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ISOLATION AND CHARACTERISATION OF N-GLYCANS OF OVINE AND HUMAN LUTEINIZING HORMONES

Jun Hiyama

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry, University of Auckland, 1991
ACKNOWLEDGEMENTS

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I am very much indebted to Ms Susan Buglass and Mr Francis Tan for their assistance in the preparation of this thesis.

Finally, I thank my parents and family for their understanding and patience.
ABSTRACT

Gonadotrophic hormones are heterodimeric glycoproteins and their N-glycans attached to specific amino acid residues are currently thought to play important roles in hormonal biosynthesis, secretion and function. The studies reported in this thesis aimed at isolation and characterisation of structural properties of the N-glycans on ovine and human luteinizing hormones.

Initially, chromatographic methods were developed using reverse-phase HPLC for the analytical separation of the three human pituitary glycoprotein hormones and their subunits. Separation of intact oLH and its subunits was also effected by a single HPLC step.

A preparative procedure was developed for the efficient purification of hLH and hTSH from crude human pituitary extracts using hydrophobic chromatography which gave highly purified hormones in good yields and with high biological activities. This method did not significantly influence the hormones' extensive charge heterogeneity and it offered potential advantages in the characterisation of their carbohydrate structures.

A preparative scheme was developed for the isolation of the N-linked oligosaccharides from each glycosylation site of o- and hLH. Charge heterogeneity of oligosaccharides, which were released by hydrazinolysis from subunits and glycopeptides, was characterised by anion-exchange HPLC.

$^1$H-NMR analysis showed that the structures of all three N-glycans on hLH were highly heterogeneous but mainly diantennary complex-type, with site-specific patterns of terminal sialylation and sulphation as well as core-fucosylation.
Sulphated/sialylated and/or disialylated oligosaccharides were the major components at each site. A set of new mono- and disialylated oligosaccharides with the terminal sequence NeuAcα2-6GalNAcβ1-4GlcNAcβ1-2Manα1-3 was identified. This finding suggested unique site-specific terminal sialylation of oligosaccharides at Asn 78 (hLHα) by an unknown α2-6 sialyltransferase(s) in the human pituitary gonadotroph cell.

Each glycosylation site in oLH had a distinct set of oligosaccharides ranging from mainly monosulphated hybrid-type at the two sites of oLHα to predominantly disulphated diantennary complex-type on oLHβ. Core-fucosylation also differed at each site. These results suggested that processing of the oligosaccharides of the α- and β-subunits by α-mannosidase II and α1-6 fucosyltransferase was differently regulated by protein structure in oLH.

Whereas hCG, hLH and oLH share similar biological activities, no apparent relationship between their N-glycan structures was found, which suggested that specific branching and peripheral structures of N-glycans on LH and hCG may not be essential for biological function, although the N-glycan nearer the N-terminus of the α-subunit of hCG has been implicated in hormonal activity.
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<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-cyclic monophosphate</td>
</tr>
<tr>
<td>CM</td>
<td>carboxymethyl</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fuc</td>
<td>L-fucose</td>
</tr>
<tr>
<td>Gal</td>
<td>D-galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine (2-acetamido-2-deoxy-D-galactose)</td>
</tr>
<tr>
<td>Glc</td>
<td>D-glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylgulosamine (2-acetamido-2-deoxy-D-glucose)</td>
</tr>
<tr>
<td>GlcNAc-ol</td>
<td>N-acetylgulosaminitol</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>HF</td>
<td>hydrogen fluoride</td>
</tr>
<tr>
<td>^1H-NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Man</td>
<td>D-mannose</td>
</tr>
<tr>
<td>NeuAc</td>
<td>N-acetylneuraminic acid (sialic acid)</td>
</tr>
<tr>
<td>NIADDK</td>
<td>National Institutes of Arthritis, Diabetes and Digestive and Kidney Diseases</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health, Bethesda, Maryland, U.S.A.</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>RP</td>
<td>reverse-phase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulphate</td>
</tr>
<tr>
<td>TPCK</td>
<td>L-1-tosylamido-2-phenylethyl chloromethyl ketone</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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CHAPTER 1 General Introduction

The pituitary gonadotrophins, luteinizing hormone (LH; lutropin) and follicle-stimulating hormone (FSH; follitropin), play central roles in the mammalian reproductive process. Synthesized in the anterior pituitary gland and secreted into the circulation, these hormones control gonadal functions in male and female, primarily in the production of steroid hormones and gametogenesis.

In the male, LH stimulates the Leydig cells of the testis to produce testosterone, which, with FSH, is responsible for spermatogenesis by Sertoli cells during sexual maturation. In the female, rhythmic releases of FSH and LH control the ovarian cycle during her fertile life; FSH stimulates the production of estrogens in the granulosa cells for the maturation of ovarian follicles, LH induces ovulation as well as causing the follicle cells to form corpora lutea, which secrete progesterone. In the human and some other mammals, another gonadotrophic hormone, chorionic gonadotrophin (CG), is secreted by the developing placenta during the early stages of pregnancy to maintain the corpus luteum for the continuous production of progesterone. The biological activities of LH and CG are similar.

In common with other polypeptide hormones, the gonadotrophins exert their actions by binding to specific receptors on the target cell membranes. This causes activation of adenylate cyclase via the GTP-binding protein that leads to cellular responses including steroidogenesis (Hunzicker-Dunn & Birnbaumer, 1985).

These three gonadotrophins and thyroid-stimulating hormone (TSH; thyrotropin) which is synthesized in the anterior
pituitary gland, are structurally related glycoproteins and they constitute the family of hormones generally termed glycoprotein hormones.

In recent years, there has been growing interest in the structure of the carbohydrate moieties of the glycoprotein hormones because of their potential biological roles in the regulation of hormonal activity. However, detailed characterisation of oligosaccharides in gonadotrophins, particularly those from human pituitaries, has been complicated by their heterogeneous structures (microheterogeneity) which are closely associated with charge heterogeneity of these hormones.

1.1 Heterogeneity of Human Gonadotrophins

There is abundant evidence for charge heterogeneity of LH and FSH isolated from human pituitary glands (Reichert, 1971; Roos et al., 1975; Stockell Hartree et al., 1985). Weise et al. (1983) separated seven isoforms of hLH (pI 5.9 - 8.8) from crude pituitary extracts by isoelectric focusing on columns and showed that the charge heterogeneity of this hormone is not solely due to differences in sialic acid content, unlike hCG (Graesslin et al., 1972). When these isoforms were characterised by immunoassay, receptor-binding activity, cAMP accumulation and testosterone production in mouse Leydig cells, they showed a marked decrease in receptor-binding and biological activities with increasing acidity (Lichtenberg et al., 1984).

Similar observations were obtained with purified hFSH which showed a pattern of multiple peaks in the pH range 3.5 - 5.5 on electrofocusing. More acidic fractions were more active in an in vivo assay, however less acidic species showed higher activity in an in vitro bioassay (Zaidi et al., 1982a; Wide,
Six major forms (pI 3.8 - 5.4) have been isolated from purified urinary hCG preparations by isoelectric focusing and characterised in respect of in vivo biological potency and sialic acid content (Graesslin et al., 1972; Nwokoro et al., 1981). It was found that isohormones with higher sialic content had the lower pIs and higher potencies and that hCG isohormones result primarily from varying degrees of sialylation. The higher potencies of more acidic forms were related to lower metabolic clearance rates from the circulation (Rosa et al., 1984; Lefort et al., 1984).

Charge heterogeneity of hLH and hFSH were also reported for pituitary and plasma samples. Reader et al. (1983) reported that the pituitaries of postmenopausal women contain more acidic forms of hLH than those of healthy menstruating women, which was consistent with earlier findings of higher circulating concentrations of acidic hLH in plasma from post-menopausal women (Strollo et al., 1981). The median charge of hLH and hFSH in individual pituitary glands was related to sex and age, with more acidic forms at higher ages (Wide, 1985b); the variable charge on hFSH was ascribed to differences in sialic acid content. Warner et al. (1985) reported that while the plasma concentrations of immunoreactive hLH were similar in young and older men, biological activity was significantly lower in the latter. Similar age-dependent variations in serum bioactive hFSH were observed for men, which suggested changes in the circulating forms of this hormone with increasing age (Tenover et al., 1987). These observations suggested that the secretory patterns of the gonadotrophin isohormones alter under certain physiological conditions (Wilson et al., 1990).
Studies of charge heterogeneity revealed that the potencies of purified hormone preparations measured by immuno- and bioassays depend not only on the degree of purity but also on the composition of the isoforms present. A number of highly purified hLH preparations were found to have significantly different distributions in biological and immunological activities on electrofocusing (Zaidi et al., 1982b). The isoform profiles of all preparations were markedly different from those of hLH present in pituitary extracts, particularly in that they lacked acidic species (pI < 6.5) that were abundant in the pituitaries of postmenopausal women. It was also found that these hLH preparations varied markedly in their immunoreactivities and their biological potencies, in vitro and in vivo (Storring et al., 1982). Similar results were also observed for a number of highly purified hFSH preparations (Storring et al., 1981).

In the well-established purification procedures, hLH is isolated by a multiple chromatographic system of CM-cellulose and DEAE-cellulose from structurally related hFSH and hTSH which are more acidic (Parlow et al., 1965; Stockell Hartree, 1966, 1975; Bates et al., 1968). These charge separations are further complicated by the charge heterogeneities of hFSH and hTSH.

The above studies indicated that these conventional methods select charged forms of hLH and hFSH and although they are widely used for the purification of these hormones, the biochemical basis of gonadotrophin heterogeneity remains to be fully understood. However, in the case of hLH, contemporary evidence strongly suggests that greater proportions of different isoforms result from their varying contents of sialic acid and sulphate that exist in the attached carbohydrates (Green &
Fig. 1.1 Amino acid sequence of human α subunit deduced from nucleotide sequences (Fiddes & Goodman, 1979).

The sequence of hLHα isolated from the intact hormone is identical but shows N-terminal heterogeneity: 95% of hLHα is reported to have three residues missing at the N-terminus (Keutmann et al., 1978). Carbohydrate is attached to Asn 52 and Asn 78.
<table>
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<td>Ser-Arg-Glu-Pro-Leu-Arg-Pro-Trp-Cys-His-Pro-Ile-Asn-Ala-Ile-Leu-Ala-Val-CH</td>
</tr>
<tr>
<td>Glu-Lys-Glu-Gly-Cys-Pro-Val-Cys-Ile-Thr-Val-Asn-Thr-Thr-Ile-Cys-Ala-Gly-</td>
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<td>Tyr-Cys-Pro-Thr-Met-Met-Arg-Val-Leu-Gln-Ala-Val-Leu-Pro-Pro-Leu-Pro-Gln-</td>
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<td>Val-Val-Cys-Thr-Tyr-Arg-Asp-Val-Arg-Phe-Glu-Ser-Ile-Arg-Leu-Pro-Gly-Cys-</td>
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<td>Pro-Arg-Gly-Val-Asp-Pro-Val-Ser-Phe-Val-Pro-Ala-Leu-Ser-Cys-Arg-Cys-</td>
</tr>
<tr>
<td>Gly-Pro-Cys-Arg-Arg-Ser-Thr-Ser-Asp-Cys-Gly-Gly-Pro-Lys-Asp-His-Pro-Leu-</td>
</tr>
<tr>
<td>Thr-Cys-Asp-His-Pro-Gln-Leu-Ser-Gly-Leu-Leu-Phe-Leu</td>
</tr>
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</table>

**Fig. 1.2** Amino acid sequence of hLHβ subunit deduced from nucleotide sequences (Talmadge et al., 1984).

This sequence contains an additional seven residues at the C-terminus (underlined) as compared with the sequence of hLHβ isolated from the intact hormone (Keutmann et al., 1979). Heterogeneity at the C-terminus and within some hLHβ molecules is reported (Stockell Hartree, 1985). Carbohydrate is attached to Asn 30.
Baenziger, 1988b). Although there is also heterogeneity in the polypeptide parts of this hormone e.g. at the N-terminal region of α-subunit (Keutmann et al., 1978), its contribution to overall heterogeneity appears to be relatively small.

1.2 Structure and Biosynthesis of Gonadotrophins

The glycoprotein hormones share many structural similarities. Each member of this family is a heterodimer, consisting of two non-covalently bonded subunits, designated α and β. Both subunits are single polypeptide chains glycosylated at specific residues and they are highly cross-linked with intramolecular disulphide bonds (Pierce & Parsons, 1981; Sairam, 1983; Ryan et al., 1987).

Within each species, the α-subunits of all the hormones have essentially identical polypeptide structures (common subunit), while the β-subunits are different and are regarded as being hormone-specific, because they confer biological specificity on each dimeric hormone. The α-subunits show considerable homology among different species. The β-subunits also show regions of homology between hormones and among different species; in particular, the β-subunits of hLH and hCG are highly homologous (85%) through the first 114 residues and these two hormones show common biological activities with identical receptor specificity.

The complete amino acid sequences of these subunits from several mammalian species are known and those of human LH are shown in Figures 1.1 and 1.2.

The α-subunits contain five, and β-subunits contain six disulphide bonds within each subunit. The location of the 10 half-cysteines in the α-subunit and 12 half-cysteines in the β-
**PITUITARY**

Single $\alpha$ and $\beta$ genes

Mature $\alpha$ and $\beta$

![Diagram of Pituitary Structures]

**PLACENTA**

Single $\alpha$ and seven $\beta$ genes

$\alpha$ is identical with pituitary $\alpha$

$\beta$ is highly homologous with LH$\beta$

![Diagram of Placental Structures]

**Fig.1.3** Structures of human pituitary and placental gonadotrophins.

The common $\alpha$-subunits have two N-glycosylation sites and the hormone-specific $\beta$-subunit has one (hLH) or two (hFSH, hCG). Only the hCG$\beta$-subunit contains a carboxyl terminal extension that has four O-glycosylation sites.

CHO, carbohydrate moiety
subunits is highly conserved among the various hormones as well as between species. Although there is good agreement between laboratories on several disulphide bonds in each subunit (α7-31, α10-32, β93-100, β26-110 and β23-72), the remaining assignments are still uncertain (Ryan et al., 1988).

Each gonadotrophin subunit possesses N-linked sugar chains (N-glycans) attached to specific Asn residues. The α-subunits of all hormones contain two, and the β-subunits contain one or two N-glycans. Only the β-subunit of hCG has four O-linked sugar chains (O-glycans) additionally attached to serine residues in the C-terminal extension.

The structures of the N- and O-linked oligosaccharides on hCG are known in detail. The N-linked oligosaccharides of LH and FSH from several animal species including humans have recently been characterised (described later).

The structural organization of the pituitary and placental gonadotrophins is schematically shown in Fig.1.3. The α- and β-subunits are encoded by separate genes, with a single gene specifying the common α-subunits (Charib et al., 1990). The LH-β and FSH-β genes are expressed in the same pituitary gonadotroph cell (Liu et al., 1988). Unlike the β-subunits of the pituitary hormones, the hCG β-subunit is not encoded by a single gene. There are at least seven hCG β-genes, two of which are expressed in the syncytiotrophoblast of the placenta.

The separate mRNAs from the α and β genes are translated in the rough endoplasmic reticulum. Signal peptides are removed and oligosaccharides are transferred cotranslationally en bloc from a dolichol precursor to the Asn residues at the sites of glycosylation, which are Asn 52 and Asn 78 in the α-subunits and Asn 13 and Asn 30 (hCG), Asn 7 and Asn 24 (FSH) or Asn 30 (LH).
in the β-subunits, in the case of human gonadotrophins. Combination of the α- and β-subunits occurs within the endoplasmic reticulum before extensive processing of the N-linked oligosaccharides in the Golgi (Hoshina & Boime, 1982).

N-glycosylation of both subunits is necessary for proper disulphide bridge formation and folding of each subunit, prior to combination of the subunits (Strickland & Pierce, 1983; Beebe et al., 1990). In the case of hCG, O-glycosylation occurs at four serine residues (121, 127, 132 and 138) of its β-subunit in the Golgi, after dimer formation (Hanover et al., 1982).

Mature LH and FSH are stored in separate secretory granules and selectively released into the circulation upon stimulation with releasing factors (Childs et al., 1983). On the other hand, hCG is released constitutively following synthesis, since the human placental trophoblast cells lack such secretory granules (Hoshina et al., 1982).

In addition to the intact hormones, substantial amounts of uncombined or free α subunits are synthesized and secreted from the pituitary (Parsons & Pierce, 1983) and placental tissues (Nishimura et al., 1983; Blithe & Nisula, 1985; Kawano et al., 1988). O-linked oligosaccharides have been found in the free α-subunit isolated from bovine pituitaries and the N-linked oligosaccharides on the free hCG α appear to differ in structure from those on native hCG. These alterations in glycosylation are thought to be responsible for the inability of free α-subunits to reassociate with the β-subunit, which distinguishes them from the combined α-subunits.

Individual subunits are generally considered to be biologically inactive, because neither α- nor β-subunit has appreciable receptor binding activity. Full biological activity
of the hormone is restored after reassociation to form the α-β complex (Sairam, 1983). Studies of various hybrid molecules, which have been prepared from the α and β subunits of different hormones and species, show that the β subunit not only determines hormonal specificity but that the species of origin affects the affinity for receptor binding (Strickland & Puett, 1981). The species of origin of the α subunit also affects potency. Regardless of the hormone specificity of the β subunit, both subunits in associated forms are thought to contribute to the binding sites for receptor interaction, with the common α subunit playing a more dominant role in the formation of the active α-β complex (Moyle et al., 1982; Milius et al., 1983). The subunit association appears to be a complex process that involves conformational changes in both subunits to generate binding sites for receptor interaction. Circular dichroic (CD) spectra of free subunits are known to differ from spectra observed in their heterodimers (Ryan et al., 1988). It is assumed that similar regions in the β subunits are involved in binding to α subunits, while the variable regions bind to the receptor. The rates of dissociation and reassociation of subunits vary considerably from hormone to hormone (Reichert et al., 1973; Reichert & Ramsey, 1975). This appears to imply that the conformational changes in each subunit that occur on subunit association are dependent upon the structure of the β-subunit. As yet no native gonadotrophic hormones have been successfully crystallised; however, recent advances in crystallisation of desialylated hCG (Lustbader et al., 1989) are expected to facilitate the elucidation of the tertiary structure of this and other glycoprotein hormones.
Asn-linked oligosaccharides

A

\[
\begin{align*}
\text{Man}^\alpha_1 \\
\text{Man}^\beta_1-4\text{GlcNAc}^\beta_1-4\text{GlcNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1-2\text{Man}^\alpha_1 \\
\pm\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1-2\text{Man}^\alpha_1 \\
\text{Man}^\beta_1-4\text{GlcNAc}^\beta_1-4\text{GlcNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1-2\text{Man}^\alpha_1
\end{align*}
\]

B

\[
\begin{align*}
\text{Fuc}^\alpha_1 \\
\pm\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1-2\text{Man}^\alpha_1 \\
\text{Man}^\beta_1-4\text{GlcNAc}^\beta_1-4\text{GlcNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1-2\text{Man}^\alpha_1
\end{align*}
\]

C

\[
\begin{align*}
\text{NeuAc}^\alpha_2 \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-3\text{GalNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-3\text{GalNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1 \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-3\text{GalNAc} \\
\text{NeuAc}^\alpha_2 \\
\text{GalNAc}
\end{align*}
\]

Serine-linked oligosaccharides

\[
\begin{align*}
\text{NeuAc}^\alpha_2 \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-3\text{GalNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-3\text{GalNAc} \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-4\text{GlcNAc}^\beta_1 \\
\text{NeuAc}^\alpha_2-3\text{Gal}^\beta_1-3\text{GalNAc} \\
\text{NeuAc}^\alpha_2 \\
\text{GalNAc}
\end{align*}
\]

Fig. 1.4 Proposed structures of the Asn-linked oligosaccharides of urinary hCG (Endo et al., 1979).

Structures of serine-linked oligosaccharides on hCGβ (Cole et al., 1985; Damm et al., 1987) are also shown.
1.3 N-Glycan Structures of Gonadotrophins

The N-glycans of hCG were the first gonadotrophin oligosaccharides to be characterised in detail. Endo et al. (1979) reported that >90% of the Asn-linked sugar chains of urinary hCG were sialylated oligosaccharides that consisted of mono- and diantennary complex-type structures (Fig.1.4). Core-fucosylation was only found in a portion of the diantennary chains. All sialic acid residues were α2-3 linked to galactose residues, whereas sialic acids in α2-6 linkage were more common in many serum glycoproteins. In the Japanese study, the Asn-linked oligosaccharides were released from intact hCG by hydrazinolysis and were radiolabelled by reduction with NaB\(^{3H}\). Oligosaccharide mixtures were first separated by net charge and anionic oligosaccharides were desialylated to facilitate structural analysis. After fractionation by paper chromatography, neutral oligosaccharides were structurally characterized by Bio-Gel P-4 gel chromatography, followed by sequential exoglycosidase digestion and methylation analysis. Identical disialylated structures were proposed for the four Asn-linked sugar chains by Kessler et al. (1979). Fucose was found only in glycopeptides derived from hCG β. In a subsequent study, Mizuochi and Kobata (1980) examined the distribution of specific oligosaccharide structures on the two subunits of hCG and they compared placental hCG with urinary hCG. Placental hCG contained more neutral oligosaccharides than the hormone extracted from urine, which was consistent with the possibility that asialo forms of hCG are cleared from the circulation more rapidly; apart from that, the oligosaccharides on hCG from the two sources were almost identical.
However, when the oligosaccharides from each subunit were analysed by P-4 column chromatography after sialidase digestion, it was found that the α-subunit contained mono-(A) and diantennary (B) structures in equal amounts, while the β-subunit contained the two diantennary structures (B and C) in equal amounts. Because both subunits each have two N-linked sugar chains, these results led Mizuochi and Kobata to suggest site-specific distribution of the three asialostructures on hCG, with monoantennary and fucosylated diantennary structures being present only in the α and β-subunit, respectively. However, it remained unclear whether or not the diantennary structures were fully sialylated.

Recently, the N- and O-glycan structures of urinary hCG were studied using $^1$H-NMR (Damm et al. 1987). In this investigation, the Asn- and Ser-linked oligosaccharides were released sequentially from intact hCG and each subunit by enzymatic digestion (PNGase F) and treatment with alkaline borohydride. Following anion-exchange chromatography, the oligosaccharides were directly analysed by 500 MHz $^1$H-NMR. The monoantennary monosialo and diantennary disialo compounds were identified in a molar ratio of approximately 1:1 in the α-subunit, and monoantennary monosialo and diantennary disialo compounds in 1:4 in the β-subunit. 50% of the diantennary disialo compounds of the β-subunit were fucosylated (Fuc α1-6 linked to the proximal GlcNAc residue), while no fucose was detected in the α-subunit. Only traces of diantennary monosialo compounds were present in either subunit, in contrast to the earlier studies.

These results agree in general with the structures proposed
**Fig. 1.5** Proposed structures of the Asn-linked oligosaccharides (asialo-forms) of hFSH (Renwick et al., 1987).

The relative abundance is indicated by percentage of total oligosaccharides.
earlier for the hCG subunits (Mizuochi & Kobata, 1980), although the precise structures of the β-subunit were not identical. The possibility of site-specific glycosylation is interesting, particularly in view of the regulation of oligosaccharide biosynthesis; however, the oligosaccharide structures of individual N-glycan chains of this hormone remain to be identified.

Studies of the pituitary gonadotrophins have been more difficult than in the case of hCG, because of the limited availability of purified hormones and the more heterogeneous and complex nature of their oligosaccharides. This is particularly true of the human hormones, moreover their carbohydrate structures were largely unknown until recently. Earlier studies of LH and FSH from human, ovine and bovine pituitaries (Hara et al., 1978; Parsons & Pierce, 1980; Bedi et al., 1982) in which conventional methods were used for structural analysis, did not, yield definitive structures, although some important features were found for several hormones; e.g. terminal O-sulphation in o-, b- and hLH.

In 1987, Renwick et al. reported the structures of Asn-linked oligosaccharides from hFSH in detail (Fig. 1.5). Sequential lectin column chromatography was used to purify as well as characterise the neutral or asialo oligosaccharides, in addition to the analytical procedures used previously for hCG (Endo et al., 1979). 95% of the four sugar chains on hFSH were acidic oligosaccharides that contained 1-3 sialic acid residues. The asialo oligosaccharides were a heterogeneous mixture of di, tri, and tetraantennary complex-type oligosaccharides, with and without a fucose residue α1-6 linked to the proximal GlcNAc. In addition, some portions of the di- and triantennary structures
(30% and 15%, respectively) contained bisecting GlcNAc.

The monoantennary sugar chain found in hCG was not found in hFSH. The presence of di-, tri- and tetraantennary chains in about 45%, 50%, and 5%, showed that the di- and triantennary complex-type structures were the predominant forms, which were even more complex with some bisecting GlcNAc structures. Core-fucosylation was present in ~50% of the total oligosaccharides. Furthermore, the sugar chains of hFSH differed from those of hLH which were reported to contain significant amounts of sulphated diantennary complex oligosaccharides (Parsons & Pierce, 1984).

Green and Baenziger (1988a,b) published the results of their structural studies on the Asn-linked oligosaccharides on the human, bovine and ovine FSH. The oligosaccharides were released from each hormone by N-glycanase digestion and reduced with NaB[3H]₄. The ³H-labelled oligosaccharides were then fractionated by anion-exchange HPLC into populations that differed in the number of sialic acid and/or sulphate moieties. The anionic oligosaccharides were structurally characterized using various analytical techniques, including amine-adsorption HPLC, endo- and exoglycosidase digestion, and lectin affinity chromatography. The oligosaccharide structures of hFSH proposed by Green and Baenziger were similar to those reported earlier (Renwick et al., 1987) in that di- and triantennary complex chains were the two major components, however their precise structures and distributions were significantly different, particularly with the triantennary oligosaccharides. In addition, the tetraantennary chain reported earlier was not found, while significant amounts of sulphated structures (~10%) were present. Both α2-3 and α2-6 linked sialic acids were
identified, with the former being more predominant in hFSH, but their precise position and linkage in each oligosaccharide remained to be determined.

A possible explanation for the significant differences between the two studies would be that they reflected differences in the degree of purity and nature of charge-heterogeneity between the two hormone preparations, if one assumes that experimental error and variations associated with the analytical methods were minimal.

While certain structural features of hFSH oligosaccharides were delineated in these studies, the oligosaccharide structures present at each of the four glycosylation sites in this hormone remain to be identified.

Bovine and ovine FSH have identical α- and β- subunits (with respect to protein structure) with two N-linked glycans on each subunit; Asn 56 and Asn 82 in the α and Asn 6 and Asn 23 in the β-subunit.

Studies of acidic sugar chains from b- and oFSH (~70% of total in each species) showed that bFSH contained mostly diantennary complex-type oligosaccharides while oFSH contained both di- and triantennary chains. In contrast to hFSH, b- and oFSH contained significant amounts of sulphated oligosaccharides (60% and 20% of the acidic oligosaccharides respectively). These results demonstrate that the N-glycans of FSH differ considerably between species, not only in their branching structures but also in respect of terminal sialylation and sulphation.

All glycoprotein hormones from bovine, ovine and human pituitaries contain sulphate, with the exception of hFSH whose charged groups are sialic acid residues (Renwick et al., 1987).
The contents of sulphate and sialic acid in these hormones vary with the hormone and the species; e.g. hLH contains both sulphate and sialic acid, while b- and oLH contain sulphate exclusively. Since placental hCG is devoid of sulphate, sulphation of the pituitary glycoprotein hormones is also considered to be tissue-specific.

