PHYSIOLOGICAL ASPECTS OF THE ß-CORE hCG FRAGMENT

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A thesis submitted to the University of Adelaide for the degree of Doctor of Philosophy.

November, 1991

DEDICATION

This thesis is dedicated to my wife Dora, and my children Matheus, Kalinka, and Cecilia who supported me with love and encouragement; and to my parents who had to forego our companionship. Also, it is dedicated to all members of the Reproductive Medicine Unit at The Queen Elizabeth Hospital. They were, directly or indirectly, kind collaborators and very close friends.

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ACKNOWLEDGEMENTS

I am particularly grateful to Dr. Robert John Norman for his enthusiastic supervision, guidance, and stimulus. He provided support and tireless constructive discussion throughout this research.

I thank Professor Colin Douglas Matthews who opened his unit for my training in Reproductive Medicine and made possible the realization of this study.

I wish to express my special acknowledgement to Mr. Fred Amato who performed the majority of iodinations, the High Performance Liquid Chromatography and ion-exchange chromatography experiments. Additionally, he provided an example of a meticulous scientist and was a source of advice and friendship.

I am also indebted to Dr. Margaret Tyler from Deakin Research Ltd, CSIRO Division of Food Processing (Sydney, NSW), who performed the amino acid analysis and sequencing experiments; Mrs. Michele Kolo who carried out the immunoassays for establishing the reference range for ßC-hCG throughout pregnancy; Mr. Alan Gilmore who performed the iodination of many gonadotrophins and antibodies used in this study; Dr. Jim Wang who provided statistical assistance.

I thank the members of the Laboratory of Endocrinology, Mrs. Jenny McLoughlin, Miss. Tracey Lloyd, Miss. Anne White, Miss. Jenny Bowman, and Mr. Alan Gilmore for their guidance and friendliness.

I thank Mr. Manohara Singh Hullan, and all members of the Department of Clinical Photography, The Queen Elizabeth Hospital, for the majority of the illustrations included in this thesis.

I express my gratitude to Mrs. Helen Holmes who provided tireless secretarial assistance and taught me the exact meaning of "I CAN".

I am indebted to Mrs. Kathy Govas who was a meticulous proof-reader of this thesis.

I thank the National Council of Research and Technological Development of Brazil and The University of Adelaide for financial support during the time of my candidature.

DECLARATION

I declare that the material reported in this thesis has not been applied or accepted for the award of any other degree or diploma in any University and, to the best of my knowledge, contains no material previously published or written by another person, otherwise proper reference is made in the text. The experiments reported in this thesis were performed by myself and any assistance received from others is specifically pointed out and acknowledged.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Sebastiao Freitas de Medeiros

Abstract

Heterogeneity in the primary structure of human chorionic gonadotrophin (hCG) is common. Several fragmented forms, either secreted by trophoblastic or non-trophoblastic tissue or resulting from the peripheral metabolism of intact hCG or its α and ß subunits, have been identified in different pathophysiological conditions. This study was performed to analyse in detail a native small fragment of hCG/ßhCG subunit, the ß-core hCG fragment (ßC-hCG), which is found in large amounts in urine and may be of clinical importance. The aims were to purify the fragment, to analyse its protein and carbohydrate structure, to develop direct assay methods for its measurement, and to examine its distribution in body fluids and the relationship between the fragment and the intact hCG molecule during pregnancy.

ßC-hCG was shown to be very stable under the majority of laboratory conditions used to handle the samples. Thus, samples may be stored without preservative at room temperature before transfer to the laboratory. From the amino acid analysis, the molecular weight of the protein portion of ßC-hCG molecule was estimated to be Mr 10218. The analysis of the structure of reduced ßC-hCG demonstrated that it exists in two forms in urine. One yields two peptides with molecular weights of Mr 8900 and Mr 7500 and represents the majority of molecules. The other, yielding an additional third peptide with molecular weight of approximately Mr 3500, was present in small quantities. Although HPLC separation of its reduced and carboxymethylated-peptides resulted in three peaks, two of them were sequenced and indicated to be the ß6-40 and ß55-92 peptides of the ßhCG subunit. Analysis of the carbohydrate composition using several lectins demonstrated the presence of N-linked oligosaccharide type sugar branches, partially or totally trimmed in their antennae.

A specific polyclonal antibody against ßC-hCG was raised and two new assay methods (radioimmunoassay and immunoradiometric assay) were validated for its detection. Both assays are sensitive and do not cross-react significantly with the majority of the related glycoproteins. However, cross-reaction with ßLH subunit is significant with the radioimmunoassay. Comparison between these two assays and two others previously published indicated high correlation coefficients (r=0.960) and good agreement. Since the radioimmunoassay cross-reacts with the ßLH, the immunoradiometric assay is considered to be more appropriate for ßC-hCG detection in non-pregnant individuals.

The peripheral metabolism of hCG to ßC-hCG was investigated both in vivo and in vitro. Intramuscular injection of hCG in male volunteers increased the levels of ßC-hCG in urine by 220% during the first 24h, decreasing thereafter to undetectable levels by 72h. Moreover, granulosa cells cultured in the presence of hCG were able to degrade the intact molecule to an immunoreactive form of ßC-hCG.

A reference range for ßC-hCG and the ratio between ßC-hCG/hCG was established in urine throughout the human pregnancy. The earliest increase of ßC-hCG and hCG in urine collected daily from women whose pregnancy resulted from artificial insemination was detected respectively 12 and 11 days following the estimated time of ovulation. The pattern of urinary ßC-hCG between the 6th and the 41st week of gestation was similar to that of hCG and the ratio between both glycoproteins varied between 1.65 and 9.57 throughout pregnancy. The fragment was found in the serum of pregnant women, the urine of newborn and non-pregnant individuals, amniotic fluid, follicular fluid, and seminal plasma. The ratio between ßC-hCG and hCG in these fluids was

variable. A high molecular weight species of immunoreactive ßC-hCG was detected in serum, amniotic fluid, and seminal plasma.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Many hormones do not exist as a single type of molecule in biological fluids. On the contrary they often consist of a mixture of molecular forms with different physicochemical, biological, and immunological properties. There are several possible reasons for this heterogeneity, including genetically determined structural changes of the protein, modifications induced by the hormonal milieu, existence of prohormones, pharmacologically induced isohormones, polymerization of native forms, association with other proteins or peptides, degradative metabolic products, or artefacts during purification *(Rennels and Hood, 1964; Goodman et al, 1972; Wildt et al, 1973; Rajaniemi and Vanha-Pertulla, 1973; Bogdanove et al, 1974; Bogdanove et al, 1975; Graesslin et al, 1984; Buckingham and Wilson, 1985; Vogel et al, 1986; Barnes et al, 1986; Padmanabhan et al, 1988; Veldhuis et al, 1989; Lahlou et al, 1990).* During the past few years considerable progress in understanding the heterogeneous nature of many hormones has been made and much new information has accumulated. In addition to this increase in our knowledge about the chemical structure and physiological significance of many unusual forms, a number of clinical disturbances associated with structural abnormalities of some hormones have been documented. The pathophysiological mechanisms of these disturbances however are less well defined. Many reports refer either to the existence of molecules with immunoreactivity and no bioactivity or an otherwise unbalanced bioactivity/immunoactivity ratio *(Nusynowitz and Klein, 1973; Park et al, 1976; Gabbay et al, 1976; Axelrod et al, 1979; Silva de Sa' et al, 1980; Given et al, 1980; Beitins et al, 1981; Silva de Sa' and Rebar, 1981; Lobo et al, 1983;*

Plourde et al, 1985; Mavroudis et al, 1988). Even though the majority of the hormones present some degree of heterogeneity, this phenomenon is more remarkable in the structure of gonadotrophins.

Pituitary and placenta secrete a family of glycoproteins able to express their biological activity at the gonadal level. The pituitary gonadotrophins luteinizing hormone (LH), follicle stimulating hormone (FSH) and placental choriogonadotrophin hormone (hCG) are structurally related and consist of alpha and beta subunits. As a single gene encodes the alpha subunit for all these hormones the amino acid sequence of this subunit is virtually identical in all gonadotrophins within an animal species. Their beta subunits arise from separate genes, differ in amino acid sequence, and confer the biological specificity to each hormone. The human choriogonadotrophin molecule (hCG) is produced by the trophoblastic cells from where it is secreted into serum and urine of pregnant women. hCG can also be ectopically secreted by a large number of normal and abnormal tissues. In addition to the intact molecule and its free alpha and beta subunits, several altered forms of this gonadotrophin have been identified in different physiological and pathophysiological conditions *(Vaitukaitis and Ebersole, 1976; Chen et al, 1976; Good et al, 1977; Franchimont et al, 1978; Braunstein et al, 1979; Matsuura et al, 1980; Yazaki et al, 1980; Ruddon et al, 1981; Mann and Karl, 1983; Borkowski et al, 1984; Norman et al, 1985; Nagelberg et al, 1985; Hay, 1986; Endo et al, 1987; Hay, 1988; Collins and Wong, 1989; Takeuchi et al, 1990; Hoermann et al, 1990*). Although atypical forms are identified more frequently in abnormal conditions, they have also been found in normal trophoblastic tissues and detected in organic fluids of normal pregnant women. Thus hCG also exists not as a single entity but as a family of isoforms and its heterogeneous nature has been demonstrated on the basis of charge, size, and immuno- and biological activities. The physiological

importance and full clinical significance of the various molecular forms remain to be determined.

1.2 Chemistry and structure of the standard hCG molecule

Two dissimilar glycoprotein subunits, designed alpha and beta, are joined non-covalently by simple hydrogen bonding to form the heterodimeric whole molecule of hCG. The hCG purified from urine of pregnant women is approximately 38000 daltons in molecular weight (*Bahl, 1969a; Bahl, 1969b*) and consists of 92 amino acid residues in its alpha subunit and 145 in the beta subunit. The whole hCG molecule is rich in carbohydrate with a proportion as high as 30% of its molecular weight due to the sugar content. The oligosaccharide units are covalently bound to the two peptide chains; approximately 17% of the total oligosaccharides are neutral or sialylated monoantennary complex-type oligosaccharides with fucosylated and nonfucosylated trimannosyl cores. Therefore, both alpha and beta subunits of hCG are glycoproteins, each consisting of a single polypeptide chain to which are attached the oligosaccharide moieties *(Bell et al, 1969; Morgan and Canfield, 1971; Bahl et al, 1972; Ross, 1977; Kessler et al, 1979a; Kessler et al, 1979b; Endo et al, 1979; Mizuochi and Kobata, 1980; Pierce and Parsons, 1981; Tyrey, 1982; Puett, 1986; Cole, 1987).*

The alpha hCG subunit has a molecular weight of approximately 14900 daltons, of which the protein portion represents 10200 and the carbohydrate 4700 daltons. It is species-specific and in the same species is virtually identical to the alpha subunit of the other glycoprotein pituitary hormones *(Pierce, 1971; Sairam et al, 1972; Bahl, 1972; Bellisario et al, 1973; Morgan et al, 1975; Kennedy and Chaplin, 1976; Mise and Bahl, 1980; Fiddes and Goodman, 1981; Strickland et al, 1985).* However, the alpha hCG subunit

amino acid sequence possesses an additional three NH_2 terminal residues resulting in a polypeptide chain of 92 amino acids with 10 cysteine residues and 5 interchain disulfide bonds between residues 7-31; 10-32; 28-60; 59-87; and 82-84 (Mise and Bahl, 1980) (Figure 1.1). Although the alpha subunits are considered similar with respect to their amino acid sequences, there exists significant differences in the composition and complexity of the oligosaccharide chains *(Maghuin-Rogister et al, 1975; Vaitukaitis et al, 1976; Cox, 1981; Nilsson et al, 1986).* Two bulky complex-type carbohydrate moieties are attached N-glycosidically to the asparagine residues at positions

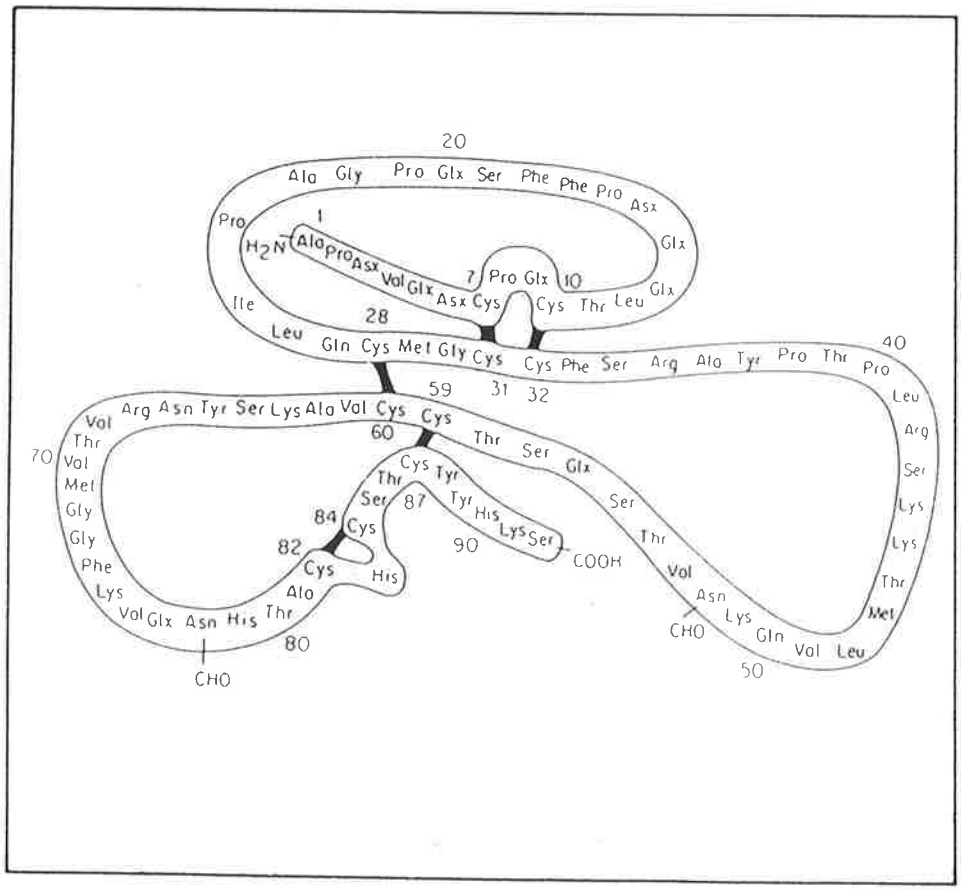


Figure 1.1 Primary sequence of αhCG and locations of the disulfide bonds. From Mise and Bahl, 1980.

52 and 78. While the alpha hCG oligosaccharides are mainly sialic acid, galactose, N-acetylglucosamine and mannose, the oligosaccharides of alpha LH, and alpha FSH contain sialic acid, galactose, N-acetylgalactosamine and sulfate *(Bahl, 1969; Closset et al, 1975; Liu and Ward, 1975; Sairam and Li, 1977; Bahl et al, 1978; Endo et al, 1979; Moore et al, 1980; Tolvo et al, 1982; Green et al, 1985; Green et al, 1986; Baezinger and Green, 1988; Green and Baezinger, 1988).* To facilitate this comparison, Table 1.1 shows their carbohydrate composition. The basic structure of the N-linked oligosaccharide of hCG shown in Figure 1.2 includes an inner chitobiosyl -4 GlcNAcB1-4Glc NAc 1-linking the asparagine residue of the polypeptide chain to a mannosyl residue of the pentasaccharide core Man 3 GlcNAc 2 containing an extension of two identical tetrasaccharide antennae ending in N-acetylneuraminyl groups.

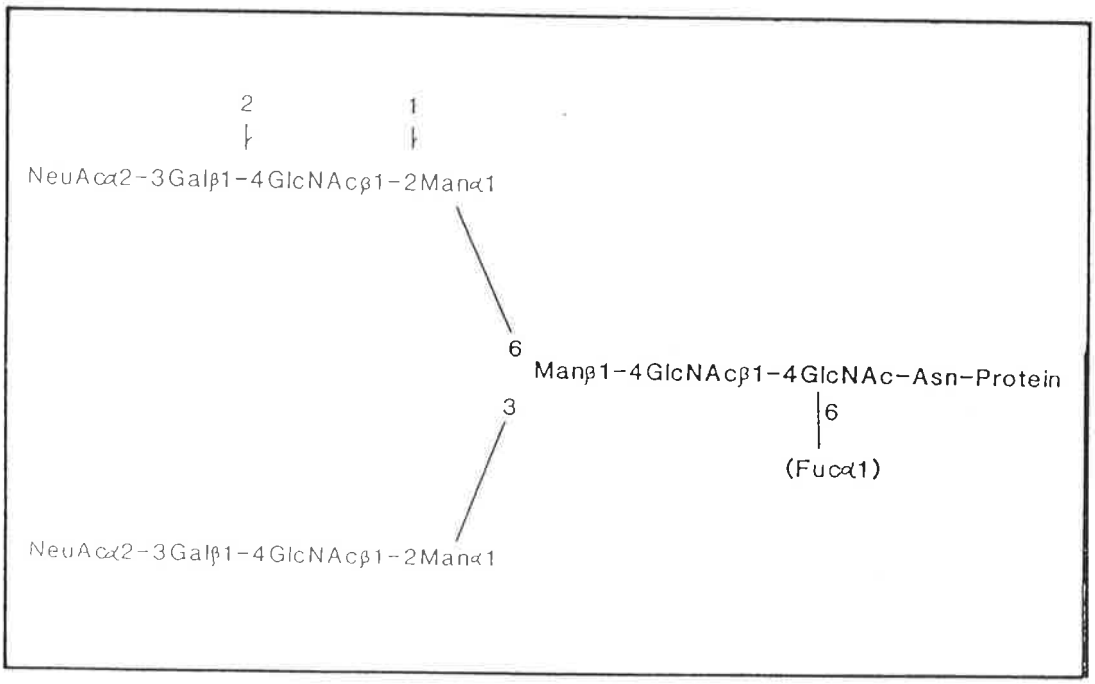


Figure 1.2 The standard asparagine-linked carbohydrate chains in hCG structure. Proposed by Kessler et al, 1979a.

Table 1.1 Structures of N-linked oligosaccharides on the pituitary glycoproteins and placental human choriogonadotrophin

Glycoprotein	Peripheral branches[a]		Inner core
hFSH[b]	SA Gal GlcNAc- SO_4 Gal NAc Glc NAc-		
hLH, hTSH[c]	Gal NAc Glc NAc-	Man	Man-Glc NAc Glc NAc - Asn ± Fuc
	SO_4 Gal NAc Glc NAc-		
	Gal Glc NAc-	Man	
	SA Gal Glc NAc-		
hCG	SA Gal Glc NAc-		

a SA - sialic acid; Gal - galactose; Glc NAc - N-acetyl glucosamine, Man - mannose; Gal NAc - N-acetyl galactosamine; Fuc - fucose; SO_4 - sulfate.

b Mostly non-sulfated

c hLH and hTSH contain dibranched complex oligosaccharides which bear Gal NAc Glc NAc (sulfated or non-sulfated) on one branch and Gal Glc NAc or SA Gal Glc NAc on the other.

The beta subunit of hCG which confers the immunological and biological specificity to the whole hCG has a molecular weight of 23000 daltons with 16000 accounted by the protein portion and 7000 by the carbohydrate of the molecule. The beta hCG amino acid chain has 145 residues and although structurally similar to the beta subunit of other glycoprotein hormones, its composition is different and specific. Despite sharing 81% of the sequence of amino acids with the beta subunit of LH up to position Ser-121 (Figure 1.3),

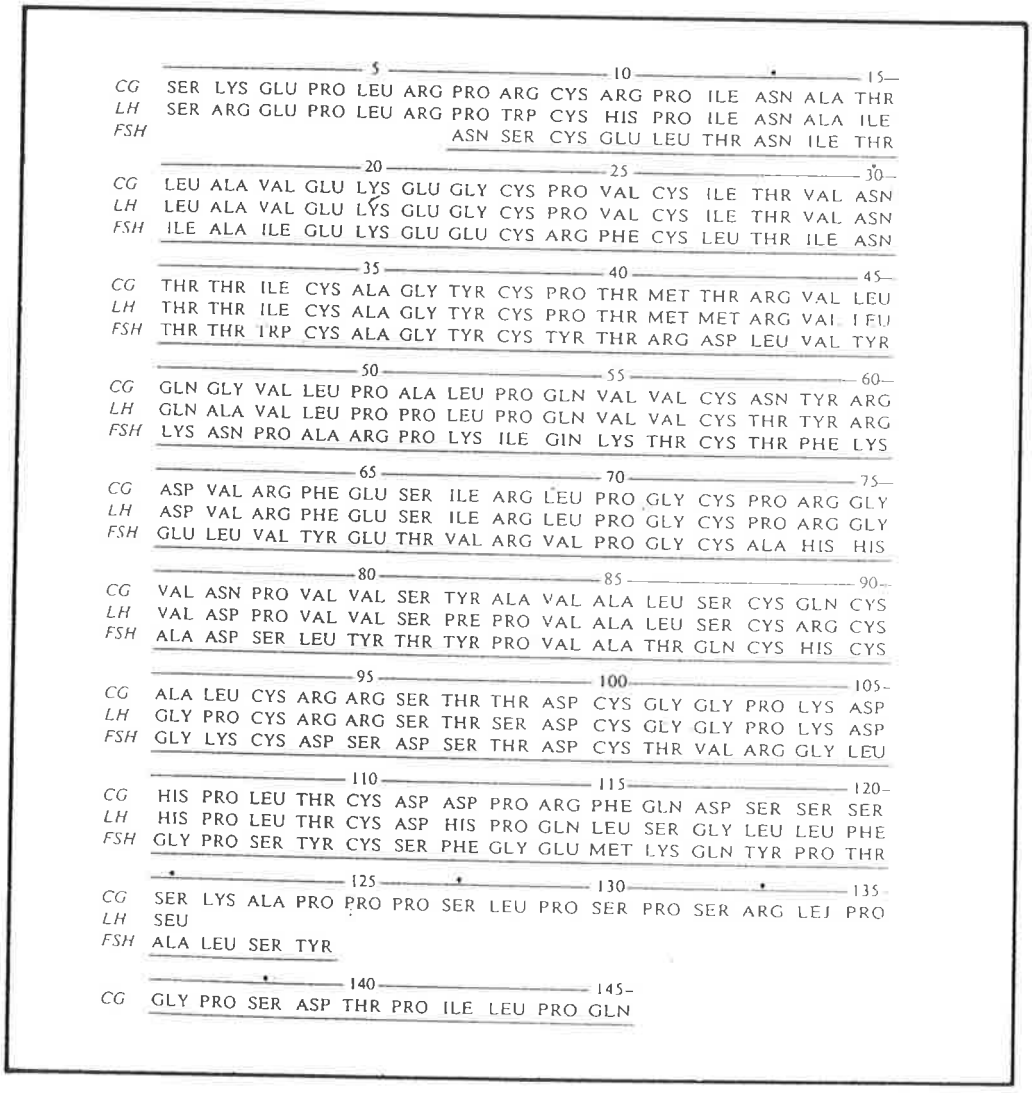


Figure 1.3 The amino acid sequences of hCG, hLH and hFSH ß-chains. The asterisks indicate the position of carbohydrate units in hCG. From Gray, 1988.

there are an additional 24 amino acid residues and a unique carbohydrate composition (*Bahl et al, 1972; Shome and Parlow, 1973; Morgan et al, 1973; Carlsen et al, 1973; Closset et al, 1973; Morgan et al, 1975; Keutmann and Williams, 1977; Birken and Canfield, 1977; Strickland and Pearce, 1985*). The complete amino acid chain of the beta subunit contains six disulfide bridges between half-cysteines located at positions 23-72, 26-110, 34-88, 38-57, 9-90, and 93-100 (*Mise and Bahl, 1981*) (Figure 1.4).

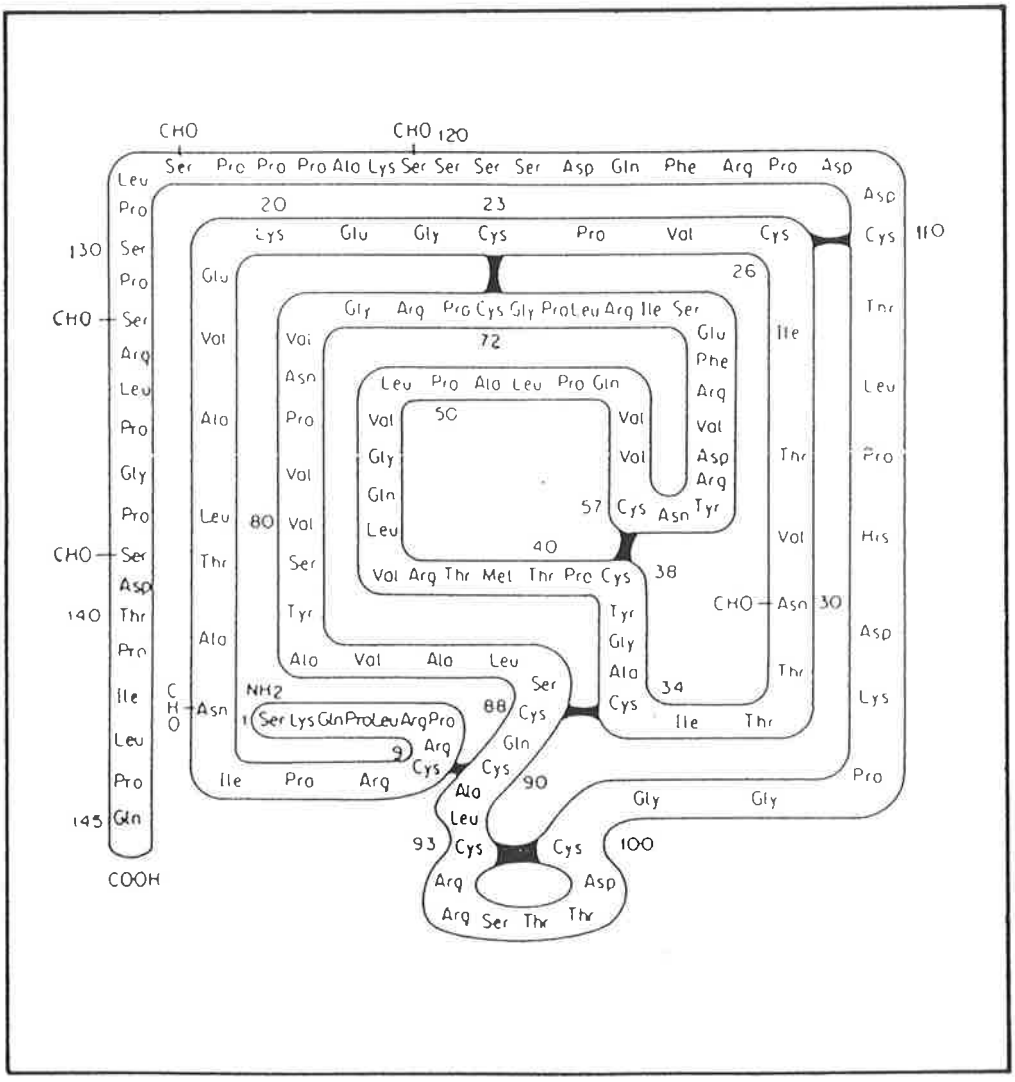


Figure 1.4 The amino acid sequence of hCG-ß showing the locations of disulfide bonds and carbohydrate moieties From Mise and Bahl, 1981.

The carbohydrate content of beta hCG is completely different from that of the beta subunits of other glycoproteins being composed of two asparagine-linked and four serine-linked carbohydrate units. The two complex asparagine units are located at positions 13 and 30 and contain fucosylated and non-fucosylated biantennary complex-type sugar chains terminating in galactose and sialic acid in equimolar ratio. The four unique O-glycosydically linked oligosaccharide moieties (Figure 1.5) are attached to serine residues at positions 121, 127, 132, and 138 in its carboxy-terminal peptide extension and contain sialic acid, galactose, and N-acetylgalactosamine *(Bahl et al, 1978; Endo et al, 1979; Kessler et al, 1979a and 1979b; Cole et al, 1985; Wagh and Bahl, 1981; Cole, 1987; Baezinger and Green, 1988).*

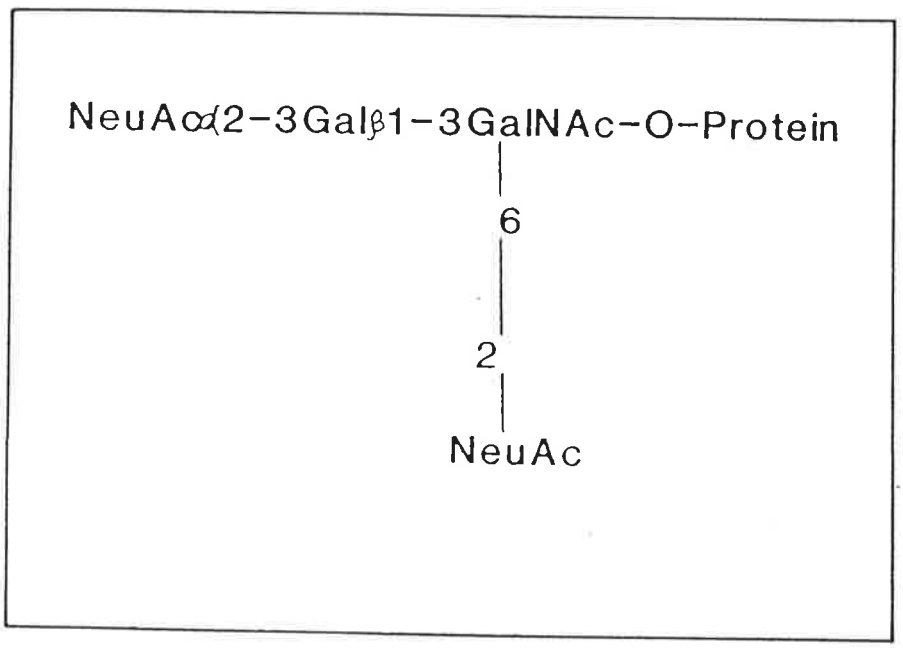


Figure 1.5 The standard serine-linked carbohydrate chain of hCG Proposed by Kessler et al, 1979b.

Proper conformation of whole hCG is necessary for recognition by its specific receptor and for transduction of hormone signal into the cells. Very little is known about the conformational structure of hCG molecule and the available information derives primarily from spectroscopic studies and chemical or enzymatic modification of amino acid chains or sugar residues. Circular dichroism spectra of hCG has shown that though there is little alpha

helicity and an appreciable amount of beta turns, the largest portions of the polypeptide chains are present as aperiodic structures. It seems that the lack of alpha helicity is due to the high prolyl and cysteinyl content and to the large amount of sialic acid *(Hilgenfeldt et al, 1974).* Part of the beta structure may arise from intermolecular hydrogen bonding involving a pleated sheet arrangement between the alpha and beta subunits. The extension of the carboxy-terminal glycopeptide region to the surface of hCG molecule suggests the presence of more beta turn in the point of each O-serine oligosaccharide attachment. The extent of beta structure for the intact hCG, alpha hCG, and beta hCG is thought to be about 28%, 21% and 13%, respectively. As the sum of the beta-structure of both subunits, after being corrected for the respective molecular weight of each subunit, is only 60% of that of the native hormone, substantial conformational changes occur during the dissociation of the dimeric hCG to the biologically inactive subunits. In this process approximately 20 residues are transferred from beta-conformation to an aperiodic conformation. In addition, the circular dichroism difference spectrum indicates that the local environment of disulfide bonds and of tyrosyl residues is altered when the subunits dissociate *(Combarnous and Maghuin-Rogister, 1974; Holladay and Puett, 1975; Ingham et al, 1976; Giudice and Pierce, 1978; Birken et al, 1986).*

These indirect approaches have provided many hints to the conformation of gonadotrophins but problems with crystallizing them have prevented x-ray crystallographic analysis of their three dimensional structure *(Ryan et al, 1987).* The spatial conformation of hCG is believed to be a function of the amino acid sequence and unsuccessful attempts to crystallize the native molecule is thought to be due to the variation in the length and composition of the carbohydrates or to the negatively charged surface sugars *(McPherson,*

1985; Lustbader et al, 1989). Recently chemical and enzyimatically deglycosylated hCG forms were successfully crystallized. At least in these forms it was demonstrated that large and single crystals may be seen as stout rounded faces bipyramids with underlying hexagonal shape (*Harris et al, 1989; Lustbader et al, 1989*). Although deglycosylation does not affect the immunological activity of hCG, subunit reassociation, circular dichroism or receptor binding, it is known that deglycosylation may induce significant conformational changes in hCG and may impede postreceptor events *(Thotakura and Bahl, 1982; Keutmann et al, 1983; Keutmann et al, 1985; Matzuk et al, 1989).* Therefore, despite the significance of these studies, definition of the exact tridimensional structure of the whole hCG molecule, ultimately responsible for binding and activation of specific receptors, awaits further studies. It is expected that advances in the knowledge of the tridimensional structure of hCG will follow in the near future.

1.3 Biosynthesis and secretion of hCG

The biosynthesis and processing of hCG resemble that of other glycoprotein hormones *(Schachter, 1978; Weintraub et al, 1980; Chin et al, 1981; Phillips et al, 1981; Hoshima and Boime, 1982; Weintraub et al, 1983).* There is evidence that both subunits of hCG dimer influence the processing of each other. There appears to be two levels of regulation in this processing; while the cell type determines the sort of oligosaccharide processing on alpha and beta subunits, the alpha-beta combination modulates the extension of this processing *(Corless et al, 1987).* The competent cell synthesizes each subunit of hCG independently at an unbalanced rate as a response to the gene transcription of alpha and beta mRNAs so that the alpha subunit is usually found in excess over the beta subunit *(Vaitukaitis, 1974).* Besides the

co-ordinate production of both subunit polypeptides by cleavage of two distinct signal peptides the assembly process of the native hCG molecule prior to its release requires post-translational glycosylation of these peptide chains, processing of the oligosaccharide moieties, formation of disulfide bonds within each subunit, and proper non-covalent association of alpha and beta subunits *(Bielinska and Boime, 1979; Birken et al, 1981).* These events occur as the hCG molecules are translocated through the cell along the secretory pathway from this site of synthesis in the rough endoplasmic reticulum (RER) to the cell surface.

1.3.1 Biosynthesis of alpha and beta polypeptide chains

The mechanisms of synthesis of the protein portion of hCG are similar to the classical mechanisms of secretory protein biosynthesis and resemble those of other glycoproteins *(Weintraub et al, 1980; Chin et al, 1981; Chappel et al, 1983).* The two subunits that form the whole molecule are transcribed from separate genes *(Kohler et al, 1971; Daniels-McQueen et al, 1978).* While the alpha subunit is encoded by a single gene *(Fiddes and Goodman, 1981; Boothby et al, 1981)* the beta subunit is encoded by a family of at least six genes arranged in a cluster on chromosome 19 *(Fiddes and Goodman, 1981; Boorstein et al, 1982; Talmadge et al, 1982; Naylor et al, 1983; Policastro et al, 1983; Graham et al, 1987).* However only two beta hCG genes are proved to be functional genes encoding the correct ßhCG amino acid sequence *(Fiddes and Talmadge, 1984).* After transcription of the genetic message the translation of the mRNA into the nascent peptide moieties of α and ß subunits takes place in the membrane-bound ribosomes of the RER *(Sharon, 1984).* Each translated product is synthesized as a slightly larger molecular weight immature peptide, named pre-alpha or pre-beta forms, containing the specific sequences and the signal peptide extension *(Giudice et al, 1979; Landefeld, 1979; Godine et al, 1980; Ruddon*

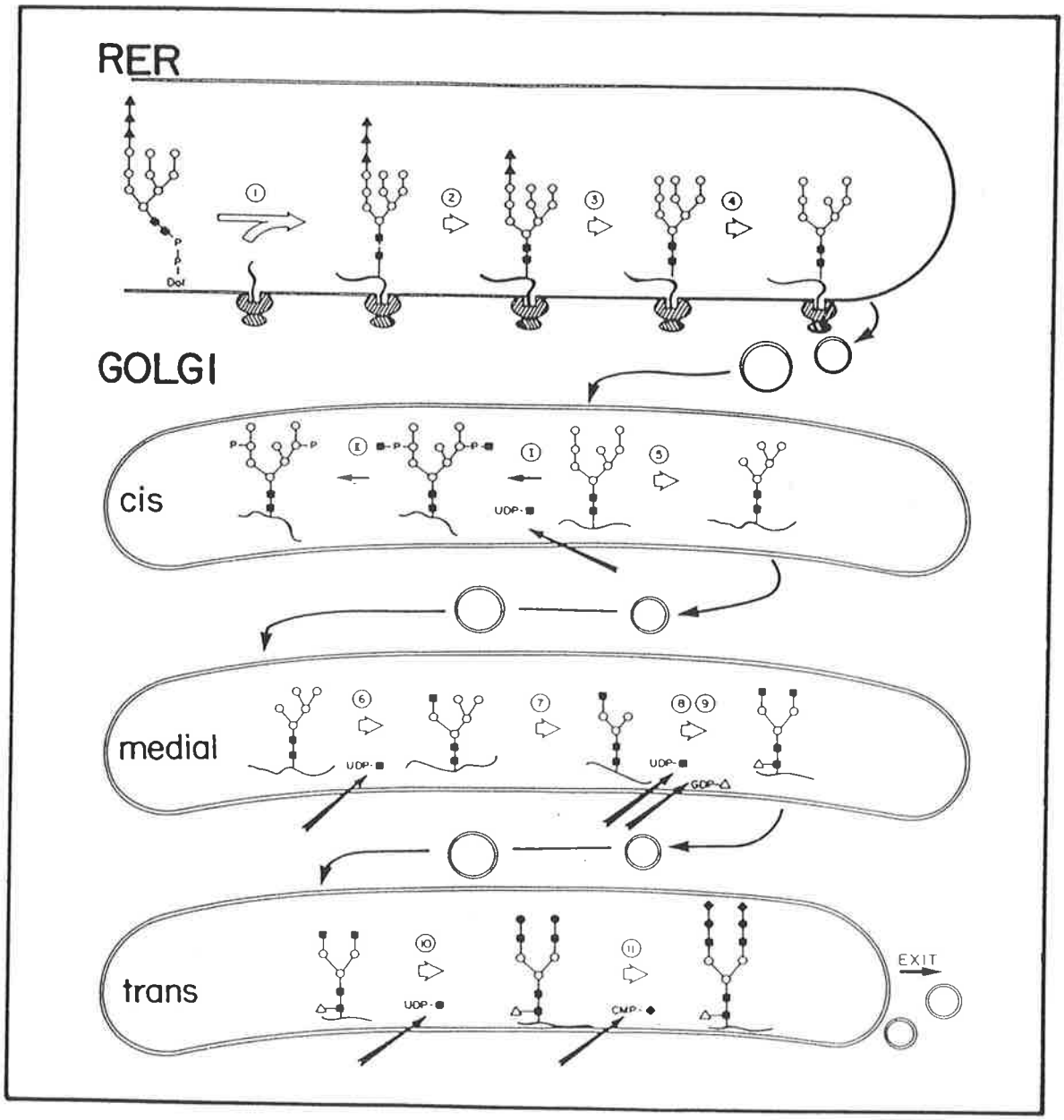


Figure 1.6

Schematic pathway of oligosaccharide processing on newly synthesized glycoproteins. The reactions are catalyzed by the following enzymes: (1) oligosaccharyltransferase, (2) α-glucosidase I, (3) α-glucosidase II,, (4) ER α1,2-mannosidase, (I) *N*-acetylglucosaminylphospatransferase, (II) *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, (5) Golgi α-mannosidase I, (6) *N*-acetylglucosaminyltransferase, I, (7) Golgi α−mannosidase II, (8) *N*-acetylglucosaminyltransferase II, (9) fucosyltransferase, (10) galactosyltransferase, (11) sialyltransferase. The symbols represent: ■ , *N*-acetylglucosamine; ○ , mannose; ▲, glucose; △ , fucose; ● , galactose; ◆, sialic acid. From Kornfeld and Kornfeld, 1985 .

et al, 1987). These signal peptide chains of pre-alpha and pre-beta subunits contain 24 and 20 amino acids, respectively *(Birken et al, 1981).*

The processing of immature subunits to the mature state involves co-translational signal peptide cleavage and removal by microsomal signal peptidase while the peptide chain is still resident upon the ribosomes *(Jackson and Blobel, 1977; Giudice et al, 1979; Godine et al, 1981; Pierce et al, 1981).* Just before the oligosaccharide moieties are attached to the polypeptide chain the newly synthesized protein is released into RER channels and transported to the Golgi.

1.3.2 Glycosylation of hCG polypeptide chains

The addition of carbohydrate chains to hCG subunits during the biosynthesis process is also analogous to other glycoproteins and involves a series of co- and post-translational modifications brought about by the sequential action of a number of enzymes before the subunits are secreted *(Birken et al, 1978; Sakakibara et al, 1986).* The carbohydrate moieties are covalently attached to the hCG molecule by two mechanisms: N-linkage and O-linkage. The N-linked glycosylation of α and ß subunits involves the transfer "en bloc" of the pre-assembled oligosaccharide unit to specific sites of asparagine residues on the polypeptide chains, while the O-linked glycosylation of beta subunit involves the addition of the linkage sugar directly to the polypeptide chains followed by the stepwise addition of each monosaccharide individually *(Bielinska and Boime, 1979; Endo et al, 1979; Wagh and Bahl, 1981; Hubbart and Ivatt, 1981; Kornfeld, 1982; Weintraub et al, 1985; Green et al, 1986; Shylaja and Seshadri, 1989).*

The N-linked oligosaccharide precursor transferred from the lipid carrier dolichol phosphate by activity of a oligosaccharyl transferase is keep a large polysaccharide rich in mannose residues. Immediately after being attached to

the nascent peptide, it undergoes a number of co-translationally and post-translationally processing reactions including removal of glucose and mannose residues and addition of N-acetyl glucosamine, fucose, galactose and sialic acid. The processing, involving a well-organized sequence of steps, as depicted in Figure 1.6, is led by the RER and the Golgi enzymes. It is completed only after the assembly of the dimer hCG, which occurs shortly before its secretion *(Marshall, 1974; Kessler et al, 1979; Lennarz, 1980; Beyer and Hill, 1982; Staneloni and Leloir, 1982; Kornfeld and Kornfeld, 1985; Lennarz, 1987; Hurbain-Kosmath et al, 1987).* The O-linked oligosaccharide does not arise from the dolichol ester intermediate but rather occurs one residue at a time, directly on the beta subunit polypeptide chain. The addition of the O-linked moieties takes place in the Golgi and the sequential reaction of residual incorporation involving specific enzymes consists of the attachment of N-acetylgalactosamine to serine residues 121, 127, 132, and 138, linkage of galactose residue to N-acetylgalactosamine, and further attachment of sialic acid to galactose. The ultimate structure of this carbohydrate unit arises from addition of sialic acid to linkage N-acetylgalactosamine *(Young et al, 1979; Kessler et al, 1979; Peters et al, 1984; Cole et al, 1985; Cole, 1987a; Cole, 1987b).*

1.3.3 Assembly and secretion of the whole molecule of hCG

Following the addition of carbohydrate units the association of alpha and beta subunits to form the intact dimer occurs in the RER at the time both subunits still contain N-linked high mannose-type oligosaccharides as immature forms *(Hoshima and Boime, 1982; Peters et al, 1984).* The combination reaction between both subunits does not reach completion and significant fractions of the intracellular alpha and beta precursors do not combine. The dimerization process takes place between the alpha and the most disulfide-bond formed beta subunits. The uncombined forms, either

excessive alpha or less disulfide-bonded beta subunits, can subsequently be processed and secreted as mature free subunits. It has been suggested that a conformational change in the beta subunit controls the final oligosaccharide processing in the combined hormone, but it is also possible that both subunits of hCG influence each other giving rise to its specific structure *(Green et al, 1985; Corless et al, 1987; Ruddon et al, 1987).* Folding and assembly of the subunits into the functional hormone appear to be dependent on the carbohydrate moieties and their specific positions on the peptide chains *(Bielinska et al, 1989).* Although the carbohydrates are not obligatory for facilitation of the formation of the correct tertiary structure of alpha subunit during the folding process, they may prevent the formation of non-active disulfide bonds and ensure that certain portions of the polypeptide chain remain on the surface of the molecule during the folding reaction avoiding incorrect interaction *(Goverman et al, 1982; Weintraub et al, 1983; Strickland and Pierce, 1983; Strickland et al, 1985).* Ultimately, the oligosaccharide moieties are located at positions that are predicted to be beta turns and the beta turns are crucial elements in folding *(Lewis et al, 1973; Crawford et al, 1973; Aubert et al, 1976; Chou and Fasman, 1977; Beeley, 1977).* Use of inhibitors of carbohydrate addition in vitro does not interfere with the secretion of the subunits but seems to block the assembly of the dimer *(Ruddon et al, 1979).* As chemical or enzymatic deglycosylation methods used to remove the O-linked sugar can also affect the N-linked moieties, and the intracellular inhibitors of O-glycosylation are not available, the significance of the O-linked oligosaccharides attached on the carboxy terminal of the beta subunit is not completely understood. In any case, they do not appear to affect the assembly or secretion of the hCG dimer *(Matzuk et al, 1987).* The importance of site-specific N-linked oligosaccharides of the alpha subunit on the assembly and secretion of the hCG molecule has been extensively studied. The oligosaccharide branch from Asn-78 assures normal secretion of alpha

subunit and the Asn-52 carbohydrate is essential for the combination with the beta subunit. In addition, the Asn-30 oligosaccharide on the beta subunit is important for secretion but not assembly and the Asn-13 branch influences mainly the assembly and dimer formation *(Matzuk and Boime, 1988)*. Thus, the two N-linked oligosaccharides of both alpha and beta subunits are critical for the efficient assembly and secretion of the hCG molecule. In relation to the importance of the protein portion on the assembly of hCG, it is known that the polypeptide sequence 27-40 of the alpha subunit has an important role in the interaction between subunits. Moreover, while alpha Tyr-37 is a critical residue for proper combination of alpha and beta subunits, the Tyr-65 is involved in holding both subunits in native conformation *(Hum et al, 1974)*. The Phe-33, Lys-45, and Glu-60 residues also are involved in the interaction of subunits (*Nishimura et al, 1986*). The final conformation of hCG molecules is stabilized by the 6 disulfide bonds and by proper readjustment after subunit assembly *(Schwarz et al, 1986)*. The terminal regions are charged, more flexible than the middle portions, accessible to the surface, and significant for both the folding pathway and stabilization of the quaternary structure *(Thornton and Sibanda, 1983)*.

hCG is mainly a product of placental syncytiotrophoblast cell activity. However, it can be secreted by a number of normal non-placental tissues and trophoblastic and certain non-trophoblastic neoplasms. The primary events leading to the induction of its secretion remain unresolved. This process involves a serial of steps in which cells make up and release the whole hCG molecule as well as free alpha and free beta subunits. Unlike the other glycoprotein hormones, there is no certain releasing factor specific to hCG, although it is accepted that cAMP activates the genetic transcription of DNA sequences which code the synthesis of alpha and beta polypeptide chains into the ribosomes attached to RER membrane. There is also no clear

understanding of the intimate mechanisms involved in the post-translated glycosylation, processing of the carbohydrate chains, transport to the Golgi apparatus, combination of subunits, and release in circulation. However, there is a body of evidence indicating that after the primary signal these events can be modulated by a variety of factors, including the cAMP analogues, epidermal growth factors, GnRH, phorbolesters, insulin, glucocorticoids, and some polyamines *(Khodr and Siles-Khodr 1980; Wilson and Jawad 1982; Carson et al, 1983; Ilekis and Benveniste 1985; Feinman et al, 1986; Morrish et al, 1987; Ullhoa-Aguirre et al, 1987; Benoit et al, 1988; Moore et al, 1988; Sibley et al, 1991; Ren and Braunstein, 1991).* The principal modulators with recognized inhibitory activity are several progestational steroids and prolactin (Yuen et al, 1980; Wilson et al, 1980; Wilson et al, 1984).

1.4 Structure-biological function relationship

To express its biological activity the hCG molecule binds to a specific receptor on the surface of target cells, stimulates the adenylate cyclase system and steroidogenesis. Biological activity is dependent on expression of the primary, secondary, tertiary, and probably quaternary structure. The signal transduction is likely due to conformational changes in one or both subunits *(Goverde et al, 1968; Mori, 1970; Moyle et al, 1975; Hattori et al, 1978; Rebois and Liss, 1987; Matzuk et al, 1979; Sairam and Schiller, 1979; Shinohiga and Chen, 1982; Moudgal and Li, 1982; Thotakura and Bahl, 1982; Sairam and Manjunath, 1982; Keutmann et al, 1983).* Since the dissociation of the subunits and the loss of either specific peptide regions or some sugar residues affect both the immunological and biological activities, it is accepted that besides the conformational structure of the dimer, biological function is dependent on particular regions of the polypeptide chains and certain

carbohydrate branches or residues. However the relative importance of both the chemical compounds and the conformational structure assumed after formation of the alpha-beta dimer upon its ability to bind to specific receptors and to express its biological activity is not yet completely known.

In a dissociated state the hCG subunits are devoid of almost all biological activity *(Swaminanthan and Bahl, 1970; Morgan and Canfield, 1971; Braunstein et al, 1972b; Rayford et al, 1972; Morgan et al, 1974; Catt et al, 1977)*, but their further recombination restores nearly 80% of the original activity *(Canfield et al, 1971; Pierce et al, 1971; Bahl, 1972b)*. These observations indicate some conformational alterations after the subunits association and demonstrate that both subunits give the correct conformational feature of the native hormone *(Bewley et al, 1972; Garnier et al, 1974; Holladay and Puett 1975; Strickland and Puett, 1982)*. Therefore, although the biological specificity is conferred by the beta subunit, significant hormone activity is expressed only after formation of the alpha-beta heterodimer. Even though the receptor binding regions are thought to be located at the surface of the beta subunit, specific amino acid groups on the alpha subunit appear critical for activity, indicating that the active site of the whole molecule includes residues or structural regions from both subunits *(Merz and Dorner, 1979; Tyrey, 1982)*. The reason for the requirement of the alpha-beta combination for expression of hormonal activity is not known. There appears to exist at least three major roles for the alpha subunit after combination with the beta subunit: carrying some of the recognition sites necessary for binding to the receptor, induction of an active conformation of the beta subunit, and stabilization of the hormone-receptor complex *(Milius et al, 1983)*.

The disulfide bonds on alpha and beta subunits assure the stability of the tertiary structure of the hCG molecule. In synergism with the carbohydrate units, closely situated half-cystinyl bonds confer resistance to hydrolysis by proteolytic enzymes *(Mise and Bahl, 1981)*. Detailed studies concerning the significance of specific disulfide bonds on the hCG molecule are yet not available. While the most accessible bonds appear not to be essential for hCG biological activity, the reduction of the inner ones inactivates the hormonal expression. As the open chain Ala 93-100 of the beta subunit is inactive, it appears that the 93-100 disulfide bond on this subunit gives the essential conformation for binding *(Keutmann, 1989)*. In addition, alteration of any Cys residue on the beta subunit generates different structural forms that are unable to assemble with the alpha subunit *(Suganuma et al, 1989)*. Information about the role of the polypeptide residues or portions on the function of hCG are based on several studies including the use of synthetic peptides, chemical or enzymatic modifications of polypeptide moieties and behaviour of monoclonal antibodies directed to particular regions of the hCG molecule. Several studies using either polyclonal or monoclonal antibodies have demonstrated that epitopes on both subunits of the intact hormone are important for the native hormone to interact with the receptors *(Pierce et al, 1979; Ji and Ji, 1981; Moyle et al, 1982; Strickland and Puett, 1982; Ehrlich et al, 1985; Norman et al, 1985; Bidart et al, 1987; Dighe et al, 1990)*. A number of chemical and enzymatic experimental data support the hypothesis that there are at least two peptide regions, named receptor-determinant loops, within the beta subunit which confer receptor specificity *(Keutmann et al, 1983; Willey et al, 1986; Keutmann et al, 1987)*. These receptor-determinant loops are located between cysteine residues 38-57 and 93-100 of the beta subunit. Experimentally, some peptide fragments of hCG, synthesized by chemical means, retain the ability to bind to hCG-specific receptors and to stimulate steroidogenesis *(Manjunath and Sairam, 1982; Keutmann et al,*

1987). The residues 87-92 of the alpha subunit play an important role in receptor binding and biological expression *(Hum et al, 1977; Parsons and Pierce, 1979; Frankenne et al, 1983; Bellet et al, 1984; Caraux et al, 1985; Grass-Masse et al, 1986; Charlesworth et al, 1987; Bidart et al, 1987; Bidart et al, 1988; Troalen et al, 1988).* The surface able to activate the adenylate cyclase would include the majority of the alpha subunit and the Asp-99 residue contained in the loop 93-100 of the beta subunit. The carboxy-terminal extension of ßhCG and associated O-linked oligosaccharides are not important for receptor binding or in vitro signal transduction, but are critical for in vivo biological response *(Matzuk et al, 1990; Chen and Puett, 1991).* Despite the putative importance of some protein components for hCG to express its biological activity, Campbell and Moyle (1989) has shown that no particular peptide portion in the hCG is the primary determinant of binding specificity. In addition, recent studies, based on the analogy between hCG and the serine protease chymotrypsin offer a putative tertiary model in which the alpha and beta subunits of hCG apportion a receptor binding region and an agonist-activity region placed to different but adjacent areas of the molecule *(Willey et al, 1986; Willey and Leidenberger, 1989).*

In a general way the carbohydrate moieties of hCG are needed for folding and acquisition of the correct conformation of the whole molecule, interaction between the alpha-beta subunits, and protection against proteolytic degradation. Additionally, they have a very relevant role in the biological activity of the hormone. In relation to the ability of hCG to bind the target cell, the N-linked carbohydrates on alpha, beta or both subunits has little effect. In fact the removal of certain sugar residues increase the affinity of hCG to its specific receptors *(Govermann et al, 1982; Manjunath and Sairam, 1982; Chen et al, 1982; Kalyan and Bahl, 1983; Keutmann et al, 1983; Matzuk et al, 1989; Thotakura et al, 1990).* The role of the individual oligosaccharides on

both alpha and beta hCG have been extensively studied in receptor binding and signal transduction. Some carbohydrates located on both subunits are critical to the biological expression of hCG. The absence of the Asn 30 from the beta subunit or a single oligosaccharide from Asn 78 from alpha do not alter the production of cAMP or steroidogenesis. In contrast, the Asn 13 from beta subunit plays an important role in steroidogenesis and the presence of the alpha Asn 52 oligosaccharide is critical for both the cAMP response and steroidogenesis. In addition to be related to the isoelectric point, charge, and plasma half life, the sialic acid content of hCG has major significance in the receptor binding ability and in in vitro and in vivo biological activities. The biological activity of hCG decreases with progressive desialylation, and the partial or complete removal of carbohydrate moieties internal to sialic acid by glycosidases or chemical treatment also decreases the adenylate cyclase and steroidogenic activities *(Van Hell and Schuurs, 1970, Braunstein et al, 1971; Brossner et al, 1971; Van Hall et al, 1971; Graesslin et al, 1972; Channing et al, 1978; Brand et al, 1980; Amano et al, 1989; Kobata, 1989; Ji and Ji, 1990).*

1.5 Heterogeneity of hCG

1.5.1 Heterogeneity of whole hCG

hCG pleomorphism has been disclosed by several techniques such as gel filtration, affinity chromatography, ion exchange chromatography, isoelectric focusing, and chromatofocusing. Differences in the amino acid composition *(Weintraub et al, 1975; Lee et al, 1977; Benveniste et al, 1979)*, carbohydrate residues *(Puett et al, 1978; Choy et al, 1979; Ruddon et al, 1980; Dean et al, 1980)*, or both have been proposed. Certainly quantitative differences in carbohydrate content account for most of the observed heterogeneity. Since the internal monosaccharides of the carbohydrate cores are relatively homogeneous, this variation in carbohydrate structure often occurs in the

peripheral residues. However, the nature and extent of the sugar heterogeneity remain to be completely clarified. It is suggested that some tissues may be unable to either synthesize the complete carbohydrate chain (hypoglycosylated forms) or trim the highly glycosylated precursor forms (hyperglycosylated variants). Besides the heterogeneity due to modifications in the sugar content, there exist some forms in which the polypeptide chains also show variations. It is thought the heterogeneous polypeptide chain may arise as a consequence of structural variation at the genetic level or from peptide-chain modifications of precursor molecules at various stages of cleavage during or subsequent to the biosynthesis *(Bahl et al, 1972; Morgan et al, 1973; Bellisario et al, 1973; Vaitukaitis, 1974; Weintraub et al, 1975; Lee et al, 1977; Benveniste et al, 1979; Story et al, 1981; Cole et al, 1982; Hussa et al, 1986).*

In any case, any modification of the native hCG may result in molecules with different charge, size, molecular weight, circulating half life, immunological properties and in vitro or in vivo biological activities. The earliest studies reporting different physicochemical properties between the hCG from normal pregnant women and from patients with trophoblastic disease date from 1959 and 1960. After zone electrophoresis and ion exchange chromatography followed by paper electrophoresis separation Reisfeld et al, (1959) and Reisfeld and Hertz, (1960) showed different distribution in the biological activity of hCG obtained from serum of normal pregnant women or women with trophoblastic disease. Following these early observations, heterogeneity in charge, size and/or chemical composition has been demonstrated among hCG isolated from different patients *(Vaitukaitis, 1973; Weintraub and Rosen, 1973; Muggia et al, 1975; Papapetrou and Nicopoulou, 1986)* as well as different tissues *(Vaitukaitis, 1974; Weintraub et al, 1975; Vaitukaitis and Ebersole, 1976; Lee et al, 1977)*, from urine *(Puett et*

al, 1978; Choy et al, 1979), and from the lysates and culture media of several eutopic and ectopic cell lines *(Lieblich et al, 1976; Hussa, 1977; Kanabus et al, 1978; Benveniste et al, 1979; Ruddon et al, 1980)*. Recently peptide variability have been demonstrated among individual samples of hCG. Approximately 25% of hCG molecules in urine and serum of normal pregnancy women or women with trophoblastic disease has the ß-polypeptide chain nicked at the linkages 44-45, 46-47, or 47-48, and 15% miss the ß-subunit carboxy-terminal region (*Kardana et al, 1991; Cole et al, 1991*). Additionally, 7%-8% of the αhCG subunits as part of the dimer is nicked between residues 70-71. Moreover, the percentage of peptide bond nicking in the various hCG standard preparations have been shown to be as high as 10%-20% (*Birken et al, 1991*). The principal immunoreactive forms of hCG or its subunits are summarized in Table 1.2. Variable degrees of desialylation have been demonstrated in both highly purified or crude preparations of hCG, hCG from placental extracts, urine and serum from normal pregnant women, urine and semen from patients with trophoblastic neoplasms or other sources *(Chan et al, 1974; Merz et al, 1974; Choy et al, 1979; Yasaki et al, 1980; Nishimura et al, 1981; Nwokoro et al, 1981; Taliadouros et al, 1982; Hay, 1986; Kobata, 1987; Cole, 1987; Ullhoa-Aguirre et al, 1990)*. Approximately 6 to 12 different charged forms of hCG have already been identified by isoelectric focusing separation and the most desialylated forms of hCG are found mainly in the urine of patients with trophoblastic neoplasms *(Graesslin et al, 1976; Nwokoro et al, 1981`; Hay, 1986)*. In addition to hCG with different charges, several reports have appeared demonstrating the existence of large molecular species of hCG as well *(Vaitukaitis, 1974; Chan et al, 1974;*

Table 1.2 Heterogenous forms of hCG, ßhCG, αhCG, and source of detection

	Protein	Source	Reference
hCG			
	Hyperglycosylated form	Pregnancy, tumours	Fein et al, 1980
	Abnormal bi, triantennary forms	Trophoblastic tumours	Mizuochi et al, 1983
	Additional peptide forms	Placental extracts	Maruo et al, 1980; Good et al, 1977
	Asialo hCG	Choriocarcinoma	Nishimura et al, 1981
	Hypoglycosylated forms	Pregnancy, tumours	Dufau et al, 1972; Puett et al, 1978
ßhCG			
	Free ßhCG	Pregnancy, tumours	Cole et al, 1983; Fan et al, 1987
	Pre-beta forms	Placenta	Godine et al, 1982; Ruddon et al, 1987
	Additional peptide forms	Placenta	Hussa et al, 1987
	Hyperglycosylated beta	Pregnancy, tumours	Fein et al, 1980; Cole et al, 1981
αhCG			
	Free αhCG	Pregnancy, tumours	Dean et al, 1980
	Pre-alpha form	Placenta	Sakakibara et al, 1986; Tominaga et al, 1991
	Hyperglycosylated form	Pregnancy, tumours	Cole et al, 1984; Fein et al, 1980
	Large alpha peptide	Gastric carcinoid cells, choriocarcinoma	Weintraub et al, 1975; Benveniste et al, 1979
	Peptide deleted form	Pregnancy, tumours	Weintraub et al, 1975; Birken et al, 1978

Good et al, 1977; Tojo et al, 1977; Patillo et al, 1979; Maruo et al, 1984; Tominaga et al, 1989).

