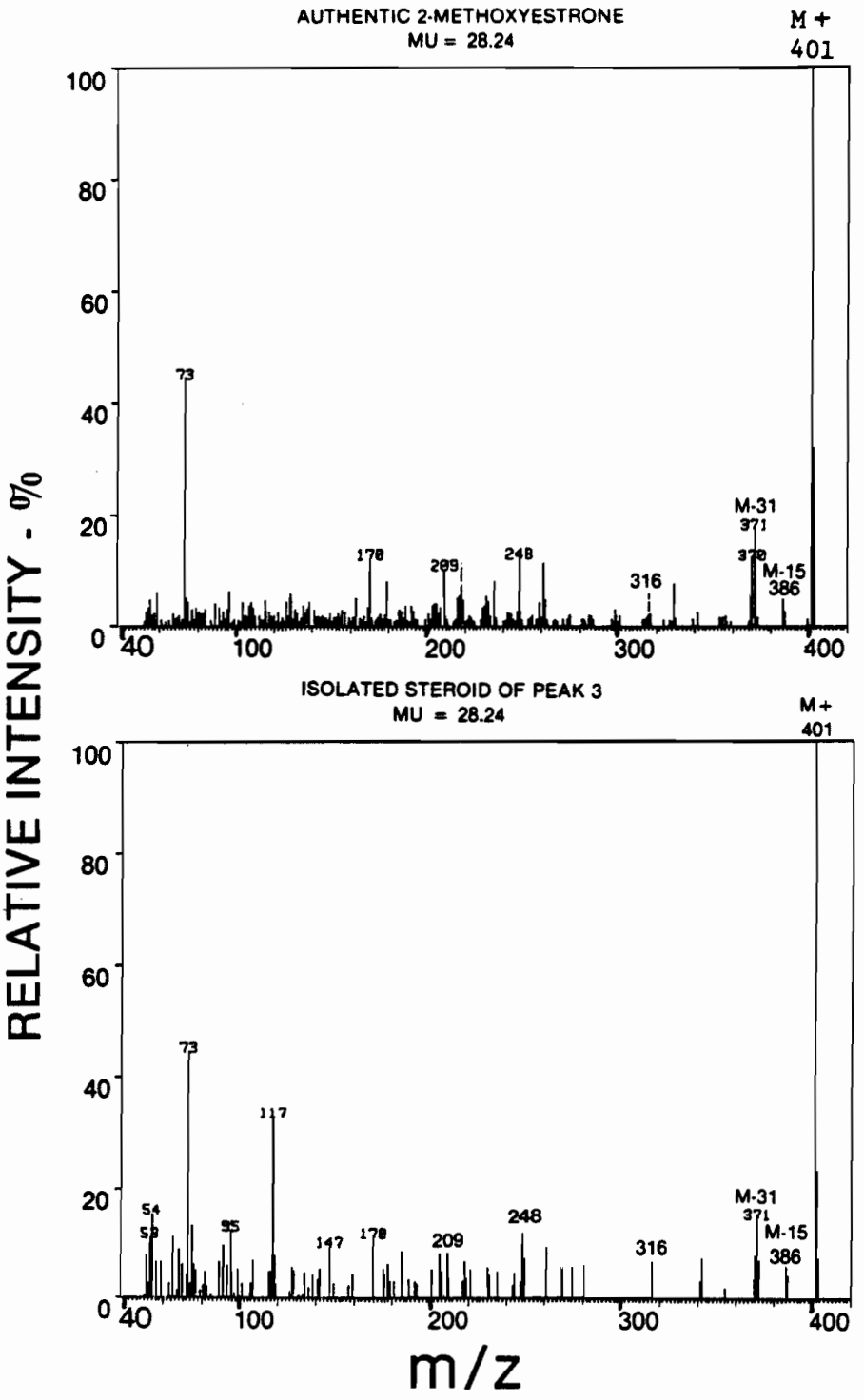


Figure 3-28. Mass spectra of authentic 2-methoxyestrone methyloxime trimethylsilyl ether and the methyloxime trimethylsilyl ether derivative of the isolated steroid of peak 3. The methylene unit value (MU) obtained on gas chromatogram is indicated.

are shown. 2-ME$_1$ was only a minor component in the peak 3 preparation. This may be explained by its labile nature and the long delay (6 months) involved in analysis after purification. The standard 2-ME$_1$ was also contaminated, showing several peaks on GC analysis.

Two of the steroids (3β-hydroxy-5β-pregnan-20-one and 20α-hydroxy-5α-pregnan-3-one) detected in the pregnancy urine as contaminants of the SHBG-bindable peaks have not previously been reported to occur in the human. The mass spectra of the methyloxime-trimethylsilyl ethers of these steroids and their corresponding standards are shown in Fig. 3-29 and Fig. 3-30, respectively. In the mass spectra of 20α-hydroxy-5α-pregnan-30-one, the M+(419), M-15(404), M-15-31(373) ions which are present in low intensity in the standard are absent in the sample (Fig. 3-30). This is due to the small amount of sample utilized for the GC-MS analysis.

Identification of each compound was based on both the GC elution and the MS data. The ion mass of the molecular ion and that of major fragment ions obtained by MS and the MU values obtained by GC are indicated on each mass spectrum. The results obtained from GC-MS suggest that, except for the 19-nor steroid isolated from peak 2, the retention times on GC and the fragmentation patterns on MS of steroids isolated from each peak were the same as those of their corresponding (postulated) standards.

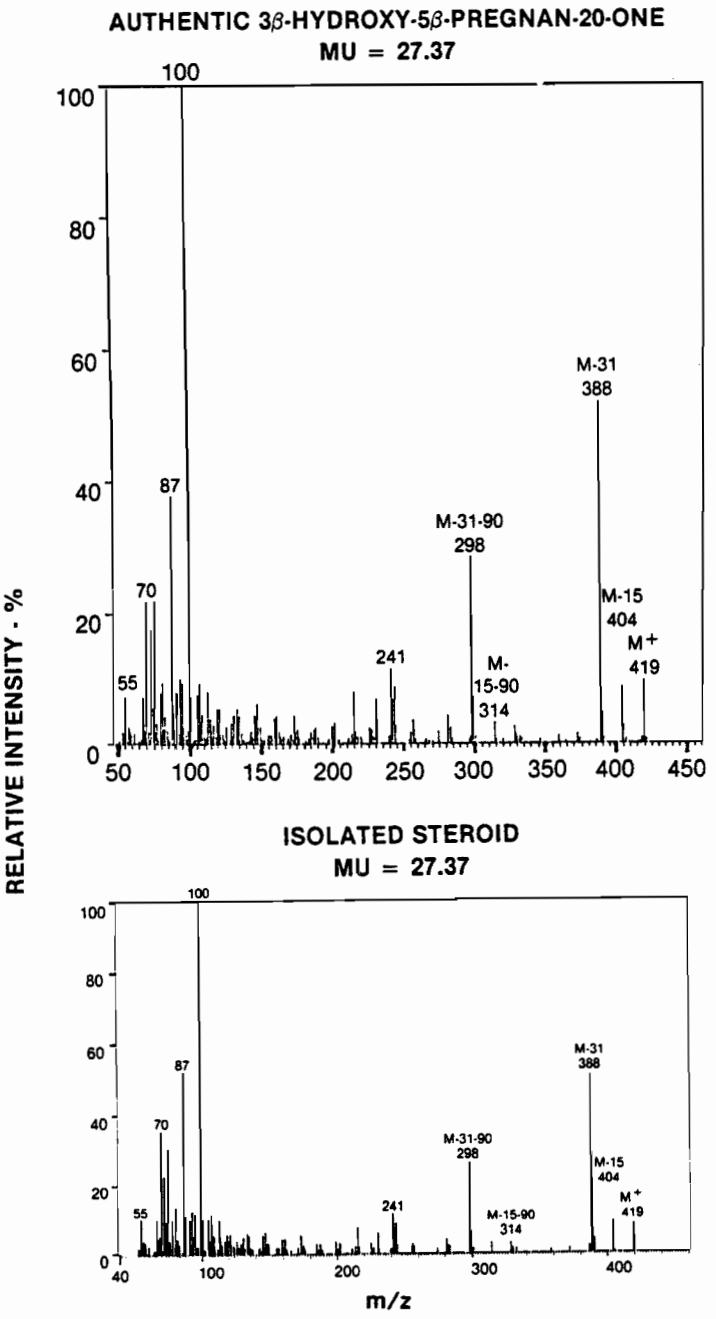


Figure 3-29. Mass spectra of the methyloxime trimethylsilyl ether derivatives of authentic 3β-hydroxy-5β-pregnan-20-one and of the isolated steroid. The methylene unit value (MU) obtained on gas chromatography is indicated.

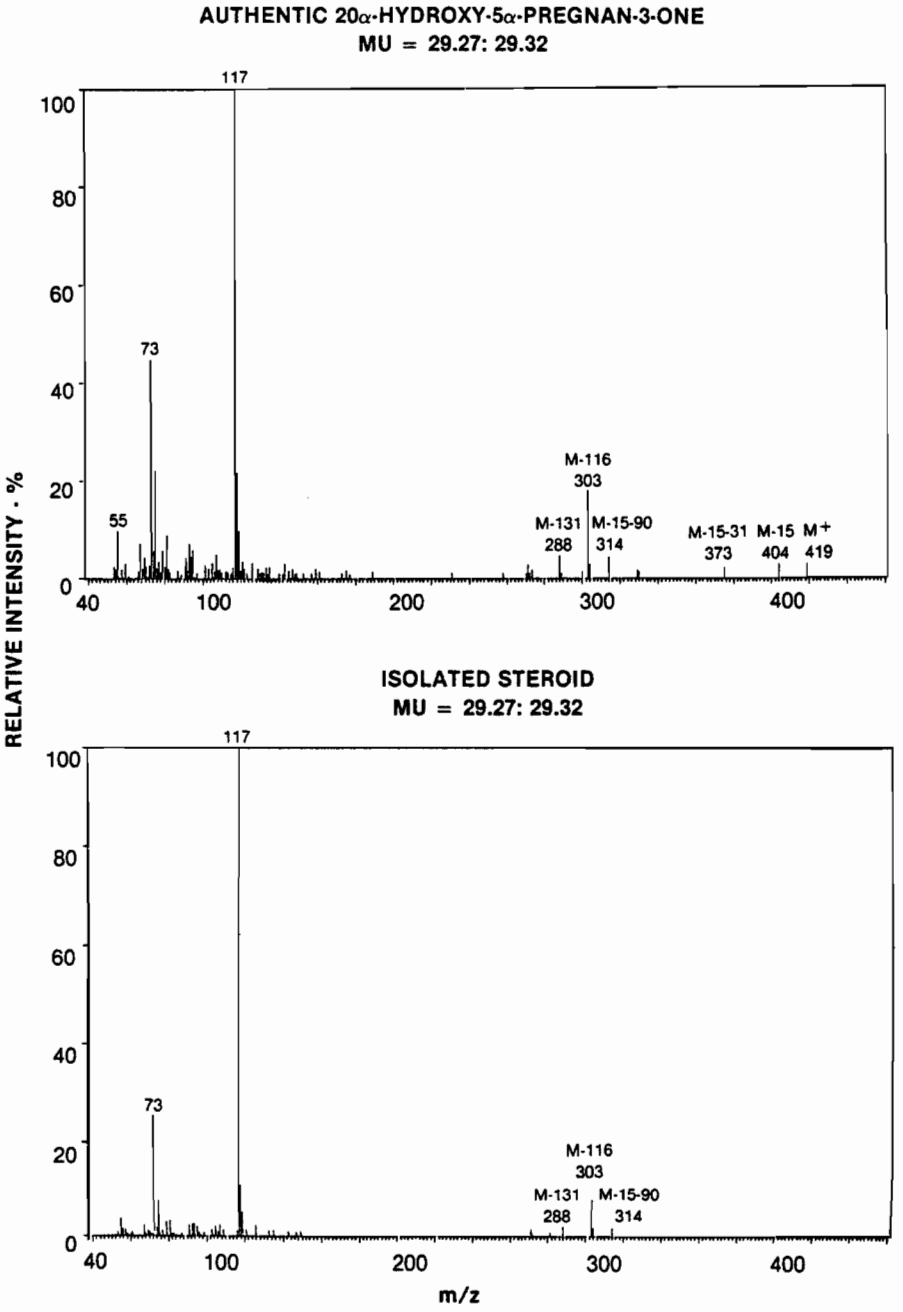


Figure 3-30. Mass spectra of the methyloxime trimethylsilyl ether derivatives of authentic 20α-hydroxy-5α-pregnan-3-one and of the isolated steroid. The methylene unit value (MU) obtained on gas chromatography is indicated.

CHAPTER IV: DISCUSSION

4.1 Study of the Significance of SHBG in the Transport of Estradiol and Testosterone

4.1.1 Introduction

The significance of steroid binding to SHBG is poorly understood. It is generally believed that the interaction of a hormone with specific steroid-binding plasma proteins such as SHBG is important in determining the availability of that hormone to target cells. Whatever the true function of SHBG is, whether it is the regulation of the metabolic clearance rate of its ligands by protecting them from cellular metabolism or whether it is direct participation in the mechanism of steroid hormone action, it is important to establish the fraction of hormone bound to SHBG in plasma (or serum). The study of estradiol and testosterone binding to SHBG is important not only for the elucidation of the mechanism of action of these classical sex steroids but also because the concentration of SHBG is regulated by these two steroids.

