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**GnRH BINDING SITES
IN THE HUMAN PLACENTA**

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PhD Thesis

University of Edinburgh

August 1995



I hereby declare that this thesis was composed by myself
and that the work contained herein is my own

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August 1995

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ABBREVIATIONS

^{125}I -GnRH	mono-iodinated gonadotrophin-releasing hormone
ACTH	adrenocorticotrophic hormone
BDU	bromodeoxyuridine
BSA	bovine serum albumin
Ci	Curie
cpm	counts per minute
CRH	corticotrophin-releasing hormone
Da	daltons
DCC	dextran-coated charcoal
EDTA	ethylenediaminetetra-acetic acid disodium salt
EGF	epidermal growth factor
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
g	gram
GnRH	gonadotrophin-releasing hormone
HCl	hydrochloric acid
hCG	human chorionic gonadotrophin
hCT	human chorionic thyrotrophin
hPL	human placental lactogen
HPLC	high performance liquid chromatography
IGF	insulin-like growth factor
IgG	bovine γ -globulins
k	kilo ($\times 10^3$)
l	litre
LH	luteinising hormone
μ	micro ($\times 10^{-6}$)
m	milli ($\times 10^{-3}$)
M	molar
n	nano ($\times 10^{-9}$)
NaCl	sodium chloride
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEG	polyethyleneglycol
PG	prostaglandin
PMSF	phenylmethylsulphonylfluoride
s.d.	standard deviation
SET	sucrose-EDTA-Tris buffer
TGF	transforming growth factor
TLC	thin layer chromatography
TRH	thyrotrophin-releasing hormone
Tris	tris(hydroxymethyl)aminomethane [TRIZMA Base]
TSH	thyroid stimulating hormone

ABSTRACT

The aim of this thesis was to investigate GnRH binding activity in human trophoblast tissue from pregnancies terminated between 6 and 18 weeks and in placentae from full-term, uncomplicated deliveries. Binding sites for GnRH in the human placenta differ from the well-characterised pituitary GnRH receptor. The pituitary superagonists Buserelin and Tryptorelin were bound by placental membranes, as were the salmon and chicken-II isoforms of GnRH, however placental binding of the GnRH agonist analogues was not enhanced compared to the two isoforms, which were not bound by the pituitary receptor. Previous studies of GnRH in the human placenta have been confined to a limited range of gestational ages and the pre-term placental tissue studied was often obtained by suction curettage, which is often contaminated by foetal tissue. Therefore, the availability of medically, as opposed to surgically, terminated early placental tissue was utilised in this study to provide a good source of very early trophoblast tissue.

Specific activity of the binding of GnRH, GnRH isoforms and GnRH agonist analogues to the human placental GnRH binding site was found to vary with stage of gestation. The high levels observed in early trophoblast (6-8 weeks) fell to around the detection limit of the assay at 12-18 weeks, before increasing to maximal levels by term. Rebinding data suggested that differential tracer degradation could not account for gestational age-related variations in binding, and levels of binding were not related to contamination of the membrane fractions by non-villous tissue. Although the specific activity of binding to placental binding sites varied with gestation, specificity was unchanged: Tryptorelin, Buserelin, chicken GnRH-II, salmon GnRH > mammalian GnRH » lamprey GnRH, chicken GnRH-I.

Buserelin, Tryptorelin and chicken GnRH-II were displaced from the membrane binding site by cytosolic extracts of placenta of all stages of gestation. Cytosol fractions from 12-18 weeks displaced iodinated GnRH tracers from the binding sites of membrane preparations from the same stage of gestation to a greater extent than an excess of non-iodinated Buserelin, but did not displace tracer from term membrane binding sites to the same degree. Placental membrane binding of tracer and the displacement effect of cytosol were reduced in the presence of protease inhibitors, however this was due to the presence of ethanol (as a solvent) in the assay. The nature of this ethanol effect remains unidentified.

Placental cytosol of all stages of gestation displaced binding of chicken GnRH-II from specific chicken GnRH-II antibodies. Protease inhibitors and ethanol had no effect on this competitive binding. Placental cytosol was also able to bind chicken GnRH-II, but not Tryptorelin, at physiological pH. At low pH, chicken GnRH-II binding was enhanced at all stages of gestation and cytosol demonstrated the ability to bind GnRH agonist tracer - gel exclusion fractionation suggested that the endogenous ligand dissociated from the cytosolic binding site at acid pH. In addition, human placental cytosol of all stages of gestation contained a high molecular weight (~60kDa) mitogenic activity, demonstrated in culture with dispersed ovine pituitary cells using direct (cytometer, Coulter counter) and indirect (tritiated thymidine, BDU) counting methods.

In conclusion, the effect of gestational age on GnRH binding to placental membranes may be related to changes in receptor number or down-regulation of receptor binding. However, the distinct pattern of GnRH binding observed for placental membrane binding sites suggested that placental GnRH, the placental GnRH binding site or both differed from the well-characterised pituitary form. In addition, GnRH-like activity was observed in the placenta, but was not characterised.

CHAPTER 1

INTRODUCTION

1.1 ENDOCRINOLOGY OF THE HUMAN PLACENTA

The placenta secretes large quantities of steroid, peptide and glycoprotein hormones, with hormone production varying with stage of gestation (Simpson and MacDonald, 1981). Placental hormone secretion may be affected by factors of maternal, foetal and placental origin, and thus functions in a paracrine fashion, as well as its well-recognised function as an endocrine organ. Release and release-inhibiting factors, neuropeptides, neurotransmitters, cytokines, growth factors and prostanoids have been described to play a role in control of placental hormonogenesis, with complex and delicately balanced interactions between these factors which are not fully understood.

1.1.1 Hypothalamic-like Peptides in the Human Placenta

The placenta contains hormonal activities with similarities to the hypothalamic-pituitary axis with releasing and release-inhibiting hormones, which were localised in cytotrophoblast cells of the placenta (Petraglia, 1991), acting to control local production of placental hormones by syncytiotrophoblasts. Placental equivalents of GnRH, corticotrophin-releasing hormone (CRH), growth hormone release-inhibiting hormone (somatostatin) and thyrotrophin-releasing hormone (TRH) have been identified in the human placenta (Siler-Khodr, 1983).

The presence of GnRH-like activity in the human placenta, which stimulated pituitary LH release *in vivo* was described by Gibbons *et al.* (1975). Placental GnRH is thought to be distinct from the hypothalamic decapeptide and is discussed in detail in Section 1.2. GnRH in the placenta has been shown to have an effect on secretion of hCG, progesterone, oestrogen and prostaglandins, but to have no effect on hPL secretion (Siler-Khodr *et al.*, 1986a,b,c). Release of placental GnRH is activated by Ca²⁺-dependent cell membrane depolarisation and adenylate cyclase/cAMP (Petraglia *et al.*, 1987a), similar to GnRH release in the hypothalamus. GnRH release in

placental cultures was modulated by steroid hormones and opiates (Petraglia *et al.*, 1990a), suggesting similar interactions are possible in the *in vivo* placenta.

Placental TRH activity was also described by Gibbons *et al.* (1975) but no physiological role has been confirmed in the placenta for this peptide. hCT has been isolated from placental extracts, but does not appear to be identical to pituitary TSH.

A chorionic somatostatin-like activity has been localised in first trimester cytotrophoblast cells (Kumasaka *et al.*, 1979; Zhang *et al.*, 1991) with gestational changes in placental somatostatin levels inversely correlated to hPL. This led to the hypothesis that hPL release was inhibited by placental somatostatin-like activity via the action of dopamine (Macaron *et al.*, 1978a), although subsequent studies failed to demonstrate a direct action on hPL secretion (Macaron *et al.*, 1978b). When injected into the uterus of early pregnant rats, somatostatin antiserum increased the implantation rate of blastocysts (Zhang *et al.*, 1991), which suggests an inhibitory role for somatostatin in blastocyst implantation.

Chorionic CRH with placental ACTH, β -endorphin and MSH release-stimulating activity has also been described (Margioris *et al.*, 1987). Pregnant women have measurable CRH levels in their systemic circulation, whereas CRH was not detected in non-pregnant females (Thomson *et al.*, 1988), and maternal serum CRH correlates with CRH mRNA levels in the placenta, suggesting the placenta is a major source of circulating CRH (Petraglia, 1991). CRH mRNA was expressed from early gestation, increasing more than 20-fold during the last 5 weeks of pregnancy (Frim *et al.*, 1988). CRH was localised to the cytotrophoblast of term placental villi (Petraglia *et al.*, 1987c) and was identical to hypothalamic CRH (Petraglia *et al.*, 1990a). Stimulation of placental CRH release was analogous to the hypothalamus: noradrenaline and acetylcholine increased CRH release from both.

1.1.2 Peptide Hormones of the Human Placenta

1.1.2a Human Chorionic Gonadotrophin (hCG)

hCG has a similar structure to the pituitary gonadotrophins FSH and LH and to TSH. These glycoprotein hormones are comprised of two dissimilar subunits, designated α and β . The α -subunits of these hormones are interchangeable, with biological activity determined by the β -subunit, although sequence homologies exist between β -subunits. The amino acid sequence of hCG has been described (Bahl *et al.*, 1972) and the molecular weights reported as 10,200Da for the α -subunit and 15,500Da for the β -subunit. 30% of hCG is carbohydrate which probably helps to prolong its half-life.

The α and β subunits are synthesised independently, with production of the β -subunit the rate-limiting step for formation of hCG. hCG is secreted almost exclusively into the maternal circulation and can be detected in the maternal serum as early as 6 days after ovulation (Braunstein *et al.*, 1976). Peak maternal serum concentrations were observed at 8 weeks, dropping rapidly after 11 weeks to a nadir by 18 weeks and staying low for the rest of gestation. At term, hCG levels declined and free α -subunits appeared in the plasma, suggesting uncoordinated synthesis of the subunits (Vaitukaitis, 1974). Hence, hCG levels can not be correlated with increasing trophoblastic mass, but correspond closely with the rate of differentiation of cytotrophoblasts into syncytiotrophoblasts (Hay, 1988).

hCG was localised, using immunofluorescence, to the syncytiotrophoblast (Khodr and Siler-Khodr, 1978a), whereas GnRH, which has a similar release profile to hCG, was localised to cytotrophoblasts (Khodr and Siler-Khodr, 1978a). If the placenta was analogous to the hypothalamic-pituitary axis, then GnRH released from cytotrophoblasts would stimulate hCG release from syncytiotrophoblast via plasma membrane receptors. *In vitro*, GnRH was shown to increase hCG secretion from placental explants (Khodr and Siler-Khodr, 1978b), although injections of GnRH had no detectable effect on circulating levels of hCG in early pregnant women (Seppala *et*

al., 1980). Patterns of hCG secretion were reviewed by Bramley (1987) who found basal hCG release from placental explants peaked at 9-10 weeks, coinciding with peak levels of hCG in maternal circulation, but showed minimal response to exogenous GnRH. From 10-17 weeks, maternal hCG levels and hCG release from placental explants fell, but responsiveness of placental cultures to GnRH rose. In term placental explants, hCG content fell to low levels but GnRH was still able to stimulate hCG release. GnRH-stimulated hCG release from mid gestation placental explants (Siler-Khodr *et al.*, 1987) and perfused first trimester placental explants (Barnea *et al.*, 1991) was inhibited by GnRH antagonists.

In addition to GnRH, dibutyryl cyclic AMP (Haning *et al.*, 1982; 1988) and EGF (Barnea *et al.*, 1990) both increased hCG release from placental explants, but no effect was observed with dibutyryl cGMP, AMP, insulin, progesterone, adrenaline or PG. Dynorphin (1-13) stimulation of hCG release from trophoblast cultures was described by Zhang *et al.* (1991). Dopamine inhibited hCG secretion *in vitro* whereas the dopamine antagonist pimozide stimulated hCG secretion (Macaron *et al.*, 1978a). This is analogous to control of LH secretion by the pituitary, where dopamine modulates the secretion of hypothalamic GnRH. Licht *et al.* (1992) described stimulation of hCG release from perfused first trimester placenta by GABA, which was independent of GnRH. The action of GABA was inhibited by bicuculline, an antagonist of GABA_A suggesting the involvement of GABA_A receptors, analogous to GABA stimulation of LH release in the pituitary (Virmani *et al.*, 1990).

1.1.2b Human Placental Lactogen (hPL; hCS, human chorionic somatomammotrophin)

hPL is a single chain polypeptide of 191 amino acids with molecular weight 22,300kDa (Simpson and MacDonald, 1981). Unlike hCG it contains no carbohydrate residues. hPL is similar to the anterior pituitary hormones, prolactin and growth hormone, and shares lactogenic and somatotrophic properties. hPL has one-hundredth

of the potency of growth hormone in promoting growth but it is produced in large quantities (1g per day in term placenta). Therefore considerable physiological effects could be possible in pregnancy. Little hPL crosses to the foetal circulation and its precise role has yet to be elucidated (Simpson and MacDonald, 1981).

Hoshina *et al.* (1982) localised hPL to the syncytiotrophoblast. hPL can be detected in the maternal circulation 5-10 days after implantation and increasing serum levels correlate with increasing trophoblast mass. No effect of somatostatin on hPL release was detected (Macaron *et al.*, 1978b).

1.1.2c Inhibin

Inhibin is a glycoprotein which preferentially suppresses FSH secretion by the anterior pituitary (de Kretser and Robertson, 1989). It is produced by the granulosa cells of the ovarian follicle and the corpus luteum in the human female. In the male, inhibin is produced by the Sertoli cells of the testes.

Inhibin has been isolated as a heterodimer of two dissimilar subunits, designated α and β (Burger, 1988), which are linked by disulphide bonds. Variations in the β -subunit give rise to two forms of inhibin, designated Inhibin A and Inhibin B (Robertson *et al.*, 1985), comprising α - β_A and α - β_B respectively. Dimers of the β -subunits, which stimulate FSH secretion (Vale *et al.*, 1986), have been designated Activin A, the homodimer of the β_A subunit, and Activin A-B, consisting of one β_A and one β_B subunit (Burger, 1988).

Suppression of FSH secretion by inhibin is thought to be due, at least in part, to tonic suppression of FSH synthesis (Farnworth *et al.*, 1988), possibly at the level of inhibition of transcription of the FSH β gene (Mercer *et al.*, 1987). GnRH-stimulated release of FSH is also decreased by inhibin, possibly by reduced responsiveness of the gonadotroph to GnRH (Wang *et al.*, 1990). Inhibin is thought to act beyond the GnRH receptor, possibly at the level of protein kinase-C and calmodulin, transducers for calcium, the second messenger for GnRH (Huckle and Conn, 1988).

In the human female, inhibin levels are cyclical, peaking in the late follicular phase and again in the mid luteal phase before declining prior to menstruation (de Kretser and Robertson, 1989). In conception cycles, the late luteal decline does not occur and levels increase during pregnancy. Inhibin immunoactivity and bioactivity was identified in extracts from human placentae by McLachlan *et al.* in 1986. This placental inhibin-like activity was neutralised by anti-inhibin antisera and had a parallel immunoactivity to inhibin isolated from human follicular fluid. However differences in bioactivity : immunoactivity ratios suggest differences may exist between placental and ovarian inhibins.

Placental inhibin was localised to the cytotrophoblast of term placenta, using immunohistochemistry, by Petraglia *et al.* (1987b) and, from studies of placental monolayer cultures, a putative role for inhibin in the control of hCG secretion in the placenta was proposed (Petraglia *et al.*, 1989). Inhibin alone had no effect on basal hCG secretion, but reduced GnRH-stimulated hCG release. Conversely, activin strongly potentiated the stimulation of hCG release by GnRH: activin action was dose-related and reversed by inhibin. Activin increased GnRH and progesterone release from placental cultures, whereas inhibin alone had no effect but reversed the effects of activin. The functional antagonism of inhibin and activin mirrors their effects on FSH secretion in the pituitary (de Kretser and Robertson, 1989).

1.1.2d Placental ACTH

Pregnancy is associated with increased maternal plasma cortisol levels and the presence of bioactive and/or immunoactive ACTH has been reported in placental extracts. The human placenta produces the proopiomelanocortin precursor molecule for ACTH and contains the ACTH release-related hormones β -lipotrophin and β -endorphin (Simpson and MacDonald, 1981; Petraglia *et al.*, 1990a). Release of ACTH, β -endorphin and MSH from placental cell cultures was stimulated by CRH, apparently acting via a specific CRH receptor (Petraglia *et al.*, 1987c).

1.1.3 Neuropeptides and Neurotransmitters in the Human Placenta

β -endorphin is generated in the pituitary by cleavage of β -lipocortin, which is formed when proopiometelanocortin is cleaved to release ACTH. Using immunogold-silver histochemistry, β -endorphin was identified in the syncytiotrophoblast (Zhang *et al.*, 1991). β -endorphin immunoactivity in the placenta was high at 6 weeks but had decreased by 10 weeks, with low levels at term (Zhang *et al.*, 1991). Other opioids were isolated in the placenta by HPLC of methanol extracts of acidified first trimester cytosol, with methionine-enkephalin > dynorphin (1-13) > β -endorphin > leucine-enkephalin (Zhang *et al.*, 1991). Interestingly, plasma levels of methionine-enkephalin are not significantly different in pregnant and non-pregnant women (Petraglia *et al.*, 1990a). When injected into the uterine horn of early pregnant rats, dynorphin antiserum decreased blastocyst implantation rate, whereas β -endorphin antiserum had no effect on implantation (Zhang *et al.*, 1991). Opioids were shown to inhibit placental GnRH release *in vitro* (Petraglia *et al.*, 1990b) and stimulate hPL from perfused placental villi (Belisle *et al.*, 1988) via placental opiate receptors. In addition, dynorphin stimulated hCG release (Barnea *et al.*, 1991a), whereas β -endorphin inhibited hCG release (Barnea *et al.*, 1991b) from first trimester placenta.

Neuropeptide Y is extensively distributed in the brain and plays a role in regulation of the hypothalamic-pituitary-gonadal axis. Neuropeptide Y and its specific binding sites have been found in the cytotrophoblast cells of the term placenta and stimulates CRH from placental preparations in a dose- and time-dependent manner (Petraglia *et al.*, 1990a).

Despite the absence of nerve endings in the trophoblast, neurotransmitter metabolising enzymes and receptors have been described in the placental tissue, suggesting a role for circulating neurotransmitters in placental function (Petraglia *et al.*, 1990a). Zhang *et al.* (1991) also detected four neurotransmitters in first trimester placental villi: noradrenaline, adrenaline, 5-HT (localised to syncytiotrophoblast by

immunogold-silver method) and DOPAC, a dopamine metabolite. Quantitatively, noradrenaline appeared to be the main neurotransmitter in the placenta.

1.1.4 Steroid Hormones in the Human Placenta

In the human, the corpus luteum, under the influence of hCG produced by the trophoblast, is the dominant source of progesterone and oestradiol until approximately weeks 6-8 of pregnancy, when the placenta becomes the primary source of these steroid hormones (Simpson and MacDonald, 1981): late in pregnancy, 250-600mg progesterone, 15-20mg oestradiol and 50-100mg oestriol are secreted by the placenta per day. These steroid hormones are required in the establishment of pregnancy, with a role in ovum transport, proliferation and differentiation of the endometrium, decidualisation and implantation (reviewed by Albrecht and Pepe, 1990). Progesterone is essential in the maintenance of pregnancy, with lack of progesterone associated with termination of pregnancy (Simpson and MacDonald, 1981).

Synthesis of oestrogens by the human placenta is dependent on availability of C-19 steroid precursors, as the human placenta is unable to convert acetate *de novo* into oestrogen: 17β -oestradiol and oestrone are formed from DHA and its sulphate DHAS, while oestriol is formed from 16α -hydroxy-DHA and its sulphate (Simpson and MacDonald, 1981). Progesterone is formed mainly from maternal LDL cholesterol (Simpson and MacDonald, 1981), but Haning *et al.* (1982) found no effect of exogenous LDL cholesterol on progesterone release by term placental explants. However, they did observe that DHAS increased oestradiol output, which was suppressed by dbcAMP, and that aromatase inhibitor decreased oestradiol release in a dose-dependent fashion. In mid-gestation placental explants, Siler-Khodr *et al.* (1983; 1987) effected suppression of progesterone release with the GnRH antagonist [N-Ac-Pro¹,D-p-CI-Phe²,D-Nal(2)^{3,6}]-GnRH.

In placental monolayers (Branchaud *et al.*, 1983), DHA was required for oestradiol and oestriol production and GnRH had an inhibitory effect on progesterone

and oestrogen output. However, stimulation of progesterone, oestrone and oestradiol from mid-gestation placental explants by GnRH has also been reported (Siler-Khodr *et al.*, 1986b).

1.2 GONADOTROPHIN-RELEASING HORMONE IN THE HUMAN PLACENTA

1.2.1 Characterisation of GnRH in the Human Placenta

GnRH is a decapeptide which is synthesised and stored in the hypothalamus, and acts on the pituitary gland via a specific plasma membrane receptor to stimulate the synthesis and release of LH and FSH (Hazum and Conn, 1988). In 1975, Gibbons *et al.* described the *in vitro* synthesis by homogenates of human placenta, of GnRH-like activity which was chromatographically identical to GnRH standards on CM-cellulose and TLC. Using placental fragments, the synthesis of immunoactive GnRH was also demonstrated (Siler-Khodr and Khodr, 1979), with release of GnRH from the placental tissue exceeding the initial tissue GnRH content. *De novo* synthesis of GnRH by human placenta was confirmed by tritiated amino acid incorporation (Khodr and Siler-Khodr, 1980; Tan and Rousseau, 1982).

GnRH immunoactivity was detected in the human placenta throughout gestation (Siler-Khodr and Khodr, 1978; Zhang *et al.*, 1991); total GnRH content was low during 6-7 weeks gestation, increased from 8 to 23 weeks, appeared to plateau until 36 weeks, then increased significantly by term. No difference was detected between male and female pregnancies. When expressed per mg of tissue, peak levels of immunoactive GnRH were detected in early pregnancy, with levels actually decreasing in mid-gestation and term placentae. This correlated with levels of immunoactive GnRH measured in maternal circulation (Siler-Khodr *et al.*, 1984) which were highest in first half of pregnancy and were significantly higher in pregnant compared with nonpregnant females.

Using immunofluorescence and immunohistochemical techniques (Khodr and Siler-Khodr, 1978a; Miyake *et al.*, 1982; Zhang *et al.*, 1991), GnRH was strongly localised in the cytotrophoblast of first trimester tissue, decreasing through gestation to low levels at term, possibly reflecting changing cytotrophoblast cell number with advancing gestation. No staining of the syncytiotrophoblast was found at any stage.

Release of GnRH from cultured placental cells was stimulated by K^+ or veratridine, a sodium ionophore (Petraglia *et al.*, 1987a). This effect was reversible by calcium antagonists such as cobalt, EGTA and verapamil, suggesting cell membrane depolarisation is involved in mediating GnRH release, analogous to hypothalamic GnRH release. dbcAMP, forskolin, theophylline and theobromine also increased GnRH release, as did prostaglandins E_2 and $F_{2\alpha}$ and adrenaline. The latter was inhibited by propranolol, suggesting the involvement of β -adrenergic receptors. Petraglia *et al.* (1989) described the effects of inhibin and activin on GnRH release from placental cell cultures: activin increased GnRH release, whereas inhibin alone had no effect but antagonised the stimulatory effect of activin. Further investigations (Petraglia *et al.*, 1990b) found no effect of the steroids oestradiol, oestriol or progesterone on GnRH release from cultured placenta, but observed stimulation of cAMP-induced GnRH release by oestradiol and oestriol, and inhibition of the same by progesterone. Stimulation of GnRH release from placental cell cultures by activin was augmented by oestriol and decreased by progesterone or tamoxifen. Agonists for the three classes of opiate receptor had no effect on GnRH release from placental cell cultures (Petraglia *et al.*, 1990b), but morphine, a μ -receptor agonist, and U-50,488H, a κ -receptor agonist, decreased cAMP-induced GnRH release. These effects were reversed in the presence of specific μ and κ receptors antagonists, and paralleled inhibition of GnRH release in the hypothalamus by dynorphin and β -endorphin.

Early evidence (Lee *et al.*, 1981) suggested that term placental GnRH was indistinguishable from synthetic GnRH after fractionation of methanol extracts by HPLC, although high molecular weight forms of GnRH were detected in rat placental

extracts (Gautron *et al.*, 1981). Tan and Rousseau (1982) identified biosynthetic GnRH in the placenta as chemically identical to hypothalamic GnRH on HPLC of partially purified peptide, but postulated the existence of high molecular weight GnRH-like proteins in placenta. Zhang *et al.* (1991), using methanol extracts of acidified placental cytosol, found high molecular weight GnRH and hypothalamic GnRH immunoactivity.

Studies using antibodies raised against the C-terminal of GnRH (Siler-Khodr and Khodr, 1978, 1979; Khodr and Siler-Khodr, 1978a, 1980; Seppala *et al.*, 1980; Lee *et al.*, 1981; Miyake *et al.*, 1982) may have failed to detect any modifications of the N-terminal of the molecule which may give rise to a distinct placental form of GnRH. Gautron *et al.* (1989) used two antibodies, to the C and N termini of GnRH respectively, to assay human placental extracts after chromatographic fractionation and concluded that placental immunoactive GnRH levels were over-estimated and that placental GnRH was not identical to hypothalamic GnRH.

Siler-Khodr (1987) noted that apparent GnRH immunoactivity exceeded the GnRH decapeptide content of the placenta and concluded that acid extraction procedures resulted in higher recoveries of placental GnRH-like immunoactivity, compared with extraction at neutral pH, due to denaturing of larger peptides. GnRH-like immunoactivity was attributable, in part, to a high molecular weight glycoprotein which had reduced potency in pituitary bioassays but which enhanced the release of hCG and prostaglandins from placental explants to a greater extent than the hypothalamic decapeptide (Siler-Khodr, 1987). This placental activity inactivated GnRH (and TRH, angiotensin II and, in the presence of DTT, oxytocin and somatostatin) (Siler-Khodr *et al.*, 1989) and was identified as a 58kDa post-proline protease unique to the placenta (Kang and Siler-Khodr, 1992).

Seeburg and Adelman (1984) cloned and sequenced cDNA for the precursor of GnRH (preproGnRH) from human placenta. The precursor gene had an unusually long 5' untranslated region (>1,000 nucleotides). The cDNA coded for a protein of 92

amino acids, in which the decapeptide was preceded by a 23 amino acid signal peptide and was followed by a Gly-Lys-Arg sequence, typical of an enzymatic cleavage site, then a 56 amino acid extension. This C-terminal GnRH-associated peptide (GAP) had both gonadotrophin-releasing (Millar *et al.*, 1986) and prolactin-inhibiting (Nikolics *et al.*, 1985) activity in cultured human pituitary cells. Seeburg *et al.* (1987) suggested tissue-specific forms of GnRH may arise by differential processing of preproGnRH: in placental preproGnRH mRNA, the first intron is not spliced out. In addition, promoter regions may differ in the placenta and hypothalamus (Adelman *et al.*, 1986).

1.2.2 GnRH Binding Sites in the Human Placenta

Superagonist analogues of GnRH, such as Buserelin, had equilibrium association constants (K_a) between 4 and 8 fold greater ($K_a = 4.8 \times 10^9 M^{-1}$) than the natural decapeptide ($K_a = 6.6 \times 10^8 M^{-1}$) in the pituitary radio-receptor assay (Clayton and Catt, 1980)

In the placenta, receptor affinity for analogues like Buserelin were comparable with the affinity of the native hormone ($K_a = 5.5 \times 10^7 M^{-1}$ and $6.2 \times 10^7 M^{-1}$, respectively) (Currie *et al.*, 1981), using crude membrane preparations. Purification of placental membranes by sedimentation on a sucrose gradient (Belisle *et al.*, 1984) gave an 8-fold enrichment of membrane markers but no improvement in receptor affinity ($5.5 \times 10^5 M^{-1}$ to $1.1 \times 10^7 M^{-1}$) for GnRH or its agonist analogues. Iwashita *et al.* (1986) performed binding studies on particulate and solubilised receptor preparations, using the GnRH agonists [D-Lys⁶]- and [D-Ala⁶]des-Gly¹⁰-GnRH-*N*-ethylamide, and obtained affinity constants of $1.6 \times 10^6 M^{-1}$ and $5.4 \times 10^5 M^{-1}$ respectively, compared with $K_a = 1.1 \times 10^6 M^{-1}$ for the decapeptide. Placental binding sites were saturable, of a single class and specific for GnRH (Currie *et al.*, 1981; Belisle *et al.*, 1984; Iwashita *et al.*, 1986). However Belisle *et al.* (1987), using suspensions of intact placental cells, described two classes of binding sites with association constants of $4.7 (\pm 2.2) \times 10^5 M^{-1}$ and $1.78 (\pm 0.8) \times 10^8 M^{-1}$ respectively for GnRH agonists, although the high affinity

sites still fell short of pituitary GnRH receptor affinity constants and the estimated number of high affinity sites was 1,000 times less than low affinity sites.

Binding sites in the placenta with a high specificity but low affinity for GnRH agonist analogues suggest the existence of an endogenous GnRH-like ligand in the placenta which may have a higher affinity for these sites. Alternatively, high local concentrations of GnRH may allow the placental receptor to operate with a much lower affinity than that required in the pituitary. High affinity binding sites may be difficult to demonstrate due to degradation of the synthetic analogue at unprotected cleavage sites, by placental proteases such as the unique placental post-prolinase described by Siler-Khodr *et al.* (1989) and Kang and Siler-Khodr (1992).

The molecular weight of the placental GnRH receptor was estimated to be 53,700 (\pm 2,700) Da by Iwashita *et al.* (1986). The receptor migrated as a single band on SDS-gel electrophoresis, with similar results under reducing and non-reducing conditions unlike the pituitary receptor. Escher *et al.* (1988) used photolabelled analogues of GnRH to identify a 58kDa GnRH-binding subunit on SDS-gel electrophoresis. This appeared to be associated with other proteins, possibly signal transduction G-proteins.

The human pituitary GnRH receptor has been cloned (Reinhart *et al.*, 1992; Kaiser *et al.*, 1992; Kakar *et al.*, 1992) and its function characterised (Anderson *et al.*, 1993). In immortalised α T3-1 pituitary gonadotroph cells, it was shown that these GnRH receptors are coupled to the phosphatidyl inositol second messenger pathway via a specific G-protein. To date, however, the placental GnRH receptor has not been analysed.

1.2.3 Role for GnRH in the placenta

A bolus injection of GnRH at 8-18 weeks pregnancy had no effect on circulating concentrations of hCG, hPL, pregnancy specific β -1-glycoprotein or pregnancy protein 5 (Seppala *et al.*, 1980; Skarin *et al.*, 1982). This may be due to

low GnRH concentrations reaching the placenta which were unable to activate the low affinity placental receptors in order to elicit a response. However, injection of anti-GnRH was followed by termination of pregnancy in early pregnant baboons (Das *et al.*, 1985). Zhang *et al.* (1991) injected anti-GnRH into the uterus of early pregnant rats and found decreased blastocyst implantation, suggesting that GnRH may have a role in implantation.

In vitro, GnRH stimulated hCG release from placental explants (Khodr and Siler-Khodr, 1978b; Haning *et al.*, 1982; Siler-Khodr *et al.*, 1986a) in a dose-related fashion (Siler-Khodr and Khodr, 1981). GnRH stimulated release of hCG most dramatically at mid term (Siler-Khodr *et al.*, 1986a), when secretion of hCG was decreasing. GnRH also increased hCG secretion in short-term placental cultures (Belisle *et al.*, 1984), with the increase in release 4-fold higher in mid-term cultures compared with term. Similar results were obtained with perfused dispersed trophoblast cells (Butzow, 1982): GnRH increased hCG secretion by up to 4-times basal levels. This effect was specific for GnRH; neither TRH nor oxytocin stimulated hCG release from cultured placentae (Belisle *et al.*, 1984), and the involvement of GnRH receptors has been implicated, as inhibition of hCG release from first trimester (Barnea *et al.*, 1991) and mid-gestation (Siler-Khodr *et al.*, 1983) placental explants by GnRH antagonists has been described. Mathialagan and Rao (1989) observed a role for extracellular calcium in GnRH-stimulated hCG release from placental explants, and a corresponding involvement of calmodulin. GnRH stimulation of LH secretion in the pituitary was also demonstrated to involve calcium (Clapper and Conn, 1985) and calmodulin (Hart *et al.*, 1983). In the pituitary, activation of the GnRH receptor has been associated with phosphoinositide hydrolysis (Marian and Conn, 1979). However, Belisle *et al.* (1989) failed to show any effect of GnRH on phosphoinositol turnover, suggesting Ca²⁺-mediated GnRH-stimulation of hCG release involves activation of protein kinase-C.

GnRH had no effect on hPL secretion from placental explants (Khodr and Siler-Khodr, 1978b; Siler-Khodr *et al.*, 1986a).

Reports of GnRH effects on placental steroid secretion were inconsistent: Wilson and Jawad (1980) reported suppression of progesterone production in a dose-related manner by GnRH in 12 and 22 week and term placental explants, and inhibitory effects of GnRH on both progesterone and oestrogen have been reported in term monolayer cultures (Branchaud *et al.*, 1983), although Haning *et al.* (1982) found GnRH had no effect on progesterone or oestradiol secretion by term placental explants. However, inhibition of progesterone release from mid-gestation placental explants by a GnRH antagonist has been documented (Siler-Khodr *et al.*, 1983) and GnRH was found to stimulate the release of progesterone, oestradiol and oestrone from mid-gestation and term placental explants (Siler-Khodr *et al.*, 1986b).

Using placental explants, Siler-Khodr *et al.* (1986c) found a dose-related inhibition of release of prostaglandins E and F and 15-keto-prostaglandin F by GnRH at 13 weeks gestation, followed by stimulation of release at 15-17 weeks gestation and at term.

1.3 GROWTH FACTORS

The human placenta produces and is a target for many growth factors. Insulin-like growth factors (IGFs), epidermal growth factor (EGF), fibroblast growth factors (FGF), transforming growth factors α and β (TGF- α and - β) and platelet-derived growth factor (PDGF) have all been described in the human placenta.

IGFs have structural and biological similarities to insulin and act on specific cell membrane receptors to stimulate proliferation of various cell types. The human placenta expresses mRNA for both IGF-I and II and placental cells carry IGF receptors (Fant *et al.*, 1986). Binding sites for IGF-I and -II are different and, in the placenta, two distinct IGF-I receptors are present suggesting the possibility of tissue-specific isoforms of the IGF-I receptor (Petraglia *et al.*, 1990a). IGFs and insulin increased

hPL release from placental cell cultures and are thought to have a role in growth and development. IGF receptor levels are higher in early placenta, suggesting IGFs regulate the early phase of foetal growth, with insulin influencing later growth (Petraglia *et al.*, 1990a).

High levels of EGF receptors have been detected in the human placenta (Lai and Guyda, 1984), although there is no evidence of placental EGF synthesis. Placental EGF receptor levels are significantly higher in early pregnancy than in term pregnancy (Maruo *et al.*, 1987), correlating with differentiation of cytotrophoblast into syncytiotrophoblast. In early placental cell cultures, EGF was noted to induce differentiation, resulting in increased hCG and hPL release (Morrish *et al.*, 1987).

Frolik *et al.* (1983) purified TGF- β from human placenta, which inhibited trophoblast differentiation in monolayer cultures (Morrish *et al.*, 1991) in a dose-dependent manner (Graham *et al.*, 1992).

Basic FGF is present in human placenta (Gospodarowicz *et al.*, 1985) and stimulates synthesis of hCG β -subunit (Petraglia *et al.*, 1990a). FGF stimulates angiogenesis in other tissues and may be associated with the high degree of neovascularisation during the initial stages of placental development.

The first trimester placenta actively expresses *sis*-proto-oncogene, which is the structural gene for a component of PDGF (Goustin *et al.*, 1985) and PDGF expression was demonstrated throughout pregnancy, with especially high levels at mid-trimester (Taylor and Williams, 1988).

1.4 PLACENTAL DEVELOPMENT AND DIFFERENTIATION

In the human, the blastocyst attaches to the endometrium after 7 days of gestation (Moll and Lane, 1990). Implantation mimics neoplastic behaviour but is highly regulated, with invasive activity peaking at about 12 weeks gestation and declining rapidly thereafter. The placenta derives from trophoblast of the blastocyst and differentiates into either villous or extravillous trophoblast (Ringler and

Strauss, 1990). The latter forms the main invasive element and anchors the placenta. Cytotrophoblast and syncytiotrophoblast cells are different lineages of the same trophoblast cell type. During pregnancy a permanent differentiation process from cytotrophoblast to syncytium occurs (Fant *et al.*, 1986; Kliman *et al.*, 1986). Cytotrophoblast cells of the chorionic villi fuse to form syncytiotrophoblast, the main source of placental hormones such as hCG and hPL. Growth of the placenta is most rapid during the first trimester, with replication of cytotrophoblast cells under stimulation of growth factors such as IGF-II and PDGF (Ohlsson, 1989; Taylor and Williams, 1988). Maturing cytotrophoblasts lose mitotic activity and then fuse to form multinucleated syncytiotrophoblast, terminally differentiated trophoblast cells. During the second and third trimesters, placental growth slows and the relative ratio of syncytiotrophoblast to cytotrophoblast increases dramatically (Ringler and Strauss, 1990).

1.5 AIMS OF THIS STUDY

Previous investigations of GnRH in the human placenta have focussed on a limited range of gestational ages. Moreover, early trophoblast from suction terminations tended to be contaminated with foetal tissue. Medical termination of pregnancy leads to the expulsion of the intact gestational sac and we therefore investigated placental GnRH activity in normal placentae from terminations of pregnancy from 6-18 weeks and from full-term uncomplicated deliveries.

Preliminary investigations of the nature of high molecular weight mitogenic and GnRH-like activities were undertaken.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Chemicals and reagents were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England, or from BDH Chemicals Ltd., Poole, Dorset, England, unless indicated.

2.2 PREPARATION OF HUMAN PLACENTAL MEMBRANES

2.2.1 Collection of Tissue

Placentae were collected from the Simpson Memorial Maternity Pavilion, Edinburgh, Scotland, and were transferred to the laboratory on ice. Preparation of the placental tissue was carried out at 4°C.

Term placental tissue was obtained from uncomplicated pregnancies after vaginal delivery at 37-42 weeks gestation. Pre-term placental tissue was obtained after therapeutic abortion carried out between 6 and 18 weeks gestation. Early pregnancies (6-9 weeks) were terminated by vacuum aspiration of the conceptus or by pre-treatment with the anti-gestagen mifepristone (Mifegyne[®], 600mg orally; Roussel Laboratories Ltd., Uxbridge, Middlesex, England) followed by medical induction of labour with gemeprost, a prostaglandin E₁ analogue (Cervagem[®], 1mg vaginal pessary; May & Baker, Dagenham, Essex, England) (Cameron and Baird, 1988). Early pregnancy is dependent on progesterone, which is essential for successful implantation of the embryo and for the maintenance of the endometrium and quiescence of the myometrium. Mifepristone has a high binding affinity for the progesterone receptor but is devoid of progestational agonist activity and has been used for the medical induction of early pregnancy, although mifepristone alone is associated with a high incidence of incomplete abortion (Avrech *et al.*, 1991). Analogues of prostaglandins elicit powerful uterine contractions and induce complete abortion in over 90% of women. However, their widespread use is limited by unpleasant side effects. As mifepristone sensitises the uterus to prostaglandins,

mifepristone and prostaglandins in combination allow the dose of prostaglandin to be reduced, minimising side effects (World Health Organization, 1991).

From 9 to 18 weeks gestation, pregnancies were terminated by prostaglandin induction with gemeprost vaginal pessaries. Vaginal pessaries (1mg) were inserted every 3 hours until the conceptus was expelled or until 5 pessaries had been given. If abortion had not occurred within 24 hours of the first pessary, a maximum of 5 further pessaries was given (Rodger and Baird, 1990). All second trimester placental tissue was obtained within 48 hours of the first pessary.

2.2.2 Preparation of Placental Membranes

2.2.2a Variation of the Method of Currie *et al.* (1981)

Trophoblast was dissected from maternal membranes, washed in ice-cold PBS (Flow Laboratories, Irvine, Scotland) to remove blood, blotted on filter paper and weighed. Term placental cotyledons were teased apart with forceps to remove large blood vessels, and placental villi were minced finely with scissors. Minced tissue was homogenised (1g tissue per 2ml) in either PBS or isotonic SET (0.3M Sucrose - 1mM EDTA - 15mM Tris, pH 7.4) using a Polytron homogeniser (2 x 10 seconds at setting 8 interspersed with 1 minute on ice). Cellular debris was pelleted by centrifugation at 1,000xg for 15 minutes at 4°C, and placental membranes were obtained by centrifugation of the supernatant at either 30,000xg or 100,000xg for 1 hour at 4°C in a Sorvall OTD50 ultracentrifuge. Membrane pellets were gently re-suspended in SET by gentle homogenisation with a loose fitting glass Dounce hand homogeniser and stored at -70°C. Supernatants from the ultracentrifugation step were stored at -70°C as placental extract.

Trophoblast prepared in the presence of protease inhibitors was processed as above in SET containing 2mM EDTA, 2mM N-ethyl maleimide, 2mM PMSF and 1.46µM Pepstatin A. Membrane pellets were resuspended in SET containing the same concentrations of protease inhibitors.

2.2.2b Variation of the Method of Smith and Brush (1978)

Cotyledons from different regions of term placenta were dissected free from maternal membranes, rinsed in PBS, blotted and weighed. Tissue was spread out and agitated gently in ice-cold 0.9% (w/v) NaCl for 30 minutes with a magnetic stirrer, ensuring the chorionic villi were well irrigated. The saline wash was centrifuged at 1,000xg for 15 minutes at 4°C and the supernatant re-centrifuged at 30,000xg for 1 hour at 4°C. The placental membrane fraction was resuspended in SET and stored at -70°C.

2.3 PREPARATION OF RAT PITUITARY HOMOGENATES

Pituitaries were excised from immature (28-35 day old) Sprague-Dawley rats, following CO₂ asphyxiation, and homogenised (2 glands per ml) in isotonic SET using a glass Dounce homogeniser. Aliquots were stored at -20°C until required.

2.4 GnRH ISOFORMS AND AGONIST ANALOGUES

The GnRH agonist analogue Buserelin was the gift of Dr J Sandow, Hoechst AG, Frankfurt, Germany. Chicken GnRH-I and -II were obtained from Peninsula Laboratories, Belmont, California, USA, and mammalian GnRH from Ayerst Laboratories Ltd, Andover, Hants, England. Salmon and lamprey isoforms of GnRH were the gifts of Dr J King, MRC Regulatory Peptides Research Unit, University of Cape Town, RSA.

2.4.1 Iodination of GnRH Isoforms and Agonist Analogues

GnRH isoforms and synthetic agonist analogues were labelled with radio-iodine using a modification of the glucose oxidase/lactoperoxidase method of Miyachi *et al.* (1973). 5µg of hormone or analogue was incubated in a 75 x 12mm borosilicate glass tube for 4 minutes at room temperature with 0.2M phosphate buffer, pH 7.4; 0.5µg lacto-peroxidase in 0.1M sodium acetate, pH 5.6; 5U glucose oxidase (Miles

Laboratories Inc., Elkhart, Indiana, USA); 0.1% (w/v) glucose; and 1mCi radio-labelled sodium iodide (Amersham International plc, Amersham, Bucks., England). By this iodination method, peroxide is generated by the glucose/glucose oxidase system, to avoid potential damage to the hormone or analogue by addition of relatively high concentrations of hydrogen peroxide to the reaction. In the presence of peroxide, lactoperoxidase oxidises radio-iodide to radio-iodine and tyrosine residues are radio-iodinated.

The reaction was stopped with 500 μ l 10mM acetic acid and the reaction products were isolated, as described below.

2.4.2 Purification of Iodinated GnRH Isoforms and Agonist Analogues

The iodination products were transferred to a 1 x 45cm Sephadex G25 (Fine) column (Pharmacia Ltd., Milton Keynes, Bucks., England) equilibrated with 0.1% (w/v) BSA in 10mM acetic acid. The column was pre-adsorbed with 5% (w/v) BSA in 10mM acetic acid, to minimise non-specific binding of the reaction products to the column. Fractions were eluted with 10mM acetic acid-0.1% (w/v) BSA and 2ml aliquots were collected in glass tubes containing 10mM acetic acid-5% (w/v) BSA. Elution of radioactivity was determined with a Mini-assay Type 6-20 counter.

All GnRH isoforms and agonist analogues studied gave a characteristic elution profile of 4 peaks of radioactivity (Figure 2.1). Peak I corresponded to a high molecular weight component which eluted in the void volume of the column, V_0 , and peak II was un-incorporated radio-iodide which eluted in the total column volume, V_t . Elution of peaks III and IV was retarded. Relative elution volumes (REV) were calculated, using the formula $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the peak. Peak III eluted at a similar position (REV = 2.6 ± 0.2) for Buserelin, Tryptorelin and chicken GnRH-II (mean \pm s.d.; n=11), and for other GnRH isoforms (Bramley *et al.*, 1992). However, peak IV was retarded to different degrees for the various tracers (Figure 2.1). For Buserelin, REV_{IV} was 3.9 ± 0.7 (mean \pm s.d.; n=5). Peak IV from

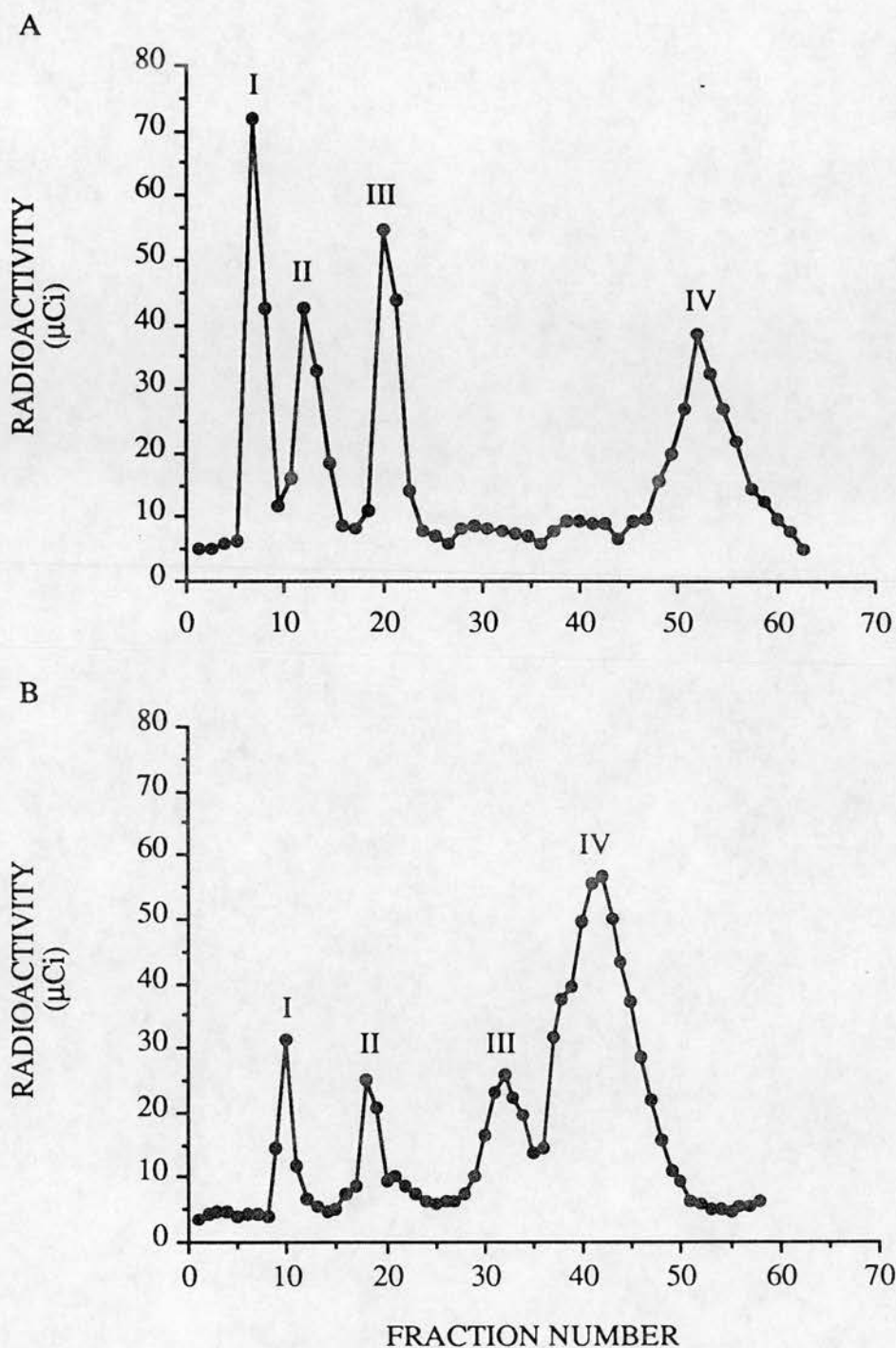


Figure 2.1 Representative elution profiles for products of GnRH iodination

GnRH isoforms and agonists were radiolabelled by the lactoperoxidase method and purified on 1 x 45cm Sephadex G25 (Fine) column, eluted with 10mM acetic acid-0.1% (w/v) BSA. Fractions were collected and counted. Four peaks of radioactivity were detected, with active iodinated hormone eluting in peak IV. Data shown were representative elution profiles of the iodination products for chicken GnRH-II (A) and Buserelin (B).

Tryptorelin and chicken GnRH-II iodinations was retarded more strongly on the column with REV_{IV} of 8.9 ± 0.2 (mean \pm s.d.; n=3) and 8.0 ± 1.0 (mean \pm s.d.; n=4), respectively.

The binding activity of the peaks of radioactivity from agonist iodinations was determined in the rat pituitary and human placental receptor binding assays (Bramley *et al.*, 1992). Peak III bound poorly to GnRH receptors, while Peak IV bound well. A similar profile of binding activity was observed for GnRH isoforms using an anti-GnRH antibody (EL-14) to determine activity. When subjected to ion-exchange chromatography on Sephadex QAE-A25, peak IV was not retarded, whereas peak III bound strongly, suggesting this iodinated species carried a strong negative charge. Sharpe and Fraser (1980) suggested that peak III represented di-iodinated, inactive GnRH and peak IV, mono-iodinated active hormone. However, thin layer chromatography of peaks III and IV suggested that both peaks contained mono-iodinated GnRH (Bramley *et al.*, 1992). Therefore, peak III appeared to represent a charged, mono-iodinated form of GnRH, with poor binding activity. Fractions containing active, mono-iodinated GnRH (Peak IV) were pooled and stored at -20°C .

The elution of unlabelled hormone, as determined by inhibition of binding of ^{125}I -GnRH agonist binding to rat pituitary receptors in the presence of aliquots of Sephadex G25 fractions, was always well separated from the active tracer peak (Bramley *et al.*, 1992).

2.5 MEASUREMENT OF ^{125}I -GnRH BINDING ACTIVITY

2.5.1 ^{125}I -GnRH Binding to Placental Membrane Preparations

Placental membrane preparations ($\sim 1\text{mg}$ protein per tube, unless stated) were incubated in $12 \times 75\text{mm}$ polystyrene tubes with $100,000\text{cpm}$ iodinated GnRH agonist or isoform tracer, in the presence and absence of 10^{-5}M unlabelled Buserelin. Incubations were carried out in triplicate, in a total volume of 1.0ml with 40mM Tris-HCl, pH 7.4, containing 0.5% (w/v) BSA, for 1 hour at 20°C .

After incubation, bound and free hormone were separated by precipitation with ice-cold 0.5ml 0.5% (w/v) IgG and 1ml 25% (w/v) PEG at 4°C, followed by centrifugation at 2,500xg for 10 minutes at 4°C (Bramley *et al.*, 1985). Supernatants were aspirated and radioactivity bound to the pelleted membranes was determined using a Packard Crystal 5400 Series Multidetector Gamma Counting System with a counting efficiency of 75%.

Specific binding of ^{125}I -GnRH agonist was determined by subtracting radioactivity bound in the presence of 10^{-5}M unlabelled hormone (non-specific binding) from binding in the absence of unlabelled hormone (total binding). Specific binding, corrected for membrane protein content, was expressed as % binding of the total radioactivity added to the incubation to allow comparison between assays and GnRH tracers.

Controls without tissue \pm unlabelled hormone were always included to correct for binding of tracer to the assay tubes and its displacement by high concentrations of cold agonist. This was always found to be negligible (< 1% of total counts added).