It has been suggested that these large molecular forms could represent an intermediate component of hCG biosynthesis, a product of post-translational changes in the carbohydrate moieties, molecules with additional peptide residues as a result of disulfide interchange with other peptides, or molecules with extended polypeptide chain in which disulfide bonds are lacking. The aggregation of hCG molecules or non-specific binding of hCG to subcellular fractions are possibilities that cannot be eliminated. Besides the large forms, smaller molecules of hCG have been found in urine from women with trophoblastic neoplasms and placental extracts *(Vaitukaitis, 1973; Fein et al, 1980).*

1.5.2 Heterogeneity of αhCG subunit (αhCG)

In addition to the native alpha hCG subunit, other forms with a different size, molecular weight and ability to combine to the native beta hCG have been reported. The differences may be due to a modified polypeptide chain or to heterogeneous carbohydrate moieties. About 70% of the amino acid chains of alpha molecules from the placenta start with the sequence Ala-Pro-Asp-Val-NH_2, 20% represent a variant in which Ala-Pro-Asp is lacking and commence with Val-NH_2 and another 10% represent a different variant that miss Ala-Pro and start with Asp-Val-NH_2 *(Birken et al, 1978).* Besides differences in the length of the amino acid chain at the alpha NH_2 terminal, other variations in different amino acid sequences have also been noted *(Weintraub et al, 1973; Weintraub et al, 1975; Lee et al, 1977; Birken et al, 1978; Bielinska and Boime, 1978; Ruddon et al, 1981).* Although these reports consider the possibility of loss or addition of certain amino acids in the alpha subunit chain, the heterogeneity of this subunit reflects mainly

excessive or poor glycosylation *(Dean et al, 1980; Fein et al, 1980).* Forms with fucose, increased sialic acid content, unusual triantennary N-linked carbohydrate moieties or containing an O-linked oligosaccharide chain attached to Tyr-39 have been described *(Ruddon et al, 1981; Cole et al, 1984; Peter et al, 1984; Blithe and Nisula, 1985; Lustbader et al, 1987; Cole, 1987).* The alpha subunit dissociated from the hCG dimer binds to Concanavalin A and consists primarily of monosialylated forms. The free alpha form, in contrast, is heterogeneous and consists of di and trisialylated molecules that may or may not bind Con A *(Blithe and Nisula, 1985).* The secretion of a mature, large form of free alpha hCG has been reported in crude commercial hCG preparation, pregnancy urine, serum, placenta, pituitary and various neoplasms or tumor cell culture systems *(Hussa et al, 1977; Hoshima and Boime, 1982; Weintraub et al, 1983; Parsons et al, 1983; Porcillico et al, 1983; Cole et al, 1984; Blithe and Nisula, 1985; Corless and Boime, 1985; Cole, 1987).* This form is larger than that which forms hCG dimer and does not combine with the ßhCG to form the intact hormone *(Weintraub et al, 1977; Hussa, 1981; Cole et al, 1983; Bielinska et al, 1989).* The existence of heterogeneity in alpha hCG from non-gestational sources has also been described and the urinary alpha hCG subunit from post-menopausal individuals has a larger molecular weight than the alpha dissociated from intact hCG both from placenta or pituitary *(Prentice and Ryan, 1975; Fein et al, 1980; Kourides et al, 1980; Papapetrou and Anagnastopoulos, 1985).* Although many reports provide evidence of alpha subunit heterogeneity, the biological significance of the different molecules and the factors that induce their synthesis and secretion remain to be clarified.

1.5.3 Heterogeneity of ßhCG subunit (ßhCG)

Heterogeneity of the ßhCG subunit also is extensively described. Even the highly purified urinary ßhCG is not homogenous *(Kessler et al, 1979; Cole*

et al, 1985). More accentuated heterogeneity, both in immunological behaviour and chemical properties, than that seen in standard preparations has been described in the ßhCG subunit secreted by normal and abnormal trophoblast and various tissues, usually malignant *(Vaitukaitis, 1973; Donaldson et al, 1980; Norman et al, 1985; Collins and Wong, 1989).* The commonest forms to date characterized in both benign or malignant conditions were classified by Cole and Hussa (1984) and are presented in Table 1.2. Several studies suggest that some heterogeneous forms of ßhCG may be a result of different gene expression *(Policastro et al, 1978; Boorstein et al, 1982; Fiddes and Talmadge, 1984; Graham et al, 1987; Jameson et al, 1987).* In both intracellular and extracellular beta hCG molecules the principal alterations detected in the protein core are a decrease in disulfide bonds *(Peters et al, 1984; Ruddon et al, 1987)*, presence of additional peptide residues *(Cole et al, 1982; Nishimura et al, 1988)*, existence of alternative amino acid sequences *(Talmadge et al, 1983)*, deletion of amino acid residues at the carboxy terminal *(Hussa et al, 1986)* and deletion of all the carboxy terminal extension *(Cole et al, 1982; Kardana et al, 1991).* However, only the forms with altered disulfide bonds or deletion of the carboxy terminal portion are well characterized and the existence of the other forms as native molecules remain to be proven. Beta hCG with altered sugar moieties is more widely documented and beta molecules with large O-linked oligosaccharide chains at the carboxy terminal extension *(Nalgeberg et al, 1985)*, high mannose content *(Sakakibara et al, 1986; Ruddon et al, 1987)*, high sialic acid content *(Benveniste et al, 1979; Dean et al, 1980; Fein et al, 1980; Cole and Hussa, 1981)* and poor in sialic acid *(Story et al, 1981)* have been studied. Different intracellular forms of beta hCG subunit with high mannose oligosaccharide moieties have been detected in first trimester placental tissue and choriocarcinoma cell lines as immature intermediate forms *(Ruddon et al, 1980; Ruddon et al, 1981; Sakakibara et al, 1986; Tominaga et al, 1989)* or

pre-beta forms *(Godine et al, 1982; Ruddon et al, 1987)*. Although small amounts of intracellular mature forms with complex-type oligosaccharides can be found, the major intracellular forms of beta subunit exist as smaller molecular species *(Ruddon et al, 1981; Sakakibara et al, 1986; Ruddon et al, 1987)*. Moreover, a large beta form, probably a precursor of the mature hCG, was found in a wheat cell-free polyribosomes system *(Maruo et al, 1984)* and may be a result of an aggregation process *(Tominaga et al, 1989)*. Mature intracellular forms of ßhCG which contain complex-type oligosaccharide chains, can be poorly disulfide-bonded and may *(Sakakibara et al, 1986; Tominaga et al, 1989)* or may not *(Ruddon et al, 1981; Ruddon et al, 1987)* combine with the native alpha hCG subunit. The molecules that do not achieve the appropriate conformation to associate with alpha are secreted as larger free forms *(Swaninathan and Bahl, 1970; Hilgenfeldt et al, 1972; McFarlane et al, 1979; Cole et al, 1982; Maruo et al, 1984; Nishimura et al, 1988)*. Free forms of hCG beta subunit have been detected in normal pregnancy serum *(Franchimont et al, 1971; Cole et al, 1984; Hay, 1985; Ozturk et al, 1987)*, pregnancy urine *(Hagen et al, 1975; Reuter et al, 1980, Norman et al, 1985)*, cultures of normal trophoblast *(Taliadouros et al, 1982; Ozturk et al, 1987)*, benign and malignant trophoblastic disease *(Khoo and Daunter, 1980; Ashitaka et al, 1980; Braunstein et al, 1983; Fan et al, 1987; Ozturk et al, 1988; Hay, 1988)*, cultures of various choriocarcinoma cell lines *(Rosen et al, 1980)*, and many non-trophoblastic tumours *(Rosen et al, 1975; Kahn et al, 1977; Tormey et al, 1977; Sheth et al, 1981; Van Nagell et al, 1981; Mohabeer et al, 1983; Rodenberg et al, 1985; Heyderman et al, 1985)*. While some authors described a normal size free beta subunit *(Cole et al, 1983; Lustbader et al, 1987; Ozturk et al, 1987)*, others described a free form larger than the beta hCG standard *(Franchimont et al, 1971; Thotakura and Bahl, 1986)*, suggesting the existence of two types of free beta hCG *(Gaspard et al, 1980; Mann and Karl, 1983; Norman et al, 1985)*. Some of these free

beta hCG molecules are able to combine with the alpha subunit to form the active dimer *(Hussa, 1980; Cole et al, 1983; Thotakura and Bahl, 1986).*

1.6 Native fragments of hCG/ßhCG molecules

In addition to the unusual forms of hCG/ßhCG previously described, some native fragments or fragmented forms have also been detected in body fluids (Table 1.3). A glycosylated small fragment composed by the 48-145 polypeptide chain portion of the ßhCG subunit as a result of reduction of the native molecule was recently found in crude preparations of urinary hCG, highly purified standard hCG, and in urine of patients with trophoblastic tumours *(Nishimura et al, 1988; Bidart et al, 1988).* Several forms of ßhCG fragmented at different linkages within the 38-57 polypeptide loop has been described in standard reference preparations, urine and serum from pregnant women or from patients with trophoblastic neoplasms *(Kardana et al, 1991; Cole et al, 1991).* Even though the ßhCG subunit can be nicked at linkages 44-45 and 46-47, a nicked beta subunit of hCG (N-ßhCG) consisting of two polypeptide chains composed of residues 1-47 disulfide-bridged to residues 48-145 as a result of nicking of the ßhCG at Gly-47 and Val-48 is the primary ß-nicked form *(Puisieux et al, 1990).* This fragmented N-ßhCG combines with the native alpha to form the dimer hCG molecule and the resultant hCG molecule has a reduced ability to bind the specific receptor and decreased ability to stimulate steroidogenesis. This form represents approximately 10% of the hCG dimer present in urine of normal pregnant women *(Sakakibara et al, 1990; Birken et al, 1991).* The nature of the enzyme that accounts for the native production of N-ßhCG remains to be investigated. Birken et al (1991) showed that human leucocyte elastase is capable of cleaving the ß44-45, ß48-49, and ß51-52 peptide bonds, suggesting that this enzyme may account for some of these cleavages.

Table 1.3 Native fragments of hCG/ßhCG and source detection

Fragmented form	Source	Reference
N-ßhCG*	Pregnancy, tumours	Puisieux et al, 1990; Sakakibara et al, 1990
Asialo CTP of ßhCG	Choriocarcinoma	Amr et al, 1983
ßhCG minus-CTP**	Cervical carcinoma, trophoblastic tumours	Nalgeberg et al, 1985
ß 48-145 peptide	Pregnancy, trophoblastic disease	Nishimura et al, 1988; Bidart et al, 1988
ßC-hCG***	Pregnancy, tumours	Vaitukaitis, 1973, Schoreder and Halter, 1983

* Nicked ßhCG, ** carboxy-terminal peptide, *** Beta-core hCG

Besides the large ßhCG subunit form without the carboxy terminal extension related to or produced by cervical carcinoma *(Nalgeberg et al, 1985)* and the N-ßhCG *(Sakakibara et al, 1990; Puisieux et al, 1990)*, the existence of a native asialo-carboxy terminal peptide fragment (CTP) has also been demonstrated *(Amr et al, 1983)*. This carboxy terminal peptide fragment of hCG or ßhCG molecules does not have wide distribution, but its description is limited nearly exclusively to the urine of patients with gestational trophoblastic neoplasia and only exceptionally may be found in the urine during normal pregnancy *(Amr et al, 1984)*. Chemically the beta CTP-fragment contains all the 115-145 amino acids sequence of the carboxy terminal region of the intact beta hCG and the four O-linked carbohydrate moieties existent in the native beta hCG but differentiates from that by lacking the terminal sialic acid residues. It is recognized by the antisera directed to the ßhCG carboxy terminal amino acid sequence *(Matsuura et al, 1978)* but does not interact with the hCG/LH receptors from the Leydig cell membranes *(Amr et al, 1983)*. Although the mechanism of appearance of this fragment is not completely explained, the infusion of asialo-hCG, but not of intact hCG, in normal subjects induces a significant increase of this fragment in the urine, suggesting that originates from the peripheral metabolism of asialo-hCG *(Amr et al, 1985)*.

1.7 Beta Core hCG fragment (ßC-hCG)

The presence of a small molecular weight immunoreactive form of hCG in the urine of pregnant women was first reported by Matthies and Diczfaluzy (1971). Further studies showed that this molecule may be found, usually in small amounts, in the sera of pregnant women *(Franchimont et al, 1972; Schroeder and Halter, 1983; Birken et al, 1988; Kato and Braunstein, 1988; Cole and Birken, 1988; Wehmann et al, 1989; Alfthan and Stenman, 1990;*

Kardana and Cole 1990), pregnancy urine *(Vaitukaitis, 1974; Good et al, 1977; Wehmann et al, 1984; Blithe et al, 1988; Akar et al, 1988; Wehmann et al, 1988)*, medium from cultured placental extracts *(Vaitukaitis 1974; Good et al, 1977; Fein et al, 1980; Cole and Birken, 1988)*, purified preparations of hCG and ßhCG *(Wehmann et al, 1988; Krichevsky et al, 1991)*, crude commercial preparations of hCG *(Sowers et al, 1979; Taliadouros et al, 1982; Koyama et al, 1986)*, various trophoblastic and non-trophoblastic neoplasms *(Vaitukaitis, 1973; Hattori et al, 1980; O'Connor et al, 1988; Norman et al, 1988)* and urine of normal subjects *(Akar et al, 1988; Wehmann et al, 1989).*

Even though this fragment has been detected in various fluids in the body, it was initially recognized in and associated to cancer states. Urines from patients with trophoblastic *(Vaitukaitis, 1973; Masure et al, 1981)* or various non-trophoblastic cancers *(Vaitukaitis, 1973; Hattori et al, 1980; Papapetrou et al, 1980; Masure et al, 1981)* exhibit variable amounts of this fragment, occasionally being the predominant molecular form accounting for the hCG immunoreactivity in urine *(Papapetrou and Nicopoulou, 1986).* Despite the huge amount found in urine of patients with neoplastic disease, only trace amounts were found in tumour extracts or serum, indicating that this fragment could be a end-product of hCG/ßhCG excretion. In addition to the presence of ßC-hCG in malignant conditions, several studies have shown that the most abundant hCG immunoreactivity detected in the urine of pregnant subjects is due to this small fragment of hCG/ßhCG molecules. In fact, ßC-hCG has been reported to account for 15% to 750% of the total hCG immunoactivity in this fluid, either when estimated by assays using antibodies that also recognize free beta or intact hCG *(Schroeder and Halter, 1983)* or when estimated by specific assays *(O'Connor et al, 1988; Birken et al, 1988).* Beta-core hCG does not combine with intact alpha subunit and does not bind to the rat hCG/LH Leydig cell receptors or to ovarian hCG/LH radioreceptor assay

(Masure et al, 1981; Schroeder and Halter, 1983). It is also devoid of biological activity in both in vitro *(Schroeder and Halter, 1983; Kato and Braunstein, 1988)* and in vivo bioassays *(Matthies and Diczfaluzy, 1971).* Birken et al (1988) recently purified ßC-hCG from pregnancy urine using gel filtration followed by immuno-affinity and ion exchange chromatography demonstrating a glycoprotein with apparent molecular weight of Mr 12000-17000 with a protein core containing 73 amino acids structured in two polypeptide chains, the beta amino acids 6-40 joined to beta amino acids 55-92, linked non-covalently by disulfide bonds and lacking the carboxyterminal extension, the immuno determinant of beta hCG. They also demonstrated that the two polypeptide chains contain nine half-cysteine residues, and raised the possibility of the existence of either a free thiol or a third undetected disulfide-bonded peptide as part of the intact beta core fragment. ßC-hCG retains the usual two biantennary-type N-linked oligosaccharide chains attached at Asn-13 and Asn-30, but unlike the intact ßhCG subunit, these N-linked carbohydrate moieties have been shown to be partially trimmed to a variable extent in peripheral residues *(Blithe and Akar, 1987; Kato and Braunstein, 1988).* Given the heterogeneity of hCG fragments, the single report by Birken needs confirmation when purification is performed by different methods. Moreover, the controversial carbohydrate sequences and the existence of other peptides needs further analysis. Therefore because of discrepancies between the published results, studies to clarify the possible existence of a third peptide in the beta core hCG protein structure and to determine the extent of trimming in the N-linked sugar moieties are needed.

Running parallel with the knowledge acquired in relation to the basic aspects of this fragment, efforts to establish its clinical significance have been published. The most promising clinical application of ßC-hCG is in the monitoring of malignant disease. In particular, the fragment has been shown

to have a sensitivity of 74% and specificity of 92% for some gynaecological cancers *(Wang et al, 1988)* and may be a more sensitive marker of hCG production by tumours than serum hCG *(Nam et al, 1990; Norman et al, 1990)*. It may also be an indicator of therapeutic effectiveness *(Cole et al, 1988)*. Besides the importance of ßC-hCG as a tumour marker the existence of this fragment in huge quantities in pregnancy urine associated with the fact that ßC-hCG molecules may be recognized by antibodies directed against the whole hCG/ßhCG subunit or that the antibodies raised against this fragment exhibit variable cross-reactivities with hCG/ßhCG have considerable clinical significance. The recent findings of ßC-hCG in pregnancy serum implicate this fragment as a potential cause of discordant results in hCG/ßhCG detection in both blood and urine. Despite the low levels of ßC-hCG detected in serum a preliminary report suggests the existence of immunological differences between serum and urinary ßC-hCG molecules *(Krichevsky et al, 1991)*. While hCG is a universally recognized early marker of clinical pregnancy, the role of ßC-hCG as a marker for normal pregnancy, ectopic pregnancy, and spontaneous abortion has not been explored. The possibility that this fragment may offer an advantage over the current methods used for the early diagnosis of pregnancy awaits confirmation. Since ßC-hCG may be useful for the development of new methods for diagnosis of normal or abnormal pregnancy, the influence of exogenous hCG used to induce ovulation on the concentrations of ßC-hCG in the urine needs to be evaluated as well. Although widely documented, the mechanisms that give rise to the beta-core molecules remain unclear. While some experiments using animal models show that, after intravenous injection, the kidney, liver, and ovary accumulate hCG and ßhCG subunit molecules and within 30-60 minutes the kidney process these molecules to beta-core *(Markkanen et al, 1979; Lefort et al, 1984; Lefort et al, 1986)*, other observations in humans show that, after injection of hCG or ßhCG, beta-core molecules are excreted in urine

Wehmann and Nisula, 1981; Wehmann et al, 1984). Together, these studies support the hypothesis that ßC-hCG fragment is a degradation product of hCG and ßhCG subunit. The recent findings suggesting a nicked ßhCG subunit as an intermediate product of the hCG/ßhCG processing further degraded to ßC-hCG also support this hypothesis *(Puisieux et al, 1990).* In this case, the detailed evaluation of the amount of ßC-hCG molecules excreted after intramuscular injection of hCG and the time needed for the disappearance of ßC-hCG is of great importance. Other investigators, however, attribute to ßC-hCG the status of a product directly secreted by trophoblast or other tissues as a result of an alternative processing pathway *(Masure et al, 1981; Kardana et al, 1988; Cole and Birken, 1988).* Both possibilities may respond by the existence of ßC-hCG fragment in the biological tissues and fluids and further studies concerning the distribution of this fragment and the mechanisms by which the kidney process hCG and ßhCG glycoproteins are required.

The majority of the problems currently related to the ßC-hCG studies arise from the high cross-reactivity of certain antibodies with other closely related glycoproteins. Because the ßC-hCG protein portion represents approximately half of the amino acids of the ßhCG subunit, it retains the beta antigenic conformation or epitopes that make it recognized by antisera directed to the beta subunit. In addition to having a unique epitope, the antibodies raised against this fragment unfortunately cross-react with the whole ßhCG subunit *(Akar et al, 1988; Krichevsky et al, 1988; Birken et al, 1988; Kardana et al, 1989; Alfthan and Stenman, 1990; Krichevsky et al, 1991)*, making a valid interpretation of results difficult, without prior chromatographic separation. Given the large amounts of ßC-hCG in urine and its potential application it is essential to develop assay systems able to distinguish ßC-hCG molecules from related glycoproteins without the prior use of complex separation

procedures. A further difficulty in the ßC-hCG measurement is the absence of a reference preparation. The need for immunological standardization is also important given the early discrepancies noted in the ßC-hCG values from different laboratories, notably in non-pregnant individuals. Certainly the use of different standard preparations by the different laboratories impedes accurate comparison of the results obtained by different investigators.

Therefore, because of the unclear aspects relating to the origin and chemical structure of ßC-hCG, the difficulties noted with the various available systems for its measurement in body fluids, the lack of information concerning the distribution and ratio between this fragment and related glycoproteins in the various biological compartments during normal or abnormal pregnancies and its potential clinical application both in pregnant and non-pregnant individuals, the current study was undertaken.

The aims were:

1) Purify ßC-hCG and confirm its protein and carbohydrate structure.

2) To develop assay methods for ßC-hCG that would permit direct measurement in body fluids without prior chromatography.

3) To examine the distribution of ßC-hCG in various body fluids to assist in the further understanding of its physiology.

4) To examine the relationship of ßC-hCG to intact hCG and its free subunits in pregnancy.

CHAPTER TWO

MATERIALS AND METHODS

To fulfil the aims of this study, i.e., verify the stability of the glycoprotein, purify a standard preparation of the fragment from pregnancy urine, analyse its physicochemical properties, and validate specific systems for its detection in various compartments in the body, several analytical methods were used. To avoid unnecessary repetition in the next chapters, the instruments, reagents, and each method used are detailed as follows:

2.1 Apparatus

- Centrifuges Beckman models J-6B and GPR; Beckman Instruments Pty, Gladesville, NSW, Australia.
- Dynavac freeze drier; Dynavac Pty, Wantirna South, Vic, Australia.
- CO_2 incubator (model 3029); Forma Scientific Division of Mallinckrodt, Inc., Marietta, Ohio, USA.
- Wescor 5100 c vapour pressure osmometer; Wescor Inc., Logan, Utah, USA.
- Varian DMS 200 Spectrophotometer; Varian Techtron Pty., Mulgrave, Vic, Australia.
- Wallac 1260 Multigamma II counter; LKB Produkter AB, Bromma, Sweden.
- Searle Delta 300 Scintillation counter; Searle Inc., Des Plains, IL, USA.
- Hetofrig cooler system; Heto Lab Equipment, Heto Birkerod, Denmark.
- Protean II double slab cell; Bio-Rad Laboratories, Richmond, CA, USA.
- Trans-blot Electrophoretic cell; Bio-Rad Laboratories, Richmond, CA USA.
- RATEK Orbital mixer OM 5; Ratek Instruments, Mitcham, Vic, Australia.

- Beckman creatinine analyzer 2; Beckman Instruments, Inc., Fullerton, CA, USA.
- Magnetic stirrer; Stansen Scientific Pty, Adelaide, Australia.
- Activon pH meter (model 209); Activon Scientific Products Company Pty, Bright, Vic, Australia.
- Fast Protein Liquid Chromatography (FPLC) system including Pharmacia Chromatography Rack-II, a Gradient programmer GP-250, two High Precision P-500 pumps, a single path ultraviolet-1 (UV-1) monitor, injector Valve V-7, two-channels recorder REC-482, and Fraction Collector FRAC-100; Pharmacia LKB Biotechnology and Pharmacia Separation Division, Uppsala, Sweden.
- Milli Q water system; Millipore Corporation, MA, USA.
- Amicon cell ultrafiltration and Amicon YM-2 62 mm membranes; Amicon, Lexington, MA, USA and Amicon division, Danvers, MA, USA.
- Whatman GF/A Glass microfiber filters; Whatman International, Maidstone, England.
- PICO-TAG amino acid analysis system, Waters Associated Inc., Milford, MA, USA.
- 470 A automatic gas-phase protein sequencer and 120 A PTH analyser; Applied Biosystems Inc., Foster City, CA, USA.
- Olympus inverted research microscope (model IMT); Olympus, Tokyo, Japan.
- Ultra cold freezer series 100; Kelvinator Commercial Products, Manitowoc, Wis, USA.
- High Pressure Liquid Chromatography (HPLC) system including two HPLC pumps 501, U6K manual injector, fix wavelength UV absorbance detector (model 441) controlled by the baseline 810 chromatography workstation programme; Waters Associated Inc., Milford, MA, USA.

2.2 Chemicals and reagents

Reagents leucopeptin A, iodoacetic acid, alpha-1 antitrypsin, phenanthroline, disodium ethylenediamine tetracetic acid (EDTA), polyoxyethylene sorbitan monolaurate (Tween), Chloramine T, haemoglobin, tris (hydroxy methyl) aminomethane, diaminobenzidine (DBA), bovine serum albumin (BSA), cytochrome C, vitamin B12, bromophenol blue, phenol red, glutaraldehyde, guanidine, 2-merceptoethanol, sodium dodecyl sulphate (SDS), nickel chloride (NiCl2), silver nitrate, glucose, Sigma cell type 20 cellulose, PTH-amino acid standards, phenol, and 1,1' carbonyldiimidazole were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ammonium acetate, ammonium sulphate, ammonium hydrogen carbonate, sodium chloride, hydrochloric acid, sodium hydroxide, magnesium chloride (MgCl2), disodium hydrogen orthophosphate, acetone, sodium metabisulphite, sodium dihydrogen orthophosphate, sodium azide (NaN3), acetic acid and butanol were purchased from Ajax Chemicals (Sydney, NSW, Australia). Methylene chloride was provided by Waters Associated Inc. (Milford, MA USA). Ammonium persulphate, tetramethylethylenediamine (TEMED), glycine, acrylamide, methylene-bis-acrylamide, low molecular weight protein standard kits, biotinylated and pre-stained low molecular weight protein standard kits (containing BSA, Mr 67000; Ovoalbumin, Mr 43000; Carbonic anhydrase, Mr 31000; Soybean trypsin inhibitor, Mr 21000; Cytochrome, Mr 14700, Protein assay kit, and Econo-Pac 10DG desalt column were provided by Bio-Rad Laboratories (Richmond, CA, USA). Beckman creatinine reagent kits were purchased from Beckman Instruments, Inc., Fullerton, CA, USA.

Percoll, glycerol, blue dextran, aldolase, ovalbumin, ribonuclease A, superfine Sephacryl S-200, Sephadex G-75, Concanavalin A (Con A)-Sepharose 4B, and the prepacked Superdex TM 75 and Mono Q HR 5/5 columns, were bought from either Pharmacia Fine Chemicals or Pharmacia AB Laboratory Separation Division (Uppsala, Sweden). Calcium chloride (CaCl2), manganous chloride (MnCl2), and dithiothreitol were purchased from Riedel de Haen AG (Seezle-Hannover, Germany), Mallinckrodt Chemical Works (St. Louis, MO, USA), and Eastman Kodak Co. (Rochester, NY, USA), respectively. Trifluoracetic acid (TFA), phenylisothiocyanate (PITC), and Iodogen-iodination reagents were provided by Pierce (Rockford, IL, USA). Sodium tetraborate, citric acid, acetonitrile (Far UV) and hydrogen peroxide were obtained from BDH Chemicals-Analar (Port Fairy, Vic, Australia). Polyethyleneglycol 6000 and sterile pyrogen free sodium chloride solution (0.9%) were purchased from Laboratory Supply Pty. Limited (Adelaide, SA, Australia) and David Bull Laboratories (Mulgrave, Vic, Australia), respectively. Formaldehyde was bought from May and Baker Australia (West Footscray, Vic, Australia). Methyl-α-D-mannopyranoside (αMP), and goat anti-rabbit immunoglobulin G (GARGG) were purchased from Calbiochem (La Jolla, CA, USA). Penicillin and BSA were purchased from Whittaker Bio-Products Inc. (Walkersville, MD, USA) and Boehringer Mannheim (West Germany), respectively.

Freund's adjuvants (complete and incomplete) were purchased from Commonwealth Serum Laboratories (Melbourne, Vic, Australia). Tandem-R hCG immunoradiometric assay kits were bought from Hybritech Inc. (San Diego, CA, USA). The Spectria direct estradiol coated tube radioimmunoassay kits were provided by Farmos Diagnostica (Gulunsalo, Finland). Sodium iodine 125, [3H] iodoacetic acid, streptavidin horse-peroxidase, magnetic Amerlex-M donkey anti-rabbit and Amerlex-M ßhCG

radioimmunoassay kits were obtained from Amersham International (Amersham, UK). Bioclone ßhCG, αhCG, Progesterone, and LH IRMA kits were obtained from Bioclone Australia Pty. (Sydney, NSW, Australia).

Sodium carbonate, trypan blue, Eagle's minimum essential medium (MEM), and fetal calf serum (FCS) were purchased from Flow Laboratories Inc. (Sydney, NSW, Australia). The commercial preparations of hCG, Pregnyl and Profasi, were purchased from Organon Pharmaceuticals (Lane Cove, NSW, Australia) and Commonwealth Serum Laboratories (Parkville, Vic, Australia), respectively. Normal rabbit serum (NRS), and heat inactivated normal goat serum (NGS) were provided by the South Australian Department of Agriculture. Biotinylated Concanavalin A (Con A), Lens culinaris agglutinin (LCA), Ulex europaeus agglutinin I (UEAI), Wheat germ agglutinin (WGA), Dolichos biflorus agglutinin (DBA), Ricinus communis agglutinin I (RCAI), Soybean agglutinin (SBA), Peanut agglutinin (PNA), and Elderberry bark agglutinin (EBA), were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

2.3 Antisera

Anti-ßC-hCG polyclonal antibody RW 37 was provided by Robert Wehmann from National Institutes of Health (NIH), Bethesda, MD, USA. The monoclonal antibodies 2/6, 3/6, 11/6, and 32H2 were provided by Dr. R.J. Norman. Biotinylated immunoglobulin G (IgG) anti-rabbit was bought from Vector Laboratories Inc. (Burlingame, CA, USA).

2.4 Hormones

Highly purified hCG (CR125), ßhCG (CR 125), αhCG (CR 119), LH (68/40), ßLH (AFP3282B), LH/FSH (LER907) were provided by Salvatori Raiti (National Institutes of Health [NIH], National Institute of Child Health and Human Development, Bethesda, MD, USA). Beta core hCG fragment (ßC-hCG reference I) was a gift from Steven Birken of Columbia University School of Medicine. Adrenocorticotropin (ACTH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Third International Standard for hCG (75/537) was provided by World Health Organization, WHO International Laboratory for Biological Standards, N.I.B.S.C., Blanche Lane, Ridge, Near Potters Bar, Hartfordshire, England.

2.5 Chromatographic methods

2.5.1 Gel Chromatography

2.5.1.1 Econo-Pac 10 DG chromatography for ßC-hCG desalting

Purified ßC-hCG samples were desalted on a Econo-Pac 10 DG column previously packed with polyacrylamide gel (Bio-Gel P-6 desalting) using Milli Q water as buffer. After equilibrating the gel, the 3ml-sample of ßC-hCG reconstituted in Milli Q was eluted with eight millilitres of this water. One millilitre fractions were collected and analysed for ßC-hCG immunoactivity. The fractions containing desalted ßC-hCG were frozen at -70^{o}C, lyophilized and stored at -20^{o}C until electrophoresis.

2.5.1.2 Sephacryl S-200 chromatography

A number of chromatographic procedures were performed at room temperature (RT) on a Sephacryl S-200 (40-120mm dry bead) 2.6 x 87.5cm (463ml) column bed equilibrated with 0.2M ammonium acetate containing

0.02% sodium azide, pH 6.8, at a flow rate of 50ml/h. The column was extensively washed with buffer before any chromatographic procedure and regularly calibrated with standard molecular weight markers (BSA, Mr 67000; ovalbumin, Mr 34000; chymotrypsinogen A, Mr 25000; cytochrome C, Mr 14700; ribonuclease A, Mr 13700; vitamin B12, Mr 1350). The elution volume of ßC-hCG was determined by chromatography of [^{125}I]-ßC-hCG. Blue dextran was used to determine the void volume (Vo) and the total gel volume (Vt) was estimated by the dimensions of the column using the equation **Vt = a x h**, where **a** is the area of cross section of the column and **h** is the height of the column. Iodinated preparations of highly purified hCG, ßhCG, and αhCG were also used as internal markers. Fractions of 4.2ml were collected and monitored for optical density (OD) at 280nm and gonadotrophin immunoactivity. The gel filtration partition coefficients (Kav) were calculated from the equation **Kav = (Ve-Vo)/(Vt-Vo)**, where Ve is the elution volume of the protein. Any elution volume was taken as the position of the apex of the peak of each protein.

2.5.1.3 Sephadex G-75 chromatography

Chromatography on Sephadex G-75 superfine gel was performed in an 1.6 x 85cm column (bed volume = 170ml). Approximately 2-3ml-sample volume was applied at all times. The gel was equilibrated with 0.15M ammonium bicarbonate buffer (pH 7.4, containing 0.02% NaN3), at a flow rate of 6ml/h. [125]I-ßC-hCG, and several standard proteins were used to mark the column bed. Blue dextran and ^{125}I were used to determine the void volume (Vo) and total volume (Vt), respectively. The Andrews' diagram (*Andrews, 1965*), constructed by plotting log of molecular weight versus Kav, was used to estimate the apparent molecular weight of unknown. Fractions of 2ml were collected and monitored for ßC-hCG immunoreactivity. Fractions

containing ßC-hCG fragment were pooled, aliquoted, lyophilized and stored at -20C.

2.5.1.4 Superdex 75 chromatography

Chromatography on Superdex 75 was effected in a prepacked 1.6 x 60cm column at a linear flow rate of 60ml/h. The column was equilibrated and run with 0.2M ammonium acetate buffer, pH 6.8, containing 0.02% sodium azide. Several standard proteins with known molecular weight were used for calibration. Blue dextran and ^{125}Iodine were used to determine the void volume (Vo) and the total bed volume (Vt), respectively. Radiolabelled hCG and αhCG were also used as internal markers. Gel filtration partition coefficients (Kav) were calculated from the equation **Kav = (Ve-Vo)/(Vt-Vo)**, where Ve is the elution volume. Fractions of 1ml were collected and analysed for gonadotrophin hormones using specific assays.

2.5.2. Affinity chromatography using Con A lectin

Affinity chromatographic procedures were performed on Con A covalently coupled to Sepharose 4B in an 8.4ml column bed previously equilibrated with 0.2M ammonium acetate (containing 0.15M NaCl, 1mM MnCl2, 1mM CaCl2, 1mM Mg Cl2, and 0.02% NaN3, pH 6.8). Approximately 2-3ml samples were applied and permitted to penetrate the residue under hydrostatic pressure. The flow was stopped for 60 minutes and re-established after this period of time. After washing the resin at a flow rate of 8ml/h with 80ml (approximately 10 bed volumes) of buffer to elute the non-absorbed substances, the Con A absorbed - glycoproteins were eluted with approximately 60ml of 0.2M alpha methyl D-pyranoside in the running buffer. The eluted fractions were collected in 2.1ml aliquots which were monitored for optical density at 280nm, radioactivity (in case of radiolabelled standards) and gonadotrophin

immunoactivity. The amount recovered on any chromatographic procedure was usually higher than 80%.

2.5.3 Ion exchange chromatography

Two hundred microlitres of samples of ßC-hCG were chromatographed on a 1ml bed column of a strong anion exchange hydrophilic resin whose beads are 10mm in size (Mono Q), using a Fast Protein Liquid Chromatography (FPLC) system, with Tris-HCl 0.02M, pH 8.0 (starting buffer). The column was run at a flow rate of 1ml/minute under a pressure, given by both pumps, of 1.5 MPa. Five minutes after sample injection, a linear gradient from 0 to 1M NaCl in starting buffer was initiated and completed in a volume of 15ml. Sample and buffers were filtered through a 0.22µm filter membrane supplied by Millipore. Fractions of 1ml were collected and monitored for ßC-hCG immunoactivity.

2.5.4 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was performed on a Brownlee C RP-8 spheri-5 column connected to a Waters HPLC system comprised by a U6K manual injector, two HPLC 501 pumps, fixed wavelength UV absorbance detector and a chromatography workstation programme. After manual injection samples were eluted at a flow rate of 0.6ml/minute, under control of the baseline programme. At all times the elution was started with 0.1% TFA buffer (Buffer A), after 10 minutes a stepwise gradient of 0-70% with 0.1% TFA in 80% acetonitrile buffer (Buffer B) was developed in the next 60 minutes and of 70-100% in the following 10 minutes. Fractions of 0.6ml were collected and either immediately processed or stored at -20^{0}C for further analysis.

2.6 Protein measurement

The protein content of pooled and concentrated fractions of each chromatographic step during purification of ßC-hCG was measured with the Bio-Rad protein assay kit, using the micro-assay procedure, 25mM phosphate (pH 7.4) as buffer and BSA as standard. This assay is a colorimetric one based on the colour change of Coomassie blue G-250 in response to various concentrations of protein (*Bradford, 1976*). The assay was performed as recommended by the manufacturer: 0.8ml of standards ranging from 1µg/ml to 25µg/ml (1.2 to 20µg protein per tube) or unknown or sample buffer (blank) and 0.2ml of concentrate dye reagent were added to appropriate labelled test tubes. The tubes were vortexed carefully to avoid excess foaming, left to stand up for 15 minutes and then the optical density was read at 595nm. The unknowns were read off from the standard curve.

2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified ßC-hCG was desalted on an Econo-Pac 10DG column previously packed with polyacrylamide gel (Bio-Gel P-6 desalting) using Milli Q water as buffer. The fractions containing desalted ßC-hCG were lyophilized and kept frozen at -20^{o}C. Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the desalted ßC-hCG and protein standards (Bio-Rad) was performed in 18% acrylamide slab gels under both non-reducing and reducing conditions at low temperature according to the method of Laemmli (1970) by using the Protean II double Slab Cell. ßC-hCG and protein standards (BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, cytochrome C, and adrenocorticotrophin) were dissolved (1:1) in sample buffer containing 125mM Tris HCl, pH 6.8, 10% glycerol, 2% SDS,

0.001% bromophenol blue. For reducing conditions 2.5% of 2-mercaptoethanol was added just prior to use and the sample buffer-protein solution was boiled for 4 minutes before loading. The gels were run at a constant voltage of 120 volts for 8h and silver stained according to the method of Morrissey (1981). This procedure is detailed in Appendix I.

2.8 Immunoblotting

SDS-PAGE for immunoblotting, was performed under reducing conditions at low temperature, according to the procedure described above. Gels were pre-equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% vol/vol methanol) for 15 minutes prior to electrophoretic transfer. Protein transfer onto nitrocellulose blotting paper (0.45u, Bio-Rad) was monitored with both pre-stained and biotinylated molecular weight standards (Bio-Rad) and performed at low temperature during 2h at 100 volts, 0.5 Amp, in a Trans-blot Electrophoretic Cell (Bio-Rad) according to the procedure described by Towbin et al (1979). After transferring, the nitrocellulose papers were soaked in 10mM sodium phosphate, 150mM NaCl buffer, pH 7.6, for 15 minutes, incubated for 1h in blot blocker (phosphate buffer containing 0.1% sodium azide, 5% heat inactivated normal goat serum, 2.5% BSA), 2h in the primary antibody (DeM3) at 1:200 dilution (or normal rabbit serum 1:200 diluted as control), 1h in the secondary antibody (biotinylated IgG anti-rabbit) at 1:500 dilution and for a further 1h with streptavidin horseradish-peroxidase in phosphate buffer containing 0.05% Tween 20 (3ul/ml). The colour reaction was developed with a substrate solution of diaminobenzidine (DBA) in 0.1M Tris-HCl buffer, pH 7.5 (1mg/ml), distilled water containing H_2O_2 (1.33ul per ml) and 10ul 8% NiCl2 until the bands appear and was stopped by rinsing the blots in several changes of water. Each incubation step was preceded by 2-3

x 5 minutes washing in phosphate buffer-Tween 20. The procedure for immunostaining is summarized in Appendix II.

2.9 Lectin blotting

SDS-PAGE and nitrocellulose transfer of protein standards, hCG and ßC-hCG were performed by using procedures similar to those described for the immunoblots. After transfer the gels were pre-equilibrated in 150mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 25mM Tris-HCl buffer (TBS) for 10 minutes and incubated in blot blocker (TBS containing 10mg/ml BSA) for 1h. After 15 minutes washing, the blots were incubated with the various lectins at a concentration of 10µg/ml for 1h. Biotinylated *Con A, Lens culinaris* agglutinin *(LCA), Ulex europaeus* agglutinin *I (UEAI), Wheat germ* agglutinin *(WGA), Dolichos biflorus* agglutinin *(DBA), Ricinus communis* agglutinin *I (RCA-I), Soybean* agglutinin *(SBA), Peanut* agglutinin *(PNA) and Elderberry bark* agglutinin *(EBA)* were prepared in TBS containing 5mg/ml haemoglobin. Following TBS washing the blots were further incubated for 1h with streptavidin-horseradish peroxidase. The colour reaction was developed until the bands appeared and the reaction was stopped by water-rinsing and the blots air-dried. The colour reaction procedure is shown in detail in Appendix III.

2.10 Amino acid analysis and sequence analysis

2.10.1. Hydrolysis and amino acid analysis of ßC-hCG

Previously desalted, Con A bound pure ßC-hCG (0.25nmol) was hydrolyzed in the gas-phase for 24h at 108^{0}C with 6 N HCl containing 1% (vol/vol) phenol. Hydrolysates were dried, and derivatised with

phenylisothiocyanate to produce phenylthiocarbamyl amino acids. Analyses were carried out on a PICO-TAG amino acid analysis system.

2.10.2 Reduction, S-Carboxymethylation, and sequencing of ßC-hCG

The amino acid sequence was determined by Edman degradation (*Hewick et al, 1981*) with an Applied Biosystems 470A automatic gas-phase protein sequencer coupled to a 120A PTH Analyser using a standard PTH program. Purified ßC-hCG (12nmol), dissolved in 500µl of 6M guanidine hydrochloride, 1mM EDTA and 1M Tris adjusted to pH 9.0, was added to an Eppendorf tube containing 1mg dithiothreitol. The tube was flushed with nitrogen, capped , wrapped in aluminium foil, and incubated at 37^{o}C for 4h. A solution of 0.5mCi iodo [2-^{3}H] acetic acid (241mCi/mmol) in 30µl of 0.5M NaOH was added and mixed in the dark, under nitrogen for 15 minutes at room temperature. After adding 50µl of 2-mercaptoethanol the reaction mixture was loaded onto a Brownlee C RP-8 spheri-5 column connected to a Waters HPLC system equipped with a 214nm fixed wavelength detector. The elution was started with 0.1% trifluoroacetic acid buffer (Buffer A) at a flow rate of 0.6ml/minute. After 10 minutes, a gradient was developed with 0.1% TFA in 80% acetonitrile buffer (Buffer B) starting from 0% B to 70% B in the next 60 minutes, and then to 100% B in the following 10 minutes. A 10µl aliquot of each 0.6ml fractions collected was counted in a Searle Delta 300 scintillation counter to determine the presence of [^{3}H] S-carboxymethyl cysteine. Fractions with radioactivity were pooled, lyophilized and stored at -20^{o}C until sequenced.

2.11 Immunoassays

Several assays either radioimmunoassays (RIA) or immunoradiometric assays (IRMA) were used for measurement of the different gonadotrophin

hormones throughout this study. Their characteristics and procedures used to prepare some of the reagents are detailed in the following paragraphs.

2.11.1 Iodination of monoclonal antibodies

2.11.1.1 Iodination of monoclonal antibody 32H2

This antibody was iodinated by the Chloramine T Method (*Campbell and Johnson, 1978*). Twenty micrograms of the lyophilized antibody was resuspended in 50ml of 0.05M phosphate buffer, pH 7.4 in an Eppendorf tube. Five hundred microcuries of sodium ^{125}I and 30µg of Chloramine T (2.5mg per ml of 0.05M phosphate buffer) were added and mixed. After 30-60 seconds 0.1ml (containing 0.25mg) of sodium metabisulphite (2.5mg/ml buffer solution) was added, mixed and followed by the addition of 0.2ml phosphate buffer. This mixture was loaded on the Bio-Rad Econo-Pac^{TM} 10DG column pre-equilibrated with 0.5M phosphate containing 0.15M NaCl, 0.05% BSA, and 0.02% NaN3, pH 7.4 . The sample was eluted with the same buffer and 0.5ml fractions were collected. The fractions containing the labelled antibody were pooled, aliquoted, and stored at -20^{o}C. The mean specific activity obtained in several iodinations was about 18.2µCi/µg.

2.11.1.2 Iodination of monoclonal antibody 11/6

This antibody was iodinated by the Iodogen method (*Campbell and Johnson, 1978*). Twenty micrograms of lyophilized antibody was resuspended in 0.1ml of 0.05M phosphate buffer, pH 7.4. Iodogen was initially reconstituted in methylene chloride at a concentration of 1mg/ml. Ten microlitres of the iodogen solution were added to the bottom of a cryovial containing 40µl of methylene chloride, mixed and evaporated under nitrogen. After drying, 50µl of the antibody preparation and 10µl containing 0.5m Ci of sodium ^{125}I were added and the contents were gently mixed for 10 minutes. After this period of time 200µl of 0.05M phosphate buffer was added and the

mixture was transferred to an Eppendorf tube, left for another 10 minutes, and then loaded onto the Bio-Rad Econo-Pac 10 DG column. The sample was eluted with 0.05M phosphate buffer containing 0.15m NaCl, 0.05% BSA, and 0.02% NaN3 buffer, pH 7.4. Fractions of 0.5ml were collected and those with the radiolabelled antibody were pooled, aliquoted, and stored at -20^{o}C. The mean specific activity obtained during various procedures was approximately 20μCi/μg.

2.11.2 Iodination of standard glycoprotein preparations

2.11.2.1 Iodination of Beta LH subunit

The iodination of ßLH subunit was performed by the Chloramine T method. Five micrograms of the lyophilized ßLH (AFP3282B) preparation was reconstituted in 5μl of 0.05M phosphate buffer, pH 7.4 into an Eppendorf tube. Five microlitres containing 0.5 mCi of sodium ^{125}I and 10μl of Chloramine T (2.5mg/ml) were added and mixed. After 30 seconds 25μl of sodium metabisulphite (2.5mg/ml buffer) was added, mixed and followed by the addition of 200μl phosphate buffer. The mixture was loaded on the Bio-Rad Econo-Pac 10DG column pre-equilibrated with 0.05M phosphate buffer containing 0.15M NaCl, 0.05% BSA, and 0.02% NaN3, pH 7.4, and eluted with the same buffer. Fractions of 0.5ml were collected, and those containing the radiolabelled hormone were pooled, aliquoted, and stored at -20^{o}C. The mean specific activity obtained was 90μCi/μg.

2.11.2.2. Iodination of ßC-hCG fragment

The purified ßC-hCG preparation was iodinated by the Iodogen method. Iodogen was reconstituted in methylene chloride to give a concentration of 1mg/ml. Ten microlitres of this preparation were added to the bottom of a cryovial containing 40μl of methylene chloride, mixed and evaporated under nitrogen. After drying, 5μl of the ßC-hCG preparation (5μg) and 10μl

containing 0.5 mCi of sodium ^{125}I were added and the contents were mixed gently for 10 minutes. Then, 200µl of 0.05M phosphate buffer was added and the mixture was transferred to an Eppendorf tube, left for another 10 minutes and loaded onto the Bio-Rad Econo-Pac 10DG column. The sample was eluted with 0.05M phosphate buffer containing 0.15M/NaCl, 0.05 BSA, and 0.02% NaN3 buffer, pH 7.4. Fractions of 0.5ml were collected and those containing the radiolabelled protein were pooled, aliquoted, and stored at -20^{o}C. The specific activities of this preparation during various procedures ranged from 160 to 250µCi/µg.

2.11.2.3 Coupling of antibodies 2/6 and 3/6 to cellulose

Activation of cellulose (*Bethell et al, 1979*) was performed by adding 5g of cellulose (Sigma cell type 20) to 25ml of acetone containing 0.61g 1.1 carbonyldiimidazole (0.15mol/l) and vortexed vigourously on a magnetic stirrer for 60 minutes. After this period of time the mixture was filtered through a glass microfibre filter (1.6mm particle retention) and the activated imidazole carbamate cellulose recovered was washed with 3 x 100ml acetone and air dried. Each monoclonal antibody was precipitated from ascitic fluid by adding 2ml of saturated ammonium sulphate to 3ml of ascitic fluid on ice. Each precipitate was centrifuged at 3290 x g for 15 minutes, the supernatants were discarded and the antibodies were reconstituted in 20ml 0.1M borate buffer, pH 9.0. Twenty millilitres of 0.1M borate buffer containing 1mg of antibody (2/6 or 3/6) and 0.581 g activated cellulose was mixed on a RATEK rotary mixer at room temperature for approximately 15-16 hours and centrifuged at 1200 x g for 20 minutes. The supernatants were stored at -20^{o}C for further coupling reactions and the pellets containing either 2/6 or 3/6 antibodies were reconstituted in 50ml of 0.5M ammonium bicarbonate buffer, pH 7.8. These preparations were washed twice with 50ml of this buffer by rotating on the RATEK mixer for 20 minutes and centrifuging at 1200 x g for 20 minutes.

Each pellet was resuspended in 50ml of 0.1M ammonium acetate buffer, pH 4.0, mixed on the RATEK mixer for another 60 minutes and centrifuged at 3290 x g for 20 minutes. After repeating this procedure once the remaining pellets were washed with 2 x 50ml of 0.05M phosphate, 0.5% BSA, 0.02% NaN3, 0.15M NaCl, pH 7.4. The final pellets were resuspended in 200ml phosphate buffer and stored at $4^{o}C$. The amount of each antibody coupled to the activated cellulose was determined by the amount of binding to ^{125}I-hCG with either a zero standard or a high standard at neat, 1:1 and 1:4 dilutions of the antibody.

2.11.3 Radioimmunoassays

2.11.3.1 Beta hCG subunit (ßhCG) RIA

ßhCG immunoreactivity was evaluated by using Amerlex M ßhCG radioimmunoassay kits. This assay employs a first antibody directed against epitopes on the beta subunit of hCG that also recognizes conformational-dependent sites. Intact hCG was used as a standard and radiolabelled tracer and a second antibody bound to magnetic polymer particles was used for separation of bound and free beta subunit. This assay therefore does not distinguish the intact hormone from free beta subunit and ßC-fragments. The total material measured is referred to as ßhCG. The assay was performed exactly as described by the manufacturer (Appendix IV) and the results were expressed in terms of the Second International Standard (61/6). Intra and inter assay variability ranged from 2.4 to 5.7% and from 4.8 to 9.4%, respectively.

2.11.3.2 Beta-core hCG fragment (ßC-hCG) RIA-II

The RIA for ßC-hCG was performed with purified ßC-hCG (Ref. I) as standards and radiolabelled preparation, and ßC-hCG directed antiserum RW37 as first antibody. The assay was designed to give approximately 30%

of total activity bound in the absence of unlabelled ßC-hCG. One hundred microlitres of standards or samples were incubated with 0.1ml of diluted antiserum (final dilution 1:96000) and 0.3ml of phosphate buffer (0.05M, 0.5% BSA, 2.5% NRS, pH 7.4) at 4°C for 2h. ^{125}I-ßC-hCG (radioiodinated by the Iodogen method, specific activity 26.8µCi/µg, 20000cpm/0.1ml) was then added and the mixture incubated at 4°C for 18-20h. After this incubation period 0.2ml of goat anti-rabbit IgG (at 1:10 dilution, 2.5 units) were added and the tubes were incubated for an additional 6 h at 4°C. The tubes were centrifuged at 3290 x g for 15 minutes, the supernatants aspirated and the radioactivity of the pellets counted for 60 seconds. The results were expressed in pmol/l. Intra and inter-assays coefficients of variation were less than 5% and 10% respectively. On a weight basis, the cross-reactivities with purified hCG, ßhCG and αhCG were reported to be 0.07%, 0.25%, and <0.03%, respectively (*Wehmann et al, 1988*).

2.11.3.3 Luteinizing Hormone beta subunit (ßLH) RIA

Human luteinizing hormone beta subunit (ßLH) was quantified by radioimmunoassay employing reagents provided by A.F. Parlow and distributed by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK). For displacement curves highly purified human Beta LH-1-4 (AFP-3282B) was used as standard and iodinated preparations. This reference material was iodinated by the chloramine T method, specific activity 90µCi/µg, 30000cpm/tube. Anti-human beta LH-1 was used at a final dilution of 1:75000 as first antibody and goat anti-rabbit IgG was used to separate bound from free beta LH subunit. One hundred microlitres of standards or samples, 0.1ml of radiolabelled ßLH, 0.1ml of antiserum anti-human ßLH-1, and 0.1ml 0.05M phosphate buffer, pH 7.4, containing 0.15M NaCl, 0.5% BSA, 0.02% NaN3 were pipetted into appropriate tubes, vortexed and incubated for 24h, at 4°C. After this first incubation, 0.1ml (1.25 units) of goat

anti-rabbit IgG was added and the tubes were incubated for 6h at 4°C. The tubes were centrifuged at 3290 x g for 20 minutes at 4°C, the supernatants were aspirated and the pellets were counted for 60 seconds in a LKB multigamma counter. The cross-reactivity of this assay with hLH, on a weight basis, is reported to be 14.7%. When intact hCG and ßC-hCG were used to compete with ßLH subunit in this system the cross-reactivities, on a molar basis, were estimated to be 8.2% and 11.3%, respectively. Inter-assay variability between 32.0pmol/l and 1050pmol/l ranged from 4.0% to 11.0%. The inter-assay variation in this range was 7.2% and 10.6%. The sensitivity was 8.2pmol/l.

2.11.3.4 Progesterone Bioclone RIA

The Bioclone progesterone RIA kit, designed for the measurement of progesterone without chromatographic separation or extraction of the samples, is a double antibody radioimmunoassay system. The assay uses a polyclonal anti-progesterone antiserum raised in sheep as first antibody and a donkey anti-sheep second antiserum to separate antibody-bound from free hormone. The assay was performed as recommended by the manufacturer and a summary of the protocol is shown in Appendix V. The standards in this assay are calibrated in nmol/l and the results were also expressed in nmol/l (1nmol/l = 3.18ng/ml). On a molar basis the cross-reactions with 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, pregnenolone, testosterone, and estradiol are reported to be 76%, 2%, 0.3%, <0.1%, and <0.1%, respectively. The intra-assay coefficient of variation between 3.3nmol/l and 159nmol/l ranged from 4% to 14.5% and the inter-assay variation between 5.2nmol/l and 59.3nmol/l ranged from 5.3% to 11.4%.

2.11.3.5 Estradiol [^{125}I] coated tube RIA

The Spectria direct estradiol [^{125}I] coated tube radioimmunoassay is designed for measurement of unconjugated 17ß-estradiol without chromatography or extraction of the sample and based on the double antibody RIA technique. The assay uses a rabbit polyclonal antiserum against estradiol as first antibody and tubes coated with the second (anti-rabbit) antibody and was performed as advised by the manufacturer (Appendix VI). Estradiol values obtained were expressed in Standard International units, nmol/l (1pg/ml = 272.4nmol/l). The cross-reactivities of this assay with estrone, estriol, 2-hydroxyestradiol, 16-oxoestradiol, 16-hydroxyestrone, progesterone, and testosterone are reported in 20%, 0.45%, 0.63%, 0.41%, 0.21%, <0.001%, and <0.001%, respectively. Intra-assay variation between 0.11nmol/l and 9.45nmol/l ranged from 7.1% to 17.3%; the inter-assay variability between 0.54nmol/l and 4.31nmol/l ranged from 7.8 to 12.1%.