While there is general agreement that SHBG is a significant binder of plasma T *in vivo* (Mercier-Bodard et al, 1970; Murphy, 1968; Vermeulen, 1969; Ritzen et al, 1974), there is conflicting evidence as to the role of SHBG in the transport of E_2 *in vivo* (Murphy, 1968; Fisher et al, 1974; Vigersky et al, 1979). Further, only a few studies have described the distribution of E_2 and T into SHBG-bound, albumin-bound and unbound fractions in plasma or serum. The serum distribution of E2 and T in newborn infants has not been reported and with the single exception of the study of Dunn et al (1981) who used a computer simulation technique, there have been no reports on the distribution of T and E2 in pregnant women. The reported values of percentage of plasma T and E2 bound to SHBG

differ widely. Discrepancies are mainly due to methodological difficulties in making the measurements under physiological conditions. In all the studies so far reported, the percentage of T and E2 bound to SHBG was determined either under non-equilibrium conditions or was estimated indirectly.

In the present study new, more direct methods were developed for the determination of SHBG-bound and unbound fractions of E2 and T in undiluted serum by equilibrium dialysis at 37°C under isosmotic conditions. Using these methods the distribution of E2 and T into SHBG-bound, albumin-bound and unbound fractions was described in the serum of non-pregnant women, pregnant women, men and newborn infants. The results showed that SHBG was an important binder of serum E2 in both non-pregnant and pregnant women but not in men or newborn infants whereas T was significantly bound to SHBG under all circumstances.

4.1.2 Evaluation of dialysis procedure used

Preliminary studies carried out to test the validity of the method showed that no adsorption or other loss of ^{3}H-T and ^{3}H-E_2 occurred during the dialysis procedure (Table 3-1). The ^{3}H-T and ^{3}H-E_2 preparations used for the dialysis procedure were more than 98% pure. If the impurities in the tritiated steroid are not strongly bound to SHBG, these will be distributed equally on both sides of the dialysis membrane and result in an overestimation of the unbound fraction. Although it has been suggested that dilution of plasma may cause a reduction in the error caused by the presence of impurities in tracer preparations (Riverola et al, 1968; Vermeulen et al, 1971), the disadvantages of using diluted plasma are several which will be discussed below.

For the estimation of the serum distribution of a steroid, it is

important that only very small quantities of the tracer steroid be added to the serum for equilibration. Since the concentration of SHBG-bound ligands in the serum of pregnant women, newborn infants, non-pregnant women and adult men were estimated to be 32.5, 18.0, 2.1, and 10.8 ng/ml testosterone equivalents respectively, the amount of ^{3}H-T or ^{3}H-E_2 added (60-70 pg/ml serum) to serum represented less than 0.2% (pregnant women), 0.4% (newborn infants), 3.3% (non-pregnant women) or 0.7% (adult men) of total SHBG-bindable ligands present.

In the evaluation of the method it was also shown that equilibrium was achieved by 20 hours of dialysis at 37°C and that the SHBG binding sites remained intact during the course of dialysis (Fig. 3-2). In addition, evidence was presented to show that heating the serum at 60°C for 1 hour denatured SHBG (i.e. abolished specific binding) whereas albumin binding remained unaltered. Further, it was demonstrated that the dialysis procedure was highly precise (Table 3-2, 3-3).

In many previous studies when equilibrium dialysis was employed, high dialysate:sample ratios such as 10:1 have been used (Vigersky et al, 1979; Wu et al, 1976; Kley et al, 1977; Vermeulen et al, 1971; Forest et al, 1971). During dialysis, since the steroid concentration in the sample compartment changes as the unbound steroid diffuses across the semipermeable membrane, thus shifting the equilibrium, it is important to keep the volume of the dialysate small. As illustrated in Figure 4-1, a high volume of dialysate in relation to that of the sample will result in an exaggerated dissociation of the protein-steroid complex as more and more unbound steroid diffuses across the membrane to the dialysate compartment. This will cause a significant reduction in the steroid concentration in the sample compartment. Thus the concentration of steroid in the sample

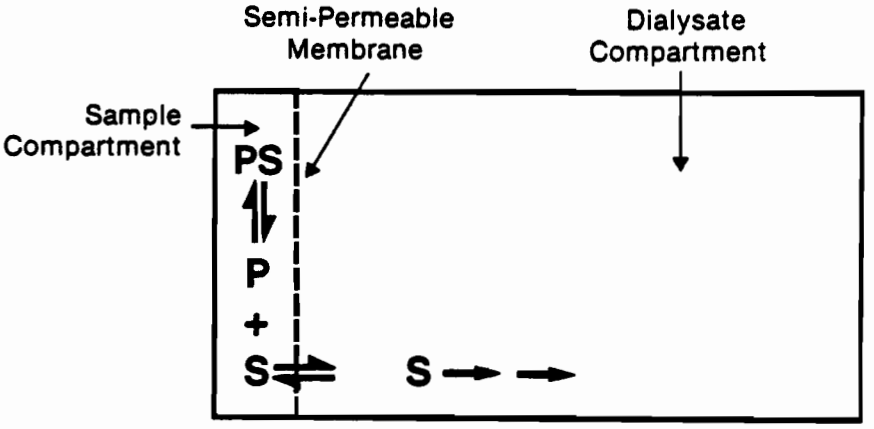


Figure 4-1. The limitation in using a high dialysate/sample ratio. During dialysis, the unbound steroid (S) in the sample equilibrates with the dialysate while the protein-steroid complex (PS) and the unbound protein (P) remain in the sample compartment. When the volume of dialysate is too large compared to that of the sample, the magnitude of change in the concentration of steroid in the sample compartment will be too high due to enhanced diffusion of unbound steroid out of the sample compartment.

compartment at the end of dialysis will be quite different from that of the original sample (Smith and Jubiz, 1980). In the present study, a sample:dialysate ratio of 1:1 was used and thus changes in the equilibrium during dialysis were minimized. A further decrease in the dialysate:sample ratio (1:9) did not alter the percentage T specifically bound to SHBG at least for late pregnancy serum (Table 3-2).

Although, testosterone has been shown to bind to CBG at high T concentrations (Shanbhag et al, 1973) and at low temperatures (Ritzen et al 1974) the results obtained in the present study showed that testosterone binding by CBG in undiluted serum at 37°C was insignificant at least for pregnant women and newborn infants (Table 3-4). This is consistent with the reports of others. Burke and Anderson (1972) using steady state gel filtration demonstrated that very large concentrations of cortisol (2.8 μM) caused no significant increase in unbound T levels at 37°C over a wide range of SHBG and T concentrations. In addition, Ritzen et al (1974) using steady state polyacrylamide gel electrophoresis have demonstrated that T binding by CBG in pregnancy serum was negligible at 37°C. Furthermore, Vermeulen (1977) by considering the association constants of SHBG, albumin, CBG and α1-acid glycoprotein for T and other competing steroids, and the serum concentration of these proteins and steroids, has shown that, for practical purposes, T binding by CBG is insignificant in human serum at 37°C.

When undiluted serum is dialysed against plain buffer, a true equilibrium cannot be reached because of the fluid shift from the buffer to the sample compartment due to the higher osmotic pressure of the serum. Fluid shift across the membrane will lead to a decrease in the concentration of protein in the sample. Although a correction factor for

the dilution effect has been used by many investigators, the increase in the sample volume can vary from sample to sample which would make the calculation of correction factor tedious and prone to error (Kley et al, 1977). In order to avoid fluid shift across the membrane, in many previous studies 1:5 diluted serum or plasma was used (Forest et al, 1973; Riverola et al, 1968; Chopra et al, 1972, 1973). However, in most of these studies the percentage unbound steroid in whole serum was calculated by linear extrapolation back to the undiluted state, using values obtained with 1:5 diluted serum. This is incorrect according to the law of mass action (Moll and Rosenfield, 1978). Moll and Rosenfield (1978) have also demonstrated that alteration of serum protein concentration (dilution of plasma) can actually alter binding site characteristics.

For the determination of percentage of T and E2 bound to SHBG, when native serum is dialysed against the same serum heated at 60°C for 1 hr, the only difference between the sample and the dialysate is the heat denaturation of heat-sensitive proteins in the dialysate. Since the concentrations of albumin are identical on the two sides, the percentage of SHBG bound can be directly determined. A direct experimental approach to determine the fraction of T and E2 specifically bound to SHBG in whole serum has not been described previously.

A pitfall in this procedure is that the ligand/SHBG ratio is doubled in the dialysis system, since the heated side contains the same concentration of ligand and no (native) SHBG. It is thus biased against binding by SHBG. Removal of steroids (e.g. by stripping) from the heated side would result in reducing the ligand/SHBG ratio compared to that in the pre-dialysis sample; such a change would bias the results in favour of SHBG binding.

For the determination of % unbound T and E2 in whole serum, since undiluted serum was dialysed against isosmotic dextran solution, changes in the concentration of protein in the sample compartment due to fluid shift were avoided. In addition, a buffer to sample ratio of 1:1 ensured that alteration in the total concentration of steroid in the sample compartment during the course of dialysis was minimal.

Data were also obtained to show that non-specific binding of T and E_2 by dextran was negligible. Furthermore, in the presence of SHBG which has an affinity of $1.4 \times 10^9\ M^{-1}$ and $6.4 \times 10^8\ M^{-1}$ for T and E_2, respectively, and albumin which has an affinity of $3.7 \times 10^4\ M^{-1}$ and $5.1 \times 10^4\ M^{-1}$ for T and E_2, respectively (Burke and Anderson, 1972), the influence of non-specific binding by dextran, if any, on % binding of T and E_2 in serum will be insignificant. The procedure for the determination of percentage unbound steroid in the present study is biased in favour of steroid binding since the dialysate consisted of isosmotic dextran solution devoid of any steroids. However, it is doubtful that this would affect the values for % unbound steroid significantly since the sample:dialysate ratio was low (1:1). Thus the true values for % unbound steroid should, if anything, be higher, than those reported in the present study.

The percentage of E_2 and T bound to albumin in the presence of SHBG was estimated indirectly from the experimentally determined values of percentage of tracer specifically bound to SHBG and of percentage of tracer unbound in whole serum. Thus the errors in the determination of this parameter will depend on the errors accrued in the measurement of SHBG-bound and protein-unbound fractions.

4.1.3 Comparison of the present methods with those previously described.

A detailed critical analysis of all the major procedures so far reported on T and E2 binding in serum has already been presented in Chapter 1 (Section 1.4.2). Each method has one or more drawbacks including non-equilibrium conditions, dilution, high dialysate-sample ratio, large sample requirement, low temperature, variable increases in sample volume or alteration in the concentration of low molecular weight substances in the sample compartment. Although results obtained under non-physiological conditions may give an index of binding, they may not provide the true value. Furthermore, in early studies, no attempts were made to describe the serum distribution of E_2 and T into SHBG-bound, albumin-bound and unbound fractions.

More recently, a few studies have described the distribution of E_2 and T in undiluted plasma at 37°C. A critical analysis of the methods used in these studies is given below. Moll et al (1981) employed a flow dialysis technique (section 1.4.2.10) to measure the percentage of unbound T and E_2 in undiluted plasma at 37°C. The fractions of T bound to SHBG and to albumin were then estimated under flow dialysis conditions by mathematical modelling using binding parameters obtained from a non-equilibrium charcoal assay. The fractions of E_2 bound to SHBG and albumin were estimated indirectly using equations relating the values of percentage of unbound T and E_2 obtained using flow dialysis procedure and the data on T binding to SHBG obtained by the charcoal adsorption method. A major disadvantage of this method is that it requires 10 ml of plasma. In addition, the method is tedious and involves addition of internal standard during dialysis and needs extraction and chromatographic

separation of steroids.

Dunn et al (1981) used a computer simulation technique to describe the fractions of E_2, T and 19 other steroids bound to SHBG, CBG and albumin in adult men, non-pregnant women and pregnant women. They determined the binding affinities of these endogenous steroids for both SHBG and CBG at physiological temperature and pH using a Concanavalin A Sepharose method. Together with the reported values for the binding affinities of these steroids to albumin, the total concentration of each steroid in plasma and the plasma concentration of SHBG, CBG and albumin, a computer program was developed to solve the complex interactions between the steroids and SHBG, albumin and CBG. The prediction of the in vivo plasma distribution of each steroid using this model will obviously depend on the accuracy of the binding parameters used for the calculation. The values for the binding parameters, except for the binding affinities of the steroids to SHBG and CBG, were obtained from the literature and vary depending on the laboratory and method used. As the authors have pointed out, small differences in the binding affinities may have profound effects on the calculated values of the distribution of steroids if those steroids have high affinity for plasma proteins. In addition, the plasma distribution values calculated by computer simulation will critically depend on the type and number of steroids included in the calculation. This is especially important in pregnancy since a major part of material bound to SHBG consists of unknown ligands (see section 3.2.1).