2.5.2 ^{125}I -GnRH Binding to Rat Pituitary Homogenates

Binding activity of iodinated GnRH analogues to rat pituitary homogenates was determined as described in Section 2.5.1, except incubation was for 4 hours at 4°C and non-specific binding was determined in the presence of 10^{-6}M unlabelled Buserelin.

2.5.3 Determination of the Specific Activity of ^{125}I -GnRH

The specific activity of GnRH agonist tracers was determined by self-displacement from rat pituitary homogenates. Self-displacement curves were obtained by incubating immature rat pituitary homogenates, as described in Section 2.5.2, with increasing concentrations of unlabelled hormone to determine the concentration of

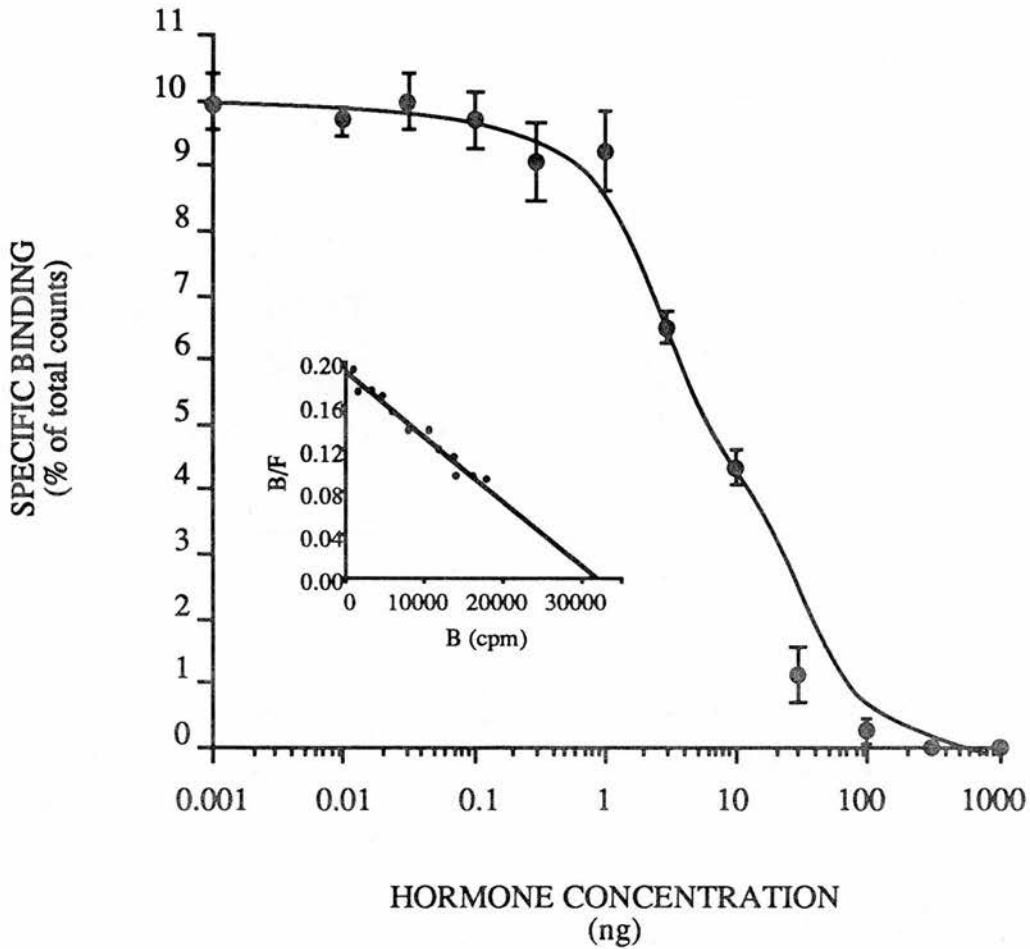


Figure 2.2

Binding inhibition curve for Buserelin

Aliquots of rat pituitary homogenate were incubated at 4°C for 4 hours with 100,000cpm iodinated Buserelin in the presence of increasing concentrations of unlabelled Buserelin.

Bound and free hormone were separated by PEG precipitation and binding determined. Binding was expressed as % of total counts added, corrected for non-specific binding to assay tubes.

Inset: Scatchard plot of the binding data.

Data shown were means \pm s.d. of triplicate estimates from a single representative experiment.

unlabelled hormones required to reduce specific binding by 50%, i.e. $IC_{50\text{ cold}}$ (Figure 2.2).

In addition, specific binding of ^{125}I -labelled GnRH agonist to rat pituitary homogenate was measured at a range of tracer concentrations in the absence of unlabelled GnRH analogue. Bound hormone was recovered by PEG precipitation and (hormone bound)/(total hormone added), [B/T], was plotted against \log_{10} (hormone added). The concentration of tracer required to decrease [B/T] to 50% was calculated ($IC_{50\text{ hot}}$) and the specific activity of the tracer was calculated by $IC_{50\text{ hot}}/IC_{50\text{ cold}}$, and expressed as $\mu\text{Ci}/\mu\text{g}$. Specific activity for GnRH agonist binding to rat pituitary assays ranged from 160-880 $\mu\text{Ci}/\mu\text{g}$ (Bramley *et al.*, 1992).

Specific activity of GnRH isoforms was determined using self-displacement from the anti-GnRH antibody EL-14 and ranged from 60-1104 $\mu\text{Ci}/\mu\text{g}$ (Bramley *et al.*, 1992).

Scatchard plots for GnRH agonist binding to rat pituitary homogenate indicated the presence of a single class of binding sites with affinity constants (K_a) similar to previously published data (Figure 2.2, inset).

2.6 MEASUREMENT OF ^{125}I -GnRH INACTIVATION

GnRH isoform and agonist analogue re-binding to placental membrane preparations was determined by incubating excess tracer (300,000cpm) with placental membranes in the presence and absence of 10^{-5}M unlabelled Buserelin, as in Section 2.5.1. Bound and free tracer were separated by high speed centrifugation (30,000 $\times g$) for 10 min at 4°C and the supernatant was carefully aspirated. Specific binding to the placental membranes was determined and expressed as % of total counts added. Supernatant was adjusted with Tris-BSA buffer to give 100,000cpm per tube and was re-incubated with fresh placental membranes for 1 hour at 20°C and bound and free hormone were separated by IgG/PEG precipitation, as described in Section 2.5.1. 10^{-5}M unlabelled Buserelin was added to the supernatant from membranes incubated in

the presence of unlabelled Buserelin in the first incubation, to measure non-specific binding in the second incubation. Specific binding was expressed as % of total counts and tracer inactivation (decrease in binding following first incubation) was determined.

2.7 MEASUREMENT OF ¹²⁵I-EGF BINDING ACTIVITY TO PLACENTAL MEMBRANE PREPARATIONS

Unlabelled mouse EGF and ¹²⁵I-EGF were the generous gift of Dr K. Brown, AFRC Institute of Animal Physiology, Babraham, Cambridge, England.

Membrane protein (~0.1mg) was incubated in 12 x 75mm polystyrene tubes with 50,000cpm ¹²⁵I-EGF in 1.0ml total volume with 40mM Tris-HCl, pH 7.0, containing 1% (w/v) BSA and 2mM manganese chloride. Non-specific binding was determined in the presence of 100ng EGF. After incubation at 37°C for 3 hours, bound tracer was precipitated with IgG/PEG, separated from free by centrifugation and counted, as described in Section 2.5.1.

2.8 ANTI-CHICKEN GnRH-II ANTIBODY BINDING ASSAY

Anti-chicken GnRH-II antisera (chII-Ab) was kindly gifted by Dr P. Sharp, The Roslin Institute, Roslin, Midlothian, Scotland (Sharp *et al.*, 1987; 1989). This antibody was specific for chicken GnRH-II: the synthetic GnRH analogues, Buserelin and Tryptorelin, were not recognised by the antibody (Figure 2.3a), and ¹²⁵I-chicken GnRH-II was not displaced from chII-Ab by the mammalian and chicken GnRH-I isoforms of GnRH (Figure 2.3b). Although displacement was observed in the presence of salmon and lamprey GnRH, half maximal activity was of the order of 2,500 and 25,000 times the 50% self-displacement of chicken GnRH-II, respectively.

The effects of placental cytosol on binding of ¹²⁵I-chicken GnRH-II to antibody were determined by incubating ~30,000cpm ¹²⁵I-chicken GnRH-II with antibody in a total volume of 1ml with 40mM Tris-HCl containing 0.1% (w/v) BSA, pH 7.4, for 16 hours at 4°C, in the presence or absence of increasing concentrations of

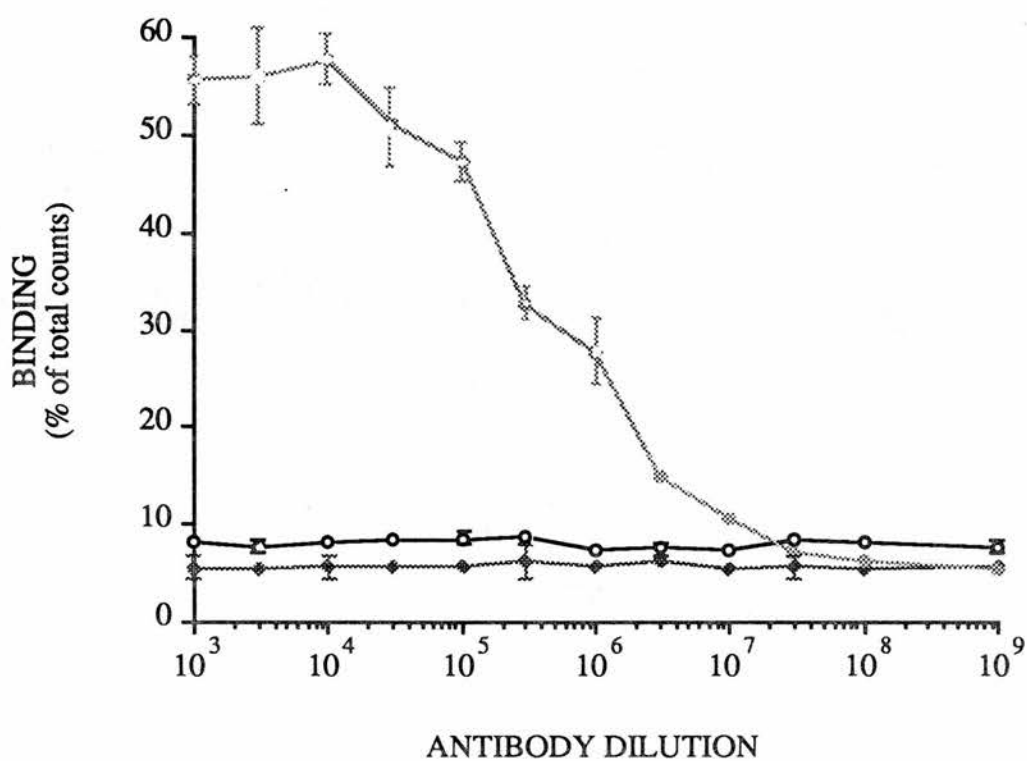


Figure 2.3a Binding of iodinated Buserelin, Tryptorelin and chicken GnRH-II to anti-chicken GnRH-II antibody

Antibody dilutions from 1:1000 to 1:1,000,000,000 were incubated with ~30,000cpm iodinated Tryptorelin (○), Buserelin (◆) and chicken GnRH-II (◐) in 0.1% (w/v) BSA-40mM Tris-HCl, pH 7.4, at 4°C for 16h.

Bound and free tracer were separated by PEG precipitation and tracer-antibody complexes in the pellet determined. Binding was expressed as % of total counts.

Data shown were means ± s.d. of triplicate determinations from a single representative experiment.

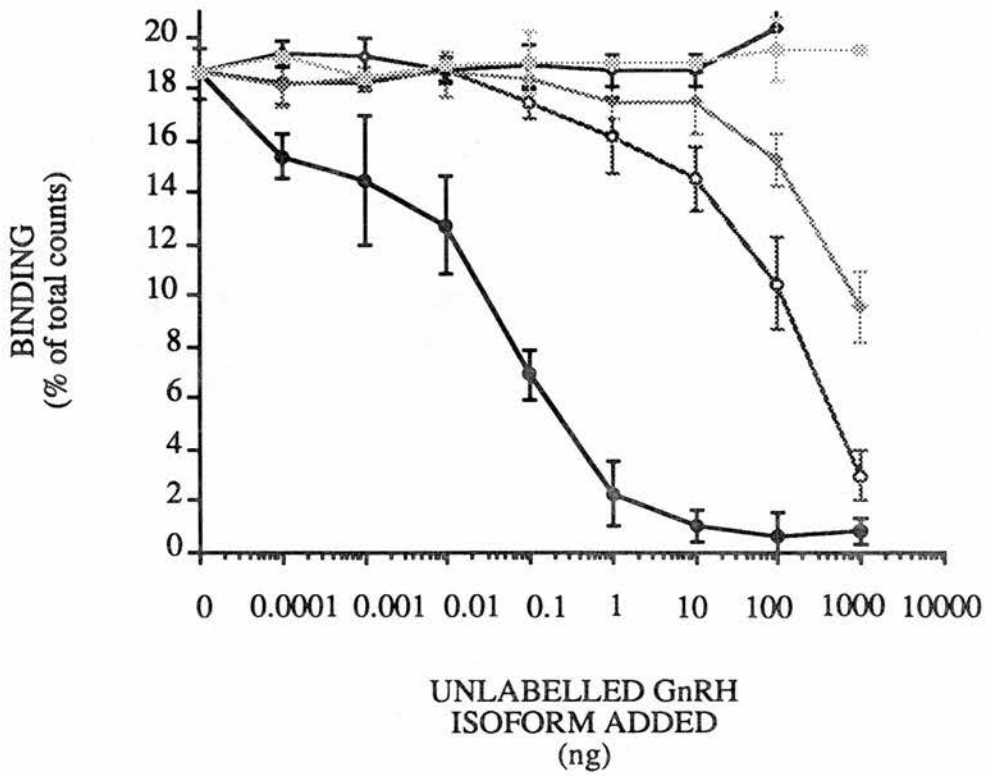


Figure 2.3b Displacement of chicken GnRH-II from anti-chicken GnRH-II antibody by GnRH isoforms

Antibody (1:20,000 dilution) was incubated with ~30,000cpm iodinated chicken GnRH-II in 0.1% (w/v) BSA-40mM Tris-HCl, pH 7.4, in the presence of increasing concentrations of unlabelled chicken GnRH-I (◇) and -II (●), salmon GnRH (○), lamprey GnRH (※) and mammalian GnRH (⊞) for 16h at 4°C. Bound and free hormone were separated by PEG precipitation and binding to antibody determined and expressed as % of total counts added.

placental cytosol. The bound and free tracer were separated by PEG precipitation and tracer recovered in the pellet determined using a Packard Crystal 5400 Series Multidetector Gamma Counting System.

2.9 DETERMINATION OF CYTOSOL BINDING BY DEXTRAN-COATED CHARCOAL (DCC) SEPARATION

Placental cytosol was incubated with ~30,000cpm ¹²⁵I-chicken GnRH-II in 1ml (total volume) 0.1% (w/v) BSA - 40mM Tris-HCl, pH 7.4 for 2 hours at 4°C. Bound and free tracer were separated by addition of 500µl DCC (0.25% (w/v) charcoal and 0.025% (w/v) dextran in 0.1% (w/v) BSA - 40mM Tris-HCl, pH 7.4) and centrifugation at 2,500xg for 15 minutes at 4°C. Supernatant, containing radioactivity not adsorbed to DCC, was decanted and counted in a Packard Crystal 5400 Series Multidetector Gamma Counting System.

2.10 DETERMINATION OF PROTEIN

The concentration of protein in placental membrane and cytosol preparations was determined by the method of Lowry *et al.* (1951). 5-10µl of membrane or cytosol was assayed in alkaline copper solution with Folin-Ciocalteu phenol reagent, using crystalline bovine albumin as standard. A coloured complex was formed between the alkaline copper-phenol reagent and tyrosine and tryptophan residues of the protein molecules.

Absorbance at 750nm was measured using a Pye Unicam SP6-500 UV spectrophotometer and protein values were expressed as mg protein/100µl. Assay limit of detection was 10µg protein per tube.

2.11 DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY

The method of Bessey *et al.* (1946) measured hydrolysis of colourless *p*-nitrophenol phosphate by alkaline phosphatase in serum samples, resulting in the liberation of yellow *p*-nitrophenol.

Using a modification of this method, ~0.05mg placental membrane protein was incubated with 3mM *p*-nitrophenol phosphate (substrate) at 37°C for 15 minutes, in 1.0ml total volume with 0.2M piperazine-HCl at pH 10.3, containing 1mM magnesium acetate and 0.01mM zinc sulphate. The reaction was stopped by addition of 3ml 0.1N NaOH-1mM EDTA solution, and *p*-nitrophenol liberated was measured by absorbance at 420nm using a Pye Unicam SP6-500 UV spectrophotometer.

2.12 STATISTICAL ANALYSIS

Statistical significance of differences between means was estimated by Student's t-test.

CHAPTER 3

GnRH BINDING SITES IN THE HUMAN PLACENTA

3.1 INTRODUCTION

Placental binding sites for GnRH differ from pituitary GnRH receptors in their affinity and specificity for the hormone and its agonist analogues. In the pituitary, the GnRH analogue Buserelin ([D-Ser(tBu)⁶]-des-Gly¹⁰-GnRH N-ethylamide) is a superagonist with an affinity constant of $4.8 \times 10^9 \text{ M}^{-1}$, approximately ten times the receptor affinity for mammalian GnRH, the native hormone (Clayton and Catt, 1980). However superagonist analogues do not possess higher binding affinity for the placental binding site, which has been found to have affinities in the range $5.5 \times 10^5 - 6.2 \times 10^7 \text{ M}^{-1}$, similar to values quoted for the native hormone (Currie *et al.*, 1981; Bélisle *et al.*, 1984; Iwashita *et al.*, 1986). In addition, certain isoforms of GnRH will bind specifically to placental but not to pituitary membranes (Bramley, 1989).

While the human placenta expresses mRNA for GnRH (Seeburg and Adelman, 1984) and a physiologically active decapeptide identical to the hypothalamic hormone has been isolated from placental extracts (Lee *et al.*, 1981; Tan and Rousseau, 1982), the major GnRH-like activity in the placenta may differ from the native hormone (Siler-Khodr, 1987). Differential transcription of the GnRH gene in the hypothalamus and placenta gives rise to an unusually long 5'- untranslated region of placental GnRH mRNA. In the placenta, the first intron is not removed from the primary gene transcript, suggesting that different promoters are involved in GnRH expression in the two tissues (Adelman *et al.*, 1986; Radovick *et al.*, 1990). If endogenous placental GnRH is structurally different from the hypothalamic decapeptide, then this may explain the comparatively low affinity of the placental receptor for the native hormone (and agonist analogues derived from it).

Previous studies have used variations of the pituitary radio-receptor assay to measure GnRH agonist binding to placental membrane preparations (Currie *et al.*, 1981; Bélisle *et al.*, 1984; Iwashita *et al.*, 1986), with incubations of 60 - 90 minutes at 20 - 22°C in the presence of 10^{-5}M unlabelled agonist used for placental binding assays. Placental membranes tended to be prepared by differential centrifugation of

homogenised tissue with various refinements introduced to attempt to maximise binding affinity. Iwashita *et al.* (1986) homogenised the tissue in buffer containing the kallikrein inhibitor aprotinin, and Bélisle *et al.* (1984) obtained a 30-fold biochemical enrichment of the membrane preparation by sedimentation on a continuous sucrose density gradient without improving the low affinity binding observed by Currie *et al.* (1981). It was therefore necessary to validate the binding assay and placental preparation methods used in this study, prior to further investigation of the hormone binding site.

Unless otherwise stated, the GnRH binding assay used was the method described in 2.5.1 and the placental preparation method used was the variation on the method of Currie *et al.* (1981), as described in 2.2.2a.

3.2 BINDING ACTIVITY OF ¹²⁵I-BUSERELIN TO TERM PLACENTAL MEMBRANES

3.2.1 Effect of Time and Temperature of Incubation on ¹²⁵I-Buserelin Binding to Term Placental Membranes

Term placental membranes were incubated with ¹²⁵I-Buserelin at 4°C, 20°C and 37°C for up to 6 hours, to determine the stability of the receptor and/or ligand.

From Figure 3.1, specific binding was dependent on both the time and temperature of the incubation. At 4°C, binding reached maximal levels slowly, and considerable inter-assay variation was observed in the maximal binding levels achieved and the duration of incubation required to achieve equilibrium. Ligand-receptor association was rapid at 37°C, peaking in under 30 minutes, but quickly fell to indeterminable levels. At 20°C, however, equilibrium was reached after 30 minutes and binding remained stable for up to 90 minutes incubation, before slowly declining. Therefore, incubation of 20°C for 60 minutes was selected as standard to assay for placental binding, to give optimal binding of GnRH analogue to placental membranes.

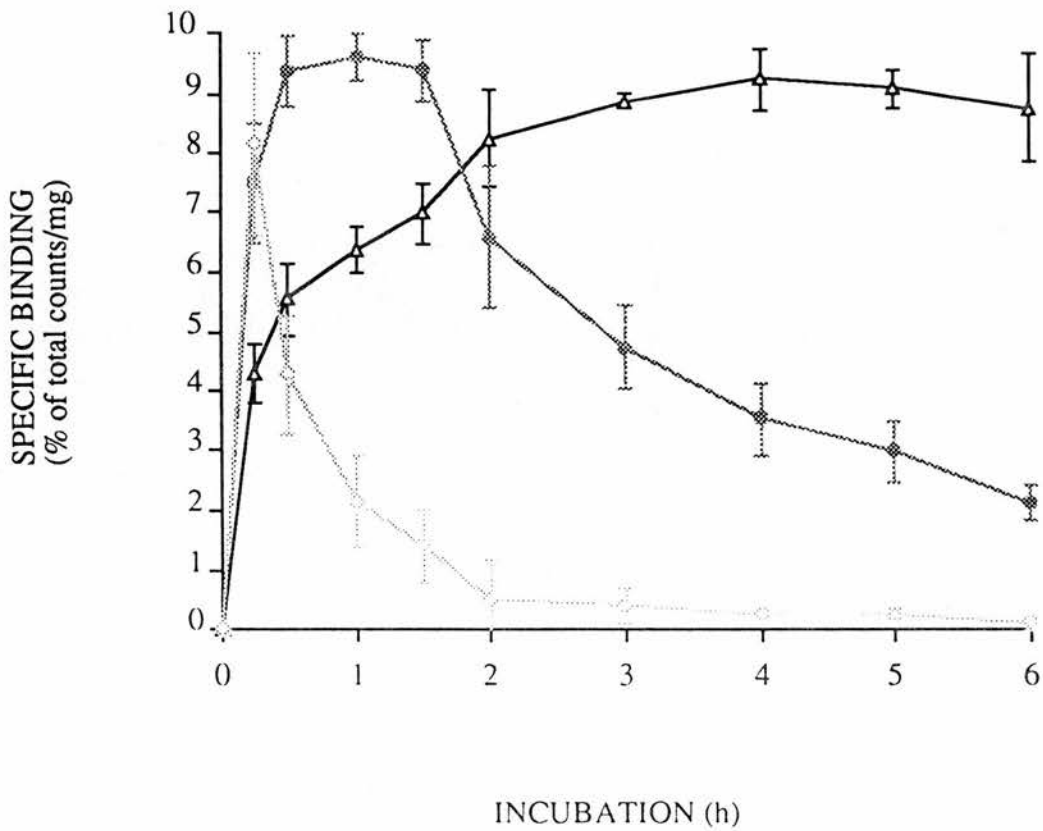


Figure 3.1 Effect of time and temperature of incubation on the binding characteristics of radio-labelled Buserelin

Term placental membranes (~1mg protein/tube) were incubated with 100,000cpm radio-labelled Buserelin in the presence and absence of 10 μ M uniodinated Buserelin at 4°C (Δ), 20°C (\bullet) and 37°C (\diamond) for up to 6h. Bound and free hormone were separated by PEG precipitation, specific binding was determined and expressed as % of total counts, corrected for protein concentration. Data shown were from a representative term placenta; points are mean \pm s.d. for one experiment in triplicate.

3.2.2 Effect of Pre-incubation of Placental Membranes on ¹²⁵I-Buserelin Binding

Term placental membranes were incubated at 4°C, 20°C and 37°C for up to 6 hours prior to assay, as described in 2.5.1, for ¹²⁵I-Buserelin binding (Figure 3.2).

Binding of ligand to membranes was not affected by up to 6 hours pre-incubation at 4°C, when compared to controls thawed and assayed immediately. At 20°C and 37°C, binding was stable for up to 2 hours pre-incubation, decreasing after 6 hours to 79% and 37% of control binding, respectively.

All placental membranes were frozen after preparation until required for assay, when aliquots were thawed at room temperature and stored on ice, typically for less than 10 minutes, before adding to the incubate. From Figure 3.2, membrane binding was unaffected by this procedure. As all buffers were equilibrated to 20°C before assay, the addition of small volumes of ice-cold membranes had a negligible effect on the temperature of the binding assay.

3.2.3 Effect of Method of Tissue Preparation on ¹²⁵I-Buserelin Binding

3.2.3a Preparation by variation of the method of Currie *et al.* (1981)

Placental membranes were prepared from individual term placentae by the method of Currie *et al.* (1981), by homogenisation in PBS and centrifugation for 1 hour at 100,000xg, or by homogenisation in SET and centrifugation for 1 hour at 30,000xg or 100,000xg (for details see 2.2.2a). Specific binding of ¹²⁵I-Buserelin tracer to the three types of membrane preparations was compared by assay as described in 2.5.1.

No significant difference between the binding activity of membranes prepared in SET or PBS was found (Table 3.1), allowing comparison of membranes prepared under any of these conditions, although SET appeared to facilitate membrane pellet formation at a lower centrifuge speed, as binding per mg protein did not increase in membranes sedimented by greater centrifugal force.

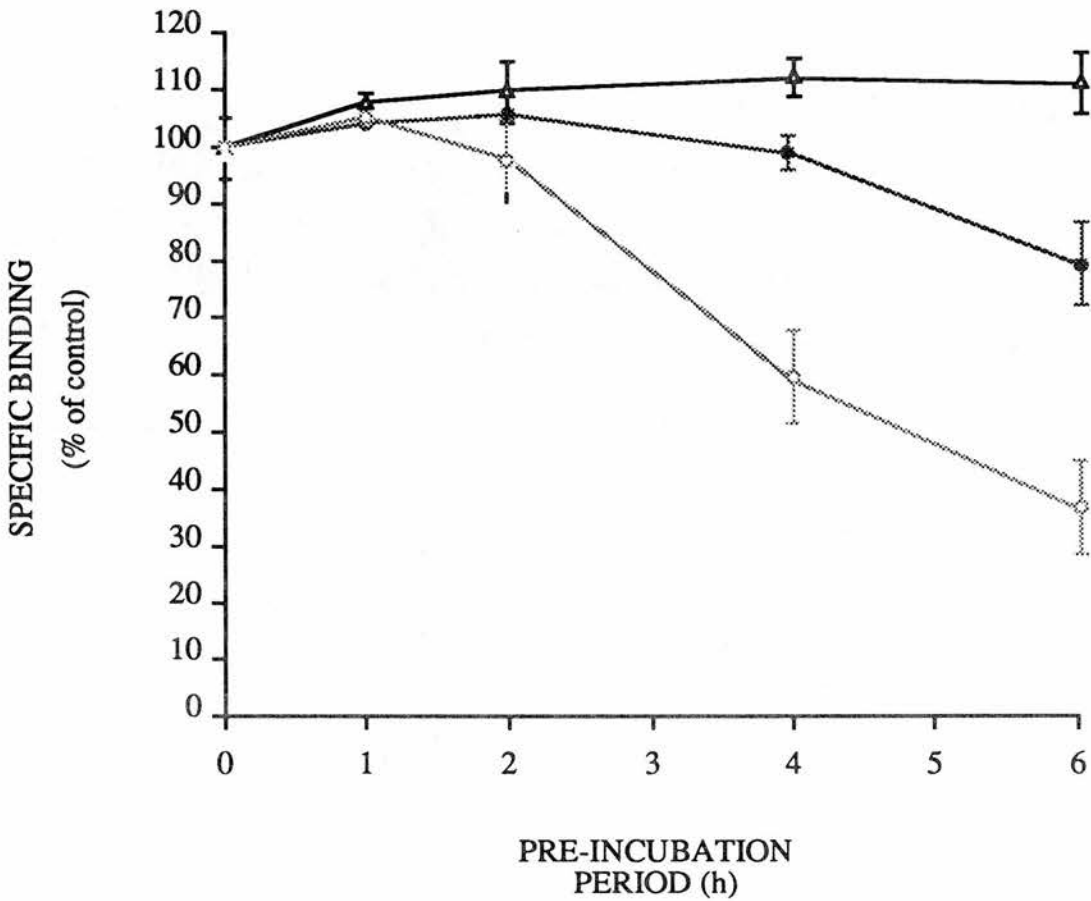


Figure 3.2 Effect of pre-incubation of membranes on binding of radio-labelled Buserelin

Term placental membranes (~1mg protein) were incubated at 4°C (▲), 20°C (◆) and 37°C (⊙) for up to 6h, then incubated for 1h at 20°C with 100,000cpm radiolabelled Buserelin, in the presence and absence of 10μM uniodinated Buserelin.

Bound and free hormone were separated by PEG precipitation and specific binding was determined. Specific binding was corrected for protein, and expressed as % of control binding (no pre-incubation).

Data shown were mean ± s.d. of triplicate determinations from a representative term placenta.

METHOD (n)	I (9)	II (3)	III (6)
SPECIFIC BINDING (% total counts/mg)	15.9 ± 1.6	15.2 ± 1.3	17.1 ± 2.1
protein (mg)	0.941 ± 0.075	1.002 ± 0.080	0.897 ± 0.249

Table 3.1 Comparison of method of membrane preparation: variation of the method of Currie *et al.* (1981)

Membranes were prepared from a term placenta, by the method of Currie *et al.* (1981), homogenised in SET and centrifuged at 30,000xg (method I) or 100,000xg (method II) or homogenised in PBS and centrifuged at 100,000xg (method III). Placental membranes (~1mg protein) were incubated with 100,000cpm ¹²⁵I-Buserelin for 1h at 20°C, in the presence and absence of 10µM uniodinated Buserelin. Bound and free hormone were separated by PEG precipitation and specific binding determined. Specific binding was expressed as % of total counts added, corrected for protein concentration. By student's t-test, binding was not significantly different. Data represents mean ± s.d.; n = number of binding assays.

METHOD (n)	I (5)	II (5)	III (5)
SPECIFIC BINDING (% total counts/mg)	3.8 ± 0.5	3.8 ± 0.3	4.0 ± 0.4
protein (mg)	1.042 ± 0.282	1.065 ± 0.220	1.017 ± 0.190

Table 3.2 Comparison of method of membrane preparation: variation of the method of Smith and Brush (1978)

Membranes were prepared from a term placenta by the method of Currie *et al.* (1981), homogenised in SET and centrifuged at 30,000xg (method I) or homogenised in PBS and centrifuged at 100,000xg (method II) or by the method of Smith and Brush (1978) in SET and centrifuged at 30,000xg (method III). Placental membranes (~1mg protein) were incubated with 100,000cpm ¹²⁵I-Buserelin for 1h at 20°C, in the presence and absence of 10µM uniodinated Buserelin. Bound and free hormone were separated by PEG precipitation and specific binding determined. Specific binding was expressed as % of total counts added, corrected for protein concentration. By student's t-test, binding was not significantly different. Data represents mean ± s.d.; n = number of binding assays.

3.2.3b Comparison of membrane preparation by the methods of Currie *et al.* (1981) and Smith and Brush (1978)

Cotyledons from individual term placentae were processed by the method of Currie *et al.* (1981) by homogenisation in PBS and centrifugation for 1 hour at 100,000xg, or by homogenisation in SET and centrifugation for 1 hour at 30,000xg (2.2.2a), or by the method of Smith and Brush (1978) as described in 2.2.2b.

Membranes prepared by all three methods demonstrated similar levels of specific GnRH agonist binding per mg of protein (Table 3.2). No enrichment of the membrane binding site was observed in NaCl-washed preparations, and placental membranes were routinely prepared by the former method.

3.2.4 Effect of Placental Membrane Protein Concentration on ¹²⁵I-Buserelin Binding

Increasing concentrations of term placental membranes were incubated with ¹²⁵I-Buserelin under standard assay conditions (see 2.5.1).

Total binding, non-specific binding and specific binding of Buserelin increased with protein concentration in a linear manner at low concentrations (Figure 3.3). However, at protein concentrations greater than ~1.5mg, binding began to plateau. When binding was expressed as specific binding/mg protein, the non-linear behaviour of high levels of protein had an appreciable effect on interpretation of specific binding activity and so assays were routinely standardised to 1.0 (\pm 0.2) mg protein.

3.2.5 Effect of Uniodinated Buserelin on ¹²⁵I-Buserelin Binding to Placental Membranes

The affinity of ¹²⁵I-Buserelin for the term placental membrane binding site was determined by self-displacement assay, where term placental membranes were incubated with ¹²⁵I-Buserelin in the presence of increasing concentrations of uniodinated hormone for 1h at 20°C.

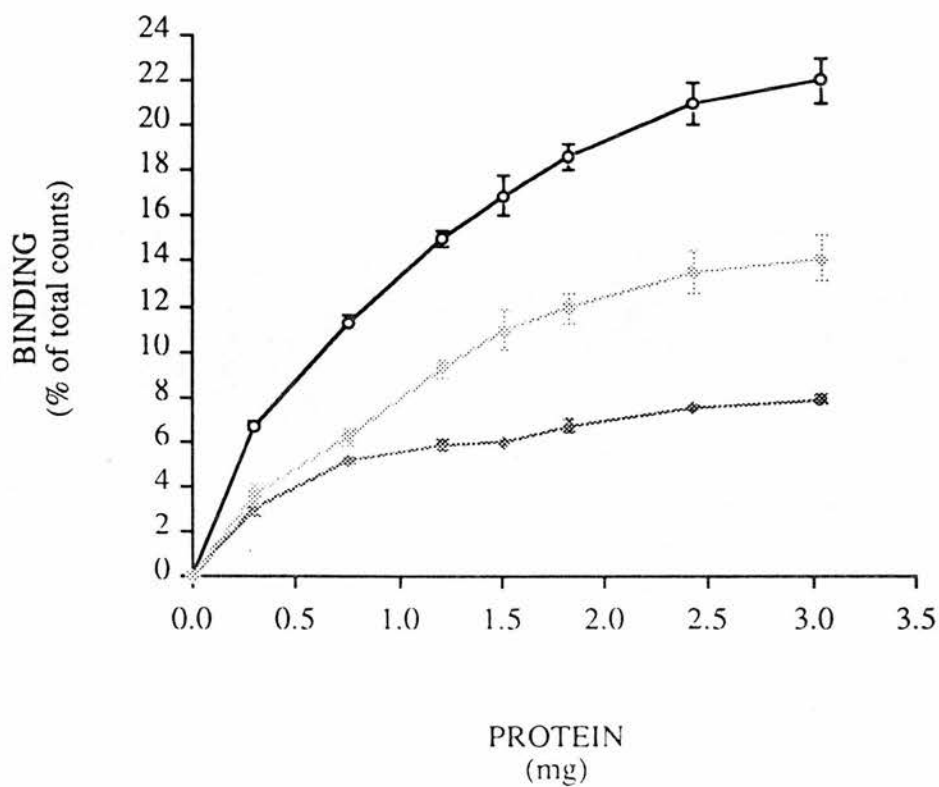


Figure 3.3 Effect of protein concentration on the binding characteristics of radiolabelled Buserelin

Increasing volumes of term placental membranes were incubated at 20°C for 1h with 100,000cpm iodinated Buserelin, in the presence and absence of 10 μ M unlabelled Buserelin. Bound and free hormone were separated by PEG precipitation and specific binding was determined. Total binding (○), non-specific binding, ie binding in the presence of unlabelled hormone (◆) and specific binding (◐) were expressed as % of total counts added. Data shown were from a representative term placenta; points are mean \pm s.d. for one experiment performed in triplicate.

Iodinated Buserelin was displaced from term placental membranes by increasing concentrations of cold hormone (Figure 3.4). Absolute levels of binding of the labelled agonist to placental membranes varied between individual placental membrane preparations, but displacement by cold hormone was consistently observed in the presence of concentrations of uniodinated Buserelin in excess of 30ng, with 50% displacement observed with approximately 300ng uniodinated Buserelin. Binding remaining at 100 μ g uniodinated Buserelin represented non-specific binding of the radio-labelled hormone to membranes and test tube.

3.2.6 Effect of Protease Inhibitors on 125 I-Buserelin Binding to Term Placental Membranes

Bramley (1987) described the rapid inactivation of Buserelin immunoactivity by placental membranes at 20°C, and Kang and Siler-Khodr (1992) have described a cytosolic chorionic protease which specifically degrades the proline-containing molecules GnRH, thyrotrophin releasing hormone and angiotensin II in the placenta. The specificity of placental binding sites for analogues of GnRH has been demonstrated (Bramley *et al.*, 1992) but, as GnRH binding was shown to plateau at high levels of membrane protein (Figure 3.3), the possibility that the low affinity binding of GnRH in placental membrane preparations may represent, at least in part, binding to a degradative protein was investigated by determination of specific binding in the presence of protease inhibitors.

3.2.6a Effect of a 'cocktail' of inhibitors on 125 I-Buserelin binding

Term placental membranes were incubated with 125 I-Buserelin under standard assay conditions, as described in 2.5.1, in the presence of the protease inhibitors EDTA (2mM), N-ethyl maleimide (2mM), PMSF (2mM) and Pepstatin A (1.46 μ M). This 'cocktail' of inhibitors had activity against the four major classes of tissue proteases (Barrett, 1977).

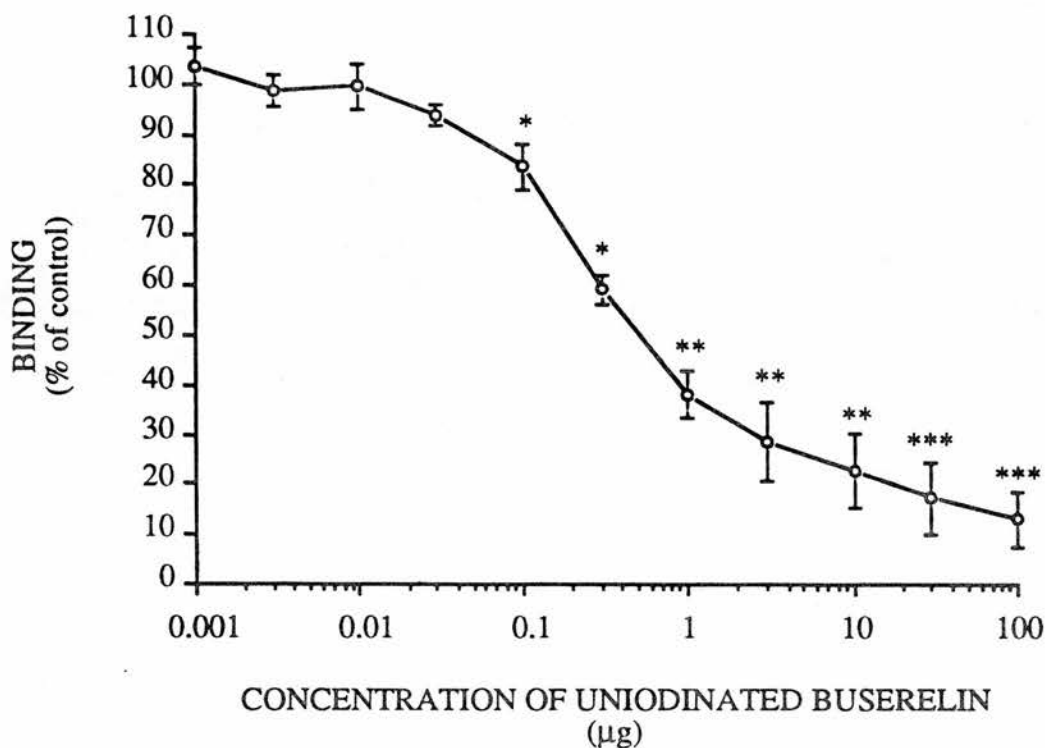


Figure 3.4 Effect of unlabelled Buserelin on the binding characteristics of radio-labelled Buserelin

Term placental membranes (~1mg protein) were incubated with 100,000cpm radio-labelled Buserelin at 20°C for 1h in the presence of increasing concentrations of unlabelled hormone.

Bound and free hormone were separated by PEG precipitation. Binding was determined, corrected for non-specific binding to the tube, and was expressed as % of control binding, ie binding in the absence of unlabelled Buserelin.

Data shown were the mean \pm s.d. of three displacement assays performed in triplicate.

*P<0.05; **P<0.01; ***P<0.005 when compared to binding in the absence of unlabelled Buserelin.

INHIBITOR	CLASS OF PROTEASE INHIBITED
EDTA	metallo-proteases
N-ethyl maleimide	thiol proteases
PMSF	serine proteases
Pepstatin A	carboxyl proteases

The presence of the 'cocktail' of protease inhibitors, with combined activity against a broad spectrum of proteases, reduced binding to negligible levels in membrane preparations from term placentae (Table 3.3), irrespective of levels of binding activity in the absence of inhibitors. This suggested that GnRH binding represented binding to degradative enzymes, not binding to membrane receptor sites.

3.2.6b Effect of individual inhibitors on ¹²⁵I-Buserelin binding

To identify which protease inhibitor was responsible for the reduction of binding shown in Table 3.3, term placental membranes were incubated with ¹²⁵I-Buserelin under standard conditions (see 2.5.1), in the presence of each individual protease inhibitor. In addition, membranes were incubated as a control with ethanol (10% v/v), the vehicle for PMSF.

Binding of Buserelin to placental membranes in the presence of Pepstatin A and EDTA was comparable to binding in the absence of inhibitors (Figure 3.5). Binding was decreased by ~25% in the presence of N-ethyl maleimide. This was significant ($P < 0.05$) and suggested that a component of binding may be due to binding of GnRH to thiol protease(s). Binding was reduced to negligible levels in the presence of the inhibitor 'cocktail' and PMSF. However, ethanol, the vehicle for PMSF which has low solubility in water, also reduced binding to non-detectable levels.

PLACENTAL PREPARATION	incubated without inhibitors (% total counts/mg)	incubated with inhibitors (% total counts/mg)
A	2.6 ± 0.9	0.1 ± 0.3
B	15.0 ± 1.5	0.0 ± 0.5
C	8.1 ± 0.6	0.4 ± 0.1
D	3.5 ± 0.9	0.2 ± 0.2
E	3.9 ± 0.6	0.2 ± 0.1

Table 3.3 Effect of protease inhibitors on ¹²⁵I-Buserelin binding to placental membranes

Term placental membranes (~1mg protein) were incubated with 100,000cpm ¹²⁵I-Buserelin, in the presence and absence of 10μM uniodinated Buserelin, for 1h at 20°C, in the presence and absence of a 'cocktail' of protease inhibitors. Bound and free hormone were separated by PEG precipitation and specific binding determined.

Specific binding was expressed as % of total counts added, corrected for protein concentration.

Data shown represent mean ± s.d. of triplicate determinations for five individual term placental membrane preparations.

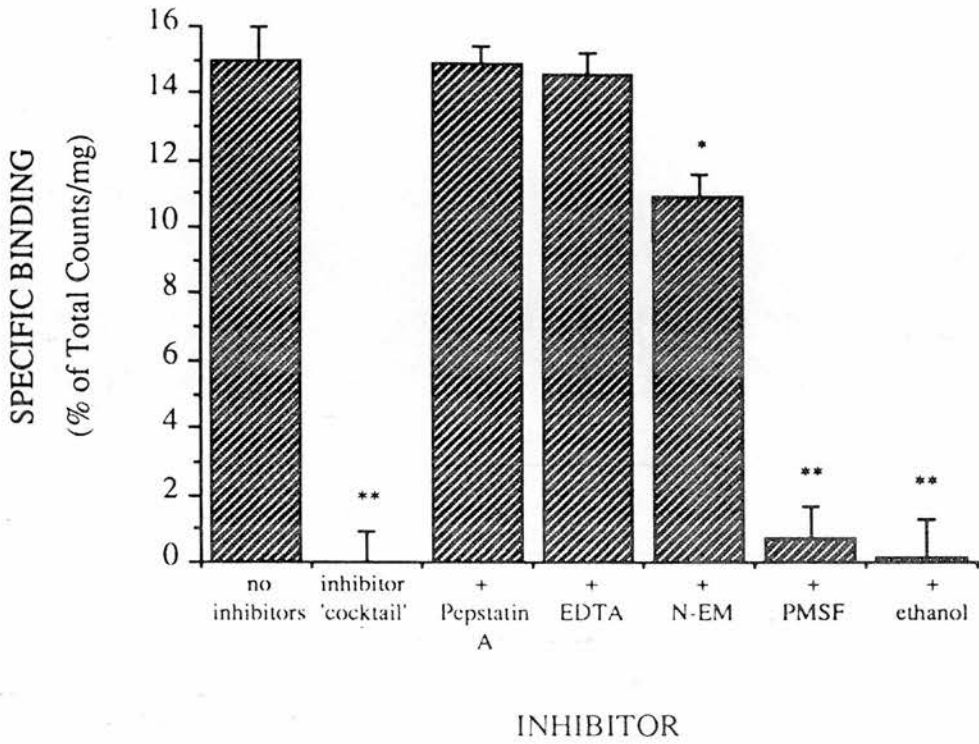


Figure 3.5 Effect of individual protease inhibitors on the binding characteristics of radio-labelled Buserelin

Term placental membranes (~1mg protein) were incubated, in the presence and absence of 10 μ M uniodinated Buserelin, with 100,000cpm iodinated Buserelin at 20°C for 1h in the presence of protease inhibitors.

Bound and free hormone were separated by PEG precipitation. Specific binding was determined and expressed as % of total counts added per mg protein.

Data shown were from a representative term placental membrane preparation, assayed in triplicate - error bars represent s.d.; asterisks denote significant differences from control i.e. binding in the absence of inhibitors (*P<0.05; **P<0.001).

3.2.6c Effect of ethanol on ¹²⁵I-Buserelin binding

To investigate the effect of ethanol on Buserelin binding, term placental membranes were incubated under standard conditions (see 2.5.1) in the presence of increasing concentrations of ethanol.

Binding of Buserelin was reduced to half maximal levels by the presence of 1% (v/v) ethanol in the assay medium and reduced to negligible levels in the presence of 20% (v/v) ethanol (Figure 3.6).

3.2.7 Effect of Preparing Placental Membranes in the Presence of the 'Cocktail' of Protease Inhibitors

Term placental membranes from individual placentae were prepared in the presence and absence of the protease inhibitor 'cocktail', as described in 2.2.2a. To allow direct comparison, individual placentae were dissected, placental tissue was randomly divided into two lots of approximately equal weight and homogenised in the presence or absence of protease inhibitors. Membranes prepared in the presence of inhibitors were resuspended and stored in inhibitor-containing medium, while membranes prepared in the absence of inhibitors were resuspended and stored in medium alone. Inhibitor-containing medium contained 1% (v/v) ethanol.

Membranes were incubated with ¹²⁵I-Buserelin under standard assay conditions (see 2.5.1). The effect of preparing membranes with or without inhibitors is shown in Table 3.4.

Binding of iodinated Buserelin to membranes prepared in the presence of protease inhibitors was reduced by 20-45%, compared to membranes from the same placenta prepared without inhibitors. This difference in binding was statistically significant ($P < 0.01$). The final concentration of ethanol in the assay was 0.1% (v/v).

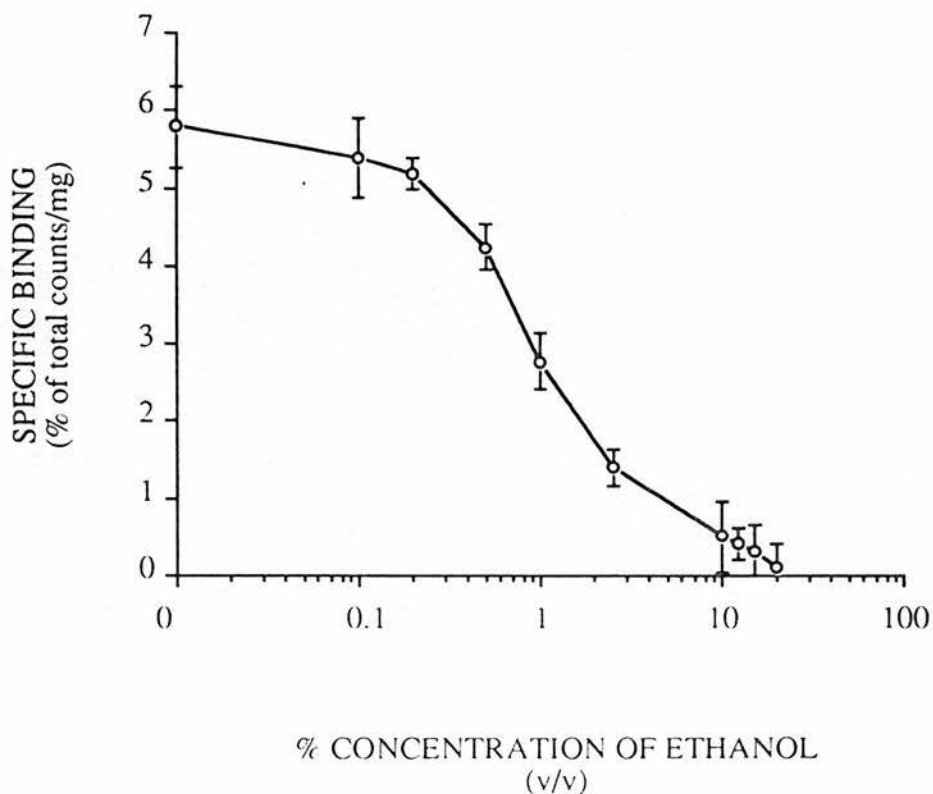


Figure 3.6 Effect of ethanol on the binding characteristics of radio-labelled Buserelin

Term placental membranes (~1mg protein) were incubated with 100,000cpm radio-labelled Buserelin, in the presence and absence of 10 μ M uniodinated Buserelin, at 20°C for 1h with increasing concentrations (0-20% (v/v)) of ethanol.

Bound and free hormone were separated by PEG precipitation, specific binding was determined and expressed as % of total counts added, corrected for protein concentration.

Data shown were from a representative term placental membrane preparation; each point represents mean \pm s.d of triplicate determinations.

PLACENTAL PREPARATION	prepared without inhibitors (% total counts/mg)	prepared with inhibitors (% total counts/mg)
A	14.2 ± 1.3	10.9 ± 0.7
B	15.0 ± 0.6	8.1 ± 1.2
C	21.8 ± 1.0	12.5 ± 0.5
D	16.9 ± 0.8	10.2 ± 0.5
E	12.5 ± 0.6	10.3 ± 0.9

Table 3.4 Effect of preparing placental membranes in the presence of protease inhibitors on the binding characteristics of ^{125}I -Buserelin

Term placental membranes were prepared in the presence and absence of a 'cocktail' of protease inhibitors (2mM EDTA, 2mM N-ethyl maleimide, 2mM PMSF and 1.46 μM Pepstatin A).

Placental membranes (~1mg protein) were incubated with 100,000cpm ^{125}I -Buserelin for 1h at 20°C in the presence and absence of 10 μM uniodinated Buserelin. Bound and free hormone were separated by PEG precipitation and specific binding determined.

Specific binding was expressed as % of total counts added, corrected for protein concentration.

Data shown represent mean \pm s.d. of triplicate determinations for five individual term placental membrane preparations.

3.2.8 Summary

The optimal assay conditions for determining binding of iodinated GnRH analogues to placental membranes were confirmed to be incubation with tracer at 20°C for 1 hour, with non-specific binding measured in the presence of 10⁻⁵M uniodinated hormone. Membranes were stable for up to 6 hours at 4°C and for 2 hours at 20°C, and hormone binding was not affected by storing thawed membranes on ice prior to assay. Membrane protein was standardised at ~1mg due to the non-linear effect of high protein concentrations on ligand binding.

Placental membranes were routinely prepared as described in 2.2.2a, by homogenisation in SET and centrifugation at 30,000xg or homogenisation in PBS and centrifugation at 100,000xg. Preparing membranes by the method of Smith and Brush (2.2.2b) did not improve the specific activity of GnRH binding.

Binding was significantly decreased in the presence of N-ethyl maleimide and ethanol in the incubate. 2mM N-ethylmaleimide was thought to inhibit tracer binding to thiol protease activity in the membrane preparation. The nature of the ethanol effect on binding was uncertain but was observed to be dose-dependent.