2.11.4 Immunoradiometric assays

2.11.4.1 Intact hCG IRMAs

Two different IRMA systems were used in this study.

Assay I This intact hCG immunoradiometric assay (hCG IRMA-I) employs a combination of two monoclonal antibodies (3/6 against hCG and ßhCG as solid phase and 11/6 against intact hCG as tracer) and was described in detail elsewhere (*Norman et al, 1985*). The radiolabelled 11/6 antibody was prepared by the Iodogen method (*Campbell and Johnson, 1978*) as described previously. Equal volumes (0.1ml) of hCG standards (WHO, 3rd IS) or samples and 3/6 antibody coupled to cellulose were mixed on a RATEK rotary mixer for 2h at room temperature. After this period of incubation, 0.1ml of uncoupled cellulose (0.6g cellulose/100ml of 0.05M phosphate, 0.5% BSA, pH 7.4 buffer)

was added to each tube, the tubes were centrifuged at 3290 x g for 10 minutes and the liquid phase aspirated. Radiolabelled 11/6 antibody (0.1ml, 100000cpm) and buffer (0.2ml) were added and the tubes incubated again with continuous mixing at room temperature overnight. After centrifugation at 3290 x g for 10 minutes and aspiration of the supernatant, the pellets were washed once with assay buffer containing 0.1% Tween 20 (0.5ml) and the radioactivity counted in the LKB Multigamma counter. The high dose hook effect was avoided by diluting each sample 10, 100, 1000 times before assaying, and the results were taken from the dilution that fitted in the best point of the standard curve. This assay exhibits negligible cross-reactivity with ßhCG (<0.1%), ßC-hCG (0.26%), αhCG (0.1%), hLH (0.15%), and ßLH (<0.1%). The calculated sensitivity is 1.25pmol/l and the intra-assay coefficient of variation between 5pmol/l and 3000pmol/l ranged from 2.3% to 5.5%. The inter-assay variation between 4.7pmol/l and 4190pmol/l ranged from 3.5% and 12.7%. This system is calibrated against the Third International Standard (WHO, 75/537) and the results are expressed in pmol/l (1pmol/l = 0.022IU/l).

Assay II Tandem-R hCG IRMA (hCG IRMA-II). This assay used the Tandem-R hCG kit reagents and was performed following the instructions recommended by the manufacturer (Appendix VII). The assay is specific to the α:ß dimer and uses a monoclonal antibody directed to a unique antigenic site on the hCG molecule as the capture antibody and a radioiodinated hCG monoclonal antibody directed against a different epitope of hCG for detection. Inter- and intra-assay coefficients of variation in the

best fit of the curve ranged from 5.8% to 12.8% and from 3.5% to 12.2%, respectively. The results were expressed in terms of the Third International Standard, World Health Organization (#75/537) and the hook effect phenomenon was avoided as described above.

2.11.4.2 Beta hCG subunit IRMA

Free beta hCG subunit was estimated by using a free ßhCG subunit IRMA kit from Bioclone. This assay employs two monoclonal antibodies directed against different ßhCG epitopes, one bound to magnetisable polystyrene particles as the solid phase and the other labelled with ^{125}I as tracer. The assays were performed as recommended by the manufacturer and the procedure is summarized in Appendix VIII. The cross-reactivities with intact hCG, αhCG, and LH were stated to be 0.3%, <0.01% and 0.1%, respectively, by the manufacturer. The cross-reaction with ßC-hCG was found to be <0.01%. Assay sensitivity was 13.5pmol/l. The hook effect was avoided by effecting serial dilution of samples before assay. The intra-assay variability in a range between 100pmol/l and 14600pmol/l was 2.5% and the inter-assay variation between 30pmol/l and 6300pmol/l was approximately 3.2%. This assay is calibrated against the first International Reference Preparation IRP (#75/551) and the results are expressed in pmol/l (1 IU/l = 45pmol/l).

2.11.4.3 Alpha hCG subunit IRMA

Free alpha hCG subunit was determined by using a free α-glycoprotein subunit IRMA kit from Bioclone. In this assay two monoclonal antibodies against different epitopes of alpha subunit are used in a two-site immunoradiometric system. All assays were performed as described by the manufacturer (Appendix IX) and the results were expressed in pmol/l in terms of first International Reference Preparation IRP (#75/569). The hook effect

was avoided as described previously. The minimum amount of free alpha subunit detected was 2pmol/l and the cross-reactivities with ßhCG, hCG and ßC-hCG were 1.1%, 0.18% and <0.01%, respectively. Between 23.5pmol/l and 2509pmol/l the intra-assay variability ranged from 3.5% to 5.1% and the inter-assay variation between concentrations of 25.4pmol/l and 2180pmol/l ranged from 4.0% to 5.0%.

2.11.4.4. Beta core hCG IRMA-II

This ßC-hCG IRMA (IRMA-II) was performed with the monoclonal antisera 2/6 and 32H2, directed against core epitopes of ßhCG subunit and ßC-hCG. Their characteristics have been presented elsewhere (*Norman et al, 1985*). The radiolabelled 32H2 antibody was prepared by the Chloramine T method (*Campbell and Johnson, 1978*) as described previously. The procedure of this assay is similar to that of intact hCG using 3/6 and 11/6 antibodies. Equal volumes (0.1ml) of ßC-hCG standards (Ref. I) or samples and 2/6 antibody coupled to cellulose were mixed on a RATEK rotary mixer for 2h at RT. After this period of incubation, the tubes were centrifuged at 3290 x g for 10 minutes and the liquid phase aspirated. Radiolabelled 32H2 antibody (0.1ml, 100000cpm) and buffer (0.2ml, 0.05M phosphate, 0.5% BSA, pH 7.4) were added and the tubes incubated again with continuous mixing at room temperature. After centrifugation at 3290 x g for 10 minutes and aspiration of the supernatant, the pellets were washed once with assay buffer containing 0.1% Tween 20 (0.5ml). After washing, centrifugation and decanting as above, the pellets were counted in the LKB Multigamma counter for 60 seconds. The high-dose hook effect phenomenon was avoided by diluting each sample 10, 100, and 1000 times before assaying, and the results were taken from the dilution that fitted in the best point of the standard curve. In the working range, the intra-assay coefficients of variation ranged from 1.9% to 2.6 % and the inter-assay coefficients of variation from 8.4% to 12.2%. This

assay showed high ability to bind purified ßhCG (CR 125) and, on a molar basis, the cross-reaction was about 100% up to 2000pmol/l. A slight binding was detected with purified hCG (CR 125) and αhCG (CR 119) at doses higher than 5 and 40nmol/l, and the cross-reactions were estimated in 0.6% and 0.1% respectively.

2.11.4.5 Human Luteinizing Hormone IRMA

The human luteinizing hormone immunoreactivity was evaluated with a commercial LH IRMA from Bioclone. This assay is constructed with two mouse monoclonal antibodies which sandwich hLH molecules by binding to different epitopes. After diluting the samples to avoid hooking this assay was performed exactly as described by the manufacturer (Appendix X), and the results were expressed in pmol/l in terms of first International Reference Preparation for immunoassasy (#68/40). The minimal detectable doses is 0.2pmol/l and the cross-reactivities, on a molar basis, are 0.5% for intact hCG, 0.2% for ßhCG, 10.0% for αhCG, <0.01% for ßLH and <0.01% for ßC-hCG. The intra-assay variability between 160pmol/l and 2000pmol/l ranged from 4.0% to 4.4% and the inter-assay coefficient of variation between 160pmol/l and 2660pmol/l ranged from 4.5% to 6.3%.

2.12 Granulosa-Lutein Cell Culture

Granulosa cells were collected from mature follicles of women at the time of oocyte retrieval for in vitro fertilization and embryo transfer (*Parry et al, 1989*). Blood-free follicular fluid from a single patient was pooled and aliquoted to 10ml into a 10ml plastic screw-top tube (Disposable Products Ltd, Adelaide, SA) and centrifuged at 100 x g for 5 minutes to separate cells. Supernatant follicular fluids were aspirated leaving 0.5ml covering the cell pellet. All cells were pooled and equal volumes were transferred into tubes

containing 5ml of modified Eagle's minimum essential medium (with Earle's salts and glutamine, 5.5mmol glucose, 25mmol sodium bicarbonate, pH 7.4, 280 mOsmol). One hundred IU of penicillin per millilitre was added as bactericide. After mechanical dispersion by repeated pipetting in a siliconized Pasteur pipette for 5 minutes, the cells were washed twice with MEM prior to being layered upon 50% Percoll columns to be isolated from red blood cells. Columns were centrifuged at 100 x g for 30 minutes. Cells were collected in 5ml MEM, again mechanically dispersed, filtered through a silk screen to remove cell clumps, washed twice with MEM, resuspended in 5ml of medium supplemented with 10% fetal calf serum (FCS) and counted in a haematocytometer. Viability was assessed by the vital stain trypan blue at 0.4% (volume/volume) concentration, and was about 95%. Cells were equally divided in two 25ml-tissue culture flasks (Falcon, Los Angeles, CA, USA) at a concentration of approximately 2.4 x 10^5 cells/ml and cultured at 38.5^{o}C in 95% O_2, and 5% CO_2. Cells were initially cultured for 48h and the experiments were started after microscopic observation of morphological signs of luteinization. Cultured media were changed every two days and the spent media were stored at -20^{o}C until chromatography.

2.13 Granulosa cell in vitro bioassay

The biological activity of ßC-hCG was tested in vitro by incubating the highly purified preparation of this fragment with human granulosa cells and measuring the amount of progesterone and estradiol secreted in the cultured medium (*Goldsmith et al, 1981*). The method of recovering and preparation of the granulosa cells is described above in section 2.12 The viability of the cells was evaluated with 0.4% (vol/vol) trypan blue vital stain in normal saline. Two hundred microlitres of the isolated granulosa cell suspension containing approximately 21 x 10^4 cells was layered into the bottom of 12 wells of a

multi-well tissue culture plate (Flow Laboratories). Following 0.8ml of culture medium (modified Eagle's minimum essential medium with Earle's salt and glutamine, 5.5mmol glucose, 25mmol sodium bicarbonate, 100IU of penicillin, 280mOsmol, pH 7.4) supplemented with 10% FCS was then added to each well and the cells were cultured in a humidified atmosphere of 95% O_2 and 5% CO_2, at 38.5^{o}C, for 48h. After observation of morphological signs of luteinization using an inverted phase contrast microscope, the culture medium was aspirated and discarded. One millilitre of fresh medium either neat or containing 0.5µg of hCG (Profasi) or 0.5µg of ßC-hCG was added to each set of four wells and incubated again for a further 96h under the same conditions. The culture medium was replaced every 24h and stored at -20^{o}C until assayed for progesterone and oestradiol. The amount of steroid measured in the culture medium was expressed as the amount produced per cell.

CHAPTER THREE

EVALUATION OF THE STABILITY OF IMMUNOREACTIVE ß-CORE FRAGMENT OF HCG (ßC-HCG) UNDER DIFFERENT LABORATORY CONDITIONS.

3.1 Introduction

Despite some advances in our understanding about the structure and occurrence of ßC-hCG, little is documented about its stability. Several investigators have recommended immediate processing of the samples, careful storage conditions and the use of complex preservatives (*Cole et al, 1988; Wang et al, 1988*). Small but significant differences have been noted in gel chromatography partition and sodium dodecyl sulphate polyacrylamide gel electrophoresis mobility among various preparations, differences that could result from the different methods of preparation and preservation of the molecule rather than from the inherent microheterogeneity of this molecule. There is no literature relating to the stability of the ßC-hCG under a variety of conditions usually employed by different laboratories to handle the samples. Because the procedures used for purification of proteins involve their submission to several chemical and physical conditions, it was of interest to identify the factors that could damage this fragment during its purification. This study was conducted using a pure preparation of ßC-hCG (Birken's ßC-hCG, reference I) to evaluate the possible effects of storage conditions on ßC-hCG and to establish the basis for further studies. In addition, the stability of intact hCG and ßhCG immunoreactivities in urine stored under the same conditions as these analytes was also analysed during the purification of ß-core.

3.2 Methods

3.2.1 Collection of urine samples

Early morning urine samples from eleven healthy pregnant women between 8 and 30 weeks' gestation were collected at the antenatal clinic and immediately sent to the laboratory. All samples were initially centrifuged at 1850 x g for 10 minutes, at 4^{o}C, to remove debris before any assay or treatment.

3.2.2 Design of the study

To examine the stability of ßC-hCG, ßhCG, and intact hCG, the effects of preservative, storage temperature, successive freezing-thawing cycles and a range of pH were evaluated (Figure 3.1). Because it is thought that several hydrolases existing in the urine may affect these molecules by proteolytic digestion, the samples were immediately divided in aliquots (2ml) with either 0.05ml 1M Tris-HCl, (pH 8.2) or 0.05ml preservative (1M Tris-HCl, pH 8.2 10mM disodium ethylenediamine tetracetic acid, 2mM phenanthroline, 2mM iodoacetic acid, 5mg/l leucopeptin A, 25mg/l alpha-1 antitrypsin and 0.4% sodium azide at a final dilution 1:40) (*Wang et al, 1988*).

Portions with and without preservative were further divided in 0.1ml aliquots and either assayed fresh or stored at room temperature, 4^{o}C or -20^{o}C and assayed at different intervals. To examine the effect of freezing-thawing, the aliquots (0.1ml) of urine from all patients were submitted to six successive cycles of freezing (by putting them in a freezer at -20^{o}C until frozen for at least 1 hour) and thawing to room temperature. The effect of pH on ßC-hCG immunoreactivity was evaluated in six fresh urine samples with original pH in the range of 5.4-6.8 by adjusting them to pH 5, 6, 7, and 8 either with 1M HCl or 2M NaOH and assaying for ßC-hCG.

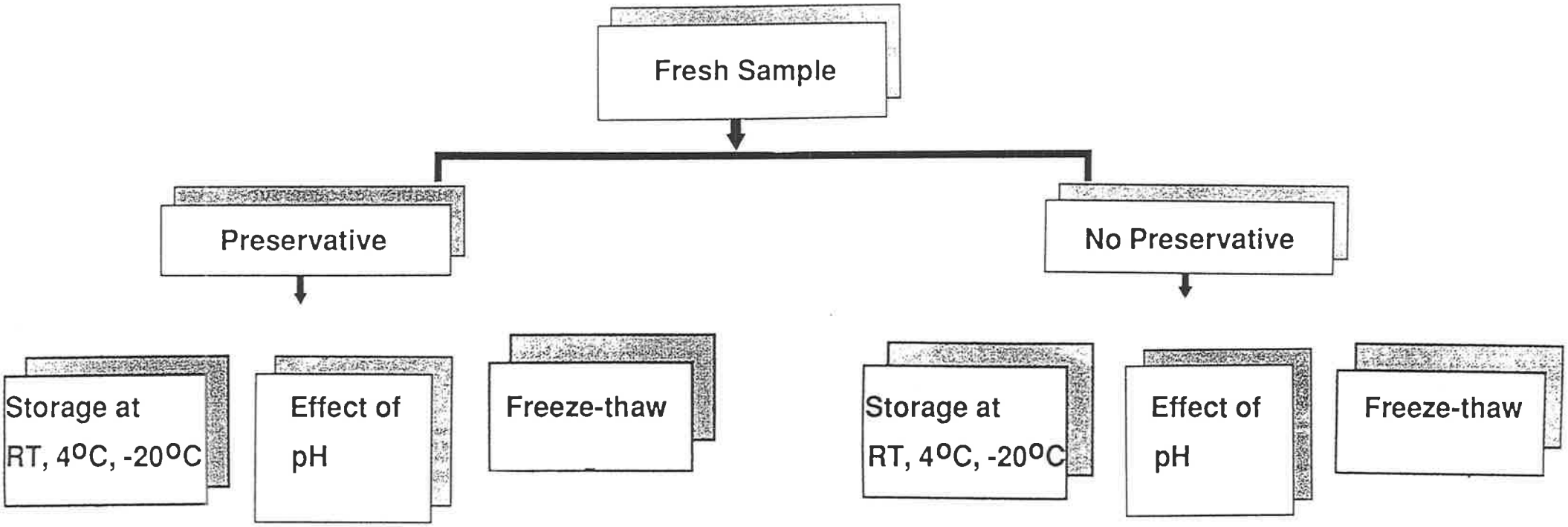


Figure 3.1 Simplified diagram for evaluating the immunoreactive stability of the ßC-hCG fragment

3.2.3 Evaluation of immunoactivity

Beta-core hCG immunoreactivity was examined by both radioimmunoassay (RIA) and immunoradiometric assay (IRMA) systems. The procedures used, the characteristics, and the source of each assay system were detailed in Chapter II, sections 2.11.3.2 (ßC-hCG RIA-II) and 2.11.4.4 (ßC-hCG IRMA-II). These assays were calibrated against the ßC-hCG standard preparation purified by Dr. Steven Birken from Columbia University (ßC-hCG reference I) and the results were expressed in pmol/l. The intact hCG immunoreactivity was measured using a commercial kit (Hybritech Inc., San Diego, CA, USA) specific to the a:ß dimer, (hCG IRMA-II, Chapter two, section 2.11.4.1), and the results were expressed as International Units (IU/l), in terms of the Third International Standard, World Health Organization (#75/537). The stability of total ßhCG immunoreactivity was also evaluated using reagents of a commercial radioimmunoassay kit (Amersham International, Amersham, England) that do not distinguish the intact hormone from free beta subunit or beta-core fragments and the total material measured is referred to as ßhCG (see Chapter two, section 2.11.3.1). The results were expressed as IU/l, in terms of the Second International Standard (61/6).

3.2.4 Analysis of data

Dose inhibition curve and potency estimates in the assays were analyzed by a computerized spline function system provided by LKB (Wallac 1260 Multigamma II counter). For group comparisons of the data, a Kruskal-Wallis one-way analysis of variance was performed. Wilcoxon nonparametric test was used where appropriate, with significance tested at the 5% level. To compensate for the different starting values, each result was standardized with the original result under fresh conditions as a percent of original.

3.3 Results

3.3.1 ßC-hCG fragment

The ßC-hCG levels in fresh samples taken between 8 and 30 weeks after the last menstrual period ranged from 9.1±0.8 to 317.0±9.5nmol/l (mean ± SD).

3.3.1.1 Preservative

The importance of inhibitors on the ßC-hCG immunoreactivity was tested. Untreated samples and samples containing preservative were assayed and compared. The results (Table 3.1) showed that the ßC-hCG levels were not altered, even after 6 months of storage, in samples both with and without preservative, regardless of the temperature at which the samples were kept (P=0.392).

Table 3.1 Effect of Time and Storage Temperature on ßC-hCG Immunoreactivity in Urine of Healthy Pregnant Women with and without Preservative*

Sample	Day 0 RT	Day 1		Day 7		Day 14		Day 21		Day 180 -20C
		RT	4C	RT	4C	RT	4C	RT	4C	
No preservative										
1	46.0	38.8	36.5	36.5	35.3	33.6	34.0	34.3	32.8	34.2
2	21.1	26.7	24.0	31.8	26.3	32.7	26.0	28.1	25.8	25.3
3	9.1	8.9	9.0	9.7	8.9	9.0	8.9	8.3	9.3	10.0
4	20.6	18.6	20.0	23.8	18.7	31.8	20.3	33.8	29.4	22.8
5	150.0	188.0	158.0	149.6	168.0	156.3	160.3	125.3	144.0	194.5
6	255.0	270.0	290.0	250.0	240.0	284.0	272.0	254.0	244.0	231.0
7	20.0	NA	NA	15.5	15.4	20.1	19.7	19.1	20.4	NA
8	95.0	NA	NA	89.0	95.0	80.0	94.0	86.0	93.0	NA
9	194.0	NA	NA	185.0	165.0	187.0	172.0	193.0	187.0	NA
10	317.0	NA	NA	209.0	240.0	210.0	210.0	168.0	235.0	NA
11	11.2	NA	NA	6.8	6.3	6.3	6.6	5.9	5.5	NA
Median	46.0	NA	NA	36.5	35.3	33.6	34.0	34.3	32.8	NA
Preservative										
1	NA	32.3	36.5	34.8	34.5	35.0	35.8	32.8	34.6	43.0
2	NA	25.9	25.8	27.3	26.8	28.9	25.7	27.9	24.7	22.8
3	NA	8.8	9.8	9.6	9.3	10.2	9.1	10.2	8.9	11.5
4	NA	18.7	19.5	17.7	20.2	13.5	19.3	18.6	16.5	19.5
5	NA	173.0	161.0	140.6	156.6	144.0	155.3	111.0	162.3	216.0
6	NA	284.0	266.0	234.0	248.0	298.0	244.0	256.0	298.0	282.0
7	NA	NA	NA	15.3	16.0	21.7	18.5	20.1	19.9	NA
8	NA	NA	NA	109.0	101.0	99.0	107.0	104.0	102.0	NA
9	NA	NA	NA	211.0	191.0	176.0	191.0	187.0	190.0	NA
10	NA	NA	NA	239.0	236.0	218.0	267.0	231.0	240.0	NA
11	NA	NA	NA	10.4	6.3	6.6	7.6	6.5	8.2	NA
Median	NA	NA	NA	34.8	34.5	35.0	35.8	32.8	34.6	NA

RT = room temperature; NA = not availabe
Data are presented as nmol/l.
*F test: P = .516, P = .197 and P = .392 for effects of preservative, temperature, and days, respectively.

3.3.1.2 Temperature

The influence of temperature was evaluated specifically by keeping the samples either at room temperature of 4ºC for 3 weeks and at -20ºC for 6 months (Table 3.1), or by submitting them to successive freezing-thawing cycles (Table 3.2). These tests showed that the urinary ßC-hCG levels were unchanged at different storage temperatures (P=0.197) and during repeated freezing and thawing before assay (P=0.408) either with or without preservative.

Table 3.2. Effect of Repeated Freezing at -20ºC and Thawing at Room Temperature on ßC-hCG Immunoreactivity in Urine With or Without Preservative

	1x		2x		3x		4x		5x		6x	
Sample	NP	P	NP	P	NP	P	NP	P	NP	P	NP	P
1	32.0	35.6	36.8	35.5	35.0	36.3	37.5	36.8	26.5	36.8	33.5	38.5
2	22.9	25.3	25.9	26.3	26.6	28.0	25.8	26.1	24.4	24.7	25.5	26.2
3	9.2	9.0	9.4	9.3	9.7	9.6	9.1	8.7	9.4	10.1	9.8	9.5
4	19.8	18.6	19.7	19.9	19.8	20.1	19.8	20.4	20.4	19.0	19.5	20.3
5	162.8	156.3	169.0	142.3	170.0	181.3	170.0	162.3	154.0	178.0	159.0	162.0
6	250.0	264.0	270.0	270.0	260.0	272.0	282.0	264.0	244.0	265.0	265.0	270.0
7	20.6	20.1	17.1	18.9	18.6	19.4	18.7	20.4	17.7	17.0	19.9	19.0
8	98.0	110.0	98.0	108.0	96.0	100.0	91.0	111.0	92.0	99.0	106.0	99.0
9	192.0	199.0	193.0	185.0	166.0	188.0	177.0	182.0	188.0	191.0	176.0	191.0
10	241.0	286.0	233.0	257.0	235.0	250.0	229.0	233.0	253.0	238.0	213.0	222.0
11	6.5	7.1	5.7	6.5	6.9	5.4	8.1	7.6	5.6	5.7	7.4	7.8
Median	32.0	35.6	36.8	35.5	35.0	36.3	37.5	36.8	26.5	36.8	33.5	38.0

NP = no preservative; P = with preservative.
Data are presented as nmol/l.
F test: P= .441 and *P* = .408 for effects of preservative and freeze-thaw, respectively.

Table 3.3 presents the close comparison between the results of ßC-hCG immunoactivity obtained with both immunoassay systems (IRMA, RIA) in several storage conditions. Although there were no differences between groups with respect to preservative or no preservative when the samples were kept at 4ºC for three weeks, the IRMA system consistently showed lower immunoreactivity (P=0.001).

Table 3.3. Comparison of ßC-hCG Immunoreactivity in Pregnancy Urine Measured by Radioimmunoassay and Immunoradiometric Assay*

Sample	Day O		Day 7		Day 14		Day 21	
	RIA	IRMA	RIA	IRMA	RIA	IRMA	RIA	IRMA
No preservative								
1	21.1	20.0	16.3	15.4	19.5	19.7	19.9	20.4
2	98.0	95.0	98.0	95.0	102.0	94.0	93.0	93.0
3	198.0	194.0	191.0	165.0	202.0	172.0	183.0	187.0
4	322.0	317.0	421.0	240.0	346.0	210.0	342.0	235.0
5	11.0	11.2	13.8	6.3	8.3	6.6	8.6	5.5
Median	98.0	95.0	98.0	95.0	102.0	94.0	93.0	93.0
Preservative								
1	20.2	16.2	19.7	16.0	19.1	18.5	18.5	19.9
2	122.0	109.0	90.0	101.0	114.0	107.0	106.0	108.0
3	204.0	199.0	211.0	191.0	207.0	191.0	208.0	190.0
4	338.0	324.0	324.0	343.0	236.0	365.0	267.0	366.0
5	12.5	11.4	16.9	6.3	11.5	7.6	9.1	8.2
Median	122.0	109.0	90.0	101.0	114.0	107.0	106.0	108.0

RIA = radioimmunoassay; IRMA = immunoradiometric assay.
Data are presented as nmol/l.
* Specimens were measured on day of collection and then weekly up to 21 days of storage at 4 °C with or without preservative. $P < .001$ for radioimmunoassay or immunoradiometric assay comparison. F test: $P=.228$ and $P=.110$ for effects of days and preservative, respectively.

3.3.1.3 pH

Because the urinary samples may have a large pH range among subjects under normal conditions (5.4 to 6.8 in six of our patients) we evaluated the possible influence of pH on the ßC-hCG immunoreactivity and on the assay by correcting every sample to a pH range 5-8 one week before analysis (Table 3.4). During this period, the samples were kept at 4ºC. There were no differences in ßC-hCG levels at any pH value evaluated (P=0.620).

Table 3.4. Influence of pH adjustment on ßC-hCG immunoreactivity in urine of pregnant women *

	pH level **				
Sample	Original***	5	6	7	8
1	46.0 (6.4)	56.9	39.5	47.8	63.8
2	21.0 (6.0)	23.2	24.1	24.1	24.0
3	9.1 (6.8)	9.0	9.0	8.9	9.0
4	20.6 (6.6)	20.2	18.0	21.6	20.1
5	150.0 (6.3)	129.7	166.7	103.0	145.7
6	259.0 (5.4)	208.5	206.7	283.0	278.0
Median	33.5	40.5	31.8	36.0	43.5

* Specimens were kept at 4 ºC for 7 days, without preservative.
** F-test, P=0.620
*** Initial pH given between brackets in first column.

3.3.2 Evaluation of Intact hCG and ßhCG subunit stabilities.

Excluding the pH study and limiting the observation to 3-4 weeks, we also performed simultaneous evaluation of the stability of intact hCG and ßhCG. The results (Table 3.5) showed that urinary hCG immunoreactivity was not altered when specimens were kept at room temperature or 4ºC for up to 21 days of collection, either in the presence or absence of preservative. The total ßhCG immunoreactivity (Table 3.6) was also unchaged in most of these storage conditions. However, even though no difference could be found regarding preservative use when samples were stored at 4ºC in the absence of preservative (P=0.002) (see discussion).

Table 3.5. Effect of Time and Storage Temperature on Intact hCG Immunoreactivity in Urine of Healthy Pregnant Women with and without Preservative *

Sample	Day 0	Day 7		Day 14		Day 21	
	RT	RT	4 °C	RT	4°C	RT	4°C
No preservative							
1	4,239	4,220	4,232	3,014	4,116	4,061	4,338
2	20,164	19,362	19,235	17,328	16,259	17,066	17,442
3	14,300	13,048	12,778	13,779	13,204	13,654	13,721
4	11,506	12,526	13,382	12,777	11,924	11,614	13,769
5	830	1,112	1,108	1,020	1,012	1,030	1,246
Median	14,300	12,526	12,778	12,777	11,924	11,614	13,721
Preservative							
1	4,032	4,317	4,270	4,189	4,453	3,862	4,424
2	18,306	20,536	18,981	18,304	19,406	19,517	18,957
3	13,187	13,569	13,193	13,696	12,993	13,721	14,941
4	11,509	13,357	12,911	12,660	12,355	12,624	14,311
5	782	1,253	1,109	1,012	1,052	1,060	1,130
Median	11,509	13,357	12,911	12,660	12,355	12,624	14,941

RT = room temperature
Data are presented as IU/l.
* F test: $P = .516$, $P = .267$, and $P = .373$ for effects of preservative, temperature, and days, respectively.

Table 3.6 Effect of Time and Storage Temperature on ß-hCG Immunoreactivity* in Urine of Healthy Pregnant Women with and without Preservative+

	Day 0	Day 7		Day 14		Day 21	
Sample	RT	RT	4°C	RT	4°C	RT	4°C
No preservative							
1	4,898	4,487	4,498	4,339	4,740	4,576	4,523
2	24,428	20,241	20,168	18,548	21,504	19,463	18,050
3	39,903	39,941	40,785	37,411	40,425	40,337	38,370
4	53,634	50,650	50,133	48,313	49,626	49,151	49,128
5	2,221	2,008	1,857	1,901	2,003	1,954	1,930
Median	24,428	20,241	20,168	18,548	21,504	19,463	18,050
Preservative							
1	4,424	4,334	4,747	4,931	4,840	4,716	4,582
2	22,509	21,850	21,311	22,674	22,693	20,681	20,368
3	36,754	41,316	41,401	38,958	40,196	39,828	42,005
4	50,886	52,165	52,731	51,755	55,468	53,414	49,761
5	2,244	2,062	2,103	1,999	2,174	2,152	2,119
Median	22,509	21,850	21,311	22,674	22,693	20,681	20,368

RT = room temperature
Data are presented as IU/l.
* Measurement procedure does not distinguish intact, free ß subunit, and ßC-hCG molecules.
+ *F* test: *P* = .823, *P* = .182 for effects of temperature and days, respectively. *P* = .117 and *P* = .002 for preservative at room temperature and 4°C, respectively.

Immunoreactive hCG and total ßhCG were also shown to be stable when submitted to successive freezing-thawing cycles before assay (P=0.999 and P=0.951, respectively), both in the presence (P=0.741) or absence (P=0.931) of preservative. We conclude that total ßhCG immunoreactivity is not influenced by repetitive freezing-thawing or the temperature at which the samples are stored. The addition of preservative may be useful specifically to protect the immunoactivity of the free ßhCG subunit.

3.4 Discussion

The results revealed that ßC-hCG immunoreactivity is very stable in urine and does not require the use of preservative to impede the possible action of proteolytic enzymes, suggesting that there is no interference in the

immunologic determinants of ßC-hCG molecules for at least 6 months when the samples are kept without preservative. Thus, it appears that the ßC-hCG molecules are not cleaved by urinary hydrolases as judged by changes in immunoactivity, and addition of protease inhibitors to the urine samples to minimize or prevent its degradation or cleavage during the collection and processing does not seem necessary. It is a normal practice in most laboratories to keep fluids containing hormones at low temperatures. We analyzed the ßC-hCG immunoreactivity at room temperature, $4^{o}C$, and $-20^{o}C$ in the eleven samples at different intervals. Our results showed that in an unfrozen state the ßC-hCG immunoactivity was not affected for at least 21 days when left either at room temperature or $4^{o}C$. At $-20^{o}C$ the ßC-hCG molecules remain stable for at least six months. Clearly, urine can be stored at room temperature overnight before transfer to the laboratory, and any delay does not invalidate the measurement. Chromatographic procedures to purify the molecule would not demand the addition of preservatives and fractions could be stored at $4^{o}C$ or $-20^{o}C$ until further procedures.

In addition, because thawing of previously frozen samples is often necessary, we submitted the ßC-hCG samples to six successive cycles of freezing-thawing and measured the ßC-hCG immunoactivity after every cycle. The results showed the remarkable stability of this glycoprotein under sudden changes of temperature. We also evaluate the influence of pH on ßC-hCG molecules and on the method used for quantification (RIA and IRMA). The results permit to conclude that the ßC-hCG molecules were not affected by a pH range 5-8 and that the different pH of samples does not interfere in the assays. Landy et al, (1990) has also demonstrated that adjustment in the pH of urinary samples does not affect the immunoreactivities of the free alpha luteinizing hormone subunit and follicle stimulating hormone. Even though the specific ßC-hCG immunoassays we have used exhibit excellent correlations,

the urinary ßC-hCG immunoreactivity was consistently higher when the RIA was used. Considering the different cross-reactivities of the antibodies, this finding would not be expected. Despite the existence of a number of reports showing that pregnancy urine contains a very small amount of free beta hCG subunit to cross react with the antibodies used, recent evidence suggests that the urinary free beta is fragmented (*Puisieux et al, 1990*), and we can not eliminate the possibility that the polyclonal antibody RW37, while not cross-reacting with the purified free beta or intact hCG, may recognize other clipped fragments found in considerable amounts in urine from pregnant women.

There are very few reports about the stability of immunoreactives hCG, ßhCG, and αhCG in body fluids when exposed to routine or experimental laboratory conditions. The stability of intact hCG and its free alpha subunit has been evaluated only in plasma, serum, whole blood specimens (*Rao et al, 1983*), and highly purified reference preparations (*Storring et al, 1980*). In our study, using the same samples used for ßC-hCG evaluation, we demonstrated that α:ß dimer molecule is also not broken down by proteases in urine; its immunoreactivity is unmodified. Our results partially differ from those of McCready et al (1978). They observed a decrease in the urinary hCG immunoreactivity when the samples were maintained at room temperature or 4^{o}C for one to seven days but also noted no modification neither after successive freezing and thawing cycles nor when the samples were stored at -20^{o}C. In turn, the stability of hCG/ßhCG demonstrated in the present study is in complete agreement with the findings of Landy et al (1990) demonstrating that various cycles of freezing and thawing do not alter the immunoreactivity of a subunit, LH, and FSH in urine. In addition, Ozturk et al (1988) demonstrated that the immunoreactivity of hCG, ßhCG and αhCG in amniotic fluid remained unmodified when stored at -20^{o}C, 4^{o}C, and at room temperate for two weeks.

Taken together, all these observations are in complete agreement with the results of Rao et al (1983) showing that both immunoreactivity and the ability of hCG to bind a specific receptor are not changed by serum proteases. Additionally, all these data permit us to presume that any hCG fragment detected in these fluids most probably results from direct secretion or an active tissue catabolic process rather than artefactual breakdown in the urine. Since the immunoreactivity of hCG remained unchanged by temperature differences or repetitive freezing and thawing, both in this study and in the previous reports (*Rao et al, 1983; Ozturk et al, 1988*), it can be concluded that the storage of hCG at various temperatures will not affect the subsequent ability of the hormone to bind to receptor or specific antibodies. Therefore, the results suggest that hCG and its metabolites exposed to different temperatures maintain their conformational structure and epitopes when restored to physiological temperatures. However, Forastieri and Ingham (1982) showed that, the intact hCG molecules are thermodinamically unstable at 37°C and, even though as a slow process, spontaneous dissociation of the α:ß dimer may occur at high temperature.

Although the RIA used to measure ßhCG immunoreactivity has an antibody similar to SB6, which is both a conformational and site specific rabbit polyclonal antibody against the ß-hCG subunit and unable to distinguish free beta from beta subunit as part of the whole hormone or even from ßC-hCG fragment (*Vaitukaitis et al, 1972*), our results indicate that ßhCG was also stable in the form of intact hormone or its principal fragment in most of the conditions investigated. However, the samples kept at 4°C without preservative showed some decrease in total beta immunoreactivity, suggesting that preservative can be useful when ßhCG is to be determined. Despite the stability of the highly purified preparation (*Storring et al, 1980*),

there is evidence that the free form of ßhCG subunit is different from the combined ßhCG in its susceptibility to proteolytic cleavage, and it would be more sensitive to the action of intracellular proteases (*Ruddon et al, 1989; Puisieux et al, 1990*). Instability of the intracellular, uncombined or in solution, ßhCG was also demonstrated by Peters et al (1984) and Beebe et al (1990). The possibility that the free ßhCG form is much more susceptible of degradation is reinforced by the knowledge that while most of the free beta hCG molecules found in serum and urine of pregnant women and patients with trophoblastic tumours are nicked, only 10% of ßhCG as part of the dimer hormone is altered (*Bidart et al, 1988; Sakakibara et al, 1990*). However, it seems unlikely that the cleavage of this subunit at a single site of its polypeptide chain may modify its immunoactivity (*Puisieux et al, 1990*). A number of studies have demonstrated that temperatures higher than those used in this study and some proteases can destroy the biological activity of gonadotrophins without affecting immunological recognition (*McArthur, 1968; Ward et al, 1986; Birken et al, 1987*). Because the method we used to measure ßhCG has lower specificity and the same alteration could not be demonstrated when the samples were kept at room temperature, the possible ßhCG instability in urine deserves further investigation. The ahCG subunit was not evaluated in this study but, together with whole hCG, this subunit has already been shown to be stable in the whole blood, plasma, serum, urine and amniotic fluid when evaluated under similar storage conditions (*Rao et al, 1983, Ozturk et al, 1988; Landy et al, 1990*).

CHAPTER FOUR

PURIFICATION AND CHARACTERIZATION OF THE IMMUNO- REACTIVE ßC-hCG FRAGMENT OF HCG.

4.1 Introduction

The previous analysis of the primary structure of this fragment by Birken et al (1988) suggests it is a glycoprotein with an apparent molecular weight of Mr 12000-17000 whose protein core contains 73 amino acids structured in two polypeptide chains linked non-covalently by disulfide bonds with carbohydrate moieties consisting of two partially trimmed N-linked complex-type oligosaccharide chains attached at Asn-13 and Asn-30 (*Birken et al, 1988; Blithe et al, 1989*). However in these studies some questions relative either to the peptide or carbohydrate composition remain poorly understood: Firstly, there are discrepancies in the reported carbohydrate composition of the beta core molecule. Blithe et al (1989) suggested that N-linked oligosaccharide chains terminated with the Man-Man branching whereas Birken et al (1988) described further sugar molecules in their preparation. These differences could alter the half-life and clearance of the beta core molecule and may be attributable to the different ways of purifying the molecule. Blithe et al (1989) used gel chromatography followed by lectin and ion exchange chromatography with final molecular sieving. Birken et al (1988) used gel filtration followed by immuno-affinity and ion exchange chromatography. Secondly, the amino acid sequence was based on only one method of purification which could exclude other sequences. Each group also used crude preparations of precipitated pregnancy urine which could account for heterogeneity. Other evidence for heterogeneity comes from observations that during preparation for sequencing of the protein core, two unidentified

peaks were found in the purified protein and a third component could not be excluded (*Birken et al, 1988*). Given the possible heterogeneous nature of the beta core of hCG it was decided to purify this fragment from fresh pregnancy urine, characterize this preparation and compare it with other preparations purified by different methods.

4.2 Methods

4.2.1 Collection and processing of urine samples

Twenty four hour urine samples from healthy pregnant women in the first trimester were collected aseptically, kept at 4^{o}C at home and brought to the laboratory early the following morning. All patients had been informed that the urine collection was for research purposes and had given consent; the protocol was approved by the Ethical Committees of the University of Adelaide and The Queen Elizabeth Hospital. One litre of the urine sample was immediately centrifuged as described in Chapter Three (section 3.2.1) to remove debris and was further concentrated at room temperature by pressure ultrafiltration in an Amicon cell with a YM-2 membrane (Mr 2000 cut-off) to a final volume of 8-10ml. This urine concentrate was the source of ßC-hCG for purification.

4.2.2 Protocol for purification of urinary ßC-hCG

Urinary ßC-hCG fragment was purified by sequential chromatographic steps on Sephacryl S-200 superfine, Concanavalin A-Sepharose 4B, Mono Q HR 5/5 anion exchange and Sephadex G-75 superfine. Figure 4.1 presents a comprehensive flow-chart summarizing the experimental design for the ß-fragment purification. The descriptions of reagents used, equipment, origin of materials, and the major methods are found in Chapter Two. Throughout the purification procedure ßC-hCG immunoreactivity was monitored using the

immunoradiometric assay I (ßC-hCG IRMA I), which uses the monoclonal antibodies 2/6 and 32H2, also described in detail in Material and Methods (Chapter Two, section 2.11.4.4). Intact hCG, ßhCG and αhCG immunoreactivities were followed with specific IRMAs also detailed in Chapter Two: the assay for hCG detection used the antibodies 3/6 and 11/6 (section 2.11.4.1); ßhCG and αhCG were measured using commercial IRMA kits provided by Bioclone (sections 2.11.4.2 and 2.11.4.3, respectively). Estimation of the protein content of samples in each step of the purification was performed with the Bio-Rad Protein assay kit microassay procedure (Chapter Two, section 2.6), by determination of absorbance at 595nm compared to a standard of bovine serum albumin.

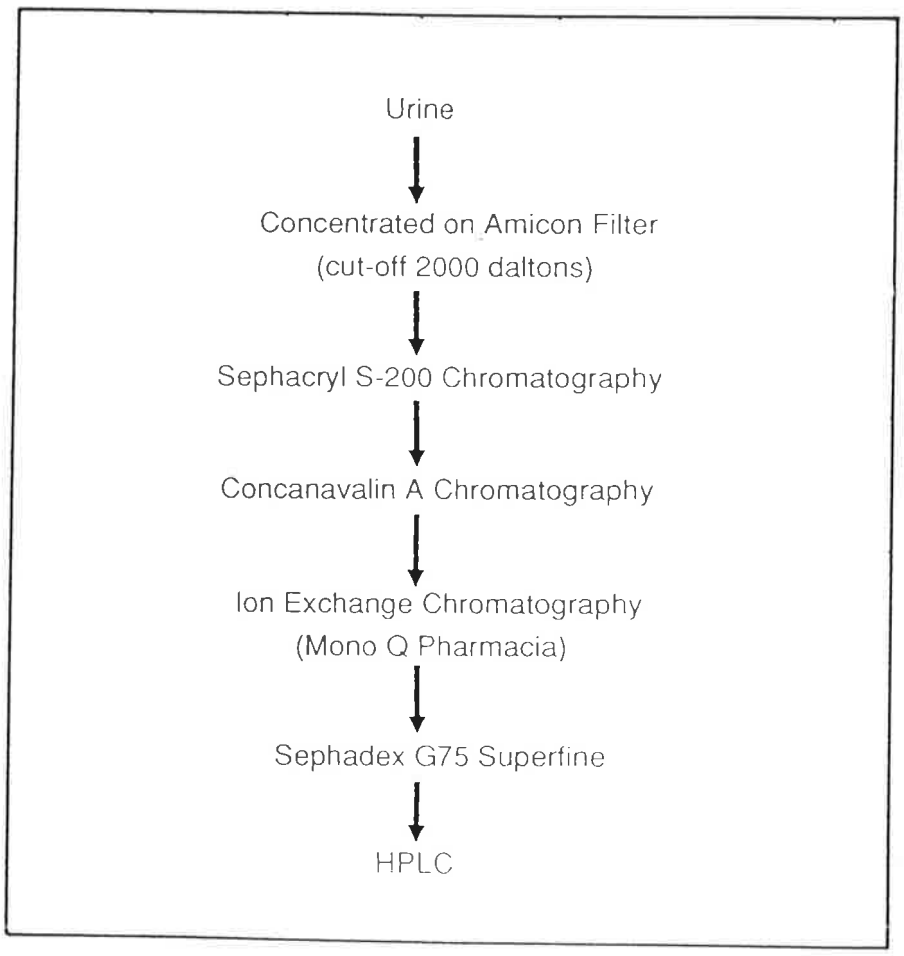


Figure 4.1

Schematic diagram of the experimental design for ßC-hCG purification.

4.2.3 Protocol for characterization of purified ßC-hCG

The purified preparation of ßC-hCG was analysed for the degree of purity by HPLC separation and SDS-PAGE under non-reducing and reducing conditions (see details in Chapter Two, sections 2.5.4 and 2.7, respectively). Molecular weight was also estimated on SDS-PAGE. The immunological characteristics were examined by Western blot. The biological activity was examined by testing its ability to stimulate steroidogenesis in vitro (section 2.13). The carbohydrate composition was investigated by lectin blots using several lectins and the peptide composition and structure was determined by amino acid analysis and sequencing. The methodology, reagents, and equipments involved in this process was presented in Chapter Two and appendixes I-III. A simplified diagram is shown in Figure 4.2.

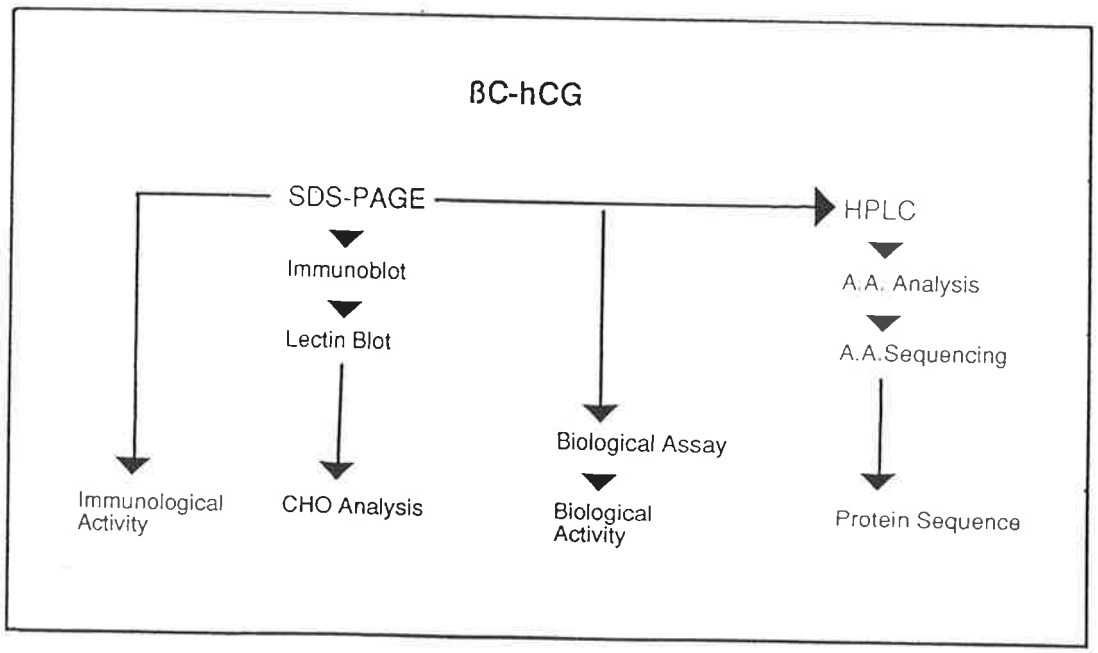


Figure 4.2

Diagram of the experimental design for characterization of the ßC-hCG fragment.

4.2.4 Analysis of data

Protein peaks eluted from Sephacryl S-200 and Sephadex G-75 columns were determined from their elution volumes (Ve) and the coefficients of partition were calculated by the equation **Kav = (Ve-Vo)/(Vt-Vo)**. Absorbance in the fractions eluted from the columns was examined at different wavelengths using a spectrophotometer; immunoactivity was monitored by specific immunoradiometric assays. Dose inhibition curves and potency estimates in the assays were analysed by a computerized spline function system provided by LKB (Wallac 1260 Multigamma II counter). Molecular weights on gel filtration column eluate were determined by the diagram of Andrews (*Andrews, 1965*), and by Ferguson plot on SDS-PAGE (*Shapiro et al, 1967*). Comparison of progesterone and oestradiol levels between control, hCG and ßC-hCG groups in the granulosa-lutein cell bioassay was made using one-way analysis of variance and the differences were considered statistically significant at $P<0.05$. At each day the levels were compared with the 95% confidence intervals.

4.3 Results

4.3.1 Purification of ßC-hCG

4.3.1.1 Sephacryl S-200 chromatography

The elution volumes and coefficients of partition of standard proteins on this column are summarized in Table 4.1. Figure 4.3 shows the straight line constructed with the log molecular weight of these proteins and their respective coefficients of partition. Even though gel filtration is not totally reliable for determining molecular weight of glycoprotein hormones, the molecular weight for any unknown was estimated using this relationship.

Table 4.1 - Elution volumes (ml) and coefficients of partition (Kav) of standard markers on Sephacryl S-200 and Sephadex G-75 columns*

Marker	Molecular Weight	Sephacryl S-200		Sephadex G-75	
		Ve	Kav	Ve	Kav
Blue dextran (Void Volume	2000000	198	-	48	-
Thyrosine (Thy)	669000	-	-	50	0.016
Aldolase (Ald)	158000	235	0.139	51	0.024
Bovine serum albumin (BSA)	67000	262	0.240	64	0.132
Ovoalbumin (Ovo)	45000	276	0.293	69	0.173
Chymotrypsinogen (Chy)	25000	317	0.447	84	0.297
Cytochrome C (Cyt)	14100	347	0.560	104	0.462
^{125}I-βC-hCG (βC)	10000	364	0.624	108	0.495
Vitamin B_{12} (B_{12})	1350	446	0.932	148	0.826
^{125}Iodide (total volume)	150	-	-	169	-

*Sephacryl S-200 chromatography was performed with 0.2M ammonium acetate, 0.02% NaN3, pH 6.8, at a downward flow rate of 50ml/h; Sephadez G-75 chromatography was developed with 0.15M ammonium bicarbonate, 0.02% NaN3, pH 7.4 at a downward rate of 6ml/h.

Initial chromatography of pregnancy urine concentrate on Sephacryl S-200 is shown in Figure 4.4A. Elution of intact hCG immunoactivity, was detected between 240-272ml (Kav = 0.29). A very small immunoactive peak was also observed at approximately 310ml elution (Kav = 0.42) in a region later revealed to be the elution position of the alpha subunit. The ßhCG immunoactivity was distributed in a large peak between 242-300ml partially overlapping with intact hCG. The elution of the free beta form corresponded to a peak seen at 278ml. A little beta immunoreactivity was observed between 305-320ml representing alpha hCG cross-reaction. Free alpha hCG activity, was detected between 290-340ml with a peak at 313ml (Kav = 0.43). In addition small quantities of alpha immunoactivity were observed between 350-364ml, a position later shown to be the same as ßC-hCG elution.

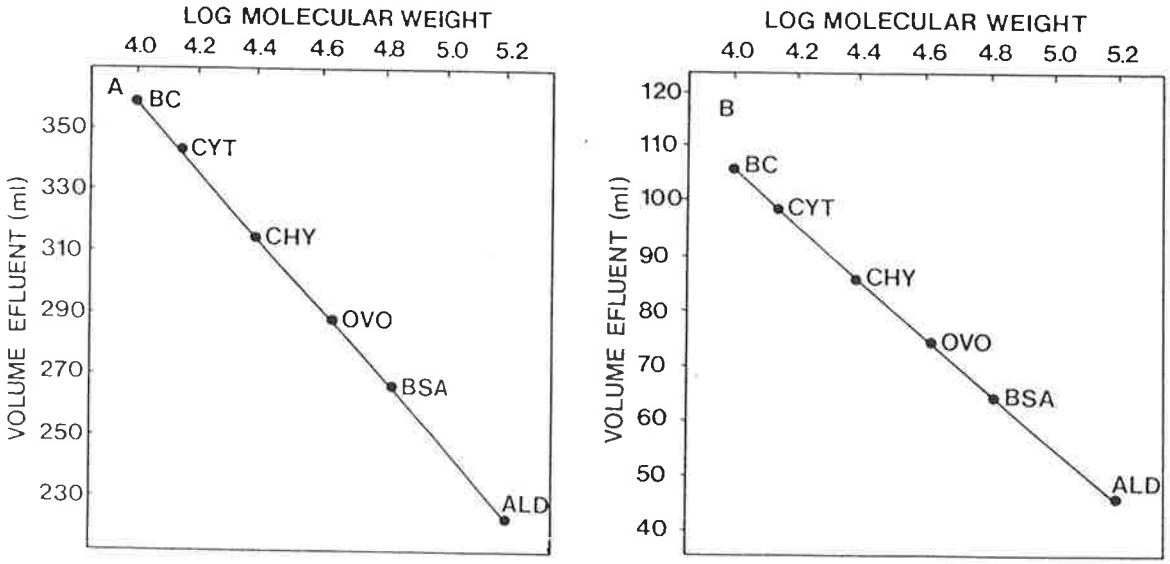


Figure 4.3

Relationship of the log molecular weight of a set of 6 different standard proteins to their elution volumes on Sephacryl S-200 (Panel A) and Sephadex G-75 (Panel B) columns. Molecular weights ranged from Mr 158000 to 10000. Molecular weights of unknowns on each column were taken from the respective lines.

Further chromatography of one litre of urine concentrate by ultrafiltration (Figure 4.4B) monitored for ßC-hCG immunoactivity by using IRMA, showed a peak of immunoreactivity between fractions 79-92 (332-386ml Ve = 360ml; Kav = 0.61) with nearly the same partition coefficient as purified [^{125}I]-ßC-hCG (Kav = 0.62). A small amount of ßC-hCG immunoreactivity, reflecting the ability of the assay to cross-react with free ßhCG subunit, was detected in the region of elution of this subunit. Fractions corresponding to the positions of intact hCG or αhCG elution did not exhibit ßC-hCG immunoreactivity. Fractions of 80-91 (336-382ml) showing ßC-hCG immunoactivity were pooled, concentrated by pressure ultrafiltration in the Amicon cell, and submitted to additional purification by affinity chromatography on Con A-Sepharose.

4.3.1.2 Concanavalin A-Sepharose 4B chromatography

The majority of the ßC-hCG immunoreactivity pooled from Sephacryl S-200 showed high affinity to Con A and could be eluted by addition of 0.2M alpha methyl-pyranoside to the starting buffer (Figure 4.4C). The remainder of the ßC-hCG immunoreactivity, approximately 22-44% among several specimens, did not bind to Con A and eluted after the void volume in a broad peak with the equilibration buffer (Figure 4.4C), suggesting it was constituted by non-reactive and weakly-reactive molecules. After concentration and reapplication to the column this material was again incapable of binding to Con A lectin. Figure 4.4.D shows that this material eluted within and after the void volume as a wide peak, indicating that the Con A - unbound ßC-hCG molecules are not homogeneous. As a trimannosyl core is essential for glycoproteins to react with this lectin and the composition of the antennae dictate their affinity, it may be concluded that the ßC-hCG eluted from Sephacryl gel in a broad range of molecular weight consists of a mixture of microheterogeneous molecules. The Con A-bound and Con A-unbound

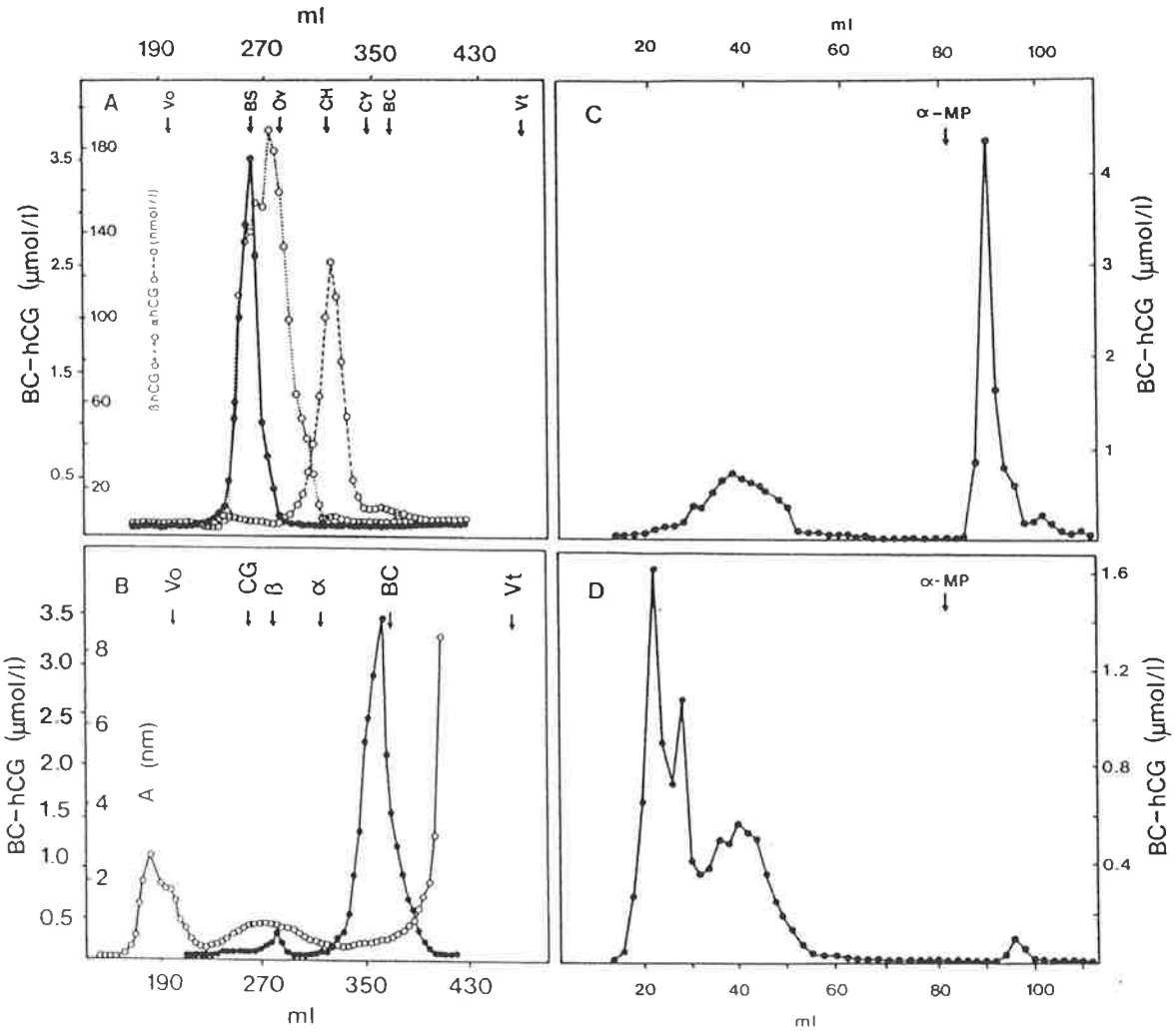
Figure 4.4

A Sephacryl S-200 gel filtration (2.6 x 87.5cm column) of 200ml of urine obtained from a healthy first trimester pregnancy woman. The sample was concentrated to 8ml by ultrafiltration and eluted at a downward flow rate of 50ml/h with 0.2M ammonium acetate, pH6.8, at room temperature. Fractions of 4.2ml were collected and assayed for intact hCG (●-●), ßhCG (○···○) and αhCG (○ - ○). Arrows indicate the position of the void volume (Vo), total volume (Vt), and the elution positions of [^{125}I]-ßC-hCG, and marker proteins: bovine serum albumin, Mr 67000 (BS); ovoalbumin, Mr 43000 (Ov); chymotrypsinogen A Mr 25000 (Ch); Cytochrome C, Mr 13000 (Cy).