Södergard et al (1982) have employed a mathematical model, the principle of which was similar to that of the computer model developed by Dunn et al (1981), for the estimation of the distribution of E_2 and T in male and female plasma at 37°C. The model was based on the knowledge of

the binding capacity of SHBG, concentration of albumin and the concentrations of steroids that bind significantly to SHBG and the association constants of these steroids to SHBG and albumin. They have included the binding parameters of 5 major sex steroids, namely T, E_2, DHT, 5α-androstane-3α,17β-diol and 5-androstene-3β,17β-diol, in the calculation. It was assumed that these steroids accounted for all the ligands of SHBG present in plasma. The binding parameters of T, E_2 and DHT for SHBG and albumin were determined using a method based on the equilibrium partition of bound and unbound steroid in an aqueous 2 phase system (Shanbhag et al, 1973). Values for other parameters used in the calculation were taken from the literature. As already mentioned for the computer simulation technique of Dunn et al (1981), exclusion of ligands having high affinity or significant plasma concentration from the calculation or errors in the measurement of any of the binding parameters included in the calculation may result in grossly inaccurate values of plasma distribution of E_2 and T obtained using mathematical modeling.

The major advantages of the method used in the present study over the previously reported ones are the following: (i) no shift of fluid across the membrane, (ii) requires only small amounts of undiluted serum (ii) carried out at 37°C, (iii) low dialysate-sample ratio, and (iv) direct estimation of % SHBG-bound and unbound fractions. Little theoretical manipulation is therefore required.

4.1.4 Percentage of estradiol and testosterone bound to SHBG

The major conclusion from the present study is that SHBG does play a significant role in the transport of estradiol in vivo in women, especially during pregnancy. The study also showed that estradiol binding to SHBG was minimal in adult men and undetectable in newborn infants.

Testosterone was significantly bound to SHBG in all four types of sera. The present study has resolved the conflicting reports in the literature on the significance of SHBG in the transport of estradiol in women and men. It described for the first time the percentage of estradiol and testosterone bound to SHBG in newborn infants.

Estradiol was significantly bound to SHBG in women despite the fact that the experimental procedure was biased against steroid binding to SHBG. This finding is not in agreement with that of Vigersky et al (1979) who have reported that SHBG is not an important binder of plasma E2 in men and women under '<u>in vivo</u>' conditions. Although they used 3 different methods, each procedure had one or more drawbacks. They employed equilibrium dialysis at 37°C but 1:5 diluted, pooled plasma and a ratio of sample:dialysate of 1:10 were used. Using steady state polyacrylamide gel electrophoresis they were not able to demonstrate E2 binding to SHBG but it is not stated whether albumin binding of E2 was observed. When polyacrylamide gel electrophoresis is done under steady state conditions, steroid binding to albumin should be demonstrable.

Based on their data from dissociation rate analysis, the above authors reported that the $t_{\frac{1}{2}}$ of dissociation of ^{3}H-T but not ^{3}H-E2 was shortened by pre-incubating the plasma with saturating amounts of non-radioactive DHT. However, close examination of their data reveals that pre-incubation of plasma with saturating amounts of E2 shortened the $t_{\frac{1}{2}}$ of dissociation of ^{3}H-T from 10.8 min to 2.4 min, and that of ^{3}H-E2 from 8.9 min to 4.9 min. Furthermore, the $t_{\frac{1}{2}}$ of both E2 and T in whole plasma was much higher than that for albumin alone. These results suggest that plasma does contain saturable binding sites for E2 and that there is competition between T and E2 for binding sites. The authors, however, have not

commented on the reduction of $t_{\frac{1}{2}}$ of ^{3}H-T in plasma saturated with E2 and attributed the reduction of $t_{\frac{1}{2}}$ of ^{3}H-E2 in E2-saturated plasma to unknown saturable non-androgen binding sites. Since several extensive studies have demonstrated that SHBG is the only high-affinity binding protein for both T and E2 in human plasma (section 1.4.1), it is reasonable to conclude that the reduction of $t_{\frac{1}{2}}$ of ^{3}H-T and ^{3}H-E2 in plasma pre-incubated with saturating amounts of E2 is due to competition of T and E2 for SHBG binding sites.

Simultaneous determination of the percentage of E2 and T bound to SHBG in the sera of non-pregnant women, pregnant women, men and newborn infants has not been described previously. Comparison of percentage of E2 and of T bound to SHBG in the above sera showed that there were remarkable differences in the values (Fig.3-3). These differences may be explained by considering the differences in the concentrations of total SHBG-bindable ligands and the SHBG binding capacity in various types of sera. The concentration of total SHBG-bindable ligands in testosterone equivalents, obtained in the present study as determined by competitive binding to SHBG in the serum of non-pregnant women, pregnant women, newborn infants and men, and the values for the concentration of SHBG binding sites in those sera reported by Anderson et al (1976) are shown in Figure 4-2 (top). The SHBG binding capacity was calculated by Anderson et al as the mass of specifically bound DHT per ml of plasma under conditions in which all binding sites were occupied. Since there is only one binding site per molecule of SHBG, the value will remain approximately the same when expressed as mass of T specifically bound. The spectrum of values for total SHBG-bindable ligands in serum obtained in the present study was: pregnant women > newborn infants > men > nonpregnant women and that for the

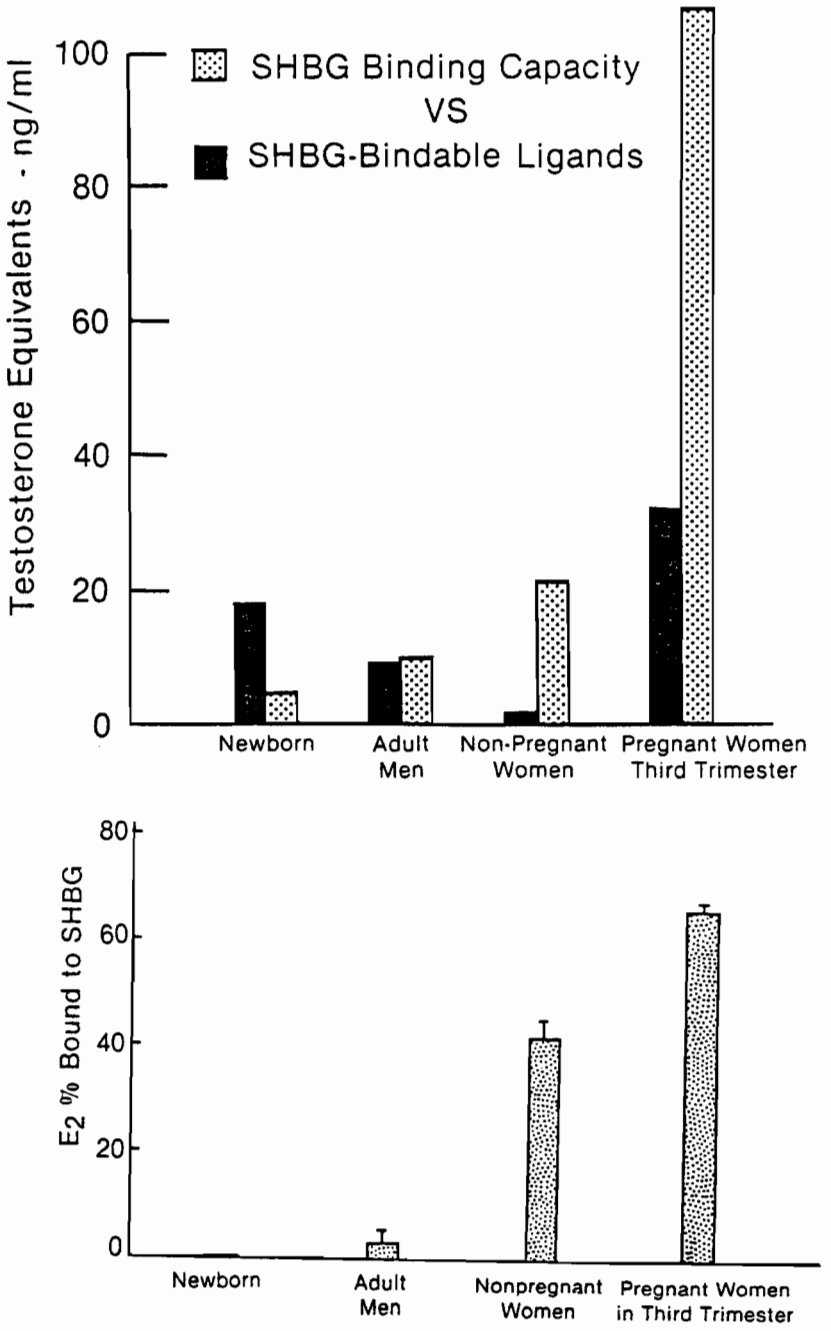


Figure 4-2. TOP: Comparison of the SHBG binding capacity and the concentration of total SHBG-bindable material in different types of sera. Values for SHBG concentration (stippled bars) in sera were taken from Anderson et al., 1976. The concentration of SHBG-bindable material (solid bars) was measured as the total concentration of all ligands in serum that can displace ^{3}H-testosterone from the SHBG binding sites when non-radioactive testosterone was used as the standard. BOTTOM: Percentage of estradiol bound to SHBG in different types of sera. The data are taken from Figure 3-3.

concentration of SHBG binding sites in serum: pregnant women > non-pregnant women > men > newborn infants. In the serum of non-pregnant and pregnant women, the SHBG binding capacity far exceeded the concentration of total SHBG-bound material present. However, in adult men, the concentration of SHBG-bindable material and SHBG binding capacity were approximately equal. In the newborn infants, on the other hand, the concentration of SHBG-bindable ligands was at least 3 times that of the binding capacity of SHBG. Thus, the number of unoccupied binding sites is high in the serum of non-pregnant and pregnant women while it is minimal in the serum of adult men and virtually non-existent in the serum of newborn infants.

The results obtained in the present study for the % E_2 and % T bound to SHBG in the 4 types of sera are consistent with the above explanation, i.e. the major factors that determine the binding of E_2 and T to SHBG in whole serum are the concentration of SHBG binding sites and the concentration of total SHBG bindable ligands. The data shown in Fig. 4-2 (bottom) indicate that the percentage of E_2 bound to SHBG in different types of sera varied with the concentration of unoccupied binding sites in those sera. The failure to demonstrate E_2 binding to SHBG in the newborn infants can be explained by the low levels of SHBG binding capacity and the high concentration of other competing ligands in their sera. In late pregnancy serum, although the total concentration of SHBG-bindable ligands is approximately 15-fold higher than that in non-pregnancy serum, the 5-fold or higher SHBG binding capacity results in an increased percentage of SHBG-bound E_2 compared with that in non-pregnancy serum. [Data on the nature of these unknown competing substances present in pregnancy serum and on the evidence which shows that these substances are

in fact bound to SHBG under physiological conditions have been presented in section 3.2.].

Changes in the percentage of T binding to SHBG in different types of sera paralleled the changes in the percentage binding of E_2 to SHBG. However, since the affinity of E_2 for SHBG is only half and for albumin is double that of T (Burke and Anderson, 1972), the presence of competing ligands and the differences in the concentrations of SHBG resulted in more pronounced changes in the percentage of E_2 binding relative to changes in percentage of T binding. Thus changes in the concentration of SHBG binding sites and of competing ligands will play a more important role in the transport and distribution of E2 than of T.

Other factors may also be of some importance. These include the individual concentration and binding affinity of each of the competing ligands for SHBG and albumin. In addition, the concentrations and the affinities of other binding proteins that may exist for the competing ligands may be important. Although unlikely, another possible explanation for the different SHBG-bound values in different types of sera is that the nature of SHBG in serum may vary among groups. While it is true that extensive microheterogeneity with respect to size and charge has been reported for purified SHBG, the purified protein may differ from native SHBG (Petra et al, 1983; Cheng et al, 1983). Further, to date there have been no reports of any differences in the binding characteristics of SHBG within the same species.

4.1.5 Comparison of the values for the percentage SHBG-bound E2 and T with those previously reported

Values for the fraction of E2 and T bound to SHBG obtained in the present study and in the 3 other major studies that have been reported to

date (Moll et al, 1981; Dunn et al, 1981; Sodergard et al, 1982) are shown in Tables 4-1 and 4-2. Values for the albumin-bound fraction and the unbound fraction are also shown. The latter two will be discussed later.

The value for % E2 bound to SHBG in non-pregnant women obtained in the present study (41.7 ± 2.8) is comparable to that reported by Moll et al, 1981 (41 ± 10), Dunn et al, 1981 (37.2) and Sodergard et al, 1982 (45.4). However, the % E2 bound to SHBG in pregnant women in the present study (66.2 ± 0.4) is lower than the value of 87.8 reported by Dunn et al (1981), the only previous study describing this parameter. Although the percentage of E2 bound to SHBG in newborn infants was not investigated in above mentioned studies, Abramovich et al (1978) using steady state polyacrylamide gel electrophoresis, have reported that E2 binding could be demonstrated only to albumin in fetal serum at mid-pregnancy and at term. This is consistent with the failure to show E2 binding in the serum of newborn infants in the present study. The value for % E2 bound to SHBG in men obtained in the present study (2.7 ± 1.5) was considerably lower than that reported by Moll et al, 1981 (21 ± 7), Dunn et al, 1981 (19.6) and Sodergard et al, 1982 (27.9).