Structural studies of the Asn-linked oligosaccharides of LH and other pituitary hormones were hampered by the lack of adequate methods for the separation of sulphated and sialylated oligosaccharides. For this reason, applications of $^1$H-NMR spectroscopy, the most rigorous method for the structural analysis of the more common sialylated oligosaccharides, were limited. There was also no effective means for the removal of sulphate residues without causing damage to the underlying oligosaccharide structures, since suitable sulphatases were unavailable.

These problems have largely been solved in recent years, by the development of HPLC techniques and methanolysis for removal of sulphate. Anion-exchange HPLC was developed to separate anionic oligosaccharides according to the number and types of acidic moieties (Baenziger & Natowicz, 1981), and ion-suppression amine adsorption HPLC was devised for the separation as well as the structural characterization of different species of anionic oligosaccharides (Mellis & Baenziger, 1983).

The use of such methods together with lectin affinity chromatography and endo- and exoglycosidase digestion allowed Green et al. (1985a) to examine the structures of metabolically labelled oligosaccharides from bLH which were a mixture of neutral, mono- and disulphated species. The structure of the
Fig. 1.6 Proposed structures of the Asn-linked oligosaccharides of hLH (Green & Baenziger, 1988a,b).

The relative abundance is indicated by percentage of total oligosaccharides.
disulphated oligosaccharide (S-2) was characterized and confirmed using methylation and $^1$H-NMR analysis.

The proposed structure for S-2 was identical with other diantennary complex oligosaccharides in its common pentasaccharide core with the two α-Man residues, each substituted by GlcNAc in β-1-2 linkage. Its unique feature lay in the presence of β1-4 linked SO$_4$-GalNAc at both non-reducing termini, where β1-4 linked Gal is commonly found in sialylated diantennary structures. The sulphate residue was shown to be located in the C-4 position of GalNAc in a subsequent NMR study (Vandana et al., 1987).

More recently, structures of the Asn-linked oligosaccharides from human, bovine and ovine LH have been reported (Green and Baenziger, 1988a,b). Oligosaccharides were released from individual hormones by digestion with N-glycanase and reduced with NaB[3H]$_4$. 3H-labelled oligosaccharides were fractionated by anion-exchange HPLC and the structures of sulphated and sialylated oligosaccharides were determined using the same approaches employed previously for the identification of S-2. However, more rigorous techniques such as $^1$H-NMR and methylation analysis were not used due to the limited amounts of purified hormones available for study.

The N-glycans of hLH consisted of a heterogeneous mixture of neutral, sulphated, sialylated, and sulphated/sialylated oligosaccharides (Fig.1.6). The acidic oligosaccharides (~85% of total) had mainly diantennary complex-type structures, which were derived from disialylated (N-2), sulphated/sialylated (S-N), and disulphated (S-2) structures, with S-N being most abundant (~20%). They were distinct in respect of the structure
of the two peripheral branches and S-N was unique in that it possessed both sialylated and sulphated branches. Some N-2 contained bisecting GlcNAc. The remaining acidic oligosaccharides were sulphated hybrid- and sulphated or sialylated monoantennary complex-type structures (~10% each). The amounts of all the individual structures were relatively small.

On the other hand, the oligosaccharides from b- and oLH were less heterogeneous. Their acidic oligosaccharides were exclusively sulphated and consisted mainly of monosulphated hybrid- and mono- or disulphated diantennary complex-type structures. Although the types of the major structures were similar, their proportions differed between the two hormones; the contents of hybrid- and diantennary structures were 25% and 40%, respectively, for bLH and 45% and 23% for oLH.

Thus, the Asn-linked oligosaccharides of LH differed significantly in both branching and peripheral structures between the three hormones, which indicated species-specific oligosaccharide processing for LH, as observed for FSH.

The oligosaccharide structures for hTSH and bTSH have also been reported (Green and Baenziger, 1988a,b). The types of sulphated and sialylated oligosaccharides on hTSH were similar to those of hLH, but were more sulphated, with S-2 and S-N the major components. The bisected structures present in sialylated oligosaccharides on hLH were not found. Like bLH, bTSH contained exclusively sulphated oligosaccharides, which were mostly derived from the diantennary disulphated structure.

It is evident that the Asn-linked oligosaccharides of the pituitary and placental gonadotrophins exhibit structural heterogeneity derived from variations in both branching and
peripheral structures. There are significant differences in the types and distributions of sialylated and sulphated oligosaccharide structures among the three human hormones and also among the different animal species. It should be noted, however, that there are also some similarities between hormones. For example, most of the acidic oligosaccharides on hLH and its placental analogue, hCG, have similar branching structures, i.e. di- and monoantennary complex (4:1), although hLH has some bisected structures. Their branch structures differ mainly in respect of their terminal sialylation/sulphation and sialic acid linkages. For hLH and hFSH, both contain a high proportion of diantennary complex oligosaccharides, although their overall branching patterns and terminal sialylation/sulphation are different.

According to Green and Baenziger (1988b), LH and FSH from all three species (bovine, ovine and human), contain substantially different proportions of sulphated and sialylated oligosaccharides, with sulphated structures dominant in LH and sialylated structures in FSH. These differences were considered to characterize the differential glycosylation of LH and FSH, which are believed to be synthesized within the same cells (Liu et al., 1988). However, this may not necessarily be the case for human and ovine hormones, since hLH and oFSH contain sulphated and sialylated oligosaccharides in about equal amounts (Green & Baenziger, 1988b). Therefore, differences in the underlying oligosaccharide structures should also be considered, when one assesses the glycosylation patterns of the two hormones.

All the gonadotrophic hormones contain multiple sites of N-glycosylation, therefore, as previously suggested for hCG
(Mizuochi & Kobata, 1980), it is possible that the individual N-glycans on each hormone have a distinct set of oligosaccharide structures, which may be related to their function(s). It is also known that glycoproteins have heterogeneous oligosaccharide structures even at a single glycosylation site (Kobata, 1984). Thus the heterogeneity within and between glycosylation sites may have been reflected in the overall structural heterogeneity observed for each hormone.

In this respect, structural characterization in most of the previous studies was carried out on total oligosaccharides released from each hormone which obviated the assignment of specific carbohydrate chains to their specific sites of attachment in each subunit. It was therefore necessary to determine the oligosaccharide structures unambiguously and to define their specific attachments to glycosylation sites in each hormone.

1.4 Biosynthesis of N-Glycans on Pituitary Gonadotrophins

Asn-linked oligosaccharides are broadly classified into three types designated as high mannose, hybrid and complex on the basis of their component sugars (Kobata, 1984). All three types of oligosaccharides are derived from a common lipid-linked precursor, Glc₃Man₉GlcNAc₂-P-P-dolichol (Kornfeld & Kornfeld, 1985). Subsequent to cotranslational transfer of the oligosaccharide chain to nascent protein, a series of processing reactions is carried out by membrane-bound glycosidases and glycosyltransferases in the endoplasmic reticulum and Golgi apparatus, leading to the formation of either high mannose, hybrid or complex chains. The general pathways and the enzymes
Responsible for the synthesis of the three classes of common sialylated oligosaccharides are well-characterised (Kornfeld & Kornfeld, 1985).

Oligosaccharide processing of glycoproteins depends largely on the activities and specificities of Golgi enzymes in the cell that is animal species- and tissue-specific (Rademacher et al., 1988). However, protein structure also plays a key role in directing oligosaccharide processing, typically through steric hindrance (Hubbard, 1988). Many glycoproteins with more than one glycosylation site exhibit site-specific glycosylation; each site has characteristic oligosaccharides that differ from those at other sites (Hsieh et al., 1983; Swiedler et al., 1985; Parekh et al., 1987). Relatively little is known concerning the detail of influences exerted by protein structures and their significance in regulating oligosaccharide synthesis.

Previous structural studies of Asn-linked oligosaccharides attached to pituitary glycoprotein hormones have revealed that the pituitary cells (gonadotrophs and thyrotrophs) contain two unique glycosyltransferases, viz. GalNAc- and sulphotransferases, and that they synthesize various sulphated oligosaccharides as well as more common sialylated oligosaccharides. Both hormone- and species-specific differences in the types and distributions of sulphated and sialylated oligosaccharides have been described (Green & Baenziger, 1988b) and it was considered that both types of oligosaccharides are synthesized from common synthetic intermediates, since they contain the identical core structure (Man₃GlcNAc₂).

A general pathway for the synthesis of sulphated and sialylated oligosaccharides on the pituitary glycoprotein
Fig. 1.7 Proposed pathway for synthesis of sulphated and sialylated oligosaccharides on the pituitary glycoprotein hormones (Green et al., 1986a).
hormones has been proposed by Green et al. (1986a) (Fig. 1.7) based on the established synthetic pathway for sialylated Asn-linked oligosaccharides.

Since the addition of GalNAc (and sulphate) to the intermediate GlcNAc$_2$Man$_5$GlcNAc$_2$ (I) prevented further processing by α-mannosidase II and GlcNAc-transferase II in vitro (Green et al., 1986a), it was assumed that competition between GalNAc-transferase and α-mannosidase II for the same substrate determines whether or not the final product becomes hybrid-type or complex-type. In the latter case, various forms of sulphated and/or sialylated diantennary and sialylated triantennary complex oligosaccharides, e.g. S-2, S-N, N-2, and N-3, are synthesized by further processing of the common intermediate GlcNAc$_2$Man$_3$GlcNAc$_2$ (II), that involves competition between GalNAc-, Gal-transferases and GlcNAc-transferase IV.

Although hormone- and species-specific glycosylation of the pituitary hormones were discussed in terms of the competition between these transferases, the question of how protein structure affects oligosaccharide synthesis in each hormone remains unanswered.

The hormone- and tissue-specificity of oligosaccharide sulphation was examined using a cell-free sulphation system (Green et al., 1984). In this model, bLH, bFSH, hLH, and hTSH added exogenously were all sulphated in their oligosaccharides with bovine pituitary membranes, but hFSH and hCG were not. Rat liver membranes also showed weak sulphotransferase activity towards bLH, however human placental membranes showed no activity (Green et al., 1985b).

Using the cell-free system, it was determined that the sequence
GalNAcβ1→4GlcNAcβ1→2Mana is the minimal structural requirement for the addition of sulphate to the pituitary glycoprotein hormones (Green et al., 1985c). Sulphotransferase activity was enriched in the Golgi fraction but was absent in the secretory granule fraction of bovine pituitary, which suggested that sulphation occurs before the hormones are transported to the secretory granules.

Smith and Baenziger (1988) have reported that GalNAc transferase in the bovine pituitary gland can specifically recognise the human α-subunit. They showed that agalacto-oligosaccharides on hCG-α were better substrates for GalNAc-transferase than Gal-transferase, in contrast to those on other glycoproteins examined. The highly selective activity of GalNAc-transferase for hCG-α with its high affinity suggested that this enzyme recognises features of the polypeptide in a similar manner to GlcNAc-phosphotransferase for lysosomal acid hydrolases (Kornfeld, 1987). However, this putative recognition marker has not been identified for any sulphated pituitary hormone.

Whereas all three human gonadotrophins share an α-subunit, the oligosaccharides on this common subunit appear to be significantly different, although their precise structural differences remain to be characterised. For hLH and hFSH, which are generally assumed to be synthesized within the same cell, their marked differences in terminal sulphation/sialylation were attributed to the hormone-specific β-subunits (Green et al., 1986b).

To explain the relative absence of sulphated oligosaccharides in FSH, it has been postulated that the FSHβ-
subunit limits access of GalNAc-transferase to the recognition marker present on the α-subunit (Smith & Baenziger, 1988). Similar effects of the associated β-subunit in limiting the extent of processing of hCGα-subunit by GlcNAc transferase have been reported (Lustbader et al., 1987; Corless et al., 1987a,b). However, in the case of bLH, the dimerization of LHα- and LHβ-subunits does not seem to affect the synthesis of sulphated oligosaccharides significantly, since the glycosylation patterns of free α and LHα are similar (Green & Baenziger, 1988b).

The differential terminal sulphation/sialylation of LH and FSH has been suggested to aid in the sorting of these hormones into separate secretory granules for their nonparallel release from the pituitary cells (Baenziger & Green, 1988). Although this is an interesting idea, it should again be noted that LH and FSH show considerable species variations in this respect.

1.5 Functions of N-glycans on Gonadotrophins

To date, various functions have been suggested for the N-glycan moieties of the gonadotrophins; they include not only those that are non-specific, such as stabilisation of protein conformation and regulation of metabolic clearance from the circulation, but also specific roles associated with hormonal function. In the latter case, it is most likely that the N-glycan moieties of the gonadotrophins are essential for the expression of cellular responses, such as cAMP production and steroidogenesis. The roles of individual N-glycans in hormone secretion, assembly and function have been addressed in recent studies and their functions in gonadotrophins are reviewed in this section, with particular reference to their participation in biological function.
In the biosynthesis of gonadotrophins, subunit assembly does not require post-translational modification of N-linked oligosaccharides, since it occurs early in the secretory pathway, probably in the endoplasmic reticulum. (Hoshina & Boime, 1982). However, the initial glycosylation of each subunit is important to ensure correct folding and disulfide bond formation (Strickland and Pierce, 1983) as well as for proper subunit assembly and secretion (Matzuk and Boime, 1988). Concerning the latter aspects, mutagenesis studies have shown that the two N-glycans on hCGα have different intracellular functions; glycosylation site 78 plays a role in the integrity of the hormone, while site 52 is important for the assembly of intact hCG.

The significance of sialic acids in the in vivo biological activity of gonadotrophins is well known (Gottschalk et al., 1960; Van Hall et al., 1971a; Braunstein et al., 1971) and terminal sialylation is considered to protect the hormones against rapid clearance from the circulation by hepatic asialoglycoprotein binding lectins (Van Hall et al., 1971b; Van Lenten & Ashwell, 1972). Removal of sialic acids from hCG, for example, reduced its plasma half-life in the rat from 50 min to 1 min (Van Hall et al., 1971b). Some species of LH such as bovine and ovine contain sulphate groups and little or no sialic acid but sulphate is considered to have a similar role in regulating the metabolic clearance of these hormones.

Several investigations have suggested that sialic acids are also important for the full expression of the hormonal activity of hCG, since desialylation reduces its in vitro biological activity (Moyle et al., 1975; Amir et al., 1987; Amano et al., 1989). On the other hand neuraminidase-treated recombinant bLH,
that contained sialylated oligosaccharides in contrast to native bLH, were reported to have increased steroidogenic activity. (Smith et al., 1990).

Amir et al. (1987) reported that desialylation of hCG enhances its binding activity for hCG receptors, while it changes hCG from a full agonist to a partial agonist in testis. Their study suggested that sialic acid in the β-subunit of hCG has a predominant role in these effects.

The role of N-glycosylation of gonadotrophins in biological function has been intensively studied using chemically or enzymatically deglycosylated hormones (Thotakura & Bahl, 1982; Manjunath & Sairam, 1982; Chen et al., 1982). In these experiments, chemical methods, such as short treatment with HF, were more widely employed to assess the effects of deglycosylation on three aspects of gonadotrophin action, i.e. receptor-binding, adenylate cyclase activation and steroidogenesis.

Various hormones were shown to retain full receptor-binding activity after deglycosylation with HF, whereas some hormones such as hCG and oFSH showed increased activity (Sairam, 1989). In contrast, the ability of such deglycosylated hormones to stimulate adenylate cyclase and steroidogenesis in whole cells or membrane preparations was markedly reduced, which indicated that receptor-binding and cellular activation are dissociable by modification of N-glycans. These deglycosylated hormones were also shown to act as competitive inhibitors of biologically active native hormones. These observations have led to the concept that N-glycosylation has an obligatory role in steroidogenic function of gonadotrophins, by coupling the hormone-receptor complex to the adenylate cyclase system.
(Sairam, 1989). Although this view is now widely held, a few studies suggest that loss of steroidogenic activity of HF-treated hormones is due to factors other than carbohydrate removal. Cole et al. (1987) reported that o- and hLH enzymatically deglycosylated with endopeptidase F retained significant steroidogenic activity.

Several studies have addressed the role of the N-glycans in the individual subunits using differential deglycosylation and reassociation. Sairam and Bhargavi (1985) used HF-treated oLH and oFSH to show that the N-glycans on the α subunit were more critical for biological activity than those on the β-subunit when assessed by cAMP accumulation. Similar results were observed for hCG reconstituted from enzymatically or chemically deglycosylated subunits (Goverman et al., 1982; Keutmann et al., 1983; Karyan & Bahl, 1983), but others reported that the N-glycans on both α and β subunits of hCG were required for adenylate cyclase activation, when HF-treated subunits were used (Shimohigashi & Chen, 1982).

The importance of N-glycosylation of the α subunit in signal transduction has been confirmed in recent studies that employed site-directed mutagenesis, which has also revealed the site-specific function of the individual N-glycans on hCG (Matzuk et al., 1989). The results showed that presence of a single N-glycan at Asn 52 of the hCGα subunit is critical for both production of cAMP and steroidogenesis, while the absence of the N-glycans from the β subunit and/or Asn 78 of the α has no effect on these responses. As the α subunit is common to all glycoprotein hormones, it is postulated that glycosylation at Asn 52 plays an essential role in hormone function (Sairam, 1989).
At present, it is not clear how the N-glycan on the α subunit contributes to post-receptor-binding events. The glycan moiety may be directly involved in the activation of adenylate cyclase through interaction with cell membrane lectins as suggested by Calvo and Ryan (1985). Alternatively, glycosylation of the α subunit may be important to maintain the conformation of the hormone that is required for the formation of productive receptor-hormone complex (Sairam, 1989). This concept is supported by several lines of immunological and chemical evidence that indicates conformational changes in deglycosylated hormones (Hattori et al., 1988; Sairam et al., 1988; Merz, 1988). Such altered hormones may not lead to positive coupling with the adenylate cyclase.

In addition, new insight into the mechanism of gonadotrophin action has been provided by the two recent observations. Willey and Leidenberger (1989) have reported that hCG is structurally and functionally related to a serine protease, α-chymotrypsin. They suggested that loss of agonism with deglycosylated hCG may be simply due to a change in peptide geometry as the glycosylated positions of the α subunit are spatially close to the catalytic domain of the hormone.

A thioredoxin-like catalytic activity of LH and FSH has also been found by Boniface and Reichert (1990), who proposed that disulfide-isomerization or redox reactions may produce structural changes in the receptor required for signal transduction.

As yet, little is known about the functional significance of the specific oligosaccharide structures attached to the gonadotrophins, although a few recent reports have suggested that they are important for the expression of full biological
activity (Hattori & Wakabayashi, 1989; Amano et al., 1990).

1.6 Aims of Present Study

Detailed knowledge of the carbohydrate components of glycoproteins is a vital prerequisite for the proper understanding of their roles in the structure and function of the intact native molecules. This is especially true for the gonadotrophins whose N-glycans play essential parts in biological function.

Structural features of N-linked oligosaccharides of the pituitary and placental gonadotrophins from several species have been reported in recent years. However, specific details of their oligosaccharides remain unclear and such information is required to establish the basis of structure-function relationships.

The main aim of the present study was to develop a preparative scheme for the isolation and characterisation of the N-glycans from each of the three glycosylation sites of human LH. However, in the light of reports that hLH prepared by conventional purification procedures does not accurately reflect the content of naturally occurring forms of this hormone in pituitary glands, it was decided to develop a new method of purification.

It was also decided to isolate and characterise the N-glycans of ovine LH with the aim of studying the reported species-specificity in the glycosylation of LH in more detail.
CHAPTER 2  Separation of Human Pituitary Glycoprotein Hormones and Their Subunits by Reverse-Phase HPLC

2.1 Introduction

The glycoprotein hormones of the human pituitary gland, hFSH, hLH and hTSH, are closely related in structure. Each hormone is a heterodimer in which the α-subunit is non-covalently associated with the hormone-specific β-subunit (Pierce & Parsons, 1981).

Similarities in size, structure and considerable heterogeneity in charge (Wide, 1985a; Johnston et al., 1987) have caused problems in the isolation and purification of these glycoproteins and no simple chromatographic procedure has been developed for the analytical or preparative separation of these pituitary hormones.

Reverse-phase chromatography offered potential advantages but its usefulness was uncertain. Reverse-phase HPLC has proved effective for the isolation of subunits (Putterman et al., 1982; Parsons et al., 1984), but its application to intact hormones has been limited due to ready dissociation into subunits. A partial or complete loss of biological activity following reverse-phase HPLC was also reported with some hormones (Wilks & Butler, 1984; Hallin & Khan, 1986). On the other hand, several investigators described this method as effective for the separation of individual hormones (Bristow et al., 1983; Grego & Hearn, 1984), although no resolution of hFSH, hLH and hTSH was achieved. These various results indicated that the chromatographic conditions were worth fuller investigation for the successful separation of these labile hormones.

The aim of this study was to develop a reverse-phase HPLC
procedure for the separation of all three glycoprotein hormones and their subunits.

2.2 Experimental Procedures

2.2.1 Materials

Purified human FSH, LH and TSH were prepared by DEAE-Sephacel chromatography and reverse-phase HPLC (Hiyama et al., 1988) from the Sephadex G-100 glycoprotein fraction obtained according to Chapman et al. (1981). Acetonitrile (HPLC grade), trifluoroacetic acid (TFA, reagent grade) and orthophosphoric acid (AnalaR grade) were obtained from BDH Chemicals (Palmerston North, New Zealand). Triethylamine (TEA, analytical grade) was from Riedel-de Haen AG (Seelze-Hannover, F.R.G.). Water was purified by a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical grade.

2.2.2 Reverse-Phase HPLC

A Tosoh HPLC system (Tosoh, Tokyo, Japan) consisting of a model CCPM pump with microprocessor control and a Rheodyne 7125 injector with a 2 ml sample loop was used with a Uvicord S model 2138 uv monitor (LKB, Bromma, Sweden). Wide-pore (300 Å) Vydac Protein C₄ columns (5 μm, 0.46 x 15 cm, 1 x 25 cm, Separations Group, Hesperia, CA, U.S.A.) and a wide-pore (300 Å) SynChropak C₅ column RP-1 (6.5 μm, 0.46 x 10 cm, SynChrom, Lafayette, IN, U.S.A.) were used for separations. Cartridge columns packed with SynChropak RP-1 (0.46 x 1 cm) were supplied by Alltech (Auckland, New Zealand). Samples of each or a mixture of the three hormones were prepared in 0.1 M NaH₂PO₄ buffer, pH 7.0 (1.0 mg each hormone/ml solution). When separating subunits, hormones were dissociated prior to HPLC as follows: hFSH was
treated with 0.1% TFA, pH 2.3, for 30 min at 25°C and hLH and hTSH were incubated for 20 h at 37°C with 6 M guanidine HCl in 0.1 M NaH₂PO₄ buffer, pH 7.0, that contained 0.02% (w/v) sodium azide (1.0 mg hormone/ml). Samples were chromatographed on a Vydac C₄ column with and without a C₁ column in series, using a linear gradient of acetonitrile in various buffers in the pH range 2.5 to 7.0. Detailed chromatographic conditions are described in the legends for figures. Eluates were monitored at 226 nm. Subunit peaks were identified by amino acid analysis or by comparison of retention time with those of the hormonal subunits.

2.2.3 Analytical Methods

Recovery of intact hormones after reverse-phase HPLC was determined by specific radioimmunoassays using Amerlex-M kits for hFSH (IM 3071), hLH (IM 3081) and hTSH (IM 3161) (Amersham Australia, Sydney, Australia). Each of the hormone peak fractions was collected and diluted to 3.0 ml with water. Aliquots of this solution were further diluted with Tris-HCl buffer, pH 7.4, that contained 0.9% NaCl and 0.1% BSA, to yield a final concentration of 2.5-10 ng/ml. Controls were prepared by diluting unfractionated hormones (100 µg) as above. The yield of each subunit was calculated from protein concentrations determined with protein assay kits (Bio-Rad, Richmond, CA, U.S.A.). Controls were prepared as above from dissociated hormones (100 µg). Samples and controls were assayed using 0.01 M NaH₂PO₄ buffer (pH 7.0) as diluent and bovine γ-globulin as reference. The following Mr values (kDa) were used to calculate percentage yields by mass; α 14.6, hFSH-β 18.0, hLH-β 14.8, hTSH-
2.3 Results and Discussion

As noted earlier, all three pituitary glycoprotein hormones are unstable in acidic solution (pH < 4) at 25°C, and they readily dissociate into their subunits (Reichert & Ramsey, 1975; Jacobson et al., 1978); this is especially true in the case of hFSH. Because of this, the pH of mobile phases used for reverse-phase HPLC of the intact hormones is limited to the range where dissociation does not occur or where it is minimal. Various mobile phases in the pH range 7-7.8 have been employed with alkylphenyl, C1, C4, and C18, alkyl-chain bonded silica columns in studies of the behaviour of the individual hormones (Grego & Hearn, 1984). Bristow et al. (1983) reported that hTSH, which was stable at pH 7.0, was significantly dissociated into subunits at pH 5-6, during analysis on a C4 column in NaCl-containing buffers. Because this hormone is stable in aqueous buffers at pH 5-7, the marked effect of mobile phase pH must be ascribed to pH-dependent influences of protein-ligand interactions on the association of subunits.

Initially, the influences of the various mobile phase conditions on the separation and stabilities of the three intact hormones were studied using C4 and C18 columns at pH 5-7. It was found that these hormones were more stable on shorter alkyl-chain packings (C4) and that gradient elutions with acetonitrile in 0.1 M triethylamine phosphate buffer (TEAP) at pH 6-7 gave good separations of hLH and hTSH. The dissociation of these hormones was noticeably decreased upon increasing the pH of the mobile phase to 6.5, above which no significant differences were observed. However, under these conditions hFSH was very
Fig. 2.1 Reverse-phase HPLC of hFSH, hLH and hTSH and their subunits on a Vydac C₄ (0.46 x 15 cm) column.

Mobile phase A, 0.1 M TEAP (pH 6.5); mobile phase B, 0.1 M TEAP in 60% (v/v) acetonitrile (pH 6.5). The 40 min linear gradient from 12 to 44% (v/v) acetonitrile is indicated by the broken line. Flow-rate, 0.8 ml/min. Samples: (a) intact hormones (25 μg each); (b) dissociated hormones, hFSH (10 μg), hLH and hTSH (25 μg each) prepared as described in Experimental Procedures. The subunit peaks from each hormone are indicated.
unstable, although its hydrophobic interaction with this column packing was apparently less than that of hLH and hTSH. Its dissociation was slightly reduced upon increasing the initial concentration of acetonitrile for gradient elutions from 0-18%; above this range, hFSH was not effectively retained by C₄. These preliminary results indicated that the pH of the mobile phase and the initial concentration of acetonitrile were important variables that affected the separation of the three hormones. Acetonitrile (12%, v/v) was chosen for initial gradient elution during the separation of hFSH, hLH and hTSH, to ensure the retention of hFSH, which was eluted first from the C₄ column.

The elution profiles of the three hormones obtained with a Vydac C₄ column with a linear gradient of 12-44% acetonitrile in 0.1 M TEAP, pH 6.5, are shown in Fig.2.1a. The extent of dissociation of each intact hormone was assessed by detection of its α and/or β subunit peaks, which were clearly resolved from their respective intact hormones except in the case of hFSH (Fig.2.1b). hTSH and hLH were eluted almost as a single peak without significant formation of subunits. In contrast, hFSH was eluted as two discrete peaks that corresponded in retention time to those of its subunits. Because hFSH-α and hTSH were well resolved, it was possible to separate all three intact hormones with partial dissociation of hFSH. These results suggested that, despite this limitation, this procedure would be useful for the separation of intact hLH and hTSH.