3.3 BINDING ACTIVITY OF ¹²⁵I-BUSERELIN TO PRE-TERM PLACENTAL MEMBRANES

3.3.1 Comparison of ¹²⁵I-Buserelin Binding to Pre-term and Term Placental Membranes

The binding of Buserelin to early, mid- and full term placental membranes, under the standard assay conditions described above, was compared. Mean binding ± standard deviation of the mean was calculated for Buserelin binding of membranes prepared from placentae from early, mid- and full term gestation and a summary plot of binding with stage of gestation is shown in Figure 3.7.

For membranes prepared from placentae of 6-10 weeks gestation, mean binding was 5.1±2.6% of total counts/mg. For mid-term placental membranes (11-18

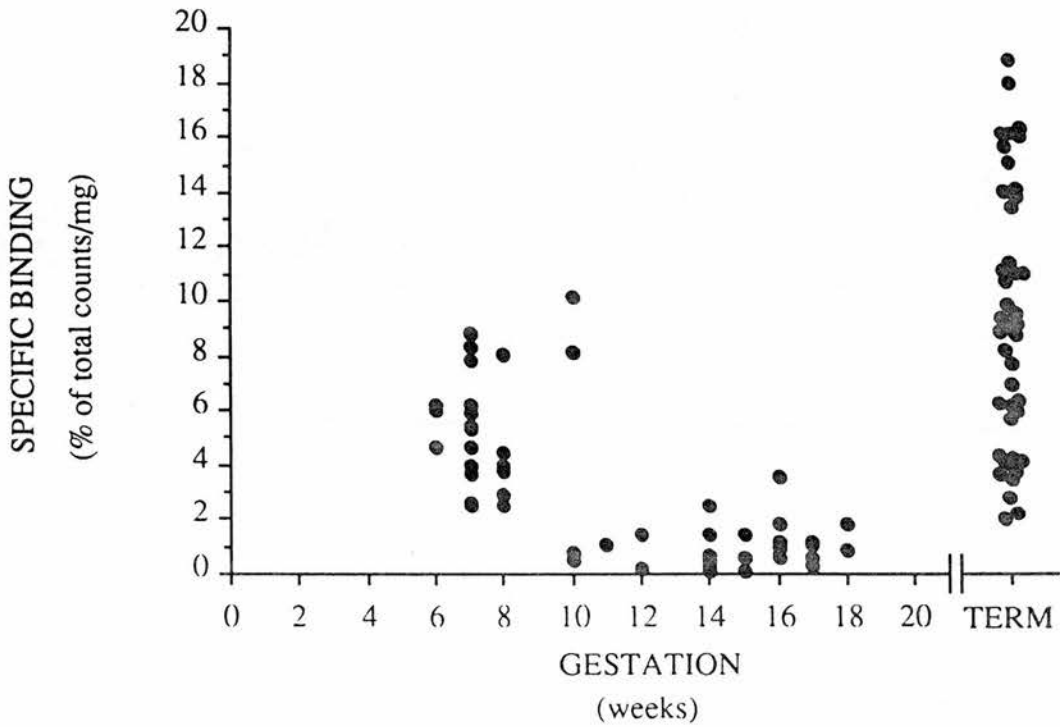


Figure 3.7

Comparison of binding of radio-labelled Buserelin to pre-term and term human placental membranes

Pre-term (ranging from 6-18 weeks gestation) and full term placental membranes (~1mg protein) were incubated with 100,000cpm iodinated Buserelin at 20°C for 1h, in the presence and absence of 10µM uniodinated Buserelin.

Bound and free hormone were separated by PEG precipitation. Specific binding was determined and expressed as % of total counts added, corrected for protein concentration.

Each point represents mean of triplicate determinations. n=27 for 6-10 weeks; n=23 for 11-18 weeks; n=43 for term. Error bars have been omitted for clarity.

weeks gestation), binding fell to $1.0 \pm 0.8\%$ of total counts added per mg protein. In comparison, binding for term placental membranes was $9.2 \pm 4.7\%$ of total counts corrected for protein concentration. Average protein concentration was 0.97 ± 0.22 mg.

The difference between the binding levels observed at 6-10 weeks gestation, 11-18 weeks gestation and full term were significant ($P < 0.001$ for all three pairs of t-tests).

3.3.2 Protein Concentration and ^{125}I -Buserelin Binding to Pre-term Placental Membranes

Specific binding of increasing concentrations of pre-term placental membranes was determined after incubation with ^{125}I -Buserelin for 1 hour at 20°C .

For placental membranes of 6-10 weeks gestation, specific binding of Buserelin increased with protein concentration in a linear manner at low concentrations (Figure 3.8). However above $\sim 2\text{mg}$ protein, binding began to plateau. For early pre-term placentae, however, the small size of the tissue meant that only low levels of membrane protein were usually recovered.

For mid-term gestation membranes, low levels of binding ($< 2\%$ of total counts) were observed at all protein concentrations and Buserelin binding did not increase with increasing protein concentration.

3.3.3 Self-displacement of Buserelin from Pre-term Placental Membranes

The effect of incubating pre-term placental membranes with ^{125}I -Buserelin in the presence of increasing concentrations of uniodinated hormone, under standard assay conditions (as 2.5.1), is shown in Figure 3.9.

As for term placental membranes, radio-labelled Buserelin was displaced from pre-term placental membranes by concentrations greater than 30ng of uniodinated hormone. However, maximal displacement of labelled agonist from pre-term placental

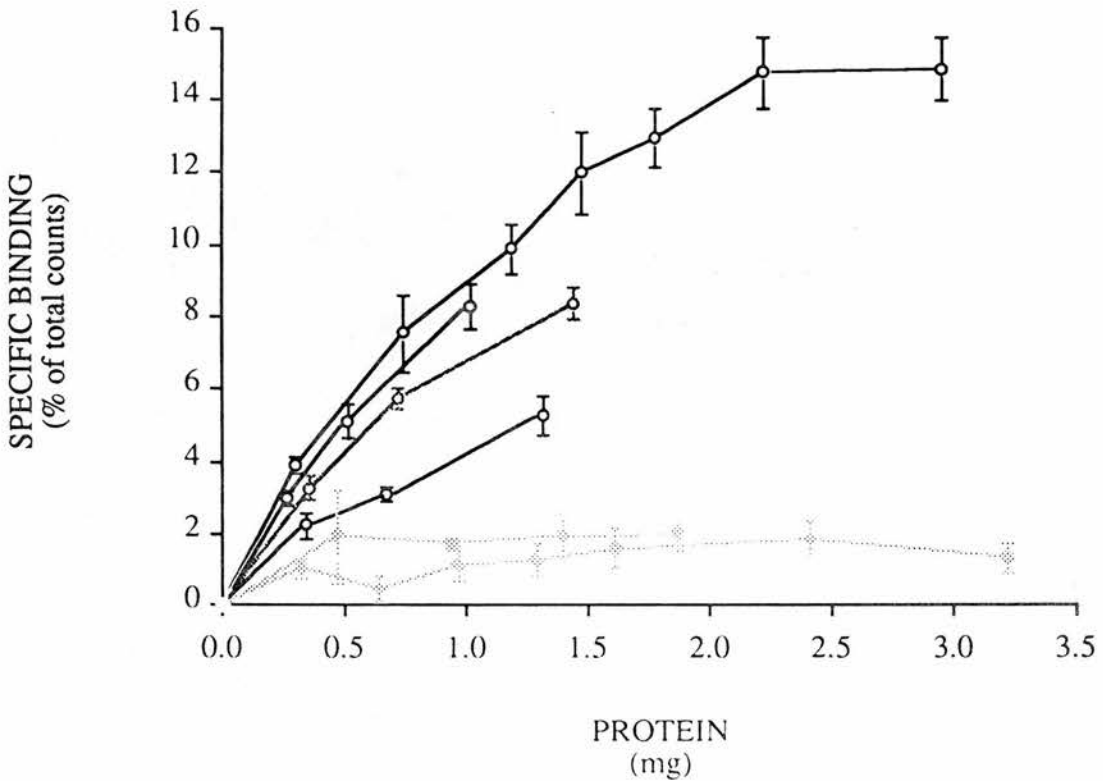


Figure 3.8 Effect of protein concentration on the binding of radio-labelled Buserelin to pre-term placental membranes

Increasing volumes of early and mid-term placental membranes were incubated with 100,000cpm iodinated Buserelin at 20°C for 1h, in the presence and absence of 10µM Buserelin.

Bound and free hormone were separated by PEG precipitation. Specific binding was determined and expressed as % of total counts added.

Data shown were mean ± s.d. for membranes prepared from four representative placentae of 6-10 weeks gestation (○) and from two representative placentae of ~16 weeks gestation (*).



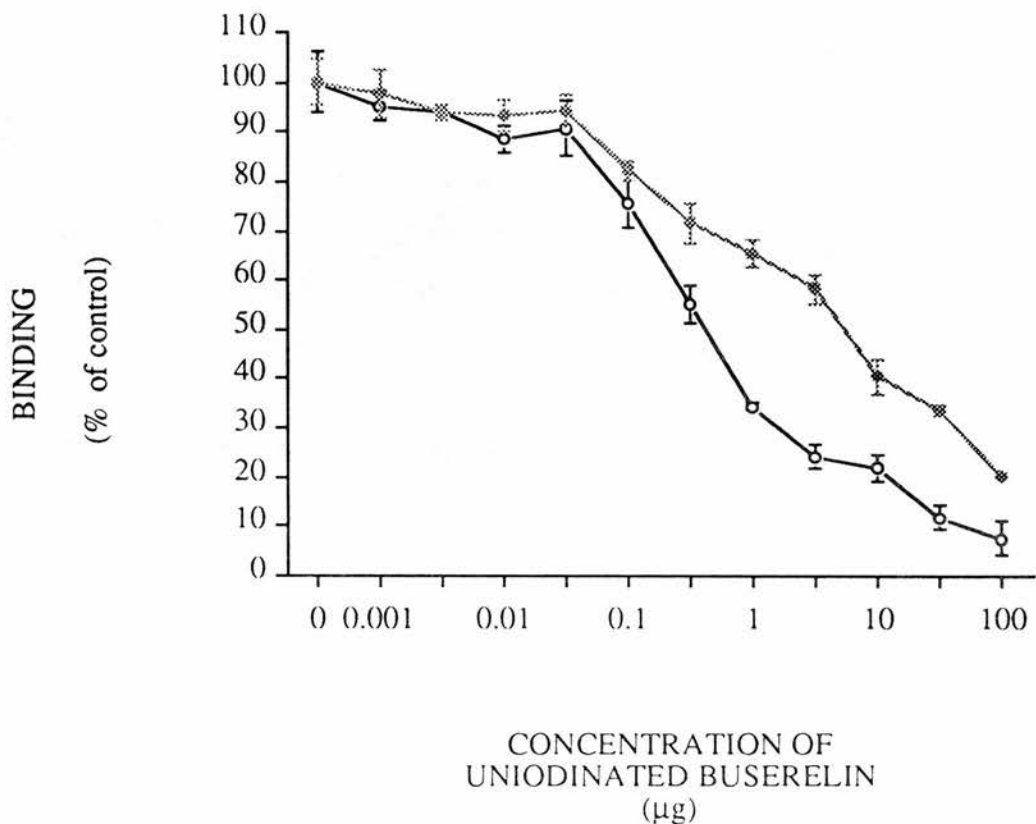


Figure 3.9

Displacement of iodinated Buserelin from pre-term placental membranes by uniodinated analogue

Early and mid-term placental membranes (~1mg protein) were incubated with 100,000cpm radio-labelled Buserelin at 20°C for 1h in the presence of increasing concentrations of unlabelled Buserelin.

Bound and free hormone were separated by PEG precipitation. Binding was determined, corrected for non-specific binding to the tube and expressed as % of control binding in the absence of 'cold' hormone.

Data shown were representative curves for Buserelin binding to membranes prepared from early placentae (○) and from mid-term placenta (◊). Each point was mean ± s.d. for triplicate determinations from a single experiment.

membranes varied with gestational age. 10^{-5} M uniodinated Buserelin displaced 90% of labelled agonist from early gestation placental binding sites and displaced 80% of binding at mid-term. Half-maximal self-displacement of Buserelin from early placental membranes was consistent with displacement from term placental membranes, i.e. ~300ng. At mid-term, 50% displacement was approximately ten-fold higher.

3.3.4 Protease Inhibitors and Buserelin Binding to Pre-term Placental Membranes

Buserelin was incubated with early and mid-term placental membranes in the presence and absence of the protease inhibitors listed in 3.2.6a.

GnRH agonist binding to early placental membranes was inhibited by the presence of the mixture of protease inhibitors (Table 3.5). However, insufficient membrane protein was available to investigate the particular inhibitory factor(s) involved. Binding of Buserelin to mid-term placental membranes was very low in the absence of protease inhibitors and the inclusion of inhibitors in the incubation failed to increase binding.

3.3.5 Summary

Binding of the GnRH agonist Buserelin to human placental membranes varied throughout gestation. The high levels of GnRH binding observed in early trophoblast (6-10 weeks gestation) fell to almost non-specific levels by 11-18 weeks, returning to high levels again at term.

At both early and mid gestation, Buserelin binding increased in a linear fashion at low protein concentrations, but began to plateau at higher protein levels. However, maximal binding at mid-term was markedly lower and levelled off at lower protein concentrations. However, incubation in the presence of the cocktail of protease inhibitors did not increase levels of GnRH binding to mid-term placental membranes.

GESTATION (weeks)	incubated without inhibitors (% total counts/mg)	incubated with inhibitors (% total counts/mg)
7	8.2 ± 0.3	1.8 ± 0.4
13	0.8 ± 0.6	0.4 ± 0.2
15	0.1 ± 0.4	0.2 ± 0.3
16	1.2 ± 0.8	0.8 ± 0.5

Table 3.5 Effect of protease inhibitors on ^{125}I -Buserelin binding to pre-term placental membranes.

Pre-term placental membranes (~1mg protein) were incubated with 100,000cpm ^{125}I -Buserelin, in the presence and absence of 10 μM uniodinated Buserelin, for 1h at 20°C in the presence of a 'cocktail' of protease inhibitors. Bound and free hormone were separated by PEG precipitation and specific binding determined.

Specific binding was expressed as % of total counts added, corrected for protein concentration.

Data shown represent mean \pm s.d. of triplicate determinations.

3.4 CHARACTERISATION OF BINDING ACTIVITY OF GnRH ISOFORMS TO PLACENTAL MEMBRANES

The five isoforms of GnRH investigated were designated mammalian GnRH (the hypothalamic decapeptide), chicken GnRH-I and II, salmon GnRH and lamprey GnRH. Their primary structure is shown in Figure 3.10. Mammalian GnRH has been found in the hypothalamic tissue of various mammalian species, e.g. mice, rats, pigs, sheep and humans, and in human placental tissue. Of the two forms of GnRH which have been sequenced from chicken brain, chicken GnRH-II appears to have evolved earlier than chicken GnRH-I and has been found in species from all major vertebrate groups, including primitive mammals such as marsupials (King *et al.*, 1989). It has been postulated that chicken GnRH-II has a role as a neurotransmitter or neuromodulator (Katz *et al.*, 1990).

3.4.1 Comparison of Isoform Binding to Rat Pituitary Homogenate and Human Placental Membrane Preparations

Human placental membranes and rat pituitary homogenates were incubated under the standard assay conditions described in 2.5.1 with iodinated isoforms of GnRH and the GnRH agonist analogue Buserelin.

Binding of the iodinated GnRH isoforms and Buserelin to human placental membrane preparations of 7, 11, 14 and 40 weeks gestation and to homogenate of rat pituitaries is compared in Figure 3.11. Rat pituitary specifically bound Buserelin, but binding of the five GnRH isoforms studied was negligible (<1% of total counts added). Specific binding of Buserelin to placental membrane preparations was less than that observed with rat pituitary homogenate. However, specific binding of salmon GnRH and chicken GnRH-II to placental membrane preparations was comparable to binding of Buserelin. Placental membrane binding of mammalian GnRH was low but detectable, while binding of chicken GnRH-I and lamprey GnRH was negligible (<1%

	1	2	3	4	5	6	7	8	9	10
LAMPREY	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH ₂
CHICKEN II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH ₂
SALMON	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH ₂
CHICKEN I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH ₂
MAMMALIAN	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH ₂
BUSERELIN	pGlu	His	Trp	Ser	Tyr	D-Ser(tBu)	Leu	Arg	Pro	NH ₂

Figure 3.10

Primary structure of five isoforms of GnRH and a synthetic agonist analogue of GnRH

GnRH isolated from species from classes of *Aves* (chicken), *Osteichthyes* (salmon), *Agnatha* (lamprey) and *Mammalia* (human, porcine, murine, ovine, rat) is highly conserved (Sherwood, 1987).

All characterized forms of GnRH are 10 amino acids in length. The terminal residues and positions 2, 4 and 9 are invariant. The pyro-glutamate and N-terminal glycine may protect the molecule against degradation; substitution of Gly¹⁰ by ethylamide further decreasing the susceptibility of buserelin to degradation. Positions 2 and 9 are thought to stabilize the conformation of the molecule (a β turn is hypothesized at positions 5 and 6).

Changes at positions 3 and 7 are conservative, between strongly hydrophobic amino acids; both tyrosine and tryptophan are aromatic amino acids.

Positions 5-8 are thought to be involved in receptor binding, and most evolutionary amino acid substitutions have occurred in this region. The most striking changes are at positions 5 and 8 where both strongly hydrophilic (histidine, lysine and arginine) and strongly hydrophobic (tyrosine and leucine) residues are found.

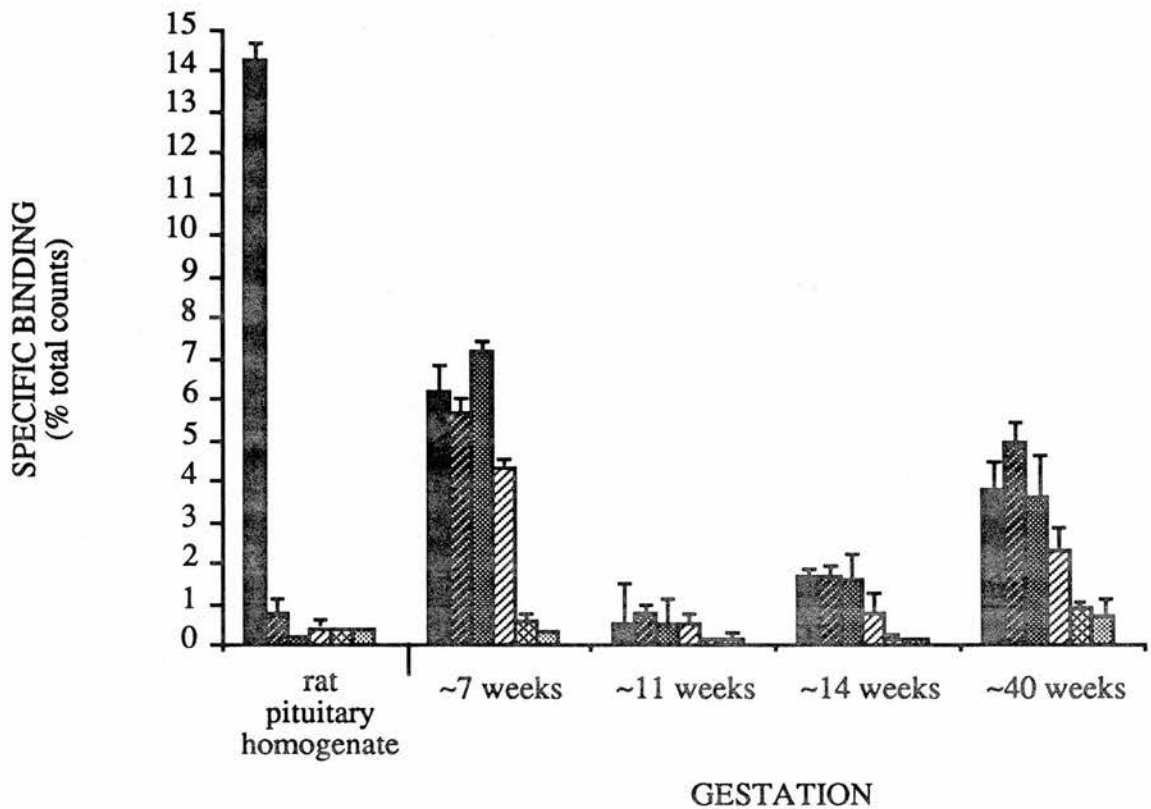


Figure 3.11 Comparison of binding of GnRH isoforms and Buserelin to human term placental membrane preparations and rat pituitary homogenate

Human placental membrane fractions and rat pituitary homogenate were incubated under standard assay conditions in the presence and absence of excess unlabelled Buserelin with 100,000cpm of iodinated Buserelin (■), salmon GnRH (▧), chicken GnRH-II (▨), mammalian GnRH (▩), lamprey GnRH (▩) and chicken GnRH-I (▩). Bound and free hormone were separated by PEG precipitation and specific binding determined, corrected for protein and expressed as % of total counts added \pm s.d.

of total counts added). Lower levels of binding were observed for all isoforms and Buserelin at mid-gestation.

At all stages of gestation, the pattern of binding activity to placental membranes was: Buserelin, salmon GnRH, chicken GnRH-II

> mammalian GnRH >> lamprey GnRH, chicken GnRH-I

3.5 CHARACTERISATION OF BINDING ACTIVITY OF GnRH AGONIST ANALOGUES TO PLACENTAL MEMBRANES

The binding activity of the GnRH agonist analogues Buserelin ([D-Ser(tBu)⁶]-des-Gly¹⁰-GnRH N-ethylamide) and Tryptorelin ([D-Trp⁶]-des-Gly¹⁰-GnRH N-ethylamide) was compared. The structures of the two analogues and their relation to mammalian GnRH is shown in Figure 3.12.

	1					6				10
MAMMALIAN GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH ₂
BUSERELIN	pGlu	His	Trp	Ser	Tyr	D-Ser (tBu)	Leu	Arg	Pro	NHEt
TRYPTORELIN	pGlu	His	Trp	Ser	Tyr	D-Trp	Leu	Arg	Pro	NHEt

Figure 3.12 Primary structure of mammalian GnRH and two synthetic analogues of mammalian GnRH

3.5.1 Comparison of GnRH Agonist Binding to Rat Pituitary Homogenate and Human Placental Membranes

Human placental membranes and rat pituitary homogenates were incubated under standard conditions (as described in 2.5.1) with the iodinated GnRH agonist

analogues Buserelin or Tryptorelin. Binding of the two agonist analogues of mammalian GnRH are compared in Figure 3.13.

Rat pituitary bound both analogues of mammalian GnRH, but specific binding of Tryptorelin was significantly greater than Buserelin by 42 - 49% ($P < 0.002$; $n=4$). The rat pituitary homogenates were standardised for protein concentration (0.22-0.32mg protein).

Term placental membranes specifically bound both agonists, with specific binding of Tryptorelin ranging from 20 - 35% greater than specific binding of Buserelin. This was statistically significant ($P < 0.05$; $n=10$).

Both analogues also bound specifically to pre-term placental membranes, with a general trend of greater binding of Tryptorelin compared to Buserelin. However, binding of GnRH analogues to pre-term preparations was more variable than binding to term placental membranes and, with those preparations which exhibited lower levels of GnRH binding, Tryptorelin did not consistently exceed Buserelin binding. Preparations of mid-term membranes exhibited significantly lower levels of binding of both analogues when compared to membranes from early gestation placentae ($P < 0.05$; $n=8$).

3.5.2 Effect of Protein Concentration on Agonist Binding to Placental Membranes

Increasing concentrations of term placental membranes were incubated under standard binding assay conditions (see 2.5.1) with ^{125}I -Buserelin or ^{125}I -Tryptorelin.

At low concentrations of membrane protein, specific binding of Tryptorelin increased with membrane protein in a linear manner. Binding began to plateau above 1.5mg protein (Figure 3.14), similar to binding curves observed with Buserelin.

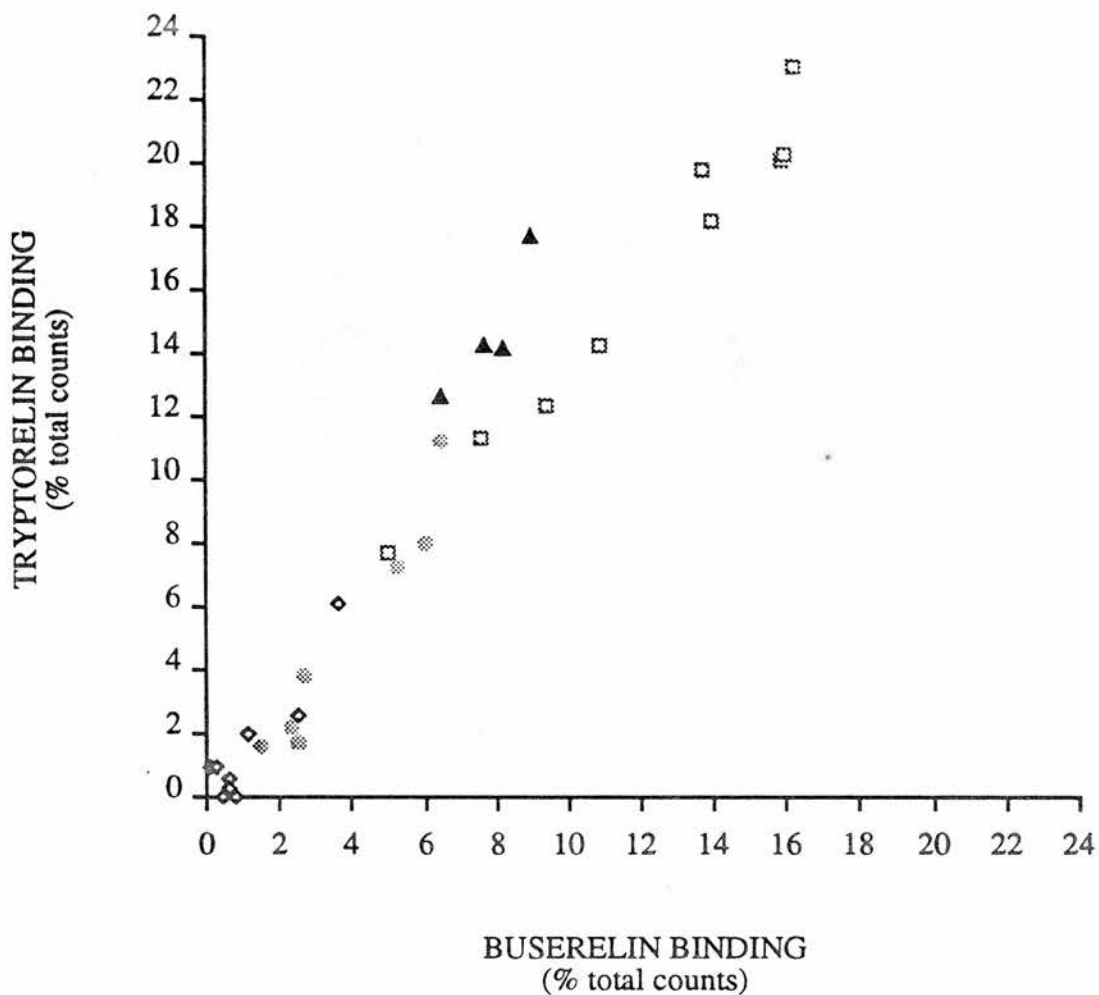


Figure 3.13

Comparison of Buserelin and Tryptorelin binding to human placental membranes and rat pituitary homogenates

Rat pituitary homogenate (▲ ; ~0.27 mg protein) and human placental membranes (◆ early, ◇ mid, □ term; ~1mg protein) were incubated under standard conditions with 100,000cpm iodinated Buserelin or 100,000cpm iodinated Tryptorelin, in the presence and absence of excess unlabelled Buserelin.

Bound and free hormone were separated by PEG precipitation. Specific binding was determined and expressed as % of total counts added. Each point represents mean for triplicate determinations; error bars have been omitted for clarity.

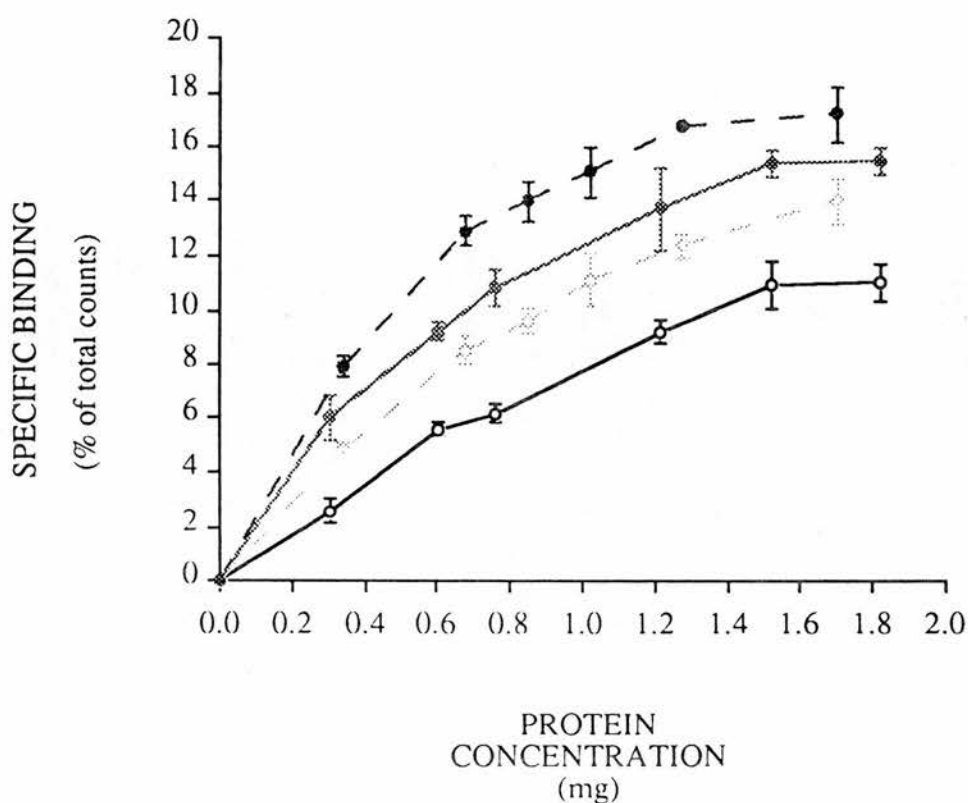


Figure 3.14 Effect of protein concentration on binding of the GnRH agonists Buserelin and Trytorelin to term placental membranes

Increasing volumes of term placental membranes were incubated at 20°C for 1h with 100,000cpm iodinated Buserelin (◐) and with 100,000cpm iodinated Trytorelin (●), in the presence and absence of 10μM unlabelled Buserelin.

Bound and free hormone were separated by PEG precipitation, specific binding was determined and expressed as % of total counts added.

Membranes were prepared from a single term placenta, as described in Section 2.2.2, homogenised in SET and centrifuged at 30,000xg (dashed line) and homogenised in PBS and centrifuged at 100,000xg (solid line).

Each point is mean ± s.d. for triplicate determinations from a single experiment.

3.5.3 Comparison of the Effect of Membrane Preparation on Agonist Binding

The preparations shown in Figure 3.14 were prepared from the same term placenta by the method described in 2.2.2a, homogenised in SET and centrifuged at 30,000xg or homogenised in PBS and centrifuged at 100,000xg. The difference in binding of ^{125}I -Buserelin and ^{125}I -Tryptorelin to the two membrane preparations was not statistically significant.

3.5.4 Summary

Binding of Tryptorelin to rat pituitary homogenate and human term placental membranes was significantly greater than Buserelin binding to the same preparations ($P < 0.002$ and $P < 0.05$, respectively). Binding to pre-term membranes was more variable and, especially with those preparations which bound lower levels of hormone, binding of Tryptorelin did not always exceed the levels of bound Buserelin.

Binding of Tryptorelin to term placental membranes increased with protein concentration in a similar manner to that of Buserelin, although levels of binding were consistently higher. As for Buserelin, placental membrane binding was not affected by preparation in SET or PBS.

3.6. TRACER DEGRADATION BY PLACENTAL MEMBRANES

In order to determine if gestational age-related differences in the degradation of Buserelin by placental membrane preparations were responsible for the marked reduction in binding observed between 11 and 18 weeks, the ability of iodinated Buserelin to rebind to placental membranes, following an incubation with placental membranes, was investigated.

^{125}I -Buserelin was incubated with placental membranes under standard conditions and bound and free tracer were separated by high-speed centrifugation, as described in Section 2.6. Unbound tracer was then re-incubated with either fresh

membranes of the same preparation or with a term placental membrane preparation used as a standard.

From Figure 3.15, re-binding to the same preparation was usually slightly less than binding to the first incubation, but the difference was always <1.2 % of total counts. Re-binding of tracer to a standard term membrane fraction was within $\pm 5\%$ of control binding for the term preparation. Tracer previously incubated with mid-term placental membranes, which bound low levels of iodinated hormone, bound to term standard membrane preparations with no loss activity. Re-binding to mid-term membranes, however, was still low.

3.7 RECOVERY AND PURITY OF PLACENTAL MEMBRANES

The purity of the membrane fraction recovered at each stage of gestation was compared in order to determine if contamination of mid-term membranes with non-villous tissue could account for the decreased binding observed at mid-gestation. The placental surface membrane markers EGF receptors and alkaline phosphatase activity (Smith and Brush, 1978) were determined to assess any correlation existed between GnRH and EGF binding, alkaline phosphatase activity and stage of gestation.

3.7.1 Binding of EGF to Pre-term and Term Placental Membranes

Increasing concentrations of term and pre-term placental membranes were incubated with ^{125}I -EGF under standard binding conditions for the growth factor, described in Section 2.7.

From Figure 3.16, specific binding of EGF to placental membranes increased with protein concentration in a similar manner at all stages of gestation. At low levels of protein, binding increased in a linear manner, but began to plateau above 0.2mg of membrane protein.

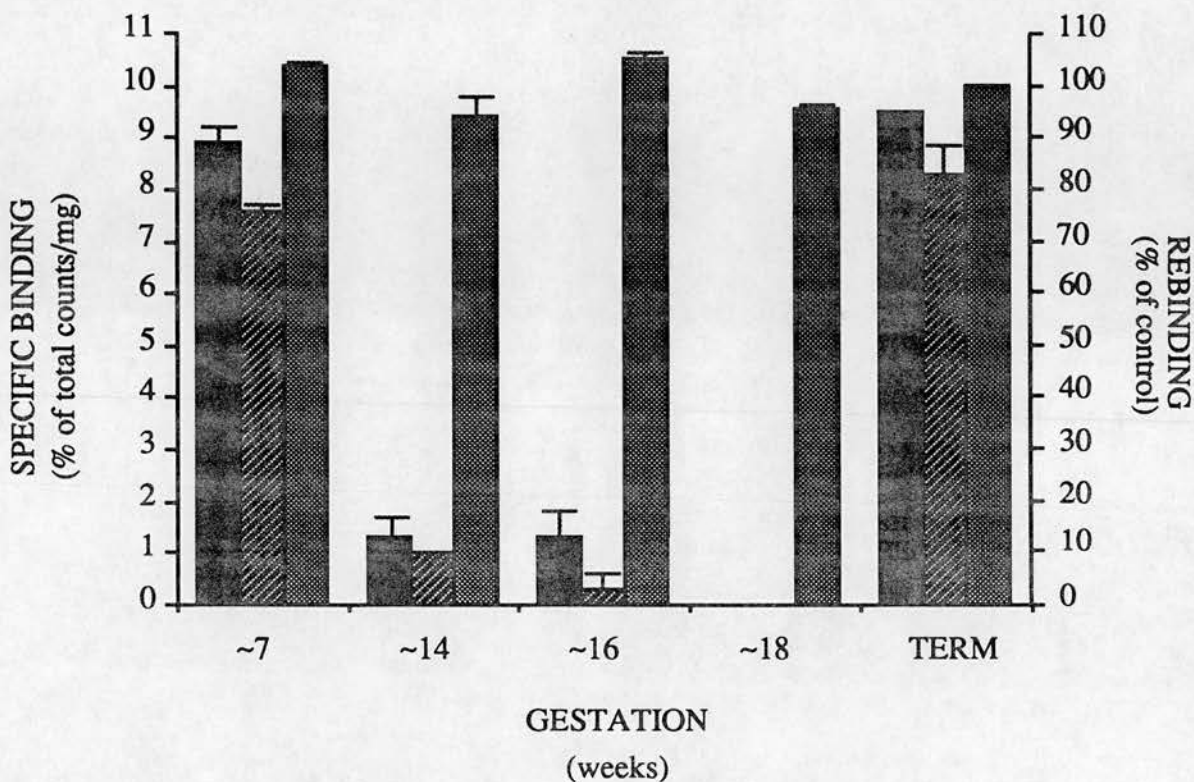


Figure 3.15

Degradation of iodinated Buserelin by placental membranes

Pre-term and term placental membranes (~1mg protein) were incubated in duplicate at 20°C for 1h with 300,000cpm radio-labelled Buserelin, in the presence and absence of uniodinated Buserelin. Bound and free tracer were separated by centrifugation at 30,000xg, specific binding was determined and expressed as % of total counts added, corrected for protein concentration (■).

100,000cpm of the unbound tracer recovered from the first incubation was then incubated with either fresh placental membranes from the same preparation (▨) or a term placental membrane standard (■) for 1h at 20°C, in the presence and absence of unlabelled Buserelin. Bound and free hormone were separated by PEG precipitation and specific binding determined and corrected for protein concentration. Re-binding to membranes of the same preparation was expressed as % of total counts added and binding to the term standard was expressed as % of control binding, defined as binding of 100,000cpm of first incubation tracer.

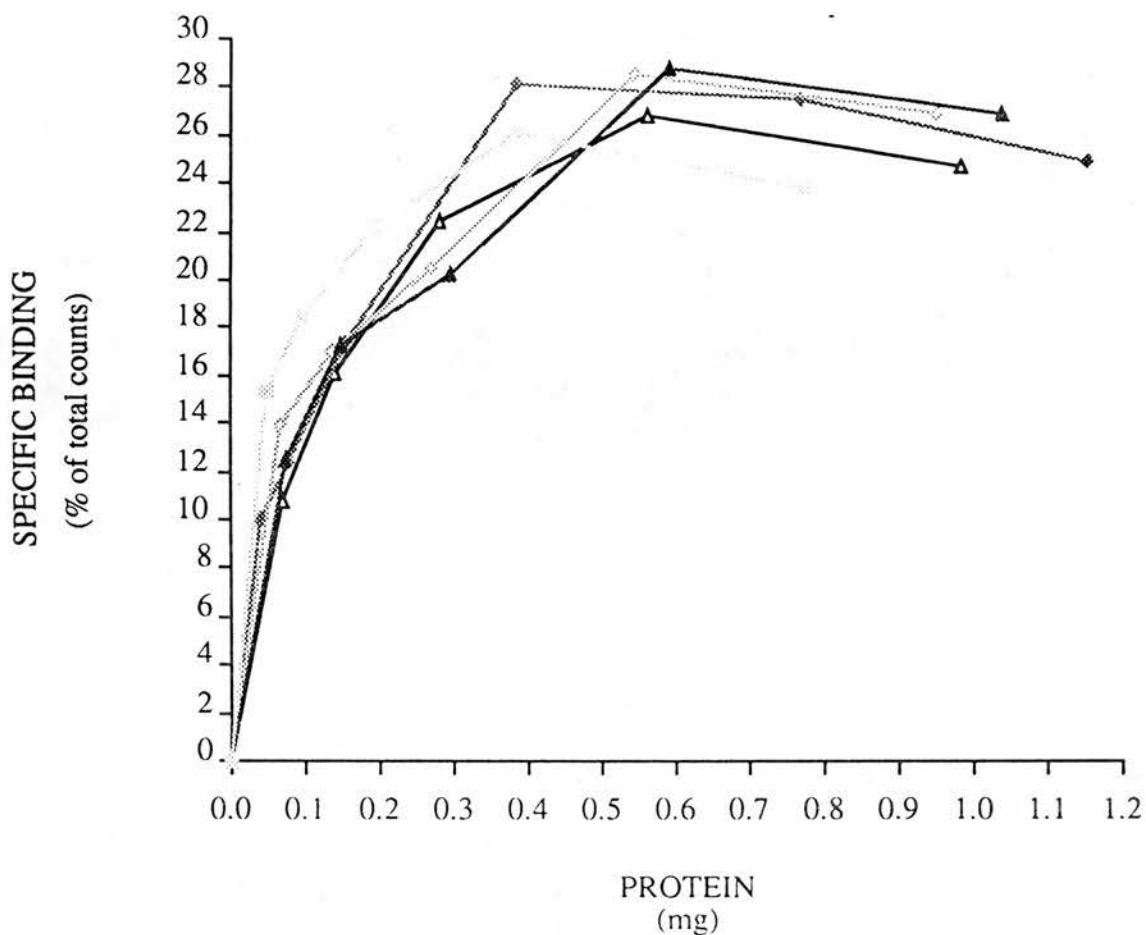


Figure 3.16 EGF binding to placental membranes

Increasing concentrations of pre-term and term placental membrane protein were incubated with 50,000cpm iodinated EGF for 3h at 37°C in the presence and absence of unlabelled EGF.

Bound and free EGF were separated by PEG precipitation, and specific binding was determined and expressed as % of total counts added.

Data shown were for binding to membranes of 8 weeks (◆), 12 weeks (◇), 14 weeks (▲), 16 weeks (△) and 40 weeks (◻) gestation.

Points are mean for triplicate determinations; error bars have been omitted for clarity.

3.7.2 Alkaline Phosphatase Activity in Pre-term and Term Placental Membranes

Activity of alkaline phosphatase in placental membrane preparations was determined by a variation of the method of Bessey *et al.* (1946).

At all stages of gestation, alkaline phosphatase activity increased linearly with membrane protein (Figure 3.17). Levels of enzyme activity were much greater in membranes prepared from term placentae, compared to membranes prepared from pre-term placentae. However, there was no statistically significant difference between alkaline phosphatase activity in early and mid-term placentae.

3.7.3 Comparison of Recovery of Placental Membranes and GnRH Binding

Placental membranes were assayed for GnRH binding, EGF binding and alkaline phosphatase activity to determine if differences in membrane recovery could account for gestational age-related differences in GnRH binding (Table 3.6).

No relation was observed between levels of specific binding of GnRH, alkaline phosphatase activity and specific binding of EGF to placental membrane preparations of different gestational age. This suggests that differences between GnRH binding with gestational age are not caused by differences in recovery of the placental villous membranes.

3.8 DISCUSSION

Binding sites for GnRH in the human placenta differ from the well-characterised pituitary GnRH receptor. The pituitary GnRH superagonists Buserelin and Tryptorelin were bound by the placenta, as were the salmon, chicken-II and mammalian isoforms of GnRH. However, placental binding of the GnRH agonists was not enhanced compared to the two isoforms, which were not bound by the pituitary receptor (Figure 3.11) and competed poorly for the pituitary binding site

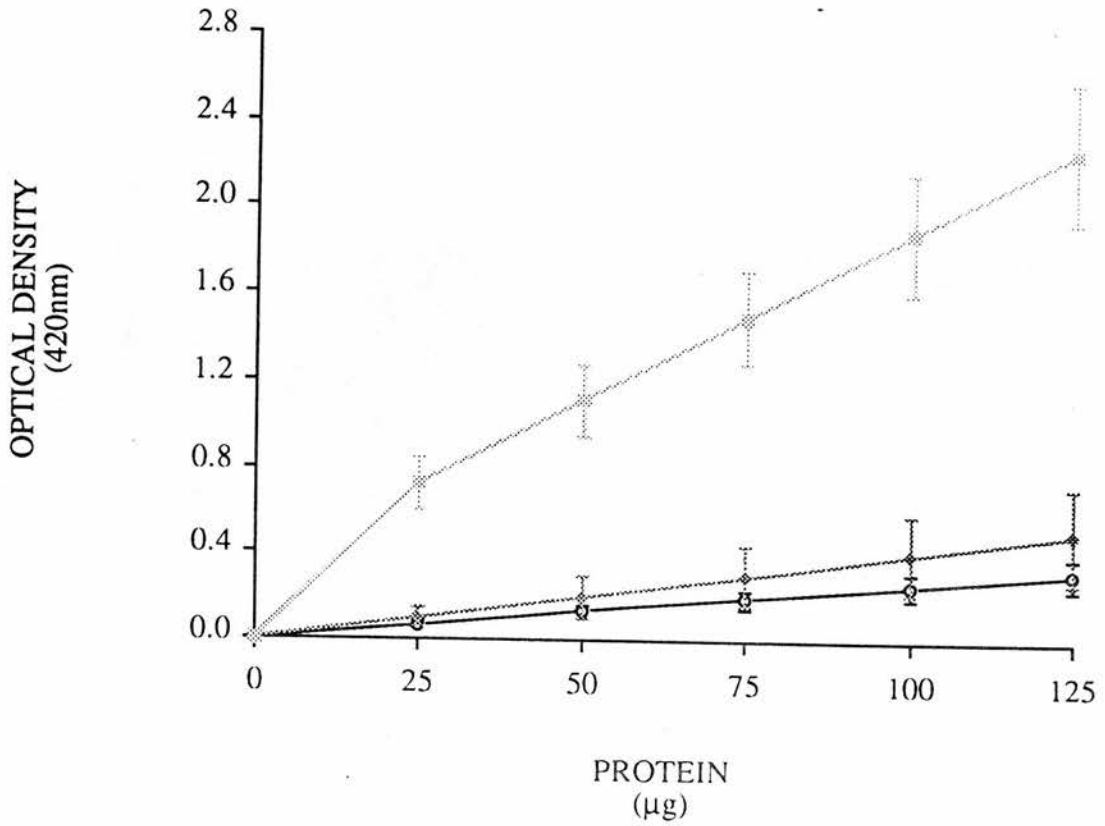


Figure 3.17 Alkaline phosphatase activity in term and pre-term placental membrane preparations

Increasing concentrations of placental membrane protein were incubated with *p*-nitrophenol phosphate for 15 minutes at 37°C. The hydrolysis reaction was stopped by dilution with alkali and optical density at 420nm was determined.

Optical density reflected liberation of *p*-nitrophenol by alkaline phosphatase activity in the membrane preparation.

Membranes were prepared from early (○), mid-term (◆) and term (■) placentae.

Points represent mean \pm s.d.; n=5, 6 and 8 for early mid and term placental membranes, respectively.

GESTATION (weeks)	GnRH BINDING (% total counts /mg)	EGF BINDING (% total counts /0.1mg)	ALKALINE PHOSPHATASE (OD ₄₂₀)
8	3.9 ± 0.6	16.0 ± 1.0	0.091 ± 0.008
12	0.8 ± 0.2	20.5 ± 0.6	0.067 ± 0.014
14	1.9 ± 0.4	16.8 ± 1.3	0.318 ± 0.042
16	3.6 ± 0.6	15.5 ± 0.9	0.343 ± 0.060
40	3.9 ± 0.2	19.1 ± 0.8	1.091 ± 0.092

Table 3.6 GnRH binding activity, EGF binding activity and alkaline phosphatase activity of placental membrane preparations of early, mid and term gestation

Placental membranes were incubated with ¹²⁵I-Buserelin and ¹²⁵I-EGF under standard assay conditions. Bound and free hormone were separated by PEG precipitation and specific binding determined. Specific binding was expressed as % of total counts added, corrected for protein concentration.

The alkaline phosphatase activity of the placental membranes was measured by standard assay, and the three parameters compared.

Data were mean ± s.d. for triplicate determinations.

(Bramley *et al.*, 1992). Differences in binding could not be accounted for by differences in tracer degradation between the two tissues: isoforms of GnRH were capable of appreciable levels of binding to EL-14 anti-GnRH antibody after incubation with rat pituitary or human placental membranes, irrespective of whether or not they bound to the tissue of the first incubation (Bramley *et al.*, 1992). This antibody is specific for GnRH isoforms and does not bind GnRH analogues as it recognises both N- and C- terminal regions of the GnRH molecule, which suggests that appreciable levels of intact isoform molecules remained after the first incubation.

GnRH binding in the placenta appeared to be specific for molecules with the structure of GnRH, as Buserelin binding was not inhibited by a range of proteins, steroids, peptides and hormones (Bramley, 1987). However, unlabelled GnRH antagonist analogues did not inhibit Buserelin binding in the human placenta except at concentrations 200- to 50,000-fold higher than observed for pituitary GnRH receptors (Bramley *et al.*, 1992).

Low affinity binding sites for GnRH have been reported to be due to binding of degradation resistant analogues of GnRH to proteases (Clayton *et al.*, 1979). However, Menzies and Bramley (1992) demonstrated that the placental GnRH binding site was distinct from GnRH degrading activity when human placental membranes were centrifuged on a continuous sucrose density gradient. In addition, analogue and isoform binding were localised to the same region of the gradient as the plasma membrane markers, EGF receptors and alkaline phosphatase activity.

As previous studies have looked at placental GnRH binding at a limited range of gestational ages, placentae were obtained at 6-18 weeks from therapeutic abortions and from normal term pregnancies and the binding characteristics of placental GnRH receptors at early, mid and term gestation were determined.

Levels of binding of GnRH, GnRH isoforms and synthetic agonist analogues in the human placenta varied throughout gestation. The high levels of binding observed in early trophoblast (6 - 10 weeks gestation) fell to non-determinable levels by 18

weeks, increasing to maximal levels at term (Figure 3.7). The profile of GnRH binding activity in pre-term placentae parallels levels of hCG in the maternal serum (Braunstein *et al.*, 1976). However, Siler-Khodr and Khodr (1978) found that levels of apparent GnRH immunoactivity in the placenta increased with gestational age but that, relative to weight, highest concentrations were found from 12-28 weeks. This correlated with the presence of immunoactive GnRH in the maternal serum (Siler-Khodr *et al.*, 1984) but, unfortunately, the GnRH levels at earlier pregnancy were not determined.

Although relative binding changed with gestation, the profile of tracer specificity was the same at each stage, i.e. Tryptorelin, Buserelin, chicken GnRH-II, salmon GnRH > mammalian GnRH » lamprey GnRH, chicken GnRH-I (Figure 3.11). Rebinding data (Figure 3.15) suggested that increased levels of degrading enzymes at mid-gestation could not account for variations in agonist binding at different stages of pregnancy, as tracer incubated with mid-term placental membranes was capable of binding to a term placental membrane fraction with no appreciable loss in specific activity. Decreased binding at 11-18 weeks could not be accounted for by contamination of villous membrane preparations by membranes of other tissues at mid-gestation, as no corresponding decrease in EGF receptors or alkaline phosphatase activity, both markers for villous plasma membrane, was found. As moderate levels of binding were measured in early placental membranes of 10 weeks gestation, which were obtained by termination of pregnancy by prostaglandins alone, the method used for terminating pregnancy did not appear to be related to gestational age-related differences in binding.

From the above, differences in GnRH binding with gestation can only be explained by: a) increased degradation of the GnRH receptor at mid gestation, which would be very difficult to measure as levels are already very low; b) decreased expression or synthesis of the GnRH receptor at mid gestation, which requires an antibody to the GnRH receptor or a cDNA probe for the gene, neither of which are presently available; or c) differential occupancy of the GnRH receptor at mid gestation.

From Figure 3.8, at mid gestation, binding activity did not increase with increasing concentrations of membrane protein, which suggested that, if receptor number is not decreased at mid-gestation, the receptor site could be occupied by an endogenous ligand with a greater affinity for the placental binding site than the pituitary superagonist. Investigation of GnRH binding was therefore extended to include placental cytosol and the putative GnRH-like ligand for the placental GnRH receptor, described in Chapter 4.

CHAPTER 4

GnRH-LIKE ACTIVITY IN THE HUMAN PLACENTA

4.1 INTRODUCTION

Many studies have demonstrated a role for GnRH in the stimulation of hCG secretion (Khodr and Siler-Khodr, 1978b; Butzow, 1982; Siler-Khodr *et al.*, 1986a; Elkind-Hirsch *et al.*, 1989; Currie *et al.*, 1992) and the pattern of GnRH binding to pre-term placental binding sites correlates with hCG levels in the maternal serum (Hay, 1988), peaking at 8-10 weeks and falling by mid-gestation. However, Siler-Khodr *et al.* (1984) measured circulating GnRH levels in maternal serum and found highest levels occurred at 7 to 17 weeks, declining significantly in the second half of gestation. In addition, the immunoactive GnRH content of the human placenta was highest at 12-22 weeks (Siler-Khodr and Khodr, 1978).

