CORTICOTROPHIN RELEASING FACTOR IN PREGNANCY

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BY

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ABSTRACT

Using a "two-site" immunoradiometric assay Corticotrophin Releasing Factor (CRF) has been measured in maternal plasma and elevated levels observed in the third trimester of pregnancy. The dilution curve of this CRF paralleled the IRMA standard curve. This CRF-like material eluted on reverse phase high pressure liquid chromatography with a retention time identical to that of synthetic CRF and had equipotent bioactivity with the synthetic peptide.

Third trimester maternal plasma contains a carrier substance for CRF with a molecular weight in the region of 40,000. The binding capacity of the carrier is not saturated but most of the CRF in late gestational plasma is bound.

In a group of 72 pregnant women CRF levels rose from a median of 20 pg/ml at 28 weeks to 1320 pg/ml at 40 weeks and 1732 pg/ml during labour. There was a strong correlation ($r=0.81, P < 0.001$) between gestational age and CRF levels. The rate of rise of CRF (pg/ml) per week was associated with weight gain ($r= 0.36$, $P < 0.05$) but with no other obstetric variable. There was an association between umbilical cord and maternal plasma CRF levels ($r= 0.54$, $P < 0.01$).

CRF levels were elevated after accidental antepartum haemorrhage at 28 weeks ($P < 0.03$). In twin pregnancies maternal CRF levels were significantly raised throughout the third trimester (28-32 weeks, $P < 0.01$; 34-36 weeks, $P < 0.001$). In cases of pregnancy induced

hypertension (28 weeks, $P < 0.001$; 32-36 weeks, $P < 0.001$ and 38-40 weeks, $P < 0.01$), preterm labour and premature rupture of the membranes (28 weeks, $P < 0.004$; 30-32 weeks, $P < 0.002$ and 34-36 weeks, $P < 0.001$), CRF levels were significantly raised.

Maternal plasma CRF levels did not correlate with total cortisol levels and did not display diurnal variation. Short term variation in plasma CRF levels had a Coefficient of Variation (CV) of 5.3 ± 0.52 pg/ml and the effect of posture had a CV of 7.1 ± 0.52 pg/ml. During and between uterine contractions in labour the CV was 4.5 ± 1.4 pg/ml and postpartum the half life of CRF was estimated at 65 minutes.

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THESIS OUTLINE

Corticotrophin Releasing Factor (CRF) is a 41 amino acid peptide produced by the hypothalamus which responds to stress signals and effects the release of adrenocorticotrophic hormone from the anterior pituitary; this in turn stimulates the adrenal cortex to produce cortisol which exerts a negative feedback on the hypothalamus and pituitary gland. CRF has been identified in sites outside the brain, the placenta being one. In the non-pregnant state CRF levels are low or undetectable in plasma but in the third trimester of pregnancy elevated levels, 1 to 2 orders of magnitude greater than the non-pregnant state, have been reported.

This thesis has the following aims:

1. To characterise maternal plasma CRF by physicochemical studies and assess its bioactivity.
2. Further physiological studies provoked by (1) determine whether maternal plasma CRF has a binding protein.
3. Describe maternal and fetal plasma levels of the peptide throughout pregnancy and parturition.
4. Describe maternal plasma CRF levels in abnormal pregnancy states
5. Describe maternal plasma CRF levels in relation to changes in the hypothalamic pituitary adrenal axis that occur normally in pregnancy.
6. Assess the variability of plasma CRF measurements.

CHAPTER 1

CHARACTERISATION OF PLACENTAL AND MATERNAL PLASMA CORTICOTROPHIN RELEASING FACTOR

INTRODUCTION

1.1. CORTICOTROPHIN RELEASING FACTOR (CRF)

1.1.1. The Nature of CRF

Harris (1948) hypothesized that stress-induced activition of the central nervous system is transformed from a neural into a humoral signal in the hypothalamus and that chemical transmitter substances elaborated therein are transported via the adenohypophyseal portal circulation to stimulate adrenocorticotrophic hormone (ACTH) secretion from the corticotroph. The hypothalamic substance has been termed Corticotrophin releasing factor as it is a potent secretagogue of ACTH with subsequent increased adrenocorticotrophic activity.

CRF bioactivity consists of a number of substances, primarily CRF and Arginine Vasopressin (VP) which interact for the coordinated control of the hypothalamic pituitary adrenocortical (HPA) axis, encompassing regulation of the diurnal rhythm and stress response.

1.1.2. Evidence for Corticotrophin Releasing Factors

The term 'Corticotrophin-releasing Factor' was coined by Saffran & Schally (1955) for the active component of their posterior pituitary and hypothalamic extracts which was able to release ACTH when incubated with pituitary tissue *in vitro*.

Both *in vivo* and *in vitro* bioassays have been used in the detection of CRF and these involve the stimulation of the adenohypophysis with

subsequent measurement of released ACTH by radioimmunoassay (RIA) or bioassay.

IN VIVO assays involve the blockade of endogenous CRF by either hypothalamic lesioning (Lymangrover & Brodish 1973), pharmacological blockade (Vernikos-Danellis 1964, Buckingham 1980) or heterotopic pituitary grafting (Kendall et al 1966). Hypothalamic lesioning and heterotopic pituitary grafting were not consistently precise and pharmacological blockade was non-specific. All assays were hampered by the lack of satisfactory techniques for the determination of ACTH until the direct estimation by RIA (Berson & Yallow 1968, Landon & Greenwood 1968) and a precise bioassay (Sayers et al 1971) were brought into practice.

IN VITRO studies. The dispersed cultured pituitary cell and dispersed pituitary cell suspension assays developed in the 1970's (Takebe et al 1975, Lowry 1974) have major advantages over the studies in the 1950's but there is the potential for non-specific ACTH release (Buckingham 1980).

1.1.3. VP & OTHER CRFs

The multifactorial regulation of ACTH secretion involves the stimulatory effects of several hormones. The primary control of ACTH secretion is mediated by CRF but VP is probably one of several hypothalamic factors which interact and synergise with CRF to produce full ACTH-releasing activity (Gillies & Lowry 1982). There is strong evidence that VP is a regulator of ACTH secretion: An area of CRF bioactivity is observed in the position of VP after separation of the active constituents of hypothalamic extracts by chromatography (Gillies et al 1984). VP is released in superfused isolated rat hypothalamic extracts as well as CRF

(Gillies et al 1984). When both CRF and VP are administered in vitro (Gillies et al 1982) or in vivo (Lamberts et al 1984, Liu et al 1983) a highly significant synergism is observed. Plasma VP levels are unaltered when ovine CRF (oCRF) is infused into normal males suggesting CRF does not have an influence on VP secretion (Conaglen et al 1984). Experiments in rats have shown a co-expression of CRF and VP immunoreactivity in some parvocellular neurosecretory neurones of the hypothalamus after adrenalectomy (Sawchenko et al 1984). Again, in the rat, it has been suggested that there is obligatory co-secretion of CRF, VP and associated neurophysin in the median eminence (Whitnall et al 1985). Immunoneutralisation techniques demonstrate synergism between VP and CRF (Linton & Lowry 1982) and anti-VP immunoglobulins reduce the ACTH response to ether stress (Linton et al 1985) suggesting that VP is exerting its main action by potentiating the effects of CRF .

In the rat adrenaline, noradrenaline, serotonin (5-HT) and angiotensin 11 (A11) have all been reported to release ACTH and/or enhance the response to VP or CRF (Gillies et al 1984) but do not fulfill the criteria for a true CRF. In man it seems unlikely that A11 or adrenaline has any effect upon ACTH secretion in vivo (Haller et al 1986, Al Damlugi et al 1987). 5-HT is a modulator of ACTH secretion and acts centrally as a neurotransmitter, stimulating the release of both CRF and VP (Bruni et al 1982). Oxytocin (OT) may potentiate the release of CRF (Antoni et al 1983). OT and CRF are co-localised in magnocellular hypothalamic neurones (Sawchenko et al1984). OT is present in rat hypophyseal blood at higher concentrations than in peripheral blood indicating secretion in the median eminence (Gibbs 1985).

Figure 1

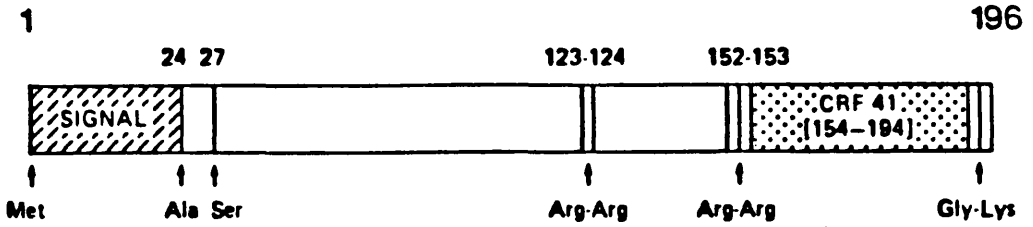


Figure 1 The structure of human pre-pro-CRF (Shibahara et al 1983)

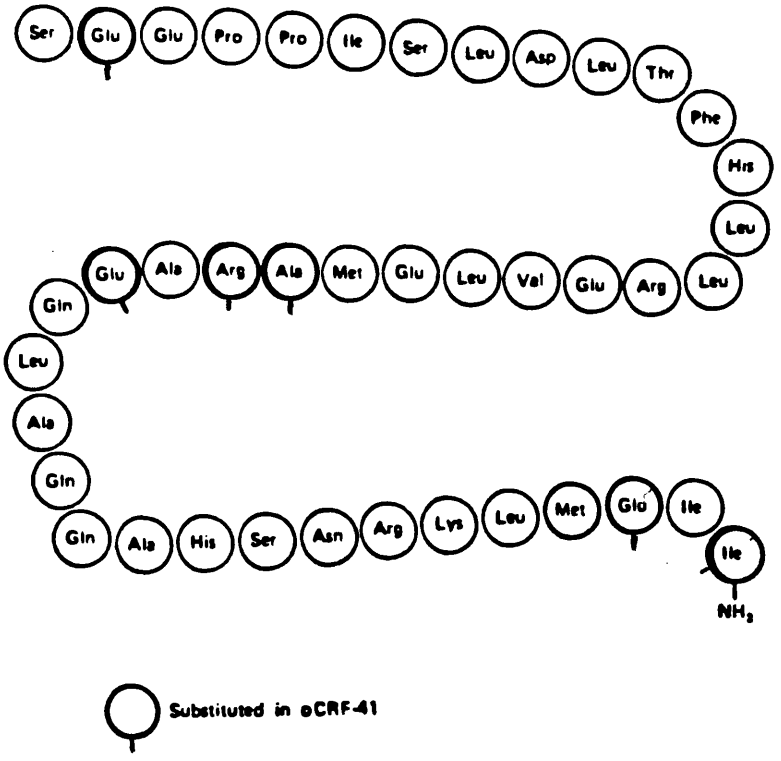


Figure 2 The primary structure of human CRF (Shibahara et al 1983)

CRF is by far the most potent secretagogue of ACTH in vitro , VP second followed by OT and then A11. Based solely on in vitro activity of some of these peptides, it is difficult to suggest that OT and A11 may have a physiological role in ACTH regulation. However, due to the known in vivo interactions of these peptides with CRF, with VP and perhaps monoamines,it is possible that these substances may play a physiological role under certain conditions (Negro-Vilar et al 1987).

1.1.4. THE CRF MOLECULE

It was not until 1981 that oCRF was characterised from ovine hypothalami as a 41 amino acid peptide (Vale et al 1981) and the precursor gene for both oCRF and human CRF (hCRF) has now been isolated and sequenced (Furutani et al 1983, Shibahara et al 1983). Human pre-pro-CRF is synthesised as a 196 amino acid precursor (Fig 1). The sequence of hCRF was deduced from the structure of the CRF precursor gene by Shibahara et al (1983)(Fig 2) and is formed by cleavage of the C-terminus (154-194) from Pre-pro-CRF. hCRF is identical to rat CRF (rCRF), differs from oCRF by 7 amino acids, with bovine CRF being only 1 amino acid different from oCRF. The biological activity depends on the integrity of the amino (N) terminal end of the molecule. Chromatography of rat hypothalamic extracts on sephadex G-50 demonstrates a region of CRF bioactivity coinciding with CRF immunoactivity (Linton & Lowry 1982, Gillies et al 1984).

1.1.5. Neural pathways for CRF

Immunohistochemical staining localises the CRF in nerve fibres surrounding the capillaries of the primary portal plexus in the zona

externa of the median eminence. CRF appears to be expressed in at least 3 morphologically and functionally distinct cell types in the paraventricular nucleus (PVN) (Swanson et al 1987, Nieuwenhuyzen-Kruseman et al 1984). There is also evidence, both morphological and immunocytochemical, for the existance of VP containing pathways in the hypothalamus, from the parvocellular division of the PVN with its fibres terminating at the portal plexus in the zona externa (Zimmerman et al 1977). Ultrastructural analysis of the nerve terminals that contain CRF and VP show these peptides are copackaged in the same neurosecretory granules (Whitnall et al 1985) but there may be functionally distinct sub populations of parvocellular CRF containing neurones which can be distinguished on the basis of their ability to express VP. The presence of CRF has also been identified in several other brain regions, including the cortex, thalamus, cerebellum, pons, medulla and spinal cord (Petrusz et al 1985) where it may function in a neurotransmitter or neuromodulatory role.

1.1.6. Mechanism of action of CRF & VP

Each regulator of ACTH secretion activates the corticotroph by a different mechanism that involves binding to plasma membrane receptors with the formation of intracellular messengers that lead to ACTH release or secretion. High affinity receptor sites for CRF and VP are found on anterior pituitary corticotrophs (Antoni et al 1984, Wynn et al 1983). CRF probably exerts its ACTH-releasing effect via both cyclic AMP (cAMP) (Bilezikjian & Vale 1983) and the calcium-calmodulin system (Murakami et al 1985). Mechanisms for VP are less well characterised but may involve the phosphatidylinositol system (Bilezikjian & Vale 1987). VP also

requires calcium-calmodulin (Murakami et al 1985). The ACTH-releasing effects of VP and OT are mediated by the same receptor which has unique pharmacological specificity (Antoni 1987).

1.1.7. Biological actions of CRF

Studies of the in vitro and in vivo actions of both oCRF and hCRF have been plentiful following the characterisation and availability of synthetic peptides.

1.1.7 i. CRF Bioactivity in vitro

Both oCRF and rCRF cause a potent log dose related specific stimulation of ACTH and other proopiomelanocortin (POMC) related peptides, β-endorphin (β-end) and β-lipotrophin (β-LPH), in many vertebrates (Turkelson et al 1981, Gillies et al 1982, Chan et al 1982).

1.1.7 ii. CRFin vivo studies in normal humans

Both o & h CRF have been administered to volunteers and a rapid rise is seen in ACTH 30-60 minutes after administration, followed by a cortisol peak at 60-90 minutes (Nakahara et al 1983, Stalla et al 1986, Tsukada et al 1984a, Schulte et al 1984, Schopohl et al 1986, Grossman et al 1982). An infusion of oCRF induces a polyphasic response in plasma ACTH and cortisol (DeBold et al 1983) and it is known that the response to oCRF is determined by the level of circulating steroids (Lytras et al 1984). Thus corticosteroid feedback acts, at least in part, at the pituitary level in man to block or attenuate the response to exogenous CRF. The effect of CRF on plasma ACTH is prolonged and related to the dose of CRF administered and lasted up to 7 hours at the highest dose tested (DeBold et al 1983). This group also noted that the high doses of

oCRF blunted or even abolished circadian increase in pituitary-adrenal activity the following day.

Schopohl et al (1986) stated that there was no desensitisation or depletion of the CRF pool after releasing hormone stimulation which is not seen with other releasing hormones. A constant high plasma level of CRF achieved by infusion of oCRF in the non-pregnant state does not alter the circadian rhythm but there is no ACTH or cortisol response to a bolus injection of CRF (Schulte et al 1985). There appears to be pituitary down-regulation in the third trimester of pregnancy (Schulte et al 1988) although Suda (Personal communication) claims that down regulation may not be complete.

Studies using oCRF demonstrate a half life of 5.6-11.6 minutes (DeBold et al 1983, Schulte et al 1984) and this reflects distribution into a volume representing the plasma space. The second half life of 46-73 minutes (Schulte et al 1984, Tsukada et al 1984b) is likely to represent metabolic clearance, and is considerably in excess of the reported half life of the smaller hypothalamic peptides TRH and LHRH. A third phase of clearance lasting about 3 hours has been described (DeBold et al 1983, Nicholson et al 1983). hCRF has a phase 1 clearance of around 4 minutes and a phase 2 clearance of around 25 minutes is only apparent when high doses of hCRF are administered (Schurmeyer et al 1984). The administration of oCRF is also associated with a rise in other ACTH-related peptides, including β-lipotropin (βLPH), γ-lipotropin (γLPH), β-endorphin (βend) and pro γ-melanocyte stimulating hormone (γ-MSH) (Jackson et al 1984).

1.1.8. Other CNS effects of CRF

CRF acts within the brain as a mediator of a number of visceral and endocrine functions and behaviours. Intracerebroventricular administration increases plasma levels of catecholamines and glucose (Brown et al 1982a). CRF has been shown to elevate the mean arterial blood pressure when injected into the cerebral ventricles (Brown et al 1982b). CRF also inhibits LH and GH secretion (Rivier & Vale 1984a & b) consequently exerting a deleterious effect on reproductive function. In the rat, CRF is a potent inhibitor of sexual receptivity in the female (Sirinathsinghji et al 1983). CRF acts within the CNS to modify the sympathetic and parasympathetic nervous systems, cardiovascular function, pancreatic function and carbohydrate metabolism; therefore CRF may have a role in intergrating the neuroendocrine, autonomic, cardiovascular and metabolic responses to stressful stimuli (Brown & Fisher 1985). CRF liberated directly into the CNS may have a neurotropic action important for mobiliSing behavioural responses to stress (Koob & Bloom 1985).

1.1.9. Distribution of CRF outside the brain

Immunocytochemistry has demonstrated CRF like activity (CRF-LI) to be associated with glucagon (A) cells of the pancreas in man, with epithelial cells of the stomach and to a lesser extent with the duodenum, jejunum and ileum of the rat, monkey and cat (Petrusz et al 1985). In man, Nieuwenhuyzen-Kruseman (1984) has demonstrated CRF-LI in the mucosal cells of the gastric antrum.

CRF-LI has been demonstrated by RIA in the adrenal gland and to a lesser extent in the liver, lung, stomach, duodenum and pancreas in man (Suda

et al 1984a, Petrusz et al 1985). CRF has also been identified in the adrenal medulla and posterior pituitary (Hashimoto et al 1983), from tumours of the lung, ovary and adrenal gland including a phaeochromocytoma (Suda et al 1984b, Suda et al 1986). CRF-LI is present in low concentrations in cerebrospinal fluid (Tomori et al, 1983).

The first description of circulating CRF-LI in man came from Stalla et al (1984) who could not detect CRF-LI in normal subjects but reported CRF-LI in patients with Addison's disease and Nelson's syndrome. Low levels of immunoreactive CRF have been detected in normal human plasma by RIA , with prior immmunoadsorption of the peptide (Suda et al 1985) and with a two site IRMA specific for CRF (Linton et al 1987). Whether the CRF detected in the human circulation originates from the hypothalamus or from a peripheral source such as the gut (or, indeed, a combination of the two) is controversial. While Suda et al (1985) found that plasma CRF levels were affected by stress, negative feedback and circadian rhythm, suggesting a predominantly hypothalamic source, Charlton et al (1986) were unable to demonstrate diurnal variation or detect changes in plasma CRF after dexamethasone treatment. Cunnah et al (1987) also did not find an increase in plasma CRF during insulin-induced hypoglycaemia. Both Cunnah et al (1987) and Linton et al (1987) found plasma CRF levels in patients with hypothalamic-pituitary-adrenal disorders to be within the normal range, with no correlation between plasma CRF and ACTH levels.

Gibbs & Vale (1982) showed CRF-LI in rat hypophyseal portal plasma in a concentration of 104.9 ± 9.7 pmoles/ml and in peripheral plasma, 27.5±

6.8 pmoles/ml. These high concentrations have not been recorded in peripheral plasma of rat or human until the report by Sasaki et al (1984) which described high levels of CRF-LI in maternal plasma in the third trimester of pregnancy (Chapter 3).

Fig 3

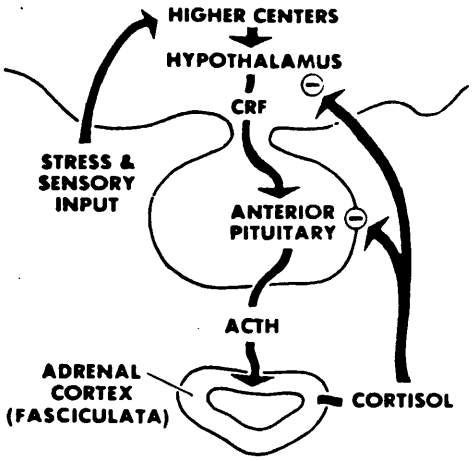


FIGURE 3 The hypothalamic-pituitary adrenal axis.

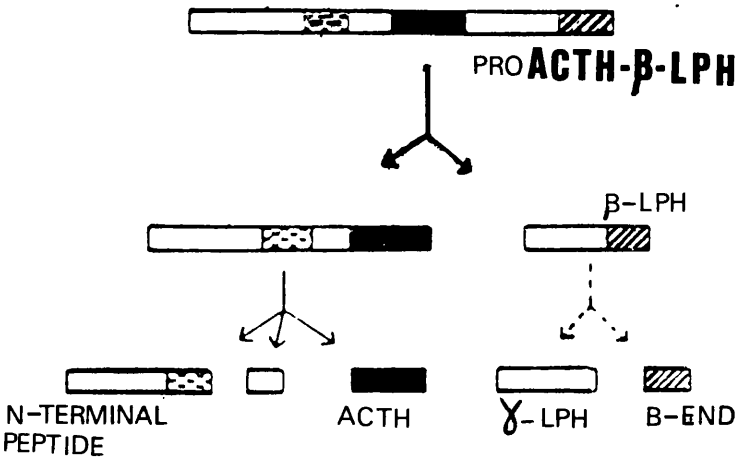


FIGURE 4. Processing of ACTH/β-LPH precursor in the anterior lobe of the pituitary (Imura et al 1983).

1.2.THE HYPOTHALAMIC-PITUITARY ADRENAL AXIS (Fig 3)

1.2.1. ACTH

The amino acid sequence of ACTH was determined by Shepherd et al (1956) but the sequence for human and porcine ACTH subsequently revised by Riniker et al (1972).

1.2.2. ACTH related peptides (Fig 4)

There is a common precursor to ACTH, β-LPH and related peptides which is composed of three segments; a carboxyl terminal segment containing a β-LPH-like peptide, a middle segment containing an ACTH -like peptide and an amino terminal glycopeptide segment with 16K molecular weight (Eipper & Mains 1978, Nakanishi et al 1979). The precursor protein is termed pro-opiomelanocortin (POMC) based on the three biological activities present (namely, opiate activity, melanotrophic activity and adrenocorticotrophic activity, the three peptides being β-LPH, α-MSH and ACTH. β-LPH is further cleaved to β-end and γ-LPH in the intermediate lobe of the pituitary whereas the conversion is limited in the anterior lobe (Imura et al 1983). ACTH is further split into N- and C-terminal fragments in the intermediate lobe and brain. The C-terminal fragment is corticotrophin-like intermediate lobe peptide (CLIP), the N-terminal fragment being α-MSH. The presence of these POMC related peptides have been reported in the brain, adrenal gland, gonads, placenta and gastrointestinal tract (Krieger et al 1980).

1.2.3. Glucocorticoid Regulation.

ACTH regulates glucocorticoid synthesis in the cells of the zona fasciculata of the adrenal cortex. ACTH also maintains the steroidogenic capacity of this zone of the adrenal cortex. The hormone exerts its action through specific receptors on the adrenocortical cell plasma membrane. The interaction of ACTH with these receptors results in activation of adenylate cyclase, and the consequent increase in intracellular cAMP concentration leads to the cell-specific physiological response, namely, acute stimulation of steroidogenesis and the long-term induction of steroidogenic enzymes through the action of cAMP-dependent protein kinase (Ramachandran et al 1988).

The glucocorticoid steroid hormones exert a wide range of effects throughout the body, including a negative feedback influence on the synthesis and release of both CRF and ACTH.

1.2.4. Factors controlling the HPA axis

i. stress: The systemic stressors, such as hypoxia, immobilisation and ether, act directly on the hypothalamus (Allen et al 1973). The neural stressors such as pain, surgical stress and injury involve the integrity of the nervous system. Impulses from peripheral nerves ascend to the brain stem and pons, then to the medial basal hypothalamus where there are two phases of neuronal response. The first being arousal and activation of the posterior hypothalamus resulting in autonomic reactions and the second activation of the anterior hypothalamus, causing release of hypothalamic factors, such as CRF and VP which induce the pituitary corticotrophs to release ACTH (Smelik 1970). Surgical stress can be blocked by antero-lateral deafferentation (Makara et al

1969) or transection of the lateral basal region of the retrochiasmatic area (Palkovits et al 1976). It was also observed by Vernikos-Danellis (1965) that 1-2 minutes after stress there is an increased CRF bioactivity in the median eminence of the rat.

ii. Circadian rhythm: The existance of a circadian periodicity of plasma corticosteroids is well documented (Ixart et al 1977). The concentrations of plasma corticosteroids usually rise during sleep to reach a maximal level around the time of waking. Increased cortisol levels are accompanied or immediately preceeded by elevations in plasma ACTH levels (Berson & Yalow 1968) but this is not always the case (Krieger & Allen 1975) and these discrepancies may be due to changes in pituitary responsiveness to CRF and adrenal gland sensitivity to ACTH (Nicholson et al 1985). Anterior hypothalamic lesions (Slusher 1964) and suprachiasmatic lesions (Moore & Eichler 1972) obliterate circadian variations, suggesting the 'master' clock lies within the hypothalamus.

iii. Corticosteroid negative feedback mechanism: The negative feedback control of corticosteroids on the HPA axis activity is well established and several hypotheses have been proposed for this feedback (Sayers & Sayers 1947, Jones et al 1972, Yates & Maran, 1974).

1.3. HPA AXIS DURING PREGNANCY

The pituitary enlarges in pregnancy with a decrease in acidophils and basophils and an increase in prolactin secreting Lactotrophes (Albrecht 1980) so that the weight of the pituitary increases by one third. Once pregnancy is established and human chorionic gonadotrophin (HCG) is

secreted, pituitary function may not be essential for the maintenance of pregnancy. These conclusions are made from observations on patients with spontaneous pituitary necrosis and those with prepregnancy and antenatal hypophysectomy (Albrecht 1980).

1.3.1. ACTH is produced both in the maternal corticotroph and placenta. There is a tendancy for maternal plasma ACTH to rise in the second and third trimesters (Rees et al 1975, Genezzani et al 1975, Carr et al 1981) but to be significantly lower than in non-pregnant subjects (Mukherjee & Swyer 1972, Carr et al 1981). All studies show a rapid rise in labour. A diurnal rhythm in plasma ACTH has been demonstrated (Carr et al 1981).

1.3.2. Cortisol Binding Globulin (CBG) like ceruloplasmin and thyroid binding globulin (TBG), increases in pregnancy. The elevation is oestrogen mediated, levels returning to normal values 2-3 weeks post partum (Doe et al 1964). CBG is an α1 glycoprotein with a Ka for cortisol in the order of $6x10^{8}M^{-1}$ (Seal & Doe 1962). Circulating Cortisol is largely protein bound, mainly to CBG and is inactive (Sandberg 1963), functioning as a circulating reservoir of hormone which keeps a supply of free cortisol available to the tissues. CBG concentration increases from non pregnant values of 17-25μg% to 45-50μg% at 6 months without any apparent changes in the affinity constant (Doe et al 1964). Despite the increase in CBG and relative decrease in albumin during pregnancy, the distribution of cortisol between CBG bound (75-84%), albumin bound (11-15%) and free hormone (6.9-7.2%) remains unchanged (Rosenthal et al 1969).