Various unsuccessful attempts were made to reduce the dissociation of hFSH by modification of mobile phase parameters such as flow-rate, gradient slope and buffer composition. The substitution of the less eluotropic methanol for acetonitrile
Fig. 2.2 Elution profiles of hFSH on C₁ and C₄ columns in series.

Columns: SynChropak RP-1 (0.46 x 1 cm) and Vydac C₄ (0.46 x 15 cm). Mobile phases A and B are the same as in Fig. 2.1. The 45 min linear gradient from 0% (a), 6% (b) and 12% (v/v) acetonitrile (c and d) with a slope of 0.8% (v/v) acetonitrile per min are indicated by the broken lines. Flow-rate, 0.8 ml/min. Samples: (a-c) hFSH 10 μg, (d) dissociated hFSH, 20 μg.
Fig. 2.3 Separation of hFSH, hLH and hTSH on analytical C1 and C4 columns in series.

Columns: SynChropak RP-1 (0.46 x 1 cm) and Vydac C4 (0.46 x 15 cm). Mobile phase A, 0.1 M TEAP (pH 6.5); mobile phase B, 0.1 M TEAP in 60% (v/v) acetonitrile (pH 6.5). The 40 min gradient from 12 to 44% (v/v) acetonitrile was applied at a flow-rate of 0.8 ml/min as shown in Fig. 2.2c. Samples: a mixture of hFSH, hLH and hTSH (10 μg each).
and the use of Na\(^+\) as a less hydrophobic counter-cation were also ineffective. The results suggested that the C\(_4\) column was too strongly hydrophobic for the separation of hFSH by reverse-phase HPLC.

When other ligands such as C\(_1\), alkylphenyl and cyanopropyl silicas were examined with the view of replacing C\(_4\), only C\(_1\) showed a noticeable decrease in the dissociation of hFSH under the conditions used in Fig.2.1. However its lower selectivity did not permit the effective separation of the three hormones. Because the magnitude of solvent-ligand interactions is expected to decrease with less retentive ligands and with increasing organic solvent modifier in the mobile phase, it was decided to insert a small C\(_1\) cartridge column in series with the C\(_4\) column. Fig.2.2 shows the effects of a coupled C\(_1\) column on the elution of hFSH, which was examined at various initial concentrations of acetonitrile (0-12\%) without changing the solvent system and gradient slope as in Fig.2.1. Although these effects were small at 0% acetonitrile (Fig.2.2a); at 12% acetonitrile, hFSH was eluted as a major peak with the same retention time as its \(\beta\)-subunit, only a small \(\alpha\)-subunit peak was detected (Fig.2.2c, d). This showed that the rapid subunit dissociation of this hormone was greatly reduced and that the intact hormone was eluted with its \(\beta\)-subunit. When a mixture of hFSH, hLH and hTSH was chromatographed, all three hormones were separated from each other without significant dissociation (Fig.2.3), which indicated that this modified procedure was effective for the separation of all three intact hormones.

The results suggest that reverse-phase HPLC can provide a rapid and sensitive method for the analysis of purity of hormone
Fig. 2.4 Preparative separation of hFSH, hLH and hTSH on C₁ and C₄ columns in series.

Columns: SynChropak RP-1 (0.46 x 10 cm) and Vydac C₄ (1 x 25 cm). The elution conditions were the same as in Fig. 2.3. Samples: (a) hFSH (25 µg); (b) hFSH (25 µg), hLH (50 µg) and hTSH (15 µg).
preparations, for which no single procedure is available at present. Because the solvent system used does not interfere with the detection at lower wave-length, it is possible to analyse hormones in <1 μg levels with detection limits at 50-200 ng at 206 nm.

Preparative applications of this procedure were attempted with larger C₄ (1 x 25 cm) and C₁ (0.46 x 10 cm) columns, because of the limited capacity of the C₁ cartridge. When a mixture of the three hormones was chromatographed without changing the gradient elution conditions, good separation of all three hormones was obtained (Fig.2.4b). The average recovery of hFSH, hTSH and hLH (100 μg each) from the columns was 56, 66 and 76%, respectively, by RIA. This procedure has been found useful for the microscale isolation of each pituitary hormone from partially purified preparations that contained the three glycoproteins in various amounts (Hiyama et al., 1988). Although no attempts were made to determine the effect of reverse-phase HPLC on biological activity in this study, it has recently been confirmed that hLH and hTSH retained high biological activity after purification on a C₄ column, according to the procedure described (Hiyama et al., 1990). The effect on hFSH has yet to be determined. These results show that reverse-phase HPLC is also effective for the preparative separation of intact pituitary glycoprotein hormones.

The α- and β-subunits of hFSH, hLH and hTSH can be rapidly separated on a Vydac C₄ column using a linear gradient of acetonitrile in 0.1 M TEAP, pH 6.5 (Fig.2.1b). This reverse-phase-HPLC method will be particularly useful for purity analysis of isolated subunits of these hormones, since no rapid and effective procedure is currently available. In this
Fig. 2.5 Separation of the subunits of hTSH under modified elution conditions.

Column: Vydac C₄ (0.46 x 15 cm). (a) A 40 min linear gradient from 18 to 42% (v/v) acetonitrile was applied in 0.1 M TEAP, pH 6.5, at 0.8 ml/min. (b) A 40 min linear gradient from 18 to 30% (v/v) acetonitrile was applied in 0.1 M TEAP, pH 3.0 at 0.8 ml/min. Samples: dissociated hTSH (20 μg).
In respect, SDS-polyacrylamide gel electrophoresis has limitations in resolution of the two subunits of either hormone, due to their similar Mr and band broadening caused by their attached carbohydrates (Segrest & Jackson, 1972). While the three α-subunits, which have virtually identical amino acid sequences, were eluted very closely as previously noted (Parsons et al., 1984), the separation of three β-subunits was readily obtained. In the case of hLH, the intact hormone and its subunits were almost completely separated from each other (Fig. 2.1a, b). Thus, this method may provide a convenient means of preparing reassociated hormones, including hybrids consisting of hLHβ and α-subunits from other hormones, since the chromatographic behaviour of such hormones can be expected to be similar to that of hLH. The average yield of subunits from 100 µg of each hormone pretreated was as follows: hFSH-α 69%, -β 67%; hLH-α 52%, -β 71%; hTSH-α 63%, -β 76%. These yields were significantly higher than those previously reported by Parsons et al. (1984) (α 50%, β 10-30%), who used a C18 column and 0.1 M sodium phosphate, pH 6.8/acetonitrile system. The low recoveries with C18 could be ascribed in part to the fact that the ligand is too hydrophobic.

Because the resolution of hTSH subunits was not sufficient to apply this procedure to large-scale separation, the effects of gradient conditions and mobile phase pH were further examined in 0.1 M TEAP/acetonitrile system. As a result, complete separation of the subunits was obtained at pH 6.5 using a shallower linear gradient (Fig. 2.5). The resolution of the subunit was also increased at pH 2.5-3.0 with the elution order reversed. In this acidic system, however, the resolution of hLH subunits was decreased and no obvious separation of hFSH subunits was effected. It appeared that the β-subunit of hFSH
was not eluted due to strong adsorption to the column, an observation also noted in the TFA/acetonitrile system.

In conclusion, this study has shown that reverse-phase HPLC is useful for the analytical and preparative separation of human pituitary FSH, LH and TSH and their subunits.
CHAPTER 3 Purification of Human Pituitary LH and TSH by Hydrophobic Chromatography

3.1 Introduction

The glycoprotein hormones of the human pituitary gland, follicle-stimulating hormone (hFSH), luteinizing hormone (hLH) and thyroid-stimulating Hormone (hTSH), have been isolated and purified by various methods, such as ion-exchange, gel-filtration (Parlow et al., 1965; Stockell Hartree, 1966; Bates et al., 1968), lectin affinity (Dufau et al., 1972) and immunoaffinity chromatography (Jack et al., 1987). Ion-exchange chromatography has been most widely used, however, this method appears to have inherent disadvantages because of the considerable charge heterogeneity of these hormones in pituitary tissue (Wide, 1985a). First, these glycoproteins have overlapping charges which make their complete separation difficult and laborious. Secondly, purification of these hormones as single fractions may be difficult (Stockell Hartree et al., 1972; Loeber, 1977) and because of this, certain charged forms may be selected or rejected during purification, as suggested in the case of hLH (Storring et al., 1982; Zaidi et al., 1982b).

Selective purification further complicates the characterization of these heterogeneous hormones, including the structural analysis of their carbohydrate chains, because variation in the content of sialylated and/or sulphated oligosaccharides is the likely major cause of charge heterogeneity in these hormones (Stockell Hartree, 1989).

This chapter describes an alternative method for the purification of hLH and hTSH by hydrophobic chromatography, in
which the effect of charge on separation is minimal. The new procedure involves the isolation of hLH and hTSH by hydrophobic-interaction chromatography and reverse-phase HPLC from crude glycoprotein hormone fractions prepared according to Chapman et al. (1981). Although a few publications have appeared on the behaviour of these hormones on reverse-phase HPLC (Bristow et al., 1983; Grego & Hearn, 1984), their separation by this method has not been reported. Highly purified, biologically active hLH and hTSH have been obtained by the following procedure.

3.2 Experimental Procedures
3.2.1 Materials
Human pituitary glands, collected under a national pituitary collection programme sponsored by the Department of Health and the Medical Research Council of New Zealand, were stored at -20°C before further use. Ovine LH (oLH-26-AFP-5551B) was supplied by NIADDK, NIH, and fetuin from fetal calf serum was purchased from Calbiochem (San Diego, CA, U.S.A.). All chemicals were of analytical grade unless otherwise indicated. Acetonitrile was HPLC grade (BDH). [3H]-sodium borohydride (25 mCi, 600 mCi/mmol, New England Nuclear) was dissolved in freshly distilled dimethylformamide (DMF) and stored at -10°C. Anhydrous hydrazine was prepared from hydrazine hydrate (99.5%, BDH) according to Takasaki et al. (1982) and stored at 4°C.

Sephadex G-15 (fine), Sephadex G-25 (medium), Sephadex G-100 (fine), phenyl-Sepharose CL-4B, Superose 12 (HR 10/30) gel-filtration column (1 x 30 cm) and a Mono Q (HR 5/5) anion-exchange column (0.5 x 5 cm) were bought from Pharmacia
(Australia) Pty Ltd. (Auckland, New Zealand). Vydac Protein C₄ reverse-phase columns (5 μm, 1 x 25 cm and 0.46 x 15 cm) were obtained from the Separations Group (Hesperia, CA, U.S.A.). SynChropak RP-1 cartridge columns (0.46 x 1 cm) were supplied by Alltech (Auckland, New Zealand). A MicroPak AX-5 aminopropyl column (5 μm, 0.4 x 30 cm) was from Varian Associates. AG 50W x8 or x4 (H⁺ form, 100-200 mesh) were from Bio-Rad.

3.2.2 Hormone Extraction and Gel-Filtration Chromatography

Eighteen hundred glands were processed in three batches of 600, (batch nos. 05-07) as described by Chapman et al. (1981) with the following modification. The glycoprotein fraction obtained by precipitation with 75% (v/v) ethanol was dissolved in 0.1 M NH₄HCO₃ (700 ml) and centrifuged at 8000 rpm for 30 min to remove any undissolved material. The supernatant fraction was concentrated to about 100 ml by ultrafiltration (Millipore membrane filter PTGC) and applied to a column (5 x 120 cm) of Sephadex G-100 which was eluted with the same buffer, at a flow rate of 50 ml/h. Fractions of 20 ml were collected and assayed for hLH, hTSH and hFSH by RIA; those that contained most of the three hormonal activities were pooled, freeze-dried and stored at -20°C. All procedures were carried out at 4°C.

3.2.3 Hydrophobic-Interaction Chromatography

The glycoprotein hormone fraction (420 - 520 mg) from the Sephadex G-100 step was dissolved in 50 mM NaH₂PO₄ buffer (50 ml, pH 7.0) containing 1 M (NH₄)₂SO₄ and was applied to a column of phenyl-Sepharose CL-4B (2.6 x 24 cm) in the same buffer mixture.
After washing with starting buffer (380 ml), the adsorbed glycoproteins were eluted stepwise with decreasing concentrations of (NH₄)₂SO₄ before a final application of 40% (v/v) ethanol/50 mM NaH₂PO₄ (pH 7.0). Each elution step was carried out with three column volumes (380 ml) of buffer at 300 ml/h using two Pharmacia P-500 pumps with a LCC-500 liquid chromatography controller. The eluate was monitored at 280 nm and 20 ml fractions were collected. Fractions eluted at each step were gathered in three consecutive pools, 1(100 ml), 2(140 ml) and 3(140 ml), and their contents of hFSH, hLH and hTSH were determined by RIA. Fractions that contained immunoreactive hLH and hTSH were combined, ethanol was removed by rotary evaporation (<30°C) and the solution was desalted by ultrafiltration (Millipore PTGC) before lyophilization. The dried material was stored at -20°C. All procedures were carried out at room temperature (about 20°C).

3.2.4 Reverse-Phase HPLC

Fraction IV-2 obtained by phenyl-Sepharose chromatography was dissolved in 0.1 M NaH₂PO₄ buffer (pH 7.0) and filtered through a 0.2 µm syringe filter to give a final sample concentration of 10 mg/ml. Samples (1 - 1.5 ml) were injected onto a Vydac C₄ column (1 x 25 cm) and elution was effected by mixing mobile phase A (0.1 M triethylamine phosphate; pH 6.5) and B (0.1 M triethylamine phosphate in 60% (v/v) acetonitrile; pH 6.5), creating a linear 60 min gradient of acetonitrile (20-100% B) at a flow rate of 1.2 ml/min. Eluates were monitored at 280 nm.

Each of the two major peak fractions was collected and identified by its activity in RIA. The same procedure was
repeated six to ten times, and each of the hormone fractions was pooled separately, desalted by gel-filtration on a Sephadex G-25 column (1.6 x 55 cm) in 1% (w/v) \( \text{NH}_4\text{HCO}_3 \), after removal of acetonitrile by rotary evaporation (<30°C), and freeze-dried. The preparations of hLH and hTSH were stored at -20°C.

A Tosoh HPLC system (Tokyo, Japan) consisting of a model CCPM pump with microprocessor control and a Rheodyne 7125 injector with a 2-ml sample loop was used with an LKB Uvicord-S model 2138 U.V. monitor (Bromma, Sweden).

### 3.2.5 Analytical HPLC

Samples of purified hLH and hTSH were examined on an analytical Vydc C₄ column (0.46 x 10 cm) at 0.8 ml/min under the same elution conditions used for preparative separations. (When crude samples were analysed by this method, a RP-1 cartridge column was connected with the C₄ column to separate hFSH with minimal dissociation.) Separation of hormonal subunits was effected by reverse-phase HPLC under the same conditions as above, following complete dissociation of each hormone in 6 M guanidine HCl in 0.1 M NaH₂PO₄ buffer (pH 7.0) containing 0.02% (w/v) sodium azide for 20 h at 37°C. Subunits isolated from each pretreated hormone were collected and freeze-dried after desalting as above. The subunits were identified by amino acid analysis and characterized by partial N-terminal sequencing (nine residues) using an Applied Biosystems (Foster City, CA, U.S.A.) gas-phase sequencer with on-line HPLC analysis.

Samples of hLH and hTSH were applied to a Superose 12 column and eluted with 0.1 M \( \text{NH}_4\text{HCO}_3 \) (pH 7.8) at 0.5 ml/min; the effluent was monitored at 280 nm. Fetuin (Mr 48000) was used as reference compound. Samples of hLH and hTSH dissolved in 20 mM
piperazine-HCl (pH 9.6, starting buffer) were loaded onto a Mono Q anion-exchange column and eluted for 5 min under starting conditions, followed by a linear gradient of 0-300 mM NaCl for 35 min at a flow-rate of 1 ml/min (Johnston et al., 1987).

3.2.6 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Samples of hLH and hTSH (20 µg each) were analysed by SDS-PAGE according to the procedure described by Segrest and Jackson (1972), using 12.5% (w/v) polyacrylamide slab gels (15 x 15 x 0.15 cm). Electrophoresis was at 30 mA for 4 h at 4°C. The gels were stained with 0.1% (w/v) Coomassie Blue R250, destained with 5% (v/v) acetic acid and scanned at 610 nm by a Quick Scan Jr densitometer (Helena Laboratories, Beaumont, TX, U.S.A.). Apparent molecular weights were determined by comparison with low molecular weight protein standards (Sigma MW-SDS-70, Dalton Mark VII-L).

3.2.7 RIA

Column fractions and hormone preparations from each step of purification were diluted to concentrations appropriate for assay in 25 mM Tris-HCl buffer (pH 7.4) containing 0.9% (w/v) NaCl and 0.1% (w/v) BSA. Amerlex-M hFSH (IM 3071), hLH (IM 3081) and hTSH (IM 3161) RIA kits (Amersham Australia) were used for the specific immunoassay of hFSH, hLH and hTSH in accordance with the manufacturer's instructions. Samples were assayed in triplicate in multiple dilutions, and hFSH, hLH and hTSH activities were expressed in terms of the second International Reference Preparation (IRP) 78/549, the first IRP 68/40 and the first IRP 68/38, respectively. The specificities of the assays in terms of percent cross-reactivity by weight were <0.1% for
hLH and hTSH in the hFSH assay, 0.7% for hFSH and 3.8% for hTSH in the hLH assay, and 0.12% for hFSH and 0.22% for hLH in the hTSH assay. Cross-contamination (percentage by weight) of hLH and hTSH preparations with other glycoprotein hormones was estimated on the assumption that the specific activities of pure hFSH, hLH and hTSH were 8150 IU/mg, 8290 IU/mg and 7480 mIU/mg respectively, which were the highest values obtained in this laboratory. For hLH the activity was also calculated on the basis of protein content assuming an absorption coefficient (A\text{\textsuperscript{1%}}\text{cm}) at 280 nm of 6.0 for hLH, for comparison with published data (Storring et al., 1982).

3.2.8 Biological Assay

The biological activity of hLH was measured by ovarian ascorbic acid depletion (OAAD) according to the method of Fukushima et al. (1964) using NIH-LH-S1 as standard. The assay was performed with four animals in each group and the design was 3 x 2; the index of precision was 0.17. Bioassay of hTSH was carried out as described by McKenzie (1958). The release of thyroidal radiiodine in mice was determined in response to graded doses of hTSH 3 h after i.p. injection of control or test solutions of hormone, which were administered in increments of three-fold dilution. Five animals were used for each dose; the index of precision was 0.19. Potency was expressed in terms of the human TSH MRC Research Standard (T1 70/9). The hLH bioassay was kindly performed by Dr J. Powell and Dr V. Stevens of the Department of Obstetrics and Gynecology, Ohio State University, Columbus, OH, U.S.A. Determination of hTSH activity was performed by Dr D.S. Munro of the Academic Division of Medicine,
3.2.9 Analysis of Asn-linked Oligosaccharides on hLH and hTSH

Asn-linked oligosaccharides were released from hLH and hTSH by hydrazinolysis, re-N-acetylated and reduced with NaB\(^{3\text{H}}\)\(_4\) according to Takasaki et al. (1982) modified as follows. Each hormone (ca. 2 mg), dissolved in anhydrous hydrazine (0.5 ml), was heated at 100°C for 10 h. After evaporating hydrazine under reduced pressure, the residue was dissolved in saturated NaHCO\(_3\) (1.5 ml) and re-N-acetylated by the addition of acetic anhydride (150 μl) dropwise with stirring, after which the reaction continued for 30 min at room temperature. The oligosaccharides were desalted and freed from peptides by elution on a column (1.6 x 30 cm) of AG 50W x 8 (H\(^+\) form, 100-200 mesh) in water. The first peak eluted was collected, evaporated and the oligosaccharides were further purified by gel-filtration chromatography on a Sephadex G-15 column (1.6 x 40 cm) in water. The oligosaccharides eluted in the void peak were collected and freeze-dried.

When required, the oligosaccharides were dissolved in 0.2 M sodium borate buffer, pH 9.8 (200 μl) chilled in ice-water, and about 5 x excess of NaB\(^{3\text{H}}\)\(_4\) (750 μCi/75 μl DMF) was added before incubation at 30°C for 4 h. To ensure complete reduction, NaBH\(_4\) (7 mg) was added and incubated for 2 h at 30°C. The reaction was stopped by the addition of 1 M acetic acid (0.5 ml) and the mixture was passed through a column (2 ml) of AG 50W-X4 (H\(^+\) form, 100-200 mesh) which was washed with water (10 ml). The eluate and washings were combined and dried under reduced
pressure, borate was removed by repeated evaporation with methanol. The $^3$H-labelled reduced oligosaccharides were freed from reaction products and radioactive impurities by gel-filtration on a Sephadex G-15 column (1.6 x 70 cm) in water. The yields of radioactive oligosaccharides were 6.68 x 10$^6$ cpm (hLH) and 8.11 x 10$^6$ cpm (hTSH).

The labelled oligosaccharide mixture (1-2 x 10$^6$ cpm) from each hormone was analysed by anion-exchange HPLC on a MicroPak AX-5 under conditions that separate oligosaccharides according to net anionic charge (Green & Baenziger, 1986); a linear gradient was applied at a flow-rate of 0.5 ml/min with buffer A, 2.5 mM KH$_2$PO$_4$ (pH 4.0) and buffer B, 250 mM KH$_2$PO$_4$ (pH 4.0). Buffer A and B were prepared by adjusting the pH of 2.5 mM and 250 mM H$_3$PO$_4$ to 4.0 with 2N KOH, respectively. The linear gradient programme was 0-50%B (0-25 min), 50-100%B (25-30 min) and 100%B (30-35 min). Fractions of 500 μl were collected and each was diluted to 1500 μl with water. Radioactivity was determined by a LKB 1209 Rackbeta liquid scintillation counter (LKB-Wallac, Wallac Oy, Finland) with 3 ml scintillation fluid containing 0.4% (w/v) 2,5-diphenyl oxazole (PPO), 33% (v/v) Triton X-100 in toluene. Recovery of labelled oligosaccharides ranged from 92-97%.

Mono-, di-, and tri-charged fractions were separately pooled, desalted on G-15 in water, then treated in 2 M acetic acid at 100°C for 30 min for selective desialylation (Hortin et al., 1986). These samples were analysed by HPLC under the same conditions following removal of acetic acid by G-15 and rotary evaporation.

The relative contents of sialylated and sulphated
Fig. 3.1 Analytical reverse-phase HPLC profile of the glycoprotein hormone fraction obtained by Sephadex G-100 chromatography.

Columns: SynChropak RP-1 (0.46 x 1 cm) and Vydac C4 (0.46 x 15 cm) in series. Mobile phases and elution conditions were the same as described in Fig. 2.3. Sample: glycoprotein hormone fraction (500 μg). The elution positions for hFSH, hLH, hTSH and α-subunits are indicated.
oligosaccharides in each charged fraction were calculated from the differences in recovery of radioactivity before and after acid treatment. Under these hydrolytic conditions, almost all sialic acid but no sulphate was removed from anionic oligosaccharides. Mono- and disulphated and tri- and tetrasialylated oligosaccharides prepared from ovine LH and fetuin, respectively, were used as reference compounds to locate the relative elution positions of anionic oligosaccharides.

3.3 Results

3.3.1 Purification of hLH and hTSH

The glycoprotein precipitate was first fractionated by gel-filtration on Sephadex G-100 to obtain a glycoprotein hormone-rich fraction. Fractions (no. 36-55, total vol. 400 ml), which contained 80-90% of the hFSH, hLH and hTSH in pituitary homogenates, as determined by RIA, were pooled and freeze-dried. The average yield of this crude preparation of glycoprotein hormones was 472 mg and was the starting-material for the purification of hLH and hTSH.

An analytical profile of the crude glycoprotein hormone preparation obtained by reverse-phase HPLC is shown in Fig.3.1. Three distinct peaks were observed at the elution positions for hFSH, hTSH and hLH (17, 28 and 33 min respectively). Of several other peaks separated, the peak with a retention time close to that for the α-subunits from intact hormones, appeared to represent an unassociated or free α-subunit. The large peak that was eluted after hLH has been characterised and recently identified as α1-acid glycoprotein (Kralicek, 1989). Although this globulin appeared to have been derived from blood that contaminated the pituitary glands, its actual origin remains
Fig. 3.2 Hydrophobic-interaction chromatography of the glycoprotein fraction obtained from Sephadex G-100 chromatography of human pituitary glands.

A sample (420 mg) was applied to a phenyl-Sepharose CL-4B column equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1 M (NH₄)₂SO₄. Stepwise elution was effected with 380 ml buffer changes (all at pH 7.0) as indicated by arrows. Fractions of 20 ml were collected and the fractions eluted at each step (II, III and IV) were pooled as shown. Fraction IV-2 (hatched area) was used for the isolation of hLH and hTSH.
uncertain.

Preliminary attempts to isolate hLH and hTSH directly from the crude glycoprotein hormone fraction by reverse-phase HPLC suggested that, while this approach is useful on an analytical or micro scale, it might not be readily adaptable for preparative purposes. The problems lay in the strong adsorption of contaminants to the matrix and increased back pressure. It was also found that hFSH was partially dissociated into its subunits under conditions developed for the separation of the other hormones. Hydrophobic-interaction chromatography on phenyl-Sepharose, however, resulted in the efficient separation of hLH and hTSH from hFSH and strongly hydrophobic contaminants.

The glycoprotein hormone fraction (422-522 mg) was applied to the phenyl-Sepharose CL-4B column at 3-4 mg of total loading per ml of gel in 1 M (NH₄)₂SO₄/50 mM NaH₂PO₄ buffer (pH 7.0) and the adsorbed proteins were eluted by stepwise decreases in concentration of (NH₄)₂SO₄ (Fig.3.2). Under these conditions about 90% of the load and all of the hFSH, hLH and hTSH were adsorbed. Nearly all the immunoreactive hFSH was eluted in a single fraction (fraction II-2) in 0.2 M (NH₄)₂SO₄, whereas most of the remaining hormones were not detected until the application of buffer containing ethanol. Between 90 and 100% of the immunoreactive hLH and hTSH recovered from the column was found in a single pooled fraction IV-2 (hatched area) which was eluted with 40% (v/v) ethanol/50 mM NaH₂PO₄, pH 7.0.

The average yield of this fraction was 106 mg (22% of the total amount loaded). A partial purification (three-fold for hLH and hTSH) was attained in this step, as estimated from the specific immunoreactivities of hLH and hTSH. Contamination by hFSH was very low (<2 IU hFSH/mg), which showed that the
Fig. 3.3 Reverse-phase HPLC of fraction IV-2 obtained from phenyl-Sepharose chromatography of human pituitary glands.

Portions (10 mg) of fraction IV-2 were applied to a Vydac C4 column (1 x 25 cm) and eluted with a linear gradient of acetonitrile (20-100%, v/v, mobile phase B; broken line) in 0.1 M TEAP, pH 6.5, at 1.2 ml/min. Mobile phase A, 0.1 M TEAP (pH 6.5); mobile phase B, 0.1 M TEAP in 60% (v/v) acetonitrile (pH 6.5). The two major peak fractions were collected as indicated.
separation of hFSH from hLH and hTSH was >99% complete.

The rapid separation of hLH and hTSH in fraction IV-2 was achieved by reverse-phase HPLC on a Vydac C₄ column in a linear gradient of acetonitrile in triethylamine phosphate buffer (pH 6.5) (Fig.3.3). A minor peak, which was eluted just before hTSH, corresponded in position to that of the α-subunits, and no significant amounts of hLH and hTSH were detected in fractions after the hLH peak. The sample loading for each separation was kept at 10-15 mg to maintain a near baseline separation of the two hormones.