B Elution profile of one litre of urine concentrated to 8ml by ultrafiltration after chromatography on Sephacryl S-200. The procedure was as in **A**. Each fraction was monitored for optical density at 280nm and assayed by IRMA for ßC-hCG immunoreactivity. The arrows labelled with CG, ß and α indicates the points of elution of urinary intact hCG, ßhCG and αhCG respectively.

C Concanavalin A- Sepharose chromatography of concentrated immunoreactive ßC-hCG fractions obtained by gel filtration of pregnancy urine on Sephacryl S-200 (**B**). The unbound/weakly bound material was eluted from a 1x10cm column at a flow rate of 8ml/h with 0.2M ammonium acetate, containing 0.15M NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, 1mM Mg Cl_2, and 0.02% NaN_3, pH 6.8. The bound material was eluted by the addition of 0.2M alpha methyl D-pyranoside at the point indicated by the arrow (α-MP). Each 2.1ml-fraction collected was analysed by IRMA by ßC-hCG immunoactivity.

D Concanavalin A-Sepharose rechromatography of a concentrate of the Con A-unbound immunoactive ßC-hCG material purified as described in **C** and pooled from several individuals. All the procedures were performed as indicated in **C** and the arrow also indicates addition of 0.2M methylpyranoside.


A
B
C
D
ml
Vo
BS
δγ
CH
CγBC
Vt
CG
β
α
α-MP
BC-hCG (µmol/l)
A (nm)
βhCG α-•-• αhCG α-○-○ (nmol/l)

materials were separately pooled, concentrated by ultrafiltration pressure, and subjected to anion exchange chromatography.

4.3.1.3 Ion exchange chromatography

The chromatography of Con A-bound material on a strong anion exchange column (Mono Q) and measurement of ßC-hCG immunoreactivity in 0.5ml collected fractions showed that most of this fragment (approximately 98%) eluted early before starting the salt gradient (Figure 4.5A). Considering that the negative charges are given mainly by sialic acid, this result suggests these ßC-hCG molecules, while retaining a mannosyl core, lack most peripheral carbohydrate residues or else contain it in a very low concentration. The immunoactive unbound-Con A ßC-hCG showed slightly different behaviour on anion exchange: as much as 12% eluted only after starting the salt gradient (Figure 4.5B). For comparison, the iodinated ßC-hCG preparation reference I (Birken) purified by different methods was also chromatographed. This material exhibited more accentuated differences on Mono Q: approximately 40% of the radioactivity was eluted only after addition of salt to the starting buffer (Figure 4.5C), indicating it is a preparation more heterogeneous than the material presented in this study. Because iodinated trace quantities of the ßC-hCG preparation may not be representative and because the iodination may alter its behaviour on anion exchange, this result has to be interpreted with caution.

4.3.1.4 Sephadex G-75 chromatography

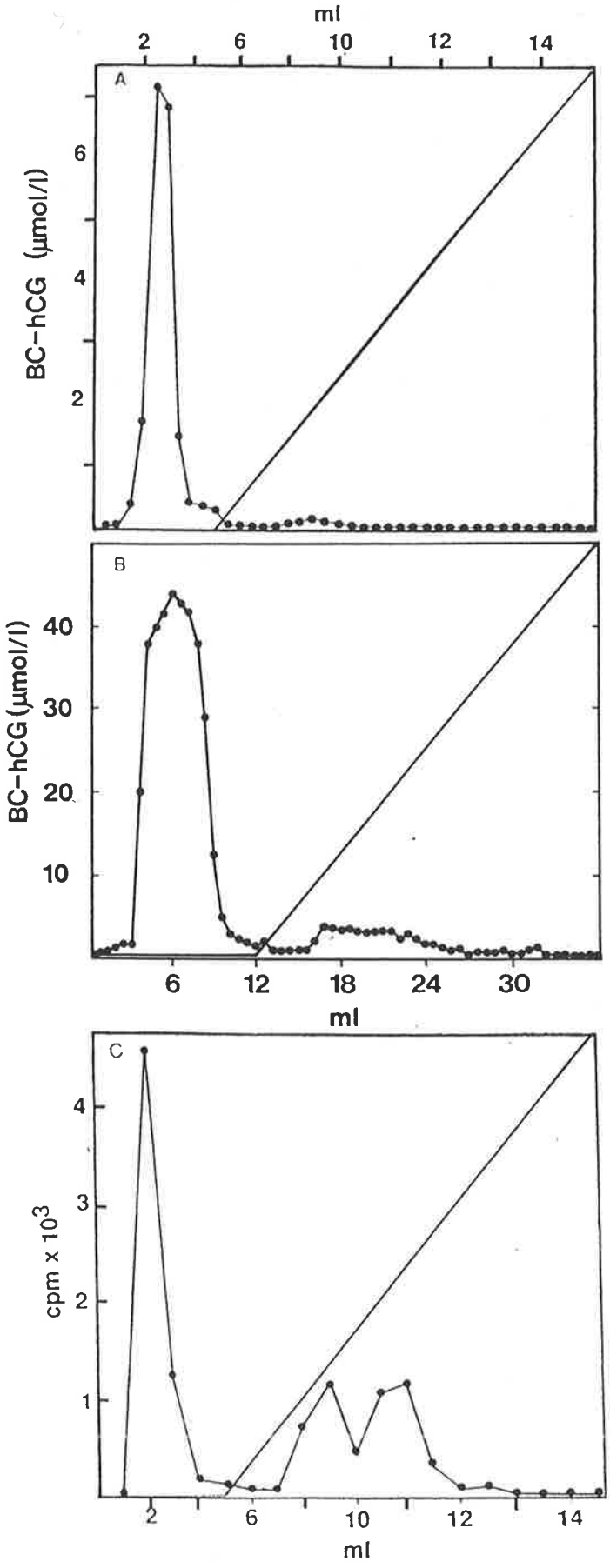
The elution volumes and coefficients of partition of standard proteins on this column are also found in Table 4.1. Figure 4.3 also depicts the relationship of the log molecular weight of standards to their coefficients of partition in this column. The Con A-bound ßC-hCG pooled from Mono Q unbound eluate showed a peak of immunoactivity between 94-116ml (peak

Figure 4.5

A Ion exchange chromatography of Con A-bound ßC-hCG. Two hundred microlitres of concentrated sample was applied to a 1ml-bed Mono Q HR 5/5 column and eluted, by using a Fast Protein Liquid Chromatography (FPLC) system, at room temperature, with 0.02M Tris-HCl, pH 8.0, at a flow rate of 1ml/min. Five minutes after sample injection, a linear gradient from 0.1M NaCl in starting buffer was initiated and completed in a volume of 15ml. The diagonal line indicates the salt gradient (0-100%). Fractions of 0.5ml were collected and assayed for ßC-hCG.

B Ion exchange chromatography profile of Con A-unbound ßC-hCG pooled and concentrated from several individuals. The procedure was as in **A**, except that the salt gradient was initiated 20 minutes after sample injection and the linear gradient was initiated and completed in a volume of 40ml. Fractions of 1ml were collected and assayed for ßC-hCG immunoreactivity.

C Ion exchange profile of ~4pmol of a ßC-hCG preparation (purified by Dr. Steven Birken) labelled with ^{125}Iodine. The procedures were as in **A**. Fractions of 1ml were collected and counted for ^{125}I radioactivity.


ml
2
6
10
12
14
A
BC-hCG (µmol/l)
6
4
2
B
BC-hCG (µmol/l)
40
30
20
10
6
12
18
24
30
ml
C
cpm x 10³
4
3
2
1
2
6
10
12
14
ml

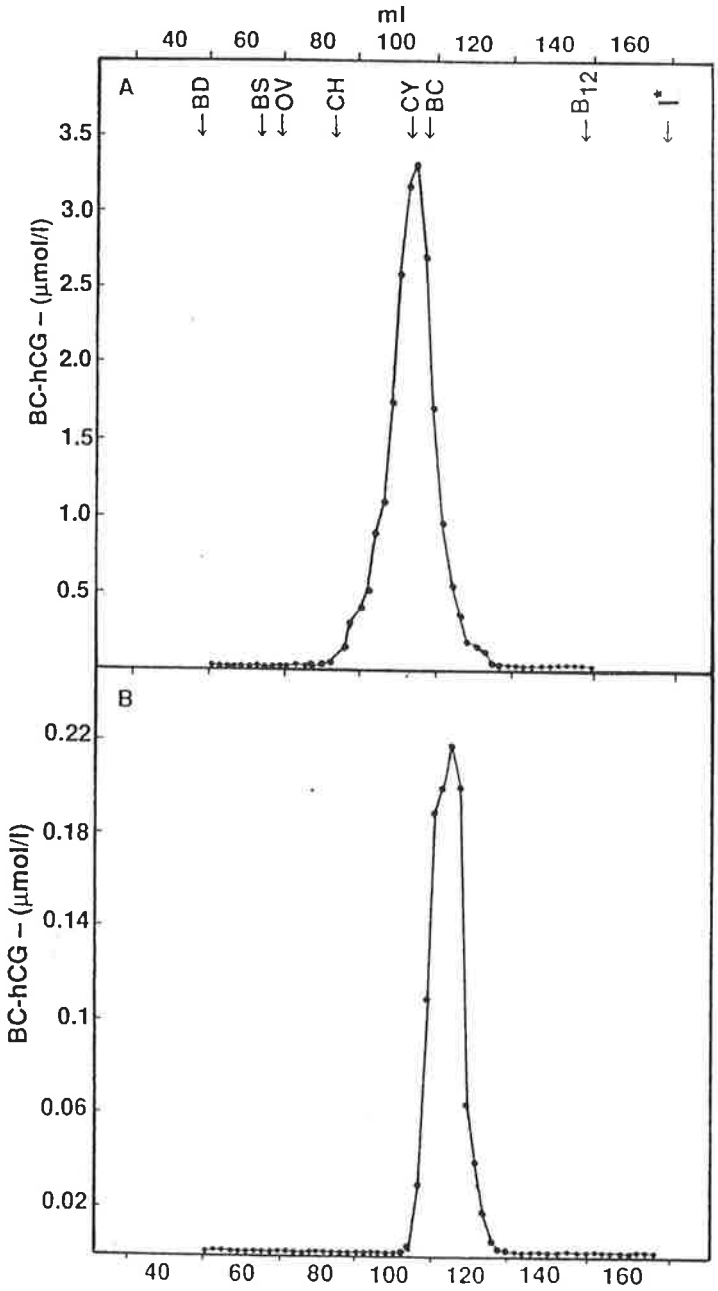


Figure 4.6.

Elution profile of immunoreactive ßC-hCG on Sephadex G-75 column. **(A)** The Con A-bound ßC-hCG (obtained from ConA-bound, mono Q unbound fractions) was filtered at room temperature on a 1.6 x 85cm column of Sephadex G-75 superfine gel with 0.15M ammonium bicarbonate, pH 7.4, at a downward flow rate of 6ml/h. Fractions of 2ml were collected and assayed for ßC-hCG immunoactivity. Arrows indicate the position of the void volume (Vo), and the elution points of [^{125}I]-ßC-hCG (BC), free [^{125}I] (I*), and internal markers: bovine serum albumin Mr 67000 (BS); ovalbumin Mr 43000 (Ov), chymotrypsinogen A Mr 25000 (CH); cytochrome C Mr 13000 (CY), and vitamin B12 Mr 1350 (B12). **(B)** Con A-unbound ßC-hCG (obtained from Con A-unbound, mono Q unbound fractions) was filtered as in **A**.

105ml, Kav = 0.47) with an apparent molecular weight of Mr 12800 when chromatographed on this Sephadex G-75 superfine column (Figure 4.6A). The ßC-hCG unbound to Con A that also did not interact with the anion exchange resin (Con A-unbound, Mono Q non-reactive ßC-hCG) eluted between 103-117ml (peak 113ml, Kav = 0.51) with an apparent molecular weight of Mr 10000 (Figure 4.6B). This slightly higher partition coefficient suggests that the majority of the Con A-unbound ßC-hCG is lacking any sugar moiety instead of being incompletely trimmed in its oligosaccharide chains. The results suggest that the residual 12% of this material, able to exchange negative charges with Mono Q, retains some monosaccharides extending the trimmanosyl core. However, these ßC-hCG molecules were not submitted to further characterization. The fractions eluted from Sephadex G-75 containing either the Con A-bound or Con A-unbound ßC-hCG (and unbound to Mono Q) were pooled, aliquoted, lyophilized and stored at -20C.

4.3.1.5 Recovery of urinary ßC-hCG during the purification sequence

Table 4.2 shows the protein content, yield and purification factor at each purification step. Because the Con A-unbound material was pooled from several patients after the affinity chromatography procedure, the recovery could not be accurately estimated for this material.

4.3.2 Characterization of purified ßC-hCG

The degree of purity of the yielded ßC-hCG preparation was analysed by SDS-PAGE under non-reducing and reducing conditions and reverse phase HPLC profile. Its molecular weight was also estimated by SDS-PAGE and compared to the molecular weight of the reference I material under the same conditions. Molecular weight was estimated using the Ferguson plotgraph (*Shapiro et al, 1967*) constructed with several standard proteins and

TABLE 4.2 purification of ß-core hCG from urine of pregnant women

SAMPLE Procedure	Total ß-core hCG activity* (ug)	Protein** (ug/ml)	Total Protein (mg)	Specific Activity (ug/mg protein)	Yield (%)	Purification (fold)
Original Urine sample	1950	47.5	47.5	41.0	100	(1.0)
Conc. urine Amicon filter (2,000 cut-off)	1741	1650	14.0	124.3	89.2	3.0
Pool Eluant Sephacryl -200	1413	204	7.7	183.5	72.4	4.4
Pool Eluant Con A	713	21	0.8	891.2	36.5	21.7
Pool Eluant Mono Q	257	-	-	-	13.2	-
Pool Eluant Sephadex G-75	180	2.2	0.04	4500. 0	9.2	109.7

* Activity by IRMA
** Determined by Bio-Rad Protein assay kit
(-) Insufficient sample for measurement

depicted in Figure 4.7. The immunological characteristics of the purified ßC-hCG preparation were evaluated by testing its ability to bind a specific polyclonal antibody using Western blots (Chapter Two, section 2.8). Its bioactivity was tested in vitro using granulosa cells (Chapter Two, section 2.13). The sugar composition was studied by examining binding to several lectins with different affinities for various monosaccharides or oligosaccharides moieties (*Osawa and Tsuji, 1987*).

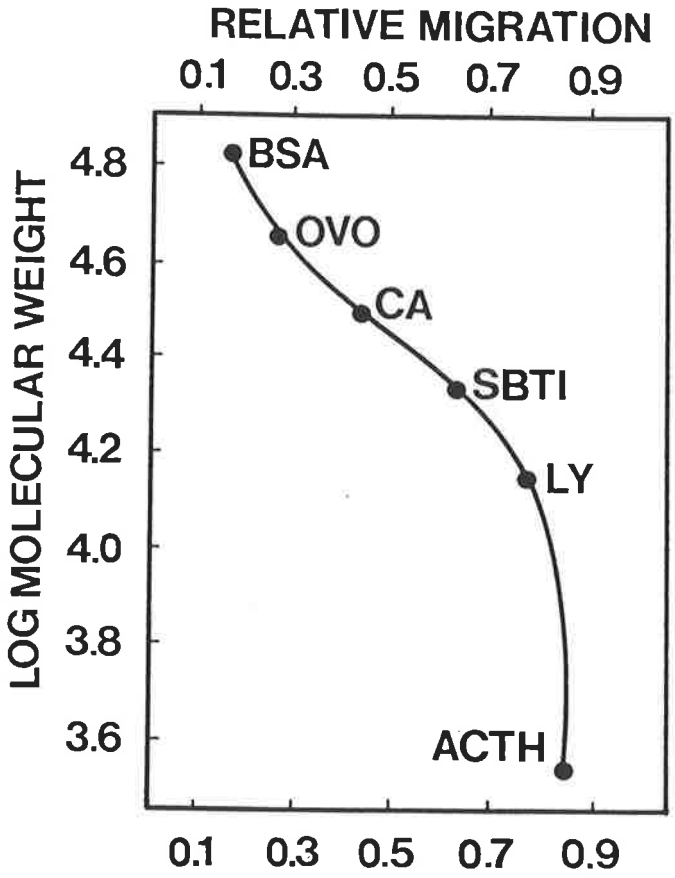


Figure 4.7

Ferguson plotgraph generated by plotting relative mobility (Rf) against log molecular weight of standard proteins (BSA, Mr 67000; Ovoalbumin, Mr 43000; carbonic anhydrase, Mr 31000; Soy bean trypsin inhibitor, Mr 21500; lysozyme, Mr 14400; adrenocorticotrophin, Mr 1350) subjected to electrophoresis as described in Chapter Two, section 2.7. The molecular weight of unknown was determined by finding its Rf and reading the log molecular weight from the ordinate.

Protein composition was established by amino acid analysis after hydrolysis. Sequencing was determined after reduction, 3 [H] S-carboxymethylation and reverse phase HPLC of the reduced peptides (*Birken et al, 1988*). The results obtained are presented in the following paragraphs.

4.3.2.1 High Performance Liquid Chromatography

Approximately 12nmol of lyophilized ßC-hCG preparation (Con A-bound material) was reconstituted in 0.2ml 0.1% TFA buffer, filtered on a micro-spin filter (0.2µm cut-off) and chromatographed on HPLC under the conditions detailed in Chapter Two. The elution was monitored with a UV detector at 214nm wavelength (Figure 4.8A) and 0.6ml fractions were collected. Ten microlitres of each fraction were taken off, diluted, and assayed for ßC-hCG immunoreactivity. Figure 4.8B shows that this material eluted in a unique peak, denoting to be a pure preparation.

4.3.2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein standards, ßhCG, αhCG, ßC-hCG reference I preparation, and the Con A-bound ßC-hCG prepared as described above were submitted to electrophoresis on 18% SDS-PAGE gel without chemical reduction as shown in Figure 4.9A. The purified ßhCG subunit (lane 2) showed a thin single band with apparent molecular weight of Mr 28000. Highly purified αhCG (CR 119) subunit showed faster migration and stained as a single band with apparent molecular weight of Mr 23400. The ßC-hCG purified by using the procedures described here and the reference I preparation used for comparison exhibited similar electrophoretic mobility. Both preparations showed intense staining single bands corresponding to an apparent molecular weight of Mr 15850 (ranging from 14390-17380). However the two lanes corresponding to the reference preparation were slightly broader, suggesting greater heterogeneity.

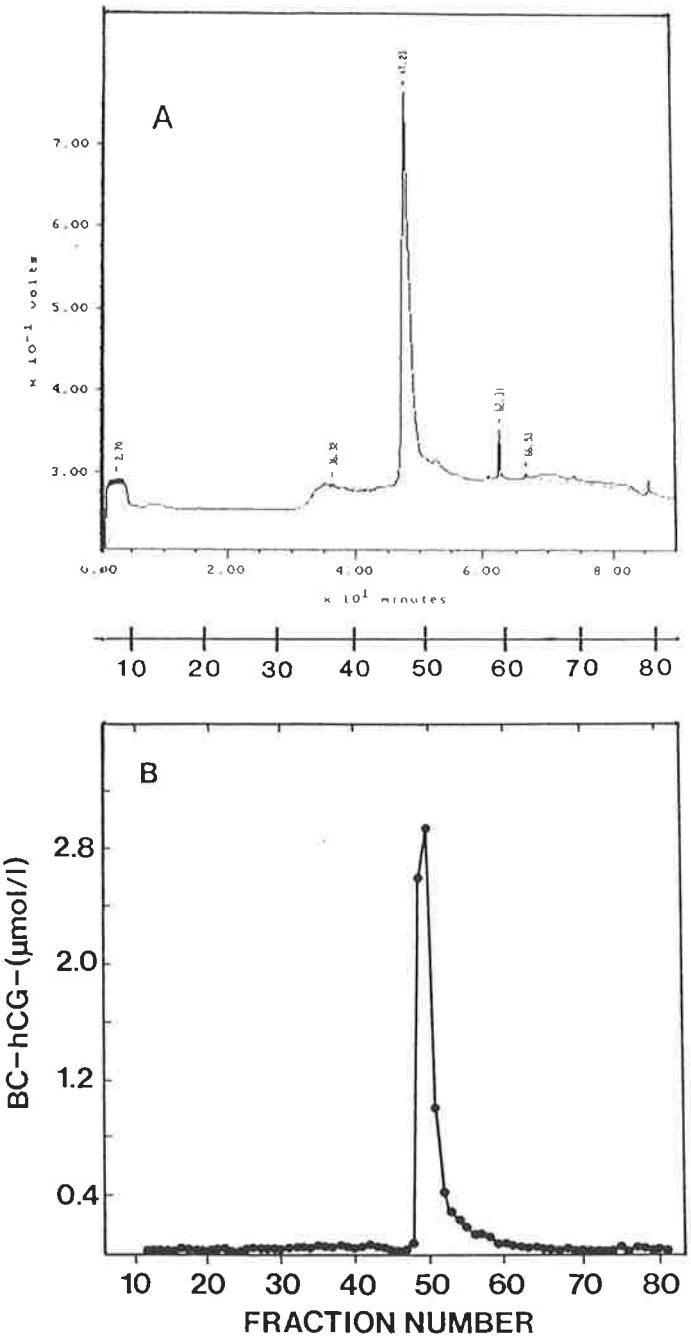


Figure 4.8

HPLC chromatography of Con A-bound ßC-hCG following ion exchange and gel filtration. Approximately 1.5nmol of native material was loaded onto a Brownlee RP-8 column connected to a Waters HPLC system and eluted with 0.1% TFA over 10 minutes at a flow rate of 0.6ml/minute. A gradient of 0-70% of 0.1% TFA in 80% acetonitrile was developed during 60 minutes and to 100% over the following 10 minutes. Fractions of 0.6ml were collected and monitored for OD at 214nm **(A)** and assayed for ßC-hCG immunoactivity **(B)**.

After reduction with 2-mercaptoethanol both preparations showed two thin bands corresponding to molecular weights of Mr 8900 and Mr 7500 (Figure 4.9B, lanes 2 and 4). By loading a greater amount a third less intensive band corresponding to about Mr 3500 was seen in our material (lane 3). Because the available amount of the reference preparation was limited this small band could not be further resolved. The purified Con A-unbound ßC-hCG, electrophoresed under non-reducing conditions, showed faster migration (Figure 4.9C, lane 3) than the Con A-bound material (lane 4) and had an apparent molecular weight estimated as Mr 10250 (ranging from 9550-11200). In addition both materials showed a slightly staining second band with an molecular weight of approximately Mr 7500, suggestive of either a co-purified byproduct of hCG/ßhCG excretion or a result of some degradation during their purification.

4.3.2.3 Western immunoblots of ßC-hCG

Immunoblots of the Con A-bound ßC-hCG form following SDS-PAGE under reducing conditions as described in Chapter Two, sections 2.7 and 2.8, showed three bands immunostained with a specific rabbit polyclonal antibody raised against this preparation and described in detail in the next chapter. The two bands corresponding to a molecular weight of Mr 8900 and Mr 7500 seen with silver nitrate were easily recognized by this antiserum (Figure 4.10A, lane 4). In addition to the 8900 and 7500 bands the fast moving third band was equally immunostained. The reference preparation of ßC-hCG, shown for comparison in Figure 4.10A, (lane 3) bound the antibody in a similar pattern. However, due to the small amount loaded, the bands with lower molecular weight could not be reproduced in detail in this preparation. These results strongly suggest that a third small chain may be present in the structure of some ßC-hCG molecules and, in addition, indicate that the ßC-

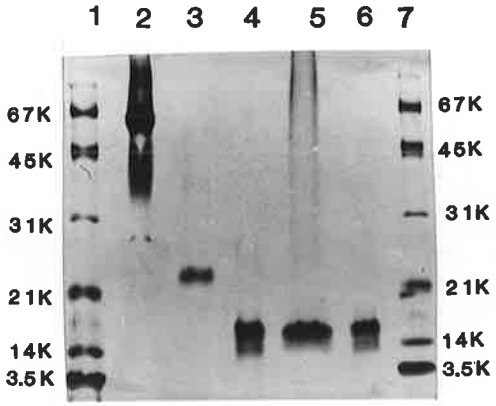
Figure 4.9

A SDS-PAGE of ßC-hCG and related glycoproteins under non-reducing conditions as described in materials and methods. The gel was silver stained according to the method of Morrissey (1981). Lanes 1 and 7 display standard markers; lane 2 was loaded with 2µg ßhCG (CR125) but showed some BSA contamination; lane 3, αhCG (CR119); lanes 4 and 6, ßC-hCG reference I; lane 5, Con A-bound ßC-hCG purified in the present study.

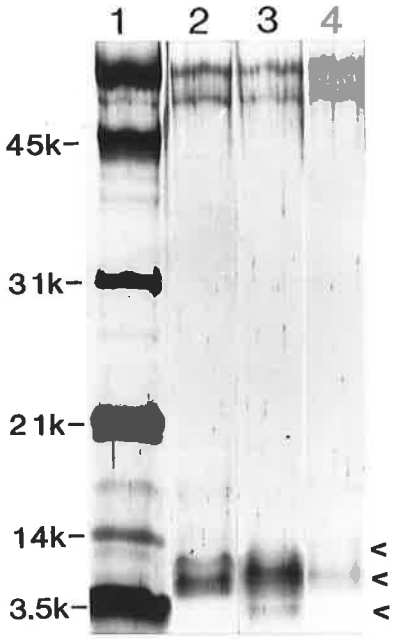
B Discontinuous electrophoresis of ßC-hCG after reduction with 2.5% 2-mercaptoethanol. Lane 1, standard markers; lanes 2 and 3 1µg and 2.5µg of Con A-bound ßC-hCG; lane 4, 1µg ßC-hCG reference. The gel was silver stained and the arrows on the right side indicate the migration positions of the reduced peptide bands.

C SDS-PAGE of hCG (CR125) (lane 2), Con A-unbound ßC-hCG (lane 3), and Con A-bound of ßC-hCG (lane 4) under non-reducing conditions. Lanes 1 and 5 display protein markers. The gel was stained with silver stain reagents.

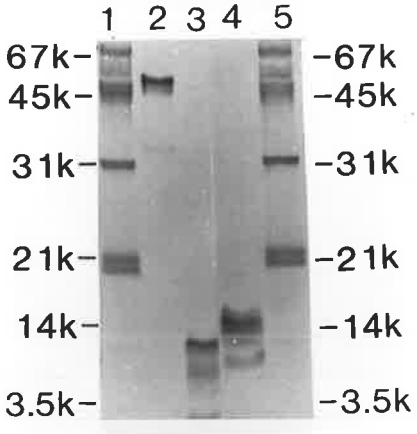
A


1 2 3 4 5 6 7
67K
45K
31K
21K
14K
3.5K
67K
45K
31K
21K
14K
3.5K

B


1 2 3 4
45k-
31k-
21k-
14k-
3.5k-
<
<
<

C


1 2 3 4 5
67k-
45k-
31k-
21k-
14k-
3.5k-
-67k
-45k
-31k
-21k
-14k
-3.5k

hCG antiserum employed may recognizes epitopes at different regions in the whole ßC-hCG molecule. The Con A-unbound ßC-hCG form was analysed under similar conditions and its Western blot showed the same pattern with the rabbit polyclonal antibody raised against the Con A-bound form, immunostaining the three bands. In addition to show the lectin blots Figure 4.11 (lanes 3, 4, and 5) compares the immunoblots of both Con A-bound and unbound forms with this antibody.

4.3.2.4 Lectin blots of ßC-hCG

Equipment, reagents, and procedures for the lectin blots were presented in Chapter Two, section 2.9. Qualitative evaluation of carbohydrate composition of ßC-hCG was assessed by blotting this fragment with several lectins which specifically recognize either peripheral monosaccharide residues *(Ricinus communis* agglutinin, *Wheat germ* agglutinin, *Peanut* agglutinin, *Elderberry bark* agglutinin*)*, the trimannosyl core *(Con A, Lens culinaris* agglutinin*)* and fucose *(Lens culinaris)* of N-linked oligosaccharide moieties or the tetrasaccharide structure found in the O-linked carbohydrate chains of whole ßhCG subunit *(Soybean* agglutinin, *Dolichos biflorus* agglutinin*)*. The binding of the ßC-hCG on nitrocellulose membrane with biotinylated Concanavalin A, specific for the trimmanosyl core of N-linked oligosaccharides (*Dufau et al, 1972; Baezinger and Fiete, 1979*), was strong in both Con A-bound and reference ßC-hCG preparations. Under reducing conditions this lectin revealed that the sugar moiety is attached to the Mr 7500 band (Figure 4.10A, lanes 5 and 6). Although the Mr 8900 molecular weight band did not bind Con A resin, this lectin also bound to the Mr 3500 molecular weight band in both preparations, indicating this method can detect small amounts of carbohydrates.

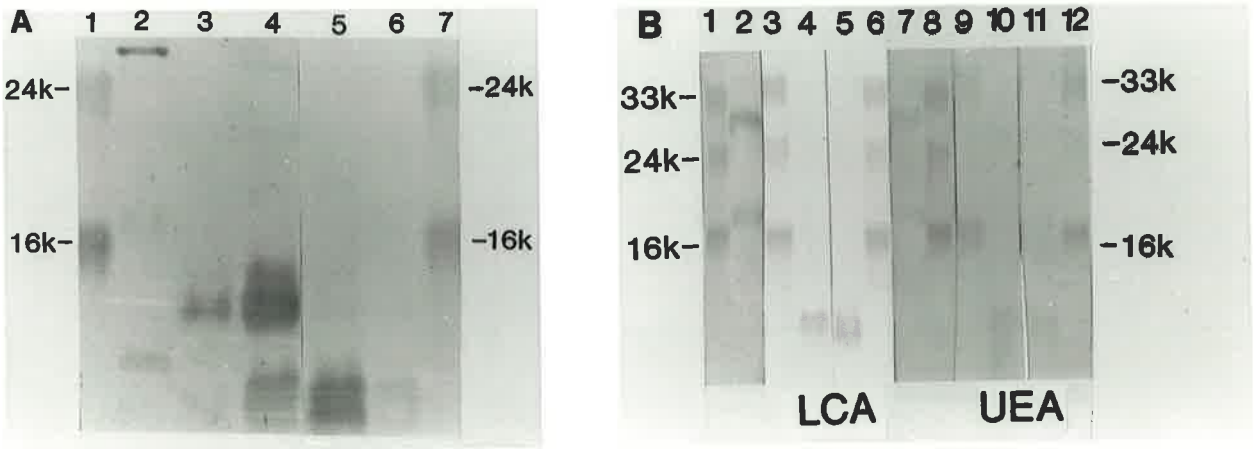


Figure 4.10.

A Western immunoblotting and Con A blotting of ßC-hCG electrophoresed in 18% polyacrylamide gel after reduction with 2.5% 2-mercaptoethanol. Immunodetection of the Con A-bound ßC-hCG (lane 4) and Birken's preparation (lane 3) was performed with DeM3 polyclonal anti-ßC-hCG antiserum (1:100). Lanes 5 and 6 depict the Con A binding abilities of the ßC-hCG preparation presented in this study and of the reference material, respectively. The immunoblots and the lectin blots were visualized by avidin-biotin-peroxidase complex. Lanes 1, 7 and 2 show pre-stained and biotinylated standard markers.

B Binding of Lens culinaris on reduced hCG (lane 2), Birken's ßC-hCG reference (lane 4) and Con A-bound ßC-hCG (lane 5); Ulex europeaus binding on reduced hCG (lane 7), ßC-hCG reference (lane 10) andConA-bound ßC-hCG (lane 11). Pre-stained markers are depicted in lanes 1, 3, 6, 8, 9 and 12.

The interaction of the Con A-bound and the reference preparation of ßC-hCG with *Lens culinaris* agglutinin (LCA) having the same specificity to that of Con A but needing a fucose residue linked to the N-acetylglucosamine at the reducing end (Fuc α1-6 NAcGlc) (*Kornfeld et al, 1981*), took place only on the Mr 7500 apparent molecular weight band. The reaction was less intense (Figure 4.10B, lanes 4 and 5), indicating either the existence of ßC-hCG molecules in which the fucose residue is lacking in their N-linked sugar moieties or a lower affinity of this lectin to the immobilized sugar unit. The faster migrating Mr 3500 band did not bind LCA and appears to be depleted of fucose in its oligosaccharide branch. Although both preparations have shown similar patterns with Con A and LCA blotting, small differences could be observed; while the reference preparation appeared to bind more strongly to Con A, the current preparation purified as described appeared to be richer in fucose residues.

The inability of Con A-bound and reference preparations of ßC-hCG to bind *Ulex europaeus* agglutinin I (UEAI), specific to Fuc α1-2 Galß1-4NAcGlc sequence (Pereira et al, 1978) confirms the structure Man ß1-4NAcGlc(α1-6 Fuc)1-4NAcGlc present in their chitobiose core (Figure 4.10B, lanes 10 and 11). ßC-hCG did not bind either *Ricinus communis* agglutinin (RCA) specific to galactose (Galß1-4NAcGlc)-linked at non-reducing end of N-glycosidic units (*Wu et al, 1986*) or *Wheat germ* agglutinin (WGA) specific to structures with a high density of terminal non-reducing NAcGlc or NAc NeuAc (*Gallagher et al, 1985*), indicating the absence of terminal sialic acid, galactose, and and N-acetylglucoasamine residues. *Soybean* agglutinin (SBA) and *Dolichos biflorus* agglutinin (DBA) which specifically bind α or ß linked galactose and α or ß N-acetylgalactosamine (Galα1-3NAcGal or Galß1-4NAcGal), respectively, did not react with any ßC-hCG preparation, reflecting the

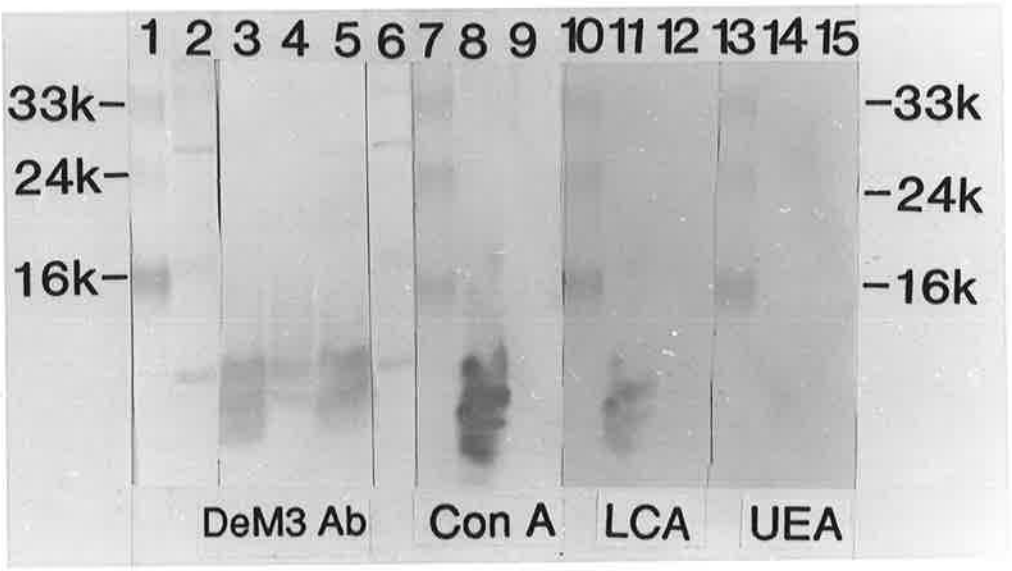


Figure 4.11

Immunoblotting and lectin blotting of both Con A -bound and Con A-unbound ßC-hCG preparations electrophoresed and blotted as described in Chapter Two, sections 2.8 and 2.9. The antiserum DeM3 was used for immunodetection. Lanes 3 and 4 show the binding of two different amounts of the Con A-unbound ßC-hCG (2mg and 1mg, respectively; see discussion) and lane 5 shows the binding of the Con A-bound material. Lanes 8 and 9 compare the abilities of Con A-bound and Con A-unbound ßC-hCG preparations to bind this lectin after blotting. Lanes 11 and 14 show the staining of Con A-bound ßC-hCG with Lens culinaris and Ulex europeaus lectins, respectively. Lanes 12 and 15 show the behaviour of the Con A-unbound material with these two lectins. Lanes 1, 7, 10, and 13 depict pre-stained markers and lanes 2 and 6 show biotinylated standards.

absence of O-glycidic chains on their carbohydrate composition (*Pereira et al, 1974; Baker et al, 1983*). The apparent discrepancies between the reported carbohydrate composition of the reference preparation (*Birken et al, 1988*) and that noted here may be due to the different methods used by the supplier for its analysis or to batch-to-batch variation. The abilities of reduced Con A-bound ßC-hCG and reference preparations to bind several lectins are summarized in Table. 4.3.

As expected, the ßC-hCG form that did not bind to either Con A or Mono Q resin on chromatographic procedures also did not bind the Con A lectin when immobilized on nitrocellulose membrane (Figure 4.11, lane 9). Further, this Con A-unbound ßC-hCG was unable to bind LCA (lane 12), UEA-I (lane 15) or any other lectin (Table 4.3), indicating absence of any sugar moiety in its structure. The Con A- weakly reactive forms, which did bind to Mono Q, could not be analysed in the present study but based on the findings of Birken et al (1988) and on the notion about the types of Asn-linked oligosaccharides of many glycoproteins that are weakly bound to Con A, this ßC-hCG form may have either intact or a spectrum of partially trimmed oligosaccharides. The highly purified intact hCG (CR 125) was also reduced, blotted on nitrocellulose membrane, and tested for various lectins as control. Although αhCG and ßhCG differentiate only in relation to fucose residues on their N-linked oligosaccharide moieties, they showed different affinities for Con A on lectin blots, as judged by the intensity of staining (Figure 4.10B, lane 2). The results seen with other lectins are presented in Table 4.3.

4.3.2.5 Amino acid analysis of ßC-hCG

The amino acid composition of the Con A-bound ßC-hCG form was examined and compared with the published sequence on material obtained after different purification procedures. The analysis of the protein content of

TABLE 4.3 Lectin binding of dissociated αhCG, ßhCG, and ßC-hCG preparations

Lectin	Specificity	hCG subunits α	hCG subunits ß	ßC-hCG (prepared in this paper)	ßC-hCG (reference)	ßC-hCG Con A-unbound Mono Q-nonreactive
Con A	Trimannosyl core	+	±	+	+	-
LCA	Trimannosyl core 1-6 linked fucose	+	+	+	+	-
UEA-I	1-2 linked fucose	±	±	-	-	-
RCA	Galactose ß1-3 NAc Gal Galactose ß1-4 Glc NAc	NT*		-	-	-
WGA	N-acetylglucosamine	±	-	-	-	-
SBA	α or ß N-acetylgalactosamine	NT		-	-	-
DBA	N-acetylgalactosamine	NT		-	-	-
PNA	Galactose ß1-3 Gal NA_c Galactose ß1-4 Glc NA_c	NT		NT	-	-
EBA	Sialic acid	NT		-	NT	-

* Not tested

\+ Strongly, ± weakly, - no stained

the unique peak eluted on HPLC chromatography (Figure 4.8B) was performed five times and its amino acid composition is shown in Table 4.4, yielding a molecular weight of Mr 10218. This result is closely related to the findings of Birken et al, (1988) and the presence of trace amounts of methionine also suggests some noncovalently bound impurity. The low yield of valine may be due to resistance of Val-Val bonds to hydrolysis. The amino acid composition of the Con A-unbound form was not determined and the possibility that the Con A-bound and Con A-unbound forms differentiate only in the sugar composition deserves further investigation.

4.3.2.6 Amino acid sequence of ßC-hCG

The sequence analysis of the Con A-unbound form was not determined. The major Con A-bound form was sequenced after reduction, S-carboxymethylation, and reverse phase HPLC (see Chapter Two, sections 2.10.2). In addition to the early elution of free labelled-iodoacetic acid three peaks of radioactivity were resolved (Figure 4.12). Each radiolabelled peak was pooled, lyophilized and sequenced. Peak I was similar to that first noted by Birken (*Birken et al, 1988*) but did not give any sequence. Peak II (Table 4.5) was composed of RCM ß-peptide 6-40 of the whole ßhCG subunit but in cycles 14 and 35 glutamic acid and threonine gave low yields. The apparent molecular weight of this peptide is Mr 5000. Peak III (Table 4.6) also was shown to be the RCM ß- peptide 55-92 of ßhCG subunit and has an apparent molecular weight of Mr 5300. These results corroborated those previously published (*Birken et al, 1988*).

4.3.2.7 Biological assay

The ability of ßC-hCG to stimulate ovarian steroidogenesis *in vitro* was tested by incubating human granulosa-lutein cells for 6 days with either hCG (0.5µg/ml Profasi, Serono), ßC-hCG (0.5µg/ml) or vehicle alone.

TABLE 4.4 Amino acid composition of Con A-bound ßC-hCG compared to a reference preparation

Amino acid[a]	ßC-hCG	
	Present study	Reference[b]
Aspartic acid	5.03	5.56 (5)[c]
Glutamic acid	4.37	4.82 (4)
Serine	3.87	2.68 (3)
Glycine	4.70	4.79 (4)
Histidine	0.00	0.30 (0)
Arginine	6.92	6.00 (7)
Threonine	4.82	5.40 (5)
Alanine	6.43	5.54 (6)
Proline	7.17	7.27 (7)
Tyrosine	3.06	2.10 (3)
Valine	7.75	8.86 (10)
Methionine	0.16	0.12 (0)
Half-cystine	0.00[d]	6.20 (9)
Isoleucine	3.71	3.41 (4)
Leucine	3.87	3.93 (4)
Phenylalanine	1.07	1.16 (1)
Lysine	1.07	1.39 (1)

a *Results expressed as residues per mol of peptide*

b *From Birken et al, 1988.*

c *Numbers in parentheses indicate expected values*

d *Not accurately determined*

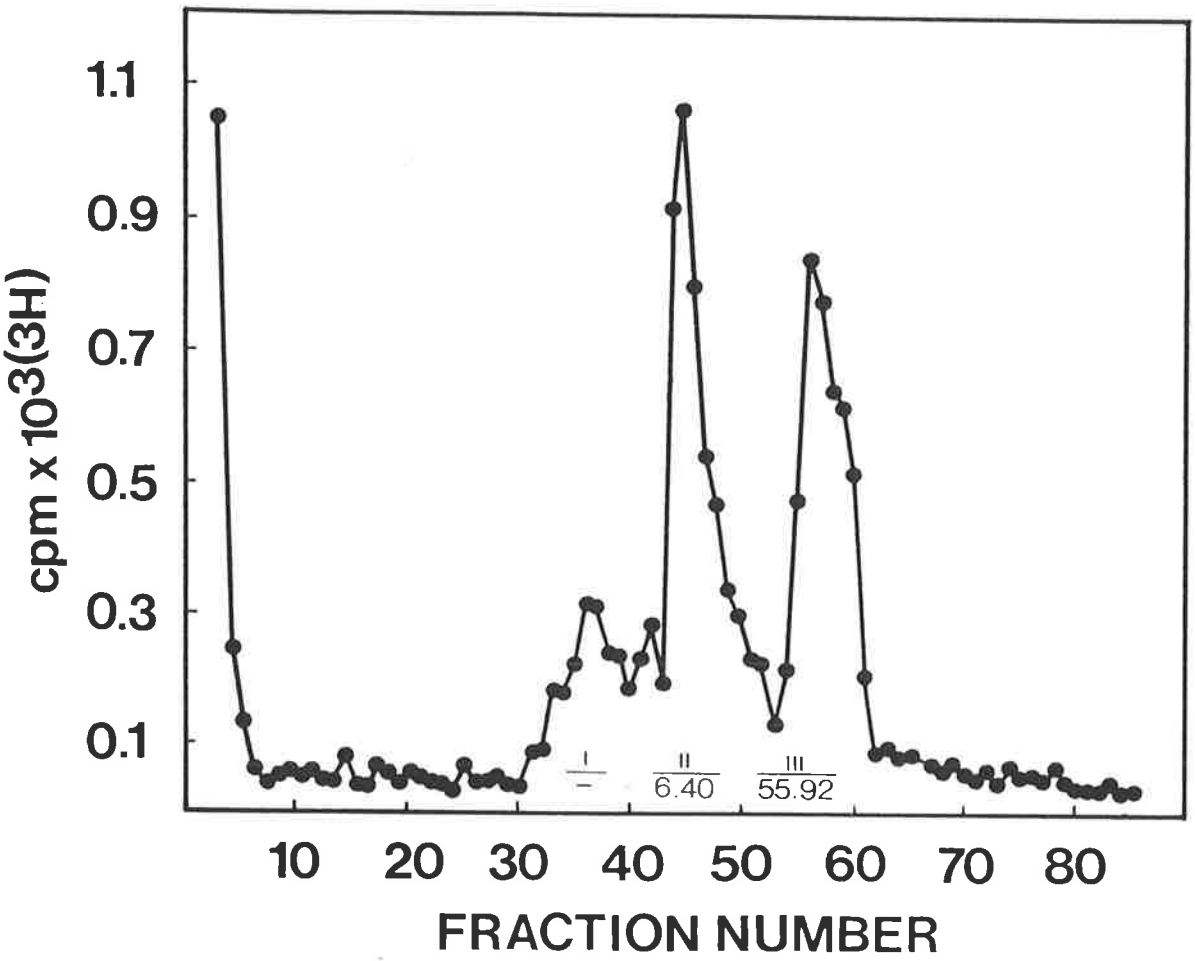


Figure 4.12

HPLC chromatography of reduced and carboxy methylated (RCM) Con A-bound ßC-hCG. Approximately 12nmol of RCM material was loaded onto a Brownlee RP-8 column connected to a Waters HPLC system and eluted with 0.1% trifluoroacetic acid (TFA) during 10 minutes at a flow rate of 0.6ml/minute. Then, a gradient of 0-70% of 0.1% TFA in 80% acetonitrile was developed during 60 minutes and to 100% in the following 10 minutes. Fractions of 0.6ml were collected and 10 microlitres of each fraction were analysed in a scintillation counter to determine the presence of [^{3}H] S-carboxymethyl cystein.

Table 4.5 Sequence analyses of the RCM-polypeptides isolated from the ßC-hCG fragment. Peak II of figure 4.12

Identified amino acid positions	PTH* amino acids recovered											
	Arg	Pro	Arg	Rcm-Cys**	Arg	Pro	Ile	(Asn)	Ala	Thr	Leu	Ala
1 Arg	4.6	1.8	4.6									
2 Pro		29.3	1.0									
3 Arg		11.1	3.9	-								
4 RCM-Cys			1.5	-	0.6							
5 Arg				-	6.5	1.4						
6 Pro					3.2	19.5	1.6					
7 Ile						14.2	20.9	0.4				
8 (Asn)							12.0	CHO***	1.6			
9 Ala									18.8	-		
10 Thr									14.0	4.3	1.1	
11 Leu										2.9	14.8	6.7
12 Ala											10.6	18.8

	Ala	Val	Glu	Lys	Glu	Gly	RCM-Cys**	Pro	Val	RCM-Cys	Ile	Thr	Val	(Asn)	Thr
13 Val	11.3	10.3													
14 Glu		-	-	-***											
15 Lys				10.5	5.9	2.4									
16 Glu					10.9	2.4									
17 Gly					7.0	9.7									
18 RCM-Cys					3.1	8.3	-								
19 Pro								7.7	0.6						
20 Val								7.4	4.3	-					
21 RCM-Cys									5.1	-	1.0				
22 Ile										-	5.3	0.5			
23 Thr											5.2	1.5	1.6		
24 Val												1.9	3.9	-	
25 (Asn)												-	3.4	CHO	1.2

	Thr	Thr	Ile	Rcm-Cys**	Ala	Gly	Tyr	RCM-Cys	Pro	Thr
26 Thr		1.6	-							
27 Thr		2.1	1.2							
28 Ile		1.8	2.3	-						
29 RCM-Cys			2.9	-	1.0					
30 Ala				-	2.4	2.8				
31 Gly					2.8	3.8				
32 Tyr					-	2.7	1.7	-		
33 RCM-Cys							2.1	-	0.8	
34 Pro									1.4	-
35 Thr									1.8	-****

* Phenylthiohydantoin

** Carboxymethylcystein

*** PTH-Asn attached to N-linked carbohydrate was not recovered

**** Glu and Thr gave very low yields at cycles 14 and 35.

Table 4.6 Sequence analyses of the RCM-polypeptides isolated from the ßC-hCG fragment. Peak III of figure 4.12

Identified amino acid positions	PTH* amino acids recovered												
	Val	Val	Rcm-Cys**	Asn	Tyr	Arg	Asp	Val	Arg	Phe	Glu	Ser	Ile
1 Val	153	153											
2 Val		149	-	-									
3 RCM-Cys			-	3.1	-								
4 Asn				83.3	3.3								
5 Tyr				21.6	88.2	1.5							
6 Arg					23.7	16.0	16.0						
7 Asp						6.6	43.5	0.6					
8 Val							16.2	80.3	2.4				
9 Arg								25.8	19.0	0.6			
10 Phe									9.7	82.8	0.7		
11 Glu										29.5	56.1	0.2	
12 Ser											19.2	10.7	1.5

	Ser	Ile	Arg	Leu	Pro	Gly	RCM-Cys	Pro	Arg	Gly	Val	Asn	Pro	Val
13 Ile	3.9	52.0	5.9											
14 Arg		16.0	18.4	1.4										
15 Leu			8.4	52.3	-									
16 Pro					45.8	2.5								
17 Gly					24.1	44.1	-							
18 RCM-Cys						17.4	-	7.8						
19 Pro							-	45.3	1.1					
20 Arg								24.7	14.2	4.7				
21 Gly									8.9	29.9	2.8			
22 Val										17.3	29.8	0.9		
23 Asn											12.4	24.6	3.5	
24 Pro												16.3	26.4	7.6

	Pro	Val	Val	Ser	Tyr	Ala	Val	Ala	Leu	Ser	RCM-Cys	Gln	RCM-Cys	Ala	Leu
25 Val	20.3	23.0	23.0												
26 Val		25.9	25.9	0.4											
27 Ser			21.1	3.4	0.9										
28 Tyr				2.3	13.5	1.6									
29 Ala					9.5	15.0	4.5								
30 Val						12.0	14.0	-							
31 Ala							-	17.8	1.0						
32 Leu								-	11.7						
33 Ser									8.1	1.6	-				
34 RCM-Cys										1.0	-	5.5			
35 Glyn											-	10.0			
36 RCM-Cys												10.8		1.7	
37 Ala														3.8	1.4
38 Leu														3.5	2.1

* Phenylthiohydantoin

** Carboxymethylcystein

The concentrations of progesterone (P) and oestradiol (E_2) in the culture medium replaced on a daily basis were measured by specific assays. As shown in Figure 4.13A, E_2 accumulated in the culture media in response to hCG and was significantly greater in comparison with ßC-hCG or vehicle from day 3 up to day 6 ($P<0.001$ for any day). The concentrations of E_2 achieved with ßC-hCG were not statistically different from those obtained without addition of any hormone. Cells incubated with hCG significantly increased P levels on days 3, 4, 5, and 6 of culture ($P<0.001$ at all times) as compared to those cultured with ßC-hCG or without stimulation (Figure 4.13B).

4.4 Discussion

Essentially this chapter reports the structure, composition, and characteristics of ßC-hCG purified from fresh pregnancy urine specimens and compares it with those proposed by other groups using different protocols. Previous analyses of the carbohydrate composition of ßC-hCG have shown this fragment has two trimmed complex-type biantennary sugar units attached to Asn 13 and Asn 30 but is lacking O-linked sugar moieties present on the carboxy-terminal extension of the whole ßhCG subunit (*Blithe et al, 1988; Birken et al, 1988; Blithe et al, 1989*). These studies agreed that there are two N-linked oligosaccharides, trimmed in their complex antennae with the trimannosyl core Man3NAcGlc 2-linked to asparagine residues on ßC-hCG. However, there is conflicting evidence relating to the extent of carbohydrate trimming. These studies evaluated different preparations obtained by different methods of purification and small differences have already been detected even between batches (*Birken et al, 1988*). In the current study the first step during the purification sequence (Sephacryl S-200) revealed a microheterogeneous population of immunoreactive ßC-hCG with an apparent

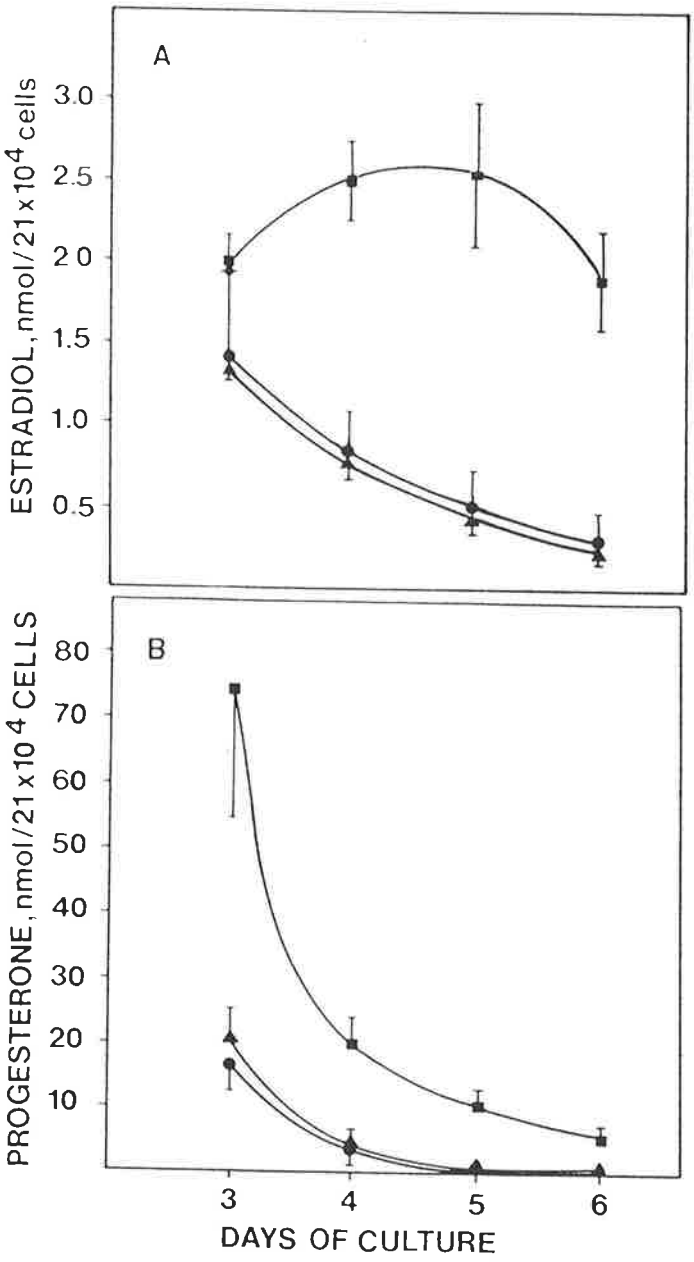


Figure 4.13

A Effect of hCG (■), ßC-hCG (●) or vehicle (▲) on E_2 production by cultured human granulosa lutein cells. Each point represents the mean of four replicates and the vertical line represents the 95% confidence interval. F test: $P<0.001$ for effects of hCG in comparison with ßC-hCG and basal conditions, and $P>0.05$ for effects of ßC-hCG compared to basal conditions.

B Progesterone production of granulosa-lutein cells with vehicle (▲) hCG (■) or ßC-hCG (●). F test: $P<0.001$ for effects of hCG compared to ßC-hCG and basal conditions and $P>0.05$ for effects of ßC-hCG compared to basal conditions.

molecular weight ranging from Mr 7000-19000. These results are similar to those of Blithe et al, (1988) who showed that ßC-hCG eluted from a Sephadex G-100 column in a range of Mr 10000-21000. Evidence that this peak is composed by heterogeneous molecules of immunoreactive ßC-hCG is supported by the differential affinity to bind Con A. Starting from crude preparations of hCG (Organon) or acetone-precipitated pregnancy urine Blithe et al, (1988) reported that 16% and 3% of the ßC-hCG molecules sieved after initial gel filtration were, respectively, incapable of binding to Con A. By using ultrafiltration to concentrate neat samples obtained from several individuals, it was found in the current study that 22% to 44% of the ßC-hCG eluted from Saphacryl S-200 does not have the ability to bind Con A lectin even after a second chromatographic step (Figures 4.4C and 4.4D). Together these studies suggest, as judged by the Con A behaviour, that some differences in the ßC-hCG composition may be due to the source (i.e. different individuals) or methods for preparation of the fresh sample (acetone precipitated or ultrafiltrated urines). Additionally, the discrepancy noted in the sugar composition of the ßC-hCG preparation purified by Birken's group (*Birken et al, 1988*) and that purified by Wehmann's group (*Blithe et al, 1988; Blithe et al, 1989*) reinforces the possibility that the composition of the sieved preparation depend on the methods used for its purification, and emphasizes the heterogeneous nature of ßC-hCG fragment. Taking the sugar content, the findings of this and other studies using different methodology are summarized in Table 4.7.