1.3.3. Total Cortisol Total cortisol levels increase in pregnancy mainly as a result of the increased CBG levels, a doubling of the cortisol concentration occuring in the first trimester and a 2-3 fold increase at term (Abou-Samra et al 1984, Doe et al 1969, Rosenthal et al 1969, Cousins et al 1983, Carr et al 1981). Vleugels et al (1986) demonstated a linear increase in plasma cortisol from the first trimester to term whereas most groups demonstrate that plasma cortisol levels plateau in the third trimester.

The concentration of corticosteroids in maternal plasma increases during labour (Haddad & Morris 1986, Carr et al 1981) probably due to increased maternal cortisol production in labour (Midgeon et al 1968). Plasma cortisol levels appear to be similar in induced or spontaneous labour (Haddad & Morris 1986). Demey-Ponsart et al (1982) noticed that the total cortisol levels were higher postpartum than antepartum which may be because of an immediate post partum decline in placental steroids cross reacting with CBG. This explanation is consistent with the normalisation of the cortisol bound CBG/true CBG ratio.

The circadian rhythm of cortisol is maintained during pregnancy (Cousins et al 1983, Carr et al 1981). The diurnal variation of plasma cortisol has been reported as increased in subjects in late gestation compared with those in early gestation (Carr et al, 1981) and non pregnant subjects (Nolten et al 1980). However Cousins et al (1983) showed a reduced variation with advancing gestation, suggesting that this may be secondary to a placental source of ACTH increasing as pregnancy advances which is not suppressed by increased free cortisol levels.

Plasma free cortisol is the biologically active moeity and several groups have demonstrated a three fold rise from the first to third

trimester (Nolten & Rueckert 1981, Rosenthal et al 1969, Demey-Ponsart et al 1982) whereas Abou-Samra et al (1984) showed a significant rise to occur only in the third trimester. Nolten & Rueckert (1981) and Demey-Ponsart et al (1982) demonstrated diurnal free cortisol variation. An alternative measure of free cortisol is urinary free cortisol (UFC) excretion which has been reported as increased throughout pregnancy (Cousins et al 1983).

The mechanism that leads to an increased production of cortisol in pregnancy is not understood. However, several investigators (Nolten & Rueckert 1981, Johnstone & Campbell 1974 ,Campbell et al 1970) have demonstrated that maternal adrenal responsiveness to short and long term infusions of ACTH more than doubled as pregnancy advanced and they postulated that the raised levels of ACTH in pregnancy enhanced the adrenal response (Genezzani et al 1975, Rees et al 1975, Newnham et al 1983). Rees et al (1975) also demonstrated diminished suppressibility of the free cortisol index by dexamethasone as pregnancy advances. In the presence of a circadian rhythm and resistance to dexamethasone suppression the control of cortisol in pregnancy is normal with resetting of the maternal feedback to a higher level. This resetting with lack of manifestations of hypercortisolism might result from refractoriness to cortisol, indicating that elevated free cortisol levels are required to maintain homeostasis.

Progesterone may modify the hypothalamo-pituitary sensitivity to cortisol negative feedback control on the secretion of ACTH-related peptides (Abou-Samra et al 1984). Campbell et al (1970) also demonstrated that the synacthen response was normal in the first and

second trimester but enhanced in the third trimester, then diminished in labour.

1.3.4. Cortisol metabolism Cope and Black (1966) calculated that cortisol production was increased 1.5 fold in pregnancy but this was not found by Midgeon et al (1968) and it was not until 1980 (Nolten et al 1980) that an accurate estimation of a 2-2.5 fold increase in cortisol production during pregnancy was made.

1.4. THE FETAL HPA AXIS

The hypothalamic nuclei are fully differentiated and the pituitary is in its classic shape and form by the 14th week of gestation. Capillary connections between the median eminence and the anterior pituitary have been detected in 11 week fetal specimins (Winter 1982) but the hypothalamic-hypophyseal system may be functional before full morphological differentiation has occured (Gluckman et al 1980).

1.4.1. CRF. Immunoreactive and bioactive CRF-LI activity is present in the human fetal hypothalamus from at least the beginning of the second trimester (Ackland et al 1986). Human fetal pituitaries *in vitro* can respond at mid-gestation to synthetic CRF with increasing ACTH and gonadotrophin production (Gibbs et al 1983, Blumenfeld et al 1986). In fetal sheep, Rose et al (1985) demonstrated that after administration of oCRF a rise in plasma ACTH occured which could be blocked by elevations in fetal plasma cortisol levels within a physiologic range, suggesting that cortisol modulates ACTH release by the fetal pituitary gland late in gestation. Single injections or short infusions of oCRF raise plasma ACTH in the ovine fetus (Wintour et al 1984). Continuous infusions of oCRF into an immature fetus can accelerate maturation of a number of organs and systems culminating in the preterm delivery of a viable lamb (Wintour et al 1986). Fetoscopic plasma samples have detected CRF and ACTH in the second trimester. There was a positive correlation between maternal and fetal CRF. In fetal plasma there was a weak inverse correlation between CRF and ACTH (Economides et al 1987). CRF has been demonstrated in umbilical cord plasma and a close correlation between maternal and fetal CRF levels noted by several groups (Goland et al 1986, Stalla et al 1987, Sasaki et al 1987, Nagashima et al 1987).

Goland et al (1986) found no correlation between umbilical cord CRF and ACTH.

1.4.2. **ACTH** By 9 weeks the pituitary contains corticotrophic activity (Kastin et al 1968). Fetal plasma ACTH is detectable at 12 weeks, peaks at 34 weeks then decreases at term (Winters et al 1974) but levels are higher than in the mother. There is evidence that ACTH does not cross the placenta (Miyakawa et al 1976), suggesting the fetal plasma ACTH is placental or fetal in origin. The decrease at term may be due to increased sensitivity of the HPA axis to cortisol or increased sensitivity of the definitive adrenal cortex to ACTH. Consequently an increase in cortisol would decrease ACTH release. Fetal plasma ACTH levels increase in labour, indicating a fetal response to the stress of parturition (Arai et al 1976). In labour maternal and fetal ACTH levels do not show a significant correlation (Arai et al 1976, Miyakawa et al 1976,Winters et al 1974). The concentration of ACTH in anancephaly (Miyakawa et al 1976) suggests that the fetal hypothalamus is important in stimulating ACTH secretion. The administration of betamethasone and dexamethasone alters the HPA axis in the fetus (Arai et al 1976) demonstrating a feedback mechanism.

1.4.3. **Cortisol & Cortisone** There is a significant transfer of cortisol across the placenta to the fetus where 80% is converted to cortisone. The fraction that is transfered without conversion contributes between 25-50% of fetal plasma cortisol (Beitins 1973). The fetal adrenal also actively secretes cortisol by ACTH stimulation and at term this represents 50-75% of fetal cortisol.

The concentration of cortisol in the maternal compartment is 5-8 times higher than that of cortisone whilst in the fetus cortisone predominates

(Talbert et al 1977). Perfusion studies in the mid-trimester show that cortisol is converted to cortisone but at term cortisone is converted to cortisol for lung maturation (Murphy 1979). Fetal plasma cortisol and cortisone levels increase from mid pregnancy to term (Murphy & Diez D'aux 1972). Nahoul et al (1985), using cordocentesis, demonstrated that fetal cortisone correlates with maternal and fetal cortisol while fetal dehydroepiandrosterone sulphate (DHEAS) is inversely correlated with maternal cortisone at 20-30 weeks. Cortisol levels rise steeply immediately prior to the onset of labour but this has not been attributed to the stress of labour (Murphy 1982). However, Ohrlander et al (1976) suggested that the rise in fetal cortisol during labour might mainly reflect maternal stress. Norman et al (1983) using twins as a model, showed that a rise in cord cortisol is primarily fetal in origin after the onset of labour.

Cortisol levels are 3-4 times higher in the mother. This may be due to the 3-4 fold increase in maternal CBG, the proportion of unbound cortisol being higher in the fetus (Talbert et al 1977). Another reason for the lower fetal cortisol levels may be its increased metabolic clearance, as has been demonstrated in the Rhesus monkey (Mitchell et al 1978)

1.5 Placental peptides resembling hypothalamic and pituitary peptides

Human placental lactogen (hPL) and human chorionic gonadotrophin (hCG) have marked homologies to the pituitary peptides GH and LH respectively (Li et al 1971, Closset et al 1973) (Chapter 3). Recently it has been noted that the placenta synthesises a number of peptides resembling hypothalamic and pitutary hormones. Their presence in the placenta has been attributed to similar embryological origins of the tissues which share these peptides (Pearse 1977). The placental concentrations of these peptides are several orders of magnitude lower than described for their original sites of production (Krieger 1982) and much lower than those of hCG and hPL. Their locus of action is unknown but could be on the maternal system, maintaining pregnancy and initiating labour; acting directly on the fetus; or having a paracrine role in the regulation of placental protein expression and secretion.

Structural relatedness to a similar peptide in another tissue has been investigated mainly by immunoassay with only the occasional confirmation by bioassay, physiochemical characterisation or sequencing. Immunocytochemistry has been utilised for peptide localisation but only sequence analysis or messanger RNA (mRNA) identification and translation provides absolute identification.

1.5.1. ACTH

The placental production of a corticotrophic like substance has been postulated for many years (Jailer & Knowlton 1950) but it was not until the 1970's that studies demonstrated the bioactive and immunoreactive ACTH-like activity of the placenta (Rees et al 1975, Genazzani et al 1975, Liotta et al 1977). The ACTH concentrations in the placenta are

several orders of magnitude less than in the anterior pituitary but comparable to those observed in tumours associated with ACTH secretion (Rees and Ratcliffe 1974).

Physicochemical characterisation of immunoreactive ACTH indicates a large proportion of the immunoreactivity is present in the high molecular weight void volume material although material with the same elution volume of ACTH 1-39 is also present. Similar findings for lipotropin and endorphin-like immunoreactivity have been demonstrated (Nakai et al 1978, Odagiri et al 1979)

Liotta and Krieger (1980) demonstrated the production of β-LPH, ACTH and precursor glycoprotein by culturing trophoblast and pituitary cells. They noted the different processing of these precursors in the two tissues, trophoblastic processing being similar to that in the hypothalamus and intermediate lobe of the pituitary.

Al-Timini and Fox (1986) demonstated specific localisation of ACTH in the syncytiotrophoblast by immunohistochemical staining which was less intense than in the pituitary. Staining suggests that there is little variation in placental ACTH production throughout pregnancy which is in agreement with immunoassay studies (Liotta et al 1977, Odagari et al 1979, Demura et al 1982).

Placental superfusion studies have demonstrated <u>in vitro</u> secretion of ACTH although this was in tissue fragments rather than by the placenta as an organ (Mulder et al 1986). This group hypothesised that placental ACTH stimulates chorion/amnion prostacyclin production which in turn inhibits myometrial contraction. Placental ACTH exhibits both paracrine activity by locally stimulating placental progesterone and oestradiol

production (Barnea 1986) and endocrine effects in augmenting maternal and fetal adrenal steroidogenesis (Simpson & MacDonald 1981).

The placental production of ACTH is reflected in maternal plasma levels which tend to rise in the second and third trimester but remain within the normal non-pregnant range until labour, when a sharp rise is observed. The oral administration of dexamethasone does not significantly alter placental ACTH content (Liotta et al 1977), which adds weight to the hypothesis that placental ACTH is not subject to the normal feedback control.

1.5.2. OTHER POMC RELATED PEPTIDES

Placental immunoreactive β-end, β-LPH and α-MSH have been identified in term placentae (Demura et al 1982, Liotta & Krieger 1980, Nakai et al 1978, Clark et al 1978). Similar to ACTH, high molecular weight forms have been reported for β-LPH and β-end like immunoreactivity (Nakai et al 1978, Odagiri et al 1979).

Maternal plasma β-end levels have been shown to rise progressively during pregnancy (Genazzani et al 1981), peak at 32 weeks (Evans et al 1985), remain unchanged (Goland et al 1981) or decrease (Hoffman et al 1984) . Maternal plasma levels increase in the later stages of labour (Goland et al 1981, Genazzani et al 1981, Hoffman et al 1984).

Radioimmunoassays used for the determination of maternal plasma β-LPH concentrations have lacked specificity and the literature is contradictory. Using a C-terminal assay maternal plasma β-LPH concentration have been reported as increased during the second trimester, returning to non-pregnant levels thereafter (Genazzani et al 1981) whereas measurement of N-terminal β-LPH shows a significant increase in the third trimester (Abou-Samra et al 1984, Browning et al

1983). Abou-Samra suggested this was due to increased processing of β-LPH to γ-LPH and β-end in pregnancy.

Immunoreactive α-MSH activity has been reported in term placental extracts (Clark et al 1978) but is within the non-pregnant range in maternal plasma in the third trimester of pregnancy (Thody et al 1985).

1.5.3. **Prolactin** has been localised to the syncytiotrophoblast and decidua using immunohistochemistry (Al-Timimi and Fox 1986) which is in agreement with bioassay and immunassay studies (Golander et al 1978, Demura et al 1982). Maternal plasma prolactin levels appear to rise dramatically throughout pregnancy (Clements et al 1977).

1.5.4. **Follicle Stimulating Hormone** (FSH) is synthesised by explants of first trimester placental tissue (Maruo 1976). Immunohistochemistry has localised FSH to the syncytiotrophoblast and the intensity of staining tends to diminish as pregnancy advances (Al-Timini & Fox 1986). Plasma FSH levels have not been detectable after the first two weeks of pregnancy (Jeppsson et al 1977).

1.5.5. **Luteinizing Hormone** shows a similar pattern of trophoblastic localisation to hCG whereas **Growth Hormone** has less marked staining in the syncytiotrophoblast than hPL (Al-Timimi & Fox 1986).

1.5.6. **Thyroid Stimulating Hormone** (TSH) immunoreactivity and bioactivity have beeen described (Hershman and Starnes 1969). The demonstration of a placental thyrotrophic substance that differs from hTSH and hCG, human chorionic thyrotropin (hCT), has been reported (Kanazawa et al 1976). Whilst maternal plasma hCT levels rise during pregnancy plasma hTSH concentration remains in the non-pregnant range. The physiological role of hCT is unclear.

1.5.7. Hypothalamic releasing hormones of the placenta

Ion-exchange chromatography demonstrates that extracts of human placentae and chorionic membranes contain material similar to thyroid hormone releasing hormone (TRH) and luteinizing hormone releasing hormone (LHRH). Homogenates of term placentae cause TSH and LH release when bioassayed in rats. After dialysis of placental homogenates synthesis of biologically active TSH- and LH-releasing factors has been shown to occur *in vitro* following the addition of appropriate precursor amino acids and cofactors (Gibbons et al 1975, Shambaugh et al 1979, Siler-Khodr & Khodr 1979, Khodr & Siler-Khodr 1980). However, the TRH like activity may be due to a molecule somewhat different from TRH (Youngblood et al 1980). Radioimmunoassay and immunofluorescence techniques localise LHRH in the cytotrophoblast and villous stroma (Khodr & Siler-Khodr 1978 a, b and 1980, Siler-Khodr & Khodr 1978), the LHRH content being greatest at 8 weeks (Miyake et al 1982). It has been suggested by these researchers that LHRH controls HCG production.

Low affinity LHRH receptors have been demonstrated in the placenta but their significance is not known (Currie et al 1981, Belisle et al 1984). Iwashita et al (1986) hypothesised that LHRH receptors could serve as low affinity regulatory sites for locally formed LHRH or related peptides within the placenta. He suggested that the placental LHRH system may have a significant role in the maintenance of pregnancy. Maternal plasma LHRH concentrations have been reported as low in pregnancy (Makino et al 1980, Hayashi 1982).

1.5.8. CRF

CRF like immunoreactivity and bioactivity has been demonstrated in extracts of the human term placenta (Shibasaki et al 1982, Sasaki et al 1984a,b & c, Schulte & Healy 1985). The molecular size of the placental CRF-like material was said to be slightly larger than that of ACTH (Shibasaki et al 1982). Subsequently, partial characterisation of a purified term placental CRF-like peptide revealed it to be chromatographically indistinguishable from rCRF in molecular sieve and multiple reversed phase high pressure liquid chromatography (HPLC) systems (Sasaki et al 1984a & b, Schulte & Healy 1985, Cunnah et al 1987) although multiple size classes of immunoreactive CRF have also been demonstrated.

Immunohistochemistry has demonstrated CRF localisation in the cytotrophoblast, decidua and amnion of early pregnancy (Saijonmaa et al 1988). Indirect immunofluorescence staining of term placentae showed CRF localisation in the cytotrophoblast (Petraglia et al 1987).

Placental CRF mRNA is similar in size to rCRF mRNA; it is under significant gestational regulation, levels being low in the first 2 trimesters and rising approximately 20 fold in placentae of 35-40 weeks gestation; CRF mRNA appears to be localised to the syncytiotrophoblast which is at variance with immunohistochemical studies (Frim et al 1987, Usui et al 1987, Suda et al 1988, Grino et al 1987).

CRF has been shown to be secreted in vitro by placental fragments and has the same chromatographic profile as synthetic CRF (Grino et al 1987, Saijonmaa et al 1988). Petraglia et al (1987) elegantly demonstrated that CRF stimulated secretion of peptides containing the ACTH sequence in the placenta in a dose dependent manner, as it does in the pituitary.

This effect was reversed by a CRF antagonist and mimicked by dibutyryl cAMP and forskolin. Glucocorticoids did not influence placental ACTH release but OT and prostaglandins stimulated ACTH and CRF secretion from cultured placental cells. They concluded that CRF may be involved in the paracrine regulation of placental ACTH secretion.

CRF-LI was first reported in third trimester maternal plasma by Sasaki et al (1984c). Subsequently many groups have demonstrated the presence of CRF-LI in maternal plasma in the third trimester (Table 1). There is variation between groups in terms of absolute levels measured and this may reflect, in part, the different assay and extraction systems used. All studies demonstrate significantly elevated maternal plasma CRF levels in the third trimester of pregnancy, with a rapid decline post partum (Chapter 3).

Gel filtration of third trimester pooled plasma revealed 2 major peaks of immunoreactive CRF, a high molecular weight peak and one at the position of endogenous rCRF (Sasaki et al 1987) and synthetic CRF (Cunnah et al 1987). Reversed phase HPLC of maternal plasma has indicated 2 (Sasaki et al 1987) or 3 (Cunnah et al 1987) peaks of immunoreactive CRF are present in maternal plasma, one of which coelutes with CRF. Maternal plasma CRF stimulated the release of ACTH from rat anterior pituitary cells in bioassay (Sasaki et al 1987, Goland et al 1987).

FIG. 5

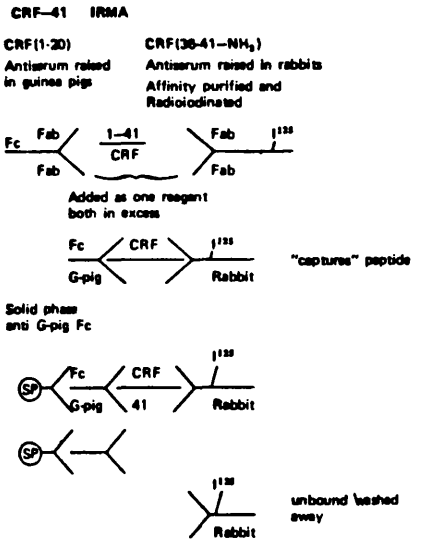


FIG. 5. CRF IRMA

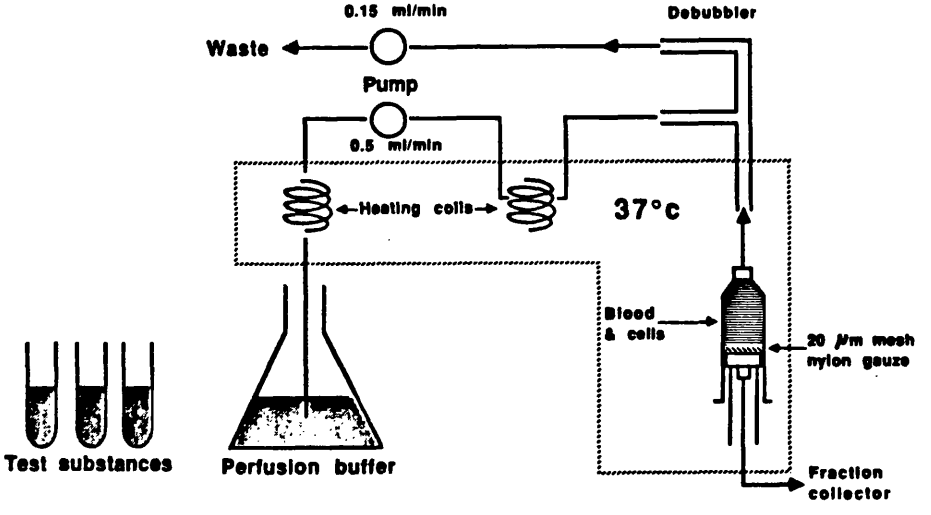


FIG. 6. Isolated pituitary cell column perfusion apparatus. Perfusion buffer or test substance was pumped through two heating coils at 37°C, past a debubbler, over the cell column and to the fraction collector.

1.6. DIRECT MEASUREMENT OF MATERNAL PLASMA CRF BY "TWO-SITE" IRMA

Although immunocytochemistry and RIA have provided much valuable information on the localisation of CRF in man, both techniques have shortcomings, limited specificity being a particular problem. Studies to date have used SepPak C_{18} extracted human maternal plasma or immunoadsorption with subsequent RIA to detect CRF-LI activity. In an attempt to overcome cross-reactivity the first section of the thesis reports the modification of an IRMA (Linton & Lowry 1986) to allow the direct measurement of CRF in small volumes of plasma without the need for prior extraction along with characterisation of maternal plasma CRF.

MATERIALS AND METHODS

1.6.1. IRMA FOR CRF (Fig 5)

i. Antibodies The immunization procedures and antibody characterisation have been reported in detail previously (Linton and Lowry 1986). Briefly an N-terminal CRF antibody was raised in a guinea pig immunized with CRF-(1-20)-NH2 (gift of Dr Jean Rivier), while a C-terminal antiserum was raised in a rabbit immunized with rCRF (gift of Dr Jean Rivier) for the first and two subsequent booster injections and then with CRF-(36-41)-NH2 (CRB, Cambridge,UK, Lot JCT 1055) for the third booster injection. The anti-N-terminal CRF was used in the IRMA as the crude antiserum. The anti-C-terminal CRF was affinity-purified (Hodgkinson & Lowry 1982) on an immunoadsorbent of epoxy-activated Sepharose 6B (Pharmacia, Piscataway, New Jersey) to which CRF-(36-41)-NH2 had been coupled following the manufacturer's recommendations.

ii. Iodination The resulting high affinity C-terminal CRF immunoglobulins (IgGs) were iodinated using glucose oxidase with lactoperoxidase (Tower et al 1977).

iii Standards Standards were prepared by adding a known weight of synthetic CRF (Bachem Inc, California) to human plasma which had previously been treated to remove peptidase activity. This treatment consisted of two freezing and thawing cycles and overnight incubation at 37°C to eliminate endogenous CRF, then at 54°C for 1 hour. After cooling, phenylmethylsulfonylfluoride, hydoxymercuribenzoate, N-ethyl-matheimide, and EDTA (each at a final concentration of 1mM), and bacitracin and thiomersal (both 0.001%) were added, and the plasma was mixed for 2 hours at room temperature. The pH was adjusted to 7.4 with 2M phosphoric acid, then the plasma was centrifuged (2h;4°C;10,000 X g) and any precipitate discarded. Standards were stored in aliquots of this treated human plasma (100ng/ml) at -70°C. (General laboratory chemicals from BDH Chemicals Ltd, Poole, Dorset).

iv. Assay procedure Duplicate 200-µL aliquots (Eppendorf Multipipette, Anderman and Co. Kingston upon Thames, Surrey) of plasma samples or standard (1-6,000pg/ml; diluted in treated human plasma) were incubated overnight at room temperature with 200µl of the CRF IRMA reagent mixture. This consisted of [^{125}I] rabbit anti-CRF-(36-41)-NH2 IgG (100,000cpm/200µl) and guinea pig anti CRF-(1-20) serum (1:5,000) in 0.005M sodium phosphate buffer, pH7.4, containing human serum albumin (Blood products Laboratory, Elstree, Herts.) (0.5%,wt/vol), normal rabbit serum (NRS)(1%,vol/vol) (ILS Ltd, London), and sodium azide (0.01%). Seperation of CRF-bound from free labeled IgG was performed with 100µl sheep anti guinea pig Fc region Ig coupled to a Dynosphere

solid phase (XP-6501, Dyno Industrier AS) and allowed to incubate for 20 minutes. The contents of the tube were then washed with 2 mls of bicarbonate buffer (1 ml 0.01 M $NaHCO_3$ containing 0.15 M NaCl and 0.01% Triton X-100) and spun for 30 minutes (MSE Mistrel Coolspin centrifuge UK), the supernatant aspirated and the wash and aspiration repeated. Radioactivity is bound to the solid phase only in tubes containing CRF, as the peptide acts as a link between the radiolabeled rabbit IgG and the unlabeled guinea pig antibody. The counts in the solid phase pellet are thus directly proportional to CRF (LKB 1261, Multigamma with RIACAL, LKB,UK). Interference by protein in some plasma samples when a liquid phase second antibody was employed for assay seperation was reduced to a minimum in the solid phase system. To check for such interference in the IRMA, nonspecific binding for individual plasma samples was tested by omitting the anti (N-terminal CRF)-guinea-pig link antibody from the IRMA reagent mixture.

v. PLASMA COLLECTION Blood from normal men and women (aged 19-65) was collected into ice-cold lithium-heparin tubes between 0800 and 1000 h after an overnight fast (Disposable needles and syringes, Gillette Industries, Iselworth, Middlesex. Heparin tubes, Western Laboratory, Farnham, Surrey.). Blood samples from women (n= 55) in the 39th and 40th week of pregnancy were collected at antenatal clinics held between 0830 and 1630 h (Chapter 3). After centrifugation (10 min;4°C;3000 X g), the plasma was decanted, flash frozen, and stored at -70°C until assay (Microcentrifuge tubes, Elkay, UK).

1.6.2.i. **IMMUNOADSORPTION OF PLASMA CRF** The oCRF immunoadsorbent was prepared as described previously (Beny et al 1985). 5 mls pooled maternal plasma, collected at 39-40 weeks gestation, were diluted with 5 mls 0.9% saline containing mercaptoethanol(0.01%)(Sigma,UK) and ascorbic acid (1mg/ml)(BDH) and mixed overnight at 4°C with 0.25g immunoadsorbent. After centrifugation, the immunoadsorbent was washed twice with 10 mls saline/mercaptoethanol/ ascorbic acid before elution of the retained CRF with four successive 1 ml aliquots of saline/mercaptoethanol/ascorbic acid containing 20% acetonitrile, adjusted to pH 3.2 with HCL . After removal of the acetonitrile under a stream of nitrogen (British Oxygen Company, London), the CRF was further purified by loading onto a column of C_4 silica which had been primed with polypep 1mg/ml (Sigma Chemicals, USA) with 0.1% Trifluoroacetic acid(TFA)(Sigma, UK) and acetone (BDH). The CRF was subsequently eluted with 80% acetonitrile in 0.9% saline containing mercaptoethanol (0.01%) and ascorbic acid (1mg/ml). The acetonitrile was again removed. Each 0.25g CRF immunoadsorbent was re-used a further 3 times to extract CRF from a total of 60 mls of the maternal plasma pool. The immunoadsorption efficiency of CRF averaged 61% from maternal plasma (as measured pre- and post- immunoadsorption directly by IRMA), while recovery of CRF immunoactivity after C_4 silica purification was better than 98%. This gave an overall extraction efficiency of 60% from maternal plasma. Following purification, the extract from normal human plasma was diluted 1:5 with 0.05M sodium phosphate containing human serum albumin(0.25%), Triton X-100 (0.05%) and sodium azide (0.01%) and assayed for CRF by IRMA. Extracts from maternal plasma were either stored at -70°C or submitted to HPLC directly.