The higher values for % E2 bound to SHBG in pregnant women and adult men reported in the previous studies may be due to the indirect methods used. In all these studies percentage of E2 bound to SHBG was determined indirectly using a physicochemical model (Moll et al, 1981), computer simulation (Dunn et al, 1981) or a mathematical model (Sodergard et al, 1982). As mentioned earlier (section 4.1.3), the estimation of distribution of steroids in plasma by these authors depended critically on the number and nature of ligands included in the calculation and the accuracy of binding parameters obtained in different laboratories. In the

TABLE 4-1. Serum distribution of estradiol obtained in the present study and in those reported in the literature.

Reference	% E_2 bound to SHBG				% E_2 bound to Albumin				% Unbound			
	non-preg. women	preg. women	newborn infants	adult men	non-preg. women	preg. women	newborn infants	adult men	non-preg. women	preg. women	newborn infants	adult men
Present study	41.7±2.8	66.2±0.4	undetec-table	2.7±1.5	56.3±2.8	32.5±0.4	95.6±0.2	94.8±1.5	1.98±0.13	1.29±0.09	4.38±0.17	2.50±0.27
Moll et al, 1981	41±10	ND Φ	ND	21±7	59±10	ND	ND	79±7	2.62±0.43	ND	ND	3.40±0.35
Dunn et al, 1981	37.2*	87.8	ND	19.6	61.0*	11.7	ND	78.0	1.82*	0.49	ND	2.32
Sodergard** et al, 1982	45.4	ND	ND	27.9	52.5	ND	ND	69.7	2.03	ND	ND	2.45

Φ Not done
* Follicular phase values were used for calculations
** Maximum concentrations (reported in literature) of androgen metabolites were used for calculations

TABLE 4-2. Serum distribution of testosterone obtained in the present study and in those reported in the literature.

Reference	% T bound to SHBG				% T bound to Albumin				% T Unbound			
	non-preg. women	preg. women	newborn infants	adult men	non-preg. women	preg. women	newborn infants	adult men	non-preg. women	preg. women	newborn infants	adult men
Present study	77.7±3.5	79.3±2.7	28.8±4.9	30.3±5.0	18.7±3.5	18.1±2.7	65.2±5.0	65.3±5.0	3.63±0.11	2.64±0.21	6.09±1.06	4.41±0.43
Moll et al, 1981	56±9	ND$^{\phi}$	ND	33±9	44±9	ND	ND	67±9	1.89±0.44	ND	ND	2.80±0.41
Dunn et al, 1981	65.9*	95.4	ND	44.3	30.6*	3.60	ND	49.9	1.37*	0.23	ND	2.23
Sodergard** et al, 1982	61.5	ND	ND	42.7	37.0	ND	ND	55.3	1.48	ND	ND	2.01

ϕ Not done
* Follicular phase values were used for calculations
** Maximum concentrations (reported in literature) of androgen metabolites were used for calculations

estimation of percentage steroid bound to SHBG in pregnant women by Dunn et al, the unrecognized SHBG ligands which are present in high concentrations in pregnancy serum were omitted from the calculation of the computer program. Similarly, the calculation of percentage steroid bound to SHBG in adult men, Dunn et al (1981) and Södergard et al (1982) included only a limited number of steroids. For example, 5 α-androstane-3 β, 17 β-diol was left out in both studies.

It should be considered here, however, that the present study is biased against steroid binding to SHBG. In the dialysis system used for the determination of percentage steroid bound to SHBG, the heated side (dialysate) contained the same concentration of ligand and no SHBG. Thus although E_2 binding by SHBG was undetectable in the newborn serum by the method used in the present study, SHBG may play a minimal role in the transport of E_2 in newborn infants. The concentration of SHBG-bindable ligands is greater than SHBG binding capacity in their sera (Fig. 4.2). Similarly, the values for percentage of E_2 bound to SHBG in adult men may be somewhat higher than those obtained in the present study. However, they are unlikely to be as high as the values reported in the previous studies. The values of percentage E_2 bound to SHBG obtained for non-pregnant and pregnant women would most probably not be affected by the higher ligand/SHBG ratio in the dialysis system since the SHBG binding capacity in the sera of both non-pregnant and pregnant women is several fold higher than the concentration of total SHBG-bindable ligands present. Indeed, changing the ratio of native serum:heated serum from 1:1 to 9:1 for dialysis did not alter percentage of T bound to SHBG for the serum of pregnant women (Table 3-2).

Despite the fact that the method used for the determination of the %

bound SHBG fraction was biased against steroid binding to SHBG, the % T bound to SHBG in non-pregnant women in the present study (77.7 ± 3.5) was higher than that reported by Moll et al, 1981 (56 ± 9), Dunn et al, 1981 (65.9) and Sodergard et al, 1982 (61.5). The % T bound to SHBG obtained in pregnant women in the present study (79.3 ± 2.7) was lower than that (95.4) in the only other study that has described % T bound to SHBG in pregnancy serum (Dunn et al, 1981). The percentage of T bound to SHBG in adult men in the present study (30.3 ± 5) was similar to that of Moll et al, 1981 (33 ± 9). However, it was lower than that reported by Dunn et al, 1981 (44.3) and Sodergard et al, 1982 (42.7). As mentioned earlier for E2 binding to SHBG, the higher values obtained in pregnant women by Dunn et al (1981) and adult men by Dunn et al (1981) and by Sodergard et al (1982) is most likely due to the failure to include all the SHBG-bindable ligands in the calculations. The percentage of T bound to SHBG in the serum of newborn infants was not investigated in previous studies.

4.1.6 Percentage of unbound estradiol and unbound testosterone in serum

Comparison of the % unbound values of E2 and T in different types of sera (Fig.3-3) shows that the % unbound values in those sera are reciprocally related to the values of percentage bound to SHBG. These results suggest that the extent of SHBG-binding of E2 and T largely determine the % unbound E2 and T in those sera.

The percentage of unbound E2 in whole serum in non-pregnant women obtained in the present study (1.98 ± 0.13) is comparable to that obtained by Dunn et al, 1981 (1.82) and by Sodergard et al, 1982 (2.03) and is slightly lower than that reported by Moll et al, 1981 (2.62 ± 0.43). In pregnant women, the % unbound E2 obtained in the present study (1.29 ±

0.09) is markedly higher than that reported by Dunn et al, 1981 (0.49), the only other study which has estimated this parameter. In adult men, the % unbound E2 obtained in the present study (2.50 ± 0.27) was similar to that of Dunn et al, 1981 (2.32) and Sodergard et al, 1982 (2.45) and slightly lower than that reported by Moll et al, 1981 (3.40 ± 0.35). Percentage of unbound E2 in the serum of newborn infants has not been determined in previous studies.

The only major discrepancy when the values for the % unbound E2 in different types of sera obtained in the present study are compared with those reported in previous studies is in the markedly higher value obtained in pregnant women. Dunn et al (1981), the only other group to have studied this parameter, reported a value which is less than half of that obtained in the present study. As mentioned earlier, the omission of unrecognized SHBG-bindable ligands present in large concentrations in pregnancy serum from their calculations would account for the lower value.

Although the values for % E_2 unbound in the present study were similar to those of previous studies, the % unbound T for all types of sera in the present study was higher than those in previous studies (Moll et al, 1981; Dunn et al, 1981 and Södergard et al, 1982). This may be due to the differences in the methods employed or the subjects studied.

It should be mentioned here that metabolism of T during dialysis, although unlikely, was not tested in the present study. Metabolism of T to non-binding metabolites could result in higher % unbound T values. Similarly it is also not known whether more frequent, rigorous purification of ^{3}H-T used would decrease % unbound T values.

4.1.7 Percentage of estradiol and testosterone bound to albumin

The percentage of E2 and of T bound to albumin was derived

from the experimentally determinated values of % bound to SHBG and % unbound in whole serum. A discussion of the values obtained for the latter two parameters has already been presented above.

The ability of albumin to bind approximately 96% of E2 in the absence of the influence of SHBG in the sera of pregnant women and newborn infants (Fig 3-4) may be explained by the fact that although the affinity of albumin for E2 is four orders of magnitude lower than that of SHBG (Burke & Anderson, 1972), the binding capacity of albumin in serum is far greater than the concentration of albumin ligands present.

The similar values for the % E2 bound to albumin in the presence and absence of SHBG in the serum of newborn infants is as expected since there was no detectable binding of E2 to SHBG in those sera. By contrast, in late pregnancy serum which has a high concentration of SHBG, the % E2 bound to albumin in the absence of SHBG was markedly higher than that observed in the presence of SHBG.

The higher values observed for % T than E2 bound to albumin in the absence of influence of SHBG in the sera of both newborn infants and pregnant women is consistent with the report that the affinity of albumin for E2 is greater than that for T (Burke and Anderson, 1972). Although the % of T bound to albumin in the absence of SHBG was markedly higher than that in the presence of SHBG in both newborn infants and pregnant women, the magnitude of difference between the value obtained in the absence and the presence of SHBG was much greater for pregnant women. This is presumably due to the 20-fold higher concentration of SHBG in the serum of pregnant women than in the serum of newborn infants (Anderson et al, 1976).

4.1.8 Total, SHBG-bound, albumin-bound and unbound concentrations of estradiol and testosterone in serum

The accuracy of the concentrations of SHBG bound, albumin-bound, and unbound E2 and T in serum is dependent on the accuracy of the total concentrations of E2 and T measured in serum. The total levels of T obtained in the present study were in agreement with those noted in previous studies for non-pregnant women (Rosenfield, 1975; Moll et al, 1981), pregnant women (Rivarola et al, 1968; Bammann et al, 1980), newborn infants (Saez and Bertrand, 1969; Abramovich et al, 1978) and adult men (Kley et al, 1977; Moll et al, 1981).

The total concentrations of E2 measured in the present study were in good accord with those reported for pregnant women (Antonipillai and Murphy, 1977; Tulchinsky et al, 1972) and newborn infants (Antonipillai and Murphy, 1977). For non-pregnant women and adult men, although chromatography was not done, the measured E2 levels were comparable with those reported for non-pregnant women (Wu et al, 1976; Wright et al, 1978) and for men (Moll et al, 1981; Wright et al, 1978). These values, however, were higher than those reported more recently for non-pregnant women (McKennna et al, 1983) and men (Nagasue et al, 1985). The higher values in the earlier studies may be explained by the cross reactivity of the antibody with other steroids. Since E2 levels measured in the present study were influenced by the presence of estrone, the total, SHBG-bound, albumin-bound and unbound values for non-pregnant women and men can be up to 40% higher than the true values.

4.1.9 Physiological significance of the binding of estradiol and testosterone to SHBG

The results obtained in the present study established the values

for the fraction of E2 and T bound to SHBG under near physiological conditions. While these data do not directly answer the question as to the the physiological significance of steroid binding to SHBG, they do provide the basis for further studies on its role in the mechanism of steroid hormone action.

One of the basic questions to be answered in this area is whether it is the fraction bound to specific plasma binding proteins such as SHBG or the unbound fraction which is the physiologically active moiety (see Section 1.3.5 for details). According to the first hypothesis which is widely held, the SHBG-bound fraction of steroid cannot enter cells and acts as an inert reservoir for the unbound fraction which is the physiologically active one. According to the more recent second hypothesis, the SHBG-bound fraction can enter the target cells and constitutes the active moiety, i.e. interacts with DNA, while the unbound hormone which can diffuse freely into cells gets metabolized (Siiteri et al, 1982).

Comparison of the levels of SHBG-bound and unbound estradiol in the sera of non-pregnant women, pregnant women, men and newborn infants (Table 3-7) shows that the concentration of unbound E2 in newborn infants (382 ± 132 pg/ml) was even higher than in pregnant women (213 ± 64 pg/ml) while the concentration of SHBG-bound E2 was undetectable in newborn infants. Similarly, the unbound T concentration in the newborn infants (87.2 ± 20.3 pg/ml) was almost double that in pregnant women (46.3 ± 9.7 pg/ml) while the SHBG-bound T concentration in newborn infants (0.42 ± 0.09 ng/ml) was the same as in non-pregnant women (0.44 ± 0.15 ng/ml). Thus, the newborn is exposed to very high concentrations of unbound E2 and unbound T. The lack of any adverse effects on the newborn despite the exposure to these very high levels is inconsistent with the concept that

it is the unbound hormone which is the physiologically active portion. The protection of the newborn from the high levels of E2 and T can be explained if one considers that it is the SHBG-bound fraction which is physiologically active, since the SHBG-bound E2 was undetectable and SHBG-bound T was very low in newborn infants. However, it is possible that other factors are involved, e.g. some other hormone, also elevated during pregnancy may oppose the action of E2 and T, or there may be a low sensitivity of target tissues.