We have shown that the decrease in GnRH binding at mid gestation cannot be accounted for by changes in affinity of the binding site, increased degradation of GnRH or changes in recovery of the membrane binding sites at this stage of pregnancy. From Figure 3.8, increasing membrane protein concentration did not result in increased binding of GnRH at mid-gestation, suggesting that GnRH receptors may be down-regulated at this stage, possibly by high levels of an endogenous GnRH-like factor.

Menzies and Bramley (1992) demonstrated that placental cytosol contained activity which rapidly inactivates GnRH-like molecules, whereas the GnRH binding site was associated with placental cell-surface membrane fractions. Therefore, the existence of GnRH-like activity in placental cytosol which could interfere with GnRH binding to placental membrane receptors but was distinct from degradative activity was investigated.

4.2 BINDING ACTIVITY OF ¹²⁵I-GnRH AGONIST ANALOGUES TO HUMAN PLACENTAL MEMBRANES IN THE PRESENCE OF PLACENTAL CYTOSOL

Placental membranes were obtained by differential centrifugation of homogenates of placental tissue and were recovered by a high-speed centrifugation

step (Smith and Brush, 1978). The supernatant obtained from this step represented the placental cytosolic fraction.

4.2.1 Effect of Increasing Concentrations of Placental Cytosol on Membrane Binding

Binding of ^{125}I -Buserelin to mid-gestation and term placental membrane preparations was measured in the presence of placental cytosol of the same gestation. In addition, the effect of mid-term placental cytosol on Buserelin binding to term placental membranes was determined.

Binding of Buserelin to mid-term placental membranes was decreased to levels of non-specific binding to the tube (assay blanks) in the presence of placental cytosol from the same preparation (Figure 4.1a). However, the maximal decrease of Buserelin binding to term placental membranes by term placental cytosol was only by ~15% of binding in the absence of placental cytosol. In contrast, cytosol extract from mid-gestation placentae decreased binding of Buserelin to term placental membranes to levels of non-specific binding to the tube. The difference in Buserelin binding to term placental membranes in the presence of term and mid-term placental cytosol was statistically significant ($P < 0.02$).

When ^{125}I -Buserelin binding in the presence of ~1.5mg placental cytosol was compared with binding in the presence of 10^{-5}M unlabelled Buserelin (Figure 4.1b), mid-term cytosol consistently decreased binding of mid-term placental membranes to levels below that observed with excess Buserelin ($P < 0.05$). This was not observed for term binding sites.

4.2.2 Effect of Time and Temperature of Incubation

Placental membranes and cytosol were incubated with ^{125}I -Buserelin at 4°C , 20°C or 37°C for up to 6 hours (Figure 4.2) to determine the effect of cytosol on tracer and receptor stability.

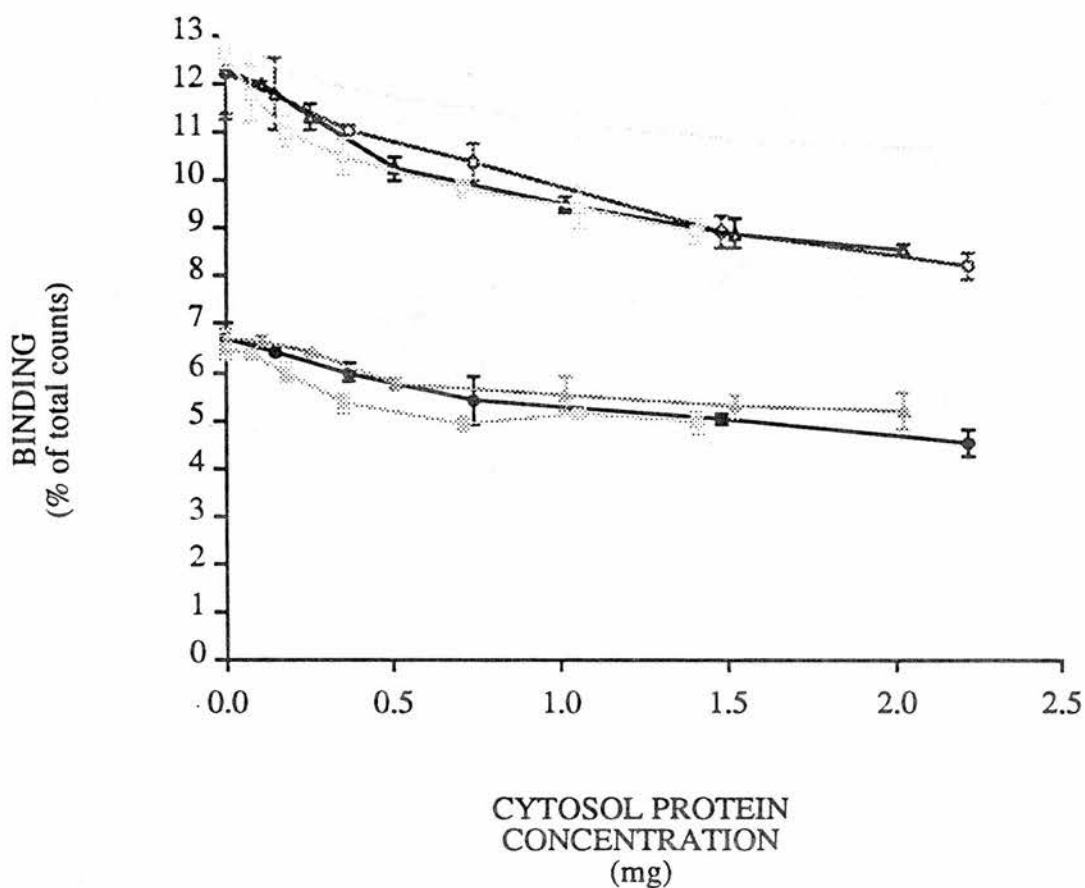


Figure 4.1a Displacement of Buserelin from placental membranes by placental cytosol

Placental membrane fractions (~1mg membrane protein) were incubated for 1 h at 20 °C with 100,000cpm iodinated Buserelin in the presence of increasing concentrations of placental cytosolic extract.

Bound and free hormone were separated by PEG precipitation and binding expressed as % of total counts added.

Buserelin binding is shown in the presence of 13 week cytosol (●, ○), 15 week cytosol (▲, △), 16 week cytosol (■, □) and term cytosol (○). Closed symbols represent binding to membranes of same gestation and open symbols represent binding to term placental membranes. Values shown represent mean ± s.d. for one representative experiment in triplicate

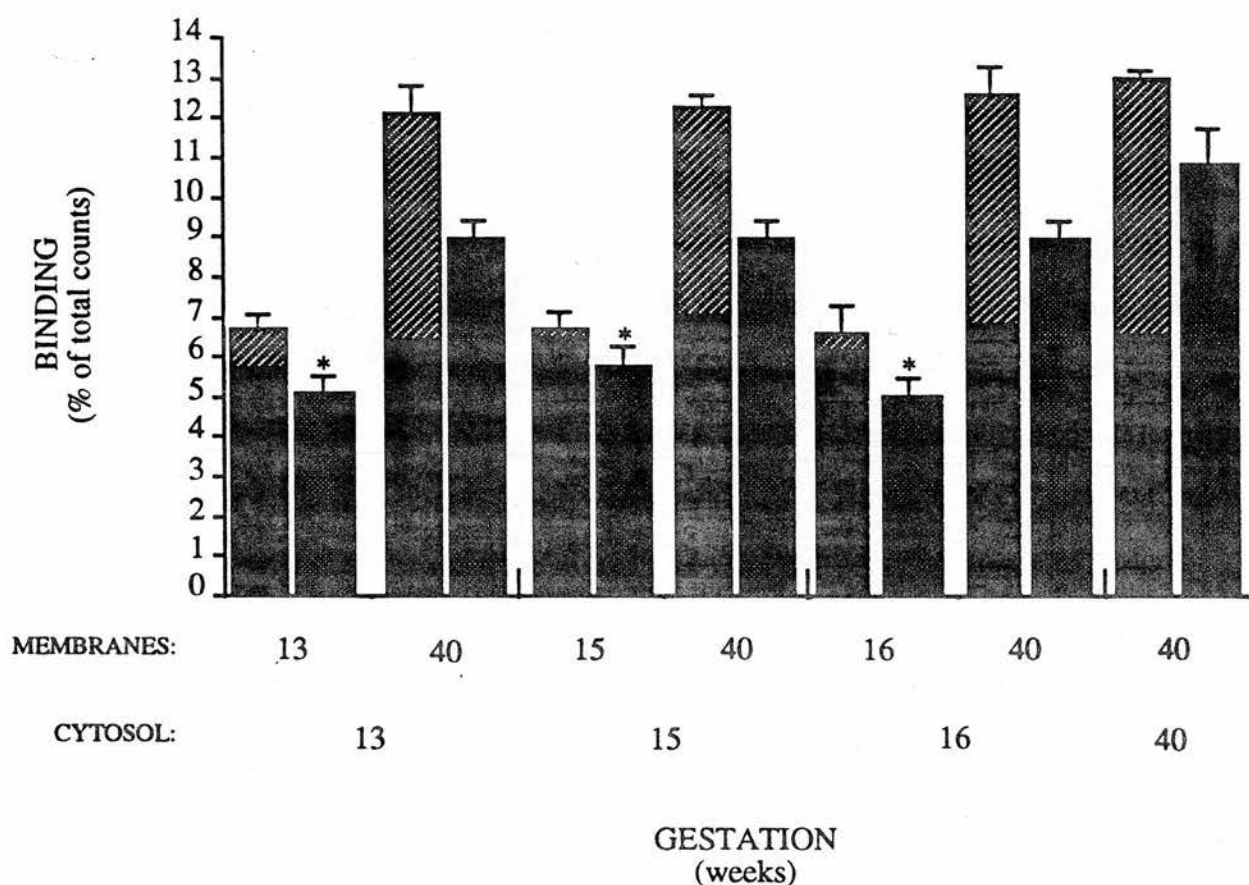


Figure 4.1b Displacement of Buserelin from placental membranes by placental cytosol

Placental membrane fractions (~1mg membrane protein) were incubated for 1 h at 20 °C with 100,000cpm radio-labelled Buserelin in the presence and absence of unlabelled Buserelin or in the presence of placental cytosolic extract (~1mg cytosol protein).

Bound and free hormone were separated by PEG precipitation and binding determined. Non-specific binding (■), specific binding (▨) and binding in the presence of cytosol (▩) were expressed as % of total counts.

Data were means ± s.d. of triplicate determinations from a representative experiment. *P<0.05 when compared to non-specific binding in the presence of unlabelled Buserelin.

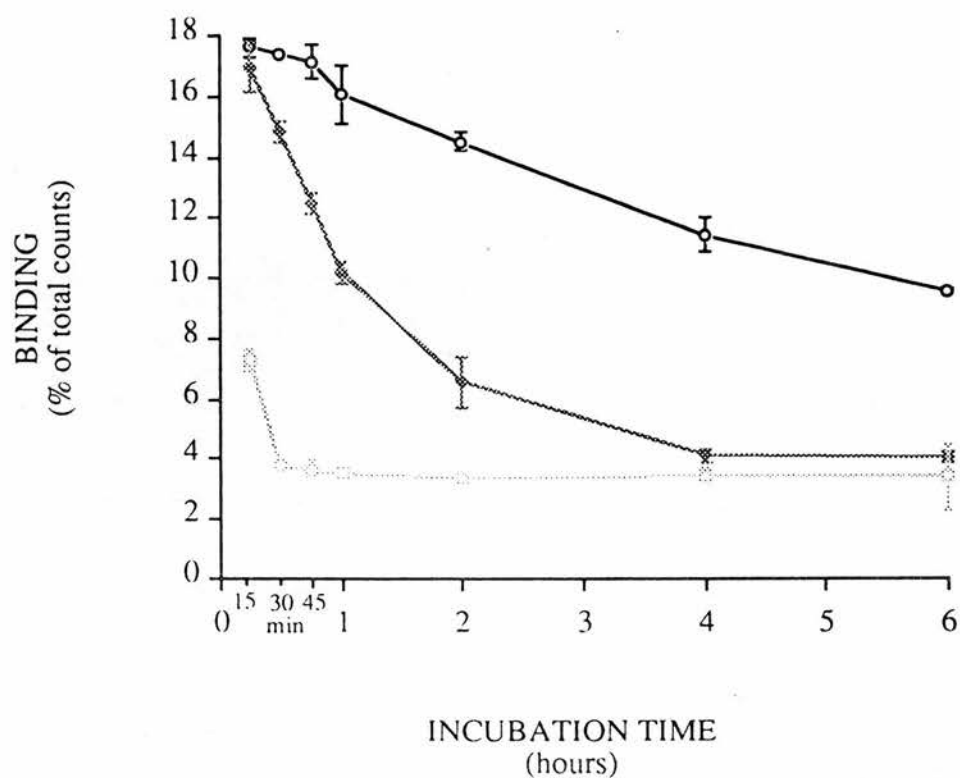


Figure 4.2

Effect of time and temperature of incubation on the displacement of Buserelin from placental membranes by placental cytosol

Placental membrane fractions (~1mg protein) were incubated with 100,000cpm radio-labelled Buserelin at 4°C (○), 20°C (◆) and 37°C (◻) in the presence of placental cytosolic extract (~1mg protein). Bound and free hormone were separated by PEG precipitation and binding expressed as % of total counts added. Data shown were mean ± s.d. for a single representative experiment performed in triplicate.

At 37°C, binding was reduced to levels of non-specific binding to the tube within 30 minutes. At 20°C, the temperature of the standard binding assay, binding was reduced by 50% after 1 hour and to non-specific levels by 4 hours. Binding was stable for up to 1 hour at 4°C, then decreased slowly; maximal decrease in binding was reached in excess of 6 hours incubation.

4.2.3 Effect of Pre-incubating Placental Membranes with Placental Cytosol

Term placental membranes were incubated with placental cytosol for up to 6 hours at 4°C, 20°C and 37°C, then assayed for Buserelin binding to membranes under standard conditions (1 hour at 20°C). Binding was calculated as % of binding in the presence of cytosol with no pre-incubation.

After 6 hours pre-incubation at 4°C and 20°C, no effect of pre-incubation was observed suggesting that cytosol had no degradative effect on the placental membrane-bound binding site at low temperatures (Figure 4.3). At 37°C, however, binding increased by 85% after 4 hours incubation.

4.2.4 Effect of Protease Inhibitors

The displacement effects observed in 4.2.1-3 could be accounted for either by the presence in the placental cytosol of a ligand with a greater affinity for the GnRH binding site than pituitary GnRH analogues, or by GnRH degradative activity associated with the cytosolic fraction (Menzies and Bramley, 1992) which reduced the level of tracer capable of binding to the membrane binding site. In order to determine if the effect of the cytosol was due to enzymatic activity, the binding of Buserelin to placental membranes was assayed in the presence of placental cytosol and protease inhibitors with activity against the four major classes of proteases (see 3.2.6 for description of activities).

In the presence of 2mM PMSF, 2mM EDTA, 2mM N-ethyl maleimide and

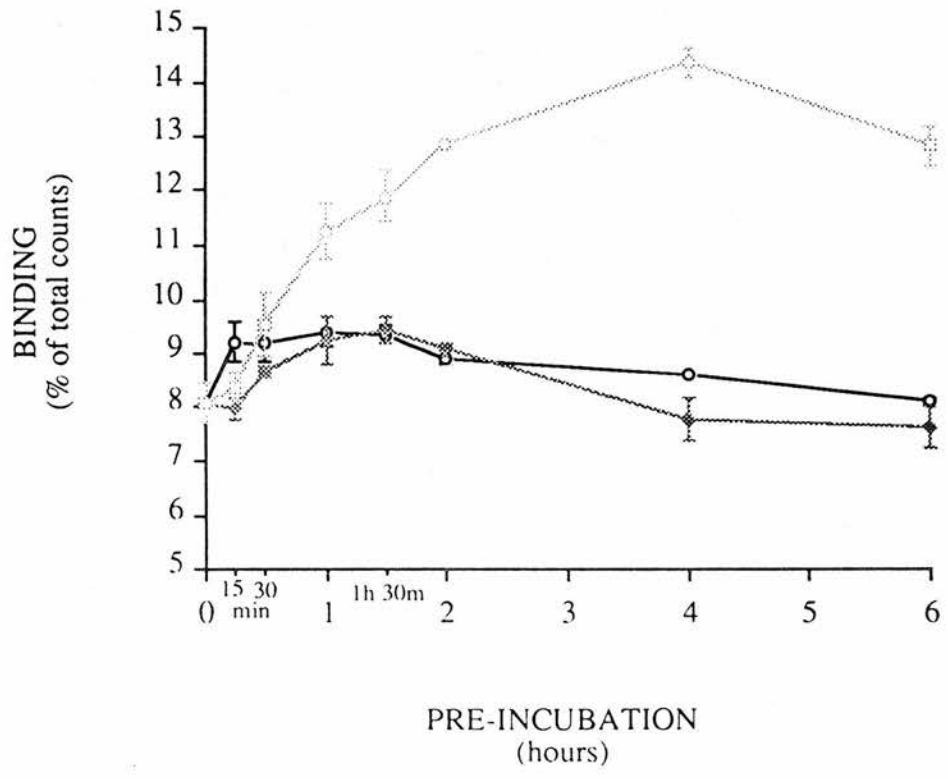


Figure 4.3 Effect of pre-incubation of placental membranes with placental cytosol on Buserelin binding

Term placental membrane fractions (~1mg protein) were incubated with placental cytosol (~1mg protein) at 4°C (○), 20°C (◆) and 37°C (◻) for up to 6h, then incubated with 100,000cpm iodinated Buserelin at 20°C for 1h. Bound and free hormone were separated by PEG precipitation and binding expressed as % of total counts added. Data shown were means ± s.d. from triplicate estimations for a single representative experiment.

1.46 μ M Pepstatin A, early, mid- and term cytosol did not decrease binding of GnRH analogues to placental membranes of the same age (Figure 4.4).

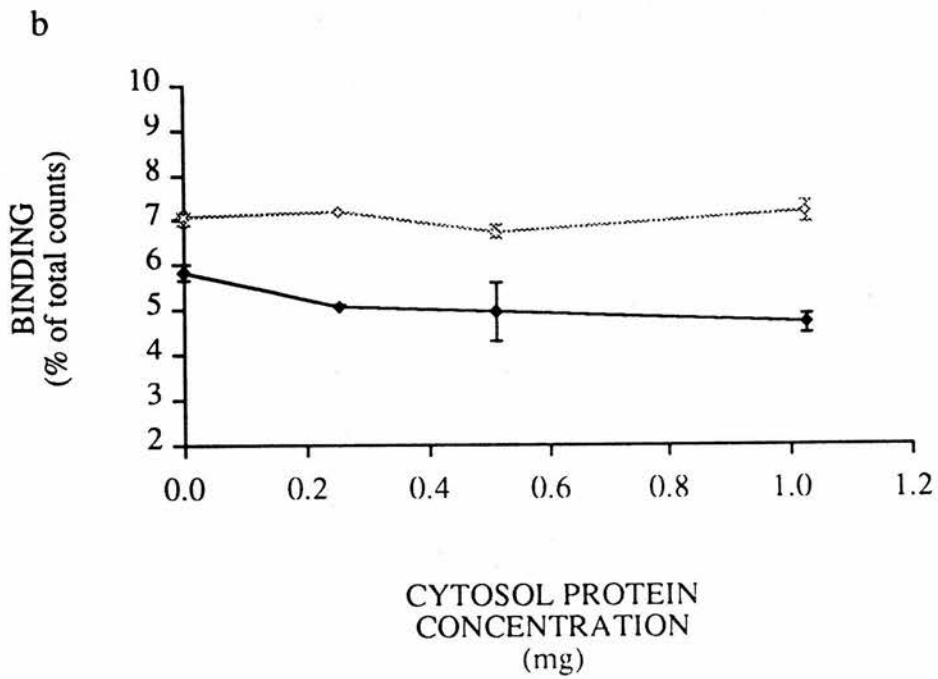
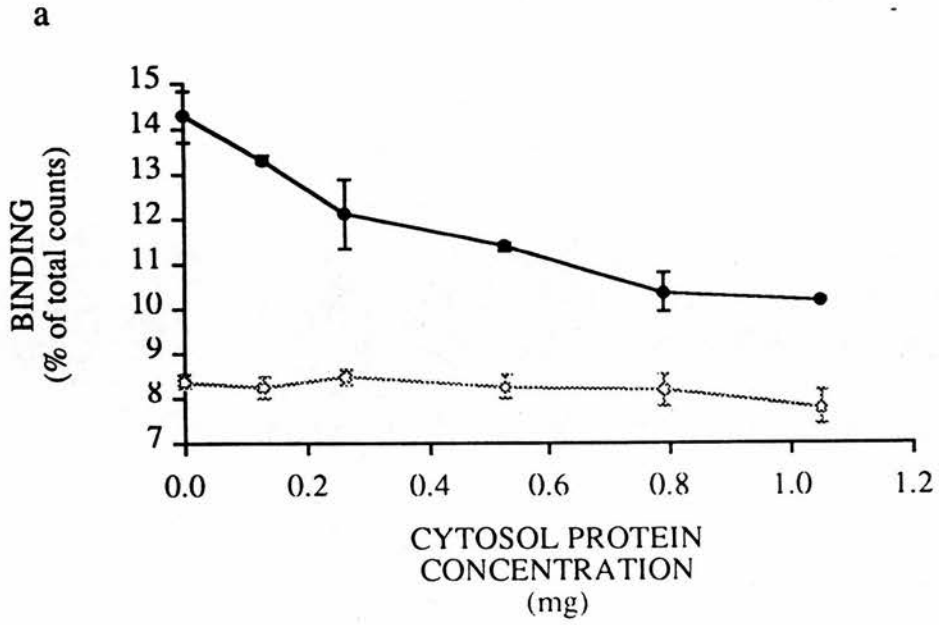
When the effect of individual inhibitors was investigated (Figure 4.5), EDTA and Pepstatin A had no effect on either Buserelin binding or on the reduction of binding in the presence of placental cytosol. Specific binding was significantly decreased in the presence of N-ethyl maleimide ($P < 0.05$) but no effect of N-ethyl maleimide was observed on Buserelin binding with cytosol. However, PMSF and ethanol, the vehicle for PMSF, reduced both specific binding and binding in the presence of placental cytosol to levels of non-specific binding ($P < 0.01$). Thus no specific effect of the protease inhibitors on Buserelin binding in the presence of cytosol was identified, although the presence of ethanol was observed to interfere with Buserelin binding, as previously described (3.2.6).

4.2.5 Effect of Placental Cytosol on the Binding Activity of Tryptorelin and Chicken GnRH-II

As chicken GnRH-II has an affinity for the placental GnRH receptor which is comparable to synthetic agonist analogues of GnRH (as discussed in Section 3.4), the effect of placental cytosol on the binding of iodinated Tryptorelin, Buserelin and chicken GnRH-II to placental membrane preparations of the same gestation was compared (Figure 4.6).

At all stages of pregnancy, binding of each GnRH analogue was decreased in the presence of placental cytosol. Lower concentrations of cytosol were required to inhibit chicken GnRH-II binding, compared with Buserelin and Tryptorelin which both incorporate modifications to protect the molecule from proteolytic degradation.

The effect of placental cytosol on the binding of Buserelin and chicken GnRH-II to placental membranes was compared in the presence and absence of inhibitors of the four major classes of protease activity, in order to determine if increased susceptibility of chicken GnRH-II to degradation by cytosolic proteases could account



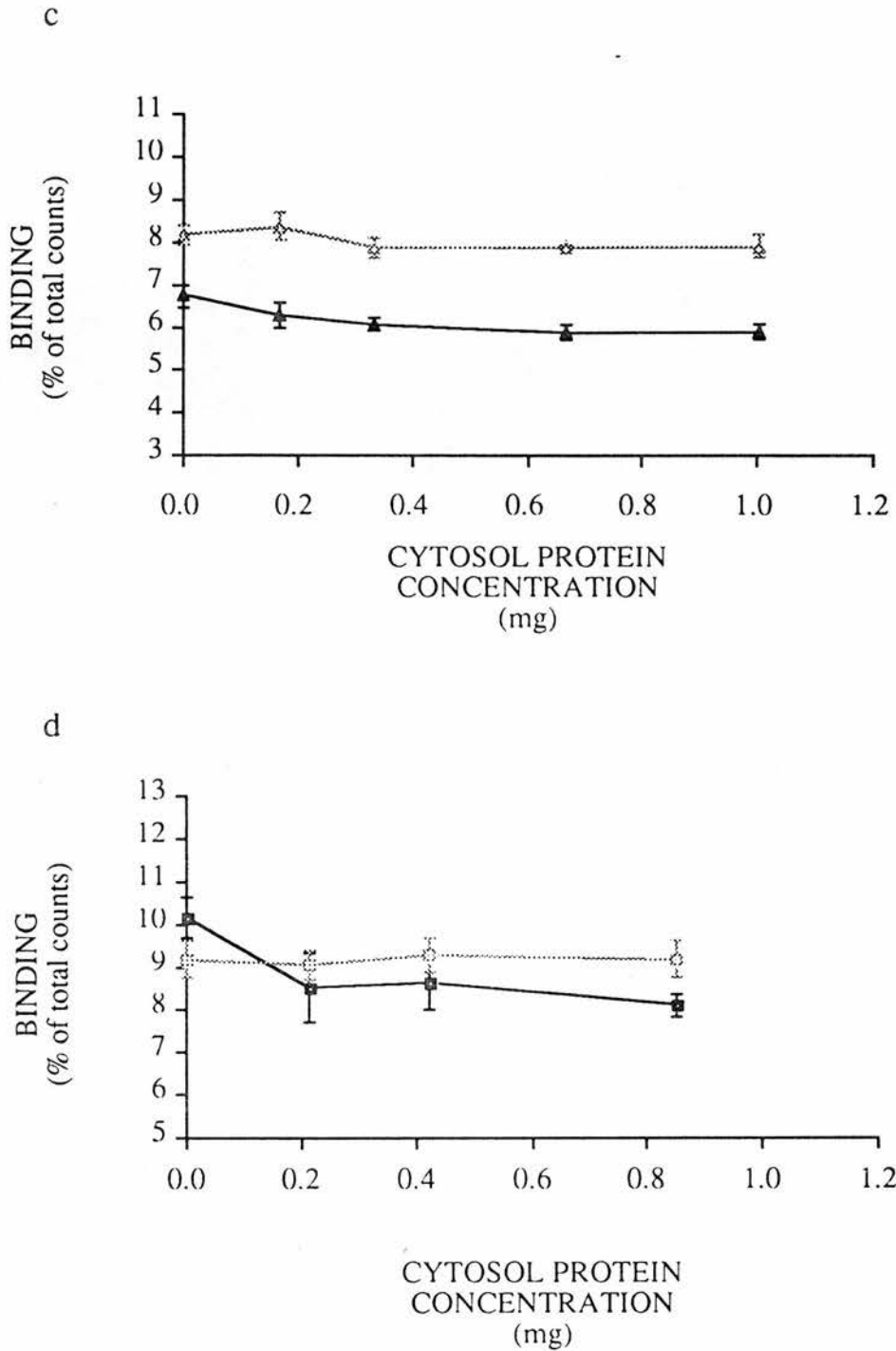


Figure 4.4 Effect of protease inhibitors on Buserelin displacement from placental membranes by placental cytosol

Placental membranes were incubated at 20°C for 1h with 100,000cpm iodinated Buserelin and increasing concentrations of cytosol fraction in the presence (open symbols) and absence (closed symbols) of a 'cocktail' of protease inhibitors.

Data shown were for a) 8 week cytosol and 7 week membranes, b) 15 week cytosol and membranes, c) 18 week cytosol and membranes and d) term cytosol and membranes. Data represents the mean \pm s.d. of triplicate determinations for individual experiments.

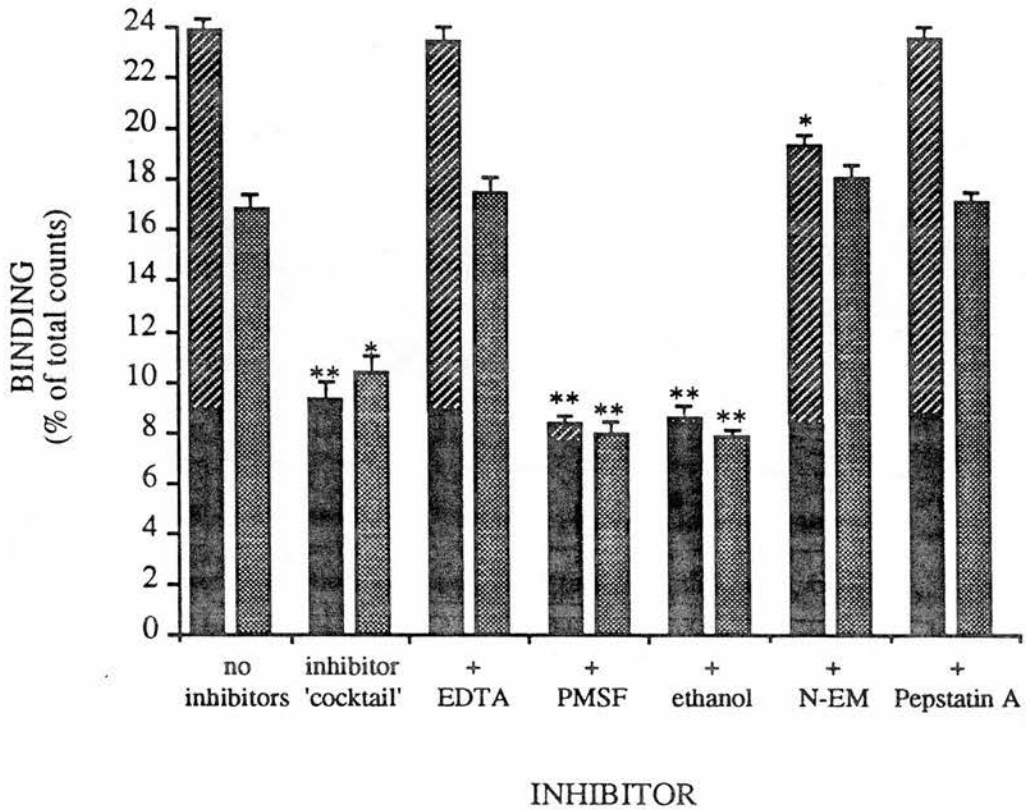
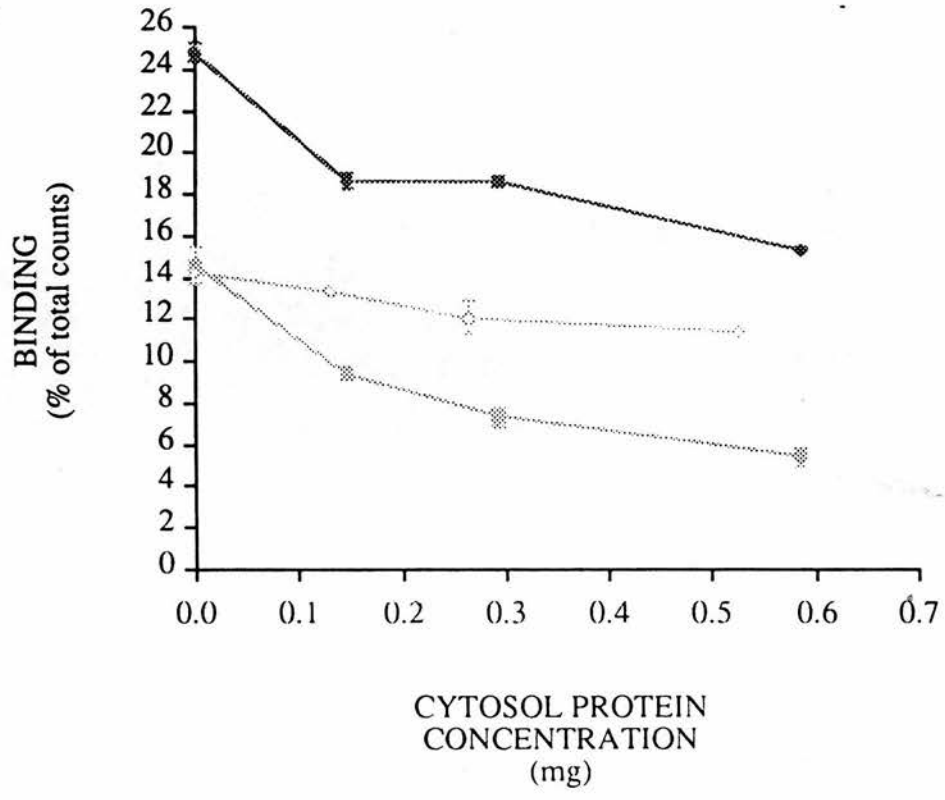


Figure 4.5 Effect of individual protease inhibitors on the displacement of Buserelin from placental membranes by placental cytosol

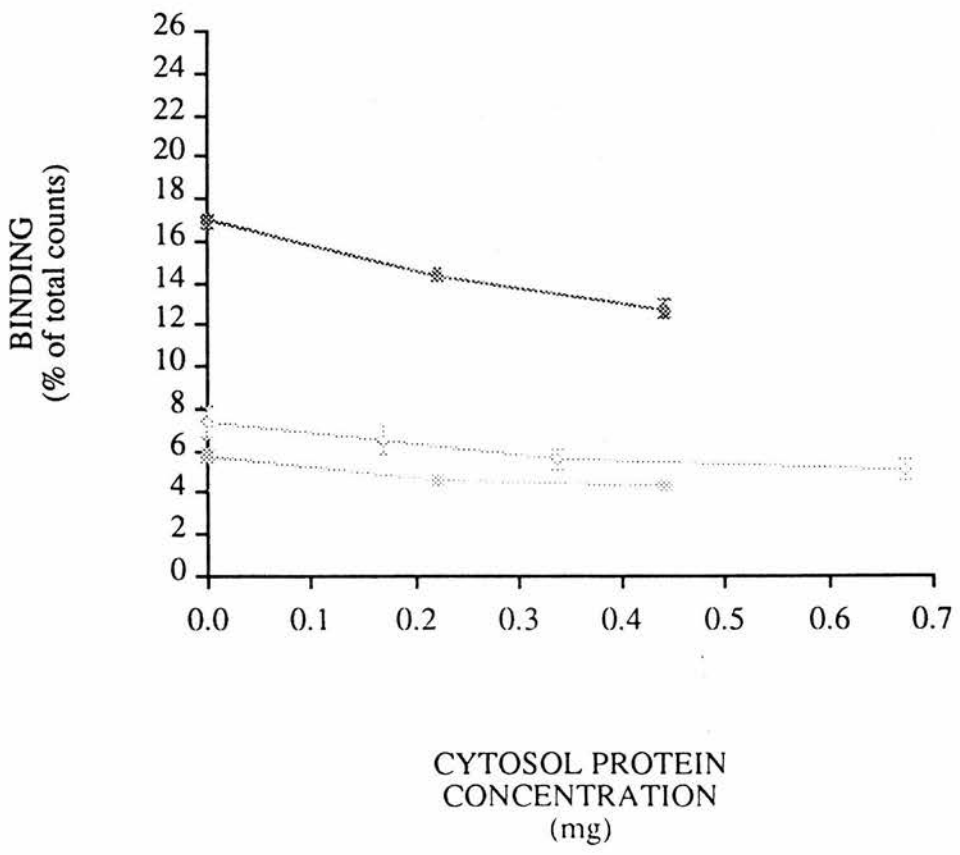
Placental membranes (~1mg protein) were incubated for 1h at 20°C with radioiodine-labelled Buserelin, in the presence and absence of 10µM uniodinated Buserelin or placental cytosolic extract (~1mg protein).

Bound and free hormone were separated by PEG precipitation and non-specific binding (■), specific binding (▨) and binding in the presence of placental extract (■) were expressed as % of total counts. Data shown were means ± s.d. of triplicate determinations from an individual, representative term placental membrane and term placental cytosol determination. *P<0.05; **P<0.01 when compared to binding in the absence of protease inhibitors.

a.



b.



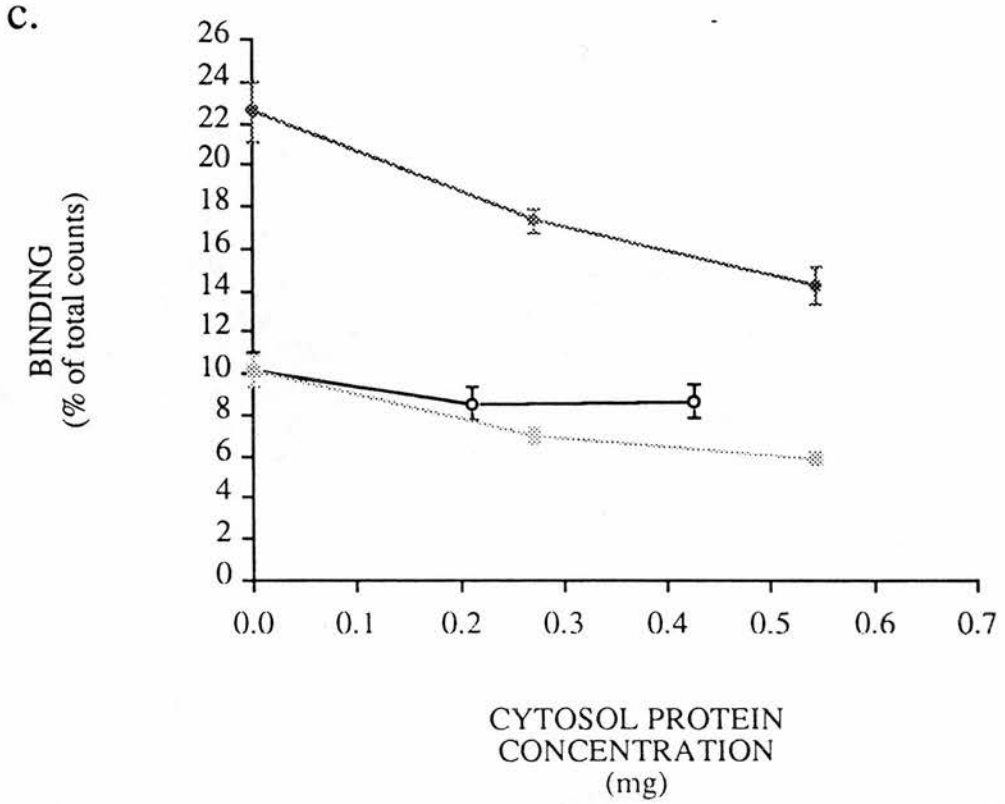


Figure 4.6

Comparison of binding of Buserelin, Tryptorelin and chicken GnRH-II to placental membranes in the presence of placental cytosol

7 week (a), 16 week (b) and term (c) membranes (~1mg protein) were incubated under standard conditions with 100,000 cpm iodinated Buserelin (○), Tryptorelin (●) and Chicken GnRH-II (✱) in the presence of increasing concentrations of cytosol of matched age.

Bound and free hormone were separated by PEG precipitation. Binding was determined and expressed as % of total counts.

Data shown were for an individual representative experiment for each gestation; each point is mean \pm s.d. of triplicate determinations.

for this effect of placental cytosol. From Figure 4.7, no conclusive pattern was observed: with protease inhibitors increasing binding in the presence of cytosol for chicken GnRH-II binding with mid-gestation placental membranes and cytosol alone.

4.3 GnRH BINDING ACTIVITY OF HUMAN PLACENTAL CYTOSOL

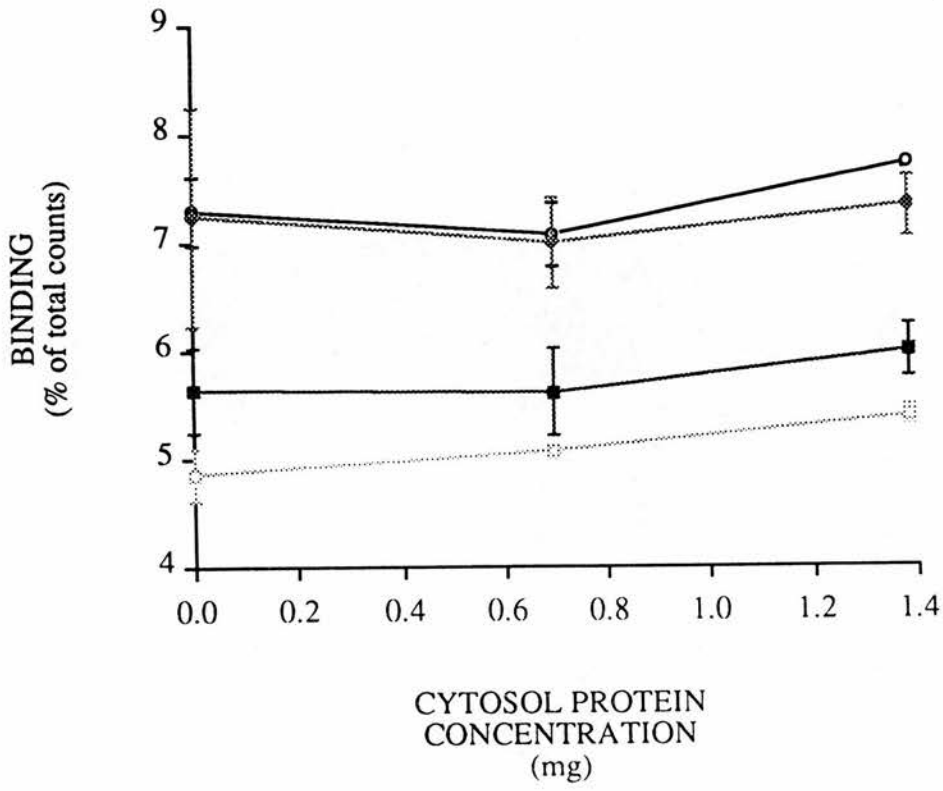
The GnRH binding activity of cytosolic extract in the standard GnRH binding assay was negligible: for a representative term placental preparation, binding of the cytosol was 0.3% of total counts/mg protein compared with 3.6% of total counts per mg for the placental membrane fraction. Measurement of the effects of cytosol on binding to placental microsomes required large amounts of placental membranes, and assays were complicated by differentiation between effects of cytosol on membranes and general effects of assay conditions on membrane binding. However, the availability of specific anti-chicken GnRH-II antibodies allowed the development of a radio-immunoassay, as described in Section 2.8, which did not require the use of placental microsomes.

4.3.1 Effects of Human Placental Cytosol on Binding of Chicken GnRH-II to Anti-chicken GnRH-II Antibody

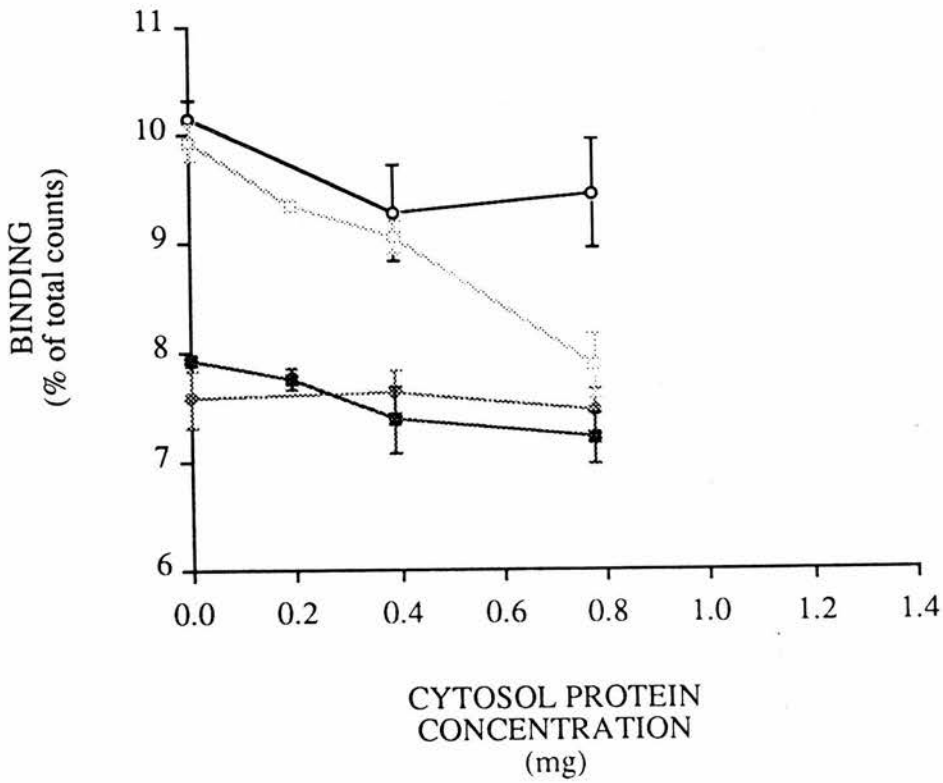
The displacement of iodinated chicken GnRH-II from chII-Ab by unlabelled chicken GnRH-II and human placental cytosol of all stages of gestation was compared in the presence and absence of protease inhibitors, as described in 2.8.

Placental cytosol and unlabelled hormone both displaced ^{125}I -chicken GnRH-II from chII-Ab (Figure 4.8). The effects of cytosol on chII-Ab binding were dose-dependent and correlated well with the inhibition of ^{125}I -chicken GnRH-II binding to placental membranes, shown in Figure 4.6. Protease inhibitors in the assay medium did not interfere with the binding of chII-Ab to tracer, hormone or placental cytosol, however displacement of cytosol extracted in inhibitor-containing medium was three-

a.



b.



c.

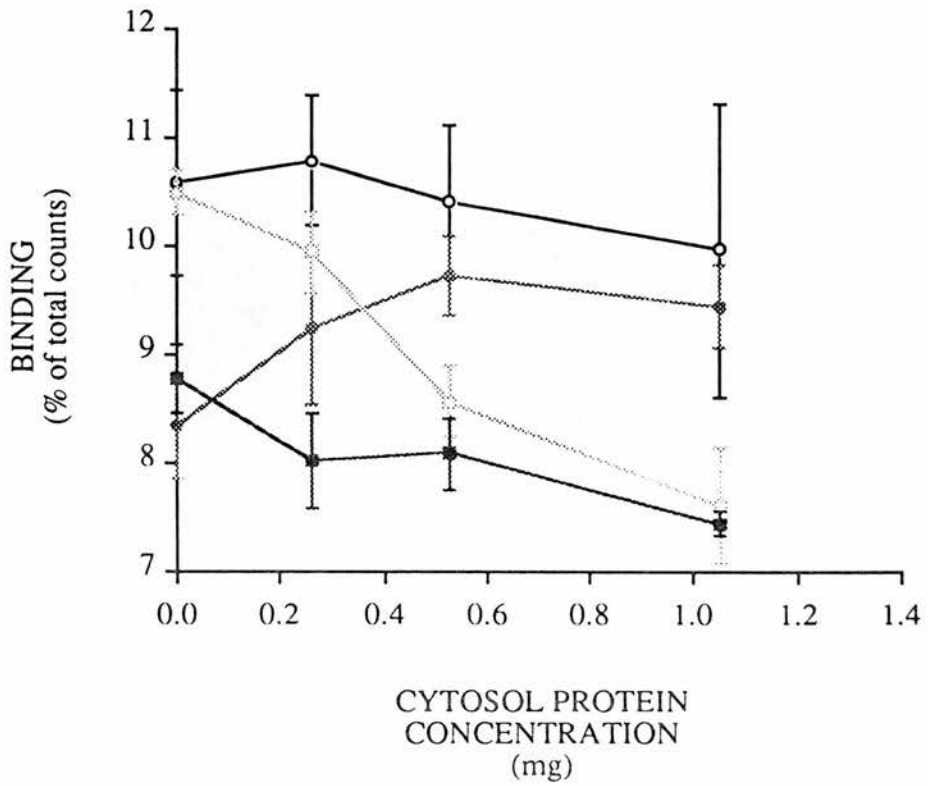


Figure 4.7

Effect of protease inhibitors on Buserelin and chicken GnRH-II binding to placental membranes in the presence of placental cytosol

13 week (a) and 40 week (b, c) membranes (~1mg protein) were incubated under standard conditions with 100,000cpm iodinated Buserelin (circles) and chicken GnRH-II (squares) in the presence of increasing concentrations of 13 week (a), 18 week (b) and 40 week (c) cytosol. Bound and free hormone were separated by PEG precipitation and binding expressed as % of total counts added. Binding in the presence and absence of protease inhibitors is represented by closed and open symbols respectively.

Data shown were means \pm s.d. from a single experiment performed in triplicate and were representative for each gestation.

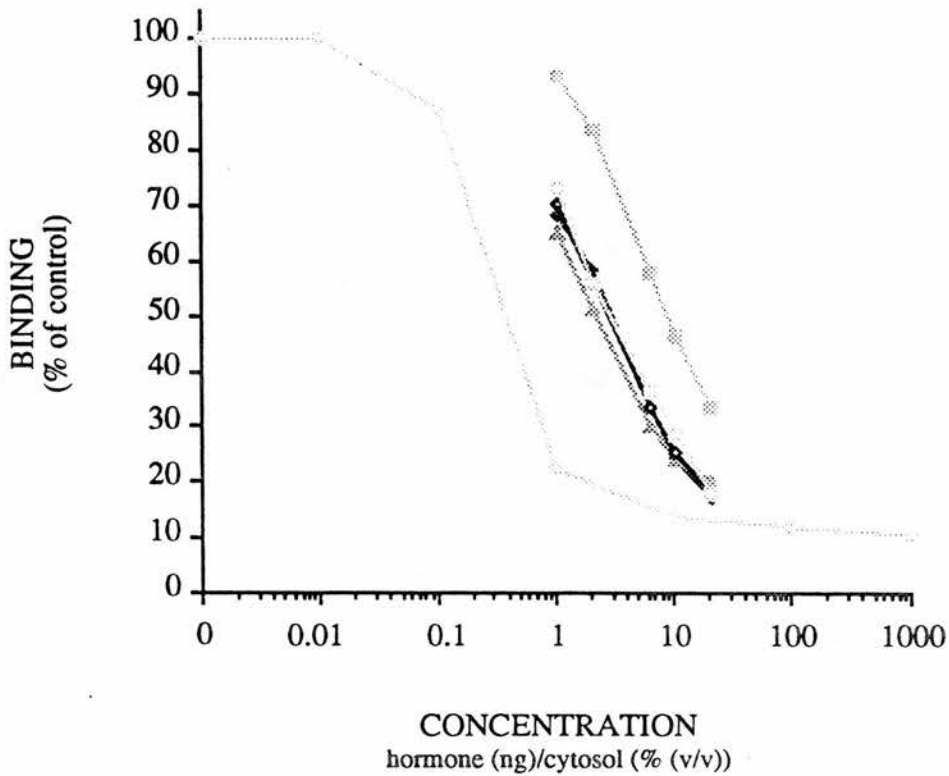


Figure 4.8 Displacement of iodinated chicken GnRH-II from chII-Ab by placental cytosol

Anti-chicken GnRH-II antibody (1:50,000 dilution) was incubated with 30,000cpm iodinated chicken GnRH-II and increasing concentrations of unlabelled chicken GnRH-II (\odot), pre-term human placental cytosol (8 weeks \blacklozenge ; 15 weeks \blacktriangleleft ; 16 weeks \blacklozenge) or term human placental cytosol (\blackast , \odot) for 16h at 4°C, in the presence and absence of protease inhibitors.

Bound and free iodinated hormone were separated by PEG precipitation, binding determined and expressed as % of control binding (in the absence of cytosol, unlabelled hormone or inhibitors).

Data shown were means \pm s.d. for triplicate estimations in the absence of protease inhibitors. Protease inhibitors had no effect on binding and data was not shown for clarity. Term placental cytosol represented extract obtained from one placenta prepared in the presence (\blackast) and absence (\odot) of protease inhibitors.

fold less, compared with cytosol prepared in the absence of inhibitors (Figure 4.8).

4.3.2 Binding Activity of Fractionated Human Placental Cytosol to Anti-chicken GnRH-II Antibody

Term placental cytosol was fractionated by gel exclusion chromatography on a Sephadex G-100 column eluted with PBS and the activity of individual fractions was determined using the chII-Ab assay in the presence and absence of protease inhibitors.

Placental cytosol fractions from gel permeation chromatography had little activity in the chII-Ab assay (Figure 4.9). However, in the presence of inhibitors of protease activity, displacement of chicken GnRH-II from chII-Ab was observed by placental cytosol fractions, corresponding to an apparent M_r of 60-80,000. As inhibition of chicken GnRH-II binding was observed only in the presence of protease inhibitors, a component of the inhibitor mixture was thought to either activate the endogenous ligand or interfere with chicken GnRH-II binding to the antibody in the presence of placental cytosol.