1.6.2.ii. HPLC (Gilson model 802C, LKB fraction collector) The extracted maternal plasma CRF samples were diluted 1 in 50 in assay buffer (4-12ng/500µl) and loaded onto a column (75 x 4.6 mm) of Ultrapore RPSC C_3 (Beckman Instruments, USA) and eluted by a linear gradient from 0% to 60% acetonitrile in 0.1% TFA at room temperature on four seperate occasions. The solvent flow rate was 1ml/min and 1 ml fractions were collected. HPLC of added synthetic hCRF immunoadsorbed from a pool of plasma from normal subjects following the procedure outlined above and human brain extract (10 mg wet wt/ml in 50mM HCL containing 1mg/ml ascorbic acid and 0.01% mercaptoethanol) was similarly performed for comparison with maternal plasma CRF. Each elution was monitored by CRF IRMA. Column recoveries approached 100 %.

1.6.3. CORTICOTROPHIN RELEASING ACTIVITY OF THE EXTRACT

The ACTH releasing activity of maternal plasma CRF was assessed using the Rat-perfused anterior pituitary cell bioassay (Gillies and Lowry 1978)(Fig 6) on two seperate occasions. HPLC fractions containing the CRF peak were pooled, dried under nitrogen and reconstituted in Earle's Balanced Salt Solution (EBSS)(Flow Labs, UK). The effluent from the column was collected and assayed for ACTH and CRF by IRMA using standards diluted in EBSS.

1.6.4. RESULTS

1. ASSAY CHARACTERISTICS Figure 7 shows an IRMA standard curve for CRF in peptidase-inactivated human plasma. The counts per min bound were directly proportional to the CRF concentration. The assay sensitivity was 1 pg/ml, with nonspecific binding (NSB) of 0.12% and maximum binding

FIG. 7.

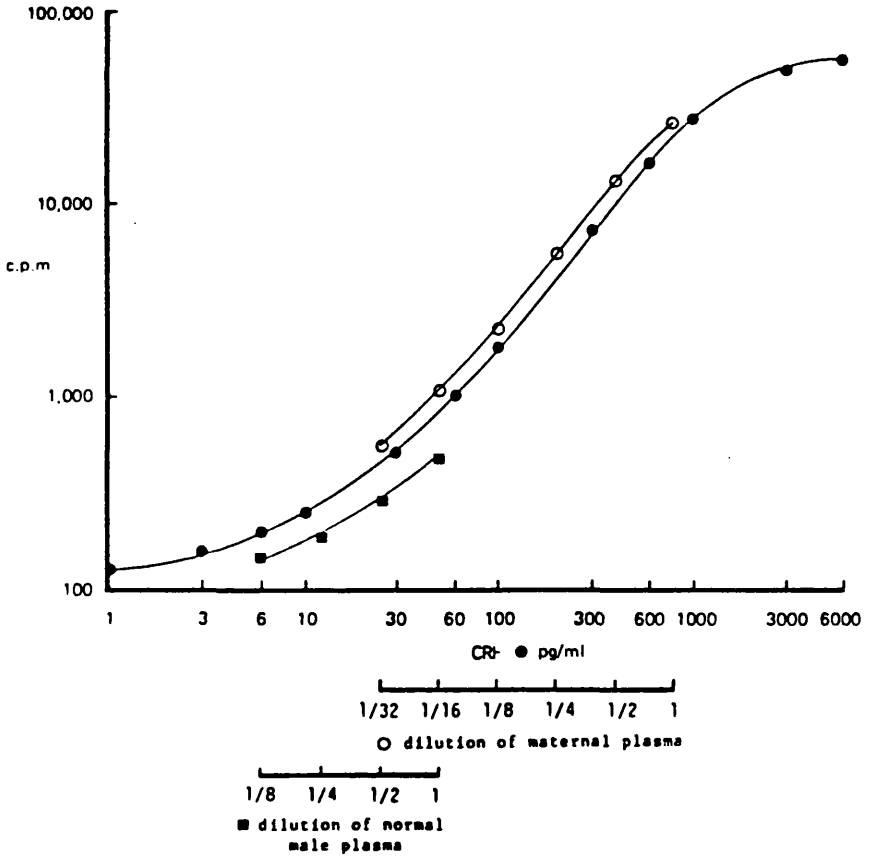


FIG. 7. CRF 'two-site' IRMA standard curve in heat-treated human plasma containing enzyme inhibitors (●) and in dilutions of plasma from a normal male (■) and from a woman who was 39 weeks pregnant (○). The data are the means of duplicate determinations.

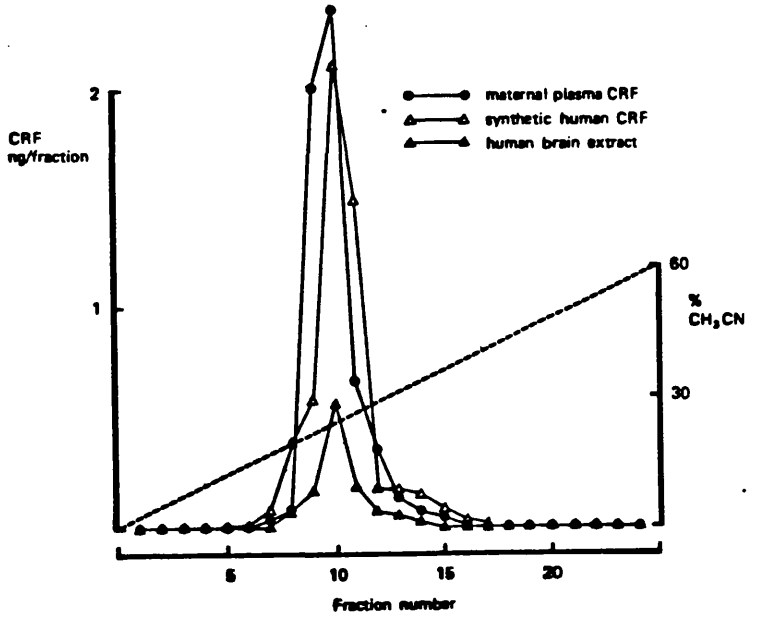


FIG. 8. HPLC of CRF IRMA activity of extracts of maternal plasma (●) pooled normal plasma containing synthetic CRF (Δ) and human brain extract (▲) on ultrapore RPSC.

of 56%, which occured at approximately 6 ng/ml. The CRF IRMA is highly specific for human (and rat) CRF, with no detectable cross-reactivity with ovine CRF, sauvagine, urotensin, CRF-(36-41)-NH_2, and CRF-(1-20)-NH_2 or nonrelated peptides such as oxytocin, angiotensin 11, somatostatin, substance P, neurotensin, peptide-histidine-isoleucine, LHRH, ACTH, and β-end over the range 50 pg-500 ng/tube (Bachem Inc, California, USA).

ii. CRF IN PLASMA A total of 370 pg CRF-LI was detected in the extract of pooled normal human plasma (45 ml) obtained by immunoadsorption and C_4 silica purification. Serial dilutions of this extracted CRF-LI paralleled the synthetic CRF standard curve (data not shown). Recovery of synthetic CRF (25 pg/ml) added to pooled normal human plasma previously exposed to the CRF immunoadsorbent was 93%. Endogenous CRF-LI could be measured directly in plasma using the CRF IRMA. The circulating plasma CRF levels in normal men averaged 16 ± 7(± standard deviation (SD)) (n=41) and 14 ± 7 (±SD)(n=27) pg/ml in normal non-pregnant women, with no significant difference between the sexes. The overall range was 2-28 pg/ml. The dilution curve of CRF in a normal male plasma paralleled that of synthetic hCRF (Fig 7). The intra- and inter- assay coefficients of variation (CV) for a normal human plasma pool containing 13 pg CRF/ml were 2.7% and 6% respectively (n= 10 samples in 8 assays).
In contrast to the low basal plasma levels in normal subjects, a grossly elevated CRF concentration of 1462 ± 752 pg/ml (± SD)(n=55- the first 55 of the normal pregnancy group discussed in chapter 3) was found in plasma of women during the 39-40th weeks of pregnancy. The intraassay CV for a maternal plasma pool containing 460 pg/ml was 1.9%, while the

FIG. 9.

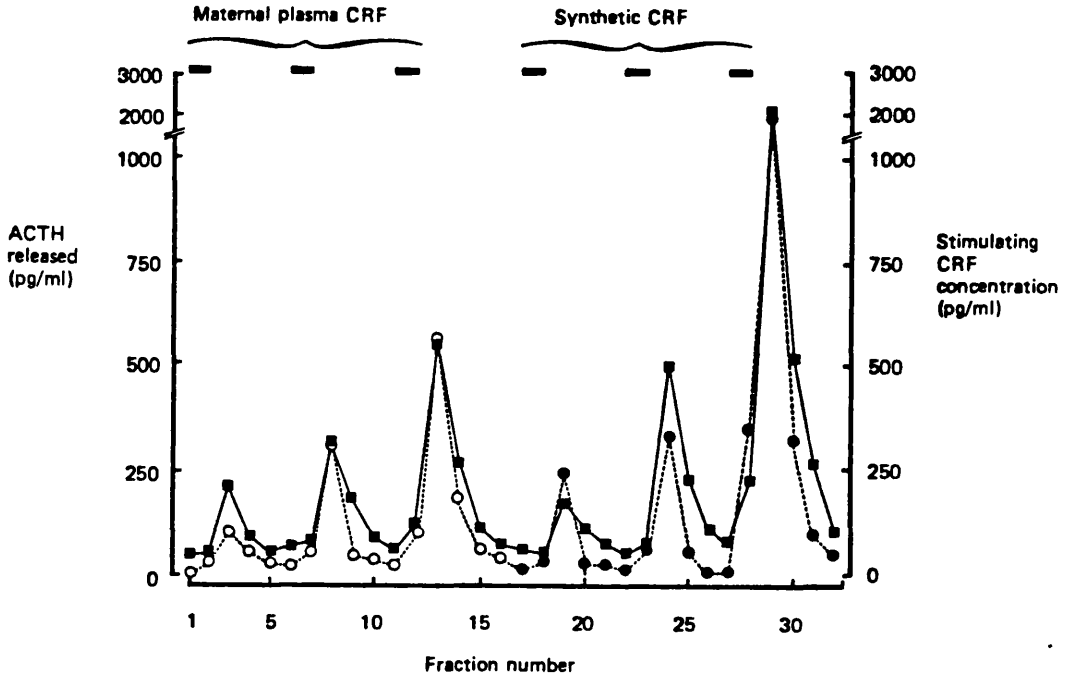


FIG. 9. ACTH-releasing activity (■) of extracted maternal plasma CRF (●) and synthetic CRF (○) in the perfused anterior pituitary cell bioassay. Both CRF and ACTH were measured in the same fractions by IRMA.

interassay CV was 5.7% (n=12). As shown in Fig 7, the dilution curve of CRF-LI in maternal plasma was parallel to that of synthetic CRF.

iii. HPLC CRF immunoadsorbed from maternal plasma, purified on C_4 silica and analysed by HPLC eluted in the position of synthetic hCRF (Fig. 8). The CRF-LI in a human brain extract also was found to coelute with the CRF from maternal plasma in the same HPLC system.

iv. BIOASSAY The CRF from maternal plasma in the HPLC fractions was bioassayed using perfused isolated rat adenohypophysial cells. This extracted CRF released ACTH in a dose-dependent fashion similar to synthetic CRF (Fig. 9). The ratio of exogenous CRF to released ACTH assayed in the same column effluent fractions was 1.17 ± 0.20 (SD)(n=12) for maternal plasma CRF and 1.22 ± 0.34 (SD)(n=9) for synthetic CRF. However, only 44 ± 8% of the maternal plasma CRF and 45 ± 13% of the synthetic CRF applied to the pituitary cells emerged in the effluent.

1.6.5. DISCUSSION

The IRMA for hCRF developed by Linton & Lowry (1986) has been adapted to allow the direct measurement of CRF in a small volume of unextracted plasma. The modifications introduced included a plasma base in which CRF is stable, allowing the serial dilution of standards and plasma samples. The glucose oxidase/lactoperoxidase radioiodination procedure was used as it allows greater incorporation of ^{125}I into the anti (C-terminal CRF)-IgG without affecting affinity or stability. To achieve low nonspecific binding and, hence, maximal sensitivity, 1% NRS was included into the reagent mixture to dilute any binding of labelled IgG to the

solid phase. In addition, only high affinity second antibody IgGs were coupled to the dynosphere particles, allowing a small mass of solid phase to bind all of the guinea pig link antibody within 30 minutes. An initial incubation of sample with IRMA reagent mixture for 16 h was sufficient to allow complete binding of the two primary antibodies to any CRF present. However, the IRMA can be completed after 3 h, although this results in fewer counts bound, and hence, sensitivity is compromised.

A sensitivity of 1 pg/ml was achieved with overnight incubation. This allowed detection of the low levels of CRF in the peripheral circulation of humans, estimated at 16 pg/ml for men and 14 pg/ml for women. These values are somewhat higher than the value of 6 pg/ml previously reported by Suda and colleagues using immunoadsorption to extract the CRF (Suda et al 1985). In their study, the recovery of CRF added to plasma in vitro averaged 90%, similar to the findings of 93% in this study. However, this study observed that the extraction efficiency of endogenous CRF was lower (74% for normal plasma and 61% for maternal plasma), although the reason for this is not clear. A further point of difference is that the immunoadsorbent used in this study was made of oCRF rather than hCRF antibody. The levels reported here are also lower than those of Cunnah et al (1987) using vycor-extracted samples. This discrepancy may be due to greater specificity of the IRMA. This immunoassay depends on the use of two antibodies directed against opposite ends of the CRF molecule, and requires the presence of (at least part of) both the 1-20 and 36-41 sequences of CRF in the same peptide (Fig. 5). Consequently, fragments of CRF that cross-react in a

RIA may not be detected by the IRMA (Linton & Lowry 1986), resulting in the measurement of lower hormone levels.

It was in 1981 that oCRF was characterised (Vale et al 1981) and in the relatively short period of time since then, the structural relatedness of placental CRF to hCRF has been determined in as much detail as other hypothalamic and pituitary peptides produced by the placenta. This comparison has employed, in the main, immunoassay and immunohistochemical techniques which although they provide valuable information on localisation of CRF, have several shortcomings, limited specificity being a particular problem.

CRF-LI immunoreactivity and bioactivity have been demonstrated in the placenta (Shibasaki et al 1982, Sasaki et al 1984a,b & c, Shulte & Healy 1985). Placental CRF has been shown to be chromatographically indistinguishable from hCRF although multiple peaks of immunoreactive CRF have been demonstrated. Similarly in late gestational maternal plasma two major peaks of immunoreactive CRF have been reported with gel filtration and HPLC, one of which coelutes with CRF (Sasaki et al 1987, Cunnah et al 1987). Because of the shortcomings of the RIA interpretation of these findings is speculative.

Evidence for the presence of CRF in the placenta using immunocytochemistry has produced conflicting results. It is not clear at what stage of gestation CRF is detectable or the exact site, although the syncytiotrophoblast has been the common site in both studies (Petraglia et al 1987, Saijonmaa et al 1988). Saijonmaa and coworkers did, however, discuss the possible technical errors in their study, which highlights the lack of specificity in immunohistochemical

research. More direct evidence, in the form of northern blot hybridization studies of total RNA , revealed a single band of CRF mRNA in placental syncytiotrophoblastic tissue (Frim et al 1987). The in vitro studies of Petraglia and coworkers (1987) have also demonstrated a possible paracrine role for CRF in the placenta.

High maternal plasma CRF concentrations during the third trimester of pregnancy have been reported by several groups using extracted plasma and RIA (Table 1). In an attempt to overcome the cross-reactivity problem of the RIA, a "two-site " IRMA has been used. The RIA results are corroborated by these direct measurements with this specific IRMA for CRF. Maternal plasma CRF was extracted with an oCRF rather than hCRF antibody to ensure that all the extracted peptide originated from maternal plasma and not from sequestrated synthetic hCRF used in raising the antibody. Additionally the CRF IRMA did not detect the oCRF peptide (Linton & Lowry 1986). Besides improving specificity, the IRMA offers other advantages above conventional RIA, including a greater working range, increased precision, sensitivity and speed (Linton & Lowry 1986).

Further evidence in this study that maternal plasma CRF is indeed CRF is demonstrated by its coelution with synthetic CRF on HPLC and its equipotent bioactivity. Unlike the studies using RIA only 1 peak, in the position of synthetic CRF and CRF from human brain extract is observed. This may be due to the specificity of the IRMA which does not detect fragments in the high molecular weight peaks as has been reported by those groups employing RIA. It is interesting to note that as much as 55% of the stimulating CRF (whether extracted from maternal plasma or

the synthetic CRF) disappeared after short exposure (< 1 min) to the adenohypophysial cells, presumably due to metabolism.

This study has demonstrated that elevated maternal plasma CRF concentrations are present in the third trimester of pregnancy, that this CRF dilutes in parallel with synthetic CRF, has identical HPLC characteristics as synthetic CRF and human brain CRF and that it is bioactive. The aims of the study are now to further investigate the physiological characteristics of maternal plasma CRF (Chapter 2) and to document the maternal plasma levels of CRF throughout pregnancy, labour and the puerperium in order to identify a possible role for CRF in pregnancy.

CHAPTER 2

EVIDENCE FOR A SPECIFIC CARRIER SUBSTANCE FOR CRF IN LATE GESTATIONAL MATERNAL PLASMA

2.1. INTRODUCTION

Substantially elevated plasma levels of CRF exist in the maternal plasma during the third trimester of pregnancy. Despite this, pathological levels of ACTH are not present, with merely a small increase in plasma ACTH in the third trimester. Whilst it has been established that CRF extracted from peripheral maternal plasma is biologically active (Chapter 1) it is uncertain whether this is the case for the circulating form in pregnancy. Orth & Mount (1987), whilst investigating plasma interference in their CRF RIA, identified a CRF-carrier protein in normal male plasma and also suggested that its presence may account for near normal ACTH levels in late gestational plasma.

This study describes the evidence for a specific carrier substance for CRF in late gestational maternal plasma and investigates the ACTH-releasing activity of the CRF-carrier complex in the perfused isolated rat pituitary cell bioassay.

2.2. Plasma collection and treatment

Blood from normal men(N=6) and women in the 39th and 40th weeks of pregnancy(N=24) were collected between 0830 and 1630 into ice-cold lithium heparin tubes. After centrifugation, (10 mins,4°C,3,000xG) the plasma was decanted and pooled into the two groups. Aliquots from each pool were flash frozen and stored at -70°C.

Upon thawing, each plasma aliquot was recentrifuged prior to incubation and chromatography. Maternal plasma was chromatographed on Sephadex G50 (Pharmacia) alone, after the addition of 8M urea and heating at 37°C for 1 hour, and after incubation with synthetic CRF (5 ng/ml) at 4C for 5 min, 4h or 18h. For comparison, normal male plasma was similarly pre-incubated with synthetic CRF.

Sephacryl S-200 chromatography (Pharmacia) was performed with maternal plasma alone, after incubation for 5 mins at 4C with ^{125}I-hCRF-41, ^{125}I-ACTH, ^{125}I-LHRH or ^{125}I-Vasopressin , and after 4h incubation with synthetic hCRF, oCRF or [Met(O)^{21,38}]CRF.

2.3. Column Chromatography

1 ml plasma volumes were loaded on to a sephadex G50 (Pharmacia) column (1.0x 80.0 cm) developed at 3 ml/h at 4C with 50mM Tris/HCL (pH7.4) containing ascorbic acid (1mg/ml) and NaCl(0.15M). Chromatography under dissociating conditions was performed in this column buffer containing 2M urea. The resulting 30 min fractions were stored at -20°C until assayed directly by CRF IRMA using standards diluted in the appropriate column buffer. Subsequent work was carried out using a Sephacryl S-200 (Pharmacia) column (1.5 x 94.0 cm) with load volumes of 1.5 ml and an elution rate of 6 ml/h. 20 min fractions were collected and stored at -20°C awaiting CRF IRMA and bioassay.

2.4. Bioassay

Fractions containing the carrier-bound CRF peak from Sephacryl S-200 chromatography of late gestational maternal plasma or male plasma incubated with CRF (4h at 4°C) were pooled and serially diluted with bioassay buffer (EBSS containing ascorbic acid (30µg/ml), trasylol(100 Kallikrein inactivator U/ml) and human serum albumin (2.5 mg/ml)) and

then tested in the perfused anterior pituitary cell bioassay (Chapter 1, Gillies & Lowry 1978) and the effluent from the pituitary column assayed for ACTH (Hodgkinson et al 1984) and CRF by IRMA using standards prepared in bioassay buffer.

A more detailed study of the ACTH-releasing activity of endogenous CRF from an S-200 chromatogram of late gestational maternal plasma was then carried out. Each chromatogaphic fraction was diluted 1:4 with EBSS and tested in turn for bioactivity as described above.

2.5. **RESULTS**

Sephadex G50 chromatography of unextracted maternal plasma (containing 1.1 ng endogenous CRF) revealed two discrete immunoreactive CRF peaks, the major peak (93% of total CRF content) eluting in the void region and the second in the position of synthetic CRF. Following pre-treatment of the maternal plasma with 8M urea, all of the immunoreactive CRF eluted in the position of synthetic CRF (Fig 10). Chromatography of maternal plasma preincubated with synthetic CRF for 5 min, 4h (data not shown) or 18h also resulted in two immunoreactive CRF peaks, with proportionately more CRF appearing in the void the longer the incubation period (38%,51% and 71% of total CRF content respectively)(Fig 11). As shown in Fig 12, chromatography of normal male plasma pre-incubated with synthetic CRF in a similar fashion also resulted in a time-dependent binding of added CRF to material eluting in the void region of 65%, 94% (data not shown) and 97%.

Sephacryl S-200 chromatography of maternal plasma pre-incubated with ^{125}I-labelled hCRF showed that the peptide was binding with a substance eluting between serum albumin and free CRF, with a molecular weight in the region of 40,000 daltons. Labelled ACTH, LHRH and VP were not bound

to a carrier but eluted in their expected positions as the free peptides. Human[Met(o)^{21,38}]CRF was also bound by the carrier, mimicking the unoxidised human peptide, although oCRF was not (data not shown).

Fig 10 .

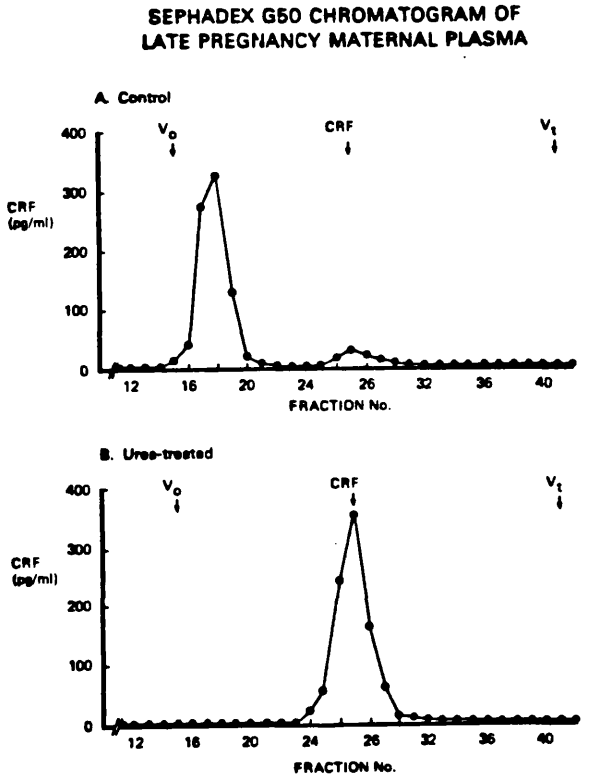


Fig 10. Sephadex G50 chromatograph of late gestational maternal plasma (a)Control; (b) Maternal plasma treated with 8 M urea (37°C, 1 h) before chromatography in column buffer containing 2M urea.

Preliminary investigations on the ACTH-releasing activity of plasma CRF using crude maternal plasma demonstrated the need for further purification, since male plasma containing little endogenous CRF-41 released ACTH, even in high dilution. A partially purified form of the carrier-bound CRF complex from Sephacryl S-200 chromatography was thus used for bioactivity testing. Dilutions of pooled chromatographic fractions containing carrier-bound CRF, whether the endogenous carrier-

Fig.11.

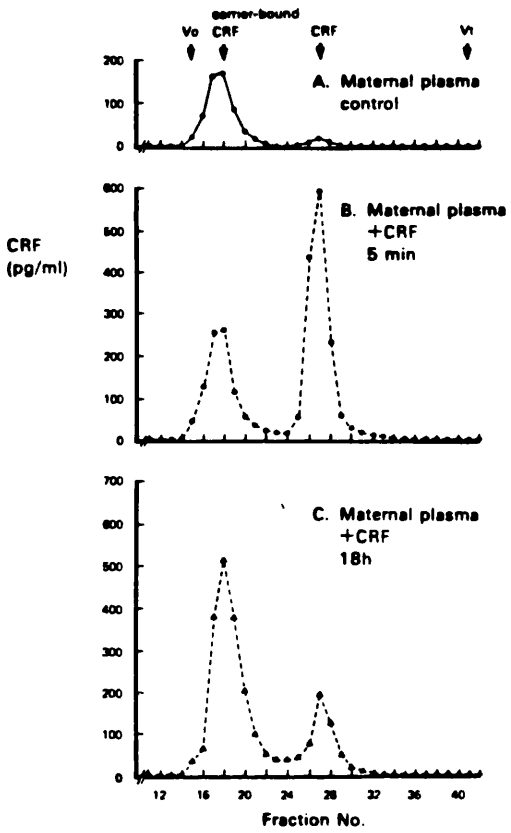


Fig.11. Sephadex G50 chromatogram of late gestational plasma alone (a) or incubated at 4°C with synthetic CRF for 5 min (b) or 18 h (c) before chromatography.

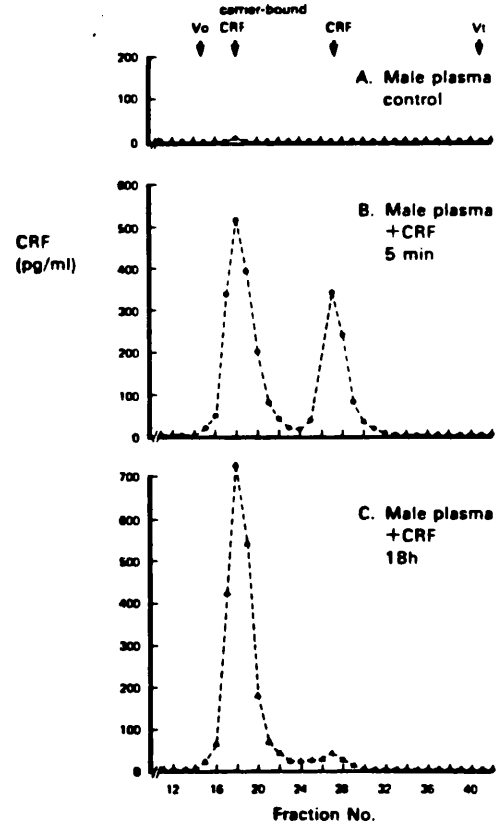


Fig.12. Sephadex G50 chromatogram of normal male plasma alone (a) or incubated at 4°C with synthetic CRF for 5 min before chromatography.

Fig. 13.