An often-used argument in favour of the concept that it is the unbound hormone which is active is that the pregnant woman, despite having very high levels of E2 and T suffers no adverse effects because there is an associated increase in the % bound fraction due to the elevated levels of SHBG. However, data obtained in the present study show that, in the pregnant women, the unbound concentration of E2 (213 ± 64 pg/ml) is at least 70 times higher than in nonpregnant women (3.1 ± 2.0 pg/ml- an overestimate). Similarly, the unbound concentration of T in pregnant women (46.3 ± 9.7 pg/ml) is more than double that in nonpregnant women (20.5 ± 6.9 pg/ml). The levels of SHBG-bound E2 (10.9 ± 3.2 ng/ml)and SHBG-bound T (1.4 ± 0.3 ng/ml) in the pregnant women are also much higher than in the non-pregnant women (0.065 ± 0.042 ng/ml and 0.44 ± 0.15 ng/ml for SHBG-bound E2 and T, respectively). It may be that there is increased action of E2 and T to meet the requirements of pregnancy. Similarly, both unbound E2 and SHBG-bound E2 concentrations in non-pregnant women (3.1 ± 2.0 pg/ml and 0.065 ± 0.042 ng/ml, respectively) are higher than in men (1.1 ± 0.2 pg/ml and 0.001 ± 0.0006 ng/ml, respectively). Also, both unbound T and SHBG-bound T concentrations in men (334 ± 99 pg/ml and 2.30 ± 0.74 ng/ml, respectively) are higher than in non-pregnant women (20.5 ± 6.9

pg/ml and 0.44 ± 0.15 ng/ml, respectively). Since the concentration of unbound E2 occupies only a small fracton of intracellular receptors in target tissues in non-pregnant women, the amount of unbound steroid alone may be insufficient for estrogen action (Siiteri et al, 1982). If one assumes that SHBG-bound E2, which is present in much higher concentrations is available for intracellular action, it would explain the discrepancy between the amount of E2 available versus the intracellular E2 receptor concentration. The discrepancy between unbound concentration and intracellular receptor levels is also present for T, though to a lesser degree.

In comparison with the previously described methods, the procedure developed in the present study for the estimation of the serum distribution of E2 and T is not only simple, reproducible and more direct but also more closely approximates physiological conditions. In addition, the method is applicable to the study of the distribution and transport of various endogenous steroids bound to SHBG in a variety of physiological situations. The data obtained in the present study resolved the controversy as to the significance of SHBG in the transport of E2 in undiluted serum at 37°C. In addition, comparison of the values obtained for the distribution of E2 and T in different types of sera suggested that the major factors influencing the serum distribution of E2 and T are the levels of SHBG-binding sites and the concentrations of competing ligands, and that these factors affect the binding of E2 to SHBG more than that of T. Further, the present study suggests that the first hypothesis on the role of SHBG cannot explain all the results obtained. It is suggested that the second hypothesis, which evokes a more direct role for SHBG in the mechanism of steroid hormone action, requires exploration. It may be that

SHBG has a dual function- one involving the regulation of metabolic clearance rate of steroid hormones by protecting them from cellular metabolism; and the other involving a direct participation in the mechanism of steroid action.

4.2 Characterization of Certain Previously Unrecognized Ligands of SHBG in Pregnancy

4.2.1 Introduction

The "total androgen levels" as measured by competitive binding to SHBG in pregnancy serum have been shown to be 10-15 fold higher than in non-pregnancy serum (Diez d'Aux and Murphy, 1974). While the total SHBG-bindable activity in the serum of non-pregnant women could be accounted for by steroids known to bind to SHBG, these steroids accounted for only approximately half of the SHBG-bound material measured in the serum of pregnant women (Fig. 4-3). This discrepancy suggested that as yet unrecognized substances might be bound to SHBG in pregnancy serum. Since available evidence indicates that there is a positive relationship between the affinity of steroids to specific steroid-binding plasma proteins such as SHBG and CBG and the biological potency of those steroids, it was reasoned that the unknown material bound to SHBG in pregnancy serum might be of physiological importance i.e. may play a role in the maintenance of pregnancy, fetal development, or in the mechanism of parturition. Thus it seemed pertinent to investigate the nature and properties of these previously unrecognized ligand(s).

In the present study, in order to characterize these substances, maternal serum was analyzed by detailed chromatography on Sephadex LH-20. It was found that a substantial part of this material eluted in the non-polar region and consisted of four major peaks: 1a, 1b, 2 and 3 which

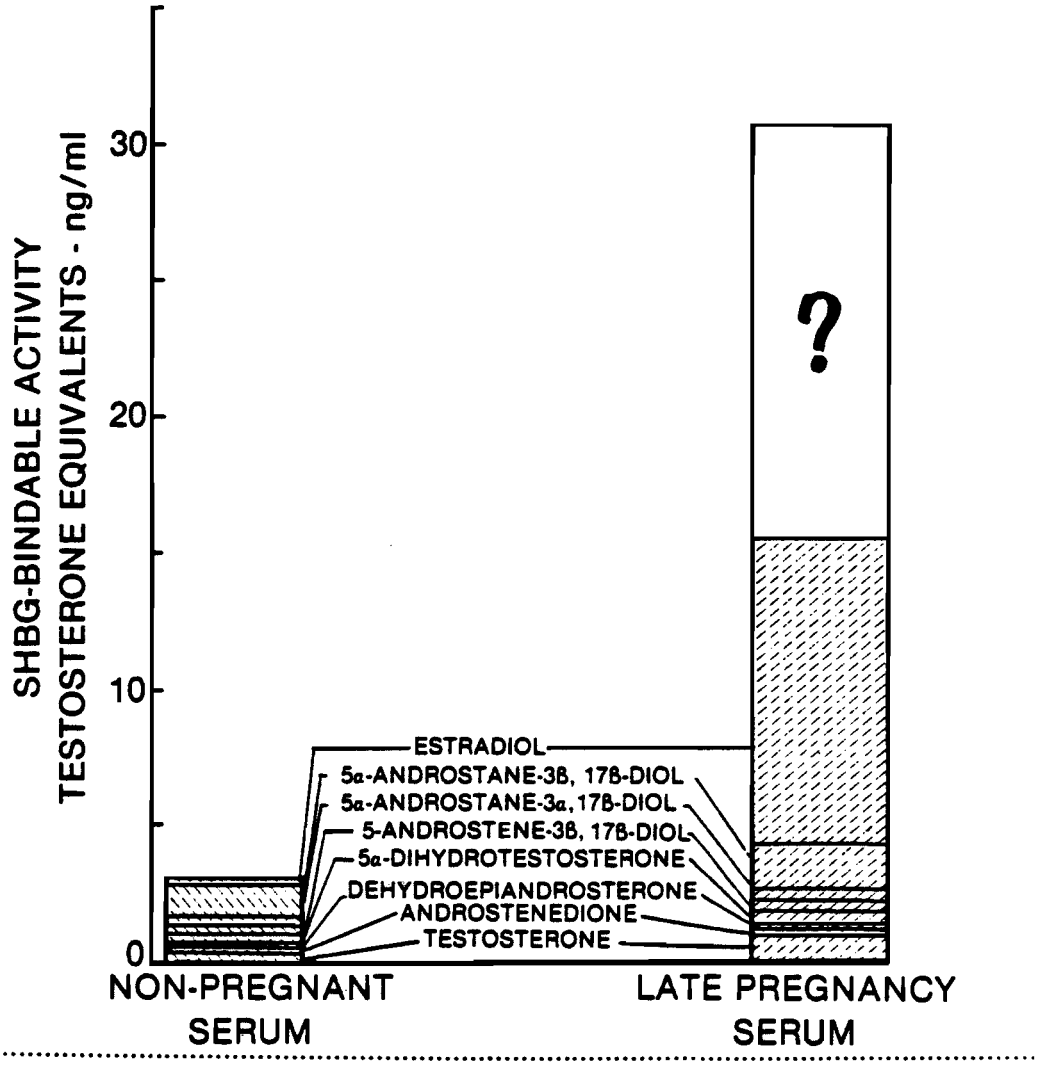


Figure 4-3. The concentration of total SHBG-bindable material in sera of non-pregnant women and pregnant women in third trimester. The figure shows that the total SHBG-bindable activity in the sera of non-pregnant women can be accounted for by the sex steroids known to bind to SHBG. However, in the sera of pregnant women, recognized sex steroids account for only half of the total SHBG-bindable activity measured.

did not correspond to any of the steroids thought to bind to SHBG. Comparison of levels in maternal, fetal and placental compartments suggested that all four peaks were of placental origin. Peak 1a, 1b and 3 were positively identified as 5α-pregnane-3,20-dione, progesterone, and 2-methoxyestrone, respectively on the basis of elution pattern on Sephadex LH-20 and high performance liquid chromatography (HPLC), binding characteristics and gas chromatography-mass spectrometry (GC-MS) analysis. The material eluting as peak 2, which was strongly bound to SHBG under physiological conditions, was identified as a 19-nor androgen, i.e. 17β-hydroxy-1,5-estradiene-3-one, on the basis of the above type of evidence and, in addition, its solubility properties and UV spectrum; however this steroid was not available for direct comparison on MS. Since this compound has a structure similar to those of progestins such as 19-nor T and since the levels of peak 2 decreased significantly close to term and in association with premature labour, it may be involved in the maintenance of pregnancy and its decrease in the initiation of parturition.

4.2.2 Methodology used for the detection and quantitation of unknown SHBG-bound material

Since any substance capable of displacing ^{3}H-T from its specific binding sites of SHBG will be measured in the assay used for the detection of the SHBG-bound peaks, the material eluting as each peak may correspond to either a substance(s) which has a high affinity for SHBG or to a substance(s) which has only a low affinity but is present in large quantities. Although no claim for the purity of these peaks can be made, studies towards the identification of material eluting as each peak were attempted on the assumption that a single compound may account for most of

the SHBG-bindable activity of each peak. It is entirely possible that one or more compounds which do not bind to SHBG may co-elute with the SHBG bindable substance.

The unknown substances eluting as peaks 1a, 1b, 2 and 3 defined in maternal serum by Sephadex LH-20 chromatography were assumed to be steroidal in nature since they are organic solvent extractable, bindable to SHBG, and dialyzable. In addition, since the elution profiles of SHBG-bound materials in the region of low polarity detected in the cord serum, maternal urine and placenta were similar to those of maternal serum, it was assumed that the peaks 1a, 1b, 2 and 3 of cord serum, maternal urine and placenta were the same as those of maternal serum. Again, it is entirely possible that different substances may have similar retention times even on a detailed chromatogram. However, although possible, it is unlikely that these substances will also have the same protein-binding characteristics.

The levels of the SHBG-bindable peaks in all serum, tissue and urine samples were measured in terms of testosterone equivalents since the identities of the peaks were unknown. In other words, the concentration of each peak was expressed as the amount of non-radioactive T required to displace the same amount of ^{3}H-T as the unknown material. Thus, the concentration of the unknown substance measured will depend on the affinity of that substance for SHBG as well as the amount present in the sample.

4.2.3 Methods employed for the identification of unknown SHBG-bound material

The identification of the material eluting as each peak was attempted using 4 different approaches: (i) elution pattern on a Sephadex LH-20 column (60 × 0.9 cm), and silica gel HPLC, (ii) binding

characteristics i.e. the levels predicted on the basis of cross-reactivity in different competitive protein-binding assays were compared to the actually measured amounts in those assays, (iii) comparison of the expected serum levels of the unknown material to the known serum levels of steroid standards and (iv) GC-MS. The nature of peak 2 was studied using two other tools, namely UV absorption spectrometry and solubility in 0.1N NaOH.

Identification of an unknown substance by GC-MS requires that the GC elution and MS of that substance are the same as those of the reference standard. Thus it is very useful to have some notion about the possible identity of the unknown. In addition, since absolute purity of a substance prepared from biological samples is extremely difficult to achieve, interpretation of the MS data can be greatly facilitated if the tentative identity is known. Therefore a tentative identification of the SHBG-bound material eluting as peaks 1a,1b,2 and 3 was attempted using three powerful tools, namely relative elution volume, binding affinities to various proteins, and agreement between predicted and measured serum levels.

Sephadex LH-20 has been used successfully to give excellent separations of many closely related steroids (Murphy, 1975). In the present study, detailed chromatography of samples on Sephadex LH-20 was employed using a solvent system known to give very good resolution of steroids of lower polarity even on a short Sephadex LH-20 column. Thus, if the elution pattern on the Sephadex LH-20 column (60 × 0.9 cm) of the unknown peak matched that of one of the more than 45 possible steroids (androgen, estrogen and progesterone metabolites of low polarity) tested, then that steroid was considered as a possible candidate for that unknown peak. Similarity of the relative elution volumes of the unknown and the

reference steroids were also checked on HPLC.

Further studies were then attempted to compare the binding properties of the unknown with that of the standard under consideration. The principle involved in comparing the apparent binding affinities to various specific steroid binding proteins as a means of verifying the identity of ligands has been described in detail by Murphy (1973). While several ligands may have closely similar binding affinities for any particular protein, 2 of these ligands having the same binding affinity for two or three different proteins with widely different binding properties, although possible, is extremely unlikely. Substances with very similar structures (e.g. T and DHT, 5α- and 5β-DHP, cortisol and prednisolone) can be readily distinguished in this way). Thus, if the values for the concentration of the unknown in 2 different binding assays predicted on the basis of the cross-reactivity of the standard in those assays are the same (within experimental error), it can be considered as strong evidence that the identity of the material being measured is the same as that of the standard.

