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Corticosteroid-binding Globulin In Fetal And Adult Sheep

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CORTICOSTEROID-BINDING GLOBULIN IN FETAL AND ADULT SHEEP

by

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ABSTRACT

In the ovine fetus, plasma cortisol is bound by its high affinity binding protein corticosteroid-binding globulin (CBG). Plasma CBG concentrations increase during late gestation, but the stimulus for this increase and its consequences in the fetal sheep are not known. These studies were undertaken to examine the stimulus for an increase in fetal plasma CBG, the mechanism of this increase, and the physiological significance of the increase.

Chronically catheterized fetal and adult sheep were used to examine the changes in late gestation of plasma CBG concentrations, plasma CBG glycosylation, total and free cortisol, and effects of glucocorticoids on plasma CBG. An ovine CBG cDNA was cloned and sequenced, allowing the utilization of Southern and Northern blot analysis to examine tissue distribution of sites of CBG biosynthesis and effects of glucocorticoid administration on hepatic CBG mRNA abundance. Maternal CBG was purified and the possibility of transplacental transfer of CBG from the ewe to the fetus was investigated. The ability of CBG to modulate glucocorticoid negative feedback was examined in fetal pituitary cells in culture.

Molecular analysis of the CBG cDNA demonstrated a deduced length of the mature peptide of 408 amino acids, with five consensus sites for *N*-glycosylation. Hepatic CBG mRNA abundance increased to reach highest concentrations at day 140 of gestation. The liver is the major site of CBG production. In the fetus, extra-hepatic sites of CBG biosynthesis include the pituitary, adrenal, kidney and

lung. There is a change in CBG glycosylation in neonatal sheep from a fetal type to an adult-like type, but no transplacental transfer of CBG in late gestation. In fetal sheep, CBG biosynthesis is stimulated by glucocorticoids while in the adult, glucocorticoid administration produced a decrease in CBG biosynthesis. CBG was able to attenuate negative feedback of glucocorticoids on both basal and CRH-stimulated ACTH output in fetal pituitary cell in culture.

Conclusions from these studies include i) CBG biosynthesis is a function of glucocorticoid concentrations, and ii) in the fetus, CBG attenuates glucocorticoid negative feedback at the pituitary, allowing a progressive increase in fetal plasma ACTH, and a sustained drive toward parturition.

For my parents

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ABBREVIATIONS USED

AAG	α1-acid glycoprotein
ACTH	Adrenocorticotrophin
bp	base pair(s)
cAMP	Adenosine 3',5'-cyclic monophosphate
CBC	Corticosteroid binding capacity
CBG	Corticosteroid-binding globulin
cDNA	complementary Deoxyribonucleic acid
CHO	Chinese hamster ovary
CPM	Counts per minute
CRH	Corticotrophin releasing hormone
CTP	Cytidine 5'-triphosphate
DMEM	Dulbecco's modified eagles medium
DPBS	Dulbecco's phosphate buffered saline
DPM	Disintegrations per minute
EMG	Electromyographic
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
GR	Glucocorticoid receptor
HPA	Hypothalamo-pituitary-adrenal
kb	kilobase
kDa	kilodalton
mRNA	messenger Ribonucleic acid
oCRH	ovine Corticotrophin releasing hormone
PAGE	Polyacrylamide gel electrophoresis
PMSG	Pregnant mare serum gonadotrophin
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHBG	Sex-hormone binding globulin
SON	Supraoptic nucleus
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

In clinical obstetrical practice preterm labour is one of the most common problems (Creasy, 1994). However, the etiology of many preterm deliveries is unknown. Therefore, an understanding of the mechanisms leading to parturition is necessary to provide possible preventative measures to minimize fetal morbidity and mortality. In species such as the sheep, the process of birth results from the sequential maturation of the hypothalamo-pituitary-adrenal (HPA) axis. The key intermediate in this pathway is cortisol and the concentration of this hormone in fetal plasma increases during late gestation (term = approximately 145 days). This increase in fetal plasma cortisol is critical for parturition (Liggins, Fairclough, Grieves, Kendall and Knox, 1973).

Fetal plasma cortisol and the increase in its concentration in late gestation has a number of very important functions, which include preparation of the fetus for extrauterine existence (Liggins, 1976). Cortisol has been associated with maturation of the lung (Liggins, 1976: Challis, Kendall, Robinson, and Thorburn, 1977: Liggins, 1976), the switch from a fetal to an adult type of haemoglobin (Wood, Pearce, Clegg, Weatherall, Robinson, Thorburn and Dawes, 1972: Wintour, Smith, Bell, McDougall and Cauchi, 1985), and maturational changes in the gut, pancreas and brain (Liggins, 1976: Challis *et al.*, 1977). Glucocorticoids are known to induce enzyme activity, such as placental 17 alpha-hydroxylase and C_{17-20} lyase, which promote a switch from a progesterone to an estrogen dominated uterine environment (Flint, Anderson, Steele and Turnbull, 1975).

Therefore, in the sheep, fetal cortisol acts to prepare the fetus for birth, and to trigger parturition, thus ensuring fetal preparedness for both.

In the plasma of the fetal sheep, cortisol is present in several forms. Plasma cortisol may either be bound to plasma proteins or 'free' (unbound). It has generally been thought that only the 'free' fraction of cortisol is biologically active, and thus the binding of cortisol by plasma proteins would affect its bioactivity. Therefore, the plasma binding proteins of cortisol would be important in modulating the biological activity of cortisol. In plasma, cortisol may be bound to albumin or it may be bound to its high affinity binding protein corticosteroid-binding globulin (CBG).

In the introductory chapter of this thesis, I will address the hypothalamo-pituitary-adrenal (HPA) axis, possible sites of negative feedback of glucocorticoids within the HPA axis, and discuss the high affinity binding protein of cortisol, CBG.

1.2 The hypothalamo-pituitary-adrenal axis

The idea of involvement of the fetus and association with the duration of pregnancy, has been around since ancient times. Hippocrates thought that the fetus was responsible for parturition by pushing out of the uterus when its food stores were depleted. The association of aberrations of gestational length in anencephalic fetuses was first reported by Rea (1898); and Malpas (1933) deemed the fetal contribution to the initiation of parturition lacking in anencephalic fetuses. The association of prolonged gestation with abnormalities in the central

nervous system was also found in other species. In Guernsey cows, prolonged gestation of up to 200 days beyond term was associated with fetuses afflicted by genetically determined malformations of the brain (Kennedy, Kendrick and Stormont, 1957). The fetuses afflicted with this birth defect were immature, with cranial and central nervous system defects, including an aplastic adenohypophysis (Kennedy *et al.*, 1957). In the sheep, ingestion of the teratogen *Veratrum Californium* on the fourteenth day of gestation, produces an increase of gestational length of many weeks (Binns, James and Shupe, 1964). Ultimately, the pelvic ligaments of the ewe rupture and maternal death follows. Upon delivery of the fetuses by Cesarean section, the fetuses have cyclopian deformities and attain giant proportions, and common to these fetuses is total absence or anomaly of the pituitary gland (Binns *et al.*, 1964).

These anecdotal studies suggesting a fetal component in the initiation of parturition were the basis for the pioneering studies examining the HPA axis in the sheep. In the late 1960's, Liggins and co-workers (Liggins, Kennedy and Holm, 1967) provided direct evidence that the fetal HPA axis was integral in the preparation for birth, and in the timing of parturition. These studies demonstrated that fetal hypophysectomy, in which more than 70% of the volume of the gland was destroyed as a result of electrocoagulation of the fetal pituitary, resulted in prolonged gestation. All hypophysectomized fetuses were delivered by cesarean section, from 10 to 34 days beyond expected term. At the time of delivery, the ewe did not display any sign of impending parturition such as udder enlargement

(Liggins *et al.*, 1967). In further studies, Liggins and Kennedy (1968) showed that electrocoagulation of the fetal sheep hypophysis produced a marked decrease in the fetal adrenal weight compared to normal controls, and that the earlier in gestation the fetuses were hypophysectomized, the greater the difference in adrenal weights. However, administration of adrenocorticotrophin (ACTH: 0.1 mg or 0.25 mg/24 hour) to chronically catheterized singleton fetal sheep of gestational age 88-129 days led to premature delivery of the fetus within seven days (Liggins, 1968). The adrenals of the animals that received ACTH treatment were at least as large as those of normal fetuses at term. In addition, except for the most premature fetuses (gestational age approximately day 110), they were viable, indicating the lung maturing properties needed for extrauterine existence that were associated with activation of the fetal HPA axis. The administration of cortisol (50 mg/24 h) to singleton fetuses produced premature parturition within 70 hours (Liggins, 1968).

The timing of parturition was not altered with maternal hypophysectomy (Denamur and Martinet, 1961), nor was the timing of birth modified by maternal administration of ACTH (0.1 mg/24 hours) or cortisol (50 mg/24 hours for 120 hours then 100 mg/24 hours for 72 hours; Liggins, 1969). Therefore, a functioning fetal HPA axis is imperative for birth. However, the contributions of the placenta and maternal factors in the initiation of labour and timing of parturition are also important.

The concentration of ACTH in the plasma of the ovine fetus increases over

the last third of gestation (Rees, Jack, Thomas and Nathanielsz, 1975; Jones, Boddy and Robinson, 1977). In samples of plasma collected from chronically catheterized fetuses over periods of five days from day 110 of gestation to term (term = 145 days), Norman, Lye, Wlodek and Challis (1985) demonstrated an increase in the concentration of fetal plasma ACTH from 16.7 ± 2.9 pg/ml at day 110-115 to 34.8 ± 8.7 pg/ml at day 141-145. Using regression analysis of the mean concentrations at each gestational age Norman *et al.* (1985) showed a mean rise of approximately 5 pg/ml every five day sampling interval from day 110 to term. The pulsatility and amplitude of pulses of ACTH secretion have been measured in fetal plasma during late gestation (Brooks and Challis, 1991; Apostolakis, Longo, Veldhuis and Yellon, 1992). Brooks and Challis (1991) observed no change in the pulsatility of ACTH from 3-4 days before parturition to 1-2 days before parturition, nor was there any change in the amplitude of these pulses over the times studied. Apostolakis *et al.* (1992) also demonstrated no change in ACTH pulse frequency or pulse amplitude from day 133 to day 146.

In the sheep, there have been many studies describing the prepartum increase in fetal plasma cortisol over the last third of gestation (Bassett and Thorburn, 1969: Brown, Coghlan, Hardy and Wintour, 1978). A detailed study over the last twenty days of gestation (Magyar, Fridshal, Elsner, Glatz, Eliot, Klein, Lowe, Buster and Nathanielsz, 1980) demonstrated that there was a daily increase in fetal plasma cortisol concentrations of 0.5 ng/ml per day at 11.8 ± 1.0 days before parturition. The rate of the increase in fetal cortisol concentrations

was 1 ng/ml per day at 9.3 ± 0.9 days preceding parturition and further increased to 2 ng/ml per day at 8.2 ± 1.1 days before parturition. Final fetal plasma cortisol concentrations from the study of Magyar *et al.* (1980) just before parturition were approximately 100 ng/ml. The pulsatility of fetal cortisol has also been examined in relation to increasing gestational age. In fetuses at 3-4 days and 1-2 days before parturition, there was a decrease in frequency of plasma cortisol pulses (9.39 ± 0.67 vs 6.59 ± 0.81 pulses/6 h) but an increase in the amplitude of cortisol pulses (33.17 ± 6.15 vs 57.17 ± 9.36 ng/ml: Brooks and Challis, 1991). In contrast, in studies of fetuses at days 133, 137, 142 and 146 of gestation, Apostolakis *et al.* (1992) demonstrated a progressive increase in pulse frequency over the four gestational ages (2.2 pulses/2 h at day 133 to 4.8 pulses/2 h at day 146). However, in agreement with Brooks and Challis (1991), Apostolakis *et al.* (1992) showed an increase in fetal plasma total cortisol pulse amplitude from 10 ± 2 ng/ml at day 133 to 44 ± 13 ng/ml at 146 days.

1.2.1 Hypothalamus

The hypothalamic nuclei of primary importance in control of fetal HPA function are the paraventricular nucleus (PVN) and the supraoptic nucleus (SON), which are positioned within the anterior portion of the hypothalamus. The PVN and SON comprise the majority of the hypothalamic parvocellular neurosecretory and magnocellular neurosecretory systems (Everitt and Hokfelt, 1986). The magnocellular neurons of the PVN project to the posterior pituitary, and regulate

systemic arginine vasopressin (AVP) and oxytocin. The parvocellular neurons of the PVN project to the median eminence, for modulation of anterior pituitary function, and to autonomic centres in the caudal brain and spinal cord (Swanson and Sawchenko, 1983).

The existence of a hypothalamic substance capable of eliciting ACTH release from rat pituitaries was first demonstrated in 1955 (Saffran and Schally; Guilleman and Rosenburg). The 41 amino acid polypeptide structure of corticotrophin releasing hormone (CRH) in the sheep was first described by Vale, Speiss, Rivier, and Rivier in 1981. Since then, the amino acid structures of human, rat, pig and goat CRH have been determined. Subsequently the ovine (Roche, Crawford, Fernley, Tregear and Coghlan, 1988) and rat (Jingami, Matsukura, Numa and Imura, 1985) nucleotide sequences have been established. The area of greatest numbers of CRH mRNA expressing cells is in the parvocellular divisions of the paraventricular nuclei (Kovacs and Mezey, 1987). The existence of a hypothalamic-pituitary portal vasculature is present by day 45 of gestation in the fetal sheep, thus allowing hypothalamic factors to reach the anterior pituitary (Levidiotis, Wintour, McKinley and Oldfield, 1989). In fetuses in which the PVN has been lesioned, there is prolonged pregnancy, suggesting the signal role of CRH in the correct timing of parturition (McDonald and Nathanielsz, 1991; Gluckman, Mallard and Boshier, 1991).

1.2.1.1 Control of CRH secretion

There are many higher centres within the brain that project to the parvocellular neurons within the PVN. In the rat, there are projections from the nucleus of the solitary tract to the parvocellular PVN (Sawchenko, 1987) as well as direct projections from the central nucleus of the amygdala to the caudal, lateral, and medial PVN (Gray, Carney, and Magnuson, 1989). The parvocellular region of the PVN also receives afferent input from the arcuate nucleus, as well as the anterior, medial, posterior and lateral hypothalamus (Mezey and Palkovits, 1991).

In a comprehensive study of CRH localization in the fetal sheep hypothalamus, immunoreactive-CRH (irCRH) was found in the PVN and median eminence from 49 days of gestation (Watabe, Levidiotis, Oldfield and Wintour, 1991). The hypothalamic content and concentration of CRH in the fetal sheep were shown to increase significantly between 48 and 140 days of gestation. In the human, irCRH in the fetal hypothalamus is detectable as early as 12-13 weeks. However the concentration of irCRH in the fetal hypothalamus does not change with gestational age (Ackland, Ratter, Bourne and Rees, 1986).

Using *in vitro* perifusion it has been shown that in the ovine fetal hypothalamus, CRH release under basal conditions was significantly higher at day 140 than at day 100 of gestation (Brooks, Power, Jones, Yang and Challis, 1989). These studies demonstrated that in the sheep fetus, CRH is released at both day 100 and day 140, and that dexamethasone inhibited the basal release

of CRH at day 140 but not day 100. Furthermore, CRH release was stimulated when the nerve terminals were depolarized by potassium, even in the presence of dexamethasone, suggesting that the effect of dexamethasone on basal CRH release is not at the nerve terminals (Brooks *et al.*, 1989). In the adult rat, release of CRH from perifused hypothalami was decreased by dexamethasone whereas it was increased by potassium and from hypothalami of adrenalectomized rats (Suda, Yajima, Tomori, Sumitomo, Nakagami, Ushiyama, Demura and Shizume, 1986). Recently, the ontogeny of hypothalamic CRH mRNA abundance in fetal sheep has been investigated. The message of pre-pro CRH mRNA by *in situ* hybridization was detected in the fetal sheep hypothalamus from day 100, increased to day 125 and decreased in abundance towards term (Myers, Ding and Nathanielsz, 1991). In contrast, Matthews and co-workers (Matthews, Han, Lü and Challis, 1994), also using *in situ* hybridization, have shown a progressive increase in the abundance of CRH mRNA in the fetal sheep hypothalamus with greatest amounts just before term. CRH mRNA increases in the PVN after fetal adrenalectomy, and decreases with fetal glucocorticoid treatment (Myers *et al.*, 1991).

1.2.2 Pituitary

The pituitary gland consists of anterior, intermediate and posterior lobes (pars distalis, pars intermedia and pars nervosa, respectively). The anterior lobe contains at least five different types of secretory cells (Ganong, 1989), including

corticotrophs that secrete ACTH. Pro-opiomelanocortin (POMC) is the precursor protein of ACTH and also several other structurally related peptides such as pro-gamma-melanocyte-stimulating hormone, beta-lipotropin hormone and beta-endorphin (Mains and Eipper, 1979: Mulvogue, McMillen, Robinson and Perry, 1986: Figure 1.1, page 40).

ACTH has been detected in fetal plasma as early as day 59 of gestation (Alexander, Britton, Forsling, Nixon and Ratcliffe, 1973). By immunohistochemistry irACTH-specific staining is observed within the fetal anterior pituitary as early as day 40 of gestation (Perry, Mulvogue, McMillen and Robinson, 1985). By day 90 of gestation, there are three distinct cell types within the fetal pituitary. The most numerous corticotrophs are large, columnar and variably stained, often occuring in clusters, and are called 'fetal corticotrophs' (Perry *et al.*, 1985). The less numerous 'adult corticotrophs' are stellate, darkly-staining cells. The third type of cell is an 'intermediate' type which does not fit into the typical description of either the adult or fetal corticotrophs, yet stains for ACTH, and the number of cells of this type does not change with gestation. The number of adult corticotrophs increase after day 130, and by day 140, this cell type has become the predominant corticotroph. The switch from the fetal to the adult type of corticotroph in normal gestation is provoked by fetal cortisol infusion (2 mg/day) from day 109-115 of gestation, and a change from the fetal to the adult type of corticotroph is blocked by fetal adrenalectomy at day 120 of gestation (Antolovich, Perry, Trahair, Silver and Robinson, 1989). Thus, it

appears that functioning fetal adrenals are intimately involved during maturation of the fetal pituitary corticotrophs. In extracts of fetal sheep pituitaries, culture pituitary extracts, and cell-derived incubation media, Brieu and Durand (1989) identified three forms of irACTH: 'big' ACTH, 'intermediate' ACTH and 'little' ACTH, with the proportion of 'little' ACTH released by pituitary cells increasing over gestation. During gel filtration chromatography, 'big' ACTH eluted in the void volume, 'intermediate' ACTH between 'big' ACTH and human $ACTH_{1-39}$, and 'little' ACTH coeluted with human $ACTH_{1-39}$ (Brieu and Durand, 1989). Furthermore, the authors showed that in cultured adrenal cells the stercidogenic activity of 'big' ACTH was much lower than that of 'intermediate' or 'little' ACTH. Thus the change in corticotroph cell types (Perry *et al.*, 1985) from fetal to adult, coupled with an increase in the bioactivity and proportion of 'little' ACTH suggests that fetal corticotrophs synthesize 'big' ACTH and adult corticotrophs produce 'little' ACTH (Brieu and Durand, 1989).

1.2.2.1 Control of ACTH secretion

There have been several studies on the hypothalamic factors that are able to elicit the release of ACTH from the fetal pituitary. It is well established both *in vivo* (Wintour, 1984; Wintour, Bell, Fei, Southwell, Tregear, and Wang, 1984) and *in vitro* (Durand, Cathiard, Dacheux, Naaman, and Saez, 1986) that ACTH is secreted from pituitary corticotrophs in response to CRH. Arginine vasopressin has also been demonstrated to elicit ACTH release and may also synergize with

CRH in the regulation of ACTH secretion (Gillies, Linton and Lowry, 1982).

1.2.2.2 Control of ACTH secretion by CRH

The first report of CRH elevating plasma ACTH concentrations in the fetal sheep was by Wintour *et al.* (1984). They demonstrated that a bolus injection of 0.1 or 1.0 μg CRH into chronically catheterized fetal sheep between day 118 and term produced an elevation in plasma ACTH concentrations. This finding was expanded upon by others (Norman *et al.*, 1985; Pradier, Dalle, Davicco, LeFaivre, Barlett and Delost, 1985; Hargrave and Rose, 1986), and from these studies it is clear that the response of ACTH release to CRH administration changes with gestational age. In fetuses of gestational age day 110-115, while the initial increase in the concentration of plasma ACTH over the first 30-60 minutes is small, the concentration continues to increase for a further 240 minutes (Norman *et al.*, 1985). At later gestational ages (day 120-125) there is a greater initial response followed by a prolonged increase in plasma ACTH concentrations *in vivo* for at least 240 minutes after the CRH challenge.

The effects of CRH administration on ACTH release have also been observed in fetal sheep pituitary cells *in vitro* (Durand *et al.*, 1986). In fetal pituitary cells maintained in short term culture, a CRH challenge induced an increase in ACTH output above basal between day 63 and day 144 of gestation. The response was maximal at day 63, declined to day 115, and then remained constant until day 144.

1.2.2.3 CRH receptors

From the above results, the ACTH response to a CRH challenge is clearly variable during gestation. The response pattern may be a function of the CRH receptor population within the pituitary, and this possibility has recently been investigated (Lü, Yang and Challis, 1991). In fetal sheep anterior pituitary membrane preparations, a single class of CRH binding sites was found which displayed a high affinity (Kd = 1 nmol/l) for CRH, and this affinity did not change during all ages studied (day 65 to term). CRH binding site numbers, however, increased from day 65 of gestation to reach maximal numbers at day 125-130, and then the number of sites declined towards term. Thus, the changes in CRH receptor number may account, at least in part, for the changes in ACTH responsiveness to CRH during gestation.

1.2.2.4 Control of ACTH secretion by AVP

The ability of AVP to elicit ACTH release has been shown both *in vitro* (Durand *et al.*, 1986) and *in vivo* (Norman *et al.*, 1985). A bolus injection of AVP provokes a significant ACTH release that is transient, such that ACTH concentrations return to basal levels within 30-60 minutes (Norman and Challis, 1987b). This rapid return of ACTH concentrations back to basal concentrations is likely a result of the short half-life of AVP (2-10 minutes; Wiriyathian, Porter, Naden and Rosenfeld, 1983). The release of ACTH after AVP administration is age related, as it is greatest in younger fetuses (day 115), with the magnitude of

the response decreasing towards term (Norman and Challis, 1987b; MacIsaac, Congiu, Levidiotis and Wintour, 1989).

1.2.2.5 CRH and AVP

CRH and AVP synergize with each other (Gilles *et al.*, 1982) in the control of ACTH secretion. This synergism is likely due to the different second messenger systems used by the different secretagogues. CRH effects are mediated through activation of cAMP-dependent protein kinases (Durand *et al.*, 1986), while the effects of AVP are modulated via phosphatidylinositol turnover (Bilezikijian, Blount and Vale, 1987; Todd and Lightman, 1987). It has been suggested that this synergism is a result of AVP inhibiting phosphodiesterase activity, thereby preventing the breakdown of cAMP (Antoni, 1986).

1.2.2.6 Other corticotrophin releasing factors

There are other agents that posses the ability to elicit ACTH release. These include the endogenous opioids (Bousquet, Lye and Challis, 1984; Brooks and Challis, 1988). Substances such as oxytocin, epinephrine, norepinephrine, and angiotensin (Vale, Vaughan, Smith, Yamamoto, Rivier and Rivier, 1983) have also been shown *in vitro* to posses weak ACTH releasing properties. Prostaglandin E_2 has been suggested to play a role in fetal HPA function (Thorburn, Hooper, Rice, and Fowden, 1988). Thus, there are factors other than CRH and AVP that can stimulate ACTH release, but the function of these

substances within the fetus, and their role in the control of plasma ACTH concentrations remains to be elucidated.

1.2.3 Adrenal

The adrenal glands of the fetal sheep are recognizable as early as day 28 of gestation (Wintour, Brown, Denton, Hardy, McDougall, Oddie and Whipp, 1975), and zonation within the adrenal cortex is evident by day 60 of pregnancy (Webb, 1980) In the late gestation sheep fetus, the zona glomerulosa and fasciculata, but not the zona reticularis are present (Webb, 1980; Robinson, Rowe and Wintour, 1979). Fetal adrenal weight increases exponentially during the last third of gestation (Liggins, 1969; Boshier and Holloway, 1989), and this increase is mainly due to an increase in the size of the zona fasciculata (Boshier, Holloway and Liggins, 1981). It has been suggested that there are two stages of cellular hypertrophy (day 124-136 and day 143-birth). There is also one major period of adrenal cortical DNA replication between days 135-143 (Durand, Bosc and Locatelli, 1980). The growth rate of the zona fasciculata has been examined using relative changes in total steroidogenic cell volume, total cell number and individual cell volume (Boshier and Holloway, 1989). Growth of the zona fasciculata was divided into three phases, with the first phase occurring between day 53 and day 100, and being characterized by marked cell hyperplasia. The second phase of growth from day 100 to day 130 of gestation was characterized by a decrease in cell proliferation from phase 1, but a small increase in cellular

hypertrophy. The final growth phase, which occurred between day 130 of gestation and 48 hours postpartum, featured cellular hypertrophy from day 130 of gestation to term, and maximal cell multiplication after birth (Boshier and Holloway, 1989).

1.2.3.1 ACTH control of cortisol secretion

The response of the fetal adrenal to ACTH changes dramatically during gestation. These changes have been divided into three separate phases (Manchester and Challis, 1982; Glickman and Challis, 1980; Wintour *et al.*, 1975; Madill and Bassett, 1973). The fetal adrenal secretes the greatest amount of cortisol per gram wet weight, or per cell *in vitro*, at day 50-60 of gestation compared to all other times during pregnancy. The response is lost during mid-gestation (day 75-110), but adrenal responsiveness reappears as term approaches. This loss of responsiveness during midgestation is suggested to involve a functional block at G_s protein coupling (Manchester and Challis, 1982; Saez, Durand and Cathiard, 1984). The triphasic response of the fetal adrenal has also been correlated with the presence of the mRNA's for $P450_{scc}$ and $P450_{17\alpha}$ using Northern blot analysis. In early gestation both mRNAs are present, the mRNAs are undetectable during mid-gestation, and there is a reappearance of both the $P450_{scc}$ and $P450_{17\alpha}$ mRNAs towards term (Tangalakis, Coghlan, Connell, Crawford, Darling, Hammond, Haralambidis, Penschow and Wintour, 1989).

1.2.3.2 ACTH receptors

ACTH receptors have been identified in subcellular preparations of sheep fetal adrenal glands (Durand, 1979). The number of ACTH binding sites increases 5-fold between day 123 and birth, with the majority (80%) of the increase in binding occurring after day 140. Durand (1979) also ascertained that there was not only an increase in the number of receptors due to hyperplasia, but an increase in the number of receptors per cell. However, there was no change in the binding affinity of the receptors for ACTH during gestation. There was an increased ACTH binding to membrane preparations from adrenals of fetal sheep after treatment *in vivo* with $ACTH_{1-24}$ (Durand, Locatelli, Cathiard, Dazord, and Saez, 1981). This increase in ACTH receptor number was further correlated with increased adenylate cyclase responsiveness *in vitro* (Durand *et al.*, 1981). In rat fetal adrenal membrane preparations after *in vivo* ACTH treatment, there was an increase the number of its own receptors (Chatelain, Durand, Naaman, and Dupouis, 1989). In ovine fetal adrenal cells, dexamethasone treatment has been shown to increase the number of ACTH receptors (Darbeida and Durand, 1990). Thus it appears that regulation of the number of ACTH receptors on fetal adrenal cortical cells is likely the result of an interplay of endocrine, paracrine and autocrine factors.

1.3 Negative feedback within the HPA

1.3.1 Hypothalamus

The hypothalamus is the site, at least in part, of glucocorticoid feedback within the ovine fetus (Figure 1.2, page 42). Receptors for glucocorticoids have been demonstrated in both the hypothalamus and pituitary (Brooks *et al.*, 1989; Yang, Jones and Challis, 1990). However, the number of glucocorticoid receptors (GR) was greatest in pituitary at all gestational ages studied (day 60-70, 100-110, 125 and term). The number of GR's in the fetal hypothalamus increased from day 60-70 to day 100-110, then decreased by day 125, and again increased at term (Yang *et al.*, 1990). It is interesting that GR numbers increase towards term in the presence of increasing endogenous glucocorticoid concentrations, suggesting that the control of GR number is unresponsive to glucocorticoids near term, or that the hypothalamus is not exposed to increasing levels of endogenous glucocorticoids. Implants of either dexamethasone or cholesterol have been placed near the fetal PVN (McDonald, Hoffman, Myers and Nathanielsz, 1990). These authors showed that dexamethasone, but not cholesterol, blocked the hypoxemia- and hypotension-induced increase in plasma ACTH by inhibiting CRH release from the hypothalamus. Thus it was suggested that glucocorticoids can exert their negative feedback effect in the PVN at the level of the CRH and AVP producing neurons (McDonald *et al.*, 1990'

1.3.2 Pituitary

In the sheep fetus, GR's are prese. in greatest abundance in the pituitary, are saturable with binding of [^{3}H]triamcinolone (a synthetic glucocorticoid), and display a single class of high affinity (Kd = 2-3 X 10^{-9} M) binding sites (Yang *et al.*, 1990). Glucocorticoids may inhibit either the production or release of ACTH from corticotrophs. The inhibition of ACTH production may occur via an interruption of signal transduction in the CRH receptor (Abou-Samra, Catt and Aguilera, 1986), or a reduction in the number of CRH receptors (Hauger, Millan, Catt, Aguillera, 1987; Lü *et al.*, 1991). Using fetal pituitary cell culture, Lü and co-workers (Lü, Yang and Challis, 1994) have demonstrated not only a decrease in ACTH release after glucocorticoid treatment, but also a reduction in POMC mRNA as determined by Northern blot analysis.

The negative feedback effects of glucocorticoids on pituitary function have been demonstrated as an inhibition of ACTH secretion in the last third of gestation after CRH stimulation (Norman and Challis, 1987a; Rose, Hargrave, Dix, Meis, LaFave and Torpe, 1985). Glucocorticoid negative feedback has also been demonstrated during hypoxia with pretreatment with cortisol (Akagi, Berdusco and Challis, 1990), in which the hypoxemia-induced rise in ACTH was blocked by the cortisol infusion. In addition, removal of endogenous cortisol by bilateral adrenalectomy of the fetus led to a large increase in fetal ACTH concentrations in the last third of gestation (Wintour, Coghlan, Hardy, Hennesy, Lingwood, and Scoggins, 1980).

The existence of glucocorticoid negative feedback in conjunction with GR's at the pituitary and hypothalamus would appear to bring about a paradox in the late gestation ovine fetus. As stated earlier (1.1), the fetal plasma concentration of ACTH increases during the last third of gestation (Norman *et al.*, 1985), and the fetal plasma total ('free' plus bound) cortisol concentration also increases during late gestation (Bassett and Thorburn, 1969). However, without knowledge of the state of cortisol (i.e. bound or 'free') the significance of the elevation in plasma cortisol, and how the elevation relates to negative feedback and the onset of parturition, is unclear. The negative feedback of cortisol on the pituitary, if all the plasma cortisol was 'free', would drive fetal plasma ACTH concentrations down. However, fetal plasma ACTH concentrations continue to increase throughout the last third of gestation. Therefore, either plasma glucocorticoids are not transported to the fetal pituitary to exert negative feedback, or the efficacy of glucocorticoid negative feedback decreases during the last third of gestation. In the ovine fetus, examination of the high affinity binding protein of cortisol, corticosteroid-binding globulin (CBG), demonstrated that while the concentration of total cortisol ('free' and bound) was increasing, the concentration of CBG also increased. Therefore the 'free' or unbound plasma cortisol did not increase until approximately twenty-four hours before birth (Ballard, Kitterman, Bland, Clyman, Gluckman, Platzker, Kaplan, and Grumbach, 1982). The plasma steroid binding protein CBG, therefore, maintained a low 'free' plasma cortisol, thus suppressing the negative feedback effect of glucocorticoids at the level of the hypothalamus

and pituitary. This low negative feedback then allows for the concomitant increase in plasma ACTH and cortisol concentrations, and the sustained drive towards parturition.

1.4 Steroid Binding Proteins in Plasma

Steroid binding proteins in plasma have many roles. The obvious role is the binding of plasma steroid hormones, thus reducing the metabolic clearance of the hormone and modulating hormonal activity (Siiteri, Murai, Hammond, Nisker, Raymoure and Kuhn, 1982). Another role of steroid binding proteins involves the delivery of steroid directly to target tissues (Siiteri *et al.*, 1982). A further role involves the maintenance of a large pool of steroid which may by used when needed (Tait and Burnstein, 1964). Finally, steroid binding proteins may interact with hormone target cells, and therefore actively elevate steroid entry into cells (Siiteri *et al.*, 1982; Rosner, 1990). Thus, the investigation of steroid binding proteins is integral to delineating the mechanisms by which steroid hormones act and in particular, the function of CBG, with regards to mediating the various effects of cortisol in the fetal preparation for birth.

1.4.1 Low affinity binding proteins

The two most prevalent steroid binding proteins in plasma are albumin and α1-acid glycoprotein (AAG; also called orosomucoid). These two proteins have relatively low steroid binding affinities, with albumin having an association

constant (K_a) for cortisol of between 10^4 and 10^5 M^{-1} at 37 C, and AAG having an association constant (K_a) for progesterone of approximately 10^5 M^{-1} at 37 C (Westphal, 1971; Westphal, 1986). Due to this low affinity, very little of the binding of steroid hormones that have specific high affinity binding proteins, is associated with albumin and AAG (Dunn, Nisula and Rodbard, 1981). Dunn *et al.* (1981) have also shown that most steroids which lack high-affinity binding proteins are predominately transported by albumin due to the high plasma concentration of the protein. The ratio of AAG:albumin in human serum is so low that AAG does not play a significant role in the steroid binding ability of serum (Westphal, 1986).

1.4.2 High affinity binding proteins

Corticosteroid-binding globulin and sex hormone-binding globulin (SHBG or sex-steroid binding protein or testosterone-estradiol binding globulin) are two high affinity steroid binding proteins that are present in serum. These two proteins have high affinities for their respective ligands, CBG having an association constant (K_a) of approximately 10^8 M^{-1} at 37 C for cortisol, and SHBG an association constant (K_a) of approximately 10^8 - 10^9 M^{-1} at 37 C for estradiol, testosterone and dihydrotestosterone (Westphal, 1971; Westphal, 1986). The concentration of the two binding proteins is much lower than the concentration of albumin and AAG, however, the high binding affinities permit them to transport most of the biologically active preferred ligand.

1.5 Corticosteroid-binding globulin (CBG)

It is generally considered that CBG provides not only a vehicle for steroid transport, but that the concentration of CBG may also change the bioavailability of steroids (Siiteri *et al.*, 1982). It has also been suggested that CBG may participate directly in steroid delivery to target tissues by interaction with specific membrane proteins (Rosner, 1990). In addition, CBG is implicated in delivery of cortisol to sites of inflammation by interaction with the plasma protease elastase, that cleaves CBG, decreasing its affinity constant and thus releasing the bound steroid (Hammond, Smith, Paterson and Sibbald, 1990).

1.5.1 Introduction and discovery of CBG

High-affinity binding of cortisol to some components of human plasma was discovered more than thirty years ago (Daughaday, 1956). In the following year, Sandberg and Slaunwhite (1957), from results of cortisol binding activity in whole plasma, concluded the existence of a high affinity cortisol binding protein. Also in 1957, Bush (1957) identified a high-affinity low capacity binding protein by using equilibrium dialysis with increasing amounts of cortisol added to plasma. He also identified a second binding protein which possessed low affinity but high capacity (Bush, 1957). Shortly after that, Daughaday (1958), using paper electrophoresis, showed the migration of [^{14}C]cortisol in the α-globulin region. The presence of this protein was confirmed by Slaunwhite and Sandberg (1959), who termed the binding protein "transcortin" which has subsequently been termed

CBG.

CBG also binds other steroids, such as progesterone (Westphal, 1986), and in humans the association affinity is approximately the same as that for cortisol. CBG has one steroid binding site per molecule (Mickelson, Harding, Forsthoefel and Westphal, 1982). The molecular sizes of CBG in the guinea pig, rat, dog, sheep, human, cynamologous monkey, squirrel monkey and turtle have been estimated using sedimentation coefficients determined by sucrose density gradient centrifugation (Kato, Hsu and Kuhn, 1988). The molecular size of CBG in these species ranged from approximately 44 to 60 kDa, except in the squirrel monkey where the protein had a molecular weight of approximately 120 kDa (Kato *et al.*, 1988). The large size of CBG in the squirrel monkey in relation to the other species is thought to arise from the existence of CBG as a dimer in this species (Kato *et al.*, 1988). The cDNA sequences for human (Hammond, Smith, Goping, Underhill, Harley, Reventos, Musto, Gunsalus and Bardin, 1987), rat (Smith and Hammond, 1989), and rabbit (Seralini, Smith and Hammond, 1990) CBG are known, and there are sequence homologies with α1-proteinase inhibitor (α1-PI) and thyroxine binding globulin (TBG) (Hammond *et al.*, 1987) both of which are members of the serine proteinase inhibitor (SERPIN) superfamily (Travis and Salvasen, 1983). Furthermore, the CBG gene has been localized to chromosome 14 in the human genome (Seralini, Berube, Gagne and Hammond, 1990), where α1-PI and TBG are also localized (Seralini *et al.*, 1990). Using the CBG cDNAs for the various species, the protein sequences have also been

deduced. The mature polypeptide is approximately 384 amino acids in length and possesses four to six consensus sites for glycosylation through asparagine residues (*N*-glycosylation; Hammond *et al.*, 1987; Smith and Hammond, 1989; Seralini *et al.*, 1990) depending on the species studied. The physicochemical properties of human CBG have been extensively studied and are contrasted with those of the protein from rat and rabbit in Table 1.1 (page 46).

The existence of a cortisol specific high-affinity binding protein in plasma of adult sheep was shown by Reich (1960), Seal and Doe (1963), and Linder (1964). The affinity constant for cortisol of CBG from adult animals has been determined to be as 0.87 X 10^8 M^{-1} at 37 C (Paterson and Hills, 1967), with a plasma corticosteroid-binding capacity (CBC) of 24 ng/ml. Furthermore, there is no difference in the affinity for cortisol of CBG from pregnant and nonpregnant adult sheep.