As ninety eight percent of Con A-bound ßC-hCG molecules (presumably with a trimmamosyl core in their sugar moiety) were unable to bind a strong anion-exchange resin, it was concluded that they are partially trimmed and deprived of negative charges (i.e. sialic acid) in their antennae extension. However, a very small amount (1.5%) of Con A-bound ßC-hCG retains

peripheral sugar residues with negative charges in sufficient quantity to interact with the Mono Q resin (Figure 4.5A). Though it is not possible to compare results with those of Birken et al's study (1988), the Con A-bound ßC-hCG behaviour shown on Mono Q in the current study was similar to that shown by Blithe et al (1988) using DEAE-Sephacel. Thus, nearly 90% of the Con A-bound ßC-hCG did not bind any anion exchange resin. Because 88% of the Con A-unbound ßC-hCG did not bind any lectin specific to N-linked sugar moieties, it is possible that is lacking in any oligosaccharide sequence. Since the remaining 12% of the Con A-unbound (or Con A-weakly bound) ßC-hCG showed further the ability to bind to a strong anion exchange resin (Mono Q), it appears to be well glycosylated. Theoretically, the ßC-hCG molecules weakly bound to Con A may have galactose-ended or galactose-sialic acid-ended antennae (*Baezinger and Fiete, 1979*). Because Birken's group did not use Con A chromatography to purify ßC-hCG and their preparation differed from the Wehmann's preparation by containing molecules with galactose and sialic acid-ended antennae (*Birken et al, 1988; Blithe et al, 1988*), it is quite clear that the sugar moieties of ßC-hCG are heterogenous and the use of Con A in any sequence of purification may predetermine the carbohydrate content in the terminal product. The possibility that, in addition to different sugar composition, the Con A-bound and Con A-unbound ßC-hCG molecules have different immunologic characteristics was also suggested by the low recovery reported by Birken et al (1988) during an immuno-affinity second step used for ßC-hCG purification.

Although an average molecular weight of Mr 17500 has been reported for ßC-hCG (*Wehmann et al, 1989*), during the last step of purification the ßC-hCG prepared in this study showed an apparent molecular weight of

Table 4.7 Carbohydrates detected with different methods on N-linked oligosaccharides of ßC-hCG

Carbohydrate	Methods of Analysis		
	Lectin* (binding ability	Fluorimetric** (molar ratio)	Dionex*** (molar ratio)
Trimmanosyl core	+	5.2	6.2
Fucose	+	ND****	1.5
Galactose	+	2.3	0.2
Glucosamine	-	7.7	3.6
Sialic acid	-	ND	0.2

* Current study and Blithe et al, 1988
** Birken et al, 1988
*** Blithe et al, 1989
**** Non-detected

approximately Mr 12800. Other previous studies (*Birken et al, 1988; Cole and Birken, 1988*) reported a pure preparation with an apparent molecular weight of Mr 12000-15000. After iodination, the same preparation showed a molecular weight of Mr 11200 in a pre-calibrated Sephadex G-75 column. The difference in size may be attributed primarily to the different conditions used to filter this fragment and, secondly, to small differences among the molecules selected by the different methods used. It was demonstrated here that the majority (~88%) of the Con A unbound ßC-hCG has a smaller molecular weight than the Con A-bound, as estimated by SDS-PAGE and gel filtration on Sephadex G-75. The apparent molecular weight of Mr 10000 found for this Con A-unbound ßC-hCG is consistent with the calculated molecular weight of the agalacto ßC-hCG fragment purified by Birken's group (*Birken et al, 1988*).

The observation of a single band on SDS-PAGE without chemical reduction and on HPLC demonstrates the degree of purity of the Con A-bound ßC-hCG preparation and validates the methodology employed in this study. This preparation and that purified by Birken's group used as a reference, exhibit almost the same mobility when evaluated under similar conditions. The apparent molecular weight of Mr 14390-17380 (average 15850) for both preparations seen on SDS-PAGE exceeds the initial estimate of Mr 12000-15000 for the reference preparation but is similar to the ßC-hCG preparation purified by Blithe et al (1988). Under reducing conditions the comparison between both preparations was more difficult: while the fast moving band with approximately Mr 3000-4000 could be easily seen in our preparation, it was not demonstrated in the reference possibly because of the insufficient amount used. However this band was clearly visible in Birken's report (*Birken et al, 1988*). A third band with molecular weight of approximately Mr 6000 shown in Blithe's (*Blithe et al, 1988*) but not in Birken's preparations was stained in our

preparation. Though different protocols have been used for ßC-hCG purification the current methodology is comparable to that of Blithe's group. As this preparation was shown to be similar to that of Blithe et al (1988) and slightly different from that used as reference, some discrepancies among preparations may be due to the different methods utilized. The results obtained in this study and those reported by Blithe et al, (1988) indicate that reduction of the disulfide bonds on ßC-hCG may result in more than two amino acid chains. It is noteworthy that this third band has not been included in the unique ßC-hCG structure so far proposed.

Immunoblots of reduced Con A-bound and Con A-unbound ßC-hCG showed all three bands seen with silver stain demonstrating both forms have similar peptide structure and the same ability to bind the specific antibody. The characteristics of the antibody used here have been extensively studied (see Chapter Five). The result suggests this antibody recognizes several different epitopes on the ßC-hCG molecule. Because the amount of the reference preparation blotted was three times smaller, the bands with an apparent molecular weight of Mr 7500 and Mr 3500 could not be clearly demonstrated in this experiment. Therefore, at present it is not possible to know whether this result denotes insufficient amount of the antigen used or different affinities of the antibody for the two preparations. Because the third band seen with SDS-PAGE after reduction of the current preparation was not consistent with the structure proposed for the ßC-hCG fragment, the analysis of its amino acids and carbohydrates were performed under reducing conditions. Because the clear advantage of direct reaction of the lectins with specific sugar components blotted onto nitrocellulose membrane, the carbohydrates on ßC-hCG were studied by lectin blot. However, this method does not permit any quantitative estimate. The behaviour of both Con A-bound and Con A-unbound ßC-hCG molecules on the blots was consistent

with those shown during their purification. However, the Con A lectin did not stain the Mr 8900 apparent molecular weight band of the reduced Con A-bound ßC-hCG, this observation being consistent with the previously proposed structure in which the large peptide chain (residues 55-92) has no attached sugar moiety (*Birken et al, 1988*). Con A lectin bound strongly to both Mr 7500 and Mr 3500 molecular weight bands, denoting that both have at least the mannosyl core characteristic of N-linked oligosaccharides. This lectin also stained the Mr 3500 band on the preparation used as reference, although this band could not be demonstrated with silver staining. The present result coupled to the fact that a third peak of RCM-ßC-hCG was separated on reverse phase HPLC in this study and by Birken et al, suggests a third glycosylated peptide as part of the amino acid structure of some ßC-hCG molecules.

The Con A-bound ßC-hCG preparation stained less intensively with LCA than with Con A, suggesting that fucose residues may be lacking in some ßC-hCG molecules. This result is consistent with the studies of Blithe et al (1988, 1989) demonstrating by affinity chromatography that only 61% of ßC-hCG molecules were bound to this lectin and, by acid hydrolysis followed by HPLC, that each ßC-hCG molecule contains 1.4 residues of fucose. Interestingly, even though Birken et al (1988) were not able to detect fucose in their preparation using the periodate oxidation method, the present study showed that their preparation bound LCA with nearly the same intensity. Together these studies demonstrate a variable amount of fucose on the Con A-bound ßC-hCG form and suggest this variability may depend on either the source or methods used to purify this fragment or the methods of analysis. Under reducing conditions only the band of intermediate mobility, with apparent molecular weight Mr 7500 on both the Con A-bound ßC-hCG and reference preparation, bound LCA. The faster moving Mr 3500 band, containing the

trimannosyl core as judged by Con A affinity, appeared to be lacking fucose in both preparations examined and may be either a more extensively deglycosylated form of the ß6-40 peptide chain sequenced by Birken et al (1988) or even a copurified small fragment of hCG. Since *Ulex europaeus* lectin has no affinity for the oligosaccharides N-acetyllactosamine-type proposed for ßC-hCG (even if these structures possess fucose residues), the study effected on Con A-unbound and Con A-bound ßC-hCG with this lectin was consistent with the known structure.

Variable amounts of sialic acid, galactose, and N-acetylglucosamine residues were reported to be present on Birken's preparation (*Birken et al, 1988*). The results obtained in the present study by blotting the Con A-bound and Con A-unbound ßC-hCG forms with EBA, RCA, and WGA lectins indicating absence of these monosaccharides were consistent with those of Blithe et al (1989). Interestingly, the ßC-hCG blotted in this study as a reference, did not bind RCA and WGA lectins as well, denoting different sensitivities between methods or likely differences between batches. Moreover, the significant amount of the Con A unbound ßC-hCG form separated from this (and Blithe's) sieved preparation by the anion exchange step could also explain these differences. Both Con A-bound and Con A-unbound forms of ßC-hCG did not bind neither SBA nor DBA, showing them to be lacking in any O-linked oligosaccharides, consistent with the amino acid sequence.

Essentially the affinities of both Con A-bound and Con A-unbound ßC-hCG preparations reported here for the different lectins are consistent with the structure proposed by Blithe's group. Birken's preparation used as standard showed similar behaviour; therefore it can be concluded that, in addition to the intrinsic ßC-hCG heterogeneity, the differences reported by those

investigators were likely due to the different methods they have used. Actually, these differences among preparations could be expected because the Con A affinity-step results in an enrichment of a specific class of ßC-hCG molecules possessing similar carbohydrate determinants recognized by the immobilized lectin. The present report also using Con A as the second step during the ßC-hCG purification sequence revealed a sieved preparation similar to Blithe's preparation. However, approximately 12% of the initially Con A-unbound or Con A-weakly bound ßC-hCG molecules were shown to be able of binding a strong anion exchange resin thereby appearing to preserve peripheral negatively charged monosaccharides, and emphasizing the heterogeneous nature of the carbohydrate branches in ßC-hCG. Considering these studies, a heterogeneous carbohydrate structure for urinary ßC-hCG molecules is suggested (Figure 4.14). However, the ratio of each carbohydrate unit present remains to be determined by direct analysis.

The amino acid analysis of the Con A-bound ßC-hCG preparation gave results comparable to those reported by Birken et al (1988). In addition, there was confirmation that peptides 6-40 and 55-92 of ßhCG subunit are the unique components of the majority of the ßC-hCG molecules (Figure 4.15). While its reduction and carboxy-methylation also yields a third peak after HPLC separation, the present study could not elucidate its nature because it did not give any sequence. As the sequencing procedures did not reveal any contamination, it was apparently difficult to accommodate this result with those shown by the material purified in this study, and that reported by Blithe et al (1988), after electrophoretic separation under reducing conditions. However, the failure in sequencing the third peak could be due to insufficient yield of material. This possibility is supported by several factors. Firstly, SDS-PAGE of the reduced Con A-bound ßC-hCG preparation did not exhibit a

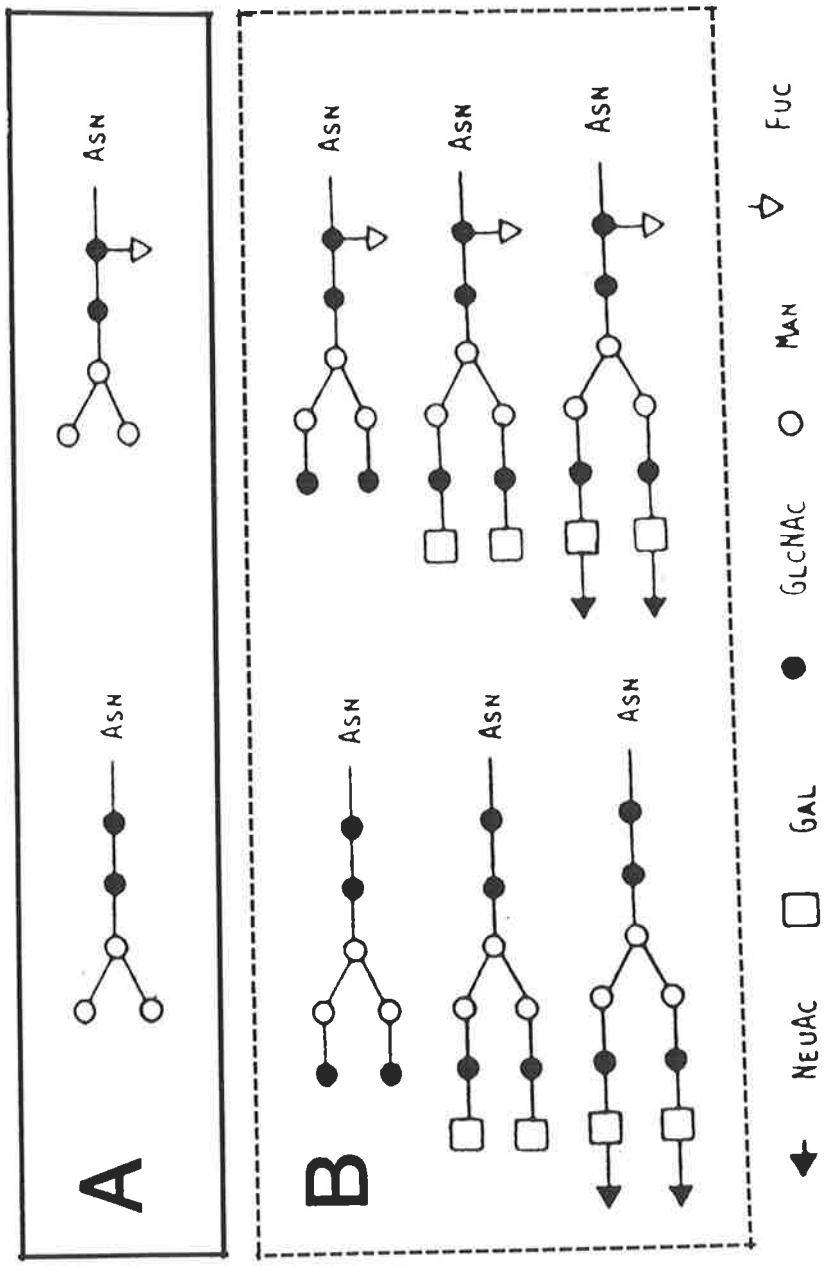


Figure 4.14

Proposed structures of carbohydrates on N-linked moieties of ßC-hCG **A**, Basic branches attached to Concanavalin A-bound form. **B**, Carbohydrate units found on Concanavalin A-weakly bound and Concanavalin A-unbound ßC-hCG forms.

third band with silver stain when small quantities were loaded, but this band was easily immuno- or lectin-stained. Secondly, despite its presence in the original report, the reference preparation used in small amounts did not show this small band as well. Thirdly, a clearly different ratio between the peaks of the two sequenced RCM-peptides and the non sequenced third peak was noted. Fourthly, a different byproduct of free unusual forms of ßhCG molecules, carrying extra peptide residues or alternative amino acid sequences, could explain the existence of a peptide in such small amount as to give no sequence (*Talmadge et al, 1983; Nishimura et al, 1988*). Fifth, the RCM-material that could not be sequenced could be another small fragment of ßhCG co-purified in small amounts. In addition this third peak of RCM-material, presumably the Mr 3500 band seen with SDS-PAGE, could be a peptide unrelated to ßhCG structure. Possibilities for this molecule could be a result of disulfide bridging between the free thiol at cystein 26 and other peptides in a mechanism similar to that proposed to explain the large molecular forms of intact hCG (*Good et al, 1977*). Alternatively, during the removal of the carboxy terminal extension, the free thiol of cystein 26, then exposed on the ßC-hCG surface, could be easily bridged to another free thiol belonging to ßhCG resulting in a homodimer (*Suganuma et al, 1989*). Intracellular disulfide-bonded beta-beta intermediates have also been demonstrated in the presence of free thiol (*Ruddon et al, 1987*). Assuming this possibility this peptide could be predicted to be either as ßhCG 41-55 peptide or, imitating the in vitro tryptic cleavage of ßhCG, the ßhCG 96-114 peptide or other sequence of this portion.

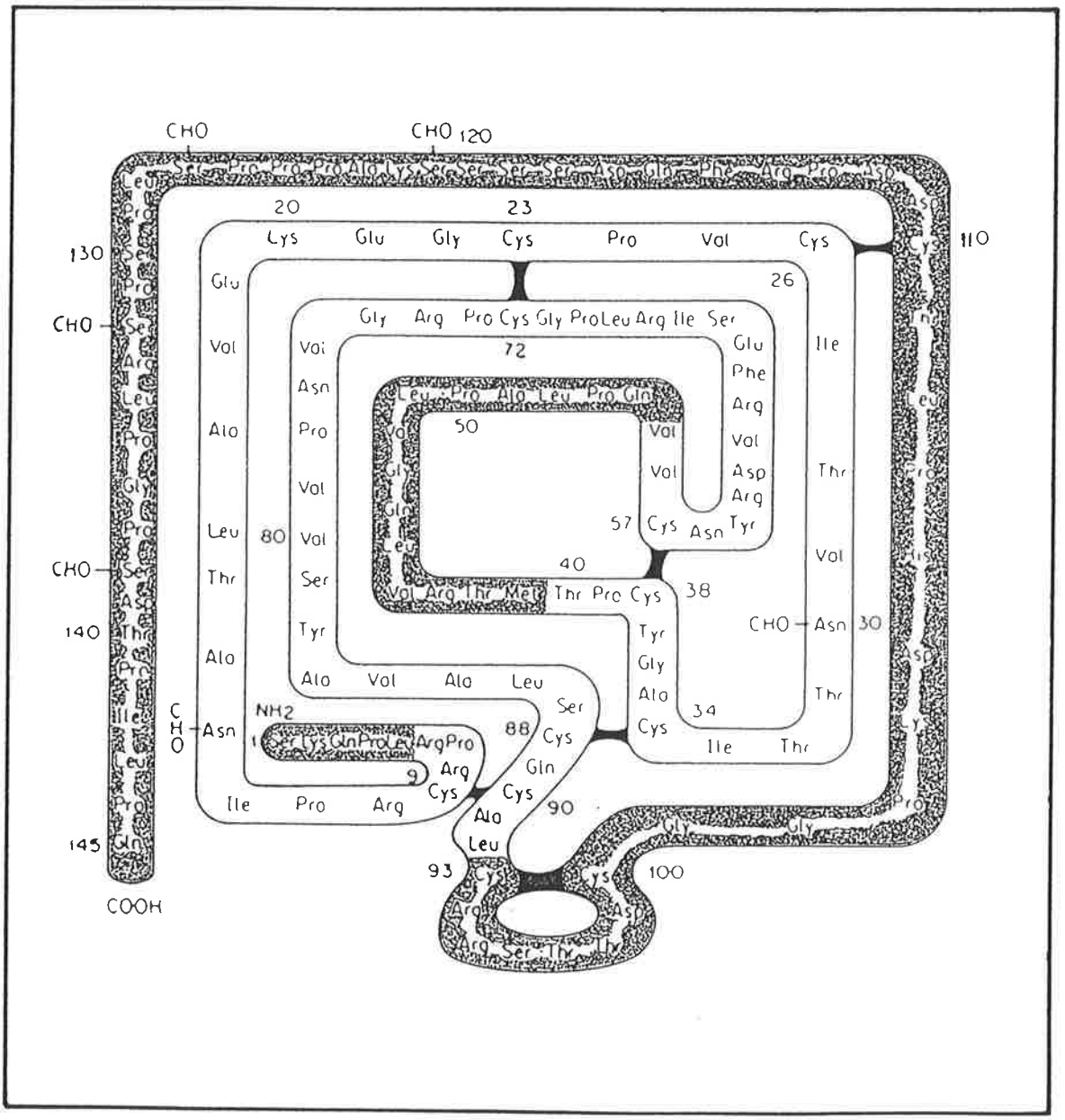


Figure 4.15

Proposed aminoacid sequence of ßC-hCG showing the locations of carbohydrate moieties (CHO) and disulfide bonds. Shaded areas indicate the peptides of ßhCG absent from the fragment.

Additionally, the RCM-material that could not be sequenced could be a small fragment of ßhCG copurified in small amount.

Although deglycosylated hCG interacts with the hCG receptor, the single removal of carbohydrate units results in significant decrease of biological activity in vitro (*Moyle et al, 1975*) and in vivo (*Kalyan et al, 1982; Matzuk et al, 1990*). The dissociated subunits are also devoid of any bioactivity. Early reports have also demonstrated that a small immunoreactive fragment of the ßhCG subunit was not able to bind to hCG receptors (*Masure et al, 1981; Schroeder and Halter, 1983*) or to express any biological activity both in vitro (*Masure et al, 1981; Kato and Braunstein, 1988*) and in vivo bioassays (*Matthies and Diczfaluzy, 1971*). The inability of our ßC-hCG preparation to stimulate lutein-granulosa cells steroidogenesis confirms those studies and indicates it is free of intact hCG contamination.

CHAPTER FIVE

DEVELOPMENT AND VALIDATION OF IMMUNOASSAYS FOR THE ßC-HCG FRAGMENT.

5.1 Introduction

Because unusual forms or fragments of hCG and its subunits may be secreted in a large number of physiological and pathological states, several new diagnostic tools for detection of these unusual molecular forms have been developed (*O'Connor et al, 1988; Krichevsky et al, 1988; Akar et al, 1988; Norman et al, 1990; Lee et al, 1991*). Understanding the physiology and clinical significance of ßC-hCG demands adequate, reliable assays. Despite the development of a few radioimmunoassays and attempts to sandwich this fragment between antibodies able to bind two different epitopes, the majority of the assays so far designed for ßC-hCG detection remain limited by the intrinsic cross-reactivity of the antibodies with the free beta hCG subunit (*Krichevsky et al, 1991*). Studies have demonstrated that ßC-hCG retains at least the beta subunit conformational immunological determinant of hCG/ßhCG molecules. Because of this cross-reactivity, a valid interpretation of ßC-hCG results is difficult without prior chromatogrpahic separation. Bearing in mind these difficulties, the aims of this study were (1) to produce a specific polyclonal antibody using a pure ßC-hCG preparation as immunogen, (2) to develop specific radioimmunoassays or immunoradiometric assays to detect this fragment, (3) to validate these new assays in several biological fluids, (4) to compare them to current assay systems employed for measurement of ßC-hCG, and (5) to establish a reference range for non-pregnant individuals.

5.2 Methods

5.2.1 ßC-hCG Antibody Production

Urinary ßC-hCG fragment used as immunogen was purified from pregnancy urine as described in chapter four. This highly pure preparation (ßC-hCG reference II) was calibrated for radioimmunoassay against the ßC-hCG preparation purified by Dr. Steven Birken (ßC-hCG reference I), so that 1µg reference II is equal to 1µg reference I. ßC-hCG reference II was diluted in saline and thoroughly mixed, at 1:2 proportion, with complete Freund's adjuvant and used to immunize five New Zealand white female rabbits by subcutaneous multiple site injection as recommended by Vaitukaitis et al, (1971). Fifty micrograms of ßC-hCG with complete adjuvant was injected into each rabbit, and the animals were boosted every 4 weeks with 25-30µg immunogen mixed with incomplete Freund's adjuvant. Two weeks after primary immunization the rabbits were bled fortnightly and antibody titres determined by testing serial dilutions of each serum for their ability to bind highly purified radiolabelled ßC-hCG, ßhCG and hCG. The antibodies were characterized by saturation and competition binding radioimmunoassays. The resultant dose response curves were analysed by Scatchard and Hill plots (*Scatchard, 1949; Hill, 1910*) as in appendix XI.

5.2.2 Immunoassays

5.2.2.1 ßC-hCG Immunoassays

5.2.2.1.1 ßC-hCG Radioimmunoassays

ßC-hCG radioimmunoassay II (RIA-II) was performed with the RW37 polyclonal antibody (*Wehmann et al, 1988*). This assay was performed as described by Akar et al (1988) with a few small modifications already presented in Chapter two (section 2.11.3.2). ßC-hCG radioimmunoassay I (RIA-I) is presented in the current study and will be detailed in section 5.3.2.1.

5.2.2.1.2 ßC-hCG Immunoradiometric assays

The two-site immunoradiometric assay II (IRMA-II) for ßC-hCG detection employing the antisera 2/6 and 32H2 has been described previously (De Medeiros et al, 1991) and the major procedures and characteristics are given in detail in Chapter two (section 2.11.4.4). ßC-hCG immunoradiometric assay I (IRMA-I) is also presented in this study and is further described (section 5.3.2.2).

5.2.2.2 Intact hCG and Free Beta Subunit Assays

Intact dimeric hCG was measured with specific immunoradiometric assay using the combination of 3/6 monoclonal antibody as solid phase and 11/6 as tracer. The detailed procedure is found in Chapter two (section 2.11.4.1). Free beta hCG subunit was estimated by using a free ßhCG subunit IRMA provided by Bioclone Australia Pty (Chapter two, section 2.11.4.2).

5.2.3 Gel Chromatography

The chromatographic procedures during the validation of the new assays were performed on Sephacryl S-200 superfine 463ml bed volume column described in Chapter two, section 2.5.1.2.

5.2.4 Collection and Processing of Samples

Early-morning urine samples from 47 healthy pregnant women between 20-40 weeks gestation were collected at the antenatal clinic of the Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital, and sent immediately to the laboratory. No preservatives were used as ß core hCG is highly stable (*De Medeiros et al, 1991*). In order to establish the reference ranges for non-pregnant subjects with the ßC-hCG IRMA-I developed in this study, early morning urine samples from 238 apparently healthy individuals,

aged 12-88 years, were collected. To compare the performances of the new ßC-hCG RIA (ßC-hCG RIA-I) with the IRMA-I, 30 non-pregnant subjects, aged 20-54 years, gave their first voided urine samples. All samples were initially centrifuged at 1850 x g for 10 minutes to remove debris and stored at -20^{o}C until assay. To validate the new ßC-hCG assays two hundred millilitres of urine from a healthy first trimester pregnant woman were also collected, centrifuged, and concentrated by pressure ultrafiltration in an Amicon unit with a YM-2 membrane, 2000MW cut-off (Amicon, Lexington, MA, USA) to a final volume of 8ml. This concentrate was chromatographed on Sephacryl S-200 and the eluted fractions were analysed for intact hCG, ßC-hCG, and ßhCG.

In order to evaluate the effect of different matrices on ßC-hCG detection, a healthy male volunteer provided serum and plasma samples. These were processed and stored at -20^{o}C until use. Follicular fluid was obtained from patients undergoing in vitro fertilization for infertility at the Reproductive Medicine Unit, The Queen Elizabeth Hospital, and stored in a frozen state until use. hCG (5000IU, Profasi, Serono) had been given to induce ovulation 36 hours earlier. All patients gave consent for this study which was approved by the Ethical Committees of the University of Adelaide and The Queen Elizabeth Hospital.

5.2.5 Analysis of Data

Statistical analysis of the radio- and immunoradiometric assays were performed as described by Rodbard (1974). Dose response curves and potency estimate of the RIA's were analysed by logit-log and 4-parameter logistic. IRMAs responses were initially determined by a weighed linear regression according to the Volund's procedure (*Volund, 1978*) and further fitted by the 4-parameter logistic model. In addition to the competition binding experiments, cross-reactivities were also estimated by measuring the total

fitted by the 4-parameter logistic model. In addition to the competition binding experiments, cross-reactivities were also estimated by measuring the total ßC-hCG immunoreactivity in the fractions eluted from Sephacryl S-200 containing the native hCG and ßhCG molecules. The molar proportion between the total immunoreactivity obtained with ßC-hCG assay and that obtained with the homologous assays at each peak was calculated by the formulas: **ßC-hCG/hCG x 100** and **ßC-hCG/ßhCG x 100**. In order to calculate the molar ratios, the molecular weights for intact hCG, ßhCG, and ßC-hCG used were 38000, 22200, and 10000, respectively. Correlation/agreement between the different ßC-hCG assays was performed using a computerized linear regression analysis estimating the difference between any two assays against their mean (Bland and Altman, 1986). The comparison between RIA I and IRMA I was performed using student's t-test for unpaired samples, applied at the 5% level of significance. Also applied at 5% level of significance, the age effect on ßC-hCG and trend for a rising with age were analysed using two-sided student's t-test and chi-square test. The reference range for ßC-hCG concentrations in non-pregnant individuals was determined as 10 and 90th centiles of the ranked positive values.

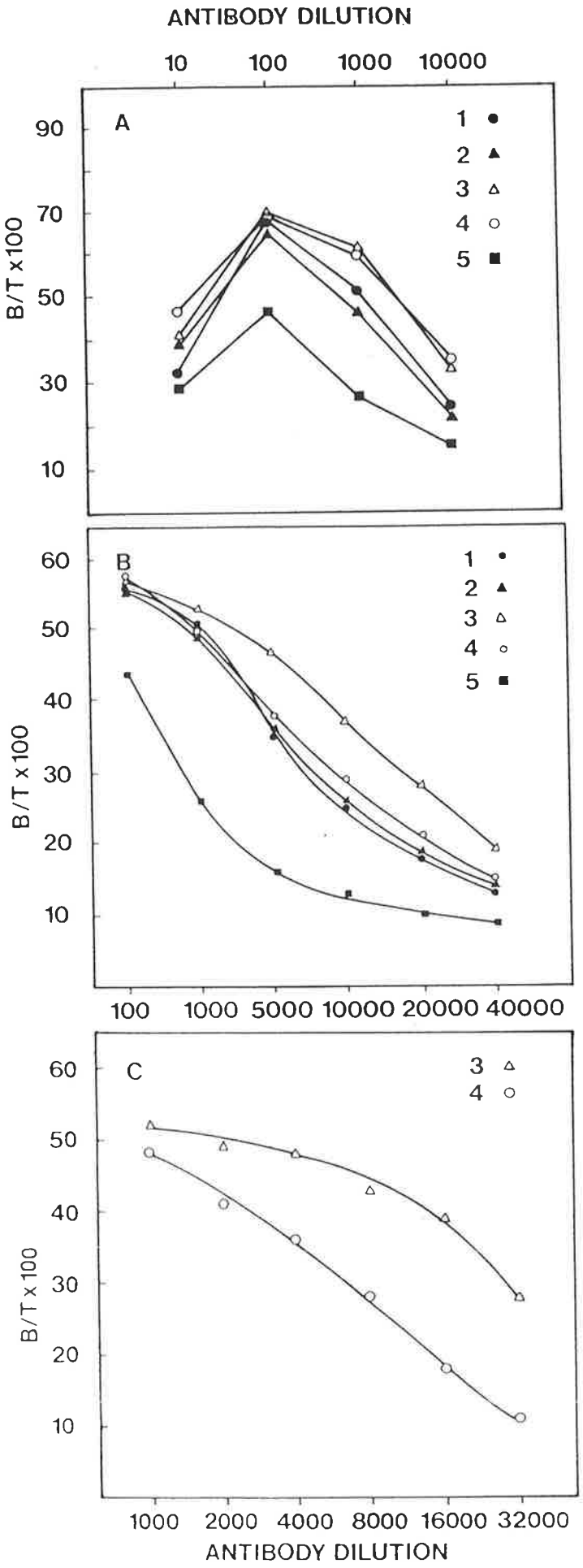
5.3 Results

5.3.1 ßC-hCG antibody production

All rabbits produced antibodies to the injected antigen. Tests performed at 75, 95, and 120 days after primary immunization showed that sera from animals DeM3 and DeM4 had consistently higher titres and binding to an iodinated preparation of ßC-hCG (Figure 5.1). These preliminary evaluations also revealed that both antisera had nearly the same capacity to differentiate ßC-hCG from intact hCG and alpha and beta subunits. Because 25-35% binding of [125I] ßC-hCG resulted from an initial dilution of 1:20000 of DeM3 antiserum and 1:10000 dilution of DeM4 antiserum, obtained 95 days after the primary immunization, the former antibody was used for further studies. Saturation binding, examined by adding increasing amounts of [125I]

Figure 5.1

ßC-hCG-binding data using different antisera against a pure preparation of ßC-hCG antigen. Curves DeM1, DeM2, DeM3 DeM4, and DeM5 denote the code of each animal. One hundred microlitres containing a fixed amount of [^{125}I]-ßC-hCG (20000cpm), 0.1ml of each antisera at different dilutions and 0.1ml of 0.05M phosphate buffer (containing 0.5% BSA, 0.02% NaN3, pH7.4) were incubated for 18h at 4^{o}C; then, 0.1ml of goat anti-rabbit IgG (1.25 units) was added, mixed, and the tubes incubated for 6h. After separation of bound and free hormone by centrifugation, the pellets were counted in a gamma counter for 60 seconds. Data obtained 75 days (**A**), 95 days (**B**), and 120 days (**C**) after the primary immunization.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ßC-hCG into a constant concentration of antibody, achieved a maximum binding with 6.18 x 10-11mol/l of ßC-hCG with an affinity constant (Kd) of 0.83 x 10^{10}l/mol (Figure 5.2A, insert). The mean affinity constant of this antiserum, assessed by Scatchard's analysis with measurement of its ability to bind a fixed amount of [125I]-ßC-hCG in the presence of increasing concentration of unlabelled ßC-hCG, was estimated in 1.21 x 10^{10}l/mol (Figure 5.2A). The total number of binding sites (R) was 6.38 x 10^{-11}mol/l and the concentration of antibodies in the neat antiserum was estimated in 6.3 x 10^{-7}mol/l (102µg/ml). The mass of DeM3 anti-ßC-hCG antibody was estimated assuming that the antibody has a molecular weight of Mr 160000 and valence of 2, the antigen has a molecular weight of Mr 10000, the dilution factor (initial dilution) of 1:20000, and the number of antibody sites as 6.38 x 10^{-11}mol/l (*Abraham, 1975; Rodbard, 1989*). The Hill model used to test for classes of antibodies with different affinities for ßC-hCG, evaluated in the range between 2.7pmol/l and 1400pmol/l, gave a coefficient nH of 1.1, indicating the existence of slight positive co-operativity among interacting binding sites (Figure 5.2B). Additional characteristics of this antiserum were evaluated by a competitive radioimmunoassay described in the following section.

5.3.2. Immunoassays for ßC-hCG

5.3.2.1 Radioimmunoassays

Radioimmunoassay I (RIA-I) for ßC-hCG molecules was performed with polyclonal antibody produced by the rabbit DeM3 and obtained 95 days after primary immunization. This antibody was used at a final dilution of 1:80000 which gave a 30% maximum binding of labelled ligand. ßC-hCG reference II (preparation characterized in Chapter four) was used as the standard and iodinated by the Iodogen method (*Campbell and Johnson, 1978*)

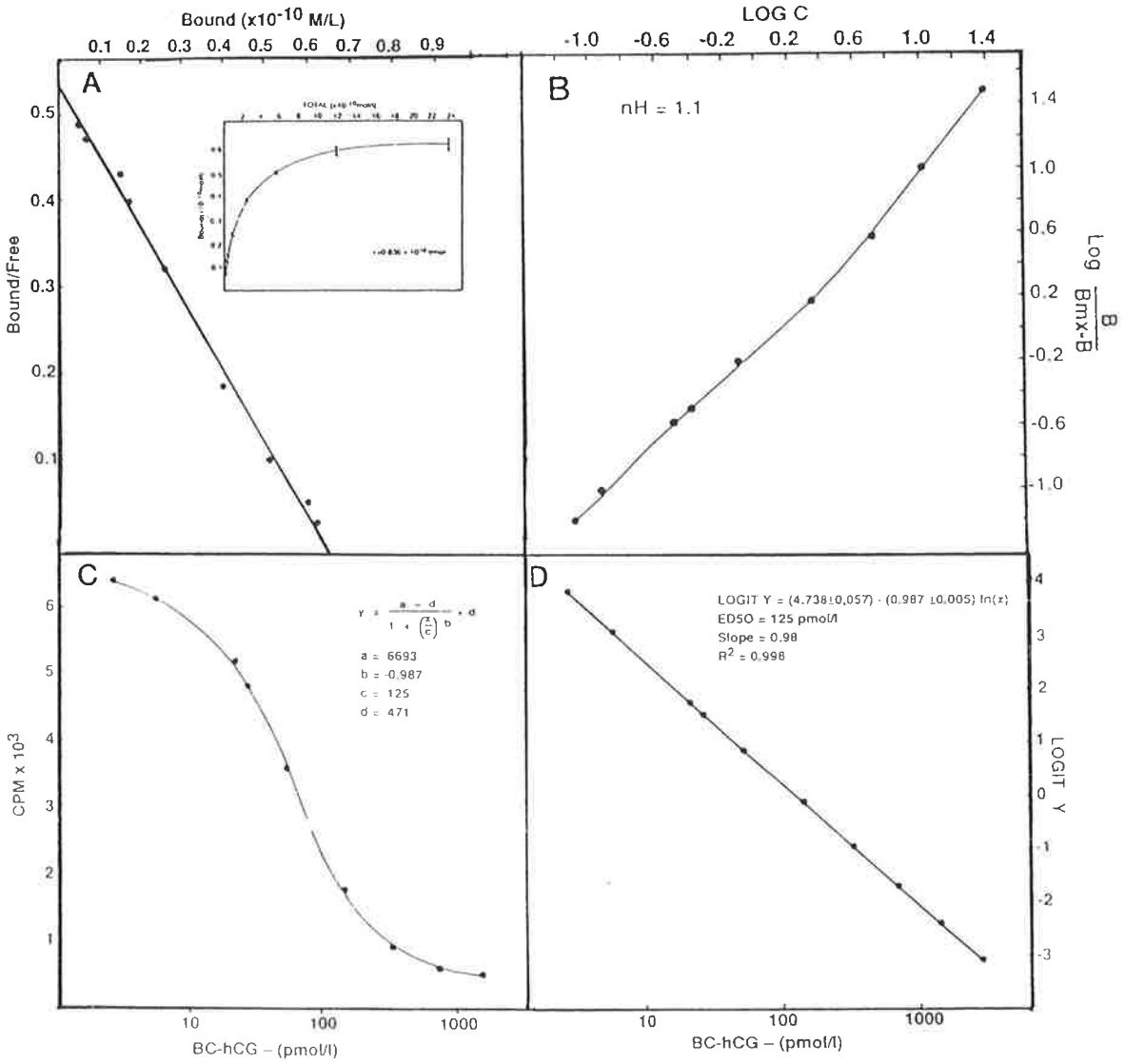


Figure 5.2

(A) Scatchard plot of ßC-hCG competition binding data, corrected for non-specific binding, using DeM3 polyclonal antiserum. Each point is the mean of two replicates at each dose level and the fitted straight line minimizes the sum squared residuals. Ordinate: ratio of bound (B) and free (F) fractions. Abscissa: specific amount bound. The total number of antibody sites (R), x intercept, is 0.638×10^{-10}mol/l; the slope -K is -0.823, and the y intercept (K.R) is 0.525. Insert, saturation binding curve for this antiserum with [125I] ßC-hCG; maximum binding (Bmx) 0.618×10^{-10}mol/l; Kd=0.836×10^{10}l/mol.

(B) Hill plot of the competitive binding assay. Bmx of 0.638×10^{-10}l/mol was estimated by Scatchard analysis. Ordinate: log B/Bmx-B; abscissa: log C, where C is the free fraction. The slope, nH, is 1.1.

(C) Typical dose-response curve for RIA of ßC-hCG; the data from A were fitted by four-parameter logistic. Parameters a, b, c, and d are the expected response at zero dose of analyte, slope, ED_{50} and the upper asymptote observed response when a concentration of analyte 100-fold higher than ED_{50} was used, respectively. Ordinate: transformed y response (cpm); abscissa: dose (pmol/l) in log scale.

(D) Logit-log transformed data of A. Ordinate: logit y; abscissa: dose in pmol/l. The equation for the line is $y = 4.738 \pm 0.05 - 0.987 \pm 0.05$ lnx, where lnx is the inverse of x.

with resultant specific activity of 189uCi/µg (section 2.11.2.2). One hundred microlitres of standard or sample were incubated with 0.1ml of the diluted antiserum and 0.1ml of assay buffer (phosphate buffer, 0.05M containing 0.5% BSA, pH 7.4) for 2h at 4°C. After this period of incubation 0.1ml of [^{125}I] ßC-hCG (20000cpm) was added, and incubated for 18-20h at 4°C. One hundred microlitres of goat anti-rabbit IgG (GARG, 1.25 units) were added and the mixture incubated for an additional 6h, at 4°C. After addition of 0.5ml 5% polyethylene glycol 6000, and incubation for twenty minutes, free and bound ligand were separated by centrifugation at 3290 x g, at 4°C, for 15 minutes. Following aspiration of the supernatant the pellet was counted for radioactivity for 60 seconds using an automated gamma counter system (LKB Wallac 1260 Multigamma II counter). The results were expressed in pmol/l of ßC-hCG reference material II. The dose response curves of this assay (ßC-hCG RIA-I) were analysed by the four parameter logistic as shown in Figure 5.2C. An iteratively weighted linear regression of the logit-log plot of the standards gave a negative slope of -0.987 (Figure 5.2D). The non-specific binding (NSB), estimated by adding an amount of standard 100-fold higher than the ED50, was 2.3% of the total counts added. The least detectable dose calculated as the amount of unlabelled antigen which resulted in a response two standard deviation higher than that of the zero standard evaluated by a one sided student's t test was 5pmol/l. The ED85, ED50 and ED15 were 28pmol/l, 125pmol/l and 650pmol/l, respectively, and the working range for any clinical application was considered between ED85 and ED15. Specific tests performed with intact hCG, ßhCG αhCG, LH/FSH (LER 907), LH (68/40) and ßLH (AFP3282B) showed a cross-reactivity, on a molar basis, measured at 50% inhibition levels of 3.8%, 10.5%, 0.03%, 2.0%, 7.0% and 48% respectively (Figure 5.3A). The high cross-reactivity of this antiserum with the free ßLH subunit indicates the strong similarity between the primary structure of these glycoproteins. Although the Scatchard plot was linear up to

1400pmol/l, some deviation from parallel competition for hCG and ßhCG may be a result of either non-identity in the binding sites, over-estimation of non-specific binding or failure to reach equilibrium at low ligand concentrations. The departure from linearity of LH and ßLH subunit at levels higher than 500pmol/l also denotes the presence of a second class of antibodies. The clinical significance of hCG and ßhCG subunit cross-reactivities in this radioimmunoassay was evaluated by chromatography of 200ml of urine (concentrated to 8ml) from a healthy pregnant women on a Sephacryl S-200 column and measurement of ßC-hCG, ßhCG and intact hCG immunoreactivities in the fractions with specific assays. This procedure (shown in Figures 5.3B and 5.3C) revealed that the true cross-reactivities of the ßC-hCG RIA-I with intact hCG and ßhCG, on a molar basis, are 0.5% and 5.6% respectively. In addition, under the conditions reported here, the ßC-hCG RIA-II showed similar cross-reactions with hCG (0.8%) and ßhCG (5.4%). Figure 5.3C compares the ßC-hCG immunoreactivity in the fractions employing both radioimmunoassays. Because it is unlikely that hCG and ßhCG cross-reactivities are significant at physiological concentrations, it was concluded that for most clinical purposes, this assay system can be used without previous chromatography of the samples.

The influence of different matrices on the assay performance was tested for urine, serum, plasma, and follicular fluid by adding increased amounts of pure ßC-hCG (reference II) in aliquots of these biological fluids before assay. Table 5.1 sums up the recovery along the working range of the assay, indicating moderate additional effect with serum, plasma and follicular fluid when the levels are below 30pmol/l. Urine itself did not show any effect on ßC-hCG estimation. The highest recovery seen when plasma and follicular

Table 5.1 Recovery of purified ßC-hCG from different matrices with ßC-hCG RIA-I

ßC-hCG Added (pmol/l)	URINE		SERUM		PLASMA		FOLLICULAR FLUID*	
	Mean Rec (pmol/l)	%	Mean Rec (pmol/l)	%	Mean Rec (pmol/l)	%	Mean Rec (pmol/l)	%
3	2.8	93.0	3.9	130.0	3.8	126.6	4.4	146.6
6	5.7	95.0	10.1	168.3	10.1	168.3	11.4	190.0
15	14.9	99.3	18.9	126.0	18.5	123.3	22.3	148.6
30	30.1	100.3	37.8	126.0	32.5	108.3	43.1	143.6
60	59.9	99.8	68.6	114.3	54.0	90.0	83.4	139.0
150	149.8	99.8	146.4	97.6	115.6	77.0	166.0	110.6
299	299.8	100.2	267.9	89.6	195.5	65.4	307.9	102.9
598	600.9	100.4	509.5	85.2	495.7	82.9	549.5	91.9
1192	1197.9	100.5	1034.6	86.8	948.8	79.6	1019.1	85.5
2392	2394.3	100.1	2430.2	101.5	2270.0	94.9	2365.6	98.9

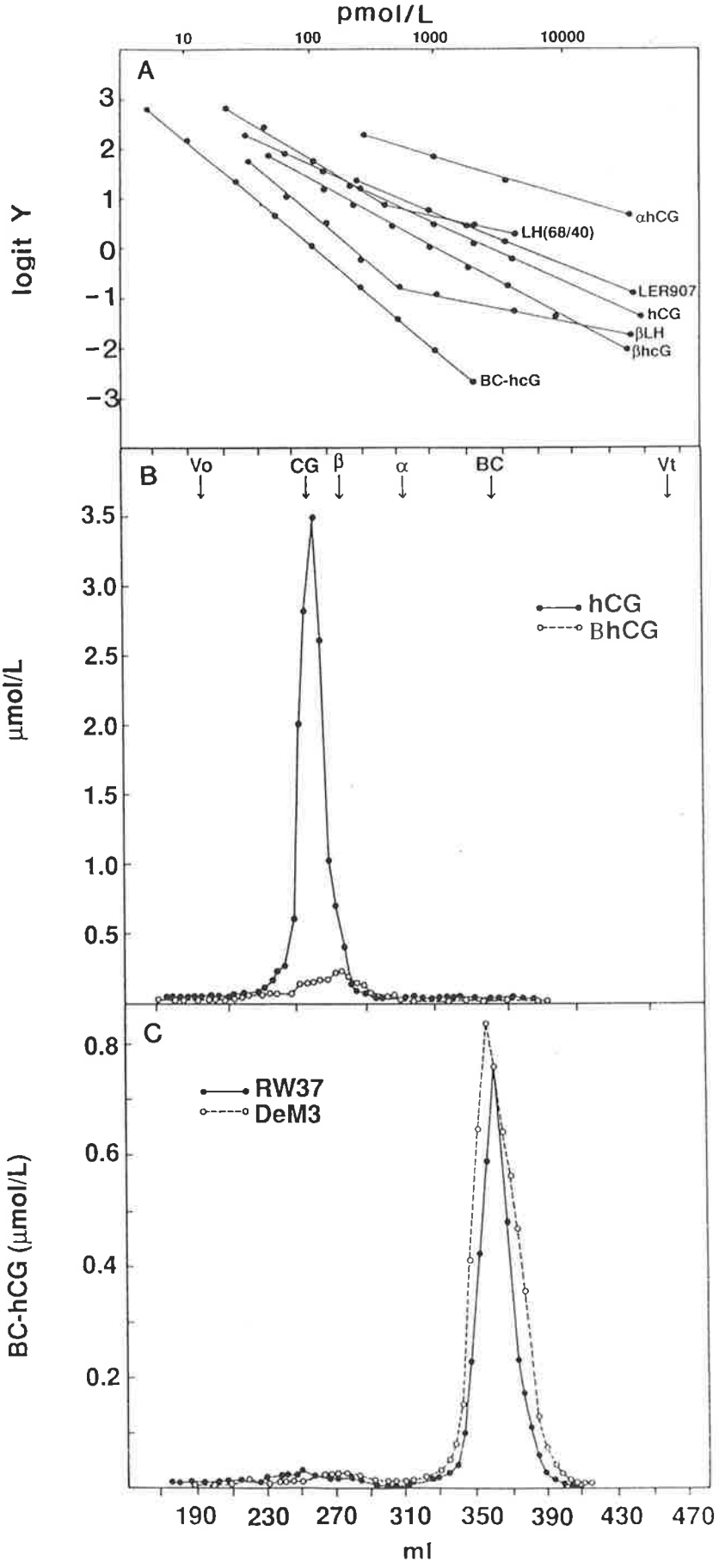
* **Sample, originally containing 7.4 pmol/l ßC-hCG, collected 36h after injection of 5000 IU of hCG (Profasi, Serono) intramuscularly. Results were corrected by subtracting the basal concentration from the observed response.**

Figure 5.3.

(A) Logit-log plot of inhibition binding data of hCG (CR 125), ßhCG (CR 125), αhCG (CR 119), LH (68/40), ßLH (AFP-3282B), and LH/FSH (LER 907) in the ßC-hCG RIA I. The cross-reactivities, on a molar basis were estimated at 50% inhibition levels. Ordinate: logit y; abscissa: dose in pmol/l.

(B) Gel chromatography of a concentrate of 200ml urine obtained from a first trimester pregnant woman. Eight millilitres concentrate-sample was applied to a 2.6 x 87.5cm column of Sephacryl-S-200 and eluted at room temperature with 0.2M ammonium acetate buffer, pH6.8, at a flow rate of 50ml/h. Fractions of 4.2ml were collected and assayed for hCG (●—●) and ßhCG (○—○). The arrows indicate elution positions of internal markers: Vo, void volume; Vt total bed volume; ßC, [^{125}I]=ßC-hCG; CG, α and ß are hCG ßhCG and αhCG respectively.

(C) Elution profile of ßC-hCG determined in the same fractions with RW37-RIA (●—●) and DeM3-RIA (○—○).


pmol/L
10
100
1000
10000
A
logit Y
3
2
1
0
−1
−2
−3
αhCG
LH(68/40)
LER907
hCG
βLH
βhcG
BC-hcG
B
Vo
CG
β
α
BC
Vt
μmol/L
3.5
3.0
2.5
2.0
1.5
1.0
0.5
hCG
BhCG
C
BC-hCG (μmol/L)
0.8
0.6
0.4
0.2
RW37
DeM3
190
230
270
310
350
390
430
470
ml

fluid were used as matrices denotes the existence of some nonspecific protein binding. In addition, dilution of either pregnant and non-pregnant urine samples in the assay buffer demonstrated parallelism with the reference preparation (Figure 5.4). The intra-assay variability estimated along the working range (ED15-ED85) of the assay ranged between 2.8% and 7.3% and the inter-assay variation ranged from 2.3% to 10.0% (Table 5.2).

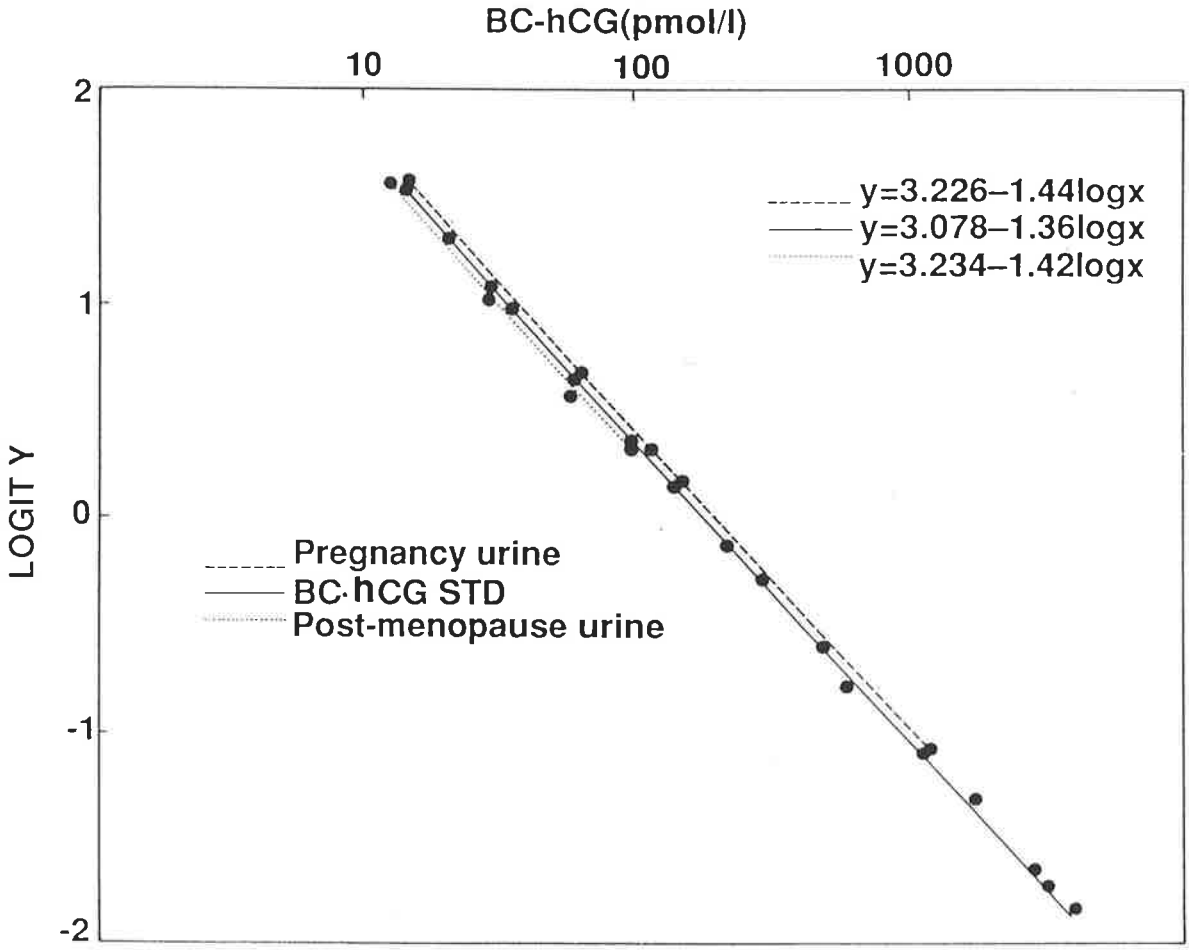


Figure 5.4

Logit-log fitted binding data of ßC-hCG standards (●—●), and native ßC-hCG from pregnant (●---●) and non-pregnant (●····●) women diluted in assay phosphate-buffer. Ordinate: logit y; abscissa: concentration of ßC-hCG (pmol/l). Equation for each line is inserted.

Table 5.2 **Evaluation of the precision of ßC-hCG RIA-I and ßC-hCG IRMA I**

Radioimmunoassay I*						Immunoradiometric assay I**					
Intra-assay			Inter-assay			Intra-assay			Inter-assay		
mean level (pmol/l)	SD	CV%	QC (pmol/l)	SD	CV(%)	mean level (pmol/l)	SD	CV%	QC (pmol/l)	SD	CV%
4.6	0.6	13.8	27.0	1.1	4.0	3.2	0.3	9.3	21.6	1.4	6.4
4.7	0.3	7.3	167.0	10.0	6.2	23.9	1.4	5.8	126.6	5.0	3.9
52.1	2.8	5.3	316.0	7.0	2.3	29.2	1.6	5.4	267.2	13.8	5.1
320.0	9.0	2.8	614.0	61.0	10.0	56.6	2.7	4.7	569.5	33.1	5.8
676.0	23.0	3.4				144.2	5.8	4.0	1349.0	127.0	9.4
2105.0	227.0	10.7				318.1	9.1	2.8			
3142.0	559.0	17.7				732.3	34.0	4.6			
						1506.1	125.7	8.3			
						2159.3	372.6	17.2			

* Antibody DeM3, n=10

** Antibodies DeM3 and 32H2, n=15

The characteristics of the polyclonal antibody RW37 as well as all procedures to validate the radioimmunoassay specific to ßC-hCG employing this antiserum have been described by Wehmann's group (*Akar et al, 1988; Wehmann et al, 1989*). For comparison under the same conditions the mean affinity of this antibody, evaluated by Scatchard analysis, for the ßC-hCG reference II (purified in this study and characterized in Chapter Four) was estimated in 2.0 x 10^{10}l/mol, the total number of sites (R) was 5.63 x 10^{-11}mol/l, and the Hill coefficient (nH) was 0.86. It is concluded that RW37 and DeM3 antisera have similar mean affinities for the standard ßC-hCG preparation (2.0 x 10^{10}l/mol vs 1.2 x 10^{10}l/mol, respectively). However the Hill coefficients indicate that both antibodies contain a second class of antibody sites, resulting either in slight positive co-operatively (DeM3) or negative co-operativity (RW37).

5.3.2.2 Immunoradiometric assay

ßC-hCG immunoradiometric assay I (IRMA-I) was designed in a liquid phase system by using 32H2 monoclonal antibody and the DeM3 polyclonal antiserum at 1:5000 initial dilution. Purified ßC-hCG reference II, was employed as a standard. [^{125}I] 32H2 antibody was labelled by the chloramine T method with a specific activity of approximately 18uCi/µg (section 2.11.1.1). Equal volumes (0.1ml) of standards or samples, [^{125}I] 32H2, DeM3 antibody, and assay buffer (0.05M phosphate, 0.05% BSA, pH 7.4) were mixed and incubated at room temperature for 3h. Magnetic donkey anti-rabbit Amerlex-M (0.5ml) was added, mixed, and the mixture was incubated at room temperature for an additional 15 minutes. The tubes were centrifuged at 3290 x g for 5 minutes, placed on a magnetic rack and the supernatant decanted. After washing with assay buffer containing 0.1% Tween 20, centrifuging and decanting as above, the pellets were counted in the LKB multigamma counter for 60 seconds. Precautions against high-dose

hooking were taken by diluting each sample 10, 100 and 1000 times before assaying, and the results were taken from the dilution that fitted the best point of the standard curve (28 to 650pmol/l).