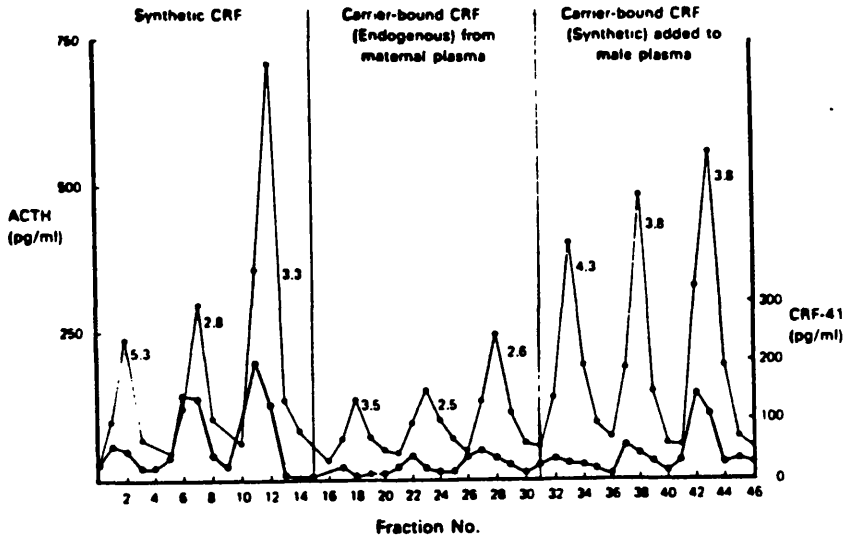


Fig. 13. ACTH-releasing activity of dilutions of synthetic CRF and pooled carrier-bound CRF peaks from Sephacryl S-200 chromatography of late gestational maternal plasma or normal male plasma incubated with synthetic CRF. Both ACTH (—•—•—) and CRF (_•_•_) are measured in the bioassay effluent.

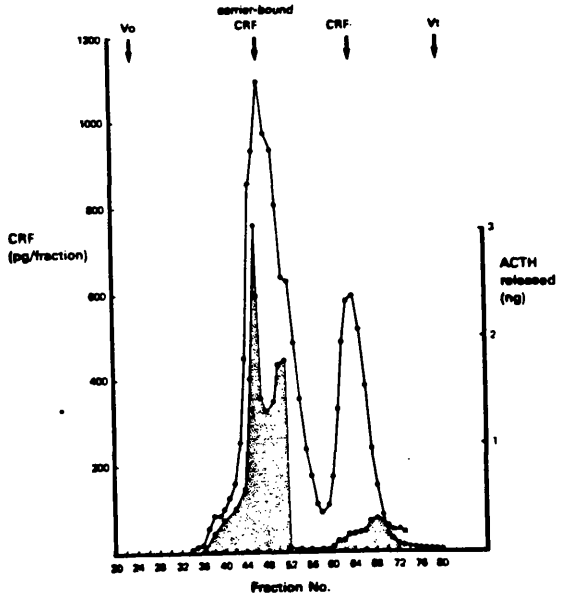


Fig. 14. Sephacryl S-200 chromatogram of late gestational maternal plasma showing CRF content and ACTH-releasing activity (Shaded area) of each fraction.

peptide complex from maternal plasma, or the synthetic peptide incorporated into the CRF carrier protein from male plasma, released ACTH in a dose dependent fashion as did the synthetic peptide (Fig 13). Figure 14 shows the chromatographic profile of late gestational maternal plasma on Sephacryl S-200. Again, two peaks of CRF like immunoreactivity were obtained, with 76% of the total endogenous CRF content bound to the carrier in the pooled plasma used in this case. Both peaks of CRF like immunoreactivity were associated with ACTH-releasing activity. However, a dip in bioactivity occurred coincident with the fractions containing the highest concentrations of CRF in the carrier-bound peak. This result was obtained on two further occasions in both a repeat bioassay and with fractions from a different chromatogram. Although fractions containing the free CRF peak were bioactive, this activity continued for several later eluting fractions.

2.6. DISCUSSION

Chromatographic evidence is presented showing that most of the endogenous CRF in maternal plasma is bound to a high molecular weight substance with an apparent molecular size of approximately 40,000 daltons. CRF is the first hypothalamic peptide for which a circulating carrier protein has been shown to exist, although large molecular weight carriers for other non-steroidal hormones have been identified, eg thyroxine binding globulin (mol. wt 59000, Raouf et al 1980), somatomedin binding globulin protein (mol. wt 40,000, Kaufman et al 1977) and growth hormone-binding globulin (Baumaun et al 1986). In pregnancy, placental steroid hormones circulate in complex with the three major plasma proteins (testosterone-binding globulin,

corticosteriod-binding globulin (CBG) and albumin, which rise in pregnancy (Dunn et al 1981).

That the high molecular weight CRF peak is not merely a CRF precursor is shown by its dissociation following urea treatment and by the incorporation of synthetic CRF into the peak in a time-dependent fashion. The peak is also unlikely to be due to polymerization of several (8-10) CRF molecules, since further peaks of intermediary-sized aggregates of the peptide would also be expected. Specificity of this CRF carrier is demonstrated by lack of binding to ACTH or LHRH, two other peptides also secreted by the placenta during pregnancy, or to VP, a hypothalamic peptide with ACTH-releasing activity. However, although the oxidized hCRF molecule, [Met(O)^{21,38}]CRF, does bind to the carrier, oCRF does not, indicating that at least one binding site is likely to be in the C-terminal half of the peptide where species-specificity resides. Thus binding is not dependent on full biological potency since the methionine analogue of hCRF displays poor ACTH-releasing activity (Rivier et al 1983) and oCRF is equipotent with the unoxidized human peptide.

Our control studies indicate that the carrier for CRF is also present in normal male plasma. Recently, Orth and Mount (1987) have demonstrated the existence of a plasma CRF binding protein by its inhibition of binding of ^{125}I-CRF to CRF antibody, whilst Ellis et al (1988) have also shown that ^{125}I-labelled CRF added to human plasma results in a reversible, time-dependent alteration in its molecular size. Suda (1988) demonstrated that most of maternal plasma CRF is bound, has reduced bioactivity and that the estimated molecular weight of the binding protein is 38000 which is smaller than the reported molecular

size of the pituitary CRF receptor. Unlike the situation in RIA, carrier-bound CRF in this study is measured as a positive signal in the IRMA, i.e. an increase in bound counts. It is perhaps surprising that the CRF IRMA detects bound CRF since IRMA is generally regarded as being a highly specific method for the determination of a particular polypeptide. Whilst it may be that the binding site for the carrier protein in the mid-portion of the CRF molecule and is not affected by the binding of amino and carboxy terminal CRF IRMA antibodies, the stable configuration of three large molecular weight molecules binding to CRF is unlikely. It is more probable that the high affinity assay antibodies in the IRMA dissociate the peptide from its carrier, rather than measure the CRF carrier complex intact, since the affinity of the IRMA antibodies are of the same order of magnitude as that quoted by Orth & Mount for the CRF carrier protein (Kd of 2 X 10^{-10}M). Although affinity estimates were not carried out in this study, it is likely that the CRF carrier in maternal plasma is similar to that observed by both groups in normal male plasma, as the carriers have the same elution positions on Sephacryl S-200 chromatography. The different rates of incorporation of added CRF into the carrier CRF peak is probably due to the presence of endogenous CRF already occupying binding sites on the carrier in maternal plasma or to different amounts of binding protein being present. However, it is not yet known whether the amount of CRF carrier increases with increasing plasma CRF concentrations during pregnancy. The oCRF peptide has here been shown not to bind to the carrier in both maternal and male plasma, corroborating the findings of Orth & Mount for male plasma.

It has been shown that degradation of synthetic hCRF by fresh human plasma in vitro is limited (Linton et al 1987), with no degradation of endogenous CRF, and suggested that the peptide may be protected by a carrier substance. Whilst investigating whether CRF would bind to albumin, it was surprising to find that, in a serum albumin preparation from human plasma (Blood products Laboratory, Elstree, Herts., UK) degradation was rapid and total, with 5ng of added CRF/ml disappearing within 4 min at 4°C. It is possible that the CRF carrier is removed during purification of the albumin from serum, leaving the degrading enzymes present to attack CRF. In support of this, it has been shown previously that the CRF degrading ability of rat plasma is greater than that of human plasma and further chromatographic studies indicate that rat plasma does not contain a CRF carrier. It is proposed that the carrier's presence in the circulation prevents the degradation of CRF, apart from any role it may have in neutralising any of the ACTH-releasing activity of the circulating peptide. Prolonging the life of CRF in plasma implies that circulating CRF serves some, as yet unknown physiological function.

Initial studies on the bioactivity of pooled chromatogaphic fractions containing the CRF carrier indicated that these diluted fractions could release ACTH, at least in the isolated rat anterior pituitary cell bioassay. This led to the belief that the binding of CRF to its carrier protein does not mask the ACTH-releasing ability of the peptide. Since this implies that circulating CRF in late gestational plasma is bioactive, ACTH levels must be kept in check by a mechanism other than neutralisation by a binding protein. It is possible that the maternal pituitary gland is desensitised by the high concentration of CRF in the

third trimester of pregnancy. Although down regulation of CRF-receptors on corticotrophs has been demonstrated in vitro (Buckingham et al 1985) the situation in humans in vivo appears to be more complicated. Schulte et al (1985) found that following a constant high plasma level of CRF achieved by infusion of the synthetic peptide in the non-pregnant state, the circadian rhythm was unaffected but there was no response to a subsequent bolus injection of CRF. The same group (Schulte et al 1988) gave a standard CRF test (1μg/kg) to pregnant women in the third trimester and did not demonstrate a rise in plasma ACTH, suggesting down regulation of the pituitary during pregnancy but similar work by Suda (Personal communication) using 100μg CRF demonstrated a smaller than usual increase in ACTH in the third trimester and concluded that down regulation may not be complete. It is therefore not clear whether the near normal ACTH levels found in late pregnancy may thus be the result of both negative feedback effect of elevated cortisol found at this time and the stimulating action of the high levels of plasma CRF. Alternatively, negative feedback of cortisol may in fact stop maternal pituitary ACTH release. The ACTH detected in the maternal circulation may originate from the placenta, as a result of the stimulatory action of high levels of CRF (Petraglia et al 1987).

An assessment of the ACTH-releasing activity of the chromatographic fractions of late gestational plasma, however, throws some doubt on the initial observations. Whilst bioactivity is associated with the carrier-bound CRF peak, reduced ACTH release is reproducibly obtained with the fractions containing the highest concentrations of CRF and thus its carrier. Whilst this may be due to an unknown inhibitary substance, it

is felt that another explanation is more likely. It may be that, in the fractions on the sides of the peak of carrier-bound CRF immunoreactivity, the more dilute CRF-carrier complex dissociates when the equilibrium is in favour of high affinity receptors of the pituitary corticotrophs, allowing the free peptide to stimulate ACTH secretion. Where the high concentrations of carrier-bound CRF occur (i.e. at the apex of the peak), CRF carrier complex formation is favoured such that fewer corticotroph receptors are activated, resulting in a decrease in ACTH release. Whilst the fractions eluting in the position of free CRF stimulated ACTH release, several later eluting fractions were also bioactive. This non-specific ACTH secretion may be due to other plasma constituents, and to the small amount of endogenous ACTH present in late gestational maternal plasma which elutes just after free CRF.

The question whether CRF carrier protein can mask the peptide's ACTH-releasing ability in the circulation during pregnancy thus remains unanswered. Further development of this research will be to purify the carrier-protein from human plasma using affinity purification and chromatographic techniques followed by amino acid and sequence analysis for characterisation of the carrier-protein. Antibodies to the carrier-protein can be raised and used in an immunoassay to allow its direct measurement in human plasma. Further <u>in vitro</u> studies using purified carrier-protein can be carried out to ascertain whether the carrier has any role in preventing the degradation of circulating CRF or masking its ACTH-releasing activity.

CHAPTER 3

MATERNAL & UMBILICAL CORD PLASMA CRF IN NORMAL PREGNANCY

3.1. INTRODUCTION

The human placenta secretes many proteins and steroids into the maternal circulation and their measurement has been used to assess fetal well being. The development of specific and accurate diagnostic tests for the identification of the fetus at risk of death or damage in utero has been an elusive goal. Consequently recently identified placental proteins need to be investigated for their potential use in predicting the outcome of abnormal pregnancy. Initially the normal range in maternal plasma at different stages of pregnancy has to be defined.

3.1.1. PLACENTAL STEROIDS AND PROTEINS: MATERNAL PLASMA LEVELS

The principle steroid hormones produced by the placenta are the oestrogens (Loriaux et al 1972) and progesterones (Lin et al 1972, Buster et al 1979), maternal plasma levels being several orders of magnitude higher during pregnancy. The ability of the placenta to utilise fetal dehydroepiandrosterone and its sulphate in the production of oestrogens introduced the concept of the fetoplacental unit (Diczfaluzy 1969). Consequently, the measurement of oestrogens, particularly oestriol, became commonplace in the assessment of fetal wellbeing (Chapter 4). Oestriol is mainly secreted into the maternal compartment in the unconjugated form, plasma levels rising steadily to term (Loriaux et al 1972) although Buster et al (1979) describe a plateau at term.

The glycoprotein hCG has an α subunit similar to the anterior pituitary hormones FSH, LH and TSH and a β subunit which has close sequence homology with LH (Closset et al 1973). In vitro studies have shown hCG release from the placenta with LHRH stimulation (Siler-Khodr & Khodr 1978). hCG production increases exponentially in early pregnancy peaking at 7-10 weeks, declining to constant levels thereafter (Braunstein et al 1976).

hPL is a peptide with a similar amino acid sequence and gene pattern to GH (Li et al 1971, Fiddes et al 1979). Maternal plasma hPL levels begin to rise at around 80 days and unlike the steroid hormones of the placenta plateau at 35 weeks (Singer et al 1970, Letchworth et al 1971, Gordon et al 1977).

With the use of appropriately absorbed hyperimmune rabbit antiserum to human third trimester plasma distinct pregnancy-associated plasma proteins have been identified (Lin et al 1974a). Circulating levels of PAPP-A increase throughout pregnancy but steeply from 30 weeks till labour with no evidence of a plateau in the last few weeks of gestation (Lin et al 1974b, Pledger et al 1984). Maternal plasma PAPP-B levels are low in the first 5 months of pregnancy and rise gradually till the 8th month, then steeply till the 9th month when a plateau is reached (Lin et al 1978). Maternal plasma SP1 levels rise with gestational age, reaching a plateau at 37 weeks, levels then tending to fall at around 40 weeks (Towler et al 1976, Gordon et al 1977). Maternal plasma PP5 levels rise from 8 weeks till 36-37 weeks, then decrease slightly (Nisbet et al 1981).

Of the hypothalamic and pituitary peptides produced by the placenta, only ACTH has been investigated in any detail, maternal plasma levels tending to rise as gestation advances with a rapid rise in labour. Measurements of maternal plasma levels of the other POMC related peptides has produced conflicting results but none are significantly elevated in the third trimester whereas prolactin levels rise dramatically throughout pregnancy. The maternal plasma levels of the other pituitary and hypothalamic peptides, except CRF, are not elevated, above the non-pregnant range, in the third trimester.

3.1.2. Using an RIA with prior extraction of CRF from maternal plasma, several groups have reported elevated levels of CRF in the third trimester of pregnancy (Table 1). Having conducted a pilot study of maternal plasma CRF levels in pregnancy at St Thomas's Hospital (Campbell et al 1985) it was decided that these findings should be confirmed prior to embarking on a study of CRF in pregnancy using the IRMA.

The study is descriptive and designed to determine the levels of plasma CRF during normal pregnancy and parturition in maternal and umbilical cord plasma and to investigate the relationship between these plasma levels and various obstetric variables to determine its potential usefulness as an indicator of obstetric outcome. The study also examines the relation between umbilical cord and maternal plasma CRF at delivery.

TABLE 1

AUTHOR	MATERNAL PLASMA CRF (pg/ml) ± SE		LABOUR
	28 - 34 weeks	35 - 45 weeks	
Campbell 1985	100 - 250 (5)	200 - 400 (9)	
Cunnah 1987	550 ------	9300 (6)	
Goland 1986	270 ------	± 68 (18)	
Goland 1987	223 ± 25 - 344 ± 33 (5)	1127 ± 80 - 4934 ± 3097 (5)	
Laatikainen 1987	32.8 ± 4.8 - 63.6 ± 9.2 (Pmol/L) (74)	187 ± 39 (Pmol/L)	NO INCREASE
Sasaki 1964	68.7 ± 23.6 (15)		2215 ± 329 (9) EARLY
Sasaki 1987	263 ± 41 (14)	800 ± 163 (20)	4409 ± 591 (28) DELIVERY
Smulte 1985	200 ------	9000 pg/ml	
Stalla 1987			To 7100 ± 3900 (7)

TABLE 1 Maternal plasma CRF levels reported by other investigators using extracted maternal plasma and RIA.

3.2. MATERIALS AND METHODS

3.2.1. PLASMA COLLECTION

All women gave their informed consent to participate and the study was approved by the Ethical Committee at St Thomas's Hospital.

All samples were obtained using an 18G needle and 10 ml syringe in an antecubital vein. In labour an 18G venflon was sited at the wrist under local anaesthesia (1% Lignocaine) and kept patent with Heparin solution. All samples were collected into plastic heparinised tubes with ascorbate on ice to minimise degradation of CRF and centrifuged at 4°C as quickly as possible. Plasma was snap-frozen on solid CO_2 and stored at -70°C in 1ml aliquots.

CRF RIA Random samples were taken from women in the antenatal clinic at 28 weeks (n=5) and at 38-40 weeks (n=9).

CRF IRMA: Antenatal samples were taken between 0830 and 1630 hrs. Gestational age was calculated using a combination of the subject's last menstrual period and a 16 week ultrasound scan. Single estimations of plasma CRF were made in 20 subjects in the first and second trimesters who subsequently had a normal pregnancy and delivery. 94 subjects had plasma samples taken sequentially from 28 weeks till delivery of whom 72 had a normal pregnancy with a spontaneous vaginal delivery at term (>38 weeks) of a baby weighing more than 2.5 Kgs. It was not possible to take a sample each fortnight in some patients. Details of the subjects antenatal care were documented as in Appendices 1 and 2. Data was subsequently entered onto the computer.

Labour. In subjects who had been followed sequentially antenatally samples were taken from a venflon throughout labour and details of the labour were recorded (Appendix 2). Patients undergoing elective or

emergency caesarean section had blood taken at the time of skin and uterine incisions. After delivery samples were taken for up to 24 hours to estimate the clearance of CRF from the maternal plasma.

Umbilical cord venous and arterial samples were collected after vaginal delivery or caesarean section (24 cases). Cord blood was obtained by double clamping the umbilical cord near the fetal umbilicus and close to the vaginal introitus. This portion of cord was excised and venupuncture of the artery and vein performed using an 18G needle and the plasma dealt with in the same way as described for maternal plasma.

3.2.2. **DEMOGRAPHIC DATA.** Details of the patients' age, race, past obstetric history, blood group, sickle status, smoking habit, weight gain in pregnancy, weight loss at term and maternal weight were recorded (Appendix 2) along with details of the gestational age at the time of onset of labour, whether labour was spontaneous or induced and the length of the first stage, fetal sex, weight and the placental weight.

3.2.3. **STATISTICS** Initially, in keeping with the other reports on maternal plasma CRF levels, the mean, standard deviation (SD) and Standard error (SE) of the mean were calculated at each stage of gestation. CRF data were tested for significance using Student's t test to compare values between groups at each gestational age and to compare venous and arterial samples. Subsequently the Mann-Whitney U Test and Spearman's rank correlation were used to analyse the data as the distribution of CRF at each fortnight of the third trimester was non gaussian and highly skewed to the right, which was not corrected with log transformation. Hence expression of CRF levels in terms of medians

was used and a cumulative frequency plot of CRF values at each fortnight of gestation displayed this. The rate of rise of CRF/week (pg/ml) in individual subjects was calculated, using simple linear regression slopes for a minimum of 3 antenatal values for each subject. To compare antenatal and labour values in the same subjects the Wilcoxon one sample test was used. Statistical analysis was performed using the statistical package, MINITAB .

3.2.4. CRF RIA. An RIA for oCRF employing direct radioiodination of the non-tyrosine containing peptide (Linton & Lowry 1982) was used and will be outlined.

i. CRF Antiserum. CRF antiserum was raised as described by Linton and Lowry (1982).

ii. Iodination of CRF. Synthetic oCRF (Universal Biologicals) was iodinated using a modification of the iodogen method of Salacinski et al (1981) (Linton & Lowry 1982). Fractions 17 and 18 (Fig. 15) were used for the assay.

iii. First Antibody Curves. Figure 16 represents the first antibody curves of fractions 17 and 18 of the iodination profile .

iv. Extraction of CRF from maternal plasma Because of interference from plasma proteins resulting in inhibition of binding it was not possible to use the RIA to measure CRF directly in plasma. Therefore samples were extracted using the method similar to that described for ACTH (Rees et al 1971) employing heat activated Vycor glass powder (Cunnah et al 1987) in which assay recovery rates varied between 30 and 55%. The inter-assay CV was 11.4% (mean concentration 500pg hCRF/ml) and the intra-assay CV was 10.2 (mean concentration 52ng/ml)

FIG. 15.

THE IODINATION OF rCRF

Fraction Number	% Conc. Methanol + 1% TFA	Counts	Fraction Number	% Conc. Methanol + 1% TFA	Counts
1	0	62 610	12	50	6 366
2	0	38 231	13	50	4 306
3	0	16 657	14	60	6 874
4	10	9 225	15	60	3 455
5	10	6 512	16	70	13 406
6	20	7 095	17	70	52 251
7	20	8 339	18	70	50 954
8	30	5 582	19	70	17 893
9	30	6 979	20	80	15 979
10	40	10 050	21	80	3 487
11	40	6 051			

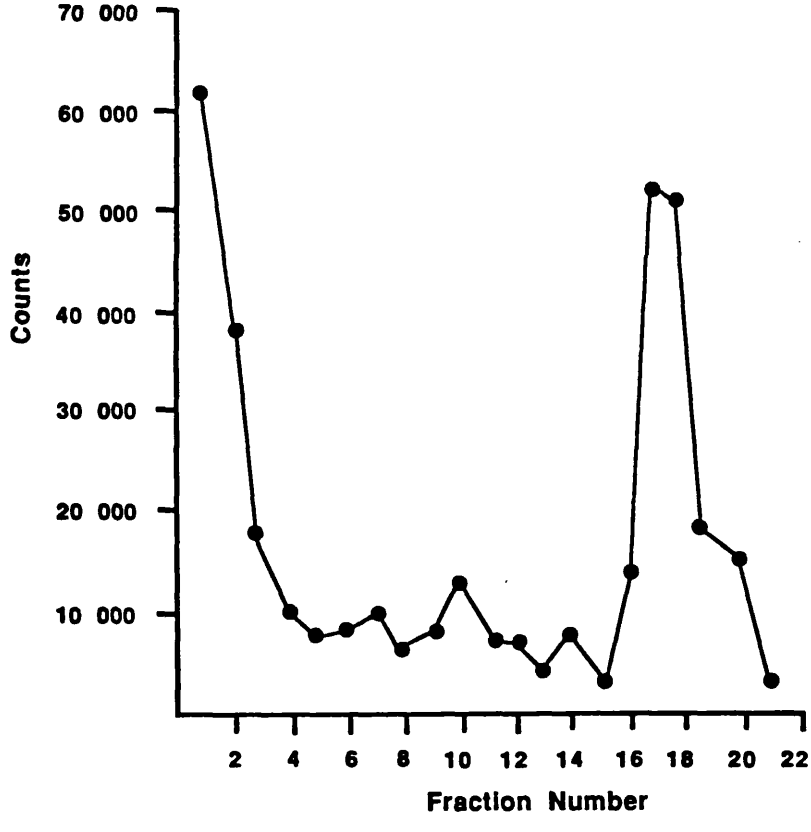


FIG. 15. The iodination profile of rCRF. Fractions 17 and 18 were used for the first antibody curves.

FIG. 16.

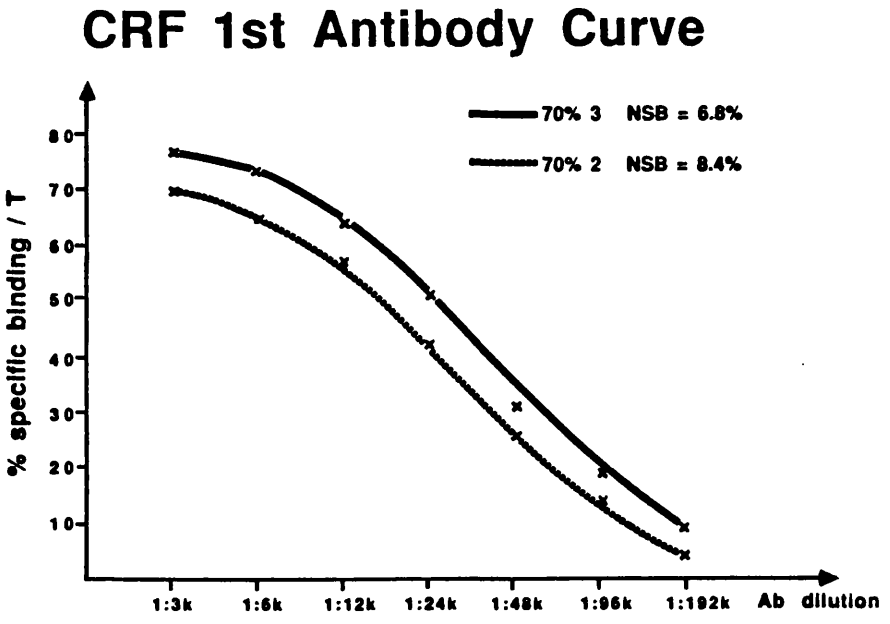


FIG. 16. CRF first antibody curves for fractions 17 and 18 of the iodination profile. NSB= non specific binding. Ab = antibody.

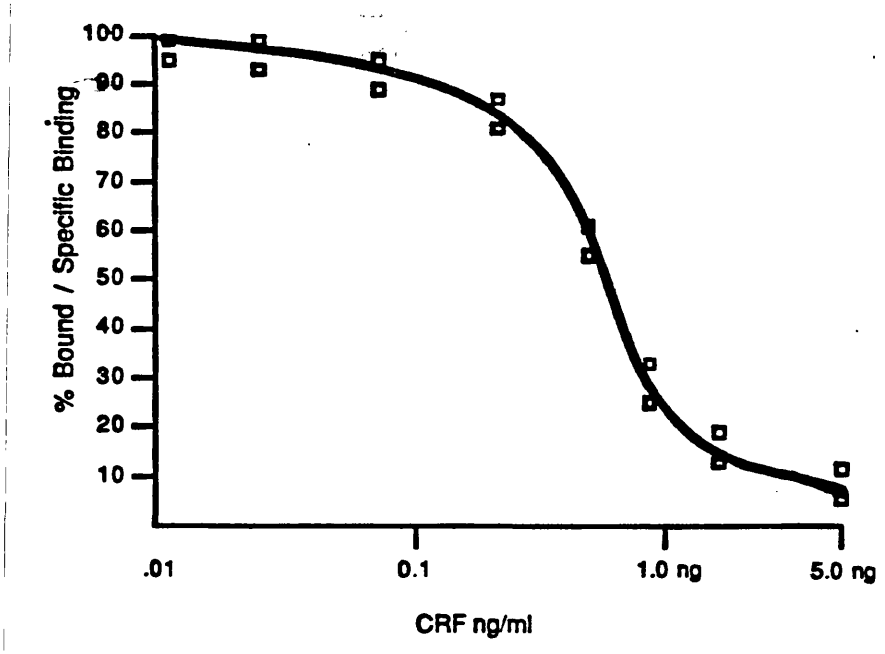


FIG. 17. RIA Standard curve. Duplicate readings at each concentration.

iv. Assay Standard Curve. Standard CRF(1µg/100µl)(CRB/Peninsula) was diluted in CRF buffer to give a concentration of 5ng/tube and double diluted to give 10 points from 5ng to 0.00975ng/tube(in duplicate)(Fig. 17). The assay procedure was as described by Linton & Lowry (1982). 200µl samples of maternal plasma were used in triplicate. The sensitivity of the assay is 100 pg/ml.