1.5.2 Species distribution, sites of synthesis and localization of CBG

The presence of a high affinity binding protein that interacts specifically with cortisol or corticosterone is virtually ubiquitous in plasma samples taken from 131 vertebrate species (Seal and Doe, 1965). This list of species includes members of the fish, amphibian, reptilian, avian and mammalian families (Seal and Doe, 1965). Indeed, the presence of a protein that possesses similar properties to CBG has been demonstrated within the unicellular eukaryote, *Candida albicans* (Loose and Feldman, 1982). Subsequently, this protein has

been demonstrated to be unrelated to members of the steroid-thyroid-retinoic acid receptor gene superfamily (Malloy, Zhao, Madani and Feldman, 1993).

Within the literature there has been one case report of human CBG deficiency (Roitman, Bruchis, Bauman, Kaufman and Laron, 1984) but this claim has not been verified. However, a study examining over 10,000 subjects did not detect any samples that displayed a total lack of CBG (Rosner, Darnstadt and Toppel, 1973). These findings indicate that CBG is necessary for independent existence, and that a null mutation is likely lethal *in utero* (Rosner *et al.*, 1973).

The liver has long been thought to be a major site of plasma CBG production (Westphal, 1983). In 1966, Gala and Westphal showed that in the rat there was a decrease in plasma concentrations of CBG after subtotal hepatectomy. In patients with liver disease, there is a reduced plasma concentration of CBG, indicating a hepatic component in plasma CBG concentrations (Doe, Fernandez and Seal, 1964). Hepatic production of CBG was substantiated by Weiser, Do and Feldman (1979), who demonstrated synthesis and secretion of CBG by liver slices *in vitro*, and Perrot-Applant and Milgrom (1979) who showed *in vitro* translation of CBG by guinea pig hepatocytes. *In vitro* translation of CBG was also demonstrated by Wolf, Armstrong and Rosner (1981) using rat liver. Furthermore, it has been shown that the human hepatoma-derived cell line HepG2 secretes CBG in culture (Khan, Aden and Rosner, 1984), and fetal rat hepatocytes in culture also secrete CBG (Vranckx, Plas, Ali, Martin, and Nunez, 1985; Ali, Vranckx and Nunez, 1986).

The major site of CBG mRNA abundance in the adult is the liver (Hammond *et al.*, 1987; Smith and Hammond, 1989), and plasma CBG concentrations in the rat and rabbit correlate well with hepatic CBG mRNA abundance (Smith and Hammond, 1991; Seralini *et al.*, 1990).

Extra-hepatic sites of CBG production have also been demonstrated. CBG mRNA has been identified in monkey testis and kidney, and a CBG cDNA has been cloned from a human lung library (Hammond *et al.*, 1987). More recently, Seralini *et al.* (1990) using a rabbit CBG cDNA, found small amounts of CBG mRNA in the maternal lung, spleen and ovary, and in the fetal kidney of the rabbit. CBG mRNA has also been localized in the liver and exocrine pancreas of fetal mice (Schrocchi, Orva, Smith, Han and Hammond, 1993) and in liver and kidney of neonatal mice (Schrocchi, Hearn, Han and Hammond, 1993).

The protein has been localized using immunohistochemistry or binding assay to many tissues and fluids within different species. In humans, CBG has been measured in bronchioalveolar fluid (Loric, Egloff, Domingo, Lacronique, and Degrell, 1989), cerebrospinal fluid (Predine, Brailly, Delaporte and Milgrom, 1984), milk (Rosner, 1976) and in amniotic fluid (Challis and Bennett, 1977). In human tissues or cells CBG has been localized to lymphocytes (Werthamer, Samuels and Anaral, 1973), liver (Amoral, Lin, Samuels, and Werthamer, 1974) and the uterus (Gueriguian, Sawyer and Pearlman, 1974).

CBG has also been localized in tissues within the adult rat, such as brain and pituitary (Kuhn, Green, Raymore and Siiteri, 1986), kidney (Feldman, Funder

and Edelman, 1973; Kuhn *et al.*, 1986), skeletal muscle (Mayer, Kaiser, Milholland and Rosen, 1975), the thyroid (Kuhn *et al.*, 1986) and the uterus (Milgrom and Baulieu, 1970). The protein has also been localized in rabbit lung (Giannopoulus, 1976).

The expression of CBG in extra-hepatic sites in most cases is very low when compared to the liver, and the significance of these sites is not known.

1.5.3 CBG in the fetal sheep

The plasma concentration of CBG in the adult ewe was examined by numerous individuals or groups during the early and mid 1960's (Reich, 1960; Seal and Doe, 1965; Linder, 1964). The presence of CBG in fetal sheep plasma was first demonstrated by Liggins *et al.* (1973), and by Fairclough and Liggins (1975). Fetal plasma corticosteroid-binding capacity (CBC) was 28 ± 6 ng/ml at 122 days of gestation (term 145 days), and plasma CBC increased over the last fourteen days of gestation to 85 ± 14 ng/ml on the day of parturition. The affinity constant of fetal sheep CBG for cortisol was also examined, and found to be 1.15 ± 0.37 X 10^8 M^{-1}. The ontogeny of CBG within the sheep fetus from day 75 of gestation to term was also examined by Ballard *et al.* (1982). It was shown that the observed increase in CBC in late gestation did not occur in hydrancephalic, stalk sectioned or hypophysectomized fetuses. After parturition there appeared to be two phases of decrease in plasma CBC involving a rapid decrease phase over the first four days, followed by a much slower decline until approximately 14

days of age. There was also no effect of fetal infusion of hydrocortisone (24 mg/day for 2 days), prolactin (1 mg/day for 5-8 days) or of 17β-estradiol (0.1 mg/kg/day for 3-6 days) on the fetal plasma CBC. The role of CBG was postulated to maintain a low 'free' plasma cortisol, and as calculated using the quadratic equation of Tait and Burnstein (1964), the plasma 'free' cortisol did not increase until after 135 days of gestation, and reached a maximum concentration within 24 hours of parturition (Ballard *et al.*, 1982). From their results, Ballard *et al.* (1982) concluded that the plasma CBG concentration in the fetal sheep was regulated by pituitary hormones, and speculated that the effect involved the placenta (Ballard *et al.*, 1982). Challis, Nancekievill and Lye (1985) showed that the prepartum increase in CBG could be mimicked by fetal infusion of $ACTH_{1-24}$ (66.7 ng/min for 15 min every 2 hours). This effect was blocked when ACTH (same dose as above) was infused together with the 11β-hydroxylase inhibitor metopirone (500 mg/day), which blocks the conversion of 11-desoxycortisol to cortisol. The metopirone-induced suppression of the ACTH stimulation was overcome by administration of exogenous cortisol (13.3 μg/min for 15 min every 2 hours or 133 μg/min for 15 min every 2 hours), or with administration of dexamethasone (0.56 μg/min for 15 min every 2 hours for 50 hours, then 5.6 μg/min for 15 min every 2 hours for a further 50 hours). From these studies the authors concluded that in fetal sheep, ACTH treatment significantly elevated plasma CBC, and that this elevation may be glucocorticoid-mediated.

1.5.4 Glycosylation of CBG

Human CBG contains only *N*-linked sugar chains of the *N*-acetyllactosamine type (Akhrem, Avvakumov, Akhrem, Sidorova and Strel'chyonok, 1982; Strel'chyonok, Avvakumov, Matveentseva, Akhrem and Akhrem, 1982); three of these chains are biantennary and two are triantennary (Figure 1.3, page 44). Using the human hepatic CBG cDNA (Hammond *et al.*, 1987) to deduce the primary structure of CBG, the protein was shown to possess six possible consensus sequences for *N*-glycosylation (-Asn-X-Ser\Thr, where X is not Pro; Montreuil, 1980; Bause, 1983). The six possible sites that have been predicted by the cDNA and the five sugar chains described by the biochemical data (Akhrem *et al.*, 1982) suggest that either one of the sites on human CBG is not utilized or a few of them are partially utilized.

The carbohydrate component of CBG has been postulated to facilitate many biological functions. The half-life of CBG within the circulation of the rat has been linked with the content of the terminal sialic acid residues, such that their removal results in a more rapid clearance of the protein from the circulation (Hossner and Billiar, 1981). The co-translational glycosylation appears to be essential for proper folding of the newly formed CBG (Ghose-Dastidar, Ross and Green, 1991), and carbohydrates have been demonstrated to influence the interaction of CBG with plasma membranes of the human liver (Strel'chyonok and Avvakumov, 1983) and the placental syncytiotrophoblast (Strel'chyonok and Avvakumov, 1991). Enzymatic deglycosylation of CBG does not influence steroid

binding (Mickelson *et al.*, 1982). Using site-directed mutagenesis, Avvakumov, Warmels-Rodenheiser and Hammond (1993) have modified a human CBG cDNA in order to study the biological significance of individual oligosaccharides linked to CBG by systematically eliminating individual or various combinations of consensus sites on the CBG cDNA. The mutant CBG cDNAs were then expressed in chinese hamster ovary (CHO) cells, and the secretion rate and steroid binding affinity of the resultant recombinant proteins were measured. The authors demonstrated that all six potential consensus sites were able to be used. Removal of sugar chains generally led to a reduction in the secretion of recombinant CBG, but removal of all *N*-glycosylation sites did not prevent production of the protein. Furthermore, the oligosaccharide attached at site Asn^{238} was essential for steroid binding, suggesting that the interaction of this sugar chain with the polypeptide may be essential for the creation of a high affinity steroid-binding site. The glycosylation site in a position homologous to Asn^{238} in human CBG is present in cDNA deduced amino acid sequences of CBG from other species (Hammond, Smith and Underhill, 1991), strengthening the likelihood of the Asn^{238} being essential for steroid binding.

1.5.5 Effect of pregnancy on maternal and fetal plasma CBG

The profiles of CBG in various species vary widely during gestation. In pregnant women, it has been demonstrated that plasma CBG levels increase approximately two-fold over nonpregnant values by the end of the second

trimester and remain relatively constant until birth (Doe *et al.*, 1964; Moore, Kawagoe, Davajan, Mishell and Nakamura, 1978). Other authors found that in human pregnancy, plasma concentrations of CBG increase throughout gestation to reach highest concentrations before birth (Murao, Yasuda, Shibukawa, Takahashi, Sawada, Kaeda, Hasegawa and Kitao, 1986). The observed increase in plasma CBG concentration has been attributed to elevated serum estradiol concentrations during pregnancy (Brien, 1981). The plasma profiles of CBG in pregnant women are in contrast to those in sheep where plasma concentrations of CBG in the ewe do not change during gestation (Patterson and Hills, 1967; Fairclough and Liggins, 1975). In the rat, maternal plasma levels of CBG undergo only minor changes during gestation (Gala and Westphal, 1965; Gewolb and Warshaw, 1986; Smith and Hammond, 1991). In the rat fetus, plasma CBG is high on day 17 and falls to term (day 21; Smith and Hammond, 1991). The hepatic CBG mRNA abundance is highest in mid-gestation and also falls to term (Smith and Hammond, 1991; Elfahime, Plant, Rene, Felix and Koch, 1992).

A pregnancy-associated variant of human CBG, which exhibits only triantennary carbohydrate moieties, has been described by Strel'chyonok, Avvakumov and Akhrem (1984). This variant does not interact with Concanavalin A, and thus elutes in the non-bound (or Con A non-retarded) fraction during Concanavalin A chromatography (Strel'chyonok *et al.*, 1984). The pregnancy associated variant was found in all serum samples tested from the sixth month of pregnancy to term. At term, the variant concentration in the maternal blood

accounted for 7-14% of the total CBG blood concentration (Avvakumov and Strel'chyonok, 1987). The molecular variant persisted in the postpartum period for a period of at least 40 days and the authors suggested that the factors initiating its biosynthesis still existed in the female after delivery (Strel'chyonok and Avvakumov, 1990).

Differences in charge microheterogeneity have been observed in CBG purified from pregnant and nonpregnant female rabbits (Seralini, Underhill, Smith, Nguyen and Hammond, 1989). However, after iodination of the two isoforms there was no difference in the half-lives in serum of the two proteins. The authors demonstrated transfer of [^{125}I]CBG from the mother to the fetus and accumulation of maternal [^{125}I]CBG in the fetal urine and the amniotic fluid, suggesting preferential removal of maternal [^{125}I]CBG from the fetal circulation by the fetal kidney (Seralini *et al.*, 1989).

1.5.6 Tissue binding sites for CBG

Binding sites for CBG bound to cortisol or corticosterone have been described in membrane preparations prepared from human liver (Strel'cyonok and Avvakumov, 1983) and prostate (Hryb, Khan, Romas and Rosner, 1986), rat spleen (Singer, Khan and Rosner, 1988) and rat kidney (Hsu, Siiteri and Kuhn, 1986). The interactions of CBG with membrane proteins have been demonstrated to be specific, saturable and temperature dependent. In membrane preparations of human endometrium, Avvakumov, Krupenko and

Strel'chyonok (1989) demonstrated binding sites that displayed 2-3 fold greater affinity for CBG bound with progesterone than CBG bound with cortisol. The authors speculated that this may be a mechanism regulating steroid delivery to the endometrium (Avvakumov *et al.*, 1989). In experiments using membrane preparations of human placental syncytiotrophoblast, two classes of CBG binding sites were described (Avvakumov and Strel'chyonok, 1988). One class of binding site was present in low concentration and displayed a high affinity for pregnancy-associated variant CBG (see section 1.5.5). The other class of binding site was present in high concentration and displayed greatest affinity for normal CBG (Avvakumov and Strel'chyonok, 1988). The membrane binding protein for CBG has been purified from human decidual membrane preparations and appears to be a sialoglycoprotein composed of four 20 kDa subunits (Avvakumov *et al.*, 1989; Krupenko, Avvakumov and Strel'chyonok 1991). A single class of membrane receptors for CBG has recently been demonstrated in rat hepatic membranes (Maitra, Khan and Rosner, 1993). In contrast to human hepatic membrane CBG binding sites, where both CBG bound to steroid and 'free' CBG interacted with the membrane protein (Strel'chyonok and Avvakumov, 1991), only CBG that was not complexed with a ligand was able to bind to the receptor. In membrane preparations from the rat liver, addition of cortisol or corticosterone, which bind to CBG, non-competitively inhibited CBG binding to the receptor (Maitra *et al.*, 1993). The authors (Maitra *et al.*, 1993) were unable to account for the differences between their findings and the findings of Strel'chyonok and

Avvakumov (1991) in human hepatic membranes other than to speculate on species differences or differences in temperature at which the experiments were conducted (37 C vs 4 C; Maitra *et al.*, 1993 and Strel'chyonok and Avvakumov, 1991, respectively).

1.5.7 Other factors affecting plasma CBG concentrations

Administration of either natural or synthetic glucocorticoids in humans depresses serum CBG levels (Schlechte and Hamilton, 1987; Frairia, Agrimonti, Fortunati, Fazzari, Gennari and Berta, 1988). The administration of progestins, however, increases serum CBG concentrations by more than 20% (Gala and Westphal, 1965) in both males and in females. Estrogen administration to women, whether endogenous (pregnancy) or exogenous, produces an increase in plasma CBG (Doe *et al.*, 1964; Sandberg, Woodruff, Rosenthal, Neinhouse and Slaunwhite, 1964). There is no difference in plasma CBG concentrations between men and women, and also no variation during the menstrual cycle (Rosner, 1990). There is a moderate influence of diet on plasma CBG concentrations with a high carbohydrate diet producing a fall in plasma CBG concentrations of approximately 20% (Anderson, Rosner, Khan, New, Pang, Wissel and Kappas, 1987).

Plasma CBG concentrations undergo a dramatic decrease after the onset of septic shock, and this decrease is associated with an increase in total and 'free' cortisol concentrations (Savu, Zouaghi, Carli, and Nunez, 1981; Pugeat,

Bonneton, Perrot, Rocle-Nicolas, Lejeune, Grenot, Dechaud, Brebant, Motin and Cuilleron, 1989).

In the human there is no diurnal variation in serum CBG concentrations (De Moor, Heirwegh, Heremans and Declerck-Raskin, 1962; Doe *et al.*, 1964). In the rat, however, plasma CBG levels may vary by approximately 20% (Hsu and Kuhn, 1988). The plasma concentrations of CBG peak at the beginning of the nocturnal period, and reach their nadir at the onset of the light period. There is a correlation between CBG concentrations and plasma concentrations of corticosterone. Furthermore, these cyclic changes are not observed following adrenalectomy (Hsu and Khun, 1988).

1.5.8 Proposed function of plasma CBG in the fetal sheep

Within the fetal sheep, the presence of CBG has been postulated to maintain a low 'free' plasma cortisol concentration. It is speculated that this low 'free' plasma cortisol concentration maintains a low negative feedback effect of cortisol on the HPA axis. The low negative feedback effect allows for the progressive increase in plasma total cortisol concentrations and a sustained drive toward parturition. However, the stimulus for an increase in fetal plasma CBG, as well as the sites of CBG production are unknown. Furthermore, the glycosylation of proteins may affect their bioactivity, and the possible changes in glycosylation of CBG in the fetus throughout gestation and in adult life may change.

This thesis will address the following questions: i) What is/are the stimulus/stimuli for an increase in plasma CBG in the fetal sheep and does this stimulus/stimuli also effect adult plasma CBG?: ii) What is(are) the mechanism(s) for an increase in the concentration of fetal plasma CBG?: iii) What are the sites of production of CBG within the fetal sheep and adult sheep?: iv) What are the patterns of glycosylation of CBG throughout gestation and in the adult?, and v) What is the physiological significance of CBG within the fetal sheep?.

Figure 1.1 Diagrammatic representation of the processing of pro-opiomelanocortin (POMC) within the human pituitary. A: presumed initial precursor molecule. ACTH: adrenocorticotrophin; LPH: lipotrophin; MSH: melanocyte stimulating hormone; CLIP: corticotrophin-like intermediate lobe peptide; JP: joining peptide (from Goodyer, 1989).

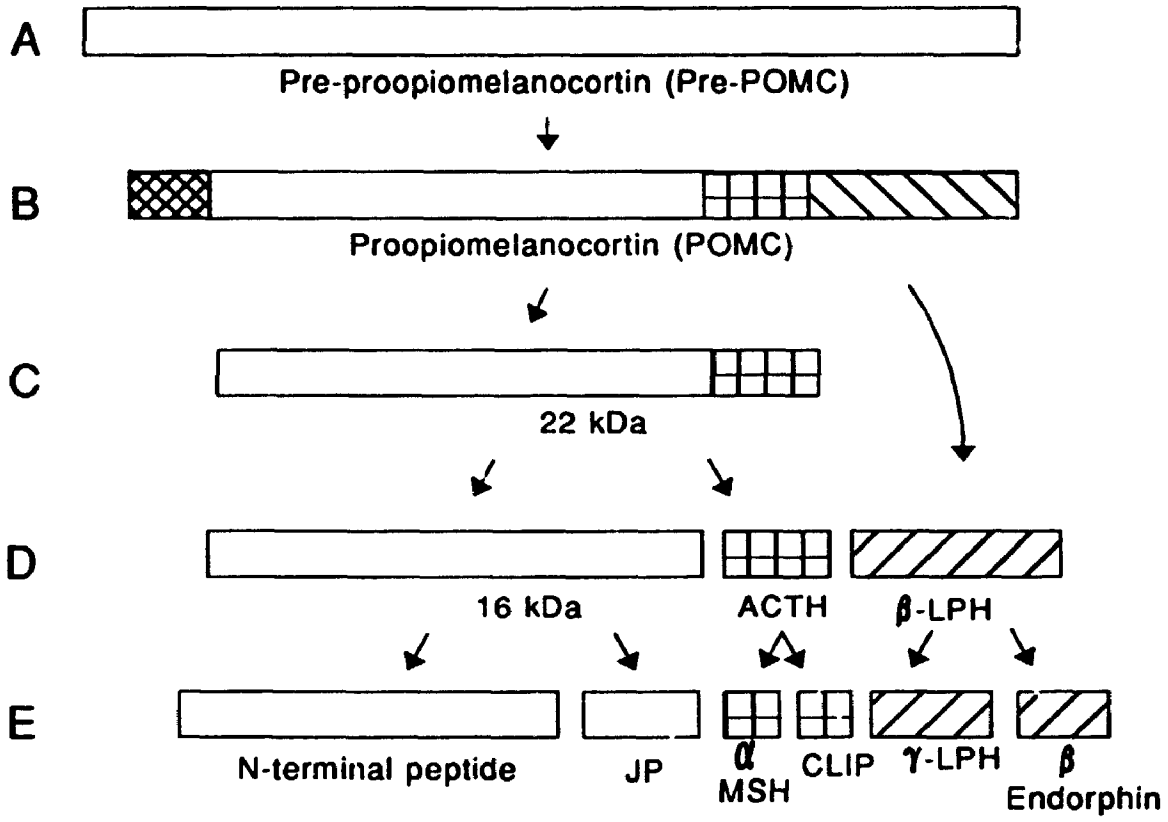

A
Pre-proopiomelanocortin (Pre-POMC)
B
Proopiomelanocortin (POMC)
C
22 kDa
D
16 kDa
ACTH
β-LPH
E
N-terminal peptide
JP
α MSH
CLIP
γ-LPH
β Endorphin

Figure 1.2 Possible sites of glucocorticoid negative feedback within the fetal hypothalamo-pituitary-adrenal axis in the fetal sheep. (CRH = corticotrophin releasing hormone, and ACTH = adrenocorticotrophin)

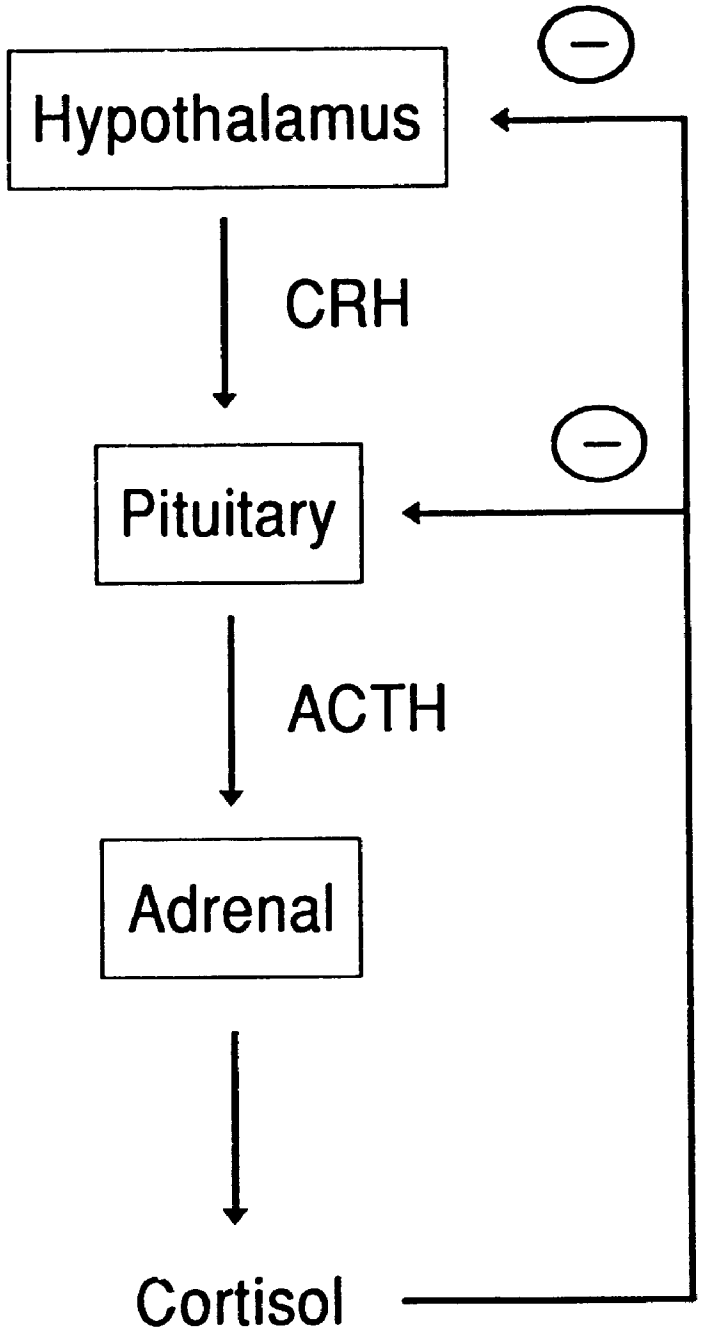

Hypothalamus
CRH
Pituitary
ACTH
Adrenal
Cortisol

Figure 1.3 Structure of the carbohydrate chains of human CBG. A = biantennary and B = triantennary. Both kinds of the oligosaccharides are of the *N*-acetyllactosamine type and, on the protein are linked to the amino acid asparagine (Asn). GlcNAc = N-acetylglucosamine; Fuc = fucose; Man = mannose; Gal = galactose; and NeuAc = N-acetylneuraminic acid.

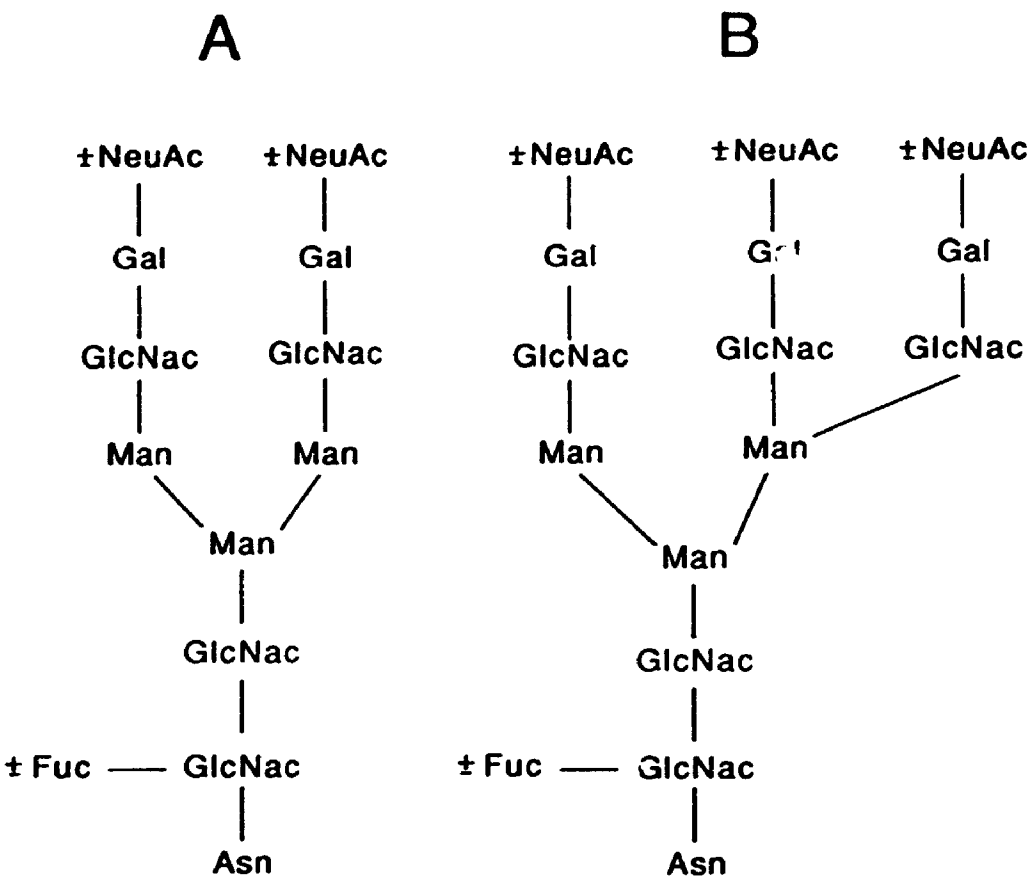

A
±NeuAc
±NeuAc
Gal
Gal
GlcNac
GlcNac
Man
Man
Man
GlcNac
± Fuc
GlcNac
Asn
B
±NeuAc
±NeuAc
±NeuAc
Gal
Gal
GlcNac
GlcNac
GlcNac
Man
Man
Man
GlcNac
± Fuc
GlcNac
Asn

Table 1.1 Comparison of physicochemical data between human, rat and rabbit CBG. Values within the table were obtained from the following: a = Kato *et al.*, 1988, b = Seralini *et al.*, 1990, c = Hammond *et al.*, 1987, and d = Smith and Hammond, 1989.

	Human	Rat	Rabbit
Molecular weight (kDa)	59.5[a]	53.8[a]	60[b]
Percent Carbohydrate	27[c]	28[d]	29[b]
# of consensus glycosylation sites	6[c]	5[d]	4[b]

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Animals

2.1.1 Time-dated pregnant sheep

Mixed breed nonpregnant and time-dated pregnant sheep and their fetuses were used in these studies. As fetal sheep of known gestational age were essential for these experiments (at regular intervals throughout the year), the ewes' estrous cycles were manipulated in the following manner. A vaginal sponge containing 60 mg medroxyprogesterone acetate (Upjohn, Orangeville, Ont.) was implanted in each ewe for 14 days. After removal of the sponge, the ewes were injected with 500 units of pregnant mares serum gonadotrophin (PMSG: Synkron Corp., London, Ont.). Two days after removal of the sponge and PMSG injection, the ewe was placed with a ram equipped with a brisket harness to mark the ewe at the time of mating. Time of mating was taken as day 0 of pregnancy, unless the ewe returned in heat at a later date.

2.1.2 General surgical procedures

2.1.2.1 Fetal surgery

All protocols used in animal studies within this thesis were approved by the Animal Care Committees of St. Joseph's Health Centre, and the University of Western Ontario, in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

In studies involving pregnant animals, surgery was performed between days 94-125 of gestation (term is approximately 145 days). Before surgery, ewes

were fasted for a minimum of 24 hours and a maximum of 36 hours. The sheep were brought into the preparation rooms and general anaesthesia induced using 40 ml of 2.5% sodium pentothal (Abbot Laboratories, Montreal, Que.), injected into the ewe via jugular venipuncture. Animals were intubated quickly and anaesthesia maintained with 1-2.5% halothane (Wyeth-Ayerst, Montreal, Que.), accompanied by a 50:50 mixture of nitrous oxide and oxygen at a flow rate of 3 l/min. Pre-operative procedures consisted of removal of all wool from the abdominal area and scrubbing the area (three times) with an iodine soap (Proviodine, Rougier Inc., Chambly, Que.). Following cleaning of the abdominal area, the animal was transferred into the operating room, and an iodine/alcohol solution was applied to the surgical field.

Surgery was performed using standard aseptic techniques. The uterus was exposed via a lower abdominal midline incision. Protocols within individual chapters will define the number of fetuses from each ewe that were catheterized, and the site of the fetal catheterization. The uterus was opened and either the fetal head or fetal hindlimbs were exteriorized. Babcock clamps were then used to fasten the edges of the uterine incision to the fetus to prevent loss of amniotic fluid and/or exposure of the umbilical cord. In these studies, either the fetal carotid arteries and jugular veins, or the fetal femoral arteries and veins were catheterized. Between 4 - 8 cm of catheter was introduced into each vessel. All fetal vessels that were cannulated were tied using 2-O silk (Ethicon, Johnson & Johnson Medical Products, Peterborough, Ont.), and all fetal catheters were

anchored using 2-O silk. An open ended amniotic fluid catheter was attached to the anchoring sutures of the vascular catheters. All catheters were filled with heparin saline (1:250, v/v; 20 international units/ml; 0.9% sodium chloride) at the time of surgery, and the catheter dead space (catheter volume) was recorded. The uterus was closed using 2-O silk. Uterine electromyographic (EMG) leads (Cooner Wire Co., Chatsworth, CA) were attached to the uterus to monitor uterine electrical activity. The abdomen of the ewe was closed in two layers to prevent herniation. The abdominal wall was closed with 2-silk using interrupted figure 8 sutures. The incision through the abdominal skin was closed with 0-vicryl (Ethicon, Johnson & Johnson Medical Products, Peterb- ough, Ont.) using a mattress suture. All catheters and the uterine EMG leads were exteriorized through the flank of the ewe. The trochar perforation was then sewn shut with a purse-string suture, and each catheter tied into place. The maternal femoral artery and vein were also catheterized, and the catheters advanced 20 cm to lie in the maternal abdominal aorta and inferior vena cava, respectively. The incision for the femoral catheters was then closed with 0-vicryl.

2.1.2.2 Adult non-pregnant surgery

In adult non-pregnant ewes, pre-surgical procedures were similar to pregnant animals. The uterus was exposed via a lower abdominal midline incision. The uterine horns were exteriorized, ligatures (2-silk) placed around the ovaries and tightened to prevent any loss of blood, and the ovaries were

removed to eliminate ovarian steroids. The abdominal wall and skin were closed using 2-silk and 0-vicryl, respectively, in a similar fashion to the pregnant ewe. The femoral artery and vein of the ewe were also catheterized, and the catheters advanced 20 cm to lie in the maternal abdominal aorta and inferior vena cava, respectively. The incision for the femoral catheters was then closed with 0-vicryl.

2.1.3 Post-operative care of animals

The animals were allowed to recover for at least five days after the date of surgery before experimentation. During the recovery period, catheters were filled and flushed daily with sterile heparinized saline to maintain patency. To assess fetal and maternal health, a 0.5 ml arterial blood sample was collected daily, and fetal blood pH, pCO_2, and pO_2 were determined using an acid blood ga analyzer (ABL-3, Radiometer, Copenhagen). At the time of surgery, and for three days post operation, the ewe received daily intramuscular injections of 4 ml Pen-di-strep (200,000 IU penicillin G and 250 mg dihydrostreptomycin/ml: Rogar, London, Ont.). Crystapen (1 ml: 1,000,000 IU Penicillin G; Wyeth-Ayerst, Montreal, Que.) was injected into the fetal vein and amniotic cavity at the time of surgery and daily for 3 days post-operation. Following recovery from surgery, catheters were flushed every day or every other day between experimental periods to maintain their patency.

Recordings of uterine EMG activity were made using a Grass EEG amplifier (Model 7 P511J, Grass Instruments, Quincy, Mass.) on a four-channel

Grass polygraph (Model 78D, Grass Instruments, Quincy, Mass.).

From the day of surgery until euthanasia, the animals were housed in individual metabolism cages. The metabolic cages allow the ewe to move backwards and forwards and to lie down. The animal housing rooms were maintained on a 14-hour light (0600 to 2000 hours) and a ten-hour dark cycle. Hay and water were provided *ad libitum*, and at 0800 hours and 1500 hours, the ewes received 0.4 kg of grain concentrate (14% Ewe fitting and nursing chow, Masterfeeds, London, Ont.).

2.1.4 Blood sampling of experimental animals

Prior to obtaining fetal or maternal blood samples, a previously heparinized syringe was attached to the stopcock and a volume of blood equal to twice the dead space of the catheter (approximately 2 ml fetal and 8 ml maternal; previously determined at the time of surgery) was drawn into the syringe. If it was the first sample of the day, the syringe with the volume of blood was discarded. The blood sample was collected into a second heparinized syringe that had also been attached onto the stopcock. After obtaining the blood sample, the dead space volume plus 1 ml heparinized saline, or at least 4 ml of heparinized saline, was infused into the animal.

Once the arterial blood samples were obtained, they were immediately transferred into polystyrene tubes kept on ice. The samples were kept on ice for not more than 20 min until centrifugation. The samples were centrifuged at 2000

X g for 10 min at 4 C, following which the plasma was separated and then stored at -20 C until subsequent analysis.

2.1.5 Euthanasia of fetal and adult sheep

At the end of the experimental protocol or when the animals were in active labour, as judged by uterine EMG tracings (Figueroa, Mahan, Poore and Nathanielsz, 1985), as called for in individual protocols, the ewes were killed with an overdose of Euthanyl (20 ml; 20% pentobarbital; MTC Pharmaceuticals, Cambridge, Ont.). The fetuses were delivered by cesarean section, and killed by administration of 5 ml Euthanyl via cardiac puncture.

2.1.6 Tissue collection after euthanasia

After death of the adult or fetus was ascertained by lack of heartbeat, tissues for subsequent Northern blot analysis were collected, cut into approximately 1 cm^2 pieces, flash frozen in liquid nitrogen and put into Poly Q scintillation vials (Beckman Instruments (Canada) Inc, Toronto, Ont.), and maintained at -70 C until total RNA extraction. Liver and lung were consistently taken from the respective right lobes.

2.2 Assays

2.2.1 [^{3}H]Cortisol purification

[1,2,6,7-^{3}H]Cortisol (60 µl; specific activity 80-100 Ci/mmol) was separated

from free [^{3}H] and breakdown products of the labelled steroid using one-half of a thin layer chromatography (TLC) plate (20 cm X 20 cm, Fisherbrand, Fisher Scientific, Toronto, Ont.), and the solvent system chloroform:ethanol (95:5). Non-labelled cortisol (20 µl; 1 mg/ml) was added on the other half of the TLC plate to estimate the relative distance the labelled steroid had moved up the plate (R_f). The TLC plate was allowed to dry, and the non-labelled cortisol visualized by UV light. The corresponding area to the unlabelled steroid on the [^{3}H]cortisol half was scraped off the plate into a test tube and the powder was mixed with 4 ml of ethyl acetate (J.T. Baker, Toronto, Ont.). After vortexing (20 sec), the tube was centrifuged (10 min at 2000 X g) and the supernatant decanted into a second test tube. The pellet was mixed with a second 4 ml of ethyl acetate, vortexed and centrifuged, and the supernatant was added to the second test tube. An aliquot (10 µl) of the supernatant was then added to 4 ml of Scintisafe Plus liquid scintillation cocktail (Fisher Chemicals, Toronto, Ont), and counted in a beta counter (Beckman model LS 5000TD, Beckman Instruments (Canada) Inc., Toronto, Ont.).

2.2.2 Measurement of plasma cortisol concentrations

The concentration of cortisol in plasma was measured using radioimmunoassay (RIA) techniques described previously (Challis, Patrick, Cross, Workewych, Manchester and Power, 1981). Samples of plasma (100 µl) were put into test tubes (16 X 125 mm), and extracted using 5 ml of diethyl ether.