A typical dose response curve for ßC-hCG binding fitted by the 4-parameter logistic is shown in Figure 5.5A. Initial estimates to fit the logistic model used Volund's method: after excluding the extremes, transforming the response to logit, and determination of weights, they were determined by weighted linear regression (r=0.967, Figure 5.5B). ßC-hCG bound up to a maximum of 27% of the total counts and gave a greatest signal noise at concentrations of 1400pmol/l. Binding equivalents to 5% (ED5), 50% (ED50), and 95% (ED95) of the maximum binding were obtained with 5pmol/l, 150pmol/l, and 1500pmol/l of pure ßC-hCG, and ED5 and ED95 levels were established as the limits for the working range of the standard curve. The non specific binding (NSB) was 2.6% of the total counts added. The least detectable dose, defined as the ßC-hCG concentration bound that was 2SD higher than the mean of the binding in 10 replicates containing only assay buffer (zero dose tubes), determined by one tail student's t test, was 1.5pmol/l. The concentrations of related glycoproteins capable of inducing a signal noise of 10% in this assay were 48pmol/l, 260pmol/l, 330pmol/l, 1100pmol/l, 75pmol/l, and 260000pmol/l for ßC-hCG, ßhCG, hCG LH(LER 907), αhCG and ßLH, respectively (Figure 5. 6A). Excess of hCG and ßhCG cross-reactants resulted in a loss of signal, reflecting insufficient amount of residual non-specific antibody to maintain the bound fraction. The ßhCG subunit showed increasing signal up to 2200pmol/l, corresponding to a maximum binding of 16%. The intact hCG gave increasing signal noise up to 2600pmol/l, a maximum binding of 6%. On a molar basis, and based on the amount of cross-reactant necessary to induce a signal of 10% the cross-

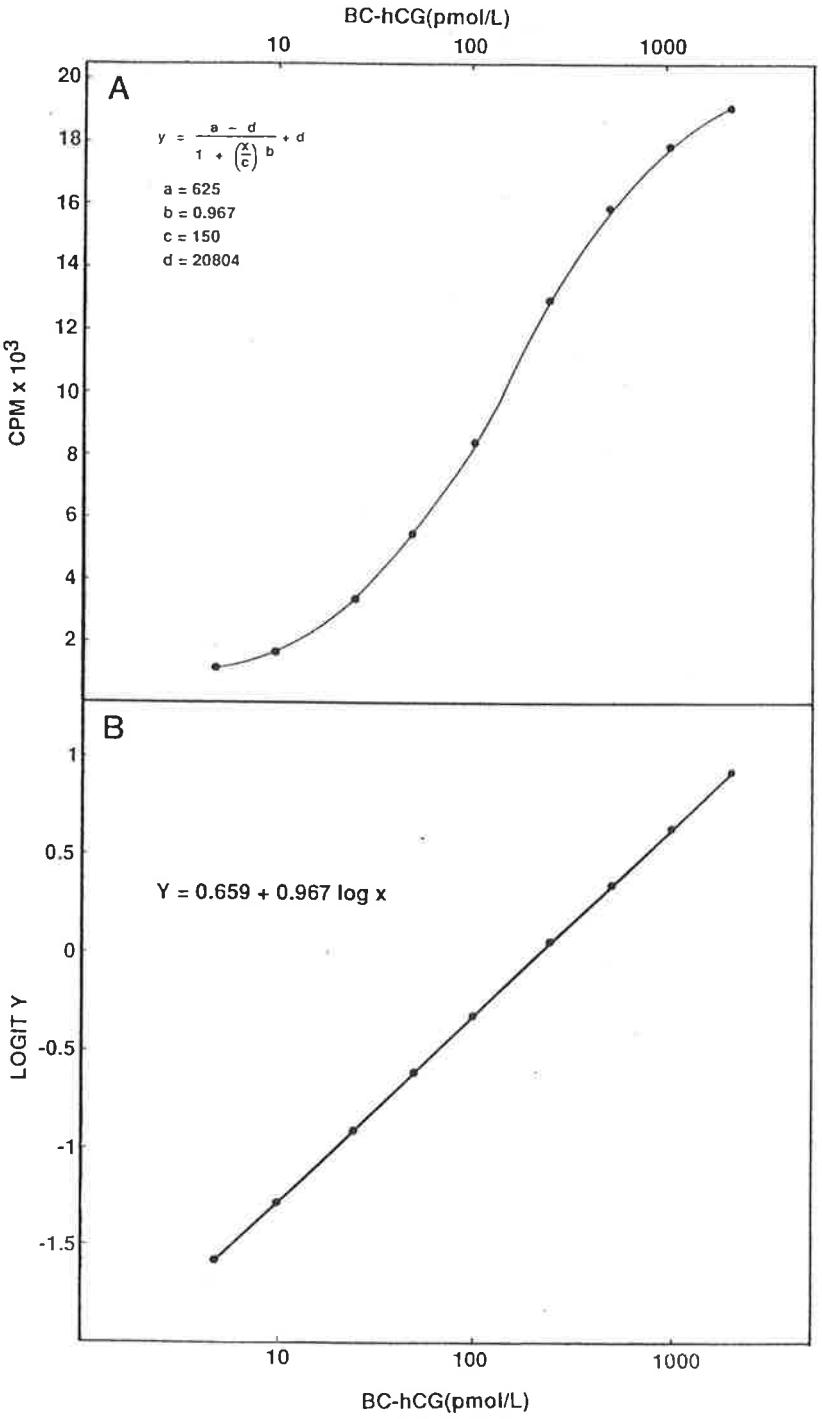


Figure 5.5.

(A) Typical dose-response curve for the liquid-phase IRMA-I of ßC-hCG with 32H2 and DeM3 antisera fitted by the four-parameter logistic. Parameters a, b, c, and d (insert) are defined by Rodbard (Rodbard, 1974); the slope was determined as presented in panel B.

(B) Same data as in panel A fitted by a logistic linear model (Volund, 1978). The straight line was determined by iteratively-weighted linear regression using equation n[(y-a)/d] [(a+b-y)/b] to determine the weights, where n = number of standard points, a = minimum y response, d = maximum y response, b = ED_{50}.

reactivities of ßhCG, hCG, LH/FSH (LER 907), αhCG and ßLH were estimated to be 18.4%, 14.5%, 4.3%, 0.6% and 0.018%, respectively. However because intact hCG and αhCG cross-reactivities in ßC-hCG assays may be overestimated due to contamination of the reference preparations used as cross-reactants and because some discrepancies were noted concerning ßC-hCG contamination of the ßhCG CR 125 reference preparation (*Wehmann et al, 1988; Krichevsky et al, 1991*), the effect of ßC-hCG contamination on the ßhCG cross-reactivity was re-examined. Eight micrograms of highly purified ßhCG-CR125 were chromatographed on Sephacryl S-200 and the fractions were assayed for free ßhCG and ßC-hCG molecules. Although ßC-hCG was not present in the ßhCG-CR125 preparation (Figure 5.6B), the ratio between the areas of ßhCG and ßC-hCG peaks obtained with both ßhCG and ßC-hCG assays showed that the ßhCG cross-reactivity in this assay was as low as 2.9%. Because of these different results the clinical significance of ßhCG and intact hCG cross-reactivities in this assay was further evaluated by measuring their immunoactivity in the same urine chromatographic fractions used to evaluate the RIA. The results suggested that the ßhCG and hCG cross-reactivities with this method are 5.3% and 2.1% respectively (Figure 5.6C). Because of non-parallelism and the low maximal signal noise given by the related glycoproteins in this system, it is concluded that the amount of cross-reactant capable of inducing a signal noise of 10% is not an accurate method to determine their cross-reativities. The intra-assay variability between 32pmol/l and 1506pmol/l ranged from 2.8% to 9.3% and the inter-assay variation between 21.6pmol/l and 1349pmol/l ranged from 3.9% to 9.5 (Table 5.2).

Non specific matrix effects were tested in the IRMA-I for plasma, serum, urine, follicular fluid, and MEM containing 10% FCS by performing specific inhibition curves with addition of increasing amounts of purified ßC-

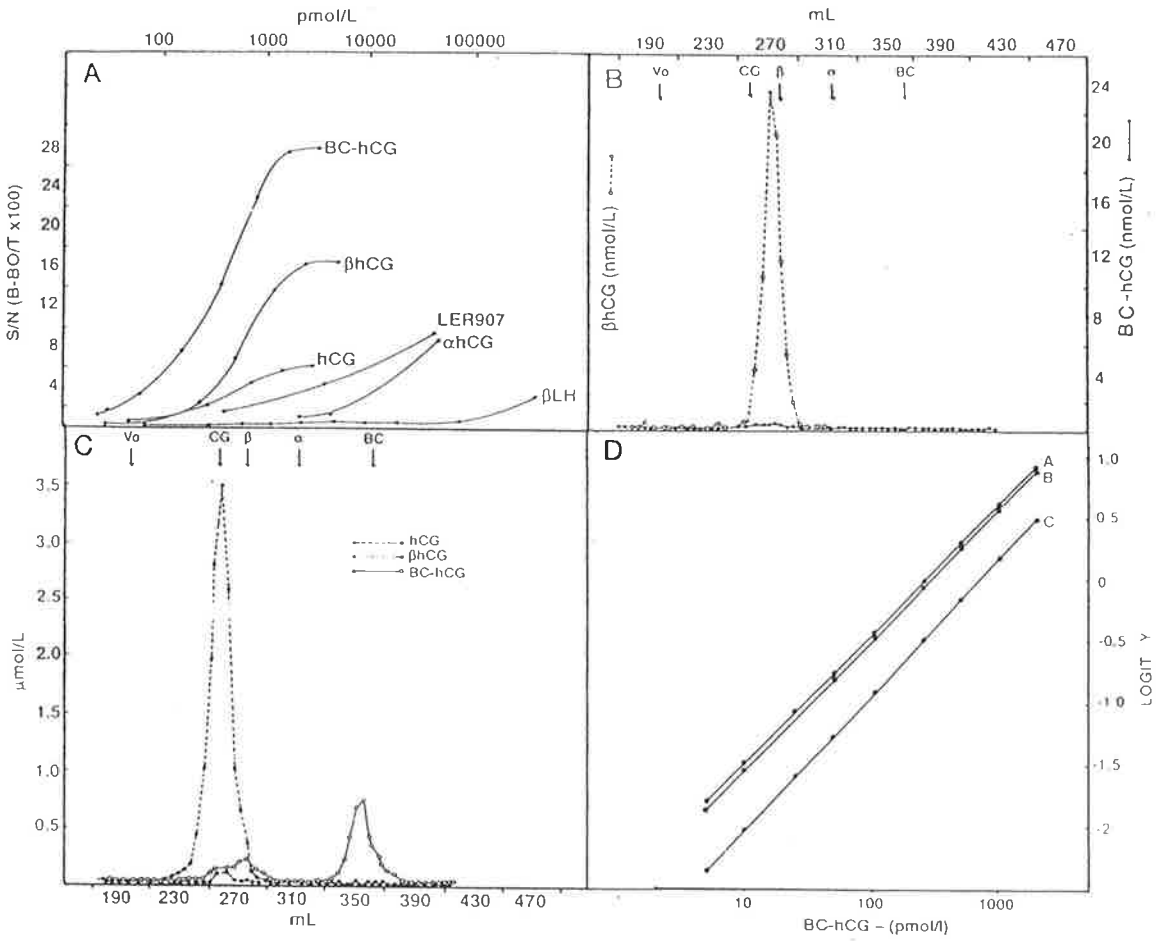


Figure 5.6.

(A) Binding data of hCG (CR 125), ßhCG (CR 125), αhCG (CR 119), LH/FSH (LER 907), and ßLH (AFP-3282B) in the ßC-hCG IRMA-I. Ordinate: signal noise ratio. Abscissa: concentration of analytes (pmol/l). See text for cross-reactivity comments.

(B) Sephacryl S-200 chromatography of ßhCG (CR 125). Eight micrograms were filtered as in legend of Fig. 5.3B. Fractions of 4.2ml were collected and assayed for ßC-hCG (●—●) with the IRMA-I and for ßhCG (O–O) with the assay described in Materials and Methods.

(C) Elution profile of ßC-hCG (—) obtained with the IRMA-I in the same fractions used to construct Fig. 5.3B. The profiles of hCG (- - -) and ßhCG (• • • •) are repeated to facilitate comparison.

(D) Comparative binding data of two standard preparations and native ßC-hCG from pregnancy urine diluted in assay buffer with ßC-hCG IRMA-I. The lines A, B, and C were fitted using the logistic linear model (Volund, 1978). The equations for A (Birken's ßC-hCG reference), B (our ßC-hCG preparation), and C (serial dilution of a pregnancy urine sample containing 191.2 nmol/l of immunoreactive ßC-hCG) are y = -2.506 + 1.03 log x, y = -2.589 + 1.05 long x, and y = -3.123 + 1.10 log x, respectively.

Table 5.3 Recovery of purified ßC-hCG added to different matrices with ßC-hCG IRMA I

ßC-hCG Added (pmol/l)	URINE		SERUM		PLASMA		FOLLICULAR FLUID*		MEM + 10% FCS	
	Mean Rec	%	Mean Rec	%	Mean Rec	%	Mean Rec	%	Mean Rec	%
5	12.0	240.0	13.0	260.0	50	>1000	12.5	750.0	11.0	220.0
10	11.6	116.0	13.0	130.0	49.0	490.0	18.2	182.0	10.5	105.0
25	28.0	112.0	20.0	80.0	47.0	188.0	32.0	128.0	25.5	102.0
50	60.0	120.0	41.0	82.0	50.0	100.0	55.0	110.0	47.5	95.0
100	82.0	82.0	77.0	77.0	120.0	120.0	115.0	115.0	115.0	115.0
250	247.5	99.0	235.0	94.0	235.0	94.0	255.0	102.0	275.0	110.0
500	385.0	77.0	470.0	94.0	470.0	94.0	490.0	98.0	485.0	97.0
1000	820.0	82.0	930.0	93.0	950.0	95.0	1000.0	100.0	1050.0	105.0
2000	1500.0	75.0	1600.0	80.0	1800.0	90.0	1800.0	90.0	2340.0	117.0

* Sample, originally containing 7.4 pmol/l ßC-hCG, collected 36h after injection of 5000 IU of hCG (Profasi, Serono) intramuscularly. Results were corrected by subtracting the basal concentration from the observed response.

hCG in these fluids. Table 5.3 shows the recovery of ßC-hCG at different levels with this assay. Between 14pmol/l and 1000pmol/l the mean recovered ßC-hCG from urine, follicular fluid, serum, MEM, and plasma were 95%, 108%, 86%, 104%, and 115% respectively. Variable interference of these matrices with recovery higher than a hundred percent was occasionally observed at very low molar concentrations, under the established work range. The accuracy of this assay was assessed by diluting a urine sample containing 191.2nmol/l of ßC-hCG in assay buffer to a range of 5pmol/l up to 4000pmol/l before assay. All diluted samples were assayed for ßC-hCG in triplicate and the responses were compared to the reference preparations I and II. The results shown in Figure 5.6D demonstrate parallelism with standards along the dose response curve. In addition, the same pregnant urine sample was diluted 100-800 times with urine from a healthy male volunteer and assayed for ßC-hCG. The recovery, after correction for dilution, ranged between 83% to 101% (Table 5.4). The effect of the sample volume on the concentrations of ßC-hCG examined in an unchromatographed pregnancy urine sample showed a linear response when 0.05ml up to 0.5ml of sample were used per tube (Figure 5.7).

5.3.3 Comparison of ßC-hCG Immunoassays

Because the validation of a new assay requires the comparison of its performance with a established system and because of the different cross-reactivities with related glycoproteins exhibited by the assays evaluated in this study, a comparison of urinary ßC-hCG concentrations obtained with the two radioimmunoassays and the two immunoradiometric assays was made by assaying unchromatographed samples from 47 healthy pregnant women (20-40 weeks of gestation) with each assay system. The values obtained here has shown they are highly correlated with each other (Figure 5.8).

Table 5.4 Recovery of native ßC-hCG from urine obtained from a normal pregnant woman (originally containing 191200pmol/l) after dilution in normal male urine negative for the fragment. After correction for dilution, the observed and expected values determined with the ßC-hCG IRMA-I were compared.

Dilution factor	Expected mean level (pmol/l)	Observed mean level (pmol/l)	Corrected mean level (pmol/l)	SD	CV(%)	Recovery (%)
1:100	1912	1688	168800	410	2.4	88.2
1:200	956	885	177100	16400	9.2	92.6
1:400	478	483	193300	2600	1.3	101.0
1:800	239	198	158600	11900	7.5	82.9

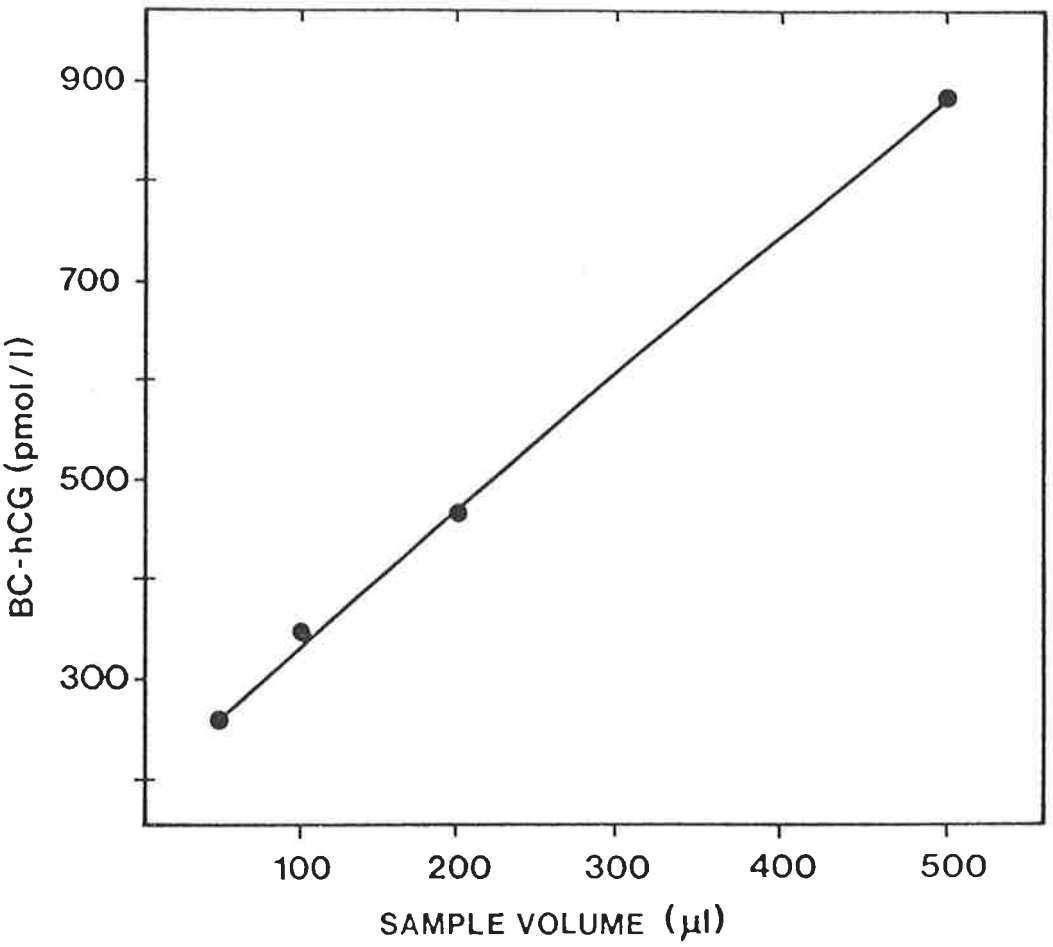


Figure 5.7.

Effect of sample volume on the concentrations of ßC-hCG measured by IRMA-I. Ordinate, concentrations of ßC-hCG (pmol/l). Abscissa, sample volume (µl).

As the correlation coefficient may not give an appropriate measure of agreement (Bland and Altman, 1986), the difference between any two assays against their mean was also analysed. Provided pregnancy urine is used, the results obtained here (Table 5.5) indicate the differences would not be clinically important.

5.3.4 Measurement of ßC-hCG Levels in Nonpregnant Subjects

Reference values from an unselected group of 238 apparently healthy women aged 12-88 years were established on early morning urine using the IRMA-I. A large majority of subjects had no detectable ßC-hCG in urine.

Taking a cut-off value of 5pmol/l as the lower limit of the assay, only 21 women (8.8%) showed detectable ßC-hCG: 3 out of 61 women (5.1%) between 30-39 years, 3 out of 47 (6.8%) between 40-49 years and 15 out of 77 (19.4%) over 50 years (Figure 5.9). Therefore all patients exhibiting ßC-hCG immunoreactivity in urine were over 30 years old. Analysis of the age effect on ßC-hCG gave a correlation coefficient r=0.497 (t=2.49, p<0.05) and a simple chi-square of 18.5 (3df, p<0.001) for a rising trend with age. The problem of variable urine dilution was minimized by correcting all results for

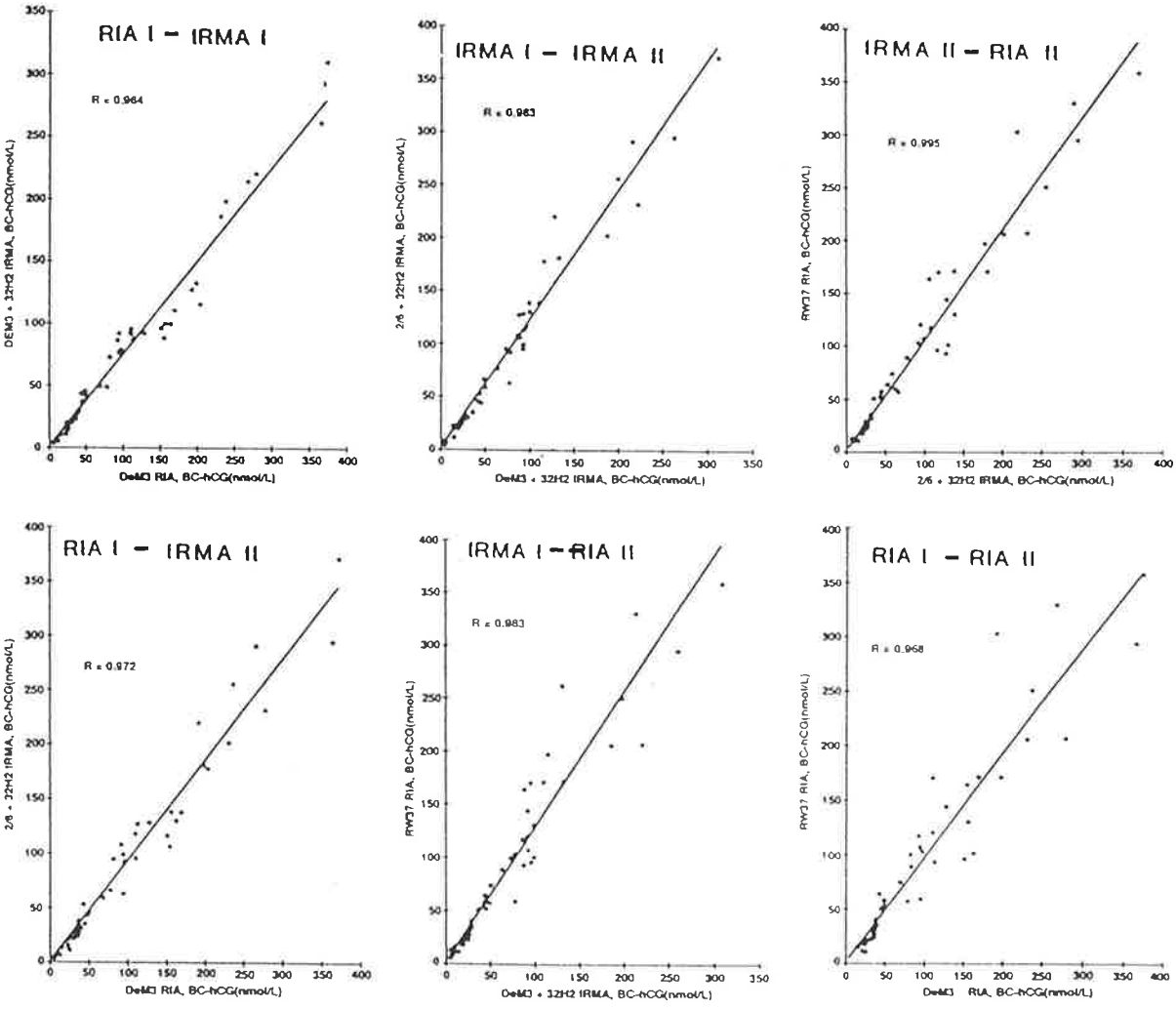


Figure 5.8

Correlation of ßC-hCG levels (nmol/l) obtained with different combinations of two RIAs and two IRMAs in urine samples from 47 healthy pregnant women.

Table 5.5 - Comparison of ßC-HCG assays in pregnancy urine using mean difference against their mean

ASSAYS	MEAN DIFFERENCE	SD
IRMA I vs RIA II	-7.0	14.4
IRMA II vs RIA II	-22.9	21.1
IRMA I vs IRMA II	11.0	13.0
RIA I vs IRMA I	6.2	17.0
RIA I vs IRMA II	14.0	17.9
RIA I vs RIA II	-1.5	13.0

* Results are given in nmol/l; Samples from 47 healthy pregnant women.

creatinine concentration in the neat sample. Although the small number of individuals with detectable ßC-hCG did not permit a full analysis of the data, a cut-off of 0.610pmol ßC-hCG per mmol creatinine and 6.6pmol/l of ßC-hCG was taken as the upper limit for normal values (95th centile).

Because the ßC-hCG radioimmunoassays showed a tendency to give higher readings than the specific immunoradiometric assays when urine samples from the pregnant subjects were examined (usually with a gradient between 0.7-0.75; P=0.05) and some reports using the RIA-II have reported a high proportion of non-pregnant subjects as having measurable ßC-hCG

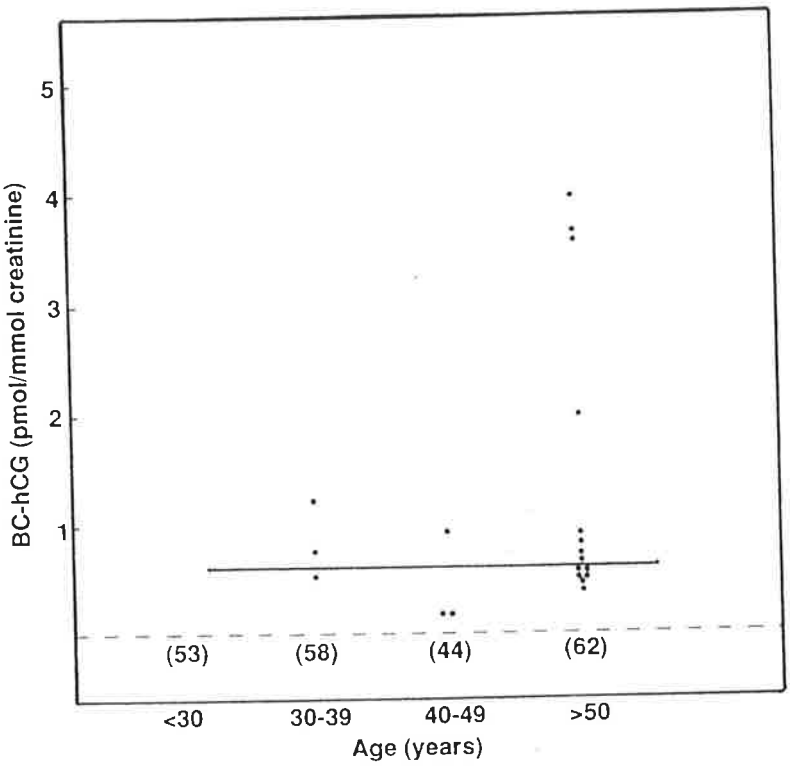


Figure 5.9

Concentration of ßC-hCG (pmol/l) in urine of non-pregnant subjects, determined by IRMA-I, after correction for creatinine excretion (mmol/l). The number (in parenthesis) under the dashed line indicate number of individuals examined in each group depicted in the bottom. The cut-off line indicates the upper limit for normal values in urine from normal non-pregnant individuals, expressed as pmol ßC-hCG per mmol creatinine.

(*Akar et al, 1988; Wehmann et al, 1989; Lee et al, 1991*), urines from a group of 30 healthy non-pregnant women, aged 20-54 years, were analysed by both RIA-I and IRMA-I. Employing this radioimmunoassay, 25 (83.3%) exhibited ßC-hCG at levels higher than 28pmol/l (which is the limit of detection of the assay, ED85), and 24 (95%) had ßC-hCG/creatinine ratios higher than 4.0. With the immunoradiometric assay, only six women (20%) had detectable ßC-hCG (P=0.05). Of these 6 subjects, 3 showed ßC-hCG/creatinine ratios higher than 0.61, which is the upper limit for normal values. Moreover, 4 out of 6 (66.6%) with detectable ßC-hCG were over 50 years old and 2 (33.3%) were 37 and 48 years when IRMA derived results were used. In contrast, the women who had detectable ßC-hCG with the RIA were scattered across all age ranges.

5.4 Discussion

Several immunoassays, either RIA or IRMA, for ßC-hCG fragment have been reported (*Krichevsky et al, 1988; Akar et al, 1988; Alfthan and Stenman, 1990; Lee et al, 1991; Krichevsky et al, 1991; DeMedeiros et al, 1991*). The majority of these assays exhibit minimal cross-reactivity with intact hCG, αhCG subunit, and intact LH but exhibit varying degrees of cross-reaction with ßhCG subunit within a range of 0.1%-100% (*Krichevsky et al, 1991; DeMedeiros et al, 1991*). The large amount of ßC-hCG in urine demands that any assay system to quantify this fragment should firstly be validated in this biological fluid. The present study reports the development of two new immunoassays, one RIA and one IRMA, able to distinguish ßC-hCG molecules from related glycoproteins in urine samples without prior use of any separation procedure in most clinical conditions. In addition, availability of antibodies for two of the published assays specifically designed for ßC-hCG detection enabled a comparison of characteristics.

Competition binding experiments, using the same standards, showed that the rabbit polyclonal antibody DeM3, employed in the new assays, possesses the same order of affinity (10^{10}l/mol) for ßC-hCG as the polyclonal anti-rabbit RW37 previously published by Wehmann et al (1989). The minimal detectable dose of 5pmol/l with the RIA presented here was comparable with the 2.5pmol/l reported with the RIA employing the antiserum RW37. Both RIA's do not bind to alpha and beta subunits as part of the dimer hCG and, except for the high cross-reactivity of the DeM3 antibody with the ßLH subunit, they do not bind to other related glycoproteins in significant proportion. It is also of interest to note that because ßC-hCG is a contaminant of the hCG (CR125) and αhCG (CR119) preparations, the cross-reactivities of free alpha and intact hCG with the ßC-hCG assays described in this study may have been over-estimated. Although this study and that of others (*Wehmann et al, 1988*) were not able to show any ßC-hCG contamination in the ßhCG (CR125) preparation to account for its cross-reactivity with the ßC-hCG assays, Krichevsky et al (1991) demonstrated that some contamination may exist. They have also shown a significantly higher cross-reactivity of this purified ßhCG preparation (CR 125) than three other ßhCG references in their ßC-hCG assay. The cross-reaction exhibited by the DeM3 antibody with human LH free beta subunit was significant and consistent with the cross-reactivities showed by most antibodies described by Krichevsky et al (1988). While this cross-reactivity can be eliminated using a two site IRMA, use of a single antibody such as in a RIA may lead to highly significant cross-reaction with ßLH, especially in postmenopausal women. The extent of interference of ßLH with RW37 was not described by the authors, but may be important in explaining the discrepancy noted concerning the prevalence of ßC-hCG molecules in non-pregnant individuals (see section 5.3.4).

As mentioned above the apparent drawback of some ßC-hCG assays is also a significant cross-reactivity observed with free ßhCG subunit. Notwithstanding the use of a highly pure preparation of ßC-hCG as immunogen, the polyclonal antibody DeM3, was shown to cross-react more than RW37 (on a molar basis, 10% vs 0.5%) with the standard ßhCG (CR125) in the competitive binding assays. However the true ßhCG cross-reactivities of these antibodies were not significantly different when chromatographed fractions from pregnant urine were analysed (5.6% vs 5.4%). This discrepancy could be due to an unequal estimation of the ßhCG amount used in the competitive assay. Because the ßhCG (CR125) subunit is only slightly contaminated with ßC-hCG molecules, most of its cross-reactivity with the ßC-hCG assays reflects the high similarity between these two glycoproteins. When this question was addressed by assaying ßC-hCG in unextracted fresh urine samples collected from 47 healthy pregnant women, the RW37 and DeM3 RIAs exhibited excellent correlation (r=0.968) and high degree of agreement (mean difference, d=-1.5$\pm$13.9nmol/l) with each other. In addition, the parallelism observed between ßC-hCG standards and diluted unextracted samples containing low (non-pregnant) and high (pregnant) levels of ßC-hCG with DeM3 antibody-RIA suggests that the samples may be accurately measured by diluting them in the assay buffer. Together these findings clearly indicate that because of the low levels of free ßhCG subunit found in physiological states, despite different cross-reactivities with this subunit, both RIAs may be equally employed in most clinical conditions. However, in any condition where the free beta subunit is expected to exist in high concentrations, chromatographic validation should be considered before sample analysis with any ßC-hCG radioimmunoassay.

The IRMA for ßC-hCG employing the monoclonal antibody 32H2 and the polyclonal DeM3 in a liquid phase system (IRMA-I) also showed high sensitivity and accuracy, excellent correlation with both RIAs (r=0.983 with

RW37-RIA; r=0.964 with DeM3-RIA) and ability to discriminate ßC-hCG molecules from most of related glycoproteins. The binding curves of cross-reactants tested were usually non-parallel to the standard binding, with the result that the calculated extent of cross-reactions varied with the dose. Because of decreasing signal noise ratio with increased dose or inadequate amount of reagents, 50% of maximum standard binding was not obtained with any cross-reactant. Therefore the cross-reaction levels were taken from the point at which any analyte gave a signal noise ratio equal to 10% of the standard in the binding curves. Estimating from the respective standard curves this assay showed apparent higher cross-reactivity with the free ßhCG than the RIA using DeM3 antibody (18% vs 10%). Interestingly, determining the ratio between ßC-hCG and ßhCG immunoreactivities with homologous assays (ßhCG IRMA and ßC-hCG-DeM3/32H2 IRMA) in the fractions eluted from the Sephacryl column with either ßhCG subunit separated from pregnancy urine or standard ßhCG (CR 125) estimated the true cross-reactivities as 5.3% and 2.9%, respectively. This cross-reactivity is similar to that obtained with the DeM3-RIA I and antibodies described by O'Connor et al (1988). Although this assay has some cross-reaction with free beta subunit, several advantages over the RIA should be noted. Firstly, the sensitivity is enhanced to 1.5pmol/l of analyte, permitting its use in a number of conditions in which ßC-hCG molecules are found in small concentrations. Second, the incubation time was dramatically reduced from 24h to 4h. Thirdly, the use of donkey anti-rabbit immunoglobulin bound to magnetic particles facilitates easy separation of bound and free molecules in a liquid phase. Fourthly, the assay offers some advantage when the departure from linearity and high cross-reactivity of ßLH in the RIA are considered and could be a more appropriate assay for ßC-hCG estimation in non-pregnant subjects where ßLH concentrations could be high.

During the validation procedure of both assays, non-specific matrix effects denoted by high recovery could be seen with the use of some biological fluids. With the RIA-I a mean recovery of 120-150% was observed with plasma, serum and follicular fluid. When the IRMA-I was used a recovery higher than 100% was observed up to 25pmol/l with the use of plasma, up to 10pmol/l with serum, and up to 5pmol/l with MEM, follicular fluid, or urine. Because samples were selected with minimum ßC-hCG immunoactivity and below the sensitivities of the assays (male urine, serum, plasma, and follicular fluid) to test their influence on ßC-hCG estimation, the results demonstrated the existence of non-specific binding. The non-specific effect of these matrices on other ßC-hCG immunoassays has not been extensively studied previously but the non-specific urine effect seen up to 5pmol/l with the IRMA-I is identical to that reported with the RIA established by Akar et al (1988). As the direct determination of ßC-hCG in serum has shown concentrations as low as 12-28pmol/l (*Wehmann et al, 1989, Alfthan and Stenman, 1990*), the results of the current study suggest that direct ßC-hCG measurement in both serum or plasma, should be preceded by concentration or extraction of the analyte.

Varying potencies should be expected for ßC-hCG molecules evaluated with several assay systems employing antibodies with different affinities and different abilities to discriminate ßC-hCG from ßhCG. Additionally, the use of different standard preparations by different laboratories makes it difficult to compare quantitative ßC-hCG accurately. While O'Connor et al (1988), Alfthan and Stenman (1990), and our group have used Birken's reference preparation to calibrate samples, Wehmann's group (*Akar et al, 1988; Wehmann et al, 1989*) have used its own preparation. The results obtained from 47 pregnant women using the two immunoassays reported here (RIA-I, IRMA-I) and two assays previously validated were compared. It was found that all have the same ability to measure ßC-hCG directly in pregnancy urine

compared. It was found that all have the same ability to measure ßC-hCG directly in pregnancy urine samples with values ranging from 5.6nmol/l to 372.5nmol/l, when the same standard preparation is used. As shown in Figure 5.8 and table 5.5 excellent correlation coefficient ($r > 0.960$) and high degree of agreement ($d < 23$nmol/l) were found with all four methods. This observation suggests that free beta hCG subunit cross-reaction has little influence on the results when pregnancy urine is to be analysed. This study also highlights the necessity for establishing an international reference preparation, so that future clinical studies can be compared. As mentioned above the reference standard from Birken (*Birken et al, 1988*) was chosen in the present study to calibrate all the samples.

Even though the assays examined exhibited excellent correlation with each other, it was noted that ßC-hCG immunoreactivity was slightly but consistently higher when any of the two RIAs were used (mean difference between 6.2nmol/l and 14.0nmol/l with DeM3-RIA). While this may reflect the different affinities of the antibodies for ßC-hCG and its related molecules, it is possible that the polyclonal antibodies, even they do not cross-react significantly with the free beta subunit or intact hCG, may recognize other clipped fragments recently found in large amounts in pregnancy urine (*Puisieux et al, 1990; Sakakibara et al, 1990*).

Application of ßC-hCG assays to measure this fragment in non-pregnant individuals either in normal or abnormal conditions requires previous knowledge of a normal range. As the IRMA designed with DeM3 and 32H2 antibodies showed better sensitivity and lower cross-reactivity with ßLH subunit this assay was chosen to analyse the urine samples obtained from 238 apparently healthy individuals. Within the defined limit of detection of the assay (5pmol/l) 21 individuals (8.8%) showed measurable ßC-hCG, 15 (71.4%) of these being over 50 years. Because of the small number of subjects with detectable ßC-hCG in urine, it was only possible to use the 95

percentile as the criterion to differentiate any abnormal non-pregnant individuals from the normal range for the female population. It was found that 226 individuals (95%) had less than 0.61pmol of ßC-hCG per mmol creatinine per litre of urine and this value was established as the upper limit for the normal female population with this assay. Without correction for creatinine the levels of ßC-hCG in those 21 subjects where detectable fragment was present ranged from 5pmol/l to 34pmol/l. These results, even using a different standard preparation, are much lower than those reported by Akar et al (1988). Using a limit of detection of 282pmol/l they found detectable levels, in approximately 46% of the female population with mean levels of 384pmol/l regardless the age. If their levels were assumed to be the limit of detection, all 238 individuals examined here would have undetectable levels of ßC-hCG. The present results also differ from those investigators with respect to age; while we could find no patient under 30 years with ßC-hCG values above 5pmol/l, they reported the presence of ßC-hCG fragment in half of the 28 premenopausal women aging 19-32 years old. Our results also disagreed with respect to the lack of correlation between ßC-hCG levels and age: while they determined a mean value of 357pmol/l and 412pmol/l for women between 19-32 years and 48-66 years respectively, the current study found clear correlation between age and ßC-hCG concentration in urine ($r=0.497$, $p<0.05$). On the other hand, the results obtained in another study (*O'Connor et al, 1988*) using a different IRMA were consistent with a much smaller proportion of individuals, pre and postmenopausally, with measurable amount of ßC-hCG in their urine. These investigators could detect ßC-hCG in very few normal subjects (6%) with levels within 30-48pmol/l. This discrepancy between the IRMA and RIA was analysed in 30 non-pregnant subjects. Using RIA-I, ßC-hCG could be detected in as many as 25 out of 30 (83%) women; levels ranged from 28.4pmol/l to 228.0pmol/l. With the IRMA, ßC-hCG could be detected in only 6 women (20%; levels ranging between 5.2pmol/l to

16.4pmol/l). Notwithstanding the fact that there was only a small number of subjects with measurable ßC-hCG, not allowing any real comparison or categorisation, the values demonstrated that absolute results obtained with IRMA and RIA are not strictly comparable.

If age is really significant then either pituitary hCG secretion increases with age, ßC-hCG assays cross-react with LH, FSH subunits or fragments which levels increase at menopause or there is an age-related decline in ßC-hCG clearance. In fact evidence that immunoreactive ßC-hCG in postmenopausal urine exceeds that of immunoreactive hCG suggests that the beta core molecules in these individuals could also originate from the fragmentation of other glycoproteins (*Iles et al, 1991*). The differences observed between the IRMA-ßC-hCG measurement effected here and the RIA-ßC-hCG measurements performed by Wehmann's group and by us could, at least in part, be also explained by the existence of fragments from other gonadotrophins cross-reacting with the polyclonal antibodies. Whether the ßC-immunoreactivity detected in the urine of pre and postmenopausal women with ßC-hCG RIAs is ßC-hCG, beta core LH (ßC-LH), or a mixture of both deserves further consideration.

In summary, two new immunoassays for ßC-hCG measurement have been established and their use in urine, serum, follicular fluid, and in a widely used culture medium has been validated. It is suggested that the use of polyclonal antibodies in RIA systems may overestimate ßC-hCG results when samples from non-pregnant individuals are analysed. In addition, our findings clearly denote the necessity for the establishment of an international reference preparation so that clinical studies may be compared between different investigators.

CHAPTER SIX

DISTRIBUTION AND METABOLISM OF ßC-HCG FRAGMENT.

6.1 Introduction

Because of the potential clinical importance of ßC-hCG, a precise knowledge of the distribution and ratio of this molecule in relation to other related glycoproteins in the compartments of the body is needed. Despite the advances in understanding about its structure and occurrence, the mechanisms by which beta core molecules appear remain unclear. Experiments using animal models have shown that after intravenous injection, kidneys, ovary and liver accumulate hCG and ßhCG subunit and degrade them to ßC-hCG molecules. Experiments in humans have shown that infusion of pure hCG or ßhCG results in the appearance of some beta-core molecules in urine (*Wehmann and Nisula, 1980; Wehmann and Nisula, 1981; Lefort et al, 1986*). Because ßC-hCG is usually detected in large amounts in urine and only in small quantities in serum, these studies support the hypothesis that ßC-hCG fragment is a product of peripheral degradation of hCG/ßhCG. However, other investigators postulate that ßC-hCG is secreted by trophoblast or other tissues as a result of either an alternative processing pathway or an abnormality in the biosynthetic sequence (*Cole and Birken, 1988; Kardana and Cole, 1990*). As one hypothesis does not necessarily exclude the other, both mechanisms may explain the existence of this fragmented form in biological fluids. There are no studies examining the distribution of ßC-hCG in fluids other than urine and blood and this chapter addresses the controversy regarding ßC-hCG by analysis of its distribution in serum, urine, follicular fluid, amniotic fluid and semen, including the relative proportions of ßC-hCG and related glycoproteins in these fluids.

6.2 Methods

6.2.1 Collection and processing of samples

6.2.1.1 Urine

Three twenty-four hour urine samples (one from a healthy pregnant woman at 22 weeks, another from a non-pregnant 34 years old volunteer during the early follicular phase, and another from an apparently healthy postmenopausal 64 years old woman), were collected, kept at 4^{o}C at home without preservative and brought to the laboratory early in the following morning. Two or four hundred millilitres of each sample were centrifuged at 1850 x g for 15 minutes, at 4^{o}C, to remove debris and concentrated by pressure ultra-filtration to a final volume of 8-10ml in an Amicon Unit using a YM-2 membrane with Mr 2000 cut-off (Amicon). These 8-10ml urinary concentrate were filtered on Sephacryl S-200 and the fractions were assayed for hCG, ßhCG, αhCG, ßC-hCG and hLH or ßLH. Additional samples from 20 newborns delivered at The Queen Elizabeth Hospital, Department of Obstetrics and Gynaecology, were collected, processed as described above to remove debris, and kept frozen at -20^{0}C until assayed. For chromatography 9.5ml of urine, pooled from three subjects, were lyophilized, resuspended in 1ml of 0.2M ammonium acetate buffer containing 0.02% NaN3, pH 6.8, and chromatographed on a Superdex 75 column. One millilitre fractions were collected and assayed for hCG, ßhCG and ßC-hCG.

6.2.1.2 Serum

Twenty millilitres of blood were taken by venupuncture from eight healthy first trimester pregnant women, at the time blood was taken for routine analysis during their antenatal care. After coagulation at room temperature, the samples were centrifuged for 15 minutes, at 4^{o}C, at 1850 x g and serum

separated and stored at -20^{o}C until processing. Eight millilitres of serum, pooled from those eight women, were chromatographed on a Sephacryl S-200 column and the eluted fractions were analysed for hCG, ßhCG, αhCG and ßC-hCG immunoactivity.

6.2.1.3 Amniotic fluid

Amniotic fluid was obtained by transabdominal amniocentesis from second trimester pregnant women (16-20 weeks of pregnancy), referred for genetic counselling. Only clear samples, uncontaminated with blood, were used. All samples were immediately centrifuged (1850 x g for 10 minutes, at 4^{o}C) and stored at -20^{o}C. Before processing amniotic fluid from several women was thawed to room temperature, pooled to make up 200ml total volume, and concentrated by pressure ultrafiltration to a final volume of 8ml. This concentrate material was filtered on a Sephacryl S-200 column and the eluted fractions were assayed for hCG and related molecules.

6.2.1.4 Follicular fluid

Follicular fluid samples were obtained from 50 patients undergoing treatment for infertility in the IVF programme at the Reproductive Medicine Unit, The Queen Elizabeth Hospital, Adelaide. Patients were hyperstimulated with a standard regimen, previously described in detail, for induction of multiple follicular development (*Kerin and Warnes, 1986*). Basically either clomiphene citrate (Clomid, Merrell Dow) or human menopausal gonadotrophin (HMG) or both were started early in the follicular phase and administered until two or more leading follicles had reached at least 16mm in diameter. Thirty-six hours after injection of 5000 IU of hCG (Pregnyl, Organon or Profasi, Serono) the oocytes were retrieved under transvaginal ultrasound guidance. After oocyte recovery the uncontaminated follicular fluids from the follicles of each patient were pooled, centrifuged for 10 minutes at 1850 x g,

aliquoted in 5ml polypropylene tubes (Johns Professional Products, Adelaide, SA, Australia) and kept frozen at -20°C until assayed. Follicular fluid (60ml) pooled from several patients was concentrated to 8ml by pressure ultrafiltration, and stored at -20°C until chromatography.

6.2.1.5 Seminal plasma

Human semen samples were collected from 74 patients attending the Reproductive Medicine Unit, The Queen Elizabeth Hospital, as part of the in vitro fertilization or related programmes. The remaining semen, after routine tests had been performed, was aliquoted (2ml), centrifuged at 10000 x g for 15 minutes, at room temperature, and the supernatant seminal plasma was aspirated from the spermatozoal pellet and stored at -20°C until assay. To validate the measurement of ßC-hCG directly in seminal plasma, 6.5ml of this fluid resulting from 0.2ml aliquots pooled from several subjects was filtered on Sephacryl S-200. To decrease the viscosity, this sample was submitted to three successive cycles of freezing (by putting it in a freezer at -20°C for at least 3 hours) and thawing (to room temperature), and centrifuged at 10000 x g for 15 minutes at each freeze-thaw cycle. This procedure has been shown to leave hCG and related molecules undamaged (see Chapter three).

6.2.2 Gel chromatography

Concentrated samples of urine, amniotic fluid, follicular fluid, (8-9.5ml) or semen samples (6.5ml) were filtered at room temperature on a Sephacryl S-200 column. The chromatographic procedures were described in detail in Chapter Two (section 2.5.1.2). Fractions of 4.2ml were collected and monitored for gonadotrophic immunoactivity. The Sephacryl S-200 fractions containing the high molecular weight material showing ßC-hCG/hCG immunoactivities were pooled, lyophilized, treated with 3M ammonium thiocyanate for 13-14 hours at 4°C and re-chromatographed at room

temperature on a Superdex 75 column under the conditions detailed in the Chapter Two (section 2.5.1.4). Fractions of 1ml were collected and assayed for ßC-hCG and hCG.

6.2.3 Metabolism of hCG in vivo

Five normal male volunteers, aged 20-45 years, participated in this study after giving informed consent. Initially, baseline blood and urine specimens were collected from each subject. A commercial hCG preparation (1500 IU, Profasi, Serono) was administered intramuscularly (Profasi contains no detectable ßC-hCG [K. Govas and R.J. Norman, unpublished observation] Appendix XXII). Blood and urine samples were collected for hCG and ßC-hCG determination before exogenous hCG administration, and at 2, 24, 48 and 96 hours after injection. Urine and serum samples were centrifuged at 1850 x g for 15 minutes, at 4^{o}C, and stored at -20^{o}C until analysis.

6.2.4 Immunoassays

ßC-hCG was estimated by either the immunoradiometric assay (IRMA-I) or the radioimmunoassay (RIA I) or both. These assays were detailed in Chapter Five. Intact hCG was measured by the IRMA which uses 3/6 and 11/6 antibodies (Chapter Two, section 2.11.4.1). Free alpha and beta hCG subunits, and LH were analysed by the respective IRMAs provided by Bioclone Australia (see Chapter Two, sections 2.11.4.2, 2.11.4.3 and 2.11.4.5). Human luteinizing beta subunit was estimated by RIA employing reagents provided by A.F. Parlow and distributed by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK). The characteristics of this assay were also described in section 2.11.3.3.

6.2.5 In vitro metabolism of ßC-hCG

The production of ßC-hCG in vitro was investigated using ovarian granulosa-lutein cells as a model and intact hCG as substrate. The cells were

cultured in the presence of 0.5µg of hCG (Profasi, Serono) per millilitre of medium for 48 hours (section 2.13). To control the eventual incorporation of the injected hCG by granulosa cells in vivo and release of ßC-hCG fragment in the culture medium, granulosa cells were also cultured without addition of hCG in the medium. Further, to evaluate the effect of culture conditions without cells on the hCG molecule, this hormone was incubated with medium alone at 38.5^{o}C for 48h. The cells were initially cultured for 48h and the experiments were started after microscopic observation of morphological signs of luteinization and cellular proliferation. Cultured medium was changed every two days and the spent media were stored at -20^{o}C until chromatography on Sephacryl S-200 column. Fractions of 4.2ml were collected and assayed for ßC-hCG (using the IRMA-I) and intact hCG (using the IRMA designed with the antibodies 3/6 and 11/6). The high molecular weight ßC-hCG immunoreactive material was processed as before and chromatographed on Superdex 75.

6.2.6 Analysis of data

Gel filtration partition coefficients (Kav) were calculated from the equation: **Kav = (Ve-Vo)/(Vt-Vo)**, where Ve is the elution volume. The molar proportion between the total immunoreactivity obtained with the ßC-hCG assay(s) and those obtained with the homologous assays at each peak was calculated by the formula: hormone A in hormone A assay/hormone B in hormone B assay. In order to calculate the different molar ratios, the molecular weights for intact hCG, ßhCG, αhCG, LH, ßLH and ßC-hCG were Mr 38000, Mr 22200, Mr 14900, Mr 28000, Mr 15200, and Mr 10000, respectively. Dose inhibition curves and potency estimates in the assays were analysed by a computerized spline function system provided by LKB (Wallac 1260 Multigamma II counter). The levels of ßC-hCG determined in the samples of urine, amniotic fluid, and semen were expressed as mean $\pm$ SD. Statistical comparison between

excretion of ßC-hCG (or hCG) at different times were performed by non-parametric Mann-Whitney U test, applied at the 5% level of significance.

6.3 Results

6.3.1 ßC-hCG and related molecules in urine

Chromatographic separation of 200ml of urine collected from a single second trimester pregnant woman with estimation of hCG, ßhCG, αhCG and ßC-hCG in the fractions with specific assays showed that their eluted peaks contained 782nmol, 89nmol, 33nmol, and 287nmol, respectively (Figure 6.1A). The molar ratios between ßC-hCG/hCG, ßC-hCG/ßhCG and ßC-hCG/αhCG estimated by IRMA were 0.367, 3.22, and 8.69, respectively. When measured using RIA-I, the ßC-hCG peak was 526nmol, giving a ßC-hCG/hCG ratio of 0.672 (Figure 6.1B). As mentioned in Chapter Five the difference noted between radioimmunoassay and immunoradiometric assay is probably due to the differential abilities of the antibodies to recognize ßC-hCG molecules or other related glycoproteins.

The amount of hCG, ßhCG, ßC-hCG, intact LH and their proportions in 200ml of postmenopausal urine was evaluated by chromatographing a concentrated sample of urine obtained from a single 64 years old individual. Specific assay-detection in the fractions estimated the amount of LH, hCG and ßC-hCG as 113.5pmol, 2.6pmol and 36.7pmol, respectively (Figure 6.1C). The free ßhCG subunit, measured by a specific assay, was undetectable in all fractions. The calculated ratios between ßC-hCG/hCG, ßC-hCG/LH and hCG/LH were 14.1, 0.323, and 0.023, respectively. This result demonstrates that after the menopause the amount of ß-core is 14 times higher than that of

intact hCG and that for every molecule of LH approximately 0.023 molecule of hCG is found.

Chromatographic separation of these glycoproteins in the concentrate of 400ml of urine from a 37 years old premenopausal woman, collected in the early follicular phase, showed peaks of LH, hCG, and ßC-hCG containing 37.8pmol, 0.9pmol, and 4.1pmol, respectively (Figure 6.1D). The ratios between ßC-hCG/LH, ßC-hCG/hCG, and hCG/LH were estimated in 0.108, 4.55, and 0.023, respectively. These ratios demonstrated to exist 0.1 molecules of ß-core and 0.023 molecules of hCG per molecule of LH. This molar ratio between hCG and LH is similar to that seen after the menopause. The results also indicate that before the menopause the amount of ßC-hCG in relation to LH is smaller than after the menopause (0.108 vs 0.323).

Nine and a half millilitres of urine, pooled from three newborns were lyophilized, resuspended in 1ml of 0.2M ammonium acetate buffer, and chromatographed on Superdex 75. Specific assay detection in the fractions estimated the amount of ßC-hCG and intact hCG in approximately 0.858pmol and 1.06pmol, respectively, indicating a relative proportion of ßC-hCG/hCG of 0.809 in this fluid. The free ßhCG immunoactivity totalled 93.4pmol, and the ratio ßC-hCG/ßhCG is 0.009 (Figure 6.2). Further, urine samples obtained from 20 newborns were analysed for the presence of ßC-hCG, ßhCG, and intact hCG without extraction. While the fragment was present in urine of 15 subjects (75.0%), with the levels ranging from 11.8pmol/l to 389.7pmol/l, the intact hormone was detected in 88% of samples, concentrations ranging between 16.7pmol/l and 112.9pmol/l. The free beta subunit was detected in 95% of samples and the levels ranged from 352.0pmol/l to 383.8pmol/l.

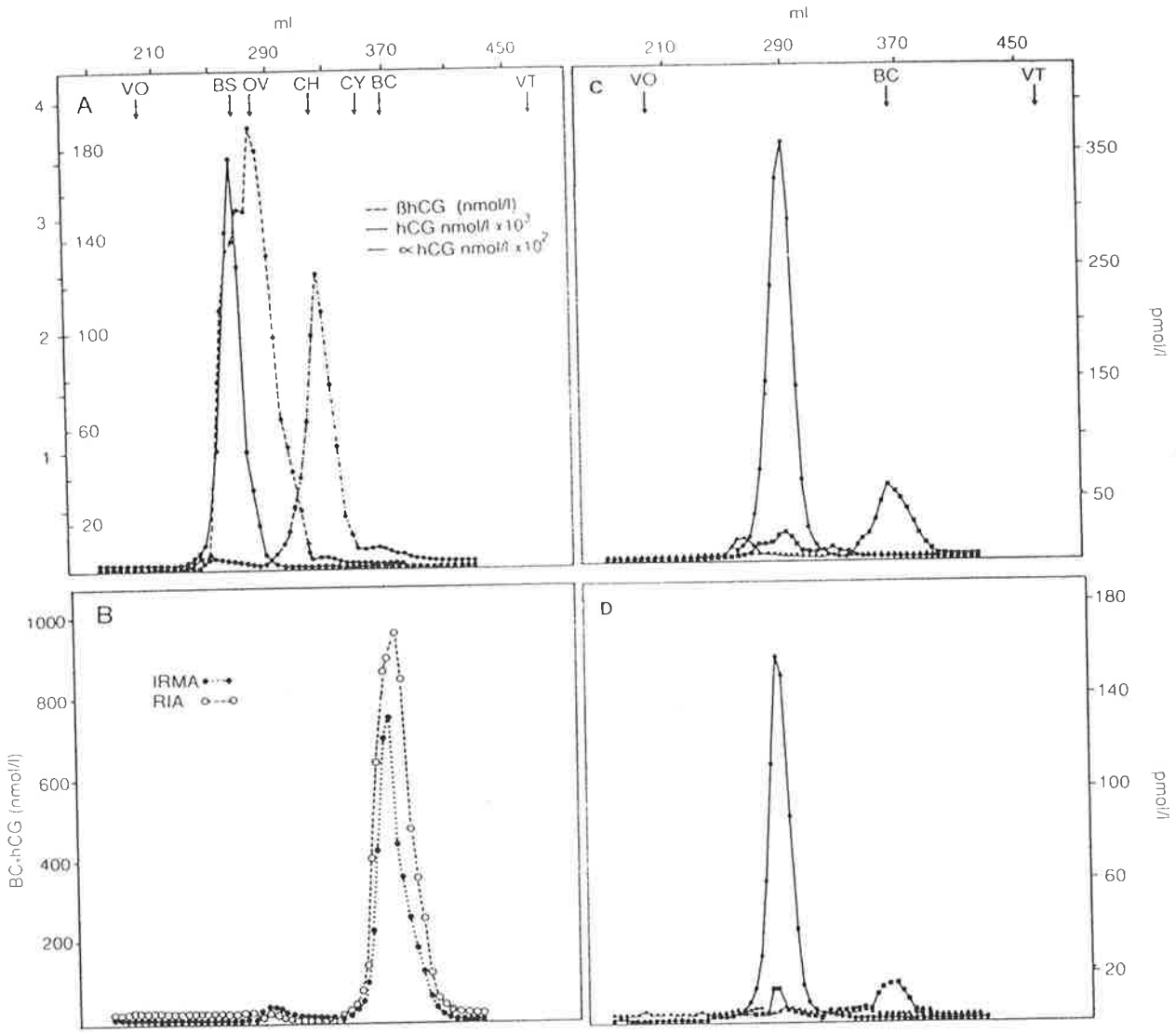


Figure 6.1

A Sephacryl S-200 gel filtration of urine obtained from a healthy first trimester pregnancy woman on a 2.6x 87.5cm column. The sample (200ml) was concentrated to 8ml by ultrafiltration and eluted at a downward flow rate of 50ml/h with 0.2M ammonium acetate, pH 6.8, at room temperature. Fractions of 4.2ml were collected and assayed for intact hCG (●—●), ßhCG (●–●) and αhCG (●··●). Arrows indicate the position of the void volume (Vo), total volume (Vt), and the elution positions of [^{125}I]-ßC-hCG, and marker proteins: bovine serum albumin, Mr 67000 (BS); ovoalbumin, Mr 43000 (Ov); chymotrypsinogen A, Mr 25000 (Ch); Cytochrome C, Mr 13000 (Cy).