3.2.5. CRF IRMA as described in chapter 1.

FIGURE 18.

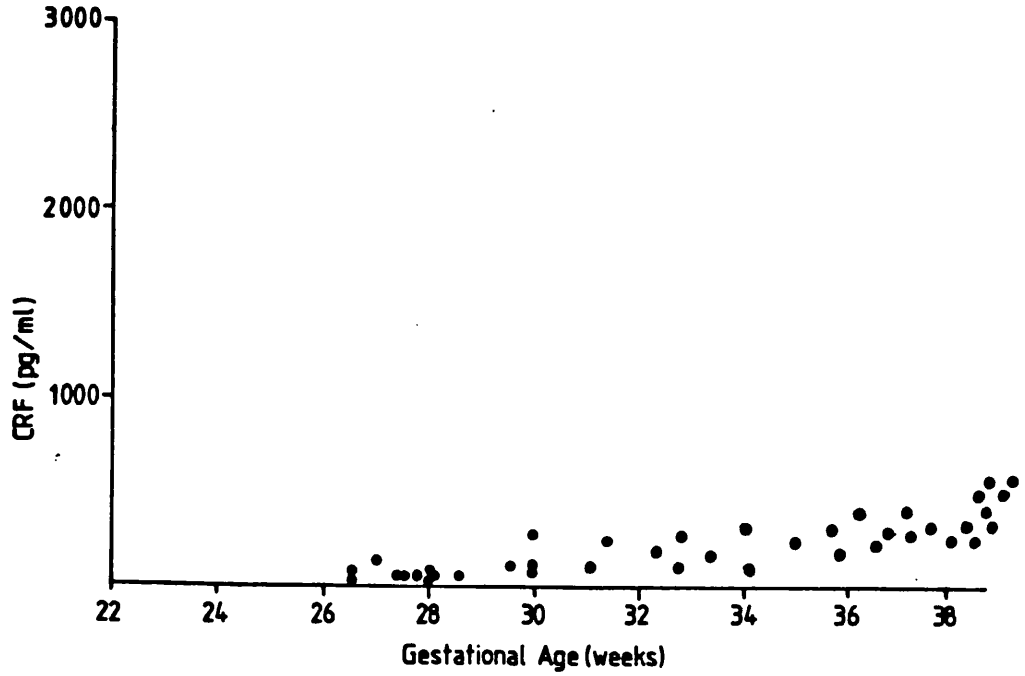


FIG. 18. RIA maternal plasma CRF concentrations from 26-40 weeks.

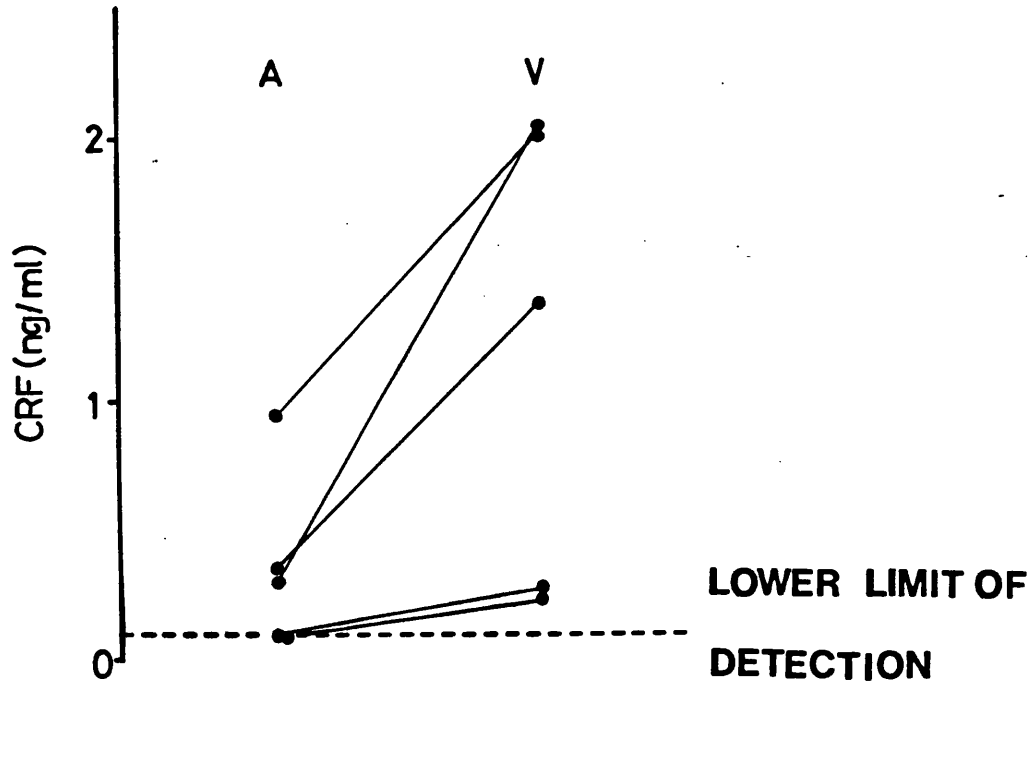


FIG. 19. RIA CRF concentrations in matched venous (V) and arterial (A) umbilical cord samples collected after vaginal deliveries.

3.3. RESULTS

3.3.1. CRF RIA

During pregnancy CRF immunoreactivity was below the detection limits of the assay (100pg/ml) until 27 weeks. At 28 weeks the levels ranged from 100-250 pg/ml (n=5) and rose to term. The plasma concentration at 38-40 weeks ranged from 200-400 pg/ml (n=9)(Fig 18). CRF was also detectable in umbilical cord blood. Umbilical artery levels ranged from undetectable to 950 pg/ml whereas venous levels were higher, between 100 pg/ml and 2100 pg/ml (Fig 19).

3.3.2. CRF IRMA

In the first (n=20) and second (n=20) trimesters CRF was not detectable above the non-pregnant range (2-28 pg/ml).

94 subjects were followed sequentially from 28 weeks, some subjects having estimations from 20 weeks, however. Of this group, 72 patients had a normal pregnancy with a spontaneous vaginal delivery at term of a baby weighing more than 2.5 Kgs (Appendix 3). Initial analysis of the data included the calculation of the mean, SD and SE (Table 2). The SDs and SEs were large and rank order analysis of the data showed a wide range of values at each gestational age (Appendix 4) and a non-gaussian distribution which was highly skewed to the right (Fig 20). The skewed distribution was not corrected by log transformation of the data, hence expression of CRF levels in terms of medians was considered most valid (Table 3) and a cumulative frequency plot of CRF values at each fornight displays this (Fig 21)

TABLE 2

GESTATION	NUMBER	MEAN	SD	SE
26	12	17.4	20.4	9.1
28	36	50	92	15
30	13	87	121	35
32	37	177	205	36
34	37	347	334	56
36	46	676	591	95
38	40	1062	748	121
40	19	1462	752	182
Labour	65	1668	816	101

Table 2 The mean, SD and SE plasma CRF in 72 normal subjects sampled sequentially from 26 weeks gestation.

Figure 20: Histograms of CRF values (pg/ml) at each fortnight of gestation. N and * represent number of observations at middle of each interval.

<26 weeks	N	
0	1	*
5	3	***
10	0	
15	3	***
20	0	
25	1	*
30	0	
35	0	
40	0	
45	2	**
50	2	**
28 weeks	N	
0	19	*******************
50	9	*********
100	5	*****
150	1	*
200	0	
250	0	
300	0	
350	0	
400	0	
450	1	*
500	1	*
30 weeks	N	
0	4	****
50	6	******
100	2	**
150	0	
200	0	
250	0	
300	0	
350	0	
400	0	
450	1	*
32 weeks	N	
0	9	*********
100	19	*******************
200	1	*
300	6	******
400	0	
500	1	*
600	0	
700	1	*
800	0	
900	0	
1000	1	*

FIG. 20

34 weeks	N	
0	10	**********
200	13	*************
400	10	**********
600	0	
800	2	**
1000	1	*
1200	0	
1400	0	
1600	1	*
36 weeks	N	
0	6	******
200	7	*******
400	9	*********
600	5	*****
800	5	*****
1000	3	***
1200	2	**
1400	1	*
1600	2	**
1800	3	***
2000	0	
2200	0	
2400	0	
2600	2	**
2800	1	*
38 weeks	N	
0	3	***
400	9	*********
800	9	*********
1200	12	************
1600	1	*
2000	1	*
2400	1	*
2800	3	***
3200	2	**
40 weeks	N	
400	3	***
800	2	**
1200	6	******
1600	4	****
2000	2	**
2400	0	
2800	2	**
3200	1	*
42 weeks	N	
1400	1	*
1600	1	*
800	1	*
3000	1	*

TABLES 3 & 5

WEEKS GESTATION	NUMBER OF PATIENTS	MEDIAN	MIN.	MAX.	10th - 90th CENTILE
20 - 26	12	15	1	49	3 - 48
28	36	20	1	520	4 - 121
30	13	37	1	466	17 - 68
32	38	78	1	965	34 - 306
34	37	260	16	1600	55 - 440
36	46	593	44	2732	95 - 1720
38	41	960	44	3315	295 - 2605
40	20	1320	239	3040	485 - 2649
42	4	2208	1450	3022	1660 - 2750

TABLE 3. The minimum, mediam, maximum and 10th-90th centile for maternal plasma CRF concentrations (pg/ml) from 20-42 weeks.

CERVICAL DILATION	NUMBER OF PATIENTS	MEDIAN	MIN.	MAX.	10th - 90th CENTILE
2 cm	14	1772	453	3186	530 - 2660
3 - 5 cm	12	1691	226	3349	407 - 1928
6 - 9 cm	13	1675	526	2756	666 - 2335
10 cm	21	1699	83	2698	306 - 2430

TABLE 5. The minimum, median, maximum and 10th-90th centile for maternal plasma CRF concentrations (pg/ml) at 4 stages of cervical dilation.

CRF median values rose dramatically in the third trimester from 20pg/ml at 28 weeks to 1320 pg/ml at 40 weeks. There was a strong correlation between the weeks gestation and CRF levels ($r=0.81$, $P<.001$)(Fig 21). Because of the skewed distribution there was a wide range of values at each gestational age and the rate of rise of CRF varied between subjects. It was therefore decided to analyse various parameters with the rate of rise CRF (pg/ml)/week. This was calculated using simple regression slopes (Appendix 5) for a minimum of 3 antenatal values in the third trimester for each subject (n=48). All fetal and maternal parameters entered into the computer were analysed in relation to the rate of rise in pg/ml CRF/week (Table 4). The only significant observation was that CRF levels were significantly associated with maternal weight gain in pregnancy ($r=0.36$, $p<.05$)(Fig 22)

PARAMETER	SIG.(MW)	r VALUE
Age	NS	0.15
Race(cauc v negro)	0.12	
Prev preg(0 v 1-5)	0.16	
Prev misc(0 v 1,2)	0.8	
No Terminations(0 v 1-4)	0.7	
Rhesus status	0.9	
Smoking status	0.66	
Maternal weight gain	<.05	0.36
Weight loss at term	0.56	
Maternal weight	NS	0.11
Maternal weight-fetal & placental weight	NS	0.1
Maternal weight gain-weight of fetus & Placenta	<.05	0.34
Gestation at Delivery	NS	0.34
Spontaneous v Induced	0.1	
Log length 1st Stage	NS	-0.21
Fetal sex	0.32	
Fetal weight	0.32	0.1
Placental weight	NS	0.02

TABLE 4 Correlation of various obstetric parameters with the rate of rise CRF(pg/ml)/week.(n=48). Sig (MW)= Mann Whitney Significance. NS= not significant

Fig. 21.

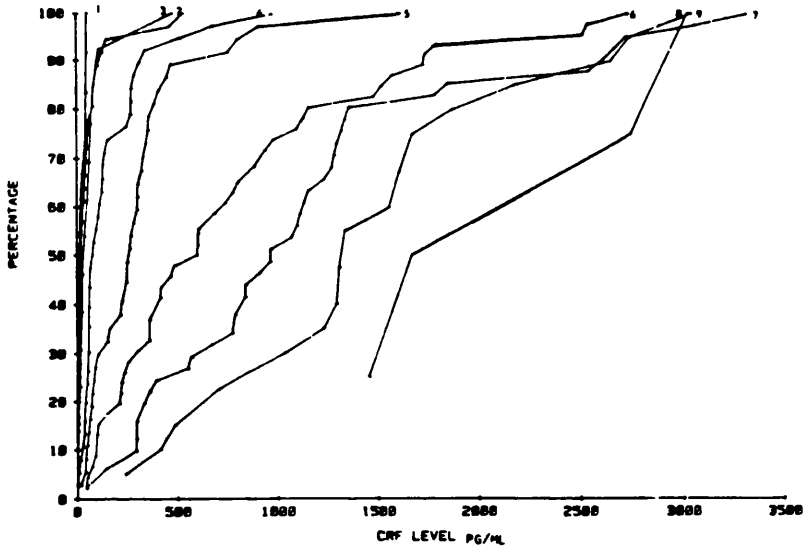


FIG 21. Cumulative frequency plot of CRF concentration from 20-26 weeks(1) and at fortnightly intervals to 42 week(9). • = 1 observation.

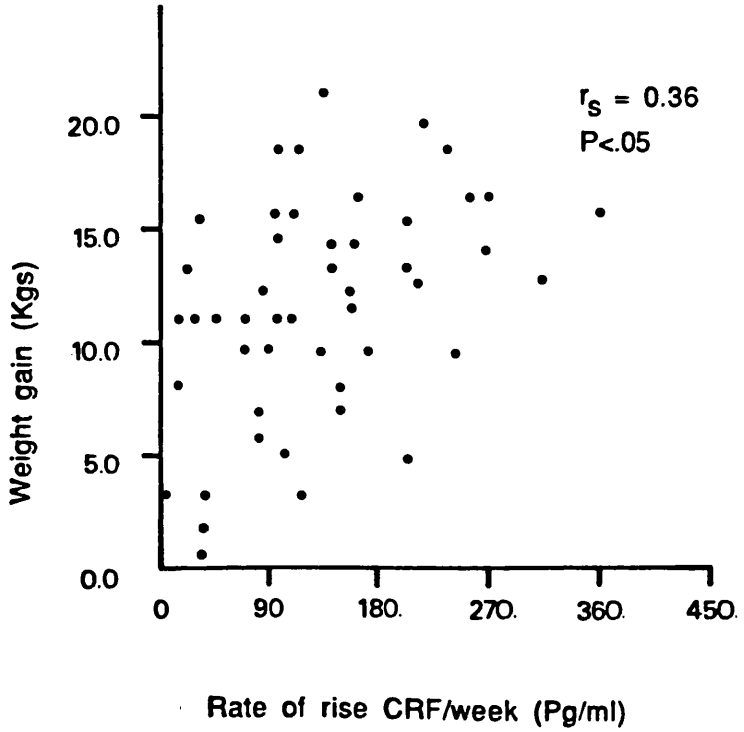


FIG. 22. Rate of rise CRF/Week vs maternal weight gain in pregnancy. • = one observation.

Table 5 and appendices 6 and 7 shows the CRF values obtained in labour in normal subjects who had been followed sequentially from 28 weeks and delivered at term (38-42 weeks)

The median value of CRF in labour was 1732 pg/ml and this was statistically elevated (P<.04) compared with the antenatal values at 40 weeks. However as there is a strong correlation between gestational age and CRF values, it is not clear from this data whether the elevation in labour was due to the process of parturition or the passage of time. In 11 subjects in whom the last antenatal value and a labour value were within a few days of each other, there was a significant difference between them (P< 0.001, Wilcoxon test (median antenatal, 1117 pg/ml; median labour 1849 pg/ml)).

There was no significant difference in CRF values at different stages of cervical dilation.

In keeping with a placental product, levels of CRF fall dramatically post partum (Fig 23) and levels were in the normal non pregnant range 24 hours post partum in all patients (Chapter 6).

UMBILICAL CORD STUDIES The umbilical cord venous CRF values ranged from 8-237 pg/ml whereas maternal plasma CRF ranged from 260-3564 pg/ml and there was a significant correlation between them (Fig 24)($r=0.54, p<0.001$). Matched arterio-venous samples showed a varied picture (Fig 25). In spontaneous vaginal deliveries the mean venous level was 19.6± 16 pg/ml and arterial 15±21 pg/ml and in caesarean section samples the venous 10.7±16 pg/ml and arterial 7.3±8.8 pg/ml. In 8 of 9 samples from vaginal deliveries and 4 of 7 caesarean sections the venous levels were slightly higher but comparing mean venous and arterial levels the difference was insignificant.

FIG. 23

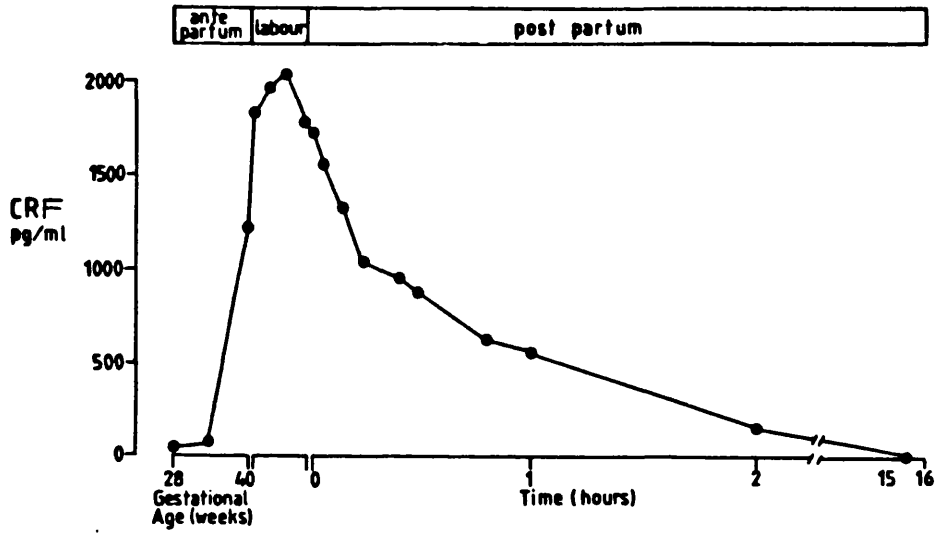


FIG 23. Plasma CRF levels measured sequentially in one woman during gestation, in spontaneous labour, and postpartum.

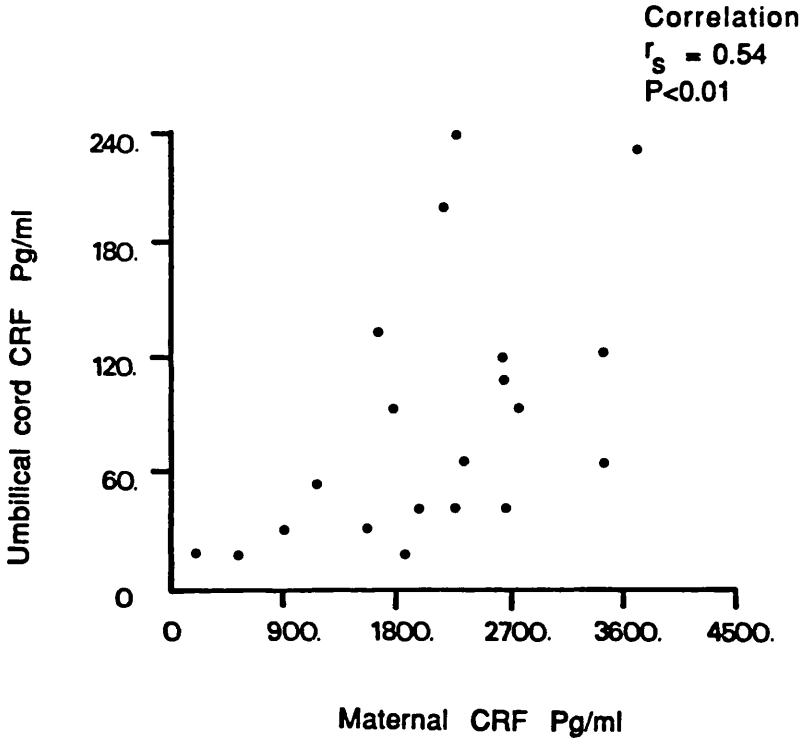


FIG. 24. Matched Maternal and umbilical vein CRF levels at delivery.

FIG. 25.

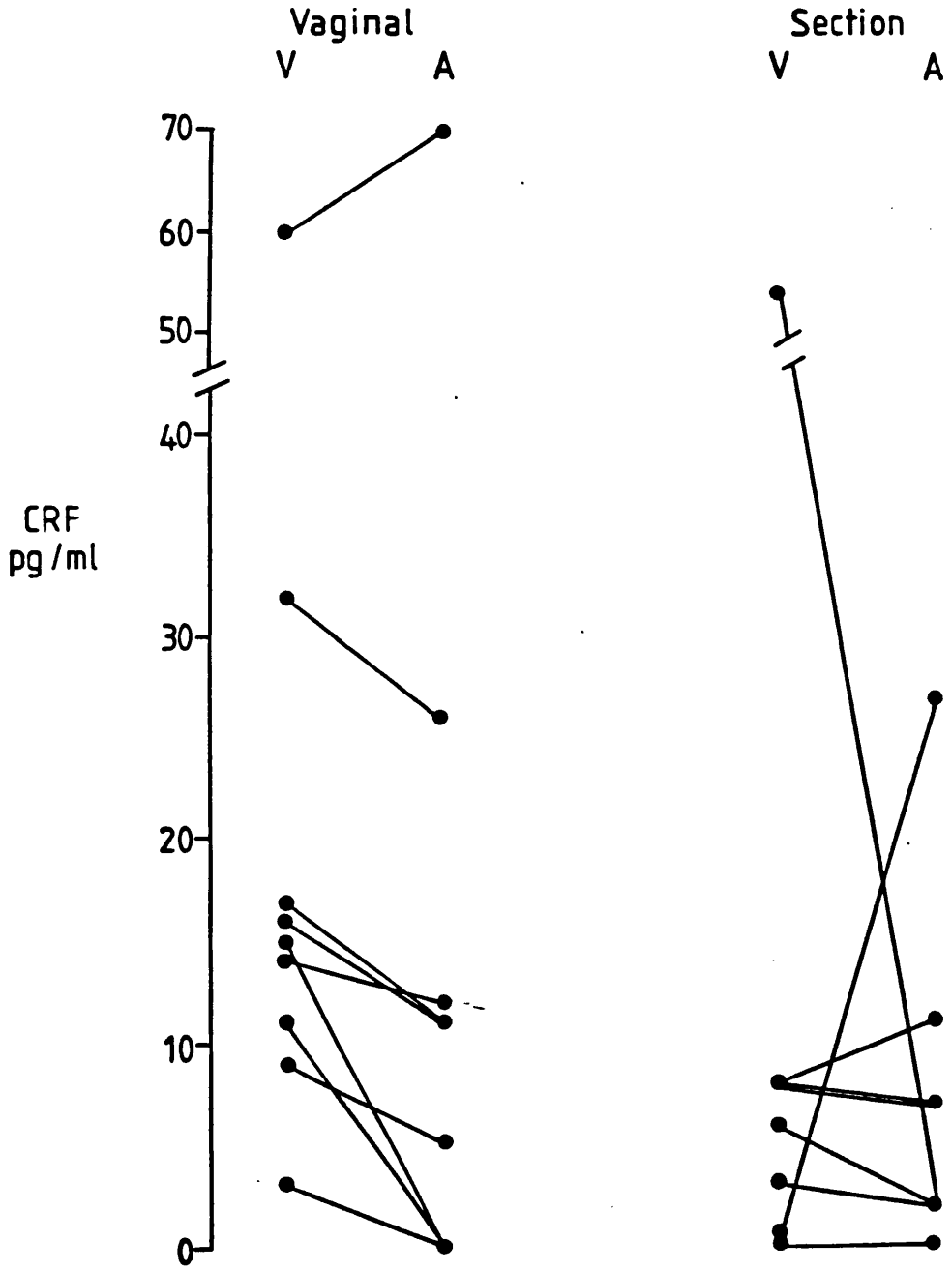


FIG. 25. CRF concentrations in matched venous (V) and arterial (A) umbilical cord samples collected after spontaneous vaginal deliveries and caesarean sections.

3.4. DISCUSSION

The placenta produces a wide range of steroids and proteins, amongst them peptides resembling hypothalamic and pitutary hormones. Most of these products can be measured in non-pregnant plasma but the concentration may be several orders of magnitude less than in the pregnant state and the concept of pregnancy specific is by and large naive. The measurement of placental products has been studied extensively to examine placental function and fetal wellbeing. Biochemical tests of fetal wellbeing were in widespread use until recently, when biophysical methods of assessment became more fashionable. The identification of a new placental protein does, however, need investigation in order to identify a possible physiological role and to assess its use as a biochemical marker of fetal wellbeing.

In contrast to the low levels of CRF observed in peripheral plasma of normal subjects (Suda et al 1985) there have been several reports demonstrating elevated levels of CRF in maternal plasma in the third trimester of pregnancy (Table 1) using RIA with prior extraction of the peptide. There is a variation between groups in terms of the absolute levels measured and this may reflect, in part, the different assay and extraction systems used. Also the timing of the samples in the third trimester may explain, in part, the wide range of values obtained. The first report by Sasaki (1984c) did not specify gestational age at sampling, levels being relatively low but in a subsequent report (Sasaki et al 1987) this group's range was in agreement with others (Campbell et al 1985, Goland et al 1986 & 1987, Laatikainen et al 1987). Two groups

recorded wide ranges to 9,300 pg/ml at term (Schulte & Healy 1985, Cunnah et al 1987). All these reports were on a small number of subjects and were random samples that did not describe the changes in plasma CRF throughout pregnancy. The first aim of this study was to describe the changes in plasma levels throughout normal pregnancy and labour.

The results of the pilot study , using the RIA, are in agreement with most groups but because of the poor extraction procedure it was decided to embark upon the larger study with the 'two-site' IRMA.
This study confirms the observations made with RIA that maternal plasma CRF is raised in the third trimester. It is the first report of the sequential measurement of maternal plasma CRF in pregnancy and parturition. Despite a large number of subjects with accurately dated gestational ages, the range of values at each fortnight of the third trimester is large. This demonstrates that,like most placental products which have been used clinically, the distribution of values at each gestational age is skewed to the right. Although a description of the mean, standard error and deviation reflects the wide range of values, the expression of medians and centiles defines the range in normal pregnancy more realistically. Unlike studies on hPL and SP 1 (Letchworth et al 1971, Gordon et al 1977) the distribution does not become gaussian after logorithmic transformation. This may be because the number of observations is still relatively small and with a larger sample size, this transformation could be of use.

Using RIA, CRF was below the detection limit till the second (Goland et al 1986 & 1987, Laatikainen et al 1987) or third trimester (Sasaki et al

1985) which is in agreement with this study. CRF appears to rise above the non-pregnant range from 28 weeks which is unlike all other placental products which begin to rise early in pregnancy. Northern blot analysis of total RNA content of the placenta has demonstrated that mRNA for CRF is present only at low levels in the first and second trimester, rising approximately twenty to forty fold in placentae of 35-40 weeks gestation (Frim et al 1987). It is not clear why the levels of mRNA should rise only in the third trimester, further in vitro studies being indicated to elucidate the mechanism that switches on its production. The fact that mRNA rises in the third trimester to term suggests that it may have a role in parturition .