After vortexing (20 sec), the tubes were shaken (1 h), and the aqueous phase was separated from the organic phase by rapid freezing in a tray containing acetone and dry ice. The organic phase was tipped into a second set of tubes (12 X 75 mm) and dried, and samples were reconstituted in 1 ml phosphate buffered saline with gelatin (PBSG: $NaH_2PO_4 \cdot 2H_2O$ 0.4 M, $Na_2HPO_4 \cdot 7H_2O$ 0.6 M, NaCl 0.15 M, NaN_3 0.015 M and 0.1% gelatin w/v; pH 7.1). Four hundred microlitres of the reconstituted sample was then used in the assay. The reconstituted sample was incubated with rabbit anti-cortisol antiserum (100 µl; final antibody dilution 1:60,000, titrated to give a B/B_0 of approximately 40%) and [^{3}H]cortisol (approximately 30,000 dpm) overnight at 4 C. The bound and 'free' cortisol were separated using dextran-coated charcoal (DCC; 0.625% w/v dextran, 6.25% w/v charcoal). The antibody used was generated by this laboratory and showed the following cross-reactivities: cortisol sulphate, 76%; 11-desoxycortisol, 35%; 21-deoxycorticosterone, 33%; cortisone, 0.6%; corticosterone, 0.84%; progesterone, 0.31%; pregnenolone, 17α-hydroxypregnenolone, 11α-hydroxyprogesterone, less than 0.1%. The mean assay sensitivity was 0.4 ± 0.09 ng/ml (n=5). The intra- and inter-assay coefficients of variation were 9 and 13% respectively.

2.2.3 Measurement of percent free cortisol in fetal plasma

The percentage of 'free' cortisol in samples of fetal plasma was measured by the method of Hammond, Nisker, Jones and Siiteri (1980). Samples of fetal

plasma (450 µl) were pipetted into glass tubes containing approximately 3 X 10^5 dpm [^{3}H]cortisol, previously added in ethanol and dried under nitrogen, and 30,000 dpm [^{14}C]glucose (NEN Research Products, DuPont Canada Inc., Mississauga, Ont.) added in distilled water (5 µl). The plasma and labelled cortisol and glucose were incubated for 1 hour at 37 C, and then spun at 500 X g for 1 min. Aliquots of the samples (200 µl each) were dispensed into tubes which had dialysis membranes at the base (inner chamber; Figure 2.1, page 65). These tubes were inserted into a second set of tubes (outer chamber) which previously had three disks of filter paper (Whatman 3M; Fisher Scientific, Toronto, Ont.) placed at the bottom, so that the bottom of the dialysis membrane was in contact with the disks of filter paper (Figure 2.1, page 65). The entire assembly was then spun at 2000 X g for 1 hour at 39 C. After centrifugation, an aliquot (30 µl) of the plasma from the inner chamber was added onto a separate filter disk. The filter disk containing plasma from the inner chamber, and the disks of 3M paper from the outer chamber, were added to separate scintillation vials. Distilled water (350 µl) was then dispensed into all of the vials. The samples were vortexed, scintillation fluid (4 ml) was added and the radioactivity within each vial measured using a beta counter. Samples of [^{3}H]cortisol and [^{14}C]glucose were counted individually. Both radioactive labels used emit beta particles, and the spillover of energy of the beta particle from the ^{14}C window into the ^{3}H window was corrected during the free cortisol calculation. The calculation for the percentage 'free' cortisol is as follows:

$$\% \text{ free cortisol} = \frac{[^3H]\text{cortisol}}{[^{14}C]\text{glucose}}_{\text{ultrafiltrate}} \div \frac{[^3H]\text{cortisol}}{[^{14}C]\text{glucose}}_{\text{plasma}} \times 100$$

2.2.4 Measurements of plasma corticosteroid-binding globulin

2.2.4.1 Corticosteroid binding capacity (CBC) in undiluted plasma

Previously, this laboratory has validated an assay to measure the CBC of fetal and adult sheep plasma (Challis *et al.*, 1985), based on the method of Ballard *et al.* (1982). Briefly, duplicate aliquots (50 µl) of plasma were added to glass tubes containing [^{3}H]cortisol (30,000 dpm) and non-radioactive cortisol (16 ng), previously added in ethanol and dried under air. The tubes were agitated briefly, and then incubated at 37 C for 30 min, then at 4 C for 12 h. Bound and free cortisol were separated using dextran-coated charcoal (90 µl; DCC, 0.625% dextran (Dextran T70, Pharmacia Fine Chemicals, Baie d'Urfe, Que.), 6.25% charcoal (Norit A, Fisher Scientific, Toronto, Ont.), in tricine assay buffer (0.15 M tricine, pH 7.4)) followed by centrifugation (10 min at 2,000 g). For each sample non-specific binding of [^{3}H]cortisol was determined in the presence of non-radioactive cortisol (1 µg). An aliquot of the supernatant (100 µl) was then added to 4 ml scintillation cocktail and subjected to liquid scintillation counting. The CBC was calculated from the fraction of bound [^{3}H]cortisol, minus non-specific binding, multiplied by the total concentration of cortisol (endogenous plus unlabelled plus [^{3}H]cortisol) in 50 µl plasma samples. The assay did not measure cortisol binding to albumin since this complex dissociates rapidly after the addition

of charcoal, and the unbound cortisol is absorbed and precipitated with the charcoal upon centrifugation.

2.2.4.2 Corticosteroid binding capacity in diluted plasma

The concentration of CBG in samples of diluted plasma was measured using the method of Hammond and Lähteenmäki (1983). Samples of diluted plasma were incubated in DCC (0.025% dextran, 0.25% charcoal in PBSG) for 30 min at 20 C to remove endogenous steroids. After centrifugation (10 min at 2,000 X g) the samples (100 µl) were incubated at 4 C overnight (12 to 16 hours) with a saturating amount (10 nM) of [^{3}H]cortisol in the presence or absence of 2 µM cortisol, with a final volume of 300 µl. Separation of CBG-bound steroid was achieved by incubation (10 min at 0 C) with 600 µl DCC suspension followed by centrifugation (10 min at 2,000 X g at 4 C). The supernatants were decanted into 4 ml of scintillation cocktail and counted using a liquid scintillation counter. Under these conditions, dissociation of normal sheep CBG-bound [^{3}H]cortisol (off-rate) is 30% during DCC separation of bound and free. The CBG concentration in diluted plasma was calculated by subtracting the non-specific binding from the total binding and multiplying by the efficiency of counting. This number was then multiplied by the conversion from µmole to pmole, by 10 (to give per ml), by the off-rate, and by the initial dilution factor.

2.2.5 Bradford protein assay

All protein determinations were made using the Bradford protein assay (Bradford, 1976; BioRad Laboratories, Richmond, CA) Briefly, after Concanavalin A chromatography, samples of eluate from the Concanavalin A column (100 µl) were mixed with 2 ml of diluted Bradford reagent (according to the manufacturers instructions: diluted 4:1 with distilled water and filtered using Whatman #1 filter paper (Fisher Scientific, Toronto, Ont.)). The absorbance of the solution was then read using a spectrophotometer (at 595 nm; CDU-64, Beckman Instruments (Canada) Inc., Toronto, Ont.).

2.2.6 Concanavalin A chromatography of fetal and adult sheep plasma

Details of individual Concanavalin A chromatography methodologies are contained within the chapters (chapters 4, 6 and 7) in which the experiments were performed. Concanavalin A was purchased from Sigma Chemical Co. (Mississauga, Ont.).

2.3 Northern blot analysis

2.3.1 Extraction of total RNA

Total RNA was extracted using the LiCl/Urea method. Frozen tissue (liver 0.5-0.75 grams, all other tissues approximately 1 gram) were placed into falcon tubes (50 ml; Falcon Labware, Becton Dickinson and Co., Lincoln Park, N.J.)

kept on ice, and containing 3 M LiCl/urea (20 ml) and heparin (0.2 ml). The tissue was homogenized using a polytron homogenizer (PT-15; Brinkman Instruments, Rexdale, Ont.). The falcon tubes were then capped and allowed to stand overnight (4 C). After the overnight incubation, the homogenate was then transferred to Nalgene centrifuge tubes (30 ml; Nalge Co., Rochester, N.Y.) and spun at 31,000 X g for 30 minutes. The supernatant was decanted and the pellet resuspended in heparinized LiCl/Urea (as above; 20 ml). After vortexing (1 min) the tubes were allowed to stand for 60 min (4 C), and then centrifuged at 31,000 X g for 30 min. The supernatant was again decanted and the resultant pellet was dissolved in Tris-SDS-heparin (TSH; 10 ml) buffer (10 mM Tris/HCL, 0.5% SDS and 100 U/ml heparin), and allowed to stand for 60 min at room temperature. The solution was transferred to a new set of falcon tubes (50 ml), and an equal volume (10 ml) of chloroform:isoamyl alcohol (24:1) was added and vortexed several times. The mixture was then centrifuged at 2,700 X g for 20 min (10 C). The aqueous layer on top was transferred to a second set of falcon tubes (50 ml), and a further 4 ml of TSH buffer was added to the first set of falcon tubes. After vortexing the tubes were again centrifuged for 20 min (2,700 X g at 10 C) and the aqueous layer was added to the second set of tubes. The RNA was precipitated from the TSH by the addition of 3 M sodium acetate (1:10 v/v) and 100% ethanol (2.5:1 v/v), and the solution placed overnight at -20 C. The tubes were then centrifuged at 2,700 X g for 30 min (0 C). The supernatant was decanted and 3 ml of 100% ethanol was added to the pellet, and vortexed.

After vortexing, the tubes were centrifuged for 30 min (2,700 X g at 0 C), the supernatant was again decanted, and the pellet dried under vacuum. The RNA pellet was resuspended in 400 µl of DEPC water (autoclaved double distilled water treated with 0.02% diethly-pyrocarbonate). The dissolved RNA was transferred to Eppendorf tubes (1.5 ml), 95% ethanol (1 ml) added, and the Eppendorf tubes stored at -70 C.

The amount and purity of the total RNA were determined using a spectrophotometer (CDU-64, Beckman Instruments (Canada) Inc. Toronto, Ont.). The absorbance of the solution was measured at 260 nm (for nucleic acid concentration), and 280 nm (for protein concentration). A ratio of the absorbances at 260 nm and 280 nm of approximately 2:1 was considered acceptable for subsequent analysis.

2.3.2 Determination of integrity of RNA

The integrity of the RNA preparation was assessed using 1% agarose formaldehyde gel electrophoresis. Samples of total RNA (10 µg) were run on the agarose formaldehyde gel and stained with 300 ml of an ethidium bromide (0.1%) 2β-mercaptoethanol (0.1%) solution (2 h). The gel was then destained (1 h) in 2β-mercaptoethanol (0.1%) and visualized under ultraviolet (UV) light. RNA was considered intact, and suitable for Northern blot analysis, if two distinct bands representing the 18S and 28S ribosomal RNAs, were clearly evident.

2.3.3 Preparation of ^{32}P-labelled cDNA probes

The CBG cDNA probe (see chapter 3), and 18S rRNA cDNA probe were labelled using the random primer technique (Feinberg and Vogelstein, 1983). The cDNA was denatured by heating in boiling water for 5 min and then cooling on ice for two minutes. The cDNA (100 ng) was then added to the reagent mix (10 µl; provided with an oligolabelling kit, Pharmacia Biotech Inc., Baie d'Urfe, Que.), [^{32}P]dCTP (5 µl; 3000 Ci/mmol), Klenow fragment (1 µl; provided with the oligolabelling kit) and distilled water to a final volume of 50 µl. The labelling reaction mixture was then incubated for 60 min (37 C). The labelled probe was purified using a Sephadex G-50 minicolumn (Nick column; Pharmacia Biotech Inc., Baie d'Urfe, Que.). The specific activities of the labelled probes were 1-2 X 10^8 cpm/µg.

2.3.4 Northern blot hybridization

Cellular RNA (20 µg) samples were electrophoresed through a 1% agarose gel in the presence of formaldehyde (Rave, Crkvenjakov and Boedtker, 1979), and transferred to a nylon membrane (Zetaprobe, Bio-Rad, Mississauga, Ont.) by capillary blotting (Thomas, 1980; Figure 2.2, page 67). The resulting Northern blots were exposed to ultraviolet light for 5 min, baked at 80 C for 1 h and prehybridized for 24 h at 42 C in the presence of 50% formamide (Smith and Hammond, 1989). Hybridization was performed under the same conditions in the presence of ^{32}-labelled (see above) ovine CBG cDNA (10^6 cpm/ml buffer). The

blots were then washed to high stringency with 0.1 x SSC (1 x = 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 42 C for 30 min and autoradiographed against XAR-5 film (Kodak Eastman Co., Rochester, N.Y.) using a Cronex Hi-Plus intensifying screen (DuPont, Wilmington, DE.) at -70 C. To ensure that approximately equal amounts of intact RNA were present in all lanes, the blots were immersed in boiling stripping buffer (5 times in 200 ml; 0.5% SDS, 0.1% SSC) to remove the hybridized probe, and rehybridized with a ^{32}P-labelled cDNA for mouse 18S ribosomal RNA (Smith and Hammond, 1991).

2.3.5 Quantification of RNA

The relative intensity of the signals achieved by autoradiograms of the Northern blots were quantified using laser densitometry (Ultrascan XL, LKB, Bromma, Sweden). The relative densities of the hybridization signals were expressed as arbitrary absorbance units. All autoradiographic signals obtained were within the linear response of the x-ray film. To correct for differences in efficiency of loading and transfer, all absorbances were expressed as the ratio of the signals of CBG cDNA to 18S rRNA.

2.4 Statistical analysis

Statistical analysis for each set of data is described in the methods section of the individual chapters in which the data are presented.

Figure 2.1 Diagram of an ultrafiltration vial. Plasma incubations (200 µl) are pipetted into the inner tubes. the vials capped, and the entire assembly is then centrifuged at 2000 X g for 1 h at 37 C. Free [^{3}H]cortisol and [^{14}C]glucose pass through the dialysis membrane with the ultrafiltrate, and are absorbed by the three filter paper disks below the dialysis membrane. (taken from Hammond *et al.*, 1980)

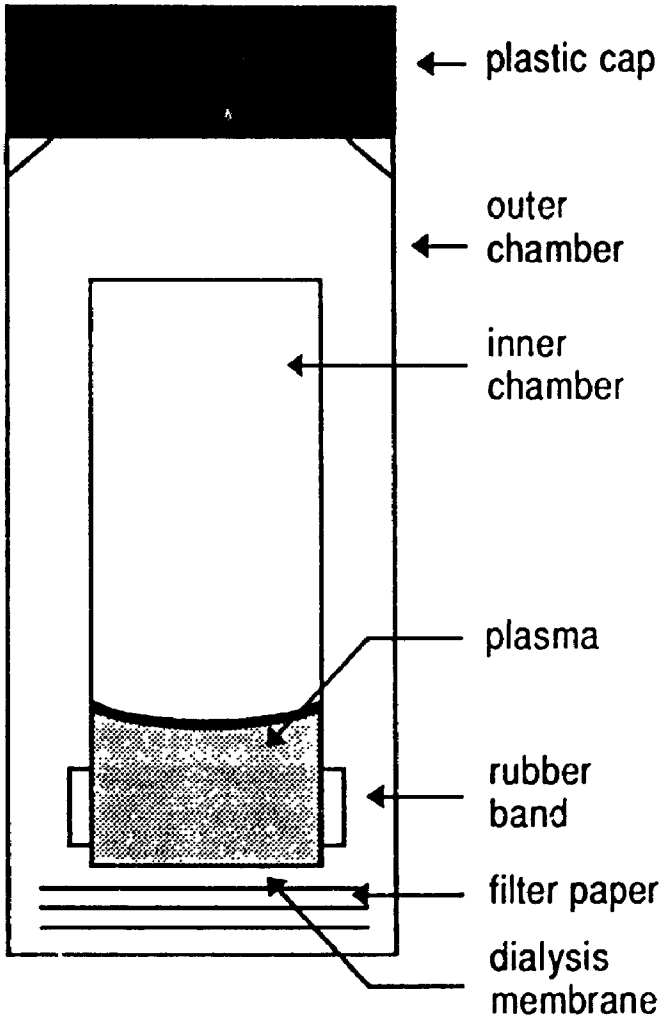
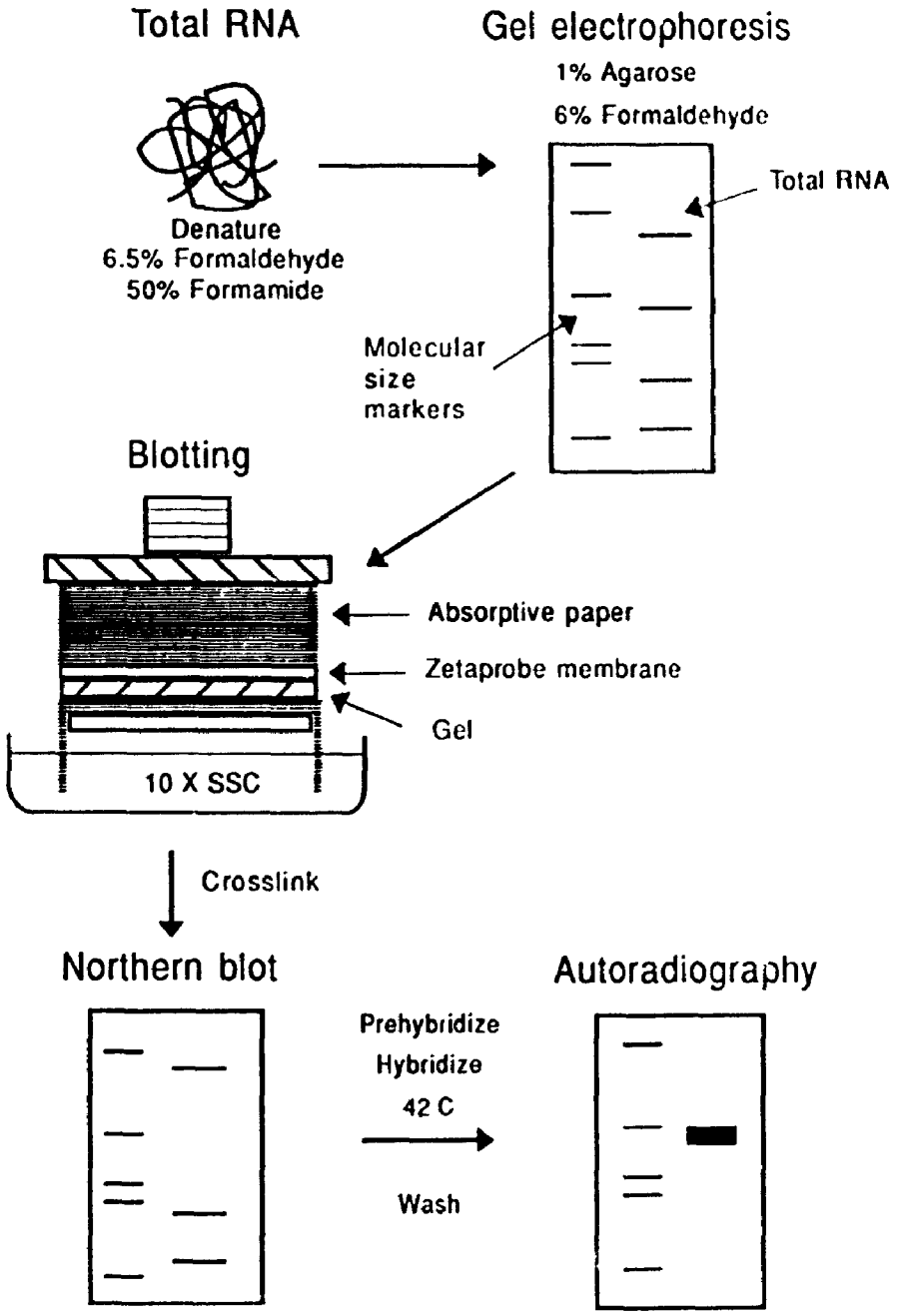

plastic cap
outer chamber
inner chamber
plasma
rubber band
filter paper
dialysis membrane

Figure 2.2 Diagrammatic representation of procedure to perform Northern blot analysis, hybridize with a labelled cDNA and perform autoradiography to quantify the abundance of mRNA within the sample of total RNA.

Northern blot analysis



CHAPTER 3

OVINE CORTICOSTEROID-BINDING GLOBULIN: cDNA CLONING AND PEPTIDE PURIFICATION

3.1 Introduction

During the studies described in this thesis, measurements of CBG mRNA abundance were conducted using the technique of Northern blotting. Amounts of CBG mRNA within selected tissues of the fetal and adult sheep were determined. In addition, the effects of manipulating the fetal or adult endocrine environment, in response to particular glucocorticoid concentrations, on tissue CBG mRNA content, were established. In order to undertake such analyses, it was necessary to clone and sequence an ovine CBG cDNA.

Within other studies to be described in this thesis, the possibility of transplacental transfer of CBG from the ewe to the fetus was examined. Therefore, CBG was purified from pregnant ewes, and iodinated to measure possible transfer of [^{125}I]CBG between the maternal and fetal compartments.

3.2 Materials and methods

All chemicals that were used in the experiments within this chapter, unless stated otherwise, were obtained from Sigma Chemicals Co. (Mississauga, Ont.).

3.2.1 Ovine CBG cDNA cloning and sequencing

3.2.1.1 Isolation of ovine CBG cDNA

A λgt10 ovine liver cDNA library (Clontech Laboratories Inc., Palo Alto, CA) was screened with a rat CBG cDNA (Smith and Hammond, 1989) at reduced stringency (40% formamide, 37 C). Two separate positive plaques were cloned,

recombinant phage were purified (Maniatis, Fritsch and Sambrook, 1982), and their cDNA inserts subcloned in pBluescript (SK$^+$) vectors (Stratagene Cloning Systems, La Jolla, CA.).

3.2.1.2 Preparation of single-stranded DNA templates

After subcloning into pBluescript SK$^+$, single stranded templates were purified using the protocol supplied with pBluescript (Stratagene Cloning Systems, La Jolla, CA.).

3.2.1.3 CBG sequencing reactions and gel electrophoresis

The dideoxy chain-termination method (Sanger, Nicklen and Coulson, 1977) using reagents obtained from Pharmacia (^{35}S; Pharmacia Biotech Inc., Baie d'Urfe, Que.) or Promega (T7 DNA Polymerase Sequencing System; Promega Biotech, Madison, WI.) was used to sequence the purified templates. The products of the reactions were resolved by 7.9% polyacrylamide gel electrophoresis (PAGE) in the presence of 7 M urea. Gels were fixed in 10% methanol/10% glacial acetic acid (v/v) in water, dried under vacuum and subjected to autoradiography (room temperature) using Kodak XAR-5 film (Figure 3.1, page 77). The nucleotide and deduced peptide sequence of ovine CBG is displayed in Figure 3.2 (page 79).

3.2.1.4 CBG primary structure and species sequence homology

The mature cDNA-deduced amino acid sequences from the sheep, human, rat and rabbit were aligned (Figure 3.3, page 81) and their overall homology was obtained using the PALIGN program of PC/Gene (Intelligenetics Incorporated, Geneva, Switzerland) which utilizes the method developed by Myers and Miller (1988).

3.2.2 CBG purification

3.2.2.1 Collection of maternal blood

Blood was collected from anaesthetized adult animals via femoral artery cannulation. Approximately 4 litres of blood was collected and allowed to clot overnight (4 C). Serum was pooled, and endogenous steroids were removed by treatment with DCC (0.625% dextan w/v, 6.25% charcoal w/v).

3.2.2.2 Purification of ovine CBG

The protocol used to purify CBG from adult sheep is shown in Figure 3.4 (page 83), and was as follows: affinity chromatography was performed using a HACA-DAEOX-Sepharose CL4B column (HACA-11β-hydroxy-androst-4-ene-3-oxo-17β-carboxylic acid, DAEOX- Diaminoethyloxirane) which bound CBG and albumin. The CBG and albumin were eluted from the column with 0.5 mg/ml cortisol in 10 mM Tris/HCl (pH 7.4). This was followed by Reactive Blue Sepharose CL-6B chromatography to remove albumin (Robinson, Langley and

Hammond, 1985). The final stages of purification involved fast protein liquid chromatography (FPLC) using a Mono Q (Pharmacia Biotech Inc., Baie d'Urfe, Que.) ion exchange column followed by a Superose 12 (Pharmacia Biotech Inc., Baie d'Urfe, Que.) gel filtration column.

3.2.2.3 Gel-electrophoresis of ovine CBG

Fractions (1 ml) from the Superose 12 gel filtration column that contained corticosteroid-binding activity were pooled and purity of the ovine CBG preparation was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples (1, 2 and 5 μg) were incubated (95 C for 4 min) in the presence of β-mercaptoethanol (0.1 M) and 1% SDS and applied to an SDS-polacrylamide gel consisting of a 4% acrylamide stacking gel and a 7.5% acrylamide separating gel (Laemmli, 1970). Following electrophoresis (100 volts for approximately 1 h), gels were fixed in 30% methanol/10% glacial acetic acid, and proteins stained with Coomasie Brilliant Blue (Figure 3.5, page 85, lanes A, B and C). Pre-stained SDS-PAGE size-standards were obtained from Bio-Rad Laboratories (Richmond, CA.).

3.2.2.4 Iodination of purified ovine CBG

Samples of purified ovine CBG (approximately 50 μg) in 0.1 M borate buffer (pH 8.0) were added to [^{125}I]Bolton-Hunter reagent (100 μCi), which had been previously placed and dried in eppendorf tubes (1.5 ml). The mixture was

then incubated overnight (4 C; 12-16 h). Radiolabelled proteins were separated from free iodine and other low molecular weight (M_r) material on a Sephadex G-25 column (PD-10; Pharmacia Biotech Inc., Baie d'Urfe, Que.), which had been pre-equilibrated with 0.1 M Tris-HCl (pH 8.0), containing 0.1 M NaCl and 0.3% gelatin. Labelled CBG was then eluted with the pre-equilibration buffer, and purity of labelled CBG in the void volume was then assessed by SDS-PAGE followed by autoradiography (Figure 3.5, page 85, lane D).

3.3 Results and discussion

The sheep CBG cDNA sequence which was obtained from two independent clones included the entire coding region for the sheep CBG precursor polypeptide. It also contained the amino-terminal sequence (20 residues) of purified sheep CBG which began 22 residues from the AUG codon for the initiating methionine (Figure 3.2, page 79, underlined). As in the CBG precursor polypeptides of other species (Hammond *et al.*, 1987; Smith and Hammond, 1989; Seralini *et al.*, 1990), these first 22 amino acids are predominantly hydrophobic, and represent a signal peptide that is cleaved during the process of secretion. This information has allowed us to deduce that the mature protein consists of 408 amino acids with a polypeptide molecular weight of 45,546, and contains 5 consensus sites (Bause, 1983) for *N*-glycosylation. One of these (position 237 in the sheep sequence) is conserved in all species examined to date (Hammond *et al.*, 1991), and glycosylation at this position has

been shown to be an absolute requirement for steroid binding (Avvakumov *et al.*, 1993). The sheep CBG cDNA sequence displayed greater than 75% homology within the coding regions for rat, rabbit and human CBG (Hammond *et al.*, 1987; Smith and Hammond, 1989; Seralini *et al.*, 1990; Figure 3.3, page 81).

Purified ovine CBG from maternal serum displayed an average M_r of 57,000 (Figure 3.5, page 85) by SDS-PAGE, and this value is in agreement with a molecular weight of 57,500 for this protein derived using sedimentation coefficients (Kato *et al.*, 1988).

The ovine CBG primary structure exhibits 68% sequence identity with human CBG, but includes 25 additional residues at the C-terminus. This is unusual, but sequence analysis of two independent clones indicated the presence of a Glu (GAA) codon in the position of the TAA stop codon (at amino acid 384 in the human peptide; Figure 3.3, page 81) tnat is present in the CBG cDNAs of other species (Hammond *et al.*, 1987; Smith and Hammond, 1989; Seralini *et al.*, 1990). In addition, sequence alignment between the immediate 3' region of the ovine cDNA with the 3' non-coding region in the human CBG cDNA (Hammond *et al.*, 1987), demonstrated at least 60% sequence homology, as indicated in Figure 3.2 (page 79, boxed region).

The primary structure of ovine CBG exhibits 68%, 69% and 56% sequence homology with the primary structures of the human, rabbit and rat, respectively (Figure 3.3, page 81). The degree of homology between these species is likely a function of the evolutionary distances at which the CBG sequences diverge

from one another. Within the aligned primary structures of the sheep, human, rabbit and rat, there are well conserved regions (such as amino acids 43-48), and it is likely that these regions are involved in the maintenance of evolutionarily-conserved activities of the proteins, such as steroid binding.

The sequence comparison between the sheep, human, rabbit and rat primary structures exhibit poor homology between amino acids 333 and 354. CBG is a member of the serine proteinase inhibitor (SERPIN) superfamily (Travis and Salvasen, 1983), and the region of poor homology includes the centre for specific interaction with the serine protease, human neutrophil elastase (Pemberton, Stein, Pepys, Potter, and Carrell, 1988; Hammond *et al.*, 1990). It has been suggested that serine proteases are rapidly evolving, and these reactive centres have co-evolved with the serine proteases in order to maintain the appropriate inhibitory activity on the serine proteases (Hammond *et al.* 1991).

Figure 3.1. Autoradiograph of sequencing gel which encodes amino acids 166 (Ser = Serine) to 198 (Phe = Phenylalanine) of the mature peptide. (A=adenine, T=thymine, G=guanine, C=cytosine).

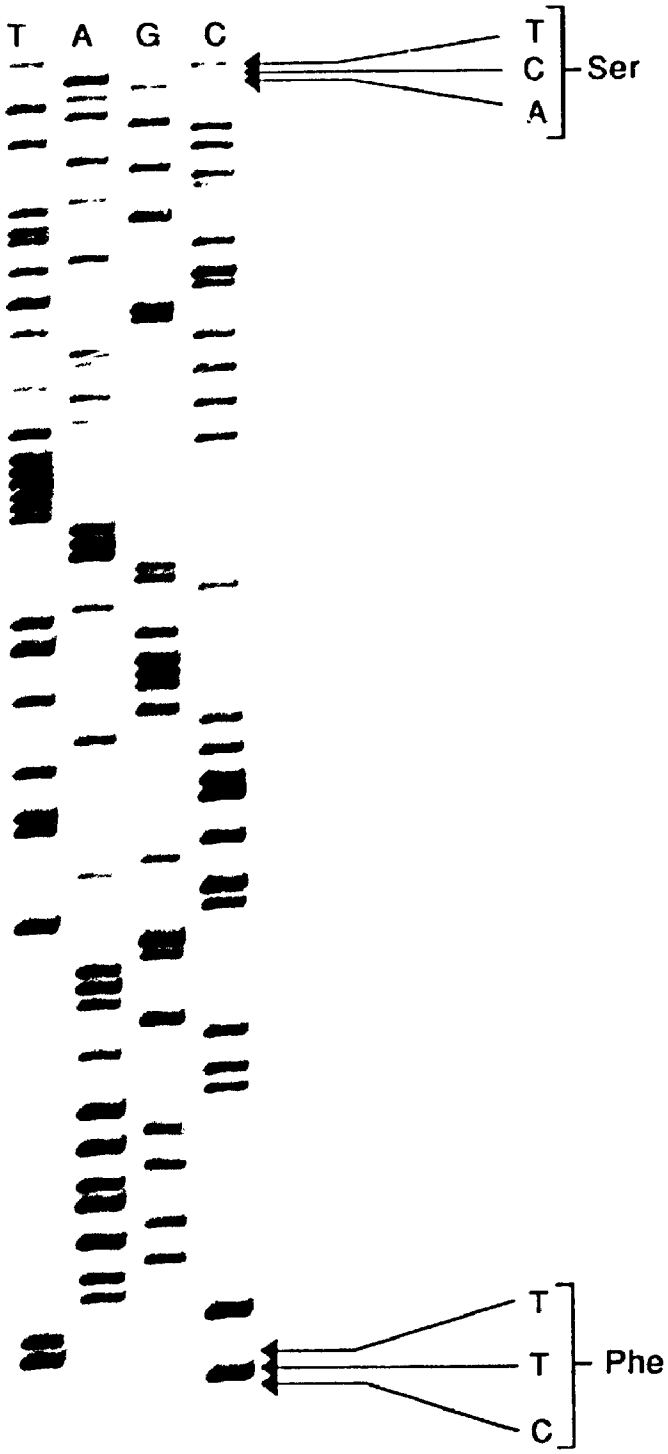

T
A
G
C
T
C
A
Ser
T
T
C
Phe

Figure 3.2. Ovine CBG cDNA nucleotide sequence and deduced amino acid sequence o' the precursor peptide. The underlined amino acids were identified by amino-terminal analysis. The CBG sequence contains 5 consensus sites for *N*-glycosylation (•). Amino acid sequence number is on the left and cDNA sequence on the right. The sequence within the 3' region encoding part of the C-terminal portion of ovine CBG that shares homology with the 3' non-coding region of the human CBG cDNA sequence is boxed. Nucleotide sequences within the box that align between the ovine and human cDNAs are underlined. The sequence was obtained from two independent cDNA clones, and the arrow at Asn-19 represents the 5' terminus of the shorter (1.2kb) cDNA.

```
                                                                                GCCAGCAGTTGCAGCCTGAGCCCACTGCAGACCGACCTGGCCATCCTGGACA      52

-22     Met Leu Leu Thr Leu Tyr Thr Cys Leu Leu Trp Leu Ser Thr Ser Gly Leu Trp Thr Ile
        ATG CTG CTC ACG CTG TAC ACC TGT CTC CTC TGG CTG TCT ACC AGT GGG CTC TGG ACC ATT      112

-2      Gln Ala Lys Gly Thr Asp Thr Asp Val Ser Thr Arg Asn Pro His Arg Asp Leu Ala Arg
        CAG GCT AAG GGC ACT GAC ACT GAC GTG AGC ACC AGG AAC CCT CAC CGG GAC TTG GCT CCA      172
        ---->

19      Asn Asn Val Asp Phe Ala Phe Thr Leu Tyr Lys His Leu Val Ala Ser Ala Pro Gly Lys
        AAC AAT GTT GAC TTT GCC TTT ACC CTG TAT AAG CAC TTA GTG GCC TCA GCT CCA GGC AAG      232

39      Asn Val Phe Ile Ser Pro Val Ser Ile Ser Met Ala Leu Ala Met Leu Ser Leu Gly Ala
        AAT GTC TTC ATC TCC CCT GTG AGC ATC TCC ATG GCC TTA GCC ATG CTG TCC CTG GGT GCC      292

59      Arg Gly Tyr Thr Arg Glu Gln Leu Leu Gln Gly Leu Gly Phe Ser Leu Val Glu Met Ser
        AGA GGC TAC ACA CGG GAG CAA CTT CTC CAA GGC CTG GGC TTC AGC CTT GTT GAG ATG TCC      352
                                                                          ●

79      Glu Ala Glu Ile His Gln Ala Phe Arg His Leu His His Leu Leu Arg Glu Ser Asn Thr
        GAA GCT GAG ATC CAC CAG GCC TTC CGG CAT CTC CAC CAC CTC CTC AGG GAG TCA AAC ACC      412

99      Thr Leu Glu Met Thr Met Gly Asn Ala Leu Phe Leu Asp His Ser Leu Glu Leu Leu Glu
        ACC TTG GAA ATG ACC ATG GGC AAT GCC TTG TTC CTT GAC CAC AGC CTG GAG CTT CTG GAG      472

119     Ser Phe Ser Ala Asp Thr Lys His Tyr Tyr Glu Leu Glu Ala Leu Thr Thr Asp Phe Gln
        TCG TTC TCA GCA GAT ACC AAG CAC TAC TAC GAG CTG GAG GCC TTG ACC ACA GAT TTT CAG      532
                                                                  ●

139     Asp Trp Ala Gly Ala Ser Arg Gln Ile Asn Glu Tyr Ile Lys Asn Lys Thr Gln Gly Lys
        GAC TGG GCA GGA GCC AGC AGG CAA ATC AAC GAG TAT ATC AAG AAT AAG ACG CAA GGG AAA      592

159     Ile Val Asp Leu Phe Ser Glu Ser Asp Ser Ser Ala Met Phe Ile Leu Val Asn Tyr Ile
        ATT GTG GAC TTG TTC TCG GAG TCA GAT AGC TCA GCC ATG TTC ATC CTG GTC AAC TAC ATC      652

179     Phe Phe Lys Gly Met Trp Val His Ser Phe Asp Leu Glu Ser Thr Arg Glu Glu Asn Phe
        TTT TTT AAA GGC ATG TGG GTG CAC TCC TTC GAC CTG GAA AGC ACC AGA GAA GAG AAC TTC      712

199     Tyr Val Asn Glu Ala Thr Thr Val Trp Val Pro Met Met Phe Gln Ser Asn Thr Ile Lys
        TAT GTG AAT GAG GCG ACG ACA GTG TGG GTG CCC ATG ATG TTC CAG TCG AAC ACC ATC AAG      772
                ●                                                           ●

219     Tyr Leu Asn Asp Ser Val Leu Pro Cys Gln Leu Val Gln Leu Asp Tyr Thr Gly Asn Glu
        TAC CTG AAC GAC TCG GTG CTC CCC TGC CAG CTG GTA CAG CTG GAC TAT ACA GGC AAT GAG      832

239     Thr Val Phe Phe Val Leu Pro Val Lys Gly Lys Met Asp Ser Val Ile Thr Ala Leu Ser
        ACT GTC TTC TTC GTG CTC CCA GTC AAG GGG AAG ATG GAC TCG GTC ATC ACT GCG CTG AGC      892

259     Arg Asp Thr Ile Gln Arg Trp Ser Lys Ser Leu Thr Met Ser Gln Val Asp Leu Tyr Ile
        CGG GAC ACC ATT CAG AGG TGG TCC AAG TCC CTA ACC ATG AGC CAG GTG GAT CTG TAC ATC      952

279     Pro Lys Ile Ser Ile Ser Gly Ala Tyr Asp Leu Gly Gly Ile Met Gly Asp Met Gly Ile
        CCA AAG ATC TCC ATC TCT GGA GCC TAC GAC CTT GGG GGC ATC ATG GGG GAT ATG GGC ATT     1012
                                ●

299     Ala Asp Leu Leu Ser Asn Arg Thr His Phe Ser Gly Ile Thr Gln Glu Ala Leu Pro Lys
        GCA GAC TTG CTC AGC AAC CGG ACA CAT TTC TCA GGC ATC ACC CAA GAG GCC CTG CCG AAG     1072

319     Val Ser Lys Val Val His Lys Ala Ala Leu Gln Val Asp Glu Lys Gly Leu Glu Ala Ala
        GTG TCA AAG GTG GTC CAC AAG GCC GCG CTG CAG GTC GAT GAG AAG GGC TTG GAG GCG GCC     1132

339     Ala Pro Thr Arg Val Ser Val Thr Ala Ala Pro Gly Pro Leu Thr Leu Arg Phe Asn Arg
        GCC CCT ACC CGG GTC AGC GTG ACG GCA GCG CCC GGG CCG CTC ACC CTC CGC TTC AAC CGG     1192

359     Pro Phe Ile Ile Met Ile Phe Asp Asp Phe Thr Trp Ser Ser Leu Phe Leu Gly Lys Val
        CCC TTC ATC ATC ATG ATC TTC GAC GAC TTC ACG TGG AGC AGC CTC TTC CTG GGC AAG GTT     1252

379     Val Asn Pro Thr Glu Gly Ala Leu Pro Gly Ala Lys Leu Arg Leu Thr Arg Ala Pro Arg
        GTG AAT CCG ACC GAA GGT GCA CTC CCA GGA GCC AAG CTG CGT CTG ACG CGG GCA CCC AGA     1312

399     Ala His Arg Lys Gly Trp Glu Gly Ser Pro ---
        GCC CAC AGA AAG GGG TGG GAG GGC TCC CCC TAA TCCTCCCCAGTGCCCCCTCTTGTAATAAATCACTG     1380

        TCATTGCTACTGGTGA(n)                                                           1411
```

Figure 3.3. Phylogenetic comparison of CBG primary structure, using one letter representation of amino acids, between human, sheep, rabbit and rat. Different letters represent different amino acids between species and a hyphen represents the same amino acid between species. Primary sequence homology between the sheep and human, rabbit and rat was 68%, 69%, and 56%, respectively.

```
          1         10         20         30         40         50
HUMAN     MDPNAAYVNMSNHHRGLASANVDFAFSLYKHLVALSPKKNIFISPVSISM
SHEEP      KGTDTD-STR-P--D--PN------T-------SA-G--V---------
RABBIT    A--PGGDISTRSPP----P----------RQ--SSA-DR--C-----V--
RAT             STNES--S-----PT------N--QR----N-D--TL--------

         60         70         80         90        100        110
ALAMI CLGTCGHTRAQLLQGLGFNLTERSETEIHQGFQHLHQLFAKSDTSLEMTMGNALF
--------AR-Y--E--------S-V-M--A----A-R---H-LRE-N-T----------
--------AS----T----------MP-A-------Y--H-LGE--------------
----V---■SAQ-QS■■--S-------T--A----S--Y-NY-LKQ---G---N----M-

        120        130        140        150        160        170
LDGSLELLESFSADIKHYYESEVLAMNFQDWATASRQINSYVKNKTQGKIVDLFSGLDSP
--H----------T-----L-A-TTD-----G------E-I-------------ES--S
--H----------RR-----A--TD----PR-C----E-IE-------A---L--EN-
-LQK-K-KD--L--V-Q-----A--ID-E--TK--Q---QH--D------EHV--D----

        180        190        200        210        220        230
AILVLVNYIFFKGTWTQPFDLASTREENFYVDETTVVKVPMMLQSSTISYLHDSELPCQL
-MFI---------M-VHS---E---------N-A-T-W----F--N--K--N--V-----
---I----------AH---PQ--E-KS----D--T-M----F----VK----PV---R-
-SFI------LR-I-EL--SPEN----D---N--ST------V--GS-G-FR--VF----

        240        250        260        270        280        290
VQMNYVGNGTVFFILPDKGKMNTVIAALSRDTINRWSAGLTSSQVDLYIPKVTISGVYDL
--LD-T--E----V--V----DS--T-------Q---KS--M---------IS---A---
--LD------A---------VD----------Q---KS--YRL-H-----AS---A-E-
I--D------A------Q-Q-D----------D--GKLM-PR--N-----FSM-DT---

        300        310        320        330        340        350
GDVLEFMGIADLFTNQANFSRITQDAQLKSSKVVHKAVLQLNEEGVDTAGSTGVTLNLTS
-GIMGD------LS-RTH--G---E-LP-V-------A--VD-K-LEA-AP-R-SVTAAP
RGA-AA--------------S-S-EGP--V---L-------D-H-GVEVAA--GP-Q-V-
K----DLN-K--L---SD--GN-K-VP-TLTM■----M---D-GN-LPNSTN-AP-H-R-

        360        370        380
KPIILRFNQPFIIMIFDHFTWSSLFLARVMNPV
G-LT----R--------D--------GK-V--TEGALPGAKLRLTRAPRAHRKGWEGSP
E-LT-N--R--L-L---D--------GK-VI-A
E-LDIK--K---LLL--K------MMSQ-V--A
```

Figure 3.4. Diagrammatic representation of CBG peptide purification. Purification procedure on the right describes the column used in the purification, and the procedure on the left lists the function of the different columns. Parentheses on the right indicate approximate initial starting and finishing amount (mg) and the percent recovery (%) of the protein after purification.