4.3.3 Effect of Individual Protease Inhibitors on Cytosol Binding to Anti-chicken GnRH-II Antibody

Inhibition of binding of ^{125}I -chicken GnRH-II to chII-Ab by high molecular weight fractions of placental cytosol in the presence of protease inhibitors was investigated to determine the effect of individual protease inhibitors.

Pooled active fractions of cytosol consistently failed to inhibit binding of chicken GnRH-II to anti-chicken GnRH-II antibody in the absence of protease inhibitors (Figure 4.10), and inhibitors had no effect in the absence of cytosol (Figure 4.8). However, in the presence of the high molecular weight fractions and PMSF, Pepstatin A, or ethanol, the vehicle for the protease inhibitors, chicken GnRH-II binding to anti-chicken GnRH-II antibody was demonstrated. Thus, ethanol appeared to be involved in interference of antibody-tracer binding.

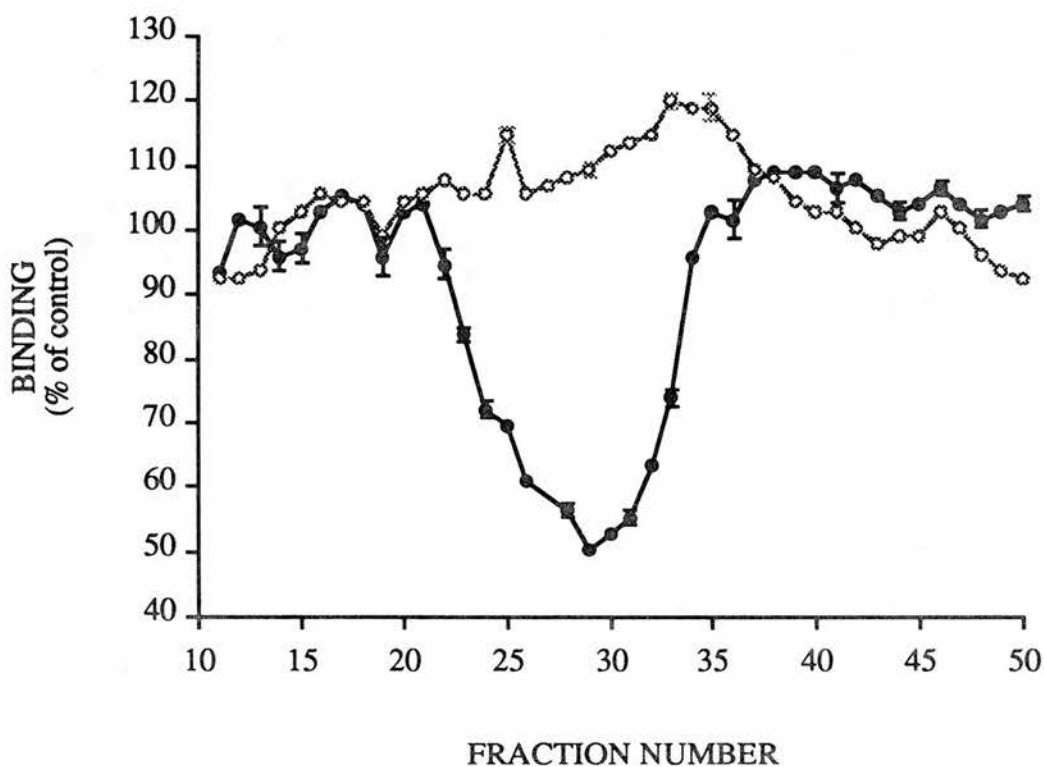


Figure 4.9 Effect of fractionated human placental cytosol on binding of iodinated chicken GnRH-II to chII-Ab

4ml human term placental cytosol was chromatographed on a 1.6cm x 50ml column of Sephadex G-100 (Fine) equilibrated and eluted with PBS. 2ml fractions were collected and 30 μ l aliquots assayed for inhibition of anti-chicken GnRH-II antibody (1:50,000 dilution) binding to 30,000cpm iodinated chicken GnRH-II in the presence (●) and absence (○) of a 'cocktail' of protease inhibitors (2mM EDTA, 2mM PMSF, 2mM N-EM and 2 μ M Pepstatin A).

After incubation at 4°C for 16h, bound and free iodinated hormone were separated by PEG precipitation. Binding was determined and expressed as % of control binding (in the absence of cytosol or inhibitors).

Data shown were means \pm s.d. for triplicate estimations from an individual representative experiment.

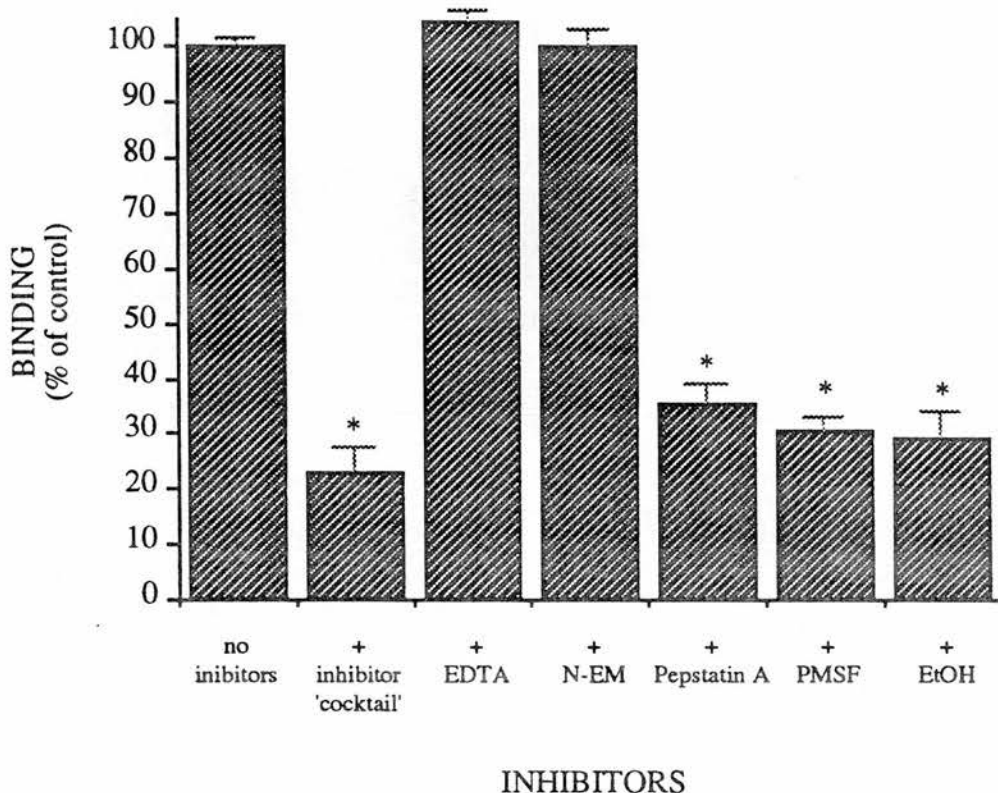


Figure 4.10 Effect of individual protease inhibitors on binding of chicken GnRH-II to ChII-Ab

Anti-chicken GnRH-II antibody (1:50,000 dilution) was incubated with 30,000cpm iodinated chicken GnRH-II and pooled, active high molecular weight fractions of human placental cytosol for 16h at 4°C. The effect of individual protease inhibitors (2mM EDTA, 2mM PMSF, 2mM N-EM and 2µM Pepstatin A) was determined.

Bound and free iodinated hormone were separated by PEG precipitation, binding determined and expressed as % of control binding (in the absence of cytosol or inhibitors).

Data shown were means ± s.d from an individual representative experiment. *P<0.01 when compared to binding in the absence of protease inhibitors.

4.3.4 Effect of Ethanol on Cytosol Binding to Anti-chicken GnRH-II Antibody

Ethanol had no effect on chicken GnRH-II binding to chII-Ab. However, a dose-dependent inhibition of chicken GnRH-II binding to chII-Ab was observed in the presence of pooled active fractions of placental cytosol in the presence of ethanol was dose-dependent (Figure 4.11). Tracer binding was reduced by 50% in the presence of 30 μ l of fraction with 1% (v/v) ethanol in the assay.

Increasing concentrations of the high molecular weight fraction of placental cytosol were assayed for displacement activity in the presence and absence of 1% ethanol in the chII-Ab assay (Figure 4.12). No displacement was seen with cytosol fraction in the absence of ethanol, or with pooled fractions of low molecular weight cytosol in the presence or absence of ethanol. However, in the presence of ethanol the high molecular weight fraction of placental cytosol decreased ¹²⁵I-chicken GnRH-II binding to antibody in a dose-dependent manner. Ethanol had no effect on the self-displacement of ¹²⁵I-chicken GnRH by unlabelled hormone, nor on the displacement activity of pre-term and term cytosol (Figure 4.8).

4.3.5 Binding of ¹²⁵I-chicken GnRH-II to an Endogenous Placental Cytosol Ligand

In order to determine if an endogenous GnRH binding protein was present in cytosolic extracts, increasing volumes of placental cytosol were incubated with iodinated chicken GnRH-II and separated by PEG precipitation, as described in Section 2.5.1, or by DCC separation, as described in Section 2.9.

PEG precipitation of tracer-ligand complexes indicated very low levels of tracer binding to the cytosol (Figure 4.13). However, as the cytosolic ligand was not pelleted with microsomes by differential centrifugation, it would presumably be a very small molecule and when complexed with tracer, may not be large enough to be pelleted by PEG.

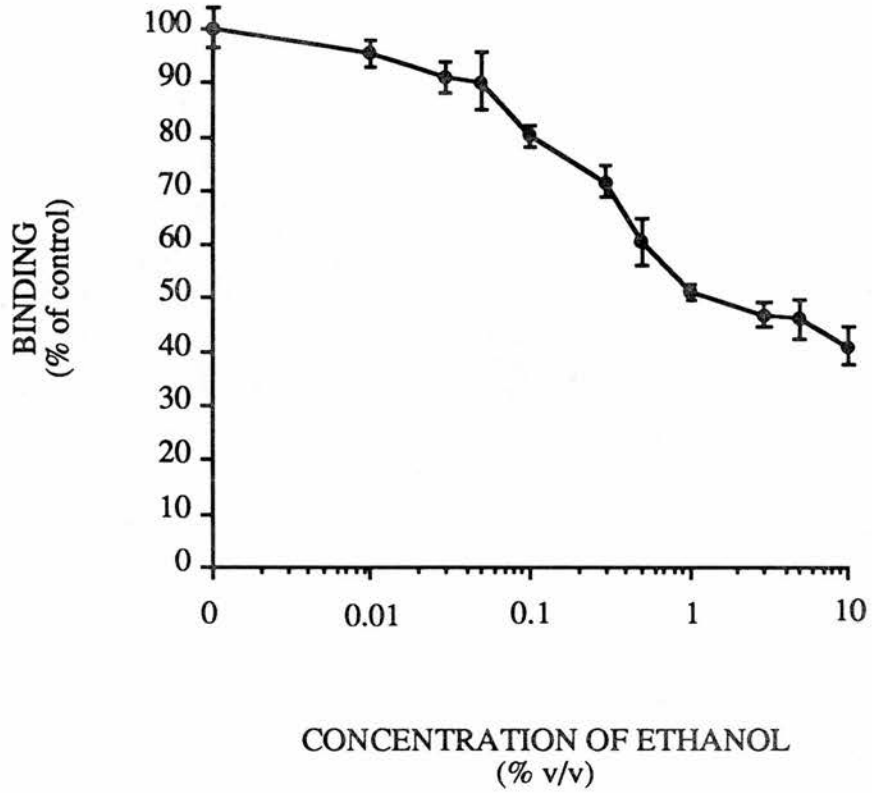


Figure 4.11 Effect of increasing concentrations of ethanol on chicken GnRH-II binding to ChII-Ab

Anti-chicken GnRH-II antibody (1:20000 dilution) was incubated with 30,000cpm radio-labelled chicken GnRH-II in the presence of the high molecular weight fraction of placental cytosol and increasing concentrations of ethanol, for 16 h at 4°C.

Bound and free hormone were separated by PEG precipitation and binding determined. Binding was expressed as % of control, with no ethanol.

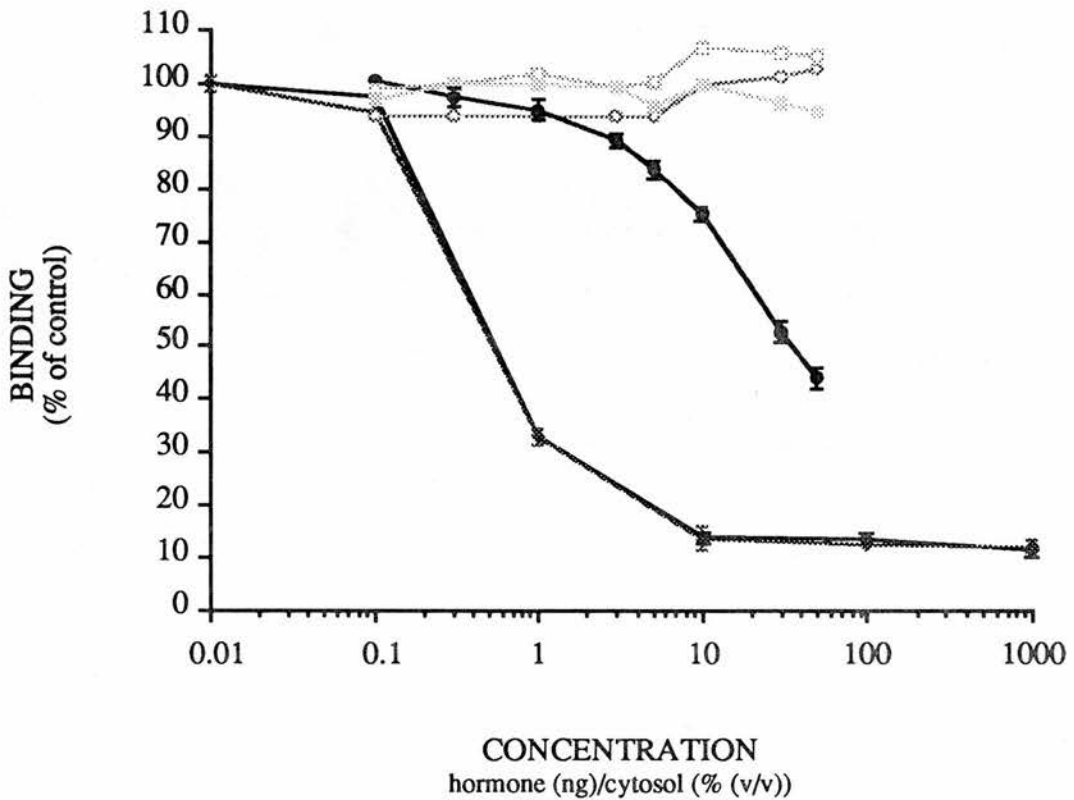


Figure 4.12 Effect of ethanol on displacement of iodinated chicken GnRH-II from ChII-Ab

Anti-chicken GnRH-II antibody (1:50,000 dilution) was incubated with 30,000cpm iodinated chicken GnRH-II and increasing concentrations of unlabelled chicken GnRH-II (♦, ♦), pooled fractions of 'active' cytosol (◊, ●) or pooled fractions of 'inactive' cytosol (◐, ◑) for 16h at 4°C, in the presence (◓, ◔, ◕) and absence (♦, ◊, ◐) of ethanol (1% v/v).

Bound and free iodinated hormone were separated by PEG precipitation, binding determined and expressed as % of control binding \pm s.d (in the absence of cytosol, unlabelled hormone or inhibitors).

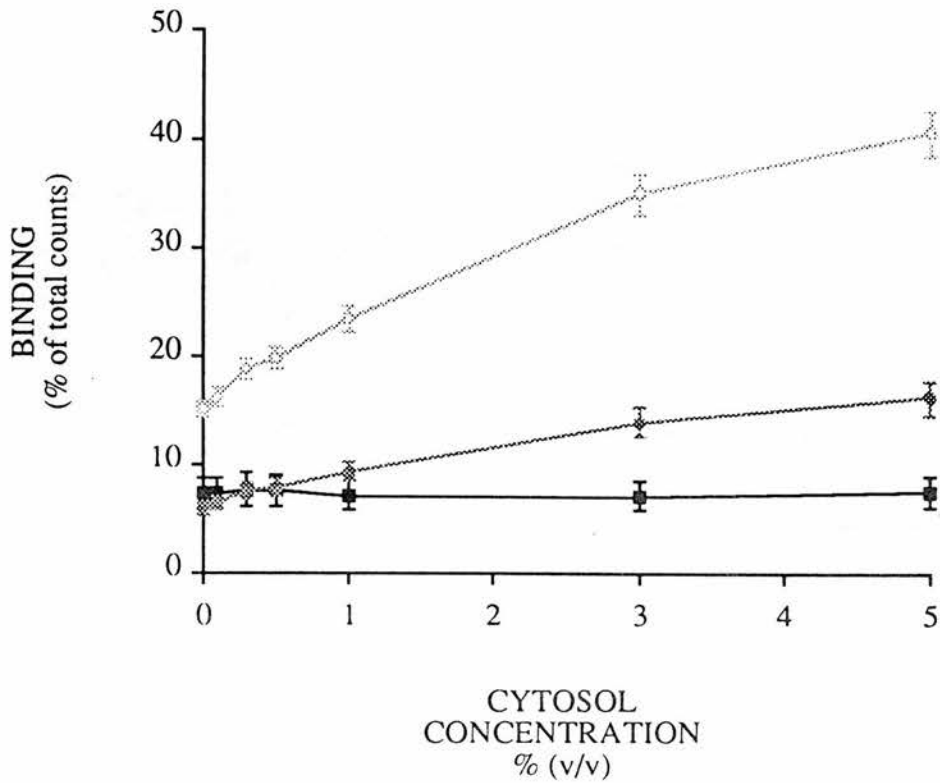


Figure 4.13 Binding of chicken GnRH-II tracer to binding sites in placental cytosol

Increasing volumes of placental cytosol were incubated with 30,000cpm iodinated chicken GnRH-II for 2h at 4°C.

Bound and free labelled hormone were separated using :

a) PEG which precipitates large tracer-ligand complexes or

b) DCC which adsorbs small molecules such as free tracer.

The radioactivity of the PEG precipitate (■), DCC precipitate (✱) and DCC supernatant (○), which contained the tracer-ligand complexes, was determined.

Data shown were mean \pm s.d. of triplicate determinations for a representative experiment.

Separation of unbound and bound ^{125}I -chicken GnRH-II by adsorbing free tracer with DCC showed that binding of chicken GnRH-II increased in a dose-dependent fashion in the presence of increasing concentrations of placental cytosol (Figure 4.13).

4.4 ISOLATION OF CYTOSOLIC CHICKEN GnRH BINDING ACTIVITY

Gel exclusion chromatography separates molecules on the basis of their size, whereas ion-exchange chromatography utilises the attraction between oppositely charged particles to separate biological compounds which carry a net positive or negative charge. Cation exchangers, eg. CM-Sepharose (Pharmacia Ltd., Milton Keynes, Bucks, England), possess negatively charged groups and will attract positively charged molecules. Conversely, anion exchangers, such as DEAE-Sepharose (Pharmacia Ltd, Milton Keynes, Bucks, England), have positively charged groups which will attract negatively charged groups.

A variety of functional groups can be attached covalently to sepharose gels and can be used to isolate specific molecules according to their affinity. For example, the carbohydrate-binding lectin gel Concanavalin A-Sepharose can be used to isolate glucose/mannose-containing glycoproteins.

4.4.1 Properties of Binding of Placental Cytosolic Binding Proteins to Ion-exchange and Lectin-binding Gels

Placental cytosol was incubated with CM-Sepharose CL-6B, DEAE-Sepharose CL-6B and Concanavalin A-Sepharose 4B were incubated for 3 hours at room temperature, on a rotating board. Changes in salt concentration or pH alter the association of compounds with the gels and so the effect of increasing concentrations of salt on cytosol GnRH binding activity to each type of gel was determined. In addition, the effect of the sugar α -methyl-D-mannoside on the association of the placental GnRH binding protein with Concanavalin A was investigated.

Association of cytosol GnRH binding activity with the gel was determined by assay of eluted fractions using the DCC chicken GnRH-II binding assay (Figure 4.14). Cytosol GnRH binding activity was adsorbed to DEAE-Sepharose, and eluted by 0.3-0.5M NaCl. Association of the binding protein with Concanavalin A was observed only at high salt concentrations but cytosol GnRH binding activity was eluted by increasing concentrations of α -methyl-D-mannoside. No adsorption was observed with CM-Sepharose incubation and controls (cytosol incubated with increasing salt or sugar) discounted any effect of NaCl or α -methyl-D-mannoside on cytosol binding in the DCC assay.

As the cytosol GnRH binding activity was retained with DEAE-Sepharose at physiological pH, this activity appeared to be negatively charged under these assay conditions. Association with Concanavalin A appeared to occur only in the presence of high salt but the cytosol GnRH binding activity was eluted from the gel in the presence of α -methyl-D-mannoside.

4.4.2 Isolation of Cytosol GnRH Binding Activity: NaCl

Adsorption of the binding protein to DEAE was utilised to attempt to isolate the cytosolic GnRH binding activity. Cytosol was incubated with the gel, then the gel was washed with sequentially higher concentrations of salt. The supernatants isolated at each step were assayed for cytosol binding activity in the DCC assay (Figure 4.15).

GnRH binding activity was negligible in the supernatant from the incubation with gel alone, from the distilled water (dH₂O) wash and from the Tris-HCl wash. Binding increased ten-fold in the supernatant from the 0.5M NaCl wash, then fell to negligible levels by the 3M NaCl wash. However, maximal binding activity of the DEAE purified ligand was <50% of the maximal activity of the cytosol. Moreover, the

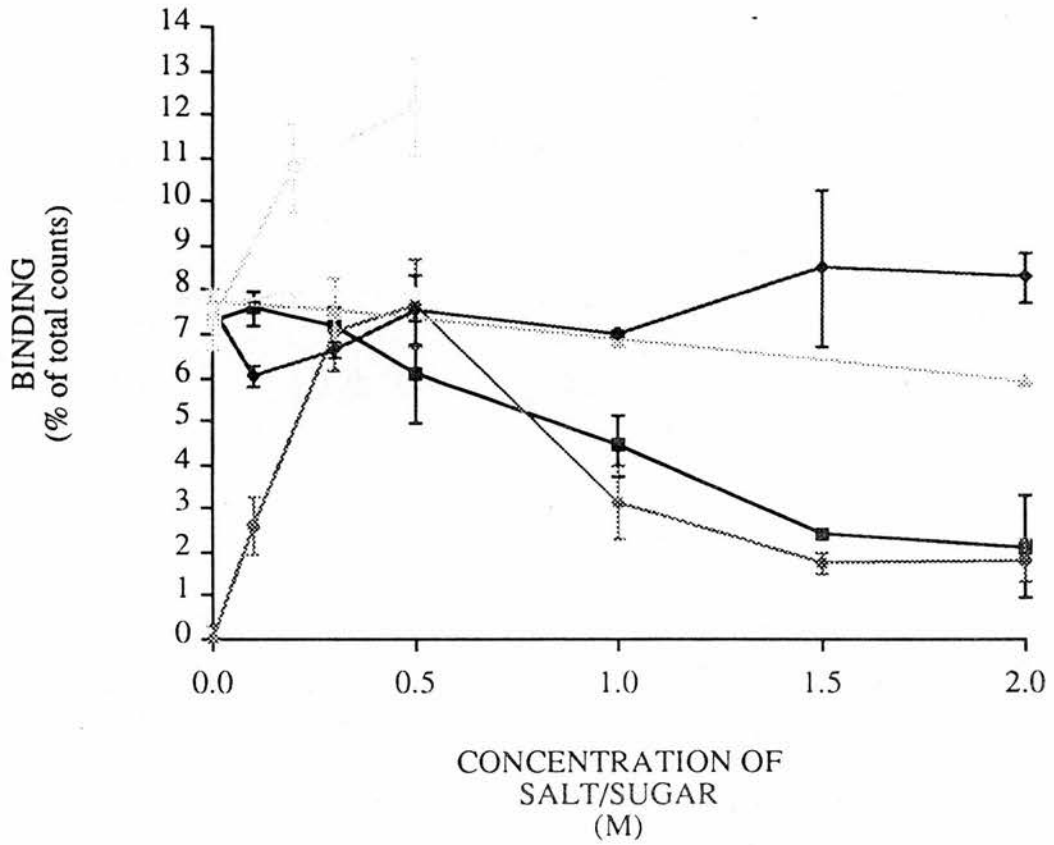


Figure 4.14

Association of cytosol with ion-exchange and lectin-linked gels

Term placental cytosol was incubated, with rotation, for 4h at 20°C with DEAE-Sepharose CL-6B (*), CM-Sepharose CL-6B (○) or Concanavalin A-Sepharose 4B (■) in the presence of increasing NaCl concentration or α-Me-D-Mannoside (○, Con A only). The effect of salt (◆) and sugar (○) on cytosol was also determined. Gels (and adsorbed molecules) were packed and supernatant assayed for chicken GnRH-II binding (incubated with 30,000cpm iodinated chicken GnRH-II for 2h at 4°C and binding activity determined by DCC separation). Binding was corrected for non-specific binding to tube and expressed as % of total counts.

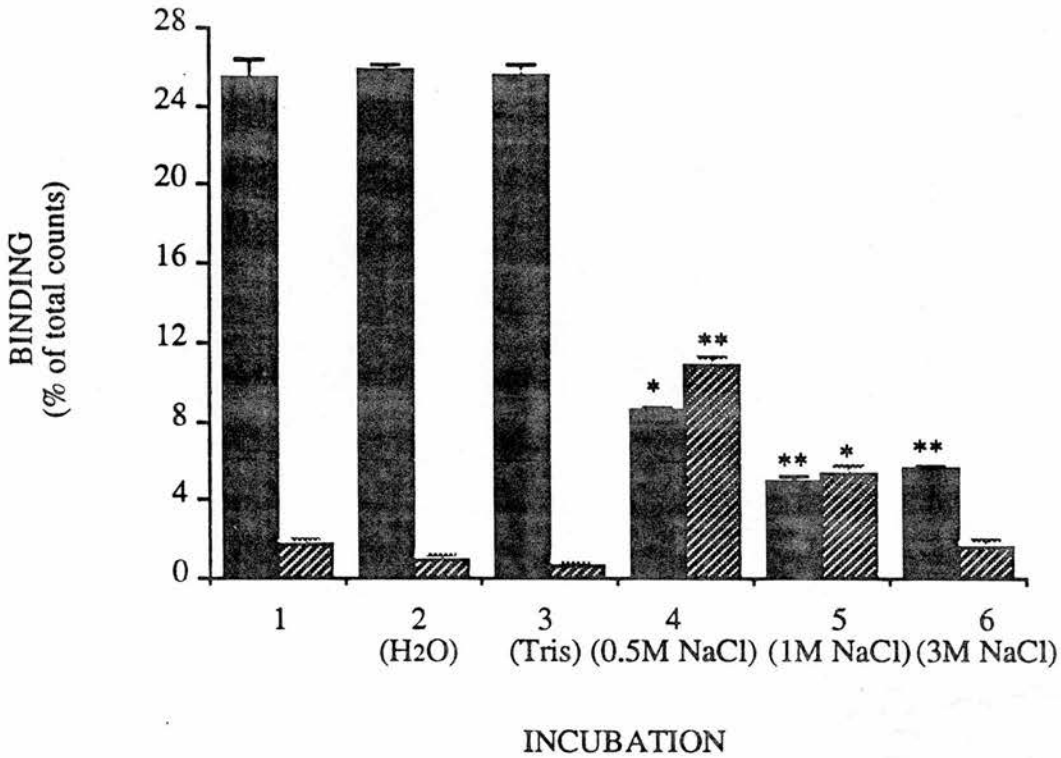


Figure 4.15 Isolation of the endogenous cytosolic GnRH binding protein by increasing salt concentration

The effect of increasing concentrations of salt on the binding of cytosol to DEAE gel was determined.

Cytosol was incubated alone (■) and with DEAE-Sepharose CL-6B (▨) for 4h at 20°C with rotation (Incubation 1). The gel was then packed and the supernatant assayed for binding of ¹²⁵I-chicken GnRH-II by DCC separation.

The gel was then washed sequentially with distilled water (2), 40mM Tris-HCl, pH7.4 (3), 0.5M NaCl (4), 1M NaCl (5) and 3M NaCl (6) under the same incubation conditions and the supernatant from each stage assayed for GnRH binding activity. Cytosol alone acted as the control for incubations 2 and 3, but incubations 4, 5 and 6 were incubated at the same salt concentration as the gel.

Binding was corrected for non-specific binding to the tube and expressed as % of total counts added.

Data shown were mean ± s.d. for triplicate determinations for an individual experiment. *P<0.05; **P<0.01 when compared to binding activity after Incubation 1.

binding activity of placental cytosol was decreased significantly following prolonged incubation in the presence on NaCl.

4.4.3 Isolation of Cytosol GnRH Binding Activity: pH

The reduction in activity observed using NaCl to displace the binding activity from the positively charged groups of DEAE-Sepharose suggested that salt effects were complicating the purification of the GnRH ligand. As the net charge exhibited by biological materials is dependent on the pH of the solution, the selective desorption of the bound molecule can be achieved by changes in pH, as well as changes in ion concentration.

Cytosol was incubated to equilibrium with DEAE-Sepharose and the gel was washed with dH₂O and 40mM Tris-HCl. Gel was then incubated with citrate buffer of pH ranging from 3-8.5 (Figure 4.16). Binding activity was low in the supernatant from the acidified washes, but was increased in the pH 6.5 and alkaline (pH 8.0 - 8.5) washes.

4.4.4 Use of Gel Exclusion Chromatography to Isolate Cytosolic GnRH Binding Activity

Gel exclusion chromatography separates molecules on the basis of their size and shape: smaller molecules enter the 3-dimensional network of pores in the gel and elute slowly, whereas large molecules pass through the interstitial spaces of the gel and elute quickly from the gel. Sephadex is prepared by cross-linking dextrans to varying degrees to give differences in porosity, giving Sephadex G25 a fractionation range of 1×10^3 - 5×10^3 daltons. Sephadex G25 columns were equilibrated to pH 3.5, 6.5 or 10 with citrate buffer, cytosol was applied to the column and eluted with the equilibration buffer, to determine the effect of pH on binding activity.

Fractions were assayed for chicken GnRH-II binding in the DCC assay at pH 3.5, 6.5 and 10 (Figure 4.17). Binding activity was eluted in the same position for all

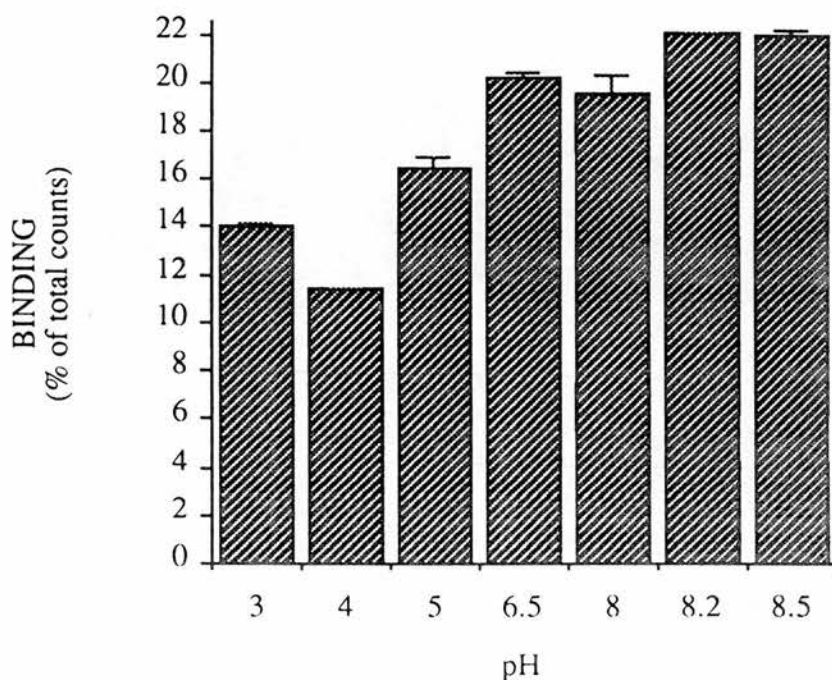


Figure 4.16

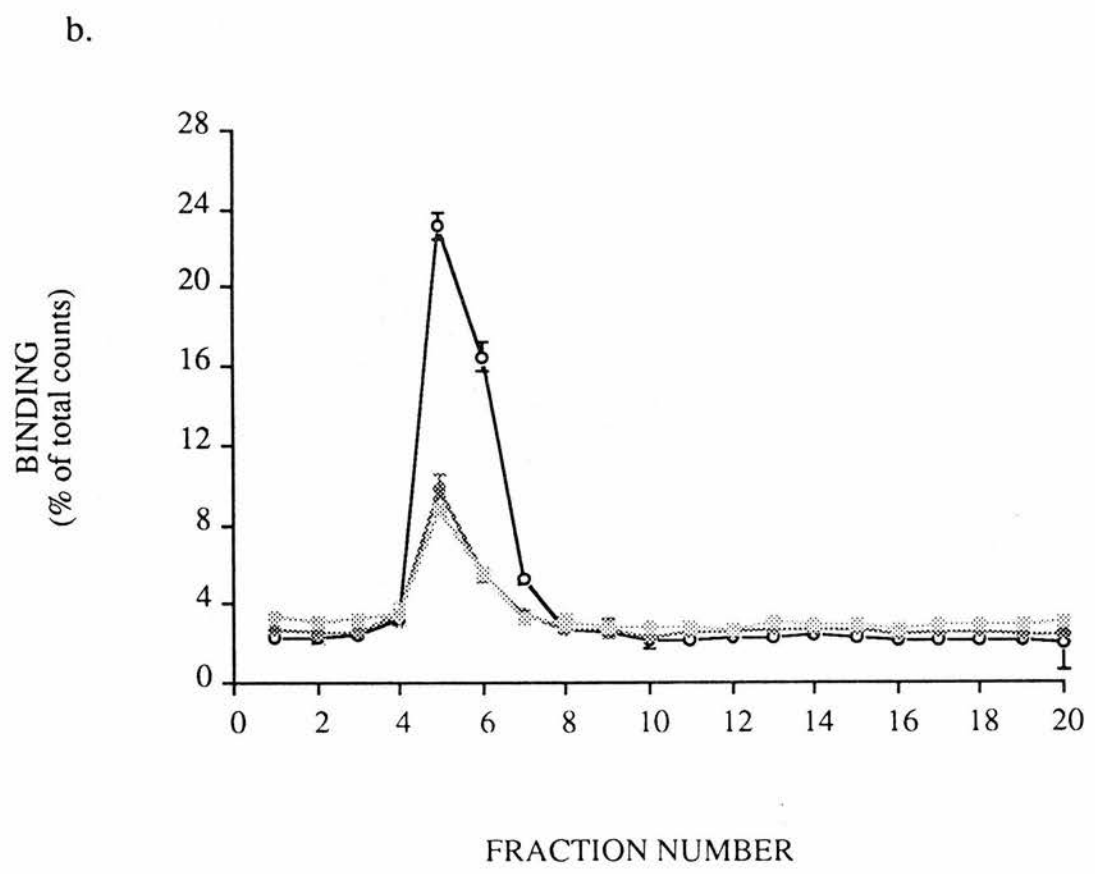
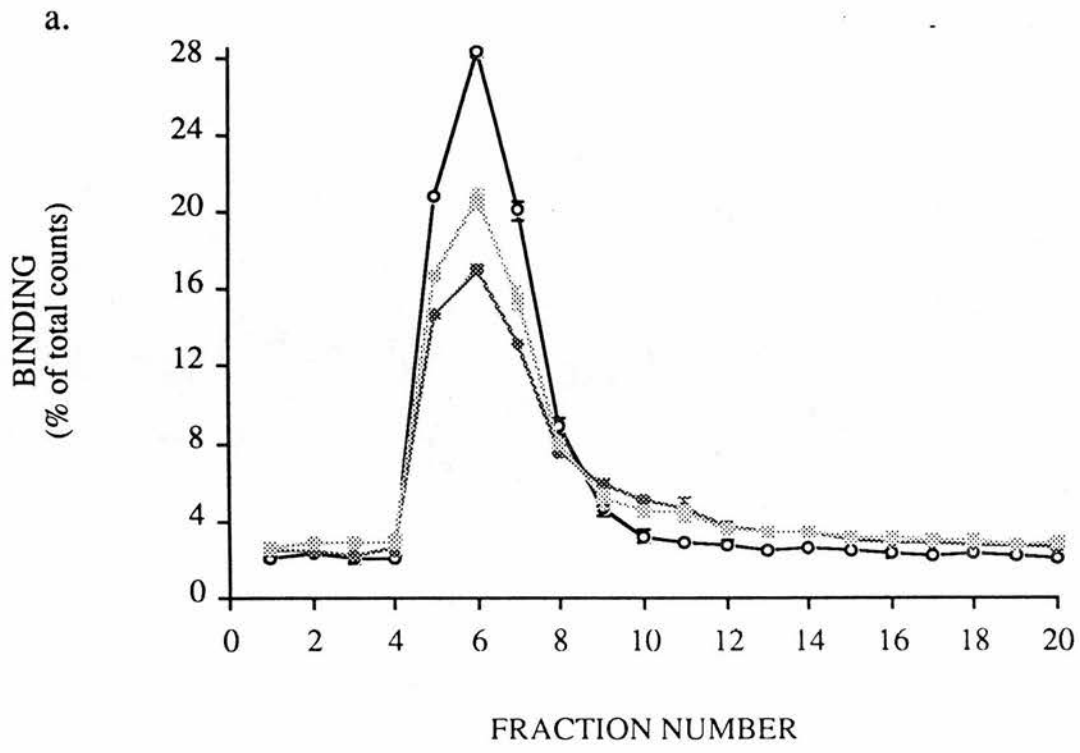
Isolation of the endogenous cytosolic GnRH binding protein by increasing pH

In order to determine the effect of pH on the binding of cytosol to DEAE gel, cytosol was incubated with DEAE-Sepharose CL-6B for 4h at 20°C with rotation. The gel was then packed and the supernatant assayed for binding of iodinated chicken GnRH -II by DCC separation.

The gel was then washed sequentially with distilled water, 40mM Tris-HCl (pH 7.4), and citrate buffer under the same incubation conditions and the supernatant from each stage assayed for GnRH binding activity. Citrate buffer ranged from pH 3 - 8.5.

Binding was corrected for non-specific binding to the tube and expressed as % of total counts added.

Data shown were mean \pm s.d. for triplicate determinations.



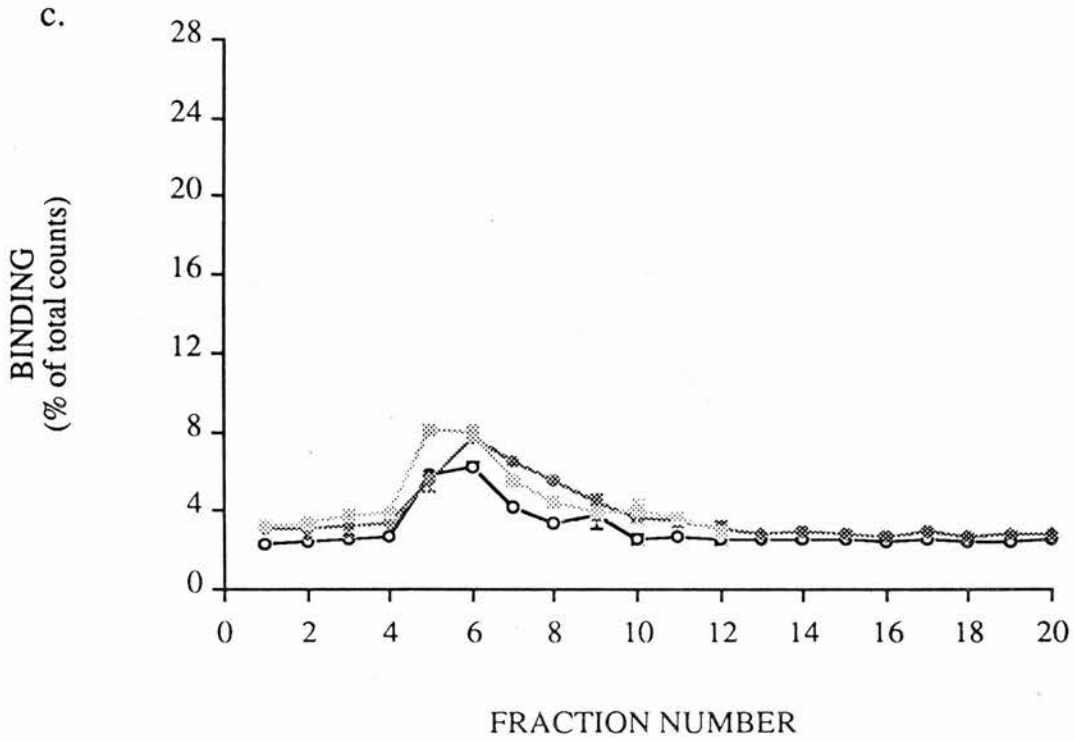


Figure 4.17 Effect of pH on gel permeation chromatography of term placental cytosol

Placental cytosol was eluted from a 20 x 1cm Sephadex G25 (Fine) column with 0.2M citrate buffer of pH 3.5 (a), 6.5 (b) or 10 (c). 2ml fractions were collected and assayed in duplicate for ^{125}I -chicken GnRH-II binding at pH 3.5 (○), 6.5 (✱) and 10 (✻), by incubation overnight at 4°C. Controls were included to determine the effect of pH on binding activity of unfractionated cytosol. Bound and free tracer were separated by DCC and unadsorbed (bound) activity determined and expressed as % of total counts added. Data shown were from a representative experiment; each point is mean \pm s.d. for triplicate determinations.

pHs. High levels of binding activity were found in the active fractions of cytosol eluted at pH 3.5 at all assay pHs, with maximal binding at assay pH 3.5. For cytosol eluted from Sephadex G25 at pH 6.5, binding activity was maximal in the pH 3.5 assay with significantly lower levels of activity in the assays at pH 6.5 and 10. Binding activity was reduced in the cytosol fractions eluted at pH 10 at all assay pHs.

Chicken GnRH-II binding activity of unfractionated cytosol was also increased when assayed at pH 3.5, and acidified cytosol and active fractions also acquired the ability to bind ^{125}I -Buserelin. This was absent at neutral pH. Increased GnRH binding of cytosol at acid pH suggested that the endogenous placental GnRH-like molecule may dissociate from the cytosolic binding site at acid pH, increasing the number of cytosolic binding sites available for the iodinated tracers.

4.4.5 Cytosolic Binding Activity of Pre-term and Term Placental Cytosol

Typically, hormones and growth factors can be dissociated from their binding proteins/receptors at low pH. Cytosol from mid-gestation and term placentae was assayed at pH 3.5 and 6.5 for chicken GnRH-II and Buserelin binding in the DCC assay (Figure 4.18). Binding of chicken GnRH-II to placental cytosol was significantly enhanced when incubated at pH 3.5, compared with binding at pH 6.5 ($P < 0.001$). Levels of binding to term placental cytosol were greater than binding to pre-term cytosol ($P < 0.05$).

Levels of Buserelin binding to placental cytosol at pH 6.5 were comparable with assay blanks, which reflect non-adsorbed tracer present in the DCC-stripped supernatant. However, when incubated at pH 3.5, Buserelin binding was high (note difference in scale) with term placental cytosol binding greater than pre-term cytosol ($P < 0.05$).

As binding is increased at low pH, this may represent dissociation of endogenous GnRH-like ligand from the cytosolic binding protein, allowing increased

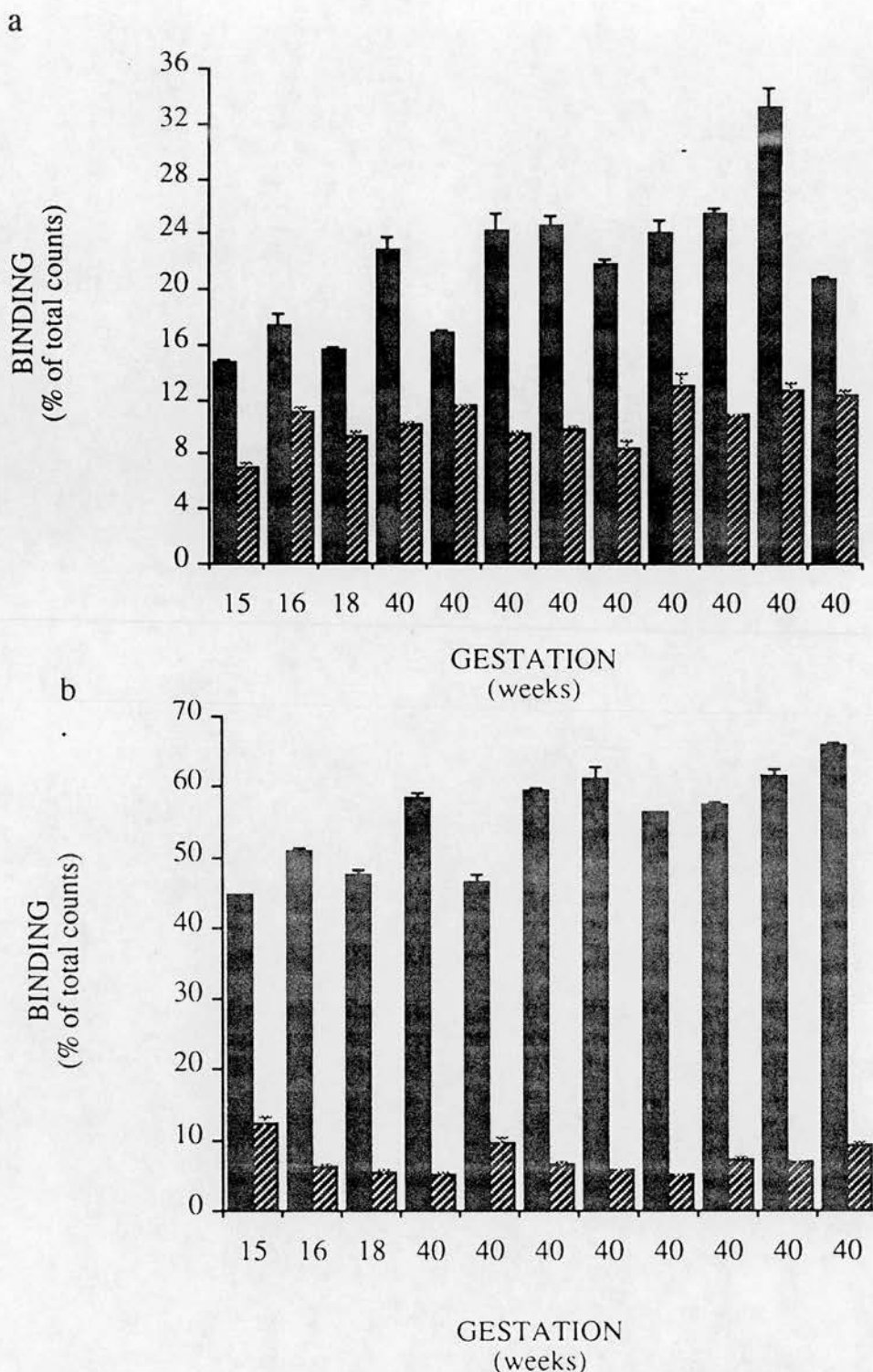


Figure 4.18

Binding activity of mid-gestation and term placental cytosol at pH 3.5 and pH 6.5

Placental cytosol was incubated at 4°C for 16h with ^{125}I -chicken GnRH-II (a) or ^{125}I -Buserelin (b) in 0.2M citrate buffer, pH 3.5 (■) or pH 6.5 (▨).

Bound and free tracer were separated by DCC and bound tracer determined and expressed as % of total counts.

Data shown were mean \pm s.d. for determinations in triplicate

binding as more binding sites are available. At neutral pH, binding is low, as most binding sites are occupied by the endogenous ligand, which may have a greater affinity than either the GnRH isoform or agonist. However, binding to acidified term placental cytosol was greater than binding to acidified pre-term cytosol, therefore there was no evidence to suggest that increased occupancy was the reason for depressed binding at mid-gestation.

4.5 DISCUSSION

Attempts to extract immunoactive placental GnRH from placental extracts were reviewed by Siler-Khodr (1983). The following differences were identified between placental and hypothalamic GnRH: hypothalamic GnRH was soluble in acetone, methanol and ethanol, whereas placental GnRH was not; placental GnRH was heat-labile but hypothalamic GnRH was heat stable; extracts of placental GnRH lose all immunoactivity in the presence of acid, base, urea and guanidine, while hypothalamic GnRH is stable under these conditions. Neutral extraction of placental extract indicated that immunoactive placental GnRH had a molecular weight of ~60kDa (Siler-Khodr, 1987).

Placental cytosol was observed to reduce binding of iodinated GnRH analogues to placental membrane binding sites in a concentration-, time- and temperature-dependent manner (Figures 4.1 and 4.2). However this activity was attenuated in the presence of protease inhibitors, suggesting that membrane binding activity was reduced through degradation of the tracer by placental cytosol enzymatic activity. Indeed membrane binding of chicken GnRH-II tracer was more susceptible to inhibition by placental cytosol than Buserelin and Tryptorelin, GnRH analogues which were modified to protect degradation of the molecule. On the contrary, the inhibition of GnRH binding to placental membranes by cytosolic fractions in the presence of protease inhibitors was found to be due to a non-specific effect of ethanol on binding, as previously discussed (Chapter 3).

Binding of chicken GnRH-II to a specific anti-chicken GnRH-II antibody was inhibited by placental cytosol in a dose-dependent manner. Protease inhibitors and ethanol had no effect on this activity, suggesting the possibility that a cytosolic molecule may compete with chicken GnRH-II for the antibody binding site. When placental extract was fractionated under neutral conditions on Sephadex G25, the inhibitory activity of cytosol was eluted as a high molecular weight (~60-80 kDa) fraction. However, inhibitory activity was only observed in the presence of ethanol. Activation by ethanol was dose-related and specific for high molecular weight fractions of placental cytosol.

The GnRH-like ligand was present in placental cytosol at all stages of gestation and appeared to be bound to a cytosolic binding protein. Binding of chicken GnRH-II to this cytosolic binding site was dose-dependent. Chromatography on DEAE-Sephadex suggested that the cytosolic binding activity was negatively charged at neutral pH. However, attempts to isolate this protein were hampered by loss of activity on extended incubation with salt.

Fractionation and assay of this binding activity at acid pH resulted in an approximately three-fold increase in cytosolic binding of chicken GnRH-II. At acid pH, placental cytosol also bound significant levels of Buserelin. Binding of both chicken GnRH-II and Buserelin to term placental extract was significantly higher than binding to placental extract from mid-gestation, although this was determined using a small number of cytosol samples. Thus, the presence of GnRH-like activity in human placental cytosol was suggested by the displacement of GnRH analogues from specific membrane binding sites. This GnRH-like activity appears to be associated with a specific binding protein.

Several authors have identified GnRH-degrading activity in placental cytosol (Siler-Khodr *et al.*, 1989; Bramley and Menzies, submitted). However, inhibition of cytosolic activity appeared to be unaffected by protease inhibitors, although it was sensitive to ethanol in the incubate.

CHAPTER 5

DESCRIPTION OF A NOVEL GROWTH FACTOR ACTIVITY IN THE HUMAN PLACENTA

5.1 INTRODUCTION

A non-steroidal gonadal activity which specifically suppressed FSH secretion by the anterior pituitary was first described in 1923 and since then much direct evidence for the existence of inhibin has been published (de Kretser and Robertson, 1989). A component of suppression of FSH secretion by pituitary gonadotrophs by inhibin may involve GnRH, possibly via reduced responsiveness of the gonadotroph to GnRH (Wang *et al.*, 1990).

Inhibin has been localised in the human placenta (Petraglia *et al.*, 1987b) and a possible role for inhibin in the endocrinology of the placenta proposed (Petraglia *et al.*, 1989). Activin was found to increase the release of GnRH and progesterone from placental monolayer cultures and potentiated GnRH-stimulation of hCG release. Inhibin alone had no effect on these parameters but reversed the effects of activin. Although the production of activin has not been demonstrated in the human placenta, excess β -subunit mRNAs, compared with α -subunit mRNAs, have been demonstrated in the human placenta (Meunier *et al.*, 1988), which suggests that the formation of $\beta\beta$ dimers is likely.

Tsonis *et al.* (1986) developed an extremely sensitive bioassay for inhibin based on inhibition of FSH secretion by cultured ovine pituitary cells. This assay system was 30-40 times more sensitive than rat inhibin bioassays. However, when applied to determine the bioactivity of crude human placental extracts, the results of this bioassay were complicated by the mitogenic effect of the placental extract on the cultured ovine pituitary cells.