About 100 mg fraction IV-2 was chromatographed successively in each batch, after which the column was thoroughly cleansed before application of the next sample.

Cross-contamination was estimated from the recovered immunoreactivities to be 1-2%, which showed that the separation of hLH and hTSH was near complete in a single run. Average yields of hLH and hTSH were 27.0 and 12.4 mg respectively per 600 glands. The average recovery of immunoreactivity from reverse-phase HPLC was 83% for hLH and 77% for hTSH; overall recoveries from the starting material were 62% (hLH) and 58% (hTSH).

The yields and activities of various preparations of hLH and hTSH are summarized in Table 3.1.

3.3.2 Purity

Purity of the isolated hormones was assessed by analytical reverse-phase HPLC, SDS-PAGE, gel-filtration HPLC and RIA. The reverse-phase HPLC procedure was used because it affords high resolution of the subunits from each intact hormone, thereby facilitating detection of any subunits in hormone preparations.
Table 3.1 Purification of hLH and hTSH.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Yield (mg)</th>
<th>hLH</th>
<th>hTSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific activity (IU/mg)</td>
<td>Recovered activity (KIU) (%)</td>
</tr>
<tr>
<td>Glycoprotein hormone fraction from G-100</td>
<td>472±29</td>
<td>616±40</td>
<td>293±36 (100)</td>
</tr>
<tr>
<td>Fraction IV-2 from phenyl-Sepharose CL-4B</td>
<td>106±3</td>
<td>2055±82</td>
<td>218±15 (74)</td>
</tr>
<tr>
<td>Reverse-phase HPLC:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hLH</td>
<td>27±2</td>
<td>6770±370</td>
<td>182±10 (62)</td>
</tr>
<tr>
<td>hTSH</td>
<td>12±2</td>
<td>411±72</td>
<td>5±1    (2)</td>
</tr>
</tbody>
</table>

Yields and activities are given as means ± S.E. of three batches (600 glands/batch). Specific activities of various preparations of hLH and hTSH measured by RIA are expressed in terms of the first IRP (68/40) for hLH and the first IRP (68/38) for hTSH, on dry weight basis.
Fig.3.4 Analytical reverse-phase HPLC of purified hLH and hTSH.

(a) Reverse-phase HPLC of hTSH and hLH. Each hormone (25 μg) was applied to a Vydac C₄ column (0.46 x 15 cm) and eluted using the same conditions as in Fig.3.3 except that the flow-rate was 0.8 ml/min. The elution positions for the subunits of each hormone are indicated.

(b) Separations of the subunits of hTSH and hLH by reverse-phase HPLC. Each hormone (50 μg) was pretreated for dissociation as described in Experimental Procedures, and chromatographed under the same conditions as in (a). The subunit fractions were collected as indicated.
Fig. 3.5 Analysis of purified hLH and hTSH by SDS-polyacrylamide gel electrophoresis and gel-filtration HPLC.

(a) SDS-polyacrylamide gel electrophoresis of hTSH and hLH (20 μg each) under non-reducing conditions. The gels were scanned by a densitometer. Migration positions of molecular weight markers are indicated.

(b) Gel-filtration HPLC of hTSH and hLH. Each hormone (400 μg) was applied to a Superose 12 column and eluted in 0.1 M NH₄HCO₃ (pH 7.8) at 0.5 ml/min. The elution position for fetuin (Mr 48000) is indicated by an arrow.
When 25-100 µg was analysed, both hLH and hTSH were eluted almost as single peaks with slightly detectable subunits (about 1%) (Fig.3.4a), showing that no significant dissociation of either hormone occurred during isolation. Fig.3.4b shows the separation of the subunits of hLH and hTSH effected by reverse-phase HPLC, following pretreatment of the hormone (50 µg each) as described in Experimental Procedures. Partial N-terminal sequence analysis showed that, except for the α-subunit of hLH, all subunits had single sequences in agreement with those described (Pierce & Parsons, 1981): hLHa, Ala-Pro-Asp-Val-Gln and Val-Gln-Asp-(Cys)-Pro; hLHb, Ser-Arg-Glu-Pro-Leu; hTSHA, Val-Gln-Asp-(Cys)-Pro; hTSHb, Phe-(Cys)-Ile-Pro-Thr. The α-subunit of hLH gave two sequences, one of which was in accord with that described for the complete N-terminal sequence (Pierce & Parsons, 1981), whilst the other corresponded to the same sequence minus the first three residues (Ala-Pro-Asp). The absence of any other discernible sequences in the isolated subunits showed that contamination of both hLH and hTSH preparations was negligible.

Both hLH and hTSH (20 µg) migrated as a single band on SDS-PAGE under non-reducing conditions (Fig.3.5a), showing that each preparation was homogeneous. The apparent Mr values for hLH and hTSH were 38000 and >65000, respectively.

Although the Mr of glycoproteins is overestimated by this method, the high Mr for hTSH was unexpected, and aggregation of this hormone preparation was suspected. Analysis of hLH and hTSH (400 µg) by gel-filtration HPLC (Fig.3.5b) showed that each hormone was eluted almost as a single peak with very similar positions, indicating that both hormones were homogeneous. The chromatographic behaviour of hTSH did not suggest the presence
Fig. 3.6 Anion-exchange HPLC of purified hLH and hTSH.

Each hormone (hLH 360 μg, hTSH 450 μg) was applied to a Mono Q column in 20 mM piperazine HCl (pH 9.6) and eluted isocratically for 5 min, followed with a 35 min linear gradient of NaCl from 0 to 300 mM at 1.0 ml/min. The gradient is only shown in the case of hTSH.
of aggregates of this hormone, and the electrophoretic behaviour of hTSH remains to be resolved.

Purified hLH and hTSH had specific activities of 6770 IU/mg (7420 IU/mg protein) and 5980 mIU/mg respectively, as determined by RIA. The hLH preparation contained 0.33 mIU hTSH/mg and 0.3 IU hFSH/mg and hTSH had 411 IU hLH/mg and 0.9 IU hFSH/mg. Cross-contamination of hLH with hTSH and hFSH was estimated to be <0.2% and <0.1%, by weight, and contamination of hTSH with hLH and hFSH was <2% and <0.1% respectively.

3.3.3 Biological Activity

The biological activity of hLH measured in the OAAD assay was 5.8 x NIH-LH-S1 (95% confidence limits 5.3-6.1). The hTSH preparation had a potency of 7.1 U/mg in terms of the human TSH MRC Standard (95% confidence limits 5.1-9.8).

3.3.4 Charge Heterogeneity

Highly purified preparations of hLH and hTSH are heterogeneous with respect to charge. The degree of charge heterogeneity of hLH and hTSH was examined by anion-exchange HPLC which was stated to be effective in separating isoforms with close isoelectric points (Johnston et al., 1987). Fig.3.6 shows the chromatographic profiles when hLH and hTSH were chromatographed on a Mono Q column under the conditions described in Experimental Procedures. Both hLH and hTSH gave broad elution profiles without separation into well-defined peaks, which indicated extensive charge heterogeneity.

The Asn-linked oligosaccharides of hLH and hTSH were characterized with respect to the number of sialic acid and sulphate residues, because variation in their content is
Fig. 3.7 Anion-exchange HPLC of Asn-linked oligosaccharides from hLH.

(a) [3H]-Labelled oligosaccharides from hLH were analysed by anion-exchange HPLC at pH 4.0 on a MicroPak AX-5 column as described in Experimental Procedures. Fractions of 500 μl were collected. The elution positions of neutral, mono-, di-, tri- and tetra-charged oligosaccharides (A0, A1, A2, A3 and A4, respectively) are indicated by brackets.

(b) Mono-, di-, and tri-charged oligosaccharides collected from (a) were analysed by anion-exchange HPLC using the same conditions after removal of sialic acid residues by acid hydrolysis as described in Experimental section. The types of sialylated and/or sulphated oligosaccharides that were originally present in A1, A2 and A3 fractions were characterised as indicated.
Fig. 3.8 Anion-exchange HPLC of Asn-linked oligosaccharides from hTSH.

[^3H]-labelled oligosaccharides from hTSH were analysed by anion-exchange HPLC before (a) and after (b) removal of sialic acid residues by acid hydrolysis as described in Fig. 3.7. The types of sialylated and/or sulphated oligosaccharides in mono-, di- and tri-charged fractions (A1, A2 and A3, respectively) were characterised as indicated in (b).
Fig. 3.9 Relative contents of neutral, sialylated, sulphated, and sulphated/sialylated oligosaccharides in hLH and hTSH.

The percentages of neutral, mono-(N1), di-(N2) and tri-(N3) sialylated, mono-(S1), di-(S2) and tri-(S3) sulphated, monosulphated/monosialylated (SN), monosulphated/disialylated (S1N2), and disulphated/monosialylated (S2N1) are presented for each hormone. The distributions of neutral, sialylated, sulphated and sulphated/sialylated oligosaccharides reported for different hLH and hTSH preparations (Green & Baenziger, 1988b) are given in parentheses for comparison.
considered to be the major source of charge heterogeneity in these hormones (Stockell Hartree, 1989). Fig. 3.7 shows the separations of radiolabelled oligosaccharides from hLH according to net negative charge, effected by anion-exchange HPLC at pH 4.0 on a MicroPak AX-5 column, before and after selective acid hydrolysis, which removed only sialic acid from each charged species. The profiles obtained from similar analyses with hTSH are shown in Fig.3.8. Relative contents of neutral, sialylated and sulphated oligosaccharides calculated for hLH and hTSH are given in Fig.3.9.

The results show that both hLH and hTSH contained various types of sialylated and sulphated oligosaccharides with one, two or three anionic charges. Besides, the content of each type is relatively low, indicating the large contribution of anionic oligosaccharides to the charge heterogeneity of hLH and hTSH. Sialylated oligosaccharides are relatively abundant in hLH, while sulphated oligosaccharides are plentiful in hTSH; disialylated oligosaccharides and monosulphated oligosaccharides were the most abundant species in hLH and hTSH, respectively (about 30% in each case). The contents of sialic acid and sulphate in hLH and hTSH calculated from these data are given in Table 3.2. The ratio of sialic acid to sulphate content was 2.6 in hLH and 0.7 in hTSH.

3.4 Discussion

Hydrophobic chromatography, i.e. hydrophobic-interaction and reverse-phase chromatography, is widely used for the separation of proteins. Reverse-phase HPLC has been particularly useful for the purification of glycoproteins because their separations under normal conditions are not
<table>
<thead>
<tr>
<th>Hormone</th>
<th>Sialic acid (A)</th>
<th>Sulphate (B)</th>
<th>Ratio (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hLH</td>
<td>3.6(2.2)</td>
<td>1.4(1.7)</td>
<td>2.6(1.3)</td>
</tr>
<tr>
<td>hTSH</td>
<td>1.6(1.5)</td>
<td>2.4(2.5)</td>
<td>0.7(0.6)</td>
</tr>
</tbody>
</table>

**Table 3.2** Content of sialic acid and sulphate in hLH and hTSH

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a Sialic acid and sulphate contents were calculated from the oligosaccharide compositions (Fig.3.9) and a value of 3 mol for the total oligosaccharides per mol hormone.

b The sialic acid and sulphate contents in different hLH and hTSH preparations, calculated from the published data (Green & Baenziger, 1988b) in the same way, are given in parenthesis for comparison.
significantly influenced by charge and glycosylation (Rosner & Robbins, 1982; Dahms & Hart, 1986). Previous studies have demonstrated the usefulness of reverse-phase HPLC in separating the subunits of bovine and human glycoprotein hormones (Parsons et al., 1984), however application of this method to the purification of the intact hormones has been very limited to date.

One of the major problems is the ready dissociation of non-covalently bound subunits, which leads to a loss of biological activity. In such cases, the isolation of intact glycoprotein hormones from their mixtures is difficult because the shared α-subunits, which are virtually identical in their peptide components but which may differ in their carbohydrate structures, are eluted at or near the same position. Although active hormones could be reconstituted from isolated subunits (Pierce & Parsons, 1981), such preparations cannot be expected to retain their original structures. There have also been reports of reduced biological activity after reverse-phase HPLC of hFSH (Hallin & Khan, 1986), hTSH (Bristow et al., 1983) and hCG (Wilks & Butler, 1984), although conditions that did not cause dissociation were employed in the case of hTSH. A partial loss of biological activity in the case of hCG was attributed in part to irreversible denaturation, which suggested that reverse-phase HPLC is of limited use in preparing biologically active hormones. However, these problems may have been caused by the chromatographic conditions used. The present study has shown that reverse-phase HPLC with a wide-pore C₄ column under the described conditions gives effective separation of hLH and hTSH without any significant dissociation and that this procedure is of value for the final purification of these hormones. The two
hormones were almost completely separated in one step in this system, which was difficult to accomplish by anion-exchange chromatography (Parlow et al., 1965; Stockell Hartree, 1966). When combined with hydrophobic-interaction chromatography on phenyl Sepharose CL-4B, reverse-phase HPLC provides an effective means for the simultaneous purification of hLH and hTSH from crude preparations. An effective separation of hFSH and most contaminants from hLH and hTSH by phenyl-Sepharose chromatography suggested that hydrophobic-interaction chromatography was most effective for partial purification.

The immunoactivity of hLH on the basis of protein content (7420 IU/mg protein) was comparable with 5770–9180 IU/mg protein (IRP 68/40) reported for various highly purified hLH preparations (Storring et al., 1982). The degree of cross-contamination in hLH was low compared with these preparations; the immunoactivities of hFSH and hTSH were 0.3 IU/mg protein and 33 mIU/mg respectively, against 9–68 IU hFSH/mg protein and <1–305 mIU hTSH/mg.

Both hLH and hTSH retained high biological activity during their purification, and in the case of hLH its potency (5.8 U/mg) compared favourably with 4.8 – 5.5 U/mg reported for highly purified preparations (Stockell Hartree, 1966; Bates et al., 1968). The hTSH preparation also showed high potency, but definitive comparison with other preparations was not possible because different assay procedures and reference materials were used by other workers. The biological evidence suggests that the relatively harsh chromatography conditions used in this study do not adversely affect the overall yield or the biological activity of either hormone. However, the possibility of partial loss of activity cannot be excluded, as was found
with hTSH, which retained about 60% of its biological activity after reverse-phase HPLC under similar conditions (Bristow et al., 1983).

The average yields for three preparations (45 mg hLH and 20 mg hTSH per 1000 glands) were higher than 27-35 mg for hLH and comparable with 14-23 mg for hTSH obtained by conventional methods (Bates et al., 1968; Stockell Hartree, 1975). The average amounts of hLH and hTSH originally present in the gland were estimated from their recoveries to be about 80 μg and 40 μg, respectively.

Despite the extensive charge heterogeneities of hLH and hTSH, both hormones can be obtained as highly purified forms in single fractions by the present procedure. This method will therefore be particularly useful for the investigation of charged forms originally present in pituitary tissue. In this regard, hormone preparations obtained by ion-exchange chromatography may not retain their original isoforms. This possibility has been suggested by Zaidi et al. (1982b) who reported significant variations in charge distribution among a number of highly purified hLH preparations. These hLH preparations were found to lack relatively acidic forms (pI <6.5) which were characteristically rich in pituitaries from postmenopausal women. They were also found to exhibit variable activities in radioimmunoassays and bioassays (Storring et al., 1982).

Modification of charge heterogeneity will also affect characterisation and structural analysis of the carbohydrate components of these glycoprotein hormones. When the compositions of Asn-linked oligosaccharides in hLH and hTSH were compared with those reported (Green & Baenziger, 1988b), several
significant differences were found for both hormones (Fig. 3.9). The two hLH preparations in particular differed in the relative contents of neutral, monosulphated and disialylated oligosaccharides (>10%). As a result, the total amounts of sialylated oligosaccharides in hLH purified by the conventional procedure was significantly lower, whereas those of sulphated oligosaccharides were similar, which reflected in the lower ratio of sialic acid to sulphate content, i.e. 1.3 vs 2.6 (Table 3.2). Furthermore, significant amounts of tri-charged oligosaccharides were found only in the preparation obtained by the present method. The absence of these higher charged species and lower total contents of sialic acid and sulphate in the NIH preparation of hLH (3.9 mol/mol hormone vs. 5.0 mol/mol hormone) appeared to reflect the preferential purification of more alkaline forms by ion-exchange chromatography (Storring et al., 1982). In the case of hTSH, the two preparations showed relatively similar oligosaccharide patterns except that the presence of tri-charged species were not reported earlier.

In conclusion, the hydrophobic chromatographic procedure now described provides a useful rapid alternative for the purification of hLH and hTSH. This method allows purification of these hormones without any significant influence of their extensive charge heterogeneity, a potential advantage in the characterisation of glycoprotein hormones with different carbohydrate structures.
CHAPTER 4  Isolation and Characterisation of N-Linked Glycans of Human LH

4.1 Introduction

Structural studies of the gonadotrophin N-glycans have become increasingly important with the recognition of their significant roles in hormone biosynthesis, secretion and function. However, structural elucidation of N-glycans remains incomplete for most of the hormones so far examined (Chapter 1).

For the human gonadotrophins, the N-glycans from urinary hCG have been intensively studied by several groups using various methods and the presence of mono-, diantennary, sialylated complex-type oligosaccharides has been established (Endo et al., 1979; Mizuochi & Kobata, 1980; Damm et al., 1987). In contrast to hCG, structural characterisation of hLH and hFSH has been very limited, due in part to the relative scarcity of purified hormone preparations. In the case of hLH which contains three N-glycosylation sites, two in the α-subunit (Asn 52 and Asn 78) and one in the β-subunit (Asn 30), the presence of sulphated oligosaccharides in addition to sialylated oligosaccharides (Parsons & Pierce, 1980; Green et al., 1985b) and their overall heterogeneity further complicated the determination of their precise structures.

Green and Baenziger (1988a,b) characterised the N-linked oligosaccharides of LH isolated from human, bovine and ovine pituitaries, using 3H-labelled oligosaccharides. In contrast to β- and ßLH, that contained exclusively mono- and disulphated oligosaccharides as acidic components, hLH was found to contain a heterogeneous mixture of acidic oligosaccharides that consisted of mono- and disulphated, mono- and disialylated, and
sulphated/sialylated oligosaccharides. Most of these oligosaccharides were diantennary complex-type.

Although detailed structures of the N-linked oligosaccharides of hLH have been reported in these studies, it appeared that limitations in analytical methods and the availability of materials precluded the establishment of definitive conclusions. Structural identification in these experiments relied solely upon the specificities of glycosidases and lectins.

Moreover, these studies did not examine structural aspects at specific glycosylation sites and such information is required for complete definition of the N-glycans of this hormone. Furthermore, structures determined with hLH prepared by conventional purification procedures may not accurately reflect those present in naturally occurring forms of hLH (Zaidi et al., 1982b; Storring et al., 1982).

The availability of human pituitary glands and the development of an efficient alternative purification method for hLH afforded the possibility of isolating each of the three N-glycans of this hormone in quantities that facilitated detailed structural analysis by high-field NMR spectroscopy. Because of complications that might have arisen from considerable oligosaccharide heterogeneity and peptide heterogeneity caused by imperfect proteolysis, purification to homogeneity of micromole quantities of each N-glycan chain required refined chromatographic procedures.

This chapter describes a scheme developed for the isolation and purification of the N-linked oligosaccharides from each glycosylation site of hLH together with the results of structural characterisation by $^1$H-NMR analysis.
4.2 Experimental Procedures

4.2.1 Materials

Human pituitary glands, collected under the auspices of the National Hormone Committee of the Medical Research Council of New Zealand, were stored at -20°C. The glycoprotein fraction obtained by ethanol extraction from an acetone-dried powder of human pituitary glands was supplied by Dr A. Stockell Hartree.

Sephadex G-15, G-25, G-100, phenyl-Sepharose CL-4B, AG 50W-X4, Vydc Protein C₄(0.46 x 15 cm, 1 x 25 cm) and MicroPak AX-5 (0.4 x 30 cm) HPLC columns were the same as described in Chapter 3. TPCK-treated trypsin (Type XIII, from bovine pancreas), iodoacetic acid and DL-dithiothreitol were from Sigma. Acetonitrile (HPLC grade, BDH), NaBH₄ (Analytical Grade, BDH), \(^{[3]H}\)-NaBH₄(600 mCi/mmole, New England Nuclear), hydrazine (99.5%, BDH) were used. All other chemicals were analytical grade unless otherwise indicated.

4.2.2 Purification of hLH and its Subunits

Two sources of human pituitary glands were used for the preparation of a large quantity of purified hLH. Frozen glands (2400) were processed in 4 batches of 600 and subjected to gel-filtration on Sephadex G-100 as described in Chapter 2. The second source of hormone was the glycoprotein fraction (4.15 g) obtained by ethanol extraction from an acetone-dried powder of about 1,800 glands (Stockell Hartree, 1975). This material was chromatographed in 2 batches (2.22 g and 1.93 g) on Sephadex G-100 to obtain the corresponding glycoprotein hormone fraction.

A total of 3271 mg of the partially purified preparations
was fractionated in six applications on a phenyl-Sepharose CL-4B column and fraction IV-2 was subjected to reverse-phase HPLC for the isolation of hLH and hTSH (Chapter 3).

Purified hLH (215 mg) was dissolved in 0.1 M NaH₂PO₄ buffer, pH 7.0 (21.5 ml) that contained 6 M guanidine HCl and 0.02\% sodium azide, and was incubated for 20 h at 37°C. Dissociated subunits were isolated at pH 6.5 by reverse-phase HPLC on a Vydac C₄ column (1 x 25 cm) using the same elution conditions as employed for the isolation of hLH (Chapter 3). Twenty mg or less of the dissociated sample was applied to the column and two major peak fractions that corresponded to hLHα- and β-subunits were collected. The same procedure was repeated, the subunit fractions were pooled separately and desalted on a Sephadex G-25 column (1.6 x 55 cm) in 1% NH₄HCO₃ after the removal of acetonitrile by rotary-evaporator. The void peak was collected and freeze-dried. The purity of each subunit was analysed by reverse-phase HPLC as described in Chapter 3 and then preparations were stored at -20°C until required.

4.2.3 Separation of Glycopeptides of the α-Subunit of hLH

Separation of the two glycosylated Asn residues (Asn 52 and Asn 78) of the α-subunit was effected by reverse-phase HPLC after tryptic digestion.

The hLH α-subunit was first reduced and S-carboxymethylated according to (Lustbader et al., 1987). The α-subunit (62 mg) was dissolved in reducing buffer (12 ml) (6 M guanidine HCl, 1 M Tris-HCl, 1 mM EDTA, pH 8.2), dithiothreitol (60 mg) was added, the vessel purged with N₂ and incubated for 4 h at 37°C. After the sample had cooled to room temperature, iodoacetic acid (180
mg) in 0.5 N NaOH (1.8 ml) was added dropwise in the dark with stirring and the reaction was allowed to continue for 30 min at room temperature; β-mercaptoethanol (160 μl) was added to stop the alkylation. The alkylated hLHα (RCM-hLHα) was desalted on a Sephadex G-25 column (1.6 x 55 cm) in 1% NH₄HCO₃ and freeze-dried.

The RCM-hLHα (60 mg) was dissolved in 0.2 M NH₄HCO₃ (12 ml) and treated with TPCK-treated trypsin (1.2 mg) in 1 mM HCl (120 μl) (2% w/w of substrate) at 37°C for 2 h. Tryptic digestion was stopped by rapid immersion in liquid N₂ and the sample was stored at -20°C until separation. The tryptic digests of RCM-hLHα were applied in aliquots (1.5 ml) to a Vydac C₄ column (1 x 25 cm), and were eluted using a linear gradient of acetonitrile in 0.1 M TEAP, pH 6.5 (mobile phase A; 0.1 M TEAP, pH 6.5, B; 0.1 M TEAP in 60% (v/v) acetonitrile, pH 6.5). A 90 min-linear gradient from 5-95% B was used at a flow-rate of 1.2 ml/min. The eluants were monitored at 226 nm. Each of the collected fractions was desalted on a Sephadex G-15 (fine) column (1.6 x 45 cm) in water after the removal of acetonitrile. The peaks eluted in the void volume were collected separately and freeze-dried. These HPLC fractions were analysed by ¹H-NMR for the presence of carbohydrate and for peptide homogeneity. The glycopeptides were identified by N-terminal amino acid sequence analysis.

4.2.4 Liberation of N-Linked Oligosaccharides from hLHα Glycopeptides and hLHβ.

The two α-subunit-derived glycopeptides, αGP I (10.8 mg) and αGP II (15 mg) that contained Asn 52 and Asn 78 respectively, and the β-subunit (48 mg) were thoroughly dried
over \( \text{P}_2\text{O}_5 \) under reduced pressure before hydrazinolysis according to Takasaki et al. (1982). Each sample was heated in anhydrous hydrazine (1 ml) at 100°C for 10 h in a tube closed with a teflon-sealed cap. The reaction mixture was evaporated under reduced pressure and the residue was freed from residual hydrazine by repeated evaporation with toluene. The residue was dissolved in saturated \( \text{NaHCO}_3 \) solution (5 ml) and re-N-acetylated by the dropwise addition of acetic anhydride (0.5 ml) at 4°C and at room temperature for 3 h with stirring. The reaction mixture was then applied in aliquots (1-1.5 ml) to a column (1.6 x 70 cm) of Bio-Rad AG50W-X8 (H\(^+\) form, 100-200 mesh) and was eluted with sugar-free water. The first peak fraction containing oligosaccharides was collected and dried by rotary evaporation. The oligosaccharides were redissolved in water (2 ml) and added dropwise to 0.3 M sodium borate buffer pH 9.8 (4 ml) containing \( \text{NaBH}_4 \) (10 mg). Reduction was carried out at 4°C for 30 min and then at 30°C for 3 h. Reduced oligosaccharides (alditols) were freed from reaction products by gel-filtration on Sephadex G-15 in water, and freeze-dried.

4.2.5 Separation of Oligosaccharides by HPLC

Oligosaccharide alditols were separated at pH 4.0 by anion-exchange HPLC (Green & Baenziger, 1986) on a MicroPak AX-5 column (5 \( \mu \)m, 0.4 x 30 cm) with the elution conditions modified as follows: Buffer A; 3 mM \( \text{KH}_2\text{PO}_4 \), pH 4.0: Buffer B; 300 mM \( \text{KH}_2\text{PO}_4 \), pH 4.0. Linear gradient; 0-15% B (0-20 min), 15% B (20-40 min), 15-50% B (40-60 min), 50% B (60-70 min), 50-100% B (70-80 min) and 100% B (80-100 min); flow-rate, 0.5 ml/min. Each sample dissolved in Buffer A (1.5 ml) was applied to the column in three aliquots (0.5 ml each). The eluant was monitored at
206 nm. Peak fractions were collected, desalted on Sephadex G-15 in water, and freeze-dried.

Major fractions obtained from anion-exchange HPLC were further separated at pH 4.0 by ion-suppression amine-adsorption HPLC (Green & Baenziger, 1986) on a MicroPak AX-5 column (5 μm, 0.4 x 30 cm) with the elution conditions modified as follows; Buffer A; 1% (v/v) TEAP in 70% (v/v) acetonitrile, pH 4.0, Buffer B; 1% (v/v) TEAP, pH 4.0. The linear gradient programme and flow-rate were the same as those used for anion-exchange HPLC. Peak fractions were collected, desalted and freeze-dried before analysis.