Protein purification

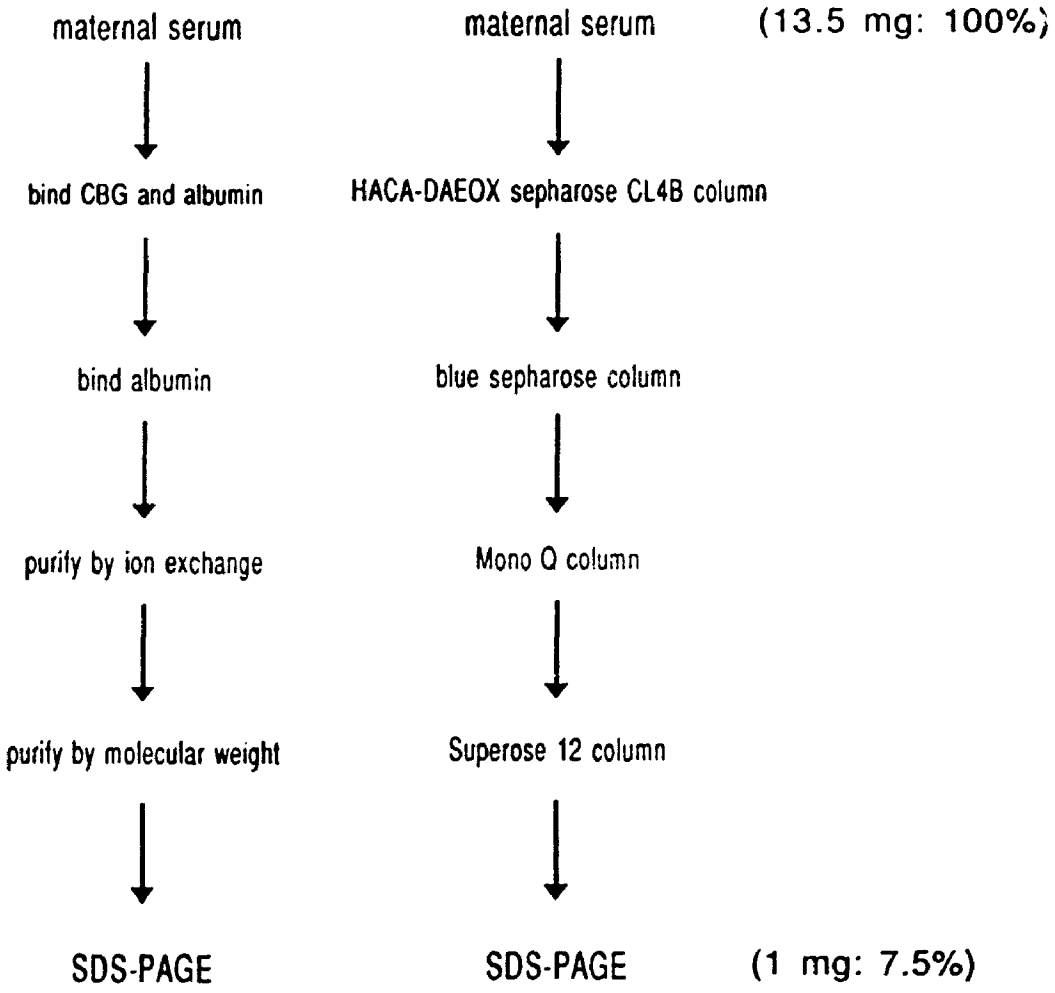
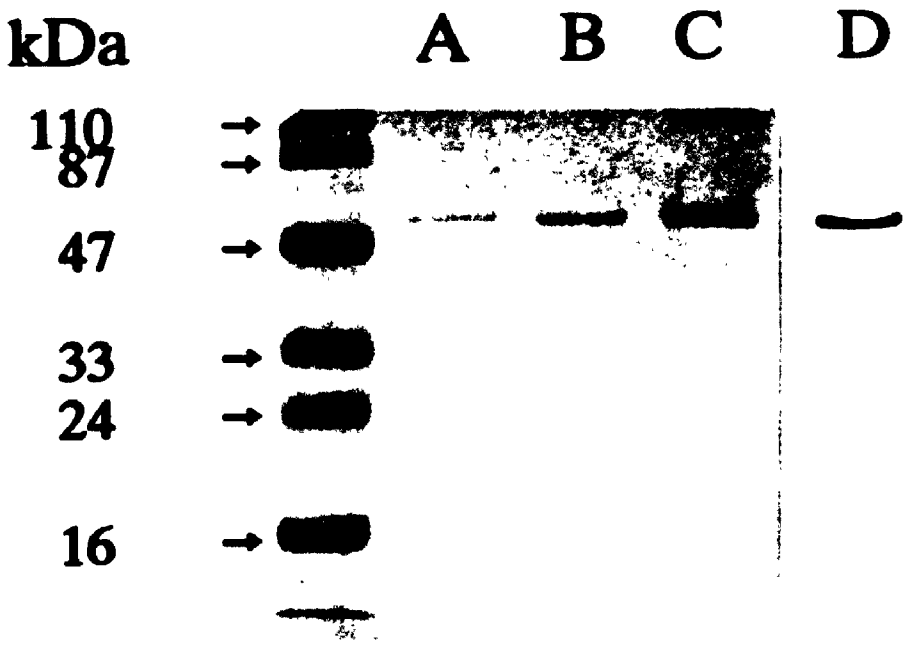


Figure 3.5. Purified ovine CBG demonstrating an average M_r of 57,000. The lanes labelled A, B, and C were loaded with 1, 2, and 5 µg of purified CBG respectively. Lane D: Autoradiogram of purified CBG after iodination, also demonstrating a M_r of 57,000.


kDa
A
B
C
D
110
87
47
33
24
16

CHAPTER 4

CONCENTRATION AND GLYCOSYLATION OF CBG IN FETAL PLASMA AND SITES OF CBG BIOSYNTHESIS DURING LATE GESTATION AND EARLY NEONATAL LIFE

4.1 Introduction

Corticosteroid-binding globulin is the major plasma binding protein for glucocorticoids in sheep (Fairclough and Liggins, 1975; Ballard *et al.*, 1982), and it is likely that CBG influences the HPA axis by modulating the bound:free ratio of plasma cortisol. Fetal plasma cortisol concentrations increase during late gestation (Bassett and Thorburn, 1969; Magyar *et al.*, 1980), but indirect measurements have indicated that the percent free cortisol in fetal plasma only increases approximately twenty-four hours before spontaneous labour (Ballard *et al.*, 1982). In the ovine fetus, plasma CBG concentrations increase during the last two weeks of gestation, but whether this reflects altered expression of the CBG gene in the fetal liver or other tissues, or a change in its half-life is not known. In all species examined to date, the liver is the major site of CBG biosynthesis, but in the fetal mouse (Scrocchi *et al.*, 1993a), fetal rabbit (Seralini *et al.*, 1990) and rat (Kraujelis, Ulinskaitė and Meilus, 1991), CBG is also produced in the kidney. However, there is no information about possible extra-hepatic sites of CBG synthesis in fetal and adult sheep. Therefore, the experiments in this chapter have determined changes in plasma CBC and sought sites of CBG production in the fetus. In addition, they have measured alterations in CBG mRNA abundance with gestation and neonatal life (within 48 h of birth). Glycosylation of proteins may affect their plasma half-life (Smith *et al.*, 1988; Hossner and Billiar, 1981), and may influence interaction with cell membranes (Avvakumov and Strel'chyonok, 1988). Therefore, we have also studied changes

in CBG glycoforms in fetal sheep plasma during late gestation and in lambs during the first month of life. Because free cortisol is generally considered the bioactive form of the glucocorticoid (Siiteri *et al.*, 1982), I have measured the percent and absolute free cortisol in plasma and related it to total cortisol and CBG capacity.

4.2 Materials and Methods

4.2.1 Materials

Unless specified, chemicals were obtained from Sigma Chemical Co. (Mississauga, Ont.). Non-radioactive cortisol was obtained from Synkron Corporation (London, Ont.), and [1,2,6,7-^{3}H]cortisol (specific activity 80-100 Ci/mmol) was purchased from NEN Research Products (DuPont Canada Inc., Mississauga, Canada) and purified by TLC in the solvent system chloroform:ethanol (95:5) before use. [^{32}P]dCTP (specific activity 3000Ci/mmol) was purchased from ICN Biomedicals (ICN Biomedicals Inc., Irvine, CA.).

4.2.2 Animals

In all our present experiments, 'animals at term' indicates the early stages of labour. Early stage of labour was based on uterine electrical activity using the criteria of Figueroa *et al.* (1985). Electrical activity in myometrium was assessed using electromyograph leads attached to the myometrium, as described previously (Challis *et al.*, 1981). Four pregnant ewes at each of gestational day

100, day 125, day 140. term (approximately day 145) in labour, and four lambs within 48 h of spontaneous birth were used. Animals were killed with a dose of Euthanyl (20% pentobarbital; MTC Pharmaceuticals, Cambridge, Ontario, Canada) suitable for euthanasia. Liver, kidney and lung from fetal and newborn animals were collected quickly, frozen in liquid nitrogen, and stored at -70 C. For examination of other possible extra-hepatic sites of CBG synthesis, placentomes were collected at day 125 and day 140 of gestation. Hypothalamus, pituitary and adrenal tissue from fetal sheep at day 125 and day 140, and from pregnant adult ewes (day 140) was collected, frozen in liquid nitrogen and stored at -70 C.

To examine the changes of fetal plasma CBC in late gestation, six fetal sheep were implanted with indwelling polyvinyl catheters into the fetal carotid artery and jugular vein, between day 110 and day 112, and under general anaesthesia, as described previously (see chapter 2). At the time of surgery the maternal femoral artery and vein were also catheterized. To measure uterine activity indicative of labour (Figueroa *et al.*, 1985), electromyographic leads were attached to the myometrium. Following surgery, both fetal and maternal catheters were filled with heparin-saline and flushed daily. Post-surgical management of the animals was as described previously (see chapter 2). After at least 5 days recovery, samples of fetal blood (3 ml) were collected daily until term. Blood gases were determined daily using an ABL-3 blood gas analyzer (Radiometer, Copenhagen, Denmark) to monitor fetal and maternal health. Fetal sheep were considered healthy if the pH was between 7.31 and 7.38, pO_2 above

18 mmHg and a pCO_2 between 40 and 60 mmHg. At term, the ewes were put into animal runs to lamb. Blood samples (3 ml) were taken from the lambs for 5 days post-partum via venipuncture. Blood (3 ml) was again taken from lambs at 1 month of age, also by venipuncture. Blood was collected into heparinized syringes kept on ice, transferred to chilled plastic tubes and centrifuged at 2500 rpm for 10 min (4 C). Plasma was stored at -20 C until analysis for CBC, total and free cortisol, and differences in CBG glycoforms.

4.2.3 Measurements of plasma cortisol and percent free cortisol

Plasma cortisol concentrations were measured by radioimmunoassay after extraction with diethyl ether. The antibody characteristics and assay validation for cortisol measurements in plasma of fetal and adult sheep have been described previously (Challis *et al.*, 1981; see chapter 2). Percent free cortisol was measured using the procedure of Hammond *et al.* (1980; see chapter 2).

4.2.4 Measurement of plasma CBC

The CBC of undiluted fetal and maternal sheep plasma was determined by the procedure of Ballard *et al.* (1982) with modifications (Challis *et al.*, 1985; see chapter 2).

4.2.5 CBG mRNA abundance by Northern blot analysis

Northern blot analysis was performed as described in chapter 2.

4.2.6 Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis

Reverse transcription (RT) was conducted on total RNA, using an RT kit supplied by Gibco/BRL (Gibco/BRL Canada Inc, Mississauga, Ont.), according to the manufacturer's instructions. Reverse transcription was followed by polymerase chain reaction (PCR) using primers designed to produce a 416 base pair (bp) fragment of the CBG cDNA. Samples (10 µl, except liver 2 µl) of the PCR products were electrophoresed through a 1.2% agarose gel, and transferred to a nylon membrane (Zetaprobe, Bio-Rad, Mississauga, Ontario) by capillary blotting (Thomas, 1980). Post-blotting procedures for crosslinking of DNA, prehybridization, hybridization using the ^{32}P-labelled ovine CBG cDNA, and autoradiography were performed as described in chapter 2.

4.2.7 Concanavalin A binding assay

Samples of fetal plasma were obtained every five days of gestation between day 125 and term. Samples were also taken from lambs at 2 and 5 days post-partum and at 1 month of age. Aliquots (20 µl) of these plasma samples were diluted with 140 µl of 50 mM Tris, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$; pH 7.0, and loaded onto 0.5 ml Con A sepharose columns by allowing the sample to enter the gel bed. The sarnple was then followed by 3 ml of the same buffer, and the eluate was collected as a single fraction (fraction 1). Proteins were removed from the column using 3 ml of 50 mM Tris and 0.5 M

NaCl containing 5% α-D-mannopyranoside (w/v), which was also collected as a single fraction. The binding capacities of fractions 1 and 2 were determined using a saturation assay that utilized dextran-coated charcoal to separate bound and free steroid (Hammond and Lähteenmäki, 1983; see chapter 2). The percentage of Con A-retarded CBG (contained in fraction 2) was expressed as a function of the total CBG (CBG contained in fractions 1 + 2).

4.2.8 Western blot analysis

Samples (10 µl) of diluted plasma (1:500 with 100 mM Tris, pH 7.4) were analyzed for immunoreactive CBG (irCBG) by Western blotting (Towbin, Staehelin and Gordon, 1979). Samples were subjected to discontinuous (4% and 7.5% acrylamide in the stacking and separating gels, respectively) SDS-PAGE. Proteins were then transferred electrophoretically (2 h at 300 mA) onto an Immobilon membrane (Millipore, Bedford, MA.). Non-specific binding of protein to the membrane was inhibited by preincubation (15 min at room temperature) of the blot with a blocking solution (4% bovine serum albumin (BSA), 0.05% Tween-20, 50 mM Tris pH 8, 150 mM NaCl). The blots were washed 3 times in 50 mM Tris pH 8, 50 mM NaCl and incubated overnight (4 C) with primary antibody (diluted 1:500) in 1% BSA, 0.05% Tween-20, 50 mM Tris pH 8, 50 mM NaCl. The antibody was raised in New Zealand White rabbits against purified ovine CBG using the methodology of Vaitukaitis, Robbins, Niechlag and Ross (1971). After the overnight incubation the blot was washed in 50 mM Tris (pH

8) and 50 mM NaCl for 5 min, then with 0.1% NP40, 50 mM Tris (pH 8) and 50 mM NaCl for 5 min, then again with 50 mM Tris (pH 8) and 50 mM NaCl for 5 min. Specific antibody-antigen complexes were detected with an alkaline phosphatase-labelled anti-rabbit secondary antibody detection system (Protoblot Immunoblotting System, Promega Corporation, Madison WI.) according to the manufacturers instructions.

4.2.9 Data Analysis

Changes in fetal and neonatal plasma CBC throughout gestation and early neonatal life were analyzed by a one-way analysis of variance (ANOVA) corrected for repeated measures. Statistical significance was determined as $P < 0.05$ and effects of individual times during gestation or neonatal-life on plasma CBC were assessed by the Student's t-test. Differences in relative abundance of hepatic CBG mRNA abundance throughout gestation and neonatal life were assessed using a one-way ANOVA followed by the Student's t-test. Con A-binding at different times in gestation and neonatal life, were assessed by a one-way ANOVA, followed by the Student's t-test.

4.3 Results

4.3.1 Plasma CBC, total cortisol and percent free cortisol in fetal and neonatal sheep

The CBC of fetal sheep plasma was 23.3 ± 4.6 ng/ml at day 115 of

gestation (Figure 4.1, page 105). Fetal plasma CBC began to increase by day 135, and there was a significant ($P < 0.05$) increase by day 140 from the day 135 values (57.9 ± 12.6 ng/ml vs 34.8 ± 4.5 ng/ml respectively). Plasma CBC was highest at term. There was a rapid decrease during the first five days post-partum to 14.4 ± 1.4 ng/ml. There was a further decrease in the plasma CBC to 5.7 ± 1.4 ng/ml at one month post-partum.

The total amount of cortisol in fetal plasma increased progressively from day 125 to term (Table 4.1, page 120). The percentage free cortisol did not change significantly from day 125 to day 145 (Table 4.1, page 120). However, there was a significant ($P < 0.05$) increase in plasma free cortisol from day 130 to day 135 (0.11 ± 0.02 ng/ml vs 0.48 ± 0.07 ng/ml, respectively) and day 140 to term (0.70 ± 0.17 ng/ml vs 5.78 ± 1.44 ng/ml, respectively; Table 4.1, page 120).

4.3.2 Abundance of CBG mRNA in liver and other tissues

The CBG cDNA hybridized with a single transcript of 1.8 kb in total RNA samples from fetal and newborn livers (Figure 4.2, page 107; Figure 4.3, page 109). Fetal hepatic CBG mRNA abundance was similar at days 100 and 125. It increased significantly ($P < 0.05$) at day 140, but was decreased at term, when it was not different from that observed in day 100 and 125 fetuses, or in newborns (Figure 4.3, page 109).

There was no detectable CBG mRNA in total RNA from the kidney and

lung at day 100, 125, 140, term and neonatal life (n=4 for all categories) with exposure times of 24 h and 2 weeks (Figure 4.4, page 111). CBG mRNA was detectable in the pituitary at day 125 (n=2) and 140 (n=2) of gestation and in the adult ewe (n=1), but was much less abundant than in the liver (Figure 4.5, page 113). CBG mRNA was also detectable in the fetal adrenals at day 125 but not at day 140 or in the adult. There was no detectable CBG mRNA by Northern blot analysis in total RNA from hypothalamus at day 140 or in the adult, or total RNA from placentome at day 125 and 140 (Figure 4.5, page 113).

4.3.3 Abundance of CBG mRNA by RT-PCR and Southern blot analysis

A 416 bp RT-PCR product generated using total RNA, from tissues at day 140 of gestation and which hybridized with the CBG cDNA was observed in greatest abundance in the liver and the pituitary. However, the abundance in the liver was much greater (approximately 40 fold) than in the pituitary (Figure 4.6, page 115). There was a low abundance of the CBG cDNA fragment in the adrenal, lung, kidney and hypothalamus, and no signal in placentome.

4.3.4 CBG elution profiles of fetal, neonatal and adult sheep using Concanavalin A chromatography

In fetuses from gestational age day 125 to day 140, the relative proportion of Con A-retarded CBG remained relatively constant at approximately 10%

(Figure 4.7, page 117). The proportion of Con A-retarded CBG increased at term and, by 2 days post partum, was elevated significantly from the day 140 values (10.2 ± 3.3% vs 24.9 ± 4.8%, respectively). By one month of age, the proportion of Con A-retarded CBG (approximately 72%) was similar to that in maternal plasma (approximately 71%; Figure 4.7, page 117).

4.3.5 Plasma irCBG by Western blot analysis

The irCBG in plasma from fetal and adult sheep exhibited two predominant electrophoretic isoforms typical of CBG (Figure 4.8, page 119). There was an increase in irCBG which peaked at term and was decreased in the adult. There was also a shift in the predominance of the two electrophoretic forms between the fetal and adult CBG. In the fetal plasma samples, the predominant band was the larger M_r isoform whereas in plasma samples from a one month old lamb and the adult ewe, the predominant band was the lower M_r isoform. The CBG antisera complexed with purified CBG, but did display some cross reactivity to other proteins in fetal and adult plasma. CBG is a member of the SERPIN superfamily, and this cross reactivity may be with other SERPINs.

4.4 Discussion

The fetal plasma CBC values of the late gestation sheep (Figure 4.1, page 105) are similar to those previously reported (Fairclough and Liggins, 1975; Ballard *et al.*, 1982). One exception is the study by Ali, Bassett, Jones and Wynn

(1992) suggesting that the plasma CBC decreased prior to birth, increased at one day post partum and decreased thereafter. The reason for the discrepancy between the results contained in this chapter and previously in the literature, and the study of Ali *et al.* (1992) is unclear. There is a good relationship between the increase in plasma CBG levels and the increase in hepatic CBG mRNA abundance at day 140, however at term, hepatic CBG mRNA abundance began to decrease before the plasma CBC began to decrease after birth.

In the present study, plasma cortisol concentrations increase concomitantly with an increase in plasma CBC, and therefore the increase in fetal plasma cortisol may stimulate the increase in fetal plasma CBC. The reason for the rapid decline in plasma CBC post partum is likely due to a decrease in hepatic biosynthesis, but may also involve a decrease in the plasma half-life of the protein. In both the late gestation fetal sheep and the neonatal lamb, the liver is the major site of CBG biosynthesis. There is some variability in the hepatic CBG mRNA abundance of animals at day 140, term and in the newborn lamb, and this is reflected in the standard error. It is not know when, between day 140 and term, that the fall in CBG mRNA abundance occurs, but presumably it is very late in gestation as the plasma CBC continues to increase until term.

In the kidney and lung in both the ontogeny Northern blot (Figure 4.4, page 111) and the multi-tissue blot (Figure 4.5, page 113) there appeared to be a very weak signal. However there are three arguments that cause me to believe that the signal is non-specific binding of the labelled CBG cDNA to other RNA on

the blot. Firstly, on the original autoradiographs, the signal is slightly higher (greater than 1.8 kb = CBG) than the signal from the liver. Secondly, the signal very closely parallels that of the 18S rRNA used to estimate amount of RNA loaded onto each lane and therefore the signal may be non-specific binding of the CBG cDNA to 18S rRNA. Thirdly, the signal of the CBG cDNA hybidized to RNA from the kidney and lung is very close to the signal of CBG cDNA hybridized to RNA from the pituitary, however, by RT-PCR the amount of CBG mRNA in the pituitary far exceeds the amount of CBG mRNA in the kidney and lung.

The existence of extra-hepatic sites of CBG production in the fetal sheep is consistent with data from the fetal rabbit where CBG mRNA was also present in the kidney (Seralini *et al.*, 1990). In the embryonic mouse CBG mRNA distribution was limited to the liver and the exocrine pancreas (Scrocchi *et al.*, 1993b), and in the mouse the liver message was absent until about two weeks postnatally, while the kidney begins to express CBG mRNA immediately after birth (Scrocchi *et al.*, 1993a). The postnatal expression of CBG mRNA by the mouse kidney is transient, and by six weeks the liver is again the major site of CBG production (Scrocchi *et al.*, 1993a). CBG gene expression was not observed within the placenta at day 125 or day 140 of gestation, and this lack of CBG gene expression in the placentome is consistent with the observation of Scrocchi et al. (1993b) in the mouse placenta, and lack of any difference in the plasma CBC of matched fetal sheep umbilical artery and vein samples (Jacobs, Han, Hammond and Challis, 1991). Using immunohistochemistry, the peptide

has been localized in the mouse placenta, at the trophoblast decidual interface (Scrocchi *et al.*, 1993b), suggesting uptake of CBG from the circulation.

This is the first report of CBG mRNA in the fetal adrenal, although Basset (1987) in the adult rat, has reported a CBG-like protein of adrenal origin that is released in conjunction with corticosterone. The CBG synthesized within the fetal adrenal may be important for local regulation of glucocorticoid concentrations but is unlikely to contribute significantly to systemic plasma CBG concentrations. Adrenal CBG production may protect the cells within the medulla from high glucocorticoid concentrations, and if so, provides a local function for CBG produced in the adrenal. Using immunohistochemistry, CBG has been localized within the pituitary of the guinea pig (Perrot-Applanat, Racadot and Milgrom, 1984) and rat (Kuhn *et al.*, 1986). Pituitary CBG biosynthesis may work in concert with plasma CBG concentrations in regulating the intracellular concentration of free cortisol in corticotrophs, and therefore provide a mechanism to block negative feedback and promote ACTH production within the late gestation fetal sheep.

In the fetus, the distribution of CBG between Con A-retarded and Con A non-retarded glycoforms was approximately 10% and 90%, respectively. Sheep CBG has five possible sites for *N*-glycosylation (see chapter 3), but the number of these sites that are utilized, and the type of carbohydrate moieties linked to them, is unknown. The *N*-linked oligosaccharides associated with glycoproteins ma, . ..prise a variety of branched structures (Strel'chyonok and Avvakumov,

1990). The Con A-binding properties of fetal sheep CBG did not change from day 125 of gestation to day 140. Thus the relative proportion of biantennary chains associated with fetal CBG probably remains constant. The increase in the proportion of Con A-retarded CBG at term, and in the newborn, may reflect production of predominantly biantennary CBG, and possible differences in the plasma half-life of the different glycoforms of CBG. By one month of age, the predominant form of CBG is Con A-retarded, which is similar to the adult and therefore, the major form of production of CBG after birth is likely to contain at least one biantennary carbohydrate group. The existence of membrane-bound proteins that interact with CBG has been reported (Strel'chyonok and Avvakumov, 1991; Maitra *et al.*, 1993), and the Con A-retarded form may interact with this receptor(s), possibly delivering cortisol to these sites.

The amount of irCBG in fetal plasma increases between day 115 and term as determined by Western blot analysis. The amount of irCBG then decreased post-partum and was approximately equal in one month old lambs and in the adult ewe. This data corroborates the observed profile of plasma CBC in the fetal sheep, lamb and adult ewe (Figure 4.1, page 105). The switch from a predominantly high M_r isoform of CBG in fetal plasma to a primarily low M_r isoform did not appear to occur until after parturition. This change occurred at the same time as the change in Con A binding properties of the fetal and neonatal sheep (Figure 4.7, page 117). Therefore, it may be that the change in predominance of isoforms is related to a switch from a triantennary type of

glycosylation (Con A non-retarded) in the fetus to a glycosylation pattern that contains at least one biantennary carbohydrate group (Con A-retarded) on neonatal and adult CBG.

The absolute and percent free cortisol are somewhat lower than those reported by Ballard *et al.* (1982), who used the quadratic formula of Tait and Burnstein (1964) to derive their free cortisol values. However, in these experiments the total cortisol and the percent free cortisol were measured directly, and these values have been used to calculate the absolute free cortisol. This difference may explain the discrepancy in values between the two studies. The concentration of cortisol in fetal plasma increased by approximately four fold between day 130 and 135. This increase was accompanied by an doubling of plasma CBC and a four fold increase in the absolute free cortisol concentration in fetal plasma. In plasma from fetal sheep at day 135 and day 140 there was a doubling of plasma cortisol (9.1 ± 1.7 ng/ml to 19.1 ± 2.2 ng/ml). Plasma CBC increased by 1.5 times, and free cortisol remained relatively constant. Thus, the large increase in plasma total cortisol concentations was accompanied by a very small increase in free cortisol in plasma. There was an increase in cortisol concentrations in fetal plasma from day 140 to 145 of approximately 4 fold. Over this time period there was also an increase in plasma CBC (1.3 times) and an increase in the absolute free cortisol over the same time span of approximately 8 fold. Therefore, in late gestation as term approaches, the plasma cortisol concentration exceeds the CBG binding capacity for glucocorticoids. This results

in a large increase in free cortisol concentrations. These results therefore support the action of CBG in maintaining a low free cortisol during late gestation, until the total cortisol in plasma exceeds the binding capacity of CBG.

It has been demonstrated that the fetal sheep liver possesses 11β-hydroxysteroid dehydrogenase (11-βHSD; Yang, Hammond and Challis, 1992). In late gestation this enzyme favours reduction (cortisone to cortisol), and 11β-HSD mRNA abundance and enzyme activity are increased after fetal glucocorticoid treatment (Yang, Berdusco and Challis, 1994). Therefore the liver possesses the ability to actively synthesize cortisol, and CBG may function in an local fashion in the liver, to transport cortisol out of the hepatocyte. Fetal plasma cortisol is necessary for the maturation of fetal tissues and for the induction of several enzymes (Bassett and Thorburn, 1969; Thorburn and Challis, 1979), and therefore, CBG may function to protect some tissues by removing or restricting access of glucocorticoids from tissues such as the liver and pituitary, while delivering cortisol to other glucocorticoid-sensitive tissues such as the placenta and lung.

The results in this chapter indicate that plasma CBG increases in conjunction with an increase in hepatic CBG mRNA abundance to maintain a constant percent free cortisol. However, the absolute free cortisol increases in a biphasic pattern with a large increase at term. In total RNA extracted from selected tissues (liver, lung, kidney, hypothalamus, pituitary, adrenal and placenta), the liver is the major site of CBG production. There was no change

in the percentage of Con A-retarded CBG until term, and by one month of age the percentage of Con A-retarded CBG was similar to the adult. The function of the change in CBG glycosylation patterns between the fetus and adult is unclear, however, it may involve delivery of cortisol via CBG receptors to specific glucocorticoid-sensitive tissues. Extra-hepatic sites of CBG biosynthesis may include the fetal adrenal and pituitary. Production of CBG by these tissues is unlikely to contribute significantly to systemic CBG concentrations but may play a role in mediating intracellular glucocorticoid concentrations.

Figure 4.1 Plasma CBC in chronically catheterized fetal sheep during the last third of gestation, until one month of age. There was a significant ($P < 0.05$) increase in CBC at day 140 and term (day 0) over all other gestational ages. Results are mean ± SEM (n=minimum of 4). Where no error bars are visible the data point is larger than the SEM.

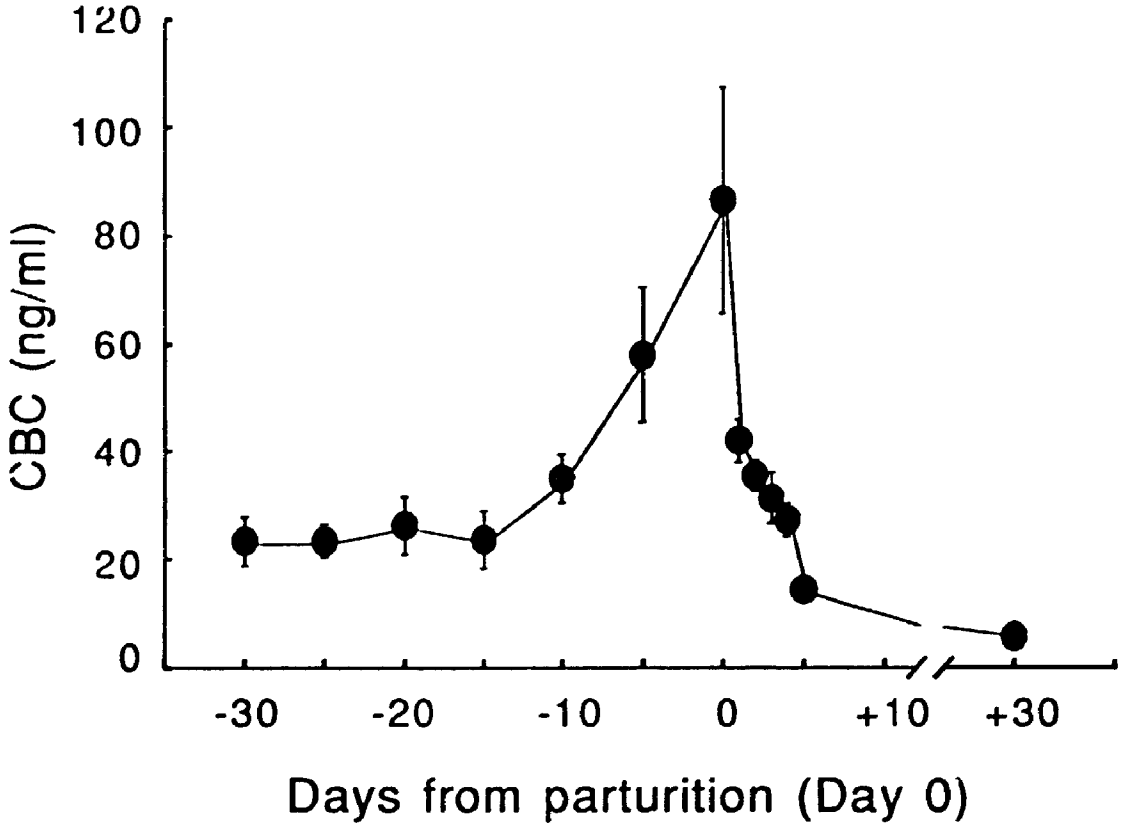

120
100
80
60
40
20
0
CBC (ng/ml)
-30
-20
-10
0
+10
+30
Days from parturition (Day 0)

Figure 4.2 Representative Northern blot of entire nitrocellulose membrane probed using a labelled CBG cDNA demonstrating a single CBG mRNA transcript. Molecular size of the CBG mRNA was determined by lambda *HindIII* molecular size markers and is shown on the right. The autoradiogram was exposed for 5 days. Therefore, in all subsequent Northern blots only the area on the autoradigaph containing the CBG mRNA signal will be shown.