B Elution oprofile of immunoreactive ßC-hCG, determined by IRMA-I (●··●) and RIA-I (○–○), in the same fractions.

C Gel chromatography of a concentrate of 200ml urine obtained from a postmenopausal woman. Eight millilitres of concentrate-sample were applied to the Sephacryl S-200 column and eluded as in **A**. Fractions of 4.2ml were collected and assayed for hCG (▲—▲), LH (●—●), and ßC-hCG (■—■). The arrows indicate the position of internal markers: Vo, void volume; Vt, total volume; ßC, [^{125}I]-ßC-hCG.

D Chromatography of a concentrate of 400ml urine obtained from a premenopausal woman. Eight millilitres of concentrate-sample were filtered on Sephacryl S-200 column as described in **A**. Fractions of 4.2ml were assayed for hCG (▲—▲), LH (●—●), and ßC-hCG (■—■).

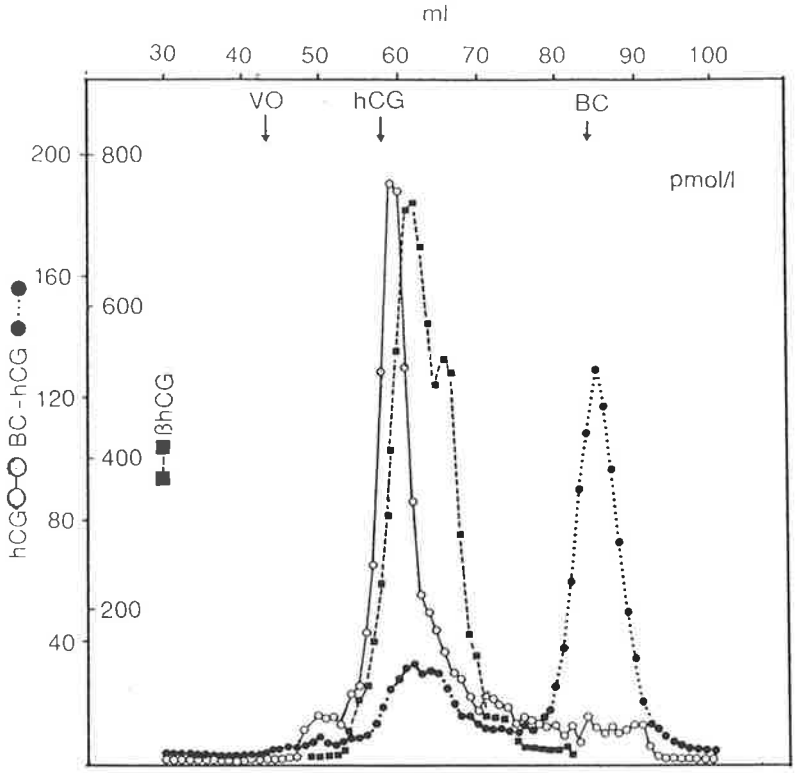


Figure 6.2

Elution profile of ßC-hCG (●), ßhCG (■), and hCG (○) in pooled urine of three newborns chromatographed on Superdex 75. One millilitre sample was eluted with 0.2M ammonium acetate buffer, at room temperature, at a flow rate of 60ml/h. One millilitre fractions were collected and assayed for ßC-hCG, ßhCG, and hCG. Note different scales.

6.3.2 ßC-hCG and related molecules in pregnancy serum

Because of the difficulty in obtaining a large volume of blood from a single individual and because the majority of reports point to the existence, if any, of very small amounts of ßC-hCG in serum during the pregnancy, the presence of this molecule in this biological compartment was investigated by chromatography of 8.0ml of serum pooled from 8 pregnant women. Fractions eluted were assayed for intact hCG, its free alpha and beta subunits and ßC-hCG (Figures 6.3A and 6.3B). By estimating the area under each peak the total amount of hCG, ßhCG, αhCG and ßC-hCG were calculated to be 166.1pmol, 3.2pmol, 9.4pmol and 1.3pmol, respectively. The amounts of ßC-hCG, ßhCG, and αhCG relative to intact hCG were 0.008, 0.019, and 0.0561, respectively.

Fractions eluting close to the void volume showing ßC-hCG and hCG immunoactivities (indicated by bar in Figures 6.3A and 6.3B) were pooled, treated with a dissociating agent (3M ammonium thiocyanate) for 15 minutes at room temperature, and refiltered under the same conditions. Even though significant ßC-hCG immunoactivity was still detected close to the void or at positions corresponding to the volume of elution of intact hCG and ßhCG, a sharper peak appeared at the position of ßC-hCG elution (Kav = 0.65). This peak was demonstrated to contain 20.9pmol (therefore, higher amount than that initially eluting as authentic ßC-hCG molecules), denoting a true ratio between serum ßC-hCG/hCG of 0.125 (Figure 6.3C). However, this procedure did not completely dissociate all high molecular weight ßC-hCG immunoreactivity. In addition, the ßC-hCG immunoreactive materials eluting near the void volume and at the position of the intact hCG were increased as well.

6.3.3 ßC-hCG and related molecules in amniotic fluid

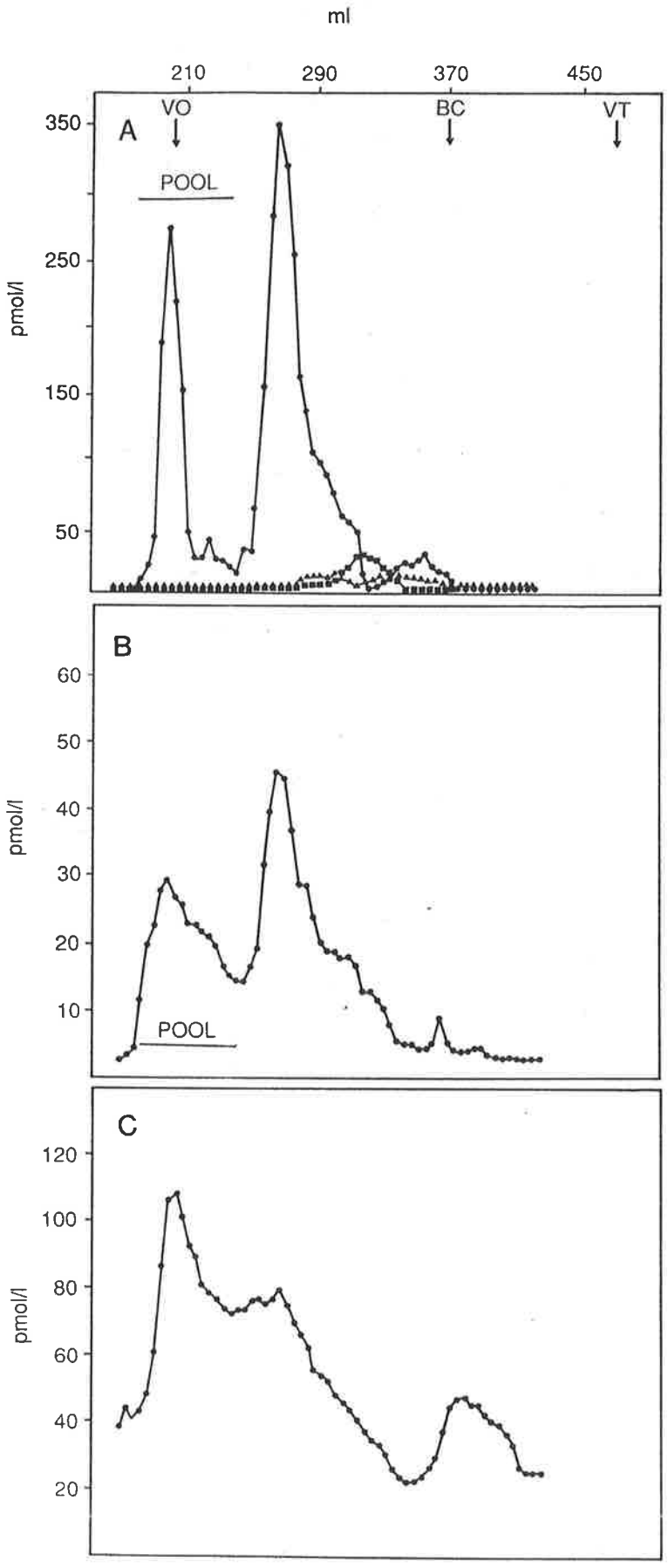
The presence of ßC-hCG molecules in amniotic fluid was investigated by filtering a concentrate of this fluid, pooled from several second trimester pregnant women, and measuring ßC-hCG immunoactivity in the eluted fractions. Figure 6.4A shows that this fragment, in a free state, was present in very small quantities either when measured by IRMA (75.4pmol) or RIA (100.9pmol). Other ßC-hCG-related molecules were also examined in the fractions and the total amounts of hCG, ßhCG and αhCG were 9.24nmol, 12.0nmol, and 108.9nmol, respectively (Figure 6.4B). The relative

Figure 6.3

A Elution profile of hCG (●—●), ßhCG (▲—▲), and αhCG (■—■) in pooled serum of eight first trimester pregnancy women chromatographed on Sephacryl S-200 as in figure 6.1.

B Elution profile of ßC-hCG, analysed by IRMA, in the same fractions.

C Elution profile of the pooled fractions with high molecular weight material immunoreactive for ßC-hCG (shown in panel B) pretreated with 3M ammonium thiocyanate and rechromatographed on Sephacryl S-200 column.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290
370
450
VO
BC
VT
A
POOL
350
250
150
50
pmol/l
B
60
50
40
30
20
10
POOL
C
120
100
80
60
40
20

distributions of hCG, ßhCG, and αhCG were 0.07, 0.093, 0.836 and 0.01, respectively (Figure 6.4B). Contrasting with the small amount of ßC-hCG, the free alpha subunit was detected in huge quantities in relation to hCG: only 0.085mol of the whole hormone was found per mol of the free alpha. The free ßhCG subunit was also found in higher proportion than intact hCG (0.76 mol of the intact hormone per mol of ßhCG). The ratio between ßC-hCG and intact hCG was 0.008 when the fragment was evaluated by IRMA and 0.01 by RIA.

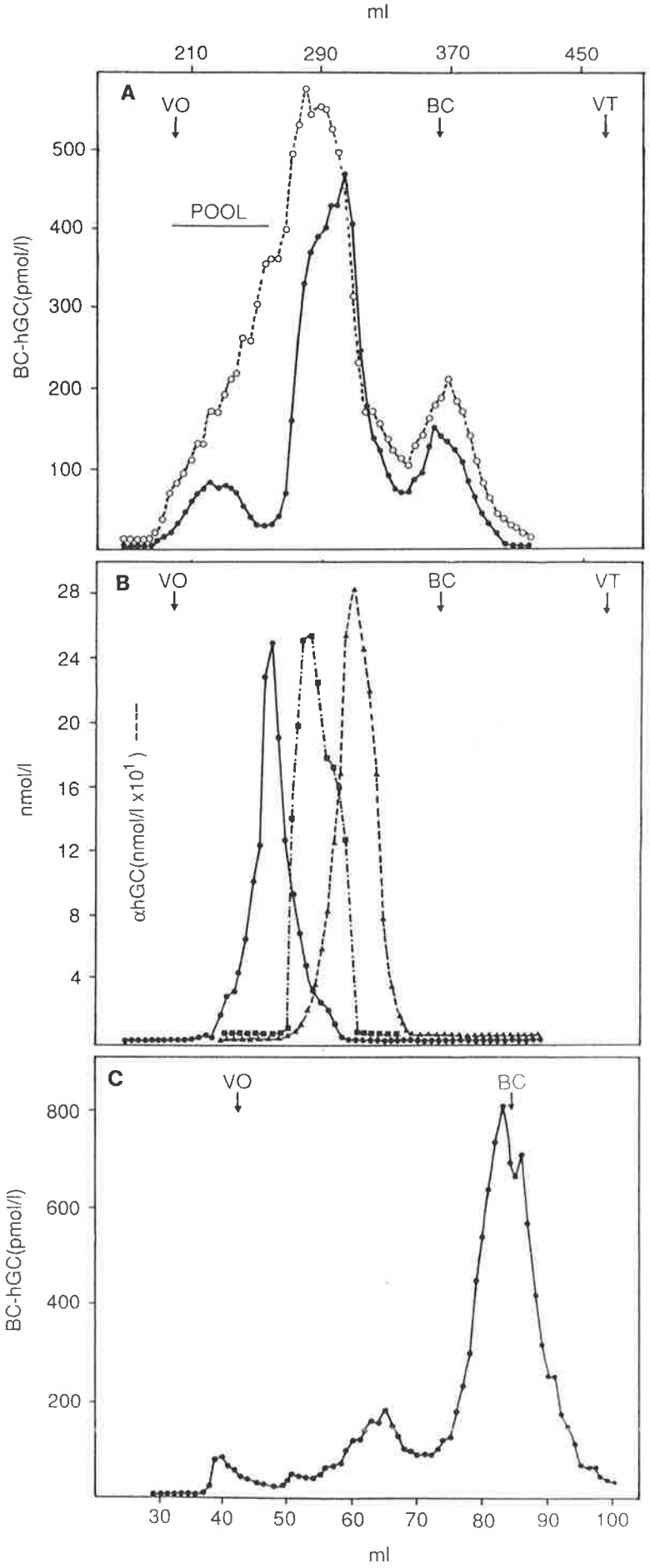
The significant amount of high molecular weight ßC-hCG immunoreactivity detected in this compartment (marked with bar in Figure 6.4A) representing approximately 55.7pmol was also pooled, treated with 3M ammonium thiocyanate for 14h, at 4°C, and filtered in a small bed column of Superdex 75 to minimize loss and dilution of the sample. The total amount of immunoreactive ßC-hCG subsequently recovered as authentic ß-core was 167.7pmol (Figure 6.4C), an amount 350% higher than the applied. This result denotes that majority of the ßC-hCG epitopes were masked in the complex. Approximately 29pmol (14.4%) of the ßC-hCG immunoreactivity were recovered at the positions of intact hCG and ßhCG elution (see discussion). As a result, 83.4% of the high molecular weight material was composed of authentic ßC-hCG molecules. As little as 2% of ßC-hCG immunoactivity (4.2pmol) did not migrate after this extended ammonium thiocyanate treatment, remaining as a high molecular weight complex. Considering both the free form of ßC-hCG and the molecules as part of the high molecular weight complex, the true ratio between this fragment and the whole hCG in the amniotic fluid is 0.026.

Figure 6.4

A Elution profile of immunoreactive ßC-hCG measured by RIA (○) and IRMA (●) in amniotic fluid concentrate-sample chromatographed on Sephacryl S-200.

B Elution profile of hCG (●), ßhCG (■), and αhCG (▲) in the same fractions.

C Superdex 75 chromatography of ammonium thiocyanate-dissociated pooled fractions with ßC-hCG immunoactivity (marked by bar in panel B). Two millilitres-sample was eluted with 0.2M ammonium acetate buffer, at room temperature, at a flow rate of 60ml/h. One millilitre-fractions were assayed for ßC-hCG using the IRMA.


ml
210
290
370
450
A
VO
BC
VT
POOL
BC-hGC(pmol/l)
500
400
300
200
100
B
VO
BC
VT
nmol/l
αhGC(nmol/l x10¹)
28
24
20
16
12
8
4
C
VO
BC
BC-hGC(pmol/l)
800
600
400
200
30
40
50
60
70
80
90
100
ml

6.3.4 ßC-hCG and related molecules in ovarian follicular fluid

The presence and relative distribution of ßC-hCG and related glycoproteins were also studied in the follicular fluid obtained at the time of oocyte recovery for in vitro fertilization from several women who had received 5000IU of hCG, intramuscularly, 36h before. Sixty millilitres of follicular fluid were pooled from different patients, concentrated, and filtered on Sephacryl S-200 as described in Chapter Two (section 2.5.1.2). The filtered fractions were analysed for ßC-hCG, hCG, ßhCG, LH and ßLH with specific assays. The amount of each glycoprotein recovered was 100pmol, 53.3pmol, 5.7pmol, 8.6pmol, and 0.8pmol, respectively. The concentration of ßC-hCG, estimated by IRMA, exceeded that of hCG (being 1.87mol of ß-core per mol of intact hCG), ßhCG (17.5mol of ß-core per ßhCG mol), LH (11.6mol of ß-core per mol of LH) and ßLH (125mol of ß-core per mol of ßLH). Using ßC-hCG RIA-I, 103.7pmol of ßC-hCG were recovered, denoting an increased ßC-hCG/hCG ratio of approximately 3-4% (Figures 6.5A and 6.5B). ßC-hCG fragment was analysed directly in 50 individual samples of neat follicular fluid obtained from different women by both RIA-I and IRMA-I. Thirteen samples (26%) were positive for ßC-hCG, levels ranging from 5.2pmol/l to 23.9pmol/l (13.1$\pm$5.7), by IRMA and 48 (96%) showed detectable ßC-hCG, ranging from 7.0pmol/l to 28.5pmol/l (19.4$\pm$5.2), when the RIA was used (see discussion).

6.3.5 ßC-hCG and hCG-like molecules in seminal plasma

The presence of ßC-hCG and the proportion of ßC-hCG and intact hCG were investigated, after Sephacryl S-200 chromatography, in a pool of seminal plasma obtained from 27 healthy male individuals. The results demonstrated that the majority of the ßC-hCG immunoreactive material found in this fluid eluted as a high molecular weight form (Figure 6.6). A significant amount, however, spread from the elution position of pure hCG. All ßC-hCG immunoreactivity in this fluid totalled approximately 51.4pmol.

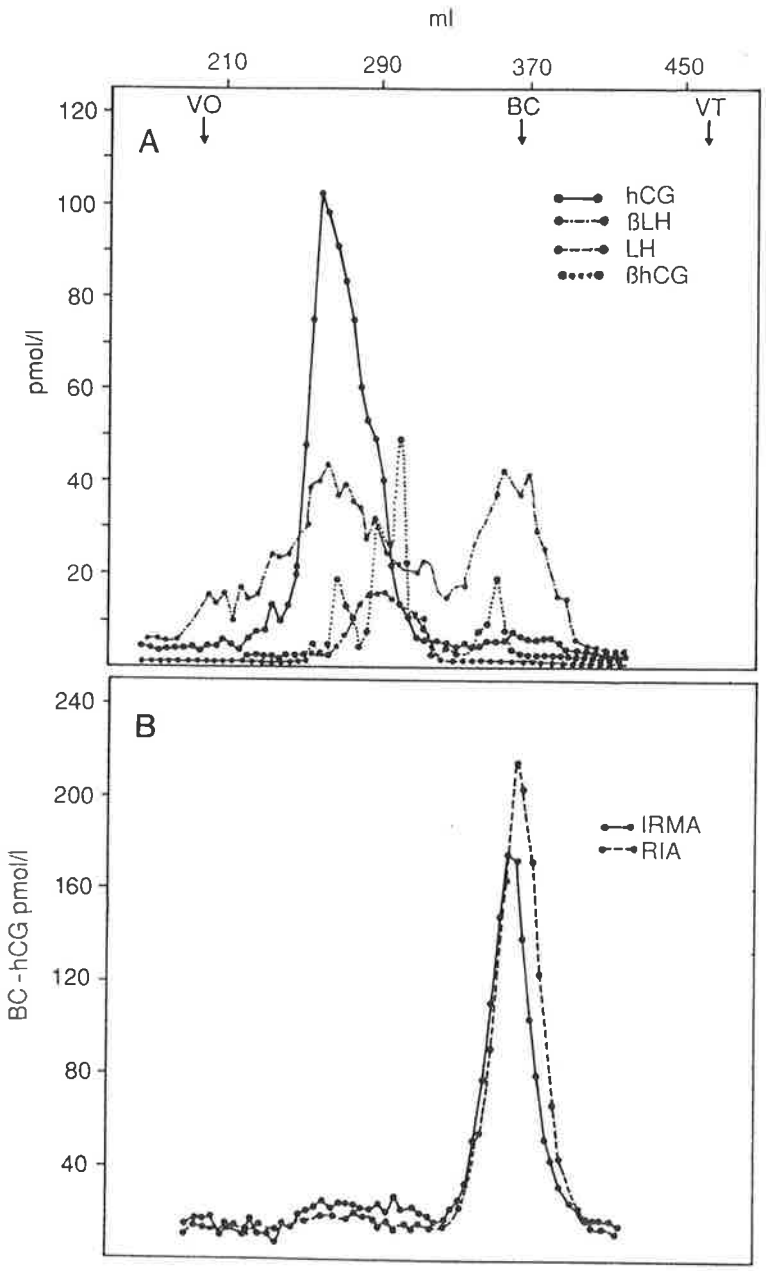


Figure 6.5

Elution profile of hCG (●—●), ßhCG (●··●), LH (●–●), ßLH (●-●) (panel A), and ßC-hCG (panel B), as measured by IRMA (●—●) and RIA (●–●), in follicular fluid filtered on Sephacryl S-200.

The pattern obtained with a specific assay for hCG in the same fractions, also depicted in Figure 6.6, showed that, in addition to a small peak of authentic hCG, some hCG immunoreactivity appeared as a higher molecular weight material. The total intact hCG immunoreactivity in the fractions was 13.9pmol.

This high molecular weight material showing ßC-hCG and some hCG immunoreactivity was pooled, lyophilized, dissociated in 3M ammonium thiocyanate for 14h, at 4^{o}C, and chromatographed on the Superdex column. Each fraction was assayed for ßC-hCG and the resultant peak was estimated to contain approximately 183.1pmol (Figure 6.6, inserted), demonstrating that the dissociated high molecular weight material generated 3.5 times more ßC-hCG than the amount loaded. However, only half of the material yielded eluted in the position of authentic ßC-hCG. The other half, seen in the ascendent limb of the ß-core peak may represent a non-dissociated dimeric form of ßC-hCG or an uncharacterized intermediate form of hCG cross-reacting with the ßC-hCG assay. Seventy four samples of neat seminal plasma obtained from different individuals were also assayed for ßC-hCG. This fragment could be detected in 42 out of 74 samples (56.7%) with the IRMA-I, levels ranging from 5.5pmol/l to 59.5pmol/l (24.9$\pm$15.2). The ßC-hCG RIA was not tested because of the insufficient volume of samples.

6.3.6 Metabolism of hCG to ßC-hCG molecules

6.3.6.1 In vitro conversion of hCG to ßC-hCG

To investigate whether granulosa cells degrade intact hCG to ßC-hCG in vitro, granulosa-lutein cells were cultured for 48h in the presence of supraphysiological concentrations (13nmol/l) of a commercial preparation of intact hCG known to be uncontaminated with ß-core (K. Govas and R.J. Norman, unpublished observation). To exclude nonspecific metabolism these experiments were controlled by culturing granulosa cells without addition of

hCG or incubating hCG without granulosa cells under the same conditions. All cultured fluids were filtered on Sephacryl S-200 and the eluted fractions were analysed for ßC-hCG immunoreactivity. Figure 6.7A shows that 140.3pmol (34.2%) of the ßC-hCG immunoactivity eluted near to the void volume as a high molecular weight form, 259.5pmol (63.3%) represented

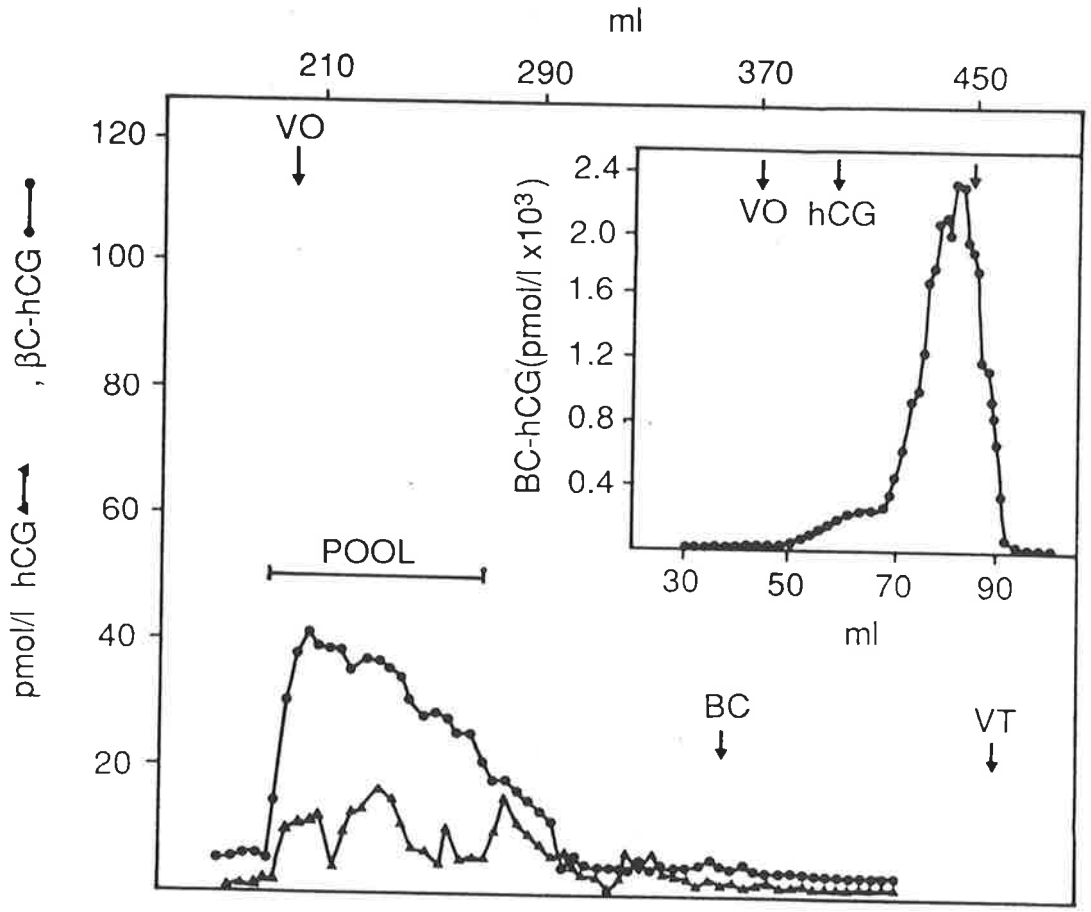


Figure 6.6

Sephacryl S-200 chromatography of seminal plasma showing the elution profiles of hCG (▲) and ßC-hCG (●). Insert: elution profile of the pooled fractions containing the high molecular weight ßC-hCG immunoreactive material after ammonium thiocyanate dissociation and separation on Superdex 75.

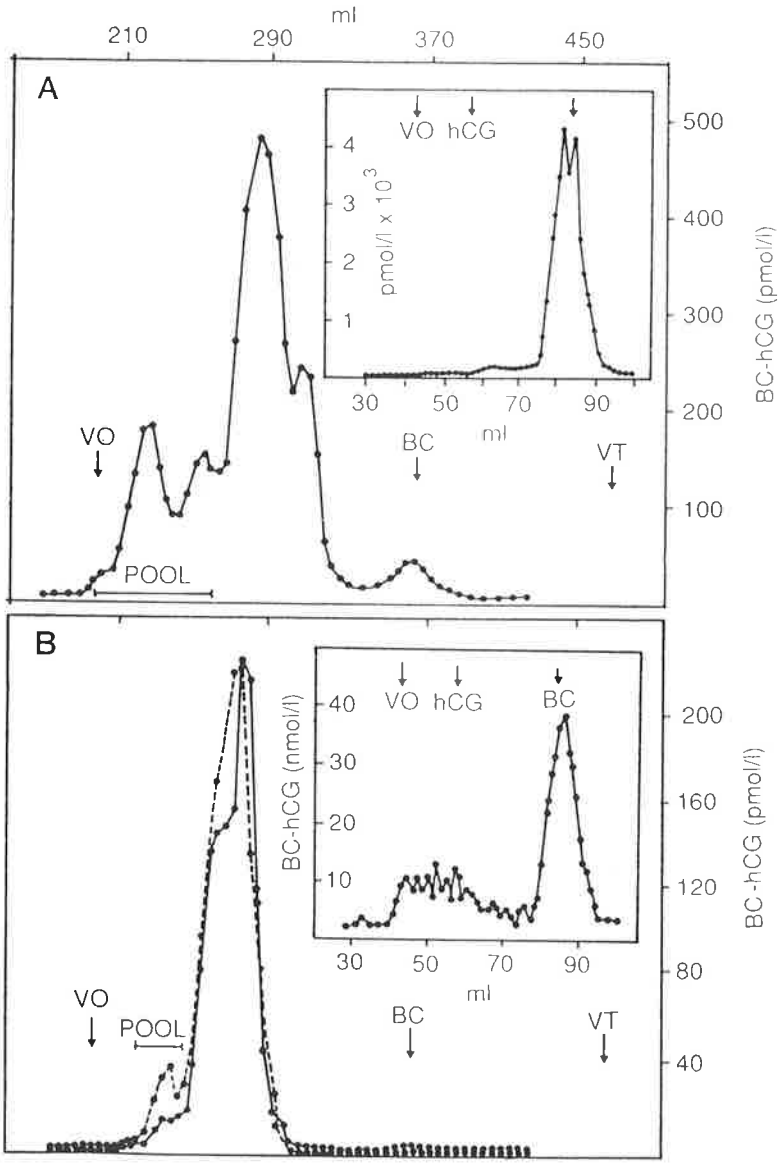


Figure 6.7

A Elution profile of ßC-hCG in 48h granulosa cell cultured medium after Sephacryl S-200 chromatography. Insert: elution profile of the high molecular weight ßC-hCG immunoreactive material after ammonium thiocyanate dissociation and Superdex 75 chromatography.

B Elution profile of hCG, on Sephacryl S-200, after 48h of incubation at 38.5C in the absence of granulosa cells. Insert: elution profile, on Superdex 75, of ammonium thiocyanate dissociated pooled fractions showing ßC-hCG activity.

hCG/ßhCG cross reactivity and 9.9pmol (2.5%) eluted in the position of radiolabelled pure ßC-hCG. The fractions exhibiting ßC-hCG immunoreactivity (approximately 140pmol) with high molecular weight were pooled, lyophilized, resuspended and incubated for 14h, at 4°C in 3M ammonium thiocyanate and filtered again on the Superdex 75 column.

Analysis of ßC-hCG in the eluate showed that the dissociated high molecular weight material was composed mainly of ßC-hCG molecules, denoting active degradation of hCG to ßC-hCG by the granulosa cells (Figure 6.7A, insert). This experiment generated five times more authentic ßC-hCG (693.5pmol) than the quantity loaded (140.3pmol), also indicating that the majority of the epitopes were not available in the high molecular weight form. In the control experiments a very small amount of ßC-hCG was generated by incubating the hCG preparation at 38.5°C for 48h in the absence of cells (Figure 6.7B). In addition, a small peak of ßC-hCG immunoreactivity (approximately 0.94pmol) appeared near the void volume and was treated with 3M ammonium thiocyanate and filtered as before. The insert in Figure 6.7B shows, in expanded scale, that approximately 5.6pmol of free ßC-hCG was dissociated from the high molecular weight material. The elution pattern of the hCG preparation without incubation, also depicted in Figure 6.7B, confirmed the previous observation that this preparation is not contaminated with ßC-hCG. The incubation of granulosa cells without hCG did not produce any ßC-hCG.

6.3.6.2 In vivo metabolism of hCG to ßC-hCG

The metabolism of hCG was studied in vivo by injecting five healthy male volunteers, aged 20-45 years, with 1500IU im of the same commercial preparation of hCG (Profasi) which had been shown to be free of ßC-hCG

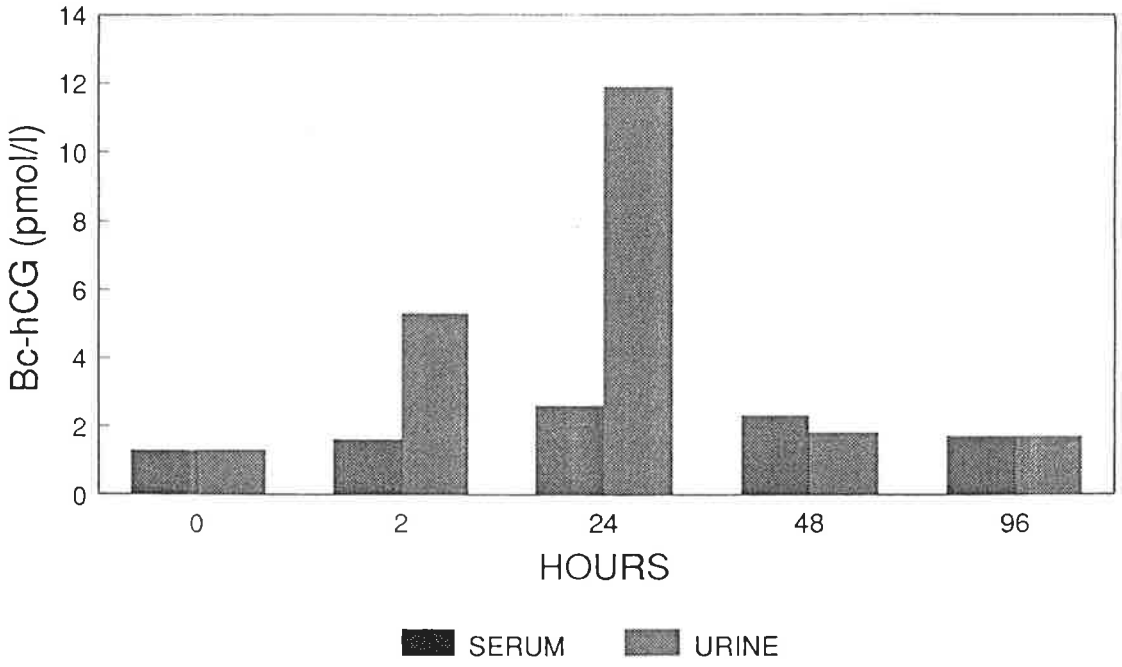


Figure 6.8

Comparison of ßC-hCG levels (mean ± SD) in serum and urine after intramuscular injection of hCG in male volunteers.

contamination. ßC-hCG was measured in urine 2h, 24h, 48h and 96h after injection. Five millilitres of blood samples were also obtained before the injection (0h) and then at 2h, 24h, 48h and 96h afterwards to monitor the circulating ßC-hCG levels (Figure 6.8). The pre-injection concentration of ßC-hCG in serum and urine were under the limit of detection of the assay (<5pmol/l).

After 24h and 48h of injection the ßC-hCG levels in serum, although very low, increased and remained in a range between 1.5pmol/l (sensitivity of the assay) and 3.3pmol/l. In turn, the urinary levels of ßC-hCG increased from the undetectable levels to 5.3±2.4pmol/l in the first two hour period of collection and to 11.8±6.3pmol/l in the next 22h, representing an increment of 221.6% (P=0.036).

6.4 Discussion

The beta core fragment of hCG has been presumed to represent post-translational processing of the intact hCG and ßhCG subunit molecules. Despite some evidence for production by the placenta, the balance of evidence favours peripheral metabolism of hCG/ßhCG for its origin. The current study analysed the amount of ßC-hCG and its proportion relative to related glycoproteins in several fluids in the body, and in addition, investigated whether this fragment may result from the peripheral conversion of injected hCG or is produced, in vitro, by ovarian tissue. It has been reported that, when determined by specific assays, ßC-hCG accounts for 15%-740% of the immunoactivity in pregnancy urine (*Cole and Birken, 1988; O'Connor et al, 1988*). After chromatography of 200ml of urine obtained from a single second trimester pregnant woman, the amount of ßC-hCG represented 37% and 67% that of intact hCG, when determined by IRMA or RIA, respectively. Even though limited to a single individual, these findings are in agreement with earlier reports, either after separating ßC-hCG from related molecules by molecular size (*Schroeder and Halter, 1983; Wehmann et al, 1990*) or after direct measurement in neat urine (*O'Connor et al, 1988; Cole and Birken, 1988; Krichevsky et al, 1991*). The different results observed with IRMA and RIA have been noted in several fluids and detailed discussion was addressed in Chapter Five. As concluded from Figure 6.1B, the discrepancy is, at least partially, due to the different affinities of the antibodies used. The quantities of ßC-hCG in pregnancy urine exceeded those of free ßhCG and free αhCG subunits by approximately 3.2 and 8.6 times, respectively. This observation has practical implications for the measurement of this fragment and explains the excellent correlation and agreement noted among some ßC-hCG assays, despite the different abilities of the antibodies in discriminating the ßC-hCG molecules from the hCG subunits.

Using an intact hCG assay with very low cross-reactivities with LH (0.15%), ßLH (<0.1%), α-subunit (0.1%), and ßC-hCG (0.26%) to determine hCG in chromatographed postmenopausal urine to separate hCG from LH, the results indicate the existence of one molecule of hCG per 45mol of LH. It remains uncertain as to whether this ratio would be similar to that obtained using hCG assays in which antibodies are directed against the carboxy-terminal extension. In addition it was demonstrated that the amount of ß-core molecules in postmenopausal urine may be as much as 14 times higher than that of hCG-like material, suggesting that this beta fragment does not originate solely from the hCG metabolism. Akar et al (1990), using a similar criterion to determine the ratio of ß-core to hCG in postmenopausal urine, showed that, in molar amounts ßC-core exceeds those of hCG by approximately 6 times. The difference between the present study and that report denotes the variability among individuals. In addition, Akar et al have raised the hypothesis that this ß-core would be an end-product of LH molecules cross-reacting with their radioimmunoassay for ßC-hCG. The literature indicating the existence of a free ßLH-core in urine of both pre- and postmenopausal women is extensive (*Young et al, 1975; Silva de Sa' et al, 1988).* We have partially purified a ß-core like material from postmenopausal women using a ßLH assay, which cross-reacts (11.3%) with pure ßC-hCG, to monitor the ß-core molecules at each step during the purification procedure (*De Medeiros et al, 1989*). As compared with ßC-hCG from pregnancy urine, this ß-core material was shown to have a smaller molecular weight on gel filtration, nearly the same mobility on SDS-PAGE and different abilities to bind Con A lectin and a strong anion exchange resin, suggesting a different sugar composition in its carbohydrate moieties. However, a different identity for this material will be provided only by determining its amino acid sequence. The significance of the possible interference of urinary free ßLH, if any, with the ß-core assays in non pregnant

individuals is also required. It was shown in Chapter Five that the ßC-hCG radioimmunoassay using a polyclonal antibody raised against pure ßC-hCG cross-reacts significantly with the free ßLH subunit (unlike the IRMA used here) and overestimates the ßC-hCG-like material in non-pregnant individuals.

The analysis of the molar ratio between hCG, ßC-hCG and LH in urine obtained from a subject before the menopause demonstrated the existence of one mol of hCG per 45 molecules of LH. However, the molar amount of ßC-hCG to LH was smaller (0.108 vs 0.326), suggesting that the total amount of ßC-hCG immunoreactivity found in postmenopausal women may result from the degradation of other related glycoproteins. Because the amount of FSH in urine after the menopause is even higher than that of LH, any fragmentation of this gonadotrophin could also contribute to the final amount of ß-core fragment detected in non-pregnant states. While an early report of Akar et al (1988) did not show any difference between the ßC-hCG levels in urine of pre and postmenopausal women, the present study and that of Krichevsky et al (1991) have shown that the urinary concentrations of ßC-hCG in non-pregnant individuals, is highly correlated with the age, tending to be higher after the menopause). Therefore the possibility that ß-core is a more generalized fragment and that the results obtained with the ßC-hCG assays may, in fact, represent the pooled end-product of different glycoproteins deserve further investigation.

The amount of ßC-hCG in pregnancy serum is still in dispute. While the majority of reports have shown no ß-core in serum (*Masure, et al, 1981; Schroeder and Halter, 1983; Kato and Braunstein, 1988*), two recent publications, using very sensitive assays, have demonstrated, after gel separation, that ßC-hCG is present in serum at concentrations ranging from 2.9pmol/l to 125pmol/l (*Wehmann et al, 1990; Alfthan and Stenman, 1990*), representing 0.028-0.3% of the intact hCG levels in this fluid. More recently

Kardana and Cole (1990) demonstrated that the majority of the immunoreactive ßC-hCG detected in serum is found as a high molecular weight-complex rather than as free form. Additionally they showed that this complex can be dissociated into a higher proportion of free molecules, providing a ratio of ßC-hCG to hCG of 0.18-0.91 throughout the pregnancy. Our data showing a relative molar ratio of ßC-hCG/hCG of 0.008 and 0.125, respectively before and after dissociation of the high molecular weight ßC-hCG immunoreactivity, in part confirm the original observation of those investigators. This complex may result from either ßC-hCG binding to serum proteins or polymerization of the monomer form. In any case the resultant complex is less sensitive to ßC-hCG antibodies. Moreover, the measurement of ßC-hCG in neat serum samples is a problem as the ßC-hCG epitopes appear hidden and the levels of intact hCG are high. The present study shows that direct analysis of ßC-hCG in serum may be complicated by matrix effects, giving high results when the levels of this fragment are below 30pmol/l (see Chapter Five); the addition of ammonium thiocyanate as a dissociating agent may however inhibit the antibody binding in a direct assay (personal observation). Any investigation of serum ßC-hCG levels on neat serum on a large scale requires the development of strategies to overcome these drawbacks.

The presence and relative concentrations of hCG and its subunits in the amniotic fluid is well known and this is the first description of the presence of immunoreactive ßC-hCG in this biological compartment. The lower levels of hCG in relation to the high concentrations of free beta and free alpha subunits, are consistent with previous studies (Clements et al, 1976; Barbieri et al, 1986; Ozturk et al, 1988). The relative amount of free alpha hCG and free beta hCG differs from the data of Ozturk et al (1988) but this apparent discrepancy could be due to the different methodology used to estimate the

different glycoproteins. While they have analysed unchromatographed samples, we measured the molar ratio of each hormone after gel separation. Even though a pool of amniotic fluid obtained from different individuals at different times of their pregnancy was used in the present study, it seems unlikely the difference was due only to different ages of gestation. Considering only the authentic ßC-hCG molecules the ratio of free ßC-hCG to hCG was found to be similar to that noted in serum, suggesting an even distribution between the two compartments. However, when the total ßC-hCG immunoreactivity, including the authentic molecules and the dissociated-high molecular weight complex material in both fluids was considered, the amount of ßC-hCG in the serum exceeded that found in amniotic fluid. The source of ßC-hCG in amniotic fluid may reflect active secretion from trophoblastic tissue and passive diffusion from maternal compartment. Because of the low molar concentration relative to hCG, a polarized secretion of ßC-hCG from placenta to the amniotic cavity, as proposed to explain the high levels of free hCG subunits (Ozturk et al, 1988), is unlikely. The immunoreactive ßhCG-small fragment reported in extracts of placenta (Vaitukaitis, 1974; Good et al 1977), and the release of huge amounts of ßC-hCG in placental culture medium reported by Birken and Cole (1988) indicate that placental tissue may synthesize this fragment and could favour the notion of placental transfer. These results, however, are not consistent with Wehmann et al (1990), who were unable to detect ßC-hCG molecules in the medium of trophoblast culture, suggesting another source for the ßC-hCG present in amniotic fluid. Because some studies have shown hCG in fetal tissues and cord blood serum (Crosignani et al, 1972; Huhtaniemi et al, 1978), it is also possible that the ßC-hCG found in amniotic fluid originates from the fetal urine. The hypothesis that fetal tissue (i.e. renal parenchyma) could metabolize intact hCG to a ßC-hCG fragment further excreted into amniotic fluid was tested either by measuring the fragment after gel separation or directly in first voided urine

obtained from newborn subjects. The results showed that the majority of the samples analysed contained hCG, ßhCG, and ßC-hCG, indicating that the fetal urine may be the source of the ßC-hCG found in amniotic fluid.

Because the bulk of information suggests that ßC-hCG is primarily a degradation product of the ßhCG subunit metabolism and because several experimental studies have shown that ovarian tissue can internalize hCG and degrade it to small fragments (*Conn et al, 1978; Amsterdam et al, 1979; Campbell et al, 1981*), the possibility that an injected preparation of hCG free of ßC-hCG contamination could be metabolized to ßC-hCG in this tissue with further appearance of this fragment in follicular fluid was also investigated. The results demonstrated that an immunoreactive ßC-hCG-like material is found in large amounts in follicular fluid of pre-ovulatory follicles of gonadotrophin-hyperstimulated cycles following hCG injection. The data also indicated that this fragment is detected in quantities that exceed those of both hCG and LH molecules, suggesting either ßC-hCG accumulates in this biological compartment or represents a pool of fragments of different gonadotrophins cross-reacting with the ßC-hCG assays. However, this question was not addressed in the present study and a similar evaluation of follicular fluid obtained from follicles of spontaneous cycles is needed. While approximately 25% of follicular fluid samples collected from peri-ovulatory follicles after hyperstimulation had detectable amounts of ßC-hCG by a specific immunoradiometric assay, in as much as 96% immunoreactive ßC-hCG was detected with the radioimmunoassay. Because a matrix effect was observed with follicular fluid with these immunoassays (see Chapter Five), caution is recommended in the interpretation of these results. As ßC-hCG and the whole ßLH subunit are differentiated in only a few amino acids it is understandable that ßC-hCG RIAs may detect a fragment of ßLH or even the

whole ßLH subunit. Although the existence of a similar core for the ßLH subunit is likely, its study is made difficult by the absence of specific assays.

The current study also demonstrated that cultured human granulosa cells metabolize intact hCG to ßC-hCG. This result is consistent with the extensive amount of data showing that ovarian tissue from different species may degrade hCG in vitro while localizing significant metabolism to the granulosa cells (*Rajaniemi and Vanha-Pertulla, 1973; Campbell et al, 1980; Zimniski et al, 1982*). While the ovary is not the major site of metabolism of hCG, variable amounts of injected radiolabelled hCG preparations are internalized and degraded by granulosa/theca lutein cells (*Braendle et al, 1973; Markkanen et al, 1979; Landefeld et al, 1981*). Unfortunately, because these studies used radiolabelled preparations, the nature and characteristics of all end-products could not be followed. Zimniski et al (1982a, 1982b) using a non-radioactive preparation, showed by radioimmunoassay that rat ovarian extract supernatants contained a small ßhCG fragment after single supra-physiological injection of hCG. Using human granulosa cells previously exposed to hCG the present study also demonstrated the release of immunoreactive ßC-hCG fragment in the cultured medium. The prolonged exposure of ovarian cells to LH/hCG in vitro or in vivo may induce refractoriness to a subsequent challenge (*Marsh et al, 1983*) either by loss of binding sites (*Conti et al, 1976*) or alteration in the receptor affinity (*Lamprecht et al, 1977*). However, our results indicated that human granulosa cells in vitro incorporate and metabolize intact hCG even after prolonged exposure to LH/hCG. This observation is consistent with the results of Habberfield et al, (1986) showing that, in rat testis Leydig cells, rapid recycling of the LH/hCG receptors occurs on the cell surface. A similar mechanism could occur in the human granulosa cells, maintaining the capacity of these cells to bind fresh hormone.

Serum from men with non-seminomatous testicular germ cells contains hCG, αhCG and ßhCG like-native subunits and a high molecular weight form of ßhCG (Mr >70000) (*Mann and Karl, 19*83). The free beta hCG subunit has also been detected in serum of normal men or serum and urine of male patients with germinal cell tumours (*Saito et al, 1988*). In addition, a ßC-hCG-like fragment has been reported in urine from healthy male individuals (*Akar et al, 1988; Kardana et al, 1988*). Using either RIAs or IRMAs with variable specificities, FSH, LH, intact hCG-like material and free ßhCG-like subunits have also been detected in normal testicular tissue, sperm cells, and seminal plasma (*Braunstein et al, 1975; Braunstein et al, 1979; Asch et al, 1979; Krause, 1979; Brotherton, 1989*). Using a ßC-hCG specific IRMA, an immunoreactive ßC-hCG material was detected in 42 out of 74 seminal plasma samples (56.7%) obtained from different men from infertile couples, but otherwise healthy individuals. The levels ranged between 5.5pmol/l to 59.5pmol/l and did not correlate with any characteristics of the spermatozoa (data not shown). Chromatographic separation of one pool of some ßC-hCG positive seminal plasma samples demonstrated that all immunoreactive ßC-hCG material in this biological fluid, with a native apparent molecular weight > Mr 70000, can be dissociated generating higher quantities of smaller ß-core immunoreactive material. It means that this material is not only composed of the monomeric ßC-hCG form, but could represent a mixture of monomer, dimer forms or other fragments cross-reacting with the ß-core assay. In addition to a peak of authentic hCG material in these fractions, intact hCG immunoreactivity was also found in the material with high molecular weight. This observation is consistent with that noted in several biological fluids in which a high molecular weight form of immunoreactive intact hCG can be detected.

Using a highly specific assay Brotherton (1989) showed that intact hCG was present in 36.8% of seminal plasma samples, levels ranging from 0.8pmol/l to 71.6pmol/l, and did not correlate with any semen parameter. In turn, Saito et al (1988) examined the free beta hCG immunoreactivity in seminal plasma of 109 individuals and correlated their findings with the seminal characteristics. They reported ßhCG levels ranging from 40.5 to 94.5pmol/l, 65.3 to 123.8pmol/l and 94.5 to 238pmol/l for azoospermic, mild and severe oligozoospermic, and normozoospermic individuals, respectively, suggesting a positive correlation between sperm counts and seminal hCG-like levels. It is very unlikely, however, that only ßhCG correlates with seminal characteristics and further studies will be necessary. Because hCG-like and related molecules present in normal body fluids or tissues are probably locally produced it is of interest to consider the source of these glycoproteins in seminal plasma. Although the present study and that of Brotherton (1989) did not address this matter, Saito et al (1988) provided evidence that most of the ßhCG molecules are synthesized by germinal cells of the testis and a small portion may be synthesized by the prostate gland.

Wehmann's group has studied the metabolic fate of hCG, αhCG and ßhCG in the human (*Wehmann and Nisula, 1980; Wehmann and Nisula, 1981*). They showed that only 21.7% of intact hCG and approximately 1% of αhCG and ßhCG subunits are excreted in urine without any modification. Therefore, the majority of the injected material is either excreted by the renal system with such extensive modification that it is undetected by specific assays against the original material or goes into alternative metabolic pathways in the body. Although experimental studies using radiolabelled gonadotrophins have indicated that other tissues (i.e. liver, ovary) can clear these hormones from the blood stream to a lesser extent, the kidneys were confirmed to be the most important organ for their metabolism and excretion

(*Markannen and Rajaniemi, 1979; Birken et al, 1990*). Together, these previous studies strongly indicate that the majority of gonadotrophin molecules are in fact modified by the renal parenchyma before being excreted in urine. However, the clearance of each hormone and the final metabolic products depend on the degree of heterogeneity of the primary molecule. The principal product of hCG/ßhCG found in urine is the ßC-hCG fragment (*Lefort et al, 1986*) but a spectrum of intermediate products may also be excreted. Recently the metabolism of this fragment was studied by Wehmann's group who showed that after intravascular injection of a pure preparation only 8.15% of the amount injected appeared in the urine, suggesting that this fragment may be cleared from the plasma compartment by other ways (*Wehmann et al, 1989*). Even though limited to a few cases, to a short sampling time, and using a different route of administration, the data of the present study confirmed the increment of ßC-hCG in the urine after injection of intact hCG and reinforce the importance of the renal parenchyma in its origin. The large amount of ßC-hCG produced by placental tissue (*Cole and Birken, 1988*) may also be cleared by different tissues or be slowly released in urine. The recent evidence that ßC-hCG in serum is associated in a high molecular weight complex may, at least partially, impede the rapid renal excretion of ßC-hCG released from the placenta, explaining why the serum ßC-hCG does not influence the amount found in pregnant urine.

CHAPTER SEVEN

URINARY CONCENTRATIONS OF ßC-hCG FRAGMENT THROUGHOUT PREGNANCY

7.1 Introduction

Although widely distributed in body fluids ßC-hCG fragment is found in large quantities only in the pregnancy urine, where it has been reported to account for 15% - 750% of the total hCG immunoreactivity (*O'Connor et al, 1988; Cole and Birken, 1988*). In small series of unchromatographed urine samples from pregnancy, the concentrations of ßC-hCG were reported to range between 1.1 nmol/l to 400 nmol/l (*Akar et al, 1988*). Using four different assays, concentrations ranging from 5.6 nmol/l to 372.5 nmol/l in samples, taken between 20-40 weeks after the last menstrual period, have been described in section 5.3.3. The absolute concentrations and the pattern of ßC-hCG secretion throughout pregnancy, remain to be determined.

Data on the relative concentrations of ßC-hCG and intact hCG are either incomplete or inconsistent. While some investigators have found lower levels of ßC-hCG than intact hCG in the first trimester (*Cole and Birken, 1988; Kato and Braunstein, 1988*), others have reported that the ßC-hCG levels are higher than those of hCG at all stages of pregnancy (*Schroeder and Halter, 1988; Krichevsky et al, 1991*). Equal or higher ratios between both molecules over the second or third trimester were reported as well (*Cole and Birken, 1988; Kato and Braunstein, 1988*). Because ßC-hCG is a putative end-product of the intact hCG metabolism, the relationship between serum hCG and urinary ßC-hCG has been the subject of analysis. Schroeder and Halter (1983) showed that although the ratio between both glycoproteins in urine

varies over a broad range during pregnancy, the concentrations of ßC-hCG appear to parallel those of hCG. Krichevsky et al (1991) demonstrated that when the level of hCG falls, so does that of the ßC-hCG. Even though these studies indicate a relationship between ßC-hCG and hCG concentrations in pregnancy, Cole and Birken (1988) surprisingly found that while the concentration of ßC-hCG in urine was low between 5-9 weeks and increased between 11-19 weeks of pregnancy, the concentrations of hCG dropped from 7-11 to 12-19 weeks, indicating that the levels of ßC-hCG in urine are not directly related to the levels of hCG in either serum or urine.

While ßC-hCG is found in highest concentration in pregnancy urine, its physiological significance in this fluid and its potential use in the development of new methods for diagnosis of normal or abnormal pregnancy have not been investigated. The significance of ßC-hCG to pregnancy testing is not known either. The aim of this study was to determine a reference range for immunoreactive ßC-hCG throughout pregnancy, to analyse the relative proportion of urinary ßC-hCG to intact hCG, and to investigate the potential role of this fragment in the early diagnosis of human pregnancy.

7.2 Methods

7.2.1 Collection and processing of samples

Early morning urine samples from patients treated on the artificial donor insemination programme at the Reproductive Medicine Unit (The Queen Elizabeth Hospital), were collected on a daily basis from day 4 after insemination to menses or one week after confirming pregnancy. After collection the samples were kept at home (at $4^{o}C$) and bought to the laboratory usually within 2-3 days. Each sample was centrifuged at 1850 x g

for 10 minutes to remove debris and stored at -20^{o}C until assay for ßC-hCG and intact hCG. All patients had been informed that the urine collection was for research purposes and had given consent; the protocol was approved by the Ethical Committees of the University of Adelaide and The Queen Elizabeth Hospital. To establish a reference range for urinary ßC-hCG throughout pregnancy, single urine samples from 741 healthy pregnant women between 6-41 weeks gestation, were collected at random from overtly healthy subjects at the antenatal clinic of the Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital. As ßC-hCG had been shown to be highly stable, no preservatives were used in either study. All samples were initially centrifuged at 1850 x g for 10 minutes and stored at -20^{o}C until assay for ßC-hCG and hCG.

7.2.2 Creatinine determination

Creatinine content in urine samples was analysed according to the Jaffe's principle (*Heinegard and Tiderstorm, 1973*) using Beckman creatinine reagent kits. All tests were performed following the procedures recommended by the manufacturer (Beckman leaflet).

7.2.3 Immunoassays

ßC-hCG was estimated by immunoradiometric assay (IRMA I) using the antibodies DeM3 and 32H2 and intact hCG was also analysed by the specific immunoradiometric assay (hCG IRMA I) designed with the antibodies 3/6 and 11/6 (see chapter two, section 2.11.4.1 and Chapter five, section 5.3.2.2, for detail). The high-dose hook effect phenomenon was avoided by diluting each sample 100, 500 and 1000 times before assay, and results chosen were those that fitted to the best point of the standard curves.

7.2.4 Analysis of data

In determining the reference intervals for intact hCG and ßC-hCG, gestational age was established, in all cases, using the last menstrual period (LMP) and/or ultrasound. Because the data were not normally distributed the reference ranges were estimated by ranking and taking the 10th, 50th and 90th percentiles for the values obtained at each week. Comparison between the levels of different weeks was made using analysis of variance and the non-parametric Mann-Whitney U test. Two samples were considered statistically different with a probability level of $P<0.05$. To compare any increase in the levels of hCG or ßC-hCG in the subjects whose pregnancy resulted from donor insemination, ovulation was defined as occurring 24h after the LH surge. The results of each hormone following the estimated time of ovulation in this group are presented as mean and 95% confidence intervals. The rise in hCG or ßC-hCG was defined at the time that their mean level were statistically higher than the mean of the baseline values.

7.3 Results

Figure 7.1 and table 7.1 show the urinary levels of ßC-hCG throughout the pregnancy and the results are indicated in nmol ßC-hCG per mmol creatinine. A wide fluctuation in the ßC-hCG immunoactivity was noted among individuals and tended to increase as the magnitude of the measured value increases. Weekly analysis demonstrated that ßC-hCG reached its maximum level between the eighth and the fifteenth week. The median

Table 7.1 Percentiles 10, 50, and 90 of the weekly ßC-hCG and hCG levels* in urine of pregnancy women.