Many studies of placental products used in the assessment of fetal wellbeing show a general tendancy for maternal plasma levels to rise from early pregnancy, reaching a plateau near term. This is in keeping with the hypothesis that the potential for placental synthesis and release of placental peptides into the maternal circulation is a direct function of the total mass of trophoblast and its maternal blood supply (Gordon & Chard 1979). The notable exception is PAPP-A, in which the maternal plasma level pattern is very different from known fetal (Alpha fetoprotein), fetoplacental (oestriol) or placental (hCG & hPL) products (Bishof et al 1982). PAPP-A shows a characteristic increase at term when other parameters level off or even decrease. PAPP-A also rises in labour (Smith et al 1981) and it has been postulated that the behavour of PAPP-A in late pregnancy is a consequence of uterine activity. Others have, however, taken this evidence as an indication of an extra placental source of PAPP-A (Bishof et al 1982). The general consensus is

that PAPP-A is localised (but not necessarily synthesised) at the apical border of the syncytiotrophoblast (Stabile et al 1988). The sharp rise in CRF values to term is similar to PAPP-A (Smith et al 1979). At present the site of localisation of CRF in the placenta is unclear but further localisation studies will perhaps explain why maternal plasma CRF levels rise in such a manner.

Although the range of values at each gestational age is wide (Table 3) the median values rise sharply and there is an order of magnitude difference between the beginning and end of the third trimester. The levels of CRF reached at term are similar to those having previously been reported in the hypophyseal vessels (Gibbs & Vale 1982). The strong correlation of plasma CRF with gestational age ($r=0.81$, $P<0.001$)(Fig 21) has been reported by others (Laatikainen et al 1987, Sasaki et al 1987) but these studies were not on sequential samples.
A significant rise in plasma CRF with advancing gestational age is demonstrated in the cohort generally but the rise in some subjects is not as great as in others. The rate of rise of CRF (pg/ml)/week was calculated on a minimum of 3 antenatal CRF measurements using simple linear regression slopes (appendix 5). Only half the subjects had significant rates of rise, which reflects the wide variation in CRF measurement in the third trimester .

There is a significant difference ($P<0.04$) between the median values at 40 weeks and during labour but this does not take into account the time of onset of labour and consequently it is not clear from these data whether the rise is due to the process of parturition or the passage of time. In an attempt to exclude the time factor 11 patients in

whom the last antenatal and labour samples were taken within a few days of each other were studied. There was a significant difference (P<0.001) between antenatal and labour values. The numbers are small and further studies into the rate of rise of CRF and its relationship to the onset of parturition are indicated. There was no rise in CRF levels during labour although the trend was for an increase in the levels towards full dilation (Appendix 6). Certainly the marked rise in CRF reported by Sasaki et al (1987) during labour has not been confirmed (Laatikainen et al 1987). Stalla et al (1987) showed that the highest CRF levels were seen in subjects who had the quickest labours (n=5) and whose second stage was shortest, but the standard deviation was large and the numbers small.

The effect of various correlates on maternal plasma CRF levels may provide useful information in suggesting various factors which could control the placental production and secretion of CRF. Studying plasma oestriol, most investigators have found no correlation with birth weight (Perry et al 1986), placental mass (Masson 1973), parity, sex of infant, maternal age or weight (Klopper & Billewicz 1963, Hay & Lorscheider 1976).

Synthesis of hPL is related to placental mass and although some investigators have found little relationship, most have shown a significant correlation between hPL with placental weight (Genazzani et al 1971, Gordon et al 1977, Letchworth et al 1971, Singer et al 1970). Considering the pregnancy specific proteins, no really significant correlation between SP1 and birth weight or placental weight has been observed (Chapman et al 1981, Towler et al 1977, Lin et al 1976) but with PAPP-A there is a significant correlation with placental weight

(Lin et al 1976). This is interesting as it is known placental weight increases with gestational age but slows in the last 8-10 weeks; This is mirrored in the pattern of plasma levels of oestriol and hPL but not PAPP-A which increases to term. This discrepancy may be because PAPP-A levels do not actually relate to placental mass or that there is an extra-placental source of PAPP-A (Bishof et al 1982). The lack of correlation between CRF and a number of clinical variables makes the the interpretation of CRF values less complex but sheds little light on possible controlling mechanisms for its release.

A significant difference in CRF levels in subjects who gain weight excessively may indicate that an abnormal pregnancy state such as diabetes, pregnancy induced hypertension with oedema or gross oedema alone may influence the CRF levels but its significance has to be tempered by the fact that weight is not accurately measured in the antenatal period and further studies on weight gain need to be undertaken.

In keeping with a placental origin for CRF, levels fall dramatically post partum (Sasaki et al 1985 & 1987, Goland et al 1987, Laatikainen et al 1987) and this will be described in detail in chapter 5 .

The findings of low plasma levels of CRF in the umbilical cord at delivery is in agreement with other groups (Sasaki et al 1987, Goland et al 1986) and tends to imply that there is limited transfer, if any, from the placenta to the fetus, as would be expected of a placental product (Grudzinskas et al 1978). There is also evidence that maternal ACTH does not cross the placenta. Therefore umbilical cord CRF may reflect, in part, endogenous fetal hypothalamic CRF; In the second trimester,

when maternal plasma CRF levels are still in the normal non-pregnant range, CRF levels are low in fetus (3.3 pg/ml)(Economides et al 1987); at delivery umbilical cord CRF levels are still in the adult non-pregnant range reaching only 3-4% of maternal CRF levels; CRF is present in the human fetal hypothalamus from at least the beginning of the second trimester (Ackland et al 1986) and the HPA axis exhibits negative feedback control. This evidence is in direct contrast to Goland et al (1986) who reported elevated levels of CRF in both mother and fetus and suggested a possible modulating role for this hormone in the maternal and fetal HPA axis during pregnancy.

There is ,however, a significant correlation between maternal and fetal plasma CRF ($r=0.54$, $P< 0.01$) which is in agreement with the other groups and may reflect a common source in the placenta. There is no statistically significant arterio-venous difference in CRF cord plasma levels which one might expect of a peptide of placental origin. These data are at variance with groups that have recorded significant arterio-venous differences (Goland et al 1986, Sasaki et al 1987) but the sample size in all these studies has been small. Further evidence for the presence of CRF in the fetal compartment comes from RIA estimations in amniotic fluid which suggest that CRF levels in this compartment probably reflect fetal CRF concentrations (Stalla et al 1985, Laatikainen et al 1988). There is a need to establish whether umbilical cord plasma CRF is of placental or fetal origin. Physicochemical characterisation of fetal CRF may shed further light on this matter. Of particular interest would be whether fetal CRF is carrier bound.

The relationship of fetal plasma CRF with POMC related peptides in fetal plasma has been studied. No correlation between fetal plasma β-end or

ACTH and fetal CRF has been reported (Goland et al 1986), which may reflect a placental source for the peptide, whereas Economides (1987) found a weak inverse relationship between fetal CRF and ACTH. Nagashima et al (1987) recorded high levels of cortisol, DHEAS and ACTH in cord blood, demonstrating increased adrenal steroid synthesis in the fetus and attributed this to high circulating fetal CRF levels. Again, more in vitro studies and cordocentesis sampling during pregnancy will help to assess the importance of placental and/or fetal CRF in the fetal HPA axis.

The physiological role of placental CRF has yet to be determined. It has been suggested that trophoblastic products have no function whatsoever (Gordon & Chard 1979) and at best, most of the supposed functions are speculative rather than definitive. This viewpoint will not help ascertain any role for CRF and speculation seems warranted. The possible down regulation of the pituitary in pregnancy is speculative, however, Suda et al (1988) felt that placental CRF alone could be responsible for the known changes in the HPA axis in late pregnancy. Another complicating factor in this argument is whether the CRF carrier protein can mask the peptide's ACTH releasing ability. It may transpire that in pregnancy the HPA axis is not driven by CRF-41 but by VP or one of the other substances with ACTH-releasing activity, the high circulating levels of CRF in pregnancy assuming a lesser role in the control of the axis. The effect of placental CRF on the maternal HPA axis will be further discussed in Chapter 5.

Other evidence for a physiological role for placental CRF is emerging. The in vitro studies of Petraglia have strengthened the observations that placental peptides related to their hypothalamic counterparts may

be important in the control of hormonogenesis in the chorionic system (Krieger 1982, Petraglia et al 1987). CRF could have a paracrine role in the placenta, controlling placental ACTH production . Petraglia demonstrated that OT and prostaglandins stimulate placental ACTH and CRF secretion and that CRF may mediate prostaglandin stimulation of ACTH secretion. Placental CRF, or ACTH that is under its control, could act on the maternal HPA axis to produce the changes observed in pregnancy. Although no evidence exists in man of a fetal signal heralding the onset of parturition, this should be further investigated in the knowledge that maternal plasma CRF levels rise rapidly in the third trimester to term. Placental CRF or ACTH could stimulate the fetal HPA axis and thereby act as a signal for the onset of parturition. The evidence for a fetal HPA axis signal comes from the classic works of Liggins (1967) and more recently the observations of Wintour et al (1986) that the infusion of oCRF into an immature fetus can accelerate maturation of a number of organs and systems culminating in the preterm delivery of a viable lamb. Unfortunately in man research has been hampered because of difficult access to the fetus and lack of suitable animal models, except the rhesus monkey. Perhaps with the advent of cordocentesis, valuable information can be obtained to unravel the sketchy data on the human fetal and maternal HPA axis and its involvement in parturition.

Although evidence that fetal ACTH triggers parturition in man is unclear (Anderson 1969, Honnebier & Swaab 1973), fetal adrenal hyperplasia has been noted in preterm babies (Anderson et al 1971). It is also known that fetal DHEAS levels increase till term (Parker et al 1982) indicating increased adrenal activity. The existing data on hormonal influences involved in parturition indicate that Prostaglandin E_2 has a

major role in the initiation of labour, whilst Prostaglandin $F_{2\alpha}$ is subsequently important in stimulating contractions. The steroid hormones, oestrogen and progesterone play only a facilitatory role in the initiation of labour with salivary oestriol:progesterone ratios having been reported as changing prior to the onset of labour (Darne et al 1987). The stimulation of the uterine muscle during labour results from an interaction of OT and Prostaglandin $F_{2\alpha}$. Recent evidence suggests that OT is most important in the initial phase of labour, whereas increased Prostaglandin $F_{2\alpha}$ is essential for the progression of labour (Fuchs & Fuchs 1984). A study of these hormonal factors in relation to fetal and maternal plasma CRF levels may add further evidence for a mechanism in the initiation of parturition.

CHAPTER 4

MATERNAL PLASMA CRF LEVELS IN ABNORMAL PREGNANCY STATES

4.1. INTRODUCTION

The reduction of the perinatal mortality rate remains one of the obstetrician's main aims. Prematurity, intrauterine growth retardation (IUGR) and congenital abnormality along with antepartum haemorrhage (APH), Pregnancy induced hypertension (PIH) , diabetes mellitus and multiple pregnancy all contribute to perinatal and maternal morbidity and mortality. The accurate assessment of fetal wellbeing and consequently placental function remains unattainable in these conditions.

Biochemical tests based on deviation of a result from the normal range in various abnormal pregnancy states have been used extensively but have not provided the clinician with a sensitive test of high predictive value. They have largely been abandoned for the biophysical methods of assessment which are non-invasive, reproducible, have high patient acceptance, are easy to perform and are a more sensitive test with higher predictive value for IUGR (Gennser & Persson 1986). There is still a place for biochemical tests as biophysical assessments are not 100% sensitive, are time consuming, require expensive equipment, experienced operators, are not applicable to a whole population and biochemical parameters may antecede changes in the biophysical profile of the fetus.

Some attempt to categorise placental products has been undertaken but requires constant updating (Chard 1982). Group 1 products are trophoblast derived, maternal plasma levels being dependant on

trophoblastic mass and uteroplacental blood flow. Maternal plasma levels of these products tend to be reduced in pathological states, eg hPL, oestriol, hCG, SP1. Group 2 products are under the same control with the possibility of control by some other factors also, levels tending to be higher in certain abnormal pregnancy states, eg PAPP-A, PP5. This study will attemp to categorise CRF in terms of this model.

Chard (1976) outlined criteria required of a biochemical test of fetal wellbeing and although not all criteria have been fulfilled in tests, some tests have entered into clinical practice. This study has so far established that CRF satisfies Chard's criteria in the following respects:

1.CRF is quantitatively much more abundant in maternal plasma than in the non-pregnant state. It is possibly qualitatively different as a carrier protein has been identified for CRF in pregnancy, although the carrier may also be present in the non-pregnant state but in different quantities.

2. CRF is easily accessible in plasma.

3. CRF is easily measured in a fast assay but at present this is not a cheap process.

The criteria that still need to be satisfied are:

Is the level of CRF altered by maternal and metabolic processes?

The initial plan is to determine whether CRF levels are significantly altered in a variety of abnormal pregnancy states. This will determine whether further studies investigating the sensitivity, specificity and predictive value of CRF as a screening test for fetal wellbeing are warranted. It is important that critical assessment of such a screening

test be carried out. In the past this has only been the case with hPL and SP1 (Gordon et al 1977, Grudzinskas et al 1981, Westergaard et al 1984a).

4.2. MATERIALS & METHODS

The observed range in normal pregnancy was constructed from a cohort of 72 normal subjects followed sequentially from 28 weeks to term (Chapter 3).

CRF estimations were made in a variety of pathological pregnancies:

Three patients with hydatidiform moles were sampled prior to evacuation of the uterus. Fourteen twin pregnancies were sampled randomly (24 samples) and of these 3 delivered prematurely. 28 diabetics, 14 insulin dependant and 14 diet controlled were sampled sequentially (62 samples). A comparison of CRF levels in diet and insulin controlled diabetics and gestational diabetics was made. Four patients with an accidental antepartum haemorrhage (APH)(11 samples) were also studied.

63 subjects hospitalised with PIH , with a blood pressure greater than 140/90 on two occasions were sampled at least once (103 samples). Ten of these subjects had significant proteinuria. Of the original group of normal subjects sampled sequentially in the antenatal clinic 7 developed PIH.

Subjects admitted in preterm labour or with ruptured membranes prior to 37 completed weeks were sampled (21 subjects,39 samples) and the group was comprised only of non-hypertensive premature deliveries, 5 of which had been sequentially sampled from 28 weeks.

TABLE 6.

WEEKS OF GESTATION	< 28	30+32	34+36	38+40
NORMAL PREGNANCIES				
N	48	51	83	61
MEDIAN	20	60	351	1126
TWIN PREGNANCIES				
N	5	4	12	3
MEDIAN	87	505	1935	1597
SIGNIFICANCE	< 0.01	< 0.01	< 0.001	< 0.4
DIABETIC PREGNANCIES				
N	15	18	26	15
MEDIAN	26	141	522	813
SIGNIFICANCE	1	< 0.16	< 0.6	< 0.16
ANTEPARTUM HAEMORRHAGE				
N	3	5	3	
MEDIAN	133	60	493	
SIGNIFICANCE	< 0.03	< 0.5	< 0.7	
POLYHYDRAMNIOS				
N	3	6	5	3
MEDIAN	15	153	307	706
SIGNIFICANCE	0.95	0.25	0.9	0.75
PREGNANCY INDUCED HYPERTENSION (PIH)				
N	14	23	45	23
MEDIAN	68	840	1253	1896
SIGNIFICANCE	< 0.001	< 0.001	< 0.001	< 0.01
PRETERM LABOUR				
N	10	11	14	
MEDIAN	86	320	1198	
SIGNIFICANCE	< 0.001	< 0.001	< 0.001	

TABLE 6. CRF values (pg/ml) in twin pregnancies, diabetic pregnancies, pregnancies complicated by APH, polyhydramnios, PIH and preterm labour

Seventeen samples were taken from patients with polyhydramnios. This group contained diabetics but most had no ascertainable reason for the increased amniotic fluid volume.

Subjects delivering a fetus of less than 2.5 Kgs at term were compared to normal subjects.

Two cases of intrauterine death were studied (23 and 40 weeks).

The maternal plasma samples were assayed for CRF using the 'two-site' IRMA. The distribution of CRF at each two week period of the third trimester in normal pregnancy was non-gaussian and CRF levels are expressed in terms of medians. The mann-Whitney U Test was used to analyse the data. From the observed range in normal pregnancy the 10th and 90th centile was calculated.

4.3. RESULTS

1. HYDATIDIFORM MOLE: In the three cases studied the levels were in the non-pregnant range(15, 18 & 20 pg/ml).

2. TWIN PREGNANCY: Fourteen pregnancies were studied and the levels were significantly higher at all stages of gestation except at 38-40 weeks. Of these pregnancies, three delivered prematurely (Table 6).

3. DIABETIC PREGNANCIES: There was no statistical difference between normal and diabetic pregnancies in the 28 subjects studied (Table 6). There were 14 diet and 14 insulin controlled diabetics. Of the insulin controlled diabetics some were gestational and some established diabetics .There was no statistical difference between either group and the normal range.

FIG. 26.

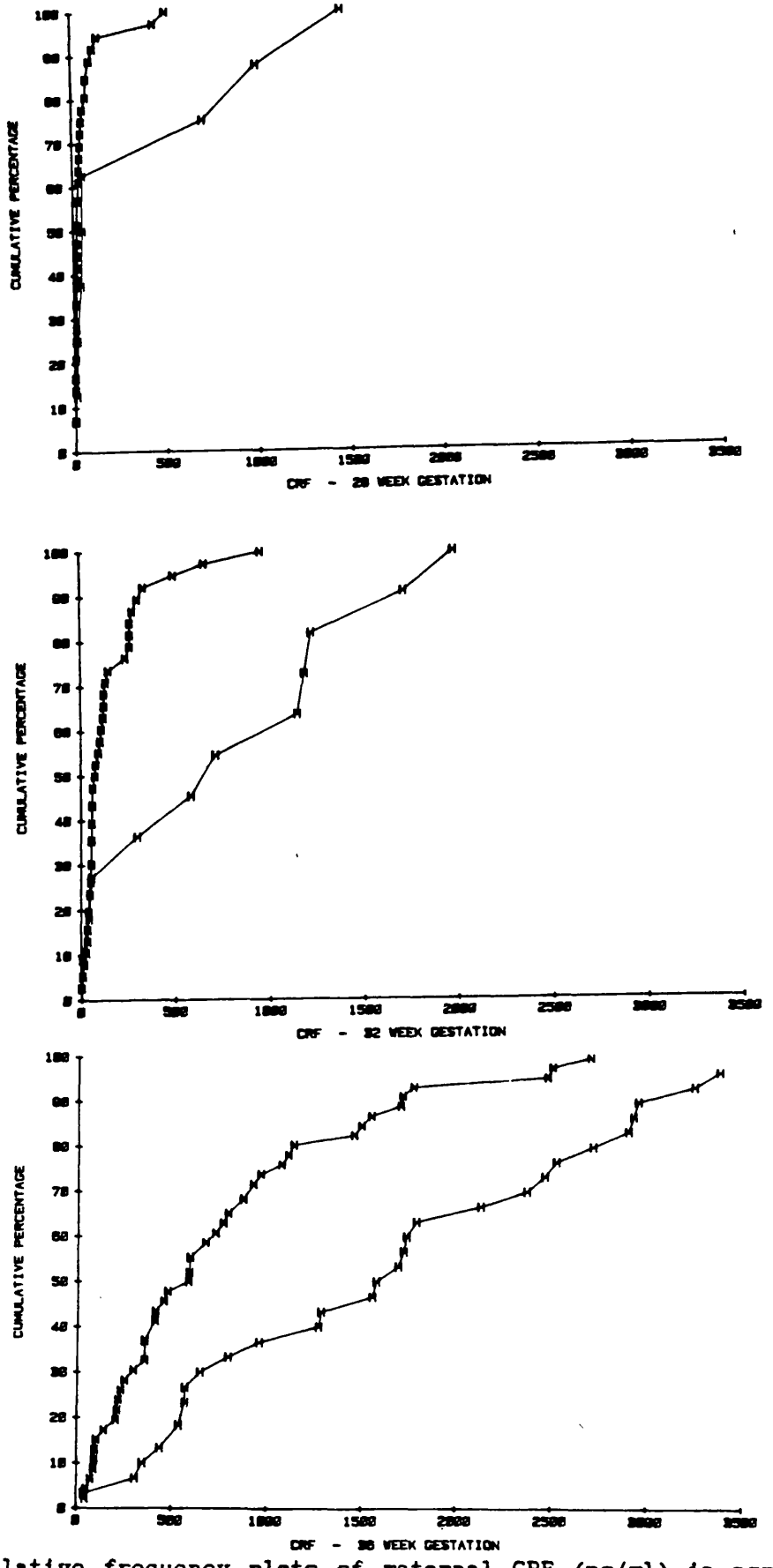


FIG 26 Cumulative frequency plots of maternal CRF (pg/ml) in normal(N) pregnancies and pregnancies associated with PIH (H) at 28,32 & 36 weeks. N & H represent one observation.

FIG. 27

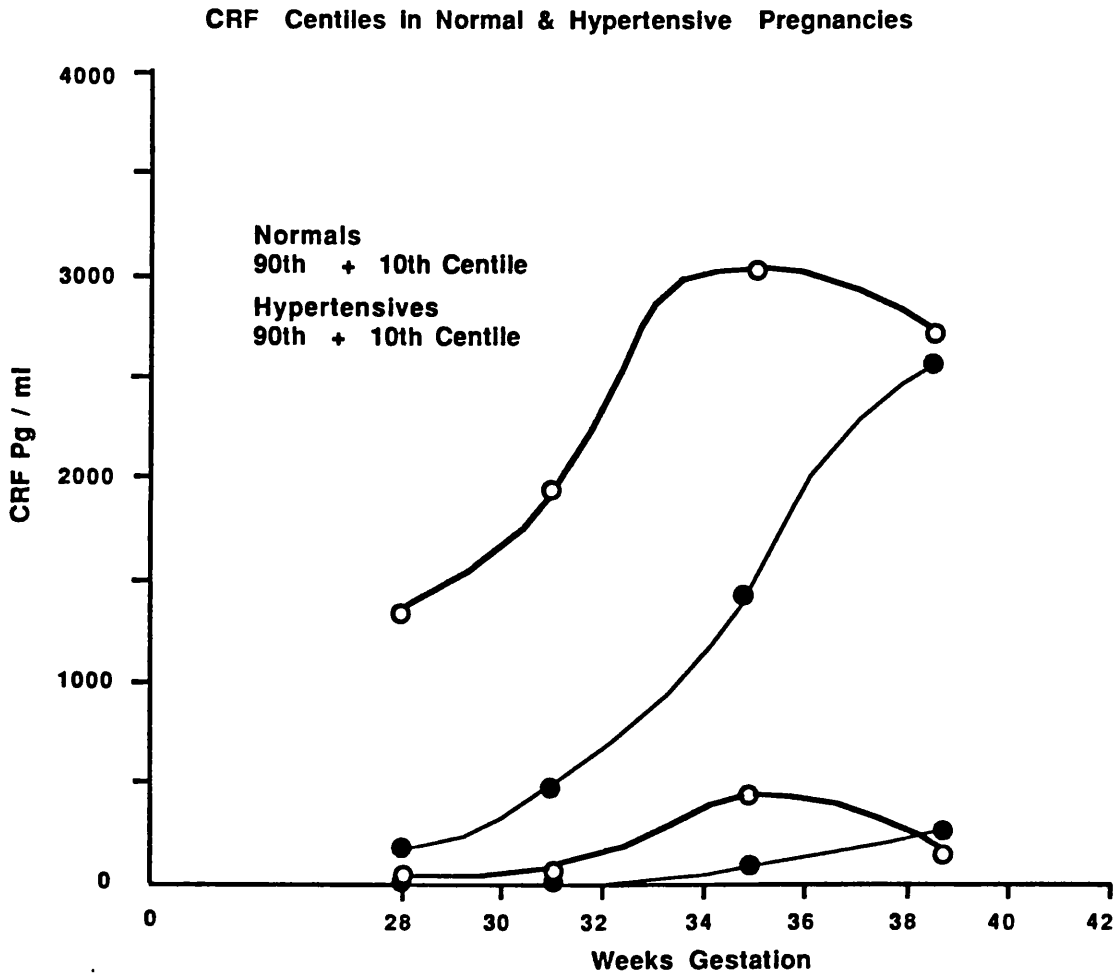


FIG 27 CRF centiles in normal (●) and hypertensive (o) pregnancies.

4. APH: Of the three samples taken in cases of accidental APH at 28 weeks there was a significant increase in CRF (table 6) but not for the rest of the third trimester.

5. POLYHYDRAMNIOS: There was no significant difference in CRF levels in the presence of polyhydramnios (Table 6)

6. PIH: 63 subjects (103 samples) of whom 10 had significant proteinuria, were sampled. The CRF values at all stages of pregnancy were significantly raised (Table 6) (Fig 26). There was no difference between proteinuric and non-proteinuric PIH. In the subjects followed sequentially through pregnancy, 4 had levels above the 90th centile up to 11 weeks prior to the development of signs or symptoms of PIH . However, the CRF values were within the normal range in another 3 subjects. Fig 27 displays the overlap in CRF values between the 2 groups.

7. PRETERM LABOUR: The CRF values in patients who went into preterm labour but with no signs of PIH, demonstated a highly significant increase at all stages of gestation (Table 6)(Fig 28). This group was comprised of patients admitted with premature rupture of the membranes or established preterm labour who subsequently delivered a preterm infant. Of the subjects followed sequentially through pregnancy, 5 went into preterm labour, 4 of whom had levels above the 90th centile prior to the development of signs or symptoms of preterm labour.

8. LOW BIRTHWEIGHT INFANTS: The CRF values in subjects delivering a fetus less than 2.5Kg at term were compared to normal subjects and although the numbers were small there was a significant increase at 30+32 weeks and 34+36 weeks (Fig 29).

FIG. 28.

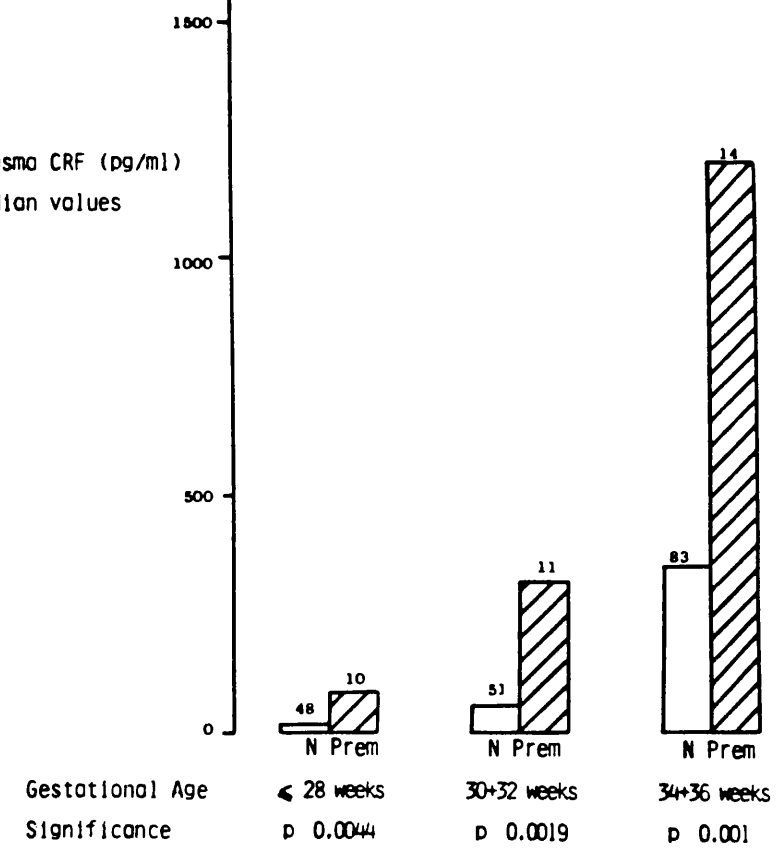


FIG. 28. CRF median values in Normal (N) vs non-hypertensive preterm deliveries (prem). The number of subjects in each group indicated above column. Significance with Mann Whitney U test.