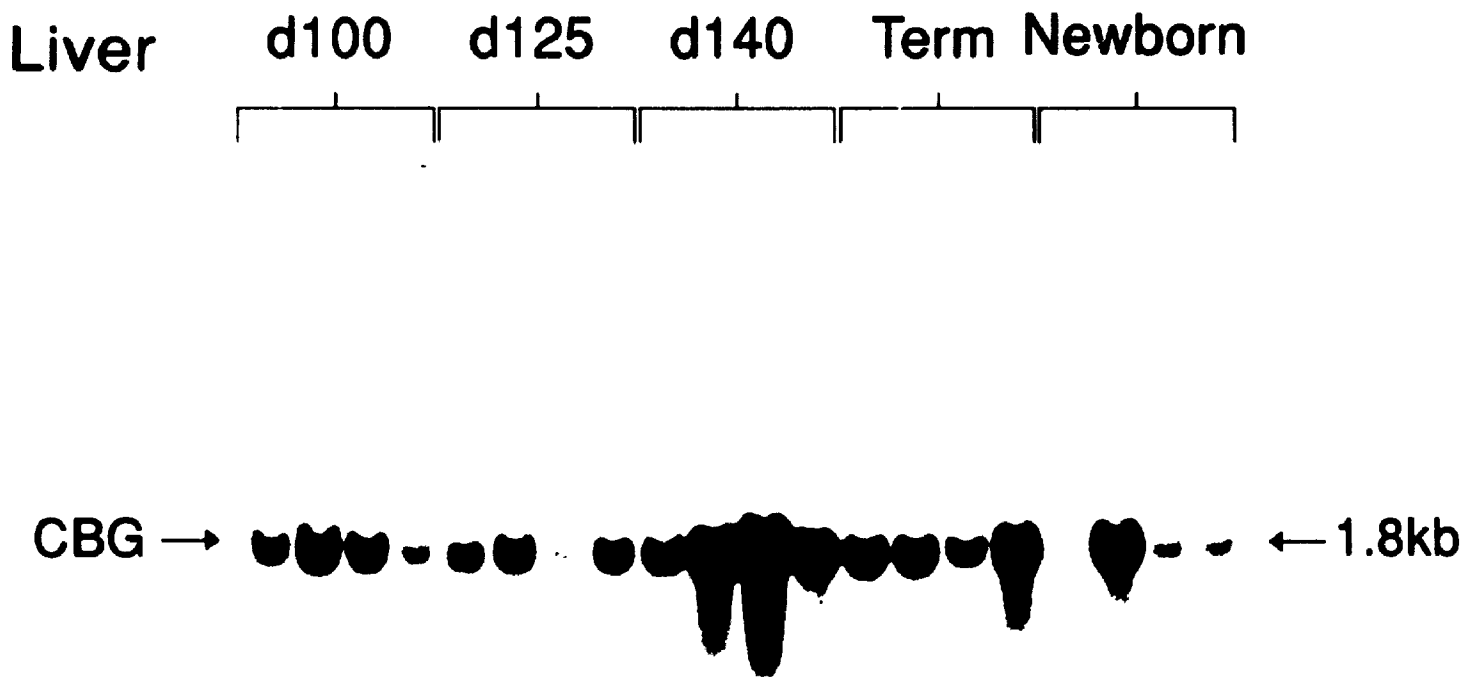

Liver
d100
d125
d140
Term
Newborn
CBG →
←1.8kb

Figure 4.3 (upper panel) Northern blot of the hepatic CBG mRNA abundance in individual fetal sheep at day 100, day 125, day 140, term and in newborn fetuses (within 48 h of birth). The autoradiograms were exposed for 24 h (A) and 5 days (B). Molecular size of the CBG mRNA was determined by lambda *HindIII* molecular size markers and is shown on the right. 18S rRNA was used to account for differences between samples in the efficiency of loading and transfer of total RNA. (lower panel) Histogram representation of the mean ratio of the signals for CBG mRNA:18S rRNA. The exposure time of 5 days (B) was used to determine the CBG mRNA abundance. There was a significant increase ($P < 0.05$) in CBG mRNA abundance at day 140 over values at day 100, day 125 and in newborns. Results are mean ± SEM (n=4 in all groups).

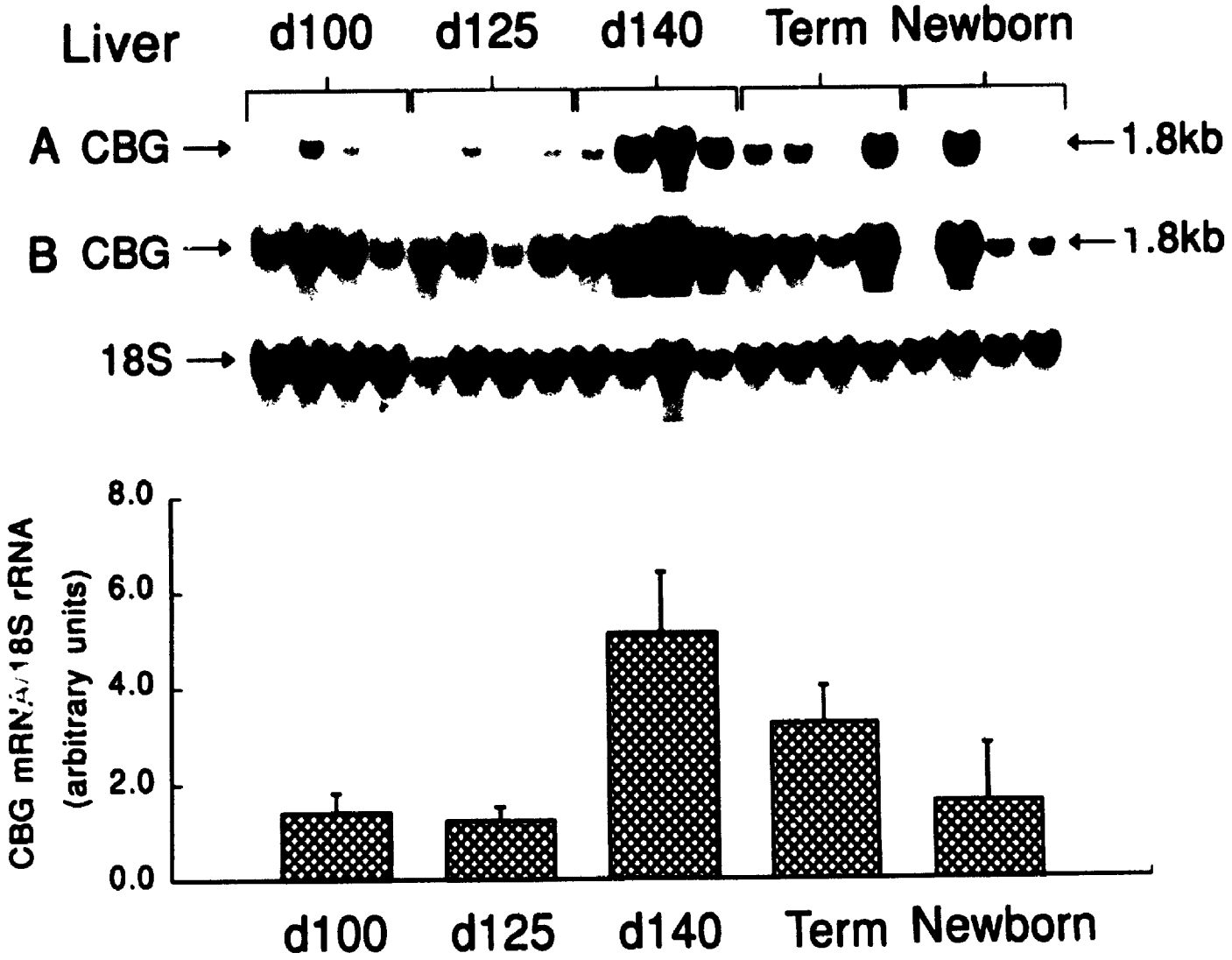

Liver
d100
d125
d140
Term
Newborn
A CBG
B CBG
18S
1.8kb
1.8kb
CBG mRNA/18S rRNA
(arbitrary units)
8.0
6.0
4.0
2.0
0.0
d100
d125
d140
Term
Newborn

Figure 4.4 Northern blot of kidney and lung CBG mRNA abundance in fetal sheep at day 100, day 125, day 140, term and in newborn fetuses (within 48 h of birth; n=4 for all groups). The autoradiograms were exposed for 24 h (A) and 14 days (B). Total RNA extracted from fetal liver at day 140 of gestation was loaded as a positive control, and is shown on the right. Molecular size of the CBG mRNA was determined by lambda *HindIII* molecular size markers and is shown on the right. 18S rRNA was used to account for differences between samples in the efficiency of loading and transfer of total RNA.

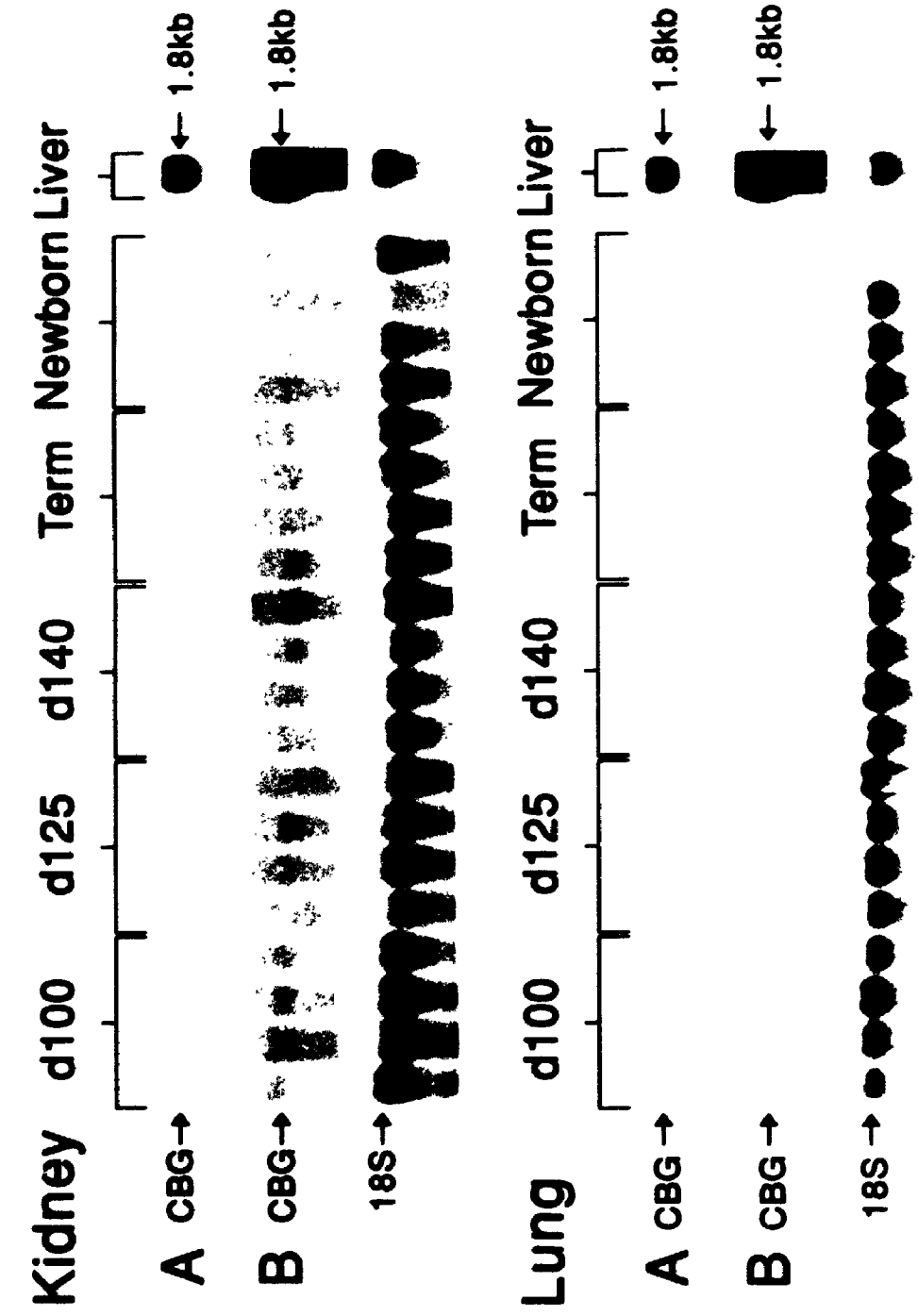

Kidney
d100
d125
d140
Term Newborn Liver
A CBG→
← 1.8kb
B CBG→
← 1.8kb
18S→
Lung
d100
d125
d140
Term Newborn Liver
A CBG→
← 1.8kb
B CBG→
← 1.8kb
18S→

Figure 4.5 Northern blot of CBG mRNA abundance in fetal tissues at day 125 (number 1), day 140 (number 2), and in adult tissues (number 3). The autoradiograms were exposed for 24 h (A) and 14 days (B). Due to the large number of samples, they were run as two blots (1, liver, kidney and lung; 2, hypothalamus, pituitary, adrenal and placenta). Molecular size of the CBG mRNA is shown on the right. 18S rRNA was used to account for differences between samples in the efficiency of loading and transfer of total RNA. CBG mRNA was present in liver, pituitary and adrenal. In the liver, the abundance was much greater in the fetus at day 140 than day 125 or in the adult. In the pituitary and adrenal, the abundance was greatest at day 125. (Hypoth = hypothalamus, Pit = pituitary, and Placen = placenta).

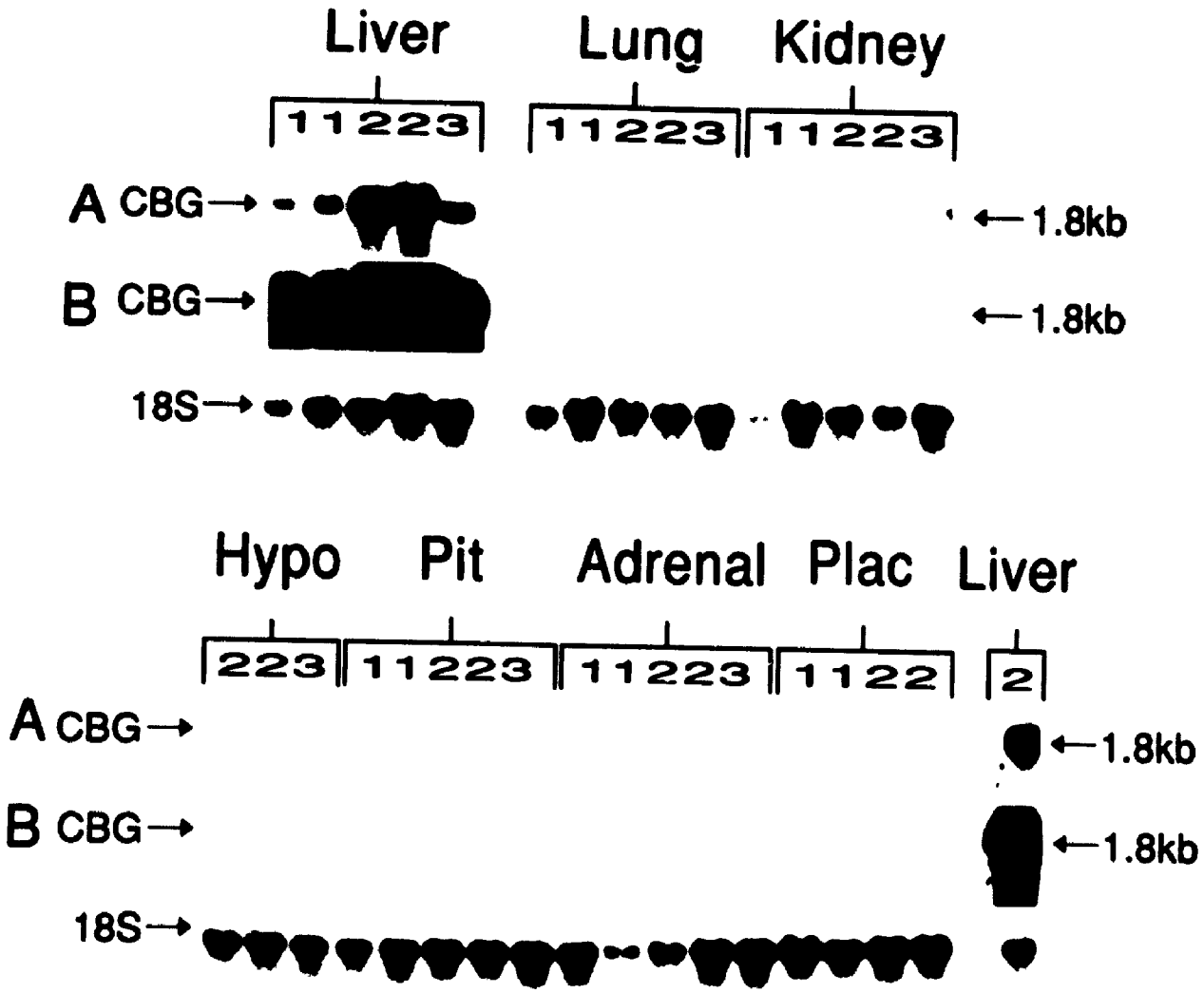

Liver
Lung
Kidney
11223
11223
11223
A CBG
B CBG
18S
1.8kb
1.8kb
Hypo
Pit
Adrenal
Plac
Liver
223
11223
11223
1122
2
A CBG
B CBG
18S
1.8kb
1.8kb

Figure 4.6 Southern blot of CBG cDNA fragment (416 base pairs) from RT-PCR generated using total RNA samples from fetal sheep tissues (n=2) at day 140 of gestation. RT-PCR products were present in liver, pituitary, adrenal, lung, kidney and hypothalamus, but not placentome. Abundance was greatest in liver and then pituitary. Panel A was exposed for 2 h and panel B was exposed for 5 days.

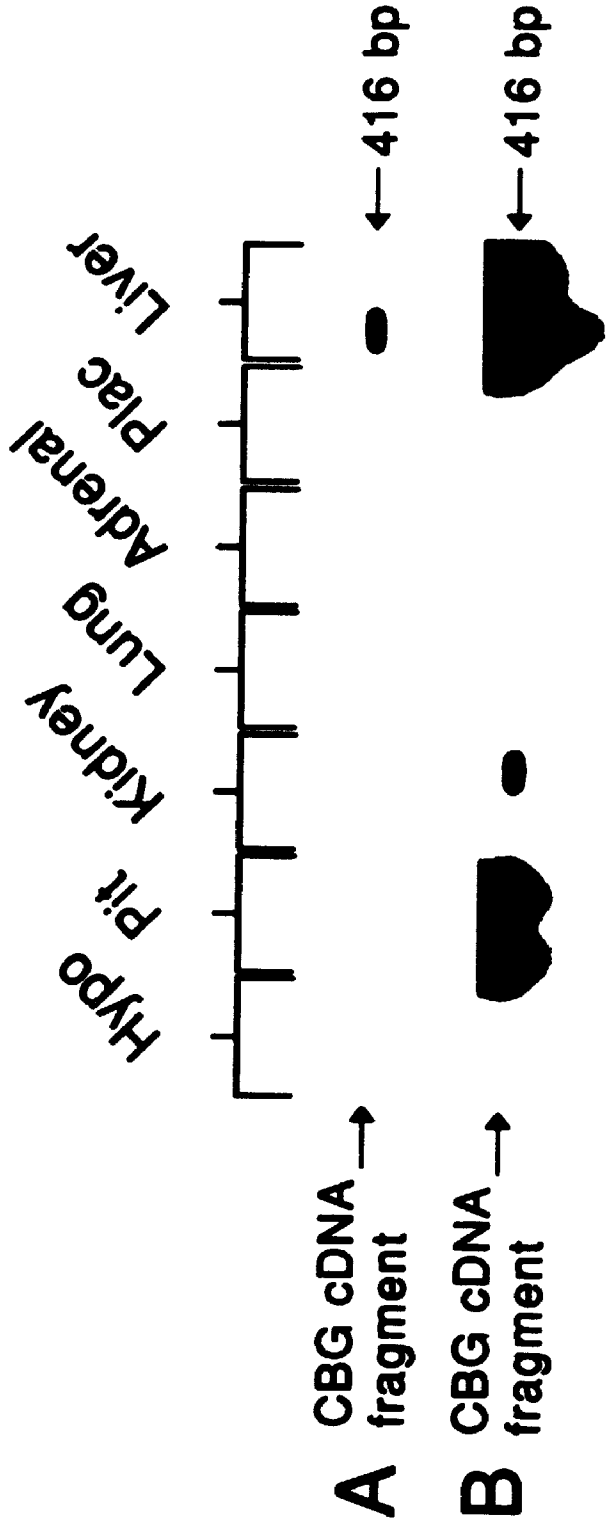

A
CBG cDNA fragment
B
CBG cDNA fragment
416 bp
416 bp
Hypo
Pit
Kidney
Lung
Adrenal
Plac
Liver

Figure 4.7 Concanavalin A-retarded CBG as a percentage of total CBG (Con A non-retarded + Con A-retarded) during late gestation fetal and in neonatal sheep. Elution percentages of CBG were approximately equal between Con A-retarded and non-retarded fractions from day 125 to term. After parturition there was a significant ($P < 0.05$) increase in the percentage of the Con A-retarded fraction. However, by one month of age the percentage of Con A-retarded sheep was similar to that of adult sheep. Data is represented as means ± SEM (n=minimum of 4). Where no error bars are visible the data point is larger than the SEM.

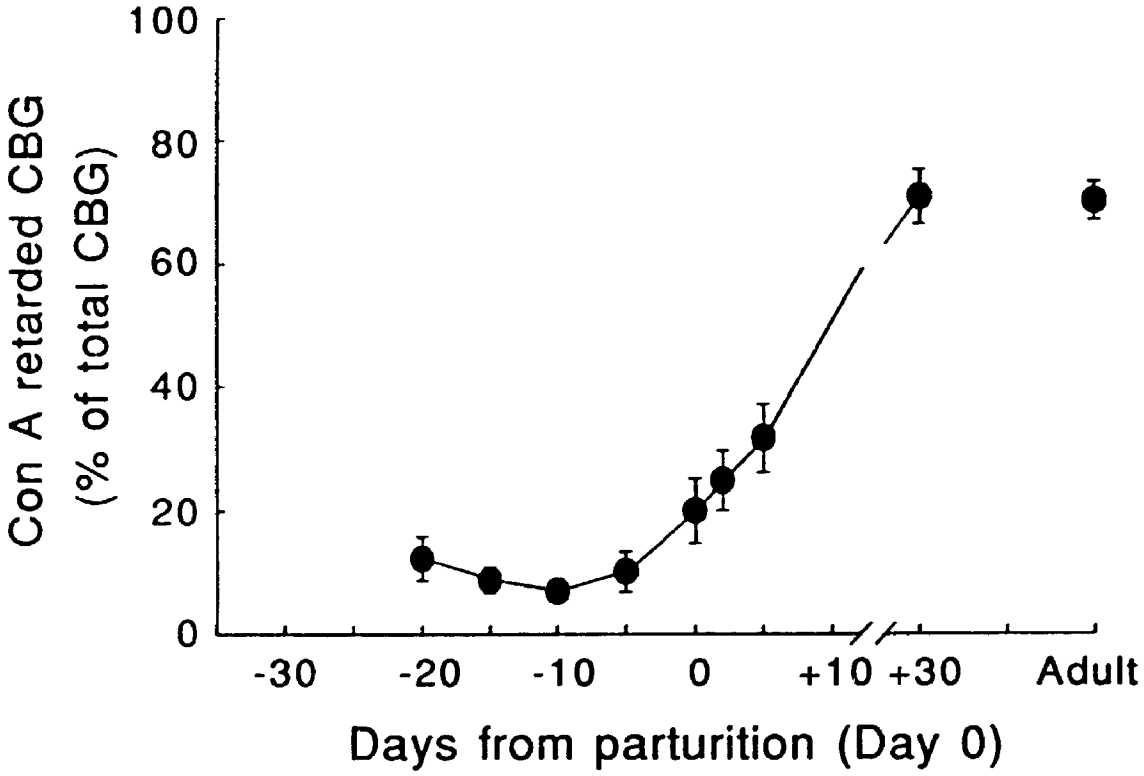

Con A retarded CBG
(% of total CBG)
100
80
60
40
20
0
-30
-20
-10
0
+10
+30
Adult
Days from parturition (Day 0)

Figure 4.8 Western blot of immunoreactive CBG in fetal, neonatal and adult sheep plasma. 10 µl of diluted plasma (1:500 in 10 mM Tris, pH 7.4) was loaded onto each lane. Molecular weight (M_r) markers are shown on the left.

Western blot analysis

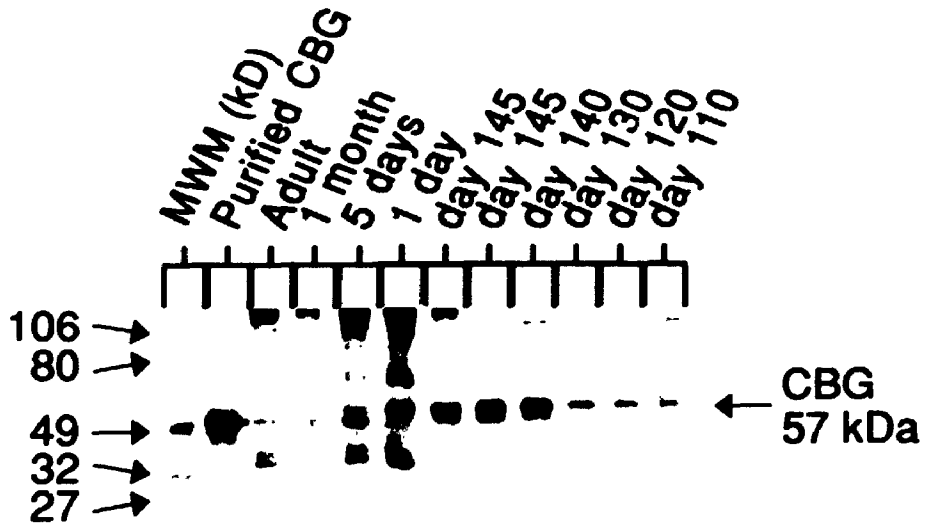


Table 4.1 Total cortisol concentrations, percent free cortisol and free cortisol concentrations in late gestation fetal sheep

Days from parturition	[Cortisol] (ng/ml)	CBC (ng/ml)	% Free cortisol	Free cortisol (ng/ml)
-20	1.6 ± 0.4[a]	26.2 ± 5.3[a]	6.1 ± 0.8[a]	0.09 ± 0.03[a]
-15	1.9 ± 0.3[a]	23.6 ± 5.3[a]	6.2 ± 1.1[a]	0.11 ± 0.02[a]
-10	9.4 ± 1.7[b]	34.9 ± 4.5[a]	5.4 ± 0.5[a]	0.48 ± 0.07[b]
-5	19.1 ± 2.2[c]	57.9 ± 12.6[b]	4.7 ± 0.6[a]	0.70 ± 0.17[b]
0	85.5 ± 10.9[d]	86.5 ± 20.7[b]	6.5 ± 1.3[a]	5.78 ± 1.44[c]

Different letters within columns indicate a significant difference (n=5: $P < 0.05$: Students t-test).
Values are mean ± SEM.

CHAPTER 5

EFFECT OF CBG ON GLUCOCORTICOID-MEDIATED SUPPRESSION OF BASAL AND CRH-STIMULATED ACTH RELEASE FROM OVINE FETAL PITUITARY CELLS IN CULTURE

5.1 Introduction

In species such as the sheep, parturition is triggered by the fetus through mechanisms that lead to the increased secretion of cortisol from the fetal adrenal glands (Bassett and Thorburn, 1969; Nathanielsz, Comline, Silver and Paisey, 1972). This prepartum increase in fetal plasma cortisol concentrations is associated with a concomitant increase in plasma CBG (Fairclough and Liggins, 1975; Ballard *et al.*, 1982; see chapter 4). Thus, the free cortisol concentration in plasma is maintained at a relatively low and constant level (Ballard *et al.*, 1982; see chapter 4). It has been suggested (Ballard *et al.*, 1982; Challis *et al.*, 1985) that the effect of CBG is to maintain a low negative feedback effect of corticosteroids on fetal hypothalamic and pituitary function. In this way, ACTH secretion is maintained, fetal adreno-cortical function is activated, and cortisol output continues to rise until it eventually exceeds the circulating corticosteroid-binding capacity. This enables unbound cortisol to act on glucocorticoid-sensitive tissues, including the placenta, to alter the output of progesterone, estrogen and prostaglandin, resulting in parturition (Challis and Mitchell, 1988; Thorburn and Challis, 1979; Liggins *et al.*, 1973).

A key feature of this hypothesis is that the binding of cortisol by CBG affects glucocorticoid negative feedback on the pituitary. To test this hypothesis, ovine CBG was purified, and the ability of CBG to inhibit negative feedback of cortisol on basal and CRH-stimulated ACTH output by ovine fetal pituitary cells maintained in tissue culture was examined. The ability of CBG to affect negative

feedback actions of dexamethasone in this system was also examined. Dexamethasone is a synthetic glucocorticoid that does not bind to CBG (Challis *et al.*, 1985); hence comparison with results of cultures treated with cortisol would allow the specificity of the CBG effect to be assessed.

5.2 Materials and methods

5.2.1 Steroids

Dexamethasone was obtained from Pharma Science (Montreal, Que.), and non-radioactive cortisol was obtained from Synkron Corporation (London, Ont.). [1,2,6,7-^{3}H]Cortisol (specific activity 80-100 Ci/mmol) was purchased from NEN Research Products (DuPont Canada Inc., Mississauga, Ont.) and purified by thin layer chromatography in the solvent system chloroform:ethanol (95:5) before use (see chapter 2).

5.2.2 Purification and gel-electrophoresis of ovine CBG

The protocol used to purify CBG from sheep serum is described in detail in chapter 3.

5.2.3 Pituitary collection and cell dispersal

Pituitary glands were obtained from fetal sheep in late gestation (day 142-144). Pregnant ewes and their fetuses were killed with an overdose of Euthanyl (MTC Laboratories, Cambridge, Ont.). The fetal pituitary cells were collected and

dispersed for tissue culture as described by Brooks and Gibson (1992). Briefly, the pituitary was removed and kept on ice in Dulbecco's Phosphate Buffered Saline (DPBS; GibcoBRL, Toronto, Ont.) with gentamycin (0.5 mg/ml; GibcoBRL, Toronto, Ont). The posterior and intermediate pituitary were removed by blunt dissection, and the anterior pituitary was then chopped into blocks (1-2 mm^2) and agitated with DPBS with gentamycin (0.5 mg/ml; 15 ml) containing trypsin (0.5%; GibcoBRL, Toronto, Ont.) at 37 C for 30 min. Medium was then removed and the tissue washed 4 times with Dulbecco's minimum Eagles medium (DMEM; GibcoBRL, Toronto, Ont.) + 10% charcoal-stripped fetal bovine serum (FBS; GibcoBRL, Toronto, Ont.). The FBS had been previously stirred with a charcoal suspension (6.5% w/v) for 48 h at 4 C, then centrifuged at 2000 X g for 60 min, and the supernatant passed through a 0.45 μm filter (Millipore, Bedford, MA.). Tissue fragments were then mechanically dispersed using silicone-coated pipettes with sequentially smaller bore sizes (2 mm, 1 mm and 0.5 mm). After mechanical dispersal the cells were passed though a nylon mesh (160 μm; B&SH Thompson, Mississauga, Ont.) to remove large tissue fragments and pieces of connective tissue. The filtrate was centrifuged and resuspended in DMEM + 10% FBS (2 times). The cells were counted using a hemacytometer (American Optical, Buffalo, N.Y.). The viability of the cells at the beginning of the culture was assessed using trypan blue (0.4% in 0.9% NaCl) exclusion, and was greater than 95% in each experiment. Cells were then plated at 300,000 per well in 1 ml DMEM + 10% fetal bovine serum (FBS) on 24-well plates (Falcon

Labware, Becton Dickinson and Co., Lincoln Park, N.J.) which had been coated with laminin (GibcoBRL, Toronto, Ont.). The cells were then maintained at 37 C in a water saturated atmosphere (95% air; 5% CO_2) for 96 hours.

5.2.4 Experimental protocol

Cells were maintained in culture for 96 h, with one change of medium (DMEM + 10% FBS) at 48 h. This allows attachment of the cells as a monolayer to the culture well, and allows the cells to recover from the enzymatic dispersion (Yang *et al.*, 1990). After 96 h, the cells were cultured for a further 6 h in DMEM + Ham F-12 (GibcoBRL, Toronto, Ont.) without added serum in the presence of cortisol (0-10^{-6}M) or dexamethasone (0-10^{-6}M) in the presence or absence of CBG. All treatments were preincubated for 15 min to allow time for the CBG to interact with cortisol. CBG was added at a concentration of 2.2 X 10^{-7} M, which was derived using a CBC assay (see chapter 2; diluted plasma assay), and using 57,000 as the molecular weight for ovine CBG (see chapter 3). The concentration of CBG was selected using estimates of plasma CBG measurements at day 140 of gestation in the fetal sheep (Fairclough and Liggins, 1975; Ballard *et al.*, 1982; see chapter 4). At the end of 6 h, the medium was harvested. Then the cells were incubated for a further 2 h in the presence of the steroid ± CBG treatments as indicated above and with the addition of 10 nM $oCRH_{1-41}$ (Bachem Co., San Francisco, CA.). The medium was collected and stored at -70 C until analysis. Results are expressed per culture well, or per

300,000 cells.

5.2.5 Measurement of immunoreactve ACTH in media from pituitary cell cultures

Immunoreactive ACTH (irACTH) in cell culture media was measured using a double antibody radioimmunoassay kit (Incstar, Stillwater, Min.). Samples were thawed only once, at which time the ACTH measurements were conducted. The primary antibody was raised in rabbits against synthetic human $ACTH_{1-39}$, and the second antibody was anti-rabbit IgG raised in goats.

The culture media to be assayed (20 µl) was diluted with serum free DMEM to a final volume of 100 µl. The diluted media was then incubated with primary antibody (200 µl) and [^{125}I]ACTH (200 µl) overnight (4 C). After the overnight incubation, the secondary antibody was added (500 µl). The tubes were vortexed gently and incubated for 20 min at room temperature. The tubes were then centrifuged (760 X g at 4 C) for 20 min, the supernatant aspirated off and the precipitate counted in a gamma counter.

The primary antibody showed less than 0.01% cross-reactivity with beta-lipotropin, beta-endorphin, met and leu-enkephalin, alpha-MSH, vasopressin, oxytocin, human growth hormone and follicular stimulating hormone (Cross-reactivities were given by Incstar, Stillwater, Min.). The mean assay sensitivity was 6.5 pg/ml. The intra-assay coefficients of variation (CV) for the two pool samples included in the kit were 8% and 4% respectively. Measurements of

irACTH from plasma pools included in each assay were 46.12 ± 6.28 pg/ml (S.D., n=13; interassay CV = 14%), and 143.39 ± 8.64 pg/ml (n=13; interassay CV=9%).

5.2.6 Measurement of cortisol in pituitary cell culture media

Cortisol was measured by RIA (see chapter 2) in samples of tissue culture medium before and after incubation of the cells for 6 h. In cell cultures where exogenous cortisol was not added, the concentration of cortisol in the medium was undetectable (less than 0.4 ng/ml).

5.2.7 Effect of CBG on ACTH output

The IC_{50} and IC_{25} were calculated as the concentration of dexamethasone or cortisol required to produce 50% and 25% inhibition of ACTH output, respectively, before or after oCRH stimulation and in the presence or absence of CBG. The basal outputs of ACTH in the presence of CBG alone and with CBG + CRH were used as the maximal ACTH outputs for the calculation of the IC_{50} and IC_{25}.

5.2.8 Data analysis

Comparisons between IC_{25} values were made by one-way ANOVA and Student-Newman-Kuels test. Data was initially tested for normality (Kolmorogov-Smirnov test). Comparisons between output of ACTH at individual steroid

concentrations with or without CBG in data passing the normality test were made using the Student's t-test. Comparisons between output of ACTH at individual steroid concentrations with or without CBG in data that failed the normality test were made using the Wilcoxon Signed-Rank test.

5.3 Results

5.3.1 Effect of pre-treatment of cells with steroids ± CBG for 6 hours on ACTH output

The mean basal output of ACTH during the 6 h treatment period was 244 ± 99 pg/300,000 cells (mean ± SEM; n=4; Figure 5.1, page 136). There was a significant stimulation ($P < 0.05$) of ACTH output in the presence of CBG alone. Cortisol and dexamethasone produced a dose-dependent inhibition of basal ACTH output. Addition of CBG completely attenuated the inhibitory effect of cortisol. However, ACTH output in the presence of dexamethasone (10^{-7}M) + CBG was not significantly different from that in the presence of dexamethasone alone ($P > 0.05$; Figure 5.1, page 136).

The data from the four individual experiments are shown in Figure 5.2 (page 138). The trends of three experiments were similar, but one experiment gave variable results. The absolute ACTH output varied considerably among the four experiments.

5.3.2 Effect of CRH ± steroids ± CBG on ACTH output

Corticotrophin releasing hormone (10 nM) produced a significant increase in ACTH output from 128 ± 23 to 599 ± 139 pg/300,000 cells (n=4; Figure 5.3, page 140). There was a further small stimulation of ACTH output to 771 ± 201 pg/300,000 cells in the presence of CRH + CBG, however this was not statistically different from ACTH output in the presence of CRH alone. Cortisol and dexamethasone both produced a concentration-dependent inhibition of CRH-stimulated ACTH output (Figure 5.3, page 140). The addition of CBG produced a significant attenuation of the negative feedback effects of cortisol at 10^{-7} and 10^{-6} M. At 10^{-7} M dexamethasone there was no significant difference in the output of ACTH from cells treated with CRH either in the absence or presence of CBG (Figure 5.3, page 140).

The data from four individual experiments are presented in Figure 5.4 (page 142). The ACTH output trends were consistent throughout all four experiments, however, the absolute ACTH output varied between animals.

5.3.3 Effect of CBG on ACTH output

In the experiments to examine the effect of glucocorticoid ± CBG on both the basal and CRH-stimulated ACTH output, the order of efficacy in mediating glucocorticoid negative feedback was dexamethasone > cortisol > cortisol + CBG (Table 5.1, page 143).

The dose-response curve for cortisol inhibition (IC_{25}) was significantly

displaced ($P < 0.05$) in both the basal and CRH-stimulated ACTH outputs (Table 5.1, page 143). The IC_{25} for basal ACTH output increased from 4.85×10^{-8} M to greater than 1×10^{-6} M, and the IC_{25} for CRH-stimulated ACTH output increased from 3.36×10^{-8} M to 7.18×10^{-7} M in the presence of CBG.

The IC_{50} for the basal ACTH output due to cortisol negative feedback could be calculated in only two experiments. The largest decrease in mean value of all experiments was a decrease of ACTH output of 47%, at a cortisol concentration of 1×10^{-7} M. The addition of CBG shifted the IC_{50} for basal ACTH output due to cortisol negative feedback to greater than 1×10^{-6} M. In the study of CRH-stimulated ACTH output, the presence of CBG moved the mean IC_{50} from approximately 2×10^{-7} M to greater than 1×10^{-6} M (Figure 5.3, page 140).