4.2.6 Quantitative Analysis of Oligosaccharides

A small fraction of total oligosaccharides (3%) was radiolabelled by reduction with 5X excess of NaB[3H]₄ (300-350 μCi) in 0.2 M sodium borate buffer, pH 9.8 (200 μl) at 30°C for 4 h, according to Takasaki et al. (1982) except that borate buffer was used instead of 0.05 N NaOH. To complete reduction, NaBH₄ (7 mg) in 0.2 M sodium borate buffer (160 μl) was added and incubated for 2 h at 30°C. The reaction was stopped by the addition of 1 M acetic acid (0.5 ml). The reaction mixture was passed through a column (2 ml) of AG 50W-X4 (H⁺ form, 100-200 mesh) which was washed with water (10 ml). The eluate and washings were combined and dried by rotary evaporation. Borate was removed by repeated evaporation with methanol. The ³H-labelled reduced oligosaccharides were freed from reaction products and radioactive impurities by gel-filtration on a Sephadex G-15 column (1.6 x 70 cm) in water. The yields of radioactive oligosaccharides were 5.16 x 10⁶ cpm (α Asn 52), 7.03 x
$10^6$ ($\alpha$ Asn 78), and 8.05 x $10^6$ cpm ($\beta$ Asn 30).

$^3$H-labelled oligosaccharide alditols (1.2-1.6 x $10^6$ cpm) were fractionated by anion-exchange and amine-adsorption HPLC under conditions identical with those used for preparative separations. Fractions (300 µl) were collected. Each fraction was diluted to 1300 µl with water and radioactivity was determined by a LKB 1209 Rackbeta liquid scintillation spectrometer with 3 ml scintillation fluid containing 0.4% (w/v) 2,5-diphenyloxazole, 33% (v/v) Triton X-100 in toluene. The relative amount of each oligosaccharide was calculated on the basis of recovered radioactivity. In the case of mixtures, the ratio of components was determined by integration of $^1$H-NMR spectra after assignment of characteristic signals to individual structures.

4.2.7 $^1$H-NMR Spectroscopy

Oligosaccharide alditols were subjected to $^1$H-NMR spectroscopic analysis at various stages of isolation and fractionation. Samples were repeatedly exchanged with $^2$H$_2$O (99.8 atom % $^2$H, Aldrich) and spectra were recorded in 0.4 ml $^2$H$_2$O (99.96 atom % $^2$H, Aldrich) in a Bruker AM-400 spectrometer operating at 400 MHz in the Fourier-transform mode. The probe temperature was 298 K. Chemical shifts (δ) were measured in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate (indirectly through acetone at 2.225 ppm). $^1$H-NMR analyses were performed by Dr G. Weisshaar of the Department of Biochemistry, University of Auckland and further experimental details have been described (Weisshaar et al., 1991).
Purified hLH

Dissociation

Separation of subunits by reverse-phase HPLC

α-Subunit

Reduction/alkylation
Tryptic digestion

Separation of glycopeptides by reverse-phase HPLC

β-Subunit (Asn 30)

αGPI (Asn 52)  αGPII (Asn 78)

Hydrazinolysis/re-N-acetylation
Cation-exchange chromatography
Free-reducing oligosaccharides
Reduction with NaBH₄ or NaB[³H]₄
Gel-filtration chromatography
Oligosaccharide alditol mixtures
Anion-exchange HPLC
Amine-adsorption HPLC
Oligosaccharide alditols (components)

Fig. 4.1 Scheme for the isolation and purification of N-glycans from each of the three glycosylation sites of hLH.

αGPI, glycopeptide α(52-63); αGPII, glycopeptides α(76-91)
4.3 Results

The approach taken for the isolation and purification of the N-glycans of hLH is summarized in Fig.4.1. The two subunits were first isolated from purified hLH by reverse-phase HPLC to separate one glycosylation site (Asn 30) of the β-subunit from the other two sites in the α-subunit; these (Asn 52 and Asn 78) were then resolved as glycopeptides by reverse-phase HPLC after tryptic digestion following reduction and S-carboxymethylation. The N-linked oligosaccharides were released by hydrazinolysis, re-N-acetylated and the free reducing oligosaccharides were reduced with NaBH₄ to give oligosaccharide alditols to facilitate structural characterisation. The oligosaccharide alditol mixtures from each site were purified with respect to charge and size by anion-exchange and ion-suppression amine-adsorption HPLC respectively, in preparation for structural analysis by ¹H-NMR. The relative amounts of oligosaccharides present in the HPLC fractions were determined using radiolabelled oligosaccharides prepared by reduction with NaB[³H]₄.

4.3.1 Purification of hLH and its Subunits

The yield of hLH in grams is given in Table 4.1 along with that of hTSH. 223.0 mg hLH and 122.3 mg hTSH were prepared from approximately 4200 glands (54 mg hLH and 31 mg hTSH per 1000 glands). The yields of both hormones were significantly higher than previously obtained from frozen glands. Improved yields were obtained from acetone-dried pituitary powder as the yields of both hormones obtained from frozen glands were similar to the earlier result. Chemical purity of the hLH preparations from both sources was comparable to that achieved previously.
Table 4.1  Yields of hLH and hTSH isolated from frozen human pituitary glands (batch nos. 08-11) and acetone-dried powder (batch nos. 12 & 13).

<table>
<thead>
<tr>
<th>Batch no. (glands)</th>
<th>Sephadex G-100 fraction mg</th>
<th>Phenyl-Sepharose fraction IV-2 mg</th>
<th>Reverse-phase HPLC hLH mg</th>
<th>Reverse-phase HPLC hTSH mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>08 (600)</td>
<td>513</td>
<td>220</td>
<td>60.2</td>
<td>27.1</td>
</tr>
<tr>
<td>09 (600)</td>
<td>470</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (600)</td>
<td>470</td>
<td>196</td>
<td>50.1</td>
<td>22.6</td>
</tr>
<tr>
<td>11 (600)</td>
<td>455</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 (965) 2.22g</td>
<td>728</td>
<td>205</td>
<td>51.6</td>
<td>35.9</td>
</tr>
<tr>
<td>13 (839) 1.93g</td>
<td>635</td>
<td>218</td>
<td>61.1</td>
<td>36.7</td>
</tr>
<tr>
<td>Total (3271)</td>
<td>3271</td>
<td>839</td>
<td>223.0</td>
<td>122.3</td>
</tr>
</tbody>
</table>

Phenyl-Sepharose fractions IV-2 from two batches (08/09 and 10/11) were combined before ultrafiltration.
Fig. 4.2 Preparative separation of hLHα and hLHβ by reverse-phase HPLC.

Purified hLH (215 mg) was pretreated for dissociation as described in Experimental Procedures. Portions (20 mg) of the dissociated hLH were applied to a Vydac C₄ column (1 x 25 cm) and eluted with a linear gradient of acetonitrile (20-100%, v/v, mobile phase B; broken line) in 0.1 M TEAP (pH 6.5) at 1.2 ml/min. Mobile phase: A, 0.1 M TEAP; B, 0.1 M TEAP in 60% acetonitrile. The two subunit peak fractions were collected as indicated. The small peak that eluted at 40 min corresponded in retention time to intact hLH.
Preparative isolation of hLH subunits effected by reverse-phase HPLC is shown in Fig.4.2. The α- and β-subunits were completely separated from each other. Each subunit preparation gave only a single peak that corresponded to hLHα- or β-subunit on analytical reverse-phase HPLC that resolves hLH and its two subunits. The extent of cross-contamination estimated from the detection limits in this method was <1% and each subunit preparation was assessed to be essentially pure. The overall yields of the subunits from 215 mg hLH were: α 71 mg (66%) and β 68 mg (63%), which were significantly higher, particularly for the β-subunit, than those previously reported by Parsons et al. (1984) (α:50%, β:10%).

4.3.2 Separation of Glycopeptides of hLH α Subunit

Preliminary experiments showed that 0.1% TFA-acetonitrile, an acidic mobile phase previously described for the separation of hCG-α glycopeptides that resulted from tryptic digestions (Lustbader et al., 1987), was ineffective in preparation of micromole quantities of purified hLHα glycopeptides. However, it was found that the near-neutral buffer system, 0.1 M TEAP-acetonitrile (pH 6.5) resolved these glycopeptides effectively and without the potential risk of losing sialic acid residues that are relatively abundant in hLH (Chapter 3).

Separation of tryptic digest of RCM-hLH α is shown in Fig.4.3. As indicated in the figure, eleven fractions were collected and analysed by 1H-NMR, which indicated that three peak fractions (3, 4 and 8) contained glycopeptides. The N-terminal sequence analysis of fraction 3 gave a sequence: (Asn)-Val-Thr-
Fig. 4.3 Preparative separation of tryptic glycopeptides from hLHα by reverse-phase HPLC.

Portions (10 mg) of the tryptic digests of RCM-hLHα were applied in two aliquots (5 mg each) to a Vydac C₄ column and eluted with a 90 min linear gradient of acetonitrile (3-57%, broken line) in 0.1 M TEAP (pH 6.5) at 1.2 ml/min. Mobile phases: A, 0.1 M TEAP; B, 0.1 M TEAP in 60% acetonitrile. Fractions were collected as indicated.
Ser-Glu-Ser-Thr-Cys-Cys-Ala-Lys-COOH, identifying the
glycopeptide as α(52-63) (designated αGP I) that contained N-
glycosylated Asn 52. Peak fraction 4 was identified as α(76-91)
(designated αGP II) that contained the second glycosylated Asn
78, from its partial N-terminal sequence: Val-Glu-(Asn)-His-Thr
and the presence of 3 His and 2 Tyr residues determined by $^1$H-NMR
analysis. The small fraction 8 was identified as α(68-91), from
its partial sequence: Val-Thr-Val-Met-Gly-Gly-Phe-Lys-Val-Glu-
(Asn)-His-Thr and $^1$H-NMR results. The presence of the two
glycopeptide fractions, both of which contained Asn 78,
indicated that cleavage between Lys 75 and Val 76 was not
complete under the conditions used. $^1$H-NMR of these glycopeptide
fractions gave a number of chemical shifts that distinguished
between fractions 3 and 4 (and 8), including Asn-linked GlcNAc
H-1 and NAc (δ 5.066 and δ 2.010, respectively for αGP I vs δ
5.018 and α 2.005 for αGP II), and aromatic protons of His and
Tyr which were detected only in fractions 4 (and 8).

The absence of these characteristic signals associated with
fraction 3 in fraction 4, and vice versa, indicated little or no
cross-contamination. $^1$H-NMR analysis of fractions 3 and 4 showed
no evidence of peptide heterogeneity, although each glycopeptide
was eluted in multiple peaks. Therefore the peak-splitting
observed was considered to represent carbohydrate heterogeneity
in the main. The yields of glycopeptides from 62 mg hLHα were
10.8 mg (72%) for αGPI (fraction 3) and 15.1 mg (90%) for αGPII
(fractions 4 and 8 combined).

4.3.3 Characterisation of Charge Heterogeneity of
N-Glycans of hLH

The previous study has shown that hLH contains neutral,
Fig. 4.4 Anion-exchange HPLC of radiolabelled oligosaccharides obtained from each glycosylation site of hLH.

$^3$H-Labelled oligosaccharides from Asn 52 and Asn 78 of hLHa (a and b, respectively) and from Asn 30 of hLHβ (c) were analysed by anion-exchange HPLC at pH 4.0 as described in Experimental Procedures. Buffers: A, 3 mM KH$_2$PO$_4$ (pH 4.0); B, 300 mM KH$_2$PO$_4$ (pH 4.0). The gradient is indicated by broken line in (c). Fractions of 300 μl were collected up to fraction 85 and fractions 86-130 that contained tri-charged oligosaccharides were pooled (not shown in this figure). Peak fractions were characterised as indicated: N1, monosialylated; S1, monosulphated; SN, sulphated/sialylated, N2, disialylated, and S2, disulphated oligosaccharides.
Fig. 4.5 Relative amounts of neutral, mono-, di- and tri-charged oligosaccharides at each glycosylation site of hLH.

The percentage of the total oligosaccharides represented by neutral (A0), mono- (A1), di- (A2) and tri- (A3) charged species is indicated for each site. Di-charged populations consisted of disialylated (N2), sulphated/sialylated (SN) and disulphated (S2) oligosaccharides.
mono-, di- and tri-charged oligosaccharides in a percent molar ratio of 7:28:55:10 (Chapter 3). To determine if certain types of charged oligosaccharides were more prevalent at a specific glycosylation site, the charge distributions of oligosaccharides were examined at each site using $^{3}$H-labelled oligosaccharides prepared by reduction with NaB[$^{3}$H]$_{4}$.

Fig. 4.4 shows the anion-exchange separations of $^{3}$H-labelled oligosaccharides on a MicroPak AX-5 column effected by the elution conditions described in Experimental Procedures. By modifying the reported gradient programme (Green & Baenziger, 1986), it was possible to separate each of identically charged oligosaccharides into several sub-fractions; three distinct peaks were resolved from the di-charged fraction which corresponded to disialylated (N2), sulphated/sialylated (SN), and disulphated (S2) oligosaccharides that were characterised by $^{1}$H-NMR (described later). Recovery of labelled oligosaccharides was between 88 and 100%.

The relative amounts of neutral, mono-, di- and tri-charged oligosaccharides at each site of hLH, calculated from recovered radioactivities, are shown graphically in Fig. 4.5. The results showed that ~90% of total oligosaccharides at all three sites were acidic and that their charge distributions were similar, with di-charged oligosaccharides being most abundant. However, the relative amounts of the three types of di-charged species differed noticeably; SN was more plentiful at $\alpha$Asn 52, N2 at $\alpha$Asn 78, and both SN and N2 were abundant at $\beta$Asn 30. These results indicated that terminal sialylation and sulphation of hLH N-glycans were somewhat site-dependent.

The relative proportions of various anionic charges that can arise from random combination of the three N-glycans were
Fig. 4.6 Distribution of anionic charges derived from the three N-glycans on hLH.

The relative proportion of various anionic charges calculated from the charge distribution of oligosaccharides at each glycosylation site is presented as a percentage of the total.
Fig. 4.7 Preparative anion-exchange HPLC of oligosaccharide alditols from each glycosylation site of hLH.

Oligosaccharide samples obtained from hLH αGPI (a), αGPII (b) and hLHβ (c) were applied to a MicroPak AX-5 column and eluted with a gradient of 3-300 mM KH$_2$PO$_4$, pH 4.0, as indicated by the broken line in (c): 0-15% B in 20 min, isocratically (15% B) for 20 min, 15-50% B in 20 min, isocratically (50% B) for 10 min, 50-100% B in 10 min and isocratically (100% B) for 20 min. Buffers, A (3 mM KH$_2$PO$_4$, pH 4.0) and B (300 mM KH$_2$PO$_4$, pH 4.0). One-third of total sample was applied in each run and six peak fractions were collected from each sample as indicated.
calculated from the data in Fig. 4.5 to assess the contribution of anionic oligosaccharides to the charge heterogeneity of hLH. The results (shown in Fig. 4.6) indicated that, although hLH molecules can theoretically contain 0-9 anionic charges from oligosaccharides a large portion of hLH molecules has in effect 3-6 such anionic charges, with a mean of 4.5, which corresponds to the total sialic acid and sulphate content previously estimated for hLH (3.6 and 1.4 mol/mol hormone, respectively (Chapter 3).

4.3.4 Preparative Separation of N-Linked Oligosaccharides

Following hydrazinolysis, re-N-acetylation and reduction, the oligosaccharide alditols obtained from αGPI, αGPII and the β-subunit were subjected to anion-exchange HPLC on a MicroPak AX-5 column using the modified elution conditions described above. As indicated in Fig. 4.7, six fractions were collected from each sample. Retention times and $^1$H-NMR analysis indicated neutral, monosialylated (N1) and monosulphated (S1), disialylated (N2), sulphated/sialylated (SN), and tri-charged oligosaccharides in fractions 1, 2, 3, 4, 5 and 6, respectively.

All fractions except S2 (fraction 5) were too heterogeneous for structural analysis by $^1$H-NMR and the three major fractions (2, 3 and 4) derived from each of the three glycosylation sites, were further purified by ion-suppression amine-adsorption HPLC that separates anionic oligosaccharides essentially according to size and linkage, e.g. NeuAc α2-3 versus α2-6 (Green & Baenziger, 1986). The HPLC chromatograms obtained from anion-exchange fractions A1-4 (derived from αGPI), A2-3 (αGPII) and B-2 (hLHβ) are shown in Fig. 4.8. Under the applied conditions,
Fig. 4.8 Preparative ion-suppression amine-adsorption HPLC of major anion-exchange fractions.

Chromatograms of fractions A₁-4, A₂-3 and B-2 are shown in (a), (b) and (c), respectively. Each sample was applied to a MicroPak AX-5 column and eluted by a linearly decreasing concentration of acetonitrile in 1% (v/v) TEAP, pH 4.0. Buffers: A, 1% TEAP in 70% acetonitrile; B, 1% TEAP. The gradient programme indicated by the broken line in (c) was the same as described in Fig.4.7. Fractions were collected as indicated.
amine-adsorption HPLC effectively resolved each fraction into a number of distinct peaks. Main peak fractions, of which oligosaccharides were ultimately identified by $^1$H-NMR analysis, are indicated in the figure. The relative amounts of oligosaccharides present in each fraction estimated by measurements of radioactivity were very low (<20% of total oligosaccharides per site). Together, these results indicated that all three N-glycan chains on hLH are highly heterogeneous mixtures of oligosaccharides that differ not only in the number of sialic acid and sulphate residues, but also in their linkages and underlying structures.

4.3.5 Structural Analysis of N-linked Oligosaccharides by $^1$H-NMR

The separation step described above yielded a total of 27 distinct fractions of reduced oligosaccharides from each glycosylation site. Of these, 10 fractions (S2 and 9 subfractions in Fig.4.8) were subjected to structural analysis by 400 MHz $^1$H-NMR spectroscopy to determine the complete primary structures of constituent oligosaccharides. Structures of oligosaccharides in many other minor fractions, each of which represents less than 2% per site, could not be determined due to insufficient material (estimated to be <100 µg from the detection limits of $^1$H-NMR). Neutral and tri-charged fractions were analysed without further separation.

Structural identification was mainly based on one- and two-dimensional $^1$H-NMR spectroscopy of intact oligosaccharide alditols, with reference to published spectral data of structurally related compounds (Carver & Grey, 1981;
Vliegenthart et al., 1983) and recently reported data on sulphated N-glycans (Weisshaar et al., 1990). For the interpretation of $^1$H-NMR spectra in terms of structural assignments, the characteristic signals of a number of protons, namely the anomeric protons (H-1), H-2 and H-3 protons of the constituent monosaccharide residues, the H-3 protons of the NeuAc residues, the H-5 and H-6 protons of the Fuc residues, and N-acetyl methyl protons were used (Vliegenthart et al., 1983). In addition, chemical shift data of H-1, H-2 and H-3 of the three, core mannose residues were used to recognise substitution patterns in the core (Geyer et al., 1984; Bhattacharyya et al., 1984).

The $^1$H-NMR spectra of all oligosaccharide fractions examined in this study revealed that they all had the following reduced pentasaccharide core structure in common.

$$\text{Man}_{4}^1\alpha 1-6\text{Man}_{1}^{3}\beta 1-4\text{GlcNAc}_{1}^{3}2\beta 1-4\text{GlcNAc-o1}$$

A set of characteristic chemical shifts; the H-2 of GlcNAc1 at $\delta$-4.25 (quartet in the Man H-2 region, 4.0 < $\delta$<4.3), the H-1 of GlcNAc2 (doublet at $\delta$-4.63) and two NAc singlets (at $\delta$-2.055 for GlcNAc1 and $\delta$-2.08 for GlcNAc2) were all typical of the reduced N, N'-diacetyl chitobiose unit (Paz-Parente et al., 1982; Paz-Parente et al., 1983). The three Man H-1 signals at $\delta$-5.12 (Man4), $\delta$-4.92 (Man4') and $\delta$-4.78 (Man3), and three Man H-2 signals (4.0< $\delta$<4.3) were further evidence for the pentasaccharide core unit. From the set of Man chemical shifts, it was also inferred that the oligosaccharides present in the
Fig. 4.9 400 MHz $^1$H-NMR spectrum of SN type oligosaccharides derived from Asn 52 of hLHa.

Sample: fraction $A_1-4c$ (αGPI). Labelling of sugar residues refers to structure on top of the spectrum. Assignments in brackets correspond to the minor fucosylated compound (15%). N-acetyl (NAc) signals and the spectral region of 4.08-4.73 ppm are expanded.
subfractions from N2, SN and S2 were all of diantennary structures, while those from N1 and S1 were either mono-, diantennary or a mixture of diantennary and hybrid structures (described below).

1. SN type oligosaccharides

Two main subfractions were obtained from the SN fraction (c and d in Fig. 4.8a). The $^1$H-NMR spectrum of the major peak fraction from αGPI (A$_1$-4c) is shown in Fig. 4.9 with the assignments of proton signals, established by 2D-correlated spectroscopy. The chemical shift data are given in Table 4.2. The set of H-1, H-2 and H-3 chemical shifts for Man3, Man4 and Man4' indicates the diantennary complex-type structure. It is evident that the sialylated N-acetyllactosamine unit is attached in β1-2 linkage to Man4' from the typical shifts for Man4' H-1, GlcNAc5' H-1 and NAc, Gal6' H-1, H-2 and H-3, and NeuAc H-3a and H-3e (a, axial; e, equatorial, Table 4.2). The set of chemical shifts for NeuAc H-3 protons, i.e. H-3a $\delta = 1.801$ and H-3e $\delta = 2.759$, indicates that the NeuAc residue is α2-3 linked to the Gal6' residue (Vliegenthart et al., 1983).

In contrast to the Manα1-6 branch, the Manα1-3 branch consists of SO$_4$-4GalNAcβ1-4GlcNAcβ1-2 linked to Man4, as determined from a number of characteristic chemical shifts for this structural element (Weisshaar et al., 1990), i.e. Man4 H-1 ($\delta = 5.111$), GlcNAc5 H-1 and NAc ($\delta = 4.557$ and 2.046, respectively) and GalNAc H-1, H-4 and NAc ($\delta = 4.585$, 4.692 and 2.069, respectively). The Fuc H-1, H-5 and H-6 signals with lower intensity show the presence of submolar amounts of Fuc α1-6 linked to GlcNAc1, indicating that this oligosaccharide is
Table 4.2  Proton chemical shifts of oligosaccharide aldolts isolated from hLH.

Chemical shifts (δ) were measured at 400 MHz in 2H2O at 298 K with acetone as internal reference (δ = 2.225 ppm). The shorthand notation of the structures refers to the numbering of the corresponding sugar residues in the formula given in Figs. 4.9 and 4.10, except that: 3N', 3NeuAc'; 6N, 6NeuAc; 6N', 6NeuAc'; Fucol-6. Values for the fucosylated compound S-3' are given in square brackets, if they differ significantly from the chemical shifts for corresponding protons in the nonfucosylated compound. (n.d., not determined)

1, Measured at 303 K. 2, Determined by 1-step relayed COSY of fraction A2-3. 3, Values may have to be interchanged.

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partially fucosylated, i.e. a mixture of fucosylated and non-fucosylated compounds. The extent of fucosylation determined from the relative intensities of the two GlcNAc2 NAc signals for the two compounds was 15%. The diantennary structure inferred from the above spectral data is shown in Fig.4.9 (denoted \( S^{-3}N \)). It has been confirmed by carbohydrate composition and methylation analysis (Weisshaar et al., 1991). \( S^{-3}N \) was the major component at \( \alpha \text{Asn} 52 \) (18%) and \( \beta \text{Asn} 30 \) (15%), however, it was 90% fucosylated in the latter case. Smaller amounts of \( S^{-3}N \) were present at \( \alpha \text{Asn} 78 \) (5%).

The \(^1\)H-NMR spectrum of the smaller peak fraction (A\(_1\)-4d) showed essentially the same proton signals as \( S^{-3}N \), except those for Man4' H-1, GlcNAc5' H-1 and NAc, Gal6' H-1, NeuAc H-3a and H-3e (Table 4.2) which are characteristic for the NeuAca2-6Galβ1-4GlcNAcβ1-2 linked to Man4' (Vliegenthart et al., 1983). This indicates that the oligosaccharide is an isomer of \( S^{-3}N \), in which NeuAc is attached in \( \alpha2-6 \) linkage (Fig.4.9). This oligosaccharide (denoted \( S^{-6}N \)) was detected in small amounts (2-5%) at each glycosylation site.

Monosulphated oligosaccharides, structurally derived from \( S^{-3}N \) and \( S^{-6}N \), were detected in various amounts at all three glycosylation sites. \( S1-6' \) (with non-reducing terminal Gal6'), \( S1-5' \) (terminal GlcNAc5') and \( S1-4' \) (terminal Man4') were baseline separated by amine-adsorption HPLC (Fig.4.8c) and identified by \(^1\)H-NMR as described for the same oligosaccharides isolated from ovine LH (Chapter 5). While fractions B-2b, B-2d and the corresponding subfractions of A\(_1\)-2 and A\(_2\)-2 were essentially pure, approximately 50% of fraction A\(_1\)-2c consisted of a monosulphated hybrid-type oligosaccharide with Man4'.
Fig. 4.10 400 MHz $^1$H-NMR spectrum of N2 type oligosaccharides derived from Asn 78 of hLHa.

Sample: fraction $A_2$-3d (aGPII). Labelling of sugar residues corresponds to structures on top of the figure; letters A and B (in parentheses) refer to compounds N2/A and N2/B, respectively. N-acetyl (NAc) signals are expanded and resolution-enhanced.
substituted in the 3-position by another α-Man residue (S1-A, Table 4.3).

2. **S2 type oligosaccharides**

   The anion-exchange fractions A\textsubscript{1}-5, A\textsubscript{2}-5 and B-5 (Fig.4.7) contained a pure disulphated, diantennary oligosaccharide (S2, Table 4.3) with or without Fuc α1-6 linked to GlcNAc1. Both branches of S2 consist of SO\textsubscript{4}-4GalNAcβ1-4GlcNAc attached in β1-2 linkage to Man4 and Man4', respectively and its structure was identified as described for the same oligosaccharide isolated from ovine LH (Chapter 5). S2 was relatively abundant at αAsn 52 and βAsn 30 (7% and 10% respectively).

3. **N2 type oligosaccharides**

   Human LH contained relatively large amounts of disialylated oligosaccharides, particularly at αAsn 78 (32%, Fig.4.5). Two main subfractions (d and f) were obtained from the N2 fractions by amine-adsorption HPLC and the \textsuperscript{1}H-NMR spectrum of the major peak fraction from αGPII (A\textsubscript{2}-3d, Fig.4.8) is shown in Fig.4.10. Characteristic H-3 (two H-3a and three H-3e) signals of both α2-3- and α2-6-linked NeuAc residues indicated that this fraction was a mixture of two disialylated oligosaccharides, both diantennary in type (Table 4.2). The ratio of α2-3/α2-6 NeuAc was 1:1. The Manα1-6 branch of both oligosaccharides consisted of NeuAcα2-3Galβ1-4GlcNAcβ1-2 linked to Man4', as determined from a number of characteristic chemical shifts for the sialylated N-acetyllactosamine unit (Vliegenthart et al., 1983); i.e. Man4' H-1, GlcNAc5' H-1 and NAc, Gal6' H-1, H-2 and H-3 and NeuAc H-3a and H-3e (Table 4.2). Similarly, the presence of NeuAcα2-6Galβ1-4GlcNAcβ1-2 linked to Man4 indicated that one oligosaccharide had
a disialylated N-acetyllactosamine-type structure with α2-6 and α2-3 NeuAc residues attached to Gal6 and Gal6', respectively.