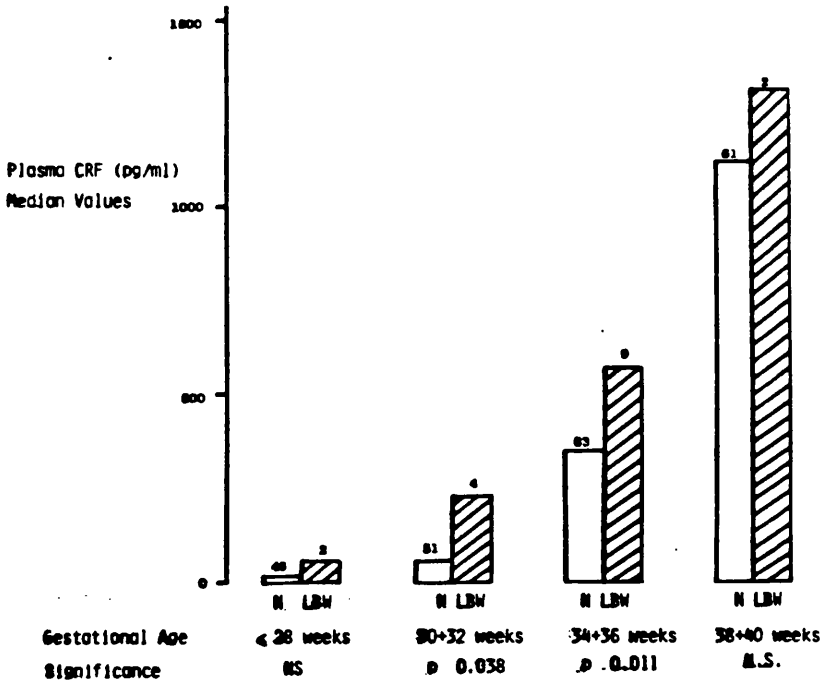


FIG. 29. CRF median values in Normal (N) vs Low Birthweight Infant (LBWI) pregnancies. Number of subjects in each group is indicated above column. Significance with Mann Whitney U test.

9. INTRA UTERINE DEATH. 2 cases were studied. The first subject had been followed sequentially and the last antenatal value was the same as that when she presented in labour with an intra uterine death (37pg/ml at 37 weeks and at 40 weeks. These values are below the 10th centile for gestational age). The second case presented in labour at 23 weeks and the CRF value was 279 pg/ml which is above the non-pregnant range at this gestation.

4.4. DISCUSSION

In the past 20 years the measurement of oestriol and hPL in maternal plasma has been used widely to assess fetal wellbeing. Despite many reports on maternal plasma oestriol measurement it was not until recently that the sensitivity, predictive value and effect of oestriol measurement on the perinatal mortality rate was assessed (Beisher et al 1983). hPL has undergone more rigourous scrutiny (Letchworth 1976, Chard & Klopper 1982) and the effectiveness of its use to decrease perinatal mortality has been addressed (Spellacy et al 1975). There have also been many studies on the levels of hPL in abnormal pregnancy states (Letchworth 1976) and quantification of its usefulness (Grudzinskas et al 1981, Letchworth et al 1978). In general maternal plasma hPL is decreased in pathology, elevated levels usually indicating optimal pregnancy outcome (Grudzinskaset al 1983). In IUGR hPL levels are decreased (Lilford et al 1983, Obiekwe et al 1984) but the sensitivity of a single measurement is only in the region of 30% with a specificity of 90%. The clinical efficiency of the test is increased only slightly with serial determinations (Obiekwe et al 1984).

More recently the pregnancy specific proteins have been similarly investigated and compared with hPL in the identification of IUGR but they appear to be at best no better than hPL (Chapman et al 1981, Gordon et al 1977, Grudzinskas et al 1983, Pledger et al 1984, Salem et al 1981a, Westergaard et al 1984 a,b & c). Of interest is the evidence that type 2 placental products, PP5 and PAPP-A, tend to be elevated in abnormal pregnancy states which is similar to the observations on CRF in abnormal pregnancy .

Normal CRF levels in cases of hydatidiform mole is not unsuspected as CRF, unlike hPL, SP1 and PAPP-A, is not above the non-pregnant range prior to 28 weeks and the levels of CRF mRNA in the placenta are low until the third trimester (Frim et al 1987). Further analysis of the effect of complications of early pregnancy, such as threatened and spontaneous miscarriage, on placental and maternal plasma CRF should be undetaken.

The raised plasma CRF levels in twin pregnancies is in agreement with observations on other placental products (Genazzani et al 1971, Nisbet et al 1981b, Singer et al 1970, Towler et al 1977, Westergaard et al 1985) and may reflect the increased placental mass in twin pregnancy although this study found no correlation between placental mass and CRF in singleton pregnancy. Westergaard et al (1985) noted that there was a distinction between hPL levels in singleton and twin pregnancy but this was less so for oestriol and SP1 which was surprising considering the association between SP1 and birthweight in singletons (Gordon et al 1977). Conversely, the levels of PAPP-A and PP5 have a poor correlation in singleton pregnancies with birthweight (Obiekwe et al 1981) but were very much higher in twin pregnancies . A similar situation is seen with

CRF which implies factors that control the synthesis and metabolism of CRF which are independent of those that influence fetal growth, although a relationship with birthweight can not be excluded because of the findings of elevated levels of CRF in mothers delivering low birthweight infants.

Diabetic patients have an increased perinatal and neonatal mortality and morbidity due to antenatal complications. A biochemical marker which, in conjunction with glucose control assessment, could predict fetal compromise would be a useful adjunct. Previously, hPL levels have been reported as elevated in diabetic pregnancies by some groups (Chard & Klopper 1982, Singer et al 1970) but not by others (Genezzani et al 1971, Spellacy et al 1971). Pledger et al (1982) reported elevated levels of SP1 in diabetic pregnancies. PP5 levels have been shown to be low in the 1st and 2nd trimester and high in the third (Nisbet et al 1981b) and reports on PAPP-A levels are variable (Stabile et al 1988). CRF levels show no significant difference from the normal range except when accompanied by severe PIH (1 case). The numbers of subjects studied is relatively small, further data being required to elucidate this point.

Similarly the data on polyhydramnios is inconclusive and difficult to interpret. However, with the finding of raised levels of CRF in some subjects who go into preterm labour it may be expected that if polyhydramnios was an associated feature of the premature labour, that levels would be raised in such cases.

The number of cases with accidental APH is small and, again, difficult to interpret. The significantly raised levels of CRF in the 28 week group is of interest as PP5, a type 2 placental product, has also been

shown to be elevated in abruption (Salem et al 1981b), but SP1, a type 1 product, to be normal (Towler et al 1977). A prospective study of CRF levels will determine whether CRF is elevated prior to the accidental abruption and of possible use in the prediction of the pathology.

The initial observations on CRF in PIH indicate that levels are elevated, which is similar to PP5 (Nisbet et al 1981b, Salem et al 1982) in PIH and in eclampsia (Salem et al 1983). PAPP-A has also been reported as elevated by some groups (Lin et al 1977,Toop & Klopper 1981) but not by others (Stabile et al 1988, Westergaard et al 1984b). In all these studies, apart from Westergaard, the numbers of subjects studied was small and the effect of different anticoagulants on the measurement of PAPP-A may underly the discordance between studies (Stabile et al 1988). SP1 levels are normal in PIH (Towler et al 1977, Westergaard et al 1984b). hPL levels are variable in PIH and the problem has been the lack of documentation of the severity of the PIH and timing of the samples, making interpretation of the results difficult (Letchworth 1976). Only Singer et al (1970) reported the levels as being raised, other groups reporting low levels (Genazzani et al 1971,Morrison et al 1980). Westergaard in a prospective study found the levels of hPL to be in the normal range in PIH unless associated with IUGR which is in agreement with others (Letchworth 1976, Morrison et al 1980). Similarly oestriol levels have been found to be decreased in PIH, especially in association with severe disease with IUGR (Hardy et al 1981).

It is not certain whether the rise in CRF levels is primary or secondary to PIH. CRF has been documented as acting as a peripheral vasodilator (Brown & Fisher 1985) and elevated levels of CRF may be a compensatory

response to the underlying pathology. Further prospective studies will determine the use of CRF measurement in the prediction of PIH . The study of Placental and plasma CRF in relation to other known endocrinological control mechanisms such as prostacyclin and thromboxane B_2, catecholamines, kallikrein kinin system, renin-angiotensin-aldosterone system may shed further light on a physiological role for the peptide in PIH (Carr & Gant 1983, Sharp & Symonds 1986)

The elevated levels of CRF in patients in preterm labour, and more importantly in those some weeks prior to preterm labour, is of considerable interest as it raises the possibility of a clinical application in the screening for, or prediction of, preterm labour. The numbers of cases studied is small and the aetiology of the preterm labour is mixed, making interpretation of the data difficult. Darne et al (1987) reported that elevated oestriol:Progesterone ratios some weeks prior to spontaneous labour at term and one to four days before delivery in idiopathic preterm labour. This group correctly state that any predictive test must be positive some weeks before delivery in order that any possible preventative treatment can be initiated.

PP5 has been shown to be elevated in preterm labour (Salem et al 1981) as has PAPP-A (Salem et al 1981) but Westergaard et al (1984c) found no difference in hPL, SP1 or PAPP-A levels in subjects who went into preterm labour compared with normal subjects. This latter report is in agreement with previous reports on hPL and SP1 (Towler et al 1977). The possible role of CRF in the initiation of labour has been discussed and may play a similar role in idiopathic preterm labour. Preliminary studies have indicated that CRF may have a positive inotropic effect on

the human myometrium (Quartero et al 1988) but there are no reports of CRF receptors in the myometrium.

The main use of biochemical tests has been in the prediction of the fetus affected with IUGR. Oestriol measurements were used extensively to detect the at risk fetus but the shortcomings of low oestriol values led clinicians into unnecessary fear and intervention and have consequently fallen into disrepute (Lauersen et al 1983). hPL levels have been more carefully assessed and at best its sensitivity to predict IUGR has been quoted as 30% (Obiekwe et al 1982, Lilford et al 1983) to 54% (Westergaard et al 1984a), but this sensitivity can be improved with serial determinations (Obiekwe et al 1984). PAPP-A has been reported as low at 20 weeks gestation in IUGR but to be of no clinical use in the prediction of IUGR (Westergaard et al 1984a). PP5 is similarly of no use clinically (Obiekwe et al 1980) but SP 1 measurement has been advocated by some groups (Towler et al 1977, Gordon et al 1977, Chapman et al 1981, Westergaard et al 1984a, Grudzinskas et al 1983) but its superiority over hPL disputed. The finding of raised levels of CRF at 30 to 36 weeks in subjects delivering a low birthweight fetus at term is of interest but the numbers studied are too small to assess its usefulness. There were only two cases of intra-uterine death in this study. The levels of CRF were in the pregnancy range but were not elevated. This would suggest that the placenta was still producing CRF but that the levels would not have been of predictive value.

Raised levels of a placental product in pathological states indicates that CRF behaves in a similar manner to type 2 products and can not be compared to the more established type 1 products. Raised levels of CRF

in the two abnormal pregnancy states which are major causes of maternal and perinatal mortality and morbidity is of importance. Further studies are required to assess plasma CRF levels in pregnancy, especially where there is a high risk of preterm labour or PIH, in order that the sensitivity, specificity and predictive value of its measurement can be calculated. These studies may also point to a mechanism for CRF in various pathological pregnancy states.

CHAPTER 5

MATERNAL HPA AXIS STUDIES

5.1. **INTRODUCTION** It has been established that the placenta synthesises CRF which circulates in the maternal plasma in the third trimester of pregnancy attached to a binding protein. *In vitro* studies indicate that CRF extracted from maternal plasma is bioactive and that placental CRF effects the release of ACTH from the placenta. Maternal plasma ACTH tends to rise during pregnancy but remains within the normal range whereas total cortisol rises substantially reaching a plateau in the third trimester. The increased production of CBG accounts for most of this increase but the plasma free cortisol moeity is also elevated in the third trimester and exibits diurnal variation (Chapter 1). This section of the study looks at the relationship between maternal plasma CRF, total plasma cortisol and CBG levels in the third trimester in an effort to explain the changes in the maternal HPA axis during pregnancy.

5.2. **MATERIALS AND METHODS**

5.2.1. SUBJECTS Maternal plasma CRF and total cortisol were measured in many of the subjects included in the cohort used to establish the normal range of CRF values during pregnancy. Plasma CBG was estimated on some of these subjects but also on mothers with abnormal pregnancies. Plasma samples were taken as described in chapter 2. Diurnal variation in plasma cortisol and CRF was estimated in 10 patients hospitalised with suspected PIH, APH, IUGR and patients brought in for social reasons. An 18 guage venflon was inserted under local anaesthesia at the wrist at 0600h and kept patent with heparin/saline solution. Samples were taken at 0645h, 0700h, 0715h, 1145h, 1200h and 0015h. The mean AM and PM

FIG. 30.

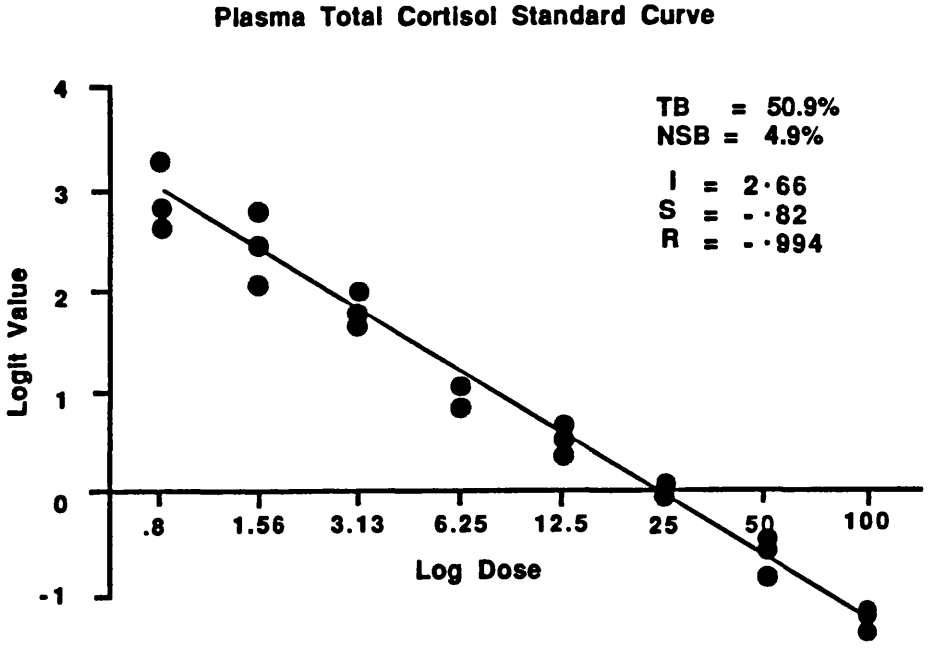


FIG 30. Standard curve of plasma total cortisol. Log dose in nMol/L. I = Intercept, S = Slope of curve, R = Correlation coefficient.

values are represented in the results. 24 hour urine collections were made on 6 patients.

5.2.2. CORTISOL ASSAY An in house RIA was used to determine venous plasma total cortisol. Cortisol was extracted with ethanol. Cortisol was iodinated with [^{125}I] iodide using chloramine-T. Standards and unknowns were incubated with labelled antigen and antibody (sheep anticortisol antiserum (HP/5/631/19) (Guildhay Antisera) for 1 h. Standards of low, medium and high Cortisol values were obtained from the Quality control centre, Cardiff. Precipitation of the antigen-antibody complex was achieved using donkey antisheep serum [500 µL of a 1:100 (vol/vol) solution in 8% (wt/vol) polyethylene glycol (PEG 6000, BDH) in phosphate buffer in the presence of normal sheep serum (1:2000, vol/vol). The sensitivity of the assay was less than 3 nMol/L,and inter- and intraassay CVs were 19% and 7.9% respectively. The data are presented as nmoles/L.

5.2.3. CBG ASSAY An IRE Transcortin RIA-100 kit was used (IRE-UK). The sensitivity of the assay was 0.25 ± 0.002 µg/ml and the inter- and inra-assay CVs were 3.3-7.7% and 2.8-4.5% respectively.

5.3.4 URINARY FREE CORTISOL (UFC) ASSAY A Gamma-BCT KIT (RIA UK) was used to estimate UFC in 24 hour collections. These were kindly performed in the Department of Chemical Pathology, St Mary's Hospital, London. The sensitivity of the assay was 7 nmol/L.

5.3. RESULTS

5.3.1 TOTAL CORTISOL A standard curve of plasma total cortisol is displayed in Fig. 30. The assays total binding (TB) was in the region of 51% and non specific binding (NSB) 5%. Fig 31 and Table 7 display the

results. There is no correlation between CRF and cortisol antenatally: Cortisol levels plateau in the third trimester and rise sharply only in labour whereas CRF rises steadily in the third trimester, with only a small rise in labour (Chapter 3). Postpartum, CRF levels fell to non-pregnant values within 24 hours, whereas Cortisol levels remained high for several days.

WEEKS GESTATION	NUMBER OF OBSERVATIONS	CRF Pg/ml MEAN	SEM	MEDIAN	TOTAL CORTISOL nmol/L MEAN	SEM
<28	14	17.4	9.1	15	689	52
28	56	50	15	20	709	30
30-32	52	177	36	78	704	31
34	39	347	56	260	688	28
36	47	676	95	593	759	39
38-40	54	1062	121	960	833	38
LABOUR	47	-	-	1709	1283	71
POSTPARTUM						
24 hours	15	-	-	-	618	67
48 hours	12	-	-	-	733	65
72 hours	11	-	-	-	526	88

TABLE 7 Total cortisol and CRF levels in maternal plasma in the third trimester of pregnancy and postpartum.

5.3.2. CBG Both CRF and CBG levels were estimated sequentially in 11 subjects. Fig. 32 demonstrates that there is no correlation between the two parameters. In all 11 subjects CRF rose in the third trimester.

FIG. 31

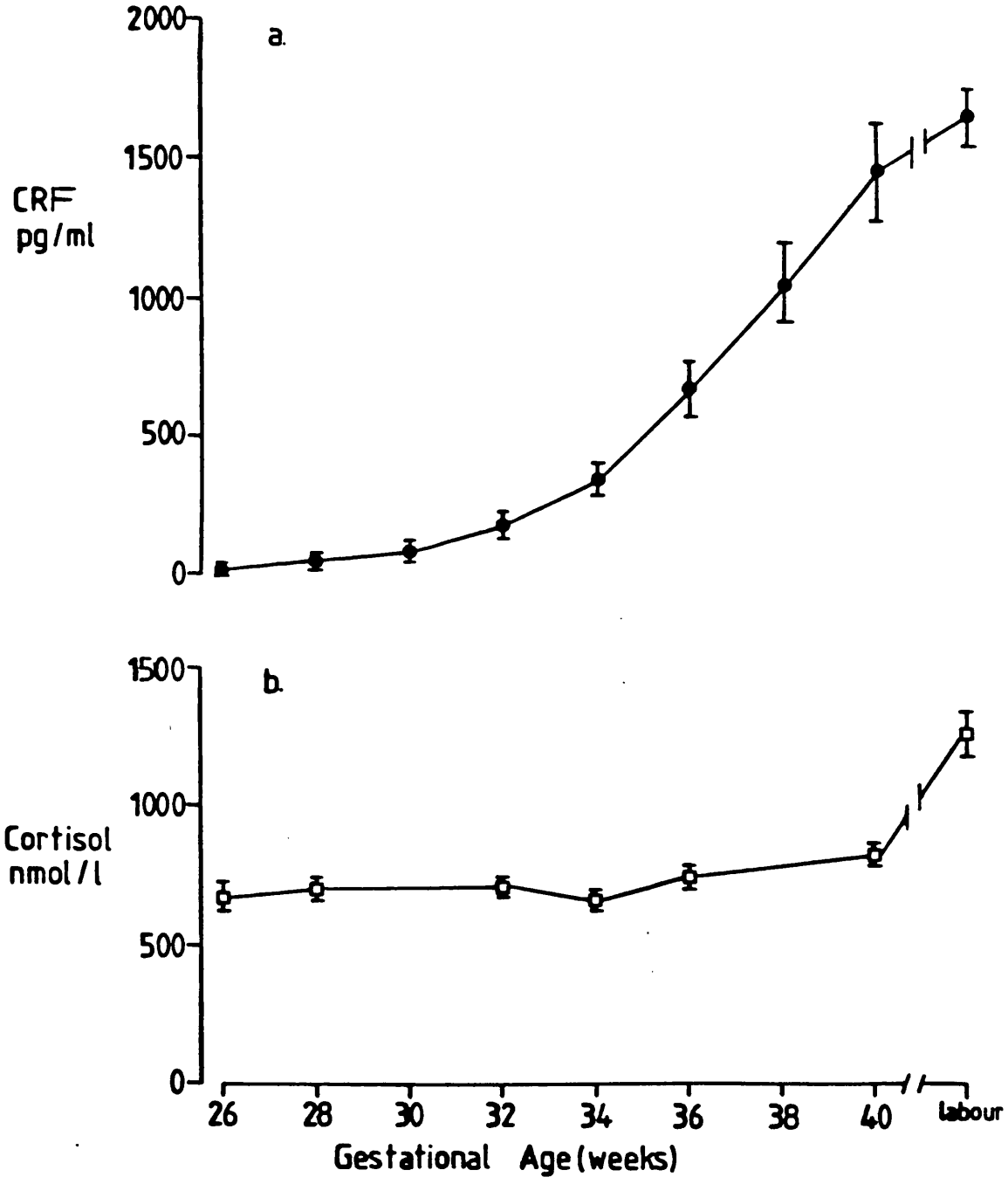


FIG. 31 Mean ± SE CRF (a) and Cortisol (b) from 26 weeks gestation to term and during labour (at full dilation) in normal pregnant women. Each point represents mean value at each gestational age.

FIG. 32.

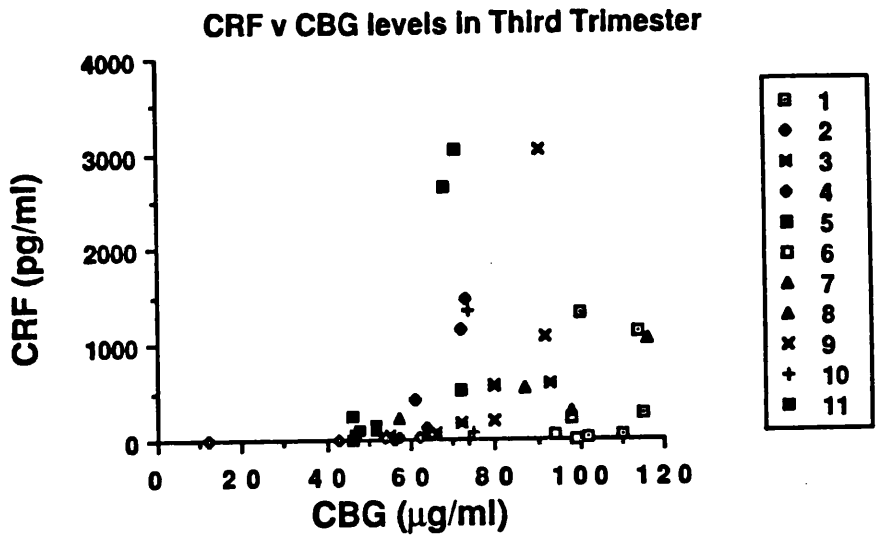
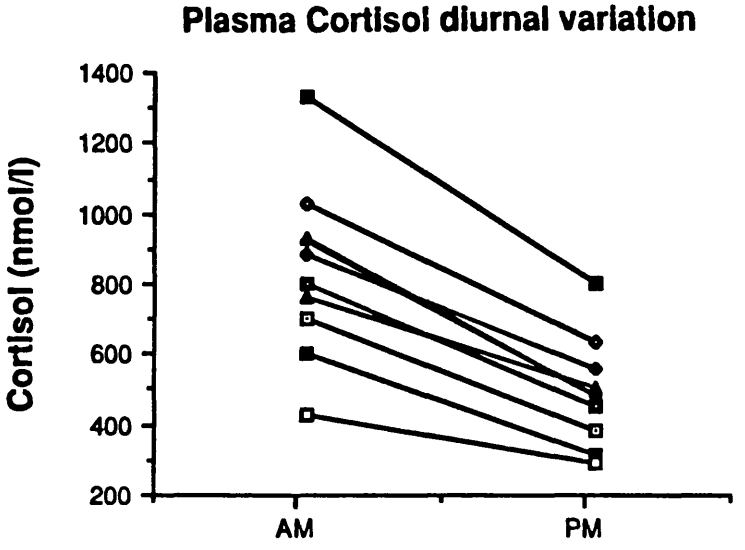


FIG. 32 Plasma CRF and CBG levels in 11 subjects in the third trimester of pregnancy. Each symbol represents one subject.

	AM		PM		
gestation (weeks)	CRF pg/ml	cortisol nmol/l	CRF pg/ml	cortisol nmol/l	UFC nmol/24hrs
29	295	767	375	418	1490
38	2429	1000	2452	600	4140
26	165	571	151	283	
30	1030	400	1058	258	600
29	1360	900	1210	450	1915
32	1844	735	2159	470	1420
38	2713	1300	2982	770	1440
41	542	670	436	350	
37	650	850	518	530	
36	800	890	508	450	

TABLE 8 Maternal plasma cortisol and CRF diurnal values in 10 patients.

FIG. 33



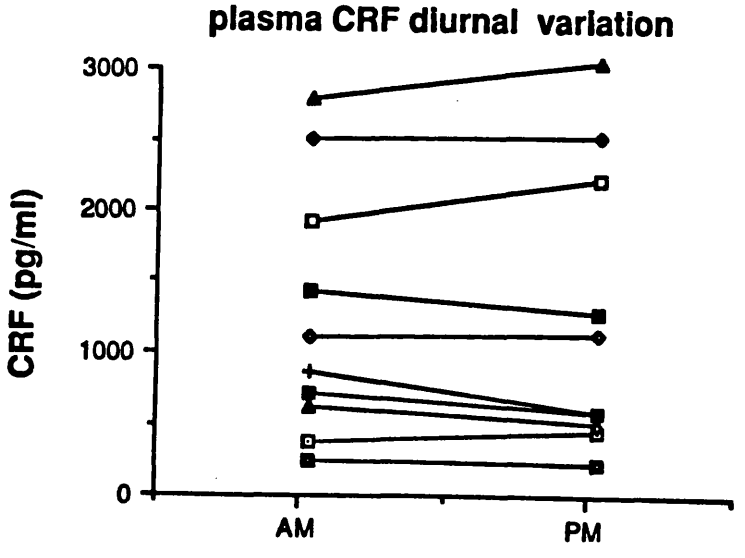


FIG. 33 Plama cortisol and CRF diurnal variation in 10 subjects. Each symbol represents one subject.

whereas CBG reached a plateau in the third trimester (second trimester 71.8 ±17.9 μg/ml, third trimester 97.5 ± 14.4 μg/ml).

5.3.3. DIURNAL VARIATION The diurnal variation of plasma CRF and total cortisol was assessed in 10 patients, of whom 6 had 24 hour UFC estimations performed. Total cortisol levels were consistently higher in the mornings and there was significant diurnal variation ($P < 0.0005$, paired T test). There was no statistical difference ($P < 0.3$, paired T test) between AM and PM CRF values, the changes being variable (Table 8 and Fig. 33). UFC levels were raised in all cases but were within the normal range for pregnancy (50-200 nMol/24 hours).