5.4 Discussion

Cortisol inhibits basal and CRH-stimulated output of ACTH by fetal sheep corticotrophs, presumably through glucocorticoid Type II receptors, since dexamethasone is also highly effective in this system, and these cells show extensive binding of the synthetic glucocorticoid triamcinolone (Yang *et al.*, 1990). The negative feedback action of cortisol was diminished in the presence of CBG, whereas CBG had little effect on the negative feedback effect of dexamethasone. Because dexamethasone does not bind, or binds only poorly to ovine CBG (Challis *et al.*, 1985), this result attests to the specificity of the effect observed in the present studies.

The purification and purity of the CBG preparation used in the current experiments has been described previously (see chapter 3). The protein exhibits an average molecular weight of 57 kDa on SDS-PAGE. This agrees with estimates of the molecular weight of ovine CBG derived from sedimentation coefficients (Kato *et al.*, 1988). Thus it is unlikely that our results reflect contamination of our CBG preparation with other plasma proteins. Due to technical difficulties of obtaining enough fetal plasma to purify CBG, the CBG was purified from adult ewes. The effect of CBG in mediating glucocorticoid negative feedback was assessed on fetal pituitary cells. The patterns of glycosylation are different in adult and fetal sheep (see chapter 4), and how this difference may affect possible interaction between CBG and pituitary CBG membrane receptors requires further investigation.

In fetal sheep plasma, cortisol is bound predominantly to CBG, with less than 10% present as free (unbound) steroid (Ballard *et al.*, 1982; see chapter 4). It is generally considered that the free steroid fraction represents that which is biologically active. The present results suggest that the binding of cortisol by CBG effectively removes bound cortisol from the biologically active pool, and thus diminishes its negative feedback efficacy at the pituitary. In this way, pituitary POMC gene expression and ACTH secretion is sustained (Matthews *et al.*, 1994), and further stimulation of the secretion of cortisol from the fetal adrenal gland occurs. Because fetal cortisol stimulates further CBG biosynthesis (see chapter 7), a low free cortisol concentration in plasma is maintained, and

pituitary-adrenal activation occurs in a manner that has been characterized as a positive-feedforward cascade (Challis and Brooks, 1989).

It was possible that CBG could have enhanced the negative feedback action of cortisol. Binding sites for CBG have been measured on membrane fractions from rat pituitary glands (Singer *et al.*, 1988), and from the human placenta (Avvakumov *et al.*, 1989). Moreover, CBG-like activity has been identified in the pars distalis of the rat (Khun *et al.*, 1986), and in corticotrophs of the pars distalis and pars intermedia of the guinea pig (Perrot-Applant *et al.*, 1984). Cell membrane binding of CBG has been suggested as a mechanism for glucocorticoid delivery to some target cells (Hammond, 1990). CBG mRNA is present in the fetal pituitary (see chapter 4), and it is therefore possible that the pituitary produces CBG, although the function of pituitary CBG is not known at this time. The present results are in contrast to the enhancement by albumin of the negative feedback potency of cortisol on basal or CRH-stimulated ACTH output by perfused pituitary cells from adult sheep (Kemppainen, Zerbe and Sartin, 1991). The reason for this discrepancy is not known, as it would be expected that any protein which bound glucocorticoid would produce a decrease in negative feedback efficacy. The authors (Kemppainen *et al.*, 1991) suggested that the formation of a cortisol-albumin complex facilitates steroid entry into corticotrophs, compared with entry of free steroid. However, the authors did not account for the CBG, the major glucocorticoid binding protein in the sheep (Ballard *et al.*, 1982).

Within the tissue culture system I have used, the ability of CBG to block negative feedback of cortisol on pituitary cells would require that CBG inhibit the ability of cortisol to interact with the glucocorticoid receptor. Therefore, the ratic of the dissociation constants (Kd) of the CBG-cortisol complex and the cortisol glucocorticoid receptor complex should be approximately 1:1 or greater. The Kd of the CBG-cortisol complex is 0.89 X 10^{-8} M (Fairclough and Liggins, 1975) and the glucocorticoid receptor in the fetal pituitary has a somewhat higher dissociation constant for [^{3}H]triamcinolone of 2-3 X 10^{-8} (Yang *et al.*, 1990). The dissociation constant of triamcinolone for the glucocorticoid receptor is much greater than that of cortisol (Rose, Kute and Winkler, 1985) which would mean that the Kd's of the CBG-cortisol and glucocorticoid receptor-cortisol interactions are approximately equal. Therefore, CBG would be able to bind cortisol, thus limiting the entry of free cortisol into the fetal pituitary cells.

CBG alone produced a small but significant stimulation of basal ACTH output. This enhancement of ACTH output by CBG alone is likely not due to its binding endogenous cortisol in the tissue culture medium, thereby removing a basal inhibitory influence on ACTH, since cortisol was undetectable in media which did not have cortisol added to it. It is possible that CBG binds to the pituitary cell membranes, with subsequent activation of adenylate cyclase, as has been described by CBG-cortisol interaction, induction of adenylate cyclase activity and accumulation of cAMP within the MCF-7 carcinoma cell line (Nakhla, Khan and Rosner, 1988).

I have suggested that inhibition by CBG of the negative feedback action of cortisol is important in sustaining ACTH release leading to the pre-partum activation of adrenal function in the fetal lamb. The present study supports this hypothesis, and suggests that CBG may affect the negative feedback action of cortisol not only on basal ACTH, but also on CRH-stimulated ACTH release by ovine pituitary cells *in vitro*.

Figure 5.1 Basal release of irACTH from pituitary cells cultured for 6 h in media alone (◊), or in media containing cortisol (▽), dexamethasone (○), cortisol + CBG (▼), dexamethasone + CBG (●) or CBG alone (♦). Data are represented as percent control for each experiment (mean ± SEM; n=4). Significant differences ($P < 0.05$) in ACTH output between cortisol and cortisol + CBG are indicated by an asterisk.

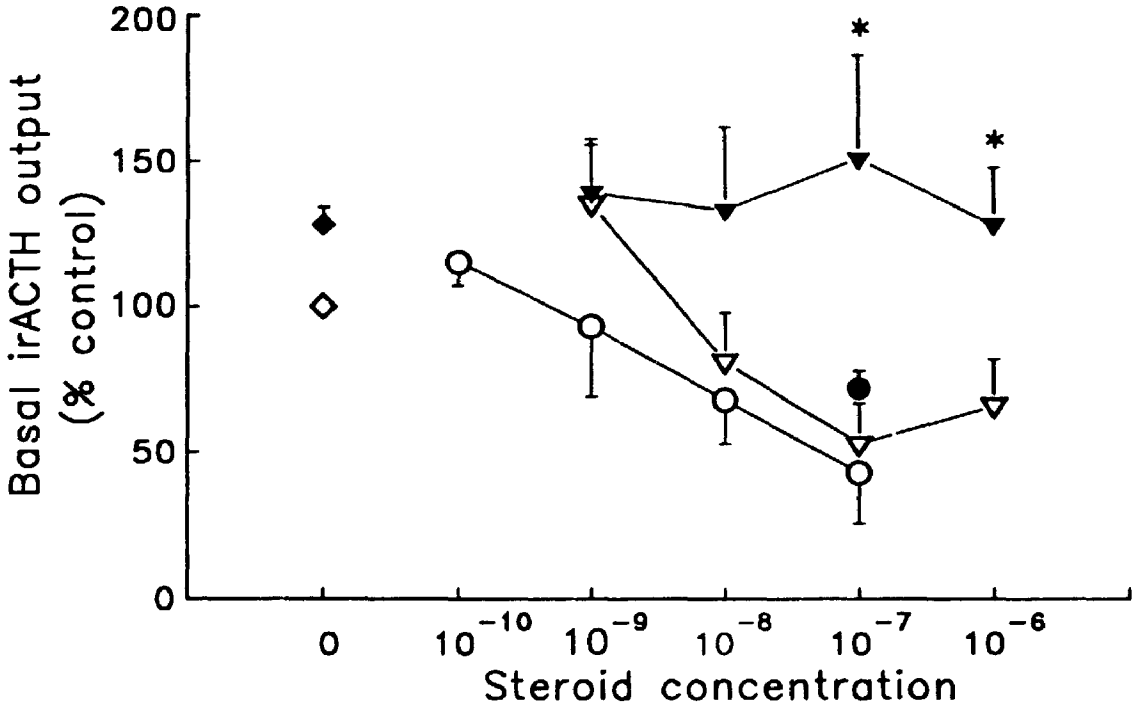

200
150
100
50
0
Basal irACTH output
(% control)
*
*
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
Steroid concentration

Figure 5.2 Basal release of irACTH from pituitary cells cultured for 6 h in media alone (◊), or in media containing cortisol (▽), dexamethasone (○), cortisol + CBG (▼), dexamethasone + CBG (●) or CBG alone (♦). Individual data from four separate experiments is presented.

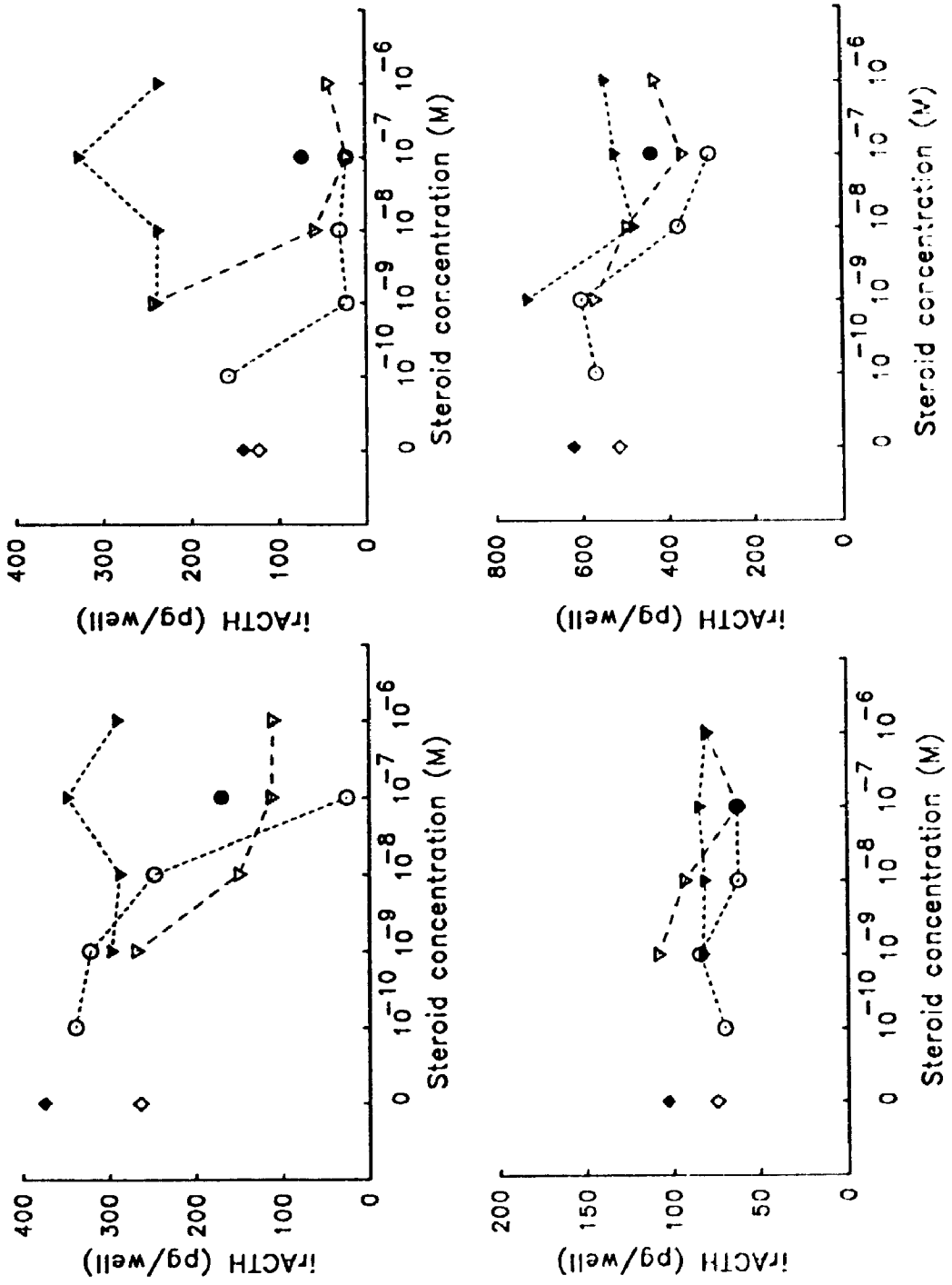

irACTH (pg/well)
Steroid concentration (M)
0
100
200
300
400
800
600
50
150
200
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}

Figure 5.3 CRH-stimulated release of irACTH from pituitary cells cultured for 2h in media alone (◊), or in media containing CRH (△), cortisol + CRH (▽), dexamethasone + CRH (○), cortisol + CBG + CRH (▼), dexamethasone + CBG + CRH (●) or CBG + CRH (▲). Data are represented as the percent control for each experiment (mean ± SEM; n=4). Significant differences ($P < 0.05$) in ACTH output between cortisol and cortisol + CBG are indicated by an asterisk.

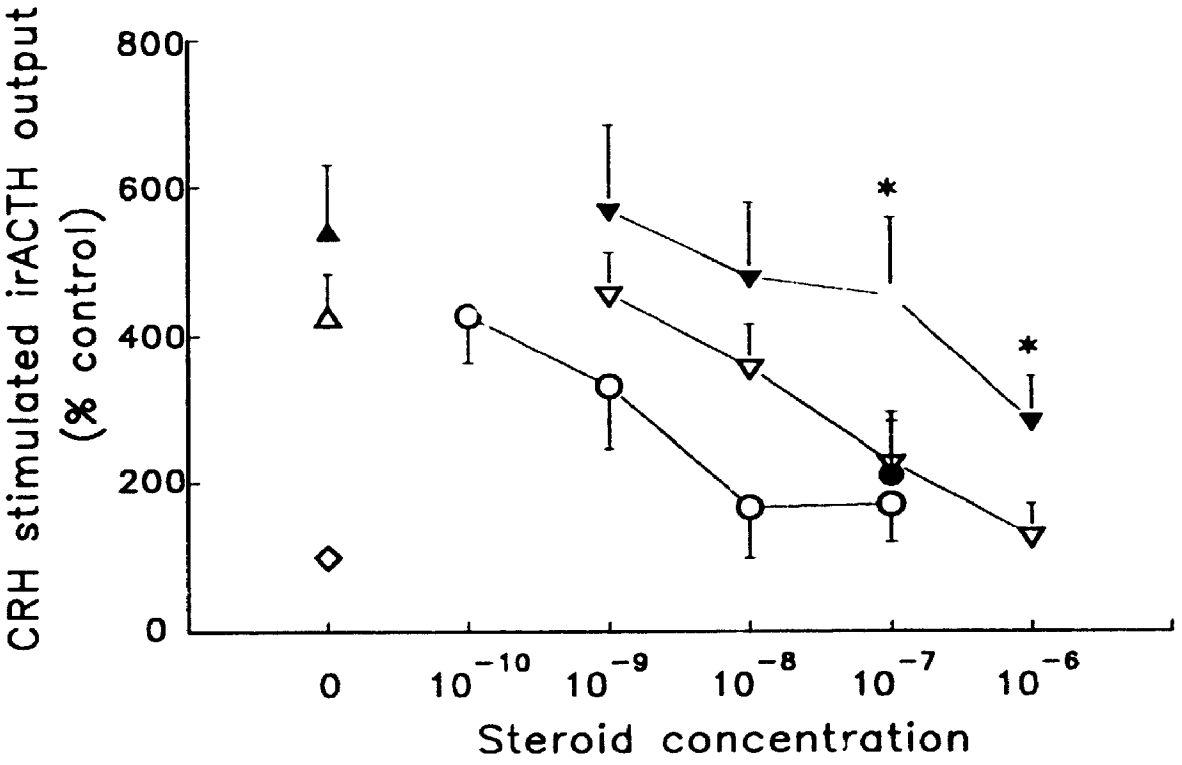

CRH stimulated irACTH output
(% control)
800
600
400
200
0
*
*
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
Steroid concentration

Figure 5.4 CRH-stimulated release of irACTH from pituitary cells cultured for 2 h in media alone (◊), or in media containing CRH (△), cortisol + CRH (▽), dexamethasone + CRH (○), cortisol + CBG + CRH (▼), dexamethasone + CBG + CRH (●) or CBG + CRH(▲). Individual data from four separate experiments is presented.

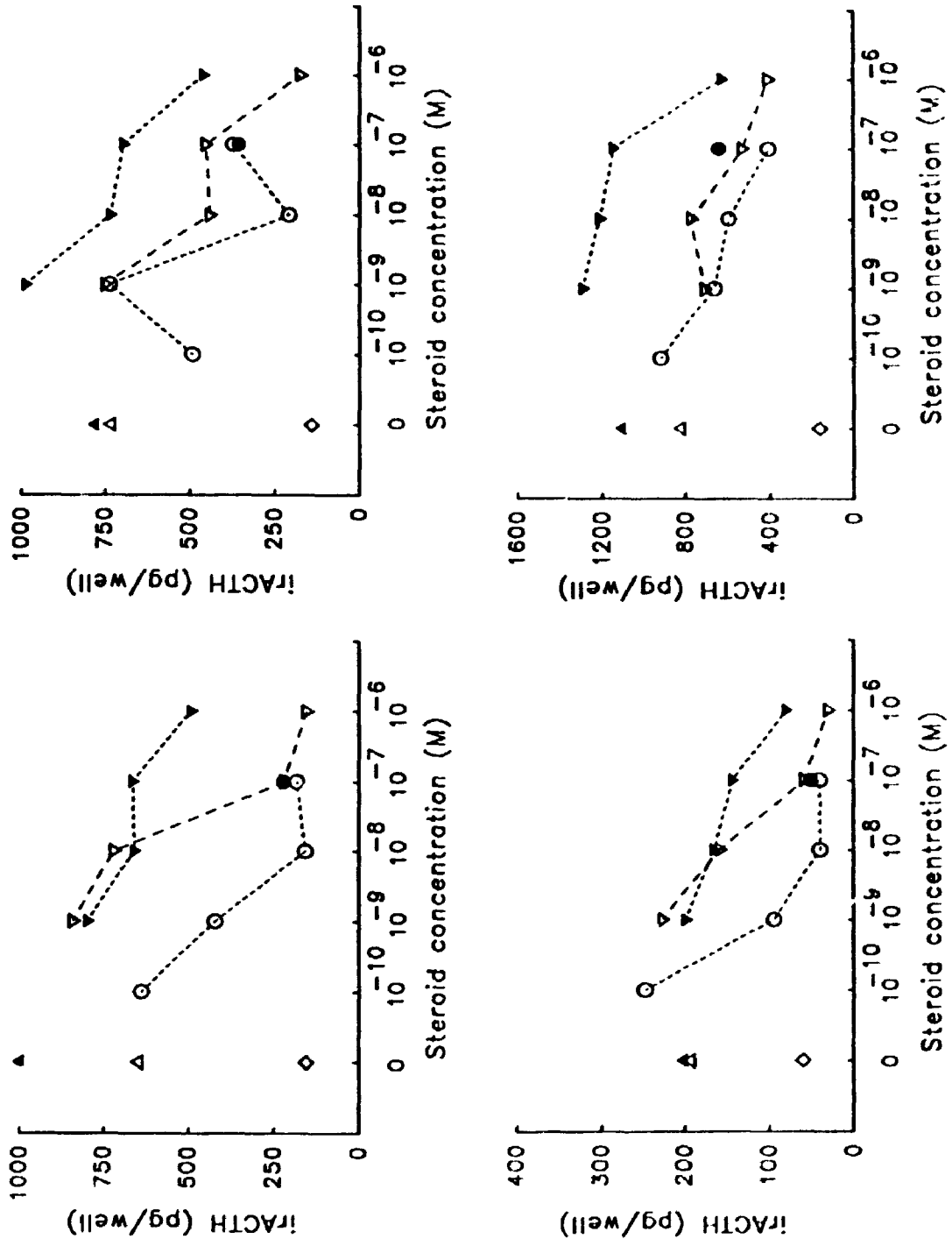

irACTH (pg/well)
1000
750
500
250
0
1600
1200
800
400
400
300
200
100
0
0 10^-10 10^-9 10^-8 10^-7 10^-6
Steroid concentration (M)

Table 5.1 Effect of presence of CBG on glucocorticoid inhibition (IC_{25}) of ACTH output from dispersed fetal pituitary cells in culture

	IC_{25}		
	Cortisol (M)	Cortisol (M) + CBG	Dexamethasone (M)
Basal ACTH	4.85×10^{-8} (2.12)	$> 1.0 \times 10^{-6}$	5.78×10^{-9} (1.9)
CRH-stimulated ACTH	3.36×10^{-8} (1.4)	7.18×10^{-7} (2.0)	3.99×10^{-9} (2.09)

Data is presented as mean ± (SEM).
n=4 for all experiments.

CHAPTER 6

EFFECT OF DEXAMETHASONE TREATMENT ON CBG BIOSYNTHESIS IN FETAL AND ADULT SHEEP AND TRANSPLACENTAL TRANSFER OF CBG IN FETAL SHEEP AT DAY 130 OF GESTATION

6.1 Introduction

Parturition in sheep is triggered by the fetus through increased secretion of cortisol from the fetal adrenal gland (Bassett and Thorburn, 1969; Brooks and Challis, 1988). Activation of the fetal HPA axis occurs during late gestation, and is associated with an increase in fetal plasma cortisol and CBG concentrations (Ballard *et al.*, 1982). It has been suggested that this increase in circulating CBG helps maintain a relatively low free cortisol concentration in plasma (Ballard *et al.*, 1982; Fairclough and Liggins, 1975), which may reduce the efficacy of glucocorticoid negative feedback at the level of the hypothalamus and/or pituitary (see chapter 5), thereby accounting for the concurrent increase in plasma ACTH (Challis *et al.*, 1985). The factors responsible for stimulating the rise in plasma CBG in the fetus are unclear, but it is known that the CBC in fetal plasma increases in response to fetal ACTH administration (Challis *et al.*, 1985). This effect appears to be glucocorticoid-mediated (Challis *et al.*, 1985), and may involve one or more of the following: increased CBG biosynthesis, a decrease in the clearance of CBG from the fetal circulation, and/or transplacental transfer of CBG from the ewe to the fetus. To examine these possibilities, I determined the effects of fetal glucocorticoid treatment on the fetal plasma CBC and the abundance of CBG mRNA in the fetal liver, since this is the major site of CBG production (Smith and Hammond, 1991; Seralini *et al.*, 1990; see chapter 4). Measurements of changes in the glycosylation of fetal plasma CBG were also made, because this may affect its plasma half-life (Hossner and Billiar, 1981).

Furthermore, because maternal CBG is transferred to the fetal compartment in the rabbit (Seralini *et al.*, 1989), I also examined the transplacental transfer of [^{125}I]CBG from the ewe to the fetus in the presence or absence of fetal glucocorticoid administration. To assess the specificity of glucocorticoid effects in fetal sheep, I also determined changes in plasma CBC and hepatic CBG mRNA in adult ewes treated with dexamethasone.

6.2 Materials and methods

6.2.1 Materials

Unless stated otherwise, chemicals were obtained from Sigma Chemical Co. (Mississauga, Ont.). Dexamethasone (10 mg/ml) was obtained from Pharma Science (Montreal, Que.), and non-radioactive cortisol was obtained from Synkron Corporation (London, Ont.). [1,2,6,7-^{3}H]Cortisol (specific activity 80-100 Ci/mmol) was purchased from NEN Research Products (DuPont Canada Inc., Mississauga, Ont.) and purified by thin layer chromatography in the solvent system chloroform:ethanol (95:5) before use. [^{32}P]dCTP (specific activity 3000 Ci/mmole) was purchased from ICN Biomedicals (ICN Biomedicals Inc., Irvine, CA.).

6.2.2 Purification and gel-electrophoresis of ovine CBG

The methodology for purification and gel-electrophresis of ovine CBG is described in detail in chapter 3.

6.2.3 CBC in plasma and hepatic CBG mRNA abundance

6.2.3.1 Surgical protocol

In experiments to examine effects of fetal glucocorticoid administration on fetal CBG biosynthesis, six pregnant ewes of mixed breed with known insemination dates and carrying twins were used. Between day 118-123, and under general anaesthesia, both fetuses were implanted surgically with polyvinyl catheters inserted 6-8 cm into the carotid artery and jugular vein (Challis *et al.*, 1981). The maternal femoral artery and vein were also catheterized. To assess changes in uterine contractility indicative of labour in response to fetal glucocorticoid treatment, uterine EMG leads were attached to the myometrium (Brooks, Hapak, Lye and Challis, 1988). Following surgery, catheters were filled with sterile heparin-saline and flushed daily. Post surgical management of the animals was as described previously (see chapter 2).

The effect of glucocorticoid treatment was examined in ovariectomized adult sheep, in which vinyl catheters were inserted 18-20 cm into a femoral artery and vein, as described previously (Challis *et al.*, 1981; see chapter 2).

Blood gases were determined daily using an ABL-3 blood gas analyzer (Radiometer, Copenhagen, Denmark) to monitor fetal and maternal health (Table 6.1, page 177).

6.2.3.2 Experimental protocol - glucocorticoid treatment of fetal sheep

At least 5 days post-surgery, and beginning on day 130 of gestation, dexamethasone was infused as pulses of 2 μg/min for 15 min every 2 h in 1 ml saline into the jugular vein of one of the twins. The other twin was infused with saline (1 ml) for 15 min every 2 h as control. Blood samples (3 ml) were collected on successive days from the carotid artery of each fetus at -15 min, 0 min, +2 h and +5 h relative to the pulse of dexamethasone or saline, which began at 10:00 am. Blood was collected into heparinized syringes, transferred to chilled plastic tubes and centrifuged immediately at 1,500 x g for 10 min. The plasma was stored at -20 C until analysis.

6.2.3.3 Euthanasia and collection of tissues

The dexamethasone and saline infusions were continued for 96 h at which time the mother and fetuses were killed by administration of Euthanyl (MTC Pharmaceuticals, Cambridge, Ontario). The right lobe of the fetal liver was collected quickly, frozen in liquid nitrogen, and stored at -70 C for subsequent Northern blot analysis.

6.2.3.4 Experimental protocol - glucocorticoid treatment of adult sheep

Following a recovery period of at least 5 days after surgery the ewes were

treated with dexamethasone (2 mg/day or 10 mg/day, n=4) or saline (n=5) given as bolus iv injections at 9:00 am for four successive days. Blood samples (5 ml) were collected from the femoral artery at -40 min, -10 min, +6 h and +12 h relative to the bolus injection. Blood was collected into heparinized syringes, transferred to chilled plastic tubes and centrifuged immediately at 1,500 x g for 10 min. The plasma was stored at -20 C until analysis.

6.2.3.5 Euthanasia and collection of tissues

After 72 h of treatment, the animals were killed with a dose of Euthanyl appropriate for euthanasia, and a portion of the right lobe of the liver was rapidly excised, frozen in liquid nitrogen, and stored at -70 C for subsequent Northern blot analysis.

6.2.4 Transplacental transfer of maternal [^{125}I]CBG

6.2.4.1 Surgical protocol

The transplacental transfer of CBG was examined in singleton or twin pregnant sheep, at day 130 of gestation. Between day 118-123, and under general anaesthesia, fetuses were implanted surgically with polyvinyl catheters inserted 6-8 cm into the femoral artery and femoral vein (Challis *et al.*, 1981), and the fetal bladder was catheterized for collection of fetal urine. The maternal femoral artery and vein were also catheterized.

6.2.4.2 Experimental protocol

Singleton fetuses received either dexamethasone or saline, and in twins, one fetus received dexamethasone (30 μg in 1 ml saline over 15 min every 2 h) and the other fetus received saline (1 ml over 15 min every 2 h). After 48 h of dexamethasone or saline infusion to the fetus, the iodinated maternal CBG ($9x10^6$ dpm/kg maternal body weight) was given as a bolus (in 2 ml heparinized saline) into the maternal femoral vein, and the catheter flushed with 10 ml saline. Maternal (5 ml) and fetal (3 ml) blood and fetal fluids (1 ml) were collected as before, to 24 h relative to the injection of labelled protein. Radioactivity in aliquots (1 ml) of fetal and maternal plasma, amniotic fluid, and fetal urine was measured by a gamma counter (Beckman Gamma 5500B, Beckman Instruments (Canada) Inc., Mississauga, Ont.) and counts per minute (CPM) expressed as a percentage of maternal plasma CPM at +2 minutes. At 24 h after the maternal bolus injection the animals were killed by administration of a dose of Euthanyl suitable for euthanasia. To ensure that the radioactivity in maternal plasma was present as intact labelled CBG, samples (20 μl) of plasma at +2 min, +30 min, +1 h, +2 h, +8 h and +24 h were electrophoresed on a SDS-PAGE (4% and 7.5% acrylamide in the stacking and resolving gels, respectively) gel. The gel was dried and autoradiography was performed.

6.2.5 Measurement of plasma cortisol and CBC

Plasma cortisol concentrations were measured by radioimmunoassay after

extraction with diethyl ether (see chapter 2). The antibody characteristics and assay validation for fetal sheep plasma cortisol measurements have been described previously (Challis *et al.*, 1981). The CBC of fetal and maternal sheep plasma was determined by the procedure of Ballard *et al.* (1982) with modifications (Challis *et al.*, 1985; see chapter 2).

6.2.6 Measurement of hepatic CBG mRNA abundance by Northern blot analysis

The methodology to conduct Northern blot analysis is described in detail in chapter 3.

6.2.7 CBG elution profiles using Concanavalin A chromatography

Fetal or maternal plasma (250 µl) was applied to 5 ml Con A Sepharose columns and allowed to enter the gel bed. Running buffer (12 ml; 50 mM Tris, pH 7.5, 0.5 M NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$ and 1mM $MnCl_2$) was added to the columns, followed by 18 ml of elution buffer (running buffer + 5% dimethylformamide + 5% α-D-mannopyranoside). The protein content of all fractions was determined using the Bradford protein assay (Bradford, 1976; see chapter 2). The first twelve fractions containing proteins that did not interact with Con A were pooled as peak A, and fractions containing protein that eluted with α-D-mannopyranoside were pooled as peak B. Samples (2 ml) of peaks A and B were dialysed overnight at 4 C against running buffer to remove

dimethylformamide and α-D-mannopyranoside, and aliquots (100 µl) were used to determine their CBC (Hammond and Lähteenmäki, 1983; see chapter 2).

6.2.8 Data analysis

Changes in fetal and adult plasma CBC in response to glucocorticoid treatment were analyzed by a two-way ANOVA corrected for repeated measures. Data was initially screened for interaction at a significance level of $P < 0.05$. Effects of individual times of treatment were assessed by the Student's t-test. Values represent mean ± SEM. Differences in relative abundance of CBG mRNA in liver after glucocorticoid or saline treatment were assessed using the Mann-Whitney U test. Differences in the elution patterns of CBG during Con A chromatography were determined using a one-way ANOVA followed by the Student's t-test. Transplacental transfer data were assessed by a two-way ANOVA ($P < 0.05$).

6.3 Results

6.3.1 Effect of dexamethasone treatment on fetal plasma CBC

Plasma CBC was similar in dexamethasone (30.0 ± 2.4 ng/ml) and saline-treated fetuses during the pre-treatment period. It rose significantly to 55.6 ± 7.7 ng/ml within 48 h of starting dexamethasone ($P < 0.05$), and continued to increase with treatment to maximum values of 92.6 ± 11.1 ng/ml at 96 hours (Figure 6.1, page 160). Plasma CBC was unchanged in saline-treated fetuses

(Figure 6.1, page 160).

6.3.2 Effect of dexamethasone treatment on fetal hepatic CBG mRNA abundance

By Northern blot analysis, a single CBG transcript of 1.8 kb was identified in liver RNA extracts of dexamethasone (n=4) and saline-treated (n=5) fetuses, however there was considerable variation between the signals obtained for dexamethasone-treated fetal sheep. After 96 hours of dexamethasone treatment, the mean relative abundance of CBG mRNA in the fetal liver was approximately 4-fold greater than in livers of control animals (3.6 ± 1.6 vs 0.9 ± 0.2; mean ± SEM, arbitrary units; $P < 0.05$; Figure 6.2, page 162).

6.3.3 Effect of dexamethasone treatment on non-pregnant adult plasma CBC

Twenty-four hours after administration of dexamethasone (10 mg/day) to adult sheep, plasma CBC was decreased from 18.5 ± 2.3 ng/ml to 4.7 ± 0.7 ng/ml ($P < 0.05$), and these depressed values were sustained through 4 successive days of treatment (Figure 6.3, page 164). There was no effect on plasma CBC during treatment of adult sheep with 2 mg/day dexamethasone, nor was there any effect of saline treatment on plasma CBC (Figure 6.3, page 164).

6.3.4 Effect of dexamethasone treatment on hepatic CBG mRNA abundance in the non-pregnant adult

The relative abundance of CBG mRNA in the liver of adult sheep treated with dexamethasone (10 mg) daily for 72 h was significantly less than that in saline-treated controls ($P < 0.05$; Figure 6.4, page 166).

6.3.5 Effect of dexamethasone treatment on fetal CBG glycosylation

Con A chromatography elution profiles of CBG were determined from fetal plasma samples taken before and after 96 h of dexamethasone or saline treatment. In control fetuses, specific binding of [^{3}H]cortisol in successive eluate fractions from the Con A columns was almost entirely in peak A (Figure 6.5, page 168, upper panel). After 96 h dexamethasone treatment, there was an increase in [^{3}H]cortisol binding in the Con A-bound fractions (Figure 6.5, page 168, middle panel). In contrast, [^{3}H]cortisol binding in plasma from a pregnant ewe was almost exclusively in Peak B (Figure 6.5, page 168, lower panel). The amount (mean ± SEM) of [^{3}H]cortisol bound in peak B was expressed as a percentage of the total amount of [^{3}H]cortisol bound in peaks A and B, in animals treated for 96 h with either dexamethasone (n=4) or saline (n=5) on day 130. This demonstrated that there was a significant increase ($P < 0.05$) in the amount of [^{3}H]cortisol bound in peak B in the dexamethasone-treated fetuses, and no difference in the saline-treated controls (Figure 6.6, page 170).

6.3.4 Transplacental transfer of CBG

6.3.4.1 Maternal half-life of labelled CBG

At 48 h of fetal treatment with dexamethasone or saline, [^{125}I]CBG was administered as a single bolus injection into the maternal femoral vein. The labelled protein disappeared with at least two volumes of distribution and an initial half-life of ~13 hours (Figure 6.7, page 172). Maternal plasma examined by SDS-PAGE and autoradiography, displayed a signal corresponding in M_r to the [^{125}I]CBG injected into the ewe (Figure 6.8, page 174). These results indicate that the radioactivity in the maternal circulation was likely to be intact CBG, and not breakdown products of the peptide.

6.3.4.2 [^{125}I]CBG transfer to fetal fluids

There was negligible radioactivity in samples of fetal plasma, urine or amniotic fluid obtained for up to 24 h after injection of [^{125}I]CBG into the mother, and this result was not altered by pre-treatment of the fetuses with dexamethasone (Figure 6.9, page 176).

6.4 Discussion

Treatment of fetal sheep with dexamethasone for 96 h increases the plasma CBC, and this is likely due, in part, to an increase in hepatic CBG biosynthesis. It may also be influenced by a glucocorticoid-induced change in the glycosylation of the protein that could increase its plasma half-life. There was

negligible transplacental transfer of CBG from the ewe to the fetus, indicating the lack of a maternal contribution to circulating fetal CBG, even during fetal glucocorticoid treatment. The effects of dexamethasone on fetal plasma CBC contrast markedly with those in the adult sheep, in which the dexamethasone treatment decreased plasma CBC and hepatic CBG mRNA abundance, as in adults of other species (Smith and Hammond, 1989).

The increase in fetal plasma CBC after dexamethasone treatment was similar to that seen at term (Ballard *et al.*, 1982), or after intra-fetal ACTH treatment (Challis *et al.*, 1985), and provides evidence that glucocorticoids are responsible for this increase in fetal plasma CBC. This is accompanied by a significant increase in CBG mRNA accumulation in the fetal liver. Additional studies are required to establish the time course of changes in hepatic CBG mRNA abundance, and their relationship to plasma CBC. The increase in hepatic CBG mRNA contrasts with previous results (Jacobs *et al.*, 1991) in which it was reported that there was no change in the liver CBG mRNA abundance after fetal treatment with ACTH. However, the labelled cDNA used in that study (Jacobs *et al.*, 1991) was subsequently determined to have the same sequence as the sheep α1-antitrypsin cDNA isolated by others (Brown, Dziegielewska, Foreman, Saunders amd Wu, 1989). In contrast to the effects of glucocorticoids in the fetus, significant decreases in both plasma CBC and liver CBG mRNA abundance were observed in adult sheep after treatment with dexamethasone at 10 mg/day. There was no effect of dexamethasone treatment (2 mg/day) on

adult plasma CBC levels demonstrating a dose response between administration of 2 and 10 mg/day. The administration of the glucocorticoids was different for the fetus and adult, with the fetus receiving an IV dose of glucocorticoid every two hours and the adult receiving a daily IV bolus. However, these disparate results indicate that dexamethasone functions differently in adults and fetuses with respect to its effects on plasma CBG levels and CBG biosynthesis in the liver. The reason for this is not known but the effect presumably occurs at the genomic level because the rate of CBG gene transcription is repressed by dexamethasone in adult rats (Smith and Hammond, 1992). This may involve a difference in the number of glucocorticoid receptors in fetal and adult livers, or their interaction with other transcription factors that may be developmentally regulated.

Examination of the Con A-binding properties of CBG in fetal plasma revealed an increase in the proportion of Con A-binding species after dexamethasone treatment. This indicates that glucocorticoid treatment alters the carbohydrate composition of fetal CBG, with the appearence of biantennary oligosaccharides. Furthermore, the appearance of a Con A-binding form of CBG in the fetus after dexamethasone treatment, suggests that glucocorticoids may modulate the expression of glycosyltransferases in the fetal liver that are involved in the processing of oligosaccharides. The functional significance of a change in the glycosylation of CBG in the fetus is unclear, but it may serve to increase the plasma half-life of the protein (Hossner and Billiar, 1981). In addition, the

carbohydrate composition of CBG may influence its interaction with plasma membrane binding sites on some tissues (Avvakumov and Strel'choyonuk, 1988; Singer *et al.*, 1988).