Sugar analysis revealed that fraction A2-3d contained N-acetylgalactosamine, substituted in the 6-position according to the results of methylation analysis (Weisshaar et al., 1991). Based on these results, it was concluded that fraction A2-3d contained a mixture of two disialylated diantennary structures (denoted N2/A and N2/B), as shown in the figure. The structures of N2/A and N2/B were confirmed by fast-atom-bombardment mass spectrometry (Weisshaar et al., 1991). The ratio of (N2/A)/(N2/B) was 1:2, as determined from the integrals of the signals for Gal6 H-1 (N2/A) and GalNAC H-1 (N2/B).

The 1H-NMR data of N2/B are included in Table 4.2. Characteristic signals for N2/A described above were essentially identical with those reported for the same compound isolated from bovine fetuin (Green et al., 1988). N2/B was the major single component at αAsn 78 (12%), while it was present in only minor quantities at the other sites. The ratio of (N2/A)/(N2/B) in fractions A1-3d and B-3d was approximately 3:1 and the fucose content in these fractions was 25% and 100%, respectively. An isomer of N2/A with both NeuAc residues in an α2-6 linkage to Gal was detected in small amounts at each glycosylation site of hLHα. 1H-NMR spectra of the anion-exchange fractions indicated that 10-20% of the disialylated structures had a bisecting GlcNACβ1-4 linked Man3.

Monosialylated oligosaccharides that were coeluted with monosulphated oligosaccharides in anion-exchange HPLC fractions were successfully separated by amine-adsorption HPLC (Fig. 4.8c). However, each of these subfractions was a mixture of at least
two components that differed in respect of the branch location and linkage of sialic acid attached to Gal or GalNAc, different incomplete branches, the presence of bisecting GlcNAc and the extent of core-fucosylation.

The major oligosaccharides were found to be structurally derived from N2/A and N2/B, i.e. with NeuAcα2-6Gal or NeuAcα2-6GalNAc in the Manα1-3 branch and terminal Gal6' (N1/A-6' and N1/B-6') or terminal Man4' (N1/A-4' and N1/B-4'). N1/A-6' and N1/B-6' were both eluted in fraction B-2k and N1/A-4' and N1/B-4' in fraction B-2g (Fig.4.8c). The chemical shifts for the α1-3 chain were essentially identical with the respective chain in N2/A and N2/B. 1H-NMR data for N1/B-6' and N1/B-4' are given in Table 4.2.

Small quantities of tri-charged oligosaccharides were detected at each glycosylation site (Fig.4.7). The 1H-NMR spectrum of fraction B-6 indicated mainly sialylated triantennary structures (bifurcated at Man4) with an overall ratio of NeuAcα2-3/NeuAcα2-6 being approximately 2:1; 4-sulphated GalNAc was also detected. Neutral oligosaccharides that were present at each glycosylation site (7-12%) were mainly diantennary structures.

The oligosaccharides identified at each of the three glycosylation sites of hLH and their distributions are summarised in Table 4.3.

4.4 Discussion

Preparative purification of glycopeptides from any glycoprotein is problematic because of complications that arise from imperfect proteolysis and influences of site-heterogeneity of oligosaccharides. In addition, recoveries of glycopeptides
Table 4.3 Oligosaccharide structures of the N-glycans of hLH.

Relative abundances are given as % of total oligosaccharides at each glycosylation site. The individual structures are composed of either of the three core-structures (in bold type), extended at the Gl-6 linked mannose residue by the listed sequences. For designation of the components S2, S-3N etc.) see text. The extent of core-fucosylation, determined for major structures (see text) and presumably applying to all structures at a given glycosylation site, is within the following ranges: 15-25% at Asn 52, 0-10% at Asn 78 and 90-100% at Asn 30.

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2 4 6 tricharged oligosaccharides

9 12 7 neutral oligosaccharides
are usually low. Reverse-phase HPLC has been widely used in the isolation of glycopeptides, since this mode of separation has some effect in suppressing the adverse influences of oligosaccharides on glycopeptide separations (Rosner & Robbins, 1982; Swiedler et al., 1985). However, its effectiveness also depends on the chromatographic conditions to be used. For example, separation of tryptic sialylated glycopeptides from bovine fetuin on octyl RP-HPLC with 0.3 M boric acid-TEA/acetonitrile (pH 7.0) was significantly affected by the degree of sialylation (Rice et al., 1990). In this case, although nearly complete resolution of glycopeptides was achieved in preparative RP-HPLC, recoveries were low (~35%). Analytical separation of tryptic sialylated glycopeptides from recombinant hCG α-subunit by C4 RP-HPLC with an TFA/acetonitrile system was reported (Lustbader et al., 1987), but because of inadequate resolution, this procedure was not readily applicable to the isolation of micromole quantities of glycopeptides required in the present study.

Some of these heterogeneity problems have been overcome by optimizing RP-HPLC conditions. Chromatography on C4 RP-HPLC in phosphoric acid buffered to pH 6.5 with TEA was effective for the preparation of purified glycopeptides containing each of the two glycosylation sites on hLH α-subunit. High protein concentrations (5 mg/ml) and 2% (w/w) of trypsin were used for digesting reduced and alkylated hLHα, but complete tryptic cleavage between Lys 75 and Val 76 could not be achieved, which resulted in the two glycopeptides containing the same linkage site (Asn 78). Kinetic studies of tryptic digestion at 37°C for longer incubation times (up to 6 h) did not show any significant changes in analytical separation profiles, which indicated that
the above peptide bond is particularly resistant to trypsin, perhaps due to steric hindrance by glycosylation of Asn 78. Recently, Pollak et al. (1990) reported that a higher trypsin/substrate ratio, 10% (w/w), was necessary to effect complete cleavage of hCG$\alpha$. The yields of purified glycopeptides obtained by the present procedure were high (70-90%), which was critical for the subsequent isolation and structural analysis by $^1$H-NMR of the highly heterogeneous oligosaccharides from hLH.

Hydrazinolysis was employed to release oligosaccharides from each glycosylation site of hLH. $^1$H-NMR analysis of the oligosaccharide and other fractions from cation-exchange chromatography did not show the presence of glycopeptides, which indicated the quantitative release of oligosaccharides under the reaction conditions used. It has been reported that the reducing end of oligosaccharides after hydrazinolysis and re-N-acetylation of glycoproteins is $\beta$-acetohydrazide GlcNAc and that mild acid hydrolysis is necessary to generate reducing GlcNAc (Bendiak & Cumming, 1986). In the present study, almost all of the oligosaccharides obtained from cation-exchange chromatography contained reducing GlcNAc as analysed by $^1$H-NMR, which indicated that relative short exposure (<40 min) to the acidic ion-exchange resins is an effective means of acid hydrolysis.

Heterogeneous populations of oligosaccharides must be efficiently fractionated with high yield and good resolution prior to characterisation. The N-glycan chain from each glycosylation site of hLH was a highly complex mixture of neutral, sialylated and sulphated oligosaccharides. Although they were effectively purified with respect to charge and size by anion-exchange and amine-adsorption HPLC, most of the major
oligosaccharide fractions were still mixtures of two or more compounds. Structural characterisations of individual oligosaccharides in mixtures were greatly facilitated by $^1$H-NMR spectroscopy without which analysis would have been a formidable task beyond the limited resources of our laboratory. Significant amounts of new mono- and disialylated structures were identified in such mixtures and will be described later.

Oligosaccharides from each glycosylation site displayed a distinct anion-exchange HPLC profile due to a specific pattern of sialylation and sulphation. This was particularly prominent for the di-charged species that consisted of disialylated, sulphated/sialylated, and disulphated oligosaccharides; oligosaccharides from Asn 78 (α) were more sialylated than those from Asn 52 (α) and Asn 30 (β). However, despite these differences in patterns of sialylation and sulphation, the distributions of net negative charges were similar, which suggested that the contributions of the three N-glycan chains to the charge heterogeneity of hLH may be similar. Assuming no contribution of peptide heterogeneity and that the glycosylation at the three sites are independent processes, an attempt was made to account for the charge heterogeneity of hLH by the distribution of anionic charges on the three N-glycan chains. It was estimated that most of hLH molecules contain 3–6 anionic residues (sialic acid and/or sulphate), which predicted that the contribution of N-linked oligosaccharides to the overall charge of hLH is within the range of 3 in terms of net negative charge. This appeared to correspond well to the range of pIs reported for hLH isohormones (pI 5.9-8.8) in crude human pituitary extracts (Weise et al., 1983), although the existence of a number of isohormones in this range could not be explained in
this approach.

The oligosaccharide structures of the N-glycans present on hLH have been characterised at the individual glycosylation sites in this study. It was found that all three N-glycans consisted of a heterogeneous mixture of sialylated and sulphated oligosaccharides that are predominantly diantennary complex-type. The presence of all of the 12 sialylated and sulphated structures previously reported for hLH (Green & Baenziger, 1988a,b) was confirmed by $^1$H-NMR, with the exception of two sulphated hybrid-type structures (S-1(A) and S-1(C) in the above ref). In addition, a set of new mono- and disialylated structures $\text{N1/B-4', N1/B-6'}$ and $\text{N2/B}$, (Table 4.3) was found in the present study. The content of these structures was relatively high (15% of the anionic oligosaccharide population in hLH), particularly at one site (Asn 78) of the $\alpha$-subunit. These oligosaccharide could not be resolved from the corresponding mono- and disialylated ($\text{N1-A4', N1/A-6'}$ and $\text{N2/A}$) during the amine-adsorption HPLC separations, hence, they may not have been readily detected in earlier studies. The presence of small amounts of tri-charged species in our preparation appeared to be associated with the method of purification used to minimise the potential risk of selective isolation of certain charged-forms.

Structural heterogeneity of N-glycans at each site is considerable, with more than 14 distinct structures present in fucosylated and non-fucosylated form. Bisecting GlcNAc structures were also detected in a small portion of mono- and disialylated oligosaccharides. Although almost all structures were commonly found at the three sites, there were two noticeable quantitative differences between sites; first,
Fig. 4.11 Biosynthetic pathway of N-linked oligosaccharides on human LH.

Each of the precursor oligosaccharides (Glc₃Man₂GlcNAc₂) attached to Asn 52, Asn 78(hLHα) and Asn 30(hLHβ) is processed to GlcNAc₂Man₃GlcNAc₂ [I] by sequential action of glucosidases, α-mannosidase I, GlcNAc transferase I, α-mannosidase II and GlcNAc transferase II (steps 1-5, respectively). Core-fucosylation (step 6) appears to occur following these reactions (Kornfeld & Kornfeld, 1985). The subsequent processing of the common intermediate [I] that involves GalNAc- and GaI transferases and sulpho- and sialyltransferases yields various diantennary complex-type oligosaccharides in different amounts depending on the glycosylation site.
sulphated/sialylated structures, e.g. S-3N, were abundant at Asn 52 (α) and Asn 30 (β), while disialylated structures, e.g. N2/B, were more prevalent at Asn 78 (α). Secondly, core-fucosylation was almost complete in the β-subunit, however it was only partial at both sites in the α-subunit.

The finding that hLH contained mono- and disialylated diantennary complex-type oligosaccharides with the terminal sequence NeuAcα2-6GalNAcβ1-4GlcNAcβ1-2Manα1-3, is important because this had not been reported for N-glycans. The results suggest the presence of a hitherto unknown α2-6 sialyltransferase in human pituitary cells that specifically sialylates GalNAcβ1-4GlcNAcβ1-2, although this sequence has been considered to be the sole substrate for sulphation (Green et al., 1985c). This sialyltransferase appears to act preferentially on GalNAc in the α1-3 branch, since no such terminal sequence was detected in the α1-6 branch. Also, the fact that sub-terminal GalNAc is either sialylated or sulphated, and not both, indicates that the two terminal reactions are mutually exclusive. Furthermore, these results have revealed that terminal glycosylation of the oligosaccharides on hLH is more complex than proposed previously (Baenziger & Green, 1988).

The major biosynthetic pathway for the N-glycans present on hLH deduced from the structural data reported in this thesis (Table 4.3) is illustrated in Fig.4.11. The sialylated and/or sulphated diantennary complex-type oligosaccharides that dominate at all three sites are derived from the common precursor GlcNAc2Man3GlcNAc2(I) by competitive action of Gal- and GalNAc-transferases on each of the two terminal GlcNAc residues (step 7), followed by termination with sialic acid (for Gal and GalNAc) or sulphate (for GalNAc). The ratio of the three
intermediates (IIa-c), that reflects the relative activities of Gal- and GalNAc-transferases, is relatively constant at all three sites, i.e. IIa:IIb:IIc = 10:50:20. These results suggest that they have apparent branch-specificity; GalNAc and Gal are preferentially added to GlcNAc residues in the α1-3 and α1-6 branches, respectively, with the result that IIb is the major product. It has been shown that GalNAc is more efficiently incorporated into the human α-subunit than Gal, which suggested that the action of GalNAc-transferase requires specific recognition of certain polypeptide structures (Smith & Baenziger, 1988). Its greater substrate-specificity (or affinity) may promote the addition of GalNAc to the α1-3 branch. Because of this strict substrate-specificity, however, its action on the α1-6 branch, that is relatively inaccessible for transferases in general, may be sterically more hindered than that of Gal-transferase.

Subsequent sialylation and sulphation produce disulphated, sulphated/sialylated and two types of disialylated structures as fully processed forms. Only this last step and core-fucosylation appear to be susceptible to the influence of polypeptide structures, as reflected in the observed site-specific glycosylation. From the major intermediate (IIb), either S^{-3}N (and S^{-6}N) or N2/B is formed by the competitive action of sulphotransferase and GalNAcα2-6 sialyltransferase on the GalNAc in the α1-3 branch (step 8). The relative activities of these two enzymes will determine the amounts of the two single major components. More common disialylated structures (N2/A and N2/C) are produced from the lesser intermediate (IIc) by Galα2-3 and α2-6 sialyltransferases (step 9). In almost all mono- and disialylated structures, sialylation in the α1-3
branch occurred in the α2-6 linkage, while that in the α1-6 branch occurred in the α2-3 linkage. Similar branch-specificity has been reported for the α2-6 sialyltransferase from bovine colostrum (Joziasse et al., 1985). Disulphated structures (S2) are produced from the other lesser intermediate (IIa) by exclusive action of sulphotransferase (step 10).

As discussed above, it seems that competition between Gal- and GalNAc transferases and that between sulpho- and various sialyltransferases are the two major factors that determine the ultimate branch structures of the diantennary complex-type oligosaccharides present on hLH.

Unlike hLH, bLH contains exclusively sulphated oligosaccharides and the terminal sulphation has been associated with the presence of GalNAc transferase activity in the pituitary cells (Green et al., 1985c). It has also been shown in vitro that GalNAc transferase in bovine pituitary membranes preferentially adds GalNAc to agalactooligosaccharides on hCGα (Smith & Baenziger, 1988), hCG and bLH (Smith & Baenziger, 1990), although Gal transferase is also active. These results may suggest that the relative activities of the two enzymes in human and bovine pituitary cells are significantly different. Alternatively, their substrate specificities may differ between the two species. In this regard, however, the possibility cannot be ruled out that the apparent relative activities of these enzymes estimated from the present data also reflected alterations in glycosylation of this hormone among individuals that may occur under different physiological and/or pathological influences (Wilson et al., 1990). Although it is difficult to assess these effects, the present results suggest that these influences are limited mainly to alterations in the branch
structures.

The site-specific biological roles of the N-glycans of hCG have recently been demonstrated (Matzuk et al., 1989) and it is of particular interest to examine the structural features of the individual N-glycans on this hormone and LH. The implication of the N-glycan structures of hLH with respect to their potential function is discussed in Chapter 6, along with hormone- and species-specific N-glycosylation.
CHAPTER 5 Isolation and Characterisation of N-Glycans of Ovine LH

5.1 Introduction

Ovine LH is structurally homologous with human LH and is composed of two nonidentical subunits, α and β. The common α-subunit (96 amino acids) is glycosylated at Asn 56 and Asn 82, while the hormone-specific β-subunit (119 amino acids) is glycosylated at Asn 13.

Earlier studies found that LH from ovine, bovine and human pituitaries contained substantial amounts of O-sulphated N-acetylhexosamines (Parsons & Pierce, 1980; Bedi et al., 1982), but detailed structural information about the N-linked oligosaccharides of these hormones has not been available until recently. The disulphated diantennary structure containing the characteristic peripheral sequence \( \text{SO}_4^\text{2}\text{-4GalNAcβ1-4GlcNAcβ1-2Manα} \), was first determined for the major oligosaccharide isolated from hLH (Green et al., 1985a). More recently, Green and Baenziger (1988a,b) have reported that the mono- and disulphated oligosaccharides are also present in oLH; various sulphated structures and their distributions were described, however, the structures of the three N-glycan chains were not defined with respect to the linkage sites. In contrast to hLH, oLH was reported to contain both hybrid-type and complex-type oligosaccharides in this study, thus it appeared to be a suitable model for the assessment of effects of protein structure on N-linked oligosaccharide processing in LH.

This chapter describes the separation and structural characterisation by \(^1\text{H}-\text{NMR}\) analysis of the N-linked oligosaccharides from each of the three linkage sites of oLH.
This study has revealed that each glycan chain has a distinct spectrum of oligosaccharide structures, ranging from monosulphated hybrid-type to disulphated diantennary complex-type. The results suggest that the N-linked oligosaccharide processing of the α- and β-subunit in oLH is differently regulated.

5.2 Experimental Procedures

5.2.1 Materials

Highly purified ovine LH (NIADDK-oLH-26-AFP-5551B) was provided by the National Hormone and Pituitary Program (National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Maryland, U.S.A.). All of the chromatographic materials, packed HPLC columns and chemicals used were the same as those described in Chapter 4.

5.2.2 Isolation of Subunits of oLH

Ovine LH (80 mg) was dissolved in 0.1 M NaH₂PO₄ buffer, pH 7.0 (8 ml) that contained 6 M guanidine HCl and 0.02% sodium azide, and was incubated for 20 h at 47°C. Dissociated subunits were separated at pH 6.5 by reverse-phase HPLC on a Vydac C₄ column (1 x 25 cm) using the same elution conditions as those employed for the isolation of the subunits of hLH (Chapter 4). Twenty mg of the treated sample was applied to the column and two major peak fractions were collected that contained these subunits; these were desalted on a Sephadex G-25 column (1.6 x 55 cm) in 1% NH₄HCO₃, freeze-dried and stored at -20°C until required. Each subunit was identified by amino acid analysis and its purity was examined by reverse-phase HPLC and SDS-polyacrylamide electrophoresis as described in Chapter 3.
5.2.3 Tryptic Digestion and Separation of Glycopeptides of the α-Subunit of oLH

The α-subunit of oLH was reduced and carboxymethylated prior to tryptic digestion as follows: α-subunit (34 mg) was dissolved in reducing buffer (6 ml, 6M guanidine HCl, 1 M Tris-HCl, 1 mM EDTA, pH 8.2), dithiothreitol (30 mg) was added before the vessel was purged with N₂ and incubated for 4 h at 37°C. After the sample had cooled to room temperature, iodoacetic acid (90 mg) in 0.5 N NaOH (0.9 ml) was added dropwise with stirring and the reaction was allowed to continue for 30 min in the dark at room temperature. After 30 min, β-mercaptoethanol (80 μl) was added to react with any residual iodoacetic acid, the alkylation products were desalted on a column of Sephadex G-25 (1.6 x 55 cm) in 1% NH₄HCO₃ and freeze-dried.

The reduced and carboxymethylated oLHα (RCM-oLHα) was dissolved in 0.2 M NH₄HCO₃ (6 ml) and digested with TPCK-treated trypsin (600 μg) in 1 mM HCl (60 μl) (2% w/w of substrate) for 2 h at 37°C. The digestion mixture was chromatographed in water on Sephadex G-25 (1.6 x 55 cm) to remove small peptides and the preparation was freeze-dried after concentration by rotary-evaporation.

The tryptic digests of RCM-oLHα dissolved in water (6 ml) were applied in aliquots to a Vydac C₄ column (1 x 25 cm) and eluted using a linear gradient of acetonitrile in 0.1 M TEAP, pH 6.5 (mobile phase A; 0.1 M TEAP, pH 6.5, B; 0.1 M TEAP in 60% (v/v) acetonitrile, pH 6.5). A 60 min-linear gradient from 5-65%B was used at a flow-rate of 1.2 ml/min. Peak fractions were collected and analysed by ¹H-NMR to locate the glycopeptides and to check the homogeneity of their peptide components, after
desalting in water on a Sephadex G-15 column (1.6 x 45 cm).

The glycopeptide mixture was further separated on a Vydac C\textsubscript{4} column (1 x 25 cm) using a linear gradient of acetonitrile in 0.1% (v/v) TFA (mobile phase A; 0.1% TFA, B; 0.1% TFA in 60% (v/v) acetonitrile). A 60 min-linear gradient from 5-65% B was used at a flow-rate of 1.2 ml/min. Peak fractions were collected, neutralized immediately with 10% NH\textsubscript{4}HCO\textsubscript{3} and desalted before analysis by \textsuperscript{1}H-NMR as described above. Glycopeptides were identified by N-terminal amino acid sequence analysis.

5.2.4 Liberation of N-Linked Oligosaccharides from Glycopeptides of the α and β Subunits of oLH

The two α glycopeptides, αGPI (5.9 mg) and αGPII (6.5 mg) that contained glycosylated Asn 56 and Asn 82, respectively, and the β-subunit (25 mg) were subjected to hydrazinolysis at 100°C for 10 h. After hydrazinolysis, each sample was re-N-acetylated and purified by cation-exchange chromatography on AG50W x 8 in water. Oligosaccharides were then reduced with NaBH\textsubscript{4} (10 mg) in 0.2 M sodium borate buffer, pH 9.8 (6 ml), and purified following the procedures described previously (Chapter 4).

5.2.5 Separation of Oligosaccharides by HPLC

The reduced oligosaccharide mixture obtained from each sample was separated by anion-exchange and ion-suppression amine adsorption HPLC on a MicroPak AX-5 column (0.4 x 30 cm) under the following elution conditions.

Anion-exchange HPLC: Buffer A; 3 mM KH\textsubscript{2}PO\textsubscript{4}, pH 4.0: Buffer B; 300 mM KH\textsubscript{2}PO\textsubscript{4}, pH 4.0. Linear gradient; 0-20% B (0-12 min) and
isocratic at 20% B: Flow-rate; 0.5 ml/min.
Amine-adsorption HPLC: Buffer A; 1% (v/v) TEAP in 70% (v/v) acetonitrile, pH 4.0: B; 1% (v/v) TEAP, pH 4.0. Linear gradient 0-20% B (0-12 min) and isocratic at 20% B: Flow-rate; 0.5 ml/min.

The eluant was monitored at 206 nm. Peak fractions were collected, desalted on Sephadex G-15 in water and freeze-dried before analysis.

5.2.6 Quantitative Analysis of Oligosaccharides

A small fraction (3%) of total oligosaccharides obtained from each sample was radiolabelled by reduction with NaB[3H]₄ (190-380 μCi) in 0.2 M sodium borate buffer, pH 9.8 and purified as described previously (Chapter 4). The yields of radioactive oligosaccharides were \(1.83 \times 10^6\) cpm(α Asn 56), \(1.80 \times 10^6\) cpm (α Asn 82) and \(1.12 \times 10^6\) cpm (β Asn 13).

\(^3\text{H}\)-labelled oligosaccharide alditols (1.2-1.8 \times 10^6\) cpm) were fractionated by anion-exchange and amine-adsorption HPLC under the conditions used for preparative separations. Fractions (300 μl) were collected. Each fraction was diluted to 1300 μl with water and radioactivity was measured by liquid scintillation spectrometry. The relative amount of each oligosaccharide was calculated on the basis of recovered radioactivity. In the case of mixtures, the ratio of component oligosaccharides was estimated by integration of \(^1\text{H}-\text{NMR}\) spectra after assignment of characteristic signals to individual structures.
Fig. 5.1 Preparative separation of oLHα and oLHβ by reverse-phase HPLC.

Ovine LH (80 mg) was pretreated for dissociation as described in Experimental Procedures. Portions (20 mg) of the dissociated hormones were applied to a Vydac C₄ column (1 x 25 cm) and eluted with a 60 min linear gradient from 12-60% acetonitrile in 0.1 M TEAP, pH 6.5 at 1.2 ml/min. Mobile phases: A, 0.1 M TEAP; B, 0.1 M TEAP in 60% acetonitrile. The two subunit peak fractions were collected as indicated.
Fig. 5.2 Analytical reverse-phase HPLC of purified oLHα and oLHβ.

Each subunit preparation (10 μg) was applied to a Vydac C4 (0.46 x 15 cm) column and eluted using the same conditions as in Fig. 5.1 except that the flow-rate was 0.8 ml/min.
5.2.7 $^1$H-NMR Spectroscopy

Oligosaccharide alditols obtained at various stages of purification were analysed by $^1$H-NMR spectroscopy using the same experimental procedures described previously (Chapter 4).

5.3 Results

5.3.1 Isolation of the Subunits of oLH

Preliminary experiments indicated that the HPLC method used for the separation of hLH subunits could also be applied to the effective isolation of intact oLH and its subunits, but the dissociation conditions were only partially successful. Preparative isolation of the two subunits of oLH was achieved by reverse-phase HPLC following dissociation with 6 M guanidine HCl (pH 7.0) at 47°C, 20 h (Fig.5.1). Two major peaks with retention times of about 30 and 42 min were identified as the α- and β-subunits respectively, from their amino acid compositions (Table 5.1). The small peak that was eluted at 35 min corresponded in retention time to that of intact oLH, which suggested that a fraction of treated hormone remained undissociated. When each subunit was analysed by reverse-phase HPLC at pH 6.5, no cross-contamination was detected in either subunit, although both α- and β-subunits were eluted in more than single peaks (Fig.5.2). Because these small peaks were not observed in hormone preparations analysed under the same conditions, they were considered to represent heterogeneous forms of each subunit, that differed in part of their polypeptide and/or carbohydrate moieties. Similar peak heterogeneity observed with hCG subunits on reverse-phase HPLC has been attributed to reversible oxidation of methionine.
Table 5.1 Amino acid compositions of the $\alpha$ and $\beta$ subunits of OLH.

<table>
<thead>
<tr>
<th></th>
<th>OLH-$\alpha$</th>
<th>OLH-$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol/mol protein</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>9.7(10)</td>
<td>2.2( 2)</td>
</tr>
<tr>
<td>His</td>
<td>2.4( 3)</td>
<td>2.1( 3)</td>
</tr>
<tr>
<td>Arg</td>
<td>3.1( 3)</td>
<td>8.6( 8)</td>
</tr>
<tr>
<td>Asx</td>
<td>5.6( 6)</td>
<td>5.5( 5)</td>
</tr>
<tr>
<td>Thr</td>
<td>9.1( 9)</td>
<td>7.4( 7)</td>
</tr>
<tr>
<td>Ser</td>
<td>6.9( 6)</td>
<td>8.3( 8)</td>
</tr>
<tr>
<td>Glx</td>
<td>8.3( 8)</td>
<td>6.7( 6)</td>
</tr>
<tr>
<td>Pro</td>
<td>7.0( 7)</td>
<td>20.0(20)</td>
</tr>
<tr>
<td>Gly</td>
<td>3.3( 4)</td>
<td>7.7( 7)</td>
</tr>
<tr>
<td>Ala</td>
<td>7.7( 7)</td>
<td>8.1( 8)</td>
</tr>
<tr>
<td>Val</td>
<td>5.6( 5)</td>
<td>8.3( 8)</td>
</tr>
<tr>
<td>Met</td>
<td>2.8( 4)</td>
<td>2.3( 3)</td>
</tr>
<tr>
<td>Ile</td>
<td>2.3( 2)</td>
<td>4.0( 5)</td>
</tr>
<tr>
<td>Leu</td>
<td>3.0( 2)</td>
<td>11.8(12)</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.7( 5)</td>
<td>1.5( 2)</td>
</tr>
<tr>
<td>Phe</td>
<td>3.6( 5)</td>
<td>3.3( 3)</td>
</tr>
</tbody>
</table>

a Amino acid compositions are normalised to 7.0 mol and 20.0 mol of proline for OLH$\alpha$- and $\beta$-subunit, respectively. Average of 2 analyses.

b Theoretical values from the sequences for OLH$\alpha$- and $\beta$-subunit (Pierce & Parsons, 1981) are given in parentheses.
Fig. 5.3 Preparative separation of tryptic glycopeptides from oLHα by reverse-phase HPLC.