Similarly, comparison of predicted and true levels of a substance in serum is useful in providing supportive evidence i.e. on the basis of its binding activity for SHBG, one can calculate how much would be required to account for the peak observed. If the amount known reliably to be present is much less, then it cannot account for the binding activity and another substance must be sought.

The identity of the SHBG-bound peaks 1a,1b,2 and 3 defined in maternal serum was confirmed by GC-MS analysis using the corresponding peak purified from hydrolysed pregnancy urine since 1) the elution profiles of SHBG-bound material were similar for maternal serum and urine, 2) the

concentrations of contaminants such as fatty acids and cholesterol which elute in the non-polar region are lower in urine than in serum or placenta and 3) the levels of peak 2 and peak 3 were high in the conjugated fractions of maternal urine and 4) urine is readily available.

All the peaks prepared from pregnancy urine and purified by HPLC were converted to methyloxime-trimethylsilyl ethers before GC-MS since these derivatives are easy to prepare, exhibit low polarity and high thermal stability which results in high intensities of molecular ions. The relative retention times of these steroid derivatives on the GC column depend on molecular weight, shape and polarity.

Although the GC-MS analysis of pregnancy urine peaks showed that these preparations were purer than those obtained from placenta and maternal serum, several contaminants were still present. Almost all the contaminants were pregnane derivatives such as pregnanolones, as expected, since 1) glucuronidated and sulfated pregnane derivatives have been shown to be major progesterone metabolites in pregnancy urine (Dobriner et al, 1948; Baillie et al, 1980) and 2) the relative elution volumes of pregnanolones on Sephadex LH-20, although higher than those of the unknown SHBG-bound peaks (Table 3-16), are close enough that massive amounts will interfere. Each of the contaminants in the peak preparations could be ruled out as a substance accounting for the unknown SHBG-bound material in pregnancy serum on the basis of its elution pattern on Sephadex LH-20, cross-reactivity, and maternal serum levels when known. An SHBG-bound peak was considered identified when the GC elution and MS of the steroid isolated matched those of a reference standard whose relative elution volume, binding characteristics and serum levels were indistinguishable from those of the unknown peak.

Although none of the pregnanolones identified by GC-MS could account for the peaks in pregnancy serum, these compounds are of physiological interest. They have been shown to have profound anesthetic properties even at low doses (Gyermek et al, 1967; Holzbauer, 1975). Little information is available on the levels of these compounds in maternal serum or urine. This is mainly due to the difficulty involved in their chromatographic separation and due to the lack of adequate detection methods. Eight isomers are possible for the pregnanolone structure: 4 each for the 3α/β-hydroxy-5α/β-pregnan-20-one structure and the 20α/β-hydroxy-5α/β-pregnan-3-one structure. To date, the presence of only 3 of these compounds (3α-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one and 3β-hydroxy-5α-pregnan-20-one) has been demonstrated in the human (Dobriner et al, 1948; Axelson and Sjöval, 1974; Mickan and Zander, 1979; Milewich et al, 1978; Laatikainen and Peltonen, 1975). The presence of 20α-hydroxy-5α-pregnan-3-one and 3β-hydroxy-5β-pregnan-20-one detected in the present study has not previously been shown to occur in the human although these steroids have been demonstrated in rat ovarian venous plasma (Ichikawa et al, 1971) and in certain other mammalian organs (Lin et al, 1980).

4.2.4 Identification of peaks 1a and 1b

Although a large number of androgen, estrogen, and progesterone metabolites of low polarity (Table 3-16) were tested for their relative elution volume on Sephadex LH-20, only 5α-DHP and P co-eluted with peak 1a and 1b, respectively. In addition, when peaks 1a and 1b were further fractionated on HPLC, the largest SHBG-bound peaks co-eluted with ^{3}H-5α-DHP and ^{3}H-P, respectively. Since the chances of a substance having similar binding affinities (i.e. measuring the same) in two different assays with

widely differing binding profiles is remote, the behaviour of peak 1a as 5α-DHP and peak 1b as P in SHBG and guinea pig PBG assays provided strong evidence for their identity. In addition there was good agreement between maternal serum levels of peak (1a + 1b) and the levels predicted from the known concentrations of (5α-DHP + P) in maternal serum.

The increase ($p < 0.001$) in the maternal serum levels (ng/ml, TE) of peak (1a + 1b) from 12-24 weeks of gestation (1.1 ± 0.3) to 30-38 weeks (2.5 ± 1.2) and to 39-42 weeks of gestation (2.1 ± 1.2) reflects the increase in the levels of 5α-DHP and P during gestation. Maternal P levels have been shown to rise 3-4-fold from the first to the third trimester of pregnancy (Tulchinsky et al, 1972; Stoa and Bessesen, 1975). The 5α-DHP concentration in the maternal circulation has also been shown to rise approximately 3-fold from 27 to 42 weeks gestation (Stoa and Bessesen, 1975; Milewich et al, 1975).

The high levels of peak (1a + 1b) in the placenta reflect the large amounts of P and 5α-DHP present in that tissue. The similar concentration of peak (1a + 1b) in placentae of 14-16 weeks gestation (55-62 ng TE/gm tissue) and 36-42 weeks gestation (45-58 ng TE/gm tissue) is consistent with the report that the endogenous concentration (μg/gm tissue) of P in placenta (6.0 ± 0.5, 7.8 ± 0.6 and 7.1 ± 3.0 at 12-20 weeks, 33 weeks and term gestation respectively) did not differ markedly throughout pregnancy (Milewich et al, 1978). Although the placental content of 5α-DHP has not been reported, it has recently been demonstrated that the human placenta is very efficient in synthesizing 5α-DHP from P *in vitro* (Milewich et al, 1979). This is in agreement with the high levels of peak 1a measured in the placental tissue in the present study.

The low concentration (1.2 ng/ml or 1.7 μg/24 hr TE) of peak (1a + 1b) in the unconjugated fraction of maternal urine obtained in the preliminary study is consistent with the low levels of P and DHP expected in urine. There is only one report on the concentration of P in urine; Chattoraj et al (1976) observed a value of 15.6 ± 9.7 μg/24 hr in the last trimester of pregnancy. As far as I am aware the levels of 5 α-DHP in pregnancy urine have not been reported. The only descrepant observation concerning the identity of peak 1a and 1b was the material detected as peak (1a + 1b) in the conjugated fractions of maternal urine in the preliminary study. This material cannot be accounted for by 5 α-DHP and P since the conjugation of these steroids to form glucuronide and sulfate derivatives cannot occur. Although it is possible that the SHBG-bindable activity observed in the conjugated fractions may be due to some other unrecognized ligand(s) of SHBG, only a single sample was studied. The activity measured in the assay was most likely an artifact since GC-MS of peak 1a and 1b prepared from hydrolyzed pregnancy urine (containing steroids from the unconjugated fraction and those hydrolysed from the conjugated fraction) showed that 5 α-DHP and P, but none of the contaminants (Table 3-18), could account for peak 1a or 1b.

The presence of the contaminants detected by GC-MS can be explained by the very high levels of these compounds in maternal urine. Cholesterol cannot account for peak 1a since its elution volume on Sephadex LH-20 was much lower than that of peak 1a (data not shown) and since its cross-reactivity is very low (0.001%, Murphy, 1969). 5 β-DHP, present in large amounts in maternal urine (Shearman, 1955; Dobriner et al, 1948), was ruled out since it has been reported to be undetectable in pregnancy serum (Milewich et al, 1975), and since its cross-reactivity was only 1% (Table

3-16). Similarly, 20α-hydroxy-5α-pregnan-3-one, also present in high amounts in pregnancy urine (Dobriner et al, 1948; Baillie et al, 1980), was ruled out since its relative elution volume was greater than that of peak 1a (Table 3-16), its concentration in pregnancy serum is low, i.e. only a few nanograms (Axelson and Sjövall, 1974; Baillie et al, 1980) and its cross-reactivity was < 1% (see Table 3-16).

The contaminants detected in the peak 1b preparation (20α-hydroxy-5α-pregnane-3-one and a 17-hydroxy C21 steroid whose presence was detected by the appearance of some low mass ions in the MS of P) were ruled out for the same reasons.

Thus the SHBG-bound materials eluting as peak 1a and 1b in maternal serum were identified as 5α-DHP and P, respectively since 1) the GC elution and MS data of the major steroid isolated from peak 1a and 1b of maternal urine were the same as that of 5α-DHP and P, respectively and 2) since the relative elution volume on Sephadex LH-20, binding characteristics and known serum levels of peak 1a and 1b were similar to those of 5α-DHP and P, respectively and not to those of any of the contaminants detected by GC-MS.

The above data suggest that a significant portion of material bound to SHBG in pregnancy serum consists of weakly bound steroids lacking a 17β-hydroxyl group. (It is generally believed that only those steroids which have a 17β-hydroxyl group are capable of binding to SHBG). The physiological significance, if any, of the low affinity binding of P and DHP to SHBG on the mechanism of action of these steroids is not known. It seems unlikely that the unbound concentrations of these steroids in serum would be significantly influenced by binding to SHBG. Similarly, the fraction of these steroids complexed to SHBG also would be low. Thus, the

binding to SHBG may have little or no consequence on the action of these low affinity ligands. However, since these substances occupy a significant portion of total SHBG-binding sites, they might influence the binding of other substances to SHBG.

4.2.5 Identification of peak 3

2-ME1 , but not any of the more than 45 steroids of low polarity tested, had the same relative elution volume as peak 3 on detailed chromatography on Sephadex LH-20. In addition, on HPLC of peak 3, the largest SHBG-bound peak eluted in the same position as 2-ME1. Further, the binding characteristics and agreement between serum levels of peak 3 and the levels predicted from known serum concentrations of 2-ME1 provided strong evidence that peak 3 could be accounted for as 2-ME1. This was confirmed by GC-MS.

Although the high affinity binding of 2-ME1 to SHBG has not been reported, enhancement of binding to SHBG by the presence of a 'methoxy' group at C-2 has previously been demonstrated for estradiol (Dunn et al, 1980). The increased binding of estrone due to the presence of a methoxy group at C-2 is in contrast to the reported reduced affinity for binding to SHBG by the presence of a 7α-methyl or a 17α-methyl group (Murphy, 1970; Cunningham et al, 1981). On the other hand, addition of a methyl group at C-4 has been shown to enhance binding (Cunningham et al, 1981). The cross-reactivity measurements of various methoxy- and acetoxyestrogens in the SHBG assay in the present study showed that, while the binding was considerably enhanced by the presence of a 2-methoxy group, it was decreased by the presence of a 4-methoxy, 3-methoxy, 3- or 17-acetoxy group (Table 3-16).

The levels of peak 3 measured as 2-ME1 throughout gestation are

in agreement with the limited information available (Emons et al, 1979) on the maternal levels of 2-ME_1 (Table 3-10). The absence of any significant difference in the serum levels of peak 3 between women at30-38 weeks gestation and 39-42 weeks gestation is consistent with the report of Wotiz and Chattoraj (1964) who observed no increase in the urinary levels of 2-ME_1 measured by a less sensitive gas chromatographic procedure in the last 2 months of pregnancy.

The concentration of 2-ME_1 in cord serum has not been studied systematically. Berg et al (1983) measured the levels of 2-ME_1 in newborn cord serum using an RIA procedure and observed a value of 1.6 ng/ml. Adlercreutz and Luukkainen (1970), the only other group to measure 2-ME_1 levels in cord serum, obtained a value of 1.2 ± 0.8 ng/ml using a less sensitive gas chromatographic procedure. The level obtained for peak 3 in cord serum in the present study, for 6 pools of cord serum collected at term, was 1.3 ± 0.7 ng/ml TE. If peak 3 is 2-ME_1 this will be equivalent to 1.6 ng/ml. Thus, the concentration of peak 3 in cord serum is similar to the values reported for 2-ME_1 in cord serum samples.

The excretion pattern of peak 3 observed in the preliminary study in various fractions (minimal amounts in the unconjugated, low amounts in the sulphate and high amounts in the glucuronide) in a maternal urine sample is in agreement with the limited data available on the excretion pattern of 2-MEl in urine during pregnancy. Ball et al (1977) found that following oral administration of labelled 2-MEl, only negligible amounts of radioactivity were excreted in the unconjugated fraction and that more radioactivity was found in the glucuronide than in the sulfate fraction. The total excretion of peak 3 of 284 µg/24 hr TE (i.e. 302 µg/ 24 hr if peak 3 is 2-ME_1) obtained in the preliminary study in maternal

urine is comparable to the values reported for 2-ME1 (Hobkirk and Nilsen, 1963; Gelbke and Knuppen, 1976; Ball et al., 1979).