The half-life of [^{125}I]CBG in adult sheep was approximately 13 h which is similar to that found in the rat (Smith and Hammond, 1991) and rabbit (Seralini *et al.*, 1989). In contrast to the rabbit (Seralini *et al.*, 1989), no transplacental transfer of [^{125}I]CBG was detected from the maternal compartment into fetal plasma or urine, and this was unaffected by glucocorticoid treatment. This may be due to differences in placentation between the two species (Amoroso, 1952). Thus, it seems unlikely that maternal CBG contributes significantly to the dexamethasone-induced increase in fetal sheep CBG concentrations. This observation is consistent with previous findings that the umbilical arterial CBC in late gestation pregnant sheep is not different from that in the umbilical vein (Jacobs *et al.*, 1991).

The present results support the hypothesis that increasing plasma cortisol concentrations are responsible for the prepartum increase in plasma CBG levels and demonstrate that this is mediated through mechanisms which likely include an increase in CBG biosynthesis. Furthermore the change in glycosylation patterns observed after fetal dexamethasone treatment may also serve to increase plasma CBG by decreasing the metabolic clearance of the protein in the fetus.

Figure 6.1 Changes in fetal plasma CBC before and during intra-fetal treatment (starting at time 0) with dexamethasone (●-●; n=5) or saline (○-○; n=5). Values are mean ± SEM and level of significance is $P < 0.05$. Significant increases are indicated by the asterisk.

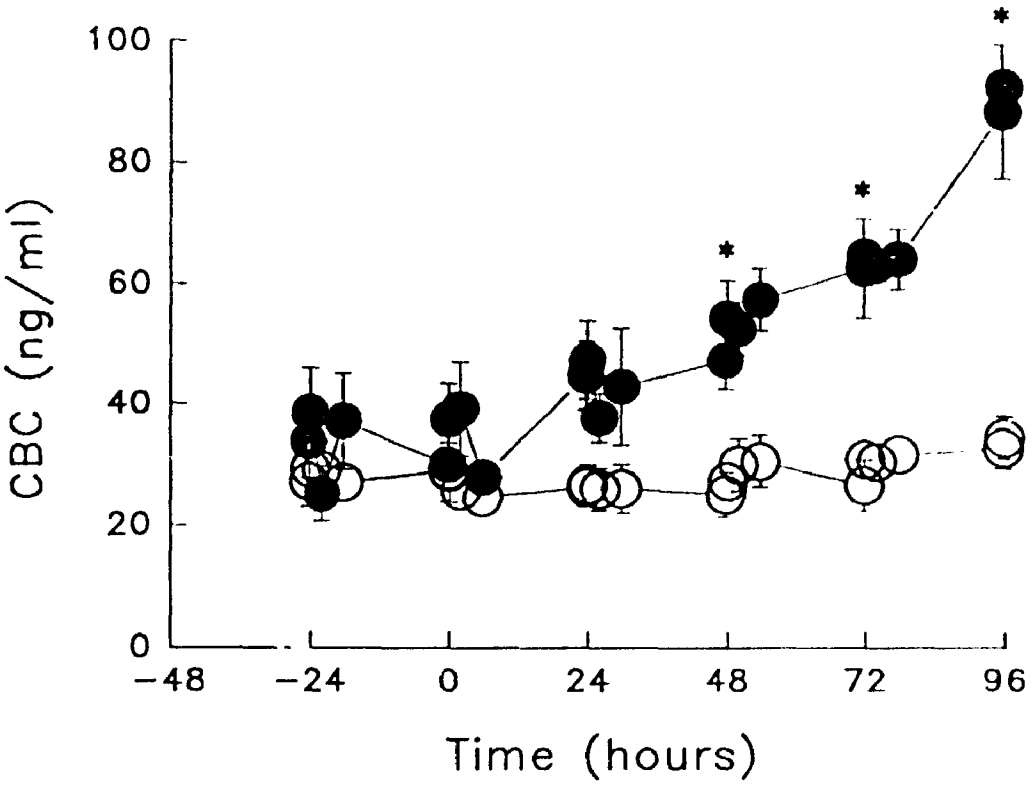

100
80
60
40
20
0
CBC (ng/ml)
−48
−24
0
24
48
72
96
Time (hours)
*
*
*

Figure 6.2 (Top panel) Northern blot of fetal hepatic CBG mRNA in individual fetuses after dexamethasone (n=4) or saline (n=5) treatment. The fetal liver autoradiogram was exposed for 21 h. Molecular size of the CBG mRNA was determined by lambda *Hind*III molecular size markers and is shown on the right. An 18S rRNA cDNA was used as a control for the amount of RNA analyzed. (Bottom panel) Histogram of mean fetal liver CBG mRNA abundance after dexamethasone or saline treatment, relative to 18S rRNA. Histogram data is represented as the ratio of CBG mRNA/18S rRNA ± SEM and significance was $P < 0.05$.

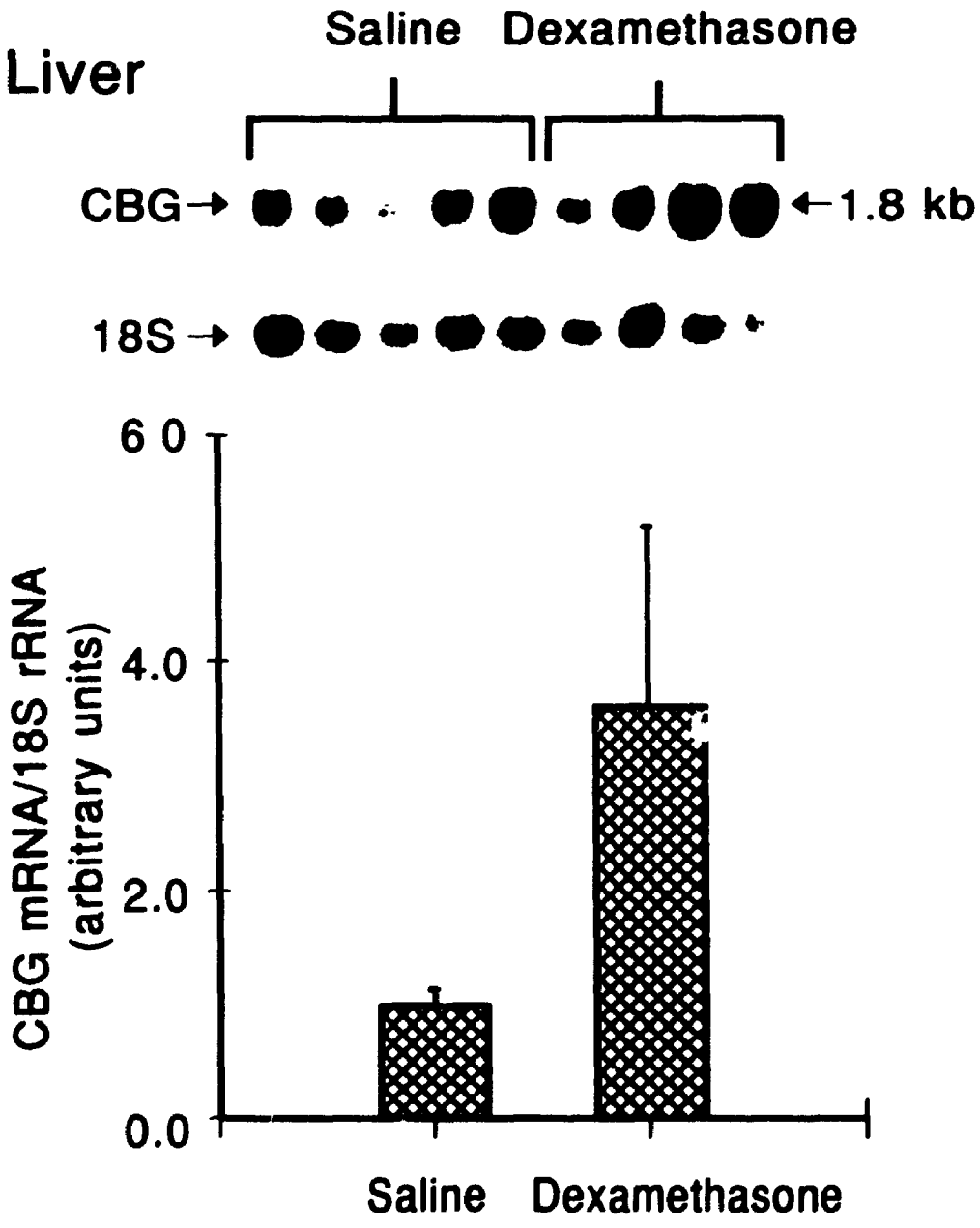

Liver
Saline
Dexamethasone
CBG→
←1.8 kb
18S→
6 0
4.0
2.0
0.0
CBG mRNA/18S rRNA
(arbitrary units)
Saline
Dexamethasone

Figure 6.3 Changes in adult plasma CBC before and during treatment (starting at time 0) with dexamethasone (●-●, 2 mg/day; ▲-▲, 10 mg/day) or saline (○-○). Values are mean ± SEM and level of significance is $P < 0.05$. Significant decreases are indicated by the asterisk.

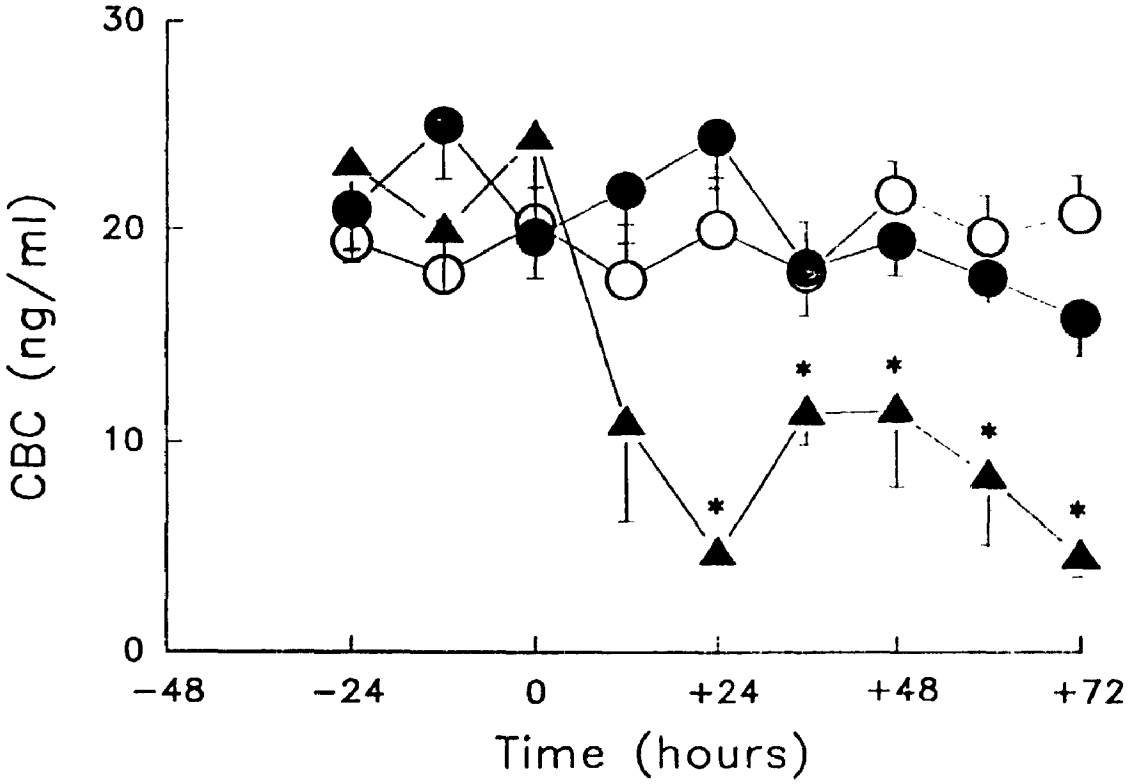

30
20
10
0
CBC (ng/ml)
−48
−24
0
+24
+48
+72
Time (hours)

Figure 6.4 (Top panel) Northern blot of adult hepatic CBG mRNA in individual animals after dexamethasone (10 mg/day) or saline treatment. The adult liver autoradiogram was exposed for 3 days. Molecular size of the CBG mRNA was determined by lambda *Hind*III molecular size markers and is shown on the right. An 18S rRNA cDNA was used as a control for the amount of RNA analyzed. (Bottom panel) Histogram of adult liver CBG mRNA abundance after dexamethasone or saline treatment, relative to 18S rRNA. Histogram data is represented as the ratio of CBG mRNA/18S rRNA ± SEM and significance was $P < 0.05$.

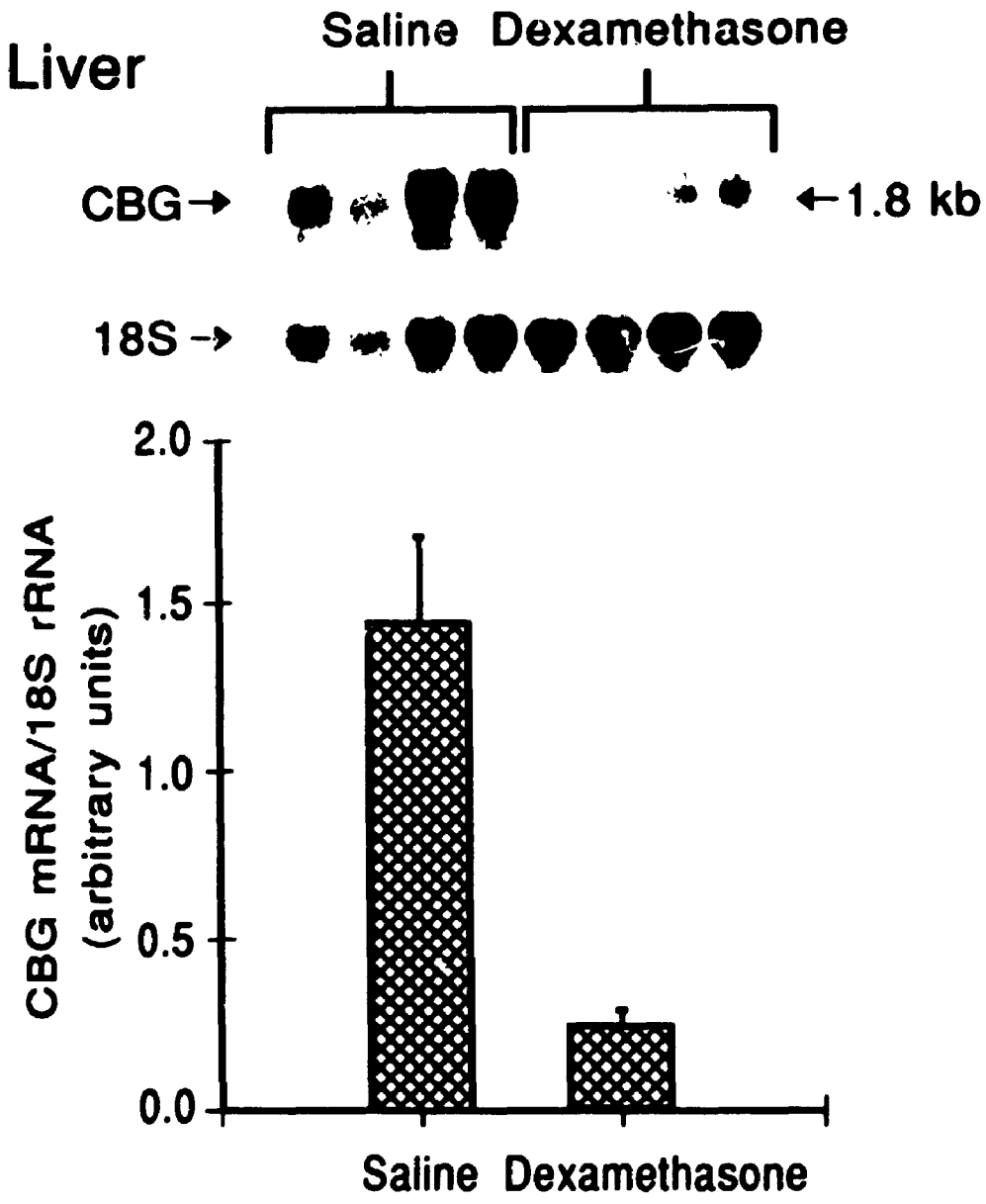

Liver
Saline
Dexamethasone
CBG→
←1.8 kb
18S →
2.0
1.5
1.0
0.5
0.0
CBG mRNA/18S rRNA
(arbitrary units)
Saline
Dexamethasone

Figure 6.5 Representative elution profiles from 5 ml Con A columns of protein (▲-▲) and [^{3}H]cortisol binding (Δ-Δ), after 96 h of saline treatment (top panel) and after 96 h of dexamethasone treatment (middle panel), compared to the elution profiles of maternal plasma (bottom panel). In the fetal plasma after 96 h of dexamethasone treatment, there was a shift of CBG elution from non-Con A binding (first 12 fractions; peak A), to Con A bound (last 18 fractions; peak B). In the adult, CBG eluted almost exclusively in the Con A bound fractions (peak B).

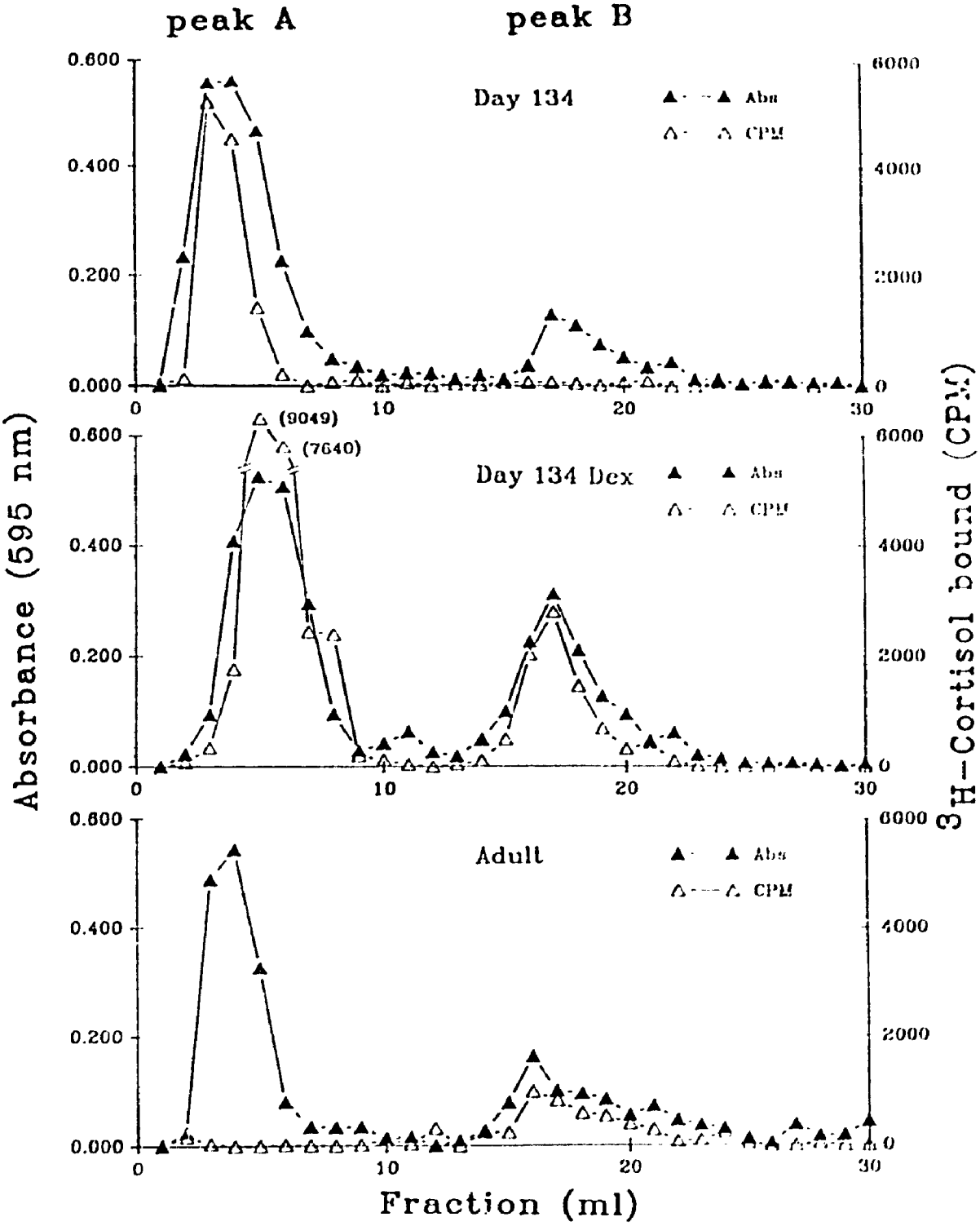

peak A
peak B
Day 134
Abs
CPM
Day 134 Dex
Adult
(9049)
(7640)
Absorbance (595 nm)
3H-Cortisol bound (CPM)
Fraction (ml)
0.600
0.400
0.200
0.000
6000
4000
2000
0
10
20
30

Figure 6.6 Elution fractions containing protein were pooled for the non-Con A bound proteins (peak A) and the Con A bound proteins (peak B). The percentage of [^{3}H]cortisol bound in peak B over the total [^{3}H]cortisol in peak A+B was then calculated. In the day 130 fetal sheep there was between 4.5% to 7.5% of total bound [^{3}H]cortisol in peak B. This increased significantly ($P < 0.05$) to 13.1% after 96 h of dexamethasone treatment, but was not changed with 96 h of saline treatment (5.5%). In the adult sheep, between 75%-85% of [^{3}H]cortisol was contained in peak B.

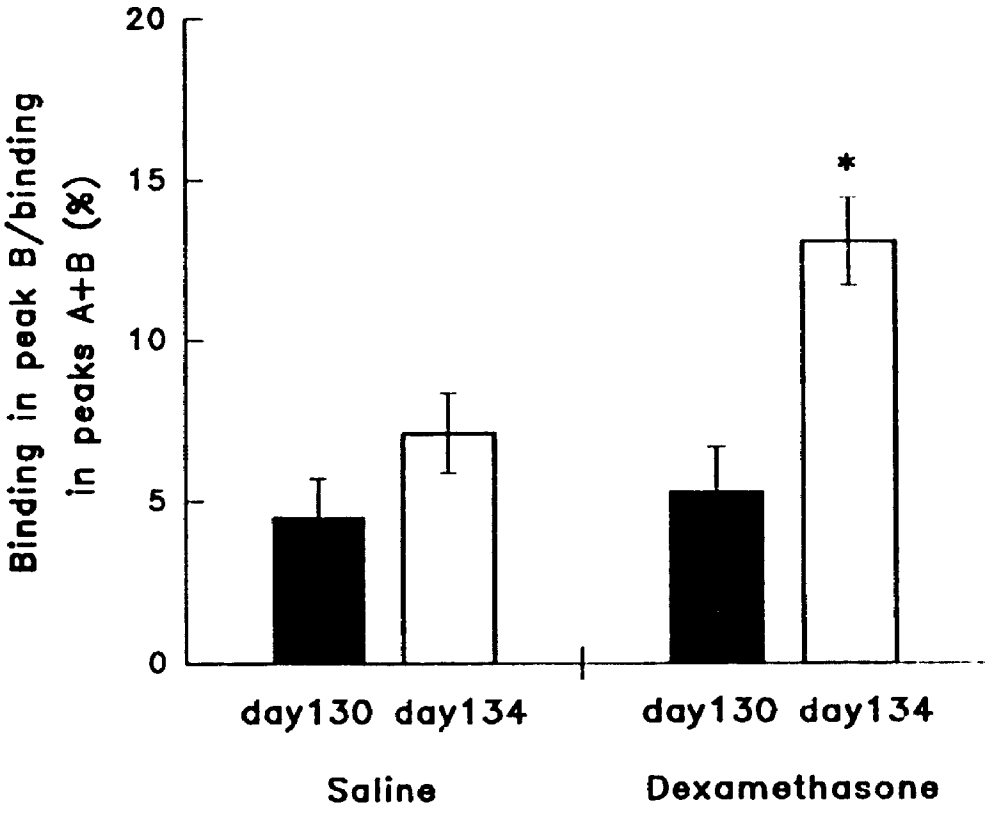

Binding in peak B/binding
in peaks A+B (%)
20
15
10
5
0
*
day130 day134
day130 day134
Saline
Dexamethasone

Figure 6.7 Disappearance of labelled CBG from the maternal vascular compartment. Half-life of labelled CBG was approximately 13 hours. Data is presented as counts per minute as a percentage of CPM in the maternal plasma at + 2 min (log scale) after the ewe received a bolus injection of [^{125}I]CBG (%CPM at + 2 min ± SEM; n=5). Where no error bars are visible the SEM is smaller than the data point.

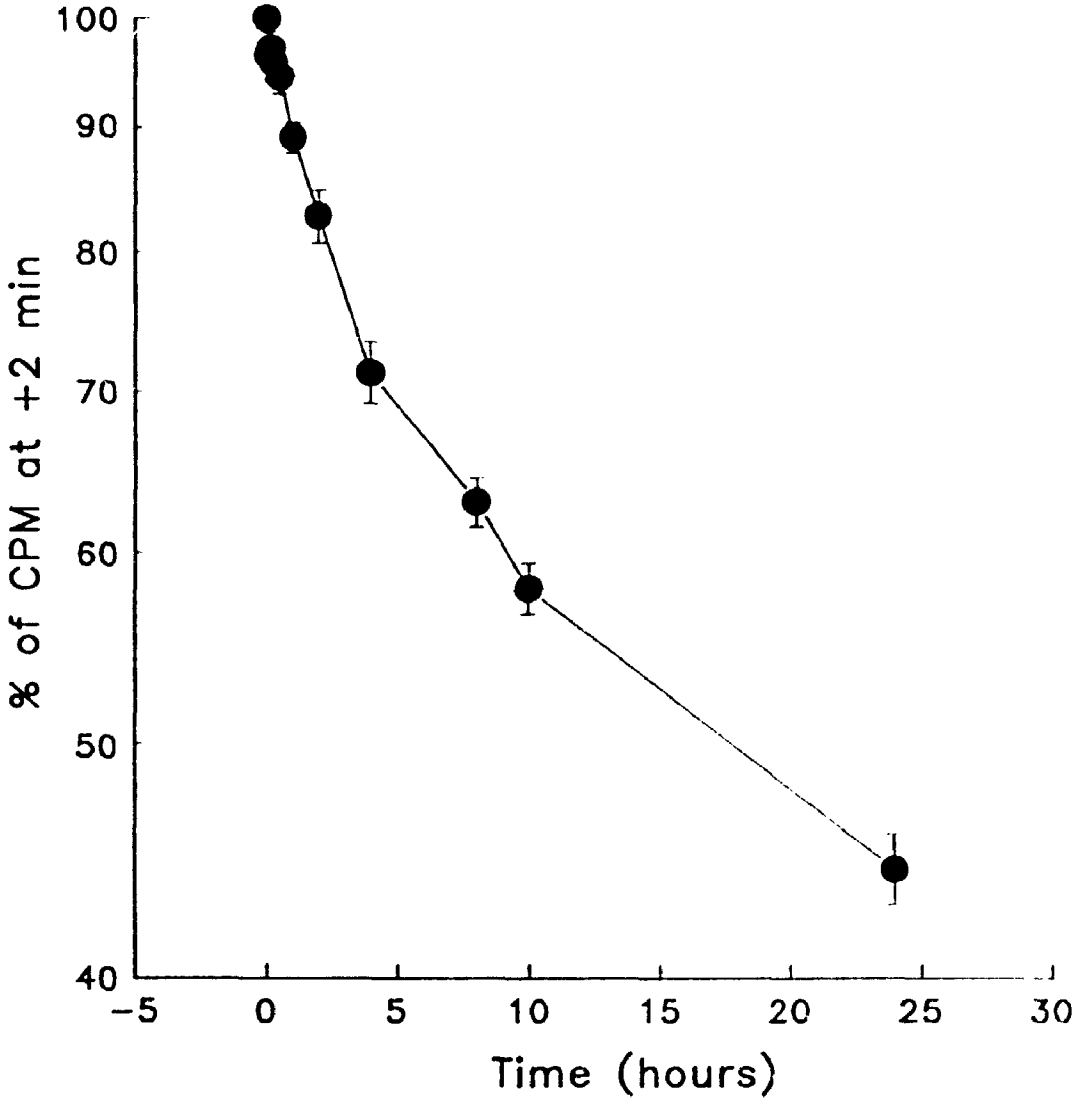

100
90
80
70
60
50
40
% of CPM at +2 min
−5
0
5
10
15
20
25
30
Time (hours)

Figure 6.8 Autoradiogram of a SDS-PAGE gel loaded with plasma (20 µl) samples from a ewe injected with [^{125}I]CBG from +2 min to 24 h after the injection. The protein was purified from pregnant ewes. The exposure time for autoradiography was 7 days. The positions of M_r markers are shown on the left.

[^{125}I]CBG in maternal plasma

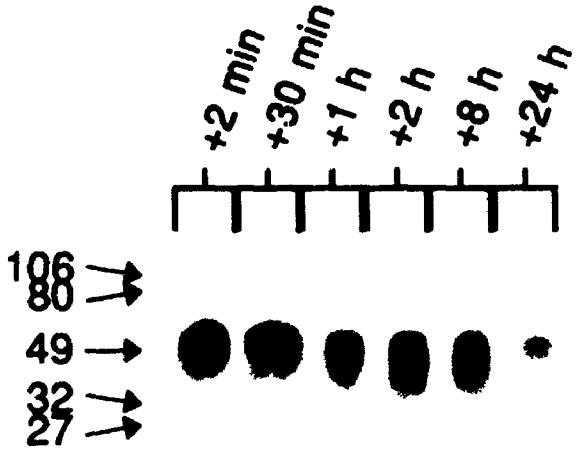


Figure 6.9 Lack of appearance of labelled CBG into either fetal plasma, fetal urine or amniotic fluid in dexamethsone (▲-▲) or saline-treated (□-□) fetal sheep (mean ± SEM). Data is presented as CPM as a percentage of the maternal plasma CPM at + 2 min after the ewe received a bolus injection of [^{125}I]CBG. Maximum counts (100 cpm) were approximately double the background counts.

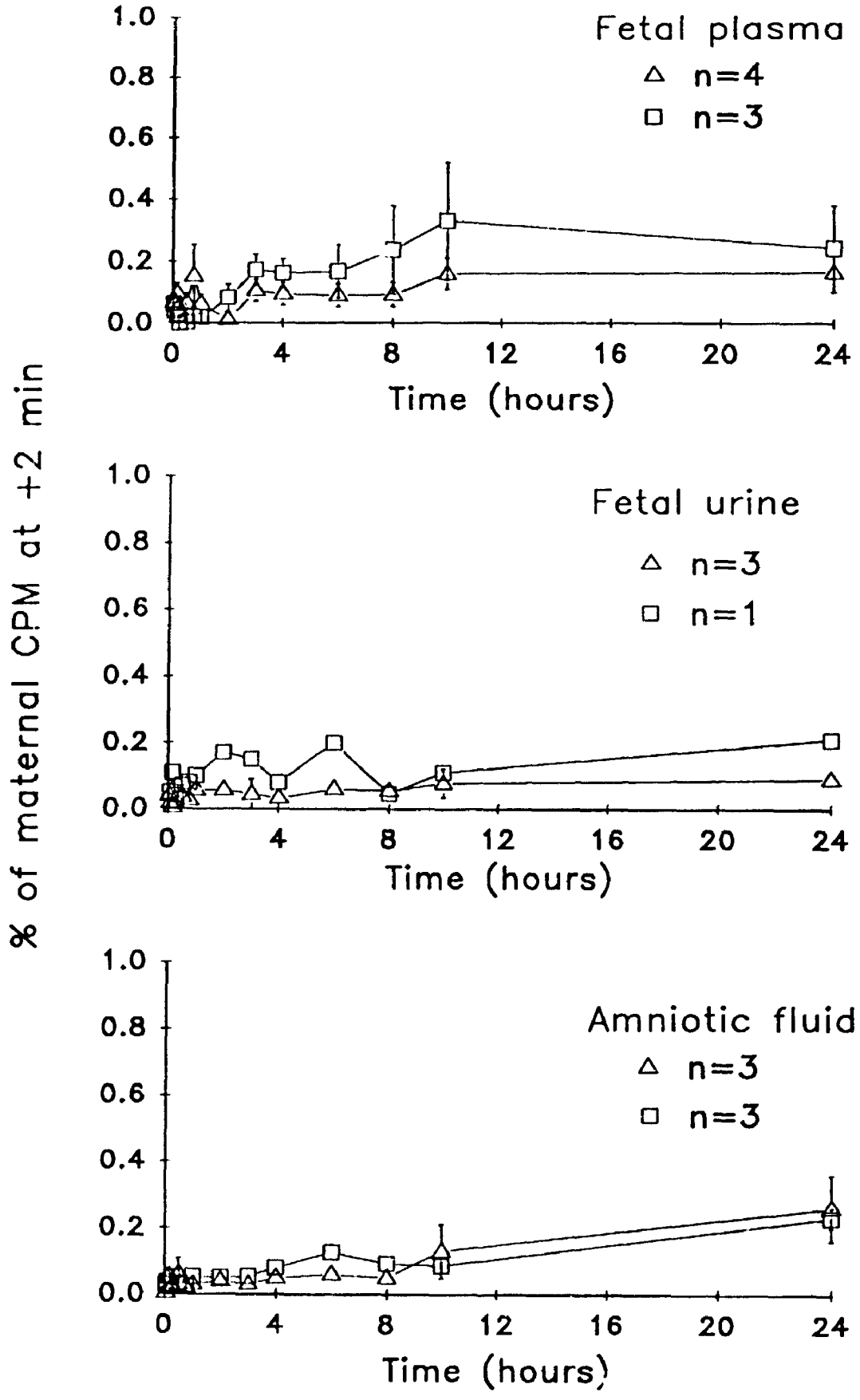

Fetal plasma
△ n=4
□ n=3
Fetal urine
△ n=3
□ n=1
Amniotic fluid
△ n=3
□ n=3
% of maternal CPM at +2 min
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
24
Time (hours)

Table 6.1 Blood gas data (pH, pCO_2 and pO_2) for dexamethasone and saline-treated fetal sheep

Dexamethasone-treated

Time (hours)	pH	pCO_2	pO_2
-24	7.35 ± 0.02	50.4 ± 0.8	22.8 ± 1.6
0	7.34 ± 0.02	50.9 ± 0.7	23.4 ± 0.9
+24	7.33 ± 0.01	51.4 ± 1.1	23.5 ± 1.3
+48	7.35 ± 0.01	51.9 - 1.1	22.0 ± 0.9
+72	7.32 ± 0.01	55.3 ± 4.2	21.0 ± 0.6
+96	NA	NA	NA

Saline-treated

Time (hours)	pH	pCO_2	pO_2
-24	7.34 ± 0.01	46.6 ± 2.4	25.2 ± 1.8
0	7.35 ± 0.01	50.7 ± 0.8	23.7 ± 1.0
+24	7.35 ± 0.01	51.0 ± 2.3	25.3 ± 2.3
+48	7.35 ± 0.01	51.4 ± 1.6	26.2 ± 1.9
+72	7.36 ± 0.02	54.9 ± 3.0	23.5 ± 1.7
+96	NA	NA	NA

Data represents mean ± SEM (n=5).
NA = not available.

CHAPTER 7

EFFECT OF CORTISOL TREATMENT ON CBG BIOSYNTHESIS AND GLYCOSYLATION IN FETAL SHEEP AT DAY 100 OF GESTATION

7.1 Introduction

During late gestation there is a concomitant increase in basal ACTH and cortisol concentrations in the systemic plasma of the fetal sheep (MacIsaac, Bell, Mcdougall, Treager, Wang and Wintour, 1985; Norman *et al.*, 1985). This occurs despite the negative feedback effects of exogenous glucocorticoid on plasma ACTH concentrations (Wood, 1987). These endocrine changes, which reflect maturation of the fetal HPA axis, are essential components of developmental events that lead to parturition (Liggins *et al.*, 1973; Thorburn and Challis, 1979). They also contribute to the stimulus to organ maturation in the fetus in preparation for extra-uterine life (Liggins, 1977).

The prepartum increase in the concentration of cortisol in fetal plasma is accompanied by an increase in the concentration of its high affinity plasma binding protein, CBG (Fairclough and Liggins, 1975; Ballard *et al.* 1982; see chapter 4). I have provided evidence that glucocorticoids stimulate the rise in plasma CBG in the late gestation fetus (see chapters 4 and 6), thereby maintaining a relatively low free cortisol concentration in fetal plasma (Ballard *et al.*, 1982; see chapter 4). In this way the negative feedback effects of glucocorticoids on the hypothalamus and pituitary are attenuated, allowing pituitary ACTH secretion to continue rising, presumably in response to sustained inputs to the hypothalamus from higher centres (Challis and Brooks, 1989; Brooks and Challis, 1991; see chapter 5), and increased pituitary responsiveness (Wintour *et al.*, 1984; Lü *et al.*, 1991). These changes have been characterized

as components of a positive feedforward glucocorticoid-mediated cascade (Challis and Brooks, 1989).

In chapter 6, I demonstrated that dexamethasone, infused at a rate of 360 µg/day produced a three-fold increase in plasma CBG concentrations in fetuses at d130-134 of pregnancy. This was associated with an increase in the abundance of CBG mRNA in the fetal liver, and with an alteration in the distribution of glycosylated species of CBG in fetal plasma. However, these experiments used a relatively high amount of synthetic glucocorticoid, given at a time in gestation when prepartum activation of the fetal HPA axis may have already been initiated. To address these points, the present experiments were conducted on fetuses at day 100 of pregnancy, when the fetal HPA axis is relatively unresponsive (Wintour *et al.*, 1975) and the plasma CBG concentration is lower than at any other time during gestation (Ali *et al.*, 1992). I infused physiological amounts of cortisol calculated to achieve plasma cortisol concentrations characteristic of fetuses at approximately day 125 of pregnancy (Hennessy, Coghlan, Hardy, Scoggins and Wintour, 1982), and examined the effects on plasma CBG concentrations, patterns of CBG glycosylation, and on the abundance of CBG mRNA in the fetal liver.

7.2 Materials and methods

7.2.1 Materials

Chemicals and non-radioactive steroids were obtained from Sigma

Chemical Co. (Mississauga, Ont.). [1,2,6,7-^{3}H]Cortisol (specific activity 80-100 Ci/mmol) was purchased from NEN Research Products (DuPont Canada Inc., Mississauga, Ont.) and purified by TLC in the solvent system chloroform:ethanol (95:5) before use.