(a) Portions (5 mg) of the tryptic digests of RCM-oLHα were applied to a Vydac C4 column (1 x 25 cm) and eluted with a 60 min linear gradient of acetonitrile (3-39%) in 0.1 M TEAP, pH 6.5 at 1.2 ml/min.

(b) The glycopeptide fraction indicated in (a) was applied to a Vydac C4 column (1 x 25 cm) and eluted with a 60 min linear gradient of acetonitrile (3-39%) in 0.1% TFA at 1.2 ml/min. The two major peak fractions were collected as indicated.
residues (Pollak et al., 1990). Subunit preparations were also analysed by SDS-PAGE which revealed a single broad band for each subunit with close mobility. Therefore it was impossible to assess any cross-contamination by this procedure. The subunit yields from 80 mg of oLH were: α 34 mg (85%) and β 25 mg (62.5%).

5.3.2 Separation of Glycopeptides of oLH α-Subunit

Tryptic digests of reduced and carboxymethylated α-subunit were first fractionated on a Vydac C4 column in 0.1 M TEAP-acetonitrile system, pH 6.5 (Fig.5.3a). When peak fractions were analysed by 1H-NMR, only those indicated in the figure were found to contain glycopeptides, which were virtually free of contaminating peptides. It was evident that the major glycopeptide fraction contained the two glycosylation sites of the α-subunit (Asn 56 and Asn 82), from the distinguishable chemical shifts for Asn-linked GlcNAc H-1 and NAc. However, it was not clear whether or not the glycopeptides contained separate sites. The combined glycopeptide fraction was effectively separated into two major peaks with the 0.1% TFA-acetonitrile system (Fig.5.3b). The first peak was identified as α (80-95) that contained N-glycosylated Asn 82 (αGPII), from its partial N-terminal sequence: Val-Glu-(Asn)-His-Thr and the presence of 2 Thr, 3 His and 2 Tyr residues determined by 1H-NMR. The second peak was identified as α(56-67) that contained the glycosylated Asn 56 (αGPI), from its sequence: (Asn)-Ile-Thr-Ser-Glu-Ala-Thr-Cys-Cys-Val-Ala-Lys-COOH. 1H-NMR analysis also revealed no significant cross-contamination in either glycopeptide fraction. The peptide moiety of αGPI was homogeneous, however αGPII showed some degree of heterogeneity.
Fig.5.4 Anion-exchange HPLC of radiolabelled oligosaccharides from each glycosylation site of oLH.

$^3$H-Labelled oligosaccharides from Asn 56 and Asn 82 of oLHa (a and b, respectively) and from Asn 13 of oLHβ (c) were analysed by anion-exchange HPLC at pH 4.0 as described in Experimental Procedures. Buffers: A, 3 mM KH$_2$PO$_4$ (pH 4.0); B, 300 mM KH$_2$PO$_4$ (pH 4.0). The oligosaccharides were eluted with the gradient indicated by the broken line in (c) and fractions of 300 µl were collected. Peak fractions were characterised as indicated: S1, monosulphated; SN, sulphated/sialylated; S2, disulphated oligosaccharides.
at the C-terminal end of the peptide chain, which was considered to be responsible for the peak-splitting found on HPLC. The yields of glycopeptides from 34 mg oLH-α were 5.9 mg (76%) for αGPI and 6.5 mg (74%) for αGPII.

5.3.3 Characterisation of Charge Heterogeneity of N-Glycans of oLH

The charge distributions of oligosaccharides at the individual glycosylation sites on oLH were examined by anion-exchange HPLC at pH 4.0, using ³H-labelled oligosaccharides obtained from glycopeptides αGPI and αGPII and from the β-subunit. Fig.5.4 shows the elution profiles obtained for oligosaccharides from each sample, under the conditions described in Experimental Procedures. The linear gradient programme previously used for the separation of hLH oligosaccharides (Chapter 4) was slightly modified, because the oLH preparation (oLH-26) did not contain any tri-charged oligosaccharides (Chapter 3). All acidic oligosaccharides were eluted under isocratic conditions with 60 mM phosphate buffer, pH 4.0 (20% Buffer B) which resolved the di-charged population into two components, sulphated/sialylated (SN) and disulphated (S2) oligosaccharides, as indicated in the figure. The mono-charged fractions from all three sites contained exclusively monosulphated (S1) oligosaccharides. The types of anionic moieties in S1, SN and S2 fractions were characterised by ³H-NMR analyses of the corresponding fractions obtained from preparative separations under identical chromatographic conditions (described later). The overall recovery of labelled oligosaccharides was >95%.
Fig. 5.5 Relative amounts of neutral, mono- and di-charged oligosaccharides at each glycosylation site of oLH.

The percentage of the total oligosaccharides represented by neutral (A0), mono- (A1) and di- (A2) charged species is indicated for each site. Mono-charged populations were exclusively monosulphated (S1) oligosaccharides and di-charged populations were composed of sulphated/sialylated (SN) and disulphated (S2) oligosaccharides.
Fig. 5.6 Distribution of anionic charges derived from the three N-glycans on oLH.

The relative proportions of various anionic charges calculated from the charge distribution of oligosaccharides at each glycosylation site is presented as a percentage.
The relative amounts of neutral, mono- and di-charged oligosaccharides at each glycosylation site of oLH, calculated from the radioactive content of respective fractions, are shown in Fig. 5.5. The results revealed that there were significantly different charge distributions between the two subunits, with monosulphated oligosaccharides predominant in the α-subunit and disulphated oligosaccharides in the β-subunit. However, the distribution patterns for the two glycosylation sites on the α-subunit were similar. The results for the α- and β-subunits were consistent with those reported for the oLH preparation (oLH-24) from the same source (Green & Baenziger, 1988a,b).

To assess the contribution of oligosaccharides to the charge heterogeneity of oLH, the anionic charges that can arise from the three N-glycans were estimated from the data in Fig. 5.5 assuming random combinations. The results are shown in Fig. 5.6, which indicates that most of oLH contained 2-5 anionic moieties, exclusively sulphate. It is suggested that the sulphated oligosaccharides contributed to the overall charge of oLH within the range of 3, in terms of net negative charge.

5.3.4 Preparative Separations of N-linked Oligosaccharides

After cleavage of the N-linked oligosaccharides from glycopeptides αGPI and αGPII and from the intact β-subunit by hydrazinolysis, followed by re-N-acetylation and reduction, the resulting oligosaccharide alditols were fractionated by anion-exchange HPLC under elution conditions identical with those used for the analytical separations (Fig. 5.7). Five fractions were collected from each sample and they were subjected to $^1$H-NMR
Fig. 5.7 Preparative anion-exchange HPLC of oligosaccharide alditols from each glycosylation site of oLH.

Oligosaccharide samples obtained from oLH αGPI (a), αGPII (b), and oLHβ (c) were applied to a MicroPak AX-5 column and eluted with a gradient of 0-20% B as indicated by the broken line in (c), using buffer A (3 mM KH₂PO₄, pH 4.0) and buffer B (300 mM KH₂PO₄, pH 4.0) at 0.5 ml/min. Five peak fractions were collected as indicated.
Fig. 5.8 Preparative ion-suppression amine-adsorption HPLC of major anion-exchange fractions from oLH αGPI and αGPII.

Fractions A₁-3 (αGPI) and A₂-3 (αGPII) (a and b, respectively) were applied to a MicroPak AX-5 column and eluted by a linearly decreasing concentration of acetonitrile (70-56%, v/v) in 1% (v/v) TEAP, pH 4.0. Buffers, A, 1% TEAP in 70% acetonitrile (pH 4.0); B, 1% TEAP (pH 4.0). Gradient, 0-20% B in 12 min, then isocratic (20% B); flow-rate, 0.5 ml/min. Three fractions were collected from each sample as indicated.
analysis, which together with the elution positions, indicated the presence of neutral, monosialylated (N1), monosulphated (S1), sulphated/sialylated (SN), and disulphated (S2) oligosaccharides in fractions 1, 2, 3, 4 and 5, respectively.

By this single separation step, oligosaccharides in all fractions except neutral and S1 were purified to homogeneity or near homogeneity. The major fractions from αGPI and αGPII (A₁-3 and A₂-3, respectively) were too heterogeneous for direct analysis by ¹H-NMR and were further separated by ion-suppression amine-adsorption HPLC, which yielded three subfractions A₁-3a – A₁-3c and A₂-3a – A₂-3c, respectively (Fig.5.8).

5.3.5 Structural Analysis of OLE N-Glycans by ¹H-NMR

The above separation procedures yielded a total of 7 distinct fractions of reduced oligosaccharides from each glycosylation site. These fractions were examined by 400 MHz ¹H-NMR spectroscopy to determine the complete primary structures of constituent oligosaccharides. Structural analysis of neutral oligosaccharides, which accounted for 13%, 16% and 4% of the total oligosaccharides at αAsn 56, αAsn 82 and βAsn 13, respectively, was not possible due to the paucity of samples and severe heterogeneity.

The ¹H-NMR spectra of all fractions examined in this study showed the typical features of N-glycosidically linked oligosaccharides. In addition, the set of H-1 and H-2 signals of the three core mannose residues indicated the presence of diantennary complex-type oligosaccharides in SN and S2 fractions and of either hybrid-type or mono- or diantennary complex-type oligosaccharides in the subfractions obtained from S1 fractions.
Fig. 5.9 400 MHz $^1$H-NMR spectrum of S2 type oligosaccharides derived from Asn 13 of oLHβ.

Sample: fraction B-5 (oLHβ). Labelling of sugar residues refers to structure at the top of the figure. N-acetyl (Nac) and Fuc CH$_3$ proton signals are expanded.
as described below.

1. **S2 type oligosaccharides**

The major portion of oligosaccharides from the β-subunit was eluted in the S2 fraction on anion-exchange HPLC and the $^1$H-NMR spectrum of this fraction (fraction B-5 in Fig.5.7c) is shown in Fig.5.9. Most assignments of the proton signals are based on 2D COSY (correlated spectroscopy) and 2D NOE (nuclear overhauser enhancement) spectroscopy (Weisshaar et al., 1990) and the chemical shifts are given in Table 5.2. The spectrum shows the typical features of diantennary N-linked oligosaccharides; the presence of the reduced pentasaccharide core structure is evident from a set of characteristic chemical shifts of GlcNAc-ol H-2 ($\delta = 4.215$) and NAc ($\delta = 2.056$), GlcNAc2 H-1 ($\delta = 4.715$) and NAc ($\delta = 2.097$), and three Man H-1 signals, at $\delta = 5.110$ (Man4), $\delta = 4.918$ (Man4') and $\delta = 4.769$ (Man3) (Paz-Parente et al., 1982) (the numbering of monosaccharides is indicated in Fig.5.9). The pair of chemical shifts of GlcNAc2 also suggests that a fucose residue is attached in α1-6 linkage to the proximal GlcNAc1 (Cahour et al., 1984). The set of Man H-2 signals at $\delta = 4.247$ (Man3), $\delta = 4.178$ (Man4) and $\delta = 4.104$ (Man4') is indicative of the diantennary type of branching (Geyer et al., 1984); i.e. both Man4 and Man4' residues are substituted at the C-2 position by another monosaccharide in β-linkage, which is normally a β1-2 linked GlcNAc in N-acetyllactosamine-type structures. The spectrum shows additional anomeric proton signals in the region $\delta = 4.5-4.6$, all doublets with $J_{1,2}$ coupling constants (~8 Hz), indicating the β-configuration. The doublet signals at $\delta = 4.558$ were ascribed to
Table 5.2  Proton chemical shifts of oligosaccharide alditols isolated from oLH.

Chemical shifts (δ) were measured at 400 MHz in ²H₂O at 298 K with acetone as internal reference (δ = 2.225 ppm). Structures on top of the table are given in shorthand notation: 1, 2, 5, 5', GlcNAc; 3, 4, 4', A, B, Man; GN, GN', GalNAc (6, 6'); G', Gal (6'). Values in parentheses refer to the respective structure without fucose; n.d., not determined.

a, Measured at 303 K.  b, Tentative assignment.
<table>
<thead>
<tr>
<th>Protons</th>
<th>Residue</th>
<th>SO(_4)</th>
<th>SO(_4)</th>
<th>GcNAc2</th>
<th>GcNAc2</th>
<th>Man3</th>
<th>Man3</th>
<th>Man4</th>
<th>Man4</th>
<th>Man4*</th>
<th>ManA</th>
<th>GlcNAc5</th>
<th>GlcNAc5</th>
<th>GalNAc8</th>
<th>GalNAc8*</th>
<th>Gal6'</th>
<th>Fuc</th>
<th>ppm</th>
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</table>
the H-1 of GlcNAc5 and 5'β1-2 linked to Man4 and 4', respectively, from 2D COSY experiments, which revealed that the H-2 resonates at the expected position for 4-substituted GlcNAc. The other two anomeric signals ($\delta = 4.586$, $\delta = 4.590$) were ascribed to GalNAc residues from the chemical shifts of their H-2 and coupling patterns of their H-3 ($J_{3,4} = 2.5$ Hz) and H-4 ($J_{4,5} < 1$ Hz), that indicated the galactose configuration (H-3 axial, H-4 equatorial). The occurrence of the characteristic downfield shift of GalNAc H-4 ($\delta = 4.692$ vs. $\delta \sim 3.9$ for unsubstituted GalNAc) (Vandana et al., 1987) indicated that both GalNAc6 and 6' residues are each substituted in the C-4 by a sulphate group.

The occurrence of six N-acetyl methyl singlets in the region of $\delta = 2.0 - 2.1$ indicated the presence of six N-acetylated amino sugars. The N-acetyl signals of the peripheral GlcNAc and GalNAc residues were observed at $\delta = 2.040$ (GlcNAc5'), $\delta = 2.046$ (GlcNAc5), $\delta = 2.069$ (GalNAc6) and $\delta = 2.076$ (GalNAc6').

The disulphated diantennary structure (denoted S2), inferred from the above spectral data, is shown in Fig.5.9. The S2 oligosaccharide was the major component of the N-glycan at βAsn 13 in the β subunit. It was also identified in lesser amounts at both glycosylation sites of the α-subunit, however, with a significantly different content of Fuc α1-6 linked to GlcNAc1 (approximately 15% at Asn 56 and 50% at Asn 82).

2. **S1 hybrid-type oligosaccharides**

For both glycosylation sites on oLHa, the major portion of oligosaccharides was eluted in the anion-exchange fractions A1-3 and A2-3, at a position expected for monosulphated N-glycans.
Fig. 5.10 400 MHz $^1$H-NMR spectrum of Sl hybrid-type oligosaccharides (Sl-AB) derived from Asn 56 of oLHa.

Sample: fraction A$_1$-3c (oGPI). Labelling of sugar residues refers to structure at the top of figure. N-acetyl (NAc) signals are expanded. The NAc signal marked by * probably has to be ascribed to GlcNAc2 in fucosylated Sl-AB (approximately 15% fucose $\alpha$1-6 linked to GlcNAc1).
Three subfractions were obtained from each of these fractions by amine-adsorption HPLC (a-c, Fig.5.8).

The $^1$H-NMR spectrum of fraction $A_1-3c$, derived from $\alpha$GPI (Asn 56), is shown in Fig.5.10, and chemical shift data are included in Table 5.2. The presence of a hybrid-type structure with a reduced, nonfucosylated N,N'-diacetylichitobiose unit is readily inferred from the typical H-1 and H-2 shifts of five Man residues (3, 4, 4', A and B, see Fig.5.10 for denotation) and from the characteristic signals for GlcNAc-ol (H-2, NAc) and GlcNAc2 (H-1, NAc). The chemical shifts for Man residues were essentially the same as those reported for structurally related hybrid-type oligosaccharide alditols (Mutsaers et al., 1985). In addition, the spectrum showed characteristic signals virtually identical with those found for the sequence $SO_4$-4GalNAcβ1-4GlcNAcβ1-2Manα1-3Man in $S_2$ oligosaccharide (Table 5.2). The N-acetyl signals of the peripheral GlcNAc5 and GalNAc6 residues were observed at $\delta = 2.046$ and $\delta = 2.070$.

The monosulphated hybrid structure (denoted $S_1-AB$), which is consistent with these spectral data, is shown in Fig.5.10. This structure was confirmed by 2D COSY (Weisshaar et al., 1990). $S_1-AB$ oligosaccharide(s) was one of the two major components at $\alpha$Asn 56 (23%), however it was a minor component at $\alpha$Asn 82 (3%).

A related hybrid structure, without mannose B (denoted $S_1-A$), was identified in the major subfractions $A_1-3b$ and $A_2-3b$ and it was found to be the predominant oligosaccharide at both glycosylation sites of oLHα (27% at Asn 56 and 35% at Asn 82); chemical shift data are included in Table 5.2. The content of fucose $\alpha1-6$ linked to GlcNAc1 in $S_1-A$ was markedly different
between the two sites, ~10% at Asn 56 and 50% at Asn 82. Only small quantities of a corresponding monosulphated hybrid structure with a terminal Man\(\alpha1-6\)Man\(4'\) (\textit{ManB}) were detected in fractions A\(1-3a\) and A\(2-3a\). No hybrid structures were found in a fraction B-3 (oLH\(\beta\)).

3. S1 complex-type oligosaccharides

Significant amounts of monosulphated complex-type oligosaccharides were also present in fractions A\(1-3a\), A\(2-3a\) and B-3. All structures contained the Man\(\alpha1-3\) branch with the sequence SO\(_4^-\)4GalNac\(\beta1-4\)GlcNac\(\beta1-2\)Man, as was evident from the highly conserved, characteristic signals of GalNac6 H-1 and NAc, GlcNac5 H-1 and NAc, and Man4 H-1 (Table 5.2). However, these monosulphated structures contained distinct Man\(\alpha1-6\) branch structures; unsubstituted terminal Man\(4'\) (denoted S1\(-4'\), chemical shifts included in Table 5.2) was found in various amounts (5-8%) at all three glycosylation sites. The presence of non-reducing terminal GlcNac5\(\beta1-2\) linked to Man\(4'\) (denoted S1\(-5'\)) was also suggested in fractions A\(2-3b\) and B-3, from the occurrence of an NAc signal at \(\delta = 2.052\) (Vliegenthart et al., 1983), however relatively low amounts and heterogeneity of these fractions did not permit definitive identification. A monosulphated oligosaccharide with a terminal Gal6\(\^\) (denoted S1\(-6'\)) was also found in fractions A\(2-3c\) (9%) and B-3 (11%), as inferred from the characteristic shifts including Gal6\(\^\) H-1 (Table 5.2). S1\(-6'\) is structurally related to the sulphated/sialylated diantennary oligosaccharide (SN), which was eluted in fractions A\(1-4\), A\(2-4\) and B-4. Although SN was present in minute amounts in oLH (3-6%), it could be identified by
comparison of the $^1$H-NMR spectrum with that of the same compound isolated from hLH.

Traces (3-4%) of a monosialylated N-acetyllactosamine-type oligosaccharide were eluted in fractions A$_1$-2, A$_2$-2 and B-2 (Fig.5.7), partially separated from monosulphated fractions. It contained sialic acid α2-3 linked to Gal6 (α1-3 branch) and terminal Gal5' (α1-6 branch).

The structures of S2 and S1-AB, as well as the presence of sulphate groups in these oligosaccharides have recently been confirmed by fast-atom-bombardment mass spectrometry (Dell et al., 1991). The oligosaccharides identified at each of the three glycosylation sites of oLH and their distributions are summarised in Table 5.3.

5.4 Discussion

The preparative procedures for the isolation of the individual N-glycan chains from ovine LH were basically the same as those employed for hLH, except that two reverse-phase separations were required to prepare purified glycopeptides from oLH α-subunit. The glycopeptide mixtures isolated from the tryptic digests of RCM-oLHα in the first RP-HPLC with 0.1 M TEAP, pH 6.5, were almost completely separated into the two glycopeptide fractions on the basis of peptide by the second RP-HPLC using 0.1% TFA. These RP-HPLC procedures were effective in achieving the expected separations of each glycopeptide in high purity as well as in good yield (~75%). In contrast to the case of hLH, most of the anionic oligosaccharides present at each site of oLH (>93%) contained only one or two sulphate groups and their relatively low heterogeneity facilitated the purification
Table 5.3 Oligosaccharide structures of the N-glycans of oLH.

Relative abundances are given in % of total oligosaccharides at each glycosylation site. The main structure shown below is common to all compounds, with Manα1-6 being substituted by the sequences listed as R. The extent of core-fucosylation of structures was within the following ranges: 10-15% at Asn 56, 45-55% at Asn 82, and 85-95% at Asn 13. n.d., not determined. a, structure not confirmed.

<table>
<thead>
<tr>
<th>R-Manα1</th>
<th>Fucα1</th>
</tr>
</thead>
<tbody>
<tr>
<td>/</td>
<td>\</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Manβ1-4GlcNAcβ1-4GlcNAc</td>
<td></td>
</tr>
<tr>
<td>3 \</td>
<td>/</td>
</tr>
</tbody>
</table>

SO₄⁻⁴GalNAcβ1-4GlcNAcβ1-2Manα1

<table>
<thead>
<tr>
<th>R</th>
<th>α subunit</th>
<th>β subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asn 56</td>
<td>Asn 82</td>
</tr>
<tr>
<td>SO₄⁻⁴GalNAcβ1-4GlcNAcβ1-2</td>
<td>(S2)</td>
<td>16</td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2</td>
<td>(SN)</td>
<td>4</td>
</tr>
<tr>
<td>Galβ1-4GlcNAcβ1-2</td>
<td>(S1-6')</td>
<td>n.d.</td>
</tr>
<tr>
<td>GlcNAcβ1-2</td>
<td>(S1-5')ᵃ</td>
<td>n.d.</td>
</tr>
<tr>
<td>H</td>
<td>(S1-4')</td>
<td>6</td>
</tr>
<tr>
<td>[Manα1-6]Manα1-3</td>
<td>(S1-AB)</td>
<td>23</td>
</tr>
<tr>
<td>Manα1-3</td>
<td>(S1-A)</td>
<td>27</td>
</tr>
<tr>
<td>Neutral oligosaccharides</td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

ᵃ Structure not confirmed.
of oligosaccharides by anion-exchange and amine-adsorption HPLC prior to structural characterisation. In particular, several major sulphated oligosaccharides in oLH that included monosulphated hybrid-type and disulphated diantennary complex-type structures, were purified in quantities sufficient to allow the detailed structural analysis of these new classes of oligosaccharides by $^1$H-NMR and fast-atom-bombardment mass spectrometry (Weisshaar et al., 1990; Dell et al., 1991). This was indispensable for the characterisation of various structurally related sulphated oligosaccharides that were present in much lower amounts in hLH.

Oligosaccharides from each glycosylation site of oLH were characterised by anion-exchange HPLC to assess their contributions to the overall charge heterogeneity of this hormone. Four species, i.e. neutral, monosulphated, sulphated/sialylated, and disulphated oligosaccharides, were obtained from each of the three sites. However, their distinct distribution patterns (Fig.5.5) indicated that oligosaccharides on the β-subunit of oLH contained, on average, more negatively charged groups than those on oLHα. These data suggested that the glycosylation of Asn 13 (β-subunit) contributes more to the charge heterogeneity of oLH than the other sites on the α-subunit.

If one assumes no peptide heterogeneity and that the glycosylations of the three sites are independent of each other, charge heterogeneity of oLH can be correlated with the distribution of anionic charges arising from random combination of the three N-glycan chains. The number of sulphate groups on oLH molecules estimated on these assumptions ranged from 2-5
(Fig.5.6), and the large portion of oLH (~80%) was estimated to contain 2-4 sulphate groups, which appeared to correspond well with the relative distribution of oLH isoforms in the pI range of 8.0-9.8 observed for the pituitary extracts by chromatofocusing (Keel & Grotjan, 1990). However, the presence of four major isohormones with slightly different pIs in this range and the presence of significant amounts of more acidic species (pI <7.0) suggested that the pI of a given oLH isohormone cannot be explained simply by the number of sulphate residues and that other factors such as peptide heterogeneity must also be considered. In addition, sulphation of oligosaccharides at the three sites on the individual oLH molecules may not be a random process. It remains unclear how the glycosylation patterns observed at the three sites are related to each other, although it is also important to understand the structural basis for charge heterogeneity.

\(^{1}\text{H-NMR} \text{ analysis has shown that the N-linked oligosaccharides present on oLH are mainly sulphated hybrid- and diantennary complex-type (Table 5.3).} \text{ Most of the oligosaccharide structures previously reported for oLH (Green \& Baenziger, 1988a,b) were confirmed by \(^{1}\text{H-NMR, however two monosulphated structures, S-1 (C) and S-1 (F) in the above reference, were not detected at any site in the present study. It was also noted that the relative contents of the neutral and three most abundant oligosaccharides were quite different, although the hormone used in both studies was from the same source; for example, the contents of S2, S1-A and S1-AB, which accounted for 28, 21 and 9% respectively, of the total oligosaccharides on oLH in the present study, had been reported}
to be 13, 9 and 27%. Although these results may in part reflect possible variation in charge heterogeneity between different batches of hormone preparation, the reason for the large differences is not clear.

The present study reveals that each of the three glycosylation sites on oLH has a distinct oligosaccharide population (Table 5.3). The prevalent oligosaccharides at the two sites of the α-subunit were monosulphated hybrid-type, S1-AB and S1-A at Asn 56 and S1-A at Asn 82. On the other hand, sulphated complex-type, mostly diantennary (S2) chains were predominant at Asn 13 of the β-subunit. In addition, another significant difference between sites was observed in the extent of core-fucosylation, which was almost complete in the β-subunit but was only partial at the two sites of the α-subunit. These results suggested that the oligosaccharide processing of the α- and β-subunits in oLH was differently regulated in at least two stages (discussed below).

Various hybrid- and complex-type oligosaccharides are present in oLH, however they all have the common structural element, the core Man$_3$GlcNAc$_2$ with Man$_\alpha$1-3 extended by the sequence SO$_4$-4GalNAcβ1-4GlcNAcβ1-2. Therefore, the structural heterogeneity observed within and between sites is derived exclusively from variation in the peripheral structures in the Man$_\alpha$1-6 branch. Aside from the site-specific hybrid structures, all three sites show similar heterogeneous patterns for complex oligosaccharides, indicating that the processing of this branch is not strictly regulated. This type of site-heterogeneity appears to be associated with the flexible orientation of the Man$_\alpha$1-6 arm in complex oligosaccharides, which permits specific interactions between parts of this arm and proximal GlcNAc
Fig. 5.11 Biosynthetic pathway of N-linked oligosaccharides on ovine LH.