Week following LMP**	ßC-hCG			hCG		
	10	50	90	10	50	90
6(10)***	9.2	10.7	22.7	3.4	6.5	8.5
7(9)	4.0	14.9	24.4	3.0	5.0	16.2
8(18)	15.1	23.8	45.9	4.6	8.9	20.5
9(16)	7.5	24.9	55.2	3.0	6.1	13.1
10(22)	23.4	39.0	52.5	6.0	8.0	16.6
11(17)	7.2	27.8	40.6	4.4	6.7	14.1
12(25)	13.3	24.4	48.7	1.1	4.3	9.4
13(15)	14.4	24.9	46.1	0.8	2.6	4.9
14(26)	4.8	20.2	47.3	0.7	2.8	5.6
15(22)	8.8	15.1	40.1	1.1	1.9	3.7
16(19)	4.5	14.3	29.4	0.5	3.0	4.7
17(31)	5.5	11.6	23.7	0.9	2.0	4.0
18(17)	6.3	11.1	19.0	0.6	1.6	3.0
19(25)	2.7	8.1	18.2	0.4	1.3	3.9
20(18)	2.3	5.3	18.1	0.5	1.2	2.1
21(29)	3.4	6.7	21.3	0.4	1.9	4.2
22(31)	1.5	4.1	15.9	0.3	1.3	3.6
23(28)	1.2	5.8	9.4	0.6	1.3	3.9
24(21)	1.6	3.5	12.3	0.3	1.3	3.6
25(29)	0.9	3.6	11.7	0.3	1.4	3.2
26(19)	1.4	4.6	9.2	0.3	1.2	2.8
27(28)	0.6	4.6	13.9	0.3	1.8	4.1
28(16)	2.2	3.7	7.4	0.3	1.5	3.3
29(31)	1.0	3.9	12.9	0.4	1.5	5.1
30(18)	1.9	6.8	12.8	0.9	2.8	5.2
31(18)	1.2	4.9	10.8	0.9	1.5	4.4
32(16)	2.7	6.3	15.5	0.4	1.9	4.8
33(18)	2.4	6.4	12.1	0.6	1.8	4.0
34(18)	2.8	6.8	11.3	0.6	1.9	5.2
35(18)	2.4	4.8	11.3	0.5	2.2	4.6
36(18)	2.3	8.6	11.9	0.5	3.2	4.2
37(18)	1.5	3.9	12.1	0.1	1.1	3.2
38(20)	1.5	3.6	9.8	0.3	1.2	3.1
39(17)	1.5	4.0	13.3	0.6	1.6	2.7
40(18)	1.0	5.8	12.2	0.2	1.4	2.7
41(25)	1.4	4.4	8.6	0.7	1.7	4.0

* Levels are given as nmol of ßC-hCG or hCG per mmol creatinine
** Last menstrual period.
*** Numbers in parenthesis denote total individuals examined at each week

concentrations rose gradually from 10.7 (9.2-22.7) to 39.0 (23.4-52.5) nmol ßC-hCG per mmol creatinine from week 6 to week 10 of gestation and then decreased to achieve the lowest level between the 20th and 29th week, at which time the levels ranged from 3.9 to 5.3 nmol ßC-hCG per mmol creatinine. A slight statistically non significant increase was observed between the 30th and 36th week, representing an approximately 1.4-fold higher level than that noted between the 20-29th week. The concentrations of ßC-hCG remained relatively constant during the next five weeks, and varied between 3.6 to 5.8 nmol per mmol creatinine.

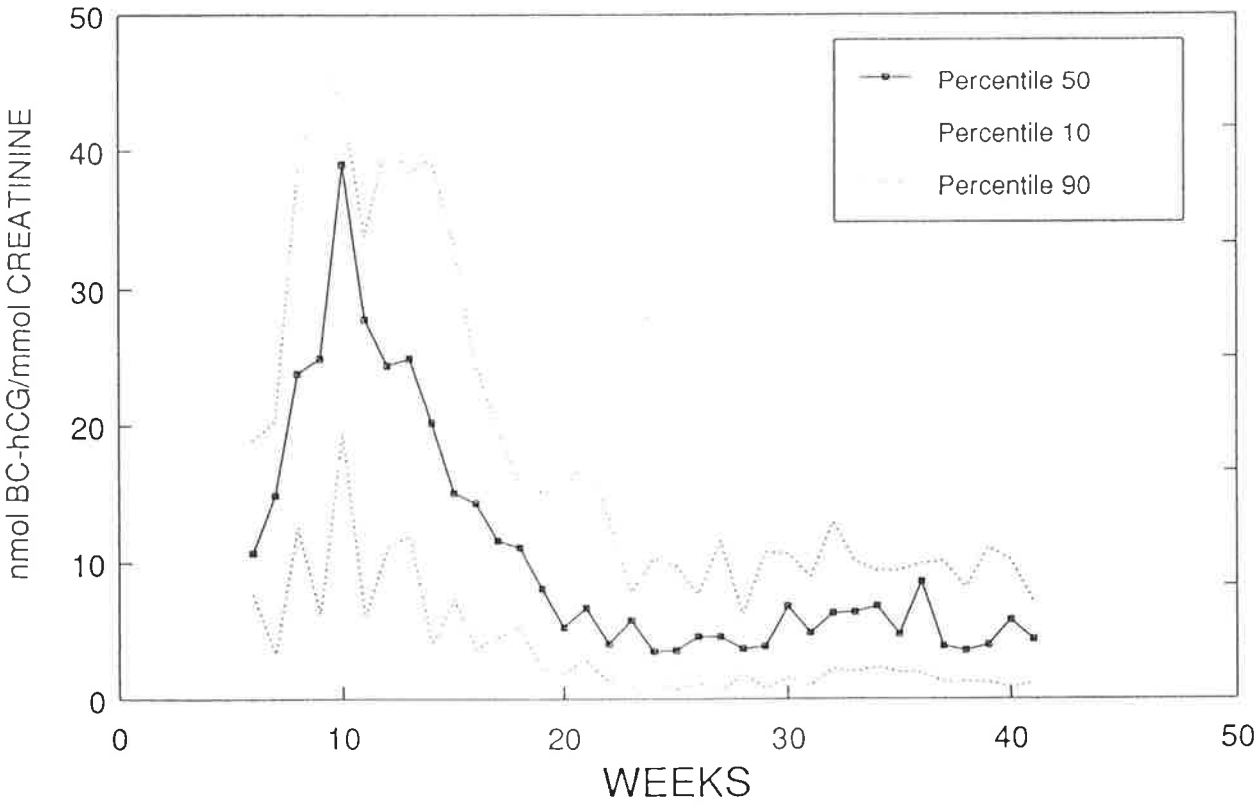


Figure 7.1

Percentiles (10,50, and 90) of the weekly ßC-hCG concentrations (nmol ßC-hCG per mmol creatinine) in 741 normal pregnancies relative to the last menstrual period.

Figure 7.2 illustrates the urinary concentrations of hCG, on a weekly basis between the 6th and the 41st week of pregnancy. The results were corrected for creatinine excretion as well and are expressed in nmol hCG per mmol creatinine (Table 7.1). The median concentrations rose from week 6, peaked between the 8th and the 12th week (levels ranged from 4.3 to 8.9 nmol hCG per mmol creatinine), and then decreased until the 20th week.

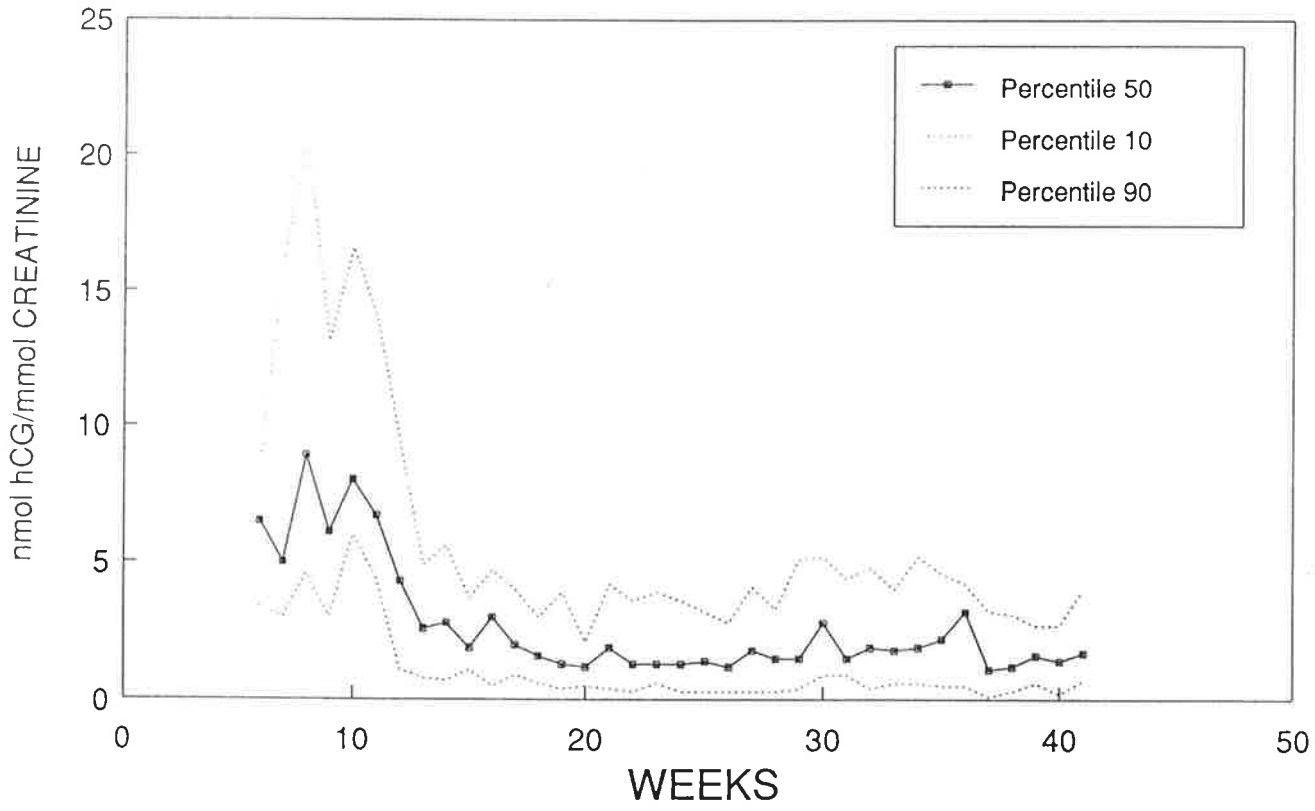


Figure 7.2

Percentiles (10, 50 and 90) of the weekly hCG concentrations (nmol hCG per mmol creatinine) in 741 normal pregnancies, relative to the last menstrual period.

The concentrations remained constant between the 20th and the 30th week, levels ranging from 1.2 to 1.9 nmol hCG per mmol creatinine. Between the 30th and 36th week the levels increased again, remaining between 1.5 and 3.2 nmol hCG/mmol creatinine. However, a comparison of the mean levels between the 20-29th week (1.44 ± 0.24) and the 30th-36th week (2.18 ± 0.6) demonstrated this small increase is not significant ($P>0.05$). Even though the concentrations were lower, the pattern of urinary hCG was similar to that of ßC-hCG. The ratio between ßC-hCG and hCG varied from 160% to 957% between the 6th and the 41st week of pregnancy and the highest amounts of ßC-hCG, in relation to the intact hormone, were detected between the 9th and the 19th week (Figure 7.3).

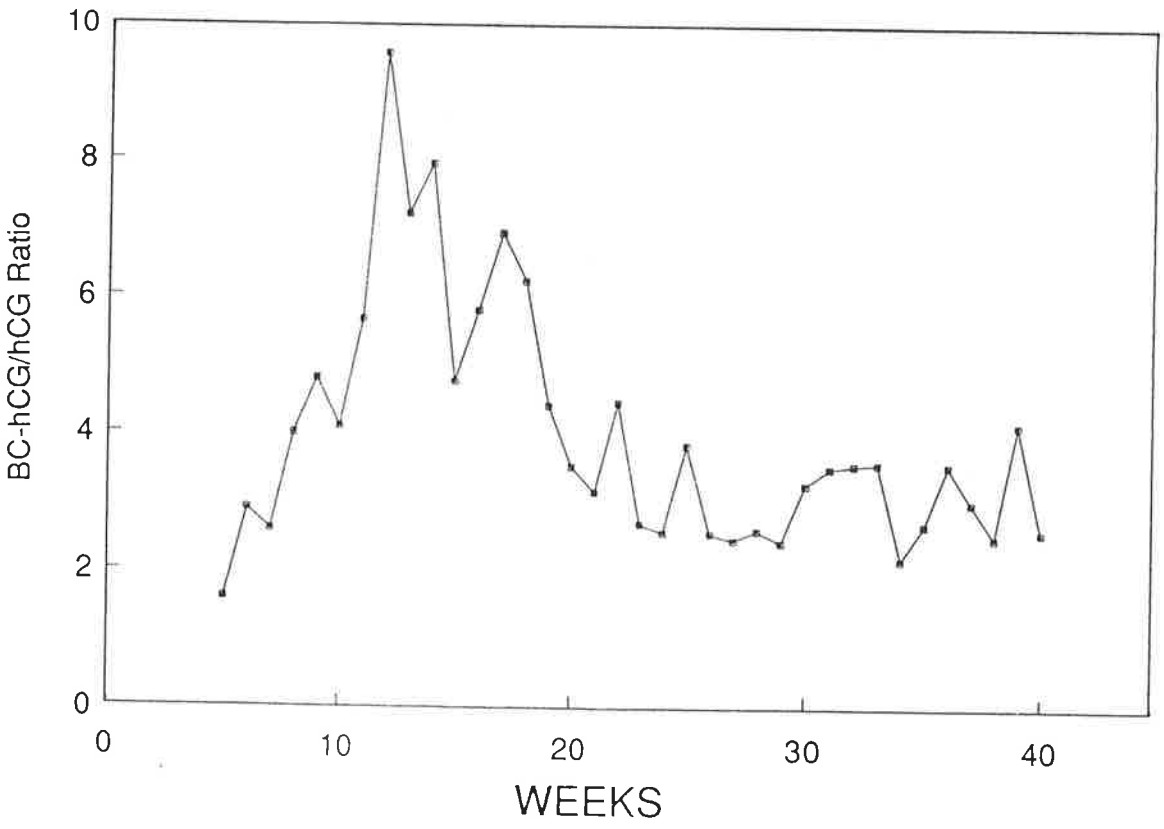


Figure 7.3

Relative proportion between ßC-hCG and intact hCG levels during pregnancy. The absolute values of both hormones were initially corrected for creatinine excretion.

The baseline concentration of ßC-hCG following artificial donor insemination (n=6) was 3.3 ±1.4pmol/l (a level between the sensitivity [2.5pmol/l] and the limit of detection [5.0pmol/l] of the assay) and on day 12 was 6.5±2.1pmol/l ($P<0.05$). Therefore, the first evidence of pregnancy, based on the appearance of ßC-hCG in urine, was detected 12 days after the estimated time of ovulation. Taking the 95% confidence intervals for daily measurements following day 12, a positive result was achieved by day 15 (Figure 7.4A). This result indicates that the increment in the levels of ßC-hCG in the early stage of pregnancy is slow and the doubling time is approximately 24-48h (Table 7.2).

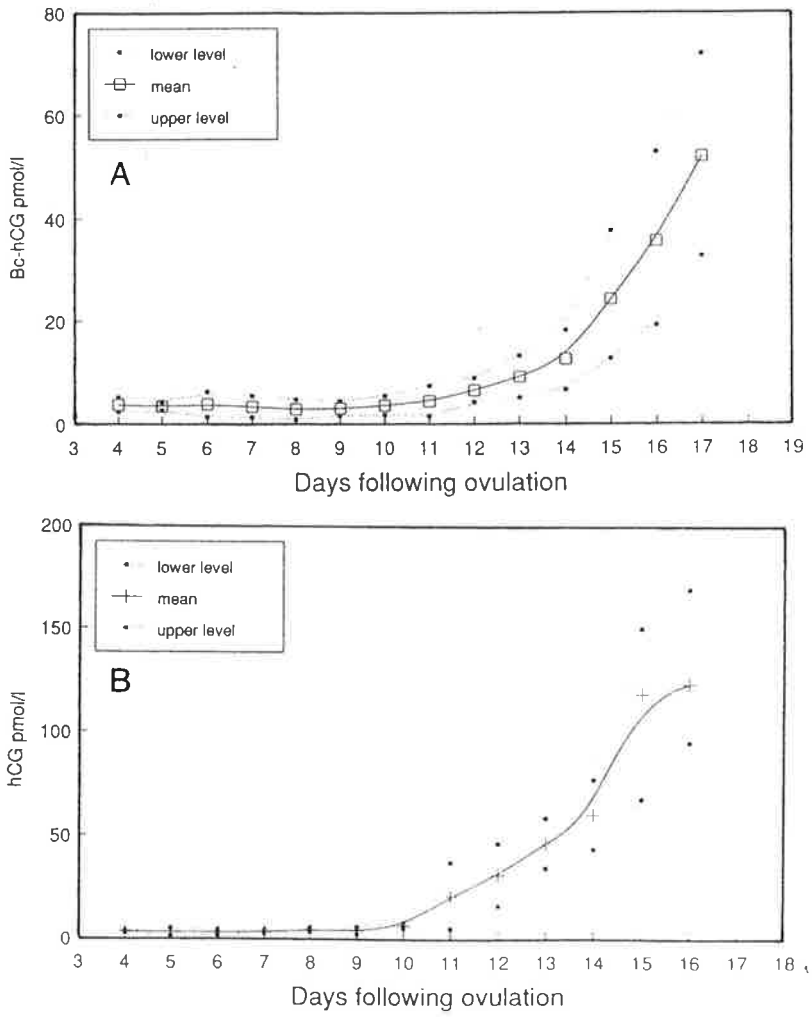


Figure 7.4

(A) Urinary ßC-hCG and **(B)** hCG levels (mean and 95th confidence intervals) in six women whose pregnancy resulted from artificial insemination

The baseline levels of hCG after artificial insemination were 3.5+1.1pmol/l (between the sensitivity [1.5pmol/l] and the limit of detection [5.0pmol/l] and increased to 6.3±0.5pmol/l on day 10 ($P<0.05$), signalling the existence of pregnancy. The positive result, taking the 95% confidence intervals for daily measurement following day 10, was confirmed on day 12 (Figure 7.4B). The increment in the concentrations of hCG was rapid, with the doubling time of 24-36h. In non-conception cycles (n=5) both hCG and ßC-hCG remained under the limit of detection of the assays up to the start of the next menstrual period.

Table 7.2 Mean and upper and lower results of hCG and ßC-hCG concentrations following artificial insemination (n=6)*

Day after Insemination	hCG Lower	Mean	Upper	ßC-hCG Lower	Mean	Upper
+4	2.9	3.4	3.7	2.4	3.8	5.2
5	1.2	3.1	4.9	2.7	3.5	4.3
6	1.6	2.8	4.0	1.5	3.9	6.3
7	2.4	3.2	4.0	1.3	3.4	5.5
8	3.1	4.2	5.3	1.0	2.9	4.8
9	2.2	3.9	5.6	1.6	3.0	4.4
10	5.0	6.3	7.5	1.8	3.6	5.5
11	4.8	18.1	32.4	1.5	4.4	7.4
12	16.1	31.2	46.3	4.2	6.5	8.8
13	34.5	46.6	58.7	5.1	9.1	13.1
14	43.7	60.4	77.2	5.5	12.5	18.1
15	67.6	118.0	150.0	12.6	24.2	37.5
16	95.0	123.0	169.0	19.1	35.6	52.8

* Results are given in pmol/l

7.4 Discussion

In addition to being widely distributed in other body fluids, the ßC-hCG molecule accounts for the majority of the hCG immunoreactivity in urine of pregnant women. Because many assays directed against the hCG molecule or its free beta subunit may also detect this fragment as a significant cross-reactant, discordant results in some hCG/ßhCG detection procedures have been reported (*Filstein et al, 1983; Hussa et al, 1985*). Therefore, in addition to any physiological interest, the ßC-hCG fragment has considerable clinical importance with respect to measurement of urinary hCG. The hCG assay used here is highly specific to the intact hormone and, on a molar basis, exhibits a cross-reactivity with ßC-hCG of 0.26%. On the other hand, the ßC-hCG assay cross-reacts with the free beta subunit of hCG and intact hCG in 5.3% and 2.1%, respectively (see Chapter five, section 5.3.2.2). The results presented here must be interpreted under the notion of these cross-reactivities, although the high molar ratio of ßC-hCG to hCG makes significant errors unlikely.

Besides the ability of different assays to discriminate ßC-hCG from related glycoproteins, the establishment of a reference range for ßC-hCG throughout the pregnancy is essential for subsequent clinical studies. Because ßC-hCG is found in huge amounts in urine and its measurement in serum has major problems this study established a reference range for ßC-hCG in urine. Even though hCG was not measured in serum, a close relationship between serum and urinary values for hCG have been reported (*McCready et al 1978; Wu et al, 1987;, Norman et al, 1988*). The current study, therefore, compares urinary ßC-hCG with hCG concentrations. Some guidelines for adequacy in their determination were followed because correct interpretation and clinical application of ßC-hCG require accurate reference

ranges. Possible problems resulting from the interference of other substances in urine with the assays, the influence of the standard preparations, the affinities of antibodies used, sensitivity and specificity of the assays, the need for serial sample dilution because the high levels of ßC-hCG and hCG, and the variability with volume of urine or the time of voiding, were all factors considered. The assays used here are not affected by the urine matrix itself and do not crossreact significantly with related molecules (however, fragments such as nicked forms of ßhCG were not tested). The intact hCG standard may be slightly contaminated with ßC-hCG (*Wehmann et al, 1988*) but the ßC-hCG used is highly pure and was calibrated against Birken's reference preparation. Care was taken to maximize the probability that this reference population would be representative of normal pregnancy women. Despite this, a cross-sectional investigation should be complemented with a longitudinal assessment of changes during normal pregnancy. In the current study the concentrations of ßC-hCG and hCG corrected for creatinine excretion is reported in nearly 750 urine samples collected at random from different individuals between the 6th and the 41st weeks of gestation. The molar ratio between ßC-hCG and hCG along this period is also presented. To investigate the occurrence of ßC-hCG in the early diagnosis of pregnancy, the concentrations of hCG and ßC-hCG in urine were measured from women whose pregnancy resulted from artificial insemination, following a well documented LH surge.

The concentrations of ßC-hCG during the first half of pregnancy and the ratio between the fragment and the intact hCG have not been well characterized previously. Schroeder and Halter (1983), used the difference between a specific radioreceptor assay to measure intact hCG and a radioimmunoassay able to detect both hCG and ßC-hCG, and claimed that in 70% of 94 women between the 3rd and 24th week of pregnancy approximately 70% of the urinary hCG immunoreactivity was due to ßC-hCG

and in almost 98% the fragment represented at least 30% of the hCG amount. In addition, these investigators showed that the activity of both proteins peaked at the 8th week, varying widely among individuals over a range of 9% and 98%. Even though well conducted, this study compared ratios from two distinct methods giving results that are not strictly comparable (*Hussa, 1987*). Analysing individual sample after size exclusion chromatography, Kato and Braunstein (1988) reported that ßC-hCG represented 25% to greater than 100% of the total hCG immunoactivity between the 6th and 20th week. However, because of the laborious protocol, they limited the study to a few samples. After correcting the results for creatinine excretion and including 300 subjects at this period, this study demonstrated that the median concentrations of ßC-hCG are higher than those of hCG, peaking between the 8th and 15th week, and gradually decreasing afterward to achieve the lowest level after the 20th week of gestation. Krichevsky et al (1991) have followed a single individual taking samples twice a week and their results, after correcting the values for creatinine excretion, were similar to the findings of this study. Before the 20th week our results indicate that the ßC-hCG levels exceeded those of hCG by approximately 1.6-9.5 fold, representing 167% - 957% of the total hCG immunoactivity content in pregnancy urine. This amount is higher than that found in the majority of reports so far published (*Schroeder and Halter, 1983; Kato and Braunstein, 1988; Akar et al, 1988; O'Connor et al, 1988*) but is comparable to that reported by Cole and Birken (1988) who found an excess of ßC-hCG of approximately 1.1 to 7.4 fold. The difference noted between studies may be explained by the use of different assays.

Previous studies have indicated that between the 20th and 41st week the concentrations of ßC-hCG relative to the intact hCG may be equivalent (*Krichevsky et al, 1991*), lower (*Schroeder and Halter, 1983*), higher (*Cole and*

Birken, 1988), or virtually the only form present in pregnancy urine (*Kato and Braunstein, 1988*). Analysing urines of 441 different subjects for both hormones during this period of gestation the present study demonstrated that, when corrected for creatinine excretion, the ßC-hCG quantity exceeded that of hCG at each time point, representing approximately 218%-440% of the hCG immunoactivity.

The source of ßC-hCG has been controversial (see Chapter five). The demonstration that granulosa cells in culture can degrade hCG to a immunoreactive ßC-hCG fragment and the possible degradation of hCG by different tissues in the body and further release of its end-products into urine deserves consideration. If the hypothesis that urinary ßC-hCG represents a metabolite of hCG/ßhCG is accepted, this would imply a close and unchanged ratio between serum and urinary concentrations of hCG and ßC-hCG. A relationship between urinary levels of both proteins would be expected as well. While the serum concentrations of hCG were not analysed, similar patterns for hCG and ßC-hCG concentrations throughout pregnancy were found. Lacks of parallelism between serum hCG and urinary ßC-hCG or an absence of relationship between both hormones in urine have been reported (*Cole and Birken, 1988; Kato and Braunstein, 1988*) but the results were limited to a few cases and were not corrected for creatinine excretion, preventing an adequate comparison.

Higher concentrations of ßC-hCG in pregnancy urine, indicate either significant contribution of other maternal compartments (i.e. placental production, enhanced peripheral conversion of hCG/ßhCG in the kidney) to the total amount of the ßC-hCG fragment found in urine or a change in the catabolism of hCG to ßC-hCG over the gestation. Since a few studies have suggested that placental tissue may synthesize large amounts of ßC-hCG and

a high molecular weight immunoreactive form of ßC-hCG found in serum could be excreted after dissociating to a monomeric form, the possibility that sources other than the renal catabolism of hCG account for the large amount of ßC-hCG in pregnancy urine can not be eliminated (*Cole and Birken, 1988; Kardana and Cole, 1990*). Additionally, if direct synthesis by placental tissue is accepted, a varying production of ßC-hCG at different stages of pregnancy may exist.

The kidney plays a dual role in hCG excretion: elimination of unaltered hCG molecules and processing of the intact molecule to small fragments within the lysosomes of the proximal tubule cells (*Nisula et al, 1989*). Because the maternal kidney does not synthesize hCG, the higher levels of ßC-hCG in pregnancy urine suggests that the majority of hCG molecules taken up by the renal system are degraded by the renal parenchyma and excreted as ßC-hCG fragment. Also, the mechanisms that modulate glomerular filtration and tubular uptake could vary over the pregnancy, explaining the changing ratio between ßC-hCG and hCG in pregnancy urine. Although little is known about the mechanisms controlling the rate of tubule cells uptake, microheterogeneity of hCG is speculated to account for the sort of end-products excreted. The presence of sialic acid and the charge of the hCG/ßhCG molecules appears to influence this process (*Markkanen and Rajaniemi, 1973; Nisula et al, 1989*). In particular, the findings of Hay (1986) demonstrating that forms of hCG become less acidic with the advance of gestation indicate variation in the way that the kidney processes intact hCG. The less acidic molecule may cross the glomerular basement membrane more rapidly than acid forms (*Brenner et al, 1978*). Additionally, as desialylated hCG is cleared rapidly by the liver and acid forms are preferentially uptaken by the kidney, the metabolic fate of hCG may shift during the pregnancy resulting in an unbalanced proportion between

the amount of intact and fragmented forms of hCG in urine, explaining the higher concentrations of ßC-hCG.

As compared with the intact hCG immunoactivity, a slower decrease in the concentrations of ßC-hCG between the 12th and 20th week was noted (Figure 7.1), resulting a highest ratio between both molecules over this period. Because hCG and ßC-hCG peaked at the same period of pregnancy, this discrepancy may be due to the slow release of ßC-hCG into the urine (*Wehmann and Nisula, 1980; Wehmann et al, 1984*). Wehmann et al (1989) speculated that this slow release of ßC-hCG into the urine either originates from a slow turnover of the renal parenchyman pool or from an extra renal compartment of pre-sequestrated ßC-hCG molecules.

Measurement of intact hCG and ßC-hCG in urines collected daily from six women whose pregnancy resulted from artificial insemination demonstrated that the earliest increase of each hormone was detected 11 and 12 days respectively after the estimated time of ovulation. This result, is consistent with the findings of Wehmann and Nisula (1981) who suggested that ßC-hCG is a metabolic product of hCG with delayed appearance in urine. The suggestion is that ßC-hCG does not offer any gain over the well established methods for diagnosis of pregnancy with respect to early detection. This does not discount the value of ßC-hCG in the urine of subjects with gestational trophoblastic disease however.

CHAPTER EIGHT

Concluding Remarks

Several altered forms of human chorionic gonadotrophin have been identified in different physiological and pathological conditions. In addition to the normal or abnormal molecules of intact hCG and its free alpha and beta subunits, a number of native fragments have also been detected in body fluids. The full physiological significance and clinical importance of the majority of these altered forms and fragments, however, are not yet established. Because of the large amount found in pregnancy urine, the wide distribution, and potential clinical application, this study was carried out to analyse in detail a native small fragment of the hCG/ßhCG subunit, the ß-core hCG fragment (ßC-hCG). Because little is known about its physical properties, the stability of this fragment was initially tested under different laboratory conditions to set up the basis for further studies. It was demonstrated that the immunoreactive ßC-hCG levels are not altered, even after six months of storage, in samples both with and without preservative, regardless the temperature at which the samples are stored. Additionally, the ßC-hCG immunoreactivity is not altered by submitting the samples to successive freezing-thawing cycles or by correcting the pH of samples within a range 5-8. These results indicate that the chromatographic procedures to purify the molecule would not demand the addition of preservatives and fractions could be stored at 4°C or -20°C until further procedures. Moreover, for the clinical purpose of ßC-hCG detection urine can be stored at room temperature overnight before transfer to the laboratory, and any delay does not invalidate the measurement. It is concluded that ßC-hCG is a very stable molecule.

ßC-hCG was purified from fresh pregnancy urine specimens. Its structure and composition were analysed and compared with those previously proposed by other groups using different protocols. SDS-PAGE separation of reduced ßC-hCG demonstrated it is present in urine as two forms: one, representing the majority of the molecules, yields two major bands with apparent molecular weights of Mr 8900 and Mr 7500, and another, present in small quantities, yields an additional third band with molecular weight of approximately Mr 3500. The molecular weight of the agalacto ßC-hCG was estimated to be Mr 10218 from the amino acid analysis after HPLC separation. Moreover, HPLC separation of its RCM-peptides resulted in three peaks, but only two of them were sequenced and demonstrated to be the ß6-40 (Mr 5000) and ß55-92 (Mr 5300) peptides of the ßhCG subunit. The third peak, present in small amount, did not give any sequence. Analysis of the carbohydrate composition demonstrated that 56%-78% of ßC-hCG molecules of molecular weight Mr 12800 are able to bind Con A. Lectin blots indicated that while 98% of ßC-hCG molecules are lacking all the peripheral monosaccharides and terminate in mannose, 1%-2% retain some sugar residues on their antennae. Approximately 22%-44% of the ßC-hCG molecules do not bind Con A, of which 88% appears to be deprived of any sugar units, do not interact with Mono Q resin, and have a molecular weight of approximately Mr 10000 (Con A and anion exchange non-reactive ßC-hCG form). The remaining 12% appears to be incompletely trimmed in the oligosaccharide chains and binds to Mono Q (Con A non-reactive, anion exchange-reactive form). It was concluded that 1) a third peptide chain may be present, even in a small proportion of the molecules, in the amino acid structure of ßC-hCG, 2) ßC-hCG has significant microheterogeneity on its N-linked oligosaccharide moieties.

The highly pure preparation of Con A-bound ßC-hCG was used to raise a specific polyclonal antiserum in rabbit. Two new methods, one radioimmunoassay (RIA) and one immunoradiometric assay (IRMA), were validated for detection of ßC-hCG in body fluids and their performance were compared with two other assays designed for ßC-hCG quantification. The RIA using the new raised polyclonal antibody (mean affinity constant of 1.21 x 10^{10}l/mol) is sensitive to 5pmol/l and has significant cross-reaction only with the free ßLH subunit. The IRMA, designed in a liquid phase, used the same polyclonal antibody associated with a ^{125}I-labelled mouse monoclonal antibody (32H2) raised against ßhCG, is sensitive to 1.5pmol/l, and does not cross-react significantly with any related glycoprotein. Both assays exhibited some non-specific interference, usually up to 30pmol/l, when serum, plasma, and follicular fluid were used as matrices. Urine did not interfere with ßC-hCG quantification in any assay. Comparison between these two assays and two others previously published was made by measuring ßC-hCG in urine from healthy pregnant women (n=47). They have shown good agreement and correlation coefficients (r) higher than 0.960 with any combination. Therefore, despite different affinities of the antibodies and different cross-reactivities with related glycoproteins, it is concluded that the four assays examined may be equally employed to detect ßC-hCG in pregnancy urine. Analysis of urine from non-pregnant subjects indicated that ßC-hCG increases with age, suggesting an age-related decline in ßC-hCG levels, an increase in the pituitary hCG secretion or cross-reactivity of ßC-hCG assays with other glycoprotein fragments. The IRMA-I showed detectable ßC-hCG in 8.8% of non-pregnant subjects. When the RIA-I was used, the ßC-hCG positivity was as high as 88.3%. Because of the high cross-reactivity of the RIA with free ßLH or ß-fragments of other glycoproteins, the IRMA appears to be more appropriate for ßC-hCG detection in pregnant and non-pregnant individuals.

The distribution and ratios between ßC-hCG and its related glycoproteins in several compartments in the body, the ability of granulosa cells to degrade intact hCG to ßC-hCG in vitro, and the conversion of injected hCG to this fragment in vivo were also examined. Using IRMA and RIA the ratios between ßC-hCG and intact hCG in the urine of a late-second trimester pregnant woman, after chromatographic separation, were 0.367 and 0.672, respectively, indicating that the detected levels of this fragment in urine may be higher when RIA is used. ßC-hCG/hCG ratios in post- and premenopausal urines were 14.1 and 4.5, respectively. The amount of ßC-hCG to urinary LH was smaller before (0.108) than after (0.323) the menopause, suggesting other sources contribute to total ßC-hCG immunoreactivity in the urine of non-pregnant individuals as well. A ratio of 0.023 for hCG/LH was found in both pre- and postmenopausal urines.

A significant amount of a high molecular weight ßC-hCG immunoreactive material was found in serum of pregnant women after size separation. The ratio between the total ßC-hCG immunoactivity and hCG in this fluid was estimated as 0.019. The conclusion is that the majority of the ßC-hCG immunoactivity in serum is given by a high molecular weight complex. Moreover, it was demonstrated that amniotic fluid contained this high molecular weight form of immunoreactive ßC-hCG as well, and the authentic ßC-hCG/hCG ratio of 0.01 in this fluid increased to 0.026 when the high molecular weight form was also considered.

ßC-hCG is the principal glycoprotein found in follicular fluid after hyperstimulated folliculogenesis and intramuscular injection of 5000 IU of hCG. Granulosa cells cultured in the presence of hCG were able to degrade the intact molecule to both high molecular weight and authentic immunoreactive forms of ßC-hCG. Using the IRMA-I, ßC-hCG was detected

in 13 out of 50 follicular fluid samples (26%). The specific ßC-hCG RIA-I could detect the fragment in 48 samples (96%). The mean concentrations of ßC-hCG in this fluid with IRMA and RIA were 13.1±5.7 and 19±5.2pmol/l. Further, it was demonstrated that, after gel filtration, a wide range of molecular weight material immunoreactive for ßC-hCG is present in human seminal plasma and assaying 74 samples of this fluid by direct IRMA, ßC-hCG was detected in 42 (56.7%).

Intramuscular injection of 1500 IU of hCG in five male volunteers increased the levels of ßC-hCG in urine by approximately 220% during the first 24h (P=0.036 for ßC-hCG levels at 2h and 24h) decreasing thereafter to undetectable levels in the next 72h. Although certain increase in the ßC-hCG immunoactivity in serum was observed at the first 24h, the levels remained under the limit of detection of the assay at all times. It was concluded 1) ßC-hCG fragment is widely distributed in body fluids and a dissociable high molecular weight material immunoreactive for ßC-hCG is found in some biological compartments. The nature of the high molecular weight is unclear but could represent a ßC-hCG-binding protein complex, oligomeres or other fragmented forms of hCG/ßhCG cross-reacting with the ßC-hCG assays; 2) granulosa-lutein cells are able to degrade intact hCG to a small ßC-hCG immunoreactive form in vitro. These results support previous studies indicating the peripheral metabolism of intact hCG to ßC-hCG as the principal source for this fragment but also support the possibility of a high molecular weight associated form that may be produced by some tissues.

To establish a reference range for immunoreactive ßC-hCG and the ratio between ßC-hCG/hCG in urine throughout the pregnancy, both hormones were measured in samples obtained from 741 pregnant women between 6 and 41 weeks' gestation. Weekly analysis demonstrated that ßC-

hCG reached its maximum level between the eighth and the fifteenth week. The median concentrations rose gradually from 10.7 to 39.0 nmol ßC-hCG per mmol creatinine from week 6 to week 10 of gestation and then decreased to achieve the lowest level between the 20th and 29th week. A slight but statistically not significant increase was observed between the 30th and 36th week. Even though the concentrations were lower, the pattern of urinary hCG between the 6th and the 41st week of gestation was similar to that of ßC-hCG. The ratio between ßC-hCG and hCG varied between 1.67 to 9.57 throughout the pregnancy.

It was demonstrated that the earliest increase of ßC-hCG and hCG in urines collected daily from women whose pregnancy resulted from artificial donor insemination was detected respectively 12 and 11 days after the estimated time of ovulation. Although ßC-hCG is an efficient marker for monitoring gestational trophoblastic disease or some non-trophoblastic neoplasms in women, the suggestion is that the fragment does not offer any benefit over the well established methods for early diagnosis of pregnancy.

APPENDICES

APPENDIX I

SILVER STAIN

Silver staining method according to Morrissey (Anal Biochem **117:** 306-310, 1981). Gentle but thorough agitation important throughout the procedure.

Step 1. Prefix for 30 min with:

50%	Methanol	75	ml.
10%	Acetic acid	15	ml.
	ddH_2O	60	ml.

Step 2. Reswell for 30 min in:

5%	Methanol	10	ml.
7%	Acetic acid	14	ml.
	ddH_2O	176	ml.

Step 3. Fix for 30 min. in 10% glutaraldehyde (Biological Grade)

10%	Glutaraldehyde (25% aqueous soln.)	60	ml.
	ddH_2O	90	ml.

Step 4. Rinse in dd H_2O. Running deionized H_2O or several changes of water, for 2 hours. More conveniently, soak in large volume of water overnight followed by a fresh water rinse the next day for 30 min.

Step 5. Soak in 5 ug/ml dithiothreitol for 30 min.

Dithiothreitol	1.0	mg.
dd H_2O	200	ml.

Step 6. Pour off DTT solution and without rinsing, add 0.1% silver nitrate. Treat for 30 min. Agitation very important.

Silver nitrate	200	mg.
dd H_2O	200	ml.

Step 7. Rinse: **once** rapidly with small amount of **dd H_2O**
twice rapidly with small amount of **developer**
Soak in developer until desired level of staining is achieved.

Developer	37%	Formaldehyde	50ul	100ul
	3%	Sodium carbonate	3g	6g
		Final volume:	100ml	200ml

Step 8. Stop Solution. Add 5 ml 2.3M citric acid directly to developer. Agitate for 10 min. Discard soln. and wash with several changes of distilled H_2O for 30 min. For storage, soak gel for 10 min in 0.03% sodium carbonate to prevent bleaching (30mg sodium carbonate/ 100ml H_2O). Store in Seal-a-Meal bags or wrap in cellophane.

Citric acid	44.19g
ddH_2O	100 ml.

APPENDIX II

IMMUNOSTAINING BLOTS

Protocol:

1. Wash blots in 3 x 5 minute changes of PBS/AZ.
2. Incubate 1-2 hours in blot blocker.
3. Wash in 3 x 5 minute changes of PBS/NGS/TW/AZ.
4. Incubate in the primary antibody for 1-2 hours at 25^{o}C or overnight at 4^{o}C.
5. Wash in 3 x 5 minute changes of PBS/NGS/TW/AZ.
6. Incubate in the secondary antibody for 1 hour at 25^{o}C.
7. Wash in 3 x 5 minute changes of PBS/NGS/TW.
8. Incubate with Vectastain reagents or Streptavidin in PBS/TW for 1 hour at 25^{o}C.
9. Wash for 5 minutes in PBS/TW.
10. Wash in 2 x 5 minutes changes of PBS.
11. Incubate in the colour reaction solution until the bands appear.
12. Stop the colour reaction by rinsing the blots in several changes of distilled water.
13. Air dry the blots.

Notes:

a) PBS=150 mM NaCl, 10 mM sodium phosphate, pH 7.6.
b) AZ = 0.1% sodium azide.
c) NGS = 1% heat-inactivated normal goat serum.
d) TW = 0.05% Tween 20 (add 1 ml of 10% TW 20 to 200 ml of PBS).
e) Use one of two blot blockers:

   i) 5% NFDM (non-fat dry milk) in PBS
   ii) 2.5% BSA (Sigma # A-7030), 5% NGS, 0.1% AZ in PBS.

f) Dilute the primary and secondary antibodies (1:100-2000) in PBS/NGS/TW/AZ. The 2nd Ab is biotinylated.
g) Sodium azide is an irreversible inhibitor of peroxidase, so it must be omitted from #7 onwards.
h) Use 10-30 ul of Streptavidin per 10 ml PBS/TW.

APPENDIX III

LECTIN STAINING OF BLOTS

Protocol:

1. Wash blots in 2 x 5 minute changes of TBS.
2. Incubate in blot blocker for 1 hour at 25°C.
3. Wash in 3 x 5 minute changes of TBS.
4. Incubate in the various lectins for 1 hour at 25°C.
5. Wash in 3 x 5 minute changes of TBS.
6. Incubate with Vectastain reagents for 1 hour at 25°C.
7. Wash in 3 x 5 minute changes of TBS.
8. Incubate in the colour reaction solution until the bands appear.
9. Stop the colour reaction by rinsing the blots in several changes of distilled water.
10. Air dry the blots.

Notes:

a) TBS = 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.5.
b) Blot blocker = TBS + 10 mg/ml BSA (Sigma # A-7030).
c) Prepare lectins in TBS + 5 mg/ml Hemoglobin (Sigma # H-2500).
d) Prepare Vectastain reagents about 30 minutes before use:
   - Mix one drop of A with 1 drop of B and let stand 30 minutes
   - Add 15 ml of TBS + 0.05% Tween 20.
e) Prepare the colour reaction solution immediately before use:
   - Prepare the following:
     A: 20 mg DAB + 20 ml Tris-HCl (pH 7.5)
     B: 26.6 ul of 30% H_2O_2 + 20 ml of distilled water
     C: 0.4 ml of 8% $NiCl_2$
   - Mix well and use.

APPENDIX IV

Amerlex-M ßHCG RIA Kit - Assay protocol*

	Standards (mIU/ml 2nd IS)**							UNKNOWNS
	0	4	10	25	50	100	200	
Tube number	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16
Standard	100	100	100	100	100	100	100	-
Unknowns	-	-	-	-	-	-	-	100
Anti-ßhCG serum	100	100	100	100	100	100	100	100
	Vortex mix, cover tubes and incubate for 30 minutes at 37°C							
^{125}I-labeled hCG	50	50	50	50	50	50	50	50
	Vortex mix, cover tubes and incubate for 45 minutes at 37°C							
Amerlex-M second antibody reagent	1000	1000	1000	1000	1000	1000	1000	1000
	Vortex mix and incubate for 10 minutes at room temperature. Separate for 15 minutes using the Amerlex-M Separator. Decant supernatant solutions, drain, blot and count.							

* All volumes are given in microlitres

** 1 mIU/ml = 45 pmol/l

APPENDIX V

BIOCLONE PROGESTERONE RIA PROCEDURE

TUBE DESCRIPTION	STANDARD OR SAMPLE (µl)	PROGESTERONE ANTISERUM (µl)	125I PROGESTERONE (µl)	PRECIPITATING SOLUTION (µl)
TC	-	-	200	-
NSB	50 (0 STD)	-	200	500
0 nmol/l STD	50	200	200	500
1 nmol/l STD	50	200	200	500
2 nmol/l STD	50	200	200	500
5 nmol/l STD	50	200	200	500
10 nmol/l STD	50	200	200	500
20 nmol/l STD	50	200	200	500
50 nmol/l STD	50	200	200	500
100 nmol/l STD	50	200	200	500
SAMPLE	50	200	200	500

Vortex and incubate at 37°C for 30 minutes.

Incubate at room temperture for 15 minutes.
Vortex then centrufuge for 20 mins at 3290 X G.
Decant supernatant, drain, blot and count pellet.

APPENDIX VI

SPECTRIA ESTRADIOL COATED TUBE RIA

Procedure	Total	NSB	Standard	Control sera	Unknown
Pipette sample		100*	100	100	100
Pipette estradiol [125I]	200	200	200	200	200
Pipette estradiol antiserum			200	200	200
Pipette distilled water		200			
Mix		X	X	X	X
Incubate 3h/37°C		X	X	X	X
Decant		X	X	X	X
Wash with washing solution		1000	1000	1000	1000
Count for 1 min	X	X	X	X	X

* All volumes are given in microlitres.

APPENDIX VII

Tandem-R hCG IRMA

Assay Procedure

1. Label tubes.
2. Introduce one bead into each tube after blotting the residual droplet which remains on the bead after removal from the container. Do not permit the beads to dry.
3. Pipette calibrators, controls, and specimens (0.1ml) into each tube.
4. Pipette 0.1ml of tracer antibody into each tube.
5. Shake the test rack to ensure mixing and cover the tubes.
6. Incubate at 37^0C for 1 hour.
7. Wash the beads by dispensing 2ml of wash solution into each tube and decanting the liquid from each tube.
8. Count each tube in a gamma counter for 60 seconds.

APPENDIX VIII

BIOCLONE BETA hCG IRMA*

Assay Procedure

1. Set up and label the test tubes in duplicate for the Total Counts (TC), free ßhCG standards (0, 0.5, 2.5, 5.0, 25, 100 and 500IU/l) and patient samples.

2. (a) Pipette 100 µl of free ßhCG Standards into appropriately labelled tubes.

   (b) Pipette 100 µl of samples into appropriately labelled tubes.

3. Suspend the anti-free ßhCG coated magnetisable particles (blue-green) by swirling the contents of the bottle until no sediment can be seen on the bottom - do not shake this reagent vigourously.

4. Pipette 500 µl of ^{125}I anti-free ßhCG (yellow) into all tubes. Set TC tubes aside.

5. Pipette 500 µl of anti-free ßhCG magnetisable particles (blue-green) into all tubes except TC.

6. Vortex tubes gently and incubate for 1 hour at 37^{0}C.

7. Separation of the sandwich from unbound antibody label may be achieved by using a magnetic separation.

   1) Place tubes into magnetic separation rack and ensure that all tubes are in contact with the magnetic baseplate. Leave for 15 minutes.

   2) After separation do not remove rack from magnetic baseplate. Decant the supernatant and keeping the magnetic baseplate inverted allow tubes to drain onto absorbent paper for two minutes.

   3) Remove the rack from its magnetic baseplate. Wash the tubes by adding 500 µl wash buffer to all tubes. Vortex, sediment on magnetic baseplate, decant, and blot as above.

8. Count all tubes for one minute in a gamma counter.

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* 1 IU/l = 45 pmol/l

APPENDIX IX

BIOCLONE FREE αhCG IRMA*

Assay Procedure

1. Set up and label the test tubes in duplicate for the Total Counts (TC), free α-glycoprotein standards (0, 0.1, 0.5, 2.5, 5.0, 25, and 100 IU/l) and patient samples.

2. (a) Pipette 50 µl of free α-glycoprotein Standards into appropriately labelled tubes.
   (b) Pipette 50 µl of patient samples into appropriately labelled tubes.

3. Suspend the anti-free α-glycoprotein coated magnetisable particles (blue-green) by swirling the contents of the bottle until no sediment can be seen on the bottom - do not shake this reagent vigourously.

4. Pipette 500 µl of ^{125}I anti-free α-glycoprotein (yellow) into all tubes. Set TC tubes aside.

5. Pipette 500 µl of anti-free α-glycoprotein magnetisable particles (blue-green) into all tubes except TC.

6. Vortex tubes gently and incubate for 1 hour at room temperature.

7. Separation of the sandwich from unbound antibody label may be achieved by using either magnetic separation or centrifugation.

   A. Magnetic Separation

   1) Place tubes into magnetic separation rack and ensure that all tubes are in contact with the magnetic baseplate. Leave for 15 minutes. Precision can be improved by increasing the time of sedimentation to 20 minutes.

   2) After separation do not remove rack from magnetic baseplate. Decant the supernatant and keeping the magnetic baseplate inverted allow tubes to drain onto absorbent paper for two minutes.

   3) Remove the rack from magnetic baseplate. Wash the tubes by adding 500 µl wash buffer to all tubes. Vortex, sediment on magnetic baseplate, decant, and blot as above.

   OR

   B. Centrifugation

   1) Centrifuge all tubes for 5 minutes at 3290 x g at 4^{0}C. Decant the supernatant and allow tubes to drain onto absorbent paper for 2 minutes.

   2) Wash the tubes by adding 500 ml wash buffer to all tubes. Vortex, centrifuge, decant and blot as above.

8. Count all tubes for one minute in a gamma counter.

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* 1 IU/l = 67.1 pmol/l

APPENDIX X

BIOCLONE HUMAN LUTEINIZING HORMONE IRMA*

Assay Procedure

1. Set up and label a sufficient number of tests tubes in duplicate for the Total Counts (TC, hLH standards (0, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 500 IU/l), and patient samples.

2. (a) Pipette 200 µl of hLH Standards into appropriately labelled tubes.
   (b) Pipette 200 µl of patient samples into appropriately labelled tubes.

3. Pipette 500 µl of ^{125}I-anti hLH (yellow) into all tubes. Set TC tubes aside.

4. Vortex tubes gently and then incubate for 30 minutes at 37^{o}C.

5. Suspend the anti-hLH coated magnetisable particles (blue-green) by swirling the contents of the bottle until no sediment can be seen on the bottom - do not shake this reagent vigourously.

6. Pipette 500 µl of anti-hLH magnetisable particles (blue-green) into all tubes except TC.

7. Vortex tubes gently and then incubate for 30 minutes at 37^{o}C.

8. Separation of the sandwich from unbound antibody label may be achieved by using either magnetic separation or centrifugation.

   A. Magnetic Separation

   1) Place tubes into magnetic separation rack and ensure that all tubes are in contact with the magnetic baseplate. Leave for 15 minutes.
   2) After separation do not remove rack from magnetic baseplate. Decant the supernatant and keeping the magnetic baseplate inverted allow tubes to drain onto absorbent paper for two minutes.
   3) Remove the rack from magnetic baseplate. Wash the tubes by adding 1.0 ml wash buffer to all tubes. Vortex, sediment on magnetic baseplate, decant, and blot as above.

   OR

   b. Centrifugation

   1) Centrifuge all tubes for 5 minutes at 3290 x g at 4^{o}C. Decant the supernatant and allow tubes to drain onto absorbent paper for 2 minutes.
   2) Wash the tubes by adding 1.0 ml wash buffer to all tubes. Vortex, centrifuge, decant and blot as above.

9. Allow tubes to drain thoroughly (2-3 minutes) before turning upright.

10. Count all tubes for one minute in a gamma counter.

---

* 1 IU/l = 4.2 pmol/l

APPENDIX XI

A. Scatchard plot - ßC-hCG RIA-I

Molecular weight of antigen = Mr 10000
Volume cytosol = 0.1ml
Volume Incubation = 0.4ml
Specific activity tracer = 189.2 µCi/µg

$1\mu Ci \quad - 2.2 \times 10^6$ cpm - $1\mu g$

$189.2\ \mu Ci$ - x - $1\mu g$

$\chi = 416.26 \times 10^6$ cpm - 1mg

1000 cpm - $\chi 1$

$\chi 1 = 1000$ cpm $= 2.402 \times 10^{-6}\mu g$

$= 0.24 \times 10^{-11} g$

Standard (x10-11g)	H* (x10-11g)	Bound x10-11g/ml	Bound x10-10mol/l	Free x10-10mol/l	B/F
2.7	4.39	3.9	0.039	0.079	0.492
5.7	"	5.4	0.054	0.114	0.477
22.0	"	13.3	0.133	0.306	0.435
28.0	"	15.6	0.156	0.383	0.406
55.0	"	24.1	0.241	0.748	0.322
146.0	"	38.6	0.368	2.119	0.182
333.0	"	50.4	0.504	5.116	0.098
700.0	"	59.0	0.590	5.901	0.052
1400.0	"	61.8	0.618	22.277	0.027

*125I-radiolabelled antigen; total count
**Minus the nonspecific binding (0.0018x10-10mol/l), an amount of the unlabelled antigen 140-fold higher than ED_{50}.

B Hill Plot

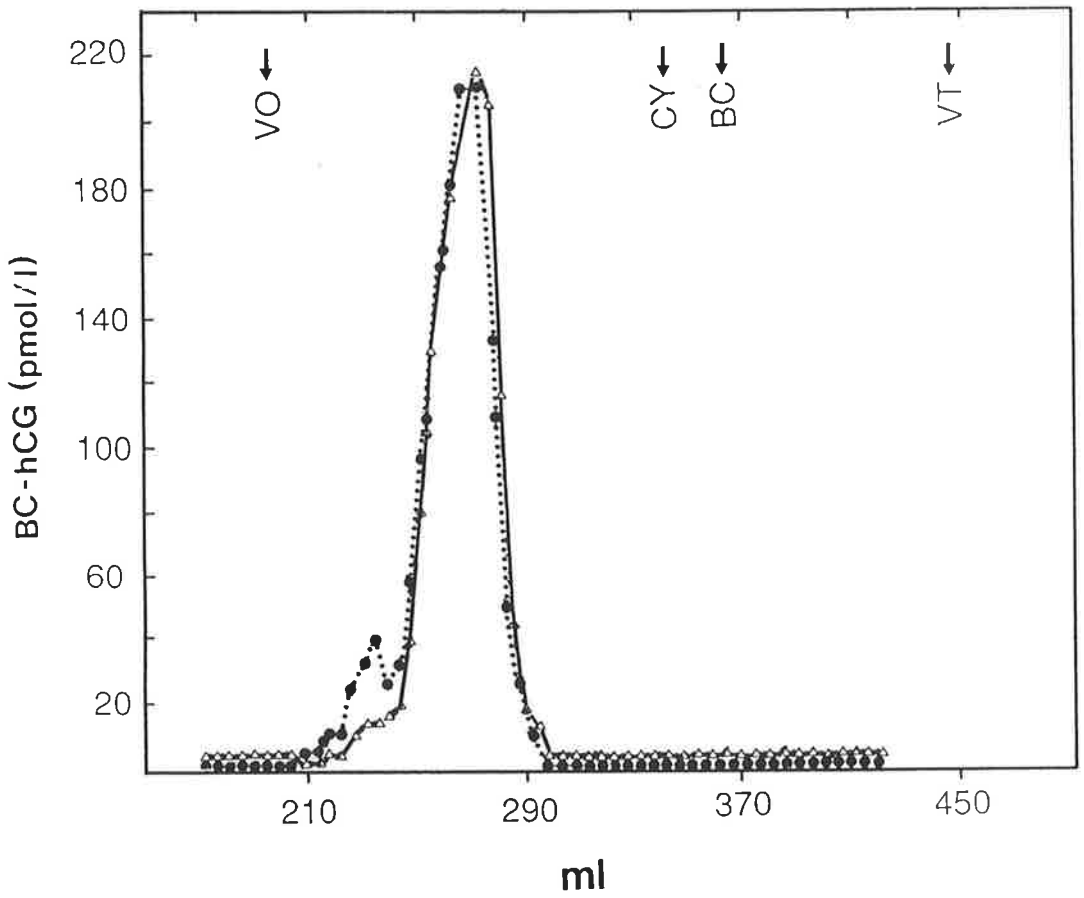
$$\log \left(\frac{B}{B_{max} - B} \right) \text{ vs } \log [\text{Free}]$$

B max = 0.638 x 10^{10}mol/l, from Scatchard plot (R)
Slope = $\eta H = 1$, homogogeneous
= $\eta H > 1$, positive co-operativity
= $\eta H < 1$, heterogeneity or negative co-operativity

Standard number	Log $\left(\frac{B}{B_{max-B}} \right)$	Log [Free] (C)
1	-1.187	-1.101
2	-1.033	-0.942
3	-0.578	-0.513
4	-0.490	-0.416
5	-0.216	-0.126
6	0.184	0.326
7	0.576	0.708
8	1.086	1.047
9	1.485	1.357

$\eta H = 1.08$

APPENDIX XII



Sephacryl S-200 chromatography of Profasi (Serono) on a 2.6 x 87.5cm column. The sample (7ml) containing 35IU of hCG was eluted at a downward flow rate of 50ml/h with 0.2M ammonium acetate, pH 6.8, at room temperature. Fractions of 4.2ml were collected and assayed for ßC-hCG using RIA-I (●····●) and IRMA-I (△——△). Arrows indicate the position of the void volume (Vo), total volume (Vt), and the elution position of [^{125}I]-ßC-hCG.

APPENDIX XIII

PUBLICATIONS

Material contained in this thesis has been published or submitted for publication as follows:

DeMedeiros SF, Amato,F, Norman RJ. Stability of immunoreactive ß-core fragment of hCG. Obstet Gynecol 77: 53-59, 1991.

DeMedeiros SF, Amato F, Matthews CD, Norman RJ. Molecular heterogeneity of the beta core fragment of hCG. Submitted for publication in Endocrinology.

DeMedeiros SF, Amato F, Matthews CD, Norman RJ. Comparison of specific immunoassays for detection of ß-core fragment of hCG in body fluids. Submitted for publication in Journal of Endocrinology.

DeMedeiros SF, Amato F, Bacich D, Wang L, Matthews CD, Norman RJ. Distribution of the beta core hCG fragment in body fluids. Submitted for publication in Journal of Endocrinology.

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