7.2.2 Animals

7.2.2.1 Surgical protocol

Experiments were conducted in 12 pregnant sheep with known insemination dates. At day 94-95 of pregnancy, and under general anaesthesia, polyvinyl catheters were implanted into the fetal carotid artery and jugular vein, using techniques described previously (Challis *et al.* 1981), and a catheter was placed in the amniotic cavity for post-operative administration of antibiotics (see chapter 2). Polyvinyl catheters were also implanted into a maternal femoral artery and vein. Following surgery, catheters were filled with sterile heparin-saline and flushed daily. The post-surgical management of the animals was conducted as described previously (see chapter 2).

7.2.2.2 Experimental protocol

Beginning on day 100 of pregnancy, either cortisol (1 mg/24 h; 0.5 ml/h) or saline (0.5 ml/h) was infused into a fetal jugular vein for 100 h. There were 6 fetuses from separate pregnancies in each group. Blood samples (2.0 ml) were withdrawn from the carotid artery catheter at 10.00 h each day beginning

24 h before the start of the infusions, and continuing at 24 h intervals thereafter. An additional sample was collected at 100 h from the start of the infusions. Samples were collected into heparinized syringes. An aliquot (0.2 ml) was taken for blood gas determinations (Table 7.1, page 198) using an ABL-3 blood gas analyzer (Radiometer, Copenhagen, Denmark). The remaining blood was transferred to chilled plastic tubes and centrifuged immediately at 1500 x g for 10 min. Plasma was separated and stored at -20 C until analysis.

7.2.2.3 Euthanasia and collection of tissues

After infusion for 100 h the mother and fetus(es) were killed by administration of a dose of Euthanyl suitable for euthanasia. The right lobe of the fetal liver was collected quickly from 4 fetuses in each group, frozen in liquid nitrogen, and the liver stored at -70 C.

7.2.3 Measurement of plasma cortisol and CBC

Plasma cortisol concentrations were measured by radioimmunoassay after extraction with diethyl ether. The antibody characteristics and assay validation for fetal sheep plasma cortisol measurements have been described previously (Challis *et al.*, 1981). The combined inter- and intra-assay coefficient of variation was 12%. The CBC was determined in 50 µl aliquots of undiluted fetal plasma using the procedure of Ballard *et al.* (1982) with modifications (Challis *et al.*, 1985; see chapter 2).

7.2.4 Measurement of hepatic CBG mRNA abundance by Northern blot analysis

The methodology for Northern blot analysis to measure hepatic CBG mRNA abundance is described in chapter 2.

7.2.5 CBG elution profiles using Concanavalin A chromatography

Plasma samples (20 µl) were applied to Con A Sepharose columns (0.3 ml) and allowed to enter the gel bed. The fraction excluded from Con A (Fraction A; Con A non-retarded) was collected with 480 µl running buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$). The columns were then washed with a further 500 µl of running buffer. The fraction which bound to the lectin (Fraction B; Con A-retarded) was eluted with elution buffer (500 µl; running buffer + 5% dimethylformamide + 5% α-D-mannosepyranoside). Samples were dialysed overnight (4 C) against running buffer, lyophilized, and reconstituted in Tricine assay buffer for CBC assay as described (Challis *et al.*, 1985), using 0.32 ng cortisol/50 µl sample to achieve saturation, and 200 ng cortisol/50 µl to assess non-specific binding. Results are expressed as specific binding of [^{3}H]-cortisol in the Con A-retarded and Con A non-retarded fractions.

7.2.6 Data analysis

Changes in plasma cortisol and CBC in the 2 groups of fetuses were analysed by a two-way ANOVA corrected for repeated measures. Values are

presented as mean ± SEM. Statistical differences were assessed by Bonferroni's t-tests with significance as $P < 0.05$. Differences in the abundance of CBG mRNA and of glycosylated forms of CBG were determined using Student's t-test ($P < 0.05$).

7.3 Results

7.3.1 Fetal plasma cortisol concentrations achieved with cortisol treatment

Basal cortisol concentrations in the two groups of fetuses were similar (~2 ng/ml). Plasma cortisol rose to 6 ng/ml within 24 h of beginning the cortisol infusion, and remained elevated during the experimental period ($P < 0.05$; Figure 7.1, page 191).

7.3.2 Effect of cortisol treatment on plasma CBC

The plasma CBC was similar (~19 ng/ml) in the two groups of fetuses prior to infusions (Figure 7.2, page 193). During intra-fetal cortisol administration, plasma CBC rose progressively, and by 72 h was significantly higher than in the saline infused fetuses, or in comparison to pre-infusion values ($P < 0.05$).

7.3.3 Effect of cortisol treatment on hepatic CBG mRNA abundance

By Northern blot analysis a single CBG transcript of 1.8 kb was identified in total RNA extracts of the liver from cortisol and saline-infused fetuses. The

mean abundance of CBG mRNA was significantly elevated (approximately 3 times; $P < 0.05$) in the livers of fetuses that had been treated with cortisol compared to those of control animals or prior to cortisol infusion (Figure 7.3, page 195).

7.3.4 Effect of cortisol treatment on CBG elution profiles using Concanavalin A chromatography

The elution profiles of CBG were measured using Con A chromatography of plasma samples collected before and after 100 h of either cortisol or saline infusion. In plasma from fetuses treated with cortisol, there was a significant increase in the proportion of CBG that eluted in fraction A (Con A non-retarded; $P < 0.05$), but no significant change in the amount of CBG that eluted in fraction B. Saline infusion for 100 h did not alter the elution profile of CBG from samples of fetal plasma (Figure 7.4, page 197).

7.4 Discussion

The rate of cortisol infusion used in this study was calculated from published estimates of metabolic clearance rates for cortisol in fetal sheep (Hennessy *et al.*, 1982), and was designed to elevate plasma cortisol concentrations by about 4 ng/ml. In this way, these studies attempted to mimic the early increase in plasma cortisol values in the fetal lamb associated with HPA maturation at about day 125 of pregnancy (Magyar *et al.*, 1980; Challis and

Brooks, 1989; see chapter 4). These physiologic increases of plasma cortisol stimulated a rise in the plasma CBC, which reflects plasma CBG, and an increase in the abundance of hepatic CBG mRNA. Thus natural, as well as synthetic glucocorticoids (see chapter 6), stimulate CBG biosynthesis in the fetal sheep, even at times during pregnancy when the HPA axis is regarded as unresponsive.

I have demonstrated that the liver is the major site of CBG production in the ovine fetus (see chapter 4). The design of the present study does not allow the determination the temporal relationship between exogenous glucocorticoids, and the rise in plasma CBG and hepatic CBG mRNA. Further studies are also required to determine whether glucocorticoid administration results in greater CBG expression within a given population of hepatocytes, or recruitment of more liver cells to produce CBG, or both. These stimulatory actions of cortisol in the fetus are in marked contrast to effects in adults of various species where glucocorticoids decrease plasma CBG and hepatic CBG gene expression (Smith and Hammond, 1989; see chapter 6).

The plasma CBG concentration of the fetal lamb rises progressively during the last 20-25 days of gestation, while in the mother it is relatively unchanged (Fairclough and Liggins, 1975; Paterson and Hills, 1967). These patterns suggest independence of CBG synthesis and/or clearance between the fetus and mother. The pattern of concentration change in plasma CBG is known for the fetus in some other species. In the baboon (Oakey, 1975) and rhesus monkey (Beamer,

Hagemenas and Kittinger, 1973), the serum CBC falls during late pregnancy. Serum CBG and hepatic CBG mRNA abundance increase in the fetal rabbit between day 10 and day 22 of pregnancy, then decrease towards term (day 31), while maternal serum CBG and hepatic CBG mRNA abundance increase to day 28-30, but fall prepartum (Seralini *et al.*, 1990). In the fetal rat, serum CBG and hepatic CBG mRNA decrease dramatically between day 15 and term (day 21) (Smith and Hammond, 1991; Elfahime *et al.*, 1992). The abundance of CBG mRNA in fetal mouse hepatocytes increases between day 11 and day 16, but falls to undetectable levels by day 19 (Scrocchi *et al.*, 1993). There is no direct information in these studies for mechanisms controlling hepatic CBG gene expression. Glucocorticoids decrease CBG gene expression in adult rats, and may be responsible for the fall in fetal plasma CBG that occurs in rodents prepartum (Smith and Hammond, 1991). At earlier times of pregnancy, it has been suggested that the fetal CBG gene is relatively unresponsive to corticosteroids (Smith and Hammond, 1991). In contrast, previous studies had indicated that the sheep fetus during late gestation responded to exogenous glucocorticoids with an increase in CBG gene expression (see chapter 5). The present study suggests that the fetal liver is sensitive to physiologic changes in systemic concentrations of naturally occurring glucocorticoids as early as day 100 (0.66) of pregnancy. Glucocorticoid receptor (GR) mRNA is present in the fetal liver at this time (Berdusco, Yang, Challis and Hammond, 1993), however the CBG promoter lacks glucocorticoid response sequences (Hammond *et al.*, 1991).

The difference in response pattern of the CBG gene to glucocorticoids between the fetus and adult must presumably relate therefore to complex transcriptional control and to actions on intermediary regulatory proteins between the adult and the fetus.

Corticosteroid-binding globulin contains five consensus sites for N-linked glycosylation in adult sheep, and sheep CBG exhibits a molecular weight of approximately 57 kDa (see chapter 3). This reflects the presence of up to five *N*-linked carbohydrate chains added to the 45.5 kDa mature protein. Greater than 70 % of the CBG in adult plasma is retained by Con A chromatography, indicating the presence of at least one biantennary oligosaccharide chain (see chapter 4). Fetal sheep plasma contains predominantly glycosylated species of CBG that are devoid of biantennary oligosaccharides, but post-translational modification leading to the formation of a more adult-like CBG glycoform occurs with dexamethasone administration to fetuses at day 130-134 (see chapter 5). In the present study cortisol had no effect on the elution profiles of CBG from Con A columns, the pattern suggesting persistence of CBG species that contained all tri- or tetra-antennary oligosaccharides, and were essentially devoid of biantennary chains. It is not yet clear whether the glycosyl transferases responsible for post-translational modification of CBG are less responsive to glucocorticoids at day 100 than at day 130, or whether there are temporal differences in responsiveness to glucocorticoids between the genes for glycosyl transferases and CBG in fetuses. Studies will need to examine dose response

relationships of these genes to cortisol at different gestational ages. It is interesting that a variant form of CBG in which the five sugar chains were all triantennary *N*-linked oligosaccharides has been found during human pregnancy in maternal, although not in fetal serum collected at term (Strel'chyonok and Avvakumov, 1990). It will be of interest to characterize the CBG species in fetal serum at earlier times of human gestation since these modifications may alter rates of clearance from the circulation, and the interaction of CBG with cell surface receptors (Strel'chyonok and Avvakumov, 1990).

The classical role of circulating CBG is believed to involve binding of plasma glucocorticoids. As total fetal cortisol concentrations increase during late gestation, an increase in CBG capacity maintains the free cortisol concentration (Ballard *et al.*, 1982). In the fetus, as in adults, the free steroid fraction is thought to be responsible for the negative feedback effects of glucocorticoids on hypothalamic CRH and pituitary POMC gene expression (Myers *et al.*, 1991). It has been suggested that the rise in CBG in the plasma of the fetal lamb during late pregnancy maintains a low negative feedback, thereby allowing sustained ACTH secretion and perpetuation of a positive feedforward maturational cascade (Challis and Brooks, 1989). The present study supports the view that cortisol itself stimulates CBG biosynthesis in the fetus, and that this action is effective before the time when the potential for negative feedback exists (Wintour *et al.*, 1980). I suggest that this action of cortisol may be critical to subsequent maturation of the fetal HPA axis, and to the onset of parturition.

Figure 7.1 Changes in the concentration of cortisol in plasma of sheep fetuses infused with cortisol (•-•; n=6) or saline (o-o; n=6). Cortisol infusions began at time 0. Values are mean ± SEM. When no SEM is shown, it is smaller than the size of the data point. Significant differences between the two groups at individual times are indicated, *, $P < 0.05$.

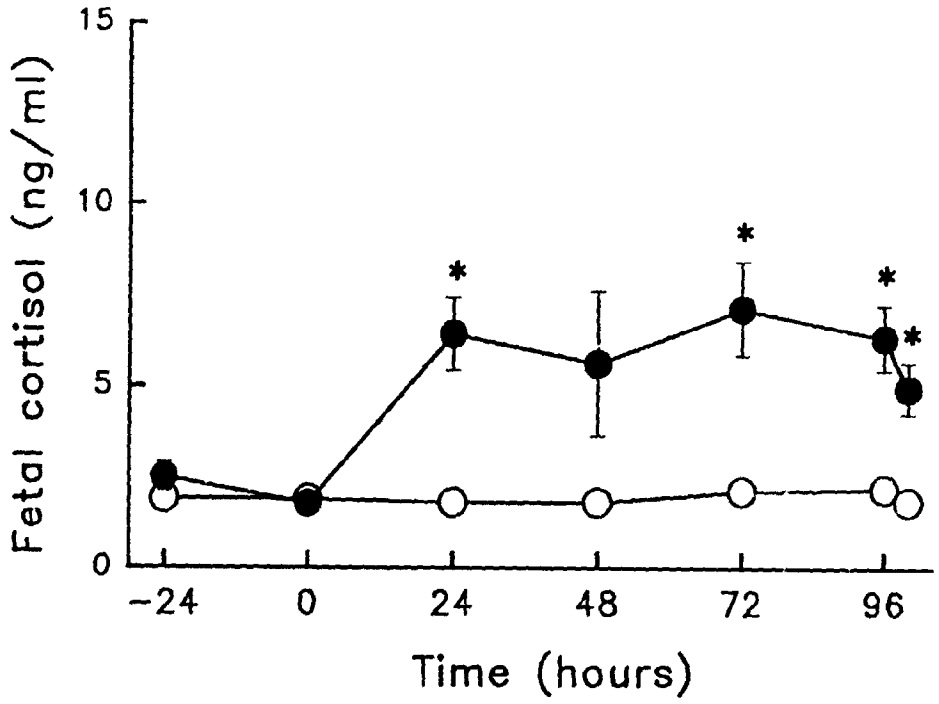

15
10
5
0
Fetal cortisol (ng/ml)
*
*
*
*
−24
0
24
48
72
96
Time (hours)

Figure 7.2 Changes in the CBC of plasma from sheep fetuses treated with cortisol (•-•; n=6) or saline (o-o; n=6). Cortisol infusions began at time 0. Values are mean ± SEM. Significant differences between the two groups at individual times are indicated, *, $P < 0.05$.

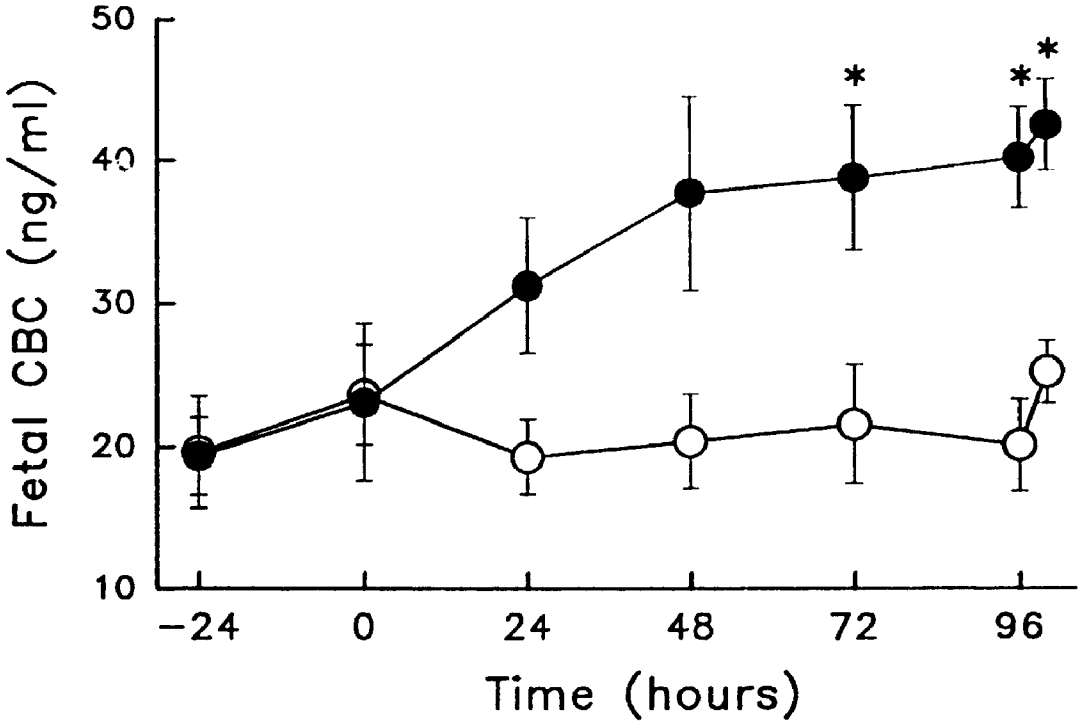

50
40
30
20
10
Fetal CBC (ng/ml)
−24
0
24
48
72
96
Time (hours)
*
*
*

Figure 7.3 Cortisol effects on CBG mRNA abundance in the liver of individual fetal sheep at day 100 of gestation. The upper panel shows the Northern blot analysis of CBG mRNA and 18S ribosomal RNA in the liver of four individual fetuses infused with cortisol or saline for 100 h. The histograms show the ratio of CBG mRNA:18S rRNA in the two groups of fetuses (n=4 both groups). There was a significant increase in CBG mRNA abundance in the cortisol-treated fetal sheep compared to saline controls. Values are mean ± SEM.

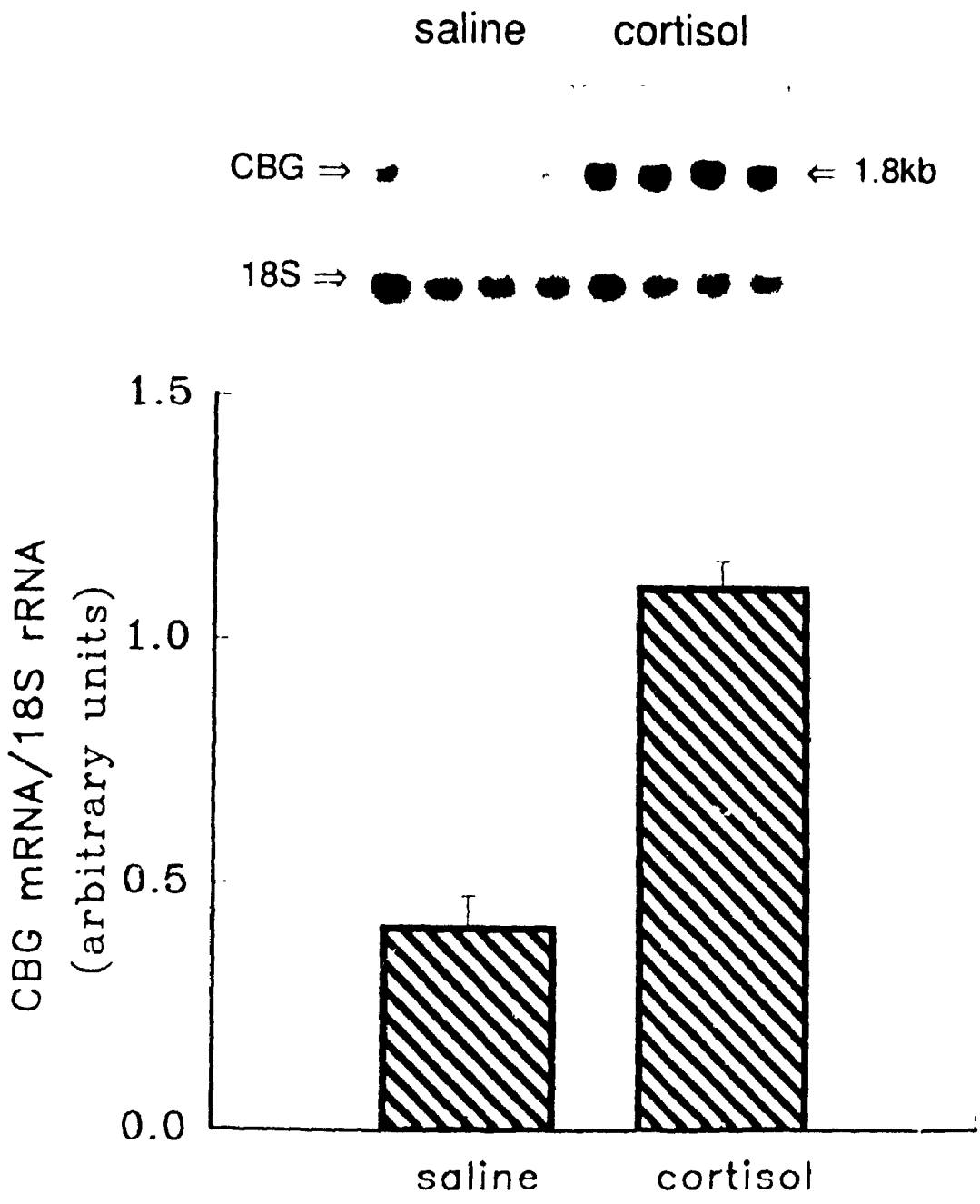

saline
cortisol
CBG ⇒
⇐ 1.8kb
18S ⇒
CBG mRNA/18S rRNA
(arbitrary units)
1.5
1.0
0.5
0.0
saline
cortisol

Figure 7.4 Specific cortisol binding to samples of fetal plasma collected before (0 h) or after (100 h) either cortisol or saline infusion. Samples were subjected to Con A chromatography, and binding of [^{3}H]cortisol measured in the Con A-retarded (solid bars) and Con A non-retarded (open bars) fractions. All values are mean ± SEM; n=6 fetuses in the cortisol or saline treatment groups. **, $P < 0.05$, cortisol vs saline treatment at 100 h.

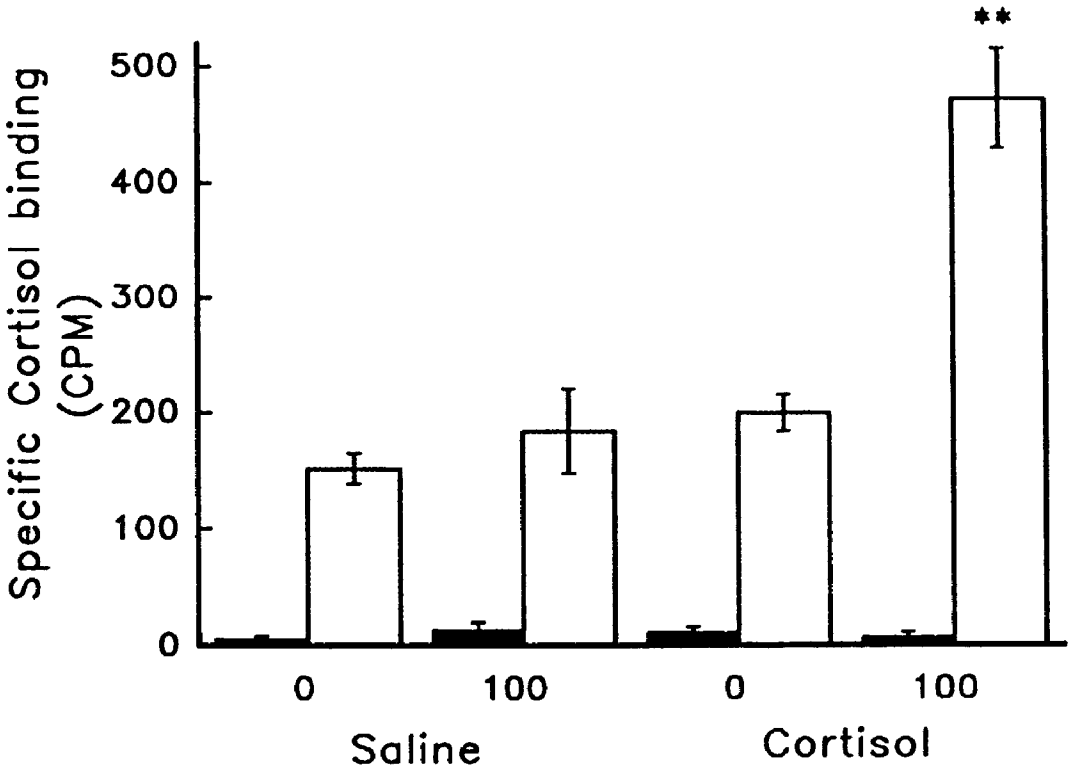

**
500
400
300
200
100
0
Specific Cortisol binding
(CPM)
0
100
0
100
Saline
Cortisol

Table 7.1 Blood gas data (pH, pCO_2 and pO_2) for cortisol and saline-treated fetal sheep

Cortisol-treated

Time (hours)	pH	pCO_2	pO_2
-24	7.35 ± 0.01	50.9 ± 0.8	25.9 ± 0.9
0	7.32 ± 0.01	52.5 ± 1.8	27.7 ± 2.5
+24	7.35 ± 0.01	52.6 ± 1.1	26.1 ± 1.5
+48	7.31 ± 0.02	54.1 ± 1.2	26.2 ± 1.1
+72	7.33 ± 0.01	51.7 ± 1.6	25.6 ± 1.5
+96	7.34 ± 0.01	50.8 ± 1.3	25.6 ± 1.2
+100	7.33 ± 0.01	53.7 ± 1.7	24.6 ± 1.2

Saline-treated

Time (hours)	pH	pCO_2	pO_2
-24	7.34 ± 0.01	47.5 ± 1.0	26.4 ± 2.4
0	7.33 ± 0.02	50.3 ± 2.3	23.8 ± 1.6
+24	7.32 ± 0.02	51.6 ± 1.6	26.0 ± 2.7
+48	7.32 ± 0.01	51.6 ± 0.8	23.8 ± 1.9
+72	7.33 ± 0.01	50.3 ± 0.7	24.5 ± 2.1
+96	7.32 ± 0.02	51.8 ± 1.4	26.4 ± 1.9
+100	7.34 ± 0.02	49.7 ± 1.3	24.4 ± 2.5

Results are mean ± SEM (n=6).

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS and FUTURE DIRECTIONS

8.1 Overall discussion and conclusions

In the fetal sheep, cortisol plays a crucial role in the maturation of the fetus and in the initiation of labour, leading to the birth of a viable fetus. Previous work has demonstrated that maturation of the fetal HPA axis is central to the cascade of events that ultimately result in parturition. Interruptions in the HPA axis, such as lesioning of the PVN or hypophysectomy, lead to a prolongation in pregnancy. The fetal plasma concentrations of the end product of the HPA axis, cortisol, increase in the fetus during the last two to three weeks of gestation, and this increase in cortisol is accompanied by an increase in its high affinity binding protein CBG. The work within this thesis has been a study of molecular and physiological aspects of CBG. The results of this thesis are summarized as follows

In chapter three, the ovine CBG cDNA was determined and the deduced sequence of the mature peptide was presented. The CBG cDNA encoded a predominantly hydrophobic 22 amino acid leader sequence and displayed five possible consensus sites for *N*-glycosylation. The deduced peptide was 408 amino acids in length, which is somewhat longer than the mature peptides from the human, rat and rabbit. However, there was homology in both the CBG cDNA (75%) and peptide (68%) between the human and sheep. Chapter three also demonstrated the electrophoretic mobility, under denaturing conditions, of purified ovine CBG. Ovine CBG has an average molecular size of 57 kDa, and using the deduced peptide structure has a carbohydrate component of approximately 26

percent.

The ontogeny of CBC in fetal and neonatal plasma and the pattern of glycosylation of fetal CBG during late gestation and neonatal life were determined in chapter four. The possible sites of biosynthesis of CBG in selected tissues of the fetus and newborn were investigated, and changes in liver, lung and kidney CBG mRNA abundance at day 100, 125, 140, term and in newborns were established. Plasma CBC increased after day 130, and reached highest levels at term. Post partum, the levels decreased rapidly, and by five days were not different from adult levels. The hepatic CBG message was highest at day 140. At term, hepatic CBG mRNA levels had begun to decline and these continued to decline after birth, when hepatic CBG mRNA abundance was not different from values at day 100 and day 125. The decrease in hepatic CBG mRNA abundance at term presumably gives rise to the rapid post partum decline in plasma CBC. The glycosylation patterns of CBG began to change at term from a fetal to an adult type. The change continued post partum, and by one month of age, the pattern of glycosylation was not different from that in the adult.

Free cortisol in the plasma of the fetal sheep did not increase between day 125 and day 130. It increased to day 135 and remained unchanged until day 145, when the free cortisol concentration was higher than at any other time in gestation. Therefore, increase during late gestation in fetal plasma CBC was instrumental in buffering an increase in free cortisol during a time when total cortisol concentrations were rising dramatically.

Extra-hepatic sites of CBG biosynthesis were found to include the hypothalamus, pituitary, lung, kidney, and adrenal, but not placenta. The liver had the strongest message, followed by the pituitary. CBG production by the liver is most likely for maintenance of systemic plasma CBG concentrations. The purpose of CBG synthesis in extra-hepatic sites may be involved with either local effects of CBG alone, or through a CBG-steroid complex mediating cell processes, however, this remains to be investigated.

The ability of CBG to block glucocorticoid negative feedback on fetal pituitary cells was examined in chapter 5. CBG was able to block the cortisol, but not dexamethasone-induced suppression of basal and CRH-stimulated ACTH release. The ability of CBG to block negative feedback of cortisol but not dexamethasone attests to the specificity of binding of glucocorticoids. These data confirm previous studies in which CBG was demonstrated to bind cortisol, but not to bind dexamethasone (Challis *et al.*, 1985; Ali *et al.*, 1992). In these experiments there was a slight enhancement of ACTH output in the presence of CBG alone. CBG mRNA is present in the fetal pituitary (see chapter 4), and the function of pituitary CBG may be to sequester free glucocorticoid which is able to diffuse from the blood into the fetal corticotroph cell and thereby attenuate glucocorticoid negative feedback.

It has been suggested that an increase in fetal plasma glucocorticoid concentration stimulates an increase in fetal plasma CBC. In chapter 5 it was demonstrated that dexamethasone, a synthetic glucocorticoid, given to the fetus

in pulses (one pulse over 15 min every two hours), produced a significant increase in plasma CBC by 48 h. The mechanism of this increase was postulated to be a function of one or more of the following: an increase in CBG synthesis and secretion, an alteration in transplacental transfer, or a change in the plasma half-life of the protein.

The abundance of the hepatic CBG mRNA was increased after 96 h of dexamethasone treatment. Therefore, the observed increase in plasma CBC is likely due, at least in part, to an increase in CBG gene transcription resulting in an increase in CBG mRNA and an increase in protein production. There was also a change in the glycosylation of CBG after 96 h of dexamethasone treatment with a switch to glycoforms resembling those of adults. This change is similar to the change in fetal CBG glycosylation observed in response to maturation during late gestation as described in chapter 4.

In fetal sheep treated with either saline or dexamethasone, there was negligible transplacental transfer of [^{125}I]CBG from the ewe to the fetus. Therefore it is unlikely that glucocorticoids influence transplacental transfer of CBG. Furthermore, it appears that the fetal sheep is relatively autonomous with regards to its plasma CBG concentrations. This lack of transplacental transfer is unlike the rabbit (Seralini *et al.*, 1990) and it may be speculated that this difference is related to differences in placentation between the two species (Amoroso, 1952).

In chapter 6, the effects of glucocorticoid treatment on plasma CBC and

hepatic CBG mRNA in the fetus were contrasted with those in the adult. Unlike the fetus, treatment of the adult with glucocorticoids resulted in a rapid and sustained decrease in plasma CBC. After 72 h of dexamethasone treatment there was a decrease in hepatic CBG mRNA abundance, and the effect of glucocorticoids on plasma CBG concentrations is likely a consequence of a glucocorticoid action on CBG gene expression. Therefore, the effect of glucocorticoids on plasma CBC is opposite in the fetus and adult. Furthermore, the mechanism of this disparity is likely at the level of gene transcription because dexamethasone treatment also has opposite effects on hepatic CBG mRNA abundance. The stability of the ovine CBG mRNA message during dexamethasone treatment has not been examined, but in the adult rat, treatment with dexamethasone produces a decrease in transcription rate and half-life of the CBG mRNA (Smith and Hammond, 1992).

Dexamethasone treatment at day 130 of gestation stimulates an increase in plasma CBC, increases hepatic CBG mRNA abundance, and changes the glycosylation pattern of fetal CBG. In chapter 7, a small increase in fetal plasma cortisol concentrations was produced, and the effect of this increase on fetal plasma CBC, fetal hepatic CBG mRNA abundance, and patterns of glycosylation was established. The increase in fetal plasma cortisol concentrations was produced at day 100, a time during gestation when the fetal HPA has been suggested as being unresponsive to glucocorticoids.

An elevation of fetal plasma cortisol concentrations from approximately 2

to 6 ng/ml for 100 h (which changes values of cortisol in the fetus from those at approximately day 100 to the concentrations observed in fetuses at approximately day 125) produced a three fold increase in plasma CBC and a three fold increase in fetal hepatic liver CBG mRNA abundance. However, there was no change in the pattern of glycosylation of CBG after cortisol treatment. It remains to be elucidated if the lack of effect of cortisol treatment on changing CBG glycoforms is a function of differences in response to dexamethasone vs cortisol, or if the response of the CBG gene to glucocorticoids is more sensitive than the genes for the glycosyl-transferase enzymes.

8.2 Proposed model for CBG regulation in the fetal sheep

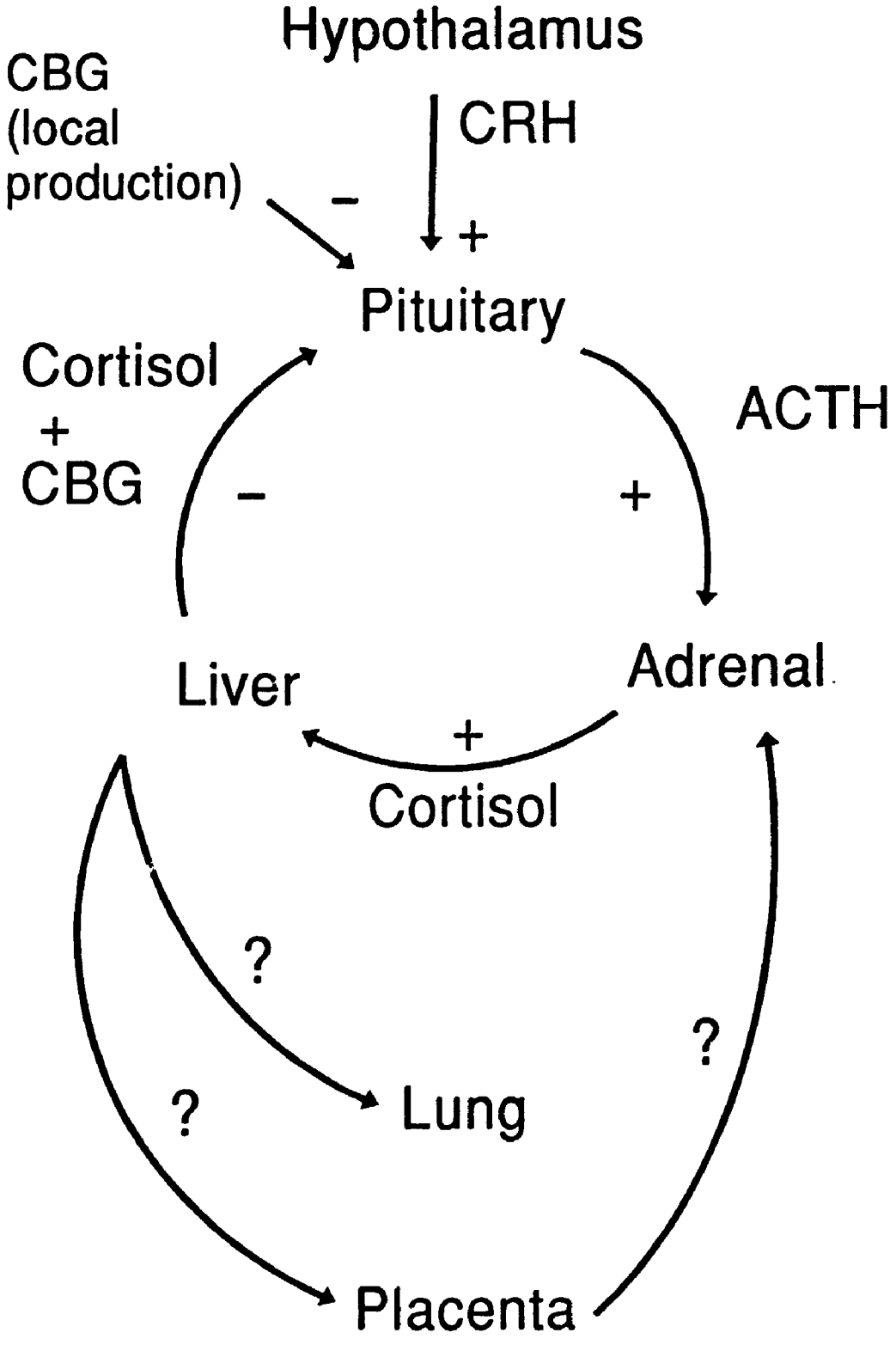
In the fetal sheep, the plasma concentration of cortisol is likely the major determining factor for the plasma concentration of fetal plasma CBG. The protein then binds cortisol, and prevents cortisol from reaching the hypothalamus and pituitary, thereby attenuating the negative feedback effect of glucocorticoids at the level of the hypothalamus and pituitary. This then allows for a progressive increase in ACTH concentrations in fetal plasma, and a sustained drive toward parturition. A proposed scheme for the actions of CBG in the fetal sheep is given in Figure 8.1 (page 208).

8.3 Future experiments and new directions

The results of the work contained within this project have raised a number

of additional questions. In the quest for answers to these questions several further experiments may be designed: i) What is the time course for the observed increase in hepatic CBG mRNA during glucocorticoid treatment? ii) In tissue culture experiments, does the addition of CBG affect the glucocorticoid-induced maturation of tissues? iii) What is the localization of CBG mRNA in the pituitary, and is this message contained within the corticotrophs?

Figure 8.1 Diagram illustrating the proposed action of CBG within the fetal sheep. +; stimulation; -; inhibition; ?; possible CBG interactions.


Hypothalamus
CBG
(local
production)
CRH
–
+
Pituitary
Cortisol
+
CBG
–
ACTH
+
Liver
Adrenal
+
Cortisol
?
?
?
Lung
Placenta

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