Each of the precursor oligosaccharides (Glc$_3$Man$_5$GlcNAc$_2$) attached to Asn 56, Asn 82(oLHa) and Asn 13(oLHP) is processed to GlcNAc$_1$Man$_5$GlcNAc$_2$ [I] by sequential action of glucosidases, $\alpha$-mannosidase I and GlcNAc transferase I (steps 1-3, respectively). Whereas the two mannosyl residues of the intermediate[I] are removed by $\alpha$-mannosidase II at Asn 13 (step 4), this processing is markedly limited at Asn 56 and Asn 82. This leads to the synthesis of hybrid-type oligosaccharides in the $\alpha$-subunit, while diantennary complex-type oligosaccharides are produced in the $\beta$-subunit. Core-fucosylation (step 6) is also partially limited in the $\alpha$-subunit.
residues (Carver & Brisson, 1984). Such interactions may limit the accessibility of the oligosaccharide chain to glycosyltransferases, resulting in various incomplete structures. On the other hand, site-specific glycosylation is considered to reflect another level of control, which involves the influence of a local polypeptide structure on the oligosaccharide processing of a particular site(s) of a glycoprotein.

Fig. 5.11 shows the biosynthetic pathway for the N-glycan chains of oLH, which is deduced from the structural data reported in this thesis (Table 5.3). Complex and hybrid oligosaccharides, both found in oLH, are synthesized from the common oligosaccharide intermediate, GlcNAc\textsubscript{1}Man\textsubscript{5}GlcNAc\textsubscript{2} (I) (Kornfeld & Kornfeld, 1985). Because Asn 13 of the β-subunit carries predominantly diantennary complex oligosaccharides with core-fucosylation, there appears to be little hindrance to the oligosaccharide processing at this site, which is catalysed by α-mannosidase II (step 4), GlcNAc-transferase II (step 5) and α1-6 fucosyltransferase (step 6). The oligosaccharide intermediate, GlcNAc\textsubscript{2}Man\textsubscript{3}GlcNAc\textsubscript{2} (II) is processed mainly by sequential action of GalNAc-transferase (step 7) and sulphotransferase (step 8) to yield disulphated diantennary complex (S\textsubscript{2}) as the major final product, suggesting that these enzymes are highly active in the ovine pituitary cell. However, Gal- and α2-3 sialyltransferases are also involved at least in the processing of the Manα1-6 branch (in steps 7 and 8, respectively), as indicated by the significant amounts of sulphated/sialylated (SN) and its incomplete structures. These results are consistent with the apparent branch-specificity of GalNAc-transferase observed in hLH (Chapter 4).
In contrast, the relative abundance of monosulphated hybrid oligosaccharides at Asn 56 and Asn 82 in the α-subunit indicates that processing of the oligosaccharide intermediates at both sites by α-mannosidase II (step 4) is markedly affected, whereas the subsequent processing of the Manα1-3 branch is similar to that for the β-subunit. The degree of interference estimated from the percentage of hybrid structure in total anionic oligosaccharide is slightly greater at Asn 56 than at Asn 82 (~60% and 45%, respectively). For these sites, core-fucosylation of oligosaccharides by α1-6 fucosyltransferases (step 6), that occurs at the late stages of processing (Kornfeld & Kornfeld, 1985), is also partially hindered.

There is ample evidence to suggest that the polypeptide structure can influence the extent of oligosaccharide processing (Hsieh et al., 1983; Swiedler et al., 1985; Hubbard, 1988), and site-specific effects on the activity of α-mannosidase II and fucosyltransferase in the α-subunit are probably due to steric hindrance by the local polypeptide structure which limits the accessibility of oligosaccharide chains to these enzymes. In this regard, competition between α-mannosidase II and GalNAC transferase has been suggested to explain the presence of monosulphated hybrid oligosaccharides on bLH (Green et al., 1986a), but it seems less likely from the complete absence of these structures in the oLH β-subunit.

Because association of the α- and β-subunits occurs prior to oligosaccharide processing (Hoshina & Boime, 1982), it may influence the processing of each subunit differently. oFSH has predominantly complex-type (di- and triantennary) oligosaccharides (Green & Baenziger, 1988a,b), even although oLH and oFSH are generally assumed to be synthesized within the same
pituitary cell and share a common α-subunit. This suggests that the oligosaccharide processing of the α-subunit is regulated (or limited) by the association with the hormone-specific β-subunit.

It is of interest that, in contrast to oLH, hLH contained predominantly diantennary complex oligosaccharides in its α-subunit (Chapter 4). This may reflect in part different influences of protein structures on the activity of α-mannosidase II in oLH and hLH, although the two hormones show high structural homologies. Alternatively, the species-specific glycosylation could be explained by different activities and/or substrate-specificity of the processing enzymes in different species (Williams & Lennarz, 1984). However, little is known about the specificity of α-mannosidase II in ovine and human pituitary gonadotrophs. In this regard, oLH and bLH have been shown to have different glycosylation patterns, whereas they have identical protein structures (Green & Baenziger, 1988b). Their results, that oLH contained more hybrid oligosaccharides and less diantennary complex oligosaccharides than bLH, suggested that the activities of processing enzymes, particularly that of α-mannosidase II, are lower in ovine pituitary cells than in bovine cells. However, the results of the present study showed that the overall glycosylation patterns for oLH were rather close to those reported for bLH, which suggested that additional studies are necessary for confirmation of species-specific glycosylation between the two hormones.

The N-glycan chains present on the α-subunit appear to play a predominant role in the signal transduction of oLH (Sairam & Bhargavi, 1985). The presence of structurally unique monosulphated hybrid oligosaccharides in the α-subunit of oLH suggests that these structures may serve an important function.
The structures of the N-glycan chains on the α-subunits in relation to their function are discussed in Chapter 6 in comparison with hLH and hCG.
CHAPTER 6 Structure and Function of N-Glycans in Gonadotrophic Action

A great deal has been learned about the structure and function of the N-glycans of the gonadotrophins in recent years. N-glycan moieties have been shown to be essential for the hormonal stimulation of target cells. Studies with selectively deglycosylated α- and β-subunits have demonstrated that glycosylation of the α-subunit is necessary for coupling receptor-binding to adenylate cyclase activation (Sairam, 1989). Glycosylation of the α-subunit has also been implicated in the hormonal regulation of gonadotrophin secretion and/or biological activity (Krummen and Baldwin, 1988; Wilson et al., 1990) and site-directed mutagenesis has provided an important observation that links the structure and function of N-glycans in gonadotrophins; a single N-glycan on hCGα was found to play a critical role in hormonal function (Matzuk et al., 1989).

Structures of the N-linked glycans on the human, bovine and ovine gonadotrophins have been reported (Renwick et al., 1987; Damm et al., 1987; Green and Baenziger, 1988a,b). In these studies, however, the oligosaccharide structures were not completely defined with respect to glycosylation site, which has made it difficult to assess structure-function relationships. In this chapter, structural aspects of N-glycan function are examined in respect of LH and hCG, based on recently acquired structural data. The potential mechanisms by which N-glycosylation influences the biological activity of gonadotrophins are also discussed in the light of currently available information.
6.1 Structure-Function Relationships of N-Glycans of hCG and LH

Site-specific glycosylation of hCG was first suggested by Mizuochi and Kobata (1980) who found that distinct oligosaccharide structures were systematically distributed between the two subunits of hCG. Kobata (1988) suggested that the monoantennary sialylated oligosaccharide on the α-subunit may be important in the biological activity of this hormone, an opinion based on the earlier observation that the glycopeptides from hCGα specifically inhibited hCG-stimulated adenylate cyclase activity (Calvo and Ryan, 1985). It was speculated that site-specific functions in hCG could be inferred from site-specific structures of N-glycans. In fact, it has recently been demonstrated by Matzuk et al. (1989) that the steroidogenic activity of hCG is regulated by the glycosylation of a specific Asn residue (Asn 52) of the α-subunit.

The structures of each of the four N-glycans on hCG have recently been identified in our laboratory (Weisshaar, Hiyama and Renwick, in press). This study has revealed that the distribution of oligosaccharide structures is not so rigidly site-specific as previously thought (Kobata, 1988). Although the monoantennary complex structure was present exclusively on the α-subunit of hCG, it was distributed between both glycosylation sites of this subunit. The disialylated diantennary complex structure was also found in significant amounts at both sites of the α-subunit and it was noted that three distinct types of structure, sialylated hybrid, monoantennary and diantennary complex, were present at the hCGα Asn 52 in a percent molar ratio of approximately 30:50:20 (Table 6.1). These results seemed to indicate that glycosylation at
Table 6.1: Structures of the oligosaccharide components and their relative abundances at the four N-glycosylation sites of hCG.

<table>
<thead>
<tr>
<th>Structures</th>
<th>hCGα Asn 52</th>
<th>hCGα Asn 78</th>
<th>hCGβ Asn 13</th>
<th>hCGβ Asn 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td></td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td></td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NeuAcα2-3Galβ1-4GlcNAcβ1-2Man</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Approximately 25% and 100% of disialylated dimannosyl oligosaccharides carry a fucose residue α1-6-linked to GlcNAc-1 at Asn 13 and Asn 30 of hCGβ, respectively; no fucose was found in hCGα.
this site is not so tightly regulated as one would expect from its alleged crucial function, although the influence of protein structure on oligosaccharide processing at this site appeared greater than at the other three sites.

These data did not give any clear indication concerning the relationships between structure and function of the N-glycans. Specific oligosaccharide structures may not be required for the biological activity of hCG, although glycosylation of Asn 52 is reported to be essential (Matzuk et al., 1989). In this case, all three types of structures present at Asn 52 of hCG could be assumed to contain the structural element(s) required. The sialylated monoantennary complex oligosaccharide, which predominates at this site, may represent such a common structure (Table 6.1). It is also possible that only the pentasaccharide core moiety is necessary for the N-glycan to be functional, since removal of the three core mannosyl residues by chemical deglycosylation led to an almost complete loss of hCG activity (Sairam, 1989).

Conversely, the specific oligosaccharide structures may be essential for the hormonal function of hCG. In such a case, the α1-6 linked moieties must be responsible for the functional differences, since the structural differences between the oligosaccharides lie only in this part. The major conformational difference between hybrid and diantennary complex oligosaccharides has been shown to exist in the orientation and the flexibility of the α1-6 linked moiety (Brisson and Carver, 1983). It has been reported that glycopeptides or free oligosaccharides derived from hCGα-subunit have a specific inhibitory effect on hCG-stimulated adenylate cyclase, which suggests the involvement of a membrane lectin in the activation
of the receptor-adenylate cyclase system (Calvo and Ryan, 1985). Since hybrid and monoantennary oligosaccharides are present specifically in hCGα and since they both contain terminal α-mannosyl residues, their interactions with a mannose-specific lectin could be suspected. Membrane lectins known to exist in various mammalian tissues and cells are highly specific for sugar residues such as mannose and galactose (Barondes, 1981). Therefore, if such interactions are involved in the mechanism of gonadotrophic action, hCG molecules with a certain type(s) of oligosaccharides will be more biologically active than hCG with other oligosaccharides. Although such a membrane lectin has not been identified in any target cells, a lectin-like sequence in the receptor has recently been reported (McFarland, 1989). It is therefore, important to see if specific oligosaccharides interact with purified receptors.

The role of glycosylation of the α-subunit in adenylate cyclase activation is common to all gonadotrophic hormones (Sairam and Bhargavi, 1985). Since this is a basic and conserved function of N-glycans, the oligosaccharides synthesised at the specific site of the common α-subunits may have structural feature(s) conserved between hormones and across species. This possibility was examined in the cases of human and ovine LH which share the same biological activity as hCG. When the N-glycans of hCG, hLH and oLH were compared in respect of their functionally important glycosylation sites, it was apparent that these three hormones have quite different oligosaccharides in different degrees of heterogeneity (see Tables 4.3, 5.3, 6.1). Significant structural differences that existed between the hormones in the peripheral moieties could be attributed to either incomplete processing or species- and
Table 6.2  Branching structures of N-glycans present at the potentially functional sites of hCG, hLH and oLH
(Asn 52 on hCGα and hLHα, and Asn 56 on oLHα)

<table>
<thead>
<tr>
<th>Branching type</th>
<th>hCG</th>
<th>hLH</th>
<th>oLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>% total oligosaccharides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\ M ±F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>/ M-M-GN-GN</td>
<td>Hybrid</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>/ R-GN-M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M ±F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\ M-GN-GN</td>
<td>Monoantennary complex</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>/ R-GN-M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-GN-M ±F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\ ±GN- M-GN-GN</td>
<td>Diantennary complex</td>
<td>18</td>
<td>54</td>
</tr>
<tr>
<td>/ R-GN-M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M = Man; GN = GlcNAc; F = Fuc; R, R' = variable peripheral sugars
tissue-specific terminal glycosylation (Green and Baenziger, 1988b) or both.

Terminal sulphation of oligosaccharides has been found only in the pituitary-derived glycoprotein hormones and it is also species-specific (Baenziger and Green, 1988). The roles of terminal sulphates on LH and sialic acids on hCG appear to be similar in that they modulate the biological activity of that hormone (Smith et al., 1990). Terminal sialic acids, but not their linkages to galactose residues, are important for full expression of the biological activity of hCG (Amano et al., 1989). However, in contrast to HF-treated hCG which has lost biological activity (Chen et al., 1982; Majunath and Sairam, 1982), neuraminidase-treated hCG retains significant biological activity (Moyle et al., 1975; Amir et al., 1987). These observations suggest that terminal glycosylation is not essential for the function of N-glycans.

When the N-glycans on the α-subunit Asn 52 (or 56 for oLH) of hCG, hLH and oLH were assessed in respect of their branching structures, it was found that each hormone had quite different branching patterns (Table 6.2). Although hybrid, mono- and diantennary complex structures were all present in each hormone, their relative proportions differed markedly; diantennary complex and hybrid oligosaccharides were predominant in hLH and oLH, respectively, while monoantennary complex oligosaccharides were predominant in hCG. There was no apparent relationship between the branching structures of the three hormones, which suggested that specific branching structures of N-glycans on LH and hCG are not essential for their function.

On the basis of these results, it is tempting to speculate that the core pentasaccharide portion of N-glycans plays the
crucial role in hormonal function while branching and peripheral sugar structures may play a modulatory role in regulating hormonal activity. There is some evidence to suggest that specific oligosaccharide structures are important for the expression of full hormonal activity. Amano et al. (1990) examined the biological activity (cAMP production) of hCGs from patients with invasive mole and choriocarcinoma in comparison with normal hCG. These hCGs have different N-linked oligosaccharide structures from those found in hCG from healthy women (Mizuochi et al., 1983; Endo et al., 1988). Complete desialylation caused a marked decrease in biological activity of all three samples and the desialylated modified hCGs showed 20-35% lower biological activity than desialylated hCG from healthy women. However, the data also indicated that the influences of different neutral oligosaccharide structures were small compared with those of terminal sialic acids.

Inhibitors of oligosaccharide processing were employed by another group to assess the contribution of high-mannose, hybrid- and complex-type oligosaccharides to the hormonal activity of rat LH (Hattori and Wakabayashi, 1989). Luteinizing hormone that contained high-mannose and hybrid oligosaccharides were shown to be 40-60% less potent than LH that contained complex oligosaccharides in the activation of adenylate cyclase of Leydig cells, whereas their receptor-binding abilities were significantly increased. These two studies suggest that incomplete or modified oligosaccharide processing impairs the full hormonal function of LH and hCG. However, available data also suggest that the effects of oligosaccharide structures, at least that of terminal sialic acid, may differ between different hormones; unlike hCG, desialylation of rat LH increases both
receptor-binding ability and biological activity (Hattori et al., 1985).

The role of the peripheral moieties of N-glycans of hCG and LH in hormonal function therefore remains unclear. It has been difficult to assess the biological properties of these hormones with respect to their specific oligosaccharide structures; this is in part due to incomplete characterisation of the N-glycans concerned. Furthermore, oligosaccharide heterogeneity at the same glycosylation sites on the gonadotrophins, has complicated the interpretation of structure/function relationships in these hormones. In order to establish the biological role(s) of specific oligosaccharides, hormonal preparations must be homogeneous with respect to the oligosaccharide in question. In view of complications encountered with pituitary hormones, urinary hCG which displays low degrees of oligosaccharide heterogeneity (Table 6.1) appears to be a more suitable candidate for structure/function studies of N-glycans. It may be possible to purify the α-subunit of hCG to homogeneity in terms of oligosaccharides present at Asn 52, by using Concanavalin A lectin affinity HPLC. Such α-subunits would be useful for the examination of the role(s) of hybrid, mono- and diantennary complex oligosaccharides in the hormonal action of hCG. Studies of this sort are particularly important, in view of the potential role of the α-subunit N-glycans in regulating the biological action of hCG.

6.2 Mechanism(s) of N-Glycan Functions

Although recent studies have convincingly shown that glycosylation of the α-subunit is essential for the biological activity of gonadotrophins (reviewed by Sairam, 1989), the
important question of how glycosylation is involved in hormonal action remains unsolved. Glycosylation may be intimately involved with the molecular events that lead to the activation of gonadotrophin hormone receptors after interaction with the hormone and several hypotheses have been proposed to explain this critical role.

The N-glycans may be directly involved in the activation of target cell responses by interaction with the receptor or other membrane components (Ryan et al., 1987). Direct involvement of membrane lectins in the activation of the receptor-adenylate cyclase system was suggested by Calvo and Ryan (1985), who found that glycopeptides or free oligosaccharides from the hCGα-subunit specifically inhibited activation of adenylate cyclase by hCG, without inhibiting hormone-receptor binding. On the other hand, a recent report indicates that the N-glycans may perturb hormone-receptor interaction through the carbohydrate-binding site of the receptor (Thotakura et al., 1990). In this study, N-linked oligosaccharides from various glycoproteins were shown to inhibit the binding of hCG to its receptor. These observations indicate that the N-glycans of gonadotrophins may directly participate in the hormone-receptor interaction for adenylate cyclase activation. Although, a soybean lectin-like sequence has been found in the putative hormone-receptor region of LH/hCG receptors (McFarland et al., 1989), its functional significance remains unknown.

There is some evidence to suggest that direct interaction of hCG oligosaccharides with the receptor is unlikely; for example, cross-linking and binding experiments have shown that the oligosaccharide chains of both subunits of hCG are positioned on the outer face of the receptor-hCG complex
(Petäjä-Repo et al., 1991). In addition, removal of oligosaccharides does not change the binding ability of hCG (Matzuk et al., 1989), which suggests that there is no significant contribution of oligosaccharides to high affinity receptor-binding.

Non-covalent association of the α- and β-subunit is necessary for high-affinity receptor binding, and both subunits of hCG and LH have been shown to interact with receptors (Ji and Ji, 1981; Moyle et al., 1982; Kusuda and Dufau, 1986; Petäjä-Repo et al., 1990). In addition, a more recent study suggests that hCG binds to its ovarian receptor mainly through the peptide moiety of its α-subunit (Petäjä-Repo et al., 1991). The role of the α-subunit may involve some common function for the glycoprotein hormones, such as adenylate cyclase activation (Milius et al., 1983). Therefore, it is possible that the N-glycans of this subunit may contribute to receptor-binding and/or activation by maintaining its active subunit conformation.

Enzymatically or chemically deglycosylated hCG and LH were shown to be potent competitive inhibitors of the native hormones, indicating the integrity of the binding domain of the modified hormones (Sairam, 1983). They retained full receptor-binding capability but their capacity to stimulate adenylate cyclase and steroidogenesis was either markedly reduced or abolished. Selective deglycosylation of the β-subunit had little effect on cAMP production, however, deglycosylation of the α-subunit virtually abolished this function (Keutmann et al., 1983; Karyan and Bahl, 1983; Sairam and Bhargavi, 1985). These observations were interpreted as indicating that glycosylation of the α-subunit plays a critical role in the coupling of the
hormone-receptor complex and adenylate cyclase system (Sairam and Bhargavi, 1985).

However, hormone-receptor interaction and adenylate cyclase activation may actually be undissociable events in gonadotrophin action. It should be noted that chemical deglycosylation of hCG was consistently associated with increased affinity for the receptor (Sairam, 1983). Besides, the effect of deglycosylation by this method on the binding properties of hormones was different, depending on which subunit was deglycosylated; gonadotrophin receptor showed increased affinity for hCG only when its α-subunit was deglycosylated (Keutmann et al., 1983; Karyan and Bahl, 1983).

Recent studies indicate that receptor-binding of chemically deglycosylated hCG is more rapid and remains tight (Hattori et al., 1988); this preparation was also shown to have an altered conformation and subunit interaction (Merz, 1988). Furthermore, immunological evidence has revealed that the conformational change in the deglycosylated hormone occurs only in the hCGβ-subunit (Hattori et al., 1988). Therefore, deglycosylation of the gonadotrophins may change the nature of the hormone-receptor interaction and leads to the formation of unproductive receptor-hormone complex on the cell surface (Sairam, 1989). These results could imply that glycosylation of the α-subunit is involved in the hormone-receptor interaction either directly or indirectly. It remains unclear, however, whether or not these changes solely reflect the removal of oligosaccharides from the hormone; the chemical reaction may be in part responsible, since non-glycosylated hCG obtained by mutagenesis did not seem to have such an increased receptor-binding affinity (Matzuk et al., 1989).
In this regard, it is noteworthy that chemically deglycosylated hCG, which is bound to its receptor, can be converted to a fully active agonist upon binding to an anti-hCGβ antibody (Rebois and Liss, 1987; Hattori et al., 1988). These observations seem to support the idea that the loss of agonism in the deglycosylated hCG is due to an altered hCG-receptor interaction resulting from a change of the conformation of the β-subunit. Since selective deglycosylation of hCGβ-subunit did not impair both binding and biological activities of hCG (Keutmann et al., 1983), these studies may, collectively, indicate that glycosylation of the hCGα-subunit is important in the maintenance of the conformation of receptor-binding domains in the β-subunit. If the α-subunit oligosaccharides interact with the β-subunit, then certain antibodies against the β-subunit may restore the agonistic properties of the deglycosylated hormone.

The binding of gonadotrophic hormones to their specific receptors displays a high degree of affinity and specificity (Roche and Ryan, 1985). However, recent studies suggest that the actual details are more complex. Two types of anti-lutropin receptor antibodies, one with agonistic and the other with antagonistic properties, have been found by monoclonal antibody techniques (Rodestá et al., 1983). This study suggested that there are at least two sites within the receptor for LH interaction, one of which represents the functional binding site. The finding of agonist antibodies also suggested that the cross-linking of receptors may be required for activation of the adenylate cyclase system by the native hormone (Rodestá et al., 1983) and this process may be catalysed by a thioredoxin-like activity of gonadotrophic hormones as suggested by Boniface and Reichert (1990). This process may also involve the interaction
of hCG oligosaccharides with membrane lectins (Ryan et al., 1987).

In another study, it has been reported that occupancy of high-affinity hCG binding sites in rat testes, which is generally assumed to be coupled to steroidogenesis, is not necessarily related to the elicitation of this biological response (Bhalla et al., 1987a, b). This study has shown that there are two types of Leydig cell with different characteristics for hCG binding and biological response. Steroidogenesis was evoked only in cells which did not bind hCG with high affinity.

Although more studies are required to substantiate these observations, they seem to provide an explanation for the biological inactivity of deglycosylated hCG which bound to its receptor with high affinity (Sairam, 1983). It has also been reported for selectively desialylated hCG that higher binding-affinity to the receptor is not correlated with higher biological activity (Amir et al., 1987). If biological responses are not directly associated with high-affinity binding of the hormone to a single receptor, it will be difficult to assess the effects of glycosylation on binding and biological activity.

Another interesting aspect of these observations (Bhalla et al., 1987a, b) would be that it supports the dissociation model previously proposed for LH/hCG receptor interaction (Bhalla et al., 1979). In this model, the interaction of the hormone with functional receptors triggers the dissociation of the regulatory subunit of the receptor, and this leads to the activation of adenylate cyclase. In fact, specific proteolytic cleavage of LH/hCG receptors has been observed when they were bound to hCG
(Kellokumpu and Rajaniemi, 1985). This might also be related to the earlier findings that treatment of ovarian membranes with serine proteases activated adenylate cyclase (Reichert and Ryan, 1977). However, these observations have been interpreted to be due to activation by endogenous membrane proteases.

The essential part of hormonal function may lie in the intrinsic protease activity of gonadotrophins. This attractive idea has been revived by the recent finding of structural and functional similarities between hCG and α-chymotrypsin (Willey and Leidenberger, 1989). It has been suggested that the α-subunit is required in the formation of the substrate pocket for catalytic reactions, which involves also part of the β-subunit. This hypothesis not only provides new insight into the mechanism of gonadotrophic action but may also account for the site-specific role of N-glycans in the biological activity of hCG. The loss of agonistic properties of hCG that followed the removal of oligosaccharides from the hCG α-subunit Asn 52 (Matzuk et al., 1989) may be due to structural alterations in the putative catalytic domains; this glycosylation site is located in part of the substrate pocket in the proposed tertiary structure model (Willey and Leidenberger, 1989).

If hCG is a unique protease whose substrate is its specific receptor, then one could speculate that hormonal activity depends on two distinct interactions between the hormone and its receptor; one involves the hormone-specific β-subunit for recognition of the receptor (substrate) and the second involves the common α-subunit for the catalytic action of the hormone. In accordance with this model, one could further speculate that there are two potential mechanisms in which the N-glycans of the α-subunit are involved in the hormonal function; they may be
concerned in both of the above hormone-receptor interactions, which seem to offer a plausible explanation for several, apparently conflicting observations in previous studies. It remains to be shown, however, whether or not the gonadotrophic hormones have intrinsic protease activity.

Present knowledge is insufficient for a proper understanding of why the N-glycans of the α-subunits of hCG and LH are of vital importance to hormonal function; glycosylation of the α-subunit may be directly or indirectly involved in the mechanism of biological action. Limited information regarding the nature of hormone-receptor interactions and their relationship to adenylate cyclase activation also makes it difficult to define the role of hormonal glycosylation in these events. Although the mechanism(s) of N-glycan function remains unclear because of conflicting evidence, most previous studies either suggest or do not exclude the possibility that the N-glycans may play an important role in the hormone-receptor interaction. However, one thing is certain and that is, if one wishes to investigate the mechanism(s) of action of any biological molecule such as a hormone, one must define its structure unambiguously.

The experiments reported in this thesis have revealed the nature of the carbohydrate structures attached to specific amino acid residues in the hormonal subunits of ovine and human luteinizing hormones and while this information is important, it is insufficient. Our complete understanding of the biological action of these hormones must await the elucidation of the three-dimensional structures of their native forms. In this regard, as mentioned earlier, hCG currently appears to be the candidate of choice because of its greater degree of
oligosaccharide homogeneity. While the fully glycosylated hormone may resist attempts at crystallization, recent developments in $^1$H-NMR spectroscopy, combined with molecular graphics, may enable speedier resolution of these complex structures.
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Microheterogeneity of luteinizing hormone in pituitary glands
from women of pre- and postmenopausal age.


PUBLICATIONS

Main parts of the experimental results described in this thesis have been reported in the following publications.

J. Hiyama & A.G.C. Renwick (1990)
Separation of human glycoprotein hormones and their subunits by reversed-phase liquid chromatography.
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NMR investigations of the N-linked oligosaccharides at individual glycosylation sites of human lutropin.

Site-specific N-glycosylation of human chorionic gonadotropin-structural analysis of glycopeptides by one- and two-dimensional 1H-NMR spectroscopy.
Glycobiology (in press).