As far as I am aware, the endogenous levels of 2-ME_1 in placenta have not been measured. However, Jakowicki et al (1973) using mass spectrometry and gas chromatography have detected unconjugated 2-ME_1 in extracts of human term placenta. The high endogenous levels of peak 3 in mid-gestation placenta obtained in the present study are consistent with the reported metabolism of estrogen in that tissue (Smith and Axelrod, 1969; Chao et al, 1981; Troen et al, 1961). Although the placental endogenous level of peak 3 was very high at midgestation, the level was low in one placenta obtained at 36 weeks gestation and undetectable in 3 placentae obtained at term. The cause for the observed decrease in the placental content of peak 3 from mid-gestation to term is not clear. It suggests that the synthesis of 2-ME_1 by the placenta decreases close to term. Since the placental synthesis of estrogen is believed to increase progressively from early to term gestation (Tulchinsky et al, 1972), the decreased placental content of 2-ME_1 can be due to a decrease in either the 2-hydroxylation or 2-methylation of estrogen, or to an increase in the further metabolism of 2-ME_1 such as demethylation back to 2-hydroxyestrone in the placental tissue.

It is intriguing that the decreased peak 3 content of placenta close to term is not reflected in the maternal serum levels. Possible explanations include a slow metabolism of peak 3 in maternal serum because of its high affinity binding to SHBG or to a decrease in the rate of demethylation. Also, extensive conversion of 2-hydroxyestrone to 2-ME_1 by red blood cells in the vascular compartment has recently been demonstrated (Bates et al, 1977).

Confirmation of the identity of SHBG-bound material eluting as peak 3 in maternal serum was provided by data obtained from GC-MS of urinary peak 3 preparation. The contaminants detected by GC-MS (Table 3-18) were ruled out as peak 3 in pregnancy serum for the following reasons. Although androsterone eluted close to 2-ME_1 on Sephadex LH-20 column, its levels (unconjugated) have been reported to be low, (i.e. only a few nanograms per ml) in serum (Gandy and Peterson, 1968) and its cross-reactivity in the SHBG assay is only 1% of that of T (see Table 3-16). 5α-Pregnane-3α,20α-diol cannot account for peak 3 since its relative elution volume on Sephadex LH-20 was much higher than that of 2-ME_1 (Table 3-16). The 3rd contaminant, a pregnanolone whose exact structure could not be elucidated, was ruled out because the cross-reactivities of all the eight possible pregnanolones in the SHBG assay were low and since the levels of these compounds in unconjugated form in pregnancy serum are relatively low, i.e. a few ng/ml as shown by Axelson and Sjövall (1974) using GC-MS (see Table 3-16)

4.2.6 <u>The physiological role of 2-methoxyestrone and significance of its binding to SHBG</u>

Catechol estrogens which have recently gained attention as potential mediators of estrogen action are major products of estrogen metabolism in the human (Ball and Knuppen, 1980; Fishman, 1983). They are highly unstable compounds and are rapidly metabolised to their corresponding monomethyl ethers in the liver (Ball and Knuppen, 1980) and vascular compartment (Bates et al, 1977) by the enzyme catechol-o-methyl transferase (COMT). 2-Methylation is the predominant pathway for catechol estrogen metabolism and 2ME_1 is quantitatively the most important metabolite (MacLusky et al, 1981). The concentrations of primary estrogens

have been known to vary in health and disease (e.g., levels of estriol in pregnancy urine). Thus, it is conceivable that alterations in estrogen metabolism also may correspond to specific disease states. For example, Fishman et al (1965) have demonstrated that 2-hydroxylation of radioactive estradiol is sensitive to thyroid hormone status in the human. Although the biological importance of catechol estrogens remains controversial, they have been shown to have a broad spectrum of physiological and pharmacological effects. These effects include growth of peripheral target organs such as uterus, inhibition of COMT in liver and brain tissues, and release of gonadotropins and prolactin (for review see Ball and Knuppen, 1980), stimulation of prostaglandin production (Kelly and Abel, 1980) and potentiation of sexual behaviour (Ball et al, 1980). However, since these steroids are rapidly cleared from the circulation, their physiological role as potential mediators of estrogen action has been questioned. The metabolic clearance rate of 2-hydroxyestrone has been reported to be the highest of any known steroid i.e. 40,000 litres per day (Merriam et al, 1980). This rapid clearance is mainly due to the rapid rate of its methylation to form 2-MEl by the COMT present in the red blood cells (Longcope et al, 1982).

Available evidence suggests that 2-ME_1 also has certain biological effects. Oral administration of 2-ME_1 has been shown to decrease serum cholesterol in the rat (Gordon et al, 1964) and rats treated with 2-ME_1 had increased concentrations of plasma renin substrate (Martucci, 1982). However, the biological effects of 2-ME_1 are believed to be brought about by the 2-hydroxyestrone or 2-hydroxyestradiol formed from it (Martucci, 1983). Demethylation of 2-ME_1 in the human was first suggested by Hobkirk and Nilsen (1963). More recently, Ball et al (1977)

demonstrated that 2-ME$_1$ and 4-ME$_1$ administered orally to healthy male subjects were demethylated to a significant extent. This observation has recently been confirmed by Longcope et al (1983) who concluded that although 2-ME$_1$ may have little biological activity, it can act as a pool of potentially active 2-hydroxyestrone in tissues. Such selective demethylation in tissues in close proximity to cellular components may be biologically important. Thus the binding of 2-MEl to SHBG would be of great physiological significance in regulating the amount of unbound 2-MEl available for demethylation by tissues.

4.2.7 A radiotransinassay for 2-methoxyestrone

Very little information is available on the concentration of 2-ME$_1$ in the blood of any species. Determination of the concentration of 2-ME$_1$ is especially useful as the parent 2-hydroxyestrone is difficult to measure because of its extremely labile nature. Although the levels of 2-ME$_1$ have been estimated in pregnancy urine by several investigators using methods not sensitive enough to detect less than microgram amounts, there have been only two reports (Emons et al, 1979; Berg et al, 1983) on the measurement of 2-ME$_1$ in plasma. Both used radioimmunoassay procedures that were sensitive enough to detect picogram amounts, but there are several limitations to these procedures.

Both methods were expensive and time-consuming since they involved the preparation of radioactive ligand (radioactive 2-ME$_1$ is not available commercially), synthesis of antigen, purification of antigen before and after conjugation to BSA, and preparation and purification of antibody. Another limitation of the above 2 methods was the high cross-reactivity (44% in the method of Emons et al, 1979 and 100% in the method of Berg et al, 1983) of 2-methoxyestradiol. However, overestimation

of 2-ME$_1$ levels in serum would be minimal since the levels of 2-methoxyestradiol are believed to be much lower than those of 2-ME$_1$.

In the present study, a sensitive radiotransinassay for the determination of 2-ME$_1$ levels was developed by taking advantage of the high affinity binding of 2-MEl to SHBG. The present method requires only readily available inexpensive reagents such as ^{3}H-testosterone, non-radioactive 2-methoxyestrone and a well characterized binding protein of unlimited supply, i.e. SHBG of late pregnancy human serum.

The sensitivity of the assay (20 pg) was similar to that of Emons et al, 1979 (25 pg) and that of Berg et al, 1983 (10 pg). While the accuracy and precision of the present method were satisfactory, they can be further improved by the addition of ascorbic acid as an antioxidant (Ball et al, 1979) in the assay buffer and in the column solvent used for Sephadex LH-20 chromatography.

Although the specificity of SHBG is less than that of the antibody preparations used in the two RIA procedures, chromatographic separation of competing ligands ensured that substances other than 2-ME$_1$ were not measured. In addition, unlike the antibody preparation whose binding characteristics may be different from batch to batch, the binding properties of human late pregnancy serum SHBG remain constant and the binding properties of this protein have been characterized extensively (Murphy, 1969; Murphy, 1970; Cunningham et al, 1981; also see Tables 1-2 and 3-16). Thus, the present method offers a convenient, practical means for the measurement of 2-ME$_1$ levels in body fluids and tissues. The maternal serum levels of 2-ME1 obtained in the pesent study were comparable to those obtained by the two RIA methods.

The chief disadvantage of the present procedure is the requirement of purification of the sample on a long (60 × 0.9 cm) Sephadex LH-20 column. However, substitution of Sephadex LH-20 chromatography by automated high pressure liquid chromatography (HPLC) separation would considerably reduce the amount of time required for processing. Aten et al (1982) have recently described an excellent method for the separation of various catechol and methoxy estrogens by HPLC using a Lichosorb Diol column.

4.2.8 Identification of peak 2

Extensive studies done toward the identification of the SHBG-bound material eluting as peak 2 in maternal serum suggested that it 1) is steroidal in nature; 2) is non-polar; 3) does not correspond to any of the possible androgen, estrogen or progesterone metabolites tested; 4) is of placental origin; 5) is bound to SHBG under physiological conditions i.e. in undiluted serum at 37°C; 6) is not bound to human CBG or guinea pig PBG; 7) is not a lipoidal derivative of a more polar steroid; 8) is alkali-soluble when partitioned between 0.1N NaOH and methylene chloride i.e. behaves like an estrogen; 9) has a UV absorption maximum at 232 nm; 10) is a 19-nor androgen (17ε-hydroxy-estradiene-3-one) as suggested by GC-MS analysis of urinary peak 2 preparation; and 11) may be involved in the initiation of parturition since its levels fell in association with premature or term labour.

The facts that peak 2 was extractable with organic solvents, had chromatographic properties similar to those of several classical steroids such as progesterone and androstenedione, was dialyzable, was bindable to SHBG and was heat stable strongly suggested that the material eluting as peak 2 is steroidal in nature. The non-polar character of this steroid was

suggested by its relatively low elution volume on Sephadex LH-20 and silica gel HPLC.

Although more than 45 non-polar metabolites of androgens, estrogens and progesterone were tested for their relative elution volume by detailed chromatography on Sephadex LH-20, only 5α-DHDOC eluted in the same position as peak 2 (Tabel 3-16). However, peak 2 could not be accounted for by 5α-DHDOC as demonstrated by the wide differences between the measured values and those predicted on the basis of cross-reactivity of 5α-DHDOC in 3 different competitive protein-binding assays using proteins with widely different binding profiles i.e. SHBG, guinea pig PBG, and human CBG (Table 3-17). None of the methoxy estrogens had the same relative elution volume as peak 2. Although 2,4-dimethoxyestrone was not tested, consideration of the relative elution volumes of various mono-methoxyestrogens and the 2,4-dimethoxyestradiol suggests that its elution volume would be less than that of peak 2. In addition, the methoxyestrogens are insoluble in 0.1N NaOH while peak 2 is soluble. Further, GC-MS analysis of peak 2 did not show the presence of any methoxyestrogens. Since none of the known steroids could account for peak 2, it was concluded that the material eluting as peak 2 is a substance which has not been isolated previously.

The fact that the levels of SHBG-bound material eluting as peak 2 in early gestation placenta were several-fold higher than in maternal serum, cord serum and the four major steroidogenic tissues (liver, testis, ovary and adrenal) of the fetus suggested that the placenta is the source of peak 2. The placental origin of peak 2 would explain the absence of this material in the serum (Figure 3-6) and urine (data not shown) of non-pregnant women.

The similar behaviour of peak 2 and T in the SHBG assay (i.e. their approximately parallel binding curves) suggested that the binding properties of peak 2 are similar to those of T in a highly diluted system, at 4°C under non-equilibrium conditions. The strong binding of peak 2 to SHBG under physiological conditions, (i.e. using undiluted serum at 37°C, by equilibrium dialysis) was shown by the preferential accumulation of peak 2 with respect to T in the compartment containing native SHBG. Since peak 2 binds poorly to human CBG and guinea pig PBG, but strongly to SHBG, it is very likely that it is structurally related to other SHBG-bound steroids such as testosterone or estradiol.

Recent evidence has suggested that fatty acid esters of estradiol normally circulate in human blood (Janocko and Hochberg, 1983). Natural occurrence of lipoidal derivatives of pregnenolone, dehydroisoandrosterone and allopregnanolone have also been reported (Mellon-Nussenbaum and Hochberg, 1980; Albert et al, 1982). Peak 2 is not a lipoidal derivative of a more polar steroid as shown by a similar elution pattern of peak 2 before and after saponification of pregnancy serum. Thus it is most likely an unconjugated steroid of low polarity.

Since it is reasonable to assume that serum and urine peak 2 are the same, the high excretion of peak 2 observed in the preliminary study in the conjugated (sulfate) fraction and only minimal amounts in the unconjugated fraction of urine (Table 3-12) suggests that peak 2 has at least one hydroxyl group on its molecule. More than one hydroxyl group is unlikely since that would render it too polar.

In addition, peak 2 behaved like an estrogen as suggested by its solubility in alkali when it was partitioned between methylene chloride and 0.1N NaOH. Serum, urine and placental peak 2 were soluble in 0.1N NaOH

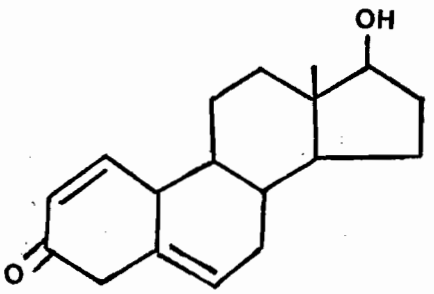
(Table 3-15b) which strengthens the argument that the material eluting as peak 2 is the same in all three compartments. The differences in solubility among the samples can be explained by differences in the degree of purity of the preparations. The solubility in alkali of peak 2 is not due to contamination with estrogens since estrogens elute far away and since the solubility of purified peak 2 was much higher than that of the crude peak 2. It is also not due to methoxyestrogens since they are insoluble in alkali (Klyne, 1957; present study, data not shown). Since peak 2 is a non-polar steroid, the solubility in alkali may be explained by a rearrangement of the steroid in the presence of 0.1N NaOH to form a steroid with a phenolic ring A.