As growth factors and protooncogenes have a pivotal role in the control of the development of the human placenta throughout gestation (Ohlsson, 1989), initial characterisation of the factors in placental cytosol with mitogenic activity in the ovine pituitary was carried out.

5.2 METHODS

5.2.1 Ovine Pituitary Cell Culture

Ovine pituitary cells were cultured according to the method of Tsonis *et al.* (1986) with slight modification.

The heads of freshly slaughtered, sexually mature, female sheep were obtained from Edinburgh Meat Plant, Edinburgh, Scotland. The pituitary glands were removed, weighed and washed in Dulbecco's PBS (DPBS) (Flow Laboratories, Irvine, Scotland) containing 7.5mM glucose. A maximum of 3g pituitary tissue was chopped into 3-4 mm blocks under sterile conditions in a laminar flow cabinet and, using aseptic techniques, was washed with DPBS supplemented with 7.5mM glucose and 0.1% (w/v) BSA. The blocks of tissue were then incubated in a siliconised spinner flask with 0.5% (w/v) trypsin in DPBS supplemented with 7.5mM glucose and 0.1% (w/v) BSA for 30 minutes at 37°C. The enzyme solution was discarded and the tissue washed and stirred gently for 30 minutes at 37°C with Dulbecco's Modification of Eagles Medium (DMEM) (Flow Laboratories, Irvine, Scotland) supplemented with 10% (v/v) lamb serum, 2.5% (v/v) foetal bovine serum, 2mM glutamine, Penstrep (250 IU penicillin and 250µg streptomycin) (all from Flow Laboratories, Irvine, Scotland) and 10mM NaHCO₃. The media was discarded and the tissue washed and incubated with Ca²⁺ and Mg²⁺ free DPBS (Flow Laboratories, Irvine, Scotland) supplemented with 2mM EDTA and 0.1% (w/v) BSA in a spinner flask for 10 minutes at 37°C.

The pituitary cells were mechanically dispersed in Ca²⁺ and Mg²⁺ free DPBS using siliconised Pasteur pipettes of decreasing bore (5-2mm) and the harvested cells centrifuged twice at 500xg for 10 minutes at room temperature in DMEM supplemented as above. The pelleted cells were resuspended in 20ml supplemented DMEM and any undispersed cells and clumps of cell debris removed. An aliquot of cell suspension was counted using a Neubauer haemocytometer and viability was assessed by trypan blue exclusion. Average viability for 16 ovine pituitary cell preparations was 86.8% (±4.0). Cells were diluted as required in DMEM,

supplemented as above, and added to individual chambers of multiwell plates. Ovine pituitary cell cultures were incubated at 37°C in a 5% CO₂, water-saturated atmosphere. In the standard bioassay protocol (Tsonis *et al.*, 1986), the cells were incubated for 48 hours prior to the removal of the medium to allow the cells to adhere to the culture plate and for basal hormone secretion to stabilise. Medium was replaced with sterilised sample in supplemented DMEM and incubated for 48 hours under the same culture conditions.

5.2.2 Comparison of Cell Counting Methods

5.2.2a Haemocytometer

200,000 ovine pituitary cells per well were pre-incubated for 48 hours, in 600µl of supplemented DMEM, as described above, then incubated with sample and supplemented DMEM in a final volume of 600µl for 48 hours under the same incubation conditions.

Media was removed and the cells were washed with Ca²⁺ and Mg²⁺ free DPBS. The cells were recovered from the culture plates by gentle trypsinisation (0.2% (w/v) trypsin in Ca²⁺ and Mg²⁺ free DPBS containing 0.08% (w/v) EDTA) for 30 minutes at 37°C. The activity of trypsin was neutralised by addition of DMEM and the cell suspension was centrifuged at 400xg for 10 minutes at room temperature. The ovine pituitary cells were then resuspended in 400µl DMEM and aliquots counted using a Neubauer haemocytometer and the number of cells per well was calculated.

5.2.2b Coulter counter

Cells were cultured and resuspended as described in Section 5.2.2a and aliquots counted in Isoton II (Coulter Electronics Ltd., Luton, Bedfordshire, England), using a Coulter Counter Model D Industrial automatic cell counter with a 200µm nominal diameter orifice, allowing the number of cells per well to be determined.

Attenuation, threshold and aperture current were set according to a calibrated size analysis.

5.2.2c Tritiated thymidine incorporation

Incorporation of tritiated thymidine into DNA by ovine pituitary cells was measured to assess levels of replication according to the method of Das (1981).

Cells were prepared as described in Section 5.2.1 and incubated with 2mCi/mmol [³H]-thymidine (Amersham International plc, Amersham, Bucks., England) for 24 hours at 37°C, during the period of active proliferation. Medium was aspirated and the cells were washed with Ca²⁺ and Mg²⁺ free DPBS. The cells were then incubated at 4°C for 20 minutes with 5% trichloroacetic acid (TCA) and the acid-insoluble radioactivity sticking to the culture wells was washed with 5% TCA and methanol. Insoluble material was dissolved with 0.5M NaCl, neutralised with HCl and the solution assayed for radioactivity in 4ml scintillant (Ecoscint®, National Diagnostics, Atlanta, Georgia, USA) on a RackBeta Liquid Scintillation Counter.

5.2.2d Bromodeoxyuridine incorporation

5-bromo-2'-deoxyuridine (BrdU) is an analogue of thymidine and, like thymidine, is incorporated into the DNA of proliferating cells. Uptake of BrdU can be measured using a monoclonal antibody to BrdU, which incorporates a nuclease to allow the antibody access to the incorporated BrdU. This assay was a modification of the procedure kindly supplied by Dr P. Perros, Endocrine Unit, Department of Medicine, University of Newcastle upon Tyne.

Cells were plated at a concentration of 10,000 cells per well in 96-well culture plates and incubated for 48 hours at 37°C in serum supplemented DMEM, as described in Section 5.2.1. The supplemented medium was then replaced with DMEM supplemented only with 2mM glutamine, 1% (v/v) Penstrep and 10mM NaHCO₃. After 24 hours of serum restriction, cells were incubated with sample diluted in serum free DMEM for 48 hours. BrdU (Amersham International plc, Amersham, Bucks.,

England) was then added to the incubation and, after 6 hours, medium was aspirated, the plates were washed with Ca^{2+} and Mg^{2+} free DPBS and the cells were fixed with absolute methanol. At room temperature, cells were washed with DPBS containing 0.5% (w/v) casein, then incubated for 18 hours with monoclonal anti-BrdU (Amersham International plc, Amersham, Bucks., England) diluted in DPBS-casein. Casein was included in the diluting and washing buffer to minimise non-specific binding to the culture plates. The unbound antibody was removed, the cells washed four times with DPBS-casein and incubated with anti-mouse IgG conjugated with horseradish peroxidase (Scottish Antibody Production Unit, Law Hospital, Carlisle, Scotland) diluted in DPBS-casein for 2 hours. The conjugate was aspirated, the plates washed twice with DPBS-casein, then twice with DPBS alone, as casein forms a precipitate with the substrate, and the cells were incubated with 2.2mM o-phenylenediamine in 30% H_2O_2 and 25mM citric acid-50mM Na_2HPO_4 , pH 5, for 30 minutes. O-phenylenediamine is a colourless substrate which is converted to a coloured product by peroxidase. The reaction was stopped by the addition of 20% sulphuric acid and the optical density at 492nm was determined using a Titertek Multiskan MCC.

5.2.3 Sterilisation of Samples

Under sterile conditions, all samples were passed through 0.2 μm PTFE Minisart filters. Filtrate was collected in autoclaved glass tubes and stored at -20°C until assay.

5.3 COMPARISON OF CELL COUNTING METHODS

Ovine pituitary cells were cultured in 24- or 96-well plates, according to the protocol for each counting method, in the presence of increasing concentrations of term placental cytosol. Cell number or incorporation was determined and expressed as % of control cultures in the absence of cytosol (Figure 5.1).

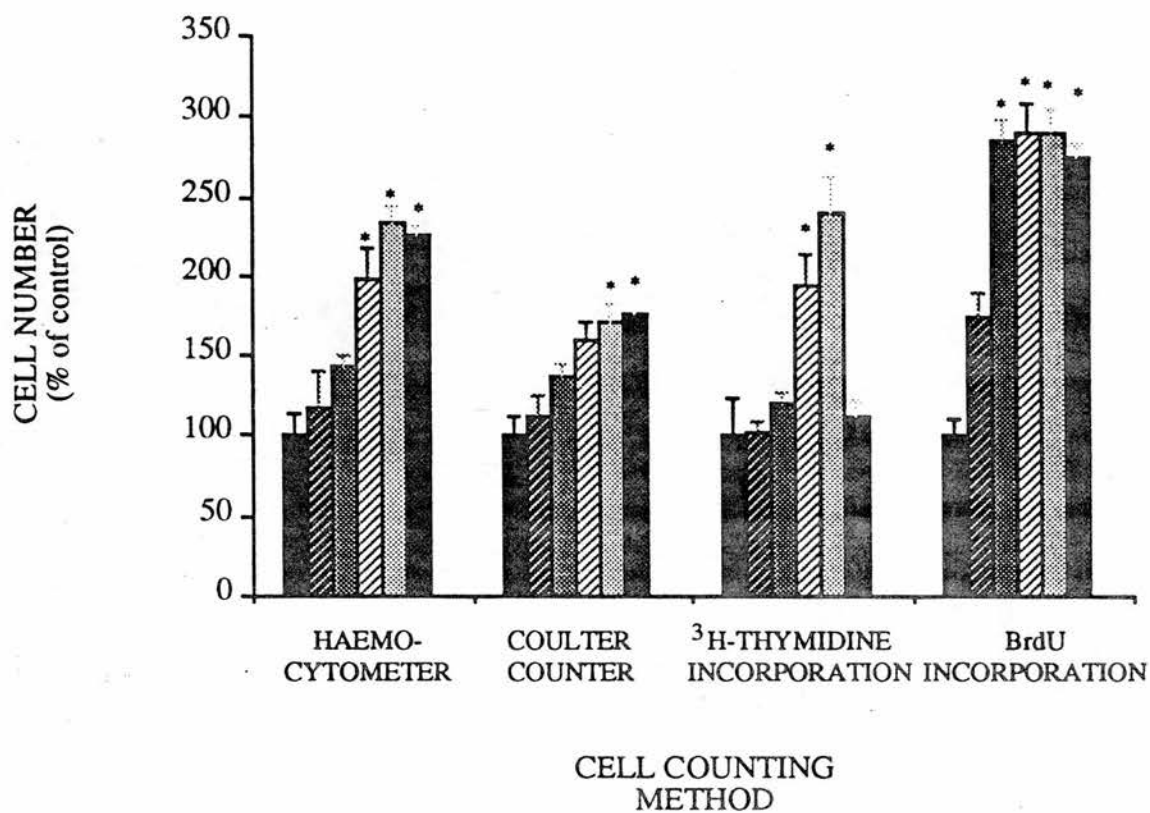


Figure 5.1 Comparison of cell counting methods

Ovine pituitary cells were cultured as described in 5.2, with 2% (■), 4% (▨), 10% (▧), 15% (▩) and 20% (■) solutions of sterilised term placental cytosol for 48 hours.

Cell number was determined by haemocytometer, Coulter Counter, tritiated thymidine incorporation and bromodeoxyuridine incorporation. Cell number was expressed as % of control, i.e. cells cultured in the absence of placental cytosol (■).

*P<0.05 when compared to cells cultured in the absence of placental cytosol.

Cell counting by haemocytometer and Coulter counter gave similar levels of stimulation, with cell number increasing progressively with cytosol concentration, although the haemocytometer may have been less accurate at higher cell counts due to the large correction factor involved. Incorporation of tritiated thymidine was more variable, with a narrow concentration window for detection of effect, whilst incorporation of BrdU was the most sensitive assay for the mitogenic effect of placental cytosol, reaching a plateau at cytosol concentrations in excess of 4% (v/v).

5.4 EFFECT OF CULTURE TIME ON CELL PROLIFERATION

The effect of incubation time on the response of ovine pituitary cells to placental cytosol was determined by pre-incubating cells for 24 or 48 hours, then adding extract from pre-term or term placental tissue and incubating for 24 or 48 hours (Figure 5.2).

Cells pre-incubated for 24 hours and incubated with placental cytosol for 24 hours showed no increase in cell number with any cytosol preparation. A similar lack of response was observed with cells pre-incubated for 48 hours and incubated with cytosol for 24 hours. Thus, incubation of 24 hours with placental cytosol appears to be insufficient for cell replication to occur.

After 48 hours incubation, an increase in cell number, compared with control, was observed with all cytosol preparations for both pre-incubation times, although 48 hour pre-incubation gave significantly increased cell number compared with 24 hour pre-incubation.

No effect of stage of pregnancy on mitogenic activity was detected in the ovine pituitary cell assay due to variation between the activity of samples of similar gestation.

5.5 FRACTIONATION OF PLACENTAL CYTOSOL

Term human placental cytosol was fractionated on a 50 x 1.6cm column of Sephadex G100. Columns were calibrated using the molecular weight standards blue

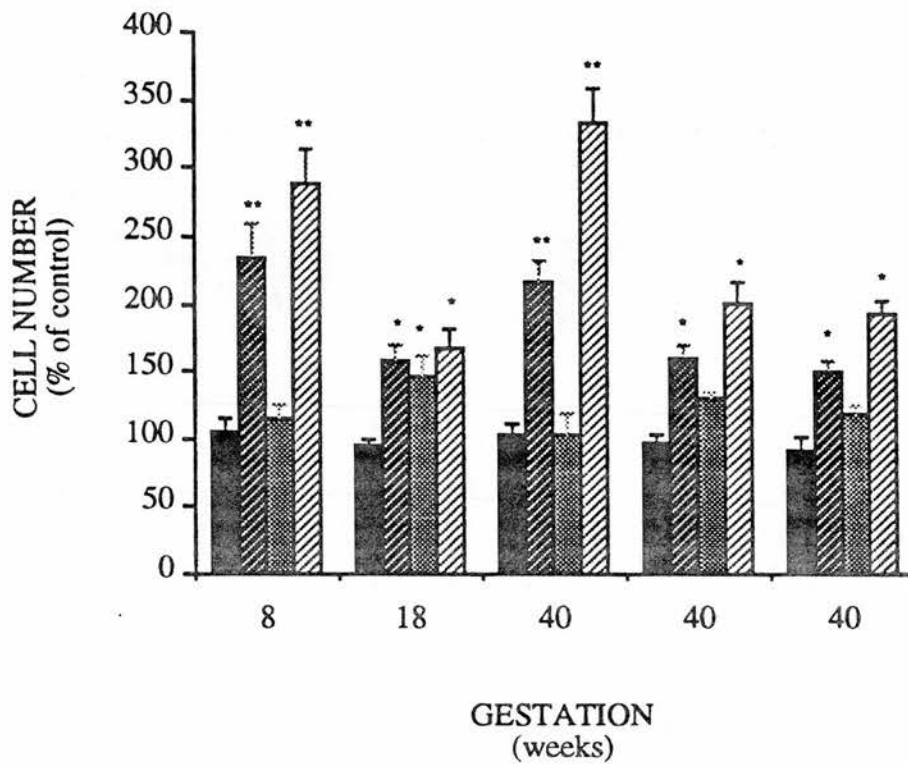


Figure 5.2 Mitogenic activity of pre-term and term placental cytosol with incubation time

Ovine pituitary cells were cultured as described in 5.2, and pre-incubated for 24 hours (■, ▣) or 48 hours (▤, ▥), then incubated with 10% sterilised term placental cytosol for 24 hours (▤, ▥) or 48 hours (▥, ▦).

Data shown were obtained from a single experiment by determining cell number using a haemocytometer but were representative of other methods. Cell number was expressed as % of control, i.e. cells cultured in the absence of placental cytosol.

*P<0.05; **P<0.01 when compared to cells incubated in the absence of cytosol.

dextran, which eluted in the void volume of the column, bovine serum albumin (MW 67,000), cytochrome c (MW 12,500) and ^{125}I . 2ml fractions were collected and assayed for protein concentration and mitogenic activity in the ovine pituitary cell assay (Figure 5.3).

The mitogenic activity in human placental cytosol corresponded to a high molecular weight fraction ($M_r \text{ } \text{Å} \text{ } 60,000$). Cell number was increased by a factor of 2.8.

5.6 EFFECT OF ACIDIFICATION OF MITOGENIC FRACTIONS

In order to investigate if the high molecular weight growth factor was bound to a binding protein, term placental cytosol was fractionated on a column of Sephadex G100 and fractions containing the high molecular weight mitogenic activity were pooled, lyophilised and reconstituted in 0.1M acetic acid. Acidified extract was then fractionated on an acidified Sephadex G100 column, using 0.1M acetic acid as the eluting buffer. The acidified fractions were dried down under nitrogen and reconstituted in PBS, but no mitogenic activity was observed in the reconstituted fractions in the ovine pituitary cell assay.

5.7 AMMONIUM SULPHATE PRECIPITATION OF TERM PLACENTAL EXTRACT

On ice, granular NH_4SO_4 was added to term placental extract, slowly with constant stirring, to 25% saturation. After 1 hour, precipitated protein was pelleted by centrifugation at 10,000xg and resuspended in PBS. NH_4SO_4 was then added to the supernatant to 50% saturation and the pellet recovered as before. This was repeated to obtain a precipitate for 75% NH_4SO_4 saturation. Resuspended precipitate from each stage was dialysed against PBS, before sterilisation and assay.

Mitogenic activity was absent and low levels of protein were precipitated by up to 25% NH_4SO_4 (Figure 5.4). Marked proliferation of ovine pituitary cells was

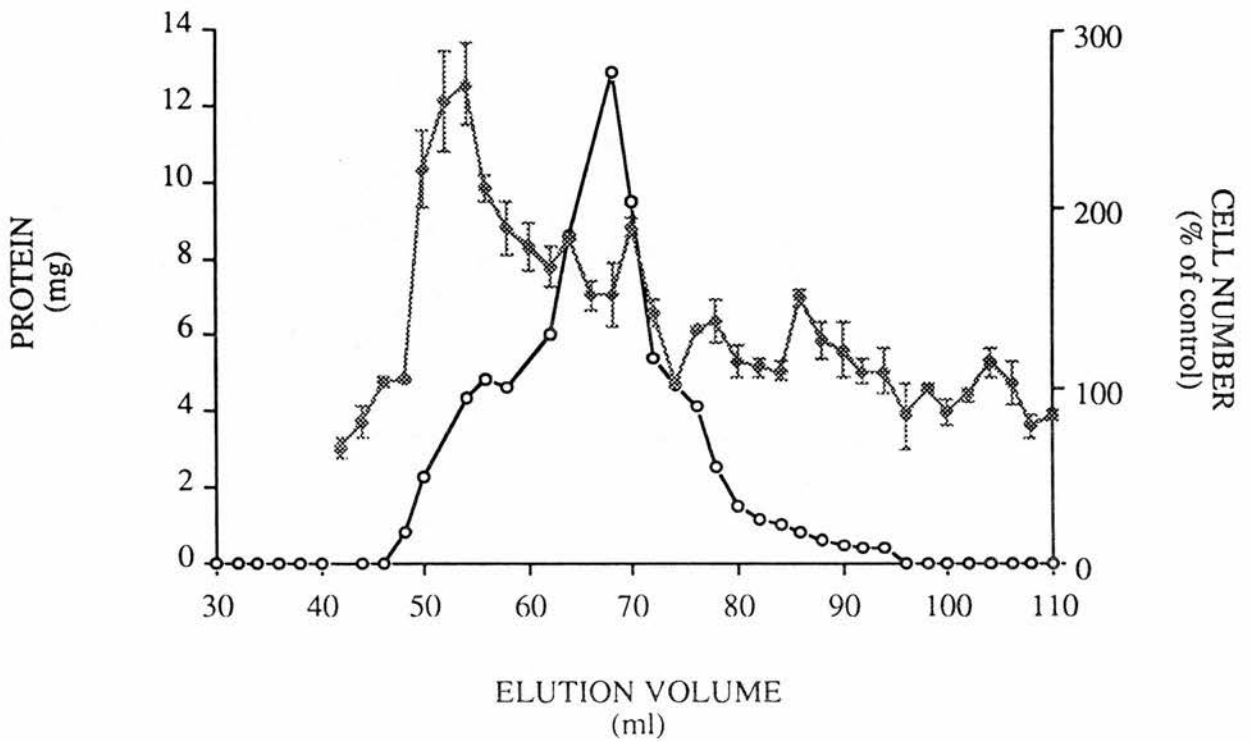


Figure 5.3 Mitogenic activity of fractionated placental cytosol

Sterilised fractions of term placental cytosol, prepared by gel exclusion chromatography on Sephadex G-100, were incubated with cultured ovine pituitary cells for 48 hours. Cell number was determined (\diamond) and expressed as % of controls incubated without cytosol.

Data shown were obtained from a single experiment by Coulter counter, but were representative of data obtained by other counting methods. Values are mean \pm s.d. for 8 determinations per fraction. Protein concentration (\circ) was determined by the method of Lowry *et al.* (1951). Values are mean \pm s.d. for triplicate determinations - error bars are not distinguishable on the graph.

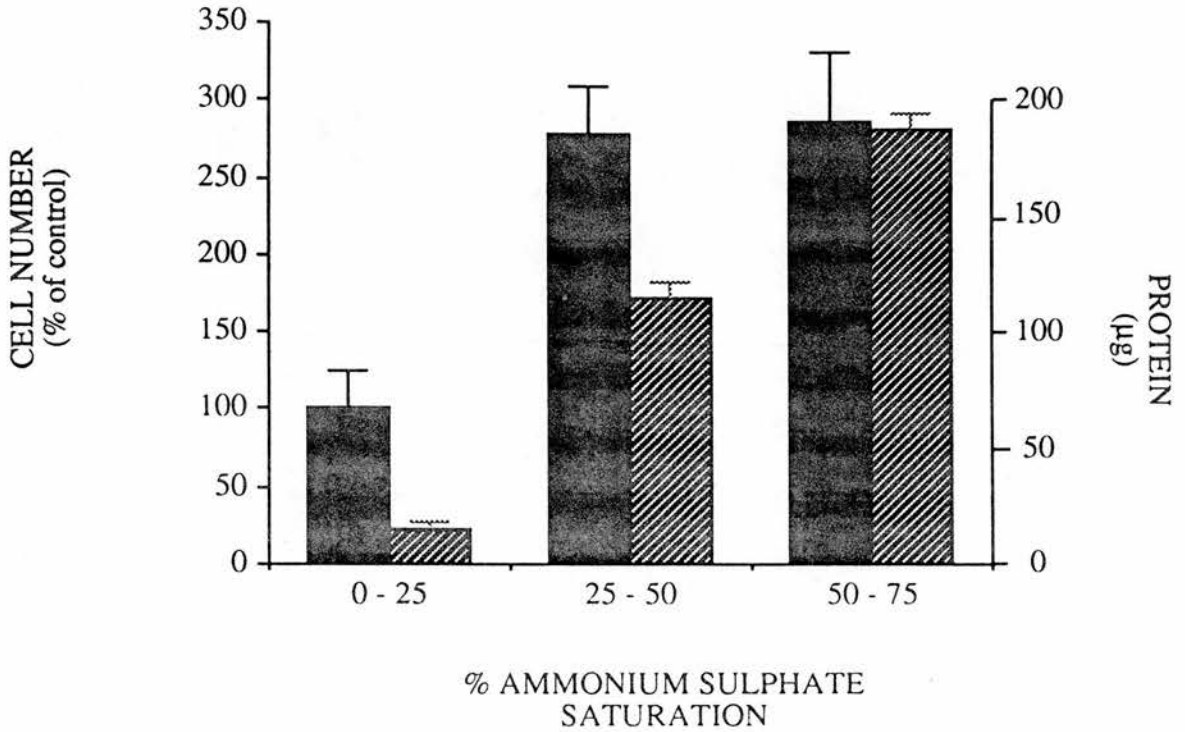


Figure 5.4 Ammonium sulphate purification of term placental cytosol

Term placental cytosol was incubated with increasing concentrations of ammonium sulphate and the precipitated protein assayed for mitogenic activity in the ovine pituitary cell bioassay (■).

Data shown were determined by BrdU incorporation, which was representative of other methods of determination, and expressed as % of control, in the absence of cytosolic protein. Protein (▨) was determined by the method of Lowry *et al.* (1951).

apparent with the protein precipitates obtained with 25-50% NH_4SO_4 saturation and 50-75% NH_4SO_4 saturation, with BrdU incorporation increasing almost 3-fold on addition of these reconstituted proteins. This suggests that the mitogenic activity is 'salted-out' around 50% NH_4SO_4 saturation, with the range of effective NH_4SO_4 concentrations overlapping the two steps, or that more than one protein, with a spectrum of salting-out points, is responsible for the mitogenicity of the placental cytosol. This could be determined by choosing different ranges of NH_4SO_4 saturation, e.g. 0-20%, 20-40%, etc.

Protein concentration was maximal in the precipitate obtained from the addition of 50-75% NH_4SO_4 . Thus, mitogenic activity was greater, per mg of protein, in the precipitate formed by 25-50% NH_4SO_4 saturation of placental cytosol.

5.8 DISCUSSION

In the human placenta, various growth factors, receptors and oncogene products appear in a developmentally regulated fashion during pregnancy. Ohlsson (1989) described placental development as a 'pseudomalignant process' and reviewed a variety of possible growth stimulatory pathways in the human placenta. In early pregnancy, the trophoblast is highly invasive, proliferates rapidly and there is a lack of cell contact inhibition. This is curbed in a temporal fashion and the 'pseudomalignant' characteristics of the placenta are lost by mid-gestation.

The purification of a novel peptide growth factor from human placenta was described by Sen-Majumdar *et al.* (1986a;b). This growth factor was distinct from the major classes of peptide growth factors with respect to its molecular weight, binding specificity and antigenicity. The 34kDa growth factor was expressed at a high level during the first trimester, but levels were significantly lower by term. It was associated with cell types of an invasive and proliferative nature and was proposed to influence cell growth in an autocrine manner.

The mitogenic activity observed when human placental cytosol was cultured with ovine pituitary cells was not evident until after 24 hours incubation and was eluted from Sephadex G100 as a high molecular weight fraction (~60kDa). This is much larger than other placental peptide growth factors, such as EGF (~6-7kDa) or PDGF (~25-30kDa). However, acidification and fractionation of the high molecular weight activity, to determine if the putative growth factor was bound to a binding protein, resulted in a loss of bioactivity. Ammonium sulphate precipitation suggested that either the mitogenic activity precipitated around 50% saturation or that more than one cytosolic protein, with a wider range of precipitation concentrations, was involved in this activity. No difference of activity with gestation was determinable from the preliminary results obtained, due to individual variation of cytosol preparations.

Comparison of cell counting methods suggested that the routinely used methods (direct count of cell number by haemocytometer or Coulter counter, or indirect methods such as determination of BrdU incorporation by ELISA) gave similar profiles in comparative assays. BrdU incorporation proved to be a very effective method of screening a large number of samples: less cells were required per well and more samples per plate with more replicates were possible as 96-well plates were used as opposed to 24-well plates.

Initial purification of the cytosolic mitogenic activity requires further investigation of the behaviour of salt precipitates of placental cytosol, to identify if a narrow range of salting out points were responsible for the increase in ovine pituitary cell number when cultured with placental cytosol, or if a variety of precipitated proteins were involved. Mitogenically active precipitates could be further purified by SDS-free (non-denaturing) gel electrophoresis to isolate active bands to give purified mitogenically active protein for further identification.

CHAPTER 6

DISCUSSION

6.1 SUMMARY, DISCUSSION AND CRITICAL ANALYSIS OF RESULTS

Much evidence is available in support of a paracrine role for GnRH in the human placenta (Bramley, 1989). The GnRH gene has been identified in the human placenta (Seeburg and Adelman, 1984) and, using in situ hybridisation, GnRH mRNA has been found in placenta from first trimester to term (Kelly *et al.*, 1991; Duello *et al.*, 1993). GnRH has a role in modulation of hCG release in the placenta (Siler-Khodr *et al.*, 1986a) and, in agreement with paracrine activity, levels of GnRH mRNA were 0.1-1% of that of β -hCG (Kelly *et al.*, 1991).

Differentiation between paracrine and autocrine actions of GnRH would require demonstration of GnRH receptors on the cytotrophoblasts and a response of the cytotrophoblast to GnRH. No evidence is presently available to confirm such a role for GnRH in the cytotrophoblast.

Many similarities have been observed between GnRH activity in the pituitary and in the placenta. For example, in the hypothalamic-pituitary axis, opioid regulation of LH release was modulated by GnRH and a similar system was demonstrated for opioid regulation of hCG secretion in the placenta (Cemerikic *et al.*, 1994). In pituitary gonadotrophs, GnRH receptor activation produced a biphasic rise in cytosolic calcium concentration (Anderson *et al.*, 1992), characterised as an initial transient increase - involving both IP₃-mediated release of calcium ions from intracellular stores and an influx of extracellular calcium via second messenger-operated calcium channels - followed by a smaller secondary plateau involving extracellular influx via protein kinase C-activated calcium channels. In the human placenta, Neki *et al.* (1993) found that GnRH-mediated hCG release was inhibited by protein kinase inhibitors, which suggested that the GnRH receptor-second messenger system was similar for both sites.

In accordance with previous studies (Currie *et al.*, 1981; Belisle *et al.*, 1984; Iwashita *et al.*, 1986), pituitary superagonists, such as Buserelin and Tryptorelin, were found to have similar affinities for the placental GnRH binding site as the hypothalamic mammalian GnRH decapeptide. In addition, the salmon and chicken GnRH-II

isoforms were observed to bind to the human placental receptor with affinities comparable to the synthetic agonists (Bramley, 1989).

It was demonstrated that specific binding of GnRH, its isoforms and synthetic analogues to the placental binding site was dependent on stage of gestation. High levels of specific binding were observed with membrane fractions isolated from early trophoblast (6-10 weeks), but between 11-18 weeks levels of specific binding fell to almost non-detectable levels. Maximal levels of GnRH binding were seen with term placental membranes. Although the specific activity of GnRH binding varied with gestation, the specificity of the binding site was unchanged: at all stages of gestation, the placental receptor bound comparable levels of Buserelin, Tryptorelin, salmon GnRH and chicken GnRH-II. Specific binding of mammalian GnRH, i.e. the hypothalamic decapeptide, was decreased but still significant, whereas binding of the lamprey and chicken GnRH-I isoforms was on the detection limits of the binding assay.

Although membrane preparations slowly degraded binding sites (Figure 3.2; Bramley, 1989) and Clayton *et al.* (1979) suggested that low affinity extra-pituitary binding sites represented binding of degradation-resistant GnRH analogues to tissue proteases, differential tracer binding at different stages of gestation could not be accounted for by changes in tracer degradation: tracer incubated to equilibrium with mid-gestation placental membranes was capable of binding to term placental membranes with little loss of activity (Figure 3.15). Menzies and Bramley (1992) demonstrated by membrane purification that the GnRH binding site was equilibrated with the microsomal fraction, whereas GnRH degrading activity was present in the cytosolic extract. In addition, the binding site co-localised with EGF receptors and alkaline phosphatase activity (both markers for plasma membranes) on sucrose density gradient fractionation. No correlation was observed between GnRH and EGF binding, alkaline phosphatase activity and stage of pregnancy, suggesting that reduced recovery of placental membranes was not responsible for the observed changes in binding with stage of gestation.

Specific binding of GnRH tracers to placental membranes was not altered by inhibitors of serine, carboxyl or metallo-proteases at any stage of gestation; in particular, binding activity to mid-term placental membranes was not enhanced. However, N-ethyl maleimide, a thiol protease inhibitor, significantly reduced binding (by ~25%), suggesting that a component of detectable GnRH binding may involve binding of GnRH to thiol proteases. Ethanol was observed to have a dramatic, dose-dependent effect on GnRH binding to placental membranes, with 1% (v/v) ethanol in the assay reducing binding to 50% of maximal levels. As ethanol was used as a vehicle for some insoluble protease inhibitors, this could have masked a specific effect and repeat experiments using a wider range of protease inhibitors, especially soluble serine inhibitors, may reveal additional forms of non-receptor binding.

Standardisation of the method used to obtain all preterm placental tissue would have removed one variable between the comparators. However, the treatment regimes used to induce termination of pregnancy were governed by medical considerations. The same applied to term placentae: tissue was simply classified as vaginal delivery, forceps, etc. Details of drugs used during labour were not available to us, owing to confidentiality of patients' notes, nor was background information like alcohol consumption. These factors could conceivably affect binding activity of individual tissues, as demonstrated by the inhibition of binding in the presence of low concentrations of ethanol in the assay.

Gestational age-related differences in GnRH binding activity in the human placenta may be due to decreased receptor number at mid-gestation. However, no GnRH receptor antibody or cDNA probe for the receptor was available to allow further investigation of receptor numbers.

Pituitary GnRH receptors have now been cloned and their molecular function was discussed by Davidson *et al.* (1994). The GnRH receptor had the characteristic structure of a G-protein-coupled receptor, i.e. comprising a single polypeptide chain with seven hydrophobic, transmembrane domains connected by hydrophilic extra- and

intra-cellular loops. The sequence of mouse, human, rat and sheep GnRH receptors shared >85% amino acid identity, with the transmembrane regions and extracellular loop being the most highly conserved areas (>90%). As attempts to isolate the placental GnRH receptor using a probe for the pituitary GnRH receptor gene have been unsuccessful (Eidne, K., *pers. commun.*), cloning of the placental receptor may allow elucidation of the differences between the pituitary and placental systems.

Placental GnRH was initially thought to be very similar to the hypothalamic hormone (reviewed by Siler-Khodr, 1983), with respect to its immunological, physiochemical and biological activities. However, more recent attempts to isolate placental GnRH suggested that this tissue may contain more than one GnRH-like activity and that high molecular weight GnRH is elaborated by the human placenta (Siler-Khodr, 1987). In the human placenta and other reproductive tissues, GnRH gene transcripts are derived from a transcriptional start site which is upstream from the hypothalamic start site (Dong *et al.*, 1993), supporting the possibility of placental GnRH existing in a different form from the hypothalamic decapeptide.

The presence of placental cytosol decreased binding of Buserelin, Tryptorelin and chicken GnRH-II to placental membranes in a dose-, time- and temperature-dependent manner. This effect was observed at all stages of gestation, with inhibition of binding to mid-gestation placental membranes by mid-gestation placental cytosol exceeding displacement of tracer by an excess of unlabelled hormone. Inhibition of binding by cytosol did not appear to be due to protease activity as the presence of a range of protease inhibitors did not reverse the observed decrease in binding, suggesting a component of the placental cytosol was competing with GnRH analogues and isoforms for the placental binding site.

Binding of chicken GnRH-II to a specific anti-chicken GnRH-II antibody was also decreased in the presence of placental cytosol. Again, protease inhibitors had no effect on this inhibition, suggesting that the cytosol activity was sufficiently similar to chicken GnRH-II to be recognised by and compete for the antibody binding site. After

fractionation under neutral conditions, inhibitory activity was only detectable in the presence of ethanol, and was associated with a high molecular weight fraction (~60-80kDa). Ethanol had no effect on binding of tracer to the antibody or on the inhibition by unfractionated cytosol or by cytosol fractions pooled from inactive regions of the eluate.

GnRH binding activity was observed in placental cytosol from all stages of gestation. However, binding activity was potentiated by assay and/or fractionation at acid pH, compared with neutral or alkaline conditions. It was hypothesised that at low pH, endogenous placental GnRH-like activity was displaced from its binding protein. However, insufficient pre-term samples were obtained to determine whether this GnRH-like activity could account for decreased binding at mid-gestation.

Although it could be demonstrated that a component of placental cytosol was recognised by the placental membrane GnRH binding sites and the specific anti-chicken GnRH-II antibody, it was not known if the cytosolic GnRH-like substance possessed biological activity and was capable of eliciting a response upon binding to the GnRH binding site *in vivo*.

Human placental cytosol of all stages of gestation possessed a high molecular weight factor which displayed mitogenic activity in culture with dispersed ovine pituitary cells. From gel permeation chromatography, this activity had a molecular weight of ~60kDa, which was much larger than reported molecular weights for other growth factors found in placental extracts, such as EGF (~6-7 kDa) and PDGF (~25-30 kDa). Mitogenic activity was lost on fractionation of acidified fractions. However, ammonium sulphate precipitation suggested that this activity was precipitated around 50% saturation.

Various methods of evaluating cell proliferation were employed. Initial determinations of cell number was by cell counting using a haemocytometer or a Coulter counter. This was time consuming and may have been influenced by correction factors required to calculate cell number per well from cells per aliquot.

Estimation of proliferation by determination of BrdU incorporation was a very effective and sensitive method of screening large numbers of samples and gave similar profiles to direct cell counting methods.

6.2 FURTHER INVESTIGATIONS

Studies of placental GnRH activity have been conducted with analogues of the hypothalamic decapeptide. As placental GnRH appeared to be a distinct form of GnRH, these analogues may be only partial agonists for the placental GnRH receptor. Thus, placental GnRH data may need to be re-interpreted if it can be demonstrated that "placental GnRH" is different from hypothalamic GnRH.

In order to fully elucidate the nature of decreased binding observed at mid-gestation, GnRH receptor expression and degradation throughout pregnancy could be determined. Differential occupancy of GnRH receptors by the hypothesised higher-affinity endogenous ligand could not be confirmed in this study. However, screening larger numbers of cytosol fraction from all gestations would give a clearer result.

Further investigation of the inhibitory effect of ethanol on GnRH binding would be pertinent as this may have implications on alcohol abuse in pregnancy. As GnRH has a modulating role on growth factor activity in pregnancy, this may be linked to low birth weights associated with foetal alcohol syndrome.

GnRH-like activity may be isolated from DEAE-Sepharose if optimal conditions for displacement of the GnRH-like factor from the gel were determined. The activity eluted from a column of the gel with 0.5M NaCl. However, prolonged incubation with high salt concentrations decreased control binding significantly, suggesting incomplete recovery of activity was obtained. More detailed investigation of salt conditions may provide high yield of intact GnRH-like activity from the placenta.

Further characterisation of the mitogenic activity in placental cytosol would extend ammonium sulphate precipitation to include a wider range of concentrations to

identify whether one or more activities are present. Non-denaturing gel electrophoresis of the samples obtained would provide a more accurate determination of molecular weight and better resolution of individual activities.

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PUBLICATIONS

Human Placental Gonadotrophin-Releasing Hormone (GnRH) Binding Sites: I. Characterization, Properties and Ligand Specificity

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SUMMARY

Radioiodinated gonadotrophin-releasing hormone tracers were prepared from mammalian (*m* GnRH), salmon (*s* GnRH), lamprey (*l* GnRH) and the two forms of chicken GnRH (*ch* GnRH I and *ch* GnRH II), and also from the GnRH agonist (GnRH_A) analogues, Buserelin ([D-Ser(tBu)⁶] 1-9 GnRH ethylamide) and Tryptorelin ([D-Trp⁶ GnRH] 1-9 ethylamide) and a GnRH antagonist (GnRH_{ANT}; [Ac 3,4-dehydro-Pro¹, D-p-F-Phe², D-Trp^{3,6}] LHRH). Specific binding of hormone tracers was compared in homogenates and membrane fractions from human placenta and rat pituitary.

GnRH agonist tracers bound readily to pituitary and placental binding sites. Binding of *m* GnRH to rat pituitary membranes was low compared to agonist binding, whereas other GnRH iso-forms were not bound. Binding of ¹²⁵I-labelled *m* GnRH to human placental membranes was also low compared to that of Buserelin, and *l* GnRH and *ch* GnRH I tracers bound poorly. However, human placental membranes bound *s* GnRH and *ch* GnRH II to the same extent as GnRH_A. Studies of the inactivation of GnRH tracers following incubation with rat pituitary and human placental membranes demonstrated that, although GnRH isoforms were degraded at different rates in these tissues, the differential ability of GnRH isoforms to bind to pituitary or placental binding sites was not related to differences in degradation of tracers, but rather to differences in ligand specificity.

Specific binding of ¹²⁵I-labelled GnRH agonists (GnRH_A) and mammalian GnRH (*m* GnRH), *s* GnRH and *ch* GnRH II tracers to human placental membrane fractions increased linearly with increasing membrane protein at low concentrations. Binding was dependent on both the duration and temperature of incubation, and pH profiles of ¹²⁵I-labelled GnRH_A, *s* GnRH and *ch* GnRH II binding to placental membranes were similar. Once bound *s* GnRH formed a tighter complex with

placental receptors than GnRH_A, though ¹²⁵I-labelled s GnRH was inactivated more rapidly than agonist tracer during incubation with placental membranes.

Binding of GnRH tracers was specific for molecules with the GnRH structure. Deletions of amino acid residues at positions 1-3 and/or deamidation at Gly¹⁰ reduced binding potencies for both human placental and rat pituitary binding sites, indicating that both ends of the GnRH molecule were required for optimal binding. Modifications which conferred increased agonist activity led to markedly increased receptor binding potency in the rat pituitary, but only slightly increased potency for placental receptors. In contrast, GnRH antagonist analogues had increased potency towards pituitary receptors, but much reduced potency towards human placental binding sites.

These studies highlight further the differences between human placental and rat pituitary GnRH receptors.

INTRODUCTION

The gene for GnRH was first cloned and sequenced from human placenta (Seeburg and Adelman, 1984; Seeburg et al, 1987), and both GnRH (Osathanondh and Elkind-Hirsh, 1981; Tan and Rousseau, 1982; Zhuang et al, 1991) and GnRH-like peptides (Siler-Khodr, 1987; Mathialagan and Rao, 1986a; Zhuang et al, 1991) have been isolated from human placenta. GnRH stimulates the release and synthesis of chorionic gonadotrophin (hCG) in a dose-dependent fashion (Butzow, 1982; Mathialagan and Rao, 1986b; Siler-Khodr and Khodr, 1981; Belisle et al, 1984, 1986; Siler-Khodr et al, 1986a; Kim et al, 1987; Iwashita et al, 1989; Merz et al, 1991), and the stimulatory effects of GnRH in vitro could be blocked by high levels of a GnRH antagonist (Siler-Khodr et al, 1983; 1986d; 1987) indicating the involvement of GnRH receptors. Moreover, GnRH also stimulated the release of prostaglandins (Siler-Khodr et al, 1986c,d) and steroids (Siler-Khodr et al, 1983; 1986b) from placental tissue explants and placental cells in vitro [though others found no effect (Haning et al 1988) or inhibition of steroid secretion (Wilson and Jawad, 1980; Branchaud, Goodyer and Lipowski, 1983), differences perhaps explained by modulation of GnRH effects by placental steroids (Ahmed and Murphy, 1988; Barnea and Kaplan, 1989; Wilson, Jawad and Dickson, 1980; Siler-Khodr et al, 1986b; Iwashita et al, 1989) or gestational age (Siler-Khodr et al, 1986a; 1987)].

The human placenta binds radiolabelled GnRH analogues with high specificity (Currie, Fraser and Sharpe, 1981; Belisle et al, 1984, 1986; Iwashita et al, 1986; Mackiewicz et al, 1987; Escher et al, 1988), though the affinity of these binding sites for GnRH agonists is low (K_d , 10^{-6} to 10^{-7} M) relative to that of the pituitary receptor (K_d , 10^{-10} M; Hazum, 1985; Clayton and Catt, 1981). Since circulating levels of hypothalamic GnRH (ca 10^{-11} M) are far too low to activate placental receptors, it has been suggested that these binding sites respond in a paracrine fashion to locally produced GnRH-like molecules (Siler-Khodr, 1987). Similar 'low affinity' extrapituitary GnRH binding sites have been demonstrated recently in human cancers of the prostate (Kadar et al, 1988), endometrium (Pahwa et al, 1991), breast (Eidne, Flanagan and Millar, 1985; Fekete, Wittliff and Schally, 1989) and ovary (Pahwa et al, 1989; Emons et al, 1989).

Previous studies have highlighted differences between human extrapituitary GnRH binding sites and pituitary receptors, related to the mid-chain region of the GnRH molecule (Bramley et al, 1986; Bramley, 1989). Although both placental and pituitary tissues express

Table 1. Structures of the GnRH agonist, buserelin and GnRH isoforms

	Amino sequence									
	1	2	3	4	5	6	7	8	9	10
Porcine/ovine GnRH	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly-NH ₂
Chicken I GnRH								-Gln-		
Chicken II GnRH					-His-			-Trp	-Tyr-	
Salmon GnRH								-Trp-	Leu-	
Lamprey GnRH			-Tyr-		-Leu	-Glu-	Trp-	Lys-		
Buserelin						D-Ser(tBu)				-EtAmide
Tryptorelin						D-Trp ⁶				-EtAmide
GnRH antagonist	AcΔ ^{3,4} Pro-D-pF-Phe-D-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH ₂									

the gene for the precursor for mammalian GnRH (Seeburg et al, 1987), there is evidence for the existence for differential processing of prepro-GnRH in the two tissues. Furthermore, hypothalamic forms of GnRH differ in different species (King & Millar, 1987; see Table 1). Moreover, different isoforms of GnRH may co-exist in some species, and their localization may differ in hypothalamic and extrahypothalamic tissues (Branton, Phillips and Yan, 1986).

We wished to examine whether human extrapituitary receptors recognized m GnRH preferentially, or whether other isoforms of GnRH were also capable of binding. We have therefore compared the ligand specificities of human placental and rat pituitary GnRH binding sites.

MATERIALS AND METHODS

Materials

Sephadex G25 (fine) was obtained from Pharmacia Ltd, Milton Keynes, Bucks, UK and from Sigma Chemical Co. Ltd, Poole, Dorset, UK. Sephadex G25 QAE-A was from Pharmacia. All other fine chemicals and reagents were from Sigma or from BDH, Poole, Dorset, UK. Radiolabelled sodium iodide (Na¹²⁵I) was from Amersham International plc, Bucks, UK.

Chicken GnRH I and II were purchased from Peninsula Laboratories, Belmont, CA, USA, and m GnRH from Ayerst Laboratories Ltd, Andover, Hants, UK. Salmon and lamprey GnRH were generous gifts of Dr J. King, MRC Regulatory Peptides Research Unit, University of Cape Town, RSA, and the GnRH agonist, Buserelin ([D-Ser (tBu)⁶] 1-9 GnRH ethylamide), was the kind gift of Dr J. Sandow, Hoescht AG, Frankfurt, Germany. All other GnRH analogues and the GnRH antagonist ([Ac 3,4-dehydro-Pro¹, D-p-F-Phe², D-Trp^{3,6}] LHRH) were purchased from Sigma. The structures of GnRH isoforms and analogues are shown in Figure 1. Peptides identical to different regions of the human gonadotrophin-releasing hormone-associated protein (GAP) (amino acid sequences 14-26, 14-37, 28-36, 38-49, 51-66 and 54-66) were generously supplied by Dr K. Eidne, MRC Reproductive Biology Unit, Edinburgh.

Tracer preparation and characterization

Peptides were radioiodinated using the glucose oxidase/lactoperoxidase method (Sharpe and Fraser, 1980), and were purified by chromatography on Sephadex G25 columns (1 × 40 or 1 × 60 cm) in 0.01 M acetic acid-0.1 per cent bovine serum albumin (BSA). Relative elution volumes were calculated from the formula $(V_e - V_o) / (V_t - V_o)$, where V_e is the elution

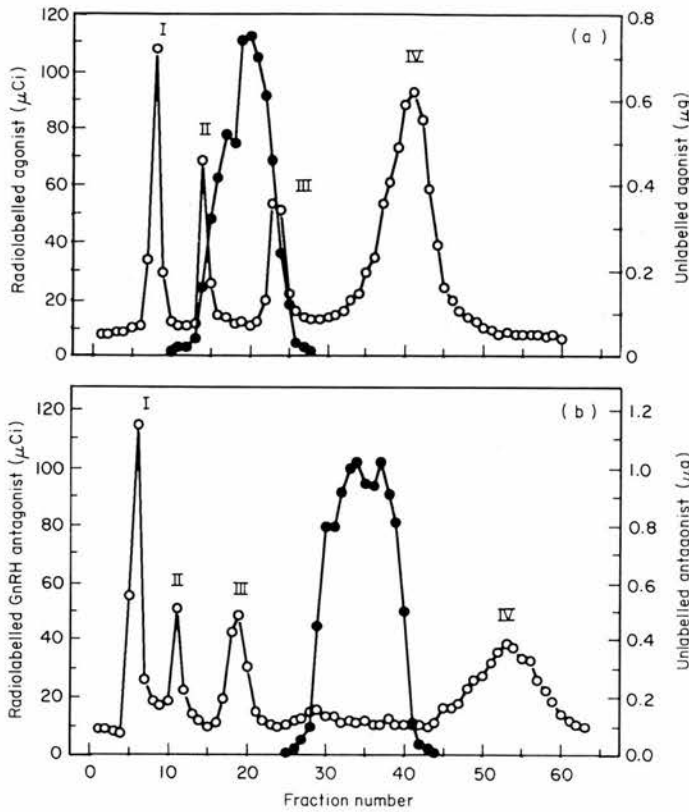


Figure 1. Purification of ^{125}I -labelled GnRH_A and GnRH_{ANT} tracers by chromatography on Sephadex G25. Radioiodinated GnRH_A (Buserelin; a) or GnRH_{ANT} (b) were prepared on Sephadex G25 (fine) columns (1 × 40 cm) equilibrated and eluted with 0.01 M acetic acid-0.1 per cent BSA. Fractions (2 ml) were collected and counted (o), and aliquots (5–50 μl) incubated with 100 μl of rat pituitary membranes (4°C for 4 h) for measurement of inhibition of binding of ^{125}I -labelled GnRH analogue by uniodinated GnRH analogue (●). Analogue concentration was estimated by comparison of inhibition of binding with standard curves constructed with unlabelled GnRH_A or GnRH_{ANT}.

volume of the peak, V_0 , is the void volume of the column (Dextran Blue), and V_t is the total column volume (Na^{125}I). In some experiments, aliquots of the purified peaks from Sephadex G25 chromatography were rechromatographed on Sephadex QAE-A25 equilibrated and eluted with 0.1 M ammonium bicarbonate buffer, pH 7.8. Relative elution volumes from QAE-A25 were calculated relative to elution of ^{125}I -iodide.

The elution positions of unlabelled GnRH analogues were determined by measuring the inhibition of specific binding of GnRH analogue tracer to rat pituitary homogenate in the presence and absence of aliquots of Sephadex G25 fractions (5–50 μl). Comparison of inhibition of binding by eluate fractions with displacement curves for unlabelled agonist or antagonist enabled calculation of the mass of unlabelled analogue in each fraction.

Specific activities of ^{125}I -labelled GnRH tracers

Self-displacement assay of GnRH_A tracers by binding to immature female rat pituitary homogenates gave values for specific activity of binding ranging from 160 to 880 Ci/g. Specific activities of radioiodinated GnRH isoforms were estimated by self-displacement

assay (Clayton, 1983), using an anti-GnRH antibody (EL-14), generously provided by Dr W. E. Ellinwood, Oregon Health Sciences University, Portland, Oregon. This antibody recognizes both N- and C-terminal regions of the GnRH molecule, and therefore binds all isoforms of GnRH, but not GnRH analogues (see below). Values for specific activities of binding to antibody calculated by self-displacement assay for three separate tracer preparations of all five GnRH isoforms ranged from 60 to 1104 Ci/g.

Thin layer chromatography of radioiodinated GnRH digests

Aliquots of ^{125}I -labelled GnRH tracer fractions from Sephadex G25 chromatography were dried under a stream of nitrogen. Ammonium bicarbonate buffer (200 μl of 0.1 M) was added, followed by 5 μg of *Streptococcus griseus* protease. Tubes were sealed under nitrogen, then incubated at 37°C for 48 h. Unlabelled mono- and di-iodotyrosine standards were added (2 μg), and aliquots (100 000 ct/min) were spotted onto prewashed glass-silica TLC plates (Sigma) equilibrated and run with 295 ml t-butyl alcohol, 160 ml t-amyl alcohol, 75 ml 7.5 M ammonium hydroxide, 35 ml methyl ethyl ketone and 85 ml distilled water (Nett and Adams, 1977). TLC plates were air-dried, stained with ninhydrin to localize amino acids and mono- and di-iodotyrosine standards, and autoradiographed (Kodak XR-4 film; 20 h).