5.4. DISCUSSION

Maternal plasma CRF rises significantly with advancing gestational age, levels being several orders of magnitude greater than in the non-pregnant state (Chapter 3). Extracted maternal plasma CRF is bioactive but CRF circulates in a carrier bound form and its bioactivity in this form may not be the same as the *in vitro* studies. It is well established that plasma ACTH levels rise during pregnancy but remain within the non-pregnant range whereas total and free plasma levels of cortisol are raised during pregnancy (Chapter 1). This HPA profile does not fit with the negative feedback hypothesis which explains the workings of the HPA axis.

This study confirms observations by many groups that maternal plasma total cortisol rises during pregnancy reaching a plateau in the third trimester and only rising significantly during labour. This rise is, in part, due to raised plasma free cortisol as demonstrated by elevated 24 hour UFC concentrations. The majority of the increase is explained by increased production of CBG and these results confirm this. There is no

correlation between plasma CRF and Cortisol and despite the raised levels of CRF, cortisol demonstrates diurnal variation. Previously, evidence that as pregnancy advanced plasma cortisol was not suppressed by dexamethasone along with the observation that diurnal variation in plasma cortisol is maintained led to the hypothesis that there was a resetting of the maternal pituitary feedback mechanism. This is most likely to be correct in the light of in vivo studies (Shulte et al 1988) but the downregulation of the pituitary may be incomplete (Chapter 1). The other alternative ,as discussed in chapter 3, is that plasma CRF may not drive the HPA axis during pregnancy, the carrier protein reducing its bioactivity. Perhaps both downregulation of the pituitary and reduced plasma CRF bioactivity account for these changes.

A model for the regulation of the maternal HPA axis in the third trimester based on these results could be as follows: High plasma CRF levels do not cause ACTH release because the CRF is carrier bound but hypothalamic CRF could still drive the pituitary ACTH release because there is not sufficient time for it to bind to the carrier protein in sufficient quantities. Alternatively, OT or VP may act as CRFs in pregnancy, CRF having a modified role. The low/normal plasma ACTH levels may be mainly placental in origin, the high levels of cortisol feeding back on the pituitary, shutting it down. The high levels of cortisol with low ACTH levels may also be due to altered adrenal sensitivity to ACTH.

Further investigation of maternal plasma CRF, ACTH, total and free cortisol is required ,especially in relation to the administration of exogenous steroids and, if agreed by ethics Committee's, synthetic CRF. In vitro studies in the monkey may also provide useful information.

CHAPTER 6

VARIABILITY OF MATERNAL PLASMA CRF

6.1. INTRODUCTION

For a single measurement of CRF to be useful as a tool in the assessment of fetal wellbeing the episodic variation of the peptide needs to be determined along with factors which might influence those variations.

6.2. MATERIALS AND METHODS

Twelve subjects in the third trimester of pregnancy admitted with either unstable lie, sciatica, placenta praevia or for the investigation of PIH but with normal blood pressure whilst in hospital or admitted for social reasons were recruited to the study. To determine short term fluctuations of plasma CRF, venous samples were obtained at 5-10 minute intervals for a period of one hour (n=110). To establish the effect of different postures on plasma CRF levels, samples were obtained at 20-30 minute intervals for a period of three hours with a change of posture every hour. Samples between and during uterine contractions were obtained (n=26), during the first stage of labour. In three patients plasma samples were taken at 5-10 minute intervals post partum for a minimum of 2 hours to calculate the plasma clearance of CRF (n = 8, 13 and 10). All blood samples were collected through an 18 guage heparinised intravenous cannula and stored as described in chapter 3.
Data are described as mean ± SD and CVs expressed as a percentage. Plasma half life was calculated using linear regression modelling, with log CRF (pg/ml) and time in hours.

6.3. RESULTS

In 9 subjects, CRF measurement in different positions showed a CV of between 2.6-14.2% with a mean of 7.1 ± 1.5 (Table 9, Fig 34).

In 11 subjects short term (5-10 minute) variation in maternal plasma CRF levels demonstrates a CV of between 2.8 to 8% (Table 10) with a mean of 5.3 ± 0.52.

During and between contractions, CRF was measured in 5 patients and the CV ranged from 1.4-9.1% with a mean of 4.5 ± 1.4(Table 11).

The half life (T 1/2)in 3 subjects was 65 minutes (R=0.95)(Fig. 35).

6.4. DISCUSSION

This study describes the fluctuations in time of plasma CRF in pregnancy, during labour and immediately post partum. As a close correlation between CRF levels and gestational age has been shown (Chapter 3), measurement 24 hours or more apart add little information to the fluctuation in time of CRF as a steady increase will be observed. None of the fluctuations over time were statistically significant and were within the variation expected with the IRMA (Interassay CV 5%). Previously studies on oestriol and hPL have demonstrated day to day variation of around 15% CV for oestriol (Masson and Wilson 1972,Klopper et al 1977) and 4.4% for hPL (Towler et al 1976).The circadian variation of PP5 is 13.3% and day to day variation 9.6% (Obiekwe et al 1979). The circadian variation of SP1 is 8.8%. day to day variation 6.1% and intrapartum variation 11% (Grudzinskas et al 1979). Gordon and Chard (1979) postulated that the main factors controlling the secretion of placental products are the trophoblastic mass and the uteroplacental

FIG. 34.

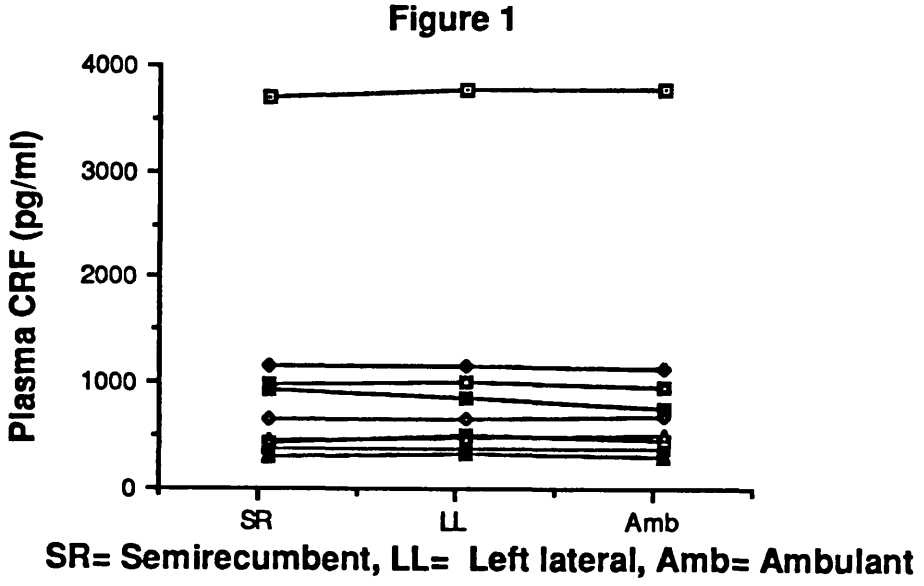


FIG. 34. Variation of maternal plasma CRF levels with posture in 9 subjects.

Patient	1	2	3	4	5	6	7	8	9
Gestational age (weeks)	33	37	39	31	41	33	29	36	37
Semi recumbent									
mean	3602	1047	870	270	555	322	209	352	818
n	4	3	3	3	3	3	3	4	3
Left lateral									
mean	3662	1064	904	276	566	402	236	380	759
n	2	2	2	3	3	3	3	3	3
Ambulant									
mean	3680	1035	855	278	589	364	208	391	861
n	2	2	2	2	2	2	3	2	3
St. sign.	no	no	no	no	no	no	no	no	no
c.v.	3.0	2.6	5.1	5.0	3.7	13	14.2	6.6	11.3

TABLE 9 Variation of maternal plasma CRF levels with posture in 9 subjects.

Table 10.

Patient	1	2	3	4	5	6	7	8	9	10	11
Gestational age	T	29	T	39	37	39	31	41	3rd	3rd	37
No. obs (n)	5	7	7	12	9	12	12	12	10	11	12
Mean	2632	202	866	3675	1098	889	264	553	798	2341	833
SD	97	16.3	65.6	102	54.3	30.5	13.5	28.9	45.8	118	68.5
c.v.	3.7	8.0	8.5	2.8	4.9	3.4	5.1	5.2	5.7	5.0	8.2

Table 10. Short term variation in maternal plasma CRF concentration.

No. of obs.	mean	SD	SEM	c.v.
5	2957	41.8	18.7	1.4
7	67	1.9	0.7	2.8
4	864	57.2	28.6	6.6
4	2834	258	129	9.1
6	1275	33.3	13.6	2.6

TABLE 11 CV of maternal plasma CRF levels in the first stage (S1) of labour.

FIG. 35

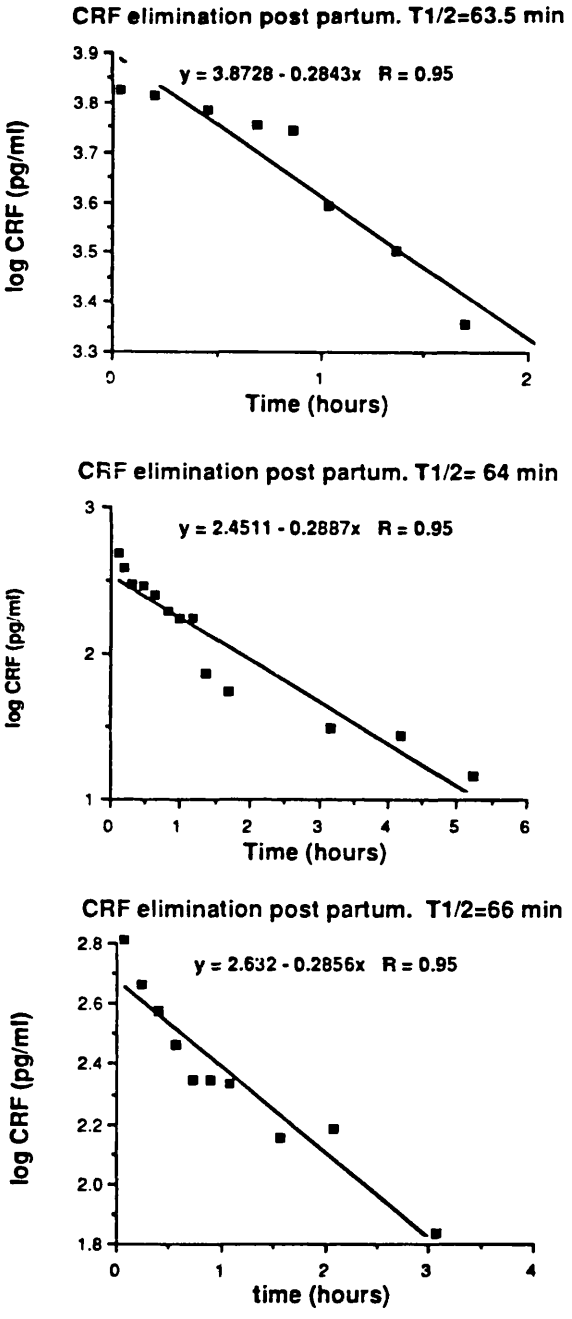


Fig. 35. Maternal plasma CRF half-life (T 1/2) in 3 subjects using linear regression models.

blood flow. They suggested that the effect of posture is relevant to the measurement and these observations demonstrate no effect of posture on maternal plasma CRF levels.

During labour, no excessive fluctuations in plasma CRF concentrations were found during or between contractions. From these data it may be surmised that placental CRF is not subject to wide fluctuations. Therefore for the assessment of the clinical usefulness of the concentration of CRF as a screening or diagnostic tool a single measurement may be sufficient to estimate the CRF levels at a given gestational age but an estimation of the rate of rise of CRF (pg/ml) may be more useful (Chapter 3).

The evidence cited in chapter 1 suggested that CRF was produced by the placenta and postpartum clearance of maternal plasma CRF should be rapid as is the case with other placental products such as hPL and oestriol but unlike peptides such as PAPP A which is also produced by the decidua and has a prolonged half life. Maternal plasma CRF has a half life of about one hour which is in the same region as the second phase of clearance of oCRF from non-pregnant plasma. The straight line relationship between log CRF and time suggests a phase 1 elimination of maternal CRF. In the non-pregnant state after administration of oCRF 2 or possibly 3 phases of elimination have been observed (Chapter 1), the second phase with a half life of 46-73 minutes probably representing metabolic clearance. Human CRF has a more rapid clearance of 25 minutes. These data are preliminary and although the data suggests one phase of clearance, more samples at shorter intervals in the postpartum period

are required to establish the pharmacokinetics of plasma CRF. The presence of CBG maintains elevated levels of cortisol for several days postpartum (Table 7) whereas plasma CRF levels return to non-pregnant levels within 24 hours but the prolonged half life of CRF may be due to the presence of the CRF binding protein.

APPENDICES

APPENDIX 1

APPENDIX 1

Name: _____ Ref: _____

Hospital no: _____

Age: _____

LMP: Working EDD:

ADD:

Risk factor(s):

GESTATION	CRF	FUNDAL HT	WT (kg)	B.P.	Prot	Remarks

Admission/Puerperium:

APPENDIX 2

CRF Project

Patient number	1	____ 1-3
Age	2	____ 4-5
Race Cauc -1	3	__ 6
Negro-2		
Asian-3		
Medit-4		
Marital Status	4	__ 7
Married -1		
Single -2		
Cohab -3		
Separat -4		
Divorced-5		
Employment yes=1	5	__ 8
no =2		
Employment partner yes=1,no=2	6	__ 9
Obstetric History		
Number of pregnancies (9= >9)	7	__ 10
No. premature labours (< 37/40)	8	__ 11
No. Caesarean sections	9	__ 12
Previous PIH yes=1,no=2	10	__ 13
No. spont. abortions <24/40	11	__ 14
No. TOP's	12	__ 15
Current pregnancy		
Booking < 16/40 =1	13	__ 16
Booking > 16/40 =2		
Rhesus neg. yes=1,no=2	14	__ 17
Sickle pos. y/n	15	__ 18
β-Thal.pos. y/n	16	__ 19
Booking Hb <10 g/dl y/n	17	__ 20
Proteinuria y/n	18	__ 21
Endocrine disease Thyroid=1	19	__ 22
adrenal=2		
nil =3		
Amniocentesis y/n	20	__ 23
Operations y/n	21	__ 24
Smoking y/n	22	__ 25
Vomiting y/n	23	__ 26
Cervical suture y/n	24	__ 27
Overall weight gain (kg)	25	____ 28/9
Weight loss at term y/n	26	__ 30
Maternal weight (kg)	27	____ 31/2
Normal pregnancy,labour & baby >2.5 kg yes=1,no=2	28	__ 33

APPENDIX 2

Special subgroups yes=1, no=2	29	⌊__⌋ 34
polybydramnios y/n	30	⌊__⌋ 35
hydatiform mole y/n	31	⌊__⌋ 36
steroid therapy y/n	32	⌊__⌋ 37
twins y/n	33	⌊__⌋ 38
Antepartum haem. y/n	34	⌊__⌋ 39
plac.praevia=1	35	⌊__⌋ 40
abruption = 2		
other = 3		
Small for dates y/n	36	⌊__⌋ 41
clinically = 1	37	⌊__⌋ 42
ultrasound = 2		
Hypertension y/n	38	⌊__⌋ 43
< 28/40 = 1	39	⌊__⌋ 44
> 28/40 = 2		
rise diastolic > 20 mm Hg y/n	40	⌊__⌋ 45
rise systolic > 30 mm Hg y/n	41	⌊__⌋ 46
B/P >140/90 y/n	42	⌊__⌋ 47
proteinuria y/n	43	⌊__⌋ 48
medication y/n	44	⌊__⌋ 49
chem.path abnormal y/n	45	⌊__⌋ 50
HPL abnormal y/n	46	⌊__⌋ 51
Oestriol abnormal y/n	47	⌊__⌋ 52
Diabetes y/n	48	⌊__⌋ 53
gestational y/n	49	⌊__⌋ 54
insulin y/n	50	⌊__⌋ 55
control - good = 1	51	⌊__⌋ 56
poor = 2		
Labour		
gestation (weeks)	52	⌊__⌋ 57/8
spontaneous onset y/n	53	⌊__⌋ 59
SROM = 1, ARM = 2	54	⌊__⌋ 60
induction y/n	55	⌊__⌋ 61
augmentation y/n	56	⌊__⌋ 62
Stage I (hours)	57	⌊__⌋ 63/4
Stage II > 1 hour y/n	58	⌊__⌋ 65
epidural y/n	59	⌊__⌋ 66
pethidine y/n	60	⌊__⌋ 67
Delivery spontaneous = 1	61	⌊__⌋ 68
forceps = 2		
ventouse= 3		
LSCS= 4		
emergency LSCS=5		
elective LSCS= 6		
fetal distress y/n	62	⌊__⌋ 69
breech/unstable lie y/n	63	⌊__⌋ 70
Baby male=1, female=2	64	⌊__⌋ 71
twins: 2x male=1, 2x female=2		
male/female=3	65	⌊__⌋ 72
weight (kg)	66	⌊__⌋ 73
		dec.point
fetal length abnormal y/n	67	⌊__⌋ 74
head circumf. abnormal y/n	68	⌊__⌋ 75
placental weight (grams)	69	⌊__⌋ 76/9
CARD 1	70	⌊1⌋ 80

APPENDIX 2

CRF project card 2

Patient no.		|_|_|_|	1-3
CRF values antenatal	20-26 wks	|_|_|_|_|	4-7
	28	|_|_|_|_|	8-11
	30	|_|_|_|_|	12-15
	32	|_|_|_|_|	16-19
	34	|_|_|_|_|	20-23
	36	|_|_|_|_|	24-27
	38	|_|_|_|_|	28-31
	40	|_|_|_|_|	32-35
	42	|_|_|_|_|	36-39
ACTH values antenatal	20-26 wks	|_|_|_|	40-42
	28	|_|_|_|	43-45
	30	|_|_|_|	46-48
	32	|_|_|_|	49-51
	34	|_|_|_|	52-54
	36	|_|_|_|	55-57
	38	|_|_|_|	58-60
	40	|_|_|_|	61-63
	42	|_|_|_|	64-66
Cortisol values antenatal			
	20-26	|_|_|_|_|	67-70
	28	|_|_|_|_|	71-74
	30	|_|_|_|_|	75-78
		|XX|	79
	CARD 2	|2|	80

CRF project card 3

Patient No.		|_|_|_|	1-3
Cortisol antenatal	32	|_|_|_|_|	4-7
	34	|_|_|_|_|	8-11
	36	|_|_|_|_|	12-15
	38	|_|_|_|_|	16-19
	40	|_|_|_|_|	20-23
	42	|_|_|_|_|	24-27
CRF values in labour	2 cm	|_|_|_|_|	28-31
	3-5 cm	|_|_|_|_|	32-35
	6-9 cm	|_|_|_|_|	36-39
	10 cm	|_|_|_|_|	40-43
ACTH values in labour	2 cm	|_|_|_|	44-46
	3-5 cm	|_|_|_|	47-49
	6-9 cm	|_|_|_|	50-52
	10 cm	|_|_|_|	53-55
Cortisol values in labour			
	2 cm	|_|_|_|_|	56-59
	3-5 cm	|_|_|_|_|	60-63
	6-9 cm	|_|_|_|_|	64-67
	10 cm	|_|_|_|_|	68-71
CRF values in cord blood:	arterial	|_|_|_|	72-74
	venous	|_|_|_|	75-77
		|XX|XX|	78-79
	CARD 3	|3|	80

APPENDIX 3

ANTENATAL VALUES ON 72 NORMAL SUBJECTS

Patient Number	20-26	28	30	32	34	36	38	40	42
1		7		56	266	1120	1333	1866	
2			17		41	71	293	413	
3			107	280	319	800	960		
4.	5	16		120		413	1146		
5.		25	51	666		933	959	1333	
6.		4	25		69		392		
7.		12		34					
8.	5				100	206	667	1600	
9.		37							
10.				266				1306	
11.		13		40	73	233	333	485	
12.		1	15	40	55	93	360		
13.		13				880		1293	1666
14.					280	600	1280		
15.	1	5		14	16	44	140		
16.		25		267	906	1729	2666	2649	
17.		39			400		1226	1666	
18.			27	500					
19.		13							
20.		1		1	39	88	140	239	
21.		7	1	60	87	253	773		
22.				63	353	880			
23.		1		27	147	300			

APPENDIX 3 (cont'd.)

Patient Number	20-26	28	30	32	34	36	38	40	42
24.		1		7	48	95	547	1306	1450
25.		15		60	220	480	786	1600	
26.			80		746		1106	1550	2750
27.				147	300	460			
28.		20		57	81	360	906		
29.	14					360		2176	
30.	49		466		1600		2532		
31.	27		68		440	591			
32.		10		133	797	2605			
33.		17		36	260	600	786		
34.		57		240		2732			
35.		36		125		973	1126		
36.			5			102	44		
37.		20							
38.		80		306		1085		3040	
39.		520					3053		
40.		453		965			1845		
41.		144			379		1352		
42.		44	60		297	682	839		
43.				55			296	704	
44.					461		2726		
45.	44					1562			
46.	45								
47.						595			

APPENDIX 3 (Cont'd.)

Patient Number	20-26	28	30	32	34	36	38	40	42
48.						2929			
49.	49		466	-	1600		2532		
50.						734			
51.									
52.						48			
53.					159				
54.		48		93	326	415	563	1033	
55.	48			75				1288	
56.		121		109	243	360	3315		
57.	3				61	219	295		
58.	16			102	340	1787		2743	
59.		80		337	247	1470	1267		
60.		33		51	212		838		
61.		19				1720	1775		
62.		52		124			1294		
63.		100							
64.						143			
65.						1509			
66.				48					
67.					243				
68.						1149			
69.							1062		
70.		77		264	299	359			
71.	13			80	351		1090		
72.				56		774	1315		

APPENDIX 4

CRF RANK ORDER ANALYSIS

20 - 26	28	30	32	34	36	38	40
1	1 x 4	1	1	16	44	44	239
3	4	5	7	39	48	140	413
5	5	15	15	41	71	140	485
5	7	17	27	48	88	293	704
13	7	25	34	55	93	295	1033
14	10	27	36	61	95	296	1228
16	12	37	40	69	102	333	1293
27	13	51	40	73	143	360	1306
44	13	60	48	81	206	392	1306
45	13	68	51	87	214	547	1333
48	15	80	55	100	219	563	1550
49	16	107	56	147	233	667	1600
	17	466	56	159	253	773	1600
	19		57	212	300	786	1600
	20		60	220	359	786	1666
	20		60	243	360 x 3	838	1866
	25		63	243	413	839	2176
	25		75	247	415	906	2649
	33		80	260	460	959	2743
	36		93	266	480	960	3040
	39		102	280	591	1062	
	44		109	297	592	1090	
	48		120	299	600	1106	
	52		124	300	600	1126	
	57		125	319	682	1146	
	77		133	326	734	1226	
	80		147	340	774	1267	
	80		240	351	800	1280	
	100		264	353	880	1294	
	121		266	379	880	1315	
	144		267	400	933	1333	
	453		280	440	973	1352	
	520		306	746	1085	1775	
			337	797	1120	1845	
			500	906	1149	2532	
			666	1600	1470	2605	
			965		1509	2666	
					1562	2726	
					1720	3053	

APPENDIX 4

CRF RANK ORDER ANALYSIS Cont/.

36	38
1729	3313
1787	
2503	
2529	
2732	

APPENDIX 5

APPENDIX 5

CRF SLOPE ESTIMATES

Patient Number	Slope Estimate	SE Slope	t value	P value
1	166	33.03	5.03	<.01
2	40.3	12.48	3.23	<.05
3	111.3	18.59	5.99	<.01
4	81.1	27.54	2.94	NS
5	107.3	15.11	7.1	<.01
6	36.9	12.54	2.94	NS
7	-	-	-	-
8	90.6	46.88	1.93	NS
9	-	-	-	-
10	-	-	-	-
11	40.3	8.135	4.96	<.01
12	29.2	10.42	2.8	<.05
13	114.2	7.092	16.1	<.01
14	250	51.96	4.8	NS
15	8.8	3.587	2.40	NS
16	255.4	39.99	6.34	<.01
17	134.9	28.53	4.73	<.05
18	-	-	-	-
19	-	-	-	-
20	19.5	4.439	4.4	<.05
21	65.9	22.06	2.99	<.05
22	204.3	34.21	5.97	NS
23	35.8	12.6	2.84	NS
24	113.8	29.52	3.86	<.05
25	122	32.97	3.7	<.05
26	193.5	42.8	4.52	<.05
27	78.3	1.01	77.48	<.01
28	79.1	32.29	2.45	NS
29	131.4	93.13	1.41	NS
30	214.6	26.04	8.24	<.05

APPENDIX 5 (Cont'd.)

Patient Number	Slope Estimate	SE Slope	t value	P value
31	59.47	13.83	4.3	<.05
32	295.9	82.33	3.59	<.05
33	81.9	20.19	4.06	<.05
34	334.4	166.6	2.01	NS
35	120.6	28.65	4.21	NS
36	7.5	9.027	1	NS
37	-	-	-	-
38	241.5	69.23	3.49	NS
39	-	-	-	-
40	134.8	5.1	27.38	<.05
41	114.4	55.81	2.05	NS
42	83.6	14.4	5.81	<.05
43	71.7	32.74	2.19	NS
44	-	-	-	-
45	-	-	-	-
46	-	-	-	-
47	-	-	-	-
49	-	-	-	-
50	-	-	-	-
51	-	-	-	-
52	-	-	-	-
53	-	-	-	-
54	76.7	16.51	4.64	<.01
55	87.5	39.22	2.23	NS
56	248.2	152.2	1.63	NS
57	22	8.669	2.62	NS
58	202	67.77	2.99	NS
59	138.8	52.74	2.63	NS
60	80.5	29.81	2.7	NS
61	186.2	30.54	6.1	NS
62	129.8	48.41	2.68	NS
63	-	-	-	-
64	-	-	-	-
65	-	-	-	-

APPENDIX 5 (Cont'd.)

Patient Number	Slope Estimate	SE Slope	t value	P value
66	-	-	-	-
67	-	-	-	-
68	-	-	-	-
69	-	-	-	-
70	35	4.515	7.76	<.05
71	85.3	35.02	2.44	NS
72	205.5	22.52	9.13	NS

CALCULATION OF SLOPES

Take all patients with more than three antenatal CRF values (n=48).Take CRF values at 26,28,30,32,34,36,38,40&42 weeks and calculate rate of rise using the formula;

$$Y = a + bx$$

where Y is CRF, a is a constant, b the slope and x the gestation .

t_{n-2} = Slope(b) /Standard error of(b)

95% confidence interval of slope=slope estimate±(t_{n-2} x SE slope).If SE(b) is greater than slope then the rate of rise is not significant.

APPENDIX 6

Appendix 6

LABOUR VALUES

PATIENT NUMBER	2 cms.	2-5 cms.	5-9 cms.	10 cms.
2	453		666	693
3	2000	1866	2400	1800
4	1430			
6			800	959
7	837			
8		1650	1732	1630
12		407		480
13		1732	1600	1666
15		226		224
16			2335	2658
17	1733	1759	1675	1699
18			526	
23				306
24	1533	1499		1866
25		1566	1666	1660
26	3186	3088		
27		1253	1213	1400
28	2991			
32			2194	2348
34				2239
36				83
39	2660		2756	2698
40	2176			
41	2313			2192
42		1928		2430
43	530			
44		3349		
54	530		1964	2199
55				1780
56	1812			

APPENDIX 7

RANK ORDER CRF VALUES IN LABOUR

2 cms.	2-5 cms.	5-9 cms.	10 cms.
453	226	526	83
530	407	666	224
530	1253	800*	306
837*	1499*	1213	480
1430	1566	1600	693
1533	1650	1666	956
1733	1732	1675	1400
1812	1759	1732	1630
2000	1866	1964	1660
2176	1928*	2194	1666
2318	3088	2335*	1699
2660	3349	2400	1780
2991		2756	1800
3186			1866
			2192
			2199
			2239
			2348
			2430
			2658
			2698

*interquarter range

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