The GC-MS analysis of urinary peak 2 suggested that it has a 19-nor-androgen-like structure (17ε-hydroxyestradiene-3-one). Although six steroids (Table 3-18) were detected by GC-MS of peak 2, all but the 19-nor compound could be ruled out as contaminants. 20α-hydroxy-5α-pregnan-3-one (a contaminant of peak 1a and peak 1b preparations as well) and 3β-hydroxy-5β-pregnan-20-one cannot account for peak 2 in pregnancy serum since their relative elution volumes on Sephadex-LH20 were higher than that of peak 2, their cross-reactivities were low (Table 3-16) and their levels in the unconjugated form in maternal serum are low (Axelson and Sjovall, 1974; Baillie et al, 1980). The presence of these compounds in peak 2 prepared from urine can be explained by the expected high levels of pregnane metabolites in hydrolysed pregnancy urine (Dobriner et al, 1948; Baillie et al, 1980).

The elution pattern and cross-reactivity of a third contaminant, ε-hydroxy-5α-pregnan-3,20-dione, are not known. However, studies of cross-reactivities of peak 2 and a large series of pregnane derivatives

have shown that all the pregnane compounds cross-reacted several fold higher in the guinea pig PBG assay (data not shown) than in the SHBG assay while the reverse was true for peak 2. Thus it is very unlikely that a pregnane compound would account for peak 2 in pregnancy serum. The fourth compound, identified as 5α-DHDOC, had an elution pattern identical to that of peak 2 on Sephadex LH-20. However, detailed cross-reactivity studies have shown that this compound cannot account for peak 2 in pregnancy serum (see data presented in Table 3-17). Although the full structural identity of the fifth compound, an 11-ketopregnanolone, was not known, this compound was also ruled out since, as stated above, cross-reactivity studies suggested that peak 2 could not be accounted for by a pregnane compound. In addition, it has been shown that the presence of a keto group on C-11 essentially eliminates binding of steroid to SHBG (Cunningham et al, 1981).

Proof of the complete structure of the sixth compound was not possible by GC-MS since a reference steroid was not available. The same conclusion that it is a 17ε-hydroxy-estradiene-3-one was reached by Dr. P.V. Fennessey of the University of Colorado and Dr. C. Shackleton of the University of California, both of whom conducted GC-MS of peak 2 independently. On the basis of the known properties of peak 2, the following complete structure was deduced:



17β-Hydroxy-1,5(6)-estradiene-3-one

This compound would be expected to bind strongly to SHBG since it contains a 17β-hydroxy group and a double bond at C5-6. Since a 17α-hydroxy structure would bind only weakly to SHBG and since peak 2 binds strongly to SHBG, the configuration at C17 must be β. The presence of the double bond at C1-2 (1-en-3-one structure) was indicated by the UV absorption maximum at 232 nm. Since a 1,4-diene structure would actually be estradiol, and since a similar compound, 5-estrene-3β,17β-diol (i.e. 19-nor-5-androstene-3β,17β-diol) which differs only by the absence of the double bond at C1-2 has been shown to bind strongly to SHBG (Murphy, 1970; Goertz et al, 1974), it was concluded that the structure was the above. Also, this neutral structure has the same number of carbon, hydrogen, and oxygen atoms as estradiol and would be expected to rearrange in 0.1N NaOH to form estradiol, which would account for the solubility of peak 2 in 0.1N NaOH. The preliminary data showing the shift in elution volume on Sephadex LH-20 of peak 2 to the position of estradiol after exposure to 0.1N NaOH appear to confirm this structure.

4.2.9 Physiological role of peak 2

An interesting observation in the present study was that the level of peak 2 fell in association with term or premature labour. In the absence of labour there was a significant decrease in the concentration of peak 2 between 30-38 weeks and 39-42 weeks gestation. In accordance with this finding, peak 2 was undetectable in placentae obtained at term, although the levels of peak 2 were high in placentae of early gestation. Furthermore, the levels of peak 2 were significantly lower in the serum of pregnant women in spontaneous labour than in women of the same gestational age (30-38 weeks) who were not in labour (Table 3-13) while the levels of peak (1a + 1b) and peak 3 remained unchanged in those two groups of women.

Similarly, cord serum samples from male and female infants at term collected at cesarean section in the absence of labour or at spontaneous delivery had no detectable peak 2 activity while a cord serum sample at 22 weeks gestation (no labour) had low but significant amounts.

As far as I am aware, the concentration of no other steroid in maternal serum has been shown unequivocally to decrease close to term. Although a few early reports (Csapo et al, 1971; Turnbull et al, 1974) have suggested that there was a significant decrease in the levels of progesterone before the onset of labour, the majority of subsequent reports failed to confirm this finding (Boroditsky et al, 1978; Mathur et al, 1980; Hartkainen et al, 1981; Anderson et al, 1985).

This decrease in the serum levels of peak 2 in association with actual (premature and term) and impending (term) spontaneous labour may reflect a decrease in synthesis or an increase in metabolism. Although it cannot be ruled out that the decreased level of peak 2 in serum of women in spontaneous labour is simply associated with or secondary to labour, it seems likely it has a causal relationship to the onset of labour.

Factors regulating the termination of pregnancy in the human are not well delineated. The onset of parturition is thought to involve a reduction in the influence of progesterone and an increase in the influence of estrogen on the uterus (MacDonald et al, 1978). As stated earlier, a decline in the circulating levels of progesterone prior to labour has not been shown unequivocally in the human although it is possible that the tissue levels are more critical for target cell action. In addition, progesterone administration does not prolong human pregnancy (Wood et al, 1963) and estrogen administration does not result in initiation of labour (Ryan, 1980). Administration of cortisol does not induce labour in women

(Flint and Ricketts, 1979) although it does so in sheep.

While the concentration of several steroid hormones has been shown to increase (e.g. progesterone, estradiol, cortisol) or remain unaltered or slightly altered (e.g. androstenedione, testosterone) throughout the course of pregnancy, a significant decrease in the concentration of a steroid near the time of onset of labour has not been established previously in the human. Recently, Mathur et al (1980) showed that the maternal concentrations of pregnenolone and its sulfate, dehydroepiandrosterone and its sulfate, 17-hydroxypregnenolone sulfate and cortisol were significantly higher during labour than before labour, presumably due to the increased adrenal activity associated with the stress of labour. In contrast, they have observed that the plasma levels of estradiol, progesterone and 17-hydroxyprogesterone which are produced almost entirely by placenta at term (see Tulchinsky and Simmer, 1972) were not significantly different in women during labour from those of women not in labour. Since changes in the concentration of steroid or steroid metabolites in relation to labour and advancing gestational age are of potential importance in the events that lead to labour, the significantly decreased levels of peak 2 in maternal circulation in premature labour and close to term may be of physiological significance in the initiation of parturition.

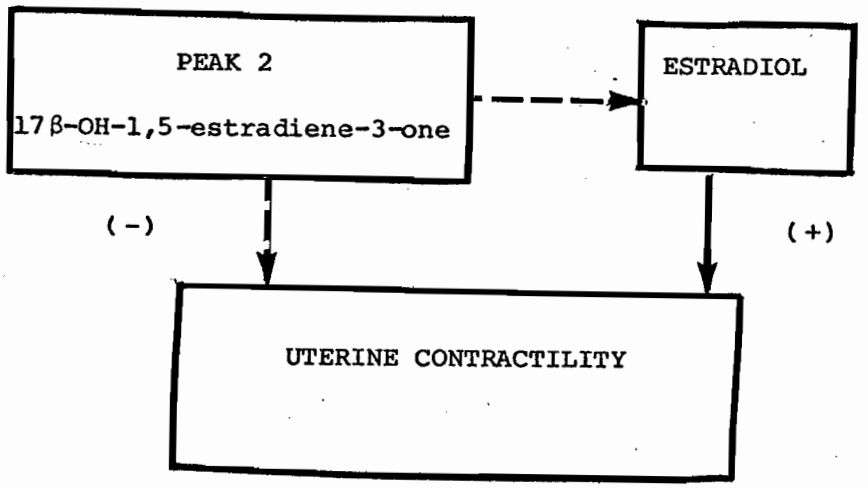
The presence of a naturally-occurring estradiene compound in pregnancy has not been reported previously. The possible biosynthetic pathway of this steroid is unknown. My own view is that it represents an alternative pathway for estradiol synthesis. The generally accepted pathway for aromatization involves three successive enzymatic hydroxylations to form a 19-hydroxyl intermediate, a 19-aldehyde

intermediate, and a 2β-hydroxylated 19-aldehyde intermediate. The formation of the latter compound has been demonstrated to result in the spontaneous synthesis of estrogens (Goto and Fishman, 1977). Formation of 19-nor T and 19-nor androstenedione from T by placental microsomes obtained from baboons has recently been reported (Milewich and Axelrod, 1979). Thus it is possible that Δ4- and Δ5- 19-nor androgens are formed from DHEA(S) in the human placenta. Unlike the 2β-hydroxylation (or dehydrogenation) of Δ4 steroids, which would result in rapid synthesis of estrogen, the 2β-hydroxylation (or dehydrogenation) of Δ5-19-nor androgens such as 19-nor-5-androstene-3,17-dione or 19-nor-5-androstene-3,17-diol may result in the formation of the above estradiene compound, which is stable and may therefore get into the circulation. It could also be converted to estradiol, either enzymatically or by acid-base catalysis.

Recent evidence suggests that the natural occurrence of 19-nor steroids is more common than previously thought. For example, 19-nor-deoxycorticosterone and 19-nor-corticosterone have been shown to be naturally-occurring substances in rat and man (Dale et al,1981; Dale et al, 1985; Gomez-Sanchez et al, 1979). As stated already, formation of 19-nor T and 19-nor androstenedione by baboon placental microsomes has also been demonstrated (Milewich and Axelrod, 1979). Several 19-nor-steroids which were synthesized and first evaluated in the 1940s and 1950s have been shown to have high biological potency. For example, 19-nor-T has been demonstrated to have higher anabolic activity than T, and 17α-ethinyl-19-nor-T (norethisterone) has been shown to be 5 times as potent as 17α-ethinyl-T (ethisterone) as an oral progestogen (Fieser and Fieser, 1959).

Since the estradiene compound has structural similarity to the above 19-nor androgens and since the levels of peak 2 decreasd significantly close to term and in association with premature labour, it is speculated that this compound has progestational activity and may therefore be involved in the maintenance of pregnancy and its decrease in the initiation of labour. The levels of this compound may decrease close to term due to increased conversion to estradiol, a steroid which has long been known to increase spontaneous muscle activity. Thus an increase in uterine contractility caused by both the decreased levels of the estradiene compound and the increased levels of estradiol would lead to the onset of labour.

POSTULATED ROLE OF PEAK 2
IN THE MAINTENANCE OF PREGNANCY



CLAIMS TO ORIGINAL RESEARCH

1. New, more direct methods were developed for the estimation of the SHBG-bound and of the unbound fractions of estradiol and testosterone in undiluted serum by equilibrium dialysis at 37°C under iso-osmotic conditions.
2. Using the above methods, the distribution in serum of estradiol and testosterone into SHBG-bound, albumin-bound and unbound fractions was described for non-pregnant women, pregnant women, adult men and newborn infants.
3. It was found that SHBG was an important binder of serum estradiol in both non-pregnant and pregnant women. Estradiol binding to SHBG was minimal in men and undetectable in newborn infants. Testosterone was significantly bound to SHBG under all circumstances.
4. The present study resolved the conflicting evidence on the role of SHBG in the serum distribution of estradiol in non-pregnant women and in men. The fraction of estradiol and testosterone bound to SHBG in undiluted serum at 37 C was measured for the first time in pregnant women and newborn infants.
5. Detailed chromatographic analysis of maternal serum on Sephadex LH-20 showed that a major part of the SHBG-bound material eluted in the non-polar region and that it consisted of 4 major peaks (peaks 1a, 1b, 2 and 3) which did not correspond to any of the steroids thought to bind to SHBG.
6. Peaks 1a, 1b and 3 were identified as 5α-pregnane-3,20-dione, progesterone and 2-methoxyestrone, respectively.

7. A radiotransinassay was developed for the measurement of 2-methoxyestrone (2-ME_1). This method has the advantages of requiring only readily available inexpensive reagents.
8. Data regarding the relative binding affinities of a group of steroids of low polarity for SHBG were reported for the first time.
9. The presence of 20α-hydroxy-5α-pregnan-3-one and 3β-hydroxy-5β-pregnan-20-one was detected for the first time in the human.
10. Peak 2 was identified as a 19-nor androgen i.e. 17β-hydroxy-1,5-estradiene-3-one, a steroid not previously described.
11. It was found that the levels of peak 2, which was strongly bound to SHBG, decreased significantly close to term and in association with premature labour.

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