Tissue

Placenta

Human placentae were obtained by elective caesarian section at term from several normal women. Placentae were washed extensively in ice-cold isotonic phosphate-buffered saline (PBS: Flow Laboratories, Irvine, Scotland) to remove blood. Placental tissue was gently spread out, and pieces of villous tissue (ca. 1 g) were cut from different central regions of the placental plate, taking care to avoid blood vessels and fibrous connective tissue. Villous pieces (10–20 g) were rinsed once more in cold PBS, then homogenized in ice-cold 0.3 M sucrose-10 mM Tris-1 mM EDTA, pH 7.4 (SET buffer; 5 ml/g) using a Polytron homogenizer (two 10 sec bursts at full speed, separated by a 1 min cooling period in ice). After filtration through four layers of cheesecloth, homogenates were fractionated immediately, or stored frozen (2 ml aliquots) in liquid nitrogen.

Rabbit placentae were obtained from two late-pregnant New Zealand White rabbits. Animals were sacrificed by Nembutal overdose and placentae cleaned of decidual tissue and membranes. Tissue from the placental plate was dissected, weighed, minced and homogenized in SET medium (Polytron) and stored frozen at -20°C .

Pituitary

Rat pituitaries were obtained from immature (28–35 day) female Sprague-Dawley rats killed by CO_2 asphyxiation. Pituitaries were excised and homogenized (2 glands/ml) in ice-cold SET medium, using a loose-fitting all-glass Dounce homogenizer. Aliquots (2 ml) were snap-frozen in solid CO_2 , and stored at -20°C until required.

Rabbit pituitaries were recovered from three virgin New Zealand White does following Nembutal overdose and were homogenized in SET medium (2 ml/pituitary) and stored at -20°C .

Tissue fractionation

Homogenates were centrifuged at 1000 g for 10 min (4°C), and the resulting pellet resuspended in SET medium. Supernatants were then centrifuged at 50 000 g for 60 min in a Sorvall OTD-50 refrigerated ultracentrifuge. Pellets were gently rehomogenized (loose

Dounce) in SET medium (membrane fraction). Supernatants (cytosol fraction) were also kept. Fractions were stored in aliquots of 2 ml at -20°C until required.

Assays

Protein was measured by the method of Lowrey et al (1951) with crystalline bovine serum albumin as a standard.

Binding assays

Receptor binding. Triplicate aliquots (20–200 μl) of placental tissue fractions were incubated in a 1 ml system containing 40 mM Tris-HCl, pH 7.4, 0.5 per cent bovine serum albumin (BSA) and 100 000 ct/min of ^{125}I -labelled GnRH analogue or GnRH isoform tracer. After incubation at 20°C for 1 h, bound hormone was recovered by polyethyleneglycol (PEG) precipitation (Bramley et al, 1985), and ^{125}I bound to the pellet counted in a Packard 'Crystal' gamma-counter at an efficiency of 75 per cent. Non-specific binding was measured in duplicate in the presence of 10 μg unlabelled Buserelin, and was normally 3–5 per cent of total counts added. The difference between binding in the presence and absence of unlabelled GnRH_A represented specific binding (normally 5–25 per cent of total counts added). Controls without tissue, with and without unlabelled GnRH_A, were included to correct for displacement of tracer from assay tubes by cold analogue (0.5–1 per cent of total counts added). Binding of GnRH tracers to rat pituitary tissue fractions was performed as described above for placental tissue (100 000 ct/min added), except that incubation was performed at 4°C for 4 h.

Antibody binding. Binding of ^{125}I -labelled GnRH tracers (20–30 000 ct/min added) to anti-GnRH antibody (1:10 000–1:50 000 dilution) was performed in the presence or absence of an excess of the appropriate unlabelled hormone. Incubation was for 6 h at 4°C . Ice-cold bovine γ -globulin (0.5 ml; 0.5 per cent w/v) was added and bound hormone recovered by PEG precipitation (Bramley, Menzies and Baird, 1985).

Specificity studies

Concentrations of peptides, proteins, GnRH or GnRH analogues required to reduce the specific binding of radiolabelled GnRH or GnRH_A by 50 per cent (IC_{50}) were estimated from displacement curves with increasing concentrations of the appropriate GnRH analogue and tracer. Values for experiments where $n > 1$ were expressed as means \pm SEM.

Inactivation of GnRH analogues

Quintuplicate aliquots (100 μl) of human placental or rat pituitary tissue fractions were incubated in Eppendorf tubes at 37°C for 30 min (or as shown) in a 1 ml system containing 40 mM Tris-HCl, pH 7.4, 0.5 per cent BSA and 600 000 ct/min ^{125}I -labelled GnRH or GnRH_A. Tubes were immediately placed in ice-water, and centrifuged at 30 000 g for 10 min (4°C). Supernatants were carefully aspirated, and pellets counted for ^{125}I . Supernatants were counted, and their volumes adjusted with incubation buffer to give either 90 000 ct/min/ml for receptor assays (GnRH analogues, m GnRH, s GnRH, ch GnRH II) or 20 000 ct/min/ml for antibody assay (all GnRH isoforms). Triplicate aliquots of supernatants (1 ml) and duplicate aliquots containing the appropriate unlabelled GnRH (10 μg) were then added to: (a), fresh human placental membranes and incubated at 20°C for 1 h (^{125}I -labelled GnRH_A, s GnRH, ch GnRH II, m GnRH); (b), rat pituitary membranes (^{125}I -GnRH_A) and incubated at 4°C for 4 h or (c), EL-14 antibody (1:10 000 dilution; all

^{125}I -labelled GnRH isoforms) and incubated at 4°C for 6 h. After incubation, bound hormone was recovered by PEG precipitation, and ^{125}I measured in the pellets by gamma-counting. Three controls were always included:

- tissue fraction incubated with tracer at 0°C for 30 min during the first incubation;
- tracer incubated without tissue fraction during the first incubation at either 0°C or
- tracer incubated without tissue fraction at 37°C for 30 min.

Values of specific binding for all three controls always agreed to within ± 5 per cent. Inactivation of tracer during the first incubation was reflected by a reduction in the specific binding of the unbound tracer (supernatant) fraction, following re-incubation with fresh receptor or antibody. Inactivation was expressed as a percentage of binding to controls.

Statistics

Statistical significance of differences between means was estimated by Student's *t*-test.

RESULTS

Characterization of ^{125}I -labelled GnRH tracers

Since it is vital to ensure that all studies of GnRH binding were conducted with tracer fractions having maximal receptor binding activity, we first characterized the different radioiodinated forms produced during GnRH tracer preparation. Typical elution profiles for ^{125}I -labelled GnRH_A [Buserelin; Figure 1(a)] or GnRH_{ANT} [Figure 1(b)] following chromatography on Sephadex G25 showed four distinct activity peaks. The first peak (I) was due to high molecular weight material which eluted in the column void volume (V_0); peak II was free ^{125}I -iodide which eluted in the total volume of the column (V_t). The retarded peak III appeared to be due to damaged forms of ^{125}I -labelled GnRH analogue which bound poorly to rat pituitary and human placental receptors (Table 2). Peak IV was retarded strongly and bound well to rat pituitary and human placental receptors (Table 2). Similar data were obtained for binding of the four peaks with m GnRH, ch GnRH II, GnRH_{ANT} (Table 2) and other GnRH isoforms (not shown). Unlabelled GnRH agonist eluted between peaks II and III [Figure 1(a)] and unlabelled antagonist, between peaks III and IV [Figure 1(b)].

Elution profiles for peaks III and IV following Sephadex G25 chromatography of

Table 2. Binding of different GnRH tracer peaks to receptors and anti-GnRH antibody

Peak	Tracer bound specifically (% total)					
	Rat pituitary		Human placenta		Antibody	
	GnRH _A	GnRH _{ANT}	GnRH _A	ch GnRH II	m GnRH	ch GnRH II
I	0.01	0.02	0.02	0.01	0.05	0
II	n.d	n.d	n.d	n.d	n.d	0
III	0.3	0.2	0.6	0.05	2	3
IV	6.8	10.6	8.3	4.5	68	73

Radioactive peaks from radioiodination and Sephadex G25 chromatography of GnRH_A, GnRH_{ANT}, m GnRH and ch GnRH II were incubated (100 000 ct/min) with rat pituitary (100 μl) or human placental membranes (100 μl), or (20 000 ct/min) with anti-GnRH antibody, EL-14 (1:10 000). Specific binding was measured as described and expressed as a percentage of total counts bound. Values are means of three different preparations for each GnRH analogue. n.d. = not done.

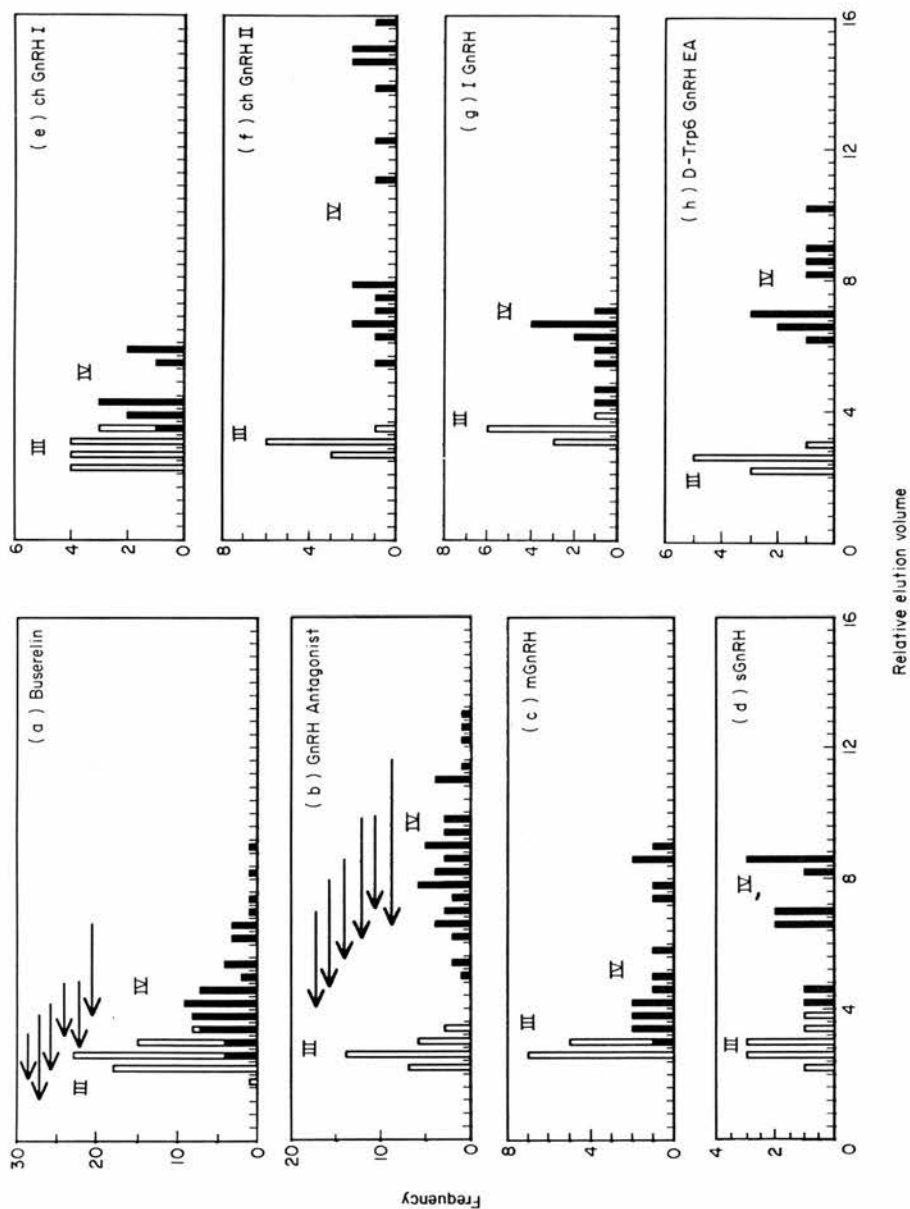


Figure 2. Sephadex G25 elution profiles for GnRH analogues and isoforms. Radiolabelled tracers (prepared from 10 μ g analogue or GnRH isoform) were chromatographed on Sephadex G25, and relative elution volumes of each tracer peak and of unlabelled hormone were measured as described in the legend to Figure 1. Relative elution volume (REV) for peaks III (\square) and IV (\blacksquare) were calculated as described in the 'Materials and Methods' section, and REV plotted against frequency. Horizontal arrows indicate the separation between the relative elution volumes of unlabelled analogue (tip of arrow) and the REV of Peak IV (125 I-labelled tracer in that purification experiment (blunt end) for Buserelin (a) and GnRH_{ANT} (b).

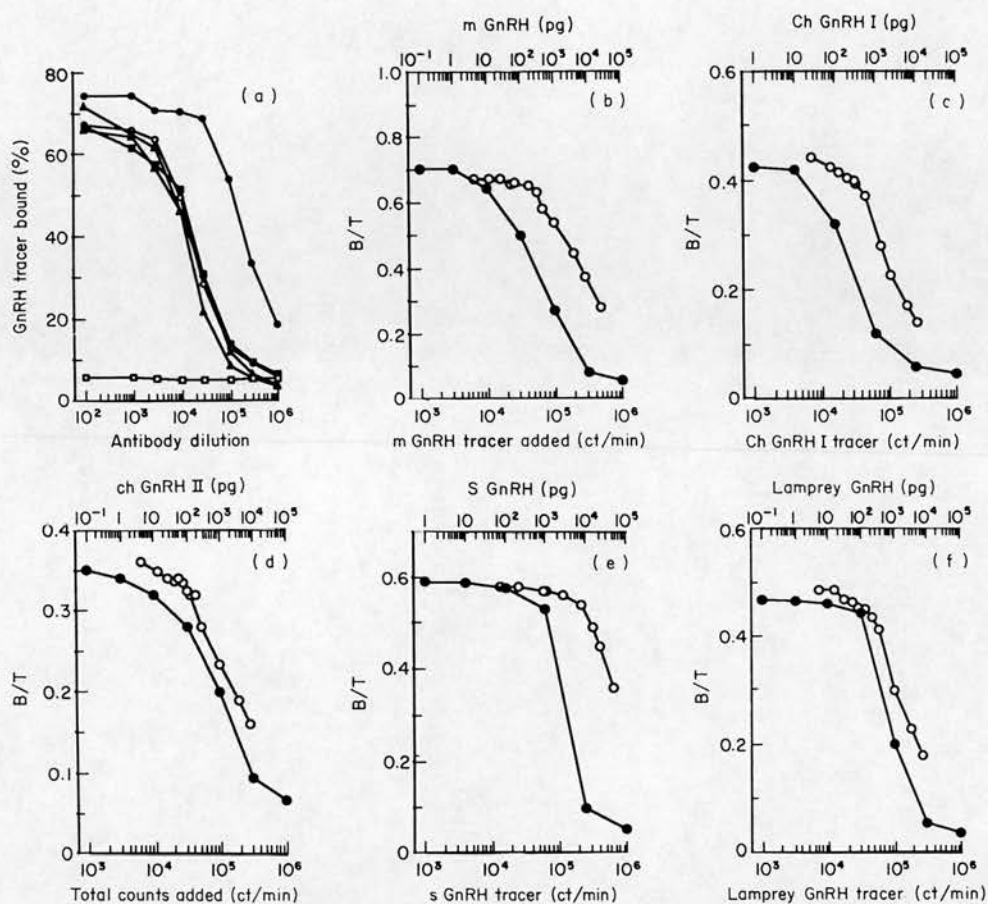


Figure 3. Antibody binding and self-displacement assay of ¹²⁵I-labelled GnRH isoforms. (a). Aliquots of ¹²⁵I-labelled GnRH isoforms and GnRH_A (peak IV) were incubated with increasing dilutions of anti-GnRH antibody (EL-14) for 6 h at 4°C, and tracer bound measured by PEG precipitation. Binding was expressed as a percentage of total counts added. (●), m GnRH; (○), ch GnRH II; (▲), ch GnRH I; (△), l GnRH; (■), S GnRH; (□), Buserelin. (b). Binding of GnRH isoform tracers to EL-14 antibody (1:10 000) in the presence of increasing concentrations of unlabelled GnRH isoform (●) or with increasing GnRH tracer concentrations (○). (b), m GnRH; (c), ch GnRH I; (d), ch GnRH II; (e), s GnRH; (f), l GnRH.

radioiodinated tracers from a number of tracer preparations are shown in Figure 2. Peak III always eluted at a similar position (V_e ; 2-3) for both GnRH agonists [Figures 2(a,h)] for GnRH_{ANT} [Figure 2(b)] and for all GnRH isoforms [Figures 2(c-g)]. However, the elution volumes of peak IV tracers for the different isoforms of GnRH differed from one another. Moreover, V_e values varied between tracer preparations for the same GnRH form (Figure 2). (This variation was related in part to different adsorption properties of various batches of Sephadex G25 from the different suppliers; unpublished data). Horizontal arrows in Figures 2(a,b) indicate the separation between the elution volumes of unlabelled (arrow head) and ¹²⁵I-labelled (Peak IV; arrow base) Buserelin [Figure 2(a)] and GnRH_{ANT} [Figure 2(b)]. Unlabelled hormone was always well separated from the tracer peak (IV). Hence, GnRH tracers (peak IV) should be minimally contaminated with unlabelled hormone.

For each ¹²⁵I-labelled GnRH isoform preparation, the tracer fraction eluting in peak IV bound well to EL-14 anti-GnRH antibody [65-75 per cent binding at 1:1000 dilution; Figure 3(a)], whereas peaks I, II and III bound poorly (Table 2). Moreover, as predicted from

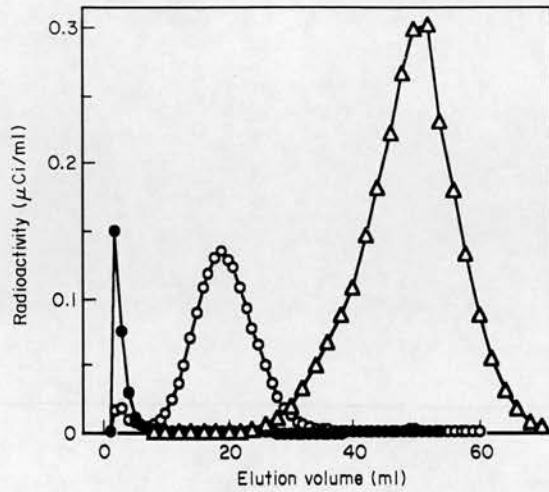


Figure 4. Purification of Buserelin tracer fractions on Sephadex QAE-A25. Fractions of peak III (○) and peak IV (●) were prepared as described in the legend to Figure 1, and aliquots applied to columns (1 × 2 cm) of Sephadex QAE-A25 packed into the barrel of a 5 ml plastic syringe. ^{125}I -labelled sodium iodide was also chromatographed as an internal and external standard (△). Columns were eluted with 0.1 M ammonium bicarbonate (pH 7.8) and 2 ml fractions collected and counted for ^{125}I .

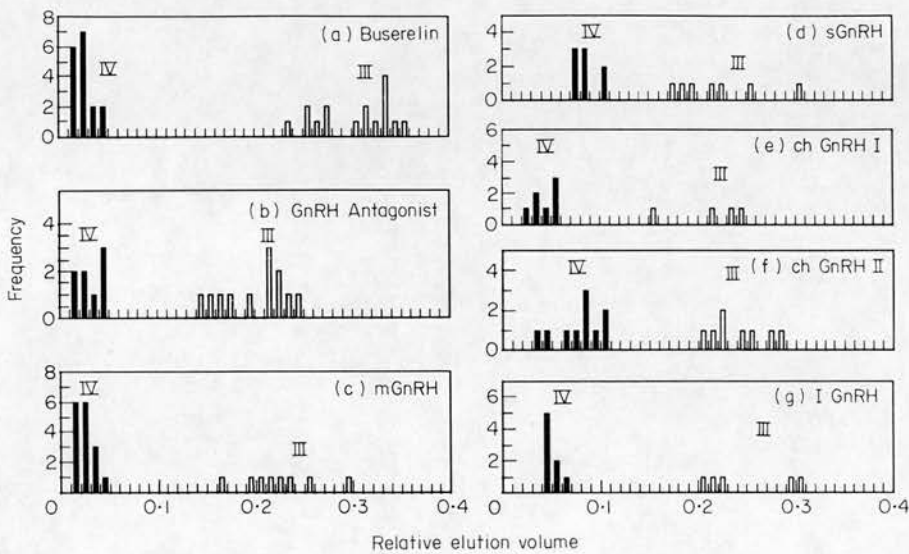


Figure 5. Sephadex QAE-A25 elution profiles for GnRH analogues and isoforms. Fractions from peak III (□) and peak IV (■) from chromatography of ^{125}I -labelled GnRH tracer preparations were pooled and rechromatographed on Sephadex QAE-A25 columns. Fractions were counted for ^{125}I , and frequency of elution volume (relative to ^{125}I -iodide) plotted.

the requirement of this antibody for both N- and C-terminal regions of GnRH, ^{125}I -labelled tracers from peak IV of GnRH agonist [Figure 3(a)] and GnRH_{ANT} preparations (not shown) did not bind to antibody, although they showed excellent binding to receptors (Table 2). Typical self-displacement curves for ^{125}I -labelled m GnRH [Figure 3(b)], ch GnRH I [Figure 3(c)], ch GnRH II [Figure 3(d)], s GnRH [Figure 3(e)] and l GnRH tracer preparations [Figure 3(f)] are illustrated. Differences in maximal binding of different GnRH isoform tracers to antibody were related to the specific activity of the tracers.

Peaks III and IV of a Buserelin tracer preparation were subjected to ion exchange chromatography on Sephadex QAE-A25 (Figure 4). Material from peak IV was not retarded by the gel, suggesting that this peak lacked strong negative charges, and hence, consisted of radioiodinated (uncharged) Buserelin. In contrast, peak III was bound quite strongly (Figure 4), suggesting that this material carried a negative charge. This could arise either by opening of the N-terminal pyro-Glu, or by loss of the terminal amine group. Profiles from a number of GnRH tracer fractions chromatographed on QAE-A25 showed a similar pattern (Figure 5); tracers from peak IV consistently failed to bind to the gel, whereas material from peak III was always retarded.

Previous studies have suggested that peaks III and IV represent GnRH with di-iodinated and mono-iodinated Tyr⁵ residues respectively (Sharpe and Fraser, 1980; Nett and Adams, 1977). Digestion of peaks III and IV by prolonged incubation with *Streptococcus griseus* protease (37°C for 48 h) followed by thin layer chromatography and autoradiography indicated that both peaks III and IV contained largely mono-iodinated tyrosine, with minimal di-iodotyrosine (Figure 6).

Binding of GnRH analogues

Specificity. Having established for all GnRH tracer preparations that material eluted in peak IV was bound with high affinity to rat pituitary homogenate (GnRH agonists and antagonist) or to EL-14 antibody (GnRH isoforms), we studied the specific binding of radioiodinated GnRH agonist (Buserelin) and GnRH_{ANT} to rat pituitary [Figure 7(a)] and to human placental membranes [Figure 7(b)]. As expected, GnRH agonist and antagonist tracers bound well to rat pituitary receptors, and binding of both tracers was displaceable by excess cold agonist or antagonist. GnRH agonist tracers also bound well to human placental membrane fractions, and there was a good linear correlation ($r = 0.93$) between the abilities of different ^{125}I -labelled GnRH agonist preparations to bind to pituitary and placental membranes (data not shown), suggesting that the same radioiodinated species binds to both receptors. Specific binding of GnRH agonist tracer to placental membranes increased with increasing protein concentration [Figure 7(b)]. In contrast, although total binding of ^{125}I -labelled GnRH_{ANT} increased with increasing concentrations of human placental membranes, GnRH_{ANT} binding was not displaceable by excess unlabelled agonist or antagonist [Figure 7(b)], even after prolonged preincubation before addition of antagonist tracer (data not shown).

Binding and inactivation of ^{125}I -labelled GnRH and GnRH isoform tracers

Specific binding of GnRH tracers to rat pituitary [Figure 8(a)] and human placental membranes [Figure 8(b)] was measured, and the unbound tracer fraction was assessed for its ability to rebind to fresh placental or pituitary receptors, or to EL-14 GnRH antibody.

Pituitary. ^{125}I -labelled GnRH_A bound well (~8 per cent of total counts added) to both tissues, and much of the unbound tracer was still capable of rebinding specifically to fresh



Figure 6. ^{125}I -labelled monoiodo- and diiodo-tyrosine in peaks III and IV. Pooled fractions from peaks III and IV of Sephadex G25-purified GnRH isoforms and agonist were digested with protease, and mono- and diiodo-tyrosine separated by thin layer chromatography. Radioactivity was localized by autoradiography. Arrows indicate the positions of monoiodo- and diiodo-tyrosine standards.

Lane 1, m GnRH (IV); lane 2, m GnRH (III); lane 3, ch GnRH I (IV); lane 4, ch GnRH I (III); lane 5, s GnRH (IV); lane 6, s GnRH (III); lane 7, l GnRH (IV); lane 8, l GnRH (III).

receptors in both systems [80–90 per cent of controls incubated without membranes; Figures 8(a,b)]. Much lower levels of m GnRH tracer were bound to rat pituitary membranes in the first incubation (~1 per cent of total counts added), and the proportion of tracer capable of rebinding (40–50 per cent of controls incubated without membranes) was reduced compared to GnRH_A [80–90 per cent of controls; Figure 8(a)]. However, appreciable quantities of tracer which were still capable of rebinding to fresh placental receptor or antibody were detectable in the unbound tracer fraction after the first binding incubation with pituitary membranes. Other GnRH isoforms bound poorly to rat pituitary membranes [<0.4 per cent of total counts added; Figure 8(a), solid bars], though once again, the unbound tracer fraction still contained appreciable levels of GnRH tracer capable of rebinding to human placental

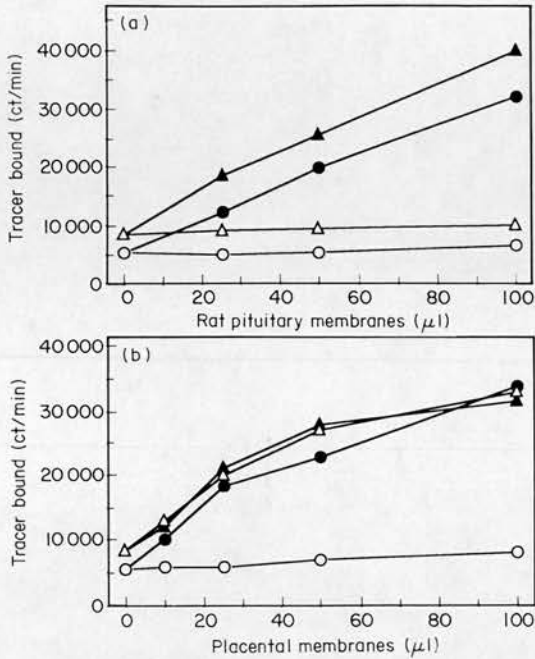


Figure 7. Binding of radiolabelled GnRH_A and GnRH_{ANT} by rat pituitary and by human placental membranes. Triplicate aliquots of rat pituitary (a) and human placental (b) membrane fractions were incubated with 100 000 ct/min of ¹²⁵I-labelled GnRH_A (●, ○) or GnRH_{ANT} (△, ▲) in the absence (▲, ●) or in the presence (△, ○) of 10 μg of unlabelled GnRH_A or GnRH_{ANT} respectively. After incubation (pituitary, 4°C for 4 h; placenta; 20°C for 1 h), bound and free hormone were separated by PEG precipitation, and bound hormone measured.

receptors [25–35 per cent relative to controls; Figure 8(a); stippled bars] or antibody [25–50 per cent relative to controls; Figure 8(a); open bars]. Moreover, estimation of the proportions of tracer rebound by pituitary receptors, and to anti-GnRH antibody, correlated well.

Placenta. GnRH_A bound well to human placental membranes (~8 per cent of total counts added), and most of the unbound tracer fraction was capable of rebinding to fresh placental or pituitary receptors [~70 per cent relative to controls; Figure 8(b)]. Once again, m GnRH tracers bound to placental membranes at lower levels than GnRH_A [~4 per cent of counts added; Figure 8(b), solid bars], and rebinding in the second incubation was reduced (~30–50 per cent relative to controls) compared to GnRH_A [Figure 8(b)], suggesting greater inactivation of m GnRH tracer than GnRH_A tracer during incubation with placental membranes. Lamprey GnRH and ch GnRH I also bound poorly to human placental membranes [solid bars; Figure 8(a)]. However, s GnRH and ch GnRH II bound specifically to placental tissue at similar levels to GnRH_A [solid bars; Figure 8(b)]. Moreover, whether or not different GnRH isoforms bound to placental membranes in the first incubation bore no relationship to the degree of inactivation during incubation. Thus, s GnRH bound well to placental membranes [Figure 8(b); solid bars] whereas ch GnRH I did not, but the proportion of tracer capable of rebinding was about 20–30 per cent for both tracers [Figure 8(b); open bars]. Similarly, ch GnRH II bound well to placental membranes [Figure 8(b); solid bars], whereas l GnRH did not. However, both tracers were rebound by antibody at 50–60 per cent of controls [Figure 8(b); open bars]. Furthermore, the inability of GnRH

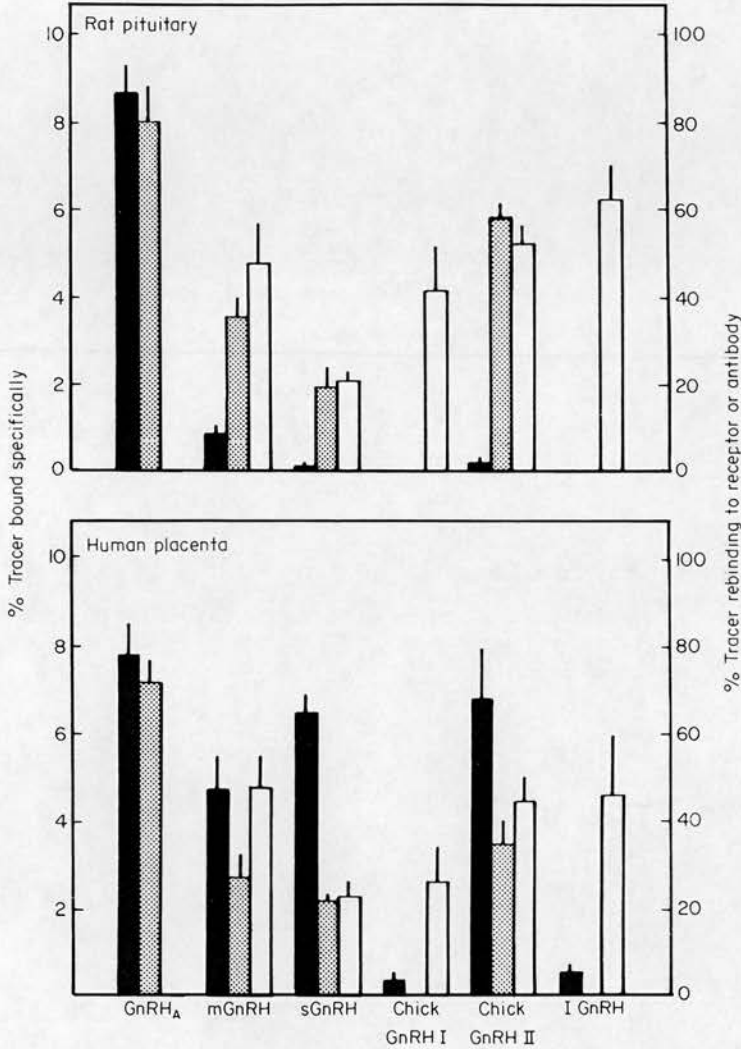


Figure 8. Inactivation of ¹²⁵I-labelled GnRH tracers following incubation with rat pituitary or human placental membranes. Quintuplicate aliquots of rat pituitary (a) or human placental membranes (b) were incubated with 600 000 ct/min of ¹²⁵I-labelled GnRH_A or GnRH isoforms under the usual incubation conditions, and bound hormone (■, left-hand scale) recovered by centrifugation at 30 000 g for 10 min. Supernatant fractions were then adjusted to either 90 000 ct/min/ml with binding incubation buffer, and reincubated with fresh placental membranes, or adjusted to 20 000 ct/min/ml and incubated with EL-14 antibody (1:10 000). Bound hormone was recovered by PEG precipitation, and binding was expressed as a percentage of tracer added which was capable of binding to fresh placental membranes (□) or to antiGnRH antibody (□) relative to the mean of the three binding controls (right-hand scale). Bars are means ± SEM of GnRH tracer bound (2–8 separate experiments) using four different ¹²⁵I-labelled GnRH tracer preparations to the same pituitary and placental membrane preparations.

isoforms which bound to placenta but not to rat pituitary receptors was not due to greater degradation of tracer by rat pituitary membranes, since similar proportions of tracers were capable of rebinding to antibody following incubation with both tissues [compare open bars in Figures 8(a,b)]. Thus, although GnRH isoforms were inactivated to a greater extent than GnRH_A during incubation with placental or pituitary tissues, appreciable levels of all tracers

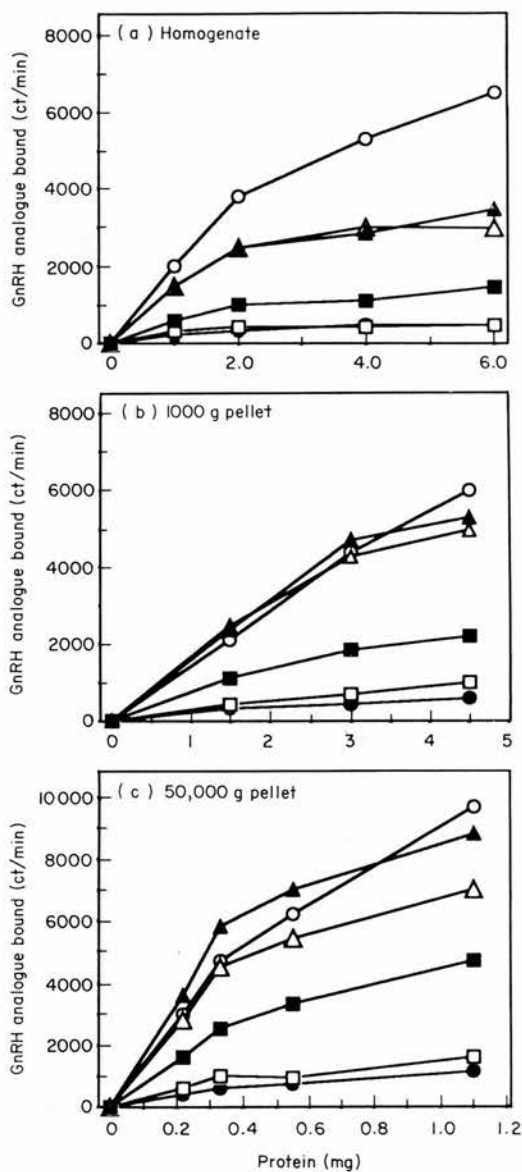


Figure 9. Binding of ¹²⁵I-labelled GnRH isoforms and GnRH_A to human placental homogenates and membrane fractions. Radioiodinated GnRH isoforms (100 000 ct/min/ml) were incubated for 1 h at 20°C with aliquots of (a), human placental homogenate, (b), 1000 g pellet or (c), 50 000 g pellet and specific binding measured in triplicate in the presence and absence of unlabelled GnRH_A. Tracers were: (○), GnRH_A [Buserelin]; (●), ch GnRH I; (△), s GnRH; (▲), ch GnRH II; (□), l GnRH; (■), m GnRH.

remained which were still capable of re-binding to receptor [stippled bars; Figure 8(a,b)] or anti-GnRH antibody [open bars; Figure 8(a,b)] after preincubation with either tissue.

Differential binding of GnRH tracers was not related to differential saturation of binding, since specific binding of all GnRH tracers increased with increasing receptor concentration (Figure 9). Furthermore, specific GnRH binding activity (per mg. protein) was greater for placental 50 000 g membrane fractions [Figure 9(c)] than for 1000 g pellet [Figure 9(b)] or

Table 3. Specific activity of ^{125}I -labelled GnRH_A binding and percentage ligand inactivation during incubation

	Homogenate	1000 pellet	50 000 pellet
Specific binding (pg per mg protein)	7.2 ± 0.5	8.8 ± 0.8	24.3 ± 3.8
Tracer inactivation (% control)	46 ± 7	28 ± 4	13 ± 6

Specific binding of ^{125}I -labelled GnRH_A (600 000 ct/min/ml) was measured following incubation at 20°C for 1 h with triplicate aliquots (100 μl) of human placental homogenates or membrane fractions, in the presence or absence of 10 μg of unlabelled Buserelin. After centrifugation at 30 000 g for 10 min, supernatant fractions were carefully aspirated, ^{125}I -GnRH_A bound by each pellet was counted, and the fraction of the unbound tracer in each supernatant fraction which was capable of rebinding to fresh placental membranes was measured and expressed as a percentage of controls. Figures shown are means ± SEM for four separate experiments.

homogenate [Figure 9(a)]. Salmon GnRH (s GnRH) and chicken GnRH II (ch GnRH II) binding reached a plateau at lower tissue levels than for GnRH_A [particularly with placental homogenate; Figure 9(a)]. However, chicken GnRH I (ch GnRH I) and lamprey GnRH (l GnRH) bound poorly at all protein concentrations for all fractions, and mammalian GnRH (m GnRH) binding levels were intermediate. Purification of placental membranes enhanced specific activity of GnRH binding (per mg protein) over that of 1000 g membranes and homogenate, but increasing binding activity was associated with progressively lower levels of GnRH_A tracer inactivation (Table 3). Membranes prepared at 50 000 g were therefore used routinely for most further studies.

Effects of pH

Binding of ^{125}I -labelled GnRH_A, s GnRH and ch GnRH II to placental membranes varied markedly with pH (Figure 10). Binding was low at both high and low extremes of pH, but a broad plateau was observed between pH 6 to 8. Maximal binding levels for all three GnRH tracers were similar, and all three showed inflection points at similar pH values (pH 5.8 and 8.1).

Effects of time and temperature

The effects of temperature and duration of incubation on the binding of GnRH agonists to placental membranes have been published previously (Currie, Fraser and Sharpe, 1981; Belisle et al, 1984; 1986; Iwashita, Evans and Catt, 1986). Specific binding of s GnRH tracer to placental membranes was dependent on time and temperature of incubation [Figure 11(a)]. Binding at 4°C increased to a plateau at 1 to 2 h, and declined slowly thereafter. At 20°C, binding was greatest at 1 h, and declined more rapidly with time, whereas at 37°C, maximal binding was observed at the earliest time measured (15 min), and by 4 h had declined dramatically such that little specific binding was detectable. Specific binding of ch GnRH II followed a similar pattern [Figure 11(b)]. Binding at 4°C was slower than for s GnRH, maximal levels being reached only after 4 h. However, binding at 20 and 37°C were very similar to s GnRH.

Inactivation of ^{125}I -labelled GnRH tracers

The rapid decrease in s GnRH and ch GnRH II binding with duration of incubation at 20 and 37°C [Figure 11(a,b)] may be due to inactivation of receptor and/or tracer. Inactivation

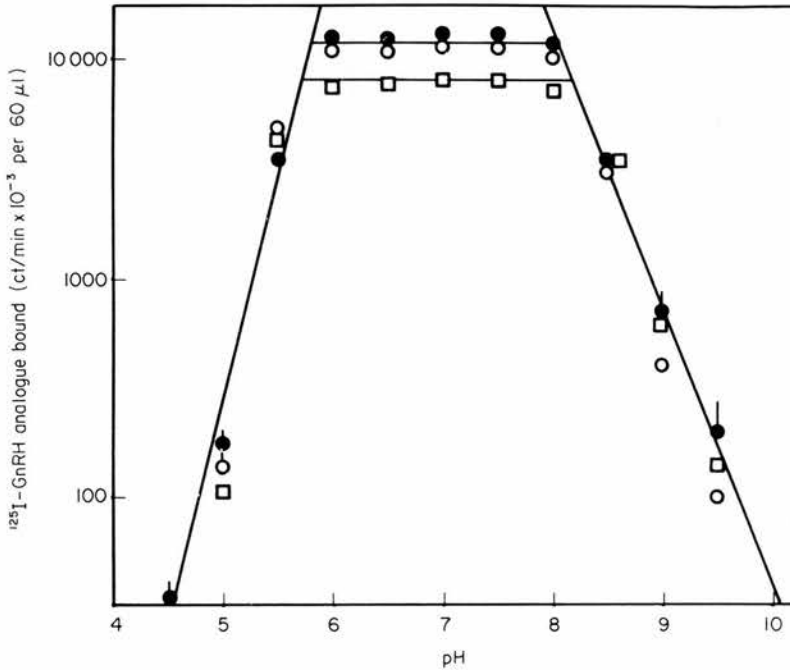


Figure 10. Effects of pH on binding of ^{125}I -labelled GnRH analogues to placental membranes. Aliquots ($100\ \mu\text{l}$) of human placental 50 000 g pellet were incubated with ^{125}I -labelled sGnRH (○), chGnRH II (□) or GnRH_A (●) for 1 h at 20°C at a range of different pH values. Specific binding was measured and plotted on a logarithmic scale against pH.

of receptor was dependent on both time and temperature of incubation, but was too slow (T_{50} , 190 min at 30°C) to account for the rates of loss of binding observed in Figure 11 (data not shown). We therefore compared the rate of degradation of GnRH_A and sGnRH tracers by placental membranes at 4 and 30°C (Figure 12).

Rates of binding of sGnRH to placental membranes at 4°C [Figure 12(a)] were similar to data shown in Figure 11(a). However, following exposure of ^{125}I -labelled sGnRH to placental membranes at 4°C , the binding ability of the unbound tracer fraction decreased sharply with time at 4°C (T_{50} , 20 min), and was almost completely abolished within 15 min at 30°C [Figure 12(c)]. At 4°C , binding of GnRH_A was much slower, and was still increasing after 2 h [Figure 12(b)]. Following incubation of GnRH_A tracer with placental membranes at 4°C , the unbound tracer fraction was still capable of high binding when incubated with fresh placental membranes [Figure 12(d)]. At 30°C , GnRH_A binding was high at 15 min, and remained elevated for up to 60 min, decreasing slowly with time thereafter [Figure 12(b)], and the receptor binding activity of the unbound fraction decreased more rapidly than at 4°C , with a T_{50} of 3.5 h. Rates of tracer inactivation were much lower for GnRH_A than for sGnRH at both temperatures.

Dissociation rates

Radiolabelled GnRH_A and sGnRH were incubated to equilibrium at 4°C with placental membranes, then excess unlabelled GnRH_A was added, and the rate of dissociation of both tracers measured at 4, 20 and 37°C (Figure 13). At 4°C , dissociation rates for GnRH_A [Figure 13(a)] and sGnRH [Figure 13(b)] were low. At 20°C , dissociation of GnRH_A was

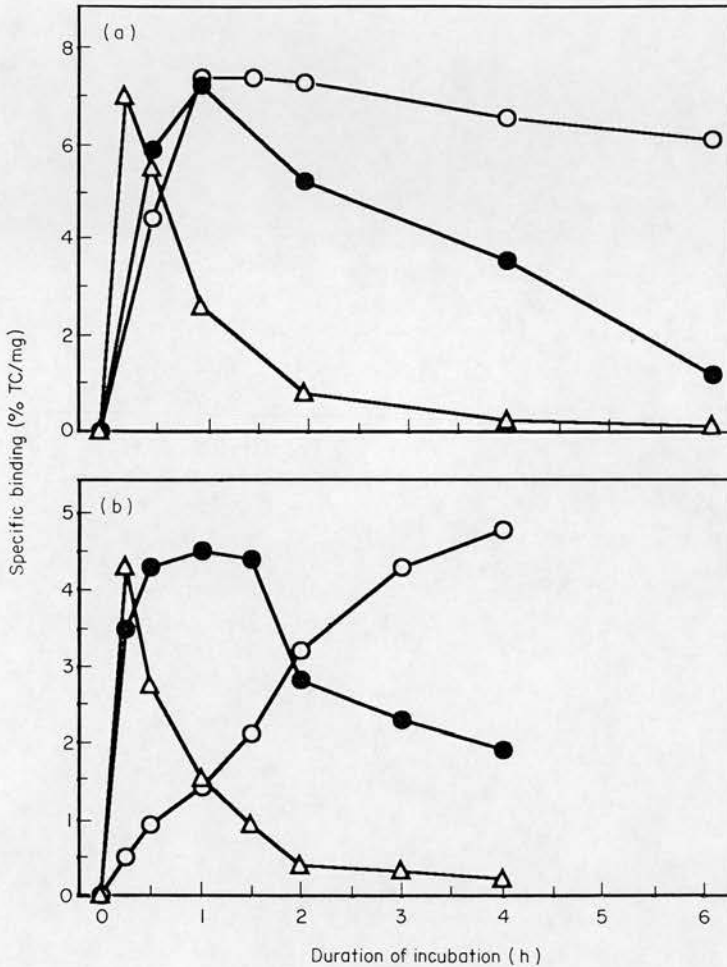


Figure 11. Effects of temperature and duration of incubation on binding of ^{125}I -labelled s GnRH (a) and ch GnRH II (b) to human placental membranes. Specific binding of ^{125}I -labelled s GnRH (a) and ch GnRH II (b) to human placental membranes was measured following incubation at 4°C (○), 20°C (●) or 37°C (△) for various periods of time. Points are means \pm SEM for three separate experiments in triplicate.

more rapid [T_{50} , 20 min; Figure 13(a)] than for s GnRH [T_{50} , 50 min; Figure 13(b)], while the dissociation rate of s GnRH at 37°C [T_{50} , 20 min; Figure 13(b)] was similar to that of GnRH_A at 20°C [Figure 13(a)].

Specificity of placental GnRH binding sites

A range of proteins (BSA, bovine IgG, FSH, LH, hCG, PRL), steroids (androgens, oestrogens, progesterone), di- and tri-peptides, chemotactic peptides and peptide hormones (angiotensins I, II and III, vasopressin, oxytocin, EGF, Substance P, neurotensin, met- and leu-enkephalins, TGF- β , TRF, insulin, bradykinin, somatostatin and eledoisin) failed to inhibit binding of GnRH_A to placental binding sites (data not shown). Furthermore, a number of peptidase inhibitors specific for the four major classes of tissue peptidases (Barrett, 1977) failed to inhibit placental GnRH_A binding (see Bramley, Menzies and Baird, 1986). The GnRH-related peptide, yeast α_1 -mating factor, and a series of peptides derived

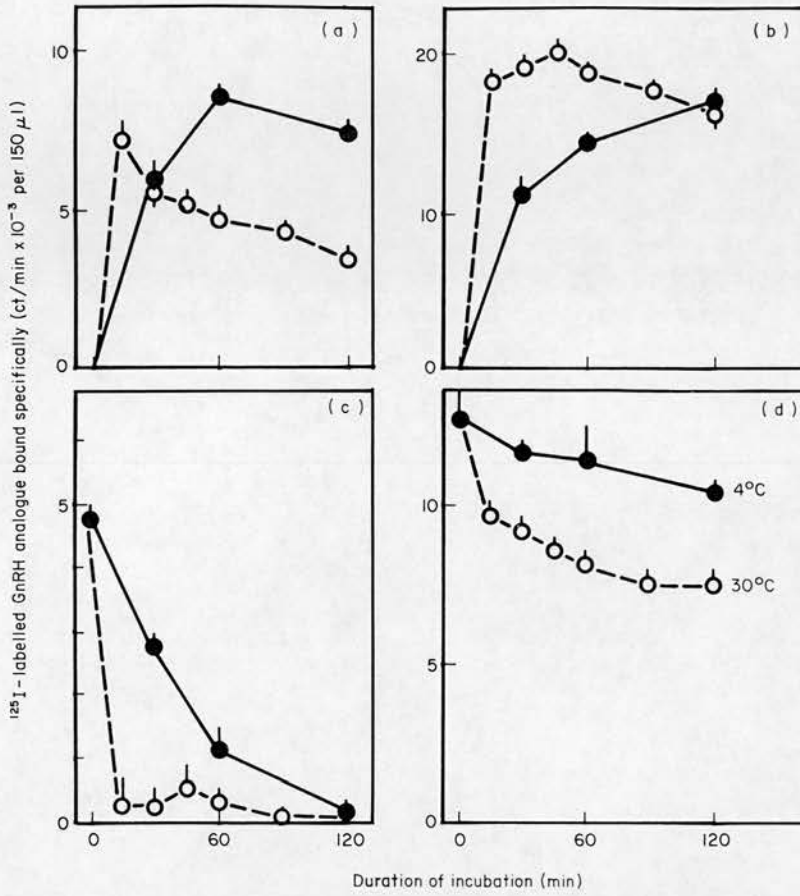


Figure 12 Binding and inactivation of ^{125}I -labelled GnRH_A and s GnRH by placental membranes. Aliquots of placental membrane fractions were incubated with 600 000 ct/min of ^{125}I -labelled s GnRH (a, c) or GnRH_A (b, d) at 4°C (●) or 30°C (○) for various times. Hormone bound specifically (a, b) was measured by centrifugation at 30 000 g for 10 min. Supernatants were recovered and diluted to 100 000 ct/min/ml with incubation buffer, then 1 ml aliquots were reincubated with fresh placental membranes at 20°C for 1 h. Specific binding was measured and the decrease in binding relative to controls (inactivation of tracer; c, d) was calculated.

from the gonadotrophin-releasing hormone-associated peptide (GAP) region of the human GnRH precursor protein (Millar, Wormald and Milton, 1986) also failed to inhibit GnRH_A binding to human placental membranes (data not shown).

The specificity of human placental GnRH binding sites was studied with radiolabelled GnRH_A, s GnRH and ch GnRH II as binding ligands (Table 4). For comparison, the affinities of these peptides in competing for ^{125}I -labelled GnRH_A binding sites in the rat pituitary were also measured. Concentrations of GnRH and GnRH agonists required to compete for GnRH_A binding to rat pituitary receptors (Table 4) were in line with values published previously (Clayton and Catt, 1981; Loumaye, Naor and Catt, 1982; Bramley, Menzies and Baird, 1986). The concentrations of GnRH from different species required to inhibit GnRH_A, s GnRH and ch GnRH II binding to human placental membranes were similar to levels required to displace agonist binding from rat pituitary (with the exception of m GnRH). Loss of N- or C-terminal amino acids led to loss of potency (as reported

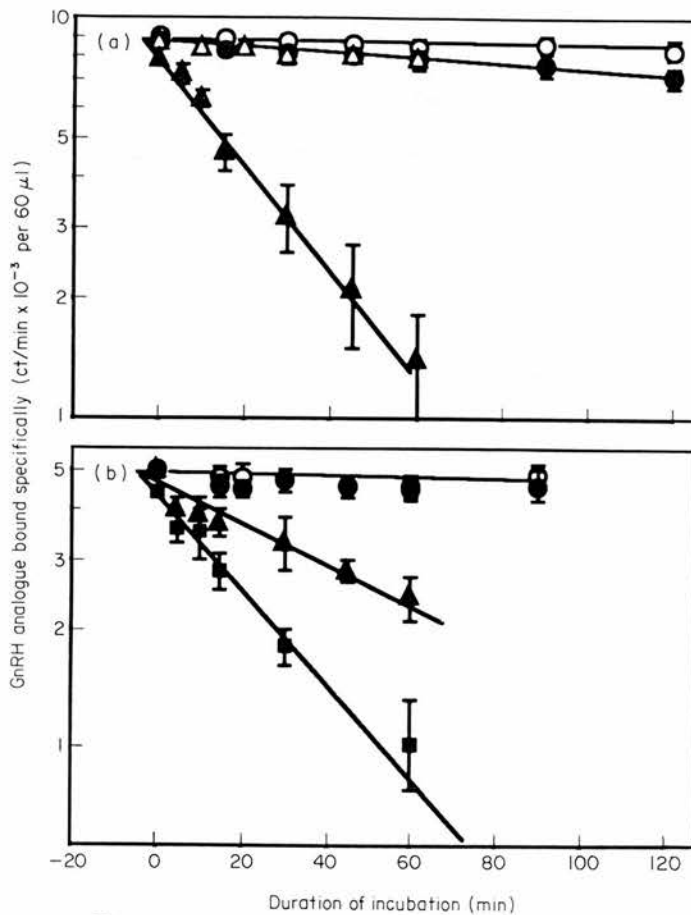


Figure 13. Dissociation of ^{125}I -labelled GnRH_A and s GnRH from human placental membranes. Aliquots of human placental 50 000 g membrane fractions were incubated to equilibrium (1 h at 20°C) with either ^{125}I -labelled GnRH_A (a) or s GnRH (b). Then 10 μl of medium alone (O, Δ) or medium containing 10 μg of unlabelled GnRH_A was added (●, ▲), and incubation was continued at 4°C (O), 20°C (Δ) or 37°C (□) as indicated. Specific binding was measured by PEG precipitation at various times thereafter, and binding plotted against duration of incubation.

previously for rat pituitary and human luteal GnRH binding sites; Bramley, Menzies and Baird, 1986), whereas substitution of D-amino acids at Gly⁶ (with or without ethylamide substitution at position 10) led to increased GnRH agonist activity, and markedly increased receptor binding potency relative to m GnRH in the rat pituitary. However, these structural alterations did not enhance agonist potency for human placental GnRH receptors appreciably (Table 4).

GnRH antagonist analogues with enhanced potency towards rat pituitary receptors had markedly reduced potencies for placental binding sites. Antagonist concentrations required to displace GnRH binding to placental membranes were 200- to 50 000-fold higher than for the rat pituitary receptors.

GnRH binding and inactivation in rabbit placenta

No specific GnRH binding to homogenates of rabbit placenta was observed with any GnRH isoform tracer under the same incubation conditions which allowed measurement of high

Table 4. Binding potencies of GnRH analogues and isoforms for ¹²⁵I-labelled GnRH binding sites in human placenta and rat pituitary

GnRH analogue Tracer	Concentration required to inhibit binding by 50% (ng)			
	Rat pituitary ¹²⁵ I GnRH _A	¹²⁵ I-GnRH _A	Human placenta ¹²⁵ I-s GnRH	¹²⁵ I-ch GnRH II
GnRH forms				
m GnRH	180 ± 30 (8)	1456 ± 530 (8)	2500 (2)	400 (2)
ch GnRH I	1550 ± 160 (6)	2300 ± 670 (5)	4000 (2)	1800
ch GnRH II	220 ± 130 (3)	740 ± 512 (5)	900	250 (2)
s GnRH	600	300 (2)	300	500
l GnRH	12 000	>10 000 (2)	>10 000 (2)	>10 000 (2)
[3-10] GnRH	4000 ± 600 (3)	>10 000 (2)	>10 000 (2)	>10 000 (2)
[Gly(OH) ¹⁰]GnRH	>10 000 (4)	>10 000 (3)	>10 000 (2)	1900 (2)
[des pGlu ¹]GnRH	4000	30 000 (2)	6000	20 000
Agonist analogues				
[D-Ser ⁶ (Bu) ⁶]EtA	0.3 ± 0.03 (23)	243 ± 166 (17)	387 ± 155 (4)	200 (2)
[D-Ala ⁶]EtA	0.5 ± 0.1 (5)	867 ± 234 (6)	750 ± 200 (3)	400 (2)
[D-Ala ⁶ N-MeLeu ⁷]EtA	0.8 ± 0.2 (3)	367 ± 115 (3)	-	795 (2)
[D-Trp ⁶] GnRH	0.3 (2)	268 ± 40 (6)	-	700 (2)
[D-Trp ⁶] EtA	0.6 ± 0.3 (5)	336 ± 153 (5)	400	200 (2)
[D-Phe ⁶] EtA	0.2 (2)	533 ± 258 (6)	-	710 (2)
1-9 GnRH EtA	3.0 (2)	1000 (2)	900	680
Antagonist analogues				
A	13 ± 5 (3)	>10 000 (3)	>10 000	>10 000
B	0.85 (2)	>10 000 (3)	10 000 (2)	15 000 (2)
C	1.0 ± 0.4 (3)	>10 000 (2)	-	>10 000 (2)
D	0.23 ± 0.1 (5)	>10 000 (4)	>10 000 (2)	>10 000 (2)
E	60 (2)	>10 000 (3)	-	-
F	0.6 ± 0.3 (5)	>10 000 (2)	>10 000 (2)	>10 000 (2)

Specific binding of ¹²⁵I-labelled GnRH analogues was measured to rat pituitary homogenate or human placental membranes in the absence and in the presence of increasing concentrations of unlabelled GnRH antagonist analogues, and the concentrations required to reduce specific binding by 50 per cent was calculated. Figures are means ± SEM for (n) separate experiments (except where n = 1).

- A, [D-Phe^{2,6} Pro³] GnRH;
 B, [AcD-pCl-Phe^{1,2} D-Trp³ D-Arg⁶ D-Ala¹⁰] GnRH;
 C, [D-pGlu¹ D-Phe² D-Trp^{3,6}] GnRH;
 D, [Ac-dehydro 3,4-Pro¹ D-p-F-Phe² D-Trp^{3,6}] GnRH;
 E, [D-Phe² D-Ala⁶] GnRH;
 F, [Ac D-Trp¹ D-p-Cl-Phe² D-Trp³ D-Arg⁶ D-Ala¹⁰] GnRH.

A range of proteins (BSA, bovine IgG, FSH, LH, hCG, PRL), steroids (androgens, oestrogens, progesterone), di- and tri-peptides, chemotactic peptides and peptide hormones (angiotensins I, II and III, vasopressin, oxytocin, EGF, Substance P, neurotensin, met- and leu-enkephalins, TGF-β, TRF, insulin, bradykinin, somatostatin and leleoisin) failed to inhibit binding of GnRH_A to placental binding sites (data not shown).

levels of binding to human placental receptors (Table 5). However, preincubation of tracer with rabbit placenta induced almost complete inactivation of all forms of GnRH, and unbound tracers were incapable of rebinding to either fresh human placental or rat pituitary receptors, or to GnRH antibody (Table 5).

DISCUSSION

Binding studies of placental GnRH receptors have used radiolabelled GnRH agonists (Currie, Fraser and Sharpe, 1981; Belisle et al, 1984, 1986; Iwashita et al, 1986) and

Table 5. Tracer binding and inactivation by pituitary and placental tissue

	Hormone tracer					
	Buser	mGnRH	sGnRH	chGnRH I	chGnRH II	IGnRH
Human placenta						
% Specific binding	7.8	4.8	6.5	0.4	6.8	0.2
% Rebinding:						
Antibody	—	48	23	27	45	46
Placental Receptor	68	—	22	—	25	—
Pituitary Receptor	74	—	—	—	—	—
Rat pituitary						
% Specific binding	6.7	0.9	0.1	0	0.2	0
% Rebinding:						
Antibody	—	48	18	42	52	61
Placental Receptor	70	—	21	—	58	—
Pituitary Receptor	91	—	—	—	—	—
Rabbit pituitary						
% Specific binding	5.6	0.5	0	0.1	0	0
% Rebinding:						
Antibody	—	10	12	16	25	29
Placental Receptor	60	—	10	—	19	—
Pituitary Receptor	40	—	—	—	—	—
Rabbit placenta						
% Specific binding	0	0.2	0	0.1	0	0.1
% Rebinding:						
Antibody	—	1	8	0	12	2
Placental Receptor	0	2	9	—	8	—
Pituitary Receptor	0	2	5	2	6	3

Rat and rabbit pituitary, and human and rabbit placenta were homogenized in SET and triplicate aliquots (100 μ l) were incubated in Eppendorf tubes (2 ml) with 600 000 ct/min of 125 I-labelled GnRH_A (Buserelin) or with GnRH isoforms in Tris-BSA buffer for 1 h at 20°C, with and without excess hormone (10 μ g/tube). Tubes were centrifuged at 30 000 g for 10 min (4°C) and supernatants carefully removed. Pellets were counted for 125 I-GnRH_A or GnRH isoform bound specifically. Supernatants were adjusted to 90 000 ct/min/ml, and incubated with fresh rat pituitary (4 h at 4°C), or adjusted to 20 000 ct/min/ml and incubated with anti-GnRH antibody (1:50 000 final concentration). Tracer bound specifically was measured by PEG precipitation, and expressed as a percentage of controls incubated: (a) at 4°C and (b) at 20°C without addition of ovarian or placental tissue, and (c) with tissue, but incubated on ice for 1h. All controls agreed to within ± 5 per cent. Specific binding of GnRH_A and GnRH isoform tracers in the second incubation were expressed as a percentage of control binding.

antagonists (Mackiewicz et al, 1987; Escher et al, 1988) as binding ligands. Agonist analogues bound to placental membranes with high specificity, but with low apparent affinity (K_d , 10^{-6} to 10^{-7} M) compared to pituitary GnRH receptors (K_d , 10^{-10} M). However, human placental binding sites did not discriminate between GnRH and a range of GnRH superagonists with D-aminoacid substitutions at Gly⁶, with or without replacement of glycinamide with ethylamide at position 10 (Currie, Fraser and Sharpe, 1981; Belisle et al, 1984; Iwashita, Evans and Catt, 1986). Furthermore, the differences observed between binding of GnRH and its analogues to rat pituitary and human placental GnRH binding sites were not species-dependent, since the properties, affinities and specificities of human pituitary receptors (Clayton and Huhtaneimi, 1982; Wormald, Eidne and Millar, 1985) were similar to those of rat pituitary (Hazum, 1985; Clayton and Catt, 1981; Loumaye, Naor and Catt, 1982), but quite distinct from those of human placental GnRH receptors (Currie, Fraser and Sharpe, 1981; Iwashita, Evans and Catt, 1986; Belisle et al, 1984). Thus, the

differences observed herein must reflect the distinct hormonal specificities of pituitary and extra-pituitary GnRH receptors.

In this study, we have shown marked differences between human placental and rat pituitary receptors. Firstly, human placental membranes bound s GnRH and ch GnRH II to the same extent as GnRH_A [Figures 8(b), 9], and binding of these two isoforms (and GnRH_A) increased with purification of placental membranes (Figure 9; Table 3). In contrast, these two isoforms were not bound appreciably by rat pituitary receptors (Table 5), and they competed poorly for pituitary GnRH_A binding sites (Table 4). Differences in the abilities of ¹²⁵I-labelled GnRH isoforms to bind to rat pituitary and human placental receptors may reflect differential inactivation of tracer during the binding incubation. Thus, if l GnRH and ch GnRH I were degraded more rapidly during incubation with placental membranes than s GnRH or ch GnRH II, the former tracers would appear to be incapable of binding. Similarly, all GnRH isoforms may be inactivated rapidly by rat pituitary tissue, and therefore appear to be unable to bind, whereas agonist (being resistant to proteolytic inactivation) may bind well. Indeed, differential inactivation of GnRH_A and m GnRH has been shown to occur in rat pituitary membranes (Clayton et al, 1979) and inactivation of GnRH_A tracer was always less than inactivation of m GnRH and other GnRH isoforms in both rat pituitary and human placenta (Figure 8). However, measurements of binding and degradation for each tracer by both human placental and rat pituitary membranes in these same incubations [Figures 8(a,b)] showed clearly that, although differences in inactivation of GnRH isoforms by rat pituitary and human placental membranes did occur, differential binding of GnRH isoforms was unrelated to the degree of tracer inactivation which occurred during preincubation with membranes. Thus, levels of rebinding of ch GnRH II and l GnRH tracers to antibody after exposure to human placental membranes was similar [open bars; Figure 8(b)], even though initial binding of these tracers to placenta was markedly different [solid bars; Figure 8(b)]. Hence, differential binding of GnRH isoforms must reflect differences in placental ligand specificity rather than differences in tracer inactivation rates.

Human placental GnRH receptors bound s GnRH and ch GnRH II well compared to m GnRH, l GnRH and ch GnRH I, suggesting a preference for the Trp⁷ group. The low potency of l GnRH (which also has a Trp⁷ residue) may be due to the additional replacement of Trp³ with a Tyr residue (Table 4). Alternatively, differences in binding may be related to the overall hydrophobicity of the peptides (although differences in potency did not appear to correlate with hydrophobicity and hydropathy plots of the different forms of GnRH: data not shown). It will be of interest to compare the binding potencies of synthetic GnRH peptides with specific amino acid substitutions in these and other positions to human placental and rat pituitary membranes.

Secondly, a ¹²⁵I-labelled GnRH antagonist tracer, which had a high potency for rat pituitary receptors and which was displaceable by either cold agonist or antagonist [Figure 7(a)], bound to human placental membranes, but in a non-displaceable manner [Figure 7(b)]. Moreover, GnRH_{ANT} binding was not displaceable by either GnRH agonists or antagonists, with or without preincubation, in the presence or absence of various GTP analogues (data not shown). This observation suggests that apparent size differences reported for complexes of solubilized placental GnRH-receptors radiolabelled with either GnRH agonist and antagonist may be due to binding of antagonist tracer to non-displaceable binding sites, rather than to differences in receptor-G-protein involvement (Escher et al, 1988). Furthermore, although there are reports of the partial reversal of GnRH effects in human extrapituitary tissues by GnRH antagonists (Siler-Khodr, Kuehl and Vickery, 1984; Siler-Khodr et al, 1986d), the molar ratio of antagonist to GnRH required was high, and a different

GnRH antagonist ([D-Phe^{2,6},Pro³] GnRH) failed to affect hCG secretion by placental tissue *in vitro* (Belisle et al, 1984). Thus, GnRH antagonists, which have played such an important role in establishing whether extrapituitary GnRH effects in rat gonadal tissues were exerted through GnRH receptors, may be of only limited use in defining GnRH effects in human extrapituitary tissues.

A paracrine role for GnRH-like peptides has been proposed in the human placenta (Siler-Khodr, 1987). It is suggested that GnRH-like peptides produced by the cytotrophoblast act on receptors on the syncytiotrophoblast membrane and regulate placental hCG, steroid and prostaglandin secretion. Both GnRH (Osathanondh and Elkind-Hirsch, 1981; Tan and Rousseau, 1982; Zhuang et al, 1991) and GnRH-like peptides have been extracted from placenta (Siler-Khodr, 1987; Mathialagan and Rao, 1986a; Zhuang et al, 1991). However, although the gene for human GnRH precursor is expressed in human placenta (Seeburg and Adelman, 1984), in contrast to the hypothalamus, the first intron is not spliced out. Furthermore, there appear to be differences in promoter regions (Seeburg et al, 1987). Differences in prepro-GnRH precursor processing may give rise to prepro-GnRH-derived peptides which are extended at either the N-terminal (signal peptide) or at the C-terminal (GnRH-GAP) region. It will be of interest to examine the placental receptor binding activity of extended forms of GnRH.

In the frog central nervous system, m GnRH was localized exclusively in the hypothalamic region, whereas teleost GnRH was localized exclusively in the spinal cord (Branton, Phillips and Yan, 1986). Thus, within the same species, the form of GnRH expressed in different tissues varied. Since we have shown that human placenta binds s GnRH and ch GnRH II to much higher levels than other GnRH forms, it is possible that the genes for s GnRH and/or ch GnRH II are expressed in addition to m GnRH in the human placenta. These forms may not be detected under stringent binding conditions with probes raised to the m GnRH sequence (see Seeburg et al, 1987). Thus, the placental GnRH receptors may recognize preferentially s GnRH- or ch GnRH-like molecules.

GnRH-like peptides have been described in placental tissues of the rat (Gautron, Pattou and Kordon, 1981) and rabbit (Nowak, Wiseman and Bahr, 1984), suggesting a possible paracrine role in these species also. We were unable to demonstrate binding of any form of GnRH to rat (not shown) or rabbit placental tissues under conditions where binding was readily measured in human placental homogenates (Table 5). However, attempts to measure GnRH binding to these tissues were confounded by the very high rate of degradation of all forms of GnRH by these tissues (Table 5). Thus, the demonstration of GnRH binding sites in these tissues must await binding assays which eliminate the effects of tracer degradation.

The properties of human placental and luteal GnRH binding sites appear to be very similar to one another (Bramley, 1987; 1989), but distinct from these of the pituitary gland (Clayton and Huhtaneimi, 1982; Wormald, Eidne and Millar, 1985). Since the corpus luteum and placenta are required for the establishment and continuation of human pregnancy, a better understanding of the physiological role of GnRH-like peptides in human extrapituitary tissues may offer exciting prospects for the regulation of human fertility, from ovulation throughout a large part of early pregnancy.

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Human Placental Gonadotrophin-Releasing Hormone (GnRH) Binding Sites: III. Changes in GnRH Binding Levels with Stage of Gestation

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SUMMARY

We have measured the levels of gonadotrophin-releasing hormone (GnRH) binding sites in human placental villous membrane fractions obtained at different stages of gestation. There was a marked decrease in the specific activity of ¹²⁵I-labelled GnRH binding to membrane fractions obtained between 10-20 weeks gestation, but there was no change in either affinity or ligand specificity of these binding sites. The observed decrease in binding was not due to contamination of placental villous membranes by membranes from other tissues, since there was no gestation-dependent decrease in the specific activity of epidermal growth factor receptor or alkaline phosphatase activity in villous membrane fractions between 10-20 weeks of gestation. Furthermore, incubation of GnRH tracer with membranes from different stages of gestation, followed by re-incubation of the unbound tracer fraction with fresh membranes, demonstrated unequivocally that decreased GnRH binding to 10-20 week membranes was not due to increased degradation of GnRH tracer. We conclude that the observed changes in GnRH receptor levels between 10-20 weeks gestation must reflect either decreased expression/synthesis (or increased catabolism) of placental GnRH receptors, or increased occupancy (or down-regulation) of placental GnRH receptors by an endogenous GnRH-like ligand.

INTRODUCTION

Human placental membranes bind radiolabelled gonadotrophin-releasing hormone (GnRH) with high specificity (Currie, Fraser and Sharpe, 1981; Belisle et al, 1984, 1986;

Iwashita, Evans and Catt, 1986; Mackiewicz et al, 1987; Escher et al, 1988; Bramley, McPhie and Menzies, 1992), though the affinity of these binding sites for GnRH agonists is low (K_d , 10^{-6} to 10^{-7} M) relative to that of the pituitary GnRH receptor [K_d , 10^{-10} M (Hazum, 1985)]. Since the circulating level of hypothalamic GnRH is thought to be too low (around 10^{-11} M) to activate these 'low affinity' receptors, it has been suggested that placental GnRH receptors must respond to locally-produced GnRH. Indeed, GnRH can stimulate the release and synthesis of human chorionic gonadotrophin (hCG) and other placental steroids and prostaglandins in a dose- and gestation-dependent fashion (see Siler-Khodr, 1987 for review).

Our previous studies have highlighted a number of differences between human extra-pituitary GnRH binding sites and rat and human pituitary GnRH receptors (Bramley, 1987, 1989; Bramley, Menzies and Baird, 1986; Bramley, McPhie and Menzies, 1992). Thus, although both placental and hypothalamic tissues express the gene for the precursor for mammalian GnRH (Seeburg et al, 1987), different promoters are probably used, and differential processing of prepro-GnRH may give rise to different forms of 'GnRH' in the two tissues. Thus, differential processing of the mammalian prepro-GnRH gene in the human placenta may give rise to other (possible larger) forms of GnRH which have a preferential affinity for the placental and luteal GnRH receptor. Furthermore, there is increasing evidence in several species that different isoforms of GnRH may coexist in different tissues (Sherwood, Lovejoy and Coe, 1993).

We have therefore extended our previous studies to examine the concentrations of GnRH binding sites in placental villous membranes in human placental tissues from different stages of gestation.

MATERIALS AND METHODS

Sephadex G25 (fine) was obtained from Pharmacia Ltd, Milton Keynes, Bucks, UK and from Sigma Chemical Co. Ltd, Poole, Dorset, UK. Sephadex G25 QAE-A was from Pharmacia. All other fine chemicals and reagents were from Sigma or from BDH, Poole, Dorset, UK. Radiolabelled sodium iodide [125 I]Na, was from Amersham International plc, Bucks, UK.

Chicken GnRH I and II were purchased from Peninsula Laboratories, Belmont, CA, USA, and m GnRH from Ayerst Laboratories Ltd, Andover, Hants, UK. Salmon and lamprey GnRH were generous gifts of Dr J. King, Department of Chemical Pathology, University of Cape Town, RSA, and the GnRH agonist, Buserelin {[D-Ser(tBu)⁶] 1-9 GnRH ethylamide}, was the kind gift of Dr J. Sandow, Hoescht AG, Frankfurt, Germany. All other GnRH analogues were purchased from Sigma.

Tracer preparation and characterization

Peptides were radioiodinated using the glucose oxidase/lactoperoxidase method (Sharpe and Fraser, 1980), and were purified by chromatography on Sephadex G25 columns (1 × 40 or 1 × 60 cm) in 0.01 M acetic acid–0.1 per cent bovine serum albumin (BSA) (Bramley, McPhie and Menzies, 1992).

Specific activities of 125 I-labelled GnRH tracers

Self-displacement assay of GnRH_A tracers by binding to immature female rat pituitary homogenates gave values for specific activity of binding ranging from 350–1105 Ci/g.

Specific activities of radioiodinated GnRH isoforms were estimated by self-displacement assay (Clayton, 1983), using an anti-GnRH antibody (EL-14), generously provided by Dr W. E. Ellinwood, Oregon Health Sciences University, Portland, OR. This antibody recognizes both N- and C-terminal regions of the GnRH molecule, and therefore binds all isoforms of GnRH, but not GnRH analogues (Bramley, McPhie and Menzies, 1992). Values for specific activities of binding to antibody calculated by self-displacement assay for three separate tracer preparations of ch GnRH II ranged from 85–1100 Ci/g.

Tissue placenta

Human placentae were obtained by elective caesarean section at term from several normal women, and from several women with 10 min of spontaneous vaginal delivery, with or without induction of labour. Placental tissue was also obtained from women undergoing elective termination of pregnancy between 6 and 22 weeks gestation. Early terminations (6–9 weeks) were generally by vacuum aspiration or by treatment with Mifepristone (RU486) followed by induction of labour using Gemeprost pessaries (Norman et al, 1992). From 9–22 weeks gestation, pregnancies were generally terminated using prostaglandin alone. All terminations were performed with full informed consent of the patient, following counselling for termination of pregnancy.

Placental villous tissue was carefully recovered and dissected free of other tissues, then washed extensively in ice-cold isotonic phosphate-buffered saline (PBS: Flow Laboratories, Irvine, Scotland) to remove blood. Pieces of villous tissue were either frozen in liquid nitrogen or homogenized immediately in ice-cold 0.3 M sucrose–10 mM Tris–1 mM EDTA, pH 7.4 (SET buffer; 5 ml/g) using a Polytron homogenizer (two 10 sec bursts at full speed, separated by a 1 min cooling period in ice). After filtration through four layers of cheesecloth, homogenates were fractionated immediately. There was no difference in GnRH binding concentrations between membranes prepared immediately, or membranes isolated from tissue which had been frozen in liquid nitrogen for several months (unpublished data).

Tissue fractionation

Homogenates were centrifuged at 1000 *g* for 10 min (4°C), and the resulting pellet resuspended in SET medium. Supernatants were then centrifuged at 50 000 *g* for 60 min in a Sorvall OTD-50 refrigerated ultracentrifuge. Pellets were gently rehomogenized (loose Dounce) in SET medium (membrane fraction). Supernatants (cytosol fraction) were also kept. Fractions were stored in aliquots of 2 ml at –70°C until required.

Assays

Protein was measured by the method of Lowry et al (1951) with crystalline BSA as a standard.

Binding assays

Receptor binding. Triplicate aliquots (5–200 μ l) of placental tissue fractions (approximately 1 mg membrane protein per tube) were incubated in a 0.5 ml system containing 40 mM Tris-HCl, pH 7.4, 0.5 per cent BSA and 100 000 ct/min of ¹²⁵I-labelled GnRH analogue or GnRH isoform tracer. After incubation at 20°C for 1 h, bound hormone was recovered by polyethyleneglycol (PEG) precipitation (Bramley, Menzies and Baird, 1985), and ¹²⁵I bound to the pellet, counted in a Packard 'Crystal' gamma-counter at an efficiency of 75

per cent. Non-specific binding was measured in duplicate in the presence of 10 µg unlabelled Buserelin and was normally 3–5 per cent of total counts added for Buserelin and chicken GnRH II, and 8–12 per cent for Tryptorelin tracers. The difference between binding in the presence and absence of unlabelled GnRH_A represented specific binding (normally 5–35 per cent of total counts added). Controls without tissue, with and without unlabelled GnRH_A, were included to correct for displacement of tracer from assay tubes by cold analogue (0.5–1 per cent of total counts added).

Antibody binding. Binding of ¹²⁵I-labelled GnRH tracers (20–30 000 ct/min added) to anti-GnRH antibody (1:10 000–1:50 000 dilution) was performed in the presence or absence of an excess of the appropriate unlabelled hormone. Incubation was for 6 h at 4°C. Ice-cold bovine γ-globulin (0.5 ml; 0.5 per cent w/v) was added and bound hormone recovered by PEG precipitation (Bramley, McPhie and Menzies, 1992).

Inactivation of GnRH analogues

Quintuplicate aliquots (100 µl) of human placental tissue fractions were incubated in Eppendorf tubes at 20°C for 60–120 min (or as shown) in a 0.5 ml system containing 40 mM Tris-HCl, pH 7.4, 0.5 per cent BSA and 600 000 ct/min ¹²⁵I-labelled GnRH analogue or GnRH_A. Tubes were immediately placed in ice-water, and centrifuged at 30 000 *g* for 10 min (4°C). Supernatants were carefully aspirated, and pellets counted for ¹²⁵I. Supernatants were counted, and their volumes adjusted with incubation buffer to give 90 000 ct/min/ml. Triplicate aliquots of supernatants (1 ml) and duplicate aliquots containing the appropriate unlabelled GnRH (10 µg) were then added to fresh human placental membranes and incubated at 20°C for 1 h. After incubation, bound hormone was recovered by PEG precipitation, and ¹²⁵I measured in the pellets by gamma-counting. Three controls were always included:

- (a) tissue fraction incubated with tracer at 0°C for 1 h during the first incubation;
- (b) tracer incubated without tissue fraction during the first incubation at either 0°C or
- (c) tracer incubated without tissue fraction at 20°C for 1 h.

Values of specific binding for all three controls always agreed to within ± 7 per cent. Inactivation of tracer during the first incubation was reflected by a reduction in the specific binding of the unbound tracer (supernatant) fraction, following re-incubation with fresh receptor or antibody. Inactivation was expressed as a percentage of binding to controls.

Statistics

Statistical significance of differences between means was estimated by Student's *t*-test.

RESULTS

Comparison of the specific binding of ¹²⁵I-labelled ch GnRH II and the GnRH agonists, Tryptorelin and Buserelin indicated that all three tracers were bound to placental membranes equivalently (Figure 1) with several different tracer preparations to a number of placental membrane preparations over a wide range of specific binding activities. Indeed, the binding of Buserelin tracer to term placental membranes could be displaced by similar levels of unlabelled ch GnRH II, Buserelin or Tryptorelin [300–1000 nM analogue; Figure 2(a)], and the levels of the unlabelled Buserelin required to inhibit binding of ¹²⁵I-labelled Buserelin to placental membranes from early, mid-gestation and term placentae were also

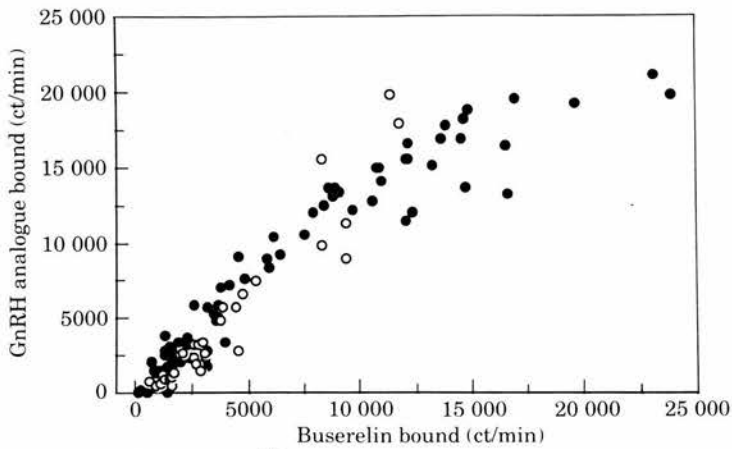


Figure 1. Comparison of specific binding of ^{125}I -labelled Buserelin with binding of radiolabelled Tryptorelin (●) and ch GnRH II (○) tracers to human placental membrane fractions. Specific binding of several tracer preparations for each different GnRH agonist tracer was measured to human placental membrane fractions prepared from a number of placentae at different stages of gestation, as described in Materials and Methods section.

very similar [Figure 2(b)]. Furthermore, inhibition of the binding of ^{125}I -labelled ch GnRH II (Bramley, McPhie and Menzies, 1992) and Tryptorelin tracers (data not shown) occurred with similar levels of unlabelled ch GnRH II, Tryptorelin and Buserelin, suggesting that all three tracers bound to the same placental binding site.

As shown in Figure 2(b), ^{125}I -labelled GnRH agonist binding varied markedly from different membrane fractions. It is possible that low GnRH binding was associated with villous membranes from pathological placental tissues, or to a gestation-dependent change in GnRH binding. We therefore measured radiolabelled Buserelin binding to villous membranes prepared from 105 normal or pathological placentae (Table 1). Whilst there was wide variation in GnRH binding by villous membranes from different stages of gestation, changes in GnRH binding were observed which appeared to be related to the stage of gestation at which the placenta was recovered (Figure 3). A number of possible causes were examined for the marked reduction in GnRH binding to placental membranes observed at 10–20 weeks gestation.

Changes in receptor affinity or specificity

Differences in binding were not related to the use of Buserelin as a tracer, since the binding of radiolabelled Buserelin and Tryptorelin was similar for villous membranes from 7 week, 16 week and term placentae, despite marked differences in membrane binding activity (Figure 4). Furthermore, radiolabelled tracers prepared from GnRH isoforms which bind to human term placental membranes (ch GnRH II, s GnRH, m GnRH) and GnRH isoforms which do not (ch GnRH I, l GnRH; Bramley, McPhie and Menzies, 1992) showed no change in GnRH analogue binding specificity to placental membrane fractions from different stages of gestation (Figure 5). This indicated that there had been no change in GnRH analogue specificity, despite marked changes in specific binding activity at different stages of pregnancy [note the scale changes between Figures 5(a), (b) and (c)]. This was further confirmed using several different GnRH tracers and placental membrane preparations from different stages throughout pregnancy (Figure 6). At all stages of ges-

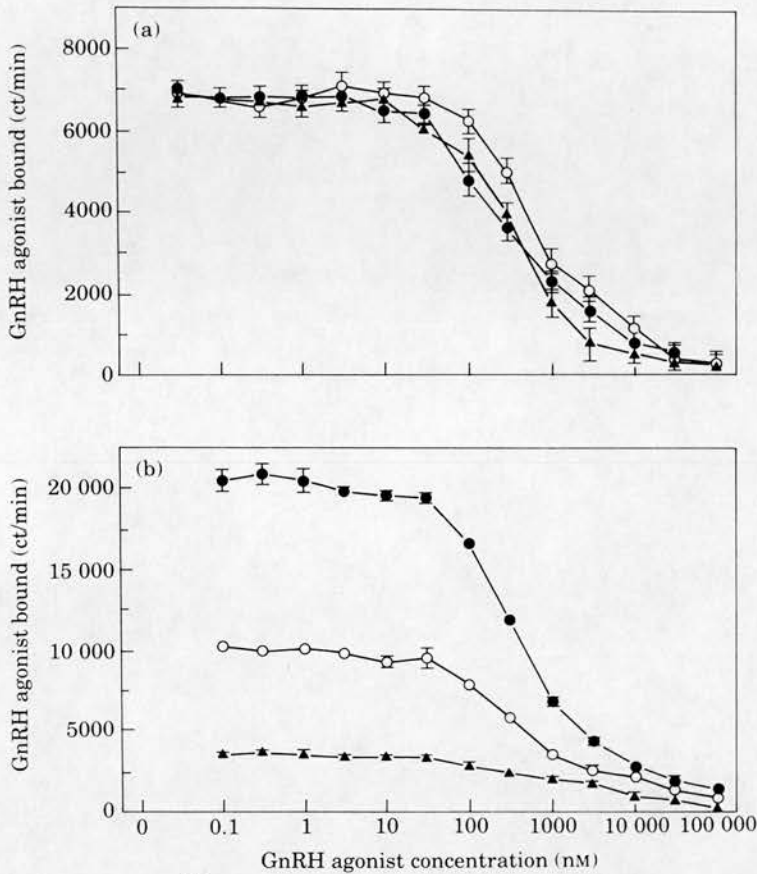


Figure 2. Competition curves for ^{125}I -labelled Buserelin tracer binding to human placental membranes: (a) Term membranes in the absence, or in the presence of increasing concentrations, of unlabelled Buserelin (○), Tryptorelin (●) or ch GnRH II (▲); (b) Term (●), 7 weeks gestation (○) or 16 weeks gestation (▲) membranes in the absence or presence of increasing concentrations of unlabelled Buserelin. Points are means \pm s.d. for one representative experiment in triplicate.

tation, GnRH agonist, ch GnRH II and s GnRH bound similarly, whilst m GnRH bound less well, and ch GnRH I and l GnRH bound poorly.

Villous membrane contamination

The decrease in GnRH binding to placental membranes recovered at 10–20 weeks gestation could be due to contamination of villous tissue by membranes of other tissues, leading to an artefactual reduction of specific binding per mg (membrane protein). This is due to suction termination leading to the extensive fragmentation of fetal and placental tissues. However, membranes prepared from villous tissue from early pregnancies terminated by RU486/Gemeprost treatment, by suction termination or by PG treatment alone gave similar levels of GnRH binding at 6–16 weeks (Figure 3). This also indicates that the decline in GnRH binding activity was not due to the use of prostaglandin to induce abortion, though this requires confirmation by others, as the number of suction terminations between 10 and 20 weeks was low.

Moreover, if contamination by non-villous tissue was responsible for low GnRH binding

Table 1. Levels of placental villous plasma-membrane markers in membrane fractions isolated at different stages of gestation

Stage of gestation (weeks)	GnRH _A Binding ^a	mEGF Binding ^b
6-8	5.1 ± 0.5 (27)	14.9 ± 5.9 (6)
10-20	1.0 ± 0.2 (23)**	22.4 ± 2.1 (7)
21-38 ^c	7.7 ± 0.9 (12)**	n.d.
Term	9.2 ± 0.7 (43)	48.4 ± 9.7 (7)*

^a Human placental villous membrane fractions were assayed for specific binding of GnRH_A (Buserelin; % tracer bound/μg protein) or ^b mEGF (ct/min/μg protein).

^c Abnormal placentae. All placental tissue was from women with pregnancy complicated by either severe hypertension or intrauterine growth retardation.

Figures are means ± SEM for (n) measurements at each stage.

* $P < 0.05$; ** $P < 0.01$ when compared to group at previous stage of gestation.

n.d., not determined.

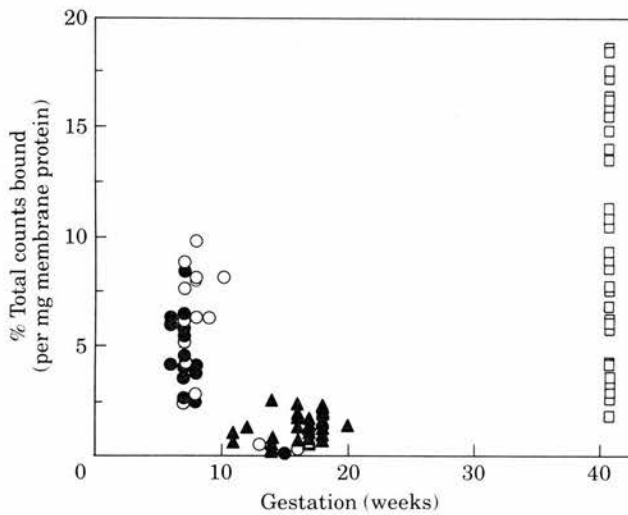


Figure 3. Specific binding of radiolabelled Buserelin to human placental villous membranes from different stages of gestation. ¹²⁵I-labelled Buserelin binding to microsomal fractions from human placentae was measured as described in materials and methods, and specific binding adjusted for membrane protein content. Stage of gestation was estimated from LMP and CRL measurements by ultrasound. (○), Suction termination of normal pregnancy; (●), RU486/Gemeprost induced termination; (▲), prostaglandin only; (□), normal spontaneous vaginal delivery.

at 10-20 weeks, other markers of villous surface membranes should also be reduced by a similar amount. In contrast to the marked fall in GnRH binding observed at 10-20 weeks gestation, there was no marked reduction in the levels of epidermal growth factor (EGF) receptor (a marker of placental villous membranes; Menzies and Bramley, 1992) in membrane fractions prepared from placentae at 10-20 weeks of gestation (Table 1). Similar results were obtained for alkaline phosphatase activity (data not shown). Hence, the reduction of GnRH binding levels at 10-20 weeks could not be accounted for in terms of contamination of villous membranes of other tissues at this stage of gestation.

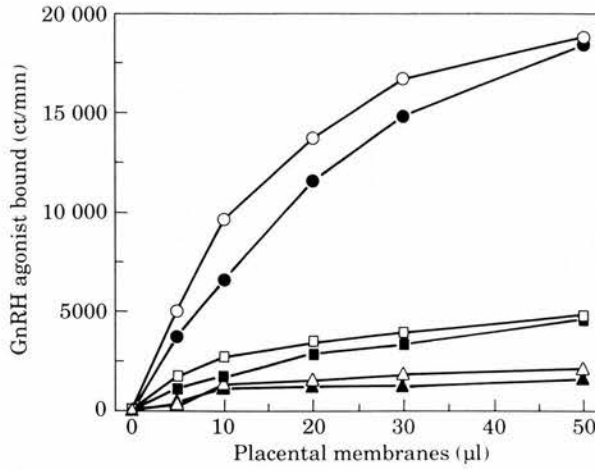


Figure 4. Comparison of specific binding of Buserelin (●■▲) and Tryptorelin (○□△) to membranes from term (●○), 7 weeks gestation (■□) or 16 weeks gestation (▲△) human placentae. Specific binding was measured as described in Materials and Methods section. Points are means of triplicate determinations.

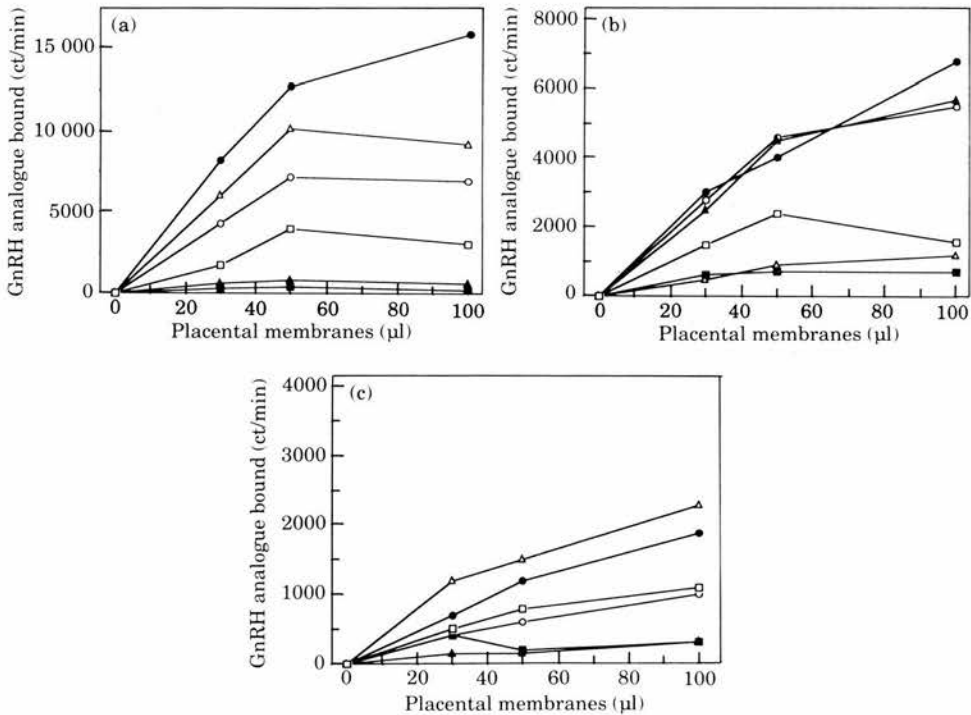


Figure 5. Specificity of human placental GnRH binding sites from different stages of gestation. Specific binding of a range of radiolabelled GnRH analogues was measured to aliquots of placental villous membrane fractions prepared from: (a) term; (b) 7 weeks gestation; (c) 16 weeks gestation, as described in Materials and Methods section. (●), Buserelin; (○), Salmon GnRH; (▲), l GnRH; (△), ch GnRH II; (■), ch GnRH I; (□), m GnRH. Note the marked scale changes between term, 7 and 16 week placental membranes.

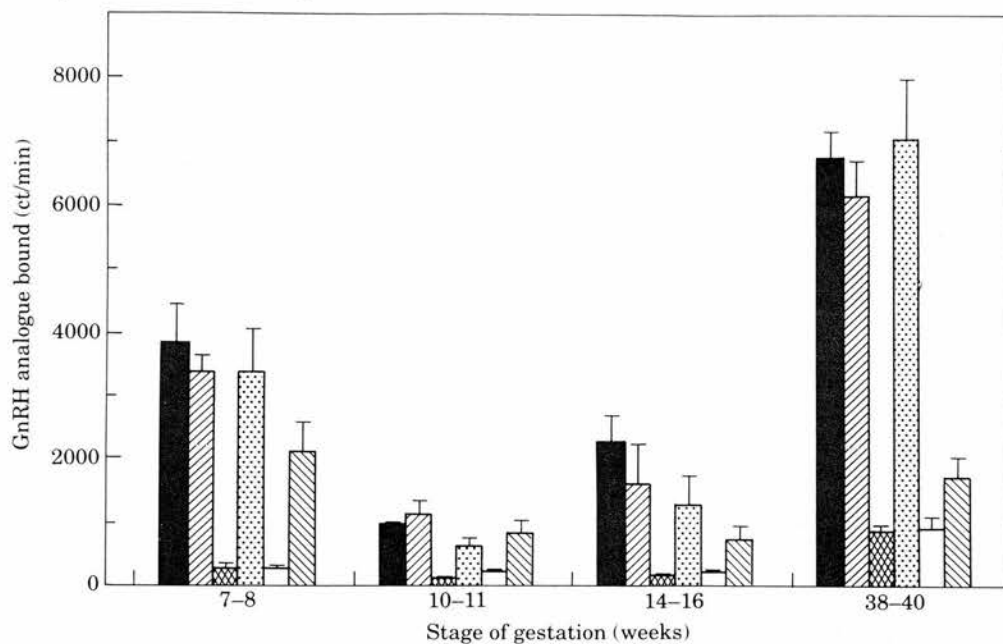


Figure 6. Specificity of human placental GnRH binding sites from different stages of gestation. Specific binding of a range of radiolabelled GnRH analogues was measured to aliquots of placental villous membrane fractions prepared from: 7-8 weeks; 10-11 weeks; 14-16 weeks; 38-40 weeks gestation, as described in Materials and Methods section. (■), Buserelin; (▨), Salmon GnRH; (▤), l GnRH; (▧), ch GnRH II; (□), ch GnRH I; (▩), m GnRH.

Degradation of GnRH tracers

Placental levels of GnRH-degrading enzymes may be greatly increased between 10-20 weeks of gestation, leading to increased degradation of ^{125}I -labelled GnRH tracers during the binding assay incubation at these stages of gestation. We therefore incubated radiolabelled Buserelin tracer with placental membranes prepared from villous tissue obtained at different stages of gestation, and separated the bound and unbound tracer fractions by high-speed centrifugation. The unbound tracer fraction was then re-incubated under standard conditions with either; (a) placental membranes from the same stage of pregnancy, or (b) with a term placental membrane fraction as a receptor source, and specific GnRH agonist binding measured. Once again, GnRH agonist binding to placental villous membranes during the first incubation was low in microsomes prepared from between 10 and 20 weeks gestation [Figure 7(a) and (b); though two preparations of 16 week placenta showed quite high binding levels]. On re-incubation of the unbound tracer fraction with the same membranes, similar levels of GnRH binding were observed in the second incubation (■). However, re-incubation of unbound tracer with term membranes gave similar levels of binding whatever the stage of gestation of the placental membrane fraction used in the first incubation [Figure 7(a) and (b)].

Furthermore, measurement of ^{125}I -labelled GnRH binding to 16 week and term placental membranes in the presence or absence of inhibitors of serine-, metallo-, thiol- or carboxypeptidases had little or no effect on GnRH binding to term membranes, and failed to 'rescue' low binding to 16 week membranes (Table 2). Thus, low levels of GnRH binding at 10-20 weeks gestation cannot be accounted for by increased degradation of GnRH tracer during the assay. Binding was abolished by phenylmethylsulphonyl fluoride:

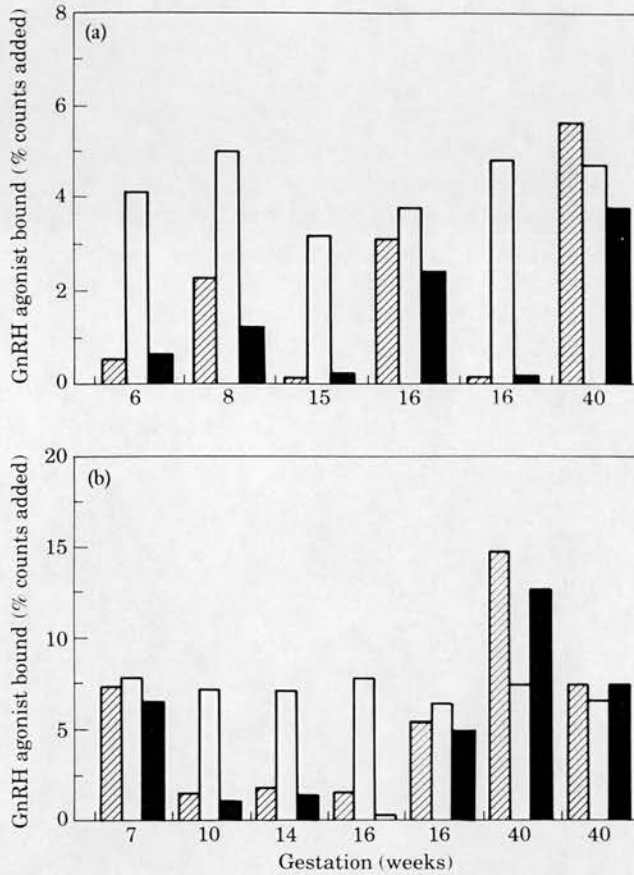


Figure 7. Re-binding of radiolabelled Buserelin tracer following pre-incubation with placental membranes from different stages of gestation, as shown. (▨), binding to membranes during first incubation; (□), re-binding of unbound tracer fraction from first incubation to a preparation of term placental membranes; (■), re-binding of unbound tracer from first incubation to same membranes as first incubation.

this was shown to be due to inhibition of placental GnRH binding by the ethanol solvent used (Table 2). However, the serine protease inhibitor, soybean trypsin inhibitor, did not inhibit GnRH binding.

DISCUSSION

In previous studies, we (Bramley, McPhie and Menzies, 1992) and others (Currie, Fraser and Sharpe, 1981; Belisle et al, 1984; Iwashita, Evans and Catt, 1986; Mackiewicz et al, 1987; Escher et al, 1988) have demonstrated highly specific, moderate-affinity GnRH binding sites in human placental membrane fractions. The properties of these binding sites differ from those of pituitary GnRH receptors in several important respects:

- (a) Placental GnRH binding sites do not discriminate between GnRH and agonist analogues with super-activity in the pituitary gland (Currie, Fraser and Sharpe, 1981;

Table 2. Lack of effect of protease inhibitors on GnRH binding to 16 week and term human placental membrane preparations

Inhibitor	¹²⁵ I-labelled GnRH binding (ct/min/50 µl membranes)			
	16 Week gestation		Term	
	Bo	NSB	Bo	NSB
Control	8118	6804	24074	8882
EDTA ^a	7206	6976	23710	8910
N-EM ^a	7868	7668	19553	7438
Pepstatin A ^b	8613	7668	23848	8766
SBTI ^b	8221	6786	25016	8902
PMSF ^a	7782	7271	8333	7629
Ethanol	7868	7668	8561	8385

Aliquots (50 µl) of placental membranes from either a 16 week (RU486 termination) or a spontaneous vaginal delivery term placenta were incubated as described in the Materials and Methods section in the presence of ¹²⁵I-labelled Buserelin under standard incubation conditions (20°C for 1 h) in the absence or presence of different protease inhibitors. Phenylmethylsulphonyl fluoride (PMSF) was dissolved in ethanol, and 10 µl added to the 1 ml assay system. Values are means of triplicate determinations; similar values were obtained in two (16 week) and three (term) separate experiments. Bo, binding in the absence of unlabelled GnRH agonist (10 µg); NSB, binding in the presence of excess unlabelled GnRH agonist; EDTA, ethylenediamine-N,N'-tetraacetic acid; N-EM, N-ethylmaleimide; SBTI, soybean trypsin inhibitor.

^a Final concentration, 10 mM; ^b Final concentration, 1 µg/ml.

Iwashita, Evans and Catt, 1986; Bramley, 1987). Hence, rat pituitary and human placental binding sites have similar potencies for GnRH, but different potencies for GnRH agonists (Bramley, 1989);

(b) Pituitary GnRH receptors do not bind non-mammalian isoforms of GnRH, whereas human placental GnRH binding sites bind ch GnRH II, s GnRH (but not ch GnRH I and 1 GnRH; Bramley, McPhie and Menzies, 1992);

(c) A number of GnRH antagonist analogues with high affinity for pituitary GnRH receptors bind poorly (Bramley, McPhie and Menzies, 1992) to human placental binding sites [though Barnea et al (1991) reported abolition of GnRH- and GnRH agonist-dependent release of hCG by superfused placental explants using a GnRH antagonist analogue].

This report now documents changes in the levels of these GnRH binding sites in membrane fractions isolated from placentae from different stages of gestation. Specific GnRH binding activity was significantly reduced in membranes from placentae recovered between 10–20 weeks gestation, compared to 6–9 weeks and term placental membranes (figure 3). These differences could not be accounted for by:

(a) The use of radiolabelled Buserelin as a GnRH agonist tracer (since Buserelin, Tryptorelin and ch GnRH II tracers bound equivalently to human placental membranes; Figures 1 and 4);

(b) Changes in GnRH binding site affinity with gestational age [since binding of three GnRH analogue tracers was competed by similar concentrations of unlabelled analogue (300–1000 nM; Figures 2 and 3)]. Furthermore, the binding specificity for GnRH agon-

ists and GnRH isoforms did not change throughout gestation, despite marked differences in specific binding activity (Figure 6);

(c) Increased degradation of GnRH by membrane fractions from 10–20 week placentae (since re-binding experiments demonstrated that unbound GnRH tracer exposed to membranes from placentae of differing gestational age bound equally well to term placental membranes; Figure 7). Furthermore, GnRH binding levels to 16 week and term placental membranes were unaltered when assays were conducted in the presence of broad-spectrum inhibitors of serine-, metallo-, thiol- and carboxypeptidase proteases (Table 2). Note that inclusion of peptidase inhibitors did not increase GnRH binding to 16 week placental membranes, suggesting that protease degradation of tracer and/or receptor was not responsible for low binding at this stage of pregnancy. However, we cannot rule out that the use of EDTA in the homogenization buffer may have inactivated some placental metallopeptidases during membrane preparation;

(d) Contamination of placental villous membranes by membranes from other tissues, since the levels of EGF receptor (and alkaline phosphatase, data not shown) did not show a similar marked decrease at this stage of gestation (Table 1);

(e) The use of prostaglandin or RU486 to induce termination of pregnancy, since no significant differences were observed in GnRH binding activity for placental membranes prepared from suction terminations or from RU486 or prostaglandin terminated pregnancies between 6–16 weeks in this study (Figure 3; though numbers were low in some groups at some stages of gestation). Furthermore, similar GnRH binding levels were observed in term placental membranes obtained after spontaneous vaginal delivery (SVD) (with or without induction of labour, with or without oxytocin treatment) and after elective caesarean section (data not shown), suggesting that labour *per se* has little effect on GnRH binding.

Decreased GnRH binding levels at 10–20 weeks gestation may reflect either suppression of GnRH receptor expression or synthesis, increased receptor catabolism and/or down-regulation, or increased occupancy of GnRH binding sites by an endogenous GnRH-like ligand. The former explanation must await the isolation, sequencing, cloning and analysis of the human placental GnRH-binding site gene and the raising of specific immunochemical reagents for detection of the placental GnRH-binding site. Mixing experiments involving pre-incubation of membranes from 10–20 week and term placenta suggest that there was no marked increase in receptor catabolism at this stage of gestation (Bramley and Menzies, unpublished data). However, human placental cytosol fractions (assayed under conditions which would be expected to eliminate placental protease activities) can inhibit GnRH binding to placental membranes in a time-, temperature- and dose-dependent manner (Bramley and Menzies; submitted). Experiments are now in progress to isolate this factor.

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